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Molecular quantification of sea lice in and around sea cages

A study comparing the molecular quantification method qPCR against a conventional method

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Preface

This thesis was written to fulfil my education as a teacher with Master of Science at the Norwegian University of Science and Technology, NTNU under guidance of Professor Rolf Erik Olsen at the Department of Biology, NTNU. The experimental work was carried out at NTNU Centre of Fisheries and Aquaculture (Sealab) and in the laboratory for functional genomics (FUGE lab) at NTNU Gløshaugen from January 2017 until June 2017. The thesis was part of the research project *Taskforce lakselus* which is a collaboration between NTNU and the aquaculture industry, including the following companies; Marine Harvest, SalMar, Lerøy Midt, Måsøval-gruppen, Bjørøya, Midt-Norsk Havbruk, Salmonor, Sinkaberg-Hansen, Ervik Laks & Ørret, Emilsens fisk and Åkerblå.

Writing a master thesis can hardly be described in one word. The process has been hard, stressful and frustrating, but at the same time filled of happiness, joy and pride. There are many people that needs to be thanked for me being where I am now. First, I would like to thank my main supervisor, Rolf Erik Olsen - for believing in me and for encouragement during the process. Thanks to my co-supervisors, Kjell-Inge Reitan and Per Winge for helpful discussions and Lone Sunniva Jevne, Torfinn Sparstad and Mari-Ann Østensen for guidance and help through the planning and completion of the laboratory work. Thanks to Salmar Farming at Makrellskjæret and Norstraumen, for great collaboration and help when collecting samples. To my family and my amazing husband, Vegard - you have been my most important support during this process. When I have lost interest, you have kept me motivated.

To all my study colleagues and great friends at the university; Thanks for all the laughter and the long lunches. The last five years would never been the same without you.

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Abstract

The aquaculture production of atlantic salmon (*Salmo salar*) have been growing in a steady pace, but for further expansion of the industry it is necessary to solve environmental challanges, including those related to sea lice. The two most common species that are affecting salmonids in Norway are *Lepeophtheirus salmonis* (Krøyer) and *Caligus elongatus* (Nordmann), both of them included in this study. Identification of these pathogens involve preservation of samples and conventional methods like microscopy, a process being time consuming. The first part of this study looked at the effect of different preservation methods due to schrinkage, in addition to investigate if the DNA amount varied at the different planktonic stages. The larval size (length and width) at the nauplius I stage was found to decrease in samples preserved on 4 % formalin. Larvae at the nauplius II stage were wider when preserved on 4 % formalin, while no significant differences was observed due to length. At the copepodid stage, no significant differences in neither length or width was observed. The results did not indicate differences in DNA amount at the different life stages.

The second part and main objective of the thesis, focused on comparing two different quantification methods; counting of sea lice larvae in plankton samples using microscope and quantification of larvae using th molecular method, qPCR. As a part of this, the primers for *L. salmonis* and *C. elongatus* developed by McBeath (2006) was tested. Analyses revealed that the primer for *C. elongatus* did not manage to detect all the larvae in known samples. In contrast, the primer for *L. salmonis* was species specific, even managing to detect trace amounts of DNA in field samples. However, when comparing the counted amount of sea lice under microscope with the estimated amount found using qPCR, the results did not correspond to each other.

Sammendrag

Akvakulturindustrien er i stadig vekst, og produksjonen av atlantisk laks (*Salmo salar*) øker jevnt. For fremtidig vekst i industrien er det derimot viktig å løse flere ulike miljøutfordringer, blant annet problematikken rundt lakselus. To arter, *Lepeophtheirus salmonis* og *Caligus elongatus* er spesielt truende for næringen, begge er inkludert i denne studien. For å kunne identifisere patogenene må feltprøver preserveres og analyseres. Analysene gjøres i dag blant annet ved hjelp av mikroskopiske undersøkelser, et arbeid som er svært tidkrevende.

Denne masteroppgaven var delt i to deler. I del en ble det studert hvordan ulike preserveringsmetoder påvirker larvestørrelse, i tillegg til å undersøke om DNA-mengden varierer mellom individer som befinner seg på ulike stadier i den planktoniske delen av livssyklusen. Resultatene fra studien viser at man på nauplius I-stadiet ser en nedgang i størrelse (både lengde og bredde) hos larver som er preservert med 4 % formalin, sammenlignet med levende larver. På nauplius II-stadiet var luselarvene preservert på formalin bredere enn de levende larvene, men det ble ikke observert signifikant forskjell i lengde. Det ble ikke observert signifikante størrelsesforskjeller i hverken bredde eller lengde hos individer som befant seg på copepodittstadiet. Resultatene fra studien indikerer at det ikke er noen forskjell i DNA mengde på de ulike livsstadiene.

Den andre delen av studiet inkluderte hovedmålet med oppgaven; å sammenligne ulike kvantifiseringsmetoder, i dette tilfellet manuell telling av luselarver ved hjelp av mikroskop og den molekylærbiologiske metoden qPCR. Som følge av dette ble primerne for *L. salmonis* og *C. elongatus* utviklet av McBeath et al. (2006) testet. Analysene avslørte at primeren for skottelus ikke klarte å detektere alle individene i en kjent positiv prøve. Primeren for *L. salmonis* klarte derimot å detektere individer, og i tillegg også svært små mengder DNA når DNA fra lakselus ble blandet med DNA fra andre organismer. Resultatene viser at antall lus telt i en prøve ved hjelp av mikroskop ikke korresponderer med estimert mengde lus funnet ved hjelp av qPCR i den tilsvarende parallelprøven, og at metoden trenger mer finjustering.

Contents

List of Figures

List of Tables

Abbreviations

AC	Amplification curve
C. elongatus*/ *C. e	*Caligus elongatus*
Copepodid	The third and final planktonic stage
Cq value	The point at which the AC crosses the vertical threshold line during qPCR
ds DNA	Double-stranded DNA
L. salmonis*/ *L. s	*Lepeophtheirus salmonis*
Maturated water	Filtered sea water maturated in respect to bacterial growth
MQ water	Milli-Q water. An unique combination of optimized pure water with pure quality
Nauplius (plural: nauplii)	Larvae in the first and second stage after hatching
Negative samples	A sample assumed not to contain sea lice larvae
Planktonic stages	The nauplius I, nauplius II and copepodid stage
Positive samples	A sample containing sea lice larvae
PCR	Polymerase Chain Reaction
qPCR	Real-time Polymerase Chain Reaction
Salmon lice	*L.s*
Sea lice	Lice of both *L.s* and *C.e*
Sea lice larvae	Nauplius I, II and copepodid
SYBR Green I	A stain available for detecting ds DNA
Unknown samples	Plankton samples from net haul

1 Introduction

1.1 The aquaculture industry in Norway

The aquaculture industry in Norway is growing in a steady pace and in 2015 this industry represented 67 % of the total export value of sea food of nearly 74,5 billion NOK. The aquaculture production of atlantic salmon (*Salmo salar*), has increased steadily through many years, and has been around 1,2 million tons the last three years. For further expansion of the industry it is necessary to solve environmental challenges, including those related to sea lice (Svåsand et al., 2016). There are major issues related to these pathogens, and it is necessary to gain more information about, and trying to understand their distribution and life cycle. Today, sea lice detection and identification involves sample collection and conventional methods like microscopy. The samples are manually scanned followed by morphological examination of each individual (McBeath et al., 2006). Separating and identifying the different planktonic stages is difficult and a time consuming process (McBeath et al., 2006; Schram, 2004), and plankton samples are likely to contain small numbers of sea lice nauplii and copepodids (Pike & Wadsworth, 1999). Hence, it is desirable to develop alternative methods of identification.

Sea lice belongs to the family Caligidae and are marine ectoparasitic copepods. Parasitic copepods like these are common on both cultured and wild marine fish, and are a major health hazard for farmed finfish (Hamre et al., 2013). Mature sea lice attached to farmed fish, produces planktonic larvae that can be spread by the water currents in the area and infect wild salmonids, like the atlantic salmon and the sea trout (*Salmo trutta*) (Aldrin, Storvik, Kristoffersen, & Jansen, 2013; Pike & Wadsworth, 1999; Svåsand et al., 2016). The most common species of sea lice affecting salmonids in Norway are *Lepeophtheirus salmonis* (Krøyer) and *Caligus elongatus* (Nordmann). Both of these pathogens may cause extensive damage when present in high numbers, causing problems for the increasing aquaculture industry

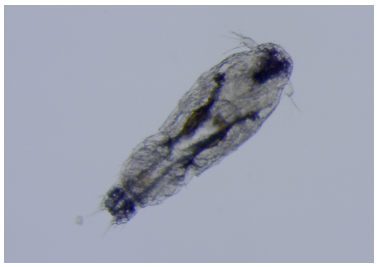
(McBeath et al., 2006; Pike & Wadsworth, 1999). Many different sea lice species are found along the coast of Norway, but *L. salmonis* is considered to be the most economically important one (McBeath et al., 2006). *L. salmonis* is salmonid specific, while *C.elongatus* has a broad host range, including both non-salmonid teleosts and elasmobranchs (Costello, 2009; Pike & Wadsworth, 1999; Schram, 2004).

1.2 Life cycle and development stages

The salmon louse life cycle consists of eight stages (Eichner, Harasimczuk, Nilsen, Grotmol, & Dalvin, 2015). First, there are three stages where the larvae is pelagic; nauplius I (1.1a), II (1.1b) and copepodid (1.1c). These stages have been difficult to study, because of their small size and low abundance. Consequently, there is little knowledge about the movement of these larvae in and around cage-culture sites (Pike & Wadsworth, 1999). During the first three planktonic stages the louse do not eat. The copepodid larvae has a mouth-like structure, but this structure does not become fully developed until later (Jones & Beamish, 2011). After the three planktonic stages, the copepodid attaches to the host and start a parasitic life style, feeding on the host's mucus, skin and blood (Eichner et al., 2015) going through the following stages: chalimus I and II, pre-adult I and II and the adult stage.

(a) *L. salmonis*. Newly hatched nauplius I larvae.

(b) Nauplius II larvae of *L. salmonis*.



(c) Copepodid larvae of *L. salmonis*.

Figure 1.1: The three planktonic stages of *L.salmonis*. The pictures are taken in the microscope Leica MS05 C, 0.78-16.0x using the Carl Zeiss Microscopy Gmbtt camera (2011) at 5.0 magnification. Pictures are edited using "Pictures" available on Macbook Pro computers.

The salmon louse is an antrophod (Eichner, n.d.), and develops a rigid outer shell through each molting, which is build up of a chitin containing cutikula and sclerotised proteins (Eichner et al., 2015; Fiskeridirektoratet, 2015). The body is segmented, and by changing the outer shell every moulting, the growth is taking place in waves. Moulting is a complex event, when the old cuticula is degraded and a new one produced (Eichner et al., 2015). When the salmon louse changes the outer shell, residues of the old structure, called exuvuia can be

seen in the microscope (Eichner, n.d.). Chitin contributes by giving the shell its hard, protective structure. Chitin is a polysaccharide consisting of acetylglucosamin residues (Eichner, n.d.). However, chitin can be deacetylated into chitosan, able to bind negatively charged DNA via an electrostatic interaction. This process creates nano-size complexes called polyplexes, shown in Figure 1.2 (Mao, Sun, & Kissel, 2010).

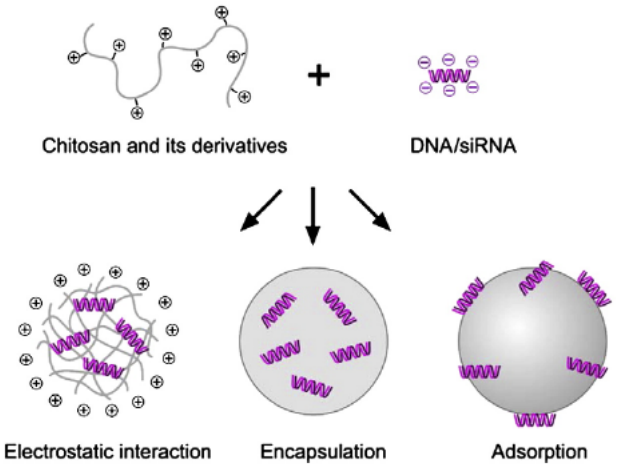


Figure 1.2: How chitosan-based DNA nanoparticles can be made based on three different mechanisms (Mao et al., 2010).

1.2.1 Temperature dependent development

Temperature is known to affect the development rates of sea lice larvae, and lower temperatures tend to increase body size (Gravil, 1996). This relationship between temperature and body size has also been reported by Tully, 1989 (cited in Gravil, 1996). According to Ritchie, Mordue, Pike, and Rae (1993), there are observed two generation types of *L. salmonis*. The winter type producing many small eggs, and a summer type producing fewer larger eggs.

1.3 Behavior and dispersal of the larvae

The nauplius I larvae, the nauplius II larvae and the copepodid are all positively phototactic, and migrate vertically. The copepodid seems to infest hosts in the dark as well. All of them migrate from the depths towards the surface during the day, before they again descends towards the depths at night. The Atlantic salmon migrate downwards during the day and up again during the night, making it possible for an infection by the parasite (Pike & Wadsworth, 1999).

It is difficult to understand the dynamics of fjord areas, and it is necessary to use several different methods to collect information, because of the complexity of the spatial and temporal variability (Jones & Beamish, 2011). Different models show that sea lice may spread several kilometer from the infection site (Asplin, Boxaspen, & Sandvik, 2011; Svåsand et al., 2016). The currents in the fjords and along the coastline is complicated, and the driving forces are many and will vary from one location to another. The most important driving forces are wind, fresh water runoff, tidal currents and stratification in the water column. The strongest currents are in the upper water layers (10-20 m) and the driving forces can be influenced by the topography of the land and the earth rotation, for instance (Svåsand et al., 2016).

1.4 Morphological analyses of sea lice larvae

In the process of gaining more information about the pathogens distribution, dispersal and life cycle, the first step is to confirm the presence of the sea lice. When quantifying the number of larvae on the basis of morphological examination, individuals of different species can be separated from one another based on characteristics due to morphology, like differences in pigmentation. With good knowledge this can be done accurately, although being time consuming. However, there are many challenges related to assessing the presence of the sea lice using a microscope, like their small sizes and variation in body size (Pike & Wadsworth, 1999; Schram, 2004). Variation in body size has been stated in earlier publications, where the measurements of sea lice larvae seems to vary (Johnson & Albright, 1991; Pike & Wadsworth, 1999; Schram, 2004).

1.4.1 Effects of formalin preservation

For practical analyses in the laboratory, preservation of samples are necessary. Several chemicals can be used, for example formaldehyde solutions. According to Steedman, 1976 (cited in Thibault-Botha and Bowen, 2004), a solution of 4 % formalin leads to an osmotic pressure more than double of the surrounding seawater, leading to a length and width loss due to the osmotically driven loss of water, further leading to changes in the cell volume (Ohman and Snyder, 1991 cited in Jaspers and Carstensen, 2009). Because of this, samples preserved on formalin needs to be re-calibrated to account for size changes due to formalin fixation. A shrinkage like this due to preservation, has been reported by Thibault-Botha and Bowen (2004) for different species, and the degree of shrinkage depends on the species and the species composition, in example surface/volume ratio and water content. It is expected that zooplankton being gelatinous, having a high water content and no hard covering or strong

structural features, will have larger size losses compared to the individuals having this, like the crustacean zooplankton (Thibault-Botha & Bowen, 2004)

In other words, different factors may affect the individuals studied in morphological analyses, thus making the process difficult and time consuming (McBeath et al., 2006; Schram, 2004). Because of this, it could be advantageously to use alternative methods for identification of the pathogens.

1.5 Molecular methods for quantification of sea lice larvae in plankton samples

1.5.1 Polymerase Chain Reaction, PCR

According to Garibyan and Avashia (2013), one of the most important medical applications of the classic PCR method is detection of pathogens, and this method can be used instead of identification using microscopy. Polymerase Chain Reaction, PCR, is a method enabling amplification of a specific DNA fragment, and this enzymatic assay can be performed using source DNA from different tissues and organisms (Pelt-Verkuil, Belkum, & Hays, 2008). Only trace amounts of DNA are needed to generate enough copies to be analysed using different conventional laboratory methods (Garibyan & Avashia, 2013).

Conducting a PCR assay requires template DNA, primers, nucleotides and the key enzyme DNA polymerase. The bases adenine (A), thymine (T), cytosine (C) and guanine (G) are building blocks that DNA polymerase uses to build the PCR product. Primers are short DNA fragments with a sequence complementary to a specific gene location on the target DNA, making it possible to detect this sequence and amplify it (Pelt-Verkuil et al., 2008). The DNA polymerase build on from the extension point of the primers (Garibyan & Avashia, 2013).

The assay is performed in a PCR machine, a thermal cycler, and goes through the following three processes; denaturation, annealing and extending. In the first step, the temperature raises above the melting point of the two complementary DNA strands of the target DNA. Because of increase in temperature, the two strands separate before the temperature is lowered to allow the primers to bind to the target DNA segments (annealing). The temperature is then raised again, allowing DNA polymerase to extend the primers by adding nucleotides to the DNA strand (extension). The steps are repeated, doubling the number of copied DNA molecules each cycle (Figure 1.3)(Garibyan & Avashia, 2013; Pelt-Verkuil et al., 2008).

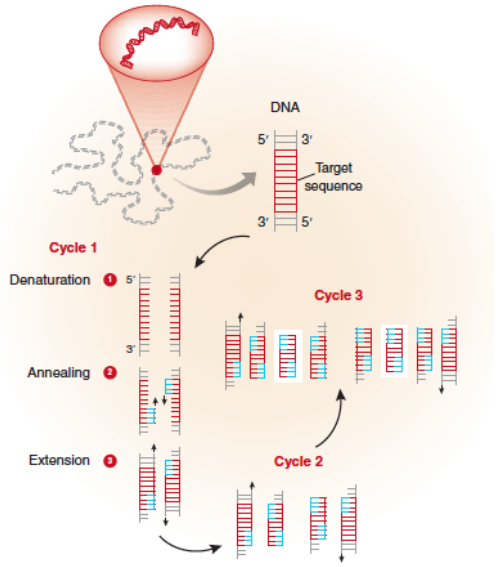


Figure 1.3: Figure that presents the principle behind the polymerase chain reaction. (Garibyan & Avashia, 2013)

1.6 Real-time Polymerase Chain Reaction, qPCR

According to Garafutdinov, Galimova, and Sakhabutdinova (2017), the PCR is a powerful tool, but it is imperative that it can be used properly in quantification analysis, because PCR by itself is not an accurate quantitative assay. Many amplification steps can lead to small differences in amplification efficiency which can result in dramatic differences in product yield (Raeymaekers, 1995). Quantitative real-time PCR is used for assessing the initial number of DNA, RNA or mRNA target molecules in a nucleic acid extract (Pelt-Verkuil et al., 2008), and according to Evrard, Boulle, and Lutfalla (2009), this assay has superseded upon conventional PCR techniques in many areas. The technique is rapid, sensitive and highly specific (McBeath et al., 2006).

For different quantitative PCR analysis', different detection systems can be used (Heid, Stevens, Livak, & Williams, 1996). One example is fluorescent labelling of the PCR products. Quantitative real-time PCR is based on "live" monitoring of the PCR product by detection and quantification of a fluorophore that is incorporated into the DNA product being amplified. By using a thermocycler with a fluorescence detector, the emissions by the fluorophore measured cycle by cycle during PCR, are directly proportional to the amount that has been generated (Evrard et al., 2009).

Quantitative real-time PCR is a method having a limited sample throughput, and is therefore not well suited for analysis of large amounts of samples (Heid et al., 1996). However, it is

regarded as the most sensitive form of PCR-based quantification (Pelt-Verkuil et al., 2008), and is a good alternative when working with minute amounts of DNA as will be the case with samples from sea-pen systems.

1.7 Aim of project

There are little knowledge about the movement of larvae in and around cage-cultured sites (Pike & Wadsworth, 1999). For further parasite control, more knowledge within the field is needed, which the project *Taskforce lakselus* aim contributing to by conducting strategic research to seal knowledge related to sea lice. The process of separating out sea lice larvae using conventional methods like microscopy, can be both difficult and time consuming (Schram, 2004). Using molecular methods, such as qPCR, in the identification process of sea lice larva can contribute to achieve more knowledge about the pathogens distribution.

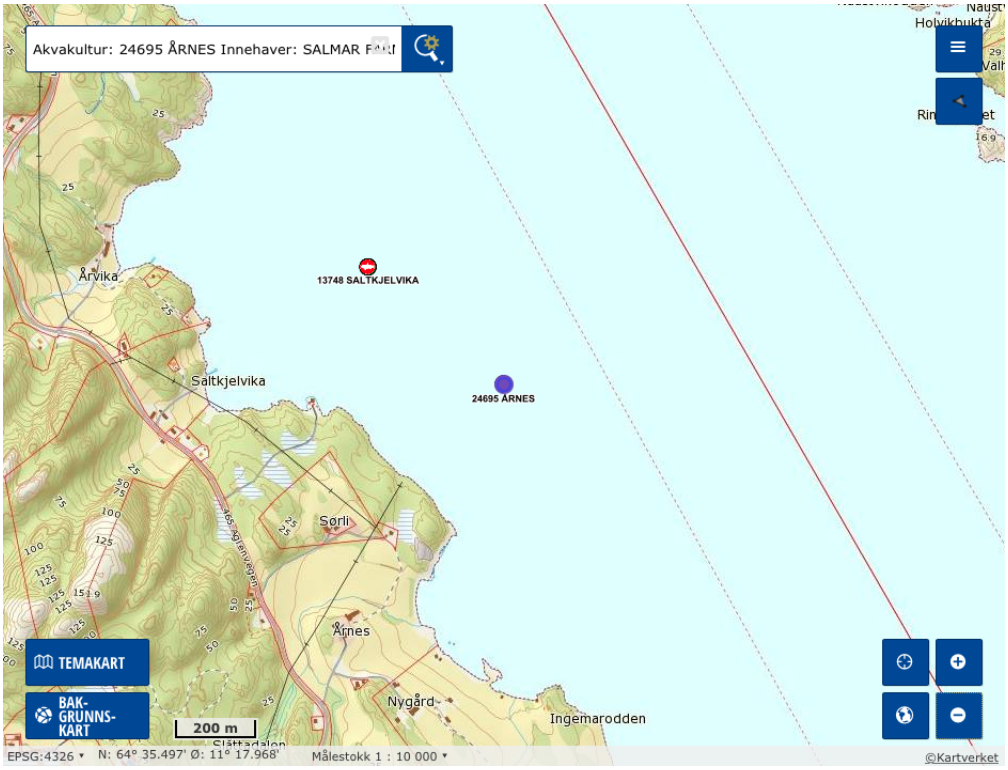
This master thesis and the experiments mentioned in this project, aimed contributing to the project *Taskforce lakselus* and its goals. The effect of formalin preservation was studied, and body measurements of *L. salmonis* larvae conducted. The qPCR method published by McBeath et al. (2006) was investigated and developed. A standard curve that can be used in qPCR to quantify the amount of sea lice larvae in a unknown plankton was further created. The estimated amount of salmon lice larvae in unknown samples found by manually counting, was compared with the estimated amount of lice found using qPCR. It was also desirable to investigate whether or not it was possible to detect different DNA levels at the different planktonic stages in individuals of *L. salmonis*.

2 Materials and methods

In the present study ovigerous sea lice were collected and hatched in the laboratory at NTNU Centre of Fisheries and Aquaculture (Sealab). The hatched individuals was preserved both using 96 % ethanol for PCR analyses and 4 % buffered formalin for morphometric examination. Length and width measurement was conducted. During traditional PCR analysis with primers designed by McBeath et al. (2006), a PCR product was made, and diluted for appropriate standard samples. The developed standard samples were used as a standard curve, which was further used in quantification of sea lice larvae in positive, negative and unknown samples.

2.1 Sample collection and preparation for qPCR analyses

Twenty five ovigerous lice of *L. salmonis* were collected from sea-farmed Atlantic salmon, on a salmon farm located at Årnes (N: 64° 35.763' E: 11° 16.406'), while fifteen ovigerous lice of *C. elongatus* were collected from atlantic salmon at another salmon farm, Makrellskjæret (N: 64° 32.949' E: 10° 44.051'), located at Nordstraumen, Flatanger. Both the fish farms were drifted by Salmar Farming. The fish was anaesthetized using Benzoak vet. (ACD Pharmaceuticals AS) in a bath, and both the bath and the body surface of the fish were examined for ovigerous lice. At the laboratory, the egg strings were removed using a scalpel. Both egg strings from one female were added to a glass containing maturated and filtered sea water. The eggs were incubated at 10°C and samples were taken out from the following development stages; mature egg strings, nauplius I, nauplius II, and the infectious copepodid stage. The samples were examined three times a day and half of the sea water in the glass were exchanged with new, maturated sea water every afternoon. This part of the study were done together with my co-supervisor Lone Sunniva Jevne, and Margrete Slåtsve Øvrelid, another student at the university master program Marine Coastal Development.



(a) Location Årnes (N:64 °35.763' E: 11 °16.406') where 25 ovigerious lice of *L. salmonis* were collected from atlantic salmon (Kartverket, n.d.).

(b) Location Makrellstjæret (N: 64 °32.949' E: 10 °44.051') where 15 ovigerious lice of *C. elongatus* were collected from atlantic salmon (Kartverket, n.d.).

Figure 2.1: Overview of the two different locations where ovigerious lice of *L. salmonis* and *C. elongatus* were collected for use in qPCR analysis.

During the egg hatching experiment, lice of the two known species (*L. salmonis* and *C. elongatus*) were used with the purpose of getting individuals at all the development stages from nauplius I until copepodid. These individuals were meant for further use in qPCR analysis, microscope studies, length measurement and imaging. The samples for the qPCR analysis were stored in 96 % ethanol, and two samples of each stage were preserved using 4 % formalin buffered with sodium tetraborate 20 g/L. The samples fixated with formalin was used in photo and length measurements.

2.2 Length measurements and photos

The examination of the larvae were done with the microscope Leica MS05 C, 0.78-16.0x and the photos taken with Carl Zeiss Microscopy Gmbtt camera (2011) at 5.0 magnification. For length measurement, the software Zen Digital Imaging for Light Microscopy (2012) at 5.0 magnification were used. Some of the pictures were then analysed, using the software ImageJ, which is an open source image processing program for multidimensional image data (Schindelin, Rueden, & Hiner, 2015). At the nauplius I stage, 29 anaesthetized individuals and 15 fixated individuals were measured, while it at the nauplius II stage were 18 anaesthetized and 20 fixated. The respective number of individuals at the copepodid stage were ten and nine. Statistical analyses were carried out to see if there was any significant difference in the measurements made of live lice anesthetized with MS-222 and the ones fixated on formalin. Length measurement and photo work were done together with Margrete Slåtsve Øvrelid.

2.3 DNA extraction

2.3.1 Positive samples

Twenty individuals of each stage (mature eggs, nauplius I, nauplius II and copepodid) were counted in the microscope and placed in preweighed 2.0 mL PCR tubes. Two replicate tubes for each stage were made. The total DNA in the samples was extracted using the DNeasy Tissue Kit (Qiagen) following the manufacturer's animal tissue protocol (Appendix 5). As much ethanol as possible was removed without disturbing the plankton using a 10 µL pipette and the remaining ethanol were allowed to evaporate. The samples were resuspended in 180 µL of Buffer ATL (Qiagen), crushed in a tissuelizer, added 20 µLProteinase-K (Qiagen) and lysed at 56°C for one hour. The protocol was followed, with these modifications; during the sec-

ond washing step, after adding 500 µL of Buffer AW2, the sample were centrifuged for one minute before discard of the flowthrough and further centrifuged for another two minutes before discard of both flowthrough and collection tube. During the elution step, 100 µL of RNase-free water were pipetted directly onto the DNeasy membrane instead of 200 µL Buffer AE as recommended in the protocol. This to increase the final DNA concentration in samples. The presence and concentration of DNA was estimated using a Qubit Fluorometer from Thermo Fisher Scientific. All samples were stored at 4°C until qPCR analyses and later stored at -20°C. After analyses, it was discovered that the actual eluted volume was not 100 µL. After investigation of 24 different samples, conducted by my co-supervisor Lone Sunniva Jevne, the average eluted volume was 83.625 µL. It was further assumed that 83.625 µL contained the extracted DNA amount of 20 individuals, and this was used in the calculations. Pipetting 5 µL of this eluate into the well during qPCR analyses, corresponds to an amount equivalent to 1.2 individuals.

2.3.2 Negative samples

For investigation of the results from an assumed negative sample, DNA was extracted from a sample presumed not containing any *L. salmonis* larvae. This based on the lack of sea lice when the replicate were counted under microscope by Margrethe Slåtsve Øvrelid. The same extraction procedure was conducted (DNeasy Tissue Kit, Qiagen). These samples are from here referred to as negative samples.

2.3.3 Unknown samples

The unknown field samples were placed in tubes. The samples were centrifuged before ethanol was removed. This step was repeated until almost all the ethanol had been removed. The resulting material was placed on a 100 µm net towel and the remaining ethanol were allowed to evaporate before measurements of dry weight. The material was further crushed using liquid nitrogen in a porcelain mortar, and placed in 2 mL PCR tubes. The total DNA was further extracted using the DNeasy Tissue Kit (Qiagen) in the same manner as for positive and negative samples.

2.4 Polymerase Chain Reaction, PCR analysis

To obtain enough of the amplicon sequence, a PCR reaction was performed to amplify a specific DNA fragment from *L. salmonis*. Amplification was performed on 63°C, 45 cycles. The *L.*

salmonis primer designed by McBeath et al. (2006) was used (Figure 2.2), and the procedure adapted. The resulting PCR product was cleansed using Wizard® Genomic DNA Purification Kit (Promega). Gelelectroforesis using the Low DNA Mass ladder were run on a 1 % agarose gel to confirm amplification of the right amplicon. The PCR product was diluted for appropriate standard samples for use in the qPCR assay, creating the standard curve presented in the results.

L.s. GGGCACCT**GACATAGCTTTCCCCCGCTTA**AACAATATAAGATTTTGGTTTTTAAT**ACCCT**
C.e. GTGCGCCAGATAT**GGCATTTCCTCGCCTGAATA**ATATAAGTTTTTGATTTTTGAT**ACCGT**

L.s. **CTTTGAGTTTATTACT**TATAAGGG**CATTAGTAGAAAGTGGTGCAGGAACT**GGGTGAACAG
C.e. **CACTAACACTACTACT**TTTAAGRG**CTCTTGTTGAAAGGGGTGCAG**GTACAGGGTGAACAG

Figure 2.2: Primers designed by McBeath et al. (2006). Amplicon size are 102 bp for *L. s.* and 92 bp for *C. e.*. The sequences are single-stranded and shown in 5'-3' direction. The primers are highlighted in bold with arrows indicating forward and reverse direction (McBeath et al., 2006).

Molecular weight of the PCR product was estimated using the online calculator from the web page www.bioinformatics.org (Bioinformatics, n.d.). By using the estimated molecular weight of the PCR product, standard samples was created. The DNA amount in the PCR product was found by using a Qubit Fluorometer. The number of DNA copies per µL in the PCR product were calculated by using equation 2.1. For all calculations, see Appendix 5.

$$copy\ number = \frac{amount \times (number/mole)}{length of bp \times (ng/g) \times (g/mole of bp)} \tag{2.1}$$

All dilution steps was carried out using MQ water, to avoid excessing the DNA amount during the PCR assay and thereby prevent inhibition during the analysis.

2.4.1 Development of new standard curve using TOPO-4 plasmid

While developing the standard curve, an unexpected error was observed. A new standard curve was developed by using a TOPO-4 plasmid with the amplicon sequence inserted. This work was performed by Mari-Ann Østensen and my co-supervisor, Lone Sunniva Jevne, who also work on the project *Taskforce lakselus*. The resulting standard curve confirmed the assumed error, and was used in further calculations, instead of the standard curve developed in the present study. A selection of the first standard samples (PCR product) were analysed together with these standard samples (plasmid product). A linear regression was used for

calculations of the actual concentration in the samples by inserting the cq value of each sample, as this is calculated independently of the standard.

2.5 Quantitative Real-Time PCR, qPCR analysis

Real-time PCR (qPCR) analyses was carried out with DNA extracted from both *L. salmonis* and *C. elongatus*. A mastermix containing SYBR® Green I, a double stranded (ds) DNA binding dye which detects PCR product as it accumulates during the PCR assay (Sigma Aldrich, 2010), and the primers for *L. salmonis* and *C. elongatus* designed by McBeath et al. (2006) was used. The amplification was carried out on a Roche LightCycler® 96 System, programmed with the following cycle profile: 45 cycles with 95 °C for 10 seconds, followed by 63 °C for 10 seconds and 72 °C for 10 seconds.

2.5.1 Positive, negative and unknown samples

The assay was performed in 96-well plates, adding 5 µL DNA template from the extracted sample into each well. This amount is equivalent to 1.2 individuals of sea lice by calculations (Appendix 5). In addition to this, 15 µL of a master mix made of primers (both reverse and forward), SYBR Green I Mastermix and MQ water was added. The primers were prepared in advance by using 10 µL of both the forward and the reverse primer (100 µM) together with 180 µL H_2O, creating a solution with a concentration of 5 µM of both the primers. The samples containing eggs, nauplii II and copepodids was diluted in 10-fold serial dilutions (1:1, 1:10, 1:100). The samples containing nauplii I were diluted 10-fold down to 1:10 000 (1:1, 1:10, 1:100, 1:1000, 1:10 000). The PCR efficiency for each pair of primers were assessed using LinRegPCR, a software that uses the raw fluorescence data, and calculates the PCR efficiency in each reaction by a method that relies on linear regression (Ruijter et al., 2009). A standard curve was made by analysing the prepared standards together with DNA extracted from a known number of individuals. To not overload the Qubit Fluorometer, a 1:100 dilution of the solution was made prior to analysis, before further calculations for the standard samples. For calculations of standard samples, see Appendix 5.

2.6 Data analysis and statistics

When conducting statistics on length and width measurements, a parametric test was implemented (ANOVA). The results from this test did not satisfy the requirements for parametric

testing. Because of this, a non-parametric test (Mann-Whitney) was conducted. All calculations and statistical analysis were carried out using Sigmaplot 13.0 from Systat Software, Inc., San Jose California USA (www.systatsoftware.com) and Microsoft Excel 15.17 (2015).

3 Results

The thesis consisted of two parts. The first one included measurements of both size and amount of DNA at the planktonic stages. The size measurements were conducted on individuals fixated on formalin or anesthetized with MS-222, to investigate the difference in shrinkage. The DNA amount in 20 individuals were measured by Qubit and qPCR. The second part was to develop a standard curve using a PCR product, for quantification of sea lice larvae in both known and unknown samples. The quantitative results from molecular analysis was compared with manually counting using microscope. The results will be utilized in dispersal studies in the project *Taskforce lakselus*.

3.1 Measurement of sea lice larvae

Length and width measurements of formalin fixated (4 %) and anesthetized (MS-222) *L. salmonis* larvae are presented in Table 3.1. The results from the present study revealed no significant increase in length between live larvae at the nauplius I stage and live larvae at the nauplius II stage ($p>0.05$). However, an increase in length was observed when the live larvae moulted into copepodid ($p<0.001$). Comparing cephalothorax width, live larvae at the nauplius II stage were skinnier than both live larvae at the nauplius I stage ($p<0.001$) and live larvae at the copepodid stage ($p<0.001$).

Comparing the live nauplii I larvae with the ones fixated on 4 % formalin, a statistically significant difference in both length and width between the two groups was observed ($p<0.001$). The fixated larvae were both shorter and skinnier compared to the live larvae. Comparing cephalothorax width of the nauplii II larvae, the anaesthetized larvae was skinnier than the fixated ones ($p<0.05$). There was no statistical significant difference in length ($p>0.05$). When the anaesthetized copepodids were compared with the fixated ones, difference in neither width ($p>0.05$) nor length ($p>0.05$) was observed.

Table 3.1: Measurements (µm) of live (anaesthetized with MS-222) and fixated (4 % formalin) *L. salmonis* larvae. Statistically significant differences between live and fixated larvae at the nauplius I and nauplius II stage are marked in bold.

L. salmonis	**Nauplius I**	**Nauplius II**	**Copepodid**
Length±SD, range			
MS 222	**515**±14, 457-529	526±21, 485-558	696±22, 670-727
Formalin	**467**±10, 443-477	527±12, 503-548	687±18, 651-709
This study, 2017			
Width±SD, range			
MS 222	**211**±7, 203-225	**188**±8, 163-199	244±6, 235-250
Formalin	**198**±8, 185-213	**193**±6, 182-205	250±12, 226-264
This study, 2017			

Figure 3.1 illustrates the observed differences between the anesthetized larvae and the fixated ones at the nauplius I stage. No evident differences between the individuals anaesthetized with MS-222 and the ones fixated on 4 % formalin can be seen at the nauplius II stage or the copepodid stage.

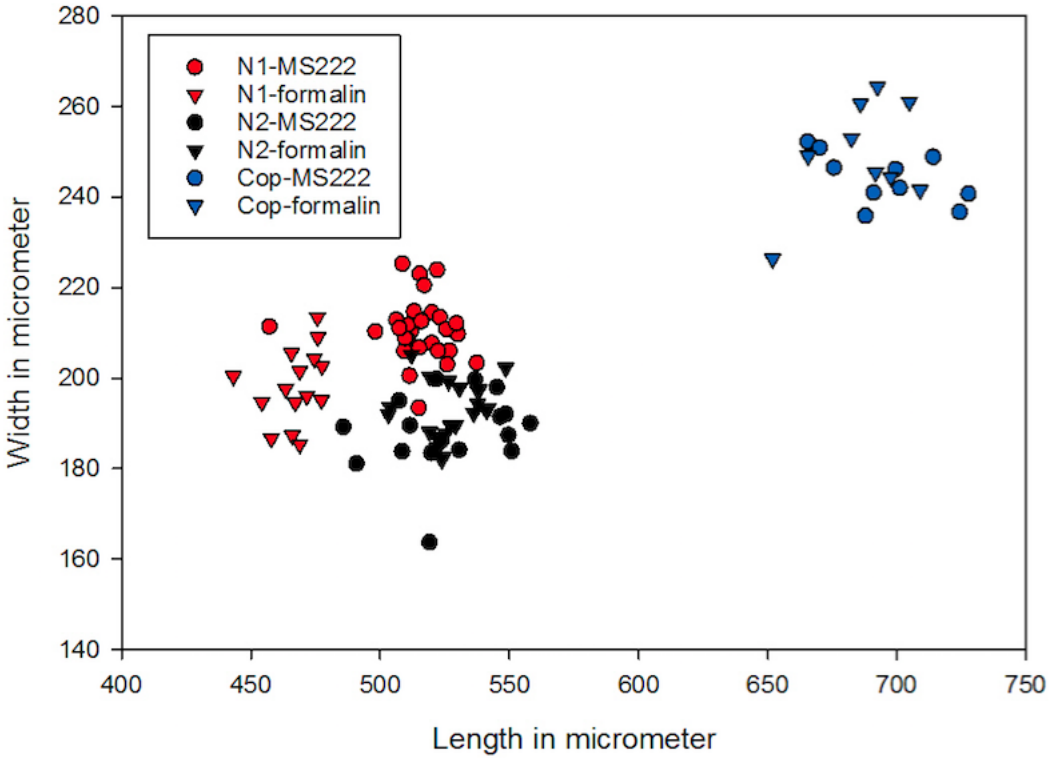


Figure 3.1: Width in µm as a function of length in µm of *L. salmonis* larvae anesthetized with MS-222 and fixated on 4 % formalin.

3.2 qPCR analyses

3.2.1 Detection of small amounts of DNA

The primers designed by McBeath et al. (2006) were tested on samples containing a known number of *L. salmonis* larvae and *C. elongatus* larvae. The primer for salmon lice was able to detect lice in samples at all the different development stages, and was species specific. However, the primer for *C. elongatus* failed to detect all the lice found in the analysed samples. Further work on *C. elongatus* was therefore abandoned.

The DNA extracted from *L. salmonis* was diluted with DNA extracted from other organisms from a field sample, and the estimated DNA concentrations of *L. salmonis* are presented in Table 3.2. It was also possible to detect DNA from the target species when the sample was diluted with extracted DNA from other organisms from a field sample and further with water. Estimated DNA concentrations for these analyses are presented in Table 3.3. The presented results confirms that this method can detect even small amounts of DNA in a precise matter.

Table 3.2: Estimated concentration (copy number) of *L. salmonis* DNA when detected in samples diluted with DNA extracted from other organisms from a field sample (10 fold dilution).

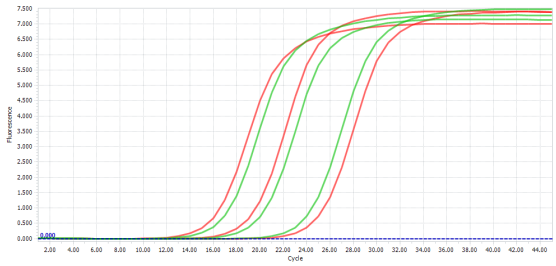
DNA:DNA field sample	**Corresponding number of lice**	**Cq**	**Concentration**
1:10	0.12	22.27	12 224.75
1:100	0.012	27.63	349.59
1:1000	0.0012	32.24	16.44
1:10 000	0.00012	34.72	3.17

Table 3.3: Estimated concentration (copy number) of *L. salmonis* DNA when detected in samples diluted with DNA from other organisms from a field sample (10 fold dilution), further diluted 10-fold with water.

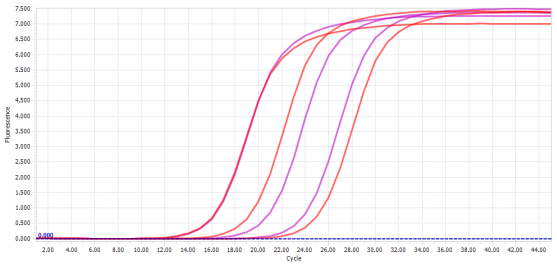
DNA:DNA field sample:H_2O	**Corresponding number of lice**	**Cq**	**Concentration**
1:10:10	0.012	20.97	28 949.42
1:10:100	0.0012	25.34	1596.17
1:10:1000	0.00012	28.46	201.61
1:10:10 000	0.000012	31.49	27.03

3.2.2 DNA amount at different development stages

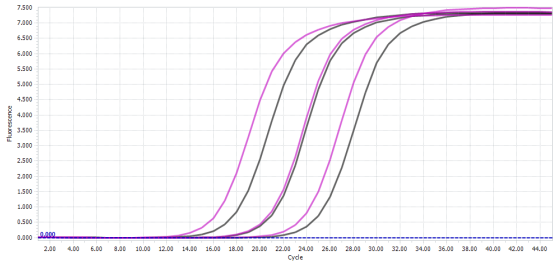
The qPCR analyses of samples containing extracted DNA from 20 individuals at the different life stages, gave the resulting amplification curves (AC) presented in Figure 3.2. The estimated mean cq values for the non-diluted samples containing eggs, nauplii I, nauplii II and copepodids were 14.98, 14.08, 14.21 and 15.05 respectively. An overview of the AC for all the different stages in 10-fold dilutions down to 1:100 are presented in Figure 3.2d. Cq bars for the different development stages are found in Figure 3.3.



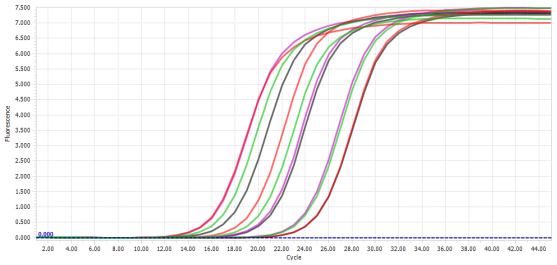
(a) AC for sample containing 20 eggs (green) *versus* AC for sample containing 20 individuals at the nauplius I stage (red).



(b) AC for sample containing 20 individuals at the nauplius I stage (red) *versus* AC for sample containing 20 individuals at the nauplius II stage (purple).



(c) AC for sample containing 20 individuals at the nauplius II stage (purple) *versus* AC for 20 individuals at the copepodid stage (black).



(d) AC for all stages. Eggs:green, n1:red, n2:purple and copepodid:black.

Figure 3.2: Overview of amplification curves (AC) at the different life stages of *L. salmonis*. All graphs include samples diluted 10-fold from 1:1 down to 1:100.

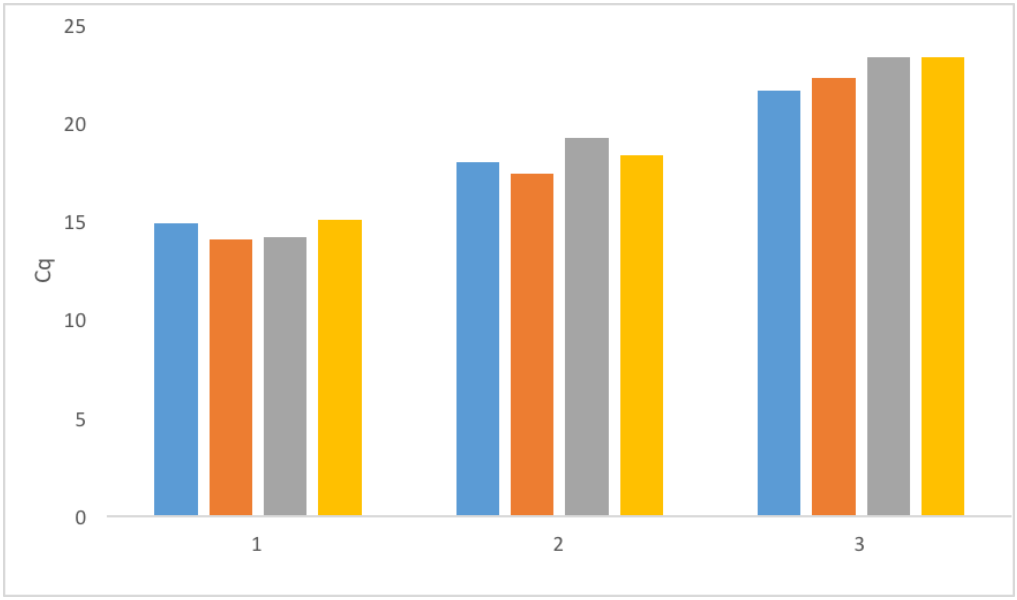


Figure 3.3: Cq-bars for the following development stages of *L.salmonis*: egg (blue), nauplius I (orange), nauplius II(grey) and copepodid(yellow). x=1 refers to dilution 1:1, x=2 to dilution 1:10 and x=3 to dilution 1:100.

3.2.3 Standard curve

The developed PCR product had a mean DNA amount of 31.7 ng/µL (Appendix 5, Table 1) when measured using the Qubit Fluorometer. The molecular weight of the double stranded primer amplicon was 63045.12 g/mole. The number of DNA copies in the PCR product was calculated to 3.03×10^{11}/ml, giving a number of 3.03×10^{8} DNA copies/µL in the PCR product. This was used in calculations when making the standard sample of 100 000 000 DNA copies per well (5 µL). For all calculations, see Appendix 5. The resulting standard curve is presented in Figure 3.4.

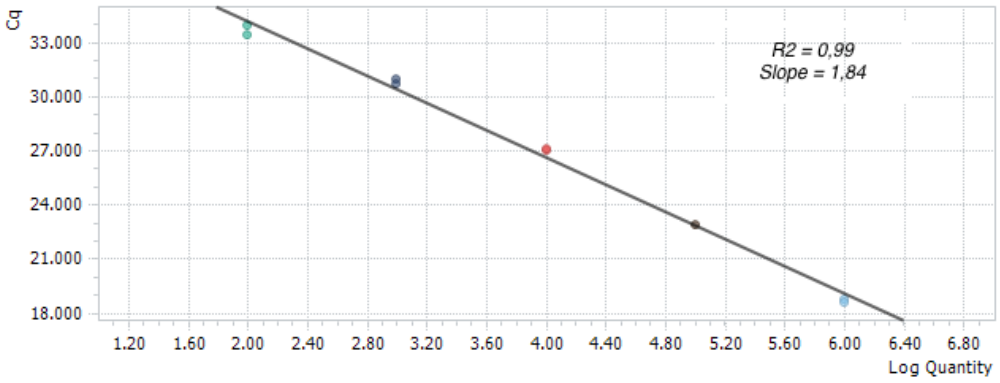


Figure 3.4: Standard curve developed in Roche LightCycler® 96 Instrument using the produced PCR amplicon consisting of 102 bp.

Comparison of the cq values for the different standard samples (developed using PCR product and the TOPO-4 plasmid) are presented in Figure 3.5. The standard samples made using the PCR product were incorrect, and the new standard curve presented in Figure 3.6 was used for further quantification of *L. salmonis* larvae in unknown field samples. The x axis represent the log of the initial number of DNA copies added. The log concentration of the standard samples are plotted on the x axis against cycles during the analysis on the y axis.

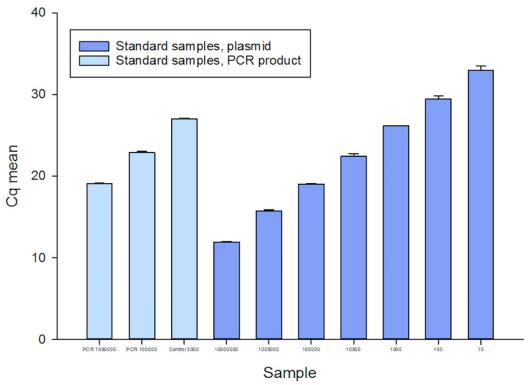


Figure 3.5: Cq values for the different standard samples made by using the TOPO-4 plasmid and the 102 bp PCR product.

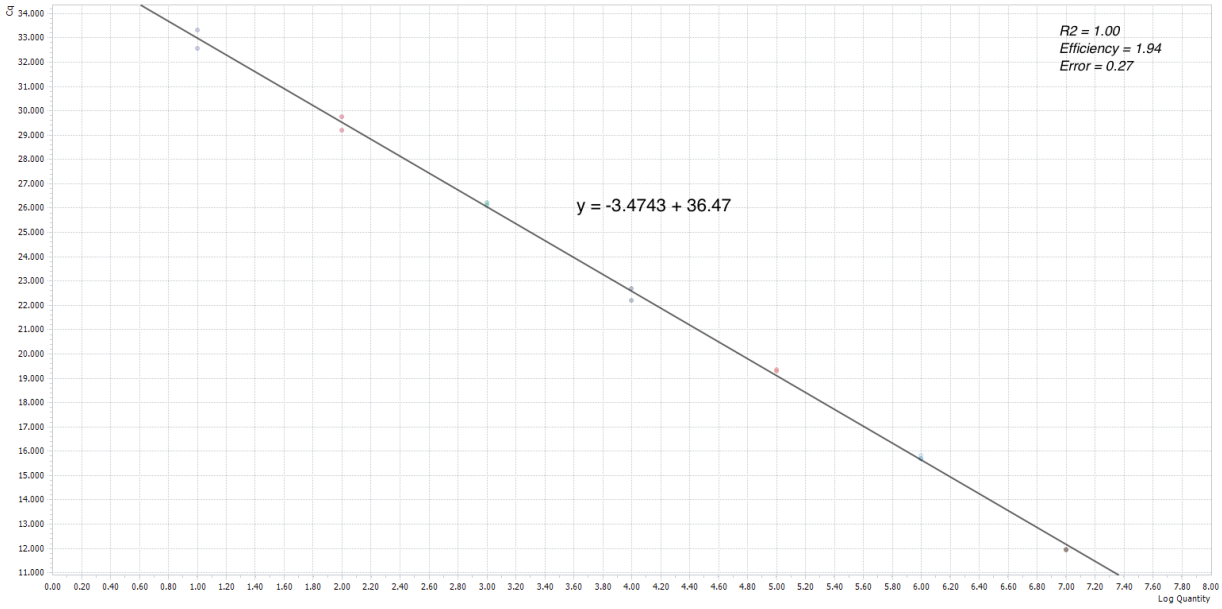


Figure 3.6: The resulting standard curve using the TOPO-4 plasmid. Standard samples were run in the Roche LightCycler® 96 Instrument and used in quantification of sea lice in field samples. y = -3.4743 + 36.47. R^2=1.00. Effiency=1.94. Error=0.27.

A selection of the first standard samples (using PCR product) were analysed together with the standard samples (using plasmid product). A linear regression of the ratio between cq values and concentration of the standards (plasmid product) is presented in Figure 3.7.

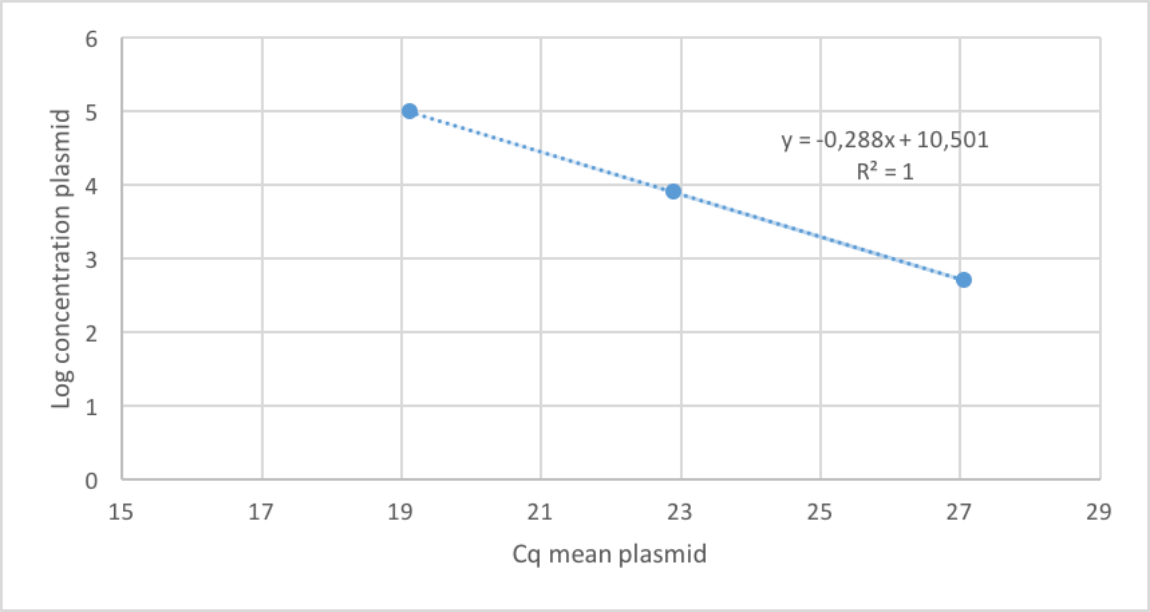


Figure 3.7: Linear regression presenting the ratio between cq values and concentration for the conducted standards made using the TOPO-4 plasmid.

3.3 Using the standard curve in quantification of sea lice in field samples

Positive, negative and unknown samples were run through qPCR analyses. A positive sample contained 20 individuals of *L. salmonis*. A negative sample was assumed not to contain any *L. salmonis* larvae, based on the lack of sea lice when the replicate was counted under microscope. The unknown samples were collected from different sea-pen systems, and the replicate samples were counted. This part of the thesis aimed to compare the amount of individuals detected by counting and the amount detected using qPCR.

3.3.1 Positive field samples

From the regression presented in Figure 3.7, the DNA amount in each of the 5 µL wells was calculated (corresponding to 1.2 individuals of *L. salmonis*), by using the cq value for each 10-fold dilution down to 1:100 000. The results are presented in Table 3.4.

Table 3.4: Estimated number of DNA copies in a known positive sample containing 1.2 individuals of *L. salmonis* diluted 10-fold down to 1:100 000.

Sample dilution	Cq mean	y-value	Concentration	Corresponding amount of lice
1:1	14.08	6.45	2 792 286	1.2
1:10	17.29	5.52	332 261	0.12
1:100	20.94	4.47	29 512	0.012
1:1000	24.20	3.53	3338	0.0012
1:10 000	27.15	2.58	380	0.00012
1:100 000	30.30	1.59	39	0.000012

An undiluted sample equivalent to 1.2 individuals of *L. salmonis* contained 2 792 286 DNA copies. As presented in Table 3.4, the plasmid standard making it possible to detect down to 0.000012 individuals with a concentration of 39 DNA copies.

3.3.2 Negative field samples

A sample presumed to contain no sea lice, based on the lack of sea lice when the replicate were counted under microscope, was analysed using qPCR. The analysis confirmed the assumption of no sea lice DNA. The results are presented in Table 3.5. Only trace amounts of DNA were estimated in 5 µL of the sample. With cq values of approximately 34 amplification cycles, the sample contain less DNA than possible for detection using the developed standard curve in quantification.

Table 3.5: Estimated number of DNA copies in a negative field sample not expected to contain *L. salmonis* larvae.

Sample dilution	Cq mean	y-value	Concentration	Corresponding amount of lice
1:1	34.43	0.59	3.85	1.2
1:10	34.14	0.67	4.66	0.12
1:100	33.88	0.74	5.54	0.012

3.3.3 Unknown field samples

The estimated number (using qPCR) of sea lice in unknown samples and the corresponding amount of sea lice in the replicate samples counted under microscope, are presented in Table 3.6. The counted amount presented in the table refers to the number of individuals counted in the actual replicate sample. Calculations are found in Appendix 5, Table 9.

Table 3.6: Estimated amount of individuals in unknown field samples and corresponding number of lice counted in parallel samples. The DNA concentration refers to the copy number in the sample.

Sample ID	**DNA concentration sample**	**Corresponding number of lice**	**Counted**
Ins2/C/1	43 998	0.015	1
Out2/C/1	263 333	0.09	1
Ins1/A/1	50 692	0.018	3
Ins2/CC/1	260 561	0.09	7
Ins1/B/1	Negative	Negative	1
Out2/B/1	Negative	Negative	2
Ins2/AA/1	Negative	Negative	2

4 Discussion

4.1 Measurements of the salmon lice larvae

4.1.1 Comparing length and width measurements of live larvae

The length of the *L. salmonis* nauplii I larvae, fits well into the existing literature on nauplii sizes reported by Schram (2004). However, the larvae measured in this study is wider in size. Looking at the nauplius II stage, another trend is observed. In the present study, both the length and width of the largest larvae measured, was smaller than some of the biggest ones measured by Schram. This also applies when comparing with the results from Johnson & Albright. The result in this study presents measurements indicating that the live larvae at the nauplius II stage are smaller in size than reported earlier. Length and width of the copepodids measured in the present study were close to the measurements reported in the earlier publications. The measured dimensions of *L. salmonis* from both this study and from the earlier publications are included in Table 4.1 for comparison.

In other words, the size of the larvae appears to vary from study to study. However, the present study was implemented in a short period of time, and it will be necessary to gain more data to get a reliable conclusion. In addition, there may be several reasons for these discrepancies, like the studies being conducted in different countries, which may include local adaptations. As stated in earlier publications, temperature is known to affect developmental rates, and lower temperatures tend to increase body size (Gravil, 1996).

Table 4.1: Measurements (µm) of live (anaesthetized with MS-222 in this study) *L. salmonis* larvae from different publications.

L. salmonis	**Nauplius I**	**Nauplius II**	**Copepodid**
Length±SD	515±14	526±21	696±22
Width±SD	211±7	188±8	244±6
N	29	18	10
This study, 2017			
Length±SD	511±24	606±10	684±16
Width±SD	188±8	205±10	229±7
N	30	22	15
Schram, 1993			
Length±SD	540±40	560±10	700±10
Width±SD	220±10	200±10	280±10
N	25	16	25
Johnson & Albright, 1991			

4.1.2 Comparing measurements of live and fixated larvae

The live nauplii I in the present study were both longer and wider compared with the ones fixated in formalin. A size reduction is expected when larvae are preserved in 4 % formalin, because of negative osmotic pressure leading to changes in cell volume (Steedman,1976 cited in Thibault-Botha and Bowen, 2004; Ohman and Snyder, 1991 cited in Jaspers and Carstensen, 2009). The fixated nauplii II were however similar in length, but slightly skinnier compared to the anesthetized ones. For the copepodids, there were no difference in neither length nor width.

It may be assumed that the size differences seen at the nauplius I stage is greater because of the lack of a hard protecting outer shell. As the louse develops into a nauplius II larvae, the outer shell becomes more rigid, and at this point, smaller size differences between the anaesthetized and fixated individuals were seen as well. When comparing individuals at the copepodid stage, no significant difference in size was observed. It can be assumed that the outer shell now contains a lot of chitin and thus has evolved into a fairly strong structure protecting the larvae from the change in osmotic pressure, further leading to the larvae not being as exposed to size differences as in the previous stages. Research also points out that larger size losses are expected in individuals being gelatinous, which have a high water content and no hard covering (Thibault-Botha & Bowen, 2004).

4.2 Method trial

When testing the primers designed by McBeath et al. (2006), the primer developed for *L. salmonis* was species-specific. However, the primer for *C.elongatus* showed variable effect and was not able to detect all the lice found in the samples. Since the project went over such a short period of time, new primers were not developed for this thesis. It is difficult to say something specific about why the primer did not work optimally. The primer published by McBeath et al. (2006) may have been incorrect. We may also speculate about how many subspecies that exists of both the two species. There are probably small differences in the DNA of different subspecies, and a single base change could affect the results due to the primer not being able to recognize the target sequence. In further studies it will be necessary to check this out more carefully and develop new primers. It could be beneficial to test different primers against a large number of lice from different locations, to look for genetic variations.

4.3 DNA amount at the different development stages

The result indicate small, but not remarkable differences in DNA amount at the different life stages, and neither the amplification curves nor the resulting cq bars indicate a clear trend of increase or decrease in DNA amount.

It is conceivable that individuals in some samples have developed at an abnormal pace or that some may have died. This could to some extent have led to degradation of DNA, already before the extraction, and may thus provide incorrect information during the analyses. The small differences observed can be related to the already existing size differences in the collected individuals. For example, two individuals at the copepodid stage were not necessarily collected at the exactly same time, which can give individually differences due to size. There is also a possibility that the DNA level increase slightly, due to the energy pack each larvae carries through the life cycle before it starts to nurture itself. Nevertheless, it is unlikely that this should lead to significant differences in DNA levels at the free-living stages.

The extraction process may also have effected the DNA output. It is difficult to assume anything regarding how the chemicals used affect the salmon louse and the chitin in the outer shell. According to Mao et al. (2010), chitin can bind negatively charged DNA if deacetylated to chitosan - a compound being positively charged. Hypothetical, chemicals during the extraction could have led to deacetylation of chitin, which may have contributed to a less effective extraction process at the copepodid stage compared to the earlier stages.

Investigation of more samples will be needed to say something certain about the DNA amount at the different planktonic stages, as well as checking if the DNA levels change from early to late in each of the different stages. In the present study, this was not conducted due to limited time of the thesis.

4.4 Development of standard curves

The standard curve developed using the TOPO-4 plasmid gave results where the measured concentrations using qPCR were close to the respective calculated concentrations. In contrary, after discussion with experienced professors, it was suspected that the standard samples using the PCR product were incorrect, calculating the number of DNA copies in the sample a 10's power too high. The standard sample (PCR product) which was expected to contain one million DNA copies, had nearly the same cq value as the standard sample (plasmid) containing 100 000 copies. Similarly, the standard expected to contain 100 000 copies, gave the same result at the sample containing 10 000 copies. A control expected to contain 5000 copies had a cq value lying between the cq values for the samples of 100 and 1000 DNA copies, indicating that the sample contained approximately 500 DNA copies.

Several reasons can be discussed when pondering about why the use of a plasmid gave a more reliable result than the first standard curve developed using the PCR product. When working with the PCR product, small sample amounts may have led to the samples being in the lower range of what the machine was able to measure. This may have given non-reliable results. Errors during measurements were critical for all subsequent calculations, since the number of copies in the standard solution and the following dilution of the standard samples was calculated based on the measured DNA concentration. In addition, the TOPO-4 plasmid consist of over 3000 bp, included the current amplicon sequence. The increase in length is believed to reduce the probability of errors during measurements. This argues that the plasmid should be used in further studies.

Taken into account that considerable errors may occur during measurements, it will be advisable making a 10-fold dilution series of the product before conducting measurements. This in order to compare the measurements and exclude any outliers to get credible results. In this thesis, the Qubit Fluorometer was chosen as quantification method because this fluorometric quantification which measure DNA via fluoroscent dyes (ThermoFisher, n.d.), was considered more specific than spectrophotometrically measurement using Nanodrop.

Creating a standard curve for use in qPCR based on the number of DNA copies in a sample using the TOPO-4 plasmid, represents a potential good methodology for quantification of

pathogens in plankton samples. This method may provide accurate results even with just trace amounts of DNA, in addition to being less time consuming compared to detection using traditional methods such as microscopy. With only parts of an individual in a plankton sample, it will be difficult to detect this under microscope. These parts will, however, be possible to detect using molecular quantification.

4.5 Quantification of larvae in field samples

A consistently smaller amount of DNA than expected was estimated using qPCR, when comparing the number of *L. salmonis* larvae counted manually in the field samples with the estimated number in the replicate samples using qPCR. Trace amounts were detected in most samples, and the dilution having a similar melting point curve as the standard was used. Some samples had cq values exceeding the detection range of the standard curve. One sample got particular attention. Using microscope, seven individuals of *L. salmonis* were counted, while the qPCR analysis gave a result of 0.09 individuals in the replicate sample. However, it is not provided that qPCR analyses underestimate the number of individuals in the samples investigated, and many factors may have contributed to the results.

It is also possible that individuals have been identified as the wrong species when counted under microscope. As stated by Pike and Wadsworth (1999), the identification process can be difficult. In addition, the parallel samples analysed using qPCR were not counted manually. One can further not assume that the two replicate samples did contain the same number of lice in the first place.

During the investigation of known positive samples, DNA was extracted from *L. salmonis* larvae. In a net haul sample, the proportion of DNA could be completely different compared to a sample isolated from one species, which in turn can affect the reaction. Using qPCR, the different species provides a complex sample that may be difficult to analyse. PCR inhibition was observed when analysing undiluted samples, causing the reaction to slow down and giving rise to high cq values indicating smaller DNA amounts. However, 10-fold dilution appeared to remove inhibitory effect. The basis for this assumption is the analyses carried out by mixing extracted DNA corresponding to a known number of individuals with extracted DNA from a field sample. The primer did manage to detect DNA from *L. salmonis*, but when comparing the results with the estimated DNA amount in positive samples, a smaller DNA amount was estimated, indicating that the samples were inhibited. However, a good correlation with the estimated DNA amount in positive samples was observed when diluting the samples further, 10-fold in water. Inhibition of samples may influence the final concentration of generated PCR product, and hence estimated copy number (Pelt-Verkuil et al., 2008).

4.6 Improvement of the method

In addition to the sources of error that have already been discussed, the following factors are worth some attention. If the present study had been conducted in a larger research context, with more time available for carrying out laboratory work and obtaining necessary material, more replicates per sample and more samples in total should have been investigated. This to obtain more reliable data. In addition, the elution step during extraction was conducted using 100 µL of MQ water and a 100 % yield in the total finished elution volume of 100 µL was assumed in the first place. In further work it will be necessary to measure the actual eluted amount in all examined samples to get more precise calculations.

5 Conclusion

In the first part of the present study, the effect of formalin preservation was studied. Measurements of larvae preserved on 96 % ethanol and 4 % formalin were conducted. The results indicate a significant smaller larval size of individuals being at the nauplius I stage, while only small differences were observed at the nauplius II stage and within individuals who have reached the copepodid stage. This can probably be seen in connection with the development of the rigid outer shell, protecting the larvae from being as influenced by the change in surrounding osmotic pressure. It was also investigated whether it was possible to detect any differences in DNA amount at the different development stages, which the results from this study did not indicate.

The second part was to compare two different methods used for quantification of sea lice larvae in plankton samples. Manual counting of larvae under microscope was compared with estimated number of larvae using qPCR. For the qPCR analyses, a standard curve was created based on the number of DNA copies and further used to estimate the number of sea lice in different samples. The results indicate that it is possible to detect the number of lice in a sample using qPCR. However, the field samples used were likely too complex and inhibited, which may have led to undesirable results. Nevertheless, several different experiments with the primer used, showed that it is possible to detect DNA from *L. salmonis* when mixed with other DNA. qPCR is believed to present a potentially good methodology for detection of pathogens in field trials, if the problems with inhibition of samples is taken into account and solved. It will however be necessary to investigate more replicates and samples to make certain conclusions. In addition, the number of individuals in the samples should be counted in advance, giving an indication of how many individuals that should be expected in the samples. Without this being done, it is concluded that it is difficult to say anything certain about the DNA amount estimated using qPCR, and the method needs refinement before further studies.

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Appendices

DNA extractions

DNeasy Blood and Tissue Handbook, procedure from Qiagen

The DNAeasy 96 protocol for purification of Total DNA from Animal Tissues is enclosed. The procedure is given by Qiagen.

Protocol: Purification of Total DNA from Animal Tissues (Spin-Column Protocol)

This protocol is designed for purification of total DNA from animal tissues, including rodent tails.

Important points before starting

- If using the DNeasy Blood & Tissue Kit for the first time, read "Important Notes" (page 15).
- For fixed tissues, refer to the pretreatment protocols "Pretreatment for Paraffin-Embedded Tissue", page 41, and "Pretreatment for Formalin-Fixed Tissue", page 43.
- All centrifugation steps are carried out at room temperature (15–25°C) in a microcentrifuge.
- Vortexing should be performed by pulse-vortexing for 5–10 s.
- Optional: RNase A may be used to digest RNA during the procedure. RNase A is not provided in the DNeasy Blood & Tissue Kit (see "Copurification of RNA", page 19).

Things to do before starting

- Buffer ATL and Buffer AL may form precipitates upon storage. If necessary, warm to 56°C until the precipitates have fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Preheat a thermomixer, shaking water bath, or rocking platform to 56°C for use in step 2.
- If using frozen tissue, equilibrate the sample to room temperature. Avoid repeated thawing and freezing of samples since this will lead to reduced DNA size.

Procedure

1. **Cut up to 25 mg tissue (up to 10 mg spleen) into small pieces, and place in a 1.5 ml microcentrifuge tube. For rodent tails, place one (rat) or two (mouse) 0.4–0.6 cm lengths of tail into a 1.5 ml microcentrifuge tube. Add 180 µl Buffer ATL. Earmark the animal appropriately.**

   Ensure that the correct amount of starting material is used (see "Starting amounts of samples", page 15). For tissues such as spleen with a very high number of cells for a given mass of tissue, no more than 10 mg starting material should be used.

We strongly recommend to cut the tissue into small pieces to enable more efficient lysis. If desired, lysis time can be reduced by grinding the sample in liquid nitrogen* before addition of Buffer ATL and proteinase K. Alternatively, tissue samples can be effectively disrupted before proteinase K digestion using a rotor–stator homogenizer, such as the QIAGEN TissueRuptor, or a bead mill, such as the QIAGEN TissueLyser (see page 56 for ordering information). A supplementary protocol for simultaneous disruption of up to 48 tissue samples using the TissueLyser can be obtained by contacting QIAGEN Technical Services (see back cover).

For rodent tails, a maximum of 1.2 cm (mouse) or 0.6 cm (rat) tail should be used. When purifying DNA from the tail of an adult mouse or rat, it is recommended to use only 0.4–0.6 cm.

2. **Add 20 µl proteinase K. Mix thoroughly by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a thermomixer, shaking water bath, or on a rocking platform.**

   Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h or, for rodent tails, 6–8 h. If it is more convenient, samples can be lysed overnight; this will not affect them adversely.

   After incubation the lysate may appear viscous, but should not be gelatinous as it may clog the DNeasy Mini spin column. If the lysate appears very gelatinous, see the "Troubleshooting Guide", page 47, for recommendations.

   Optional: If RNA-free genomic DNA is required, add 4 µl RNase A (100 mg/ml), mix by vortexing, and incubate for 2 min at room temperature before continuing with step 3.

   Transcriptionally active tissues such as liver and kidney contain high levels of RNA, which will copurify with genomic DNA. For tissues that contain low levels of RNA, such as rodent tails, or if residual RNA is not a concern, RNase A digestion is not necessary.

3. **Vortex for 15 s. Add 200 µl Buffer AL to the sample, and mix thoroughly by vortexing. Then add 200 µl ethanol (96–100%), and mix again thoroughly by vortexing.**

   It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol can be premixed and added together in one step to save time when processing multiple samples.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure. Some tissue types (e.g., spleen, lung) may form a gelatinous lysate after addition of Buffer AL and ethanol. In this case, vigorously shaking or vortexing the preparation is recommended.

4. **Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x *g* (8000 rpm) for 1 min. Discard flow-through and collection tube.***

5. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x *g* (8000 rpm). Discard flow-through and collection tube.***

6. **Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x *g* (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.**

   It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

   Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000 rpm).

7. **Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at ≥6000 x *g* (8000 rpm) to elute.**

   Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield (see Figure 2, page 21).

8. **Recommended: For maximum DNA yield, repeat elution once as described in step 7.**

   This step leads to increased overall DNA yield.

   A new microcentrifuge tube can be used for the second elution step to prevent dilution of the first eluate. Alternatively, to combine the eluates, the microcentrifuge tube from step 7 can be reused for the second elution step.

   Note: Do not elute more than 200 µl into a 1.5 ml microcentrifuge tube because the DNeasy Mini spin column will come into contact with the eluate.

* Flow-through contains Buffer AL or Buffer AW1 and is therefore not compatible with bleach. See page 8 for safety information.

Measurement of DNA amount using Qubit Fluorometer

Table 1: Estimated DNA amount of the diluted PCR product (1:100) of *L. salmonis* in µg/ml and ng/ml. All measurements were done using Qubit Fluorometer.

Stage	µg/ml	ng/ml
1	0,023	23
2	0.024	24
3	0.0488	48,8
4	0.031	31
Average	**0.0317**	**31.7**

PCR and qPCR analysis

Calculation for standard samples

The following molecular weight, presented in Table 2, were found for the *L. salmonis* primer amplicon.

Table 2: Estimated molecular weight of the primer amplicon for *L. salmonis* using online calculator.

	g/mole
L. salmonis ss	31535.56
L. salmonis ds	**63045.12**

For further calculations of the DNA copy number in the PCR product, equation 1, 2, 3 and 4 was used.

$$copy\ number = \frac{amount \times (number/mole)}{lengthofbp \times (ng/g) \times (g/moleofbp)} \tag{1}$$

$$copy\ number = \frac{ng \times (number/mole)}{(ng/g) \times 63045.12 g/mole} \tag{2}$$

$$copy\ number = \frac{31.7\text{ng/ml} \times (6.022 \times 10^{23})}{10^{9}\text{ng/g} \times 63045.12\text{g/mole}} \tag{3}$$

$$copy\ number = \frac{3.027948872 \times 10^{11}}{ml} \tag{4}$$

Giving a number of 302801969.7 DNA copies per µL in the diluted 1:100 PCR-product. For further calculations of the standard containing 100 000 000 DNA copies, the law of dilution (5) was used. The standard sample contained 5 µL of the diluted PCR product and the estimated amount of water, see Table 3.

$$C1V1 = C2V2 \tag{5}$$

Table 3: Calculations of the needed amount of water and PCR product making the standard solutions, using the law of dilution.

	Amount	**Unit**
C1	302801969.7	copies/ µL
V1	5	µL
C2	200 000	copies/ µL
V2	7570	µL
Added H2O	7565	µL

Table 4: Overview of the standard samples used in the qPCR analysis. The standards was diluted 10 fold, from 200000 copies/µL, down to 0.02 copies/µL.

	µL standard	Concentration/ µL	Copy number	µL after dilution
1	7570	200000	1000000	6570
2	1000	20000	100000	900
3	1000	2000	10000	900
4	1000	200	1000	900
5	1000	20	100	900
6	1000	2	10	900
7	1000	0.2	0.1	900
8	1000	0.02	0.01	900

Table 5: Estimated amount of individuals in 5 µL of DNA eluate. 5 µL eluate was pipetted into each well on the qPCR plate.

Amount of eluate	**Amount lice**	**Relationship**
83.625 µL	20	0.23916293
5 µL	1.2	

Data from qPCR anaysis of standard samples made from PCR product

Table 6: Results from the qPCR analysis of standard samples of DNA from *L. salmonis*, using PCR product. Standard samples are based on the number of DNA copies in the sample, from 1 000 000 copies down to a 100 copies.

Standard sample	**Cq1/Cq2**	**Cq mean**	**Cq error**	**Conc mean**	**Slope1/2**
Copy #					
1.000.000	18.61/18.76	18.69	0.11	$1{,}232 \times 10^6$	5.74/5.54
100.000	22.88	22.88	0.00	9.635×10^4	5.37
10.000	27.09/26.99	27.04	0.07	7.710×10^3	5.70/5.52
1000	30.72/30.92	30.82	0.14	7.777×10^2	5.88/5.58
100	33.89/33.43	33.36	0.33	1.397×10^2	5.00/5.60

Data from qPCR anaysis of standard samples made using plasmid

Table 7: Results from the qPCR analysis of standard samples of DNA from *L. salmonis*, using the TOPO-4 plasmid. Standard samples are based on the number of DNA copies in the sample, from 1 000 000 copies down to a 100 copies.

Standard sample	**Cq1/Cq2**	**Cq mean**	**Cq error**	**Conc mean**	**Slope1/2**
Copy #					
10.000.000	11.96/11.91	11.94	0.04	1.150×10^7	5.37/5.56
1.000.000	15.67/15.83	15.75	0.11	9.187×10^5	5.76/5.74
100.000	19.35/19.27	19.31	0.06	8.671×10^4	5.57/5.53
10.000	22.18/22.67	22.43	0.35	1.114×10^4	5.87/5.73
1000	26.20/26.12	26.16	0.06	9.256×10^2	5.19/5.68
100	29.20/29.73	29.47	0.37	1.051×10^2	5.63/5.62
10	32.55/33.32	32.94	0.54	1.072×10^1	5.40/5.47

Calculations of DNA amount in unknown field samples

Data for the field samples being investigated is presented in Table 8 .

Table 8: Received data for the unknown field samples investigated in the present study. DWen refers to total dry weight of the sample before the extraction and DWeDNA to the dry weight of extracted DNA.

Sample ID	**Date**	**Location**	**DWen**	**DWeDNA**	**Eluated volume**
Ins1/A/1	24.06.16	Bukkholmen	0.6992	0.0216	87
Out2/C/1	24.06.16	Bukkholmen	0.5179	0.0254	82
Ins2/CC/1	24.06.16	Bukkholmen	1.2259	0.0234	80
Out2/B/1	28.06.16	Bukkholmen	0.0812	0.0229	88
Ins1/B/1	28.06.16	Bukkholmen	0.1361	0.0216	88
Ins2/C/1	28.06.16	Bukkholmen	0.3524	0.0234	83
Ins2/AA/1	27.06.16	Bukkholmen	0.4679	0.0224	78

Calculations of DNA amount in all of the different unknown samples are found in Table 9. The total number of DNA copies in the whole sample are calculated using equation 6 and the corresponding number of individuals by equation 7.

$$Total\#ofcopies = \frac{Conc5\mu L/5 \times eluatedvolume \times DWen}{DweDNA} \tag{6}$$

$$Amountoflice = \frac{total\#insample}{2792286} \tag{7}$$

where 2 792 286 is estimated number DNA copies in 1.2 individuals of *L. salmonis*. In the results the estimated number of DNA copies of 1.2 individuals is presented in Table 3.4.

Table 9: Received data for the unknown field samples investigated in the present study and calculations of DNA amount and the corresponding number of *L. salmonis* individuals.

Sample ID	**Date**	**Dilution**	**Conc/**5 µL	**Conc/**µL	**DWen**	**DWeDNA**	**Eluated volume**	**total # in sample**	**Amount of lice**
Ins1/A/1	24.06.16	1:10	90	18	0.6992	0.0216	87	50 692	0.018
Out2/C/1	24.06.16	1:100	788	158	0.5179	0.0254	82	263 333	0.09
Ins2/CC/1	28.06.16	1:100	176	35	0.3524	0.0234	83	43 998	0.015
Out2/B/1	28.06.16	-	-	-	-	-	-	-	-
Ins1/B/1	28.06.16	-	-	-	-	-	-	-	-
Ins2/C/1	24.06.16	1:100	310	62	1.2259	0.0234	80	260 561	0.09
Ins2/AA/1	27.06.16	-	-	-	-	-	-	-	-