Exposing infected fish to recirculating aquaculture systems: consequences to biofilter functionality and potential disinfection strategies

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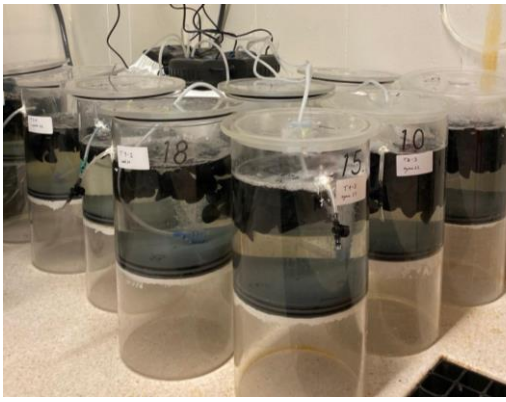

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Resumo

A crescente implementação de sistemas de aquacultura de recirculação (RAS) na produção de salmão-do-Atlântico (*Salmo salar*) tem suscitado preocupações quanto à persistência de agentes patogénicos e à gestão de surtos, com destaque para o Vírus da Anemia Infecciosa do Salmão (ISAV). Este estudo teve como objetivo avaliar a sobrevivência e infetividade do ISAV em 3 RAS de pequena escala, o seu impacto na funcionalidade dos biofiltros e a eficácia de estratégias de desinfeção pós-surtos. Para tal, os peixes (peso inicial: 50 g; comprimento furcal: 17 cm) foram inoculados por via intraperitoneal com 0.1 mL ($1.0 \times 10^4 TCID_{50}$/peixe) da estirpe patogénica HPR-Δ de ISAV.

Recolheram-se amostras de água, zaragatoas dos tanques e biofiltros, bem como amostras de coração e da região média do rim de peixes moribundos, que foram posteriormente analisadas por Real Time (RT)-qPCR, cortes histológicos e imunohistoquímica. Após a mortalidade, os sistemas foram limpos e 10 peixes *naïve* (de tamanho semelhante) foram adicionados aos RAS. Em paralelo 75 biochips de cada RAS e 4 L de água foram divididos em biorreatores submetidos a três tratamentos: nenhum tratamento (controlo), troca completa da água (100%) ou ozonização até 700 mV. O RNA viral foi mais eficientementente detetado nas amostras de zaragatoas e na água do que nos biofiltros após a morte dos peixes, mas a concentração decresceu nestas amostras, quer nos tanques, quer nos biorreatores. Embora a ozonização tenha eliminado rapidamente o vírus, este também se dissipa com rapidez na ausência do hospedeiro. A imuno-histoquímica e a histologia dos tecidos dos peixes confirmaram a presença do ISAV no coração e no rim, mas a histopatologia tradicional foi frequentemente inconclusiva devido à natureza aguda da doença. No entanto, como os peixes *naïve* não foram infectados, pode-se inferir que o *fallowing* ocorre naturalmente e que o vírus não persiste em sistemas RAS. A investigação microbiológica em curso avaliará os efeitos ecológicos dos tratamentos nas comunidades microbianas dos biofiltros. Este estudo confirma a sobrevivência do ISAV na infraestrutura de sistemas RAS, porém com baixa capacidade de infectividade, e apresenta evidências que apoiam o uso do ozono como uma medida eficaz de desinfecção.

Palavras-chave: Vírus da Anemia Infecciosa do Salmão (ISAV), Sistemas de Recirculação em Aquacultura (RAS), Biossegurança, Persistência de patogénios.

Abstract

The increasing use of recirculating aquaculture systems (RAS) in the farming of Atlantic salmon (*Salmo salar*) has prompted concern regarding pathogen persistence and outbreak control, specifically with respect to Infectious Salmon Anaemia Virus (ISAV). This research assessed ISAV survival and infectivity in 3 small-scale RAS setting, its effect on biofilter function, and the effectiveness of post-outbreak disinfection strategies. Atlantic salmon (initial body weight: 50 g; fork length: 17 cm) were injected intraperitoneally with 0.1 mL ($1.0 \times 10^4 \mathrm{TCID}_{50}/\mathrm{fish}$) of ISAV HPR-Δ strain.

Water, tank swab, and biofilter samples were taken, along with heart and mid-kidney organ samples from moribund fish, which were examined by RT-qPCR, histology, and immunohistochemistry. After death of the fish, the systems were cleaned, and 10 naïve fish (similar size) were introduced into the RAS systems. In parallel, 75 biochips from each RAS and 4 L of water were divided into bioreactors which were subjected to one of three disinfection strategy treatments: no treatment (control), 100% water exchange and ozonation to 700 mV. ISAV RNA was more efficiently detected in swab and water samples than biofilter samples after fish mortality, but the concentration decreased over time both in the tanks and bioreactors. Although ozonation promptly eliminates the virus, it also dissipates swiftly in the absence of the host. Immunohistochemistry and histology of fish tissues confirmed ISAV in the heart and kidney, but traditional histopathology was often inconclusive because of the acute nature of the disease. However, since naive fish were not infected, it can be inferred that fallowing occurs naturally, and that the virus does not persist in RAS. Ongoing microbiome research will continue to characterize the ecological impacts of disinfection treatments on beneficial microbial populations that underpin RAS water quality and stability. This study confirms the survival of ISAV in RAS infrastructure but with low infectivity capacity and presents evidence supporting the use of ozone as an effective disinfection measure.

Keywords: Infectious Salmon Anaemia Virus (ISAV), Recirculating Aquaculture Systems (RAS), Biosecurity, Pathogen persistence.

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List of Abbreviations

ARGs	ANTIMICROBIAL RESISTANCE GENES
ASK	ATLANTIC SALMON KIDNEY
BSA	BOVINE SERUM ALBUMIN
cDNA	COMPLEMENTARY DNA
Ct	CYCLE THRESHOLD
DNA	DEOXYRIBONUCLEIC ACID
DO	DISSOLVED OXYGEN
D.P.I.	DAYS POST-INFECTION
FBBS	FIXED BED BIOFILTERS
FBS	FETAL BOVINE SERUM
FDO	FLUORESCENT DISSOLVED OXYGEN
HE	HEMAGGLUTININ-ESTERASE
HPR	HIGHLY POLYMORPHIC REGION
H&E	HEMATOXYLIN AND EOSIN
IDS	INTELLIGENT DIGITAL SENSOR
IFAT	IMMUNOFLUORESCENCE ANTIBODY TEST
IG	IMMUNOGLOBULIN
IHC	IMMUNOHISTOCHEMISTRY
IP	INTRAPERITONEALLY
IPNV	INFECTIOUS PANCREATIC NECROSIS VIRUS
ISA	INFECTIOUS SALMON ANEMIA
ISAV	INFECTIOUS SALMON ANEMIA VIRUS
LB	LYSIS BUFFER
MBBS	MOVING BED BIOFILTERS
ORP	OXIDATION-REDUCTION POTENTIAL
PAA	PERACETIC ACID
PBS	PHOSPHATE-BUFFERED SALINE
pH	POTENTIAL OF HYDROGEN
RAS	RECIRCULATING AQUACULTURE SYSTEMS
RNA	RIBONUCLEIC ACID
RPM	REVOLUTIONS PER MINUTE
RT-qPCR	REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION

SD	STANDARD DEVIATION
TAN	TOTAL AMMONIA NITROGEN
TCID	TISSUE CULTURE INFECTIOUS DOSE
UV	ULTRAVIOLET
VHSV	HAEMORRHAGIC SEPTICAEMIA VIRUS
WOAH	WORLD ORGANISATION FOR ANIMAL HEALTH

1. Introduction

1.1. Atlantic salmon (*Salmo salar*)

Norway is the biggest producer of Atlantic salmon (*Salmo salar*), which is one of the most consumed and in-demand finfish species worldwide. In fact, this country provides more than 50% of the global production of farmed salmon and, in 2023, Norway alone produced about 1.6 mill tons of farmed salmon, which represents a significative share of the global aquaculture production of 94 mill tons in aquatic animal farming (FAO, 2024; Norwegian Directorate of Fisheries, 2024).

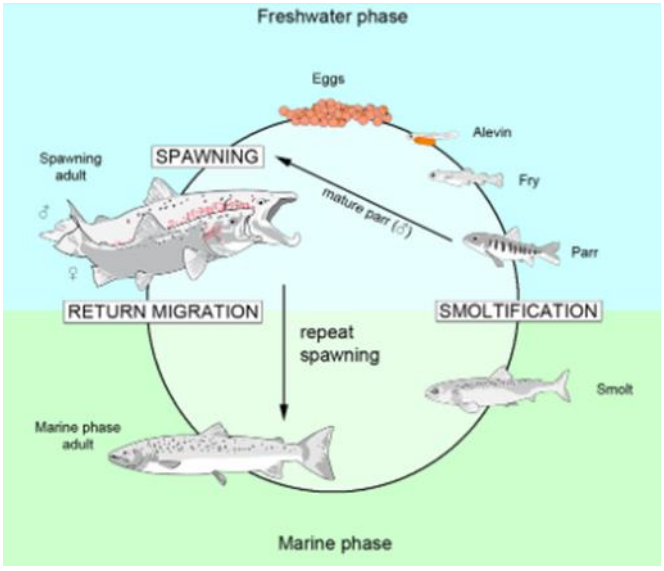


Figure 1 - Salmon life cycle (Mobley *et al*., 2021).

Farm production of Atlantic salmon is divided into a two-stage process where smolts (a stage represented in Figure 1) are reared indoors and a grow-out stage occurs in sea cages, resulting in market-sized fish. In recent years, there has been a transition from traditional flow-through systems to recirculating aquaculture systems (RAS) for smolt production for stocking into Norwegian sea-cages, where approximately 70% of smolts are produced by RAS production (Meriac, 2019). Salmon aquaculture now represents approximately 80% of the global salmon market, primarily in sheltered areas of the oceans, such as fjords, due to biological, thermal, and environmental factors that constrain the species (FAO, 2024).

As Atlantic salmon research production increases, the need to optimize the knowledge of biosecurity RAS becomes more imperative in the context of sustainable and ethical salmon farming (Ahmed *et al.,* 2021; Lekang, 2013).

1.2. Recirculating aquaculture system - RAS

Aquaculture production technologies are advancing rapidly, especially in land-based or onshore systems. Among the innovations, RAS is gaining prominence. These systems are designed to recycle water by incorporating multiple H_2O treatment processes (Timmons *et al*., 2010; Lekang, 2013). These setups comprise ammonia and nitrite elimination and may include solid waste and carbon dioxide removal and disinfection, among other treatments. In such systems, 95–99% of water used is typically recirculated back into the system and reutilized, maximising the efficiency of food production and minimising waste and environmental impacts, particularly for the farming of finfish (Fudge *et al*., 2023; Murray *et al*., 2014).

Moreover, RAS offers several advantages over open water and flow-through production systems when managed effectively, making it one of the most environmentally friendly methods of fish production at a commercial level due to close-to-market production, that minimizes CO_2 footprint on transportation (Li, X. *et al.,* 2023; Martins *et al.,* 2010). These benefits also include year-round production, flexibility in species selection and location, efficient water utilization and consumption, heat conservation, and reduced fish stress, leading to higher survival rates and productivity. In particular, when compared to flow-through systems, RAS is beneficial for Atlantic salmon production. In fact, flow-through systems rely on a continuous intake of fresh water, being more dependent on the quality and stability of the external source, which may undergo limited treatment when compared to RAS. This makes RAS less vulnerable to seasonal changes, contamination, or pathogen ingress. Although installation and operational costs are generally higher, tanks and equipment in RAS have a longer average lifespan compared to the flow-through ones, which allows for extended amortization periods. Flow-through systems may require more frequent maintenance or replacement due to wear from continuous water flow and possible lack of tight environmental (Ebeling *et al*., 2012; Roque d'Orbcastel *et al.,* 2009). However, there are some limitations to RAS. In fact, low water usage, high feeding rates, and high-density production, unless properly controlled, can lead to the accumulation of small particles and organic materials, which are a risk factor for difficult-to-manage disease outbreaks (Dahle *et al*., 2023; Fernandes *et al*., 2017).

Although biosecurity measures reduce the likelihood of pathogens entering the system, once inside, harmful bacteria can attach to biofilms, leading to persistent infections since biofilm removal is difficult (Schoina *et al*., 2022). High mortality rates are also induced by elevated levels of suspended solids, carbon dioxide, nitrites, and ammonia (Hjeltnes *et al*., 2012). Oxygen consumption and particulate matter can rise as heterotrophic bacteria break down organic matter, and if not controlled, this can hinder the process of nitrification in the biofilter by allowing these bacteria to outcompete nitrifying bacteria (Dahle *et al*., 2023).

Even though there are many biosecurity protocols in place to prevent the entry of pathogenic organisms, these are poorly documented, and the spread of disease caused by bacteria, viruses, fungi, and parasites has been increasing over the past few years. In addition, RAS presents some issues, such as highly qualified personnel requirements, continuous monitoring of water conditions, and the rise in manufacturing cost due to expensive power-consuming equipment and water treatments involving pumping for ideal working conditions. (Badiola *et al.,* 2018; Ayuso Virgili *et al*., 2023).

1.2.1. Water Quality

Water quality control is a critical issue in RAS, since water is re-used in the system. It can be broadly classified based on physical-chemical parameters, including turbidity, alkalinity, temperature, salinity, pH and dissolved oxygen levels (Su *et al.,* 2020; Tziortzioti *et al*., 2019; Amijar *et al*., 2020). By maintaining these parameters at optimal levels, fish growth and the reduction of risk of infection are promoted, since even slight changes in water parameters may harm aquatic animals (Lindholm-Lehto, 2023; Tolentino *et al*., 2021; Yusoff *et al*., 2024) Salmonids require oxygen saturation levels of approximately 80% (which is equivalent to above 8 mg L^{-1}), with temperatures between 8°C and 14°C for optimal health conditions. The ideal pH range is generally from 6.0 to 8.5 (Marine Institute, 2017; Remen *et al*., 2016). Moreover, ammonia and nitrite concentration, which are fish metabolism and organic matter decomposition waste products, also define water quality as these products can accumulate and become poisonous to fish health if not properly managed (Eding *et al.,* 2006).

1.2.2. Biofilter

One of the primary functions responsible for water quality maintenance is carried by biofilters (an integral part of RAS) that promotes the development of autotrophic ammonia-oxidizing bacteria and archaea like *Nitrosomonas*, and nitrite-oxidizing bacteria and archaea such as *Nitrobacter*, occurring in high density biofilms and colonizing filter surfaces (Stewart *et al*., 2008). These microorganisms execute the key reaction of nitrification in two stages: i) the aerobic oxidation of toxic ammonia to nitrite, and ii) oxidation of nitrite to less toxic nitrate. While their presence can strain the carrying capacity of the system through increased oxygen requirements, space competition, and ammonia production, heterotrophic bacteria in the biofilm assist in the breaking down of both dissolved and particulate organic matter and by excreting molecules that help generate a physical biofilm, further maintaining water quality (Qi *et al*., 2025).

The biofilter ensures the removal of ammonia from fish metabolism and waste, maintaining a stable environment for aquatic organisms, and the establishment of moving bed bioreactors for treating RAS wastewater has been increasingly seen as a sustainable approach (Qi *et al*., 2025).

However, regular maintenance is required for optimal performance of a biofilter. This includes monitoring and adjusting key water parameters like flow rate, pH, carbonate alkalinity, and temperature, while cleaning or replacing the biofilter should be avoided in order to prevent damaging the microbial biofilm. These factors have an influence on the effectiveness of the biological filtration. Indeed, nitrifying bacteria have good growth at pH 7.0-8.0 and their optimum temperature is between 20-30°C, with any great and abrupt change of the environment (such as fluctuation of pH, temperature) possibly leading to unsustainable conditions for the biofilter microorganisms, affecting negatively the performance and efficiency of the biofilter. Nitrifying bacteria work best in a high oxygen concentration, usually higher than 2 mg/L dissolved oxygen (DO) (Park & Noguera, 2004). However, for optimal functioning, nitrifying bacteria require a level of DO close to saturation (~7-9 mg/L at low temperatures) (Xu W. *et al*., 2020).

Nitrosomonas obtains carbon from inorganic sources such as bicarbonate or carbonate for cell synthesis during nitrification (Horan, 2003). Both pathways produce hydrogen ions (H^+), which have an acidifying effect, influencing pH decrease and possibly inhibiting nitrification if not appropriately buffered. By ensuring that the carbonate alkalinity is kept above 100 mg/L as $CaCO_3$, it is possible to stabilize the pH and keep the activity of

nitrifiers (Jafari *et al*., 2024). Another important parameter connected to alkalinity is the concentration of dissolved CO_2. Although the bicarbonate buffer prevents pH alteration, the overloading of CO_2 from fish respiration, microbial metabolism and organic matter mineralization is likely to disturb the balance of the carbonate system and may lead to increased acidity and reduced ability to carry oxygen in the blood of the fish due to the Bohr effect (Brauner *et al*., 2017; Fivelstad *et al*., 1999). High levels of CO_2 (>20 mg/L) may also cause chronic stress and physiological injury in fish and demand active removal of CO_2 from the system via degassing towers or aeration units (Fivelstad *et al*., 2013). To maintain appropriate alkalinity levels, a balance must be achieved: although sufficient bicarbonate should be available to support both microbial processes and chemical buffering, carbon dioxide levels should be controlled in order to ensure good water quality and fish health. In this way, alkalinity must be routinely manipulated by operators through additions of sodium bicarbonate or other alkaline materials, especially following heavy ammonia loads or water changes (Colt, 2006; Martins *et al*., 2010; Timmons & Ebeling, 2010).

Given these requirements, implementation and continual management of biological filtration in RAS can be expensive, especially in larger operations, as there is a significant investment in the initial infrastructure and continuous time and resource expenditures in the maintenance of the systems.

Excessive nitrite and ammonia concentrations can be harmful to fish and disrupt biofilter efficiency, as free ammonia concentrations greater than 10 mg/L have been found to suppress nitrification processes (Nowak *et al*., 1994). As mentioned beforehand, one of the primary features of RAS is the biofilter, which employs nitrifying bacteria and archaea to convert toxic ammonia into less toxic nitrate (Aalto *et al*., 2022). However, it can take 3 to 8 weeks before these bacteria are able to mature/colonize a new system (Rojas-Tirado *et al*., 2017; Navada *et al*., 2021). In order to accelerate this process, aquaculture managers prefer inoculation of biofilters through the addition of bacteria from mature systems or by adding ammonia directly into the system before the introduction of fish (Yanong *et al*., 2015).

Disturbance of the biofilter process, such as power failure, may lead to interruptions in the nitrogen cycle, leading to toxic ammonia and nitrite peaks (above 1 mg/L and 0.5 mg/L respectively) that are deadly to the fish by affecting the water flow and oxygen supply, hence the necessity for constant operation and servicing of biological filtration systems (Ebeling *et al.,* 2006; Gutiérrez *et al*., 2019, Schreier *et al*., 2010, Timmons *et*

al., 2018). Disease prevention, correct maintenance strategies, and a gradual increment of feed input - no more than an increase of 20–25% per day - are key for a RAS biofilter maturation. This is technically and economically challenging and usually requires significant modifications of, not only the filter design, but also the filtration process (Timmons & Ebeling, 2010). Moreover, overstocking in RAS systems should be avoided, as it leads to an accumulation of waste products, which not only harm aquatic organisms but can also disrupt the nitrogen cycle within the filter, reducing its efficiency (Liu *et al.,* 2021).

1.3. Biosecurity in RAS

Both effluent and intake water disinfection in Norwegian aquaculture are often subject to regulations. Authorized methods include ultraviolet (UV) irradiation, ozone (O_3 - a highly reactive oxidizing agent, utilized for controlling pathogens such as bacteria, viruses, and parasites), sodium hydroxide (NaOH), formic acid (HCOOH), chemical precipitation, and others, all requiring approval from the Norwegian Veterinary Institute (Veterinærinstituttet, 2017).

RAS consist of numerous interconnected components, such as pipes, production tanks, and mechanical and biological filters, all of which can harbour pathogens if not properly maintained. The United States Environmental Protection Agency (2020) shapes disinfection protocols to ensure the health of aquaculture systems that comprise strategies such as chemical disinfection of surfaces and equipment that could reduce the risk of pathogen accumulation (Timmons *et al*., 2018).

The biofilter may, however, serve as a potential reservoir for pathogens. The biofilm matrix and organic matter that accumulate in biofilters can constitute a protective niche for the pathogens, protecting them from disinfection methods and environmental stresses, making it possible for a longer survival. Research pertaining to viral dynamics, pathogen transmission routes, and effective decontamination methods are considerably lacking. The scarcity of this type of research leaves a significant gap in biosecurity practices for RAS operators, as the risk of re-infection and cross-contamination between production cycles remains an unresolved challenge (Blancheton *et al*., 2013; Pietrack *et al*., 2024).

To address this, the implementation of chemical disinfection between fish batches has been proposed as a strategy for commercial production. Hofstad (2023) also pointed out

that biofilter disinfection affects the microbial dynamics of RAS but its effect on opportunistic pathogens was still unknown. RAS-specific disinfection strategies include continuous water disinfection, occasional disinfection of equipment and surfaces, which can be conducted by using chemical disinfectants such as peracetic acid (PAA), formalin, hydrogen peroxide (H_2O_2), copper sulphate, and chloramine-T. Such disinfectants have great efficacy against a wide range of pathogens, but also can be harmful to biofilter activity, and consequently nitrification. Although ozone and PAA differ chemically, both are frequently used for disinfection in RAS posing similar challenges: both disinfectants, especially at high concentrations or multiple exposures, have been demonstrated to inhibit nitrification by damaging the microbial populations associated with ammonia and nitrite oxidation (Mugwanya *et al*., 2022; Summerfelt *et al*., 2009).

Ozone is a very potent, non-specific oxidizing agent that degrades rapidly in water, also in comparison to PAA. As beneficial as ozone is for the disinfection of water, its residual level needs to be carefully monitored to avoid passing on to the biofilter where it can break down nitrifying bacteria. Aqueous ozone concentrations ranging from 1.2 to 3.6 µg/mL have been found to considerably reduce viable bacterial cells within biofilms after just 30 seconds of application, according to studies (Bialoszewski *et al*., 2011). While this highlights the effectiveness of ozonation as a disinfection method, such treatments must be accurately regulated, often using chemical degradation or UV treatment, in an attempt to prevent unrequired effects for biofilm action and maintain effective nitrification. However, studies have shown that mild ozonation, in RAS studies with an oxidation-reduction potential (ORP) of around up to ~340 mV (with no residual ozone detected), can stimulate biological nitrification without damaging biofilter performance (Mugwanya *et al*., 2022; Summerfelt *et al*., 2009). In contrast, PAA requires higher concentrations (typically higher than 2 mg/L) to provide similar antimicrobial action. However, PAA also alters the structure of the biofilm and reduces its capacity to degrade nutrients at concentrations around 1 mg/L. Therefore, while ozone is more reactive and toxic at lower concentrations, PAA is less reactive but still able to cause long-term nitrification problems at higher concentrations. Moreover, its longer permanence in the water may negatively affect the biofilter and fish, mainly in RAS, in which the water is continuously reused (Pedersen *et al*., 2013; Suurnäkki *et al*., 2019). Qi *et al*. (2025) concluded that careful management of disinfection strategies, including controlled dosing of ozone and PAA, is essential to avoid negative impacts on biofilm activity and ensure healthy fish production.

Preferably, disinfection strategies in RAS should be highly efficient in killing pathogenic microorganisms, without adverse effects on fish health and welfare. Furthermore, since the biofiltration is based on certain bacteria that convert toxic ammonia and nitrite into less toxic nitrate, it is important that the disinfectants do not potentially inhibit this nitrification process. This equilibrium is difficult to achieve, and therefore, the disinfection of RAS is a complex issue (Mota *et al.*, 2022).

According to Lazado and Good (2021), a comparative study of disinfection strategies in Norwegian and North American RAS facilities revealed key insights into their application and efficacy. Figure 2 compares the utilization of disinfectants in North America and Norway. While interpreting the results, it is important to take note that the USA have more facilities than Norway, which may influence the overall distribution and diversity of disinfectant usage. In Norway, PAA is the predominant disinfectant, with chlorine being also very significant, whereas in North America, PAA is utilized in relatively few facilities, with chlorine being the most widespread disinfectant, followed by quaternary ammonium compounds, formalin, and hydrogen peroxide. These disinfectants are used primarily between production cycles without fish in the system since it may impact biofilm bacteria and fish health (Lazado & Good, 2021).

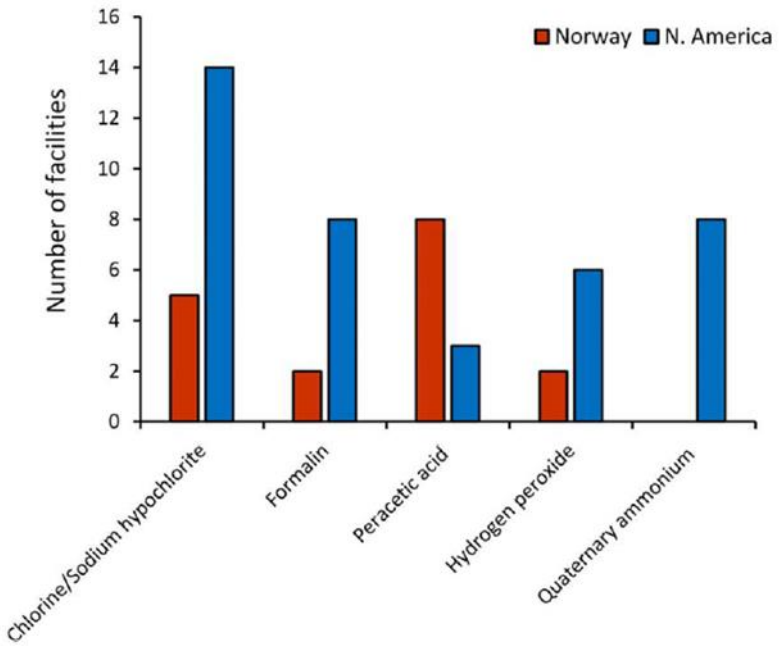


Figure 2 - Chemical disinfectants used between production cycles in RAS facilities: Norway vs North America (Lazado & Good, 2021).

Although, UV is used predominantly for pathogen control, there are other beneficial aspects of ozone since it can remove organic carbon, turbidity, algae, odour and taste from water and even alter colour. Ozone degrades nitrogen compounds such as ammonia and ammonium, increasing the microbiological and chemical quality of water while eliminating other potentially harmful substances or microorganisms. Moreover, ozone is often integrated with complementary treatment methods to optimize water quality. For instance, Rurangwa and Verdegem (2015), describe that ozone is commonly paired with protein skimmers to enhance the removal of dissolved organic material and fine particles. Ozone facilitates the flocculation of organic particles, thereby making them removable by skimmers, and consequently increasing the clarity of water and reducing organic loads. In addition to skimmers, ozone is employed in combination with biologic filters to stimulate nutrient recycling: by oxidizing organic substances to simpler compounds which can be treated in the biofilter, it improves the nitrification and also decreases the organic load. Likewise, ozone enhances UV disinfection systems by removing suspended organic matter, thereby reducing light-scattering particles and allowing more efficient penetration of UV rays.

Notwithstanding the application of ozone in RAS should also be regulated to mitigate unsought implications, as mentioned beforehand. There are indeed probes and automated controls in RAS that regulate ozone dosing to ensure safety. ORP sensors (mV) monitor the total oxidants, maintaining levels around 300 mV in freshwater and automatically switch off ozone generators upon reaching setpoints. Additionally, ambient ozone gas detectors and interlock systems protect operators by detecting leaks and shutting down the system >0.1 ppm ozone exposure levels (Lindholm-Lehto, 2023).

Qi *et al*. (2025) emphasized that the first application of ozone amplified endogenous respiration activity of biofilm by 45–74%. Nevertheless, during the succeeding four weeks, this elevated respiration activity weakened, with respiration activity returning to control values at the end of the experiment, suggesting that the stimulating influence of ozone on biofilm metabolism was short lived. Remarkably, ozone removal enhanced water clarity but did not have the significant impact on nitrification potential or the metabolic status of the biofilm. This finding emphasizes the potential of ozone in enhancing water quality without damaging the efficiency of crucial biofilter elements when properly used, for example at the appropriate dose. Typically, mild ozonation corresponds to an ORP range of approximately +200 to 300 mV in freshwater systems and +250 to 400 mV in saltwater or brackish systems (Waller, 2024). Use of ozone has also been linked to the relative dominance of antimicrobial resistance genes (ARGs) in

the water. Huang *et al*. (2024) commented that treatment with ozone might boost the occurrence of acquired ARGs and intrinsic efflux-mediated ARGs, specifically those within the resistance nodulation cell division family. Interestingly, the bacterial genera with co-occurrence of efflux and non-efflux ARGs dominated the lists, where many genera became dominant among the microbial community upon treatment with ozone. This suggests that although ozone can be used effectively for pathogen control, it may also be involved in the development of antimicrobial resistance in aquaculture systems, as discussed by Huang *et al*. (2024) and Lu *et al*. (2022).

1.4. Infectious salmon anaemia (ISA)

ISA is an infectious disease that mainly affects farmed Atlantic salmon, with associated huge financial losses in the aquaculture industry, both currently and especially during the late 1990s and early 2000s. In fact, during this time, large-scale outbreaks led to massive fish mortalities, resulting in substantial financial damage both at the industry level and for individual farms. These outbreaks also prompted trade restrictions and increased operational costs due to stringent biosecurity measures and disease management efforts (Kibenge *et al*., 2001; Mardones *et al*., 2014). Losses are not only due to direct mortality but also due to disease control, including compulsory culling of the stock, disinfection procedures, reduction of production and market exclusion.

The limited data available suggest that ISAV may persist in compartments such as biofilters even after outbreaks since viral RNA was isolated from RAS samples, but it is unclear if the virus is infectious and viable (Christiansen *et al*., 2021).

The Infectious salmon anaemia virus (ISAV) belongs to the genus *Isavirus* of the family Orthomyxoviridae and infects primarily salmonids, a specificity not described for the other members of the virus family. ISAV shares structural similarity with the influenza virus, being enveloped within a lipid layer (capsid) into which the genome, comprising 8 segments of negative-sense single-stranded RNA, is folded. As in other *orthomyxoviruses*, this segmentation allows for genetic reassortment, which may facilitate evolutionary change and adaptation (Rimstad & Markussen, 2020; Stallnecht & Brown, 2009).

The virus invades the skin and gills of fish and then the circulatory system. Macroscopic post-mortem examination of dead fish typically shows pale gills, in addition to clinical signs of ascites and necrosis of organs such as the heart, spleen, and kidney (Rimstad

et al., 2011). Nonspecific clinical signs comprise lethargy, anorexia, swimming abnormalities, exophthalmia and skin darkening (Fuhong *et al*., 2024). The disease tends to appear more frequently during spring/early summer and late autumn, periods associated with environmental changes that can influence host immunity and viral stability.

There are primarily two genetic variants of ISAV: i) HPR-0, non-pathogenic variant without a deletion in the highly polymorphic region (HPR) of the hemagglutinin-esterase (*HE*) gene, which is often encountered in sub-clinical and/or transient infections; and ii) HPR-Δ (pathogenic variant), which carries a deletion in the HPR of the *HE* gene. This form is more virulent due to improved receptor binding and immune evasion capabilities, causing overt disease and facilitating rapid transmission between farms. HPR-Δ can mutate from HPR-0, through genetic changes (deletions) in the *HE* gene mentioned beforehand, which causes a transient infection without clinical signs. The HPR deletion may increase the virulence of the virus by amplifying its capacity to enter host cell receptors, enhancing its capability to escape detection by the host immune system and enhancing replication and transmission within the host and among fish populations (WOAH, 2021). The surveillance of ISAV HPR-0 (as a potential precursor to the development of pathogenic ISAV HPR-Δ infection) and of ISAV HPR-Δ infection is rendered challenging given that these might be sub-clinical over a prolonged period before typical clinical signs develop. This suggests that seemingly healthy fish may still carry the virus without exhibiting symptoms (Gagné & LeBlanc, 2018; Mugimba *et al*., 2021). Successful ISAV control relies on early detection and prompt removal of the infected fish to prevent further spread.

Histopathological examination continues to be a primary diagnostic tool, tending to show common erythrophagocytosis, congestion of the liver and spleen, and degeneration of endothelial cells. Current diagnostics also utilize Real Time (RT) qPCR to identify viral RNA in symptomatic, as well as asymptomatic fish, while virus isolation in cell culture, although more laborious, is still the diagnostic gold standard. While ISAV has been traditionally linked to open-net pens and flow-through systems, it has been reported in RAS, as well in various system compartments, including tank walls, biofilms, suspended solids, and biofilters, particularly when there are biosecurity lapses. However, the exact reservoirs and their role in viral persistence or transmission are still unclear (Davidson *et al*., 2017; Rimstad *et al*., 2011). Theoretically, RAS should offer high levels of protection due to their controlled environment, but outbreaks have revealed vulnerabilities, especially due to insufficient treatment of intake water, improper disinfection of

equipment, or via personnel due to improper hygiene. In one documented case, ISAV outbreaks in RAS were associated with the introduction of fish carrying undetected HPR-0 strains, which later evolved into virulent HPR-Δ forms within the closed system (Christiansen *et al*., 2017). Furthermore, poor design or maintenance of biofilters and insufficient water treatment have been identified as contributing risk factors for viral persistence in RAS environments (Blancheton *et al*., 2013; Gupta *et al*., 2024). Viral particles may become embedded within biofilms or adsorbed onto surfaces, potentially allowing prolonged survival despite water treatment protocols. However, the infectivity of ISAV in these environmental matrices, and the extent to which such locations act as true reservoirs capable of initiating new infections are yet to be definitively established (Rimstad *et al*., 2011; Vike *et al*., 2009). Moreover, the extraction and detection of ISAV from RAS components pose technical challenges, often requiring aggressive or specialized methods such as scraping, enzymatic digestion, sonication, or mechanical scraping to recover viral material effectively (Ferrera *et al*., 2010).

These findings emphasize the importance of routine molecular surveillance, strict quarantine procedures, and validated water treatment protocols (for example, UV, ozone, PAA) in RAS, not only to prevent the entry of ISAV but also to mitigate the risk of viral amplification within the system (Gagné & LeBlanc, 2018; Godoy *et al*., 2008; Thorud & Djupvik, 1988).

The Norwegian Food Safety Authority has implemented stringent measures, such as monitoring and the euthanasia of infected fish in fish farms, which have been effective in preventing large-scale regional epidemics, despite the significant economic losses incurred by the industry (Norwegian Food Safety Authority, 2024).

ISAV remains infectious in aquatic environments with a pH between 5 and 9 (Falk *et al*., 1998). Rimstad and Mjaaland (2002) detected stability and infective ISAV in seawater at 4°C for up to four months. Moreover, a study conducted by Vike *et al.* (2014a) investigated the infectivity of the virus under various conditions. The research found that ISAV loses infectivity rapidly when exposed to natural seawater or sterile seawater with ultraviolet radiation, with infectivity lost within 3 hours. In sterile seawater without UV exposure, the virus remained infectious for less than 24 hours.

1.5. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) as ISA identification tool

Quantitative PCR is a widely used tool in industry and aquaculture research, in particular to analyse gene expression and microbial populations. RT-qPCR combines the method of PCR amplification and the technique of fluorescent dye detection, which enables the quantitation and identification of specific DNA sequences in real-time. This is particularly beneficial in the monitoring of fish health, the detection of microbial populations in RAS and in the description of pathogens in farmed salmonids, including Atlantic salmon (Gleerup *et al*., 2024).

qPCR amplifies a target DNA sequence using short oligonucleotide primers, a thermostable DNA polymerase and a fluorescent label such as SYBR Green (which binds double stranded DNA, as described above) or amplicon-specific DNA probes (e.g. TaqMan). The extent of the fluorescence increase upon amplification is directly proportional to the amount of the product amplified. The cycle threshold (Ct) value, the cycle of PCR in which the fluorescence is above the background threshold, is a quantitative value: lower Ct values correspond to higher initial template concentrations (Arya *et al*., 2005).

RT-qPCR is used to first reverse transcribe RNA into complementary DNA (cDNA) in order to enable quantification of transcript abundance. This method is very sensitive and allows for the detection of low-abundance mRNA transcripts and thus is employed in tracking immune responses, stress markers, and other physiological pathways in aquaculture fish following infection or exposure to stress (Bellec *et al*., 2022; WOAH, 2009).

RT-qPCR is extremely sensitive and can detect as few as 1–10 copies of target DNA per reaction in optimized circumstances (Bustin & Nolan, 2004). This sensitivity also requires strict contamination control procedures and carefully designed assay components. Specificity of RT-qPCR is very much dependent on primer and probe design: primers must bind specifically to the target sequence without dimerizing or binding to non-target sequences, and probes must bind specifically within the amplified sequence for proper signal generation. These features make RT-qPCR capable of detecting residual low levels of bacterial contamination (*Aeromonas salmonicida*, *Tenacibaculum spp*., *Flavobacterium psychrophilum*, which frequent in water or biofilm), viral (such as ISAV, Salmonid Alphavirus and Infectious Pancreatic Necrosis Virus), that may be present in the water, in the mucus or in fish tissues. Also, parasites (e.g. *Ichthyophthirius multifiliis*,

Parvicapsula pseudobranchicola or *Desmozoon lepeophtherii*) can be identified using RT-qPCR. In RAS, RT-qPCR can be used as a robust technique for assessing profiling microbiota and for the early detection and sampling of pathogen expansion, before the presentation of clinical disease (Cerda *et al*., 2024; Hodneland *et al*., 2005; Howell *et al*., 2019; Lewin *et al*., 2020; López-Lastra *et al*., 1994; Weli *et al*., 2017).

To ensure reproducible and reliable results, RT-qPCR assays must be carefully validated. Some of the most significant considerations include: i) primer/probe specificity, verified by in silico analysis (for example BLAST) and experimental validation, ii) no formation of primer-dimers, essential for SYBR Green assays to prevent false positives, iii) reference gene stability, required for appropriate normalization in RT-qPCR. Good assay design assures both sensitivity (limit of detection) and specificity (discrimination between target and non-target sequences), both of which are required for biologically relevant interpretation, especially if one is working with complicated microbial populations or subclinical infections (Taylor *et al*., 2019).

1.6. Objectives

The objectives of this study were:

1. To strengthen biosecurity in RAS facilities by generating new knowledge on the sensitivity of ISAV to different biosecurity strategies and their survival and persistence within RAS environments.

2. To investigate the establishment and maintenance of ISAV under conditions relevant to RAS, including its potential for persistence in water or biofilms, and if possible, to differentiate if it is infective.

3. To characterize the efficacy of different strategies (ozone, water exchange) against ISAV.

4. To examine development of ISAV infection, including histopathological changes in target organs (mainly heart and kidney), clinical signs, and mortality patterns, to better understand the disease progression and inform health monitoring strategies in RAS systems.

2. Materials and methods

This thesis was part of the Biosikkerhet i RAS (BRAS) project (https://www.fhf.no/prosjekter/prosjektbasen/901792/), primarily focusing on Work Packages 2 and 3.

Three small-scale RAS were established at Marineholmen RASLab, each with a total water volume of 200 L, including a 100 L single-bottom-drain fish tank. The water salinity was adjusted to 15 ppt (brackish water), and the daily water exchange rate was maintained at approximately 10% (20 L). Each RAS was equipped with two filter bags for mechanical filtration, which were cleaned daily, a protein skimmer, and a moving bed biofilter containing around 1100 matured biofilter chips (biochips) (approximately 10 L). These chips provided an active surface area of 6.25 m² for biological filtration and were pre-matured in 40 L containers by feeding ammonium chloride (NH_4Cl) and sodium bicarbonate ($NaHCO_3$).

The biofilter chips were aerated using an air pump (Luftpump Super 8500, Pondteam, Norway) to ensure effective biological activity and water circulation. Each system was equipped with automatic sensors for continuous monitoring of pH, temperature, and salinity, with additional daily manual measurements of oxygen levels. Ammonia, nitrite, nitrate and alkalinity rate concentrations were assessed weekly to assess water quality and biofilter performance. This daily maintenance follow-up were performed by Marineholmen RASLab personnel.

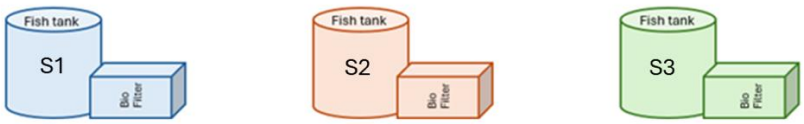


Figure 3 - Lay out of the Lab scale RAS.

Figure 4 - Picture of one of the Lab scale RAS.

2.1. Phase I: Infection

All procedures involving live fish were performed in accordance with Norwegian Food and Safety Authorities (FOTS Id: 30918) and were conducted with full compliance with National and international guidelines for animal welfare, including the principles of the Three Rs: replacement, reduction, and refinement.

Since the focus of this study was the effect of biosecurity measures, the day the systems were disinfected was defined as Day 0. On Day -26 (October 24th), 28 smolt-stage Atlantic salmon juveniles (initial body weight: 50 g, fork length: 17 cm) were introduced to each tank, and were maintained at 12°C. A photoperiod of 14 hours light/10 hours dark was maintained, and fish were fed three times daily with commercial fish feed (30 g daily), using an automatic feeder. The fish were allowed to acclimate for 2–3 weeks before the infection procedure on November 5th (Day -14).

The fish were monitored twice a day, and the spreadsheet documenting water quality parameters (i.e. salinity, temperature) was updated as described in section 2.2. Before any handling and to relieve suffering and stress, the fish were immersed in a solution of 100 mg/L tricaine methanesulfonate (Finquel: MS-222) buffered with 100 mg/L sodium bicarbonate for 2 min in order to be fully anaesthetized. Afterwards, the fish were intraperitoneally injected (IP) with 0.1 ml of ISAV (1.0×10^4 $TCID_{50}$ (50% Tissue Culture Infective Dose)/fish) of the strain Glesvær. During the experiment, any fish displaying signs of lethargy or other indications of distress were euthanized to reduce suffering.

Fish were intended to remain in the RAS system until the end of the viraemic phase, approximately three weeks post-challenge, at which point they would have been euthanized with an overdose of anaesthetic (200 mg/L tricaine methanesulfonate buffered with sodium bicarbonate), for over 10 minutes (Neiffer & Stamper, 2009). However, all fish died within two weeks post-challenge, on Day 00 (November 19th).

All euthanized fish were immediately sampled for histopathology. Organs, including the heart, mid-kidney, and spleen, were preserved in 10% neutral buffered formalin. The liver, pancreas, second gill, and pseudo-branchiae of control and moribund fish were also sampled. Samples from the heart and mid-kidney were stored in RNAlater and sent for RT-qPCR testing to confirm the presence of ISAV.

Euthanized and dead fish were separated into bags according to their tank (S1, S2, S3), their condition (dead or moribund), and whether they had been sampled. The date and other relevant details (any signs of external ISAV infection, for example) were recorded. Fish were stored carefully in a single layer within each bag to avoid stacking, and the bags were carefully stored in the freezer at -20°C, in case some extra confirmation was necessary. Daily records of the number of dead and moribund fish from each tank were maintained.

All nets used for fish handling were thoroughly rinsed with new water before use. After handling, nets were immersed in a disinfectant solution (30% chlorine solution) and stored in a designated orange bucket for each respective tank. The euthanasia solution was kept in the disinfectant bucket until the end of the day to ensure that no further moribund fish required euthanasia. At the end of each day, the euthanasia solution was disposed of via the drainage system.

2.1.1. Tank sampling

On Days -07 and 00, from each experimental system, water samples (2 samples of 1 L), swabbed tank wall biofilms (2 samples), and biofilter samples (2 samples of 5 biochips) were collected for ISAV detection, using RT-qPCR and cell culture method (Table 1). The cell culture method would give an indication if the virus detected is infectious at the time of sampling. Biochips were collected on Day -14 (infection day) to perform RT-qPCR, in order to confirm that the biofilters were negative for ISAV. Five biochips from each tank were also sampled on day 14 for microbiome analysis.

Table 1. Sampling for assessing ISAV establishment, adapted from BRAS project group

Days	Sample Name	Tank 1 (S1)	Tank 2 (S2)	Tank 3 (S3)
D-07	Water	2 x 1 L	2 x 1 L	2 x 1 L
	Swab vessel wall biofilm	2 x 1	2 x 1	2 x 1
	Bio-filter	2 x 5	2 x 5	2 x 5
D00	Water	2 x 1 L	2 x 1 L	2 x 1 L
	Swab vessel wall biofilm	2 x 1	2 x 1	2 x 1
	Bio-filter	2 x 5	2 x 5	2 x 5

2.2. Phase II: Bioreactors and Naïve fish

As mentioned before, on Day 00, the first phase of the trial was terminated due to high mortality and the euthanasia of moribund fish according to ethical considerations. At that point, 225 biochips were taken from each RAS and transferred to lab-scale bioreactors (nine bioreactors of 4 L and 75 biochips each). Each RAS system (both water and biochips) was split into three bioreactors corresponding to each of the following treatments, that were performed on the same day as represented in Figure 5 and 6:

- Treatment 1 (Control): 4 L of water transferred from the original fish tanks; no treatment was performed. This treatment group contains reactors S1R1, S2R1, S3R1.

- Treatment 2 (Water Exchange): 4 L of new fresh water was added (1.5 ppt) in the first 24h, then new 4 L of 15 ppt water was added. This treatment group contains reactors S1R2, S2R2, S3R2.

- Treatment 3 (Ozone): New seawater was added (since ozone works best with seawater), ozonated to 700 mV for 24 h, then the water was removed and replaced with 4L 15 ppt water. This treatment group contains reactors S1R3, S2R3, S3R3. This treatment group had 200 biochips in each bioreactor.

The RAS systems that were emptied for fish, were flushed, lightly scrubbed, and refilled with new 15 ppt water. No further treatments were applied to the RAS.

Water in each RAS and the bioreactors was manually replenished each day, particularly during sampling events, accordingly to the volume of water that was displaced due to sampling, in order to keep the same volume of water.

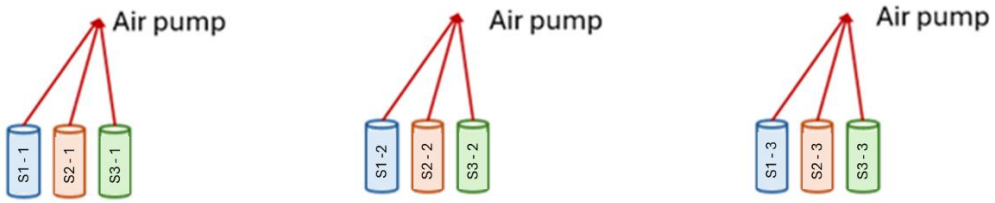


Figure 5 - Bioreactors and their division according to tank and treatment.

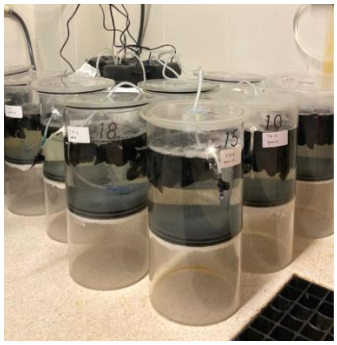


Figure 6 - Picture of bioreactors and their division according to tank and treatment.

2.2.1. Tank and bioreactors sampling

After the fish death, the assembly of the bioreactors and respective disinfection strategies was carried out, water, swabs and bio-filter samples were collected twice a week, from both reactors and RAS. From the reactors, it was taken 200 mL of water and 5 biochips. No swab samples were performed.

The sampling was repeated until two consecutive negative results from RT-qPCR confirmed virus eradication both for the bioreactors and the RAS.

The biochips sampling for microbiome was performed during all the duration of the experiment, mainly when the bioreactors and tanks trials were terminated, but those results will be not presented in this thesis.

New fish,10 per tank with similar size as the ones utilized in phase I, were added to the RAS systems on days 03 to tank S1, 07 to tank S2, and 10 to tank S3 post-challenge to evaluate potential infection to naïve fish and to assess how fallowing might happen and affect a possible survival of ISAV in the system and further infection to new fish. On Day

49, the experiment was terminated, and the fish were euthanized and sampled as described previously.

2.2.2. Tanks and bioreactors water quality and analysis

Water quality analysis, salinity, temperature, pH, nitrite, nitrate, alkalinity, and ammonium readings were analysed twice a week by Marineholmen RASlab personnel and NIVA researchers.

The pH was measured with a VWR pH pen. The probe was inserted into each tank to take the readings. The probe was rinsed with MiliQ water and ethanol 70% and wiped with Kimtech ® between readings from different tanks and bioreactors. While aiming to maintain a pH of around 7.5 for optimum nitrification, if the pH levels decreased or were below 7.0, sodium bicarbonate ($NaHCO_3$) was added to prevent the pH value from going lower than 6.5 and compensate for alkalinity loss caused by nitrification.

The salinity and temperature were measured with a multiprobe handheld device (WTW Multi 3620 IDS, Xylem, USA) installed with a salinity (Tetracon 925). Similar to the pH meter, the probe was inserted into the tank/bioreactor to measure the salinity levels and temperature. The probe was rinsed with MiliQ water and ethanol 70% between readings from tanks/bioreactors. DO was also measured using FDO 925 optical oxygen IDS sensor.

The nitrogen compounds were controlled by using test kits from Merck – Sigma-Aldrich (Darmstadt, Germany) - Spectroquant ammonium (1.14752.0001), nitrite (1.14776.0002), nitrate (1.09713.0002), and nitrate in sea water (1.14942.0001). These kits were respectively based on International Standard Organization (Eaton *et al*., 2012; ISO, 1984a; ISO, 1984b). The amount of measured nitrogen compounds was evaluated using NOVA 60A Spectroquant® from Merck – Sigma-Aldrich (Darmstadt, Germany) (Eaton *et al*., 2012; ISO, 1984a; ISO, 1984b; ISO,1986). These parameters were controlled in both the tanks and bioreactors.

Alkalinity (determined by titration using Mquant Alkalinity test kit (1.11109.0001) (Merck – Sigma–Aldrich, Darmstadt, Germany) and expressed as mg/L as $CaCO_3$), nitrite and nitrate measurements were taken every day. Consistently after sampling, the displaced volume was immediately replaced with a solution containing TAN and HCO_3^-, giving a final concentration of 3 mg of TAN per liter and 1.3 mmol/L HCO_3^- to each bioreactor. This solution was prepared using NH_4Cl (CAS no. 12125-02-9) and $NaHCO_3$ (CAS no.

144-55-8) and was added to compensate for nutrient loss and maintain water chemistry stability after each sampling.

Throughout all the procedures, the reagents and sampling were handled carefully, respecting the safety protocols of the laboratory.

2.3. Sample processing

The sample processing was performed according to Figure 7. Swab samples from the tank walls of each RAS were collected using sterile techniques and placed into 2 mL Eppendorf tubes containing NucliSENS Lysis Buffer. These samples were immediately frozen at -20 ºC to preserve nucleic acids and prevent degradation during storage.

Biofilter/Biochips samples were shaken at 250 rpm for 1 hour at room temperature on 50 mL of Phosphate-Buffered Saline (PBS) +10% Fetal Bovine Serum (FBS) to extract any virus remaining in the biofilter.

Afterwards, biofilter samples were processed by filtering the sample through a 0.45 µm filter using a vacuum pump system (SHENCHEN V6-3L by Baoding Shenchen Precision Pump Co., Ltd.), which efficiently trapped viral particles. The water samples were directly filtered, which was critical to concentrate the viral material (Weli *et al*., 2025).

Each filter was then cut into two equal pieces which were placed into separate Petri dishes: one containing L15 medium (supplemented with 10% FBS (Sigma-Aldrich), 10 µg/mL gentamicin (Sigma-Aldrich), and 2 mM L-glutamine) for virus cultivation and the other one was treated with NucliSENS lysis buffer (LB) for molecular analysis. The samples were shaken (350 rpm for 30 min) to facilitate the extraction of the particles from the filter into the medium and stored in Eppendorf tubes for further analysis. The L15 medium that extracted the possible virus from the filter was filtered through 0.45 µm filter using a 2 mL syringe, which were washed beforehand with L15 medium to prevent the virus from binding to the dry filter matrix, ensuring that the viral material was not captured and allowing a cleaner sample to be extracted. The samples were stored at -20°C to preserve the integrity of the viral content until further analysis.

In order to check if there was any infectious virus in the sample, L15 extracts were cultured in Atlantic salmon kidney (ASK) II cell plates, with 200 µL of the extract added to each well. Each sample extract had replicates (3 or 4 cell culture plate wells) and 10 µL of virus stock (1.0×10^6 $TCID_{50}$ /mL) was placed in the wells for positive control, alongside 190 µL of L15 medium with gentamicin and L-glutamine to prevent bacterial

contamination and to support cell growth, respectively. The plates were then sealed to avoid desiccation and were incubated for 9 days at 12°C, after which the wells were emptied and 200 µL of 80% acetone was added to fix cells for 10 min. Afterwards, the wells were emptied again, and the plate was allowed to dry in the hood for 20 min. Both the plate and the lysis buffer samples were stored at -20°C and were analysed, thus providing crucial information to determine the presence and virus load of ISAV in different environmental compartments.

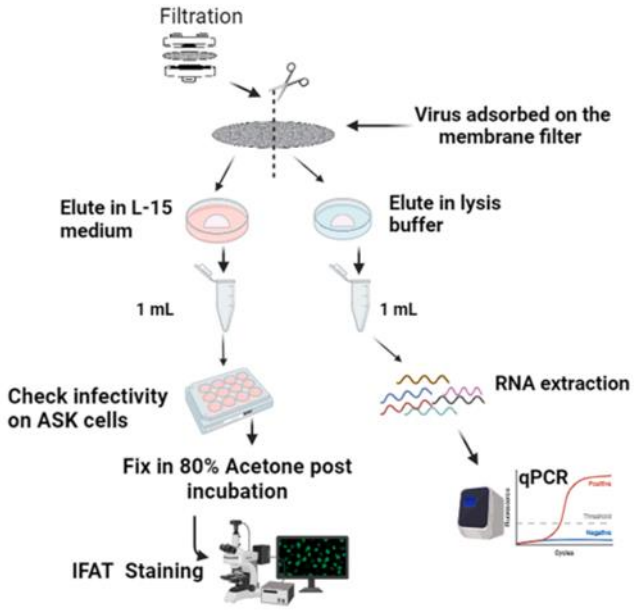


Figure 7 - Graphic representation of the processing of the biofilter and water samples (Weli *et al*., 2025)

Immunofluorescence Antibody Test (IFAT) Staining was then performed by the project group. The ASK II cells were incubated with an ISAV-specific mouse monoclonal antibody (p10), diluted 1:50 in PBS with KCl (pH 7.2) after fixation and drying. After primary antibody incubation, which was conducted on a shaker for 1 hour to allow the antibody to distribute evenly, the cells were washed three times in PBS containing 0.1% Tween-20 for 1 min per wash and with PBS containing KCl (pH 7.2). Afterwards, the cells were treated with Alexa Fluor 488 goat anti-mouse IgG (H+L) antibody (Invitrogen) (1:200) for 1 hour on a shaker in the dark, in order to inhibit photobleaching. Following secondary antibody incubation, the cells were again rinsed three times with PBS with 0.1% Tween-20 and 50 µL of PBS with KCl (pH 7.2) was added to all wells. The stained cells were analysed using a Zeiss fluorescence microscope, and image-based analysis was performed to visualize and quantify the ISAV-specific staining.

2.4. Real-Time qPCR

RNA was isolated with an input of 500 µL of each sample separately, both from the RAS and bioreactors:

- NucliSENS Lysis Buffer eluate water samples
- NucliSENS Lysis Buffer eluate bio-filter samples
- Swab samples in NucliSENS Lysis Buffer

The RNA isolation and RT-qPCR was carried out by Engineers at NVI, Ås to detect and quantify ISAV using the AriaMx RT-qPCR system (Agilent Genomics, USA). The ISAV-M-segment 8-specific primers

- F-primer: 5'-CTACACAGCAGGATGCAGATGT-3'
- R-primer: 5'-CAGGATGCCGGAAGTCGAT-3'
- Probe: 5'- 6 FAM-CATCGTCGCTGCAGTTC-MGBNFQ-3') (Snow *et al*., 2006)

were used to generate a 105-bp product.

RNA was reverse-transcribed to cDNA according to the instructions of the manufacturer, by using the High-Capacity RNA-to-cDNA kit (Applied Biosystems Inc., U.S.). RT-qPCR was carried out in 96-well plates using RNAse-free water and no-RT as negative controls. The conditions were as follows: 95 °C for 10 minutes for enzyme activation, followed by 40 cycles of 95 °C for 15 seconds (denaturation) and 60 °C for 60 seconds (annealing and extension), with fluorescence acquisition at the end of each cycle. All RT-qPCR analysis were performed using 2–3 experimental replicates (cell culture wells). Cycle threshold (Ct) values were obtained, being considered positive below 40.00 for fish organs and between 20.00 and 40.00 for the rest of the samples, since environmental samples are more diluted for ISAV. Below or above that number, the results were considered artefacts, and amplification curve each sample in the range below Ct 20, and the ones between Ct 35-40 was checked to make sure that none of the false positives were included, or real positive samples were excluded (Rimstad & Markussen, 2020). For fish organs, background noise is not expected, and all values up to Ct 40 were considered as real amplification.

2.5. Histopathology and Immunohistochemistry

This study evaluated the confirmation of infection in the organs that are the primary target of ISAV by identifying the primary organs or tissues affected, including the gills, kidney, liver, and heart. The presence of ISAV was confirmed using specific antibodies and compared with results from RT-qPCR. Pathological lesions, such as necrosis, inflammation, and cellular damage, were associated with the presence of the virus. The slides were fixed in formalin and trimmed appropriately to fit properly into embedding cassettes, which were subsequently dehydrated by exposure to a gradient series of ethanol and xylene to dissolve ethanol and to allow efficient paraffin permeation of the tissues. After dehydration and clearing, tissues were further infiltrated under vacuum with molten paraffin wax to guarantee full infiltration, after which, the tissues were embedded in paraffin blocks at an embedding station. Correct orientation of the tissue during embedding was critical to enable the desired plane of sectioning to be achieved during microtomy. The paraffin blocks were then cooled to solidification in order to be cut with a microtome.

With the help of the engineers at NVI, the tissues were sectioned to 2 µm, hematoxylin and eosin stained (H&E), and used for initial evaluation. The sections were deparaffinized with xylene and rehydrated in graded alcohols to remove paraffin and prepare the tissue for aqueous reagents. Endogenous peroxidase activity was inhibited by incubation with hydrogen peroxide to prevent nonspecific background staining. Antigen retrieval was then performed by boiling the sections in a target retrieval buffer to unmask epitopes that may have been altered during formalin fixation. Finally, protease digestion at 40 °C was applied to further expose antigenic sites and enhance antibody binding efficiency.

Immunohistochemistry (IHC) was executed by the engineers at NVI. The specific antigens of ISAV were identified with an antigen-antibody reaction, visualized by enzymatic staining and enzymatic digestion with trypsin was used to uncover antigens hidden during fixation. The slides were treated with a solution of Tris buffer containing calcium chloride and trypsin at 37°C and were cooled with ice cold Tris buffer to stop enzyme reaction. To avoid nonspecific binding, the slides were blocked with 5% dry milk in Tris buffer.

The primary antibody incubation included dilution of specific antibodies of ISAV (pooled serum) in 2.5% BSA and overnight incubation at 4°C in a humid chamber. Biotionylated secondary antibodies: goat anti-rabbit Ig, were then applied, and the detection system

was finalised with streptavidin-alkaline phosphatase. Visualization was achieved using ImmPACT® Vector® Red, with incubation times based on kit recommendations. Tissue sections were counterstained with hematoxylin and rinsed thoroughly.

Finally, slides were prepared for microscopic analysis. ImmPACT® Vector® Red-stained slides were dehydrated and mounted using xylene-based media. Positive controls, consisting of tissues known to express ISAV, and negative controls, processed without the primary antibody or with irrelevant antibodies, verified staining specificity and accuracy. The sections were assessed by the Researchers in the project group and trained me to be able to do the assessment as part of this thesis.

2.6. Statistical analysis and Bioinformatics

The statistical analyses were performed on SPSS 30.0 software package for Windows (IBM® SPSS® Statistics, New York, USA). To test the normality and homogeneity of the results, the Shapiro-Wilk and Levene test were used respectively. The data from the RAS systems were analysed by a randomized complete block design Two-way ANOVA. The sample type and days were considered fixed factors. In case of significant interactions, one-way ANOVA was performed for each factor, and a significance level of 0.05 was used to reject the null hypothesis. For the bioreactors, a Three-way ANOVA was performed, where the bioreactor replicates were considered a Random factor and Sample type, Day and treatments were considered fixed factors. In case of significant interaction between three factors, Two-way ANOVAs were performed. Differences among means were assessed using Tukey's multiple comparison test.

3. Results

3.1. Water quality

Water quality parameters were monitored closely during the trials by Marineholmen RASLab personnel and NIVA researchers. During Phases I and II, water salinity remained around 15 ppt, with temperatures ranging from 12 to 13 °C. The pH levels fluctuated between 7 and 8, and DO levels ranged from 93% to 99% (Tables 2 and 3). Alkalinity was slightly higher in Phase I compared to Phase II. Nitrogen compound concentrations were comparable across the three RAS systems (Figure 8). TAN levels peaked on day -33, while nitrate concentrations were elevated from day -29 to -15, gradually declining thereafter until the end of the experiment. Similarly, nitrite levels were highest on day -35 and decreased steadily throughout the trial.

In the bioreactors the water salinity also remained around 15 ppt, with temperatures of 15°C. The pH remained stable at approximately 7.7, and DO levels were consistently maintained between 97% and 98% (Table 4). The TAN spiked in the ozone-treated group, peaking on Day 10. In contrast, TAN levels remained low in both control and water exchange treatments throughout the monitoring period. After Day 10, nitrite accumulated in the control and water exchange groups, peaking on Day 17. Nitrate concentrations increased steadily in all treatments. However, after Day 10, nitrate levels continued to rise in the control and water exchange groups, reaching their highest levels by Day 23.

Table 2. Salinity, water temperature, pH and DO of the three RAS during Phase I. Values are presented as mean ± SD.

Tanks	Salinity(‰)	Water temperature(°C)	pH	DO(%)	Alkalinity(mg/L as $CaCO_3$)
S1	15.6 ± 0.4	12.5 ± 0.5	7.9 ± 0.2	95.5 ± 4.6	98.6 ± 22.3
S2	15.1 ± 0.3	12.2 ± 0.7	7.8 ± 0.3	95.1 ± 3.7	86.7 ± 19.9
S3	15.3 ± 0.3	12.6 ± 0.6	7.8 ± 0.2	93.6 ± 6.1	82.1 ± 23.6

Table 3. Salinity, water temperature, pH and DO of the three main fish Tanks during Phase II. Values are presented as mean ± SD.

Tanks	Salinity(‰)	Water temperature(°C)	pH	DO(%)	Alkalinity(mg/L as $CaCO_3$)
S1	15.0 ± 0.1	12.4 ± 0.6	7.2 ± 0.2	98.5 ± 1.1	75.1 ± 24.5
S2	15.0 ± 0.1	12.4 ± 0.3	7.2 ±0.2	98.5 ± 1.3	57.7 ± 21.7
S3	14.9 ± 0.4	13.0 ± 0.9	7.3 ± 0.2	98.5 ± 2.1	60.0 ± 17.6

Figure 8 - Total Ammonia Nitrogen (TAN), Nitrite (NO_2^--N), and Nitrate (NO_3^--N) concentrations for samples from S1, S2, and S3 over the experimental days. Values are presented as means.

Table 4. Salinity, water temperature, pH, and DO of the three bioreactors corresponding to each treatment. Values are presented as mean ± SD.

Treatments	Salinity(‰)	Water temperature(°C)	pH	DO(%)	Alkalinity(mg/L as $CaCO_3$)
Control	14.8 ± 0.1	14.9 ± 0.4	7.7 ± 0.3	97.6 ± 2.0	65.0 ± 0.9
Water Exchange	14.6 ± 0.6	15.2 ± 0.4	7.6 ± 0.3	98.2 ± 1.9	65.0 ± 0.9
Ozone	14.5 ± 0.3	15.3 ± 0.5	7.7 ± 0.3	97.5 ± 2.0	65.0 ± 0.9

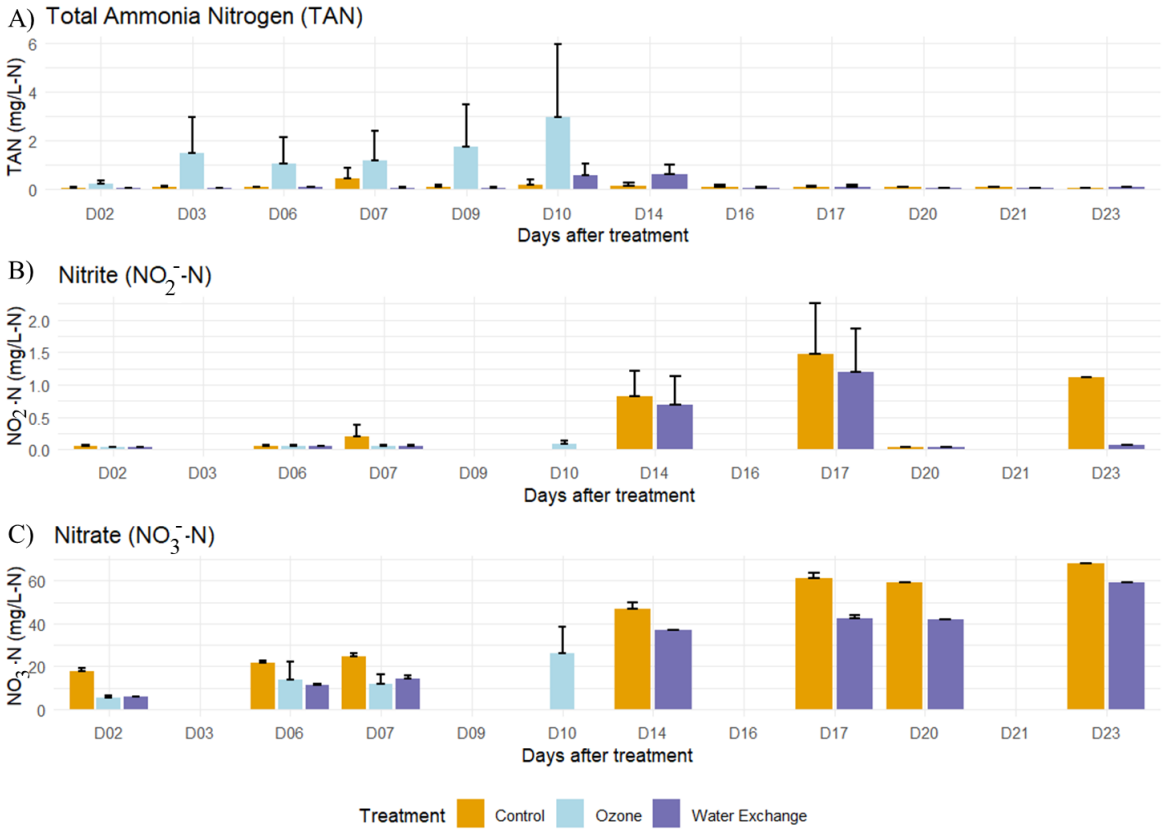


Figure 9 - Average measurements of Total Ammonia Nitrogen (TAN) (A)), Nitrite (NO_2^--N) (B)), and Nitrate (NO_3^--N) (C)) concentrations across the bioreactors of the three treatment groups: Control, Water Exchange, and Ozone over the experimental days. Values are presented as mean with ± SD.

3.2. Real-Time qPCR Phase I and II

3.2.1. RAS

Viral RNA levels in water, biofilter, and swab samples increased throughout the challenge period, peaking on the final day when phase I of the trial was terminated (Figure 10). Samples collected on Day -14 tested negative, confirming that the RAS were free of ISAV.

As observed across all RAS, Ct values were the lowest on Day 00, the same day of the death of all fish due to viral infection, which was confirmed by positive Rt-qPCR results (Figure 10). After that, the Ct value increased over time, since there was no host for the virus to replicate. When the naïve fish were introduced on days 3 to S1, 7 to S2, and 10 to S3, the fish were not reinfected, as indicated by the negative RT-qPCR results.

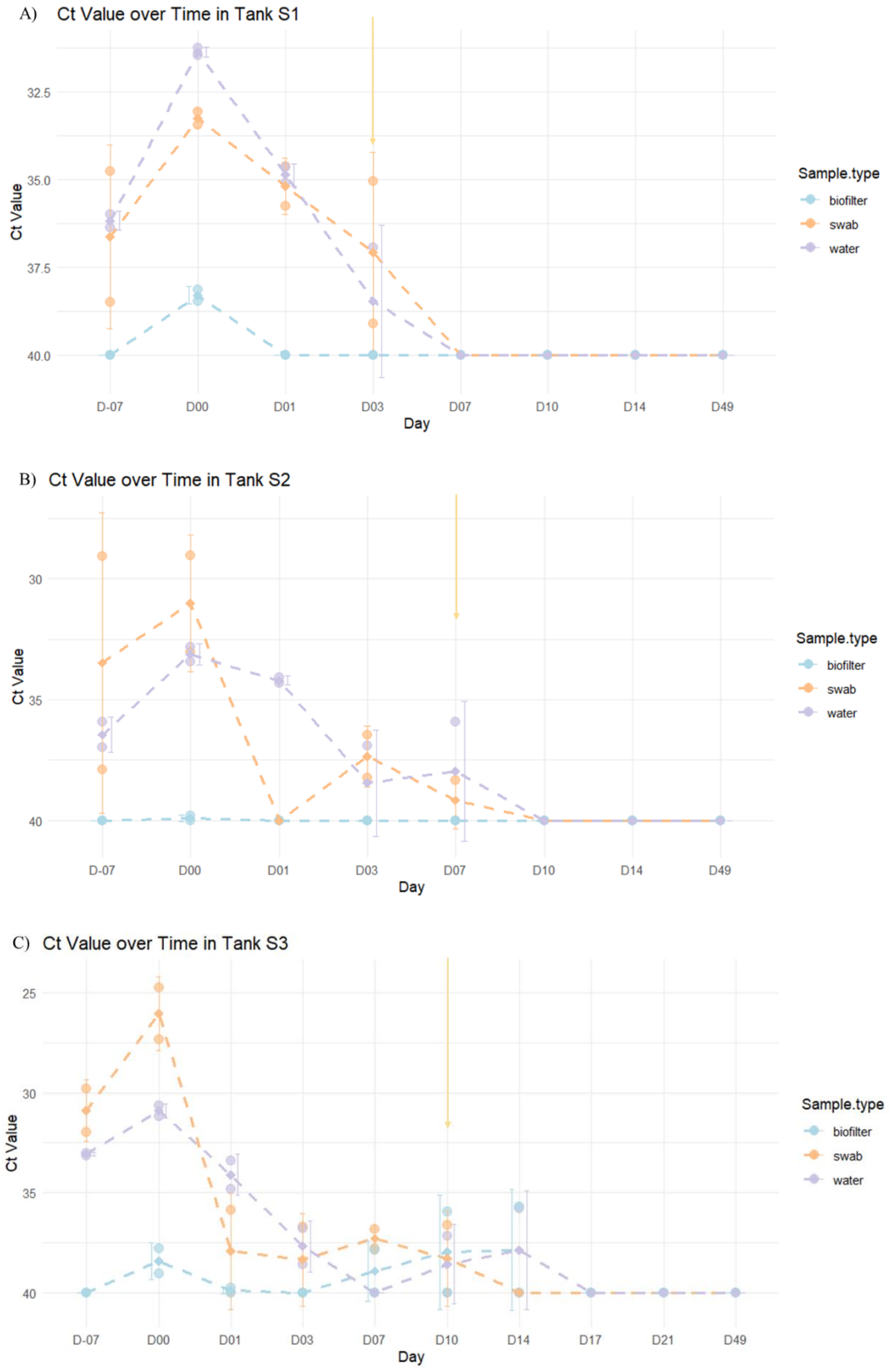
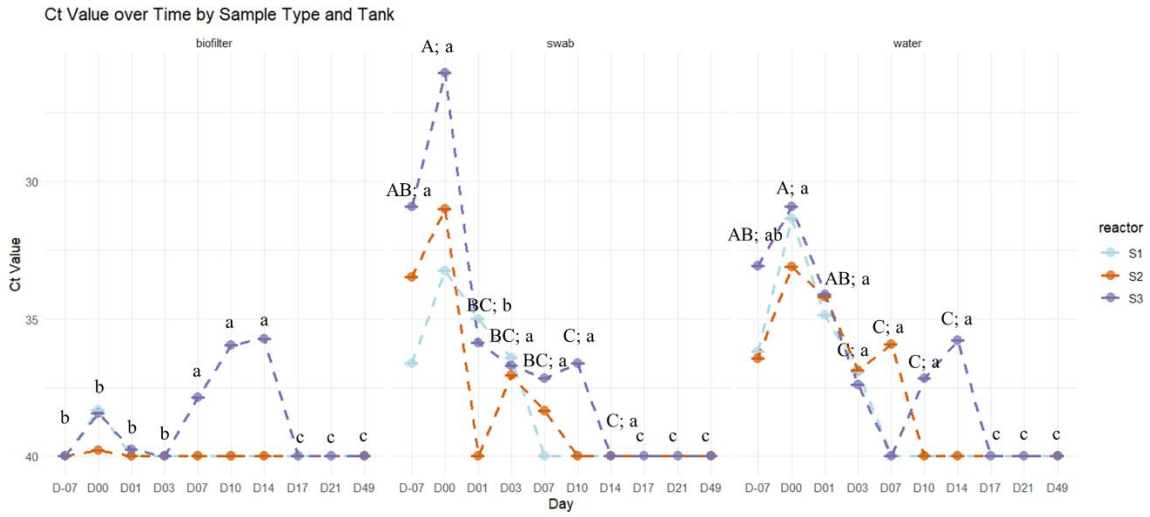


Figure 10 - Ct values of samples collected from each system, S1(A), S2(B) and S3(C), over time, according to the type of samples. Ct= 40 represents a negative result. Lower Ct values indicate higher viral loads. The arrow represents the introduction of the naïve fish. Values are presented as mean with ± SD. N = 3.

Figure 11 shows that the number of days and sample type impacted the virus presence.

The presence of the virus in the biofilter was not affected by the Day (Figure 11). Swab samples showed the highest virus concentration on day 0 and a decreased virus concentration after that day was noticed. A similar pattern is observed for water samples - Day 00 is significantly different from days -07, 03, 07, 10, 14, 17, 21, and 49. The sample type effect for each day showed that the biofilter samples were significantly different from water on days 00, 01 and 03, and from swab on days -07, 00 and 03. Water and swab samples were only different from each other on day 01. Randomized complete block design, Two-way ANOVA showed no RAS impact on Ct values.



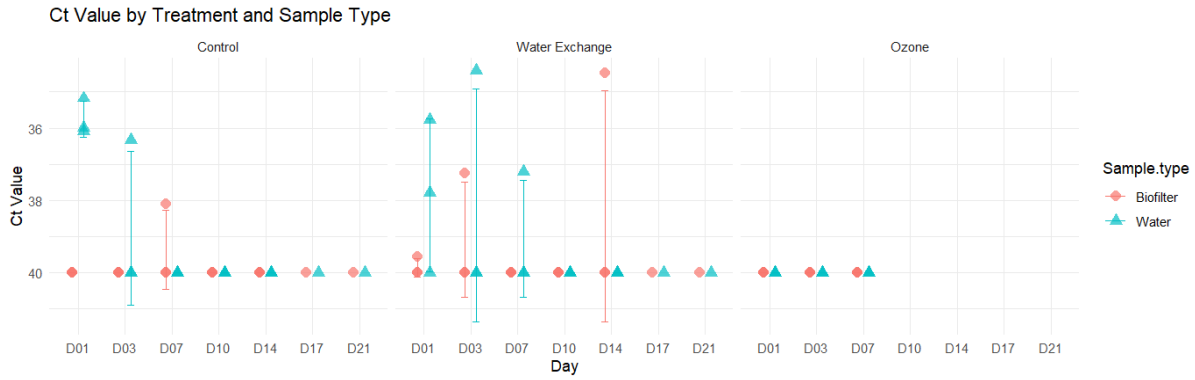
Two-way ANOVA

Factors	Significance (p value)
Days	<0.001
Sample type	0.003
Tanks	NS
Days vs Sample type	<0.001
Days vs Tanks	NS
Sample type vs Tanks	NS

Figure 11 - Ct values of the type of samples over time, from tanks S1, S2, and S3. 40 represents a negative result. Values are presented as mean with ± SD. Two-way ANOVA: NS: non-significant ($p \geq 0.05$). If the interaction was significant, one-way ANOVA was performed for each factor. The uppercase letters represent the effect of the Day for each sample type; the lower-case letters represent the effect of the sample type for each day. The absence of a letter means that there was no difference (biofilter, in this case). N= 3.

3.2.2. Bioreactors

The Three-way ANOVA represented in Table 3, showed that the treatment, days and sample type impacted the Ct values. However, an interaction between Treatment, Days and Sample Type was found (Figure 12). Treatment and days did not impact the virus concentration in Biofilter samples (most values were negative, with a few sporadic positive values), these parameters impacted the results observed for Water samples, which is shown by a decrease of the Ct values of the water samples overtime. The Tukey tests confirmed that there was no difference between the Control and Water Exchange treatments and between Water Exchange and Ozone, but Ozone decreased the Virus concentration compared to the control group (Figure 12). Further, the ISAV concentration decreased from day one to 7. In the Ozone treatment, no differences were observed since the Ct values were 40 or above since Day 01, meaning the virus was absent. Sample Type impacted the ability to detect ISAV, being the water sample being more effective for that purpose than the biofilter.



Three-way ANOVA

Factors	Significance (p value)
Treatment	0.009
Days	0.021
Sample type	<0.001
Treatment vs Sample type	0.026
Days vs Sample type	0.009
Treatment vs Days vs Sample type	0.049

Two-way ANOVA - Water samples		Tukey Test					
Treatment	0.018	Treatment			Days		
Days	0.017	Control	WE	Ozone	1	3	7
Treatment vs Day	NS	a	ab	b	a	ab	b

Two-way ANOVA - Biofilter samples - Treatment: NS; Days: NS; Interaction: NS

Two-way ANOVA - Sample type vs Treatment - Sample type: 0.003; Treatment: NS; Interaction: NS

Figure 12 - CT values of the bioreactors subjected to the different treatments during the trial. 40 represents a negative result. Lower Ct values indicate higher viral loads. Values are presented as mean of each replicate with ± SD. Three-way ANOVA of the effect of the Treatment, Days, Sample Type, and Tanks. Two-way ANOVA of the effect of the Days and treatments on the Water Samples. Different letters indicate differences between treatments and days. Two-way ANOVA of the effect of the Treatments and Days on the Biofilter Samples. Two-way ANOVA of the effect of the Sample type and Treatment. N = 9.

3.3. ASK II Plates and IFAT Staining

In this study, it was not possible to detect infectious virus in any of the samples (both from bioreactors and RAS) using the cell culture technique except the positive controls, as presented in Figure 13a. The dark colour of some wells in Figure 13b, can be due to the presence of cytotoxic compounds.

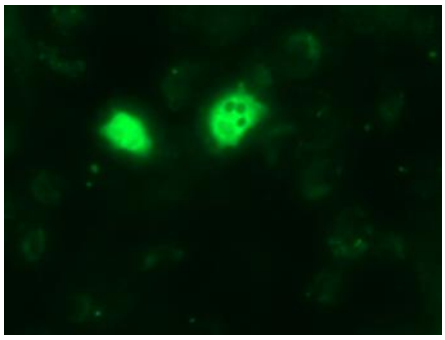


Figure 13a - Representative image of ISAV-infected cells stained by IFAT, serving as positive control and illustrating the expected fluorescence pattern.

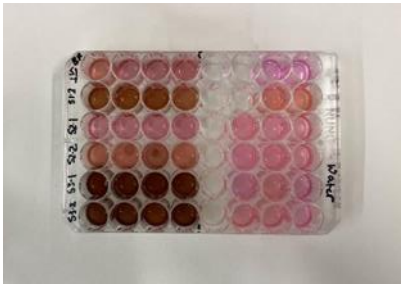


Figure 13b - Picture of ASK II cell plate inoculated with Biofilter and water samples.

3.4. Histopathology and Immunohistochemistry

Fish did not show some of the typical visual clinical signs of ISAV infection, such as pale gills. However, internal and external signs of infection were verified such as eye haemorrhage, ascites fluid, internal haemorrhage and lethargy.

The hemotoxin and eosin-stained samples did not show any noticeable changes. Although the pancreas showed some variability between samples, this organ is very variable even in the same individual, making the conclusion unclear.

The immunohistochemistry (IHC) analysis of the heart tissue revealed strong red chromogenic signals primarily in the endothelial cells of blood vessels and cardiomyocytes, which is consistent with ISAV antigen localization, confirming infection

(Figure 14). In the kidney tissue of Atlantic salmon, positive immunohistochemical staining appears as red chromogenic deposits, indicating the presence of viral antigens (Figure 15). Occasional melanomacrophages were also observed in the kidney (Figure 15).

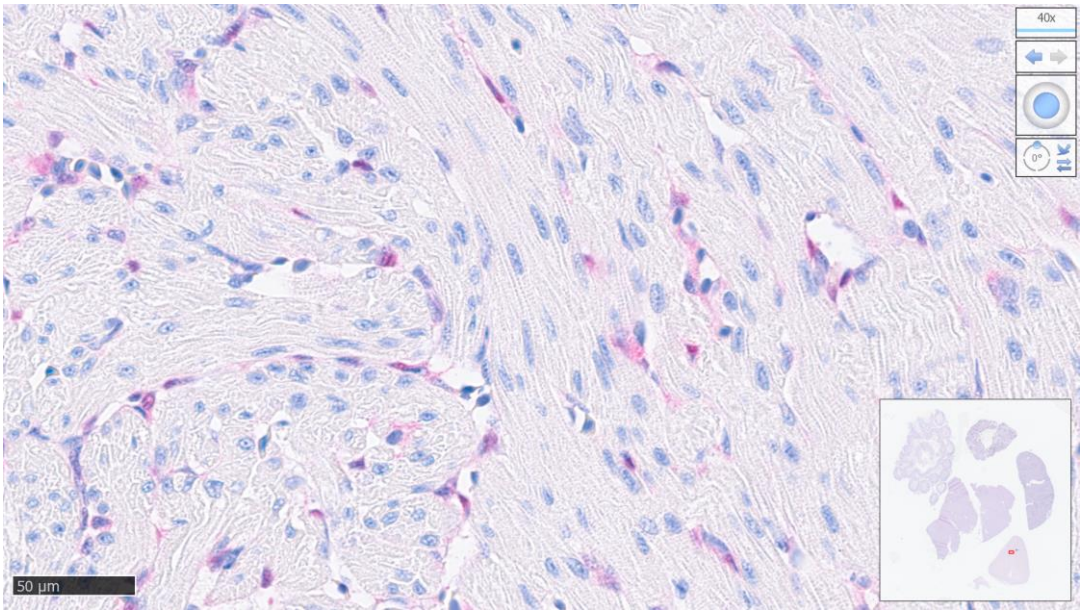


Figure 14 - Immunohistochemical detection of ISAV in slides from formalin fixed paraffin embedded heart from experimentally challenged Atlantic salmon. Positive endothelial cells are visualized with red staining of cytoplasm and nucleus. The images are zoomed x40.

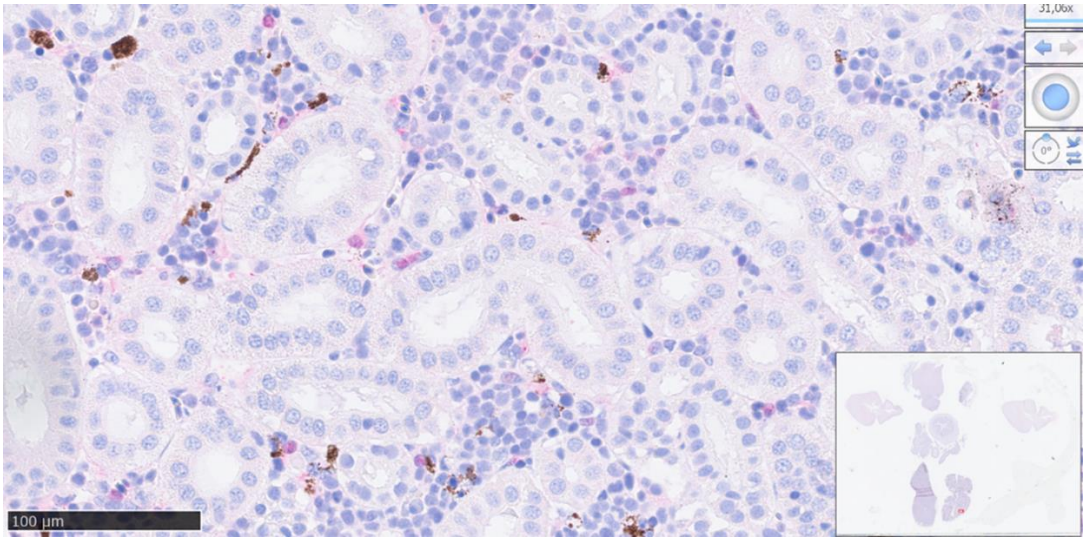


Figure 15 - Immunohistochemical detection of ISAV in slides from formalin fixed paraffin embedded mid kidney from experimentally challenged Atlantic salmon. Positive reaction in endothelial cells are visualized with red staining of cytoplasm and nucleus. The images are zoomed x31.

4. Discussion

4.1. Water quality

Based on nitrogen compound concentrations observed around Day -27, just prior to the initial stocking of fish, it can be concluded that the biofilter in every RAS system had attained a functionally mature condition. Total ammonia nitrogen (TAN) and nitrite (NO_2^--N) concentrations were continuously low in S1, S2, and S3, whereas nitrate (NO_3^--N) was on a rising trend, indicating continuous nitrification. These profiles were consistent with those of an established biofilter, with the capacity to effectively nitrify toxic nitrogenous waste products. In fact, Timmons *et al*. (2018) state that ammonia concentrations below 1 mg/L and nitrite concentrations below 0.5 mg/L are indicative of a stable biofiltration system, favourable for fish stocking. Furthermore, Schreier *et al*. (2010) and Van Rijn (2013) note that the concomitant accumulation of nitrate, along with low levels of ammonia and nitrite, is an indication of a complete nitrifying population of bacteria. The water quality noted Day -27 therefore attests to the fact that the system had developed sufficient nitrifying capacity for the safe stocking of fish with no risk of acute ammonia or nitrite toxicity.

When comparing alkalinity during Phase I and Phase II, it is possible to observe that its concentration decreased after the water exchange and introduction of new fish, which means that the water renewal diluted the concentration of chemical buffers. Moreover, when comparing phases I and II, pH lowered from ~7.8 to ~7.2, which might be related to the reduction of alkalinity (lower buffering capacity). It may also be an effect of the introduction of new water (possibly more acidic) or a more intense bacterial activity (nitrification). DO increased after the water exchange, as new water typically has a higher O_2 saturation. In phase II, the biofilter seems to have more efficiency than in phase I, which meant more nitrifying bacteria and a higher and more efficient conversion of ammonia to nitrate. There were also fewer fish in each tank, which meant less ammonia.

In the bioreactors that were treated with ozone, there were markedly elevated levels of TAN. As expected, the NO_2^--N and NO_3^--N concentrations remained similar until Day 10. This pattern is consistent with the intense oxidative and antimicrobial properties of ozone, which can disrupt microbial populations essential for nitrification, particularly ammonia-oxidizing bacteria and nitrite-oxidizing bacteria possibly explaining the higher TAN concentration during the first 10 days (Powell & Scolding, 2018). Moreover, nitrate concentration is also affected by the fact that 100% of the water was exchanged in the ozonated and Water Exchange treatments, instantly removing nitrate that has

accumulated in the system and replacing it with clean water that had much lower nitrate levels, providing an immediate decrease in measured nitrate (Timmons & Ebeling, 2010; Yusoff *et al*., 2024). The reduced conversion of ammonia into nitrite and nitrate in the ozonated reactor thus likely reflects a suppressed nitrifying biofilm or compromised microbial filter efficiency due to oxidative stress (Schreier *et al*., 2010). However, a study performed by Teitge *et al*. (2020) demonstrated that ozone disinfection in brackish aquaculture systems for Pacific white shrimp was more effective than UV irradiation in improving water quality. Specifically, ozone treatment, where the redox potential in the water was adjusted to a stable level of 350 mV, stabilized the microbial composition in the water and on biofilms of tank surfaces and shrimp carapaces, prevented an increase of nitrite, and accelerated the degradation of nitrate in the water, which is consistent with the findings observed in this study. In the current study, the redox potential was targeted as 700 mV in the water, which means higher ozone level concentrations and a stronger disinfection that may be effective in controlling a broader spectrum of pathogens. Thus, it can be argued that lower ozone concentrations in water have a milder impact on the microbiome and may contribute to improved water quality. This is in accordance with the outcomes of Spiliotopoulou *et al*. (2018) and Lazado and Good (2021), who highlighted the sanitizing potential of ozone against pathogens in RAS. The increase of TAN under ozonation could also be induced due to enhanced protein and organic matter dissimulations as ozone reacts with organic matter, forming nitrogenous byproducts (Aguilar-Alarcón *et al*., 2022). Additionally, ozone may also transiently oxidize the ammonia into more labile intermediates that are not necessarily retained as nitrite or nitrate, especially if the microbial conversion steps are impaired.

Notably, ISAV was not detected in the ozonated group. This may be due to the fact that O_3 can inactivate viruses in water by oxidative damage of viral proteins, lipids, and nucleic acids, being particularly effective against enveloped viruses, such as ISAV (Zimmer & Slawson, 2002; Shinriki *et al*., 1978). Furthermore, the microbial suppression triggered by ozone may interfere with virus-microbiome interactions, which may sometimes be a factor in viral persistence or transmission (Derome, 2019).

Bioreactors maintained high DO levels, suggesting proper aeration. Ozone did not significantly affect DO, which is expected, as ozone is an unstable gas that does not directly compete with O_2. The uniform alkalinity across bioreactors (65 mg/L) reflects controlled buffering, which stabilized carbonate levels across treatments (Summerfelt, 2003; Ebeling *et al*., 2006; Aguilar-Alarcón *et al*., 2022).

Although no studies directly examining the effects of TAN, NO_2^--N and NO_3^--N have been conducted on ISAV, it is possible to draw parallels from research on other aquatic pathogens. It has been shown that Atlantic salmon post-smolts subjected to elevated levels of ammonia respond by exhibiting signs of physiological stress. For example, exposure to water with ammonia levels ranging from 0.07 to 12.84 mg/L, showed detectable levels of the highly toxic unionized ammonia (NH_3), which can lead to stress and potential immunosuppression, making the fish more vulnerable to pathogens (Knoph & Olsen, 1994; Preena *et al.,* 2021). Furthermore, prolonged exposure to nitrite can cause methemoglobinemia, reducing the ability of the fish to transport oxygen and their immune response, which could increase the susceptibility to infection from pathogens such as ISAV. Alternatively, nitrate seems to have a lower negative impact on Atlantic salmon, with research supporting that there is no harm to the health and growth performance of Atlantic salmon under chronic exposure to nitrate up to 100 mg/L in freshwater RAS (Davidson *et al*., 2017). However, monitoring its levels remains important to ensure overall water quality. The recorded values in this experiment remained within ranges that did not impact the fish, ruling them out as factors influencing disease progression.

It has been well-documented that ISAV infectivity can be compromised by environmental stressors, particularly temperature fluctuations. However, in this experiment, the incubation temperature was steadily maintained at 12°C. Although 15°C is considered optimal for ISAV replication, the lower temperature allows the viral propagation to slow down, thereby reducing the degradation of older viral particles and preserving their detectability and stability (Christiansen *et al*., 2017; Kibenge *et al.,* 2000).

4.2. Variability in sampling

According to the World Organisation for Animal Health (WOAH) (2009), a definitive diagnosis of ISAV requires at least two independent diagnostic methods to ensure reliability and minimize false positives. In this study, by applying both molecular and histopathological approaches, it was possible to confirm the presence of ISAV in samples from infected fish, aligning with internationally accepted diagnostic criteria. In this experiment, the molecular detection strategy recommended by WOAH for ISAV surveillance was supported by performing RT-qPCR, targeting conserved regions of the ISAV genome, confirming the presence of viral RNA in affected fish. This approach is consistent with recent findings by Eckstrand *et al*. (2024) and Mugimba *et al*. (2021), who

emphasized the value of combining multiple diagnostic tools to improve the detection and characterization of ISAV in experimentally infected fish. Before the confirmation by RT-qPCR results, the infected fish died and exhibited visual clinical signs of the disease (Rimstad & Mjaaland, 2002).

Biofilter results, both positive and negative, could be attributed to the dependency on obtaining a biochip that successfully carries the virus for a positive outcome. Sampling was not discontinued until two consecutive negative results were obtained, in order to avoid “false” negatives, as the probability of obtaining two consecutive negative results without detecting the virus if the biochips are ISAV positive is low.

The diverse distribution of microorganisms in the biofilm makes disease detection, e.g., ISAV, in Moving Bed Biofilters (MBBs) difficult, not due to spatial heterogeneity, but by experimental constraints to sample from a dynamic, well-mixed environment. Lee *et al.* (2016) used metagenomic analysis in marine biofilters and found significant differences in bacterial communities depending on the type of filter and operating conditions, such as salinity. Studies in RAS have shown that the use of oxidants such as peracetic and ozone significantly reduces both ammonia and nitrite oxidation in biofilms, confirming that nitrification is impaired after intense treatments. In addition, stronger treatments slow down biofilm recolonization, as observed when microbial recovery was dose dependent (Lazado & Good, 2021; Qi *et al*. 2025). Norley & Love (2020) demonstrated a reduction in bacterial load of up to 99.8% with 120 ppm Virkon® Aquatic and the application of hydrated lime causes bactericidal effects due to the increase in pH. Similar dose-dependent effects of disinfectants on microbial surface contamination were also reported in hospital settings (Di Martino *et al*., 2021). Del Duca *et al*. (2019) and Huang *et al*. (2016) have shown that biofilms and replicated biofilters have a spatially heterogeneous distribution of microorganisms, including potential pathogens. Thus, sampling only a few media may not adequately reflect the total microbial diversity and abundance, compromising the accurate detection of pathogens. Fernandes *et al.* (2017) also report that Fixed Bed Biofilters (FBBs) create stable microenvironments that could act as microbial reservoirs and hence, possibly increase the rates of detection in targeted samples. However, such systems are strongly spatially and temporally heterogeneous, meaning that individual samples provide limited insight into the overall microbiological patterns within the reactor. In contrast, the continuous mixing of MBBs produces a more homogeneous environment and theoretically improves the chances of detecting widely spread pathogens. The same fluidity that produces homogeneity, however, also produces inconsistent sampling, due to mobile carriers, variable biofilm thickness, and

inconstant detachment. Thus, while MBBs may have a better theoretical justification for representative sampling, both MBBs and FBBs pose unique, severe challenges to accurate pathogen detection with biochips (Blanco-Cabra *et al*., 2021).

Although one of the aims of this study was to identify where ISAV establishes in the system, a goal which was successfully achieved, biofilter samples proved to be unreliable and not significant in terms of Ct values, and therefore not informative regarding the infection dynamics. The results presented in this study show the variability in ISAV detection depending on sample type, both in the RAS and bioreactors. In fact, in the swab samples is possible to attain a higher number of positive samples when compared to water and biofilter, as swab samples often yield higher detection rates of viral RNA. This is largely due to the tendency of viruses, including ISAV, to adsorb onto organic matter, biofilms, and particulate surfaces within the system. The swabbing of surfaces such as tank walls, biofilter media, and pipe surfaces enables direct sampling of these potential viral reservoirs, where viral RNA may persist for longer periods. In contrast, viral particles in the water column are typically more diluted and subject to faster degradation due to environmental factors such as temperature fluctuations, UV exposure, and microbial enzymatic activity (Luukkonen *et al*., 2014; Silverman & Boehm, 2021; Tzafesta & Shokri, 2025).

Moreover, both swab and water samples exhibited significantly lower Ct values compared to biofilter samples across all tanks, which suggests a higher concentration of detectable viral RNA in these matrices. This phenomenon was also noticed in the bioreactors when water samples and the biofilter were compared. This could be attributed to the fact that swab and water samples tend to contain more freely circulating and recently shed viral particles. As mentioned beforehand, in RAS, viral particles excreted by infected fish can be directly sampled from the tank surface or water before degradation (García *et al*., 2015; Gregory *et al*., 2009).

Furthermore, the concentration method employed, and the volume of water filtered highly influence the efficiency of virus detection in water samples. In fact, by filtering larger volumes of water, the detection sensitivity is improved, mainly for low-concentration viral particles, but can be limited by filter clogging (Bibby & Peccia, 2013; Ikner *et al*., 2012). Different viral concentration methods, such as ultrafiltration, electronegative membrane filtration, and polyethylene glycol precipitation, vary in efficiency, and the choice of method can significantly affect detection outcomes (Cashdollar & Wymer, 2013). In this study, 2 litres were filtered from a 200-liter tank and 200 mL from a 4L biofilter, which is a relevant fraction; however, in systems with low viral loads, even this volume may be

insufficient for reliable detection. Vacuum filtration enables the retention of viral particles, either free-floating or adsorbed onto larger organic or inorganic matter by drawing water through a membrane (in this case, 0.45 µm pore size) using negative pressure, being a relatively simple and rapid technique that does not require complex equipment. This could also explain why the water samples were less reliable with lower positive values than the swab samples.

Although degradation also occurs within biofilms, which are biologically active and rich in extracellular enzymes, the structural matrix may offer partial protection from environmental stressors, allowing more detectable amounts of viral RNA to remain (Blancheton *et al*., 2013; Pietrak *et al*., 2024). This likely happens more in the biofilm of the tank walls close to the air-water interface, since these are more transient communities with a less aggressive environment (due to the frequent cleaning and handling), allowing ISAV to establish more easily in contrast with the mature, stable, and functionally specialized biofilms of the biofilter (Blancheton *et al*., 2013; Schreier *et al*., 2010). Swab sampling is therefore regarded as a more sensitive method for environmental surveillance of viral pathogens in aquaculture systems, particularly when no clinical signs are present.

In contrast, biofilters host complex biofilms composed of diverse microorganisms capable of biodegrading organic matter, including microbial pathogens (Bentzon-Tilia *et al*., 2016; Derome, 2019; Schreier *et al*., 2010). It is consequently more difficult to obtain viral RNA and DNA for nucleic acid extraction, as more specialized techniques are required, such as scraping, enzymatic digestion and sonication. In addition, microbial activity in biofilters might lead to enzymatic and/or oxidative degradation of viral RNA over time, leading to reduced amounts of amplifiable viral material and hence to higher and unreliable Ct values (Coca *et al*., 2025; Davidson *et al*., 2017). Additionally, matrix complexity and extraction efficiency can influence RT-qPCR results. Biofilter materials may contain substances that partially inhibit nucleic acid extraction or the RT-qPCR reaction itself, even when standard clean-up steps are applied. Such inhibitory effects have been reported to affect the recovery and detection efficiency of viral RNA from environmental samples (Ikner *et al*., 2012). However, in this study, variation in maturity affect establishment is not known.

4.3. Mortality and ISAV persistence

ISAV was present for 17 days in tank S1, 21 days in tank S2, and 28 days in tank S3 at a temperature of 12°C, which was lower than the 15°C typically used in similar studies. The lower temperature may also have impacted the speed at which the fish succumbed to the virus, as previously mentioned, since lower temperature can reduce the immune system of the host, depending on optimum interval of the species, thereby increasing disease progression (Bowden, 2008); however, no differences were observed between all the RAS systems. The progression of Ct values over time, particularly the increase observed from Day 01 onwards, highlights a time-dependent inactivation of ISAV, likely related to fish mortality and impossibility for the virus to replicate due to the lack of a host. Based on the Ct values that were obtained and since no treatment was performed on the tanks, it is possible to conclude that fallowing occurs naturally and relatively fast, a topic that will be further addressed ahead.

There is evidence that ISAV can survive for long periods in freshwater at low temperatures, but its viability is decreased in seawater, independent of temperature conditions (Tapia & Kocan, 2013). Rimstad and Mjaaland (2002) detected infective ISAV in seawater at 4°C for up to four months, after which ISAV suffered a reduction of three logs in titter. On the other hand, Nylund *et al*. (1994) observed that, at 6°C, ISAV was infective in seawater after 20 hours. Weli *et al*. (2025) have reported that, at 15°C, the infectivity of ISAV in seawater is reduced over time, with the ISAV-Glesvær strain remaining infective after 4 days, while the ISAV-Hønsvikgulen strain was still detected after 6 days. Field observations indicate that ISAV-related mortality rates can fluctuate with temperature changes, as mortality may decrease when temperatures rise above 15°C and increase when the temperature is lower.

The 2022 isolate ISAV-Hønsvikgulen (tested at 2.0×10^6, 4.0×10^5, 8.0×10^4, and 1.6×10^4 $TCID_{50}$/L), showed a longer infectivity compared to the 1990s ISAV-Glesvær (tested at 1.0×10^6, 2.0×10^5, and 4.0×10^4 $TCID_{50}$/L) (Weli *et al*., 2025). This may be indicative that the virus has become more resistant over time or that repeated serial passage in hosts or lab conditions may alter its characteristics. Moreover, it was confirmed that the virus survives longer with higher initial concentrations. These results imply that viral concentration and temperature are both relevant parameters in ISAV persistence. In high fish density aquaculture, such sustained infectivity with elevated transmission rates would lead to shedding of higher virus concentrations would accentuate contamination of the environment during an outbreak, mainly in RAS. The recirculation of water could permit the fixation of ISAV, even at low levels, in the biofilm

on surfaces of the tanks and possibly allow the build-up of viral load over time, increasing the exposure risk for naïve fish and making early detection and biosecurity interventions especially critical (King *et al*., 2008).

In order to mimic more closely natural marine conditions, several studies have examined the effect of parameters such as temperature, virus load, and the presence of organic matter in untreated seawater on ISAV stability and infectivity. Research executed by Rimstad and Mjaaland (2002), Vike *et al*. (2014a,b) and Weli *et al*. (2025) demonstrated that the survival of the virus is influenced both by temperature and the concentration of virus present. They found evidence of infectious virus particles up to 8 days at 5°C and 10 days at 10°C, and for 6 days at 15°C; thus, longer virus shedding periods in seawater are supported by cooler temperatures and higher viral levels. In contrast, and at higher temperatures (e.g. 15 °C), the virus degrades faster as the initial concentration decreases. More recent findings also suggest that temperature plays a significant role in influencing the immune response of *Salmo salar* to ISAV. A study by Groves *et al*. (2023) reported that the lethal effect of an ISAV infection was much greater at 10°C than at 20°C, despite elevated acute mortality being recorded at the higher temperature. The study also found that virus prevalence decreased more rapidly at 20°C than at 10°C, and that genes associated with innate immune responses, such as *mx1* and *isg15*, were upregulated at 10°C, whereas genes involved in Th1 (T helper type 1) and antigen prevailing cell responses, such as *il12rb2*, were higher at 20°C.

In the present work, a concentration of 1×10^4 $TCID_{50}$ per fish was used. This is a higher concentration than the minimum infective dose reported by Gregory *et al*. (2009), who stated that 1.0×10^1 $TCID_{50}$/mL in seawater was able to infect Atlantic salmon. This concentration is, however, not higher than the one utilized by Vike *et al.* (2014b), who IP infected fish with 0.1mL of a 3×10^6 $TCID_{50}$/mL, resulting in a total dose of 3×10^5 $TCID_{50}$ per fish.

In the current experiment, the fish died after 2 weeks of infection, suggesting that water salinity, as well as a higher virus dose, may have contributed to disease progression. As the fish tested were kept in brackish water, it is possible to assume that the virus replicated faster, which could also be the result of the adaptation of the virus to this environment, as demonstrated by Tapia *et al*. (2013) that suggested that ISAV has significantly higher survival in freshwater and brackish water than in full-strength seawater.

Finally, temporal dynamics could also explain the differences. Water and surface samples may reflect acute viral shedding events, while viral RNA accumulation in the biofilter might lag or be more diffuse over time, resulting in a weaker and less consistent signal (Coca *et al*., 2025; Vike *et al*., 2014b).

In the control reactors, ISAV was completely undetectable at Day 10, with water samples already testing negative by Day 07, suggesting that the virus persisted longer in the tanks. This supports that fallowing occurs naturally and fast but depends on the site of sampling.

4.3.1. Effect of disinfection strategies on ISAV

Days and sample type did not affect the ozone treatment in any way, as the viral RNA could not be detected since Day 01, showing a very different trend when compared to the Control and Water Exchange treatments. Ozonation is commonly used in aquaculture as a method for disinfection of water and pathogen control, as well as for inactivation of viral fish pathogens. Liltved *et al*. (2006) demonstrated that ISAV is extremely sensitive to ozone treatment, with much lower doses needed for inactivation than for other aquatic viruses. In the same study, a 90% reduction of ISAV infectivity was obtained with a concentration × time value of just 1.4 (mg s)/L, whereas much higher values were required for the Infectious Pancreatic Necrosis Virus (IPNV) at 1944 mg s/L and Atlantic Halibut Necrosis Nodavirus at 1000 mg s/L. It is possible to infer that ISAV, as an enveloped virus, is far more susceptible to oxidative damage caused by ozone exposure compared to non-enveloped viruses such as IPNV, being more susceptible to degradation of its lipid membrane and nucleic acids (Dopazo, 2020). The efficiency of ozonation in ISAV inactivation suggests that its use in RAS and seawater hatcheries may be a very effective biosecurity measure. Nevertheless, precise control of ozone dosage must be maintained to inactivate the virus without causing any damage to fish health or negatively affecting water quality.

However, there was no significant difference observed between Water Exchange and Control treatment. Since in the Water Exchange group, the freshwater water was fully replaced after 24 hours with new brackish water, it is likely that the virus was not significantly affected by the change in water salinity. Although there is no specific research on how quickly osmotic change impacts ISAV, available studies suggest, as mentioned earlier, that the virus will survive for a longer duration at lower salinity (Poulson *et al*., 2016). Nevertheless, the limited exposure time to freshwater in this case

may not have been long enough to induce any effects. In aquaculture, producers aim to manage water quality by diluting contaminants, maintaining optimal environmental conditions for fish health, and even controlling pathogen proliferation, usually replacing either totally or a portion of the water in the systems with clean water. Although water exchange can improve water quality by flushing out nutrients and organic matter, it is not always the most effective method for controlling pathogens (Devaraja *et al*., 2013) as demonstrated in the presented study.

4.3.2. Naïve fish

To assess whether fallowing occurred naturally and if the remaining viral particles could still cause infection, naïve fish were introduced to S1 at Day 03, to S2 at Day 07, and to S3 at Day 10. Although exposed to virus-contaminated water over extended periods, none of the fish yielded positive results for ISAV, which indicates that the present viral load was too low to sustain infection and the degradation of the virus and fallowing occurs naturally without the need for any specific treatment. In fact, the closed nature of RAS promotes the development of complex and stable biofilms, whose continuous microbial activity and competition can impede the persistence and infectivity of ISAV, making viral particles more likely to be degraded due to enzymatic activity and microbial antagonism and reducing their overall viability over time (Wommack & Colwell, 2000). On the other hand, in flow-through systems, there is a constant input and output of water, limiting the establishment of such complex microbiomes, thus resulting in a less aggressive microbial environment. While the ongoing dilution of viral particles may diminish the risk of infection, if attachment occurs before removal, the reduced microbial pressure may allow the virus to persist and propagate more easily than in RAS. While specific data on ISAV in flow-through systems and RAS are limited, it is reasonable to extrapolate that similar mechanisms occurred in this study and therefore could reduce the viability of ISAV over time (Attramadal *et al.,* 2012; Blancheton *et al*., 2013; Huavas *et al.,* 2024; Pietrack *et al*, 2024; Schryver & Vadstein, 2014). However, as mentioned beforehand, the presence of organic matter in aquatic environments may offer a protective matrix for viruses, potentially minimizing degradation and enhancing their persistence, even in the absence of disinfection treatments, suggesting that viral fate in RAS depends on the balance between microbial pressure and physical-chemical protection mechanisms (Gerba, 1984; Poulson *et al*., 2016). Therefore, in non-axenic systems, viral degradation or stabilization can be facilitated by microbial interactions (Robledo Gonzalez *et al.*, 2023; Suttle, 2007).

Furthermore, viral stability can be modulated by water quality through the mediation of viral aggregation and dispersion. Other investigations have found that filtration and suspended solids can also influence infectivity and persistence of viruses, such as the viral haemorrhagic septicaemia virus (VHSV) (Finstad *et al*., 2012). However, as previously suggested, ISAV was found in the tank water for a considerable time, which shows that the virus may have become non-infectious, but could still be detected for a long time.

In addition, the results in the naïve fish could be due to a possible lower virus concentration in the RAS system compared to phase I, and the immune and innate antiviral response of the fish prevents infection even if the virus is in the environment (Andreassen et al., 2017; Brown *et al*. 2019). Over the course of the trial, water quality in tanks was not a limiting factor that could have negatively affected the immune system of the fish, being ruled out as a factor influencing the infection or disease progression.

4.4. Cell culture and clinical observations

Despite positive RT-qPCR results, attempts to propagate the virus in ASK II cell cultures from environmental samples collected in this study did not result in observable cytopathic effects, failing to detect viral infectivity. Viral degradation before sample processing, which could occur due to suboptimal storage or environmental conditions, is one possible explanation for this outcome. Additionally, the concentration of infectious virus in the samples may have been too low for replication to occur in cell culture. Moreover, samples heterogeneity could have affected the assay to test for infectious virus (Dannevig *et al.*, 1995; Gjessing *et al*., 2018; Rimstad & Mjaaland, 2002). While ISAV incubation conditions were carefully controlled in this study, viral concentration in the sample may still have been too low to allow successful replication in cell culture. This challenge is particularly relevant when analysing environmental samples, such as biofilters and water, as is the case in this experiment, where viral loads are typically more diluted compared to organ tissues. Moreover, there might be some components in samples that might have been toxic to cells under incubation (Ikner *et al*., 2012). Environmental conditions can decrease viral viability prior to inoculation in cell culture. Organic material, residual disinfectants, or antimicrobial agents within the water or biofilter could have adversely impacted ISAV stability. Additionally, exposure to ozone or extreme variations in salinity could have compromised viral infectivity, though RNA was still detectable

through RT-qPCR, hence the negative findings within the ozone treatment bioreactors (Weli *et al*., 2021).

Although the initial signs of ISA may be limited to only a few weak fish exhibiting anaemia and non-specific autopsy findings, the presence of anaemia combined with histopathological findings, such as erythrophagocytosis and moderate interstitial kidney haemorrhage, is indicative of ISA. Immunohistochemistry often yields positive results in the endothelium of kidney vessels, confirming viral presence (Miller *et al*., 2025).

According to published information from infection experiments, ISAV was detected throughout the body within 5-10 days post-infection (d.p.i.), with viral loads peaking around 15 d.p.i. This is followed by a temporary decline in viral levels, after which a second rise in viral replication occurs at approximately 25 d.p.i., continuing until the terminal stage of the disease. By performing an early diagnosis, it is possible to perform a culling of infected stocks to prevent further dissemination (Mikalsen *et al*., 2001; Rimstad *et al*., 2011).

In the present study, clinical observations provided early indications of ISAV infection, as infected fish exhibited anaemia, ascites, and haemorrhaging in internal organs. However, tissue samples did not reveal significant lesions typically associated with ISAV infection when histopathologically examined. This absence of characteristic histopathological changes may be attributed to the rapid progression of the disease, leading to mortality before observable tissue alterations could develop. Similar cases have been documented in previous ISAV outbreaks and even other fish pathologies, where fish succumbed so quickly that histological lesions were minimal or absent (Gjessing *et al*., 2019; Miller *et al*., 2025; Noguera *et al*., 2019).

Pietrak *et al*. (2024) demonstrated that fish testing positive for ISAV-HPR-0 often did not develop tissue damage, likely because the infection did not persist long enough to cause lesions. For instance, in one case, smolts that tested 60% positive for ISAV-HPR-0 in early May 2021, tested completely negative just three weeks later. Similarly, a group of fish exposed to ISAV-HPR-0 positive individuals in 2016 showed no signs of infection three months later. It is possible to infer by these findings that ISAV-HPR-0 infections may last only a few weeks to a few months, clearing before significant tissue damage occurs. Additionally, ISAV-HPR-0 may persist below detection limits in some fish, only becoming detectable during stress events, such as spawning or smoltification. Thus, routine histopathological examination alone may not be a reliable indicator of ISAV

infections, particularly in cases where infections are highly transient or persist at undetectable levels.

4.5. Immunohistochemistry

IHC was conducted to further confirm the involvement of ISAV in disease progression, revealing the presence of viral antigens within lesion sites. This method yielded empirical evidence by connecting ISAV to the pathology, as well as confirming the association of the viral infection with fish mortality. Intense red chromogenic signals were observed mostly in the endothelial cells of blood vessels and cardiomyocytes, which are similar to sites of ISAV antigen localization in the heart tissue. The staining pattern was predominantly cytoplasmic and membranous and consistent with the viral protein localisation in the infected state (Falk *et al*., 1998; McBeath *et al*., 2009). Nuclear staining was also noticed in some of the infected cells, especially endothelial and myocardial nuclei, indicating active viral replication as ISAV replicates within the nucleus of the host cell (Kibenge *et al*., 2000). In the kidney, positive immunohistochemical staining against viral antigens can be seen as red chromogenic deposits. These signals are predominantly present in endothelial cells of interstitial capillaries, which are putative target sites for ISAV (Aamelfot *et al*., 2012; Aamelfot *et al*., 2015; Aamelfot *et al*., 2016; Falk *et al*., 1998; Munro *et al*., 2003). Some intranuclear and cytoplasmic staining was also evident, suggesting active viral replication within the renal tissue (Kibenge *et al*., 2000). Melanomacrophages were found sporadically, which is indicative of a local immune reaction against the infection.

In immunohistochemistry, studies conducted at the NVI, the mid kidney is often preferred over the head kidney for several reasons related to tissue composition and function. The head kidney is a primary site for haematopoiesis in fish, which leads to a dense population of developing erythrocytes, immune cells and regenerative activity. The interpretation of IHC results can be interfered by this abundance of hematopoietic cells, due to non-specific staining and background noise (Kondera *et al*., 2014). The various immune cells that may be activated during infections can also potentially lead to variable staining patterns. The mid kidney has a more stable and uniform tissue architecture with fewer hematopoietic and regenerative activities compared to the head kidney and heart, allowing for clearer and more specific IHC staining. A recent study showed that while both head and mid kidneys respond to infections, the mid kidney exhibits distinct immune responses, making the results more accurate and interpretable (Sun *et al*., 2022).

Despite the spleen and gills being interesting detection tissues for ISAV, the non-specific background staining, presence of mucus, and other bacteria make these organs not useful or ideal for IHC (Aamelfot *et al*., 2012; Falk *et al*., 1998; Koppang *et al*., 2008; Moneke *et al*., 2003; Moneke *et al*., 2005).

5. Conclusion

This study assessed the consequences of exposing fish to HPR-Δ strain of ISAV on RAS, focusing on virus persistence, impact on biofilter function, and evaluation of disinfection strategies. All the initial objectives were completed.

The results confirmed that ISAV RNA can persist in water, biofilms of tank walls, and biofilters even after the removal of infected fish. Although viral RNA levels decreased over time in all systems, the persistence of detectable ISAV in environmental samples emphasizes the risk of horizontal transmission between production cycles if proper disinfection is not applied. Notably, no infectious virus was detected in ASK cell cultures from environmental samples, and naïve fish introduced after treatments remained uninfected, indicating that while viral RNA was present, its concentration was low, infectivity was likely lost, and fallowing could have contributed to this outcome.

Among the treatments tested, ozonation emerged as the most effective disinfection strategy. Compared to the control and Water Exchange, ozone treatment resulted in a significantly faster decline in ISAV RNA levels and maintained consistently negative RT-qPCR results. Importantly, ozone treatment initially compromised biofilter performance, as evidenced by impaired nitrification indicators and changes in water quality. Another key finding is that the type of sample influences the ability to detect ISAV, with water and swab samples proving more effective than those taken from the biofilter.

Immunohistochemical analysis confirmed the successful establishment of infection in challenged fish, particularly in endothelial and cardiomyocyte tissues. In contrast, traditional H&E staining showed limitations in detecting lesions, probably due to the rapid disease progression and acute mortality caused by ISAV. Immunohistochemistry thus proved to be a more sensitive method for visualizing viral antigens in situ and should be considered an essential tool in ISAV diagnostics.

The study also highlights the complexity of managing pathogen risks in RAS, especially considering the role of biofilms as both ecological buffers and potential pathogen reservoirs. While the microbiome analysis is ongoing, preliminary observations support the hypothesis that disinfection strategies, particularly ozone, may affect microbiological stability. Although careful dosing is essential, ozone remains an effective method for decontaminating the RAS system after an ISAV outbreak.

6. Future prospects

While valuable insights were provided, some limitations in the experimental setup should be addressed in future research to improve accuracy and applicability.

The bioreactors, despite being practical, lacked a chiller, which would have helped maintain a stable temperature comparable to the conditions in the tanks. Additionally, the bioreactors were deeply cleaned before their assembly. However, there was no possibility to collect biofilm swab samples, an important approach for ISAV detection, since biofilms are known to be a reservoir of microorganisms (including viruses and bacteria) and might be involved in the persistence and transmission of ISAV. The absence of detectable biofilm may be attributed to insufficient system runtime for biofilm formation or to the strong aeration, which could have hindered biofilm development. Moreover, strong aeration could have seriously impacted the development of these biofilms. Furthermore, having additional replicates would increase statistical strength. Sampling larger water volumes and doing this experience at a larger scale could improve the reliability of the results.

The fact that this study was conducted in brackish water, is a noteworthy aspect to explore. Salinity is known to impact viral stability, host immune responses, and water chemistry, all of which could affect ISAV dynamics. A next step could involve a comparison of ISAV persistency and inactivation on seawater, freshwater and brackish water, helping us understand the behaviour of the virus across different aquaculture environments. In addition, testing a range of ozone doses would also be important in order to understand and identify concentrations that effectively inactivate pathogens without harming essential system functions. The study of alternative disinfection strategies, such as the application of PAA, would also provide valuable insights into this matter.

7. References

- Aalto, S., Letelier-Gordo, C., Pedersen, L.-F., & Pedersen, P. (2022). Effect of biocarrier material on nitrification performance during start-up in freshwater RAS. Aquacultural Engineering, 99, 102292. https://doi.org/10.1016/j.aquaeng.2022.102292
- Aamelfot, M., Christiansen, D.H., Dale, O.B., McBeath, A., Benestad, S.L., & Falk, K. (2016). Localised Infection of Atlantic Salmon Epithelial Cells by HPR0 Infectious Salmon Anaemia Virus. PLoS One, 11(3), e0151723. https://doi.org/10.1371/journal.pone.0151723
- Aamelfot, M., Dale, O.B., Weli, S.C., Koppang, E.O., & Falk, K. (2012). Expression of the infectious salmon anemia virus receptor on Atlantic salmon endothelial cells correlates with the cell tropism of the virus. Journal of Virology, 86(19), 10571–10578. https://doi.org/10.1128/JVI.00047-12
- Aamelfot, M., McBeath, A., Christiansen, D.H. Matejusova I, & Falk K.I. (2015). Infectious salmon anaemia virus (ISAV) mucosal infection in Atlantic salmon. Veterinary Research, 46, 120. https://doi.org/10.1186/s13567-015-0265-1
- Aguilar-Alarcón, P., Zherebker, A., Rubekina, A., Shirshin, E., Simonsen, M. A., Kolarevic, J., Lazado, C. C., Nikolaev, E. N., Asimakopoulos, A. G., & Mikkelsen, Ø. (2022). Impact of ozone treatment on dissolved organic matter in land-based recirculating aquaculture systems studied by Fourier transform ion cyclotron resonance mass spectrometry. Science of The Total Environment, 843, 157009. https://doi.org/10.1016/j.scitotenv.2022.157009
- Ahmed, N., & Turchini, G. M. (2021). Recirculating aquaculture systems (RAS): Environmental solution and climate change adaptation. Journal of Cleaner Production, 297, 126604. https://doi.org/10.1016/j.jclepro.2021.126604
- Amijar, M.I.S.M., Ramli, N.M., Nurulhuda, K., & Aziz, S.A. (2020). Portable Spectrophotometer for Water Quality Monitoring in Recirculating Aquaculture Systems. IOP Conference Series: Earth and Environmental Science, 1359, 012028. https://doi.org/10.1088/1755-1315/1359/1/012028
- Andreassen, R., Woldemariam, N.T., Egeland, I.Ø., Agafonov, O., Sindre, H., & Høyheim, B. (2017). Identification of differentially expressed Atlantic salmon miRNAs responding to salmonid alphavirus (SAV) infection. BMC Genomics, 18(1), 349. https://doi.org/10.1186/s12864-017-3741-3
- Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., Arya, N., & Patel, H. R. (2005). Basic principles of real-time quantitative PCR. Expert Review of Molecular Diagnostics, 5(2), 209–219. https://doi.org/10.1586/14737159.5.2.209
- Attramadal, K.J.K., Salvesen, I., Xue, R., Øie, G., Størseth, T.R., Vadstein, O., & Olsen, Y. (2012). Recirculation as a possible microbial control strategy in the production of marine larvae. Aquacultural Engineering, 46, 27–39. https://doi.org/10.1016/j.aquaeng.2011.10.003
- Ayuso Virgili, G., Jafari, L., Lande-Sudall, D., & Lümmen, N. (2023). Linear modelling of the mass balance and energy demand for a recirculating aquaculture system. Aquacultural Engineering, 101, 102330. https://doi.org/10.1016/j.aquaeng.2023.102330
- Badiola, M., Basurko, O., Piedrahita, R., Hundley, P., & Mendiola, D. (2018). Energy use in recirculating aquaculture systems (RAS): A review. Aquacultural Engineering, 81, 57–70. https://doi.org/10.1016/j.aquaeng.2018.03.003
- Bellec, L., Le Du-Carré, J., Almeras, F., Durand, L., Cambon-Bonavita, M.A., Danion, M., & Morin, T. (2022). Glyphosate-based herbicide exposure: effects on gill microbiota of rainbow trout (Oncorhynchus mykiss) and the aquatic bacterial ecosystem. FEMS Microbiology Ecology, 98(8), fiac076. https://doi.org/10.1093/femsec/fiac076
- Bentzon-Tilia, M., Sonnenschein, E. C., & Gram, L. (2016). Monitoring and managing microbes in aquaculture—Towards a sustainable industry. Microbial Biotechnology, 9, 576–584. https://doi.org/10.1111/1751-7915.12392
- Bialoszewski, D., Pietruczuk-Padzik, A., Kalicińska, A., Bocian, E., Czajkowska, M., Bukowska, B., & Tyski, S. (2011). Activity of ozonated water and ozone against

Staphylococcus aureus and Pseudomonas aeruginosa biofilms. Medical Science Monitor, 17(11), BR339–BR344. https://doi.org/10.12659/msm.882044

- Bibby, K., & Peccia, J. (2013). Prevalence of respiratory adenovirus species B and C in sewage sludge. Environmental Science: Processes & Impacts, 15(2), 336–338. https://doi.org/10.1039/c2em30831b
- Blancheton, J. P., Attramadal, K. J. K., Michaud, L., Roque d'Orbcastel, E., & Vadstein, O. (2013). Insight into bacterial population in aquaculture systems and its implication. Aquacultural Engineering, 53, 30–39. https://doi.org/10.1016/j.aquaeng.2012.11.009
- Blanco-Cabra, N., López-Martínez, M. J., Arévalo-Jaimes, B. V., Martin-Gómez, M. T., Samitier, J., & Torrents, E. (2021). A new BiofilmChip device for testing biofilm formation and antibiotic susceptibility. NPJ Biofilms and Microbiomes, 7(1), 62. https://doi.org/10.1038/s41522-021-00236-1
- Bowden, T. J. (2008). Modulation of the immune system of fish by their environment. Fish & Shellfish Immunology, 25(3), 373–383. https://doi.org/10.1016/j.fsi.2008.03.017
- Brauner, C.J., Harter, T.S., & Rummer, J.L. (2017). Gas transport and exchange: interaction between O_2 and CO_2 exchange. In A.P. Farrell (Ed.), Encyclopedia of Fish Physiology: From Genome to Environment (pp. 1798–1807). Academic Press. https://doi.org/10.1016/b978-0-12-809633-8.03114-9
- Brown, R.M., Wiens, G.D., & Salinas, I. (2019). Analysis of the gut and gill microbiome of resistant and susceptible lines of rainbow trout (Oncorhynchus mykiss). Fish & Shellfish Immunology, 86, 497-506. https://doi.org/10.1016/j.fsi.2018.11.079
- Bustin, S. A., & Nolan, T. (2004). Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. Journal of Biomolecular Techniques, 15(3), 155–166
- Cashdollar, J.L. & Wymer, L. (2013). Methods for primary concentration of viruses from water samples: a review and meta-analysis of recent studies. Journal of Applied Microbiology, 115(1), 1–11. https://doi.org/10.1111/jam.12143
- Cerda, A., Rivera, M., Armijo, G., Ibarra-Henriquez, C., Reyes, J., Blázquez-Sánchez, P., Avilés, J., Arce, A., Seguel, A., Brown, A.J., Vásquez, Y., Cortez-San Martín, M., Cubillos, F.A., García, P., Ferres, M., Ramírez-Sarmiento, C.A., Federici, F., & Gutiérrez, R.A. (2024). An Open One-Step RT-qPCR for SARS-CoV-2 detection. PLOS ONE, 19(1), e0297081. https://doi.org/10.1371/journal.pone.0297081
- Christiansen, D. H., McBeath, A. J. A., Aamelfot, M., Matejusova, I., Fourrier, M., White, P., Petersen, P. E., & Falk, K. (2017). First field evidence of the evolution from a non-virulent HPR0 to a virulent HPR-deleted infectious salmon anaemia virus. Journal of General Virology, 98(4). https://doi.org/10.1099/jgv.0.000741
- Christiansen, D. H., Petersen, P. E., Dahl, M. M., Vest, N., Aamelfot, M., Kristoffersen, A. B., Jansen, M. D., Matejusova, I., Gallagher, M. D., Jónsson, G., Rodriguez, E., Fosse, J. H., & Falk, K. (2021). No Evidence of the Vertical Transmission of Non-Virulent Infectious Salmon Anaemia Virus (ISAV-HPR0) in Farmed Atlantic Salmon. Viruses, 13(12), 2428. https://doi.org/10.3390/v13122428
- Christiansen, D.H., McBeath, A.J.A., Aamelfot, M., Matejusova, I., Fourrier, M., White, P., Petersen, P.E., & Falk, K. (2017). First field evidence of the evolution from a non-virulent HPR0 to a virulent HPR-deleted infectious salmon anaemia virus. Journal of General Virology, 98(4), 595–606. https://doi.org/10.1099/jgv.0.000741
- Coca, Y., Sáez-Navarrete, C., Caro, D., Suárez, R., Bledsoe, J.W., Abanikannda, M.F., Montes de Oca, M., Pontigo, J.P., Rodríguez-Córdova, L., Burbulis, I., Kibenge, F.S.B., & Godoy, M. (2025). Microbiome re-colonization dynamics of a moving bed biofilter in an Atlantic salmon recirculating aquaculture system. Research Square, 1-34. https://doi.org/10.21203/rs.3.rs-5853176/v1
- Colt, J. (2006). Water quality requirements for reuse systems. Aquacultural Engineering, 34(3), 143–156. https://doi.org/10.1016/j.aquaeng.2005.08.011.
- Dahle, S. W., Gaarden, S. I., Buhaug, J. F., Netzer, R., Attramadal, K. J. K., Busche, T., Aas, M., Ribicic, D., & Bakke, I. (2023). Long-term microbial community structures and

dynamics in a commercial RAS during seven production batches of Atlantic salmon fry (Salmo salar). Aquaculture, 565, 739155. https://doi.org/10.1016/j.aquaculture.2022.739155

- Dannevig, B. H., Falk, K., & Namork, E. (1995). Isolation of the causal virus of infectious salmon anaemia (ISA) in a long-term cell line from Atlantic salmon head kidney. Journal of General Virology, 76(Pt 6), 1353–1359. https://doi.org/10.1099/0022-1317-76-6-1353
- Davidson, J., Good, C., Williams, C., & Summerfelt, S.T. (2017). Evaluating the chronic effects of nitrate on the health and performance of post-smolt Atlantic salmon (Salmo salar) in freshwater recirculation aquaculture systems. Aquacultural Engineering, 79, 1–8. https://doi.org/10.1016/j.aquaeng.2017.08.003
- Del Duca, A., Cesar, D., Freato, T., Azevedo, R., Rodrigues, E., & Abreu, P. (2019). Variability of the nitrifying bacteria in the biofilm and water column of a recirculating aquaculture system for tilapia (Oreochromis niloticus) production. Aquaculture Research, 50, https://doi.org/10.1111/are.14211.
- Derome, N. (2019). Microbial communities in aquaculture ecosystems: Improving productivity and sustainability. Springer Ed. https://doi.org/10.1007/978-3-030-16190-3
- Devaraja, T., Banerjee, S., Yusoff, F., Shariff, M., & Khatoon, H. (2013). A holistic approach for selection of Bacillus spp. as a bioremediator for shrimp postlarvae culture. Turkish Journal of Biology, 37, 92–100. https://doi.org/10.3906/biy-1203-19
- Di Martino, G., Pasqua, S., Douradinha, B., Monaco, F., Di Bartolo, C., Conaldi, P. G. & D'Apolito, D. (2021). Efficacy of Three Commercial Disinfectants in Reducing Microbial Surfaces' Contaminations of Pharmaceuticals Hospital Facilities. Int J Environ Res Public Health, 18 (2). doi: 10.3390/ijerph18020779.
- Dopazo, C. P. (2020). The infectious pancreatic necrosis virus (IPNV) and its virulence determinants: what is known and what should be known. Pathogens, 9(2), 94. https://doi.org/10.3390/pathogens9020094
- Eaton, A.D., Clesceri, L.S., Rice, E.W., & Greenberg, A.E. (2012). Standard Methods for the Examination of Water and Wastewater (22nd ed., SM 4500). American Public Health Association.
- Ebeling, J. M., Timmons, M.B. & Bisogni, J.J. (2006). Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia–nitrogen in aquaculture systems. Aquaculture, 257(1-4), 346-358. https://doi.org/10.1016/j.aquaculture.2006.03.019
- Ebeling, J.M., & Timmons, M.B. (2012). Recirculating aquaculture systems. In J.H. Tidwell (Ed.), Aquaculture Production Systems (Chapter 11). https://doi.org/10.1002/9781118250105.ch11
- Eckstrand, C.D., Torrevillas, B.K., Wolking, R.M., Bradway, D.S., Warg, J.V., Clayton, R.D., Williams, L.B., Pessier, A.P., Reno, J.L., McMenamin-Snekvik, K.M., Thompson, J., Baszler, T., & Snekvik, K.R. (2024). Investigation of laboratory methods for characterization of aquatic viruses in fish infected experimentally with infectious salmon anemia virus. Journal of Veterinary Diagnostic Investigation, 36(3), 319-328. https://doi.org/10.1177/10406387231173332
- Eding, E.H., Kamstra, A., Verreth, J.A.J., Huisman, E.A., & Klapwijk, A. (2006). Design and operation of nitrifying trickling filters in recirculating aquaculture: A review. Aquacultural Engineering, 34, 234–260. https://doi.org/10.1016/j.aquaeng.2005.09.007
- Falk, K., Namork, E., Rimstad, E., Mjaaland, S., & Dannevig, B. (1998). Characterization of infectious salmon anemia virus, an Orthomyxo-like virus isolated from Atlantic salmon (Salmo salar L.). Journal of Virology, 71(12), 9016–9023. https://doi.org/10.1128/JVI.71.12.9016-9023.1997
- FAO (2024). The State of World Fisheries and Aquaculture 2024: Towards Blue Transformation. Rome: FAO. Available at: https://openknowledge.fao.org/items/06690fd0-d133-424c-9673-1849e414543d (accessed: 10/11/2024).).
- Fernandes, P.M., Pedersen, L.-F., & Pedersen, P.B. (2017). Influence of fixed and moving bed biofilters on micro particle dynamics in a recirculating aquaculture system. Aquacultural Engineering, 78(A), 32–41. https://doi.org/10.1016/j.aquaeng.2016.09.002

• Ferrera, I., Massana, R., Balagué, V., Pedrós-Alió, C., Sánchez, O., & Mas, J. (2010). Evaluation of DNA extraction methods from complex phototrophic biofilms. Biofouling, 26(3), 349–357. https://doi.org/10.1080/08927011003605870. PMID: 20140796.
• Finstad, O. W., Dahle, M. K., Lindholm, T. H., Nyman, I. B., Lovoll, M., Wallace, C., & Rimstad, E. (2012). Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes. Veterinary Research, 43, 27. https://doi.org/10.1186/1297-9716-43-27
• Fivelstad, S. (2013). Long-term carbon dioxide experiments with salmonids. Aquacultural Engineering, 53, 40–48. https://doi.org/10.1016/j.aquaeng.2012.11.006
• Fivelstad, S., Olsen, A.B., Kløften, H., Ski, H., & Stefansson, S.O. (1999). Effects of carbon dioxide on Atlantic salmon (Salmo salar L.) smolts at constant pH in bicarbonate rich freshwater. Aquaculture, 178(1–2), 171–187. https://doi.org/10.1016/S0044-8486(99)00125-8
• Fudge, M., Higgins, V., Vince, J., & Rajaguru, R. (2023). Social acceptability and the development of commercial RAS aquaculture. Aquaculture, 568(12), 739295. https://doi.org/10.1016/j.aquaculture.2023.739295
• Fuhong, D., Gagné, N., Ditlecadet, D., Quinn, B. K., & Trudel, M. (2024). Modelling the dispersion of infectious salmon anemia virus from Atlantic salmon farms. FACETS, 9, 1–19. https://doi.org/10.1139/facets-2023-0156
• Gagné, N., & LeBlanc, F. (2018). Overview of infectious salmon anaemia virus (ISAV) in Atlantic Canada and first report of an ISAV North American-HPR 0 subtype. Journal of Fish Diseases, 41, 421–430. https://doi.org/10.1111/jfd.12670
• García, K., Ramírez-Araya, S., Díaz, Á., Reyes-Cerpa, S., Espejo, R.T., Higuera, G., & Romero, J. (2015). Inactivated E. coli transformed with plasmids that produce dsRNA against infectious salmon anemia virus hemagglutinin show antiviral activity when added to infected ASK cells. Frontiers in Microbiology, 6, 300. https://doi.org/10.3389/fmicb.2015.00300
• Gerba, C. P. (1984). Applied and theoretical aspects of virus adsorption to surfaces. Advances in Applied Microbiology, 30, 133–168. https://doi.org/10.1016/S0065-2164(08)70067-9
• Gjessing, M. C., Aamelfot, M., Batts, W. N., Benestad, S. L., Dale, O. B., Thoen, E., Weli, S. C., & Winton, J. R. (2018). Development and characterization of two cell lines from gills of Atlantic salmon. PLoS ONE, 13(2), e0191792. https://doi.org/10.1371/journal.pone.0191792
• Gjessing, M. C., Steinum, T., Olsen, A. B., Lie, K. I., Tavornpanich, S., Colquhoun, D. J., & Gjevre, A. G. (2019). Histopathological investigation of complex gill disease in sea-farmed Atlantic salmon. PLoS One, 14(10), e0222926. https://doi.org/10.1371/journal.pone.0222926
• Gleerup, D., Trypsteen, W., Fraley, S.I., & De Spiegelaere, W. (2024). Digital PCR in virology: Current applications and future perspectives. Molecular Diagnosis & Therapy. 29(1), 43-54. https://doi.org/10.1007/s40291-024-00751-9
• Godoy, M. G., Aedo, A., Kibenge, M. J. T., Groman, D. B., Yason, C. V., Grothusen, H., Lisperguer, A., Calbucura, M., Avendaño, F., Imilán, M., Jarpa, M., & Kibenge, F. S. B. (2008). First detection, isolation and molecular characterization of infectious salmon anaemia virus associated with clinical disease in farmed Atlantic salmon (Salmo salar) in Chile. BMC Veterinary Research, 4, 28. https://doi.org/10.1186/1746-6148-4-28
• Gregory, A., Munro, L.A., Snow, M., Urquhart, K.L., Murray, A.G., & Raynard, R.S. (2009). An experimental investigation on aspects of infectious salmon anaemia virus (ISAV) infection dynamics in seawater Atlantic salmon, Salmo salar L. Journal of Fish Diseases, 32(6), 481–489. https://doi.org/10.1111/j.1365-2761.2009.00999.x
• Groves, L., Whyte, S. K., Purcell, S. L., Michaud, D., Cai, W. C., Garber, A. F., & Fast, M. D. (2023). Temperature impacts Atlantic salmon's (Salmo salar) immunological response to infectious salmon anemia virus (ISAv). Fish and Shellfish Immunology Reports, 4, 100099. https://doi.org/10.1016/j.fsirep.2023.100099
• Gupta, S., Makridis, P., Henry, I., Velle-George, M., Ribicic, D., Bhatnagar, A., Skalska-Tuomi, K., Daneshvar, E., Ciani, E., Persson, D., & Netzer, R. (2024). Recent

developments in recirculating aquaculture systems: A review. Aquaculture Research. https://doi.org/10.1155/are/6096671

- Hjeltnes, B., Erikson, U. G., Mortensen, S., Rosten, T. W., Bøe, K. E., Hoel, K., Olsen, R. E., Rimstad, E., & Østerås, O. (2012). Risk assessment of recirculation systems in salmonid hatcheries. Norwegian Scientific Committee for Food Safety (VKM). Available online: https://vkm.no/english/riskassessments/allpublications/riskassessmentofrecirculationsystemsinsalmonidhatcheries.4.72c3261615e09f2472f46f9b.html (accessed on 5 November 2024).
- Hodneland, K., Bratland, A., Christie, K. E., Endresen, C., & Nylund, A. (2005). New subtype of salmonid alphavirus (SAV), Togaviridae, from Atlantic salmon Salmo salar and rainbow trout Oncorhynchus mykiss in Norway. Diseases of Aquatic Organisms, 66(2), 113–120. https://doi.org/10.3354/dao066113
- Hofstad, K. (2023). The Effects of Biofilter Disinfection on Nitrification Capacity and Bacterial Communities in RAS with Atlantic salmon (Salmo salar L.) fry (Master's thesis, Norwegian University of Science and Technology). Retrieved from NTNU Open Institutional Repository.
- Horan, N. (2003). Suspended growth processes. In D. Mara & N. Horan (Eds.), Handbook of Water and Wastewater Microbiology (pp. 351–360). Academic Press. https://doi.org/10.1016/B978-012470100-7/50022-4
- Howell, C. K., Atkinson, S. D., Bartholomew, J. L., & Hallett, S. L. (2019). Development and application of a qPCR assay targeting Ichthyophthirius multifiliis in environmental water samples. Diseases of Aquatic Organisms, 134(1), 43–55. https://doi.org/10.3354/dao03351
- Huang, Q., Butaye, P., Ng, P. H., Zhang, J., Cai, W., & St-Hilaire, S. (2024). Impact of low-dose ozone nanobubble treatments on antimicrobial resistance genes in pond water. Journal of Infectious Diseases and Public Health, 15, 1393266. https://doi.org/10.3389/fmicb.2024.1393266
- Huavas, J., Heyse, J., Props, R., Delamare-Deboutteville, J., & Shelley, C. (2024). Microbiomes of Tilapia Culture Systems: Composition, Affecting Factors, and Future Perspectives. Aquaculture Research. 2024, 1-26. https://doi.org/10.1155/2024/5511461
- Ikner, L.A., Gerba, C.P., & Bright, K.R. (2012). Concentration and recovery of viruses from water: A comprehensive review. Food and Environmental Virology, 4(2), 41–67. https://doi.org/10.1007/s12560-012-9080-2
- Jafari, L., Montjouridès, M. A., Diesen Hosfeld, C., Attramadal, K., Fivelstad, S., & Dahle, H. (2024). Biofilter and degasser performance at different alkalinity levels in a brackish water pilot scale recirculating aquaculture system (RAS) for post-smolt Atlantic salmon. Aquacultural Engineering, 106, 102407. https://doi.org/10.1016/j.aquaeng.2024.102407
- Kibenge, F. S., Whyte, S. K., Hammell, K. L., & others. (2000). A dual infection of infectious salmon anaemia (ISA) virus and a togavirus-like virus in ISA of Atlantic salmon Salmo salar in New Brunswick, Canada. Diseases of Aquatic Organisms, 42(1), 11–15. https://doi.org/10.3354/dao042011
- Kibenge, F.S., Gárate, O.N., Johnson, G., Arriagada, R., Kibenge, M.J., & Wadowska, D. (2001). Isolation and identification of infectious salmon anaemia virus (ISAV) from Coho salmon in Chile. Diseases of Aquatic Organisms, 45(1), 9–18. https://doi.org/10.3354/dao045009
- King, R., Flick, G., Smith, S., Pierson, M., Boardman, G., & Coale, C. (2008). Response of bacterial biofilms in recirculating aquaculture systems to various sanitizers. Journal of Applied Aquaculture, 20, 1–22. https://doi.org/10.1080/10454430802191766
- Knoph, M.B., & Olsen, Y.A. (1994). Subacute toxicity of ammonia to Atlantic salmon (Salmo salar L.) in seawater: Effects on water and salt balance, plasma cortisol, and plasma ammonia levels. Aquatic Toxicology, 30(4), 295–310. https://doi.org/10.1016/0166-445X(94)00046-8
- Kondera, E. (2014). Cell composition of the head kidney of European chub (Squalius cephalus L.). Archives of Polish Fisheries, 22, 271–280. https://doi.org/10.2478/aopf-2014-0029

• Koppang, E.O., Bjerkås, I., Haugarvoll, E., Chan, E.K., Szabo, N.J., Ono, N., Akikusa, B., Jirillo, E., Poppe, T.T., Sveier, H., Tørud, B., & Satoh, M. (2008). Vaccination-induced systemic autoimmunity in farmed Atlantic salmon. Journal of Immunology, 181(7), 4807–4814. https://doi.org/10.4049/jimmunol.181.7.4807
• Lazado, C. C., & Good, C. (2021). Survey findings of disinfection strategies at selected Norwegian and North American land-based RAS facilities: A comparative insight. Aquaculture, 532, 736038. https://doi.org/10.1016/j.aquaculture.2020.736038
• Lee, D. E., Lee, J., Kim, Y. M., Myeong, J. I., & Kim, K. H. (2016). Uncultured bacterial diversity in a seawater recirculating aquaculture system revealed by 16S rRNA gene amplicon sequencing. Journal of Microbiology, 54(4), 296–304. https://doi.org/10.1007/s12275-016-5571-4
• Lekang, O.-I. (2013). Aquaculture engineering (2nd ed.). Wiley-Blackwell, Chichester. https://doi.org/10.1002/9781118496077
• Lewin, A. S., Haugen, T., Netzer, R., Tøndervik, A., Dahle, S. W., & Hageskal, G. (2020). Multiplex droplet digital PCR assay for detection of Flavobacterium psychrophilum and Yersinia ruckeri in Norwegian aquaculture. Journal of Microbiological Methods, 177, 106044. https://doi.org/10.1016/j.mimet.2020.106044
• Li, H., Cui, Z., Cui, H., Bai, Y., Yin, Z., & Qu, K. (2023). A review of influencing factors on a recirculating aquaculture system: Environmental conditions, feeding strategies, and disinfection methods. Journal of the World Aquaculture Society, 54(3), 566–602. https://doi.org/10.1111/jwas.12976
• Liltved, H., Vogelsang, C., Modahl, I., & Dannevig, B.H. (2006). High resistance of fish pathogenic viruses to UV irradiation and ozonated seawater. Aquacultural Engineering, 34(3), 218–227. https://doi.org/10.1016/j.aquaeng.2005.05.002
• Lindholm-Lehto, P. (2023). Water quality monitoring in recirculating aquaculture systems. Aquaculture and Fisheries, 3, 113–131. https://doi.org/10.1002/aff2.102
• Lindholm-Lehto, P. (2023). Water quality monitoring in recirculating aquaculture systems. Aquaculture, Fish and Fisheries, 3(2), 113–131. https://doi.org/10.1002/aff2.102
• Liu, W., Du, X., Tan, H., Xie, J., Luo, G., & Sun, D. (2021). Performance of a recirculating aquaculture system using biofloc biofilters with convertible water-treatment efficiencies. Science of the Total Environment, 754, 141918. https://doi.org/10.1016/j.scitotenv.2020.141918
• López-Lastra, M., Gonzalez, M., & Sandino, A. (1994). A detection method for infectious pancreatic necrosis virus (IPNV) based on reverse transcription (RT)-polymerase chain reaction (PCR). Journal of Fish Diseases, 17, 269–282. https://doi.org/10.1111/j.1365-2761.1994.tb00222.x
• Luukkonen, T., Teeriniemi, J., Prokkola, H., Rämö, J., & Lassi, U. (2014). Chemical aspects of peracetic acid based wastewater disinfection. Water SA, 40(1), 1-10. https://doi.org/10.4314/wsa.v40i1.9
• Mardones, F.O., Martinez-Lopez, B., Valdes-Donoso, P., Carpenter, T.E., & Perez, A.M. (2014). The role of fish movements and the spread of infectious salmon anemia virus (ISAV) in Chile, 2007-2009. Preventive Veterinary Medicine, 114(1), 37–46. https://doi.org/10.1016/j.prevetmed.2014.01.012
• Marine Institute. (2017). CHAPTER III: Environment. The Farmed Salmonid Health Handbook (Version 2.0). Marine Institute.
• Martins, C., Eding, E., Verdegem, M., Heinsbroek, L.T.N., Schneider, O., Blancheton, J.P., d'Orbcastel, E., & Verreth, J. (2010). New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. Aquacultural Engineering, 43(3), 83–93. https://doi.org/10.1016/j.aquaeng.2010.09.002
• Meriac, A. (2019). Smolt production and the potential for solid waste collection in Norway. Nofima AS, 2–19.
• Mikalsen, A.B., Teig, A., Helleman, A.L., Mjaaland, S., & Rimstad, E. (2001). Detection of infectious salmon anaemia virus (ISAV) by RT-PCR after cohabitant exposure in Atlantic salmon, Salmo salar. Diseases of Aquatic Organisms, 47, 175–181. https://doi.org/10.3354/dao

• Miller, D. L., Pessier, A. P., Hick, P., Whittington, R. J., & Forzán, M. J. (2025). Pathology and diagnostics. In M. J. Gray & V. G. Chinchar (Eds.), Ranaviruses. Springer, Cham. https://doi.org/10.1007/978-3-031-64973-8_8
• Mobley, K., Aykanat, T., Czorlich, Y., House, A., Kurko, J., Miettinen, A., Moustakas-Verho, J., Salgado Ismodes, A., Sinclair-Waters, M., Verta, J.P., & Primmer, C. (2021). Maturation in Atlantic salmon (Salmo salar, Salmonidae): a synthesis of ecological, genetic, and molecular processes. Reviews in Fish Biology and Fisheries, 31, 523-571. https://doi.org/10.1007/s11160-021-09656-w
• Moneke, E., Groman, D.B., Wright, G.M., Stryhn, H., Johnson, G.R., Ikede, B.O., & Kibenge, F.S. (2005). Correlation of virus replication in tissues with histologic lesions in Atlantic salmon experimentally infected with infectious salmon anemia virus. Veterinary Pathology, 42, 338 -349. https://doi.org/10.1354/vp.42-3-338
• Moneke, E.E., Kibenge, M.J., Groman, D., Johnson, G.R., Ikede, B.O., & Kibenge, F.S.. (2003), Infectious salmon anemia virus RNA in fish cell cultures and in tissue sections of atlantic salmon experimentally infected with infectious salmon anemia virus. Journal of Veterinary Diagnostic Investigation. 15(5), 407-17. https://doi.org/10.1177/104063870301500502
• Mota, V., Striberny, A., Verstege, G., Difford, G., & Lazado, C. (2022). Evaluation of a Recirculating Aquaculture System Research Facility Designed to Address Current Knowledge Needs in Atlantic Salmon Production. Frontiers in Animal Science, 3, 876504. https://doi.org/10.3389/fanim.2022.876504
• Mugimba, K. K., Byarugaba, D. K., Mutoloki, S., Evensen, Ø., & Munang'andu, H. M. (2021). Challenges and Solutions to Viral Diseases of Finfish in Marine Aquaculture. Pathogens, 10(6), 673. https://doi.org/10.3390/pathogens10060673
• Mugwanya, M., Dawood, M. A. O., Kimera, F., & Sewilam, H. (2022). A review on recirculating aquaculture system: Influence of stocking density on fish and crustacean behavior, growth performance, and immunity. Annals of Animal Science, 22(3), 873–884. https://doi.org/10.2478/aoas-2022-0014
• Munro, A.L.S., & Midtlyng, P. J. (2003). Localization of ISAV antigens in kidney tissues of experimentally infected Atlantic salmon. Diseases of Aquatic Organisms, 53(3), 187–195.
• Murray, F., Bostock, J., & Fletcher, D. (2014). Review of Recirculation Aquaculture System Technologies and Their Commercial Application. Final Report, Stirling Aquaculture, Scotland, UK, 1–82.
• Navada, S., Gaumet, F., Tveten, A.K., Kolarevic, J., & Vadstein, O. (2021). Seeding as a start-up strategy for improving the acclimation of freshwater nitrifying bioreactors to salinity stress. Aquaculture, 540, 736663. https://doi.org/10.1016/j.aquaculture.2021.736663
• Neiffer, D. L., & Stamper, M. A. (2009). Fish sedation, anesthesia, analgesia, and euthanasia: Considerations, methods, and types of drugs. ILAR Journal, 50(4), 343–360. https://doi.org/10.1093/ilar.50.4.343
• Noguera, P., Olsen, A. B., Hoare, J., Lie, K. I., Lopez, M. M., Poppe, T., & Rodger, H. D. (2019). Complex gill disorder (CGD): A histopathology workshop report. Bulletin of the European Association of Fish Pathologists, 39(4), 5.
• Norley, S. E., & Love, A. (2020). The effect of Virkon® Aquatic on the bacterial count of Artemia cultures. Journal of Zoo and Aquarium Research, 8(1). https://doi.org/10.19227/jzar.v8i1.461
• Norwegian Directorate of Fisheries (2024). Statistics for aquaculture: Atlantic salmon and rainbow trout. Bergen: Norwegian Directorate of Fisheries. Available at: https://www.fiskeridir.no/English/aquaculture/statistics-for-aquaculture/atlantic-salmon-and-rainbow-trout (accessed: 24/04/2025).
• Nowak, O., Schweighofer, P., & Svardal, K. (1994). Nitrification inhibition – A method for the estimation of actual maximum autotrophic growth rates in activated sludge systems. Water Science and Technology, 30(6), 9–19. https://doi.org/10.2166/wst.1994.0248
• Nylund, A., Hovland, T., Hodneland, K., Nilsen, F., & Levik, P. (1994). Mechanisms for transmission of infectious salmon anemia (ISA). Diseases of Aquatic Organisms, 19, 95-100. 10.3354/dao019095.

• Park, H.-D., & Noguera, D.R. (2004). Evaluating the effect of dissolved oxygen on ammonia-oxidizing bacterial communities in activated sludge. Water Research, 38(14–15), 3275–3286. https://doi.org/10.1016/j.watres.2004.04.047
• Pedersen, L.-F., Meinelt, T., & Straus, D. (2013). Peracetic acid degradation in freshwater aquaculture systems and possible practical implications. Aquacultural Engineering, 53, 65–71. https://doi.org/10.1016/j.aquaeng.2012.11.011
• Pietrak, M., Warg, J., Gustafson, L., & Peterson, B.C. (2024). Intermittent detections of ISAV-HPR0 in a salmon recirculating aquaculture system, and implications for sampling. Fishes, 9(8), 325. https://doi.org/10.3390/fishes9080325
• Poulson, R. L., Tompkins, S. M., Berghaus, R. D., Brown, J. D., & Stallknecht, D. E. (2016). Environmental stability of swine and human pandemic influenza viruses in water under variable conditions of temperature, salinity, and pH. Applied and Environmental Microbiology, 82(13), 3721–3726. https://doi.org/10.1128/aem.00133-16
• Powell, A., & Scolding, J. (2018). Direct application of ozone in aquaculture systems. Reviews in Aquaculture, 10, 424–438. https://doi.org/10.1111/raq.12169
• Preena, G., Rejish Kumar, V.J., & Singh, I. (2021). Nitrification and denitrification in recirculating aquaculture systems: the processes and players. Reviews in Aquaculture, 13. https://doi.org/10.1111/raq.12558
• Qi, W., Skov, P. V., Gregersen, K. J. de J., Mousavi, S., Pedersen, L.-F., & Mota, V. C. (2025). Estimating biofilm activity on biofilter elements in recirculating aquaculture systems (RAS) for rearing Atlantic salmon parr (Salmo salar) during operation with ozone and peracetic acid. Aquaculture, 594, 741381. https://doi.org/10.1016/j.aquaculture.2024.741381
• Remen, M., Sievers, M., Torgersen, T., & Oppedal, F. (2016). The oxygen threshold for maximal feed intake of Atlantic salmon post-smolts is highly temperature-dependent. Aquaculture, 464, 582-592. https://doi.org/10.1016/j.aquaculture.2016.07.037
• Rimstad, E., & Markussen, T. (2020). Infectious salmon anaemia virus—molecular biology and pathogenesis of the infection. Journal of Applied Microbiology, 129(1), 85–97. https://doi.org/10.1111/jam.14567
• Rimstad, E., & Mjaaland, S. (2002). Infectious salmon anaemia virus. APMIS, 110(4), 273–282. https://doi.org/10.1034/j.1600-0463.2002.100401.
• Rimstad, E., Dale, O. B., Dannevig, B. H., & Falk, K. (2011). Infectious Salmon Anaemia. In P. T. K. Woo & D. W. Bruno (Eds.), Fish diseases and disorders. Volume 3: Viral, bacterial and fungal infections (2nd ed., pp. 143–165). CABI. https://doi.org/10.1079/9781845935542.0143
• Robledo Gonzalez, L., Tat, R.P., Greaves, J. C., & Robinson, C.M. (2023). Viral–Bacterial Interactions That Impact Viral Thermostability and Transmission. Viruses, 15(12), 2415. https://doi.org/10.3390/v15122415
• Rojas-Tirado, P., Pedersen, P.B., & Pedersen, L.F. (2017). Bacterial activity dynamics in the water phase during start-up of recirculating aquaculture systems. Aquacultural Engineering, 78, 24–31. https://doi.org/10.1016/j.aquaeng.2016.09.004
• Roque d'Orbcastel, E., Blancheton, J.-P., & Belaud, A. (2009). Water quality and rainbow trout performance in a Danish Model Farm recirculating system: Comparison with a flow-through system. Aquacultural Engineering, 40(3), 135–143. https://doi.org/10.1016/j.aquaeng.2009.02.002
• Rurangwa, E., & Verdegem, M. C. (2015). Microorganisms in recirculating aquaculture systems and their management. Reviews in Aquaculture, 7(2), 117-130, https://doi.org/10.1111/raq.12057
• Schoina, E., Doulgeraki, A. I., Miliou, H., & Nychas, G.-J. E. (2022). Dynamics of water and biofilm bacterial community composition in a Mediterranean recirculation aquaculture system. Aquaculture Journal, 2(2), 164–179. https://doi.org/10.3390/aquacj2020008
• Schreier, H.J., Mirzoyan, N., & Saito, K. (2010). Microbial diversity of biological filters in recirculating aquaculture systems. Current Opinion in Biotechnology, 21(3), 318–325. https://doi.org/10.1016/j.copbio.2010.03.011

• Schryver, P., & Vadstein, O. (2014). Ecological theory as a foundation to control pathogenic invasion in aquaculture. The ISME Journal, 8(12), 2360–2368. https://doi.org/10.1038/ismej.2014.84
• Shinriki, N., Ishizaki, K., Ikehata, A., Nomura, A., & Mizuno, Y. (1978). Degradation of nucleic acid with ozone. Nucleic Acids Research, 5(Suppl 2), s303–s308. https://doi.org/10.1093/nar/1.suppl_2.s303
• Silverman, A.I., & Boehm, A.B. (2021). Systematic Review and Meta-Analysis of the Persistence of Enveloped Viruses in Environmental Waters and Wastewater in the Absence of Disinfectants. Environmental Science & Technology, 55(21), 14480-14493. https://doi.org/10.1021/acs.est.1c03977
• Snow, M., McKay, P., Mcbeath, A., Black, J., Doig, F., Kerr, R., Fraser, C., Nylund, A., & Devold, M. (2006). Development, application and validation of a Taqman real-time RT-PCR assay for the detection of infectious salmon anaemia virus (ISAV) in Atlantic salmon (Salmo salar). Developments in Biologicals, 126, 133-145; discussion 325-6. PMID: 17058489.
• Spiliotopoulou, A., Rojas-Tirado, P., Chhetri, R., Kaarsholm, K., Martin, R., Pedersen, P., Pedersen, L.-F., & Andersen, H. (2018). Ozonation control and effects of ozone on water quality in recirculating aquaculture systems. Water Research, 133, 289-298. https://doi.org/10.1016/j.watres.2018.01.032
• Stallknecht, D.E., & Brown, J.D. (2009). Tenacity of avian influenza viruses. Rev Sci Tech, 28(1), 59–67. https://doi.org/10.20506/rst.28.1.1880
• Standard - No. 7890-1 (ISO,1986) Available online: <https://www.iso.org/standard/14840.html > (accessed on 30 December 2024).
• Standard - No.6777-1 (ISO, 1984a) Available online: <https://www.iso.org/standard/13273.html > (accessed on 30 December 2024).
• Standard - No.7150-1 (ISO, 1984b) Available online: <https://www.iso.org/standard/13742.html> (accessed on 30 December 2024).
• Stewart, P.S., & Franklin, M.J. (2008). Physiological heterogeneity in biofilms. Nature Reviews Microbiology, 6, 199–210. https://doi.org/10.1038/nrmicro1838
• Su, X., Sutarlie, L., & Loh, X. J. (2020). Sensors, biosensors, and analytical technologies for aquaculture water quality. Research, 2020, 8272705. http://doi.org/10.34133/2020/8272705
• Summerfelt, S. T. (2003). Ozonation and UV irradiation—an introduction and examples of current applications. Aquacultural Engineering, 28(1-2), 21-36. https://doi.org/10.1016/S0144-8609(03)00020-5
• Summerfelt, S.T., Sharrer, M.J., Tsukuda, S.M., & Gearheart, M. (2009). Process requirements for achieving full-flow disinfection of recirculating water using ozonation and UV irradiation. Aquacultural Engineering, 40(1), 17–27. https://doi.org/10.1016/j.aquaeng.2008.10.002
• Sun, R., Wang, Q., Huang, Z., Zhan, M., Zhao, Z., Wang, B., Guo, M., Yuan, L., Shi, Z., Ouyang, G., & Ji, W. (2022). Comparative study on immune function of the head and trunk kidney in rainbow trout responding to IHNV infection. Viruses, 14(12), 2663. https://doi.org/10.3390/v14122663
• Suttle, C.A. (2007). Marine viruses—major players in the global ecosystem. Nature Reviews Microbiology, 5(10), 801-812. https://doi.org/10.1038/nrmicro1750
• Suurnäkki, S., Pulkkinen, J., Lindholm-Lehto, P., Tiirola, M., & Aalto, S. (2019). The effect of peracetic acid on microbial community, water quality, nitrification, and rainbow trout (Oncorhynchus mykiss) performance in recirculating aquaculture systems. Aquaculture, 516, 734534. https://doi.org/10.1016/j.aquaculture.2019.734534
• Tapia, E., & Kocan, R. (2013). Survival of Infectious Salmon Anemia Virus (ISAV) in freshwater and seawater at different temperatures. Bulletin of the European Association of Fish Pathologists, 33(1), 11-18.
• Tapia, E., Monti, G., Rozas-Serri, M., Sandaval, A., Gaete, A., Bohle, H., & Bustos, P. (2013). Assessment of the in vitro survival of the infectious salmon anaemia (ISAV) under different water types and temperature. Bulletin of the European Association of Fish Pathologists, 33, 3–12.

• Taylor, S. C., Nadeau, K., Abbasi, M., Lachance, C., Nguyen, M., & Fenrich, J. (2019). The ultimate qPCR experiment: Producing publication quality, reproducible data the first time. Trends in Biotechnology, 37(7), 761–774. https://doi.org/10.1016/j.tibtech.2018.12.002
• Teitge, F., Peppler, C., Steinhagen, D., & Jung-Schroers, V. (2020). Water disinfection by ozonation has advantages over UV irradiation in a brackish water recirculation aquaculture system for Pacific white shrimp (Litopenaeus vannamei). Journal of Fish Diseases, 43, 1259–1285. https://doi.org/10.1111/jfd.13238
• Thorud, K., & Djupvik, H. O. (1988). Infectious anaemia in Atlantic salmon (Salmo salar L.). Bulletin of the European Association of Fish Pathologists, 8, 109–111.
• Timmons, M., Guerdat, T., & Vinci, B. (2018). Recirculating Aquaculture (4th ed.) Ithaca Publishing, USA.
• Timmons, M.B., & Ebeling, J.M. (2010). Recirculating Aquaculture (2nd ed.). Cayuga Aqua Ventures, NRAC Publication No. 401-2010, Ithaca Publishing, USA.
• Tolentino, L. K., Pedro, C., Icamina, J., Navarro, J., Salvacion, L., Sobrevilla, G., Villanueva, A., Amado, T., Padilla, M., Madrigal, G. A., & Enriquez, L. A. (2021). Development of an IoT-based intensive aquaculture monitoring system with automatic water correction. International Journal of Computing and Digital Systems, 10, 1. http://dx.doi.org/10.12785/ijcds/1001120
• Tzafesta, E. & Shokri, M, (2025). The combined negative effect of temperature, UV radiation and salinity on eDNA detection: A global meta-analysis on aquatic ecosystems. Ecological Indicators, 176, 113669. https://doi.org/10.1016/j.ecolind.2025.113669
• Tziortzioti, C., Amaxilatis, D., Mavrommati, I., & Chatzigiannakis, I. (2019). IoT sensors in sea water environment: Ahoy! Experiences from a short summer trial. Electronic Notes in Theoretical Computer Science, 343, 117–130. https://doi.org/10.1016/j.entcs.2019.04.014
• United States Environmental Protection Agency, E. (2020). Disinfection Profiling and Benchmarking. Technical Guidance Manual. Available at: https://www.epa.gov/system/files/documents/2022- 02/disprof_bench_3rules_final_508.pdf (accessed: 10/11/2024).
• Van Rijn, J. (2013). Waste treatment in recirculating aquaculture systems. Aquacultural Engineering, 53, 49–56. https://doi.org/10.1016/j.aquaeng.2012.11.010
• Veterinærinstituttet Veileder: Metoder godkjent for desinfeksjon av vann til/fra akvakulturrelatert virksomhet (2017). Available online: https://www.vetinst.no/fagomrader/desinfeksjon/veileder-metoder-godkjent-for-desinfeksjon-av-vann-til-fra-akvakulturrelatert-virksomhet (accessed on 12 November 2024).
• Vike, S., Duesund, H., Andersen, L., & Nylund, A. (2014b). Release and survival of infectious salmon anaemia (ISA) virus during decomposition of Atlantic salmon (Salmo salar L.). Aquaculture, 420–421, 119–125. https://doi.org/10.1016/j.aquaculture.2013.09.043
• Vike, S., Nylund, S., & Nylund, A. (2009). ISA virus in Chile: Evidence of vertical transmission. Archives of Virology, 154(1), 1–8. https://doi.org/10.1007/s00705-008-0251-2
• Vike, S., Oelckers, K., Duesund, H., Erga, S.R., Gonzalez, J., Hamre, B., Frette, Ø., & Nylund, A. (2014a). Infectious salmon anemia (ISA) virus: Infectivity in seawater under different physical conditions. Journal of Aquatic Animal Health, 26(1), 33–42. https://doi.org/10.1080/08997659.2013.864720
• Waller, U. (2024). Critical assessment of the process and logic behind fish production in marine recirculating aquaculture systems. Fishes, 9(11), 431. https://doi.org/10.3390/fishes9110431
• Weli, S. C., Amundsen, M. M., Dale, O. B., & Patel, S. J. (2025). Characterization of infectious salmon anaemia virus survival and infectivity in seawater. Aquaculture Reports, 40, 102504. https://doi.org/10.1016/j.aqrep.2024.102504
• Weli, S. C., Bernhardt, L. V., Qviller, L., Dale, O. B., & Lillehaug, A. (2021). Infectious salmon anemia virus shedding from infected Atlantic salmon (Salmo salar L.)—Application of a droplet digital PCR assay for virus quantification in seawater. Viruses, 13(9), 1770. https://doi.org/10.3390/v13091770

• Weli, S. C., Dale, O. B., Hansen, H., Gjessing, M. C., Ronneberg, L. B., & Falk, K. (2017). A case study of Desmozoon lepeophtherii infection in farmed Atlantic salmon associated with gill disease, peritonitis, intestinal infection, stunted growth, and increased mortality. Parasites & Vectors, 10(1), 370. https://doi.org/10.1186/s13071-017-2303-5
• WOAH - World Organisation for Animal Health. (2009). Manual of Diagnostic Tests for Aquatic Animals: Infectious Salmon Anaemia. Paris, France: WOAH. Available online: <https://www.woah.org> (accessed on 20 November 2024).
• WOAH - World Organisation for Animal Health. (2021). Infection with HRP-deleted or HPR0 infectious salmon anaemia virus. Manual of Diagnostic Tests for Aquatic Animals, Chapter 2.3.4, 282–296. Available online: https://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/current/2.3.04_ISAV.pdf (accessed on 09/12/2024).
• Wommack, K. E., & Colwell, R. R. (2000). Virioplankton: Viruses in aquatic ecosystems. Microbiology and Molecular Biology Reviews, 64(1), 69–114. https://doi.org/10.1128/MMBR.64.1.69-114.2000
• Xu, W., Xu, Y., Su, H., Hu, X., Yang, K., Wen, G., & Cao, Y. (2020). Characteristics of ammonia removal and nitrifying microbial communities in a hybrid biofloc-RAS for intensive Litopenaeus vannamei culture: A pilot-scale study. Water, 12, 3000. https://doi.org/10.3390/w12113000
• Yanong, R. P. (2015). Fish health management considerations in recirculating aquaculture systems—Part 2: Pathogens. Circular 121, 1–8.
• Yusoff, F. M., Umi, W. A. D., Ramli, N. M., & Harun, R. (2024). Water quality management in aquaculture. Cambridge Prisms: Water, 2, 1–61. https://doi.org/10.1017/wat.2024.6
• Zimmer, J. L., & Slawson, R. M. (2002). Potential repair of Escherichia coli DNA following exposure to UV radiation from both medium- and low-pressure UV sources used in drinking water treatment. Applied and Environmental Microbiology, 68(7), 3293–3299. https://doi.org/10.1128/AEM.68.7.3293-3299.2002
• Gutiérrez, X. A.; Kolarevic, J.; Takle, H. R.; Bæverfjord, G.; Ytteborg, E.; Terjesen, B. F. Effects of chronic sub-lethal nitrite exposure at high water chloride concentration on Atlantic salmon (Salmo salar, Linnaeus 1758) parr. Aquaculture Research 2019, 50 (9), 2687–2697. https://doi.org/10.1111/are.14226

8. Attachments

Attachment 1

This work was presented at Frisk Fisk conference, which took place on 11st and 12nd of March 2025.

Abstract for the Frisk Fisk conference: https://www.tekna.no/friskfisk/Info/?info=222857#effektavbrakkleggi.

Sonal Patel, Effekt av brakklegging og andre biosikkherhetsstrategier på ILAV-HPRdel i et kontrollert RAS forsøk

Type bidrag: Innlegg

Firma: Veterinærinstituttet

Medforfattere: Simon Weli (VI), Marta Guimarães Moutinho (VI, NIVA, Univeristy of Porto), Duncan Colquhoun (VI), Paulo Mira Fernandes (NIVA), Ole-Kristian Hess-Erga (NIVA), Sigmund Sevatdal (Veso Vikan), Sondre Veberg Larsen (Akvaplan niva), Melanie Andrews (Marine RASLab), Reidar Handegaard (Marine RASLab), Håkon Dale (UiB), Sonal Patel (VI)

Finansiert av: FHF- prosjektet BRAS (901792)

Kategori: Virussykdommer, Forebyggende helsearbeid og bekjempelsesstrategier, Settefisk og landanlegg

Fravær av sykdom er en forutsetning for bærekraftig og moderne produksjon av fisk. Sykdomsutbrudd og tilstedeværelse av patogene mikroorganismer i RAS kan resultere i en rekke utfordringer og føre til fiskedød og redusert fiskevelferd i den aktuelle fiskepopulasjonen og i senere livsfaser. I det FHF-finansierte prosjektet BRAS (901792) har vi undersøkt etablering, overlevelse og inaktivering av ILAV-HPRdel i et kontrollert forsøk med RAS. Effekten av bioskkerhetsstrategiene, brakklegging, vask og ozonering på ILAV-HPRdel ble karakterisert, samt om brakkleggingstiden påvirket risiko for smitte av naive fisk. Det ble satt opp tre kar med brakkvann for å simulere vanlige produksjonsbetingelser i næringen. Hvert kar var koblet til hver sin RAS-enhet med modnede biolegemer. Laksesmolt ble introdusert til hvert kar, akklimatisert og infisert i.p. med ILAV-HRPdel. Vannkvaliteten ble overvåket og det ble tatt svaberprøver av karvegg, prøver av vann, biolegemer og fisk for å bekrefte virusutskillelse til karmiljøet og om ILAV-HPRdel var til stede i biofilm. All fisk døde under den viremiske perioden, og innen to uker etter at fisk var infisert, ble første delen av forsøket avsluttet. ILAV-HPRdel RNA kunne påvises ved hjelp av PCR i de fleste prøver tatt i denne perioden. Neste fase hvor forskjellige biosikkerhetsstrategier skulle undersøkes ble startet umiddelbart etter. Biolegemene fra hver RAS-enhet ble fordelt i tre reaktorer med lufting, hvor de tre biosikkerhetstiltakene ble testet. I fiskekarene ble effekten av brakkleggingstid testet ved å re-introdusere naive laksesmolt hhv. etter 3, 7 og 10 dager. Resultatene fra forsøket viser at ILAV-HPRdel RNA kan påvises i RAS selv etter at fisk er fjernet fra systemet, og at noen av tiltakene er mer effektive enn andre.

Attachment 2

In addition to the thesis work, competences were developed in the following area: to the thesis work, competences in “DNA Isolation, 16S rRNA gene amplification, and Illumina sequencing for microbiome of biochips” were learned. The electrophoresis showing preliminary results can be seen in Figure 16.

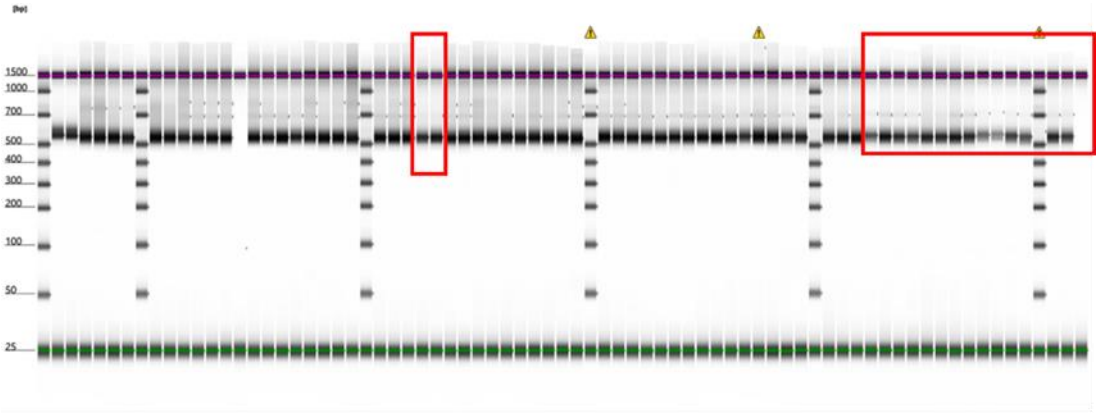


Figure 16 - Agarose gel electrophoresis of biochips samples. From left to right: First red square – reactor ozone samples; Second red square – last days samples