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Determination of peracetic acid exposure concentration without effect on Atlantic salmon parr health, welfare or growth cultured in RAS.

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Abstract

Recirculating aquaculture systems (RAS) are replacing land-based flow through systems in Norway and other producing countries. RAS has many advances but also bring some biological challenges as water disinfection that may compromise biofilters performance and fish health and welfare. For example, Denmark is using peracetic acid (PAA) as a water disinfection strategy to control pathogens for rainbow trout production. PAA is a highly reactive peroxygen compound; 100 times more effective than hydrogen peroxide and has the potential to control bacterial, and parasitic infections and has a wide range of antimicrobial effects. PAA can be applied continuously or as a pulse to the water and degrades into oxygen and water. In Norway, PAA has been used to disinfect surfaces and different types of equipment in RAS facilities for salmonids, but not for water treatment.

The effects of PAA on Atlantic salmon (*Salmo salar*) smolt and rainbow trout (*Oncorhynchus mykiss*) have already been studied, but no studies on Atlantic salmon parr have been performed to date. In this study, two trials (9 identical RAS in each trial) have been performed to identify the PAA concentration threshold that bears no effect on welfare and growth of Atlantic salmon parr.

In trial 1, which included looking at fish external welfare, histology of gills and skin, swimming behaviour, and hand feeding was used to identify threshold. In the trial, the parr were exposed to PAA concentrations of 0, 0.05, 0.1,0.2 0.4, 0.8, 1.6, 3.2, and 6.4 mg/L for 1h and re-exposed two days later with the same treatment for 1h. The two highest treatments resulted in significant histological effects at skin and gills' level. The feeding and swimming behaviour was significantly reduced with treatments of 1.6 - 6.4 mg/L PAA, respectively. Overall welfare indexes indicated that a treatment over 0.8 mg/L had a negative impact on fish wellbeing.

In trial 2, DNA standard brakes in gills was analysed, growth and external fish welfare was used to identify threshold to Atlantic salmon. Three PAA treatments in triplicates, control (no PAA), low (0.1 mg/L) and high (1.0 mg/L) were performed. The PAA was added continuously for 4 weeks after the first sampling. There was no significant difference in fish growth, external welfare score, overall welfare index, or cellular DNA damage in the gills as measured by comet assay. The latter showed that 95% of the gill cells were intact and not affected by PAA.

On the conditions tested in the present work, PAA exposure equal or below 1 mg/L seems to be safe for Atlantic salmon parr welfare and it can be considered a potential disinfectant in salmon production.

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Abbreviations

CFU	Colony-forming unit
CO_2	Carbon dioxide
DAPI	4′,6-diamidino-2-phenylindole
FOTS	Forsøksdyrforvaltningens tilsyns- og søknadssystem.
K-factor	Condition factor
LOD	Limit of determination
MBBR	Moving bed biological reactor (MBBR)
NH_3	Unionized ammonia
NH_3-N	Unionized ammonia nitrogen
NH_4^+	Ammonium
NH_4-N	Ammonium nitrogen
NO_2	Nitrite
NO_2-N	Nitrite nitrogen
NO_3	Nitrate
NO_3-N	Nitrate nitrogen
NTU	Nephelometric turbidity units
O_2	Oxygen
OWI	Overall welfare index
OWIs	Operational welfare indicators
PAA	Peracetic acid
RAS	Recirculating aquaculture system
SGR	Specific growth rate
TGC	Thermal growth coefficient
WC	Weighting categories
WF	Weighting factor
WS	Weighting score

1 Introduction

1.1 Aquaculture in Norway

Aquaculture is one of the fastest-growing food- producing sectors in the world (Aponte & Tveterås, 2019). Aquaculture in Norway dates back to 1850 with brown trout (*Salmo trutta trutta*) was hatched to be released. Around 1900 rainbow trout (*Oncorhynchus mykiss*) were imported from Denmark and the 1st attempt at pond culture took place. At the end 1960s, the first Atlantic salmon (*Salmo salar*) smolt was set out at sea. Commercial aquaculture in Norway began in the early 1970s and rapidly became one of the larger industries in this country. Since early 2000 Norway has been a world leader in Atlantic salmon farming with a contribution of about 50% of the annual global production of this species (Misund, 2021) (Aponte & Tveterås, 2019) (FAO, 2021).

Atlantic salmon is an anadromous fish, where the first life stages egg, larva and parr is in freshwater. After the parr phase, Atlantic salmon is going through a radical physiological and morphological change. Smoltification increases salinity tolerance, metabolism, downstream migratory, schooling behaviour, silvering, darkened fin margins, and olfactory imprinting to make the fish ready for a life in sea (Björnsson, et al., 2011).

Atlantic salmon production has several bottlenecks and diseases remain perennial issues. During the last decade, the ectoparasite sea lice (*Lepeophtherius salmonis*) became one of the main challenges in Norway salmon production. Sea lice feed on the mucus of the skin and blood from the hosts, resulting in skin damage, stress-induced mortality, osmoregulatory failure, immune suppression, increased risk of secondary infections, and chronic stress (Hannisdal, et al., 2020) (Lazado, et al., 2021) (Powell, et al., 2015). Other recurring issues include the accidental escape of farmed fish from sea cages and their ecological impact on wild stocks (Martins, et al., 2010), and the high fish mortality recorded during sea phase (Davidson, et al., 2016) (Bergheim, et al., 2009). Overall, all these aspects negatively affect fish health and welfare, the profitability, and the general sustainability of the industry.

1.2 Recirculating aquaculture systems (RAS)

Land-based flow through systems have been gradually replaced by recirculating aquaculture systems (RAS) (Badiola, et al., 2018). RAS is an alternative production system to the traditional open sea cages. The last years RAS are used for production of smolt, post-smolt

and up to marked-size fish (Summerfelt, et al., 2016) (Mota, et al., 2019) (Dalsgaard, et al., 2013). In 2012, the Norwegian Ministry of Fisheries gave licenses to produce smolt up to 1000 g in closed or semi-closed tanks on land or in the sea, under controlled conditions. The smolt can be kept longer in a protected environment before setting out to the traditional sea cages. This reduces the time spent in open seawater, and reduces the possibility of Atlantic salmon being exposed to sea lice (Hagspie, et al., 2018), increases fish robustness, reduces mortality during the sea phase, and decreases the overall production time (Davidson, et al., 2016) (Bergheim, et al., 2009).

The RAS consist of solid removal (mechanical filtration), biological treatment (ammonia removal by biofilter), temperature regulation, gas control (degassing of carbon dioxide and pure oxygen), and specially designed fish tank (Summerfelt, et al., 2015).

The use of RAS for production of Atlantic salmon offers several advances. RAS is re-using over 90% of the system volume per unite time (Holan, et al., 2020) and each treatment step reduces the system water exchange (Martins, et al., 2010). It gives greater control on the environment than sea cages e.g. avoiding parasites (Summerfelt, et al., 2016), controlling incoming water and water quality (Martins, et al., 2010), and no fish escapes (Kolarevic, et al., 2016). RAS have the potential of making intensive fish production more environmentally sustainable with better hygiene and disease management (Summerfelt, et al., 2009), and biological pollution control (Martins, et al., 2010).

On the other hand, the RAS technology also presents several challenges with increased running cost, need for more skilled personnel, and water quality control (Kolarevic, et al., 2016) (Noble & Summerfelt, 1996) representing some of the weakens. Fish culturing has normally a high density and feeding rate, resulting in accumulation of organic matter and small particles and which create favourable conditions for bacterial growth (Liu, et al., 2018). Poor water quality and high stocking densities in the tank can contribute to disease outbreaks (Noble & Summerfelt, 1996). Managing disease outbreaks can be a primary challenge in RAS (Martins, et al., 2010). If pathogenic bacteria enter the system they can stick to biofilms and release pathogenic bacteria capable of causing recurring diseases if not removed (King, et al., 2008). High levels of ammonia, nitrites, carbon dioxide (CO_2), and suspended solids is known to causes high mortalities in RAS (Noble & Summerfelt, 1996).

According to Nobel and Summerfelt (Noble & Summerfelt, 1996), some of the diseases that can occur with rainbow trout in RAS can be caused by bacteria (*bacterial gill disease, furunculosis, bacterial kidney disease, fin rot*), parasites (*Gyrodactylus, Chilodonella, Trichodina, Epistylis, Trichophrya, Ichthyopthirius, Ichtyobodo, proliferative kidney disease, amoebic gill infestation, Coleps*) and the fungi Saprolenia.

1.2.1 Nitrification

Biofilter has an important role in controlling water quality in the RAS (Suurnäkkia, et al., 2020) and a key role in ammonia removal (Meade, 1985). Ammonia exists in two forms unionized ammonia (NH_3) and ionized ammonium (NH_4^+) in an aqueous solution (Ebeling, et al., 2006). Concentration of NH_3 and NH_4^+ depends on temperature, pH, and salinity, e.g., low temperature and pH increase the amount NH_3 (Trussell, 1972). Sum of NH_3 and NH_4^+ is referred total ammonia- nitrogen (TAN) (Ebeling, et al., 2006). Both can be toxic to the fish, but NH_3 is more toxic at a low level than NH_4^+ (Meade, 1985).

Effectiveness of nitrification process depends on different parameters such as substrate, dissolved oxygen concentration, organic matters, temperature, pH, alkalinity, salinity, and turbulence level in the system. Impact of this parameters makes it difficult to predict the performance of the biofilters nitrification (Chena, et al., 2006).

Nitrification is a two steps process and are accomplished with the use of biofilter with nitrifying bacteria that oxidates ammonia to nitrite (ammonia-oxidizing bacteria) and nitrite to nitrate (nitrite-oxidizing bacteria) by autotrophic bacteria (Ebeling, et al., 2006) (Kuhn, et al., 2010) (Graham, et al., 2007) :

$Eq\ 1.$ $\quad NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$

$Eq\ 2.$ $\quad NO_2^- + 1.5O_2 \rightarrow NO_3^-$

It takes 4-8 weeks to establish a healthy and viable population of both ammonia-oxidizing and nitrite-oxidizing bacteria (Figure 1). Bacteria population is sensitive to changes (e.g., salinity changes) and physical stress (e.g., temperature changes) (Malone & Pfeiffer, 2006) (Emparanza, 2009). Such stress can inhibit nitrification rates resulting in spikes in either ammonia or nitrite (Kuhn, et al., 2010).

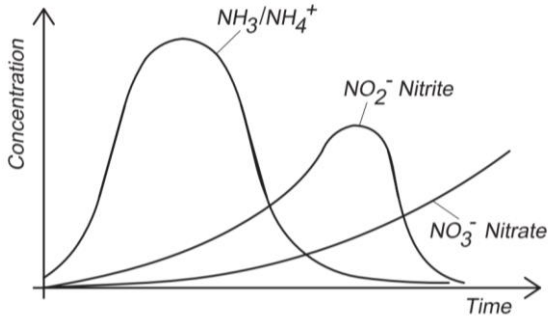


Figure 1A Schematic representation of a typical start-up curve of NH_3/NH_4^+, NO_2, and NO_3 concentration during the maturation process of the biofilter. Biofilter will require a certain start-up time before fully functional (Lekang, 2020).

1.3 Peracetic acid (PAA)

Peracetic acid (PAA, CH_3CO_3H) referred to as PAA, has gained interest in water treatment over the last few decades (Straus, et al., 2018) as a promising water sanitizer or disinfectant tool (Pedersen, et al., 2009). In aquaculture PAA has been used to disinfect water, surfaces, and to lower or eliminate the burden of fish pathogens (Straus, et al., 2018). In Danish flow-through farms with trout PAA has been added (semi-continuous application) in a concentration range of 0.10–0.15mg/L to prevent outbreak of white spot disease (Pedersen & Henriksen, 2017).

PAA is a strong disinfectant with wide-ranging antimicrobial activity and high treatment efficiency (Pedersen, et al., 2009) (Pedersen, et al., 2013). For *Ichthyophthirius multifiliis, Aeromonas salmonicida, Flavobacterium columnare, Yersinia ruckeri, Saprolegnia* spp., *Aphanomyces* spp., and infectious salmon anemia virus, the treatment dose has been under 2 mg/L in most cases (Pedersen, et al., 2013) (Straus, et al., 2018) (Soleng, et al., 2019). PAA has shown to be 100 times more effective that hydrogen peroxide (H_2O_2) (Straus, et al., 2012). PAA is approved in Europe for use in veterinary medicine, and it can be legally used to prevent and control disease outbreaks in fish

production systems in the EU (Davidson, et al., 2019) (Kitis, 2004). To avoid the recurrence of pathogens, it can be necessary to use PAA treatment in a pulse or continuous way (Gesto, et al., 2018).

PAA is available in commercial solutions as an acidic quaternary equilibrium mixture of hydrogen peroxide, acetic acid (CH_3CO_2H) and water (H_2O) in the equilibrium (Pedersen, et al., 2009):

Eq 3. $CH_3CO_2H + H_2O_2 \rightleftharpoons CH_3CO_3H + H_2O$

Various commercial compounds exist with different combinations and different concentrations of PAA and hydrogen peroxide (Pedersen, et al., 2009) (Kitis, 2004) with hydrogen peroxide contributing to the disinfection power to PAA (Kitis, 2004). Commercial products are often stabilized by acidification (Pedersen, et al., 2009) as PAA equilibrium stability and decomposition are pH-dependent (Yuan, et al., 1997). PAA is less stable than hydrogen peroxide and a diluted PAA solution is even more unstable (Kitis, 2004).

PAA at low concentrations is proven to be an efficient antimicrobial and considered as an eco-friendly alternative disinfection method (Liu, et al., 2017 a) with a low environmental impact (Kitis, 2004), because it degrades spontaneously and releases no harmful residues to the environment (Wagner, et al., 2002). Also, the PAA degradation time and kinetics make it an eco-friendly alternative compared to other disinfectants (Pedersen, et al., 2009) (Pedersen, et al., 2013) (Liu, et al., 2017 a). The decomposition products of PAA are acetic acid, hydrogen peroxide, O_2, and water (Wagner, et al., 2002). The health hazards (to humans) for 12 % PAA solution has the same health hazards as 50% hydrogen peroxide (Kitis, 2004).

The degradation rate of PAA is influenced by the amount of organic matter present in the system. The degradation rates increase with higher fish biomass and organic matter content (Pedersen, et al., 2009) (Davidson, et al., 2019).

It has been reported that exposure to PAA may induce oxidative stress in rainbow trout, common carp (*Cyprinus carpio*) and Atlantic salmon and lead to the increase of common stress markers like cortisol and glucose (Liu, et al., 2020) (Gesto, et al., 2018). With re-exposure of PAA cortisol decreases, which indicates an adaption of the stress response to PAA and supports the use of PAA as a possible welfare friendly antimicrobial agent (Liu, et al., 2017 a) (Gesto, et al., 2018).

Toxicity of PAA varies among fish species and life stages (Straus, et al., 2012) (Straus, et al., 2018). For example, in the channel catfish (*Ictalurus punctatus*) the PAA tolerance of yolk-sac fry lies around 2.2 mg/L while is 1.3 mg/L PAA for swim-up fry (Straus, et al., 2012). Finally, the fathead minnow (*Pimephales promelas*) has a PAA tolerance at 1.9 mg/ L while in the blue tilapia (*Oreochromis aureus*) the tolerance is at 5.8 mg/L (Straus, et al., 2018).

1.4 Fish welfare

There is no consensus or universal definition of animal welfare. Most animal welfare scientists and legislators agree that animal welfare relates to what the individual animals experience or perceive, and the quality of life as perceived by the animal itself (Noble, et al., 2018). The Norwegian law about animal welfare states the following: "*The animal is going to be protected from unnecessary stress, disease, and injury. Fed by good quality food that meets their nutritional, physiological, and behavioural needs based on species, age, and condition. Providing space to behaviour based on the needs of the species and the individual. The fish veterinarian is going to have regular supervision regarding fish health, and the fish must have an environment and a handling that ensures good welfare throughout their life cycle*" (LOVDATA, 2016).

Fish farmers or scientists want to document or improve fish welfare. Clear and effective methods to assess fish welfare are needed. Welfare assessment should describe fish welfare and consider the development time. Welfare indicators that are relevant should be based on science, measure welfare over extended time periods, and they must provide information on potential welfare problems and their causes. Fish behaviour should be easy to observe, and a good welfare indicator(s) should be reliable. Examples of behaviour are changes in appetite, swimming activity, ventilation rate (Martins, et al., 2012), and reduced growth (Noble, et al., 2012).

Poor health can be a result of poor fish welfare, and histology can be used to unravel health conditions in several fish tissues (Saraiva, et al., 2015). Histology is a suitable tool to prove morphological and pathological change in the tissues due to diseases, injury, infection, pollutants, and unfavourable water conditions. Knowledge of the tissue's natural condition is essential. Histological examinations include microscopy of thin tissue sections, which are

stained to detect disease or damage conditions (Flores-Lopes & Thomaz, 2011) (Bernet, et al., 2001) (Kryvi & Poppe, 2016).

1.5 Aim of the study

In this study we have investigated how long term and short term PAA exposure affected Atlantic salmon parr welfare, growth, gill and skin health. Both quantitative and semi-quantitative methods were used. The study sub-objectives were fish appetite, swimming behaviour, OWI, external fish welfare score, histology on skin and gills.

Trial 1. Investigated how short term (1 h) PAA treatments (0.0- 6.4 mg/L) exposure affected Atlantic salmon parr behaviour, welfare, and health

Trial 2. Investigated how long term (4 weeks) continuous PAA exposure (0.0, .1 and 1.0 mg/L) affected Atlantic salmon parr growth performance, welfare and health and RAS water quality.

The thesis general hypothesis is formulated as:

H0: Peracetic acid does not affect Atlantic salmon parr health, welfare, or growth.

2 Material and methods

2.1 Experimental Set-up

The experiments were performed in RAS located at the Aquaculture Research Station, Tromsø. The work consisted of two trials, each time using 9 identical RAS. An overview of the of the experiment is illustrated in Figure 2. More details are given in sections 2.2.1 and

2.3.1.

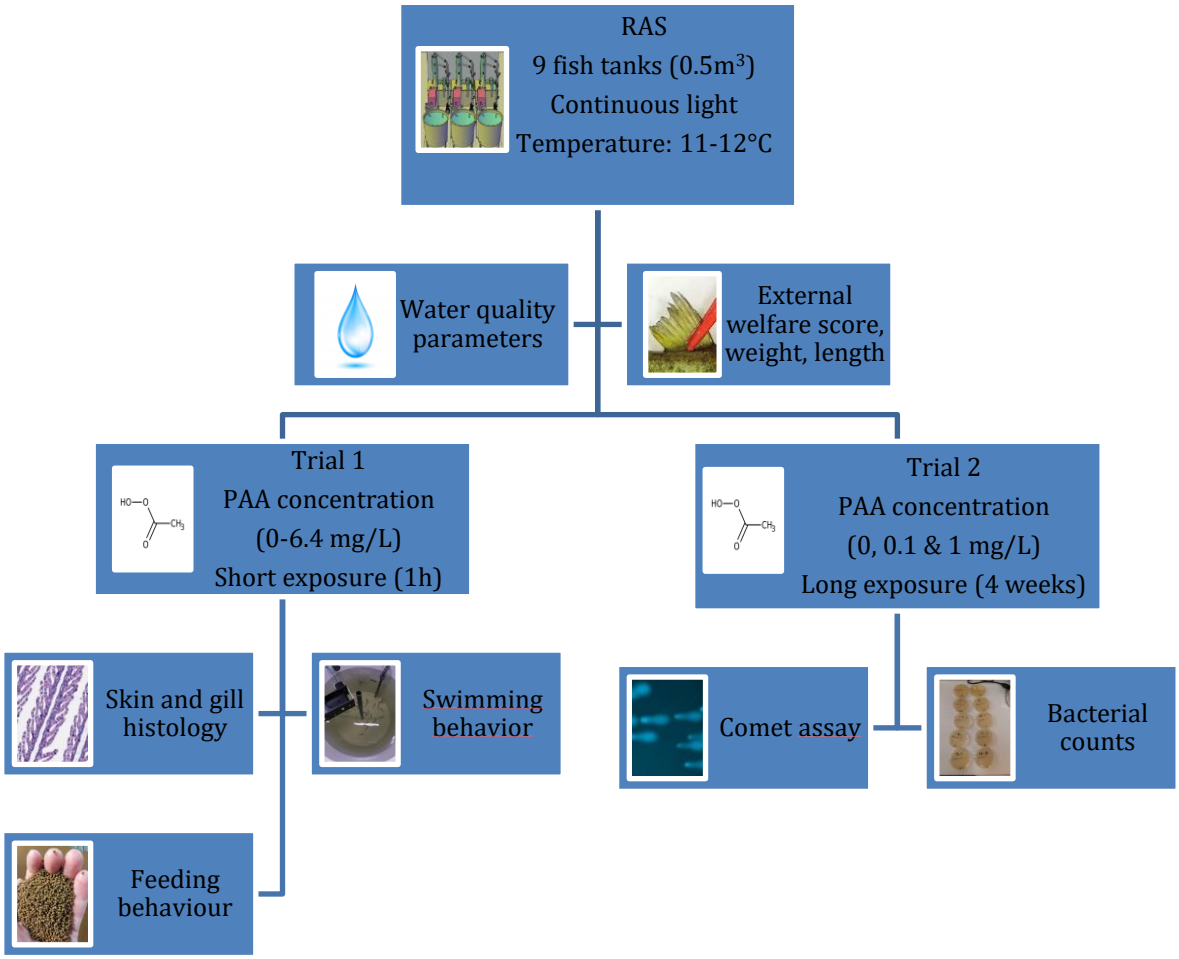


Figure 2 Experimental set-up for the two trials. PAA concentrations and analytical methods used in the study are reported.

2.2 Methods common for both trial 1 and 2

2.2.1 Recirculating *aquaculture system*

In this experiment nine identical RAS (AquaBioTech Group) were used (Figure 3). In each RAS (0.8 m^3 water total volume) water was recirculated through a fish tank (0.5 m^3 volume), pump sump, drum filter (40-micron mesh with a capacity of 5m^3/h), and nitrifying biofilter

(moving bed bioreactor 300 L 750 m^2/m^3 media). Further, water flows (3000 L/h) through a degasser to remove carbon dioxide and a protein skimmer that removes fine solids. Small particles attach to small air bubbles and produce a brown foam which are flushed out via the skimmer. Water (1500 L/h) was oxygenated and flowed through a cooling element where the water temperature was regulated before entering the fish tank. The water flowed through a drum filter before entering a bed biological reactor (MBBR). To ensure optimal pH for the nitrification process, sodium bicarbonate ($NaHCO_3$) was periodically added. Oxygen saturation and temperature were controlled and adjusted according to the operative systems of RAS, and all RAS were running at similar conditions. During the trials, the fish were exposed to continuous light (LD 24:0).

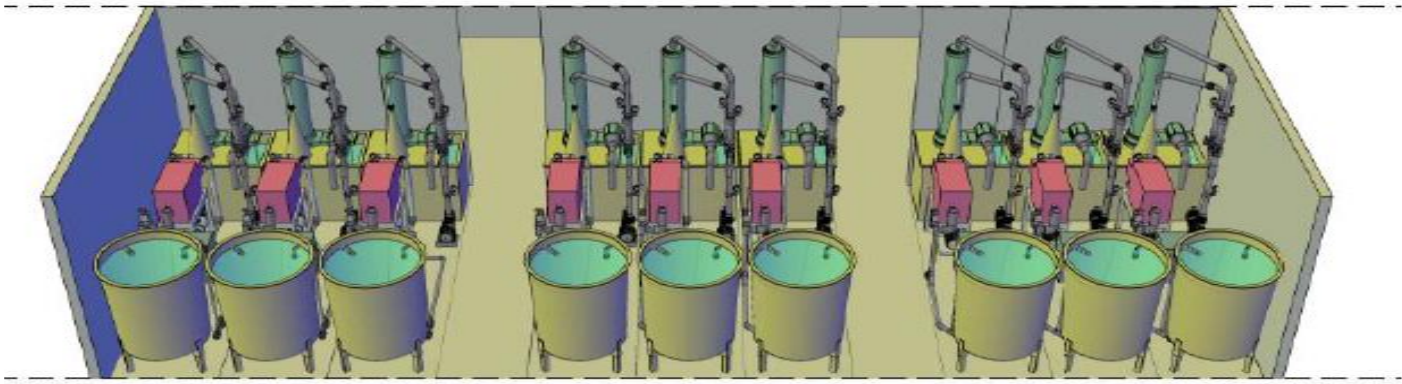


Figure 3 Overview figure of setup of the 9 identical RAS. Figure taken from (ABTG)

2.2.2 Fish and husbandry conditions

Atlantic salmon parr were raised in a freshwater flow-through system (approx.10 °C, continuous light, LD 24:00) to the experimental size of 8.5 -14 g (age 6 month) at Havbruksstasjonen (Aquaculture Research Station, Tromsø, Norway). Before experimental start up, the fish were moved to another Department at Havbruksstasjonen (Dept. Fish Health Laboratory) and acclimatized to the experimental units and feed conditions. The fish were visually inspected daily, and mortality was recorded.

Temperature, pH, and oxygen were monitored daily by automatic sensors, as well as water flow. The NH_3-N concentration was calculated from NH_4-N concentration as a function of pH temperature, and salinity (calculated by several equations on Excel sheet made and provides by Vasco C. Mota, personal communication).

2.2.3 PAA measurement

Water sample (2.5 ml) was pipetted into a cuvette, and 250 µl of N,N-diethyl-p-phenylenediaminesulphate salt (reagent 1) and 250 µl potassium iodide buffer solution (reagent 2) was added and mixed. The colour intensity of regent 1 and reagent 2 was measured in a spectrophotometer (PharmaSpec UV-1700, Shimadzu®, Japan) at λ = 550 nm, 30 seconds after adding reagent 2. Using the Y value from the standard curve to calculate the concentration of PAA based on the absorption value from spectrophotometer absorption on 550 nm. For more information read appendix section PAA measurements.

The PAA solution Aqua Des, provided by Lilleborg AS (Oslo, Norway), was used in the trials. According to the declaration for Aqua Des (provided by supplier); concentrations were given as 5% PAA, 23% HP, and 10% acetic acid. However, the actual concentration of the product was determined to be 23.37% hydrogen peroxide and 6.58% PAA when measured by Lars-Flemming Pedersen (National Institute of Aquatic Resources, Denmark).

2.2.4 *Methods for evaluation of external fish welfare score*

External welfare score was done according the FISHWELL handbook (Noble, et al., 2018). Welfare scoring includes skin damage, vertebral deformity, skin haemorrhages, lesion/wound, scale loss, dorsal fin damage, caudal fin damage, pectoral fin damage, eye haemorrhaging, exophthama, opercular damage, snout damage, upper jaw deformity, and lower jaw deformity were evaluated by a morphological scoring system (Figure 4). FISHWELL handbook standardises the scoring from different welfare indicators to a 0 to 3 scoring system. 0 is fully intact and normal, level 1 indicates minor level of operational welfare indicators (OWIs) is compromised and 3 as severe damage and bad fish welfare.

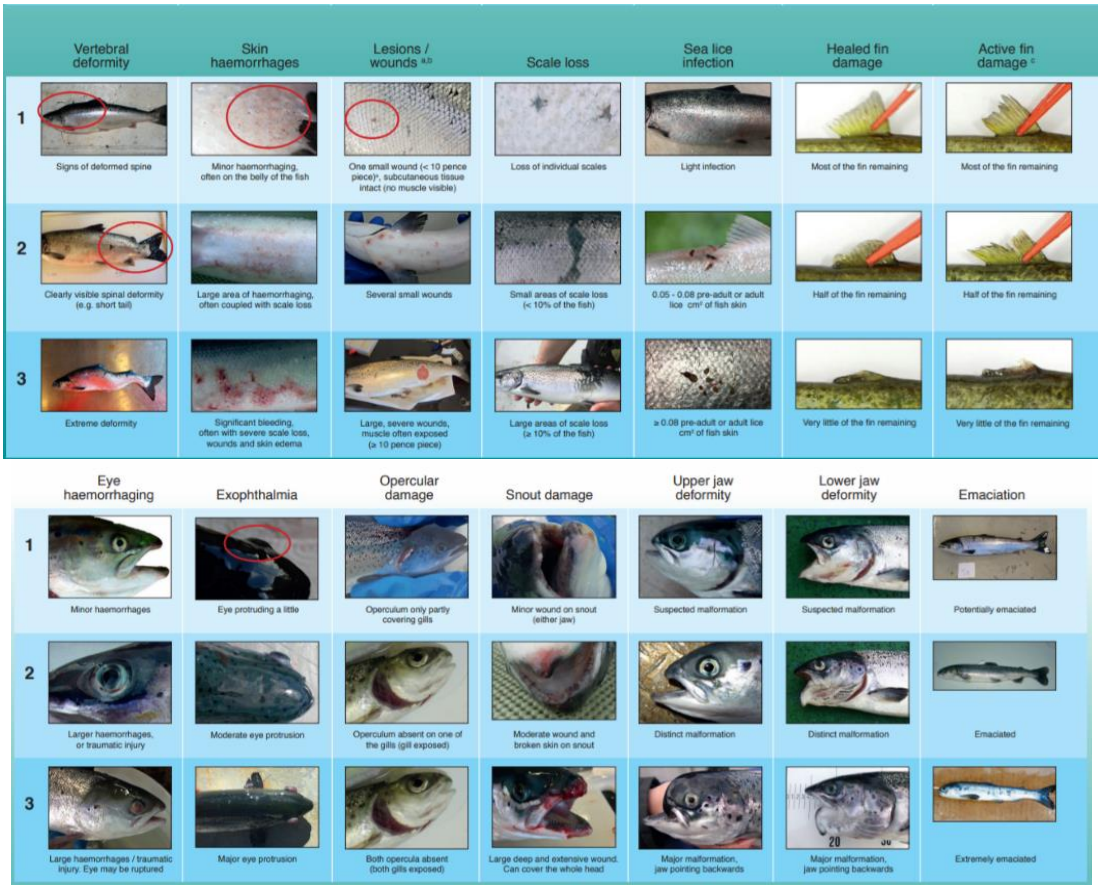


Figure 4 Morphological Operational Welfare Indicators (OWI's) for farmed Atlantic salmon used to score external fish welfare in trial 1 and 2. 0-to-3-point system where 0 indicate little or no evidence of OWIs and 3 indicates clear evidence of the OWIs (from Noble, et al., 2018).

2.2.5 The overall welfare index (OWI)

The overall welfare index (OWI) is a semantic model for overall welfare assessment for fish welfare using a set of selected welfare indicators. The model identifies how each welfare indicator contributes (positive or negative) to the overall index and which welfare indicators are compromised or fulfilled. The first step was to identify the welfare needs (physical needs as respiration, nutrition, health, and behavioural needs as safety, rest, feeding) and find literature for welfare indicators (appetite, growth, and swimming behaviour to mention something) for evaluation of each indicator. The second step was to select criteria for the indicators. They must be practical and measurable. Each indicator can be divided into levels, from good to poor welfare, and must be backed up by scientific literature. OWI has a scale from 0 to 1, 1 indicate good fish welfare and 0 indicate severe poor fish welfare. (Stien, et al., 2013). For more information see appendix section 7.3.

2.3 Trial 1 material and methods

2.3.1 Experimental design

Atlantic Salmon parr were acclimatized for 3 days prior to the onset of the experiment, (Figure 5). Fish were exposed at nine different PAA concentrations (0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 mg/L), and PAA were applied into the fish tanks. The fish were exposed for PAA for 1h and re-exposed for 1h after 48 h. During PAA exposure the fish tank was disconnected from the RAS.

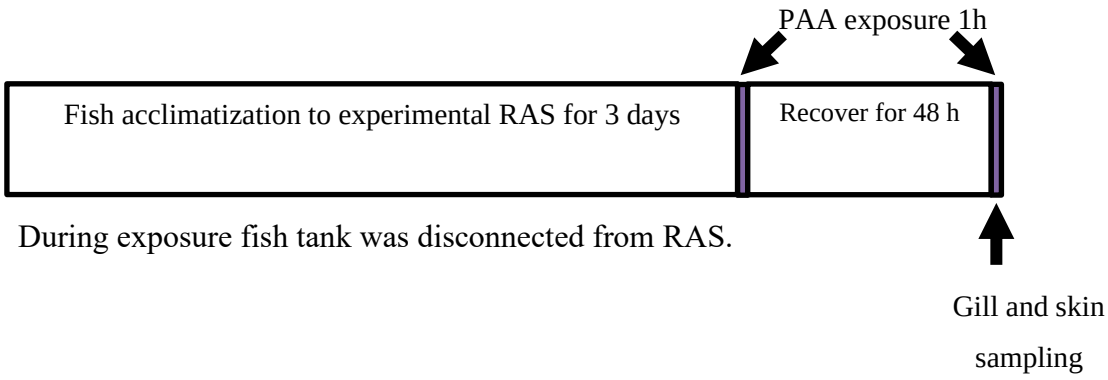


Figure 5 Experimental design. Atlantic salmon parr in 9 identical RAS were acclimatisatised for 3 days before being exposed to 9 different PAA levels for 1h and re-exposed 48 h later. After the first PAA exposure the fish were 50% hand feed for 3 days. After the re-exposure the fish were euthanized and gill and skin tissues sampled for histological examinations.

2.3.2 Fish and husbandry conditions

Each RAS was stocked with 20 fish per tank (total of 180 fish) at an average weight of 8.5g. Experimental fish were feed at ratio of 7 g/day per 20 fish, using a commercial feed (Nutra Olympic, Skretting, Norway 1mm) delivered via an automatic belt feeder (50%, 12 h) and by hand feeding (50%, 30 min). To better observe the reaction to feed after PAA treatment and measure feed interest, hand feeding was performed (3 days) every day after first PAA exposure.

2.3.3 Exposure regime of peracetic acid (PAA)

During exposure, water flow was stopped for 1h and the calculated (Eq 4) PAA dose (0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 mg/L) was added to the fish tank.

Eq 4. $C1V1 \cdot C2V2$

C1V1is initial concentration (C) and volume (V), C2V2 is final concentration (C) and volume (V).

Water samples from the fish tanks were immediately analysed for PAA (see appendix section 7.1). In the first exposure and re-exposure, PAA was measured before addition and after 10 min.

2.3.4 Water quality, sampling, and analyses

During trial 1, the oxygen saturation, pH and temperature before PAA exposure ranged from 79-103.2 %, 6.98-7.43 and, 11.4-12.9 C°, respectively, and pH adjusted if required. Before PAA exposure, water samples were measured for PAA, NH_4^+-N, NO_2-N, NO_3-N by use of a Spectrophotometer (Prove 100, Spectroquant®, Merck, Germany). The mentioned parameters, method of analyses, and frequency are presented in Table 1. After PAA exposure (1h), NH_4^+-N, pH and O_2, and temperature were measured.

Table 1 Parameters, frequency of analysis, and methodology used for water quality analysis.

Parameter	**Units**	**Frequency**	**Method of analyzing and sampling**
Temperature	°C	3 times a week	FDO 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany
Dissolved oxygen	%saturation	3 times a week	FDO 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany
pH		3 times a week	FDO 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany)
Salinity	ppt	3 times a week	FDO 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany
PAA	mg/L	Daily	(Pedersen, et al., 2013)
Ammonium (NH_4-N)	mg/L	3 times a week	*Ammonium Cell Test Kit 1.14558.001 Spectroquant* ®, Merck, Germany
Ammonia (NH_3-N)	mg/L	3 times a week	*Calculated*
Nitrite nitrogen (NO_2-N)	mg/L	2 times a week	Nitrite Test kit 1.14776.0001, *Spectroquant* ®, Merck, Germany
Nitrate nitrogen (NO_3-N)	mg/L	2 times a week	*Nitrate test in seawaer kit 1.14942.0001, Spectroquant* ®, Merck, Germany
Alkalinity	mg/L CaCO3	Weekly	*Acid Capacity Cell test to ph 4.3 (total alkalinity) kit 1.01758.0001, Spectroquant* ®, Merck, Germany
Turbidity	NTU	2 times a week	AQ4500 Turbidity Meter, Thermo Scientific®, Orion®, AQUAfast®, Thermo Fisher Scientific, US

2.3.5 Fish sampling

After the last exposure, all the fish in the tanks were humanely euthanized with overdose of benzocaine (Benzoak vet, EuroPharma, Leknes, Norway) (0.6 ml benzocaine /L), before length (mm) and weight (g) were measured. External welfare scoring was performed according to the FISHWELL handbook (Noble, et al., 2018). Samples of the dorsal skin and second gill arch (5 fish per treatment) were placed in biopsy containers (BiopSafe®, Mermaid Medical, Denmark) containing 20 ml formalin and histological slides were prepared by The Norwegian Veterinary Institute (Harstad, Norway) (see section 2.3.7).

2.3.6 Overall welfare index

OWI of Atlantic salmon parr in RAS is based on SWIM 1.0, SWIM 2.0 model, welfare indicators form FISHWELL handbook (Noble, et al., 2018) (Stien, et al., 2013) (Pettersen, et al., 2014) and on observations from the control group.

We focused on 8 potential welfare indicators (mortality, appetite, swimming behaviour, fin health as dorsal, and pelvic fin, skin health, and opercular damage) as described in Table 2. Welfare indicators as fin health, skin health, scale loss, and opercular damage are levels taken from FISHWELL handbook (Noble, et al., 2018). Welfare indicator level from appetite and swimming behaviour was based on the control group, the end point for mortality was according to FOTS (Forsøksdyrforvaltningens tilsyns- og søknadssystem). Score from mortality, appetite and swimming behaviour was based on the whole tank and not individual fish as rest of the welfare indicators. Swimming behaviour and appetite are scored by observations of the control groups being used as a reference.

Table 2 Trial 1. Welfare indicators (WI) with levels from best to worst score, information about different levels of scoring, indicator level score (IS), calculated weighting factor (WF) and relative weighting factor (RWF).

WI	Score	Levels	IS	WF	RWF
Mortality	1	0 mortality	1.00	21	0.18
	2	5-15 % mortality	0.50		
	3	≥20 % mortality	0.00		
Appetite(g)	1	80-100&%	1.00	11	0.10
	2	65-80%	0.50		
	3	0-65%	0.00		
Swimming behaviour	1	No reaction to adding PAA. Standing/swimming separate, calm, 25% or less is standing with the outlet	1.00	20	0.18
	2	Close together in the middle when adding PAA. Standing close together at the outlet, less than 25% are swimming in the surface/active swimming	0.50		
	3	Lots of activity when adding PAA More than 25% on the surface, erratic swimming, gasping for air, and abnormal swimming behaviour	0.00		
Fin health (dorsal fin)	1	Normal fin health and nothing to comment on	1.00	13	0.11
	2	Most of the fin remaining	0.67		
	3	Half of the fin remaining	0.33		
	4	Very little of the fin remaining	0.00		
Fin health (pelvic fin)	1	Normal fin health and nothing to comment on	1.00	13	0.11
	2	Most of the fin remaining	0.67		
	3	Half of the fin remaining	0.33		
	4	Very little of the fin remaining	0.00		
Scale loss	1	Normal skin health	1.00	12	0.11
	2	Loss of individual scales	0.67		
	3	Small areas of scale loss (<10 % of the fish)	0.33		
	4	Lage areas of scale loss (≥10% of the fish)	0.00		
Skin health	1	Normal skin and noting to comment on	1.00	17	0.15
	2	scare tissue, minor haemorrhaging, one small wound	0.67		
	3	Lage areas of haemorrhaging, often couple with scale loss, several small wounds	0.33		
	4	Significant bleeding, often with severe scale loss, wounds and skin damage. Muscle often exposed (≥10 pence piece)	0.00		
Opercular damage	1	Normal opercular	1.00	7	0.06
	2	Operculum only partly cover the gills	0.67		
	3	Operculum absent one of the gills (gill exposed)	0.33		
	4	Both opercula absent (both gills exposed)	0.00		
SUM				**114**	**1**

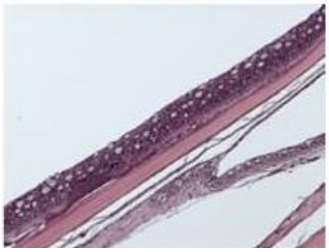
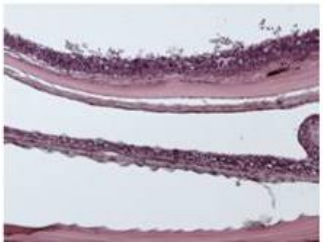
2.3.7 Histology

Skin and gill histology slides were evaluated using the Aprio image scope analysis software (Leica Microsystems, Wetzlar, Germany), scoring method described by Lazado (Lazado, et al., 2021) with some modifications

Condition of the gills was carried out by randomly selecting six gill filaments. Each gill filament has 40 lamellae and a total of 240 secondary lamellae were evaluated per fish (if not possible to count 40 lamellae, the gill filaments next to it is included). Cases of clubbing, lamellar fusion, hyperplasia, hypertrophy, lifting, hyperaemia, aneurysm, and necrosis were documented. Secondary lamellae that did not show any pathological changes were categorized as "healthy". If more than one pathology was present in the same secondary lamella, the most prominent pathology was accounted for. If the observation could not be confidently differentiated, then the secondary lamella was not included in the scoring. Then another secondary lamella was chosen in the same gill filament or, if not possible, the gill filament next to it. The evaluation of gills was carried out at 20X magnification.

A semi-quantitative approach was used to evaluate surface quality of the skin. Three random regions (1500 µm) on the skin were scored by using a 0 to 3-ponits system (see Table 3). Scoring 0 indicating healthy skin with intact and smooth surfaces and 3 indicating severely damage skin structure, disappearance of epidermal layer and rough surface. Acidic and neutral mucous cells was counted in the same regions on a magnification at 20X.

Table 3 Epidermis scoring system with description, made and given privately by Carlo Lazado.

Epidermis	Score	Description	Example	Score	Magnification
General appearance	0	Even epidermis		0, 0	5X
	1	Uneven epidermis			
	2	Parts of the epidermis is missing			
	3	Most of the epidermis is missing			
Surface	0	Smooth surface		0, 3	20X
	1	Signs of rough sells at the surface			
	2	Clear signs of rough cells, < 50% of the surface affected			
	3	All cells lining the other part of the epidermis appears rough			

2.3.8 Fish behaviour (GoPro Camera)

Recorded swimming behaviour with a GoPro camera (Hero 7 and 8) during each PAA exposure (1 h) (1st exposure 3.2 and 6,4 mg/L PAA 2nd exposure; 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 mg/L PAA). Swimming behaviour was evaluated every 5 min, using a 1 to 3-point system; 0 (no reaction to the addition of PAA to the fish tank, calm swimming or standing still) to 3 (intense activity when adding PAA, more than 25% on the surface, intense swimming activity, gasping for air, and abnormal swimming behaviour).

2.4 Trial 2 Material and methods

2.4.1 Experimental design

In trial 2, after acclimation (1 week), the Atlantic Salmon parr were continuously exposed to PAA for a period of 4 weeks, by use of a peristaltic pump (IPC high precision multichannel pump, Ismatec®, IPC, Germany) to 6 RAS/out of 9 RAS sump. RAS were randomly chosen and exposed in triplicate to different PAA treatments: control (no PAA), low (0.1 mg/L), and high (1.0 mg/L). Overview of the experimental design see Figure 6.

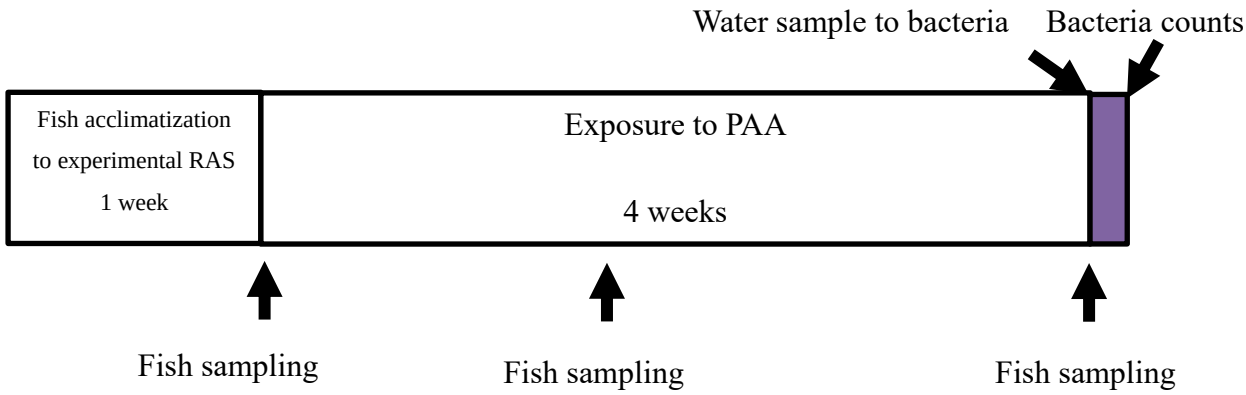


Figure 6 Overview of acclimatization, PAA exposure, sampling points, water sampling for bacteria, and bacteria counting during trial 2

2.4.2 Fish and husbandry conditions

Each RAS was stocked with 40 fish, in total of 360 fish, with an average weight of 14g. In trial 2, fish with compromised opercular was evenly distributed between the fish tanks. Feed

was provided with an automatic belt feeder with commercial diet *(*1,5 mm, Natura Olympic, Skretting, Norway*)* 24h a day (110% feed satiation over 24h)

Water exchange rate was approx. 1040L/kg feed (Eq 5), water exchange rate in fish tank was at 29 min (Eq 6), and tank hydraulic retention time 16.4 day (Eq 7).

$$Eq\ 5. \quad Water\ exch.rate\ = \frac{\text{make–up water}}{\text{kg feed}}$$

$$Eq\ 6. \quad Water\ exch.rate\ = \frac{\text{System volum}}{\text{Flow rate}}$$

$$Eq\ 7.\ HRT \quad = \frac{\text{system volume}}{\text{make–up water}}$$

2.4.3 Peracetic acid dosing trial

After first sampling and the next day, PAA exposure was performed manually (calculated by Eq 1). On the third day, the peristaltic pumps were used, and added 1.2 ml PAA and 12.2 ml PAA every 6h, for low and high exposer concentrations, respectively, for 10 days before increasing similar exposure concentration to every 3h.

2.4.4 Water quality sampling and analysis

The specific parameters, method of analysis, and frequency during trial 2 are described in Table 4. Temperature, O_2, and pH were measured continuous by automatic sensor and recorded in the range of 11.2- 13.4° C, 86.1-104.4 %, and 6.6- 7.8, respectively. The water samples were collected in the fish tanks (1-9) on the sampling day, and same day pH, O_2 and temperature was measured by Multi Meter 3630 IDS. After ten days, flushing of all RAS was performed, 2 min 3 times a week.

Table 4 Water quality parameters, frequency of measurements, and analythical methods

Parameter	Units	Frequency	Method of analysing and sampling
Temperature	°C	Biweekly	FDO 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany
O_2	%saturation	Biweekly	FDO 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany
pH		Biweekly	FDO 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany
Salinity	ppt	Biweekly	FDO 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany
PAA	mg/L	Biweekly	(Pedersen, et al., 2013)
NH_{4+}-N	mg/L	Biweekly	*Ammonium Cell Test Kit 1.14558.001 Spectroquant* ®, Merck, Germany
NH_3-N	mg/L	Biweekly	*Calculated*
NO_2-N	mg/L	Biweekly	Nitrite Test kit 1.14776.0001, *Spectroquant* ®, Merck, Germany
NO_3-N	mg/L	Biweekly	*Nitrate test in seawaer kit 1.14942.0001, Spectroquant* ®, Merck, Germany
Turbidity	NTU	Biweekly	AQ4500 Turbidity Meter, Thermo Scientific®, Orion®, AQUAfast®, Thermo Fisher Scientific, US)

2.4.5 Sampling and analysis

First sampling was performed before exposure to PAA. Three sampling events took place, 14 days apart. At 1st sampling event, six fish were collected from each tank and humanely euthanized with an overdose of benzocaine (0.6 ml benzocaine /L). All fish were given an individually number, measured body weighted, length, and external welfare status was evaluated according to FISHWELL handbook (Noble, et al., 2018). Condition factor (K-factor), Eq 8), growth, thermal growth coefficient (TGC, Eq 9) and specific growth rate (SGR/ %/d, Eq 10) were calculated form these measurements.

Condition factor was calculated as:

$$\text{Eq 8.} \qquad 100 \cdot \frac{W}{L^3}$$

W is the individual weight in grams and length is in cm.

Thermal growth coefficient was calculated as:

$$Eq\ 9. \qquad 1000 \cdot \frac{W2^{\frac{1}{3}} - W1^{\frac{1}{3}}}{T \cdot \Delta t}$$

T is the water temperature in °C and ΔT is the number of days between time T_1 and T_2.

Specific growth rate was calculated as:

$$Eq\ 10. \qquad 100 \cdot \frac{\ln Wt - \ln W0}{t - t0}$$

Wt is the average weight at time t and W0 is the initial weigh at time t0.

Gill samples from three fish from each tank were carefully collected and transferred into 1.5ml cryotubes containing 1ml cell culture media (RPMI 1640, Sigma), 20% DMSO and kept on ice. The samples were then placed in controlled freezing boxes (Corning® CoolCell®) and placed in a freezer at -80°C for 80 min to allow for slow freezing at a rate of 1°C/min prior to comet analysis (see section 2.4.7).

2.4.6 Overall fish welfare

OWI is based on the same literature as trial 1 (SWIM 1.0, SWIM 2.0 model, welfare indicators form FISHWELL handbook (Noble, et al., 2018) (Stien, et al., 2013) (Pettersen, et al., 2014). We focused on 7 potential welfare indicators as described below in Table 5.

Table 5 Trial 2. Welfare indicators (WI) with levels from best to worst score, information about different levels of scoring, indicator level score (IS), calculated weighting factor (WF) and relative weighting factors (RWF).

WI	#	Levels	IS	WF	RWF
Fin health (caudal fin)	1	Normal fin health and nothing to comment on	1,00	13	0,15
	2	Most of the fin remaining	0,67		
	3	Half of the fin remaining	0,33		
	4	Very little of the fin remaining	0,00		
Fin health (pelvic fin)	1	Normal fin health and nothing to comment on	1,00	13	0,15
	2	Most of the fin remaining	0,67		
	3	Half of the fin remaining	0,33		
	4	Very little of the fin remaining	0,00		
Fin health (Dorsal)	1	Normal fin health and nothing to comment on	1,00	13	0,15
	2	Most of the fin remaining	0,67		
	3	Half of the fin remaining	0,33		
	4	Very little of the fin remaining	0,00		
Scale loss	1	Normal skin health	1,00	12	0,14
	2	Loss of individual scales	0,67		
	3	Small areas of scale loss (<10 % of the fish)	0,33		
	4	Lage areas of scale loss (≥10% of the fish)	0,00		
Skin health	1	Normal skin and noting to comment on	1,00	17	0,20
	2	Minor haemorrhagig or one small wound (<10 pence piece) and, subcutaneous tissue intact (no muscle visible)	0,67		
	3	Lage areas of haemorrhaging, often couple with scale loss, several small wounds	0,33		
	4	Significant bleeding, often with severe scale loss, wounds and skin edema. Lage, severe wounds. Muscle often exposed (≥10 pence piece)	0,00		
Vertebral deformation	1	No signs of deformation	1,00	10	0,12
	2	Signs of deformed spine	0,67		
	3	Clearly visible spinal deformity	0,33		
	4	Extreme deformity	0,00		
Opercular damage	1	Normal opercular	1,00	7	0,08
	2	Operculum only partly cover the gills	0,67		
	3	Operculum absent one of the gills (gill exposed)	0,33		
	4	Both opercula absent (both gills exposed)	0,00		
SUM				85	1

2.4.7 Bacterial counts

At the end of the experiment, the water samples collected from the sump and fish tanks were diluted to 10^{-1},10^{-2}, 10^{-3}, and 10^{-4} times before spreading on duplicates agar plates (TSA+ and TCBS, see appendix section bacteria counts). Agar plates were first kept at 12°C for 1 week

before kept at 4°C for 1 week before counting bacteria between 25-300. Bacteria was calculated as CFU/ml (colony-forming units) as Eq 11:

$$Eq\ 11. \quad \frac{\text{Colonies} \cdot \text{Dilution factor}}{\text{Volume of culture plate}}$$

2.4.8 Comet assay

Preserved fish gill samples were removed from the freezer, thawed, gently crushed in a cell culture medium (RPMI-1640™, Sigma) containing 25% DMSO, and kept on ice. Approximately 1ml of the crushed samples were transferred into an Eppendorf tube and centrifuged at 250 x g for 10 min at 4°C. Supernatant cell culture medium containing DMSO was removed and discarded. Precipitated tissues were re-suspended in 500µl cold PBS + EDTA and further centrifuged at 250 x g for 10 min at 4°C, the resulting supernatant was then removed and discarded. This procedure was then repeated one more time using 500µl cold PBS + EDTA as a double washing step. Precipitate cells are then re-suspended in 500µl cold Ca2+ and Mg2+ free PBS and the Eppendorf tubes set on ice until use.

The obtained cell suspension was thoroughly mixed with 0.5% low melting agarose (LM agarose, Sigma) kept in molten state at 37°C at a ratio of 1:9 respectively. 50µl of this mixture were carefully pipetted onto slides pre-coated with 1% standard agarose and covered with glass coverslips. All operations were done maintaining a temperature of 37ºC. Slides with cover slips were then cooled by refrigerating at 4°C for 30 min. Cover slips were carefully removed leaving the solidified gel on the slides which were then placed on a rack and immediately immersed in a cold lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl and, pH10) for 45 min at 4°C in the dark. The slides were removed, briefly drained, and immersed in alkaline unwinding solution (200mM NaOH, 1mMEDTA and, pH>13) for 20 min at room temperature prior to single-cell electrophoresis.

850 ml of cold alkaline electrophoresis solution (200mM NaOH, 1mMEDTA and, pH>13) was poured into an electrophoresis chamber (CometAssay Electrophoresis System II, Mineapolis USA) equipped with cooling elements before carefully submerging slides into the solution. The chamber was then covered, and the electrophoresis run performed at 21 V and 0.4 A for 30 min ensuring that the temperature remained cold throughout the run.

After electrophoresis, the slides were carefully removed from the chamber, placed on a rack, and immersed in distilled water (dH2O) for 5 min and repeated once more before finally being immersed in 70% ethanol for 5 min at room temperature in the dark. Slides were then removed and left to dry for 15 min at 37ºC before staining. The latter was done by covering each slide with approximately 100µl of the DNA fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) and slides left to dry for about 5 min at 37ºC in the dark. The excess DAPI was then poured off the slides by gently tapping them. The slides were then briefly washed in dH2O and allowed to dry completely at 37ºC prior epifluorescence microscopy using a Leitz Aristoplan (Leica Microsystems GmbH, Germany) equipped with a Filter Cube A for UV (excitation filters BP 340-380 nm) and digital camera (Flexcam C1). The slides were viewed by Pictures (10x objective) were processed using Leica Application Suite v.4.2 (Leica Microsystems AG, Switzerland) and stored as tiff. files for further analysis. The COMET analysis software analysed (Trevigen®) the percentage DNA in the comet tail for each treatment.

Use of electrophoresis negatively charged DNA towards the anode results in structures that's resembling comets, the intensity if the comet tail relative to the head reflects the number of DNA breaks (Collins, 2004) (Olive, 2002) (Liao, et al., 2009). An overview of the method is reported in Figure 7.

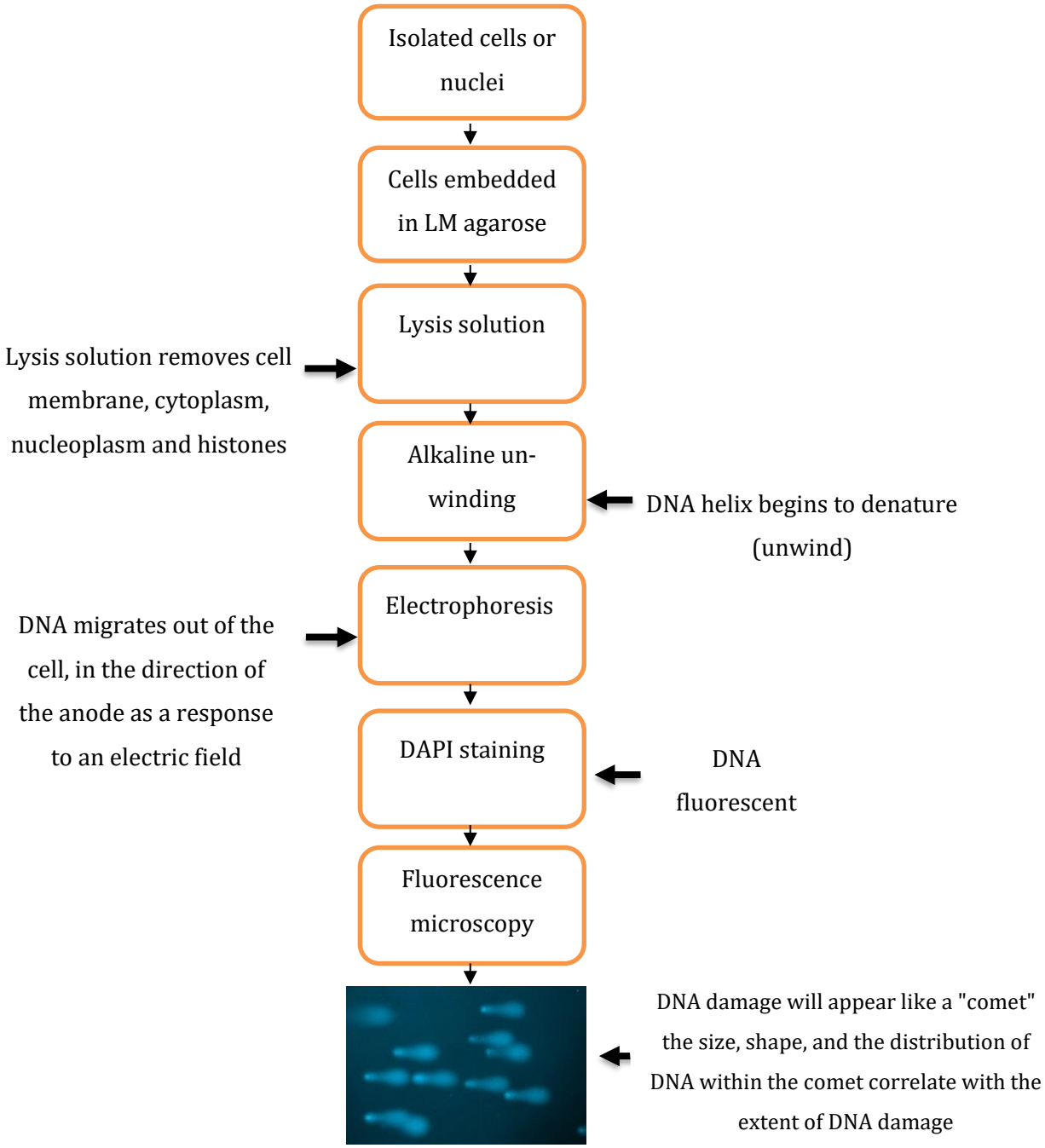


Figure 7 An overview of the comet assay methodology employed in the study.

2.5 Ethical statement

In order to estimate the threshold for Atlantic salmon parr it was necessary to perform a live fish experiment. This study was carried out in accordance with the Norwegian regulations for use of animals in experiments and was approved by the Norwegian Committee on Ethics in Animal Experimentation and Norwegian Food Safety Authority (FOTS id: 24128). PAA exposure concentration and administration method were carried out at the Tromsø Aquaculture Research Station. The experiment was designed to use the least possible number of fish (Small tanks 500L and 20 fish in each tank in in trial 1 and 40 fish in each tank in trial 2) while keeping a sufficient statistical number of individuals to answer the key questions. Before handling all fish were euthanized with an overdose of anaesthetic (Benzocaine, 0.6 ml/L).

2.6 Statistical analyses

All statistical analyses were carried out using IBM SPSS Statistics 26 (IMB, USA). Each data set was check for normal distribution and homogeneity (Shapiro and Levene tests). Weight performance, water quality, bacteria, histology and overall welfare score were compared among treatments using one-way ANOVA followed by Tukey's post- hoc test for equal variances or Games-Howell followed nonparametric statistic Kruskal Wallis-test in case of unequal variances.

External welfare score, and skin histology was check for normal distribution and homogeneity (Shapiro and Levene tests), when requirement for parametric statistics were not met, Kruskal-Wallis test was used. Sample size was too small to use a Chi-square tests. Data are presented as average ± standard deviation (SD) except comet assay appendix where the data are presented as average ± standard error (SE). Results were considered significant at $P<0.05$.

3 Results

3.1 Trial 1

3.1.1 Water quality

Water quality parameters measured for 3 days experiment are summarized in Table 6. All water quality parameters were measured in each fish tank or sampled from each fish tank. NH_3-N was calculated.

A large drop in pH with high PAA treatment was observed. With treatment 3.2 mg/L pH dropped from 7.161 to 5.752 and with treatment 6.4 mg/L the pH dropped from 7.11 to 4.77 after addition of PAA. When closing the water flow to the fish tank, the tank volume dropped from 485L to 404L.

Table 6 Summary of water quality measured in fish tanks as average± SD before and after PAA treatment, n=1.

	0 (mg/L)	0.05 (mg/L)	0.1 (mg/L)	0.2 (mg/L)	0.4 (mg/L)	0.8 (mg/L)	1.6 (mg/L)	3.2 (mg/L)	6.4 (mg/L)
Measured PAA (mg/L)	<LOD	<LOD	<LOD	0.2	0.4	1.1	2.1	4.8	9.4
NH3-N (mg/L)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
NH4-N (mg/L)	0.68±0.07	1.05±0.12	0.59±0.05	0.71±0.12	0.63±0.59	0.73±0.11	0.53±0.17	0.76±0.37	1.02±0.21
Turbidity (NTU)	0.83±0.13	0.96±0.30	0.85±0.04	0.91±0.25	0.99±0.11	0.82±0.18	0.96±0.22	0.91±0.00	1.30±0.00
NO3-N (mg/L)	0.50±0.14	1.20±0.99	0.45±0.07	0.45±0.07	0.75±0.49	0.40±0.00	0.40±0.00	0.40±0.00	0.40±0.00
NO2-N (mg/L)	0.00±0.00	0.00±0.00	0.02±0.01	0.00±0.0	0.00±0.0	0.01±0.01	0.00±0.0	0.01±0.00	0.00±0.00
O2 (%)	100.6±0.6	100.2±0.6	100.4±0.1	102.0±0.6	102.3±0.6	94.1±10.7	101.3±1.3	100.9±0.8	100.9±0.8
pH	7.15±0.20	7.14±0.14	6.94±0.38	7.04±0.38	7.15±0.24	6.99±0.42	6.93±0.42	6.46±1.00	5.94±1.66
Temperature (C°)	12.2±0.2	11.8±0.2	11.7±0.1	11.6±0.5	11.7±0.2	11.7±0.4	11.6±0.5	11.8±0.2	11.9±0.2
Salinity (ppt)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Alkalinity (mg/l CaCO3	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0

**<LOD (limit of determination) when PAA measurement is under 0.00*

3.1.2 Swimming behaviour

A normal fish swimming behaviour was observed in treatment up to 1.6 mg/L PAA, while at treatment concentrations 3.6 and 6.4 mg/L PAA, the swimming behaviour became erratic with gasping for air. Treatments 3.6 and 6.4 mg/L had a scoring at 2.8-2.9 and was significantly higher ($p<0.001$) compared to the rest of the treatments. Treatments (0-0.8) had an average score below 1.5 indicating that the fish were unstressed. Treatments group 0.05 to 1.6 mg/L PAA was not significant different from the control group (score 1.0-1.6). Swimming behaviour showed a positive linear regression ($R^2= 0.805$) between PAA concentrations and an average swimming score (see Appendix section 7.5.1). Furthermore, treatments 3.2, and 6.4 mg/L PAA were significantly different ($p< 0.001$) from the rest of the treatments.

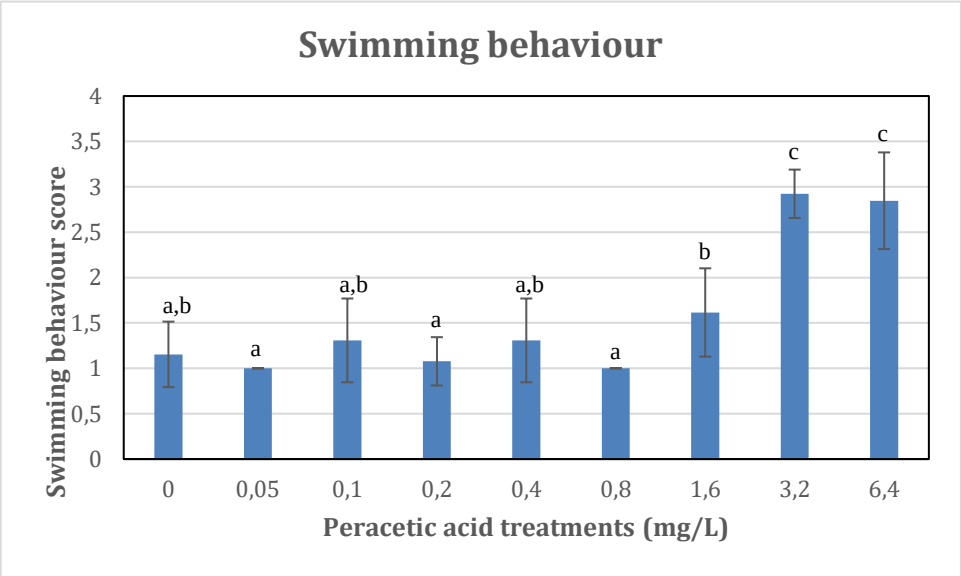


Figure 8 Swimming behaviour of Atlantic salmon parr during 1 h exposure (0, 0.05, 0 0.1, 0.2, 0.4, 0.8, and 1.6 mg/L PAA last exposure and 3.2 and 6.4mg/L PAA first exposure). Swimming behaviour was scored by a 1-to 3 ranking, where 1 is relaxed and calm whereas 3 indicates stressed fish that struggle with survival. Values are given as treatment average± SD. Different letters denote significant differences (P<0.05).

No mortality for fish exposed to PAA concentrations up to 1.6 mg/L for 1 h during PAA exposure and re- exposure was observed. At treatment with 3.2 mg/L, the 1st fish died after 50 min and four fish were dead within 1 h (Figure 9). At PAA treatment with 6.4 mg/L, the 1st fish died after 30 min, and all fish were dead after 1h.

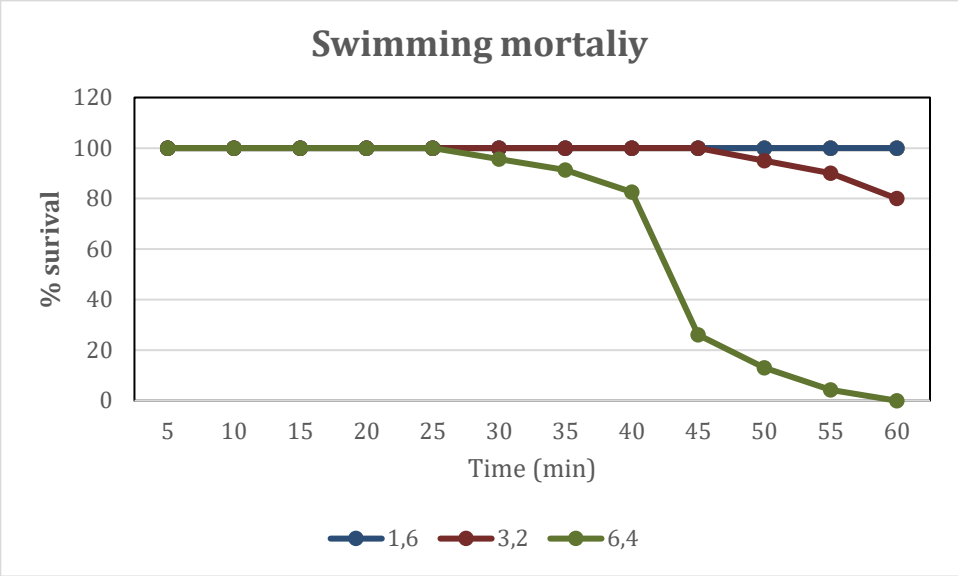


Figure 9 Mortality during 1 h peracetic acid exposure of 9 different treatments (0-6.4 mg/L). Only the treatments resulting in some level of mortality (3.2 and 6.4 mg/L) and the treatment at 1.6 mg/L as reference points are depicted in the graph.

3.1.3 Feeding behaviour

Estimation of fish appetite after PAA exposure was calculated by taking the average of hand feeding (g) during the three days (n=3), see Figure 10. Treatment 0.05 mg/L had the highest feed intake at 3.6 g over control group at 3.3 g. Treatment with 3.2 and 6.4 mg/L are not included since they were euthanizing after 1st exposure. Treatments with PAA concentrations 0.1, 0.2, 0.4 and 0.8 mg/L showed feed intake in the same range (2.5 to 2.7 g), while treatment 1.6 mg/L has the lowest feed intake (2.1g).

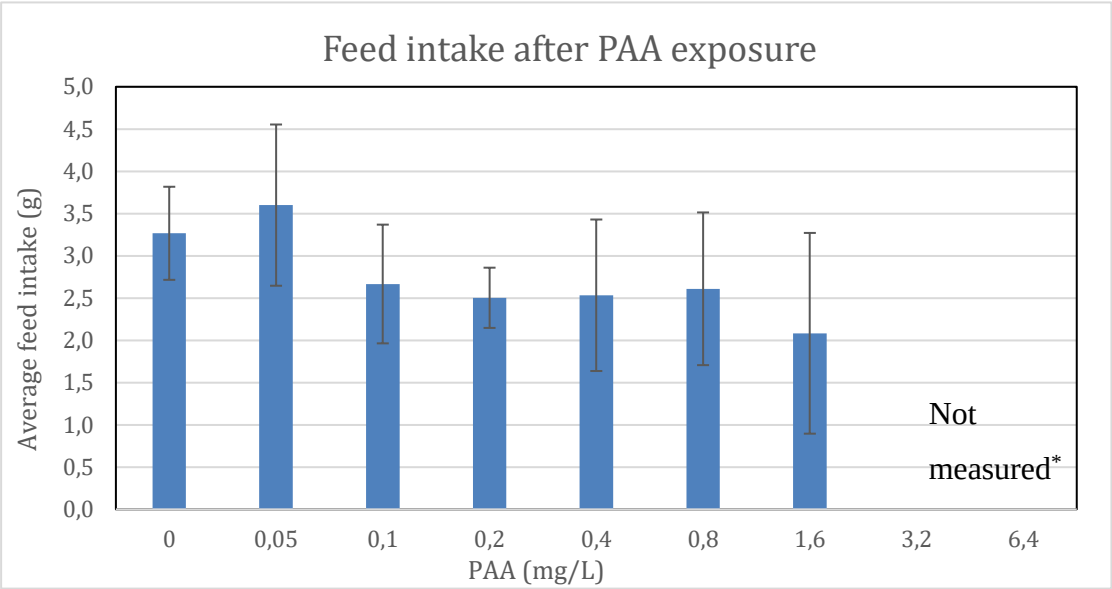


Figure 10 Average (±SD) feed intake during 3-days. Treatment PAA 3.2 and 6.4 mg/L are not shown due to early euthanization of fish. Feeding behaviour decreases as the amount of PAA increases.

3.1.4 Fish welfare

The compromised welfare parameter (skin, dorsal and, operculum damage) during PAA exposure are presented. External welfare parameters are presented as the average for specific welfare indicators for the whole tank in each treatment in Figure 11. In general, the overall welfare status was good, and three treatments have score above 1. Skin health was the only welfare parameter that has a significant difference ($p < 0.001$) between treatment 6.4 mg/L and the other treatments. During the examination, it was observed absence of skin for treatment 6.4 mg/L (Figure 12). Observation during sampling of fish from treatments 3.2 and 6.4 mg/L had a thick layer of mucus on the skin, and gills were grey/pink (Figure 12). There was no regression between dorsal fin and operculum damage during the trial but there was a positive linear regression ($R^2=0.759$) between PAA treatments and skin damage (see Appendix section 7.5.2).

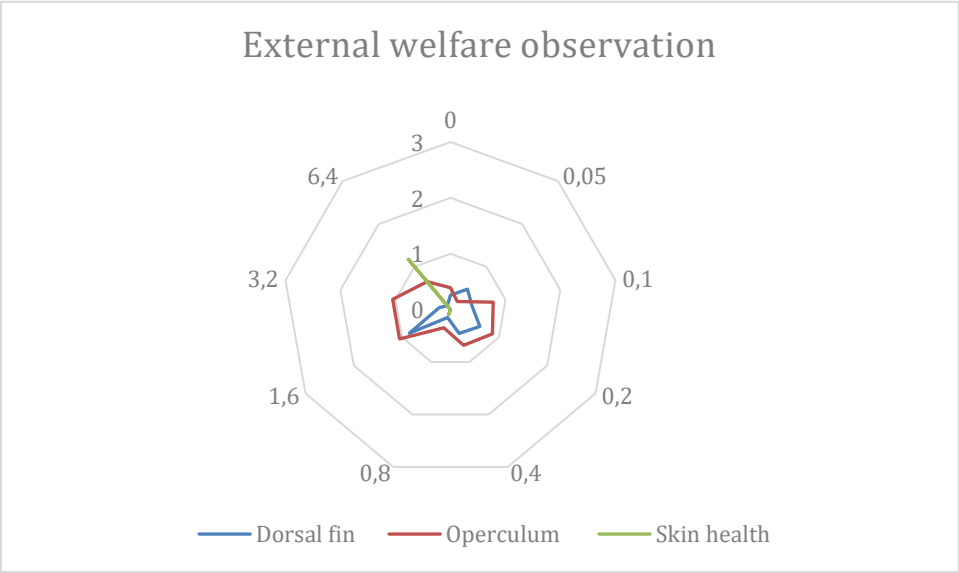


Figure 11 Average external welfare score of each treatment group (0, 0.05, 0.1, 0.2,0.4, 0.8, 1.6, 3.2 and, 6.4 mg/L peracetic acid (PAA). Dorsal fin, operculum and skin was the welfare parameter that was compromised during the trial.

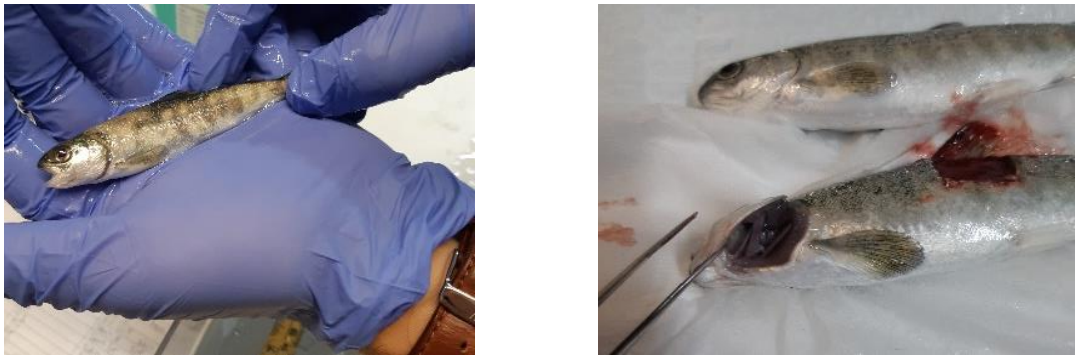


Figure 12 Fish from treatment 6.4mg/L PAA showing absence of skin and gills with a grey/pink coloration.

The overall welfare index, including mortality, appetite, swimming behaviour, dorsal fin, pelvic fin, scale loss, skin health, and opercular damage, of Atlantic salmon parr after PAA exposure are shown in Figure 13. The overall welfare index was good for treatment 0 to 0.8 mg/L showing a score over 0.9 (score 1 indicates good fish welfare). However, treatment 0.2 to 0.8 gave a significant ($p<0.05$) reduction compared to treatment 0 and 0.05. Treatment 3.2 and 6.4 mg/L had a significant ($p<0.05$) lower score than treatment 0 to 0.8 with a score below 0.5 (reduced fish welfare). Treatment 1.6 mg/L showed significant difference ($p<0.05$) compared to treatment 0 and 0.05 mg/L with a score at 0.7. The overall welfare index had a negative linear regression ($R^2=0.882$) with PAA treatments (see Appendix section 7.5.3).

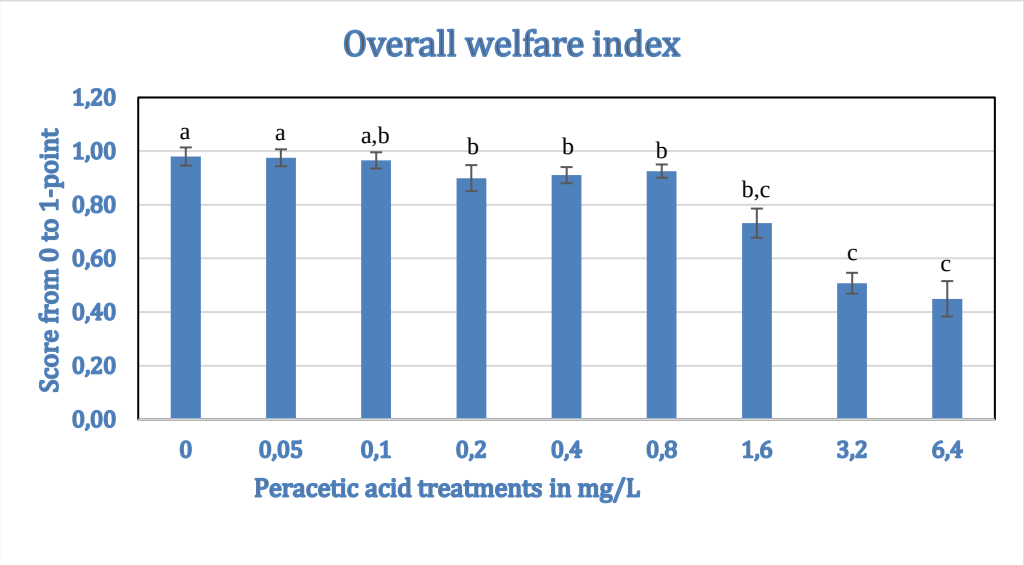


Figure 13 Overall welfare index (dorsal fin, operculum, skin health, motility, swimming behaviour, appetite, and scale loss) after 2 exposures to 9 different PAA concentrations, except for treatment 3.2 and 6.4 mg/L PAA that were exposed once. Overall welfare index scored by a 0 to-1 ranking, where 1 means good welfare while 0 indicates severely compromised conditions. Values are given as treatment average ±SD. Different letters denote significant differences (P<0.05).

3.1.5 Histology

Skin health scoring was evaluation as the quality of surface structure on the epidermis (Figure 14). The average score for general appearance was below 1 for treatment 0, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6 mg/L indicating even epidermis, see Figure 14. The score for PAA treatments 3.2 and 6.4 mg/L were around 2 indicated that parts of epidermis was missing. For general appearance, treatment 3.2 mg/L had a significant higher ($p<0.05$) score than treatment 0, 0.05, 0.1, 0.4 and, 0.8 mg/L, and treatment 6.4 mg/L was significant higher ($p<0.05$) score than treatments 0- 1.6 mg/L.

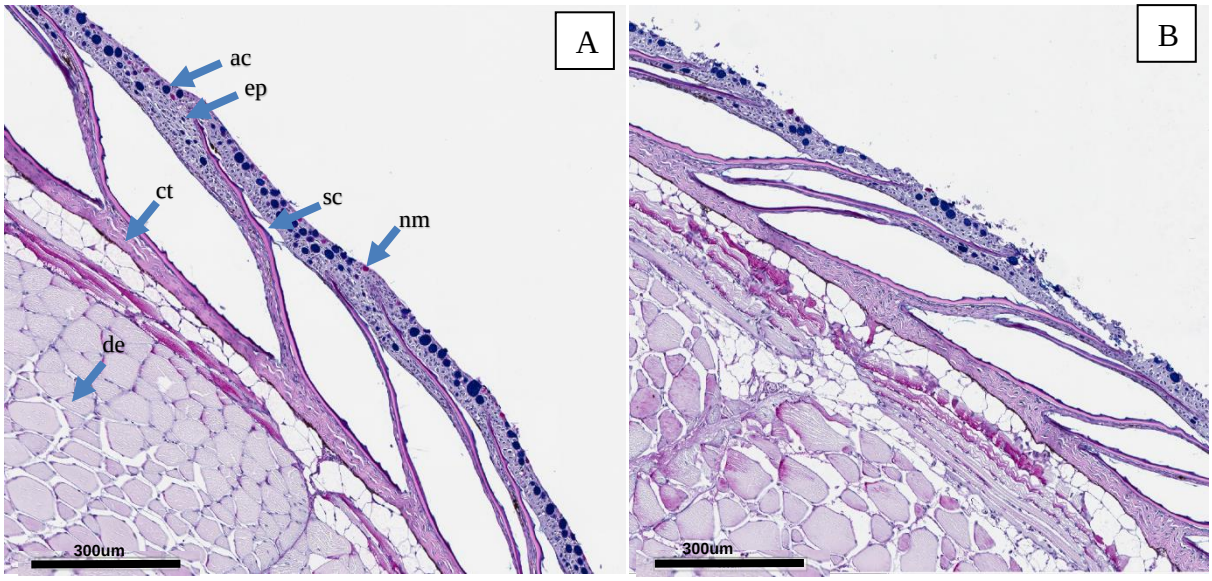


Figure 14 Histological sections of skin, ep, epidermis; sc, scale; de, dermis; ac,acetic acid mucous cells; nm, neutral mucous cells. Picture A) indicates healthy epithelium with a smooth surface. Picture B) shows a compromised epithelium with rough surface and where parts of the epidermis are missing.

Similarly, surface appearance gave an average score below 1 for treatments 0, 0.05, 0.1, 0.2, 0.4, and 1.6 mg/L, indicated by smooth, single rough cells at the surface. Furthermore, treatment 3.2 had significant higher ($p<0.05$) score that treatment 0-0.4 mg/L. Treatment 6.4 mg/L was significant higher ($p<0.05$) that treatments 0, 0.05, 0.1, 0.2, 0.4, and 1.6 mg/L. Treatments 0.8, 3.2, and 6.4 mg/L had an average score between 1 to 2 that indicates rough cells at the surface, and around 50 % of the evaluated epidermal surface was structurally compromised. Treatment 0.8 mg/L was not significant different from the rest of the treatments, but treatment 1.6 mg/L PAA had a significant lower score that treatment 6.4 mg/L. The epidermis surface had a positive linear regression between PAA treatment and general appearance ($R^2= 0.704$) and surface ($R^2=0.707$) (see Appendix section 7.5.4).

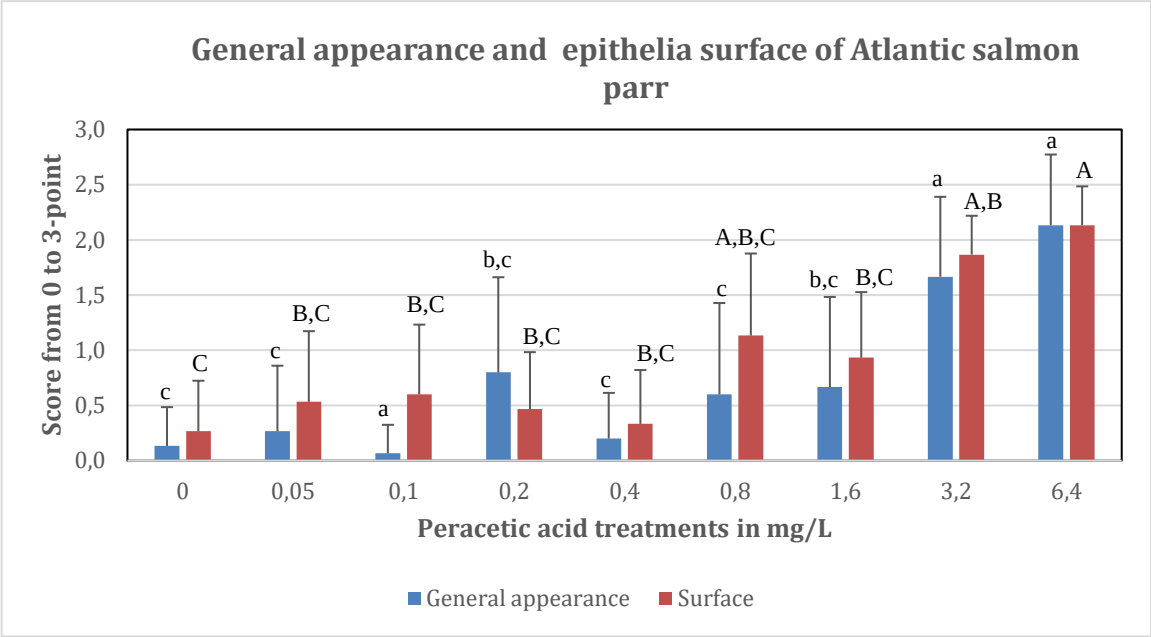


Figure 15 General appearance and epithelia surface of Atlantic salmon parr after 2. exposure to 9 different PAA concentrations (n=5), except treatment 3.2 and 6.4 mg/L PAA that was exposed once. The General appearance and epithelia surface were scored by a 0 to-3 ranking, where 0 means healthy/ intact while 3 indicates severely compromised. Values are given as treatment average ±SD. For each measurement, different letters denote significant differences (P<0.05).

The average number of acidic mucous cells after treatment with PAA 6.4 mg/L were significantly lower ($p<0.05$) than treatments 0.05, 0.1, 0.2, 1.6 and 3.2 mg/L, and after treatment 1.6 mg/L significantly higher ($p<0.05$) amount of acidic mucous cells than treatments 0.4 and 0.8 mg/L was found. Figure 16 show an increasing in acidic from 0 to 0.1 mg/L PAA, from 0.2 to 0.8 mg/L PAA descending. Treatment 1.6 mg/L PAA has the highest amount of acidic cell and treatment 6.4 mg/L PAA have the lowest one. A weak negative linear regression between PAA treatment and average number of mucus cell (R^2 =0.560) was observed too (see Appendix section 7.5.5).

As for neutral mucous cells, treatment 6.4 mg/L resulted in a significant lower ($p<0.05$) number of these cells than treatments 0.05, 0.2 and 1.6 mg/L PAA.

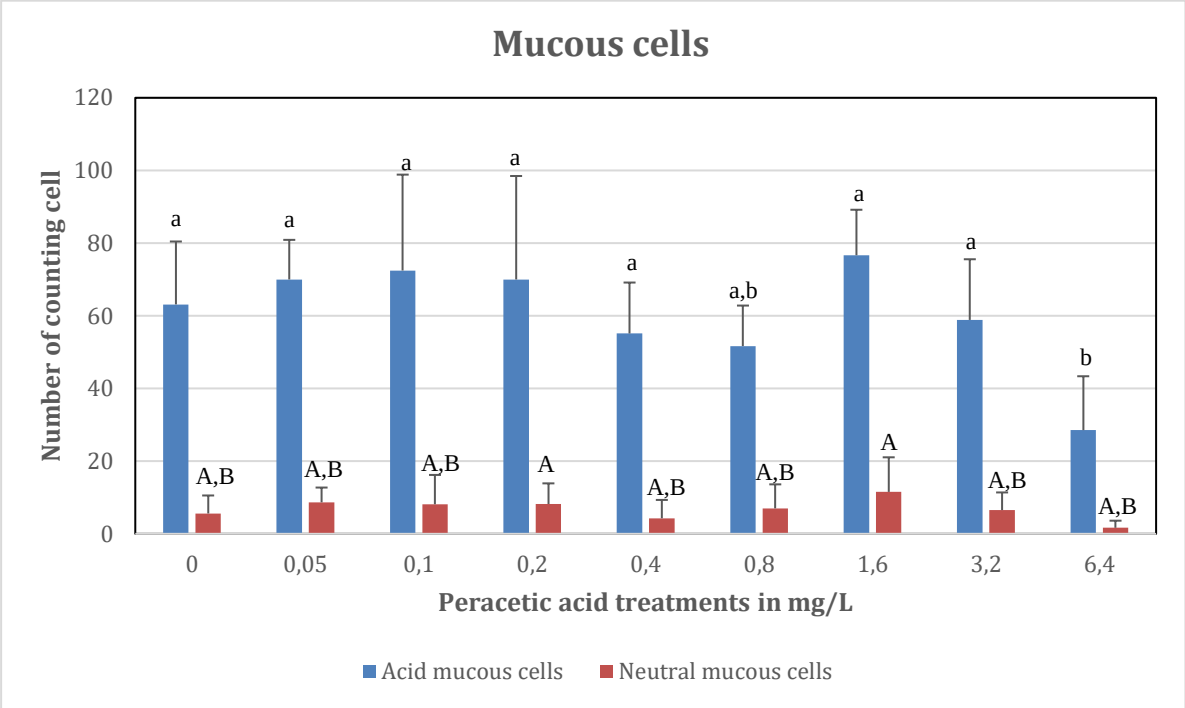


Figure 16 Acidic mucous cells and neutral mucous cells scored on the epidermis. Values are given as average ±SD, n=5. For each measurement, different letters denote significant differences (P<0.05)

The number of healthy lamellas, as well as 8 key branchial pathologies (fusion, aneurysm, epithelial lifting, clubbing, hypertrophy, hyperplasia, hyperemia, and necrosis) and "healthy" were evaluated, and the ratios of their occurrences relative to the total number of evaluated lamellas are shown in Figure 17 and 18. Gill morphology was general healthy in treatment 0 to 1.6 mg/L PAA except treatments 3.2 and 6.4 mg/L PAA. In treatments 0.1 and 6.4 mg/L it was found local inflammation.

In gill histology treatments 3.2 and 6.4 mg/L PAA are significant different ($p<0.001$) from the other treatments when it comes to healthy, hyperplasia, and necrosis on the secondary lamella. Treatment 6.4 mg/L all the secondary lamella was necrotic, and treatment 3.2 mg/L PAA secondary lamellae were in an early stage of necrotic or complete necrotic phase.

The extent of epithelial lifting in secondary lamella was significant higher ($p<0.001$) in treatments 1.6, 0.2, 0, 0.05 mg/L compared to treatments 3.2 and 6.4 mg/L. Treatment 0.4 mg/L showed significant lower ($p<0.05$) cases of cells with lifting than treatments 0.05 and 0.2 mg/L, and treatment 0.8 mg/L had a significant lower ($p<0.05$) cases with lifting than treatment 0.05 mg/L.

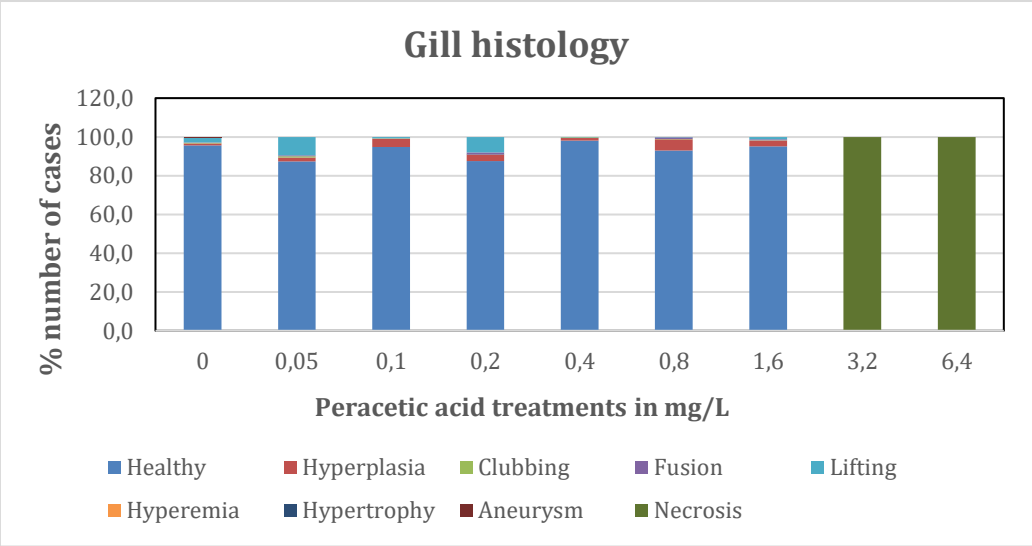


Figure 17 Histological scoring of gills in Atlantic salmon parr after 2 exposures to PAA (for treatment 3.2 and 6.4 mg/L PAA, one single exposure). The 9 common cases were quantified from 240 individual lamellae per fish (n=5). Treatment 3.2 and 6.4 mg/L was significant different from the rest.

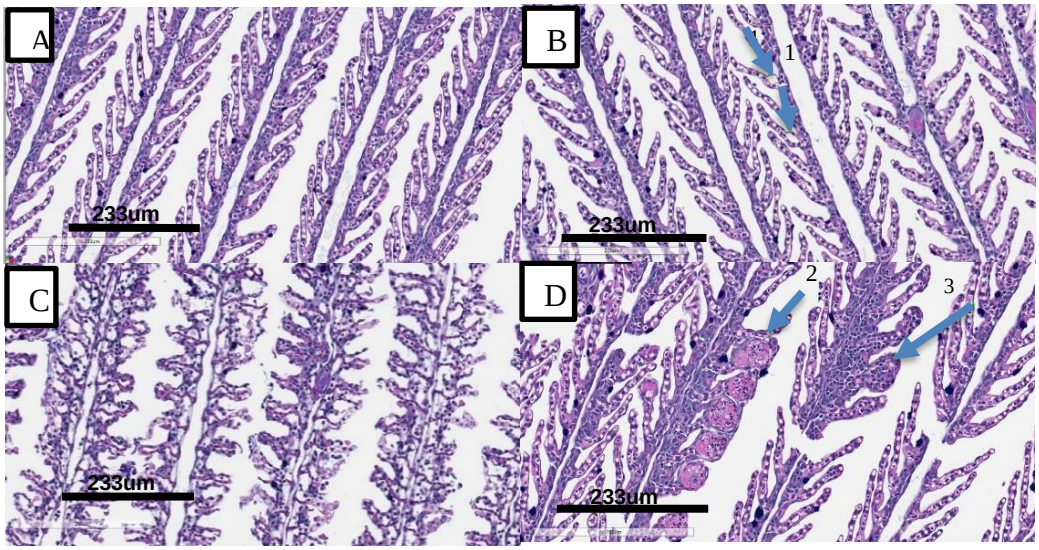


Figure 18 Some the histopathology observed in gills of Atlantic salmon parr. A) healthy gills with well-defined structure, B) epithelial lifting of secondary lamellae (arrow 1); C) necrotic gills with dead cells; D) aneurysm of secondary lamellae (arrow 2) and hyperplasia of epithelial cells (arrow 3). Scale bars represent 233 µm.

3.2 Trial 2

3.2.1 Water quality

Water quality parameters measured during trial 2 are summarized in Table 7. All water quality parameters were measured in or sampled from each fish tank, apart from bacteria counting (CFU/ml) from the sump tanks, NH_3-N and PAA was calculated. Bacteria growth was just observed on TSA+ agar. The bacteria growth in the fish - and sump tanks were significant higher ($p<0.05$) in the high treatment group (1.0 mg/L) than the lower (0.1 mg/L) treatment group and the control (no PAA) group).

No significant difference between PAA treatment and the water quality parameters. Different PAA concentration were too low to be measured in the trial. At the end of trial, there was presence of biofilm on the tank wall in the control group and the low treatment group, while in the high treatment group, the tank walls were deprived of biofilm, see Figure 19.

Table 7 Summary of water quality measured for 4 weeks. Values are given as treatment average ±SD, n=3. NH3-N and bacteria (sump and tank) CFU/ml are calculated values from bacteria count from TSA+ agar. Bacteria in sump tank is the only measurement that's not from fisk tank. For each measurement, different letters denote significant differences (P<0.05)

	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_3-N (mg/L)	0.48±0.22	0.47±0.20	0.52±0.21	0.332
NO_2-N (mg/L)	0.06±0.17	0.09±0.26	0.02±0.03	0.682
Tank Bacteria CFU/ml	6667 [a]	34825 [a]	164000 [b]	0.044
Sump Bacteria CFU/ml	6850 [a]	17100 [a]	35528 [b]	0.001

**<LOD (limit of determination) when PAA measurement is under 0.00*

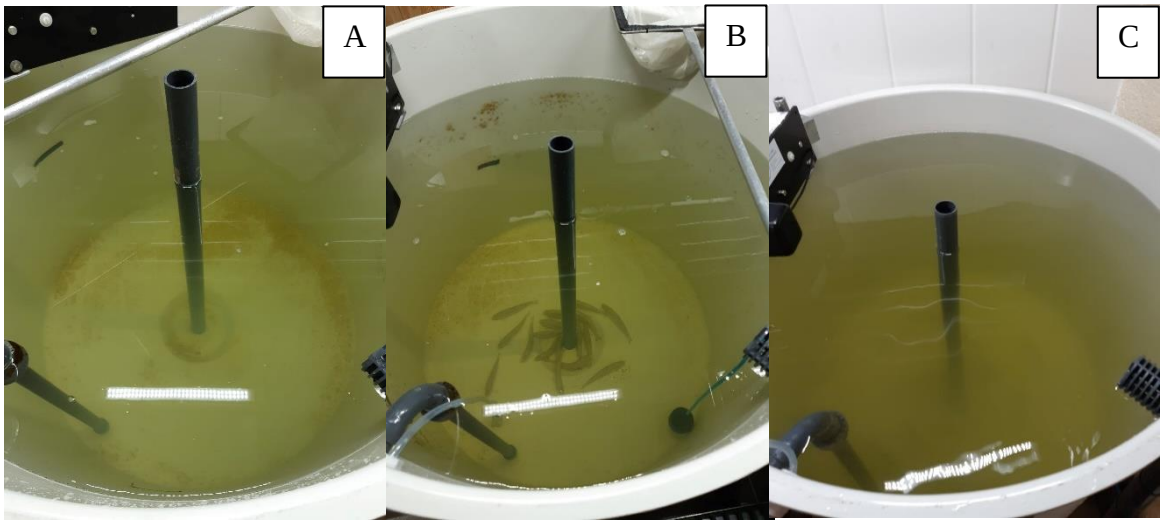


Figure 19 Picture showing the difference between A) control, B) low, and C) high treatment groups of PAA after 4 weeks. In the high treatment group, the water is brown and the is no biofilm growth in the tank compared to control and low concentration of PAA.

3.2.2 Fish performance

During trial 2 no fish mortality was recorded. The weight curves of the Atlantic salmon parr during the 4 weeks of PAA treatment, overlapped almost identically through the experiment (Figure 20). The average body weights measured at the end of the experiment were: control group 35.7 g ±5.4, low treatment group 34.6 g ±4.5 and, high treatment group 34.3 g ±5.1. No significant differences were detected in weight performance. Over the whole sampling period, the fish doubled their body weight (from 15 g to 36 g).

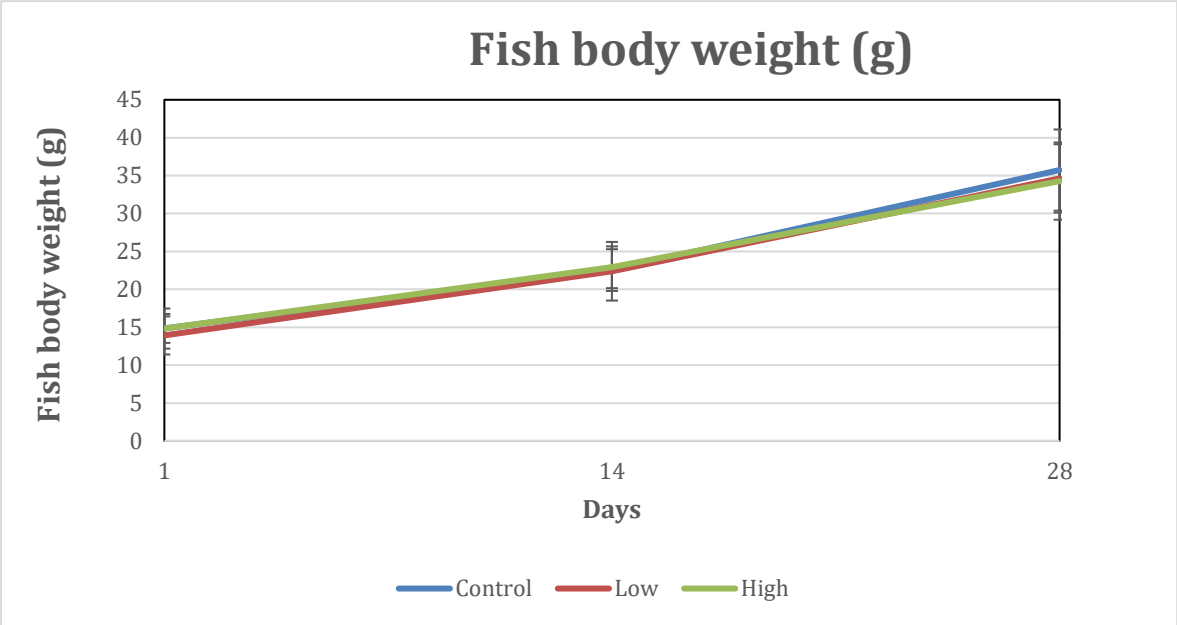


Figure 20 Growth for Atlantic salmon parr in the three treatment groups, control, low and high levels during 4-week with different peracetic acid (PAA) exposure. No significant difference between PAA treatments. Values are given as average ±SD, n=3

The relationship between PAA treatments and SGR (%/d), TGC, and K-factor at the end of the experiment was similar among the PAA treatments, and no significant difference between treatments was observed, see Figure 21, 23, and 22. The average TGC's in the experimental groups were: control group 2.8 ±0.6, low 2.5±0.3 and, high 2.5 ±0.7 concentration of PAA. The average SGR (%) between PAA treatment, control 3.5±0.6, low3.3±0.3 and high 3.4±0.8. The condition factor increased during the trial as following: control group 1.3±0.1 to1.4±0.1, low 1.4±0.1 to 1.4±0.2 and, high 1.3±0.1 to1.4±0.1. No significant difference was detected in terms of fish growth (TGC) and condition factor over the experimental period and at any PAA level.

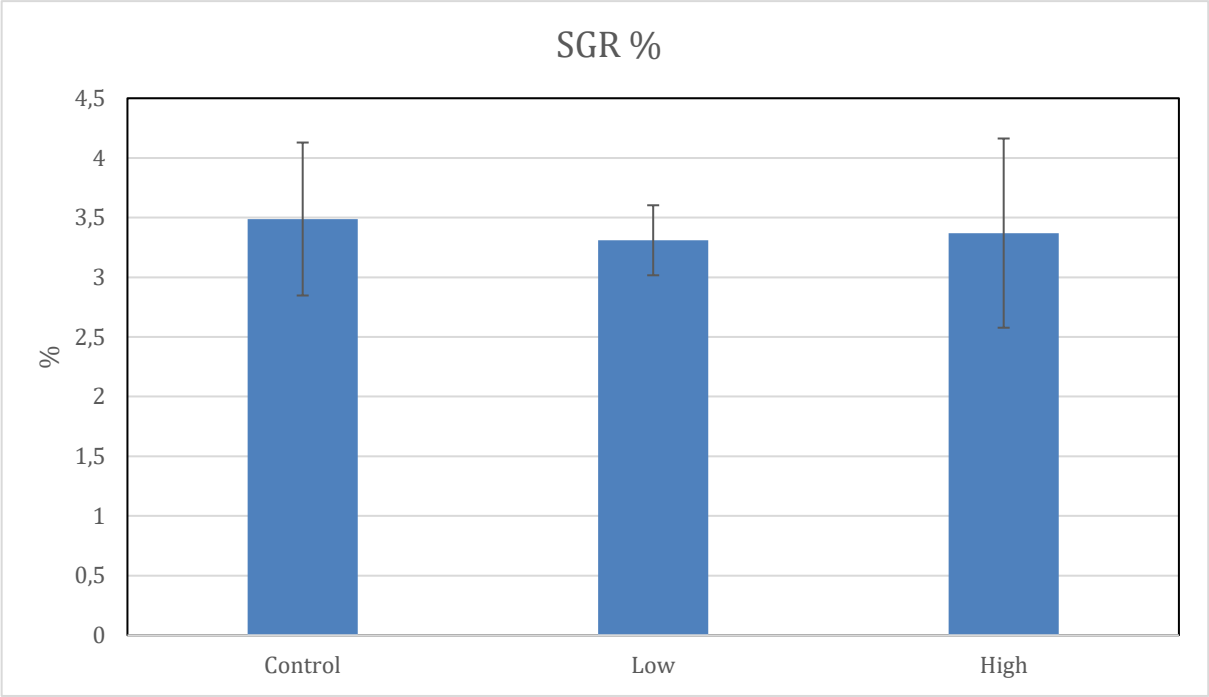


Figure 21 Specific growth rate (SGR %/d) during the trial. No significant difference among peracetic acid (PAA) treatments. Values are given in average ±SD, n=3

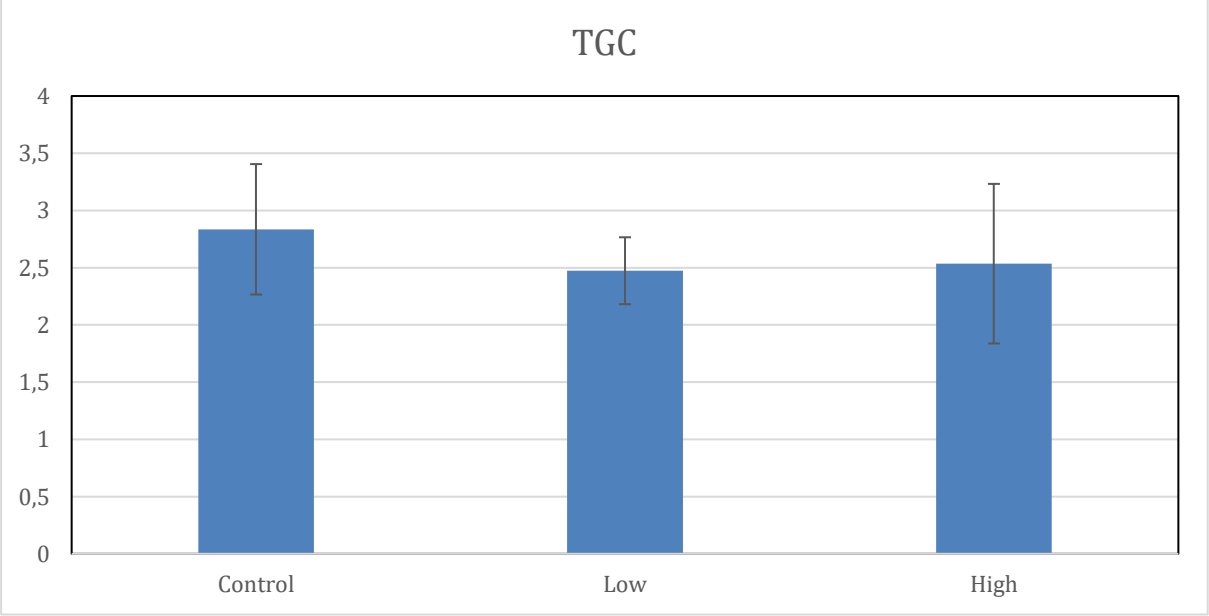


Figure 22 Thermal growth coefficient (TGC) during the trial. No significant difference among the peracetic acid (PAA) treatments. Values are given in average ±SD, n=3

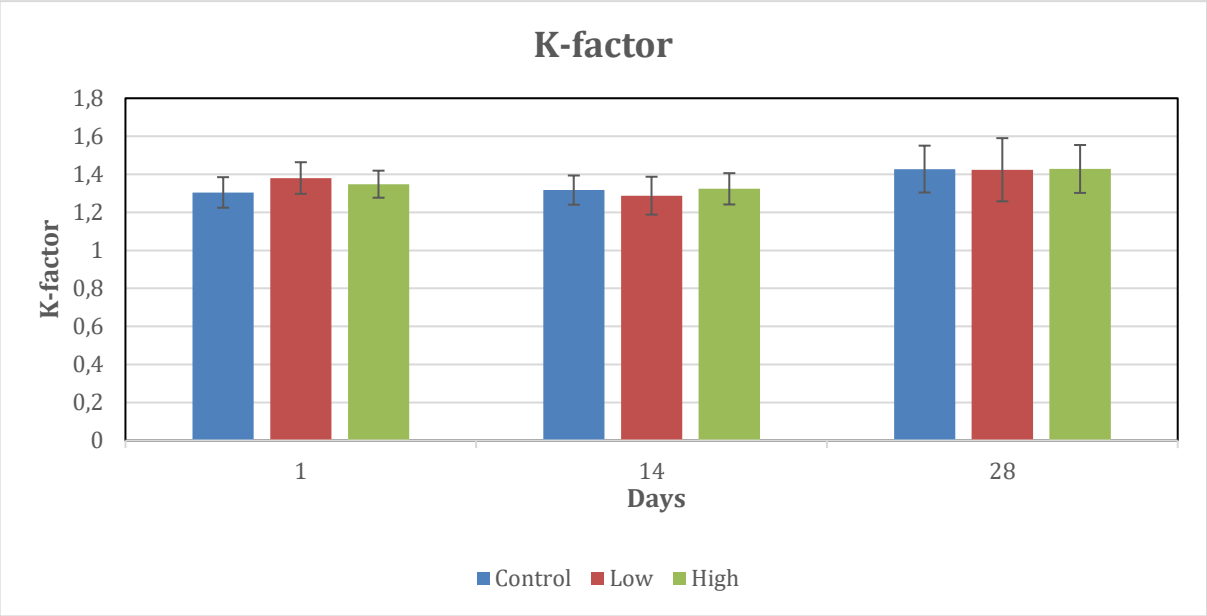


Figure 23 Condition factor for Atlantic salmon parr during 28 days with different peracetic acid (PAA) exposure. No significant difference between PAA treatments. Values are given as average ±SD, n=3

3.2.3 *Fish welfare*

External fish welfare of the first sampling showed no significant difference between control, low and high treatments, see Figure 24. In the last sampling, in the high treatment group, left pectoral fin was significantly higher than in the control group. Overall, long term exposure had no negative effect on external welfare status, with an average score below 1. Welfare indicators as lesion/wound, scale loss, eye haemorrhaging, snout damage, upper jaw deformity, and lower jaw deformity were not observed during the 4 weeks.

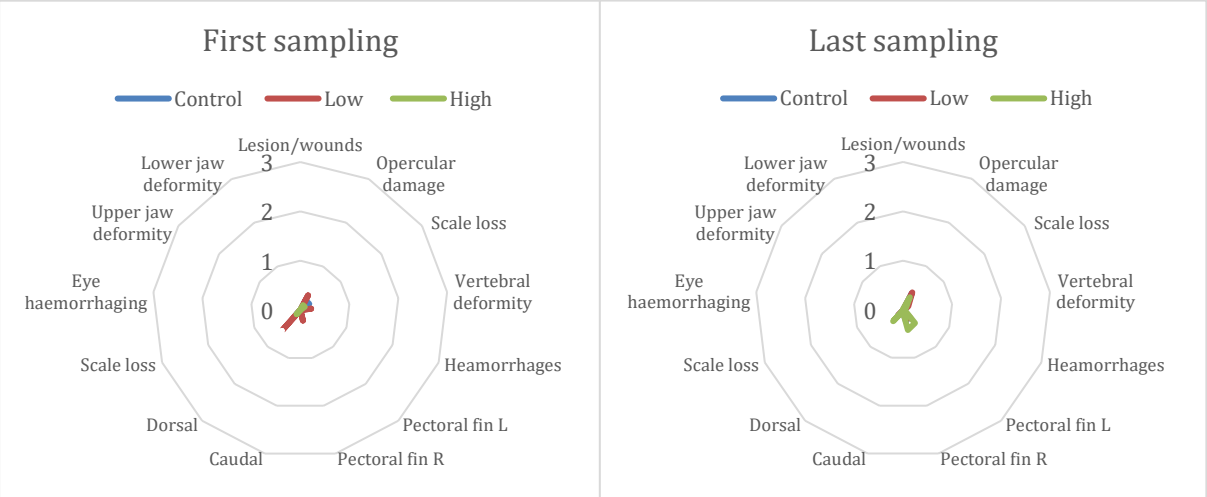


Figure 24 Average external welfare score from the first sampling and the last sampling, remained in good condition. All treatments groups have a score below 1.

The OWI (caudal fin, pelvic fin, dorsal fin, scale loss, skin health, vertebral deformation, and opercular damage) se Figure 25. At first sampling (before adding PAA), a significant difference between low and high treatment group was observed ($p<0.05$). At second sampling (day 14), no significant difference was observed, and at last sampling (day 28), high treatment was significant ($p<0.05$) lower than control and low treatment. However, all the scores were above 0.9 which indicated good welfare.

From first to last sampling point (28 days), the control group and high treatment group had a negative linear regression, $R^2= 0.564$ and $R^2=0.582$ respectively. The low treatment group had a strong positive linear regression, $R^2=0.906$ (see Appendix section 7.5.7).

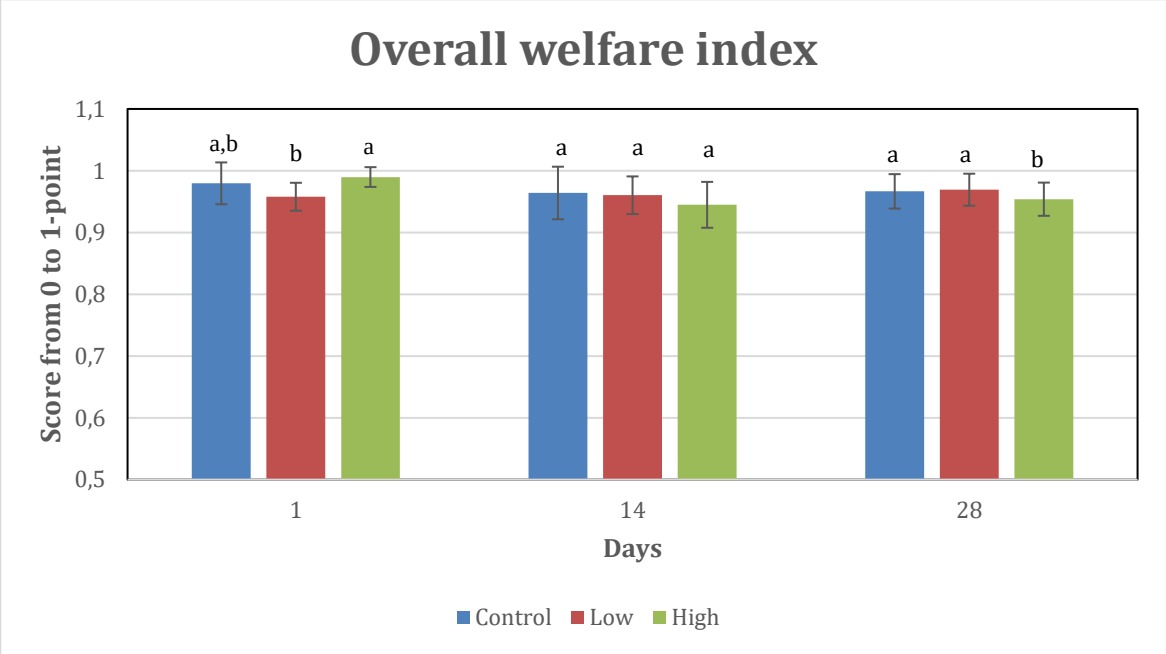


Figure 25 Overall welfare index for Atlantic salmon parr before, 14, and 28 days after PAA exposure. Overall welfare index scored by a 0- to-1 ranking, where 1 good welfare and 0 indicates severely compromised. Values are given as treatment average ± SD, n=3. Different letters indicate statistical differences (p<0.05).

3.2.4 Comet assay

Percentage DNA in the comet tail in combination with tail length are considered good indicators of nuclear DNA damage when dealing with low damage level in exposed cells (Liao, et al., 2009). In this work, 450 cells from each treatment (control, low and high) were measured resulting in a total of 1350 cells for each sampling point. Overall, the comets analysis revealed very low levels of DNA damage at all PAA concentrations. The results

(Table 8) showed value below 5% in terms of percentage DNA in tail, and comet tail lengths was below 1 µm during the trial. Interestingly, at day 14 the low treatment group showed a significant lower ($p<0.001$) value of percentage DNA in the tail than the control and the high treatment groups, but not differences in tail length.

Table 8 Overview of data on % DNA in the comet tail and the tail length from the three sampling points recorded during the trial. Values are given as average ±SD, n=3. Different letters indicate statistical differences (p<0.05).

	Control	Low	High	Day
%DNA in tail	4.29 ± 3.31	4.54± 3.27	4.54 ± 2.98	1
%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	1
Tail length (µm)	0.01 ± 0.110	0.00 ± 0,08	0.01 ± 0,10	14
Tail length (µm)	0.16± 0.50	0.12±0.50	0.19 ±0.54	28

4 Discussion

4.1 Trial 1

4.1.1 Water

In this study all RAS had same stocking density, feed input, and identical water before adding PAA. PAA application did not show any impact on oxygen content, nitrogenous compounds (NH_3-N, NH_4+-N, NO_3-N and NO_2-N), turbidity, and temperature. None of the nitrogenous compounds were measured in toxics levels (0.00 mg/L NH_3-N and 0.02 mg/L NO_2-N) (Fivelstad, et al., 1995) (Fossmark, et al., 2021). pH was affected by PAA application in the two highest treatment doses 3.2 and 6.4 mg/L pH decreased to 5.75 and 4.77, respectively.

To prevent the measured pH drop, the water should be buffered before and during the two highest PAA exposures. A good buffer will keep the pH more stable and to lower the pH more H^+ is needed (Summerfelt, et al., 2015). Mortality due to low pH is very unlikely, but if the experiment had lasted for several weeks low pH could have led to mortality (Farmer, et al., 1989).

The planned PAA doses and the measured PAA concentrations were not the same. The three first concentrations (control, 0.05, and 0.1 mg/L) were below LOD for PAA treatments. Treatments 0.2 and 0.4 mg/L were the same as originally planned but the rest of the treatments were higher than planned. In the calculation, we did not plan for the reduced tank volume by 81 L when the flow closed. This might have caused an increase in the PAA concentrations in the fish tanks. The analytical method was not able to detect the three lowest treatments (control, 0.05, and 0.1 mg/L). This might be due to the added PAA was not homogeneous mixed with the tank water when sampled, degradation of PAA, or the analysis were not performed precisely enough. PAA is reported to have half-lives from a few minutes (Pedersen, et al., 2013) and up to hour (Pedersen, et al., 2009). From previous experiments, it is expected that the method for measuring PAA is sensitive enough to measure concentration from 0 to 0.2 mg/L (Pedersen, et al., 2013). It might also had been a problem with the spectrophotometer.

4.1.1 PAA exposure affect behavioural responses

The swimming behaviour of fish in treatment 0 to 0.8 mg/L PAA were calm and most of them were standing close to the bottom or swimming calm around close to the bottom. A study by Liu and colleagues (Liu, et al., 2017 b) reported that rainbow trout exposed to 1 mg/L PAA triggers a stress response illustrated by an increase of active swimming, whereas a concentration of 0.2 mg/L did not provoke any response. Furthermore, Lazado and colleagues (Lazado, et al., 2020) reported no major behavioural changes under exposure to PAA at 0, 0.6, and 2.4 mg/L of Atlantic salmon smolts. In contrast to the study by Lazado and co-workers (2020), fish in treatments 1.6 mg/L had a more active swimming behaviour during PAA exposure. Treatment 1.6 mg/L PAA had a 40% higher score that the control group. In a study by Pedersen and colleagues (Pedersen, et al., 2009), rainbow trout were exposed to 2 and 3 mg/L PAA, the first two days they were standing close to the surface. These changes in behaviour are a response to unfavourable condition of PAA and that the fish try to escape (Lazado, et al., 2020). A possible explanation for the different results observed in Lazado and co-workers (2020) and the current study are different life stages and different PAA formulations. Different PAA formulations can led to different toxicities at the same PAA concentration, and it is shown that PAA solutions with a higher ratio of hydrogen peroxide to PAA are more toxic to *Daphnia magna* (Liu, et al., 2015). Furthermore, tolerance to PAA varies among fish species and life stages (Straus, et al., 2012) (Straus, et al., 2018).

Change in behaviour as erratic swimming, abnormal swimming behaviour, gasping for air and mortality was observed in the treatments 3.2 and 6.4 mg/L PAA. This indicates that PAA treatment were identified by the fish as danger and escape behaviour was observed. The two highest treatments were too high for Atlantic salmon parr and this was further supported by mortality in these treatments. In treatment 3.2 mg/L, 20% of the fish died during 1h and in treatment 6.4 mg/L, 100% of the fish were dead within 1h.

The fish was feed after first PAA exposure, and it was observed a 63% feed intake in treatment 1.6 mg/L compared to the to control group (100%). The linear regression between feed intake (g) and treatment group have a moderate effect (R^2=0.529), indicate reduced feed intake with increasing PAA regression. The linear regression would probably be higher if the two highest treatments (3.2 and 6.4 mg/L) were included. The lowest treatment (0.05 mg/L PAA) and control (no PAA) showed close to 40% higher feed intake compared to 1.6 mg/L. Study by Lazado and colleagues (Lazado, et al., 2020) reported no significant different of

feeding amongst Atlantic salmon smolt exposed to 0, 0.6 and 2.4 mg/L PAA. In the study by Liu and colleagues (Liu, et al., 2017 b) with rainbow trout exposed to PAA (0.2 and 1 mg/L), no uneaten pellets were observed by the daily inspections throughout the experiment. However, an experiment with rainbow trout reported reduced feeding activity (uneaten pellets) for treatment 2 and 3 mg/L PAA (Pedersen, et al., 2009). It is assumed that reduced feed intake for fish in treatment 1.6 mg/l PAA is due to PAA exposure.

4.1.2 Atlantic salmon parr welfare

External welfare scoring (0 to 3 where 0 as fully intact and 3 as severely compromised) is a systematic evaluation of morphological indicators in fish farms used as indication of welfare status (Noble, et al., 2018). Use of semi-quantitative scoring is a rough evaluation of the fish welfare, however, it can help the fish farmers to measure the impact of husbandry practices e.g., handling and treatment. In this study, we used this strategy to determine if PAA treatment influenced the external welfare of Atlantic salmon parr.

The overall external welfare score of the experimental fish, regardless of the treatments, remained in good condition. Three welfare parameters, dorsal fin, operculum, and skin were compromised during the experiment. Each welfare indicator had an average score around 1, this indicates that a minor level of operational welfare indicators was compromised. In fact, the operculum score should have been even lower since the fish had already short operculum before the trial started. We do not know the reason for the compromised operculum, but it was not linked to the experiment.

In a study by Lazado and colleagues (Lazado, et al., 2021), Atlantic salmon smolt were treated with 0.5, 2 mg/L and 4.8 mg/L PAA. The study shows same damages as in our trial, damaged skin, pectoral fin, and dorsal fin. The treatment groups had a welfare indicator score lower than 2. Skin damage was due to scale loss during transferring the fish from the holding tank to the exposure tank

Skin damage in treatment 6.4 mg/L PAA (highest concentration), was the only treatment with a significantly higher score than the other treatments. Furthermore, skin damage was the only welfare parameter that ended up with a visible negative impact and a strong negative regression compared to dorsal fin and operculum damage. A small skin injury can function as entrance for infection and can adversely affect the osmoregulation (Noble, et al., 2018).

However, absence of skin in treatment 6.4 mg/L had a direct effect on the nociception. Fish have free nerve cells throughout the skin surface and in treatment 6.4 mg/L PAA this caused a welfare concern (Noble, et al., 2012). The absence of skin in treatment 6.4 mg/L affects the physical welfare needs of Atlantic salmon parr.

The absence of skin in treatment 6.4 mg/L may have occurred during addition of PAA to the fish tank. PAA can have touched the skin before PAA was homogeneous mixed with the total tank water. To prevent this to occur we should dilute the amount with water before adding PAA to the tank.

The OWI is a scoring system ranging from 0 to 1. The general score from treatment 0 to 0.8 mg/L PAA indicates that the fish has no negative reactions to PAA treatment. However, treatment 1.6 mg/L and higher PAA doses showed a negative impact on Atlantic salmon parr. It was a strong negative regression (R^2=0.882) between treatments and OWI that support a higher PAA concentration have a negative impact on the fish welfare. This result indicates that Atlantic salmon parr has an unhealthy reaction to PAA treatments over 0.8 mg/L.

4.1.3 Impact of PAA on gill and skin

Histology is the study of the tissue's natural structure and composition used to prove morphological and pathological changes in the tissues due to for instance diseases, injury, infection, pollutants, and unfavourable water condition. Histological examinations of gills and skin are one of the tools to detect disease or damage (Flores-Lopes & Thomaz, 2011) (Bernet, et al., 2001) (Kryvi & Poppe, 2016).

Gills and skin have a large surface and are in direct contact with the environment and are the most sensitive organs (Bernet, et al., 2001). Gills are a multipurpose organ providing gas exchange, osmotic and ionic regulation, acid-base regulation, and excretion of nitrogenous waste. Blood flow through the lamella in a counter current direction of the water flow (Evans, et al., 2005).

Gills are a sensitive organ, that are easily damaged by low concentration of different kinds of pollutions (Flores-Lopes & Thomaz, 2011). During unfavourable environmental conditions, they are the first to react to structural alterations (Bernet, et al., 2001) (Raskovic, et al., 2010).

The gill epithelium is a thin and delicate layer that's directly exposed to the environment, and the structure and function of the gills can be altered in response of irritants (Dang, et al., 2020). A semi-quantification of common histopathological characteristics of the gills from treatment 0 to 1.6 mg/L, showed more than 87 % of the evaluated lamellae have a good gill health status. Lifting and hyperplasia were the most prevalent observation in treatment 0 to 1.6 mg/L. The gill alterations vary from minor (lifting and hyperplasia of the epithelium) to serious (necrosis of gill epithelium in treatment 3.2 and 6.4 mg/L).

Lifting, hypertrophy, and hyperplasia reduce the gill surface and the efficiency to take up O_2 and remove waste products (Poppe, 2002) (Strzyżewska-Worotyńska, et al., 2017) (Haddeland, et al., 2021). Lifting is a protective mechanism against the danger of unfavourable aquatic environment. Epithelial lifting is one of the initial branchial reactions to a variety of pollutants as zinc, cadmium, and acid (Smart, 1976). Such a response to stressful conditions and the presence of contamination would result in an increased diffusion distance between water and blood, causes rise to circulatory alterations (Kostić, et al., 2017). The histopathological changes due to PAA treatment 0 to 1.6 mg/L in the present study, do not pose significant gill health of concerns.

Several studies have demonstrated that lifting is gill alterations response to PAA exposure (Haddeland, et al., 2021) (Liu, et al., 2020). In a study from Haddeland and colleagues (Haddeland, et al., 2021), Atlantic salmon exposed to PAA (0, 0.6, 1.2, and 2.4 mg/L), more that 90% of histopathological observations were healthy secondar lamella. Clubbing and lifting were the most prevalent negative observations (0.5–3%). All gills were histologically categorised as functionally normal by the end of the trial period. Liu et al., (Liu, et al., 2020) reported that rainbow trout treated with 1 mg/L PAA found that "minima" hyperplasia was present more frequently, and this was significantly different from the control group.

Morphological change (cell death) and change in gill colour (turned grey/pink) in gill histology from treatments 3.2 and 6.4 mg/L PAA were observed. The strong impact of PAA attributed destroy the gill structure and resulted in cell death. When cells are dead and the structures destroyed, the gills no longer able to function as a respiratory and osmotic regulation (Evans, et al., 2005) and lead to mortality.

Skin has an important role in osmotic balance and sensory reception (Cabillon & Lazado, 2019). The skin of salmonids is composed of a multi-layered epidermis with 10-15 layers of

cells on head and 6-12 cells ventral, dorsal, and lateral region (Harris & Hunt, 1975), with living cells underlay by a collagenous dermis (O'Byrne-Ring, et al., 2003). Structure of fish scales, consisting of small rigid plates growing out of the skin of a majority of fish species, is characterized by a large variety of shape, size, and properties in order to achieve particular functions (Vernerey & Barthelat, 2010). During smoltification, studies indicate variation in the numbers of epidermal mucous cells during this period (Lazado, et al., 2021).

Histological structural evaluation of the skin showed good regression between treatment and epithelia surface quality scoring. The two highest PAA doses (3.2 and 6.4 mg/L PAA) had a negative effect on the epithelia surface. The skin sample showed that the epidemies were compromised in the two highest treatment groups with rough epithelia surface and parts of epidermis missing. When parts of the epidermis are missing, and the surface are uneven, and the first-line defence line of disease resistance against pathogens and toxic substances are reduced (Cabillon & Lazado, 2019). The histopathological changes from PAA treatment 3.2 to 6.4 mg/L in this present study is a health concern to the Atlantic salmon parr.

Histological data has shown that gills are more sensitive and are more easily damaged that epidermis (Flores-Lopes & Thomaz, 2011). Gills are the first to react to unfavourable environmental condition by structural alterations as lifting, necrosis, hyperplasia, clubbing, and fusion (Bernet, et al., 2001) (Raskovic, et al., 2010) (Haddeland, et al., 2021). Fish skin is far more complex with several layers of interconnected physical barriers (i.e., scales, epidermis, dermis) than the gills (Vernerey & Barthelat, 2010) (O'Byrne-Ring, et al., 2003).

Several studies have demonstrated that mucosal surfaces respond rapidly to environmental changes (Cabillon & Lazado, 2019) (Dang, et al., 2020). Mucosal surface is interfering between the fish and the surrounding aquatic environment (Cabillon & Lazado, 2019). The skin plays an importin role as the first line of defence of agents. Epidermis is highly dynamic tissue with a number of mucus cell located in the epidermis. These cells are responsible for production of the mucus covering the fish body (Dang, et al., 2020) (Vatsos, et al., 2010) (Harris & Hunt, 1975) (Liu, et al., 2020). The mucosal immune system is important in the ability of the fish to respond and adapt to changes, because of the direct connection with the environment. Mucosal surfaces respond exceptionally to environmental change e.g., increase in mucus cell size and numbers (Cabillon & Lazado, 2019). Skin mucous cells play an important role in disease resistance against pathogens and toxic substances (Bernet, et al.,

2001). Husbandry manipulations have been demonstrated to influence their numbers of mucosal surface (Lazado, et al., 2021).

Fish exposed to 3.2 and 6.4 mg/L PAA were covered in thick mucus compared to the rest of the treatment groups. Mucus secretion can be considered as a nonspecific response of fish, that helps the fish to swim to favourable areas and mucous cells could be used as an indicator of exposure to a stressor (Vatsos, et al., 2010) (Liu, et al., 2020). Acidic cells production increased from treatment 0.8 mg/L to 1.6 mg/L as a response to PAA treatment. Treatment 3.2 and 6.4 mg/L showed a reduction in acidic cells. This can be explained by parts of the epidermis were missing, the surface was structurally compromised, and there was no surface to secret acidic cells to protect epidermis against environmental contaminants.

However, during sampling, fish sampled from treatments 3.2 and 6.4 mg/L were covered with mucus on the skin. The increasing mucus production was a response to the unfavourable water condition and tried to protect the fish from PAA. Mucous cells are a ubiquitous element of the mucosal surface and the main producer of mucus (Esteban, 2012). However, it is yet to be established whether this static population also results in stable exudation of mucus to cover the mucosa, thereby, maintaining a biophysical barrier (Lazado, et al., 2021). Nonetheless, this mucosa layer on the fish tried to protect the fish from unfavourable water conditions.

A study from Liu (Liu, et al., 2020) showed significant reduction of epidermal mucous cell density after continuous exposure to 0.2 mg/L PAA, but no difference between the control group and rainbow trout treated with 1mg/L PAA biweekly (Liu, et al., 2020).

4.2 Trial 2

4.2.1 Water

Optimal water quality is crucial for good performance and welfare (Gorle, et al., 2018). No negative impact of PAA application was observed on the nitrogenous compound's concentrations (NH_3-N, NH_4^+-N, NO_3-N, and NO_2-N), O_2, pH, and temperature. In the present study, the RAS were given same treatment, the same flushing time, stocking density, and feed input. The first nitrification step transferring NH_4^-/NH_3 to NO_2 became a bottleneck, because the biofilter was not optimally maturated before trial 2 started. To prevent the water from becoming toxic we started to flush the system to bring new water into the system.

NH_3 can be toxic in too high concentration and cause negative effects on central nervous system, osmoregulatory performance, reduce feeding rate, reduced growth, reduced swimming activity, gill damage and histological lesion in various internal organs. It can also increase ventilation rate, and lead to a loss of equilibrium and also lead to death (Noble, et al., 2018) (Kolarevic, et al., 2013) (Thorarensen & P.Farrell, 2011). The recommended maximum safety level for NH_3 in salmonid aquaculture to support growth and welfare is 0.012 mg/L NH_3-N (Fivelstad, et al., 1995). In this study the NH_3 was above 0.012 mg/L (varied from 0.0 to 0.1 NH_3-N), however, the result shows no signs that the fish has been negatively affected by the high concentration. A study by Kolarevic (Kolarevic, et al., 2012) on Atlantic salmon *Salmo salar* parr, suggest that growth and welfare are not negative affected by long term exposure to sublethal ammonia levels up to 0.035 mg/L NH_3-N. None of the analyses showed a negative impact on the fish due to high NH_3-N. There were no signs of reduced growth, behaviour change, damage on fish (external welfare and overall welfare index) and the gills were healthy. It might have been impact on the fish physiology which was not measurable in the methods and tests included in this study.

The increasing NO_2-N levels during the trial had no signs of impacting the fish welfare, growth, behaviour, or gill health. High NO_2^- concentration result in negative effects on gills, thymus, and reduced swimming performance (Thorarensen & P.Farrell, 2011). The recommended maximum safe level 0.1 mg/L NO_2^- (Thorarensen & P.Farrell, 2011).

Despite of daily PAA input to the system, low (9.6 ml daily, 0.001% of total water volume) and high concentrations (97.2 ml daily, 0.01%of total water volume) of PAA did not cause PAA accumulation, and the measured values were below the detection limit. Furthermore, PAA was added to the sump tank, and 2/3 of PAA may have decreased before the water reach the fish in the fish tanks. The water exchange rate (retention time) in the sump tanks and in the fish tanks were 8 and 21 min, respectively (Eq 6). PAA half-life is related to stock density, temperature, organic matter, existing biofilm on the surface COD (chemical oxygen demand), and biofilter surface, and the half-life is reported to vary between hours to few minutes (Pedersen, et al., 2013) (Pedersen, et al., 2009). The PAA decay increase with increasing organic matter in the system. From earlier experiments, it is expected that the method for measuring PAA is sensitive enough to measure concentration at 0 to 0.2 mg/L (Pedersen, et al., 2013). It could be as the organic matter increased, we underdoses the system instead of compensating for the decay of PAA. The PAA concentration depend on the PAA product concentration of PAA, applied dose, stability of the product and PAA consumption

and decay in the RAS (Pedersen, et al., 2013). However, even if we assume that PAA decayed before entering the fish tank, a difference between the treatment groups in number of bacteria, the water turbidity and bacteria growth were measured, and biofilm on the tank wall was observed. This indicates that the PAA treatment influenced the water even it was not recorded via PAA measurements.

The bacteria count from agar plates (TSA+) showed a significant increase in heterotrophic bacteria growth when adding PAA, and it was also a significant difference between the control group and the high treatment group. Heterotrophic bacteria in TSA+ is unknown and TSA+ support the growth of a wide range of bacteria (Balestra & Misaghi, 1997). Furthermore, there was no *Vibrio* species growing in the system because no growth on the TCBC ager was observed (Tagliavia, et al., 2019). In previous studies, major disadvantages with use of PAA as a disinfectant for wastewater has been reported, e.g., increased organic content because of acetic acid component that potential might lead to microbial regrowth (Kitis, 2004). A study by Liu (Liu, et al., 2017 b), reported that continued application (0.2 mg/L PAA) promoted biofilm formation in a flow-through tanks used for rainbow trout, however, a pulse treatment (1 mg/L) restricted the biofilm formation. In the present study, the control group and the low PAA treatment (0.1 mg/L) showed biofilm formation in the fish tank, while in the highest PAA treatment (1 mg/L) no biofilm formation was detected during the trial. Since the types of bacteria in the water column and on the tank wall were not identified, the bacteria on the tank wall could be different from those in the water column. A high dose of PAA resulted in increased bacteria load in the water column, however, no biofilm on the tank wall was observed. A study by Davidson and co-workers (Davidson, et al., 2019) found that PAA (0.05-0.3 mg/L) increased TSS levels and did not reduced general heterotrophic bacteria and total coliform counts.

Acetic acid is compounds and decomposition product of PAA. Acetic acid is a major disadvantage because it increases organic content and potential microbial regrowth. (Kitis, 2004). Turbidity refers to the clarity of the water and is caused by suspended particles in the water, and the particles may be organic or inorganic (Parra, et al., 2018). It is likely that the increased bacteria in the water column and, increases turbidity is due to acetic acid (Davidson, et al., 2019).

4.2.2 Welfare

External welfare scoring (0 to 3) is a systematic evaluation of morphological indicators according to a ranking system. As in trial 1, operculum was compromised before the trial 2 started, and the score should have been even lower. The external welfare status of the parr remained in an overall good state, with an average score below 1 in all welfare indicators, for all three samplings. This indicates that the PAA treatment did not affect the external welfare status of the fish. It was not reported any abnormal behaviour during the trial (information provided from the staff working at Havbruksstasjonen).

The OWI (caudal fin, pelvic fin, dorsal fin, scale loss, skin health, vertebral deformation, and opercular damage) has a scoring system from 0 to1. The results showed that the overall welfare score during 4 weeks of exposure were above 0.9 for all treatments group, showing that PAA exposure did not compromise the fish welfare. The control and high treatment had a weak negative regression showing the score decreased during the 4 weeks. For the low concentration group the score increased during the trial and the regression was strong. The PAA did not have negative effect on Atlantic salmon parr.

4.2.3 Comet assay

Comet assay (single-cell gel electrophoresis) is a rapid, sensitive, cost-effective, and quantitative method for visual and measuring deoxyribonucleic acid (DNA) standard breaks in eukaryotic cells. Use of electrophoresis, negatively charged DNA is drawn towards the anode. If the negatively charged DNA contained breaks, the broken ends will migrate toward the anode and result in structures that are resembling comets (see appendix section comet assay) (Collins, 2004) (Olive, 2002) (Liao, et al., 2009). In this study DNA was undamaged, and there were no free ends, and no migration was taking place (Collins, 2004). Comet assay is not able to tell what the cause of the DNA brakes (Belpaeme, et al., 1998).

DNA cells in the gills were not damaged by benzocaine, TAN, or PAA treatments. Similarly, % DNA in the tail was almost identically from the beginning to the end of the trial. Over 95% of the DNA in all studied cells were intact. Compared to the CometAssay® Control Cells (see appendix section comet assay), the % DNA in tail in the gills were not compromised in this trial. These results demonstrate that euthanized with an overdose of benzocaine does not destroy the DNA in the gills of Atlantic salmon. The use of an overdose of benzocaine during

sampling avoids unnecessary suffering and stress. PAA treatment, NH_3, and NH_4^+ did not have a negative impact on the gill's DNA.

The first description of this method was published in 1984. Over time this method has been improved, however, yet not completely standardized (Liao, et al., 2009). Comet assay has a great potential to estimate the DNA status in fish. It has been used for freshwater fish such as carp (*Cyprinus carpio*), brown trout, zebra danio (*Brachydanio rerio*), rainbow trout, marine flounder (*Pleuronectes americanus*)) (Belpaeme, et al., 1998), turbot (*Scophthalmus maximus*), and gilthead seabream (*Sparus aurata*) (Cabrita, et al., 2010).

The comet assay has been used to study samples of blood, liver, kidney, gills (Belpaeme, et al., 1998) and sperm (Cabrita, et al., 2010). The assay can be used in all types of isolating cells and can be used to monitor genotoxic effects on aquatic species e.g., blue mussel (Rank & Jensen, 2003).

4.2.4 Fish performance

Atlantic salmon parr weight curves from PAA-treated and control overlapped almost identically throughout the trial. Therefore, growth was not affected by continuous PAA dosing. Fish performance and welfare was not affected by the not fully mature biofilter due to our method and observations. The weight of the fish in the end of the trial was doubled from the start. Davidson and colleagues (Davidson, et al., 2019) reported no different in growth in a study with rainbow trout in RAS with continuous PAA treatment (0.05 and 0.30 mg/L). Moreover, another study with rainbow trout exposed to continuous (0.2 mg/L) and pulse (1 mg/L) PAA, reported similar growth (Liu, et al., 2017 b). Lazado (Lazado, et al., 2020) reported no significant difference in weight between treatment groups exposed to PAA (0, 0.6, and 2.4 mg/L) of Atlantic salmon.

In the present study, no significant difference was seen in the K-factor, TGS, and SGR (%/d). This indicates continuous application was too low to trigger the threshold for the Atlantic salmon parr to react on PAA concentration.

5 Conclusions

On the conditions tested in the present study, PAA exposure equal or below 1 mg/L seems to be safe for Atlantic salmon parr welfare and it can be considered a potential disinfectant in Atlantic salmon production.

In our short-term study, Atlantic salmon parr seem to have a tolerance up to 0.8 mg/L (measured to be 1.1 mg/L) PAA. A 0.8 mg/L PAA treatment does not affect parr health, welfare, swimming behaviour or skin and gill histology. Conversely, higher treatments had a negative impact on the fish welfare such as increased swimming behaviour, or mortality, and reduced feeding behaviour or score in overall welfare index, and with histology showing necrotic gills filaments, lack of epidermis and rough skin surface.

Long term, i.e., 4 weeks, PAA treatment did not have any effect on Atlantic salmon parr health, welfare, growth, or gill health. High treatment dosage had higher CFU/ml in the tank and the sump tank compared to control and low PAA treatments. Furthermore, high PAA treatment prevents biofilm formation in the fish tank.

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7 Appendix

7.1 PAA measurement

2,5 ml water sample was pipetted into a cuvette, and 250 µl of reagent 1 and 250 µl reagent 2 were added and mixed. Color intensity of regent 1 and reagent 2 was measured in a spectrophotometer at λ = 550 nm, 30 second after adding reagent 2. Using the Y value from the standard curve to calculate the concentration of PAA based on the absorption value from spectrophotometer absorption on 550nm (Figure 24).

Regent 1 N,N-diethyl-p-phenylenediamine sulfate salt; 250 ml of milli-Q-water to an erlenmeyer flask, transferring 0.1274 g of Ethylenediaminotetraacetic acid Dihydrate (EDTA * $2H_2O$, E1), 1 ml of H_2SO_4 96%, 8 g of N, N-diethyl-p-phenylendiamine sulphate salt (DPD salt, D25) to the same erlenmeyer flask. Added Milli-Q water to the 500 ml line. Cover the flask with aluminum foil.

Regent 2 DPD solution; Transfer 22. 64g Na2HPO4*7 H2O (D25), 23 g KH_2PO_4 (K4), and 0.5 g KI (K9) to an erlenmeyer flask. Add 400 ml Milli-Q water. Transfer the erlenmeyer flask to a magnetic stirrer and adjust the pH to 6.5 with N_2OH. Transfer the solution to a volumetric flask and fill up to make 500 ml with Milli-Q water. Cover the flask with aluminum foil so it is stable for 3 months.

Standard curve: The absorbance values were used to calculate “exact” PAA concentration based on a standard curve. The standard curve was made by a 1000 mg/L PAA stock solution, calculated by Eq 12. 1.5 ml Aqua Des was pipetted into a 100 ml volumetric flask, then add Milli-Q water up to the line and mix the water.

$$Eq\ 12. \qquad C1V1 \cdot C2V2$$

Use a 25 ml pipette to transfer 50 ml tank water without PAA to six falcon tube. Calculate the amount of stock solution, with Eq 12. With a micropipette transfer same amount of water that's going to be added with the stock solution, out from Falcone tube. Add calculated concentration of stock solution to the falcon tube, mix the water, see Table 9. Measure the different concentrations by transfer 2.5 ml stock solution with a micropipette into a cuvette, and 250 µl of reagent 1 and 250 µl reagent 2 were added and mixed. The color intensity of regent 1 and reagent 2 was measured in a spectrophotometer at λ = 550, 30 seconds after adding reagent 2, see Figure 26.

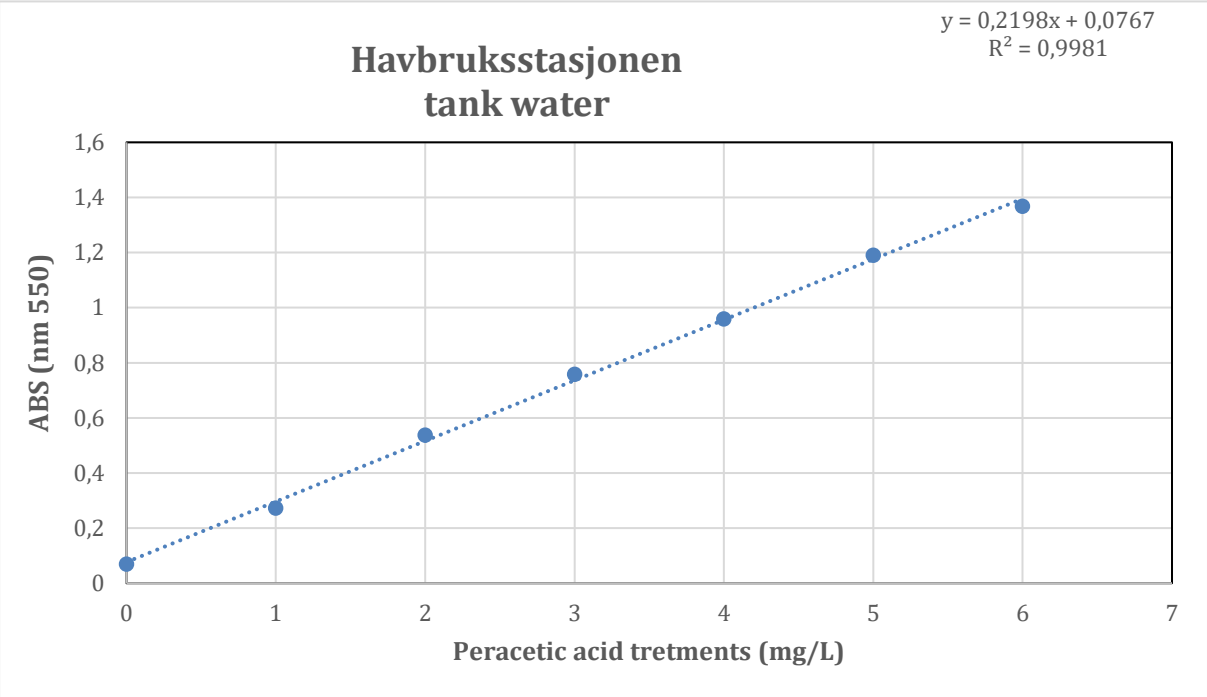


Figure 26 Standard curve for PAA measured in a spectrophotometer at λ = 550

Table 9 The different PAA concentrations used to make the standard curve. How much water is pipetted out and the amount of standard solution that is added

PAA concentration (mg/L)	Falcone tube (ml)	Water removed from Falcone tube (µl)	Stock solution added to Falcone tube (µl)
0 (water+ R1+R2)	50	0	0
1	50	50	50
2	50	100	100
3	50	150	150
4	50	200	200
5	50	250	250
6	50	300	300

7.2 Bacteria counts

Water samples from the fish tank (1-9) and water samples from the sump tank (1-9) were taken last day of the experiment. Made a dilution series from the water sample on 10^{-1},10^{-2}, 10^{-3}, and 10^{-4} for tank water and water from the sump. Dilution series were made by, transferred 900 µl distilled water to 70 Eppendorf tube, see Table 10. To dilute to 10^{-1}, transfer 100 µl water sample from RAS to Eppendorf tube, place the Eppendorf tube on a vortex/mixer. Next dilution 10^{-2}, use a pipette transfer 100 µl water sample from 10^{-1} to the next Eppendorf and mix the water and repeat it till 10^{-4}.

Table 10 Dilution series from tank water and sump tank water.

Concentration	Dilution
10^0	900 µl water sample
10^{-1}	900 µl dH_2O+ 100 µl 10^0 water sample
10^{-2}	900 µl dH_2O+ 100 µl 10^{-1} water sample
10^{-3}	900 µl dH_2O+ 100 µl 10^{-2} water sample
10^{-4}	900 µl dH_2O+ 100 µl 10^{-3} water sample

Used duplicates of TSBS and TSA+ (Tryptone Soy Agar + 1% NaCl), with the concentration 10, 10^{-1},10^{-2}, 10^{-3}, and 10^{-4}. Adding 100 µl water sample (10^0) on the agar, spread the water sample around to it dry up, the same procedure for the rest of the solution. In total 90 TCBS and 90 TCA agar. Agar plate was stored at 1-week 12°C wrapped in plastic before it was stored 1 week at 4°C before counting bacteria colony.

7.3 Overall welfare index

Sematic model prosses, the 1st step is to find the basic needs as physical needs (respiration, osmotic balance, nutrition, health, thermal regulation) and behavioural needs (behaviour control, feeding, safety, social contact, exploration, kinesis, rest, sexual behaviour, body care) that was done in SWIM 1.0 model (Stien, et al., 2013). The 2nd step to collect a list of scientific statements. The literature must obtain relevant information about good welfare and bad fish welfare. 3rd step is to make a list of measurable or observable welfare indicators (WIs) from the literature for fish welfare indicators and fish welfare (Pettersen, et al., 2014). Divided the WIs (e.g., appetite, scale loss, skin and, condition) from best to worst fish welfare and described exactly the level from the best to the worst fish welfare. Based on the ranking levels, each level is assigned an indicating score (IS). IS is calculated as (Stien, et al., 2013):

$$Eq\ 13. \qquad = \frac{NL-RL}{NL-1}$$

NL is the total number of levels of indictors, RL is the rank number of levels.

To assign weighting score (WS) we are using weighting categories (WC) (illness, pain, reduced survival, abnormal behaviour, negative performance, positive performance and, demand) from SWIM 0.1 and SWIM 0.2, see table 11. WSs is based on scientific statement of expert's judgement (Stien, et al., 2013) (Pettersen, et al., 2014).

Table 9 To be able to calculate a weighting scores (WS) we are using information about weighting categories (WC). Tables is made bay (Stien, et al., 2013) (Pettersen, et al., 2014) based on scientific statements.

WC	Brief description	Range of WS
Illness	Evidence of health problems, including increased mortality, but excluding skin lesions, fin damage and abnormalities in body shape (see 'pain')	-5 to -1
Pain	Evidence of unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage	-5 to -1
Reduced survival	Evidence of reduced survival related to physiological requirements (other than through specific health problems), for example, longevity, deprivation of food, poor environment	-5 to -1
Abnormal behaviour	Evidence of disturbed behaviour and/or apathy	-3 to -1
Negative performance	Evidence of decreased performance (that is likely to indicate negative affect), including (re)production effects, but excluding specific survival aspects related to physiological necessities, HPI activation and illness	-3 to -1
Positive performance	Evidence of healthy, fit fish, which are growing well	1–3
Demand	Evidence that the fish are willing to spend effort to obtain food or other recourses.	1-5

Weighting factor (WF) is calculated by use of literature about each welfare indicator (see below) and Eq 14 (Stien, et al., 2013) (Pettersen, et al., 2014):

$$Eq\ 14. \quad \left(\sum_{WC} max\ (\text{WSwcl})\right) \text{IL best i} - \left(\sum_{WC} max\ (\text{WSwcl})\right) \text{IL woeste i}$$

$IL_{\text{best i}}$ is the best indicator level and $IL_{\text{woest, i}}$ is the worst indicator level of welfare indicator level of the welfare indicator. WC_{wcl} is the weighting score assigned to the indicator level based on the scientific statements, *wc* identifies the weighting categories liked to the indicator level (Stien, et al., 2013) (Pettersen, et al., 2014).

Literature about each welfare indicator is used to calculate WF:

Mortality rate: high mortality indicative of illness (-5), reduce survival (-5), pain (-5) and negative performers (-3), low mortality indicates positive performers (3). The WF is calculated to 21(Eq 14, Table 11) (Stien, et al., 2013).

Appetite: poor appetite is an indicator of negative performance (-2) and illness (-3), and good appetite suggest demand (3) and positive performance (3), calculated WF of 11 (Eq 14, Table 11) (Stien, et al., 2013)

Fin condition: fin damage represent injury to living tissue and potential for inflammation and pain (-5), damage on epithelial structure can case invasion of pathogen and lead to illness (-3) and negative performance (-2). Healthy fins have a positive performance (3), WF is calculated to 13 (Eq 14, Table 11) (Stien, et al., 2013)

Skin condition: skin damage can cause pain (-5), route for pathogens that can lead to infection and illness (-3) and can reduce survival (-3). Small skin damage can lead to negative performance (-3), and normal healthy skin leads to positive performance (1). WF is calculated to 15 (Eq 14, Table 11) (Stien, et al., 2013).

Scale loss: scale loss can lead to route for pathogens that can lead to infection and illness (-3), can reduce survival (-3), pain (-2), and negative performance (-3). Normal healthy skin has a positive performance (1). WF is calculated to 12 (Eq 14, Table 11) (Noble, et al., 2018)

Vertebral deformation: no singe to vertebral deformation led to positive performance (1), and extreme deformation indicate level 3 indicates negative performance (−3), pain (−3) and illness (−3) this gives a WF of 10 (Eq 14, Table 11) (Stien, et al., 2013).

Opercula: shortened opercula are related to abnormal behaviour (-2), reduced survival (-2) and illness (-2), intact has a positive performance (1). WF is calculated to 7 (Eq 14, Table 11) (Pettersen, et al., 2014).

Behaviour: abnormal behaviour and gasping for is associated with bad fish welfare, illness (-5), reduce survival (-4), pain (-5) and negative performance (-3), with a normal swimming behaviour indicate a positive performance (3). WF is calculated to a score of 20 (Eq 14, Table 11) (Noble, et al., 2018)

The last step is to calculate relative weighting factors (RWFs, Eq 15), indicator welfare score (IWSs, Eq 16) and overall welfare index (OWI, Eq 17) is calculated as:

$$Eq\ 15. \quad WF_i \cdot \left(\sum_{j=i}^{m} WF_j\right)^{-1}$$

$$Eq\ 16. \quad IS_i \cdot RWF_i$$

$$Eq\ 17. \quad \sum_{j=i}^{m} IWS_j$$

m is the total number of indicators in the model. WF_i and WF_j are the weighting factors of the respective indicator *i* and *j*, and IS_i is the indicator score given by the assessor (fish tank) for indicator *i*. Overall welfare index have a scale from 0-1 fully intact and good fish welfare and 3 as severe damage and bad fish welfare.

7.4 Comet assay

Alkaline COMET assay's experimental conditions were verified by use of commercially available reference cells (CometAssay® Control Cells, Trevigen Inc., Gaithersburg, MD20877, USA) showing no DNA damage (negative control) and different levels of nuclear damage (low, medium, and high) following etoposide treatment. The results are shown in Figure 27 and Table 12, the results are presented as % DNA in tail (average ± Standard Error, SE).

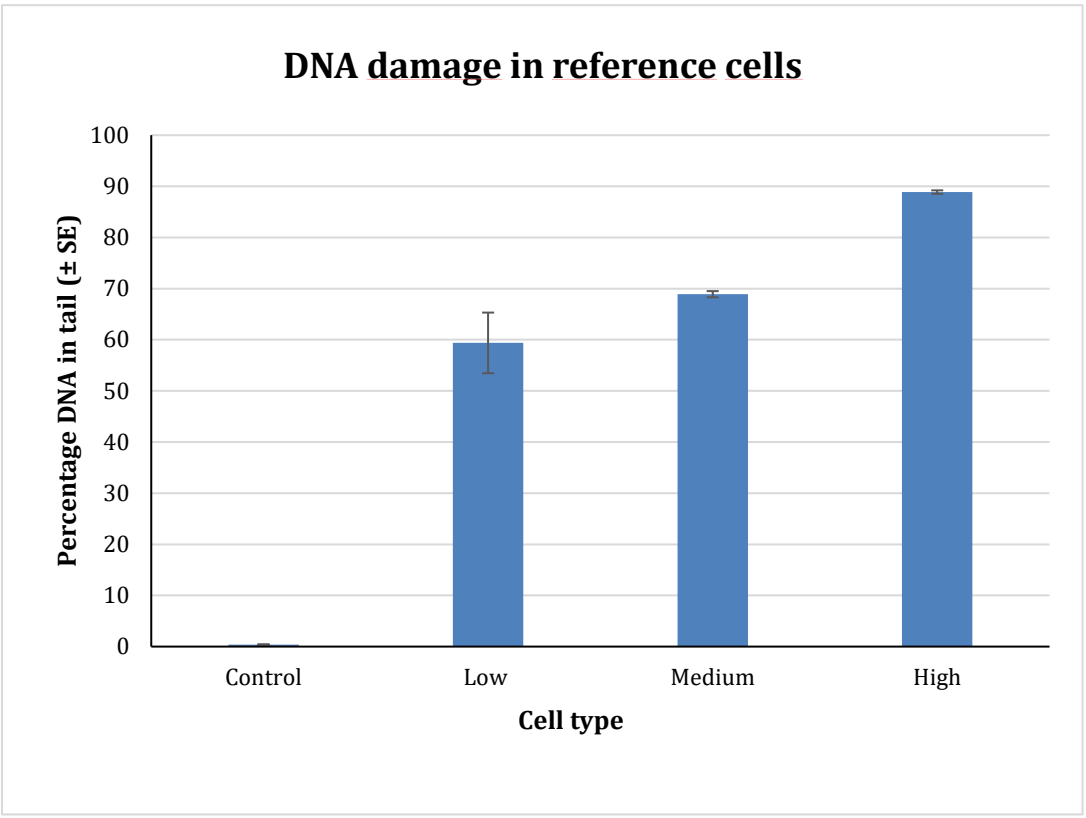
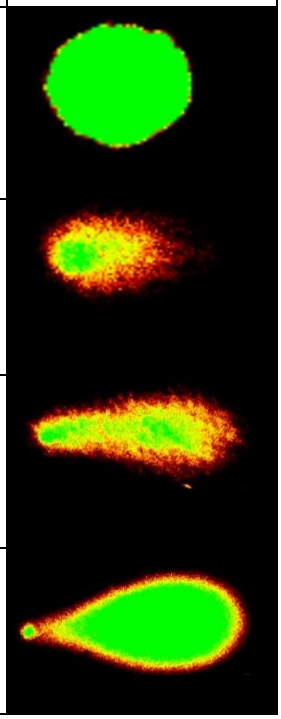


Figure 27 Show different levels of % DNA in tail with control (no nuclear damage), low, medium, and high nuclear damage form control cells. The figure is presented as average ± SE.

Table 12 Show the as average ± SE of % DNA in tail and pictures from fluorescence microscopy from Comet Assay control cells. control (no nuclear damage), low, medium, and high nuclear damage.

	% DNA in Tail (average)	Standard Error (SE)	Pictures of DNA
Control	0.39	0.06	
Low	59.38	5.93	
Medium	68.90	0.61	
High	88.87	0.34	

7.5 Figures

7.5.1 Swimming behaviour

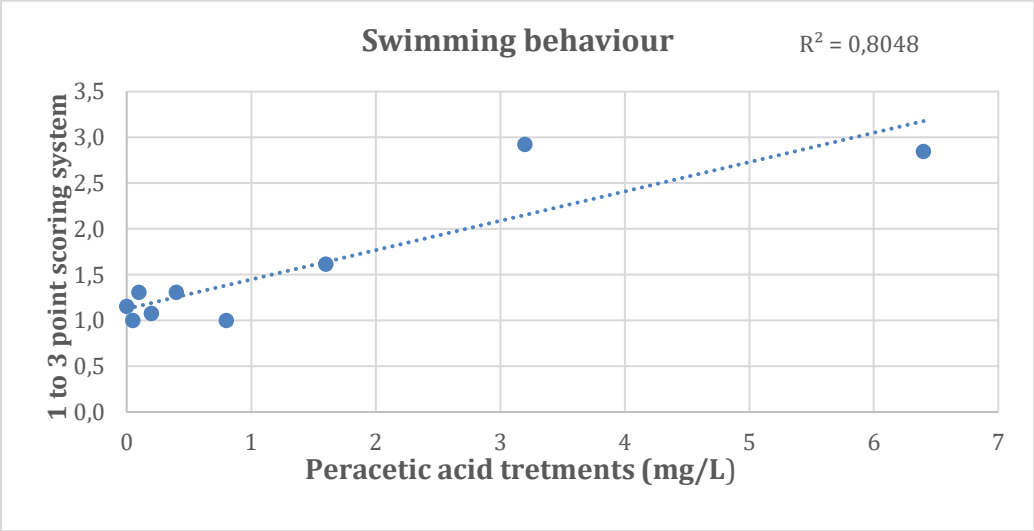


Figure 28 Positive linear regression between peracetic acid treatments and swimming behaviour, with a scored by a 1-to 3 ranking, where 1 is relaxed and come whereas 3 indicated stressed fish that struggle with survival.

7.5.2 Feeding behaviour

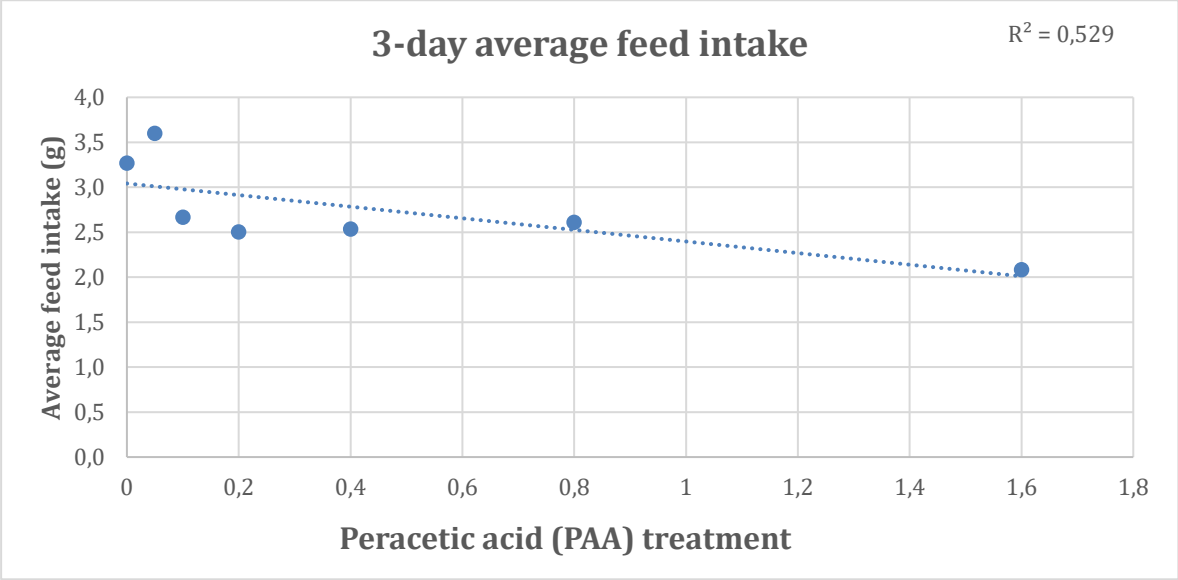


Figure 29 Negativ linerar regression between feeding behaviour and PAA treatment.

7.5.3 Fish external welfare score trial 1

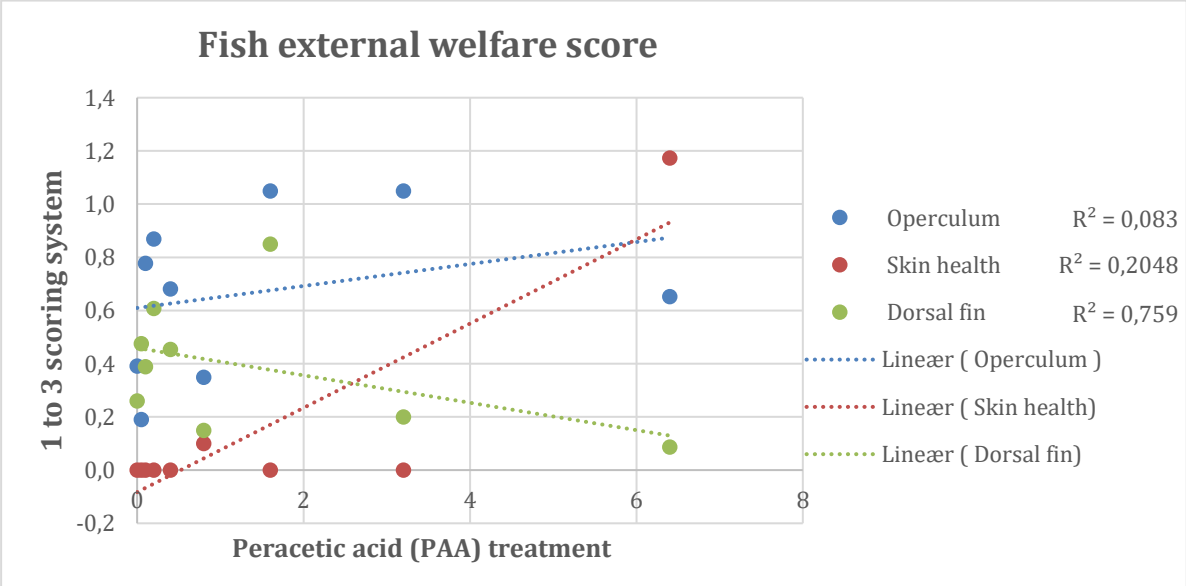


Figure 30 Linear regression between welfare parameter operculum, dorsal fin, and skin health.

7.5.4 Overall welfare index trial 1

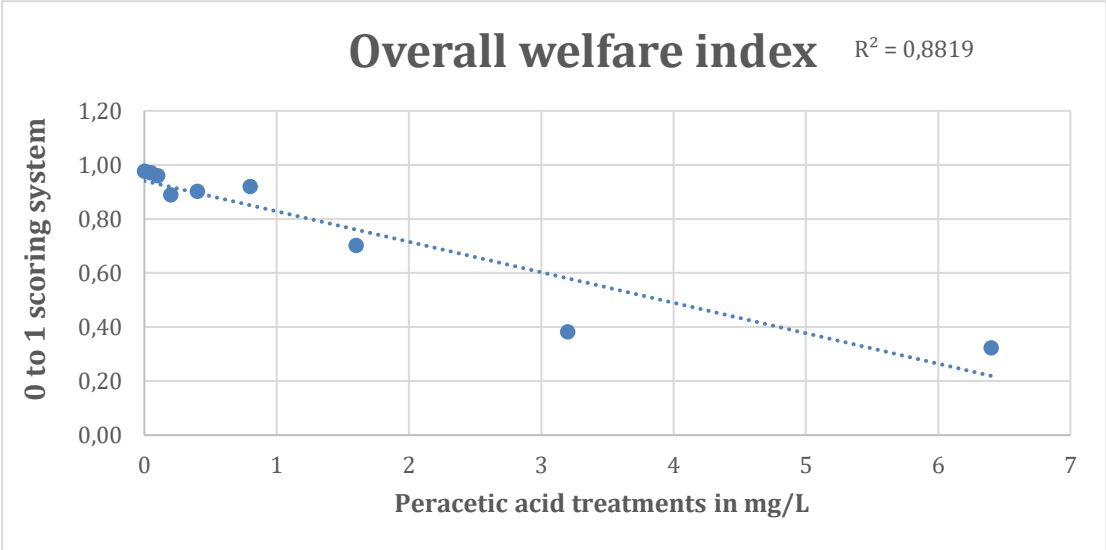


Figure 31 Negative linear regression between PAA treatment concentration and welfare index score. Score 1 indicates good fish welfare while score 0 indicates poor fish welfare.

7.5.5 Skin scoring

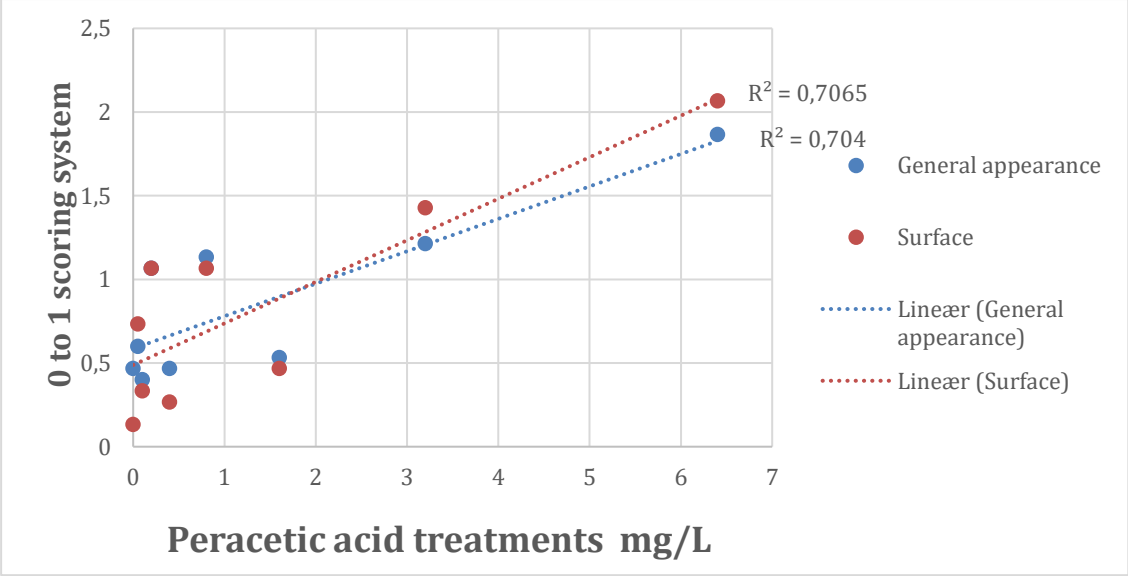


Figure 32 Portativ linear regression between PAA treatment and histology scoring from general appearance and surface.

7.5.6 Mucous cells

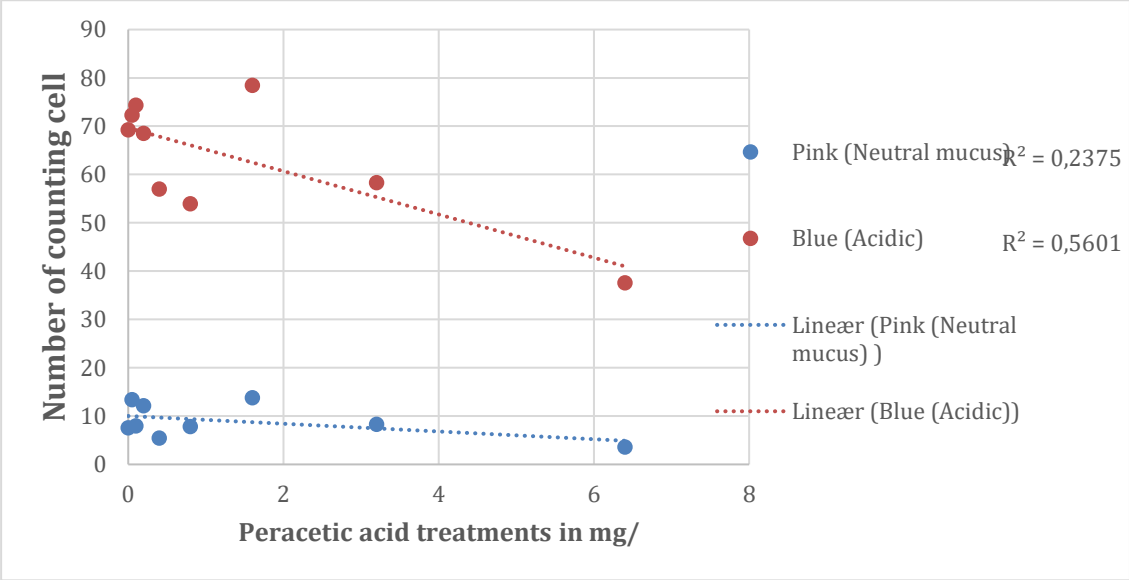


Figure 33 Negative linear regression between PAA treatment concentration and mucous cells.

7.5.7 Weight

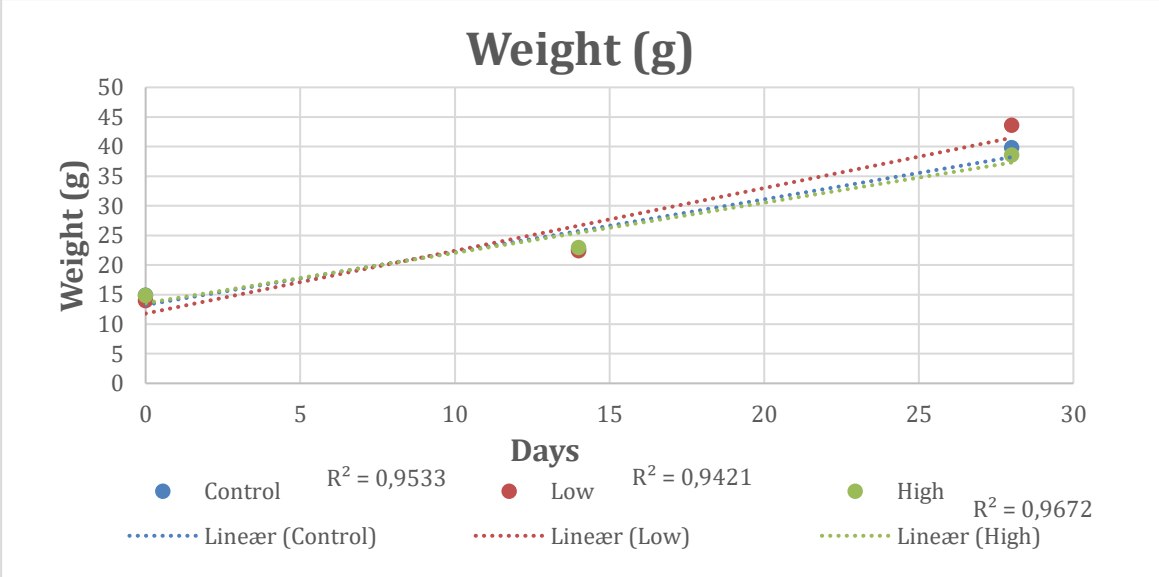


Figure 34 During the trial the fish had a positive linear growth.

7.5.8 Overall welfare index trial 2

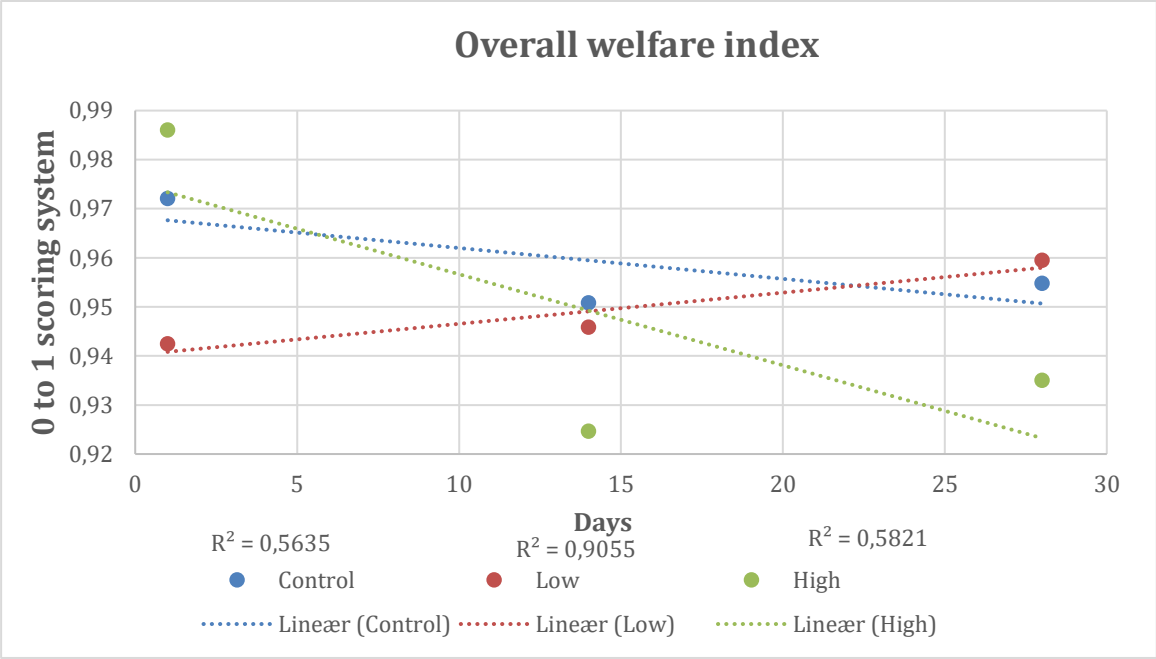


Figure 35 overall welfare index during the 4 weeks trail shows a negative linear regression for treatment group control and high and a positive linear regression for treatment group low.