UiT The Arctic University of Norway

Faculty of Bioscience, Fisheries and Economics

Comparing seed carrier transfer ratios for start-up of moving bed biofilter reactors in recirculating aquaculture systems (RAS)

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Master's Thesis in Biology - BIO-3950 – June 2023

Acknowledgement

This master's thesis started in August 2022 and was founded as a collaboration between Havbruksstasjonen in Kårvika and UiT (Faculty of Bioscience, Fisheries and Economics).
I want to give my first thanks to my wonderful supervisor, Dr. Jelena Kolarevic. She granted me the opportunity to work on the subject I was most fascinated by. These months have given me more experience than any reading in a book could do. She has not only helped me by answering my questions, but also guided me to take matters into my own hands to accomplish the goal. I am eternally grateful for the opportunity DR.Jelena Kolarević gave me and for sharing her knowledge, experience and the teaching she has provided for me.
Next, I would like to thank the people at Havbruksstasjonen in Kårvika for being helpful and supportive during my stay there. I want to give a special thanks to Chris Verstege for not only helping me on a daily basis, but also for making the hours of work a pleasant and friendly experience with many interesting conversations.
I want to thank my friends at UiT for encouraging each other to spend many hours together to work on the master. A little special thanks to Haakon Winge Hjertaas for helping with structuring my data set and debugging R code.
Last, but not least, I want to thank my family for understanding the time and effort required for completing a master thesis and supporting me throughout the process.

Abstract

Recirculating aquaculture systems (RAS) is one of the many methods utilized for fish farming. Advantages with RAS is the ability to control the environment, reduce the environmental impact on wild species, disease prevention, Year-round production etc. Fish farming with RAS is working on optimizing the production to ensure economic profitability. It is therefore needed to make the production more efficient and fish friendly.

This master's thesis examined the use of inoculum to mature maturation tanks, and differences in seeding ratios for start-up of biofilters. The inoculated maturation tank preformed substantially better than control relied on chemical activation. Results from seeding % indicated that using 10% and 15% did not give a more favourable result than 5%. The results were concluded from inspecting chemical concentrations and oxidation rates visualized from graphs.

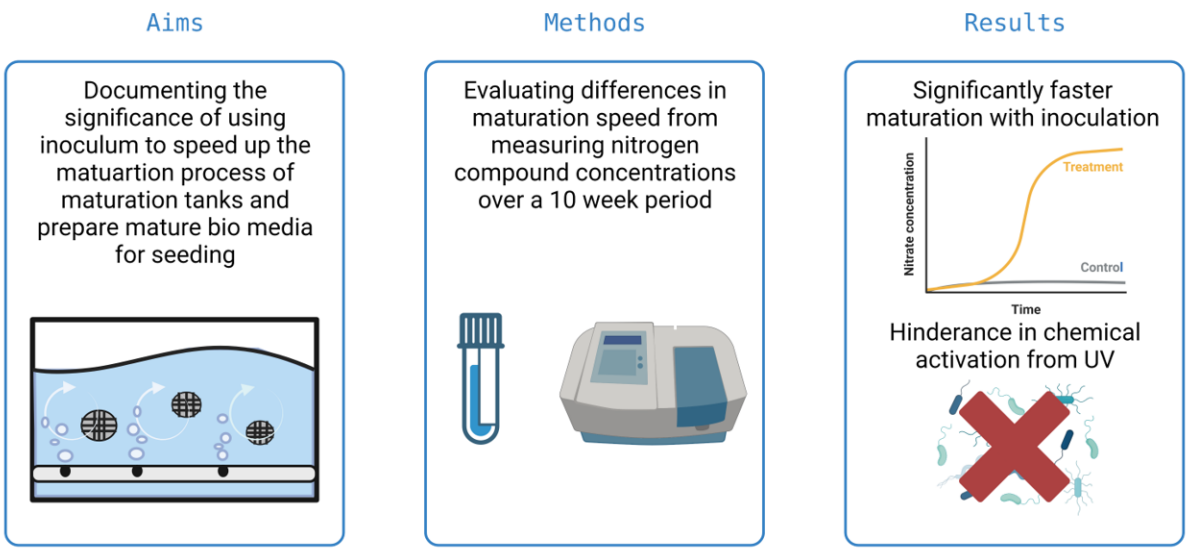

Figure 1 Graphical abstract showing aims, methods, and results of the case study. Created with BioRender.com

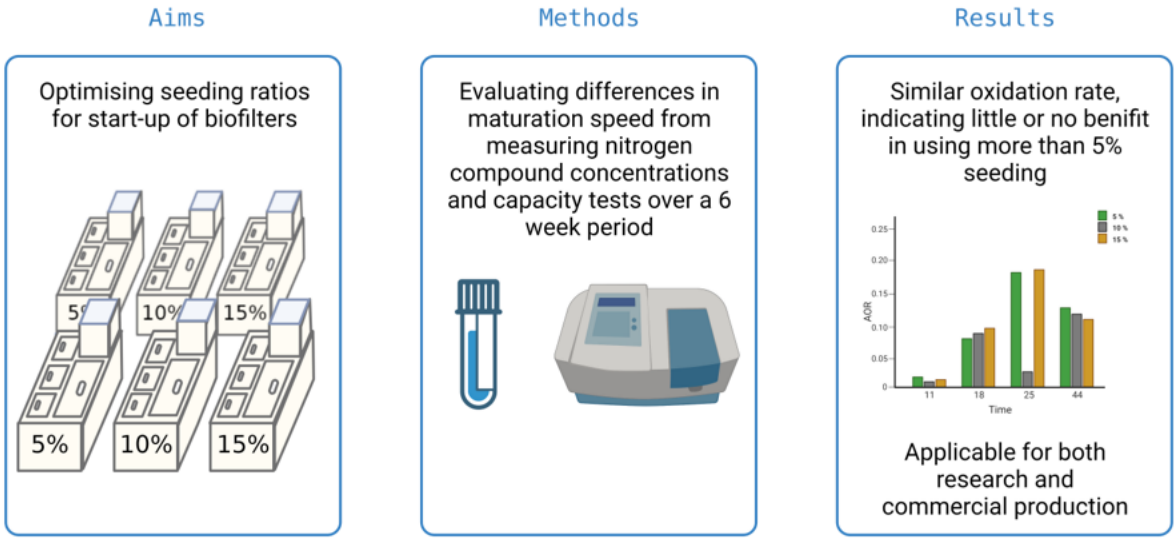

Figure 2 Graphical abstract showing aims, methods, and results of the seeding experiment. Created with BioRender.com

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1 Introduction

Norway is the leading country of Atlantic salmon production accounting for more than 50% of the total production in the world. Norway's topography has been a large factor for why aquaculture has been so successful. The topography and climate are on the other hand not as effective for self-sufficient production of neither protein rich plants or terrestrial animal production (Aas et al., 2022). The height differences in the topography and little area of arable land makes it harder to have efficient large scale animal production. Up to 40% of Norway's area can be used for grazing if all the best forests, bogs and mountainous areas are used (Grønlund et al., 2013; Rekdal, 2014). The utilization can be difficult as some areas are far from civilization and each other. Atlantic salmon and other marine species utilize food more efficient than terrestrial animals. Animals such as cattle require multiple times more food than salmon to gain the same amount of weight (Fry et al., 2018). Salmon is also a good source of omega-3 and protein which is an important part of our dietary needs (Simopoulos et al., 1999).

1.1 Aquaculture and fisheries in Norway

Norway's largest volume of fish is harvested through marine fisheries. Aquaculture harvests about half the volume of fisheries. Although fisheries are harvesting in larger volumes, aquaculture stands for 77% of Norway's total economic benefits from fish harvesting (OECD, 2021)(Figure 3). Today's marine fisheries are managed in a more sustainable way and cannot stand for further increase in seafood production. On the contrary, aquaculture has been the fastest growing and the most efficient way of producing protein for humane production in the last couple of decades.

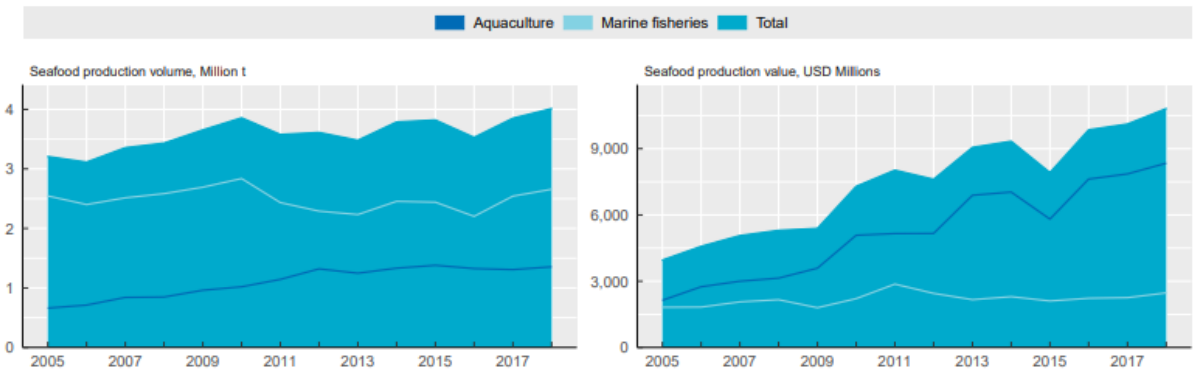

Figure 3 production volume and production value for marine fisheries and aquaculture (OECD, 2021).

As with marine fisheries, the limitation of production in aquaculture is set by factors concerning sustainability. There are different regulations to manage aquaculture. One of them is: the

purpose of the aquaculture act (2005) "*To promote the profitability and competitiveness of the aquaculture industry within the framework of a sustainable development and contribute to the creation of value on the coast."* (Affairs, 2005).

1.2 Sustainable production

One of the managing methods to keep aquaculture on Atlantic salmon (*Salmo salar*) sustainable is through a traffic light system. This system divides Norway's coast into thirteen parts. Each part is managed through the mortality rate inflicted by salmon lice on the wild salmon smolt population. The management is divided into red, yellow, and green as seen in figure 4.

- Green: Mortality of salmon smolt is below 10 %. Production can increase with 6 %.
- Yellow: Mortality of salmon smolt is between 10 % - 30 %. Production cannot increase, but do not need to decrease.
- Red: Mortality is higher than 30 %. Production needs to decrease with 6 %.

Figure 4 Norway's traffic light system for salmon lice management 29.09.2022

Salmon lice (*Lepeophtheirus salmonis*) is a copepod that has a parasitic lifecycle on salmon. Atlantic salmon is especially harmed by the parasite, and fjord aquaculture is believed to be one of the largest driving factors for the high mortality rate for the wild Atlantic salmon population (Sommerset et al., 2022). High mortality from salmon lice have stopped certain parts of Norway from increasing production in the fjords. An increase in production managed in the same way as today will likely lead to more parts of Norway getting yellow or red light, reducing the potential for more food production. Producers have therefore come up with different solutions to solve or reduce the problem.

Unique sea cages have been built to circumvent some of the issues. Submerged-, snorkel sea-cages and sea-cages with a lice skirt are some ways to keep salmon away from interacting with sea lice. Sea lice stay in the upper part of the water column, and these cages keep the fish lower in the water column, walls of the upper part of the water column, or both (Noble et al., 2018). Semi-closed cages are one method to give farmers more control of the environment the fish lives in by example restricting aspects of where in the water column water exchange is occurring. Having a flow through system with water gathered from a lower part of the water column helps against salmon lice, as salmon lice are mostly present closer to the surface. The additional physical barrier also reduces the chance for escapes to occur (Noble et al., 2018).

Waste from feed and other by-products can also affect the environment. Some cages are equipped with feed collectors and semi-closed systems can have mechanical filtering. Integrated Multi-Trophic Aquaculture uses species from different trophic levels to take up organic and inorganic waste (Chopin, 2010). The potential for sustainability is high, but there have been worries concerning food safety issues from contamination and chemicals (Rosa et al., 2020). Another method to avoid affecting wild populations is to move the production to land.

1.3 Recirculating aquaculture systems (RAS)

RAS is a land-based closed containment farming system. The water going into and out of the system can be entirely managed. In Norway this production system is mainly used for growing smolts and postsmolts, but there is research and trials done to effectively use RAS for grow out production. Land based production to grow out resolves issues related to fish escapes and salmon lice (Lekang et al., 2016), which are large problems for fjord aquaculture. In RAS a high percentage, normally between 95-99% of the water is reused through recirculation (Timmons & Vinci, 2022) as well as heat (Workshop, 2014). Production in RAS can potentially

give salmon the best environment for growth and welfare through protection from diseases and provision of optimal living conditions. Effluent water can be disposed sustainably or reused for other productions such as agriculture (Toze, 2006). The possibility to farm all year around gives a predictable food source to the markets and can cut down on travel time between producer and market, reducing transportation cost and pollution. RAS can also farm species that are not native to the local climate (Commission, 2020). The system can farm intensive having the highest production by area and least number of workers per unit out of all the other aquaculture systems (Timmons & Vinci, 2022). This is why RAS is considered as one of the sustainable options for salmon production.

The share of land-based aquaculture production from egg to post smolt using RAS technology or flow through systems depends on the producer, but there is a positive trend in converting over to RAS (Meriac, 2019). Traditionally flow through systems were the most used technology to produce smolts in Norway. However, current trends in the Norwegian farming industry show increased use of RAS for production of both smolts and postsmolts. The flow through system is simpler to manage than RAS as it does not recirculate the water. One of the reasons for the downwards trend in use of flow through systems is the need for continuous and large water intake and discharge. Water is becoming scarce, making RAS a more relevant technology that would allow for increase in production on a given water source volume. The environment in a flow through system is less controlled and with lower biosecurity, meaning problems related to welfare is more likely to occur. Waste management can also be a challenge for flow through systems as wastewater is not filtered as thorough as in RAS (Timmons & Vinci, 2022).

A robust management of RAS is needed to keep water quality at the recommended levels for fish production and it can vary depending on species and system design. All RAS facilities have these main components: Fish tank, mechanical filter, biofilter, degasser, oxygenator and heat exchange system, while optional components are also available as seen in table 1. These components are designed and built to produce fish with good welfare, but you will not get far without the knowledge on how and why to operate them.

Table 1 Main and optional components in RAS

Components	Function
Fish tank	Houses the fish and contains systems such as lights and feeder. Tank design provides good self-cleaning, optimized water flow and velocity, and provides uniform water quality.
Oxygenation	Oxygen is added to the water before it is returned to the fish tanks.
Mechanical filter (Drum filter, Belt filter)	Filters out particles from water down to ca 20-40 micron.
Biofilter (Moving bed bioreactor, fixed bed biofilter)	Oxidation of ammonia to nitrate.
Degasser	Increases the contact surface between water and air to facilitate gas exchange.
Heat exchange system	Regulates the temperature to the need of the fish or biofilter depending on the stage of the production.
UV-light (optional)	Reduces virus and bacteria count.
Ozone (optional)	Reduces turbidity, nitrite and disinfects water.
Swirl separator (optional)	Collects uneaten feed pellets and feces at the tank level.
Protein skimmers (optional)	Supportive filtering device reducing particulate organic/protein matter smaller than the mechanical filter.

1.3.1 Particle removal

Mechanical filters are a core component for RAS. The most common way to remove the particles in RAS is with a drum filter or in some cases with belt filter. Particles comes from fecal matter, fish feed and bacterial growth and have to be removed as high concentrations can be responsible for reduction in the functionality of other RAS components and can cause welfare issues for fish (Chapman et al., 1987; Muir, 1982). A drum filter can filtrate large particles down to about 20-40 µm with close to 100% efficiency. The distribution of particles in RAS consists heavily of smaller particulate organic carbon (POC) under 20 µm and dissolved organic carbon (DOC)(Chen et al., 1993). Removal of suspended solids is important to maintain core functions in RAS. POC and DOC can cause an increase in growth of heterotrophic bacteria as they get more substrate to grow on and available food. This can lead to reduction in efficiency of the biofilter (Muir, 1982). These particles can also lead to reduced welfare of fish stock due to gill irritation (Bullock et al., 1994) and stress (Lake & Hinch, 1999).

Protein skimmers are an option to reduce particulate organic/protein matter. It is not meant to replace other filtering components, only complement the filtering process. A part of the water in circulation is sent to the protein skimmer. Aeration creates bubbles and foam that particles adhere onto. These bubbles are pushed upwards into a retention tank where particles cannot move back into the system. Protein skimmer for freshwater is a newer invention, earlier only sea water skimmers have been used, as it is harder to produce the right foam fractionation with freshwater. A method to increase foam fractionation comes from including ozone in the process. Ozone breaks down larger organic molecules and increases oxygen concentrations.

1.3.2 Biofilter

A biofilter is a system containing microorganisms used to reduce concentrations of toxic nitrogen compounds. It can consist of a mixture of different microorganisms such as bacteria, fungi, algae, plants, etc. In biofilters in RAS the main focus is heterotrophic microorganisms, ammonia oxidising bacteria (AOB), ammonia oxidising archaea (AOA) and nitrite oxidising bacteria (NOB). AOBs and NOBs are needed for nitrification to occur. AOAs are also a part of the nitrification process, but not as researched as AOBs and are believed to be more adept to lower ammonia concentrations than AOBs (Erguder et al., 2009; Martens-Habbena et al., 2009). Heterotrophic bacteria compete for resources with nitrifying bacteria and are therefore selected against. Heterotrophic bacteria use organic carbon to grow and can outgrow nitrifiers. Organic matter is therefore removed using mechanical filtration to ensure higher nitrification.

Nitrifiers together with heterotrophic microorganisms form biofilms on bio media consisting of extracellular polymeric substances (EPS). 90% of the biofilm can consist of EPS with 10% being microorganisms (Flemming & Wingender, 2010). Biofilm have many functions for the microorganisms, a few of them are protection, sorption of organic and inorganic compounds, and communication and interaction between cells (Flemming & Wingender, 2010).

Bacteria needs a substrate to grow biofilm. Carrier media/bio media are designed to fit a systems filtration specification and to provide large enough area for bacterial growth. A usual material to make them from is polyethylene. Their shape and size vary depending on the needed nitrification rate. Increasing surface area heightens the potential for nitrification but can lead to problems with movement in some systems.

The two most common biofilter types are fixed bed biofilter and moving bed biofilter reactor (MBBR) (figure 5). Fixed bed biofilter has a stationary bio media through which water passes and air is added to secure aerobic conditions for nitrification. Biofilm thickens over time and nitrification can become ineffective; it is therefore necessary to backwash fixed bed biofilters to maintain optimal function over time. MBBR has individual pieces of non-corroding bio media that is kept in constant movement with a rising water current and constant aeration. Movement in the current creates a self-cleaning effect where bio media scrapes against each other and reduces biofilm build up.

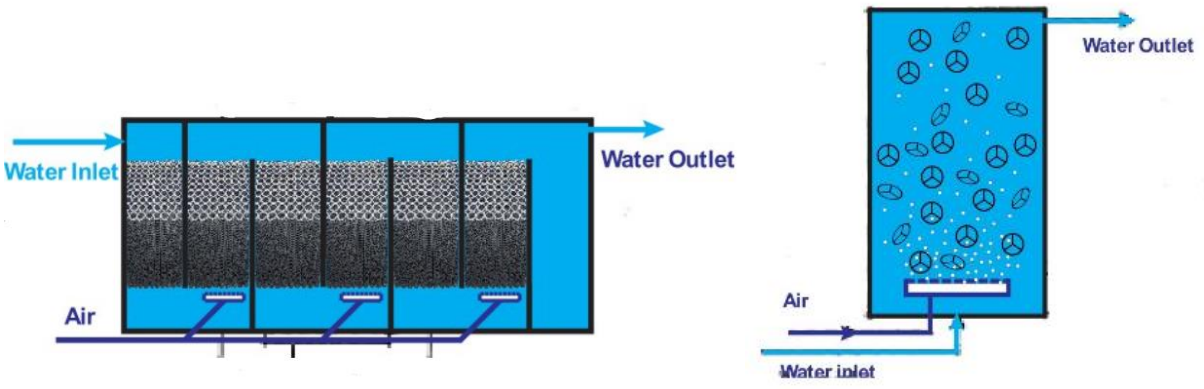

Figure 5 Fixed bed biofilter (left) and moving bed biofilter reactor (right) (EIO, 2017)

1.3.2.1 Nitrification

Fish excrete mainly ammonium (NH_4^+) from the gills (Randall & Wright, 1987). NH_4^+ acts in equilibrium with ammonia (NH_3) (Equation 1). Ammonia is toxic to fish. Sublethal concentrations of ammonia can affect fish growth and welfare, while higher concentrations can

even lead to fish mortalities (Levit, 2010). Higher pH and water temperature skews the equation to have a higher concentration of ammonia which is damaging for fish health (Levit, 2010). Salinity and water hardness is also affecting the equilibrium. Ammonias safe concentrations should stay under 0.012 mg/l with long exposure (Fivelstad et al., 1995) and under 0.1 mg/l with short (4 h) exposure (Wedemeyer, 1996).

$$NH_4^+ \leftrightharpoons NH_3 + H^+$$

Equation 1 Ammonium and ammonia equilibrium

As ammonia is continuously produced during the farming process, there is a need to limit the concentrations in the RAS water. This is done through nitrification. AOBs and NOBs are both needed to transform ammonia to the non-toxic nitrate (NO_3^-) (Equation 2).

$$NH_3\ (toxic)\ \overrightarrow{AOB}\ NO_2^-\ (toxic)\ \overrightarrow{NOB}\ NO_3^-\ (non-toxic)$$

Equation 2 Process of nitrification

Nitrosomonas, Nitrosococcus and Nitrosospira are examples of AOBs. The process of turning ammonia to nitrite is done through two steps called nitritation (Soliman & Eldyasti, 2018). AOBs use an enzyme called ammonia monooxygenase (AMO) to oxidate NH_3 to hydroxylamine (NH_2OH). AMO is not able to oxidise NH_4^+, meaning a shift in the equilibrium favoring NH_4^+ affects the efficiency of nitration (Suzuki & Kwok, 1970). The oxidation requires O_2 and $2H^+$. The result is NH_2OH and H_2O (Equation 3):

$$NH_3 + O_2 + 2H^+ = NH_2OH + H_2O$$

Equation 3 First process in nitritation

Hydroxylamine oxidoreductase (HAO) enzymes are then used to further oxidise hydroxylamine to Nitrite. Oxygen from water is used as electron acceptor to finalize the oxidation process (Equation 4):

$$NH_2OH + H_2O = NO_2^- + 5H^+$$

Equation 4 Second process in nitritation

The extra protons released in the environment can increase the acidity, changing the equilibrium of NH_4^+/NH_3 to more NH_4^+.

NOBs are the next group of bacteria needed to oxidise nitrate. Examples of NOBs are: *Nitrobacter*, *Nitrococcus* and *Nitrospina*. Nitrite and oxygen from water is needed for the oxidation (Equation 5):

$$2NO_2^- + 2H_2O = 2NO_3^- + 4H^+ + 4e^-$$

Equation 5 Nitrification process of Nitrite to nitrate

$$4H^+ + 4e^- + O_2 = 2H_2O$$

Equation 6 Concomitant conversion of oxygen to water

Concomitant conversion to water on the same side of the cytoplasmic membrane will inhibit a gradient of H^+ to occur (Equation 6). It is nevertheless accepted that there is a net transfer of $2H^+$ for each mole of NO_2^- (Mathews et al., 2000).

Nitrification has its optimal conditions. To nitrify 1g of ammonium, 4.71g of O_2 and 7.05g of calcium carbonate ($CaCO_3$) is needed (Timmons & Vinci, 2022). Table 2 shows optimal values for the different water parameters that are important for nitrification.

Table 2 Water parameters with optimal values for nitrification and fish welfare.

Water parameters	Optimal Values	References
Total Ammonia	Optimal range: 2-3 mg/L.	(Ebeling & Wheaton, 2006)
	Max conc. For salmon: 2 mg/L.	The Norwegian Food Safety Authority (Kolarevic et al., 2018).
Temperature	Optimal range: 14ºC-27ºC. Requirements of the fish stock is usually the most important factor.	(Zhu & Chen, 2002) (Chen et al., 2006)
pH	Optimal pH range for nitrifiers: 7.0 to 9.0	(Chen et al., 2006)
	Optimal pH range for fish stock: 6.2 to 7.8	The Norwegian Food Safety Authority (Staurnes et al., 1995).
Dissolved Oxygen	At least 2 mg/L of oxygen.	(Malone et al., 1998)
Alkalinity	70 mg/L of $CaCO_3$.	(Summerfelt et al., 2015)
Salinity	Higher salinity lengthens start-up period and is reported to negatively affect nitrification rate.	(Rusten et al., 2006)
Organics	Needs to be removed continuously.	(Chen et al., 2006)

1.3.3 Biosecurity

Biosecurity in RAS is at a higher level compared to the traditional sea cages, but once pathogens enter the system, it can be challenging to remove it. There is therefore a need to reduce the possibility for pathogens to enter the system. Pathogens can enter through different ways, but most common situations are with inlet water, equipment/personnel or introduction of new fish/eggs. Disinfection is one method to kill pathogens and can occur in various parts or the RAS loop. Some of the most common locations are at the intake water, inlet to rearing tank or before the biofilter.

The two most common methods to disinfect in the RAS loop are use of ultraviolet light (UV) or ozone. UV is electromagnetic radiation and have the best effect against bacteria at a wavelength of 254 nm. Bacteria are damaged over time or suffers lethal damage from alteration in their nucleic acids (Liltved et al., 1995).

Ozone can be used as disinfection but is most commonly used for improving water quality. Ozone used in disinfection changes the cell membrane leading to loss of nucleic acids and protein (Liltved et al., 1995). Water quality is improved from flocculation of fine particles and oxidation of molecules (Summerfelt et al., 2009). Managing concentrations are important as it can be hazardous to humans and fish (Summerfelt et al., 2009).

Factors such as water flow, particle density and particle size have an effect on how effective the disinfection dose is (Timmons & Ebeling, 2013). High water flow reduces the contact time UV can have on pathogens, while high turbidity can shield pathogens from UV light.

The biofilter is a natural sanctuary for bacteria. Opportunistic bacteria can create thick biofilms which increases the potential risk of hydrogen sulphide (H_2S) production to harmful levels for the fish stock (Timmons & Ebeling, 2013). Fixed biofilters need to be manually cleaned regularly to avoid thick biofilms. Biofilters can also be disinfected if there is identified a pathogen in the system, but biofilter function can be compromised during this process.

Getting correct water parameters is important for both efficiency and welfare. Fish that are stressed or not getting their daily needs can be more susceptible to disease. Good water quality is needed, with correct values of temperature, pH, dissolved oxygen, etc (Table 2). RAS is farmed intensively, but too intensive can lead to stress and injuries, which again increases the possibility to get diseases.

1.4 Biofilter start-up

Biofilters must be matured over time for the biofilm to be formed and to establish optimal nitrification. The process in which biofilters are matured is often called biofilter start-up phase. This phase is one of the first processes that must take place in a new RAS facility before fish are introduced in the system. Starting up a new biofilter using virgin carriers (not previously used) often takes longer time compared to start-up with carriers that were disinfected or already used in functional biofilters. Biofilter can be cleaned/disinfected between two production batches after which biofilter will have to be matured again. Currently it is not required by law that biofilters are cleaned/disinfected after each production cycle, but it is recommended if biosecurity issues occur during production (The Veterinary Institute, 2016).

1.4.1 Biofilter start-up methods

There are different methods to start-up the biofilter. The most common methods are inoculation with bio media from functional biofilter, addition of commercial inoculum (commercialized bacteria solution) or chemical activation.

The bio media from a mature biofilter can be transferred over to a new biofilter to give a good foundation for start-up. Inoculation using bio media from mature biofilters is advantageous as it can reduce the start-up time of the new biofilter (Navada et al., 2021). However, there is a certain risk of using bio media from mature biofilter that was in contact with fish, as opportunistic pathogens can be transferred to the new biofilter. Lately number of commercial producers in Norway have started to build maturation tanks in which biofilter is matured and maintained over time without any contact with the fish. In this way the use of bio media from maturation tanks reduces risk at the start-up and new biofilters are matured more efficiently. Seeding nitrifying bacteria has advantages in reducing stress, reducing growth time as more feed can be fed earlier, and ensures better water quality for the fish (DeLong & Losordo, 2012).

The use of commercially available inoculums can reduce time needed for maturation of biofilters. Starting a biofilter can take many weeks. Commercial farming loses out on fish production for every extra day that biofilter maturation takes. Researchers can also have schedules that do not allow for a long maturation process between experiments. Inoculum can be a means to boost the start-up process by having a solution of live nitrifying bacteria that are ready to nitrify when added to the system. The downside of this method is that you are reliable on another company for production and biosecurity of the product. Inoculum can also consist of microbiota that can be outcompeted by microbiota from intake water.

Chemical start-up relies on chemicals, water and bio media presence to start-up the biofilter. At the start, the system is usually given carbon, ammonia, nitrite, phosphate and micronutrients. Heterotrophs grow faster than nitrifiers, so heterotrophs are given carbon to start the production of biofilm that nitrifiers also need. Ammonia is the food source for AOB, while nitrite is the food source for NOB. Starting the growth of both AOB and NOB at the same time can shorten the maturation process. Phosphate and micronutrients are needed for different parts of the bacterial life cycle. The process is slower than the others, but the biosecurity is the highest. Bacteria is usually introduced from the water entering the system.

1.4.2 Optimal conditions for the biofilter start-up

During biofilter start-up phase an optimal water quality for biofilm growth must be established. Optimal values for start-up of the biofilter can differ from the required ranges of the future fish stock as the biofilter have different optimal ranges for number of water quality parameters than fish. There are four main effects that determine the optimal range for nitrifying bacteria:

- Activation/deactivation of nitrifying bacteria;
- Nutritional effect, connected with alkalinity and oxygen;
- Inhibition through free ammonia and free nitrous acid (Anthonisen et al., 1976);
- Abiotic factors, temperature, pH, salinity and water hardness.

Both H^+ and HO^- can activate/deactivate nitrifying bacteria from binding to weak basic/acid groups of the enzyme. The highest concentration of nitrifying bacteria occurs around pH=8. This is not a pH that is usable for aquaculture purpose. pH of 6.8 to 7.2 is the optimal range concerning efficiency and required levels for the fish stock (DeLong & Losordo, 2012).

Alkalinity is the mineral carbon source that nitrifying bacteria need to grow. The amount should be around 70 mg/L, but an initial start-up between 200-250 mg/L can help bacteria such as *Nitrobacter* to establish (DeLong & Losordo, 2012). The heightened amount of alkalinity will naturally go down to operational level, where there is a need to continuously add alkalinity to maintain 70 mg/L (Summerfelt et al., 2015).

Water in RAS can be 5ºC higher than the original water source as RAS creates heat from friction in pipes and pump, bacterial activity in biofilter and metabolism from fish (Kolarevic et al., 2012). Temperatures between 12-13ºC is a good temperature for smolt production (Bæverfjord et al., 2012), however, higher temperatures increase nitrification rate of biofilters. Temperature

can also affect AOB and NOB in different ways. Figure 6 shows how AOB and NOB growth is affected from different temperature values.

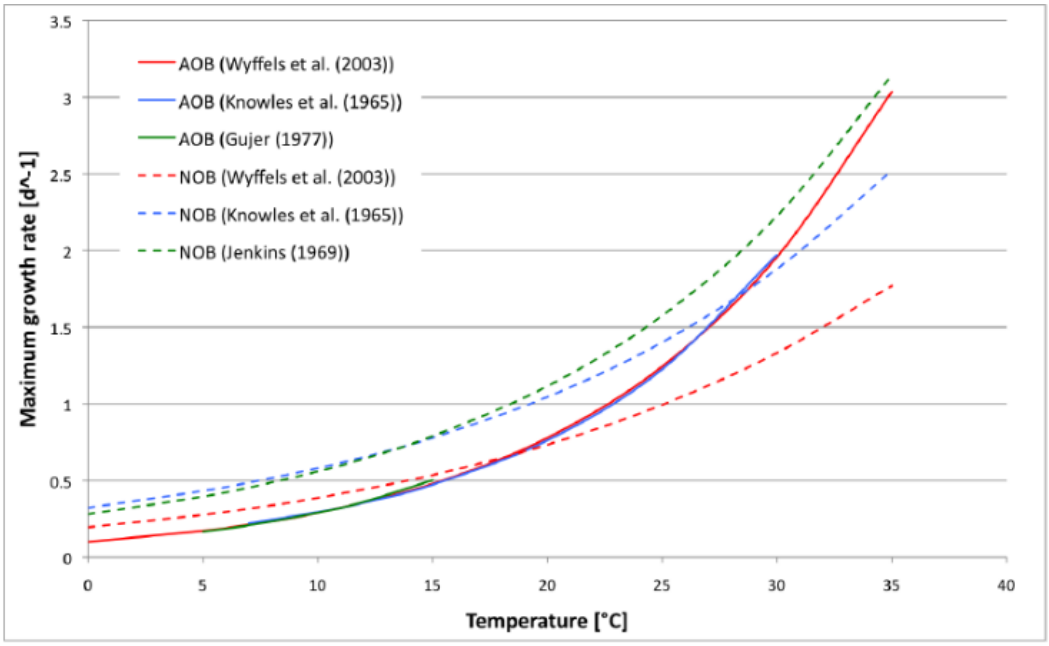

Figure 6 The relationship between maximum growth rate and temperature for AOB and NOB from different authors (De Mulder, 2014).

Postsmolt production is often done using brackish water (12ppt) in Norway. Higher salinity decreases nitrification rate. It will also take longer for the start-up phase and establishment of the mature biofilter. Growing biofilter in brackish water is proved to make the biofilter more resistant to future salinity changes (Navada et al., 2020).

With water quality at recommended levels (see table 2), ammonia, nitrite, phosphate and micronutrients can be added to the biofilters. Products such as ammonium chloride and Sodium nitrite can be used. Ammonia should stay between 3-5 mg/L. Dissolved feed can also be added to the biofilter during start-up to provide necessary nutrients for the biofilm growth. The required concentrations are lowered as the nitrification process is established. Under 2 mg/L for total ammonia nitrogen and under 0.1 mg/L for nitrite nitrogen are the recommended limits for salmon indicated from the Norwegian Food Safety Authority (Kolarevic et al., 2018). It is only after this water quality is secured that fish can be introduced to the RAS. Phosphate is essential for membrane integrity, energy metabolism and information storage. The concentrations should be above 0.5 mg/L. A micronutrient solution contain many different types of micronutrients (example of micronutrient combination in methods) and give benefits for different parts of the bacterial life cycle.

1.4.3 Monitoring of water quality during biofilter start-up

Regular monitoring of water chemistry parameters is crucial during biofilter start-up process. Most important parameters to monitor are ammonia, nitrite, nitrate, pH, alkalinity, oxygen and temperature (DeLong & Losordo, 2012). Monitoring of water quality should be conducted with a reliant measuring method. Measurements should occur in the biofilter, and should be done systematically in the same place, at the time and using the same methods/instrumentation. Having a daily graphic representation of the ammonia and nitrite values can give a good indication on how much the start-up has progressed (figure 7). Start-up is finished when ammonia and nitrite concentrations level out under required concentrations.

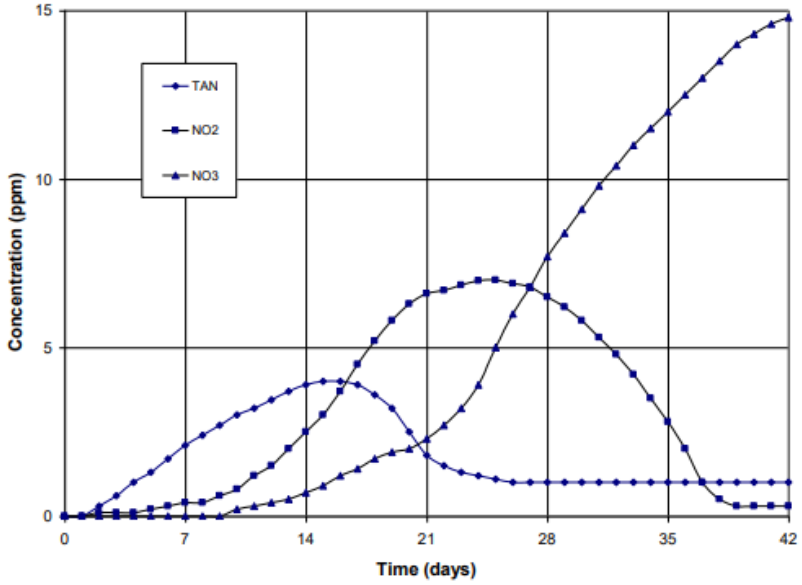

Figure 7 Simplified view on how a theoretical start-up of biofilter progresses (Timmons & Vinci, 2022).

1.5 Aims

This thesis includes two studies. Use of inoculum for maturation tank start-up is the first case study, and differences in maturation for start-up of biofilter with different seeding ratios is the main experiment. Both studies are conducted at Havbruksstasjonen (HiT) in Kårvika in a newly established RAS facility, RASforsk.

1.5.1 Start-up of maturation tanks

Shortening time to maturation is useful both for research and commercial operation. Chemical activation for start-up of a maturation tank is a common method as having high biosecurity is important when bio media is further being shared to new biofilters. A downside of chemical activation is the time needed for maturation. Using inoculum of a commercial bacteria solution is shown to speed up the process of maturation. The object of this case study is to compare

start-up of biofilters in maturation tanks using chemical activation and commercial inoculum of nitrifying bacteria (Using BioRAS TAN inoculum from the producer Novozymes).

1.5.2 Research question:

1. Would use of commercial inoculum shorten the start-up phase of new biofilters?

1.5.3 Null hypothesis:

1. Using inoculum in maturation tank will not provide a faster start-up.

1.5.4 Alternative hypothesis:

1. Using inoculum in maturation tanks will provide faster start-up.

1.5.5 Start-up of biofilters in RAS

RAS farming has been used for a short time compared to fjord farming, and therefore lack the same degree of optimisation. There are multiple ways to start-up a biofilter, one of the methods uses seeding from mature biofilters. An earlier study used 10% seeding for start-up of biofilter in RAS (Navada et al., 2021), However, there is not much research done on which seeding % achieves the best start-up efficiency over shortest period of time. The objective of this study is to optimize the biofilter seeding process during start-up phase in RAS.

1.5.6 Research Question:

1. Will different % of seeded bio media result in difference in maturation speed?

1.5.7 Null hypothesis:

1. Seeding with 15% mature bio media will not result in a faster biofilter start-up than 5% or 10% would.

1.5.8 Alternative hypothesis:

1. Seeding with 5% or 10% mature bio media will result in a faster biofilter start-up than 15% would.

2 Maturation tank start-up: experimental setup

2.1 Methods

2.1.1 Experimental design and setup

Both maturation tanks used in the first study are 4.68m^3 operating with freshwater as MBBRs (figure 8). Both MBBRs are filled with the freshwater from the surface water source in December 2022. The water has been treated with UV (280 mJ/cm^2) to prevent entrance of Salmon gill poxvirus (SGPV) that has been identified earlier in the intake water. This dose was chosen based on the recommendation from another research station were similar issues with SGPV were encountered (information provided by HiT personal). Further on, the water was recirculated in both maturation tanks through maturation process. New water was added only once on day 21 due to potential algal growth in both systems. This also coincided with technical problems when aeration in both systems stopped during weekend and was not restarted before Monday (day 44). To accomplish optimal water movement and aeration pressurised air was continuously supplied. The temperature control in tanks was not possible, leading to temperature ranges from 12-17ºC. Oxygen levels in the tanks were kept using aeration. Both tanks are filled to 40% filling degree with bio media (bio media type AnoxK Chip M from producer Anoxkaldnes). Bio media used in the maturation tanks have a high surface area of 1200 m^2/m^3 ± 1%.

Ethanol and sucrose were supplemented daily for the first weeks as a carbon source for heterotroph bacteria growth on the bio media to initiate biofilm growth. Ammonium chloride (NH_4Cl), Sodium nitrate ($NaNO_2$), phosphate and micronutrients ($FeCl_3 \cdot 6H_2O$ (55), $MgSO_4 \cdot 7H_2O$ (190), $CuSO_4 \cdot 5H_2O$ (5), $CoCl_2 \cdot 6H_2O$ (6), $NiCl_2 \cdot 6H_2O$ (6), $ZnSO_4 \cdot 7H_2O$ (34), $NaMoO_4 \cdot 2H_2O$ (5), and $MnCl_2 \cdot 4H_2O$ (42) (contents are in mg per 2L deionized water from the paper (Navada et al., 2020)) were added for growth of nitrifiers. Bicarbonate ($NaHCO_3$) was added to increase alkalinity which in turn increases and holds pH at the value of 8.5±0.2 that we want for the experiment. Ammonium chloride and bicarbonate was only added weekly at the start at an amount of 125g and 250g respectably. Throughout the experiment, the amount went up to ca 250g and 600g respectably, on a daily basis for the treatment tank. The chemical activation tank did not get any more chemicals after day 35. Tanks were manually mixed with a mixing tool the first week after chemical addition to ensure that most of the bio media has come in contact with water. Left tank had inoculum added to it on day 35 (22.02). Ammonia, nitrite, nitrate, phosphate and alkalinity were measured three times per week using water test

kits (Nitrate 114942, Nitrite 114776, Ammonium 114558, acid capacity to pH 4.3 (total alkalinity) 101758 all from Supelco) and a spectrophotometer (Spectroquant prove spectrophotometer 100 from Supelco) to get the results from the kits. Oxygen, temperature and pH were tested five times weekly with a Multi 3630 IDS SET F (with IDS-pH sensor and IDS conductivity sensor).

Figure 8 Both maturation tanks used in the experiment. The left maturation tank in the picture had inoculum added, while the right one is control.

2.1.2 Data analysis

Each maturation tank had two water samples taken, one from each lid of the tank. Chemicals were added at one side of the tank. To make sure that chemicals were mixed properly throughout the maturation tank one water sample was taken from each side of the tank, where the lids are. Water samples were analysed for ammonia, nitrite, and nitrate. Water quality results for each measured parameter were averaged for each tank and are presented as means ± standard deviation (SD) from mean. T.test was done to compare water quality in two maturation systems for post treatment phase. An assumption for using t.test is normal distribution within each group that are tested. Parameters that are not normally distributed are transformed with log10 or square root to get a normal distribution. The confidence interval for the statistical analysis were 95%.

2.2 Results

2.2.1 Nitrification activity

Pre-treatment phase of the experiment up to day 35 indicated little difference between control and treatment as shown in measured nitrogen compounds (figure 9). On Day 21 there was a drop in concentration of nitrogen compounds for both maturation tanks, which was caused by water exchange to remove a possible algae bloom. After addition of inoculum on day 37, ammonia and nitrite concentrations decreased, while nitrate increased over time in the treatment maturation tank. The control group had stable concentration of nitrogen compounds from day 35 to the end of the experiment. The two ammonium peaks on day 56 and 63 (figure 9a) are the result of spiking the water in the treatment tank in order to maintain nitrification process. Figure 10 indicates a significant difference between means for all three measured nitrogen compounds from day 35 until the end of the case study. T.tests on NH_4-N, NO_2-N and NO_3-N indicated significant difference between mean values. The last mean values for the treatment group in the experiment were 0.06, 0.1, 57.5, respectively. Mean difference between groups were 9.21, 3.35 and 27,54.

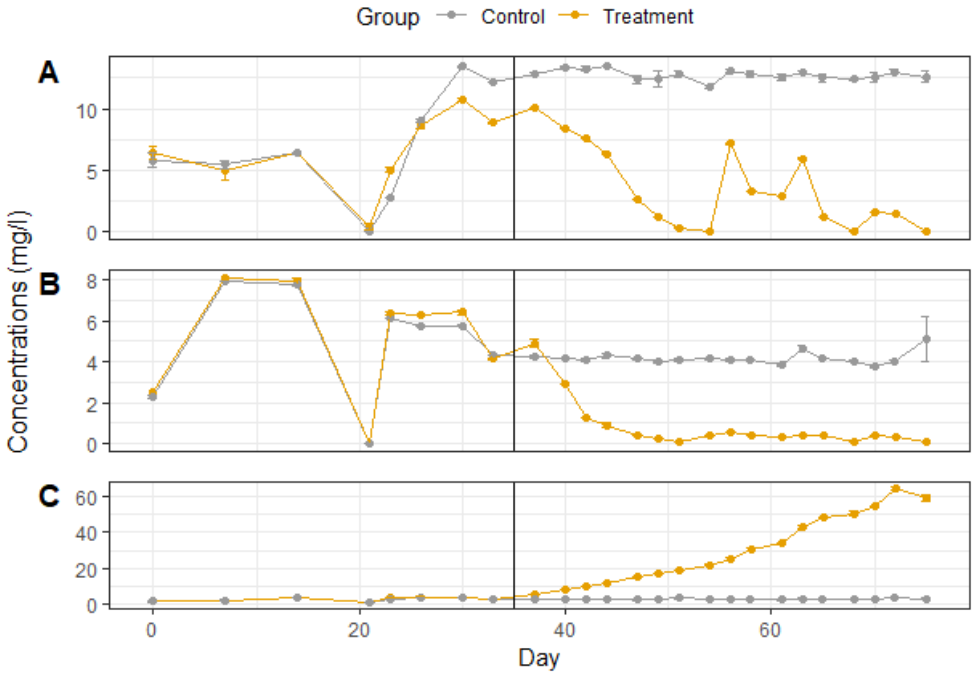

Figure 9 Three graphs are shown in the order of NH4-N (A), NO2-N (B) and NO3-N (C). Each data point represents mean value with standard deviation indicating uncertainty for both treatment and control over the time of the experiment. The black vertical line corresponds to day 35 when inoculum was added.

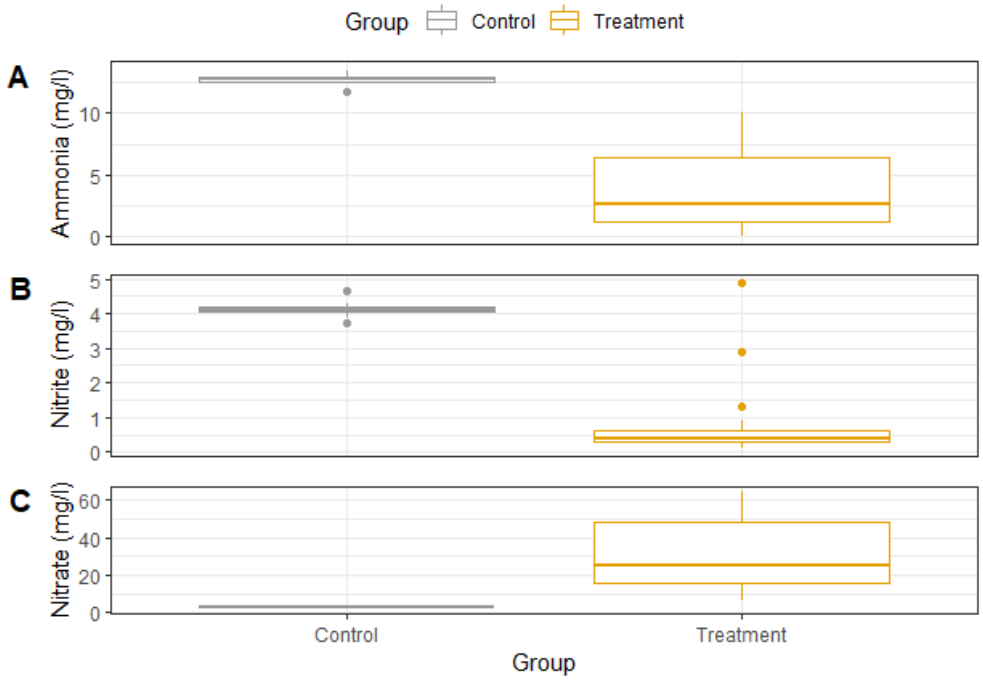

*Figure 10 Box plots for concentrations; A is ammonia (NH4-N mg/l) *, B is nitrite (NO2-N mg/l) * and C is nitrate (NO3-N mg/l) *. Control and treatment median value after inoculum was added (day 35+). * Signifies significant difference between groups.*

Temperature during this case study varied from 12.7 to 16.5, 13 to 17.50, and pH from 8.20 to 8.672, 8.54 to 8.671, oxygen saturation was between 100 to 103.2, 102.1 to 103.6 for treatment and control group, respectively. Values for pH and dissolved oxygen were similar in pre-treatment between the two maturation tanks (figure 11). Post-treatment phase (from day 35) has similar values for dissolved oxygen and temperature between treatment and control, while pH values are visibly lower in treatment after inoculation. Figure 12 is more accurately showing the differences. There is significant difference between treatment and control for pH and oxygen values with lower values measured for the treatment group after addition of inoculum.

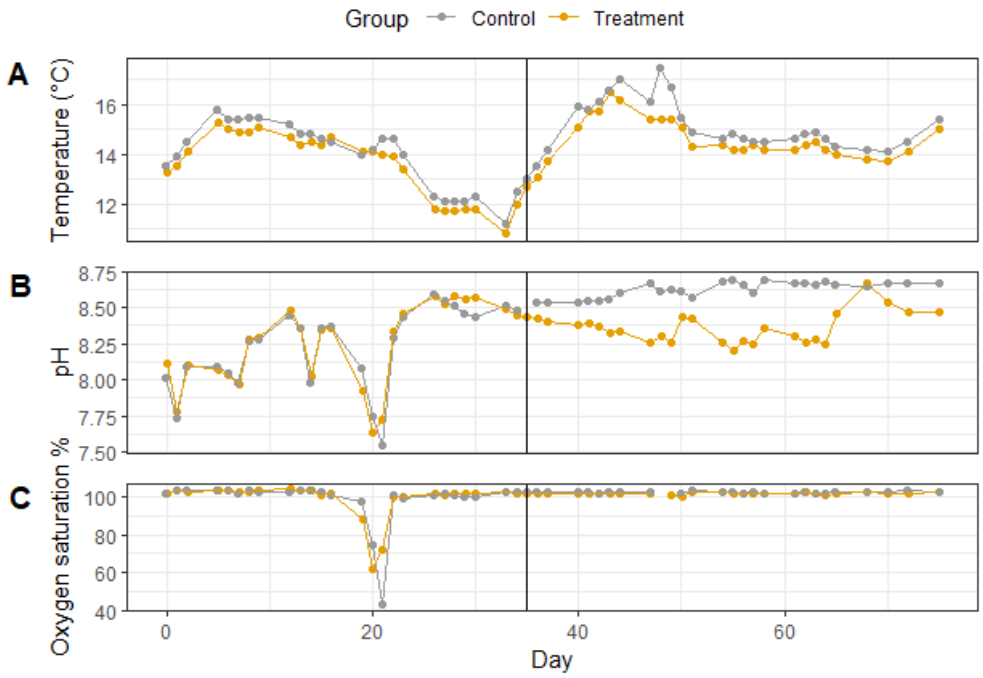

Figure 11 Three graphs are shown in the order of temperature (A), pH (B) and dissolved oxygen* (C). Each data point represents mean for both treatment and control over the time of the experiment. The black vertical line corresponds to when inoculum was added.*

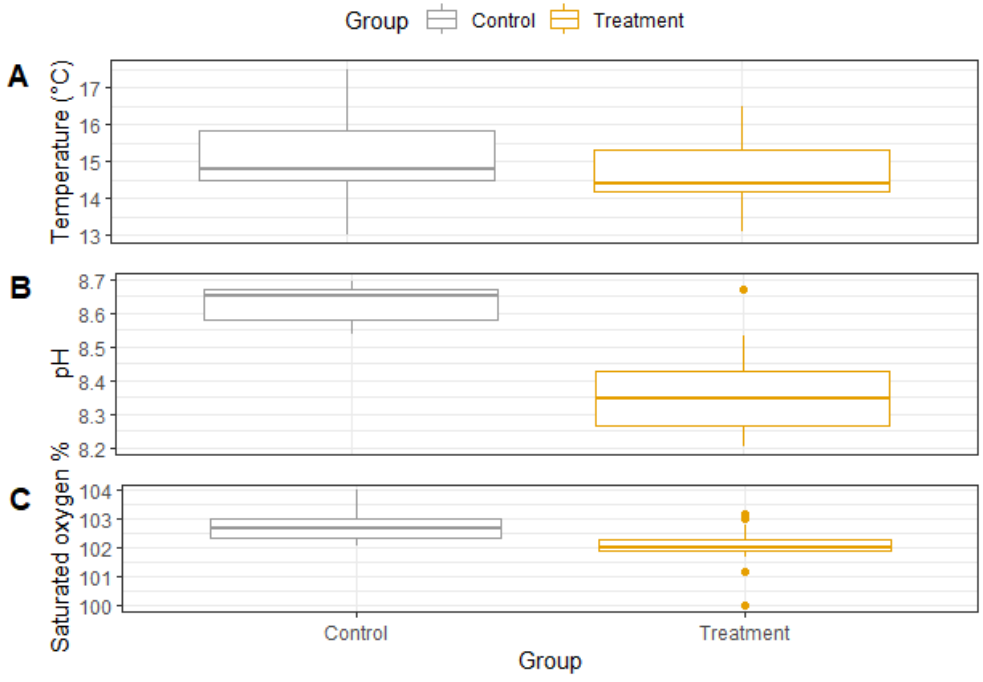

*Figure 12 Boxplot for treatment and control post-treatment phase in temperature (A), pH (B) and saturated oxygen (C). Control and treatment median value after inoculum was added. * Signifies significant difference between groups.*

2.3 Discussion

The result of the case study show that addition of the commercial inoculum can result in establishment of nitrification within 16 days of its addition in new biofilter. The goal of this

study was to document if using inoculum for start-up of maturation tanks would significantly decrease the time for "maturation" of the new biofilter. The biosecurity risk of introducing SGPV from the inlet water and the inability to use UV to treat large volumes of new water have restricted the way maturation tanks had to be started. During the first month of the study n nitrification was observed in maturation tanks. This was attributed to the treatment of the intake water with more than 10 times higher dose of UV than what is required by the Norwegian Food Safety Authority (Institute, 1997). This treatment has most likely sterilised the water that was recirculated internally in maturation tanks which prevented establishment of the functional microbial community necessary for nitrification process. This high UV dose would not be recommended to use for research in this type of studies, but in order to keep high biosecurity in the newly established RAS research facility, it was necessary to avoid entry of the potential pathogens. Results of this case study have indicated that the use of commercial inoculum can be a good strategy for biofilter start-up under stringent biosecurity conditions.

The nitrogen compound concentrations were similar to each other during the pre-treatment phase, but ammonia was about 3 mg/l higher in control right before inoculation . A reason for this could be faulty estimations when spiking the water with ammonia after exchanging water in day 21, because of human error during water tests or miss calculating the needed spiking amount. Ammonia concentrations in the control group remained at 12.74 ± 0.5 from day 35 until the end of the case study. The high concentrations of ammonia in the water can lead to inhibition of ammonia oxidation (Anthonisen et al., 1976). However, in this case UV treatment of inlet water might be the main reason for the lack of nitrification in the control group and for the observed significant differences in nitrogen compound concentrations between treatments. The analysis of the microbial community could confirm this observation.

Other relevant water quality parameters (pH, oxygen and temperature) during pre-treatment phase were similar between treatment and control. However, during post-treatment period pH and oxygen saturation were significantly lower in the group where inoculum was added. This difference between two maturation tanks in pH and oxygen saturation, was in agreement with the fact that the process of oxidating 1g ammonia requires 4.71g of O_2 and 7.05g of $CaCO_3$ (Timmons & Vinci, 2022). In this study we did not have the possibility to automatically adjust pH and oxygen and those water quality parameters were adjusted by batch addition of bicarbonate and aeration in maturation tanks that was at its maximum. Having said that, both

oxygen and pH values in both maturation tanks were in the ranges that are optimal for nitrification (Chen et al., 2006; Malone et al., 1998).

There was no temperature regulation built into the maturation tanks. The temperature of the inlet water and air temperature were therefore the regulators of water temperature in the maturation tanks and the reason why the temperature varied during the experiment. The increase in water temperature before inoculation was related to increased temperature of the inlet water. Although nitrifiers perform better at higher temperatures (De Mulder, 2014; Zhu & Chen, 2002), temperatures used in the case study are relevant for biofilter start-ups at commercial RAS facilities.

The left maturation tank was chosen for inoculation, instead of randomly assigning it as the ammonia concentration was lower compared to other maturation tank at day 35 of the experiment. Randomly assigning replicates can help to strengthen internal validity by reducing sources of bias within the study. The start-up of the maturation tanks was taking a lot longer than first expected, mainly due to the high UV dose that was used for inlet water treatment. The left tank was chosen in order to secure start-up of the next phase of experiment. The experiment of NanoRAS biofilter start-up was the main experiment, and therefore it was prioritized. A type two error is more likely to occur as we chose the more favourable replicate to become the treatment.

Statistical analysis were done to look at significant difference, undertaking applied statistics with only one replicate for each treatment would give no power in extrapolating the results to an overall population (Faber & Fonseca, 2014; Singh & Masuku, 2014). There are also many assumptions to meet while doing an analysis. Violating these can lead to inaccurate probability, or distortion of type I or type II error. Although the lack of replicates is an issue with this study (therefore called case study in this thesis), the size of the maturation tanks and conditions used in this case study could be relevant for commercial RAS facilities. The information about biofilter start-up under commercially relevant conditions are still lacking and some of the published papers on this topic include studies done without replicate biofilters (Navada et al., 2020)

2.4 Conclusion

This case study indicates that using inoculum for bio filter start-up has a significant positive result compared to chemical start-up. However, the chemical start-up in this case study was

most likely hindered by the treatment of the intake water with high dose of UV caused by the biosecurity risk of SGPV uptake to the newly opened RASforsk research facility. This treatment has most likely caused sterilisation of the water used in biofilter maturation tanks and has prevented establishment of the nitrification microorganisms in the absence of inoculum and under otherwise favourable water quality conditions.

The conditions recommended by the Norwegian Food Safety authority of 0,1 mg/L NO_2-N and < 2mg/L TAN have been established 16 days after inoculum was added to biofilter maturation tank. Subsequent spiking of the water with ammonium confirmed the ability of the biofilter to successfully oxidise ammonia, and other water quality parameters (pH and oxygen saturation) were indicative of nitrification process.

This case study lacked experimental replicates; however, it still documents biofilter start-up under conditions that are relevant for the commercial RAS facility for salmon production. It was therefore included in this thesis. In addition, the results from the start-up phase in maturation tanks at RASforsk are important for the following experiment with seeding done in the nanoRAS that is explained in this thesis.

3 3RAS biofilter start-up

3.1 Methods

3.1.1 Experimental design and setup

The experiment was preformed using MBBRs in six nanoRAS at RASforsk facility (figure 13). A nanoRAS is the name of the water treatment unit that can be connected to the fish tank to create RAS and was provided by Alpha Aqua. The nanoRAS are made to be able to handle wide range of conditions that could be relevant for commercial production now and in the future. Three nanoRAS can be connected to service larger fish tank (9m in diameter). In this experiment the nanoRAS connected to smaller tanks (1m in diameter) were ran individually. Each nanoRAS had a water volume of 2.16m^2 with the water level used in the experiment.

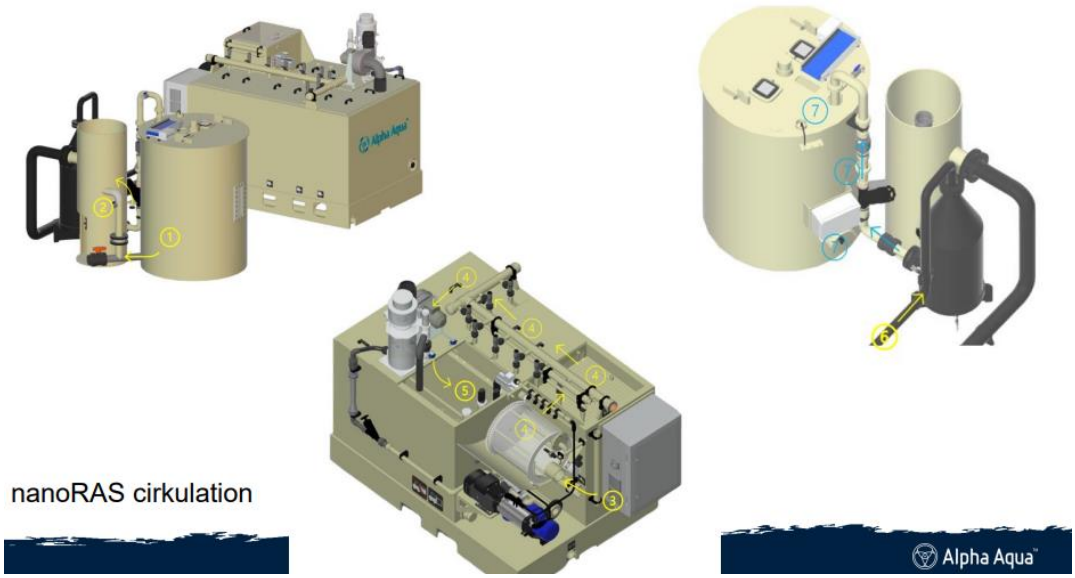

Figure 13 Overview over RAS compartments in Kårvika 1. Fish tank, 2. Swirl separator, 3. Mechanical filter (drum filter), 4. Biofilter and degasser, 5. Pump sump with temperature regulator, UV-light, water outlet and ozone adder, 6. Pressurization with oxygen, 7. Water inlet to fish tank (Images by Alpha Aqua).

All six nanoRAS are operated using UV treated inlet freshwater (280mJ/cm^2). The reactors were filled with different volumes of virgin media. The type of bio media being used are the same as in maturation tanks (bio media type AnoxK Chip M from producer Anoxkaldnes). Three treatments are run in duplicates of 15%, 10% and 5% mature bio media seeding (see table 3). The filling degree used is 40%. The seeding % is in relation to the full size e.g., 35 % virgin bio media and 5 % seeded bio media. The experiment ran for 44 days.

Table 3 Randomization of filling degree with mature bio media.

NanoRAS #	Seeding (% of mature bio media)
25	5%
26	10%
27	15%
28	10%
29	15%
30	5%

Mature biofilm carriers used for seeding were gathered from the left maturation tank that had inoculum added in the case study. The biofilm carriers from the maturation tank were newly started using inoculum and is considered mature as the nitrogen values have reached the requirements after repeated spiking of the biofilter with ammonia and nitrite over time. In preparation of the experiment, all six nanoRAS were operated with freshwater using only virgin bio media to achieve good mixing in the biofilters.

200g C_2H_6O are added for the three weeks to grow heterotrophic bacteria that help form biofilm on bio media which is important for growth of nitrifying bacteria. An addition of 21.6 ml micronutrients is needed for fundamental enzymatic actions, were added with the C_2H_6O and contains: $FeCl_3 \cdot 6H_2O$ (55), $MgSO_4 \cdot 7H_2O$ (190), $CuSO_4 \cdot 5H_2O$ (5), $CoCl_2 \cdot 6H_2O$ (6), $NiCl_2 \cdot 6H_2O$ (6), $ZnSO_4 \cdot 7H_2O$ (34), $NaMoO_4 \cdot 2H_2O$ (5), and $MnCl_2 \cdot 4H_2O$ (42) (contents are in mg per 2L deionized water from the paper (Navada et al., 2020)). 24.2g Na_2HPO_4*$12H_2O$ and 14.1g KH_2PO_4 were added once as bacteria needs phosphate to ensure normal functions such as growth. 125g HCO_{3-} is added at the start to increase to increase alkalinity which acts as a buffer for pH making it more stable. Chemical spiking of NH_4Cl started at 3mg/l NH_4-N and increased up to 30mg/l NH_4-N. All chemicals were spiked into the fish tank.

At day 1(17.04), all six MBBRs in nanoRAS were filled with mature bio media to reach a total filling grade of 40%. MBBRs were operated at temperature between 16.84-20.62 ºC, a pH of 7.10-9.00 and a water flow of 40 L/min. Oxygen saturation stayed between 33.78.-100.62 % during the experiment. Oxygen was not added in this experiment as the oxygen supply had to be removed during construction. Oxygen exchange through the air was the main factor in sustaining oxygen saturation. All nanoRAS had initially online pH probes and automatic bicarbonate feeding to ensure a stable pH level of 8.5 throughout the experiment. The system stopped working as intended and was completely stopped on day 20. The rest of the experiment had to use manual feeding (three times the amount of NH_4Cl is spiked). Temperature, dissolved oxygen, salinity, flow rate and inflow of make-up water were also measured continuously with online probes: two online oxygen probes were placed in the pump sump and fish tank (they also did the readings for temperature), salinity in pump sump, both pH probes were placed in the pump sump, and make-up water flow was measured from probes connected to the pipes of the inlet water. On day 23 and 24 33% of the total volume was emptied to dilute nitrite and nitrate concentrations in the systems. This was done as measurements of high concentrations of nitrite and nitrate were hard to measure precisely with the kits used. After day 25 NH_4Cl was added to maintain nitrification process until way 44 when the final measurements were done.

Ammonia, nitrite, nitrate, alkalinity, and phosphate were measured using testing kits (Nitrate 114942, Nitrite 114776, Ammonium 114558, acid capacity to pH 4.3 (total alkalinity) 101758 all from Supelco) and a spectrophotometer (Spectroquant prove spectrophotometer 100 from Supelco). All three nitrogen compounds were measured at nitrogen level. Water samples were collected from the inlet to the fish tank.

The experiment was operated with minimum addition of new water. Over time nanoRAS lose some of the water through evaporation and from the drum filter backlash. It was important to know the amount of water lost from each system. Water loss through evaporation increase concentrations of chemicals, while water loss from drum filter removes both water and chemicals. Each nanoRAS had different amount of water loss. Chemicals were added to all nanoRAS and were circulated for three days to estimate the dilution for each of them to correctly obtained water quality results. It was also necessary to add small amounts of new water to account for above mentioned water loss. Make up water was regulated by the water level in the pump sump. Every time the water level went below 27.5 cm in the sump, 15 L was added to the system.

3.1.2 Capacity tests for determination of nitrification performance

A capacity test is a test to measure the performance of the system in a controlled environment. In this case the performance is in oxidation rate, and the environment is controlled by stopping addition of new water to all nanoRAS, disabling drum filter and spiking each nanoRAS with NH_4Cl. Capacity tests were done on days 11, 18, 25 and 43-44. Maximum ammonia oxidation rate (AORmax) and maximum nitrite accumulation rate (NARmax) where calculated based on grams nitrogen per square meters per day. Addition of new water was stopped to all nanoRAS, drum filters were turned off and each MBBR were spiked with NH_4Cl. The spiked amount varied for each nanoRAS to try to get similar starting ammonia concentrations (in mg/l) during the capacity test. Water samples were collected every two hours, three to four times for the first three capacity tests. The final two tests (day 25 and day 44) had eight tests each, and the last one was the only with samples from two different days (day 43 – 44).

Table 4 NH_4-N added for the last 15 days of the experiment measured in mg/l.

Replicate	Day 29-36	Day 36-44
N25	17.53	22.16
N30	17	21.68
N26	17	21.68
N27	16.76	21.44
N28	22.82	27.43
N29	17	21.68

3.1.3 Data analysis

Graphs and ANCOVA are used in this thesis to discuss if there is any difference between the treatments. All statistical analyses use 95% confidence interval. There are many assumptions to account for when preforming a statistical analysis. One of the first assumptions for use of a linear regression (lm) is to randomise replicates for the treatments. This helps to justify the generalizability of the results (Nimon, 2012). A linear relationship between the response and explanatory variable is needed. There should be no or little autocorrelation. Residuals should be independent; dependant residuals can lead to positive or negative autocorrelation. Normal distribution for residuals is assumed, but it is not necessary if the sample size is large enough. Homogeneity of for variance and regression is assumed for an ANCOVA test (Nimon, 2012; Warner, 2008). At last, it is assumed there is no or low collinearity. Collinearity occurs from correlation between independent variables.

Statistical analysis and visual representation of data were done by using R studio (team, 2023) with the R software (Team, 2022). Readxl (Bryan, 2022), tidyverse (Wickham H et al., 2019) and ggpubr (A, 2023) are used to modify my data and visualising it in a better manner. MuMIn (Barton, 2023) is used for the dredge function, which generates sets of models and gives me an AICc for each model. A model with a lower AICc is usually a better model. Mctest (Imdad, 2018; Imdadullah, 2016) is used to test for collinearity.

3.2 Results

3.2.1 Nitrification activity

Oxidation of ammonia to nitrite started immediately after seeding. The systems had NH_4Cl added to reach 3mg/l of NH_4-N. The first day used about 2.5 mg/l for all systems. NH_4Cl was spiked again to reach 3 mg/l, but the same amount got used up in a day. The spiking estimate kept increasing with 1-2 mg/l until the amounts in table 4 were reached. Oxidation of nitrite to nitrate is a slower process, which resulted in nitrite accumulating in the RAS water (figure 14B). On day 23 and 24 had about 33% of the total water volume was exchanged, leading to nitrite and nitrate concentrations decreasing consequently (figure 14B and 12C). The concentrations of measured nitrogen compounds in all nanoRAS were similar during the first 25 days of the experiment. Ammonium concentrations was between 0.35 and 38.05 mg/l NH_4-N in all treatments, and the observed peaks on days 11, 18, 25 and 44 were the consequence of spiking RAS water during capacity tests. The results of the final capacity test done on day 44 (figures 14 and 15) are showing differences in concentrations of nitrogen compounds between the treatments and replicates. T15 have oxidised the most STDEVS nitrate during the experiment at 269 mg/l, T5 is second with 228 mg/l, and lastly T10 at 214 mg/l. Figure 15 C indicates that replicate 29 is the reason for the higher nitrate value in T15. Replicate 29 had the highest amount of ammonium chloride added in the last two weeks of the experiment (table 4), while having the lowest level of nitrite on the last day, 120 mg/l lower than the second lowest (figure 15 B).

There was no significant difference between treatments in measured ammonia concentration during the experiment. However, there was significant difference in nitrite concentration ($P = 0.00306$) between T15 and T5. The nitrite means ± STDEV were 51.1± for T15, 61.3± for T10 and 76.9± mg/l NO_2-N for T5. The concentration of nitrite was significantly higher in T5 compared to T15. Nitrate concentrations were significantly different between T15 and both T10 and T5. The nitrate means ± STDEV were 67.8± for T15, 56.5± for T10 and 59.6± for T5. Both T10 and T5 had significantly lower nitrate concentration than T15.

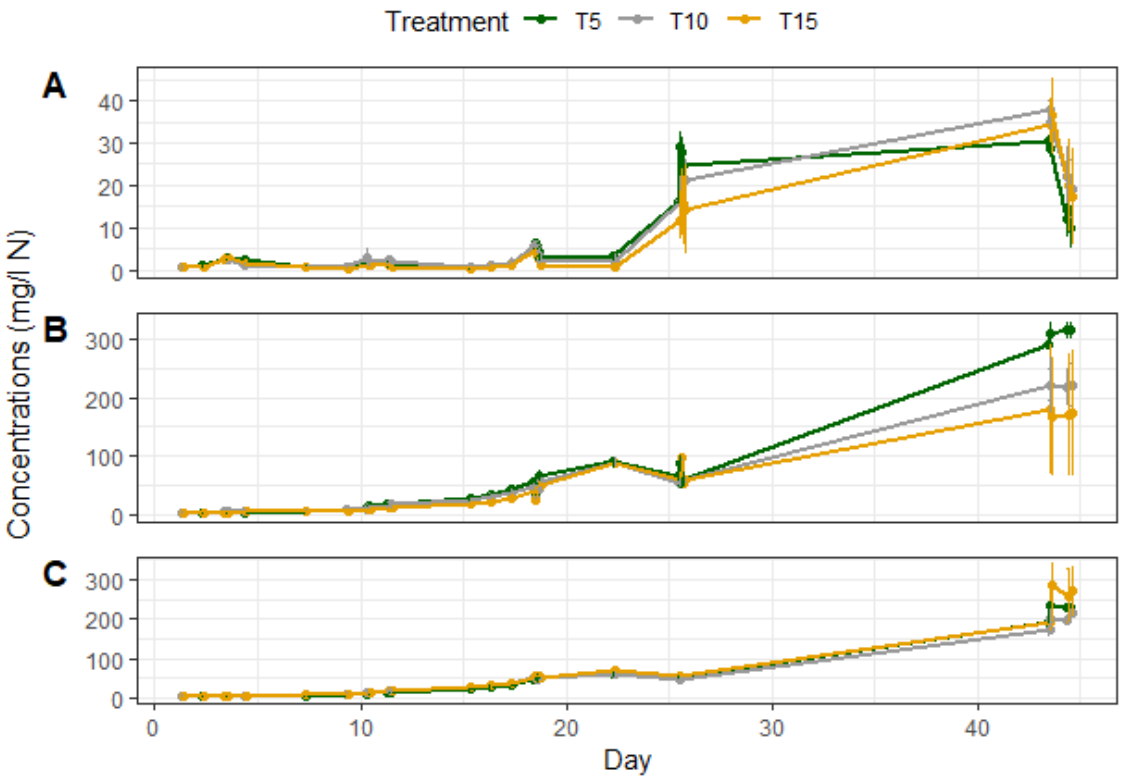

Figure 14 Average nitrogen concentrations of ammonium (n=2) (A), nitrite (B) and nitrate (C). Each data point represents mean value of replicates in each treatment with standard deviation for uncertainty.

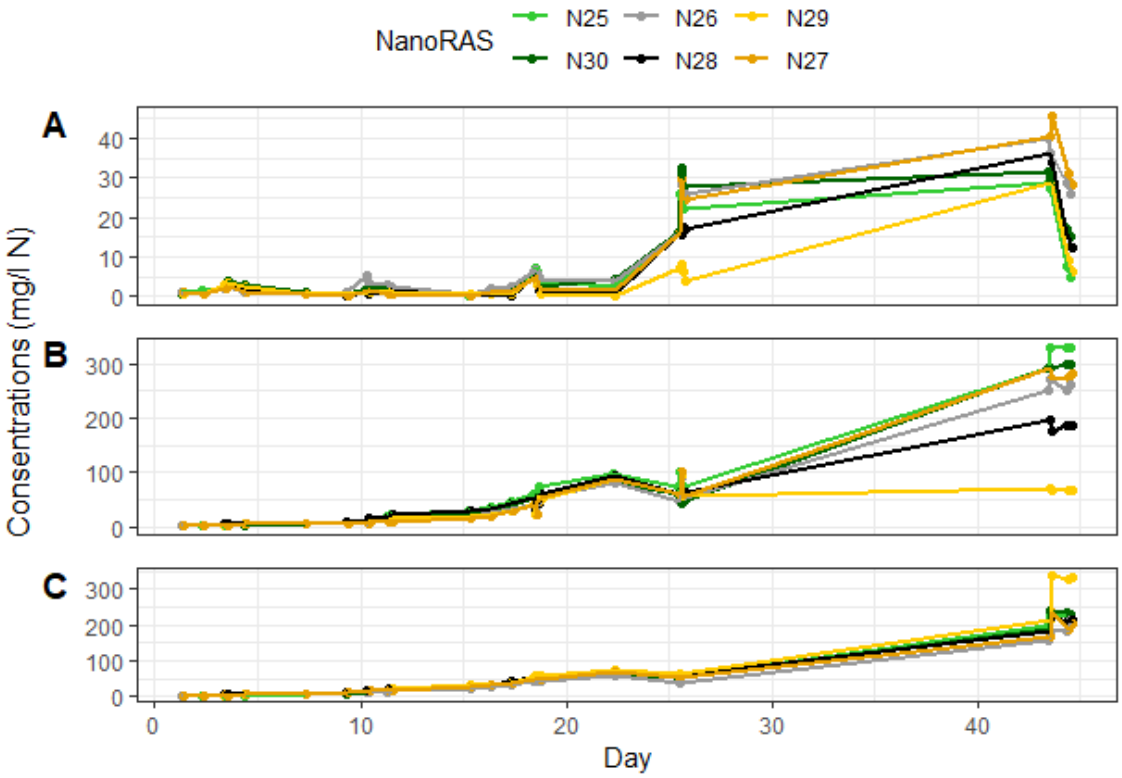

Figure 15 Concentrations of ammonia (A), nitrite (B) and nitrate (C) for each experimental nanoRAS during the experiment.

3.2.2 Capacity tests

Figure 16A indicate that the oxidation rate for ammonia was between 0.012-0.018 for treatments on day 11. It increased sharply on day 18 and doubled for T15 and T5 on day 25. Two nanoRAS from T10 showed large variation in AOR, as negative oxidation rate was recorded for system 28 (figure 16 A). On day 44 a similar oxidation rate was recorded for all treatments. Nitrite oxidation rate (figure 16 B) on day 10 showed negative oxidation for T5 and T10, while T15 has a higher NOR. On day 18 similar negative rates for all three treatments were recorded, while on day 25 positive NORs were recorded for T5. The results of the last capacity test show that the T15 was the only treatment with positive NOR. AOR linear regression line is close for T5 and T15, but T10 is lower because of the results on day 25 (14 A). Figure 17 A shows the AOR and NOR values for the replicates in each treatment. The difference for AOR on day 11 and 18 were 0.0097 gN/m^{2}d and 0.031 gN/m^{2}d respectively. The difference widened for the last two tests on day 25 and 44 with 0.232 gN/m^{2}d and 0.072 gN/m^{2}d. NOR linear regression has T15 as the best performing treatment, while T5 and T10 have a similar lower increase in NOR over time. An ANCOVA test for AOR finds significant difference between T15 and T10 (P=0.03927), with mean values of 0.97 and 0.059.

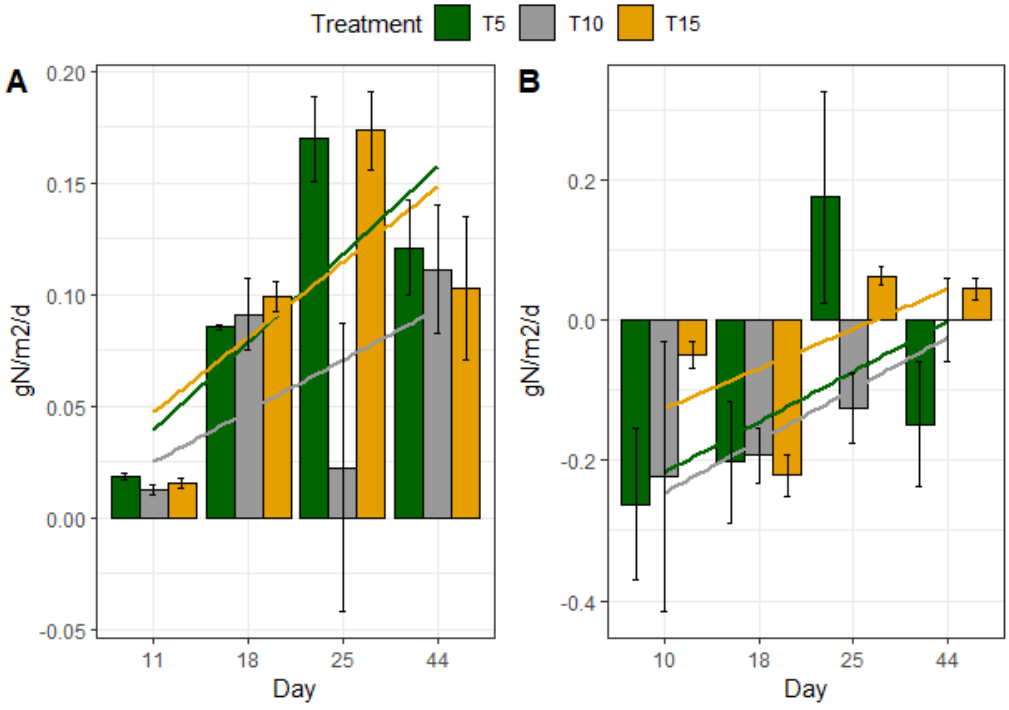

Figure 16 Ammonia oxidation rate (AORs) and nitrite oxidation rate (NORs) calculated for the capacity tests done on days 11, 18, 25 and 44. The graphs show values for each treatment (T5, T10, T15) for the total gN/m^{2}d of ammonia (A) and nitrite (B). Each data point represents mean value of replicates in each treatment with standard deviation for uncertainty. Regression lines are added to see the increase in oxidation rate over the experiment.

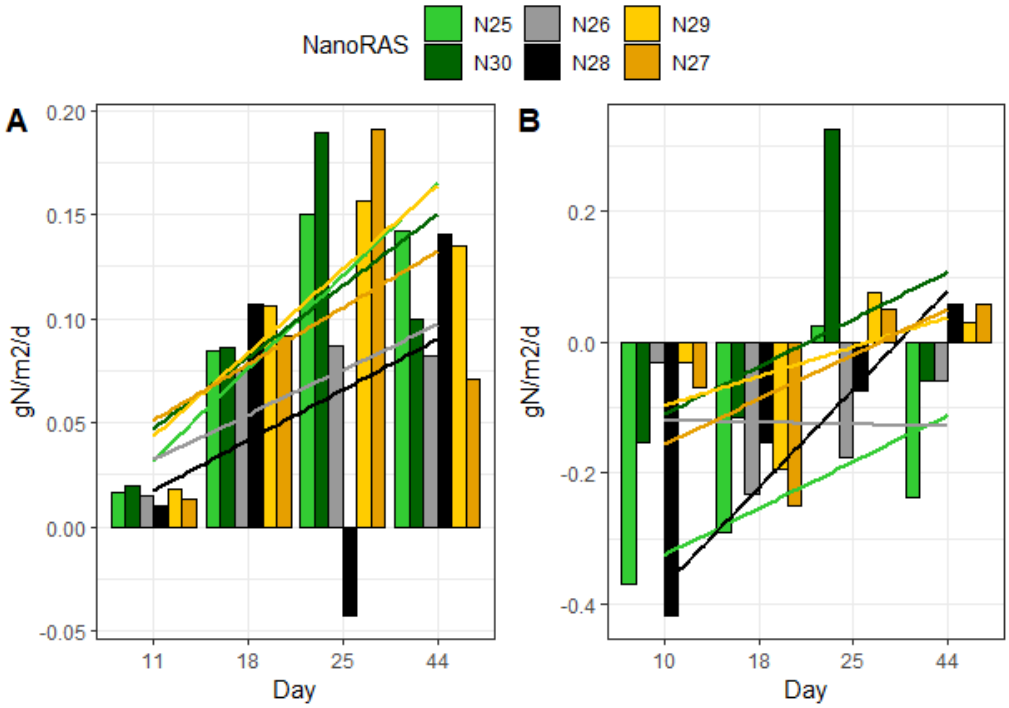

Figure 17 Ammonia oxidation rate (AORs) and nitrite oxidation rate (NORs) calculated for the capacity tests done on days 11, 18, 25 and 44. The graphs show values for each replicate (N25, N26, N27, N28, N29, N30) for the total gN/m^{2}d of ammonia (A) and nitrite (B). Each data point represents mean value of replicates in each treatment with standard deviation for uncertainty. Regression lines are added to see the increase in oxidation rate over the experiment.

3.2.3 Temperature, pH, Oxygen and make-up water

Only make-up water and pH did not have significant difference in mean value between treatments (table 6). Oxygen in sump was 3 % higher in T5, than the other treatments. Oxygen in tank was significantly different between all treatments, with a difference from lowest value to largest of 9.11 %. Temperature in the sump for T15 0.25 ºC lower that for other treatments, while temperature in tanks were 0.13 ºC higher in T5 than in other treatments.

Table 5 Average values for OXS (oxygen sump), OXT (oxygen tank), pH, TS (temperature sump), TT (temperature tank) and M (make-up water). Groups indoccates if there is significant difference in mean value between treatments. A difference in letter means significant difference.

Treatment	Average value	Groups
OXS T5	90.8	a
OXS T10	87.25	b
OXS T15	87.39	b
OXT T5	91.48	a
OXT T10	82.37	b
OXT T15	87.88	c
pH T5	8.26	a
pH T10	8.27	a
pH T15	8.33	a
TS T5	19.92	a
TS T10	19.85	a
TS T15	19.61	b
TT T5	20.10	a
TT T10	19.97	b
TT T15	19.96	b
M T5	98.48	a
M T10	109.85	a
M T15	95.97	a

In general, oxygen saturation during the experiment decreased for all treatments apart from the indicated increase in the last couple of days of experiment (figure 18). Oxygen saturation in tanks was lower than oxygen saturation in the sump, with T10 having the lowest sump oxygen saturation. Cleaning of sensors were not done regularly after day 25 of the experiment. The effect of cleaning can be seen at the end when recorded oxygen saturation increases by 20-40%

for all treatments. System 26 showed the largest drop in oxygen measured in the tank which affected the average oxygen saturation for T 10 from day 25 onwards (appendix 2)

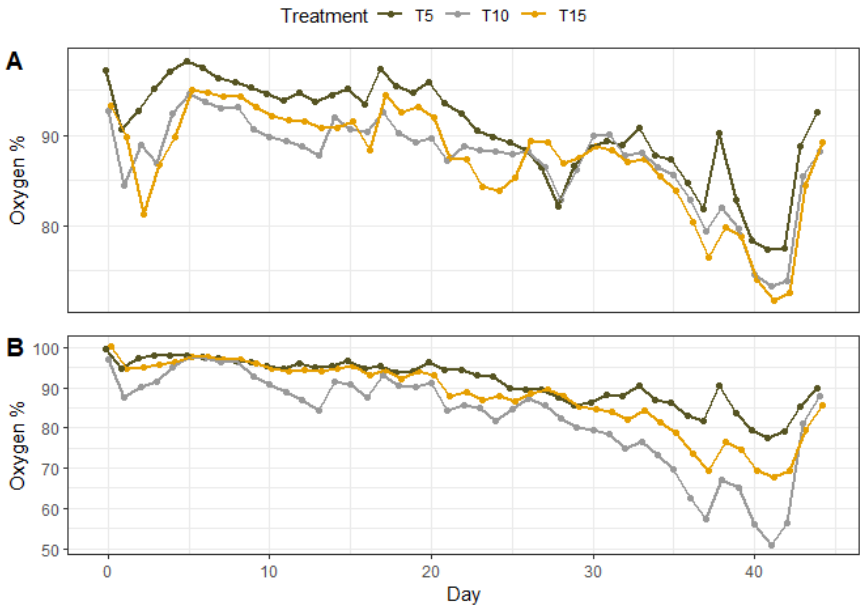

Figure 18 Oxygen saturation (%) for sump (A) and tank (B) between treatments throughout the experiment. Oxygen saturation decreases from day 20.

Temperature during the experiment were mostly stable. The only drop in the temperature was recorded on days 23 and 24 when almost one third of all system water volume was exchanged with the new cold make-up water (figure 19) in order to dilute concentrations of nitrogen compounds.

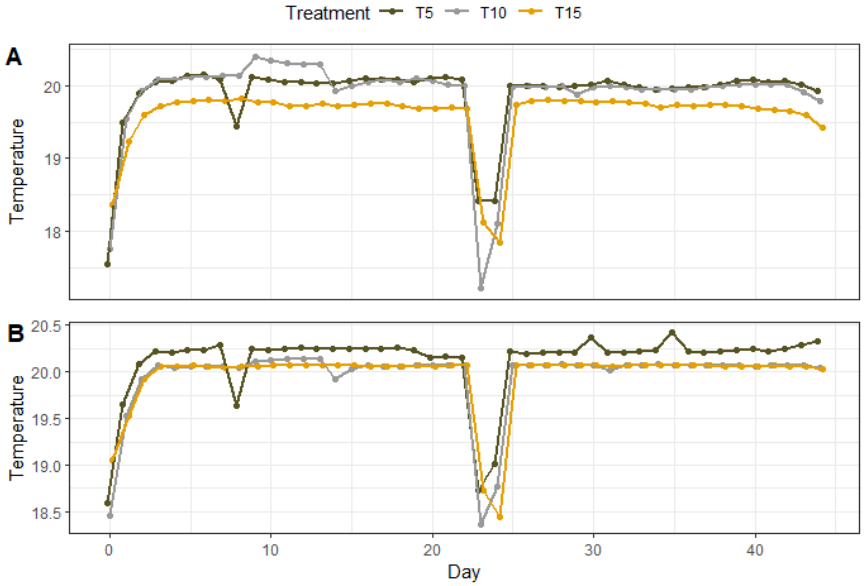

Figure 19 Temperature changes in the sump (A) and tank (B) for all treatments throughout the experiment.

The desired pH for the experiment was 8.5 which was the set point for the automatic dosing od the bicarbonate to the system. However, between days 3-14 pH decreased in all systems and treatments (figure 20) due to the issues with the centralised bicarbonate dosing systems. The dosing system was restarted on day 15, but the pH values increased in all systems after that (day 15-20) as dosing failed to stop at the desired set point of 8.5. After this automatic dosing was stopped in all systems and from day 21 onwards bicarbonate was added manually which introduced daily variation observed during this period.

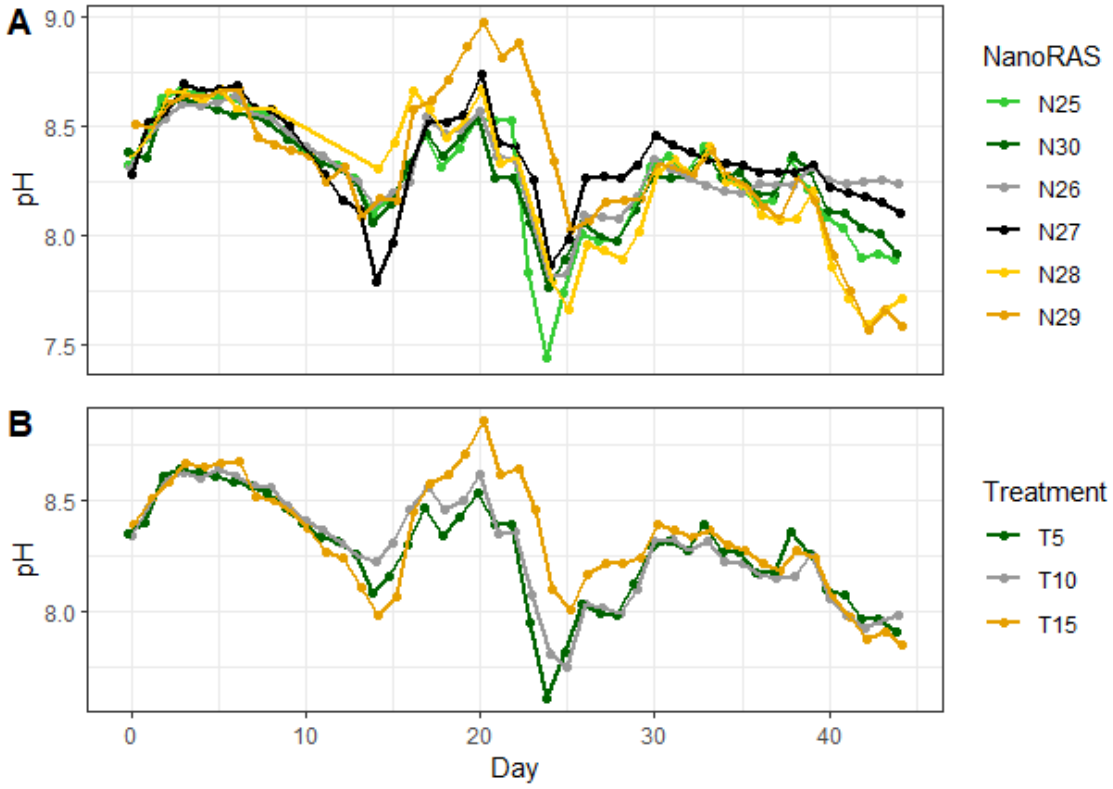

Figure 20 pH changes for replicates (A) and treatments (B) throughout the experiment. Day 3-14 shows the pH decreasing as a consequence of the automatic bicarbonate pumps not feeding. Day 15- 20 shows the rapid increase in pH when automatic bicarbonate feeding did not stop at intended level (8.5). Day 21 + shows unstable pH levels from manual feeding.

The make-up water flow was mostly stable during the experiment for all systems at 4.50, 4.69, 4.34 % total water exchange for T5, T10 and T15 respectively (excluding day 23 and 24). The higher make-up water flows were recorded on day 23 and 24 when 1/3 of water volume was exchange in all systems to dilute concentration of nitrogen compounds (figure 21).

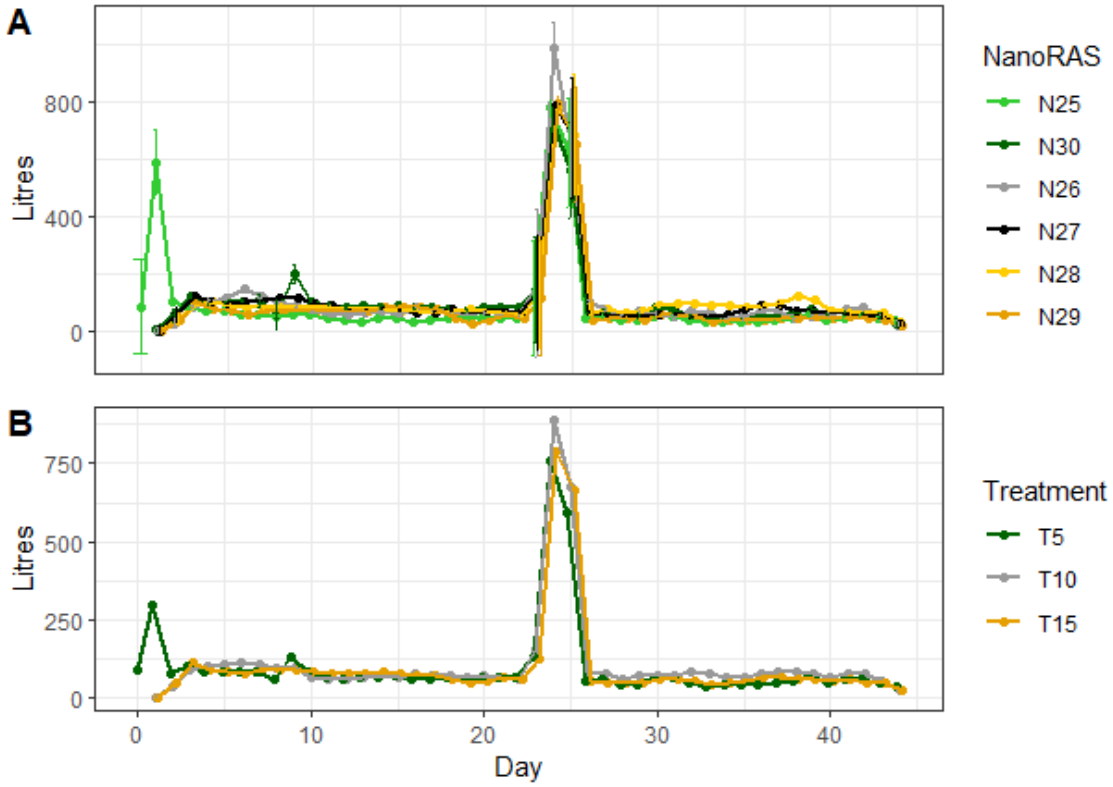

Figure 21 Changes in inflow for replicates (A) and treatments (B) throughout the experiment.

3.3 Discussion

The goal of this study was to optimise the seeding ratios for start-up of biofilters. Nitrification of ammonia and nitrite started the same day as seeding was initiated, indicating a successful seeding process. Ammonia concentrations were continuously being used up, while nitrite accumulated over time. The relation in performance between ammonia- and nitrite oxidisers continued for the first half of the experiment (day1-24), while the second half shows signs of nitrite oxidisers reaching similar oxidation rates. The three highest AOR replicates have similar mean value and come from different treatments. The difference between replicates in treatments are also similar. However, AOR did not reach the goal value of 0.3 gN/m^2d in any of the systems. NOR is harder to interpret. The nitrite oxidation rate measured is negatively affected by the production of nitrite from ammonia oxidation, which is probably the reason for why there is such a large difference between the tests. Replicate 25 and 26 looks to be performing the worst out of the replicates for NOR as their value have mostly been negative throughout the experiment. The other replicates seem to have a positive increase in NOB, meaning they start to oxidate more nitrite, than AOB can produce. Other papers (Aalto et al., 2022) have also documented a slower increase of nitrite oxidising microorganisms in relation to ammonia oxidiser. (Arvin et al., 2021) had nitrite accumulation until day 30, where it thereafter stabilised.

NOB regression lines are indicating that treatments are reaching positive oxidation or stabilising at around the last capacity test (day44).

The largest reason for the low treatment value in AOR comes from the negative value for replicate 28. There is little reason for a negative ammonium oxidation rate to be true, as that would imply that ammonium is being made in the system. The first sample taken during capacity tests might not be a good representation of the overall ammonium value, as ammonium from spiking needs time to circulate throughout the whole system. Spiking is being adding in the fish tank and measurements are collected from the inlet to the fish tank. Water samples are collected as far as possible from the location where spiking chemicals is occurring. Circulation might have been slower than estimated, resulting in patches of lower and higher concentrations. It was noticed that increasing the ammonium chloride spike, also increased the time for ammonium to stabilise in the system.

Nitrate concentration can also give an indication on the performance of NOB as there is little denitrification and water exchange to reduce nitrate concentrations. The first half of the experiment is not indicating a significant difference, but the latter half shows separation between the treatments with T15 having about 40mg/l more than the other treatments. The reason for the performance with T15 comes from replicate 29 which have about 110 mg/l higher than the rest. It is unknown why replicate 29 is showing a higher perform in concentrations than the other replicates, while in AOR and NOR it is performing similar to the other replicates.

At day 44 the AOR and NOR for T10 were 0.11 gN/m^2d and 0 gN/m^2d respectably. (Navada et al., 2020) paper had a value that looks to be 0.5 gN/m^2d for both AOR and NOR after 44 days of her experiment. The data in this experiment ranges from 20 % to 0 % of her results. (Navada et al., 2020) results are calculated with mass balance, which this experiment has not done. Calculating with mass balance gives more precision, especially for NOR. Nitrate concentration show this experiments T10 to have a value of 214 mg/l, while it is about 80 mg/l in her paper. The result on nitrate can mean that the oxidation rate for this experiment might have been higher if calculated using mass balance. Oxidation rate over time is also important to account for. This experiment might have had a higher oxidation rate for most of the experiment, while in (Navada et al., 2020) paper the oxidation rate has most of its increasing around day 40-45.

A higher mean nitrate concentration measured in T 15 RAS water during experiment is a good indication that this treatment is performing better compared to other treatments. However, this was not confirmed when comparing oxidation rates between different treatment. The reason for this could be uncertainties during sampling where chemicals are not equally distributed or inaccurate results from testing, because of human error. AOR for T15 is significantly different from T10. Day 25 is the reason for this, as T10 had close to 0 in oxidation rate, while the other treatments had their highest value for the experiment. The model used is not the best, indicated from the function dredge in r studio (appendix 1). Even though the model has a worse fit of the data, it is still important to analyse. It is probable that the low number of capacity tests, uncertainty with the tests and biological variation are the reason for why treatment is not in the best models.

There is statistical significance between many of the water parameters, but it might not have a biological significance as there is only 1-5 % difference in mean values. In addition, all measured water quality parameters measured are in the range of what is considered to be optimal for nitrification process to be established.

There were lower oxygen levels for the last half of the experiment. Oxygen concentrations between 5.18 to 7.12 mg O_2 L^{-1} have been shown to negatively affect microorganism (Spietz et al., 2015). The 75% dissolved oxygen at 20 degrees equals 6.8mg/l, while 33 % (3 mg/l) as measured for some nanoRAS could have probably affected the microorganisms. However, due to the lack of capacity from day 25 to day 44 of the experiment, oxygen probes were not clean frequent. What was seen at RASforsk indicated that cleaning twice a week would ensure that measurements were not affected. It is known that biofouling of the probes can, lead to less accurate data (Schraa et al., 2006). The increase in oxygen saturation during the last days of the experiment therefore stem from cleaning the probes. Having all this in mind, it is likely that the oxygen saturation was within the required range for nitrifiers throughout the experiment although the recordings indicate differently. It was noticed that bio media in replicate 26 did not move as usual in 2/3 chambers. The aeration was partially blocked from bio media that had managed to get stuck with the aeration pipes.

The facility at RASforsk was still under construction while the experiment was ongoing. This experiment was the first real tests on how the systems preform and are managed. Some unexpected faults and inexperience with managing the systems, ended up with some

inconsistencies between the systems from time to time. Replicas were overall run close enough with the problems that occurred.

Proving statistically significant differences can be difficult for RAS experiment. There are many assumptions that need to be met to do a statistical analysis. Violating these can lead to inaccurate probability, or distortion of type I or type II error. Each RAS unit is expensive to run and test, needs a specialised facility with the right equipment, and the number of units can be limited. Applied statistics need a sufficient number of samples for each treatment to give the statistical analysis enough power to be able to extrapolate the results to the overall population (Faber & Fonseca, 2014; Singh & Masuku, 2014). Few replicates can lead to important associations and effects not being detected, or imprecisely estimating them. This experiment has two replicates for each treatment, which is better than the first case study on maturation tanks, but still very low for statistical analysis. Comparing graphs of concentrations and oxidation rates with water parameters and observations is the main focus for discussion in this experiment. T.tests have been done on water parameters as there are a thousands of measurements on each nanoRAS, and them not being the main factor to reject the null hypothesis.

Nitrogen compound concentrations during the experiment and calculated oxidation rates (AOR and NOR) have not given solid grounds to recommend use of more than 5% of mature bio media when seeding new biofilters. Based on the hypotheses of the study, it was expected that seeding with 5% mature bio media to either show the worst nitrification performance compared to other treatment or at the best give equal results as T10 and T15. The results of this study are indicating that seeding with 5% mature bio media leads to equal or even better nitrification rates as when higher seeding rates (10 and 15%) are used. preforming as good as the others, or even better in some cases. The knowledge of this can have a use for both research and commercial production. It is important to state that the results of this study show large differences between replicates for some of the treatments, in particular T10 that might have affected the final results of this study. The use of more replicates in this experiment could have provided better grounds for comparison. Unfortunately, at the time the study was done, this was not possible dues to the technical issues art RASforsk.

There was uncertainty about how precise the test kits were from results highly oscillating concentration results during capacity tests, so a test was done to determine the uncertainty. Each sample were tested in duplicate to determine the difference during day 22. The uncertainty of

ammonia, nitrite and nitrate were respectably 2.4 % ± 1.55, 6.8 % ± 5.9, and 5.4 % ± 4.2. The uncertainty for ammonia is acceptable, while nitrite and nitrate data showed more uncertainty than wanted. The high levels of nitrite did affect the accuracy of the testing. Nitrite test kits have a maximum range up to 1 mg/l. To test for higher nitrite levels that were present in the experiment, meant that the sample had to be diluted 100-300 times, which made it easier for human error to occur.

In the maturation tanks, the rate of ammonia and nitrite oxidation seemed to be similar to each other as nitrite is usually close to zero. Ammonia oxidation rate in nanoRAS is higher than nitrite as seen from the increase in nitrite concentration over time Having a temperature of 20ºC in the system compared to the standard of 14ºC (Crouse et al., 2022), should have given the bacteria favourable conditions to grow (Wortman & Wheaton, 1991). NOB activities should be favoured over AOB in this temperature range (De Mulder, 2014). To understand better the results, we would have to do an analysis on the community composition of the microorganisms. This would give us the information about the proportions of the different types of nitrifiers. Doing a capacity test to verify the oxidation in the maturation tank should have been done before seeding. 0.3 gN/m^2d was the value wanted to claim that the system was mature. It is unknown if the maturation tank had reached the value at the time of seeding. A further matured maturation tank could have made NOB grow more abundant and possibly made the nitrite oxidation rate higher after seeding. Only bio media was used during seeding, due to the experimental design. It was undetermined if the water in the maturation tanks contained high amounts of nitrifying microorganisms. High UV affected the case study and might have influenced this experiment as well. Normal UV doses would let nitrifying bacteria enter the system through the inlet water. This could have increased the maturation speed, as new bacteria would be constantly entering.

Automatic pH has not worked as intended for most of the experiment. The centralised dosing system at the start did not dose enough bicarbonate to sustain the pH level which led to the decrease in pH under intended levels. Then another problem occurred where the system did not stop dosing, which ended in high pH for a couple of the systems. Automatic feeding was ended and manually feeding commenced from then on. It was hard to keep the intended levels with manually dosing of bicarbonate. However, there were no significant differences in pH between treatments or replicates and the recorded levels of pH were in the range considered to be optimal for nitrification.

The results of this study are relevant both for biofilter start-up in research and commercial facilities. In research facilities, it is common to disinfect biofilters between experiment to ensure good biosecurity. Knowing the adequate seeding ratios when starting biofilters for each experiment will improve the efficiency for this process.

The benefit for commercial production comes indicating that seeding more, does not necessarily give better results. The time series gives a notion for the time required for start-up from seeding in a system that had optimal water conditions for bacteria, and high UV on the inlet water.

4 Conclusion

This master's thesis resulted in the development of effectively choosing the seeding ratio for start-up of biofilter in RAS. The findings in this experiment can be used for both research and commercial production. The results are indicating that there is little or no benefit in using more than 5% seeding with mature bio media when starting up a biofilter. Difference in maturation speed between seeding ratios could not be concluded as no systems reached the maturation value of 0.3 gN/m^2d NH_4-N, but the capacity test on day 44 is indicating that the performance between treatments are similar. The technical issues that were encountered during this experiment did not allow us to follow full maturation of the biofilters in the second experiment. Therefore, there is a need to do some further research of the topic to get a more conclusive answer to the exact amount of seeding that is recommended.

5 Future considerations

Thoroughly testing the stability of the systems water parameters and getting more familiarised with the management before starting experimentation would have helped with some inconsistencies in running the experiment for this study. Having an accurate and easily reproducible estimate of how much each % seeding in the biofilter is, can ensure results that are comparable to other treatments and experiments.

Getting an accurate estimate on the performance of the bio media donor before seeding (e.g., do capacity tests to determine oxidation rate). Having automatic feeding of an ammonia source could ensure more stability and might make it easier to do capacity tests and statistical analysis. Increasing the number of replicates per treatment could make it possible to do more testing for significant differences. Having only two treatments would make it easier to get more replicates for each treatment. The seeding % efficiency might not be the same depending on the size of

the chamber. It could be interesting to research if difference with the same seeding % affects the maturation process.

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7 Appendix

Appendix 1 Dredge function in R to calculate the best combination of variables for the model. Both are indicating that there is not a good model to choose, as the model with treatment included is far down the list.

```
Global model call: lm(formula = Ammonia_rate_mean ~ Treatment + M_mean2 + TS_mean2 +
    TT_mean2 + pH_mean + OXS_mean2 + OXT_mean2, data = DredArate,
    na.action = "na.fail")
---
Model selection table
      (Int)      M_mn2   OXS_mn2    OXT_mn2    TS_mn2     TT_mn2 Trt     pH_men df logLik  AICc  delta weight
19 16.25000            -0.014910                       -0.7395000                4 24.122 -34.5   0.00  0.335
83 22.00000            -0.022120                       -1.0310000      9.069e-02 5 27.098 -34.2   0.33  0.283
23 15.74000            -0.023660  0.0062280            -0.7024000                5 25.403 -30.8   3.72  0.052
3   1.00300            -0.010230                                                 3 19.717 -30.4   4.10  0.043
27 23.54000            -0.017310             0.101500 -1.1930000                5 25.149 -30.3   4.23  0.040
1   0.08517                                                                      2 17.810 -30.3   4.24  0.040
11  2.85900            -0.010670            -0.091150                           4 21.199 -28.7   5.85  0.018
17  8.73300                                                -0.4313000           3 18.772 -28.5   5.99  0.017
9   1.76600                                 -0.084310                           3 18.692 -28.4   6.15  0.015
20 16.38000 -7.348e-06 -0.015350                       -0.7437000                5 24.135 -28.3   6.26  0.015
2   0.07065  7.388e-05                                                           3 18.597 -28.2   6.34  0.014
35  1.45000            -0.015800                                     +           5 24.027 -28.1   6.47  0.013
```

```
Global model call: lm(formula = Nitrite_rate_mean ~ Treatment + M_mean2 + TS_mean2 +
    TT_mean2 + pH_mean + OXS_mean2 + OXT_mean2, data = DredNrate,
    na.action = "na.fail")
---
Model selection table
       (Int)      M_mn2   OXS_mn2    OXT_mn2    TS_mn2    TT_mn2 Trt    pH_men df logLik  AICc  delta weight
19  38.41000            -0.037530                       -1.75200               4 13.554 -13.4   0.00  0.209
65   2.18800                                                        -0.27970  3 10.998 -13.0   0.40  0.171
10   6.07300 3.068e-04                      -0.312300                          4 12.829 -11.9   1.45  0.101
11   7.78700            -0.027050           -0.273400                          4 12.487 -11.3   2.13  0.072
3    2.36600            -0.027430                                              3  9.611 -10.2   3.17  0.043
73   4.57600                                -0.138600                 -0.23350 4 11.621  -9.5   3.87  0.030
66   1.73800 1.332e-04                                                -0.22780 4 11.605  -9.5   3.90  0.030
67   2.79700            -0.012620                                     -0.21550 4 11.553  -9.4   4.00  0.028
2   -0.15020 2.759e-04                                                         3  9.169  -9.3   4.06  0.028
81  12.76000                                              -0.53390    -0.26380 4 11.379  -9.0   4.35  0.024
1   -0.09538                                                                   2  7.138  -8.9   4.45  0.023
9    5.45700                           -0.278400                             3  8.957  -8.9   4.48  0.022
21  33.16000                 -0.0181900            -1.57800                   4 11.155  -8.6   4.80  0.019
20  36.77000 1.213e-04 -0.030600                   -1.70300                   5 14.207  -8.4   4.98  0.017
5    1.07100                 -0.0131300                                       3  8.672  -8.3   5.05  0.017
69   2.19000                 -0.0002038                            -0.27760   4 10.998  -8.3   5.11  0.016
12   7.23900 2.010e-04 -0.016010           -0.297700                          5 14.087  -8.2   5.22  0.015
18  23.73000 3.074e-04                                 -1.19100               4 10.749  -7.8   5.61  0.013
83  32.75000            -0.030270                      -1.46900    -0.08203   5 13.881  -7.8   5.63  0.013
27  33.95000            -0.035890           -0.057400  -1.48000               5 13.614  -7.2   6.17  0.010
13   5.77000            -0.0112000 -0.244100                                  4 10.426  -7.1   6.26  0.009
23  38.37000            -0.039520  0.0013600           -1.74700               5 13.564  -7.1   6.26  0.009
74   5.49900 2.298e-04                      -0.237300                 -0.11110 5 13.446  -6.9   6.50  0.008
17  18.64000                                           -0.93420               3  7.796  -6.6   6.80  0.007
4    1.59300 1.510e-04 -0.019150                                              4 10.134  -6.6   6.84  0.007
15   9.01400            -0.045300  0.0123400 -0.307800                        5 13.184  -6.4   7.03  0.006
75   6.71800            -0.019510           -0.208300                 -0.11110 5 13.012  -6.0   7.37  0.005
26   2.06200 3.040e-04                      -0.345800  0.23340                5 12.869  -5.7   7.65  0.005
14   6.07900 2.910e-04           -0.0013640 -0.306400                          5 12.852  -5.7   7.69  0.004
7    2.51400            -0.032380  0.0033400                                  4  9.643  -5.6   7.82  0.004
35   3.12200            -0.036950                                     +        5 12.654  -5.3   8.09  0.004
```

Appendix 2 Oxygen changes for sump (A) and tank (B) between replicates throughout the experiment. Similar trend with oxygen saturation decreasing in the latter half of the experiment. Shows replicate 26 (T10) having a distinctly lower oxygen saturation, than the o