Effects of prolonged application of peracetic acid-based disinfectant on a recirculating aquaculture system stocked with Atlantic salmon parr

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Abstract

The use of recirculating aquaculture systems (RAS) for land-based Atlantic salmon (*Salmo salar*) production has become increasingly common in Norway. An essential aspect of RAS is water disinfection, which plays a crucial role in ensuring water quality and biosecurity. Peracetic acid (PAA) has emerged as a promising disinfectant due to its powerful oxidative properties, broad antimicrobial spectrum, and rapid degradation into no harmful compounds. This study focused on assessing the consequences of prolonged application of a PAA-based disinfectant in a RAS stocked with salmon parr. The experiment included three treatment groups in triplicate: 0 mg/L PAA (control), 0.1 mg/L PAA, and 1 mg/L PAA, using nine-replicated RAS units with a total of 360 fish (14.8 ± 2.3 g; N=40/RAS). The study spanned 28 days, with samples collected on days 0, 14, and 28. The analyzed parameters were water quality, and several fish parameters, including external welfare indicators, gill histology, total antioxidant capacity (TAC), reactive oxygen species/reactive nitrogen species (ROC/RNC), oxidative stress biomarkers related to DNA and protein, cellular DNA damage, and global gene expression. While water quality remained relatively stable, there was an increase in bacterial populations in the groups exposed to PAA, particularly the high PAA concentration group. Fish weight did not significantly differ between the control and PAA-exposed groups. TAC, ROC/RNC, and oxidative stress biomarkers exhibited similar trends. Intriguingly, the study identified more than 400 differentially expressed genes (DEGs) in the skin, gill, and olfactory organ, with many of these DEGs associated with immune responses. Comparing the transcriptomic profiles of the three tissue organs revealed that the olfactory organ was the most reactive to PAA treatment. This study shows that calculated PAA concentrations of 0.1 mg/L and 1 mg/L in the pump-sump, contributed to an increase of bacteria whereas no detectable differences in health and

40 welfare of salmon parr were found. These findings are promising for the implementation of PAA-
41 based disinfectants in RAS to stoked with Atlantic salmon parr.

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43 Keywords: differentially expressed genes, oxidative stress, peracetic acid, recirculating aquaculture
44 systems, salmon parr, welfare

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46 Graphical abstract

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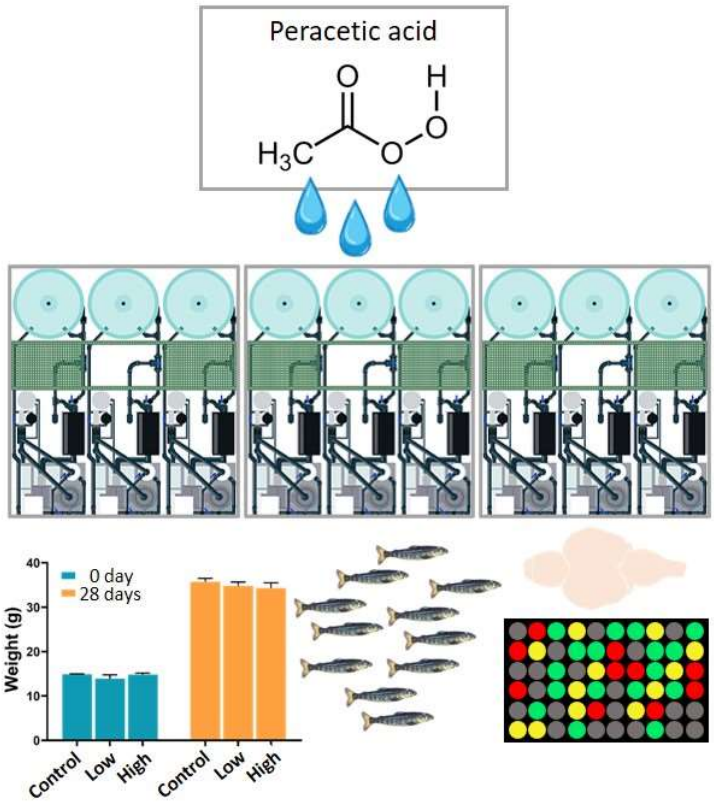

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1 Introduction

Peracetic acid (PAA), an organic compound produced by combining acetic acid and hydrogen peroxide, has gained widespread use in water disinfection for its effectiveness and lack of environmentally harmful by-products (Dominguez Henao et al., 2018; Luukkonen and Pehkonen, 2016). Over the past decade, PAA has emerged as a popular disinfectant especially against off-flavor-producing bacteria in aquaculture (Acosta et al., 2022; Carletto et al., 2022; Lazado and Good, 2021; Liu et al., 2014; Liu et al., 2017; Pedersen and Lazado, 2020; Pedersen et al., 2009).

PAA at low concentrations is efficient in rapidly eradicating the protozoan parasite (*Ichthyophthirius multifiliis*), the causative agent of white spot disease in fish (Meinelt et al., 2007). Recent research has shown that PAA at concentrations ranging from 2.8 to 9.3 mg/L (mean 24-h LC_{50}) and 1.9 to 5.8 mg/L (no-observed effect concentration) during 24-hour exposure exhibited no lethal effects on twelve different fish species (Straus et al., 2018). In recirculating aquaculture systems (RAS), periodic water disinfection with PAA temporarily reduces suspended bacterial loads, modulates fish stress responses, and provides long-term health benefits (Liu et al., 2018). The degradation of PAA in aquaculture systems varies with water quality (chemical oxygen demand, salinity, temperature), fish densities, and initial PAA concentrations (Pedersen and Lazado, 2020; Pedersen et al., 2013; Pedersen et al., 2009; Wu et al., 2024; Wu et al., 2023).

Atlantic salmon (*Salmo salar*) holds a significant position in global aquaculture production, with a worldwide output of 2.7 million tons, Norway being the largest producer (FAO, 2022). The Atlantic salmon aquaculture cycle typically involves a fresh water land-based smolt production, followed by on-growing in sea cages to reach market size (MOWI, 2023). In Norway, up to 70% of land-based smolt is produced in RAS, owing to the numerous benefits, including reduced environmental impact,

flexible location, and high biosecurity (Meriac, 2019; Mota et al., 2022b). Although several studies have examined the effects of PAA on salmon, most have focused on smolt and post-smolt stages, with varied responses observed in different life stages (Lazado et al., 2020a; Lazado et al., 2020b; Lazado et al., 2021b; Lazado et al., 2020c). For example, salmon fry weighing approximately 0.5 g exhibited a 76.6% survival rate in the presence of 0.2 mg/L PAA (DiCocco et al., 2021), while smolts weighing around 100 g survived without any abnormal behavior when exposed to 2 mg/L PAA (Lazado et al., 2020b). For Atlantic salmon fingerlings, i.e.. parr, the no-observed-effect concentration for acute PAA exposure was found to be below 1.6 mg/L for Atlantic salmon parr (Mota et al., 2022a).

This study extends our prior investigations into the acute dose-response of PAA and the repercussions of both pulse and continuous PAA application (maintained at a concentration of 1 mg/L over 4 weeks) on Atlantic salmon parr in RAS (Carletto et al., 2022; Mota et al., 2022a). The current inquiry delves deeper into the consequences of prolonged PAA application, exploring two concentrations (0.1 and 1 mg/L over 28 days), on the welfare, health, and growth performance of Atlantic salmon parr in RAS.

2 Material and methods

2.1 Experimental fish and RAS

All experimental protocols and methods involving fish were conducted with the approval of the Norwegian Food Safety Authority, under FOTS ID 24128. The experiment took place at Tromsø Aquaculture Research Station (HiT, Kårvik, Norway). Atlantic salmon underwent standard

procedures for hatching and rearing, as previously described in (Mota et al., 2022a). In brief, Atlantic salmon eyed eggs were hatched and the parr were raised in a freshwater flow-through system with conditions maintained at 7.5 °C, oxygen saturation levels above 85%, and a continuous photoperiod (LD 24:00) until they were transferred to the experimental RAS units.

2.2 Experimental design

Three experimental groups were established, each in triplicate and with different concentrations of PAA: a control group (0 mg/L), a low concentration group (0.1 mg/L), and a high concentration group (1 mg/L). The experiment spanned 28 days, during which PAA was continuously introduced into the pump sump unit using a high-precision multichannel peristaltic pump (IPC, ISMATEC®, Cole-Parmer, USA) to achieve an applied PAA dose of 0.1 or 1 mg/L in this unit. The PAA-based disinfectant product used (Aqua Des™, Aquatic Chemistry AS, Lillehammer, Norway) was a stabilized PAA solution, consisting of 5% v/v PAA, 23% hydrogen peroxide, and 10% acetic acid. This product is approved for use in aquaculture in Norway only when disinfecting aquaculture facilities, transport units and equipment (https://www.mattilsynet.no/fisk-og-akvakultur/dere-ma-bruke-godkjente-midler) but it is approved in the EU and in the USA as an aquaculture water disinfectant too. The precise PAA concentration (6.6%) in the product was verified through empirical testing by an external laboratory (DTU Aqua, through Dr. Lars-Flemming Pedersen) (Mota et al., 2022a).

A total of 360 Atlantic salmon parr (average weight 14.8 ± 2.3 g) were selected from the flow-through system holding tank and randomly distributed among nine experimental RAS units, with

40 fish per tank. The detailed technical specifications of the experimental units was earlier reported in (Mota et al., 2022b). Briefly, each RAS was composed of a cylindro-conical fish tank (V: 0.5 m^3), a drum filter with a micro-screen mesh size of 40 µm, a moving bed bioreactor (V: 0.2 m^3, bio-media area 750 m^2/m^3), a pump sum (V = 0.1 m^3), a gas exchange unit (CO_2-degasser cylinder), a low-pressure oxygen cone (0.6 bar) and a temperature control unit. The total RAS water volume was 0.8 m^3, with a water flow rate of 1500 L/h, a hydraulic retention time (HRT) of 20 minutes in the fish tank, and a photoperiod of L24:D00.

The fish were acclimated for 7 days prior to the 28 days experimental period under the following conditions: dissolved oxygen levels maintained above 85% saturation, pH levels between 7 and 7.5, a temperature range of 11.5–12.5 °C, and a salinity of 0 ppt. The fish were continuously fed (approximately 23 hours a day) with a commercial diet (1.5 mm pellet size, Nutra Olympic, Skretting, Norway) dispensed through an automatic belt feeder.

2.3 Water analysis

All water parameters were measured from the effluent of fish tank.

2.3.1. General water quality parameters

Dissolved oxygen, pH and temperature were monitored daily using a portable meter (FDO 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany). Ammonium (NH_4-N), nitrite (NO_2-N) and nitrate (NO_3-N) levels were measured daily using a spectrophotometer with the detection limit of 0.5 mg/L for ammonium and 0.02 mg/L for nitrite/nitrate (Test Kit 1.14558.001, 1.14776.0001 and 1.14942.0001, Spectroquant ®, Merck, Germany). Ammonia (NH_3-N) concentrations were

calculated based on ammonium levels, factoring in pH, temperature, and salinity (Johansson and Wedborg, 1980). Water turbidity was measured daily using a portable meter (ORION AQ4500, Thermo Scientific®, Thermo Fisher Scientific, USA). All these parameters were measured in all water samples from each fish tank.

2.3.2. PAA

The method for quantifying and validation of PAA in water was based on a modified DPD (N,N-diethyl-p-phenylenediamine sulfate salt) (Sigma-Aldrich, Oslo, Norway) photometric method (Pedersen and Lazado, 2020). Briefly, DPD reacts with PAA at pH 6.5, resulting in the formation of a red-colored complex (DPD+). The reaction is catalyzed using potassium iodide (KI) (Sigma-Aldrich, Oslo, Norway) photometric and exhibits maximum absorption at 550 nm (Pedersen et al., 2009). To determine the PAA concentration, water samples (2.5 mL) were pipetted to a cuvette (4 mL) and reagent 1 (EDTA•2H_2O, H_2SO_4 96% (Merck KGaA, Darmstadt, Germany) and DPD salt; 250 µL) was added. Thereafter, reagent 2 (Na_2HPO_4•7 H_2O, (Sigma-Aldrich, Oslo, Norway) KH_2PO_4 (Merck KGaA, Darmstadt, Germany) and KI; 250 µL) was added. The solution was mixed and allowed to equilibrate for 30 s before the absorbance at λ = 550 nm was measured in a spectrophotometer (PharmaSpec UV-1700, Shimadzu®, Japan). The detection limit and quantification for PAA was 0.1 mg/L.

2.3.3. Water bacteria

At the conclusion of the 28 days trial, water samples were collected from both the pump sump and

fish tank. To decrease the bacteria concentration, we used 10-fold serial dilutions and each dilution (100µl) was platted in technical duplicates on two different medium plates: a general-purpose, nonselective media for a wide variety of microorganisms - Tryptone Soy Agar (TSA) + 1% NaCl (REF 101112DA, VWR, Leuven, Belgium) and a selective media to isolate Vibrio species - Thiosulfate-Citrate-Bile-Sucrose (TCBS) agar (REF 101011ZA, VWR, Leuven, Belgium). The culture plates were then incubated at 12 °C for a period of 7 days. Following incubation, bacterial colonies were counted, and the results were expressed as colony forming units per milliliter of the sample (CFU/mL). Only plates with CFU counts falling within the range of 30 to 200 were considered.

2.4 Fish sampling

Three sampling events were conducted to evaluate the effects of PAA exposure treatment on the fish. The first event took place before the addition of PAA (week 0), the second occurred midway through the trial (14 days, week 2), and the final event was at the termination of the trial (28 days, week 4). At week 0, three fish were collected from each individual tank, while at weeks 2 and 4, five fish were collected from each tank. Animal euthanasia was performed by immersing the fish in a solution of benzocaine (Benzoak vet, 200 mg/ ml, EuroPharma, Norway). The length and weight of each fish were recorded, and external welfare indicators of the fish were evaluated following the guidelines outlined in Noble et al. (2018) (Noble et al., 2018). Blood samples were collected from the caudal vessels using Vacuette® vacuum tubes (Greiner Bio-One, Kremsmunster, Austria) containing a clot activator (for plasma). The collected plasma was obtained through centrifugation at 4000 x G for 10 min and stored at −80 °C until further analysis. Skin mucus was obtained using

a FLOQSwabs® swab (COPAN Diagnostics, USA) from below the lateral line, and then snap-frozen using dry ice. Sections of dorsal skin (just below the dorsal fin), the second gill arch, and the olfactory organ were dissected and divided into two portions. The first fraction was suspended in RNAlater™ (Ambion, USA), kept overnight at room temperature to aid penetration, and subsequently stored at −80 °C until RNA extraction. The other half of the dissected dorsal skin, olfactory organ, and second gill arch was stored in 10% neutral buffered formalin (BiopSafe®, Denmark). Gill clip samples (ca. 3 mm) from three fish from each tank and sampling point were collected, transferred into 1.5ml cryotubes containing 1ml cell culture media (RPMI 1640, Sigma), 20% DMSO (Hininger et al., 2004) and kept on ice. The samples were then placed in controlled freezing boxes (Corning® CoolCell®) in a freezer at -80°C allowing slow freezing (1°C/min) and stored under these conditions for further comet assay. In addition to the gills and skin, liver and dorsal fin were also collected, snap-frozen in dry ice, and stored at −80 °C.

2.5 Gill histology

The formalin-preserved gill samples were sent to an external laboratory (Norwegian Veterinary Institute, Harstad, Norway). These tissue sections were stained using Periodic Acid Schiff-Alcian Blue (AB-PAS) and delivered to Nofima as digitally scanned files. Gills were manually scored for common pathologies based on the scoring strategy earlier described (Carletto et al., 2022).

2.6 Plasma and skin mucus TAC, ROS/RNS

Only samples collected at week 4 were utilized for this analysis. Due to the limited quantity of skin

198 mucus, quantification involved pooled samples from five fish per replicate tank. A colorimetric kit
199 from Sigma-Aldrich (Burlington, MA, USA) was employed to assess the total antioxidant capacity
200 (TAC) in both plasma and skin mucus. This assay measures the concentration of small molecules
201 and antioxidant proteins, or specifically, the concentration of small molecule antioxidants. This
202 method was validated earlier for salmon (Soleng et al., 2019). The level of antioxidant capacity is
203 expressed relative to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox).

204 To determine the concentration of reactive oxygen species (ROS) and reactive nitrogen species
205 (RNS) in both plasma and skin mucus, OxiSelect™ In Vitro ROS/RNS Assay Kit (Cell Biolabs Inc.,
206 USA) was employed. This kit is suitable for detecting and quantifying the total ROS plus RNS in a
207 wide range of sample types, including plasma and cell lysates. The levels of ROS and RNS were
208 determined by measuring the oxidation level of the dichlorodihydrofluorescin DiOxyQ (DCFH-
209 DiOxyQ) probe, expressed in terms of fluorescence. Hydrogen peroxide (H_2O_2) was used as a
210 standard.

211

212 2.7 Gills, skin, dorsal fin, and liver tissue 8OHDG and DIY

213 The tissue samples were extracted and analysed following the procedure reported by (Zhang et al.,
214 2022). Briefly, the samples were thawed at room temperature, and approximately 100 mg of each
215 tissue sample was carefully weighed. Subsequently, 600 µL of methanol (VWR International AS,
216 Oslo, Norway)) containing 1% ammonium formate (Sigma-Aldrich, Oslo, Norway) (w/v) was
217 added to each sample. These mixtures were vortexed for 30 seconds, ultrasonicated for 30 minutes,
218 and then centrifuged for 5 minutes at 3,500 rpm. The resulting supernatant was meticulously

collected, and 400 μL of water was introduced. The mixture was placed in a freezer at –20 °C for 1 hour to facilitate purification. After this purification step, the supernatant (referred to as the extract) was carefully transferred for UPLC-MS/MS analysis after a final centrifugation at 10000 rpm. The analytical column ACQUITY UPLC HSS T3 (100 × 2.1 mm, 1.7 μm; Waters, Milford, CT, USA) connected to an ULTRA C18 guard column (20 × 2.1 mm; Waters, Milford, CT, USA) was utilized to separate these two biomarkers at an Acquity UPLC I-Class system (Waters, Milford, CT, USA). The analysis was done using a triple quadrupole mass analyzer (QqQ; Xevo TQ-S) with a ZSpray ESI ion source (Waters, Milford, CT, USA). More details about this method can be found in a previously published study (Zhang et al., 2022). UPLC-MS/MS data was acquired with the MassLynx v4.1 software, and quantification processing was conducted with TargetLynx (Waters, Milford, USA).

2.8 Gill comet assay

Preparation of cells was performed following the procedure reported by (Hininger et al., 2004) with the addition of a rapid crush of thawed gill tissue by use of an Eppendorf micropestle preceding cell harvest. The alkaline comet assay was performed using a CometAssay® Kit (4250-050-K, Trevigen Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, the cell suspension was then embedded in low-melting-point agarose (1:9 v/v) at 37 °C. This mixture of agarose and cells was evenly spread onto glass slides pre-coated with 1% standard agarose, covered with glass coverslips and allowed to solidify in the dark at 4 °C for 5 min. The coverslips were subsequently removed, the slides submerged in an ice-cold freshly prepared lysis solution following Hininger et al. (2004). After 1h in the dark at 4 °C, the slides were allowed to drain briefly before

241 being immersed in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, adjusted to
242 pH > 13, Sigma-Aldrich, MO, USA) for unwinding (20 min) and single-cell electrophoresis (21
243 V/400mA, 30 min) using a CometAssay® Electrophoresis System II (R&D Systems, Inc., MN,
244 USA). After electrophoresis, the slides were briefly washed in distilled water followed by 70%
245 ethanol and covered with approximately 100 µL of the DNA fluorescent dye 4',6-diamidino-2-
246 phenylindole (DAPI, Sigma-Aldrich, MO, USA). The slides were then left to completely dry at
247 room temperature in the dark. Finally, the extent of DNA damage was visualised using a Leitz
248 Aristoplan fluorescent microscope (Leica Microsystems GmbH, Germany) equipped with a Filter
249 Cube A for UV (excitation filters BP 340-380 nm) and a digital camera (Flexcam C1, Leica
250 Microsystems GmbH, Wetzlar, Germany). Percentage DNA in comets' tail and tail length (µm) were
251 measured (50 cells per sample, n=3) by CometAssay® Analysis Software (Comet 1.3d, R&D
252 Systems, Inc., MN, USA). The comet samples were coded and evaluated blind. Alkaline COMET
253 assay's experimental conditions were verified by use of commercially available reference cells
254 (CometAssay® Control Cells, Trevigen Inc., Gaithersburg, MD, USA).

255

256 2.9 Gills, skin, and olfactory organ microarray analysis

257 Total RNA extraction was performed on the gill, skin, and olfactory organ samples collected at all
258 three time points (week 0, 2, and 4) in a Biomek 4,000 Benchtop Workstation using Agencourt
259 RNAdvance™Tissue Total RNA Purification Kit (Beckman Coulter Inc, USA). The concentration
260 and purity of the RNA were determined using a NanoDrop 8,000 spectrophotometer (Thermo
261 Scientific, USA). For quality control, a representative subset of samples was further assessed using
262 an Agilent® 2,100 Bioanalyzer™ RNA 6000 Nano kit (Agilent Technology Inc, USA). All the

263 samples that were evaluated displayed an RNA Integrity Number (RIN) exceeding 8.0.

264 A custom-designed 44K Atlantic salmon DNA oligonucleotide microarray designated SIQ-6
265 (Agilent Array, ICSASG_v2) was used for microarray analysis. The One-Colour Quick Amp
266 Labelling Kit was used for RNA amplification and Cy3 labelling, using 110 ng of RNA template
267 per reaction. RNA fragmentation was measured using the gene expression hybridization kits,
268 followed by a 15-h hybridisation period within a 65°C oven with a continuous rotational speed of
269 10 rpm. Following hybridisation, the arrays were washed using Gene Expression Wash Buffers 1
270 and 2 and subsequently scanned using the Agilent SureScan Microarray Scanner and processed
271 within Nofima's bioinformatics software package Salmon and Trout Annotated Reference
272 Sequences (STARS).

273

274 2.10 Statistical analysis

275 Normality of the data distribution was assessed using a Shapiro-Wilk test, and the equality of
276 variance was analyzed with a Brown-Forsythe test for several of the data sets, including those from
277 plasma, total antioxidant capacity analysis, reactive oxygen species analysis, gene expression
278 analysis, and histological assessment (Systat Software Inc, London, UK). To evaluate differences
279 within exposure groups, a two-way analysis of variance (ANOVA) was employed. This analysis
280 considered variations due to both modes of exposure and sampling points, as well as the interactions
281 between these two factors, for gene expression and histology data. Additionally, a one-way ANOVA
282 was conducted to test for differences among treatments in the case of plasma and mucus TAC and
283 ROS/RNS analysis, gill comet assay, as well as the analysis of the oxidative stress biomarkers. The

data from the gill comet assay were arcsin-transformed before ANOVA. In instances where significant differences were observed, a Holm-Sidak method comparison was carried out. Principal component analysis (PCA) was performed using Origin 2019 (OriginLab Corporation, Northampton, MA, USA). Pearson correlation analysis was conducted with SPSS (version 28.0.1.0.0). The threshold for statistical significance was set at $p < 0.05$. All data are presented as mean ± standard deviation (S.D.).

Differentially expressed genes (DEGs) were selected by calculating t-tests between untreated controls and the two treatment groups (high and low) separately and for each of the three tissues. In addition, the mean differences between treated and un-treated groups needed to be larger than 0.79 log2 expression ratio (ER) for up-regulated genes and lower than -0.79 for down-regulated genes to be selected.

Genes which were DEGs for at least one of the six treatment groups and had a functional annotation were selected for a cluster analysis (n = 475). The analysis and visualisation were performed in R (version 4.0.2, https://www.r-project.org/). The distances between groupwise mean ERs were calculated by the dist() function (stats package, Euclidean distance), were clustered by the hclust() function (stats package, complete linkage method) and the heatmap was plotted by the heatmap.2() function (gplots package). The resulting dendrogram was cut into six clusters (cutree() function, stats package) and functional enrichments within each of these clusters were calculated by Fisher's exact tests (fisher.test(), stats package) with the alternative hypothesis set to "greater" (one-sided test).

Correlated gene expressions between individuals were calculated by the cor.test() function (stats package, Pearson method) for genes which were DEGs for at least one the groups of the compared

tissues. Correlation between tissues was defined by p-values smaller than 0.05.

3 Results and discussion

3.1 Water quality

The concentrations of PAA in all water samples from fish tank effluent were below the limit of detection (<0.1 mg/L) (Table 1). A possible explanation is the full degradation of PAA from the site where it was added to the fish tank outlet, a circuit that had a combined hydraulic retention time (HRT) of 35-40 min. A rapid degradation of PAA was previously by (Pedersen and Lazado, 2020), with decay rates ranging from 0.5 to 4 hours under different conditions. The study also revealed that the decay rates of PAA at concentrations of 0.15, 0.30, 0.60, and 1.2 mg/L were not significantly different (Pedersen and Lazado, 2020), explaining the absence of detectable PAA in all groups in the fish tank effluent.

Temperature remained quite stable across the groups, with mean values of 12.1±0.3, 12.3±0.4, and 12.5±0.7 °C in the control, low, and high groups, respectively. The pH values showed no significant differences among the three groups (p=0.520, One-way ANOVA), similar to that reported in an earlier study (Teitge et al., 2020). Turbidity was slightly elevated in the high-concentration group but the difference did not reach statistical significance (p=0.057, One-way ANOVA). The amount of bacteria has been reported to be an important factor explaining the turbidity of water in RAS (Davidson et al., 2019). The significantly increased number of bacteria in RAS treated with 1 mg/L PAA has a high turbidity. Regarding the concentrations of NH_4^+-N, NH_3-N, NO_3-N, and NO_2-N, no significant differences were observed among the three groups (Table 1). This suggests that both low

and high PAA concentrations had no negative impact on nitrification, which is in line with previous findings by Pedersen et al., who reported that addition of 1.0 mg/L of PAA only caused minor impairment of nitrification (Pedersen et al., 2009). Notably, the number of bacteria in the tank and sump was significantly higher in the high-concentration group than in the low-concentration and control group. The regrowth of bacteria after PAA disinfection was also reported in previous studies (Kitis, 2004; Zhang et al., 2019). Acetic acid and acetate, transferred directly to the water during application of PAA, are energy-rich and readily biodegradable carbon sources for many bacteria (Pedersen and Lazado, 2020).

3.2 Fish growth and welfare

The initial and final average weights of the Atlantic salmon in the three experimental groups were as follows: at the beginning, the average weights were 14.87 g (control), 13.93 g (low), and 14.83 g (high), and at the end, the weights were 35.73 g (control), 34.77 g (low), and 34.30 g (high) (Fig. 1a). These measurements were used to calculate the thermal growth coefficient (TGC), a reliable predictor of the expected mean growth of Atlantic salmon within a temperature range of 4–14 °C, commonly used in aquaculture studies (Thorarensen and Farrell, 2011). The TGC values for the control, low, and high concentration groups were 2.50, 2.49, and 2.30, respectively, and these values did not exhibit significant differences (p>0.05, One-way ANOVA). Furthermore, the specific growth rate (SGR) was calculated for each group, resulting in values of 2.1 %/day for the control, 2.2 %/day for the low concentration, and 2.1 %/day for the high concentration groups (Fig. 1b). The K-factor, which is a parameter used to assess the body shape and condition of salmon parr, was found to be 1.43 for the control, 1.44 for the low concentration, and 1.43 for the high concentration groups.

Typically, K-factors ranging from 0.8 to 2.0 are considered acceptable for assessing the overall health of the fish (Barnham and Baxter, 2003). These K-factor values reinforce that the fish maintained a healthy body shape across all groups. The welfare scores of the salmon parr did not exhibit significant changes at the three sampling time points (Fig. 1c), further indicating that both 0.1 mg/L and 1 mg/L PAA had minimal impact on the growth and overall welfare. These results collectively suggest that the prolonged application of a PAA-based disinfectant in a RAS did not significantly affect the growth or welfare of the salmon parr.

3.3 Gill histology

Over 50% of the gill lamellae examined in this study were classified as healthy (Fig. 2a). In the gills assessed, non-specific pathologies such as hyperplasia, lifting, clubbing, and fusion were observed with some frequency: significant differences were found in cases of clubbing between the fish sampled at the initial stage and at 14 days in the control group, and between the initial stage and 28 days in the 1 mg/L PAA group. However, no differences were observed in cases of clubbing between the different treatment groups at the same time points. Some of the non-specific pathologies were showed in Fig. 2b and 2c, which includes epithelial lifting, hyperplasia and fusion of lamella. There were no significant effects in gill health status in 0.1 and 1 mg/L PAA groups.

Gills play a critical and unique role in fish due to their direct contact with the external environment. They are also sensitive indicators of environmental changes and potential stressors for fish (Evans, 1987; Haddeland et al., 2021; Mota et al., 2022a). A previous acute exposure study reported that a higher prevalence of healthy lamellae was found in treatment groups exposed to PAA concentrations

of 1.6 mg/L or lower, with only minor occurrences of lifting and hyperplasia, compared to groups exposed to 3.2 and 6.4 mg/L PAA, which exhibited 100% necrosis of the lamellae (Mota et al., 2022a). Similar results regarding gill abnormalities, including hyperplasia, lifting, clubbing, and fusion, have also been observed in other studies (Carletto et al., 2022; Lazado et al., 2021b). Despite the observed significant differences in clubbing at different time points, the prevalence of clubbing was similar at the final time point, suggesting only minor effects on fish cultured in a RAS where a prolonged application of a PAA-based disinfectant is present.

3.4 TAC and ROS/RNS of plasma and mucus

It has been documented that PAA, as an oxidant, can trigger oxidative responses in organisms, in addition to its pathogen-eliminating properties (Lazado et al., 2020b). This makes it crucial to assess the effects of PAA on salmon parr before its application, as chronic oxidative stress may have detrimental consequences to fish health. TAC measures the capacity of free radical scavenging by the antioxidants present in the samples, indicating their ability to counteract various disorders and diseases induced by oxidative stress (Niki, 2010). The levels of TAC and ROS/RNS were slightly elevated in the 0.1 mg/L PAA group in both plasma and mucus, although the increase was not statistically significant. There were no significant differences in TAC and ROS/RNS levels among the treatment groups in both plasma and skin mucus (Fig. 3).

The parallel results of TAC in both plasma and mucus suggested that mucus can serve as a non-invasive method to evaluate the effects of xenobiotics in fish, which is consistent with the study (Oliveira et al., 2018). It is well established that ROS and RNS are essential to the harmful effects

391 of oxidative stress (Di Meo et al., 2016). Oxidative stress occurs due to an imbalance between
392 antioxidants and ROS/RNS (Gupta et al., 2021). The patterns observed in ROS/RNS levels in both
393 the control and treated groups are similar to the TAC results, further indicating that salmon parr
394 cultured in a RAS with a prolonged application of a PAA-based disinfectant, presents only minor
395 effects on the oxidative response.

396 3.5 Oxidative stress biomarkers of DNA and protein in tissues

397 In all groups, the concentrations of 8OHDG were generally low, ranging from <0.11 to 3.6 ng/g,
398 with median values (ranges) of 0.36 ng/g (<0.11 to 454 ng/g) in skin, 3.4 ng/g (<0.11 to 216 ng/g)
399 in gill tissue, 2.9 ng/g (<0.11 to 10 ng/g) in dorsal fin tissue, and below 0.11 ng/g in liver tissue. In
400 contrast, the concentrations of DIY were notably higher, ranging from 66 to 284 ng/g in skin tissue,
401 247 to 685 ng/g in gill tissue, <1.4 to 4050 ng/g in dorsal fin tissue, and 922 to 13176 ng/g in liver
402 tissue, with median values of 144 ng/g, 383 ng/g, 310 ng/g, and 3118 ng/g, respectively. The
403 concentrations of 8OHDG in skin were negatively correlated with those in liver ($r = -0.47$, $p =$
404 0.003). Positive correlations were observed between the concentrations of DIY in skin and gill tissue
405 ($r = 0.269$, $p = 0.006$), as well as skin and dorsal fin tissue ($r = 0.278$, $p = 0.005$). However, a negative
406 correlation was found between DIY concentrations in dorsal fin and liver tissue ($r = -0.435$, $p =$
407 0.000). Interestingly, there were no significant differences in the concentrations of these two
408 biomarkers among most of the different groups in the four types of samples. Notably, the
409 concentrations of DIY in gill tissue were significantly higher in the 1 mg/L PAA groups compared
410 to the control groups at 14 days and 28 days (Fig. 4). Principal component analysis revealed distinct
411 trends in these two biomarkers with fish growth (Fig. 5).

412 In addition to the biological assays for TAC and ROS/RNS, oxidative stress biomarkers related to

DNA and protein were analyzed using UPLC-MS/MS, an analytical method that was established in our previous study (Zhang et al., 2022). The varied concentration levels of 8OHDG and DIY in different tissues indicate diverse responses in these tissues. For example, the liver, being the primary detoxification organ in fish, exhibited higher levels of stress compared to other tissues. Apart from the sensitive gills, there were no significant changes in the other three tissues after 28 days of exposure. These results are in agreement with the findings of the TAC and ROS/RNS assays, underscoring the reliability of both chemical and biological assays. Moreover, the sensitivity of gill tissue to oxidative stress has been well-documented in previous studies (Carletto et al., 2022; Osório et al., 2022; Soleng et al., 2019). The negative and positive correlations of 8OHDG and DIY between different tissues underscore the varied impacts of oxidative stress on different tissues.

3.6 Comet assay

In the Comet assay, two parameters were measured: the tail length and the percentage of DNA in the tail (Table 2). Overall, the analysis revealed low (< 5% DNA in the tail, < 0.2 μm tail length) and comparable levels of DNA damage in control and PAA-treated groups (Table 2). Interestingly, at day 14 the low (0.1 mg/L) PAA treatment group showed a significant lower ($p<0.001$) value of percentage DNA in the tail than the control and the high treatment groups (control: 4.25±4.03%, 0.1 mg/L: 2.59±1.98, 1 mg/L: 3.87±3.10), but not differences in tail length. There is no clear explanation for this and we speculate that this was due to an error in coding the sample or most likely to a technical artefact. In contrast to the percentage of DNA in tail, comet lengths were similar across groups at the three sampling time points.

The Comet assay is a sensitive method for detecting DNA damage in individual cells (Liao et al., 2009). The data indicate that prolonged exposure to PAA has no significant genotoxic effects on gill tissues of salmon parr. Similar results were reported in a biomonitoring study, which used the same dose of PAA and did not induce clastogenic/aneugenic effects or DNA damage in haemolymph or gill cells of the filter-feeder zebra mussel (*Dreissena polymorpha*) after 20 days of exposure (Bolognesi et al., 2004).

3.7 Regulation of molecular defenses in gill, skin and the olfactory organ

A total of 475 differentially expressed genes (DEGs) were identified across the gills, skin, and olfactory organ (Fig. 6a). Sensitivity to PAA varied among these organs, with the gills and olfactory organ exhibiting notable responses. The gills showed a clear dose-dependent reaction, whereas the olfactory organ displayed a higher number of DEGs at the low dose compared to the high dose. Conversely, the skin exhibited minimal sensitivity to PAA, with only a limited number of DEGs detected compared to the other mucosal organs.

Comparison of the gill and olfactory transcriptomes revealed only one shared gene between the two organs at both exposure doses (Fig. 6b). Due to the limited DEG count, the skin was excluded from this comparison. In the olfactory organ, 291 DEGs were exclusively identified at the low dose, while 27 were exclusive to the high dose, with only 9 DEGs common to both doses. For the gills, 55 DEGs were exclusive to the high dose, 26 to the low dose, and 30 were common to both.

Functional annotation of these DEGs revealed key pathways grouped into 6 clusters affected by PAA exposure (Fig. 6c). Clusters 1, 2, and 5 stood out, with Cluster 1 showing dose-dependent

changes in the gills and significant changes only at the low dose in the olfactory organ. Genes in this cluster are primarily involved in tissue differentiation, endocrine function, xenobiotic metabolism, and lipid metabolism. Cluster 2, the largest cluster, exhibited high upregulation in the olfactory organ of fish exposed to the low dose, mainly associated with immunity. Finally, Cluster 5 included genes downregulated in both gills and olfactory organ, primarily involved in mitochondrial metabolism and erythrocyte function.

The sensitivity of the olfactory organ and minimal response of the skin mirrored earlier transcriptome studies in salmon exposed to PAA (Lazado et al., 2021a; Lazado et al., 2021b; Lazado et al., 2021c; Lazado et al., 2020c). The responsiveness of the olfactory organ is likely related to the innate nature of PAA as an irritant, which targets the mucous membranes of nasal passages. Most of the upregulated genes in Cluster 2 are related to immune responses, suggesting that PAA was a strong immunomodulator. PAA produces free radicals during degradation (Rokhina et al., 2010), which has been shown to be a potent activator of fish immunity (Biller and Takahashi, 2018; Lazado et al., 2020c). The up-regulation of these crucial genes likely played a role in maintaining the structural integrity and barrier functionality under oxidative challenge (Lazado et al., 2020c). The magnitude of transcriptional changes in salmon smolts is not entirely dependent on the dose of PAA (Lazado et al., 2020b). For instance, the magnitude of gene expression changes in salmon smolts was larger in the 0.6 mg/L group than in the 2.4 mg/L group (Lazado et al., 2020b). Additionally, the responses to PAA substantially varied in different organs indicating the differential sensitivity. Immune response at mucosal sites to various challenges is a crucial first line of defense in fish. Here we showed that salmon cultured in a RAS with a prolonged application of a PAA-based disinfectant could mount a strong immune response and this is likely to be a crucial mechanism for fish living

in an environment with the constant presence of oxygen radicals, though at very low levels.

Conclusions

This study evaluated the effects of different concentrations of PAA on salmon parr weighing between 15-35 g. A limitation of the current study is that the PAA concentration mentioned in this study (0.1 and 1.0 mg/L) are calculated for the pump sumps and not measured in these units. All the measurements were done from fish tank effluent water and, there measured PAA was below the limit of detection (<0.1 mg/L). Therefore, the observations from the current study are the result of a prolonged application of a PAA-based disinfectant in a RAS and its effects on the cultured salmon parr. Most of the water quality parameters remained unchanged, except for increased bacterial numbers, possibly because PAA by-products can serve as a nutrition source for bacteria. Fish growth performance, assessed through the parameters body weight, TGC, SGR, K-factor, and external welfare score, indicated no significant differences between the control group and the groups exposed to 0.1 mg/L and 1 mg/L PAA over the 28-day exposure period. Histological assessments of gill tissues revealed no significant differences after 28 days of PAA exposure. Two methods were employed to evaluate the oxidative stress on salmon parr. One was a biological assay measuring TAC and ROS/RNS, while the other involved a chemical analysis of oxidative stress biomarkers in DNA and proteins using UPLC-MS/MS. Both methods showed no significant differences between the control group and the exposure groups. Furthermore, the Comet assay, which assesses DNA damage, indicated no significant changes in the percentage of DNA in the tail or tail length, except for a significantly lower DNA percentage observed in the 0.1 mg/L PAA group at 14 days, compared to the control and 1 mg/L PAA group. Gene transcriptome analysis identified 475 DEGs in the gill,

skin, and olfactory organ. These DEGs were related to various categories, including cell, immune, metabolism, and tissue. The olfactory organ was the most reactive tissue to PAA treatment, exhibiting the highest number of DEGs. Notably, the number of up-regulated DEGs exceeded that of down-regulated ones. In the olfactory organ, the 0.1 mg/L PAA group had more DEGs than the 1 mg/L PAA group. In conclusion, the water quality and fish parameters measured in this study indicate that a prolonged use of a PAA-based disinfectant in RAS water in the tested conditions (0.1 and 1 mg/L) did not result in significant adverse effects on growth, water quality, histology, oxidative stress, or DNA damage.

Credit authorship contribution statement

Vasco C. Mota, Carlo C. Lazado and Alexandros G. Asimakopoulos designed the experiment. Junjie Zhang wrote the manuscript draft. Junjie Zhang, Maia Eggen, Rolf Klokkerengen, Eivind Sundfør and Derrick Kwame Odei conducted the lab analyses. Vasco C. Mota, Carlo C. Lazado, Junjie Zhang, Stefano Peruzzi and Gerrit Timmerhaus conducted data visualisation and made figures. Stefano Peruzzi, Trond Peder Flaten, Vasco C. Mota and Carlo C. Lazado offered helpful comments. All co-authors contributed to the draft and revision of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1 Summary of water quality parameters measured at the fish tank effluent

	Control	Low	High	*p*-value
Measured PAA (mg/L)	<LOD	<LOD	<LOD	-
Temperature (°C)	12.1±0.3	12.3±0.4	12.5±0.7	0.300
pH	7.64±0.15	7.70±0.11	7.60±0.07	0.520
O_2 (%)	93.9±2.1	93.6±2.3	92.7±3.5	0.849
Turbidity (NTU)	0.64±0.25	0.69±0.49	1.06±0.28	0.057
NH_4^+-*N* (mg/L)	5.64±2.34	6.23±2.42	5.04±1.93	0.130
NH_3-*N* (mg/L)	0.05±0.04	0.06±0.04	0.04±0.03	0.140
NO_2-*N* (mg/L)	0.00±0.01	0.01±0.02	0.02±0.03	0.682
NO_3-*N* (mg/L)	0.48±0.22	0.47±0.20	0.52±0.21	0.332
Tank Bacterial CFU/mL	6667[a]	34825[a]	164000[b]	0.044
Sump Bacterial CFU/mL	6850[a]	17100[a]	35528[b]	0.001

Superscript alphabets indicate significant differences, post-hoc Tukey HSD test, $p < 0.05$

values are given as treatment mean ± SD (n = 3)

PAA - peracetic acid; LOD – limit of detection; 0.1 mg/L; CFU -colony formation units

Table 2 Level of nuclear DNA damage in gill cells given as percentage of DNA in the comet tail and tail length measured (n=50 cells/sample) at the three sampling points throughout the trial (mean ± S.D., n=3).

	Control	Low	High	Day
%DNA in tail	4.29±3.31	4.54±3.27	4.54±2.98	0
%DNA in tail	4.25±4.03[a]	2.59±1.98[b]	3.87±3.10[a]	14
%DNA in tail	4.76±4.50	4.48±4.60	4.84±4.78	28
Tail length (μm)	0.01±0.11	0.01±0.11	0.01±0.09	0
Tail length (μm)	0.01±0.11	0.01±0.08	0.01±0.10	14
Tail length (μm)	0.16±0.50	0.12±0.50	0.19±0.54	28

Statistically significant differences ($p<0.05$) are indicated by distinct letters.

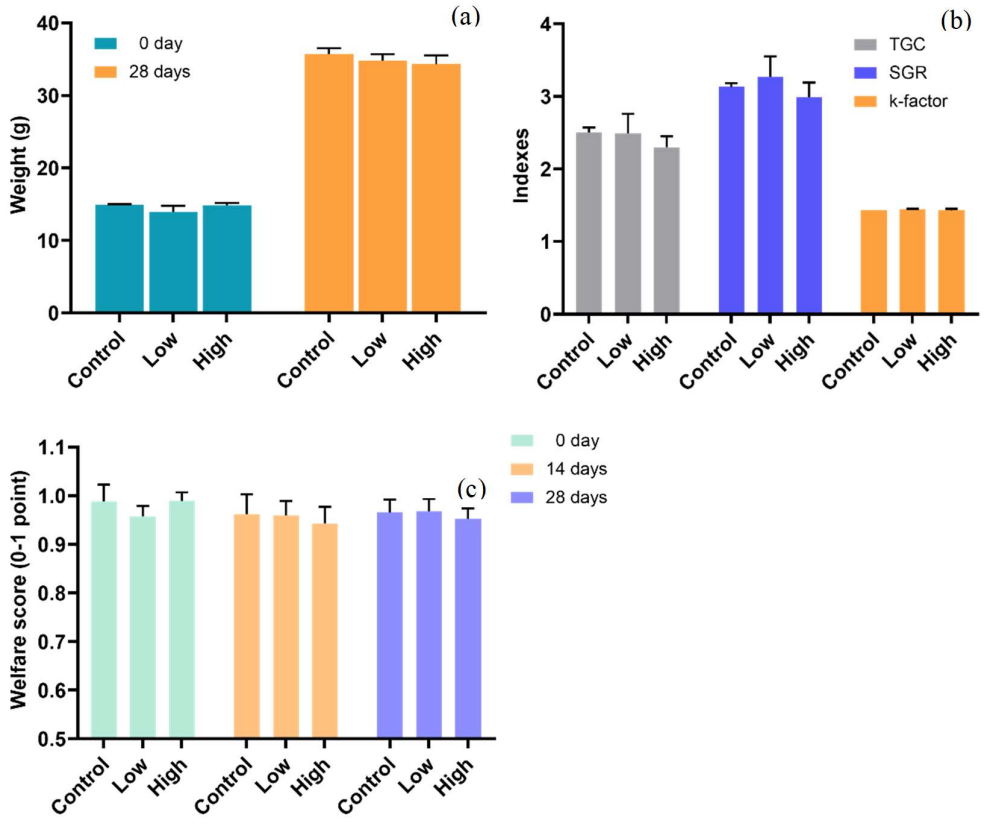


Figure 1 Fish performance metrics weight (a), growth and condition indexes (b), welfare score (c) (mean ± S.D.) to at the beginning and at the end of each PAA dosing period for PAA-treated and control RAS

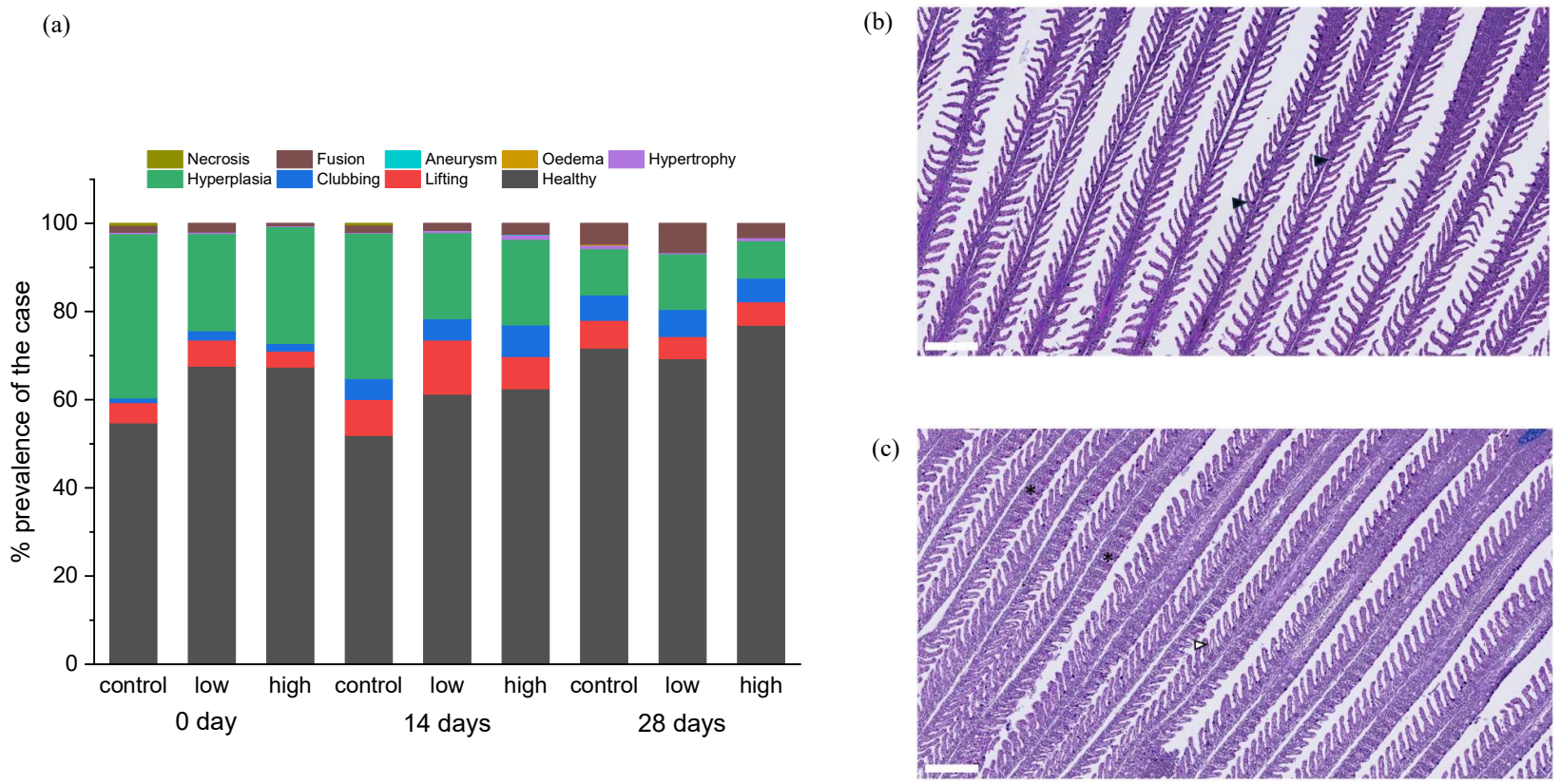


Figure 2 Changes in the structure of the gills following PAA administration. Histopathological cases are shown as a percentage of prevalence of a specific lesion to the total analysed cases (a); Representative AP/Pas-stained histological sections of healthy (b) and compromised gills (c) (Filled arrowheads show mucus cells at the base

of the secondary lamella. Unfilled arrow shows epithelial lifting. Asterisks show hyperplasia and fusion of lamellae. Scale bars = 200 μm).

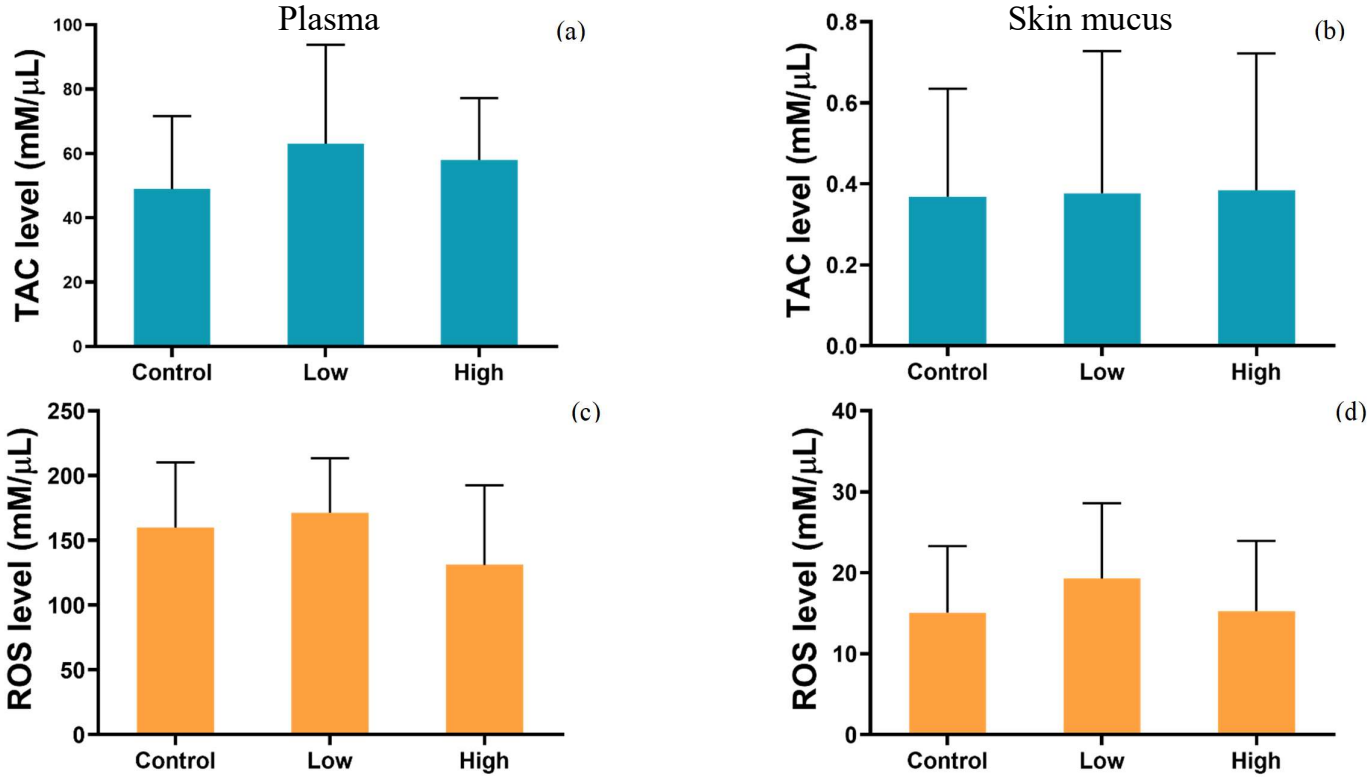


Figure 3 The level of total antioxidants (TAC) and reactive oxygen species (ROS, expressed as H_2O_2) in plasma (a: TAC, c: ROS) and skin mucus (b: TAC, d: ROS) at day 28. Values are presented as mean ± S.D. (n=9).

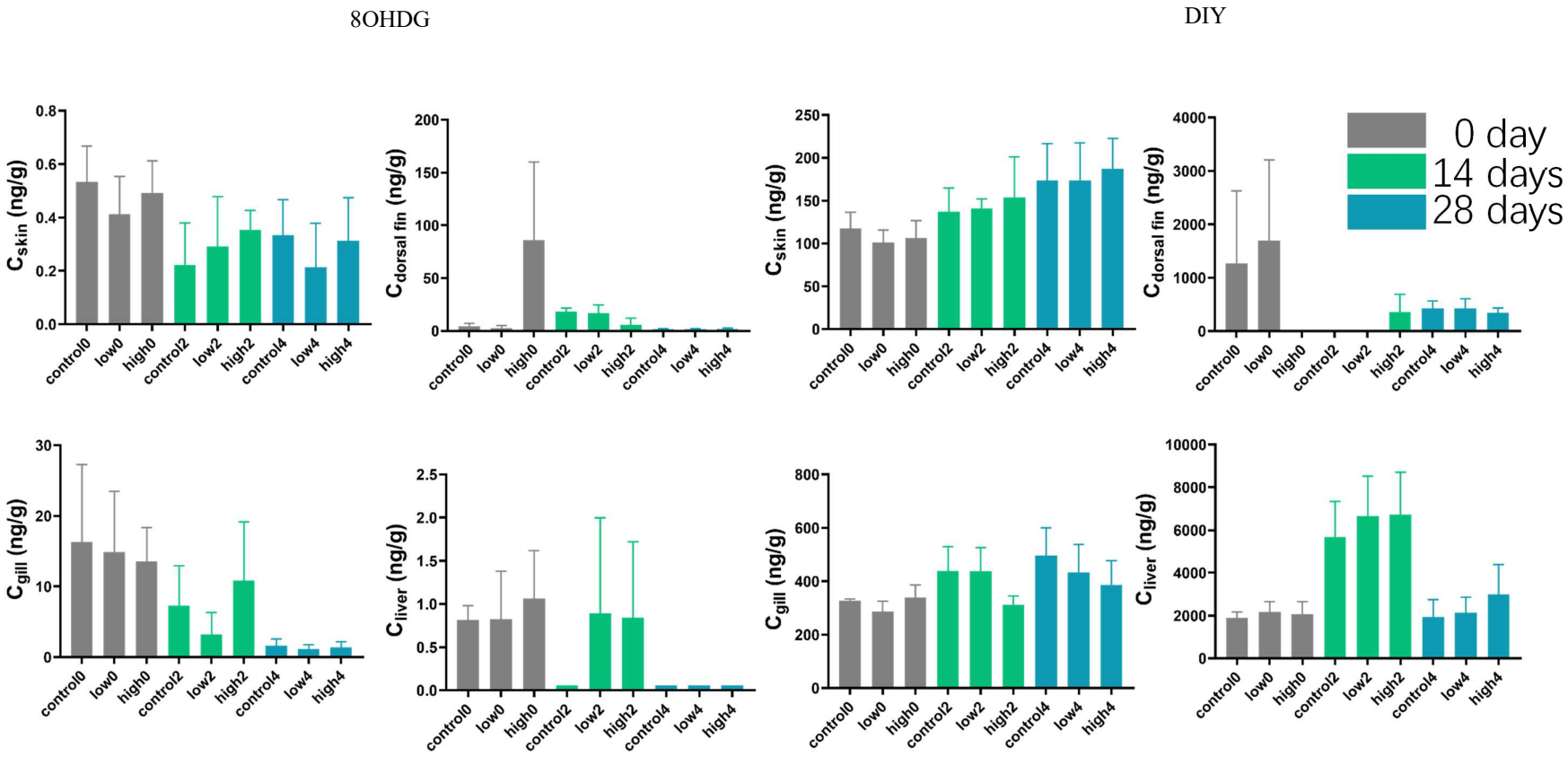


Figure 4 The level of 8OHDG and DIY in Atlantic salmon parr exposed to PAA. The levels were quantified in skin, gill, dorsal fin, and liver of experimental fish. The

left charts represent the concentration of 8OHDG and the right ones represent the concentration of DIY. Values were presented as mean ± S.D. of at least 13 fish per treatment group at a particular sampling point. No significant difference was observed over time within the treatment groups

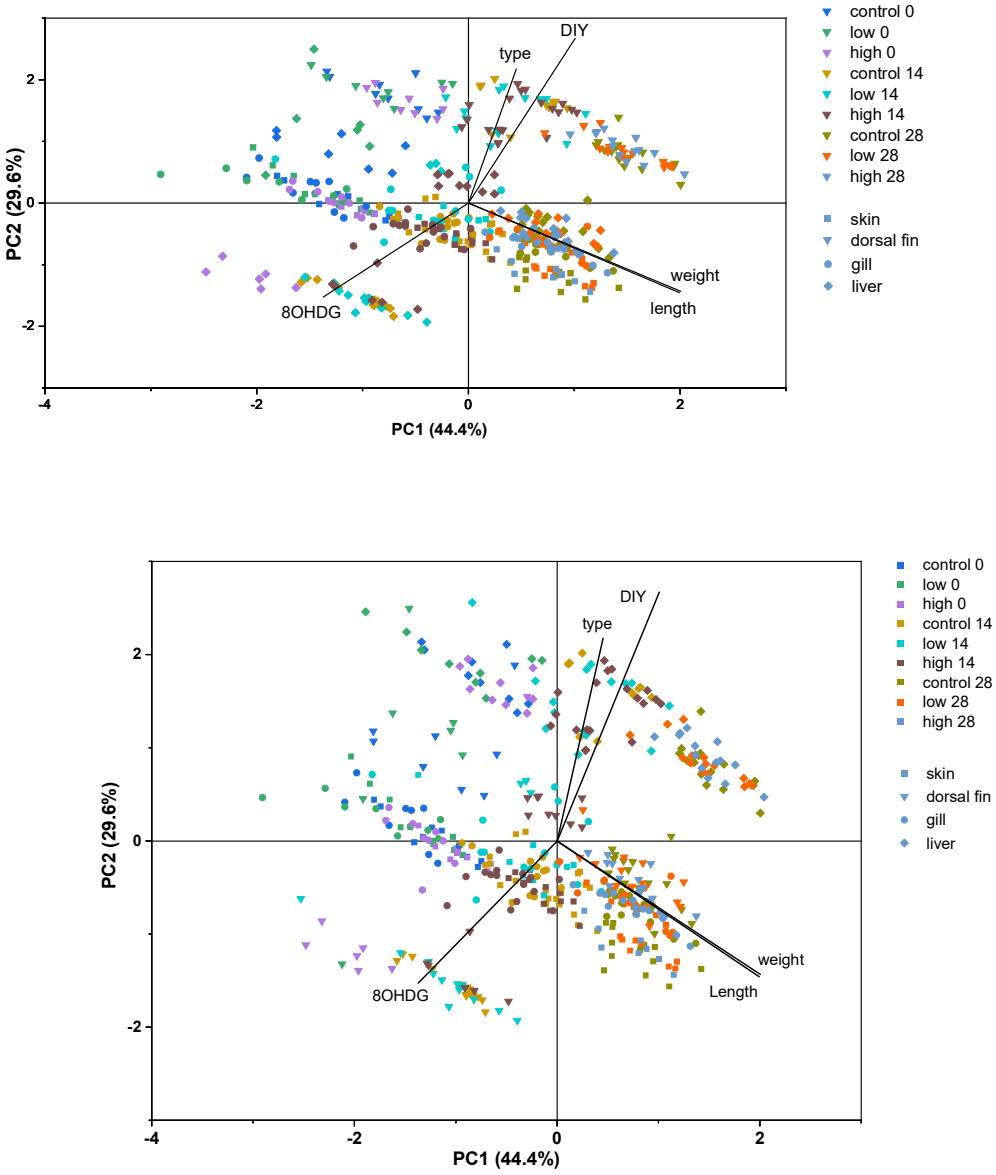
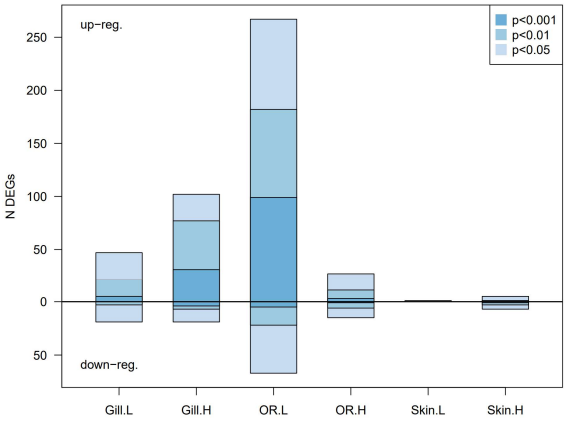
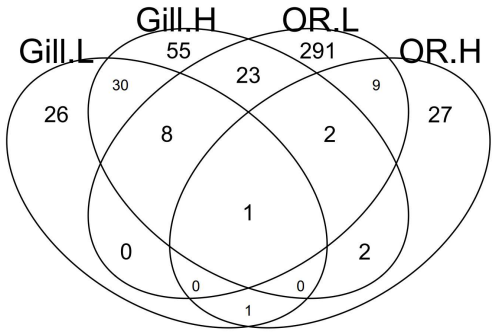


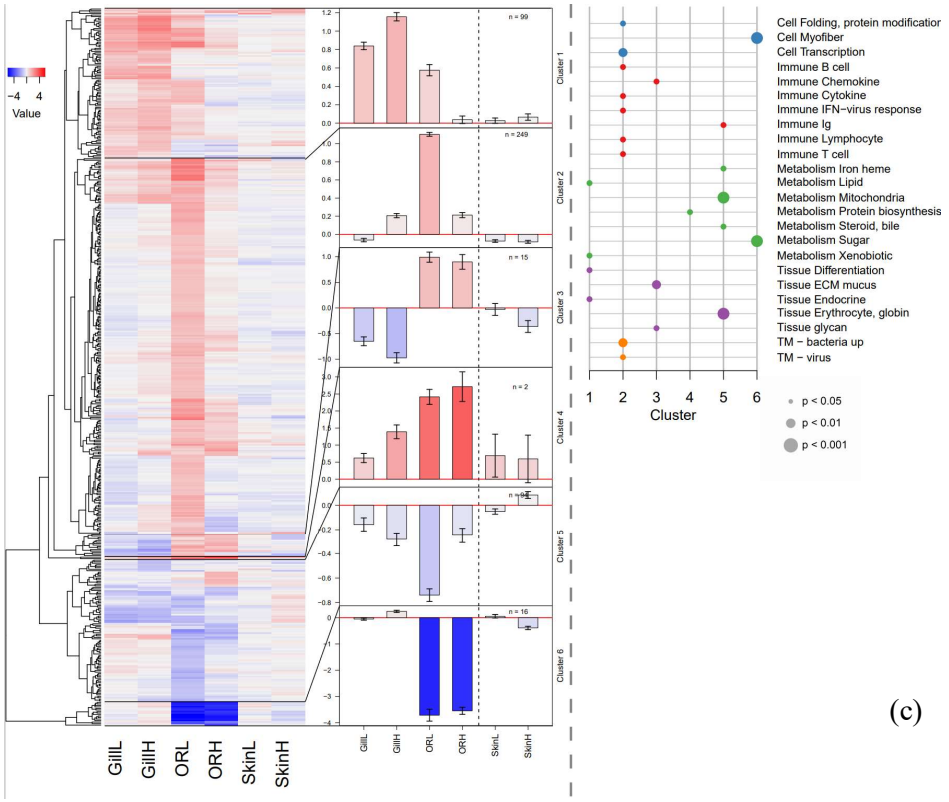
Figure 5 Principal component analysis of 8OHDG and DIY concentrations in different tissues of Atlantic salmon parr under different treatments (control, low, and high) and sampling time points (day 0, 14 and 28)


up−reg.
down−reg.
p<0.001
p<0.01
p<0.05
N DEGs
250
200
150
100
50
0
50
Gill.L
Gill.H
OR.L
OR.H
Skin.L
Skin.H

(a)


Gill.L
Gill.H
OR.L
OR.H
26
30
55
23
291
9
27
8
2
1
0
2
0
0
1

(b)


−4
4
Value
GillL
GillH
ORL
ORH
SkinL
SkinH
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
n = 99
n = 249
n = 15
n = 2
n = 16
1
2
3
4
5
6
Cluster
Cell Folding, protein modificatior
Cell Myofiber
Cell Transcription
Immune B cell
Immune Chemokine
Immune Cytokine
Immune IFN−virus response
Immune Ig
Immune Lymphocyte
Immune T cell
Metabolism Iron heme
Metabolism Lipid
Metabolism Mitochondria
Metabolism Protein biosynthesis
Metabolism Steroid, bile
Metabolism Sugar
Metabolism Xenobiotic
Tissue Differentiation
Tissue ECM mucus
Tissue Endocrine
Tissue Erythrocyte, globin
Tissue glycan
TM − bacteria up
TM − virus
p < 0.05
p < 0.01
p < 0.001

(c)

Figure 6 Transcriptome of the gills, skin, and olfactory organ of peracetic acid (PAA) -exposed salmon parr. (a) Stacked column of DEGs numbers in different organs with low and high PAA exposure, split for three different p-value levels. (b) Venn diagram for the DEGs expression variation among different PAA exposure in salmon parr, respectively. (c) The heatmap shows the down- and upregulation of differentially expressed genes (DEGs) in a colour gradient from blue to red. The dendrogram was split into six sub-clusters and the mean values for genes within these clusters were represented in bar plots (error bars show ± S.D.) in the centre. The bubble chart shows the enrichment analyses of the seven sub-clusters.

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