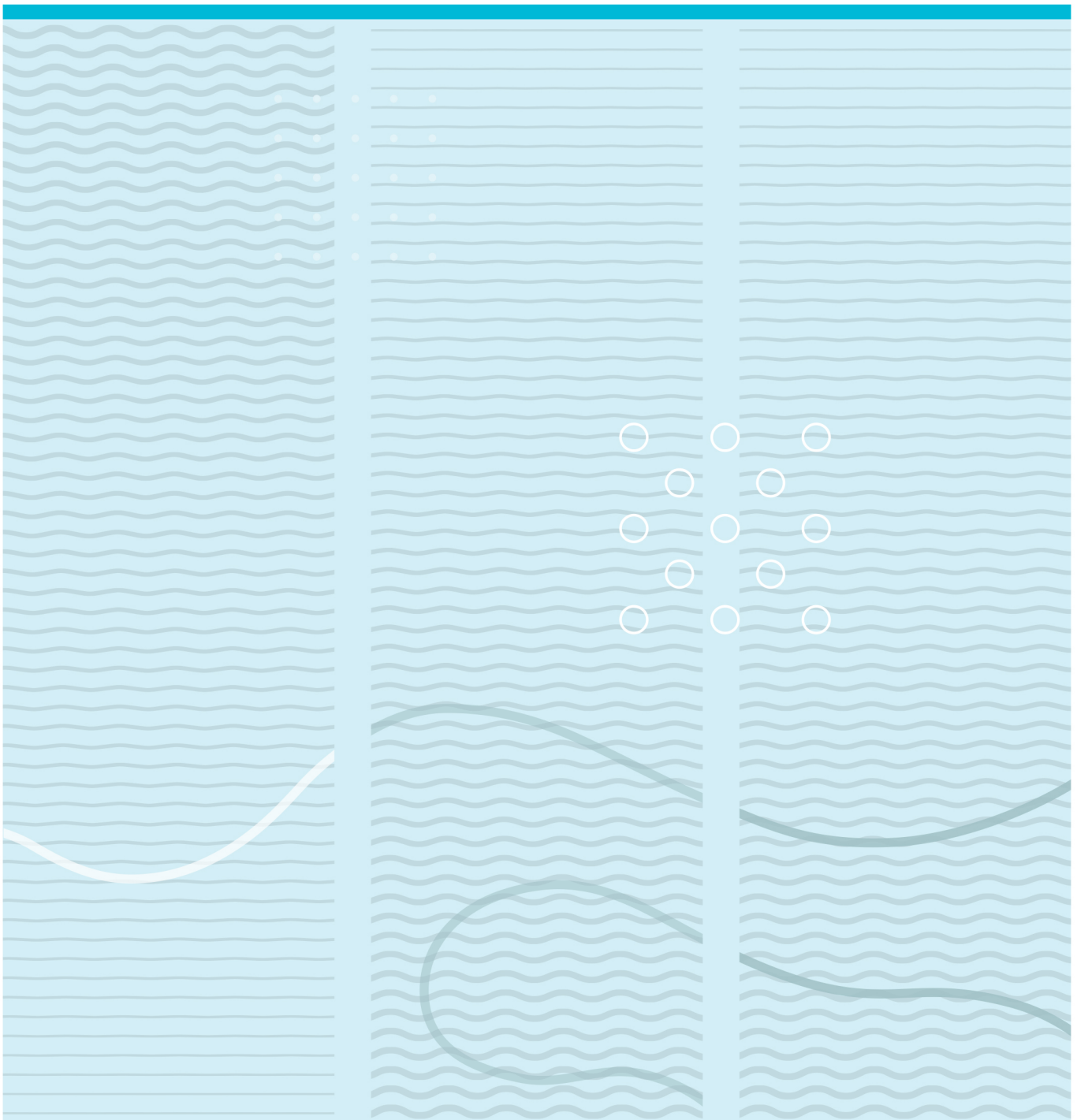# University of South-Eastern Norway

Tora Camilla Eng Aune

Method testing: Collecting and analyzing eDNA from a large lake

With bacteria and the endoparasite *Tetracapsuloides bryosalmonae* as example organisms

This thesis is worth 60 study points

Abstract

As techniques and protocols for eDNA studies develop, ideas for research are pouring in. The need for standardized methods and protocols for different environments is increasing and it´s necessary to ensure reliable data that can be tested.

This study aims to test methods to collect eDNA in a freshwater lake to detect the endoparasite *Tetracapsuloides bryosalmonae* and bacteria, to collect data that can contribute towards the standardization of eDNA sampling. eDNA were collected at five stations located around lake Norsjø in Southeast Norway, with four different sample strategies referred to as sample types, volume, mixed samples (1 and 2L), direct samples (1 and 2L), and sample station. Detection of the parasite was done with real-time qPCR and bacteria with 16S Barcoding Kit 1-24 (SQK- 16S024) from Oxford Nanopore. The parasite was only found in five samples, due to the small amount of data, the parasite was not used for any further analysis. Bacteria, on the other hand, was detected in all samples that were used for metabarcoding (except for blanks). Further statistical analysis showed that the sample stations were the most important factor in the collection of eDNA from Lake Norsjø and explained 35,6 % of the differences in beta diversity of the sequenced samples.

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Preface

This study has taken me on an incredible journey in science. I´ve grown to become a more critical, disciplined, and humble person. The subjects I found most demanding at the beginning, like chemistry and genetics, turned out to become my favorites. I´ve achieved an increased understanding of how much I don´t know and I´m grateful that my "backpack" has been filled with inspiring knowledge about environmental sciences. I wish to thank my supervisors, Assistant Professor Tone Jøran Oredalen, Associate Professor Mona Sæbø, and Associate professor Jørn Henrik Sønstebø for the exemplary guidance, professional replenishment, and help along the way. I´m very grateful for the weekly metabarcoding meetings, where I´ve learned a lot and have developed a deeper understanding and confidence in this field. I would also like to thank my cohabitant, Kristoffer Oppedal, for the general support and his assistance in the field.

Asker, 18.05.2022

Tora Camilla Eng Aune

1. Introduction

Environmental DNA (eDNA) has a great potential that can help provide a better understanding of aquatic species conservation and their ecology (Goldberg et al., 2016; Harper et al., 2019). There has been an increased use of eDNA to detect rare species (relative abundance and presence), especially for invasive or endangered aquatic species (Biggs et al., 2015; Carim et al, 2016; Dejean et al., 2012; Klymus et al., 2020; Lawson Handley et al., 2019; Rusch et al., 2018; Wilcox et al., 2015; Wilcox et al., 2016). eDNA analysis can be non-invasive, as DNA from the targeted species is detected without visual identification or capturing and reduces impact on species that are sensitive and rare (Barnes & Turner, 2016; Taberlet et al., 2012; Eichmiller et al., 2016; Goldberg et al., 2016; Harper et al., 2019). In comparison with traditional methods for surveys of aquatic species such as electrofishing, seine fishing, and visual identification, eDNA surveys often prove to be more sensitive (Kumar et al., 2020; Boussarie et al., 2018; Dejean et al., 2012; Gillet et al., 2018; Hinlo et al., 2018; Jerde et al., 2011; Nevers et al., 2018).

eDNA was described for the first time in 1987 (Taberlet et al, 2018; Ogram et al., 1987), and since then, it has become an essential part of environmental and ecological management, and several experimental protocols have been adjusted for different environments (Taberlet et al., 2018). Environmental DNA, often referred to as eDNA, is defined as the genomic DNA originating from different species present in environmental samples (Taberlet et al., 2018; Taberlet et al., 2012). Environmental samples can be collected from various substances such as water, sediments, soil, and feces or are considered the filtered material from air, water, sifting sediments, or bulk samples (Barnes & Turner, 2016; Taberlet et al., 2018). eDNA comes from different genetic materials such as skin cells, hair, feces, urine, seeds, spores (Bohmann et al., 2014; Taberlet et al. 2012). eDNA contains extra- and intracellular DNA (Pietramellara, 2009), whereas intracellular DNA comes from living organisms or cells present in environmental samples and extracellular from dead cells (Taberlet et al., 2018).

There are multiple methods to examine eDNA samples, and several terms have developed through the years (Taberlet et al., 2018). For single species detection, quantitative PCR (qPCR) with specific primers is most common (Taberlet et al., 2018;

Ficetola et al., 2008; Goldberg et al., 2011; Jerde et al., 2011; Thomsen et al., 2012). Analyses of eDNA most often depend on amplification of degraded DNA and/or low quantities (Ficetola et al, 2015). Quantitative real-time PCR (qPCR) is increasingly used to measure low values of eDNA in environmental samples like water, air, or soil (Klymus et al., 2020). The method can help researchers to detect low concentrations of target species with high levels of confidence (Klymus et al., 2020).

The description of low target concentrations of DNA is often poorly described in assay performance; this can be due to confusion around the limit of detection (LOD) and limit of quantification (LOQ) and how these parameters should be defined when using real-time qPCR (Klymus et al., 2020). "*LOD can be defined as the lowest concentration of target analyte that can be detected with a defined level of confidence, with a 95% detection rate as the standard confidence level*" (Klymus et al., 2020; Burd, 2010; Burns & Valdivia, 2008; Bustin et al., 2009; Forootan et al., 2017). To ensure this, one must run many replicated standard curves, standards with low concentrations where 95% of replicates produce positive amplification (Klymus et al., 2020).

DNA metabarcoding is another approach which can detect all species in a clade with PCR analysis (Taberlet et al., 2018), eighter based on shotgun sequencing (Taberlet et al., 2018; Deininger, 1983) or targeted PCR (Taberlet et al., 2018; Saiki et al., 1988; White et al., 1989). The term DNA metabarcoding was used for the first time in 2011 (Taberlet et al., 2018; Pompanon et al., 2011; Riaz et al., 2011) and is the identification of several taxa simultaneously with the use of metabarcode sequences of amplified eDNA (Taberlet et al., 2018). Metabarcoding uses designed genetic primers to detect different taxa simultaneously (Pedersen et al., 2015). A metabarcode is a short, taxonomically informative region of DNA, flanked next to two conserved sequences (forward- and reverse primers), as shown in fig.1.-1 below, used as primer anchors in polymerase chain reactions (Taberlet et al., 2018).
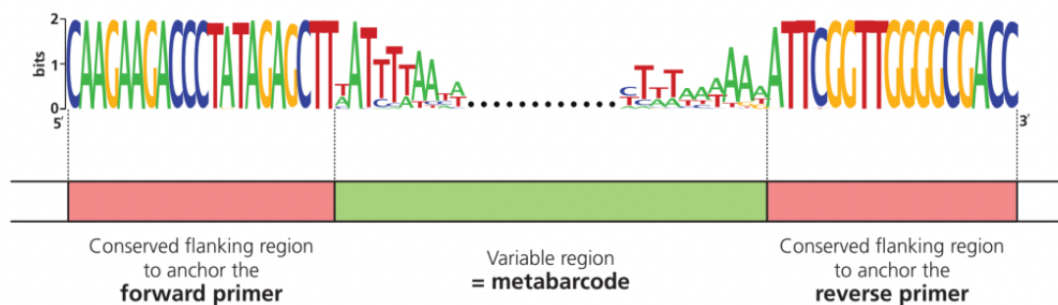
Figure 1.-1 Illustration of variable metabarcode flanked with Lumb01 primers, where the primer pair suborder earthworms (Lumbricina) (Taberlet et al., 2018; Bienert et al., 2012).

Choice of metabarcode and primers are crucial for further analysis and important decisions to consider, ensuring coverage of the taxonomic target group (Alberdi et al., 2018; Clarke et al., 2017; Taberlet et al., 2018). The COI gene (c oxidase I) is a standardized barcode sequence used for the detection within the animal kingdom (Alberdi et al., 2018; Ratnasingham & Hebert, 2017), but seems to give a poor performance as it favors several targeted taxa above others (Taberlet et al., 2018; Clarke et al., 2014; Deagle et al., 2014). 16S is a gene region on ribosomal DNA (rRNA) and is used as a metabarcode to detect archaea and bacteria (Gibson et al., 2014; Tringe & Hugenholtz, 2008), while the ribosomal 18S gene region is used for detection of microbial eukaryotes (Gibson et al., 2014; Creer et al., 2010).

Group-specific primers are used for DNA amplification prior to DNA sequencing the amplicons with next-generation sequencers, sequences are then analyzed, and multiple taxa identified (Taberlet et al., 2018). Metabarcoding approaches are split into three different procedure alternatives concerning add of nucleotide tags and the library index, with one-, two- and three-step PCR (Bohmann et al., 2021; Taberlet et al., 2018). In one simple reaction, DNA extract can be amplified and then build into a sequencing library, this constitutes a one-step PCR (Bohmann et al., 2021). For the two-step approach, the DNA extract is amplified using two sets of primers. The third approach includes metabarcoding primers carrying 5´-nucleotide tags, used to amplify the DNA extract (Bohmann et al., 2021).

eDNA metabarcoding studies are increasingly done to study both past biodiversity and present (Ficetola et al., 2015; Pedersen et al., 2015; Sønstebø et al., 2010; Thomsen & Willerslev, 2015). As the use of eDNA methods is increasing, the need for standardized protocols for specific habitat assessment grows (Harper et al., 2019). In

addition, quality assurance of the methods of choice is necessary to ensure credible results (Goldberg et al., 2016; Kumar et al, 2020). Metabarcoding of eDNA samples offers both limitations and challenges towards reliable results and requires accurately assessed laboratory and analytical workflows (Alberdi et al., 2018; Burian et al., 2021; Goldberg et al., 2016; Pompanon et al., 2012).

Independent groups of researchers have designed a variety of techniques for eDNA analysis and developed eDNA detection protocols for several taxa of aquatic macroorganisms and their environments (Goldberg et al, 2016). There are available metabarcoding protocols for freshwater ecosystems to detect fish, amphibians (Taberlet et al, 2018; Valentini et al., 2016), and diatoms (Taberlet et al., 2018; Apothéloz-Perret-Gentil et al.,2017; Visco et al., 2015; Zimmermann et al., 2015).

But challenges remain. Dickie et al. (2018) found that 95% out of 75 metabarcoding studies had eighter used unsuitable field methods or/and didn´t provide methodological information critically. Scientists could replicate only 5% of the studies, a substandard level than the general ecological studies provided (Dickie et al., 2018). Metabarcoding demands several expertise, and it´s necessary to combine all steps from sampling, theoretical knowledge, taxonomy, microbiology, bioinformatics, and statistics to minimize biases throughout the experimental process (Zinger et al., 2019).

Though several protocols for eDNA approaches have been produced, there are no standardized methods to collect samples (Kumar et al., 2020; Goodwin et al., 2017; Lear et al., 2018; Rees et al., 2014). There are several ways to collect environmental samples from water e.g., direct into the filter funnel, a sample bag, or a bottle, assured that the equipment is sterile (Laramie et al., 2015).

Usually, the content within the environmental sample originates from the local environment, but in water, extracellular DNA can originate from passive transport (Taberlet et al., 2018). eDNA could be detected several kilometers downstream of the source, depending on turbulence and water flow (Taberlet et al., 2018; Deiner & Altermatt, 2014; Jane et al., 2015). The conditions often differ within different depths and from lakesides to the middle of the water (Taberlet et al., 2018).

Several studies have tried to trace where the DNA found in environmental samples in freshwater originates from. Dejean et al., (2011) looked at two targeted species, Bullfrog tadpoles (amphibian) and Siberian sturgeon (fish) in ponds and tanks to

see how DNA detection decreased over time after removing the DNA sources. In both settings (pond and tank) the eDNA was detected for almost a month as detectability decreased (Dejean et al., 2011).

Researchers have tried to assess the impact on different filtered volumes (Bedwell & Goldberg, 2020; Muha et al., 2019). A study by Bedwell & Goldberg (2020) tested different sampling designs in lotic and lentic water systems to determine the impact of sample volume, seasonal sampling, and spatial sampling by identifying two rare yellow-legged frogs. The study showed sample volume (1L and 2L) impacted streams, but not in lakes, where filter clogging limited the results (Bedwell & Goldberg, 2020).

Minamoto et al. published a study in 2015 on different techniques for the collection of eDNA to optimize previous studies, focusing on the selection of filters, eDNA preservation, and extraction. The water characteristics should be the basis for filter selection and chosen in advance of the study. eDNA analysis could apply to various studies with adaptive combinations with different techniques (Minamoto et al., 2015).

Several techniques have been developed for filtration, including in-situ filtration with peristaltic pump (Sieber et al., 2020), peristaltic pump-head eighter driven by a motor or a cordless rechargeable driver, or a manual vacuum pump driven by hand (Laramie et al., 2015). ESP (Environmental Sample Processor) is a robotic bio-surveillance to detect eDNA, developed by the Monterey Bay Aquarium Institute (Sepulveda et al., 2020). With automatically sampling, filtration, and material preservation, it's developed to overcome the human challenges of biological monitoring, such as time consumption, difficult weather, water flow, and safety (Sepulveda et al., 2020). Regarding the choice of filter, glass fiber is popularly used in eDNA assessments (Muha et al., 2019; Wilcox et al., 2013; Jerde et al., 2011; Janosik & Johnston, 2015) and cellulose nitrate (Goldberg et al., 2013; Goldberg et al., 2011; Muha et al., 2019; Pilliod et al., 2013).

After collecting environmental samples, DNA should be extracted as soon as possible to avoid changes in microbial communities or DNA degradation (Taberlet et al., 2018). If it's not possible to extract in the field, freezing could be an alternative, but cells might break, and if the target is extracellular DNA, this could cause a problem

when extracting (Taberlet et al., 2018). Buffering the sample is a promising alternative as it stabilizes DNA and prevents further degradation (Taberlet et al., 2018).

Extraction of eDNA from freshwater can be done using several different kits, e.g. DNeasy´s Blood and Tissue Kit (Qiagen), DNA isolation kit PowerWater® and UltraClean® Soil isolation kit (Goldberg et al., 2016). DNeasy Blood and Tissue kit from Qiagen is the protocol most used for eDNA extraction from aquatic environments (Kumar et al., 2020).

MinION from Oxford Nanopore is a relatively new sequencing technology (third generation) with several advantages including sequencing speed, probability, easy to use, simplicity, and ability to read long sequences (Nygaard et al., 2020). Illumina MiSeq on the other hand has been used widely used to sequence short reads of 16S rRNA amplicons and provides high quality (Nygaard et al., 2020) The disadvantage with MinION compared with Illumina MiSeq is a comparatively low read precision (Nygaard et al., 2020). Nygaard et al. (2020) compared the two DNA sequencers and didn´t find a significant difference from family level and above using the rRNA gene 16S. At genus level and especially species level minion (Oxford Nanopore) showed a higher taxonomic resolution (Nygaard et al., 2020). Illumina Miseq is currently the most favored platform (Porter & Hajibabaei, 2018).

After the samples have been sequenced, the data are run true a taxonomically database to unread them and identify the different taxa (Yilmaz et al., 2014). SILVA and Greengenes (GG) are examples of these kinds of reference databases that classify rRNA (Nygaard et al., 2020, Yilmaz et al., 2014). Kraken 2 is a high memory-efficient classification tool for metagenomics with, a new and better alternative that replaces Kraken 1 (Wood et al., 2019).

Sampling contaminations in the lab or errors during sequencing or PCR can contribute to false positives (Ficetola, et al., 2016; Ficetola, et al., 2015). This can help draw false conclusions which could cause waste of resources (Ficetola, et al., 2016). It´s necessary to implement various quality measures in the workflow to minimize the risk of false positives (Ficetola et al., 2016).

eDNA relying on amplification prior to sequencing can detect thousands of species but does not necessarily reflect the correct species composition and occurrence of biomass abundance (Kelly et al., 2019; Lamb et al., 2019). It is well known that PCR

amplification could result in bias which now is fully exposed by high-throughput sequencing techniques of DNA (Zinger et al., 2019).

Tetracapsuloides bryosalmonae, a myxozoan endoparasite, causes proliferate kidney disease (PKD) in salmonids they host (Oredalen et al., 2022; Ferguson & Ball, 1979; Sieber et al., 2020; Seidlova et al., 2021,) and is a big threat to the declining wild population as well as farmed (Ferguson & Needham, 1978; Forseth et al., NINA, 2007; Oredalen et al, 2022; Wahli et al., 2007). It´s shown that quantitative PCR (qPCR) is a useful technique for targeting the parasite (Sieber et al., 2020). The collection of DNA varies from extracted kidney tissue samples of salmonids (Forseth et al., NINA, 2007; Oredalen et al, 2022; Seidlova et al., 2021) to eDNA samples from different water types which are more diluted and with low target concentrations of the parasite's DNA but non-invasive (Sieber et al, 2020). Invasive species is a major concern for biodiversity conservation (Fossøy et al., 2019; Sieber et al., 2022). As biodiversity declines globally, it´s necessary to streamline the distribution and density monitoring of species that are in danger of becoming extinct (Burian et al., 2021).

Sequencing-based detection of bacteria with rRNA gene 16S has been used for decades, while whole genome sequencing with high throughput is more recent (Johnson et al, 2019; Rosselli et al., 2016). Since the 1970´s rRNA sequencing with 16S has been an important factor in the identification and classification of bacteria (Rosselli et al., 2016). Aquatic research has had a main focus on detecting different human parasites including pathogens such as *Listeria monocytogenes*, *Salmonella* (Arvanitidou, 1997; Lyautey et al., 2007; Papić et al., 2019; Sieber et al., 20) and *Legionella* (Moreno et al., 2019).

1.1. Aim of study

The goal of this thesis is to investigate how different strategies of collecting environmental samples in a large freshwater lake affect detection of eDNA. First, I study how the different sampling strategies influence the probability to detect the endoparasite *Tetracapsuloides bryosalmonae* using quantitative real-time PCR. Secondly, I test how sampling affect the community of bacteria identified using 16S metabarcoding with Oxford nanopore technologies.

Hypothesis 1

The 2L mixed samples will give a higher detection rate than the 1L mixed samples.

Hypothesis 2

The 2L direct samples will give a higher detection rate than the 1L direct samples.

Hypothesis 2

The direct samples (1 and 2L) will give a higher detection rate than mixed samples (1 and 2L).

Hypothesis 4

The station factor will be the most important sampling strategy to influence the result.

2. Material and Methods

2.1. eDNA Sampling

All samples used in this study were collected on September 15th,2021, at Lake
Norsjø in Telemark, Norway. eDNA samples were taken with 1- 2 L bottles,
under the surface at knee height, 0,5 - 25 m from land covering 30-50 m of
shoreline at five different stations around the lake, illustrated in fig. 2.1- under.
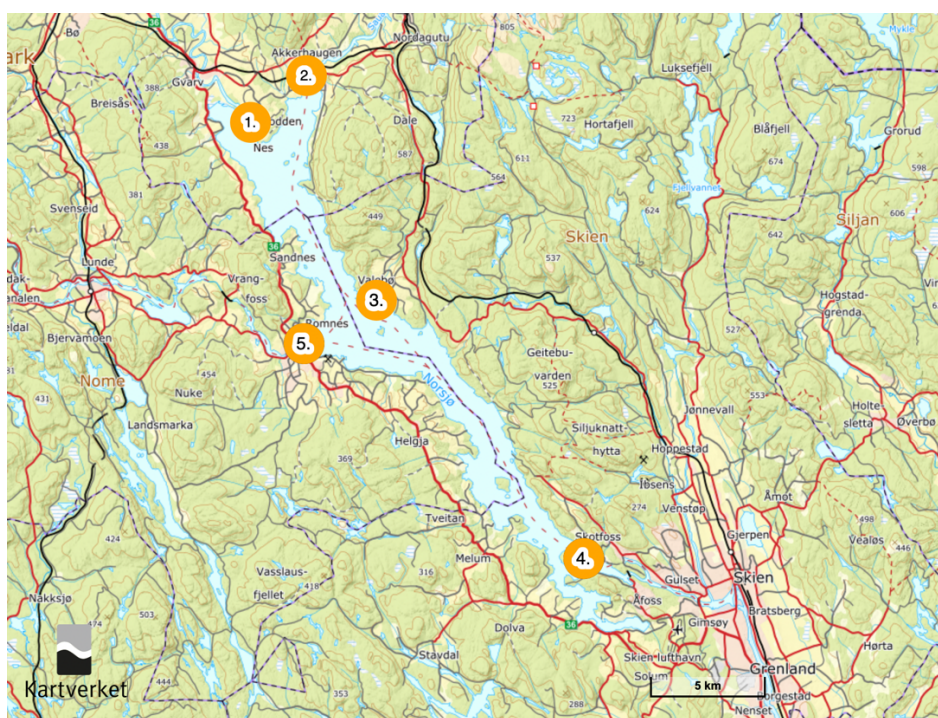
*Figure 2.1-1 Map of L. Norsjø in Vestfold og Telemark County, illustrated with sample stations 1.-
5 (norgeskart.no).*

Each sample station was picked strategically in relation to L. Norsjø´s inlets and outlets
(Fig. 2.1.-1); see appendix 1 for a closer look at each sample station and Tab. 2.1.-1 for
morphometrical parameters.

2.1-1 The morphometric parameters of L. Norsjø (Vann-nett.no)

Area (km^2)	Volume (m^3)	Altitude (m)	Maximum depth (m)	Average depth (m)	Upstream area (km^2)
55,1	5100	15	171	87	10382,2

The first station (Aslaksborg) is located at the edge of the outlet of the river Gvarvelva at the end of a bay (Norgeskart.no). The second (Akkerhaugen) is located at a top of the neighboring bay where the river Sauar flows out from Lake Heddalsvatnet. The third station, Valebø brygge, is located at a dock close to the river Brennelva´s outlet. Station four (Bjørkøya) was very shallow which made it possible to walk quite a bit into the water body to sample further out. It is placed where the lake begins to flow out to the river Farelva. Station five (Ulefoss Bridge) is located close to Ulefoss lock where the Telemark canal goes (Norgeskart.no).

eDNA samples were collected in four different ways, referred to as sample types in this study, with three biological replicates of each sample type at every station. Direct samples with 2- and 1-liter bottles and mixed samples put together in 2- and 1-liter bottles. The mixed samples were put together by five 2L eDNA samples taken at each sample station (Fig. 2.1.-2 under). 3x2L mixed samples with 400 ml, and 3x1L mixed samples with 200 ml from each of the five samples were put together with a measuring cylinder and a funnel. See appendix
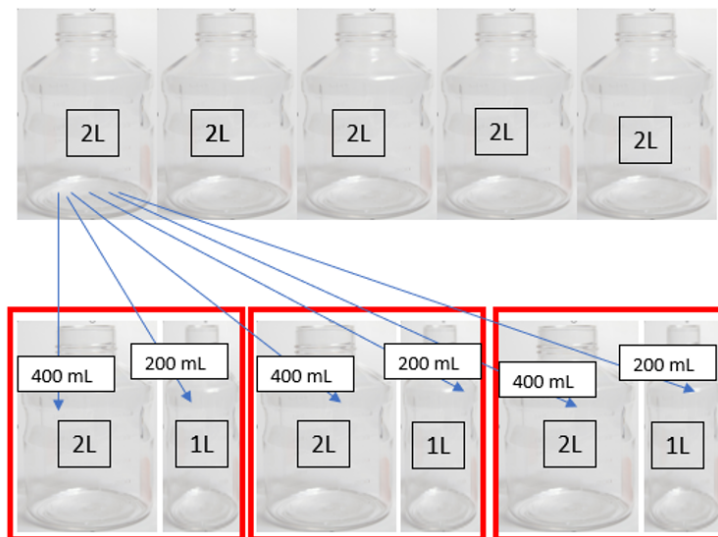
Figure 2.1.-2 Illustrating how six mixed samples (three 2L samples and three 1L samples) were put together by five 2L samples taken at different places to cover the sample site.

Bottles used to collect mixed samples, measuring cylinder, and funnel was cleaned with 50% chlorine mix and rinsed three times with distilled water prior

to use and between each station. Twelve samples were taken at each sample station giving a total of 60 samples (Fig. 2.1.-3).
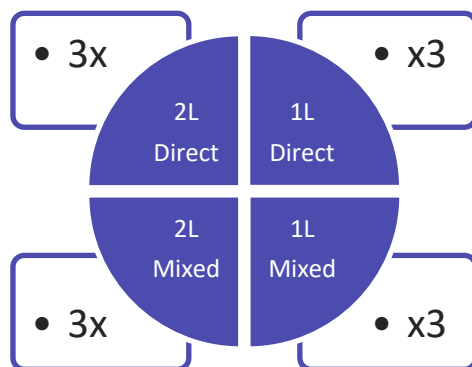
Figure 2.1.-3 Sample size brought from each station (1.-5.) at L. Norsjø.

After collection, all samples were transferred directly to cooling boxes and kept there with ice elements inside a car until they were placed in cold storage at the lab around 09:00 pm and kept at 4°C until the next day.

Prior to sampling, every bit of equipment was cleaned with a 50% chlorine mixture and then rinsed with distilled water three times. Each bottle was marked with station number, sample type, and biological replicate number. A 50/50 chlorine and distilled H_2O mixture were prepared and brought to the sample site with a distilled H_2O jug. The car used for transport was prepared with cooling boxes and ice elements to keep the samples cold.

2.2. Filtration

All samples were filtrated on September 16th, 2021, through a vacuum funnel with a cellulose nitrate filter (47mm, 0,45 µl Pall Corporation, Ann Arbor, MI, USA) into either a 0,5 L or 0,75 L glass flask. Due to slow flow true, different volumes were filtered, depending on time with a minimum of 400ml (see appendix 3.). The water container on the vacuum funnel and forceps used to remove the filters were cleaned between each filtered sample with 50% chlorine mixture, then rinsed in distilled water and dried on paper, before further use. Blank samples were made with filtered distilled water in between filtering each station with one exception (station 1-2). The filters were folded to fit in each its

1,5 ml Eppendorf tube with 1440 µl ATL-buffer and kept at room temperature until extraction.

2.3. DNA extraction

Prior to extraction, a washing station for forceps was prepared with two 50 ml tubes, one with 40 ml 50% chlorine mixture and one with 45 ml distilled water. The forceps were rolled in paper and used when dry. DNeasy´s Blood and Tissue kit were used to extract DNA from the environmental samples. The filters were stored in ATL-buffer at room temperature for 1 – 2 months prior to extraction. The volume of ATL-buffer and proteinase K that was used was larger than the ones described in the Blood and Tissue-kit protocol.

Samples (filters and ATL-buffer) were transferred to new 2 ml Eppendorf tubes with 160 µl proteinase K. They incubated in Thermomixer Comfort (Eppendorf AG, Hamburg, Germany) for 16,00 hours with 500 rpm at 56°C. Each filter was then squeezed dry by two forceps over its microliter tube and discarded. After the forceps were used, they were placed in the 50%chlorine for a minimum of two minutes, placed in distilled water for two minutes or more, and dried before use. The samples were distributed into three 2 ml tubes, 600 µl in two tubes, and 400 µl in one. A 50/50% ethanol and AL-buffer mix were prepared and then vortexed. Each tube was added double its amount (1200 µl and 800 µl) with an ethanol/AL-buffer mix. Samples were centrifuged down in a DNease´s mini spin column, 600 µl at the time, at room temperature for 1 minute at 8000 rpm, until all samples were processed. The standard protocol was then used from point 5. - 8., except that the DNA was eluted with 100 µl AE-buffer at the last step. The extraction method used in this study was inspired by Natalie Haugan´s master thesis (Haugan, 2020). During the implementation of extraction, a mistake was found in Natalie Haugan´s master thesis that may have affected four of the samples. When transporting the content over to a new tube that was described to be 1,5 ml, the volume was simply too high, and I had to change to a 2,00 ml tube instead.

2.4. DNA Concentration and Purity

The samples were measured with the spectrophotometer Thermo Scientific[TM] Nanodrop[TM] Lite to find purity (A260/A280) and concentration (ng/µl). For calibration, double distilled water was used two times before measuring the samples. The magnet, where the DNA was placed, was cleaned with tech napkins between every measurement.

2.5. Real-time qPCR (T. bryosalmonae)

Real-time quantitative PCR (qPCR) was used to detect the DNA from T. bryosalmonae. A new standard curve, made by making a $10^6 - 10^0$ dilution series with *Tetracapsuloides bryosalmonae* plasmid (P.Tbr) and TE-buffer (1 mM EDTA, 10 mM Tris), was made for each PCR run, as illustrated in figure 2.5.-1 under.
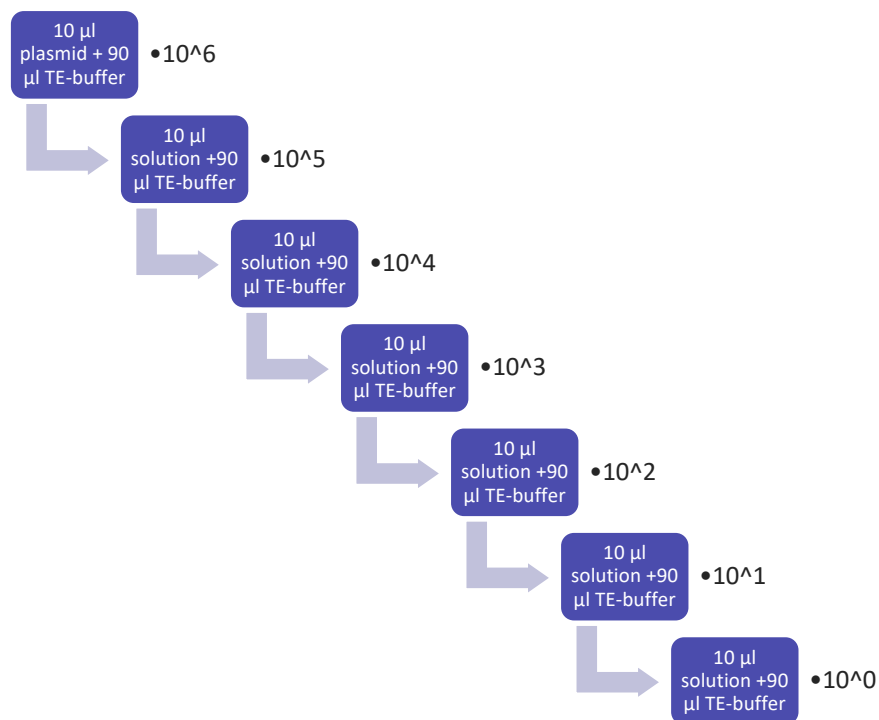
Figure 2.5.-1. Dilution series made with plasmid and TE-buffer used in qPCR to detect Tetracapsuloides bryosalmonae.

Each of the four PCR runs had three blank and two positive samples along the standard curve, the positive samples come from a *Tetracapsuloides bryosalmonae* kidney sample and the negatives are double-distilled water.

Each well contained 20 µl master mix prepared with the reagents shown in Table 2.5.-1 under and 5 µl sample. Each of the biological replicates had three technical PCR replicates. The spiking was done with 10^4 plasmids (2000 cpsa/µl) to check for inhibitors. A total of 12 samples were spiked with three PCR replicates of each sample.

Table 2.5.-1 Recipe Mastermix used to detect T. bryosalmonae

Reagents	Solution Concentration	Volume pr. Sample (µl)
TagMan Environmental Master mix 2x(VIC)		12,5
Forward primer: PKDtagf1 5' – gcgagatttgttgcatttaaaaag - 3' 73 bp (18S rDNA) Bettge et al. 2009, NINA	10 pmol/ µl	1
Reverse primer: PKDtagr1 5' – gcacatgcagtgtccaatcg - 3' 73 bp (18S rDNA) Bettge et al. 2009, NINA	10 pmol/ µl	1
TagMan probe AGTCGGACGGTTCCA	10 pmol/ µl	0,5
Doble distilled Water		5
Total		20

All four real-time qPCR runs were done following the PCR program in Table 2.5.-2 under with StepOne´s Real-Time PCR system and software (2.3) from Applied Biosystems.

Table 2.5.-2 PCR Program used to detect T. Bryosalmonae

Step	Degrees (°C)	Time	Cycles
Step 1	95	10 minutes	1
	95	15 seconds	45
Step 2	60	1 minute	
Step 3	4	10	coding

2.5.1. Limit of detection (LOD)

I´ve used a calculation done by my supervisor with the exact same ingredients and amounts as in the dilution series made for each of the qPCR tables used in this study. LOD for *Tetracapsuloides bryosalmonae* was calculated in R Studio following the procedure described in Klymus et al., (2020) with the R-script from Merkes et al., (2019). Dataset was made in Excel. The calculations are made with ten replicates of the total dilutions' series (10^6 -1 DNA copies) and ten replicates with each concentration 20, 40, 50, 60, 80, and 100 DNA copies. Curve fitting method (Merkes et al., 2019) was used to calculate effective LOD with a 95% probability of detection. With three technical replicates for each biological replicate in the qPCR, the limit of detection was estimated to be 10.3288. See Appendix 6-10 for all information regarding LOD and LOQ.

2.6. PCR Amplifying (16S)

One sample representing a biological replicate of each sampling type from stations 1. – 5. and three blank samples were used further for metabarcoding (1A, 5A, 7A, 10A, 2B, 5B, 6B, 8B, 9B, 2C, 5C, 6C, 2D, 5D, 8D, 9D, 2E, 5E, 8E, 1F, 2F, 8F, and G1). The samples had to be adjusted before amplifying so the DNA concentration was between 5-10 ng/µl. The primers were diluted to 10 µM. To amplify the region of interest, LongAmp hot Start Taq 2X Mastermix was used. The PCR was run twice to mark the fragments with UMI´s. The original DNA treads should then get marked with a unique taq and have an overhang with primers used in the next PCR. After amplification, the samples were washed with ampure to remove the rest of the enzymes, primers, and dNTP (nucleoids).

2.7. Sequencing (16S) and metabarcoding

The 16S Barcoding Kit 1-24 (SQK-16S024) from Oxford Nanopore Technologies was used to sequence the samples on a MinION using a flow cell v.9.4.1 (Oxford Nanopore Technologies). The protocol was followed except that PCR was run for 30 cycles. Raw data received from the flow cell

was translated with base calling by Guppy (from Oxford Nanopore) to DNA bases, structured in a FASTQ-file, filtered for the quality (Q Score > 8) and length (1500-2000 bp). Kraken2 (Wood et al., 2019), assigned the taxonomy from the filtered reads down to genus level with the database of the 138.1 SILVA, SSU Taxonomy, Ref NR 99 (https://www.arb-silva.de) with Kraken 2 integrated. The results files from Kraken 2 were combined in a single biome file, using the following Kraken-biome script (http://github.com/smdadoub). The biom file was further imported into R with the import_biom command in the *phyloseq* R-package.

2.8. Data analysis (16S)

The sequenced data were analyzed with the *phyloseq* method in R studio using three data sets (tax_table, OUT_table and SAM). To give an estimate of the amount of DNA within the samples I made a graph of sequencing depths and made tables in word from data read true *phyloseq* in R studio. To answer the different hypotheses, I calculated the beta diversity and alpha diversity using *phyloseq* and R-package *vegan*, and estimated the different factors sample type (mixed and direct), volume (1L and 2L), and station factor by running an ANOVA and PERMANOVA test to see if any of them were significant.

The whole workflow is illustrated in Fig. 2.8.-1 on the next page.

Sampling
- Samling eDNA in Lake Norsjø:
- 3x2L direct samples
- 3x1L direct samples
- 3X2L mixed samples
- 3x1L mixed samples

Extraction
- Filtration (Vacuum funnel and 0,45 µl nitrate filter)
- DNA extraction (DNeasy Blood and Tissue-kit from Qiagen)
- Measure DNA concentration and purity (NanoDrop™ Lite)

Single species detection
- Detection of *Tetracapsuloides bryosalmonae* with real-time qPCR
- LOD/LOQ

Metabarcoding (Bacteria)
- 16S amplification (PCR)
- DNA sequencing (PCR, MinION)
- Base calling by Guppy (Oxford Nanopore Technologies)
- Identify multiple taxa with Kraken2 and database SILVA (SSU Ref.Nr 99 138.1 data)

Statistics
- *Phyloseq* in Rstudio
- Alpha diversity
- Beta diversity
- Anova and Permanova

Figure 2.8.-1 Experimental processes for this study.

3. Results

3.1. Sampling

Table 3.1.-1 Stations at Lake Norsjø with different parameters

Station	Name	Water temperature (°C)	Weather	Sample time	Distance from land (m)	Covered Shoreline (m)
1.	Aslaksborg	12-12,5	Cloudy	10:51 - 11:18	1,5-8	Ca. 50
2.	Akkerhaugen	11,5	Cloudy/Sunny	12:08 - 12:43	0,5-5	Ca. 50
3.	Valebøbrygge	13	Sunny	14:10 - 14:37	2-7,5	Ca. 30
4.	Bjørkøya	13	Cloudy	16:34 - 17:02	5-25	40
5.	Ulefoss Bridge	12,2	Cloudy	18.15	1-2	50

3.2. DNA Concentration and Purity

The samples had a DNA concentration range between 1,7 – 89 ng/µl and the purity range was measured to be between 1,37 – 3,07 A260/A280 as shown in Tab. 3.2.-1 under with, mean, median and mode. See the full list of DNA concentration and purity in appendix 4.

Table 3.2.-1 Mean, Range, Median and Mode of DNA Concentration (ng/µl) and purity (A260/A280)

	Concentration (ng/µl)	Purity (A260/A280)
Mean	14,7	1,77857143
Range	1,7 - 89	1,37 – 3,07
Median	11,1	1,75
Mode	14,4	1,85

3.3. qPCR

Only a few samples tested positive for T. bryosalmonae and none of them were technical qPCR replicates nor biological replicates from the field. Two of them were from the same station (see table 3.3.-1). Due to few detections, I didn't do any further statistics.

Table 3.3.-1 T. bryosalmonae positive samples detected with real-time qPCR. The samples are added technical replicate number after the hyphen in the first column. For a full overview of qPCRs, see appendix 5.

Sample (Date)	Station/Method	CT-Value	Quantity	CT-Threshold
A10-2 (29.10.21)	1/1L, Mixed	33,74371	18,85345	0,009913
B7-2 (29.10.21)	2/2L, Direct	35,89061	3,757221	0,009913
D10-3 (22.11.21)	4/2L, Direct	36,63958	14,49154	0,009912
E10-3 (22.11.21)	4/1L, Mixed	36,65598	14,33497	0,009913
F4-3 (22.11.21	5/2L, Direct	37,00628	11,36769	0,009913

Spiking was done with a few samples from each station, to see if there were any inhibitors, due to the low number of detections. Four samples were marked as undetermined, and the others were around the expected quantity. The whole dilution series only showed up in the first run, and the three others lacked $10^{\wedge 1}$ and $10^{\wedge 0}$.

3.4. Metabarcoding (16S)

3.4.1. OTUs and taxa

The samples had a smaller amount of Holozoa and Eukaryota alongside bacteria and Archaea shown in Tab. 3.4.1.-1 under together with the most common taxa in phylum, class, order, family, and genus.

Table 3.4.1.-1 10 Most common taxa in sequenced samples.

Kingdom	Bacteria, Archaea, Holozoa, Eukaryota
Phylum	Bacteriodota, Cyanobacteria, Proteobacteria
Class	Alphaproteobacteria, Bacterioidia, Cyanobacteria, Gammabacteria
Order	Burkolderiales, chloroplast, Cytophagales, SAR II clade, Sphingobacteriales
Family	Burkholderiaceae, Clade III, Comamonadaceae, NS 11-12 marine group, Spirsomaceae
Genus	Acidovorax, Comamonas, Limnohabitans, Polynucleobacter, Peseudarcicella, Rhodoferax

OTUs appeared in all samples except one negative (1F), and the two other negatives contained very low amounts (8D, 5C) (see Fig. 3.4.1-1). One sample deviated from other samples (5A) and contained over a million OTUs.
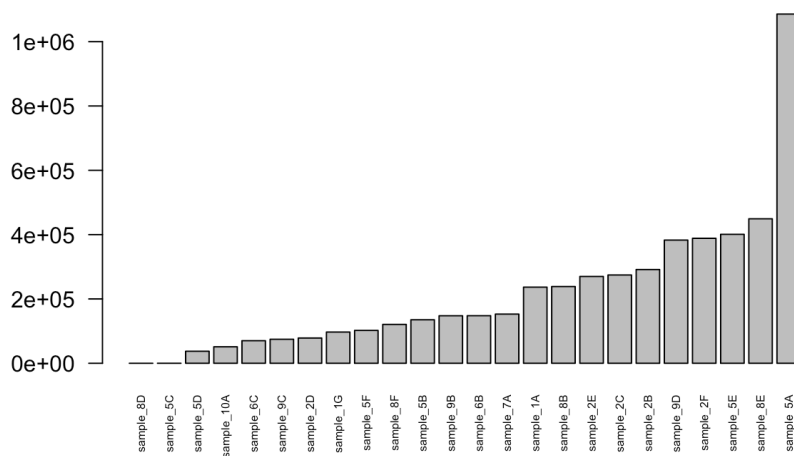
Figure 3.4.1.-1 Sorted OTUs, illustrate the variation of the amount of OUT reads found in the sequenced samples (8D and 5C are negatives).

The number of OTUs varied with high differences between samples. Fig. 3.4.1.-2 shows the abundance of class in the samples and the ones that are named from the database Kraken2 which shows a high variety in class taxa from all samples, especially sample 5A, which is a mixed 2L sample. Gram-negative bacteria in turquoise seems to be the dominant classes.
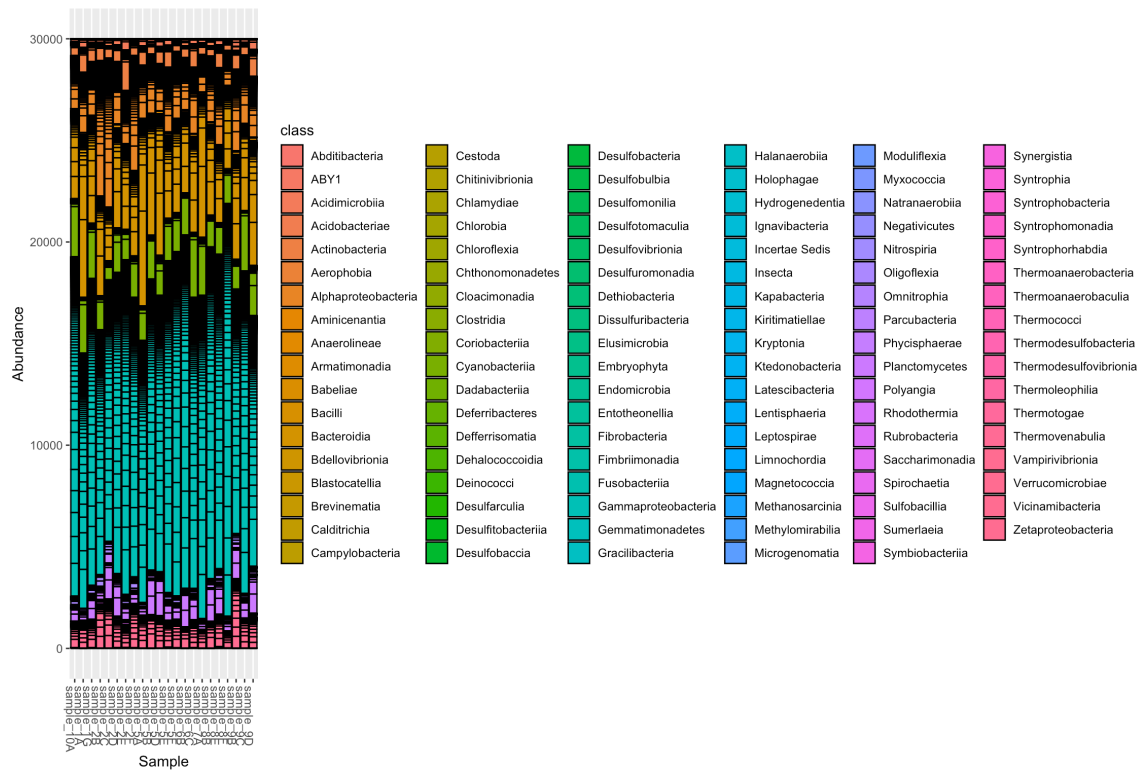
Figure 3.4.1.-2 Abundance of bacteria classes from sequenced samples with 16S

3.4.2. Alpha diversity measures

In the boxplots below (Fig. 3.4.2.-1), neither the sample type nor the volume (L) seems to illustrate significant importance for the sampling of eDNA in large freshwater lakes, with the boxes largely overlapping. However, there is a tendency that mixed samples have a slightly higher diversity than the direct samples.
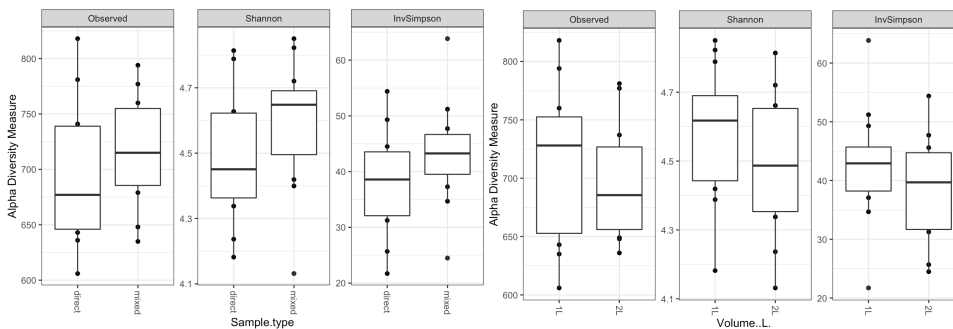
Figure 3.4.2.-1 Alpha diversity Measure: Observed, Shannon and Inverse Simpson for sample type on the left and volume (L) on the right.

The boxplot in Fig. 3.4.2-2 indicates that the station factor is more important as the variation between sample stations seems to be more significant.
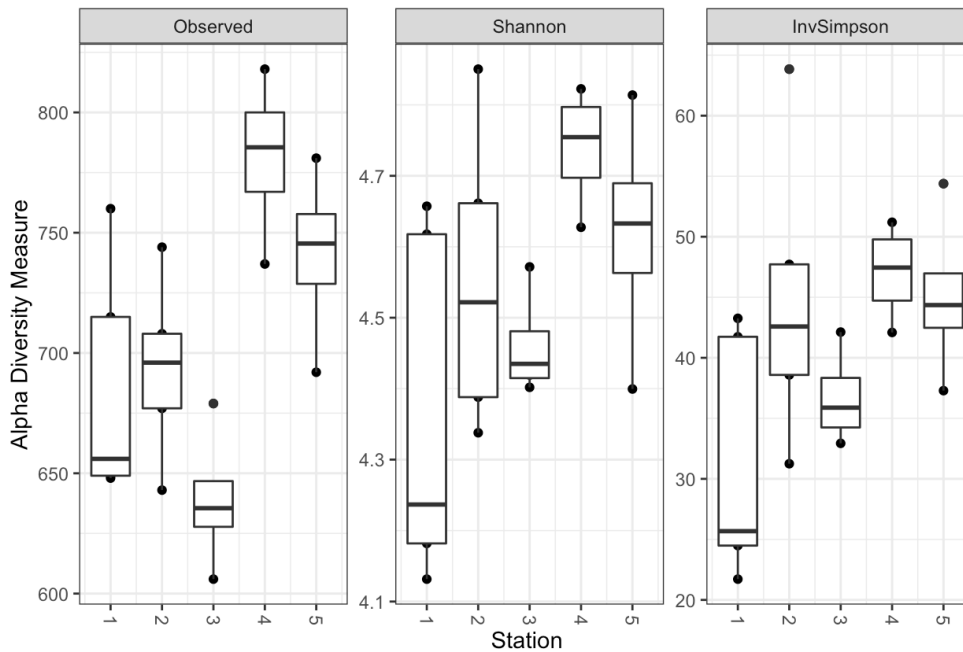
Figure 3.4.2.-2 Alpha diversity Measure: Observed, Shannon, Inverse Simpson for sample station (1-5)

3.4.3. Beta diversity measures

The ordination plot in Fig. 3.4.3-1 illustrates the beta diversity. The samples from the different stations (color) group together, which indicates that the station factor is a more important factor determining the bacterial community than sample type (shapes).
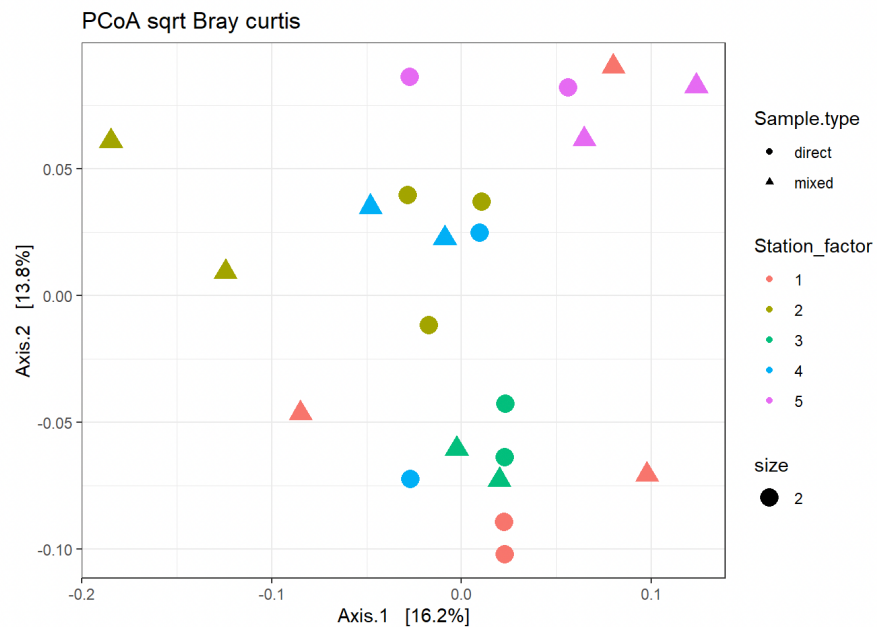
Figure 3.4.3.-1 The dot plot shows PCoA sqrt Bray curtis for sample type and station factor.

Statistical tests

In order to test the impact of the three factors, an ANOVA test was done to look at the significant difference in alpha diversity between the sample stations, shown in Tab. 3.4.3.-1 below. The test clearly shows a significant difference in observed species between sample stations but none for the two other factors (sample type and volume).

Table 3.3.4-1 ANOVA test on sequenced samples (16S) concerning alpha diversity where F-value illustrates the significant difference between the three variables station factor, sample type, and volume.

	Sum of squares	F-Value	P-value
Station-factor	136147	10.789	0.0003
Sample type	5295	1.679	0.2147
Volume	3134	0.994	0.3346

For further investigations, a PERMANOVA test was run to see if any of the three sample method variables showed a significant difference in beta diversity (Tab 3.3.4.-2). The station factor explains a total of 35,6 % of the variance and was the only significant factor (0.001).

Table 3.3.4-2 PERMANOVA test on sequenced samples (16S) concerning beta diversity where P-value illustrates the significant difference between the three variables station factor, sample type, and volume.

	Sum of squares	R^2	P-value
Station-factor	0.22609	0.35624	0.001
Sample type	0.02362	0.03722	0.439
Volume	0.02265	0.03569	0.514
Residuals	0.36229	0.57086	
Total	0.63465	1.00000	

4. Discussion

Overall, station is the most important factor determining the microbial community in Lake Norsjø explaining 35,6% of the beta diversity in the sequenced samples, while volume and sample type didn't turn out to be of any significant importance for neither the beta diversity nor the alpha diversity.

4.1. Filtration and Volume

Bedwell and Goldberg (2020) tested the impact on sampling volume (1L and 2L) at five lakes with various sizes, chose five sample stations (50 meters apart) on four different occasions from spring to early fall. Initially, they planned to filter 400 ml from the 2L samples and 200ml from 1L samples. Due to clogging of 0,45 µm CN filters, the targeted volume was not achieved on the first occasion. Meeting problems with clogged filters, they tested filers with larger pores to increase filtered volume on additional samples, 5 µm PES filters on the second occasion, and 5µm MCE filters on the third and fourth occasion. They also revisited three lakes on one occasion to test the comparison of a different filter cup with 5 µm filter (PES) to the former fitted with 0,45 µm filter (CN) and 5 µm filter (MCE) with 500 ml as the targeted filer volume. The filtered volume varied by pore size and location, but the detection rate didn´t differ by any of the factors. When testing five streams with seven sample stations with the same purpose, 2L had an increased detection rate to capture DNA from the two yellow-legged frogs with qPCR (Bedwell & Goldberg, 2020).

Muha et al. (2019) compared the effect of different filtered volumes (15, 100, 250, 1000, and 2000 mL) by collecting freshwater from a river, lake, and pond. Increased water volume showed a positive result in amplification efficiency and DNA capture. To compare quantitative assessment and species detection, they recommend that protocols for the sampling of eDNA in different freshwater deposits should be developed (Muha et al., 2019).

4.2. Real-time qPCR and detection of *Tetracapsuloides bryosalmonae*

There are many different sampling strategies concerning eDNA in freshwater (Laramie et al., 2015). The first thought with this experiment was to capture the DNA from *Tetracapsuloides bryosalmonae* to examine the different sample strategies. The different sampling stations were picked strategically nearby streams and rivers, where salmonoids often stay. During autumn e.g., Atlantic salmons return from sea to spawn in rivers (Forseth et al., 2017). Detection of species can be very difficult in several environments, especially in low-density populations, certain time periods, and development stages (Dejean et al., 2011; Ficetola et al., 2008). In-situ filtration combined with quantitative PCR is a method developed for large water samples to detect wildlife parasites in freshwater (Sieber et al.,2020). The method is sensitive for quantification, but for eDNA studies, sufficient replications, and sensitive quantification remains a challenge for parasite detection in water (Sieber et al., 2020).

The parasite didn't appear in many samples, and none of them were biological replicates or technical replicates. Four spiked samples were undetermined and could indicate inhibition or pipetting mistakes. The reason for only a few detections on Tetracapsuloides bryosalmonae may be due to uneven distribution within the lake, and a big risk of "missing" the target, despite the stations being picked to give the highest probability.

Hypolimnion could be protecting species during summer with its cold temperatures (Gaudard et al., 2018). Oredalen et al., 2022 present an alleged connection: cold water may protect the wild salmonids from developing PKD in the hypolimnion of deep freshwater lakes, and cold water in the deep may protect the farmed ones.

4.3. Detection of bacteria with 16S

The second aim was to detect bacteria by DNA metabarcoding, and the result showed high detection of multiple taxa. The choice of stations clearly showed the importance of testing different places around the lake for bacteria as the species composition varies

around the lake. The variance from each station was expected because of the inlets and outlets, but it seems like the water at each station was quite homogenous with little variance, only a few outliners. Mixed samples 1L and 2L didn´t show any significant difference and that´s not strange, they are in fact put together by the same samples (5x2L mixed samples). There were a few detections of holozoa and eukaryota in addition to bacteria and archaea. The 16S rRNA has also been used to detect arthropods (Alberdi et al., 2018, Elbrecht et al., 2016, Gibson et al., 2014).

Clade III, the second most detected family taxa in this study, is one out of six clades of *Clostridium difficile* and has been studied limited (Chen et al., 2017). *Closterium difficile* is a gram-positive anaerobic bacterium that can cause colitis and is known as the main cause of diarrhea associated with antibiotics (Bartlett et al., 1978; Chen et al., 2017). *Limnohabitans* and *Polynucleobacter* were two of the most common genus found in this study. *Limohabitans* belongs to the *Comamonadaceae* family and Polynucleobacter to *Burkholderiaceae* (Nuy et al., 2020). *Cyanobacteria*, *Alphaproteobacteria* and *Bacterioidia* were the most common class taxa detected at Lake Norsjø. The same findings were made by Nuy et al. (2020) despite variation in sample sites comparing 255 different freshwater lakes.

4.4. Biases

It´s necessary to implement various quality measures in the workflow to minimize the risk of false positives (Ficetola et al., 2016). Following an experimental design with strict procedures avoiding contaminations in the lab and field, including blanks in extraction and positive and negative PCR controls (Ficetola, et al., 2016). Technical PCR replicates are needed to insure reliable results (Ficetola, et al., 2015). Bioinformatic analysis for qPCR and especially for next-generation sequencing and metabarcoding need to be selected appropriately, to translate data to exploitable species distribution information (Ficetola, et al, 2016). Both NGS and metabarcoding can give millions of reads. By removing low-numbered sequences, preventing sequencing and PCR errors, and primer-dimers/ chimaeras, will increase the chance of a more credible result (Ficetola, et al., 2016). Tag-jumps can occur and can give false number of frequencies and further influence on incorrectly estimated diversity (Carøe & Bohmann, 2020).

5. Conclusion

This thesis contributed results that are quite clear, nor sample type (mixed/direct) or volume (1L/2L) shows significant importance when collecting eDNA samples in large freshwater lakes to detect bacteria. This clearly needs to be proven with more studies, but it could be a pointer toward a more useful way to collect eDNA samples. The most important factor turned out to be sample stations, which illustrates the importance of testing at several stations within a lake.

There were many factors that I didn't consider due to time and cost limitations, such as collecting samples over several seasons and years. It could be interesting to see how the bacterial community changed throughout the year and the presence of *Tetracapsuloides bryosalmonae,* and if the results would turn out differently when it´s done over a large time perspective. The data collected on the parasite showed too few detections to draw any conclusions.

There is great variation between studies that use eDNA methods from freshwater, and many factors to consider such as water type, season, filter size and material, and further what laboratory tests are to be carried out, thus the road to standardized protocols could be far. To compare the studies in a more sincere way, it is necessary to standardize the eDNA methods, to be able to detect species abundance, and be able to verify the implementation of the studies.

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7. Appendices

Appendix 1. Maps of each sample station (1.-5.) at Lake Norsjø. Numbers illustrate where mixed samples were collected, in numbered order from start (1) to end (5).
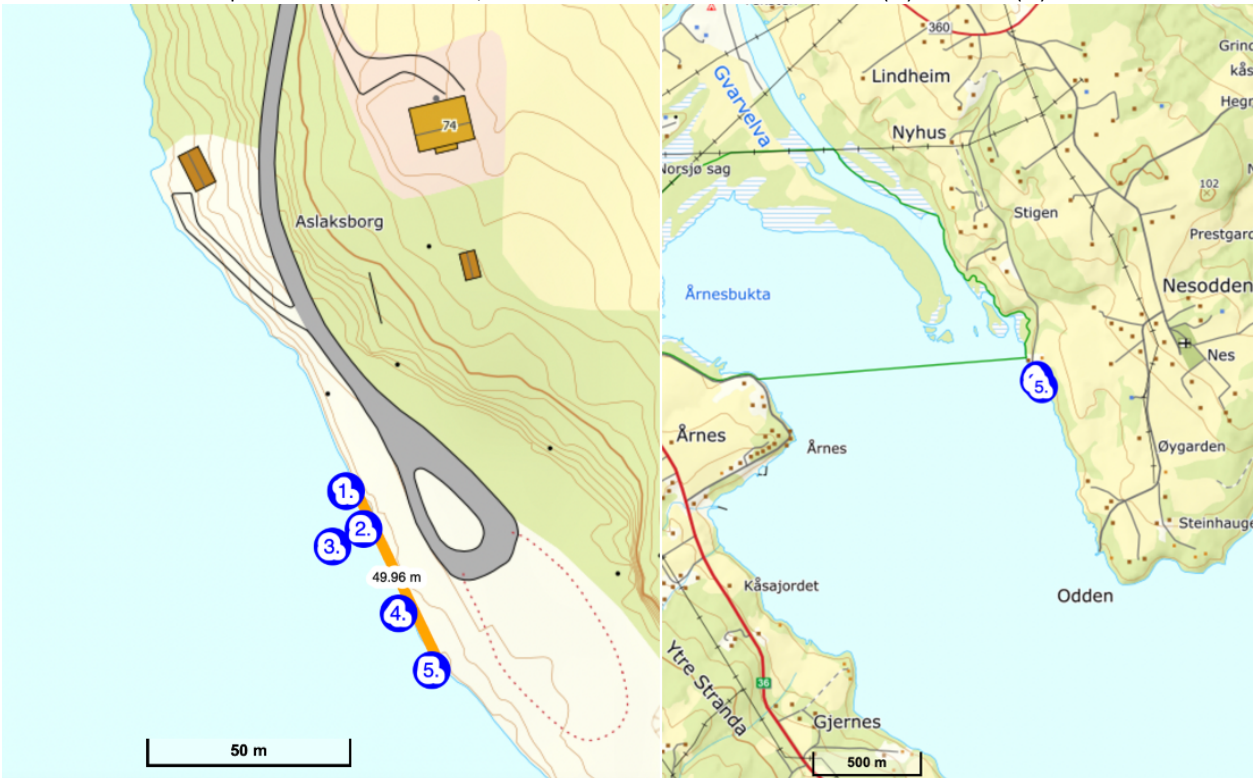
Station 1: Aslaksborg. Direct samples were collected at spot 3. (Norgeskart.no, 14.04.22). 1:50m scale to the left and 1:500 to the right.
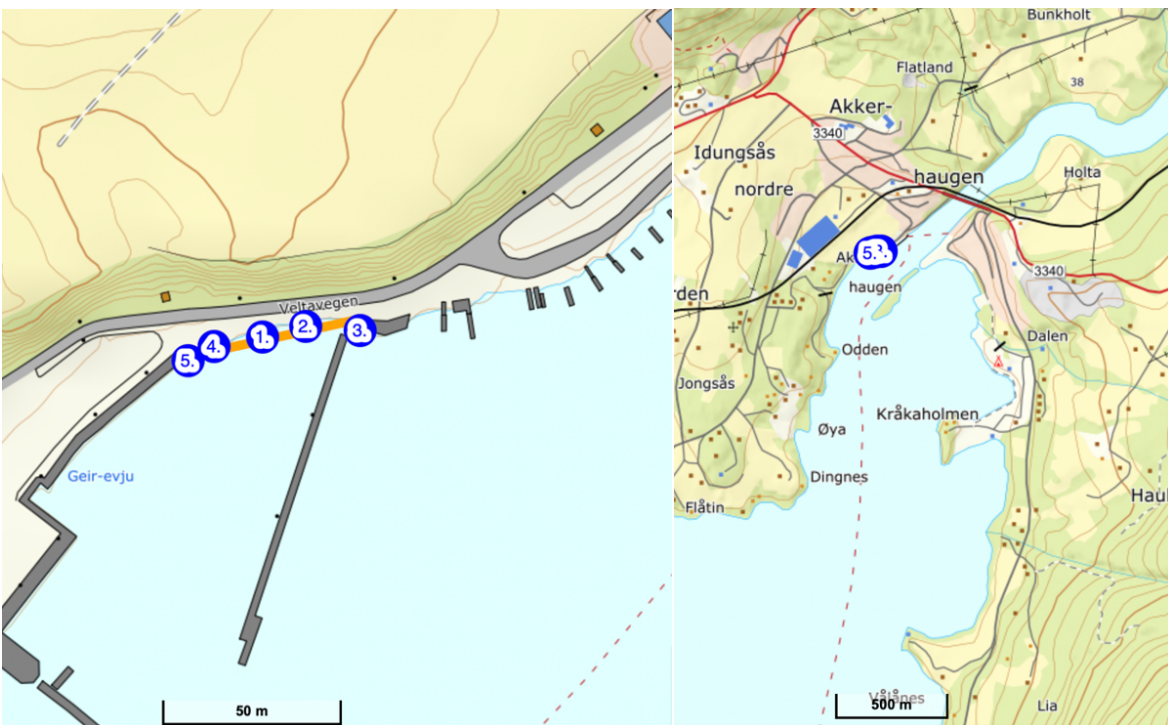
Station 2: Akkerhaugen. Direct samples were collected at spot 1. (Norgeskart.no 17.04.22). 1:50 scale to the left and 1:500 to the right.
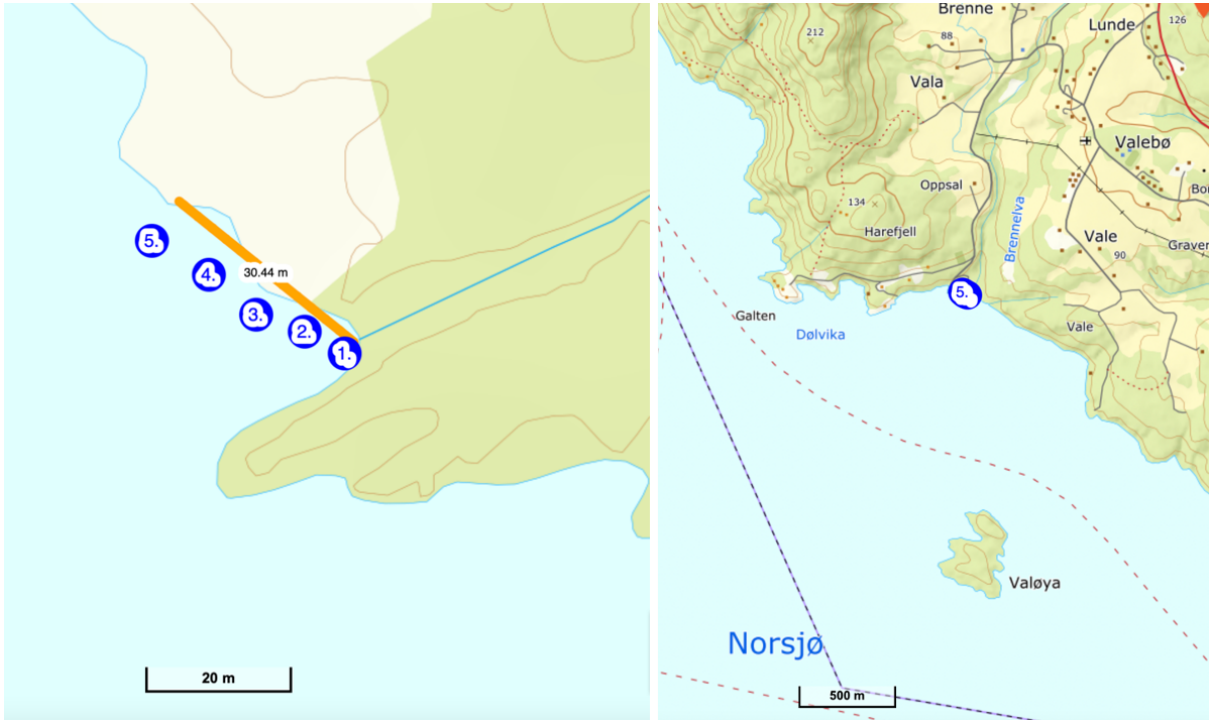
Station 3: Valebø Direct samples were collected at spot 1. (Norgeskart.no, 18.04.22).
1:20 scale to the left and 1:500 to the right.
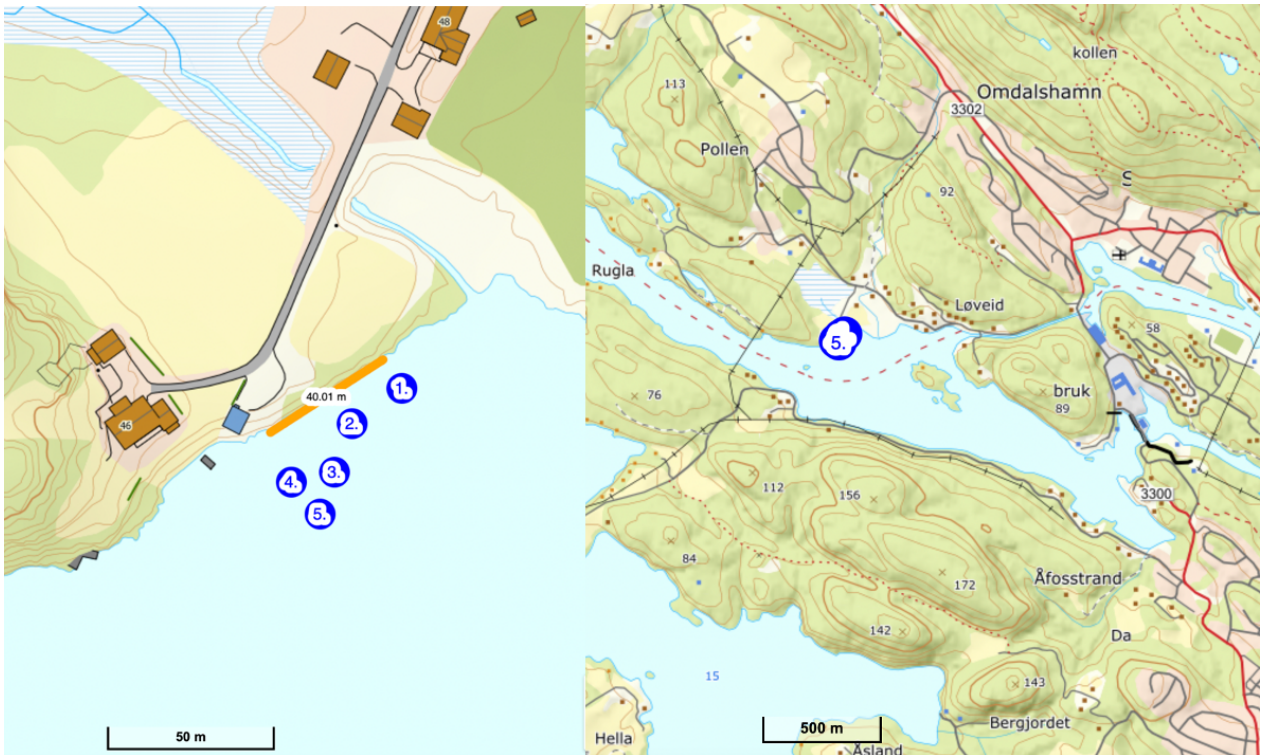
Station 4: Bjørkøya. Direct samples were collected at spot 2. (Norgeskart.no 18.04.22).
1:50 scale to the left and 1:500 scale to the right.
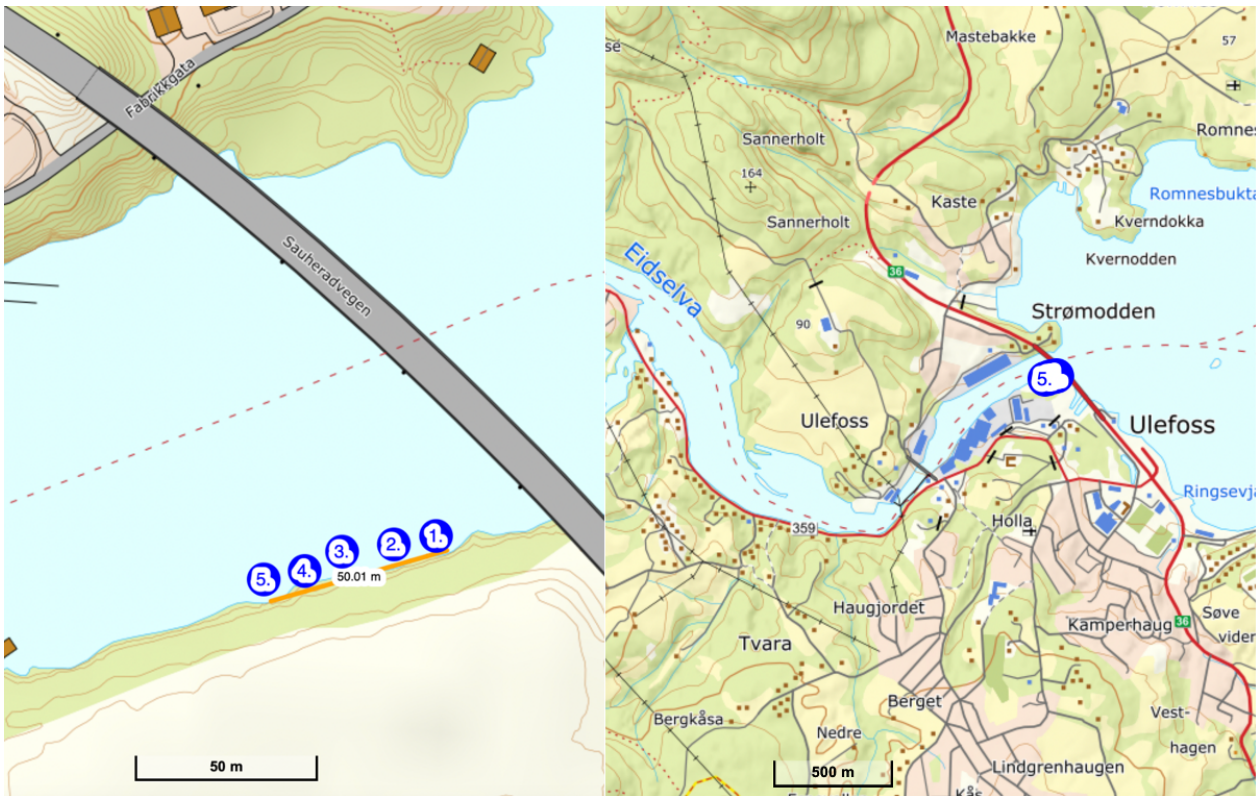
Station 5 Ulefoss bro (Bridge). Direct samples collected at spot 1. (Norgeskart, 18.04.22). 1:50 scale to the left and 1:500 to the right.

Appendix 2. Overview of eDNA sampling

Sample order	Station	Method	Volume	Water temperature	Distance from land	Comments
1	1	Direct (1)	2L	12°C	8m	Cloudy
2	1	Direct (2)	2L	12°C	8m	Cloudy
3	1	Direct (3)	2L	12°C	8m	Cloudy
4	1	Direct (1)	1L	12°C	8m	Cloudy
5	1	Direct (2)	1L	12°C	8m	Cloudy
6	1	Direct (3)	1L	12°C	8m	Cloudy
7	1	Mixed	2L	12°C	8m	Cloudy
8	1	Mixed	2L	12,5°C	1m	Cloudy
9	1	Mixed	2L	12,5°C	2m	Cloudy/Driftwood
10	1	Mixed	2L	12,5°C	1m	Cloudy/Deep
11	1	Mixed	2L	12,5°C	1,5m	Cloudy/Deep
12	2	Direct (1)	2L	11,5°C	0,5m	Cloudy/Sunny
13	2	Direct (2)	2L	11,5°C	0,5m	Cloudy/Sunny
14	2	Direct (3)	2L	11,5°C	0,5m	Cloudy/Sunny
15	2	Direct (1)	1L	11,5°C	0,5m	Cloudy/Sunny
16	2	Direct (2)	1L	11,5°C	0,5m	Cloudy/Sunny
17	2	Direct (3)	1L	11,5°C	0,5m	Cloudy/Sunny
18	2	Mixed	2L	11,5°C	0,5m	Cloudy/Sunny
19	2	Mixed	2L	11,5°C	2m	Cloudy/Sunny
20	2	Mixed	2L	11,5°C	4m	Cloudy/Sunny
21	2	Mixed	2L	11,5°C	2m	Cloudy/Sunny
22	2	Mixed	2L	11,5°C	4,5m	Cloudy/Sunny
23	3	Direct	2L	13°C	2m	Sunny/Cloudy
24	3	Direct	2L	13°C	2m	Sunny/Cloudy
25	3	Direct	2L	13°C	2m	Sunny/Cloudy

26	3	Direct	1L	13°C	2m	Sunny/Cloudy
27	3	Direct	1L	13°C	2m	Sunny/Cloudy
28	3	Direct	1L	13°C	2m	Sunny/Cloudy
29	3	Mixed	2L	13°C	2m	Sunny/Cloudy
30	3	Mixed	2L	13°C	3m	Sunny/Cloudy
31	3	Mixed	2L	13°C	3,5m	Sunny/Cloudy
32	3	Mixed	2L	13°C	5m	Sunny/Cloudy
33	3	Mixed	2L	13°C	7,5m	Sunny/Cloudy
34	4	Direct	2L	13°C	5m	Shallow/Cloudy W.
35	4	Direct	2L	13°C	5m	Shallow/Cloudy W.
36	4	Direct	2L	13°C	5m	Shallow/Cloudy W.
37	4	Direct	1L	13°C	5m	Shallow/Cloudy W.
38	4	Direct	1L	13°C	5m	Shallow/Cloudy W.
39	4	Direct	1L	13°C	5m	Shallow/Cloudy W.
40	4	Mixed	2L	13°C	5m	Shallow/Cloudy W.
41	4	Mixed	2L	13°C	5m	Shallow/Cloudy W.
42	4	Mixed	2L	13°C	15m	Shallow/Cloudy W.
43	4	Mixed	2L	13°C	18m	Shallow/Cloudy W.
44	4	Mixed	2L	13°C	25m	Shallow/Cloudy W.
45	5	Direct	2L	12,2°C	1m	Cloudy/Windy
46	5	Direct	2L	12,2°C	1m	Cloudy/Windy
47	5	Direct	2L	12,2°C	1m	Cloudy/Windy
48	5	Direct	1L	12,2°C	1m	Cloudy/Windy
49	5	Direct	1L	12,2°C	1m	Cloudy/Windy
50	5	Direct	1L	12,2°C	1m	Cloudy/Windy
51	5	Mixed	2L	12,2°C	1m	Cloudy/Windy
52	5	Mixed	2L	12,2°C	1,2m	Cloudy/Windy
53	5	Mixed	2L	12,2°C	1,5m	Cloudy/Windy
54	5	Mixed	2L	12,2°C	1,6m	Cloudy/Windy
55	5	Mixed	2L	12°C	2m	Cloudy/Windy

Appendix 3: <Filtration time and volume>

Sample	Station	Method	Start	Stop	Filtration Volume	Comments
A1	1.	Direct (2L)	10:30	10:45	～400 ml	
A2	1.	Direct (2L)	10:50	11:20	～500ml	Very slow
A3	1.	Direct (2L)	10:59	11:12	～750ml	
A4	1.	Direct (1L)	11:25	11:50	～750ml	
A5	1.	Direct (1L)	11:26	12:35	～400ml	Very slow
A6	1.	Direct (1L)	11:56	12:30	～500ml	Very slow
A7	1.	Mixed (2L)	12:32	12:52	～500ml	
A8	1.	Mixed (2L)	12:48	13:07	～500ml	
A9	1.	Mixed (2L)	12:59	13:30	～400ml	
A10	1.	Mixed (1L)	13:10	13:17	～500ml	
B1	1.	Mixed (1L)	13:19	13:29	～400ml	
B2	1.	Mixed (1L)	13:22	14:00	～400ml	
B3	2.	Direct (2L)	13:55	14:10	～500ml	
B4	2.	Direct (2L)	14:02	14:22	～400ml	
B5	2.	Direct (2L)	14:11	14:20	～400ml	
B6	2.	Direct (1L)	14:28	14:37	～500ml	
B7	2.	Direct (1L)	14:29	14:39	～400ml	
B8	2.	Direct (1L)	14:40	14:50	～500ml	

B9	2.	Mixed (2L)	14:45	14:53	∼400ml	
B10	2.	Mixed (2L)	14:56	15:03	∼500ml	
C1	2.	Mixed (2L)	15:04	15:13	∼500ml	
C2	2.	Mixed (1L)	15:09	15:28	∼400ml	
C3	2.	Mixed (1L)	15:14	15:30	∼500ml	
C4	2.	Mixed (1L)	15:33	15:45	∼500ml	
C5	-	Blank	15:57	16:05	∼500ml	
C6	3.	Direct (2L)	16:02	16:14	∼500ml	
C7	3.	Direct (2L)	16:10	16:39	∼500ml	
C8	3.	Direct (2L)	16:20	16:27	∼400ml	
C9	3.	Direct (1L)	16:30	16:41	∼500ml	
C10	3.	Direct (1L)	16:44	17:00	∼400ml	
D1	3.	Direct (1L)	16:45	16:56	∼500ml	
D2	3.	Mixed (2L)	17:02	17:14	∼500ml	
D3	3.	Mixed (2L)	17:08	17:19	∼500ml	
D4	3.	Mixed (2L)	17:18	17:30	∼500ml	
D5	3.	Mixed (1L)	17:24	17:35	∼500ml	
D6	3.	Mixed (1L)	17:35	17:45	∼500ml	
D7	3.	Mixed (1L)	17:36	17:48	∼500ml	
D8	-	Blank	17:56	18:01	∼500ml	
D9	4.	Direct (2L)	18:00	18:18	∼500ml	
D10	4.	Direct (2L)	18:03	18:20	∼500ml	
E1	4.	Direct (2L)	18:21	18:44	∼500ml	
E2	4.	Direct (1L)	18:40	19:06	∼500ml	
E3	4.	Direct (1L)	18:49	19:03	∼400ml	
E4	4.	Direct (1L)	19:05	19:27	∼500ml	
E5	4.	Mixed (2L)	19:07	19:32	∼500ml	
E6	4.	Mixed (2L)	19:33	19:56	∼500ml	
E7	4.	Mixed (2L)	19:35	19:59	∼500ml	
E8	4.	Mixed (1L)	19:57	20:11	∼500ml	
E9	4.	Mixed (1L)	20:01	20:17	∼500ml	
E10	4.	Mixed (1L)	20:12	20:30	∼500ml	
F1	-	Blank	20:34	20:40	∼500ml	
F2	4.	Direct (2L)	20:47	21:02	∼500ml	
F3	4.	Direct (2L)	20:48	21:16	∼500ml	
F4	4.	Direct (2L)	21:03	21:19	∼500ml	
F5	4.	Direct (1L)	21:20	21:30	∼500ml	
F6	4.	Direct (1L)	21:22	21:42	∼400ml	
F7	4.	Direct (1L)	21:31	21:45	∼500ml	
F8	4.	Mixed (2L)	21:47	21:57	∼500ml	
F9	4.	Mixed (2L)	21:48	22:13	∼500ml	
F10	4.	Mixed (2L)	21:57	22:07	∼500ml	
G1	4.	Mixed (1L)	22:10	22:21	∼500ml	
G2	4.	Mixed (1L)	22:14	22:44	∼400ml	
G3	4.	Mixed (1L)	22:23	22:39	∼400ml	

Appendix 4. Extraction date, concentration, purity, and sequenced samples (16S)

Sample	Extraction date	Nanodrop ng DNA/µL	A260/A280 DNA Purity	Metabarcoding (16S)
A1	26.10.2021	14,4	1,62	Sequenced
A2	26.10.2021	10,9	1,85	
A3	26.10.2021	8,5	1,72	
A4	26.10.2021	7,4	2,01	
A5	26.10.2021	5,4	1,69	Sequenced
A6	26.10.2021	7,5	1,67	
A7	27.10.2021	13,8	1,96	Sequenced
A8	27.10.2021	16,5	1,98	
A9	27.10.2021	15,2	1,97	
A10	27.10.2021	28,2	1,76	Sequenced x 2
B1	27.10.2021	13,7	1,8	
B2	27.10.2021	26,2	1,94	Sequenced
B3	27.10.2021	10,8	2,03	
B4	27.10.2021	7,5	1,95	
B5	27.10.2021	15,2	1,76	Sequenced
B6	27.10.2021	14,2	1,59	Sequenced
B7	27.10.2021	12,9	1,71	
B8	27.10.2021	17,3	1,63	Sequenced
B9	27.10.2021	5,6	1,53	Sequenced
B10	27.10.2021	5,5	1,71	
C1	27.10.2021	5,9	1,68	
C2	27.10.2021	6,7	1,79	Sequenced
C3	27.10.2021	4,8	1,66	
C4	27.10.2021	4,4	1,67	
C5	27.10.2021	3,2	1,6	Sequenced
C6	27.10.2021	6,1	1,89	Sequenced
C7	27.10.2021	7,2	1,85	
C8	27.10.2021	89	1,37	
C9	27.10.2021	15,5	1,63	Sequenced
C10	27.10.2021	9,6	1,44	
D1	17.11.2021	8,7	1,94	
D2	17.11.2021	6,7	1,85	Sequenced
D3	17.11.2021	27,1	1,09	
D4	17.11.2021	11,3	3,07	
D5	17.11.2021	4,1	2,07	Sequenced
D6	17.11.2021	4,9	2,39	
D7	17.11.2021	9,6	1,94	
D8	17.11.2021	1,7	2,74	Sequenced
D9	17.11.2021	9,4	1,72	Sequenced
D10	17.11.2021	11,5	1,71	
E1	18.11.2021	22,1	1,5	
E2	18.11.2021	16	1,58	Sequenced
E3	18.11.2021	16,9	1,48	
E4	18.11.2021	10,6	1,66	
E5	18.11.2021	13,2	1,82	Sequenced
E6	18.11.2021	11,1	1,76	
E7	18.11.2021	8	1,94	
E8	18.11.2021	14,4	1,64	Sequenced
E9	18.11.2021	11,1	1,76	
E10	18.11.2021	80,8	1,76	
F1	18.11.2021	3,5	1,5	Sequenced
F2	18.11.2021	29,8	1,57	Sequenced
F3	18.11.2021	16,6	1,83	
F4	18.11.2021	16,3	1,97	
F5	18.11.2021	10,3	1,73	Sequenced
F6	18.11.2021	10,3	1,91	
F7	18.11.2021	15,8	1,79	

F8	18.11.2021	14,5	1,52	Sequenced
F9	18.11.2021	15,8	1,85	
F10	18.11.2021	13,1	1,7	
G1	18.11.2021	14,9	1,75	Sequenced
G2	18.11.2021	48,2	1,43	
G3	18.11.2021	8,7	1,63	

Appendix 5. qPCR (*Tetracapsuloides bryosalmonae)*

qPCR 1. (29.10.21)

Sample	Sample type	CT	CT-Threshold	Quantity
	Standard	18,93117	0,009912	1000000
	Standard	21,9354	0,009912	100000
	Standard	25,28968	0,009912	10000
	Standard	28,49797	0,009912	1000
	Standard	32,99539	0,009912	10
	Standard	35,90413	0,009912	1
	Standard	35,65423	0,009912	
	Negative	Undetermined	0,009912	
	Negative	Undetermined	0,009912	
	Negative	Undetermined	0,009912	
	Positive	29,16713	0,009912	587,1002
	Positive	28,32869	0,009912	1102,277
A10-2	Mixed, 1L	33,74371	0,009912	18,85345
B7-2	Direct, 1L	35,89061	0,009912	3,757221

qPCR 2. (22.11.21)

Sample	Sample type	CT	CT-Threshold	Quantity
	Standard	19,95884	0,009912	1000000
	Standard	23,46525	0,009912	100000
	Standard	26,53773	0,009912	10000
	Standard	29,57878	0,009912	1000
	Standard	34,29133	0,009912	100
	Standard	Undetermined	0,009912	10
	Standard	Undetermined	0,009912	1
	Negative	Undetermined	0,009912	
	Negative	Undetermined	0,009912	
	Negative	Undetermined	0,009912	
	Positive	28,45015	0,009912	3280,101
	Positive	28,32677	0,009912	3559,293
D10-3		36,63958	0,009912	14,49154
E10-3		36,65598	0,009912	14,33497
F4-3		37,00628	0,009912	11,36769

qPCR 3. Spiked samples with standard 10^4

Sample	Sample type	CT	CT-Threshold	Quantity
	Standard	20,0454	0,009912	1000000
	Standard	23,12707	0,009912	100000
	Standard	26,42327	0,009912	10000
	Standard	29,28599	0,009912	1000
	Standard	33,29073	0,009912	100
	Standard	Undetermined	0,009912	10
	Standard	Undetermined	0,009912	1
	Negative	Undetermined	0,009912	
	Negative	Undetermined	0,009912	
	Negative	Undetermined	0,009912	
	Positive	28,36276	0,009912	2567,19

	Positive	28,26359	0,009912	2753,172
A7-1	Spiked	28,97654	0,009912	1665,198
A7-2	Spiked	29,04677	0,009912	1584,73
A7-3	Spiked	29,47034	0,009912	1175,49
B9-1	Spiked	28,70573	0,009912	2015,634
B9-2	Spiked	29,33594	0,009912	1292,368
B9-3	Spiked	29,75856	0,009912	959,272
D2-1	Spiked	28,96005	0,009912	1684,669
D2-2	Spiked	29,01584	0,009912	1619,671
D2-3	Spiked	Undetermined	0,009912	
E5-1	Spiked	29,73085	0,009912	978,1991
E5-2	Spiked	29,30861	0,009912	1317,516
E5-3	Spiked	Undetermined	0,009912	
F8-1	Spiked	Undetermined	0,009912	
F8-2	Spiked	29,33076	0,009912	1297,094
F8-3	Spiked	Undetermined	0,009912	
C10-1	Spiked	34,8301	0,009912	26,82802
C10-2	Spiked	36,27904	0,009912	9,655986
C10-3	Spiked	30,41495	0,009912	603,807
E3-1	Spiked	29,44983	0,009912	1192,618
E3-2	Spiked	29,24126	0,009912	1381,601
E3-3	Spiked	29,80786	0,009912	926,4902

qPCR 4. (29.11.21) Spiked samples with standard 10^4

Sample	Sample type	CT	CT-Threshold	Quantity
	Standard	19,72328	0,009912	1000000
	Standard	22,94581	0,009912	100000
	Standard	26,3096	0,009912	10000
	Standard	29,3276	0,009912	1000
	Standard	32,4172	0,009912	100
	Standard	Undetermined	0,009912	10
	Standard	Undetermined	0,009912	1
	Negative	Undetermined	0,009912	
	Negative	Undetermined	0,009912	
	Negative	Undetermined	0,009912	
	Positive	28,79072	0,009912	1469,495
	Positive	32,3693	0,009912	109,8326
D6-3	Spiked	28,07069	0,009912	2476,362
F10-1	Spiked	28,60708	0,009912	1678,701
F10-2	Spiked	29,13917	0,009912	1141,532
F10-3	Spiked	28,67469	0,009912	1598,429
A7-1	Spiked	28,76446	0,009912	1497,736
A7-2	Spiked	29,02141	0,009912	1243,235
A7-3	Spiked	29,12258	0,009912	1155,341
B8-1	Spiked	29,60532	0,009912	814,2463
B8-2	Spiked	29,18929	0,009912	1100,807
B8-3	Spiked	28,7291	0,009912	1536,615
C10-1	Spiked	28,28303	0,009912	2123,131
C10-2	Spiked	28,97289	0,009912	1287,736
C10-3	Spiked	29,18273	0,009912	1106,049
E9-1	Spiked	28,9023	0,009912	1355,332
E9-2	Spiked	28,87518	0,009912	1382,241
E9-3	Spiked	28,71909	0,009912	1547,804

Appendix 6.

PCR run for LOD with adjusted threshold (*Tetracapsuloides bryosalmonae*) - Data file 1.

Block Type: 96well
Chemistry: TAQMAN
Experiment File Name: M:\PhD-prosjekt\Data_og_Analyser\Ekstraksjon av DNA og PCR\rtPCR-køyringar\Standardcurvetest_Taq_291020_TJO.eds
Experiment Run End Time: 2020-10-29 15:45:04 PM CET
Instrument Type: steponeplus
Passive Reference: ROX

Well	Target Name	Task	Reporter	Quencher	Cᴛ	Cᴛ Mean	Cᴛ SD	Quantity	Ct Threshold
A1	Target 1	STANDARD	VIC	NFQ-MGB	20,69370651	20,75381279	0,050140049	1000000	0,019875726
A2	Target 1	STANDARD	VIC	NFQ-MGB	20,72929192	20,75381279	0,050140049	1000000	0,019875726
A3	Target 1	STANDARD	VIC	NFQ-MGB	20,71913338	20,75381279	0,050140049	1000000	0,019875726
A4	Target 1	STANDARD	VIC	NFQ-MGB	20,75178146	20,75381279	0,050140049	1000000	0,019875726
A5	Target 1	STANDARD	VIC	NFQ-MGB	20,80651474	20,75381279	0,050140049	1000000	0,019875726
A6	Target 1	STANDARD	VIC	NFQ-MGB	20,85411072	20,75381279	0,050140049	1000000	0,019875726
A7	Target 1	STANDARD	VIC	NFQ-MGB	20,76682281	20,75381279	0,050140049	1000000	0,019875726
A8	Target 1	STANDARD	VIC	NFQ-MGB	20,78225899	20,75381279	0,050140049	1000000	0,019875726
A9	Target 1	STANDARD	VIC	NFQ-MGB	20,69832611	20,75381279	0,050140049	1000000	0,019875726
A10	Target 1	STANDARD	VIC	NFQ-MGB	20,73617744	20,75381279	0,050140049	1000000	0,019875726
A11	Target 1	NTC	VIC	NFQ-MGB	Undetermined				0,019875726
A12	Target 1	NTC	VIC	NFQ-MGB	Undetermined				0,019875726
B1	Target 1	STANDARD	VIC	NFQ-MGB	26,39293861	26,504879	0,080096662	100000	0,019875726
B2	Target 1	STANDARD	VIC	NFQ-MGB	26,50298119	26,504879	0,080096662	100000	0,019875726
B3	Target 1	STANDARD	VIC	NFQ-MGB	26,51324081	26,504879	0,080096662	100000	0,019875726
B4	Target 1	STANDARD	VIC	NFQ-MGB	26,49878883	26,504879	0,080096662	100000	0,019875726
B5	Target 1	STANDARD	VIC	NFQ-MGB	26,49297523	26,504879	0,080096662	100000	0,019875726
B6	Target 1	STANDARD	VIC	NFQ-MGB	26,6782093	26,504879	0,080096662	100000	0,019875726
B7	Target 1	STANDARD	VIC	NFQ-MGB	26,4355545	26,504879	0,080096662	100000	0,019875726
B8	Target 1	STANDARD	VIC	NFQ-MGB	26,52836418	26,504879	0,080096662	100000	0,019875726
B9	Target 1	STANDARD	VIC	NFQ-MGB	26,57195473	26,504879	0,080096662	100000	0,019875726
B10	Target 1	STANDARD	VIC	NFQ-MGB	26,43377113	26,504879	0,080096662	100000	0,019875726
B11									
B12									
C1	Target 1	STANDARD	VIC	NFQ-MGB	27,60370064	27,64548874	0,070497192	10000	0,019875726
C2	Target 1	STANDARD	VIC	NFQ-MGB	27,70210648	27,64548874	0,070497192	10000	0,019875726
C3	Target 1	STANDARD	VIC	NFQ-MGB	27,79261971	27,64548874	0,070497192	10000	0,019875726
C4	Target 1	STANDARD	VIC	NFQ-MGB	27,68310738	27,64548874	0,070497192	10000	0,019875726
C5	Target 1	STANDARD	VIC	NFQ-MGB	27,57162094	27,64548874	0,070497192	10000	0,019875726
C6	Target 1	STANDARD	VIC	NFQ-MGB	27,67004204	27,64548874	0,070497192	10000	0,019875726
C7	Target 1	STANDARD	VIC	NFQ-MGB	27,64570618	27,64548874	0,070497192	10000	0,019875726
C8	Target 1	STANDARD	VIC	NFQ-MGB	27,63746262	27,64548874	0,070497192	10000	0,019875726
C9	Target 1	STANDARD	VIC	NFQ-MGB	27,55937958	27,64548874	0,070497192	10000	0,019875726
C10	Target 1	STANDARD	VIC	NFQ-MGB	27,58913612	27,64548874	0,070497192	10000	0,019875726
C11									
C12									
D1	Target 1	STANDARD	VIC	NFQ-MGB	30,84076881	30,82933426	0,181996331	1000	0,019875726
D2	Target 1	STANDARD	VIC	NFQ-MGB	30,82126808	30,82933426	0,181996331	1000	0,019875726
D3	Target 1	STANDARD	VIC	NFQ-MGB	30,64322472	30,82933426	0,181996331	1000	0,019875726
D4	Target 1	STANDARD	VIC	NFQ-MGB	31,15656662	30,82933426	0,181996331	1000	0,019875726
D5	Target 1	STANDARD	VIC	NFQ-MGB	31,05187035	30,82933426	0,181996331	1000	0,019875726

D6	Target 1	STANDARD	VIC	NFQ-MGB	30,85252953	30,82933426	0,181996331	1000	0,019875726
D7	Target 1	STANDARD	VIC	NFQ-MGB	30,80788994	30,82933426	0,181996331	1000	0,019875726
D8	Target 1	STANDARD	VIC	NFQ-MGB	30,60053062	30,82933426	0,181996331	1000	0,019875726
D9	Target 1	STANDARD	VIC	NFQ-MGB	30,61182976	30,82933426	0,181996331	1000	0,019875726
D10	Target 1	STANDARD	VIC	NFQ-MGB	30,90686417	30,82933426	0,181996331	1000	0,019875726
D11									
D12									
E1	Target 1	STANDARD	VIC	NFQ-MGB	34,72211075	33,94688416	0,458553284	100	0,019875726
E2	Target 1	STANDARD	VIC	NFQ-MGB	33,46953201	33,94688416	0,458553284	100	0,019875726
E3	Target 1	STANDARD	VIC	NFQ-MGB	33,86246872	33,94688416	0,458553284	100	0,019875726
E4	Target 1	STANDARD	VIC	NFQ-MGB	33,79940414	33,94688416	0,458553284	100	0,019875726
E5	Target 1	STANDARD	VIC	NFQ-MGB	34,27175903	33,94688416	0,458553284	100	0,019875726
E6	Target 1	STANDARD	VIC	NFQ-MGB	33,87390518	33,94688416	0,458553284	100	0,019875726
E7	Target 1	STANDARD	VIC	NFQ-MGB	34,29352188	33,94688416	0,458553284	100	0,019875726
E8	Target 1	STANDARD	VIC	NFQ-MGB	33,82167435	33,94688416	0,458553284	100	0,019875726
E9	Target 1	STANDARD	VIC	NFQ-MGB	33,11040115	33,94688416	0,458553284	100	0,019875726
E10	Target 1	STANDARD	VIC	NFQ-MGB	34,24406052	33,94688416	0,458553284	100	0,019875726
E11									
E12									
F1	Target 1	STANDARD	VIC	NFQ-MGB	34,79456329	36,76130676	1,077644587	10	0,019875726
F2	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined	36,76130676	1,077644587	10	0,019875726
F3	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined	36,76130676	1,077644587	10	0,019875726
F4	Target 1	STANDARD	VIC	NFQ-MGB	37,60251999	36,76130676	1,077644587	10	0,019875726
F5	Target 1	STANDARD	VIC	NFQ-MGB	37,46926117	36,76130676	1,077644587	10	0,019875726
F6	Target 1	STANDARD	VIC	NFQ-MGB	36,55452347	36,76130676	1,077644587	10	0,019875726
F7	Target 1	STANDARD	VIC	NFQ-MGB	37,56946564	36,76130676	1,077644587	10	0,019875726
F8	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined	36,76130676	1,077644587	10	0,019875726
F9	Target 1	STANDARD	VIC	NFQ-MGB	36,57748413	36,76130676	1,077644587	10	0,019875726
F10	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined	36,76130676	1,077644587	10	0,019875726
F11									
F12									
G1	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined			1	0,019875726
G2	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined			1	0,019875726
G3	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined			1	0,019875726
G4	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined			1	0,019875726
G5	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined			1	0,019875726
G6	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined			1	0,019875726
G7	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined			1	0,019875726
G8	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined			1	0,019875726
G9	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined			1	0,019875726
G10	Target 1	STANDARD	VIC	NFQ-MGB	Undetermined			1	0,019875726

Appendix 7.

PCR run for LOD with adjusted threshold (*Tetracapsuloides bryosalmonae*) - Data file 2.

Block Type: 96well
Chemistry: TAQMAN
Experiment File Name: M:\PhD-prosjekt\Data_og_Analyser\Ekstraksjon av DNA og PCR\rtPCR-
køyringar\Standardcurvetest_taq2_301020.eds
Experiment Run End Time: 2020-10-30 14:16:28 PM CET
Instrument Type: steponeplus
Passive Reference: ROX

Well	Target Name	Task	Reporter	Quencher	Cт	Cт Mean	Cт SD	Quantity	Ct Threshold
A1	Target 1	STANDARD	VIC	NFQ-MGB	33,90608215	34,0561409	0,380648851	100	0,019875726
A2	Target 1	STANDARD	VIC	NFQ-MGB	34,85521698	34,0561409	0,380648851	100	0,019875726
A3	Target 1	STANDARD	VIC	NFQ-MGB	34,16225052	34,0561409	0,380648851	100	0,019875726
A4	Target 1	STANDARD	VIC	NFQ-MGB	33,59631729	34,0561409	0,380648851	100	0,019875726
A5	Target 1	STANDARD	VIC	NFQ-MGB	33,66403961	34,0561409	0,380648851	100	0,019875726
A6	Target 1	STANDARD	VIC	NFQ-MGB	33,8166275	34,0561409	0,380648851	100	0,019875726
A7	Target 1	STANDARD	VIC	NFQ-MGB	34,49747086	34,0561409	0,380648851	100	0,019875726
A8	Target 1	STANDARD	VIC	NFQ-MGB	33,9577713	34,0561409	0,380648851	100	0,019875726
A9	Target 1	STANDARD	VIC	NFQ-MGB	34,00421906	34,0561409	0,380648851	100	0,019875726
A10	Target 1	STANDARD	VIC	NFQ-MGB	34,10141754	34,0561409	0,380648851	100	0,019875726
A11	Target 1	NTC	VIC	NFQ-MGB	Undetermined				0,019875726
A12	Target 1	NTC	VIC	NFQ-MGB	Undetermined				0,019875726
B1	Target 1	STANDARD	VIC	NFQ-MGB	34,42379379	34,33340454	0,720561147	80	0,019875726
B2	Target 1	STANDARD	VIC	NFQ-MGB	35,5556221	34,33340454	0,720561147	80	0,019875726
B3	Target 1	STANDARD	VIC	NFQ-MGB	34,18904495	34,33340454	0,720561147	80	0,019875726
B4	Target 1	STANDARD	VIC	NFQ-MGB	33,93672562	34,33340454	0,720561147	80	0,019875726
B5	Target 1	STANDARD	VIC	NFQ-MGB	34,76639175	34,33340454	0,720561147	80	0,019875726
B6	Target 1	STANDARD	VIC	NFQ-MGB	33,91783905	34,33340454	0,720561147	80	0,019875726
B7	Target 1	STANDARD	VIC	NFQ-MGB	34,20633698	34,33340454	0,720561147	80	0,019875726
B8	Target 1	STANDARD	VIC	NFQ-MGB	34,37149429	34,33340454	0,720561147	80	0,019875726
B9	Target 1	STANDARD	VIC	NFQ-MGB	35,07194901	34,33340454	0,720561147	80	0,019875726
B10	Target 1	STANDARD	VIC	NFQ-MGB	32,89483643	34,33340454	0,720561147	80	0,019875726
B11									
B12									
C1	Target 1	STANDARD	VIC	NFQ-MGB	34,97022247	34,84393692	0,663058758	60	0,019875726
C2	Target 1	STANDARD	VIC	NFQ-MGB	34,62305069	34,84393692	0,663058758	60	0,019875726
C3	Target 1	STANDARD	VIC	NFQ-MGB	34,69944763	34,84393692	0,663058758	60	0,019875726
C4	Target 1	STANDARD	VIC	NFQ-MGB	36,07233047	34,84393692	0,663058758	60	0,019875726
C5	Target 1	STANDARD	VIC	NFQ-MGB	34,20359421	34,84393692	0,663058758	60	0,019875726
C6	Target 1	STANDARD	VIC	NFQ-MGB	35,7782402	34,84393692	0,663058758	60	0,019875726
C7	Target 1	STANDARD	VIC	NFQ-MGB	34,94643021	34,84393692	0,663058758	60	0,019875726
C8	Target 1	STANDARD	VIC	NFQ-MGB	34,63843155	34,84393692	0,663058758	60	0,019875726
C9	Target 1	STANDARD	VIC	NFQ-MGB	33,84757233	34,84393692	0,663058758	60	0,019875726
C10	Target 1	STANDARD	VIC	NFQ-MGB	34,66004944	34,84393692	0,663058758	60	0,019875726
C11									
C12									
D1	Target 1	STANDARD	VIC	NFQ-MGB	35,53838348	35,64769745	1,007674336	40	0,019875726
D2	Target 1	STANDARD	VIC	NFQ-MGB	36,62494659	35,64769745	1,007674336	40	0,019875726
D3	Target 1	STANDARD	VIC	NFQ-MGB	34,94256592	35,64769745	1,007674336	40	0,019875726
D4	Target 1	STANDARD	VIC	NFQ-MGB	36,6292305	35,64769745	1,007674336	40	0,019875726
D5	Target 1	STANDARD	VIC	NFQ-MGB	35,21975327	35,64769745	1,007674336	40	0,019875726

D6	Target 1	STANDARD	VIC	NFQ-MGB	37,65211487	35,64769745	1,007674336	40	0,019875726
D7	Target 1	STANDARD	VIC	NFQ-MGB	35,63455582	35,64769745	1,007674336	40	0,019875726
D8	Target 1	STANDARD	VIC	NFQ-MGB	34,87833786	35,64769745	1,007674336	40	0,019875726
D9	Target 1	STANDARD	VIC	NFQ-MGB	34,8192749	35,64769745	1,007674336	40	0,019875726
D10	Target 1	STANDARD	VIC	NFQ-MGB	34,53779221	35,64769745	1,007674336	40	0,019875726
D11									
D12									
E1	Target 1	STANDARD	VIC	NFQ-MGB	37,59583664	36,18888092	0,805897176	20	0,019875726
E2	Target 1	STANDARD	VIC	NFQ-MGB	37,08460999	36,18888092	0,805897176	20	0,019875726
E3	Target 1	STANDARD	VIC	NFQ-MGB	36,22430801	36,18888092	0,805897176	20	0,019875726
E4	Target 1	STANDARD	VIC	NFQ-MGB	36,22430038	36,18888092	0,805897176	20	0,019875726
E5	Target 1	STANDARD	VIC	NFQ-MGB	35,31557846	36,18888092	0,805897176	20	0,019875726
E6	Target 1	STANDARD	VIC	NFQ-MGB	36,68016052	36,18888092	0,805897176	20	0,019875726
E7	Target 1	STANDARD	VIC	NFQ-MGB	35,72089005	36,18888092	0,805897176	20	0,019875726
E8	Target 1	STANDARD	VIC	NFQ-MGB	35,28554916	36,18888092	0,805897176	20	0,019875726
E9	Target 1	STANDARD	VIC	NFQ-MGB	36,52279663	36,18888092	0,805897176	20	0,019875726
E10	Target 1	STANDARD	VIC	NFQ-MGB	35,23476791	36,18888092	0,805897176	20	0,019875726

Appendix 8.

Input-file with standard curves (*Tetracapsuloides bryosalmonae*) used for LOD-calculator (R-file).

Well	Cq	SQ	Target
A1	20.69370651	1000000	TB1
A2	20.72929192	1000000	TB1
A3	20.71913338	1000000	TB1
A4	20.75178146	1000000	TB1
A5	20.80651474	1000000	TB1
A6	20.85411072	1000000	TB1
A7	20.76682281	1000000	TB1
A8	20.78225899	1000000	TB1
A9	20.69832611	1000000	TB1
A10	20.73617744	1000000	TB1
B1	26.39293861	100000	TB1
B2	26.50298119	100000	TB1
B3	26.51324081	100000	TB1
B4	26.49878883	100000	TB1
B5	26.49297523	100000	TB1
B6	26.6782093	100000	TB1
B7	26.4355545	100000	TB1
B8	26.52836418	100000	TB1
B9	26.57195473	100000	TB1
B10	26.43377113	100000	TB1
C1	27.60370064	10000	TB1
C2	27.70210648	10000	TB1
C3	27.79261971	10000	TB1

C4	27.68310738	10000	TB1
C5	27.57162094	10000	TB1
C6	27.67004204	10000	TB1
C7	27.64570618	10000	TB1
C8	27.63746262	10000	TB1
C9	27.55937958	10000	TB1
C10	27.58913612	10000	TB1
D1	30.84076881	1000	TB1
D2	30.82126808	1000	TB1
D3	30.64322472	1000	TB1
D4	31.15656662	1000	TB1
D5	31.05187035	1000	TB1
D6	30.85252953	1000	TB1
D7	30.80788994	1000	TB1
D8	30.60053062	1000	TB1
D9	30.61182976	1000	TB1
D10	30.90686417	1000	TB1
E1	34.72211075	100	TB1
E2	33.46953201	100	TB1
E3	33.86246872	100	TB1
E4	33.79940414	100	TB1
E5	34.27175903	100	TB1
E6	33.87390518	100	TB1
E7	34.29352188	100	TB1
E8	33.82167435	100	TB1
E9	33.11040115	100	TB1
E10	34.24406052	100	TB1
F1	34.79456329	10	TB1
F2	Undetermined	10	TB1
F3	Undetermined	10	TB1
F4	37.60251999	10	TB1
F5	37.46926117	10	TB1
F6	36.55452347	10	TB1
F7	37.56946564	10	TB1
F8	Undetermined	10	TB1
F9	36.57748413	10	TB1
F10	Undetermined	10	TB1
G1	Undetermined	1	TB1
G2	Undetermined	1	TB1
G3	Undetermined	1	TB1
G4	Undetermined	1	TB1
G5	Undetermined	1	TB1
G6	Undetermined	1	TB1
G7	Undetermined	1	TB1

G8	Undetermined	1	TB1
G9	Undetermined	1	TB1
G10	Undetermined	1	TB1
A1	33.90608215	100	TB1
A2	34.85521698	100	TB1
A3	34.16225052	100	TB1
A4	33.59631729	100	TB1
A5	33.66403961	100	TB1
A6	33.8166275	100	TB1
A7	34.49747086	100	TB1
A8	33.9577713	100	TB1
A9	34.00421906	100	TB1
A10	34.10141754	100	TB1
B1	34.42379379	80	TB1
B2	35.5556221	80	TB1
B3	34.18904495	80	TB1
B4	33.93672562	80	TB1
B5	34.76639175	80	TB1
B6	33.91783905	80	TB1
B7	34.20633698	80	TB1
B8	34.37149429	80	TB1
B9	35.07194901	80	TB1
B10	32.89483643	80	TB1
C1	34.97022247	60	TB1
C2	34.62305069	60	TB1
C3	34.69944763	60	TB1
C4	36.07233047	60	TB1
C5	34.20359421	60	TB1
C6	35.7782402	60	TB1
C7	34.94643021	60	TB1
C8	34.63843155	60	TB1
C9	33.84757233	60	TB1
C10	34.66004944	60	TB1
D1	35.53838348	40	TB1
D2	36.62494659	40	TB1
D3	34.94256592	40	TB1
D4	36.6292305	40	TB1
D5	35.21975327	40	TB1
D6	37.65211487	40	TB1
D7	35.63455582	40	TB1
D8	34.87833786	40	TB1
D9	34.8192749	40	TB1
D10	34.53779221	40	TB1

E1	37.59583664	20	TB1
E2	37.08460999	20	TB1
E3	36.22430801	20	TB1
E4	36.22430038	20	TB1
E5	35.31557846	20	TB1
E6	36.68016052	20	TB1
E7	35.72089005	20	TB1
E8	35.28554916	20	TB1
E9	36.52279663	20	TB1
E10	35.23476791	20	TB1

Appendix 9.

LOD for *Tetracapsuloides bryosalmonae* estimated with 10 dilution series $10^{\wedge}6$ - $10^{\wedge}0$.

Assay	Replicates	Estimated LOD	Lower	Upper	Standard error
TB1	1.	15.653146682659	3.01074440999749	28.2955489553206	6.3841738313018
TB1	2.	12.0416973662693	7.92377630586579	16.1596184266728	2.07947218465308
TB1	3.	10.3288583399691	9.23409855968485	11.4236181202533	0.552832965611756
TB1	4.	9.26347132627678	7.68322514535404	10.8437175071995	0.797994407841105
TB1	5.	8.51337299958718	5.87496130046525	11.1517846987091	1.3323479638169
TB1	6.	7.12622966701729	2.68286433524539	11.5695949987892	2.24381538114431

Appendix 10.

Limit of detection (LOD) plot for *Tetracapsuloides bryosalmonae.*