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Molecular responses of Atlantic salmon to peracetic acid and ozone during *Yersinia ruckeri* challenge in Recirculating aquaculture system

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Aquatic food production

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Foreword

This project was written for my master's thesis in the Aquatic Food Production program at the Norwegian University of Life Sciences (NMBU), with all lab work conducted at the Nofima center in Ås, Norway.

First, I want to thank my supervisors, Dr. Vasco Mota and Dr. Carlo Lazado, for the professional team we formed. I learned a lot from you, both scientifically and in life lessons. Thank you for guiding me and supporting me through this project; it was a great honor working with you.

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A special thank to Marianne, Vibeke, and all the wonderful people at the Nofima center who helped me throughout this project. Finally, I appreciate all my friends, who were my second family and stood by me.

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Abstract

Yersinia ruckeri (*Yersinia*) is a gram-negative bacterium and a challenging pathogen in Recirculating Aquaculture Systems (RAS), causing Enteric Redmouth Disease (ERM) in fish, including Atlantic salmon, which can lead to significant economic losses. Among the wide range of disinfectant agents used in aquaculture, ozone and peracetic acid (PAA) have shown great potential for eliminating various pathogens, including *Yersinia*, through their oxidative effects. Studying the effects of PAA, ozone, and *Yersinia* at the molecular level in fish can provide a better understanding of the physiological processes involved and improve disease management strategies in aquaculture. The two core objectives of this project were, first, to analyze the gene expression levels of Atlantic salmon parr exposed to PAA and ozone, either alone or during *Yersinia* challenge, and second, to study the effect of *Yersinia* on the gene expression of the fish.

The experimental setup involved a total of nine RAS units, divided into three sets (N=3), each designated for a specific treatment. Timepoint 1 served as the untreated group, where fish were not exposed to any disinfectant or *Yersinia*. At sampling point 2, the RAS units were exposed to continuous ozone at a concentration of 300-350 mV and semi-continuous PAA at a concentration of 1 mg/L for eight days. After introducing *Yersinia* (serotype O1) to the RAS units, samples were taken at timepoints three and five, 24 hours and 21 days post-inoculation, respectively. Samples were collected from four tissues—gill, skin, spleen, and olfactory. RNA was extracted, followed by cDNA synthesis and quantitative real-time PCR (qPCR) to compare gene expression levels.

Yersinia gene expression was below the detection level of the assay in all tissues. The number of genes that underwent significant changes between different time points was highest in gill, followed by skin, which had more changes than spleen, while olfactory had the fewest changes. Overall, *Yersinia* primarily suppressed antioxidant genes in gill. Ozone caused significant changes in gene expression, especially in gill and olfactory tissues, mainly with long-term exposure along with Yersinia. In contrast, PAA, along with Yersinia, temporarily affected the spleen and activated immune and antimicrobial responses in the skin and olfactory tissues, highlighting the suppressive effect of ozone on the examined genes compared to PAA.

1 literature review

1.1 Aquaculture in Norway

Research indicates that the global population is projected to reach 9.2 billion by the year 2050, leading to an increased demand for food, particularly protein (Lidicker Jr 2020). Utilizing water resources for aquaculture is a dependable method, considering that nearly 71% of the Earth's surface is covered in water (Panchal, Patel et al. 2021). This presents a significant opportunity for food production (Duarte, Holmer et al. 2009, Schubel and Thompson 2019). In Figure 1, the volumes of fishery and aquaculture production can be visually compared over time. According to the FAO's latest report in 2022, the total production of fisheries and aquaculture reached 214 million metric tons in 2020 (FAO 2022).

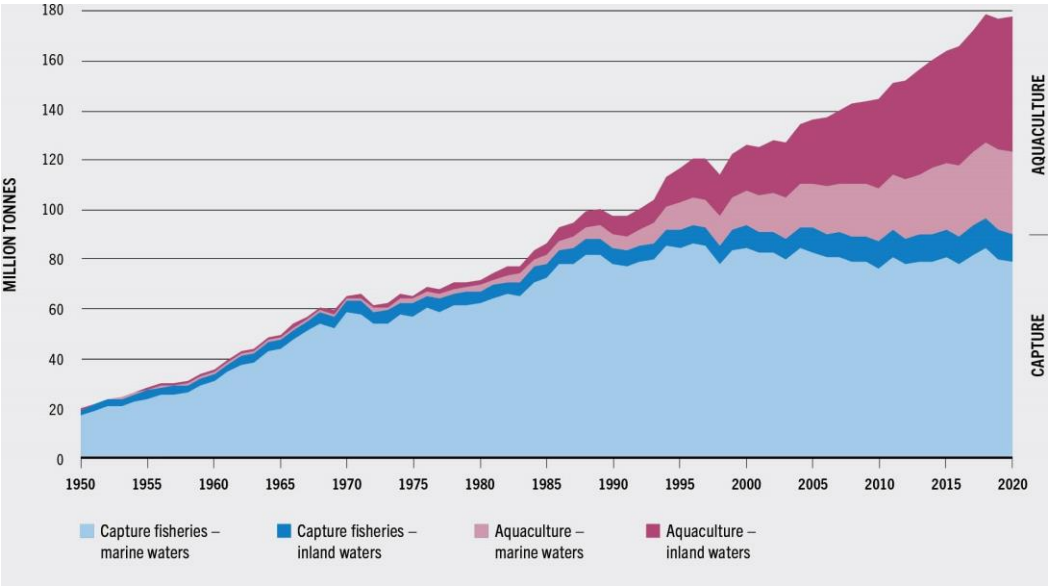

Figure 1. The illustration compares the volume of capture fisheries and aquaculture from the year 1950 till 2020, As evident from the data, 63% of the total production originated from marine waters, with the remaining 37% sourced from inland waters (FAO 2022).

The Norwegian aquaculture began in the late 1960s on a small scale and has since grown into a substantial industry (Paisley, Ariel et al. 2010). Today, it plays a significant role in global seafood

production (Hersoug, Mikkelsen and Karlsen 2019). Conversely, Norwegian aquaculture is largely dominated by Salmon and Trout production, with 95% of the output intended for export (Espinasse, Mikkelsen et al. 2023). The Norwegian aquaculture industry has experienced tremendous growth over the last 20 years, reaching a production of 1,650,000 tons of salmon in 2021 (Young 2022) and Studies indicate the potential for further production expansion in the future (Couture, Froehlich et al. 2021). In 1970, the first successful salmon pen was launched off the coast of Hitra Island in Norway by Ove and Sivert Grøntvedt. This pioneering effort was likely inspired by similar aquaculture constructions. The initial frame was constructed using wood, styrofoam, and recycled tires, but it was subsequently replaced with plastic and steel. This switch in materials allowed for the creation of larger pen structures and led to specialized producers taking over their production (Afewerki, Asche et al. 2023) Figure 2 illustrates the changes gone through the production cages through time.

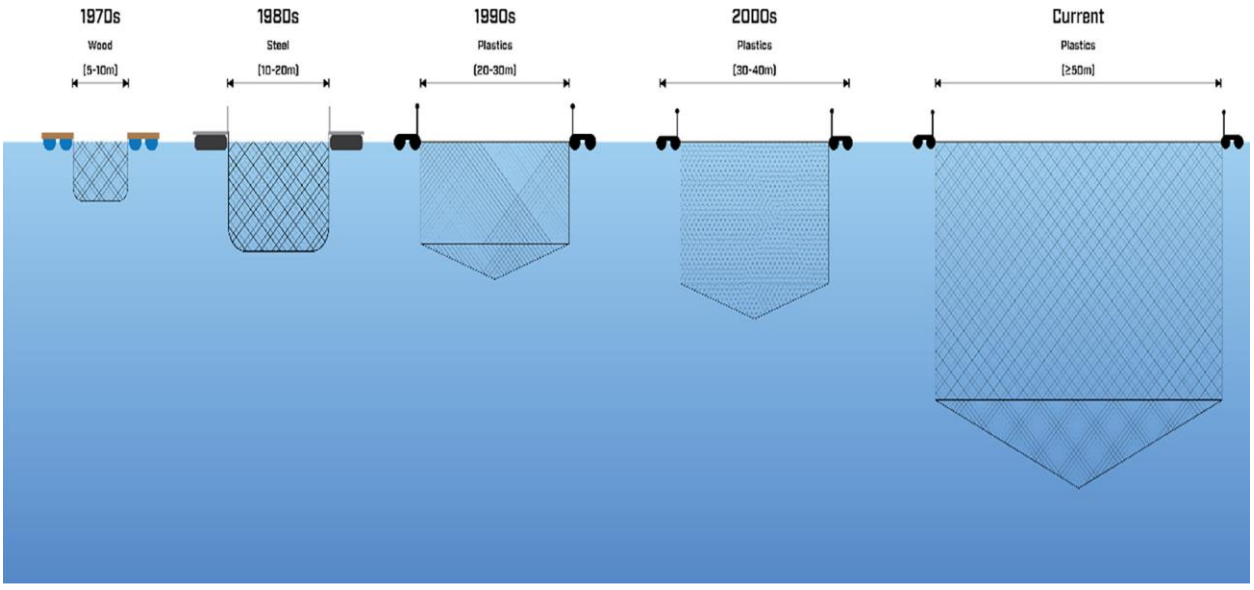

Figure 2. The illustration depicts the evolution of open net pens from 1970 to the present day. It is evident that there have been significant alterations in both size and the materials used (Afewerki, Asche et al. 2023).

1.1.1 Atlantic salmon

The salmonid subfamily *Salmoninae* includes about 30 species of fish in seven genera of which *Salmo* and *Salvelinus* (along with *Oncorhyncus*) are the best studied. The Atlantic salmon is iteroparous, meaning it may spawn repeatedly, as opposed to most species of Pacific salmon (*Oncorhynchus*), which are semelparous and die after only one spawning (Klemetsen, Amundsen et al. 2003). Diadromous fish, a varied group of migratory species, traverse between freshwater and saltwater habitats. Among them, the Atlantic salmon stands as an anadromous example. Typically, their life cycle starts with freshwater spawning, followed by a migration to the ocean where they experience accelerated growth due to existence of abundant food resources (Thorstad, Whoriskey et al. 2012). Breaking down the life stages of the Atlantic salmon into two parts, the first four stages take place in freshwater and include egg, alevin, fry, and parr. Subsequently, following smoltification, salmon move into a saltwater environment, pass through the smolt and post-smolt stages, and then head back to their initial habitat for spawning (Sahlmann 2013).

There are several aquaculture facilities and production systems which can be classified into "extensive," "intensive," and "semi-intensive" categories based on production per unit volume (Oddsson 2020). Extensive aquaculture involves low-density farming with minimal artificial inputs and technology, like pond farming without additional feeding. In contrast, intensive aquaculture achieves high production with advanced technology, but it requires substantial investment, optimal conditions, and faces a higher risk of disease outbreaks, as seen in salmon farming. Semi-intensive aquaculture combines elements of both. These systems can also be classified by species life stages, location, water supply, and usage, such as closed and open units, land-based farms, and tidal through-flow farms, each serving specific needs in the aquaculture industry. Additionally, farms can be categorized as flow-through or recirculating systems, and they may practice monoculture or integrated polyculture, combining the production of multiple species (Snow, Anderson and Wootton 2012, Lekang 2020).

According to a report by MOWI, the primary sources of protein for human consumption are fish, poultry, pork, and beef (figure 3). Salmon, however, constitutes a small portion compared to other

fish varieties. In 2022, among the 161 million tons of fish consumed globally, both farmed and wild catch of Atlantic salmon amounted to approximately 3.4 million tons (Mowi 2023).

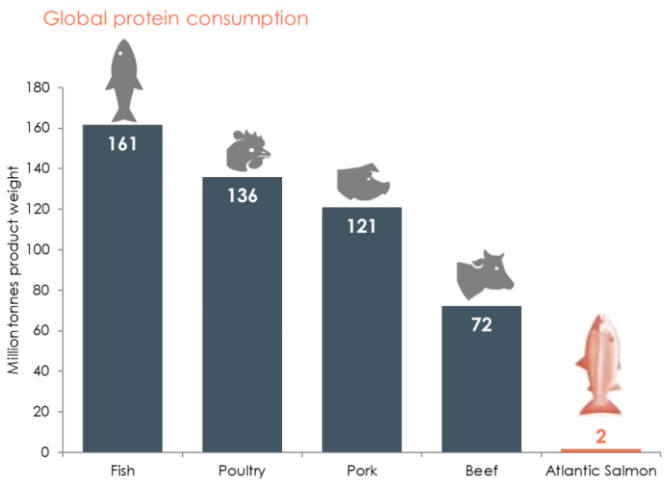

Figure 3. This figure illustrates the global consumption of animal protein in 2022, as surveyed by the FAO, measured in million tons.

Numerous studies have highlighted the advantages of incorporating seafood into one's diet, primarily because of its rich content of polyunsaturated fatty acids, notably omega-3 fatty acids like EPA and DHA. Moreover, seafood serves as an excellent source of essential nutrients such as vitamin D, taurine, selenium, a diverse range of amino acids, choline, iodine, and vitamin B12 (Lund 2013). In a clinical study conducted by Lara, Jose J., et al., the effects of a salmon diet over a 4-week period on volunteers' cardiovascular risk factors were examined. The study revealed the beneficial impact of fish consumption on blood pressure, with observed reductions in VLDL and LDL cholesterol levels, accompanied by an increase in HDL cholesterol. Furthermore, there was a significant increase in adiponectin levels (Lara, Economou et al. 2007), a protein hormone produced by adipose cells, known for its pivotal role in regulating glucose and lipid metabolism in tissues (Chandran, Phillips et al. 2003).

1.1.2 Recirculating aquaculture system – RAS

The global challenge of inadequate access to freshwater presents a significant and immediate issue, intensified by a growing economy, population, and climate-induced droughts (Rahman, Kumar and Dominguez 2022). Recirculating Aquaculture Systems (RAS) offer a sustainable alternative to conventional aquaculture methods like outdoor ponds and net pens, enabling year-round fish production in a controlled environment, ensuring product safety and environmental compatibility. Despite initial setup costs, RAS efficiently conserves resources by recycling water, achieving high production efficiency per unit of space and labor (Ahmed and Turchini 2021). In Europe, where traditional cage-based and flow-through aquaculture systems grapple with limitations like limited space due to land competition, freshwater scarcity, and pollution concerns, countries with established aquaculture sectors, such as the United Kingdom, Ireland, Italy, and notably Norway, champion RAS as a promising solution and a means to further expand the aquaculture industry. The same research also highlights the cost-effectiveness of RAS, particularly in salmon farming, with ongoing efforts aimed at improving their competitiveness compared to traditional aquaculture approaches (Badiola, Mendiola and Bostock 2012).

RAS (figure 4) is an intensive fish production method that uses a series of water treatment steps to clean and reuse the water in which the fish are raised. These systems typically include mechanisms to remove solid waste like fish waste, uneaten food, and bacterial particles, nitrifying biofilters to convert fish ammonia to nitrate, and devices for gas exchange to remove carbon dioxide and add oxygen. RAS may also incorporate UV irradiation for water disinfection, ozonation and protein skimming for fine particles and microbial control, and denitrification systems to eliminate nitrate (Ebeling and Timmons 2012, Goddek, Joyce et al. 2019).

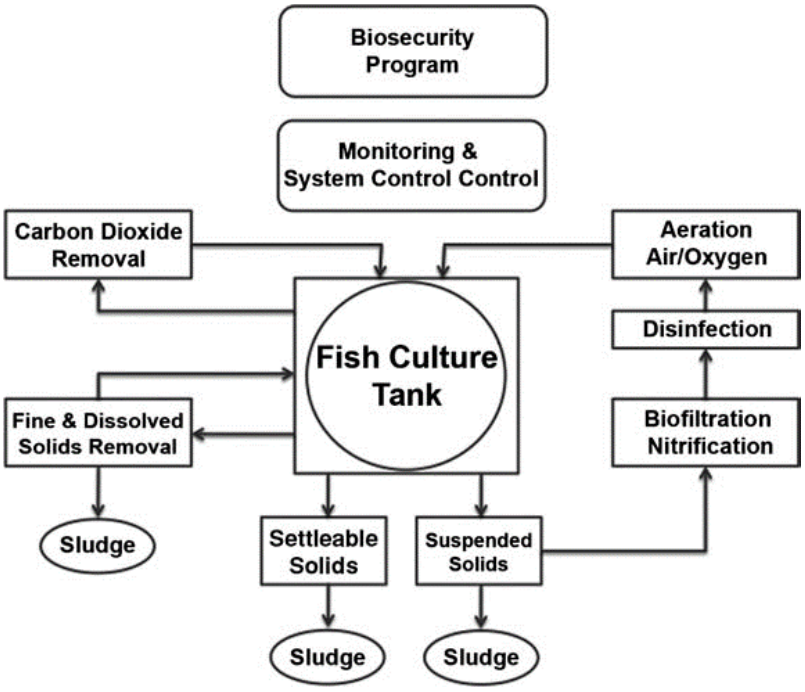

Figure 4. Recirculating aquaculture system abstract schematic (Ebeling and Timmons 2012).

The primary production facilities in a RAS building are divided into five main sections: hatchery and grow-out, breeding, long-term holding, short-term holding, and display (Yanong 2012). Some of the key species reared in RAS in Nordic countries are Atlantic salmon, rainbow trout, eel, Arctic char, pike perch, and sturgeon. Additionally, experiments are underway to rear other species, such as Nile tilapia, European lobster, and many others (Dalsgaard, Lund et al. 2013).

Wastewater in RAS mainly comprises unconsumed food, fish feces, bacteria, biofilm, and other suspended particulates, all of which can significantly impact fish health. Therefore, effective water treatment equipment is essential to filter and purify the water, allowing it to be recycled back into the system (Bao, Zhu et al. 2019). The first key step in water filtration involves removing particles without using chemicals, with various methods available for this purpose. Swirl separators and hydro-cyclones utilize centrifugal force to separate larger particles from the water. Sand filters, which use quartz sand to filter out solids, are another low-cost and efficient type of physical filtration method. However, they can sometimes suffer from media hardening over time. Microscreen drum filters offer a self-cleaning mechanism to remove particles larger than 60 µm, but they may require a significant amount of energy to operate. Parabolic screen filters, on the

other hand, can remove particles larger than 70 µm without consuming power. Foam separation equipment is yet another technique used to remove smaller particles. This method works by using air pressure to create bubbles on the water surface, allowing for the separation of finer particles (Xiao, Wei et al. 2019).

Another crucial step in water treatment within RAS is the use of biofilters which employ microorganisms to remove ammonia and nitrite from the system. Bacteria like *Nitrosomonas* and *Nitrospira* are primarily responsible for nitrification, the process of converting ammonia to nitrite. Meanwhile, Pseudomonas plays a crucial role in denitrification, reducing nitrite to nitrogen gas (Schreier, Mirzoyan and Saito 2010). There is several different equipment utilized for this purpose including Fluidized Sand Biofilters (FSBs), Moving-Bed Biofilm Reactors (MBBRs), Fixed-Bed Biofilm Reactors (FBBRs), Trickling Filters, Rotating Biological Contactors (RBCs), and Bead Biofilters (Xiao, Wei et al. 2019).

1.2 Challenges in salmon farming

Salmon farming in aquaculture faces various challenges in Norway. One significant obstacle, despite the country's extensive 103,000 km coastline and a nearly 1 million kilometer Exclusive Economic Zone (EEZ), is the surprising shortage of coastal space for aquaculture (Hersoug, Mikkelsen and Osmundsen 2021) also the negative environmental consequences associated with cage-based farming arise from the direct exposure of cages to the open environment. This exposure not only affects the stock within the cages due to environmental factors but also directly impacts the wider environment through the production activities in the cages (Lekang 2020).

Another challenge arises with the potential escape of fish, predominantly in sea cages and occasionally in land-based facilities, resulting in diverse adverse consequences. This includes a notable risk of gene pool mixing, the potential transmission of pathogens, and increased competition for mating resources between the cultured species and their wild counterparts (Naylor, Hindar et al. 2005, Føre and Thorvaldsen 2021).

Salmon lice, caused by the parasite *Lepeophtheirus salmonis*, is a significant challenge during the post-smolt period when salmon migrate from rivers to seawater (Myksvoll, Sandvik et al. 2020). This parasite attaches to the salmon's skin, feeding on its mucus, blood, and tissue, which can lead to a variety of problems. These problems include increased risk of secondary infections, skin irritation, and osmoregulatory issues, affecting the overall health and survival of the salmon (Overton, Dempster et al. 2019). The prevalence of salmon lice is closely linked to fish density in aquaculture. In high-density settings, salmon lice tend to multiply and spread at a much faster rate, potentially leading to significant economic losses due to increased treatment costs and a decrease in marketability of the affected fish (Torrissen, Jones et al. 2013).

1.2.1 Disease challenges in aquaculture

Aquaculture faces major hurdles when dealing with disease outbreaks, resulting in substantial worldwide economic losses estimated in the billions. Moreover, these diseases within aquaculture settings can affect nearby wildlife. Unlike closed systems such as recirculating aquaculture systems (RAS) that involve minimal water exchange, open systems like sea cages or ponds have the potential for parasite transfer between farmed and wild fish, posing a threat to nearby wildlife (Bouwmeester, Goedknegt et al. 2021). In Figure 5, the primary factors contributing to fish diseases are illustrated. These include environmental influences such as water quality and feed, intrinsic factors like the fish's genetics and life stage, and external factors like viruses, bacteria, and chemicals (Moreira, Schrama et al. 2021).

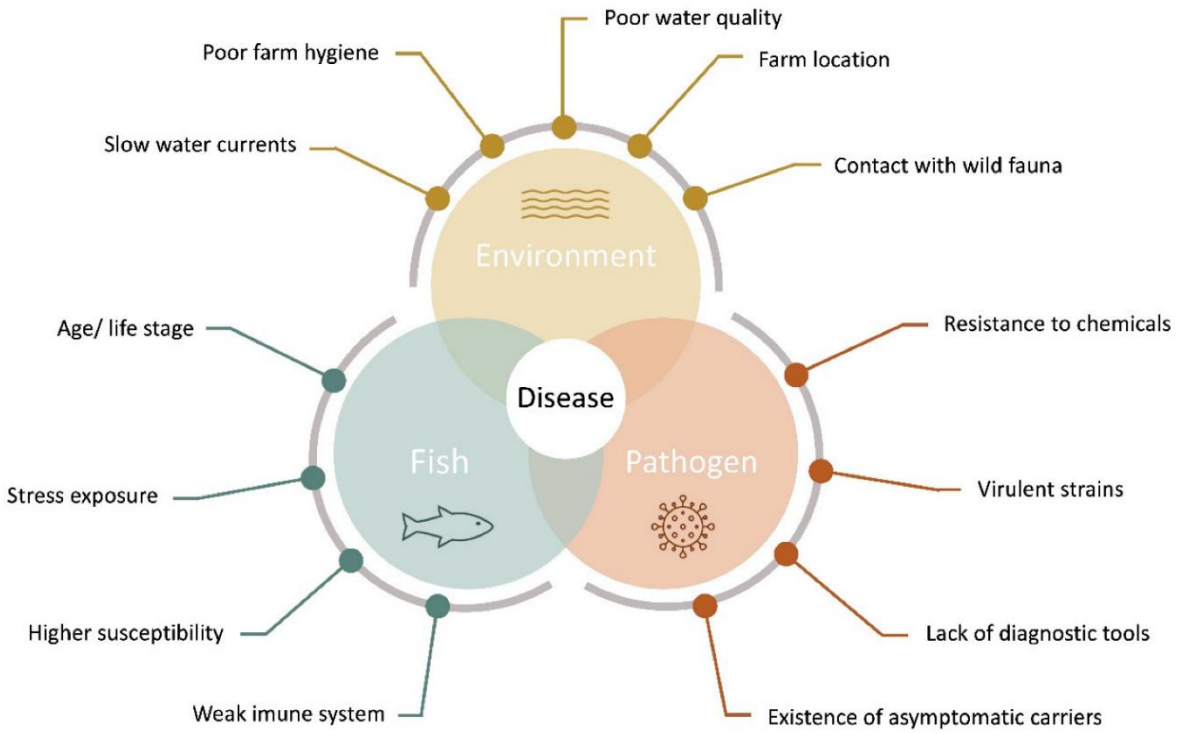

Figure 5. Diagram illustrating the key factors for assessing pathogens and host-pathogen interactions that play a role in the occurrence of fish disease outbreaks in aquaculture (Moreira, Schrama et al. 2021).

1.2.2 *Yersinia ruckeri*

Yersinia ruckeri (*Yersinia*), is a Gram-negative bacterium in the *Enterobacteriaceae* family, causes enteric redmouth disease (ERM) in *salmonids* (Kumar, Menanteau-Ledouble et al. 2015). *Yersinia* displays various serotypes, and the predominant O-serotype holds significance. A swift slide agglutination test, utilizing specific O antigens, facilitates the classification of five distinct strains: O1, O2, O5, O6, and O7, with Serotype O1a notably associated with salmonid infections. Genetic ana*ly*ses indicate substantial homogeneity among O1a strains, indicating clonal expansion (Tobback, Decostere et al. 2007, Ormsby, Caws et al. 2016).

The hallmark of yersiniosis is evident enlargement of one or both eyes, frequently accompanied by erythematous patches on the iris. The histological features have similarities to a typical case of septicemia, in which bacteria are easily detected in the blood and circulating macrophages,

and they can also gather at tissue bleeding sites (Carson and Wilson 2009). In Figure 6, certain impacts of *Yersinia* on salmon fish can be observed.

Figure 6. Salmon infected with Yersinia. In part A, swollen eyes are observable, part B depicts an enlarged spleen. And in part C, subcutaneous hemorrhages are evident. image taken from (Aas 2022).

ERM disease can impact fish across all life stages, but it is most observed in younger fish, particularly fry. In the initial stage, the bacteria attach to the skin and proceed to colonize, demonstrating the ability to form biofilms in the water. Infected fish may display altered behavior, such as swimming near the surface and a reduced appetite. Moreover, the disease can impact the functionality of enteric organs, including the spleen, swim bladder, liver, and pancreas. Histological studies have consistently shown inflammation primarily in internal organs such as the kidney, spleen, and liver (Ahmed, Soliman et al. 2021, Nakatani and Hori 2021).

The primary mode of *Yersinia* transfer occurs through infected fish, which can carry the bacteria for an extended period, surviving for nearly two months. Additionally, transmission can occur

through infected feces, and other animals, such as birds, may serve as carriers for the bacteria. Contaminated water represents another source contributing to the spread of the disease (Kumar, Menanteau-Ledouble et al. 2015).

1.3 Disinfection methods in RAS

RAS offers controlled environments and promotes water recycling by integrating engineering and water treatment procedures. It tackles issues by using water more efficiently, requiring less land, and providing advantages like resilience to outside threats and environmental control (Li, Cui et al. 2023). In aquaculture, disinfection is vital for routine biosecurity, reducing disease incidence, and eradicating diseases. It involves applying chemicals at suitable concentrations, mainly in tank holding facilities and hatcheries, with methods tailored to facility characteristics. Effective disinfection requires removing aquatic creatures, thorough cleaning, proper disinfectant use, and neutralizing chemicals. Disposing of sick populations in waterways is discouraged. The procedures encompass waste removal, prewashing, deep cleaning, disinfection, and rinsing, overseen by a qualified individual, with meticulous documentation (Hill, Berthe et al. 2013).

In Norway, the aquaculture industry operates under stringent regulations to proactively combat diseases, guided by the directives of the Norwegian Food Safety Authority (NFSA), especially in the case of salmon farming. Farmers rigorously adhere to safety protocols specified on disinfectant labels. Salmon eggs, for instance, undergo a meticulous cleaning process using iodophor, while alternative disinfection methods like ozone and glutaraldehyde are employed for eggs of other fish species (Wennberg, Martins et al. 2022). *Mycobacterium chelonae*, *Vibrio salmonicida, Moritella viscosa, Renibacterium salmoninarum, Yersinia ruckeri, Pasteurella sp., Flavobacterium psychrophilum, M. salmonipilum, M. pseudoshottsii, Pasteurella skyensis, M. marinum, M. shottsii, Aeromonas salmonicida salmonicida, M. fortuitum* are among the challenging bacteria in European aquaculture. They primarily affect salmonoids and require proper treatment to avoid destructive consequences (Sommerset, Bang Jensen et al. 2021).

Within RAS, the distribution of microorganisms differs depending on the compartment. The largest microbial reservoirs are found in biofilters, especially single sludge and fixed film biofilters. The uneven distribution of suspended free-floating microorganisms can lead to fouling in pipes and tanks (Rurangwa and Verdegem 2015). Generally, a variety of techniques are employed in RAS for water disinfection. Key strategies include the use of chlorine-based techniques, ultraviolet (UV), and ozone treatment (Ben-Asher, Ravid et al. 2019, Xiao, Wei et al. 2019), as well as the application of hydrogen peroxide (Arvin and Pedersen 2015) and peracetic acid (Liu, Pedersen et al. 2017).

1.3.1 Peracetic acid – PAA

Peracetic acid, also referred to as peroxyacetic acid (PAA), is the peroxide derivative of acetic acid (AA). With a higher oxidation potential compared to chlorine and chlorine dioxide, it serves as a potent disinfectant and oxidizing agent (Kitis 2004). Some of the reasons that make PAA an intriguing option for water disinfection include its potent sterilization capabilities, reduced production of toxic byproducts compared to classic sterilizers such as chlorine, pH independence, and ease of implementation (Ao, Eloranta et al. 2021).

In the study by Mark J. Leggett et al., it has been suggested that PAA does not directly affect DNA but rather induces damage to the inner spore membrane of microorganisms. This damage occurs through the release of DPA (dipicolinic acid) from the spore core, weakening the permeability barrier of the membrane. Additionally, PAA has been observed to affect membrane-associated proteins such as the nutrient germination receptors (GerB and GerK receptors) which are involved in the process of releasing DPA form spore (Leggett, Schwarz et al. 2015).

Research conducted over the past decade suggests that PAA effectively controls various fish pathogens, including *Ichthyophthirius multifiliis*, *Saprolegnia* spp., *Flavobacterium columnare*, *Ichthyobodo necatar*, *Aeromonas salmonicida*, *Yersinia ruckeri*, and marine microalgae *Tetraselmis chuii*, among others. The same study also revealed that semi-continues dosing of PAA, administered at concentrations of 0.05, 0.1, and 0.3 mg/L, does not significantly impact water

quality across various parameters, including total suspended solids, CO2 levels, and dissolved oxygen (Davidson, Summerfelt et al. 2019). In another study in Norwegian RAS aquaculture, salmon has exposed to a range of different dose of PAA, and survival rate has measured. The study examined the impact of PAA on fish survival and behavior. Fish exposed to PAA concentrations ranging from 0 to 1.6 mg/L showed survival, while higher concentrations (3.2 and 6.4 mg/L) led to reduced survival rates and mortality. The study suggests that concentrations below 1.6 mg/L are suitable for disinfecting water in aquaculture, whereas higher concentrations pose risks to fish health (Mota, Eggen and Lazado 2022).

1.3.2 Ozone

Since the early 20th century, ozone has been utilized as a disinfectant due to its potent oxidation capabilities, making it an asset in various industrial applications, including aquaculture. Ozone exhibits the capacity to degrade organic compounds rapidly and acts as a highly reactive agent compared to other disinfectants such as chlorine or H2O2. The rapid action of ozone allows for the utilization of lower concentrations. Additionally, ozone displays non-selectivity towards numerous microorganisms, providing another advantage of this substance (Eriksson 2005, Gardoni, Vailati and Canziani 2012).

Ozone acts in water through two primary pathways: direct reaction with compounds and indirect reaction via radicals formed during ozone decomposition. Various substances, including hydroxide ions, peroxides, and activated carbon, water pH, temperature and bicarbonate level can initiate ozone decomposition, leading to the formation of secondary oxidants such as HO• radicals (Summerfelt and Hochheimer 1997, Faria, Órfão and Pereira 2006). The primary mechanism by which ozone affects bacteria and fungi involves damaging cell membranes and cytoplasmic components. It has been widely discussed that ozone can be highly effective against both gram-negative and gram-positive bacterial strains (Thanomsub, Anupunpisit et al. 2002).

Y.Q. Zhang et al. conducted a study to assess the effects of ozone on Pseudomonas aeruginosa, a Gram-negative bacterium recognized for causing infections, especially in immunocompromised

individuals. Their research revealed that ozone damaged the cells' cytoplasmic membrane, causing the leakage of cell components like K+, Mg2+, and ATP, ultimately leading to cell inactivation and death (Zhang, Wu et al. 2011). In another study by D.B. McNair Scott et al., the effect of ozone on *E. coli* was examined. It was found that ozone had a rapid reaction with the unsaturated fatty acid components on the cell wall, potentially targeting the double bonds of fatty acids. This reaction resulted in the leakage of cellular contents (Scott and Lesher 1963). In a study by H. Liltved et al., the effect of ozone on various bacteria presents in water, including *Yersinia*, was investigated. They found that exposure to ozone at concentrations ranging from 0.15 to 0.2 mg/liter resulted in the eradication of the bacteria from the water by 99.99% within two minutes (Liltved, Hektoen and Efraimsen 1995).

1.4 Genetic applications in aquaculture

The utilization of molecular genetic applications developed in the 1980s, such as restriction enzymes and polymerase chain reactions (PCR), has revolutionized various fields of science, including aquaculture (Okumuş and Çiftci 2003). These advancements have enabled researchers to delve into molecular and DNA-level studies. In aquaculture, molecular data serves two general purposes: individual and population studies (Lo Presti, Lisa and Di Stasio 2009). Gene expression involves a series of intricate steps such as transcription, RNA splicing, translation, and post-translational modifications, all orchestrated by various enzymes and mechanisms. These processes utilize DNA sequences to generate functional proteins or RNA molecules, playing crucial roles in organismal development. Notably, gene expression can be dynamically regulated in response to changes in the environment. Gene expression ana*lys*is offers a convenient approach to investigate the molecular underpinnings of phenotypic variations, responses to environmental cues, and dynamics within populations. Various methods, including RNA expression ana*lys*is (e.g., northern blotting, DNA microarrays, real-time PCR), promoter ana*lys*is, protein expression ana*lys*is, and post-translational modification ana*lys*is, are employed for this purpose (Gibney and Nolan 2010, Chandra and Fopp-Bayat 2021).

1.4.1 qPCR and detection of expression

In 1984, Kary Mullis pioneered the Polymerase Chain Reaction (PCR), a method used for RNA expression ana*lys*is (Saiki 1985). PCR amplifies a specific segment of DNA, generating millions of copies of a target fragment within hours. Initially employed for qualitative studies, such as determining the presence or absence of a specific sequence in a sample, PCR underwent further development. In 1992, Higuchi et al. introduced the quantitative Polymerase Chain Reaction (qPCR), facilitating gene expression studies by utilizing the same PCR process. To conduct qPCR, the same materials as conventional PCR are employed, allowing for amplification and detection processes to occur simultaneously within the same vial. The PCR reaction involves cyclical temperature changes, including denaturation, primer hybridization, and elongation or polymerization stages. Ideally, DNA molecules double with each cycle if the reaction efficiency is 100%, although in optimal conditions, efficiency typically is less than that (San Segundo-Val and Sanz-Lozano 2016).

Exponential, linear, and plateau are the three main stages of the qPCR process shown in Figure 7. Initially, an abundance of reagents and enzymes causes the PCR product to grow exponentially. As the process progresses into the linear phase, product accumulation is limited by the availability of reagents. Eventually, during the last stage, primers and dNTPs are depleted, which stops product accumulation (Yuan, Reed et al. 2006).

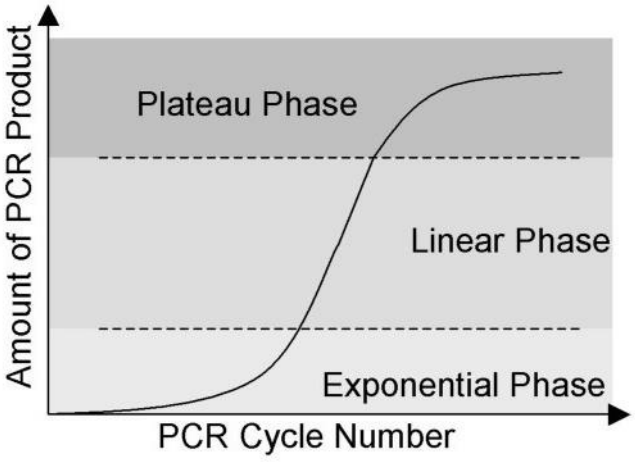

Figure 7. The main phases of qPCR reaction (Yuan, Reed et al. 2006).

It is possible to quantify real-time PCR data by two absolute or relative methods. Relative quantification compares the expression of the target gene to reference genes either within the same sample or between samples, whereas absolute quantification uses calibration curves to determine the precise transcript copy number. The efficiency calibrated model and the ΔΔCt model are two commonly used mathematical models for relative quantification. Gene expression in treatment and control samples can be compared by the efficiency calibrated model, which computes amplification efficiency from Ct values and cDNA inputs. A more generalized version of this model, called ΔΔCt, uses the amplification efficiencies of the target and reference genes to calculate expression ratios as described below: (Yuan, Reed et al. 2006, Rao, Huang et al. 2013)

ΔCt = Ct (a target gene) - Ct (a Reference gene)

ΔΔCt = ΔCt (a target sample) - ΔCt (a reference sample)

Fold change = $2^{-\Delta\Delta CT}$

1.4.2 Immune response genes

Animal immune systems are crucial for protecting them against threats such as bacteria, viruses, and parasites (Yatim and Lakkis 2015). While research on mice and humans has significantly advanced our understanding of immunity, there's a growing interest in fish immunology, using zebra fish as a model (Sullivan and Kim 2008). Studying the immune systems of fish offers valuable insights into the evolution of immunity among lower vertebrates and sheds light on the development of both innate and adaptive immunity. This exploration extends to comparative immunology, evolutionary aspects of immunity, and practical applications in aquaculture and related industries. In examining fish immunology, genes related to immune response can be categorized into three primary branches.

Firstly, innate immunity relevant genes encompass those involved in the initial and unspecific defense against pathogens. This branch includes various subcategories such as pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs) and trigger immune responses (Mogensen 2009). Additionally, antimicrobial peptides play a crucial

role in innate immunity by disrupting bacterial membranes or hindering bacterial growth (Duarte-Mata and Salinas-Carmona 2023). Complement molecules contribute to opsonization, inflammation, and pathogen *lys*is, while lectin family members recognize carbohydrates on pathogens, initiating immune responses. Cytokines, including interferons, interleukins, tumor necrosis factors, colony-stimulating factors, and chemokines, modulate immune responses (Zhu LvYun, Nie Li et al. 2013).

Secondly, adaptive immunity relevant genes form a more specific and enduring defense mechanism that evolves over time. This category includes Major Histocompatibility Complex (MHC) molecules, which present antigenic peptides to T cells, eliciting adaptive immune responses (Danchin, Vitiello et al. 2004). Immunoglobulins (Igs), comprising antibodies and B cell receptors produced by B cells, mediate humoral immune responses (Mix, Goertsches and Zett 2006). Adaptive Immunity Relevant Cytokines, such as interleukins, play pivotal roles in regulating the activation and function of various immune cells (Wang and Secombes 2013).

Lastly, negative immune regulators encompass molecules or mechanisms that temper immune responses to prevent excessive inflammation or autoimmunity. These regulators primarily involve pathways such as JAK-STAT and NF-jB signaling (Lieschke and Trede 2009, Zhu LvYun, Nie Li et al. 2013).

1.4.3 Oxidative stress genes

Oxidative stress occurs from an imbalance in the production and elimination of reactive oxygen species (ROS) within the cell which are produced by both endogenous enzymatic activities and external sources, potentially leading to cell damage or death. However, it has been discovered that ROS also serves as signaling molecules, influencing various cellular functions, impacting processes like cell growth and repair. Organisms have developed antioxidant defense mechanisms to counteract the rise in ROS products, with genes capable of expressing and aiding in the regulation of these molecules (Scandalios 2002, Dato, Crocco et al. 2013). Oxidants like hydrogen peroxide can stimulate the expression of certain genes, for instance Ras signaling pathways which

leads to the production of more ROS molecules, while antioxidants can have the opposite effect (Allen and Tresini 2000).

Various factors can induce oxidative stress reactions in fish. These include exposure to pollutants or chemicals like heavy metals and pesticides, fluctuations in environmental conditions such as water temperature, oxygen and pH levels, salinity, and the presence of disinfectants like ozone (Subaramaniyam, Allimuthu et al. 2023). Additionally, the handling and transportation of fish can also impact this process. Oxidative stress can trigger several different signaling pathways depending on the factors that cause it. For instance, during hypoxia, key pathways like AMPK, MAPK, Nrf2/Keap1, and NFκB play crucial roles in adaptation. Additionally, the transcription factor HIF regulates responses to low oxygen levels (Chowdhury and Saikia 2020). S. Reiser et al. investigated the impact of varying ozone concentrations on fish histology and the expression of oxidative stress genes over a 21-day period. They highlighted that even minimal ozone levels can result in the production of ozone-produced oxidants (OPO). Their findings indicated that ozonation induces the expression of heat shock proteins (hsp) and glutathione S-transferases (gst) mRNA in both the liver and gills of turbot. The study further discussed that prolonged exposure led to a reduction in expression levels, possibly due to cellular adaptation and cellular defensive mechanisms (Reiser, Wuertz et al. 2011).

1.4.5 Housekeeping genes

Housekeeping genes are crucial elements in the cell, maintaining critical functions with consistent expression levels across various cell types and tissues, even under different conditions. They play a vital role in cell survival and serve as reliable internal controls in experimental studies (Joshi, Ke et al. 2022). These genes are characterized by distinct genomic and evolutionary elements, including shortened exons and introns, particular repetitive sequences, and rich protein domains (Eisenberg and Levanon 2013). Olsvik, Pål A., et al., investigated the stability of six reference genes, including *18S rRNA*, *S20*, *β-actin*, *GAPDH*, *EF1AA*, and *EF1AB*, across different tissues in fish. They found that elongation factor (*EF1AA* and *EF1AB*) and *β-actin* exhibited good stability

and can be considered reliable sources for research (Olsvik, Lie et al. 2005). Additionally, in a separate study by Jorgensen, Sven Martin, et al., the stability of *RPL1, RPL2, EF1A, G6PDH, ACTB, B2M, 18S, MHC class I, Pparα*, and *LPL* was examined. They demonstrated that *EF1A* showed the highest stability in gene expression levels across various conditions, including viral infection (Jorgensen, Kleveland et al. 2006).

2 Introduction

As the population increases, the demand for food grows, and aquaculture emerges as a reliable source to fulfill both food and nutritional needs for humans, particularly serving as a significant protein source (Pradeepkiran 2019). Norway plays a pivotal role in Europe's aquaculture sector, benefitting from its expansive coastline, abundant fjords, and islands that offer protection from adverse weather conditions. Additionally, the presence of the Gulf Stream helps regulate water temperatures year-round, preventing ice formation in the Arctic waters that flow along the coastline (Paisley, Ariel et al. 2010). According to the FAO's 2022 report, it stands as the second-largest exporter of aquatic products globally (FAO 2022). Salmon and rainbow trout are among the key species in Norwegian aquaculture, playing a crucial role. Nevertheless, the aquaculture industry in Norway is predominantly centered around salmon production (Espinasse, Mikkelsen et al. 2023). Atlantic salmon stands out as a sustainable choice for aquaculture, because they easily adjust to farm environments, exhibit rapid growth to substantial sizes, provide high-quality meat, and yield a large amount of fillet (Forster 2002).

RAS is a sustainable aquaculture method that allows for significant fish production while using minimal resources, prioritizing water conservation, biosecurity, and high output (Aich, Nama et al. 2020). Disease outbreaks pose significant challenges in RAS, resulting in substantial economic losses annually (Moreira, Schrama et al. 2021). *Yersinia*, a Gram-negative bacterium, is responsible for causing ERM in fish, which can result in fish blindness, enlarged spleen and kidney, and ultimately death (Wrobel, Leo and Linke 2019). Accordingly, water disinfecting is a very crucial action in RAS, so it helps routine biosecurity and reduce disease incidence (Hill, Berthe et al. 2013). PAA, a potent and sustainable antimicrobial compound, finds extensive use in aquaculture for disinfection, ensuring robust biosecurity within freshwater systems due to its antipathogenic properties and rapid degradation (Soleng, Johansen et al. 2019). Ozone has demonstrated significant disinfection efficacy against a diverse spectrum of fish pathogens, including bacteria, viruses, and fungi, also effectively removing organic carbon from the water (Gonçalves and Gagnon 2011). These substances act as strong oxidizing agents, primarily targeting the cell

membrane, which results in the deactivation or killing of the microorganisms (Kitis 2004, Gardoni, Vailati and Canziani 2012).

Q-PCR serves various purposes, such as studying mRNA expression levels. It facilitates the identification and quantification of PCR products produced in each cycle of the process, in direct proportion to the template quantity. These instruments, utilizing diverse chemistries like TaqMan probes and SYBR Green I dye, capture fluorescent signals during PCR (Ginzinger 2002).

2.1 Aim of the study

In previous project (by S. Mousavi et al.), the effect of ozone and PAA on fish that were kept in normal water (control) and fish that were exposed to *Yersinia* bacteria has been investigated. In this project, the same scenario is examined from a molecular perspective. The main goal of this project is to analyze and compare the expression levels of genes related to immunity, oxidative stress, and microbial defense under different conditions. To enhance ana*lys*is and comprehension, the impact of PAA and ozone treatment, alone or along with *Yersinia*, on gene expression is examined by comparing changes between timepoints one, two, three, and five. Additionally, the influence of *Yersinia* on gene expression is investigated by analyzing changes across timepoints one, three, and five in control-treated RAS units.

Research Question 1: Does exposure to PAA and ozone affect gene expression in fish?

Hypothesis 0: Exposure to these disinfectants does not change the expression levels of the genes.

Research Question 2: Does *Yersinia* affect gene expression in fish?

Hypothesis 0: *Yersinia* challenge does not change the expression levels of the genes.

3 Materials and methods

3.1 Ethical statement

The project focuses on the RAS health, building upon a previous master thesis study by S. Mousavi titled "Evaluation of ozone and peracetic acid use during a *Yersinia* challenge in Atlantic salmon (*Salmo salar*) freshwater recirculating aquaculture systems." Which has been approved by the Norwegian Food Safety Authority (Mattilsynet) under FOTS ID number 28715. This experimental trial took place in the fish health laboratory of Tromsø Aquaculture research station (Havbruksstasjonen i Tromsø AS, Kårvik, Norway) (Mousavi 2023).

3.2 Experimental setup

This section summarizes the experimental trial conducted in a previous thesis project by S. Mousavi It aims to enhance understanding of the work carried out in this thesis (Mousavi 2023).

The experiment utilized nine individual RAS units, each with a capacity of 0.8 m^3, situated within the infection room of the fish health laboratory. Atlantic salmon parr were randomly distributed across these nine RAS units, as illustrated in Figure 8.

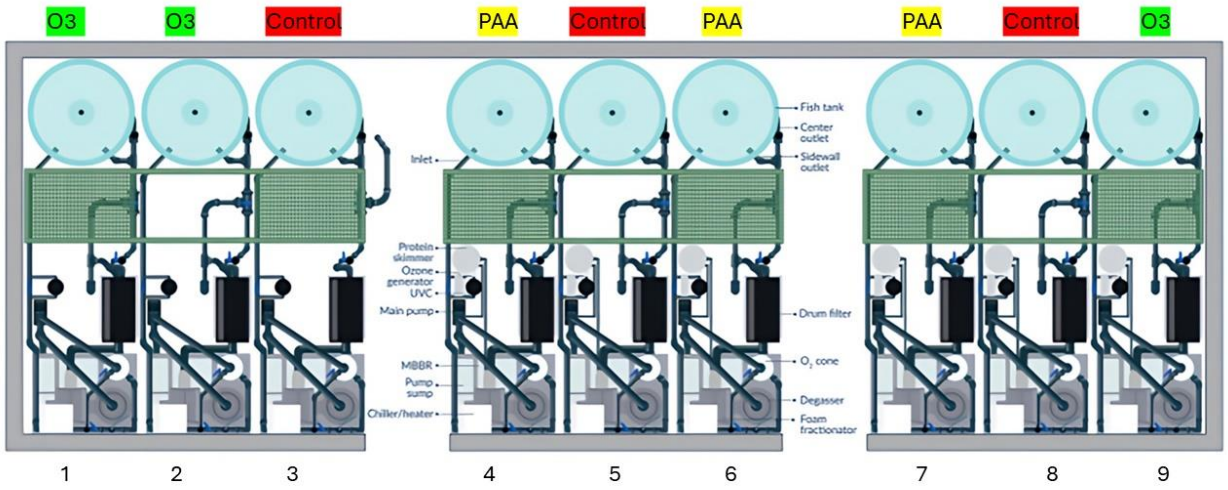

Figure 8. The illustration of trial with 9 RAS units. Figure retrieved from Mota, Striberny, et al. (2022), with modifications.

The experiment involved three different scenarios: non-disinfection (control), peracetic acid (PAA), and ozone (O3) treatments in freshwater recirculating aquaculture systems (FW-RAS) with Atlantic salmon parr during a *Yersinia* outbreak, each replicated three times (n=3). PAA was administered semi-continuously at a concentration of 1 mg/L, O3 was continuously applied at a concentration of 300-350 mV, and the control group underwent no disinfection.

In this experiment, fish were transferred to the RAS on day -29 and maintained under normal conditions for 29 days. On day 1, disinfectants (PAA and O3) were introduced into the system. Subsequently, *Yersinia* (serotype O1) was added to the units on day 9, and the fish were kept under these conditions for an additional 20 days before being harvested. Figure 9 provides an overview of the different periods and sampling points throughout the experiment.

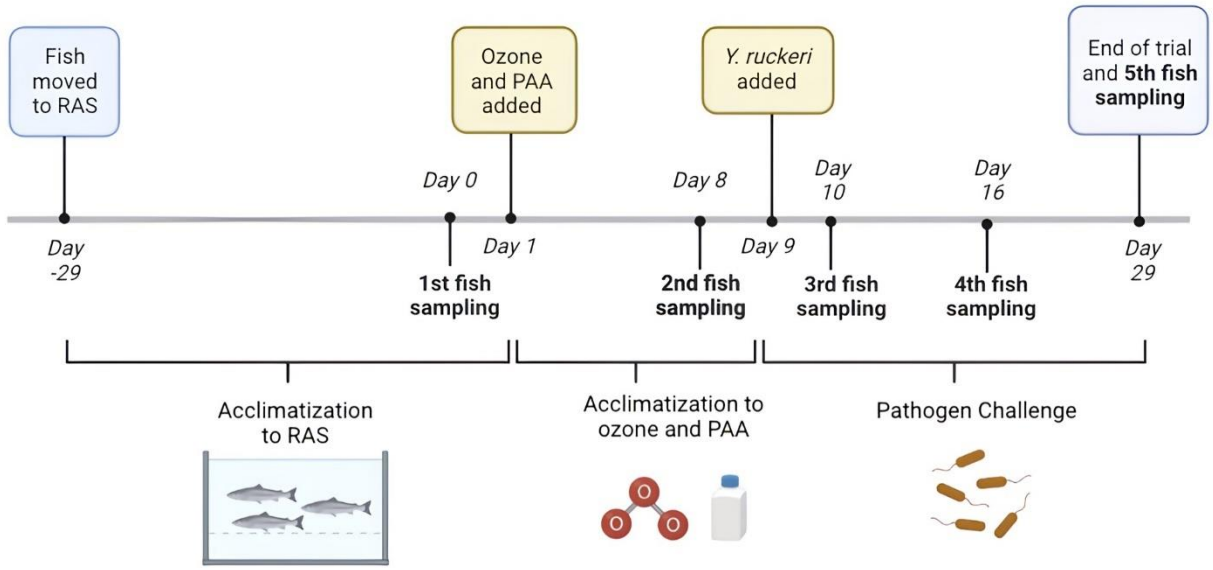

Figure 9. As depicted, the fish were acclimatized to normal water for 29 days before the introduction of disinfectants on day 1. The initial sampling occurred on day 0, prior to any treatment exposure. The second sampling point was at day 8, which was 7 days after exposure to PAA and O3 treatments. The third sampling point was on day 10, 24 h after the fish were exposed to Yersinia (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). The fourth sampling point (which was not included in our project), was scheduled for day 16. The final sampling point was conducted on day 29.the illustration retrieved from S. Mousavi et al. thesis project.

Initially, 1800 Atlantic salmon were reared in a flow-through freshwater system with 24-hour light manipulation and were fed a standard commercial parr feed supplied by the Tromsø Aquaculture Research Facility, with a body weight of 19.1 g. Subsequently, they were randomly allocated into groups of 200 fish per RAS unit. Parameters associated with water quality were continuously monitored and maintained within the recommended range for optimal conditions. To ensure proper system operation, various calculations were performed, including water exchange rates and tank hydraulic retention time.

3.3 Samples

In this project, four types of fish organs were examined: gills, skin, spleen, and olfactory. These tissues were preserved in RNA later solution (Thermo Fisher Scientific, Oslo, Norway) at -80°C. Upon receipt, they were arranged as detailed in the table below:

Table 1. Different sampling points.

Sampling	Date	Experimental period
1	29.11.2022	Before ozone and PAA (All control after 29 days acclimatization)
2	07.12.2022	8 days after Ozone and PAA, and before adding pathogen *Yersinia*
3	09.12.2022	24h after adding *Yersinia* in all 9 RAS/tanks
4	15.12.2022	8 days after adding *Yersinia* in all 9 RAS/tanks
5	28.12.2022	21 days after adding *Yersinia* in all 9 RAS/tanks

Sample number 4 was not analyzed due to the substantial amount of data on hand. Also, it was not expected that this timepoint would have a significant impact on the results and conclusions. Each sampling event comprises 45 fish, with 5 fish per RAS unit, as listed in Appendix 1. For this project, a random selection of 3 fish per RAS unit was utilized, resulting in a total of 27 fish samples per timepoint.

3.4 Cutting and *lys*is of tissues

First, the Qiagen collection tube-plate was filled with beads (2 beads per tube). Each tube was then filled with 400 µL of *lys*is LBE buffer (REF C39467, Beckman coulter, USA). At the next step, the tissues which were already inside RNA later solution were prepared. On the other hand, Falcon tubes were prepared with ddH2O and 70% EtOH to clean forceps. (instead of 5% bleach, RNase away was used). In the beginning gill has been sampled as described in the appendix 2.

After transferring the samples into the tubes, each tube was sealed with Thermal adhesive PCR film before being frozen at -40 degrees Celsius. Plate 2 contains the remaining gill samples and skin samples. After collecting the samples in the *lys*is buffer, they were stored in a -80°C freezer.

The other day, samples were thawed in a heat cupboard (B 8054, Termaks, Bergen, Norway) for approximately 15 minutes. Following this, 20 microliters of proteinase K enzyme (REF C42150) were added into each tube and securely sealed with push cap lids to ensure the samples were well-protected. Then, the samples were homogenized with the FastPrep-96™ machine (6010500, CA, USA), setting it to the maximum shaking power of 1800 for 120 seconds. Subsequently, a centrifuge machine (avanti J-30I, Beckman Coulter, CA, USA) at 1600 RPM was used for 1 minute. Finally, the samples were placed in a heat cupboard for 1 hour to allow the proteinase K enzyme to effectively digest the tissues.

3.5 From sample to cDNA

Synthesizing cDNA from a sample is an essential step toward gene expression, involving several processes outlined in detail below, including RNA extraction, concentration and purity control using methods like Nano-Drop spectroscopy, normalization to ensure equal starting material for downstream reactions, and amplification through reverse transcription PCR (RT-PCR).

3.5.1 RNA extraction

The RNA extraction robot (Beckman coulter biomek 4000, CA, USA) functioned through a series of automated steps to isolate RNA from degraded tissue samples. Initially, the samples were handled, transferring them into specialized plates. Then, specific reagents and protocols were utilized to bind the RNA to a solid support as mentioned:

• Mixed ethanol 96% (Avantor, VWR chemicals, France) with ddH2O to make 70% ethanol.

• Mixed bind solution (REF C42089, Beckman coulter, USA) with Isopropanol (Avantor, VWR chemicals, France).

• Mixed RNAse free water with DNAse buffer and DNAse enzyme (LOT 2893971, Thermo Scientific kit).

followed by one or more washing steps to remove impurities. Subsequently, the RNA was eluted from the solid support using an appropriate solution. Additionally, quality control measures were incorporated to assess the quantity and quality of the extracted RNA. Finally, the purified RNA was transferred to storage vessels for further ana*ly*sis. The whole process took approximately around 2 and a half hours.

3.5.2 Nano-drop test

Nano-drop machine (Thermo scientific, nanodrop 8000, USA) was utilized to analyze the quality and purity of the RNA samples. To prepare for this ana*ly*sis, the measurement pedestals were first cleaned with paper and water. Subsequently, they were normalized with 1.2 microliter of water per dot and further normalized with 12 microliters of elution buffer per dot. Finally, the samples were loaded into the machine and the ana*ly*sis was initiated.

3.5.2.1 RNA quality analysis

In this study, the acceptable range for the ratio of A260/A280 in Nanodrop ana*ly*sis of RNA typically fell between 1.8 and 2.1, indicating pure RNA without contamination. Similarly, for the ratio of A260/A230, the acceptable range was generally between 1.8 and 2.2, suggesting minimal contamination by substances such as salts, phenol, or carbohydrates. However, there were samples that fell out of this range due to either high or low concentration of RNA, and these samples were subsequently extracted and replaced.

3.5.3. Sample normalization

To normalize all the samples to have an equal amount of RNA for each treatment, it is necessary to dilute all the samples (4x). Therefore, the samples contain 30 microliters of Nuclease free water and 10 microliters of RNA samples.

Now, the new concentration is ¼ of the previous concentration, and there needs to be 300 ng of RNA molecule inside the solution. The final solution volume must be 20 microliters. For instance, if 3 microliters of RNA are needed, 17 microliters of RNase free water should be added.

3.5.4. DNA removal from the samples

To remove DNA molecules from samples, all the samples were treated with DNAse 1 enzyme and buffer solution. For this purpose, 1 µl of enzyme was mixed with 1 µl of buffer. Because each plate contains 96 tubes, 96 µl of each solution was needed, but due to pipetting error 110 µl of each solution was taken.

After mixing 110 µl of each solution, each tube was loaded with 2 µl of mix and then the plate was centrifuged for 30 seconds at 1600 RPM. Finally, the plates were put in the PCR machine (applied biosystems veriti 96 well thermal cycler) with 2 main cycles as follows:

30 minutes at 25°C (for activating the enzyme)

2 minutes at 75°C (for deactivation of the enzyme)

3.5.3 cDNA synthesis

To synthesize cDNA, a reverse transcription master mix is prepared (LOT 2944697, High-Capacity cDNA Reverse Transcription Kit, Thermo fisher) which contains 2.0 µL of 10X RT Buffer, 0.8 µL of 25X dNTP Mix (100 mM), 2.0 µL of 10X RT Random Primers, 1.0 µL of MultiScribe Reverse Transcriptase and 4.2 µL of Nuclease-free H_2O which totally becomes 10.0 µL of solution per reaction. For 96 tubes, multiply everything by 110 due to pipetting error.

After making this mix, 10 µL was taken for each tube and then 10 µL of RNA samples were added into the tubes. So, the final amount would be 20 µL. Then the plate was put into the PCR machine with the program that optimized for use with the High-Capacity cDNA Reverse Transcription. During this program, the PCR machine undergoes four steps. The first step is at 25°C for 10 minutes, followed by 37°C for 120 minutes, equivalent to 6 cycles of 20 minutes each. Step 3 is at 85°C for 5 minutes, and the last step is at 4°C. After the PCR is done, the cDNA is in the plates and can be stored at -20 degree C for a long time.

3.6 Realtime-PCR

Real-time PCR is a potent technique that has proven essential in biological research and clinical diagnostics for the detection and quantification of minute amounts of nucleic acid sequences. Using fluorescent technology to monitor amplification in real-time, it is possible to precisely detect changes in gene expression and evaluate disease states. Its broad use in many academic disciplines during the 1990s is a testament to its adaptability and influence (Valasek and Repa 2005).

To utilize RT-PCR (Quantstudio 5, applied biosystems), the cDNA samples were initially diluted with a ratio of 1/19 (20x) and Stock primers are diluted with the ratio of 1/9 before use, so they were (10x). Subsequently, a q-PCR master mix was prepared with 0.5 µL of Forward primer, 0.5 µL of Reverse primer and 5.0 µL of SYBR green mix (LOT 2898181, applied biosystem) per sample. Each q-PCR plate contains 384 wells, and during q-PCR, the samples were duplicated, with each tube requiring 4 µL of cDNA sample and 6 µL of the master mix illustrated in detail are described at the appendix part 3.

For the RT-PCR program, an 8-step setup was employed. The first step was regulated at 50°C for 2 minutes. Step two involved heating to 95°C for 20 seconds, followed by step three at 95°C for 1 second. Step four consisted of incubation at 60°C for 20 seconds, followed by step five at 95°C for 1 second. Step six repeated the 60°C incubation for 20 seconds. Finally, step seven entailed heating to 95°C for 1 second.

3.7 Table of primers

Table 2. Presented below is the table of primers used.

Category	*Gene name*	*Abbreviation*	*Sequence (5'-3')*	*Reference*
House keeping genes	*β-actin*	*actb*	F: CCAAAGCCAACAGGGAGAA R: AGGGACAACACTGCCTGGAT	(Sanden and Olsvik 2009)
	Elongation factor 1-α	*elf1a*	F: GAATCGGCTATGCCTGGTGAC R: GGATGATGACCTGAGCGGTG	(Garcia de la serrana and Johnston 2013)
Immune related genes	*Interleukin-1 β*	*il1b*	F: AGGACAAGGACCTGCTCAACT R: CCGACTCCAACTCCAACACTA	(Ingerslev, Rønneseth et al. 2009)
	Interleukin-8	*il8*	F: GAAAGCAGACGAATTGGTAGAC R: GCTGTTGCTCAGAGTTGCAAT	(Soto-Dávila, Valderrama et al. 2020)
	Secreted IgM	*sigm*	F: CTACAAGAGGGAGACCGGAG R: AGGGTCACCGTATTATCACTAGTTT	(Jenberie, Thim et al. 2018)
	membrane bound IgM	*migm*	F: CCTACAAGAGGGAGACCGA R: GATGAAGGTGAAGGCTGTTTT	(Jenberie, Thim et al. 2018)
Oxidative stress genes	*Glutathione S-transferase*	*gsta*	F: AGGGCACAAGTCTAAAGAAGTC R: GTCTCCGTGTTTGAAAGCAG	(Lazado and Voldvik 2020)
	Glutathione peroxidase	*gpx*	F: GATTCGTTCCAAACTTCCTGCTA R: GCTCCCAGAACAGCCTGTTG	(Solberg, Kvamme et al. 2012)
	Catalase	*cat*	F: GGGCAACTGGGACCTTACTG R: GCATGGCGTCCCTGATAAA	(Olsvik, Vikeså et al. 2013)
	Manganese superoxide dismutase	*mnsod*	F: GTTTCTCTCCAGCCTGCTCTAAG R: CCGCTCTCCTTGTCGAAGC	(Solberg, Kvamme et al. 2012)
Microbial Defense genes	*Lysozyme*	*lys*	F: CACCGACTATGGCATCTTCC R: CTGACCGCCACTGTGATGTC	(Mutoloki, Cooper et al. 2010)
	Cathelicidin	*camp*	F: AAGCCAGAAAATGCTCCAGA R: ACCCTCAGGACGACCAATTA	(Eslamloo, Caballero-Solares et al. 2020)
	Mucin 5 ac-like	*muc5ac*	F: GACCTGCTCTGTGGAAGGAG R: AGCACGGTGAATTCAGTTCC	(Sveen, Grammes et al. 2017)
Test	*Yersinia Ruckeri*	*Yersinia*	F: GCGAGGAGGAAGGGTTAAGTG R: GAAGGCACCAAGGCATCTCT	Nofima

3.8 Statistical analysis

Excel was utilized to organize the data, and the delta r between the first and second replications was computed. A delta r exceeding 0.5 was considered as an error limit, prompting us to repeat samples exceeding this threshold. The average of replications was then calculated, and the

geometric mean of two reference genes was determined. Subsequently, delta Ct (ΔCT), delta delta Ct (ΔΔCT), and $2^{-\Delta\Delta CT}$ were calculated as fold changes. Later, the data was organized by tank order, with each tank containing 3 fish. The average for each tank was calculated, so N=3. The fold change values calculated in Excel, were imported into GraphPad Prism 10.1.2 (Dotmatics, CA, USA) for visual representation. Data ana*lys*is was performed using SPSS 27.0 (IBM® SPSS® Statistics, IL, USA). Initially, an outlier test was conducted, and data with extreme outliers were excluded. Subsequently, the homogeneity of variance (Levene's test) was assessed at a 95% confidence level. Samples meeting the assumption ($p > 0.05$) underwent one-way ana*lys*is of variance ANOVA (Tukey test), with p-values calculated using a 95% confidence level. For samples not meeting the assumption of homogeneity of variance (Levene's test) ($p < 0.05$), a log transformation was applied to the data, and the test was re-run. If the assumption continued to not be met, non-parametric ana*lys*is (Kruskal-Wallis test) was performed (figure 10). A one-way ANOVA was conducted separately for both treatments (N=3) and time-points (N=3) to assess significant changes in gene expression. This approach was adopted due to the unavailability of a non-parametric test with two-way ANOVA. Consequently, the data were analyzed twice: once for evaluating timepoints and again for assessing treatments for each gene. The results from these two ana*lys*es were aggregated and depicted in a single figure for each gene. The values provided consist of means along with their respective standard deviations (SD).

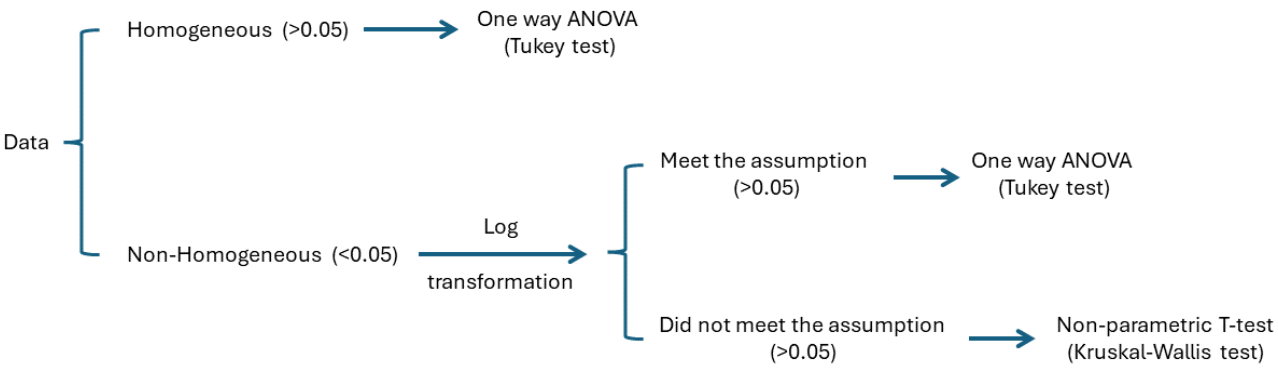

Figure 10. Methodology for analyzing data using SPSS.

4 Results

4.1 Gill

Figure 11 depicts the immune gene expression of *sigm*, *migm*, *il1b*, and *il8* across three different treatments: control, ozone, and PAA, over four different timepoints (specified in Table 1) in gill tissue. Notably, no significant changes are observed in the expression of the *migm* (Figure 11.B) and *il1b* (Figure 11.C) genes. However, the expression of the *sigm* gene (Figure 11.A) shows a significant decrease (p-value: 0.025) in the ozone treatment from timepoint 1 (before disinfection) to timepoint 5 (21 days after *Yersinia*), showing that Prolonged exposure to *Yersinia* resulted in a significant reduction in *sigm* gene expression within the ozone-treated tank.

Significant alterations are observed in the expression of the *il8* gene (Figure 11.D). Samples treated with ozone display a notable reduction in expression from timepoint 1 (pre-disinfection) to timepoints 2 (8 days post-disinfection) and 5 (21 days post-*Yersinia*), with corresponding p-values of 0.036 and 0.022, respectively. This indicates that both disinfection and long-term exposure of *Yersinia* infection led to a reduction in the expression of this gene within the ozone-treated tank.

Furthermore, in the same gene, a significant decrease is noted in the control treatment from timepoint 1 (pre-disinfection) to timepoint 3 (24 hours post-*Yersinia* exposure), with a p-value of 0.039. This suggests that short-term exposure to *Yersinia* resulted in a reduction in the expression of the *il8* gene within the control treatment.

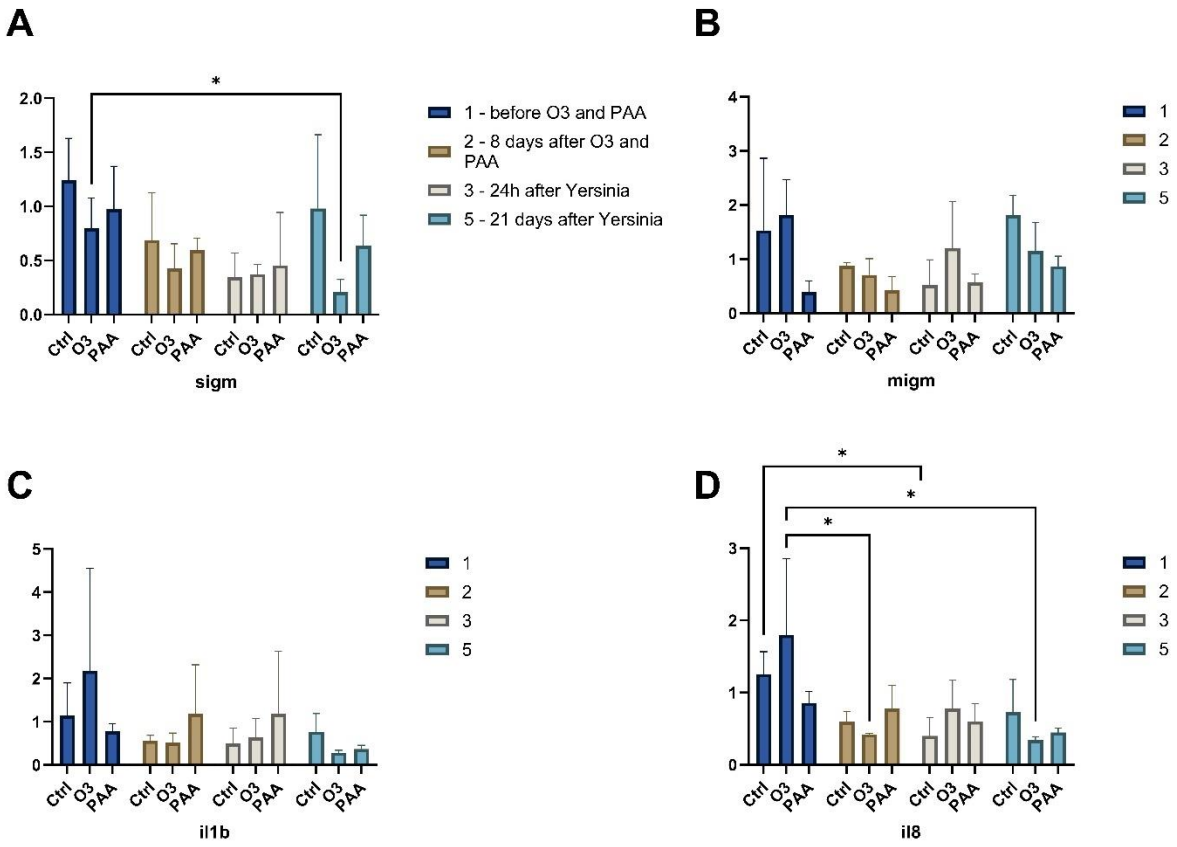

Figure 11. Expression levels of immune-related genes, sigm, migm, il1b, and il8 (A-D) in gill tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with an asterisk (*). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

Microbial defense genes, *camp*, *muc5ac* and *lys* in gill tissue are depicted in figure 12. As can be seen, there are no significant changes observed in the expression of *muc5ac* gene (figure 12.B).

However, the *camp* gene (figure 12.A) displays significant decreases in expression in ozone-treated samples from timepoint 1 (before disinfection) to timepoints 2 (8 days after disinfection) and 5 (21 days after *Yersinia*), with p-values of 0.036 and 0.028 respectively. This indicates that both disinfection and long-term exposure of *Yersinia* infection led to a reduction in the expression of this gene within the ozone-treated tank.

The *lys* gene (figure 12.C) exhibits more pronounced changes, with significant decreases observed within timepoint 1 (before disinfection) between control and PAA treatments with p-value of

0.036 and Ozone and PAA treatment with the p-value of 0.011. Another significant change is observed in the control sample from timepoint 1 (pre-disinfection) to timepoints 2 (8 days post-disinfection), 3 (24 hours post-*Yersinia*), and 5 (21 days post-*Yersinia*) with p-values of 0.002, <0.0001, and <0.0001 respectively. This suggests that disinfection, short- and long-term exposure to *Yersinia* infection, reduced the expression of *lys* in the control treatment. Additionally, a significant reduction is observed from timepoint 2 (8 days post-disinfection) to timepoint 3 (24 hours post-*Yersinia*) in the control treatment with a p-value of 0.038.

Other significant changes are observed in the ozone treatment, where the expression is reduced from timepoint 1 (pre-disinfection) to timepoints 2 (8 days post-disinfection) and 5 (21 days post-*Yersinia*) with p-values of 0.007 and 0.003 respectively. This indicates that both disinfection and long-term exposure to *Yersinia* infection led to a reduction in the expression of this gene within the ozone-treated tank.

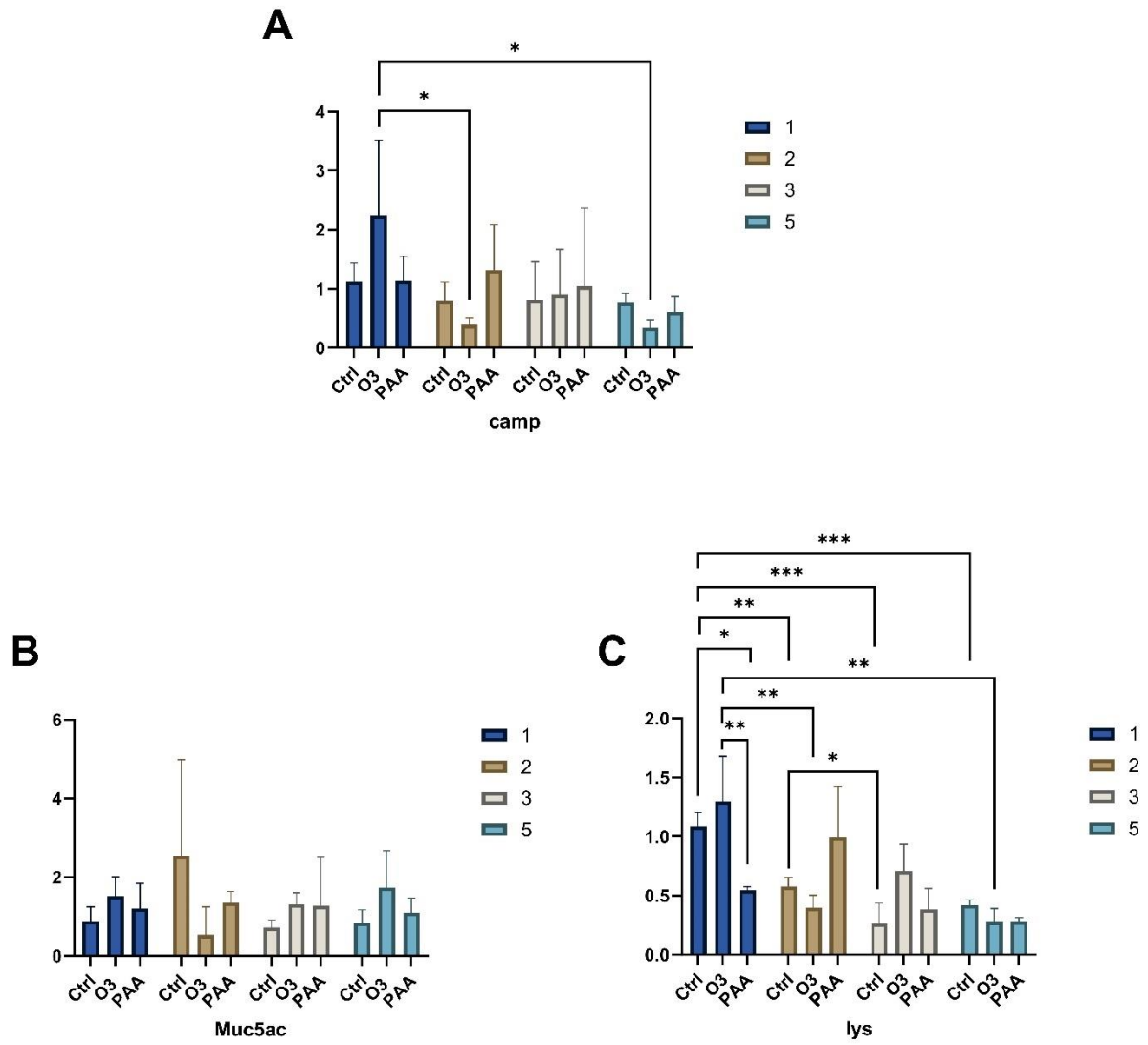

Figure 11. Expression levels of microbial defense genes including camp, muc5ac and lys (A-C) in gill tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with a single asterisk (*) and very significant changes with two asterisks (**) while extreme significant changes are denoted with three asterisks (***). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

Turning to oxidative stress genes in gill tissue, *gsta*, *gpx*, *mnsod*, and *cat* depicted in Figure 13 (A-D). In terms of the *mnsod* (figure 13.C) and *cat* (figure 13.D) genes, there are no significant changes observed. Concerning the *gsta* gene (Figure 13.A), a notable decrease is observed in control samples from timepoint 1 (pre-disinfection) to timepoints 3 (24 hours post-*Yersinia*) and 5 (21 days post-*Yersinia*) with p-values of 0.006 and 0.039 respectively. This indicates that both

short- and long-term exposure to *Yersinia* infection reduced the expression level of this gene within control treated tanks.

Additionally, there's a significant decrease in *gpx* gene expression (Figure 13.B) in control treatments from timepoint 2 (8 days post-disinfection) to timepoints 3 (24 hours post-*Yersinia*) and 5 (21 days post-*Yersinia*) with p-values of 0.003 and 0.011 respectively. This further suggests that both short- and long-term exposure to *Yersinia* infection reduced the expression level of this gene within control treated tanks.

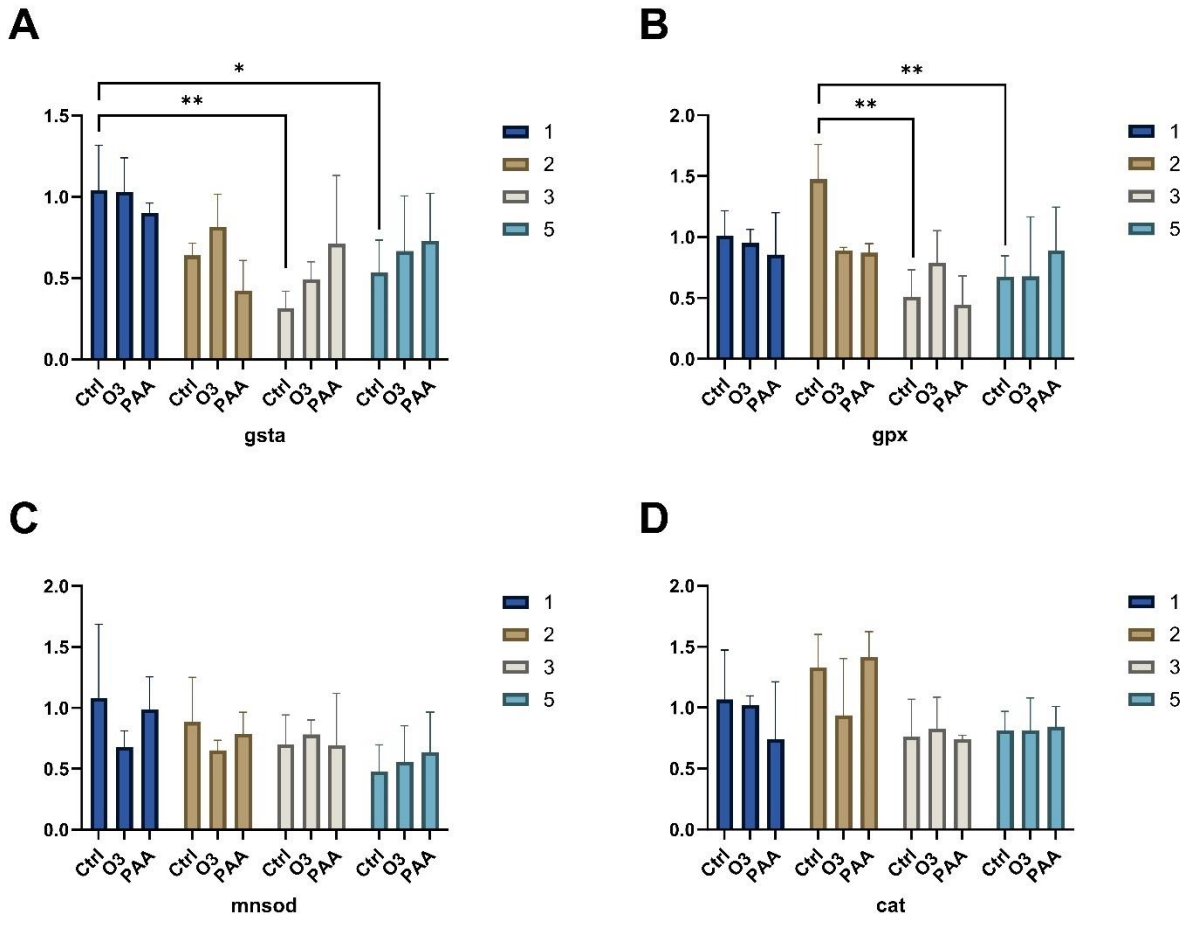

Figure 12. Expression of oxidative stress genes, gsta, gpx, mnsod, and cat (A-D) in gill tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with a single asterisk (*) and very significant changes with two asterisks (**). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

4.2 Skin

In the skin tissue, the expression of immune genes, *sigm*, *migm*, *il1b*, and *il8* across three different treatments: control, ozone, and PAA, over four different timepoints are illustrated in figure 14. As can be seen there are no significant changes observed in *sigm* (figure 14.A) and *migm* (figure 14.B) genes.

However, in the *il1b* gene (figure 14.C), a significant decrease is observed in PAA treatment from timepoint 1 (before disinfection) to timepoint 3 (24 hours after *Yersinia*) and 5 (21 days after *Yersinia*) with the p-values of 0.009 and 0.044 respectively. This further suggests that both short- and long-term exposure to *Yersinia* infection reduced the expression level of this gene within PAA treated tanks.

Concerning the *il8* gene (figure 14.D), there's a significant increase in expression from ozone to PAA treatment with p-value of 0.0257 within timepoint 5 (21 days after *Yersinia*).

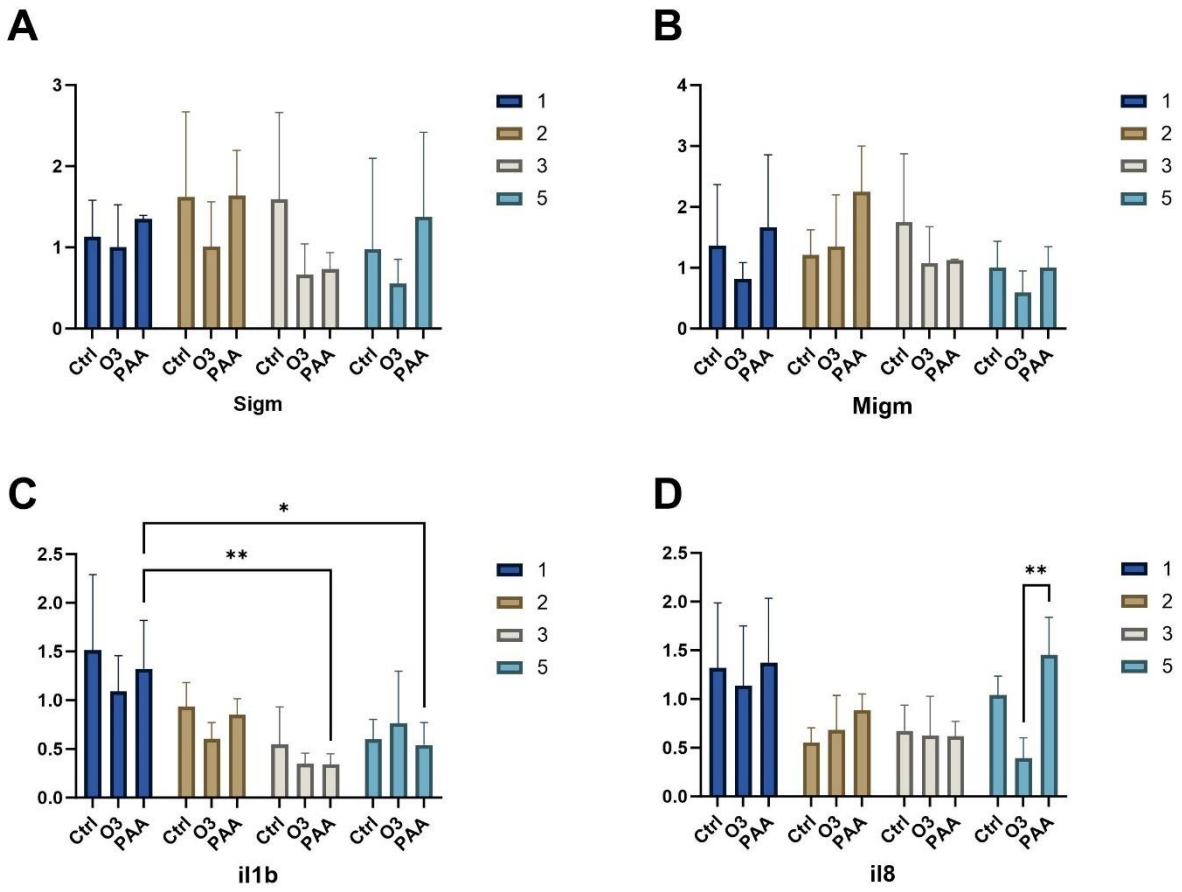

Figure 13. Expression levels of immune-related genes, sigm, migm, il1b, and il8 (A-D) in the skin tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with an asterisk (*) and very significant changes with two asterisks (**). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

Upon initial examination of microbial defense genes, *camp*, *muc5ac* and *lys* in skin tissue depicted in figure 15, there are no significant changes observed in *camp* (figure 15.A) and *muc5ac* (figure 15.B) genes.

In contrast, the *lys* (figure 15.C) gene demonstrates significant changes in expression. Within timepoint 5 (21 days after *Yersinia*) there is a significant decrease from control to ozone treatment (p-value: 0.04) and there is a significant increase from ozone to PAA (p-value: 0.012). Additionally, a significant decrease is observed in the ozone treatment from timepoint 1 (pre-disinfection) to timepoint 5 (21 days post-*Yersinia*) with a p-value of 0.037. This further underscores that long-

term exposure to *Yersinia* reduced the expression of this gene within tanks treated with ozone disinfectant.

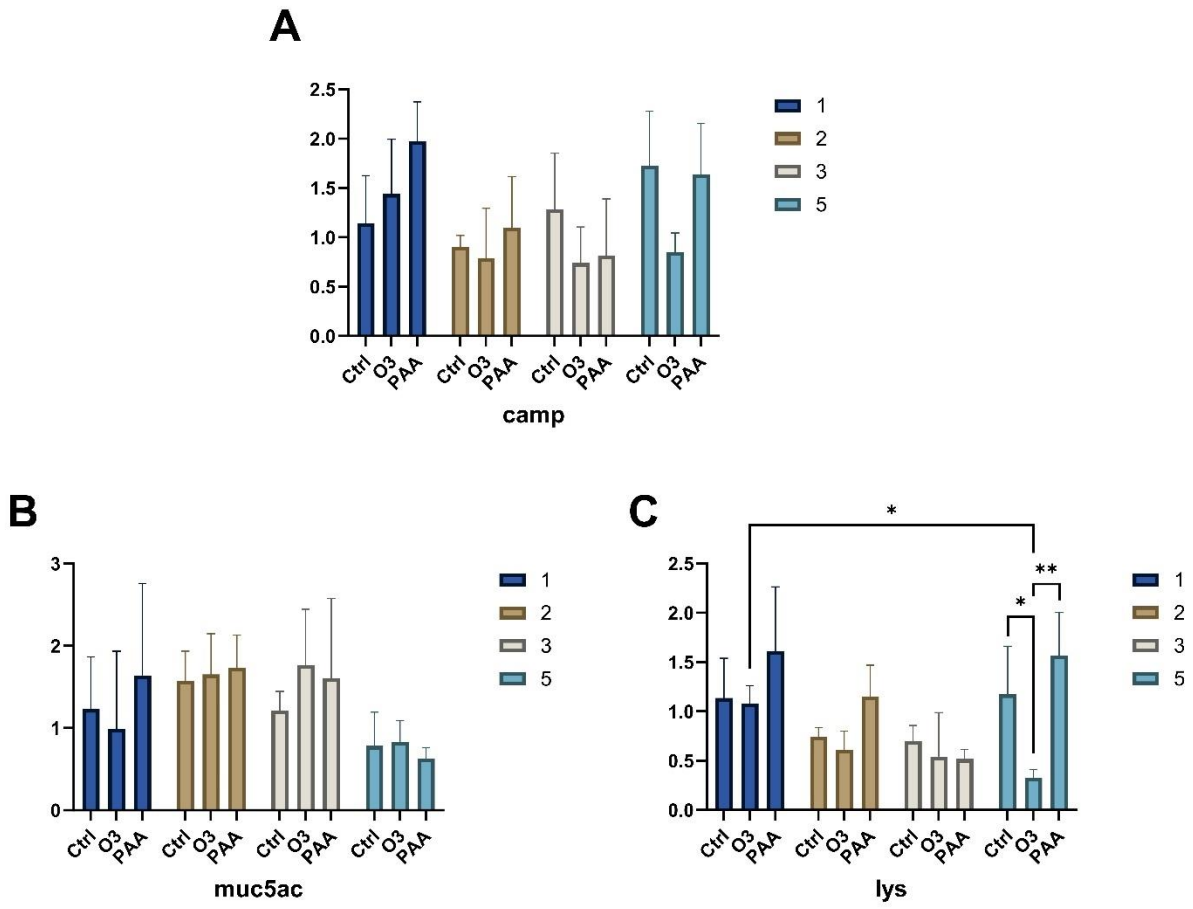

Figure 14. Expression levels of microbial defense genes including camp, muc5ac and lys (A-C) in the skin tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with a single asterisk (*) and very significant changes with two asterisks (**). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

Observing the oxidative stress gene, *gsta*, *gpx*, *mnsod*, and *cat* expressions in skin tissue, as depicted in Figure 16, there are no significant changes noted in the expression of *gsta* (figure 16.A) and *gpx* (figure 16.B) genes. However, in *mnsod* gene (figure 16.C), there is a significant decrease in ozone treatment samples from timepoint 1 (before disinfection) to timepoint 3 (24 hours after *Yersinia*) and 5 (21 days after *Yersinia*) with the p-values of 0.036 and 0.022

respectively, suggesting that both short- and long-term exposure to *Yersinia* infection reduced the expression level of this gene within ozone treated tanks.

Additionally, in the *cat* gene (Figure 16.D), within timepoint 2 (8 days after disinfection), there is a significant increase in expression from the control sample to PAA, with a p-value of 0.012. indicating the impact of PAA disinfectant over an 8-day period on elevating the expression of this gene.

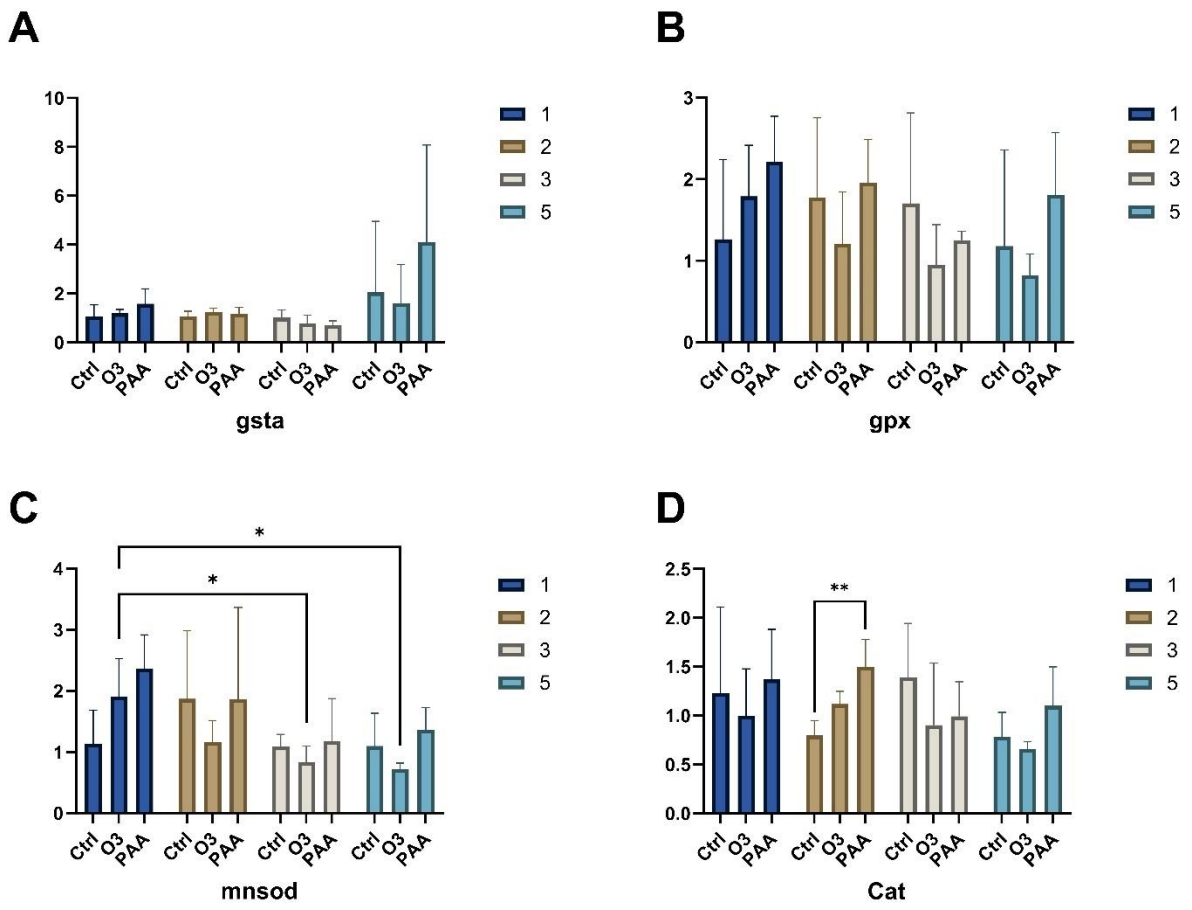

Figure 15. Expression of oxidative stress genes, gsta, gpx, mnsod, and cat (A-D) in skin tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with a single asterisk (*) and very significant changes with two asterisks (**). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

4.3 Spleen

Analyzing the immune genes *sigm*, *migm*, *il1b*, and *il8* across three distinct treatments—control, ozone, and PAA—across four varied timepoints is depicted in figure 17 for spleen tissue. As can be seen, there are no significant changes observed in *sigm* (figure 17.A), *migm* (figure 17.B) and *il8* (figure 17.D) genes.

However, significant increase is noted in the expression of the *il1b* (figure 17.C) gene in the PAA treatment from timepoint 2 (8 days post-disinfection) to 5 (21 days after *Yersinia*) with a p-value of 0.0023, suggesting that long-term exposure to *Yersinia* increased the expression level of this gene within PAA treated tanks.

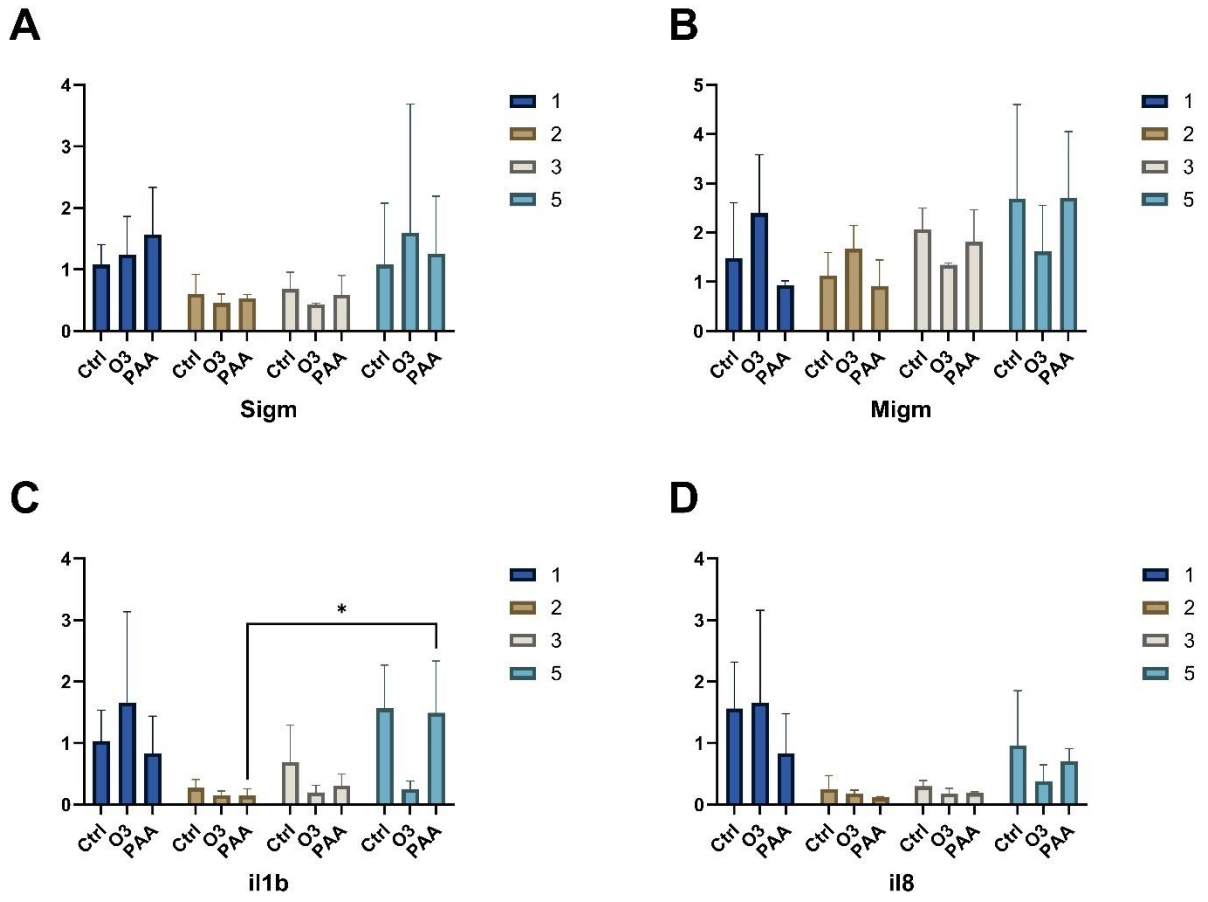

Figure 16. Expression levels of immune-related genes, sigm, migm, il1b, and il8 (A-D) in the spleen tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the

horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with an asterisk (*). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

In Figure 18, the microbial defense-related genes, *camp* and *lys*, in the spleen can be observed. The expression of the *muc5ac* gene was not studied due to expectations of its low expression level in this tissue.

In the *camp* gene (figure 18.A), there are no significant changes observed. However, In the *lys* gene (figure 18.B), a significant increase is observed in the PAA treatment from timepoint 2 (8 days after disinfection) to 5 (21 days after *Yersinia*) with a p-value 0.036, suggesting that long-term exposure to *Yersinia* increased the expression level of this gene within PAA treated tanks.

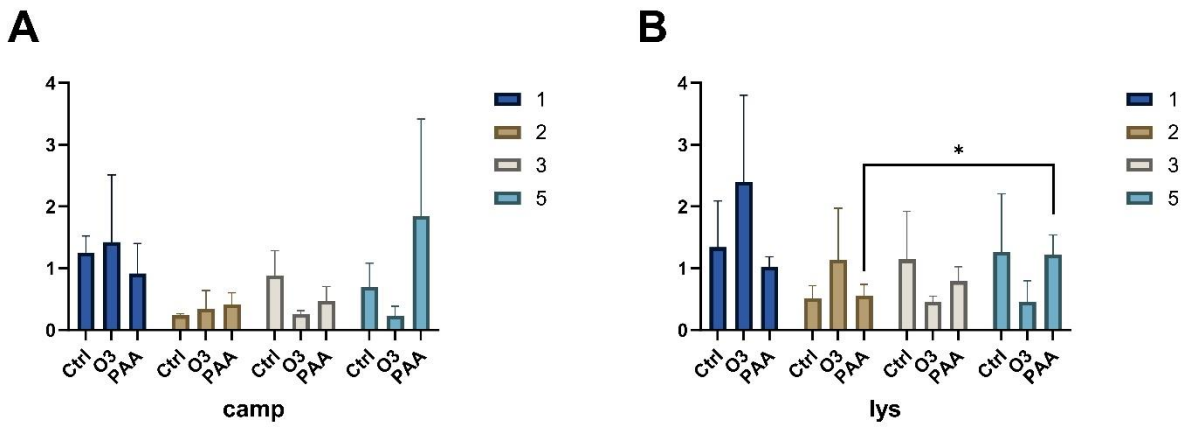

Figure 17. Expression levels of microbial defense genes including camp and lys (A and B) in the spleen tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with a single asterisk (*). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

In Figure 19, the expression levels of oxidative stress genes, *gsta*, *gpx*, *mnsod*, and *cat* in the spleen tissue are exhibited. there are no significant changes observed in the expression of the *gpx* (figure 19.B) and *cat* (figure 19.D) genes.

In the *gsta* gene (figure 19.A), PAA treatment displays a significant increase in expression from timepoint 1 (before disinfection), 2 (8 days after disinfection), and 3 (24 hours after *Yersinia*) to timepoint 5 (21 days after *Yersinia*), with p-values of 0.037, 0.029, and 0.029 respectively. Describing the long-term exposure of *Yersinia* caused a significant increase in the expression of this gene, compared to all previous timepoints in the PAA treated tanks. Additionally, there's a significant increase in expression level of the ozone treatment from timepoint 2 (8 days after disinfection) and 3 (24 hours after *Yersinia*) to 5 (21 days after *Yersinia*), with a p-value of 0.019 for both, highlights the effect of long-term exposure of *Yersinia* on the increase of *gsta* gene compared to previous timepoints.

Concerning the *mnsod* gene (figure 19.C), the graph indicates a significant decrease in expression of PAA treatment from timepoint 1 (before disinfection) to 5 (21 days after *Yersinia*), with p-value

of 0.023, showing the effect of long-term exposure to *Yersinia* decreased the expression level of this gene.

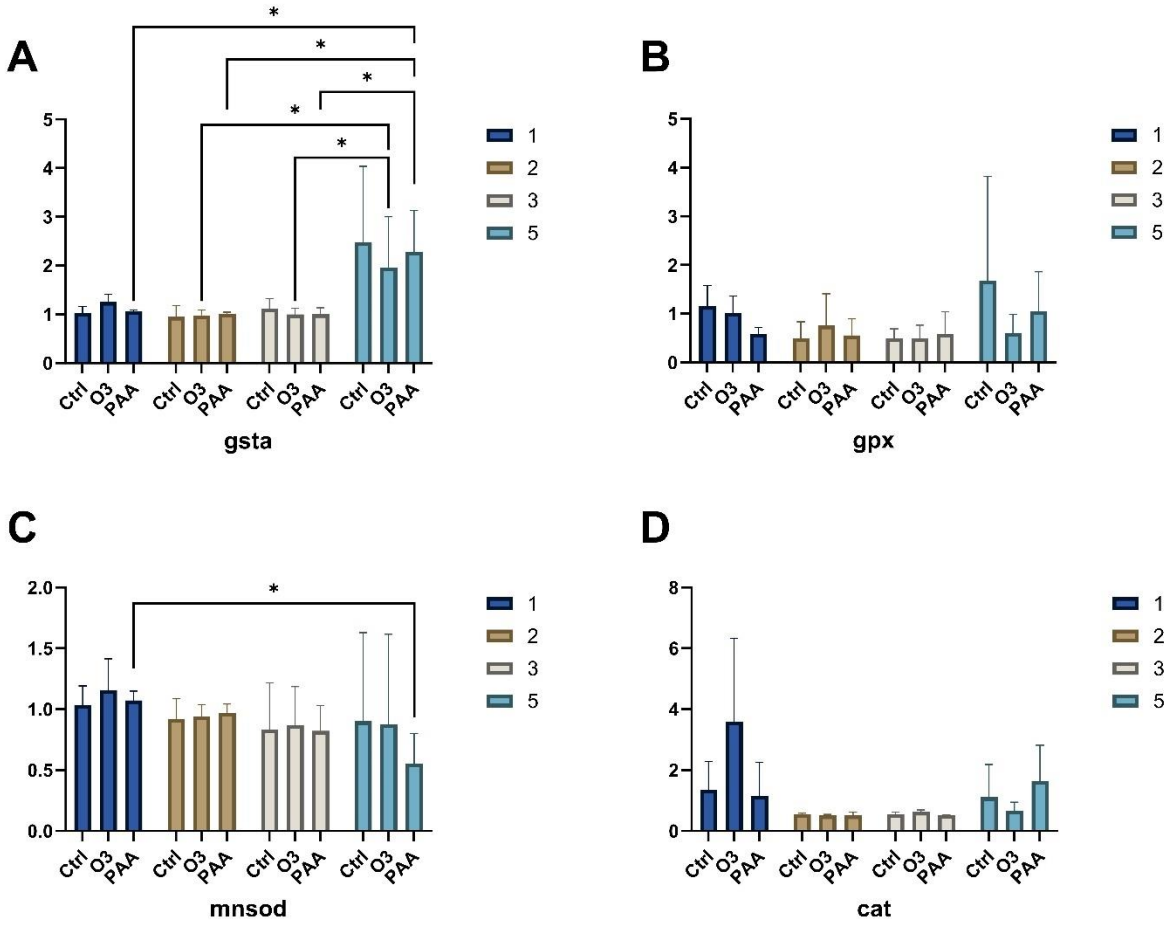

Figure 18. Expression of oxidative stress genes, gsta, gpx, mnsod, and cat (A-D) in spleen tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with a single asterisk (*). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

4.4 Olfactory epithelium

In Figure 20, the comparison is made between the expression of immune genes *sigm*, *migm*, *il1b*, and *il8* across three treatments (control, ozone, and PAA) at four different timepoints in the

olfactory tissue. It is evident that there are no significant changes in *sigm* (figure 20.A) and *il1b* (figure 20.C) genes. However, about *migm* gene (figure 20.B), there is a significant increase within timepoint 5 (21 days after *Yersinia*) between ozone and PAA treatment with the p-value of 0.046. Also, there is a significant decrease is observed with ozone treatment from timepoint 1 (before disinfection) to timepoint 2 (8 days after disinfection), 3 (24 hours after *Yersinia*), 5 (21 days after *Yersinia*) with p-values of 0.039, 0.031 and 0.002 respectively. This suggests that disinfection,

short- and long-term exposure to *Yersinia* infection, reduced the expression of *migm* gene in the ozone treated samples.

Regarding the *il8* gene (figure 20.D), there is a significant increase in expression in the third timepoint (24 hours after *Yersinia*) between ozone and PAA treatment, with a p-value of 0.042.

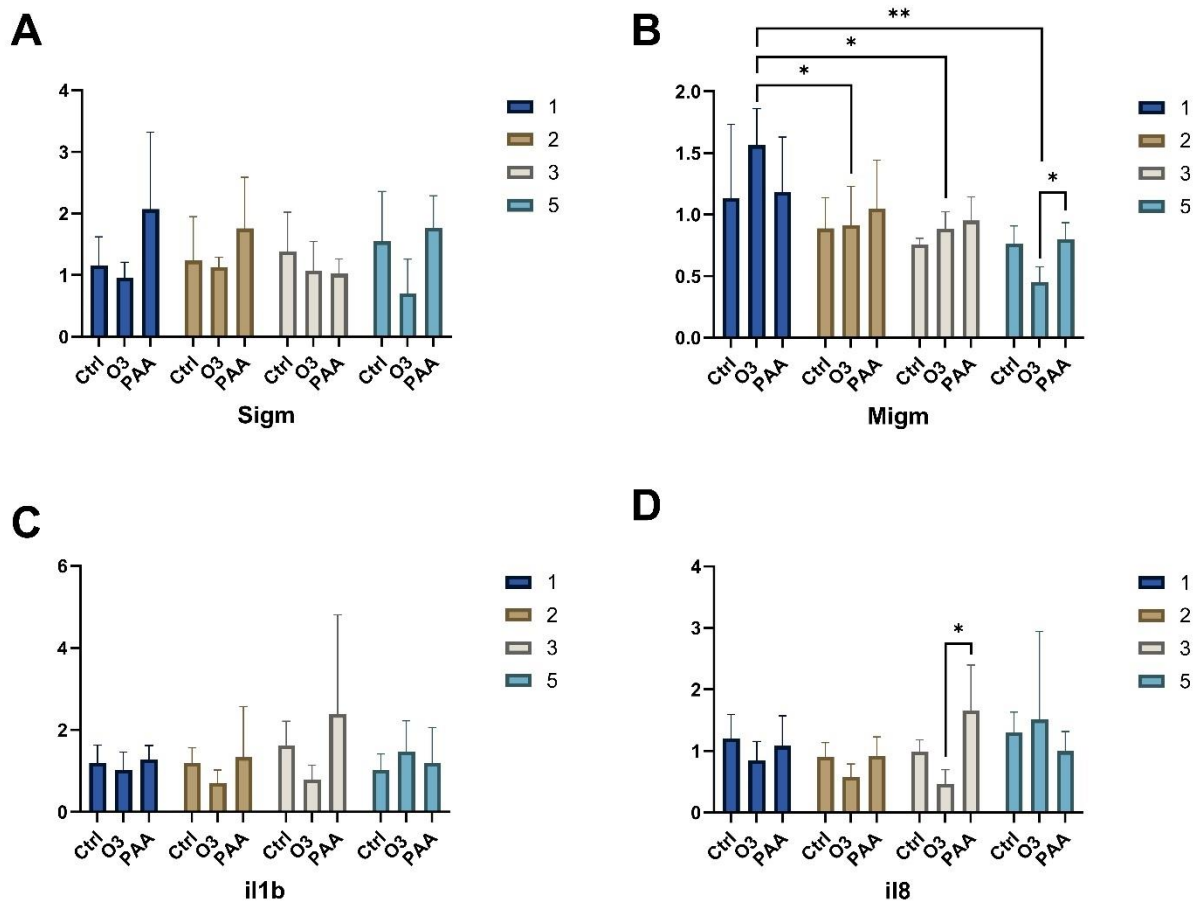

Figure 20. Expression levels of immune-related genes, sigm, migm, il1b, and il8 (A-D) in the olfactory tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with an asterisk (*) and very significant changes with two asterisks (**). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

In Figure 21, the expression level of microbial defensive genes (*camp*, *muc5ac* and *lys)* in the olfactory tissue are depicted. As can be seen there are no significant changes in *lys* (figure 21.C) and *muc5ac* (figure 21.B) genes expression.

However, there is a significant decrease in the expression of the *camp* gene (figure 21.A) in ozone treatment from timepoint 1 (before disinfection) to timepoint 2 (8 days after disinfection) and timepoint 3 (24 hours after *Yersinia*), with p-values of 0.041 and 0.038, respectively, suggesting that disinfection and short-term exposure to *Yersinia* infection, reduced the expression of *camp* gene in the ozone treated tanks.

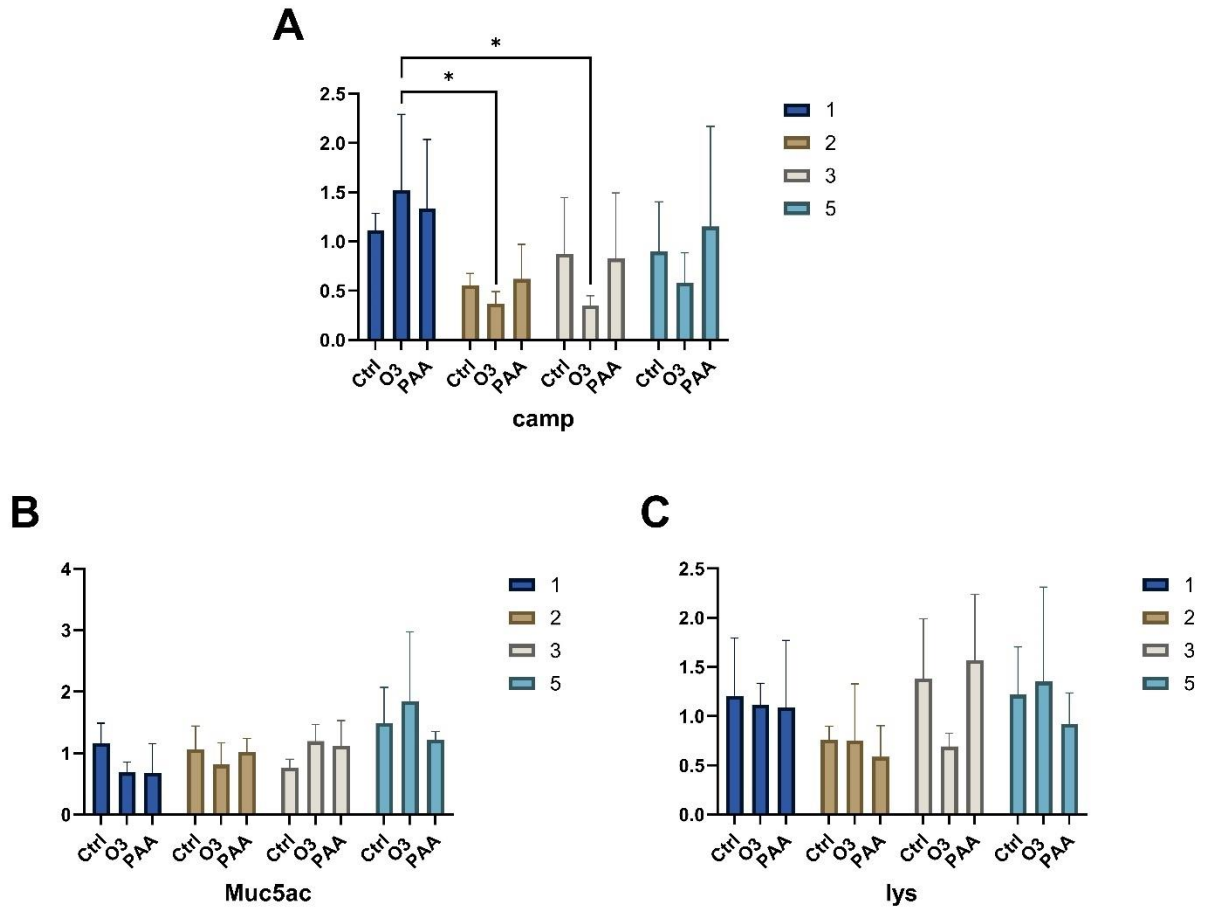

Figure 21. Expression levels of microbial defense genes including camp, muc5ac and lys (A-C) in the olfactory tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with a single asterisk (*). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

The oxidative stress genes, *gsta*, *gpx*, *mnsod*, and *cat* expression of olfactory tissue are depicted in Figure 22, where it is generally observed that there are no significant changes in *gsta* (figure 22.A), *gpx* (figure 22.B) and *cat* (figure 22.D) genes. While there is a significant increase observed with *mnsod* gene (figure 22.C) at the ozone treatment from timepoint 1 (before disinfection) to 3 (24 hours after *Yersinia*) and 5 (21 days after *Yersinia*) with the p-values of 0.023 and 0.005 respectively, pointing that both short- and long-term exposure of *Yersinia* increased the expression of this gene within the ozone treated tanks.

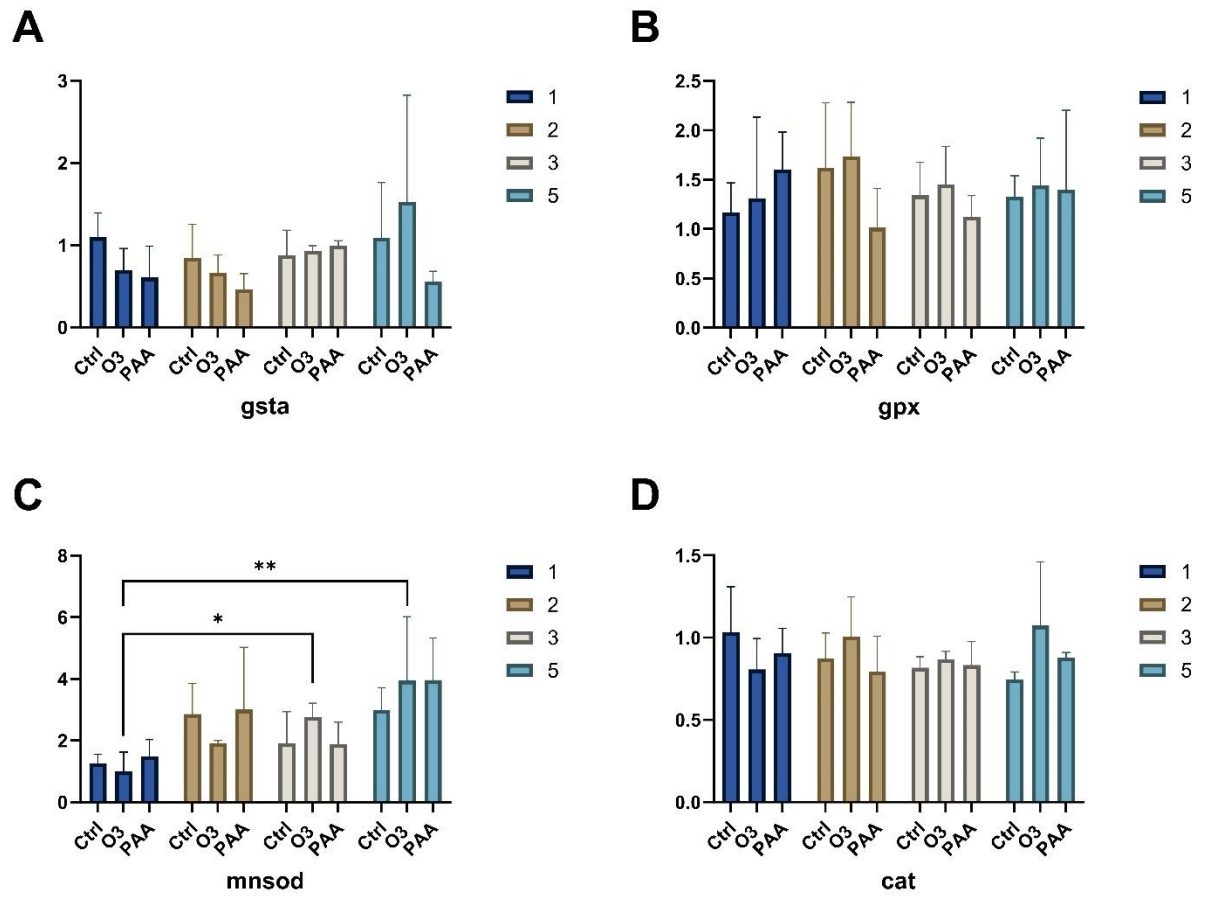

Figure 19. Expression of oxidative stress genes, gsta, gpx, mnsod, and cat (A-D) in olfactory tissue during control, ozone, and PAA treatments across four timepoints. The vertical lines represent expression levels, while the horizontal lines represent timepoints. Significant changes among treatments or timepoints are denoted with a single asterisk (*) and very significant changes with two asterisks (**). At timepoint 1, all treatments are without disinfection, ozone and PAA are merely labels for tanks.

4.5 *Yersinia*

Yersinia gene expression was below the detection level of the assay in all tissues.

4.6 Overview of results

In a general overview, when comparing the total number of significant changes observed in different tissues and expressing them as a percentage, gill tissue exhibited the most pronounced changes in gene expression and 56% of the genes has up- or down-regulated, followed by skin at 44%, and spleen at 42%, while olfactory tissue showed the least changes at 36%. However, upon closer examination, as illustrated in Table 3, microbial defense genes across all tissues displayed the most significant changes at 46%, compared to immune-related and oxidative stress genes, both at 44%.

Table 3. the percentage of genes exhibiting significant changes in each tissue.

Tissue	Immune related	Microbial defense	Oxidative stress	Average per tissue
Gill	50%	67%	50%	56%
Skin	50%	33%	50%	44%
Spleen	25%	50%	50%	42%
Olfactory	50%	33%	25%	36%
Average per gene group	44%	46%	44%	-

In the gill tissue, a reduction in expression levels was observed for *il8* (Immune response), *camp* (Antimicrobial) and *lys* (microbial defense) genes 8 days after disinfection, which were affected in ozone treated tanks. Similarly, these same genes, along with *sigm* (Immune response), exhibited reduced expression levels affected by long-term *Yersinia* infection in the ozone treated tank.

the effects of short-term exposure to *Yersinia* (comparing timepoints 1 or 2 with 3 and 5 in control-labeled tanks without disinfectants) on *il8* were noted, along with both short- and long-term exposures of *Yersinia* on *lys*, *gsta* (Antioxidant), and *gpx* (Antioxidant) genes, resulting in reduced expression in all these genes.

In the skin tissue, the expression of the *il1b* (pro-inflammatory) gene is affected and reduced by short- and long-term exposure to *Yersinia* in PAA-treated tanks. Additionally, the expression of *lys* and *mnsod* (Antioxidant) genes was reduced by long- and short-term exposure to *Yersinia* in ozone-treated tanks. Additionally, inner DEGs (differentially expressed genes between treatments within a particular timepoints) were observed in this tissue, including *il8*, *lys*, and *cat* genes, with their expression significantly higher in PAA-treated samples compared to ozone or control treatments within timepoint 5 for *il8* and *lys* and timepoint 3 for *cat* gene.

In the spleen tissue, a completely opposite effect of disinfection on gene expression compared to other tissues was observed. Here, the expression of *il1b*, *lys*, and *gsta* genes increased due to long-term exposure to *Yersinia* in PAA-treated tanks. Additionally, *gsta* expression increased with long-term exposure to *Yersinia* in ozone-treated tanks.

In the olfactory tissue, the expression of *migm*, *camp*, and *mnsod* genes decreased in ozone-treated tanks between timepoints. Additionally, in PAA-treated tanks, the *migm* and *il8* genes exhibited significantly higher expression levels compared to ozone-treated tanks at timepoints 5 and 3, respectively.

In total, 44 DEGs were observed throughout the experiment, with 36 changes occurring between different timepoints (temporal DEGs) and 8 changes happening within specific timepoints (inner DEGs). A closer examination of the temporal changes according to the treatments, as shown in Table 4, reveals that ozone-linked DEGs accounted for the largest portion of changes at 53%. The control treatment was second with 25%, and PAA-linked DEGs represented 22% of the changes.

Table 4. The table displays temporal DEGs, across various tissues based on the treatment.

Tissue	O3	PAA	Ctrl
Olfactory	19%	0%	0%
Spleen	6%	17%	0%
Skin	8%	6%	0%
Gill	19%	0%	25%
Total	53%	22%	25%

5 Discussion

The primary aim of this project was to analyze and compare the expression levels of categorized genes, including immune-related genes, microbial defense genes, and oxidative stress genes, across four tissue samples (gill, skin, spleen, and olfactory) of salmon fish under three treatments (PAA, ozone, and control) spanning four different timepoints (pre-disinfection, 8 days post-disinfection, 1 day after *Yersinia* and 21 days after *Yersinia* challenge). To manage the extensive dataset, the study can be divided into two main parts.

Firstly, the focus is on assessing changes induced by disinfection agents (ozone and PAA) by comparing significant alterations between timepoints 1 (pre-disinfection), 2 (8 days post-disinfection), 3 (1 day after *Yersinia*), and 5 (21 days after *Yersinia*). During the last two timepoints, the effect of disinfectants in the presence of *Yersinia* is analyzed.

In the second part, the investigation delves into assessing the impact of *Yersinia*, both in short- and long-term exposures, on gene expression. This ana*ly*sis includes examining gene expression patterns in control samples without the presence of any disinfectants. Data were examined and compared between timepoints 1 (pre-disinfection) or 2 (8 days after disinfection) with timepoints 3 (24 hours after *Yersinia* exposure) and 5 (21 days after *Yersinia* exposure).

5.1 Effect of ozone and PAA disinfectants on gene expression

Using ozone and PAA disinfectants against pathogens in an aquaculture system has proven to increase the survival rate of the fish (Linh, Panphut et al. 2021, Liu, Straus et al. 2024). In a study examining post-smolt salmon subjected to continuous ozone exposure (334±22 mV) for 45 days, no DEGs were detected between the control and ozone-treated groups in the skin tissue at day 45. However, microarray ana*ly*sis of gill tissue revealed 242 DEGs, primarily showing upregulation, indicating the heightened sensitivity of gill tissue in contrast to skin tissue (Lazado, Stiller et al. 2021). Another study focused on identifying the ozone concentration threshold in RAS facilities by monitoring salmon post-smolt mortality at various ozone concentrations. They also examined

the gene expression patterns in gill and skin tissues. The results revealed that fish mortality increased at ozone concentrations higher than 350 mV and that gill tissues were more sensitive to ozone exposure compared to skin tissues (Stiller, Kolarevic et al. 2020). Although limited research exists on the effect of ozonation on salmon parr genes in RAS, previous findings align with the results shown in this project. Across all tissues, the gill and olfactory organs stand out as the most impacted tissues by ozonation, underscoring their heightened sensitivity to the substance. Four genes—*sigm*, *il8*, *camp*, and *lys*—are down-regulated in the gill tissue because of ozone exposure. Additionally, ozone exposure leads to the downregulation of the *lys* and *mnsod* genes in the skin tissue. In the spleen, the *gsta* gene showed up-regulation, while the *migm*, *camp*, and *mnsod* genes in the olfactory organ are also changed significantly. In general, during the experiment, 36 temporal significant changes were observed among whole genes, where 53% of them linked to ozone disinfectant, either alone (between timepoint 1 and 2) or in combination with the *Yersinia* pathogen (comparing between timepoints 1 with 3 and 5). This observation demonstrates the strong effect of this substance on gene regulation, indicating the need for increased caution when using this disinfectant in the system. Also, most of the changes related to ozone occurred in a long-term exposure can explain that not only the concentration but also time of the exposure can be a very important parameter effecting the molecular level. The downregulation of immune genes was observed, indicating potential immune system suppression, which may increase the risk of infectious diseases in the fish. (Krasnov, Afanasyev et al. 2020) in a longer period. Even though the previous study by (Mousavi 2023) discussed that PAA and ozone treatment did not significantly affect the health and welfare of Atlantic salmon parr during the *Yersinia* challenge, this suggests that significant gene expression changes do not necessarily translate into physical changes. Generally, the sensitivity of gill and olfactory tissue in response to ozone treatment, especially with long-term exposure, can be concluded.

In contrast to ozone, a considerable number of studies have been conducted on the effect of PAA on salmon in RAS. In a study by M. Soling et al., the effects of three different concentrations of PAA on oxidative stress genes in the gill and skin tissues of Atlantic salmon smolt were examined at three different timepoints: 2 hours, 48 hours, and two weeks. The results showed that gill tissue was more responsive to PAA-induced oxidative stress than skin tissue (Soleng, Johansen et al.

2019). Additionally, in another study, salmon smolts were exposed to three therapeutic doses of PAA in two phases: initially for 5 minutes, and then again for 30 minutes after two weeks. The expression of four PAA-responsive markers in the skin samples was quantified using qPCR. The results showed that genes were modulated with higher concentrations (2.4 ppm) of PAA, but overall, there wasn't a consistent pattern observed throughout the experiment (Lazado, Haddeland et al. 2020). Another study experimented on the effect of PAA treatment on salmon smolts exposed to amoebic gill disease (AGD), with gill samples collected 24 hours, 2 weeks, and 4 weeks after treatment. Using a microarray test, most of the DEGs were related to change over different timepoints and PAA treatment and AGD infection respectively. The experiment showed that PAA had a significant immediate and temporary effect on gene regulation. The main gene clusters that exhibited significant changes were involved in immunity, metabolism, and stress responses (Lazado, Strand et al. 2022). In our project, it has been used a moderate concentration of PAA, which resulted in the least significant changes in gene expression between different timepoints compared to ozone and control treatments. Among 36 observed significant changes between timepoints, 22% of them were linked with PAA treatment, primarily in spleen tissue. Regarding temporal DEGs, only one gene, *il1b*, was downregulated in the skin tissue due to the effect of PAA in the presence of *Yersinia*. In the spleen tissue, PAA caused upregulation of *il1b*, *lys*, and *gsta* genes, and downregulation of the *mnsod* gene. all inner DEGs were associated with PAA treatment. For instance, in the skin and olfactory tissue, genes such as *il8*, *lys*, and *migm* showed significant upregulation in PAA-treated samples compared to ozone showing the effect of this disinfectant on activating the immune and antimicrobial response in the tissues. Comparing our results with previous studies, no significant changes were observed in the gill tissue, contrasting with the findings of the first study. Similar to the third study, significant changes were observed mainly in immune and oxidative stress genes. In the skin, a suppressed immune response was noted concerning temporal DEGs, while an activated immune response was observed regarding inner DEGs. In the spleen, both immune and antimicrobial responses were activated. Additionally, in the olfactory tissue, an activated immune response was observed concerning inner DEGs. This can be explained by the clinical results from (Mousavi 2023), which concluded that clinical signs such as enlarged spleens—an indicator of immune response—were observed in 31% of the

control group, compared to 16% in the PAA group and 5% in the ozone group. These findings primarily highlight the sensitivity of spleen tissue in response to PAA treatment, especially with long-term exposure to this substance.

5.2 Effect of *Yersinia* on gene expression

Transcriptomic responses of Atlantic salmon to *Yersinia* have not been studied before, but there are studies on the molecular responses of rainbow trout to *Yersinia*. In one study, rainbow trout were exposed to *Yersinia* for an hour and monitored for 30 days for mortality. The results showed that 60% of the fish died in the end of this period. Molecular ana*lys*is indicated that the fish which eventually died had slightly higher levels of cytokine expression levels (Raida, Holten-Andersen and Buchmann 2011). In another experiment, gene expression profiling in the gill and spleen of naïve and vaccinated rainbow trout was studied after exposure to *Yersinia*, with samples taken at 6-, 24-, 48-, and 72-hours post-challenge. The results showed a significant increase in the spleen index of the fish. Additionally, naïve fish exhibited a significant increase in pro-inflammatory cytokine levels following the *Yersinia* challenge (Harun, Wang and Secombes 2011). In another project, rainbow trout were exposed to *Yersinia* for 6 hours, and mortality was monitored for 21 days. Gill, liver, and spleen samples were collected for molecular response ana*lys*is at three timepoints: before the challenge, 7 days and 21 days post-challenge. The study indicated that survival could be heritable, with surviving fish likely carrying resistance genes. Gene expression ana*lys*is revealed that various immune genes were involved during the infection, and surviving fish had significantly higher upregulation of antimicrobial peptide genes like *camp* and *lys*. This upregulation was interpreted as these genes playing a crucial role in protecting against pathogens (Zuo, Karami et al. 2020). Although these studies were conducted on rainbow trout, immune, microbial, and oxidative stress genes responded to *Yersinia* exposure in our study as well. Among the 36 DEGs observed across various timepoints, 25% were solely linked to the effect of *Yersinia* on genes. Significant changes were observed in gill tissue, where the *il8* gene was downregulated within a short time. The *lys* gene showed downregulation in both short- and long-term exposures. Interestingly, changes in the *lys* gene were observed from timepoint 1 to timepoint 2, despite the

samples being the same and no *Yersinia* being present at these timepoints. Additionally, two other oxidative stress genes in the gill, *gsta* and *gpx*, were affected, showing downregulation in both short- and long-term exposures. Comparing our results with those observed in rainbow trout, the types of gene responses to the challenge followed a similar pattern in Atlantic salmon. However, in our study, gene expression was downregulated, and no significant changes were observed in tissues other than the gill, highlighting the sensitivity of gill tissue to infection. Histological evaluation of gill tissue in the previous study (Mousavi 2023) showed a prevalence of scores from 0 to 4, with 0 indicating the healthiest gills and 4 representing the most damaged tissue. Although the prevalence of higher scores increased over time, this increase was not statistically significant. Downregulation of *il8*, *lys*, and two antioxidant genes in gill tissue due to exposure to *Yersinia* indicates the suppressive effect of this pathogen on these genes over time.

In general, ozone disinfectant caused 53% DEGs, which is more than twice the changes caused by PAA or even *Yersinia* in the control samples. These findings are consistent with the mortality observed in a previous project (Mousavi 2023), where 3 out of 5 fish died after week 2 in ozone-treated RAS, compared to 2 in the control-treated RAS units. Overall, this indicates that ozone can regulate gene expression more significantly than *Yersinia* infection. Despite PAA resulting in less mortality from pathogens, it appears to be more stable treatment at the molecular level.

6 Conclusion

The goal of this study was to analyze the gene expression levels in Atlantic salmon parr in RAS exposed to PAA and ozone disinfectants, as well as those exposed to *Yersinia*. At the molecular level, temporal DEGs caused by ozone (either alone or along with *Yersinia*) were most prominent in the gill and olfactory tissues, indicating the sensitivity of these tissues to this disinfectant, especially with long-term exposure. On the other hand, PAA combined with *Yersinia* affected spleen tissue across different time points, particularly in long-term exposure, possibly due to the sensitivity of this tissue to PAA. Additionally, PAA activated immune and antimicrobial responses in skin and olfactory tissues compared to ozone, highlighting the lower efficacy of this disinfectant against *Yersinia* compared to ozone. Finally, *Yersinia* affected gill tissue by primarily suppressing antioxidant genes during both short- and long-term exposure.

Based on these observations, it could be inferred that RAS units treated with ozone disinfectant exhibited more suppressive changes in gene expression compared to those treated with PAA, which mostly activated gene expression.

According to a previous study (Mousavi 2023), no significant health and welfare differences were observed between fish reared in PAA- and ozone-treated units during the *Yersinia* challenge. This suggests that changes in gene expression may not necessarily result in physiological effects, especially detrimental ones. The current project serves as a valuable indicator for developing strategies in RAS facilities concerning disinfection methods. It underscores the importance of carefully managing the quantity and duration of substance exposure to maintain water quality and hygiene without compromising fish health.

Limitations

There was an abundance of data available across various treatments, timepoints, tissues, and genes, offering numerous scenarios for ana*lys*is and study. However, time constraints posed a significant limitation in this project, compounded by the high costs associated with the materials used for qPCR testing. Additionally, the qPCR technique is sensitive and involves numerous intricate steps, making it susceptible to human error, especially when handling a large number of samples that require meticulous pipetting and attention to detail. Conducting microarray tests could have allowed for the ana*lys*is of a broader spectrum of genes. Based on the results, longer timepoints could have been employed to further explore the effects of chemicals over extended durations.

Future research

For future research, several avenues can be pursued to enhance our understanding and application of disinfectants. One approach is to test different types of disinfectants to identify the most optimal treatment that has a moderate effect, comparing their efficacy, safety, and cost-effectiveness. Further research could also explore the combined effects of PAA and ozone, the impact of different concentrations and exposure times, and the environmental implications of their use. Since the analyzed genes *muc5ac*, *cat*, *gpx*, *migm*, and *sigm* did not show a notable response, it is suggested that future studies focus on other genes.

Implications

The findings of this project suggest that PAA and ozone disinfectants can influence the genes of salmon parr within RAS units, particularly in the presence of *Yersinia*, although these changes are not necessarily harmful. These results also shed light on the molecular effects of the *Yersinia* pathogen on fish, suggesting avenues for future research into alternative disinfectants and

comparative studies. These implications could significantly impact aquaculture companies, potentially mitigating economic losses caused by pathogen challenges within their systems.

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8 Appendix

Appendix 1.

In this table, the number of fishes and the treatments they have undergone can be observed.

Treatment	Fish number	Treatment	Fish number
Ozone	1 - 10	Ozone	136 - 145
Control	11 - 15	Control	146 - 150
PAA	16 - 20	PAA	151 - 155
Control	21 -25	Control	156 - 160
PAA	26 -35	PAA	161 - 170
Control	36 - 40	Control	171 - 175
Ozone	41 - 55	Ozone	176 - 190
Control	56 - 60	Control	191 - 195
PAA	61 - 65	PAA	196 - 200
Control	66 - 70	Control	201 - 205
PAA	71 - 80	PAA	206 - 215
Control	81 - 85	Control	216 - 220
Ozone	86 - 100	Ozone	221 - 225
Control	101 - 105		
PAA	106 - 110		
Control	111 - 115		
PAA	116 - 125		
Control	126 - 130		
Ozone	131 - 135		

Appendix 2.

In this table, the gill tissues are listed along with their corresponding numbers.

Plate 1	1	2	3	4	5	6	7	8	9	10	11	12
A	1 GL	13 GL	27 GL	41 GL	53 GL	67 GL	81 GL	93 GL	107 GL	121 GL	133 GL	194 GL
B	2 GL	16 GL	28 GL	42 GL	56 GL	68 GL	82 GL	96 GL	108 GL	122 GL	183 GL	195 GL
C	3 GL	17 GL	31 GL	43 GL	57 GL	71 GL	83 GL	97 GL	111 GL	123 GL	184 GL	198 GL
D	6 GL	18 GL	32 GL	46 GL	58 GL	72 GL	86 GL	98 GL	112 GL	126 GL	185 GL	199 GL
E	7 GL	21 GL	33 GL	47 GL	61 GL	73 GL	87 GL	101 GL	113 GL	127 GL	188 GL	200 GL
F	8 GL	22 GL	36 GL	48 GL	62 GL	76 GL	88 GL	102 GL	116 GL	128 GL	189 GL	203 GL
G	11 GL	23 GL	37 GL	51 GL	63 GL	77 GL	91 GL	103 GL	117 GL	131 GL	190 GL	204 GL
H	12 GL	26 GL	38 GL	52 GL	66 GL	78 GL	92 GL	106 GL	118 GL	132 GL	193 GL	205 GL

In this table, there are the remaining gill tissue samples and the beginning of the skin tissue samples.

Plate 2	1	2	3	4	5	6	7	8	9	10	11	12
A	208 GL	220 GL	7 SK	21 SK	33 SK	47 SK	61 SK	73 SK	87 SK	101 SK	113 SK	127 SK
B	209 GL	223 GL	8 SK	22 SK	36 SK	48 SK	62 SK	76 SK	88 SK	102 SK	116 SK	128 SK
C	210 GL	224 GL	11 SK	23 SK	37 SK	51 SK	63 SK	77 SK	91 SK	103 SK	117 SK	131 SK
D	213 GL	225 GL	12 SK	26 SK	38 SK	52 SK	66 SK	78 SK	92 SK	106 SK	118 SK	132 SK
E	214 GL	1 SK	13 SK	27 SK	41 SK	53 SK	67 SK	81 SK	93 SK	107 SK	121 SK	133 SK
F	215 GL	2 SK	16 SK	28 SK	42 SK	56 SK	68 SK	82 SK	96 SK	108 SK	122 SK	183 SK
G	218 GL	3 SK	17 SK	31 SK	43 SK	57 SK	71 SK	83 SK	97 SK	111 SK	123 SK	184 SK
H	219 GL	6 SK	18 SK	32 SK	46 SK	58 SK	72 SK	86 SK	98 SK	112 SK	126 SK	185 SK

Plate 3 containing the remaining of skin tissue samples and the beginning of the spleen tissue samples.

Plate 3	1	2	3	4	5	6	7	8	9	10	11	12
A	188 SK	200 SK	214 SK	1 SP	13 SP	27 SP	41 SP	53 SP	67 SP	81 SP	93 SP	107 SP
B	189 SK	203 SK	215 SK	2 SP	16 SP	28 SP	42 SP	56 SP	68 SP	82 SP	96 SP	108 SP
C	190 SK	204 SK	218 SK	3 SP	17 SP	31 SP	44 SP	57 SP	71 SP	83 SP	97 SP	111 SP
D	193 SK	205 SK	219 SK	6 SP	18 SP	32 SP	46 SP	58 SP	72 SP	86 SP	98 SP	112 SP
E	194 SK	208 SK	220 SK	7 SP	21 SP	33 SP	47 SP	61 SP	73 SP	87 SP	101 SP	113 SP
F	195 SK	209 SK	223 SK	8 SP	22 SP	36 SP	48 SP	62 SP	76 SP	88 SP	102 SP	116 SP
G	198 SK	210 SK	224 SK	11 SP	23 SP	37 SP	51 SP	63 SP	77 SP	91 SP	103 SP	117 SP
H	199 SK	213 SK	225 SK	12 SP	26 SP	38 SP	52 SP	66 SP	78 SP	92 SP	106 SP	118 SP

In this plate, spleen tissue samples end, and the last tissue sample, which is olfactory, starts.

Plate 4	1	2	3	4	5	6	7	8	9	10	11	12
A	121 SP	133 SP	194 SP	208 SP	220 SP	7 OE	21 OE	33 OE	47 OE	61 OE	73 OE	87 OE
B	122 SP	183 SP	195 SP	209 SP	223 SP	8 OE	22 OE	36 OE	48 OE	62 OE	76 OE	88 OE
C	123 SP	184 SP	198 SP	210 SP	224 SP	11 OE	23 OE	37 OE	51 OE	63 OE	77 OE	91 OE
D	126 SP	185 SP	199 SP	213 SP	225 SP	12 OE	26 OE	38 OE	52 OE	66 OE	78 OE	92 OE
E	127 SP	188 SP	200 SP	214 SP	1 OE	13 OE	27 OE	41 OE	53 OE	67 OE	81 OE	93 OE
F	128 SP	189 SP	203 SP	215 SP	2 OE	16 OE	28 OE	44 OE	56 OE	68 OE	82 OE	96 OE
G	131 SP	190 SP	204 SP	218 SP	3 OE	17 OE	31 OE	43 OE	57 OE	71 OE	83 OE	97 OE
H	132 SP	193 SP	205 SP	219 SP	6 OE	18 OE	32 OE	46 OE	58 OE	72 OE	86 OE	98 OE

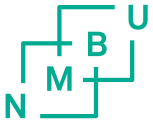
The last plate is continued with the remaining olfactory tissue samples.

Plate 5	1	2	3	4	5	6	7	8	9	10	11	12
A	101 OE	113 OE	127 OE	188 OE	200 OE	214 OE						
B	102 OE	116 OE	128 OE	189 OE	203 OE	215 OE						
C	103 OE	117 OE	131 OE	190 OE	204 OE	218 OE						
D	106 OE	119 OE	132 OE	193 OE	205 OE	219 OE						
E	107 OE	121 OE	133 OE	194 OE	208 OE	220 OE						
F	108 OE	122 OE	183 OE	195 OE	209 OE	223 OE						
G	111 OE	123 OE	184 OE	198 OE	210 OE	224 OE						
H	112 OE	126 OE	185 OE	199 OE	213 OE	225 OE						

Appendix 3.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	1	1	1	1	1	1	1	1	1	1	1	1	1	1	PC									
B	1	1	1	1	1	1	1	1	1	1	1	1	1	1	PC									
C	2	2	2	2	2	2	2	2	2	2	2	2	2	2	NT									
D	2	2	2	2	2	2	2	2	2	2	2	2	2	2	NT									
E	3	3	3	3	3	3	3	3	3	3	3	3	3	3										
F	3	3	3	3	3	3	3	3	3	3	3	3	3	3										
G	4	4	4	4	4	4	4	4	4	4	4	4	4	4										
H	4	4	4	4	4	4	4	4	4	4	4	4	4	4										
I	5	5	5	5	5	5	5	5	5	5	5	5	5											
J	5	5	5	5	5	5	5	5	5	5	5	5	5											
K	6	6	6	6	6	6	6	6	6	6	6	6	6											
L	6	6	6	6	6	6	6	6	6	6	6	6	6											
M	7	7	7	7	7	7	7	7	7	7	7	7	7											
N	7	7	7	7	7	7	7	7	7	7	7	7	7											
O	8	8	8	8	8	8	8	8	8	8	8	8	8											
P	8	8	8	8	8	8	8	8	8	8	8	8	8											

here is a schematic of each q-PCR plate. Each tube contains 4 µL of cDNA and 6 µL of master mix. PC: positive control (for positive control, 2 µL of 25 random diluted cDNA was taken so there was 50 µL of mix cDNA). NC: negative control (water).



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