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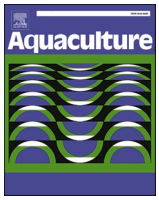

Dynamic morphometrics of mucous cells reveal the minimal impact of therapeutic doses of peracetic acid on Atlantic salmon gill health

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ABSTRACT

Mucous cells, the microscopic structural hallmark of mucosal surfaces, are highly responsive to environmental changes. Here we report how the gills of Atlantic salmon (*Salmo salar*) smolts responded to peracetic acid (PAA), a potent oxidative disinfectant and a candidate chemotherapeutant in aquaculture, through the Mucosal Mapping showing mucous cell size, volumetric density and defence activity, coupled with two-way histopathological scoring strategies. Two hundred and forty smolts were exposed to therapeutic doses of PAA on two occasions. The initial exposure included a 5-min bathing at concentrations of 0, 0.6, 1.2, and 2.4 ppm PAA. After a two-week recovery, the treatment groups were re-exposed to the same PAA concentrations for 30 min. Gill samples were collected at 2 h, 2 days, and 2 weeks after each exposure. The dynamic changes (i.e., size, volumetric density and defence activity) of the mucous cells were analysed on the distinct mucous cell populations in the gill filament and the lamella, as well as the lamellar thickness. Lamellar mucous cells were always significantly smaller (<70 μm^2) and less dense (<2% volume) than those in the filament (70–100 μm^2 and 8–11% volume) giving defence activities (combination of mean mucous cell area and volumetric density in a given tissue) of 0.1–0.4 for lamellae and about 0.6–1.4 for filaments, consistent with the functions of these branchial areas. A transient sub-acute mucous cell hypertrophy was a striking response in the gill lamella to PAA during the initial and re-exposure, particularly demonstrated by the groups exposed to 0.6 and 1.2 ppm where this size change was significant. Nonetheless, the recovery was quick, suggesting more an effect of general stress than dose of PAA. Similarly, a transient reduction in hyperplasia was noted as mucous cell density decreased on the filament, but generally significant hyperplasia was not detected, and volumetric density remained unaltered regardless of treatment doses and duration of exposure. The defence activity of the lamella and the filament demonstrated a transient tendency to decrease after the initial exposure but was minimally affected by re-exposure. Lamellar thickness was not markedly affected by the highest PAA dose and overall was positively correlated with mucous cell size. The results from two independent histopathological scorings revealed that at least 93% of the evaluated gill filaments per fish were categorised as healthy. Taken together, mucous cells in the gills of salmon smolts responded with transient hypertrophy of mucous cells to therapeutic doses of PAA however the impact was minimal, and the mucosal morphometrics were in agreement with the marginal alterations in tissue structure and integrity. The data suggest that the PAA doses used in the study are safe for salmon and do not pose substantial impact on gill mucosal health.

1. Introduction

Mucous cells, the microscopic structural hallmark of mucosal surfaces, are highly responsive to environmental changes and are especially relevant to the health of the multifunctional gills (Foyle et al., 2020). Mucosal epithelia have protected aquatic organisms for about

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half a billion years (Xu et al., 2013) and constitute the primary barrier against pathogens and other harmful compounds and stimuli (Cabillon and Lazado, 2019). While the gill structure can be remodelled in response to a variety of impacts, histological indicators of gill disease highlight hypertrophy and hyperplasia of the epithelial and mucous cells (Foyle et al., 2020; Gjessing et al., 2019). The convoluted 3D structure of the gill has been resistant to simple histological measures but the advent and application of Mucosal Mapping, a design-based stereological method (Pittman et al., 2013; Pittman et al., 2011) provides an unbiased, standard and universally applicable measure of mucosal epithelia in skin and gills that is highly sensitive, represents many fold and more surface area, is efficient and suitable for ecotoxicological studies as well as trials on stressors and diets (Dang et al., 2019; Dang et al., 2020; Lazado et al., 2020b; Pittman et al., 2013; Pittman et al., 2011).

The gill surface is estimated to 0.1–0.4 m^2/kg body weight, representing the largest organ-specific surface interacting with the environment and constitutes approximately 50% of the total surface area of the fish (Koppang et al., 2015; Streit, 1998). The two segments of the gill, filament and lamellae, have distinct mucous cell populations and discrete responses, with the filament having larger and denser cells and the respiratory surface of the lamellae having fewer and smaller mucous cells (Dang et al., 2019, 2020). Gill tissues react quickly to unfavourable environmental conditions (Poleksic and Mitrovic-Tutundzic, 1994) and lesions on the gills are visible ahead of behavioural changes (Strzyzewska-Worotynska et al., 2017). Hence, the rapid and sensitive reaction to external changes makes gills an ideal organ for fish health monitoring (Palaniappan et al., 2010) and for ecotoxicological studies (Dang et al., 2020; Stiller et al., 2020).

Stressors, such as therapeutically employed oxidants, are frequent in commercial fish farming and may have both short- and long-term effects on the remodelling of basic gill structures. Peracetic acid (PAA, CH_3COOOH) is a highly reactive peroxygen compound with a broad antimicrobial spectrum (Pedersen et al., 2013). It is considered a promising aquatic disinfectant with low environmental risk because of the absence of persistent toxic or mutagenic residuals and byproducts, minor dependence on pH fluctuation, and short contact time to deliver a potent action (Kitis, 2004). It is commercially available as an equilibrium mixture with acetic acid, hydrogen peroxide, and water (Pedersen et al., 2009). PAA produces hydroxyl radicals (•OH) and "active" oxygen from the photolysis upon decay and they are known as powerful oxidizing agents. These substances play a central role in the antimicrobial activity of PAA. Elevated levels of environmental radicals may pose health and welfare issues as they can cause oxidative stress (Lazado et al., 2020a; Soleng et al., 2019), the imbalance between the level of oxidants and the ability of the organism to sequester the radicals (Almeida et al., 2009). Oxygen metabolites have been documented to impact the physiology and morphology of mucous cells in both mammalian (Hiraishi et al., 1991; Hiraishi et al., 1993) and fish (do Carmo et al., 2018; Mozhdeganloo and Heidarpour, 2014) models, however, objective quantification of their response is currently lacking.

PAA is currently being explored as a candidate chemotherapeutant for amoebic gill disease in Atlantic salmon (Soleng et al., 2019). Here we investigate the consequences of repeated therapeutic doses of PAA and apply Mucosal Mapping to evaluate the gill tissue response in filaments and lamellae of salmon smolt.

2. Materials and methods

2.1. Ethical statement

The experimental trial was performed in accordance with national and EU legislation (2010/63/EU) on animal experimentation.

2.2. Fish and acclimation

Atlantic salmon smolts (*Salmo salar*) (average weight 150 ± 9.5 g; mean ± standard deviation) were purchased from a local supplier (Danish Salmon, Hirtshals, Denmark) and transported to the recirculation aquaculture facility of DTU Aqua (Hirtshals, Denmark). Sixty fish were stocked to each of the 8 1-m^2 holding tanks (volume approximately 600 L, density *ca* 15 kg/m^3) in a seawater recirculating aquaculture system. The fish were acclimated for 3 weeks under the following laboratory conditions: temperature – 15±1 °C; dissolved oxygen – 80-90% saturation; salinity – 33-34 ppt; photoperiod – 16 L:8D (0600–2200). The fish were fed (Biomar, EFICO Enviro, 4.5 mm) at a ratio of 1–1.5% total biomass per day which was administered via a belt feeder.

2.3. Peracetic acid exposure

Fish from the holding tanks (8) were transferred to their corresponding exposure tanks (8), with similar conditions (i.e. density, volume, water quality). After 10 mins, peracetic acid-based disinfectant (PAA; Divosan Forte™, Lilleborg AS, Norway) solution was added with aeration to achieve the following final concentrations in duplicate tanks: 0 (seawater), 0.6, 1.2, and 2.4 ppm. After an exposure to these doses lasting only 5 mins, fish were returned to their original holding tanks. Feeding was resumed two days after the PAA exposure. Fish recovered for two weeks, after which the fish were re-exposed to the same PAA concentration for 30 min. Post exposure the salmon were again kept for 2 weeks in the original tanks before final sampling. Additional details of the trials were described in earlier publications (Lazado et al., 2020a; Soleng et al., 2019).

2.4. Sample collection

Sample collection was performed prior to exposure, and at 2 h, 2 days and 2 weeks after each exposure experiment. Five fish were taken from each replicate tank and were humanely euthanised with an overdose of 20% benzocaine solution. The length and weight were measured, and an external evaluation of fish appearance was done before invasive sampling. For this study, the second gill arch from the right gill was carefully sampled from each fish, placed in a labelled histocassette and preserved in 10% neutral buffered formalin (Sigma-Aldrich, Darmstadt, Germany).

2.5. Sample processing and mucosal mapping analysis

The gill samples were processed for evaluation following Quantidoc's standard Mucosal Mapping protocol. Briefly, gills were dehydrated, embedded in paraffin, sliced *tangentially* in 3 μm sections and stained with PAS – Alcian Blue. Stained sections were digitally scanned by NanoZoomer 2.0-RS, Hamamatsu Photonics K.K. (Japan) to high-resolution NDPI image format. Mucosal Mapping was done according to Pittman et al. (Pittman et al., 2013; Pittman et al., 2011) with calibrated dedicated semi-automatic software developed for stereological image analysis of mucosa by Quantidoc AS (Veribarr™ and Mucomaster). The volumetric density in % of mucous cells in the epithelium (D) and the mean area of mucous cells at the equator (μm^2; A) on the gill filament (GF) and lamella (GL) were analysed and used to calculate the defence activity of the mucosal epithelia, or how the amount of mucus cells has changed, in each tissue according to the formula: 1/(A/D) *1000. These variables of volumetric density and defence activity are necessarily distinguished from the unfortunately misleading numerical density commonly used and the simple counts of cells as a variable. The application of design-based stereology recognises the complex 3D structure of gills and compensates for the 2D limitations of a histological slide.

The mean width of the lamellae gives some indication of the diffusion distance (the distance oxygen travels from water to blood) (Flajšhans and Piačková, 2006). This was measured on 20 random locations in the lamellae of gills in the control and 2.4 ppm PAA groups using VIS image analysis software (Visiopharm, Hoersholm Denmark).

2.6. Semi-quantitative histopathology

Gill sections were evaluated by two methods and two independent groups of evaluators. First, histopathological case scoring was performed following a previously published strategy (Reiser et al., 2010), with modifications (Stiller et al., 2020). The evaluation was carried out by randomly selecting five locations in a gill section (i.e., 2 upper half, 2 lower half and 1 middle of the whole gill section). A total of 100 lamellae were evaluated per fish. Cases of clubbing, lamellar fusion, hyperplasia, hypertrophy, lifting, hyperaemia, aneurysm and necrosis were documented. Lamellae that did not show any histopathological changes as described above were denoted as "healthy". The second strategy involved the assessment of histopathological alteration index (HAI) following the method of Poleksic and Mitrovic-Tutundzic (Poleksic and Mitrovic-Tutundzic, 1994) where gill lesions are given a score according to the severity of the alteration. Both strategies were performed by blind evaluation.

2.7. Statistics

A Linear mixed effect model (lme) was used to test differences in mean area and defence activity as well as lamellar thickness at each sampling time (R studio, Massachusetts, USA). A quasi generalised linear model (GLM quasi) was used to compare the difference in volumetric density between treatments. A Pearson correlation test was done for mucous cell area and lamellar thickness with significance set at $P \leq 0.05$.

3. Results

There was no recorded mortality during the trial and no significant differences among the treatment groups in length, weight, and K-factor. Unfortunately, the samples taken prior to PAA exposure were subject to transport and storage problems and could not be used for this analysis (Soleng et al., 2019). Statistical comparisons are therefore made to the control group and between treatments.

3.1. Mucous cell morphometry in the gill lamella (GL)

3.1.1. GL mucous cell area

The mucous cell area on the lamella varied from 27.62 μm^2 to 81.7 μm^2, with a mean mucous cell area of 49.98 μm^2. Two hours after a 5-min exposure, the range of mean mucous cell sizes in GL was 49.05 μm^2 to 52.19 μm^2 with no significant difference between the control and the 2.4 ppm PAA group (Fig. 1A, *left panel*). However, the GL mucous cell area of the group exposed to 0.6 ppm PAA was significantly larger while those exposed to 1.2 ppm PAA group did not significantly differ from the control.

Two days after 1st exposure for 5 min, the mean mucous cell size was about the same in all groups. At 2 weeks, gills exposed to 2.4 ppm PAA exhibited significantly smaller mucous cells than the 1.2 ppm and the 0.6 ppm groups. No significant differences were observed within the groups between 2 days and 2 weeks after the 1st exposure (Fig. 1A, *left panel*).

Two hours after the 2nd exposure, this time for 30 min, the mucous cell area in the groups exposed to 1.2 ppm and 0.6 ppm PAA were significantly larger than in the control group and 2.4 ppm PAA group (Fig. 1A, *right panel*). At 2 days post exposure, 0.6 ppm PAA resulted in significantly larger mucous cells than in the control and the 2.4 ppm groups. No significant dose-related differences were observed 2 weeks after 2nd exposure (Fig. 1A, *right panel*).

3.1.2. GL mucous cell density

The GL mucous cell ranged from 0.97% to 1.22% volumetric density of the lamellar epithelium (Fig. 1B). The initial exposure had no significant density impact, but the 0.6 ppm group displayed the highest variance with densities ranging from 0.8% to 3.5% and a mean of 2.1% (Fig. 1B, *left panel*).

The second PAA exposure, this time of 30 min duration, did not significantly influence the GL mucous cell density in any dose or time point (Fig. 1B, *right panel*).

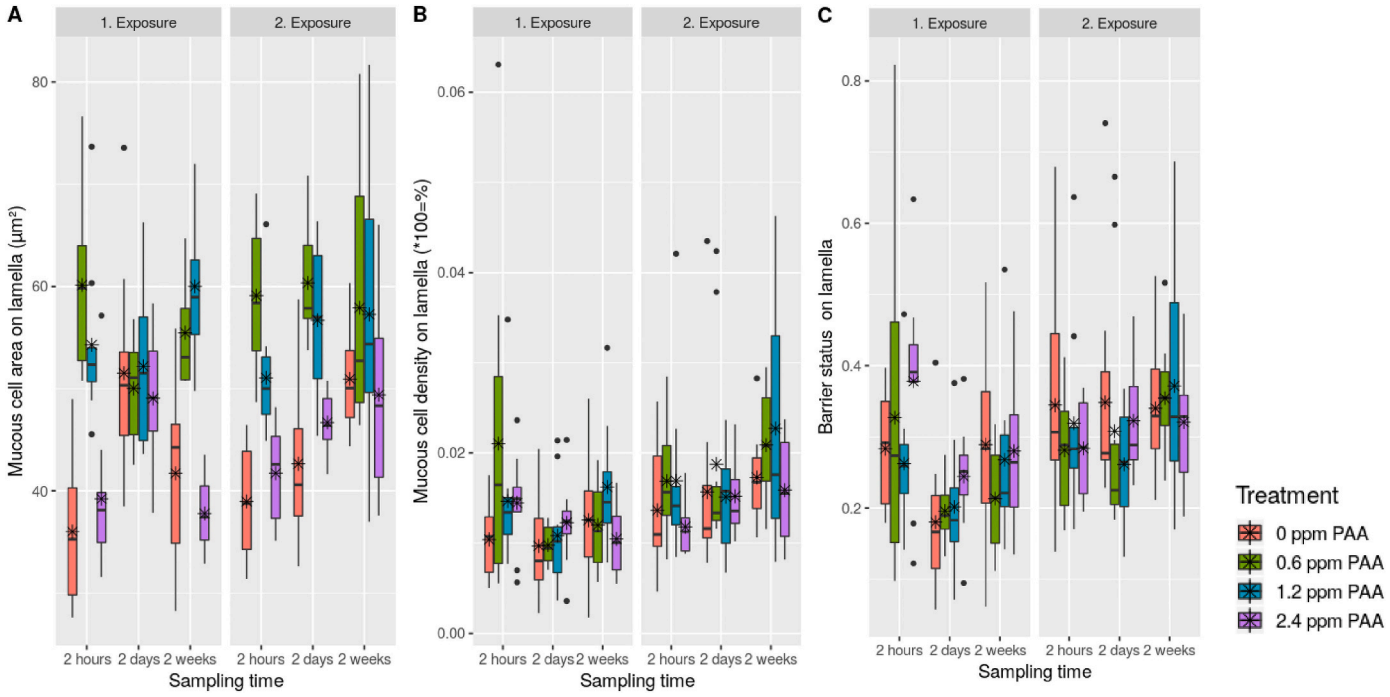

Fig. 1. Temporal morphometry of mucous cells on the gill lamella of fish treated with therapeutic doses of PAA. Fish were exposed to PAA at 2 occasions. A = mean mucous cell area at the equator; B= volumetric density of mucous cells in mucosal epithelium; C= defence activity (barrier status) of mucosal epithelium. *n*=10 fish per treatment per sampling point, two tanks per treatment.

3.1.3. GL defence activity

Two hours following the initial exposure the mean defence activities ranged from about 0.25 to 0.4. However 2 days after exposure all groups had non-significantly reduced defence activity relative to those 2 h after exposure (means between 0.17 and 0.25). The defence activity (Fig. 1C, *left panel*) returned to nearly initial levels 2 weeks after the 1st exposure (means between 0.2 and 0.3).

The 2nd exposure had no substantial impact or dose-related response on the mean GL defence activity (Fig. 1C, *right panel*).

3.2. Mucous cell morphometry in the gill filament (GF)

3.2.1. GF mucous cell area

The mucous cell area on the filament varied from 56.9 μm^2 to 141 μm^2 with a mean mucous cell area of 85.3μm^2. Dose-related significant differences following 1st exposure were not detected in the mean size of the filament mucous cells (Fig. 2A, *left panel*).

Following the 2nd and longer exposure, mucous cells in the gill filaments displayed similar response patterns to those of the 1st exposure. Nonetheless, exposure to the highest dose, 2.4 ppm, induced significantly larger mucous cell sizes at 2 days relative to 2 h after the 2nd exposure. Two weeks after 2nd exposure, all groups displayed the same filament mucous cell sizes as those immediately following the repeated treatment with no dose-related changes in response (Fig. 2A, *right panel*).

3.2.2. GF mucous cell density

The mean mucous cell density varied from 8.73% to 10.57% of the mucosal epithelium with no significant dose-related differences (Fig. 2B). A small reduction in density 2 days post 1st exposure in the 2.4 ppm group was not significantly different (Fig. 2B, *left panel*).

Exposing the fish to PAA at longer durations did not significantly affect the volumetric density of mucous cells in the gill filaments at any timepoint (Fig. 2B, *right panel*).

3.2.3. GF defence activity

Mean defence activity varied between 1 and 1.25 for all doses immediately after the 1st exposure (Fig. 2C, *left panel*). The longer-term response pattern for all groups showed a decrease in defence activity at 2 days post exposure, particularly for the high dose group (not significant) but returned to nearly initial values by 2 weeks post exposure. The control group (0 ppm) showed significantly elevated defence activity 2 weeks post exposure compared to 2 days suggesting a response to a stressor rather than treatment dose. There were no dose-related differences in filament defence activity during the 2 weeks after 2nd exposure, suggesting a stabilization of response (Fig. 2C, *right panel*).

3.2.4. Lamellar thickness

The lamellar thickness in the control and high dose groups varied between 7.8 μm and 19.6 μm with a mean of 12.22 μm (Fig. 3A). Oddly, only the control group thickened significantly from 2 h to 2 days after the 1st exposure. There was no significant difference between the control and the 2.4 PAA group 2 h after the 2nd exposure, though the gradual increase over time was not statistically significant (Fig. 3A, right panel).

The relationship between mucous cell size and the physical space it needs to occupy in the double epithelial layer of the lamella is demonstrated by the significant positive correlation between this and the lamellar thickness (r=0.47) (Fig. 3B).

3.3. Relationship between the mucous cells on the gill lamella and filament

Mucous cells were generally larger in the filament than in the lamella at all doses, exposures and sampling times. The mucous cell density was even more distinct between the two gill sites, where the filament mean density was 9.49% and the lamellar mean density was 1.48% of the mucosal epithelium (Figs. 1B,2B). Mucous cell volumetric density on the filament was significantly higher than the lamella in all instances.

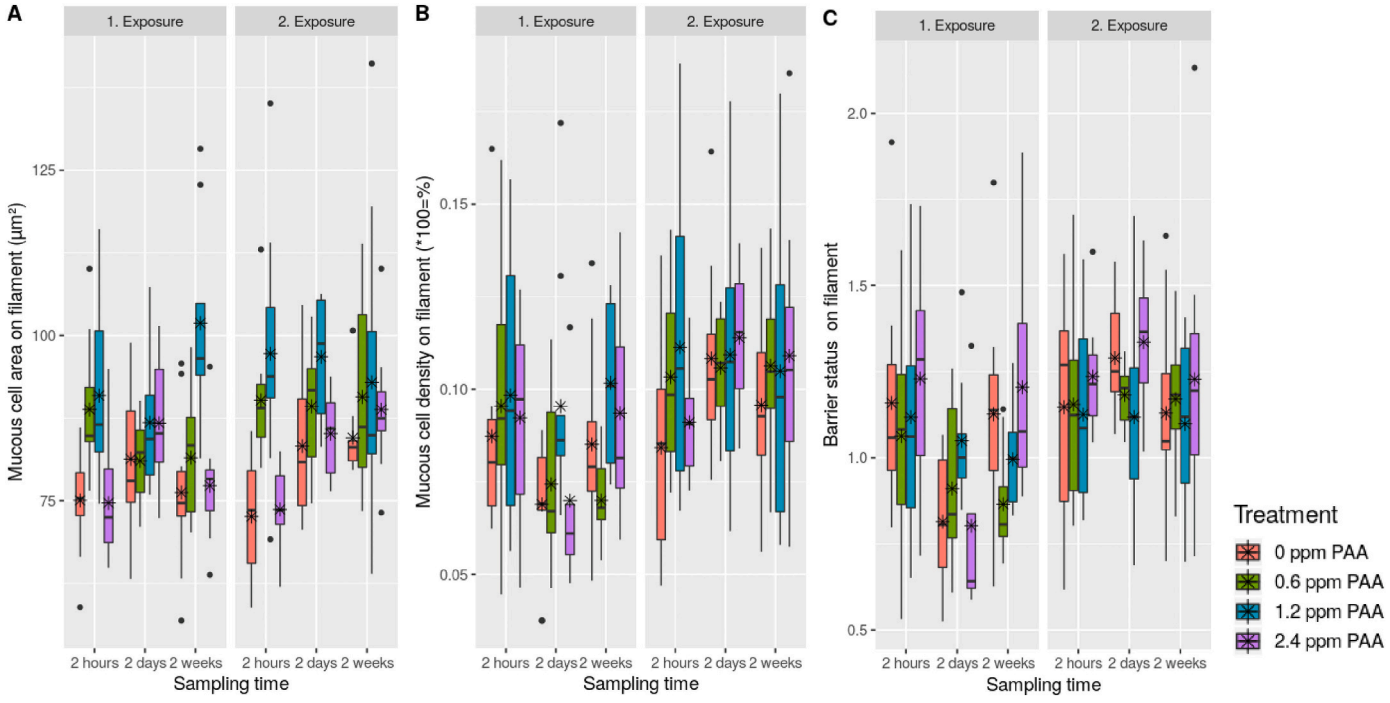

Fig. 2. Temporal morphometry of mucous cells on the gill filament of fish treated with therapeutic doses of PAA. Fish were exposed to PAA at 2 occasions. A = mean mucous cell area at the equator; B = volumetric density of mucous cells in mucosal epithelium; C = barrier status (defence activity) or the combination of area and density of mucosal epithelium. An individual box plot represents measurements from 10 individual fish (n=10) of a treatment per sampling point, two tanks per treatment.

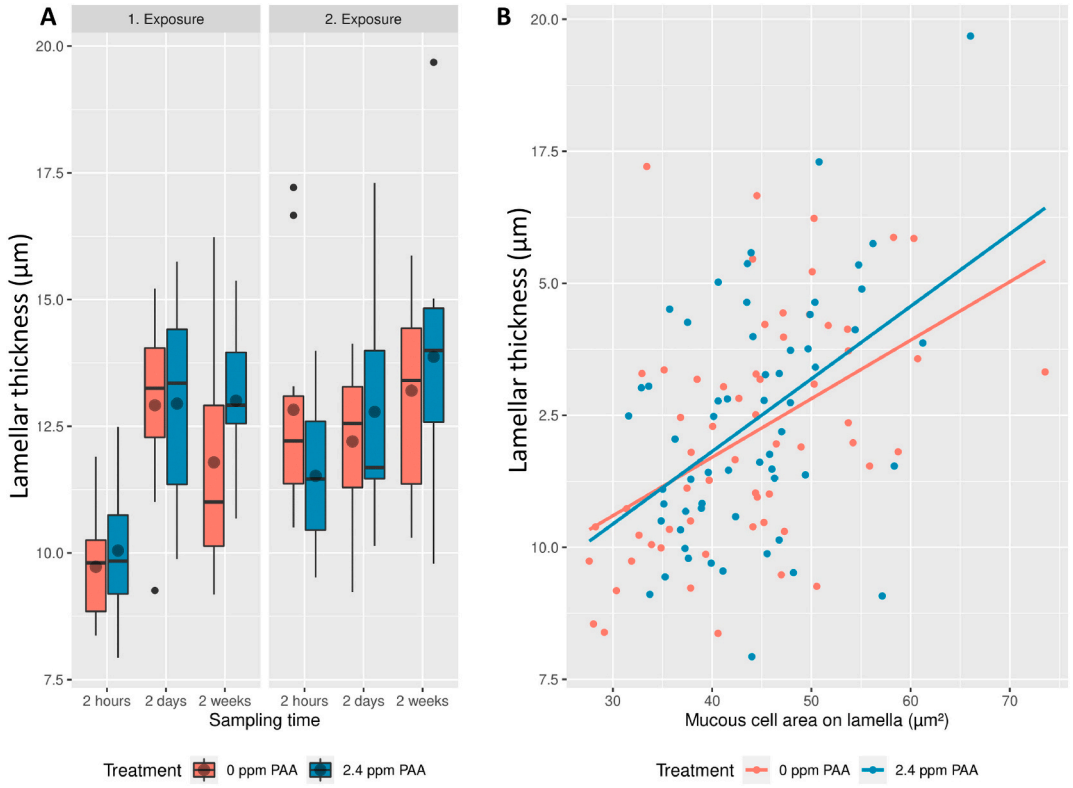

Fig. 3. A) Gill lamellar thickness and B) its correlation with mucous cell area. *N*=120 individual fish, 60 per treatment group.

3.4. Histopathological alterations

The majority of the gills analysed (*n*=225, 93.8%) were healthy and had a HAI-score between 0 and 10 indicating functionally normal gills. The remainder were moderately damaged gills (*n*=14, 5.8%) or moderate to heavily damaged gills (n=1, 0.4%). Two weeks after the 1st exposure, more alterations were induced by 2.4 ppm PAA. However, all gills were histologically categorised as functionally normal by the end of the trial period (Fig. 4).

These results agreed with the semi-quantification of common histopathological characteristics (Fig. 5A). Clubbing and lifting were the most prevalent observations, accounting for 0.5–3% of the cases (Fig. 5B). Hyperplasia was significantly more 2 weeks after 2nd exposure than the same time point after the 1st exposure but there was no relationship with dose. Lamellar clubbing during the 1st exposure increased as a function of PAA dose.

4. Discussion

Several studies have already demonstrated that mucous cells respond rapidly to environmental changes, with variations in size of mucous and epithelial cells as the most common characteristics of complex gill disease (Gjessing et al., 2019). These reproducible changes can be objectively measured using Mucosal Mapping to illuminate even subtle effects of potential therapeutics like PAA and other interventions (Cabillon and Lazado, 2019; Dang et al., 2019; Dang et al., 2020; Pittman et al., 2013; Pittman et al., 2011; Torrecillas et al., 2015).

4.1. Changes in mucous cell morphometries exhibit a dose dependent response to PAA

Exposure to the highest dose, 2.4 ppm, induced significant hypertrophy in filament mucous cells at 2 days relative to 2 h after the 2nd exposure, suggesting a latent membrane-wide development. The second highest dose 1.2 ppm PAA exhibited some hypertrophy 2 weeks after exposure. Beyond that, varying doses of PAA from 0 to 2.4 ppm did not significantly change the mucous cell sizes in the gill filament (mean 85.3 μm^2). These were smaller than those on the gill filament of commercially produced salmon (mean 97.14 μm^2) exposed to 1500 ppm of H_2O_2 (Rantty, 2016), but comparable with filament mucous cell sizes in shorthorn sculpins (*Myoxocephalus scorpius*) exposed to an environmental gradient of heavy metals (i.e., lead and zinc) (87.63 μm^2 (Dang et al., 2019). The mucous cell density on the filament ranged from 3.7% to 18.8% of the mucosal epithelium with a mean density of 9.49% with no inter-treatment differences. A sub-acute response, a significant *decrease* in defence activity on the filament, was found 2 days after fish were first exposed to the highest dose of 2.4 ppm PAA. However, this effect was only detected after the initial exposure, not the second exposure, and the fish recovered. This change may offer insight into the influence of previous exposure on the effects of PAA on mucous cell size. A study in rainbow trout demonstrated that repeated exposure to PAA lead to a form of physiological habituation (Gesto et al., 2018) and such a response had been likewise identified in salmon (Osório, 2020). The behaviour of mucous cells in the filament is pointing to a similar form of response to PAA. Since even the control group with 0 ppm PAA exhibited similar transient changes in the filament, it suggests that the mucosal changes are not induced by PAA up to 2.4 ppm but rather by other generalised stressors, for example, handling during treatment.

Some hypertrophy of mucous cells was also found in the gill lamellae. Both medium doses (1.2 and 0.6 ppm) induced significant hypertrophy of lamellar mucous cells 2 h after the initial and re-exposures. Lamellar mucous cell sizes in this study (mean 49.98 μm^2) were smaller than those found by Rantty (70.26 μm^2) (Rantty, 2016) on salmon exposed to H_2O_2 but larger than in salmon of about 100 g in a controlled feeding trial (<35 μm^2; (Bogevik et al., 2019). Healthy wild smolts have very few, spatially distributed lamellar mucous cells of about 30 μm^2 (Quantidoc unpublished data). This acute response to PAA in the respiratory surface was independent of dose or exposure time,

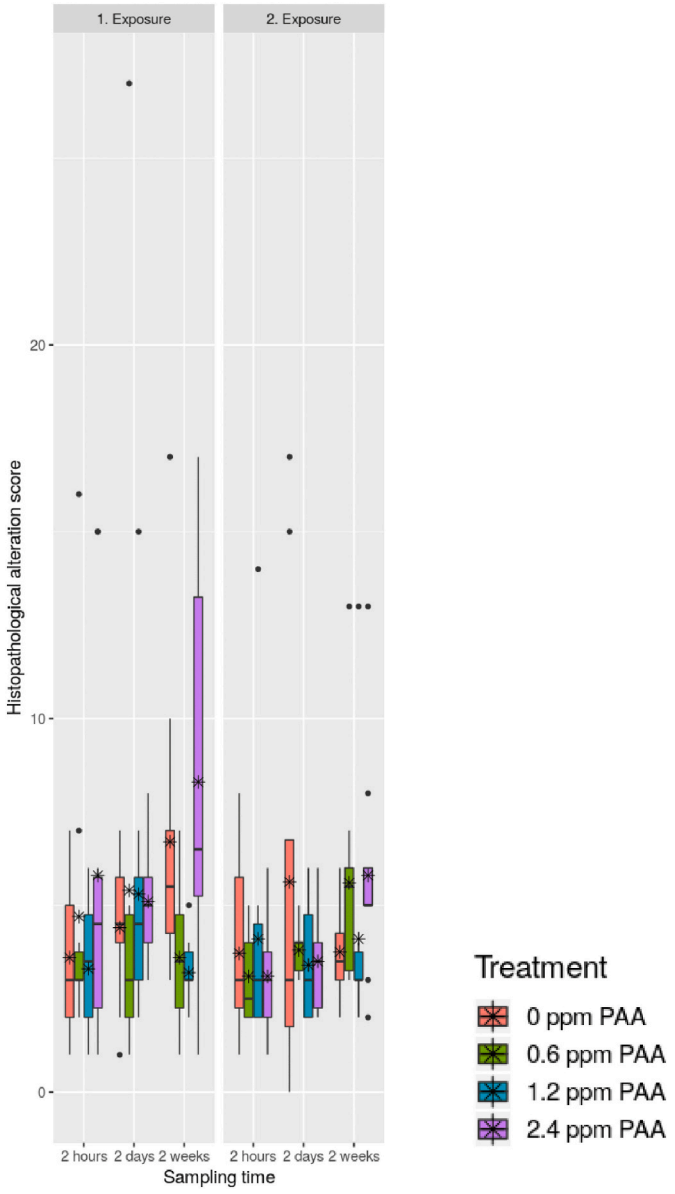

Fig. 4. Histopathological alteration index (HAI) for the gills of the treatment groups. 60 gills were analysed per treatment group, making 240 gills for all the trial.

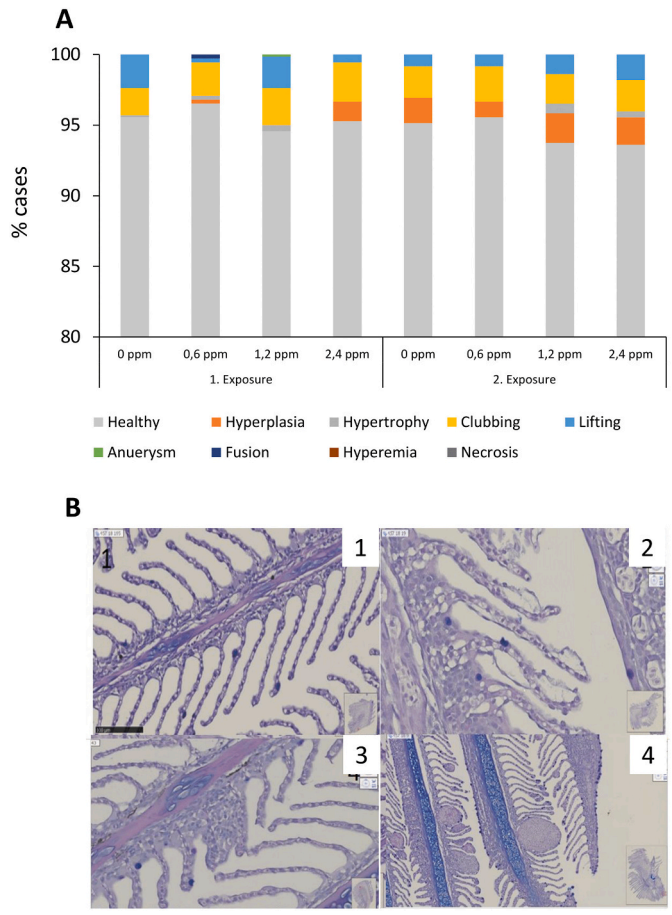

Fig. 5. Quantification of histopathological cases in the gills at 2 weeks after first and second exposure. (A) Histopathological cases are reported as number of cases relative to the total number of evaluations. (B) some of the histopathology observed in the gills of experimental fish. 1= healthy gills, 2=lifting, 3= hypertrophy, 4= aneurism.

again suggesting a conserved response to brief challenges and minimal impact of repeated doses of PAA up to 2.4 ppm. In earlier studies where fish were exposed to environmental stressors and pollutants (Dang et al., 2019; Rantty, 2016), a common response to the insults was a *reduction* of mucous cell density. Stress from acetic exposures can stimulate secretion of mucus (Humphrey, 2007; Segner et al., 1988), and short-term stress and acetic exposure can reduce mucous cells in the skin and the mucosal barrier in general (Lazado et al., 2020b; Segner et al., 2012). Both PAA and H_2O_2 generate an increased volumetric density of mucous cells a couple of days after exposure followed by a return to baseline level two weeks after. This further supports the protective and adaptive mechanisms of the mucous cells (Strzyzewska-Worotynska et al., 2017).

The impact of these mucous cell measures extends into the physiology of growth because the cells occupy space between the respiratory epithelial membranes. The lamellar thickness is positively correlated with mucous cell size in our study. Ultsch & Gros (Ultsch and Gros, 1979) hypothesised that an increase in mucous around the gill will decrease the oxygen diffusion efficiency by increasing the physical space between the outer membrane and the central capillaries. The estimated maximum diffusion distance (thickness divided by 2; mean 6.1 μm) was in agreement with previous estimates for salmon gill diffusion distances (Koppang et al., 2015; McDonald et al., 1991). There was no clear PAA dose-related change in gill mucosal thickness in this study, suggesting unchanged respiratory parameters.

4.2. *Mucous cells from the filament and lamellae demonstrate a distinct response to PAA*

Mucous cells reside in both the filament and lamella of the gills. Most mucous cell morphometry studies in fish do not often differentiate their response to stimuli, as the traditional manual approach is tedious and laborious. In addition, the traditional 2D sections often give a misleading impression of the actual variation in 3D structure of the gills. The Mucosal Mapping addresses this issue. The technique has facilitated extensive measurements of mucous cells and has identified that the two populations of the mucous cells in the gills often responded differently based on thousands of gill samples analysed from different experiments and localities; thus the distinction of the two proposed populations is highlighted in this paper. Our results show that the two mucous cell populations in the gill filament and in the gill lamellae demonstrated distinct responses to PAA. These include significant transient hypertrophy but not hyperplasia in both segments. A transient decrease in hyperplasia (decrease in defence activity) was found only in response to 2.4 ppm, suggesting that higher doses of PAA may have measurable

impacts on the gills. The significantly larger and denser mucous cells of the filament vs those in the lamellae support the hypothesis of two separate populations of mucous cells in the gills, where the filament mucous cells are associated with excretion of ions, minerals and metals (Dang et al., 2019; Leknes, 2002) and may reflect an upregulation of metabolism or an increased need for excretion of metabolic products (Bogevik et al., 2019) and those in the lamella are more integrated with functions of respiration and mucosal immunity. Thus, studies pooling cell populations from both segments may be overlooking valuable information which would be apparent when using separated measurements (Dang et al., 2020). Future studies must explore in depth the functional relevance of the distinct responses of these two mucous cell groups in the gills of salmon.

Visualising the distinction between these cell populations may be key to advancing aquaculture sustainably, especially in quantifying mucosal responses to environmental stressors. While traditional histological sections of gills show patchiness in mucous cell distribution and direct comparison is visually difficult, a standardized illustration of mucous cell size, volumetric density and defence activity is given below (Fig. 6). In this case, Quantidoc's Dicer App v2 illustrates average mucous cells in gill lamellae (Fig. 6A,B) and in gill filament (Fig. 6C,D) in a standardised 10 thousand square microns of epithelium at densities found in this study. The illustrations underscore the tissue differences in size, density and defence activity between healthy lamellae and healthy filaments.

4.3. Mucosal Mapping complements traditional histopathology

Both histopathological scorings employed in the present study indicated that 90% of the gills remained in a healthy state following PAA exposures although infrequent pathologies were observed. Alterations of gill tissue can be caused by pathogens (Munday et al., 2001), pollution (Evans et al., 2005) and particles (Randall, 1990), among other stimuli. Gills exposed to suspended materials have previously shown increased mucous production, hypertrophy and hyperplasia (Reynolds et al., 1989) and branchial histopathological alterations can reduce mucosal function (Henriksen et al., 2013; Stiller et al., 2020; Tort, 2011). Alterations can vary from minor (clubbing and lifting of the epithelium) to serious (necrosis of gill epithelium) and can be divided into direct damage of environmental stimuli, and defence mechanisms (lifting, hypertrophy and hyperplasia) resulting in reduced oxygen absorption in the gills (Strzyzewska-Worotynska et al., 2017; Velasco-Santamaria and Cruz-Casallas, 2008). The histopathological scores and the limited hypertrophy strongly corroborate that application of PAA at therapeutic doses used in the present study does not pose significant gill health concerns. Both histopathological scorings and mucous cell dynamics highlight the minimal, mostly transient and fast recovery rate from PAA-induced changes.

5. Conclusions

Taken together, the results of the present study revealed that mucous

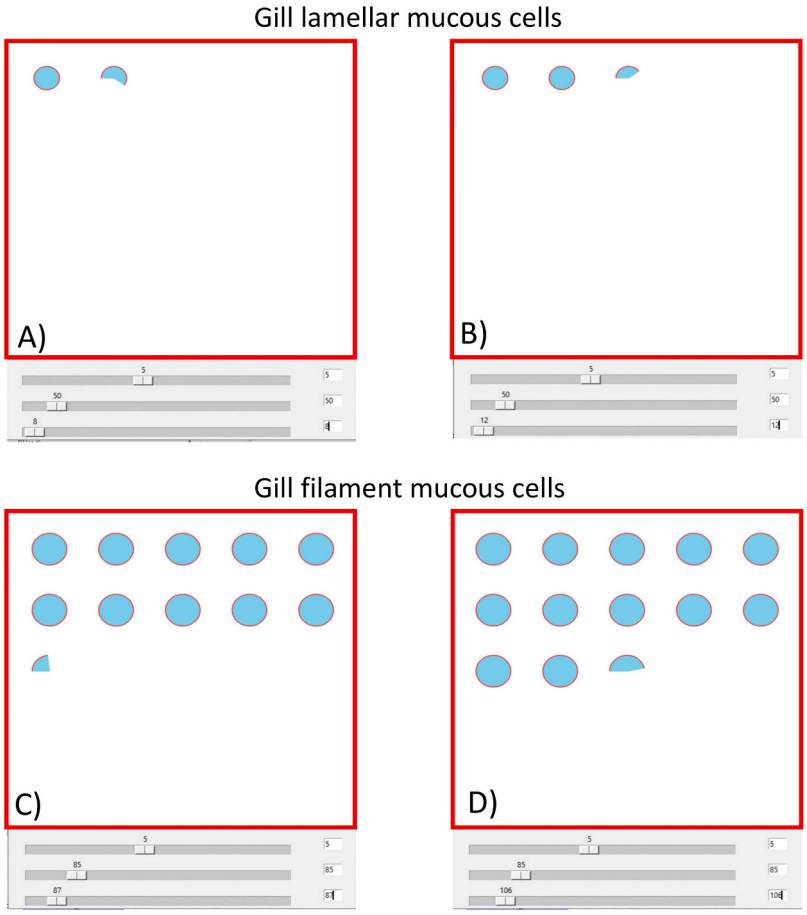

Fig. 6. Standardized representations of mucous cells in the gill lamellae and gill filament in this study, generated using Quantidoc's Dice App v2. A-B) Gill lamellar mucous cells shown at mean size of 50 μm^2 and volumetric densities of 0.8% (A) to 1.2% (B) in 10,000 μm^2 of epithelium. C-D) Gill filament mucous cells shown at a mean size of 85 μm^2 and volumetric densities of 8.7% (C) to 10.6% (D) in 10,000 μm^2 of epithelium.

cells in the gills of Atlantic salmon smolts responded minimally to therapeutic doses of PAA. Nonetheless, the changes can be characterised as adaptive responses to general stressors and the levels of oxidants present in the immediate environment, without significantly damaging gill health. A number of the significant changes in the cellular morphometries were observed during the 1st exposure, indicating the possibility that the gill mucous cells respond to the oxidant following an initial rapid reaction to PAA, and the recovery was likewise quick. These mucosal responses are in agreement with an earlier report on the physiological adaptation of salmon to repeated exposure to PAA (Soleng et al., 2019). Mucosal Mapping could reliably distinguish between the two populations of mucous cells, one in the filament and one in the lamellae, each with distinct transient hypertrophy, which caused no measurable lasting impact on functionality of healthy salmon gills. It would be interesting to explore in the future whether the changes documented in the present study are specific to a particular PAA trade product, or are a universal response to PAA-based disinfectant. The variable compositions of commercial PAA products provide limitations to the observations in the present study. Moreover, the safe thresholds for PAA vary among fish species, and factors such age, physiological status, mode of delivery, exposure duration, among others, will affect how fish respond to the oxidant challenge (Hushangi and Hosseini Shekarabi, 2018; Lazado et al., 2021; Straus et al., 2018). Hence, the responses observed here are only valid within the parameters described in the exposure trial.

Author contributions

C.C.L. and L.F.P. conceived the idea for the research. C.C.L. and L.F.P. designed the trial. S.H., C.C.L., L.F.P. and K.P. conducted the experiments and collected the samples. K.P. supervised S.H. S.H., G.M., and M.O. performed the analyses and made the visuals for the data. K.P. and O.J.M. provided the initial interpretation of results. S.H. wrote the first draft of the manuscript. All authors contributed to the writing of the draft and reviewed the final version of the manuscript.

Declaration of Competing Interest

Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by Nofima and DTU Aqua. K.P., O.J.M., G.M. and M.O. are all working for Quantidoc AS. K.P. is the Chief Scientific Officer while O.J.M. is the Chief Executive Officer of the company.

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