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Environmental DNA for assessing abundance of salmon lice (*Lepeophtheirus salmonis*) in salmon (*Salmo salar*) farms

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Photo credits: Filipe Figueiredo

1000 μ m



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Tromsø, May 2018

Magnus Nygaard

Preface

This thesis concludes my Master in Fisheries and Aquaculture management at UiT, The Arctic University of Norway, Tromsø. The project has been supervised by associated professor Kim Præbel at UiT, and Postdoc. Dr. Owen Wangensteen. Dr. Wangensteen performed the bioinformatics of the metabarcoding data in this thesis.

The aim of this thesis was to investigate an alternative way to detect salmon lice (*Lepeophtheirus salmonis*) in the field, and investigate the possibility of counting salmon lice in a commercial farming facility of Atlantic salmon (*Salmo salar*) by using methods and tools based on environmental DNA (henceforth referred to as eDNA). This was performed by sampling water in a salmon farming facility over a couple of months, collecting live salmon lice at a local salmon slaughter factory and conducting an experiment in a laboratory under a controlled environment.

This project has opened a new perspective to me and taught me things I never knew, especially when it comes to the presence and abundance of DNA in the environment and what is possible to do with it.

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Abstract

Salmon lice (*Lepeophtheirus salmonis*) is a well-known parasite in the Atlantic salmon farming industry in Norway, and causes losses of billions of Norwegian kroner to the industry every year due to fish death and salmon lice-treatment measures. A lot of resources and time is spent on counting salmon lice and treating the salmon against the parasite. All salmon lice counting means physically handling of the fish which may induce stress and cause damage to the protective mucus and skin layer, and even death.

The aim of this study was to investigate the possibility of detecting salmon lice in the field and quantifying the amount of salmon lice in an Atlantic salmon (*Salmo salar*) farm based on sequencing of the salmon lice DNA in the environment. And further to assess how this method compares to traditional manual salmon lice counting. The method used was based on metabarcoding- analysis of extracted environmental DNA from sampled and filtered water from a salmon farming facility located in the northern part of Norway, over the span of a couple of months.

DNA from salmon lice turned out to be detectable in the environment when using a fragment of the mitochondrial marker cytochrome c oxidase (Leray-XT primers), and to be of great abundance in the facility. It was also possible to quantify the abundance of the salmon lice in an experiment in a controlled environment, with the help of a standard curve. However, the comparison between manual counting and eDNA based counting did not yield a good correlation. A possible explanation for this is that the salmon lice DNA present in the water may also come from other life-cycle stages other than adult salmon lice. Despite this result, eDNA still has a promising future as a useful tool in the Atlantic salmon farming industry, not only in the context of salmon lice counting and surveillance.

Introduction

Salmon lice (*Lepeophtheirus salmonis*) in modern aquaculture.

About the salmon lice and their life cycle:

Salmon lice (*Lepeophtheirus spp.*) are parasitic copepods which are found in marine areas north of the Equator. Salmon lice can be divided into two subspecies; *Lepeophtheirus salmonis salmonis*, living in the Atlantic Ocean and *Lepeophtheirus salmonis oncorhynchi*, located in the Pacific Ocean. In order for the salmon lice to survive their entire life cycle, it requires a host. Atlantic Salmon lice in the Atlantic ocean are found on salmonids, such as Atlantic salmon (*Salmo salar*), sea trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*), while the Pacific salmon lice goes to the Pacific salmon family (*Oncorhynchus spp.*).

Salmon lice live in the ocean and thrive in salt water with salinity between 29 ppt and 36 ppt (Bricknell *et al*, 2006). The salmon lice are an ectoparasite that can live on the skin of salmon from a few hours to about 14 days, where it lives from skin, mucus and blood (Johnson and Albright, 1992). Salmon lice has a direct life cycle with only salmonids as host. Parasites affect the host's immune system, and if there are too many parasites on a single host, it can lead to serious health problems and, in the worst case, death (Johnson *et al*, 2004).

The life cycle of the salmon lice has been discussed for many years. For a long time, it was thought that the salmon lice had ten life stages, but recent research shows that it has eight (Boxaspen, 2006). The first two life stages of the salmon lice (Nauplius I and II) (Figure 1) are free living. As a free-living stage, the nauplii survives on energy reserves and are carried back and forth with the water currents (Boxaspen, 2006). The copepodite stage (3rd stage) is the infectious stage where the salmon lice find a host and adheres to it. After the copepodite stage, we find the chalimus stage. It was thought that chalimus consisted of four stages, whereas today it is thought to consists of two stages; Chalimus I and II. Chalimus I, is the first parasitic life stage, as the salmon lice are stationary bound to the salmonids skin using a filament. In the last three stages, they are motile and can move both on the individual and between individuals. These motile stages are called Pre-adults I and II, and finally, Adult (Hamre *et al*, 2013; Igboeli, Burka and Fast, 2013; Johnson and Albright, 1992; White, 1942). Adult male salmon lice reach approx. 6 mm while the female salmon lice are 12 mm (29 mm including the egg strings). Over the years, many tests have been made with salmon lice and there has been found that water flow, salinity and temperature affect the salmon lice's ability to survive and manage to infect

the fish (Bricknell *et al*, 2006; Bron, 2006; Genna, 2005; Heuch, Nordhagen and Schram, 2000). Studies have shown that the female salmon lice can live up to 210 days and produce 11 pairs of egg strings. The first pair of egg strings is shorter and contains fewer eggs than the later developing egg strings. Fecundity decreases over time (Heuch, Nordhagen and Schram, 2000; Mustafa, Conboy and Burka, 2000). At lower temperatures, the strings are longer and contain more eggs (Heuch, Nordhagen and Schram, 2000). It takes longer for the egg to hatch at low temperatures, 45,1 days at 2 °C, than at higher temperatures, 8.7 days at 10 °C. A large part of the nauplii manages to develop into copepodites at 4°C, which means they can stick to a host at low temperatures (Boxaspen and Naess, 2000). The salmon lice live longer at low temperatures. The salmon lice are productive and can produce many eggs quickly at high temperatures. The eggs hatches much faster at high temperatures, and therefore the salmon lice pressure can be high in late summer and autumn.

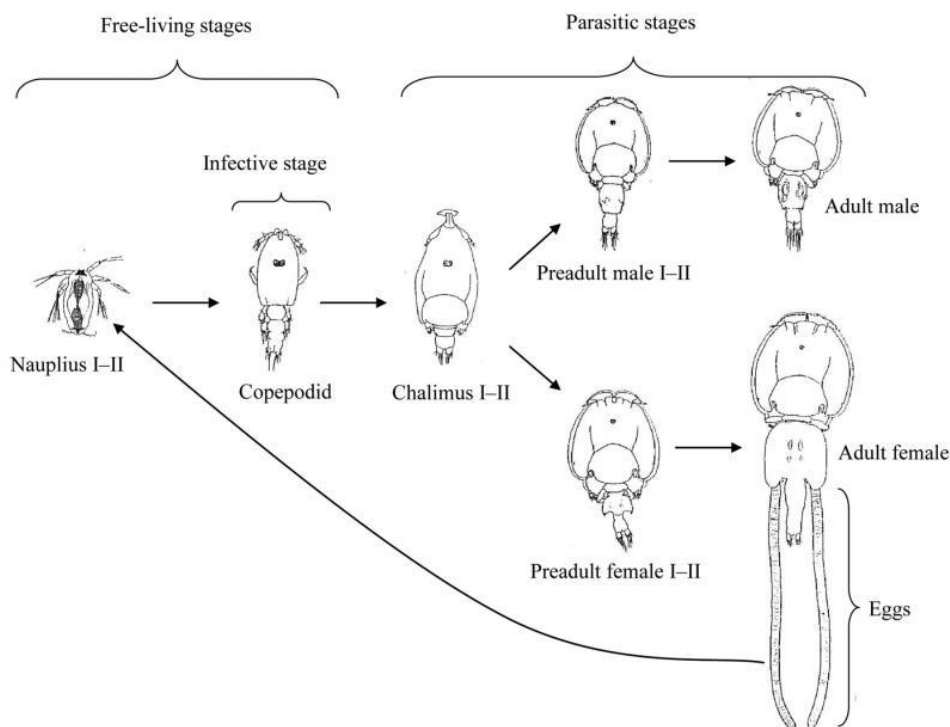


Figure 1 Life cycle of salmon lice (*Lepeophtheirus salmonis salmonis*) with the stages from egg to mature adult (Igboeli *et al*, 2013).

Challenges caused by the salmon lice

Salmon lice have a clearly negative effect on the fish just like any other disease would. Feeding on skin, mucus and blood of the fish, they ease the access of bacteria and fungi, disturb the physiological balance and stress the fish which may lead to further diseases. They may even cause death if the damages becomes too severe (Pike and Wadsworth, 1999). The salmon lice do not only affect the farmed salmon, but also the wild salmon too (Rosenberg, 2008). This increases the pressure against the salmon industry to control the number of salmon lice in the production.

Salmon lice are strictly regulated by the government regarding the amount of salmon lice per fish at each facility on a weekly basis. It is required that the average number of salmon lice do not exceed a total of 0.5 sexual mature females per fish in the whole fish farm. There are some exceptions to this limit, like in the spring when the wild smolt travels from the rivers to the sea. The limit is 0.2 sexual mature female lice per fish in the fish farm, over the span of a couple weeks to reduce the chance of infecting the wild smolt (Weblink#1 www.lovddata.no).

To keep track of the number of salmon lice in a facility, manual salmon lice counting is performed every week throughout the year. This is done manually and 10-20 salmons are caught with a casting net, anesthetized, held by hand during counting and then released back into the cage. The stages which are registered are adult females, motile stages and attached stages. In the end the total number of adult females makes up the total average number for the entire facility.

Treatment against the salmon lice

When/if the limit of average mature salmon lice per fish (0.2/0.5) are reached or exceeded, measures must be done. The salmon must be de-liced, and there are many ways to do this. But there are two main approaches for treatment: medical and non-medical treatments. The medical treatments are based on bathing the salmon in water containing different substances like pyrethrins, pyrethroids, chitin synthesis-blockers and organic phosphorus-insecticides which are taken up by the salmon lice. For example, organic phosphorous-insecticides mainly work by inhibiting the actions of acetylcholine-esterase, which blocks the decomposition of the transmitter-substance acetylcholine, which again lead to overstimulating followed by blocking of the actual receptors and leads to death (Weblink#2 www.legemiddelverket.no).

Because of possible negative effects on ecosystems, there is now focus on developing and improving the non-medical treatments (Weblink#3 www.hi.no). These treatments do not include adding non-native compounds, and are thus thought to be more environment friendly. One popular method is to add other fish species to the salmon cages, with the purpose of eating the salmon lice from the body of the salmon. The main species used as cleaner fish in Norway are lumpfish (*Cyclopterus lumpus*) and wrasse (*Labrus bergylta*).

Other methods include mechanical removal of the salmon lice, the Licelazer (produced by Stingray Marine Solutions, (Weblink#4 www.stingray.no), Skamik, mechanical removal of the salmon lice with brushes and water (Weblink#5 www.skamik.no) and Optilice® which removes the salmon lice with heated water (Weblink6 www.optimarstette.com). The lastly mentioned methods may have a negative effect on the salmon due to rough handling which again may lead to damage in the skin and mucus layer, posing a risk of infections (Weblink7 www.vetinst.no).

What could be done differently?

No matter the treatment used, there still have to be performed frequent counts of salmon lice which means handling of the fish and potential of reduced fish welfare. Handling of the fish can as mentioned earlier lead to loss of scales and damages in the mucus and skin layer, which give access to bacteria, cause diseases, slow growth and even death.

Salmon lice counting is performed every week at a facility with sea temperatures over 4 °C, during the production time of the salmon which is approximately 14-22 months (Weblink#8 www.laks.no). This mean that the fish is handled at a minimum of 56-88 times from sea setting to slaughter facilities only due to salmon lice counting. In addition, the fish will be handled many more times in the context of other necessary operations such as delousing, sorting, splitting and many more. So, if it is possible to create a tool or practice which does not include handling of the fish, one could save the fish from a lot of stress and other possible disadvantages.

If we then look at the costs for the breeding company, the cost of one salmon lice counting is: approx. 4 hours of work (depends on number of fish cages in the facility and other factors) x 215 kr/h (average hourly wage taken from personal work experience, but varies from company to company and is affected by seniority and other factors) x two - three employees

(salmon lice counting normally require two - three persons, based on own work experience), which will give: 1.720-2.580 kr per salmon lice count. Total costs during the production time for one facility will be approx. from 1.720kr x 56 times = 96.320 NOK, to 2.580kr x 88 times = 227.040 NOK. These costs should be considered as a minimum because there are more factors that affect the total cost, such as loss of salmon biomass due to stress, fuel for the boats, anaesthetics, wear and tear on the boats and other equipment, and more.

What is environmental DNA, and why it is used

All living aquatic organisms shed DNA into the water via e.g. skin, urine and faeces and sexual products (Taberlet *et al*, 2012), and such DNA is referred to as environmental DNA (hereafter eDNA). By sampling and filtering water or even faeces and mud/soil, one can detect organisms, just based on analysing the captured eDNA (Taberlet *et al*, 2012). eDNA is shed intra- and extracellular DNA from various organisms and consists of nuclear and organelle DNA (Taberlet *et al*, 2012). eDNA degrade in the environment due to abiotic (e.g. temperature, acidity and UV-radiation) and biotic factors (fungi, endonucleases, bacteria and so on), and it may also be adsorbed in inorganic and organic particles (Dejean *et al*, 2011; Levy-Booth *et al*, 2007). The type of environment also affects the degradation rate, where warm and wet conditions increase the degradation and cold and dry conditions slows the degradation rate (Willerslev and Cooper, 2005). The best way to store sampled eDNA during transport is in a sterile, airtight container inside of a cooled, dark box. Preferably a styrofoam box containing dry ice or cooling elements. For longer storage, the samples should be stored in a -80 °C freezer until extraction (Jerde *et al*, 2011).

Extraction of DNA from samples

There are several procedures to sample and extract DNA depending on the type of environment and target species as shown in Table 1.

Table 1 “Comparison of capture and extraction methods used for detecting biodiversity in water with eDNA. This selection is not exhaustive, but rather exemplifies the variability in capture methods, extraction methods, sample effort (i.e., water volume), sequencing approach, and combinations thereof across different taxa and freshwater environments.” Table from (Deiner *et al.*, 2015).

Capture method	Extraction method	Volume of water	Locus	Habitat	Targeted or metabarcode	Taxonomic group	Sequencing technology	Reference
Precipitation	Qiagen DNeasy	3 × 15 mL	Cyt b	Lentic	Targeted	Amphibian	Sanger	(Ficetola <i>et al.</i> , 2008)
Filtration	Qiagen DNeasy	5 L	Cyt b	Lotic	Targeted	Amphibian	Sanger	(Goldberg <i>et al.</i> , 2011)
Filtration	MO BIO PowerWater	2 L	d-loop	Lotic	Targeted	Fish	Sanger	(Jerde <i>et al.</i> , 2011)
Precipitation and filtration	Qiagen DNeasy	2 L	Cyt b	Lotic	Targeted	Fish	Sanger	(Minamoto <i>et al.</i> , 2012)
Precipitation	Qiagen DNeasy	3 × 15 mL	Cyt b, COI	Lentic and lotic	Targeted	Fish, amphibian, crustacean, insect, mammal	Sanger	Thomsen <i>et al.</i> (2012)
Centrifugation	QIAamp DNA stool mini kit	(250 or 500 mL)	NADH 5	Lentic	Targeted	Mammal	Sanger	(Caldwell, Raley and Levine, 2007)
Filtration	EPICENTRE	4 L	16S rRNA	Lotic	Metabarcoding	Bacteria	Roche 454 GS-FLX-Ti	(Ghai <i>et al.</i> , 2011)
Filtration	Phenol–chloroform–isoamyl	10 L	16S rRNA	Lentic	Metabarcoding	Bacteria	Roche 454 GS-FLX-Ti and Illumina GA II	Oh <i>et al.</i> , (2011)
Filtration	Phenol–chloroform–isoamyl	45 L	16S rRNA	Lentic	Metabarcoding	Bacteria	Sanger	Debroas <i>et al.</i> , (2009)
Lyophilization	MO BIO ultraclean soil DNA kit	2 L	18S	Ground water	Metabarcoding	Plant	Sanger	Poté <i>et al.</i> , (2009)

The procedure used in this thesis, water filtration with Sterivex™ sterile filter unit (Merck KGaA, armstadt, Germany. EMD Millipore Corp., Billerica, MA USA) and extraction with Qiagen's DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) was chosen based on recommendations for identification of macro-invertebrate species (Deiner *et al*, 2015).

Identification of aquatic organisms using eDNA (barcoding, metabarcoding)

Two typical methods suited for this thesis are; barcoding, which is referred to taxonomic identification of species based on single specimen sequencing of diagnostic barcoding markers (Hebert, Ratnasingham and deWaard, 2003; Figure 2).

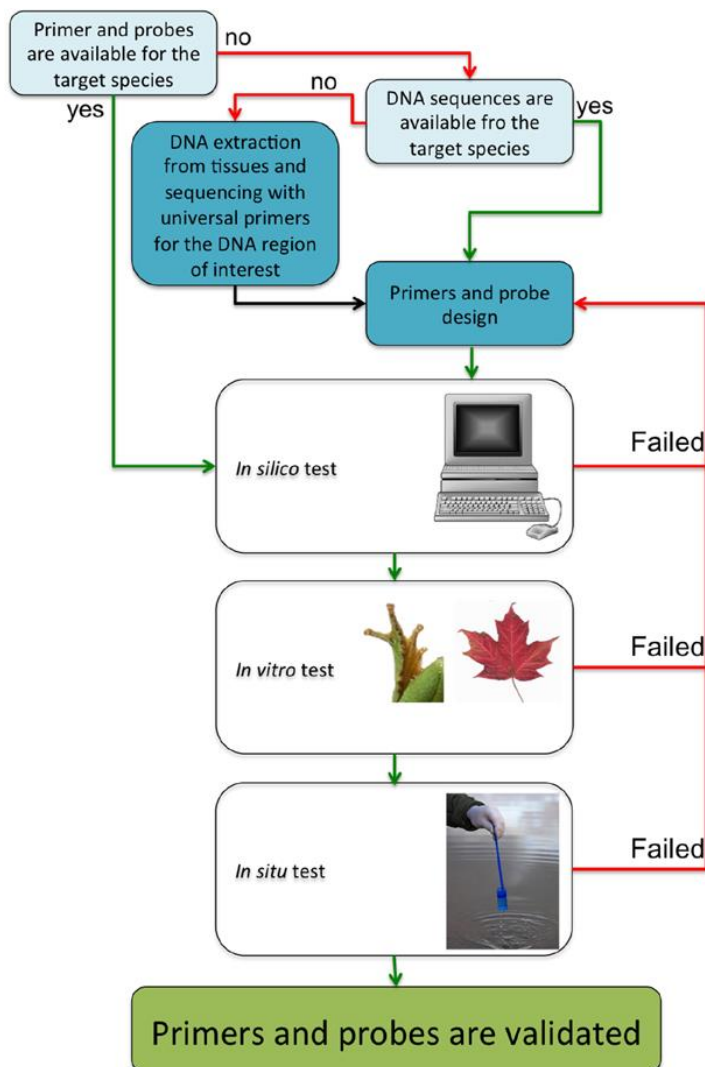


Figure 2 Procedure for testing primer (or probe) reliability, robustness and specificity (Herder et al., 2014).

Metabarcoding, which uses universal primers to amplify DNA from a group of target species (in this case metazoans), is the more general approach (Figure 3). When DNA amplification with the polymerase chain reaction (PCR) has been done, amplified fragments are sequenced with a Next-Generation Sequencer platform (NGS), before the result is compared with a reference database to identify the identity of the species included in the captured DNA (Taberlet *et al*, 2012).

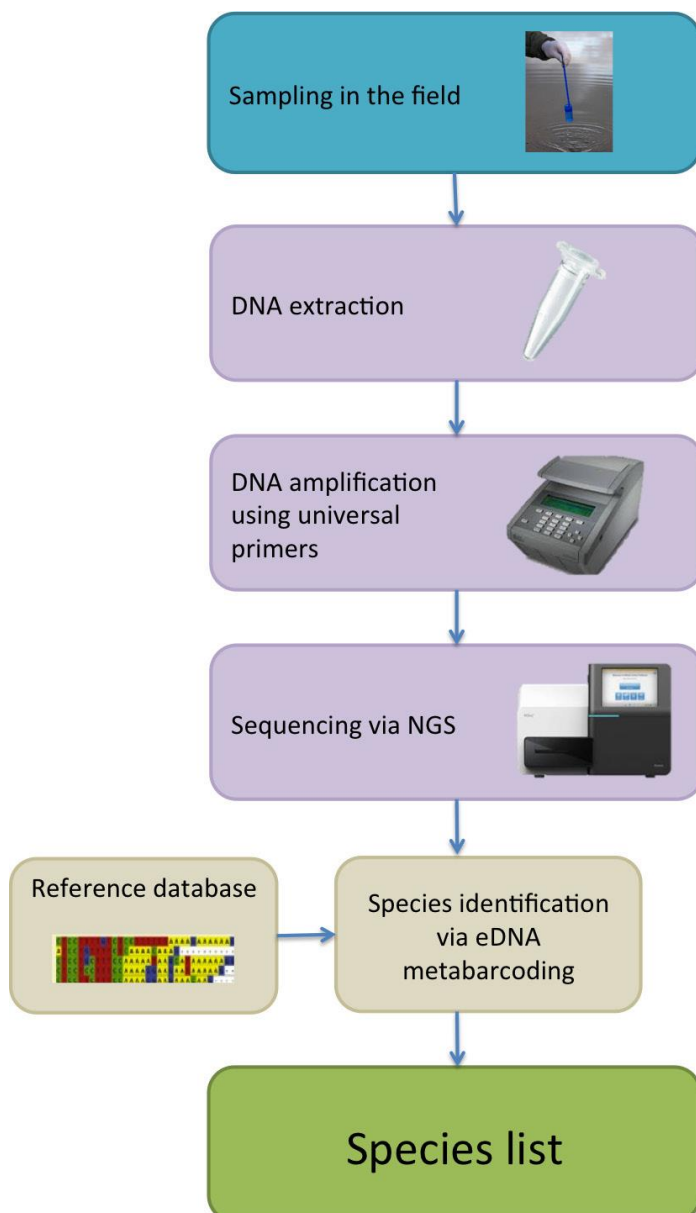


Figure 3 Methodology for analysis using an eDNA metabarcoding approach. Figure borrowed from (Valentini *et al*, 2009), modified by (Herder *et al*, 2014).

Metabarcoding was chosen in this thesis due to lack of time to design and validate novel primer/probes specific for salmon lice. Metabarcoding will also provide valuable information about which other species can be found in and around the salmon facility

Quantification of aquatic organisms using eDNA

Quantification of species with eDNA can be achieved using different approaches, despite being controversial. One approach is species-specific quantitative PCR (qPCR) where a threshold cycle that corresponds with the lowest amount of target DNA is identified. This value can then be compared to a standard curve with known amounts of DNA to identify the environmental abundance (Biggs *et al*, 2014). The qPCR method presently represents the gold-standard method for quantifying organisms using eDNA. Another approach, which this thesis is based on, is eDNA metabarcoding that use NGS to quantify the number of sequence reads after amplification. Here, the number of sequence reads are also related to a standard curve with known amounts of DNA/organisms to quantify the abundance of a particular species in the sample (Takahara, 2012).

The main advantage of using eDNA methods to detect and quantify organisms in aquatic environments is that eDNA is no-invasive and have proved more powerful in terms of detecting species than traditional methods (Ficetola *et al*, 2008; Boussarie *et al*, 2018). These are also some of the reasons of why eDNA was used in this thesis. eDNA may be a promising tool for the salmon industry, which potentially could reduce the handling of the fish and provide better welfare, by making it possible to count salmon lice without even touching the fish. There is clearly much to save by not performing manual salmon lice counting, when it comes to fish welfare and costs. Which leads to the main research question of the thesis:

Is it possible to; 1, detect salmon lice in aa aquaculture facility using environmental DNA, and 2, perform salmon lice-counting or to keep control on the amount of salmon lice in an aquaculture facility, without physically handling the fish?

Material and methods

Field procedures

Water sampling

Water was sampled at an aquaculture facility located in southern part of Skjervøy municipality, Troms, Northern Norway. The location contained salmon smolt from the 2017 generation (introduced in the sea in 2017). Seven cages had fish in it when sampling began, and eight to nine when sampling finished (approx. 800,000 – 1,000,000 fish in total in the facility). The sampling was performed every second week from start (week 29, 2017) until the sea temperature started to drop (week 45, 2017, Figure 4).

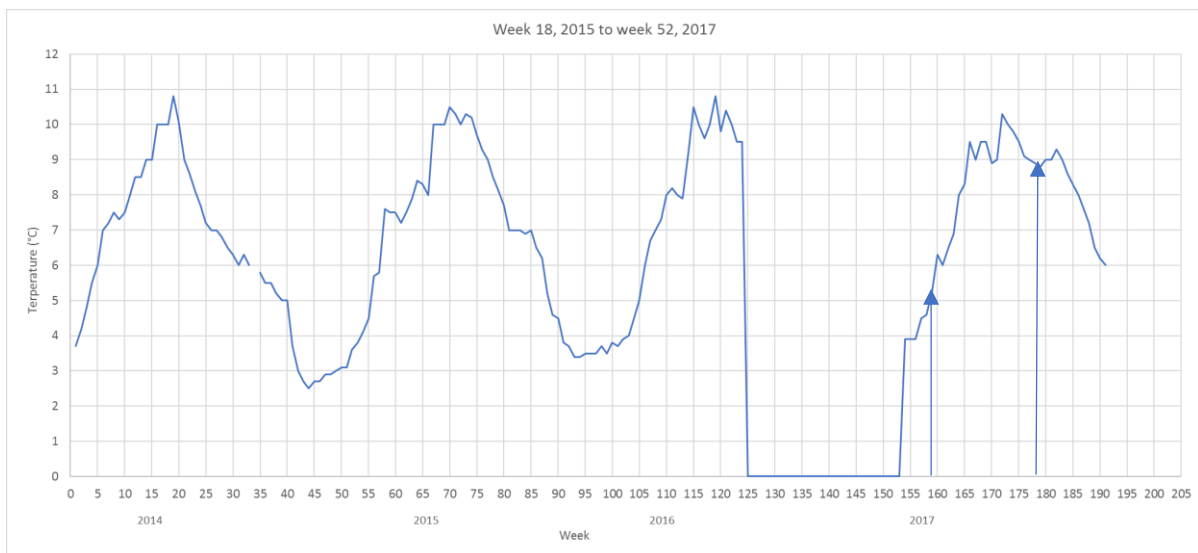


Figure 4 Logged sea temperature from the salmon farming facility; Uløybukt, from week 18, 2014 to week 52, 2017. The gap in the temperature record between 2016 and 2017 was because the facility was empty since all salmon was slaughtered, and no temperature was logged. Arrows indicate when sampling performed in this work started and ended. Curve made based on weekly recordings retrieved from Weblink#9 www.barentswatch.no.

Water was collected at 1.5 m depth in three cages containing fish. To check if it was possible to detect any drifting eDNA, we also took samples at fixed points on the northern and southern side of the middle of the salmon farm (illustrated in Figure 5), respectively at 1.5 m and 6 m depth.

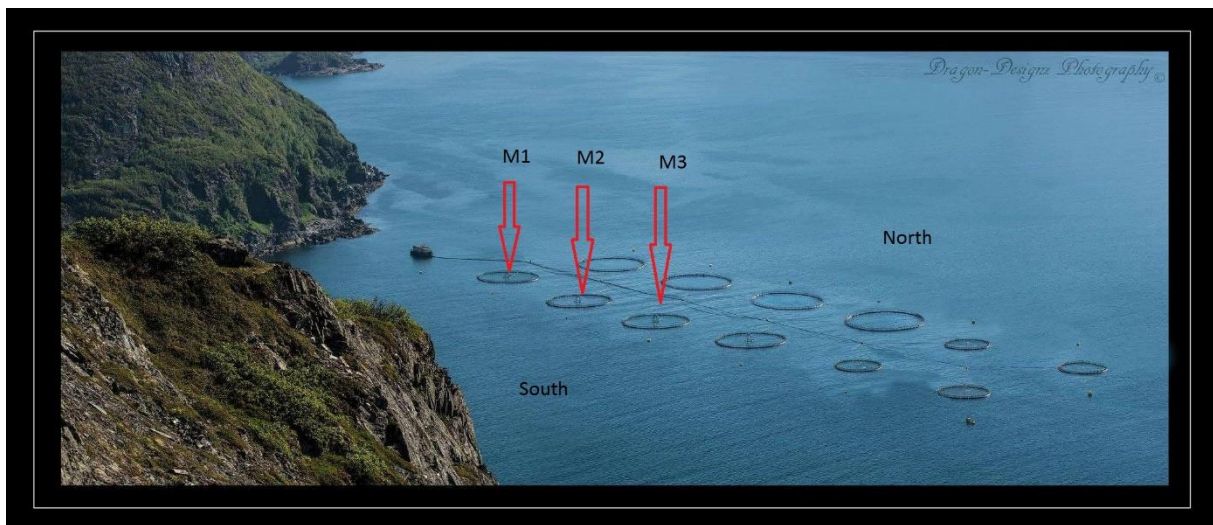


Figure 5 “Skjervøy Vest” facility, and shows the locations of where the samples were taken in the facility in Uløybukt: in M1, M2, M3, northern and southern side (photo edited by me, taken by Ronni Bless Bekkemellem/ Dragon-Design Photography).

1 L of water was collected from five locations within each fish cage, and mixed in a 5 L plastic can. The can was shaken to mix the water, before 0.5 L was filtered through each of five filters from each cage. Additionally, 3-5 L of water was collected at each depth (1.5 and 6 m) at one fixed position on the northern and southern side of the facility, before filtering (five filters x 0.5 L) to be able to monitor drifting salmon lice DNA if wanted.

The reason water was sampled from five different location at each cage was to ensure a representative sample of eDNA was caught. It was assumed that the top 5 metres of the water column inside the cages in this survey were well mixed. This was because the fish cages were mounted with salmon lice skirts which act as an aid to prevent salmon lice larvae from settling on the fish. The skirts are tied all the way around the collar of the plastic cage itself and overlap. It also hangs down five meters, so that the top of the water column is protected from the larvae drifting in the top layer of the water. There may of course be some differences in the water column due to mixing when the currents are strong, and because of thermal differences in the different water layers, and currents made by the fish itself.

Decontamination and collection of seawater

Equipment such as sampling bottles, beakers, gloves and cans were thoroughly cleaned with 10 % bleach and 50 % EtOH at the start. To be able to collect water at desired depth, a Niskin sampling bottle model 1010-2.5 L from GeneralOceanics was used. One L of water from five spots at each cage was kept in a cleaned 5 L plastic can. The sampled water from each location was filtered right away before cleaning process was repeated and moving to the next location. A blank sample was also included by filtering air around the boat used for sampling to account for contamination from this source.

Filtering for eDNA

Filtering was performed by pouring water from the 5 L can to a 1 L measuring beaker to ease the access for the syringe. A sterile 50/60 ml syringe from BD Plastipak was used to press water through the filters (Filter, Sterivex, sterile filter unit, Merck KGaA, Darmstadt, Germany. EMD Millipore Corporation, Billerica, MA USA). A total of 0.5 L of water was pressed through each of the five filters from each location to achieve a good mean value of eDNA at later analysis. After filtering, the filters were placed in new sterile 50 ml Greiner centrifuge tube and stored in a Styrofoam box containing freeze packs to delay degradation of eventual DNA in the filter. After filtering was done, filters were transported to NFH, UiT, Tromsø, and placed in an - 80 °C freezer until extraction.

Collection of live salmon lice

Live salmon lice were collected over the span of a couple days at the salmon slaughter factory of Arnøy Laks Slakteri A/S located on Lauksletta, Arnøy, in Skjervøy municipality. Over 1.000 live salmon lice were handpicked. The most appropriate spot in the production line to pick the salmon lice was right after the bleeder/stunning machine. This way it was possible to get the salmon lice as fresh as possible. The salmon lice were kept alive in 1 L plastic containers for a couple of days to make sure they emptied their gastrointestinal system and in that way, avoid the risk of getting too much host DNA in the water during the later experiment. The water in the containers were changed daily to ensure survival of the salmon lice.

Wet lab experiment



Figure 6 Dilution setup, all blue squares represents a 3 L can. The three top ones contained 3 salmon lice in 3 L of water each and were used as reservoir for “salmon lice water” which were added to the cans below. The ones below the three “reservoirs” contained the given amount of added salmon lice water to achieve a total volume of 3 L.

To make the standard curve, a setup was made which simulated the amount of salmon lice in a cage. It was based on the actual volume of one cage, number of salmon in the cage when sampling occurred, and the number of counted salmon lice done by the company. Calculations can be seen in Appendix I and II.

The experiment was done by placing a known number of salmon lice in closable cans with a known amount of filtered seawater. Filtered seawater was used to avoid contamination of DNA from too many other organisms. Only sexual mature female salmon lice were used in the experiment since those are mostly focused on in commercial salmon farming. The females with egg strings had the strings removed to avoid release of egg in the water during the experiment.

Three salmon lice were placed in five cans each, then put in a fridge and left for 24 hours. As an additional step, three salmon lice were placed in five cans each containing three

L of unfiltered sea water. Next step was to dilute “salmon lice water” from three of the filtered cans, into new cans to reach wanted concentrations of salmon lice. Appendix III and Figure 6 shows the experiment setup with the amount of filtered seawater and lice water added to the new cans. This tool (standard curve) is then used when DNA from the field samples is extracted.

The DNA from the field will be compared to the standard curve to see if there is a compliance between the amount of DNA and actual number of salmon lice in the aquaculture facility. When all of the dilutions were done, three filters were filtered from each can. Then the filters were placed in marked 50 ml centrifuge tubes and stored at -80 °C until extraction. We had access to the data from the traditional salmon lice counting performed by the workers at the aquaculture facility that was carried out each week during the summer when the water sampling was carried out. This made it possible to compare the estimated amount of salmon lice based on DNA, to the manually counted salmon lice.

The study required many steps both in the field and in the laboratory. An overview over all steps can be seen in Figure 7 below.

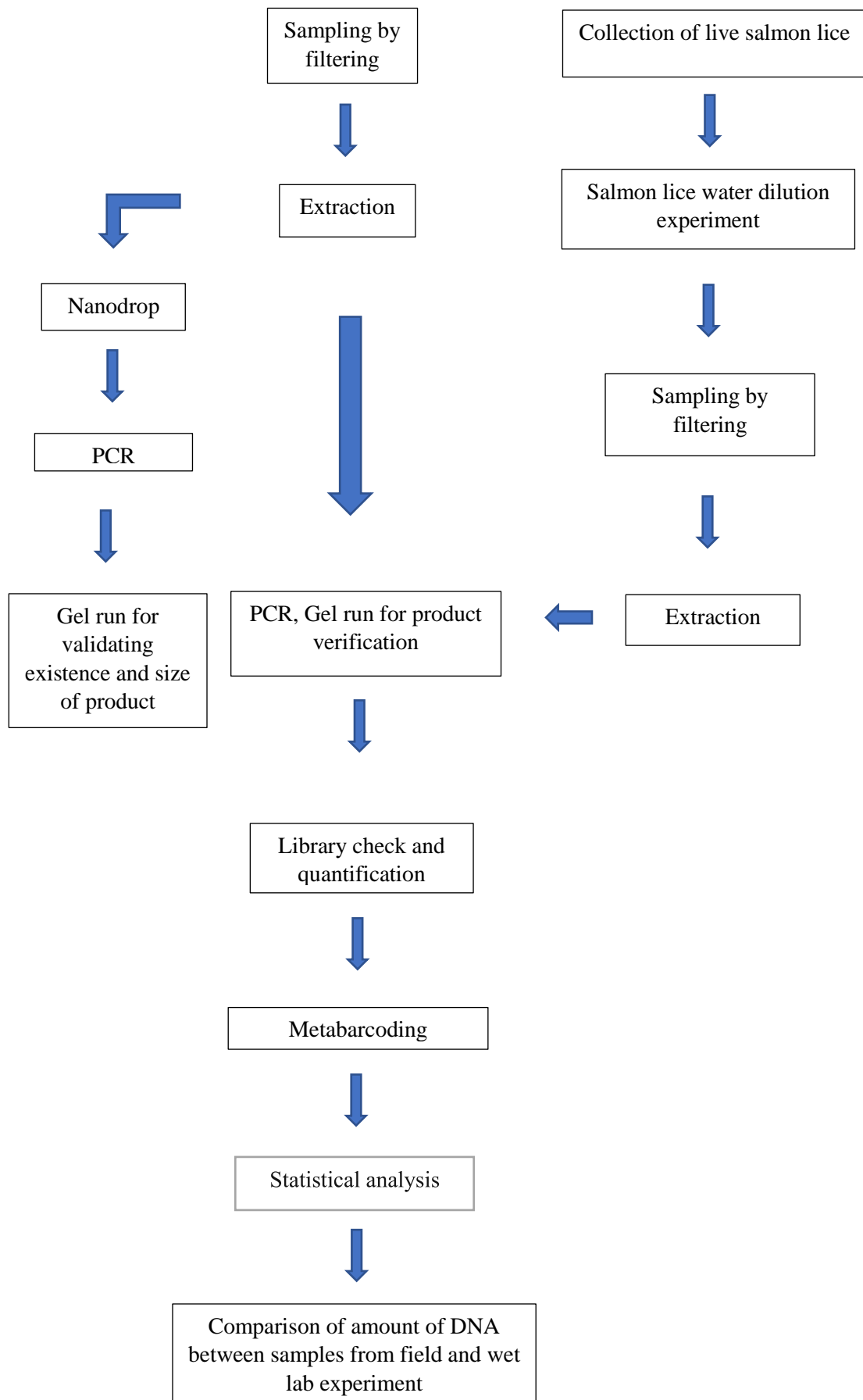


Figure 7 Overview of the steps performed from sampling of water to final analysis.

Laboratory procedures

Table 2 Sampling dates, location and average number of salmon lice manually counted in the facilities at the same week sampling occurred. Number of salmon lice is per fish retrieved from www.barentswatch.no (weblink#10).

Week	Date	Location	Average number of sexual mature female salmon lice per fish	Average number of salmon lice per fish
29	20.07.2017	Uløy	0.00	0.01
31	05.08.2017	Uløy	0.00	0.03
33	19.08.2017	Uløy	0.00	0.04
35	03.09.2017	Uløy	0.00	0.33
37	12.09.2017	Uløy	0.00	0.30
39	01.10.2017	Uløy	0.00	0.85
41	15.10.2017	Uløy	0.00	3.15
43	25.10.2017	Uløy	0.00	0.70
45	07.11.2017	Uløy	0.00	0.00

Sample dates chosen to be analysed was based on the amount of salmon lice found in traditional salmon lice counts performed by the farming company. As seen highlighted in Table 2, salmon lice appeared most abundant in weeks 29, 37, 41 and 43, and these samples was chosen for inclusion in this study. An overview of sample dates, facility, sampling locations in the facility and number of filters used at each location can be found in Appendix IV.

DNA extraction

The extraction of eDNA was performed in a locked room specially organized for eDNA extraction with strict routines for cleaning and introduction of material and people. The procedure used was: DNA extraction based on Qiagen DNeasy blood and Tissue kit (Appendix V). The stock of the extracted product was placed in marked 2 ml centrifuge tubes and placed

in an -80 °C freezer for storage. An aliquot from each sample was placed on a 96 well PCR tray for further analysis.

DNA quantitation

The concentration of DNA were measured on a NanoDrop ND-1000 spectrophotometer (Saveen and Werner ab, ©2014, Limhamn, Sweden), using the program “Nucleic Acid”. The machine was initialized by placing a 1 µl drop of sterile water. The reader was cleaned and a 1 µl drop of buffer (elution buffer) was placed on the reader to get a blank measurement. Then all aliquots were analyzed, as 1 µl samples.

PCR

PCR was prepared in a clean UV-cabinet with overflow of air to reduce the chance of contamination. The procedure used was made by associated professor Dr. Kim Præbel, NFH, UiT (Appendix VI).

Gel run

2 µ PCR products was ran on a 1 % agarose gel, added 5 µl ethidium bromide, at 200 V or 12 min. The gel was then placed in an UV camera chamber which is an apparatus that illuminates the products in the gel, making it possible to determine the presence or absence and eventually the size of the product.

Metabarcoding procedure

DNA amplification

The Leray-XT primer set (Wangensteen *et al*, 2018) was used, which is a highly-degenerated primer set targeting the 313 bp Leray fragment (Leray *et al*, 2013) of the mitochondrial marker cytochrome c oxidase subunit I (COI) from a wide selection of eukaryotic groups, including practically all metazoans. Forward primer used was miCOIint-XT (5`-GGWACWRGWTGRACWITITAYCCYCC-3`), modified by Wangenstein et al., 2018, from

the mlCOIintF primer (Leray *et al*, 2013). Reverse primer used was jgHCO2198 (5'-TAIACYTCIGGRTGICCRAARAAYCA-3'; Geller *et al*, 2013).

A single 1-step PCR protocol was used to amplify the Leray-XT fragment. The metabarcoding primers had an eight-base oligo-tag attached (each tag with at least 3 differences out of 8 bases). A variable number (2-4) of leading Ns was also added to increase the sequence variability of the amplicon sequences (Guardiola *et al*, 2015). Each forward and reverse primer had 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 had 96 such different pairs, so we could multiplex up to 96 samples in one library. Since we analysed a total of 269 samples, we prepared a total of three different metabarcoding libraries, each one labelled with a different 6-base library tag, which were pooled and analysed together within the same MiSeq sequencing run.

The PCR protocol included Amplitaq Gold 360 master mix (ThermoFisher) (Weblink#11 www.thermofisher.com), and bovine serum albumin (BSA) (Weblink#12 www.thermofisher.com). We used a standard sample volume of 5 µl of the extracted DNA for all PCR amplifications. The PCR mix can be seen in Appendix VII.

The primers were not added to the PCR master mix for aliquoting, but added to every individual sample because they were amplified with different versions of the primer set. PCR programme used can be seen in Appendix VIII and PCR plate setup in Appendix IX.

Library pooling and concentration

The amplified product was pooled in a 2 ml Eppendorf tube and homogenized thoroughly before it was purified using MinElute columns for removal of DNA fragments below 70 bp. This step did also concentrate the amplified DNA approx. 10 times. (Weblink#13

www.qiagen.com). The DNA concentration was then measured with a Qubit fluorimeter (Weblink#14 www.thermofisher.com), using a Broad-Range DNA quantification kit (Weblink#15 www.thermofisher.com).

Library preparation

A PCR-free ligation protocol was used, the NEXTflex PCR-Free DNA Sequencing Kit from BIOO Scientific (Weblink#16 www.biooscientific.com). 3 µg of DNA (up to 40 µl of the previous pool) was used as starting material. The method used for preparing a COI library was the same as described in the kit manual (Weblink#17 www.biooscientific.com).

Library quality control and quantification

The library was analysed with a Bioanalyzer (Weblink#18 www.genomics.agilent.com) to check if the ligation went well, following the manufacturer`s instructions. To be able to load the correct concentration of the library in the Illumina MiSeq to avoid under/over clustering in the flow cell, the exact concentration was determined using qPCR. The NEBNext Library Quant Kit from New England Biolabs (Weblink#19 www.neb.com) were used. The dilution of the library used was 1:5,000, 1:10,000 and 1:50,000.

Library dilution and MiSeq loading

A v3 MiSeq sequencing kit (2x300 bp) was used, but with 2x250 cycles for better error rates. The optimal final target concentration of the libraries is 18 pM, but as calculations in Appendix X show, we ended up with 16,6 pM. The loading sample including a 1 % of PhiX genomic library (for internal sequencing control for calculating error rates per cycle) (Weblink#20 www.illumina.com), was prepared and loaded into the Illumina MiSeq. Calculations in Appendix X

Bioinformatics analysis

The bioinformatics analyses were done by Dr. Owen Wangenstein and, were based on the OBITools metabarcoding software suite (Boyer *et al*, 2016). Read quality assessment was performed with FastQC. Paired-end read alignment was done with Illumina paired-end, and

reads with a paired-end alignment quality score > 40 were retained. Demultiplexing and primer removal were achieved using ngsfilter. Obigrep was applied to select all aligned reads with a length between 303-323 bp and without ambiguous bases. Obiuniq was used to dereplicate the reads and the uchime-denovo algorithm (Edgar *et al*, 2011), implemented in VSEARCH (Rognes *et al*, 2016) was used to remove chimeric sequences. Amplicon clustering was performed using the SWARM 2.0 algorithm (Mahé *et al*, 2015) with a distance value of $d=13$, which offers a conservative solution to the high variability of the COI gene (Siegenthaler *et al*, 2018). Taxonomic assignment of the representative sequences for each MOTU was performed using the ecotag algorithm (Boyer *et al*, 2016), using a local reference database (Wangenstein *et al*, 2018) containing curated COI sequences retrieved from the BOLD database (Ratnasingham and Hebert, 2007), and the EMBL repository (Kulikova *et al*, 2004). This algorithm uses a phylogenetic approach to assign sequences to the most reliable monophyletic unit, so that sequences can be assigned to different taxonomic ranks, depending on the density of the reference database. All MOTUs in the resulting dataset that were taxonomically assigned as salmon louse (*Lepeophtheirus salmonis*) which were probably split by SWARM 2.0 due to feeble links in the resulting network (Mahé *et al*, 2015) were collapsed into a single MOTU, in order to get the total of read counts from this species.

Statistical analysis

The standard curve was made by converting the reads of salmon lice of the diluted experiment samples (Appendix XI) from filtered water to unfiltered water. This was done because the ratio of salmon lice reads to total reads is lower when measured in unfiltered water (as is the case for all samples taken from the field). This conversion was achieved by introducing a correction factor, calculated from dividing the average number of salmon lice from nine samples with the same concentration of salmon lice, analysed in both filtered and unfiltered water, and after correcting for the sequencing depth between libraries 1 and 3 as explained below (Appendix XIII and XV).

Due to stochastic factors during library preparation and sequencing procedures, the three sequenced libraries had different sequencing depths (i.e. different total number of reads obtained from each library) To further standardize the libraries and the field sample reads in order to get a quantitative response (Appendix XII), we had to correct the differences in the total sequencing depth of all the libraries using a correction factor based on the average of total

reads for two internal standards that were analysed in all libraries. Library 2 and 3 were converted based on the sequencing depth of Library 1 (Appendix XIV and XVI).

The standard curve was made by plotting the corrected number of reads (Appendix XV) from the diluted sample using a Log-10 transformation of both axes. A linear model was calculated using R (R Core Team, 2017) (Figure 8). The converted field sample reads were plotted to the linear model after a Log-10 transformation to match the curve (Figure 9 and 10) and the inferred concentrations of salmon lice per salmon were calculated for all field water samples from this linear model.

Results

A total of 22,821,455 raw reads were obtained from the sequencing of all samples. 9,154 MOTUs with 10,234,572 reads remained after the initial quality filtering. Out of this, 1,210,616 reads were assigned to salmon lice.

The standard curve, based on a linear model, based on the dilution experiment (Figure 6), showed that there was good agreement between number of salmon lice reads and number of mature female lice per salmon (Figure 8). Each dilution had a total of nine replicates, except the one that were added 500 ml of “salmon lice water” which had 8 replicates.

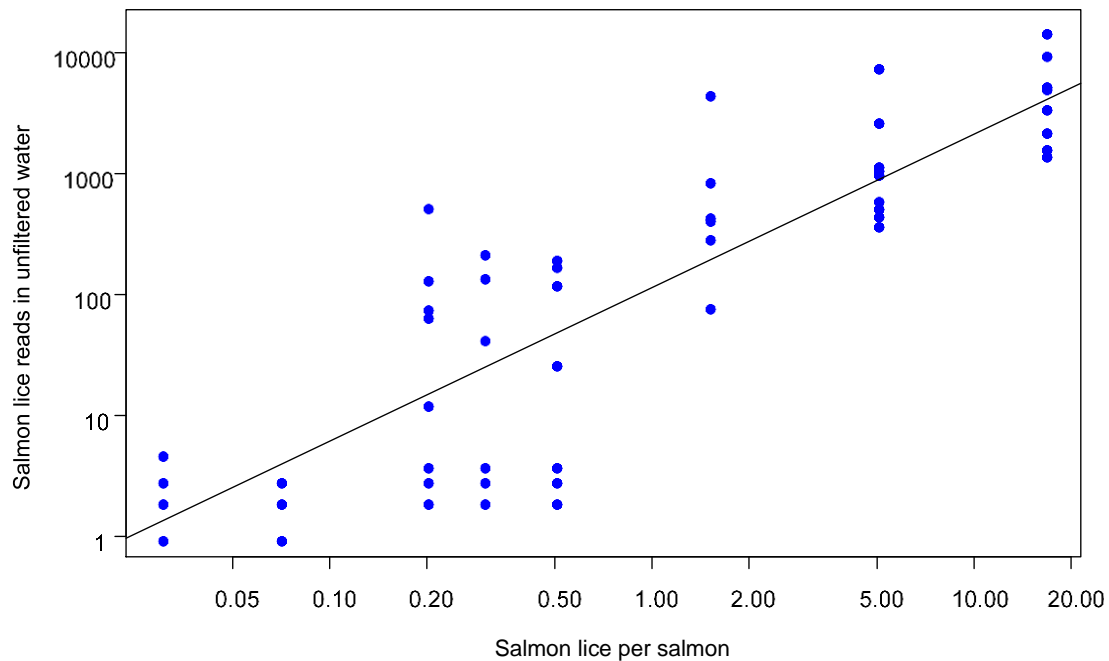


Figure 8 Standard curve based on the corrected number of reads from the dilution experiment. The number of reads increases with the amount of added salmon lice water. Both axes were Log10-transformed. Proof of correlation between the amount of salmon lice and the amount of released salmon lice DNA in the water.

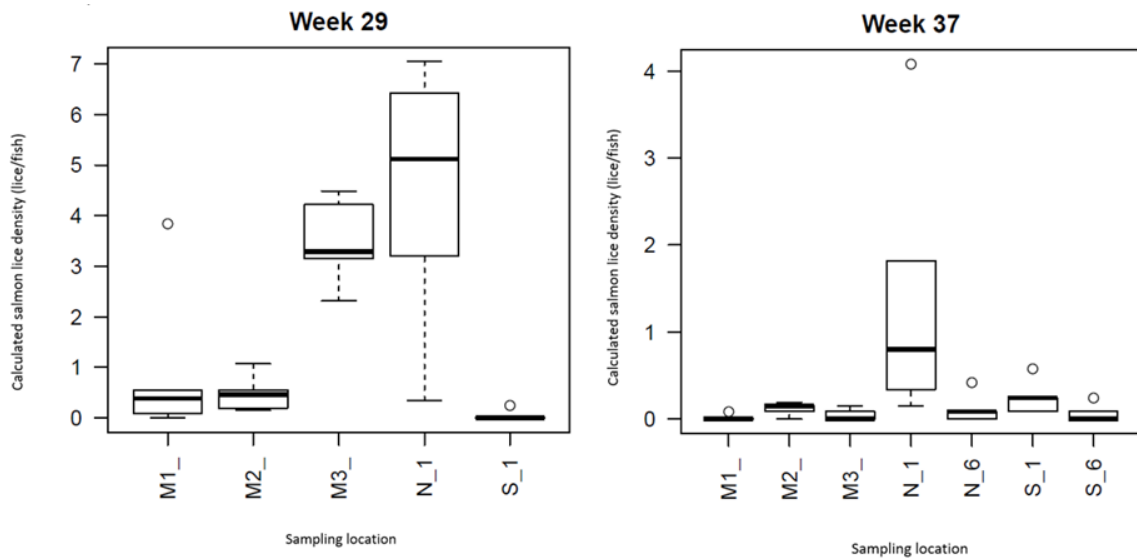


Figure 9 Average salmon lice per fish for each sampling location week 29 and 37. Results found by plotting Log-10 converted field sample reads to the standard curve in Figure 8. Some of the replicates for each location had a big difference between the number of reads, and are shown as black dots.

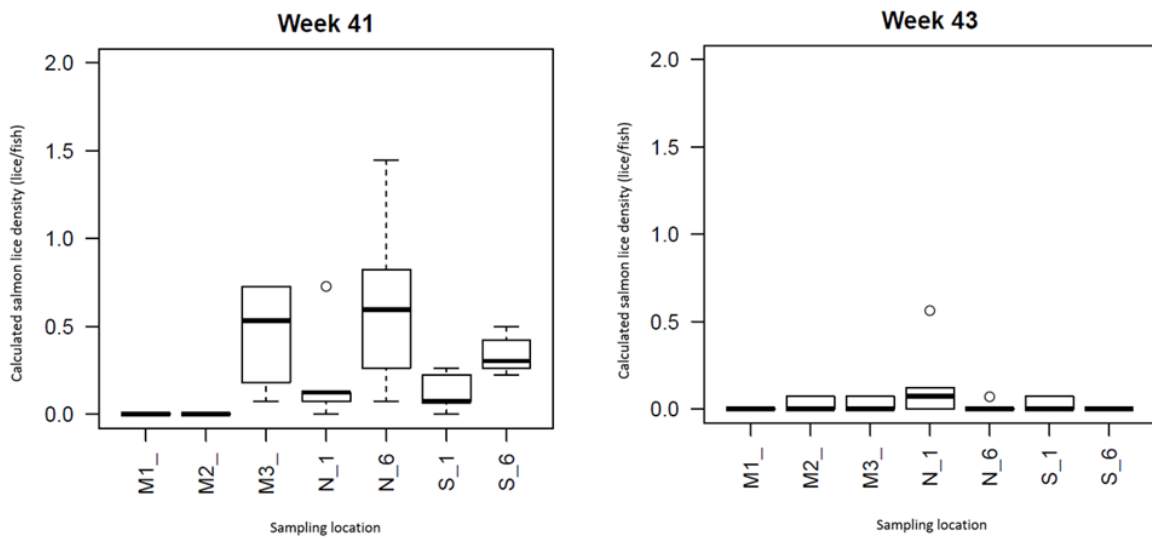


Figure 10 Average salmon lice per fish for each sampling location week 41 and 43. Results achieved by plotting Log-10 converted field sample reads to the standard curve in Figure 8. Some of the replicates for each location had a big difference between the number of reads, and are shown as black dots.

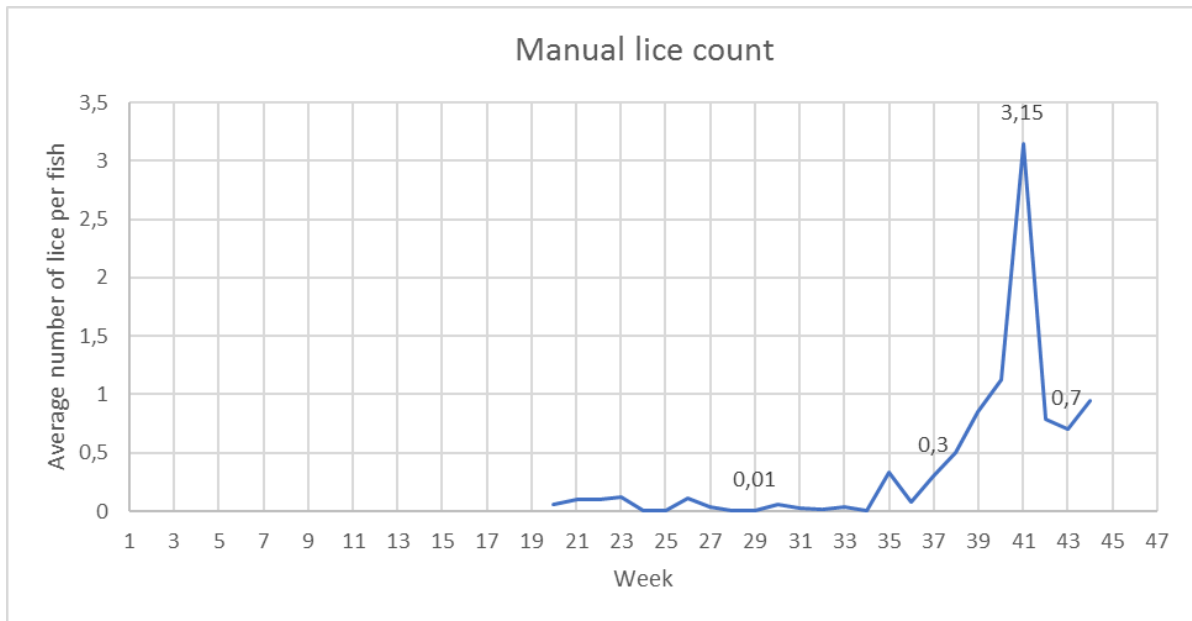


Figure 11 Weekly average number of salmon lice per fish in the facility, from week 20 to week 44, 2017.

Table 3 Comparison between eDNA based salmon lice count and manual salmon lice count summed up.

Sampling	eDNA (salmon lice per fish)	Manual count (salmon lice per fish)
Week 29	0.00 - 5	0.01
Week 37	0.00 - 0.85	0.30
Week 41	0.00 - 0.60	3.15
Week 43	0.00 - 0.15	0.70

Comparison between curve sample reads and field sample reads

By comparing the calculated number of salmon lice per salmon for the cages in week 29 (Figure 9), and the manually counted average (Figure 11), we see that M1 and M2 both estimates 0.50 salmon lice per fish vs 0.01 manually counted (Table 3). M3 estimates approx. 3.30 salmon lice per fish versus 0.01 manually counted. The northern location at 1.5 m depth estimates salmon 5 salmon lice per fish, and the southern location at 1.5 m estimates approx. 0.10 salmon lice per fish.

Estimations for week 37 (Figure 9) shows 0.00-0.20 salmon lice per fish for all sampling locations, except the northern side at 1,5 m depth with approx. 0.85 salmon lice per fish. The manual counting estimated 0.30 salmon lice per fish in the facility (Figure 11).

For week 41 (Figure 10), we see that M1 and M2 both estimates 0.00 salmon lice per fish vs 0.01 manually counted, while M3 estimates approx. 0.55 salmon lice per fish versus 0.01 manually counted. The northern and southern locations both estimates round 0.20 salmon lice per fish at 1.5 m depth. The samples from 6m depth from the northern side estimates approx. 0.60 salmon lice per fish while the southern side estimates 0.40 salmon lice per fish. The manually counting estimated 3.15 salmon lice per fish in the facility (Figure 11).

Week 43 (Figure 10), estimations from all sampling locations is around 0.00-0.15 salmon lice per fish, while the manual counting estimated 0.70 salmon lice per fish in the facility.

Discussion

The present master thesis project had two main aims; 1) to see if salmon lice DNA were detectable in the field and evaluate eDNA as a tool for quantifying the amount of salmon lice in a salmon farm, and 2) to investigate how quantification based on eDNA compares to the traditional manual counting enforced by the restrictions made by the government.

By making an experiment with a set of dilutions with increased amount of added salmon lice water in a controlled environment (Figure 6), the results showed that it was possible to make a standard curve based on the increased amount of released salmon lice DNA added with the salmon lice water (Figure 8). This further suggested the possibility of quantifying salmon lice solely based on the amount of eDNA measured from the field water samples (salmon farm facility), if the salmon lice were detectable in the field. The present project indeed showed that salmon lice DNA could be detected in the field with filtering, extraction, metabarcoding and further methods used in this project which leads to the next aim of the study.

Was the eDNA based method quantitative enough to estimate the abundance of salmon in a salmon facility and did the estimations correlate to the manually counted number of salmon lice?

Estimations of salmon lice in the field based on eDNA has not been published so far, but several studies on fresh and marine organisms have shown positive correlation between the amount of shed DNA and biomass of the target species (Nevers *et al*, 2018; Thomsen *et al*, 2016; Takahara *et al*, 2012). We found that the estimated numbers of salmon lice per fish inferred from the sampled eDNA did not correspond that well with the manually counted number of salmon lice per fish during the same weeks. The expectations would be that the eDNA based salmon lice count number would follow the manually counted number of salmon lice per fish, at least to some degree. Especially in week 41 where the manually count showed 3.15 salmon lice per fish in the facility (Table 3). Despite this divergence in salmon lice abundance between the two methods, the present study has provided the initial results for future improvement of eDNA as a tool for monitoring salmon lice abundance. In other species, such as fishes, advances are being made towards developing reliable tools for detecting biomass (e.g. Ushio *et al*. 2018). Some of the reasons for the disagreement between the eDNA counting and the result from the manual count could stem from the practice that the average number of salmon lice per fish from the manual lice counting are given for all cages in the facility pooled together (seven to nine cages). This might be a factor that affects the reason why the number

was lower in the eDNA count. Some cages might have had higher number of salmon lice than the three cages we used in the survey. Which gave a higher total average number of salmon lice in the facility. Own experience with manual salmon lice counting have shown that the average number of salmon lice often differs a lot between the cages in the same facility, even if they lie next to each other. Another reason could be that metabarcoding of eDNA may not be accurate. Peters *et al*, 2017, suggests that it might would have been more appropriate to design species specific tools like qPCR probes which might have yielded in better quantitative information than the metabarcoding sequencing. However, Ushio *et al*, (2018) showed that metabarcoding, when performed with care, have equal quantitative value as qPCR, which validates the approach taken herein.

Even though there were some overlap between abundance estimates obtained by manual counting and eDNA (in week 29 and 37), the use of eDNA as a tool for detecting abundance of salmon lice needs improvement. But, as the standard curve (Figure 8) showed, it was possible to quantify the abundance of salmon lice with environmental DNA, at least in a controlled environment.

The estimated number of salmon lice per fish (Figure 9 and 10) yielded from the standard curve could be expected to be higher with eDNA than that of manual counting, because the metabarcoding method detects DNA from all life stages of the salmon louse, while manual counting only include the attached stages (chalimus, preadult and adult, Figure 1). The DNA detected in the water may indeed come from planktonic stages that cannot be counted with the manual recount and that could have different temporal dynamic patterns than the attached stages.

One pattern that should be noticed is that the samples from the northern sample location showed the highest amount of reads and number of salmon lice in all weeks analysed (Figure 9 and 10). One of the reason for this might be transport of eDNA and early life stages of salmon lice from all cages from the farm at the northern (and southern) sample locations approx. 150 meters away from the centre of the facility (Figure 5). The currents might have led salmon lice DNA, eggs and naupli from more than the three cages used in the survey past the sampling points.

One plausible explanation for higher abundance at the northern sample location, and not southern sample location, might be that the currents have been shown to have a slightly higher flow towards north in the strait between Uløya and Ravelseidet (approx. N 69° 51.3577718', S

69° 51.440173') in Troms, where the sampling was done (Figure 12). Troms County did a survey in 2009-2010 to map the ocean currents and further investigate the possible spread of diseases between salmon farms in the same areas. This survey resulted in an interactive map-service where it is possible to see models of the currents, temperature, salinity and more in different areas of Troms County (Weblink#21 www.stroms.no).

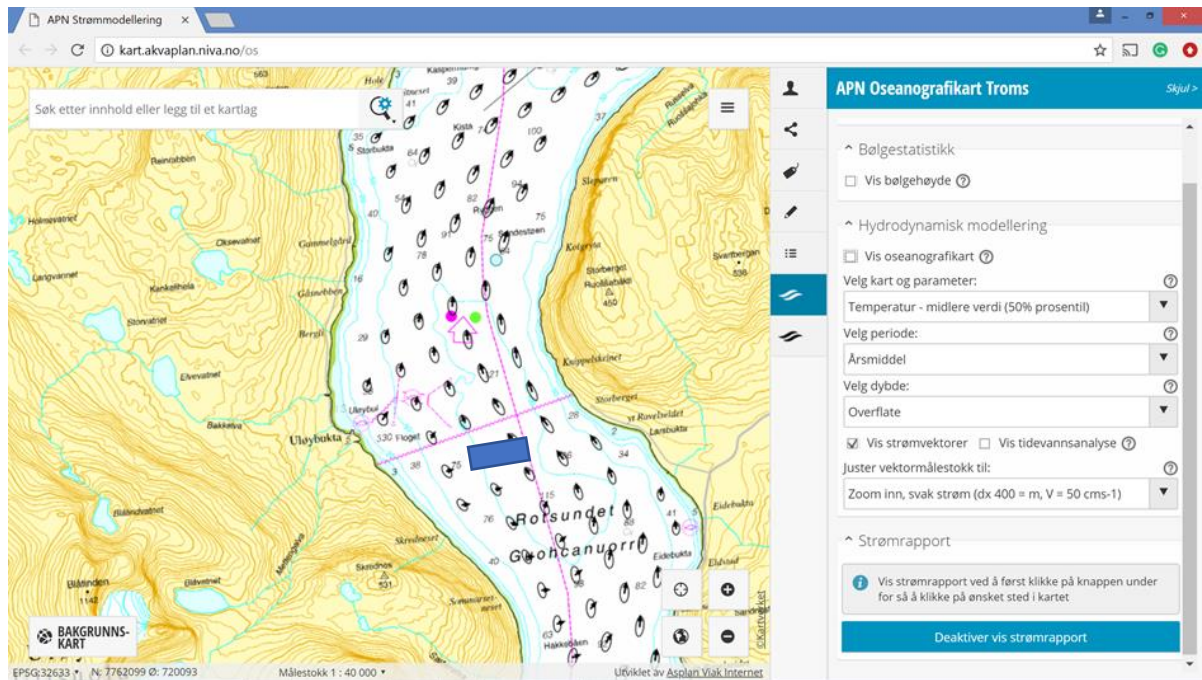


Figure 12 Map with modelling of the main direction of the current registered during the surveying period of 2009-2010. Arrows indicate the main feature of the current (Weblink# 22 www.akvaplan.niva.no/os). The blue square indicates the approximate location and direction of the farm where the sampling was performed (North upwards).

Another possibility of higher number of reads on the northern sampling location is that the tide has coincidentally been going the same direction (northward) on all four sampling days. This have unfortunately not been possible to figure out because of lacking information on the time of the day when the sampling occurred. The difference between high and low tide in the area has been recorded to be high (almost four meter) according to historical and current measurements (1952-2018) (Weblink#23 www.kartverket.no). This might have caused high movement of high water volume and carried salmon lice DNA to the northern sampling location. However, a recent study have shown that the tides scarcely affect the movement of nearshore eDNA (Kelly, Gallego and Jacobs-Palmer, 2018), and hence change the detection

limit. Thus, further studies are needed to understand why and how eDNA appeared more abundant in only one of the reference locations.

As mentioned above, the eDNA-based counting did not correlate with the manual counts, but eDNA-based counting of salmon lice may nonetheless be used as a predictive, early-detection method for future infestation outbreaks of salmon lice, but further validation is needed. Was the high peak in week 29 (Figure 9) detected by eDNA correlated to the peak in the manually counted salmon lice number in week 41 (Figure 11)? If so, have we detected an increased number of salmon lice eggs and nauplii?

In this project only a limited set of samples (week 29, 37, 41 and 43) of the total set of samples were analysed due to economic constraints. It would therefore be of great interest to see how the abundance estimates for the manual counts vs. eDNA for salmon lice changed and / or correlated in the weeks between week 29 and 37, as that would provide insights into what life stage of salmon lice that are prevalent in eDNA detection. This could even be combined with longer studies using a higher frequency of sampling events along different years to get a better picture of the temporal dynamic patterns obtained from eDNA and their correlation with patterns of manual salmon lice counts.

Future directions for using eDNA as a survey tool for salmon lice in aquaculture

Field practice

From this pilot study we have learned that the experiment design could have been changed in a couple of ways. Sampling could have been performed in all cages in the facility to get an overall average of the number of salmon lice per fish in the facility as it was done in the manual method. Also, if this had been considered at the start of the study, we would have been able to ask the farming company to save the counted average from each cage in the facility and in that way, been able to compare the results on cage level. Sampling should also have been performed over a longer period, at least one year, or even throughout the whole production time of the salmon. Samples from all sampling days should also have been extracted and analysed to get a better and broader overview of the changes in the amount of DNA and salmon lice. This way one might have been able to see differences due to natural factors such as sea temperature, changes in salinity, photoperiod and more.

Laboratory

Samples from more weeks should have been extracted to fill the gaps between the weeks extracted in this study e.g. between week 29 and 37 to be able to see if and how the estimated number of salmon lice changed according to the sea temperature, manual counting and more. These changes would however might have increased the total cost of the experiment and acquired time of the study beyond the limits of a normal master thesis.

The time and costs could have been shortened by producing a species-specific probe for salmon lice. The analysis would then have been done by qPCR and replacing the metabarcoding procedure. The bioinformatics procedure would also be shorter since there would only be one species to analyse.

The curve experiment design also had some factors that might interfered with the results. The curve was made with salmon lice in cans filled with filtered seawater instead of normal sea water. This was done because of fear of not being able to detect any salmon lice DNA in the most diluted cans. This has shown to be unnecessary and the curve should have been repeated with normal seawater only. By only using unfiltered seawater, the performed conversion between the filtered samples and unfiltered would have been unnecessary (see “Statistical analysis” in material and method, and Appendix XIII). The conversion between the three libraries to even out the sequencing depths should also be considered as a possible factor for loss of quantitative accuracy (Appendix XIV).

Conclusion

This project has shown that it is possible to detect salmon lice DNA in a farm for Atlantic salmon, by performing metabarcoding of DNA extractions from filtered water samples. The project was also able to make a standard curve, showing the number of salmon lice based on the amount of released DNA in the water. The comparison between the eDNA based salmon lice count and manual count did not correlate in such a manner that the salmon lice count could be replaced by eDNA, *per se*. Nevertheless, by performing further studies and refinement of the used methods, environmental DNA is predicted to be of great use in salmon farming in the future as a method to detect and monitor pathogens and parasites, and maybe even as a predictive early-warning tool.

Future thoughts on the use of eDNA in aquaculture regarding salmon lice and other possibilities

Manual salmon lice counting will most likely be revolutionized by eDNA tools in the future, hopefully decreasing the cost, time wasting and stress related to the traditional method. Further, if it is possible to detect the sex of the salmon lice from environmental DNA, it will be possible to predict the amount of female salmon lice in a salmon facility. It would then be possible to calculate/estimate how many sexually mature females will appear in the following days/weeks according to the temperature and existing amount of sexual mature females and pre- mature stages.

This project was a “pilot study” on the use of eDNA in the context of farming of Atlantic salmon in Norway and one of its major challenges, salmon lice. There are countless opportunities of possible studies that can be done in the future based on eDNA in aquaculture, not only on salmon farming. My predictions are that one might be able to detect diseases in fish farming long before an outbreak is visible and by monitoring the presence of the cause (bacteria, viruses, amoebas), and be able to predict possible outbreaks. This will of course require thorough field studies and over a long period, but the yield would be great if the fish farming industry is revolutionized with new molecular tools.

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Weblinks

Weblink#1 <https://lovdata.no/dokument/SF/forskrift/2012-12-05-1140> (visited 27.11.2017)

Weblink#2

<https://legemiddelverket.no/Documents/Veterin%C3%A6rmedisin/Terapianbefalinger/Behandling%20mot%20lakselus%20i%20oppdrettsanlegg.pdf> (visited 27.11.2017)

Weblink#3 https://www.hi.no/filarkiv/2014/03/havforskningsrapporten_2014_web.pdf/nb-no (visited 13.05.2018)

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Weblink#19 <https://www.neb.com/products/e7630-nebnext-library-quant-kit-for-illumina> (visited 23.04.2018)

Weblink#20 <https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/phix-control-v3.html> (visited 23.04.2018)

Weblink#21 <http://stroms.no/metoder/strommodellering> (visited 12.05.2018)

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Appendix

Appendix I Estimation of number of salmon lice based on real cage volume, number of salmon in the cage, and average number of salmon lice counted at the facility (www.barentswatch.no).

Cage M2	liter	Nr of fish	Avg lice	Tot nr of lice	lice pr l of water	l of water per lice	Added licewater ml Straight from can	Sterile water added (ml) 3000 with 3 lice
8200	8200000	120000	100	12000000	1,4634	0,6833		
	8200000		10	1200000	0,1463	6,8333	439	2561
	8200000		5	600000	0,0732	13,6667	220	2780
	8200000		3,6	432000	0,0527	18,9815	158	2842
	8200000		2	240000	0,0293	34,1667	88	2912
	8200000		1,8	216000	0,0263	37,9630	79	2921
	8200000		1,5	180000	0,0220	45,5556	66	2934
	8200000		1,3	156000	0,0190	52,5641	57	2943
	8200000		1,1	132000	0,0161	62,1212	48	2952
	8200000		0,9	108000	0,0132	75,9259	40	2960
	8200000		0,7	84000	0,0102	97,6190	31	2969
	8200000		0,5	60000	0,0073	136,6667	22	2978
	8200000		0,4	48000	0,0059	170,8333	18	2982
	8200000		0,3	36000	0,0044	227,7778	13	2987
	8200000		0,2	24000	0,0029	341,6667	9	2991
	8200000		0,1	12000	0,0015	683,3333	4	2996
			0,05	6000	0,0007	1366,6667	2	2998
			0,025	3000	0,0004	2733,3333	1	2999

Appendix II Average number of salmon lice estimated to be in the facility, based on dilutions.

Avg nr of lice	0,0	0,1	0,2	0,3	0,5	1,5	5,0	16,7	100,0
Ratio between licewater and filtered sea water	3000,0	1500,0	500,0	333,3	200,0	66,7	20,0	6,0	1,0
average calculated based on data from facility	0,1	0,1	0,3	0,5	0,9	1,8	3,8	12,0	100,0
Amount of lice water added to filtered sea water	1/3000	2/3000	6/3000	9/3000	15/3000	45/3000	150/3000	500/3000	3 lice in 3000ml filtered seawater

Appendix III Setup for the wet lab curve experiment showing the different dilutions made, volumes needed, number of cans and filters needed.

Average lice	ml	ml	ml	ml	ml	ml	Amount of sterile seawater needed each (ml)	<---- x3	Nr of filters	Nr of cans
	Can A	Can B	Can C	Can D	Can E					
100	3000	3000	3000	3000	3000	3000	3000	15000	15	5
10	500	500	500	500			2500	7500	9	3
3,6	150	150	150				2850	8550	9	3
1,8	45	45	45				2955	8865	9	3
0,9	15	15	15				2985	8955	9	3
0,5	9	9	9				2991	8973	9	3
0,3	6	6	6				2994	8982	9	3
0,1	2	2	2				2998	8994	9	3
0,05	1	1	1				2999	8997	9	3
Tot	728	728	728				26272	84816	87	29

Appendix IV Overview of sample dates, facility, sampling locations in the facility and number of filters used at each location.

Week		29	31	33	35	37	39	41	43	45	46
Location		Uløybukt, 10726	Uløybukt, 10726	Uløybukt, 10726	Uløybukt, 10726	Uløybukt, 10726	Uløybukt, 10726	Uløybukt, 10726	Uløybukt, 10726	Uløybukt, 10726	Skjervøy vest, 33097
Sample names	Date	20.07.2017	05.08.2017	19.08.2017	03.09.2017	12.09.2017	01.10.2017	15.10.2017	25.10.2017	07.11.2017	16.11.2017
M1 Blank		1	1	1	1	1	1	1	1	1	1
M1 1,5m		5	5	5	5	5	5	5	5	5	5
M1 6m						5					
M1 10m						5					
M2 Blank		1	1	1	1	1	1	1	1	1	1
M2 1,5m		5	5	5	5	5	5	5	5	5	5
M2 6m						5					
M2 10m						5					
M3 Blank		1	1	1	1	1	1	1	1	1	1
M3 1,5m		5	5	5	5	5	5	5	5	5	5
M3 6m						5					
M3 10m						5					
North Blank		1	1	1	1	1	1	1	1	1	1
North 1,5m		5	5	5	5	5	5	5	5	5	5
North 6m		5	5	5	5	5	5	5	5	5	5
North 10m						5					
South Blank		1	1	1	1	1	1	1	1	1	1
South 1,5m		5	5	5	5	5	5	5	5	5	5
South 6m		5	5	5	5	5	5	5	5	5	5
South 10m						5					
Tot nr of filters		40	40	40	40	80	40	40	40	40	40

Appendix V Protocol for extraction of eDNA from filters, based on Qiagen DNeasy blood and Tissue kit. improved by Kim Præbel, UiT.

DNA extraction

Important notes:

- Make sure that the incubator is set to 56 °C before start working.
- Always shake tubes out of the bags, don't put your hand inside the bag. Discard excess tubes.
- Only use pipette tips with barriers
- Never take anything out from the eDNA clean lab, in that case, don't bring it back. Do not bring anything **not** related to the eDNA "lab", inside the lab, while working.
- Always discard tips/tubes/gloves if you have the slightest suspicion about contamination (e.g. if the tip touches the table before entering the tube/buffer bottle, the replace it with a new one)
- Always start with the lowest concentration (i.e. blanks)

- If extracting samples from several species, sterilize everything again with ethanol.
- Do not touch the ends of the filters or the inside of the tube caps with hands or tweezers
- Always wear new cleaned lab-coat and nitrile gloves. Disinfect gloves in 10 % bleach for 5 - 10 mins followed by 50 % EtOH- wash before work starts (the gloves are not clean out of the box) and between steps/samples

DAY 1

1. Find filters in - 80 °C freezer and place them at 4 °C for gentle thawing (will take 1-2 hours).
2. Wear clean lab-coat before entering the lab. Gloves on. Clean lab bench, pipets, forceps, pens, etc. with 10% bleach from spraying bottle, incubate for 5 - 10 mins, then wipe off with tissue paper. Then flush all equipment, including gloves, with 50% EtOH.
3. To remove excess water in the filters, place the end of the filter (the end where the syringe is introduced) inside an eppendorf tube and place it in the same 50 ml centrifuge tube that contained the filter. If more than one filter in the tube, label a new tube for the second filter. Gently, slide the filter and Eppendorf tube back into the 50 ml tube. When done with the filters from one species/ station, clean (bleach+EtOH) everything again, 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 filter
5. Make extraction buffer solution for adding 2.5 x the recommended volume = 500 µl per filter. (Recommended volume: 20 µl Pk + 180 µl buffer ATL → 2.5 x proteinase k = 50 µl, 2.5 x 180 µl ATL = 450 µl tot = 500 µl. E.g. for 20 samples: = 1000 µl Pk, 9000 µl ATL
Pipet first the 9 ml with plastic pipet (sterile conditions) and place content in a 50 ml clean tube. Then pipet 1 ml (1000 µl) of PK and mix in the same tube. Mix the two solutions together with care to avoid foam.
6. Add 500 µl of the extraction solution to each filter (start with lowest concentration, the blanks), by pushing the 1000 µl tip tight to 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 clothed, then aspirate from the inlet end of the filter.
7. Then melt the incoming end with an EtOH burner and close it with forceps and make sure that it is completely sealed. Cap the lure end of the filter with a medical cap.

8. MAKE SURE YOU LABEL ALL THE FILTERS CORRESPONDING TO THE TUBES, by writing the label and the replicate letter (A, B, C...) on the filter and cover with tape.
9. Then place the filters in the rotator and fasten them with an elastic rubber spring/ band.
10. When done with all filters, transfer the rotator to the incubator oven (56 °C). Make sure that the rotator is moving (5 rpm) and not hitting the oven. Check the filters after a couple of hours and then leave them for 8 - 12 hours.
11. Always use similar incubation time for all filters within a project. Note the time when the filters were placed for incubation.

DAY 2/part 2

1. Clean table, pipettes, etc. with 10% bleach (5 - 10 min incubation) and 50% EtOH.
2. Note the time when the filters are removed from the incubator oven.
2. Reopen the sealed (melted) end of the filters with a burned red needle or tweezer. Transfer the incubated filters to a marked 2 ml tube inside a new 5 0ml centrifuge tube.
3. Centrifuge the 50 ml tubes containing the 2 ml tubes and the filters at 1700 x g for 3 minutes.
4. Carefully remove the 2 ml tubes from the 50 ml tubes with a tweezer without touching the cap or edge. Close the 2 ml tube and place it in a rack. Again, start with the lowest concentration (e.g. air → blank → real samples).
5. Label the DNeasy spin columns with the sample names and place them in order on the clean table.
6. “Measure” the approximate volume of two-three samples using a pipette with NEW tips for each sample. Round the mean volume to nearest 50 µl.
7. Add an equal volume of the AL buffer as the one determined above (6.) and ensure to mix it with the pipette immediately, using new tips for each sample.
8. Add an equal volume of 100 % EtOH as the one determined above (6.) and ensure to mix it with the pipette immediately, using new tips for each sample.
9. Locate the spin columns in front of the samples in the rack.
10. Transfer 630 µl of the sample into the corresponding spin column. Be careful not to make too many bubbles.
11. Centrifuge the columns at 15000 x g for 2 min.

12. Discard the collection tube with the eluate and transfer the spin column to a new collection tube. Take care to “leave” as much as possible of the flow-through solution in the discarded collection tube.
13. Transfer the rest of the sample to the corresponding spin column. If more than 630 μ l, three rounds of spinning is required.
14. Centrifuge the columns at 15000 x g for 2 min.
15. Discard the collection tube with the eluate and transfer the spin column to a new collection tube. Take care to “leave” as much as possible of the flow-through solution in the discarded collection tube.
16. Add 500 μ l buffer AW1 (check that EtOH has been added to the buffer) using new tips for each tube.
17. Centrifuge at 15000 x g for 2 minutes.
18. Discard the collection tube with the eluate and transfer the spin column to a new collection tube. Take care to “leave” as much as possible of the flow-through solution in the discarded collection tube.
19. Add 500 μ l buffer AW2 and centrifuge for 4 mins at 20000 x g.
20. While centrifuging, clean table, pipettes and pens (bleach+ EtOH) and label 1.5 ml Eppendorf tubes with the corresponding sample names. All info on the side and short version on the cap.
21. Transfer the spin-columns to the corresponding Eppendorf tubes. 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 20000 x g for 2 mins. Note what samples that have been centrifuged twice. Also make sure that the lid/tap of the spin column do not touch the cap of the Eppendorf tube to avoid contamination.
22. Add 75 μ l of buffer AE (elution buffer) to the spin columns. Make sure to aspirate the 75 μ l in the center of the membrane by aspirating the buffer just over the membrane (do not touch the membrane). Incubate for 1 min, then centrifuge the samples at 20000 x g for 2 minutes.
23. Discard the spin columns and transfer a 20 μ l aliquot of the extracted DNA to a PCR plate (labelled with the content. Note the locations of the samples in the plate in your lab book.
24. Freeze the DNA stock as soon as possible at - 80 °C and do only thaw the stock when absolutely necessary. Place the aliquot at 4 °C.

25. Clean the lab space, pipettes, forceps, pens, etc. in 10% bleach (5-10 min incubation) and 50% EtOH. Clean trash and floor and stock up with lab supplies. Dispose the lab coat in the “dirty-bin” at the first floor.
26. Transfer the aliquoted samples to wet ice. Perform Nanodrop/Quibit on the aliquots and proceed with PCR. When done, store at - 80 °C. Remember that it is better to store the sample at 4 °C for 1-3 days, than doing repeated freeze-thawing cycles.

Appendix VI Procedure based on “protocol for PCR of eDNA SAMPLES, v2, 090217 BY Kim Præbel. UiT.

PCR

Important notes:

- Never set up PCR in the same room where DNA/RNA have been extracted
- Make sure that the work space, pipettes, tips, tubes, etc. are absolutely clean and contaminant free. If doubt, but new (plastic)
- Before starting, clean workspace (positive flow fume hood), pipettes, centrifuge, etc. with bleach and EtOH. Place the tip boxes in the fume hood and illuminate everything (except DNA and chemicals) for half an hour with UV-light
- Always include a blank (use ultrapure water for PCR-run instead of template DNA)
- Always include a positive control. Remember to add this after all eDNA samples have been loaded, and the tubes are closed
- Only use pipette tips with barriers
- Do not bring anything not related to the eDNA “lab” inside the lab, while working
- Always discard tips/tubes/gloves if you have the slightest suspicion about contamination (e.g. if the tip touches the table before entering the tube/buffer bottle, replace it with a new tip)
- Always start with the lowest concentration (i.e. blanks)
- Only bring the aliquot taken from the original extracted samples to avoid contamination and degradation of the template

- Always work on ice with pre-chilled tubes. Covering the wet-ice with tinfoil reduces the mess with water

Master mix: Q5 (Q5 High-Fidelity 2X Master Mix create blunt end product)

Component	25 μl reaction	50 μl reaction	10 μl reaction	To 7 samples	Final concentration
Q5 High-fidelity 2X Master mix	12.50 μ l	25 μ l	5 μ l	50 μ l	1X
10 μM Forward ML3	1.25 μ l	2.50 μ l	0.50 μ l	5 μ l	0.50 μ M
10 μM Reverse ML3	1.25 μ l	2.50 μ l	0.50 μ l	5 μ l	0.50 μ M
Template DNA	variable	variable	3.50 μ l		< 1,000 ng
Nuclease-free water	to 25 μ l	to 50 μ l	0.50 μ l	15 μ l	

Note: template DNA can be diluted 5-20x or added undiluted, depending on amount water filtered, eDNA in the water, and elution volume used at the extraction

PCR program

Step	Temp	Time
Initial denaturation	98 °C	30 s
40 cycles	98 °C	5 s
Could be tested with 45 cycles	60 °C	20 s
	72 °C	60 s
Final extension	72 °C	2 min
Hold	4 °C	

Appendix VII PCR mix used in QPCR preparation

Amplitaq Gold master mix	10	μl
BSA 20 μg/μl	0.16	μl
H2O	2.84	μl
Forward primer 5 μM	1	μl
Reverse primer 5 μM	1	μl
DNA template	5	μl

Appendix VIII PCR-program used in QPCR

95 °C 10 min (denaturing the blocking antibody of Taq polymerase)

94 °C	1 min	
45 °C	1 min	x 35 cycles
72 °C	1 min	

72 °C 5 min (extension time)

Appendix IX PCR plate setup with overview over samples before pooling and metabarcoding.

04.04.2018												
Pool1												
curve	concentration											
	1	2	3	4	5	6	7	8	9	10	11	12
A	A1-A	C1-C	C2-B	A6-A	C6-C	Blank 1	A15-A	C15-C	B-C	A150-A	C150-C	E-B
B	A1-B	A2-A	C2-C	A6-B	A9-A	Blank 2	A15-B	A45-A	C-A	A150-B	C150-C	E-C
C	A1-C	A2-B	C9-B	A6-C	A9-B	Blank 3	A15-C	aA45-B	C-B	A150-C	A500-A	
D	B1-A	A2-C	C9-C	B6-A	A9-C	A-A	B15-A	A45-C	C-C	B150-A	A500-B	
E	B1-B	B2-A	C45-B	B6-B	B9-A	A-B	B15-B	B45-A	D-A	B150-B	A500-C	negativ
F	B1-C	B2-B	C45-C	B6-C	B9-B	A-C	B15-C	B45-B	D-B	B150-C	B500-A	negativ
G	C1-A	B2-C	C500-A	C6-A	B9-C	B-A	C15-A	B45-C	D-C	C150-A	B500-B	negativ
H	C1-B	C2-A	C500-C	C6-B	C9-A	B-B	C15-B	C45-A	E-A	C150-B	B500-C	negativ

05.04.2018												
Pool 2												
Sample date	25.okt					15.okt						
	1	2	3	4	5	6	7	8	9	10	11	12
A	M1 blank	M1 D	M3 B	N 1,5m E	N 6m C	S Blank	M3 D	M3 B	S 6m A	N 6m D	negativ	
B	M2 blank	M1 E	M3 C	S 1,5m A	N 6m D	N Blank	M3 E	M3 C	S 6m B	N 6m E	negativ	
C	M3 blank	M2 A	M3 D	S 1,5m B	N 6m E	M3 Blank	M2 A	M3 D	S 6m C	S 1,5m A	curve (A-A)	
D	N blank	M2 B	M3 E	S 1,5m C	S 6m A	M2 Blank	M2 B	M3 E	S 6m D	S 1,5m B	curve (C-A)	
E	S blank	M2 C	N 1,5m A	S 1,5m D	S 6m B	M1 Blank	M2 C	N 1,5m B	S 6m E	S 1,5m C	curve	
F	M1 A	M2 D	N 1,5m B	S 1,5m E	S 6m C	M3 A	M2 D	N 1,5m C	N 6m A	S 1,5m D	curve	
G	M1 B	M2 E	N 1,5m C	N 6m A	S 6m D	M3 B	M2 E	N 1,5m D	N 6m B	S 1,5m E	curve	
H	M1 C	M3 A	N 1,5m D	N 6m B	S 6m E	M3 C	M3 A	N 1,5m E	N 6m C	N 1,5m A	curve	

05.04.2018												
Pool 3												
Sample date	12.sep				12.sep	20.jul			20.jul	curve		curve
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank N	M1D	M3B	N 1,5M E	N 6M C	Blank N	N 1,5m E	M1 C	M3 A	A-A	C-C	negativ
B	Blank S	M1E	M3C	S 1,5M A	N 6M D	Blank S	S 1,5m A	M1 D	M3 B	A-B	D-A	Negativ
C	Blank M1	M2A	M3D	S 1,5M B	N 6M E	Blank M1	S 1,5m B	M1 E	M3 C	A-C	D-B	curve (A-B)
D	Blank M2	M2B	M3E	S 1,5M C	S 6M A	Blank M2	S 1,5m C	M2 A	M3 D	B-A	D-C	curve (C-B)
E	Blank M3	M2C	N 1,5M A	S 1,5M D	S 6M B	N 1,5m A	S 1,5m D	M2 B	M3 E	B-B	E-A	
F	M1A	M2D	N 1,5M B	S 1,5M E	S 6M C	N 1,5m B	S 1,5m E	M2 C	Blank 1	B-C	E-B	
G	M1B	M2E	N 1,5M C	N 6M A	S 6M D	N 1,5m C	M1 A	M2 D	Blank 2	C-A	E-C	
H	M1C	M3A	N 1,5M D	N 6M B	S 6M E	N 1,5m D	M1 B	M2 E	Blank 3	C-B		

Appendix X Calculating Library loading SLI

Concentrations from qPCR:

SLI1: 10.07 nM (94 samples)

SLI2: 3.08 nM (84 samples)

SLI3: 5.50 nM (91 samples)

Compensation for number of samples:

$$\text{SLI1: } 3.08 \times 94/84 = 3.45 \text{ nM}$$

$$\text{SLI2: } 3.08 \text{ nM}$$

$$\text{SLI3: } 3.08 \times 91/84 = 3.34 \text{ nM}$$

Dilution of SLI1 and SLI3:

$$\text{SLI1: } 10.07 \text{ nM} / 3.45 \text{ nM} = 2.92 \text{ times} \rightarrow 1 \mu\text{l SLI1} + 1.92 \mu\text{l H}_2\text{O}$$

$$\text{SLI3: } 5.5 \text{ nM} / 3.34 \text{ nM} = 1.65 \text{ times} \rightarrow 1 \mu\text{l SLI3} + 0.65 \mu\text{l H}_2\text{O}$$

SLI1: 2 μ l of library + 3.84 μ l H₂O

SLI3: 2 μ l of library + 1.30 μ l H₂O

Mixing the three libraries:

2 μ l of SLI1: 3.45 nM

+ 2 μ l of SLI2: 3.08 nM

+ 2 μ l of SLI3: 3.34 nM

= 6 μ l of pooled libraries with mean concentration of 3.29 nM

Denaturing with N₂OH:

5 μ l of pooled library

+ 0.40 μ l of PhiX (0.04 nM)

+ 5.40 μ l of N₂OH (0.20 N)

= 10.80 μ l total

Waited 5 min for denaturation to happen

Then added 989.2 μ l of HT1 hybridization buffer to get total volume of 1 ml.

The final concentrations:

$(5 \mu\text{l} \times 3.29 \text{ (n mol/L)} + 0.40 \mu\text{l} \times (0.04 \text{ nM}) \times 1000 \text{ p mol/1 nM}) / 1\text{ml} \times (1000 \mu\text{l}/1 \text{ ml})$

= 16.60 pM

Percentage of PhiX:

PhiX = $((0.04 \times 0.40) / (5 \times 3.29) + (0.40 \times 0.40)) \times 100 = \underline{0.96 \%}$

Appendix XII Total number of salmon lice reads for each field sample

Week 29			20 Jul			Week 37			12 Sep			Week 41			15 Oct			Week 43			25 Oct		
Sample name	Reads	Average reads	Sample name	Reads	Average reads	Sample name	Reads	Average reads	Sample name	Reads	Average reads	Sample name	Reads	Average reads	Sample name	Reads	Average reads						
07_20_M1_blank	0		09_12_M1_blank	1		10_15_M2_blank			10_25_M1_blar	0		10_25_M2_blar	0		10_25_M1_A	0							
07_20_M1_A	1		09_12_M1_A	0		N/A	N/A	N/A	10_25_M1_B	0		10_25_M2_A	0		10_25_M1_B	0							
07_20_M1_B	0		09_12_M1_B	0		N/A	N/A	N/A	10_25_M1_C	0		10_25_M2_B	0		10_25_M1_C	0							
07_20_M1_C	7		09_12_M1_C	0		N/A	N/A	N/A	10_25_M1_D	0		10_25_M2_C	0		10_25_M1_D	0							
07_20_M1_D	131		09_12_M1_D	0		N/A	N/A	N/A	10_25_M1_E	0		10_25_M2_D	1		10_25_M1_E	0							
07_20_M1_E	11		09_12_M1_E	1	1	N/A	N/A	N/A	10_25_M1_E	0		10_25_M2_E	1		10_25_M1_E	0							
07_20_M2_blank	363		09_12_M2_blank	2		10_15_M2_blank	11		10_25_M2_A	0		10_25_M2_A	0		10_25_M2_A	0							
07_20_M2_A	26		09_12_M2_A	2		10_15_M2_A	0		10_25_M2_B	0		10_25_M2_B	0		10_25_M2_B	0							
07_20_M2_B	9		09_12_M2_B	0		10_15_M2_B	0		10_25_M2_C	0		10_25_M2_C	0		10_25_M2_C	0							
07_20_M2_C	3		09_12_M2_C	1		10_15_M2_C	0		10_25_M2_D	0		10_25_M2_D	1		10_25_M2_D	1							
07_20_M2_D	11		09_12_M2_D	3		10_15_M2_D	0		10_25_M2_E	0		10_25_M2_E	1		10_25_M2_E	1							
07_20_M2_E	2	10.2	09_12_M2_E	2	2	10_15_M2_E	0	0	10_25_M2_E	1	1	10_25_M2_E	1	1	10_25_M2_E	1	1						
07_20_M3_A	148		09_12_M3_blank	0		10_15_M3_blank			10_25_M3_A	1		10_25_M3_A	1		10_25_M3_A	1							
07_20_M3_B	108		09_12_M3_A	0		10_15_M3_A	18		10_25_M3_B	1		10_25_M3_B	1		10_25_M3_B	1							
07_20_M3_C	102		09_12_M3_B	0		10_15_M3_B	12		10_25_M3_C	0		10_25_M3_C	0		10_25_M3_C	0							
07_20_M3_D	69		09_12_M3_C	0		10_15_M3_C	18		10_25_M3_D	0		10_25_M3_D	0		10_25_M3_D	0							
07_20_M3_E	160	117.4	09_12_M3_D	1	1	10_15_M3_D	1	1	10_25_M3_E	0		10_25_M3_E	0		10_25_M3_E	0							
07_20_N_blank	0		09_12_M3_E	2	1.5	10_15_M3_E	3	10.4	10_25_M3_E	0		10_25_M3_E	0		10_25_M3_E	0							
07_20_N_1.5m_A	284		09_12_N_blank	0		10_15_N_blank	0		10_25_N_blank	0		10_25_N_blank	0		10_25_N_blank	0							
07_20_N_1.5m_B	252		09_12_N_1.5m_A	6		10_15_N_1.5m_A	18		10_25_N_1.5m	0		10_25_N_1.5m	0		10_25_N_1.5m	0							
07_20_N_1.5m_C	189		09_12_N_1.5m_B	18		10_15_N_1.5m_B	14		10_25_N_1.5m	1		10_25_N_1.5m	1		10_25_N_1.5m	1							
07_20_N_1.5m_D	104		09_12_N_1.5m_C	142		10_15_N_1.5m_C	2		10_25_N_1.5m	13		10_25_N_1.5m	2		10_25_N_1.5m	2							
07_20_N_1.5m_E	6	167	09_12_N_1.5m_D	51	43.8	10_15_N_1.5m_D	1	5.75	10_25_N_1.5m	2	5.3	10_25_N_1.5m	0		10_25_N_1.5m	0							
			09_12_N_1.5m_E	2		10_15_N_1.5m_E	0		10_25_N_1.5m	0		10_25_N_1.5m	0		10_25_N_1.5m	0							
			09_12_N_6m_A	0		10_15_N_6m_A	21		10_25_N_6m_A	0		10_25_N_6m_A	0		10_25_N_6m_A	0							
			09_12_N_6m_B	1		10_15_N_6m_B	14		10_25_N_6m_B	0		10_25_N_6m_B	0		10_25_N_6m_B	0							
			09_12_N_6m_C	8		10_15_N_6m_C	43		10_25_N_6m_C	1		10_25_N_6m_C	1		10_25_N_6m_C	1							
			09_12_N_6m_D	8		10_15_N_6m_D	1		10_25_N_6m_D	0		10_25_N_6m_D	0		10_25_N_6m_D	0							
			09_12_N_6m_E	1	3.3	10_15_N_6m_E	5	16.8	10_25_N_6m_E	0		10_25_N_6m_E	0		10_25_N_6m_E	0							
07_20_S_blank	1		09_12_S_blank	0		10_15_S_blank	2		10_25_S_blank	0		10_25_S_blank	0		10_25_S_blank	0							
07_20_S_1.5m_A	4		09_12_S_1.5m_A	4		10_15_S_1.5m_A	5		10_25_S_1.5m	1		10_25_S_1.5m	1		10_25_S_1.5m	1							
07_20_S_1.5m_B	0		09_12_S_1.5m_B	12		10_15_S_1.5m_B	0		10_25_S_1.5m	0		10_25_S_1.5m	0		10_25_S_1.5m	0							
07_20_S_1.5m_C	0		09_12_S_1.5m_C	1		10_15_S_1.5m_C	1		10_25_S_1.5m	1		10_25_S_1.5m	1		10_25_S_1.5m	1							
07_20_S_1.5m_D	0		09_12_S_1.5m_D	4		10_15_S_1.5m_D	4		10_25_S_1.5m	0		10_25_S_1.5m	0		10_25_S_1.5m	0							
07_20_S_1.5m_E	0	4	09_12_S_1.5m_E	1	4.4	10_15_S_1.5m_E	1	2.75	10_25_S_1.5m	0		10_25_S_1.5m	0		10_25_S_1.5m	0							
			09_12_S_6m_A	0		10_15_S_6m_A	6		10_25_S_6m_A	0		10_25_S_6m_A	0		10_25_S_6m_A	0							
			09_12_S_6m_B	0		10_15_S_6m_B	5		10_25_S_6m_B	0		10_25_S_6m_B	0		10_25_S_6m_B	0							
			09_12_S_6m_C	0		10_15_S_6m_C	9		10_25_S_6m_C	0		10_25_S_6m_C	0		10_25_S_6m_C	0							
			09_12_S_6m_D	1		10_15_S_6m_D	4		10_25_S_6m_D	0		10_25_S_6m_D	0		10_25_S_6m_D	0							
			09_12_S_6m_E	4	2.5	10_15_S_6m_E	11	7	10_25_S_6m_E	0		10_25_S_6m_E	0		10_25_S_6m_E	0							

Appendix XIII Conversion between library 1 and 3, filtered to unfiltered, using two internal standards.

	Lib 1	Lib 3	Ratio (lib/lib3)	Average
	Reads	Reads		
A-B	51.504	12.806	4.02	
C-B	20.859	4.490	4.65	4.33

Appendix XIV Conversion between library 1 and 3 and library 1 and 2.

Library conversion factor with total reads			
Lib 1	Lib 3	Ratio	Average
A-B	Internal P3 A-B		
C-B	Internal P3 C-B		
177.894	36.523	4.87	
141.227	30.038	4.70	4.79

Library conversion factor with total reads			
Lib 1	Lib 2	Ratio	Average
A-A	Internal P2 A-A		
C-A	Internal P2 C-A		
171.573	26.069	6.58	
151.231	79.955	1.89	4.24

Appendix XV Filtered samples from the curve converted to unfiltered according to the correction between the sequencing depth between the libraries.

Converted from filtered to unfiltered								
Sample name	Read	average	Sample name	Read	average	Sample name	Read	average
A1_A		0	B1_A		2	C1_A		5
A1_B		1	B1_B		3	C1_B		1
A1_C		2	1 B1_C		2	2 C1_C		3
A2_A		2	B2_A		3	C2_A		2
A2_B		2	B2_B		2	C2_B		1
A2_C		2	2 B2_C		2	2 C2_C		2
A6_A		69	B6_A		4	C6_A		4
A6_B		140	B6_B		80	C6_B		2
A6_C		555	255 B6_C		3	29 C6_C		6
A9_A		231	B9_A		2	C9_A		145
A9_B		45	B9_B		3	C9_B		2
A9_C		4	93 B9_C		2	2 C9_C		50
A15_A		207	B15_A		4	C15_A		2
A15_B		128	B15_B		2	C15_B		3
A15_C		28	121 B15_C		2	3 C15_C		62
A45_A		4775	B45_A		306	C45_A		78
A45_B		440	B45_B		82	C45_B		137
A45_C		907	2041 B45_C		463	284 C45_C		292
A150_A		634	B150_A		553	C150_A		393
A150_B		1228	B150_B		475	C150_B		7907
A150_C		1056	973 B150_C		1143	724 C150_C		3712
A500_A		10081	B500_A		1500	C500_A		5348
A500_B		5637	B500_B		2326			
A500_C		3657	6458 B500_C		15436	6421 C500_C		3519

Appendix XVI Field sample reads converted by correction factors according to the correction between the sequencing depth between the libraries.

Week 29			Week 37			Week 41			Week 43		
Sample name	Reads	Average reads	Sample name	Reads	Average reads	Sample name	Reads	Average reads	Sample name	Reads	Average reads
07_20_M1_blank	0		09_12_M1_blank	5		10_15_M1_blank	76		10_25_M1_blank	0	
07_20_M1_A	5		09_12_M1_A	0		N/A	#VERDI!		10_25_M1_A	0	
07_20_M1_B	0		09_12_M1_B	0		N/A	#VERDI!		10_25_M1_B	0	
07_20_M1_C	34		09_12_M1_C	0		N/A	#VERDI!		10_25_M1_C	0	
07_20_M1_D	627		09_12_M1_D	0		N/A	#VERDI!		10_25_M1_D	0	
07_20_M1_E	53	144	09_12_M1_E	5	5	N/A	#VERDI!	0	10_25_M1_E	0	0
07_20_M2_blank	1737		09_12_M2_blank	10		10_15_M2_blank	47		10_25_M2_blank	0	
07_20_M2_A	124		09_12_M2_A	10		10_15_M2_A	0		10_25_M2_A	0	
07_20_M2_B	43		09_12_M2_B	0		10_15_M2_B	0		10_25_M2_B	0	
07_20_M2_C	14		09_12_M2_C	5		10_15_M2_C	0		10_25_M2_C	0	
07_20_M2_D	53		09_12_M2_D	14		10_15_M2_D	0		10_25_M2_D	4	
07_20_M2_E	10	49	09_12_M2_E	10	8	10_15_M2_E	0	0	10_25_M2_E	4	4
			09_12_M3_blank	0		10_15_M3_blank	4		10_25_M3_blank	4	
07_20_M3_A	708		09_12_M3_A	0		10_15_M3_A	76		10_25_M3_A	4	
07_20_M3_B	517		09_12_M3_B	0		10_15_M3_B	51		10_25_M3_B	4	
07_20_M3_C	488		09_12_M3_C	0		10_15_M3_C	76		10_25_M3_C	0	
07_20_M3_D	330		09_12_M3_D	5		10_15_M3_D	4		10_25_M3_D	0	
07_20_M3_E	766	562	09_12_M3_E	10	7	10_15_M3_E	13	44	10_25_M3_E	0	4
07_20_N_blank	0		09_12_N_blank	0		10_15_N_blank	0		10_25_N_blank	0	
07_20_N_1.5m_A	1359		09_12_N_1.5m_A	29		10_15_N_1.5m_A	76		10_25_N_1.5m_A	0	
07_20_N_1.5m_B	1206		09_12_N_1.5m_B	86		10_15_N_1.5m_B	8		10_25_N_1.5m_B	4	
07_20_N_1.5m_C	905		09_12_N_1.5m_C	680		10_15_N_1.5m_C	8		10_25_N_1.5m_C	55	
07_20_N_1.5m_D	498		09_12_N_1.5m_D	244		10_15_N_1.5m_D	4		10_25_N_1.5m_D	8	
07_20_N_1.5m_E	29	799	09_12_N_1.5m_E	10	210	10_15_N_1.5m_E	0	19	10_25_N_1.5m_E	0	23
			09_12_N_6m_A	0		10_15_N_6m_A	89		10_25_N_6m_A	0	
			09_12_N_6m_B	5		10_15_N_6m_B	59		10_25_N_6m_B	0	
			09_12_N_6m_C	0		10_15_N_6m_C	182		10_25_N_6m_C	4	
			09_12_N_6m_D	38		10_15_N_6m_D	4		10_25_N_6m_D	0	
			09_12_N_6m_E	5	16	10_15_N_6m_E	21	71	10_25_N_6m_E	0	4
			0	0							
07_20_S_blank	5		09_12_S_blank	0		10_15_S_blank	8		10_25_S_blank	0	
07_20_S_1.5m_A	19		09_12_S_1.5m_A	19		10_15_S_1.5m_A	21		10_25_S_1.5m_A	4	
07_20_S_1.5m_B	0		09_12_S_1.5m_B	57		10_15_S_1.5m_B	0		10_25_S_1.5m_B	0	
07_20_S_1.5m_C	0		09_12_S_1.5m_C	5		10_15_S_1.5m_C	4		10_25_S_1.5m_C	4	
07_20_S_1.5m_D	0		09_12_S_1.5m_D	19		10_15_S_1.5m_D	17		10_25_S_1.5m_D	0	
07_20_S_1.5m_E	0	19	09_12_S_1.5m_E	5	21	10_15_S_1.5m_E	4	9	10_25_S_1.5m_E	0	4
			0	0							
			09_12_S_6m_A	0		10_15_S_6m_A	25		10_25_S_6m_A	0	
			09_12_S_6m_B	0		10_15_S_6m_B	21		10_25_S_6m_B	0	
			09_12_S_6m_C	0		10_15_S_6m_C	38		10_25_S_6m_C	0	
			09_12_S_6m_D	5		10_15_S_6m_D	17		10_25_S_6m_D	0	
			09_12_S_6m_E	19	12	10_15_S_6m_E	47	30	10_25_S_6m_E	0	0