UiT The Arctic University of Norway

Faculty of Biosciences, Fisheries and Economics

Evaluation of ozone and peracetic acid use during a *Yersinia ruckeri* challenge in Atlantic salmon (*Salmo salar*) freshwater recirculating aquaculture systems

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Foreword

This thesis was written for my master's degree in Aquamedicine at Arctic University of Tromsø (UiT). The study was done as part of the Research Council of Norway project RASHealth – 302767 "Water disinfection strategies to improve Atlantic salmon parr production". The goal of the thesis is to evaluate two water disinfection strategies efficacy to prevent or limit a bacterial disease outbreak in Atlantic salmon in a recirculating aquaculture system (RAS). There's a lack of knowledge on how disinfection can help reduce or eliminate the risk of infectious disease outbreaks in RAS. This is relevant topic in today's aquaculture industry as the use of RAS is increasing, and bacterial outbreaks in Norwegian fish farms cause major losses in the industry.

I want to give a huge thank you to my supervisor at Nofima Dr. Vasco Mota for trusting me to be part of your project. I have learned a lot from being a part of this trial, where the research work we have done has been an exciting, and a fulfilling experience. Thanks for the knowledge you shared with me and being so kind to invite me to Denmark for the project meeting where I got to meet amazing new people. I would also like to express my appreciation to Professor Stefano Peruzzi, my supervisor at UiT. Thank you for introducing me to Vasco, and your support and advice in the writing process of the thesis.

I met many amazing people which contributed to this study, and I know the trial would not have gone as well without your contributions. I would like to give a special thanks to Chris Verstege at the Havbruksstasjonen for all your help and guidance during the trial with sampling of water quality, sharing your knowledge of RAS to me, and your hard work throughout the fish samplings. To Carlo C. Lazado at Nofima, thank you for your guidance in evaluating the histology sections, the research process, and stepping in to help in the fish samplings. Thank you to Hanne Brenne at Nofima for helping and guiding me through the lab work and being happy to step in when we needed help for the fish samplings. Thanks so much to my friend and pHd student Wanhe Qi at DTU Aqua for your contribution in the fish samplings and letting me introduce you to Tromsø for the very first time. Thank you to my friends and fellow student Jens, Anne-Marja and Mina for support, motivation, and companionship during our days at Nofima.

A special thanks to my family and friends for their love and support during my masters.

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Samaneh Mousavi

Abstract

Yersinia ruckeri (*Y. ruckeri*) the causative agent of Enteric red-mouth disease (ERM), a serious septicemic bacterial disease of salmonid fish, poses a significant challenge in salmon production. With the emergence of recirculating aquaculture system (RAS) in Norway, new biological challenges in relation to infectious disease outbreaks have arisen. There is a gap in knowledge on how potential disinfection methods in the rearing water could help reduce or eliminate the risk of infectious disease outbreaks in RAS. Ozone and peracetic acid (PAA) are two disinfectants used in aquaculture. Both are reported to inactivate a range of pathogens, including *Y. ruckeri*. There are however limitations to the use of disinfectants in RAS as the re-use of water leads to accumulation of substances added to the water, and an ideal disinfectant need to balance pathogen control, fish health and welfare and biofilter performance.

This study investigates the use of continuous ozone and semi-continuous PAA addition in RAS as a tool for preventing a disease outbreak of *Y. ruckeri* in Atlantic salmon parr. Nine replicated RAS units were stocked with Atlantic salmon parr (N=1800, ±19 g) and were treated with PAA (50.88 ml PAA product dose of 0.05% of make-up water daily), ozone (ORP: 500mV through protein skinner) or no treatment (control), in triplicates (n=3). To induce a ERM outbreak, *Y. ruckeri* was introduced through the make-up water. Health and welfare of fish were followed for 28 days after treatment started and 20 days after pathogen challenge, where water quality was monitored to see the effect of disinfectants on different water quality parameters.

Although *Y. ruckeri* introduced to the systems did not lead to significant mortalities in any treatment group, and only mild clinical signs of the disease were observed, the pathogen was detected in the spleen of fish across all treatment groups. A trend could be seen towards higher infection prevalence in the control group, but no significant difference among the treatments (control: 33.3% ± 30.6, PAA: 13.3% ± 11.5, ozone: 6.7% ± 11.5). Sub-clinical pathologies were observed toward end of trial, where mild cases of hemorrhage in liver and eyes, and enlarged spleen were recorded in some fish across treatments. Presence of enlarged spleen was the only pathology where statistically significant differences were present among groups, as the prevalence was higher in control group, followed by PAA and ozone. However, histological evaluation of spleen did not show inter-treatment differences.

Furthermore, the results indicate that the tested disinfection methods did not severely affect health and welfare of the fish as no major alterations were observed regarding growth, external welfare, stress levels (glucose, lactate, and hematocrit) or histopathological changes in gills or spleen between treatments. Regarding water quality, lower turbidity levels were measured in PAA and ozone treatments. Nitrite levels were also significantly higher in PAA treatment than the control, which may indicate that nitrite-oxidating bacterial community in the biofilter were negatively affected by this compound. The result of this study may be used in further research, with the goal of improving biosecurity measures by developing effective disinfection protocols in RAS to limit disease outbreaks.

Figure 1 shows a graphical abstract to visualize and summarize the study of this master thesis.

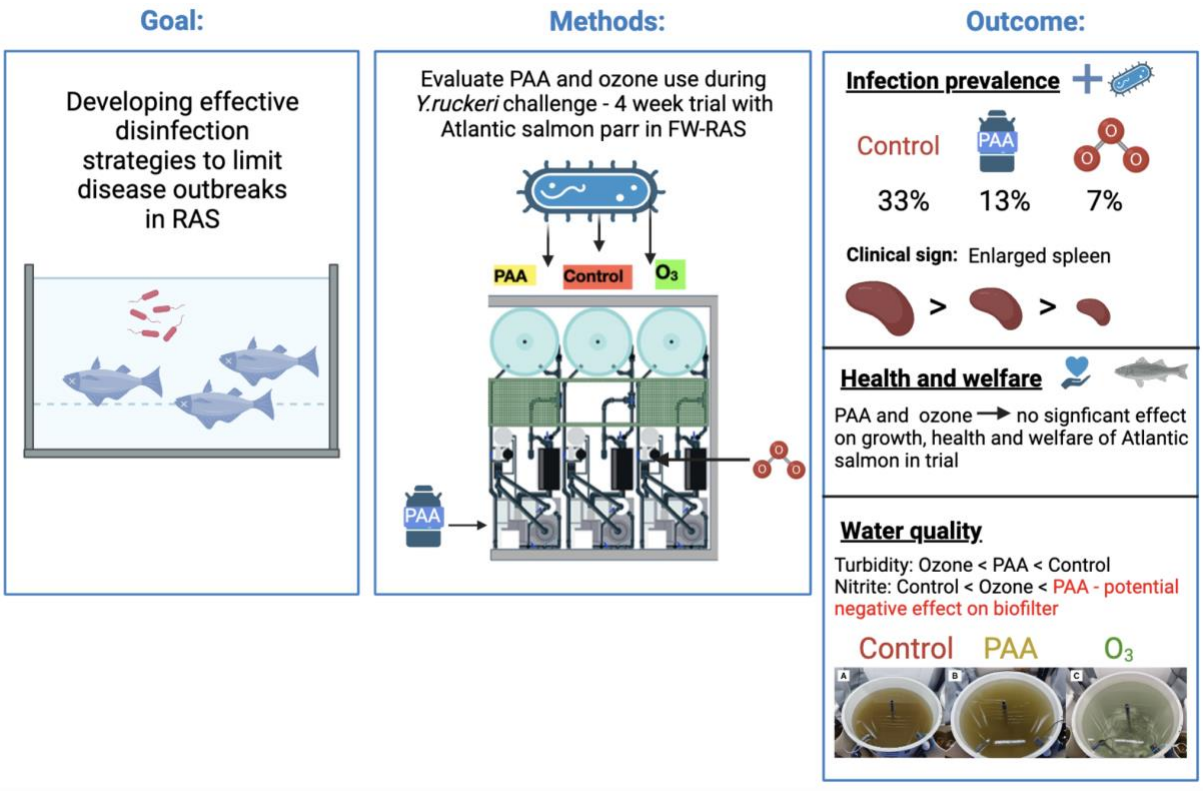

Figure 1: Graphical abstract showing goal, methods for achieving the goal, and outcome of the trial. Created with BioRender.com

1 Introduction

1.1 Aquaculture in Norway

The aquaculture industry in Norway has significantly grown the past decades and has an important role in providing nutritious food to the world and for the country's total export incomes. Today the aquaculture industry is Norway's second largest export industry behind petroleum (Regjeringen, 2021). The industry in Norway had an export that was valued of ca. 111.3 billion NOK in 2022, with a rising trend the last 10 years (Norwegian Seafood Council, 2023). Figure 2 shows a chart of production volume from total capture and aquaculture production in Norway from 1980 to 2020, where there is a clear trend with increasing part of the fish production coming from aquaculture (FAO, 2023b).

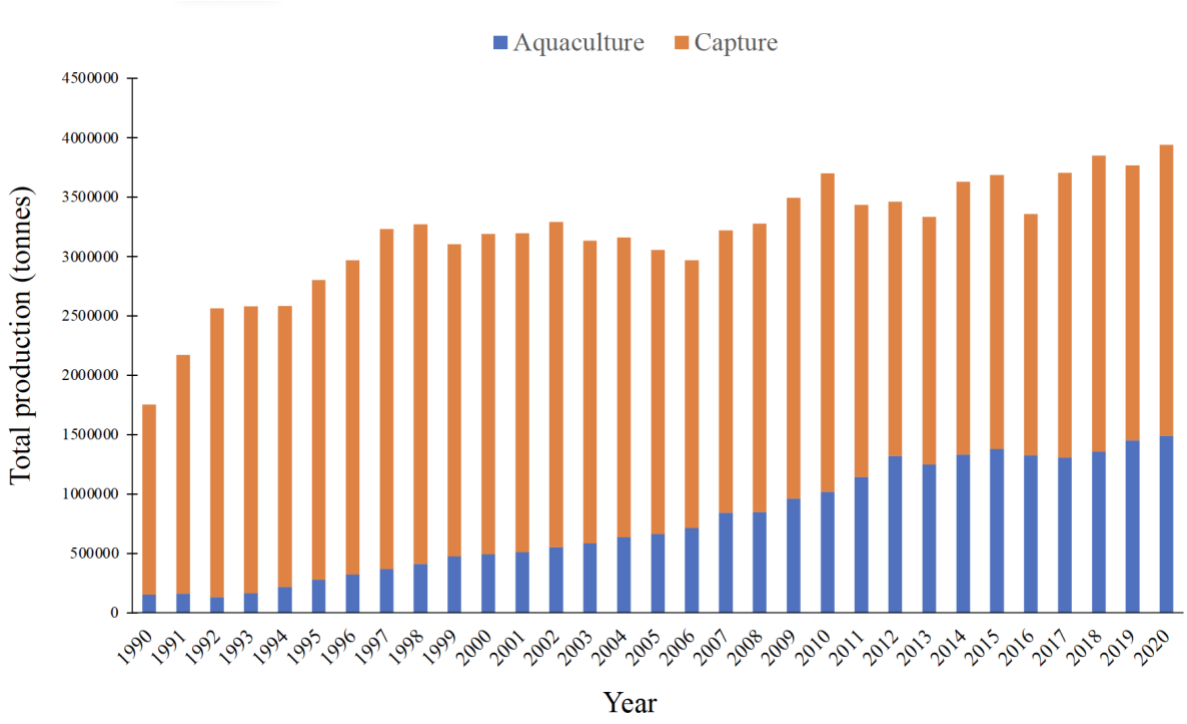

Figure 2: Chart with profile of Norway's total capture (orange bar) and aquaculture (blue bar) production in tonnes from 1990 to 2020. Data retrieved from FAO (2023b)

1.1.1 Atlantic salmon

The aquaculture industry in Norway is dominated by Atlantic salmon, *Salmo salar*, since the aquaculture industry was established at the beginning of the 1970s. Atlantic salmon, is a salmonid species naturally located to the temperate and subarctic region of the North Atlantic

Ocean (Aas et al., 2011). The species is anadromous; wild stocks live their first stages of life in the river (eggs, alevins, fry and parr) for 1-3 years before going through a physiological and morphological change called smoltification. The smolts then migrate to the ocean to feed, grow, and sexually mature for a few years before returning to their natal rivers to spawn.

1.1.2 Atlantic salmon farming in Norway

Globally Norway is an important producer of Atlantic salmon, where it is currently the world's leading producer with 52.8 % of the world's production of Atlantic salmon coming from Norway (Idoniboye, 2022). The Atlantic salmon farming industry started in Norway over 50 years ago (Norwegian seafood council, 2020). Since then, the industry has been through rapid growth with large-scale farms with intensive production that are highly industrialized. The Atlantic salmon sales in Norway have seen a rising trend, from approximately 4000 tonnes in 1980 to over 1.5 million tonnes in 2021 (Figure 3) (Fiskeridirektoratet, 2021).

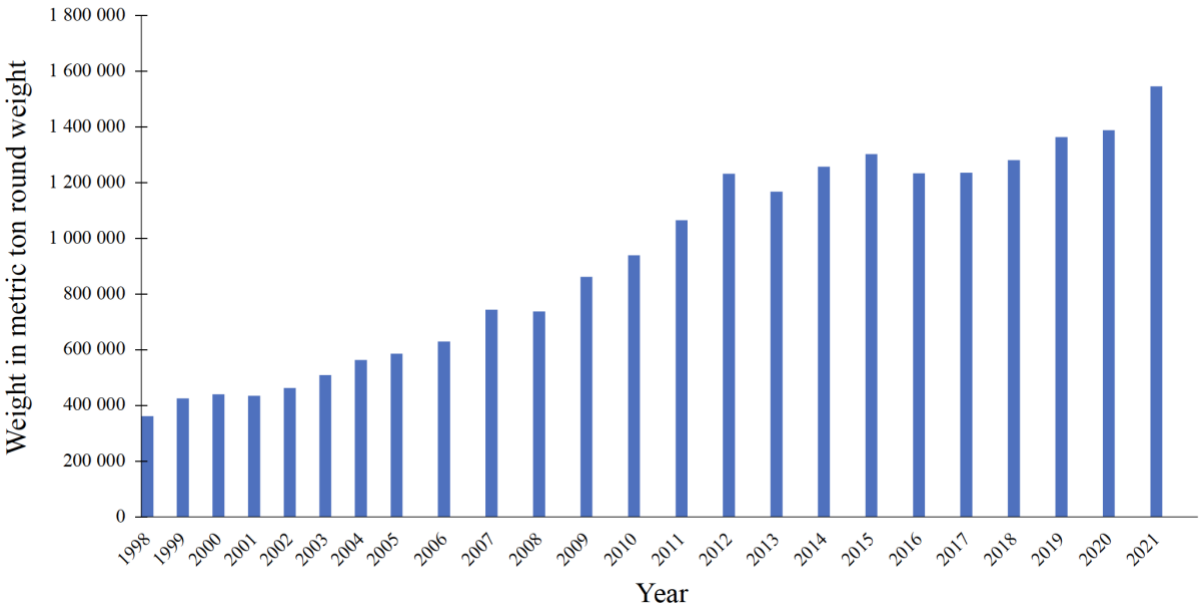

Figure 3: Total sale of Atlantic salmon from grow out production in the period 1998-2021 in Norway, measured in metric ton round weight. Data retrieved from Fiskeridirektoratet (2021).

The traditional way of producing Atlantic salmon is separated into two phases: the freshwater phase and seawater phase (Figure 4). First, salmon juveniles are produced from roe/eggs in hatcheries and juvenile land-based farms, where they develop to smolts after which they are transferred as post-smolts to sea in open net cages (Bergheim et al., 2009). Land-based farms

have an intensive production in tanks and normally utilize more technical equipment than sea cage farms (Lekang, 2007). Normal components of these farms are water inlet and outlet, water treatments facilities, production units, feeding equipment, equipment for waste and wastewater treatment and instrumentation and monitoring systems (Lekang, 2007). Most of the production of Atlantic salmon smolts has taken place in land-based flow-through farms in Norway where water pass through the system once and afterwards is discharged (with waste treatment beforehand). (Bergheim et al., 2009)

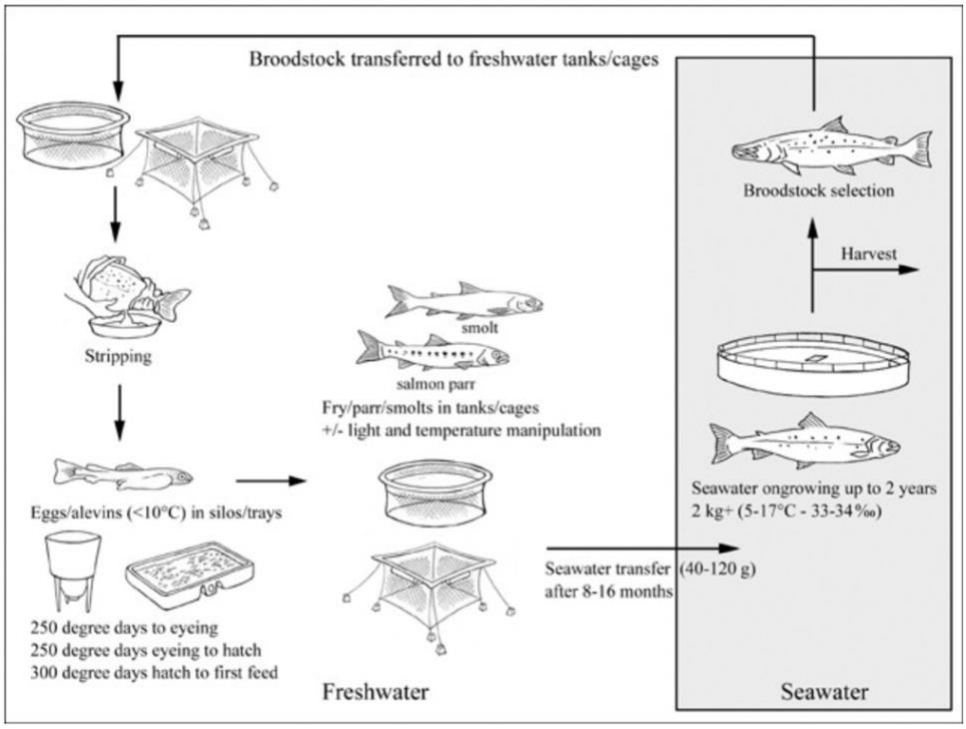

Figure 4: Traditional production cycle of Atlantic salmon, Salmo salar. Figure retrieved from FAO (2023a).

These flow-through systems (FTS) are dependent on being localized in places where freshwater resources are sufficient to cover the large volumes of high quality water they need to grow fish (Snow et al., 2012). However, suitable places for these types of farms are a limiting factor for increase of production of smolts, which has led to innovation of new land-based designs.

1.2 Challenges in salmon farming

Environmental regulations, reduced availability of water and suitable production locations, and increasing outbreaks of aquatic animal diseases related to intensive production practices,

all highlight the need for more sustainable aquaculture practices (FAO, 2020). The Norwegian aquaculture industry has been identified as big threat to wild salmonid species, as the big volume of open net-pen farmed Atlantic salmon can spread diseases and sea lice to the migrating wild salmon smolts, and escapees can alter the genetics of the wild populations (Vøllestad, 2021). These challenges are the reason the industry in Norway is heavily regulated with regard to environmental impact, e.g., the traffic light system that regulates the production capacity of farms based on sea lice levels and their impact on wild salmon population in production areas ("Produksjonsområdeforskriften," 2017).

1.2.1 Welfare challenges in land-based farms

According to the annual fish health report for 2021 the most important health problems with fish in land-based fish farms is production-related diseases, like nephrocalcinosis, fin damage, gill shortening, smoltification issues, etc (Sommerset et al., 2022). Infectious diseases are a big challenge as well and causes high losses in the industry in the form of mortalities, and reduced welfare and growth. Various fish pathogen in the forms of bacterium, virus, fungi, and parasites can breach the biosecurity of the land-based farms and cause deadly outbreaks. Some of the most common infectious diseases are infectious pancreas disease, enteric redmouth disease/yersinosis, and gill disease based on the annual fish health report for 2021 (Sommerset et al., 2022). According to the latest fish health report there is a concerning trend of increased bacterial pathogen outbreaks the last 2-3 years (Sommerset et al., 2023).

1.2.2 Yersinia ruckeri

Yersinia ruckeri (*Y. ruckeri*) is a gram-negative rod- shaped bacteria that belongs to the family Enterobacteriaceae (Ross et al., 1966). It is the fish pathogen responsible for the systemic bacterial infection Enteric red-mouth disease (ERM), also called yersiniosis (Furones et al., 1993). This disease has been reported in various fish species, however salmonids, especially rainbow trout (*Oncorhynchus mykiss*), seem to be most susceptible (Furones et al., 1993). It is one of the most significant bacterial infections in cold-water fish farms and has a broad geographical distribution where it has been responsible for significant economic losses in salmonid aquaculture worldwide the last decade (Kumar et al., 2015).

ERM is rated as the second most important infectious health challenge in juvenile Atlantic salmon farms in Norway (Sommerset et al., 2022). Symptoms of ERM in salmonid fish are many and unspecific. The signs are associated with what is typically seen in classical septicemia. This includes behavioral changes like fainting, breathing problems, abnormal swimming, swimming near the surface and loss of appetite. A typical clinical sign of ERM is subcutaneous hemorrhages, which are often seen at the corners of the mouth and in gums and tongue (Tobback et al., 2007). Other outer clinical signs include exophthalmia (Figure 5A), darkening of the skin, and lesions and hemorrhages on the gills and skin (Figure 5C). Internal signs that have been observed are splenomegaly (Figure 5B), pale liver, fluid in the abdominal cavity and lower intestine and petechial hemorrhages in internal organs, as well as histopathological changes in gills, kidney and spleen (Avci & Birincioğlu, 2005; Kumar et al., 2015; Veterinærinstituttet).

Figure 5: Clinical signs of fish infected with Y. ruckeri shown. A) shows fish with exophthalmia (pop eyes), B) shows fish with enlarged spleen (arrow), and C) shows fish with subcutaneous hemorrhages (arrow). Picture retrieved from (Aaas, 2022) credited Carlo Lazado.

There are several serotypes of the bacteria, both virulent and avirulent. In Norway the most common associated with ERM outbreaks are serotype O1 (Veterinærinstituttet). All life stages of salmonids can get ERM, but young fish are observed to have a more acute condition, while grown fish have a more chronic condition (Kumar et al., 2015). The disease in seen primarily in the freshwater phase or a short time after sea transfer (Veterinærinstituttet). Furthermore, the

transmission of *Y. ruckeri* happens horizontally, with direct contact of infected and non-infected fish. Infection can also occur when in contact with infected fish feces as infected fish shred the bacterium in the feces (Busch & Lingg, 1975; Kumar et al., 2015). The main entry route is believed to be the gills, where the pathogens spreads to other organs via the bloodstream (Ohtani et al., 2014).

Prevention of introduction of *Y. ruckeri* and outbreak of ERM in land-based farms is to ensure that all eggs have been disinfected properly, and avoiding stressful conditions, high densities, and non-optimal water quality (Leatherland & Woo, 2010). Prevention of infectious outbreaks in recirculating aquaculture systems (RAS) is especially important as the biofilm establishment of pathogens, including *Y. ruckeri*, can cause recurring outbreaks of ERM in the same locality (Terje Svåsand et al., 2017). Especially RAS has had problems with recurring outbreaks of ERM, with some cases of high mortalities (Bornø & Linaker, 2015; Hjeltnes et al., 2017).

1.3 Recirculating aquaculture system

A big challenge with increasing the production of smolts has been the lack of places to establish in-land farms as FTS are dependent on being localized places with a rich freshwater availability (Snow et al., 2012). To tackle some of these challenges, the industry is looking at new ways to produce Atlantic salmon to further expand the industry and production in a both environmentally sustainable and economically profitable way. RAS is one solution and has the past decade or so been extended in Norwegian salmon farming industry. These systems are land-based intensive aquaculture systems that consist of different water treatment units, which makes it possible to re-use most of its water. Not only are they used to raise smolts and post-smolts prior to sea transfer, but some are also raising market size salmon in RAS as well (Summerfelt & Christianson, 2014).

This way RAS offers an advantage in terms of reduced water consumption and flexibility in localization of them (Martins et al., 2010). In addition they offer reduced environmental pollution, advantages of constant, optimized temperature conditions, increased biosecurity, and controlled waste treatments (Dalsgaard et al., 2013).

RAS are able to recycle up to 90-99% of the water, where a conventional RAS has a water exchange rate of 0.1-1 m^3/kg feed while a FTS has water exchange rate above 50 m^3/kg feed

(Martins et al., 2010). This recycling of the water is possible as the system consists of advanced treatment units which are capable of removing both particulate and dissolved organic wastes like uneaten food, feces, bacteria and algae, and are dependent on that the different treatment units work correctly to create an optimal environment for the fish (Gonçalves & Gagnon, 2011).

Figure 6 shows an example of a RAS. RAS can be designed different ways, but the main elements are indoor tanks with live fish stock, a mechanical filter (e.g., drum filter) that removes feces and uneaten feed, a pump sump for movement of water throughout the system, a biofilter that removes ammonia and nitrite, as well as re-oxygenation units (e.g., oxygen cones) and CO_2 stripping units (Helfrich & Libey, 1991). RAS can also consist of disinfection systems, to provide biosecurity control, e.g., UV and ozone (Helfrich & Libey, 1991).

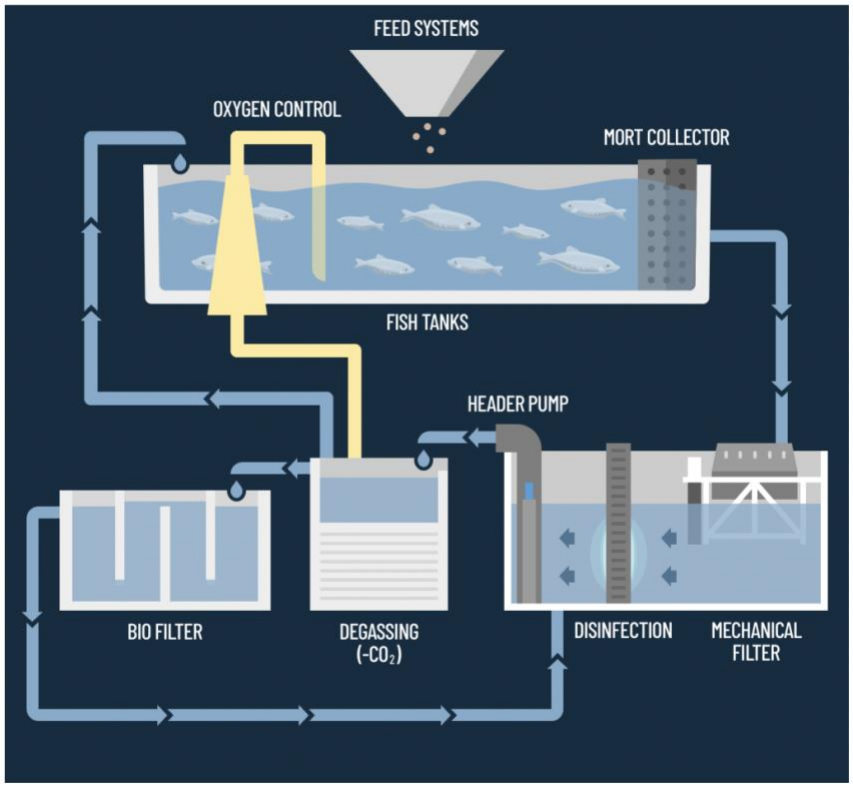

Figure 6: Example of a RAS systems with its main water treatments units. Figure retrieved from Derwent Group

A big advantage of RAS is the possibility to control its environment. However, this becomes difficult the more the water is reused because the wastes accumulate. Water quality parameters need to be monitored continuously as they can change rapidly in a relatively short time and disrupt the system (Timmons & Ebeling, 2013). Parameters like alkalinity oxygen, CO_2, ammonia, nitrate and nitrite, pH, salinity and temperature are therefore regularly controlled,

with backup systems and alarms to indicate out of tolerance conditions (Timmons & Ebeling, 2013).

1.3.1 Solids removal

Particles and organic material are added to the water through uneaten feed, feces, decaying fish, and biofilm slough from surfaces (Chen et al., 1993; Patterson & Watts, 2003). Accumulation of these particles in RAS can lead to suboptimal water quality and gill irritation for the fish (Fjellheim et al., 2016; Magor, 1988). In addition, increased organic matter can have negative effect on the system by increasing the biochemical oxygen demand and dissolved carbon dioxide levels, reducing biofilter nitrification, and increasing bacterial load and ammonia in the system (Becke et al., 2018; Bergheim et al., 1998; Wong, 2001; Zhu & Chen, 2001).

Therefore, removal of particles from the recirculating water is important. Removal of particles already starts in the rearing tanks, with self-cleaning circular tanks with an optimal diameter/ depth- ratio and an effective flow injection mechanism creating secondary radial-flow that moves settable solids to the bottom center drain of the tank (Timmons et al., 1998). Further solid removal takes place in mechanical filters (e.g., drum filters, belt filters), which removes bigger particles like feed waste and feces through fine screens (Dolan et al., 2013). Protein skimmer can also be used in RAS as the last step to remove smaller particles ≤ 30 µm. Protein skimmers removes fine solids and organic matter from the water, by using surfactants in the water that generates foam which helps remove particulate and dissolved organic matter (Timmons & Ebeling, 2013).

It's reported that high particle levels can decrease the efficiency of disinfection methods (Fjellheim et al., 2016; Liltved & Cripps, 1999). Particles can provide protection against chemical and non-chemical disinfection agents (Timmons & Ebeling, 2013). A high levels of organic matter can lead to a low concentration of oxidants available for inactivation of microorganisms (Timmons & Ebeling, 2013). Bullock et al. (1997) reported decreased efficacy of ozone on heterotrophic bacteria in recirculating rainbow trout culture systems, because of a rapid loss of oxidation caused by the levels of suspended solids.

1.3.2 Biofilter and nitrification

An important part of the water treatment system is the biological filter (biofilter), that consists of a media (e.g., plastic sheets or beads) where nitrifying microorganisms are attached (Helfrich & Libey, 1991). Nitrifying bacteria are autotrophic; they derive all energy required for growth from the oxidation of inorganic nitrogen compounds (Belser, 1979). The source of nitrogen compounds are the atmosphere, precipitation, geologic sources (soil and sediments) and agriculture (Feth, 1966). Nitrogen is essential for life, as they are the key building block of nucleic acids and amino acids. In animals most of the nitrogen is transformed to unionized ammonia (NH_3) by the protein metabolism (Wurts, 2003).

NH_3 is a highly toxic waste product from protein metabolism of fish and is excreted into the water (Fivelstad et al., 1995; Helfrich & Libey, 1991). NH_3 is also released through bacterial decomposition of organic material (Romillac, 2019). NH_3 is polar and binds H^+ in water to form ammonium ion (NH_4^+) as seen in Eq.1 (Butler, 1971). NH_3 is more toxic than NH_4^+, and the concentration of each of these forms in the water is dependent on temperature, pH and salinity, where the higher pH and temperature, the equilibrium goes towards the more toxic NH_3 (Anthonisen et al., 1976; Meade, 1985).

$$NH_4^+ \leftrightarrow NH_3 + H^+ \qquad \text{Eq. 1}$$

The sum of NH_3 and NH_4^+ is called Total Ammonium Nitrogen (TAN) (Fjellheim et al., 2016), and in RAS most of the TAN is in the form of NH_4^+ as pH and temperature are controlled. The recommended safety levels for TAN in salmonid aquaculture (not system specific) issued by the Norwegian food health authority is < 2 mg/L (Fjellheim et al., 2016), while Kolarevic et al. (2013) study suggest long-term exposure of NH_3-N levels of 35 µg/L do not affect growth or welfare of Atlantic salmon parr.

Treatment of ammonia is important in RAS, as the nitrifying bacteria in the biofilter reduce the ionized and unionized ammonia levels in the nitrification process. Nitrifying bacteria in the biofilter oxidize ammonia to nitrite (NO_2^-) which is toxic for fish, and thereafter to nitrate (NO_3^-) which is much less toxic (Aich et al., 2021; Davidson et al., 2014; Helfrich & Libey, 1991). NO_2^--N is recommended to stay below 0.1 mg/L for fish (Wedemeyer, 1996), while for NO_3^--N its recommended to stay below 100 mg/L (Bregnballe, 2022; Noble et al., 2018). Nitrifying bacteria's like *Nitrosomonas* (ammonia-oxidizing), *Nitrospira,* and *Nitrobacter*

(nitrite-oxidizing) species are responsible for this process (Bartelme et al., 2017). This overall reaction of nitrification is showed in Eq. 2 and Eq. 3 (Timmons & Ebeling, 2013).

Nitrosomonas $NH_4^+ + 1.5\ O_2 \rightarrow\ NO_2^- +\ 2H^+\ +\ H2O$ Eq. 2

Nitrobacter $NO_2^- +\ 1.5O_2 \rightarrow\ NO_3^-$ Eq. 3

There are different types of biofilters in the market, all designed with a big surface area for the nitrifying microorganisms to reside in (Fjellheim et al., 2016). One of them is moving bed bioreactors (MBBR) (Timmons & Ebeling, 2013). MBBR has media carriers that move freely in the water, which are always scrubbing against each other and this way self-rinsing. Via a course air bubble aeration system, the media is maintained in constant circulation with aerobic conditions (Timmons & Ebeling, 2013).

Many factors influence the rate of nitrification of a biofilter as nitrifying bacteria populations are sensitive to changes (Malone & Pfeiffer, 2006). Several factors can affect biofilter performance, including water temperature, pH, alkalinity, salinity, substrate concentration, dissolved oxygen, mixing regime, and competition of essential nutrient or space with heterotrophic bacteria (Chen et al., 2006). All factors need to be considered and predicting the performance of a biofilter is therefore a challenge in RAS (Chen et al., 2006).

1.3.3 Disease outbreak in RAS

Disease outbreaks in fish farms pose a major risk to the sustainability of the industry, with major economic losses in the sector. An advantage of moving most of the production cycle in land-based facilities like RAS, is that many infectious diseases in the sea phase of farming can be avoided. However, despite det high biosecurity features, the re-use of water in RAS may provide favorable conditions for certain obligate and opportunistic fish pathogens (Aich et al., 2021). If a pathogen occurs in RAS, it will recycle with the rearing water and won't get diluted as is the case in FTS, and therefore the rates of infection in RAS can be greater (Delabbio et al., 2004). This is especially a risk if you have RAS without or with poor disinfection facilities (Aich et al., 2021).

Also, it's important to note that the introduction of a pathogen in RAS can be difficult to handle because of the negative effect the treatment may have on the nitrifying microorganisms in the biofilter and its function (Noble et al., 2018). There's also difficulties with exterminating the outbreak as pathogens introduced to the system may be incorporated into the biofilm in the biofilter which may lead to recurring exposure of fish to pathogens and the presence of asymptomatic carriers (Gonçalves & Gagnon, 2011).

1.4 Disinfection methods in RAS

Many RAS facilities have disinfection systems to provide biosecurity control. The goal of biosecurity is to prevent introduction and transmission of pathogens to the fish, and having a good biosecurity is a prerequisite for a successful RAS farm (Noble et al., 2018). In RAS you can both disinfect the intake water and the recirculating water (operating water). In Norway disinfection of water in aquaculture is highly regulated. Facilities are allowed to use approved disinfection methods such as ozone (O_3), UV-irradiation, formic acid (HCOOH), sodium hydroxide (NaOH), chemical precipitation and more, where the Norwegian veterinary institute has the authority of approval (Mattilsynet, 2012; Veterinærinstituttet).

There are different disinfection methods and practices that are used in aquacultural facilities, where the most commonly used disinfectants of water in aquaculture is physical (e.g., UV irradiation) and chemical disinfectants (e.g., ozone, chlorine, peracetic acid) (International Office of Epizootics, 2009). The most used water disinfectants in RAS in Norway are UV-radiation and ozone (Fjellheim et al., 2016). There are some challenges with the use of disinfectants in RAS as there are restrictions to the efficiency of disinfection because of the high amount of substances in the water, which can have toxic effects on aquatic animals in high concentrations (Leal et al., 2018). They may also affect biofilter performance by the disturbing the nitrifying microbial population on the biofilter (Liu, Straus, et al., 2017; Pedersen et al., 2009; Pedersen & Pedersen, 2012).

The different types of disinfectants used in aquaculture have their own advantages and disadvantages, where an ideal disinfectant has a broad spectrum activity, is effective against pathogens, works in any environment, does not negatively affect biofilter, is non-toxic and relatively inexpensive, according to Timmons and Ebeling (2013). Table 1 shows an overview of pros and cons of some of the water disinfectants used in aquaculture.

Table 1: An overview of advantages and disadvantages of some different water treatment disinfectants used in aquaculture.

Disinfectant	Toxicity	Efficacy	Water quality	Biofilter	Other
Ozone	High toxicity, and risk of high residual ozone concentration, but no harmful reaction by-products in freshwater (Spiliotopoulou et al., 2018) .	Reactive oxidant, that effectively kills many pathogens (Liltved et al., 1995). 0.15-0.20 mg/l residual ozone give 99.99% inactivation of *Y.ruckeri* in freshwater *(Liltved et al., 1995).*	Improves water quality (Summerfelt & Hochheimer, 1997).	Reported improved removal efficiency in biofilter (Davidson et al., 2011; Park et al., 2015), but high residual ozone concentration can cause impairment of biofilm (*Ozone in recirculating aquaculture systems*).	Safety risk for workers because of toxicity. Complex application
UV-irradiation	No production of toxic residuals or byproducts.	Effective against various pathogens (Liltved et al., 1995). 2.7 mWs/cm^2 required for 99.999% inactivation of *Y.*	No impact	No impact	Low risk concerning safety. Ineffective in turbid water.

		ruckeri, in brackish water. (Liltved et al., 1995)			
Peracetic acid (PAA)	Toxic on aquatic organisms in high concentrations but produce little to no toxic by-products (Straus et al., 2018).	Reactive oxidant, that kills various pathogens (Pedersen et al., 2013) . Complete inactivation of *Y. ruckeri* achieved with 5 mg/L PAA in RAS water with 5 min exposure (Good et al., 2022).	Reports of no significant effect on water quality by Davidson et al. (2019), however Suurnäkki et al. (2020) reports temporary water quality improvements.	Reports of minimal effect on biofilter performance in low concentration (Davidson et al., 2019).	Worker safety risk (highly acidic). Low environmental impact.
Hydrogen peroxide (H_2O_2)	Toxic to aquatic organisms in high concentrations, but produce no toxic by-products (Gaikowski et al., 1999).	Effective against wide range of pathogens, especially various parasites (Rach et al., 2000).	Reported to improve water quality (Pedersen & Pedersen, 2012).	Reported to reduce ammonium removal efficiency, but with partial recovery (Pedersen & Pedersen, 2012).	Worker safety risk. Low environmental impact.

1.4.1 Ozone

Ozone (O_3) is an inorganic molecule used in many different areas such as a water disinfectant, pesticide action in agriculture, antibiotic, anti-inflammatory and antiviral actions in animal husbandry and fish farming (Remondino & Valdenassi, 2018). It's widely used in aquaculture because of its effectiveness in eliminating bacteria and virus, but also because it improves water quality (Timmons & Ebeling, 2013). Ozone is traditionally used to disinfect intake and discharge water but has also been used to disinfect fish eggs (Gonçalves & Gagnon, 2011). In a RAS, ozone may be applied for water treatment and processing inside the system, but is often used with other water treatment units to have the best effect, like UV lights (Gonçalves & Gagnon, 2011). Ozone is used in RAS primarily to suppress the microflora within the system and to assist water filtration and purification treatment (Powell & Scolding, 2018).

As an oxidizing agent ozone is documented to remove organic carbon, turbidity, color, odor, taste, nitrite, suspended solids, and ammonia (Rosenthal & Kruner, 1985; Summerfelt et al., 1997; Summerfelt & Hochheimer, 1997). According to a study by Davidson et al. (2011), ozone water treatment (0-10 mg O_3/L) improved water quality in a study with rainbow trout in RAS. The same study also found a slightly better removal efficiency of biofilter operated with ozone, likely due to cumulative water quality improvements initiated by ozone (Davidson et al., 2011). A more recent study of effect of ozone on post-smolt Atlantic salmon in FW-RAS also found that ozone resulted in water quality improvements that promoted Atlantic salmon growth (Davidson et al., 2021).

The oxidizing feature of ozone has also been documented reduce bacterial populations in RAS (Sharrer & Summerfelt, 2007), including inactivating a range of fish pathogens, (Bullock et al., 1997; Colberg & Lingg, 1978; Liltved et al., 1995). Table 2 shows an overview of some of the different fish pathogens ozone is reported to inactivate.

Table 2: An overview of some of the different fish pathogens ozone is reported to inactivate in distilled water, both viral and bacterial incled. Percent removal and concentration is also noted. Table based on Timmons and Ebeling (2013). Exposure time not included.

Pathogen	**Percent removal (%)**	**Conc. (mg/l)**	**Reference**
Aeromonas salmonicida	99.8-100	0.04-0.4	Colberg and Lingg (1978), Wedemeyer and Nelson (1977), Liltved et al. (1995), Liltved and Landfald (1995)
Vibrio anguillarum	99.99	0.05- 0.177 (residual)	Liltved et al. (1995), Sugita et al. (1992)
Yersinia ruckeri	99.9-100	0.01-0.20	Wedemeyer and Nelson (1977), Colberg and Lingg (1978), Liltved et al. (1995)
Infectious hematopoietic necrosis virus (IHNV) and infectious pancreatic necrosis virus (IPNV)	100	0.01	Wedemeyer et al. (1978)
Infectious salmon anaemia virus (ISAV)	99	0.33	Liltved et al. (2006)

Use of ozone in treatment or prevention of disease outbreaks in fish has been researched in a few studies. Tipping (1988) study on ozone control of Ceratomyxosis in rainbow trout (*Oncorhynchus mykiss*) had positive results with less mortality and larger fish than control group, while Bullock et al. (1997) study on ozonation (0.025–0.039 kg ozone/kg feed) effect on bacterial gill disease (BGD) in rainbow trout cultured in RAS did not manage to remove the causative agent of BGD, but did provide <1 log reduction of the bacteria in the system water and on the gill tissue which prevented BGD outbreak with reduced mortality.

On the other side, the application of ozone in RAS is expensive and quite complex, because of its unstable form (Gonçalves & Gagnon, 2011). The decomposition of ozone and rate of oxidation is highly affected by the quality of water, including pH, bicarbonate level, total organic carbon levels and temperature (Gonçalves & Gagnon, 2011; Summerfelt & Hochheimer, 1997). To get optimal disinfection with ozone the farmer is dependent having high enough ozone concentration and contact time to eliminate the specific pathogen but also be careful that the concentrations are not toxic for fish (Fjellheim et al., 2016).

1.4.2 Peracetic acid (PAA)

Peracetic acid (CH_3CO_3H) is another oxidizing disinfectant used in the aquaculture industry. It is an organic antimicrobial compound, that is used as a surface disinfectant or sanitizer for various industrial applications, including aquaculture (Davidson et al., 2019). As a water disinfection method PAA is not approved by the Norwegian veterinary institute, however it is approved in Europe by the European Union Commission for use in veterinary medicine and as a water disinfectant for aquaculture systems (Acosta et al., 2022).

The chemical can be applied continuously or intermittently to disinfect water where reports suggest the use of PAA as a both eco and welfare-friendly antimicrobial agent in fish farming (Acosta et al., 2022; Davidson et al., 2019; Gesto et al., 2018; Liu, Pedersen, et al., 2017; Pedersen et al., 2009; Pedersen & Lazado, 2020). PAA as a water disinfectant in aquaculture is especially used in Denmark in some commercial open rainbow trout RAS farms as a strategy to control fish parasites (Pedersen et al., 2013). In Norway PAA is currently only used in aquaculture as a surface disinfectant. Commercially PAA is sold in a mixture of acetic acid, hydrogen peroxide, and water in equilibrium (Davidson et al., 2019).

Contrary to ozone, there are no signs PAA gives any water quality benefits. Davidson et al. (2019) study reported that water quality was unaffected by PAA (0.05, 0.1 and 0.3 mg/L) for most tested parameters like total suspended solids (TSS), N-species, CO_2, and dissolved oxygen when added semi-continuously RAS with rainbow trout. The same study indicates that continuous PAA dosing does not lead to reduction of heterotrophic bacteria and total coliform counts (Davidson et al., 2019). Mota, Eggen, et al. (2022) study with Atlantic salmon parr in RAS with concentrations of 0.0- 6.4 mg/L exposed 1h twice also saw no significant difference throughout the 52-h trial for dissolved oxygen, temperature, turbidity, NH_4-N, NH_3-N and NO_2-N. pH however had a drop for $\leq$ 1.6 mg/L PAA due to the PAA product being acidified mixture of acidic acid and hydrogen peroxide.

Regarding PAA antimicrobial properties, several studies have indicated that the application of PAA at low concentration is an effective disinfection method against various fish pathogens, especially against ectoparasites (Abu-Elala et al., 2021; Meinelt et al., 2009; Meinelt et al., 2007). Most of the studies are however in flow-through systems, but a recent study by Good et al. (2022) focus specifically on the bactericidal activity of PAA on selected fish pathogens in RAS water, and reports that complete inactivation of *Y. ruckeri* is achieved with 5 mg/L PAA in RAS water, after 5 min exposure time. The chemical has been reported to inactivate a range of other fish pathogens, as shown in table 3.

Table 3: An overview of some of the fish pathogens PAA is reported to inactivate in vitro. Exposure time not included.

Pathogen	**Percent removal (%)**	**Nominal conc. (mg/l)**	**Reference**
Saprolegnia parasitica	55.3	0.5-10	Marchand et al. (2012)
Flavobacterium columnare	26.1	1-10	Marchand et al. (2012)
Aeromonas salmonicida	100	> 2	Meinelt et al. (2015)
Yersinia ruckeri	100	> 2	Meinelt et al. (2015)
Yersinia ruckeri **(RAS water)**	100	> 5	Good et al. (2022)
Ichthyophthirius multifiliis	>95	> 0.2250	Straus and Meinelt (2009), Meinelt et al. (2009)
Paramoeba perurans	50	> 4.8 ppm	Lazado (2019)

There are also *in vivo* studies on PAA as a water treatment against pathogens in fish farms. These studies focus mainly on PAA as water treatment against the protozoan parasite *Ichthyophthirius multifiliis* that cause white spot-disease in infected freshwater fish (Matthews, 2005). Pedersen and Henriksen (2017) study on continuous PAA application was able to prevent outbreak of the parasite in a flow-through rainbow trout fish farm with concentration range of 0.10-0.15 mg/L PAA.

Another study by Good et al. (2020) looked at PAA as control for post-vaccination *Saprolegnia spp*. infection on juvenile Atlantic salmon held in FW-RAS with 0.2, 0.5 and 1.0 mg/L PAA pulse treatment. The study concluded that PAA significantly reduced observable external saprolegniasis and led to higher survival rates in the PAA treatment groups although no elevated mortality was caused by saprolegniasis in control group (Good et al., 2020).

Furthermore, as any disinfection method there is a risk that PAA may alter the biofilter activity in RAS, because of its antibacterial properties. Reports on how the disinfectant peracetic acid (PAA) affects nitrification in RAS, indicate that low PAA additions (≤ 1.0 mg/L) only cause minor impaired nitrification, while PAA application of 2.0 and 3.0 mg/L can induce increased nitrite levels over a prolonged period (Pedersen et al., 2009). In low doses it seems that PAA can disturb the nitrification process, but only in the short-term before it adapts (Pedersen et al., 2009; Suurnäkki et al., 2020).

A challenge with PAA administration is its fast degradation time, as degradation of PAA is highly affected by the organic load, where degradation rate is seen to increase with higher organic matter content in the system, as well as positive correlation related to fish biomass and temperature (Pedersen et al., 2013; Pedersen et al., 2009; Pedersen & Lazado, 2020). This might make it difficult to reach desired doses and contact time for proper disinfection, with a discrepancy reported between delivered quantities and realized residuals (Pedersen et al., 2013).

1.5 Fish health and welfare

The fish farming industry in Norway has many challenges regarding fish health and welfare considering the mortality of farmed Atlantic salmon in the sea phase was a total of 54 million fish (15.5%) in 2021 (Sommerset et al., 2022). Welfare and health of farmed fish is affected

by factors like disease, environmental conditions, nutrition, and operating routines like handling (Sommerset et al., 2022).

To get an understanding of the fish health and welfare in a farm, welfare indicators (WI) are used as tools to see if the fish's different welfare needs are fulfilled. Welfare needs are resources the fish needs to gain enough energy to survive, grow and reproduce. These needs are usually based on either direct observations of the animals' condition and behavior or indirect resource-or environmental WI's based on what resources and environment the animal is exposed to (Noble et al., 2018). Most animal welfare assessment protocols and scientists use a combination of WI's to assess the overall welfare of groups of fish. Examples of welfare indicators are mortality, physical state of the fish (skin condition, snout damage, fin damage, eye status etc.) and behavior (swimming activity, appetite). Laboratory based WI's are also used which require access to a laboratory or other analytical facilities to provide useful information (e.g., cortisol in blood, histology). (Noble et al., 2018)

Histology is the study of microscopic anatomy of thin stained tissues and cells seen through a microscope. It is a tool that can be used to examine tissues for morphological and pathological changes and diagnose what the causes are, e.g., diseases, infection, pollutants, or injury. However, this requires a thorough understanding of normal tissue structure, which may vary among species, age and physiological and developmental stages (Bruno et al., 2013; Kryvi & Poppe, 2016).

1.5.1 O_3 and PAA effect on performance and welfare

As ozone is a highly effective oxidizing agent that is very unstable and decomposes rapidly in the water (Leynen et al., 1998), toxicity issues are a concern with the use of the chemical. Direct exposure of ozone to aquatic organisms and especially oxidants formed in ozonated seawater can be lethal (Wedemeyer et al., 1979), however most species can tolerate a certain amount of dissolved ozone (Gonçalves & Gagnon, 2011). High doses of ozone can cause severe gill damage, with gill epithelial damage and mortality registered with concentrations of 0.0093 mg O_3/ l on rainbow trout (Wedemeyer et al., 1979a).

Some studies have indicated that the water quality improvement initiated by ozone could possibly create a more optimal environment for growth in RAS (Sutterlin et al., 1984). According to a study by Davidson et al. (2011), ozone water treatment (0-10 mg O_3/L) in

RAS promoted animal survival, growth and welfare of rainbow trout. Furthermore, a recent study on the effect of ozone on post-smolt Atlantic salmon in FW-RAS with ORP levels of 300–320 mV, suggests that the fish reared in ozonated RAS grew significantly faster vs. non-ozonated RAS, which is suggested to be because of the improvement of water quality that ozone causes (Davidson et al., 2021).

The same study also saw no significant difference between treatments for histopathology in gills, skin and skeletal muscle (Davidson et al., 2021). Another study on effect of continuous ozonation (334±22 mV) on health and welfare of Atlantic salmon post-smolts in brackish water RAS saw no difference in survival, operational welfare indicators, average weight and skin health, while ozone treatment promoted better gill health status, with 350 mV suggested as the upper safe limit for ozone in salmon in brackish water (Lazado et al., 2021; Stiller et al., 2020).

PAA is reported to have minimal effect on fish welfare in low concentrations according to several studies (Davidson et al., 2019; Gesto et al., 2018; Liu, Straus, et al., 2017; Mota, Eggen, et al., 2022). However, it is an oxidative disinfectant with free radicals and reactive oxygen as intermediate products which is reported to cause oxidative stress in rainbow trout and Atlantic salmon (Liu et al., 2020; Soleng et al., 2019). Reports of increased common stress markers like cortisol and glucose indicate this (Soleng et al., 2019), however Atlantic salmon seem to physiologically adapt to these responses with habituation (Gesto et al., 2018; Lazado et al., 2020; Liu, Pedersen, et al., 2017).

For rainbow trout, a study by Davidson et al. (2019) indicate PAA doses at 0.05-0.30 mg/L semi-continuously added in RAS showed growth and survival were unaffected by these doses. A new study on PAA exposure to Atlantic salmon parr in RAS indicated that survival, swimming behavior and mucosal health were not affected below 1.6 mg/L PAA, but acute mortality, damaged skin and gill necrosis could be observed with equal or higher than 3.2 mg/L (Mota, Eggen, et al., 2022).

1.6 Aim of the study

There is an urgent need for effective and operational water disinfection strategies, as there's a lack of knowledge on how disinfection can help reduce or eliminate the risk of disease outbreaks in RAS. The primary objective of this thesis is therefore to try to find better ways

of disease management in Atlantic salmon cultured in FW-RAS, by optimizing and developing new water disinfection strategies to prevent disease outbreaks in this system.

The goal of this thesis is to evaluate two water treatment disinfection strategies: ozone and peracetic acid use during a *Y. ruckeri* challenge in Atlantic salmon RAS. This will be done by investigating fish groups in three different treatments; semi-continuous PAA treatment through PAA product dose of 0.05% of make-up water added daily in pump sump, 500 mV ozone treatment added through the protein skimmer, and control groups with no disinfection. The three different fish group's welfare and health will be followed through the trial, in addition to assessing how the disinfectants affect water quality.

The secondary objectives (SOs) of this thesis below are followed by each their hypothesis below:

SO1. Assess if ozone and PAA water treatment can prevent outbreak of *Y. ruckeri* in Atlantic salmon cultured in FW-RAS.

H0: Ozone and PAA water treatment does not prevent outbreak of *Y. ruckeri* in FW-RAS.

SO2. Asses how ozone and PAA water treatment affect Atlantic salmon parr welfare and health during a *Y. ruckeri* outbreak in FW-RAS.

H0: Atlantic salmon parr welfare and health are not affected by ozone and PAA water treatment during a *Y. ruckeri* outbreak in FW-RAS.

SO3. Assess how ozone and PAA water treatment affects water quality in FW-RAS.

H0: Water quality is not affected by ozone and PAA water treatment.

2 Materials and methods

2.1 Ethical statement

To understand and compare the efficacy of the different water disinfection methods during a pathogen outbreak in RAS, a live fish experiment was necessary. The trial was approved by the Norwegian Committee on Ethics in Animal Experimentation and the Norwegian Food Safety Authority of Norway (Mattilsynet) under FOTS ID number 28715. The Norwegian regulation for use of animals in experiments ("Forskrift om bruk av dyr i forsøk," 2015), that aims to better the animal welfare of animals used in experiments, and promote the principle of the 3R's (Replacement, Reduction and Refinement), was taken into account. The number of fish used was necessary as it was the minimum replicated sample to give reliable scientific result. Fish was handled by experienced and qualified staff in fish samplings, where all fish were euthanized with an overdose of the anesthetic benzocaine (Benzoak vet, ACD Pharmacuticals AS, Leknes, Norway, 200mg/ml) before each sampling, and humane end points were defined.

2.2 Experimental setup

The experimental trial took place in the fish health laboratory of Tromsø Aquaculture research station (Havbruksstasjonen i Tromsø AS, Kårvik, Norway). Nine identical individual RAS (AquaBioTech Group), with a total water volume of 0.8 m^3 for each, were used in the trial. These were located in one of the infection rooms of the fish health laboratory. Atlantic salmon parr were randomly distributed among these nine individual identical RAS units for this trial. Figure 7 shows a general overview of the set-up of the RAS and what water disinfection treatment they were exposed to in the trial (n = 3).

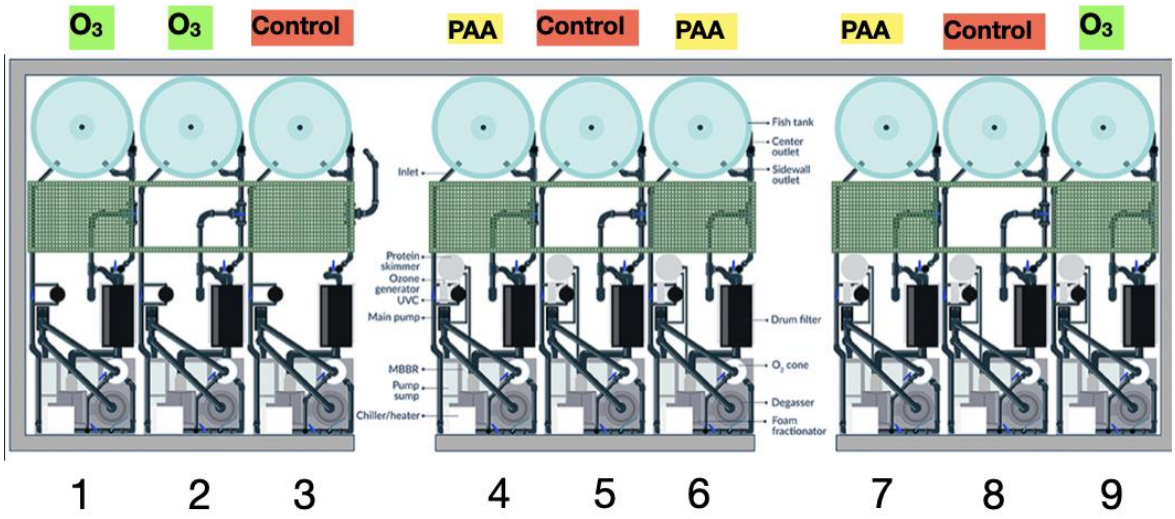

Figure 7: A sketch of the nine RAS, including what treatments they will be exposed to. Figure retrieved from Mota, Striberny, et al. (2022), with modifications.

The trial was a comparative benchmark study using non-disinfection (control), PAA- and O_3-disinfection strategies in FW-RAS with Atlantic salmon parr during a *Y. ruckeri* outbreak. The fish were exposed to these three different treatments, with three replicates per treatment, as shown in Figure 7. Semi-continuous PAA administration in three RAS (target of 1 mg/L PAA), continuous O_3 application in three other RAS (target of 300-350 mV), and control groups with no disinfection in the last three units.

Regarding experimental setup, the trial was separated into three periods. The first period was acclimatization of fish to the RAS units. This period lasted 29 days, before the disinfection treatment was started (PAA and O_3) on day 1. The acclimatization to the disinfection strategies lasted 7 days, before *Y. ruckeri* was added to all rearing units on day 9. This part consisted of administering a 24h-culture of the pathogen (5.3×10^8 cfu/ml) via the make-up water at 1% per total daily volume. Fish was followed for 20 days after this pathogen challenge.

A total of five fish samplings were done throughout the trial: a day before treatment started (day 0), a day before the pathogen challenge (day 8), a day after pathogen challenge (day 10), 7 days after pathogen challenge (day 16) and at end of the trial, 20 days after pathogen challenge (day 29). An overview of the experimental setup is shown on Figure 8.

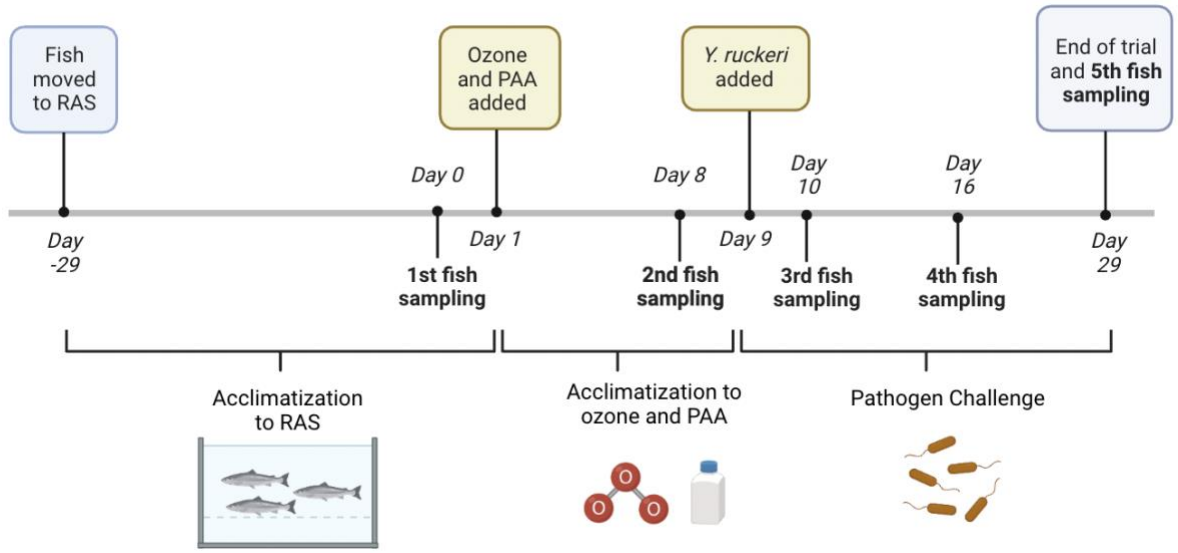

Figure 8: Shows timeline of the experiment, with specific events highlighted. The timeline is separated into three periods: acclimatization to RAS, acclimatization to ozone and PAA, and pathogen challenge. Atlantic salmon parr in 9 identical RAS were acclimatized for 29 days before being exposed to three different water disinfection treatments (O_3, PAA and control) on day 1. Y. ruckeri was administered into all systems 8 days later and exposed to this environment for 20 days. Fish samplings took place 5 times; before disinfectants were added (day 0), 24 h before pathogen challenge (day 8), 24 h after pathogen challenge (day 10), 7 days after pathogen challenge (day 16) and at the end of the trial (day 29). Figure created with Biorender.com.

2.2.1 Exposure regime of peracetic acid

A peristaltic pump (Ismatec™ IPC 4 Peristaltic Pump, Germany) was used to continuously add PAA into the pump sump of three randomly selected RAS systems (triplicates) throughout the day. A target concentration of 1 mg/L PAA was chosen for the three replicated RAS. This was done by adding 50.88 ml solution per day from the product Aqua-oxides super 15% PAA provided by the company S. Sørensen Thisted. This makes a PAA product dose of 0.05% of the make-up water daily. Concentration of PAA in the solution is listed as 15% PAA, 23 % Hydrogen peroxide (H_2O_2) and 10% Acetic acid (CH_3COOH). Concentration of PAA was 150 000 mg/L in the solution. The volume of Aqua-oxides super 15% PAA that needed to be added in the system was calculated with Eq. 4.

$$C1V1 \cdot C2V2 \qquad \text{Eq. 4}$$

C1 and V1 are respectively the initial concentration and volume, and C2 and V2 are respectively the final concentration and volume. The calculated volume was adjusted based on decay of PAA, which was accounted for in a beaker trial done in relation to this study (described in appendix 7.2). The PAA product was added every 30 minutes for semi-

continuous exposure to PAA, with 1.06 ml PAA product per 30 minutes. Water samples from fish tanks were analyzed for PAA (with chemical analysis method described in 2.5.1.1) three times a week.

2.2.2 Exposure regime of ozone

Three other RAS units were randomly chosen as triplicates to have ozone treatment. The set point for redox was 500 mV (measured from protein skimmer). The ozone generator used was Ozonizer S1000 (Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany), which generates ozone from the air. The output of ozone was 1000 mg/h. The ozone generator was connected to protein skimmer, and ozone added through there. For controlling and regulating redox, the Redox Potential Measuring and Regulating Unit (Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany) was used.

2.3 Experimental systems - RAS

Each of the nine RAS units consisted of a cylindroconical experimental tank with a volume of 0.5 m^3 (Figure 9). Furthermore, the tanks contained inlet and dual outlet drains, an emergency oxygen stone, and a sensor for oxygen and temperature (Oxyguard®, Farum, Denmark). Water left the tank through a bottom center outlet and a sidewall swirl separator that removed large solid wastes, before entering drum filters that filtered out suspended solids. These are 40-μm microscreen drum filters with a capacity of 5 m^3/h. The water then flowed through a moving bed bioreactor (200 L) that is filled with bio-media with nitrifying bacteria (20L of bio-media; 750 m^2 /m^3 specific surface area). Make-up water was added in the pump sump, where water was further pumped into three loops: degasser unit (loop 1), protein skimmer and ozone generator unit (loop 2) and oxygen saturation cone (loop 3), as shown in Figure 9. Loop 1 was off for this trial.

There was a pH probe (K01SVPHD, OxyGuard International A/S, Farum, Denmark) and water level sensors (KQ6001, ifm electronic gmbh, Essen, Germany) in the pump sump. Water was pumped (600 L/h) to the protein skimmer column in loop 2, where smaller organic compounds are removed. Ozone was also added here (Ozonizer S 1000, Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany) before the water returned to the biofilter. The water in loop 3 was pumped (1500 L/h) to an oxygen saturation cone (low-pressure)

where pure O_2 diffuses into the water. The water then entered a chiller and heater unit (TK-1000, TECO®, Ravenna, Italy) to adjust the temperature, before entering the rearing tanks. The PAA was administrated through the water inflow (pump-sump) via a peristaltic pump, while the ozone was administered via the protein skimmer.

The biomedia in the biofilters were pre-acclimatized for around 2 months by use of sodium bicarbonate ($NaHCO_3$), ammonium chloride (NH_4Cl) solutions (Permakem AS, Lørenskog, Norway), and fish feces from the fish that would be used in the experiment.

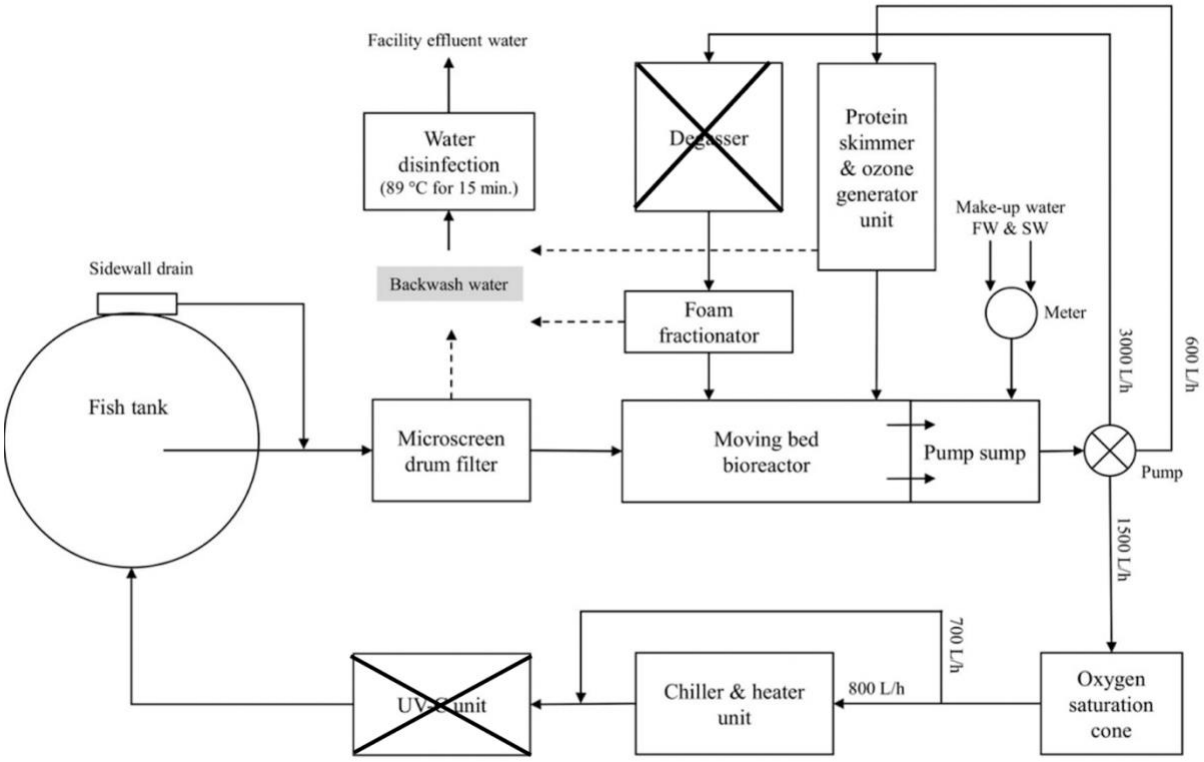

Figure 9: Flow scheme of the RAS units with different components listed, and order of them in the water loops. Note degasser loop and UV-C unit (crossed) is turned off for this experiment. Figure retrieved from Mota, Striberny, et al. (2022). Note: Swirl separator not shown.

2.4 Fish and husbandry conditions

The fish used in the experiment was Atlantic salmon parr that had been cultured at Tromsø Aquaculture Research Facility. Here they were hatched and raised in freshwater flow-through system, where they grew under 24h with daylight (LD 24:00) and were fed 24h a day at apparent satiation with a standard commercial parr feed with an automatic belt feeder.

There were a total of 1800 fish used in the trial, that were randomly distributed amongst the 9 RAS units. 200 fish were placed in each RAS unit. They were exposed to freshwater during the trial (0 ppt salinity). The starting body weight of the fish was on average 19.1 g when moved to the RAS units for acclimatization. The feed was standard commercial parr feed (Nutra RC, Skretting, Norway, 2mm) delivered via an automatic belt feeder and fed 24h a day at apparent satiation. However, at the start of the trial, in the acclimatization period of RAS, there was given a 50% feed rate, to avoid possible accumulation of TAN and NO_2^- in the system. Feed rate was slowly increased to 100% when the biofilter seemed to be handling this load (day 22 of acclimatization).

Water quality parameters in each RAS were tried to be kept on recommended values for optimal water quality for Atlantic salmon parr, with temperature, pH and oxygen saturation being measured automatically via sensors, and manual measurements taken regularly. Table 4 shows an overview of the different water quality parameters and operations, and their target values.

Table 4: Target water quality parameters at the fish tank outlet and water exchange target. Includes measurement methods used and frequency of the measurements.

Parameter	**Target value**	**Analytical method**	**Frequency**
Temperature	12-13 °C	FDO 925-P and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany	2 times a week
Dissolved oxygen	85-100 %	FDO 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany	2 times a week
pH	7.2-7.8	FDO 925 and Sentix 940 sensors, Multi 3630 IDS, WTW, Germany	2 times a week
Photoperiod	24:00 hours light	-	-
Water flow	12.5 L/min	-	-
Feed regime	100 % satiation over 24h	-	-

NH_4^+- N (Ammonium)	< 2 mg/L	Spectrophotometer (Test Kit 1.14558.001, Spectroquant ®, Merck KGaA, Darmstadt, Germany)	3 times a week
NH_3 -N (Ammonia)	< 0.035 mg/L	Calculated from the ammonium concentration as a function of pH, temperature, and salinity (Excel sheet provided by Vasco C. Mota, personal communication).	3 times a week
NO_2^- - N (Nitrite)	< 0.1 mg/L	Spectrophotometer (Test Kit 1.14776.0001, Spectroquant®, Merck KGaA, Darmstadt, Germany)	3 times a week
NO_3^- - N (Nitrate)	< 100 mg/L	Spectrophotometer (Test Kit 1.14942.0001, Spectroquant®, Merck KGaA, Darmstadt, Germany)	3 times a week
Turbidity	-	ORION AQ4500, Thermo Scientific®, Thermo Fisher Scientific, Nijkerk, the Netherlands	2 times a week
Salinity	0 ppt	Tetracon 325, Multi 3630 IDS, WTW, Germany	2 times a week
CO_2	< 12 mg/L	OxyGuard CO2 Portable, OxyGuard International A/S, Farum, Denmark	1 time a week

PAA	1 mg/L	Based on Pedersen et al. (2013) method, using Spectophotometer.	2 times a week
Oxidation reduction potential (ORP)/ Redox	300-350 mV	IDS ORP-T 900 SenTix®, WTW, Xylem Analytics, Germany	2 times a week
Total residual oxidants (TRO)	< 10 (µg/L Cl_2) * Based on brackishwater study	DR300 Pocket Colorimeter, Chlorine, Free + Total, Hatch, USA	2 times a week
Water exchange rate	500 L/kg feed	Calculated (Eq. 7)	Throughout trial
Water flow fish loop	1500 L/hour	Automatic (sensor)	2 times a week
Water flow protein skimmer loop	600 L/hour	Automatic (sensor)	2 times a week

Make-up water (L/d), water exchange (% volume/d) and water exchange rate (L/kg feed) were respectively calculated with Eq. 5, Eq. 6, and Eq. 7. Tank hydraulic retention time (HRT) was calculated with Eq. 8, make-up water (L/min/kg fish) with Eq. 9 and percentage of reused water with Eq. 10.

$$\text{Make} - \text{up water (L/d)} = \frac{\text{Water consumption (L)}}{\text{Number of days}} \quad \text{Eq. 5}$$

$$Water\ exchange\ (\%\ volume/d) = \frac{Make{-}up\ water\ (L/d) * 100}{RAS\ volume\ (L)} \quad \text{Eq. 6}$$

$$Water\ exchange\ rate\ (L/kg\ feed) = \frac{Water\ consumption\ (L)}{Cumulative\ feed\ (kg)} \quad \text{Eq. 7}$$

$$Tank\ HRT\ (min) = \frac{Fish\ tank\ volume\ (L)}{Total\ recycled\ flow\ (L/min)} \quad \text{Eq. 8}$$

$$Make - up\ water\ (L/min/\ kg\ fish) = \frac{make{-}up\ water\ (L/min)}{Fish\ biomass\ at\ end\ (g)/\ 1000} \quad \text{Eq. 9}$$

$$\%\ of\ reused\ water = \frac{Total\ recycled\ flow\ (L/min) - make{-}up\ water\ (L/min)}{Total\ recycled\ flow\ (L/min)} * 100 \quad \text{Eq. 10}$$

2.5 Samplings and analysis

Figure 10 shows an overview of the practical methods in the trial used to evaluate water quality, fish performance, health, and welfare. Note that all methods were not possible to be performed in house in this study, and some samples were therefore sent to external laboratories for analysis, including pathogen detection, producing histology sections, sequencing of bacteriology samples, and analyzing blood parameters.

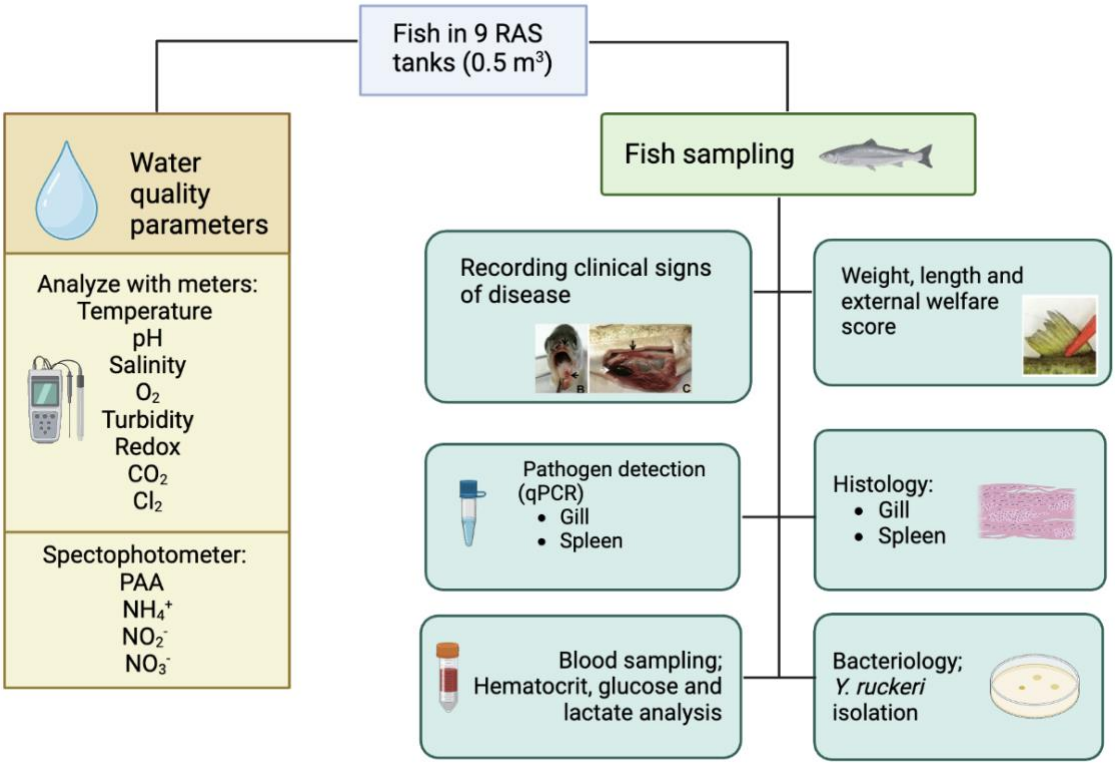

Figure 10: Show an overview of the trial, what water quality parameters will be measured, and what samplings, analysis and observation from fish will be taken. Figures of clinical signs retrieved from Kumar et al. (2015), and dorsal fin welfare scoring figure from Noble et al. (2018). Figure created with BioRender.com.

2.5.1 Water quality samplings

The water quality parameters that were manually measured or recorded are all listed on table 4, including method of analyses and frequency of measurements. Water quality was only measured for outlet fish tank water. Prior to the trial, all the measuring tools were calibrated according to their manual.

2.5.1.1 PAA measurement

The concentration of PAA was measured by a method developed by Falsanisi et al. (2006) and further modified by DTU AQUA (Pedersen et al., 2013). Before the trial started reagent 1 (R1) and reagent 2 (R2) were made in the laboratory at Nofima. They were prepared using the ingredients described in table 5.

Table 5: The contents of R1 and R2 solutions made in lab at Nofima, Tromsø.

Reagent	**Ingredients**
R1 (N,N-diethyl-pphenylenediaminesulphate salt, DPD)	N, N-diethyl-p-phenylenediamine sulphate salt (DPD), H_2SO_4 96%, Ethylenediaminotetraacetic acid Dihydrate (EDTA*2H_2O), Milli-Q water
R2 (potassium iodide buffer solution)	Na_2HPO_4*7 H_2O, KH2PO_4, KI, Milli- Q water, N_2OH (for adjusting pH to 6.5)

The principle of this measuring method is that the N,N-diethyl-p-phenylenediamine sulphate reacts with PAA at pH 6.5 to give a red color complex, that is catalyzed with potassium iodide (KI). By using a spectrophotometer (Spectroquant® Prove 100, Merck KGaA, Darmstadt, Germany) at 550 nm the absorbance of dilutions with known different PAA concentrations were measured, resulting in a standard curve that can be used to measure concentration of PAA in samples with unknown concentrations. Please refer to appendix section 7.1 for the standard curve measurement method.

For measuring PAA concentration in the sampled water, 2.5 ml of the water sample was pipetted into an empty cuvette. 250 µl of R1 was pipetted into the cuvette, before 250 µl of R2 was added. The solution was gently mixed, and after 30 second the absorbance was measured in the spectrophotometer at λ = 550 nm. Based on this absorption value (y-value) and the standard curve function made based on dilutions with known PAA concentrations (Appendix Figure 25), the concentration of PAA in the sample was calculated. The water samples were taken from the RAS units exposed to PAA, from the tank outlet water.

2.5.1.2 Turbidity measurement

The turbidity is measured with the help of a turbidimeter (ORION AQ4500, Thermo Scientific®, Nijkerk, the Netherlands) that gives measurements based on infrared ratio with a light-emitting diode (LED) that measures in nephelometric turbidity units (ntu). In addition, the UV Transmittance (UVT) for water samples from all tanks were measured with a spectrophotometer (uniSPEC 2, LLG labware, UK). This is a measurement of the amount of UV- light at 254 nm that passes through the water sample. The absorption of the water samples from each RAS was recorded twice every week and the measurement expressed as a percentage %UVT, with the formula below (Eq. 11).

$$UVT(\%) = 10^{-Abs} * 100 \qquad \text{Eq. 11}$$

2.5.2 Determine specific PAA decay rate and half-life

To understand how much the PAA demand in the RAS is, *in situ* beaker trials were done at Havbruksstasjonen i Tromsø. This was done to see the effect of turbidity (as a proxy for organic matter) on PAA decay. The results were used to help deciding PAA dosages for the systems.

Before treatment started six water samples with different turbidity were made by mixing distilled H_2O with the outlet water of a RAS unit. 1000 ml of RAS water was transferred to one glass beaker. A second glass beaker was filled with 80% RAS water and 20% distilled H_2O. Third, fourth, fifth and sixth glass beaker was respectively filled with 40%, 20%, 10% and 0% RAS water, and the rest distilled H_2O. The volume and turbidity in the different glass beakers are shown in table 6.

Table 6: Volume of RAS water and distilled water (dH_2O) in each glass beaker are described in the table. Turbidity measurement was also recorded (ntu).

Glass beaker	RAS water (ml)	dH2O (ml)	Turbidity (ntu)
1	1000 ml	0 ml	6.80
2	800 ml	200 ml	4.67
3	400 ml	600 ml	2.20
4	200 ml	800 ml	1.14
5	100 ml	900 ml	0.62
6	0 ml	1000 ml	0.01

A nominal PAA concentration of 1 mg/L PAA was added to each beaker (1 ml from the stock solution of 1000 mg/L), and the samples stirred with a magnetic stirring device. After addition of PAA the water was analyzed after 10, 15, 25, 35, 45, 60, 120, 180 and 240 min. The temperature in the room was 17 °C. Based on the PAA measurements a graph was made with concentration of PAA as a function of time. Curves for each beaker were made with exponential trendlines and functions for each (Appendix Figure 26).

According to Newman (1995) PAA degradation exhibits exponential decay. Therefore, the first-order decay rate constant (k, h^{-1}) was calculated using the Eq. 12

$$C_t = C_0 \times e^{-kt} \quad \text{Eq. 12}$$

Where C_t is the concentration of PAA at time t(h) and C_0 is the initial concentration. Half- life ($T_{1/2}$) was calculated with Eq. 13

$$T_{1/2} = ln2/k \quad \text{Eq. 13}$$

The results of this beaker trial are described in appendix section 7.2.

2.5.3 Fish sampling

In total there were 5 fish samplings as shown in the timeline on Figure 8. In all the fish samplings the following were done: 5 random fish from each tank, in total 45 fish (5 x 9), were humanely euthanized with overdose of benzocaine (Benzoak vet, ACD Pharmacuticals AS, Leknes, Norway, 200mg/ml). Each fish was given a running number where weight (g) and fork length (mm) were measured and recorded. Blood samples were taken from each fish, for analysis of hematocrit, and plasma analysis of glucose and lactate content. An external welfare score evaluation was done according to the FISHWELL handbook (Noble et al., 2018), before the fish was dissected. Specific signs of clinical disease in the individual fish were recorded, and specific tissues/organs cut and sampled for histology (in formalin) and pathogen detection (in RNA-later) (described in table 7). The last sampling an extra 25 fish were taken per tank where body weight and fork length were recorded, in addition to recording clinical disease signs.

The collected tissues were gills and spleen for histology and pathogen detection. In addition, samples for bacteriology were taken from the head kidney on the last sampling and from fish

that died throughout the trial. Table 7 is a summary of the organ samples taken, where they were collected from and in what solution they were preserved.

Table 7: Summary of the tissue samplings, how they were collected, and what they were analyzed for.

	Formalin (Histology)	**RNAlater (qPCR, Pathogen detection)**	**Blood agar plates (Bacteriology)**
Gills	Right side: 2nd gill arch	Left side: 2nd gill arch	-
Spleen	0,4x1x1 cm slice	ca. 5x5x5 mm	-
Kidney	-	-	Swabs from head kidney on blood agar plates

2.5.3.1 External welfare scoring, and clinical signs observation

The external welfare status was evaluated with a scoring scheme based on the FISHWELL handbook (Noble et al., 2018), for each of the 45 fish sampled in each sampling. The following morphological operational welfare indicators (OWI's) were scored: cataracts, exophthalmia, eye hemorrhaging, epidermal damage (lesions/wounds), scale loss, opercular damage, emaciation state, snout damage, vertebral deformity, upper/lower jaw deformity, and fin damage. The scoring range for each indicator is graded from 0 (best/normal) to 3 (worst). Only one person did the scoring throughout the trial, to ensure objectivity. Figure 11 shows all the OWI's that were used.

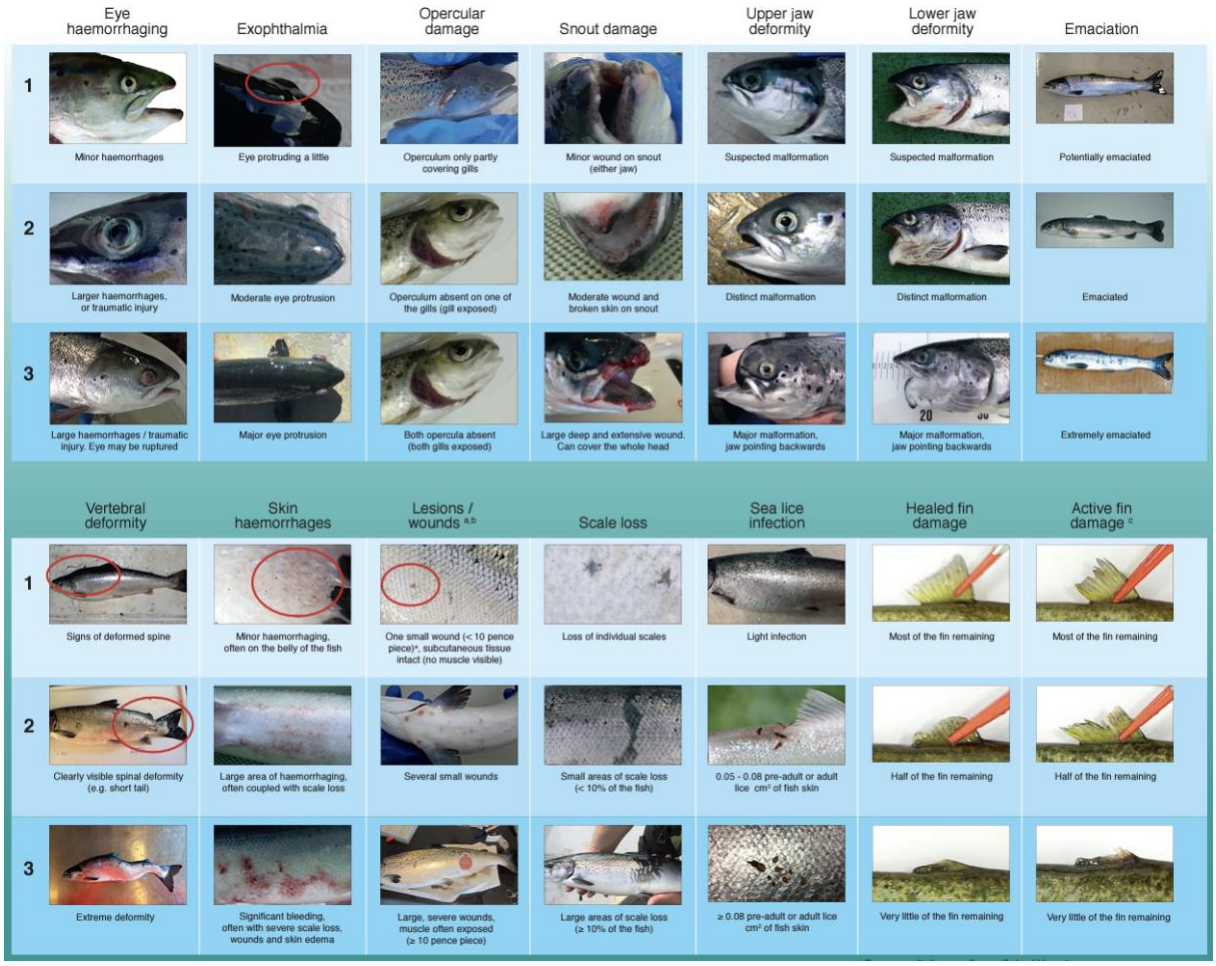

Figure 11: Poster of FISHWELL Morhological Operational Welfare Indicators (OWI's) for farmed Atlantic salmon. Level 0 for each indicator indicates little or no evidence of this OWI (normal), level 1-3 gradually increases severity of the indicator with level 1 indicating minor, and level 3 indicating clear evidence of OWI. Level 0 is not illustrated. Retrieved from Noble et al. (2018).

Clinical signs for enteric red mouth disease were recorded on the same fish in the samplings, where the presence of the following clinical signs was noted: exophthalmia, enlarged spleen, external hemorrhage, ascites, and hemorrhage in inner organs (Figure 12).

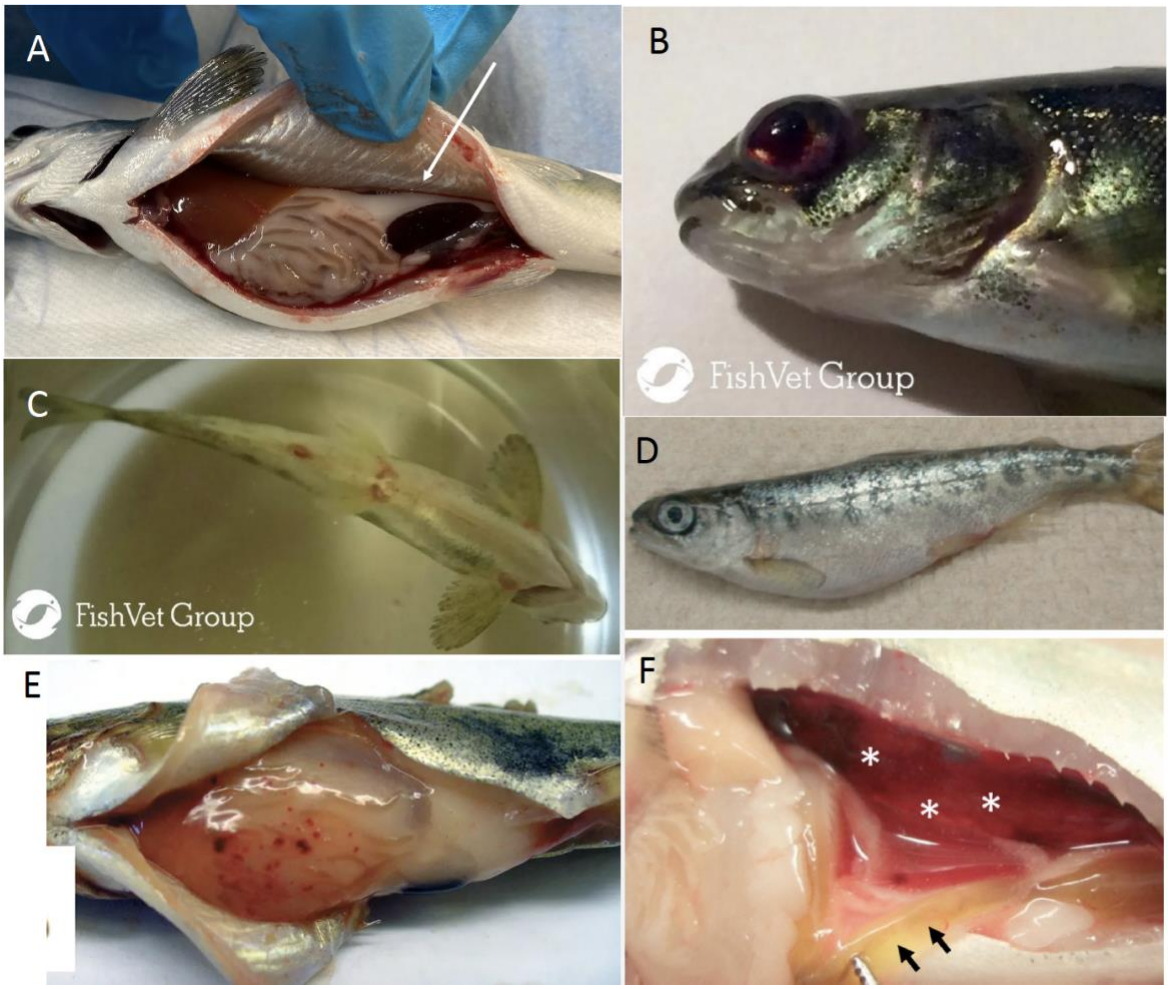

Figure 12: Fish with clinical signs of ERM with A) Enlarged spleen (arrow), B) exophthalmia with hemorrhage in eye, C) external hemorrhages at the base of fins, D) and F) ascites, and E) hemorrhages in internal organs. Picture A) was taken by Samaneh Mousavi, B) and C) was retrieved from Iglesias (2018), D) and F) retrieved from Kim and Faisal (2010), and E) retrieved form Brunt and Austin (2009).

2.5.3.2 Fish performance metrics

Calculation of condition factor (K-factor), thermal growth coefficient (TGC) and specific growth rate (SGR) was calculated from the measurements of weight and length of each fish, and are respectively shown in Eq. 14, Eq. 15, and Eq. 16.

$$K - factor = \frac{W}{L^3} \cdot 100 \qquad \text{Eq. 14}$$

W and L is respectively weight (in grams) and length (in cm) of the individual fish.

$$TGC = \frac{W2^{\frac{1}{3}} - W1^{\frac{1}{3}}}{T \cdot \Delta t} \cdot 1000 \qquad \text{Eq. 15}$$

T is the temperature in °C and Δt is the the number of days between the time of the samplings (W1 and W2).

$$SGR = \frac{ln\,Wt - \ln W0}{t - t0} \cdot 100 \qquad \text{Eq. 16}$$

W0 is the initial weight at time t0, while Wt is the average weight at time t.

2.5.3.3 Histology

The tissues analyzed for histology were gill and spleen. These tissues were collected for each sampling. The collected tissues from each individual fish (n = 5 fish/tank) were put in a neutral formalin container with 10% formalin (BiopSafe®, Mermaid Medical, Denmark), and stored at 4 °C until analysis. Samples were sent to Veterinærinstituttet (Harstad, Norway), for preparation of histology slides. Personnel there prepared the slides for us, by embedding them in paraffin, slicing in to 3-µm-thick section before they were stained with Periodic Schiff-Alcian Blue (AB-PAS). The slides were digitized using a slide scanner with a magnification of 20X (Aperiod CS2, USA), and sent back to us as slide images. The images were viewed via the software QuPath v0.4.1, for bioimage analysis.

For overall evaluation of the gill morphology (overall gill score), a semi-quantitative scoring system previously published in Alipio et al. (2023) study was used and is based on a modified scoring system developed specifically for each mucosal tissue (Appendix section 7.3 for scheme). Briefly, the scoring system grades gills from 0 to 3, with 0 indicating a normal morphology, 1- mild pathological signs, 2- moderate pathological signs and 3- severe pathological signs.

I addition, important pathological changes of the gills were recorded by following a previously published protocol (Stiller et al., 2020). Six gill filaments per fish were randomly chosen, and in each 40 lamellae were analyzed giving a total of 240 lamellae evaluated per fish. The histopathological signs that were looked for and recorded were hyperplasia, lamellar fusion, hypertrophy, and necrosis. If no pathological changes were observed for a lamella, they were recorded as “healthy”.

Pathological changes in spleen were noted and done based on a semiquantitative scoring system developed by Deshmukh et al. (2013), that is based on mathomorphological changes

in spleen (Appendix 7.4 for scheme). Histological alterations in spleen that was recorded were necrosis, red pulp hyperplasia, capsular damage, cystic/clear space in parenchyma, and stromal cell hyperplasia & hypertrophy. These parameters were scored from 0 to 3, where 0 indicated normal/minimal changes, 1- mild changes, 2- moderate changes, and 3- severe changes.

2.5.3.4 Pathogen detection

The tissues analyzed for pathogen detection were gill and spleen. The collected samples were placed in 2 ml cryotubes with 1.5 ml RNAlater, before they were stored for 24 hours in 4°C, and thereafter frozen in -80 °C. All 45 spleen samples from the last sampling (n=15 per treatment) were sent to PatoGen AS for detection of the pathogen *Y. ruckeri.* The company performed qPCR on the samples sent, and results were sent back as positive or negative detection, ct-values, and level of detection.

During the trial there was an outbreak salmon gill poxvirus (SGPV) disease in other sections of Havbruksstasjonen i Tromsø. Although in the present trial there were no clinical symptoms for this disease, 27 gill samples from fish in first and last sampling of the trial were sent to PHARMAQ for detection of SGPV (n=9).

2.5.3.5 Bacteriology

On the last sampling day bacteriology samples were collected. Swabs were taken from the head kidney of 12 fish (n=4 per treatment) and plated on blood agar plates with no NaCl. These were stored 12-15 °C for 3-4 days, to observe an eventual growth of *Y. ruckeri*. In addition, for fish that died during the trial the same procedure was done. Growth on the plates were recorded, and all bacteriology samples were sent to UNN for sequencing of *Y. ruckeri* to confirm presence of the bacteria.

2.5.3.5.1 . DNA isolation and PCR of bacteriology samples for sequencing of *Y. ruckeri*

Before sending bacteriology samples for sequencing, the DNA was extracted from the colonies grown in the blood agar and ran through PCR (polymerase chain reaction) for amplification.

First step of isolating the DNA, was to harvest the bacteria in each blood agar sample with colonies overnight. A sterile loop was used to transfer some colonies from our samples in 1 ml 0,9% NaCl for each sample. These were shaken overnight at 12 °C (24 hours). The second step was to prepare lysates. The samples were centrifuged (Eppendorf ® Centrifuge 5804, Germany) 17 000 x g for one minute to make cell pellets. The supernatant was thrown away from the samples. For DNA extraction a PureLink™ Genomic DNA Mini Kit was used (Thermo Fisher Scientific, USA). The pellet was resuspended in 180 µL PureLink™ Genomic Digestion buffer, before 20 µL of Proteinase K was added to lyse the cells. The samples were vortexed for 5 seconds, and thereafter incubated for 1.5 hours in 55 °C with vortexing two times in between.

RNase A from the kit was added to the samples and then vortexed briefly before incubation at room temperature for 2 minutes. 200 µL of PureLink™ Genomic Lysis/Binding buffer was added to the samples and mixed by vortexing. Thereafter 200 µL of 96-100% ethanol was added to the lysate. Samples were mixed with a vortex for 5 seconds to get a homogenous solution.

After the lysate was prepared, the binding of DNA was proceeded. The solution/lysate from each sample was transferred to a PureLink™ Spin Column. Samples were centrifuged at 17 000 x g for one minute, and thereafter the Spin Column transferred to new PureLink collection tubes. 500 µL of Wash buffer 1 (prepared with ethanol before using it for the first time) was added to the column. The column was centrifuged at 17 000 x g for one minute. The collection tubes were discarded, and columns transferred to new PureLink collection tubes. 500 µL of Wash buffer 2 (prepared with ethanol before using it for the first time) was added to the columns, and centrifugated 17 000 x g for three minutes.

For eluting the DNA, the spin columns were placed in sterile 1,5 mL microcentrifuge tubes, where 75 µL of PureLink™ genomic elution buffer was added to the column. After 1 minute incubation in room temperature the samples were centrifuged 17 000 x g for one minute. The columns were discarded. DNA concentration was measured using Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA), and frozen at -80 °C for 24 hours.

The samples were defrosted the next day and prepared for PCR. A master mix was made for the sequencing reaction. This consisted of pipetting 34 µL BigDye™ Terminator v3.1, 68 µL 5X sequencing buffer, 8.5 µL primer (10µM) and 59.5 µl H_2O in an Eppendorf tube. Enough master mix for 17 samples was made. Two master mixes were made: one with forward primer

and one with reverse primer. The primers used were forward primer *Y. ruckeri* 16S rRNA GCG-AGG-AGG-AAG-GGT-TAA-GTG and reverse primer *Y. ruckeri* 16S rRNA GTT-AGC-CGG-TGC-TTC-TTC-TGC.

In 15 sterile PCR tubes 10 µL of master mix with forward primer was added in each. 15 other PCR tubes were added 10 µL of master mix with reverse primer. Before adding the template (extracted DNA samples), these samples with extracted DNA were briefly vortexed. 10 µL of each template sample were pipetted into their own tubes with master mix- one with forward primer and one with reverse primer. Thereafter each sequencing mix was vortexed a few seconds to mix the solutions. The strips were put in a PCR machine (GeneAmp® PCR System 2700, Applied Biosystems, USA), with the PCR program of 96 °C for 1 minute, and 25 cycles of 96°C for 10 second, 50°C for 5 seconds and 60 °C for 4 minutes, before the program ends with 4 °C until the machine was stopped.

After PCR, the amplified DNA samples were sent to UNN for nucleotide sequencing. The results came back as DNA sequence visualization that were run on the webpage BLAST ® (program version BLASTN 2.13.0+), to find matches for the sequences in their database (*Basic Local Alignment Search Tool (BLAST)*).

2.5.3.6 Blood sampling

Blood from fish were collected by personnel in Havbruksstasjonen. Blood was taken from the caudal vein complex of the fish with a Vacutainer Glide (21G x1.5``) and Vacutainer 2 mL with clot activator. The blood samples were then rested at room temperature for a minimum of 30 minutes, before they were centrifuged at 3200 rpm for 10 minutes. 100 µL serum was then transferred to 2 mL cryotubes for analysis of glucose and lactate. The samples were stored on dry ice at -80 °C for further analysis. For analyzing glucose and lactate content in the plasma, samples were sent to the laboratory in Nofima, Sunndalsøra, where they were analyzed by personnel using a method of potentiometry with Pentra C400 (HORIBA medical, France).

The hematocrit was obtained by filling two microcapillary tubes per fish (2-3 drops of blood), from the same heparinized vacuum tubes that were centrifuged to obtain serum. These filled microcapillary tubes were then centrifuged at 12 000 rpm for 3 minutes. A scale was used to determine the % of packed cell volume (PCV)/ hematocrit, which is the calculated volume

percentage of red blood cells (erythrocytes) in your blood. Length of red blood cells and length of whole blood in mm were measured from the microcapillary tubes. Hematocrit was calculated with Eq. 17

$$\%\ hematocrit = \frac{Length\ red\ blood\ cells\ (mm)}{Lenght\ whole\ blood\ (mm)} \quad \text{Eq. 17}$$

2.6 *Yersinia ruckeri* culture

24h-culture of *Y. ruckeri* (ca. 10^8 cfu/ml) was made for the pathogen challenge in the trial and added to the pump sump of all the 9 RAS units. The strain used in the trial was *Yersinia ruckeri* 2014-70 F646 (serotype O1), that was isolated from the head kidney of infected Atlantic salmon and preserved in glycerol at -80 °C. This was originally received from the Veterinary Institute in Harstad on 20.02.15. Before the pathogen challenge the bacteria were defrosted and inoculated onto plates of Blood agar (BA) without NaCl and incubated 2-3 days at 12 °C. Thereafter, single colonies were transferred for further culturing to a 10 mL marine broth (MB) liquid media (76448 Marine Broth 2216, Sigma-aldrich, Germany) in a conical flask. The flask was incubated for 24 hours in an orbital shaker (120 rpm) at 11-12 °C (KS501 digital, IKA, Germany). The density of bacteria was measured by a spectrophotometer (LLG-uniSPEC 2 UV/VIS-Spectrometer 190-1100nm, China), where when the solution was valued 1 absorbance unit (AU), the concentration was estimated 10^9 CFU/ml with the optical density on 550 nm wavelength (OD550nm).

Thereafter, pre-cultures were made, where three bottles with 10 ml medium (marine broth, small bottles) were inoculated with 3-4 pure colonies each and incubated for 20-21 hours. Furthermore, these were inoculated in 8 bottles of 200 ml medium (marine broth, large bottles), which would be the challenge material. Ca. 7 ml from the pre-culture (per bottle) was inoculated for ca. 20-21 hours. 6 of these culture flasks were mixed in one large flask that was placed on ice. This bacterial suspension was diluted 1:10 with phosphate-buffered saline (PBS), and OD520 was measured twice. The OD520 was adjusted to 0.047 with marin broth liquid. The real OD of our suspension was 0.47. An OD520 value of 0.5 is assumed to contain 10^8 bacteria/ml.

To calculate the actual infection dose in the form of colony-forming units (cfu)/ml a ten-fold dilution series was made with PBS, and plated on BA in parallels, with 100 µl per plate.

These were incubated at 12 °C for 5 days until colonies could be seen. From the average cfu in the plates diluted by a factor of 10^6, the actual cfu/ml in our challenge material was calculated by the formula in Eq. 18:

$$Cfu/ml = \frac{No.colonies * dilution\ factor}{Volume\ of\ culture\ plated\ in\ ml} \quad \text{Eq. 18}$$

The infection dose was calculated to be 5.3 x 10^8 cfu/ml in the challenge material. The challenge material (in total 3600 ml) was brought on ice to Kårvika. Before challenge these were taken off from ice and kept at room temperature for around 30 minutes. The solution was mixed and by using a measuring cup, 400 ml of the suspension with a concentration of 5.3 x 10^8 cfu/ml was added to the pump sump of each RAS unit.

2.7 Statistical analysis

Excel was used for calculating mean and SD for all the data collected. For statistical analyses the program IBM ® SPSS ® Statistics 26 (IBM, Corp., USA) was used. Each data set obtained from the trial was expressed as mean ± SD and checked for normal distribution using the Shapiro-Wilk test and homogeneity using Levene's test. One -way ANOVA analysis was used to compare fish performance metrics, water quality, prevalence of histology scores, prevalence of the clinical scoring and prevalence of positive detection of *Y. ruckeri* between the different treatments at a timepoint. This was followed by Tukey's post- hoc test for equal variances to compare differences between the treatments. Two-way ANOVA analysis was used to determine if there were differences between sampling points and to see if there is interaction between sampling and treatment for the dependent variable. Data that was in percentages (hematocrit, and prevalence of variables) were arcsine-transformed prior to ANOVA, to fulfill assumption of homogeneity of variances. Other data that were not fulfilling assumption of homogeneity of variances were log-transformed. When $p < 0.05$ the results were considered statistically significant.

3 Results

3.1 Fish performance

The mortality throughout the experiment was minimal, with a total of 5 fish dead, representing all treatments. Three fish died from the ozone tanks and two from control. All mortalities happened the last week of the trial (>2 weeks since *Y. ruckeri* was introduced to the systems), and all were positive for *Y. ruckeri* based on bacteriological cultivation and sequencings results.

Figure 13 shows the average body weight of the fish in different treatments through time, starting from the acclimatization period (day 0-day 29), and ending 29 days after treatment started + 20 days after *Y. ruckeri* was added to the system. The initial mean weight before treatment started (day 29) was 31.43 g ± 1.48. The final mean weight (day 58) was 56.30 g ± 2.37 for control group, 55.59 g ± 5.61 for PAA group and 61.42 g ± 4.07 for ozone group. The last sampling there were observable differences in average weight with ozone being higher than PAA and control treatment as seen in Figure 13, but this was of no statistical significance ($p = 0.261$).

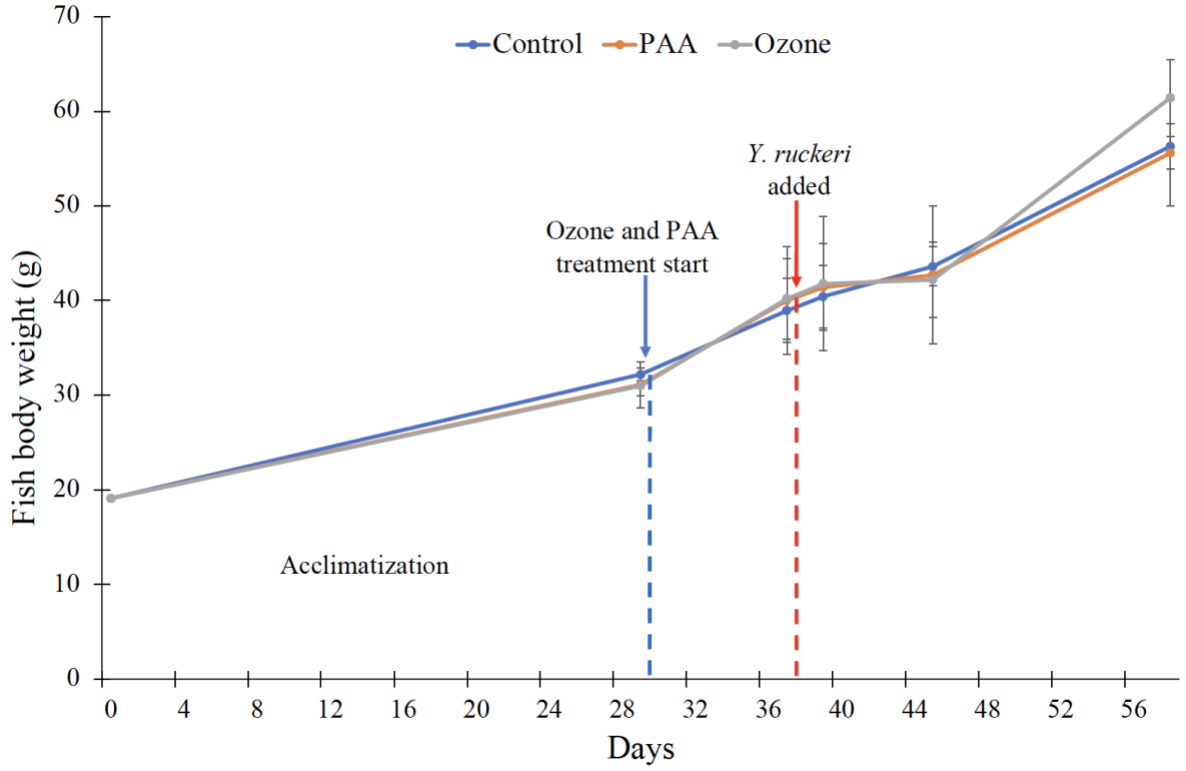

Figure 13: A graph that shows body weight of fish in grams (y-axis) for different treatments through the timeline of the trial (days in x-axis). Values are presented as mean ± SD for each treatment group*. Control group had no treatment, PAA group was treated semi-continuously with PAA (1.06 ml PAA product per 30 min) added through the pump sump, and ozone group treated with ozone continuously with ORP of 500 mV through protein skimmer. Blue dotted line represents when ozone and PAA treatment started (day 30), and red dotted line represents when Y. ruckeri was added to all systems (day 38). Days prior to day 29 was acclimatization period in RAS. Samples consisted of n=15 from 1-4th sampling per treatment, while last sampling n=90 for control and PAA each, and n=95 for ozone treatment.*

Figure 14 shows the difference between mean TGC and mean SGR for fish in different treatments from 1st sampling (before treatment started) to 5th sampling (last trial day). Mean final k-factor is also presented. TGC and SGR of the fish was higher for ozone treatment group, compared to PAA and control group, while final k-factor has less variation between treatments. There were no statistical differences among treatments for any of the above parameters.

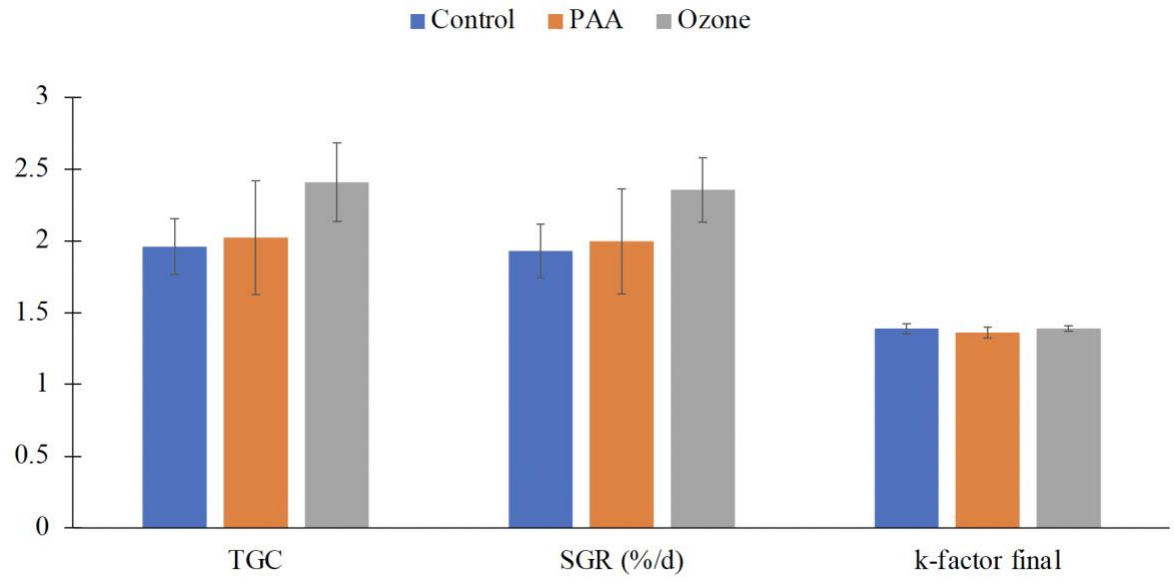

Figure 14: Bar chart which shows growth parameters; TGC, SGR (%/d), final K-factor for control, PAA and ozone treatment, presented as mean ± SD for each treatment group*. The time period is from 1st sampling (day 0) to 5th sampling (day 29), and final k-factor is from the 5th sampling. The sampling pool consisted of 45 fish for sampling 1 (n=15 per treatment), and 275 fish for sampling 5 (n=90 for control and PAA treatment each, and n=95 for ozone treatment).*

3.2 Detection of *Y. ruckeri* and SGPV

The results of detection of *Y. ruckeri* with qPCR analysis (from PatoGen) of spleen samples from the last sampling (20 days after adding *Y. ruckeri*) are summed up in table 8 as percentage of positive samples per treatment group. Of the 45 spleen samples sent for analysis (n=15 each treatment), only 8 came back positive for *Y. ruckeri*. All treatment groups had minimum one positive sample. Control had the most positive cases with 5 cases out of 15 (33.3% ± 30.6), while PAA group had the second most with 2 positive cases (13.3% ± 11.5). Ozone group only had one positive case (6.7% ± 11.5). There were no statistical differences among the treatment groups. The Ct-value from the qPCR ranged from 29.0 to 36.9.

Table 8: In total 45 spleen samples were sent (n= 15 per treatment) for qPCR detection of Y. ruckeri at the end of the trial. The percentage of positive samples per treatment group are described as average prevalence of positive samples ± SD. One way ANOVA test results shown for % positive samples (arcsine-transformed).

	Control	PAA	Ozone	P-value (ANOVA)
n total	15	15	15	-
Total positives	5	2	1	-
% positive	33.3% ± 30.6	13.3% ± 11.5	6.7% ± 11.5	0.469

The results from PHARMAQ on detection of SGPV with qPCR of the gill samples sent, showed no detection of the virus in fish from the first sampling. Results from the last sampling showed one positive detection SGPV in gills out of the 27 fish analyzed. The relative pathogen amount was described as low in this sample.

Bacteriology samples from head kidney taken the last sampling (n=4 each treatment) resulted in growth in ¾ (75% ± 35) of blood agar plates from each treatment. All the 5 collected dead fish also had growth. Results from UNN of the nucleotide sequencing in all the samples matched with several *Y. ruckeri* strains in the BLAST database.

3.3 External fish welfare

Figure 15 shows the average score of operational external welfare indicators at the first (A) and last (B) sampling point. Most external welfare indicators had a score of 0 throughout the whole trial. The indicators that were compromised were operculum damage, epidermal damage, scale loss and fin damage. A few of the indicators had mean scores above 1 (pectoral fin damage, dorsal fin damage and scale loss). There were no significant differences of the average external welfare score among treatments for any of the indicators at any of the sampling points. It appeared pectoral fin damage (right) and caudal fin damage, significantly increased from 1st sampling ($p = 0.007$ and $p = 0.034$ respectively) for all treatments, however dorsal fin damage significantly decreased from 1st sampling ($p < 0.001$).

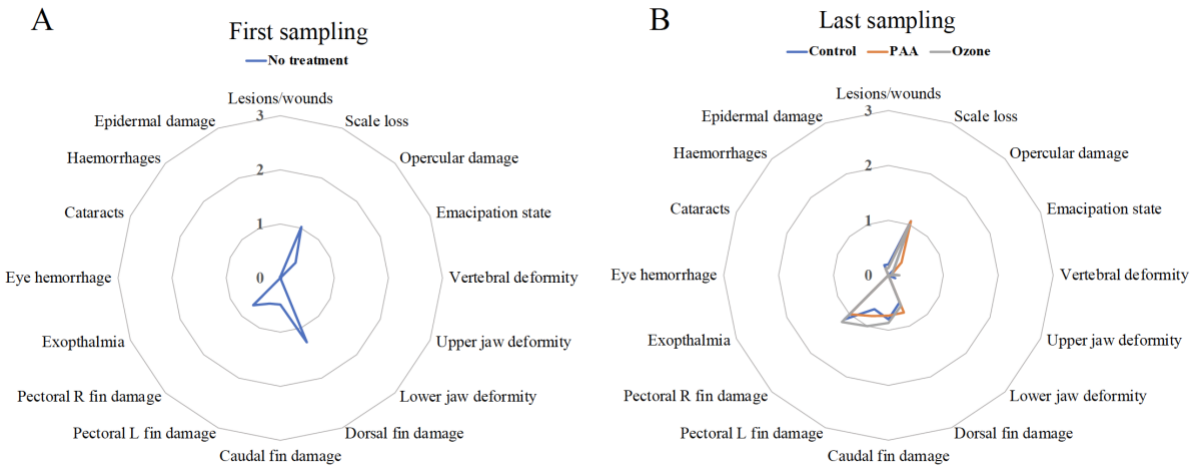

Figure 15: Radial chart showing the mean scores of each external welfare indicator before treatment had started (day 0) in A), and B) 29 days after treatment with ozone and PAA + 20 days since Y. ruckeri was added to all systems (day 29). The scoring strategy followed a 0-to-3-point system based on FISHWELL handbook (Noble et al., 2018) where higher values indicate worse conditions. 0 represents excellent condition, while 3 indicates severely compromised state for the indicator. For each sampling n=15 per treatment.

3.4 Clinical signs observations

Clinical signs of ERM were recorded throughout the trial, but only on the last sampling point there were significant observations of these. Figure 16 shows the prevalence of different clinical signs on the last sampling day, in different treatments. There were mostly mild cases of enlarged spleen, eye hemorrhage, exophthalmia, and hemorrhage in inner organs observed, which represented fish from all treatments. Ascites was not observed in any fish, and exophthalmia had low prevalence in all treatments. Enlarged spleen was observed in 31% ± 14 of the control fish, 16% ± 8 of PAA fish and 5% ± 5 of ozone fish, with a significant difference for this clinical sign between treatments. Ozone group had significantly lower prevalence of enlarged spleen than the control group ($p = 0.038$). Other clinical signs observed were eye hemorrhage and hemorrhage in the liver. The prevalence of mild eye hemorrhages ranged between 14% to 26% with the lowest prevalence in control group, but no significant difference between treatments. Prevalence of hemorrhage in liver also had no significant difference between treatments but had the highest prevalence in PAA group with

38% ± 12, and the lowest in ozone treatment with 20% ± 13.

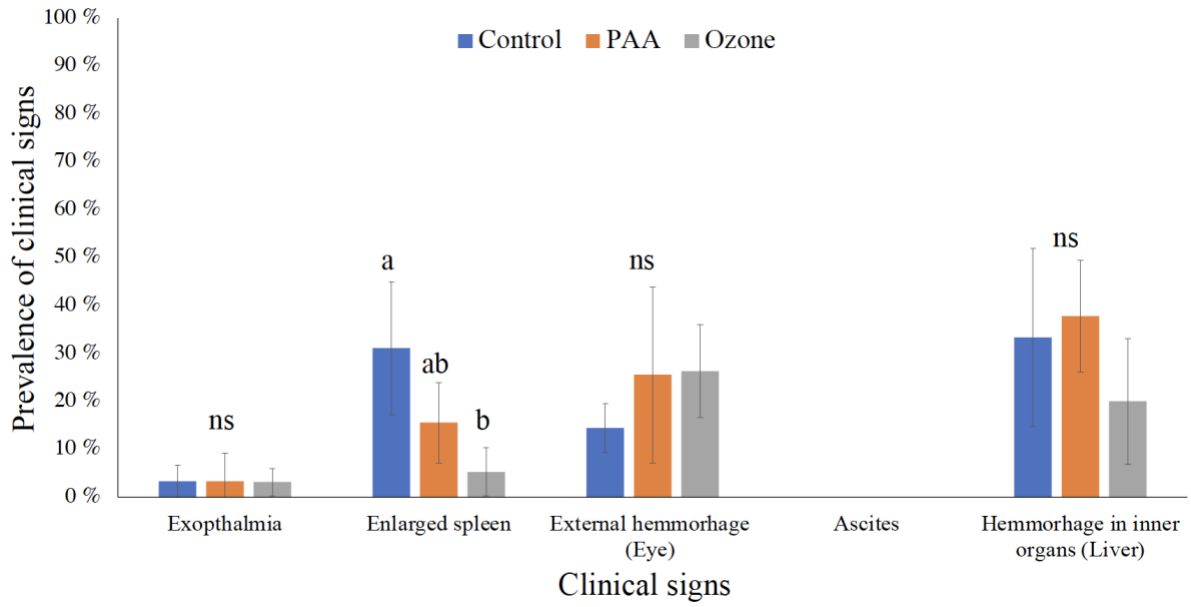

Figure 16: Bar chart that represents average prevalence ± SD (y-axis) of the clinical signs; exophthalmia, enlarged spleen, external hemorrhage, ascites, and hemorrhage in inner organs, observed in n=15 fish per treatment (control, ozone and PAA), on the last sampling (day 29). The external hemorrhage was only observed in eyes, and the hemorrhage in inner organs were only observed in the liver. ns = non-significant differences between treatments. Different letters on bars indicate which treatments are significantly different to each other within the sampling point.

3.5 Histological evaluation

3.5.1 Gill evaluation

Figure 17 show prevalence of overall gill scores from fish in different treatments for each sampling period. The histological sections of gills before treatment started (day 0) show the gill score varied from 0-2. On day 0 most of the fish had a gill score of 1 (44% ± 25). After over a week of treatment (day 8), a few of the gills from control and PAA have scored 3, however low in prevalence (<13%). After *Y. ruckeri* is introduced (day 10, 16 and 29) the prevalence of the different scores varies from 0-3 with the highest prevalence of score 3 registered in PAA group on day 16 (20% ± 20). The highest prevalence of score 0 is the ozone group on day 29 with 58% ± 18, and ozone group also register highest prevalence of score 0 at all sampling points except on day 8. Significant inter-treatment differences for each score within each sampling point was only identified on the last sampling day (day 29), where

ozone group had significantly higher prevalence of score 0 than PAA group (p = 0.043). No significant differences were found among sampling points for any score.

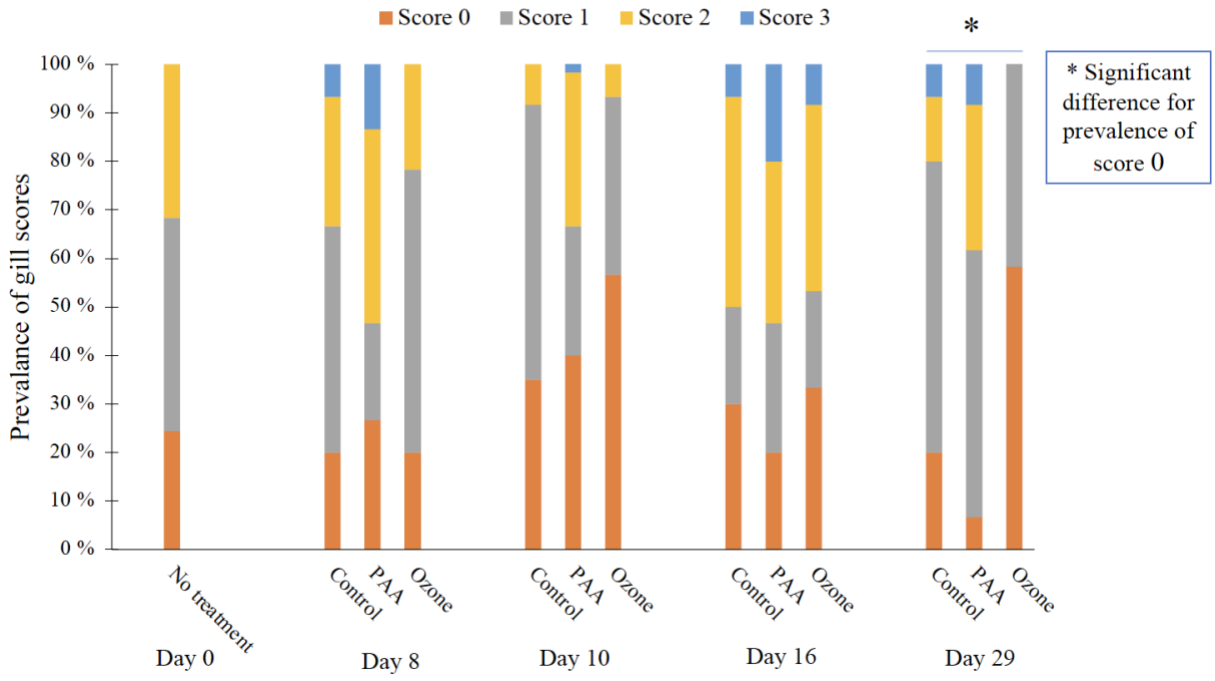

Figure 17: Bar chart of the prevalence of gill scores (y-axis) for each treatment group (control, ozone and PAA), within each of the five sampling points (x-axis), based on evaluating histology gill sections. Graded score from 0 to 3 based on Alipio et al. (2023) study, where score 0- indicates healthy gills with least amount of pathological changes observed, and score 3- indicate "worst" gill with the most pathology observed. Astriks "" denotes statistically significant differences between treatment within the sampling for score 0 (P-value<0.05). Gill histology of n=15 fish per treatment in each sampling point were evaluated.*

Pathological findings that were observed throughout the trial in all treatments were hyperplasia, hypertrophy and fusion as shown on Figure 18.

Figure 18: Histological sections of gills of Atlantic salmon from the trial stained with AB-PAS. A) shows healthy gills with well-defined structure, B) shows gill lamellae with hypertrophy (arrow), C) shows lamellae with hyperplasia and hypertrophy, and D) shows gills with fusion of lamellae (arrow). Scale bars represent 100 µm. Screenshot taken from the software QuPath v0.4.1.

Figure 19 shows prevalence (%) of some common gill pathologies that were recorded in the gills of Atlantic salmon in the last sampling. Gill morphology was relatively healthy, where minimum 80% of lamellas evaluated in all treatments was classified as healthy. Cases of hyperplasia and hypertrophy was recorded in fish for all treatments. Most cases of hyperplasia were recorded in PAA treatment group, with 14% ± 7, where control had 6% ± 4 and ozone the lowest prevalence with 4% ± 3. Hypertrophy had lower prevalence in all treatments (<5%)

and followed the same trend with most cases recorded in PAA treatment group. Fusion of lamella was only observed in PAA group, but in very low prevalence (0.2% ± 0.1). No significant differences were found between the treatments for the prevalence of these gill alteration.

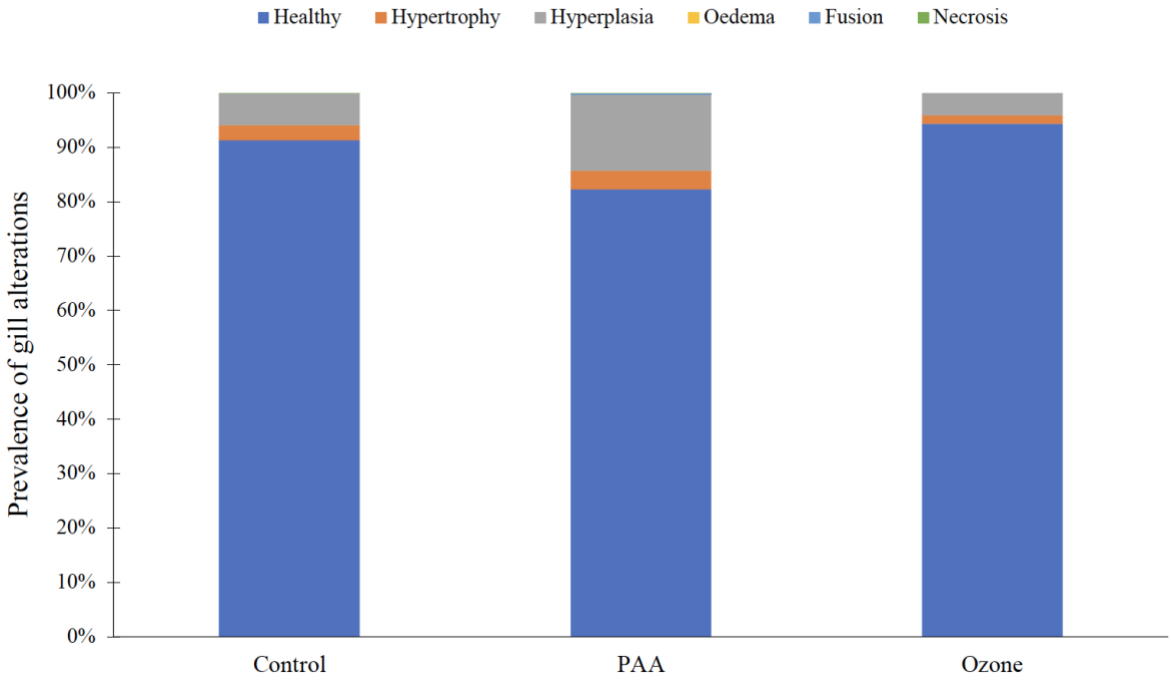

Figure 19: Bar chart with average prevalence of different gill alterations (y-axis) observed in gill histology sections on the last sampling day: hyperplasia, hypertrophy, fusion, oedema, and necrosis, including healthy gill lamellaes in control, PAA and ozone treatment. Pathologies were recorded from 240 individual lamellae per fish (n=15 gill per treatment).

3.5.2 Spleen evaluation

Histopathological changes recorded in spleen were necrosis, red pulp hyperplasia, cystic/clear space in parenchyma and stromal cell hyperplasia and hypertrophy. All treatments had some degree of these lesions, but most had mild changes (score 0-1). No clear large differences were found between treatments or samplings for necrosis, cystic/clear space in parenchyma, and stromal cell hyperplasia and hypertrophy. However, the score prevalence of red pulp hyperplasia (Figure 20), changed through samplings. Score 0 – no red pulp hyperplasia – was significantly lower for the last sampling compared to day 0, day 8 and day 10 ($p = 0.001$) for all treatments. For the first sampling (day 0), 72% ± 19 of spleens (no treatment) had no observed red pulp hyperplasia (score 0). For the last sampling (day 29) this amount had decreased to 43% ± 21 for the average in all treatments. Prevalence of score 1-3 increased the

last two samplings, where 57% of the spleen had these scores in last sampling, compared to 27% in the first sampling. No significant inter-treatment differences were present for the prevalence of the different scores within the same sampling point. Figure 21 show some of the observed spleens in the trial with scores of 0, 1, 2 and 3 for red pulp hyperplasia.

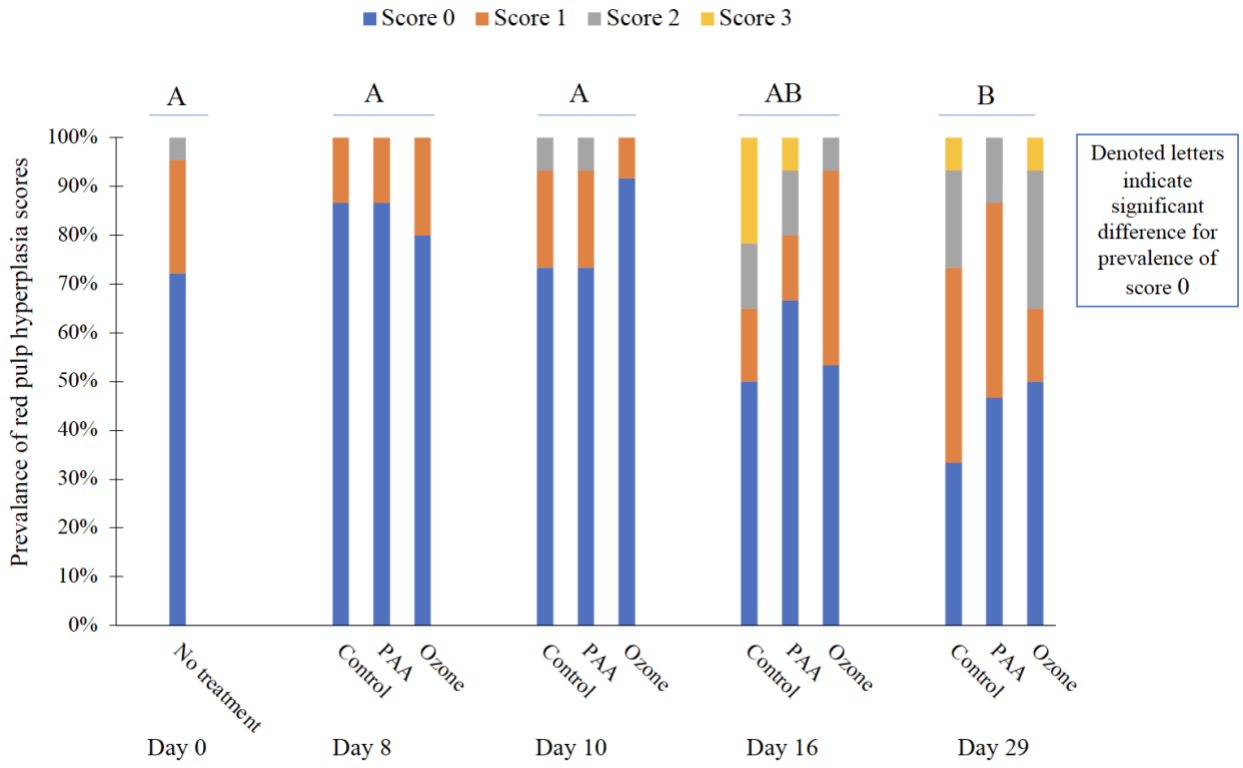

Figure 20: Bar chart with prevalence of red pulp hyperplasia score in the spleen (y-axis) in each treatment per sampling point (x-axis), based on histological evaluation of spleen sections. Score 0 (blue) indicates normal/minimal changes, score 1 (orange) indicates mild changes, score 2 (grey) indicates moderate changes and score 3 (yellow) indicates severe changes of the lesion. Spleen of n= 15 fish per treatment was evaluated per treatment. Different letters on each sampling point indicate which samplings are significantly different to each other for the score 0.

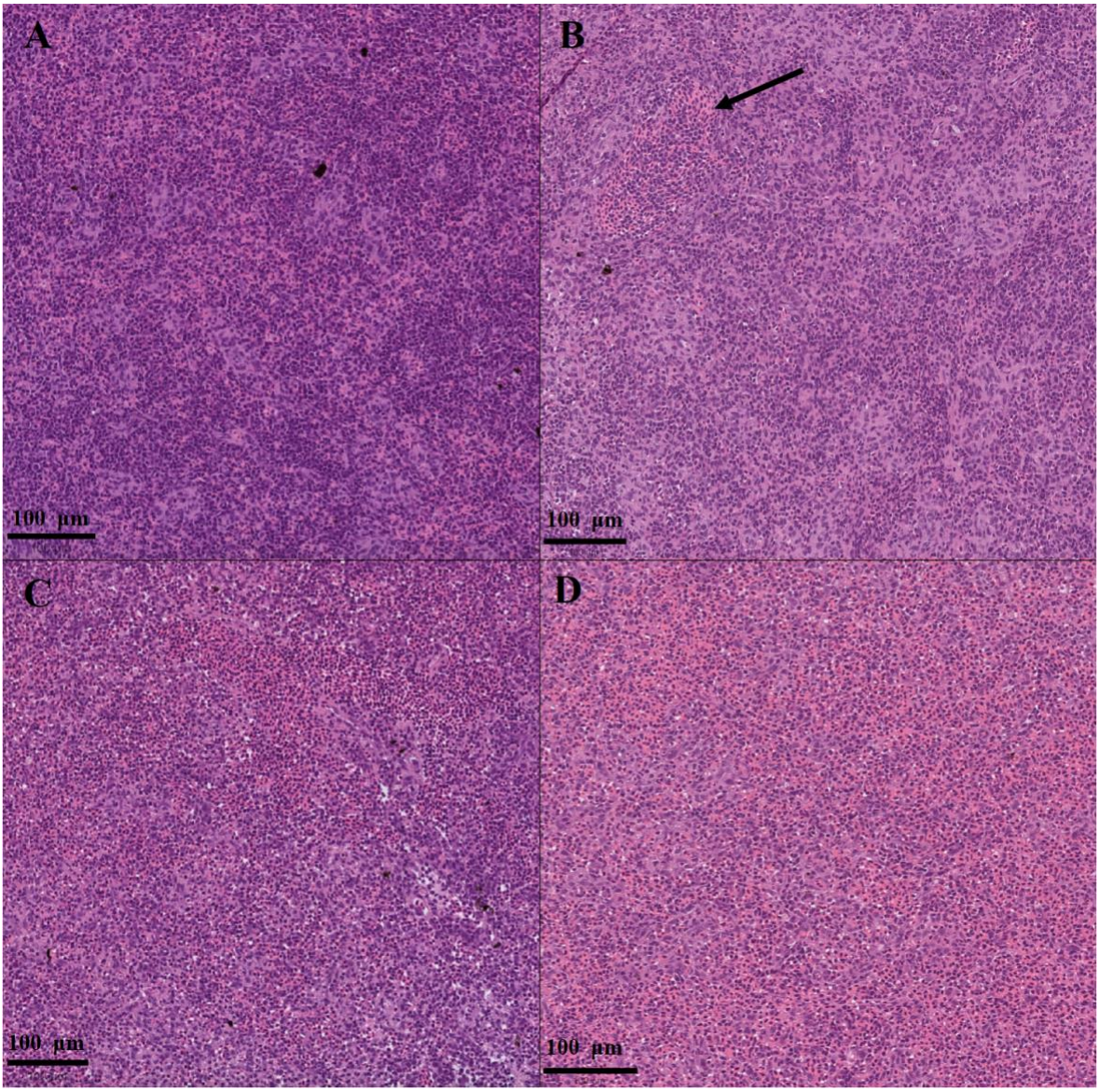

Figure 21: Histology sections of spleen from the trial. A) shows normal section of spleen (score 0), B) shows spleen with some red pulp hyperplasia (arrow), with as score of 1 for this lesion, C) shows a section with a score of 2 for red pulp hyperplasia, and D) shows a section with a score of 3 for red pulp hyperplasia. Scale bars represent 100 µm. Screenshot taken from the software QuPath v0.4.1.

3.6 Glucose, lactate, and hematocrit in plasma

Figure 22 shows the average lactate, glucose, and hematocrit levels in the plasma of fish taken at each sampling point per treatment. Plasma lactate for the control group stays relatively stable with just a small drop from 5.1 mmol/L to 4.0 mmol/L from day 8 to day 16 (1 week after *Y. ruckeri* challenge) and stabilized on 4.0-4.4 mmol/L. PAA group had lower starting levels (before treatment) but had a rise on day 10 to the same level as control group (1 day

after challenge). Ozone group on the other hand had a drop on day 10 to 3.9 mmol/L and then stabilized on 4.5-4.7 mmol/L. Significant difference was found only on day 10 between treatments, with ozone group being significantly lower from PAA and control group (p-value = 0.024). The glucose levels between treatments had minimal differences, but all had a significant drop, after 1 week of treatment (day 8) with values ranging from 5.4-5.9 mmol/L on 1st sampling to 3.9-4.2 mmol/L after 1 week of acclimatization to treatments (p-value < 0.001). The hematocrit levels did not change much through time points and had a value ranging from 35.8% to 48.7% through the whole trial.

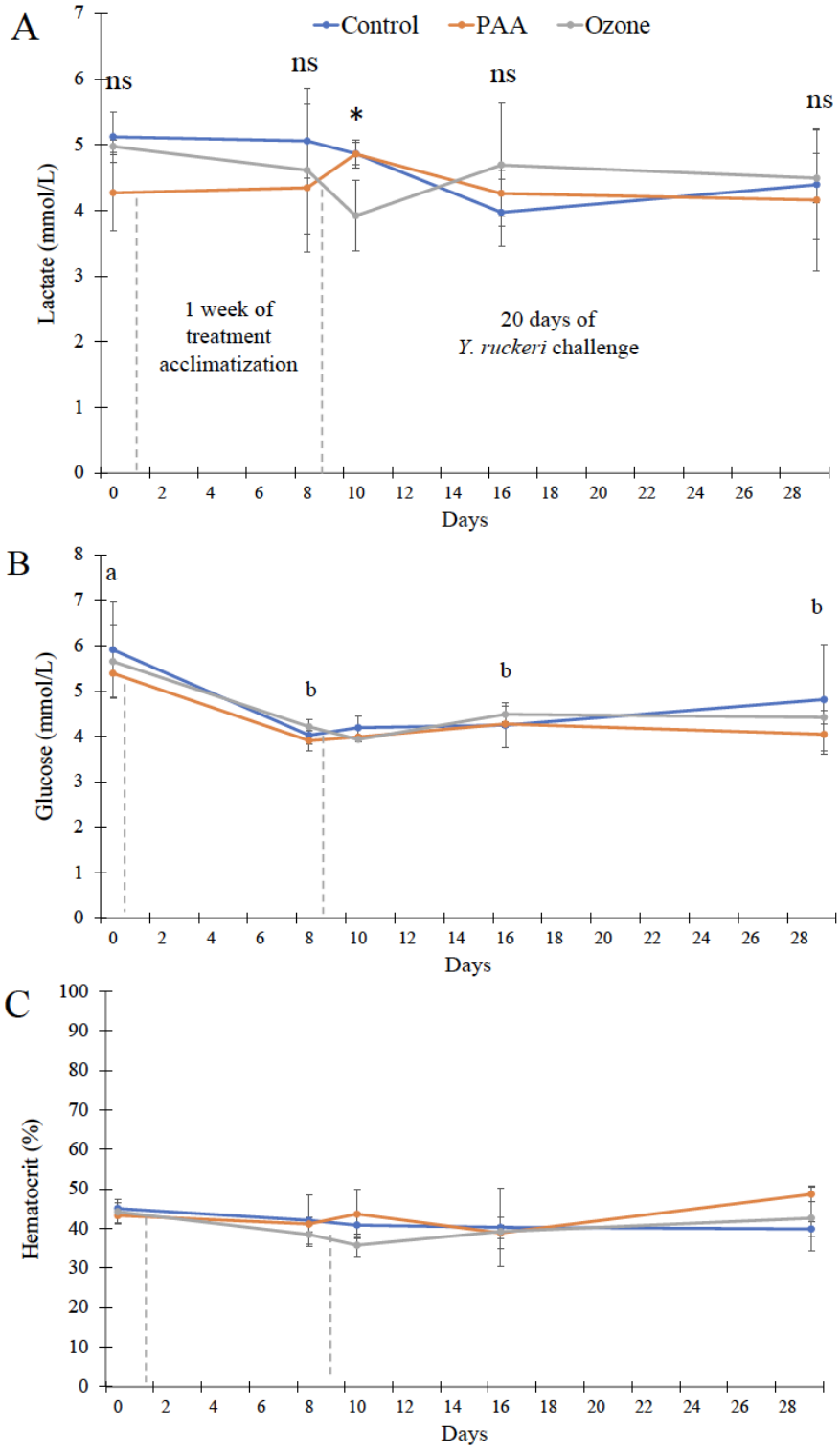

Figure 22: Graph showing changes in plasma lactate (A), glucose (B) and hematocrit (C) in the five sampling points in the trial for control, PAA and ozone group. Plasma from 45 fish per sampling point was analyzed (n=15 per treatment). First dotted line (to the left) marks when treatments started, and second line when Y. ruckeri was added to systems. Significant difference between treatments groups within a timepoint is marked with an asterisk (*), while ns = non-significant differences between treatments. Different letters denote significant difference between sampling points.

3.7 Water quality

Table 9 shows the average values of water quality parameters per treatment, measured at the fish tank outlet. Most parameters had no statistically significant difference between treatments, where the exception was the turbidity parameters and NO_2-N. All treatments were significantly different from each other for turbidity (ntu) ($p < 0.001$), with control treatment having the most unclear water (8.13 ntu ± 2.65), and ozone with the clearest water (1.02 ntu ± 0.31). The other way of measuring turbidity, UVT, significant difference was only found for ozone, where ozone treatment was significantly higher than both control and PAA treatment ($p < 0.001$).

For the nitrogen species, NH_4^+-N, NH_3-N, NO_3-N had similar concentrations while NO_2-N in PAA treatment with an average value of 0.34 mg/L ± 0.02, was significantly higher ($p = 0.003$) than control (0.23 mg/L ± 0.02) and ozone treatment (0.27 mg/L ± 0.03).

Table 9: A summary of the average values of specific water quality parameters per treatment from day 0 (ozone and PAA treatments started) to day 28 (a day before trial ended), taken from fish tank outlet. All parameters were measured manually. Values are given as treatment mean ± SD (n = 3). P-value (one way ANOVA) shown for each parameter, with significant difference at P < 0.05. An asterisk (*) indicates significant difference between treatments, and different letters denote which treatments are significantly different from each other.

Parameters	Control	PAA	Ozone	P-value (ANOVA)
O_2 (% saturation)	98.6 ± 1.9	104.3 ± 4.2	103.7 ± 5.8	0.272
Temperature (°C)	11.5 ± 0.1	11.5 ± 0.1	11.5 ± 0.1	0.710
CO_2 (mg/L)	3 ± 0	3 ± 0	3 ± 0	.
pH	7.42 ± 0.08	7.38 ± 0.16	7.45 ± 0.01	0.724
Turbidity (ntu)	8.13 ± 2.65 [a]	3.60 ± 0.68 [b]	1.02 ± 0.31 [c]	<0.001 *
UVT (%)	48.4 ± 2.8 [a]	54.9 ± 3.4 [a]	75.4 ± 2.5 [b]	<0.001 *
Redox tank outlet (mV)	218.6 ± 5.2	219.4 ± 3.0	233.9 ± 19.7	0.281
PAA (mg/L)	.	0.09 ± 0.02	.	.
Ozone (mg Cl_2/L)	.	.	0.03 ± 0.00	.
NH_4^+-N (mg/L)	0.38 ± 0.06	0.40 ± 0.08	0.47 ± 0.04	0.257
NH_3-N (µg/L)	2.19 ± 0.76	2.03 ± 0.69	2.79 ± 0.29	0.346
NO_2-N (mg/L)	0.23 ± 0.02 [a]	0.34 ± 0.02 [b]	0.27 ± 0.03 [a]	0.003 *
NO_3-N (mg/L)	28.8 ± 1.9	25.8 ± 3.2	29.9 ± 0.8	0.145

In addition to Figure 23, that shows visible comparison of the turbidity of the water in control, PAA and ozone tanks, Figure 24 show how the turbidity changed when treatment started (day

28). Until this point a steady rise in turbidity was observed in all fish tanks. After this point, control treatment turbidity kept rising until end of trial. PAA treatments turbidity dropped at the beginning of treatment, but after a few days rose steadily. Ozone treatments turbidity however sank rapidly after treatment and held this level until the end of the trial. Fish could not be observed in control and PAA tanks due to the high water turbidity.

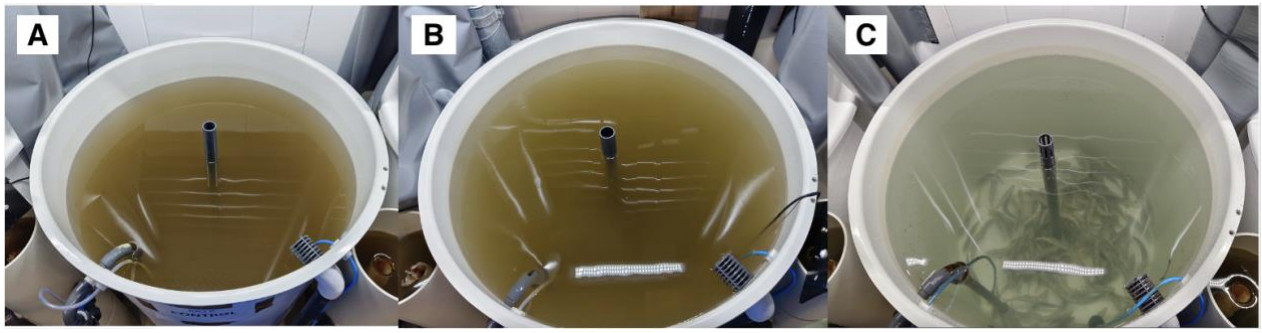

Figure 23: Fish tanks with different treatments on the last day of the trial. A) is the control treatment, B) is PAA treatment and C) is ozone treatment. Control and PAA treatments tanks are visibly more turbid than ozone treatment tank.

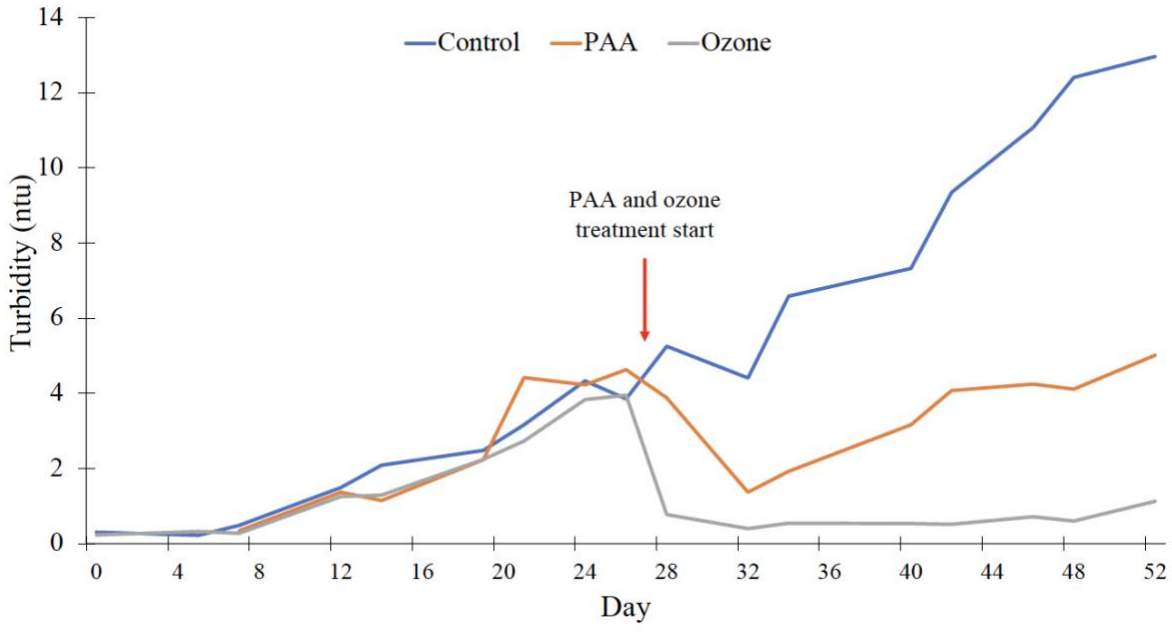

Figure 24: Graph shows the average turbidity (ntu)(y-axis) measured against days (x-axis) for the three treatments; control, PAA, and ozone (n = 3) in water samples taken from fish tank outlet. The time period is from acclimatization to RAS started (day 0), to end of trial (day 52). Day 28 marks when treatments started.

The calculated system management metrics per treatment are summed up in table 10. No difference in statistical significance was found between treatments.

Table 10: Calculated system management metrics per treatments summarized. The time period is from day 0 (day treatments started) to day 28 (a day before trial ended). Values are given as treatment mean ± SD (n = 3). P-value (one way ANOVA) shown for each parameter, with significant difference at P < 0.05.

Water exchange metrics	Control	PAA	Ozone	P-value (ANOVA)
Water exchange (% volume/d)	13.10 ± 0.05	13.26 ± 0.10	13.29 ± 0.16	0.173
Water exchange rate (L/kg feed)	324.28 ± 1.15	328.10 ± 2.39	328.86 ± 3.97	0.173
Make-up water (l/min/kg fish)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.311
HRT in system (days)	7.63 ± 0.03	7.54 ± 0.06	7.53 ± 0.09	0.172
HRT in fish tank (min)	19.53 ± 0.64	19.93 ± 0.29	19.38 ± 0.22	0.321
HRT in protein skimmer (min)	1.79 ± 0.12	1.95 ± 0.04	1.86 ± 0.00	0.112
Percentage of reused water (%)	99.72 ± 0.01	99.71 ± 0.00	99.71 ± 0.00	0.243

4 Discussion

This thesis addresses how continuous disinfection with ozone and semi-continuous disinfection with PAA in RAS can affect disease development in Atlantic salmon parr, when *Y. ruckeri* is introduced to the system through water. Fish performance, fish welfare and health, and water quality were followed for 20 days post-pathogen challenge. This section will discuss what effect the treatments had on disease development of fish, but also potential effect of ozone and PAA on welfare and health of fish, and water quality.

4.1 Impact of PAA and ozone on Y. ruckeri infection

Infection with *Y. ruckeri* is regarded as a highly problematic disease in Atlantic salmon as it generally causes systemic infection with septicemia, hemorrhages and ultimately circulatory failure (Veterinærinstituttet). This disease also causes significant economic losses which is reported in some cases to be as high as 30-70% of the stock among fish depending on size of fish, stress condition, water temperature and individual susceptibility (Furones et al., 1993; Horne & Barnes, 1999; Noga, 2010; Poppe & Bergh, 1999; Raida & Buchmann, 2008). Especially RAS has challenges with the disease, where *Y. ruckeri* in biofilm is a problem which has caused reoccurring outbreaks (Bornø & Linaker, 2015; Hjeltnes et al., 2017).

There are a few studies on how disinfection in water of RAS can affect a bacterial pathogen outbreak with Atlantic salmon parr, though to my knowledge none that look at if continuous disinfection with PAA or ozone could possibly prevent an outbreak of ERM in RAS. The present study showed a positive detection of *Y. ruckeri* in fish of all treatments, by qPCR results from detection in spleen and sequencing of bacteriology samples from head kidney. This indicates the added doses of PAA and ozone in water could not prevent Atlantic salmon parr from getting infected by *Y. ruckeri* in RAS.

Ozone's ability to effectively eliminate *Y. ruckeri* in water is reported in several studies (Colberg & Lingg, 1978; Liltved et al., 1995; Wedemeyer et al., 1979). Bullock et al. (1997) study on ozonation effect on BGD in rainbow trout cultured in RAS did similarly as the present study, not manage to remove the causative agent of the disease. However, that study did provide <1 log reduction of the bacteria in the system water and on the gill tissue which prevented BGD outbreak and reduced mortality. The possibility of ozone working to

eliminate the causative pathogen in water, which can lead to prevention of an outbreak and reduced mortality is therefore present.

A new study by Good et al. (2022) tested the bactericidal activity of PAA to *Y. ruckeri* in RAS water with different concentrations (0, 2, 5 and 10 mg/L PAA) and exposure times (0, 2, 5 and 10 minutes). The results showed that *Y. ruckeri* was completely eliminated following 5 minutes of exposure to 5 mg/L PAA. For comparison with the present trial, the concentration of PAA that was achieved was in average 0.09 mg/L in fish tank outlet, and according to Good et al. (2022) study 2 mg/L minimally reduced the CFU/20 µl after 10 minutes of exposure in RAS water. It might be questioned if this led to an elimination of *Y. ruckeri* at all in the present study. However, the concentration of PAA on the pump sump was likely higher than what measured in fish tank outlet, as PAA was added via the pump sump, and through contact could eliminate *Y. ruckeri* from the recirculating water.

The potential of PAA as treatment to prevent infectious diseases in aquaculture has been evaluated in a few studies (Abu-Elala et al., 2021; Good et al., 2020; Lazado, 2019; Pedersen et al., 2013). A study by Good et al. (2020) tried to determine if low doses of PAA (0.2, 0.5 and 1.0 mg/L) could prevent saprolegniasis in Atlantic salmon juveniles in RAS, following vaccination. The conclusion of this report was that PAA at the dosages tested, significantly reduced observable external saprolegniasis following vaccination and led to higher survival rates (Good et al., 2020).

In the present trial, the potential elimination of *Y. ruckeri* through disinfection could be supported by infection prevalence trends detected, as control had the highest prevalence (33.3%), followed by PAA (13.3%) ozone group (6.7%). This might suggest the disinfectants did reduce the load of pathogens in the water which thereby caused less fish being infected, as *Y. ruckeri* can be transmitted through water (Tobback et al., 2009). However, since there were no significant differences between the treatments, no conclusion can be made on this. Further research would be required with a bigger sample size to make this correlation clear.

In the present study there was minimal mortality for all treatment groups and observations of clinical signs, behavioral signs, and external welfare indicators did not indicate that fish were acutely sick. A full-scale acute ERM outbreak was therefore not achieved. Because of this the full potential of ozone or PAA as disinfectants in the water affect an ERM disease outbreak could not be investigated to a full extent.

4.2 Impact of PAA and ozone on growth, fish health and welfare after *Y. ruckeri* challenge

This section will consider how fish growth, health and welfare were affected by the pathogen challenge, in light of the disinfection methods used. Mortality is one of the most important welfare indicators used in aquaculture, where high or increased mortality indicate health and welfare problems at the population level in the production units. Mortality is used as a robust indicator to find differences between treatments in experimental trials with pathogen challenges. However, the present study had minimal mortalities and therefore not enough to compare if PAA or ozone treatment could potentially help increase survival of Atlantic salmon parr in a *Y. ruckeri* outbreak in RAS.

4.2.1 Growth

Low mortality does not equal to fish not having welfare issues in the systems, as many diseases can cause bad welfare, without necessarily causing death. Growth is another welfare indicator that is frequently used to assess the welfare of animals. Growth is affected by many factors, like appetite, nutrition content in feed, diseases, water quality and stress (Adams et al., 2000; Ellis et al., 2002; Huntingford et al., 2006; Jobling, 1983). A change in the populations growth could be used as an early sign of potential problems in fish farms (Noble et al., 2018). Fish with infectious diseases, including ERM, can be lethargic and lose their appetite, which can lead to reduced feeding and slower growth (Noble et al., 2018; Tobback et al., 2007).

By taking into account all the sampled fish regardless of treatment, the present study saw fish growth increased by 46% from second sampling (1 day before *Y. ruckeri* challenge) to last sampling (21 days after). No growth inhibition was observed after *Y. ruckeri* was added to system in neither control, PAA or ozone group. This suggest growth was not affected by introduction of *Y. ruckeri* in the systems.

PAA and ozone disinfection did not significantly affect growth of Atlantic salmon in the present trial. Growth curves of the fish in control, PAA and ozone group showed minimal differences between the weight of fish throughout most of the trial. Only on the last sampling

day ozone group deviated slightly from PAA and control with a final average weight of 61.42 g, compared to 56.30 g for control group, and 55.59 g for PAA, which was non-significant.

The faster growth of ozonated fish may be further indicated by SGR and TGC, which was calculated from first to last sampling point in the present trial. Although SGR, TGC and final k-factor also show no significant difference between treatments, TGC and SGR of ozone treatment was slightly higher with a TGC of 2.4 compared to 2.0 for both control and PAA group. Comparing to other studies, ozone has been observed to significantly increase growth of rainbow trout and post- smolt Atlantic salmon in RAS (Davidson et al., 2011; Davidson et al., 2021). Results from Good et al. (2011) study indicated rainbow trout reach market size faster in ozonated low-exchange RAS (ORP= 250mV) than in non-ozonated systems. A study by Davidson et al. (2021) on effects of ozone on post-smolt Atlantic salmon health in FW-RAS with ORP setpoint of 300-320mV utilized, similarly found enhanced growth of Atlantic salmon in systems with ozone. However, these fish were grown for a longer period up to 1 kg, but the trend of the last sampling may suggest that ozone group is starting to grow faster.

Davidson et al. (2021) hypothesized that the higher growth rates could be as result of cumulative improvements to the culture environment, with e.g., reduction in true color, heterotrophic bacteria counts, dissolved copper, biochemical oxygen demand, total suspended solids, and increase of UVT reported with ozone treatment. This may reduce the energetic cost of fish acclimating to water chemistry as suggested by Powell and Scolding (2018). It could also be suggested that the significantly higher turbidities measured in control and PAA tanks compared to ozone tanks could potentially make it more difficult for fish to see and catch feed pellets which could lead to less growth compared to ozone treatment, as previously suggested by Sigler et al. (1984). Considering the higher infection prevalence in control and PAA treatments, it could also be suggested that some infected fish had a lower appetite in the control and PAA groups which caused reduced growth towards end of the trial.

Growth of fish exposed to PAA did not seem to be affected by the treatment as there were little differences for growth parameters between control and PAA group, and weight curves overlapped almost identically through samplings, before and after *Y. ruckeri* challenge. The measured PAA concentration in the fish tank outlet were only 0.09 mg/L, and previous reports indicates these low concentrations of PAA do not affect growth of Atlantic salmon or rainbow trout. A study by Davidson et al. (2019) on rainbow trout performance where three target doses were evaluated (0.05, 0.10, and 0.30 mg/L PAA), and added semi-continuously in

RAS, concluded with no effect on performance metrics, including growth, survival and feed conversion ratio. Eggen (2021) thesis also found no effect of PAA on growth with Atlantic salmon parr 4-weeks exposed to 0.1 and 1.0 mg/L PAA. This study therefore further strengthens that these findings of PAA added semi-continuously in low doses in RAS do not impact growth of Atlantic salmon.

4.2.2 External welfare

Similarly, to mortality and growth, the external welfare of fish was also not significantly impacted by the pathogen challenge. External welfare scoring schemes are one of the tools that farmers, fish health personnel and scientist can use to roughly evaluate external welfare of fish (Noble et al., 2018). In this study, the tool was used to evaluate how *Y. ruckeri* introduced to the systems affected welfare and health of ozone and PAA exposed Atlantic salmon parr, compared to non-exposed fish. An outbreak of ERM should affect external welfare scoring as external hemorrhages, snout damage, lesions, exophthalmia and emancipation are all pathologies that have been reported in ERM outbreaks (Tobback et al., 2007). The present study however, found non-significant differences of the external welfare scoring, when comparing first to last sampling, which suggests external welfare was not affected by the pathogen challenge. This highlights the relatively low prevalence of infection that was recorded in some fish tanks and suggest fish were not severely clinically ill by examination of them externally.

The only external welfare indicators affected throughout the trial were fin damage, operculum damage, epidermal damage, and scale loss. The external welfare of fish was overall good as most indicators had score of 0, and generally few fish that registered score over 1 for the indicators affected. Shortening of operculum was already a problem before trial started, thus this score was most likely not a cause of the experiment.

Fin condition is frequently employed as indicator of fish welfare, and is affected by several factors, as high densities in cages/tanks and/or high levels of suspended solids can be indirect causes of fin erosion (Latremouille, 2003; Wedemeyer, 1996), and increased competition between fish can lead to aggression and fin damages, as well as abrasion with tank walls (Pelis & McCormick, 2003; Skipnes, 2014). At start of trial fish were given 50% feeding, which may have increased competitive aggression and be a cause of the higher dorsal fin

scores recorded the first sampling. Lower damage for dorsal fins in the last sampling indicates a healing effect, however the same was not seen for caudal and pectoral fin, that had an increased score the last sampling.

Predisposing factors for fin damages are suggested to be high stocking densities and suspended solid as they can trigger behavioral changes which may lead to aggression and increased foraging (Timmerhaus et al., 2021; Wedemeyer, 1996). As turbidity increased in control and PAA tanks, it could be suggested this led to higher scores for some fin damages in the last sampling. However, this is most likely not the case as ozone fish would likely have lower scores compared to PAA and ozone fish in this case. As observed PAA and ozone treatment did not under any timepoint throughout the trial affect external welfare scoring of fish as no significant differences was observed for any of the external welfare indicators between treatments.

In a study by Good et al. (2011) of ozone's effect on rainbow trout health and welfare in low exchange RAS (250 mV ORP in culture tanks), he found similar fin indices between ozone and no ozone treatment groups when evaluating fin erosion. The study by Davidson et al. (2021) on effects of ozone on post-smolt Atlantic salmon health in FW-RAS similarly to the present trial found no difference in eye cataracts, epidermal damage, operculum damage, skin hemorrhages, and snout damage between ozone and no ozone treatment parameters, and mean welfare scores were generally under 1. These findings are further strengthened by this study where Atlantic salmon parr in RAS exposed to continuous ozonation at 233.9 mV ORP in the present trial did not affect external fish welfare and had no impact on external welfare when fish was exposed to *Y. ruckeri*.

A study by Lazado et al. (2020) found cases of fin damage and scale loss was prevalent in smolts exposed to PAA, but no statistical significance between control and PAA exposed fish, with concentrations of 0.6 and 2.4 mg/L PAA (5 min exposed and after 2 weeks 30 min). Eggen (2021) thesis got results that indicate PAA concentrations of < 3.2 mg/L PAA do not seem to significantly affect external Atlantic salmon welfare in RAS. The PAA dose fish were exposed to in this trial was low with an average of 0.09 mg/L measured in culture tanks, and results further indicate this concentration do not affect external welfare of Atlantic salmon parr and had no impact on this after *Y. ruckeri* challenge.

4.2.3 Clinical signs

Even though fish might seem in good health by observing them externally, sub-clinical observation might be discovered by obduction of fish. Macroscopic clinical signs like exophthalmia, enlarged spleen, external hemorrhages, ascites and hemorrhage in inner organs are all observations that have been recorded in previous ERM outbreaks in farms (Tobback et al., 2007). The presence of these signs do not say anything about the cause as these are unspecific symptoms, however they could be a good indicator of health related problems like inflammation and/or circulatory failure (Noble et al., 2018).

In the present study these signs were not observed 1-4th sampling, only on the last sampling significant observations could be seen. The most observed signs were enlarged spleen and mild hemorrhages in eyes and liver. This suggests that pathogen challenge may have affected health of some fish as these signs suggest the sampled fish had circulatory issues that may be related to ERM. The clinical signs like mild eye hemorrhage and hemorrhage in liver were also present in fish in all treatment groups, with prevalence varying from 14%-38% and no significant difference between treatments. These two findings were however mild in severity, and not as prominent as the enlarged spleen.

The spleen is a lymphoid organ central in immune defenses and hematopoiesis (Kryvi & Poppe, 2016), where an increase in size could possibly reflect investment in immune function (SeppÄnen et al., 2009; Smith & Hunt, 2004). There is also a strong indication that *Y. ruckeri* counts are seen to increase in spleen after pathogen challenge (Timothy & Gregory, 2005; Wiens et al., 2006). A previous trial with *Y. ruckeri* challenge in rainbow trout also shows a dramatic increase in spleen weight which was interpreted as a result of influx of cells recruited by inflammatory cytokines (Wiens et al., 2006). This indicates the spleen is actively involved in immune responses against *Y. ruckeri* (Raida & Buchmann, 2008; Timothy & Gregory, 2005; Wiens et al., 2006). There is therefore a strong indication that fish in the trial showed inflammatory responses in the spleen in response to *Y. ruckeri* infection. A further indication of this is that the prevalence of fish with enlarged spleen in each treatment (Control: 31%, PAA: 16%, Ozone: 5%) aligns nicely with the positive detection of *Y. ruckeri* in the spleen (Control: 33%, PAA: 13%, Ozone: 7%).

The only clinical sign with significant difference between treatments was enlarged spleen, where ozone group had significantly lower prevalence of this observation than control group. Based on the function of the spleen, these findings together with the detection of *Y. ruckeri* in

the spleen indicates less of an immune response in Atlantic salmon exposed to ozone, compared to non-exposed fish, which is likely because the ozonated fish had lower prevalence of infection than control group. This leads back to the suggestion that ozone in the doses added for the present study led to reduction of *Y. ruckeri* in the rearing water, and subsequently less infected fish in these ozone tanks.

These correlations are unclear at this point, and further research is needed to see if this was fact was the case. However, these results might be promising in commercial RAS farms, where losses as a cause of bacterial pathogen outbreaks may be big, and information about biosecurity control of pathogens in these systems is scarce. Future trials should try mimicking an ERM outbreak with high mortality, so survival rates could be compared, which would be good indicator of ozone's ability to control such outbreaks.

Even though PAA group also had less observations of enlarged spleen and a lower infection prevalence (though more than ozone group), both findings were non-significant compared to control. It could however be suggested this disinfectant also had similar effects as ozone described above, but at a lower degree. More research is therefore also needed here, to properly assess PAA's ability to reduce *Y. ruckeri* in RAS which could lead less infection of fish and subsequently reduce the losses of Atlantic salmon parr in RAS, in case of a ERM outbreak.

4.2.4 Histological evaluation of gill and spleen

Histological sections of tissues are frequently used to evaluate morphological and pathological changes, and one of the tools for diagnosis of fish. These microscopic changes may be caused by pathogenic diseases, environmental conditions, or mechanical injuries. Gills are frequently histologically evaluated in aquaculture and research, as they are sensitive organs with large surface area that are easily affected by environmental conditions (Flores-Lopes & Thomaz, 2011). Histopathological changes in gills compromise the gills function and capacity as an important organ providing gas exchange, osmotic and ion regulation, acid-base regulation and excretion of nitrogenous waste (Kryvi & Poppe, 2016).

The scoring system used for evaluating gills gives an indication of the general gill health of a group of fish. In summary the results of the overall gill scoring indicated quite a lot of variation within the same treatment, where gill scores of 0-2 were the most prevalent and few

cases of score 3 recorded. It has been reported of a worsening of gill health, with increased scores of Atlantic salmon in ERM outbreaks and reported pathologies like oedema, hyperemia, desquamation of epithelial cells, hypertrophy and hyperplasia of epithelial cells, and fusion (Adel et al., 2015; Tobback et al., 2007; Tobback et al., 2009; Aaas, 2022). However, comparing between the sampling points in the present trial, there were no significant differences found, which indicate gill health was not significantly affected by the *Y. ruckeri* challenge. Poor gill health is frequently reported in salmon reared in RAS and is suggested to be because of the high turbidities, although no firm conclusion is made on this topic as of yet (Figenschou & Hillestad, 2019).

Only at the last sampling point a significant difference between treatments were found, where prevalence of score 0- healthy gills, in ozone treatment, was significantly higher than the PAA group. The result of this gill scoring indicate fish exposed to ozone had better gill health, compared to PAA exposed fish after 28 days continuous exposure. Furthermore, both treatments did not significantly differ against the control group, which thereby suggest at the doses added both disinfectants had minimal impacts on Atlantic salmon gill health in the present trial.

To get a better picture of the gill health a semi-quantification of common histopathological characteristics of the gills was done on the same gill samples (only for last sampling), where pathological findings like hyperplasia, hypertrophy, necrosis, and fusion were recorded. These gill alterations are unspecific defensive responses which can be caused by other factors than pathogens, like environmental factors, including pollution (Evans et al., 2005) and particles (Mansouri et al., 2018). The alterations can disturb the normal function of the gills by increasing the diffusion distance between the water and blood, which could pose consequences for fish gas exchange (Strzyżewska-Worotyńska et al., 2017; Velasco-Santamaría & Cruz-Casallas, 2008).

Through this evaluation the gill condition for all treatments could be classified as relatively good with the prevalence of healthy gill lamella at > 82% for all treatments, but still with some gill alterations, particularly hyperplasia and hypertrophy, which could compromise gill health of individual fish. Ozone had the highest prevalence of healthy lamellaes, followed by control and lastly PAA group. However, no significant difference was found for healthy lamellae between the treatments or any of the pathologies observed, which indicate the PAA and ozone treatment did not severely affect gill morphology.

A study by Liu et al. (2020) found more frequent observed minimal hyperplasia in PAA-treated rainbow trout after periodic exposure to PAA (1 mg/L), although no significant difference between continuously PAA exposed trout (0.2 mg/L) and unexposed group. Although the PAA group in the present study had recorded more cases of hyperplasia with up to 14% of lamellaes affected in comparison to 6% in control fish and 4% in ozone fish, this was non-significant. However, this trend could indicate that gills could be sensitive to PAA, even when exposed to it at low concentrations (0.09 mg/L PAA).

Previous reports suggest ozone with the present study's ORP levels (234 mV) should not dramatically affect gill health of Atlantic salmon. Davidson et al. (2021) found similar result as the present study when looking at histology of gills in post-smolt Atlantic salmon in FW-RAS exposed to ozone (300–320 mV), with no significant differences between non-exposed and ozone exposed fish and only minor subclinical histopathological findings. On the contrary, a previous study on health and welfare of Atlantic salmon post-smolts in brackish water RAS, found continuous ozonation promote better gill health status than non-ozonated groups (Lazado et al., 2021). It was suggested that this might indicate continuous ozonation provide an environment that promotes better gill health, as increased suspended solids can negatively influence the gill health status (Au et al., 2004; Lazado et al., 2021). This may be relevant in the present study as ozone exposed fish had slightly less pathologies observed and lower scores at the last sampling point, but more research is needed to make this conclusion.

The spleen is an important hemopoietic and lymphoid organ. Microscopically its normal histology in Atlantic salmon has no clear characteristics but is rather dominated by many stages of blood cell formation and spread melanomacrophages (Kryvi & Poppe, 2016). The spleen has a thin capsule and thin network of reticular cells that supports the erythrocyte-producing (red pulpa) and leucocyte-producing tissue (white pulpa). There is not much literature on the effect of ERM on the spleen, but two studies with *Y. ruckeri* challenge in rainbow trout found necrosis in the spleen of infected dead fish and in acute ill fish (Avci & Birincioğlu, 2005; Tobback et al., 2009). This pathology was minimally observed in the present study. Based on the mentioned study's it may be suggested that this pathology occurs more so in fish with an acute condition of ERM, as the present trial did not lead to significant mortality, nor severe clinically sick fish.

A study by Deshmukh et al. (2013) developed a semiquantitative scoring system for spleen based on the presence of pathomorphological alterations found microscopically. This scoring

system was used in the present study with some modification. The only significant finding from the present study was the red pulp hyperplasia observed during the trial, in all treatment groups. Although no significant difference was found between treatments in the scoring for this alteration, there were significantly more frequent observations made in all treatments towards end of trial, with > 50% of sampled spleen registering the alteration at last sampling point. To my knowledge, red pulp hyperplasia has not been linked with ERM in literature before.

The reason of this response in the present trial could be the immune system responding by producing an inflammatory response, as spleen in fish is considered the primordial secondary lymphoid organ with key immunological functions (Neely & Flajnik, 2016). This response together with the enlarged spleen could indicate the fish's immune system is working hard to fight off the infection, and *Y. ruckeri* being detected in spleen also gives this indication.

Another suggestion is that the red pulp hyperplasia may stem from the RAS system itself, as a previous study by Good et al. (2009) discovered increased splenic congestion in fish in low exchange RAS compared to FTS and high exchange RAS, which was suggested to indicate a higher degree of immune system challenge related to higher bacterial counts in the water. As the turbidity increased throughout the trial in all treatments, and consequently more organic matter, this likely increases the bacterial load in the systems and consequently red pulp hyperplasia in spleen. This may suggest the disinfectants effect on bacterial load of the systems, may not have been as significant. More research should be done in relation to spleen and the effect of RAS, and potentially disinfection to make this correlation clear.

4.2.5 Blood parameters

Acute or chronic stress in fish is unavoidable in the highly intensified fish farming industry today and is mainly caused by handling practices. If fish can't properly cope with the stressors, it may strongly affect fish physiology, including growth, reproduction, and welfare (Eslamloo et al., 2014). Fish response to stress causes alterations in blood characteristics, as the activation of the hypothalamic-pituitary-interenal axis (HPI) which results in increased blood levels of catecholamines and cortisol (Wendelaar Bonga, 1997). This results in energy source mobilization with depletion of glycogen stores which in turn increases plasma levels of glucose, and anaerobic glycolysis which increases plasma lactate (Wendelaar Bonga, 1997).

Levels of glucose and lactate are therefore frequently used in fish experiments to assess stress levels of fish. In the present study the main stressor is believed to be *Y. ruckeri* introduced to the system. However, PAA and ozone can also cause oxidative stress in organisms, as both are highly oxidizing agents (Liu et al., 2020; Rivas-Arancibia et al., 2009; Soleng et al., 2019).

As indicated by the small changes in glucose, lactate, and hematocrit levels with no significantly elevated levels of these parameters between sampling points or treatment, Atlantic salmon from all three treatment groups were likely not stressed as a result of treatments or the pathogen challenge. Glucose levels variate a lot based on feeding status, diet and other factors (Noble et al., 2018). Plasma glucose usually increases slowly and tops after ca. 3 to 6 hours on Atlantic salmon with acute stress (Noble et al., 2018; Olsen et al., 2002). The results from plasma glucose levels in the present trial therefore indicate that the treatment or *Y. ruckeri* introduction did not seem to cause acute stress in fish in the present trial.

Although, if the fish were acutely stressed during any time, it would most likely be 3-6 hours after treatment started, but sampling was not done at this time point. If this was the case, the results indicate fish most likely coped with this stressor. The significantly higher glucose levels on 1st sampling compared to rest of the samplings could be explained with feeding status, or because plasma glucose levels can vary considerably in fish as the variable is not as regulated as in mammals (Mommsen et al., 1999).

There were no elevated levels of lactate detected in the trial and no significant differences between the sampling points suggesting that neither PAA or ozone treatment, nor *Y. ruckeri* introduction altered the levels of this stress indicator. Lactate is the product of anaerobic ATP production in the cells which takes place when there is not enough oxygen available for aerobic cell metabolism. This can happen when there's reduced oxygen levels in the water (Remen et al., 2012), or in case of high physical activity (Milligan & Girard, 1993). There was significant inter- treatment difference found on day 10 (1 day after pathogen challenge), where the ozone group's lactate levels dropped, with significantly lower lactate levels than both control and PAA treatment. However, because of the fluctuations seen for this parameter from the start of the trial, it's difficult to make any conclusions based on only this.

Good et al. (2011) study also showed no significant difference between ozone treated and non-ozone treated fish for lactate and glucose levels in RAS. There are no comparable studies on effect of continuous PAA exposure on plasma stress indicators in Atlantic salmon, but

Lazado (2019) study looked at Atlantic salmon exposed to 10 ppm of PAA for 15 mins (10 ppm) and 30 mins with three times exposure, and showed no significant differences from pre-exposure group. The present study further confirms these findings, where continuous ozone and PAA in the doses used did not affect plasma stress indicators in fish. It should be mentioned that glucose and lactate are useful indicators for acute stress, but they are difficult to interpret in relation to chronic long-term exposure of stress (Mommsen et al., 1999; Noble et al., 2018).

Hematocrit is the volume percentage (%) of erythrocytes in the blood and is also regarded as key indicator of secondary stress responses (Noble et al., 2018). Erythrocytes are the oxygen transporting cells in blood, and elevated hematocrit can be seen with fish associated with physical movement, excitement and stress responses (Pearson & Stevens, 1991). Normally the percentage of hematocrit in non-stressed Atlantic salmon is 44-49% (Sandnes et al., 1988), which aligns with the values recorded from the present study. The hematocrit values were stable for all treatments throughout the trial, which further strengthens the indication that fish were minimally exposed to stress during this trial when treated with PAA or ozone, and when *Y. ruckeri* was introduced.

Infection or environmental toxins are also observed to reduce hematocrit values in fish (Witeska, 2015). *Y. ruckeri* infection generally causes development of septicemia which can cause hemorrhages externally on the fish and in internal organs which may reduce the value of red blood parameters, as previously reported in other study's with ERM outbreak (Fajardo et al., 2022; Witeska, 2015). This decrease in hematocrit was not observed in the present trial and is likely linked to the minimal signs of acutely sick fish in the present study compared to Fajardo et al. (2022), which gave an acute ERM outbreak.

4.3 Impact of PAA and ozone on water quality

The RAS units used in the trial had the same stocking density and feed input. Water exchange metrics also showed similar values between the different treatment groups. Most water quality parameters, including oxygen, temperature, CO_2, pH, redox, NH_4^+-N, NH_3-N and NO_3-N did not seem to be impacted by PAA and ozone treatment during the trial. The parameters affected were the transparency of water with the measurement of turbidity (ntu), UVT (%), and NO_2-N.

The transparency of water is used as an indicator of general water quality and can be described by turbidity and UVT, where low turbidity and high % UVT indicate fewer large particles in the water (Yao et al., 2014). The effect of ozone on the clarity of water is highly documented with reported reduction of turbidity with ozonation (Davidson et al., 2011; Rueter & Johnson, 1995). This is as an effect of ozone's ability to oxidize various deteriorating agents, like organic carbon, color, bacteria and suspended solids, and inducing microflocculation of fine particles (Davidson et al., 2011; Summerfelt et al., 1997; Summerfelt & Hochheimer, 1997; Summerfelt et al., 2009).

Depending on the system intensity of RAS, the UVT in non-ozonated water in RAS ranges from 30-60% (Davidson et al., 2011; Spiliotopoulou et al., 2018). This is in alignment with the UVT value that was measured in the control group of the present trial (48.4%). In comparison the UVT of the ozonated water in this trial was significantly higher with 75.4%. This is higher than Spiliotopoulou et al. (2018) study that saw ozone-treated water have 50-60% UVT in RAS with stocked rainbow trout. This is likely due to the set point of redox being lower with only 200 mV in Spiliotopoulou et al. (2018)'s trial. The higher ORP, the more oxidizing agents are in the water to remove and microflocculate particles in the water. Benefits of having clearer rearing water is that the feed pellets are observable by the fish to catch and eat, and therefore feed optimally and grow well (Sigler et al., 1984). In addition, observing fish for control of health, behavior and feeding activity, is difficult to do with higher turbidities.

Less is known about how PAA may affect turbidity in RAS, and previous studies have found different results. One study on the effect of PAA on water quality in RAS for rainbow trout saw a decrease in turbidity and TSS, in units with one or four additions a week of PAA applied 1.1 mg/L twice per day, but no difference in bi-weekly additions (Suurnäkki et al., 2020). Davidson et al. (2019) study found that PAA do not reduce total suspended solids levels in culture water with 0.05, 0.10 and 0.20 mg/L PAA added semi continuously. Our study made this question no clearer as no significant difference between UVT of PAA and control treatment were found, but the turbidity measured in ntu, show PAA treatment had significantly lower turbidity than control treatment (and higher than ozone). This ability of PAA to reduce particles in water is likely affected by various factors like dosing regime, concentration and RAS intensity and design.

NH_3 and NO_2 are nitrogenous compounds that are very soluble in water and in high concentration are toxic for fish. Accumulation of NH_3 in the rearing water will increase partial pressure for NH_3 and thereby reduce efflux of the compound. This can lead to elevated levels of NH_4^+ and NH_3 in plasma of fish (Hollingworth, 2002), which can cause negative physiological effects, as increased metabolic rate, reduce growth rate and disease resistance, where some symptoms of ammonia toxicity is reduced swimming activity, increased gill ventilation, gill damage, histological lesions in various internal organs and osmoregulatory disturbances (Ip et al., 2001; Randall & Tsui, 2002; Thorarensen & Farrell, 2011; Tomasso, 1994).

Kolarevic et al. (2013) indicated that growth and welfare of Atlantic salmon parr are not affected by long term exposure to sublethal ammonia levels up to 35 μg/L NH_3-N. In the present study all treatments had NH_3-N between 2-3 μg/L with no significance difference between treatments, and no signs of ammonia toxicity in relation to growth rate, fish behavior or plasma stress indicators observed. This indicates the biofilter microbial community responsible for oxidizing ammonium were not affected by PAA or ozone exposure in the present trial, and managed to keep this compound within safe levels for Atlantic salmon parr.

Nitrifying bacteria in the biofilter oxidize ammonia to nitrite (NO_2^-), which is also toxic for fish. NO_2-N levels in this study was higher in all treatments than what was the target, which is the recommended safety levels (< 0.1 mg/L). With levels up to 0.34 mg/L NO_2-N recorded in the present trial, nitrite could become toxic for fish as it is actively taken up across gills and can disrupt multiple physiological functions like ion regulation, respiration, cardiovascular system, and endocrine processes (Kroupova et al., 2012). These high levels suggest the second step of nitrification in the biofilter, where most of the compound oxidizes to the less harmful compound nitrate, has not been effective enough. It is to be noted that no signs of acute toxicity of fish were observed, like reduced swimming, brown blood or gills, or reduced growth, which all are reported with toxic nitrite concentrations (Hjeltnes et al., 2012).

It could be suggested that the biofilter start-up time was possibly too short for the establishment of nitrifying bacteria on the biofilm. A mature biofilter microbial community could possibly prevented this elevated concentration of nitrite. Future trials in the facilities that were used should take these results into consideration, for example by having a longer maturation time of the biofilter or increasing the size of biofilter to handle the toxic metabolites.

Furthermore, NO_2-N in PAA treatment was significantly higher than both control and ozone treatment. The average value of PAA treatment was 0.34 mg/L NO_2-N, which is ca. 48% higher than control treatment. This indicates that the nitrification process in the biofilter may have been negatively affected by semi-continuous PAA additions. A previous study by Pedersen et al. (2009) on RAS stocked with rainbow trout has shown disturbed nitrification with elevated nitrite levels as well, with nominal concentrations of 2 mg/L and 3 mg/L (added once), while 1 mg/L added had little effect. Suurnäkki et al. (2020) did a similar study and found pulsed PAA applications (1.1 mg/L) disrupt nitrification, but the microbial community was capable of adapting. It's difficult to draw comparison between these studies as PAA concentrations the biofilter were exposed to likely differ between trials, as different factors, like organic matter, system design, dose regime, density and temperature differs affect PAA degradation. In conclusion, the present study indicates the PAA additions affected nitrification with elevated nitrite levels detected, and caution should therefore be taken on the vulnerability of the nitrifying bacteria when using PAA in aquaculture.

In the present trial ozonation did not have any significant impact on the nitrogenous compounds in the water, which suggest ozone minimally impacted the nitrite-oxidizing bacteria in the biofilter. A previous study on ozonation control in RAS with rainbow trout found slightly lower NO_2-N concentration for ozone treatment (Davidson et al., 2011), which is due to ozone's ability to oxidize nitrite. The present trial however had slightly higher NO_2-N for ozone treatment compared to control, but with no significant difference from control treatment. Removal rates for nitrite-N in RAS biofilter can variate a lot as its effectiveness is dependent on different variables like environmental conditions, biofilter designs and management of the systems (Timmons & Ebeling, 2013).

Even though ca. 51 ml of the PAA product (150 000 mg/L PAA in product) was added daily semi-continuously x48 times each day (product dose of 0.05% of make-up water) into the systems to try achieving 1 mg/L, the actual measured PAA from fish tank outlet was only 0.09 mg/L PAA. This is likely because of the very high organic matter in the system which caused imminent degradation of PAA. This is reported in other aquaculture systems with half-life reported to vary between hours to few minutes (Liu et al., 2014; Pedersen et al., 2013; Pedersen et al., 2009). Degradation of PAA is highly affected by the organic load which leads to faster PAA degradation and is also positively related to fish biomass and temperature (Pedersen et al., 2013; Pedersen et al., 2009; Pedersen & Lazado, 2020). The PAA decay

beaker trial (Appendix 7.2) also shows increased turbidity, which was used as a proxy for organic matter, also leads to faster PAA degradation. As shown turbidity increased in PAA tanks through the trial, which may have affected the residual PAA levels at end of the trial.

A previous trial also had difficulty with measuring detectable levels of PAA when adding 1.2 ml and 12.2 ml every 3 hours semi-continuously of a PAA product with a lower concentration, of 50 000 mg/L PAA (Eggen, 2021). This should be considered in RAS farms with high organic loads that want to apply PAA to their systems, as the desired concentration needed to inactive pathogens may be difficult to achieve.

There is a wide range of ozone dosages reported in RAS, mostly given as feed ratio, the reduction potential (ORP) or total residual oxidants (TRO) as µg/L of chlorine (Cl_2) (Spiliotopoulou et al., 2018). This study measured ORP, where 300-350 mV was the target value as it's proven safe for fish and is also high enough to eliminate pathogens (Bullock et al., 1997; Stiller et al., 2020). 500 mV ORP was reached through the protein skimmer where ozone was added. However only 233.9 ± 19.7 mV in ozone treatment was achieved in fish outlet tank measurements, which was not significantly different than PAA and control treatment. The ozone demand of the water in the RAS was therefore quite high, most likely as a result of the high organic loads which results in reduced half-lives of ozone (Spiliotopoulou et al., 2018).

ORP will vary among RAS as an effect of differences in feeding, waste production, oxygen levels and treatment system. ORP could therefore be quite an unspecific measuring method of ozone. Concentration of chlorine (Cl_2) was therefore also measured in the water to control TRO, where the concentrations measured (30 µg/L Cl_2) in the fish tank outlet was higher than the target based on Stiller et al. (2020) study. His study on effect of ozone on Atlantic post-smolt in brackish water suggested that ozone doses higher than 350 mV, with measured values of 10.7 µg/L Cl_2, results in significant mortality (Stiller et al., 2020). There were no signs of ozone toxicity despite the high Cl_2 values. This observation may indicate that our study levels were within safe for Atlantic salmon parr in freshwater, but further studies are necessary here to make this conclusion.

In a RAS context where the disinfectant used need to compromise between benefits of eliminating pathogens, while not affecting welfare and health of fish, and maintaining biofilter performance to keep ammonia and nitrite levels low, farmers also must consider the specific RAS they are working with. Each RAS is different from one another in relation to

e.g., organic load, water exchange, retention times, system design, which can lead to different disinfectant demands. Therefore, it might be difficult to make the best protocols for use of disinfectants in high-intensity RAS.

4.4 Critical assessment and future research

Infection with *Y. ruckeri* can cause acute septicemia with hemorrhage and circulatory failure (Poppe & Bergh, 1999). Although infection of *Y. ruckeri* was present in all treatments, signs of an acute disease outbreak like this were minimal with fish welfare and health remaining in a relatively good state, with mostly sub-clinical pathologies observed at end of the trial. Mortality data is important to compare treatment methods against each other in disease challenges, however since this trial had minimal mortality and only mild pathologies, comparisons against treated and not-treated fish in a ERM disease outbreak was more difficult. It is therefore suggested a similar study could be done in the future, but with a more acute disease development to compare survival rates. It could be suggested that smaller fish are used as they are more suspectable to ERM (Ohtani et al., 2019), or higher infectious dose added in the water.

Furthermore, when working with pathogens, handling of samples should be done in a way that limits the chance of cross-contaminations. Gloves and scalpels were changed between tanks, and everything wiped with ethanol, to prevent cross-contamination. A limitation in this study design could be that there were no control groups without *Y. ruckeri* challenge. If this was included, it could be confirmed that there had been no cross-contamination between the tanks. Moreover, evaluation of histology sections was done by humans and subjectiveness, or bias could affect the results. Some of the gill sections had many artifacts, likely because of handling of the samples, and could therefore not be evaluated. Number of evaluated gills in some tanks was therefore less and is therefore less representative.

Some trends were noticed in this trial, and it could be suggested a higher number of fish from each treatment should have been taken, as standard deviation was too high to see any definitive significant differences in some results. However, there were limitations regarding cost and time for making this happen. Furthermore, when comparing external welfare scoring between treatments, average scores for treatment groups was used. This might not be the best

way to present the data, as some fish with very high scores or very low scores are not represented and may mislead readers.

There was an outbreak of SGPV in the research station where the trial was performed, and a positive detection of SGPV was uncovered in one gill sample of fish in this trial. It's likely that most of trial the fish were free of infection as the virus was not detected in fish from first sampling and only one fish with a low pathogen load were detected in the last sampling. Regardless, all results should take this into account as infection dynamics could be altered with co-infections. SGPV is a virus that infects gill epithelial cells in Atlantic salmon and can cause SGPV disease which is associated with high mortality, respiratory distress, and severe gill pathology (Gjessing et al., 2020; Nylund et al., 2008).

It's not known how a SPGV co-infection with *Y. ruckeri* affect development of ERM, and therefore difficult to know how this might influenced our pathogen challenge in this trial. Observation of fish health and welfare did not indicate SPGV outbreak. It should however not be excluded that it might have affected host-pathogen dynamics, infection biology, disease severity, duration of infection and host pathology, as all these can be altered with co-infections (Graham et al., 2007; Telfer et al., 2008).

For future studies, samples taken from this study could be used for gene expression analysis to detect differences in important immune genes, for analyzing how PAA and ozone affected immune responses of fish compared to control in a *Y. ruckeri* challenge. Also, organic load was high in the RAS units in this trial, and future studies should be aware of difficulty regarding reaching target disinfectant doses for eliminating pathogens in high intensity RAS. At last, the findings of this study could be used in future research for developing appropriate disinfection practices in RAS to find the most effective way of eliminating pathogens, but at the same time maintain fish welfare and health, and biofilter performance.

5 Conclusions

The goal of this study was primarily to evaluate if Atlantic salmon parr continuously exposed to ozone or PAA in RAS could prevent a disease outbreak of ERM. It was established that the two disinfectants could not prevent fish from getting infected with *Y. ruckeri*, as indicated by the positive detection of the pathogen in spleen and mild clinical signs as enlarged spleen and hemorrhages observed in all treatments. Some positive trends were detected in the ozone and PAA treatment groups, as the prevalence of infection being lower in both PAA and ozone compared to control group, however with no significant differences.

It could be suggested that control group showed a stronger immune response in the spleen with significantly more fish recording enlarged spleen than in the ozone group. PAA group also registered less enlarged spleen than control but was not significantly affected. These trends together with prevalence of infection could suggest a lower *Y. ruckeri* load in the water of PAA and ozone treated RAS. It's therefore recommended that future studies try to explore this further by inducing a significant ERM outbreak with mortalities, to see if survival rates could also possibly be affected.

It was established during the trial that continuous ozone (233.9 mV in fish tank outlet) and PAA exposure (0.09 mg/L PAA in fish tank outlet) did not significantly affect health and welfare of Atlantic salmon parr in RAS during the *Y. ruckeri* challenge, as external welfare indicators, growth, stress parameters and gill histological evaluation showed no significant differences between groups. Gill lesions such as hyperplasia and hypertrophy were however more prevalent in PAA group, which could suggest gills are sensitive to this compound even at low concentrations.

PAA and ozone treatment did affect some water quality parameters. Turbidity and UVT were noticeably lower in ozone, and to some extent in PAA treatment. Furthermore, PAA in the doses added may have influenced the nitrite-oxidating bacterial community in the biofilter as nitrite-N was significantly higher in RAS with PAA than in control and ozone RAS.

The work from this study could be valuable for developing effective disinfection protocols in RAS to eliminate pathogens without negatively affecting fish health and welfare, and still maintaining good water quality and biofilter performance.

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7 Appendix

7.1 PAA measurement

For making a standard curve for the calculation of PAA, a 10x dilution series were made, with known PAA concentrations. Initially, a stock solution was made, where the final concentration of PAA was 1000 mg/L PAA. The product we used was Aqua-oxides super with 15% PAA (S. Sørensen Thisted.), that has a concentration of 150000 mg/L PAA. To make the stock solution with 1000 mg/L PAA, Eq. 4 was used, with initial concentration being 150000 mg/L, final volume being 1 L, and final concentration being 1000 mg/L. The initial volume in the stock solution was calculated to be 6.7 ml. To make the stock solution this amount from the Aqua-oxides super was added to a 1 L volumetric flaks. Milli-Q water was then added to get a total volume of 1 L (993 ml added).

With a 25 ml pipette, 50 ml of tank water from a RAS unit (without PAA) was added to 14 falcon tubes. These will be used to make a dilution series with 7 different PAA concentrations: 0, 0.1, 0.5, 1, 1.5, 2, 2.5 mg/L PAA from the stock solution, in replicates. Volume of the stock solution that will be added in each of these to make a dilution series, was calculated with Eq. 4. Table 11 shows an overview of the dilution series, with concentration of PAA, and volume of stock solution that was added, with final volume and how much tank water was in each. Before the calculated stock solution was added into each tube, the same amount of the water was removed, to get a final volume of 50 ml in each tube. The falcon tubes were mixed, before 2.5 ml of each concentration was transferred with a micropipette to their own cuvette. 250 µl of reagent 1 was added into the cuvette, and thereafter the same amount of reagent 2 was added and mixed. After 30 second the color intensity of the solution was measured in a spectrophotometer at $\lambda = 550$. Figure 25 shows the standard curve made, based on measuring these series dilution with known PAA concentrations.

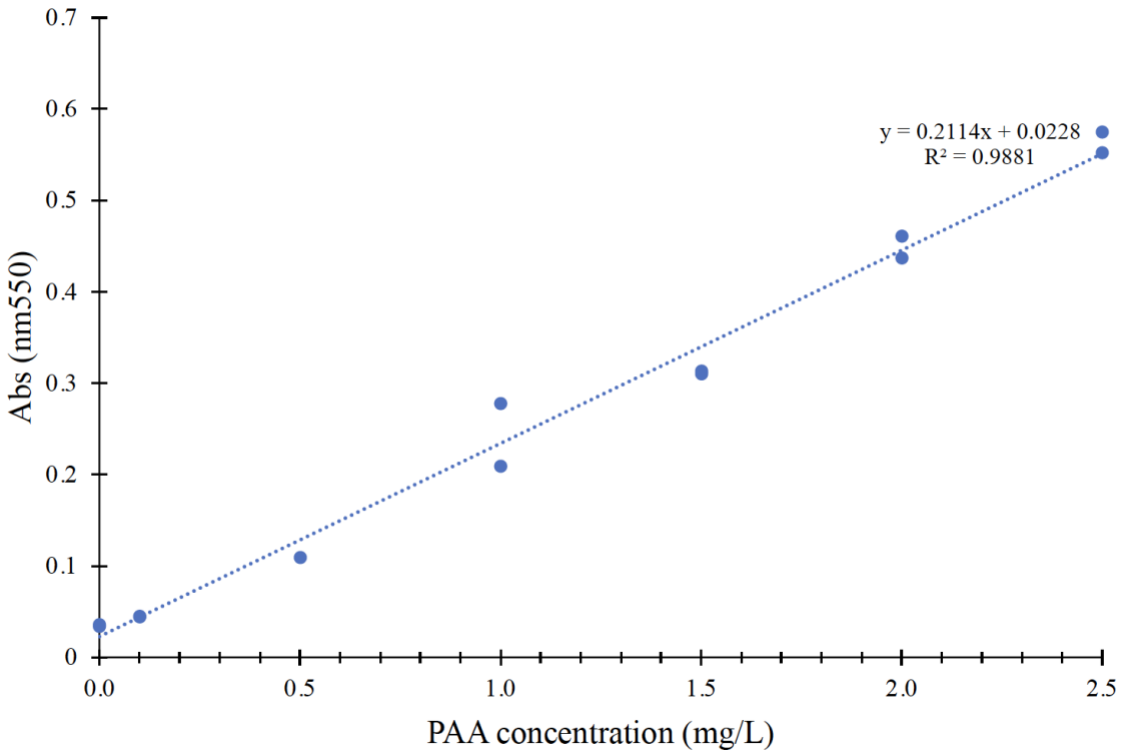

Figure 25: Standard curve where the solutions with different PAA concentrations and their absorption value (measured with spectrophotometer, λ = 550nm) was plotted. x-axis shows PAA concentration in mg/L, and y-axis shows absorption value at nm550. Based on the plot y was calculated as a function.

Table 11: An overview of the series dilution made with different concentration of PAA, including amount of stock solution, and tank water in each of the falcon tubes.

PAA concentration (mg/L)	**Stock solution (µl)**	**Final volume (µl)**	**Tank water (µl)**
0 (tank water + R1 + R2)	0	50000	50000
0.1	5	50000	49995
0.5	25	50000	49975
1.0	50	50000	49950
1.5	75	50000	49925
2.0	100	50000	49900
2.5	125	50000	49875

7.2 Determine PAA decay and half-life in trial

The results of the *in situ* beaker trial was used for deciding how much PAA should be added to the systems. The results (Figure 26) show PAA decay is highly affected by turbidity, shown with increasing the incorporation of RAS water. For all the glass beakers with different turbidity, except the distilled H_2O (0% RAS water), the PAA concentration declined exponentially over time after adding 1 mg/L PAA. Higher turbidities increased the rate of decay of PAA. The highest rate constant was the 100% RAS water (6.80 ntu) with 0.292 h^{-1}. Calculated half-life ($T_{1/2}$) decreased with higher percentage of RAS water (higher turbidity), with the lowest half-life being ca. 2.37 hours for the 100% RAS water.

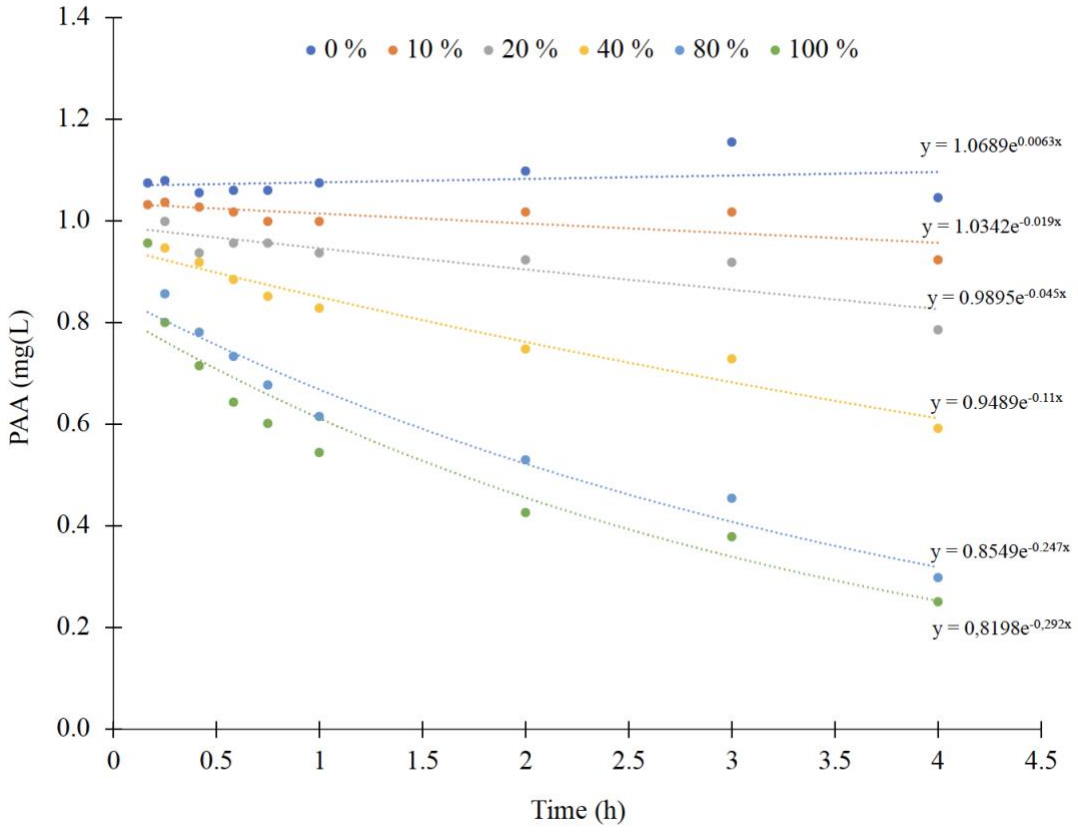

Figure 26: Graph shows the effect of PAA degradation (PAA in mg/L on y-axis) through time (hours in x-axis) in glass beakers with different percentage of RAS water from trial incorporated. 0% RAS water incoporated (blue curve) = 0.01 ntu, 10% (orange curve) = 0.63 ntu, 20% (grey curve) = 1.14 ntu, 40% (yellow curve) = 2.2 ntu, 80% (ligh blue) = 4.67, 100% (green curve) = 6.8. Det temperature of water was ca. 17 °C. Exponential trendlines set on all curves ≥ 10%, with formula for each curve.

Based on this result it was assumed PAA was completely degraded after approximately 5 hours, for the water sample with 100% RAS water. The method of calculating concentration

of PAA that needed to be added per day to achieve 1 mg/L PAA in the system is described below.

5.33 mL Aqua Des was calculated to give 1 mg/L PAA in the system based on Eq. 4, but this does not consider decay of PAA. Taking decay into account, from the beaker trial that was done, 1 mg/L PAA was consumed within ca. 5 hours in RAS water. Assuming 1 mg/L PAA is consumed in 5 hours, which is estimated to be 4.8 mg/L consumed in 24 hours, 25.564 ml needed to be added daily, which is 1.06 ml per hour. However, because of the very low detected PAA in the analytical measurement, this amount was doubled to try achieving ca. 1 mg/L with the analytical PAA measurement from the outlet water. The final volume that was pumped from the PAA product was 1.06 ml per 30 minutes, and a total of 50.88 ml PAA product added daily per system (0.05% of make-up water).

7.3 Overall gill scoring scheme for histology

Table 12: Semiquantitative gill scoring scheme for microscopic evaluation, based on a modified scoring system developed specifically for each mucosal tissue. Scoring graded from 0-3 where 1 -normal morphology, 2- mild pathological signs, 3- moderate pathological signs, and 4- severe pathological signs. Scheme retrieved from Alipio et al. (2023).

Score	Description	Pathological signs
0	Normal	Gill structures (filament and lamella) are well-defined, non-specific pathologies such as lifting, hyperplasia and clubbing account for <5% of the evaluated lamella
1	Mild	5 to 10% of the microscopic field show lesions, including increased cases of lifting, hyperplasia, and clubbing. Lamellar fusion can be observed sporadically.
2	Moderate	11 to 20% of the microscopic field show lesions, including increased cases of lifting, hyperplasia, clubbing and lamellar fusion. Sporadic cases of lamellar bleeding and aneurisms.
3	Severe	More than 20% of the microscopic field show lesions, including increased cases of lifting, hyperplasia, clubbing and lamellar fusion. Cases of bleeding and aneurism increase. Widespread multifocal proliferative gill inflammation is observed. Necrosis is observed. Severe filamental and lamellar congestion are observed.

7.4 Spleen scoring scheme for histology

Table 13: A semiquantitative scoring system for microscopic evaluation of spleen lesions, based on the presence of pathomorphological alterations. Scoring graded from 0-3, where 0- normal/minimal changes, I-mild changes, II- moderate changes and III- severe changes. Scheme retrieved from Deshmukh et al. (2013).

Parameter	**0 (normal/minimal changes)**	**I (mild changes)**	**II (moderate changes)**	**III (severe changes)**
Damage				
Capsular damage	None evident	Sparse degenerative changes ± thickened BM	Regional damage with capsular disruption	Widespread damage with capsular disruption
Necrosis	None evident	Focal/small scattered	Focally extensive to multifocal	Multifocal coalescing to diffuse
Reactive changes with negative impact				
Red pulp hyperplasia	Sinusoidal congestion ± sparse congested BVs	Frequent accumulated RBCs ± sparse congested BVs	Widely accumulated RBCs ± sparse congested BVs	Dense/diffuse accumulated RBCs ± sparse congested BVs
Cystic/clear space in parenchyma	None evident	Indistinct/scattered small vacuoles	Noticeable small clear vacuoles	Clear empty spaces with eosinophilic coagulum ± RBCs

Susceptibility— bacterial masses/colonies	None evident	Frequent in ellipsoids ± sparse in sinusoids/parenchyma	Numerous in ellipsoids frequent in sinusoids/parenchyma	Extensive in ellipsoids sinusoids/parenchyma
Protective cellular response				
Stromal cell hyperplasia & hypertrophy	Normal presence of cells	Frequent hyperplasia giving lobular appearance	Hyperplasia and hypertrophy cellular transformation	Extensive hyperplasia and hypertrophy no evidence of lymphoid cells