GIFAS

Gildeskål Forskningsstasjon a.s

Ferskvannsavlusing small-scale tests*:*

Short-term exposure tests to elucidate handling effects.

The use of freshwater to control infestations of the sea louse ***Lepeophtheirus salmonis*** K on Atlantic salmon *Salmo salar* L.

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FHF Project number: 901006

Preface

This study is part of the FHF funded project "*Bruken av ferskvann for å kontrollere infeksjoner av lakselus Lepeophtheirus salmonis K på atlantisk laks Salmo salar."*

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Previous studies undertaken at Gifas have shown that exposing Atlantic salmon to freshwater resulted in reductions of all infectious stages of *L. salmonis*, however, the studies also highlighted the need to fully elucidate the primary causal factors which contribute to these reductions. Acute changes in water quality parameters such as temperature, salinity and pH may play a crucial role in the removal of lice from the salmon. In addition, there is a need for more detailed analysis of water quality parameters before and during treatments. The use of freshwater will be limited due to supply thus studies will be undertaken to assess the potential for reducing the time salmon need to be exposed to this medium without compromising the effects. Reusing freshwater for treating larger biomasses of fish will allow for the method to become more practical commercially. Further elucidation of the mechanical/handling effects will also be undertaken in an attempt to maximise the potential treatment effects when fish are exposed to freshwater.
This study is one in a series which are aimed at attempting to fully elucidate the effects of freshwater as a biological control method in removing infectious stages of *Lepeophtheirus salmonis* from Atlantic salmon (*Salmo salar* L.). The study aimed to fully elucidate the effects on physical removal of sea lice as fish are pumped/transferred from a cage to a well containing freshwater and back to the cage. If sea lice levels can be reduced by up to 40% due to the effects of physical contact before exposure to freshwater then short-term exposure to freshwater may be sufficient if the fish are pumped back into the cage after exposure using the same method. In addition, the study attempted to determine these physical effects over a range of exposure times from 15 minutes up to one hour.

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Abstract/Summary

Atlantic salmon with an average initial weight of 2200g were used in the study. The study was conducted at GIFAS small-scale facility Langholmen. A 2x2x1 m tank was filled with freshwater. For each exposure test, lice counts were undertaken on ten fish after first handling, after exposure to freshwater and after secondary handling. This process was repeated over a range of three different exposure times (15, 30 and 60 minutes). All exposure tests were performed in duplicate.

The results from this study show clear reductions of all present infectious stages of L.*salmonis* after first and secondary handling. The results showed that the percentage reductions attained after the first handling and before exposure to freshwater were on average 45.2% for pre adults (mature males and pre-adults), 45.5% for mature females and 44.3% for all stages of L.*salmonis.*

The percentage reductions attained after the second handling and after exposure to freshwater were on average 88.5% for pre adults (mature males and pre-adults), 95.5% for mature females and 89.1% for all stages of L.*salmonis* present on the fish compared to pre-treatment counts.

Reductions in the number of attached stages of L.*salmonis* appear to be enhanced with additional handling after exposure to freshwater.

Results may indicate that fish can be exposed to freshwater under commercial-scale conditions for shorter time periods than previous studies had shown as long as the fish are passed over grader systems before and after exposure to freshwater.

Larger biomasses of fish may be treated with freshwater as a result to reductions in the time exposed to freshwater as water quality may be maintained for longer time periods.

The clearance rates recorded for all tests are not attributed to acute changes in water temperature or pH.

Results from blood analysis showed that handling in freshwater resulted in minor physiological disturbances consistent with a stress response with an elevation in blood glucose, CO2 and reduction in blood pH. Further handling and replacement of fish back into seawater resulted in an increase in blood sodium concentrations consistent with acute hyperosmolality stress. Neither the effect of acute freshwater handling nor the acute osmotic stresses are severe to conclude that the fish would not be able to recover without adverse effects

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1.0 Introduction

A series of studies have been undertaken at Gifas to assess the potential for using freshwater to remove attached sea lice from infected Atlantic salmon. The studies started in 2011 and four studies have now been completed.

The first study (report title: **The use of freshwater to control infestations of the sea louse *Lepeophtheirus salmonis* K on Atlantic salmon *Salmo salar* L. September 2011**) initially assessed the potential for using freshwater to remove attached sea lice from infected Atlantic salmon.

The study showed that exposing infected salmon to freshwater resulted in a significant reduction of both mature male and female lice after three hours and results from freshwater bioassays undertaken at the same time during the first study showed that after 1 hour exposure to freshwater, 10% of mature females were found to be dead whilst 90.9% of mature males had died as a result to exposure to freshwater. These initial small-scale studies showed that there is potential in using freshwater to delouse infected Atlantic salmon.

The second and third studies (report tilte; **Ferskvannsavlusing i brønnbåt. The use of freshwater to control infestations of the sea louse *Lepeophtheirus salmonis* K on Atlantic salmon *Salmo salar* L. April 2013 & October 2013**) were undertaken under more realistic commercial conditions.

These studies showed that a significant biomass of Atlantic salmon (up to 110 T) could be successfully deloused with freshwater. However, the studies also highlighted the need to maintain water quality parameters such as dissolved oxygen and particularly pH in order for the attached sea lice to be exposed to freshwater for sufficient time in order to be affected.

During the studies undertaken in October 2013, it was found that a super oxygenation system can maintain safe levels of dissolved oxygen. Saturation levels decreased from 124.0% at the start of the exposure study to 84.0% at which point oxygen was added and levels increased to 101.0% quickly thereafter. However, pH levels steadily decreased to 6.08 ppt during the exposure period At this point the fish were showing signs of acute stress and it was decided at this point to start pumping in seawater to safeguard the large biomass of fish and to ensure the welfare of the fish. Carbon dioxide readings on board the well boat (ranging from 19.1 to 68.4ppt) were based on pH levels and were not measured in real time. Readings from hand-held instruments measured CO_2 between 16.0 and 17.0 ppt at the later end of the study.

For carbon dioxide the safe criterion used for the Norwegian production of Atlantic salmon smolts is 15 mg L^{-1} (Fivelstad, S. 2013) provided dissolved oxygen concentrations are high. However, constant fish respiration can raise carbon dioxide levels high enough to interfere with oxygen intake by fish, in addition to lowering the pH of the water. If the cause of the stress noted in the fish was attributed to lowering of pH and/or an increased carbon dioxide concentration then some form of buffering agent may alleviate this problem.

A potential option to prevent swings in pH is to add Sodium hydroxide (NaOH), also known as caustic soda, lye/lut solution or Sodium Hydrate solution. It is a highly caustic metallic base and alkali salt which is available as a prepared solution at a number of different concentrations. Sodium hydroxide forms an approximate 50% (by weight) saturated solution with water. It is commonly used at smolt facilities which use recirculation systems to help maintain safe pH levels throughout production.

The fourth study (report title: **Ferskvannsavlusing i brønnbåt: Study 4. Water quality. December 2013)** had the aim of assessing the potential of using a buffering agent (NaOH) to maintain safe levels of pH when treating a large biomass of salmon in freshwater for a define period of time.

Results from the study show that initially there was a steady but small decrease in pH in both wells once fish transfer had been complete and prior to the addition of NaOH. The addition of NaOH commenced approximately I hr. and 30 minutes after the fish had been transferred to both wells at a

rate of 0.25 l/hr. The decline in pH slowed after the addition and in the well containing freshwater even increased slightly after 10 minutes post-addition. The decrease in pH levels continued however, as the rate at which NaOH was increased there were corresponding small increases in pH in both wells. This present study showed that there is potential for NaOH to be used as a buffering agent to control pH in wells filled with freshwater. However, further research is required to elucidate flow rates and how much to add to maintain safe levels throughout a desired treatment period of approximately three hours.
The present studies have shown clear reductions in all infectious stages of *L. salmonis* from Atlantic salmon. However, the studies have also highlighted the need to fully elucidate the primary causal factors which contribute to these reductions. To this end studies will be implemented to identify these factors. Acute changes in water quality parameters such as temperature, salinity and pH may play a crucial role in the removal of lice from the salmon. In addition, there is a need for more detailed analysis of water quality parameters before and during treatments. The use of freshwater will be limited due to supply thus studies will be undertaken to assess the potential for reducing the time salmon need to be exposed to this medium without compromising the effects. Reusing freshwater for treating larger biomasses of fish will allow for the method to become more practical commercially. Further elucidation of the mechanical/handling effects will also be undertaken in an attempt to maximise the potential treatment effects when fish are exposed to freshwater.

Aims and Objectives

Primary objective:

The aim of this study is to fully elucidate the effects on physical removal of sea lice as fish are pumped/transferred from a cage to a well containing freshwater and back to the cage. If sea lice levels can be reduced by up to 40% due to the effects of physical contact before exposure to freshwater then short-term exposure to freshwater may be sufficient if the fish are pumped back into the cage after exposure using the same method. The aim of this study is to determine these physical effects over a range of exposure times from 15 minutes up to one hour. The study will be performed at Gifas small-scale facilities using holding tanks containing freshwater exposing small groups of Atlantic salmon for each test. Sea lice infestation levels will be recorded prior to each test and immediately after transfer, after exposure and immediately after transfer back to the cage.

Secondary objectives:

Water samples will be taken from the freshwater tank before and during treatment for full chemical analysis in order to elucidate status and/or alterations in water quality over time.
In addition, blood samples will be drawn from fish exposed to freshwater and immediately before and after treatment to investigate issues relating to fish welfare.

2.0 Methods

2.1 Fish

Atlantic salmon with an average initial weight of 2200g were used in the study. All fish originate from the same group of fish and share the same genetic and environmental background. These fish have not been used in any previous trials. The fish were maintained in one cage of 125 m^3 (5x5x5m).

2.2 Experimental design

The study was conducted at GIFAS small-scale facility Langholmen on 9th and 10th July 2014. A 2x2x1 m tank was used in the study (picture 1). The tank was placed on the platform and filled with freshwater. The tank had a flow-through system deployed which allowed for continual replenishment of the freshwater throughout all exposure tests. For each exposure test, 30 fish were lifted from the cage with the aid of a wet net and deposited into a transfer tank used in the small-scale facility for grading and transferring fish from cage to cage (picture 2). The first ten fish (n= 10) entered the pipe system individually and were netted before they entered the freshwater tank. These fish were sedated and any lice present were recorded. After lice counting was completed these fish were removed. The remaining twenty fish were then passed from the transfer system through the pipe system and into the freshwater tank. After the desired exposure time to freshwater had been attained, ten fish were immediately removed (n= 10) by netting them into a separate contained for sedation and lice counting. After lice counting had been completed, these fish were removed. The remaining ten fish (n=10) were then netted from the tank and placed through the transfer system for a second time. These fish were then sedated and any lice present registered.
The pipe system was fitted with a grid to remove seawater. Thus, all fish were transferred without seawater entering the cages and were subjected to the same handling and mechanical perturbation.
This process was repeated over a range of three different exposure times (15, 30 and 60 minutes). All exposure tests were performed in duplicate

Picture 1 The tank used in the study which was filled with freshwater.

Picture 2 The holding tank used to transfer the fish from the cage to the freshwater tank. The pipe has slits at the bottom to prevent sweater entering the holding tank.

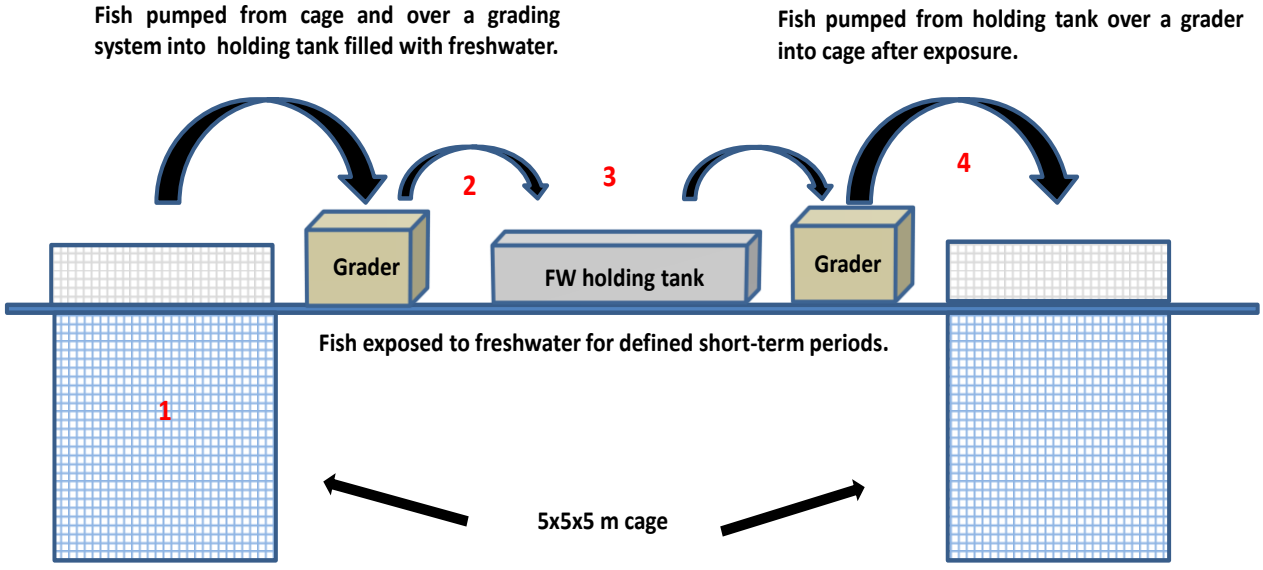

Figure 1 Diagrammatic representation of experimental set-up. Red numbers indicate when lice counts were undertaken. 1: baseline count prior to the experiment; 2: Ten fish removed and sea lice counted before they entered the freshwater tank; 3: Ten fish counted for sea lice after exposure to freshwater and 4: ten fish counted for sea lice after passing through the grading system for a second time.

2.3 Registration of sea lice levels

A lice count was undertaken the same day to assess the lice burden prior to treatment with freshwater. From the cage being used for the study, 30 fish were sedated with Bonzoak at a concentration of active substance of between 30-40mg / l (15-20ml Benzoak/100 liter) and any lice present were recorded. After counting has been complete, any lice remaining in the container was also recorded. In addition for each exposure test, sea lice infestation levels were recorded from 10 fish immediately after the fish have passed over the grader before exposed to freshwater, these fish were transferred to the holding cage after counting. A lice count was taken after the fish have been exposed to freshwater for the defined period of time (10 fish), again these fish will be transferred to the holding cage after counting. The remaining 10 fish were assessed for lice status after they have been pumped from the holding tank and had passed over the grader or the second time before being transferred into the holding cage. All salmon counted for sea lice infestation levels during the tests were netted and placed into a tank containing seawater and sedated with Benzoak 20mg/l.
Lice were registered in 4 categories for all lice counts:

- *Lepeophtheirus salmonis*: Adult female
- *Lepeophtheirus salmonis*: Preadult and males
- *Lepeophtheirus salmonis*: Chalimus
- *Caligus elongatus*

2.4 Fish welfare

To determine the level of stress on fish during treatment blood samples were drawn from randomly selected fish at different exposure times and at different stages of treatmet. Ten fish were randomly netted from the holding cage and euthanized by an overdose of Benzoak and blood samples were drawn prior to the treatments (n = 10) to establish baseline stress and osmoregulation levels. A further five fish (n = 5) were euthanized and blood samples drawn after the fish had passed over the grader and exposed to freshwater for 60 minutes. Five more fish were selected after exposure for 60 minutes and having passed over the grader for a second time (n = 5). This process was repeated for the fish exposed to freshwater for 15 minutes (n = 5) giving a total of thirty fish sampled (N = 30).
Caudal blood samples were withdrawn from euthanized fish via a 21 G heparinized needle (Sodium heparin). Blood samples were immediately injected into an EC8+ cartridge and analyzed using an i-STAT 300 series Portable Clinical Analyzer (Abbott Laboratories. Abbott Park, Illinois, USA). pH, glucose, sodium (Na+), potassium (K+), total CO2 (TCO2), partial pressure of CO2 (PCO2), bicarbonate (HCO3), haematocrit (Hct) and haemoglobin (Hb) were measured as potential indicators of stress and disturbed osmoregulation. Remaining blood was centrifuged at 5000 xg for 5 minutes and the plasma decanted into Eppendorf vials and frozen at -20^{o}C for potential further analysis.

2.5 Water Quality

Oxygen saturation (%), salinity (ppt), and pH within the freshwater tank was monitored routinely throughout for each exposure study.
In addition, water samples (1 L) were drawn immediately prior to and after the fish had been exposed to the freshwater treatment for one hour to compare differences to water chemistry after short-term exposure. Both samples were stored frozen at – 20^{0}C for later analysis by NIVA.

2.6 Statistics

For lice counts statistical significance of differences were computed from one-way or two-way analysis of variance (ANOVA) using Minitab^{TM} statistical software (Ryan & Joiner, 1994). The normality and homogeneity of the variance of all data sets was tested prior to parametric statistical

analysis. Normality was tested by graphic examination of probability plots and the Anderson-Darling test. Significant differences between treatments were determined by Tukey's multiple range test ($p < 0.05$). Differences in mean abundance of attached sea lice were detected after log transformation of the data.
For blood samples, data was analysed using one way analysis of variance with a bonferroni planned contrast against the pre-treatment values. Where assumptions of normality were not met by the data, a Kuskal Wallis non-parameteric ANOVA on ranked data was performed.

3.0 Results

3.1 Freshwater exposure tests: Pre-adult lice

The results from the freshwater exposure study for pre adult lice can be seen in figure 2. There was a significant reduction in all stages of sea lice counted after the fish had passed over the grader and before exposure to freshwater. Reductions in the average number of pre-adult lice decreased from 1,1 per fish (pre-count) to 0,45 per fish for both the sixty and thirty minute treatments ($F_{1,2}$ 169,1; $p < 0.01$ and $F_{1,2}$ 169,2; $p < 0.01$ respectively) whilst the fifteen minute treatment handling effect was a reduction to 0.35 per fish ($F_{1,2}$ 225.1; $p < 0.001$).
The levels of pre-adult lice recorded after direct exposure to freshwater were similar to those recorded after the first handling. The number of pre-adult stages recorded on the fish exposed to freshwater for sixty minutes was 0.4 per fish which was significantly lower compared to the pre count value ($F_{1,2}$ 296.3; $p < 0.001$) but not significantly lower than the counts recorded after first handling. A similar trend was recorded for fish exposed to freshwater for thirty and fifteen minutes ($F_{1,2}$ 9.0; $p > 0.05$ and $F_{1,2}$ 224.9; $p < 0.001$ respectively).
After exposure to freshwater for sixty minutes and the fish had passed over the grader for a second time, there was significantly less pre adult stages (0.5 per fish) compared to the pre count ($F_{1,2}$ 440.1 $p < 0.001$). For fish passing over the grader for a second time after thirty minutes exposure to freshwater there was an average of 0.25 pre-adult lice per fish which was significantly lower compared to the pre count value ($F_{1,2}$ 32.11 $p < 0.01$ whilst fish exposed to freshwater for fifteen minutes and having passed over the grader were found to have no attached pre adult stages present.

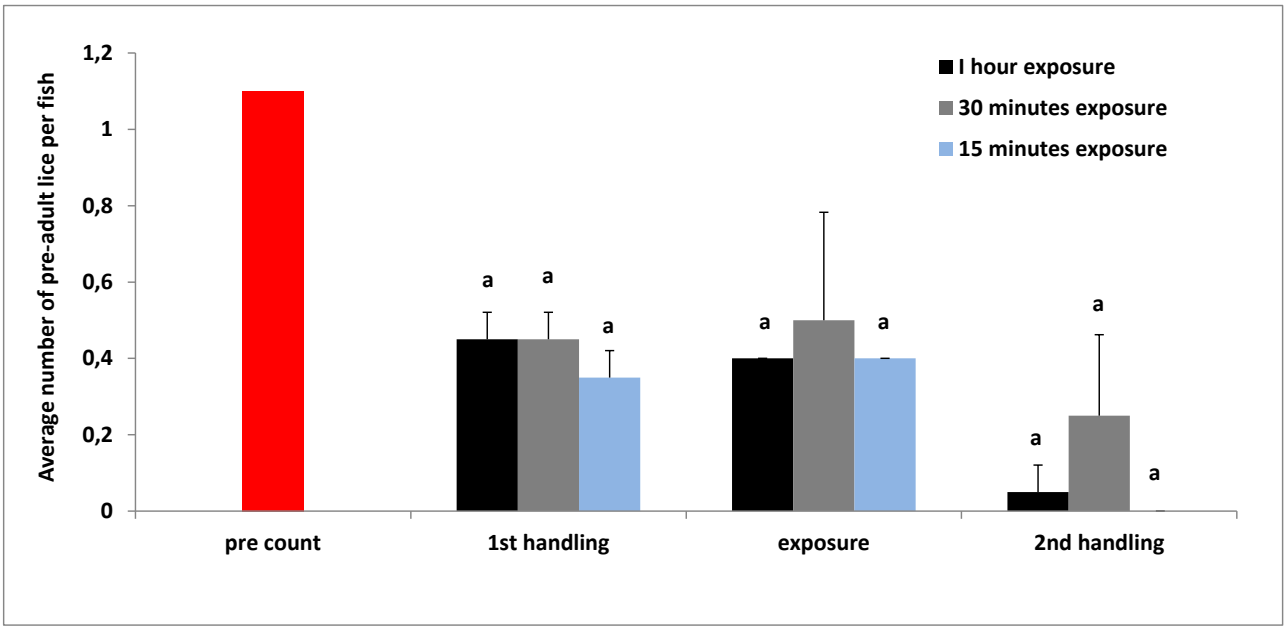

Figure 2 Average number of Pre-adult stages of *L. salmonis* per fish recorded prior to treatment (pre-count), after first hamdling, after exposure to freshwater for 60, 30 and 15 minutes and after second handling. Values represent means ± S.D. Mean values which do NOT share a letter were found to be significantly different by ANOVA and by Tukey`s multiple range test.

3.2 Freshwater exposure tests: Mature female lice

The results from the freshwater exposure study for mature female lice can be seen in figure 3. There were reductions in female stages of sea lice counted after the fish had passed over the grader and before exposure to freshwater. The average number of mature female lice decreased from 0.37 per fish (pre-count) to 0.45 and 0.20 per fish for the sixty and thirty minute tests after the first handling but not significantly so ($p > 0.05$). The first handling of fish for the fifteen minute treatments resulted in a significant reduction to 0.15 per fish ($F_{1,2}$ 225.11 $p < 0.001$).

The levels of mature female lice recorded after direct exposure to freshwater were similar to those recorded after the first handling. The number of mature female stages recorded on the fish exposed to freshwater for sixty and thirty minutes (0.2 per fish for both treatments) was lower than the pre count value but not significantly so ($p > 0.05$). There was a further significant reduction of mature female lice after exposure to freshwater for thirty minutes and after handling ($F_{1,2}$ 40.96; $p < 0.05$)

After exposure to freshwater for all treatments and the fish had passed over the grader for a second time, there was significantly less mature female stages (0.05 per fish) for the sixty minute exposure tests ($F_{1,2}$ 40.96 $p < 0.05$) whilst for both the thirty and fifteen minutes tests there were no mature female lice recorded on any of the fish ($F_{1,2}$ 225.1 $p < 0.001$).

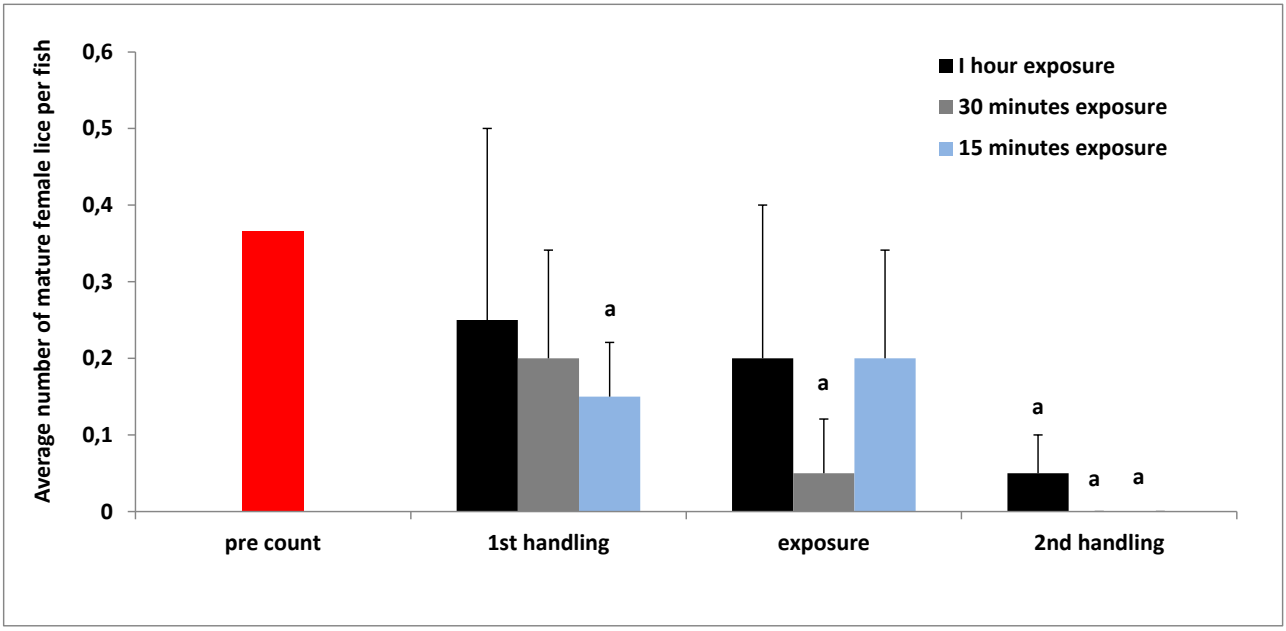

Figure 3 Average number of mature female stages of *L. salmonis* per fish recorded prior to treatment (pre-count), after first handling, after exposure to freshwater for 60, 30 and 15 minutes and after second handling. Values represent means ± S.D. Mean values which do NOT share a letter were found to be significantly different by ANOVA and by Tukey`s multiple range test.

3.3 Freshwater exposure tests: Mature male lice

The results from the freshwater exposure study for mature male lice can be seen in figure 4.

There were reductions in male stages (from 0.63 per fish for the pre-count) of sea lice counted after the fish had passed over the grader and before exposure to freshwater for both the sixty (0.55; $p > 0.05$) and fifteen minute (0.4 per fish; $F_{1,2}$ 224.1 $p < 0.001$) treatments. There was no reduction in the average number of mature male lice after first handling for the thirty minute exposure tests ($p > 0.05$).

The levels of mature male lice recorded after direct exposure to freshwater were lower to those recorded after the first handling for the sixty and thirty minute treatments (0.45 and 0.35 respectively) but not significantly so ($p > 0.05$).

There was no significant reduction of mature male lice after exposure to freshwater for fifteen minutes and after handling ($p > 0.05$).
After exposure to freshwater for all treatments and the fish had passed over the grader for a second time, there were less mature male stages (0.05 and 0.25 per fish) for the sixty and thirty minute exposure tests ($F_{1,2}$ 134.56 $p < 0.01$and $p > 0.05$ respectively) whilst for the fifteen minutes tests there were no mature male lice recorded on any of the fish ($F_{1,2}$ 876.16 $p < 0.001$).

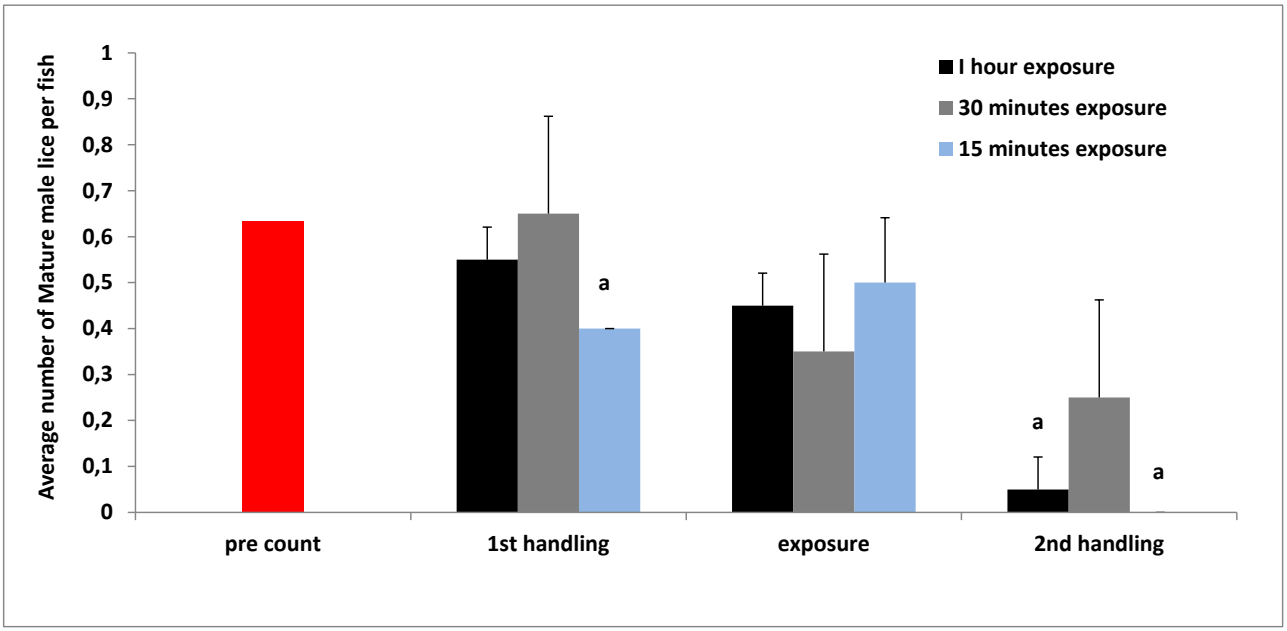

Figure 4 Average number of mature male stages of *L. salmonis* per fish recorded prior to treatment (pre-count), after first handling, after exposure to freshwater for 60, 30 and 15 minutes and after second handling. Values represent means ± S.D. Mean values which do NOT share a letter were found to be significantly different by ANOVA and by Tukey`s multiple range test.

3.4 Freshwater exposure tests: Combined pre-adult and mature male lice

The results from the freshwater exposure study for pre-adult and mature male lice can be seen in figure 5.
There were significant reductions (from 1.73 per fish for the pre-count) of combined mature male and pre-adult lice counted after the fish had passed over the grader and before exposure to freshwater for both the sixty (1.0 per fish; $F_{1,2}$ 225.1 $p < 0.001$) and fifteen minute (0.75 per fish; $F_{1,2}$ 384.16 $p < 0.01$) treatments. There was a reduction of pre-adult and mature male lice for the thirty minute exposure tests (1.1 per fish) compared to the pre count value but not significantly so ($p > 0.05$).
The levels of combined pre adult and mature male lice recorded after direct exposure to freshwater were lower to those recorded for the pre count and after the first handling for the sixty and thirty minute treatments (0.85 per fish; $F_{1,2}$ 309.76 $p < 0.01$ and 0.85 per fish $p > 0.05$ respectively). Exposure for fifteen minutes resulted in significantly lower pre-adult and mature male lice (0.9 per fish) ($F_{1,2}$ 68.89 $p < 0.05$) compared to the pre-count but there were no further reductions after first handling.
After exposure to freshwater for all treatments and the fish had passed over the grader for a second time, there were less pre-adult and mature male stages (0.1 and 0.5 per fish) for the sixty and thirty minute exposure tests ($F_{1,2}$ 265.69 $p < 0.01$and $p > 0.05$ respectively) whilst for the fifteen minutes tests there were no pre adult and mature male lice recorded on any of the fish ($F_{1,2}$ 876.16 $p < 0.001$).

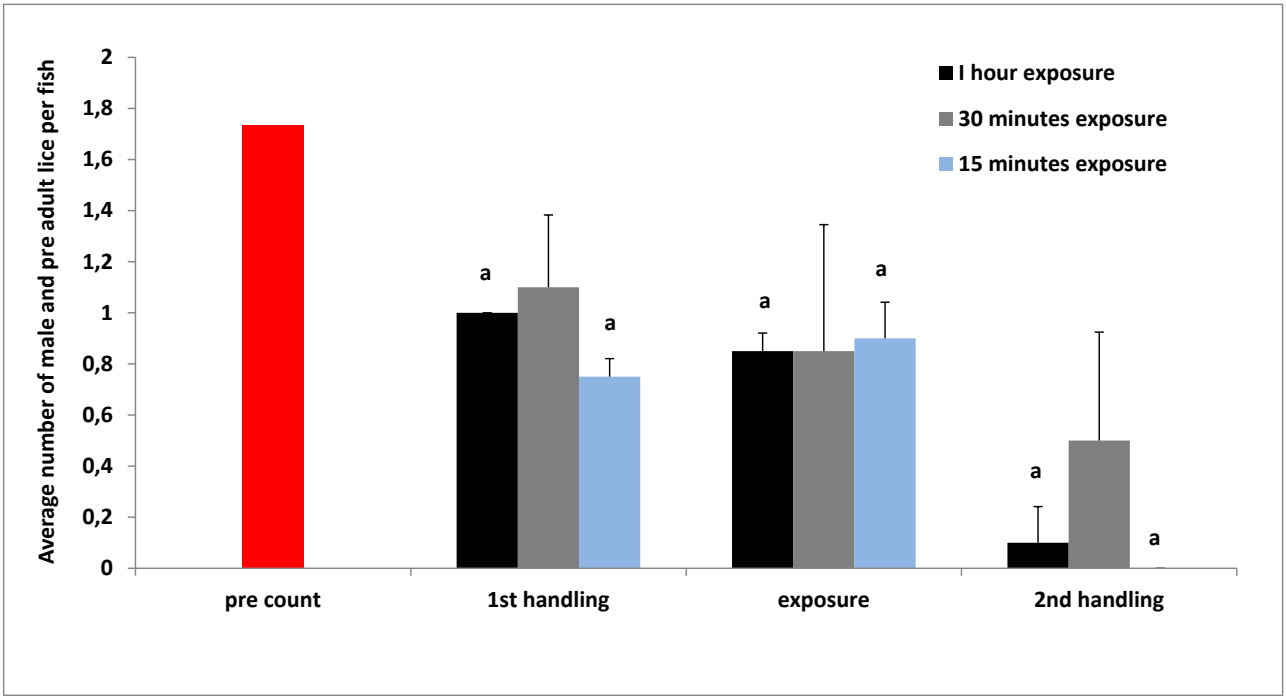

Figure 5 Average number of mature male and pre-adult stages of *L. salmonis* per fish recorded prior to treatment (pre-count), after first handling, after exposure to freshwater for 60, 30 and 15 minutes and after second handling. Values represent means ± S.D. Mean values which do NOT share a letter were found to be significantly different by ANOVA and by Tukey`s multiple range test.

3.5 Freshwater exposure tests: All stages of *L.salmonis*

The results from the freshwater exposure study for all stages of L. *salmonis* can be seen in figure 6. There were significant reductions (from 3.83 per fish for the pre-count) of all lice counted after the fish had passed over the grader and before exposure to freshwater for both the sixty (2.35 per fish; $F_{1,2}$ 876.16 $p < 0.001$) and fifteen minute (1.65 per fish; $F_{1,2}$ 1900,1 $p < 0.001$) treatments. There was a reduction in the thirty minute treatments of all stages of lice (2.40 per fish) compared to the pre count value but not significantly so ($p > 0.05$).

The levels of lice recorded after direct exposure to freshwater were lower to those recorded for the pre count and after the first handling for the sixty and thirty minute treatments (1.9 per fish; $F_{1,2}$ 93.12 $p < 0.01$ and 1.75per fish $p > 0.05$ respectively). Exposure for fifteen minutes resulted in significantly lower lice (2.0 per fish) ($F_{1,2}$ 37.21 $p < 0.05$) compared to the pre-count but there were no further reductions after first handling.

After exposure to freshwater for all treatments and the fish had passed over the grader for a second time, there were significantly less lice (0.25 per fish; $F_{1,2}$ 205.06 $p < 0.01$ and 1.0 per fish; $F_{1,2}$ 22.25 $p < 0.05$) whilst for the fifteen minutes tests there were no lice recorded on any of the fish ($F_{1,2}$ 876.16 $p < 0.001$).

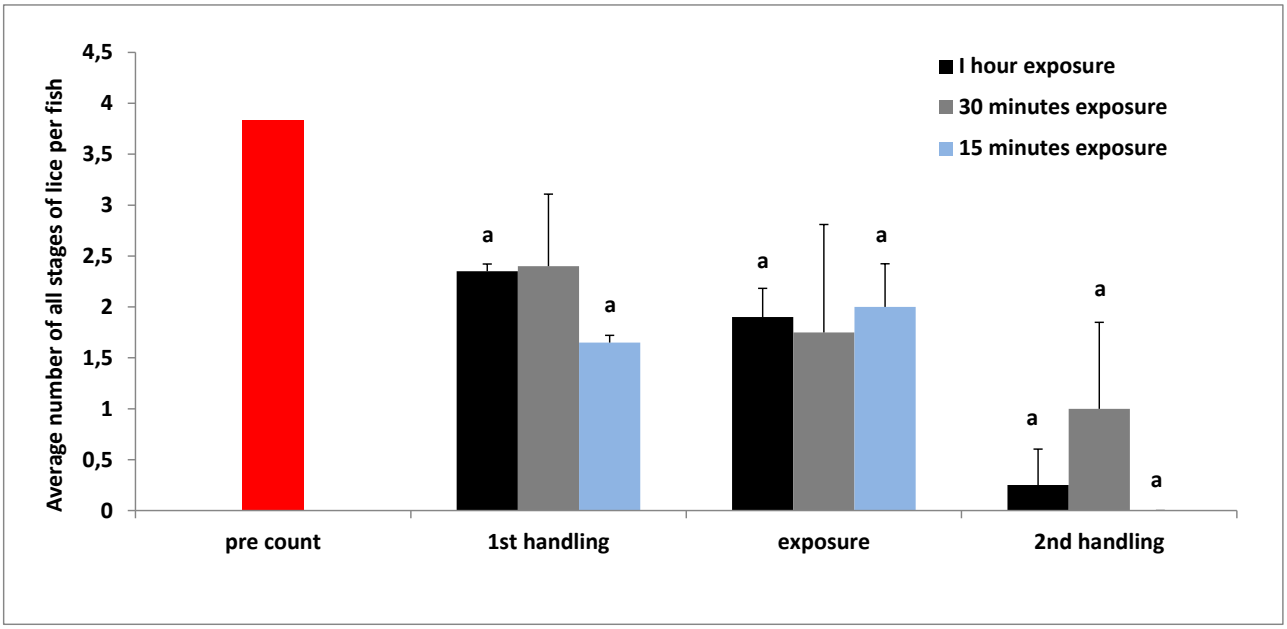

Figure 6 Average number of all stages of *L. salmonis* per fish recorded prior to treatment (pre-count), after first handling, after exposure to freshwater for 60, 30 and 15 minutes and after second handling. Values represent means ± S.D. Mean values which do NOT share a letter were found to be significantly different by ANOVA and by Tukey`s multiple range test.

3.6 Percentage reductions of L.*salmonis*

The percentage reductions for each stage of L. *salmonis* before, during and after exposure to freshwater can be seen in table1.

For pre-adult stages, the percentage reductions attained after first handling during freshwater exposure and at second handling were between 59.1 and 68.2%. After exposure to freshwater, reductions were between 54.5 and 63.6%. After second handling, percentage reductions were 77.3 and 95.5% for thirty minutes and sixty minutes treatments respectively whilst there was 100% reduction for the fifteen minute treatment after second handling.

For mature female stages, the percentage reduction after the first handling and before exposure to freshwater was between 31.8 and 59.1%. After freshwater exposure values ranged between 45.5 and 86.4% After second handing fish exposed to freshwater for sixty minutes had a total reduction of 86.4% whilst for fish exposed for thirty and fifteen minutes had 100% reduction of mature female lice.

For mature male stages, there was a13.2% reduction for the fish for the sixty minute treatment and 36.8% for the fifteen minute treatment whilst there was no percentage reduction recorded for the fish during the thirty minute treatment. The percentage reduction recorded after freshwater exposure ranged between 21.1% for the fish exposed to freshwater for fifteen minutes and 44.7%. for fish exposed for thirty minutes. After exposure and second handling, there was a 60.5% reduction for the thirty minute treatment group and 92.1 and 100.0% reductions for the sixty and fifteen minute groups respectively.

For combined mature males and pre-adult stages, there were reductions ranging from 36.5 and 56.7% after first handling. After freshwater treatment, percentage reductions ranged from 48.1 and 51.0% for the three treatments. After second handling, there was a percentage reduction of 71.0% for the thirty minute treatment group whilst the sixty minute treatment groups attained an overall reduction of 94.2% and the fifteen minute group attained 100.0% reduction.

For all stages of L.*salmonis,* the percentage reduction attained after first handling for all three treatments ranged from 37.4 to 57.0%. After freshwater exposure, the percentage reductions ranged between 47.8 and 54.3% whilst after second handling, percentage reductions for fish treated for

sixty minutes was calculated to be 93.5% whilst for the thirty minute treatments, a reduction of 73.9% was attained. For fish treated for fifteen minutes a percentage reduction of 100% was recorded.

Table 1 Percentage reduction of all stages of L. *salmonis* after first handling, exposure to freshwater for 60, 30 and 15 minutes and after second handling.

Stages	Treatment time	Percentage reduction		
		1st handling	exposure	2nd handling
Pre-adult stages	1 hour	**59,1**	**63,6**	**95,5**
	30 minutes	**59,1**	**54,5**	**77,3**
	15 minutes	**68,2**	**63,6**	**100,0**
Mature female stages	1 hour	**31,8**	**45,5**	**86,4**
	30 minutes	**45,5**	**86,4**	**100,0**
	15 minutes	**59,1**	**45,5**	**100,0**
Mature male stages	1 hour	**13,2**	**28,9**	**92,1**
	30 minutes	**0,0**	**44,7**	**60,5**
	15 minutes	**36,8**	**21,1**	**100,0**
Combined males and pre-adults	1 hour	**42,3**	**51,0**	**94,2**
	30 minutes	**36,5**	**51,0**	**71,2**
	15 minutes	**56,7**	**48,1**	**100,0**
Total lice	1 hour	**38,7**	**50,4**	**93,5**
	30 minutes	**37,4**	**54,3**	**73,9**
	15 minutes	**57,0**	**47,8**	**100,0**

3.6 Fish welfare

Blood parameters measured by iSTAT analysis area summarised in table 2. Baseline blood Na^+ levels were 155.1 mM prior to treatment. For the sixty minute treatments, after first handling and before exposure to freshwater, Na^+ levels decreased to 152.6 mM and increased to 162.4 mM after exposure to freshwater and after second handling ($p < 0.05$). For fish exposed to freshwater for fifteen minutes, blood Na^+ levels increased to 166.4 mM ($p < 0.05$).

Baseline blood K^+ levels were 4.06 mM prior to treatment. Levels increased to 4.16 mM after exposure to freshwater for sixty minutes and after second handling whilst for fish exposed for fifteen minutes, blood K^+ concentrations increased to 4.24 mM after exposure and second handling.

For total CO_2, baseline levels were found to be 9.6 mM, this increased to 10.8 mM for fish exposed for sixty minutes and after second handling whilst for fish exposed to freshwater for fifteen minutes total CO_2 levels were found to be 9.0 after second handling.

Beeline glucose levels were 78.9 mg/L and after second handling of fish exposed to freshwater for sixty and fifteen minutes, levels significantly increased to 96.6 and 89.0 mg/L ($p < 0.05$).

Baseline percentage haematocrit levels were 26.9%, this increased significantly to 27.6 and 31.0% after sixty and fifteen minutes exposure and after second handling ($p < 0.05$).

Blood pH levels decreased from 7.53 to 7.2 and 7.14 after second handling for both groups ($p < 0.05$).

Table 2 Mean (SEM) blood parameters as measured by iSTAT demonstrating the effects of handling and freshwater bath treatments on Atlantic salmon. Values with differing letters indicating significant differences from pre- treatment values.

Treatment	Na^+ mM	K^+ mM	TCO_2 mM	Glucose mg/L	Hct %	pH	PCO_2 mmHg	HCO_3^- mM	Hb g/100mL
Pre	155.1a	4.06	9,6	78.9a	26.9a	7.353a	16.68a	9.11	9.14
	(0.7)	(0.24)	(0.4)	(2.2)	(1.0)	(0.033)	(1.22)	(0.37)	(0.34)
1h 1x handling	152.6a	3.56	10.6	97.4b	27.4a	7.213b	24.12b	9.72	9.30
	(0.6)	(0.18)	(0.9)	(3.8)	(0.9)	(0.045)	(1.80)	(0.81)	(0.30)
1h 2xhandling	162.4b	4.16	10.8	96.6b	27.6a	7.202b	25.12b	9.94	9.42
	(2.5)	(0.30)	(0.8)	(4.4)	(1.9)	(0.018)	(0.99)	(0.70)	(0.63)
15 min 1x handling	158.6a	3.02	10.2	91.4b	31.6b	7.119b	28.32b	9.18	10.76
	(0.7)	(0.37)	(0.5)	(3.0)	(0.8)	(0.019)	(1.06)	(0.38)	(0.28)
15 min 2x handling	166.4b	4.24	9.0	89.0a	31.0a	7.145b	23.7b	8.14	10.54
	(1.3)	(0.72)	(0.7)	(3.5)	(0.8)	(0.038)	(2.01)	(0.61)	(0.27)
Normality	Yes	No	No	Yes	Yes	Yes	Yes	Yes	Yes
ANOVA	F 19.267	H 7.452	H 4.44	F 6.777	F 3.617	F 8.798	F 10.925	F 1.335	F 3.600
df	4,29	4	4	4,29	4.29	4.29	4.29	4.29	4.29
P value	<0.001	0.114	0.350	<0.001	0.019	<0.001	<0.001	0.284	0.019

Partial CO_2 pressure significantly increased from baseline levels of 16.68 mmHg to 25.12 and 23.7 mmHg after sixty and fifteen minute exposure tests and after second handling ($p < 0.05$).
Baseline blood HCO_3^- levels were 9.11 mM. This increased to 9.94 mM after sixty minutes exposure and second handling whilst blood HCO_3^- levels were found to be 8.14 mM after fifteen minutes exposure and second handling.
Baseline hemoglobin levels were found to be 9.14 g/100 mL. This increased to 9.42 g/100 mL after second handling of fish exposed to freshwater for sixty minutes and to 10.54 g/100 mL after second handling of fish exposed to freshwater for fifteen minutes.

3.6 Water quality

3.6.1 Temperature, Oxygen, pH and salinity

For the sixty minute exposure tests, water quality parameters are summarised in table 3. Oxygen concentrations ranged between 74.2% and 89.3% for test 1 and 84.2 and 101.4% for test 2. pH levels for test 1 decreased from 8.1 to 7.6 at the end during test 1 and ranged between 7.89 and 8.23 for test 2. Salinity was maintained between 0.16 and 0.17 ppt for test 1 and between 0.16 and 0.18 ppt for test 2. Temperature varied slightly for both tests

Table 3 Water quality parameters recorded during sixty minute exposure tests

Sixty minute freshwater exposure: Test 1				
Time	**Oxygen (%)**	**pH**	**Salinity (ppt)**	**Temperature (^{0}C)**
0	89,10	8,10	0,16	13,1
10	89,30	8,20	0,16	13,0
15	82,90	8,10	0,17	13,1
20	76,90	8,40	0,19	13,2
30	74,20	7,90	0,19	12,9
40	75,50	7,80	0,18	12,8
50	82,10	7,70	0,18	12,9
60	85,10	7,60	0,17	13,1
Test 2:				
0	84,20	7,89	0,16	13,9
10	85,20	8,10	0,16	13,8
15	84,20	8,23	0,17	13,5
20	77,70	8,08	0,17	13,4
30	101,20	8,03	0,16	13,2
40	109,30	8,01	0,17	13,2
50	105,40	8,03	0,18	13,3
60	101,40	8,01	0,18	13,4

For the thirty minute exposure tests, water quality parameters are summarised in table 4. Oxygen concentrations ranged between 95.1% and 101.2% for test 1 and 92.6 and 101.1% for test 2. pH levels for test 1 decreased from 8.17 to 7.9 at the end during test 1 and ranged between 8.1 and 7.9 for test 2. Salinity was maintained between 0.19 and 0.23 ppt for test 1 and between 0.2 and 0.23 ppt for test 2. Temperature varied slightly for both tests
For the fifteen minute exposure tests, water quality parameters are summarised in table 5. Oxygen concentrations ranged between 92.1% and 94.5% for test 1 and 92.5 and 94.5% for test 2. pH levels for test 1 varied between 8.12 to 8.16 at the end during test 1 and ranged between 7.9 and 8.1 for

test 2. Salinity was maintained between 0.2 and 0.22 ppt for test 1 and between 0.19 and 0.21 ppt for test 2. Temperature varied slightly for both tests.

Table 4 Water quality parameters recorded during thirty minute exposure tests

Thirty minute freshwater exposure: Test 1				
Time	**Oxygen (%)**	**pH**	**Salinity (ppt)**	**Temperature (^{0}C)**
0	95,10	8,17	0,20	13,30
10	95,20	8,15	0,21	13,00
15	100,10	8,10	0,20	13,10
20	101,20	8,20	0,19	13,50
25	100,40	8,10	0,21	13,20
30	98,80	7,90	0,23	13,40
Test 2:				
0	92,60	8,10	0,21	13,10
10	113,30	8,20	0,22	13,40
15	101,10	8,10	0,20	13,30
20	100,90	8,10	0,21	13,40
25	99,50	8,00	0,20	13,40
30	99,40	7,90	0,23	13,20

Table 5 Water quality parameters recorded during fifteen minute exposure tests

Fifteen minute freshwater exposure: Test 1				
Time	**Oxygen (%)**	**pH**	**Salinity (ppt)**	**Temperature (^{0}C)**
0	94,50	8,16	0,21	13,3
5	93,50	8,16	0,22	13,4
10	92,40	8,14	0,20	13,2
15	92,10	8,12	0,21	13,4
Test 2:				
0	94,50	8,10	0,19	13,1
5	94,20	8,10	0,19	13,4
10	93,90	8,00	0,21	13,3
15	92,50	7,90	0,20	13,2

3.6.2 Water chemistry

Water quality parameters measured before and after one hour exposure to freshwater can be seen in table 6. The pH remained similar after one hour exposure (7.91) compared to the pre-treatment sample (7.87).There were small increases in conductivity, alkalinity and turbidity after the one hour treatment. Total N increased from 345 to 500 µg N/L after the treatment. Nitrate (NO_3-N) and total organic carbon (TOC) concentrations remained little unchanged after exposure compared to the pre-sample. There were small elevations in chlorine (Cl); sulphate (SO_4) and CO_2 after exposure whilst levels of reactive Aluminium (Al) decreased slightly after one hour exposure. Non-labile Al concentrations increased from < 5 µg/L to 13 µg/L whilst labile Al levels decreased from > 24 µg/L to 7 µg/L after exposure.

Calcium (Ca), copper (Cu) and Iron (Fe) levels remained little unchained after one hour exposure whilst there were small elevations in Potassium (K), Magnesium (Mg) and Manganese (Mn) concentrations after one hour exposure. Sodium (Na) levels increased from 12.7 mg/L to 17.2 mg/L after exposure.

Table 6 Freshwater chemistry parameters measured prior to and after fish have been exposed for one hour.

Parameter	Measurement	Pre-sample	1hour exposure
pH	pH	7,87	7,91
Konduktivitet	mS/m	29,2	32,8
Alkalinitet	mmol/L	1,998	2,049
Turbiditet (TURB860)	FNU	12,5	15,3
Total N	µg N/L	345	500
Nitrat NO3-N	µg N/L	48	48
TOC	mg C/L	2,3	2,4
Cl	mg/L	26,2	34,6
Sulfat (SO4)	mg/L	4,21	5,37
CO2	mg/L	1,9	2,5
Al - reaktivt	µg/L	29	20
Al - ikke labilt	µg/L	<5	13
Al/ICP	mg/L	0,052	0,056
Al - labilt *	µg/L	>24	7
Kalsium (Ca)	mg/L	48,4	49,5
Kobber (Cu) ICP	mg/L	<0,002	<0,002
Jern (Fe) ICP	mg/L	0,0424	0,0486
Kalium (K)	mg/L	1,15	1,53
Magnesium (Mg)	mg/L	3,16	3,79
Mangan (Mn) ICP	mg/L	0,0076	0,0086
Natrium (Na)	mg/L	12,7	17,2

4.0 Discussion

4.1 Freshwater exposure tests

The results from this study show clear reductions of all present infectious stages of L.*salmonis* after first and secondary handling. The results showed that the percentage reductions attained after the first handling and before exposure to freshwater were on average 45.2% for pre adults (mature males and pre-adults), 45.5% for mature females and 44.3% for all stages of L.*salmonis* present on the fish. There were no *chalimus* stages present on any of the fish examined for sea lice throughout all tests. These reductions due to physical perturbation were similar with results from previous studies undertaken at Gifas. It has been shown from previous studies that as the fish are being pumped from polar circle cages to well boats that there was a reduction in the average number of infectious stages immediately after pumping and before the fish were exposed to freshwater. The percentage reductions recorded for *chalimus*, pre-adult and mature female stages (Reynolds

October 2013) were 77%, 30% and 14% respectively, giving a total reduction for all stages of 39%. Similar percentage reductions were observed from previous studies undertaken at Gifas where it was shown that transferring fish from one cage to another or crowding the fish resulted in reduction of up to 40% compared to pre-count levels of infestation (Reynolds 2011). The reductions in attached stages recorded immediately after the fish were pumped from the holding cage and before exposure to freshwater from these tests can be attributed to mechanical perturbation.: physical contact from crowding, contact with the inner surface of the pipes used to pump the fish, netting and contact with the grading system.

Previous studies undertaken at Gifas assessing the potential for freshwater to be used as an effective delousing treatment, have mainly focussed on using well-boats under commercial-scale conditions. During these studies, efficacy of treatment was assessed by counting any remaining lice on the fish immediately after finishing freshwater exposure and no lice counting had been undertaken at the point when the fish were being pumped back into the polar circle cages. These studies were imitated primarily to assess this secondary handling effect. Results from these studies showed that the percentage reductions attained after the second handling and after exposure to freshwater were on average 88.5% for pre adults (mature males and pre-adults), 95.5% for mature females and 89.1% for all stages of L.*salmonis* present on the fish compared to pre-treatment counts. The percentage reduction attained for all infective stages of sea lice found on Atlantic salmon exposed to freshwater (89.1%) would be considered to be a successful treatment outcome and infection levels would be below treatment thresholds imposed under Norwegian legislation (0.5 sexually mature females per fish). The reductions recorded after secondary handling may be attributed to exposing the sea lice to freshwater for short time periods. Direct exposure to freshwater may have affected the lice attached to the fish and weakened them allowing for the physical effects of an additional handling to remove them from the fish as they passed over the grader system. Previous studies have shown lower reductions of attached stages of L.*salmonis* when exposed to freshwater for short time periods and all well-boat studies have shown that fish had to exposed to freshwater for up to three hours to achieve effective clearance rates. The reductions recorded during these tests may be attributed to the stress effects of freshwater on attached stages of L.*salmonis*. However, the reductions may also be purely attributed to the physical effects of handling the fish two times with no contributing effects from freshwater exposure. A repeat of these tests using seawater would elucidate these effects fully.

The clearance rates recorded after the second handling were generally higher for fish exposed to freshwater for the shortest time period (fifteen minutes) For pre-adult stages, sixty minute exposure and secondary handling resulted in reductions of 94.2% overall whilst for fish exposed to thirty minutes, a reduction of 71.2% was attained after second handling. However, for fish exposed to fifteen minutes, there were no lice present on the fish after they had passed over the grader for the second time, constituting a 100% reduction of all attached stages. A similar trend was recorded for mature female stages with both the 30 minute and fifteen minute exposures resulting in a total clearance of 100% whilst for fish exposed for sixty minutes, total clearance was calculated to be 86.4%. For all stages of L. *salmonis*, fish exposed for fifteen minutes achieved a clearance rate of 100% compared to fish exposed for sixty and thirty minutes (93.5 and 73.9% respectively). If the results are partially attributed to the stress of freshwater exposure on sea lice physiology then shorter exposure times may be used commercially with well-boats. This would have the added advantage of potentially treating larger biomasses of fish with the same water than has been done in previous commercial scale tests. Fish can be exposed for a short time to freshwater and as long as they are passed over the grader system on the well-boat for a second tme prior to being returned to the cage then the freshwater may be used over a longer time period without compromising water quality.

Handing Atlantic salmon for two times during freshwater treatment may also prove challenging in that excessive physical contact with graders and/or pipes may result in surface damage to scales and excess removal of mucus from the surface of the fish. If such a system is used commercially, care

would be needed as to when fish are treated and at what size. Delousing with freshwater in late Autumn/early winter may result in wounds associated with lower water temperatures if care is not taken.

4.2 Fish welfare

A principal concern of any treatment is its effect on the welfare of the fish. Freshwater delousing may be considered a less stressful method compared to current chemical methods used in the industry at present. Results from blood analysis showed that handling in freshwater resulted in minor physiological disturbances consistent with a stress response with an elevation in blood glucose, CO2 and reduction in blood pH. Further handling and replacement of fish back into seawater resulted in an increase in blood sodium concentrations consistent with acute hyperosmolality stress. Neither the effect of acute freshwater handling nor the acute osmotic stresses are severe to conclude that the fish would not be able to recover without adverse effects (Powell, *pers comm.*)

4.3 Water quality

Previous studies undertaken at Gifas performed under commercial-scale conditions showed that there were acute differences between the temperature of the seawater (10.1 ^{0}C) and the freshwater contained within the well-boat (5.5 ^{0}C) (Reynolds October 2013). In addition, during these studies, lowering pH levels in the wells containing freshwater resulted in the fish showing signs of acute stress. The clearance rates attained for attached sea lice durig these studies constituted a treatment success and it has been proposed that these acute changes in water quality may have contributed to the reductions of attached sea lice. There is currently research underway to elucidate that if different stages of sea lice are exposed to abrupt changes in water temperature and/or decreasing pH levels then survival is compromised.

Water quality parameters recorded during these tests showed that freshwater pH levels were maintained throughout all test regimes and at no time did pH levels drop to levels recorded during previous well boat tests. In addition, the temperature in the tank containing freshwater was comparable to that of the seawater (13^{0}C for freshwater compared to 13.8 ^{0}C for seawater). Thus, the reductions recorded during these tests are not a result of acute changes in water temperature or pH and are directly as a result of handling effects and/or freshwater exposure.

Water chemistry changed little after the fish were exposed for one hour to the freshwater bath treatment. Most parameters measured after exposure were similar to those prior to treatment. In addition, the parameters measured could be considered to have no long lasting harmful effects to the fish after short-term exposure. For example, a pH below 6.0 has been shown to impair osmoregulatory abilities and seawater tolerance of *S. salar* smolts (Staurnes *et al*., 1993). The pH levels recorded before and after one hour exposure were within a range that would unlikely cause stress to the fish.

The safe levels of CO_2 for Atlantic salmon parr and smolt is 15 mg/L (Fivelstad, 2013). With regard to toxicity in fish, the two states of 'ammonia', ionized ammonia (NH_4^+) and un-ionized ammonia (NH_3), should be indicated. TAN expresses total ammonia nitrogen (NH_3-N + NH_4-N). Generally, safe levels have been stated as mg/L NH_3-N since NH_3 easily crosses the gill membrane by diffusion. For freshwater fish, an overall safe level held for many years was 25 µg/LNH_3-N (Alabaster and Lloyd, 1980), while the safe limit for Atlantic salmon can be as low as < 2 µg/L.

Ca and Na levels were slightly higher than recommended for freshwater treatment of AGD (< 10 mg /L respectively) (Powell and Kristensen 2014), however, it would be unlikely that there would be any long-lasting effects ffrom such a short exposure time.

5.0 Conclusions

The percentage reduction attained for all infective stages of sea lice found on Atlantic salmon exposed to freshwater (89.1%) would be considered to be a successful treatment outcome and infection levels would be below treatment thresholds imposed under Norwegian legislation (0.5 sexually mature females per fish).

Reductions in the number of attached stages of L.*salmonis* appear to be enhanced with additional handling after exposure to freshwater.

Results may indicate that fish can be exposed to freshwater under commercial-scale conditions for shorter time periods than previous studies had shown as long as the fish are passed over grader systems before and after exposure to freshwater.

Larger biomasses of fish may be treated with freshwater as a result to reductions in the time exposed to freshwater as water quality may be maintained for longer time periods.

The clearance rates recorded for all tests are not attributed to acute changes in water temperature or pH.

Neither the effect of acute freshwater handling nor the acute osmotic stresses are severe to conclude that the fish would not be able to recover without adverse effects.

Water chemistry is important in the survival of Atlantic salmon smolts when stressed by other pathogens such as sea lice with the main focus to date being acidifcation of freshwater and its associated implication with the mobility of toxic transitional metal ion species such as Al3+ . In particular the episodic and fluctuating effects of acidified freshwater enhances the stress effects and reduced survival of post-smolts infected with sealice (Finstad *et al.*2012). Not all freshwater can be deemed suitable or optimal for the treatment of Atlantic salmon is a parasite control regime.

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