Ultrasound as potential inhibitor of salmon louse infestation – a small-scale study

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Abstract: The effect of ultrasound exposure during controlled infection with salmon lice, Lepeophtheirus salmonis, has been studied. Salmon were placed in tanks with salmon lice copepodids for 1 hour, while simultaneously being exposed to sound frequencies of 9.3, 21 or 54 kHz. The sound transducers were operated at maximum power levels, and the 9.3 kHz transducer generated the highest sound level (220.6 dB). Only the group exposed to 9.3 kHz displayed a significant reduction in louse infestation. However, the observed effect of ultrasound was relatively small, and in a practical implementation in sea cages, the sound intensity will be lower than that used in the experiments. It is also possible that the observed reduction in infestation is due to ultrasonic cavitation effects, which are only present at a very short range from the ultrasound source. We therefore do not consider ultrasound a feasible method for preventing attachment of salmon lice copepodids on salmon in cage farms.

Keywords: salmon, salmon louse, Lepeophtheirus salmonis, copepodid, ultrasound

1 INTRODUCTION

The salmon louse, Lepeophtheirus salmonis, is a marine parasitic copepod in the Caligidae family. Its life cycle consists of 8 stages, of which three stages are free-swimming (two naupliar stages and a copepodid stage) followed by five stages where it lives parasitically on salmonids (Hamre et al., 2013; Johnson & Albright, 1991). A salmon louse lives off its host's mucus, skin and blood, creating lesions which cause osmoregulatory stress for the fish and can lead to secondary infections and higher risk of predatory mortality (Johnson & Albright, 1991; Poppe & Bergh, 1999). High infection levels can cause the death of the host fish (Skilbrei, Bjørn, & Vollset, 2015; Vollset et al., 2017). Salmon farms have the potential to produce large numbers of salmon lice that then infect wild salmonids and lead to unnaturally high levels of lice on these fish. This can reduce the number of fish returning to rivers to spawn (Krkošek et al., 2013). Salmon farms are now the largest source of salmon lice found on wild salmon in Norway (Fjørtoft et al., 2017). This is the reason that salmon lice from salmon farms and salmon escaped from salmon farms are now ranked as the most important threats to the wild salmon populations by the Norwegian scientific advisory board for salmon management (Thorsdad & Forseth, 2017). The Norwegian government has also stated that further development of aquaculture requires that the density of salmon lice is to be kept under strict control (Norwegian Ministry of Trade Industry and Fisheries, 2015). Thus, the salmon louse represents both an environmental and an economic problem for the aquaculture sector in Norway.

Several methods, both preventive and curative, are currently used for handling the salmon louse problem. Preventive methods include using feed that strengthens the salmon's immune system (Covello et al., 2012; Jensen, Provan, Larssen, Bron, & Obach, 2015), using tarpaulin skirts which prevent the louse from entering the cages (Næs, Grøntvedt, Kristoffersen, & Johansen, 2014), and using closed or semi-closed cages (Nilsen, Nielsen, Biering, & Bergheim, 2017). Curative methods include administering medicine through feed (Grant, 2002), chemical treatment of seawater (Burridge, Weis, Cabello, Pizarro, & Bostick, 2010), immersion of fish in fresh and/or heated water (Grøntvedt et al., 2015), high-pressure hosing (Gismervik, Nielsen, Lind, & Viljugrein, 2017) and using cleaner-fish

(Leclercq, Davie, & Migaud, 2014). However, all these methods have disadvantages such as high cost, decreased salmon welfare and growth, and negative effects on the surrounding environment.

Ultrasound insonification of fish cages has been proposed as a potential method for salmon louse control in fish cages, and there are already commercial products available based on this concept (H2O-Technics, 2018.; Prado, 2016; Sonic Norway, 2016). However, to the best of our knowledge, the efficacy of these systems has not yet been scientifically documented.

Using ultrasound has a lower cost than alternative methods, no direct handling of the fish is required, and there are few effects on the surrounding environment. Because there is practically no transmission of sound between the water and the air above water, the method is also safe with regard to human hearing. Note, however, that some marine mammals have very wide hearing ranges which extend into the ultrasonic frequency range (Slabbekoorn et al.,2010). Large-scale use of ultrasound in fish cages could potentially cause harm or noise-induced stress in such mammals. If ultrasound can be shown to be effective for salmon louse control while not being harmful to mammals in the vicinity, the method would be an attractive alternative to the louse control methods in use today. The main goal of the current study is to investigate whether ultrasound can affect the attachment of salmon louse copepodids on salmon in a controlled laboratory environment.

Ultrasound has been used as a method to prevent growth of unwanted organisms ("biofouling") in several different applications. At very high intensities, ultrasound induces cavitation bubbles in water, which collapse and create "micro-jets" and localized zones of high temperature and pressure (Brennen, 2005; Santos, Lodeiro, & Capelo-Martinez, 2009). Ultrasonic cavitation can be used to kill algae, bacteria and plankton (Holm et al., 2008), and has also been found to cause mortality in barnacle and zebra mussel larvae (S. F. Guo et al., 2011; Legg et al., 2015), which are relatively similar to salmon louse larvae. In a recent lab study, it was also found that exposing salmon lice to high levels of cavitation within a glass beaker resulted in 0 % survival after 20 and 60 s for lice in the naupliar and copepodid stages, respectively (Svendsen et al., 2017).

Cavitation is generally only achievable at a short distance from the ultrasound transducer, i.e. within a few centimeters from the transducer surface (Hallez et al., 2010; Moussatov, Granger, & Dubus, 2003). Directly exposing the salmon lice to cavitation effects is therefore not practically feasible – at least not in an open sea cage. However, studies have shown that moderate ultrasound intensities (194 dB re 1 μ Pa) can prevent barnacle larvae from attaching to a surface, without killing the larvae (S. Guo, Lee, Teo, & Khoo, 2012). During their host searching phase, the behavior of the salmon louse is similar to that of the barnacle larvae, in that they both probe a potential host surface before settling (Bron, Sommerville, Jones, & Rae, 1991). Ultrasound could potentially change the behavior of the salmon louse in this host searching phase, thereby avoiding infection of nearby salmon.

Ultrasound frequencies around 20 kHz have been used in a number of studies on biofouling (Legg et al., 2015), and 20 kHz is therefore taken as a reference value in the experiments described here. Three different frequencies were included in the experiment: 9.3 kHz, 21.0 kHz and 54.0 kHz. The 21 kHz frequency was chosen because it is close to the reference value, and 9.3 kHz and 54.0 kHz were included to study the effect of using frequencies above and below the reference. The exact frequencies were chosen based on the product portfolio of the transducer manufacturer. Note that the 9.3 kHz frequency is audible to humans and therefore not strictly in the ultrasound frequency range. However, the two other frequencies are in the ultrasound range, and in this work, we will use ultrasound as a shorthand for all frequencies tested.

Salmon have a hearing frequency range which only extends up to approximately 580 Hz, and ultrasound is therefore not expected to induce any hearing damage or changes in behavior. However, very high-intensity sounds may induce physical damage (Popper, 2008). In this work, care has been taken to keep the fish some distance away from the transducer to avoid cavitation damage or other adverse effects.

2 METHODS AND MATERIALS

The experiments were performed at the Aquaculture Research Station in Tromsø (Tromsø, Norway), using salmon and salmon lice copepodids, both produced locally at the station. The salmon had an average weight of 183±39g (SD) at the start of the experiments, and 20 salmon were used in each experimental group. Five experiments were performed in total: two control experiments (no ultrasound) and one experiment for each of the three ultrasound frequencies used. The experiments were approved by the Norwegian Committee on Ethics in Animal Experimentation (id 10520).

The main purpose of the experiments was to give an indication of the potential for ultrasound as a method for control of salmon lice infection. The resources for the project (mainly funding and available time and equipment at the research station) were limited, and the experiment design was therefore relatively simple, with no replicates. Also, rather than studying the effect of ultrasound at equal sound levels for different frequencies, each individual transducer was run at its maximum sound level. The rationale for this was the following: The goal is to establish whether ultrasound treatment has any significant effect on salmon louse infection, for any combination of frequency and sound level. A potential effect is assumed to be stronger at higher sound levels. The experiment should therefore be performed with as high a sound level as possible for each transducer, to increase the chance of achieving significant results, rather than performing the experiments at an equal level determined by the least powerful transducer. If a significant effect is observed, additional experiments can be performed at a later stage to study the effect of frequency and sound level in more detail.

Given that no replicates were used and that the sound levels were not equal, the experiments presented here should be considered preliminary in nature.



Figure 1: Experimental setup for controlled infection and ultrasound insonification. a) Schematic drawing of tank with wire mesh cylinder, transducer on top and six-sided sound reflector on bottom. Dimensions are shown in mm. b) Image of experimental setup. The transducer is seen in the upper left corner. The probe with black wire in the upper right corner is the hydrophone used for measuring sound levels.

2.1 CONTROLLED INFECTION IN TANKS

The salmon were placed in tanks containing 500 liters of water, as shown in Figure 1a). A wire mesh cylinder with a sound reflector was placed in the middle of the tank, and the transducer was placed in the middle of the cylinder, partially submerged in water and pointing downwards. The wire mesh cylinder was included to keep fish from potentially harmful cavitation effects close to the transducer. In the control experiments, the same cylinder was used, but without the ultrasound transducer. The sound reflector was made from steel plates and designed as a six-sided "pyramid". The purpose of the reflector was to reflect the sound outwards towards the side of the tank, to create a relatively uniform sound field.

Infection of the salmon was performed using a challenge model developed together with the Aquaculture Research Station in Tromsø. This is a bath challenge model, where the fish is challenged with a known number of lice copepodids in stagnant seawater. The research station routinely produces infective copepodids by performing controlled infections, harvesting mature eggs and incubating these until they reach the copepodid stage. The experiments were performed in the salmon louse laboratory at the research station, where factors such as water temperature are closely monitored and controlled. The water outlet from the laboratory is disinfected to prevent distribution of live copepodids to the environment.

The number of copepodids used in the experiments was quantified by stirring the container with copepodids, extracting three samples that each contained 10 ml of water, transferring these to a Petri dish and using a magnifier to count the number of copepodids. The mean value for three samples was used to estimate the density used in the challenge. Based on experience from previous preliminary experiments, the number of copepodids per experiment was set to 1200, corresponding to 2.4 per liter of water. The same production batch of copepodids was used for all the experiments.

The copepodids were added to the tank before the fish and distributed by manually stirring the water. 20 fish for each experimental group were transferred from a larger tank to the experimental tank. In the groups with ultrasound exposure, the ultrasound was turned on immediately before adding the fish. During the experiment, the oxygen saturation of the experimental tank was kept between 90 and 100 %. The salmon were kept in the tank for 1 hour.

Some differences in fish behavior were observed between the control groups and the groups exposed to ultrasound. In the control groups, the experimenters perceived the fish as relatively inactive, settling on the bottom of the tank approximately 15 minutes after the experiment started. Every few minutes the experimenters used a wooden plank to stir the water and make the fish move, to ensure a more realistic behavior and continuous exposure to lice. In the groups exposed to ultrasound, the fish were perceived as more active, continuously moving around the tub. These differences suggest that even though the fish could not hear the sound, they experienced some kind of physical effect. Note, however, that the level of activity was not objectively measured.

After the controlled infection, the fish were netted and transferred to separate tanks (one tank per experimental group). The fish were kept in the tanks for 13 days, at which time the salmon lice had reached the chalimus 2 stage where it is stationary and easily seen by visual inspection. The salmon was then euthanized by using an overdose of Benzoak (80 mg benzokain per liter of water). The fish were transferred to white examination trays, and all visible lice were removed from the fish and counted. A picture from the counting process is shown in Figure 2.



Figure 2: Salmon lice being picked from the fish and counted.



Figure 3: Ultrasound transducers used in the experiments. From left: BII-7509 (9,3 kHz), BII-7502 (21 kHz), BII-7506 (54 kHz).

2.2 ULTRASOUND INSONIFICATION AND MEASUREMENT

Transducers with center frequencies of 8.25, 20.00 and 59.45 kHz were purchased from Benthowave Instrument, Inc., Ontario, Canada. The transducers are shown in Figure 3. Each transducer included an integrated impedance matching and tuning network, yielding an input impedance close to 50 Ohm around the transducer center frequency. The key transducer parameters are listed in Table 1.

The transducers were driven by a continuous sine signal, generated by a Tektronix AFG2021 signal generator, and amplified by an E&I 1040L power amplifier. The amount of forward and reflected power to/from the transducer could be monitored though a numeric display on the amplifier. The driving frequency was chosen by sweeping the frequency in a range close to the center frequency, and choosing the frequency with the least amount of reflected power. The driving frequency for each transducer is listed in Table 2, together with the forward and reflected power. The forward power was set to 50 % or less than the maximum (instantaneous) value given by the manufacturer, to minimize the risk of damaging the transducer during long-term operation. Note that the amount of reflected power would generally increase during each 1-hour experiment, probably due to heat buildup and

subsequent de-tuning of the impedance matching network in the transducer. The reflected power is therefore listed as a range.

The sound levels in the tank were measured using a Reson TC4013 hydrophone, a Reson EC6067 conditioning charge amplifier, and a Tektronix TBS1052B oscilloscope. The hydrophone sensitivity was -213 re $1V/\mu$ Pa for the frequency range of the hydrophones, and the input capacitance of the amplifier was adjusted to yield 0.8 dB gain, resulting in a total sensitivity of -212.2 dB re $1V/\mu$ Pa. The hydrophone was not calibrated, but the sensitivity is expected to be within a few dB of the general value listed by the manufacturer.

For each transducer, the sound pressure was measured without any fish in the tank, at 12 different measurement positions, as indicated in Figure 4. The depth of the hydrophone relative to the water surface was approximately 300 mm. The hydrophone signal levels were measured as root-mean-square (RMS) values on the oscilloscope. Because the sound levels were strongly fluctuating, the recorded RMS values were calculated as mean values based on five sample recordings (recorded by "stopping" the oscilloscope). The mean and standard deviation for the sound pressure are given in Table 2. Note that because of the logarithmic nature of the dB scale, the positive and negative standard deviations correspond to different dB values. The sound level distribution measured for the different frequencies is shown in Figure 5. The sound levels for 9.3 kHz are relatively uniform around the tank, while the 21 and 54 kHz distributions show somewhat more variation. The 21 kHz distribution also has two peaks and valleys which are relatively symmetrically located, indicating the presence of a dominant acoustic mode. The shape of such a mode is a function of frequency, transducer placement and the shape of the tank, and the mode pattern could for example be caused by imperfect centering of the transducer. However, the sound levels even at the lowest points are still relatively high, indicating that all parts of the tank were exposed to high intensities of ultrasound.

Each of the transducers were tested for cavitation effects by placing a piece of aluminium foil in front of the transducer, at a few centimeters distance. In cases where the sound intensity is sufficiently high to induce cavitation, the effects of the imploding cavitation bubbles will erode the aluminium foil, creating small holes and dents (Zeqiri, Hodnett, & Carroll, 2006). While operating at the power levels given in Table 2, such effects were observed for the 9.3 and 21 kHz transducers, but not the 54 kHz transducer. An example of these effects is shown in Figure 6.

Transducer model	Center frequency [kHz]	Max. electrical input power [W]	Bandwidth [-3 dB]	Diameter and height [mm]	Weight[kg]
BII-7509	8,25	180	2,0	88 x 200	2,35
BII-7502	20,00	120	4,0	100 x 170	2,10
BII-7506	59,45	50	25	48 x 120	0,60

Table 1: Transducer specifications

Table 2: Transducer operating parameters and sound pressure levels.

Transducer model	Drive frequency [kHz]	Forward power [W]	Reflected power [W]	Sound pressure [kPa, mean ±std.dev.]	Sound pressure [dB re 1V/μPa, mean ±std.dev.]
BII-7509	9,30	60	11-14	107 ± 19	$220,6^{+1.4}_{-1.7}$
BII-7502	21,00	60	5-8	77 ± 29	$217,7^{+2.7}_{-4.0}$
BII-7506	54,00	25	2-5	46 ± 16	$213,2^{+2.7}_{-3.9}$



Figure 4: Sound pressure measurement positions, indicated by numbers 1-12.



Figure 5 Sound level distribution in tank.



Figure 6: Aluminium foil performated by ultrasonic cavitation effects.

2.3 STATISTICAL ANALYSIS OF LOUSE COUNTING RESULTS

The purpose of the controlled infection experiments was to test the following hypotheses:

- H₀: Exposure to ultrasound during controlled salmon louse infection does not affect the number of lice infections per fish.
- H₁: Exposure to ultrasound during controlled salmon louse infection causes the number of lice infections per fish to change, i.e. either to increase or decrease.

H₀ is rejected only if the number of lice in groups exposed to ultrasound is significantly different from the number of lice in control groups without ultrasound exposure (a two-tailed significance test).

If the number of lice per salmon is normally distributed within each group, the hypotheses can be tested using Student's t-test (Walpole, Myers, Myers, & Ye, 2002). Because this is a very commonly used test, it is also included in this study. However, the experimental results indicated that the number of lice may not be normally distributed. An example of this is given in Figure 7, showing a histogram of the number of lice per salmon for the two control groups combined. The distribution is highest around 5-10 lice, but also has a long tail, with up to 30 lice for some fish.

In this study we have included a Wilcoxons rank-sum test (Krzywinski & Altman, 2014; Wilcoxon, 1945), abbreviated WRS-test, as an alternative to the Student's t-test for hypothesis testing. The test belongs to a set of statistical methods called non-parametric statistics, which are useful in cases where the exact probability distribution is unknown. The WRS test has the advantage that no requirements are placed on the statistical distribution. However, it is slightly less sensitive to significant differences than the Student's t-test. The hypothesis testing was done using standard 95 % significance levels for both the t-test and the WRS-test.

The results for the two control groups were relatively similar, indicating consistency between the controls. To formally test for consistency, the t- and WRS-tests were applied to the control groups with the null hypothesis that the mean (t-test) or median (WRS-test) of the two groups are equal. The hypothesis testing yielded p-values of 0.570 and 0.957 for the t- and WRS-test respectively, and the null hypothesis was therefore not rejected (the controls were considered consistent). In the hypothesis testing regarding ultrasound treatment, the two control groups were combined into a single group.



Figure 7: Histogram of number of lice per salmon for control groups 1 and 2.

3 RESULTS

The louse counting results are presented in Figure 8, in a box and whisker plot. The boxes indicate the quartiles of the data set, and the whiskers indicate the maximum and minimum values. Two data points were considered outliers and were not included in the significance tests. These are indicated as separate points in the box-and-whisker plot.

There are large variations within each of the groups. For the control groups, the number of lice are within the range of 1-30 per fish. For the 54 kHz group, the louse count distribution appears similar to that of the control groups, with a louse count range of 3-27. For the 9.3 and 21 kHz groups, the distribution is shifted towards somewhat lower levels, with ranges of 2-15 and 1-16 lice per fish, respectively. For the ultrasound groups, the median value (Q2) seems to indicate a trend: The number of lice increases with the frequency used. Note, however, that the sound intensity was also highest at 9.3 kHz and lowest at 54 kHz.

The results of hypothesis testing are shown in Table 3. Both tests indicate that only the group exposed to 9.3 kHz is significantly different from the control (p=0.0037 and p=0.0052 for the t-test and WRS-test, respectively).



Table 3: Hypothesis testing results. Note that the outliers indicated in Figure 8 were not included in the tests. P-values for each test are indicated in parantheses.

Figure 8: Louse count statistics for all experimental groups. The boxes indicate the ranges between the first and second quartiles (red) and the second and third quartiles (green) of the data set. The whiskers indicate the maximum and minimum values. Two data points were considered outliers and are indicated as separate points (blue).

4 **DISCUSSION**

In this study, fish were exposed to ultrasound in a very limited space with a relatively high degree of sound reflection from the outer walls. As a result, the sound field is heavily influenced by reflected sound waves, leading to interference effects with strong spatial and temporal variations in sound intensity. If the transducer was placed in free space, e.g. in a fish cage on the open sea, the sound intensity would decrease monotonically with the distance to the transducer, and remain relatively constant with regard to time. Thus, the sound field generated in this study differs from what would be expected in a practical application. However, since the sound level used here is generally very high, we assume that the sound level in all parts of the water volume is equal to or exceeding that of a practical implementation in open water.

The results show that there is high variability in the number of louse infections for fish exposed to the same infection conditions. Some of the variability can probably be attributed to varying ability of the individual fish to resist infection (Gjerde, Ødegård, & Thorland, 2011), and some can be attributed to the random nature of the infection process.

Based on experience from previous salmon louse studies we know that netting of fish may cause salmon lice to fall off, causing inaccuracies in the count statistics. In this study, the fish were netted while the lice were in the chalimus 2 stage, when they are still stationary and relatively firmly attached to the fish. The netting operation was also performed in the same way for all experimental groups. The effect of netting is therefore assumed to be negligible for the results in this study.

The fish exposed to ultrasound were perceived to be more active than the fish in the control groups, even though the frequencies used were outside the hearing range of the fish. This could be an indication that high-intensity ultrasound can cause elevated stress levels, and thus reduce welfare and growth rate. Such possible effects should be taken into account in an eventual large-scale implementation of the method.

Among the groups exposed to ultrasound, only the 9.3 kHz group had a significantly lower number of infections than the control groups. One possible explanation for this is that low-frequency sound at high intensity changes the copepodid behavior during the infection stage, while higher frequency sound at slightly lower intensity does not. Another possible explanation for the lower values for the 9.3 kHz group is that the copopodids of this group were more affected by cavitation. Svendsen et al. (2017) have previously demonstrated that cavitation can kill salmon lice copepodids in seconds. The effects of cavitation are stronger at low frequencies because cavitation bubbles are more easily formed at these frequencies (Santos et al., 2009). The sound level was also higher at 9.3 kHz than at the other frequencies. While the fish were prevented from swimming into the cavitation zone near the transducer by the wire mesh, the copepodids could float freely around the whole tank, including the cavitation zone. If a number of copepodids were killed by exposure to collapsing cavitation bubbles, this would result in a reduced infection pressure and thus a lower number of infestations. If this is the case, then the observed results are due to a different effect than what the experiments were originally designed to study, namely if ultrasound can change the copepodid behavior. In an eventual follow-up experiment, the experiment design should be updated to avoid exposure of the copepodids to cavitation effects.

The results show a partial reduction in louse infection for 9.3 kHz sound at very high sound levels. In a practical implementation with lower sound levels, the effect of using ultrasound will probably also be less than that observed here. Additionally, it is possible that some of this observed reduction in infection is due to cavitation, as discussed above. If this is the case, the overall effect of ultrasound on lice infection outside the immediate vicinity of the transducer (> 1 m) may be very small.

In this study the salmon lice were exposed to ultrasound only during the short period while attaching to salmon. But if the lice were exposed to ultrasound for a longer time, for example throughout the entire period when they are attached to the salmon, they would receive a dose of ultrasound (intensity multiplied by exposure time) significantly higher than that employed in this study. The results of the present study do not rule out the possibility that long-time exposure could affect the development of the lice and reduce the ability of the lice to reproduce. Experiments with Chilean sea lice (*Calicus* rogercressyi) have indicated that nauplia of this species can be damaged by 20 kHz ultrasound, while lice in the life stages when they are attached to salmon are unaffected (Prado, 2016). Note that these results have so far not been fully documented, e.g. by publication in a peer-reviewed journal. However, if the same effect also applies to *L. salmonis* it implies that continuous ultrasound may reduce the release of infective salmon lice from salmon farms (and thus reducing overall infection pressure in the vicinity), without directly affecting the attachment of copepodids on the salmon.

5 CONCLUSION

The main purpose of this study was to investigate whether ultrasound can affect salmon louse copepodids during the host attachment process. The results show a significant reduction in number of attached lice for the groups exposed to 9.3 kHz ultrasound at 220.6 dB. The observed effect could be due to changes in copepodid behavior, but also possibly due to copepodid mortality caused by ultrasonic cavitation. The experimental design does not enable a separation of these two effects. Ultrasonic cavitation can kill salmon louse copepodids within seconds, but this effect is limited to small water volumes close to the transducer, and is therefore not practically applicable for large water volumes such as salmon cages. If the ultrasound causes a change in the copepodid behavior, the effective range of the effect will extend further than that of cavitation. However, in a practical implementation it will not be possible to achieve the same ultrasound intensity as that of this study, due to physical limitations and possibly also concerns regarding underwater noise pollution. At decreased sound intensities, the relatively small effects observed in this study are expected to become smaller. Based on the current results we therefore do not consider ultrasound a viable large-scale method for preventing attachment of salmon copepodids on salmon in cage farms.

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