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Addressing the aquatic vertebrate community of Vågsfjorden 2019 with the use of Environmental DNA

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Master's thesis in Marine Ecology and Resource Biology, Bio-3950, May 2020

Acknowledgements

Firstly, I would like to thank the many people I met during this adventure for the smiles, interesting conversations, and their help with understanding the world of biology.

I would like to thank my supervisor, Arve Lynghammar, who was always available and helpful. For a man feared by the students as a strict and orderly teacher, he was surprisingly quick with jokes, and quite literary the coolest supervisor I can ever imagine having.

My friends deserve their praise, for helping me with R, for helping me understand biological concepts, and last but not least for motivating me throughout the writing period.

The Genetics group members at UiT were always helpful. Special thanks to Owen Wangensteen for helping me understand what environmental DNA is and for teaching me some R basics. Thank you Kim Præbel, for helping me take water samples.

Finally, I would like to thank Terje Hansen from Andørja Adventures. The kind smiles, jokes, food, coffee, and not least the boat, were all key to sampling.

Thank you.

Tromsø, May 2020

Simeon Mikalsen

Abstract

In order to estimate biodiversity or do species counts, techniques such as trawling or longlining have been used. These techniques are detrimental to the ecosystem, in that they have high rates of bycatch, high mortality, and they damage the habitat. Lately techniques used to monitor, such as baited remote underwater video systems and environmental DNA have been tested as possible methods to investigate species richness and abundance, the latter which will be used in this thesis.

Using eDNA methodology, a northern Norwegian fjord was investigated in order to detect the species richness throughout a year at two depths. In addition, an attempt was made to explain the variation of eDNA reads by correlating it to biotic and abiotic factors.

The fjord showed presence of 40 different taxonomical units (MOTUs). The fish community were unevenly distributed, where a few MOTUs (*Pollachius virens, Hippoglossus* sp., *Gadus morhua, Salmo salar, and Molva molva)* were present most dates in both depths throughout the year. On contrast 19 MOTUs were present one to three times throughout the year, where 12 MOTUs were detected only once. The spring season contained more species and higher numbers of eDNA than the rest of the year. This might have been as animals gathered in order to predate upon either spawning *Gadus morhua*, their eggs, or to graze on the increased primary production during this period.

The 20m samples and 350m samples were more similar during the spring turnover period, but it is hard to say if this is a factor of turnover, as high numbers of reads were also associated with this period indicating a higher amount of biomass in total.

The use of eDNA succeeded in providing a picture of the Vågsfjorden biodiversity. For very numerous animals the method was applicable to associate with biological traits, but not for rarer animals.

1 Introduction

Oceans have been a source of food for humans for centuries, providing food, and for some people quite remarkable experiences. One of those experiences were described in Oliver Goldsmiths novels “A history of the earth and animated nature”, where he describes multiple

scenarios where he observes animal counts beyond imagination. At one point he observes herring schools so vast he believes them inexhaustible (Roberts, 2008).

Sadly, Goldsmith was wrong.

From 1970 to 2014 the global size of wildlife populations have decreased by 60% (World Wildlife Fund, 2017), this intensity varying greatly on a worldwide scale. For the oceans it was estimated by 1998 that 25 to 30% of the world's fisheries were being overexploited or depleted and 40% were heavily or fully exploited (Pauly *et al.*, 1998). In order to keep populations from crashing knowledge about fish communities ought to be monitored so that population crashes can be detected early and averted.

Some species are easier to observe and monitor than others as a consequence of their habitat and behaviour. Animals on land are for an example easier to spot than animals in the ocean. And animals that are not very abundant, and live in the deep ocean, may be very hard to quantify and/or observe. Thus, leaving knowledge gaps about the biology of these species.

An example of this is the Greenland sleeper shark, *Somniosus microcephalus*, a long lived deep-water species that is found in the Arctic oceans (Nielsen *et al.*, 2016; Mecklenburg *et al.*, 2018b). Low commercial interest in this species combined its habitat preference the shark has resulted in the failure to estimate its population status. The Norwegian Biodiversity Information Centre has currently listed the species as Data Deficient (*Norsk rødliste for arter*, 2019). Not knowing the niche and population status of this animal could have negative consequences, for the species the surrounding ecosystems as removal of large sharks has been shown to have an pervasive effect(Hussey *et al.*, 2015). A desirable outcome would be if it was possible to observe and quantify these obscure species without negatively impacting the populations.

2 Ecosystem monitoring

2.1 Current methods observing and quantifying wildlife

Some animals are observed by happenstance and reported to institutions such as Artsdatabanken so that the presence of animals may be documented. Examples of this are when fishermen catch a species that have not previously been documented in that area, such as the capture of a pink salmon in Matre in western parts of Norway (Mæstad, 2009). Other

times aquatic environments are being actively investigated in order to detect any species, or the biomass present (Bax and Eliassen, 1990). There are multiple ways of trying to detect if an animal is present in a system. To investigate Greenland sharks, they may for an example use either trawl, longlines, or use baited underwater remote video systems (BRUVS) (Nielsen *et al.*, 2014; Lydersen, Fisk and Kovacs, 2016; Devine, Wheeland and Fisher, 2018).

Trawling is a great tool to extract novel knowledge or update what we know about species distribution in large areas (Byrkjedal and Høines, 2007), or to estimate biomass (Evans *et al.*, 2000). However this tool is detrimental to the ecosystem, in particular the sea-floor (Eleftheriou, 2000; Thrush and Dayton, 2002). There are also the unavoidable selectivity, where some species avoid the trawls, and thus the biomass of these species are underestimated (Kaartvedt, Staby and Aksnes, 2012).

In order to target more specifically one may use longlines. This method has slightly lower mortality on release for sharks (Bryk, Hedges and Treble, 2018; Wheeland and Devine, 2018), and might therefore be more beneficial when investigating particular species. Though not as mortal, both methods still harm non targeted species (Bull, 2007).

In recent years, technology has allowed for new ways of sampling ecological data to be tested, one of these being Baited remote underwater video systems (BRUVS), which may give a special and temporal picture of the biodiversity and biomass. These have their own pros and cons, some of the positive sides being the ability to sample from the deep ocean, that it is non-invasive and the fact it can derive accurate length measurements of animals observed. Some of the negative sides with BRUVS include the lack of a consistent protocol, bait related biases and issues regarding counting the same individual twice (Harvey *et al.*, 2013). This methodology has been applied of the Australian coast, where the technique sampled 56 of the 82 genera observed when combined with the use of eDNA metabarcoding (Stat *et al.*, 2018).

2.2 eDNA

A new way of extracting information from the environment is the use of environmental DNA (eDNA) (Thomsen *et al.*, 2012). eDNA is DNA that has been shed from an organism, and then are sampled from the ocean, lakes or sediments (Strickler, Fremier and Goldberg, 2015).

After extracting the eDNA and using primers to amplify a sequence[1] in the lab, one may assemble a picture of the ecosystem composition. eDNA may come from e.g. faeces, saliva, eggs, sperm, blood, skin. The quantity of eDNA extracted from aquatic samples is dependent on biomass, age structure, physiology, space use, feeding behaviour, and life history traits (Ruppert, Kline and Rahman, 2019).

eDNA has until this point been used to assess biodiversity in aquatic and terrestrial environments, by either taking a water, sediment, or bulking specimen[2]. An example being species detection in the central California current, where eDNA together with trawling in 2016 and 2017 where eDNA methodology identified 48 fish taxa, and the trawling identified 28 fish taxa (Andruszkiewicz *et al.*, 2017). It also may be further applied to reconstruct ancient ecosystems, detect plant-pollinator interactions, analyse diet, and detect invasive species (Ruppert, Kline and Rahman, 2019).

As eDNA abundance has been proven to positively correlate with biomass (Klymus *et al.*, 2015; Lacoursière-Roussel *et al.*, 2015) attempts to estimate the exact shedding rate per gram has been conducted for some species (Goldberg, Strickler and Fremier, 2018). The problem with estimating populations on a large scale are that there are many factors that affect the release and degradation of eDNA (Table 1).

[1] The target site varies from study to study, and there are studies commenting on the different primers and their effects, e.g. (Hajibabaei *et al.*, 2019)

[2] When eDNA metabarcoding is applied directly to collected specimens (Ruppert, Kline and Rahman, 2019).

Table 1 Parameters affecting the degradation of eDNA.

Factor	Effect	Source
Light	Denatures DNA molecules.	(Pilliod *et al.*, 2013; Barnes *et al.*, 2014; Strickler, Fremier and Goldberg, 2015)
pH	Mediates DNA-sediment interactions.	(Barnes *et al.*, 2014; Strickler, Fremier and Goldberg, 2015)
Temperature	Increase microbial and enzymatic activity, increasing degradation.	(Zhu, 2006; Pilliod *et al.*, 2013; Strickler, Fremier and Goldberg, 2015)
Organic Phosphate	Substrate for the microbial community. Once it runs out the new substrate becomes eDNA.	(Zhu, 2006; Salter, 2018)
Oxygen	Potential to influence eDNA confirmation. Higher levels of dissolved oxygen increase degradation rates.	(Barnes *et al.*, 2014; Weltz *et al.*, 2017)
Salinity	Mediate DNA-sediment interactions. Potential to influence eDNA conformation.	(Barnes *et al.*, 2014)
Sediment	Binds to eDNA Inactivates extracellular nucleases.	(Barnes *et al.*, 2014)

There is recent proof that even in oceanic waters where many of these factors intertwine fish biomass still correlates with eDNA abundance (Salter *et al.*, 2019). However, large variations in the release of eDNA has specifically been reported as a function of spawning behaviour, where eels in laboratory tanks released between 10 and 200 times more eDNA during spawning events (Takeuchi *et al.*, 2019).

Spawning events have successfully been detected in the wild with the use of eDNA (Duke and Burton, 2020), and for fish that are demersal or benthic with pelagic eggs, such as *Gadus morhua,* and *Hippoglossus hippoglossus,* one should then be able to observe differences in distribution of eDNA in the water column as a function of spawning behaviours

2.3 Aims

The fish community of Vågsfjorden, Troms was then investigated throughout 2019 by using eDNA methodology in order to map the aquatic vertebrate community and to correlate the amount of eDNA with biotic and abiotic parameters.

3 Material and methods

3.1 Study area.

The eastern branch of Vågsfjorden (Figure 1) is approximately 50km long and 10km wide. The fjord has multiple outlets to the open ocean, with the closest being a 250m deep sill located 25km northwest of the sampling location. It is a very deep fjord, containing areas that are down to 500m deep. From low tide to high tide this region normally fluctuates between 1m and 2m (*Harstad - Kartverket*, 2019), and the fjord has a period of spring turnover from February to May[3]

There are no fine scale reports of fishery catches at a communal or local level, with the closest catch statistics being the total catch for Troms county. In 2015, 55 thousand tonnes of cod, about 28 thousand tonnes of other whitefish, and 10 thousand tonnes of pelagic fish were caught in the Troms county where the Southern Troms region (which includes Vågsfjorden) only accounted for 1% of all landings from Troms (Nyrud, Robertsen and Henriksen, 2017).

[3] Metadata used to define this period is present as appendix 8.1. Temperature and salinity for the sampling location.

The fjord is known to house spawning areas for cod, pollock, haddock, halibut, tusk, common ling, and rockfish which are commercially sought after (Fiskeridirektoratet, 2020), while the Greenland sleeper Shark has been observed in the fjord (Pers conv.).

3.2 Sampling

Andørja Adventures at Engenes provided infrastructure such as storage room and boats. Before heading out from port equipment used was washed according to a standardised protocol (Appendix 8.2), then transported by a small boat northwest to the sampling point, approximately at 68.956368, 17.080297.

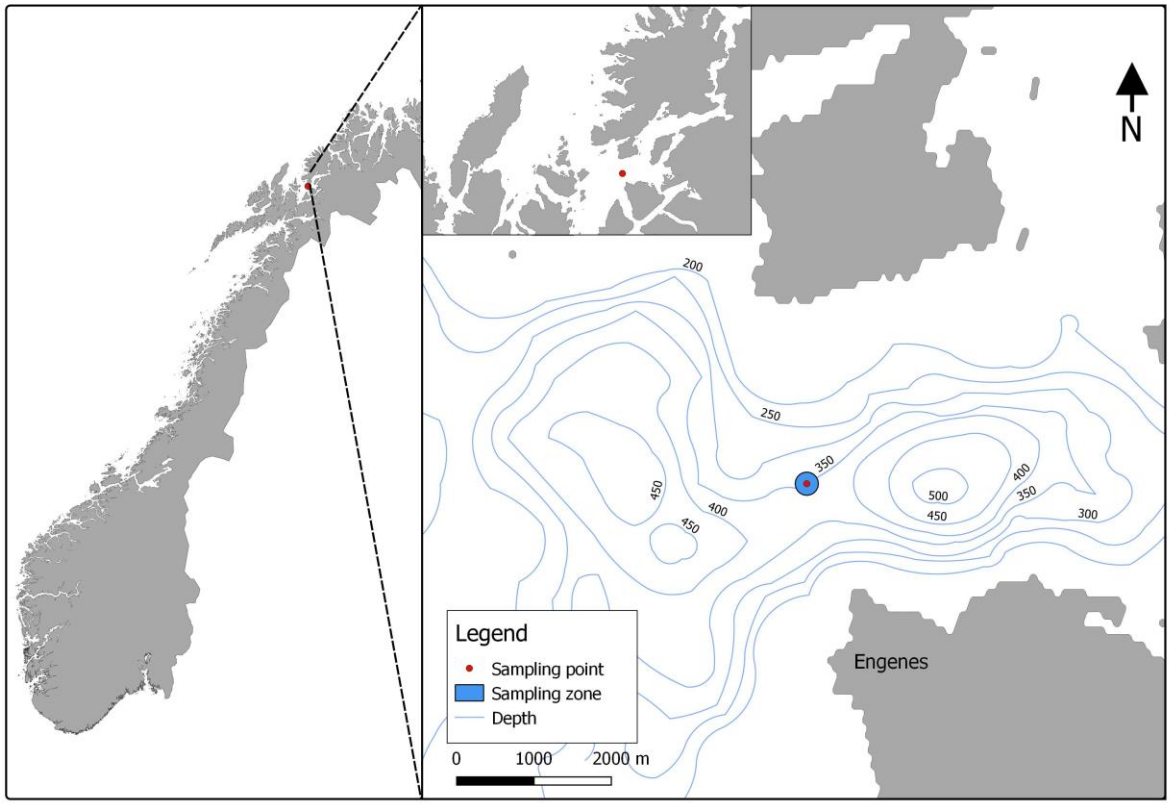

Figure 1: Map created by Gledis Guri displaying the sampling location and the approximate area expected to have been sampled from. The coordinates for the point are 68.956368, 17.080297.

After arrival at the sampling location approximately, 2-2.5l of water from both 20m and 350m were collected with a water sampler (Niskin[4]). The first sampling dates the water was stored

in 1l plastic boxes that had been cleaned prior to departure at Engenes. However, storing the water in 2.5l one-time use zip lock plastic bags proved to be more practical, and were thus used for storing the water before returning to Engenes.

Once onshore at Engenes, 1.5l of water from each dept was filtered. This was done by pushing 500ml of water through each of three 0.22um filter units (Sterivex, Cat. No: SVGPL10RC), creating one sample from that depth.

In addition to the oceanic samples (the 20m and 350m samples), 500ml of tap water from the same source that was used to clean the equipment with was filtrated in order to create a control blank.

The dates that were sampled are displayed in Table 2, and the samples were taken by either myself, my co-supervisor Kim Præbel, or the daily manager at Andørja Adventures, Terje Hansen.

Table 2 Sampling dates and who collected the samples.

Sampling date	Collected by	Notes
17.12.2018	Simeon	Were displaced.
19.01.2019	Kim Præbel	
07.02.2019	Simeon	
15.02.2019	Simeon	
15.03.2019	Simeon	The 350m sample hit the ocean floor.
04.04.2019	Simeon	
23.04.2019	Terje	
14.05.2019	Simeon	
09.07.2019	Simeon	
25.07.2019	Simeon	
17.09.2019	Kim Præbel	Lacked the shower blank sample. Also labelled 300m.
29.11.2019	Simeon	

The filters were stored in a 50ml Falcon tube, before wrapped into 4 layers of zip lock bags and stored on ice. The samples were so transported in bulk to University of Tromsø, The Arctic University of Norway, where they were kept frozen in a -80° Celsius freezer until extraction.

3.3 Lab and bioinformatics

All the eDNA samples except the ones sampled 17.12.2018 were extracted in a clean lab[5], where 3 technical replicates were created per sample in order to detect variation made by lab procedures. In addition, an extraction blank was created for each lab day by opening a new filter unit and treating it as if it contained DNA. The extraction protocol is attached as appendix 8.3. After extraction a PCR was ran using 12s primers as per (Miya *et al.*, 2015). During this step 3 technical replicates was created per existing replicate.

The remainder of the steps (Pooling, Library preparation and sequencing) were done by my co-supervisor Owen Wangensteen. A protocol displaying the process with the use of COI primers are attached in the appendix where the only difference is the use of primers.

Finally clustering of the eDNA were used for dividing the DNA strings into molecular operational taxonomic units (MOTU) where the database is the same as used in (Sales *et al.*, 2019).

3.4 Statistical analysis

Shower blanks and extraction blanks were analysed in order to detect if there were MOTUs that contained more than 10% of all their eDNA in the blank samples. If any were detected the MOTUs were excluded from further analysis.

Also, in order to cope with the stochasticity of PCR, the number of reads were transformed to a unit called relative abundance.

$$Relative\ abundance = \frac{(\frac{x_1}{y_1}+\frac{x_2}{y_2}+\frac{x_3}{y_3})}{z} \qquad \text{F.1}$$

Where x is the amount of reads from a MOTU in a lab replicate and the y value is the total amount of eDNA reads in that lab replicate. Then the fraction is averaged within the sample by adding the fractions together and divided by z, the number of technical replicates for that lab replicate.

[5] A lab specifically used for eDNA extraction with high pressure in order to avoid contamination.

The exclusion of MOTUs that contained more than 10% of their eDNA in the blanks, and the transformation of the dataset from amount of reads to relative abundance, were done in R version 3.6.1 (script attached in the appendix).

MOTUs were grouped by their biotype per (Mecklenburg *et al.*, 2018a, 2018b). For the few MOTUs that were not included in this work individual literature search were done.

A t.test were used to distinguish 20m samples and 350m samples when it came to eDNA reads, relative abundance and amount of MOTUs. It was also used to compare the amount of eDNA and the relative abundance between spawning seasons and non-spawning seasons for MOTUs detected that might have spawned in Vågsfjorden.

An F test/ANOVA test were used to examine the variability of reads and relative abundance throughout 2019. The statistical figures and the remainder of the statistical work were done in Microsoft Excel using the data analysis tools “Anova: single factor” and “t-Test: Two-Sample Assuming Equal Variances” found in the analysis ToolPack Add-inn.

4 Results

4.1 General description

The dataset had a relatively similar distribution of eDNA with a total of 6,398,339 reads in the 20m samples and 5,530,019 reads in the 350m samples throughout the year (Figure 2). The shower blanks contained 400,640 eDNA reads, and the extraction blanks from the lab contained 52,473 reads. The amount of eDNA in the 20m samples (M= 659,271, SD= 113,658,479,452) did not differ significantly from the amount of eDNA in the 350m samples (M= 485,635, SD= 143,686,770,113) where t= 1.082 and p=0.14.

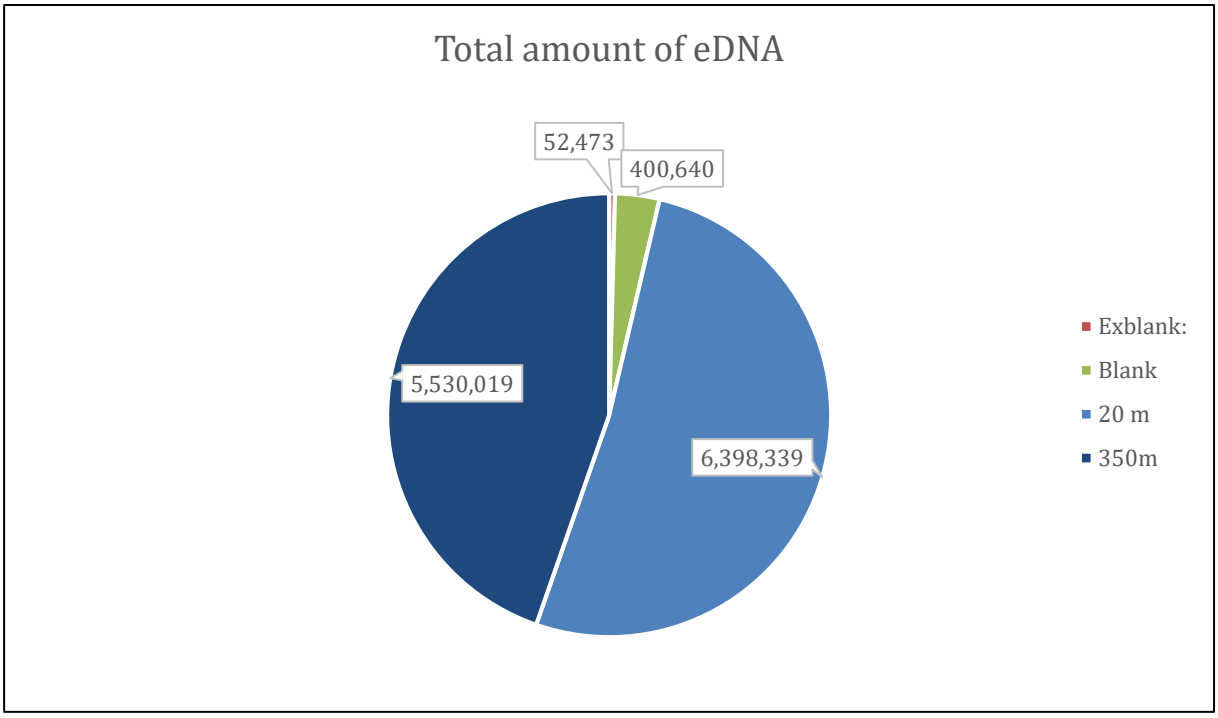

Figure 2 The total amount of eDNA in the 20m samples, 350m samples, shower blanks, and extraction blanks throughout the entire 2019.

There was a variation in eDNA throughout the year $F(9,170)=5.8$ $p=4.99e-7$. Where the variation was greater between the 350m samples $F(10,88)=6.2$, $p=4.6e-7$, than the variation in eDNA between the 20m samples $F(10,88)=3.9$, $p=0.0002$.

There are two dates, the 19th of January and 14th of May, where there are notably more eDNA in the 20m samples (M= 96,204 SD= 78 213) than in the 350m samples (M= 19,020 SD= 15,268), $t=4.1$ $p=0.0001$. On no occasion were there dates where there was statistically more eDNA in the 350m samples than in the 20m samples. The average amount of eDNA per sample throughout 2019 are displayed in Figure 3.

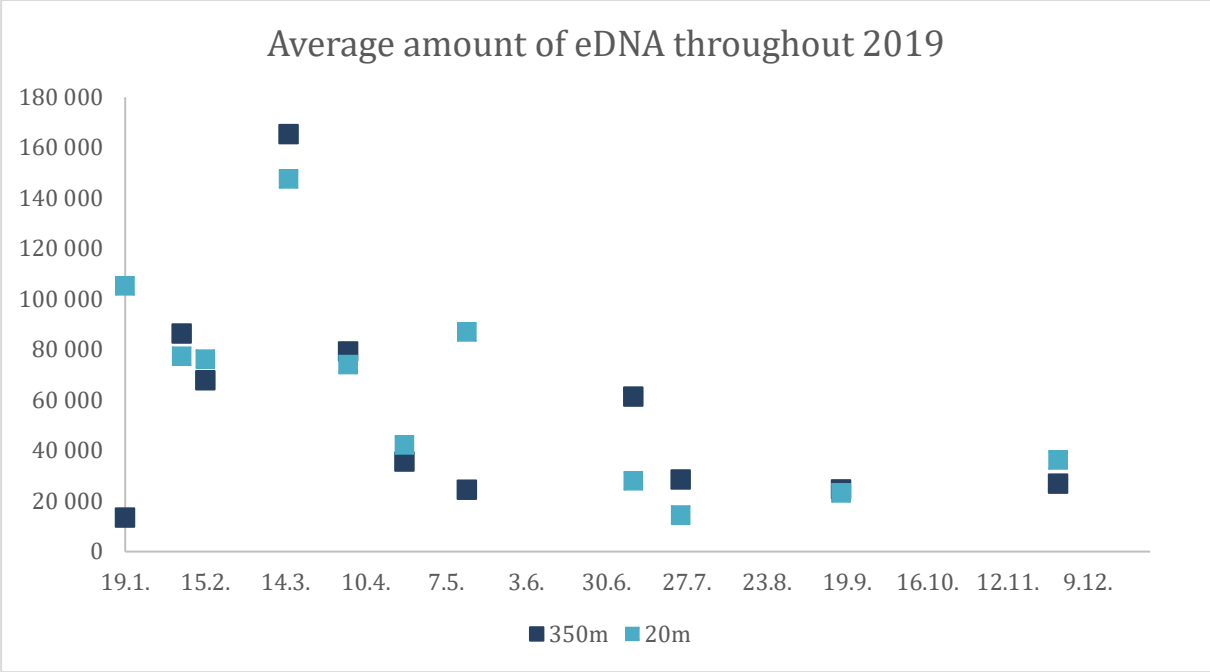

Figure 3 The average amount of eDNA found in the 9 replicates for all sampling dates for the 20m samples and the 350m samples. The x axis does not represent the actual dates sampled, for that see (Table 2) in the sampling section of material and methods

Using eDNA methodology 59 different MOTUs were detected. Out of them, 43 were identified to species level, 13 down to genus level, and 3 down to family level.

All shower blank samples contained eDNA, while only 12 of the 25 extraction blanks contained eDNA. Seven different MOTUs were found in the extraction blanks, and 20 different MOTUs were found in the shower blank samples. A table containing the MOTUs and the proportion of eDNA found from each individual MOTU is present in the appendix. The MOTUs that contained more than 10% of their total eDNA in the blanks together with MOTUs believed to be terrestrial animals were removed from further analysis.

The terrestrial MOTUs detected were *Alces alces*, *Felis catus*, *Canis lupus familiaris*, *Sus scrofa*, *Ovis*, *Bos*, *Capreolus capreolus*, *Corvus* and *Gallus gallus*.

After removal of MOTUs believed to be terrestrial animals, and removal of MOTUs abundant in the two blank samples, the 20m samples and 350m samples together contained a total of 40 different MOTUs.

In the 20m samples 28 different MOTUs were found while the 350m samples contained 34 different MOTUs. The samples shared 22 of these MOTUs. The average number of MOTUs

found in the 20m samples (M=10.6, SD=4.34) and the average number of MOTUs in the 350m samples (M=13.7, SD=3.77) were not statistically different (T=1.78, p=0.09).

4.2 Vågsfjorden's biodiversity 2019

All MOTUs found in the oceanic samples are presented in tables (Table 3, Table 4 and Table 5), where they are grouped by their biotype (Mecklenburg *et al.*, 2018a, 2018b). Information used to group *Salmo salar, Lampetra fluviatilis*[6] and *Spinachia spinachia*, were found elsewhere (Beamish, 1980; Mackney and Hughes, 1995; Skilbrei *et al.*, 2009). All MOTUs with exception of *Pleuronectes quadrituberculatus, Lampetra fluviatilis* and *Chirolophis japonicus* are MOTUs that could be expected to be found in this area. These MOTUs are expected to be *Pleuronectes platessa, Petromyzon marinus* and *Chirolopis ascanii* which are species commonly found in Norwegian waters (Pethon, 2005).

The 20m samples contained 3 MOTUs representing pelagic species that were not found in the 350m samples, while the 350m samples contained 5 MOTUs that represent pelagic species that were not found in the 20m samples. There were 9 MOTUs that represent pelagic species that were found in both the 20m samples and the 350m samples.

Among detected demersal species, there were no species that were exclusive to the 20m samples. The 350m samples contained 4 demersal MOTUs that were not found in the 20m samples. A total of 9 MOTUs of demersal fish were found in both the 20m samples and the 350m samples.

Finally, for the MOTUs assigned to the benthic community there were 4 MOTUs only found in the 20m samples, 3 MOTUs only found in the 350m samples, and 4 MOTUs found in both.

[6] Believed to be *Petromyzon marinus.*

Table 3 Pelagic species found in Vågsfjorden.

20m	350m	Present in both the 20m and 350m samples.
Belonidae sp.	*Argentina silus*	*Lampetra fluviatilis*
Phocoena phocoena	*Salvelinus alpinus*	*Clupea harengus*
	Arctozenus risso	*Salmo salar*
	Protomyctophum arcticum	*Mallotus villosus*
	Balaenoptera acutorostrata	*Maurolicus muelleri*
		Pollachius virens
		Trisopterus esmarkii
		Gadiculus argenteus
		Scomber scombrus

Table 4 Demersal species found in Vågsfjorden.

20m	350m	Present in both the 20m and 350m samples.
	Chimaera monstrosa *Trisopterus minutus* *Crystallogobius linearis* *Ammodytes* sp.	*Somniosus microcephalus* *Molva molva* *Gadus morua* *Melanogrammus aeglefinus/Merlangius merlangus* *Micromesistus poutassou* *Sebastes* sp. *Cyclopterus lumpus* *Ammodytes* sp.[7] *Anarhichas* sp.

Table 5 Benthic species found in Vågsfjorden.

20m	350m	Present in both the 20m and 350m samples.
Spinachia spinachia *Pomatoschistus* sp. *Pholis* sp. *Pleuronectes quadrituberculatus*	Coelorinchus sp. Lycodes sp. *Pleuronectidae* sp.	*Myoxocephalus scorpius* *Chirolophis japonicus* *Hippoglossus* sp. *Microstomus kitt*

[7] The two *Ammodytes* sp. found are different MOTUs.

Table 6 The total amount of times MOTUs occurred throughout the 11 sampling dates in 2019. The three rows in the table illustrates the number of occurrences in the 20m samples, in the 350m samples and the species are sorted after the total amount of occurrences in both.

Species	Total number of occurrences	Times found in 20m samples	Times found in 350m samples
Pollachius virens	22	11	11
Hippoglossus sp.	21	10	11
Gadus morhua	21	10	11
Salmo salar	20	11	9
Molva molva	19	8	11
Scomber scombrus	16	9	7
Clupea harengus	15	7	8
Sebastes sp.	14	4	10
Melanogrammus aeglefinus/ Merlangius merlangus	10	8	2
Micromesistius poutassou	9	2	7
Anarhichas sp.	7	3	4
Ammodytes sp.	7	6	1
Maurolicus muelleri	6	1	5
Chimaera monstrosa	6	0	6
Protomyctophum arcticum	6	0	6
Somniosus microcephalus	5	3	2
Cyclopterus lumpus	5	2	3
Argentina silus	5	0	5
Lampetra fluviatilis	4	2	2
Gadiculus argenteus	4	1	3
Pholis sp.	4	4	0
Chirolophis japonicus	3	2	1
Myoxocephalus scorpius	3	1	2
Mallotus villosus	3	2	2
Trisopterus esmarki	3	2	1
Microstomus kitt	3	2	1
Pleuronectes quadrituberculatus	2	2	0
Arctozenus risso	2	0	2
Coelorinchus sp.	1	0	1
Belonidae sp.	1	1	0
Phocoena phocoena	1	1	0
Spinachia spinachia	1	1	0
Salvelinus alpinus	1	0	1

Balaenoptera acutorostrata	1	0	1
Crystallogobius linearis	1	0	1
Trisopterus minutus	1	0	1
Ammodytes sp.	1	0	1
Pleuronectidae sp.	1	0	1
Lycodes sp.	1	0	1
Pomatoschistus sp.	1	1	0

The fish communities followed an uneven distribution where a few species (*Pollachius virens, Salmo salar, Gadus morhua, Hippoglossus* sp.*, and Molva molva)* were present most sampling dates at both depts throughout 2019. More MOTUs were present more rarely, where the largest group of MOTUs being the 12 MOTUs that only appeared once.
As seen in (Table 6), there were 2 MOTUs in the 20m samples that were present all sampling dates, *Pollachius virens* and *Salmo salar*. There were 4 MOTUs in the 350m samples that were present every sampling date; *Pollachius virens*, *Hippoglossus* sp., *Gadus morhua*, and *Molva molva.*

The average amount of MOTUs found in both the oceanic samples each month throughout 2019 was 6.9, with a standard deviation of 3.45. On average, 16.55 MOTUs with a standard deviation of 4.55 were detected each sampling date across both depths.

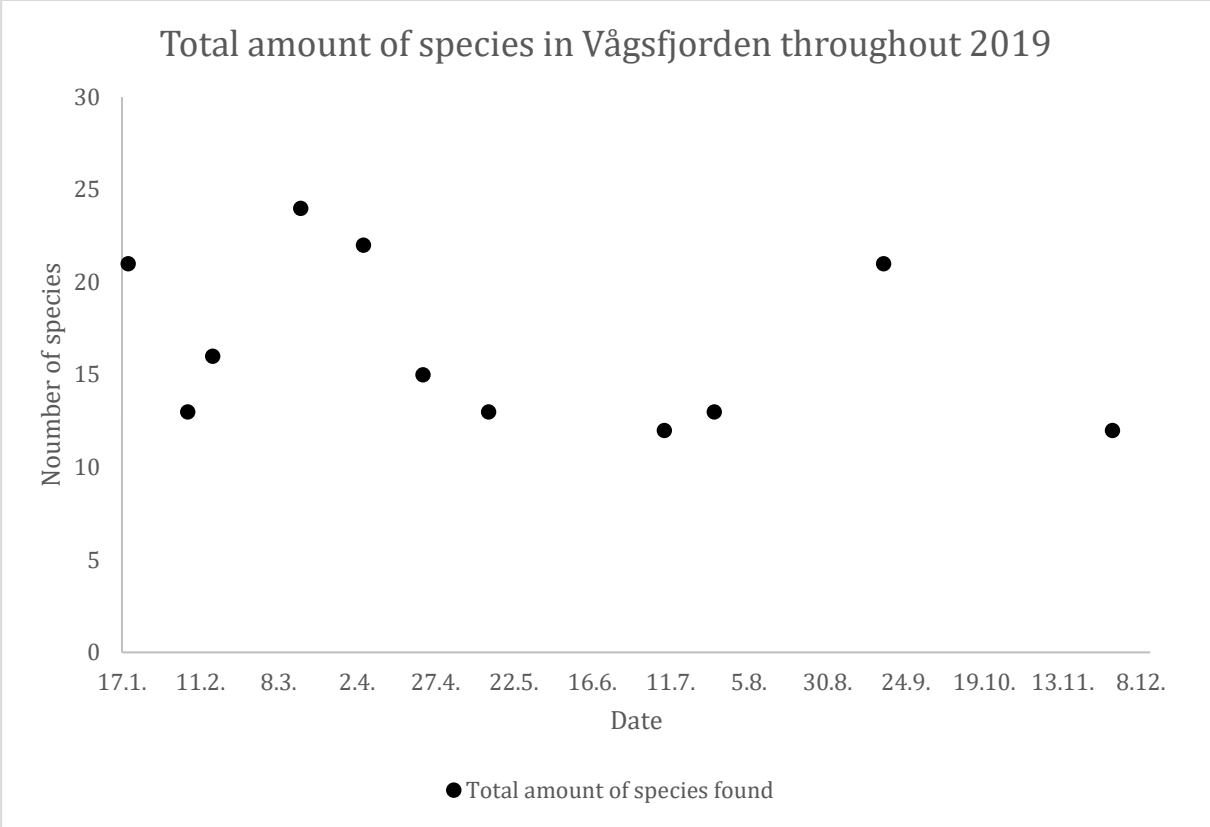

Figure 4 Total amount of species found for the 20m and 350m samples combined. The x axis does not represent the exact sampling dates. The exact sampling dates are in table x in the sampling section of material and methods.

An ANOVA test shows that the amount of MOTUs varies for both the 20m samples ($F(10,22)=4.2$ $p=0.002$) and the 350m samples ($F(10,22)=3.15$ $p=0.01$) throughout 2019. The species composition of the 20m samples and the 350m samples were statistically more similar during the spring turnover period ($M=0.5$ $SD=0.01$) than during the rest of the year ($M=0.34$ $SD=0.15$), $T=2.12$ $p=0.03$.

Table 7 Demersal species present in the 350m sample throughout 2019. A 1 means the MOTU was present and 0 means the species was absent.

	19.1.	7.2.	15.2.	15.3.	4.4.	23.4.	14.5.	9.7.	25.7.	17.9.	29.11.
Gadus morhua	1	1	1	1	1	1	1	1	1	1	1
Micromesistius poutassou	1	1	1	1	1	0	0	0	1	1	0
Trisopterus minutus	0	0	0	0	1	0	0	0	0	0	0
Melanogrammus aeglefinus| *Merlangius merlangus*	0	0	0	0	1	0	0	0	1	0	0
Molva molva	1	1	1	1	1	1	1	1	1	1	1
Coelorinchus sp.	0	0	0	0	0	0	0	0	0	1	0
Gadiculus argenteus	0	0	0	0	1	1	0	0	0	1	0
Crystallogobius linearis	0	0	0	0	0	0	0	0	0	0	1
Anarhichas sp.	1	0	0	1	0	0	1	0	1	0	0
Cyclopterus lumpus	0	0	0	1	1	0	1	0	0	0	0
Sebastes sp.	1	1	1	1	1	0	1	1	1	1	1
Chimaera monstrosa	1	1	0	1	1	0	1	0	0	1	0
Somniosus microcephalus	0	0	0	0	1	0	1	0	0	0	0

There were a lot of MOTUs that were present the 4th of April and the 14th of May, but not the 23rd of April (Table 7). There are also a difference in the appearance throughout the year in the 350m samples where MOTUs such as *Cyclopterus lumpus* and *Somniosus microcephalus* seemed to appear only at a set time of the year, while MOTUs such as *Anarhichas* sp. and possibly *Chimaera monstrosa* seemed to appear spread out through the year.

4.3 Quantitative analysis

4.3.1 Abundant MOTUs

Out of the 40 different MOTUs that remained after removing the terrestrial animals and the ones abundant in the blank samples, the most detected MOTU was *Pollachius virens* which had about 4 million reads (Table 8). This is twice as much as *Salmo salar*, the second most abundant MOTU, which contained approximately 2 million reads. The MOTUs representing *Gadus morhua*, *Clupea harengus* and *Hippoglossus* sp. all appear around 1 million reads, before *Scomber scombrus* appear with 600,000 reads.

Table 8 The MOTUs with the highest amount of eDNA reads for all samples in 2019. The table displays the MOTUs in descending order based on amount of eDNA reads.

MOTU	# reads
Pollachius virens	3,981,820
Salmo salar	2,017,408
Gadus morhua	1,123,257
Clupea harengus	1,088,808
Hippoglossus sp.	989,842
Scomber scombrus	619,226
Micromesistius poutassou	324,776
Molva molva	313,873

When looking at relative abundance (F.1)(Table 9), the same pattern is present. *Pollachius virens* had a relative abundance of 19, almost twice as much as the second most abundant MOTU *Salmo salar*. *Micromesistius poutassou* had the same relative abundance as *Sebastes* sp. even though the latter contain only two thirds (325,000 reads vs 200,000 reads). *Sebastes* sp. also has twice the relative abundance of *Molva molva*.

Table 9 The species with the highest relative abundance in Vågsfjorden.

Scientific name	Relative abundance
Pollachius Virens	19.02
Salmo Salar	11.18
Hippoglossus sp.	7.67
Gadus morhua	6.16
Scomber Scombrus	5.44
Clupea harengus	4.58
Micromesistius poutassou	2.11
Sebastes sp.	2.1
Molva molva	0.97
Mallotus villosus	0.90

When looking at the relative abundance in the 20m samples compared to the 350m samples (Table 10) there is a difference in the distribution. The relative abundance from the 2 most abundant MOTUs, *Pollachius virens* and *Salmo salar*, combinedly occupy 19.98 of the total 32 points of relative abundance from the 20m samples. In order to reach the same proportion of relative abundance in the 350m samples the 4 most abundant species needs to be added up.

A MOTU that is driving a large difference between the two depts is the high presence of *Salmo salar* which with a relative abundance of 9.59 in the 20m samples only have a relative abundance of 1.59 in the deep samples. On the contrast *Scomber scombrus, Hippoglossus* sp., *Sebastes* sp, *Molva molva* and *Chimaera monstrosa* all have a higher relative abundance in the 350m samples than in the 20m samples.

Table 10 The MOTUs in the dataset with the highest relative abundance of eDNA reads in the oceanic samples.

20m		350m	
MOTU	Relative abundance	MOTU	Relative abundance
Pollachius virens	10.39	*Pollachius virens*	8.63
Salmo salar	9.59	*Hippoglossus* sp.	4.20
Gadus morhua	3.47	*Scomber scombrus*	4.09
Hippoglossus sp.	2.99	*Gadus morhua*	3.18
Clupea harengus	1.87	*Clupea harengus*	2.71
Scomber scombrus	1.35	*Micromesistius poutassou*	2.11
Mallotus villosus	0.79	*Sebastes* sp.	1.81
Melanogrammus aeglefinus| *Merlangius merlangus*	0.51	*Salmo salar*	1.59
Sebastes sp.	0.30	*Molva molva*	0.85
Ammodytes sp.	0.20	*Chimaera monstrosa*	0.82

4.3.2 Light and temperature

There were no difference in the amount of eDNA in the 20m samples from May to August (M= 388977 SD=347 365) compared to the samples from the rest of the year (M= 655647

SD= 363 203) t=1.09, p=0.15. There was also no difference in number of reads in the 20m samples in March and April (M= 792 261 SD= 486 153) compared to June to September (M= 197 743 SD= 61 914) t=2.1, p=0.052.

4.3.3 Spawning Activities

A literature research was done to conclude that, *Pollachius virens, Gadus morhua, Hippoglossus* sp. *Scomber scombrus, Micromesistius poutassou* and *Molva molva,* potentially spawn in Vågsfjorden (Ware, 1977; Coombs, Pipe and Mitchell, 1981; Kjorsvik, Haug and Tjemsland, 1987; Dunn *et al.,* 1992). These fish, unlike *Clupea harengus,* spawn at set times of the year and it was therefore possible to compare the amount of eDNA from a species during its spawning season to outside of its spawning season to observe.

Of these six MOTUs, there are only three that have a statistically significant different relative abundance between the periods, *Gadus morhua*, *Hippoglossus* sp., and *Scomber scombrus.* *Hippoglossus* sp. did however contain a lower relative abundance during its spawning season compared to outside of it.

Table 11 Mean amount of relative abundance during and outside the 2019 spawning season for the 6 MOTUs with the highest number of reads where SD is the standard deviation, T is the t value, and P is the p value.

MOTU	Spawning	SD	Not spawning	SD	T	P
Pollachius virens	0,3	0,19	0,29	0,25	0,30	0,38
Gadus morhua	0,14	0,08	0,03	0,03	3,9	0,0004
Hippoglossus sp.	0,06	0,09	0,15	0,22	1,7	0,04
Scomber scombrus	0,03	0,05	0,12	0,04	2,3	0,01
Micromesistius poutassou	0,04	0,12	0,026	0,07	0,05	0,3
Molva molva	0,02	0,1	0,004	0,01	0,94	0,17

When comparing the amount of reads instead of relative abundance there are slight changes. One can see in (Table 12) that when the amount of eDNA were analysed instead of relative abundance, there now are only three MOTUs that have significant difference between the spawning season and non-spawning season. These being *Gadus morhua, Micromesistius poutassou* and *Molva molva*.

Table 12 Mean amount of reads during and outside the 2019 spawning season for the 6 MOTUs with the highest number of reads where SD is the standard deviation, T is the t value, and P is the p value.

MOTU	Spawning season	SD	Not spawning	SD	T	p
Pollachius virens	19 335	18 925	19 601	35 332	0,05	0,47
Gadus morhua	9293	8 763	745	1 906	8,99	1,20E-16
Hippoglossus sp.	4653	9 070	5273	13642	0,34	0,36
Scomber scombrus	3320	9600	2994	6 800	0,28	0,39
Micromesistius poutassou	2526	8906	546	3 205	2,01	0,02
Molva molva	2835	14638	137	606	1,75	0,041

In order to test if pelagic eggs could influence the distribution of eDNA, the mean relative abundance between 20m and 350m were calculated in addition to the mean amount of reads between 20m and 350m during and outside of the spawning season. Even though all of these animals have pelagic eggs there were no cases where there were higher values of either relative abundance, or higher numbers of eDNA, in the 20m samples than in the 350m samples during their spawning seasons.

Table 13 Mean relative abundance in the 20m samples and 350m samples for the 6 MOTUs with the highest number of reads during the assigned species' spawning period.

MOTU	20m M	20m SD	350m M	350m SD	T	p
Pollachius virens	0,26	0,16	0,36	0,22	1,09	0,15
Gadus morhua	0,13	0,07	0,16	0,1	0,41	0,34
Hippoglossus sp.	0,05	0,09	0,08	0,09	0,6	0,26
Scomber scombrus	0,015	0,008	0,06	0,06	1,2	0,18
Micromesistius poutassou	0,0003	0,001	0,09	0,16	2,09	0,02
Molva molva	0,004	0,005	0,005	0,006	1,13	0,13

Out of all MOTUs in (Table 13), only *Micromesistius poutassou* contained a statistically different relative abundance, where the 350m samples contained a higher mean than the 20m samples during its spawning season.

Table 14 Mean reads in the 20m samples and 350m samples for the six MOTUs with the highest number of reads during the assigned species' spawning period.

MOTU	20m M	20m SD	350m M	350m SD	T	p
Pollachius virens	17 757	18 541	20 914	19 522	0,61	0,34
Gadus morhua	10258	10 693	8606	7 321	0,85	0,2
Hippoglossus sp.	4997	11 316	4309	6211	0,3	0,37
Scomber scombrus	3015	5038	7433	14 792	1,65	0,051
Micromesistius poutassou	32	183	5092	12 129	3,07	0,001
Molva molva	271	594	356	766	0,53	0,3

Out of all MOTUs in (Table 14), only *Micromesistius poutassou* contained a statistically different amount of reads, where the 350m samples contained a higher mean than the 20m samples during the spawning season of this species.

5 Discussion

5.1 General

The MOTUs that identified *Careproctus reinhardti,* were most likely contamination from the lab. There are ongoing projects where samples taken from the arctic are extracted in the clean lab, which most likely are the source of this contamination. For MOTUs such as *Brosme Brosme, Lumpenus lampretaeformis* and *Triglops murrayi,* that contained 12%, 32% and 73%, of their eDNA in the blank samples, the source of error becomes harder to detect. Their distribution range include the northern parts of Norway (Mecklenburg *et al.*, 2018a) and *Brosme Brosme* has been caught in Vågsfjorden (Fiskeridirektoratet, 2020) indicating that perhaps *Brosme Brosme* ought to have been included in the results even though more than 10% of its eDNA was found in the blank samples.

False positives up to family level are not uncommon when dealing with eDNA methodology, where other publications have contained around 3% (Andruszkiewicz *et al.*, 2017) and 8% (Kelly *et al.*, 2014). It is possible that *Pleuronectes quadrituberculatus, Chirolophis japonicus, Lampetra fluviatilis* are false positives, but it is also possible that these are *Pleuronectes platessa, Chirolopis ascanii* and is *Petromyzon marinus,* whose distribution range include Vågsfjorden (Pethon, 2005; Mecklenburg *et al.*, 2018a). Considering that there

are some terrestrial MOTUs that also were mistyped, such as *Gallus gallus* and *Sus scrofa*, a possible error that might have occurred is mistyping in the database, or that the 12s gene are the same for these species.

The fact the blank samples contained less eDNA is to be expected as tap water typically are cleansed before arrival at households, and the extraction blanks as they in they were supposed to be empty.

When sampling the bottom of Ullsfjorden the years 1986 and 1988, 24 fish species were observed (Nilssen, Grotnes and Haug, 1992), and 12 fish species were found in Balsfjorden 1975-1976 using trawl (Bax and Eliassen, 1990). Keeping in mind that 28 MOTUs were found with trawl and 48 MOTUs found with eDNA outside of California (Andruszkiewicz *et al.*, 2017), the 40 MOTUs found in Vågsfjorden is a plausible number. Some of these MOTUs were only detected down to family level but can be identified further down as there are only one likely species present in this area that belong to this family. The MOTUs *Hippoglossus* sp., *Coelorinchus* sp. and *Pholis* sp. most likely are *Hippoglossus hippoglossus*, *Coryphaenoides rupestris* and *Pholis gunnellus.* For *Melanogrammus aeglefinus/Merlangius merlangus* these species cannot be distinguished from each other using 12s metabarcoding.

Variation in the oceanic samples are to be expected, as fjords are connected to the open ocean and allows for large populations to enter and exit. (Figure 3) shows that the 15 of march samples contained on average three times more eDNA than the average amount of eDNA for all samples. This is a period where two biological phenomena happen at the same time. The first is spring turnover causes increased vertical mixing, and in turn higher primary production (Aure *et al.*, 2007), and the second is the annual spawning of *Gadus morhua*, which spawns in fjords along these parts of Norway (Dunn *et al.*, 1992). This date was also the date when the 350m sample hit the seafloor, but this does not seem to have affected the samples much. The presence of *Chirolopis ascanii* might be a consequence of this, but as this sample contained high amounts of eDNA from MOTUs like *Clupea harengus*, a high number of animals present in the fjord at this time more likely is the reason this sample contains so much eDNA.

5.2 The biodiversity of 2019

The fact the 20m and 350m samples shared 22 of the 40 MOTUs can be explained by the high presence of species that are not restricted to either of the water depts. These are for an example *Gadus morhua, Clupea harengus* and *Pollachius virens* which can be both in the shallow water and in the deep water. Vertical mixing in the spring period also facilitates dispersion, so species found in high numbers are bound to appear in both samples.

A characteristic for species that only were detected in one of the depts is that *Chimaera monstrosa* (6), *Protomyctophum arcticum* (6) and *Argentina silus* (5) all only were found in the 350m[8] (Table 6). *Argentina silus* and *Protomyctophum arcticum* are both mesopelagic fish (Mecklenburg *et al.*, 2018a, 2018b), while *Chimaera monstrosa* is known to be found at depts greater than 200m (Moura *et al.*, 2005).

Sebastes sp., *Micromesistius poutassou* and *Maurolicus muelleri* were detected multiple times throughout the year, where they appeared one to three times in the 20m samples, and at least twice as many times in the 350m samples. *Maurolicus muelleri* have been found in large numbers around 50m dept, with fractions being at 20m and 100m depth (Giske *et al.*, 1990). *Micromesistius poutassou* is a epi-mesobenthopelagic fish (Mecklenburg *et al.*, 2018a, 2018b). All *Sebastes* sp. species found in this area are either epipelagic, mesopelagic or benthopelagic, with *Sebastes norvegicus* being Epi-mesobenthopelagic, and *Sebastes viviparus* being epibenthopelagic (Mecklenburg *et al.*, 2018a, 2018b).

It then possible that all these MOTUs were found more often in the 350m samples as a function of their habitat use, as they are species naturally are found bellow 20m. The MOTUs that also were found a few times in the 20m samples differ by belonging to species that are associated with the epipelagic layer.

Pholis sp. *Melanogrammus aeglefinus/Merlangius merlangus* and *Ammodytes* sp. were all found more times in the 20m samples than in the 350m samples (Table 6). A common element for all these MOTUs are that they all are associated with the littoral zone, so what

[8] The amount of times found noted behind the name.

might have happened are that animals that are present in the littoral zone shed eDNA which is transported to the sampling point.

The MOTUs that belonged to the species found the highest amount of times throughout the year are all species that have been reported and targeted by local fishermen (Fiskeridirektoratet, 2020). *Salmo salar* can be noted, as this species naturally would not be expected to occur throughout the year in such high numbers, but there are multiple aquaculture farms in the near proximity which likely are the source of this eDNA.

There were a bunch MOTUs that appeared less times (Table 6) but spread out across the year (Table 7). These MOTUs (such as *Chimaera monstrosa* and *Anarhichas* sp.) are likely present throughout the year, but at so low numbers that they are not always detected. MOTUs from species such as *Cyclopterus lumpus* and *Somniosus microcephalus* on the contrast have all their appearances from February to May (Table 7 & appendix 8.7), which indicates that these species only temporally visited Vågsfjorden in contrast to staying there in less numbers throughout the year.

The higher variability in observed species the 20m samples compared to the 350m samples could be a factor of higher variability in abiotic factors that the 20m samples are more exposed to. Temperature is one of the factors known to impact the degradation of eDNA (Table 1), and the temperature varied from 3 to 10 degrees Celsius down to 20m deep at the sampling location, where down at 350m deep the temperature variation were from 6 to 7 degrees[9]. The same counts for light intensity, which decrease with dept (Gallegos and Moore, 2000), but for the 20m samples vary throughout the year (Time and date AS, 2020).

From February to 1st of May the MOTU community found in the 20m samples and the MOTU community found in the 350m samples were most likely more similar as a function of spring turnover. The increased vertical fluctuation aided in transporting eDNA from not only the 20m and down, but also the opposite direction as this was the only period throughout the year *Anarhichas* sp. and *Sebastes* sp. were observed in the 20m samples.

[9] Metadata found in the appendix.

The number of demersal species from the 23rd of April likely displays an error in the dataset. Multiple species such as *Cyclopterus lumpus*, *Sebastes* sp., *Chimaera monstrosa* and *Somniosus microcephalus* all are present the sampling dates before and after, which indicates that these MOTUs are in fact not absent from the fjord, rather lacking for other reasons.

5.3 Quantitative analysis

Abundant MOTUs

MOTUs that contained high numbers of reads and high relative abundance such as *Pollachius virens, Salmo salar* and *Gadus morhua* are all species that are commercially sought after in the fjord (Fiskeridirektoratet, 2020), which could be interpreted as further evidence that as in (Salter *et al.*, 2019) biomass correlate with eDNA numbers.

MOTUs such as *Gadus morhua* and *Clupea harengus* rank high when it comes to numbers of reads (Table 8), but a lower ranking when it comes to relative abundance (Table 9), this indicates that there are periods of the year when *Gadus morhua* and *Clupea harengus* are very abundant, and then parts of the year when they are not present in large numbers in Vågsfjorden.

There are a bunch of MOTUs that have a comparatively lover amounts of read, but high values of relative abundance. Among these MOTUs are *Hippoglossus* sp., *Micromesistius poutassou, Sebastes* sp. and *Chimaera monstrosa.* Suggesting that there were lower amounts of biomass compared to for an example *Clupea harengus* but, there were a fjord population present in some numbers throughout the year.

For the 20m samples two thirds of all relative abundance belonged to *Pollachius virens* and *Salmo salar* (Table 10), where the main difference from the 350m samples is the high relative abundance *Salmo salar* occupy in these samples. Knowing that there are multiple salmon farms in the proximity, the eDNA likely are from farmed salmon that are concentrated in large amounts in the upper water layers. The lover values in the 350m samples are then likely as a function of dispersion and deration (see Table 1).

Light and temperature

Light and temperature might have influenced the amount of eDNA in the 20m samples, but if they did then there were then other variables such as biomass impacted the variation of eDNA to a larger degree. The temperature impact was almost significant, but at the same time as the three coldest days happened there were the spring turnover and *Gadus morhua* spawning, so the amount of eDNA present in the water were more likely a function of those events.

The fact that the variation of eDNA in the 350m samples were higher than in the 20m samples reaffirms that light and temperature were not important factors when it comes to the actual amount of eDNA in Vågsfjorden.

Spawning activities

Gadus morhua, *Micromesistius poutassou* and *Molva molva* all had a higher amount of eDNA within their spawning period than outside of it (Table 12). The effect was quite large, where *Gadus morhua* contained 10 times more eDNA during spawning period than outside of the spawning period. This is not the same magnitude as for the eels (Takeuchi *et al.*, 2019), but this is possibly a result of the fact that *Gaudus morhua,*spawns in a window 30 and 50 days (Brander, 1994), which was not the case in (Takeuchi *et al.*, 2019). One would thus not receive the same ratio of released eDNA just because the entire population does not spawn at the exact same time. The dispersion area is also way larger in comparison, where the oceanic currents might have spread the eDNA unevenly throughout a larger space.

It is hard to explain exactly what how much of the eDNA from *Gadus morhua* that derives from spawning activities compared to movement of biomass. Great volumes of *Gadus morhua* moved in this period of the year into the fjords to spawn, and even if they did not spawn the movement of the biomass itself would most likely be enough to increase the amount of eDNA present.

Relative abundance was used as a unit to compare biomass, and the tool worked as long as one assumed that there only was one variable that was fluctuating. However, if biomass from *Gadus* morhua and biomass from *Pollachius virens* varied, then the effect from less abundant species became harder to observe. This might be the case of *Micromesistius poutassou* and *Molva molva*, where their spawning activities were so inconsequencal on the community at

large that the effect of spawning behaviour was not witnessed with relative abundance. In addition, when investigating the MOTU *Hippoglossus* sp. there were a significant difference between the seasons, showing that the relative abundance of were higher outside of the spawning season. This was not the case, as comparing the number of reads shows (Table 12). The problem with addressing the spawning behaviour, or variation of eDNA throughout the year for that manner, by using amount of eDNA is the stochasticity of the method. In order to cope with that relative abundance was used, but the cost is the loss of data such as from the spawning behaviours of *Micromesistius poutassou* and *Molva molva*. If one is not careful, a wrong interpretation of data such as the relative abundance of *Hippoglossus* sp. when comparing the abundance within and outside of its spawning season might also happen.

Release of pelagic eggs did not impact the relative abundance in the 20m samples compared to the 350m samples to a degree large enough to be observed for any of the species in (Table 13). However without knowing the complete vertical distribution of the eggs for MOTUs such as *Gadus morhua* it may be hard to say to which degree this is true. Vertical mixing might have spread the eggs through the water column, as witnessed in some studies (Coombs, Pipe and Mitchell, 1981), thus making it impossible see to which degree gonad release impacted the release of eDNA. In (Table 14) only *Micromesistius poutassou* were more abundant, but this in the 350m samples, indicating that pelagic eggs had no impact on the distribution of eDNA.

6 Conclusion

The aquatic vertebrate society of Vågsfjorden was successfully mapped using eDNA methodology. The amount and dispersion of eDNA could be associated with some biotic and abiotic factors, but not all that were investigated.

Vågsfjorden contained 40 MOTUs throughout 2019, where a few MOTUs appeared throughout the year. These were MOTUs that are known to be either commercially sought after in this area or cultivated. Most MOTUs were found at both depts, where only a few species appeared to a large degree more often in one of the depts.

The relative abundance of the MOTUs were also calculated, and the most abundant MOTUs also belonged to species that were commercially sought after or actively cultivated in the area.

In addition, there were a statistically higher amount of reads, and higher relative abundance for *Gadus morhua* and *Micromesistius poutassou* during their respective spawning seasons. No effect pelagic eggs, light, or temperature was found

This work adds to the knowledge we have about Vågsfjorden, creating a reference dataset for the fjord containing rare species such as *Somniosus microcephalus,* and in addition creating reference numbers in terms of amount of reads found from a variety of species, some which are of commercial interest.

In order to improve the knowledge about the species richness of Vågsfjorden other studies with other tools ought to be conducted, or complimentary eDNA studies where a higher focus should be placed on spatial variation within the fjord. eDNA has proven to be usable when it comes to mapping a large set of abundant species but has limitations when it comes to less numerous species.

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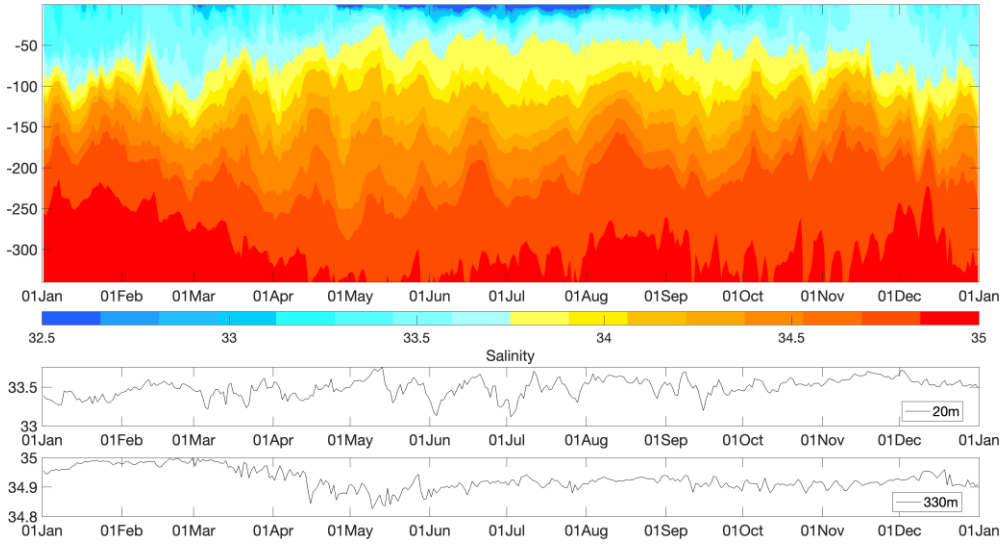
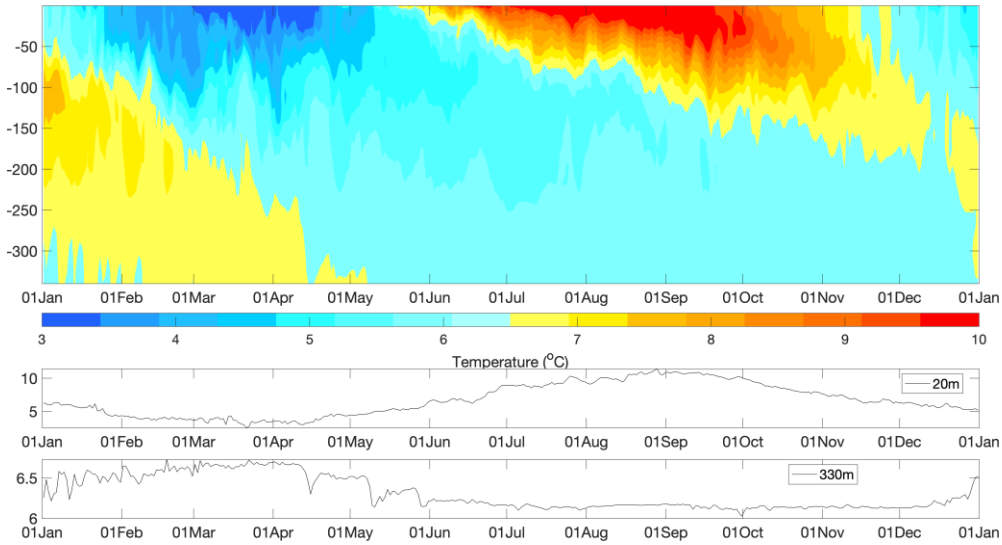
Appendix

Temperature and salinity data from Vågsfjorden 2019





Above are salinity and temperature graphs used for determining when the spring turnover period was for Vågsfjorden 2019. Coordinates 68.956368, 17.080297.

Sampling protocol

Protokoll for vannprøver, eDNA

Utstyr:

500m tau (4mm)i tilhørende lagringsboks/tønne, Vannhenter (Niskin water sampler 5l.), lodd (5kg), utløserlodd, sprøyter, filter, 3 plastposer (2.5l), l, plastposer (250ml), klor, vann.

Før du drar ut:

Utstyr: Vannhenter, plasthansker, plastpose (2.5l), balje(for lagring av utstyr på dekk) og klor.

1. Ta med vannhenter, 1 plastpose, balje og sprøyteflaske med klorvann til dusj/vask hvor det ikke har vært fisk.
2. Ta på plasthansker.
3. Rens vannhenter med ferskvann, for så å spraye og rengjøre den med klorvann.
4. Skyll på nytt med ferskvann, før så å lukke vannhenteren i dusjen/vasken slik at den ikke er åpen før vi senker den i havet senere. Husk å lukke utløpsventilen ved å dra ventilen ut og vri slik at metallpinnen holder ventilen ute.
5. Fyll opp plastposen med vann fra dusj/vask, dette er kontrollprøven som skal fortelle oss om det er forurensning i vannet/ i rommet som vi bruker til å vaske eller omgivelsene på land. Ikke ta denne med ut i båten.
6. Legg alt opp i baljen, slik at det ikke kommer i kontakt med fiskeDNA som er på båten.

På sjøen:

Utstyr: 500m tau (4mm), balje, plasthansker, vannshenter og 2 plastposer (2.5l), lodd (5kg) og utløserlodd.

1. Kjør båt til prøvetakningsområdet, Odden (ca 360 bunndyp).
2. Fest lodd til ende av tau.
3. Gjør klar vannhenter i balje, (Åpne den og fest trådene i utløsermekanisme).
4. Fest vannhenter 20m over lodd.
5. Fest ende av tau til rekka slik at vi ikke mister alt utstyr hvis man en uheldig og mister tauet.
6. Senk loddet 40m ned, slik at vannsampler er på 20m dyp. Bruk linehaler for kontrollert nedstigning.
7. Fest utløserlodd på tau, (selve loddet skal sitte på tauet i tillegg til karabinkroken som er ekstrasikring).
8. Slipp loddet slik at det synker og kolliderer med utløsningsmekanismen på vannsamleren.
9. Heis opp vannhenter med linehaler.

10. Vannhenter burde nå være lukket, hvis ikke: Fjern utløserlodd og gjenta steg 6-9. (ved problemer kan man prøve å «jokke» litt på tauet når utløserloddet er nede).
11. Plasser vannhenter i balja.
12. Ta på plasthansker.
13. Åpne plastpose(2.5l).
14. Åpne luftuttak på vannhenter.
15. Åpne utløpsventilen ved å vri på den til hullet ligger over metallpinne og så presse ventilen inn.
16. Når 2l vann er samplet tøm eventuell restvann på sjøen og gjenta steg 6 til 15 på omtrent 350m.

Sluttresultat: to poser med vann fra 20m og 350m.

Filtrering:

Utstyr: 2 Plastposer (2.5l) med vann, sprøyter, filtre, 50ml lagringsrør med blått lokk plastposer for lagring, plasthansker.

Denne prosessen skal gjøres identisk for alle prøvene. (kontroll, 20m og 350m) Det er viktig at sprøyten byttes ut når du er ferdig med en dybde.

1. Ta på plasthansker.
2. Merk rør med blått lokk med dato, løpenummer og dyp/kontroll. (totalt 3 rør)
3. Ta ut sprøyte, og klargjør filter.
4. Åpne plastposen (2.5l), fyll sprøyta med 50ml vann og lukk.
5. Fest filter på sprøyte.
6. Skyv vann gjennom sprøyte.
7. Gjenta steg 4-6 helt til 500ml har blitt filtrert.
8. Fyll sprøyta med luft til slutt, og sprøyt dette gjennom filteret 2 ganger. (Hvis det er mer vann i filtret gjør dette en eller 2 ganger til. Det er viktig at filteret er så tørt som mulig.
9. Ta filter av, og plasser dette i lagringsrør med blått lokk (50 mL), så i lagringsplastpose (250ml).
10. Gjenta til 1.5l har blitt filtrert fra samme dybde.
11. Hiv sprøyta, hansker, og papir/emballasje.
12. Når alle filtrene har blitt sikret og merket. Lagre dem i fryser.

Resultat: 3 filter fra 20m, 3 filter fra ca.350m og 1 filter fra kontroll.

Det viktige er at vi har dato og klokkeslett på når prøvene ble tatt, i tillegg til dybden på prøvene. Uhell/slurv ved markering av dato, tid og dybde vil føre til store problemer ved tolkning av resultater senere.

Vasking:

Skyll vannhenter med ferskvann → Klorvann → ferskvann. Hvis vi bruker samme litersbokser fra gang til gang så skyll disse med ferskvann. Bruk samme vannkilde som var brukt for kontrollsampler.

La vannhenteren være åpen til den er tørr, lukk den så for å unngå støvsamling. Det samme gjelder litersboksene. Lagre alt utstyr på anvist plass.

Niskin water sampler 5l.

Extraction protocoll

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

DAY 1:

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

DAY 2

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

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

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

Protocol for COI Metabarcoding

Protocol for COI metabarcoding using Leray-XT primers and Metafast library preparation (PCR-free ligation procedure) Owen S. Wangensteen. January 2018.

METABARCODING PRIMERS

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

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

DNA AMPLIFICATION

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

Primer F1: NNaacaagccGGWACWRGWTGRACWITITAYCCYCC
Primer R1: NNNNaacaagccTAIACYTCIGGRTGICCRAARAAYCA

Primer F2: NNNggaatgagGGWACWRGWTGRACWITITAYCCYCC
Primer R2: NNNggaatgagTAIACYTCIGGRTGICCRAARAAYCA

Primer F3: NNNNaattgccgGGWACWRGWTGRACWITITAYCCYCC
Primer R3: NNaattgccgTAIACYTCIGGRTGICCRAARAAYCA

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

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

AmpliTaq Gold Master Mix	10.00	µl

BSA 20 µg/µl	0.16	µl
H2O	5.84	µl
Forward primer 5 µM	1	µl
Reverse primer 5 µM	1	µl
DNA Template	2	µl

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

The PCR programme is:

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

LIBRARY POOLING AND CONCENTRATION

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

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

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

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

LIBRARY PREPARATION

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

We use 3 µg of DNA (up to 40 µl of the previous pool) as starting material. The instructions for preparing a

COI library are exactly the ones described in the kit manual:

http://www.biooscientific.com/Portals/0/Manuals/NGS/5142-01-NEXTflex-PCR-Free-DNA-Seq-Kit.pdf

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

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

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

LIBRARY CHECKING

We usually analyse the final library using either an Agilent TapeStation or Bioanalyzer, in order to check that the ligation has gone well. If you don't have any of these analyzers

available, then you could use just a gel electrophoresis to check the right migration of the fragment. Note that the library fragments are the result of a special Y-shaped adapter ligation and they will not be linear DNA. So they will migrate anomalously in all this analytical methods. The library peak will not appear at the expected size of ~ 510 bp, but it will produce a broad peak of ~ 800 bp. This strange migration behaviour is normal and won't interfere with the MiSeq sequencing.

LIBRARY QUANTIFICATION

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

For this purpose, we use the NEBNext Library Quant Kit from New England Biolabs: https://www.neb.com/products/e7630-nebnext-library-quant-kit-for-illumina We usually analyse library dilutions of 1:5000, 1:10,000 and/or 1:50,000.

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

LIBRARY DILUTION AND MiSeq LOADING

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

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

The protocol for the final sample denaturation before loading is as follows:

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

References:

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

R-script

```
setwd("C:/Users/Simeon/OneDrive - UiT Office 365/Mastergrad/Under arbeid/Mal for fremgang i R/work")

Re <- read.table("SIMX.All_MOTUs3_Curated.csv", sep=",", head=T, stringsAsFactors = F)

j1 <- Re[Re$scientific_name != "Lumpenus lampretaeformis",]

j2 <- j1[j1$scientific_name != "Brosme brosme",]

j3 <- j2[j2$scientific_name != "Leptoclinus maculatus",]
```

```
j4 <- j3[j3$scientific_name != "Sus scrofa",]

j5 <- j4[j4$scientific_name != "Homo sapiens",]

j6 <- j5[j5$scientific_name != "Atherina hepsetus",]

j7 <- j6[j6$scientific_name != "Icelus spatula",]

j8 <- j7[j7$scientific_name != "Triglops murrayi",]

j9 <- j8[j8$scientific_name != "Capra",]

j10 <- j9[j9$scientific_name != "Alces alces",]

j11 <- j10[j10$scientific_name != "Canis lupus familiaris",]

j12 <- j11[j11$scientific_name != "Felis catus",]

j13 <- j12[j12$scientific_name != "Gallus gallus",]

j14 <- j13[j13$scientific_name != "Corvus",]

j15 <- j14[j14$scientific_name != "Capreolus capreolus",]

j16 <- j15[j15$scientific_name != "Careproctus reinhardti",]

j17 <- j16[j16$scientific_name != "Bos",]

j18 <- j17[j17$scientific_name != "Ovis",]


data <- subset(j18,select=-c(SIMM24a,SIMM77c))


names(data)


sample_cols <- 17:249
```

```
renormalize <- function(db){
  total_reads <- colSums(db)
  for (i in 1:ncol(db)) db[,i] <- db[,i]/total_reads[i]
  return(db)
}

data[,sample_cols] <- renormalize(data[,sample_cols])


replicates <- read.table("Replicates_cleanlab.csv",sep=";",head=T,stringsAsFactors = F)


collapsed_data <- data.frame(matrix(ncol = length(replicates$ï..Sample), nrow=nrow(data)))
colnames(collapsed_data) <- replicates$ï..Sample


for (i in 1:nrow(replicates)){
  replicate_names <- unlist(strsplit(replicates$replicates[i],",")) # split the list of replicates for sample i
```

```
  means <- rowMeans(data[,colnames(data) %in% replicate_names]) # Calculate row means of the columns belonging to the same sample

  collapsed_data[colnames(collapsed_data)==replicates$ï..Sample[i]] <- means # Save the resulting means into the right column of collapsed_data table

}




final_column_taxo <- sample_cols[1]-1

first_column_seq <- sample_cols[length(sample_cols)]+1

collapsed_table <- data.frame(data[,1:final_column_taxo],collapsed_data,data[,first_column_seq:ncol(data)])




write.table(collapsed_table,"Clean Lab.csv",row.names=F,sep=",",quote=F)
```

Table displaying MOTUs found in the blank samples.

Species found in the extraction blank, the shower blank and species found in both. The number after the species represents the proportion of eDNA found compared to the total amount of eDNA found from that species

Extraction blank	Shower blank	Present in both
Lumpenus lampretaeformis (32%)	Atherina hepsetus (100%)	Clupea harengus (4%)

Leptoclinus maculatus *(85%)*	*Gadus morhua* *(5%)*	*Pollachius virens* *(4%)*
	Brosme brosme *(12%)*	*Careproctus reinhardti* *(100%)*
	Molva molva *(3%)*	*Sus scrofa* *(7%)*
	Icelus spatula *(30%)*	*Homo sapiens* *(21%)*
	Triglops murrayi *(73%)*	
	Hippoglossus *(0.09%)*	
	Salmo salar *(0.5%)*	
	Scomber scombrus *(0.9%)*	
	Bos *(2%)*	
	Capra *(100%)*	

	Alces alces *(0.9%)*	
	Canis lupus familiaris *(1%)*	

Table displaying amount of demersal species in Vågsfjorden throughout 2019

Table 15 Demersial species precent in the 20m sample throughout the year.

	19.1.	7.2.	15.2.	15.3.	4.4.	23.4.	14.5.	9.7.	25.7.	17.9.	29.11.
Gadus morhua	1	1	1	1	1	1	1	1	0	1	1
Micromesistius poutassou	0	0	0	1	1	0	0	0	0	0	0
Melanogrammus aeglefinus | Merlangius merlangus	1	1	1	1	1	1	0	0	0	1	1
Molva molva	1	1	1	1	1	1	0	0	0	1	1
Gadiculus argenteus	0	0	0	0	1	0	0	0	0	0	0
Anarhichas	0	0	0	1	1	1	0	0	0	0	0
Cyclopterus lumpus	0	0	0	1	0	1	0	0	0	0	0
Sebastes	0	0	1	0	1	1	0	0	0	1	0
Ammodytes	1	0	1	1	1	0	0	0	1	1	0
Somniosus microcephalus	1	0	0	1	1	0	0	0	0	0	0