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Optimization of calcium oxide treatment against salmon louse (*Lepeophtheirus salmonis*). A controlled laboratory study

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ABSTRACT

Salmon louse (*Lepeophtheirus salmonis*) infestations pose a significant challenge to the salmonid farming industry. While most conventional water-treatment protocols primarily target lice at parasitic stages once attached to the fish, preventive measures targeting planktonic/free-living stages are scarce. This study investigated the effects of fine calcium oxide (CaO) particles on salmon louse planktonic stages under controlled laboratory conditions. The study tested a range of concentrations (0.2 g/L to 0.6 g/L) exposure times (2–15 minutes) and frequency of exposures (from daily to every five days). The results indicated that the effects of CaO exposure were positively correlated with the concentration, duration, and frequency of the treatment. Exposure to CaO (10 minutes at 0.2 g/L) reduced by 60–70 % the number of nauplii reaching the copepodid stage by decreasing survivability and molting. CaO treatment (10 min at 0.6 g/L) induced up to 90 % mortality of free-living copepodids. In infection trials using copepodids that survived CaO treatment, there was a 42.1 % reduction in the number of parasitic chalimus, pre-adult, and adults on the salmon five weeks post-infection as compared to control. The LC_{50} for copepodids ranged between 0.54- and 0.36-g/L for an exposure time of 2–10 minutes, respectively. This laboratory study serves as an essential first step in validating the efficacy of CaO and establishes a foundation for future field trials to assess its potential as an antiparasitic treatment in aquaculture.

1. Introduction

The parasitic copepod family *Caligidae* (Crustacea) includes over 450 species across more than 30 genera (Dojiri and Ho, 2013; Hemmingsen et al., 2020). Among these, *Lepeophtheirus salmonis,* commonly known as "Salmon lice", is a prevalent parasite of salmonids (Delabbio et al., 2004). Its life cycle includes both a free-living and a parasitic stage and can be divided into four functional phases: i) planktonic dispersal, ii) infective copepodid, iii) host attached and iv) mobile on host (Hamre et al., 2013). Lice infestation in aquaculture produces a significant economic and ecological impact worldwide (Igboeli, Burka and Fast, 2014; Igboeli et al., 2012).

In Norwegian aquaculture, lice-related biomass growth loss per production cycle varies from 3.62 % to 16.55 %, resulting in an annual economic loss exceeding US$ 436 million (Abolofia, Asche and Wilen, 2017). This also influences wild lice population dynamics, with potential ecological consequences for wild salmon populations (Oldham et al., 2023). To combat lice pressure, farmers employ two main strategies: preventive and reactive treatments. Preventive treatments aim at reducing contact between infective copepodids and fish, while reactive treatments involve removing attached and mobile lice from the host (Barrett et al., 2020; Overton et al., 2019). Currently, Atlantic salmon (*Salmo salar*) aquaculture faces critical challenges, with traditional delousing methods showing declining efficacy (Aaen et al., 2015; Besnier et al., 2014; Fjørtoft et al., 2020, 2019, 2021; Myhre Jensen et al., 2020). Lice are developing multi-resistance to chemo-therapeutants

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(Fjørtoft et al., 2021). By 2017, farmed salmonids made up 99.6 % of the available hosts, contributing to 99.1 % of adult female salmon lice and 97.6 % of mated (ovigerous) adult female salmon lice in the coastal waters of Norway (Dempster et al., 2021). In response to these challenges, well-timed preventive measures may offer a more dynamic, efficient, and environmentally responsive strategy when applied according to natural lice population dynamics (Oldham et al., 2023). For instance, water treatments using environmentally neutral compounds (such as sodium percarbonate) has been proven successfully in controlling free-living stages of the ciliate fish parasite *Ichthyophthirius multifiliis* (Heinecke, Buchmann, 2009), thus providing support to the efficacy of well-timed water treatments. Moreover, exploring antiparasitic compounds of natural origin may help minimize the ecological impact of lice management in aquaculture.

Lime is a natural inorganic material primarily composed of oxides and hydroxides of calcium. One of these components, calcium oxide (CaO), or quicklime, has been successfully used as a controlling agent for echinoderms for the first time over a century ago (Wood, 1908). Since then, CaO particles have found applications in various settings, such as controlling sea urchin populations in Norwegian fjords (Strand et al., 2020), and managing sea urchins and starfish in commercial kelp and oyster beds in Nova Scotia and California (Bernstein and Welsford, 1982; Shumway et al., 1988). A recent laboratory study (Brooks et al., 2020) showed that the copepod *Tisbe battagliai* is extremely sensitive to exposure to CaO. When in contact with water, CaO produces calcium hydroxide, $Ca(OH)_2$, in an exothermic and alkalinizing reaction, increasing temperature and pH. This reaction results in epidermal burns and lesions in the target organisms, leading to acute and delayed mortality due to osmotic imbalances and bacterial infections (Bernstein and Welsford, 1982). Repeated exposure to 0.2 g/L CaO (0.1–0.3 mm diameter) for three consecutive weeks at both 5°C and 12°C in Atlantic salmon post-smolt within flow-through systems was proven to be safe for the fish. It did not cause mortality or histopathological damage to the skin, eyes, or intestine. While no gill inflammation or hyperplasia was observed, vascular damage and necrosis were detected in small portions of the tissue (<10 % of the surface) (Ciani et al., 2024).

Given the high efficacy of CaO particles against planktonic copepods, using quicklime as a natural antiparasitic treatment in salmonid farming shows promising potential for combating sea lice. The objective of this study is to develop and optimize an antiparasitic treatment using fine CaO particles, specifically targeting the salmon louse (*Lepeophtheirus salmonis*) under controlled laboratory conditions. This includes evaluating the CaO-induced effect on nauplii and copepodids survivability, molting, and the ability of copepodids to infect fish. The findings will provide a foundation for subsequent field trials.

2. Material and methods

The experiments were performed at the Sea Lice Research Centre (SLRC) at the University of Bergen (Norway). The experiments were performed according to EU regulations concerning the protection of experimental animals (Directive 2010/63/EU). Appropriate measures were taken to minimize pain and discomfort. The experiment was approved by the Norwegian Food and Safety Authority (FOTS ID 8589).

2.1. Experimental design

Salmon louse (*Lepeophtheirus salmonis*) wild type from an established laboratory strain, *Ls*Gulen (Hamre, Glover and Nilsen, 2009), and the resistant strain *Ls*H_2O_2 (Borchel et al., 2018), were raired under standard laboratory conditions (Salinity 34.5 ppt; pH 8; temperature 10℃). The exposure trials were performed in either rectangular box [White plastic; Height (H): 15 cm; Width (W) 8 cm; Length (L) 11 cm; Surface (S): 88 cm^2], glass beaker with magnetic stirring [Diameter (D): 9 cm; H: 15 cm; S: 63.6 cm^2; stirrer 5 cm] or flat containers [White ceramic plate (D: 26 cm; H: 2.5 cm; S: 532 cm^2] (Figure S1). When subjected to multiple CaO exposures, the lice were kept in incubators (volume 76 ml) with constant supply of fresh seawater (30 ml/min) between different trials. Atlantic salmon (*Salmo salar*) smolts, (sexually immature, mixed sex, weight 200–300 g) were reared under standard laboratory conditions and transferred to individual tanks (55 l; size 54 ×34 x 35 cm, internal measurements) during infection trials.

To evaluate the impact of CaO administration on water pH, this parameter was measured in dedicated trials using all three types of containers in the study, as well as in fish tanks (700 L), as described by Ciani et al. (2024). Based on these data, the pH was expected to remain stable between 8 and 8.7 in containers without stirring and rise up to 10 in those with stirring after 10 minutes of exposure.

Nine different laboratory trials (Fig. 1) were conducted to investigate different aspects of CaO treatment such as concentration, exposure time, frequency of treatment and administration procedure (Table S1). The protocols of the different trials are described individually in the following chapters.

2.1.1. Trial 1 – measuring CaO-induced mortality and molting arrest of nauplii-I and -II

Free-living nauplii-I (Age: day 1) and nauplii-II (Age: day 4) were divided in plastic containers filled with 0.5 L of seawater (Control: 6 replicates. Treatment: 15 replicates per stage and exposure time; 25 animals per container). Animals were given 10 minutes for acclimatization to the new conditions. The defined amount of CaO [0.1 g (0.2 g/L; 11 g/m^2)] or control substance (0.1 g fine sand particles) was added to the surface. The end of the application was considered as a treatment start. Three exposure times (2-, 5- and 10-min) were used. After exposure, the animals were collected with a 1 ml pipette, moved to a container with fresh seawater, rinsed from any particles and finally transferred to incubators with constant supply of the fresh seawater.

Mortality of the nauplii and inhibition of molting of surviving animals was measured via light microscopy after the controls reached copepodid stage (after 3 days for nauplii-I and after 24 h for nauplii-II at 10℃). Since the molts were synchronized (Fig. 2), any animals unable to molt within the expected time were considered inhibited.

2.1.2. Trial 2 – measuring CaO-induced mortality on copepodid. Optimization of concentration and exposure time

Free-living copepodids were divided in plastic containers filled with 0.5 L of seawater (3 replicates per group; 25 copepodids per container). Copepodids were given 10 minutes for acclimatization to the new conditions. Three different concentrations of CaO [0.1 g (0,2 g/L; 11 g/m^2); 0,2 g (0.4 g/L; 23 g/m^2) 0,3 g (0.6 g/L; 34 g/m^2)] or control substance (0.1 g; 0.2 g; 0.3 g of fine sand particles) were spread on the surface. The copepodids were manually transferred to new containers with fresh seawater after 2 min; 5 min and 10 min of exposure. Animals were rinsed from any particles and transferred to incubators[3] with constant supply of the fresh seawater. Copepodids were counted via light microscopy after 24 h, 48 h and 72 h for survival.

2.1.3. Trial 3 – comparing 1 × 15 min vs 15 × 1 min CaO treatments on copepodid mortality

Free-living copepodids were placed in glass beakers with magnetic stirring filled with 0.5 L of seawater (4 replicates per group; 25 animals per replicate). Animals were given 10 minutes for acclimatization to the new conditions. The defined amount of CaO [0.3 g (0.6 g/L; 47 g/m^2)] or control substance (0.3 g fine sand particles) was added to the surface. The animals were exposed to CaO 1 time for 15 minutes or 15 times for 1 minute. In 15 × 1 minute exposure group, new reactive CaO was added each time animals were transferred to a new container. The same procedure was performed in the control group. After the exposure, animals were manually collected and transferred to the container with fresh seawater, rinsed from any particles and transferred to the incubators with constant supply of the fresh water. After 72 hours, the survival rate of copepodids was assessed through counting via light

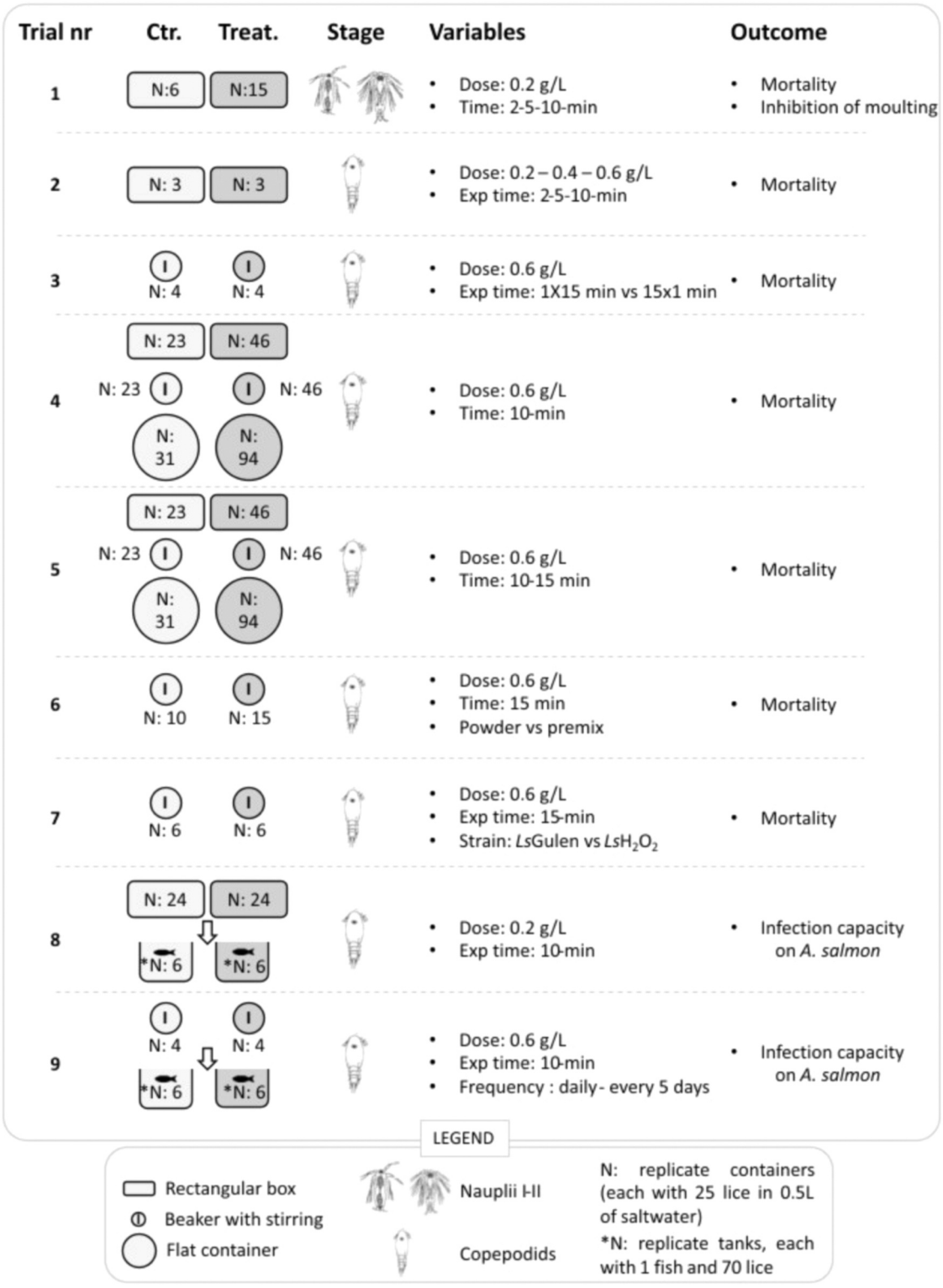

Fig. 1. Experimental design summary. The doses of CaO and the control substance (fine sand) range from 0.2 g/L to 0.6 g/L. The concentration per surface area (g/m^2) is detailed in the protocol section of the text, as it varies depending on the container used. "Exp. Time" indicates the duration of exposure to CaO or sand for the animals. The test substance is always administered as dry powder, except in trial 8, where it is pre-mixed with water in a syringe before administration. (N) indicates the number of replicate containers, each with 25 lice in 0.5 L of saltwater.

microscopy.

2.1.4. Trial 4 – measuring CaO-induced mortality on setups with different surface to volume ratio

Free-living copepodids were divided between three different types of containers (see Section 2.1 for specific dimensions, Figure S1) each filled with 0.5 L of seawater: i) Glass beakers with magnetic stirring (n = 23 control, 46 treatment; 25 cop. per container). The water was constantly stirred to simulate currents and promote particle suspension; ii) Flat containers (n = 23 control; 46 treatment; 25 cop. per container). These

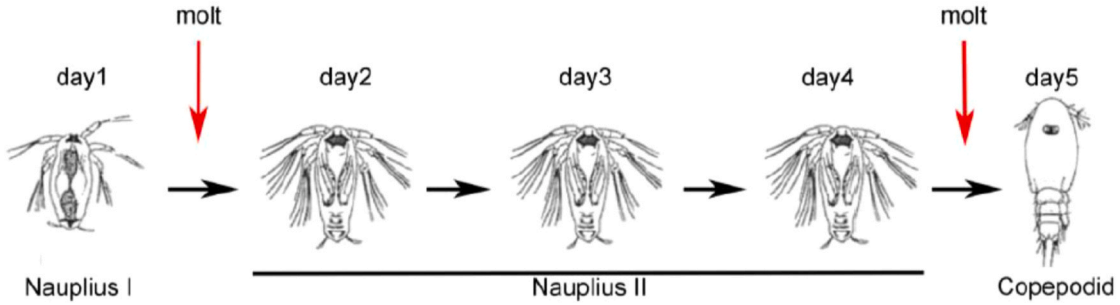

Fig. 2. Free-living stages: from hatching of the nauplius-I (day 1) to infectious copepodid stage. The red arrows indicate molts to the next stage. (Modified from Johnson and Albright, 1991).

containers have a higher surface to volume ratio (532 cm^2/0.5 L) thus simulating CaO administration in a large area.; iii) Deep plastic containers (n = 31 control; 94 treatment; 25 cop. per container). These containers have a lower surface to volume ratio (88 cm^2/0.5 L), thus simulating CaO administration in a smaller area.

Copepodids were given 10 minutes for acclimatization to the new conditions. The defined amount of CaO (0.3 g; concentration 0.6 g/L) or control substance (0.3 g fine sand particles) was spread on the surface. Given the varying surface-to-volume ratios of the containers, the concentration of CaO per surface area was 4.7 g/m^2, 0.6 g/m^2, and 3.4 g/m^2 in containers I, II, and III, respectively. After 10 minutes, animals were manually collected and transferred to containers with fresh seawater. Animals were rinsed from any particles and transferred to incubators with constant supply of the fresh water. After 72 hours, the survival rate of copepodids was assessed through counting via light microscopy.

2.1.5. Trial 5 – measuring CaO-induced mortality on setups with different surface to volume ratio modulating exposure times

This trial replicated the protocol of trial 4 using both 10- and 15-minutes CaO exposure time.

2.1.6. Trial 6 – simulating CaO exposure procedure in field, where CaO is premixed with water before use

Copepodids were placed in glass beakers with magnetic stirring filled with 0.5 L of seawater (Control: 10 replicates; Treatment: 15 replicates per technique; 25 animals per beaker). Animals were given 10 minutes for acclimatization to the new conditions. The defined amount of CaO [0.3 g (0.6 g/L; 47 g/m^2)] was administrated with two different techniques: i) spread on the surface as a powder or ii) premixed with 4 ml seawater in a 10 ml syringe and mixed for 3–5 seconds before adding it to the baker surface. The control substance (0.3 g fine sand particles) was spread on the surface. This premix simulated typical treatment conditions on the fish farm, where CaO is mixed with water just before use. The animals were exposed to CaO for 15 minutes. After defined time, animals were manually collected and transferred to the container with fresh seawater. Animals were rinsed from any particles and transferred to the incubators with constant supply of the fresh water. Mortality was documented 72 h later.

2.1.7. Trial 7 – comparing CaO-induced mortality between wild type and resistant strain (LsGulen and LsH_2O_2)

Two different *L. salmonis* strains, *Ls*Gulen and *Ls*H_2O_2 were used in this trial. Copepodids were divided in glass beakers with stirring, filled with 0.5 L of seawater (6 replicates per group each strain; 25 copepodids per beaker). They were given 10 minutes for acclimatization to the new conditions. The defined amount of CaO [0.3 g (0.6 g/L; 47 g/m^2)] or control substance (0.3 g fine sand particles) were premixed with seawater in a syringe as in Trial 8. The animals were exposed to CaO for 15 minutes then transferred to containers with fresh seawater. Animals were rinsed from any CaO particles and moved to incubators with constant supply of the fresh water. Mortality was documented 72 h later.

2.1.8. Trial 8 – measuring the effects of CaO treatment on the capacity of copepodid to infest fish

Free-living copepodids were divided in plastic containers filled with 0.5 L of seawater (24 replicates per group; 25 animals per container). Copepodids were given 10 minutes for acclimatization to the new conditions. CaO 0.1 g (0.2 g/L; 11 g/m^2) or control substance (fine sand particles; 0.1 g) were spread on the surface. After 10 min of exposure, copepodids were transferred to new containers, rinsed from any particles, then moved to incubators[3] with a constant supply of fresh seawater. Meanwhile, twelve Atlantic salmon (smolt 200–300 g) were distributed in individual tanks[5]. Healthy copepodids from treatment and control group were selected 48 h after the treatment and used to infest fish (70 copepodids per fish; 6 fish per group). Water level in the fish tanks was lowered and copepodids added to the tanks. After 10 minutes, water flow was restarted (5–6 L/min). After 5 weeks, fish were anesthetized (methomidate (5 mg/L) and benzocaine (60 mg/L) for 3 minutes) and lice collected and staged. The percentage of lice (at chalimus, pre-adult and adult stages) that was attached to the fish was compared between treated and control group.

2.1.9. Trial 9 – measuring the effects of CaO treatment frequency on the capacity of copepodid to infest fish

Free-living copepodids were placed in glass beakers with mechanical stirring filled with 0.5 L of seawater (4 replicates per group; 25 copepodids per beaker). Copepodids were given 10 minutes for acclimatization to the new conditions. The defined amount of CaO [0.3 g (0.6 g/L; 4.7 g/m^2)] or control substance (0.3 g fine sand particles) was added to the surface. After 10 min, animals were transferred to containers with fresh seawater, rinsed from any particles then moved to incubators with constant supply of fresh seawater. Copepodids were treated for 7 days with different frequencies: i) everyday ED ii) every second day ESD iii) every third day ETD iv) every fourth day EFD and v) every fifth day EFiD (Table 1). Control beakers were treated daily.

At day 9, 48-hours after the treatment week's conclusion, 100 healthy copepodids from each group were selected to infest the fish. A total of 24 fish, 4 per group, were placed in individual tanks. The water level in each tank was lowered, and copepodids were added. After a 10-minute interval, water flow was restarted (5–6 L/min). Following a 5-

Table 1
Summary of treatment (x) frequency. Counting (c) was performed on day 9. (ED) Every Day; (ESD) Every Second Day; (ETD) Every Third Day; (EFD) Every Fourth Day; (EFiD) Every Fifth Day.

Group	Days								
	1	2	3	4	5	6	7	8	9
Control	x	x	x	x	x	x	x		c
ED	x	x	x	x	x	x	x		c
ESD	x		x		x		x		c
ETD	x			x			x		c
EFD	x				x				c
EFiD	x					x			c

week period, the fish were anesthetized using the standard protocol involving methomidate (5 mg/L) and benzocaine (60 mg/L) for 3 minutes. The percentage of lice successfully attaching to the fish was compared between the treated and control groups, as was done in trial 4.

2.2. CaO formulation

Two CaO formulations were used in the study. Procalx (Franzefoss Minerals AS) was used in all trials except nr. 3–5 −6, where Brentkalk (Franzefoss Minerals AS) was used instead. Both formulations had equal particle composition (CaO ≥ 94 %; particle size: 0.1 mm - 0,6 mm) and reactivity in water ($t_{60} \leq 3$ min).

2.3. Statistical analysis

Statistical analysis were performed using Stata 17 (StataCorp, 2021). Results from all the tests are available in Supplementary file 1. The test performed were selected according to the type of data. A mixed-effect logistic regression followed by multiple comparison (Bonferroni adjustment) was used to assess statistically significant ($p < 0.05$) effect of CaO treatment (included as fixed effect) to all the trials with a dichotomous outcome such as i) mortality (alive vs dead); ii) attachment (attached vs not attached). Based on the experimental design, the duration of the treatment and the frequency were considered as fixed effects, while the cage or fish were included as random effect. Kaplan-Meyer survival index followed by log-rank test were used to validate the effects of CaO treatment and duration on mortality rates over time. LC_{50} were extrapolated from marginal predictions after mixed-effect logistic regression.

3. Results

3.1. Trial 1 − CaO-induced mortality and molting arrest of nauplii-I and -II

Exposure to CaO (0.2 g/L) increased mortality and inhibition of molting in nauplii, as compared to control. Both parameters increased with exposure time, showing the highest values after 10 min exposure to CaO (Fig. 3; Fig. 4; Tables S2 and S3).

The exposure to CaO of nauplii-I caused, in average, mortality of 36.7 % and molting arrest in 13.5 % of treated animals. The most efficient treatment time was 10 minutes, with mortality of 41.5 % and molting arrest of 20.8 % (Fig. 3; Table S2). The multilevel logistic regression test (χ^2 137.44; $p < 0.001$) confirmed that CaO treatment significantly reduced (Odds ratio 0.007; $p < 0.001$) the percentage of Nauplii-I reaching the copepodid stage. This effect was further enhanced from treatment time (Odds ratio = 0.54; $p = 0.002$). The random component of the model indicated a statistically significant variation between replicate tanks (Coeff: 1.92; $p < 0.001$).

The treatment of nauplii-II (day 4 post hatching) caused, in average, mortality of 24.1 % and molting arrest of 31.3 % of treated animals. The most efficient treatment time was 10 minutes, with mortality of 29.0 % and molting arrest of 39.2 % (Fig. 4; Table S3). When considering both mortality and molting arrest, 62.3 % of nauplii-I and 68.2 % nauplii-II were not able to molt to the following life stage after 10 min exposure to 0.1 g CaO. The multilevel logistic regression test (χ^2 126.1; $p < 0.001$) confirmed that CaO treatment significantly reduced (Odds ratio 0.01; $p < 0.001$) the percentage of Nauplii-I reaching the copepodid stage. This effect was further enhanced from treatment time (Odds ratio = 0.61; $p = 0.002$). The random component of the model indicated a statistically significant variation between replicate tanks (Coeff: 1.33; $p < 0.001$).

3.2. Trial 2 CaO-induced mortality on copepodid is concentration and exposure time dependent

No copepodid died in the control group at any concentration. On the other hand, copepodids mortality after CaO exposure was positively correlated with concentration (Log-rank χ^2 312.37; $p < 0.001$) and treatment time (Log-rank χ^2 36.85;; $p < 0.001$), ranging from 6.7 % (at day 3; exposure to 0.1 g CaO for 2 min), to 89.3 % (at day 3; exposure to 0.3 g CaO for 10 min).

When grouping by concentration (Fig. 5), the mortality rates went from 12.9 % in the 0.2 g/L CaO group to 57.8 % in the 0.6 g/L group. When grouping by exposure time, 2 minutes exposure caused death in 24 % of copepodids, 5 minutes - 33.6 % and 10 minutes - 45.9 %, (Table S4).

LC_{50} values were 0.36–0.45- 0.54-g/L (or 20.45 – 25.57–30.68 - -g/m^2) respectively for 10–5- and 2-min exposure (Fig. 6).

3.3. Trial 3–15 × 1 min CaO treatments induce higher copepodid mortality than 1 × 15 min treatment

Copepodids mortality was higher when animals were treated 15 times x 1 minute (73 %) than when they were treated once for 15 minutes (57 %) (Fig. 7; Table S5). Both treatments strategies caused

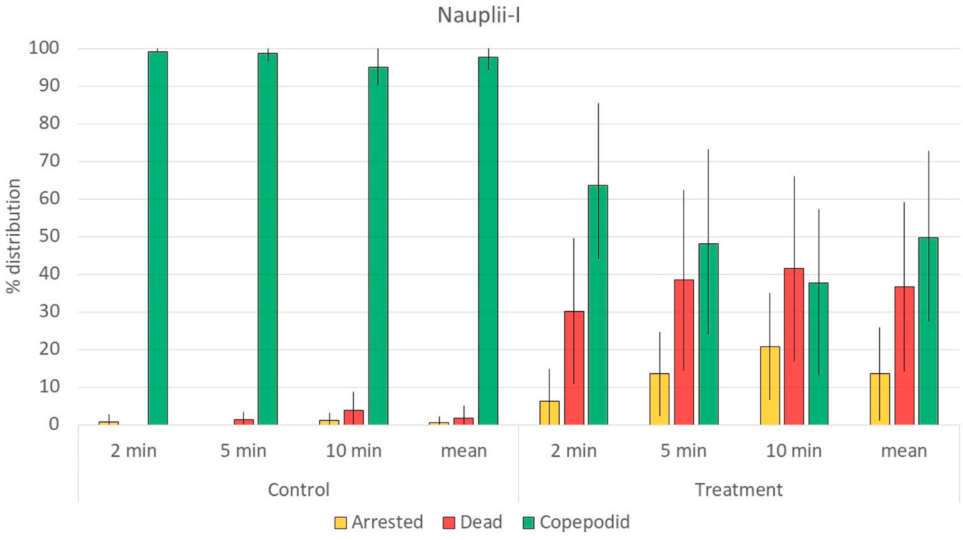

Fig. 3. Effects of CaO exposure (0.2 g/L) for 2-, 5-, or 10 minutes in nauplii-I. Mean percentage distribution ± SD of lice found dead, arrested (still at nauplii stage - inhibited molting), or at the copepodid stage 6 days post hatching in treated and control groups.

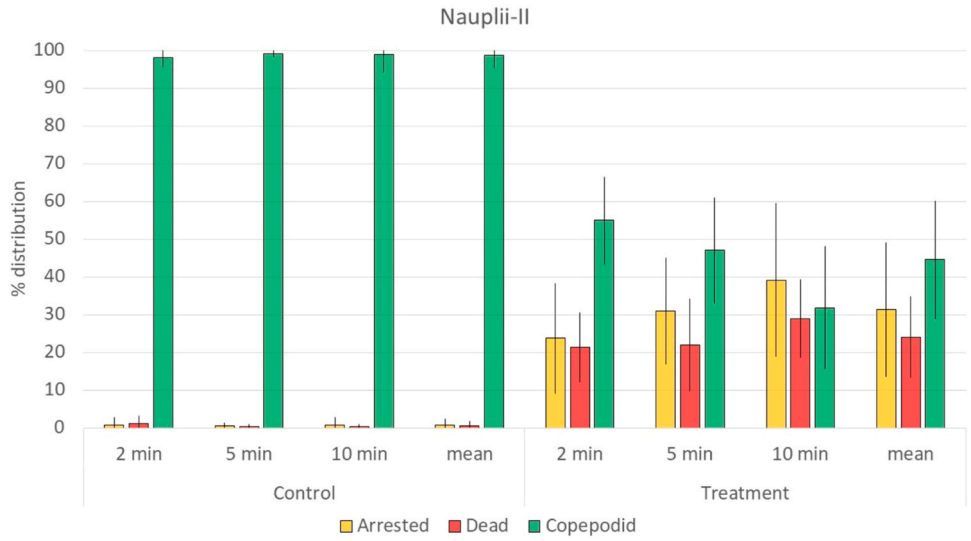

Fig. 4. Effects of CaO exposure (0.2 g/L) for 2-, 5-, or 10 minutes in nauplii-II. Mean percentage distribution ± SD of lice found dead, arrested (still at nauplii stage - inhibited molting), or at the copepodid stage 6 days post hatching in treated and control groups.

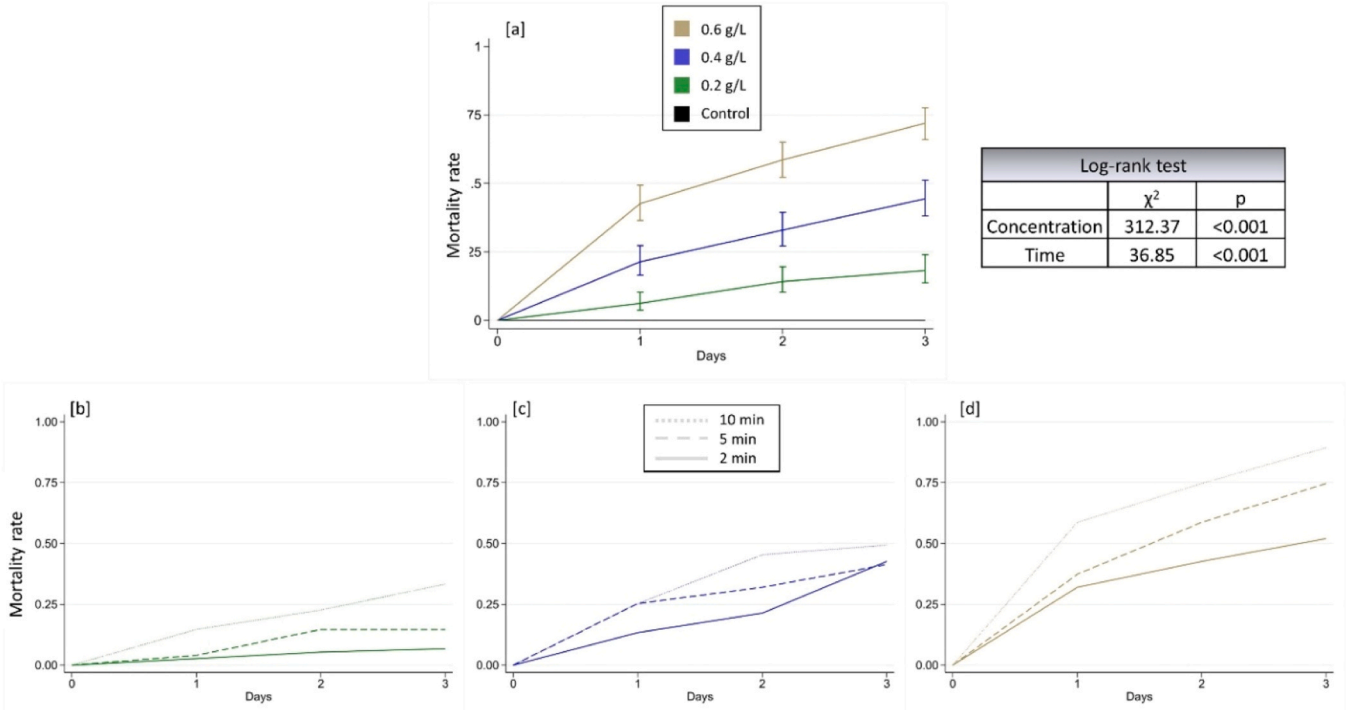

Fig. 5. [a] Kaplan-Meyer cumulative mortality index ± 95 % CI for Control; 0.2-; 0.4-; 0.6-g/L CaO groups (black, green, blue, brown lines, respectively) as average of the three exposure durations. [b-c-d] Kaplan-Meyer cumulative mortality index of [b] 0.2 g/L [c] 0.4 g/L [d] 0.6 g/L exposed for 2–4- or 6-minutes (continued, dashed, and dotted lines, respectively).

higher mortality than control group (5.2 %).

Multilevel logistic regression was used to analyze the relationship between CaO treatment and copepodids mortality. The test was strongly significant (χ^2 61.7; $p < 0.001$) indicating an effect of treatment over copepodid mortality. It was found that, the odds of mortality increased 20.8 times (95 % CI [8.3 – 51.9]; $p < 0.001$) when animals were treated with one dose for 15 min and increased 42.4 times (95 % CI [16.6–108], $p < 0.001$) when animals were treated with 15 doses for one minute. The random component of the model indicated no statistically significant variation between replicate tanks.

3.4. Trial 4 – water agitation reduces CaO-induced mortality

Copepodids mortality in CaO-treated containers was significantly higher (Mixed-effect logistic regression: χ^2 (5) 287.54; $p < 0.001$) than their respective controls across all setups (Fig. 8; Table S6). Additionally, containers without stirring showed significantly higher mortality rates (flat: 67.5 %; deep: 66.2 %) compared to those with stirring (44.1 %). The random component of the model indicated significant variation between replicates (Coeff: 0.79; 95 % CI 0.59 – 1.0).

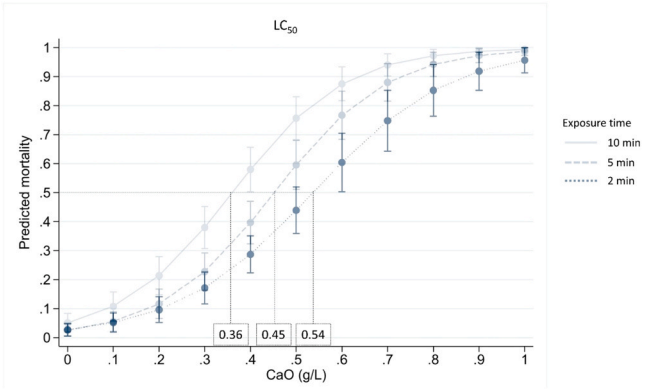

Fig. 6. The predicted mortality curves (mean ± SD) and LC_{50} values (shown in boxes above the x-axis) are extrapolated from marginal predictions following mixed-effects logistic regression. These predictions are based on the three-day survival trial after 2, 5, and 10 minutes of exposure to 0.2–0.6 g/L CaO.

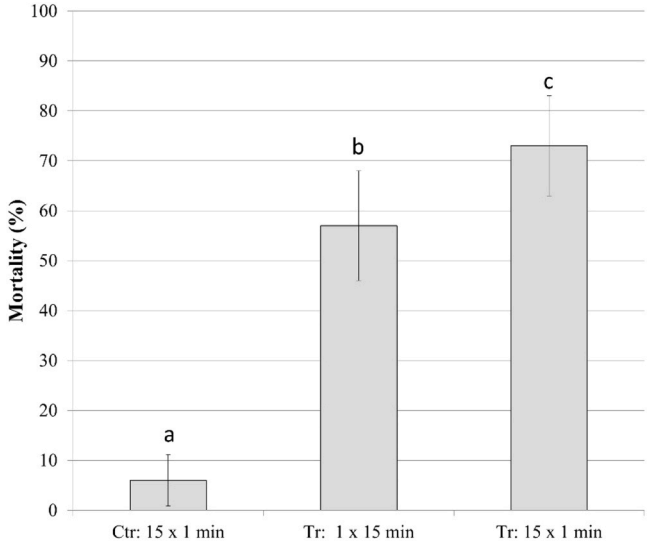

Fig. 7. Mortality rates (mean ± SD) 72 hours after exposure to 0.6 g/L CaO once for 15 minutes (1 ×15 min) or 15 times for 1 minute (15 ×1 min). Different letters denote statistically significant differences between groups according to multilevel logistic regression followed by multiple comparison between groups (Bonferroni adjustment, $p < 0.05$).

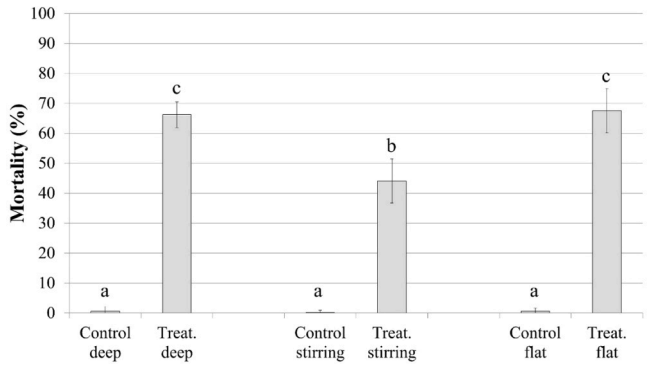

Fig. 8. Mean mortality ± SD of the different experimental setup in control and treatment group. Different letters denote statistically significant ($p < 0.05$) differences between groups according to mixed effect logistic regression followed by multiple comparison (Bonferroni adjustment).

3.5. Trial 5–10 and 15 Minutes of CaO Exposure induce comparable mortality

The mortality rates after CaO exposure followed same pattern observed in Trial 6: highest mortality in the containers without stirring (deep: 76.3 % average, flat: 77.3 % average) than with stirring (55.7 % average) (Fig. 9; Table S7) (Mixed-effect logistic regression (χ^2 (3) 25.48; $p < 0.001$). Increasing exposure time from 10 to 15 minutes had no statistically significant effects on mortality rates. The random component of the model indicated significant variation between replicates (Coeff: 0.17; 95 % CI 0.06 – 0.5).

3.6. Trial 6 – water premixing does not affect CaO treatment efficacy

No statistically significant differences in copepodid mortality were observed when premixing CaO with water compared to spreading the dry powder over the water (Fig. 10; Table S8). No mortality was recorded in control group.

3.7. Trial 7- CaO treatment is equally effective on LsH_2O_2 strain

No statistically significant differences in mortality rates were reported between *Ls*Gulen and *Ls*H2O2 strains after CaO exposure (Fig. 11; Table S9).

3.8. Trial 8 – CaO treatment on copepodid results in less infective lice on salmon

The number of lice attached to the fish (as chalimus, pre-adult or adult) five weeks after CaO exposure was 67.8 % lower in the treated group compared to the control group. Only 13.6 % of lice treated with CaO were found attached to the fish in comparison to 42.1 % the control group (Fig. 12; Table S10).

Multilevel logistic regression was used to analyze the relationship between CaO treatment and the number of parasitic lice. The test was strongly significant (χ^2 78.3; $p < 0.001$) indicating a significant effect of treatment (odds ratio 0.21; 95 % CI [0.15 – 0.30]; $p < 0.001$). The random component of the model indicated no statistically significant variation between replicate fish.

3.9. Trial 9 – reduced frequency of CaO treatment leads to lower mortality

The number of lice found on the fish (as chalimus, pre-adult or adult) five weeks after CaO exposure decreased with the frequency of CaO treatment (Multilevel logistic regression χ^2 41.42; $p < 0.001$). Treatments executed from daily to every third day induced a significant ≈ 64 % reduction in the number of parasitic lice compared to control (Fig. 13; Table S11). Treatments performed every four of five days resulted in no significant reduction. The random component of the model indicated significant variation between fish (Coeff: 0.10; 95 % CI 0.03 – 0.32)

4. Discussion

The most commonly used water-treatment protocols against salmon louse (*Lepeophtheirus salmonis*) are intended for parasitic stage and there is currently a shortage of preventive treatments for planktonic/free-living stages (Coates et al., 2021). This study provides evidence of the detrimental effects of fine CaO particles on the larval stages of the salmon louse in laboratory trials. The research highlights a substantial reduction of survivability, molting, and infectivity of the planktonic stages following CaO treatment.

This study tested the effects of particle exposure. It is worth to differentiate between dissolved and particulate CaO, as the former is relatively harmless to marine organisms and can actually contribute to

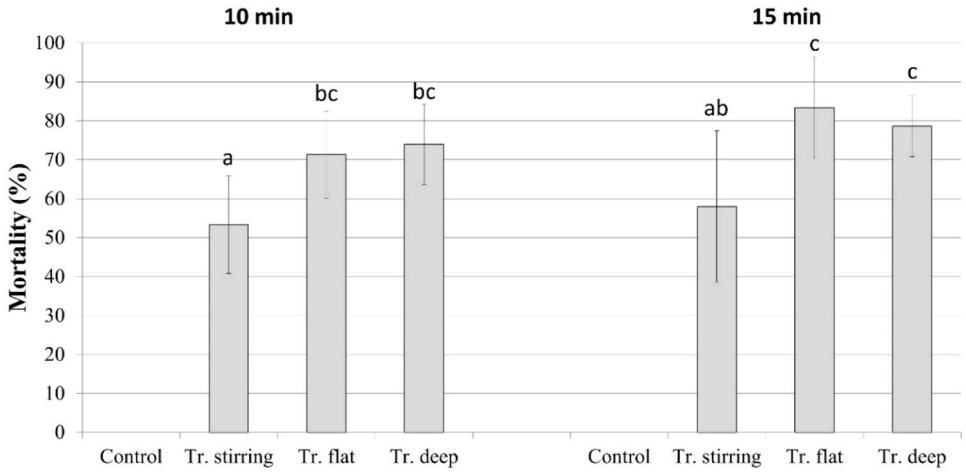

Fig. 9. Mean mortality ± SD of the different experimental setup in control and treatment group exposed for 10 or 15 minutes to 0.6 g/L CaO. Different letters denote statistically significant ($p < 0.05$) differences between groups according to mixed effect logistic regression followed by multiple comparison (Bonferroni adjustment).

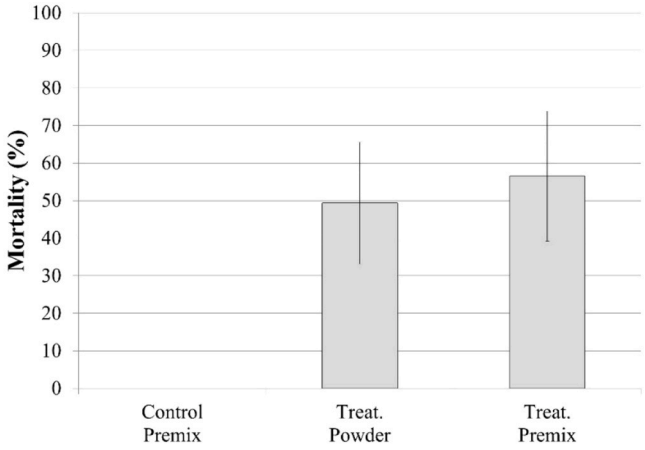

Fig. 10. Mean mortality (± SD) was compared between control and treatment groups (15 minutes exposure to 0.6 g/L CaO), administered either as dry powder or pre-mixed with water. Pre-mixing the CaO with water did not affect the mortality rates ($p > 0.05$ mixed effect logistic regression).

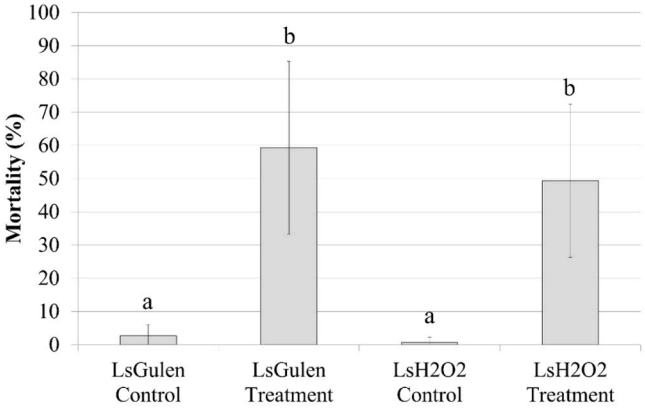

Fig. 11. Mean mortality (± SD) was measured for two different L. salmonis strains (LsGulen and LsH2O2) exposed to 0.6 g/L CaO for 15 minutes and their respective controls. Different letters indicate statistically significant differences between groups ($p < 0.05$), as determined by mixed effect logistic regression followed by multiple comparisons with Bonferroni adjustment. No significant differences in mortality rates were observed between the two strains.

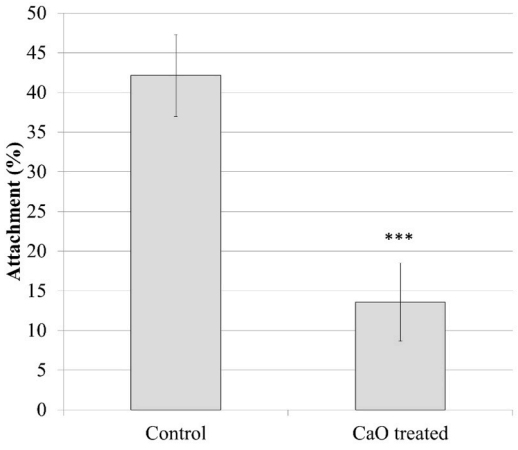

Fig. 12. The percentage of lice (chalimus, pre-adult, and adult stages) attached to the fish was measured five weeks post-infection in both control and CaO-treated groups (0.2 g/L for 10 minutes). Lice used in the infection trial were given 48 hours to recover from the treatment prior to infection. A total of 70 healthy-looking lice were used per fish in the trial. The effect of CaO treatment was evaluated using multilevel logistic regression (***$p < 0.001$), and the results are presented as mean ± SD.

their resistance against divalent metal toxicity (Das and Das, 2005). Particulate CaO, on the other hand, triggers an exothermic and alkalinizing reaction when in contact with water which can lead to epithelial burns and lesions in target organisms. This may directly kill the organism or cause infections leading to death after a few days (Bernstein and Welsford, 1982). In this study, fine CaO particles (< 0.8 mm) were chosen for their enhanced reactivity and toxicity compared to coarse particles (Brooks et al., 2020), primarily due to their increased surface-to-volume ratio. The changes in pH and temperature were not measured in real time in all trials to avoid unnecessary bias between replicates, as probe clogging from particle suspension and the small container size could interfere with particle diffusion. Instead, measurements were conducted in dedicated tests using the current setup, as well as in additional trials described previously (Ciani et al., 2024). The expected pH variation ranged from 8 to 8.75 in beakers without stirring and could rise to pH 10 in beakers with stirring.

The present study demonstrated that CaO exposure have detrimental effect on *L. salmonis* planktonic stages by increasing mortality, inhibiting molting and reducing infectivity to the salmon. The effect of CaO treatment is concentration and exposure dependent for all parameters

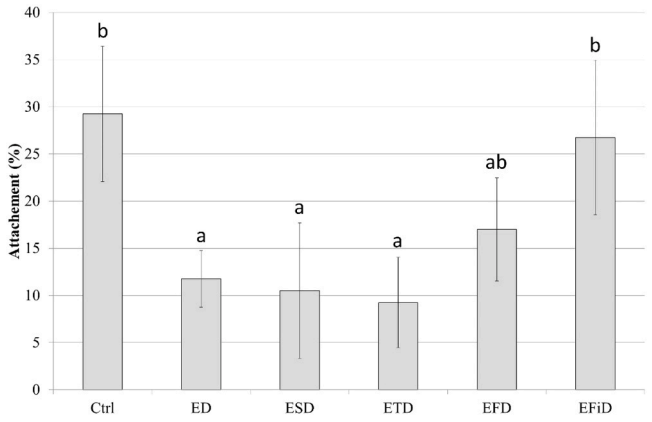

Fig. 13. The percentage of lice (chalimus, pre-adult, or adult stages) attached to the fish measured five weeks post-infection in control and CaO-treated groups (0.2 g/L for 10 minutes) according to the frequency of the treatment, from every day (ED) to every five days (EFiD). Lice used for the infection trial were given 48 hours to recover from the treatment before infection. A total of 70 healthy-looking lice were used for each fish in the trial. Different letters denote statistically significant differences ($p < 0.05$) between groups according to multilevel logistic regression followed by multiple comparison (Bonferroni adjustment).

analyzed with longer treatment at higher concentration producing the strongest results. Exposure to 0.2 g/L [11 g/m^2] CaO for 2 minutes reduced the number of nauplii-I and nauplii-II reaching the copepodid stage by 35.9 % and 43.9 % respectively as compared to control. A 10-minute exposure to the same concentration reduced these numbers to 60.3 % and 67.8 %. This outcome was attributed to both increased mortality and inhibited molting. Similarly, the mortality rates of free-living copepodids exposed to CaO ranged from 6.7 % after a 2-minute treatment with 0.2 g/L CaO to almost 90 % after a 10-minute exposure to 0.6 g/L CaO. The frequency of treatment also played a significant role, with the best results observed when treating the animals daily to every three days. Notably, constantly replacing reactive CaO particles increased copepodid mortality compared to a single treatment of the same duration. This is likely because CaO reacts rapidly with water, and introducing new CaO leads to further damage to the lice. CaO treatment also reduced the infectivity of copepodids to salmon. The exact cause of this reduction is unclear; it could be due to decreased survivability, reduced ability to attach to the host, or other factors. However, the number of parasitic lice found on salmon five weeks after exposure to copepodids that survived CaO treatment was reduced by 65 % compared to the control.

Few previous studies have tested the effects of CaO treatment on marine invertebrates. A study on *Tisbe battagliai*, another member of the copepod family, reported an LC_{50} of 3.14 g/m^2 for fine CaO particles (Brooks et al., 2020). The current investigation determined an LC_{50} of 0.36 g/L (equivalent to 20.45 g/m^2) for *L. salmonis* in copepodid stage. It's worth noting that differences in the experimental conditions may account for the disparity in these values. The earlier study exposed the animals to CaO for one hour at a water temperature of 20°C, with mortality recorded 10 days after exposure. In contrast, the current study subjected the animals to CaO from 2 to 10 minutes at 10°C, and mortality was assessed 3 days after exposure. Indeed, the variation in exposure times is a crucial factor in the differing toxicity values observed. As demonstrated by the lower toxicity associated with shorter exposure times of 5 or 2 minutes. Brooks and colleagues also showed that copepods have a higher sensitivity (lower LC_{50}) compared to other marine taxa such as echinoderm [sea urchin (*Strongylocentrotus droebachiensis*), 20.1 g/m^2; starfish (*Asterias ruben*) 22.2 g/m^2] polychaete [ragworm (*Nereis pelagica*) 29.6 g/m^2] mollusks [netted dog welch (*Hinia reticulata*) 41.9 g/m^2] or teleost [Lump sucker (*Cyclopterus sp.*) 226 g/m^2]. A laboratory study showed that five exposures to fine CaO particles (0.2 g/L) repeated over the course of three weeks did not induced mortality in Atlantic salmon post-smolts (Ciani et al., 2024). Histopathological assessment confirmed the absence of CaO-induced damage to skin, eyes or intestine. However, it indicated CaO-induced vascular damages and necrosis in limited portions of the gills, extending to less than 10 % of the gill surface in 60 % and 30 % of the sampled tissues respectively. Even though the affected area was limited, these findings raise concerns about potential long-term effects on fish health and welfare. Future research should aim to refine exposure protocols to minimize any adverse effects, ensuring that the treatment remains both effective and ethically responsible. Additionally, studies should assess behavioral responses, stress levels, and recovery post-exposure to fully evaluate the welfare implications.

This study also aimed to simulate, in a laboratory setting, certain conditions that might be encountered when applying CaO at sea. To simulate water currents, copepodids were exposed in beaker with stirring. Interestingly, administering CaO with moving water resulted in lower mortality compared to still water. This is likely due to reduced contact time between the active particles and the animals. In systems with stirring, some particles accumulated at the bottom of the containers, while the animals moved towards the surface. This suggests that, in an open sea farm, the intensity of the water currents could probably influence the efficacy of the treatment. Indeed, this hypothesis requires testing in actual field conditions. The study also simulated the administration procedure used in the field, where CaO is pre-mixed with water and sprayed over the target area using a dedicated pump to ensure product homogeneity. In the lab, this process was simulated by pre-mixing CaO with water in a syringe before distributing it into the experimental containers. Interestingly, this methodology did not compromise the treatment's efficacy when compared to traditional methods where dry CaO particles are directly applied to the surface. When transitioning from laboratory to field trials, it is crucial to acknowledge the influence of several other variables. Factors such as suspended particles, organic matter, plankton, fish or different buffering capacity of sea water could impact the treatment's effects. Moreover, controlling the treatment volume becomes challenging in a semi-closed system where water can freely circulate through the cage. Another limitation is the necessity of repeating the treatment over time. Despite these challenges, and the clear need for further research to optimize the protocol for farming conditions, the ability of CaO to target planktonic stages makes it a promising preventive measure to reduce lice pressure and decrease the need for reactive measures such as delousing.

Reactive measures aim to reduce the number of lice after they have attached to the host, whereas preventive measures focus on lowering the rate of new infections (Barrett et al., 2020). Preventive measures offer several benefits including: i) a small impact on non-target organisms (Burridge et al., 2010; Taranger et al., 2015); ii) a decrease in the need of delousing, which can otherwise stress and injure the fish; iii) the ability to combine multiple measures simultaneously (Bui et al., 2020; Gentry et al., 2020) and iv) the possibility to administrate the therapy following lice dynamics and environmental cycles. Preventive measures have been recently applied with varying efficiency, such as physical barriers [weighted median reduction (wmr) 78 %], manipulation of salmon swimming depth (wmr 26 %) functional feeds (wmr 24 %) vaccines (wmr 4 %) (Barrett et al., 2020). Chemotherapeutants have also been widely used for lice control. However, a major drawback of chemical treatment is the development of resistance (Aaen et al., 2015; Helgesen et al., 2015). For instance, hydrogen peroxide efficacy dropped from 75 % to 8 % in a farm after 8 years of use (Treasurer, Wadsworth and Grant, 2000). Resistant strains can develop an EC_{50} from 3 to 100 times higher than sensitive strains (Coates et al., 2021). The present study also showed that the strain *Ls*H_2O_2, a robust strain resistant to H_2O_2, pyrethroids, organophosphates and several other commonly used drugs (Borchel et al., 2018), was not resistant to CaO, showing similar mortality rates to the *Ls*Gulen strain. Indeed, to mitigate the eventual

development of resistance, a cautious approach to treatment is necessary, applying it only when needed and considering population dynamics rather than extensive and continuous use over time.

5. Conclusion

Exposure to fine CaO particles significantly reduced the survivability of *L. salmonis* nauplii and copepodid stages in laboratory conditions. A 10-minute exposure to 0.2 g/L CaO reduced the number of nauplii reaching the copepodid stage by 60–70 %, affecting both survivability and molting. Additionally, a 10-minute exposure to 0.6 g/L CaO resulted in almost 90 % removal of free-living copepodids. Only 13.6 % of copepodids that survived CaO treatment were found as chalimus, preadult, or adult stages on salmon five weeks post-infection, compared to 42.1 % in the control group, resulting in a 67.6 % reduction in parasitic lice on the fish. The effectiveness of the treatment depends on the concentration, length of exposure, and frequency of treatment. The highest efficacy was observed with 0.6 g/L CaO for 15 minutes, repeated at least every three days. This laboratory study represents a necessary initial step to characterize the efficacy of CaO exposure in *L. salmonis* and provides a basis for future field trials to evaluate its potential as an antiparasitic treatment in aquaculture.

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CRediT authorship contribution statement

Marit Stormoen: Writing – review & editing, Data curation. **Elia Ciani:** Writing – review & editing, Writing – original draft, Formal analysis. **Stein Ivar Antonsen:** Writing – review & editing, Resources. **Jørgensen Even:** Writing – review & editing. **Frank Nilsen:** Writing – review & editing, Resources, Conceptualization. **Komisarczuk Anna:** Writing – review & editing, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Elia Ciani reports financial support was provided by Seacalx AS. Stein Ivar Antonsen reports a relationship with Seacalx AS that includes: board membership and employment. Elia Ciani reports a relationship with Seacalx AS that includes: funding grants. The research costs were financed from Seacalx AS. The postdoc position of Elia Ciani was 50 % financed from Seacalx AS. Stein Ivar Antonsen in the CEO of Seacalx AS. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors statement

The authors declare that data will be available under request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2025.102894.

Data availability

Data will be made available on request.

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