DOI: 10.1111/are.13627

ORIGINAL ARTICLE

WILEY Aquaculture Research

# Effect of hydrogen peroxide as treatment for amoebic gill disease in Atlantic salmon (*Salmo salar* L.) in different temperatures

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**Funding information**
Akzo Nobel Pulp and Performance Chemicals

## Abstract

Amoebic gill disease (AGD) is a pathogenic disease in salmonids caused by *Neoparamoeba perurans*. Treatment of AGD infection has been through freshwater bathing of the fish. However, as the availability of fresh water is often limited, hydrogen peroxide has been introduced as an alternative treatment. This study investigated the effect of hydrogen peroxide as treatment for AGD-infected salmon (*Salmo salar L.*,) at different seawater temperatures and hydrogen peroxide dosages. In total, 600 fish were challenged with *N. perurans* and the severity of the AGD infection was measured using a gill score scale. After challenge and disease development, the fish were distributed into 12 tanks. The treatment was performed at different seawater temperatures (8°C, 12°C, 17°C) using different hydrogen peroxide doses. Each temperature included an untreated control group. Linear models were used to analyse gill score. A significant effect of treatment was found ($-0.68 \pm 0.05$) regardless of dose and temperature, suggesting that hydrogen peroxide was effective in treating AGD. When the model included dose, a negative linear relationship between dose and gill score was found. The study proved that treatment of AGD with hydrogen peroxide was successful, as gills partially recovered following treatment and further disease development was delayed.

**KEYWORDS**
Amoebic gill disease, Atlantic salmon, hydrogen peroxide, treatment

## 1 | INTRODUCTION

Amoebic gill disease (AGD) is a parasitic disease that affects salmonids in the seawater phase and is caused by the marine amphizoic amoeba *Neoparamoeba perurans* (Young, Crosbie, Adams, Nowak & Morrison, 2007). The disease attacks the respiratory system of the fish, causing lesions or white and grey mucoid spots on the gill surface, which lead to reduced gill function (Mitchell & Rodger, 2011; Rodger, 2014). The physiological characteristics of an AGD-infected salmon are lethargy, reduced swimming activity, increased respiratory rate, reduced feed intake and reduced growth (Bustos et al., 2011; Norwegian Scientific Committee for Food Safety, 2014; Steinum et al., 2008). The development of the disease is dependent on seawater temperature and salinity, which has made AGD a problem for the farmed salmon industry in areas with higher salinity and seawater temperatures (Norwegian Scientific Committee for Food Safety, 2014). The disease was first discovered in Tasmania in the mid-1980s and today the disease has spread to Northern European countries such as Ireland, Scotland and the Faroe Islands. The first AGD outbreak in Norway occurred in 2006 (Norwegian Scientific

Committee for Food Safety, 2014; Steinum et al., 2008). Since then, AGD has become more frequent along the Norwegian coast, and by 2013, over 60 different sites had registered AGD outbreaks (Bornø & Linaker, 2015). The disease is a considerable challenge for the Norwegian farmed salmon industry, causing both economic losses as well as reduction in fish robustness and health (Norwegian Scientific Committee for Food Safety, 2014; Oldham, Rodger & Nowak, 2016).

Amoebic gill disease also affects other species besides salmonids (Munday, Zilberg & Findlay, 2001), and there is a risk that AGD outbreaks from the aquaculture industry may spread to wild populations. No vaccines or other effective preventive measures exist and the current methods to treat AGD are through freshwater or hydrogen peroxide bathing of the fish (Ruane & Jones, 2013). Although fresh water is used routinely in Tasmania (Powell & Kristensen, 2014), a study suggested that hydrogen peroxide could be a suitable alternative (Adams, Crosbie & Nowak, 2012). Hydrogen peroxide may be a preferred method when logistic solutions for freshwater treatment are not available. In addition, hydrogen peroxide is often used for treatment against sea lice (Costello, 1993), meaning that a logistic system for transportation and treatment already exists and that a combined treatment for sea lice and AGD can be performed. Both freshwater and hydrogen peroxide treatment may cause mortalities as the fish is already weakened by the disease and the treatment itself can cause additional stress to the fish. Neither treatment seems to be fully effective, as AGD reappears in treated fish, both under natural and controlled experimental conditions (Hytterød et al., 2017; Powell & Kristensen, 2014). Repeated treatments are therefore necessary, sometimes several times during a life cycle. Furthermore, it is important for both economic considerations and fish welfare that the used method is both effective and gentle on the fish. The aim of this study was to estimate the effectiveness of different doses of hydrogen peroxide to treat AGD-infected Atlantic salmon at a range of seawater temperatures.

## 2 | MATERIALS AND METHODS

### 2.1 | Data material and challenge test

The experiment in this study was conducted at VESO Vikan research facility in Norway with permission from the Norwegian Food Safety Authority (FOTS ID 7654). The fish available for the experiment came from VESO Vikan Hatchery and originated from the strain Salmobreed Standard. In total, 600 hatchery-reared Atlantic salmon smolt were used in the experiment. The average live weight was 228 g at challenge and 272 g at treatment. Each fish was individually pit-tagged 4 weeks before they were challenged with *N. perurans*. Before challenge, the fish were housed in a round tank (8,800 L) to acclimatize for 20 days, with a stocking density at maximum 40 kg/m$^3$. During this time, the water had a salinity of 25‰ ± 2‰ and a temperature of 12°C ± 1°C. Two days before the challenge, the fish were acclimatized to 15°C ± 1°C and starved the last 24 hr prior to challenge. Throughout the experiment, the water flow was >70% in effluent water and the water discharge was controlled by a tube overflow system. The fish tanks were cleaned every day and the photoperiod regime for the tanks was 24 hr of light and 0 hr of darkness (L: D = 24:0). This is standard procedure in experiments carried out at VESO Vikan. Ten fish were scored before the challenge to verify that the fish were healthy and did not show any gill damage.

A non-monoclonal amoeba isolate of *N. perurans* was collected from an outbreak in Norway and isolated on 15-10-2014 and subsequently cultivated at VESO Vikan on malt yeast agar (MYA) plates covered with sterile seawater. Amoebas were grown on MYA plates and transferred to new plates weekly. The amoebas used in the trial were from the eighth plate transfer.

The 600 fish were challenged with a high concentration of *N. perurans* (150 amoebas per L) in an aerated tank containing 3,100 L sea water with a temperature of 15°C ± 1°C for 4 hr. After the challenge was finalized, the tank was refilled with sea water. To monitor the disease development, fish were examined macroscopically and gill-scored according to Taylor, Muller, Cook, Kube and Elliott (2009). This gill score scale ranges from 0 (healthy) to 5 (severe infection) and in the current study, is based on the sum of lesions over the total gill surfaces when all gill arches on both sides are examined. The severity of the AGD infection as defined by the gill score is presented in Table 1. Thirty fish were scored each week post challenge until a gill score >1.5 was reached. All fish ($n$ = 600) were then gill-scored and equally divided into twelve different tanks ($n$ = 50/tank). The average gill score before treatment was 2.3.

### 2.2 | Experiment design and treatment scheme

The treatments were performed in tanks with aerated seawater (33‰ ± 2‰) and kept at three different temperatures, 8°C, 12°C and 17°C throughout the trial period. Hydrogen peroxide doses were selected based on earlier studies including treatment for AGD and sea lice, documenting that salmonid's tolerance of hydrogen peroxide

**TABLE 1** Gross gill score system to estimate the severity of amoebic gill disease (AGD) from Taylor et al. (2009)

| Infection level | Gill score | Gross description |
|---|---|---|
| Clear | 0 | No sign of infection and healthy red colour. |
| Very light | 1 | One white spot, light scarring or undefined necrotic streaking. |
| Light | 2 | 2–3 spots/small mucus patch. |
| Moderate | 3 | 4–10 spots.<br>Over 10 spots but no more than 10% of the gill.<br>10%–20% of the gills are covered. |
| Advanced | 4 | Established lesions covering up to 50% of the gill area. |
| Heavy | 5 | Extensive lesions covering most of the gill surface. |

diminishes with increased temperature (Adams et al., 2012; Rach, Schreier, Howe & Redman, 1997; Thomassen, 1993). Hence, the charges of hydrogen peroxide varied between the evaluated temperatures (Table 2). In addition, each treatment temperature contained a control, without addition of hydrogen peroxide. The fish were acclimatized to the new temperature (1–3 days) before treatment. At the time of treatment, the water volume was reduced to 200 L for 8°C and 675 L for 12°C and 17°C. The appropriate amount of hydrogen peroxide diluted with 10 L of water was added to the tank (Table 2). After 20 min, the treatment was terminated by refilling the tank with sea water. This procedure ensured that the concentration of hydrogen peroxide in the tanks was rapidly diluted and therefore did not affect the assessment of the effectiveness of the treatments.

The tanks were aerated during treatment. After treatment, 10 fish per tank were gill-scored weekly to follow the progression of the infection until termination. The fish were anaesthetized with 10 ml benzocaine chloride (5% in propylene glycol) to 10 L water and pit-tag scanned when gill-scored. All fish in each tank were gill-scored when the experiment was terminated after 4 weeks (17°C), 6 weeks (12°C) and 8 weeks (8°C). Amoebas develop faster at higher seawater temperatures. Therefore, different time intervals between temperatures were used, due to animal welfare concerns.

During the experiment, two tanks (1 and 10) experienced some unexpected mortality due to technical issues at the research facility. The experiment in tank 10 was repeated in a new tank (11). This resulted in two doses to be tested for the full experimental period at 8°C and 17°C, as both tanks 1 and 10 were discarded from the statistical analyses.

## 2.3 | Statistical analyses

One-way analyses of variance were used for comparison of effects of treatments and temperatures. The analyses were performed in R using linear models (R Core Team, 2016). In total, the dataset consisted of 1,113 gill score records that were analysed in four different models to compare different approaches.

**TABLE 2** Experimental design showing tank number, tank size, allocation of fish, temperature and hydrogen peroxide concentration ($H_2O_2$ concentration)

| Tank number | Tank size ($m^2$) | Number of fish | Temperature (°C) | $H_2O_2$ concentration (g/L) |
|---|---|---|---|---|
| 1* | 1 × 1 | 50 | 8 | 1.5 |
| 2 | 1 × 1 | 50 | | 1.8 |
| 3 | 1 × 1 | 50 | | 1.2 |
| 4 | 1 × 1 | 50 | | 0.0 (control) |
| 5 | 1.5 × 1.5 | 50 | 12 | 1.0 |
| 6 | 1.5 × 1.5 | 50 | | 1.2 |
| 7 | 1.5 × 1.5 | 50 | | 1.4 |
| 8 | 1.5 × 1.5 | 50 | | 0.0 (control) |
| 9 | 1.5 × 1.5 | 50 | 17 | 1.0 |
| 10* | 1.5 × 1.5 | 50 | | 0.8 |
| 11 | 1.5 × 1.5 | 50 | | 0.8 |
| 12 | 1.5 × 1.5 | 50 | | 0.0 (control) |

*Excluded from the statistical analyses.

1. To test the effect of treatment compared with no treatment (control)

$$GS_{ijkmn} = \beta_1 \times SC_i + \beta_2 \times week_j + temp_k + treat_m + e_{ijklmn} \quad (1)$$

The model expresses gill score (GS) of individual *i* at week *j*, where $\beta_1$ is the fixed regression coefficient of the start score (SC), which is the GS of individual *i* before the treatment. Furthermore, $\beta_2$ is the fixed regression coefficient of week *j*, which is the week of the registered gill score. Temperature (temp) *k* was included as a fixed class effect and treatment (treat) *m* was a binary indication for whether the observation was from a treated group (1) or a control group (0). The residual (*e*) was the random deviation of the observation from the expected value based on the effects included in this model.

2. Test the effect of treatment compared with no treatment with an additional temperature-dependent effect of treatment.

$$GS_{ijkmn} = \beta_1 \times SC_i + \beta_2 \times week_j + temp_k + treat_m + temp_k \times treat_m + e_{ijklmn} \quad (2)$$

Model 2 includes the same parameters as defined in Model 1, but is extended to include an interaction effect between temperature *k* and treatment *m* ($temp_k \times treat_m$).

3. Test the effect of different doses of hydrogen peroxide, nested within temperature, assuming a linear relationship between gill score and dose.

$$GS_{ijkmn} = \beta_1 \times SC_i + \beta_2 \times week_j + temp_k + \beta_3 \times dose_m(temp_k) + e_{ijkmn} \quad (3)$$

Model 3 has substituted the binary effect of treatment (from Models 1and 2) with a linear regression of gill score on the exact dose *m* of hydrogen peroxide (within each temperature group). Here, $\beta_3$ is the fixed regression coefficient of dose *m* within temperature *k*.

4. Test the effect of different doses of hydrogen peroxide, nested within temperature with no assumption regarding the relationship between gill score and dose.

$$GS_{ijkmn} = \beta_1 \times SC_i + \beta_2 \times week_j + temp_k + dose_m(temp_k) + e_{ijkmn} \quad (4)$$

The parameters in Model 4 are the same as in Model 3, but it makes no assumption about the relationship between gill score and dose. This is because dose was included as a class variable which tested the effect of each dose within each temperature.

All the models were used for analysing gill score, ranging from 0 (healthy) to 5 (severe infection) (Table 1).

## 3 | RESULTS

All studied combinations of temperatures and hydrogen peroxide concentrations demonstrated a clear effect on AGD infection (Table 3). Figure 1a–d present the observed variation and development of gill score at different hydrogen peroxide concentrations at the different temperatures, using the gill score scale 0 – 5. One to 2 weeks after treatment, a decrease in gill score was observed for all temperature groups, followed by a slow increase due to reinfection. This occurred for all temperatures and all concentrations except for the lowest hydrogen peroxide dose at 17°C (Figure 1a–d). The AGD infection on the untreated salmon developed faster at 17°C compared with 12°C and 8°C (Figure 1a).

### 3.1 | Effect of treatment (Model 1)

There was a significant effect of hydrogen peroxide treatment when gill score was analysed in Model 1. In general, hydrogen peroxide treatment would reduce the gill score points by $-0.68 \pm 0.05$. The increase in gill score was estimated to be $0.17 \pm 0.01$ per week, which means that the reduction in gill score due to hydrogen peroxide treatment would delay further development of the disease by approximately 4 weeks (0.68/0.17). This suggests that it would take 4 weeks until the gill score would reach the same level as before treatment.

### 3.2 | Effect of treatment within each temperature (Model 2)

Since the development of AGD is highly dependent on seawater temperature, the treatment effect on gill score within each temperature was evaluated separately at each temperature in Model 2. The results suggested a more effective treatment at a lower temperature (8°C) compared to higher temperatures (12°C and 17°C). The overall effect of treatment with hydrogen peroxide, expressed as change in gill score points, was $-1.11 \pm 0.07$ at seawater temperature 8°C, $-0.33 \pm 0.17$ and $-0.58 \pm 0.19$ at 12°C and 17°C respectively. The subsequent development of AGD infection was estimated to be delayed 8.5 weeks at 8°C, 1.7 weeks at 12°C and 1.5 weeks at 17°C (Table 3). Figure 2 shows the estimated linear development of gill score with and without hydrogen peroxide treatment at the different seawater temperatures (Model 2). Fish in sea water at 8°C had the lowest estimated gill score at all stages among all the treated and untreated groups.

### 3.3 | Effect of different doses of hydrogen peroxide within temperature (Models 3 and 4)

The linear effect of different doses of treatment on gill score was also investigated (Model 3). A significant negative linear relationship between dose of hydrogen peroxide and gill score was found ($p < .0001$). This suggested that gill score decreased when the dose increased. When dose was nested within temperature, the relationship between dose and gill score was lower, but significant, at 12°C compared with both 8°C and 17°C (Table 3).

When no assumption was made about the relationship between dose and gill score, the model was fitted for each temperature, as the number of doses tested within each temperature was not the same (Model 4). The doses 1.2 g/L and 1.8 g/L of hydrogen peroxide were tested at 8°C. The doses resulted in significant reduction in gill score, relative to the control group ($p < .0001$). The difference in response between the low dose (1.2 g/L) and high dose (1.8 g/L) was 0.4 in gill score points. The significance level of difference between the doses was lower than for the control group ($p < .05$). For seawater temperature 12°C, three doses were tested, 1.0 g/L, 1.2 g/L, 1.4 g/L and a control group. No significant differences were found between the doses. The results suggested that there was a significant effect of treatment (as tested in Model 2, $p < .0001$), but no significant difference between the different doses tested ($p > .05$). At sea temperature 17°C, two doses, 0.8 g/L and 1.0 g/L, were tested along with a control group. Both doses resulted in significant reduction in gill score ($p < .0001$) and were significantly different from each other ($p < .01$). The estimated effect of the treatments was a reduction in gill score of 0.47 and 0.77 points for the doses of 0.8 g/L and 1.0 g/L respectively.

## 4 | DISCUSSION

### 4.1 | Treatment with hydrogen peroxide

This study showed that treatment of AGD with various doses of hydrogen peroxide was successful at all combinations of doses and temperatures tested. Gills partially recovered following treatment and further development of the disease was delayed in Atlantic salmon. The first studies of the effect of hydrogen peroxide as treatment for AGD were carried out in Atlantic salmon in Tasmania (Cameron, 1993, 1994; Howard & Carson, 1993). Cameron (1994) stated that hydrogen peroxide was ineffective as treatment for AGD, whereas Cameron (1993) and Howard and Carson (1993) suggested that hydrogen peroxide had only a moderate effect as treatment for

**TABLE 3** Effect of treatment measured as a binary trait within temperatures, increase in gill score per week and the linear effect of dose on gill score

| Temperature (°C) | Effect of treatment | Increase in gill score per week | Weeks delay due to treatment | Linear effect of dose |
|---|---|---|---|---|
| 8 | $-1.11 \pm 0.07$ | $0.13 \pm 0.01$ | 8.5 | $-0.71 \pm 0.05$ |
| 12 | $-0.33 \pm 0.17$ | $0.19 \pm 0.03$ | 1.75 | $-0.25 \pm 0.12$ |
| 17 | $-0.58 \pm 0.19$ | $0.37 \pm 0.05$ | 1.5 | $-0.71 \pm 0.15$ |

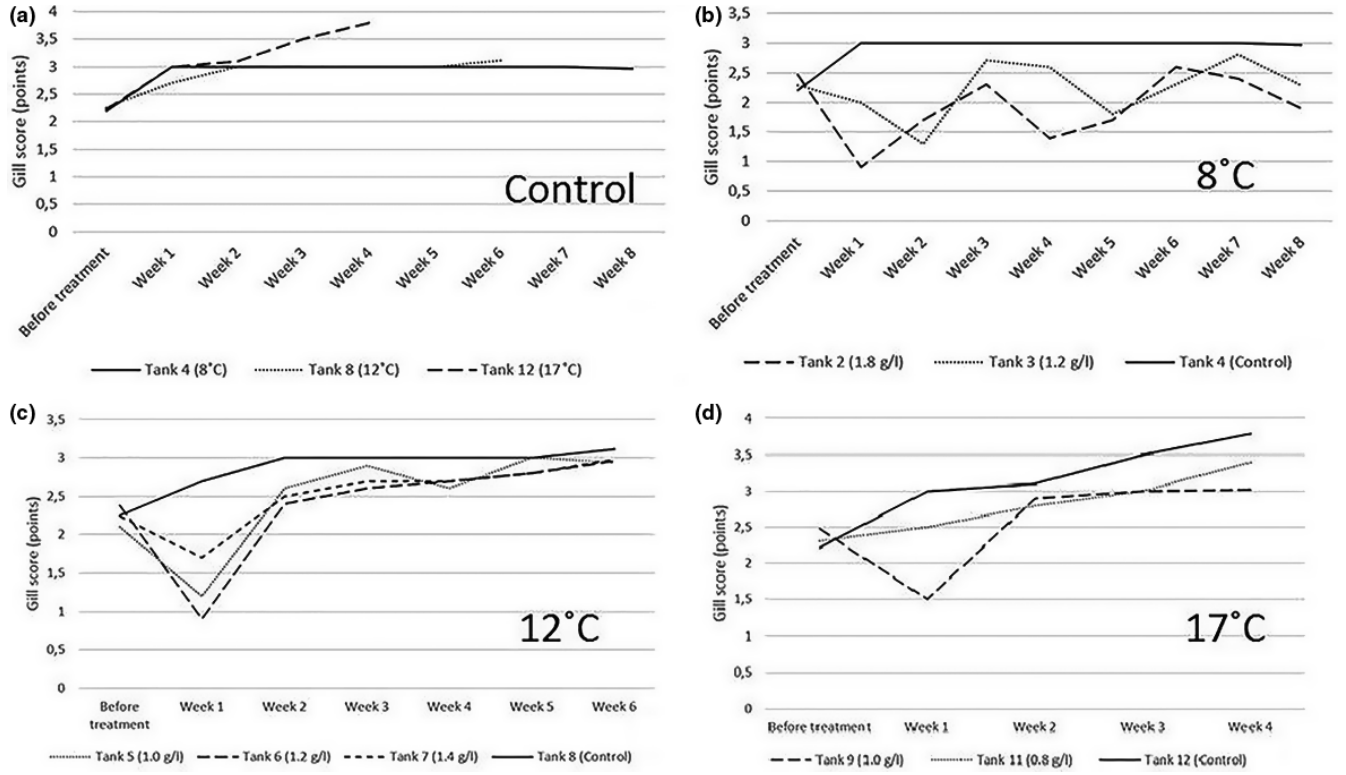


**FIGURE 1** Development of gill score points (0–5) on the fish from before treatment to experiment termination for fish in the control tanks at all temperatures not treated with hydrogen peroxide (a) and for fish in the tanks with a seawater temperature of 8°C (b), 12°C (c) and 17°C (d) treated with different doses (g/L) of hydrogen peroxide

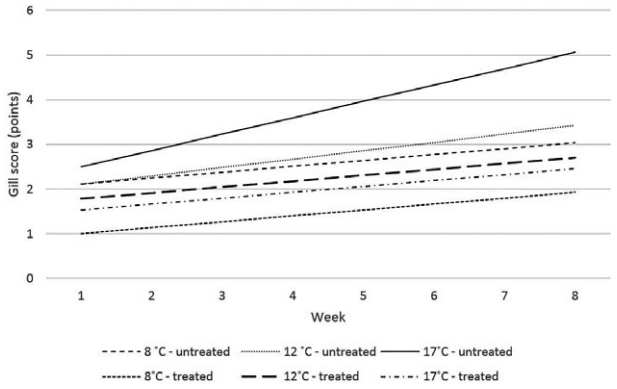


**FIGURE 2** Estimated linear development of gill score (0–5) in fish treated with hydrogen peroxide and untreated fish in seawater temperatures 8°C, 12°C and 17°C, regardless of dose

AGD. However, several factors differ between the studies and the current one. First, the concentration of hydrogen peroxide was significantly lower in these studies (0.1-0.4 g/L). Second, the strain of the pathogen was different, as the investigated strain in the studies was *Paramoeba pemaquidensis* while in the current study it was *N. perurans*. In a review of earlier literature on the effect of hydrogen peroxide treatment for AGD, Woo, Bruno and Lim (2002) concluded that the effect was questionable and unclear.

As temperature and salinity are high-risk factors regarding AGD outbreaks, AGD has been a recurring problem for a longer time in countries with higher seawater temperatures and high salinity (Clark & Nowak, 1999; Oldham et al., 2016). The first incident of AGD in Norway was in 2006, but it was not until 2013 that the disease was observed on a regular basis (Norwegian Scientific Committee for Food Safety, 2014; Steinum et al., 2008). Since AGD has become a challenge for the Atlantic salmon industry worldwide, treatments for AGD in Atlantic salmon, such as bathing with fresh water, levamisole and hydrogen peroxide have more recently been investigated in countries throughout Europe, Australia and America (Adams et al., 2012; Findlay, Zilberg & Munday, 2000; Parsons, Nowak, Fisk & Powell, 2001). Adams et al. (2012) tested the effect of both hydrogen peroxide and freshwater treatment for mild cases of experimentally induced AGD in Atlantic salmon in Tasmania. The study concluded that hydrogen peroxide was as effective as freshwater treatment in resolving mild cases of AGD in Atlantic salmon and that there was a potential for using hydrogen peroxide as treatment for AGD. Hytterød et al. (2017) investigated the effect of fresh water and hydrogen peroxide as treatment for AGD in Norwegian Atlantic salmon and showed that treatment with hydrogen peroxide had a recovering effect on AGD. The study concluded that fresh water had a better effect than hydrogen peroxide against AGD, but stated that the most gentle and effective combination of dose and duration, if hydrogen peroxide was used, was 1200 ppm hydrogen peroxide for 20 min. At water temperatures >12°C, caution must be used when treatment with hydrogen peroxide is practiced due to animal welfare issues. The same study also concluded that the effect of treatment

was not highly dose-dependent. The current study supported these results and could verify that hydrogen peroxide was beneficial for treatment of AGD and that the treatment effect was highly dependent on the seawater temperature and not necessarily dose-dependent.

Amoebic gill disease in Tasmania often occurred at seawater temperatures between 12°C to 20°C, and was mainly a summer problem in farmed salmon (Munday, Foster, Roubal & Lester, 1990). However, amoebic gill disease outbreaks have since been observed at temperatures as low as 7°C (Rodger, 2014; Steinum et al., 2008). Based on the results in the current study, the development of the disease seems to be more rapid at high seawater temperatures compared with lower temperatures, as there was a significant effect of treatment nested within temperature groups (Figure 1). In addition, treatment with hydrogen peroxide was shown to be most effective at a low temperature (8°C) and delayed the reinfection by several weeks. These findings are supported by Adams et al. (2012), which found a better response to hydrogen peroxide treatment at a seawater temperature of 12°C compared with 18°C. Powell, Attard, Harris, Roberts and Leef (2005) and Hytterød et al. (2017) also supported the results and suggested that hydrogen peroxide could be used as treatment at lower seawater temperatures. In accordance with the current study and regardless of treatment method, there is consensus in the literature that the treatment does not cure the fish, but delays the development of the disease and growth of the amoeba (Adams et al., 2012; Florent, Becker & Powell, 2007; Hytterød et al., 2017; Mitchell & Rodger, 2011). The fact that the fish were not cured could be due to the nature of the infection. In laboratorial trials, the infection might be more severe and the challenge pressure might be higher compared with a natural infection. This could make the disease more difficult to cure. In addition, Lillehammer, Boison, Gjerde, Norris and Løvoll (2015) showed that there was a low genetic correlation between gill score in challenge test and gill score in field test. This suggests that a challenge test might not be the best way to describe the disease development and treatment for AGD in the field and that the challenge model must be improved. In general, results from tank trials might be difficult to interpret for application to salmon farms, as a variety of effects would influence the disease development (Clark & Nowak, 1999).

### 4.2 | Experimental design and statistical analyses

As no replicates were performed in the experimental design of the study, it was not powerful in detecting differences between doses. Still, significant differences between the lowest and highest dose tested at 8°C and 17°C were detected. Based on the results, it seemed that there was an effect of dose of hydrogen peroxide on gill score. However, it was not possible to detect whether this effect was consequently increasing with increased dose, or if the highest doses tested in the experiment did not reduce the gill score further.

When analysing the effect of treatment with different doses, control was included as a dose at all temperatures. This means that the observed negative relationship between gill score and dose might be a result of being treated with hydrogen peroxide.

When the relationship between dose and gill score at 12°C was described as linear, there was a significant effect of dose; whereas when each dose was treated as a class variable, no significant difference in effects was estimated at this temperature. This inconsistency between the results from the different models might be due to that more doses were tested within this temperature, and that there were different ranges of doses tested at each temperature. This was mainly due to the fish' tolerance to hydrogen peroxide diminishing with increasing temperature (Rach et al., 1997).

The statistical analyses in this study were performed using the gill score scale described in Taylor et al. (2009). The current study was solely based on gill score records of the fish, and no information regarding amoeba counts was available. More information regarding the relationship between the severity of the disease and the amoeba count on the gills would be available if a histopathological analysis of the gills was performed (Adams, Ellard & Nowak, 2004).

## 5 | CONCLUSION

Hydrogen peroxide as treatment for AGD was shown to be successful for all combinations of doses and temperatures tested. The gills partially recovered following treatment and further development of the disease was delayed. However, all groups developed the disease again after treatment, suggesting that fish were not cured. The effect of treatment was most effective at a low temperature (8°C). At this temperature, the disease redeveloped more slowly compared with 12°C and 17°C, and resulted in a substantial delay of disease development by several weeks.

## ACKNOWLEDGMENTS

The authors would like to thank VESO Vikan for conducting this study and Akzo Nobel for funding the work.

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**How to cite this article:** Martinsen KH, Thorisdottir A, Lillehammer M. Effect of hydrogen peroxide as treatment for amoebic gill disease in Atlantic salmon (*Salmo salar* L.) in different temperatures. *Aquac Res*. 2018;49:1733–1739. https://doi.org/10.1111/are.13627