Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Egg string hatching success, development to copepodids and tolerance to low salinities in salmon lice (*Lepeophtheirus salmonis*) as a consequence of freshwater delousing

Master's thesis in Msc Ocean Resources
Supervisor: Yngvar Olsen
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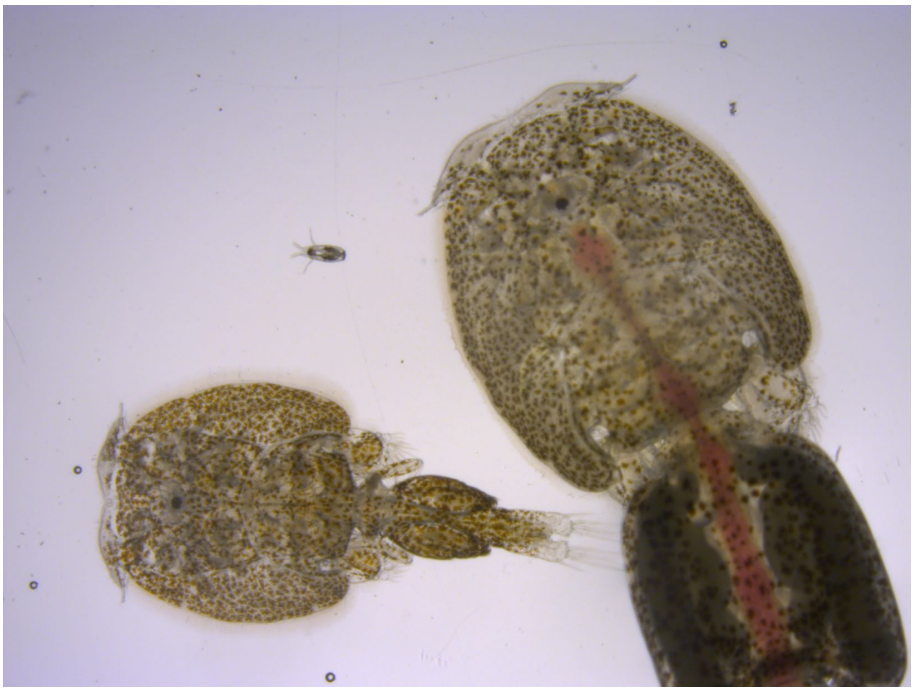
June 2022



Taskforce salmon lice

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NTNU
Kunnskap for en bedre verden

Preface

This thesis has been completed at the Department of Biology at the Norwegian University of Science and Technology (NTNU) in Trondheim between August 2021 and June 2022.

The thesis was a part of the Taskforce Salmon lice project, which is an R&D project organised as a PhD program at NTNU. The project is a collaboration between NTNU, Fiskeri- og havbruksnæringens forskningsfinansiering (FHF) and aquaculture companies SalMar, SalmoNor, Lerøy, Emilsen Fisk AS, MOWI, Måsøval Fiskeoppdrett AS, Midt-Norsk Havbruk, Erviks Laks og Ørret, Refnes Laks AS, Skretting, Sinkaberg-Hansen, AQS, Aqua Kompetanse AS, and Åkerblå. The projects overall objective is to establish fundamental knowledge on how sea lice infest farmed salmon and mechanisms of how the parasites spread within and between farmed and wild populations of salmonids.

First, I would like to thank my main supervisor Yngvar Olsen for help during the first phase of the thesis, as well as good feedback during the writing process. To co-supervisor Anna Solvang Båtnes for advice, guidance and help during fieldwork and writing. Furthermore, to my second co-supervisor Cecilie Miljeteig for help during fieldwork, as well as with statistics and writing. I would also like to thank my external co-supervisor Gro Vee for help and advice during the start of this thesis.

I would also like to thank the staff on board the well boats and aquaculture sites for help during sampling, with transportation of equipment as well as the food I got served while on fieldwork.

Finally, a special thanks to the people closest to me for their support and encouraging words in the most stressful periods during this past year.

Sondre Strand Hansen, Trondheim, June 2022

Abstract

Salmon lice (*Lepeophtheirus salmonis)* is recognized as a key constraint to the continued growth within the salmon aquaculture industry. Several newly developed methods for controlling lice infestation have emerged, and one of these is freshwater baths using well boats. Freshwater treatments have shown promising results as an alternative to the former medicinal treatments. However, efficacy of treatments is rarely 100%, meaning that lice could go through the entire treatment and potentially survive. This has raised concerns about the potential development of increased tolerance to low salinities which is unwanted for both wild fish populations and the industry. The objective of this study was to investigate how salmon lice egg strings hatched and developed to the copepodid stage following freshwater treatment, and if the treatment could potentially lead to changes in freshwater tolerance. Samples were collected from four treatments, and egg strings were collected before, every second hour during, and after treatment. The proportion of egg strings that hatched was high for before-samples (97%) and decreased following two (82%) and four (41%) hours exposure time. Egg strings treated for six hours or more did not hatch at all. Following, the results showed that hatching success (number of nauplii divided by the estimated number of eggs; %) was high before treatment (85 to 66%) and decreased with increasing exposure time, with hatching success from 0 to 22% for the four hours treated egg strings. Survival to the copepodid stage was high for egg strings sampled before treatment, and the median copepodid survival was observed to decrease rapidly following two hours of freshwater exposure. Before treatment, each egg string produced an average of 238 ± 118 copepodids, which declined to 63 ± 58 following two hours of exposure.

Bioassay results revealed differences in response to low salinities at the copepodid stage, where differences were observed both within and between the treatments followed. For the freshwater exposed egg strings, survival curves indicated decreased or similar tolerance to low salinities in the two and four hours treated egg strings compared to the Before treatment group. Copepodids (F1-generation) from parent lice (F0-generation) which had been exposed to freshwater for two and four hours indicated increased treatment tolerance compared to before treatment. However, infection to produce F2-generation lice was unsuccessful for these copepodids, and significant conclusions could not be made because of limited number of replicates. F2-generation lice from the Before treatment group were successfully reared and tolerance tested and showed similar survival and treatment tolerance compared to the F1-generation. The results obtained for hatching success and copepodid survival suggested a detrimental effect of the treatment which in turn could have resulted in decreased tolerance levels in copepodids from treated egg strings. The same pattern was not observed in the copepodids from the treated F0-generation lice. Thus, no conclusion could be made rergarding the possible increased tolerance to low salinities, however, the egg string hatching and larval development were

clearly affected by the freshwater treatment, indicating limited infestation potential from freshwater treated lice.

Sammendrag

Lakselus (*Lepeophtheirus salmonis)* er en hovedgrunnene til at veksten har stangnert i havbruksnæringen. Flere nye metoder for å kontrollere luseinfestasjon har blitt utviklet, og en av disse er ferskvannsbehandlinger ved hjelp av brønnbåt. Ferskvannsbehandlinger har vist lovende resultater som alternativ til de tidligere medisinske behandlingene. Effekten av behandling er sjelden 100%, som betyr at lus kan gå gjennom hele behandlingen og potensielt overleve. Dette har ført til bekymring for potensielt økt toleranse til lav salinitet hos lus, noe som ikke er ønskelig for både ville fiskepopulasjoner og havbruksnæringen. Målet med denne studien var å undersøke hvordan eggstrenger fra lakselus klekker og utvikler seg til kopepoditter etter ferskvannsbehandling, samt om behandlingen kan potensielt føre til endringer i ferskvannstoleranse. Prøver ble samlet fra fire behandlinger, og eggstrenger ble samlet før, hver andre time under, og etter behandling. Andelen eggstrenger som klekket var høy for før-prøver (97%) og minket som følge av to (82%) og fire (41%) timers behandlingstid. Eggstrenger behandlet med ferrskvann for seks timer eller mer klekket ikke. Resultatene for klekkesuksess (antall nauplier delt på estimert antall egg; %) var høy for før behandlinng (85 til 66%) og minket med økende behandlingstid, med klekkesuksess fra 0 til 22% for fire timers behandlede eggstrenger. Overlevelse til kopepodittstadiet var høy for eggstrenger samlet før behandling, og median kopepodittoverlevelse ble observert til å synke raskt som følge av to timer ferskvannsbehandling. Før behandling produserte hver eggstreng gjennomsnittlig 238 ± 118 kopepoditter, som sank til 63 ± 58 som følge av to timers eksponering.

Bioassayresultater viste forskjeller i respons til lav salinitet ved kopepodittstadiet, hvor forskjeller ble observert både innad og mellom behandlinger. For ferskvannseksponerte eggstrenger indikerte overlevelseskurver redusert eller lik toleranse til lav salinitet i to og fire timers eksponerte eggstrenger sammenliknet med før behandling. Kopepoditter (F1-generasjon) fra foreldrelus (F0-generasjon) som hadde vært eksponert i to og fire timer indikerte økt toleranse sammenlignet med før behandling. Men, infeksjon for å produsere F2-generasjonslus feilet for disse kopepodittene, og signifikante konklusjoner kunne ikke tas som følge av få replikater. F2-generasjonslus fra før behandling ble produsert og toleransetestet og viste lik overlevelse og behandlingstoleranse sammenliknet med F1-generasjonen. Resultatene for klekkesuksess og kopepodittoverlevelse indikerte en ødeleggende effekt av behandlingen som i sin tur resulterte i lavere toleransenivå i kopepoditter fra behandlede eggstrenger. Det samme mønsteret ble ikke observert i kopepoditter fra den behandlede F0-generasjonen. Dermed kunne ingen konklusjon tas med tanke på mulig økt toleranse til lave saliniteter. Likevel, eggstrengenes klekking og larveutvikling var tydelig påvirket av ferskvannsbehandling, som indikerer et begrenset smittepotensial fra ferskvannsbehandlet lus.

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1 Introduction

1.1 Background

The aquaculture industry and especially farming of salmonids such as Atlantic salmon (*Salmo salar*) has seen a rapid increase in production since its early development in the 1960s. The current annual production is estimated to be worth US $15.4 billion, along with job opportunities and economic growth in regions where production takes place (International Salmon Farmers Association, 2018). Norway´s long and sheltered coastline has proven to be well suited for extensive production of Atlantic salmon, making Norway the largest contributor with an estimated yearly production of 1 350 000 tonnes (Norwegian Directorate Of Fisheries, 2020). Farm sites are currently distributed all along the Norwegian coast, with varying degree of production density. The South-Western part has the highest production density, and the highest reported biomass is normally found in the period of October to December (Norwegian Directorate Of Fisheries, 2020).

The increasing production has been accompanied by various health problems caused by bacterial- and viral diseases, as well as parasitic infections mainly caused by salmon lice (*Lepeophtheirus salmonis*). Although many challenges with diseases have been mitigated through the development of vaccines and increased knowledge on husbandry, salmon lice are still regarded as the second largest threat to Atlantic salmon in Norway (Forseth et al., 2020) and a key constraint to the continued growth of the salmonid aquaculture industry. The extensive production has also improved conditions for the salmon lice population to grow and spread. It is estimated that the total cost of salmon lice is 5 billion NOK per year and counts for around 6% of the product value (Costello, 2009; Iversen et al., 2017). These estimates only cover direct costs related to infestations such as preventive measures and direct cost of treatments. However, salmon farmers can experience periods with increased mortality and reduced appetite and growth after treatments, as well as possible early harvesting of infected fish. This means that the total cost of salmon lice is most likely underestimated (Iversen et al., 2017).

1.2 *Lepeoptheirus salmonis*

Salmon lice are ectoparasitic copepods in the family *Caligidae*. The distribution is circumpolar in the Northern Hemisphere, and it classifies as a stenohaline

copepod whose survival and development are optimal in high-salinity waters (Torrissen et al., 2013). Salmon lice have a direct life cycle and only needs one host to complete its life cycle from egg to fertile adult. The salmon lice feed directly off the skin, epidermis, slime and blood of salmonids, and research shows that severe infections can lead to skin erosions, disruption of osmotic balance, increased susceptibility for diseases and reduced immunological capacity (Overton et al., 2020).

1.2.1 Life cycle and development

The life cycle comprises eight developmental stages (Hamre et al., 2013), each of which are separated by a moult (Figure 1; Igboeli et al., 2014). These eight developmental stages can in turn be divided into four main stages: a planktonic stage, a copepodid stage, a nonmotile stage, and finally a motile stage. The first two stages after the eggs hatch are the free-living nauplius I and nauplius II. The development continues to the infectious larval copepodid. These three stages comprise the free-living stages that are planktonic and lecithothropic, meaning that they rely entirely on endogenous lipid reserves. As a result, the infectious copepodid devotes it's time to searching for hosts and attachment through several adaptive behavioral traits. These include positive phototaxis, semiotaxis and rheotaxis, meaning that the copepodid display diel vertical migrations in response to light, response to waterborne gradients of host-derived chemicals and move towards vibrations originating from susceptible host (Mordue & Birkett, 2009). When settling, the copepodid anchor themselves to the host with frontal filaments. The remaining five stages of the life cycle are parasitic and will normally be completed on a single host (Hamre et al., 2013). After settlement, the copepodid starts to feed on the host while developing through the two nonmotile stages Chalimus 1 and Chalimus 2, where the lice are attached to the host through frontal filaments. The development continues through the motile pre-adult stage 1 & 2, where the lice can move around on and between hosts, followed by the adult stages where mating and production of egg strings occur (Hamre et al., 2013).

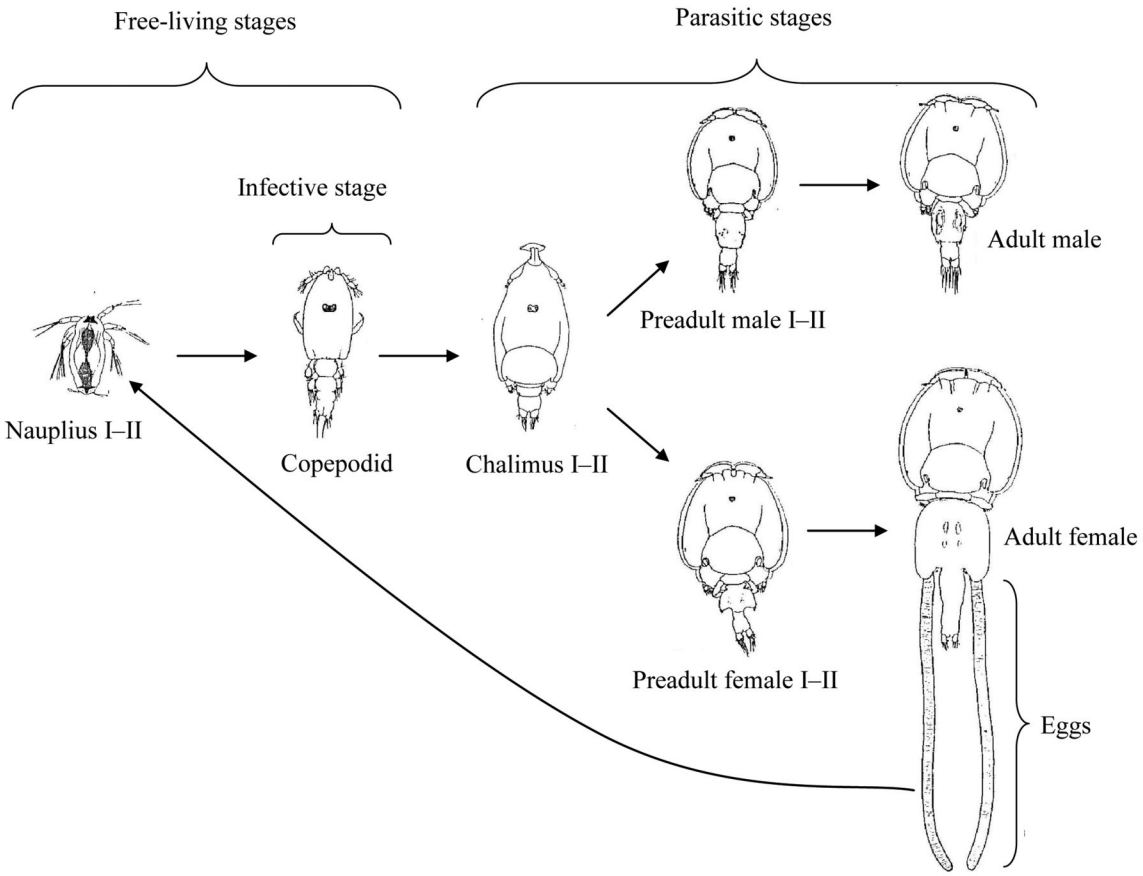


Figure 1. The life cycle of *L. salmonis* consisting of eight developmental stages, including 3 free-living and 5 parasitic attached to the host (Hamre et al., 2013; modified by Igboeli et al., 2014).

Water temperature is a key regulator of development, reproductive output, and dispersal of salmon lice (Samsing et al., 2016). For the free-living stages, temperature is especially important since they rely on their endogenous lipid reserves until they successfully attach to a host (Samsing et al., 2016; Tucker, 2000). Higher temperatures speed up the development, hence reducing the time from egg to infectious copepodid. However, elevated temperature also increases metabolic rate, which in turn can cause the larvae to expend lipid reserves quicker, hence reducing larval viability (Angilletta et al., 2004; Samsing et al., 2016). A prolonged larvae development period increases the risk of mortality but can also increase the dispersal distance and probability of a host encounter. High temperatures increase the rate of egg development and hatching activity; however, the adult female louse produces shorter egg strings with fewer eggs. The reverse occurs at lower temperatures, but the egg size diameter and viability are significantly reduced (Boxaspen, 2006; Samsing et al., 2016).

1.2.2 *L. salmonis* and salinity

The salmon lice show optimal survival and development in high-salinity waters, i.e., salinities greater than 27 ‰ (Ljungfeldt et al., 2017; Torrissen et al., 2013). Attached stages of salmon lice can compensate for their loss of ions through host-dependent mechanisms where they gain ions from the host to maintain homeostasis during freshwater exposure (Hahnenkamp & Fyhn, 1985). Free-swimming adult lice, however, start to succumb after 8 hours due to dilution of their hemolymph by osmosis. This suggests that the main detrimental effect of low salinity is related to osmotic stress and depletion of ions. However, in brackish waters with salinity > 12 ‰, adult female lice can maintain homeostasis independent of their host (Hahnenkamp & Fyhn, 1985). For the less developed free-swimming stages, research shows that the copepodid stage is highly susceptible to freshwater, with observed mortality of 96-100% after 1 hour exposure. Attached stages that are developed past the copepodid stage did however show a higher tolerance (Wright et al., 2016).

1.3 Treatments against *L. salmonis*.

The regulation of salmon lice infestation in Norwegian fish farms ("Forskrift om bekjempelse av lakselus i akvakulturanlegg") is governed by the Norwegian Directorate of Fisheries and was implemented in 2013. The purpose of the regulation is to "*reduce the occurrence of salmon lice to minimize the harmful effects on fish in aquaculture facilities and wild salmonids, as well as combating the development of resistance in salmon lice*". The regulation applies for all aquaculture facilities farming salmonids and states that the facility should develop coordinated plans for salmon lice control and combat (Norwegian Directorate Of Fisheries, 2013). This includes for example that the farmers are obliged to keep the infestation level below 0.5 adult female lice per fish, except during the annual wild salmon smolt migration in spring, where the infestation level must be kept below 0.2 adult female lice per fish. The regulation is controlled by the Norwegian Food Safety Authority.

Treatment methods for controlling lice infestations have formerly been dominated by chemotherapeutants used in two ways: bath treatments and in-feed additives (Burridge et al., 2010). However, development of resistance and reduced sensitivity to chemotherapeutants has led the industry towards non-medicinal alternatives to control salmon lice. These newly developed methods, often called non-medicinal methods (NMM), include mechanical cleaning with brushes and/or flushing, thermal treatments, as well as freshwater treatments (Guragain et al., 2021; Overton et al., 2019). For all systems, the treatment begins when the fish are crowded in the sea-cage and pumped onboard either a

well boat or other type of vessel fitted with the given delousing unit. These crowding methods and procedures vary among different farming companies, and the methods used are frequently a welfare problem for the fish (Overton et al., 2019; Sommerset et al., 2022). Medicinal and NMMs are often used in combination with one or several preventive measures farmers employ to combat the louse and is commonly referred to as Integrated Pest Management (IPM). Such preventive measures include semipermeable lice skirts around cages, special dietary feed, cleaner fish, as well as breeding programs for more lice-resistant salmon (Sommerset et al., 2022).

NMMs have shown promising results as alternatives to medicinal treatments. However, salmon lice could potentially develop tolerance towards freshwater (Ljungfeldt et al., 2017) and research has shown that temperatures used in thermal treatments during the warmest periods of the year is close to the upper thermal limits for salmonids (Roth, 2016). The increased use of NMMs have resulted in concerns about the potential of salmon lice to evolve increased tolerance towards these methods, as it previously did to the medicinal treatments.

As a result of this, the Norwegian Food Safety Authority announced in 2017 a new salmon lice regulation for aquaculture sites (Norwegian Food Safety Authority, 2017). The background for the regulation was not only the situation with possible increased tolerance towards the newly developed methods, but also the increased fish welfare problems related to treatments. The new regulation has yet to be implemented as it stands in 2022.

1.4 Freshwater treatments

Freshwater can be used in several ways to delouse salmon. One method is carried out using a snorkel-system which creates a freshwater layer in the upper part of the net pen. However, the most common and efficient method employed is freshwater baths in well boats (Gaasø, 2019; Powell et al., 2015; Reynolds, 2015).

Before the fish can be pumped onboard the well boat and start the treatment, the fish must be crowded in the pen. The two most common methods used for crowding are either with a swipe net (“orkast”) or with a ball line (“kulerekke”) (Nersten, 2021). The two methods differ in use and efficiency and are used depending on the total biomass within the pen. During crowding with a swipe net, a seine is deployed inside the net pen to collect the salmon. The ball line is pulled under the net pen and tightened to crowd the salmon in one part of the pen.

As the salmon is crowded, the well boat starts pumping the fish onboard through tubes. The fish passes through a dewatering unit which removes the seawater

hence maintaining the quality of the freshwater in the well. Cleaner fish are sorted out and handled according to the Aquaculture Operation Regulations (Norwegian Directorate Of Fisheries, 2008). The seawater that is removed during the dewatering process is filtered to collect lice and other biological particles present in the water. The time required for transferring the salmon from the pen and onboard the well boat differs depending on the crowding method, as well as the biomass of fish within the pen and the pumping capacity of the well boat.

The treatment time starts when the last salmon is transferred from the pen and into the well. Treatment times can vary depending on the total lice load as well as the distribution of different lice stages, but typically ranges from 5 to 10 hours. Since the freshwater often is reused, it is recycled continuously and filtered to remove detached parasites preventing them from resettling on the salmon. Once the treatment time has elapsed, the salmon is returned to the pen. Maintaining the quality of freshwater is important to obtain the best possible result in terms of fish welfare and parasite reduction. Hence, well boats receive their freshwater from a known reservoir and the water is quality controlled by measuring several parameters including salinity, O_2, temperature, pH, TAN, NH_3, N_2, TGP and CO_2.

1.5 Aim

The main objective of the thesis was to study egg string hatching success and the further development and survival of copepodids and to assess if freshwater delousing of salmon lice may result in increased lice tolerance to low salinities.

This was investigated by comparing treated and untreated egg strings, first with focus on hatching success, development, and reproductive success. The second part of the thesis focused on freshwater tolerance, which was investigated by using established bioassay methods to compare freshwater tolerance in treated vs untreated egg strings.

Research questions:

1. How is the hatching success and development to the copepodid stage affected by freshwater treatment?
2. What are the potential changes in treatment tolerance as a consequence of freshwater delousing?

2 Materials and method

2.1 Study area

Fieldwork was carried out in 2021 in the period between week 46 and 49 (16.11.21-7.12.21), at three different locations within Production Area 6: Nordmøre and Sør-Trøndelag (Figure 2;Norwegian Directorate Of Fisheries, 2017). For anonymity will the locations in this thesis be named 1, 2 and 3. Samples were collected from four treatments, two at Location 1, one at Location 2 and one at Location 3.

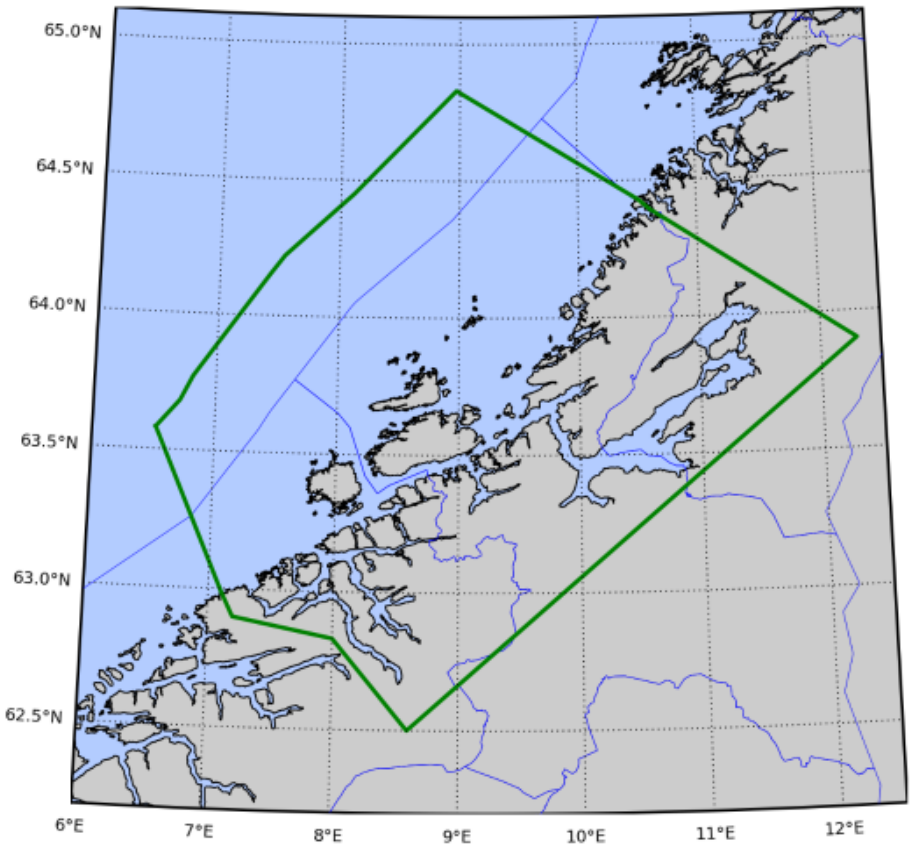


Figure 2. Map showing production area 6 outlined in green (Norwegian Directorate Of Fisheries, 2017).

2.2 Collection of salmon lice

Samples were collected before freshwater treatment (during crowding), every second hour during treatment (2, 4 and 6 hours after finished pumping/loading) and after treatment. The samples collected during treatment were mostly from the lice-filter which removes detached parasites from the treatment water, although some additional samples were collected from lice still present on fish from the well. The samples after treatment were collected when the fish exited the well-boat and returned to the pen. Collection of salmon from the pen during crowding and after treatment was done by dipnet, and the salmon were anaesthetized in a 300L tub filled with seawater and Benzoak veterinary (15-20 mL per 100L seawater), lined with a lice fabric which collected lice that detached during sedation. In each sampling, the aim was to collect egg strings (pairs or single egg strings) from 10 different individuals. However, the amount of egg strings collected differed depending on how effective the treatment was, and that a large proportion of adult female lice shed egg strings during freshwater treatment (Table 1). The difficulty with obtaining sufficient samples, as well as signs of poor hatching success from samples collected during the two first treatments (Location 1) called for an alternative sampling of preadult and adult stages of treated individuals. These were collected from fish sampled from the wells, placed in seawater, and surviving individuals were later transferred directly to Atlantic salmon reared at NTNU SeaLab. This was to see if treated preadult and adult individuals could produce egg strings which in turn could be used to investigate freshwater tolerance. Overview of number of egg strings sampled from each treatment and sampling time is listed in Table 1. Overview of preadult and adult lice sampled is listed in Table 2.

Table 1. Number of egg strings (pairs or single egg strings) sampled at the different treatments and sampling times.

Treatment/ sampling time	Before	2 hours freshwater exposure	4 hours freshwater exposure	6 hours freshwater exposure	After
1.1	10	3	5	7	5
1.2	5	10	8	11	
2.1	10	10	9	5	6
3.1	9	15	12		

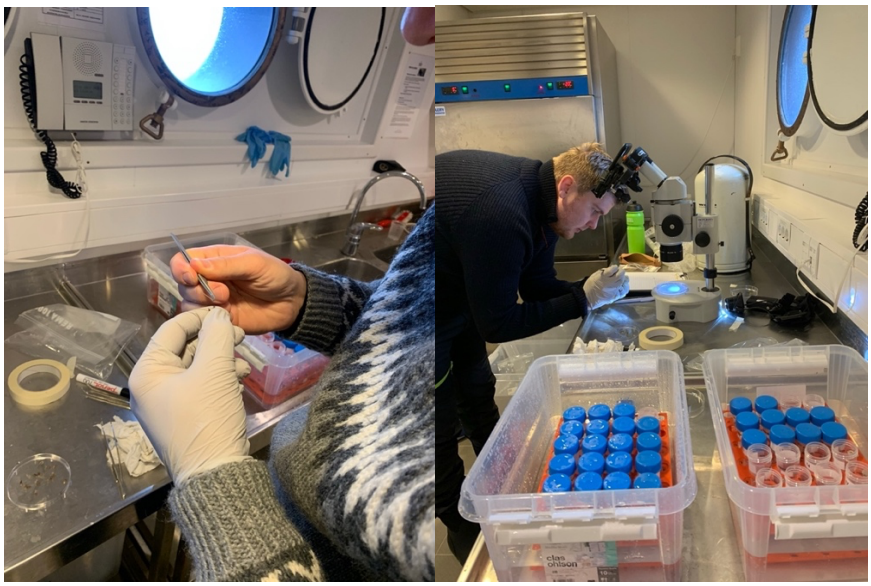
Table 2. Number of preadult and adult lice sampled from the different freshwater treatments and sampling times. The lice was assessed once returned to NTNU SeaLab and were evaluated as active or immobilized ("immob").

Treatment/ sampling time	2h preadult		2h adult		4h preadult		4h adult	
	Active	Immob.	Active	Immob.	Active	Immob.	Active	Immob.
2.1		17		7		15		4
3.1	14	22	7	4	5	25	4	5

2.3 Egg string hatching and development

2.3.1 Egg string handling and photographing

After sample collection, the egg strings were gently removed from the lice with tweezers, photographed under a stereo microscope (Wild Leitz) using a phone camera with an adapter (Celestron NexYZ), and stored in individual sample tubes (Figure 3). The sample tubes were made from 50 mL Polypropylene Conical Tubes (Falcon), with the bottom cut off and replaced with plankton mesh (Sefar Nitex, 150 µm mesh size). The sample tubes were stored in a plastic container with filtered sea water, which was regularly replaced during sampling and transport and kept outside to avoid temperature fluctuations during storage.



(3.1) **(3.2)**

Figure 3. Pictures showing 3.1) how the egg strings were removed with tweezers and 3.2) stereo microscope set up for photographing egg strings. Photos: Anna S. Båtnes

Since the stereo microscope was disassembled during transport and often between each sampling series, a photo was first taken with standard graph paper before the samples were photographed. The photographs were later used to measure egg string length and calculate the number of eggs in each egg string (see chapter 2.3.3). Egg strings were then transported to NTNU SeaLab and transferred from the sample tubes to incubator tubes and incubated with sea water flow-through. The incubator tubes were similar to the sample tubes, except for the cover which was replaced with plankton mesh (Sefar Nitex, 150 µm mesh size).

2.3.2 Incubation of egg strings

The incubators used were developed to specifically handle a high number of samples (single or pairs of egg strings from individual sea lice). The setup was derived from incubators developed for *L. salmonis* by Hamre et al. (2009), although with some modifications. For this thesis, two incubators were set up at NTNU SeaLab, each with a capacity of 112 samples. The seawater used was tempered to 12°C, and thermometers were used to monitor incubation temperature daily. The incubation temperature during the experiment period was 11.6°C ± 0.1°C. The incubator set up is shown in Figure 4.

The intake water was supplied through a reservoir placed ca. 1 meter above the incubators to allow controlled flow down to the incubators via the blue hoses (Figure 4A). From the incubator inlet, the water was split in two directions towards sides 1 and 3. Another splitter divided the water before reaching the inlet hoses. Each incubator had thus four inlets located in the bottom corners on sides 1 and 3. The location of the inlets secured upward flow through the incubator tubes towards the two outlets at the top. Flow was determined by the length and diameter of the four inlet tubes (Figure 4B), as well as the height of the reservoir. The flow was estimated to be 0.4 – 0.6 L/min per incubator (3.6-5.3 mL/min per incubator tube). Hatching activity was controlled daily in the experiment period.

Figure 4. Incubator set up. A: Intake water was transported to the reservoir (R) fitted with an overflow (OF). Blue hoses were the main inlet hoses transporting water from the reservoir to the incubator. Inlet water was first split in two directions to sides 1 and 3 via the splitter (S) before reaching another splitter which divided the water to the inlet hoses as shown in B) and into the incubator through the bottom. Outflowing water went through the outflow (O). B: Overview showing the main inlet (MI), how the water was split in two (S) and the inlet hose (IH) transporting the water into the incubator. Outflowing water went through the outlet pipe (OP). C: Close-up of incubator tubes fitted with mesh in top and bottom. Photos: Anna S. Båtnes and Mikael Furberg.

2.3.3 Estimating number of eggs

To estimate the number of eggs within each egg string, a method for measuring mean egg size and correlating it to egg string length was developed (Furberg, 2022). First, mean egg size was found by measuring the length (mm) of 30 eggs on four different areas per egg string. This was performed on 10 egg string pairs (Appendix Table A1). The measurements were then calculated to mm/egg and compared within and between egg strings (Appendix Table A2). Standard deviation (0.0022) and coefficient of variation (3.9%) was acceptable, thus these measurements were used to estimate mean egg size. Further, total length (mm) of all 10 egg string pairs were measured and number of eggs was calculated based on the mean egg size found for that particular egg string pair and the mean egg size from all 10 egg strings (Appendix Table A3). This was performed to see if a mean egg size was representative or whether it was necessary to measure egg size for all egg strings. The difference between the results when using the egg size for each egg strings as opposed to the mean from all 10 egg strings varied from -2.8% to 2.6%, which was considered to be acceptable variation. Hence, mean egg size of 56.9 µm/egg were used for estimating the number of eggs in all egg strings. All length measurements were performed using the "Segmented Line" measuring tool in ImageJ (Version 1.53;Rasband, 2018) (Figure 5).

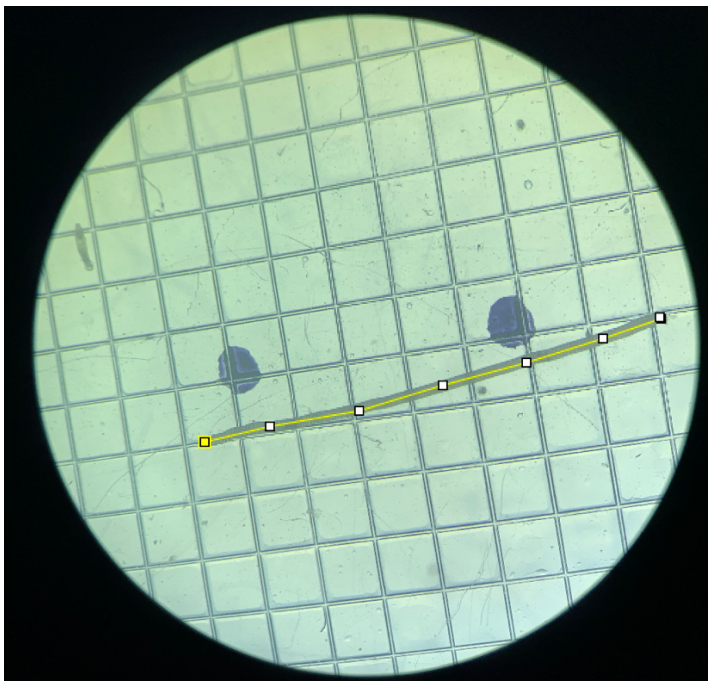


Figure 5. Example of egg string length measurement. Length measurements was performed using the "segmented line" measuring tool in ImageJ (Version 1.53;Rasband, 2018).

2.3.4 Counting nauplii and copepodids

The number of salmon lice larvae was counted within 24 hours after hatching and at the copepodid stage, 3-4 days after hatching. At the first counting, the incubator tubes content was emptied using a 1 mL plastic pipette (VWR). The material was transferred to white weighing boats (VWR) before nauplii were sorted as active or immobilized. This treatment also removed unhatched pieces of egg string and debris. The amount of nauplii which were only partially hatched and still present in the egg string was counted under a stereo microscope. After sorting, each weighing boat was photographed and the photographs were later used to count the amount of hatched nauplii. The nauplii were then returned to the incubator tube and to the incubator and allowed to develop further. At the copepodid stage, the same method was used to sort the active and immobilized copepodids before photos were taken for later counting and analysis. The counting of nauplii and copepodids was performed with ImageJ (Version 1.53;Rasband, 2018) using the "Multi-Point" tool (Figure 6).

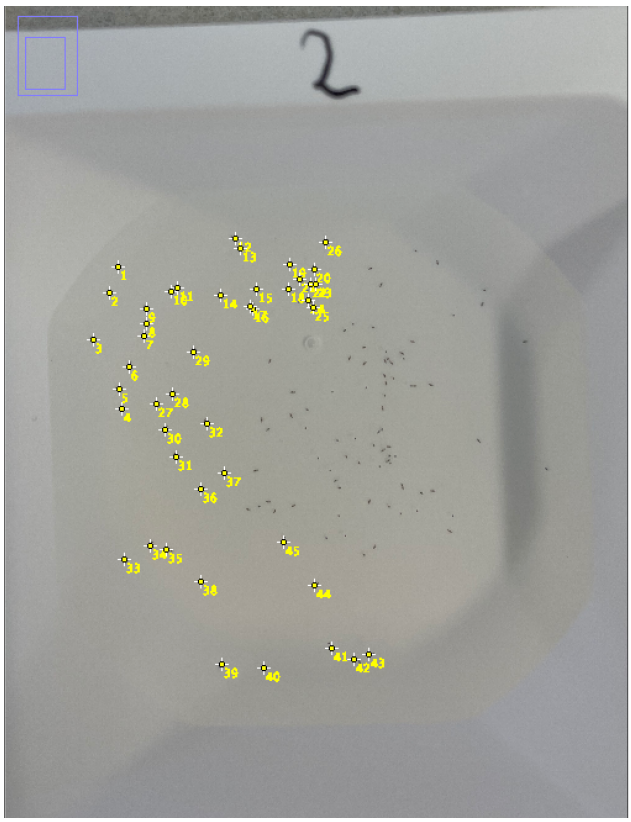


Figure 6. Example of how the nauplii and copepodids were counted using the "Multi-Point" tool in ImageJ (Version 1.53;Rasband, 2018)

2.3.5 Systematizing data and calculation of hatching success and copepodid survival

During sampling, egg strings were assigned to an individual and labeled sampling tube. When transferring the egg strings from sample tubes to the incubator, each pair or single egg string were assigned to a randomly determined incubator tube. This incubator tube and ID followed the sample through the nauplii and copepodid counting. All counting results were recorded and systemized in an Excel document (version 16.54, Microsoft Office).

The hatching success was calculated by dividing the total amount of hatched nauplii (active and immobilized) with the estimated number of eggs in the sample. One sample represented a single egg string or pair of egg strings from one female lice.

The copepodid survival was calculated by dividing the amount of actively swimming copepodids with the estimated number of eggs in the sample.

2.4 Tolerance to freshwater/lower salinities

2.4.1 Bioassay F1-generation copepodids

Bioassays to investigate freshwater tolerance were performed on copepodids from samples collected before, and following two and four hours freshwater exposure employing the method developed by Andrews and Horsberg (2020). Samples were also collected following six hours freshwater exposure and after finished treatment, but no copepodids were obtained for bioassay analysis due to lack of hatching. A total of 14 bioassays were conducted on *L. salmonis* originating from three different locations within the study area. Time of sampling and water temperature affected hatching and development time to reach the copepodid stage. However, all bioassays were conducted between day 4 and 9 after hatching.

The bioassays were performed in 50 mL Polypropylene Conical Tubes (Falcon), in two replicates per salinity level, each containing 20 actively swimming copepodids. The containers were each assigned to one of the following 10 salinities: a seawater control (32‰), 27, 23, 20, 17, 14, 11, 8, 5, and 2‰ (g/L). Copepodids and seawater was added first before addition of deionized water to achieve the given salinity. The addition of deionized water was divided in three steps over a period of 2 minutes. This method was employed to ensure a stepwise transition more representative of the natural conditions experienced by the salmon louse when moving through different salinity gradients whilst attached to the host fish. The exact volume to add at the different steps was

calculated beforehand to secure efficient and precise mixing of the different salinities. The source water used was the same as the intake water for the hatchery and was either adjusted with deionized water or red sea salt (Red Sea) to be constant at 32‰. A stopwatch was used during mixing to ensure that the amount of time between each step was consistent for all 10 salinities. The containers were then held in a temperature-controlled water bath using the outflowing water from the hatchery (Figure 4) and remained undisturbed for 24 hours.

Once 24 hours had elapsed, copepodids in each container were transferred to a white weighing boat and examined to find the number of affected and unaffected copepodids. Using methods described by Hamre et al. (2009) and Andrews and Horsberg (2020), status of the copepodids were determined by agitating the water around each copepodid and observing it for signs of normal swimming behavior. Individuals which responded quickly and were actively swimming were classified as unaffected. Individuals exhibiting abnormal movement or lack of mobility were classified as affected. Abnormal movement also included erratic swimming behavior, or inability to hold position in the water column, as well as delayed response to external stimuli.

2.4.2 Rearing of F0-generation to produce F1-generation of treated individuals

The preadult and adult lice sampled during treatment (Table 2) were assessed once returned to NTNU SeaLab after fieldwork. Surviving individuals from Treatment 3.1 were transferred to fish in two separate tanks, one for the two hours treated lice and one for the four hours treated lice. An overview of the transferred preadult and adult lice is listed in Table 3.

Table 3. Two and four hours treated salmon lice from Treatment 3.1 which was transferred to fish at NTNU SeaLab in two separate tanks.

Tank	Adult female	Preadult female	Preadult male	Adult male
6 (two hours treated)	7	8	6	0
8 (four hours treated)	5	3	0	2

2.4.3 Rearing of F2-generation

Copepodids from before and during delousing (2 and 4h) were either tolerance tested as described in chapter 2.4.1 or used to infect Atlantic salmon for rearing

of F2-generation. Atlantic salmon were reared at Taskforce Salmon Lice´s culture room at NTNU SeaLab in three 400 L tanks (100 cm length x 100 cm width x 50 cm height). The room was climate-controlled with stable temperature of 10.0 °C. Water temperature in tanks varied with season but remained in the range of 7.5 – 11.0 °C. Salinity was between 27 and 33 ‰, water flow in each tank was between 250-750 L h^{-1}, and fish were exposed to 24 h light. There were 5 fish in each tank with a size range from 200-400 g. Salmon lice load varied between fish, however, a maximum limit of 10 adult lice per fish was set (FOTS ID 15366) and was monitored by responsible personnel.

Salmon lice used for infection were reared in the hatchery until reaching the copepodid stage. 250 actively swimming copepodids (50 per fish in the tank) were then extracted from the incubator tubes and placed in white weighing ships. Water flow in the tank was reduced to 150 L h^{-1} and water level reduced to 1/3. Copepodids were spread evenly in the tank, while the flow remained low for approx. 60 minutes – to allow copepodids to locate and attach to the fish – before it was returned to normal after the 60 minutes had elapsed and infection was completed. O_2-concentration was continuously monitored during the infection process.

2.5 Statistical analysis

All statistical analyses were performed either using Excel (version 16.54, Microsoft Office) or RStudio (version 1.1.456; RStudio, 2016)

Differences in egg string length, hatching success and copepodid survival between treatments and sampling times were investigated with Mann-Whitney U test. The significance level was set to $P < 0.01$ instead of 0.05 to reduce the false discovery rate when conducting multiple tests.

For the freshwater tolerance investigations, the mortality data presented in the results were log-transformed and analyzed via probit regression to determine the concentration where half of the population was immobilized or the half maximal effective concentration (EC_{50}). The similar approach was used to determine the effective concentration where 75, 90 and 95% of the population was immobilized (EC_{75}, EC_{90}, EC_{95}). The regression was performed in Excel with the help of Finney´s table for transformation of percentage mortality to probit values (Finney, 1962). Further, the survival curves were analyzed with Paired sample T-test, while the EC_{50}-values were analyzed via One-way ANOVA. The significance level was set to $P < 0.05$, since the number of tests was lower compared to the analysis of the hatching success and development to copepodid stage.

3 Results

3.1 Background data, lice numbers and treatment effect

Background data for the four freshwater treatments followed during this study is presented in Table 4. The sea water temperature ranged from 8.0 to 10.1 C° at the different locations. The total biomass treated at the different treatments ranged from approx. 106 000 kg to 469 000 kg. The table also shows the treatment prior to and following the sampling week.

Table 4. Background data from the different freshwater treatments followed, represented with sea water temperature, mean fish weight and total biomass treated, along with the treatment before and after the sampling week.

Treatment	Temp (C°)	Mean fish weight (kg)	Biomass (kg)	Prior treatment	Sampling week	Following treatment
1.1	9.8	2.01	105 729	Week 43 Freshwater	46	Week 52 Freshwater
1.2	9.8	1.88	185 946	Week 43 Freshwater	46	Week 52 Freshwater
2.1	10.1	1.68	204 435	Week 44 Mechanical (Flushing)	46	None
3.1	8.0	3.89	468 754	Week 43 Combination (Freshwater + Thermal	49	None

An overview of lice numbers one week prior to and after the delousing event is shown in Table 5. The effect on the sessile stages (Chalimus I & II) was between 89 and 100%, whilst the effect on the motile stages (Preadult I & II + Adult males) was ranging from 13 to 100%. The effect on adult females ranged from 44 to 75%.

Table 5. Lice numbers the week prior to and after freshwater treatment at the different treatments. The numbers are listed as mean values of the lice stages sessile, motile and adult female.

Treatment	Week prior to delousing			Week after delousing			Effect		
	Sessile	Motile	Adult female	Sessile	Motile	Adult female	Sessile	Motile	Adult female
1.1	0.00	0.40	0.30	0.00	0.35	0.10	NA	13%	67%
1.2	0.00	0.75	0.45	0.05	0.00	0.25	NA	100%	44%
2.1	1.75	2.70	0.50	0.20	1.65	0.20	89%	39%	60%
3.1	0.90	1.85	0.20	0.00	0.15	0.05	100%	92%	75%

3.2 Egg string length

Median egg string length from the different sampling times at the different treatments is presented in Figure 7. The highest median egg string length before treatment was at Treatment 1.2, where median egg string length was 26.8 mm. The lowest was at Treatment 3.1, with median egg string length at 14.9 mm. No significant differences in egg string length were observed before treatment ($P > 0.01$; Appendix Table B1).

After two hours treatment time, median egg string length was again highest at Treatment 1.2 with 28.1 mm, and lowest at Treatment 3.1 with 16.1 mm. However, no significant differences in median egg string length were observed at the different treatments after two hours treatment time ($P > 0.01$; Appendix Table B2).

Samples collected after four hours treatment time showed highest median egg string length at Treatment 2.1 with 26.4 mm, and lowest median egg string length at Treatment 3.1 with 18.1 mm. There were no significant differences in median egg string length at this sampling time for the different treatments ($P > 0.01$; Appendix Table B3).

Samples collected after six hours treatment time were only obtained from Treatment 1.1, 1.2 and 2.1. The highest observed median egg string length for this sampling time was at Treatment 2.1 with 21.3 mm, while the lowest was at Treatment 1.1 with 16.9 mm. There were no significant differences in median egg string length for the different treatments at this sampling time ($P > 0.01$; Appendix Table B4).

Samples after treatment were only obtained from Treatment 1.1 and 2.1. At Treatment 1.1, median egg string length was 23.7 mm. At Treatment 2.1, median egg string length was 17.7 mm. There were no significant differences between the two treatments for this sampling time ($P > 0.01$; Appendix Table B5).

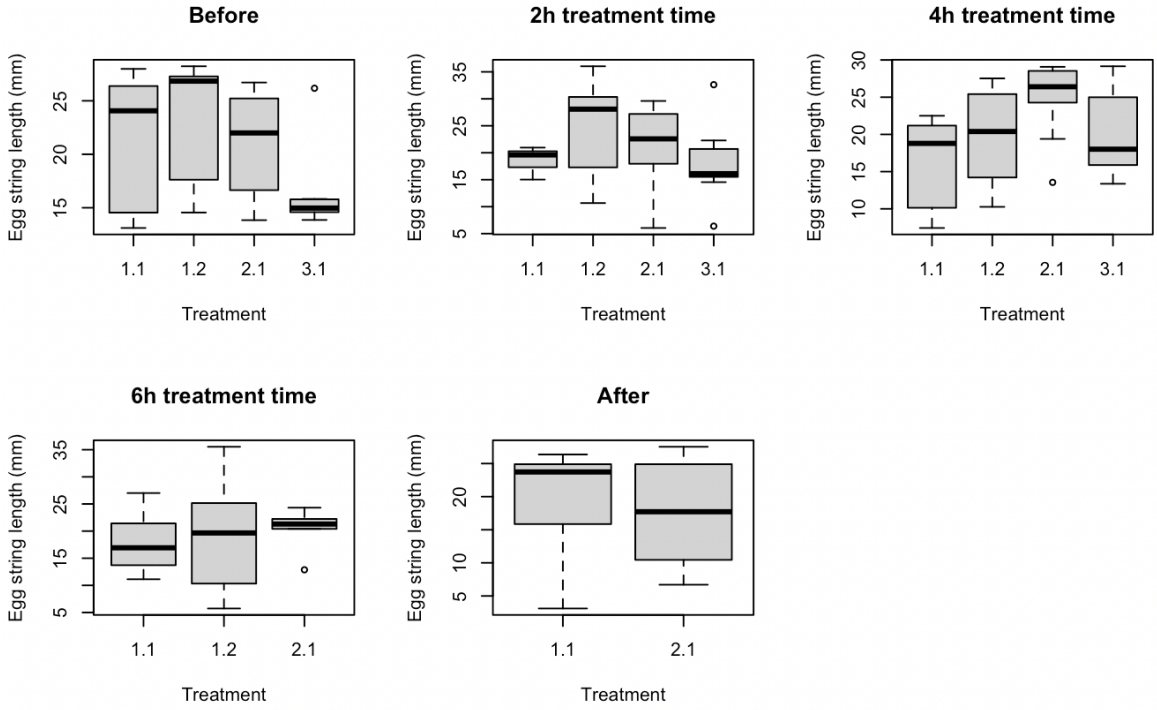


Figure 7. Boxplots showing median egg string length with interquartile and total range for the different sampling times at the different treatments.

Median egg string length from all treatments combined is presented in Figure 8. The median egg string length did not vary systematically with sampling time and varied from 18.7 mm to 21.7 mm. Observed no significant differences in median egg string length from all treatments at different sampling times ($P > 0.01$; Appendix Table B6).

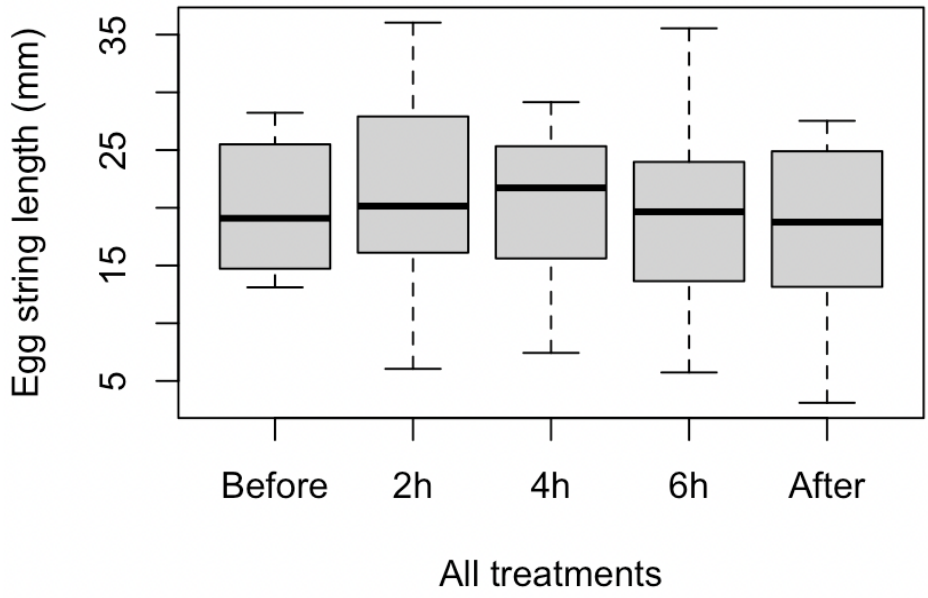


Figure 8. Boxplots showing median egg string length with interquartile and total range from all treatments at the different sampling times.

3.3 Hatching success

The number of egg strings that hatched varied between the different sampling times and is listed in Table 6. Close to all samples collected before treatment hatched, with an observed decline as the treatment time progressed. No samples hatched after six hours of treatment time.

Table 6. Overview of the number of egg strings sampled, hatched, as well as mean hatching success (number of nauplii observed per egg string) and mean number of copepodids observed per egg string for the different sampling times.

Sampling time	Before	2h	4h	6h	After
Egg strings incubated	34	38	34	23	11
Egg strings hatched	33	31	14	0	0
Proportion of egg strings hatched	97.1%	81.6%	41.2%	0.0%	0.0%
Mean hatching success: number of nauplii per egg string (±SD)	270 ± 78	94 ± 32	52 ± 47	0	0
Mean number of copepodids per egg string (±SD)	238 ± 118	63 ± 58	67 ± 71	0	0

The hatching success was found by dividing the number of hatched nauplii (active and immobilized) with the estimated number of eggs in the sample. Hatching success for the different sampling times at the different treatments is illustrated in Figure 10. The sampling times where no samples hatched (6h and

after treatment) are excluded from the figure. The highest observed median hatching success before treatment was at Treatment 2.1 with 85.4%, while the lowest was at Treatment 3.1 with 65.7%. The differences in hatching success before treatment was not significant ($P > 0.01$; Appendix Table B7).

After two hours of freshwater exposure, median hatching success was between 55.3% (Treatment 2.1) and 41.7% (Treatment 3.1), with an exception at Treatment 1.1 where median hatching success was 0.0%. However, the low hatching success observed here may partly be caused by the small sample size obtained at this treatment ($N = 3$), where only one of the three samples hatched, although with similar hatching success (43.5%) as the observed median for the other treatments. There were no significant differences in hatching success after two hours for the different treatments ($P > 0.01$; Appendix Table B8). Comparing the hatching success after two hours of freshwater exposure with the before samples revealed a significant decline in hatching success at Treatment 1.2 and 2.1 ($P < 0.01$; Appendix Table B9).

For the four hours treated egg strings, median hatching success was 0.0% at Treatment 1.1, 1.2 and 3.1. However, some samples were observed to hatch relatively successfully. The highest observed hatching success was at Treatment 2.1 with 22.0%. At Treatment 1.2, only one sample hatched with observed hatching success at 55.7%. Hatching success was also low at Treatment 3.1, even though the number of samples were highest ($N = 12$) for this sampling time. There was a significant difference in hatching success from two to four hours at Treatment 1.2 ($P < 0.01$; Appendix Table B9), but no significant differences for Treatment 1.1, 2.1 or 3.1 ($P > 0.01$; Appendix Table B9).

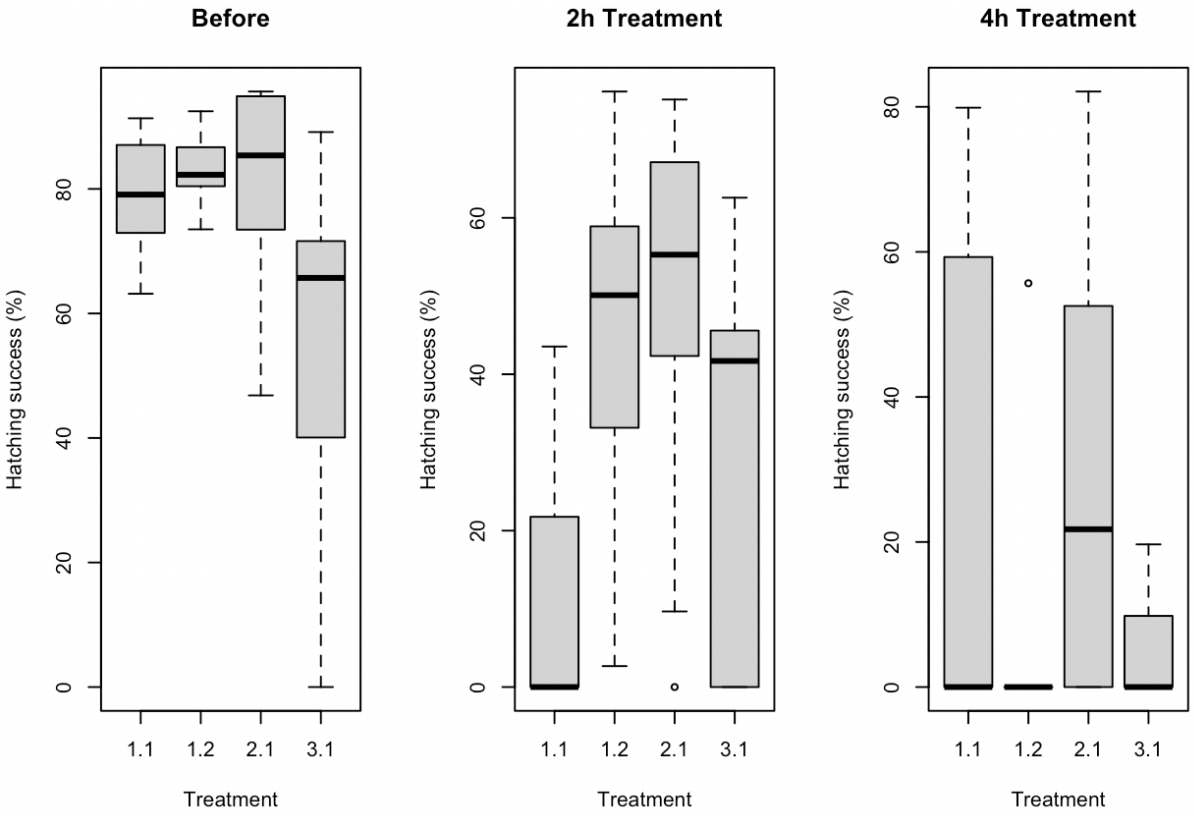


Figure 9. Boxplot showing median hatching success (number of nauplii divided by the estimated number of eggs (%)) with interquartile and total range for the different sampling times at the different treatments.

The hatching success from egg strings collected at different sampling times for all treatments combined is illustrated in Figure 11. Median hatching success was highest before treatment and declined with treatment time. After four hours of treatment, the median hatching success was 0.0%. However, some samples were observed to hatch relatively successfully, up to 80.0%. For the two last sampling times, six hours freshwater exposure and after treatment, no samples hatched. The difference in hatching success before and after two hours treatment, as well as between two and four hours treatment, were significant ($P < 0.01$; Appendix Table B10).

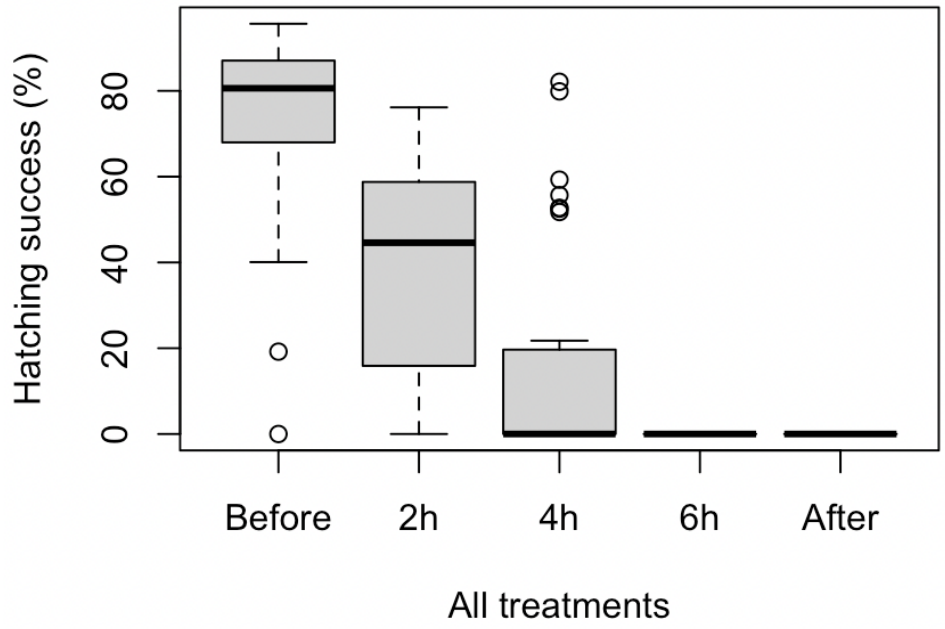


Figure 10. Boxplot showing median hatching success with interquartile and total range from all treatments at the different sampling times.

3.4 Copepodid survival

The copepodid survival was calculated by dividing the number of actively swimming copepodids by the estimated number of eggs. Egg strings that did not hatch were excluded. Copepodid survival for the different sampling times at all treatments are illustrated in Figure 12. The copepodid survival before treatment showed some variation with the highest observed at 80.1% (Treatment 2.1) to the lowest at 60.9% (Treatment 3.1). The differences in hatching success before treatment were not significant ($P > 0.01$; Appendix Table B11).

After two hours of treatment, copepodid survival was significantly lower compared to before treatment at Treatment 1.2 and 2.1 ($P < 0.01$, Appendix Table B12). There was no significant difference at Treatment 1.1, which again can be caused by the small sample size from this sampling time. The copepodid survival at Treatment 3.1 before treatment was the lowest observed, and the reduction in copepodid survival following two hours treatment was not significant ($P = 0.059$, Appendix Table B12).

Copepodid survival following four hours treatment at Treatment 1.1 was observed to increase compared to the two hours treated egg strings. The difference was however not significant ($P = 0.667$, Appendix Table B12). For Treatment 1.2 and 2.1, there was no significant differences in copepodid survival

between two- and four-hour samples (P > 0.01; Appendix Table B12), but the differences between before treatment and four hours treated egg strings were significant (P < 0.01; Appendix Table B12). The lowest median copepodid survival was observed at Treatment 3.1 with 3.2%, which was significantly lower compared to before- and two- hour samples (P < 0.01, Appendix Table B12).

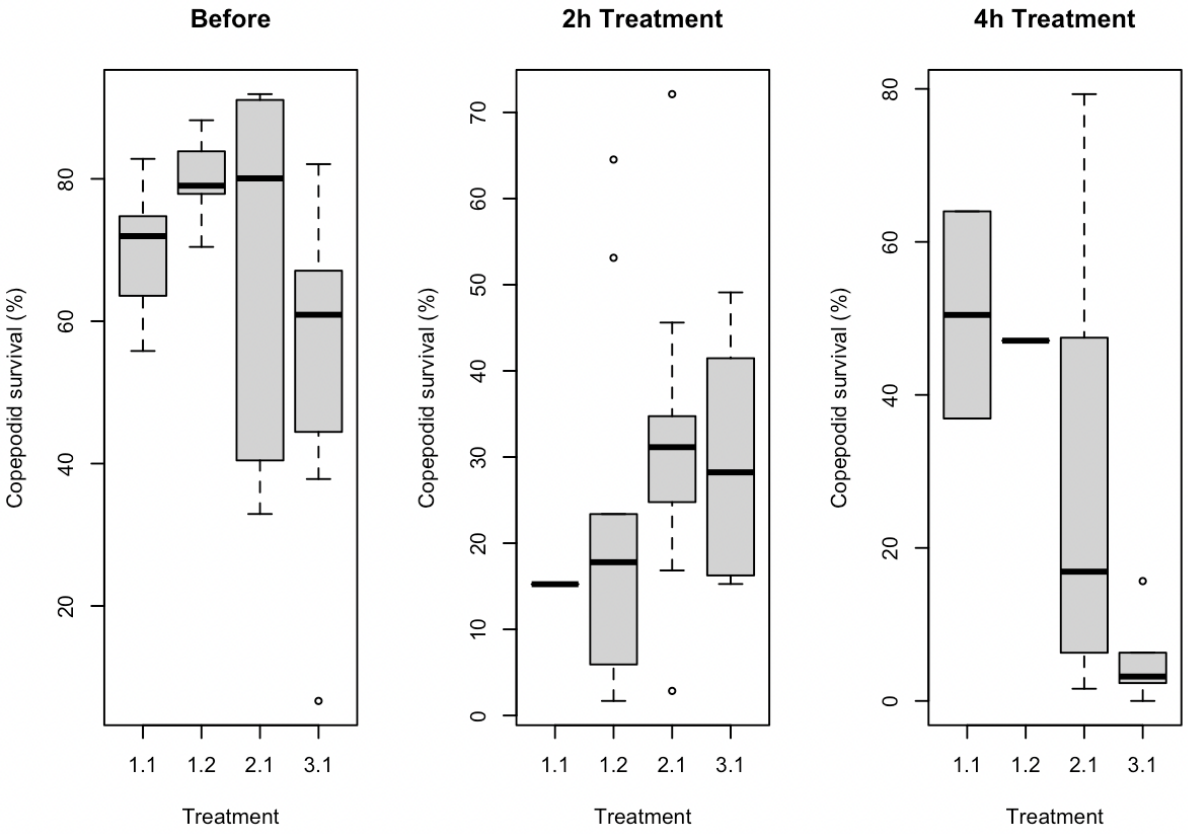


Figure 11. Boxplot showing median copepodid survival with interquartile and total range for the different sampling times at the different treatments. The boxplots are based on number of actively swimming copepodids 4 days post hatching divided by the estimated number of eggs in the egg string. Unhatched egg strings were removed from the data.

Copepodid survival from all treatments combined at the different sampling times are presented in Figure 13. Before treatment, median copepodid survival was highest with 70.4%. The median copepodid survival was observed to decline as the treatment time increased. After two hours exposure, median copepodid survival was at 23.4%, which was significantly lower compared to before treatment (P < 0.01; Appendix Table B13). The lowest median copepodid survival was after four hours treatment time at 14.8%, however not significantly different from the two hours treated egg strings (P > 0.01; Appendix Table B13).

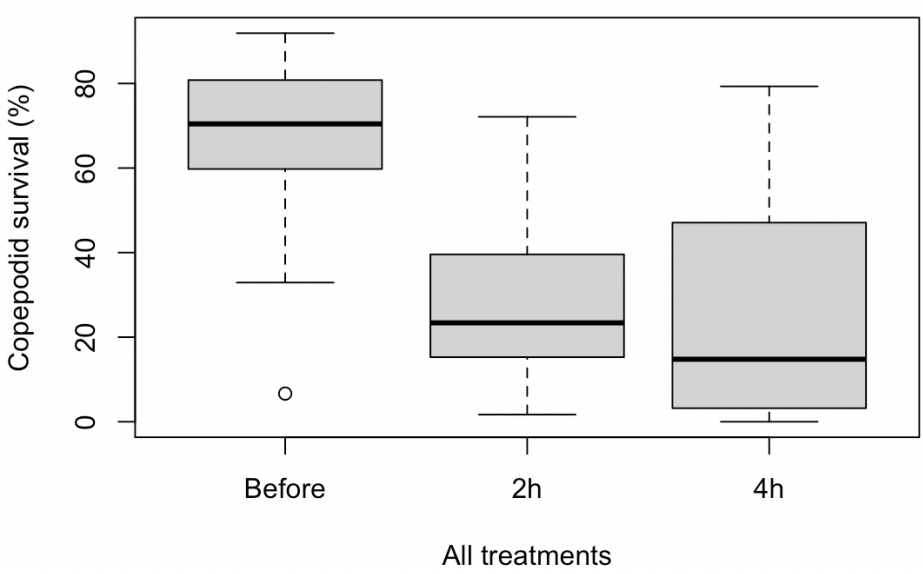


Figure 12. Boxplot showing median copepodid survival with interquartile and total range from all treatments at the different sampling times. The boxplots are based on actively swimming copepodids 4 days post hatching and divided by the estimated number of eggs in the egg string. Unhatched egg strings were removed from the data.

3.5 Number of copepodids

The median number of active copepodids per egg string recorded during counting is presented in Figure 14. The median number of active copepodids produced per egg string were observed to decline following two hours treatment time.

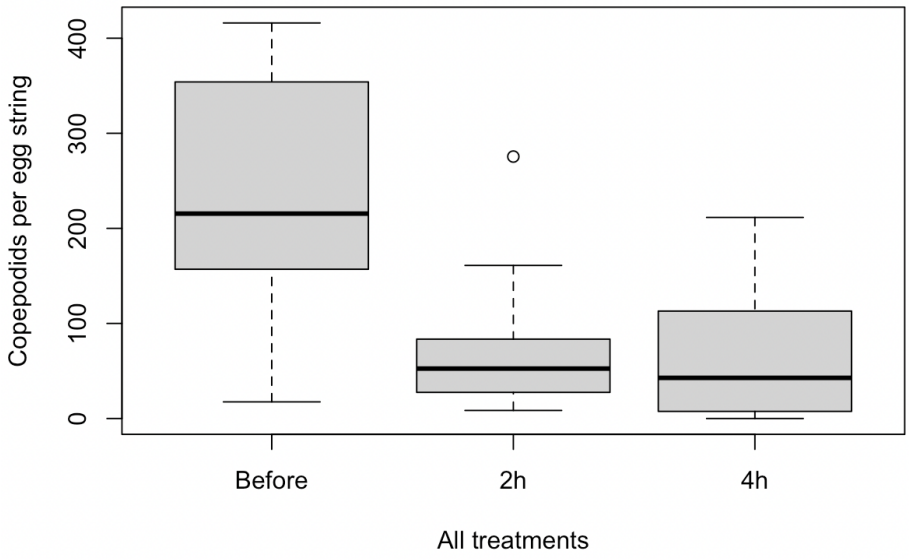


Figure 13. Median number of active copepodids per egg string for all treatments at different sampling times. The number of observations per sampling time is listed in Table 6.

The average number of copepodids per egg strings before treatment were 238 ± 118. The average amount of copepodids in egg strings exposed to freshwater for two hours were 63 ± 58, significantly lower compared to the before treatment ($P < 0.01$; Appendix Table B14). The average amount of copepodids observed for the four hours treated egg strings were similar with the two hours treated egg strings at 67 ± 71, and significantly lower compared to before treatment ($P < 0.01$; Appendix Table B14).

3.6 Survival from nauplii to copepodid stage

Figure 15 shows median survival recorded from nauplii to the copepodid stage for all treatments at the different sampling times. Median survival before treatment was 97.8% and observed to decline with increasing treatment time. Median survival for the two hours treated egg strings was 88.1%, and significantly lower compared to before treatment ($P < 0.01$; Appendix Table B15). For the four hours treated egg string, median survival was at 70.0%, which was significantly lower compared to before treatment ($P < 0.01$; Appendix Table 21), and similar to the two hours treated egg strings ($P = 0.11$; Appendix Table B15).

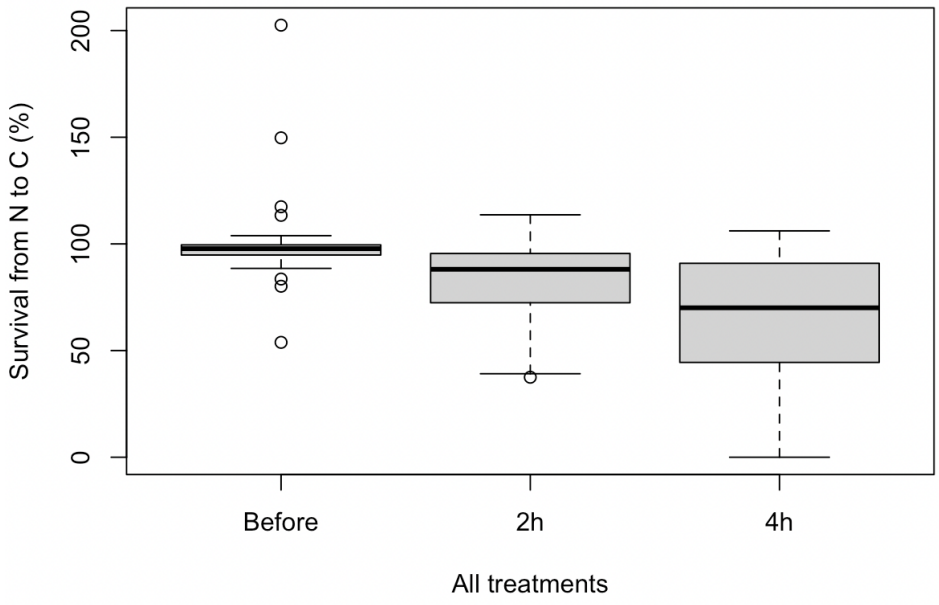


Figure 14. Median survival from nauplii to the copepodid stage (active copepodids/active nauplii). Values over 100% were most likely caused by nauplii recorded as immobilized during the first counting that recovered and displayed active behavior at the copepodid counting. Number of observations is listed in Table 6.

3.7 Freshwater tolerance

A total of 11 bioassays were conducted on salmon lice copepodids hatched from egg strings sampled before and following two and four hours of freshwater treatment. Two additional bioassays were conducted on copepodids from egg strings produced by two- and four- hours treated preadult and adult lice, as well as one for the F2-generation copepodids originating from before treatment at Treatment 3.1.

Analysis of the different treatments and sampling times revealed differences in tolerance to low salinity levels at the copepodid stage. Figure 16 illustrates the survival curves before treatment for the four treatments followed. Treatment 3.1 maintained high survival rates (> 85%) until 11‰, after which survival rapidly dropped, whereas Treatment 1.2 and 2.1 experienced a gradual reduction in survival below 85% at salinities below 17‰. Treatment 1.1 showed a decline in survival below 85% at salinity 23‰, with steady decline in survival rate as the salinity decreased.

The dose-response curve shows variation in treatment tolerance when comparing the EC_{50} from the different treatments, even Treatment 1.1 (13.5‰) and 1.2 (9.3‰) which were sampled from the same farm site (Figure 17). Treatment 1.1 had the highest EC_{50}, which was significantly different to the lowest observed EC_{50} at Treatment 3.1 (7.6‰) ($P < 0.05$; Appendix Table B20). There was also a significant difference when comparing the EC_{50} from Treatment 1.2 and 3.1, and 2.1 and 3.1 ($P < 0.05$; Appendix Table B20). Paired sample t-test were conducted between the survival curves for the different treatments, and there was a significant difference in survival for Treatment 1.1 compared to the other treatments ($P < 0.05$; Appendix Table B16). Survival at Treatment 3.1 was significantly higher compared to the other treatments ($P < 0.05$; Appendix Table B16). Survival at Treatment 1.2 and 2.1 were similar.

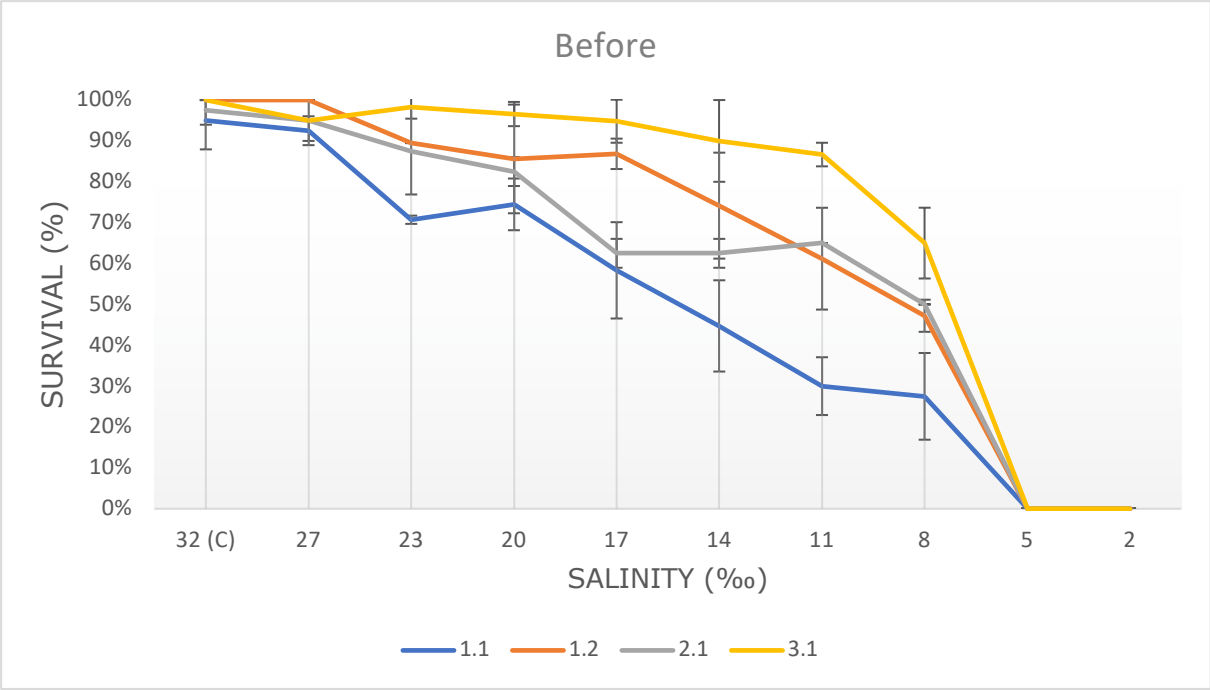


Figure 15. Survival (%, error bars are standard deviation) for salmon lice copepodids from egg strings sampled before treatment at four different treatments following 24 h exposure to a range of salinities (‰).

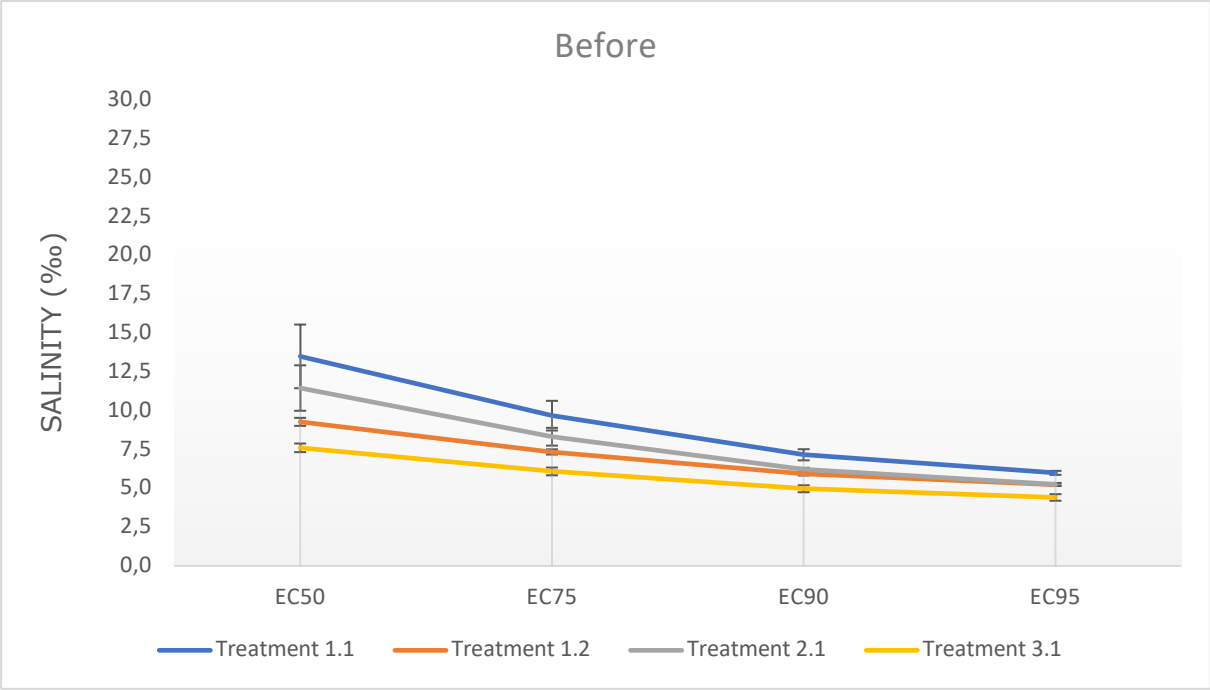


Figure 16. Dose-response curve (error bars are standard deviation) for salmon lice copepodids from egg strings sampled before treatment at four different treatments exposed to different salinities to determine the half maximal effective concentration, provided as range from EC50 to EC95.

Survival curves for copepodids hatched from two hours freshwater treated egg strings are presented in Figure 18. Observing the survival curves from each treatment across the salinity gradient revealed that Treatment 1.1, 2.1 and 3.1 maintained survival rates around 70% until 14‰, after which survival rapidly dropped for Treatment 1.1 and 2.1. Survival for Treatment 3.1 maintained at 63% at salinity 8‰ before rapidly dropping. The survival curve for Treatment 1.2 was under 50% at salinity 27‰ and continued to be low across the salinity gradient with survival under 10% at salinity 14‰.

The dose-response curve presented in Figure 19 shows the high EC_{50} from Treatment 1.2 where the median immobilizing salinity was 28‰. Comparing the EC_{50} for Treatment 1.1 and 2.1 with the Before samples revealed no significant differences in treatment tolerance ($P > 0.05$; Appendix Table B21). EC_{50} values from Treatment 3.1 increased significantly compared to the Before samples ($P < 0.05$; Appendix Table B21).

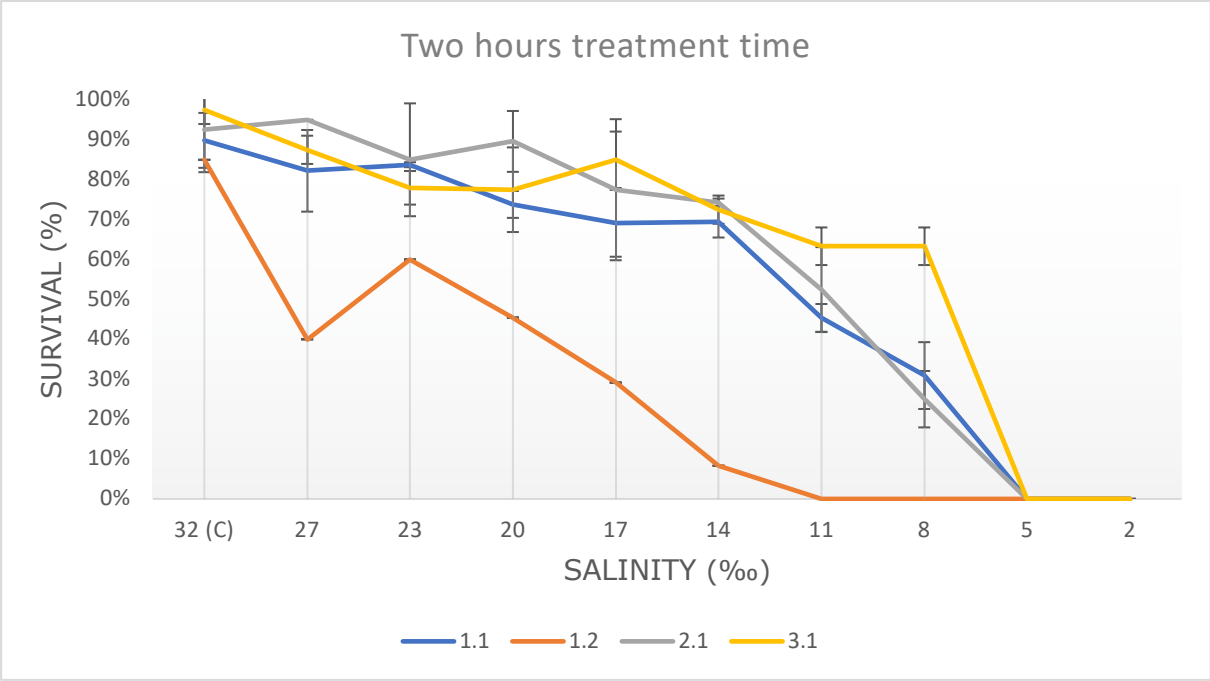


Figure 17. Survival (%, error bars are standard deviation) for salmon lice copepodids from egg strings sampled after two hours of freshwater treatment at four different treatments following 24 h exposure to a range of salinities (‰).

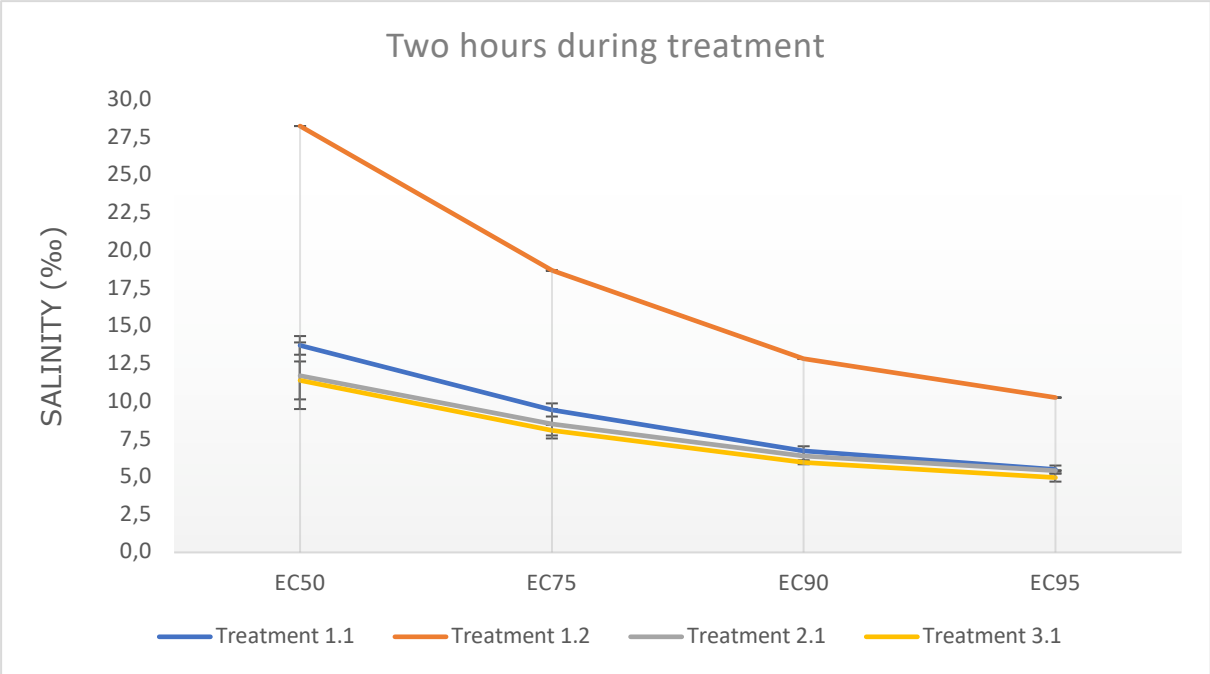


Figure 18. Dose-response curve (error bars are standard deviation) for salmon lice copepodids from egg strings sampled after two hours of freshwater treatment at four different treatments exposed to different salinities to determine the half maximal effective concentration, provided as range from EC50 to EC95.

Survival curves for copepodids hatched from four hours freshwater treated egg strings were not obtained from Treatment 1.1 (Figure 20). For Treatment 1.2 and 3.1, only one replicate was performed due to the low number of hatched samples from this sampling time. The survival was highest for Treatment 3.1, showing 80% survival at salinity 11‰, before rapidly declining. Surival was lowest for Treatment 1.2 showing values below 80% at 20‰.

Analyzing the survival curves revealed a significant lower survival at treatment 1.2 compared to before treratment ($P < 0.05$; Appendix Table B17). The dose-response curve indicated reduced treatment tolerance at Treatment 1.2 and 3.1 (Figure 21), but due to only one replicate, no analysis could determine if the differences were significant. For Treatment 2.1, treatment tolerance in copepodids from four hours treated egg strings were similar to the Before and two hour samples ($P > 0.05$; Appendix Table B21).

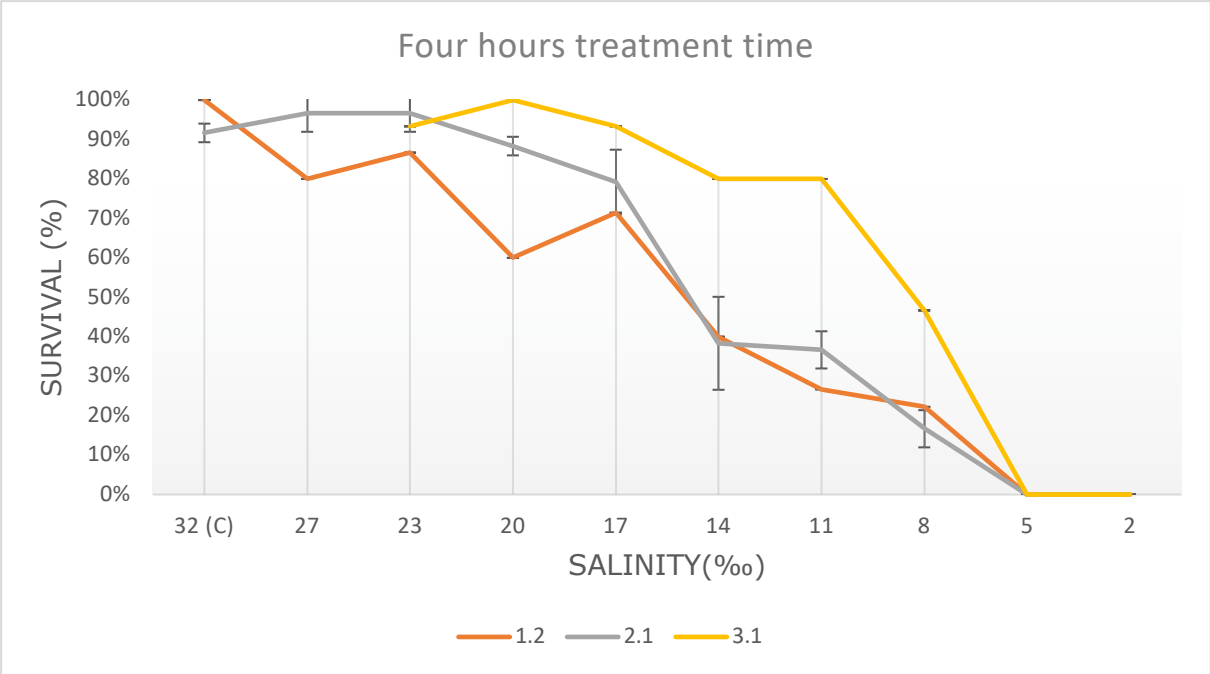


Figure 19. Survival (%, error bars are standard deviation) for salmon lice copepodids from egg strings sampled after four hours of freshwater treatment at three different treatments following 24 h exposure to a range of salinities (‰).

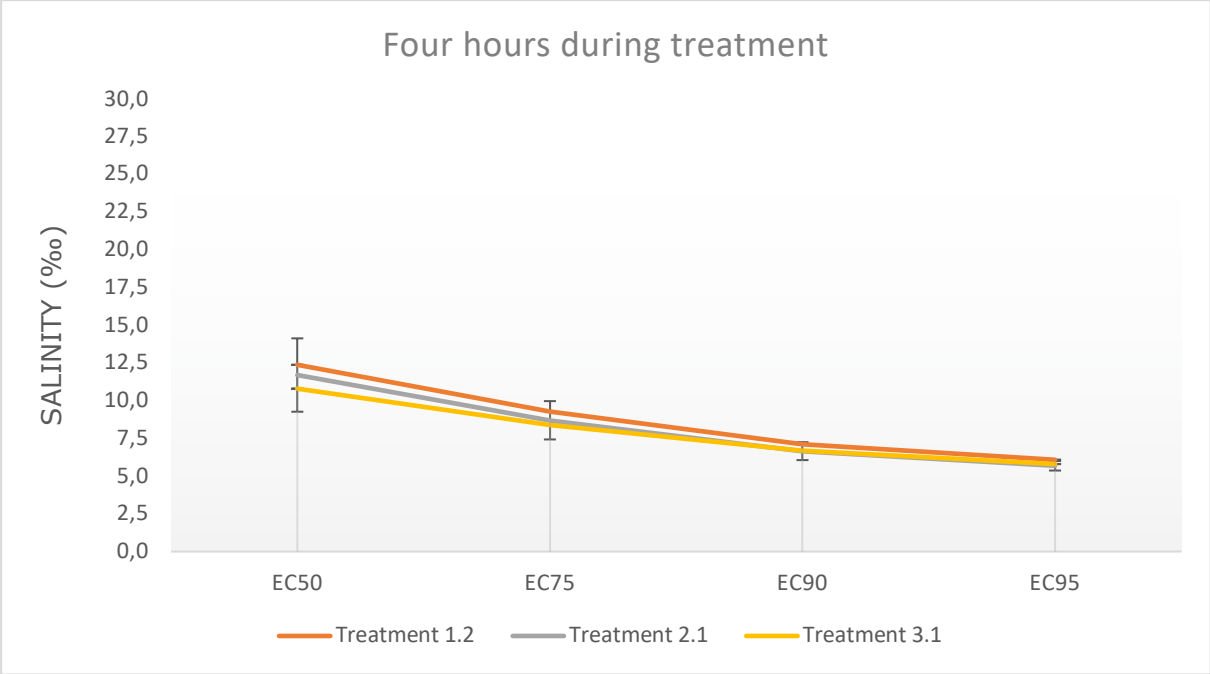


Figure 20. Dose-response curve (error bars are standard deviation) for salmon lice copepodids from egg strings sampled after four hours of treatment at three different treatments exposed to different salinities to determine the half maximal effective concentration, provided as range from EC50 to EC95.

Survival curves for copepodites from egg strings produced by two and four hours treated preadult and adult lice from Treatment 3.1 are presented in Figure 22. Observing the survival curves across the salinity gradient revealed that they were similar compared to the before samples from Treatment 3.1 (Figure 16), with high survival (> 80%) until 11‰ before rapidly dropping.

Paired sample t-tests for survival curves revealed that the copepodids originating from two hours treated preadult and adults had significantly higher survival compared to the before samples from the same farm site ($P < 0.05$; Appendix Table B18). Copepodids originating from four hours treated preadult and adults were similar to the before sample and two-hour sample ($P > 0.05$; Appendix Table B18)

The dose-response curve indicated increased treatment tolerance in both the two and four hours treated sample (Figure 23). When compared with the before sample, there was significant increase in treatment tolerance in copepodids from both the two and four hours treated preadult and adult lice ($P < 0.05$; Appendix Table B22).

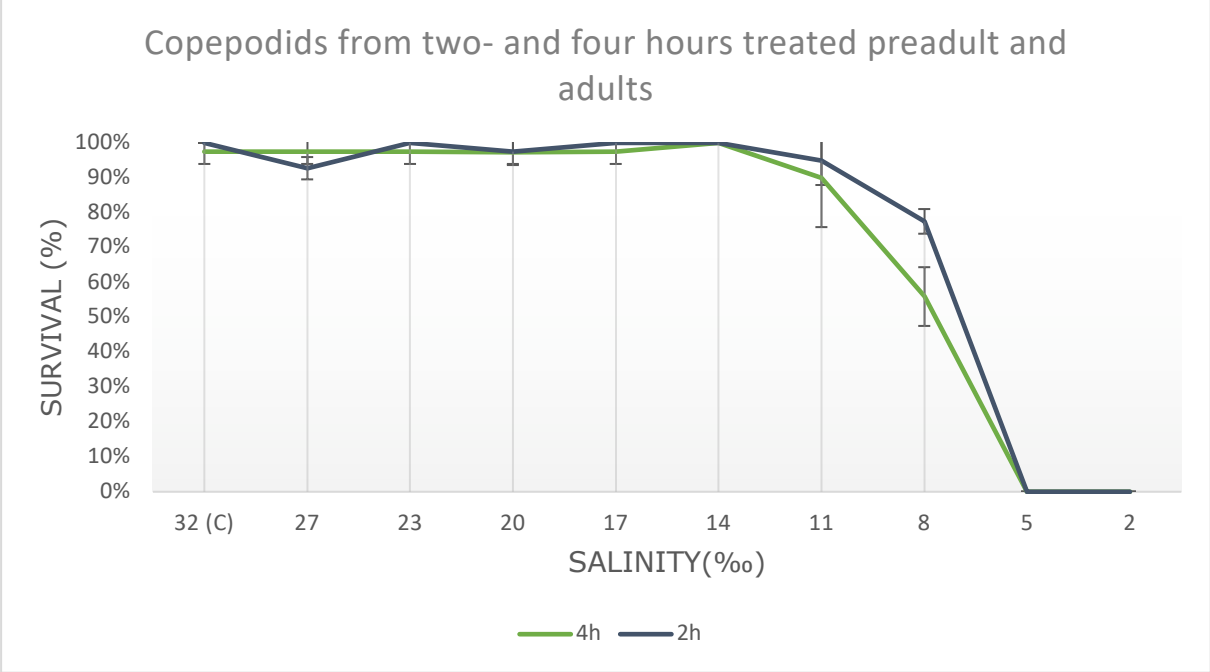


Figure 21. Survival (%, error bars are standard deviation) for salmon lice copepodids from egg strings produced by two and four hours treated preadult and adult lice at Treatment 3.1 following 24 h exposure to different salinities (‰).

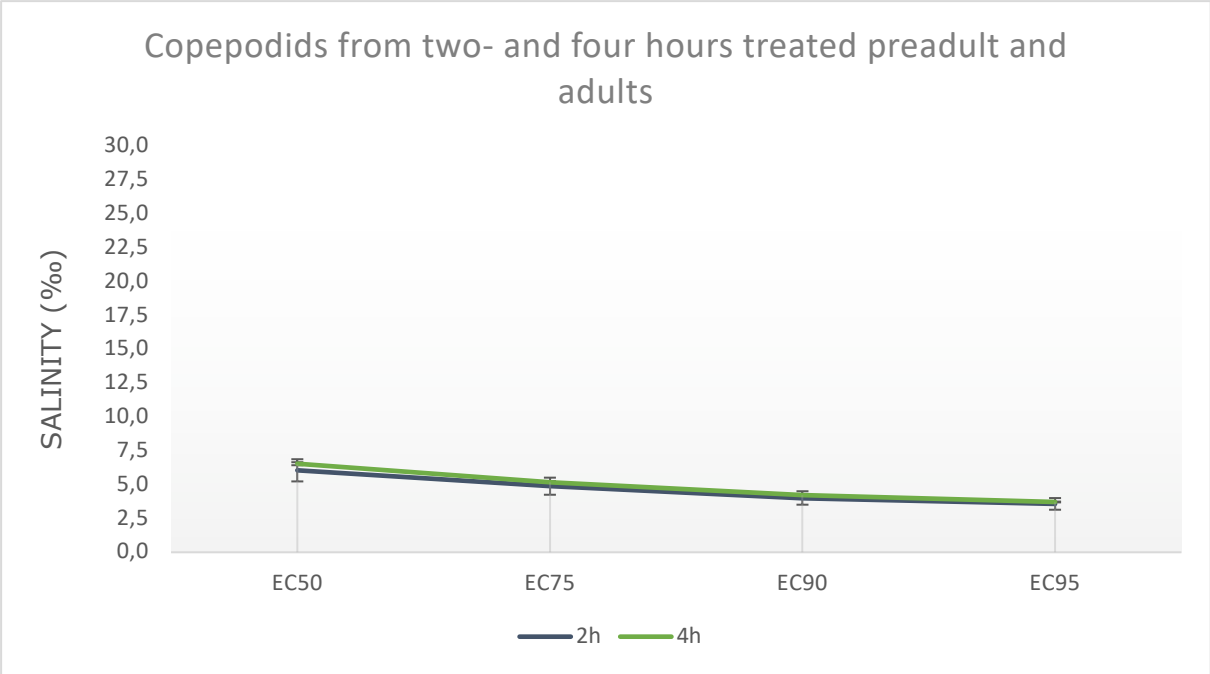


Figure 22. Dose-response curve (error bars are standard deviation) for salmon lice copepodids from egg strings produced by two and four hours treated preadult and adult lice at Treatment 3.1 exposed to different salinities to determine the half maximal concentration, provided as range from EC50 to EC95.

Copepodids from before as well as copepodids from preadult and adult lice collected at Treatment 3.1 that were not tolerance tested were used to infect Atlantic salmon to produce F2-generation lice. For F2-generation copepodids from treated preadult and adult lice, no results were obtained as it looked like the infection was successful when observing fish behavior during infection, but no lice successfully developed to adults.

Observing the survival curve for the F2-generation lice before treatment (Figure 24) revealed that the survival was 90% around 11‰ before dropping rapidly, which is very similar to results observed in the F1-generation copepodids from this sampling time (Figure 16). Two-sample paired tests revealed that there were no significant differences between the F1- and F2-generation copepodids from before treatment ($P > 0.05$; Appendix Table B19). In addition, the dose-response curve (Figure 25) revealed no significant differences in treatment tolerance ($P > 0.05$; Appendix Table B23).

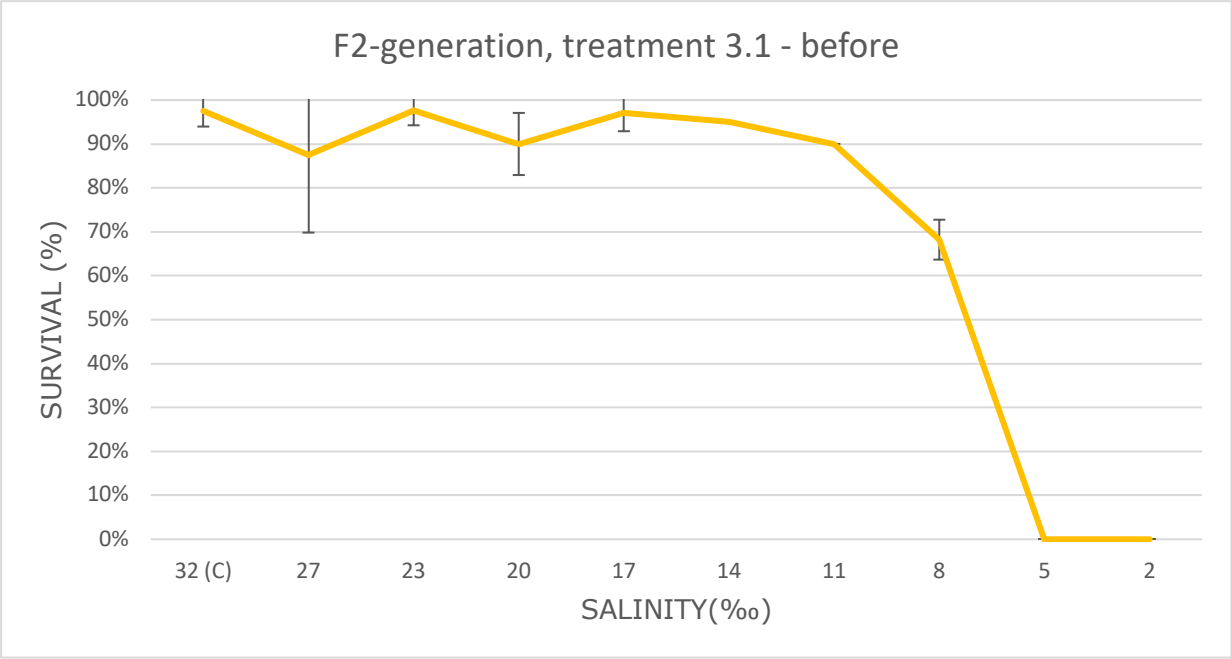


Figure 23. Survival (%, error bars are standard deviation) for salmon lice F2-generation copepodids sampled before treatment following 24 h exposure to a range of salinities (‰).

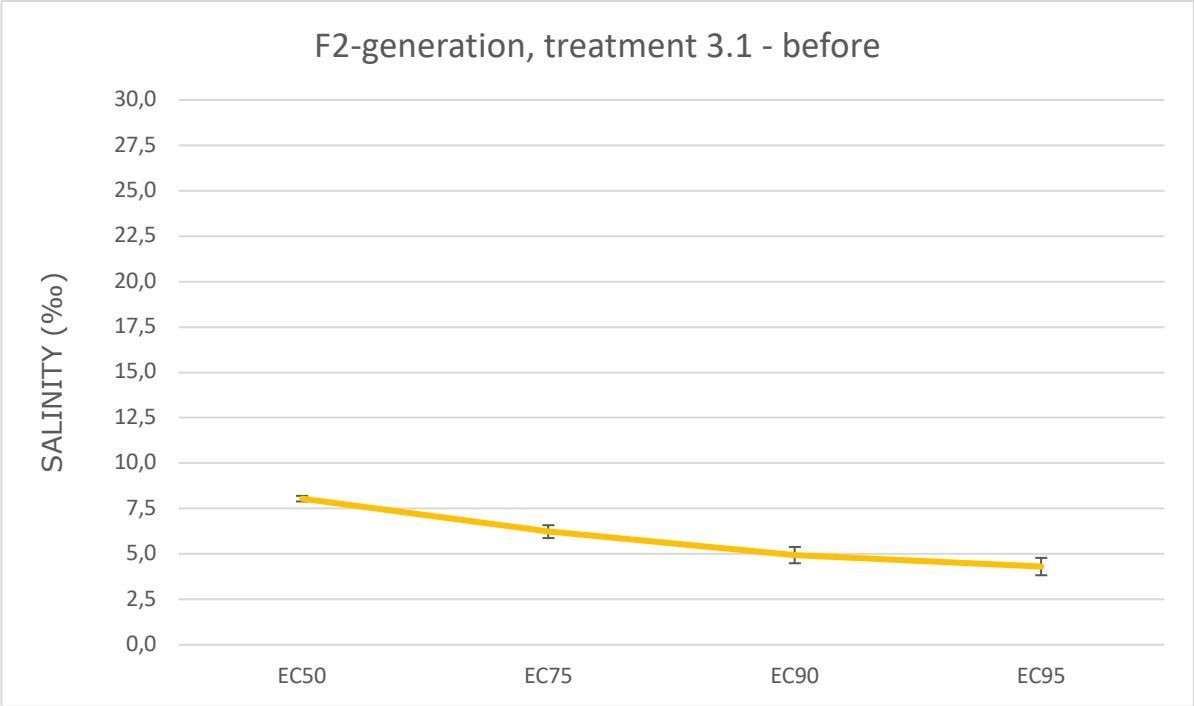


Figure 24. Dose-response curve (error bars are standard deviation) for salmon lice F2-generation copepodids sampled before treatment exposed to different salinities to determine half maximal effective concentration, provided as range from EC50 to EC95.

4 Discussion

The aim of this study was to investigate how salmon lice was affected by freshwater treatment, and if the treatment could potentially lead to changes in freshwater tolerance. The first research question focused on investigating if freshwater treatment affected hatching success and development to the copepodid stage. The research was conducted by collecting salmon lice during freshwater treatment and comparing nauplii and copepodids hatched from treated and untreated egg strings. The second research question focused on potential changes in treatment tolerance as a consequence of freshwater delousing and was investigated by using established bioassay methods to compare tolerance in copepodids from treated and untreated egg strings.

4.1 Egg string length

Egg string length was measured to estimate the number of eggs and in turn used to calculate hatching success for the different treatment groups during this study. Further, the egg string length was compared between the different treatments and sampling times to investigate base differences. However, the median egg string length did not vary systematically with sampling time (Figure 8), and although there was some variation between the different treatments and sampling times (Figure 7), no significant differences in egg string length were observed. Hence, variation in egg string length would most likely not influence the other results from this study. Earlier studies have suggested that egg string length is correlated with sea water temperatures, but the effect was apparently more evident for extremely low and high temperatures (3 and 20 °C) (Boxaspen, 2006; Samsing et al., 2016). Sea water temperature ranged from 8.0 to 10.1 °C at the three different locations in this present study, which could contribute to the similar egg string length observed which were in accordance with values found in previous studies (Eisenhauer et al., 2020; Hamre et al., 2009; Samsing et al., 2016).

The estimation of egg numbers present in the egg strings were based on the egg size measured by Furberg (2022) (56.9 ± 2.2 µm/egg, Appendix Figure 24). This number was slightly lower compared to the value 60-64 µm/egg reported by Heuch et al. (2000), and Samsing et al. (2016) who reported values of 62 ± 0.8 µm/egg. A possible explanation for this could be that photographing egg strings during this study was performed with a phone camera on a moving vessel,

whereas the other studies used camera equipment which could secure better photo quality and in turn better visualization of the eggs. Future employment of this method should consider using camera equipment that secure better photo quality than phone cameras, although this study was limited to this for practical reasons when following delousing operations on site.

4.2 Hatching success

The proportion of egg strings hatched varied between the different sampling times and were observed to decrease with increasing treatment time (Table 6). No eggs hatched from egg strings exposed to freshwater for six hours or more during this study. To our knowledge, this is the first cultivation study where the viability of freshwater treated egg strings is documented. One earlier study focused on the efficacy of freshwater on the different lice stages and showed that a mean lice reduction of 85% is expected (Gaasø, 2019). The same study also reported that the mean reduction was lowest for the adult male and female compared to the other stages, and that the reduction in females with egg strings was over 80% within 1 hour treatment time. This could suggest that the adult female sheds the egg strings relatively early during freshwater exposure. Since treatment times are normally between 5-10 hours, the findings of these studies suggest that the potential of finding viable egg strings at the end of a freshwater treatment is very small.

The highest observed hatching success (number of nauplii divided by the stimated number of eggs; %) was in the Before treatment group (85 to 66% across treatments, Figure 10). For all treatments combined, hatching success declined significantly with increasing treatment time ($P < 0.01$; Appendix Table B10). The decline in hatching success was very evident when observing the mean number of nauplii produced per egg string (Table 6). For egg strings sampled before treatment, hatching success was 270 ± 78 nauplii per egg string, which was in accordance with earlier studies where they found that the mean number of hatched nauplii per egg string was 287 ± 37 nauplii (Heuch et al., 2000). The mean number of hatched nauplii per egg string for the treated egg strings was significantly lower and reduced to 94 ± 32 following two hours exposure, and 52 ± 47 following four hours exposure.

There are currently few publications available describing hatching success of salmon lice egg strings. One publication reports a hatching success of over 80% for three different lice strains (Espedal et al., 2013). However, the method used in that study differed from the one employed here, among others because we included unhatched egg strings in the hatching success calculations and did accordingly not remove them from the data. The decision to include unhatched egg strings was made based on the assumption that there will be a possibility of

finding egg strings that do not hatch in the natural environment, as well as under laboratory conditions. Since the proportion of egg strings hatched varied greatly from untreated to treated egg strings, exclusion of unhatched egg strings during the calculations would have influenced the results, especially for the treated egg strings.

Furberg (2022) investigated hatching success in egg strings in a similar study to this one comparing other Non-Medicinal Methods (NMM) of lice treatment. The results of Furberg (2022) revealed no significant difference in hatching success before and after treatment for mechanical and thermal delousing. His results are accordingly very different compared to the results found in this study where hatching success were observed to decline with treatment time, and where egg strings exposed to freshwater for more than six hours did not hatch at all.

4.3 Development to copepodid stage

Median copepodid survival for the Before treatment group (80 to 61%, Figure 12) was generally high compared to the treated egg strings. Further, the median number of copepodids produced per egg string were observed to decline when comparing the untreated and treated egg strings. For the Before treatment group, each egg string produced an average of 238 ± 118 copepodids, which was similar to earlier values published by Hamre et al. (2009) who found an average production of 218 copepodids per egg string. The copepodid production for the egg strings exposed to freshwater during this study were observed to be significantly lower at 63 ± 58 copepodids per egg string for the two hours treated egg strings and 67 ± 71 for the four hours treated egg strings. These numbers differ slightly from the mean number of hatched nauplii per egg string (Table 6), which is explained by the different calculation methods applied. Calculation of the number of copepodids per egg string did not include unhatched egg strings.

The survival from nauplii to copepodids (Figure 15) revealed that the median survival was higher for the untreated egg strings (97%) compared to the treated egg strings. However, there was no significant difference when comparing the two sampling times of treated egg strings, although the figure revealed that the median survival was reduced from 88 to 70% from two to four hours treatment time. These results suggests that development from nauplii to copepodids is affected by the treatment and considering that the variation is greater for the four hours treated group, longer treatment time could have larger effect on development.

4.4 Investigation of tolerance to low salinities in copepodids

The use of freshwater is a widely accepted natural deterrent to the settlement of salmon lice copepodids on Atlantic salmon (Connors et al., 2008; Hahnenkamp & Fyhn, 1985; Wright et al., 2016). Previous studies have found that copepodids die following 1-3h exposure time whilst attached to the host (Bricknell et al., 2006; Stone et al., 2002; Wright et al., 2016). Free-swimming copepodids are reported to succumb after 3h at a salinity of 4‰ (Bricknell et al., 2006). Previous studies where bioassays were conducted on copepodids indicated that the copepodids remained unaffected until the salinity reached 9‰. Above this level, the active copepodids exhibited normal swimming behavior and appendage movement, as observed in the control group. This study also indicated population differences in treatment tolerance (Andrews & Horsberg, 2020).

Similarly, analysis of the different treatments and sampling times during this study revealed differences to low salinity levels at the copepodid stage. For the control group sampled before treatment, the survival curve revealed differences in survival rates, where Treatment 1.1 was significantly lower, and Treatment 3.1 significantly higher than the others. Survival rates for Treatment 1.2 and 2.1 were similar. However, analysis of the EC_{50} values only revealed a significant higher treatment tolerance at Treatment 3.1 compared to the other treatments. Since all locations sampled during this study belonged to the same production area, observing differences in tolerance levels was not expected. Previous research on genetic differences in salmon lice across regions has provided contradicting results, where some report no genetic variance (Todd et al., 1997), weak genetic differences between regions (Tjensvoll et al., 2006), to major regional distinction among populations (Dixon et al., 2004; Guragain et al., 2022; Nolan & Powell, 2008). The findings of this study suggest that there are differences in tolerance levels within the same production area, which in turn could be caused by selective pressure within the same population as a result of delousing strategies. Location 1 is relatively far from Location 2 and 3, meaning that physical barriers could influence gene flow and dispersal of lice within the same area. However, Location 2 and 3 are relatively close but with different delousing strategies in terms of the use of freshwater. Helgesen et al. (2021) observed a significant difference in tolerance to low salinities when comparing salmon lice from areas with high and low frequency of freshwater treatments. The same difference was not observed during the 2019-study (Helgesen et al., 2020), perhaps suggesting a development towards more low salinity tolerant lice in some areas. However, the limited number of farms included in the study and the relatively small differences between the two groups made it difficult to draw strong conclusions.

Observing the survival curves of the two hours treated egg strings indicated that the survival rate was lower at Treatment 1.2 and 3.1 compared to the control samples. Analysis of the EC_{50} values revealed that the treatment tolerance was

decreased, since EC_{50} values increased significantly. However, the results obtained from Treatment 1.2 was most likely influenced by the low amount of copepodids produced, which resulted in only one bioassay replicate and longer time from hatching to tolerance test, where the limit was set to 9 days post hatching.

Challenges with having enough samples and material for testing was an issue also present for the four hours treated egg strings. Here, only Treatment 1.2, 2.1 and 3.1 were tolerance tested and the survival curves revealed a significantly lower survival at Treatment 1.2 compared to before treatment (P = 0.007). For Treatment 2.1 and 3.1, the dose-response curve (Figure 21) indicated reduced treatment tolerance as the EC50 values increased compared to the before samples, however no analysis could determine if the difference was significant due to only one bioassay replicate.

This study also investigated tolerance level in copepodids produced by two and four hours freshwater treated preadult and adult lice collected at Treatment 3.1. Analysis of the survival curves revealed that the survival was higher for the two hours treated sample compared to the untreated sample (P = 0.046), while the four hours treated sample was similar with both the before (P = 0.609) and two-hour sample (P = 0.233). Further, the dose-response curve indicated increased treatment tolerance in copepodids from both the two and four hours treated preadult and adults compared to the before treatment copepodids (P = 0.042, P = 0.013). These results differ compared to the observations made earlier, where copepodids from two and four hours treated egg strings exhibited decreased tolerance compared to the untreated egg strings. The results also suggest that treated preadult and adult lice which survives two or four hours of freshwater treatment can produce new egg strings, which in turn develop normally to the copepodid stage and display similar or even higher freshwater tolerance compared to untreated lice.

The copepodids from treated preadult and adult lice which were not tolerance tested were used to infect Atlantic salmon to produce F2-generation lice. The infection was unsuccessful since only one louse was found from infection of 250 copepodids in each treatment group. Since the infections only were performed once per treatment group, it was hard to determine the cause for this unsuccessful infection. However, the same protocol was followed as for the before treatment group, and these F2-generation lice were successfully reared, hatched and tolerance tested. The results for survival rate and treatment tolerance for the F2-generation lice from Treatment 3.1 were in accordance with the observations made for the F1-generation, and no significant differences were observed (P = 0.820, P = 0.141). Previous research on the infection potential of copepodids exposed to low salinities is limited, although Bricknell et al. (2006) observed that exposure to low salinity levels seemed to compromise this ability. However, during this study, the copepodids used for infection were not exposed to low salinities before infection, it was the F0-generation collected during delousing which was exposed to freshwater. This could suggest a long-term

effect of freshwater where treated preadult and adult lice survive treatment, but infection potential of their offspring is limited. However, this infection was only performed once for the two and four hours treated lice, making it hard to draw any strong conclusions.

4.5 Infection potential

Findings in this study showcased some interesting results when it comes to the infection potential following a freshwater treatment. The poor hatching success observed for the treatment duration higher than 4 hours, and the fact that freshwater treatments normally last between 5-10 hours, suggests that the infection potential after freshwater treatment is very small. Two and four hours treated preadult and adult lice managed to reproduce and the copepodids tolerance to low salinity were observed to be similar or even higher than before treatment. However, the infection success was observed to be poor and whether this was caused by the treatment or human error during infection is hard to determine due to the limited replicates. The lice numbers (Table 5) show varying treatment effect, especially for the adult stages. This is similar to previous research (Gaasø, 2019) and is most likely explained by the host-dependent mechanism where the lice gain ions from the host to replace ions lost to the environment (Hahnenkamp & Fyhn, 1985). Since the efficacy of treatments are rarely 100%, it is reasonable to assume that some lice will go through the entire treatment and potentially survive. The lice numbers could also be influenced by detached parasites during crowding and loading, where previous research suggests that repeated use of swipe net may cause a mechanical delousing effect (Wright et al., 2016). Gaasø (2019) reported that treatments where swipe net was used to crowd salmon had lower mean number of lice attached to the fish before treatment compared to other crowding methods.

4.6 Future work and perspectives

The methods employed in this study for data collection were challenging which resulted in little material and few replicates. Rearing of copepodids is laborious and requires hatchery facilities as well as personnel for counting and husbandry. For future research, performing a controlled study where freshwater delousing is simulated under laboratory conditions, with more control of the adult females and their egg strings, could make the sampling more predictable and accurate in terms of treatment time. When following delousing operations on site, exposure time will differ depending on the time required for loading and unloading, especially between the wells. More control of data collection and exposure time

would increase accuracy and contribute to better understanding of the effect of freshwater on the lice and egg strings and indicate if the effects are as strong as observed in this study. Also, more observations from different production areas could help showcase potential differences across different areas.

Research within the aquaculture industry is in constant development and the research on salmon lice is increasing for each year. The importance of understanding evolutionary processes to help understand mechanisms and variation in low salinity tolerance is important both for the aquaculture industry and from a wild fish perspective. Since wild sea trout use fresh and brackish water for delousing, development towards more low salinity tolerant lice is unwanted. Also, since salmon lice populations are shown to exhibit considerable family-level genetic variation linking to temperature and salinity tolerance (Andrews & Horsberg, 2020; Guragain et al., 2022; Ljungfeldt et al., 2017), commercial treatments should aim for the highest possible reduction in lice numbers. Sievers et al. (2019) recommended that freshwater treatments should be incorporated within a cyclical treatment regime whereby different treatment types are applied in succession. This approach could limit the salmon lice ability to develop increased tolerance to low salinity (Groner et al., 2019), hence ensure continued efficacy of the treatment type for the protection of both wild and cultured fish (Sievers et al., 2019).

5 Conclusion

The proportion of egg strings that hatched was high for before-samples (97%) and decreased following two (82%) and four (41%) hours exposure time. Egg strings exposed to freshwater for six hours or more did not hatch at all. The hatching success (number of nauplii divided by the estimated number of eggs; %) was observed to decrease with increasing exposure time, from median 81% before treatment to 0% for egg strings exposed to freshwater for 4 hours. Similarly, the development to the copepodid stage showed that survival was generally high for egg strings sampled before treatment, and that the median copepodid survival was observed to decrease rapidly following two hours of freshwater exposure. Similarly, the mean number of active copepodids per egg string was observed to decrease as a result of freshwater exposure. Before treatment, each egg string produced an average of 238 ± 118 copepodids, which declined to 63 ± 58 and 67 ± 71 for two and four hours treated egg strings, respectively. The same pattern was observed when investigating the proportion of active nauplii that developed to active copepodids. Thus, the egg string hatching and larval development towards the copepodid stage were clearly affected by the freshwater exposure already from the first sampling point at two hours.

The bioassay results revealed differences in response to low salinity levels at the copepodid stage. For the Before treatment group, differences were observed both within and between the different locations. For treated egg strings, survival curves indicated decreased or similar tolerance to low salinities compared to the Before treatment group. The results observed for hatching success and copepodid survival could indicate a detrimental effect of the treatment which in turn resulted in decreased tolerance levels in copepodids hatched from treated egg strings.

Bioassays performed on F1-generation copepodids hatched from egg strings where F0-generation preadult and adult lice were exposed to freshwater for two and four hours indicated increased treatment tolerance. Compared with the Before-samples from the same farm site, EC_{50} decreased from 7.6‰ to 6.0‰ for copepodids from two hours treated preadult and adult lice to 6.5‰ for four hours treated copepodids. However, drawing strong conclusions was difficult due to limited replicates and low sample number, also considering that F1-generation copepodids where F0-generation had been treated did not manage to successfully infest salmon.

F2-generation lice from the Before treatment group were successfully reared and tolerance tested. The results for survival and treatment tolerance were very

similar compared to the F1-generation, which could indicate that tolerance to low salinity is inherited and observable between generations. However, alternative methods to study possible heritability of freshwater tolerance could be necessary due to the unsuccessful infection observed in this study.

No conclusion could be made regarding the possible increased tolerance to low salinities. However, the egg string hatching and larval development were clearly affected by the freshwater treatment, and the high mortality of treated preadult and adult lice suggested that a small number of older lice stages were capable of surviving treatment and producing new egg strings. Also, since the infection success of these treated preadult and adult lice were limited, infestation potential from freshwater treated lice is probably small.

References

Andrews, M., & Horsberg, T. E. (2020). Sensitivity towards low salinity determined by bioassay in the salmon louse, Lepeophtheirus salmonis (Copepoda: Caligidae). *Aquaculture*, *514*, 734511. https://doi.org/10.1016/j.aquaculture.2019.734511

Angilletta, M. J., Steury, T. D., & Sears, M. W. (2004). Temperature, Growth Rate, and Body Size in Ectotherms: Fitting Pieces of a Life-History Puzzle. *Integr. Comp. Biol*, *44*(6), 498-509. https://doi.org/10.1093/icb/44.6.498

Boxaspen, K. (2006). A review of the biology and genetics of sea lice. *ICES journal of marine science*, *63*(7), 1304-1316. https://doi.org/10.1016/j.icesjms.2006.04.017

Bricknell, I. R., Dalesman, S. J., O'Shea, B., Pert, C. C., & Luntz, A. J. M. (2006). Effect of environmental salinity on sea lice Lepeophtheirus salmonis settlement success. *Dis Aquat Organ*, *71*(3), 201-212. https://doi.org/10.3354/dao071201

Burridge, L., Weis, J. S., Cabello, F., Pizarro, J., & Bostick, K. (2010). Chemical use in salmon aquaculture: A review of current practices and possible environmental effects. *Aquaculture*, *306*(1), 7-23. https://doi.org/10.1016/j.aquaculture.2010.05.020

Connors, B. M., Juarez-Colunga, E., & Dill, L. M. (2008). Effects of varying salinities on Lepeophtheirus salmonis survival on juvenile pink and chum salmon. *Journal of fish biology*, *72*(7), 1825-1830. https://doi.org/10.1111/j.1095-8649.2008.01839.x

Costello, M. J. (2009). How sea lice from salmon farms may cause wild salmonid declines in Europe and North America and be a threat to fishes elsewhere. *Proc. R. Soc. B*, *276*(1672), 3385-3394. https://doi.org/10.1098/rspb.2009.0771

Dixon, B. A., Shinn, A. P., & Sommerville, C. (2004). Genetic characterization of populations of the ectoparasitic caligid, Lepeophtheirus salmonis (Kroyer 1837) using randomly amplified polymorphic DNA. *Aquaculture research*, *35*(8), 730-741. https://doi.org/10.1111/j.1365-2109.2004.01095.x

Eisenhauer, L., Solvang, T., Alver, M., Franklin Krause, D., & Hagemann, A. (2020). Dispersal of salmon lice (Lepeophtheirus salmonis Krøyer, 1837) egg strings from open-cage salmon farming: A neglected source for infestation dynamics. *Aquaculture research*, *51*(11), 4595-4601. https://doi.org/10.1111/are.14805

Espedal, P. G., Glover, K. A., Horsberg, T. E., & Nilsen, F. (2013). Emamectin benzoate resistance and fitness in laboratory reared salmon lice (Lepeophtheirus salmonis). *Aquaculture*, *416-417*, 111-118. https://doi.org/10.1016/j.aquaculture.2013.09.001

Finney, D. J. (1962). *Probit analysis : a statistical treatment of the sigmoid response curve* (2nd ed. ed.). University Press.

Forseth, T., Thorstad, E. B., & Fiske, P. (2020). *Status of wild Atlantic salmon in Norway 2020*. https://www.vitenskapsradet.no/Portals/vitenskapsradet/Pdf/Status%20of%20wild%20Atlantic%20salmon%20in%20Norway%202020T.pdf

Furberg, M. (2022). *Sammenlikning av mekanisk og termisk avlusing: Klekkesuksess og larveutvikling fra eggstrenger med vurdering av smittepotensial hos lakselus.* Norwegian University of Science and Technology].

Groner, M. L., Laurin, E., Stormoen, M., Sanchez, J., Fast, M. D., & Revie, C. W. (2019). Evaluating the potential for sea lice to evolve freshwater tolerance as a consequence of freshwater treatments in salmon aquaculture. *Aquaculture Environment Interactions*, *11*, 507-519. https://doi.org/10.3354/aei00324

Guragain, P., Båtnes, A. S., Zobolas, J., Olsen, Y., Bones, A. M., & Winge, P. (2022). IIb-RAD-sequencing coupled with random forest classification indicates regional population structuring and sex-specific differentiation in salmon lice (Lepeophtheirus salmonis). *Ecol Evol*, *12*(4), e8809-n/a. https://doi.org/10.1002/ece3.8809

Guragain, P., Tkachov, M., Båtnes, A. S., Olsen, Y., Winge, P., & Bones, A. M. (2021). Principles and Methods of Counteracting Harmful Salmon–Arthropod Interactions in Salmon Farming: Addressing Possibilities, Limitations, and Future Options. *Frontiers in Marine Science*, *8*. https://doi.org/10.3389/fmars.2021.701793

Gaasø, M. (2019). *Sea lice (Lepeophtheirus salmonis and Caligus elongatus) during freshwater treatment.* Norwegian University of Science and Technology]. https://ntnuopen.ntnu.no/ntnu-xmlui/handle/11250/2656633

Hahnenkamp, L., & Fyhn, H. J. (1985). The osmotic response of salmon louse, Lepeophtheirus salmonis (Copepoda: Caligidae), during the transition from sea water to fresh water. *Journal of comparative physiology. B, Biochemical, systemic, and environmental physiology*, *155*(3), 357-365. https://doi.org/10.1007/BF00687479

Hamre, L. A., Eichner, C., Caipang, C. M. A., Dalvin, S. T., Bron, J. E., Nilsen, F., Boxshall, G., & Skern-Mauritzen, R. (2013). The Salmon Louse Lepeophtheirus salmonis (Copepoda: Caligidae) Life Cycle Has Only Two Chalimus Stages. *PLoS One*, *8*(9), e73539-e73539. https://doi.org/10.1371/journal.pone.0073539

Hamre, L. A., Glover, K. A., & Nilsen, F. (2009). Establishment and characterisation of salmon louse (Lepeophtheirus salmonis (Krøyer 1837)) laboratory strains. *Parasitol Int*, *58*(4), 451-460. https://doi.org/10.1016/j.parint.2009.08.009

Helgesen, K. O., Horsberg, T. E., Stige, L. C., Norheim, K., & Tarpai, A. (2021). *The surveillance programme for resistance in salmon lice (Lepeoptheirus salmonis) in Norway 2020.* (Surveillance program report., Issue.

Helgesen, K. O., Horsberg, T. E., & Tarpai, A. (2020). *The surveillance programme for restistance in salmon lice (Lepeophtheirus salmonis) in Norway 2019.* (Surveillance program report., Issue.

Heuch, P. A., Nordhagen, J. R., & Schram, T. A. (2000). Egg production in the salmon louse [Lepeophtheirus salmonis (Krøyer)] in relation to origin and water temperature. *Aquaculture research*, *31*(11), 805-814. https://doi.org/10.1046/j.1365-2109.2000.00512.x

Igboeli, O. O., Burka, J. F., & Fast, M. D. (2014). Lepeophtheirus salmonis: A persisting challenge for salmon aquaculture. *Animal frontiers*, *4*(1), 22-32. https://doi.org/10.2527/af.2014-0004

International Salmon Farmers Association, I. (2018). *Salmon Farming - Sustaining communities and feeding the world.*

Iversen, A., Hermansen, Ø., Nystøyl, R., & Hess, E. J. (2017). *Kostnadsutvikling i lakseoppdrett - med fokus på fôr- og lusekostnader* (Nofimas rapportserie, Issue. https://nofima.no/publikasjon/1523319/

Ljungfeldt, L. E. R., Quintela, M., Besnier, F., Nilsen, F., & Glover, K. A. (2017). A pedigree-based experiment reveals variation in salinity and thermal tolerance in the salmon louse, Lepeophtheirus salmonis. *Evol Appl*, *10*(10), 1007-1019. https://doi.org/10.1111/eva.12505

Mordue, A. J., & Birkett, M. A. (2009). A review of host finding behaviour in the parasitic sea louse, Lepeophtheirus salmonis (Caligidae: Copepoda). *J Fish Dis*, *32*(1), 3-13. https://doi.org/10.1111/j.1365-2761.2008.01004.x

Nersten, M. (2021). *Fate of Lepeophtheirus salmonis and Caligus elongatus During Farmed Salmon Crowding* Norwegian University of Science and Technology]. https://ntnuopen.ntnu.no/ntnu-xmlui/handle/11250/2782504

Nolan, D. V., & Powell, R. (2008). Geographic and temporal genetic structure in Lepeophtheirus salmonis from four salmon farms along the northwest and west coasts of Ireland: results from a microsatellite analysis. *Hydrobiologia*, *617*(1), 55-63. https://doi.org/10.1007/s10750-008-9525-7

Norwegian Directorate of Fisheries. (2008). Forskrift om drift av akvakulturanlegg (akvakulturdriftsforskriften). https://lovdata.no/dokument/SF/forskrift/2008-06-17-822
Norwegian Directorate of Fisheries. (2013). Forskrift om bekjempelse av lakselus i akvakulturanlegg. https://lovdata.no/dokument/SF/forskrift/2012-12-05-1140
Norwegian Directorate of Fisheries. (2017). Forskrift om produksjonsområder for akvakultur av matfisk i sjø av laks, ørret og regnbueørret (produksjonsområdeforskriften). https://lovdata.no/dokument/SF/forskrift/2017-01-16-61?q=produksjon%20akvakultur
Norwegian Directorate Of Fisheries, N. (2020). *Foreløpig statestikk for akvakultur 2019*. https://www.fiskeridir.no/Akvakultur/Tall-og-analyse/Statistiske-publikasjoner/Statistikk-for-akvakultur
Norwegian Food Safety Authority, M. (2017). *Ny lakselusforskrift*. https://www.mattilsynet.no/fisk_og_akvakultur/fiskehelse/fiske_og_skjellsykdommer/lakselus/ny_lakselusforskrift.25694
Overton, K., Barrett, L. T., Oppedal, F., Kristiansen, T. S., & Dempster, T. (2020). Sea lice removal by cleaner fish in salmon aquaculture: a review of the evidence base. *Aquaculture Environment Interactions*, *12*, 31-44. https://doi.org/10.3354/aei00345
Overton, K., Dempster, T., Oppedal, F., Kristiansen, T. S., Gismervik, K., & Stien, L. H. (2019). Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review. *Reviews in aquaculture*, *11*(4), 1398-1417. https://doi.org/10.1111/raq.12299
Powell, M. D., Reynolds, P., & Kristensen, T. (2015). Freshwater treatment of amoebic gill disease and sea-lice in seawater salmon production: Considerations of water chemistry and fish welfare in Norway. *Aquaculture*, *448*, 18-28. https://doi.org/10.1016/j.aquaculture.2015.05.027
Rasband, W. S. (2018). ImageJ. https://imagej.nih.gov/ij/
Reynolds, P. (2015). *The use of freshwater to control infestations of the sea louse Lepeophtheirus salmonis K on Atlantic salmon Salmo salar L.*
Roth, B. (2016). *Avlusing av laksefisk med Optilice: Effekt på avlusing og fiskevelferd* (Nofima rapportserrie 59/2016, Issue. https://nofima.no/publikasjon/1408716/
RStudio. (2016). *RStudio: Integrated Development for R.* In (Version 1.1.456) RStudio. http://www.rstudio.com/
Samsing, F., Oppedal, F., Dalvin, S., Johnsen, I., Vågseth, T., & Dempster, T. (2016). Salmon lice (Lepeophtheirus salmonis) development times, body size, and reproductive outputs follow universal models of temperature dependence. *Canadian journal of fisheries and aquatic sciences*, *73*(12), 1841-1851. https://doi.org/10.1139/cjfas-2016-0050
Sievers, M., Oppedal, F., Ditria, E., & Wright, D. W. (2019). The effectiveness of hyposaline treatments against host-attached salmon lice. *Sci Rep*, *9*(1), 6976-6976. https://doi.org/10.1038/s41598-019-43533-8
Sommerset, I., Walde, C. S., Bang Jensen, B., Wiik-Nielsen, J., Bornø, G., Oliveira, V., Haukaas, A., & Brun, E. (2022). *Fiskehelserapporten 2021* (Veterinærinstituttes rapportserie, Issue. https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2022/fiskehelserapporten-2021
Stone, J., Boyd, S., Sommerville, C., & Rae, G. H. (2002). An evaluation of freshwater bath treatments for the control of sea lice, Lepeophtheirus salmonis (Krøyer), infections in Atlantic salmon, Salmo salar L. *Journal of fish diseases*, *25*(6), 371-373. https://doi.org/10.1046/j.1365-2761.2002.00370.x
Tjensvoll, K., Glover, K. A., & Nylund, A. (2006). Sequence variation in four mitochondrial genes of the salmon louse Lepeophtheirus salmonis. *Dis Aquat Organ*, *68*(3), 251-259. https://doi.org/10.3354/dao068251
Todd, C. D., Walker, A. M., Wolff, K., Northcott, S. J., Walker, A. F., Ritchie, M. G., Hoskins, R., Abbott, R. J., & Hazon, N. (1997). Genetic differentiation of populations of the copepod sea louse Lepeophtheirus salmonis (Krøyer) ectoparasitic on wild and farmed salmonids around the coasts of Scotland:

Evidence from RAPD markers. *Journal of experimental marine biology and ecology*, *210*(2), 251-274. https://doi.org/10.1016/S0022-0981(96)02696-2

Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O. T., Nilsen, F., Horsberg, T. E., & Jackson, D. (2013). Salmon lice - impact on wild salmonids and salmon aquaculture. *J Fish Dis*, *36*(3), 171-194. https://doi.org/10.1111/jfd.12061

Tucker, C. S., Sommerville, C., Wootten, R. (2000). *An Investigation into the Larval Energetics and Settlement of the Sea Louse, Lepeophtheirus salmonis, an Ectoparasitic Copepod of Atlantic salmon, Salmo salar.* (Fish Pathology, Issue.

Wright, D. W., Oppedal, F., & Dempster, T. (2016). Early-stage sea lice recruits on Atlantic salmon are freshwater sensitive. *J Fish Dis*, *39*(10), 1179-1186. https://doi.org/10.1111/jfd.12452

Appendix A

Table A1. Table showing length measurements (mm) for 30 eggs on 10 different egg string pairs. Four areas were counted per egg string.

		Egg string 1				Egg string 2			
	Counts	1	2	3	4	5	6	7	8
Egg string pair	Number of eggs	Length (mm)	Length (mm)	Length (mm)	Length (mm)	Length (mm)	Length (mm)	Length (mm)	Length (mm)
1	30	1,6	1,7	1,7	1,8	1,6	1,7	1,7	1,6
2	30	1,7	1,6	1,7	1,6	1,7	1,7	1,7	1,6
3	30	1,8	1,8	1,7	1,7	1,6	1,9	1,9	1,7
4	30	1,7	1,7	1,7	1,7	1,7	1,6	1,6	1,6
5	30	1,7	1,7	1,7	1,6	1,6	1,6	1,7	1,7
6	30	1,6	1,7	1,7	1,7	1,8	1,7	1,6	1,7
7	30	1,8	1,7	1,7	1,6	1,8	1,7	1,8	1,8
8	30	1,7	1,8	1,7	1,6	1,8	1,8	1,8	1,7
9	30	1,7	1,8	1,7	1,8	1,7	1,8	1,7	1,7
10	30	1,7	1,7	1,6	1,8	1,7	1,8	1,8	1,6

Table A2. Table showing egg size measurements from the 10 egg string pairs. The measurements were calculated to mm/egg and compared within and between egg strings. Mean egg size was found to be 56.9 µm/egg.

		Egg string 1				Egg string 2						
	Counts	1	2	3	4	5	6	7	8			
Egg string pair	Number of eggs	mm/egg	mm/egg	mm/egg	mm/egg	mm/egg	mm/egg	mm/egg	mm/egg	Mean	STD	Coefficent of variance (%)
1	30	0,0544	0,0564	0,0576	0,0585	0,0541	0,0578	0,0570	0,0534	0,0562	0,0019	3,4
2	30	0,0581	0,0544	0,0557	0,0542	0,0559	0,0561	0,0565	0,0542	0,0556	0,0014	2,4
3	30	0,0605	0,0584	0,0568	0,0557	0,0550	0,0618	0,0622	0,0583	0,0586	0,0027	4,6
4	30	0,0572	0,0554	0,0580	0,0576	0,0554	0,0540	0,0538	0,0525	0,0555	0,0020	3,6
5	30	0,0561	0,0581	0,0570	0,0533	0,0549	0,0532	0,0574	0,0561	0,0558	0,0018	3,3
6	30	0,0548	0,0559	0,0554	0,0575	0,0584	0,0565	0,0543	0,0571	0,0562	0,0014	2,5
7	30	0,0594	0,0556	0,0552	0,0546	0,0584	0,0574	0,0607	0,0599	0,0577	0,0023	4,0
8	30	0,0564	0,0612	0,0577	0,0549	0,0590	0,0591	0,0590	0,0557	0,0579	0,0021	3,6
9	30	0,0582	0,0598	0,0564	0,0599	0,0573	0,0608	0,0580	0,0561	0,0583	0,0017	2,9
10	30	0,0561	0,0561	0,0549	0,0608	0,0582	0,0598	0,0609	0,0546	0,0577	0,0026	4,5
									Total	0,0569	0,0022	3,9

Table A3. Table showing the difference in estimated number of eggs when calculated with the actual egg size for that egg string pair and mean egg size found in Table A2. The difference was between -2.8 to 2.6% for the egg string pairs examined.

Egg string pair	1	2	3	4	5	6	7	8	9	10
Actual egg size	352	598	687	747	744	587	434	471	531	600
Mean egg size (0,0569 mm/egg)	347	585	706	729	729	580	440	479	544	608
Difference (%)	1,44 %	2,22 %	-2,69 %	2,47 %	2,06 %	1,21 %	-1,36 %	-1,67 %	-2,39 %	-1,32 %

Appendix B

Table B1. Mann-Whitney U test, egg string length before treatment at different treatments.

Treatment	1.2	2.1	3.1
1.1	P = 0.309	P = 0.971	P = 0.277
1.2		P = 0.309	P = 0.059
2.1			P = 0.035

Table B2. Mann-Whitney U test, egg string length two hours during treatment at the different treatments.

Treatment	1.2	2.1	3.1
1.1	P = 0.468	P = 0.371	P = 0.864
1.2		P = 0.353	P = 0.211
2.1			P = 0.156

Table B3. Mann-Whitey U test, egg string length four hours during treatment at the different treatments.

Treatment	1.2	2.1	3.1
1.1	P = 0.284	P = 0.019	P = 0.442
1.2		P = 0.114	P = 0.792
2.1			P = 0.049

Table B4. Mann-Whitney U test, egg string length six hours during treatment at the different treatments.

Treatment	1.2	2.1
1.1	P = 0.929	P = 0.639
1.2		P = 0.661

Table B5. Mann-Whitney U test, egg string length after treatment at treatment 1.1 and 2.1.

Treatment	2.1
1.1	P = 1

Table B6. Mann-Whitney U test, egg string length from all treatments at different sampling times.

Sampling time	2h treatment	4h treatment	6h treatment	After
Before	P = 0.292	P = 0.639	P = 0.369	P = 0.745
2h treatment		P = 0.755	P = 0.252	P = 0.386
4h treatment			P = 0.261	P = 0.473
6h treatment				P = 0.971

Table B7. Mann-Whitney U test, hatching success before at all treatments.

Treatment	1.2	2.1	3.1
1.1	P = 0.439	P = 0.481	P = 0.035
1.2		P = 0.859	P = 0.029
2.1			P = 0.028

Table B8. Mann-Whitney U test, hatching success two hours during treatment at all treatments.

Treatment	1.2	2.1	3.1
1.1	P = 0.075	P = 0.074	P = 0.338
1.2		P = 0.684	P = 0.177
2.1			P = 0.085

Table B9. Mann-Whitney U test, hatching success before and during treatment at all treatments.

Treatment	1.1 2h	1.1 4h	1.2 2h	1.2 4h	2.1 2h	2.1 4h	3.1 2h	3.1 4h
1.1 before	P = 0.014	P = 0.016						
1.1 2h		P = 0.733						
1.2 before			P = 0.001	P = 0.002				
1.2 2h				P = 0.002				
2.1 before					P = 0.002	P = 0.002		
2.1 2h						P = 0.219		
3.1 before							P = 0.056	P = 0.001
3.1 2h								P = 0.035

Table B10. Mann-Whitney U test, hatching success for all treatments at different sampling times

Sampling time	2h treatment	4h treatment	6h treatment	After
Before	P = 5.828e-08	P = 3.948e-10	P = 1.473e-10	P =1.393e-06
2h treatment		P = 5.352e-4	P =3.527e-08	P = 4.228e-05
4h treatment			P = 5.492e-4	P = 0.014

Table B11. Mann-Whitney U test, copepodid survival before at all treatments.

Treatment	1.2	2.1	3.1
1.1	P = 0.083	P = 0.605	P = 0.114
1.2		P = 0.898	P = 0.011
2.1			P = 0.236

Table B12. Mann-Whitney U test, copepodid survival before and during treatment at all treatments.

Treatment	1.1 2h	1.1 4h	1.2 2h	1.2 4h	2.1 2h	2.1 4h	3.1 2h	3.1 4h
1.1 before	P = 0.2	P = 0.218						
1.1 2h		P = 0.667						
1.2 before			P = 0.001	P = 0.333				
1.2 2h				P = 0.6				
2.1 before					P = 0.003	P = 0.017		
2.1 2h						P = 0.529		
3.1 before							P = 0.059	P = 0.003
3.1 2h								P = 0.009

Table B13. Mann-Whitney U test, copepodid survival for all treatments at different sampling times.

Sampling time	2h treatment	4h treatment
Before	P = 7.220e-09	P = 7.827e-06
2h treatment		P = 0.346

Table B14. Mann-Whitney U test, number of copepodids from all treatments at different sampling times.

Sampling time	2h treatment	4h treatment
Before	P = 1.273e-07	P = 3.071e-05
2h treatment		P = 0.639

Table B15. Mann-Whitney U test, survival in percent from nauplii to the copepodid stage for all treatments at different sampling times

Sampling time	2h treatment	4h treatment
Before	P = 0.001	P = 0.0001
2h treatment		P = 0.111

Table B16. Two-sample paired test, survival curves before treatment.

Treatment	1.2	2.1	3.1
1.1	P = 0.003	P = 0.014	P = 0.006
1.2		P = 0.168	P = 0.025
2.1			P = 0.010

Table B17. Two-sample paired test, survival at all sampling times for different treatments.

Treatment	1.1 2h	1.1 4h	1.2 2h	1.2 4h	2.1 2h	2.1 4h	3.1 2h	3.1 4h
1.1 before	P = 0.167	NA						
1.1 2h		NA						
1.2 before			P = 0.001	P = 0.007				
1.2 2h				P = 0.001				
2.1 before					P = 0.748	P = 0.288		
2.1 2h						P = 0.276		
3.1 before							P = 0.007	P = 0.082
3.1 2h								P = 0.146

Table B18. Two-sample paired test, survival curves for copepodids from treated preadult and adult lice compared to before sample at Treatment 3.1.

Treatment	Tank 6 2h	Tank 8 4h
3.1 before	P = 0.046	P = 0.609
Tank 6 2h		P = 0.233

Table B19. Two-sample paired test, survival curves for F1- and F2-generation lice from Treatment 3.1.

Treatment	F2-generation
3.1 before	P = 0.820

Table B20. One-way ANOVA, median immobilizing concentrations (EC50) before treatment.

Treatment	1.2	2.1	3.1
1.1	P = 0.102	P = 0.373	P = 0.012
1.2		P = 0.182	P = 0.006
2.1			P = 0.017

Table B21. One-way ANOVA, median immobilizing concentrations (EC50) for all treatments and sampling times.

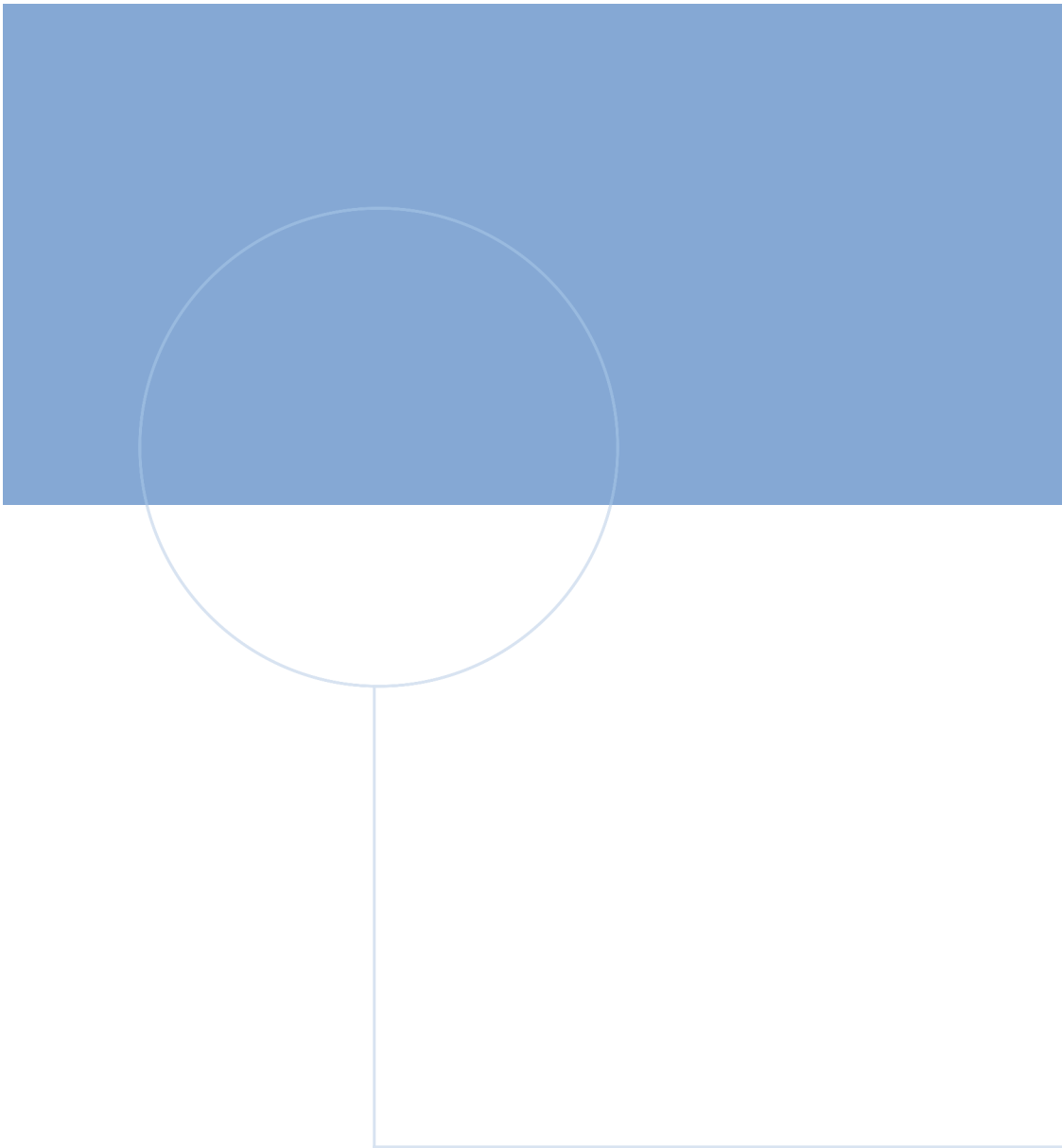
Treatment	1.1 2h	1.1 4h	1.2 2h	1.2 4h	2.1 2h	2.1 4h	3.1 2h	3.1 4h
1.1 before	P = 0.869	NA						
1.1 2h		NA						
1.2 before			NA	NA				
1.2 2h				NA				
2.1 before					P = 0.887	P = 0.912		
2.1 2h						P = 0.985		
3.1 before							P = 0.012	NA
3.1 2h								NA

Table B22. One-way ANOVA, median immobilizing concentrations (EC50) for untreated copepodids from Treatment 3.1 and copepodids from treated preadult and adult lice.

Treatment	Tank 6 2h	Tank 8 4h
3.1 before	P = 0.042	P = 0.013
Tank 6 2h		P = 0.461

Table B23. One-way ANOVA, median immobilizing concentrations (EC50) for F1- and F2-generation lice from Treatment 3.1.

Treatment	F2-generation
3.1 before	P = 0.141



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