Norwegian University of Life Sciences

Master's Thesis 2024 60 ECTS
Faculty of Science and Technology

Evaluation of Disinfectants in RAS Water

Tabassum Ali
Master of Science in Aquatic Food Production

Acknowledgment

Firstly, no amount of words can fathom how incredibly grateful I am to have so many supportive and wonderful people who helped me in this phase of my life. I am always indebted to their kindness and encouragement.

I would like to thank my main supervisor, Vasco C. Mota for guiding me, teaching me with utmost care, and being incredibly patient with me. I would also like to thank my co-supervisor, Carlo Lazado for giving me the opportunity to witness and learn firsthand about Atlantic salmon in RAS. The experience in Nofima and Havbruksstasjonen i Tromsø was one of a kind and I had never imagined encountering it, it was made possible because of him. Being under the tutelage of my amazing supervisors made it possible for me to move forward and gain invaluable insights into my field of study.

Special thanks to Lena Oksdøl Foseid, Even Bull Holmen, and Dana Byrtusova for giving me their time and showing me the ropes in the lab. I wouldn't have been able to carry out the experiment on my own if it wasn't for their immeasurable help. I would love to express my gratitude to my remarkable friends (in no particular order) Andres R. Diaz V., Tahsin M. Onee, Shabib Ally, Booshra Ahmed, Ayesha Siddiqua, Hiruni Hewage, Alvira F. Ria, Daniela C. Quevedo, and Katla M. Meyer. Their unwavering support, liveliness, and companionship gave me the strength to go forward.

Last but not least, I would like to thank my family, especially my parents, Atiya Ali and Mohammad Ali. Their unconditional love, nurturing, and countless compromises led me to where I am today. Ammu, Abbu, I am finally here and it's all because of you, Tabir is a TTMP now.

Abstract

Recirculating aquaculture systems (RAS) are being adopted by many Norwegian aquaculture companies because they are considered more sustainable, provide high biosecurity, and have a more controlled environment. One of the key characteristics of RAS is water treatment and reuse. Despite having high biosecurity, if a pathogen gains access to the system or an opportunistic pathogen emerges, the water circulation can spread the pathogens which can lead to development of fish disease. The Norwegian Food Safety Authority (Mattilsynet) enlisted several disinfectants that are approved for use in aquaculture. Seven approved disinfectants were assessed in this experiment. These include three peracetic acid (PAA) based disinfectants (Addi Aqua, Aqua Des, and Perfectoxid), two quaternary ammonium compound (QAC) based disinfectants (Virkon Aquatic and Virocid), one hydrogen peroxide (H_2O_2) based disinfectant (Free Bac ®35) and, one chlorine dioxide (ClO_2) based disinfectant (Life Clean). The efficacy of these disinfectants has been demonstrated in aquaculture surfaces and auxiliary equipment but their effectiveness in water disinfection is not fully understood yet. Therefore, the objective of this study was to test whether the above seven disinfectants had an impact on the ammonia, pH, and bacterial load of RAS biofilter water. Six small-scale biofilters were set up to simulate the MBBR in RAS. The cultured water and biomedia were collected from the research facility, Havbruksstasjonen i Tromsø, Kårvika. 8 groups of samples were created, 1 control (no disinfectants), and the other 7 were designated to treatment with the aforementioned disinfectants. The samples were taken in triplicates. The test was carried out in 35 days (1 replicate of each disinfectant carried out each week). The recommended dosage and contact time of the respective manufacturers were used in the experiment. Ammonium and pH levels were measured before and after the disinfectants were administered. Trypticase Soya Agar (TSA) and Cefsulodin-Irgasan-Novobiocin (CIN) plates were used to count bacterial colonies (CFU) before and after disinfection. The results of the study were that all three PAA-based disinfectants caused a 50% decrease in pH and a 100% reduction in ammonium levels and Perfectoxid seemed to have the strongest effect. QAC-based disinfectants also caused a considerable drop in the water's pH (Virocid 12%, Virkon: 69% reduction) and ammonium levels (Virocid: 93%, Virkon: 66% reduction). Contrastingly, H_2O_2-based disinfectant (Free Bac ®35) and ClO_2-based disinfectant (Life Clean) seemed to have no considerable impact on ammonium (Free Bac ®35: 4% increase, Life Clean: 6% decrease) or pH (Free Bac ®35: 2% and Life Clean: 1% reduction). All PAA-based and QAC-based disinfectants have caused a 100% reduction of bacterial colonies on both TSA and CIN plates. However, Free Bac ®35 and Life Clean did not cause any reduction in the bacterial load on either of the plates. In conclusion, 5 out of 7 approved disinfectants (Addi Aqua, Aqua Des, Perfectoxid, Virkon Aquatic, and Virocid) were effective in disinfecting the water whereas Free Bac ®35 and Life Clean were ineffective. These findings can be useful in future research to determine what factors could affect their inefficacy.

Table of Contents

List of Figures

List of Tables

Abbreviations

ADBAC: Alkyl Dimethyl Benzyl Ammonium Chloride

CFU: Colony Forming Units

CIN: Cefsulodin-Irgasan-Novobiocin

ClO_2: Chlorine Dioxide

DDAC: Dodecyl Dimethyl Ammonium Chloride

H_2O_2: Hydrogen Peroxide

MBBR: Moving Bed Bioreactors

$NaHCO_3$: Sodium Bicarbonate

NH_4Cl: Ammonium Chloride

PAA: Peracetic Acid

QAC: Quaternary Ammonium Compound

RAS: Recirculating Aquaculture System

TSA: Trypticase Soya Agar

1. Literature Review

1.1 Atlantic Salmon Aquaculture in Norway

Salmon is the most farmed fish in Norway. According to the Norwegian Directorate of Fisheries (2023), the fish farms yielded livestock of around 435 million salmon in 2020. Since its inception in the 1970s, the Norwegian aquaculture industry has grown exponentially. For instance, the production of farmed salmon was only 50 tonnes in 1970 whereas it was almost 860 000 tonnes in 2009. (FAO, 2011)

Atlantic salmon (*Salmo salar*) belongs to the salmonid species and is located in the subarctic and temperate regions of the North Atlantic Ocean. (Finstad, Armstrong & Nislow, 2010). Atlantic salmon is also anadromous and its first life stages (egg, larva, and parr) occur in freshwater. Following the parr phase, the Atlantic salmon undergoes a significant physiological and morphological change. This change is termed smoltification and prepares the fish for life at sea. The process increases the overall metabolism, salinity tolerance, silvering, schooling and downstream migratory behavior, and darkening of fin margins. (Björnsson, Stefansson & McCormick, 2011).

According to a report by MOWI (2023), approximately farmed salmon accounts for 80% of the global salmon harvest. Salmon farming is mainly carried out in sheltered waters such as bays or fjords. The majority of farmed salmon comes from Norway, Scotland, Canada, and Chile. Due to its large content of protein, minerals, vitamins, and omega-3 fatty acids, salmon is considered to be incredibly nutritious and healthy. Atlantic salmon is the largest species of salmonids, in terms of quantities. Around 2.5 million tonnes (GWT) of Atlantic salmon were harvested in 2021. (Figure 1)

Farmed salmon can be considered a versatile food product as can be consumed in various ways such as fresh and raw in sushi, smoked, and even in ready-made meals. Atlantic salmon are typically farmed in the seas because of biological constraints, temperature requirements, and other natural parameters. Hence, the majority of them are produced in Norway, Chile, the UK, North America, the Faroe Islands, Iceland, Ireland, New Zealand, and Tasmania. (MOWI, 2023).

As portrayed in Figure 2, since 1995, the supply of Atlantic salmon has elevated by 543% (annual growth of 7%). In the period between 2012 and 2022, annual growth was 4 %. According

to MOWI (2023), it is expected for the growth to be generally stable at 3% from 2022 to 2027. The reason behind this progression is that the industry has attained a production level where the biological restraints are constantly being pushed. Moreover, measures have been implemented to minimize the salmon industry's biological footprint and hence, the future expansion will no longer be solely influenced by the industry and regulators. Such measures call for advancement in technology, improvement in fish feed, manufacturing protocols, updated regulations, and so on.

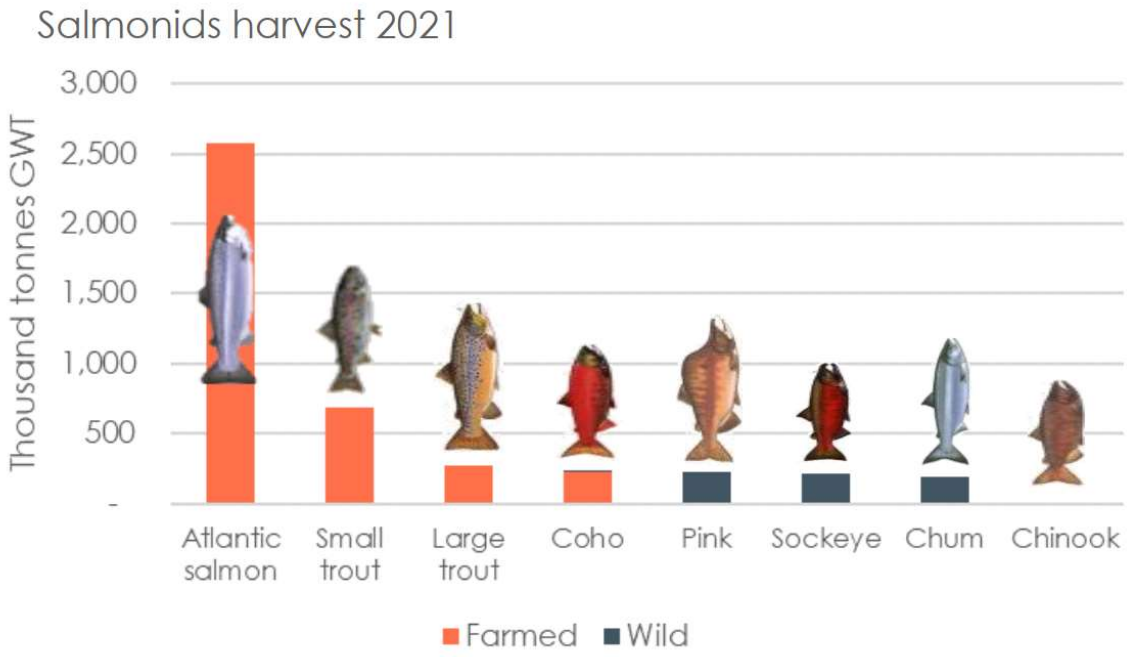


Figure 1: Salmonid Harvest 2021(MOWI, 2023)

The conventional way of producing Atlantic salmon has two phases: freshwater and seawater phases. At first, salmon fry are hatched from eggs in hatcheries and they grow into juveniles and smolts in land-based farms. When they develop into post-smolts, they are transferred to the sea in open net cages. (Bergheim et al., 2009). Land-based farms typically have intensive production in tanks and usually use more technical equipment than sea cage farms. (Hersoug, Mikkelsen & Osmundsen, 2021).The main components of these farms are water treatment facilities, water inlets and outlets, waste treatment, and monitoring setups (Lekang, 2013).

There are two types of land-based farms: flow-through system and recirculating aquaculture system (RAS). In a flow-through system, the water is continuously supplied to the system which passes through once and is treated before being discharged (Bergheim et al., 2009). On the other hand, RAS recirculates and reuses water in a closed-loop system.

However, there is a lack of suitable available sites in the sea, for expanding sea cage production and an increase in biological constraints such as sea lice and various disease-causing agents. Such predicaments are hindrances in conventional Atlantic salmon production. combination of land-based and sea-based production.(Aleynik, Adams & Davidson, 2022) Norwegian authorities are actively implementing new initiatives to curb such deterrents. Such instances are the development of a license plan for offshore Atlantic salmon production in cages, on-shore production in semi-closed facilities, and in-shore systems like RAS. (Øvrebø et al., 2022)

There are other initiatives adopted such as converting flow-through systems to RAS in land-based production, rearing larger post-smolts (150g - 600g) on land-based systems, and several plannings, operations, and construction of RAS facilities to farm approximately 137,000 tonnes market size fish in Norway. (Tian & Dong, 2023)

RAS is considered more sustainable for intensive aquaculture production due to its reduced water usage. (Bregnballe, 2022). There has been an increased implementation of RASs for producing smolts and post-smolts, partially due to high biosecurity, flexible location, and lesser adverse impact on the environment amongst many other advantages of a controlled production setting. (Lazado & Good, 2021; Martins et al., 2010; Mota et al., 2022)

It should be noted that the majority of the research publications on pathogen outbreaks in aquaculture are based on flow-through systems (FTS). Despite contributing information on the infection dynamics of numerous pathogens, it can be assumed that applying the findings of FTS in RAS settings is not simple since the biology and chemistry of the RAS environment are far more complex than FTS. On the contrary, this feature is generally based on conjectures and anecdotes as there is a lack of empirical evidence produced in controlled conditions.

Due to the rising concern regarding the biosecurity and efficacy of disinfectants without compromising the beneficial nitrifying microbes in the biofilter of RAS, this study will focus on recirculating the aquaculture system (RAS) as there is also limited research on the pathogen dynamics in this production setting.

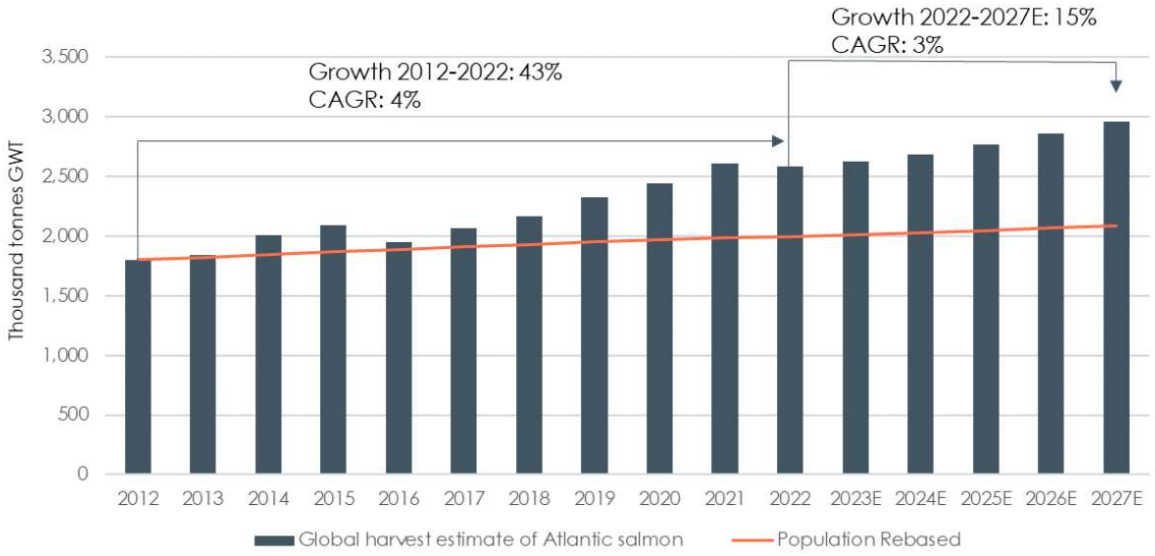


Figure 2: Growth Rate of Atlantic Salmon Harvest (MOWI, 2023)

1.2 Recirculating Aquaculture System (RAS)

The basic concept of Recirculating Aquaculture Systems (RAS) is to recycle the wastewater and accordingly, various water treatments are implemented. (Figure 3). The wastewater is typically subjected to various water treatments such as solid waste removal, carbon dioxide removal, disinfection, etc. Since building RAS facilities mostly aims for the highest degree of biosecurity and complete control of the environment there is a high rate of recirculation, of around 95-99% of the water. (Murray, Bostock & Fletcher, 2014)

Recirculating aquaculture systems have started to replace land-based flow-through systems. Closed RAS systems are becoming preferable as they are a better alternative to open sea cages. (Blancheton et al., 2007). RAS has been used for smolt and post-smolt production for the past years. (Mota et al., 2022). The Norwegian Ministry of Fisheries granted licenses for the production of smolt weighing up to 1000g under controlled conditions, in closed or semi-closed tanks on land or the sea. The smolt can be reared longer in a closed and protected system like RAS before being released into the sea. This is advantageous as it decreases the time spent in open water and exposure to sea lice. (Lekang, 2013). Hence, improving overall fish robustness, diminishing mortality in the sea phase, and reducing the general production time. The vital functions of RAS, as stated by Murray, Bostock and Fletcher (2014) are the following:

1. Allocation of the fish in a suitable physical environment in accordance with space, stock density, and water quality parameters.
2. Protection of the fish stock from pathogenic infection.
3. Ensuring the physiological requirements of the fish (mainly nutritious feed and oxygen)
4. Removal of metabolic wastes such as feces, ammonia, and carbon dioxide from the fish
5. Removal of uneaten feed and disintegrated organic compounds.
6. Maintenance of optimum temperature and water chemistry parameters within acceptable limits.

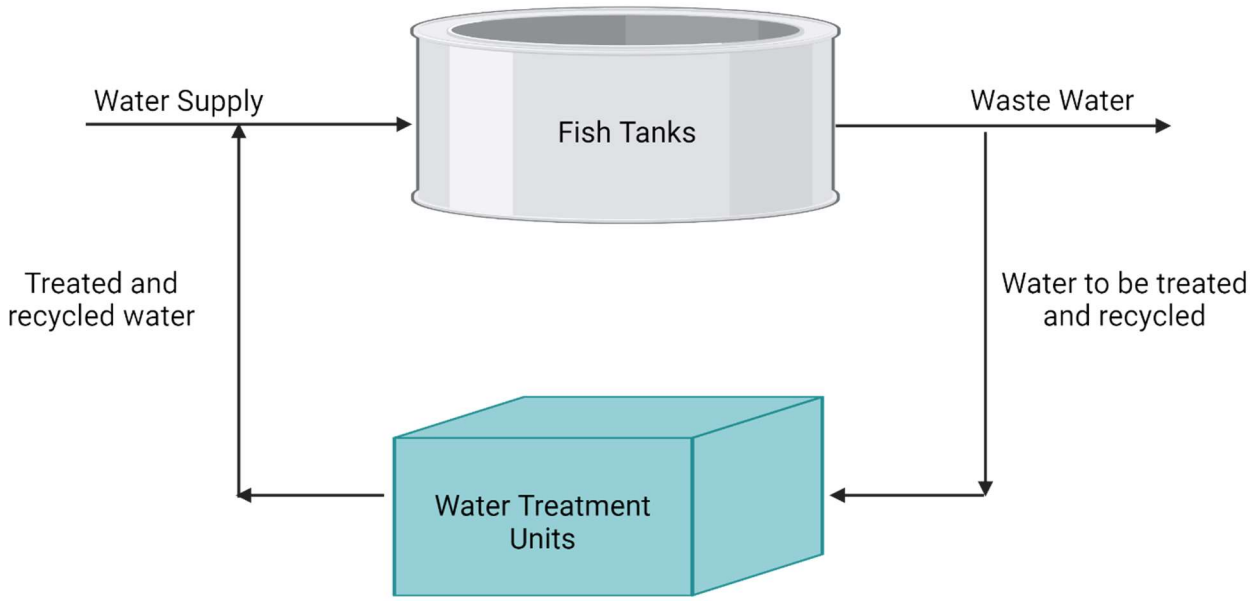


Figure 3: Basic Overview of RAS. Created with BioRender.com

Such functions are carried out by specific units of RAS. These common functions are illustrated in Figure 4. Zhang et al. (2011) discovered that RAS was built to reduce the water pollution caused by the aquaculture industry's rapid growth over the past decade. RAS technology is utilized for treating the effluent from the culture water, removing the toxic nitrogenous waste, feces, and uneaten feed, and then pumping it back into the system. (Taufik et al., 2023). RAS mainly comprises specially designed fish tanks, mechanical filtration, biological filtration, gas control, and temperature regulation. The effluent from the culture tanks in RAS is reused after being treated under a few processes such as solids removal by using a drum filter. The most crucial water quality

parameters to control in RAS are the N-compounds and solid content as they can be deleterious to the fish being farmed. (Holan, 2013).

The solids are removed from the water by particle/mechanical filtration. The ammonia is removed from the water by the biofilter and is the biological water treatment of RAS. The gas

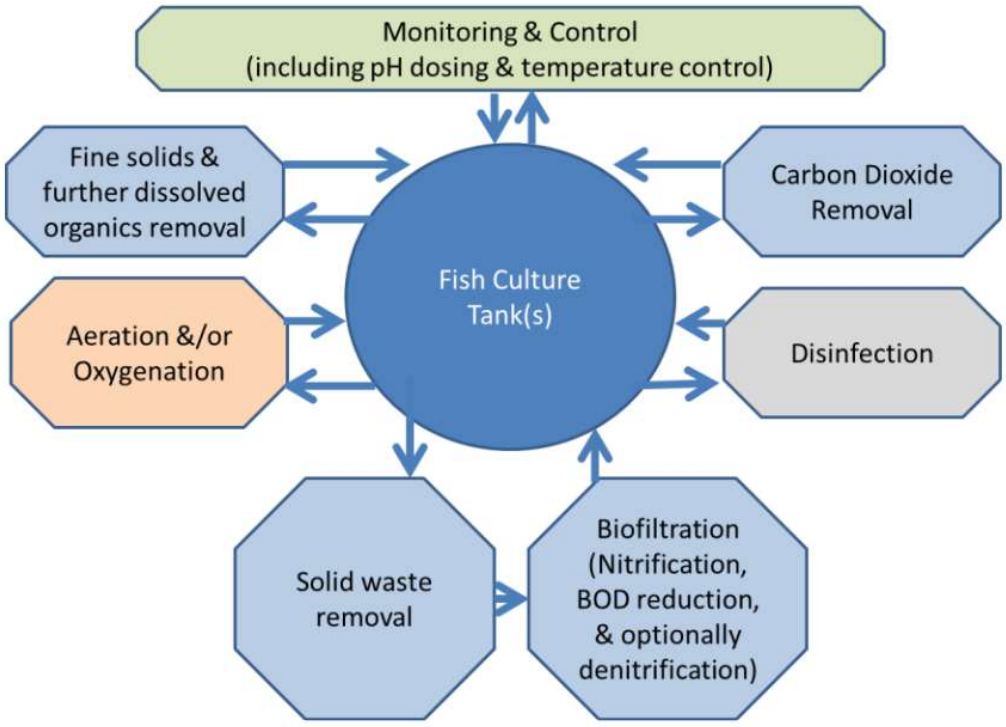


Figure 4: Common unit processes used in RAS. (Murray, Bostock & Fletcher, 2014)

control is mainly the degassing of carbon dioxide and pure oxygen in the water. Pumps are used to circulate the water in RAS and hence make production in RAS an energy-consuming process.

The RAS implementation for Atlantic salmon production has several benefits. Rearing in RAS enables control over the environment such as the quality of incoming water. Hence diseases and exposure to parasites are alleviated, and fish escapes are avoided. In comparison with the flow-through systems, RAS has more advantages as they enable heat conservation, reduced water consumption, better biosecurity, alleviation of issues related to pollution, and growth rate optimization. (Holan, 2013)

RAS has certain characteristics that enhance the mutualistic interactions between the fish and microbe. (Dahle et al., 2023; Vadstein et al., 2018). It also boosts microbial stability. Strong competition between the bacteria is established by the system's large surface area, amplified total

hydraulic retention time, and comparably stable organic loading. Such heightened contest for limited resources leads to the formation of a stable community dominated by specialized microbes growing at a gradual rate, at the expense of opportunistic microorganisms. (Attramadal et al., 2021; Dahle et al., 2023)

According to Bregnballe (2022), RAS is considered to be the most environmentally friendly method of fish production at a commercially viable level. This is because there is more control over biological pollution. Furthermore, over 90% of the system volume per unit of time is reused and each treatment process decreases the system water exchange. (Holan, Good & Powell, 2020). However, RAS also has some concerning challenges such as the requirement for more skilled personnel, water quality control, and increased production cost. The production cost is high due to the numerous water treatments and water pumps which have a high energy demand. (Lekang, 2013). Rearing fish normally requires a high feeding rate and density, resulting in the build-up of small particles and organic matter. Such accumulation can provide favorable conditions for the growth of bacteria.

Moreover, oxygen consumption and waste loading on the system can get elevated by the heterotrophic bacteria breaking down the organic matter. If not monitored, high amounts of organic matter can lead to diminished efficiency of the biofilter's nitrification due to the heterotrophic bacteria dominating the nitrifiers. (Dahle et al., 2023; Schreier, Mirzoyan & Saito, 2010). High stocking densities and improper water treatment can cause outbreaks of diseases. Managing and controlling disease outbreaks can be immensely challenging in RAS. Despite the reduced likeliness of pathogens entering the facility, if pathogenic bacteria do gain access to the system, they can adhere to the biofilm and proliferate, causing recurring diseases if not eliminated as biofilm removal is quite difficult in RAS. (Lekang, 2013). Additionally, a high rate of mortalities in RAS can be caused by elevated levels of suspended solids, carbon dioxide, nitrites, and ammonia. (Hjeltnes, 2012)

1.3 Water Quality in RAS

The most crucial factors of water quality maintenance are its source, fish density or load in the system, feeding rates, and biofilter capacity. Water from the source should be assessed and tested before being introduced into the rearing facility. Water from different resources might have various potential issues that must be considered. For instance, municipal waters in certain regions might contain chlorines or chloramines that can be toxic to fish. Despite the initial water quality of a system can be evaluated based on its source and the water treatment routines, in the long run, the water quality of RAS relies mainly on the levels of ammonia. (Yanong, 2015) As mentioned earlier, high levels of ammonia can be lethal to fish. It can take time for the nitrifiers in biofilters to initially convert the ammonia into nontoxic nitrate as they require 3 to 8 weeks to become established. Hence, specialists in aquaculture often accelerate the establishment of the biofilter's microbial community before introducing the fish, by adding ammonia directly into the system or by implanting the biofilter with bacteria from a healthy and already established community in a credible commercial source.(Yanong, 2015)

It is essential to maintain good water quality in recirculating aquaculture systems (RAS) to ensure proper rearing and growth of the fish. As stated by Su, Sutarlie and Loh (2020) water quality can be put into categories that include physical parameters, pathogens, and organic and biological contaminants. For monitoring the water quality, the main parameters are pH, temperature, salinity, dissolved oxygen levels, salinity, and oxidation-reduction potential (ORP/Redox). Optimal water quality or the parameters in the ideal range can fortify fish health, improve growth, and reduce the incidence of diseases.

Feed composition and loading also affect the water quality of RAS. Removal of particulate matter and dissolved compounds is quite arduous. The build-up of or even the mere presence of such elements can be a substrate for microbial growth, making the entire system susceptible to diseases. These microbes can cause undesirable flavor of the fish flesh. Moreover, such microorganisms can also be pathogens and increase mortality. Besides causing ailments, large populations of microbes can also deplete the oxygen levels of fish, causing more distress. (Lindholm-Lehto, 2023)

Typically, fish are often farmed in high densities in RAS. This causes the accumulation of nutrients and various organic compounds. Such components need to be monitored and maintained.

However, the available tools are usually complicated or include a significant delay between sampling and the results. There are no established regulations for which parameters to implement. Each farmer has their own customized system and parameters for maintaining the water quality. Moreover, there are no set guidelines regarding acceptable fluctuations and ranges. (Lindholm-Lehto, 2023)

The entry of obligate pathogens can be minimized or obliterated with proper biosecurity protocols such as implementing certain pathogen-free eggs, safe sources of groundwater, and secured facilities/buildings with limited access to employees following strict biosecurity measures. Obligate pathogens are the microbes that need a host cell to proliferate. Parasites like *Myxobolus cerebralis*, all major fish viruses such as Infectious Pancreatic Necrotic Virus (IPNV), and particular bacteria like *Yersinia ruckeri* are such obligate disease-causing agents of fish. (Holan, Good & Powell, 2020)

In contrast, opportunistic fish pathogens are causative agents of diseases that can reproduce in and sustain in an aquatic environment. They are usually associated with clinical diseases only when the host and/or certain environmental factors elevate their virulence and proliferation. In the enclosed containment of RAS, the obligate pathogen exposure can be removed. However, opportunistic pathogens should always be taken into account as potential risks to fish health. This is especially critical in RAS settings with the recycling of water since there is potential for the pathogens to build up to hazardous levels. (Holan, Good & Powell, 2020).

1.4 Disinfection in RAS

Every aquaculture facility has disinfection in their biosecurity measures as it is vital for reducing the probabilities of pathogen entry and endemic within the fish farms. However, there is a general deficit in established and documented disinfection strategies in salmonid aquaculture. The top three criteria for selecting a disinfectant in Norway are effectiveness against pathogens, ease of application, and user safety. (Lazado & Good, 2021).

In RAS, biosecurity is generally high but concern regarding the pathogen outbreaks has been surging. This triggered the necessity for disinfection protocols as preventive measures. Toxic nitrogenous compounds of RAS are mainly removed by the biofilter. However, some doubts are arising regarding whether the biofilter serves as a reservoir for pathogens. Hence, a strategy of

chemical disinfection between fish batches has been proposed for commercial production. As Hofstad (2023) stated in their study, the dynamics of the microbial flora of the RAS are influenced by biofilter disinfection but, how it affects the opportunistic pathogens is yet to be fully understood.

RASs possess numerous units comprising pipes, culture tanks, and mechanical and biological filtration that can harbor pathogens, acting as a reservoir if not maintained. Such instances necessitate the application of disinfection strategies. (Timmons, Guerdat & Vinci, 2018). It is ideal for applying a disinfection protocol with high efficacy without having any deleterious effect on the fish's health and well-being, boosting more favorable conditions for fish farming. Furthermore, the biofiltration unit relies on certain bacterial strains for the conversion of the toxic ammonia to the less toxic nitrate. It is also desirable to have the disinfectants not mitigate the biological filter's nitrification, making the circumstances a tad complex. (Mota, Eggen & Lazado, 2022; Timmons, Guerdat & Vinci, 2018)

The disinfection of RAS is carried out in the following components of the facility:

1. Water treatment unit of intake water to the facility.
2. Entire circuit or loop of RAS water
3. RAS surfaces and ancillary equipment
4. End of the circuit/pipe

Disinfection solutions specialized for RAS can be classified as continuous water disinfection and periodical disinfection of ancillary equipment and surfaces. The latter generally involves using chemical disinfectants such as peracetic acid (PAA), formalin, hydrogen peroxide (H_2O_2), copper sulfate, and chloramine-T on surfaces. (Pedersen, 2012). Either of the solutions or a combination of the two are implemented in Norwegian RAS currently. (Mota, Eggen & Lazado, 2022).

These chemicals are very effective against a broad range of pathogens but can also affect the fish's health and biofilter performance. Hence, chemical disinfection is generally applied at the end of the production phase without fish in the system. (Mota, Eggen & Lazado, 2022; Timmons, Guerdat & Vinci, 2018). The continuous water disinfection eliminates pathogens from the intake

water before entering the RAS system. This approach is also used for the water coming out of the system before being reused. (Timmons, Guerdat & Vinci, 2018).

For the elimination of pathogens, the intake water is subjected to mechanical filtration to remove particulates.(Schneider et al., 2005) Then continuous water disinfection is carried out which typically involves exposing the water to UV radiation and ozone (O_3). The UV destroys the microbial DNA and consequently, inactivates the microorganisms. The ozone oxidizes and kills the pathogens, it also improves water quality.(Powell & Scolding, 2018) . On the contrary, direct exposure to ozone in circulating water might be hazardous to both humans and fish because of the ozone-produced oxidants toxicity. (Lazado et al., 2021).

A combination of ozone with UV irradiation seems to be a promising approach. However, Stiller et al. (2020) stated that its implementation can result in deteriorating fish health, and even mortality, if there's a failure to monitor the ozone system and understand the complex water chemistry. Nonetheless, numerous studies and commercial applications have utilized ozone effectively in RAS freshwater, demonstrating advantages like improved water quality. (Davidson et al., 2021)

Ancillary equipment is objects and tools that support daily activities in production facilities like RAS. Such items may include buckets, ladders, nets, boots, transport containers, and so on. Besides harboring pathogens and serving as a reservoir, these materials can also act as vectors for opportunistic pathogens. According to a study by Lazado and Good (2021), six out of nine Norwegian aquaculture facilities use both H_2O_2 and PAA for disinfecting their ancillary equipment. They also use wipes or sprays of 70% ethanol on equipment that is small and with specified requirements such as probes. In the study, six facilities out of nine utilized different disinfectants for different ancillary equipment and implemented a designated cleaning/disinfection procedure for separate ancillary items. The findings of the study were that chlorine and peracetic acid (PAA) are the most used disinfectants for ancillary equipment disinfection in Norway, where PAA is the most frequently cited. (Figure 5)

In Norway, safe drinking water and food for consumers are ensured and monitored by the national governing body, the Norwegian Food Safety Authority (Mattilsynet). Mattilsynet enlisted several disinfectants that are approved for aquaculture in Norway. Such disinfectants are ADDI Aqua, Aqua Des, Perfectoxid, and Virocid. (Mattilsynet, s.a.)

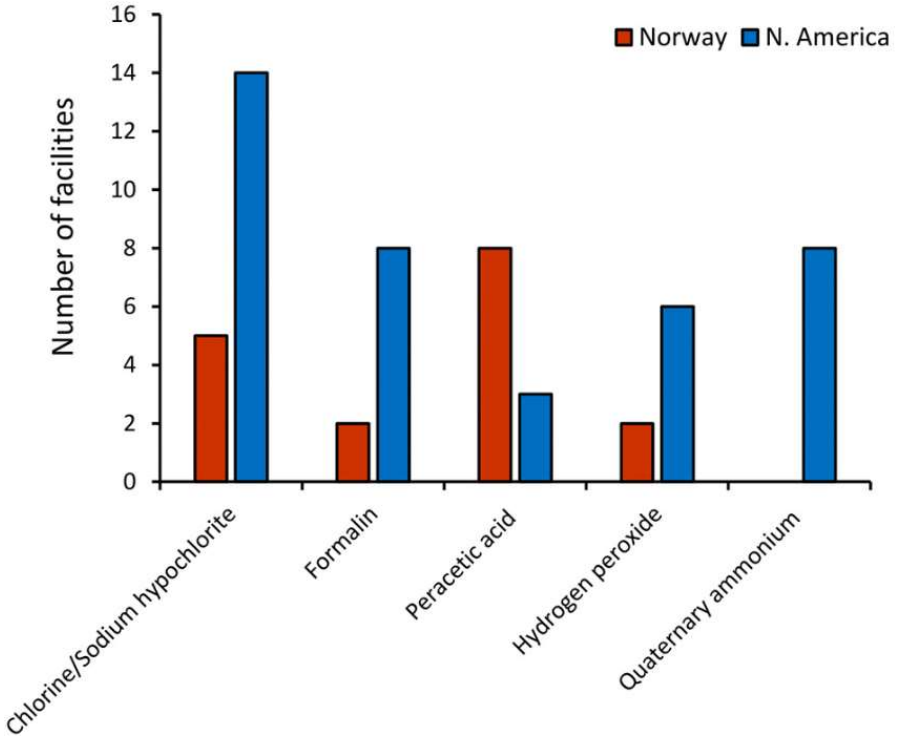


Figure 5: Summary of chemical disinfectants used in land-based RAS facilities(Lazado & Good, 2021)

1.4.1 Peracetic Acid-Based Disinfectants

Perfectoxid, ADDI aqua, and Aqua Des are peracetic acid (PAA) based disinfectants. PAA is the peroxide of acetic acid. It is a powerful oxidant and disinfectant with a wide spectrum of antimicrobial activity. It is commercially available in a quaternary equilibrium mixture of hydrogen peroxide, water, and acetic acid:

$CH_3COOH + H_2O_2 \rightleftharpoons CH_3CO_3H + H_2O$

Peracetic acid-based disinfectants are typically effective against a large range of bacteria, both gram-negative and gram-positive bacteria (mostly heterotrophs) They are also fungicidal and virucidal. (Kitis, 2004). This group of disinfectants is preferred by most fish-rearing facilities due to its effectiveness at low doses, fast decay, and lack of toxic residuals. They have active antimicrobial and parasiticidal efficacy over a broad range of temperatures, including those below 10°C. (Colgan & Gehr, 2001; Kitis, 2004; Moe Føre, Dahle & Gaarder, 2018; Pedersen et al., 2009; Pedersen & Lazado, 2020).

Both PAA and hydrogen peroxide (H_2O_2) have robust antimicrobial properties. However, PAA is a more potent disinfecting agent than hydrogen peroxide. Moreover, for the same degree of disinfection, hydrogen peroxide requires much higher doses than PAA. However, the combination of hydrogen peroxide and PAA has been found to have the most efficacy or more synergistic.(Pedersen et al., 2009).

Peracetic acid breaks down to hydrogen peroxide and acetic acid when it dissolves in water. Hence, the residual products of PAA disinfection are oxygen, water, and carbon dioxide. This makes it easy to discharge them into the receiving water bodies without concern since they are harmless to the environment. However, they must be diluted with water before being released into the environment. (Alasri et al., 1992; Kitis, 2004; Pedersen et al., 2009; Solvay, 2014). On the contrary, a requirement for implementing disinfection with PAA in aquaculture is that, at the dosages applied, it should neither cause sublethal effects to the fish treated nor compromise the robust nitrification in the biofilter. The concern regarding nitrification is crucial in extensive aquaculture as it depends on a large scale of reusing water and therefore requires biofiltration for continuous removal of nitrite and ammonium (Pedersen et al., 2009).

In Denmark, peracetic acid is used as a water disinfectant and as a strategy to mitigate pathogens in rainbow trout RAS. This chemical can be used intermittently in pulses or continuously for disinfecting the system's water. (Gesto et al., 2018; Liu et al., 2018). Furthermore, findings by Liu et al. (2018) stated that its application in RAS water demonstrated a reduction in bacterial density, regulation of fish stress response, and an overall advantageous effect on fish health in the long run. In contrast, Pedersen et al. (2009) discovered PAA dosing can diminish the nitrification performance of the RAS biofilters in some cases but the impediment can be avoided by bypassing the biofilter during PAA treatment.

Findings from a project funded by The Norwegian Seafood Research and coordinated by Nofima demonstrated promising results for using PAA for disinfecting water, indicating that Atlantic salmon smolts can resist a single and repeated exposure to comparatively high PAA doses with minimal physiological impairment. (Lazado et al., 2019). Suurnäkki et al. (2020) discovered in their study that the PAA administration lowered the rate of nitrification but also elevated the water quality by reducing ammonium levels. Moreover, Teitge et al. (2020) demonstrated a continuous decrease in pH value upon exposure to PAA.

1.4.1.1 Perfectoxid

Perfectoxid is generally used for cold disinfection (CIP surface, bathroom, and fogging). According to the manufacturer, it can be diluted with both freshwater and seawater. For disinfection against bacteria and viruses, the manufacturer recommended using 0.5% and contact time for 1 hour. (Appendix 1). Besides effectively eliminating pathogens, water treatment using this disinfectant does not cause any significant interference with fish health and welfare. (Osório et al., 2022)

1.4.1.2 Aqua Des

Aqua Des, via numerous tests, has been proven to be effective against a broad spectrum of fungal, bacterial, and viral pathogens that harm fish production.(Solvay, 2014). It can be considered to be a strong liquid broad-spectrum disinfectant. It can work against almost all types of microbes (viruses, bacteria, mold, fungi, protozoa, and parasites).

It is recommended to use for cold disinfection of surfaces, CIP, and also for mist disinfection. It is instructed to dilute with either freshwater or seawater. According to the manufacturer's protocol, 0.5% of the disinfectant is sufficient to work against viruses and bacteria with an exposure time of 30 minutes. (Appendix 1).

1.4.1.3 Addi Aqua

Addi Aqua is also a robust liquid disinfectant that is effective against a wide range of pathogens. It is urged not to be used as a concentrate by the supplier. 0.5% of this disinfectant is required to eliminate bacteria and viruses with a contact time of 30 minutes. As per the directions of the manufacturer, it is recommended to use after the pipes and tanks have been cleaned and rinsed. Similar to Aqua Des it can also be diluted with both seawater and fresh water. It is mentioned that the solution needs to be changed as required or at least every day. (Appendix 1).

1.4.2 Quaternary Ammonium Compound Disinfectants

Virkon Aquatic and Virocid (Appendix 2) are quaternary ammonium compound-based disinfectants. Quaternary ammonium compounds (QACs) are a type of disinfectant that consists of one quaternary nitrogen linked to at least one major hydrophobic substituent. Such compounds have certain bactericidal effects. (Wang et al., 2022). It is theorized that the mode of action of QAC against bacterial cells involves the disruption of the bilayer lipid membranes.

These membranes were found to be a major component of the cytoplasmic membrane of bacterial cells and the outer membrane of Gram-negative bacteria. The perturbation causes a generalized and continuous leakage of cytoplasmic contents into the surroundings. The bacterial cells leak protons and potassium ions, leading to loss of osmoregulatory functions as the low concentrations of QAC firmly attach to the anionic sites on the cell's membrane surface. (Gilbert & Moore, 2005)

QACs are commonly used in aquaculture facilities to disinfect equipment but the surfaces need to be rinsed sufficiently before contact with recycled/culture water because they are very hazardous to fish. (Yanong, 2015)

1.4.2.1 Virkon Aquatic

Virkon is a disinfectant possessing sodium dodecylbenzene sulfonate (SDBS), oxone, sulfamic acid, and inorganic buffers. (Lazado & Good, 2021). This compound contains a triple salt of potassium monosulfate which acts as an oxidizing agent. It also comprises malic acid, sodium hexametaphosphate buffer, and sodium alkyl benzene sulphonate as a surfactant. Its end products are nontoxic salts. The enzyme systems get inhibited and the cell membrane loses its integrity as a result of the chemical oxidizing proteins and other components of the cytoplasm. (Curry et al., 2005; Stockton & Moffitt, 2013)

1.4.2.2 Virocid

Virocid is a biocide that kills bacteria, viruses, mold, and yeast. Its active ingredients are alkyl dimethyl benzyl ammonium chloride (ADBAC), dodecyl dimethyl ammonium chloride (DDAC), isopropanol, and glutaraldehyde. (Mohammadi-Aragh, Linhoss & Evans, 2022). Glutaraldehyde strongly binds with the external layers of the bacterial cells and disrupts the membrane. It reacts with the functional amines and thiol groups of microbial proteins. This can aid the penetration of

biofilms, and disrupt them as proteins are vital components of the biofilm matrix. (Osland, Vestby & Nesse, 2020)

1.4.3 Hydrogen Peroxide Based Disinfectant

Hydrogen peroxide (H_2O_2) can be considered a benign chemical that has minimal impact on the environment as it can be easily broken down and does not yield any toxic byproducts. (Pedersen, 2012). In water, it disintegrates into water and oxygen, making it safe for the environment. Along with disinfection, H_2O_2 also enriches the RAS water with oxygen leading to the potential reduction in oxygen expenses in aquaculture establishments. (Bögner et al., 2020). It has been reported that 70− 100 mg/L with a contact time of a maximum of 2 hours is safe for salmonids. (Bögner et al., 2020; Pedersen et al., 2019)

On the contrary, there is limited knowledge of low-dose H_2O_2 treatment efficacy and regimens in RAS. Pedersen (2012) discovered in his research that H_2O_2 has deleterious effects on nitrifying communities in biofilters of RAS. These findings hindered its implementation in the closed-circuit system so far. It is challenging to determine an efficient and safe threshold level for H_2O_2 dosage and exposure time concerning the inhibition of nitrification of RAS biofilters because it relies on numerous parameters. Therefore, further research is necessary to determine the underlying processes of H_2O_2 degradation and inhibition. (Arvin & Pedersen, 2015).

The mode of action of H_2O_2 is based on hydroxyl radicals' formation as intermediate products before breaking down into water and oxygen. The hydroxyl radical is considered to be one of the most robust oxidizers as it is highly reactive and can disrupt membrane lipids, DNA, and other components of the cell such as detaching iron from heme proteins. Consequently, they are effective in eliminating yeasts, fungi, and viruses. They have a generally higher bactericidal effect on Gram-negative bacteria than Gram-positive bacteria. Several pathogens, including anaerobic bacteria, are more vulnerable because they do not possess the enzymes catalase and superoxide dismutase. Such enzymes disintegrate the peroxide compound. (Imlay, 2002; Keyer & Imlay, 1996; Russo, Curtis & Yanong, 2007)

1.4.3.1 Free Bac ®35

Free Bac ®35 contains 35% hydrogen peroxide. It is mainly effective against biofilm and has a long storage duration in water. In the study carried out by Skutvik (2022), the efficacy of Free Bac and chlorine were compared against *Escherichia coli* and biofilm. It was observed by the authors that the Freebac reacted slower than chlorine but still succeeded in working against the biofilm. This disinfectant can also be used in flushing the wire mesh to get rid of the biofilm. According to the manufacturer's instructions, the recommended dosage is 17 ppm with a contact time of one hour. (Appendix 3).

1.4.4 Chlorine Dioxide Based Disinfectant

Life Clean is a chlorine dioxide (ClO_2) based disinfectant. In the study carried out by Muniesa et al. (2019), active and inactive chlorine dioxides at medium dosage resulted in the elimination of the bacteria within 5 minutes. One of the common disinfection methods of seawater is the use of chlorine dioxide-based disinfectant by continuous, intermittent, or shock doses. This group of chemicals can be considered an environment-friendly alternative to chlorine due to its high and selective oxidation potential and low production of halogenated organic byproducts. (Andrés et al., 2022; Simon et al., 2014). World Health Organization (WHO) classified chlorine dioxide as a fourth-generation A1, broadly used, safe and effective disinfectant. (WHO, 2016)

1.4.3.1 Life Clean

The manufacturer claimed to be fully effective against bacteria, viruses, fungi, yeast, mycobacteria, e.g., TB, spores, e.g., *Clostridium difficile* and *Bacillus subtilis,* and multi-resistant bacteria. It is a liquid disinfectant directed to be used to disinfect surfaces. Its dosage is 300 ppm with a maximum contact time of 5 minutes, as per the manufacturer's instructions. (Appendix 3).

1.5 Biofilter in RAS

Nitrifying biofilters are crucial elements of most RAS and the operational success of the facility relies on their functionality. The byproducts from the fish protein catabolic and oxidation processes in RAS are constantly removed by the biofilters. Depending on the pH and the type of fish being reared, toxic doses of ammonia and nitrite are raising concerns among the aquaculturists. (Bartelme, McLellan & Newton, 2017)

RAS promotes the conservation of water by recirculating the treated culture water for recycling. There are numerous types of biofilters: Moving Bed Bioreactors (MBBR), rotating biological contactors, trickling filters, static bed filters, etc. Static bed filters can support substantial water volumes, but it require frequent maintenance which can be cumbersome and time-consuming. On the other hand, MBBRs need minimal maintenance but requires more time for the establishment and organization of the nitrifying bacterial community.(Interdonato, 2012)

Implementing moving bed bioreactors (MBBRs) for treating RAS wastewater has been considered to be favorable for maintaining commendable water quality, making aquaculture more sustainable. (Shitu et al., 2022). The MBBR utilizes the entire tank's volume for nitrifier growth. It also involves minimal head loss. The biomass proliferates on the bio-media that move freely in the bioreactor tank. (Rusten et al., 2006).

For nitrification, the oxygen from the air is compulsory, hence the MBBRs are typically designed for aerobic processes. Hence, a specialized coarse bubble aeration system has been designed. Special sieve arrangements have also been devised to keep the biomedia within the reactors. For optimum performance of the MBBR, it is crucial to have proper aeration grids and sieves. (Rusten et al., 2006).

One vital benefit of MBBR is that the filling fraction of the biomedia can be customized. For optimum movement of the biomedia, the recommended filling fraction is within 70%. (Rusten et al., 2006). The biomedia is typically composed of polyethylene (PEHD) with a density of 0.95 g/cm^3. The microbes primarily grow on the outer surfaces and ridges of the biomedia, maximizing the total surface area. The treatment of RAS effluent water involves multiple phases (Figure 6). The first phase is the removal of suspended and dissolved organic solids by using foam fractionator devices, sedimentation, screen filters, or settling chambers. The second phase involves the removal of toxic ammonia by nitrifying bacteria on the biomedia of the MBBR. After the nitrification

process, water is subjected to UV irradiation and ozone for disinfection and oxygenation before returning to the fish tank for reuse.(Boaventura et al., 2018; Shitu et al., 2022)

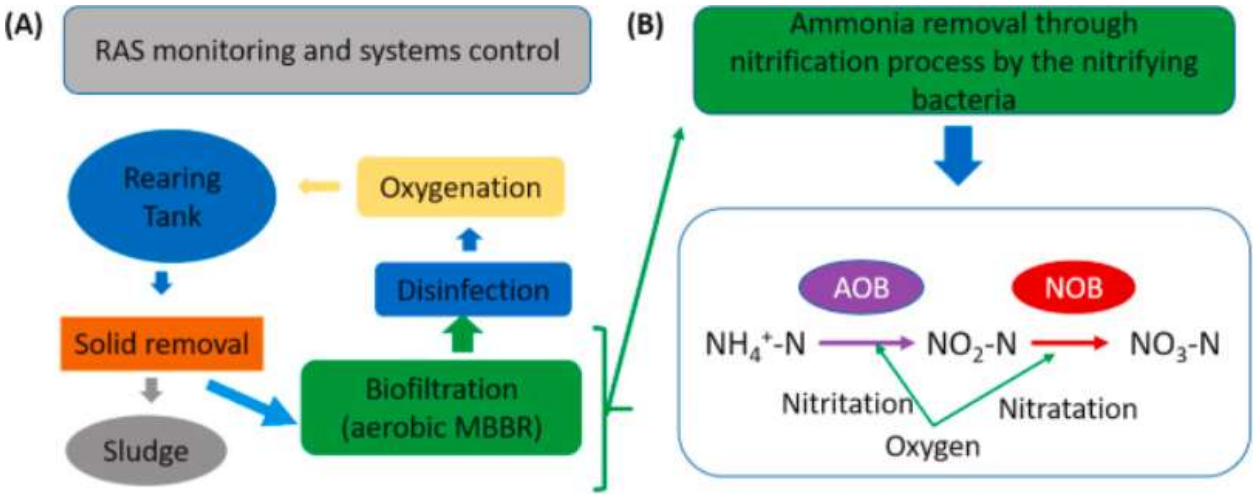


Figure 6: Simplified unit processes in RAS, and (B) the nitrification process; AOB: ammonium-oxidizing bacteria and NOB: nitrite-oxidizing bacteria.(Shitu et al., 2022)

Due to the fish metabolism and uneaten feed, fish excrete nitrogenous waste. Such nitrogenous compounds are toxic and stress inducers causing reduced appetite, diminished growth rates, and even mortality when the concentrations are high. (Nazar, Jayakumar & Tamilmani, 2013; Ruiz et al., 2020). Hence, it is vital for the RAS facility to operate under efficient biofiltration of these products.

Aerobic nitrification is the process of removal of such nitrogenous compounds in the presence of oxygen in the biofilter. Nitrification is a two-step process. The first step involves the oxidation of ammonium (NH_4) to nitrite (NO_2). The second step is the conversion of nitrite to nitrate (NO_3). (Ruiz et al., 2020; Timmons, Guerdat & Vinci, 2018). The first step of nitrification is carried out by ammonia-oxidizing bacteria (AOB). The second step is carried out by nitrite-oxidizing bacteria (NOB). (Ge et al., 2015). These nitrifying bacteria are also chemolithotrophs and they consume nitrite ions and ammonia molecules as their only source of energy for growth and metabolism. (Shitu et al., 2022; Stein & Klotz, 2016). Both of these nitrifiers are Gram-negative. Nitrosomonas, Nitrosospira, Nitrosococcus, Nitrosovibrio, and Nitrosolobus are genera of the AOB involved in RAS. On the other hand, NOB in RAS comprises of Nitrococcus, Nitrobacter, *Nitrotoga arctica, Nitrolancea hollandica,* Nitrospina*, and Nitrospira moscoviensis.*(Ge et al., 2015; Li et al., 2021; Shitu et al., 2022).

In RAS, pH 7.0 is best suited for rearing fish. Several aquaculture species can withstand pH fluctuations from 6.5 to 8.5. On the contrary, acidic water decreases the toxicity of excreted ammonia on the fish but diminishes the efficacy of the biological filter. (Zibiene & Žibas, 2018) For nitrification, the optimum pH can range between 7.0 and 9.0, where it ranges from 7.2 to 9.0 for *Nitrobacter* and 7.2 to 8.8 for *Nitrosomonas*. The backbone of the biological filter is the nitrifying bacteria, mostly *Nitrosomonas* and *Nitrobacter.* Low pH perturbs their metabolism and diminishes their efficacy to a large extent. (Chen, Ling & Blancheton, 2006). Such disruption can cause the accumulation of toxic nitrite and ammonia, leading to a significant damaging effect on the fish's health. Moreover, low pH can favor the proliferation of unwanted fungi and bacteria, leading to outcompeting with the beneficial nitrifying bacteria in the biofilter.(Michaud et al., 2006)

1.6 Media for Bacterial Count

In microbiological assessment, complex media with high nutritional content such as trypticase soy agar (TSA) is used to isolate and enumerate heterotrophic bacteria. (Bugno, Almodóvar & Pereira, 2010). It is considered to be a broad-spectrum and non-selective media as it supports the growth of a wide range of bacteria. (Balestra & Misaghi, 1997). Numerous studies assessing the efficacy of disinfectants utilize counting bacteria on these plates. (Alajlan et al., 2022; Weidmann, 2023) In contrast, Cefsulodin-Irgasan-Novobiocin (CIN) media is more selective than TSA. It is typically used for isolating mannitol-fermenting bacterial species such as *Yersinia spp.*, *Serratia liquefaciens*, *Enterobacter agglomerans*, *Aeromonas spp*., *Citrobacter* spp., and *Providencia* spp. (Tan et al., 2014)

1.7 Log Reduction

Log reduction can be used to determine the efficacy of disinfectants. It is a method of quantifying the viable bacterial colonies before and after the treatment with disinfectants. It can express the performance of the disinfectant in percentage reduction, generally in factors of 10, implementing a logarithmic reduction scale. Logarithmic reduction or log reduction is a mathematical term used for evaluating the product’s performance to show the relative number of live microbes eradicated upon contact with the disinfectant. The value can be expressed as a

function. A 1-log reduction corresponds to eliminating 90% of the target microbe or bacteria, with the bacterial count being reduced by a factor of 10. Therefore, a 2-log reduction indicates 99% removal of viable bacteria by a factor of 100, and so on. (Martínez de Alba et al., 2021)

2. Introduction

Recirculating Aquaculture Systems (RAS) are becoming more popular as an advent technology for Atlantic salmon production in Norway. Besides having the advantage of lower water consumption than traditional flow-through systems (FTS), RAS provides the ability of incredible control over environmental conditions and physiochemical water quality. This ameliorates fish welfare and improves production efficiency. The significance of microbial communities for optimizing fish welfare and chemical water quality in RAS is becoming more evident.

Despite the generally high level of biosecurity in RAS systems, concerns about pathogen outbreaks are rising, necessitating the implementation of disinfection protocols as preventive measures. While biofilters are primarily responsible for removing toxic nitrogenous compounds in RAS systems, concerns have emerged regarding their role as potential reservoirs for pathogens.

In response to these concerns, a strategy of chemical disinfection between fish batches has been proposed for commercial RAS production to enhance biosecurity and minimize the risk of pathogen transmission. Despite the potential benefits of biofilter disinfection in RAS systems, there is a lack of understanding of its impact on the dynamics of resident bacterial communities and the associated risk of pathogen invasion. (Hofstad, 2023)

Farming fish in RAS has minimal exposure to the surroundings, providing the benefit of protecting the fish from diseases and fluctuating environmental variables. Enhanced biosecurity is achieved by effectively preventing the entry of pathogens (Fisheries and Aquaculture Management Division, 2012), a stark contrast to traditional aquaculture methods.

One of the most crucial stages for water quality maintenance usually involves the removal of toxic ammonia and water disinfection.(Moreno-Andrés et al., 2020). Using chemicals has high efficiency for disinfection but they must be administered in a certain range of concentrations that does not deteriorate the nitrifying microbes in the biofilter and the fish being farmed in the facilities. Such specified ranges are typically not sufficient enough for complete disinfection.(Arvin & Pedersen, 2015; Attramadal et al., 2021; Moreno-Andrés et al., 2020)

For instance, there is limited knowledge of the efficacy of low-dose (<20mg/L) hydrogen peroxide treatment and treatment parameters such as active concentration and exposure time. Moreover, the findings on its potentially adverse effects on aquaculture organisms and the nitrifying communities in the biofilters have hindered its use in RAS. (Arvin & Pedersen, 2015; Pedersen, 2012; Schwartz et al., 2000). It was quite complicated to evaluate an efficient and safe threshold and contact time for hydrogen peroxide dose, concerning the nitrification inhibition in biofilters as it relies on numerous parameters. Hence, Arvin and Pedersen (2015) carried out an experiment to understand the underlying inhibitory and degradative mechanisms of hydrogen peroxide. In the study, they discovered that its potency and activity halt at comparatively low chemical oxygen demand (COD) concentrations.

According to Lazado and Good (2021), there are numerous applications for executing experimental validation of disinfection procedures. For instance, i) experimenting with different concentrations and contact times using the recommended protocol from the manufacturer as a point of reference; ii) assessing the effects of various factors (e.g. material quality, cleaning method) on disinfection efficacy; and iii) quantification of disinfection effectiveness either by conventional culture-reliant procedures (e.g. direct plate count) or rapid microbiological activity assessment (e.g. Bactiquant®-surface). The general lack of protocol experimental validation was supposedly reflected by the facilities' mixed responses to their confidence in the efficacy of their disinfection practices, in the study by Lazado and Good (2021)

Developing disinfection strategies for limiting pathogen outbreaks in the culture water and water treatment units in RASs without negatively affecting the fish health and welfare and the nitrifiers in the biofilters is a challenge. As mentioned earlier, it should be considered that the majority of the research institutes that study disease outbreaks typically employ flow-through systems. Hence, this study aims to elucidate the effects of the approved disinfectants on RAS water.

2.1 Objective

The aim is to determine the most effective disinfection method for fish in RAS and evaluate the water quality after disinfection in RAS.

The following research questions are formed to navigate this study:

1. Does the water quality get affected by the disinfection with the approved chemicals?2. Are the disinfectants approved by Mattilsynet effective at removing bacteria in the MBBR water?
Based on these research questions, the following hypotheses were formulated:

Hypotheses

$H_0$1: Selected chemical disinfectants approved by the Norwegian Food Safety Authority (Mattilsynet) do not impact the water quality, particularly water NH_4^+-N and pH.

$H_0$2: Selected chemical disinfectants approved by the Norwegian Food Safety Authority (Mattilsynet) are not effective against bacteria in the water of MBBR

3. Materials and Methods

3.1 Experimental Design

Seven commercial disinfectants approved by Mattilsynet (Perfectoxid, Aqua Des, ADDI Aqua, Virocid, Virkon Aquatic, Life Clean, and Free Bac ®35) (Appendix:1) were used to test their efficacy in removing bacteria from the biofilter water. Each disinfectant test and a control group (no disinfectant) were replicated three times in a total of 24 tests. Each test consisted of stocking a reactor (3L) with 40% biomedia + 60 % water for 5 days and on the 6^{th} day a selected dose of disinfectant was added to the biofilter. Water was collected immediately before adding the disinfectant and after a selected exposure time. Both biomedia and water were originally from a mature RAS stocked with Atlantic salmon smolt. The water was used for plating bacteria and key water quality analysis. After recording the measurements before and after disinfection, the whole setup was washed thoroughly and ready to be used again for experimenting with the next replicate.

3.2 Disinfectants

The disinfectant dose and exposure time used are elucidated in Table 1. The dosages for soaking equipment and boot baths instructed by the manufacturers were taken into account for this study. The manufacturer of Perfectoxid (Aco Kjemi AS) recommended a dose of 0.5% with a contact

time of 60 minutes, for eliminating bacteria and viruses. The system volume (the water volume) was 1.8L, hence, 9mL of this disinfectant was administered.

Similarly, the recommended dosages for Aqua Des (Aquatic Chemistry) and Addi Aqua (Lilleborg) were also 0.5% with a contact time of 30 minutes. Accordingly, 9mL of these disinfectants were injected. The recommended dose for Virkon Aquatic (Lanxess Deutchland GmbH) was 1% with a contact time of 30 minutes whereas, for Virocid (VESO), it's 0.25% for an exposure time of 40 minutes. Following the manufacturer's instructions, 18 mL of Virkon Aquatic (1% of 1.8L) was dispensed into the tank. Regarding Virocid, 4.5 mL was administered (0.25% of 1.8L).

Regarding Free Bac ®35, the instructions on the label stated the dosage is 17ppm (50mL per m3), accordingly 90 uL of disinfectant was pipetted into the system to disinfect 1.8 L water initially. In the first replicate, there was significant growth on both TSA and CIN plates after disinfection (discussed extensively in Results and Discussion). Hence, both the water volume and biomedia volume (total 3L) were considered in the later replicates to ensure that they were working or to make the results more valid. Therefore, the new dosage was 0.15 ml. The contact time recommended by the producer was 60 minutes.

Table 1:Dosage and Contact Time of Disinfectants Used in the Experiment

Disifectant	Treatment (nr)	Replicate nr	Water Volume (L)	Exposure Time (minutes)	Dosage (%)	Volume of disinfectant (mL)
Control	1	1	1.8	30	0.00%	0
Control	1	2	1.8	30	0.00%	0
Control	1	3	1.8	30	0.00%	0
Perfectoxid	2	1	1.8	60	0.50%	9
Perfectoxid	2	2	1.8	60	0.50%	9
Perfectoxid	2	3	1.8	60	0.50%	9
Aqua Des	3	1	1.8	30	0.50%	9
Aqua Des	3	2	1.8	30	0.50%	9
Aqua Des	3	3	1.8	30	0.50%	9
Addi Aqua	4	1	1.8	30	0.50%	9
Addi Aqua	4	2	1.8	30	0.50%	9
Addi Aqua	4	3	1.8	30	0.50%	9
Virocid	5	1	1.8	40	0.25%	4.5
Virocid	5	2	1.8	40	0.25%	4.5
Virocid	5	3	1.8	40	0.25%	4.5
VirKon	8	1	1.8	30	1.00%	18
VirKon	8	2	1.8	30	1.00%	18
VirKon	8	3	1.8	30	1.00%	18
Freebac	6	1	1.8	60	0.005%	0.09
Freebac	6	2	3	60	0.005%	0.15
Freebac	6	3	3	60	0.005%	0.15
LifeClean-	7	1	1.8	2	0.03%	0.54
LifeClean-	7	2	3	5	0.03%	0.9
LifeClean-	7	3	3	5	0.03%	0.9

The dosage for Life Clean (PartnarMéd AS) was not clearly stated on the label or bottle. There was also a lack of online resources mentioning the recommended dosage. The dosage was taken from the official microbiological efficacy sheet of Life Clean (Appendix 5). There it was mentioned 300 ppm with a contact time of 2 minutes. Therefore, 540 uL of the disinfectant was added to disinfect 1.8 L of water. Similar to the samples of Free Bac treatment, there was also significant growth on both plates after disinfection in the first replicate. To get more valid results, the total volume of the water and biomedia (3 L) was taken into account in the corresponding replicates. Hence, the new dosage was 0.9 mL. The contact time was also increased from 2 minutes to 5 minutes as it was mentioned on the label that it is the maximum contact time.

3.3 Experimental Set-up

A small-scale and simple biofilter was set up in the water labs of NMBU at Realtek in Ås. 6 identical containers of 3 L volume were used to simulate the 6 biofilter tanks (Figure 7). The tanks were fixed with flexible air stones (Hailea® Flexible Air Curtain, Tropex, Norway) that were attached to a steel valve with 6 outlets. The valve was connected to an air pump (Luftpump Super 8500, Pondteam, Norway) to circulate the biomedia. The specific surface area of the biomedia used was 750 m^2/m^3. The biomedia and water samples were fed to the tanks. 1.8L of water and

1.2L of biomedia were filled in each tank. Water loss due to evaporation was replenished by daily filling with distilled water. Ammonium chloride was administered to replenish the nutrients for the nitrifiers on the biomedia. A parameter of 10 mg/L of ammonium was set, and accordingly, 60 mg of NH_4Cl was added.

Figure 7: Experimental Set-Up of Small-Scale Biofilters

3.4 Samples Collection and Sample Preparation:

Water and biomedia samples were collected from an experimental trial at Havbruksstasjonen i Tromsø, Kårvika (Figure 9). This research facility had the approval from the Norwegian Food Safety Authority to carry out infection trials in fish utilizing pathogens related to Atlantic salmon research. There were nine RAS units set up in one of the rooms of the research station. Each unit consisted of a cylindroconical experimental tank with a volume of 0.5 m^3, an emergency oxygen stone, a dual outlet drain, a dual sensor of oxygen and temperature (Oxyguard®, Farum, Denmark), a protein skimmer, micro screen drum filter that flowed into the

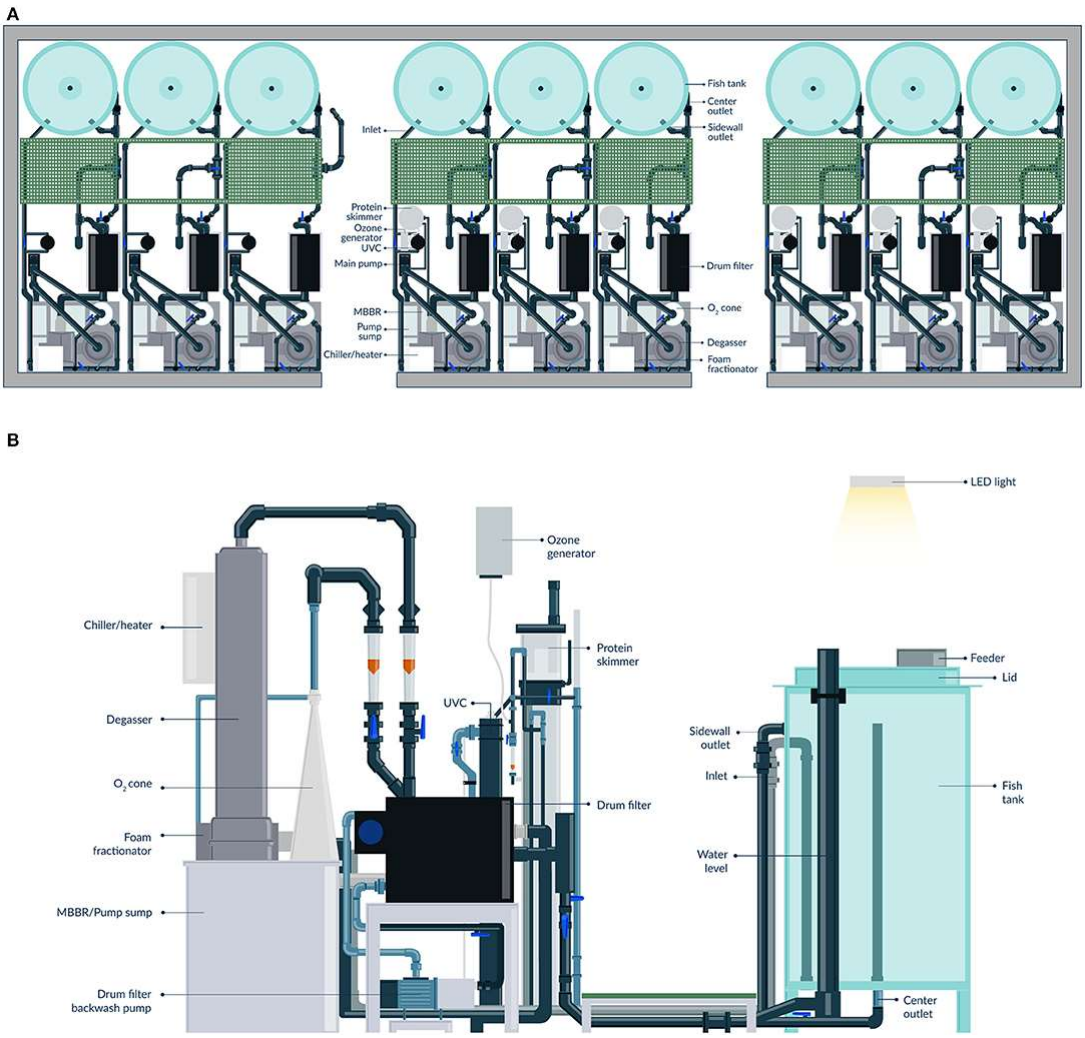


Figure 8: Sketch of the RAS research facility (A) and a replicated RAS unit (B)(Mota et al., 2022)

moving bed bioreactor (MBBR), degasser, and low-pressure oxygen saturation cone. The MBBR had a volume of 0.2 m^3 and 50% of the bioreactor was filled with biomedia (RK BioElements, RK Plast A/S, Skive, Denmark). This novel experimental RAS setup is illustrated in Figure 8.

The samples were obtained from an experimental trial (PathoRAS) led by Carlo Lazado (Senior Scientist, Nofima). The trial simulated a breach in pathogen biosecurity by infecting the Atlantic salmon with *Yersinia ruckeri* and aimed to determine the environmental factors associated with pathogen establishment in the different components of the RAS system. The experiment was comprised of three treatments. Each treatment was carried out in three tanks, totaling 9 tanks. Treatment 1 was the control treatment where the fish was fed with RAS specialized feed and there was no infection. The fish in Treatment 2 underwent infection (via intake water) with *Y. ruckeri*

and were fed feed with less water stability. The fish in Treatment 3 were also infected with *Y. ruckeri* and were fed RAS-specialized feed. The biomedia in the MBBRs were well-matured and the fish were already acclimatized to the RAS system when the samples were being collected. All of the samples were collected from the control treatment (Treatment 1). The biomedia samples were collected in large 6L Ziplock bags. 2L biomedia from each MBBR of the three controls was acquired, contributing to a total of 6L. Each sample was accumulated in triplicates. Hence, a total of 18 L of biomedia from the control treatment were collected. The biomedia was collected by using a net. The net was dipped in disinfectant and then rinsed in water before retrieving the biomedia. The biomedia samples were stored at -20°C at Nofima, Tromsø which was then transported to the water lab of NMBU (TF Fløy V), Ås and stored at the same freezing temperature. Around 7.2 L of the frozen biomedia samples were defrosted at 4°C overnight before being used in the experiment. The water samples were obtained from the inlet water of all 3 tanks of the control treatments. They were collected in large 20 L jerry cans. 6L from each of the three controls were acquired, contributing to a total of 18 L. Each sample was accumulated in triplicates. Hence, a total of approximately 54L of water was collected.

The water samples were initially stored at -20°C at Nofima, Tromsø. They were later transported and Nofima, Ås and stored at the same freezing temperature. They were finally transported to the water lab of NMBU (TF Fløy V), Ås, and stored at 4°C. To minimize errors and increase the statistical significance and reliability of the data acquired, all samples were prepared in triplicates and the triplicates were analyzed in identical conditions. Moreover, for the assessment of the protocol's validity, one blank or control of each triplicate was prepared using deionized water and processed and analyzed in the same conditions as the sample replicates. The sample standard deviation was calculated for evaluating the intervals of triplicate variation

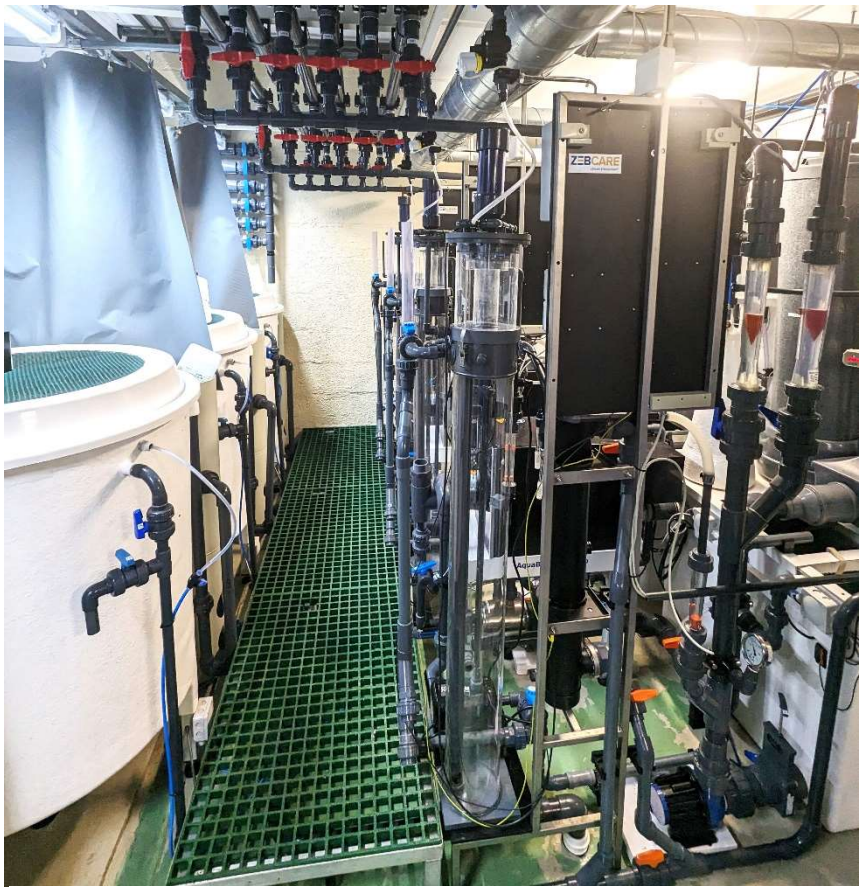


Figure 9: RAS Research Facility, Havbruksstasjonen i Tromsø, Kårvika

3.5 Water Quality Sampling and Analysis

For water quality analysis, salinity, temperature, pH, and ammonium readings were recorded daily

3.5.1 pH

The pH was measured with a pH meter (VWR® PH20) The probe was inserted into each tank to take the readings. The probe was rinsed with distilled water and wiped with Kimtech ® in between taking the readings of the six tanks. The pH levels were monitored closely. When the pH levels were decreasing or below 7.0, 0.1 g of sodium bicarbonate ($NaHCO_3$) was added to prevent the pH from going lower than 6.5 and compensate for alkalinity loss caused by nitrification. The aim was to maintain a pH of around 7.5 for optimum nitrification.

3.5.2 Salinity and Temperature

The salinity and temperature were measured with a conductivity meter (Cond 3110, VWR). Similar to the pH meter, the probe was inserted into the tank to measure the salinity levels and temperature. The probe was rinsed with distilled water and wiped with Kimtech ® in between taking the readings of the six tanks.

3.5.3 Ammonium Levels

The water lab of NMBU is equipped with Merck Spectroquant® Prove 100. Supelco Ammonium (NH_4N) Photometric Spectroquant® Test Kit (114752) was used with this device to measure the ammonium levels. The range of this kit was 0.010 - 3.00 mg/L. Since the ammonium levels in the experimental set-up surpassed the maximum values, the samples were diluted to 10^{-5}. The samples were diluted in falcon tubes. 1 mL of the sample water was mixed with 4 ml of deionized water, totaling 5 mL of diluted sample.

The preparation of samples for ammonium readings was carried out in the fume hood as a safety precaution. According to the test kit instructions, 600 uL of reagent labeled NH_4-1 and then 1 level blue micro spoon of reagent labeled NH_4-2 were added to the 5 mL sample. The mixture was then shaken thoroughly to dissolve the powder (NH_4-2) fully. The mixture looked cloudy and was then kept idle for 5 minutes for the reaction. Afterward, 4 drops of the reagent labeled NH_4-3 were added to the mixture which changed its color to yellow. The mixture was allowed to rest again for 5 minutes. The color then changed to light or dark green after 5 minutes.

While waiting for the last five minutes, the method was selected on the device by inserting the AutoSelector, and zero adjustments were carried out by measuring the absorbance of deionized water in a 10mm cuvette. After the zero adjustment was fixed, the ammonium levels of the samples were measured. After the reaction with NH_4-3, the sample mixture was then transferred to the cuvette. It was ensured that the transparent side of the cuvette was clean by wiping it with Kimtech ® paper. The cuvette was then inserted into the compartment of the device. The values appeared after a few minutes. (Appendix 4) After recording the reading, the sample was then disposed of, and the cuvette was rinsed thrice with deionized water before measuring the next sample. NH_4-1 contained sodium hydroxide. NH_4-2 contained hypochlorite ions. NH_4-3 contained 2-propanol, thymol, and sodium nitroprusside.

The ammonium levels were monitored closely. If the ammonium levels were below 4 mg/L, ammonium chloride (NH_4CL) was added according to the concentration at the time. The ammonium levels were maintained between 6 mg/L and 12 mg/L. The amount of NH_4CL to be added was calculated using the following equation:

EQ.1

$$\text{Amount of NH4Cl (mg)} = \frac{\text{Molar Mass of NH4Cl } \left(\frac{\text{mol}}{\text{g}}\right) \times \text{ System Volume (L)} \times \text{Aimed NH4} - \text{N Concentration } \left(\frac{\text{mg}}{\text{L}}\right)}{\text{Molar mass of NH4} - \text{N } \left(\frac{\text{mol}}{\text{g}}\right)}$$

3.6 Bacteria Sampling and Analysis of MBBR Water

Once the biomedia and water reached stable ammonium levels and pH values, water samples from the tanks were taken for serial dilution and ammonium measurements. Disinfectants were then administered according to the recommended dosage and after fixed periods, serial dilution and ammonium levels were measured again. Duplicate plates were prepared with samples before and after administering the disinfectants for bacterial enumeration. The bacterial count was carried out on both TSA and CIN plates. The samples were taken before and after disinfection. Serial dilution was carried out with the samples to make the counting easier. The samples before disinfection were diluted from 10^{-1} to 10^{-3}. Except for samples treated with Free Bac ® 35 and Life Clean, undiluted 100 uL of disinfected samples were plated onto TSA and CIN agar.

3.6.1 Sampling of Water

On the day before administering the disinfectants, deionized water was autoclaved for serial dilution and the plates and Eppendorf tubes were labeled. The next day, the pH and ammonium levels were measured with the pH meter and Spectroquant®, respectively. Then disinfectants were added according to the recommended dose and exposure time. After the specified time, 5 mL of water samples were taken into Falcon tubes which were later used for serial dilution and plating. The pH and ammonium levels were measured again after the disinfection.

3.6.2 Serial Dilution for Plate Count Method

Serial dilution aims to measure an estimated concentration of the sample by counting the colony-forming units (CFU) from serial dilutions of the samples. The dilution was carried out in Eppendorf PCR tubes (Figure 10). Plates and tubes for samples before disinfection were labeled as T_0 and samples after disinfection were labeled as T_1 beforehand. 100 uL of the MBBR water (sample) was taken before disinfection and then mixed with 900 uL of sterile deionized water to make a 10^{-1} dilution. Before use, the deionized water was sterilized by autoclaving at 121°C for 30 minutes with 15 psi of pressure. 100 uL of the 10^{-1} dilution sample was then mixed with 900uL of sterile deionized water to make a 10^{-2} dilution and so on. Undiluted samples of treatment after disinfection were taken except for Free Bac ®35 and Life Clean. Samples after treatment with only Free Bac and Life Clean were diluted to 10^{-3} in the latter two replicates.

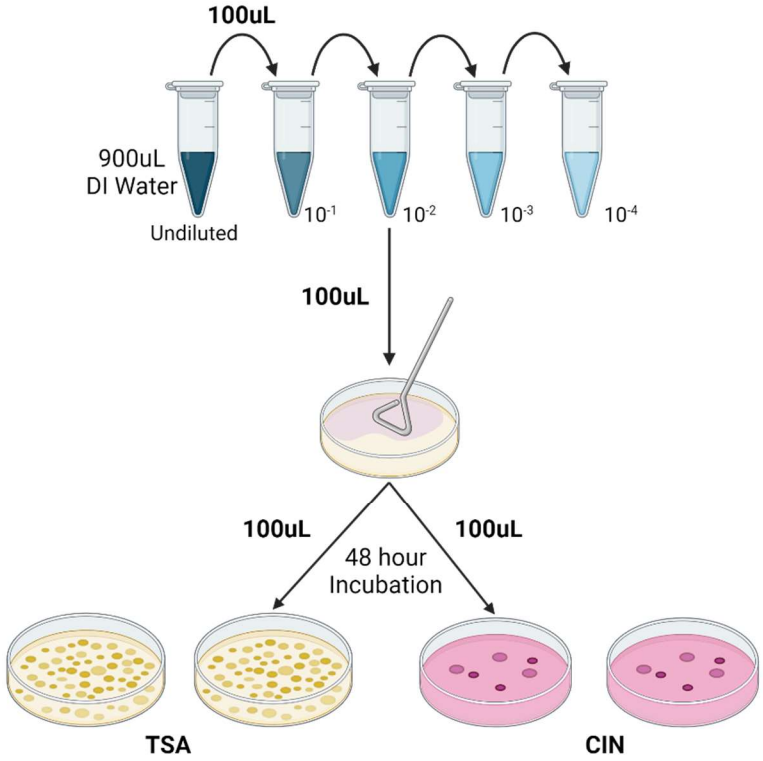


Figure 10: Serial Dilution and Plating Before Disinfection (T0) (Created with BioRender.com)

3.6.3 Plating and Incubation

The plating was carried out in duplicates for each sample. 0.1 ml of 10^{-3} and 10^{-4} diluted samples (before disinfection) and 0.1 ml samples after disinfection were spread-plated on TSA (101114ZAMP, Harm Ph, VWR, Norway) and CIN (Nofima) agar. All the plates were incubated for 48 hours in the incubator (INCU-line 68R, VWR, Norway) of the BioSpec microbiological lab at 12°C. The colonies were then counted and recorded.

3.6.4 Bacterial Count Calculation

Bacteria between the range of 30-300 colonies were counted and recorded. Bacteria were calculated as CFU/ml (colony-forming units) using the following equation:

EQ.2

$$\mathrm{CFU/ml} = \frac{\text{Colonies} \times \text{Dilution Factor}}{\text{Volume of sample on plate}}$$

3.6.5 Log Reduction

The log reduction was calculated by using the following equation:

EQ.3

$$\text{Log reduction} = \text{Log } 10 \times \frac{\text{Bacterial count of T0}}{\text{Bacterial count of T1}}$$

T0 = Before disinfection

T1= After disinfection

Based on the log reduction, the percentage reduction of bacterial count was determined using Table 2.

Table 2: Log reduction in terms of CFU and percentage reduction of bacteria. (Kochelek, 2019)

Log Reduction	Number of CFUs Remaining	Percentage Reduction
0 log	1,000,000	0%
1log	100,000	90%
2log	10,000	99%
3log	1,000	99.9%
4log	100	99.99%
5log	10	99.999%
6log	1	99.9999%

3.6.6 Data Analysis

The measurements were recorded and calculated in Microsoft® Excel® for Microsoft 365 MSO (Version 2405 Build 16.0.17628.20006). The average value of all three replicates before and after disinfection was calculated. The average values were then compared. Standard deviation was also calculated, and the average standard deviation values were calculated in pivot tables. For a better understanding and portrayal of the differences, the values were plotted and arranged in a column bar graph.

4. Results

4.1 Ammonium and pH Levels of Water.

The readings from all three replicates were used to calculate the average values and standard deviation. The measurements and analyses of pH levels and ammonium levels of the MBBR water before and after disinfection were compared to determine the disinfectant's effect on the water.

4.1.1 Ammonium Levels Before and After Disinfection

The ammonium levels decreased from 11.85 mg/L to 0.8 mg/L in all replicates of water samples disinfected with Virocid. Regarding the samples exposed to Virkon Aquatic, the ammonium levels were reduced from 8.25, 8.6, and 9.21 mg/L to 3.45, 2.95, and 2.5 mg/L respectively. The initial ammonium levels of samples ranged between 8.5 to 9.5 mg/L which dropped to values lesser than 0.1 mg/L after being treated with Addi Aqua and Aqua Des. For samples disinfected with

Perfectoxid, 2 out of 3 replicates accounted for the decrease in ammonium levels to values below 0.1 mg/L, their initial ammonium levels were 9.05 and 9.75 mg/L. The ammonium in the remaining replicate decreased from 9.25 mg/L to 0.07 mg/L.

The trend of the ammonium levels of samples after disinfection with Free Bac ®35 and Life Clean was significantly different from the rest of the disinfectants. The samples treated with the other five disinfectants experienced a decrease in ammonium levels. On the contrary, 2 of 3 replicate samples exposed to Free Bac ®35 had an increase in ammonium levels from 8.95 to 9.25 mg/L and from 5.95 to 6.65 mg/L, respectively. The remaining sample replicate encountered a meager decrease, from 10.85 mg/L to 10.8 mg/L. In the case of Life Clean, the sample replicates underwent a decrease in ammonium levels but was not as radical as the other samples. 1 out of 3 of its samples had a reduction in ammonium levels from 9.25 to 9.2 mg/L. The other two samples had ammonium levels dropped from 12.2 mg/L to 10.6 mg/L and from 10.45 to 10.1 mg/L, respectively. The ammonium levels recorded before administering the disinfectants have been summarized in Figure 11.

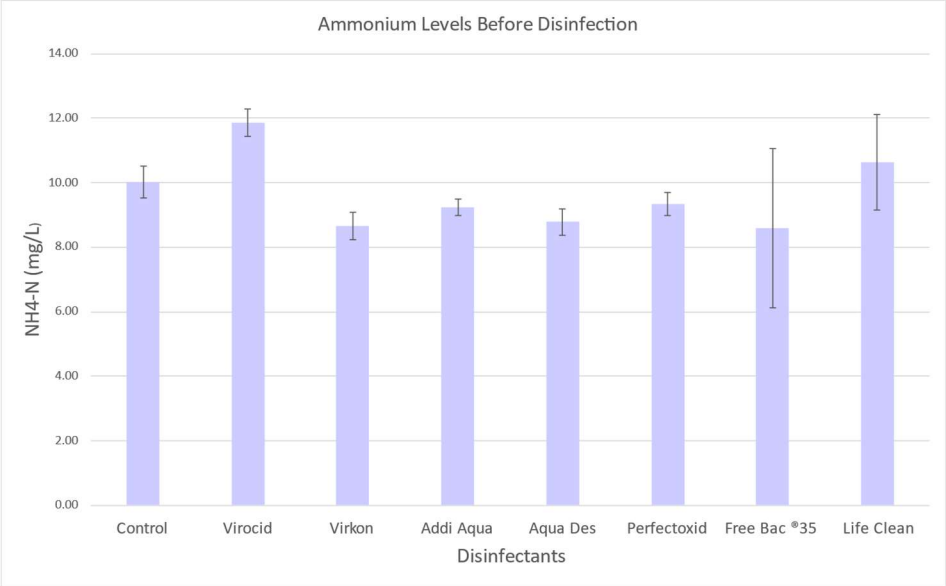


Figure 11: Ammonium Levels Before Disinfection

The average ammonium level in samples before disinfection with Virocid was 11.85 ± 0.44 mg/L. After disinfection, the average dropped to 0.8 mg/L. This indicated that there was a 93% reduction in ammonium levels by Virocid. In the context of Virkon Aquatic, the average ammonium levels of samples before disinfection were 8.65 ± 0.43 mg/L which decreased to 2.97 ±0.48 mg/L (Figure 12) after adding the disinfectant. This presented a 66% ammonium reduction by the chemical. The average ammonium levels of the samples before exposure to Addi Aqua was 9.23 ± 0.25 mg/L. After disinfection, it was reduced to 0 mg/L. In other words, Addi Aqua resulted in lowering 100% of the ammonium levels. The samples before being disinfected with Aqua Des had an average ammonium level of 8.78 ±0.41mg/L. After exposure to the chemical, it was 0 mg/L, showing a 100% reduction of ammonium levels. The average ammonium level of samples before treatment with Perfectoxid was 9.35±0.36 mg/L. It dropped to 0.02 ±0.04 mg/L, indicating a 100% decrease in ammonium levels.

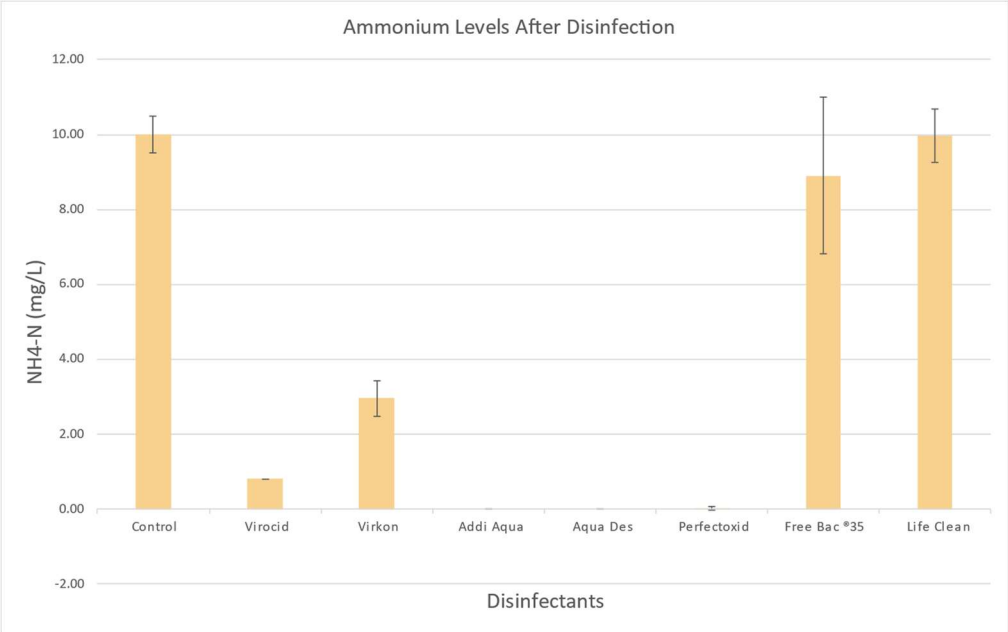


Figure 12: Ammonium levels after adding disinfectants

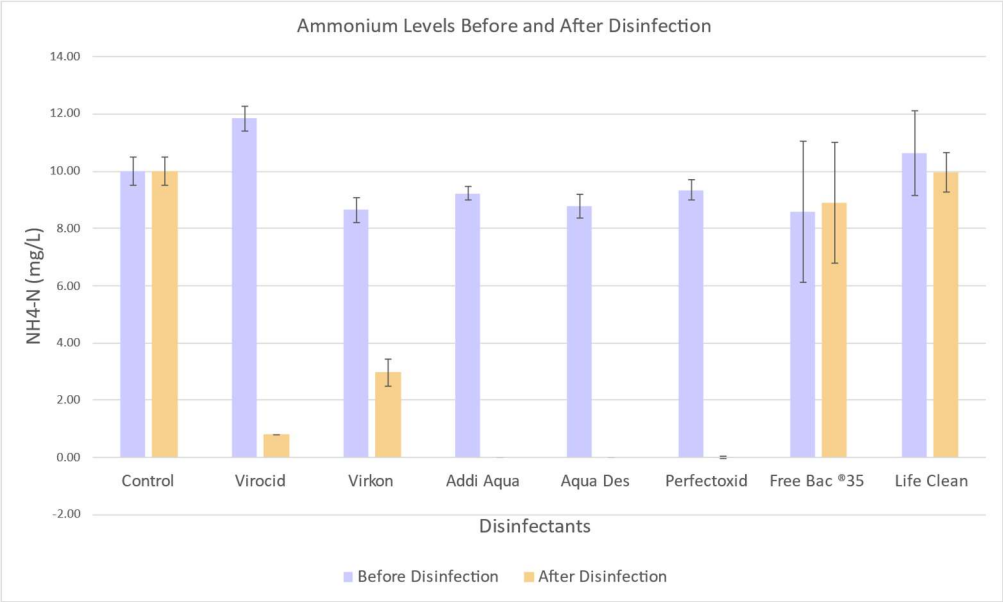


Figure 13: Summary of ammonium levels before and after adding disinfectants

Focusing on Free Bac ®35, the average ammonium level before exposure was 8.58±2.47 mg/L which increased to 8.90± 2.10 mg/L: indicating a 4% increase in ammonium levels upon contact with the disinfectant. Regarding Life Clean, the average ammonium level before disinfection was 10.63±1.48 mg/L which dropped to 9.97±0.71 mg/L after disinfection. This presented a 6% decrease in ammonium levels by disinfection with the chemical. The ammonium levels recorded before and after administering the disinfectants have been summarized in Figure 13.

4.1.2 pH Levels Before and After Disinfection

The pH levels recorded before and after administering the disinfectants have been summarized in Figure 16. The pH values of all the water samples dropped after being treated with the respective disinfectants. Out of all the disinfectants, water samples disinfected with Virkon Aquatic underwent the most drastic drop in pH Levels. From the initial pH values around 7, all the samples faced a decrease in pH levels to values between 2.36 and 2.33. Regarding the samples exposed to Virocid, the initial pH values (Figure 14) were around 7.5 which declined to values around 6.5 after disinfection (Figure 15).

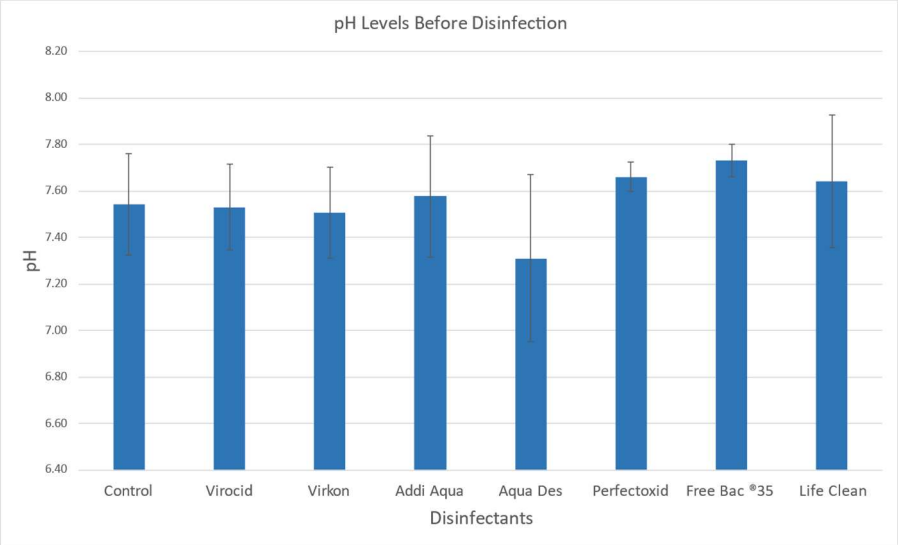


Figure 14: pH levels before adding disinfectants.

All the peracetic acid-based disinfectants caused a similar rate of pH decline. With initial pH levels between 7.2 and 7.8, the pH of samples treated with Addi Aqua reduced to values between 3.71 and 3.97. The pH of samples before being disinfected with Aqua Des was 7.31, 7.67, and 6.95. After disinfection, the pH dropped to 3.69, 3.74, and 3.44 respectively. The initial pH of samples was ranging from 7.68 to 7.71 before treatment with Perfectoxid. After disinfection, the pH dropped to values ranging from 3.67 to 3.69. The decrease of pH in samples treated with Free Bac ®35 and Life Clean was not as drastic as in the remaining samples. Regarding samples disinfected with Free Bac ®35, the pH levels declined from 7.7 to 7.57, 7.81 to 7.69, and 7.68 to7.45, respectively. Samples exposed to Life Clean had a negligible decrease in pH levels. The pH levels reduced from 7.32 to 7.25, 7.75 to 7.72, and 7.86 to 7.81 respectively.

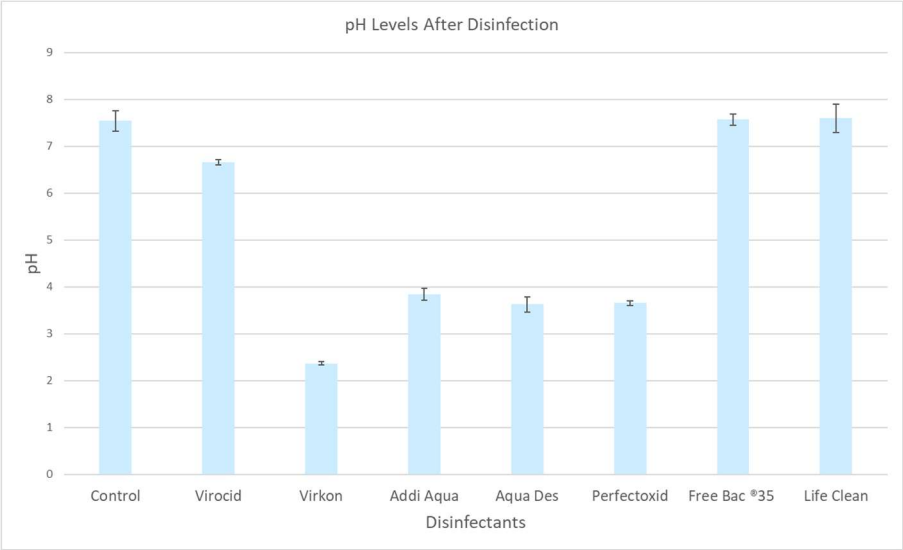


Figure 15: pH levels after disinfection

The average pH level of samples before being subjected to Virocid was 7.54±0.18 which dropped to 6.66±0.06 after adding disinfectants. Thus, there was a 12% decrease in pH levels after disinfection with Virocid. Regarding Virkon Aquatic, the initial average pH before disinfection was 7.51±0.2 which decreased to 2.36±0.04 after exposure to the chemical. This indicated a 69% reduction in pH. The samples before being disinfected with Addi Aqua had an average pH level of 7.58±0.26 which reduced to 3.84±0.13 after contact with the chemical. The pH was decreased by 49%. The initial average pH before treatment with Aqua Des was 7.31±0.36 which reduced to 3.62±0.16 after disinfection. Hence, there was a 50% decrease in pH by the chemical. The average pH before disinfection with Perfectoxid was 7.66±0.06 which dropped to 3.65±0.05 after contact with the chemical. This presented a 52% reduction in pH. The samples before being disinfected with Free Bac ®35 had an average pH level of 7.73±0.07 which was reduced to 7.57±0.12 after disinfection. Hence, indicating a 2% decrease in pH. Regarding Life Clean, the initial average pH was 7.64±0.29 which decreased to 7.59±0.30. This portrayed a 1% decrease in pH levels caused by the disinfectant.

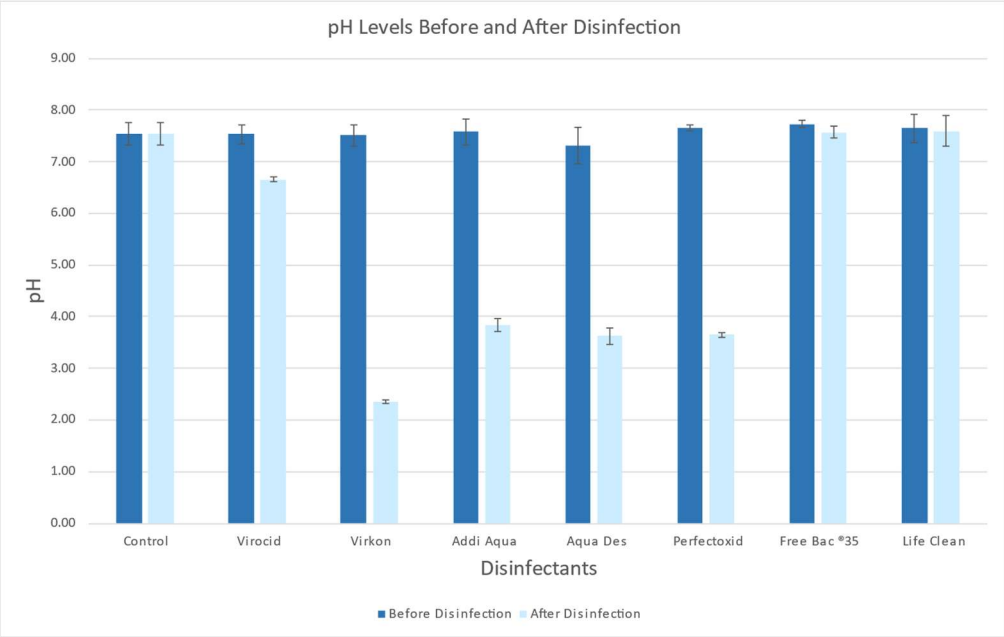


Figure 16: Summary of pH levels before and after disinfection

4.2 Log Reduction by Disinfectants

There was no bacterial growth on either of the CIN and TSA plates of water samples exposed to 5 out of 7 disinfectants. There were no colonies formed on the samples disinfected with Virocid, Virkon Aquatic, Addi Aqua, Aqua Des, and Perfectoxid (Appendix 2 and 3). On the contrary, there was bacterial growth in samples treated with Free Bac and Life Clean on both plates. The log reduction on TSA plates by Virocid, Virkon Aquatic, Addi Aqua, Aqua Des, and Perfectoxid were all 100% (Appendix 2). Figure 17 shows the comparison of the control plate before disinfection and the plate containing the sample treated with Aqua Des, demonstrating the effect of the chemical. In contrast, the log reduction on TSA by Free Bac ®35 and Life Clean were 70.66% and 57.66%, respectively. Virocid caused an average reduction of 5±0.53 log on TSA whereas Virkon Aquatic had an average log reduction of 6±0.11. Both Addi Aqua and Aqua Des had an average reduction of 6 log on TSA as well. Samples disinfected with Perfectoxid had an average log reduction of 7 whereas the samples exposed to Free Bac ®35 had an average log inactivation of 1

log on TSA. Regarding the samples treated with Life Clean, there was an average of 0 log reduction.

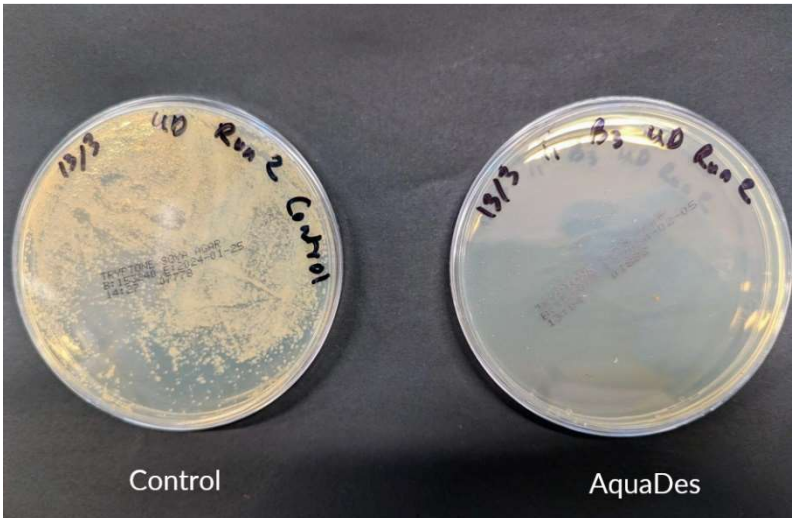


Figure 17: Bacterial colonies before and after disinfection with Aqua Des

All disinfectants except Free Bac ®35 and Life Clean caused a 100 % average log reduction (Figure 18) of the designated samples on CIN plates (Appendix 3). Free Bac ®35 caused about a 15.89 % average log reduction while Life Clean yielded about 62.65% average log reduction. Virocid, Virkon Aquatic, Addi Aqua, and Aqua Des caused an average of 4 log reduction on CIN whereas Perfectoxid had an average log reduction of 5 (Figure 19). Contrastingly, Free Bac ®35 had an average of 0 log inactivation and Life Clean caused an average of 1 log reduction.

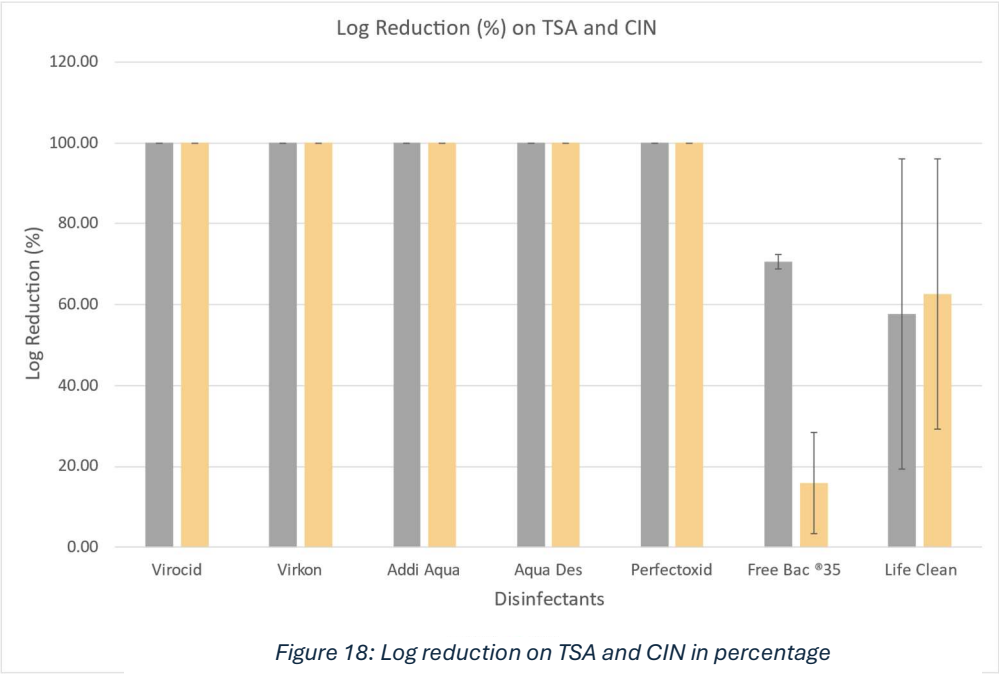


Figure 18: Log reduction on TSA and CIN in percentage

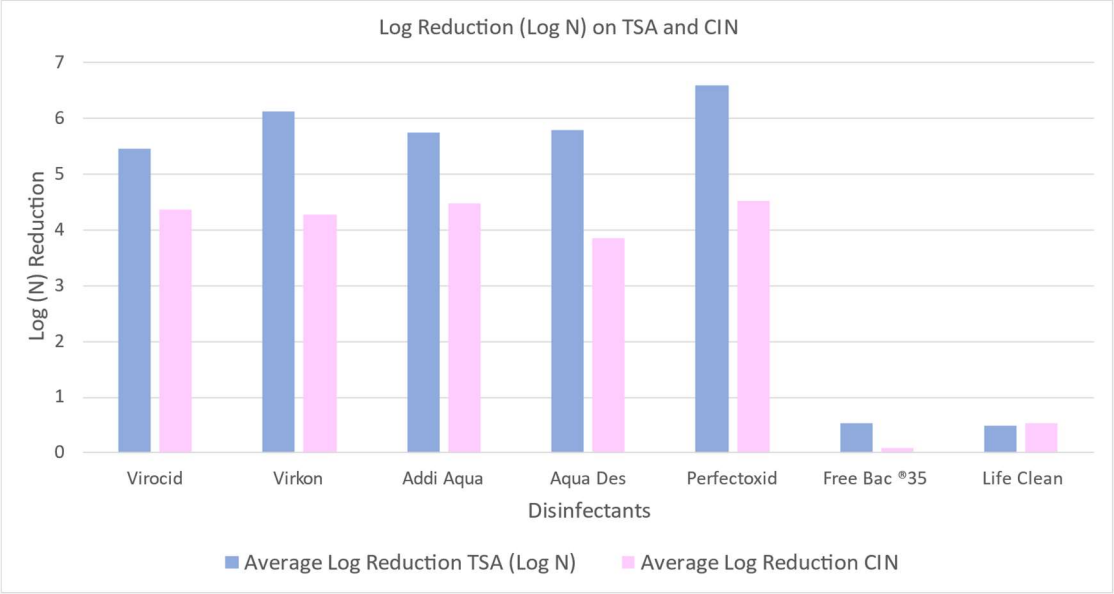


Figure 19: Log (N) reduction on TSA and CIN

4.3 Overview of Disinfectant Effects on pH

A trend between the bacterial count (CFU/mL) on TSA and the pH was noticed. Figure 20 shows bacterial count before and after disinfection against pH. It can be seen that 5 out of the 7 disinfectants achieved a bacterial reduction to 0 and had a pH decrease. The highest bacterial reduction was achieved by Perfectoxid. The pH of samples after disinfection with PAA disinfectants (Addi Aqua, Aqua Des, Perfectoxid) was between 3.6 and 3.8. The lowest pH (2.36) after disinfection was achieved by Virkon Aquatic. Samples exposed to Virocid had the highest pH (6.66) after disinfection, among the effective disinfectants. The remainder plots on the graph are the samples exposed to Life Clean and Free Bac®35.On the graph, it can be seen that there was an insignificant decrease and are in the same vicinity/range as the control sample, indicating there was no significant change caused by the two disinfectants.

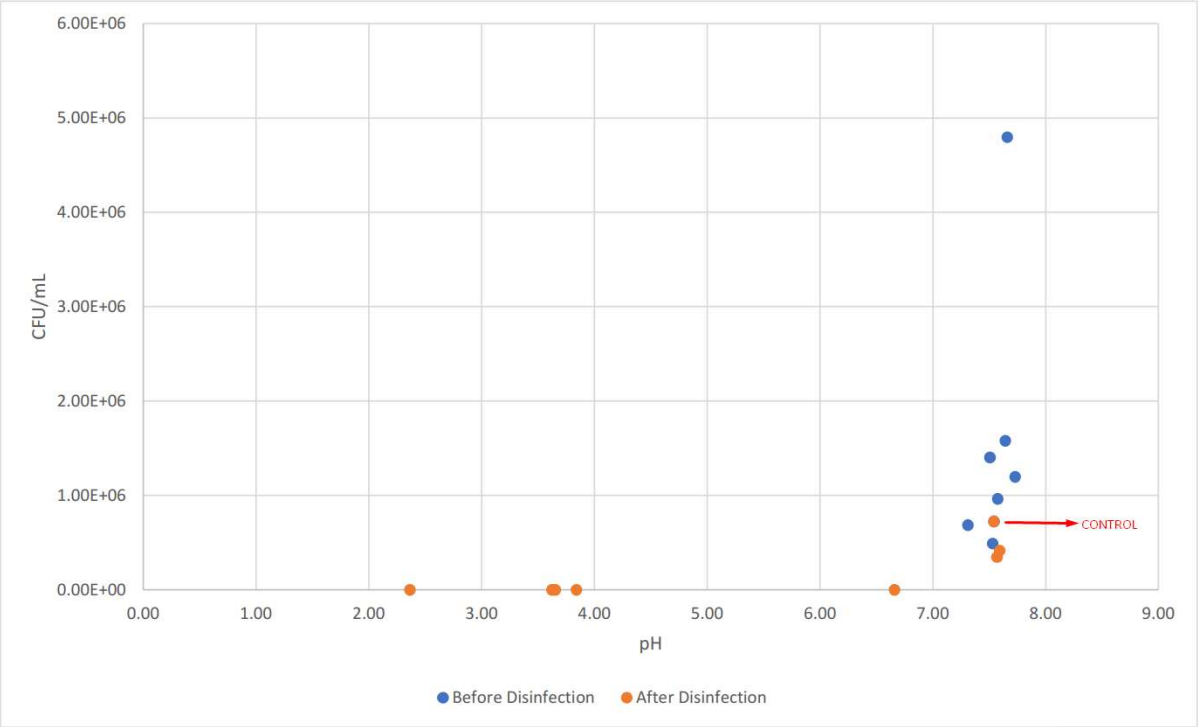


Figure 20: Overview of bacterial count and pH changes by disinfection.

4.4 Overview of Disinfectant Effects on Ammonium

Figure 21 portrays the bacterial count (CFU/mL) on TSA before and after disinfection against ammonium levels (mg/L). All 3 PAA-based disinfectants (Addi Aqua, Aqua Des, Perfectoxid) had 0 CFU/mL and 0mg/L ammonium. Their plots overlapped with each other on the graph. Among the effective 5 disinfectants, the samples exposed to Virkon Aquatic had the highest ammonium (2.97 mg/L) after exposure. Samples treated with Virocid achieved 0 CFU/mL and their ammonium level was 0.8 mg/L. It can be seen that the samples after disinfection with Free Bac ®35 and Life Clean clustered around the control plot and not at 0 CFU/mL, indicating its lack of significant impact on the sample.

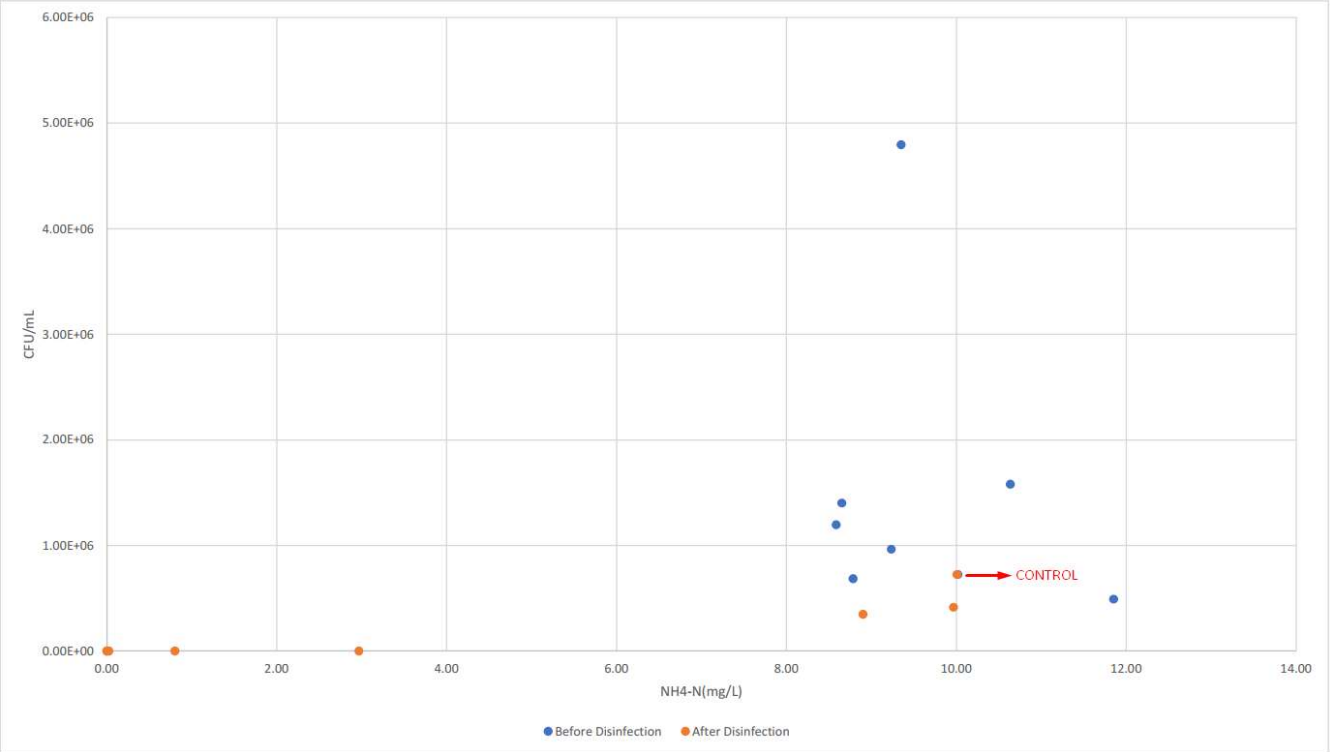


Figure 21: Overview of impact on bacterial count and ammonium (mg/L) in RAS by disinfectants.

5. Discussion

In some instances, RAS can harbor opportunistic pathogens if the water quality is not properly maintained and certain components such as pipes are not properly disinfected. Moreover, if there is a pathogen introduced or disease developed in a fish, it can proliferate and spread to the entire system if proper disinfection protocols are not maintained. Implementation of a disinfection regimen can prevent pathogen outbreaks. The water quality in RAS should be compatible with the reared fish's requirements, specifically ammonia, nitrite, nitrate, pH, temperature, and salinity. (Yanong, 2015).

The most common water quality obstacles in RAS are the toxic levels of ammonia and nitrite, usually caused by imbalances between the biofilter capacity and accumulation of uneaten feed, and high fish density. Toxic amounts of nitrite and ammonia in established RAS might be a result of the breakdown of proteins from overfeeding, overcrowding, or ineffective solids removal. Hence, along with overall proper production maintenance and rearing volume, a robust biofilter is imperative for RAS. The nitrifying microbes convert ammonia to nitrate, causing a drop in the ammonia levels and consequently, improving the water quality. The gradual decrease in pH is also

caused by the nitrifying bacteria in the biofilter generating acid (H+) as a byproduct. Hence, The nitrification activity of the biofilter is indicated by the decrease in ammonia levels as well as the pH. (Keuter et al., 2017; Yanong, 2015).

The efficacy of disinfection can be determined by the reduction of bacterial populations. The reduction of bacterial population can be demonstrated by log reduction/inactivation. Log reduction is the value that indicates the inactivation of undesirable microbes and typically relates to the percentage of microbes inactivated. For instance, a 2-log reduction corresponds to a 99 percent elimination of microorganisms. (Table 2) Most disinfectants require a minimum log reduction of 3, i.e. >99.9%. (Di Martino et al., 2021; United States Environmental Protection Agency, 2020). Visually, the efficacy of disinfectants can also be stipulated by comparing the bacterial population before and after disinfection. Significantly lower colonies on plates of samples after disinfection indicate that the disinfectant has an effect on the microbes. (Weidmann, 2023)

Any drastic changes to the pH and ammonium levels will demonstrate a hindrance in the microbial community in the biofilter. In this experiment, the ammonium levels underwent a significant decrease by all disinfectants except Free Bac®35 and Life Clean. Similarly, the pH levels also dropped significantly upon exposure to Virkon Aquatic, Addi Aqua, Aqua Des, and Perfectoxid. However, there was little or no change in pH after disinfection with Free Bac ®35 and Life Clean. The results indicated that Free Bac ®35 and Life Clean had no antimicrobial effect on the RAS water. As stated earlier, this study was intended to determine the most effective disinfectant approved by Mattilsynet and assess the change in water quality after disinfection. Moreover, all disinfectants except Free Bac ®35 and Life Clean have caused significant log reduction on both CIN and TSA plates.

5.1 Effect of Disinfectants on pH and Ammonium of Biofilter Water

As stated earlier, optimum water quality is crucial in aquaculture, specifically in closed systems like RAS where the water is recycled in closed units. Key components of upkeeping the water in such facilities are continuous removal of ammonia. Nitrifying bacteria require optimum pH conditions for oxidizing ammonium to nitrite and then to nitrate. If the pH is too low, then the nitrification would not be carried out sufficiently and can cause hindrance which can lead to substantial accumulation of ammonia and nitrite. High levels of such compounds are deleterious

to fish health and can also cause high mortality. Furthermore, other bacteria and fungi can grow in low pH which can cause them to outcompete the advantageous nitrifying bacteria in the MBBR. Parameters determining the water quality, when in the ideal range, can strengthen fish health and immunity, ameliorate growth, and reduce the likeliness of disease development. (Lindholm-Lehto, 2023; Michaud et al., 2006; Su, Sutarlie & Loh, 2020).

Findings of an experiment on 1 mg/L of PAA administration caused increased ammonia levels.(Teitge et al., 2020). Another study on PAA treatment in water did not find any significant differences to the ammonia levels. (Mota, Eggen & Lazado, 2022). However, there have been other studies that demonstrated PAA application to decrease ammonia levels. For instance, Pedersen et al. (2009) observed that there were no significant signs of ammonia accumulation in biofilters treated with 1 mg/L PAA despite diminished nitrification capacity. A study by Suurnäkki et al. (2020) discovered that PAA application reduced ammonium levels despite compromised nitrifying capacity in the biofilter. The decrease in TAN levels was the findings of an experiment by Liu et al. (2017). These findings coincide with the results obtained from the present experiment. Addi Aqua, Aqua Des, and Perfectoxid are all PAA-based disinfectants that caused a 100% reduction in ammonium levels. PAA disinfectants have been demonstrated to reduce ammonium and ammonia levels in RAS without having a significant adverse effect on the nitrification without disrupting nitrification. (Lepine et al., 2023; Suurnäkki et al., 2020). However, large doses of PAA can impact nitrification in the biofilter. (Pedersen et al., 2009). The reduction in ammonium levels could indicate that the ammonium oxidation was repressed.

Mota, Eggen and Lazado (2022) revealed PAA disinfectants greater than 3.2 mg/L caused a decrease in pH. Teitge et al. (2020) have also found PAA to decrease pH levels. Pulse treatment of PAA (0.1 mg/L) led to a transient reduction in pH immediately after the application. (Liu et al., 2017) . All of the PAA-based disinfectants used in this experiment have also decreased pH levels significantly. Addi Aqua lowered the water's pH by 49%, Aqua Des reduced pH by 50%, and Perfectoxid caused a 52% decrease. The decrease in the pH can be a result of the PAA hydrolyzing into acetic acid and water. The acetic acid in the water then releases hydrogen and acetate ions that can decrease the pH. (Kitis, 2004)

Both Addi Aqua and Perfectoxid contain hydrogen peroxide (H_2O_2) and PAA. The combination of H_2O_2 and PAA is synergistic and can effectively eliminate microbes. (Alasri et al.,

1992). Hence, Addi Aqua and Perfectoxid are found to fully reduce ammonium and pH levels. Out of the three PAA-based disinfectants, Perfectoxid seems to be the most potent disinfectant. On the contrary, Free Bac®35 contains 35% H_2O_2 but unexpectedly had no impact on the ammonium levels and pH levels found in this experiment, unfortunately. Perhaps the salinity of the brackish water has impaired its biocidal effect, or the chemical expired.

Regarding QAC-based disinfectants, literature on their use in aquaculture is quite limited, especially its effects on water quality parameters like ammonia and pH. QACs typically ionize in water and release cations which adsorbs the negatively charged bacteria.(Peyneau et al., 2022) Compared to the PAA-based disinfectants, QAC-based disinfectants caused less drastic changes in the ammonium levels and pH. Virocid reduced ammonia levels by 93% whereas 66% of the ammonia was decreased by Virkon Aquatic. Moreover, Virocid had caused a lower pH reduction (12%) than Virkon (69%) and the PAA disinfectants. Virkon contains sulfamic acid ((Lazado & Good, 2021) which has likely caused a substantial dive in the pH.

There is limited literature and research about the effects of chlorine dioxide-based disinfectants on water quality parameters. Chlorine dioxide (ClO_2) does not significantly change the pH of water. It carries out stable oxidation and possesses disinfection strength that can work in a broad range of pH (2 to10) (Gan et al., 2020). In this experiment, the ClO_2-based disinfectant, Life Clean did not seem to have any significant effect on the pH. There was only a 1% pH decrease but it didn't seem to have any effect on the water because there was also a negligible reduction in the ammonium levels (6%). Perhaps higher concentrations of the chemical were required, or it had expired or maybe the composition of the brackish water may have disrupted its effect.

5.2 Effect of Disinfectants on Bacteria in Biofilter Water

An experiment with pulse treatment of 1 mg/L PAA resulted in a substantial increase in bacterial levels in RAS water, presumably due to enhanced growth of heterotrophic bacteria (Teitge et al., 2020). In contrast, Good et al. (2022) carried out an experiment where they discovered 3-5 mg/L PAA exposure in RAS water spiked with *Yersinia ruckeri, Weissella ceti,* and *Flavobacterium columnare* caused 6 -log reduction, resulting in 0 CFU/20 µl of the pathogens. Findings from another experiment stated that 3 mg/L PAA resulted in total bacterial reduction of lab-cultured *Pseudomonas aeruginosa* by 5 $\log_{10}$.(Alasri et al., 1992). Another study reported a 5 $\log_{10}$

reduction of the total bacterial population by 100 mg/L PAA with a 30-minute exposure time at 4°C.(Verner – Jeffreys et al., 2009).

Such findings are somewhat similar to the results of this study. Samples treated with all three PAA disinfectants resulted in 0 CFU/mL. 0.5% of Aqua Des with 30 minutes contact time resulted in an average of 6 $\log_{10}$ reduction on TSA and 4 $\log_{10}$ reduction on CIN. Similarly, 0.5% of Addi Aqua with 30 minutes contact time resulted in an average of 6 $\log_{10}$ reduction on TSA and 4 $\log_{10}$ reduction on CIN. Perfectoxid (0.5%) with an exposure time of 60 minutes caused 7$\log_{10}$ reduction on TSA and 5$\log_{10}$ inactivation on CIN. The exposure time of Perfectoxid is double compared to the other two PAA disinfectants but their recommended dosages are the same. Perfectoxid had the highest log reduction compared with the Addi Aqua and Aqua Des. It can be hypothesized that Aqua Des and Addi Aqua might cause a higher log reduction of bacteria if their contact time was extended from 30 minutes to 60 minutes.

QACs can disrupt the cellular membrane of the microbe and cause leakage of its contents, killing it in the process (Percival et al., 2016). Some studies reported 35, 45, and 55 μg/ml QACs (ADBAC and DDAC) caused rapid reduction of 2to3 $\log_{10}$ microbes within 3 minutes at 25°C. (Ioannou Christopher, Hanlon Geoff & Denyer Stephen, 2007). Takasaki et al. (1994) reported there were no viable *Staphylococcus aureus* after being treated with, the minimum lethal concentration (MLC), 32 μg/ml of QAC (DDAC) with 20 seconds of contact time. QACs in another study were found to have an average of 4.75 log reduction of *S. aureus*. (Lineback et al., 2018). Comparably, QAC-based disinfectants used in this experiment have shown 100% removal of bacteria from the RAS water samples, in both TSA and CIN plates. Samples exposed to 0.25% Virocid for 40 minutes had an average of 5 $\log_{10}$ reduction on TSA and 4 $\log_{10}$ on CIN. 1% Virkon Aquatic with contact time of 30 minutes led to an average of 6 $\log_{10}$ reduction on TSA and 4 $\log_{10}$ on CIN.

A study reported hydrogen peroxide disinfectant caused an 8.73 log reduction of *S. Aureus* and an 8.51 log reduction of *P. aeruginosa.*(Lineback et al., 2018). Another author discovered a hydrogen peroxide based disinfectant (contained 4% H_2O_2) was able to have > 3 $\log_{10}$ reduction of *Clostridioides difficile* spores within a minute. (Cadnum et al., 2021). Bögner et al. (2020) reported that 15.8 mg/L H_2O_2 on a 4-hour per day basis, caused a reduction in microbes from 604.4 CFU/mL to 159.8 CFU/mL in the rearing tanks of RAS. On the contrary, Perumal et al. (2014)

presented that multi-drug resistant nosocomial pathogenic biofilms were not susceptible to several H_2O_2-based disinfectants. H_2O_2 resistance of the microbes in the RAS water is not likely in this study. However, the H2O2-based disinfectant, Free Bac®35 unexpectedly did not cause any significant reduction in the bacterial count. 17 ppm of this disinfectant only had an average of 1 $\log_{10}$ reduction in TSA and none in CIN, with a contact time of 1 hour. Perhaps the desired biocidal effect could have been achieved if a higher concentration of disinfectant was applied or the pH and salinity of the water may have disrupted its potency.

Chlorine dioxide was found to cause a 98.2% reduction of bacteria in a study.(Ma et al., 2017). Another study presented that the efficacy of ClO_2 was higher in natural waters compared to that tested in ultra-pure buffered waters.(Barbeau et al., 2005). Foschino et al. (1998) reported 3.4 mg/L chlorine dioxide to be effective in aqueous samples but ineffective against bacteria attached to steel surfaces. The author also mentioned that they were unable to fully remove the bacteria on the PVC surface despite using 14 mg/L ClO_2 for 8 min. The scenarios of these studies are somewhat similar to the results of this experiment. 300 ppm of Life Clean exposed to the sample for 5 minutes were found to have no bacterial load reduction on TSA and only an average of 1 $\log_{10}$ reduction on TSA. The negligible biocidal effect may have arisen due to the water composition of the sample. The material of the container might have caused inefficacy, or the product was probably out of date.

6. Conclusion

In conclusion, all PAA-based (Addi Aqua, Aqua DES, Perfectoxid) and QAC-based (Virkon Aquatic, Virocid) disinfectants were found to have a significant impact on the ammonium and pH levels of the MBBR water. In contrast, the H_2O_2-based disinfectant (Free Bac ®35) seemed to have a slight increase in ammonium levels but no significant alterations in the pH. The ClO_2-based disinfectant (Life Clean) did not seem to affect the pH and ammonium levels of the MBBR water. All PAA-based and QAC-based disinfectants were found to be effective against heterotrophic bacteria in the MBBR. On the contrary, the H_2O_2-based and the ClO_2-based disinfectants were not found to have any impact on the bacteria in the MBBR water. To answer the hypotheses, Addi Aqua, Aqua DES, Perfectoxid, Virkon Aquatic, and Virocid rejected both null hypotheses as they had an impact on the ammonium and pH levels, and they eliminated bacteria in the water of the

MBBR. Contrastingly, Free Bac ®35, and Life Clean accepted both null hypotheses as they did not have any substantial impact on pH, ammonium levels, and the bacteria in the culture water.

7. Limitations of Experiment

The water in the experimental setup did not undergo any water treatment of a typical RAS, lacking a complete simulation of the whole process. The temperature and water flow were not controlled in the entire setup due to a lack of equipment and time constraints. If temperature was controlled, the rate of evaporation could have been maintained and there would have been better accuracy of the results. The nitrification rate of the MBBR was not as robust as the ones in the research facility (Karvika) where the samples were obtained. This probably has been due to limited nutrients for the bacteria. The microbial load of the sample water was increasing as the initial water samples were stored at -20 °C but in the later replicates the water was stored at 4 °C. This may have caused more variation in the results. The exact recommended concentration of Life Clean was not stated in any online resources or in the package itself. The concentration of the disinfectant was decided later taken from the microbiological efficacy sheet from the manufacturer's site (Appendix 5). Due to time constraints, more research for verifying the inefficacy of Free Bac ®35 and Life Clean was not carried out. The bacterial colonies isolated on CIN plates were not further studied for identification due to limited time and resources. The measurement of ammonium levels were carried out in test kit that could read a maximum of 3 mg/L NH_4-N (Appendix 4). Spectroquant® Test Kit (100683) or any other kit with higher reading capacity could have been more appropriate; it would have increased accuracy and reduced time consumption. The appropriate kit was unable to be obtained because of the distributor's logistic issues and time constraint.

8. Implications for Science and Industry

The lack of biocidal effect of Free Bac ®35 and Life Clean was unexpected. The majority of the aquaculture industries use these disinfectants and rely on their efficacy. It would be quite catastrophic if the industries using them were unaware of the unknown factors that can make the disinfectants less effective, despite knowing basic factors such as organic matter and pH. The

material of the structures should also be taken into account when assessing the antimicrobial effect of the disinfectants.

9. Future Research

It is evident that more research is needed to study the efficacy of Free Bac ®35 and Life Clean to find out what factors caused them to be ineffective. A more comprehensive study needs to be done on quantifiable variables such as temperature, pH, salinity, and microbial load on the specified disinfectants to verify what factors cause their inefficacy. Future research should take account of other water quality parameters such as dissolved oxygen (DO), nitrite levels, salinity, total suspended solids (TSS), and so on for more accuracy. The disinfectants should also be tested at a fixed nitrification rate to pinpoint factors that could affect their efficacy. Different materials with different porosities could be taken into account for bacteria adhesion as there have been previous studies that found ClO_2 to be ineffective against bacteria on steel and PVC surfaces (Foschino et al., 1998). The Minimum Lethal Concentration (MLC) of the disinfectants could also be looked into to determine its effectiveness. More research is also needed for the use of QAC-based disinfectants in aquaculture, specifically its effect and mode of action on the ammonium levels, pH levels, and organic load in water.

References

Alajlan, A. A., Mukhtar, L. E., Almussallam, A. S., Alnuqaydan, A. M., Albakiri, N. S., Almutari, T. F., Bin Shehail, K. M., Aldawsari, F. S. & Alajel, S. M. (2022). Assessment of disinfectant efficacy in reducing microbial growth. *PLoS One*, 17 (6): e0269850. doi: 10.1371/journal.pone.0269850.

Alasri, A., Roques, C., Michel, G., Cabassud, C. & Aptel, P. (1992). Bactericidal Properties of Peracetic Acid and Hydrogen Peroxide, Alone and in Combination, and Chlorine and Formaldehyde Against Bacterial Water Strains. *Canadian Journal of Microbiology*, 38 (7): 635-642.

Aleynik, D., Adams, T. & Davidson, K. (2022). Optimizing the Connectivity of Salmon Farms. Role of Exposure to Wind, Tides, and Isolation. In, pp. 61-86.

Andrés, C. M. C., Lastra, J. M. P., Andrés Juan, C., Plou, F. J. & Pérez-Lebeña, E. (2022). Chlorine Dioxide: Friend or Foe for Cell Biomolecules? A Chemical Approach. *Int J Mol Sci*, 23 (24). doi: 10.3390/ijms232415660.

Arvin, E. & Pedersen, L.-F. (2015). Hydrogen Peroxide Decomposition Kinetics in Aquaculture Water. *Aquacultural Engineering*, 64: 1-7. doi: https://doi.org/10.1016/j.aquaeng.2014.12.004.

Attramadal, K. J. K., Øien, J. V., Kristensen, E., Evjemo, J. O., Kjørsvik, E., Vadstein, O. & Bakke, I. (2021). UV Treatment in RAS Influences the Rearing Water Microbiota and Reduces the Survival of European Lobster Larvae (Homarus gammarus). *Aquacultural Engineering*, 94: 102176. doi: https://doi.org/10.1016/j.aquaeng.2021.102176.

Balestra, G. M. & Misaghi, I. J. (1997). Increasing the efficiency of the plate counting method for estimating bacterial diversity. *Journal of Microbiological Methods*, 30 (2): 111-117. doi: https://doi.org/10.1016/S0167-7012(97)00056-0.

Barbeau, B., Desjardins, R., Mysore, C. & Prévost, M. (2005). Impacts of water quality on chlorine and chlorine dioxide efficacy in natural waters. *Water Research*, 39 (10): 2024-2033. doi: https://doi.org/10.1016/j.watres.2005.03.025.

Bartelme, R. P., McLellan, S. L. & Newton, R. J. (2017). Freshwater Recirculating Aquaculture System Operations Drive Biofilter Bacterial Community Shifts around a Stable Nitrifying Consortium of Ammonia-Oxidizing Archaea and Comammox Nitrospira. *Front Microbiol*, 8: 101. doi: 10.3389/fmicb.2017.00101.

Bergheim, A., Drengstig, A., Ulgenes, Y. & Fivelstad, S. (2009). Production of Atlantic Salmon Smolts in Europe—Current Characteristics and Future Trends. *Aquacultural Engineering*, 41 (2): 46-52. doi: https://doi.org/10.1016/j.aquaeng.2009.04.004.

Björnsson, B. T., Stefansson, S. O. & McCormick, S. D. (2011). Environmental Endocrinology of Salmon Smoltification. *Gen Comp Endocrinol*, 170 (2): 290-8. doi: 10.1016/j.ygcen.2010.07.003.

Blancheton, J. P., Piedrahita, R., Eding, E., d'orbcastel, D. E., Lemarié, G., Bergheim, A. & Fivelstad, S. (2007). Intensification Of Landbased Aquaculture Production In Single Pass And Reuse Systems. *Aquacultural Engineering and Environment*.

Boaventura, T. P., Miranda-Filho, K. C., Oréfice, R. L. & Luz, R. K. (2018). Influence of porosity of low-density polyethylene media on the maturation process of biofilters used in recirculating aquaculture systems. *Aquaculture International*, 26 (4): 1035-1049. doi: 10.1007/s10499-018-0266-y.

Bögner, D., Schmachtl, F., Bögner, M., Slater, M., Halfer, J. & Bill, N. (2020). Hydrogen Peroxide Oxygenation and Disinfection Capacity in Recirculating Aquaculture Systems. *Aquacultural Engineering*, 92: 1-11. doi: 10.1016/j.aquaeng.2020.102140.

Bregnballe, J. (2022). *A Guide to Recirculation Aquaculture: an Introduction to the New Environmentally Friendly and Highly Productive Closed Fish Farming Systems*: Food & Agriculture Org.

Bugno, A., Almodóvar, A. A. & Pereira, T. C. (2010). Enumeration of heterotrophic bacteria in water for dialysis: Comparison of the efficiency of reasoner'2 agar and plate count agar. *Braz J Microbiol*, 41 (1): 15-8. doi: 10.1590/s1517-83822010000100003.

Cadnum, J. L., Pearlmutter, B. S., Haq, M. F., Jencson, A. L. & Donskey, C. J. (2021). Effectiveness and real-world materials compatibility of a novel hydrogen peroxide disinfectant cleaner. *American Journal of Infection Control*, 49 (12): 1572-1574. doi: https://doi.org/10.1016/j.ajic.2021.08.008.

Chen, S., Ling, J. & Blancheton, J.-P. (2006). Nitrification Kinetics of Biofilm as Affected by Water Quality Factors. *Aquacultural Engineering*, 34 (3): 179-197. doi: https://doi.org/10.1016/j.aquaeng.2005.09.004.

Colgan, S. & Gehr, R. (2001). Disinfection. Peracetic Acid Gains Favor as an Effective, Environmentally Benign Disinfection Alternative for Municipal Wastewater Treatment Applications. *Water Environment & Technology*, 13 (11): 29-33.

Curry, C., McCarthy, J., Darragh, H., Wake, R., Churchill, S., Robins, A. & Lowen, R. (2005). Identification of an Agent Suitable for Disinfecting Boots of Visitors to the Antarctic. *Polar Record*, 41 (1): 39-45.

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. doi: https://doi.org/10.1016/j.aquaculture.2022.739155.

Davidson, J., Summerfelt, S., Espmark, Å. M. O., Mota, V. C., Marancik, D., Earley, R. L., Snead, A. & Good, C. (2021). Effects of ozone on post-smolt Atlantic salmon (Salmo

salar) performance, health, and maturation in freshwater recirculation aquaculture systems. *Aquaculture*, 533: 736208.

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.

Directorate of Fisheries. (2023). *Atlantic Salmon, Rainbow Trout and Trout - Grow Out Production*: Directorate of Fisheries. Available at: https://www.fiskeridir.no/English/Aquaculture/Statistics/Atlantic-salmon-and-rainbow-trout/grow-out-production (accessed: 29/3/2024).

FAO. (2011). *Fishery and Aquaculture Country Profiles. Norway*. Available at: https://www.fao.org/fishery/en/facp/nor (accessed: 20/10/2023).

Finstad, A. G., Armstrong, J. D. & Nislow, K. H. (2010). Freshwater Habitat Requirements of Atlantic Salmon. In *Atlantic Salmon Ecology*, pp. 67-87.

Fisheries and Aquaculture Management Division. (2012). *Improving Biosecurity Through Prudent and Responsible Use of Veterinary Medicines in Aquatic Food Production*. 1 ed.: FAO Fisheries and Aquaculture Technical Paper. Available at: https://openknowledge.fao.org/handle/20.500.14283/ba0056e.

Foschino, R., Nervegna, I., Motta, A. & Galli, A. (1998). Bactericidal Activity of Chlorine Dioxide against Escherichia coli in Water and on Hard Surfaces. *Journal of Food Protection*, 61 (6): 668-672. doi: https://doi.org/10.4315/0362-028X-61.6.668.

Gan, W., Ge, Y., Zhong, Y. & Yang, X. (2020). The Reactions of Chlorine Dioxide with Inorganic and Organic Compounds in Water Treatment: Kinetics and Mechanisms. *Environmental Science: Water Research & Technology*, 6. doi: 10.1039/D0EW00231C.

Ge, S., Wang, S., Yang, X., Qiu, S., Li, B. & Peng, Y. (2015). Detection of nitrifiers and evaluation of partial nitrification for wastewater treatment: A review. *Chemosphere*, 140: 85-98.

Gesto, M., Liu, D., Pedersen, L.-F., Meinelt, T., Straus, D. L. & Jokumsen, A. (2018). Confirmation that pulse and continuous peracetic acid administration does not disrupt the acute stress response in rainbow trout. *Aquaculture*, 492: 190-194. doi: https://doi.org/10.1016/j.aquaculture.2018.04.009.

Gilbert, P. & Moore, L. E. (2005). Cationic Antiseptics: Diversity of Action Under a Common Epithet. *Journal of Applied Microbiology*, 99 (4): 703-715. doi: 10.1111/j.1365-2672.2005.02664.x.

Good, C., Redman, N., Murray, M., Straus, D. L. & Welch, T. J. (2022). Bactericidal activity of peracetic acid to selected fish pathogens in recirculation aquaculture system water. *Aquaculture Research*, 53 (16): 5731-5736. doi: https://doi.org/10.1111/are.16031.

Hersoug, B., Mikkelsen, E. & Osmundsen, T. C. (2021). What's the Clue; Better Planning, New Technology or Just More Money? - the Area Challenge in Norwegian Salmon Farming.

Ocean & Coastal Management, 199: 105415. doi: https://doi.org/10.1016/j.ocecoaman.2020.105415.

Hjeltnes, B. B., Grete; Erikson, Ulf Gøran; Mortensen, Stein; Rosten, Trond Waldemar; Bøe, Knut Egil; Hoel, Kristian; Olsen, Rolf Erik; Rimstad, Espen; Østerås, Olav. (2012). *Risk Assessment of Recirculation Systems in Salmonid Hatcheries*: Norwegian Scientific Committee for Food Safety (VKM). Available at: https://vkm.no/english/riskassessments/allpublications/riskassessmentofrecirculationsystemsinsalmonidhatcheries.4.72c3261615e09f2472f46f9b.html.

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. Available at: https://hdl.handle.net/11250/3088972.

Holan, A. B. (2013). *Improved Water Quality in Recirculating Aquaculture Systems (RAS) by Applying a Membrane Bioreactor (MBR) Concept for Removal of Colloidal and Fine Suspended Solids*. Trondheim: Norwegian University of Science and Technology. Available at: http://hdl.handle.net/11250/242373 (accessed: 1/4/2024).

Holan, A. B., Good, C. & Powell, M. D. (2020). Health Management in Recirculating Aquaculture Systems (RAS). In Kibenge, F. S. B. & Powell, M. D. (eds) *Aquaculture Health Management*, pp. 281-318: Academic Press.

Imlay, J. A. (2002). How Oxygen Damages Microbes: Oxygen Tolerance and Obligate Anaerobiosis. *Adv Microb Physiol*, 46: 111-53. doi: 10.1016/s0065-2911(02)46003-1.

Interdonato, F. (2012). *Recirculating aquaculture system (RAS) biofilters: focusing on bacterial communities complexity and activity*.

Ioannou Christopher, J., Hanlon Geoff, W. & Denyer Stephen, P. (2007). Action of Disinfectant Quaternary Ammonium Compounds against Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy*, 51 (1): 296-306. doi: 10.1128/aac.00375-06.

Keuter, S., G, Q., S, B., Schulz, C. & Spieck, E. (2017). Long-term monitoring of nitrification and nitrifying communities during bio filter activation of two marine recirculation aquaculture systems (RAS). *International Journal of Aquaculture and Fishery Sciences*, 3: 51-61.

Keyer, K. & Imlay, J. A. (1996). Superoxide Accelerates DNA Damage by Elevating Free-iron Levels. *Proc Natl Acad Sci U S A*, 93 (24): 13635-40. doi: 10.1073/pnas.93.24.13635.

Kitis, M. (2004). Disinfection of Wastewater with Peracetic Acid: a Review. *Environ Int*, 30 (1): 47-55. doi: 10.1016/s0160-4120(03)00147-8.

Kochelek, C. (2019). *Misleading Math: Kill Claims and Log Reduction*. Available at: https://infectioncontrol.tips/2019/06/17/kill-claims-and-log-reduction/ (accessed: 6/5/2024).

Lazado, C. C., Timmerhaus, G., Krasnov, A., Kirste, K. H., Pittman, K., Rørmark, L. & Pedersen, L. F. (2019). *Physiological coping mechanisms of Atlantic salmon exposed to an organic peroxide*. Frisk Fisk Tromsø, Norway

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. doi: https://doi.org/10.1016/j.aquaculture.2020.736038.

Lazado, C. C., Stiller, K. T., Reiten, B.-K. M., Osório, J., Kolarevic, J. & Johansen, L.-H. (2021). Consequences of continuous ozonation on the health and welfare of Atlantic salmon post-smolts in a brackish water recirculating aquaculture system. *Aquatic Toxicology*, 238: 105935. doi: https://doi.org/10.1016/j.aquatox.2021.105935.

Lekang, O.-I. (2013). *Aquaculture Engineering*. Ås Wiley-Blackwell.

Lepine, C., Redman, N., Murray, M., Lazado, C. C., Johansen, L.-H., Espmark, Å. M., Davidson, J. & Good, C. (2023). Assessing Peracetic Acid Application Methodology and Impacts on Fluidized Sand Biofilter Performance. *Aquaculture Research*, 2023 (1): 6294325. doi: https://doi.org/10.1155/2023/6294325.

Li, W., Shi, C., Yu, Y., Ruan, Y., Kong, D., Lv, X., Xu, P., Awasthi, M. K. & Dong, M. (2021). Interrelationships between tetracyclines and nitrogen cycling processes mediated by microorganisms: A review. *Bioresource technology*, 319: 124036.

Lindholm-Lehto, P. (2023). Water Quality Monitoring in Recirculating Aquaculture Systems. *Aquaculture, Fish and Fisheries*, 3 (2): 113-131. doi: https://doi.org/10.1002/aff2.102.

Lineback, C. B., Nkemngong, C. A., Wu, S. T., Li, X., Teska, P. J. & Oliver, H. F. (2018). Hydrogen peroxide and sodium hypochlorite disinfectants are more effective against Staphylococcus aureus and Pseudomonas aeruginosa biofilms than quaternary ammonium compounds. *Antimicrob Resist Infect Control*, 7: 154. doi: 10.1186/s13756-018-0447-5.

Liu, D., Straus, D. L., Pedersen, L.-F. & Meinelt, T. (2017). Pulse versus continuous peracetic acid applications: Effects on rainbow trout performance, biofilm formation and water quality. *Aquacultural Engineering*, 77: 72-79. doi: https://doi.org/10.1016/j.aquaeng.2017.03.004.

Liu, D., Straus, D. L., Pedersen, L.-F. & Meinelt, T. (2018). Periodic bacterial control with peracetic acid in a recirculating aquaculture system and its long-term beneficial effect on fish health. *Aquaculture*, 485: 154-159.

Ma, J.-W., Huang, B.-S., Hsu, C.-W., Peng, C.-W., Cheng, M.-L., Kao, J.-Y., Way, T.-D., Yin, H.-C. & Wang, S.-S. (2017). Efficacy and Safety Evaluation of a Chlorine Dioxide Solution. *International Journal of Environmental Research and Public Health*, 14: 329. doi: 10.3390/ijerph14030329.

Martínez de Alba, A. E., Rubio, M. B., Morán-Diez, M. E., Bernabéu, C., Hermosa, R. & Monte, E. (2021). Microbiological Evaluation of the Disinfecting Potential of UV-C and UV-C Plus Ozone Generating Robots. *Microorganisms*, 9 (1). doi: 10.3390/microorganisms9010172.

Martins, C., Eding, E. H., Verdegem, M. C., Heinsbroek, L. T., Schneider, O., Blancheton, J.-P., d'Orbcastel, E. R. & Verreth, J. (2010). New Developments in Recirculating Aquaculture

Systems in Europe: a Perspective on Environmental Sustainability. *Aquacultural engineering*, 43 (3): 83-93.

Mattilsynet. (s.a.). *Godkjende Desinfeksjonsmiddel for Akvakultur*. Available at: https://www.mattilsynet.no/fisk-og-akvakultur/dere-ma-bruke-godkjente-midler (accessed: 21/11/23).

Michaud, L., Blancheton, J. P., Bruni, V. & Piedrahita, R. (2006). Effect of Particulate Organic Carbon on Heterotrophic Bacterial Populations and Nitrification Efficiency in Biological Filters. *Aquacultural Engineering*, 34 (3): 224-233. doi: https://doi.org/10.1016/j.aquaeng.2005.07.005.

Moe Føre, H., Dahle, S. & Gaarder, R. (2018). Tensile Strength of Nylon Netting Subjected to Various Concentrations of Disinfecting Chemicals. *Journal of Offshore Mechanics and Arctic Engineering*, 141: 011603. doi: 10.1115/1.4040562.

Mohammadi-Aragh, M. K., Linhoss, J. E. & Evans, J. D. (2022). Effects of Various Disinfectants on the Bacterial Load and Microbiome of Broiler Hatching Eggs Using Electrostatic Spray. *Journal of Applied Poultry Research*, 31 (3): 100278. doi: https://doi.org/10.1016/j.japr.2022.100278.

Moreno-Andrés, J., Rueda-Márquez, J. J., Homola, T., Vielma, J., Moríñigo, M. Á., Mikola, A., Sillanpää, M., Acevedo-Merino, A., Nebot, E. & Levchuk, I. (2020). A comparison of photolytic, photochemical and photocatalytic processes for disinfection of recirculation aquaculture systems (RAS) streams. *Water Research*, 181: 115928. doi: https://doi.org/10.1016/j.watres.2020.115928.

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. doi: 10.3389/fanim.2022.876504.

Mota, V. C., Eggen, M. L. & Lazado, C. C. (2022). Acute dose-response exposure of a peracetic acid-based disinfectant to Atlantic salmon parr reared in recirculating aquaculture systems. *Aquaculture*, 554: 738142. doi: https://doi.org/10.1016/j.aquaculture.2022.738142.

MOWI. (2023). *Salmon Farming Industry Handbook 2023*. Mowi Salmon Farming Industry Handbook: MOWI. Available at: https://mowi.com/wp-content/uploads/2023/06/2023-Salmon-Farming-Industry-Handbook-2023.pdf (accessed: 28/5/23).

Muniesa, A., Escobar-Dodero, J., Silva, N., Henríquez, P., Bustos, P., Perez, A. M. & Mardones, F. O. (2019). Effectiveness of disinfectant treatments for inactivating Piscirickettsia salmonis. *Preventive Veterinary Medicine*, 167: 196-201. doi: https://doi.org/10.1016/j.prevetmed.2018.03.006.

Murray, F., Bostock, J. & Fletcher, D. (2014). *Review of Recirculation Aquaculture System Technologies and Their Commercial Application*. Highlands and Islands Enterprise: University of Stirling Aquaculture. Available at:

http://www.hie.co.uk/common/handlers/download-document.ashx?id=236008c4-f52a-48d9-9084-54e89e965573 (accessed: 28/5/2024).

Nazar, A. K. A., Jayakumar, R. & Tamilmani, G. (2013). *Recirculating aquaculture systems*.

Osland, A. M., Vestby, L. K. & Nesse, L. L. (2020). The Effect of Disinfectants on Quinolone Resistant E. coli (QREC) in Biofilm. *Microorganisms*, 8 (11). doi: 10.3390/microorganisms8111831.

Osório, J., Stiller, K. T., Reiten, B. K., Kolarevic, J., Johansen, L. H., Afonso, F. & Lazado, C. C. (2022). Intermittent Administration of Peracetic Acid is a Mild Environmental Stressor That Elicits Mucosal and Systemic Adaptive Responses From Atlantic Salmon Post-smolts. *BMC Zool*, 7 (1): 1. doi: 10.1186/s40850-021-00100-x.

Øvrebø, T. K., Balseiro, P., Imsland, A. K. D., Stefansson, S. O., Tveterås, R., Sveier, H. & Handeland, S. (2022). Investigation of growth performance of post-smolt Atlantic salmon (Salmo salar L.) in semi closed containment system: A big-scale benchmark study. *Aquaculture Research*, 53 (11): 4178-4189. doi: https://doi.org/10.1111/are.15919.

Pedersen, L.-F., Pedersen, P. B., Nielsen, J. L. & Nielsen, P. H. (2009). Peracetic Acid Degradation and Effects on Nitrification in Recirculating Aquaculture Systems. *Aquaculture*, 296 (3): 246-254. doi: https://doi.org/10.1016/j.aquaculture.2009.08.021.

Pedersen, L.-F. (2012). Hydrogen Peroxide Application to a Commercial Recirculating Aquaculture System. *Aquacultural Engineering*, 46: 40–46. doi: 10.1016/j.aquaeng.2011.11.001.

Pedersen, L.-F., Rojas-Tirado, P., Arvin, E. & Pedersen, P. B. (2019). Assessment of Microbial Activity in Water Based on Hydrogen Peroxide Decomposition Rates. *Aquacultural Engineering*, 85: 9-14. doi: https://doi.org/10.1016/j.aquaeng.2019.01.001.

Pedersen, L. F. & Lazado, C. C. (2020). Decay of Peracetic Acid in Seawater and Implications for Its Chemotherapeutic Potential in Aquaculture. *Aquaculture Environment Interactions*, 12: 153-165.

Percival, S. L., Finnegan, S., Donelli, G., Vuotto, C., Rimmer, S. & Lipsky, B. A. (2016). Antiseptics for treating infected wounds: Efficacy on biofilms and effect of pH. *Crit Rev Microbiol*, 42 (2): 293-309. doi: 10.3109/1040841x.2014.940495.

Perumal, P., Wand, M., Sutton, J. M. & Bock, L. (2014). Evaluation of the effectiveness of hydrogen-peroxide-based disinfectants on biofilms formed by Gram-negative pathogens. *Journal of Hospital Infection*, 87. doi: 10.1016/j.jhin.2014.05.004.

Peyneau, M., de Chaisemartin, L., Gigant, N., Chollet-Martin, S. & Kerdine-Römer, S. (2022). Quaternary ammonium compounds in hypersensitivity reactions. *Front Toxicol*, 4: 973680. doi: 10.3389/ftox.2022.973680.

Powell, A. & Scolding, J. (2018). Direct application of ozone in aquaculture systems. *Reviews in Aquaculture*, 10: 424-438. doi: 10.1111/raq.12169.

Ruiz, P., Vidal, J. M., Sepúlveda, D., Torres, C., Villouta, G., Carrasco, C., Aguilera, F., Ruiz-Tagle, N. & Urrutia, H. (2020). Overview and future perspectives of nitrifying bacteria on

biofilters for recirculating aquaculture systems. *Reviews in Aquaculture*, 12 (3): 1478-1494. doi: https://doi.org/10.1111/raq.12392.

Russo, R., Curtis, E. W. & Yanong, R. P. E. (2007). Preliminary Investigations of Hydrogen Peroxide Treatment of Selected Ornamental Fishes and Efficacy against External Bacteria and Parasites in Green Swordtails. *Journal of Aquatic Animal Health*, 19 (2): 121-127. doi: https://doi.org/10.1577/H05-024.1.

Rusten, B., Eikebrokk, B., Ulgenes, Y. & Lygren, E. (2006). Design and operations of the Kaldnes moving bed biofilm reactors. *Aquacultural Engineering*, 34 (3): 322-331. doi: https://doi.org/10.1016/j.aquaeng.2005.04.002.

Schneider, O., Sereti, V., Eding, E. H. & Verreth, J. A. J. (2005). Analysis of nutrient flows in integrated intensive aquaculture systems. *Aquacultural Engineering*, 32 (3): 379-401. doi: https://doi.org/10.1016/j.aquaeng.2004.09.001.

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. doi: https://doi.org/10.1016/j.copbio.2010.03.011.

Schwartz, M., Bullock, G., Hankins, J., Summerfelt, S. & Mathias, J. (2000). Effects of selected chemotherapeutants on nitrification in fluidized-sand bioftlters for coldwater fish production.

Shitu, A., Liu, G., Muhammad, A. I., Zhang, Y., Tadda, M. A., Qi, W., Liu, D., Ye, Z. & Zhu, S. (2022). Recent advances in application of moving bed bioreactors for wastewater treatment from recirculating aquaculture systems: A review. *Aquaculture and Fisheries*, 7 (3): 244-258. doi: https://doi.org/10.1016/j.aaf.2021.04.006.

Simon, F. X., Berdalet, E., Gracia, F. A., España, F. & Llorens, J. (2014). Seawater disinfection by chlorine dioxide and sodium hypochlorite. A comparison of biofilm formation. *Water, Air, & Soil Pollution*, 225 (4): 1921. doi: 10.1007/s11270-014-1921-1.

Skutvik, T. S., Håvard; Kolsrud, Astrid. (2022). *Freebac som desinfeksjonskjemikalie*: NTNU (accessed: 30/5/2024).

Solvay. (2014). *Use of Aqua Des in Aquaculture*. Chemicals Sector – Peroxygens Disinfection Technology Team. Available at: https://issuu.com/aquatic/docs/use_of_aqua_des_in_aquaculture_-_a (accessed: 5/5/2024).

Stein, L. Y. & Klotz, M. G. (2016). The nitrogen cycle. *Current Biology*, 26 (3): R94-R98.

Stiller, K. T., Kolarevic, J., Lazado, C. C., Gerwins, J., Good, C., Summerfelt, S. T., Mota, V. C. & Espmark, Å. M. (2020). The effects of ozone on Atlantic salmon post-smolt in brackish water—Establishing welfare indicators and thresholds. *International Journal of Molecular Sciences*, 21 (14): 5109.

Stockton, K. A. & Moffitt, C. M. (2013). Disinfection of Three Wading Boot Surfaces Infested with New Zealand Mudsnails. *North American Journal of Fisheries Management*, 33 (3): 529-538. doi: https://doi.org/10.1080/02755947.2013.768569.

Su, X., Sutarlie, L. & Loh, X. J. (2020). Sensors, Biosensors, and Analytical Technologies for Aquaculture Water Quality. *Research*. doi: 10.34133/2020/8272705.

Suurnäkki, S., Pulkkinen, J. T., Lindholm-Lehto, P. C., Tiirola, M. & Aalto, S. L. (2020). The effect of peracetic acid on microbial community, water quality, nitrification and rainbow trout (Oncorhynchus mykiss) performance in recirculating aquaculture systems. *Aquaculture*, 516: 734534. doi: https://doi.org/10.1016/j.aquaculture.2019.734534.

Takasaki, A., Hashida, T., Fujiwara, S., Kato, K. & Nishihara, T. (1994). Bactericidal Action of A Quaternary Ammonium Disinfectant, Didecyldimethyl Ammonium Chloride, against Staphylococcus aureus. *Eisei kagaku*, 40 (4): 344-350. doi: 10.1248/jhs1956.40.344.

Tan, L. K., Ooi, P. T., Carniel, E. & Thong, K. L. (2014). Evaluation of a modified Cefsulodin-Irgasan-Novobiocin agar for isolation of Yersinia spp. *PLoS One*, 9 (8): e106329. doi: 10.1371/journal.pone.0106329.

Taufik, M., Tuan Ismail, T. I., Manan, H., Ikhwanuddin, M., Abdul Salam, A. I., Abdul Rahim, A. I., Ishak, A. N., Kamaruzzan, A. S., Draman, A. S. & Kasan, N. A. (2023). Synergistic Effects of Recirculating Aquaculture System (RAS) With Combination of Clear Water, Probiotic and Biofloc Technology: a Review. *Aquaculture and Fisheries*. doi: https://doi.org/10.1016/j.aaf.2023.07.006.

Teitge, F., Peppler, C., Steinhagen, D. & Jung-Schroers, V. (2020). Effect of disinfection with peracetic acid on the microbial community of a seawater aquaculture recirculation system for Pacific white shrimp (Litopenaeus vannamei). *Journal of Fish Diseases*, 43 (9): 991-1017. doi: https://doi.org/10.1111/jfd.13207.

Tian, X.-L. & Dong, S.-L. (2023). Land-Based Intensive Aquaculture Systems. In Dong, S.-L., Tian, X.-L., Gao, Q.-F. & Dong, Y.-W. (eds) *Aquaculture Ecology*, pp. 369-402. Singapore: Springer Nature Singapore.

Timmons, M., Guerdat, T. & Vinci, B. (2018). *Recirculating Aquaculture, 4th edition*.

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: 10/5/2024).

Vadstein, O., Attramadal, K. J. K., Bakke, I. & Olsen, Y. (2018). K-Selection as Microbial Community Management Strategy: A Method for Improved Viability of Larvae in Aquaculture. *Front Microbiol*, 9: 2730. doi: 10.3389/fmicb.2018.02730.

Verner – Jeffreys, D. W., Joiner, C. L., Bagwell, N. J., Reese, R. A., Husby, A. & Dixon, P. F. (2009). Development of bactericidal and virucidal testing standards for aquaculture disinfectants. *Aquaculture*, 286 (3): 190-197. doi: https://doi.org/10.1016/j.aquaculture.2008.10.001.

Wang, G., Yang, L., Jiang, L., Chen, J., Jing, Q., Mai, Y., Deng, L., Lin, Y., Chen, L., Chen, Z., et al. (2022). A New Class of Quaternary Ammonium Compounds as Potent and Environmental Friendly Disinfectants. *J Clean Prod*, 379: 134632. doi: 10.1016/j.jclepro.2022.134632.

Weidmann, M. (2023). Efficiency of surface inactivation disinfectants for bacteria studied in an aquaculture microbiology laboratory. *Journal of Biosafety and Biosecurity*, 5 (2): 84-86. doi: https://doi.org/10.1016/j.jobb.2023.06.001.

WHO. (2016). *CHLORINE DIOXIDE, CHLORATE AND CHLORITE IN DRINKING-WATER*: World Health Organization Press. Available at: https://cdn.who.int/media/docs/default-source/wash-documents/wash-chemicals/chlorine-dioxide-chlorite-chlorate-background-jan17.pdf?sfvrsn=cab527bb_5.

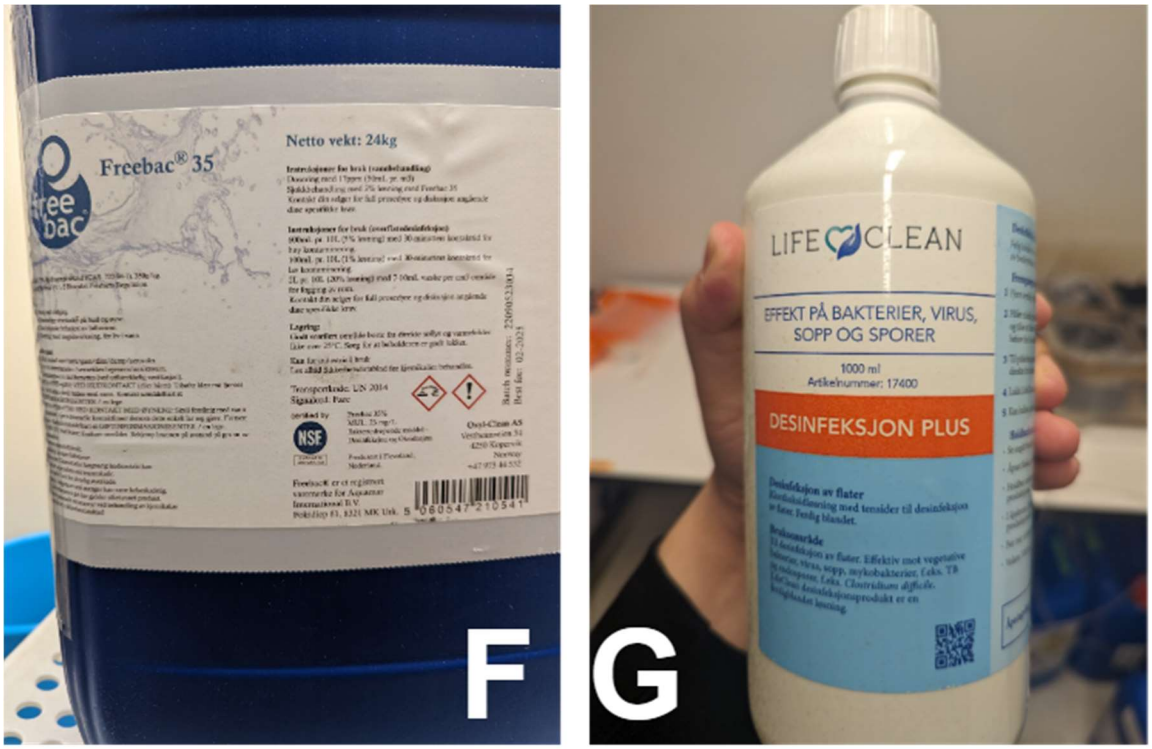
Yanong, R. P. (2015). *Fish Health Management Considerations in Recirculating Aquaculture Systems—Part 2: Pathogens 1*.

Zhang, S.-Y., Li, G., Wu, H.-B., Liu, X.-G., Yao, Y.-H., Tao, L. & Liu, H. (2011). An Integrated Recirculating Aquaculture System (RAS) for Land-based Fish Farming: The Effects on Water Quality and Fish Production. *Aquacultural Engineering*, 45 (3): 93-102. doi: https://doi.org/10.1016/j.aquaeng.2011.08.001.

Zibiene, G. & Žibas, A. (2018). *WATER QUALITY AND WASTE CONTAMINATION STUDY IN CATFISH REARING RECIRCULATING AQUACULTURE SYSTEM*, vol. 0.

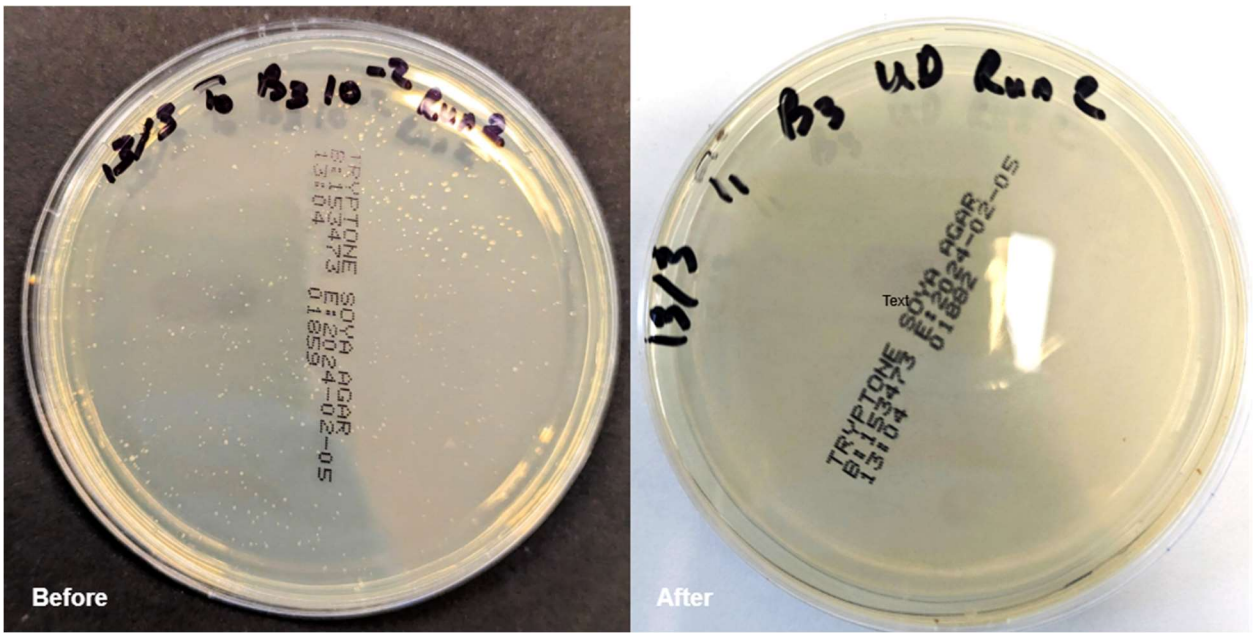
Appendix

Appendix 1. Disinfectants. (A: Addi Aqua, B: Aqua Des, C: Perfectoxid, D: Virkon Aquatic, E: Virocid, F:Free Bac ®35, G : LifeClean)

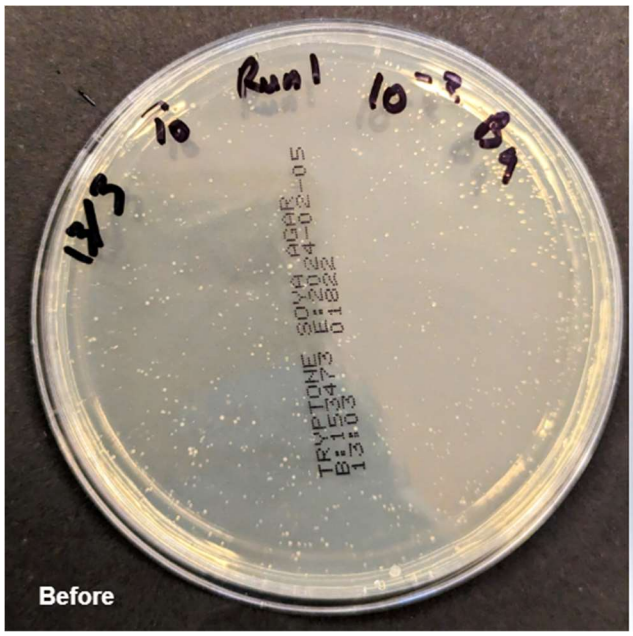


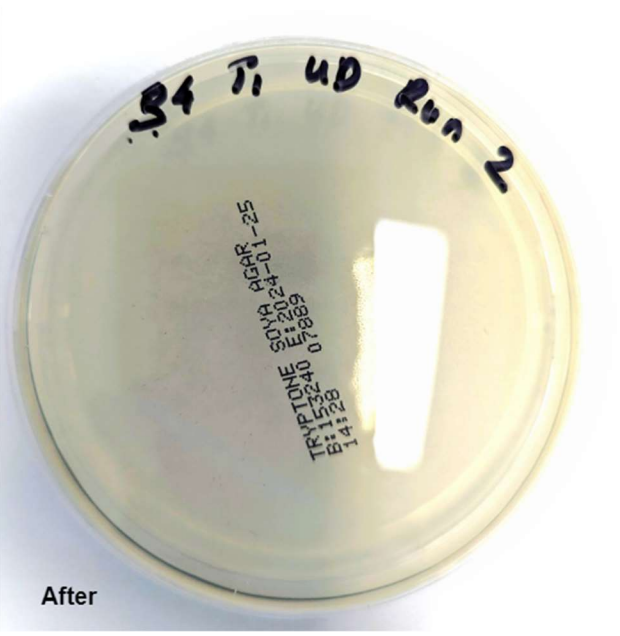
Appendix 2. TSA Plates Before and After Disinfection

A. Aqua Des

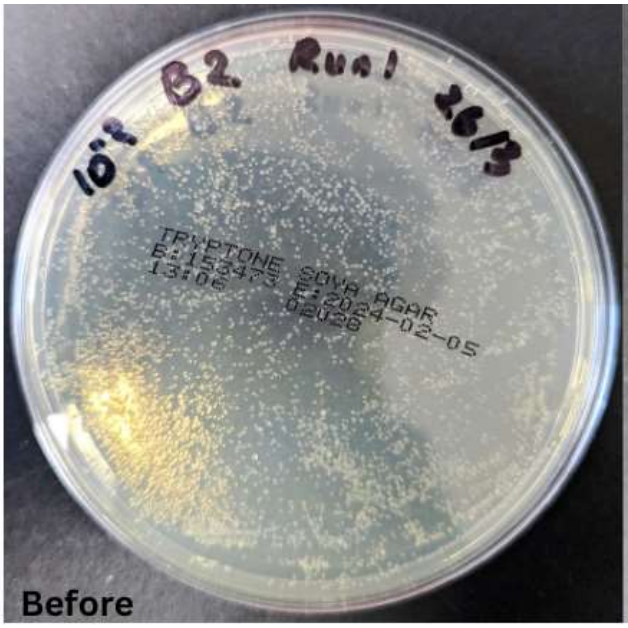


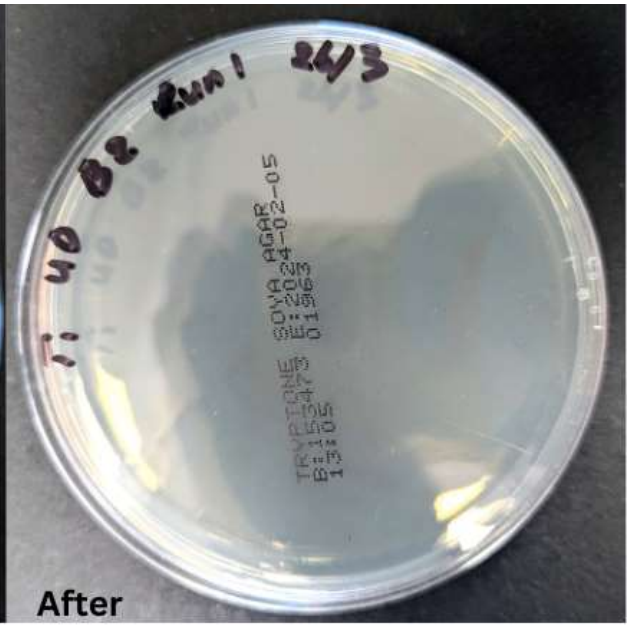
B. Addi Aqua



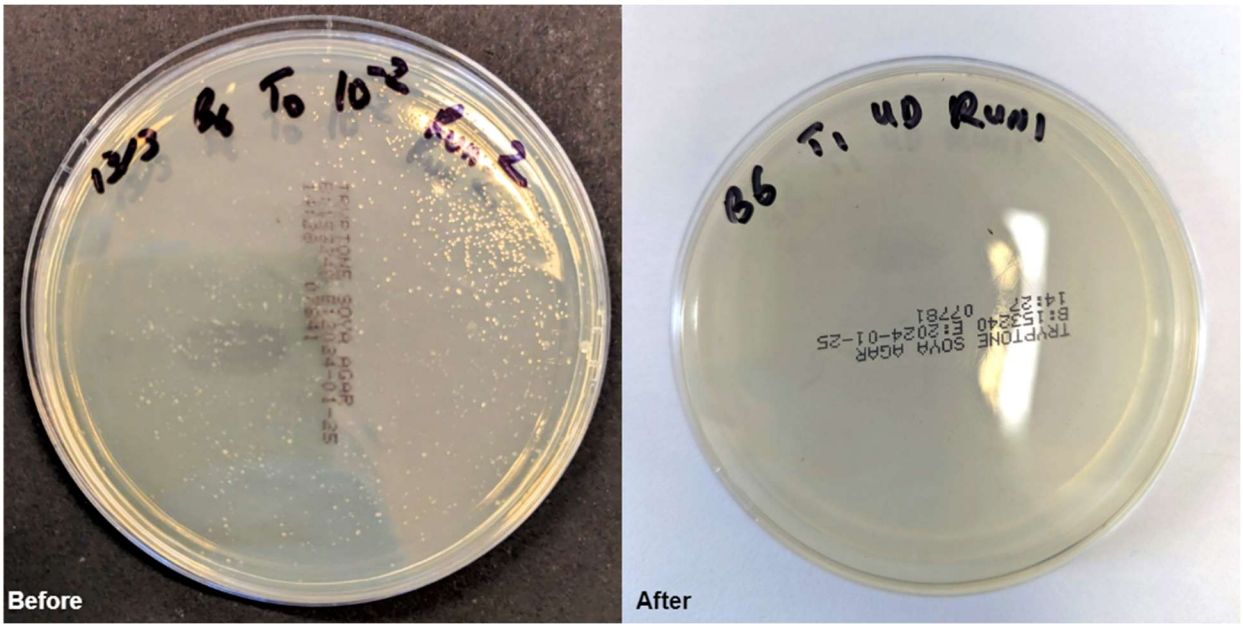


C. Perfectoxid

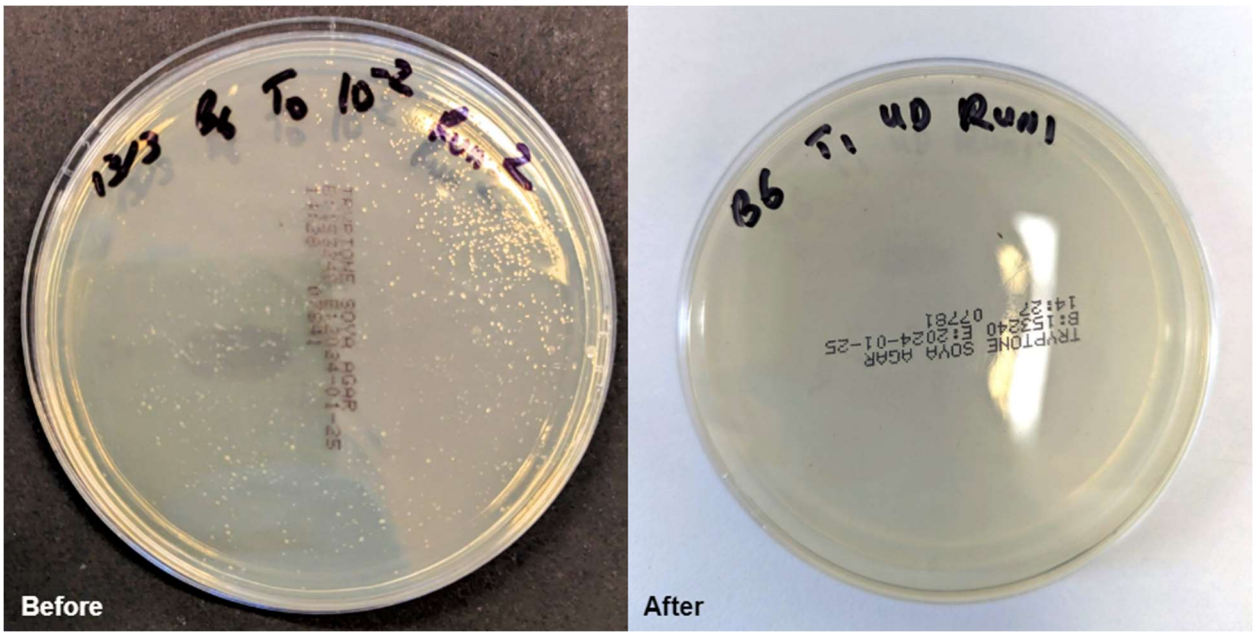




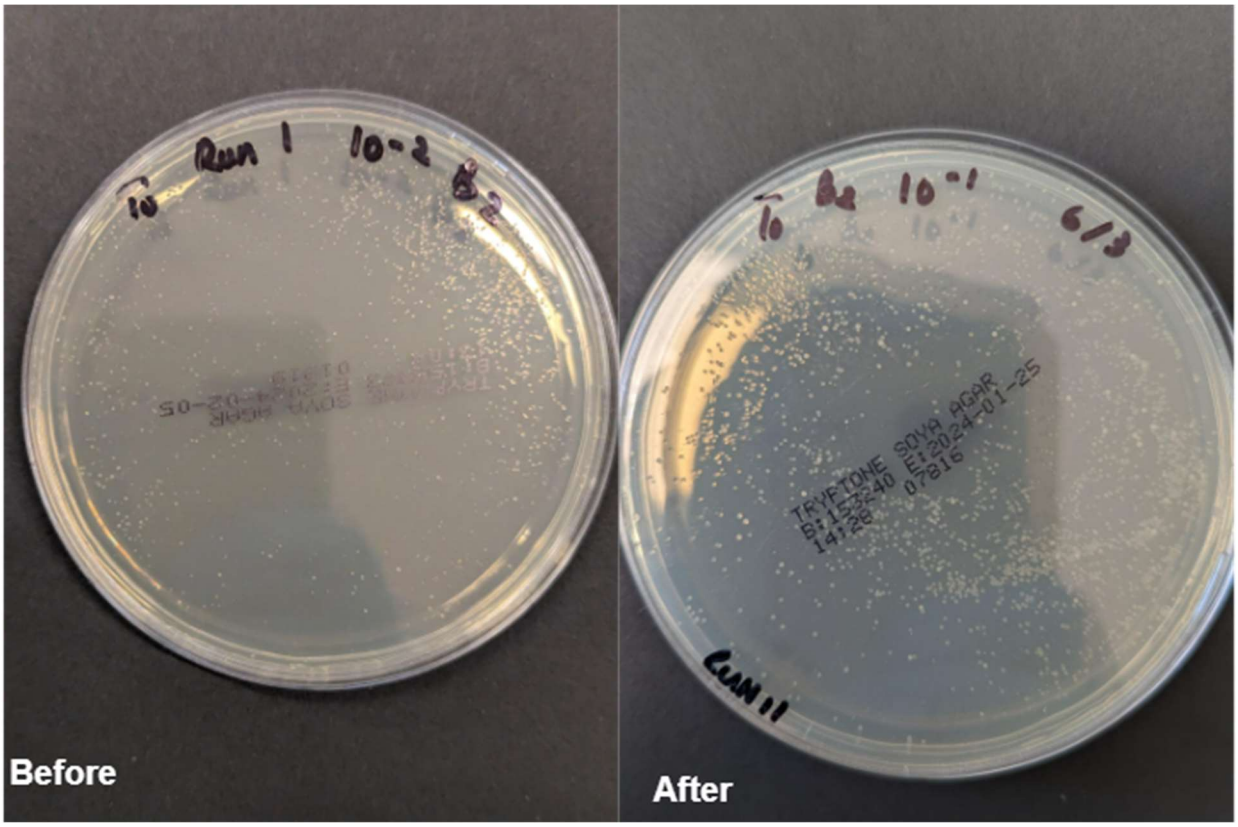
D. Virkon Aquatic



E. Virocid



F. Free Bac ®35

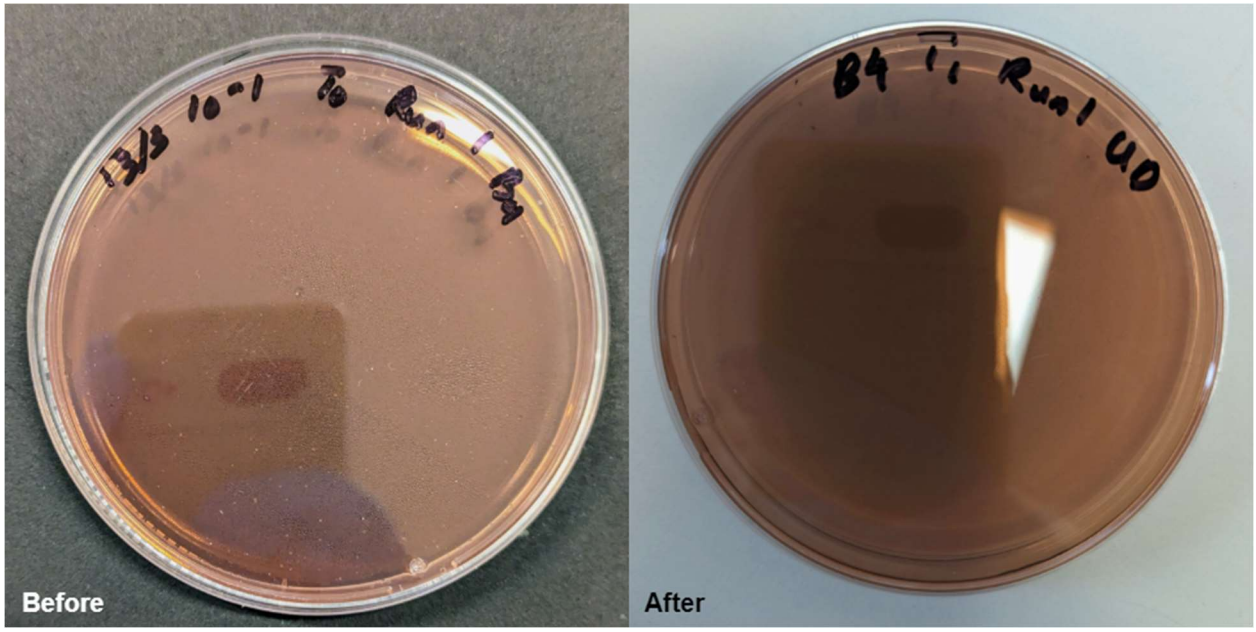


G. Comparison with TSA Control and Sample Treated With Life Clean

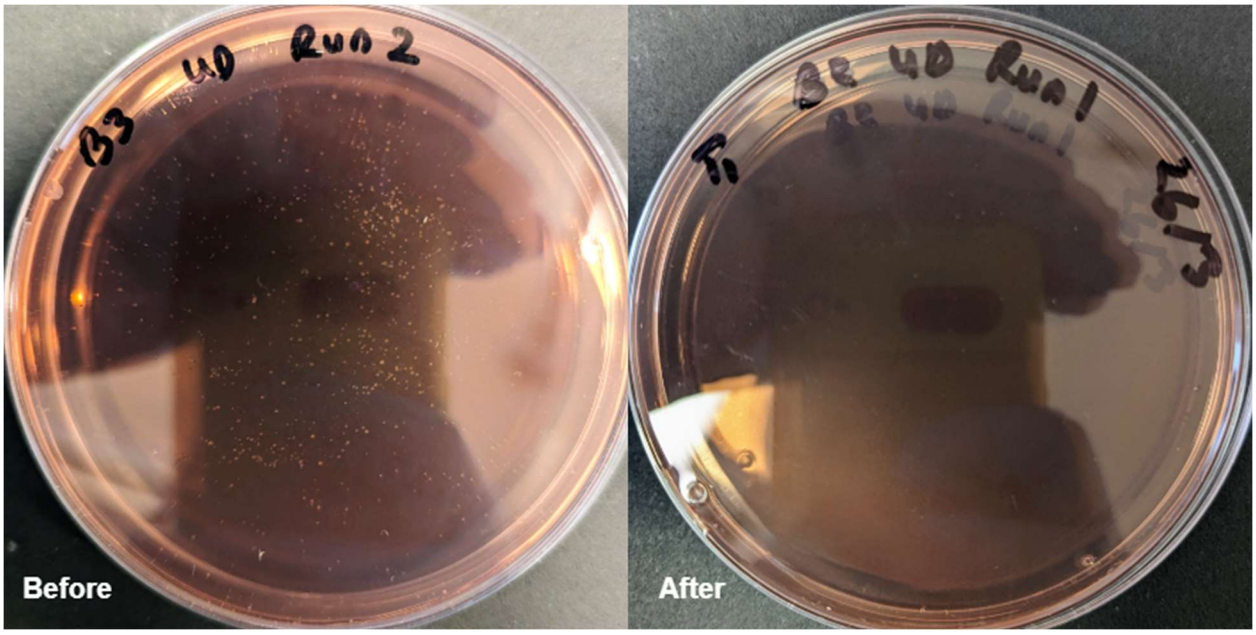
Appendix 3. CIN Plates Before and After Disinfection

A. Aqua Des

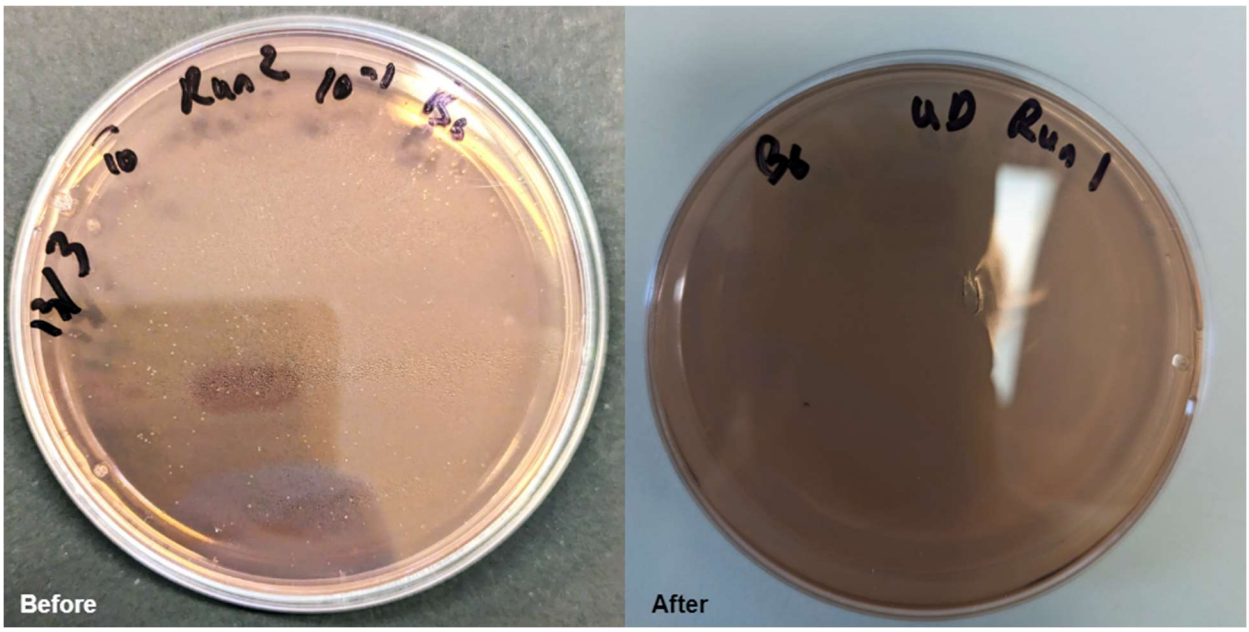
B. Addi Aqua



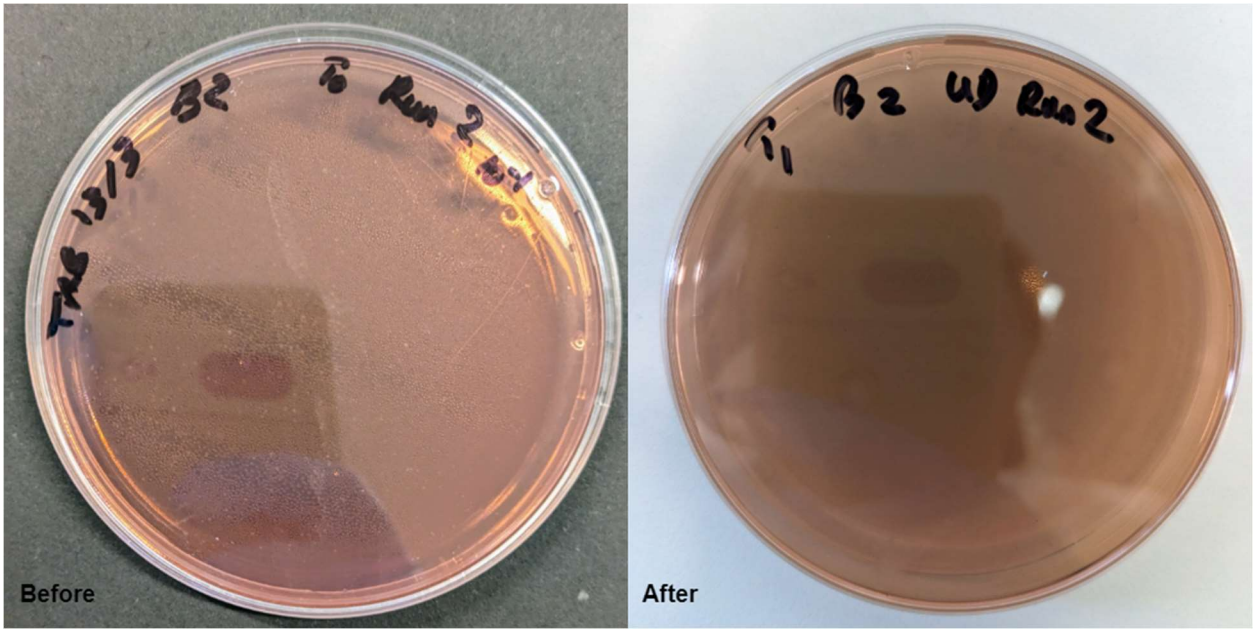
C. Perfectoxid



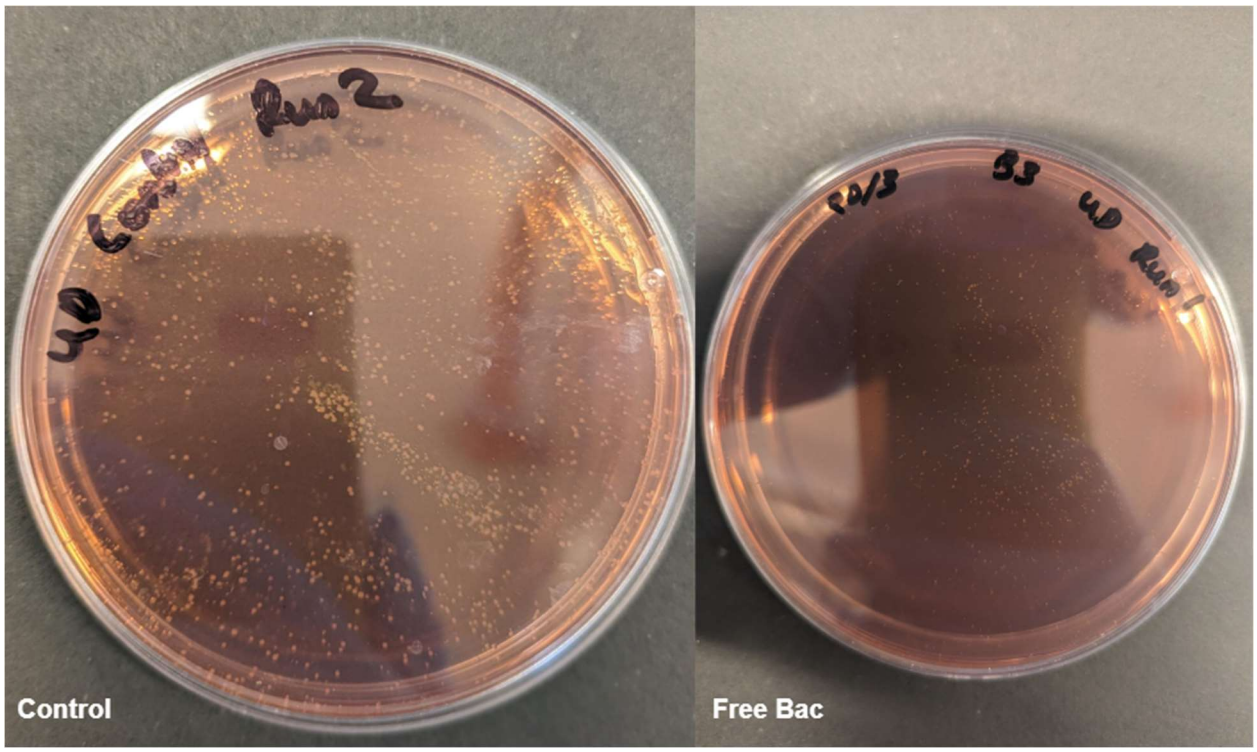
D. Virkon Aquatic



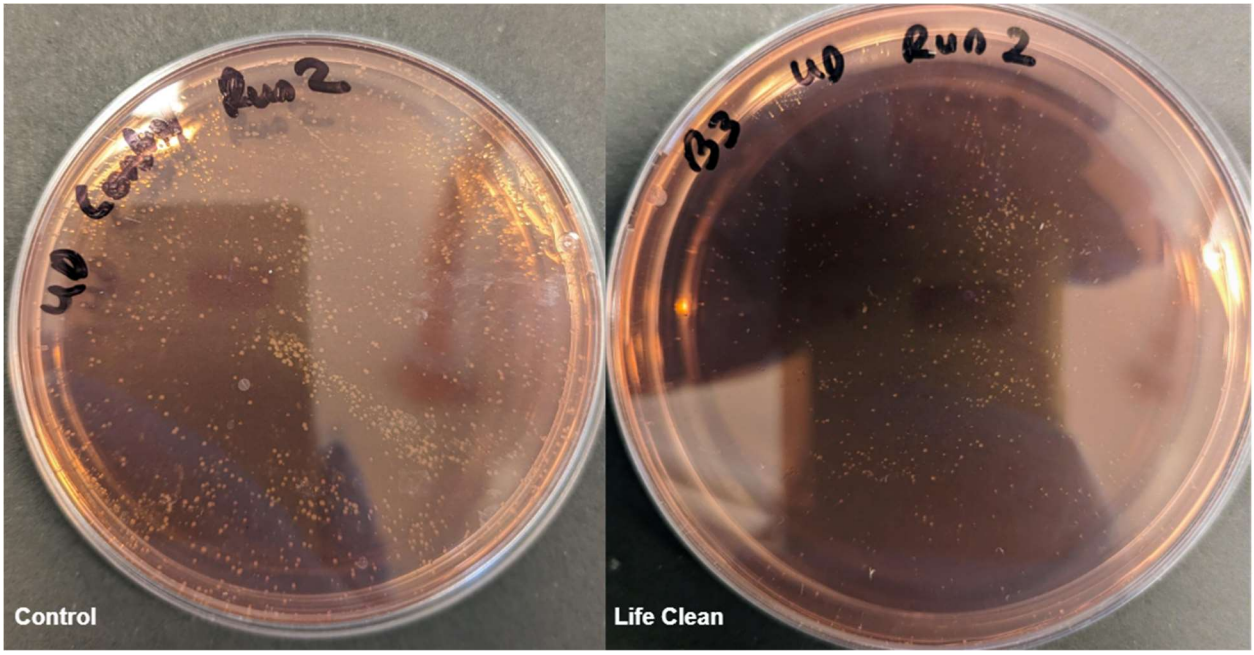
E. Virocid



F. Comparison with Control CIN plate and Samples Treated with Free Bac ®35



G. Comparison with Control CIN plate and Samples Treated with Life Clean



Appendix 4: Analytical procedure for ammonium (NH_4-N) test using Spectroquant Prove 100

Ammonium

114752

Test

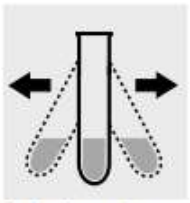
Measuring	0.05 – 3.00 mg/l NH_4-N	0.06 – 3.86 mg/l NH_4	10-mm cell
range:	0.03 – 1.50 mg/l NH_4-N	0.04 – 1.93 mg/l NH_4	20-mm cell
	0.010 – 0.500 mg/l NH_4-N	0.013 – 0.644 mg/l NH_4	50-mm cell
	0.05 – 3.00 mg/l NH_3-N	0.06 – 3.65 mg/l NH_3	10-mm cell
	0.03 – 1.50 mg/l NH_3-N	0.04 – 1.82 mg/l NH_3	20-mm cell
	0.010 – 0.500 mg/l NH_3-N	0.016 – 0.608 mg/l NH_3	50-mm cell
	Expression of results also possible in mmol/l.		

Check the pH of the sample, specified range: pH 4 – 13.
If required, add dilute sodium hydroxide solution or sulfuric acid drop by drop to adjust the pH.

Pipette 5.0 ml of the sample into a test tube.

Add 0.60 ml of **NH_4-1** with pipette and mix.

Add 1 level blue microspoon of **NH_4-2**.

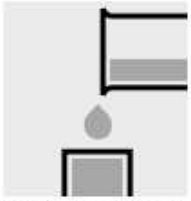


Shake vigorously to dissolve the solid substance.

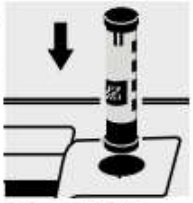
Reaction time:
5 minutes

Add 4 drops of **NH_4-3** and mix.

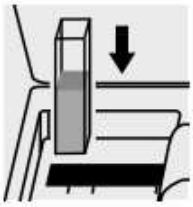
Reaction time:
5 minutes



Transfer the solution into a corresponding cell.



Select method with AutoSelector.



Place the cell into the cell compartment.

Important:

Very high ammonium concentrations in the sample produce turquoise-colored solutions (measurement solution should be yellow-green to green) and false-low readings are yielded. In such cases the sample must be diluted (plausibility check).

To measure in the 50-mm cell, the sample volume and the volume of the reagents have to be doubled for each. Alternatively, the semi-microcell, Cat.No. 173502, can be used.

Quality assurance:

To check the measurement system (test reagents, measurement device, and handling) we recommended to use Spectroquant® CombiCheck 50, Cat.No. 114695, or the Standard solutions for photometric applications, CRM, Cat.Nos. 125022, 125023, 125024, and 132227.

Ready-to-use ammonium standard solution Certipur®, Cat.No. 119812, concentration 1000 mg/l NH_4^+, can also be used after diluting accordingly.

To check for sample-dependent effects the use of addition solutions (e.g. in CombiCheck 50) is highly recommended.

Appendix 5. Microbiological Efficacy Summary of Life Clean

LIFE CLEAN

Microbiological Efficacy Summary

Document	Doc. id.	Version	Page
Assessment of antimicrobial efficiency – LifeClean Disinfectant Std and Plus	LC-TD-0052-Public	01	1 (4)

Issued by (Name/Signature) — Date

Rahma Wehelie — Rahma Wehelie — 2022-03-29

Approved by (Name/Signature) — Date

Lars Nord — 2022-03-29

	Test norm	Test type	Organism	Log reduction required	Log reduction achieved	Laboratory	Clean or dirty	Contact time	LC Std 400-200 PPM	LC Plus 800-400 PPM
Sporicidal	EN 13697:2001 (Phase 2, Step 2)	Quantitative Carrier test	*Bacillus subtilis* ATCC 6633	4 log_{10}	>4 log_{10}	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	2 min		✓
	EN 13697:2001 (Phase 2, Step 2)	Quantitative Carrier test	*Clostridium difficile* UK 027	3 log_{10}	>3 log_{10}		Clean	2 min		✓1
	EN 13704:2002 (Phase 2, Step 1)	Quantitative suspension test	*Clostridium difficile* UK 027	3 log_{10}	>3 log_{10}		Clean	1-5 min	✓	✓
					>3.3 log_{10}		Clean/Dirty	5 min	✓1, 5	✓
			Clostridium difficile UK 027		>6.1-7.5 log_{10}		Clean	5-10 min	✓2	✓
			Clostridium difficile UK 023		>4 log_{10}	Internal test at Örebro University Hospital, Sweden	Clean	2 min	✓3-4	✓
	EN 17126:2018 (Phase 2, Step 1)	Quantitative suspension test	*Bacillus subtilis*	4 log_{10}	>5.02 log_{10}	MSL Solution, UK	Clean	2-5 min	✓6	✓
			Bacillus cereus		>5.21 log_{10}					
			Clostridium difficile		>5.21 log_{10}					
	EN 17126:2018 (Phase 2, Step 1)	Quantitative suspension test	*Bacillus subtilis*	4 log_{10}	>4.08 log_{10}	MSL Solution, UK	Dirty	2 min	✓6	✓
			Bacillus cereus		>5.36 log_{10}			2 min	✓6	✓
			Clostridium difficile		>5.43 log_{10}			2 min	✓6	✓
	EN 13727:2012+A2:2015 (Phase 2, Step 2)	Quantitative suspension test	*Clostridium perfringens*	5 log_{10}	5.08 log_{10}	Hygiene Nord GmbH, Germany	Clean	2 min	100 ppm	✓
							Dirty	5 min		
Mycobactericidal	EN 14348:2005 (Phase 2, Step 1)	Quantitative suspension test	*Mycobacterium terrae* ATCC 15755	4 log_{10}	>4 log_{10}	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	1 min	✓	✓
			Mycobacterium avium ATCC 15769						✓	✓
	EN 14563:2009 (Phase 2, Step 2)	Quantitative Carrier test	*Mycobacterium terrae* ATCC 15755	4 log_{10}	>4 log_{10}	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	1 min	✓	✓
			Mycobacterium avium ATCC 15769						✓	✓
Virucidal	EN 14476:2019 (Phase 2, Step 1)	Quantitative suspension test	Poliovirus Type 1, LSc-2abo	4 log_{10}	>6.33 log_{10}	Labor-Enders Laboratory, Germany	Clean	30 sec	17.5 ppm	✓
			Adenovirus Type 5, strain Adenoid 75, ATCC VR-5		>5.33 log_{10}				17.5 ppm	✓
			Murine Norovirus Strain S99		>5.50 log_{10}				17.5 ppm	✓
			Polyomavirus SV40		>4.50 log_{10}				17.5 ppm	✓
	EN 14476:2019 (Phase 2, Step 1)	Quantitative suspension test	Bovine Viral Diarrhea Virus (BVDV) strain NADL	4 log_{10}	>5.67 log_{10}	Labor-Enders Laboratory, Germany	Clean	1 min	20 ppm	✓
			Modified vaccinia virus Ankara (MVA)		>4.58 log_{10}					
	EN 16777:2018	Quantitative Carrier test	Adenovirus Type 5	4 log_{10}	>6.51 log_{10}	Labor-Enders Laboratory, Germany	Clean	2 min	110 ppm	✓
	EN16777:2018	Quantitative Carrier test	Murine Norovirus	4 log_{10}	>6.19 log_{10}	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	2 min	✓	✓
	EN16777:2018	Quantitative Carrier test	Modified vaccinia virus Ankara (MVA	4 log_{10}	>4.97 log_{10}	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	5 min	✓	✓

	Test norm	Test type	Organism	Log reduction required	Log reduction achieved	Laboratory	Clean or dirty	Contact time	LC Std 400-200 PPM	LC Plus 800-400 PPM
Virucidal	EN16777:2018	Quantitative Carrier test	Murine Parvovirus (MVM)	4 $\log_{10}$	>4.41 $\log_{10}$	Labor-Enders Laboratory, Germany	Clean	5 min		✓
	EN 14675:2015 (Phase 2, Step 1)	Quantitative suspension test	IPN Virus Salmonid fish viral infection	4 $\log_{10}$	>4 $\log_{10}$	Norwegian Veterinary Institute, Norway	Clean	5 min	✓	✓
				4 $\log_{10}$	>4 $\log_{10}$		Dirty	5 min	✓	✓
	EN 14349:2007 (Phase 2, Step 1)	Quantitative Carrier test	Avian influenza virus, (H10N7)	4 $\log_{10}$	>4.2 $\log_{10}$	SVA	clean	1 min		✓
	EN 14349:2007 (Phase 2, Step 2)	Quantitative Carrier test	PPV, strain 89/76	4 $\log_{10}$	>5.3 $\log_{10}$	SVA	Clean	5 min	✓	✓
	EN 14476:2013+A2:2019	Suspension test	Feline coronavirus (FCoV)	4 $\log_{10}$	>4.3 $\log_{10}$	MSL solution, UK	Clean	1 min	✓	✓
	EN 14476:2013+A2:2019	Suspension test	Bovine coronavirus (BCoV)	4 $\log_{10}$	>5.5 $\log_{10}$	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	30 sec	50 ppm	✓
	EN 14476:2013+A2:2019	Suspension test	SARS-CoV-2-Covid-19	4 $\log_{10}$	>5.6 $\log_{10}$	SVA	Clean	30 sec	50 ppm	✓
	AOAC 961.02	Quantitative Carrier test	Adenovirus Type 5, strain Adenoid 75, ATCC VR-5	5 $\log_{10}$	6.1 $\log_{10}$	MicroChem Laboratory, Texas, USA	Clean	5 min	✓	✓
Fungicidal	EN 13624:2013 (Phase 2, Step 1)	Quantitative suspension test	Candida albicans ATCC 10231	4 $\log_{10}$	>4 $\log_{10}$	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	1 min	✓	✓
			Aspergillus brasiliensis ATCC 16404						✓	✓
	EN 13624:2013 (Phase 2, Step 1)	Quantitative suspension test	Candida albicans ATCC 10231	4 $\log_{10}$	>6.36 $\log_{10}$	Labor-Enders Laboratory, Germany	Clean	1 min	20 ppm	✓
	EN 13624:2013 (Phase 2, Step 1)	Quantitative suspension test	Candida auris DSM 21092	4 $\log_{10}$	>4 $\log_{10}$	Microbial Analytics Sweden	Clean	2 min	✓	✓
	EN 14562:2006 (Phase 2, Step 2)	Quantitative Carrier test	Aspergillus brasiliensis (niger) / black mold ATCC 16404	4 $\log_{10}$	>4 $\log_{10}$	Mikrolab Stockholm AB	Clean/Dirty	3/5 min	✓	✓
	EN 14562:2006 (Phase 2, Step 2)	Quantitative Carrier test	Candida albicans ATCC 10231	4 $\log_{10}$	>4 $\log_{10}$	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	1 min	✓	✓
	EN 14562:2006 (Phase 2, Step 2)	Quantitative Carrier test	Aspergillus brasiliensis (niger) / black mold ATCC 16404	4 $\log_{10}$	>4 $\log_{10}$	Dr. Brill + Partner GMBH Laboratory, Germany and	Clean	2 min		✓
				4 $\log_{10}$	>4 $\log_{10}$		Dirty	2 min		✓
	AOAC 961.02	Germicidal Spray	Trichophyton mentagrophytes	0/60	0/60	Medical Technology, Mahidol University, Thailand	Clean	30 sec	✓	✓
							Dirty	2 min	✓	✓
	EN 16615:2015 (Phase 2, step 2)	4-Field test	Candida albicans ATCC 10231	4 $\log_{10}$	>5 $\log_{10}$	RISE Research Institutes of Sweden	Clean	2 min	✓	✓
	EN 16615:2015 (Phase 2, step 2)	4-Field test	Candida albicans ATCC 10231	4 $\log_{10}$	>5.63 $\log_{10}$	Hygiene Nord GmbH, Germany	Clean	1 min	✓	✓
	EN 17387:2020	Quantitative Carrier test	Candida albicans ATCC 10231	4 $\log_{10}$	>5.47 $\log_{10}$	Labor-Enders Laboratory, Germany	Clean	5 min	✓	✓
Gram-negative Bacteria	EN 13727:2014 (Phase 2, Step 1)	Quantitative suspension test	Pseudomonas aeruginosa ATCC 15442	5 $\log_{10}$	>7.58 $\log_{10}$	Labor-Enders Laboratory, Germany	Clean	1 min	20 ppm	✓
	EN 14561:2006 (Phase 2, Step 2)	Quantitative Carrier test	Pseudomonas aeruginosa ATCC 15442	5 $\log_{10}$	>5.11 $\log_{10}$	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	1 min	✓	✓
	EN 13727:2015 (Phase 2, Step 2)	Quantitative suspension test	Pseudomonas aeruginosa ATCC 15442	5 $\log_{10}$	>5 $\log_{10}$	Food Safety Laboratory, Chung-Ang University, Seoul, South Korea	Dirty	2 min	✓	✓
	EN 13727:2015 (Phase 2, Step 2)	Quantitative suspension test[a]	Escherichia coli ATCC 25922	5 $\log_{10}$	>5 $\log_{10}$	Internal test at Örebro University Hospital, Sweden	Clean	2 min	✓	✓
	EN 13727:2015 (Phase 2, Step 2)	Quantitative suspension test	Escherichia coli K12, NCTC 10538	5 $\log_{10}$	>7.5 $\log_{10}$	Labor-Enders Laboratory, Germany	Clean	1 min	20 ppm	✓
	EN 13727:2015 (Phase 2, Step 2)	Quantitative suspension test	Campylobacter jejuni ATCC 33560	5 $\log_{10}$	>5 $\log_{10}$	Internal test at Örebro University Hospital, Sweden	Clean/Dirty	2 min	✓	✓
	EN 13727:2015 (Phase 2, Step 2)	Quantitative suspension test	Proteus mirabilis ATCC 14153	5 $\log_{10}$	>7.5 $\log_{10}$	Labor-Enders Laboratory, Germany Labor-Enders Laboratory, Germany	Clean	1 min	20 ppm	✓
	EN 13727:2015 (Phase 2, Step 2)	Quantitative suspension test	Salmonella typhimurium ATCC 14028	5 $\log_{10}$	>5.76 $\log_{10}$	Internal test at Örebro University Hospital, Sweden	Clean	2 min	✓	✓
				5 $\log_{10}$	>5.76 $\log_{10}$		Dirty	2 min	✓	✓
			Legionella pneumophila ATCC 33152	5 $\log_{10}$	>5.76 $\log_{10}$		Clean	2 min	✓	✓
				5 $\log_{10}$	>5 $\log_{10}$		Dirty	2 min	✓	✓

	Test norm	Test type	Organism	Log reduction required	Log reduction achieved	Laboratory	Clean or dirty	Contact time	LC Std 400-200 PPM	LC Plus 800-400 PPM
Gram-negative Bacteria	EN 14349:2012 (Phase 2, Step 2)	Quantitative suspension test	Aeromonas salmonicida ATCC 14174	5 log_{10}	>5.65 log_{10}	Internal test at Örebro University Hospital, Sweden	Clean	2 min	✓	✓
					>5.65 log_{10}		Dirty	2 min	✓	✓
			Yersinia ruckeri ATCC 29473		>5 log_{10}		Clean	2 min	✓	✓
					>5 log_{10}		Dirty	2 min	✓	✓
	EN 13727:2015 (Phase 2, Step 2)	Quantitative suspension test	Klebsiella pneumoniae (ESBL) CCUG 54718	5 log_{10}	> 5.76 log_{10}	Internal test at Örebro University Hospital	Clean/Dirty	2 min	✓	✓
			Acinetobacter baumannii (Clinical strain)						✓	✓
	AOAC 961.02	Germicidal Spray	Salmonella choleraesuis (ATCC 10708)	0/60	0/60	Medical Technology, Mahidol University, Thailand	Clean	30 sec		✓
			Pseudomonas aeruginosa (ATCC 15442)				Dirty	2 min		✓
	AOAC 961.02	Quantitative Carrier test	Salmonella enterica (ATCC 10708)	5 log_{10}	>6.2 log_{10}	MicroChem Laboratory, Texas, USA	Clean	5 min	✓	✓
			Pseudomonas aeruginosa (ATCC 15442)		>6.7 log_{10}					
	EN 16615:2015 (Phase 2, step 2)	4-Field test	Pseudomonas aeruginosa (ATCC 15442)	5 log_{10}	>6.83 log_{10}	Hygiene Nord GmbH, Germany	Clean	1 min	✓	✓
	EN 17387:2020 (Phase 2, step 2)	Quantitative Carrier test	Pseudomonas aeruginosa (ATCC 15442)	5 log_{10}	>6.41 log_{10}	Labor-Enders Laboratory, Germany	Clean	5 min	✓	✓
Gram-Positive Bacteria	EN 13727:2014 (Phase 2, Step 1)	Suspension test	Staphylococcus aureus ATCC 6538	5 log_{10}	>5 log_{10}	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	1 min	✓	✓
	EN 13727:2014 (Phase 2, Step 1)	Suspension test	Staphylococcus aureus ATCC 6538	5 log_{10}	>7.36 log_{10}	Labor-Enders Laboratory, Germany	Clean	1 min	20 ppm	✓
	EN 13727:2015 (Phase 2, Step 2)	Suspension test	Staphylococcus aureus ATCC 6538	5 log_{10}	>5 log_{10}	Food Safety Laboratory, Chung-Ang University, Seoul, South Korea	Dirty	2 min	✓	✓
	EN 14561:2006 (Phase 2, Step 2)	Quantitative Carrier test	Staphylococcus aureus ATCC 6538	5 log_{10}	>5 log_{10}	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	1 min	✓	✓
	AOAC 961.02	Germicidal Spray	Staphylococcus aureus ATCC 6538	0/60	0/60	Medical Technology, Mahidol University, Thailand	Clean	30 sec		✓
							Dirty	2 min		✓
	AOAC Use Dilution	Quantitative Carrier test	Staphylococcus aureus ATCC 6538	5 log_{10}	>6.71 log_{10}	MicroChem Laboratory, Texas, USA	Clean	5 min	✓	✓
	EN 14349:2012 (Phase 2, Step 2)	Quantitative Carrier test	Staphylococcus aureus ATCC 6538	5 log_{10}	>5.02 log_{10}	SVA, Sweden	Dirty	5 min		✓
	EN 14349:2012 (Phase 2, Step 2)	Quantitative Carrier test	Enterococcus hirae ATCC 10541	5 log_{10}	>5.11 log_{10}	SVA	Dirty	5 min		✓
	EN 14561:2006 (Phase 2, Step 2)	Quantitative Carrier test	Enterococcus hirae ATCC 10541	5 log_{10}	>5.11 log_{10}	Dr. Brill + Partner GMBH Laboratory, Germany	Clean	1 min	✓	✓
	EN 13727: 2015 (Phase 2, Step 2)	Quantitative suspension test	Enterococcus hirae ATCC 10541	5 log_{10}	>5 log_{10}	Food Safety Laboratory, Chung-Ang University, Seoul, South Korea	Dirty	2 min	✓	✓
	EN 13727: 2015 (Phase 2, Step 2)	Quantitative suspension test	Enterococcus hirae ATCC 10541	5 log_{10}	>7.45 log_{10}	Labor-Enders Laboratory, Germany	Clean	1 min	20 ppm	
	EN 13727:2015 (Phase 2, Step 2)	Quantitative suspension test	Listeria monocytogenes CCUG 51681	5 log_{10}	>5 log_{10}	Internal test at Örebro University Hospital	Clean/dirty	2 min	✓	✓
		Quantitative suspension test	Streptococcus equi CCUG 37782	5 log_{10}	>5 log_{10}	Internal test at Örebro University Hospital	Clean	2 min	✓	✓
				5 log_{10}	>5 log_{10}		Dirty	2 min	✓	✓
	EN 14349:2012 (Phase 2, Step 2)	Quantitative suspension test	Carnobacterium piscicola ATCC 35586	4 log_{10}	>5 log_{10}	Internal test at Örebro University Hospital	Clean	2 min	✓	✓
				4 log_{10}	>5 log_{10}		Dirty	2 min	✓	✓
	EN 16615:2015 (Phase 2, step 2)	4-Field test	Staphylococcus aureus ATCC 6538	5 log_{10}	>5 log_{10}	RISE Research Institutes of Sweden	Clean	2 min	✓	✓
			Enterococcus hirae ATCC 10541							
	EN 13727:2015 (Phase 2, Step 2)	Quantitative suspension test	Enterococcus faecium (VRE) CCUG 56431	5 log_{10}	> 5.75 log_{10}	Internal test at Örebro University Hospital	Clean/Dirty	2 min	✓	✓
	EN 16615:2015 (Phase 2, step 2)	4-Field test	Staphylococcus aureus ATCC 6538	5 log_{10}	>6.95 log_{10}	Hygiene Nord GmbH, Germany	Clean	1 min	✓	✓

Gram-Positive Bacteria	EN 16615:2015 (Phase 2, step 2)	4-Field test	Enterococcus hirae ATCC 10541	5 logs	>7.02 logs	Hygiene Nord GmbH, Germany	Clean	5 min	✓	✓
	EN 17387:2020 (Phase 2, step 2)	Quantitative Carrier test	Enterococcus hirae ATCC 10541	5 logs	7.30	Labor-Enders Laboratory, Germany	Clean	5 min	✓	✓
			Staphylococcus aureus ATCC 6538		6.95			5 min		
Parasites	N/A	In vitro sporulation method for Coccidia	Coccidia spp		N/A	VidiLab, Sweden	Dirty	N/A		✓
	Salmon lice	In vitro	*Lepeophtheirus salmonis*	N/A	N/A	ILAB, Norway	N/A	1 min	100 ppm	✓
	N/A	In Vitro	*Gyrodactylus salaris*	N/A	N/A	Norwegian Veterinary Institute, Norway	N/A	10 sec / 1 min	200 / 100 ppm	✓
	DNA Analysis	In Vitro	Plasmid DNA	N/A	N/A	SLU	Clean	10 / 2 min	✓	✓

[1]Initial *bacterial spores* 10^6
[2]Initial *bacterial spores* 10^8
[3]Initial *bacterial spores* 10^8 *and different bacteria strain*
[4]Initial *bacterial spores* 10^8 *and 200 PPM*
[5]*Bactericidal effect (Clean condition for 2 minutes and Dirty condition for 5 minutes).*
[6]*These bacteria: Bacillus subtilis, Bacillus cereus and Clostridium difficile were tested for 400 PPM for 2 minutes for clean/dirty condition.*

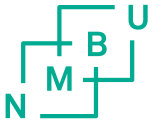
Third party test institutes

DR. BRILL + DR. STEINMANN · MIKROLAB · RISE · Region Örebro län Universitetssjukhuset Örebro · CAV · Vidilab · Veterinærinstituttet Norwegian Veterinary Institute · LABOR ENDERS Labor Prof. Dr. G. Enders MVZ GbR · Medizinische Diagnostik · micans Microbial Analytics Sweden AB

For more information, please contact:

Rahma Wehelie, PhD
Senior Scientific Officer
Phone +46 522 506 475
Mail: rahma.wehelie@lifeclean.se

LIFE CLEAN
LifeClean International AB
Kärrastrandvägen 124B
451 76 Uddevalla, SWEDEN
+46 (0)522 104 04
info@lifeclean.se
www.lifeclean.se



Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway