




Article

Acoustic Delicing of Atlantic Salmon (*Salmo salar*): Fish Welfare and Salmon Lice (*Lepeophtheirus salmonis*) Dynamics

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Abstract: Acoustic lice treatment (AcuLice) is a newly developed system, which uses a composite acoustic sound image with low-frequency sound to remove salmon lice (*Lepeophtheirus salmonis*) from Atlantic salmon (*Salmo salar*). This field study documents the stress effects on Atlantic salmon and the effect on salmon lice dynamics during large-scale use of the AcuLice system. The effect of the AcuLice treatment on salmon lice dynamics was measured by weekly salmon lice counting at the facilities from mid-summer 2019 to late-spring 2020. The number of salmon lice treatments in the same period was also compared to a reference group. In addition, the number of weeks until the first salmon lice treatment (mechanical treatment) was compared between the two groups. Apart from a slight increase in plasma glucose, no significant differences were observed in the primary, secondary, or tertiary stress responses measured. For the mature female salmon lice, a significantly lower number (mean \pm SEM) was shown for the AcuLice group (0.24 ± 0.03) compared to the reference group (0.44 ± 0.04). In addition, a lower number (mean \pm SEM) of salmon lice treatments and a longer production period before the first salmon lice treatment occurred was observed at the AcuLice facilities (33.2 ± 3 weeks) compared to the reference facilities (20.3 ± 2 weeks). These data suggest that the use of the AcuLice system reduces the need for traditional salmon lice treatments with no added stress to the fish.

Keywords: Atlantic salmon; acoustic delicing; salmon lice; fish welfare; sustainable aquaculture



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1. Introduction

The biggest pathogenic threat to Atlantic salmon (*Salmo salar* L.) aquaculture is the salmon louse (*Lepeophtheirus salmonis*) [1,2]. Salmon lice feed on blood, skin, and mucus from the salmonid, with effects ranging from mild skin damage to more serious wounds and even death [3,4]. Furthermore, infestation can lead to several negative factors such as a decreased growth rate, appetite, and feed conversion efficiency [3,5]. Salmon lice also have a negative impact on wild salmonids, i.e., contamination through escapees [6,7]. In addition, the parasite constitutes a huge economic cost for the aquaculture industry due to treatment and preventative efforts, costing the Norwegian salmon farming industry approximately Norwegian kroner (NOK) 5 billion in 2016 [8], which corresponds to about 9% of farm revenues [9]. The increasing salmon lice pressure has also led to the parasite becoming a decisive factor when it comes granting new aquaculture concessions and has affected the reputation of the aquaculture industry [10–12].

The use of bioacoustic methods to address sea lice infestation in salmonid farming is a promising innovative method [13,14]. Sole et. (2021) repeatedly exposed Atlantic salmon to 350 and 500 Hz tones in 3–4 h exposure sessions, reaching received sound pressure levels of 140 to 150 dB. The gross pathology and histopathological analysis performed

on the exposed salmon's organs did not reveal any lesions that could be associated to sound exposure. Further, [14] found that *L. salmonis* is sensitive to low-frequency sounds. Specifically, this study found that the central nervous system in all stages and the A/B cells (responsible for the secretion of the precursor of frontal filament) in the copepodit and chalimus stages of *L. salmonis* were affected by sound exposure, leading to a reduction in the capacity of the sea lice to infest its host [14]. Based on the desire to develop new and effective methods that have high efficiency, low costs, and minimal negative effects on fish while avoiding wastage, labor-intensive operations, and negative effects on the environment, a treatment called acoustic lice treatment (AcuLice) has been developed. AcuLice is a method for preventing the spread of salmon lice with the use of a complex acoustic sound image, which produces and sends out constant low-frequency sound to the water masses. The system emits sound waves and sound occurs when water molecules are set in motion and pressed closer together so that the pressure increases [15]. The sound levels generated by an acoustic source propagate in the water mass and are attenuated with distance. In water, the velocity is five times higher than in air but varies through the water column depending on the water temperature, salinity, and hydrostatic pressure. Sound is also deflected towards depths where the sound speed is lowest and thus leads to the formation of sound channels. In water, high-frequency energy is absorbed quickly while low-frequency energy is almost not absorbed. This means that 99% of the energy in a sound pulse of 100 kHz is absorbed after approximately 10 m while the corresponding distance for a sound pulse of 100 Hz is 10 km. The use of acoustic methods for delousing salmon is a good alternative in comparison with other delousing methods such as mechanical, thermal, or chemical ones as it is a sustainable, non-invasive alternative.

A previous study has shown that salmon lice react with 'aggressive behavior' if they are exposed to low-frequency sound in the frequency range 1–5 Hz [16]. This study showed that the copepodites increased their swimming activity in this frequency range and at a frequency of 3 Hz, the highest activity was observed. The frequency area of 1–5 Hz is the same as that produced by salmon when swimming. Hydrodynamic signals have been observed to be one of the factors salmon lice use to select the right host fish to infect. The AcuLice system is thought to disturb the salmon lice so that it enters a state of dormancy and ultimately dies. In a previous pilot study [17], a positive effect from the use of AcuLice regarding the removal of salmon lice over time was observed and no welfare challenges were shown in the measurements of stress, fin condition, and growth when exposed to the AcuLice treatment. Based on the positive results in the previous pilot study, it was decided to conduct a large-scale study to follow the effects in an ordinary production situation with a focus on fish welfare and health, and the effect on salmon lice.

A high density of salmon lice, including salmon lice treatments, diseases, and noise are some factors that can cause increased levels of stress in teleosts [6,18–20]. Due to the magnitude and duration of exposure to a stressor, the stress response can be divided into primary, secondary, and tertiary stress responses [21,22]. To determine whether a fish has been exposed to a stressor, the changes that occur during a possible impact can be measured. The primary response can be measured by analyzing the concentration of cortisol in blood. Since the secretion of catecholamines occurs faster than for cortisol [23], and the biological half-life of adrenaline and noradrenaline is as short as 10 min in fish blood [22], catecholamines are not a useful indicator of the primary stress response. To determine the secondary stress response, measurements of the plasma concentration of calcium, chloride, glucose, lactate, and magnesium are performed. The stress-induced homeostasis caused by the primary and secondary stress response will usually decrease to almost normal values in a chronic state of stress [24,25]. This makes measurements of these parameters challenging in the context of detecting a tertiary stress response. However, the tertiary stress response can be analyzed by measuring, for example, survival and the specific growth rate. Chronic stress occurs if the stress response is activated repeatedly or is persistent.

This study aimed to map the effect of AcuLice on the welfare, stress, and health situation of salmon in intensive farming and document the possible effect of AcuLice on the risk of infestation of salmon lice in farmed salmon from a commercial perspective.

2. Materials and Methods

2.1. Field Trial 1—Acute Stress Effects of AcuLice Treatment

2.1.1. Fish Material and Rearing Conditions

The Atlantic salmon ($N = 60$) used in field trial 1 originated from the Salmobreed strain and were reared from hatching to smolt at a recycling facility operated by Hardingsmolt AS in Tørvikbygd, Kvam, Norway. After hatching, the juveniles were fed a standard dry diet (Ewos, Skretting, Norway) in circular fiberglass tanks (rearing volume 5–50 m³) with constant light and in heated water (approximately 12–14 °C). Later (at size 6–8 g), they were transferred from the start-feeding tanks to grow-out recirculated aquaculture system (RAS) tanks (8–12 m, circular, fiberglass, volume 90–150 m³). Following transfer, the fish were reared with constant light and further fed a standard dry diet according to the temperature and fish size [26]. All groups were vaccinated at a size of 40–60 g and then transferred to new (grow-larger-out tanks) 12–15 m tanks (circular, fiberglass, volume 150–350 m³), where they were supplied with environment-temperature freshwater and reared as described above. The oxygen content in the outlet water was measured regularly and kept above 80%. A traditional photoperiod regime was conducted to stimulate parr smolt transformation [27]. After completion of parr-smolt transformation, the fish were reared for seven weeks in a semi-closed system at Koløy, Fitjar (GreenBag). When the fish reached approximately 500 g, the group was transferred to open sea cages (160 m and volume of 37,000 m³) at Brattavika, Norway (60.044° N, 5.303° E).

2.1.2. AcuLice Installation Process

The installation process for the AcuLice treatment was performed in collaboration with the equipment supplier. This involves connecting the speaker, usually in the center of the site (depth of 10–20 m), placing the processor, and connecting the component to the internet (Figure 1). The exact placement was calculated and based on the local acoustic surroundings, with the goal of minimizing the interference. During this phase, a complete requirement specification for maintenance and operation was also prepared. The system was continuously monitored electronically. Locally, at the site, there are a number of sensors that are connected to local electronic equipment that transmit via internet information to the central computer that is placed in the central data center. Once the AcuLice system was installed, it could be turned on by the equipment supplier whenever it was desired.

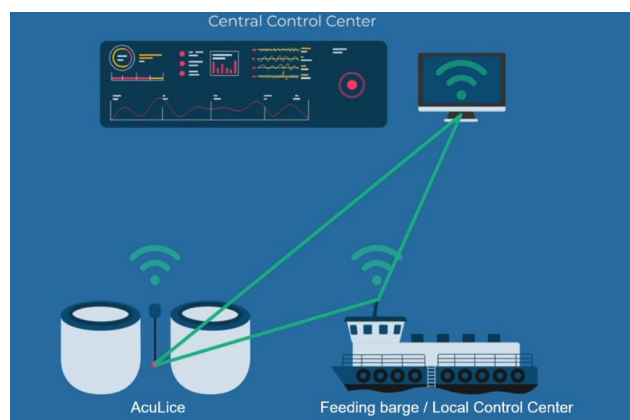


Figure 1. Set up of the AcuLice system. The AcuLice system consists of a central control room with monitoring, an electronic processor located at the facility, and a component in the sea (that sends out low-frequency sound waves). The whole system is connected to the internet.

There was a separate computer for each locality in the central data center, and based on algorithms, it continuously calculated local-specific data, for which sound images were generated at the respective location. AcuLice-source was hard wired to electronic equipment placed on a local feeding barge.

2.1.3. Experimental Design

Field trial 1 was carried out on 24 October 2019 and included a control sample from two sea cages and a treatment sample from two sea cages. The control sample (sample 1) took place prior to the start of the AcuLice treatment, and the treated group (sample 2) was carried out one hour after the start of the AcuLice. During the start of the AcuLice treatment, the farmed salmon were monitored using an underwater camera, and no changes in the fish's behavior were observed. All measurements and plasma collections were performed at the feed barge at the facility. To get to and from the edge of the sea cage, a boat was used. Daily feeding started at the same time as the AcuLice treatment.

Pellets were used to attract the fish, before the fish were captured using a hand net. Then, the fish was humanely euthanized with an anesthetic overdose of Benzocaine (Benzoak vet.® 20%, ACD Pharma AS, Leknes, Norway). The blood was collected within 1–3 min to limit the effects of stress. The blood (2 mL) was taken from the caudal vein using heparinized syringes with 21G needles. The plasma was separated from blood cells by centrifugation (4 min at 5000 rpm). The fish's size (weight (g) and length (cm)) were then measured to the nearest 0.1 g and 0.1 cm. When the first subset with N = 10 was completed, the same procedure was followed for the two next subsets: subset number 2 and 3 (i.e., only 10 fish netted at each time to minimize the stress). When all the three subsets in sample 1 were completed, the AcuLice was turned on, and sample 2 was performed one hour after. The same sampling protocol was used for all three subsets.

2.1.4. Analysis of Plasma Cortisol Concentration

Cortisol quantification from plasma was carried out using competitive ELISA (DEMEDITEC Cortisol ELISA Kit, Demeditec Diagnostics GmbH, DEH3388, Kiel, Germany) following the manufacturer's protocol. The plasma samples were analyzed in triplicates (10 µL) in a 96-well microplate. Every plate included two internal control samples and standards of a known concentration. The sample (10 µL), control, or calibrator were dispensed into each well. Enzyme conjugate (200 µL) was dispensed using a multi-channel pipette. With the help of known concentration standards, cortisol concentrations were calculated using a 4 Parameters Marquardt logistic regression with an extrapolation factor of 1 in the SparkControl Magellan v2.2.10 software.

2.1.5. Analysis of Plasma Chloride, Calcium, Magnesium, Glucose, and Lactate

The concentration of chloride in the plasma samples was measured by potentiometry using the Pentra c400 clinical chemistry analyzer with the Ion-Selective Electrode (ISE) module (HORIBA, Kyoto Prefecture, Kyoto, Japan). Calibration of the ISE module was carried out using the ABX Pentra Standard 1, ABX Pentra Standard 2, and ABX Pentra. The samples were measured using a specific electrode. Chloride in the sample induced a change in the potential difference across the electrode membrane, which was then compared with the reference electrode.

The measurements of glucose, lactate, calcium, and magnesium were analyzed using a Pentra c400 by colorimetric spectrophotometry determination. Each required reagent was calibrated using the ABX Pentra Multical and quality control was performed using ABX Pentra P and N controls, as stated in the manufacturer's protocol.

2.2. Field Trial 2: Effect of AcuLice Treatment under Commercial Conditions

2.2.1. Fish Material and Rearing Conditions

As a result of different companies being involved in field trial 2, the fish at the various facilities came from Salmobreed strain but were farmed at different hatcheries in the

Hardanger region (Hordaland, Norway). All the fish followed the general procedure for hatchery production as previously described in Section 2.1.1.

2.2.2. Experimental Facilities and Locations

Field trial 2 took place in Sunnhordaland at 9 full-scale facilities (Table 1, Figure 2) within the fjords: Bømlafjorden, Klosterfjorden, Ålfjorden, and Skåneviksfjorden; collectively, they are further referred to as Hardangerfjorden. Assignment of the facilities into reference or AcuLice treatment groups was based on the current regime of the area [28]. This was carried out to avoid infection of salmon lice released from an AcuLice-treated facility of a reference facility placed downstream. The facilities followed an ordinary production protocol for salmon farming for commercial consumption. This included weekly salmon lice counting, daily feeding (commercial dry diet fed from automatic feeders) and daily registration of sea temperature (at approximately 6–9 m depths, Figure 3), oxygen levels (at –3 m depth), and dead fish registration. All the facilities are fjord facilities with a salinity of 30–32‰. In addition, all the facilities had a density of 3–5% of cleaner fish present in the cages. The daily husbandry was conducted by the facility employees. All experimental sites followed similar feeding routines, with salmon fed according to their size and appetite.

2.2.3. Experimental Design

Each experimental group was followed from onset to the sea during the spring of 2019 until week 20 in 2020. The start-up at each locality varied due to the different times when production fish were transferred to sea cages and other company-internal conditions. The installation of the AcuLice equipment at the facilities in field trial 2 followed the general description as previously described (see Section 2.1.3). All the experimental facilities started the AcuLice treatment in mid-summer 2019. The experimental period for salmon lice counting was set from mid-summer 2019 to late-spring 2020 (a period of 43 weeks). The number of salmon lice treatments was also counted for this period. In addition, the number of weeks between the fish being transferred to sea water (SW) cages and the first salmon lice treatment (defined as mechanical delicing in the present study) was measured and calculated (see below). All equipment maintenance during the period was performed by the supplier. Daily follow-up was carried out by employees at the facility. Due to ordinary operation of the facilities included in field trial 2, the facilities had to follow the Norwegian governmental regulations on delicing if mature female salmon lice exceeded the limit of 0.5 mature female lice per salmon. Throughout the production, period delicing treatments did occur when required for all the facilities.

Table 1. Field trial 2 facilities divided into the two treatment groups (AcuLice or reference), with the site number, company that operates the facility, and coordinates of the location of the sites.

	Site Name	Site Number	Company that Operates the Facility	Coordinates (°N/°E)	
AcuLice	Breivik S	11,574	Bremnes Seashore AS	59.671	5.312
	Grimsholmen	11,559	Sjøtroll Havbruk AS	59.657	5.404
	Hattasteinen	11,511	Bremnes Seashore AS	59.628	5.252
	Hillersvik	10,300	Erko Seafood AS	59.608	5.312
	Loddetå	28,996	Bremnes Seashore AS	59.692	5.543
	Svollandsneset	22,955	Bremnes Seashore AS	59.685	5.589
Reference	Maradalen	12,134	Fjeldberg-, Nordsjø-, Sunnhordaland-& Tysnes Fjordbruk AS	59.762	5.687
	Seglberget	17,015	Fjeldberg-, Nordsjø-, Sunnhordaland-& Tysnes Fjordbruk AS	59.730	5.788
	Mælen	12,127	Fjeldberg-, Nordsjø-, Sunnhordaland-& Tysnes Fjordbruk AS	59.699	5.724

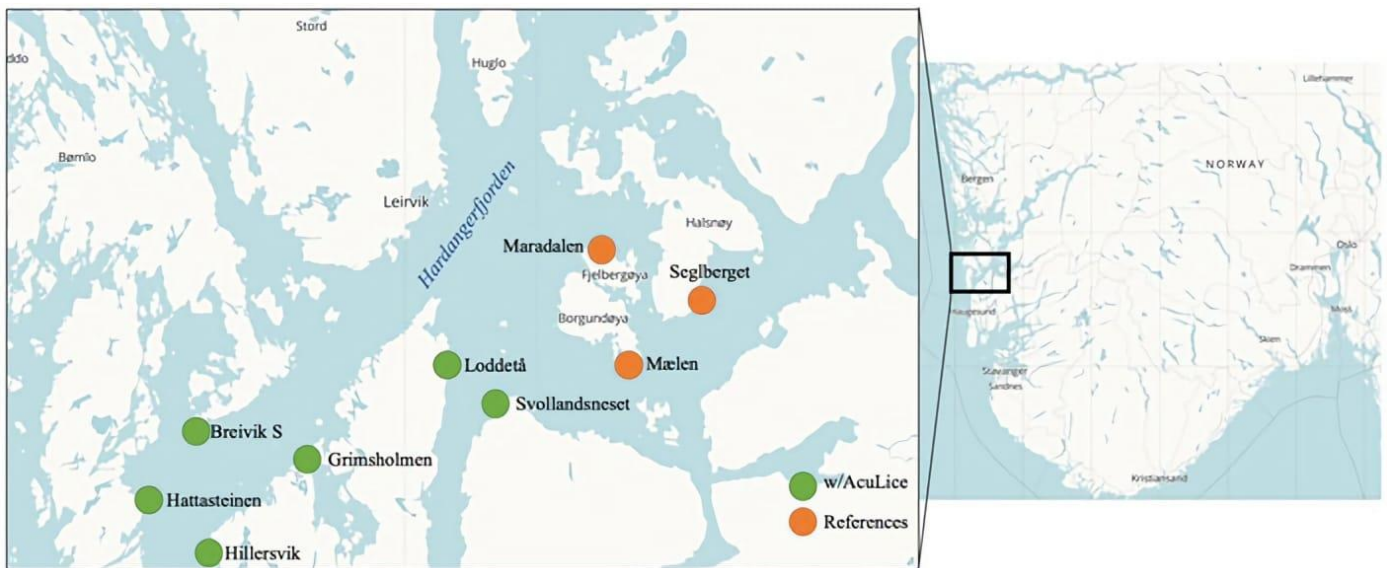


Figure 2. The facilities in experiment 2 with green dots (AcuLice treatment) and orange dots (references without AcuLice treatment) located in Hardangerfjorden.

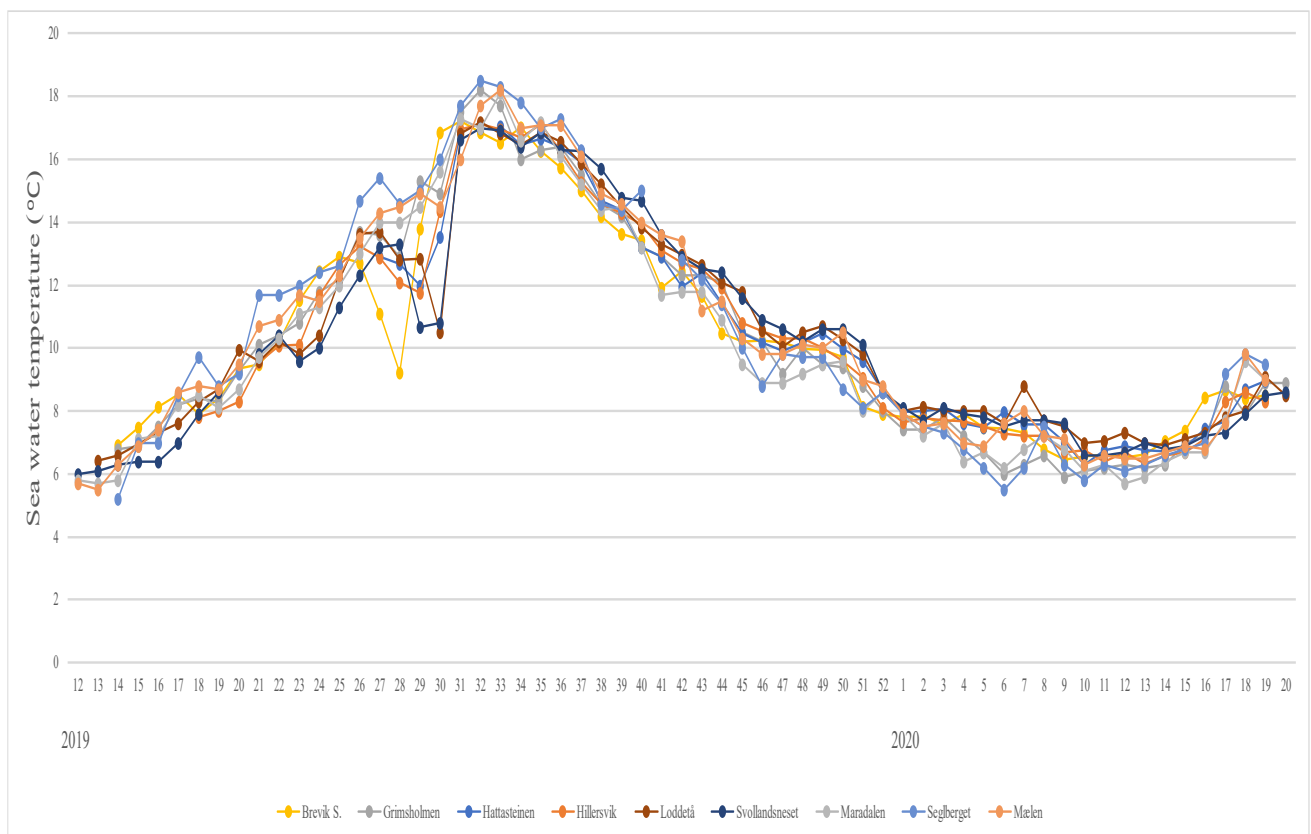


Figure 3. The sea temperature at all the facilities in experiment 2 from week 12 in 2019 until week 20 in 2020 located in Hardangerfjorden, measured at approximately a 6–9-m depth.

2.2.4. Sampling Protocol

As an integral part of the field trial, production data was collected from each locality throughout the trial period (AcuLice and reference) with a focus on weight, weekly salmon lice infestation, and number of salmon lice treatments. The facilities followed a salmon louse counting protocol in accordance with regulations [29]. Fish were randomly collected from

three to six different, randomly chosen, cages (N = 20 from each pen) at each experimental site using hand nets. The fish was then anesthetized as directed by the given agent used.

The salmon lice counting was carried out by qualified salmon lice counters by carefully examining each individual fish. Salmon lice were classified into the following stages: sessile salmon lice, mobile salmon lice, and adult salmon lice (adult male and female salmon lice). As a result of anesthetic treatment, salmon lice that fell off in the tub were counted and categorized. Subsequently, the average for each category and cage was calculated and registered in the BarentsWatch database (BarentsWatch, <https://www.barentswatch.no/fiskehelse/>, accessed on 1 February 2020).

2.2.5. Data Processing and Calculations

Data collected during the 43-week period (week 30, 2019–week 20, 2020) were processed. First, the classifications of the salmon lice life stages that were registered during salmon lice counting were split up and changed to the new categories: small salmon lice (including copepodite, *chalmus* 1 and 2 life stages) and mature female salmon lice. Total average values for each different category were calculated for the data collected in the 43-week period at each facility in field trial 2.

The specific growth rate on weight (SGR) was calculated on a bi-weekly basis for the period in the reference and AcuLice groups apart from Seglberget due to missing weight measurements. Each measurement date was based on N = 6 (AcuLice) and N = 3 (reference sites) and these data were then summarized for the whole rearing period of 43 weeks. The SGR was calculated on a group level according to the formula:

$$\text{SGR} = \frac{\ln W_2 - \ln W_1}{\Delta T},$$

where W_1 is the mean weight at the first measuring point T_1 and W_2 is the mean weight at second measuring point T_2 . ΔT is the number of days between T_2 and T_1 .

2.2.6. Data Collection

For the analysis of the number of weeks until the first salmon lice treatment was required, data for all the production groups in field trial 2 was collected from the database BarentsWatch in the period from SW transfer of Atlantic salmon until the first salmon lice treatment occurred. The number of mature female salmon lice the week before the first salmon lice treatment occurred was also retrieved from the database. Salmon lice treatment in this study was defined as mechanical salmon lice treatment solely conducted due to an excessive number of female mature salmon lice (regulated limit of 0.5 mature female salmon lice per salmon (0.2 in summer)). The requirements for salmon lice treatment were decided by the company veterinarian based on weekly salmon lice counts.

2.3. Statistical Analysis

All statistical analysis and figures were performed using the Statistica™, v.13 (TIBCO Software Inc., Palo Alto, CA, USA) software. Data in all graphical illustrations are presented as the mean value of each group and the standard error of means (SEM) for each group. Statistical outliers with values greater than 1.5 times the interquartile range were excluded from the datasets using the Tukey fence method in Microsoft® Excel v. 16.41 (Microsoft, Redmond, Washington, DC, USA). The distributions of all response variables were checked for normality and homogeneity of variance using the Shapiro–Wilk test and the Levene test. No deviations from normality or homogeneity of variances were found. A general linear model (two-way random effects nested ANOVA) analysis was fitted between each of the response variables and the predictor variables, “AcuLice sites” and “control site”, with replicate sub-samplings (random effect) as a nested factor within the predictor variables. A student *t*-test was used to analyze the specific growth rate, number of salmon lice treatments, and number of weeks from when the Atlantic salmon were transferred to SW

cages until the first salmon lice treatment occurred between the AcuLice and reference groups. A significance level of $\alpha = 0.05$ was used for all statistical models.

3. Results

3.1. Field Trial 1: Acute Stress Effects of AcuLice Treatment

The plasma glucose concentration increased (two-way nested ANOVA, $p < 0.05$, Table 2) from an initial (control) mean value (\pm SEM) of 5.75 (0.14) to 6.13 mmol L⁻¹ (0.15) at the second sampling point. The plasma glucose concentration was the only blood parameter that differed between the control and AcuLice treatment groups (Table 2).

Table 2. Average plasma concentrations (mmol L⁻¹) of cortisol, glucose, lactate, chloride, calcium, and magnesium for Atlantic salmon before starting the AcuLice treatment (control) and 1 h after starting the AcuLice device (AcuLice). Each data sample is presented as a mean \pm SEM, N = 30. Different letters and italics indicate significant statistical differences (two-way nested ANOVA, $p < 0.05$) between the experimental groups.

Plasma Variable	Control	AcuLice
Cortisol	29.72 (2.74)	35.50 (4.21)
Glucose	5.75 (0.14) <i>b</i>	6.13 (0.15) <i>a</i>
Lactate	2.70 (0.13)	2.68 (0.16)
Chloride	127.34 (0.99)	126.28 (1.10)
Calcium	2.67 (0.01)	2.68 (0.01)
Magnesium	0.89 (0.04)	0.85 (0.02)

3.2. Field Trial 2: Effect of AcuLice Treatment under Commercial Production

3.2.1. Specific Growth Rate (SGR)

The AcuLice-treated groups had a minimum value of SGR in weight of 0.32% day⁻¹ (Svollandsneset) and a maximum growth rate of 0.52% day⁻¹ (Grimsholmen, Hattasteinen) in the period from week 30, 2019 to week 20, 2020 (Figure 4). For the reference group, the minimum growth rate was 0.37% day⁻¹ (Maradalen) and the maximum was 0.48% day⁻¹ (Mælen) in the same period. Overall, there were no significant differences in the mean SGR between the reference group and the AcuLice-treated groups (Student’s *t*-test, $p > 0.05$, Figure 4) in the experimental period (week 30, 2019 to week 20, 2020). The mean SGR for the AcuLice-treated groups was 0.45% day⁻¹ and 0.43% day⁻¹ for the reference group.

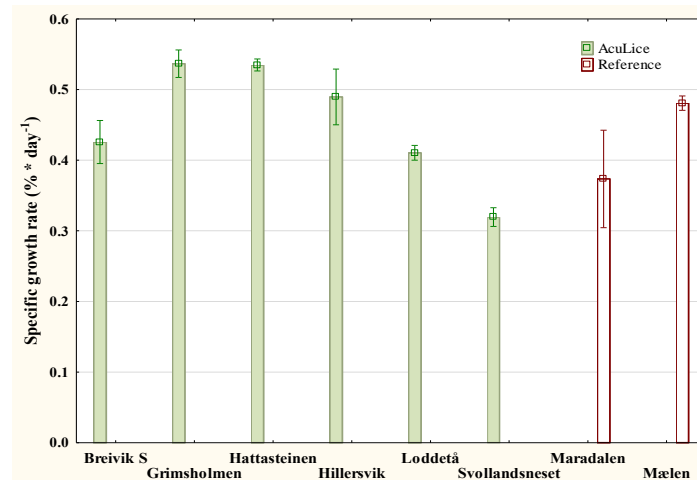


Figure 4. Mean specific growth rate (SGR (% day⁻¹)) calculated for Atlantic salmon at each experimental facility in field trial 2 in the period from mid-summer 2019 to late-spring 2020. The AcuLice-treated facilities are marked in green, and the reference group is marked in red. Data from each production facility is presented as mean \pm SEM (N = 30). Note that data from the reference facility Seglberget is missing due to missing weight measurements.

3.2.2. Effect on Salmon Lice Dynamics: Sessile and Mobile Salmon Lice

The AcuLice-treated groups showed a mean number of small (sessile and mobile) salmon lice of 0.39 (Loddetå) to 1.22 (Hillersvik) in the period week 30 in 2019 to week 20 in 2020 (Figure 5). The reference group had, in the same period, a mean number of small salmon lice of 0.07 (Mælen) to 0.24 (Maradalen).

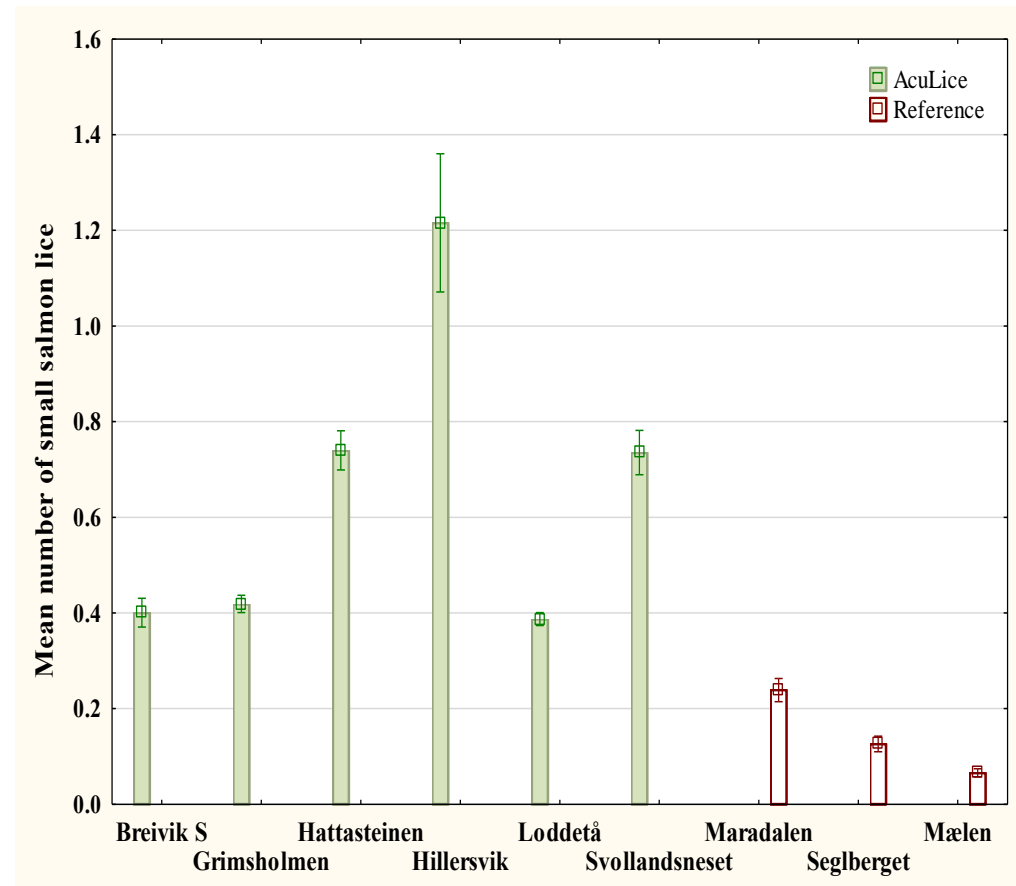


Figure 5. Mean number of small (sessile and mobile) salmon lice measured per Atlantic salmon in the period from mid-summer 2019 to late-spring 2020 at each facility. Groups of Atlantic salmon exposed to AcuLice treatment (AcuLice) compared to the reference group (reference). Green-marked columns are facilities with AcuLice treatment and red columns are the reference facilities. Data from each facility is presented as mean \pm SEM (N = 60–120).

3.2.3. Effect on Salmon Lice Dynamics: Mature Female Lice

The AcuLice-treated groups had a mean number of mature female salmon lice of 0.12 (Breivik S) to 0.31 (Hillersvik) in the period of week 30 in 2019 to week 20 in 2020 (Figure 6). The reference group had, in the same period, a mean number of mature female salmon lice of 0.39 (Maradalen) to 0.49 (Mælen).

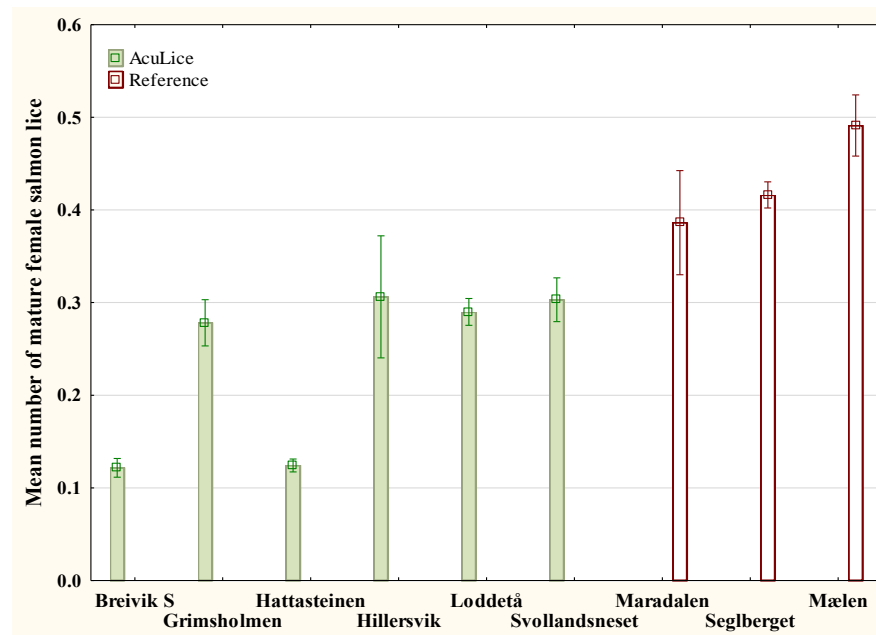


Figure 6. Mean number of mature female salmon lice measured per Atlantic salmon in the period from mid-summer 2019 to late-spring 2020 at each facility. Groups of Atlantic salmon exposed to AcuLice treatment (AcuLice) compared to the reference group (reference). Green-marked columns are facilities with AcuLice treatment and red columns are the reference facilities. Data from each facility is presented as mean ± SEM (N = 60–120).

3.2.4. Effect of AcuLice Treatment on the Salmon Lice Population Composition

A higher number of small salmon lice were observed in the AcuLice-treated groups compared to the reference groups during the experimental period (one-way ANOVA, $p < 0.001$, Figure 7). In contrast, a lower number (mean ± SEM) of mature female salmon lice were observed in the AcuLice-treated groups (0.24 ± 0.03) compared to the reference group (0.44 ± 0.04) in the same period (one-way ANOVA, $p < 0.001$, Figure 7).

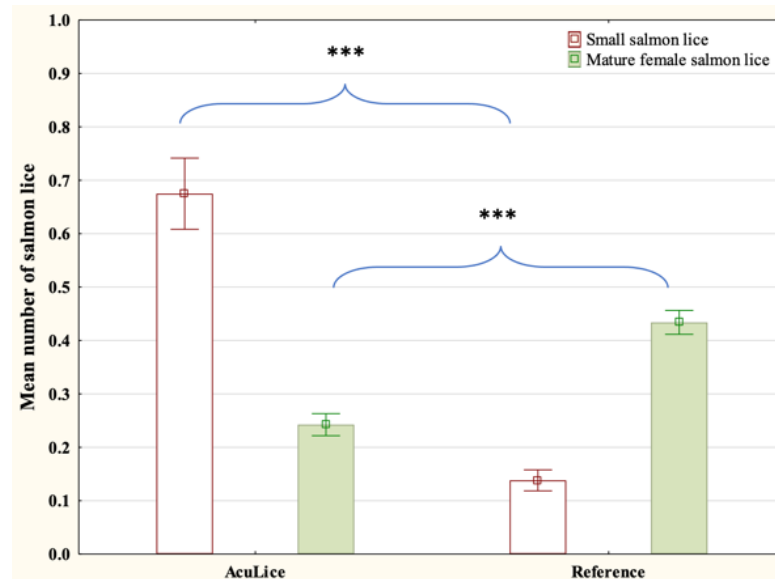


Figure 7. Mean number of small salmon lice (red columns) and mature female lice (green columns) measured per Atlantic salmon between groups exposed to AcuLice treatment compared to the reference group during the period of mid-summer 2019 to late-spring 2020. Asterisks indicate the level of significance between the two groups: *** $p < 0.001$. Data from each treatment group is presented as a mean ± SEM (N = 60–120).

3.2.5. Number of Salmon Lice Treatments in the Experimental Period

Overall, the AcuLice-treated group had a significantly lower (mean \pm SEM) number (3.1 \pm 0.6) of salmon lice treatments (Student's *t*-test, $p < 0.05$) during the 43-week period (week 30, 2019 to week 20, 2020) compared to the reference group (6.3 \pm 0.5).

3.2.6. Number of Weeks to First Salmon Lice Treatment

For the AcuLice-treated facilities, the minimum number of weeks was 22 (Grimsholmen, Figure 8) and the maximum number was 40 weeks (Loddetå). The reference group had a period of 16 to 25 weeks (Seglberget, Maradalen) before the first treatment was necessary. Overall, the mean number (\pm SEM) of weeks until the first salmon lice treatment increased significantly (Student's *t*-test, $p < 0.05$) from 20.3 (2) weeks in the reference group to 33.2 (3) weeks in the AcuLice-treated groups.

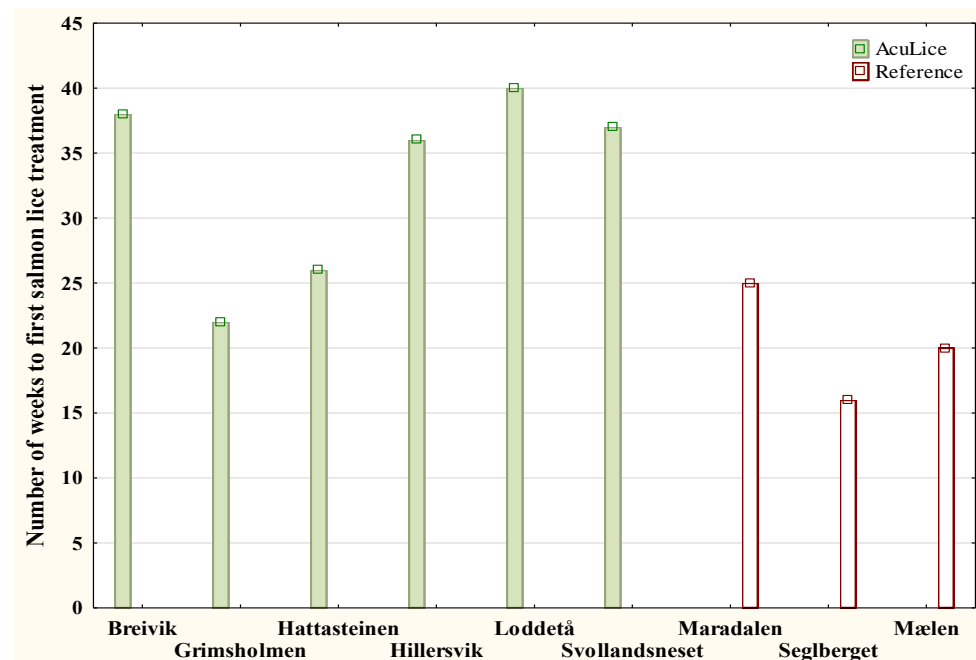


Figure 8. Mean number of weeks to the first salmon lice treatment at the experimental facilities in field trial 2. Green marked columns are facilities with AcuLice treatment and red columns are the reference facilities.

4. Discussion

4.1. Possible Stress Effects of AcuLice Treatment

The cortisol results in field trial 1 did not show any difference in the concentration between the control sampling and after one hour of treatment. Cortisol has a central role in the stress response and homeostasis related to stress, in addition to its impact on other processes, such as growth, behavior, reproduction, and osmoregulation [30,31]. The cortisol concentration increases rapidly after fish are exposed to a stressor [30] and decrease to normal levels within one to two hours in Atlantic salmon [31]. In the present experiment, samples were taken 1 h after AcuLice treatment was started. No alteration in schooling behavior (data not shown) was observed via camera in the moment the AcuLice treatment was started, which supports the findings that the fish were not stressed. If the fish were affected in this moment, it was mild stress and there is a possibility that the cortisol levels had already dropped to normal levels when the sampling took place. However, the present results show no significant difference in the concentration levels in blood between the two samplings. The observation of no behavior alteration substantiates that the Atlantic salmon in field trial 1 did not have a primary stress response.

In field trial 1, a significantly higher concentration of glucose was observed after the group was exposed to AcuLice treatment for one hour. The plasma glucose concentration is affected by an increase in cortisol levels but is also influenced by other factors such as diet and nutrient type [32]. The elevations in plasma cortisol stimulate glycogenolysis (conversion of glycogen stored in the tissue to glucose that is released into the blood), and an increase is a slow response to a stressor [33]. According to [34], the maximum concentration of glucose in the blood is achieved approximately 3–6 h after salmon are exposed to a stressor. Since the sampling took place one hour after the start of the treatment, it could indicate that the elevated concentration had either not reached the maximum concentration or that the glucose levels were influenced by other factors, such as feeding. Studies have shown that Atlantic salmon have a normal concentration of glucose in blood of around 3.3 mmol L^{-1} [33] and values under 6 mmol L^{-1} are observed to be in the normal range [35]. The mean values measured in the present study were 5.75 and 6.13 mmol L^{-1} for the control and AcuLice groups, respectively, so both can be considered to fall within the normal range for Atlantic salmon. The glucose levels in fish blood are also known to show great variability and have been considered as a poor indicator of secondary stress [32]. In addition, the low values of the lactate concentration in the plasma support the indication that the increase in glucose that occurred was due to factors other than stress, such as diet. Based on this, the increase in the plasma glucose levels found in the present study may not be directly correlated with the AcuLice treatment.

No significant difference in the plasma lactate concentration between the control and treated groups in the field trial 1 was observed. Lactate is a result of a limited amount of oxygen accessible for aerobic cell metabolism and can be achieved by hard physical activity or low oxygen levels in the water [36]. In relation to a stressor, lactate indicates that high muscle activity has occurred, which can be correlated with a fish being exposed to a stressor [37]. As a result of a stressor, lactate concentrations have been observed to be over 6 mmol L^{-1} in blood plasma [37]. This indicates that the present results, with concentration levels of around 2.7 mmol L^{-1} , are in the normal range of the lactate concentration. It also corresponds to the schooling behavior observed via the camera, which showed no changes in swimming behavior during the treatment period. No significant differences in the plasma chloride concentration between the control sampling and one hour after the AcuLice was started were found in the present study. In SW, the plasma chloride concentration increases when an acute stressor occurs due to leakage through the tight junctions of the epithelium [38]. For a non-stressed Atlantic salmon, in SW, the plasma chloride concentration has been reported to be around $135 \pm 2.5 \text{ mmol L}^{-1}$ [39]. The present observations are lower and thus indicate no elevated values associated with a stressor.

No differences in the magnesium concentration between the two experimental groups were observed. Previous studies have shown that there is a high connection between increased plasma magnesium and mortality after fish experience a stressor [40,41]. Changes in the magnesium concentration are a good indicator of acute stress [42]. The normal plasma magnesium concentration is typically less than 1 mmol L^{-1} for salmonids [40,41], which is consistent with the current values.

Overall, the findings of the current trial indicate that the secondary stress response was not activated during the one-hour treatment with AcuLice. The glucose levels did increase during the experiment, but in relation to the other parameters and the results from previous studies, this is potentially based on factors other than the treatment. Unfortunately, it was not possible to conduct physiology measurement in the second field trial and it will be important to include measurements such as cortisol in future trials on the long-term effect and effect of repetitive AcuLice treatment. Assessment of the cortisol levels at intermediate times could help to obtain a better idea of whether the stress response is activated in addition to the monitoring of behavioral parameters (aggressiveness, dominance, hierarchies, etc.).

4.2. Effect of AcuLice Treatment in the Field

No differences in SGR were found between the AcuLice and reference production facilities during the 42-week trial period. This indicates no tertiary stress response occurred in the treatment group. A chronic stress factor can have a negative effect on growth [24] and the present data indicate that this was not the case in the current study.

To find out whether the AcuLice treatment had a salmon lice removal effect, the number of salmon lice was counted weekly and categorized. In the salmon farming context, the two categories of small salmon lice and mature female salmon lice are the most relevant in connection with accumulation and the delicing limit [6,43]. Therefore, these main categories were analyzed. The results showed that there was a difference in the number of salmon lice between the two experimental groups in the 42-week study period. The AcuLice sites had a significantly larger proportion of small salmon lice in their facilities. This may indicate that the salmon lice pressure at the sites with AcuLice treatment was higher and thus had a significantly greater salmon lice impact on these facilities compared to the reference group. Based on the results that the AcuLice sites had a significantly higher number of small salmon lice, this would result in the other salmon lice stages accumulating in a larger number than at the reference sites [44]. However, the results showed that the AcuLice sites had a significantly lower number of mature female salmon lice than the reference sites. This is contrary to the expected development, where a larger number of small salmon lice should lead to more mature female salmon lice [45]. The lower proportion of mature female salmon lice may indicate that salmon lice were removed or disappeared during the salmon lice life cycle at the localities using AcuLice. However, it should be noted that the lower numbers cannot be unequivocally associated with the treatment with this experimental design due to lack of replicated control and AcuLice treatments within sites. Further studies are warranted to verify this possible effect.

A previous study [16] has observed that the anterolateral flow field from a swimming salmonid is one of the most important factors for successful infestation with a host for salmon louse. The flow field is derived from water being moved when the salmonid is swimming and is in the low-frequency range of 1–5 Hz [16,46,47]. Therefore, low frequencies in this range can be used to mask the water pressure signature from a potential host. As shown in the present study, some of the salmon lice disappeared during the AcuLice treatment and it is unclear exactly why this occurred. It is conceivable that salmon lice that infected the salmon were unsure of whether they had infected the right species and, therefore, chose to jump off while waiting for the apparently correct host from which the sound frequency originates from. Another possible reason is that the salmon lice were disturbed by the constant frequency, which caused them to stop eating the salmon skin and thus died.

The results indicated that salmon lice disappeared in the period from when they were defined as small salmon lice to the stage of mature female salmon lice. Since the study included localities that produce fish during ordinary operation, these had to follow national regulations regarding delicing, with a limit of 0.5 mature female salmon lice. An average of 3.1 delicing operations per cage were carried out in the AcuLice facilities, which is a significantly lower number of treatments compared to the reference group, with an average of 6.3 delicing operations during the period from week 30, 2019 to week 20, 2020. This suggests that the delicing was not the cause of the lower number of mature female salmon lice in the AcuLice facilities. Furthermore, it supports previous findings that AcuLice has a lower number of mature female lice, which leads to fewer salmon lice treatments.

Overall, the results indicate that the AcuLice sites experienced greater salmon lice pressure, with a significantly larger number of small lice during the period. In addition, the results suggest that salmon lice were removed from the fish during the salmon lice life cycle at the AcuLice sites and that the number of delice treatments compared to the reference sites was significantly lower. Based on these results, it appears that AcuLice influences the removal of salmon lice and has a significant effect on the reduction in the salmon lice burden during Atlantic salmon commercial production.

5. Conclusions

The Atlantic salmon group reared with low-frequency sound treatment (AcuLice) for one hour in commercial open sea cages showed minor or no acute stress responses compared to the control. Long-term field study showed changes in the salmon lice composition, number of salmon lice treatments, and number of weeks until the first needed treatment, indicating that the AcuLice treatment had a significant effect on the reduction in the salmon lice burden during Atlantic salmon commercial production.

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