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Peracetic acid as a potential treatment for amoebic gill disease (AGD) in Atlantic salmon - Stage 1

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Report

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Foreword

PERAGILL is a two-stage project funded by The Norwegian Seafood Research Fund (FHF 901472). The results presented in this report cover only the activities in Stage I.

Mention of trade names or commercial products in this report is solely to provide specific information and does not imply recommendation or endorsement from the funding agency or the parties actively involved in the experiments.

Contents

1 Summary

English summary

PERAGILL is an initiative that ultimately aims to develop an alternative treatment for the currently available therapies for amoebic gill disease (AGD) that have several practical and environmental issues. Peracetic acid (PAA) is a potent oxidant with a broad spectrum of antimicrobial activity and decays into relatively safe residuals, thus, has been widely recognised as a sustainable disinfectant in aquaculture. Earlier reports on PAA underscore its potential to address the challenges of the current AGD treatments. Hence, this project aimed to establish its credentials as a chemotherapeutant for AGD. Stage 1 documented the impacts of PAA exposure on the health and welfare of salmon, its degradation kinetics and its antiparasitic activity against the *Paramoeba perurans*, the causative agent of AGD. There were 3 *in vivo* exposure experiments performed where salmon were exposed to varying levels of PAA. Experiment 1 was designed to evaluate whether previous exposure history might desensitise the responses upon re-exposure. Salmon were exposed to different nominal concentrations (0, 0.6, and 2.4 ppm) of PAA for 5 min, followed by a re-exposure to the same concentrations for 30 min 2 weeks later. Experiment 2 explored how a stressful episode before exposure might interfere with the adaptive responses to PAA. Fish were subjected to crowding stress prior to PAA exposure at 4.8 ppm for 30 min. And lastly, Experiment 3 investigated the impacts of repeated exposures to PAA. Salmon were exposed to 10 ppm PAA either for 15 min to 30 min every 3 weeks, with 3 exposures in total. Growth performance was not affected in all exposure trials. Behavioural changes such as agitation, erratic swimming, increased ventilation and loss of balance during exposure were only observed in experiment 3. No significant mortality was recorded in all experiments, and exposed fish recovered quickly after exposure as evidenced by unaffected feeding patterns. Though there were external welfare changes (e.g. skin damage, fin damage) following exposure, the degree of alterations was not dramatically high. Histological analyses of gills and skin revealed that despite the presence of some pathologies in PAA-exposed fish, mucosal barriers can still be categorised as healthy. Repeated exposure, however, may compromise the barrier status of the gills as observed in experiment 3. PAA could trigger oxidative stress. In addition, classical players of systemic stress responses were activated by PAA exposure. The adaptive responses were robust and, in most cases, the level returned to basal concentrations hours after exposure. Crowding stress prior to exposure could interfere with the normal systemic stress and antioxidant responses to PAA. Metabolic profiling revealed that PAA concentrations in experiments 1 and 2 did not substantially alter the plasma metabolomes. Recurrent exposures, however, have a significant impact. Metabolites that were differentially affected by PAA exposure were known to be involved in protecting the cells from oxidative stress damage, suggesting that salmon were able to mount a strong protective response against PAA-induced oxidative stress. Transcriptomic profiling of the mucosal tissues (i.e., skin and gills) demonstrated that PAA could trigger a strong immunological response as several differentially expressed genes following PAA exposure have known roles in immunity. Skin transcriptome was more responsive than the gills at lower PAA dose. However, the opposite trend was identified at a higher dose. The developed gill explant culture could be used as a model to compare mucosal responses to oxidants (i.e., PAA vs. H_2O_2). PAA exhibited amoebicidal activity against *P. perurans*. Viability of the amoeba can be reduced by 50 % following exposure to 4.8 ppm PAA and higher. Toxicity of PAA towards the amoeba was influenced by different factors (i.e., density, temperature, light, culture age) at varying degrees. Toxic effect of PAA against the amoeba is rendered by disruption of the cell membrane. The decay of PAA was affected by several factors including light, fish density and salinity. It was demonstrated that PAA degrades significantly

faster compared with H_2O_2 in seawater. Taken together, the results indicate that PAA is safe for use in salmon, with promising potential as a chemotherapeutant for AGD with low environmental risk.

Norwegian summary

Det overordnede målet for PERAGILL var å etablere en alternativ behandling mot AGD. Eksisterende behandlinger har flere praktiske og miljømessige begrensninger. Pereddikksyre (PAA) er et potent oksidasjonsmiddel med bredspektret antimikrobiell aktivitet som brytes ned til relativt trygge komponenter og har derfor blitt anerkjent som et bærekraftig desinfeksjonsalternativ i akvakultur. Det har allikevel ikke blitt testet ut som potensiell behandling mot AGD. Dette prosjektet hadde derfor til hensikt å teste ut hvorvidt PAA kunne være et alternativt behandlingsmiddel.

Fase 1 i prosjektet har undersøkt effekt av PAA eksponering på laksens helse og velferd, nedbrytningskinetikk og antiparasittisk effekt mot Paramoeba peruans, agenset som forårsaker AGD. Det ble gjennomført 3 in vivo eksperimenter der laks ble eksponert for ulike nivå av PAA. Eksperiment 1 var designet for å evaluere hvorvidt tidligere eksponeringshistorikk ville dempe sensitivitetsresponsen ved gjentatt eksponering. Eksperiment 2 undersøkte hvorvidt stresshåndtering i forkant at PAA-behandlingen ville påvirke effekten av PAA. I eksperiment 3 ble effekt av PAA etter gjentatt eksponering undersøkt.

Veksthastighet var ikke påvirket av noen av eksponeringsalternativene. Adferdsendring ble kun observert i eksperiment 3. Det ble ikke registrert økt dødelighet i noen av forsøkene og den eksponerte fisken kom seg raskt etter behandlingen. Fôringsaktiviteten ble raskt gjenopptatt. Eksterne velferdsforandringer var av mild karakter. Histologisk analyse av gjeller og skinn viste at selv om det var observert noe patologi hos den eksponerte fisken var de mukosale barrierene opprettholdt. Gjentatte eksponeringer så derimot ut til å påvirke det mukosale laget i gjellene (eksperiment 3). Fiskens adaptive responser var robust og i de fleste tilfellene var fiskens basale konsentrasjon gjenopprettet kort tid etter eksponeringen. PAA eksponeringen i eksperiment 1 og 2 påvirket ikke plasma metabolomet i motsetning til gjentatt eksponering (eksperiment 3).

Transkriptomanalyse av mukosale vev (skinn og gjeller) viste at PAA trigger en sterk immunologisk respons. Den etablerte gjellekulturmodellen egner seg til å teste mukosal respons mot oksidasjonsmidler (som PAA vs H2O2). PAA hadde amøbicid effekt mot *P. perurans.* Effekten av PAA var påvirket av flere faktorer (som tetthet, temperatur, lys). PAA ødelegger amøbens cellemembran.

Nedbrytingen av PAA var påvirket av en rekke faktorer inkludert lys, fisketetthet og salinitet. Forsøkene viste at PAA nedbrytes signifikant raskere enn H2O2.

Resultatene fra forsøkene indikerer at PAA er trygt for laksen og er et potensielt lovende medikament mot AGD med lav miljømessig risiko.

2 Introduction

Diseases remain a significant bottleneck in Atlantic salmon aquaculture. Besides sea lice infection, amoebic gill disease (AGD) is a perennial parasitic issue in global salmon aquaculture, including in Norway. The causative agent of the disease is the free-living and opportunistically parasitic amoeba *Paramoeba perurans* (syn. *Neoparamoeba perurans*) [1, 2]. Cases have been documented as well in other salmonid species (*e.g.*, rainbow trout, *Oncorhynchus mykiss*; brown trout, *S. trutta*; chinook salmon, *O. tsawyschta*), Ballan wrasse (*Labrus berhylta*), sharp snout sea bream (*Diplodus puntazzo*), seabass (*Dicentrarchus labrax*), ayu (*Plecoglossus altivelis*) and blue warehouse (*Seriolla brama*) [3-6]. Acute cellular necrosis is a canonical pathological manifestation of AGD infection [7, 8]. Also, branchial epithelial hyperplasia is likewise manifested that results in the characteristic hyperplastic plaque on the gills infiltrated with inflammatory immune cells.

AGD was first reported in Tasmania, Australia in the 1980s. Outbreaks have been reported after that in other countries including the United States, Chile, Ireland, Spain, France and Japan. The first case of AGD in farmed salmon in Norway was reported in 2006 [9]. It was detected for the second time in 2012 with five positively diagnosed cases, and since then, the prevalence has been increasing. An increment of about 1000 % was documented in 2013 with 56 cases, and the number increased even more to 70 in 2014, with predictions of progressive advance northwards of the outbreaks in the following years [6]. The rate of increase and the expansion of localities where outbreaks have been identified are pointing to the imminent threat of AGD to Norwegian aquaculture. Freshwater and hydrogen peroxide (H_2O_2) bathing are the widely used approaches to treat AGD. Though freshwater bathing is effective in controlling AGD to a significant extent, the strategy entails substantial infrastructure cost and is labour expensive. One important consideration and remains a major challenge is a requirement for a nearby freshwater source [10]. Several chemotherapeutants have been explored for AGD treatment with H_2O_2 remains the most popular one. H_2O_2 is a common disinfectant in aquaculture against fungal, bacterial and protozoan infections [11]. The effectivity of H_2O_2 against AGD has been documented both in *in vitro* and *ex vivo* experiments with variable resolutions [10]. The spectrum of efficacy was limited and required a higher concentration; hence, the mortality problem is a significant issue during treatment [6, 10]. The high dose and the substantially large amount of H_2O_2 use not only for AGD but also for other parasitic infections (i.e., sea lice) have become a severe issue of the last years in Norway, mainly because it raises concerns of its environmental sustainability. To our knowledge, there are no publicly available data on the prevalence of H_2O_2 treatment for AGD in commercial production systems in Norway, though the results of laboratory-based experiments have recently been published by the Norwegian Veterinary Institute [12].

The need to find an alternative for the current treatments is timely and relevant. Peracetic acid (PAA) is a potent peroxygen compound and has gained prominence in the last ten years as a sustainable disinfectant in aquaculture [13, 14]. PAA is commercially available as an equilibrium mixture of acetic acid, H_2O_2, and water. The potential of PAA for improved biosecurity in aquaculture is based by its broad range of antipathogenic activity and rapid decay into neutral residuals (*i.e.*, carbon dioxide, oxygen, and water) [13-18]. It's fat solubility also significantly contributes to its potent antimicrobial activity [19]. Oxidative disruption of cell membranes via hydroxyl radicals is the primary mode of action of PAA [20, 21]. These radicals interrupt the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport [22, 23]. PAA oxidises enzymes thereby impairing the biochemical pathways, active transport across membranes, and intracellular solute levels [24]. It is also suggested that it can oxidise the sensitive sulfhydryl and sulfur bonds in proteins, enzymes, and other metabolites [15, 20].

These features account for why PAA is effective against a wide range of microorganisms, including *Ichthyophthirius multifiliis*, *Aeromonas salmonicida*, *Flavobacterium columnare*, *Yersinia ruckeri*, *Saprolegnia* spp., *Aphanomyces* spp., and infectious salmon anemia virus [15], where, in most cases, the effective dose is less than 2 mg L^{-1}[14].

PAA is being proposed as a chemotherapeutant against AGD. It offers several advantages that accentuate its potential for AGD treatment. First, it degrades entirely within several hours after application into harmless, neutral residuals (acetic acid and H_2O_2 and eventually to H_2O) [17]. Second, the effective concentration of PAA against various aquaculture pathogens is less than 2 mg L^{-1}. This is way below compared with H_2O_2 that requires a much higher level (over 20 mg L^{-1}) to achieve successful disinfection. Third, PAA application as routine disinfection in recirculating aquaculture systems has little impact on fish health, and evidence suggests that fish could habituate to continuous or periodic exposures and with no compromise in fish welfare [25, 26]. And lastly, PAA exhibits anti-parasitic effects. The antimicrobial activity and anti-parasitic effects remain potent over a wide temperature range, including temperatures below 10 °C [27, 28], and its antimicrobial activity is far more potent than H_2O_2 [20, 28, 29]. It remains to be documented whether PAA has an inhibitory activity against *P. perurans*.

Most of the studies on the application of PAA in aquaculture have focused on degradation kinetics, antimicrobial activity, impacts on water quality, and biofilter nitrification [14, 17, 18, 30]. Though toxicological data exist [15], the physiological responses of fish to PAA exposure are not well documented, and this might undermine its potential as sustainable prophylaxis and chemotherapeutant in aquaculture. It is inherent that in the development of new therapeutic measures that the health and welfare consequences of a new compound must be established first before its application — no published data on how salmon respond to PAA.

PERAGILL is a project led by Nofima – The Norwegian Institute of, Food, Fisheries and Aquaculture Research in collaboration with the Technical University of Denmark (DTU Aqua), The Norwegian Veterinary Institute (VI), Quantidoc As and Lilleborg AS.

FHF appointed Linda Andersen from Industrial and Aquatic Laboratory (ILAB) and Amund Litlabø from Aqua Pharma AS as members of the Reference Group.

3 Objectives

Developing new alternative methods for AGD must address safety, efficacy, and sustainability. The documented features of PAA warrant an initiative to explore its potential as a treatment for AGD. Therefore, the overarching aim of PERAGILL is to explore the potential of PAA as an alternative and sustainable treatment to amoebic gill disease, an emerging threat in the Norwegian Atlantic salmon aquaculture.

In Stage I, we aim to establish the credentials of PAA as an alternative treatment by exploring the behavioural, physiological and morphological responses of salmon to PAA with the use of an integrative toolbox, by evaluating its antiparasitic potential and by determining how PAA degrades under different production scenarios.

The sub-objectives of Stage I are as follows:

- *To assess the impact of PAA treatment on fish health and welfare.*
- *To investigate the amoebicidal activity of PAA and identify factors influencing this feature.*
- *To determine the potential environmental risk of PAA treatment.*

4 Materials and methods

4.1 Ethical statement

All fish handling procedures complied with the Guidelines of the European Union (2010/63/UE), as well as with national legislation. Experiment 3 was conducted with the approval from Mattilsynet under FOTS ID 19321.

4.2 Peracetic acid

Peracetic acid (Divosan Forte™, PAA) was supplied by Lilleborg AS (Oslo, Norway). The actual concentration of PAA in the commercial product was verified by the DTU Aqua laboratory (Hirtshals, DK) to be at approximately ~18% v/v. The solution was stored at 4°C. During each exposure, the concentration of PAA in the water was experimentally verified [17] in real-time to ensure that the fish were exposed to the target concentration from start to termination of exposure.

4.3 Exposure of salmon to different PAA concentrations

Experiments 1 and 2 were performed at the Recirculation Aquaculture Facility of DTU Aqua in Hirtshals, Denmark. Experiment 3 was performed at Havbrukstasjonen i Tromsø (HiT) in Tromsø, Norway. Salmon smolts for Experiments 1 and 2 (Experiment 1: 150.3 ± 5.6 g, mean ± SE; Experiment 2: 131.3 ± 2.3 g) were purchased from Danish Salmon A/S (Hirtshals, Denmark) while the fish used in Experiment 3 (82.03 ± 5.6 g) were produced by HiT.

4.3.1 Experiment 1

Upon arrival at the recirculation aquaculture (RAS) facility of DTU Aqua (Hirtshals, DK), fish were sorted and moved to six 1-m2 holding tanks (water volume ≈ 600 L), with 60 fish in each tank. The RAS had a 40-µm drum filter, a submerged fixed bed biofilter, and a trickling filter with a makeup water exchange at approximately 0.4 m3/h, equivalent to a retention time of 1.5 days. Internal recirculation allowed more than two-times the tank exchange per hour. Fish were acclimated for 3 weeks under stable conditions, with daily monitoring of water quality parameters, which were kept within safe limits. The tanks had no direct light above them, and the photoperiod in the experimental hall was set at 16L:8D (06.00 – 22.00), similar to the natural photoperiod in April–May 2017 (57º35'N 09º57'E). Water temperature was at 15±1°C. The fish were fed (Biomar, EFICO Enviro, 4,5 mm) at a ratio of 1.0–1.5% total biomass per day using a belt feeder. Feeding was gradually increased during the acclimation period, and feeding behaviour of the fish in terms of uneaten feed pellets was registered by daily inspection of the swirl separator.

To represent the pre-exposure fish, on the day before the first exposure, two fish from each of the holding tanks were sampled, as described in detail in Section 2.4. Feeding was temporarily ceased 24 h prior to PAA exposure. Fish were netted from the holding tank, transferred to a transportation container, and immediately thereafter into a 300-L exposure tank. Each holding tank had its equivalent exposure tank, and water quality parameters were identical between these two tanks. The fish were allowed to settle for 10 min before the PAA solution was added to the tanks to achieve the following final concentrations: 0 (seawater), 0.15, 0.30, 0.6, 1.2 and 2.4 ppm. For this report, we will only show

the data from 0, 0.6 and 2.4 ppm groups. Even PAA distribution was assured by vigorous aeration directly into the rearing tanks. The concentrations were pre-selected based on an earlier report on the toxicity of PAA for rainbow trout [15]. Each treatment group had two replicate tanks. During the exposure period, the water flow to the tanks was stopped, and the decay of PAA in the water matrix was followed. After 5 min, fish were immediately netted out of the tank and returned to their corresponding holding tank. Post-exposure samplings were carried out thereafter, as detailed in Section 4.3.3. Two days after the PAA exposure, feeding was resumed, similar to the protocol in Section 2.2. All husbandry conditions during post-exposure rearing were similar to pre-exposure conditions. Two weeks after the first exposure, the fish were re-exposed to the same concentration of PAA. The protocol used in the re-exposure experiment was identical with the approach employed in the initial exposure, with a slight modification on the duration of exposure. Instead of 5 min, fish were re-exposed to PAA at a similar concentration used in the first trial for 30 min. Fish were returned to their corresponding recovery tank, and post-exposure samplings were carried out thereafter.

4.3.2 Experiment 2

A second batch of smolts was transported to the aquaculture facility of DTU Aqua, sorted and moved to two 4-m^2 holding tanks (water volume ≈ 1500 L) in a seawater flow-through system, with approximately 100 fish in each tank. The fish acclimated for 2 weeks under stable rearing conditions, with daily monitoring of water quality parameters. Water temperature was at 11±1^oC. The photoperiod was set at 24L:0D and the dietary ration of 1–1.5% total biomass (Biomar, EFICO Enviro, 4,5 mm) per day was provided using a belt feeder.

Fish were starved for 24 h before the exposure experiment. Before the experiment was carried out, four fish were collected from each holding tank to represent the pre-exposure fish. Fish were transferred to a closed-system 500 L exposure tank to achieve a density of 15 kg/m^3. The fish were allowed to settle for 15 min before a group was subjected to crowding stress for 1 h, by lowering the water volume to attain a density of 75 kg/m^3. Aeration was provided during crowding stress. Fifteen minutes after the water level returned to the initial level, one group of the stressed fish was exposed to 4.8 ppm PAA, double the highest concentration tested in experiment 1, while the other stressed group was exposed 0 ppm (seawater) for 30 min. Likewise, another group of fish was transferred to the same exposure tank but was not exposed to crowding stress. After allowing the fish to settle for 15 min, one group was exposed to 4.8 ppm PAA, and one group was exposed to 0 ppm (seawater) PAA for 30 min. After the exposure experiment, fish were transferred to their corresponding recovery tank, similar to what was used in experiment 1. Each treatment group was represented with duplicate tanks.

4.3.3 Sampling strategies for Experiment 1 and 2

For experiment 1, sampling was conducted at 2 h, 48 h and 2 w after exposure for each occasion. For experiment 2, sampling was carried out at 1 h, 4 h, and 2 w after PAA exposure. Five fish were taken from each replicate tank and were humanely euthanised with an overdose of 20 % benzocaine solution. All fish for sampling were photographed for skin colour analysis. External welfare status was evaluated following the FISHWELL handbook. After the length and weight were measured, blood was withdrawn from the caudal artery using a heparinised vacutainer, centrifuged at 1000 x g for 10 min at 4°C, and plasma was collected and kept at –80°C until analyses. The same sampling protocol was applied for fish that were collected before exposure. Tissue samples were collected for RNA isolation. A portion of the dorsal skin and the second gill arch was dissected and transferred to RNAlater

(Ambion, USA). Tissue samples in RNAlater were left at room temperature overnight and thereafter kept at – 80°C before RNA extraction.

4.3.4 Experiment 3

Forty fish were stocked into each of the 500-L tanks with 35 ppt salinity, 10-12^{o}C. There were 9 tanks in total, 3 tanks for each treatment groups (i.e., 0 ppm, 10 ppm for 15 mins, 10 ppm for 30 mins). Fish were allowed to acclimatise for at least a week before the first exposure. Feeding was stopped 24-h before exposure and sample collection. Before PAA exposure, 3 fish were taken from each tank to represent the pre-exposure group. *The exposure protocol was as follows:* Water flow in the tank was closed. PAA was added to the tank to achieve the final concentration of 10 ppm. The 0 ppm group (Group A) served as the mock control group. Fish were exposed to PAA for either 15 (Group B) or 30 (Group C) mins. During the exposure period, fish behaviour was documented. Oxygen was supplied during exposure to facilitate mixing. After the exposure period, the water outlet was opened, and water flow was increased dramatically to ensure that the disinfectant was flushed out. At least 75 % of the water was replaced in the next 5-10 mins after exposure.

After 24 hrs, 3 fish were taken from each tank and were humanely euthanised with an overdose of anesthetics. All fish for sampling were photographed for skin colour analysis. The length and weight of fish for sampling were recorded. External welfare status was also evaluated following the FISHWELL handbook. Blood was withdrawn from the caudal artery for plasma collection. A commercially available mucus collection kit collected skin mucus. Skin and gills were sampled both for histology (formalin) and gene expression analysis (RNAlater). Liver was also sampled for gene expression analysis. PAA exposure was performed every 3 weeks and there were 3 exposure occasions in the whole experiment.

4.3.5 Analyses

Samples collected from each experiment were subjected to different downstream analyses.

a) Plasma

The levels of classical stress indicators such as cortisol, glucose and lactate were measured. The total antioxidant capacity was also determined. Plasma samples were also subjected to shotgun metabolomics by LC-MS/MS to identify metabolic disturbances following exposure. The analyses were carried out using an UPLC system coupled to a high-resolution quadrupole-orbitrap mass spectrometer (Q Exactive™ HF Hybrid Quadrupole-Orbitrap, Thermo Fisher Scientific).

b) Histology

Quantidoc performed histological analyses for gills and skin samples from Experiment 1 and 3 through the Verribarr™ method. Nofima performed histology for samples from Experiment 2 following in-house histoprocessing protocol.

c) Microarray analysis

RNA was isolated using Agencourt® RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., CA, USA) according to the manufacturer's protocol. NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific) was used to evaluate the RNA quality and quantity. The quality of the RNA was further assessed with an Agilent® 2100 Bioanalyzer™ RNA 6000 Nano kit. In all experiments, the

gills and skin were subjected to Nofima's 15-K microarray analysis to identify the global molecular responses to PAA. The array includes unique probes to protein encoding transcripts, including those involved in immunity, tissue structure, integrity and functions, cell communication and junctions, extracellular matrix, metabolic pathways, secretion of mucosal proteins and digestive enzymes [31].

d) Skin color

Individual fish photos were used to measure skin colour changes following exposure. The picture was processed in an R-script to cut out a sub-picture of the skin from belly to back with a width of 600 pixels. The pictures were further processed in an R-script by determination of the mean color (RGB) values in the picture. The overall RGB means (plus overall mean) was expressed as the mean of the RBG means in the top of the picture (back half of the skin) and RBG means in the bottom of the picture (belly half of the skin).

e) Mucus

Mucus samples from experiment 3 were subjected to metabolomics, as described above.

4.4 Exposure of *P. perurans* to PAA

4.4.1 Amoeba isolate

The amoeba (*P. perurans*) used in this study was isolated from an AGD outbreak in a commercial farm in Norway kindly provided by the Norwegian Veterinary Institute (Sigurd Hytterød).

The amoebas were routinely grown in a specific growth media, Malt Yeast Broth (MYB) in cell culture flasks (TC 25 cm2 with filter) with seawater (35 ppt) and incubated at 15 °C. The amoebic cells were split and washed with filtered autoclaved seawater every second week.

4.4.2 Viability tests and preliminary exposure

Four commercially available assay kits were tested to determine the most suitable system to study amoeba viability after PAA exposure *in vitro*; 1) Neutral Red (TOX-4, Sigma); 2) Resazurin (TOX-8, Sigma); 3) MTT (CGD1, Sigma); and 4) WST-1 (CELLPRO-RO, Roche, Switzerland). To test the ideal seeding condition before PAA *in vitro* exposure, a preliminary trial was performed by seeding a well of the microplate with either 100 or 200 amoebae/well. After that, one plate was placed in a 15 °C incubator for approximately 24 h, while the other plate was placed in the same incubator for 30 min, before PAA exposure and viability tests. The seeded amoebae were exposed to different concentrations of PAA for 30 mins. Thereafter, the viability was determined following the manufacturer's protocol of each of the viability test kits. WST-1 was selected in the different *in vitro* exposure trials because the assay readouts were reproducible.

4.4.3 PAA exposure trials

***In vitro Trial 1: Effects of different PAA concentrations and exposure durations*.** Two plates were seeded with amoeba as described above and allowed to settle for 24 h in the incubator. The amoebae were exposed to PAA at concentrations 0 (distilled water), 0.6, 2.4, 4.8 and 9.6 ppm for either 15 or 30 mins. Unexposed amoeba served as control. After the incubation period, the media with PAA was

pipetted out and replaced with new media. The viability of the amoebae was evaluated using the WST-1 assay.

In vitro Trial 2. Effects of culture age. We tested the influence of culture age of amoebae on PAA toxicity. One plate was seeded with 1-week old amoeba culture while the other plate was seeded with a 2-week old culture. Thereafter, they were exposed to the different PAA concentrations for 30 mins. The viability was determined after the exposure period.

In vitro Trial 3: Effects of different amoeba density. Four different density were tested: 200, 500, 1000 and 2000 amoebae/well. The seeded amoebae were exposed to the four concentrations of PAA as in the other *in vitro* trials for 30 mins. The viability was determined after exposure duration by WST-1.

In vitro Trial 4: Effects of light. Plates for PAA exposure were seeded with amoebae (200 amoeba/well). PAA was added similarly as with the previous trials. During incubation, one plate was covered with aluminium foil while the other plate was exposed to light. Viability was determined thereafter.

In vitro Trial 5: Effects of exposure temperature. Amoeba plates were prepared and exposed to different PAA concentrations similarly with the other *in vitro* trials. One plate was placed at 15^{o}C during exposure while the other plate was left outside the incubator (room temperature, 22^{o}C). Viability was determined thereafter.

4.5 Decay of PAA

Besides the real-time determination of the decay kinetics in experiments 1 and 2, *in situ* experiments were performed. Several beaker-based seawater systems were set-up, where parameters vary including salinity, temperature and light. The system was spiked with PAA (1 mg/L) and its decay was followed [14]. The decay kinetics of PAA and H_2O_2 in seawater was also compared.

4.6 Development of gill explant culture model

The gill explant culture was established as described earlier [32, 33], but with modifications. Briefly, blood was withdrawn from the caudal artery with a heparinised vacutainer. The entire gills were dissected out and immediately placed in chilled wash medium (i.e., Leibovitz's L-15 GlutaMax™ Supplement (Gibco, USA) with 5 % v/v fetal bovine serum [FBS, Sigma-Aldrich, USA], 1 % 100× Antibiotic Antimycotic Solution (AA, Sigma-Aldrich), 1 % 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [Hepes, Sigma-Aldrich] and 0,2 % 5000 IU/mL heparin [Biochrom, Germany]). Both the left and right gills were collected. Sterile 1× phosphate buffered saline (PBS, Gibco) was injected into the collected gill tissue through the gill arch. PBS perfusion was performed 3–4 times until the gill tissue was almost blanched, indicating the significant elimination of blood. Perfused gill tissues were gently washed with wash medium and then transferred to chilled growth medium (i.e., Leibovitz's L-15 GlutaMax™ Supplement with 10 % FBS, 1 % AA, 1% Hepes and 1 % 100× Non-essential amino acids solution [Sigma-Aldrich]), where they were cut into small pieces of approximately 1–2 mm in size. Gill fragments were placed onto each well of a 24-well CellBIND™ (Corning, USA) plate earlier seeded with 100 µl of the growth medium. The plates with the gill fragments were placed in an incubator set at 13°C overnight to allow adherence. After 24 h, each well was supplemented with an additional 200 µl of the growth medium as gently as possible to avoid

disturbing the fragments. Daily microscopic evaluation of the explants and their outgrowths were performed under a light microscope during 7 days. The culture model was used to compare transcriptional responses of the gill mucosa to PAA and H_2O_2.

5 Results and discussion

5.1 General description of salmon exposed to PAA

Narratives from earlier studies in rainbow trout suggest that erratic swimming, agitation and grasping for air are some of the typical behavioural responses of fish exposed to PAA at higher concentrations (>2 mg/L). There were no significant behavioural changes observed in both experiments 1 and 2. The fish remained calm during the whole PAA exposure period. There was no recorded mortality as well in both trials. In experiment 3, behavioural changes were observed in fish exposed to PAA. The overall sequence of response was as follows: *first 5 minutes* – abrupt swimming, minimal agitation; *the next 10 minutes* – increased ventilation, increased agitation in some fish; *the last 15 minute*s – increased ventilation was still prominent, fish were calmer, some fish (4-6) in each tank appeared to have lost balance. These behavioural responses indicate that fish might have tried to swim away from what they perceived to be foreign in the immediate environment, but eventually, they have been accustomed to it. After the water with PAA had been flushed out and replaced with new water, fish seemed to recover quite fast. Normal swimming was observed immediately after exposure, and hyperventilation stopped. These observations were noted in all the exposure occasions. There was one dead fish 24 h after the 1st and 2nd exposures in the group that was exposed to 10 ppm for 30 minutes. Nevertheless, the mortality record was not significantly different between groups.

It was also observed that fish resumed feeding right after treatment in all exposure trials. No case of emaciated fish was noted. Weight at termination in all treatment groups was not statistically different in all three experiments.

5.2 External welfare of PAA exposed fish

Overall, the PAA application did not significantly affect the overall external welfare status of salmon (Figures 1-3). Regardless of the PAA concentrations, exposure, and presence of a confounding/compounding factor (i.e., stress), the overall welfare index remains favourable with scores below 1. It appeared that fish from experiments 1 and 2 had higher cases of external welfare issues compared with the fish in experiment 3. This apparent difference might be due to the status of fish stocks used.

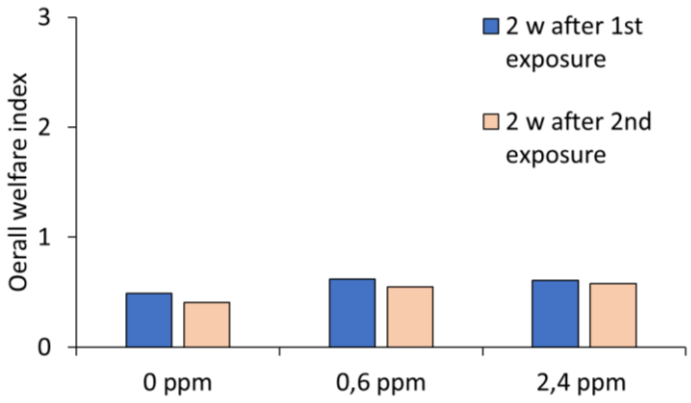

Figure 1 Overall external welfare of salmon in Experiment 1. External welfare was assessed two weeks after the initial exposure and re-exposure.

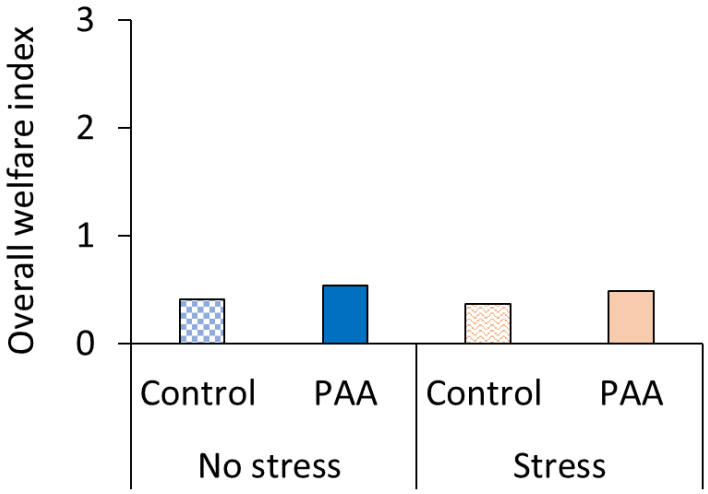

Figure 2 *Overall external welfare of salmon in Experiment 2. External welfare was assessed two weeks after exposure.*

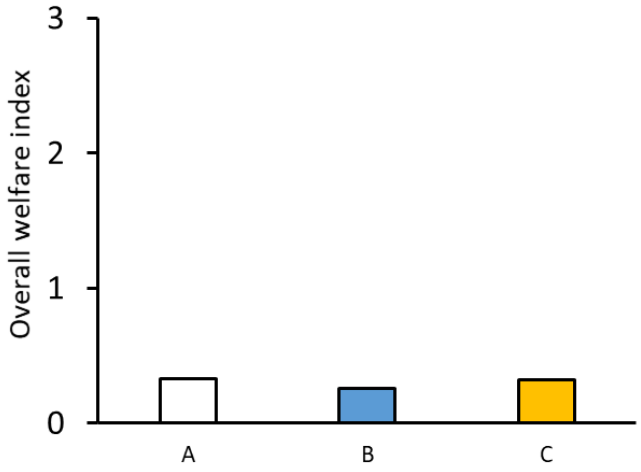

Figure 3 *Overall external welfare of salmon in Experiment 3. A = unexposed fish; B = 10 ppm, 15 mins; C = 10 ppm, 30 mins. External welfare was assessed after the 3rd exposure.*

We likewise evaluated the variations of individual external welfare indicators (Figure 4-6). Skin, pectoral fin, and dorsal fin damages were common in Experiments 1 and 2. Almost 90 % of the recorded skin damage was scale loss. In Experiment 1, it appeared that skin damaged slightly increased after the re-exposure in all groups. In Experiment 2, fin damages in no stress-control group were relatively lower compared with the other group. Caudal fin and skin damages were slightly higher in the stress-PAA group. Nonetheless, the change relative to other groups was not substantial. Most of the scores in Experiment 3 were below 1, indicating that repeated exposures to PAA did not have a dramatic impact on the welfare status. It was quite striking that cases of caudal fin damages were higher in A (control) than in the PAA exposed groups (B&C). Overall, there was no indication that PAA exposure could compromise external welfare status of salmon.

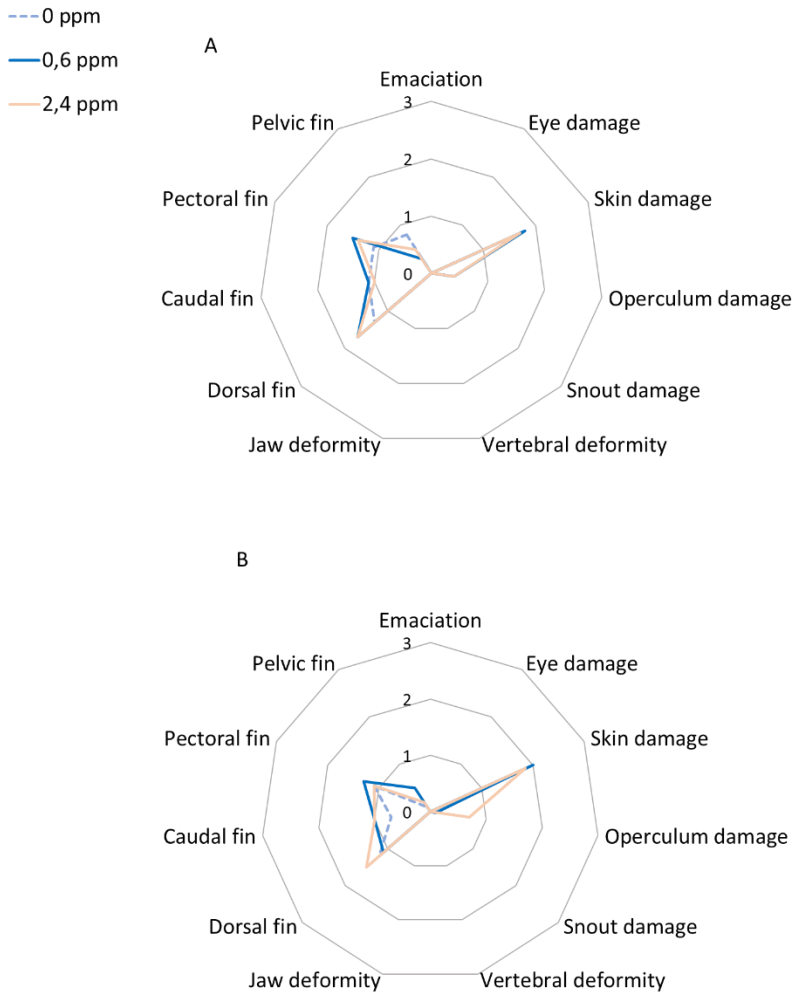

Figure 4 *Scores of individual welfare indicators two w after A) first exposure and B) re-exposure in Experiment 1. The 0 represents the "best" and 3 represents the "worst".*

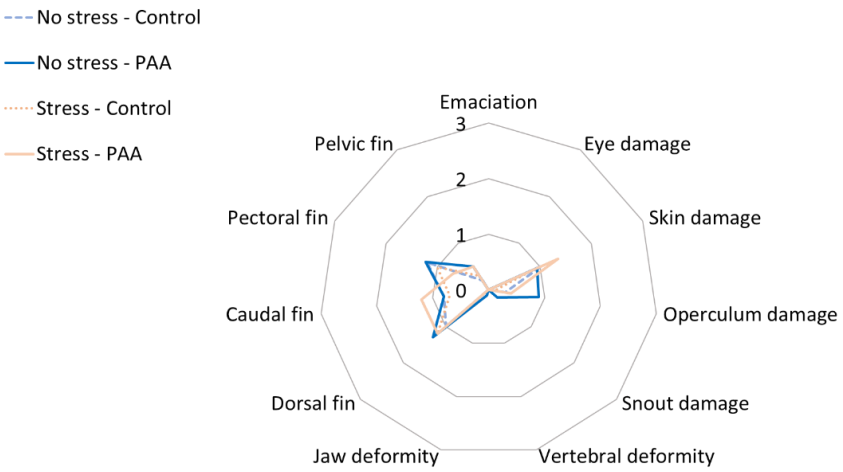

Figure 5 *Scores of individual welfare indicators two w after exposure in Experiment 2.*

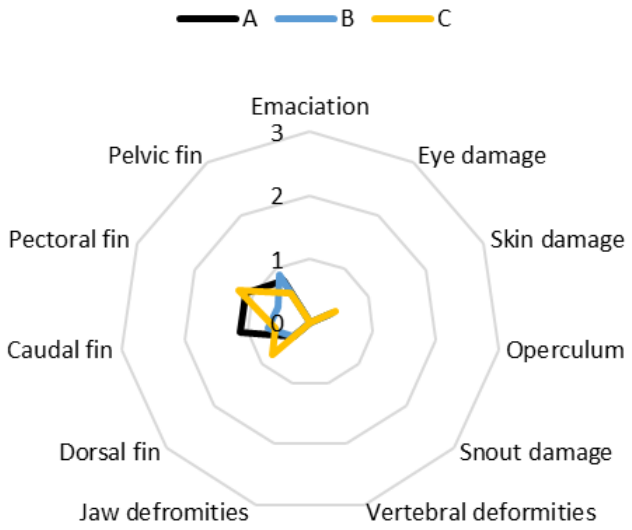

Figure 6 Scores of individual welfare indicators after the 3rd exposure in Experiment 3.

5.3 Total antioxidant capacity

As a strong oxidant, it is likely that exposure to PAA might trigger oxidative stress. Oxidative stress occurs when the balance between reactive oxygen species (ROS) and the antioxidant defence system is disturbed [34]. Excessive levels of ROS might have detrimental effects on lipid metabolism, protein synthesis, and DNA [35]; therefore, scavenging off the radical surplus must be performed effectively. The level of total antioxidant capacity in the plasma indicates whether the fish experienced oxidative hence the production of antioxidants. A 5-min exposure did not trigger significant changes in the TAC of plasma (Figure 7A). Re-exposing the fish to the same concentration at a more prolonged period significantly elevated the TAC in 2.4 ppm group that lasted for at least 48 h (Figure 7B). Such an increase was also observed in 0.6 ppm group but only after 48 h.

Experiment 2 revealed that a stressful episode before exposure interfered with the systemic antioxidative response to PAA (Figure 7C). Fish without stress history prior to PAA exposure displayed increased TAC, notably at 4 h after exposure. The increase in TAC in PAA-exposed fish indicates that systemic antioxidant repertoire against PAA-induced oxidative stress has been mobilised. Crowding interfered with the TAC system in response to PAA as the groups exposed to crowding stress before PAA exposure displayed no significant differences. It could be speculated that the stress status of fish before PAA exposure could disturb the ability of fish to mount a systemic antioxidant response to oxidative stress.

Repeated exposure to a higher dose of PAA did not have striking TAC responses 24 h after each exposure, except on the 3rd occasion, thereby suggesting that the response might be an additive effect (Figure 7D). At 24 h after 3rd exposure, TAC of Groups B and C was 10 and 12 % higher, respectively compared with the unexposed group (A). It appeared that the recovery was quite faster compared with the re-exposure changes in Experiment 1 (i.e., 0.6 and 2.4 ppm 48 h after re-exposure). The difference in temperature (15 versus 12) might have played a crucial role in the mobilisation of antioxidants and not just the level of PAA.

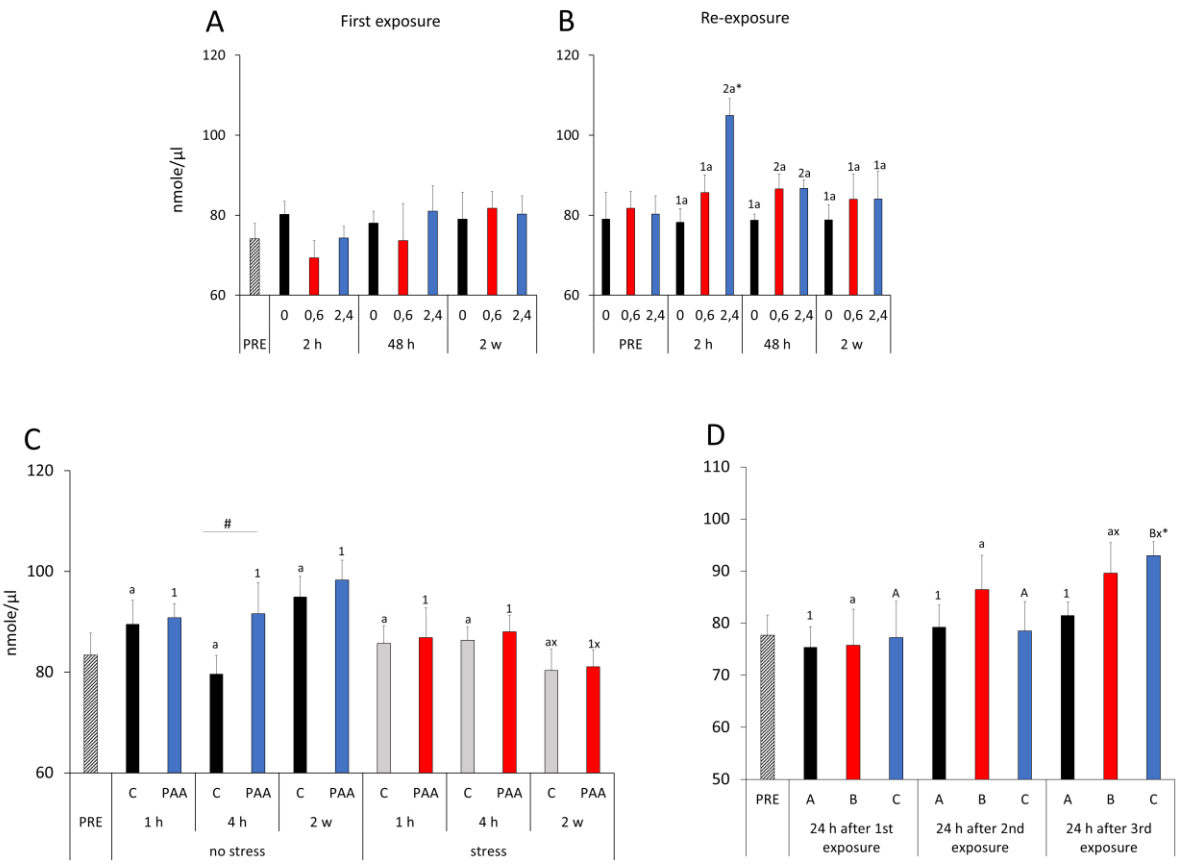

Figure 7 *Changes in the level of total antioxidant capacity (TAC) in plasma of fish from Experiment 1 (A & B), Experiment 2 (C) and Experiment 3. The level is expressed relative to Trolox standards. Values are mean ± SE of eight individual fish. Notations: For graphs A & B (Experiment 1): an asterisk (*) denotes significant difference between a treatment group and the pre-exposure group (PRE), different numbers signify significant differences between treatments within a sampling point, and different letters indicate significant differences within a treatment through time. For graph C (Experiment 2): different letters denote significant differences within control groups through time, while different numbers indicate differences within PAA exposed groups through time. The same notations are used for no stress and stress groups. x designates that the level of a particular group significantly differs between no stress and stress groups, whereas # indicates a significant difference between the control and PAA-exposed group at a particular time point. For graph D (Experiment 3): different numbers denote significant differences within Group A (control) through time, different small letters indicate differences within Group B (10 ppm, 15 mins) through time and different capital letters indicate differences within Group C (10 ppm, 30 mins) through time. asterisk (*) indicates that level is significantly different from the pre-exposure group, while x denote that the level is significantly different from Group A at a particular time point.*

5.4 Stress responses of salmon exposed to PAA

Salmon were able to mount a robust adaptive response to PAA (Figures 8 and 9). Plasma cortisol levels from Experiments 1 and 2 followed the same pattern (Figure 8A, G) - a significant increase in the early hours after stress had been triggered, then followed by a decrease and return to the baseline values thereafter, which is the classical cortisol response to stress in fish [36, 37]. A distinctive rise in cortisol level was observed in the 2.4 ppm group at 2 h post-re-exposure, but it returned to basal level thereafter (Figure 8D). This indicates that fish were able to mount an acute cortisol response to PAA. In experiment 2, all groups – regardless of treatment (control, PAA, and/or stress) – had identical

patterns in their average cortisol response, which was significantly elevated in the first 4 h after exposure. The similarities in the response of the two groups illustrate that potential interactions and additive effects did not alter the ability of fish to mount a cortisol response to a challenging condition (Figure 8G). In Experiment 3, there were no significant differences in the cortisol level in all groups at all sampling points (Figure 9A).

PAA did not significantly alter the glucose level in either of the exposure occasions in experiment 1, though temporal variability was apparent (Figure 8B,E). In experiment 2, however, prior stress history and a higher PAA dose resulted in the differential activation of glucose metabolism (Figure 8H). PAA induced increased glucose level in fish the were not exposed to stress prior to exposure. Such a change was not observed in the group that was exposed to crowding before exposure. Glucose is mobilised following a stressful event to ensure energy is provided to overcome the physiological pressure of the situation [36]. The result in the stress group suggests that the fish might have already mobilised the stored glycogen during the crowding stress [38]. Thus, no adaptive changes were identified when subjected to another stressor. In experiment 3, glucose level in the 2 groups was not affected in the 1st and 3rd exposures. However, in the 2nd exposure, Group C had significantly lower glucose level compared with the other groups in that particular time point (Figure 9B).

Lactate is known to increase as a response to a stressful condition [36]. Experiment 1 revealed that the tested PAA concentrations, exposure duration, and re-exposure did not significantly alter the plasma lactate level (Figure 8C,F). This result corroborates other stress parameters (i.e., cortisol and glucose) in this experiment and further illustrates that though PAA exposure at tested concentrations triggered stress (i.e., changes in plasma cortisol), the magnitude of the stress was not high, though there was no clear distinction that can be drawn with regards to stress versus no stress prior to PAA exposure. This not so dramatic lactate response was also identified in Experiment 3 as no significant changes were observed between groups in all time-points (Figure 9C). The corroboration of lactate response between the 3 experiments suggests that even though salmon experienced the stressful condition during PAA exposure, the magnitude was not overly high as other canonical stress indicators such as lactate were not markedly affected.

Repeatedly exposed fish in Experiment 3 were subjected to crowding stress after the 3rd exposure to evaluate whether stress responses to a secondary stressor were altered following the treatments. Analyses of these samples are on-going.

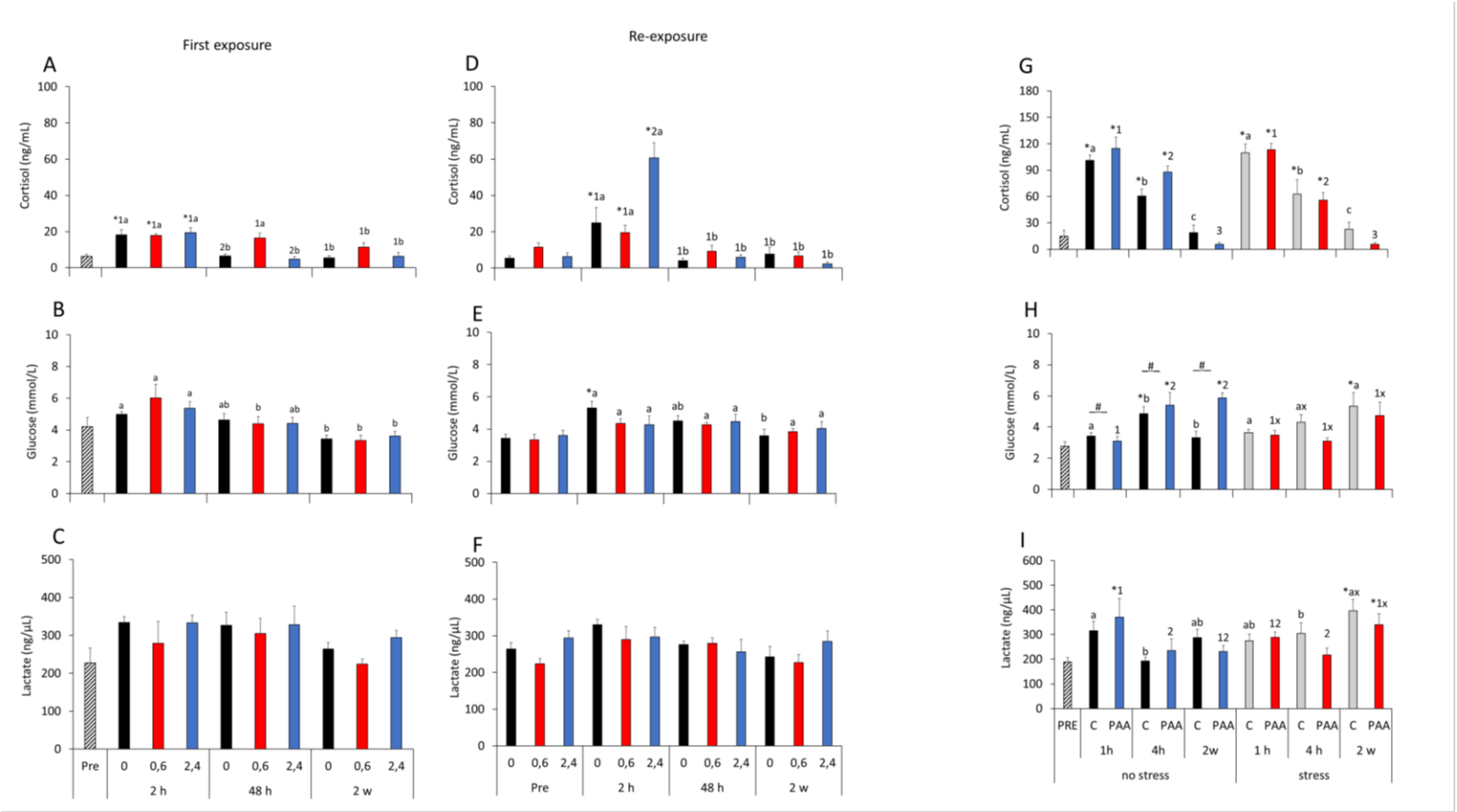

Figure 8 *Changes in the level of plasma stress indicators (cortisol, glucose and lactate) of fish from Experiment 1 (A-F) and Experiment 2 (G-I). Values are mean ± SE of eight individual fish. Notations: For graphs A & B (Experiment 1): an asterisk (*) denotes significant difference between a treatment group and the pre-exposure group (PRE), different numbers signify significant differences between treatments within a sampling point, and different letters indicate significant differences within a treatment through time. For graph C (Experiment 2): different letters denote significant differences within control groups through time, while different numbers indicate differences within PAA exposed groups through time. The same notations are used for no stress and stress groups. x designates that the level of a particular group significantly differs between no stress and stress groups, whereas # indicates a significant difference between the control and PAA-exposed group at a particular time point.*

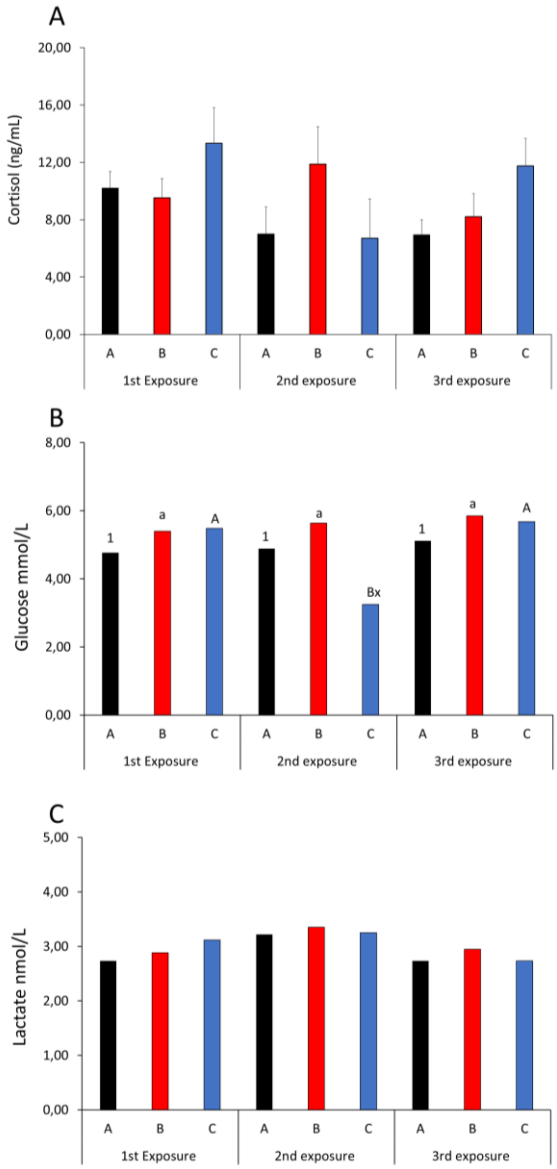

Figure 9 *Changes in the level of plasma stress indicators (cortisol, glucose and lactate) of fish from Experiment 3. Values are mean ± SE of eight individual fish. Different numbers denote significant differences within Group A (control) through time, different small letters indicate differences within Group B (10 ppm, 15 mins) through time and different capital letters indicate differences within Group C (10 ppm, 30 mins) through time. asterisk (*) indicates that level is significantly different from the pre-exposure group, while x denote that the level is significantly different from Group A at a particular time point.*

5.5 Histological evaluation of mucosal tissues

Experiment 1. Overall, the gills were in a healthy condition. Almost 94 % of all the gill filaments evaluated appeared to be healthy (Figure 10). Histopathological alterations were found side by side with healthy gill tissue. Histopathological damages included clubbing, hypertropia, hyperplasia, fusion, lifting, telangiectasias, and aneurisms (Figure 11). Though cases of hyperplasia and clubbing appeared to increase as the PAA dose increased in the initial exposure, the changes were not statistically significant. It was quite striking to observe, however that cases of lifting were high in 0 ppm group

compared with the PAA-exposed group in the initial exposure. Two weeks after re-exposure, cases of lifting were higher in the group exposed to 2.4 ppm, but not significantly different from other groups.

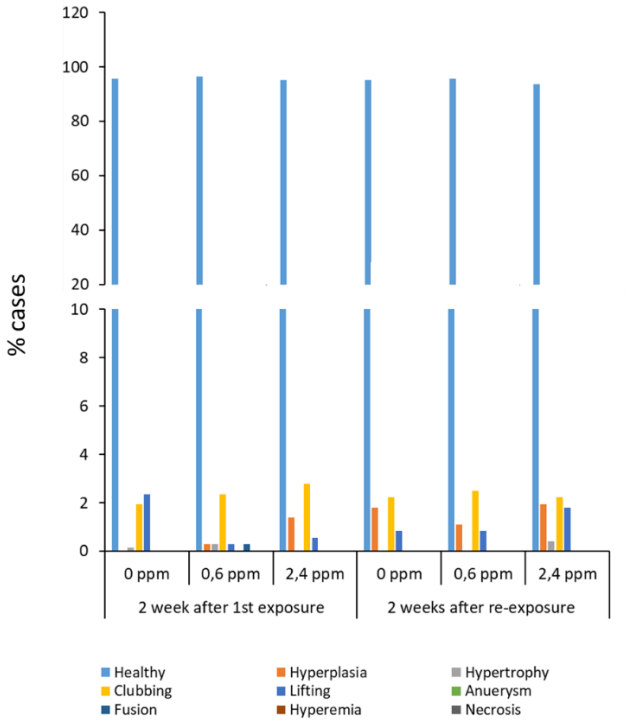

Figure 10 Histopathological cases in the gills of fish from Experiment 1. At least 120 individual filaments were evaluated per fish.

Mucosal mapping

Gills. The group exposed to 0.6 ppm PAA had significantly larger mucous cell area on the lamella than the control (0 ppm) and 2.4 ppm PAA group 2 hours after first exposure (Table 1; Figure 12). The mucous cells of the control and the 2.4 ppm increased to about the same size as the 0.6 and 1.2 ppm groups 2 days after first exposure. The following 2 weeks the control and the 2.4 ppm group decreased to the same values like 2 hours after first exposure. However, 0.6 and 1.2 ppm were stable. The re-exposure did not affect the groups exposed to 0.6 ppm and 1.2 ppm and the mucous cell area of the control and 2.4 ppm PAA had a trend towards increasing. The density of mucous cells on the lamella in all groups remained stable throughout the trial (Figure 13). The re-exposure had no immediate significant effect on the treatment groups. The group exposed to 2.4 ppm PAA increased significantly from 2 hours to 2 days after the first and second exposure. However, between 2 days and 2 weeks after second exposure, the mucous cell areas were constant across treatments. The diffusion distance increased from 2 hours to 2 days after first exposure, with a significant increase for the control. However, there was no significant difference in the rest of the trial.

Skin. The mucous cell density and the barrier status on the skin had a low difference between the first and the second exposure for the control and the group exposed to 0.6 ppm PAA. The mean mucous area tended to decrease in control between the first and the second exposure. However, the group exposed to 2.4 ppm PAA appeared to have a decrease in mean mucous area, mucous cell density and barrier status for the first exposure to the second exposure.

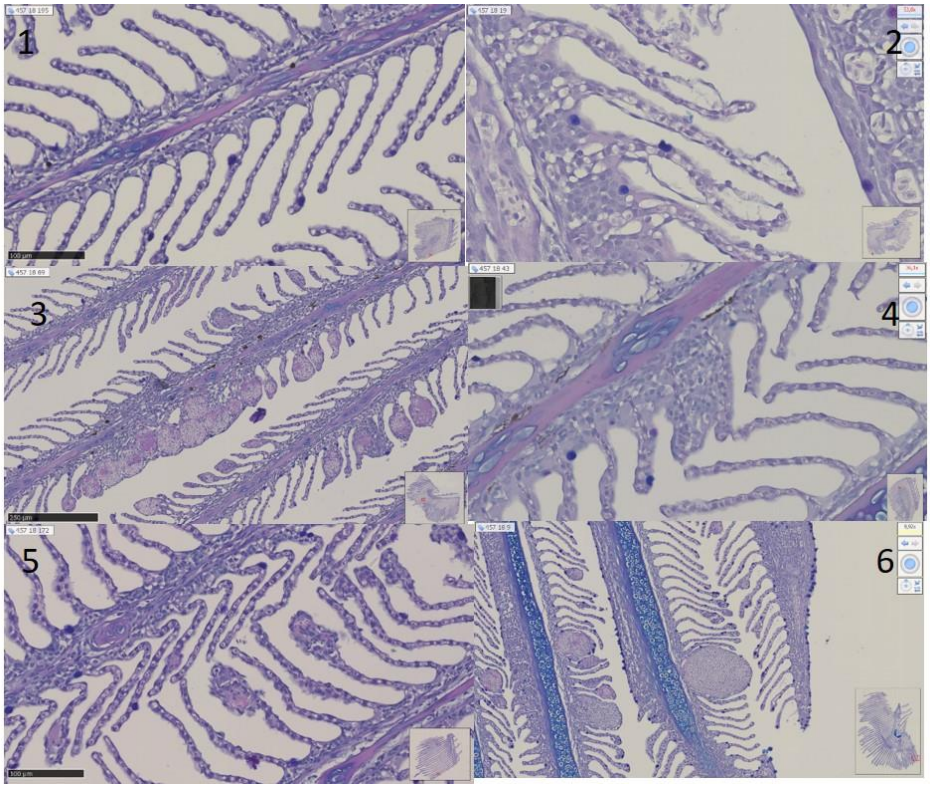

Figure 11 *Extremes of histopathological alterations in the gills of the fish in experiment 1. 1= healthy gills, 2=lifting, 3= Aneurism, 4= Hypertrophy, 5= telangiectasis and 6= Aneurism.*

Table 1 *Range of means for mucosal values and diffusion distance for all treatment groups. The mucosal measurements were done on 4 treatment groups with a total of 240 fish, and the diffusion length was measured on 120 gills at random points.*

	Diffusion length	**Mucous cell Density**	**Mean mucous cell area**	**Barrier status**
Range lamella	4.864±0.54µm to 6.94±1.35µm	0.97±0.6% to 2.27±1.44%	36.03±7.19µm^2 to 60.14±8.24µm^2	0.180±0.09 to 0.371±0.17
Range filament		6.34±1.89 % to 11.39±1.91%	72.61±9.29 µm^2 to 101.85±15.1µm^2	0.780±0.23 to 1.336±0.19
Range Skin		11.48±5.2% to 19.4±8.2%	164.78±15.7µm^2 to 200.9±49.2µm^2	0.66±0.25 to 0.94±0.20

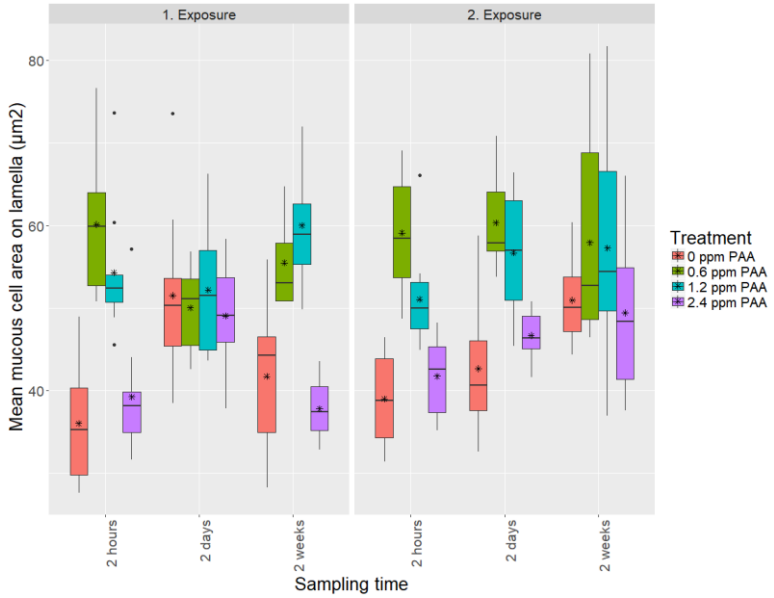

Figure 12 *The mean mucous area of the treatment groups at different sampling times. The red colour represents the control, the green represents 0.6 ppm PAA, blue represents 1.2 ppm PAA and the purple represent 2.4 ppm PAA. The graph is divided in 2 exposures. Second exposure was 15 days after first exposure.*

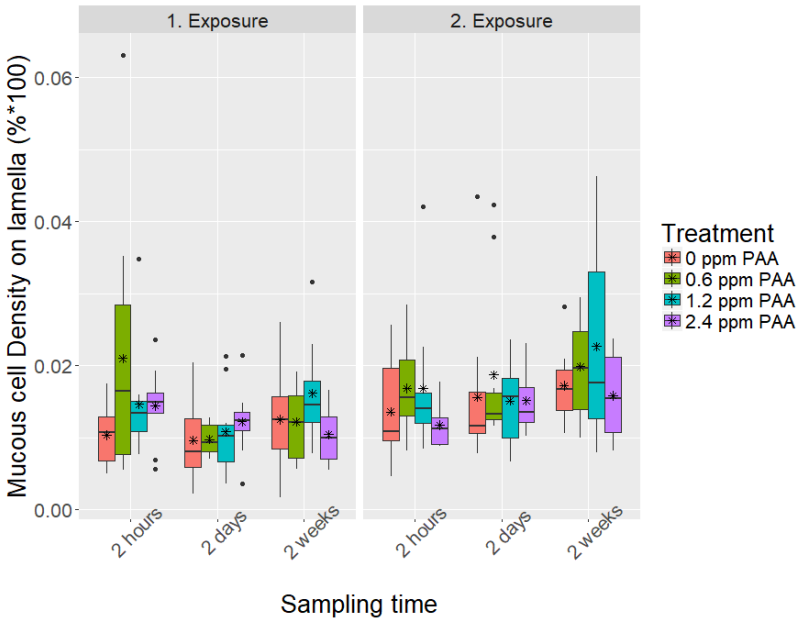

Figure 13 *Volumetric mucous cell density on the lamella. The density is given in %*100 of the mucosal epithelium. The red colour represents the control, the green represents 0.6 ppm PAA, blue represents 1.2 ppm PAA and the purple represent 2.4 ppm PAA.*

Experiment 2. Gill health was not affected as at least 93 % of the evaluated gill filaments were characterised as healthy (Figure 14). Moreover, it appeared that there was no clear interaction between prior stress and PAA exposure as the majority of the histopathological cases was almost the same between groups. Interestingly, cases of hypertrophy increased in the stress-PAA group while cases of clubbing increased in stress-control group 2 w after exposure.

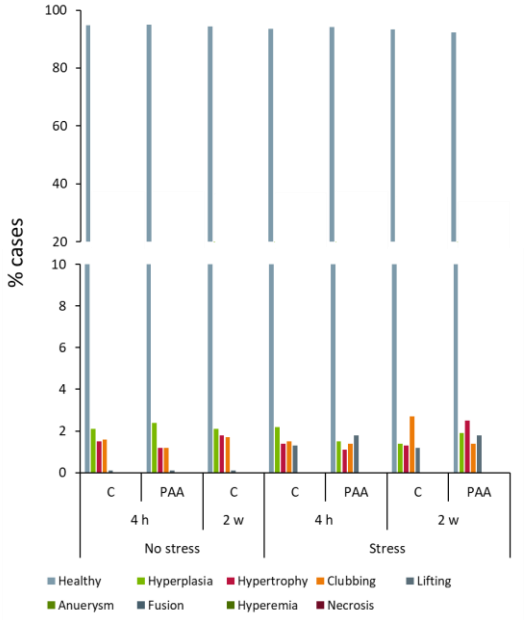

Figure 14 Histopathological cases in the gills of fish from Experiment 2. At least 120 individual filaments were evaluated per fish.

The number of mucus cells in the gills of fish that were not subjected to stress before PAA exposure did not vary between treatments and between sampling points (Figure 15). There was a clear difference, however, in the group that was exposed to crowding stress before PAA treatment, particularly at 4 h. It was identified that the total number of mucous cells and the mucous cells in the 2nd lamella significantly decreased in the PAA-exposed fish compared with the control group. This result substantiates how sensitive the mucous cells in the gills to stressors even at a short period. The decrease in the number of mucous cells may have significant implications in the mucosal immune function of the gills. The recovery was rather fast as all groups have similar mucus cell profile after 2 weeks.

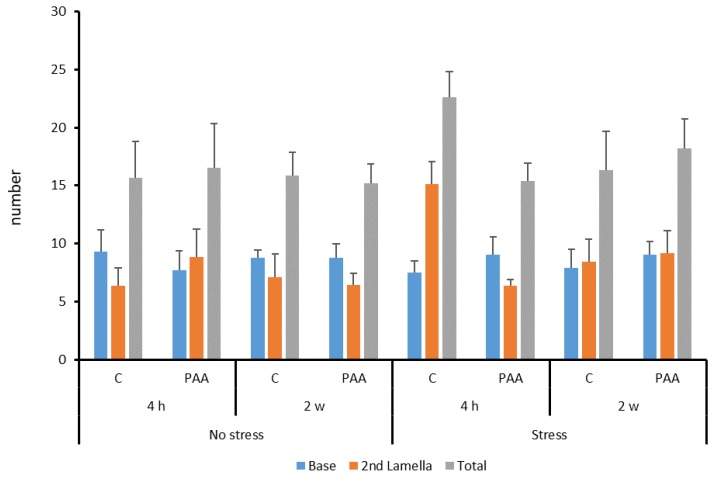

Figure 15 Mucus cells in the gills of fish from Experiment 2.

Experiment 3. Preliminary data of mucosal mapping of the gills and skin samples from Experiment 3 indicate that skin was not remarkably responsive to repetitive exposures to PAA (Figure 16). There was no significant difference between the groups in mucous cell area in the skin. Moreover, the density of skin mucous cells between the groups also exhibited no significant difference. There was no difference between the groups in barrier status in the skin. These insignificant changes may indicate two scenarios: 1) skin mucous cells were desensitised by repeated exposures, or 2) skin mucous cells were not dramatically affected by PAA. The gill mucous cells were extremely responsive to repeated exposures to PAA. Mucous cells in the gill lamellae in Group C were significantly bigger than in Group A. There was also a tendency that the mucous cells in the gill lamellae in Group B to be bigger than in Group A ($p=0.06$). There was no significant difference however, between the groups in the density of gill mucous cells. Repeated PAA exposure might compromise gill health as Group C and Group B have significantly lower Barrier Status than group A in the gill lamellae.

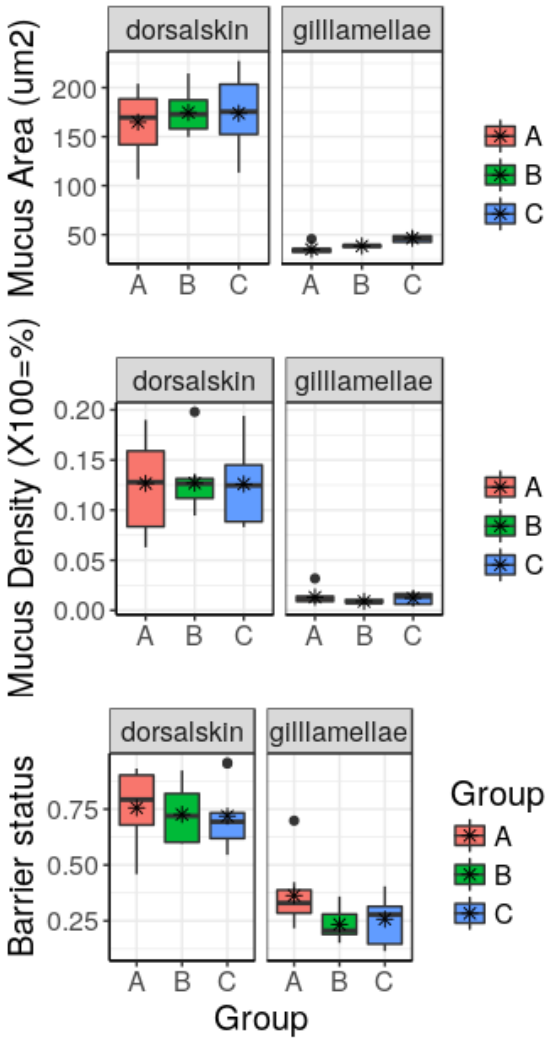

Figure 16 Mucosal features of the skin and gills after the 3rd exposure in Experiment 3. A = unexposed/control; B = 10 ppm, 15 mins; C = 10 ppm, 30 mins.

5.6 Skin colour analysis

PAA has a very high oxidation potential and hence is extremely reactive. Besides being a strong oxidant, it is also a bleaching agent. Change in skin colour following H_2O_2 has been described earlier [12]. The skin colour profiles of initial and re-exposure fish in experiment 1 were also similar (Figure 17). The tendency of the colour value to increase relative to the concentration of PAA illustrates that skin tended to lighten up at high PAA doses. Nonetheless, all recorded values were not statistically different except in the blue channel at 2 w post initial exposure. Though we could not conclusively ascertain what might have caused such a singular effect, previous observation emphasising bluish coloration on the skin after H_2O_2 treatment in salmon might shed insight into this peculiarity (S. Hytterød, pers comm.). We did not observe such a change in Experiment 2, hence, it may likely not be related to PAA dose. Experiment 2 similarly revealed that PAA exposure had little impact on skin colour (Figure 18). No significant differences were observed between groups in all channels, though the group that was not stressed and exposed to PAA tended to have slighter skin colour.

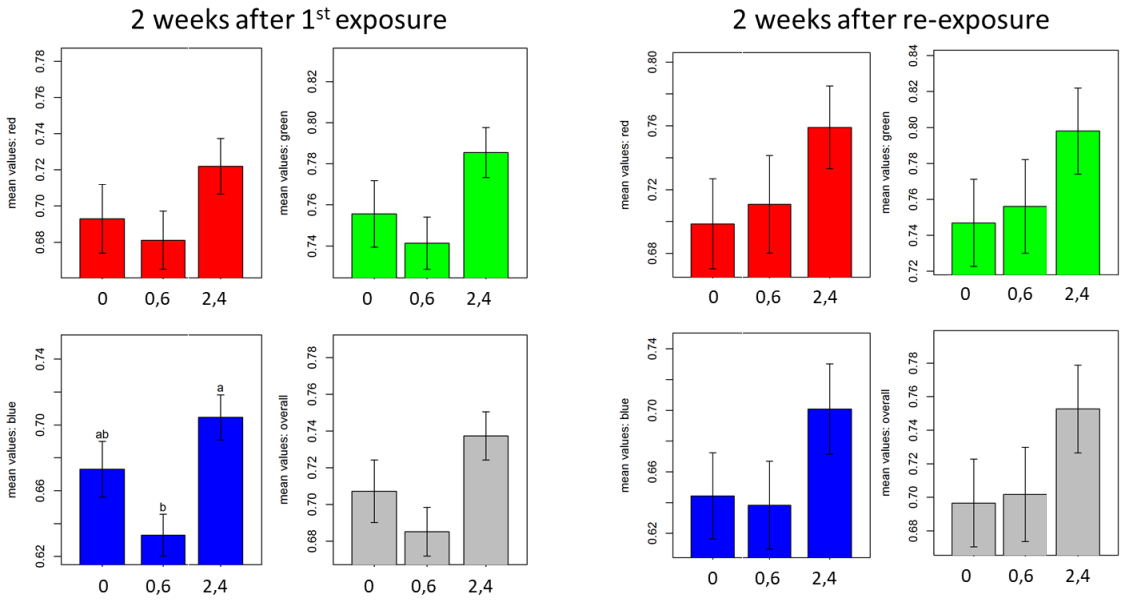

Figure 17 Skin colour profile of salmon exposed to PAA in Experiment 1. The red, blue and green channels are given including the overall RGB composite.

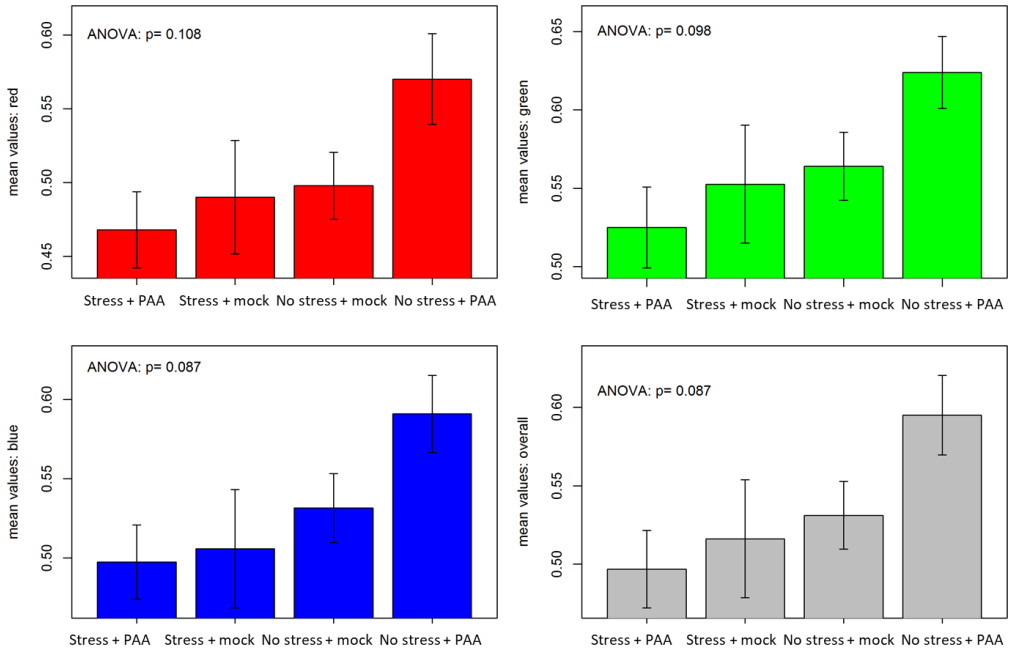

Figure 18 Skin colour profile of salmon exposed to PAA in Experiment 2.

5.7 Mucosal transcriptomics

Experiment 1. Global transcriptomic profiling reveals that there are 473 differentially expressed genes (DEG) in the gills while 679 in the skin (Figures 19-20). Cluster 1 is the overly represented group of DEGs in the gills (Figure 17). Functional classification shows that most of the genes in the group are involved in immunity and tissue structural integrity. In most cases, upregulation of the genes was latent. Nonetheless, the magnitude of change was not that high. The second most represented group

is cluster 4 where a clear tendency was observed – upregulation after re-exposure in 0.6 ppm while downregulation after re-exposure in 2.4 ppm group. Cellular processing appeared to be highly affected by PAA treatment, which was also dose-dependent. Besides the number of DEG, the magnitude of response in the skin was relatively more pronounced compared with the gills (Figure 20). Similarly with the gills, most of the DEGs are involved in immunity. The regulation of several genes involved in immunity indicates that immune defences have been activated to protect the mucosal barrier against PAA. Nonetheless, the magnitude of immunological responses is not overly dramatic illustrating that the reaction was likely more as a form of adaptation as similarly indicated by other response variables, and not as dysregulation of mucosal barrier and functionality. There are also 62 differentially expressed genes that are common in both skin and gills (Table 2). Some of these genes are known to be involved in radical scavenging to protect the cells from oxidative stress damage, such as *glutathione S-transferase 3* and *glutathione reductase, mitochondrial precursor*.

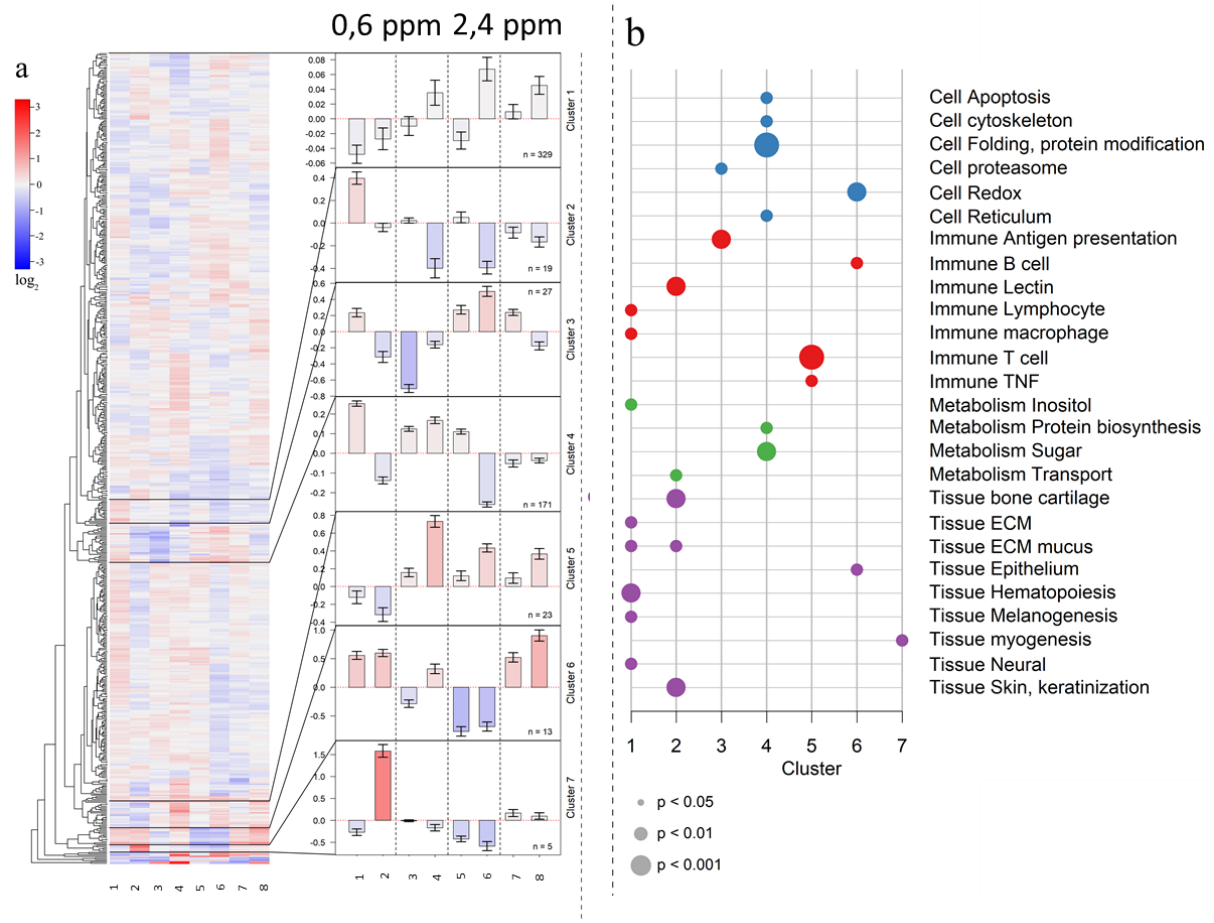

Figure 19 *Changes in the gill transcriptome following PAA exposure in Experiment 1. Note: 1, 5 = 48 h after 1st exposure; 3,7 = 48 h after re-exposure; 2,6 = 2 w after 1st exposure; 4,8 = 2 w after re-exposure. Change in expression was expressed relative to 0 ppm group.*

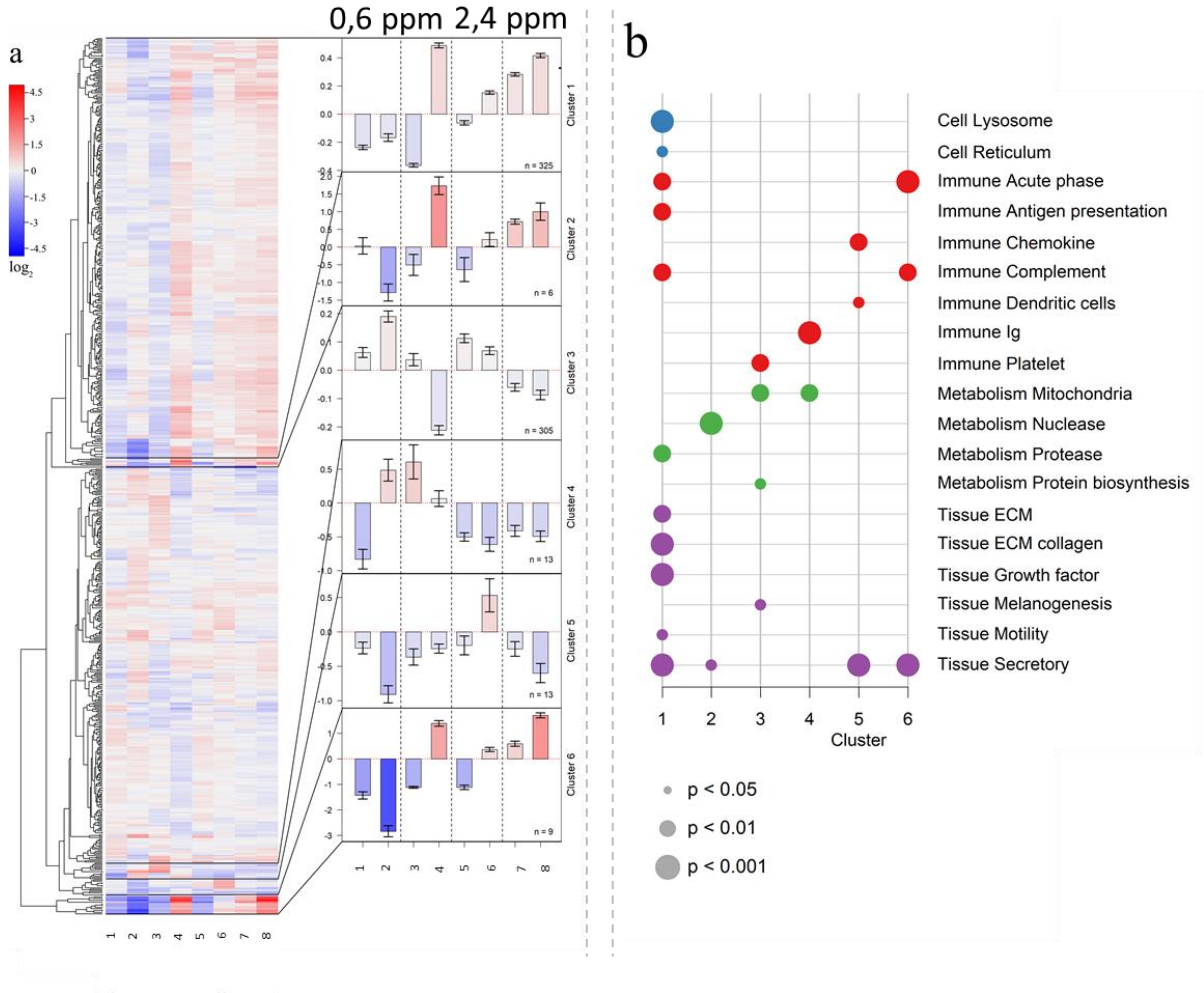

Figure 20 Changes in the skin transcriptome following PAA exposure in Experiment 1. Note: 1, 5 = 48 h after 1st exposure; 3,7 = 48 h after re-exposure; 2,6 = 2 w after 1st exposure; 4,8 = 2 w after re-exposure. Change in expression was expressed relative to 0 ppm gr.

Experiment 2. In contrast to experiment 1, the transcriptome profile in the 2nd experiment demonstrated that gills were more responsive to the treatment than the skin (Figure 21). There are 467 DEG in the gills while only 177 have been identified in the skin. It was also evident that the responses were instantaneous as a relatively higher number of DEGs have been found 4 h after exposure than at a later timepoint. Stress prior to PAA exposure might have a compounding factor as the number of DEGs in the stress-PAA group was substantially higher compared with the no stress-PAA group at 4 h after exposure in the gills. Such a tendency was absent in the skin. *Cathepsin M precursor*, *amyloid beta A4*, *Sgk1 serum/glucorticoid regulated kinase*, *apolipoprotein A-II*, *secreted phosphoprotein 2* and *albumin precursor* are some of the genes the were significantly upregulated in the gills following stress-PAA exposure. In the skin, *glutamate decarboxylase-like protein 1* and *TNF decoy receptor* are two genes that were strikingly upregulated by the treatments.

Table 2 List of DEGs that are common between skin and gills in Experiment 1.

QueryID	Gene name
Ssa#TC84485	Lactosylceramide 1_3-N-acetyl-beta-D-glucosaminyltransferase A
Ssa#TC78366	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit [DDOST 48 kDa subunit]
Ssa#TC72388	Transforming growth factor-beta-induced protein ig-h3 [Salmo salar]
Ssa#TC110861	Mucin-2
Ssa#TC104311	CD166 antigen homolog precursor [Salmo salar]
Ssa#S48403336	Rho GTPase-activating protein 33
Ssa#S48374874	Titin a - Ident 98
Ssa#S47730142	6-phosphogluconate dehydrogenase, decarboxylating [Salmo salar]
Ssa#S35708553	Glutathione S-transferase 3 [Salmo salar]
Ssa#S35693229	Sulfotransferase family cytosolic 2B member 1 [Salmo salar]
Ssa#S35688361	Chromosome-associated kinesin KIF4A
Ssa#S35687974	Importin subunit alpha-2 [Salmo salar]
Ssa#S35676520	Glutathione S-transferase 3 [Salmo salar]
Ssa#S35605514	Zinc finger protein 423
Ssa#S35601168	Fumarylacetoacetate hydrolase (Fumarylacetoacetase)
Ssa#S35599692	Glutathione reductase, mitochondrial precursor [Salmo salar]
Ssa#S35591043	Sulfotransferase family cytosolic 2B member 1 [Salmo salar]
Ssa#S35590931	Cytochrome P450 1B1
Ssa#S35580189	Collagen I alpha 2 chain
Ssa#S35543751	Cytochrome P450 2M1 [Salmo salar]
Ssa#S35531704	NADH-ubiquinone oxidoreductase chain 5
Ssa#S35502105	C-C motif chemokine 4 precursor [Salmo salar]
Ssa#S35477514	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A [Salmo salar]
Ssa#S32006209	Neuronal calcium sensor 1 [Salmo salar]
Ssa#S31963280	Coronin-1C [Salmo salar]
Ssa#S30346271	Cytochrome b
Ssa#S30283213	Novel protein similar to vertebrate leprecan-like 1 (LEPREL1)
Ssa#S30265748	Cartilage associated protein - Ident 99
Ssa#S30262342	Hemoglobin subunit alpha [Salmo salar]
Ssa#S30246737	Rho GTPase-activating protein
Ssa#KSS4038	Olfactomedin-4
Ssa#KSS3328	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit [DDOST 48 kDa subunit]
Ssa#KSS3273	Nuclear migration protein nudC [Salmo salar]
Ssa#KSS3060	Carboxypeptidase E
Ssa#GRASP223672612	CCAAT/enhancer binding protein beta-2
Ssa#GRASP223648741	Coronin-1C [Salmo salar]
Ssa#GRASP223648725	Vascular endothelial growth factor C precursor [Salmo salar]
Ssa#GRASP223648107	6-phosphogluconate dehydrogenase, decarboxylating [Salmo salar]
Ssa#GRASP223647619	CD9-1
Ssa#GRASP209736579	TNF receptor member 11B
Ssa#GRASP209735233	CDGSH iron sulfur domain-containing protein 1 [Salmo salar]
Ssa#GRASP209156075	Sodium-dependent neutral amino acid transporter B0 [Salmo salar]
Ssa#GRASP209155137	Glutathione reductase, mitochondrial precursor [Salmo salar]
Ssa#GRASP209154753	Importin subunit alpha-2 [Salmo salar]
Ssa#GRASP209154477	Histone deacetylase 2 [Salmo salar]
Ssa#GRASP209152284	Jun B-1
Ssa#EG873728	UDP-glucose 4-epimerase [Oncorhynchus mykiss]
Ssa#DY723698	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3A [Salmo salar]
Ssa#DY709730	Cadherin
Ssa#DW544960	Intraflagellar transport protein 20 homolog
Ssa#CL15Contig1	Cytochrome B
Ssa#CK991073	Hemoglobin subunit alpha [Salmo salar]
Ssa#CK878867	Glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase
Ssa#CB515710	Cytochrome P450 3A27 [Salmo salar]
Ssa#CA048665	Protein transport protein Sec31A
Omy#S48435895	C-C motif chemokine 4 precursor [Salmo salar]
Omy#S22610346	interleukin-11 [Oncorhynchus mykiss]
Omy#S18154233	ATP binding cassette G1
Omy#S15341138	Ikaros family zinc finger protein 1
Omy#S15301725	UDP-glucose 4-epimerase [Oncorhynchus mykiss]
Omy#CA356314	Reticulocalbin 3_ EF-hand calcium binding domain

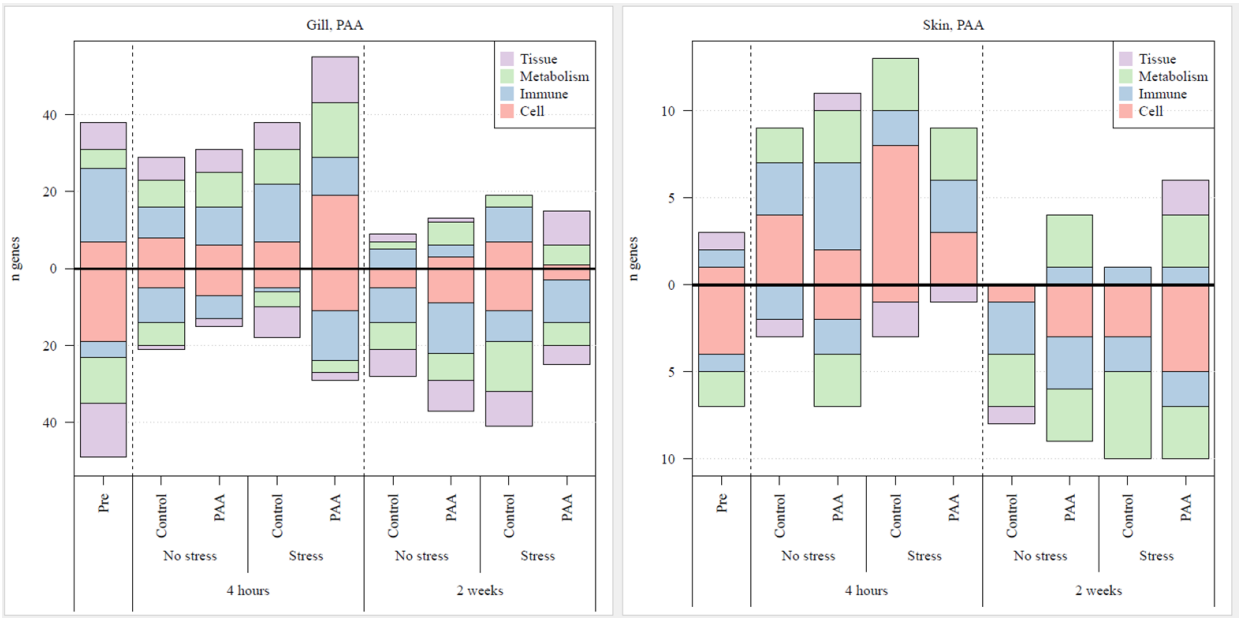

Figure 21 Functional groupings of differentially expressed genes in the gills and skin of salmon in experiment 2.

Experiment 3. Microarray analyses were performed in the gills, skin, and brain. Analyses of these samples are still on-going. These results are expected to be reported as a peer-review article beginning of 2020.

5.8 Metabolomic profiling

Experiment 1. A total of 39 compounds (with authentic standards) were identified. Additionally, 1317 features were extracted using mzMine. Overall, the metabolic profile reveals that exposing salmon to 0,6 and 2,4 ppm PAA either for 5 or 30 min did not significantly alter the plasma metabolome. The absence of significant groupings in the metabolomes of the 3 groups as well as with the pre-exposure group support that plasma metabolomes between groups are identical (Figure 22). This indicates that PAA did not trigger metabolic disturbances in salmon. The list of known metabolites further highlights that the effect of PAA on plasma metabolome was minimal (Table 3). However, there are 4 compounds in the feature group that displayed strikingly high levels (log2ratio > 2) compared with the control group at 2 w after re-exposure - 2-Amino-3-(ethanesulfinyl)propanoic acid, phosphocreatine, [(3-methylbut-3-en-2-yl)oxy]sulfonic acid and indoleacetic acid. Phosphocreatine [39] and indoleacetic acid [40] have been previously identified to have protective roles during oxidative stress. They may likely play a similar role in PAA-exposed salmon.

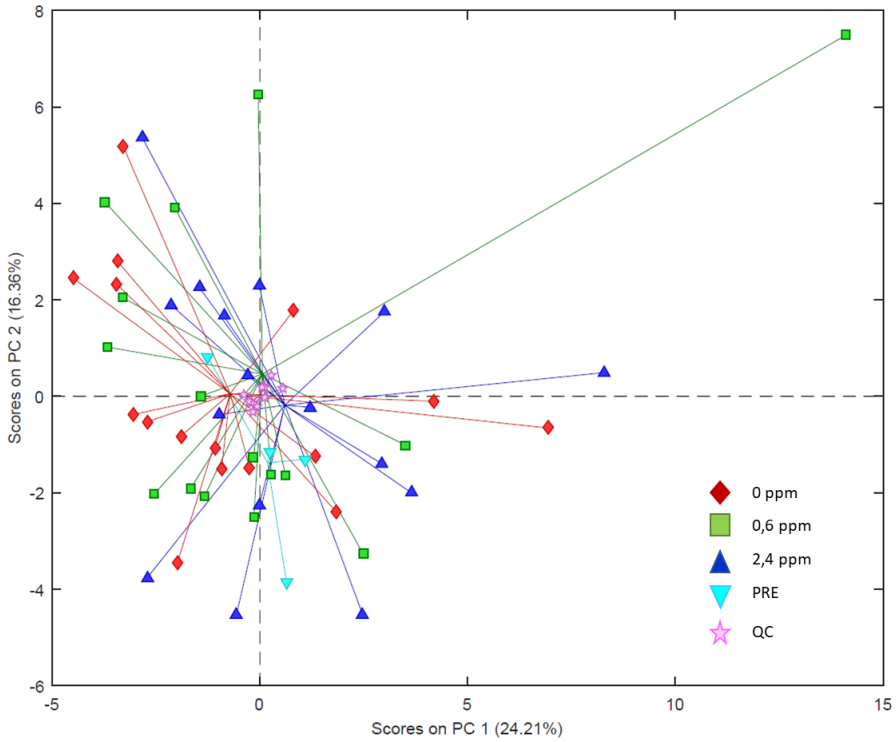

Figure 22 *Score plot from the PCA model calculated on the relative concentrations of the variables in the identified compounds in the plasma from Experiment 1.*

Experiment 2. Analysis of the samples resulted in 639 detected compounds, of these were 138 annotated on level 3, 66 on level 2b, 12 on level 2a, and 42 on level 1. Annotations on level 1 are the most confident identifications. The annotations are based on three pieces of information: accurate mass, MSMS spectra, and known retention time obtained from reference standards analysed on the same system. Annotations on level 2 are based on two pieces of information and are divided into two sublevels; Level 2a is based accurate mass and known retention time as obtained from reference standards analysed on the same system; Level 2b is based on accurate mass and MSMS spectra from an external library. Level 3 annotation is based on library searches using the accurate mass and elemental composition alone, hence, the accuracy is not so high.

The grouping of the samples does not show any relation to the treatment classes (Figure 23). Inspection of groupings in higher order PC's shows some treatment-related groupings in PC5 and PC6. In Figure 24, the plot (PC5 vs. PC6) is shown with samples coloured in three different ways. In the top left plot, it is shown that stress and no stress samples are differentiated on the diagonal going from the lower left side to the upper right. The top right plot indicates that the two timepoints are differentiated from each other on the diagonal going from the upper left part to the lower right. On the last plot, in the bottom, it can be seen that stressed fish treated with PAA (red diamonds / sample group A) are distinguished from the remaining samples, indicating that the effect of the treatment is more pronounced in stressed fish. Stress and time had a larger effect than PAA treatment. Only a small effect of PAA was indicated in the stressed salmon.

The univariate data analysis in form of multiple t-tests identified 11 compounds (guanine, xanthine, guanosine, disperse orange 3, 4-hydroxybutyric acid (GHB), 2-amino-1-propanol, N-benzylformamide, 4-hydroxybenzaldehyde, tyrosine, methionine sulfoxide and laurolactam) that significantly varied between the treatment groups.

Table 3 Changes (expressed as log2ratio relative to 0 ppm) in the identified known metabolites in the plasma of salmon in Experiment 1.

	0,6 ppm				2,4 ppm			
	1st exposure		2nd exposure		1st exposure		2nd exposure	
Metabolite	48 h	2 w	48 h	2 w	48 h	2 w	48 h	2 w
Lactic acid	-0,40	-0,19	-0,03	-0,09	-0,21	-0,20	-0,26	-0,12
Valine	0,56	0,12	0,18	-0,31	-0,36	0,18	0,16	-0,24
Leucine	0,82	0,01	0,19	-0,29	-0,15	0,31	0,02	-0,30
Isoleucine	0,58	-0,02	0,27	-0,12	-0,23	0,19	-0,09	-0,02
Hypoxanthine	-0,52	-1,06	0,29	-0,23	0,41	-0,30	-0,38	-0,66
a-Ketoglutaric acid	-0,01	-0,05	-0,35	-0,66	-0,59	0,43	-0,46	-0,14
Methionine	0,80	-0,34	0,48	0,32	0,73	0,34	-0,11	0,00
Tyrosine	0,25	-0,06	0,20	-0,33	-0,27	0,22	-0,25	-0,07
Taurine	0,50	-0,30	0,06	-0,23	0,74	-0,17	-0,27	-0,27
Citrulline	0,25	0,47	0,14	-0,63	-0,29	0,50	0,46	-0,26
Hexose	-0,04	-0,32	-0,26	0,56	-0,21	-0,32	-0,38	0,07
Inosine	-0,22	-0,70	0,03	-0,03	0,09	-0,02	-0,60	-0,23
?3-hydroxybuturylcarnitine (C4)	-0,42	0,09	0,49	0,12	-0,08	1,18	-0,05	0,09
?Succinylcarnitine (C4-DC)	-0,15	-0,08	0,33	0,14	0,27	0,53	0,27	0,45
4-aminobenzoic acid	0,13	-0,08	0,57	0,37	0,14	-0,05	0,27	0,35
Acetylcarnitine (C2)	-0,08	-0,19	0,53	-0,02	0,09	0,94	0,14	0,06
Adenine	-1,00	-0,54	-0,30	0,34	0,79	0,62	-0,02	0,37
Arginine	0,16	-0,12	-0,21	-0,22	-0,30	-0,12	-0,95	-0,91
Carnitine	0,08	-0,13	0,31	-0,18	0,14	-0,11	-0,03	-0,20
Choline phosphate (PCHO)	-0,50	-0,35	0,01	0,23	0,30	-0,01	-0,01	0,25
Creatine	0,21	0,18	0,38	0,35	0,55	0,48	-0,04	-0,01
Cyanocobalamin	-0,21	-0,36	0,15	0,54	0,33	-0,08	0,20	0,29
Cytidine	0,02	-0,41	-0,11	-0,29	-0,07	-0,34	-0,04	-0,44
Cytosine	-0,41	0,28	0,11	0,14	-0,41	0,50	0,94	0,09
Glutamic acid	-0,43	-0,23	0,03	0,35	0,13	-0,20	-0,13	0,19
Guanosine	-0,26	-0,45	0,01	0,02	-0,17	0,06	-0,19	-0,13
Histidine	0,39	0,49	-0,02	-0,63	-0,13	0,22	0,16	-0,73
Tryptophan	0,70	0,09	-0,04	-0,40	0,12	0,14	-0,01	-0,34
Lysine	1,10	-0,39	-0,06	-0,45	0,06	0,54	-0,18	-0,43
Nicotine amide	0,28	-0,20	0,07	0,23	0,36	0,10	0,06	0,13
Phenylalanine	0,15	-0,18	-0,05	-0,14	-0,11	-0,12	-0,15	-0,11
Propionylcarnitine (C3)	0,00	-0,02	0,46	-0,12	0,17	0,56	-0,03	0,12
Pyridoxal	0,24	0,24	0,16	-0,73	-0,46	0,37	0,17	-0,94
Pyroglutamic acid	-0,06	0,09	0,12	0,08	0,02	0,04	-0,17	0,33
Serine	0,02	0,13	0,10	0,21	0,19	0,38	-0,02	0,12
Sorbitol/Mannitol	-0,41	-0,08	-0,02	0,19	-0,43	-0,27	0,15	0,14
Threonine	-0,01	-0,06	0,04	0,47	0,18	0,90	-0,15	-0,11
trans-4-hydroxyproline	0,33	0,77	0,10	-0,19	0,43	0,54	0,63	-0,03
Uridine	-0,49	-0,79	0,13	0,19	-0,36	-0,54	-0,16	-0,30

The results support the findings of the PCA-models that the largest differences were related to effects of stress and time. Exposure to PAA only affected the concentration of 2-amino-1-propanol, which increased in concentration compared to the control groups in both the stressed and non-stressed salmons. However, as 2-amino-1-propanol could only be annotated to Level 2b these results should be interpreted with caution. Stress alone affected the levels of six compounds (guanine, guanosine, 4-hydroxybutyric acid (GHB), Nbenzylformamide, 4-hydroxybenzaldehyde and tyrosine) in the samples collected after 4 hours, however all of these effects had disappeared in the samples collected after two weeks. Exposure to PAA in stressed salmon resulted in significant changes in guanine, guanosine, xanthine and disperse orange 3, of which both guanine and xanthine were annotated to Level 1. Considering that xanthine can be created from guanine, these results indicate that the combination of stress and PAA exposure may interfere with this specific pathway.

Overall 12 KEGG pathways were affected by the 11 compounds with significant differences:

- Biosynthesis of secondary metabolites
- Microbial metabolism in diverse environments
- Biosynthesis of plant secondary metabolites
- Carbon metabolism
- 2-Oxocarboxylic acid metabolism
- Degradation of aromatic compounds
- Biosynthesis of alkaloids derived from shikimate pathway
- Biosynthesis of phenylpropanoids
- Biosynthesis of antibiotics
- Biosynthesis of amino acids
- Protein digestion and absorption
- Central carbon metabolism in cancer

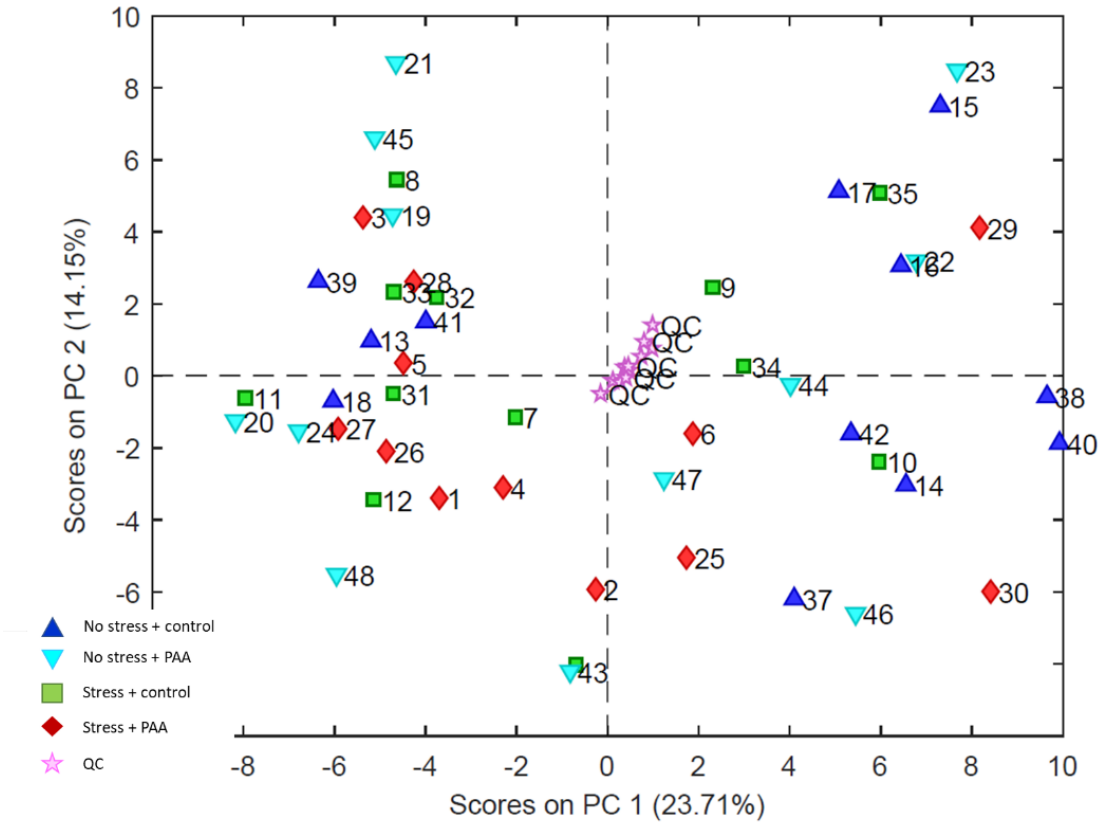

Figure 23 Score plot from the PCA model calculated on the relative concentrations of the variables in the reduced dataset in Experiment 2.

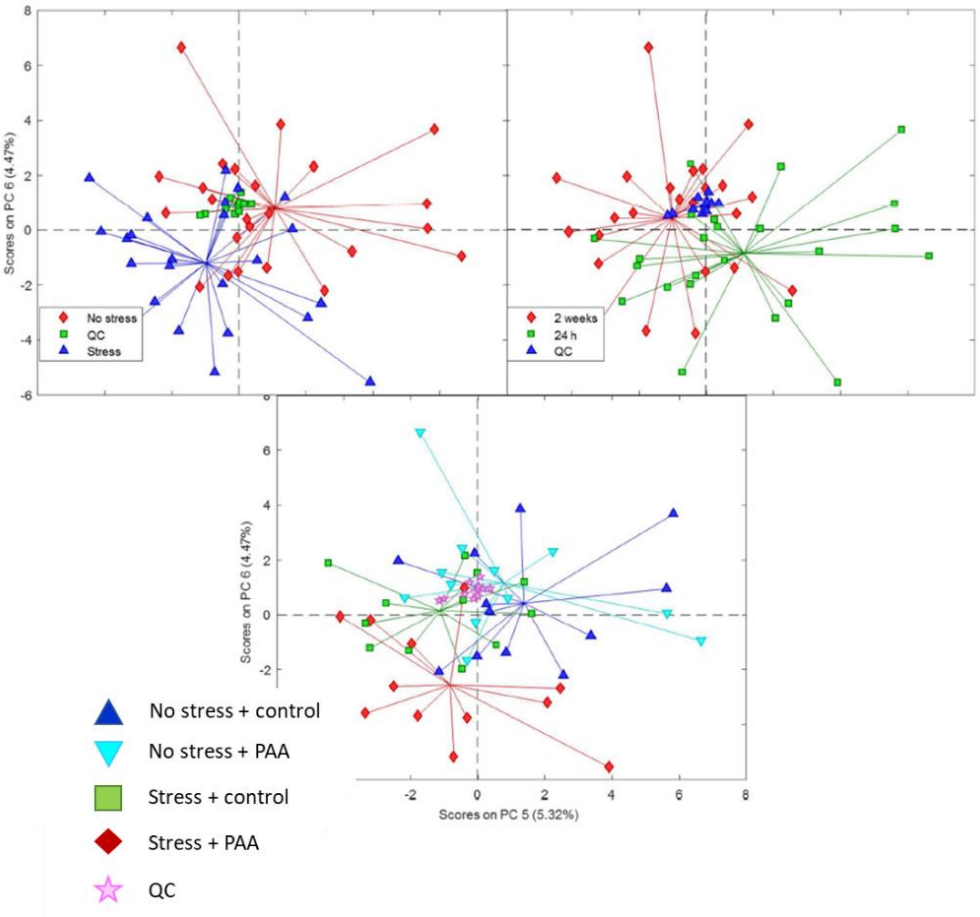

Figure 24 Score plot from higher order PCA model calculated on the relative concentrations of the variables in the reduced dataset.

Experiment 3. Plasma, liver and skin mucus after the 3rd exposure were subjected to metabolomic profiling. A total of 944 compounds were detected in the plasma samples, hereof were 197 annotated on level 3, 75 on level 2b, 23 on level 2a and 38 on level 1 (similar annotation strategies in Experiment 2). **Figure 25** illustrates that exposing salmon to a much higher PAA concentration altered the plasma metabolome, as indicated by the clustering of the two treatment classes in the upper part, separated from the control samples, which are all located in the lower part of the plot. Comparison of the control group with the two PAA treatment groups shows significant differences in several metabolites: ***Control vs. 10 ppm, 15 min***: Inosine, 7-Methyladenine, Biotinsulfoxide, 4-Acetamidobenzoic acid, Hypoxanthine, and Guanosine. ***Control vs. 10 ppm, 30 min***: 4-Acetamidobenzoic acid, Inosine, Valpromide, 7-Methyladenine, and Guanosine. Inosine, 4-acetamidobenzoic acid, 7-Methyladenine, and guanosine are the metabolites that are common to be differentially affected by PAA treatment. The first 3 metabolites were significantly higher in PAA treated groups compared with the control group. Though there is still no clear data yet whether 4-acetamidobenzoic acid and 7-Methyladenine have important roles during oxidative stress, inosine has already been reported to have the antioxidant capacity and protective role during oxidative stress [41]. It is possible that the increase in inosine level in the plasma protects the fish from oxidative damage during PAA exposure. Guanosine [41, 42] has a similar antioxidant role as inosine. Its significant reduction in the plasma after PAA exposure indicates that the treatment interferes with guanosine synthesis, thereby reducing guanosine-mediated antioxidant defence. These metabolites are potential markers of oxidative stress during PAA treatment.

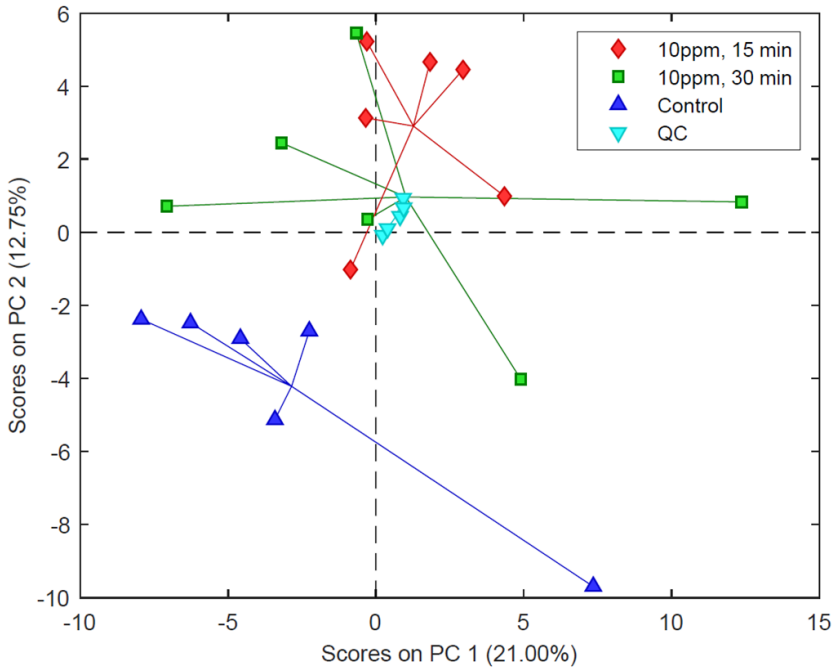

Figure 25 PCA plot of plasma metabolome of salmon in Experiment 3.

A total of 450 compounds were detected in the liver samples, hereof were 102 annotated on level 3, 73 on level 2b, 5 on level 2a and 11 on level 1. The samples classes seem to be separated with control samples to the left and treatment samples to the right in the plot (Figure 26). However, no significant differences were found between the control and treatment classes. It is worth mentioning however that cantharidin, benzoic acid and taurocholic acid are the metabolites in which the level in the plasma decreased substantially after PAA treatment.

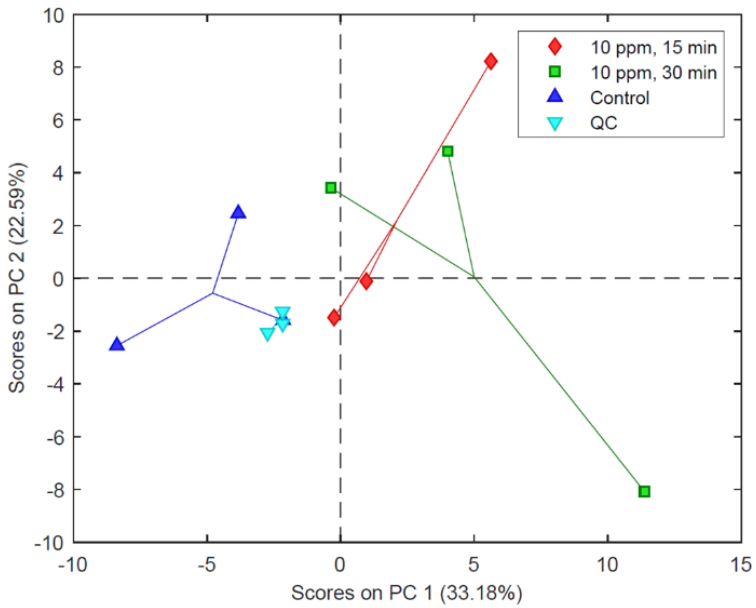

Figure 26 PCA plot of liver metabolome of salmon in Experiment 3.

A total of 2226 compounds were detected in the mucus samples, hereof were 406 annotated on level 3, 191 on level 2b, 32 on level 2a and 53 on level 1. The samples are distributed with control and 15 minutes samples in the upper right half and 30 minutes samples in the lower left half of the plot. The PCA plot reveals that the effect of the treatment on mucus metabolome was more dramatic when fish were exposed at a longer duration (Figure 27). Nonetheless, we did not identify significant differences between the control and PAA treatment groups. Inspection of the individual metabolites shows some interesting changes. Decanamide level in both PAA-treated groups was at least 2.8-fold higher compared with the control group. Benzoic acid was at least 2-fold lower in PAA treated group compared with the unexposed fish. This metabolite was identified to be substantially reduced both in the mucus and liver of PAA exposed fish. Benzoic acid has been reported to have a role during the increased level of radicals [43, 44], and its substantial reduction, albeit not significant, in both mucus and liver indicate that PAA impedes its potential antioxidant action during oxidative stress.

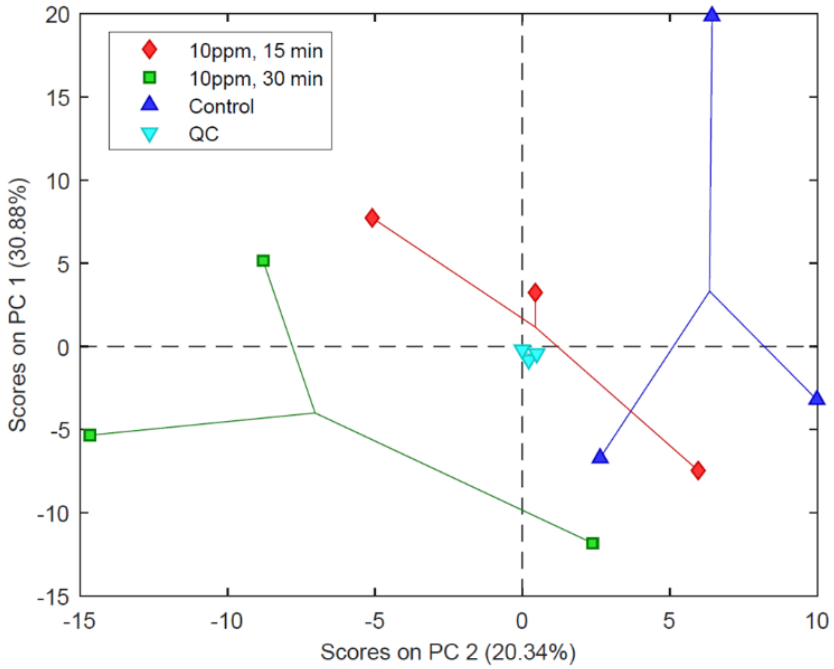

Figure 27 PCA plot of mucus metabolome of salmon in Experiment 3.

5.9 PAA decay studies

5.9.1 Degradation kinetics of PAA during exposure trials

The PAA degradation trials were made in 10 tanks at two occasions and covered nominal concentrations of 0,15 – 0,30 – 0,60 – 1,2 and 4,8 mg PAA/l. The first trial (May 1st, First Exposure Experiment) included the addition of PAA and exposure time of 5 minutes before the fish were removed, whereas the second trial (May 15th) included a 30-minute exposure time. Reduction rates were calculated as exponential decay based on PAA residual analysis of water samples collected at t= 1, 3, 5, 15, 40, 60 minutes after addition.

The change in PAA concentration over time followed exponential 1° order decay, and the deduction rate constants ranged from 0., 8 to 0,32 h^{-1} in the first trial and from 0,3 to 0,39 h^{-1} in the second trial (Figure 28).

Additional test with 4.8 ppm addition revealed similar degradation rates (0,17 and 0,30 measured in tanks with unstressed and stressed salmon exposed to PAA for 30 minutes). These reduction rates correspond to half-lives at approximately 2,3 to 4 hours.

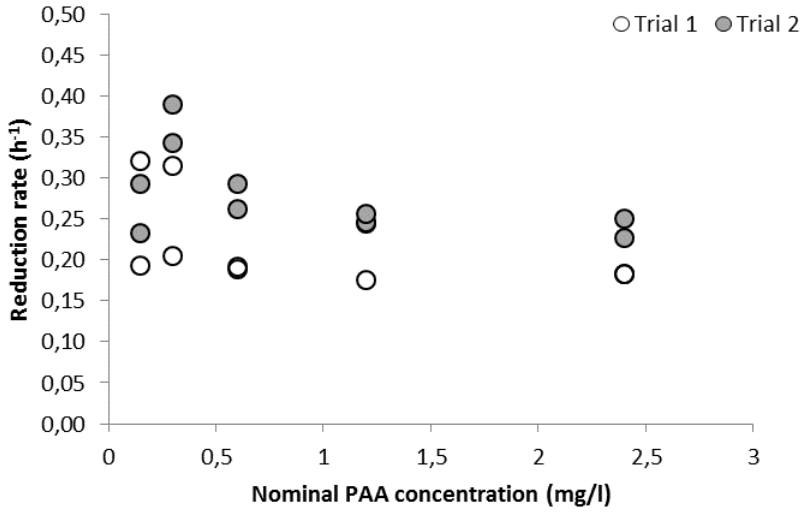

Figure 28 Calculated reduction rates k, based on $C_t = C_0 * e^{-kt}$ where C_t=is the concentration at time t (hours) with C_0 reflecting the nominal concentration. Trial 1 includes 5 min PAA exposure; trial 2 with 30 min exposure time.

5.9.2 Effects of different parameters on PAA decay kinetics

The influence of several factors including 1) temperature and 2) light (photo catalysis) on PAA degradation had been studied by spiking experiments.

Temperature. The effects of temperature on PAA degradation was investigated by controlled PAA spiking and subsequent analysis of PAA residuals over time. Briefly, 34 ‰ saltwater was incubated at 4 different temperatures and divided into temperature controlled beakers with magnetic stirring (N=12). A nominal PAA concentration equivalent to 1.00 ppm was added, and water samples were analysed after t = 0, 10, 20, 30, 45, 60, 120, 180 and 240 min as shown in Figure 29.

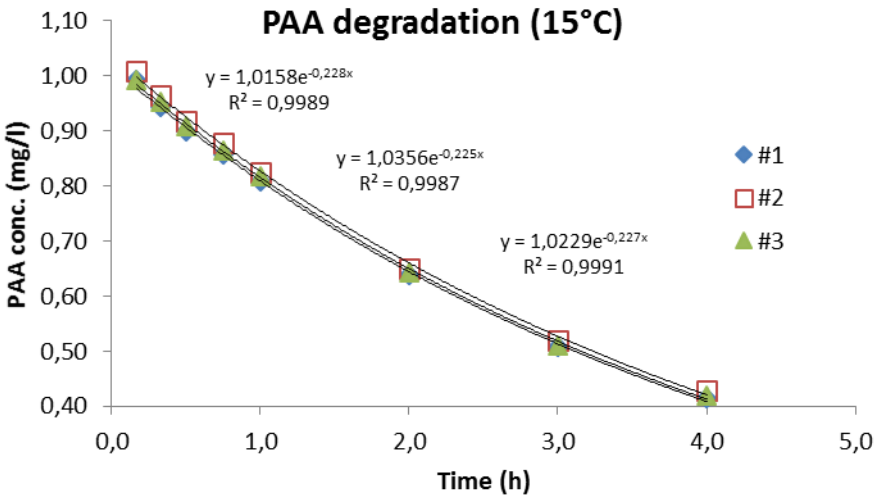

Figure 29 Degradation of PAA in 34 ‰ seawater at 15°C.

The degradation of PAA was found to be highly significantly affected by temperature ($p < 0.001$). The reduction rates were 0,87 at 5° C and 0,35 at 20 °C, corresponding with half-lives around 8 hours at 5 °C and 1,99 h at 20 °C (Figure 30). The reduction rate k in freshwater at 15 ° is < 0.05 h-1 corresponding to a half-live > 14 hours.

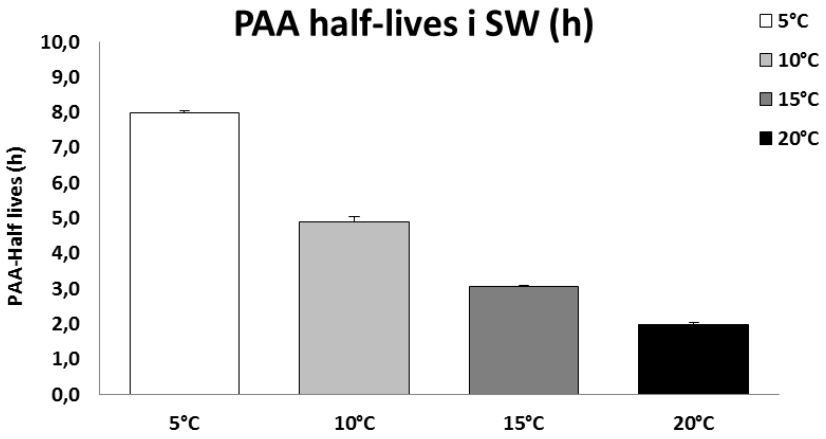

Figure 30 Estimated half-lives (mean ± std) of PAA in 34 ‰ seawater at 15°C.

Light. The effects of sunlight on PAA degradation was investigated in a similar setup as above. Seawater was divided into six 2000-ml Pyrex beakers and places in direct sunlight (light, n=3) or covered by aluminium foil (dark, n=3). The PAA residuals were determined as above, and 1° order degradation kinetics was used to calculate degradation rate constants. The effect of light on PAA is depicted in Figure 31, with the corresponding half-lives at 1,2 h and 2,1 hour in light and dark, respectively.

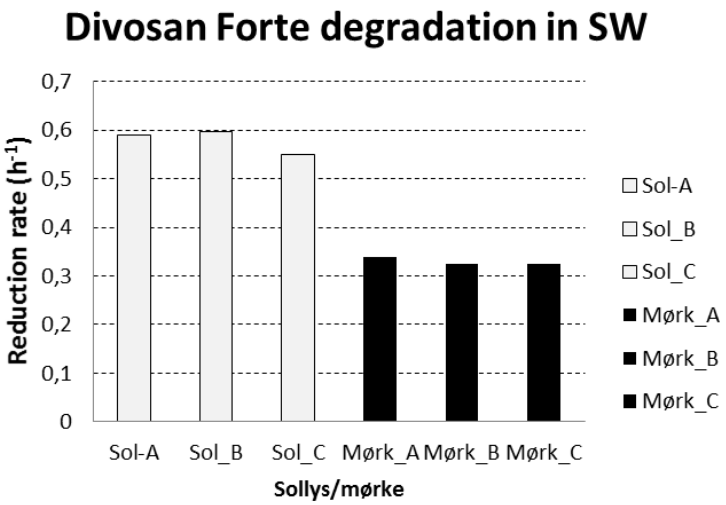

Figure 31 *Calculated reduction rates k, based on $C_t = C_0 * e^{-kt}$ where C_t=is the concentration at time t (hours) with C_0 reflecting the nominal concentration. Note: k is not corrected with the specific temperature coefficient.*

5.9.3 Comparison between PAA and H_2O_2

Since PAA is being proposed as an alternative to H_2O_2, we compared the decay kinetics between the two under *in situ* conditions (Figures 32-33).

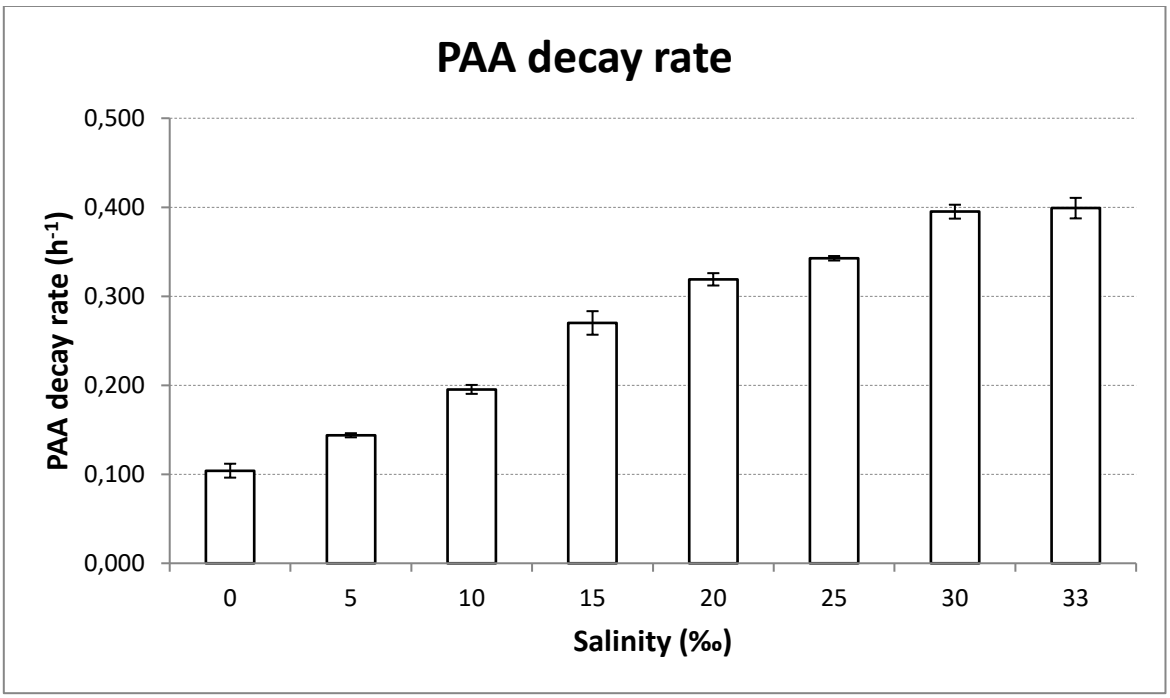

Figure 32 *Calculated first order PAA degradation rate constants (mean ± std. dev.) according to salinity, based on batch experiments performed at 20 °C with nominal PAA concentration at 1 ppm. All data are based on replicated experiments [Note: highly significant effect of salinity; all groups significant different from each other except 30 vs. 33 ppt].*

Similarly, degradation of H_2O_2 (Figure 31) was investigated at different salinities – showing a comparatively much slower degradation (half-life in the order of 10-20 hours).

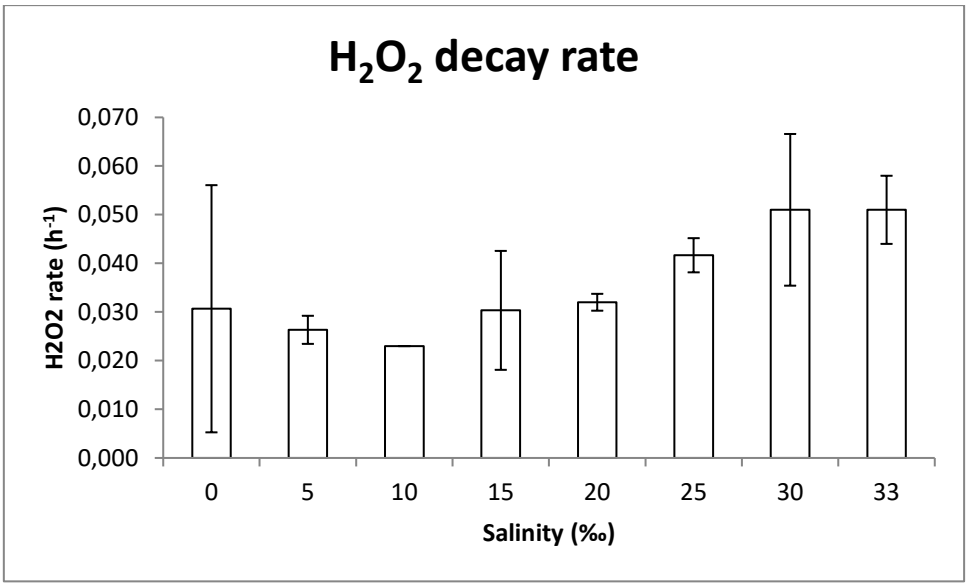

Figure 33 Calculated first order H_2O_2 degradation rate constants (mean ± std. dev.) according to salinity, based on batch experiments performed at 20 °C with a nominal H_2O_2 concentration at 1.5 ppm. All data are based on replicated experiments.

Additional completed or ongoing experiments include investigation of the effects of transition metals on PAA and H_2O_2 degradation as well as investigations of acetic acid. These data will be reported in a peer-review article expected to be sent for peer-review by September 2019, as mentioned in section 7.

5.10 The amoebicidal activity of PAA

P. perurans are susceptible to PAA, even at low concentrations (Figure 34). The amoebae were exposed to different concentrations of PAA either for 15, 30 or 60 mins. The concentrations tested were the same concentrations used for salmon exposure. The percentage viability after exposure was quantified using the WST-1 vital dye.

Exposure dose rather than exposure duration appeared to have more influence on the toxicity of PAA towards the amoeba. At 0,6 ppm and regardless of the exposure time, the viability of amoeba in culture was at least 60 %. The viability was reduced significantly to at least 25 % when exposed to 2,4 and 4,8 ppm. The viability was almost negligible when exposed to 9,6 ppm. Microplate-based viability assay was complemented with the conventional neutral red staining of individual amoeba exposed to PAA (Figure 35). The membrane integrity of amoeba is compromised by PAA, as shown by the inability of PAA-exposed amoeba to uptake the vital dye.

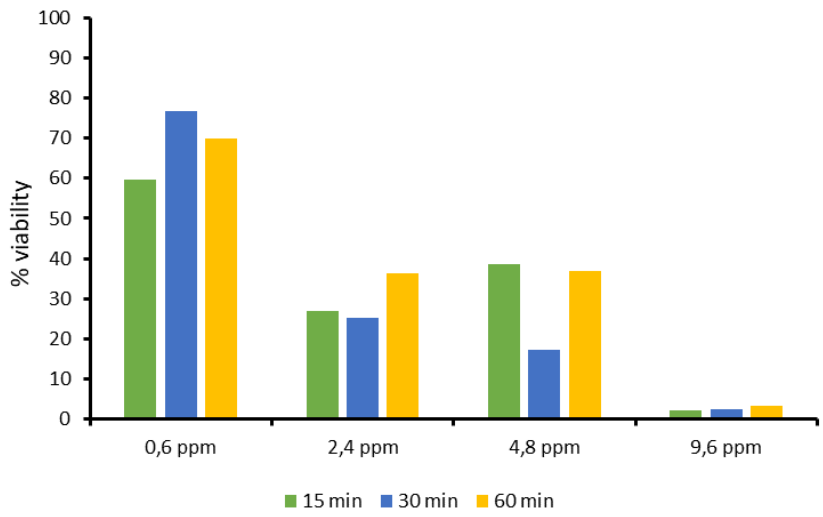

Figure 34 *Percentage viability of N. perurans exposed to different concentrations of PAA and under varying exposure duration.*

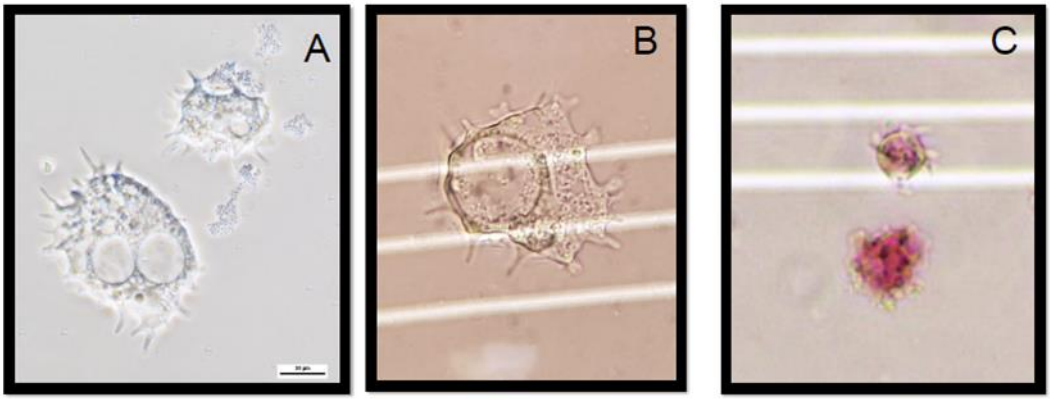

Figure 35 *Neutral red staining of amoeba.* ***A)*** *Unstained amoebas.* ***B)*** *Dead amoeba after exposure to 2,4 ppm PAA for 30 mins. Note the change in morphology.* ***C)*** *Live amoeba from the unexposed group.*

5.10.1 Factors affecting PAA toxicity towards *P. perurans*

We evaluated several factors affecting the susceptibility of P. perurans to PAA (Figures 36-39). PAA at 0.6 ppm is more toxic towards a two-week-old amoeba culture compared with a 1-week old culture, particularly at 200 amoebae/well seeding concentration (Figure 36). It appeared that amoeba density has more influence on PAA toxicity in 1-week old than 2-week old culture. Under standard PAA toxicity assay condition (200 amoebae/well, 15oC) it was shown that viability was reduced significantly at higher amoeba density in the well, especially at concentration > 4.8 ppm (Figure 37). One might expect that the denser colony might be less susceptible to a chemical, but such a case was not observed here. This might be related to metabolic competition during oxidative stress – more cells, higher metabolic requirements during stress, the thereby higher competition for limited resources.

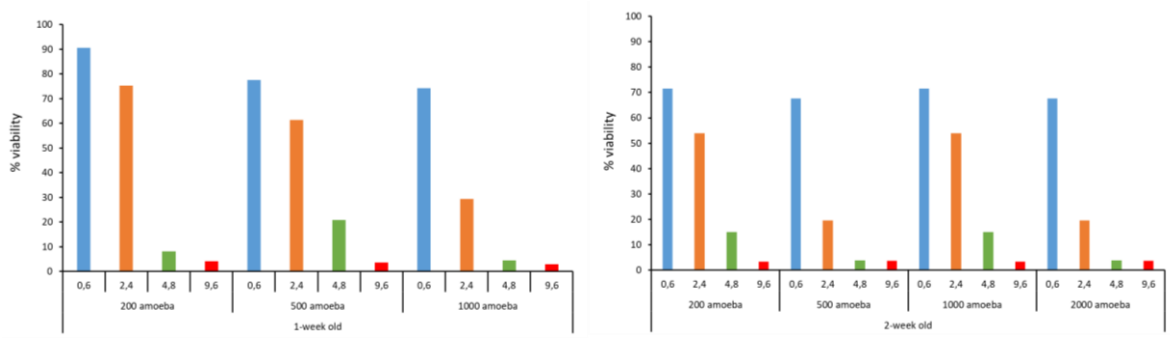

Figure 36 Effects of culture age on the toxicity of PAA towards P. perurans.

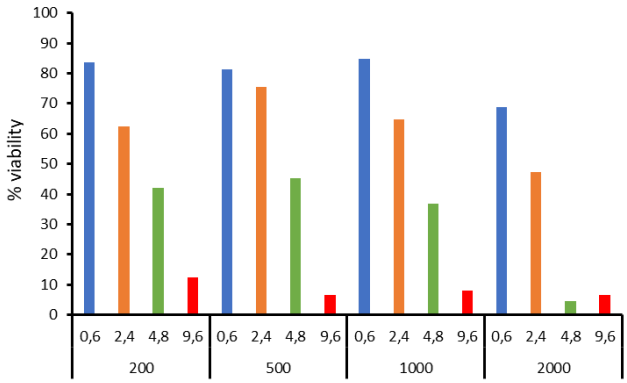

Figure 37 Effects of amoeba density on PAA toxicity.

Since light is crucial in the kinetics of decay of PAA, it was hypothesised that they might play an important function as well in its toxicity (Figure 3). One striking observation was noted in amoebae exposed to 2,4 ppm under light, where the viability was at least 15 % lower compared with the same PAA-exposed group without light. We could not establish whether exposure in the presence of light increased the toxicity since, at higher doses, the viability of amoebae either with light or without light during exposure were almost identical.

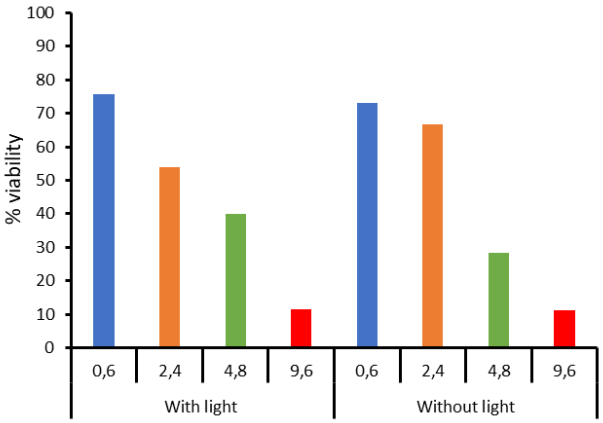

Figure 38 Effects of amoeba density on PAA toxicity.

Exposure temperature did not reveal a clear tendency, whether it had a negative or positive impact on the toxicity of PAA (Figure 37). The profile at 15°C was consistent with the other *in vitro* trials that the increase in dose resulted in reduced viability. It appeared that a relatively higher exposure temperature altered this profile, where amoeba viability at 4.8 and 9.6 ppm at room temperature was almost identical.

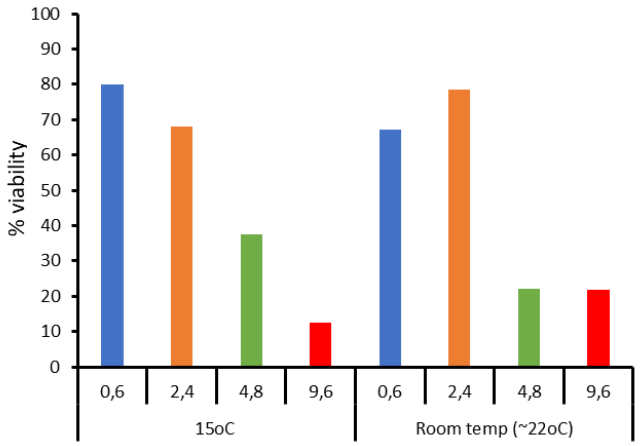

Figure 39 Effects of exposure temperature on PAA toxicity.

5.11 *In vitro* models for PAA and H_2O_2 comparison

We also developed an explant culture to compare PAA and H_2O_2, giving us initial data for the comparative study that is planned for Stage II of the project. A short-term gill explant culture was successfully established (Figure 40) to study the interactions of the molecular clock and antioxidant genes in a mucosal tissue under different environmental ROS conditions. A day after explantation, new cells surrounded the tissue fragments (Figure 40B). More cell outgrowths were observed in the next 5 days (Figure 40C), and some cells started to differentiate (Figure 40D) and acquire a typical morphology of gill epithelial cells [33]. Mucus secretion was noted to be prominent at day 7 (Figure 40E) and about 80–90 % of the explants exhibited the feature by visual inspection. These manifestations indicate that the tissue explants were physiologically and metabolically active under culture conditions, supporting the use of such a model to study key processes in the gill mucosa [33, 45].

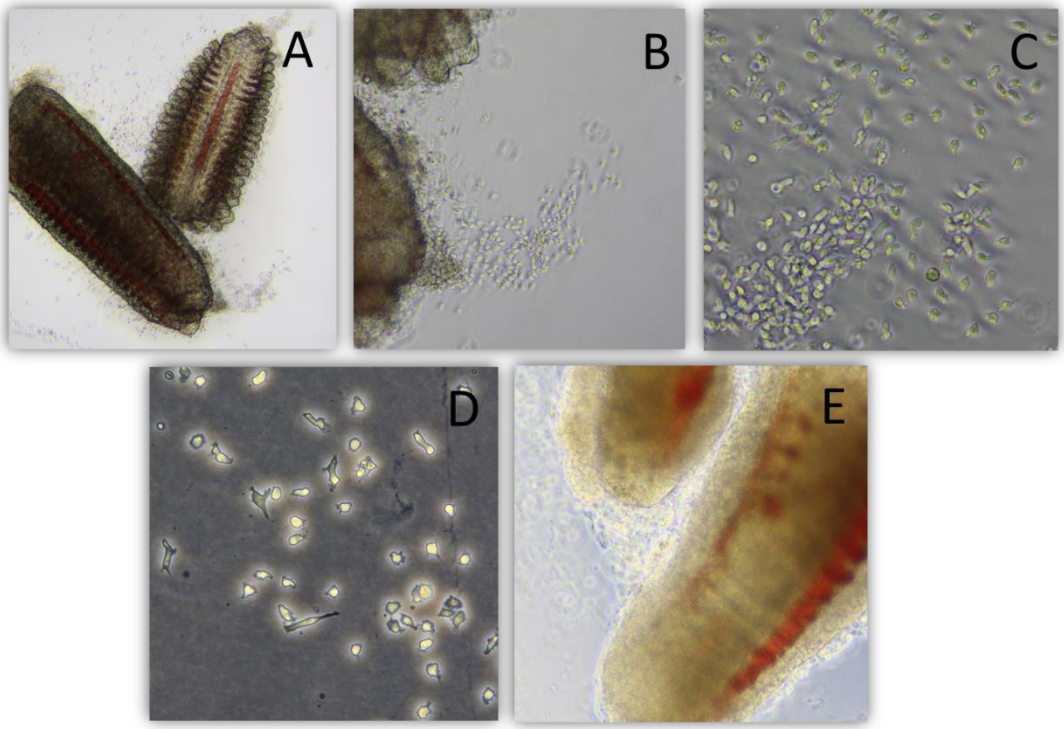

Figure 40 *Gill explants and their outgrowths. A) Gill epithelial cell outgrowths surrounded the tissue fragment 1 day after explantation (4×). B) Cells coming out of the explant, magnified (10×). C) and D) The cells outgrowths were proliferating and differentiating between day 2–6. E) Explant appeared to excrete mucus profusely at 7 days after explantation.*

The expression of key antioxidant genes in the gill explants following stimulation with PAA and H_2O_2 reveals that the model is sensitive to the two oxidants (Figure 41). Both oxidants differentially regulated the expression of the key marker genes. A recent study indicates that the toxicity of H_2O_2 towards salmon is dependent on the time of day. Therefore, were exposed the gill explant to the oxidants either during the day or during the night (Figure 42). There was a marked pattern in the transcriptional responses of the antioxidant genes to the two oxidants: When oxidative stress was induced during the day, the post-exposure profile of the antioxidant gene expression demonstrated significant upregulation. This was identified in the transcription of *gr*, *gsta*, and *mnsod*. It also appeared that an antioxidative response was already mobilised 4 h after exposure to H_2O_2, unlike in PAA, for which significant alterations were only observed 24 h post exposure. When the same stimuli were given at night, *gr*, *gsta*, *mnsod*, and *cu/znsod* were significantly downregulated 24 h after exposure. This response pattern was particularly striking when GE was exposed to H_2O_2 as the transcript level of four genes (i.e., *gr*, *gsta*, *mnsod*, and cu/*znsod*) was reduced by at least a fold compared with the unexposed group. Collectively, the differential regulation of the antioxidant genes to increased ROS level highlights the temporal sensitivity of antioxidant defence in the gills, which dictates the time-wise regulation of the magnitude and type of response to oxidative stress. The ROS scavenging potential in the gills, as indicated by heightened antioxidative state, was likely more efficient when oxidative stress or increased ROS level is encountered during the day than at night. These provide important points that will be taken into consideration in the *in vivo* trials for Stage II.

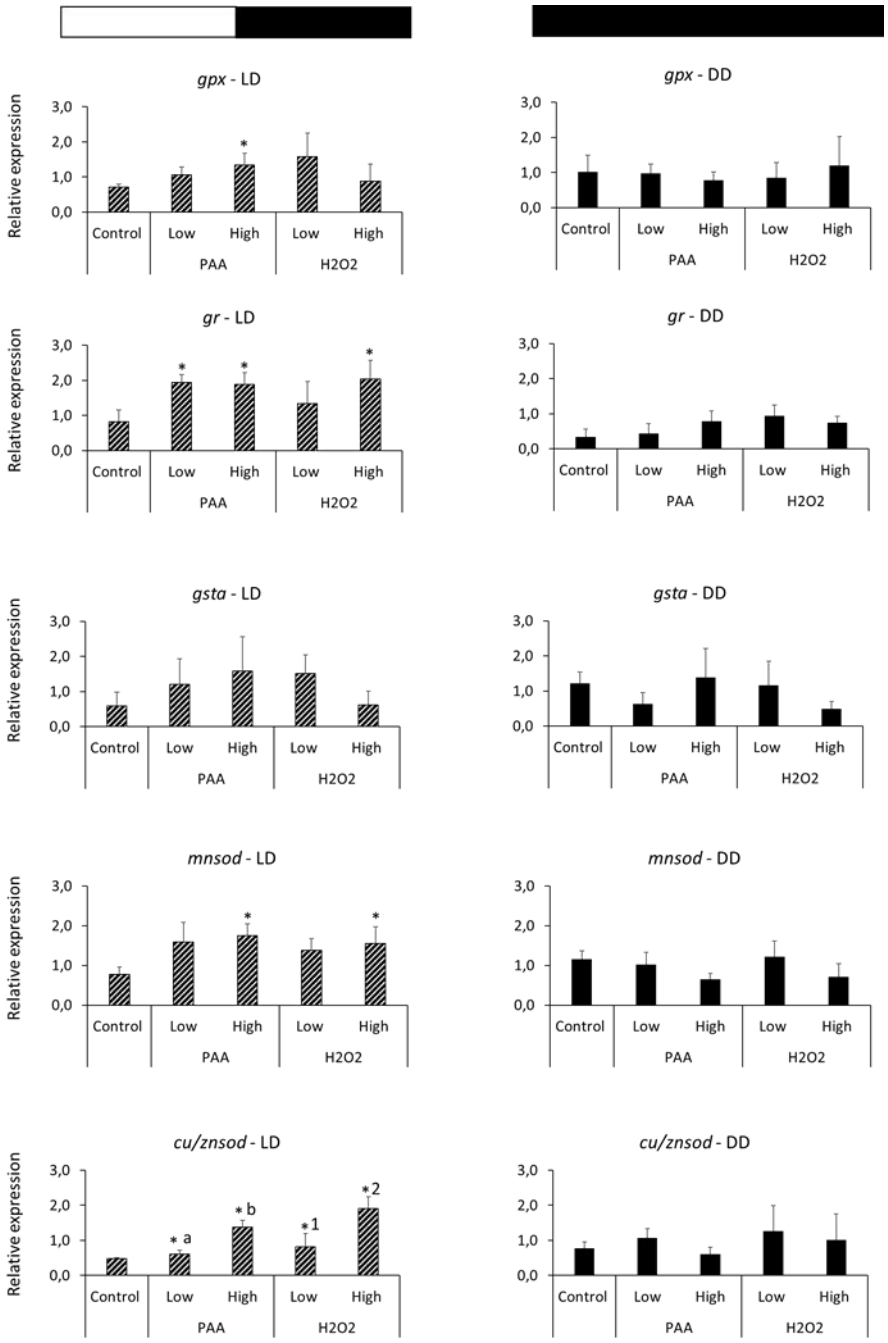

Figure 41 *Regulation of antioxidant gene expression following increased environmental ROS level in gill explants cultured either under equal length of day and night (LD) or total darkness (DD). GE cultures were exposed either to low (10 ppm) or high (100 ppm) concentrations of PAA or H_2O_2. The expression value represents mean ± SD. N = 3 wells, where each well had gill tissue fragments from three individual fish. Asterisk (*) indicates that expression displayed a significant difference from the control group. Different letters/numbers denote that a significant difference exists between the low and high group within a particular oxidant. The level of statistical difference was set at P = 0.05.*

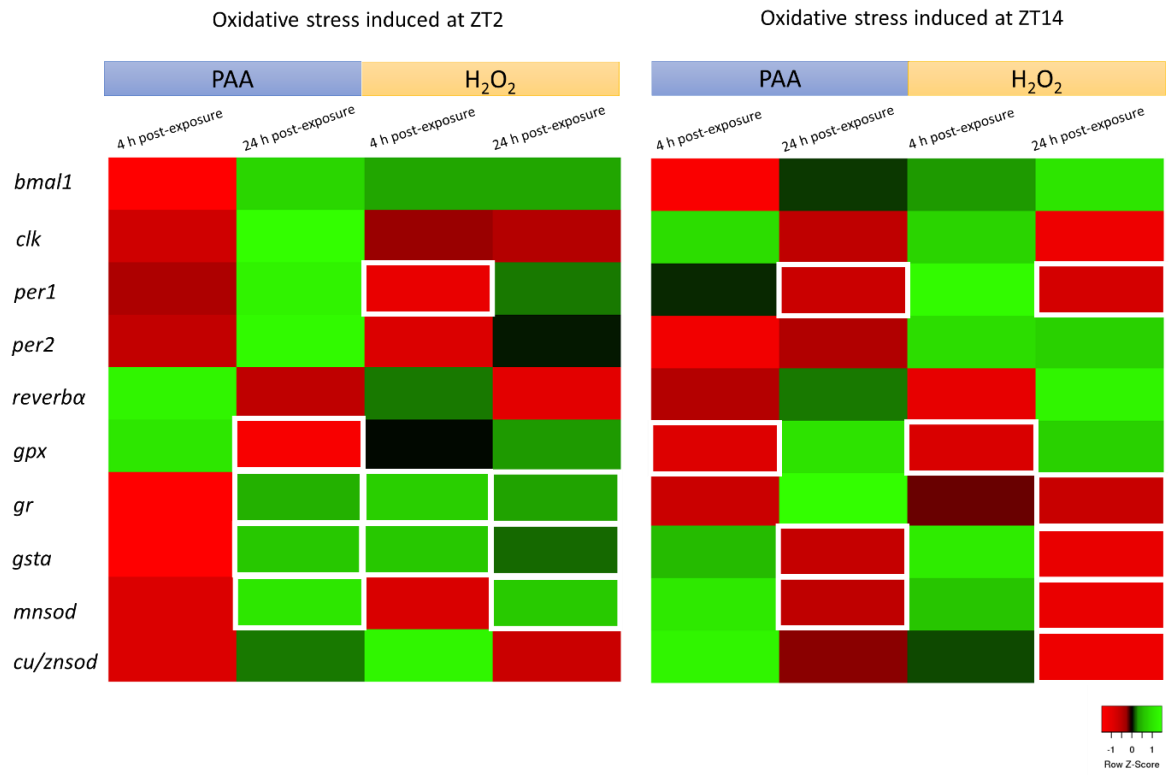

Figure 42 *Temporal sensitivity in the expression of clock and antioxidant genes in the LD-cultured gill explant exposed to oxidants either at ZT2 (day) or at ZT14 (night). Samples were collected 4 and 24 h after exposure. Expression values were expressed as the ratio between the transcript level in the treated group relative to the transcript level in the control group at that timepoint. Spectral panels enclosed in white outline indicate that the response was significantly different ($P < 0.05$) from the control/unexposed group at that particular time point. Expression value represents mean ± SD. N = 4 wells, where each well had gill tissue fragments from three individual fish.*

6 Summary and recommendations

- PAA exposure at concentrations 0.6 to 10 ppm for 30 mins did not have dramatic health and welfare consequences in salmon smolts.
- Though PAA exposure could trigger oxidative stress, salmon were able to mount robust adaptive responses to the physiological demands of PAA by activating various systemic and mucosal defences.
- PAA exhibits anti-parasitic activity against *P. perurans* at concentrations salmon could physiologically tolerate.
- PAA degrades into neutral residuals more rapidly than H_2O_2 in the water and the decay kinetics are influenced by different factors.

Therefore, the results from Stage I suggest that PAA under the tested concentrations is safe for use in Atlantic salmon. Evidence of its amoebicidal activity against *P. perurans* further supports the ensuing initiative to apply PAA to AGD-infected salmon.

7 Deliverables

A. Presentations at scientific meetings/workshops

Oral

1) Lazado, C.C. “Peracetic acid as a potential treatment for amoebic gill disease in Atlantic salmon” at the 6th Gill Health Initiative (GHI) Meeting, Oranmore, Ireland. 11-12 April 2018.

2) Lazado, C.C. “Gill health in a changing environment”. Scientific Lecture Series. Temasek Polytechnic, Singapore. July 4, 2018.

3) Pittmann, K., Okubamichael, M., Merkin, G., Haddeland, S., Lazado, C.C., Pedersen, L.F., Skjennum, F.C., Myre, O.J. “Barriers and RAS: Trading immunity for growth?”. 3rd Nordic RAS Workshop. November 19-20, 2018

4) Karin Pittman. “Slimlaget – fiskens eldgammel nærforsvar mot sykdom». Aqkva Konferansen, Bergen, Norway. January 17, 2019.

5) Carlo C. Lazado, Gerrit Timmerhaus, Aleksei Krasnov, Katrine Hånes Kirste, Karin Pittman, Lisbeth Rørmark, Lars-Flemming Pedersen. Physiological coping mechanisms of Atlantic salmon exposed to an organic peroxide. Frisk Fisk 2019. Tromsø, February 6-7, 2019.

6) Sindre Haddeland, Karin Pittman, Carlo Lazado, Lars Flemming Pedersen, Grigory Merkin, Mearge Okubamichael, Ole Jacob Myre. Bruk av Pereddiksyre i akvakultur: Effekten av gjentatt eksponering for slimceller i gjeller. Frisk Fisk 2019. Tromsø, February 6-7, 2019.

Poster

1) Sindre Haddeland. Effekten av gjentatt eksponering med pereddiksyre på slimceller i gjeller. Aqkva Konferansen, Bergen, Norway. January 17, 2019.

2) Mette W. Breiland, Sigurd Hytterød, Saima Nasrin Mohammad, Malene Soleng, Lill-Heidi Johansen, Lisbeth Rørmark, Lars-Flemming Pedersen, Carlo C. Lazado. Peracetic acid and its anti-parasitic activity against Neoparamoeba perurans, the causative agent of amoebic gill disease. Frisk Fisk 2019. Tromsø, February 6-7, 2019.

3) Malene Soleng, Lars-Flemming Pedersen, Mette W. Breiland, Lill-Heidi Johansen, Karin Pittman, Lisbeth Rørmark, Carlo C. Lazado. The role of stress in the responses of Atlantic salmon to a peroxide-based oxidant. Frisk Fisk 2019. Tromsø, February 6-7, 2019.

B. Master theses

1) Malene Soleng. «Systemic and mucosal stress responses of Atlantic salmon (*Salmo salar*) to peracetic acid». Faculty of Biosciences, Fisheries and Economics. UiT – The Arctic University of Norway. May 2019.

2) Sindre Haddeland. «Benchmarking healthy gills in Atlantic salmon (*Salmo salar*) in seawater recirculating aquaculture system after repeated peracetic acid exposure». Department of Biology. University of Bergen. June 2019.

C. Peer-reviewed papers

Under review

1) Malene Soleng, Lill-Heidi Johansen, Hanne Johnsen, Gunhild S. Johansson, Mette W. Breiland, Lisbeth Rørmark, Karin Pittman, Lars-Flemming Pedersen and Carlo C. Lazado. Atlantic salmon (*Salmo salar*) mounts systemic and mucosal stress responses to peracetic acid. Under review in Fish & Shellfish Immunology. FSIM-D-19-00849.

2) Carlo C. Lazado, Vibeke Voldvik. Transcriptional responses to induced oxidative stress are gated by the time of day in the gill mucosa of Atlantic salmon. Under review in Fish & Shellfish Immunology. FSIM-D-19-00794.

In preparation

1) Carlo C. Lazado, Gerrit Timmerhaus, Marianne Hansen, Aleksei Krasnov, Katrine Hånes Kirste, Lisbeth Rørmark, Karin Pittman and Lars-Flemming Pedersen. Peracetic acid-induced oxidative stress minimally alters the mucosal transcriptome and circulating metabolome of Atlantic salmon (*Salmo salar)*. Target submission date: August 2019; Target journal: Frontiers in Immunology

2) Lars-Flemming Pedersen, Carlo C. Lazado. Degradation fate of peracetic acid in saltwater. Target submission date: September 2019; Target journal: Reviews in Aquaculture

Planned

1) Carlo C. Lazado, Lene Sveen, Gerrit Timmerhaus, Mette W. Breiland, Aleksei Krasnov, Marianne Helén Selander Hansen, Lisbeth Rørmark, Lars-Flemming Pedersen. Crowding stress prior to peroxide exposure in Atlantic salmon – a confounding or a compounding factor? Target submission date: November/December 2019; Target journal: Frontiers in Physiology.

2) Carlo C. Lazado, Mette W. Breiland, Gerrit Timmerhaus, Sigurd Hytterød, Saima Nasrin Mohammed, Marianne Hansen, Aleksei Krasnov, Lisbeth Rørmark, Karin Pittman, Lars-Flemming Pedersen. Health and welfare consequences of repeated exposure to peracetic acid. Target submission date: December 2019/January 2020; Target journal: Scientific Reports.

8 References

[1] N.D. Young, P.B.B. Crosbie, M.B. Adams, B.F. Nowak, R.N. Morrison, Neoparamoeba perurans n. sp., an agent of amoebic gill disease of Atlantic salmon (Salmo salar), International Journal for Parasitology 37(13) (2007) 1469-1481.

[2] P.B.B. Crosbie, A.R. Bridle, K. Cadoret, B.F. Nowak, In vitro cultured Neoparamoeba perurans causes amoebic gill disease in Atlantic salmon and fulfils Koch's postulates, International Journal for Parasitology 42(5) (2012) 511-515.

[3] P.B.B. Crosbie, K. Ogawa, D. Nakano, B.F. Nowak, Amoebic gill disease in hatchery-reared ayu, Plecoglossus altivelis (Temminck & Schlegel), in Japan is caused by Neoparamoeba perurans, Journal of Fish Diseases 33(5) (2010) 455-458.

[4] B. Nowak, J. Carson, M. Powell, I. Dykova, Amoebic Gill Disease in the marine environment, Bulletin of the European Association of Fish Pathologists 22(2) (2002) 144-147.

[5] E. Karlsbakk, A.B. Olsen, A.-C.B. Einen, T.A. Mo, I.U. Fiksdal, H. Aase, C. Kalgraff, S.-Å. Skår, H. Hansen, Amoebic gill disease due to Paramoeba perurans in ballan wrasse (Labrus bergylta), Aquaculture 412(Supplement C) (2013) 41-44.

[6] M.D. Powell, P. Reynolds, T. Kristensen, Freshwater treatment of amoebic gill disease and sea-lice in seawater salmon production: Considerations of water chemistry and fish welfare in Norway, Aquaculture 448(Supplement C) (2015) 18-28.

[7] M.B. Adams, B.F. Nowak, Sequential pathology after initial freshwater bath treatment for amoebic gill disease in cultured Atlantic salmon, Salmo salar L, Journal of Fish Diseases 27(3) (2004) 163-173.

[8] M.D. Powell, M.J. Leef, S.D. Roberts, M.A. Jones, Neoparamoebic gill infections: host response and physiology in salmonids, Journal of Fish Biology 73(9) (2008) 2161-2183.

[9] T. Steinum, A. Kvellestad, L.B. Rønneberg, H. Nilsen, A. Asheim, K. Fjell, S.M.R. Nygård, A.B. Olsen, O.B. Dale, First cases of amoebic gill disease (AGD) in Norwegian seawater farmed Atlantic salmon, Salmo salar L., and phylogeny of the causative amoeba using 18S cDNA sequences, Journal of Fish Diseases 31(3) (2008) 205-214.

[10] M.B. Adams, P.B.B. Crosbie, B.F. Nowak, Preliminary success using hydrogen peroxide to treat Atlantic salmon, Salmo salar L., affected with experimentally induced amoebic gill disease (AGD), Journal of Fish Diseases 35(11) (2012) 839-848.

[11] L.-F. Pedersen, P.B. Pedersen, Hydrogen peroxide application to a commercial recirculating aquaculture system, Aquacultural Engineering 46(Supplement C) (2012) 40-46.

[12] S. Hytterød, L. Andersen, H. Hansen, S.H. Blindheim, T.T. Poppe, A.B. Kristoffersen, T.A. Mo, AGD-behandlingsstrategier - Dose-respons-studier med hydrogenperoksid og ferskvann, Veterinærinstituttet, Norway, 2017, p. 50.

[13] M. Gesto, D. Liu, L.-F. Pedersen, T. Meinelt, D.L. Straus, A. Jokumsen, Confirmation that pulse and continuous peracetic acid administration does not disrupt the acute stress response in rainbow trout, Aquaculture 492 (2018) 190-194.

[14] L.-F. Pedersen, T. Meinelt, D.L. Straus, Peracetic acid degradation in freshwater aquaculture systems and possible practical implications, Aquacultural Engineering 53 (2013) 65-71.

[15] D.L. Straus, T. Meinelt, D. Liu, L.-F. Pedersen, Toxicity of peracetic acid to fish: Variation among species and impact of water chemistry, Journal of the World Aquaculture Society 49(4) (2018) 715-724.

[16] D. Liu, S. Behrens, L.-F. Pedersen, D.L. Straus, T. Meinelt, Peracetic acid is a suitable disinfectant for recirculating fish-microalgae integrated multi-trophic aquaculture systems, Aquaculture Reports 4 (2016) 136-142.

[17] L.-F. Pedersen, P.B. Pedersen, J.L. Nielsen, P.H. Nielsen, Peracetic acid degradation and effects on nitrification in recirculating aquaculture systems, Aquaculture 296(3) (2009) 246-254.

[18] J. Jussila, J. Makkonen, H. Kokko, Peracetic acid (PAA) treatment is an effective disinfectant against crayfish plague (Aphanomyces astaci) spores in aquaculture, Aquaculture 320(1) (2011) 37-42.

[19] S.S. Block, Peroxygen compounds, Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, USA, 1991.

[20] M. Kitis, Disinfection of wastewater with peracetic acid: a review, Environment International 30(1) (2004) 47-55.

[21] L. Liberti, M. Notarnicola, Advanced treatment and disinfection for municipal wastewater reuse in agriculture, Water Science and Technology 40(4) (1999) 235-245.

[22] S. Leaper, Synergistic killing of spores of Bacillus subtilis by peracetic acid and alcohol, International Journal of Food Science & Technology 19(3) (1984) 355-360.

[23] M.G.C. Baldry, J.A.L. Fraser, Disinfection with peroxygens., JohnWiley & Sons, Inc, New York, USA., 1988.

[24] J.A.L. Fraser, A.F. Godfree, F. Jones, Use of peracetic acid in operational sewage sludge disposal to pasture, Water Science and Technology 17 (1985) 451-466.

[25] D. Liu, Effect of peracetic acid at low concentrations on fish health and water quality, 2016.

[26] D. Liu, C.C. Lazado, D.L. Strauss, L.F. Pedersen, M. Gesto, T. Meinelt, Exogenous free radicals induce morphological, redox and immunological alterations in fish. , In prep (In prep).

[27] S. Colgan, R. Gehr, Disinfection, Water Environment and Technology 13(11) (2001) 29-33.

[28] S.S. Block, Peroxygen compounds, fifth ed., Pa, Philadelphia, 1991.

[29] M.G.C. Baldry, The bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid, Journal of Applied Bacteriology 54(3) (1983) 417-423.

[30] D. Liu, D.L. Straus, L.-F. Pedersen, T. Meinelt, Comparison of the toxicity of Wofasteril peracetic acid formulations e400, e250, and lspez to Daphnia magna, with emphasis on the effect of hydrogen peroxide, North American Journal of Aquaculture 77(2) (2015) 128-135.

[31] A. Krasnov, G. Timmerhaus, S. Afanasyev, S.M. Jørgensen, Development and assessment of oligonucleotide microarrays for Atlantic salmon (Salmo salar L.), Comp Biochem Physiol Part D Genomics Proteomics 6(1) (2011) 31-38.

[32] K.S. Sobhana, K.C. George, G. Venkat Ravi, G. Ittoop, R. Paulraj, Development of a cell culture system from gill explants of the grouper, Epinephelus malabaricus (Bloch and Shneider), Asian Fisheries Science 22(2) (2009) 541-547.

[33] M.C. Gjessing, M. Aamelfot, W.N. Batts, S.L. Benestad, O.B. Dale, E. Thoen, S.C. Weli, J.R. Winton, Development and characterization of two cell lines from gills of Atlantic salmon, PLOS ONE 13(2) (2018) e0191792.

[34] A.V.F. Pedro, D. Martínez, J.P. Pontigo, C. Vargas-Lagos, C. Hawes, S. Wadsworth, F.J. Morera, L. Vargas-Chacoff, A.J. Yáñez, Transcriptional activation of genes involved in oxidative stress in Salmo salar challenged with Piscirickettsia salmonis, Comparative Biochemistry and Physiology: B Biochemistry and Molecular Biology (2018) 18.

[35] C.E. Cross, B. Halliwell, E.T. Borish, W.A. Pryor, B.N. Ames, R.L. Saul, J.M. McCord, D. Harman, Oxygen radicals and human disease, Annals of internal medicine 107(4) (1987) 526-45.

[36] B.A. Barton, G.K. Iwama, Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids, Annual Review of Fish Diseases 1 (1991) 3-26.

[37] T.G. Pottinger, T.A. Moran, Differences in plasma cortisol and cortisone dynamics during stress in two strains of rainbow trout (Oncorhynchus mykiss), Journal of Fish Biology 43(1) (1993) 121-130.

[38] S. Polakof, S. Panserat, J.L. Soengas, T. Moon, Glucose metabolism in fish: a review, Biochemical, Systems, and Environmental Physiology 182(8) (2012) 1015-1045.

[39] M.P. Cunha, M.D. Martin-de-Saavedra, A. Romero, J. Egea, F.K. Ludka, C.I. Tasca, M. Farina, A.L. Rodrigues, M.G. Lopez, Both creatine and its product phosphocreatine reduce oxidative stress and afford neuroprotection in an in vitro Parkinson's model, ASN neuro 6(6) (2014).

[40] M.L. Lecube, G.O. Noriega, D.M. Santa Cruz, M.L. Tomaro, A. Batlle, K.B. Balestrasse, Indole acetic acid is responsible for protection against oxidative stress caused by drought in soybean plants: the role of heme oxygenase induction, Redox report : communications in free radical research 19(6) (2014) 242-50.

[41] S.V. Gudkov, I.N. Shtarkman, V.S. Smirnova, A.V. Chernikov, V.I. Bruskov, Guanosine and inosine display antioxidant activity, protect DNA in vitro from oxidative damage induced by reactive oxygen species, and serve as radioprotectors in mice, Radiation research 165(5) (2006) 538-45.

[42] L.E.B. Bettio, J. Gil-Mohapel, A.L.S. Rodrigues, Guanosine and its role in neuropathologies, Purinergic Signal 12(3) (2016) 411-426.

[43] B. Velika, I. Kron, Antioxidant properties of benzoic acid derivatives against superoxide radical, Free Radicals and Antioxidants 2(4) (2012) 62-67.

[44] H. Bas, S. Kalender, D. Pandir, In vitro effects of quercetin on oxidative stress mediated in human erythrocytes by benzoic acid and citric acid, Folia biologica 62(1) (2014) 59-66.

[45] Z. Xu, F. Takizawa, D. Parra, D. Gómez, L. von Gersdorff Jørgensen, S.E. LaPatra, J.O. Sunyer, Mucosal immunoglobulins at respiratory surfaces mark an ancient association that predates the emergence of tetrapods, Nature Communications 7 (2016) 10728.

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