Comparative Assessment of Water Quality and Biofilter Performance in Recirculating Aquaculture Systems (RAS) Exposed to High and Low Particle Loading

Thesis for degree
Master of science in Aquaculture

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-*Your ego is not your amigo.*

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Abstract

Aquaculture is incorporating more and more recirculating aquaculture systems (RAS) to deal with challenges in the industry. However, technology used in today's intensive systems are relatively new, and as more facilities are being built every year, knowledge of how operational conditions affect the environment in the system is essential. When recirculating intensity in a RAS increase so does the need for particle removal, as particles tend to accumulate and creating good growth conditions for heterotrophic bacteria. In this study triplicates with individual intensive RASs were exposed to a normal (Low) or manipulated high particle loading to assess its effect on water quality in relation to the biofilter and biofilter nitrification performance. Results showed that an increased particle loading led to elevation of total particle surface area (TSA), microbial activity and increased ammonia concentration, indicating inhibited biofilter performance by heterotrophs. However, ammonia spiking trials revealed similar nitrification performance of biofilters both exposed to low and high particle loadings. Thus, implying that an increased particle loading in intensive RAS effects water quality negatively, but not necessarily biofilter performance. Bacterial environment in high particle load intensive RASs should be addressed in future studies to assess whether this can add considerable amounts of ammonium in addition to what the fish excretes, so that it may have to be considered during biofilter dimensioning.

Abbreviations

ANOVA – Analysis of Variance

AOB – Ammonia Oxidizing Bacteria

C/N-ratio – Carbon/Nitrogen-ratio

CCS – Closed Containment Systems

CO_2 – Carbon dioxide

COD – Chemical Oxygen Demand

COD_{diss} – Dissolved COD

COD_{part} – Particulate COD

COD_{tot} – Total COD

Cr^{6+} – Hexavalent chromium

DNA – Deoxyribonucleic Acid

FAO – Food and Agriculture Organization

FBBR – Fixed Bed Biofilm Reactor

FM – Final Mass

FT – Flow Through aquaculture system

H_2O_2 – Hydrogen peroxide

H_2S – Hydrogen sulfide

H_2SO_4 – Sulfuric acid

IM – Initial Mass

ISO– International Organization for Standardization

LMM – Linear Mixed effect Model

MBBR – Moving Bed Biofilm Reactor

Mili-Q – Purified water/Destilled water

MUW – Make-Up Water

$NaHCO_3$ – Sodium carbonate

NH_3 – Ammonia

NH_4^+ – Ammonium

NH_4Cl – Ammonium chloride

NH_4VO_3 – Ammonium metavanadate

NIVA – The Norwegian Institute for Water Research

NLS– Non-linear least squares

NO_2^- – Nitrite

NO_3^- – Nitrate

NOB – Nitrite Oxidizing Bacteria

NS– Standard Norge

O_2 – Oxygen

POC – Particulate Organic Carbon

PSD – Particle Size Distribution

R^2 – Coefficient of determination

RAS – Recirculating Aquaculture System

SA – Surface Area

SD – Standard Deviation

SEM – Standard Error of Mean

STR – Surface specific TAN Removal

T (T0-T7) – Sampling Day (0-7)

TAN – Total Ammonia Nitrogen

TOC – Total Organic Carbon

TSA – Total particle Surface Area

TSS – Total suspended solids

Tukey HSD – Tukey honestly significant difference

UV – Ultraviolet light

UVT – UV-transmittion

WF – Filtered Water

1. Introduction

1.1 The global aquaculture industry

Since the late 1980s, aquaculture has been the primary factor in the growth of the total fisheries and aquaculture production (Fig. 1), and in 2020, it contributed 49% of the total production of fisheries and aquaculture. In contrast, its share of the global seafood production was only 20% back in the 1990s. This considerable increase in contribution is the result of major changes in the industry, moving from small-scale to large-scale and intensive farming (Gutierrez-Wing and Malone, 2006, FAO, 2022). However, the average annual growth of the aquaculture industry has decreased from 9.5% to 3.3% over the last three decades. At the same time the production from capture fisheries has leveled off and the percentage of stock fished at biologically unsustainable levels has increased since the 1970s (FAO, 2022).

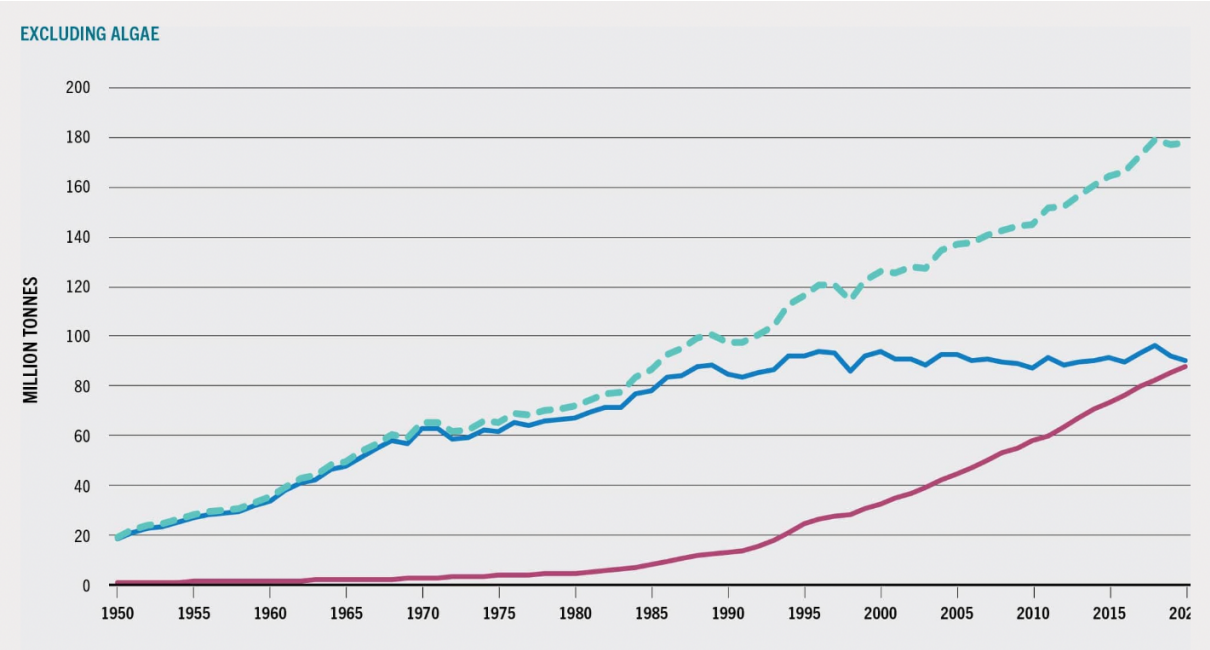

Fig 1. World capture fisheries and aquaculture production (excluding algae). *The red line represents aquaculture, the blue line represents fisheries and the dotted line total. Figure: FAO (2022).*

While the global seafood production shows stagnant trends, the global demand for aquatic food products is increasing. The global supplies of fisheries will not be able to meet this increasing demand of aquatic food. Aquaculture on the other side appears to have a significant potential to contribute (FAO, 2022). However, in order to keep up with the increasing demand the aquaculture industry will face significant challenges. The sector is dependent on using new production methods and species, as well as intensifying and modifying already used systems and production methods (Del Campo et al., 2010)

Around the globe several different farming methods are used to produce different aquatic species. Some are cultured in ponds, others in coastal areas and saltwater areas, often it comes down to the properties of the ecosystem in the given production areas and the characteristics of the animal. Due to its extensive tropical and subtropical landmass and access to freshwater, China is the world's largest producer of aquatic animals. Many freshwater species can be successfully farmed in their inland waters (Wang et al., 2015). Their most produced species is grass carp (*Ctenopharyngodon idellus*), and account for 11.8% of the global inland water aquaculture with a production of 5791.5 thousand tons in 2020 (FAO, 2022). Furthermore, Norway has an ecological advantage with their freshwater resources and coastal water properties to produce Atlantic salmon (*Salmo salar*). However, this is not the case in their neighboring country Denmark, which do not have the same advantage in their coastal ecology and a limited amount of freshwater which is needed in the production of smolt (*Salmo salar)*. These examples demonstrate how ecological factors affect where today's aquaculture is most advantageous and which species can be cultured.

On a global scale Atlantic salmon stands for 32.6% of the global finfish produced in marine and coastal waters. Most of this is produced in Norway and a significant amount in Chile (FAO, 2022). Olafsen et al. (2012), estimated a production potential of Atlantic salmon and Rainbow trout (*Oncorhynchus mykiss*) in Norwegian aquaculture that corresponds to a fivefold of the production done in 2010, by 2050. Despite desired growth, the Government points out that the industry is close to utilizing its full production capacity and current problems within the industry must be solved before the potential can be exploited (Meld.St.16, (2014-2015)). The most severe problems are related to traditional production in open net pens (Olafsen et al., 2012). This includes challenges related to salmon lice (*Lepeophtheirus salmonis*), waste production- and escapees from traditional net pen salmon farming. Concerns are also raised towards other aquaculture nations around the globe. These concerns are directed towards the impact global warming will have on the industry and the impact waste discharged from farms can have on its environment (Wang et al., 2015).

In recent years, several new production methods have been developed and tested out in Norway and other aquaculture nations. These methods aim to solve challenges and make the production potential of salmon possible. Among these are closed containment systems (CCS), offshore framing, and recirculating aquaculture systems (RAS). These production methods aim to treat the underlying challenges with traditional salmon farming in open net pens. This is done by separating the entire farming process from the natural environment it can affect and be affected by. RAS differs from the other production methods in that it can be virtually

independent of environmental characteristics in the production area, this offers some advantages: Food can be produced closer to its marked witch will have a big impact on carbon footprint, by limiting the need for transport (Ernst & Young, 2019) and seasonal variations such as temperature can be controlled throughout production to optimize growth. This allows RAS to produce a consistent volume year around close to the market, giving a competitive advantage over other production methods which are seasonal and sporadic (Ebeling and Timmons, 2012, Dalsgaard et al., 2013) Indoor production of aquatic animals in RAS has the potential to ensure a safe source of seafood, free from chemicals and heavy metals, meeting the increasing concern towards food safety from consumers (Ebeling and Timmons, 2012, FAO, 2022).

Recirculation limits water use, and disinfection of intake water ensures biosecurity. Collection of excess feed and feces prevents impact on the environment, while it also enables a separate industry through waste-water treatment (Ebeling and Timmons, 2010). Still, costs of building and maintaining RASs are high. They are complicated systems where biology and technology must work together. Constant monitoring with alarms and back-up systems are imperative to ensure that water treatment processes function as intended and provide good water quality. Insufficient performance of the water treatment processes in a RAS can lead to poor water quality and resulting challenges. Several cases of acute fish mortality have been recorded due to the poisonous gas hydrogen sulfide (H_2S), which can occur in anaerobic zones in the RAS loop (Rojas-Tirado et al., 2021, Bergstedt and Skov, 2023). Insufficient aeration of RAS-water and subsequent high CO_2 levels has also been recorded to have a negative impact on salmonids and in some cases cause mortality (Good et al., 2010, Mota et al., 2019). Another challenge of great interest is what high particle loading do to the water quality in a RAS. In Badiola et al. (2012) biofilter and solid removal was recognized as the two most difficult devices to manage within a RAS, and they influence each other. High organic loads can lead to bad performance of the different water treatment components, especially the biofilter (Zhu and Chen, 2001, Ling and Chen, 2005, Michaud et al., 2006). Good water quality is crucial to reduce cost and risk in a RAS.

1.2 The development of recirculating aquaculture systems (RAS)

Historically, the development of commercial RASs started with simple systems designed to breed freshwater species with a relatively high tolerance to poor water quality (Goldman et al., 1974, Naegel, 1977, Helfrich and Libey, 1991, Martins et al., 2010). In previous decades, several different recirculating systems have been designed and researched.

Commercial facilities have been opened with great ambitions but then later went out of business (Ebeling & Timmons, 2012). In these early systems there was little technology specifically developed for aquaculture and most solutions were obtained from wastewater treatment concepts and technology (Helfrich and Libey, 1991, Ebeling and Timmons, 2010). Several have also had a trial-and-error approach, such as model rainbow trout farms in Denmark. Here the aim was to provide documentation on specific discharges, how to- reduce the use of fresh water, increase the retention of organic matter and other nutrients as well as other relevant environmental parameters (Jokumsen and Svendsen, 2010). In later years, recirculating systems have been specifically engineered to meet the biological needs of producing fish in a land based aquatic system (Lekang, 2007). Today there are several established companies that can offer equipment and systems that are specifically adapted to the commercial production of different fish species in RAS's. Nile Tilapia (*Oreochromis niloticus*), striped bass (*Morone saxatilis*), European eel (*Anguilla Anguilla*), barramundi (*Lates calcarifer*) and European lobster (*Homarus gammarus*) are examples of other species that are being successfully cultivated in intensive RAS's (Ebeling and Timmons, 2012, Dalsgaard et al., 2013).

Although many species are produced in recirculating systems, the majority of existing systems produce salmon smolt. New systems are being built and planned, designs and solutions continue to improve and are becoming cost competitive with traditional systems used for smolt production, like flow through systems (FT) (Tidwell, 2012). Analyzes and economic studies vary greatly in their findings about the competitiveness of salmon production in RAS. There are many factors at play: Some studies believe that the fish can be produced more cheaply in RAS, while others believe that capital costs are too high compared to traditional production methods. This uncertainty suggests that there is a lack of data within the production of salmon in RAS facilities and that the experience is spread among several international players with a relatively short history (Tidwell, 2012, Bjørndal and Tusvik, 2020).

Nevertheless, RASs receive significant investment, and most commercial actors incorporate it into their production chain in one way or another. (Hilmarsen et al., 2018, Ytrestøyl, 2022). Some commercial producers aim to do the whole production cycle of Atlantic Salmon in recirculating systems. They are mainly located in countries that have a profitable consumers market, but don't have the natural environmental conditions to produce the species in their waters. By doing this, salmon transportation-related emissions to major markets can be greatly reduced (Ernst & Young, 2019).

A large share of the Atlantic salmon smolt in Norway is produced in RAS, this production method enables rapid growth in early life stages, before transfer to open net pens in

the sea (Dalsgaard et al., 2013). Over the past ten years, the size of smolt transferred to the open net pens at sea has increased steadily, and studies indicate that producers wish to increase both their production of larger smolt, and the number of fish transferred (Ytrestøyl, 2022). The major motivation for producing larger smolt is to shorten production time at sea, as this can have numerous advantages over conventional farming. Shorter production time at sea can lead to fewer salmon lice treatments. Lice treatments result in increased mortality, reduced fish welfare and growth in addition to being very costly (Gismervik, et al., 2022). The industry seldom grants new licenses, and current permits restrict growth in production volume, better utilization of existing licenses at sea is therefore another driving factor (Bjørndal and Tusvik, 2020, Ytrestøyl, 2022). Better utilization of each permit is achieved with a shorter production phase at sea, as this allows more production cycles per unit of time (Hilmarsen et al., 2018). Another motivation is that a shorter production time in the sea exposes the salmon to less infectious fish diseases, which is one of the main reasons for high mortality in the sea phase, potentially resulting in better fish welfare and greater output (Ytrestøyl et al, 2020, Walde, 2022). It has also been shown that larger post smolts can be more robust and handle transfer to sea better than smaller smolts (Terjesen et al., 2008, Øvrebø et al., 2022).

1.3 Treatment of water in RAS

An increasing degree of water recycling entails an increasing degree of complexity in a recycling system as more water treatment processes needs to be included (Fig. 2). Degree of recirculation is a term that has several different definitions, Fjellheim et al. (2016) presents three common ways of defining recirculation:

- Recirculation degree in % = (amount of water to rearing tank per hour/ (added water per hour/amount of water to rearing tank per hour)) x100
- Replacement per day in % = (added water per day/total water volume in facility) x100
- Replacement per day per kg feed = added water per day/daily feed consumption.

Knowing the amount of feed given to the fish is a crucial component to dimensioning and comprehending the requirement for water treatment, the first two definitions do not account for this. The last definition can be used to define the type of aquaculture system, where a system with 20 000 – 40 000 L make up water (MUW)/kg feed is defined as a FT-system, and a system

with very little MUW/ feed, 50-400 L/ kg, can be defined as an intensive RAS (Martins et al., 2009, Pedersen, 2022).

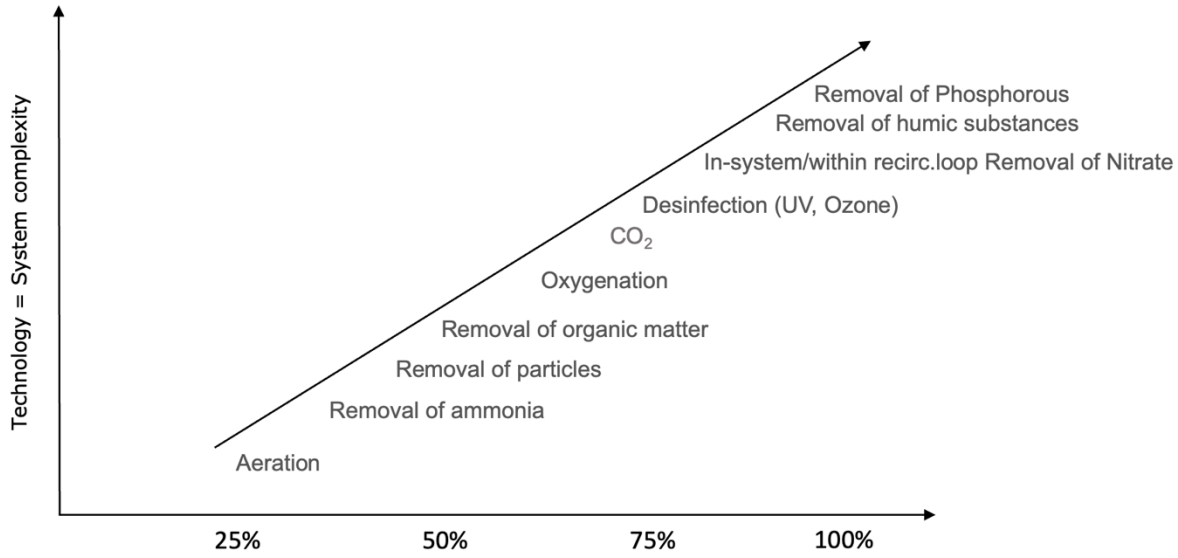

Fig 2. The relationship between degree of recycling and complexity*. Inclusion of more water treatment processes to provide an increased degree of recycling increases the need for technology which in turn increases complexity. The x-axis represents degree of recirculation in %, y-axis represents system complexity. Figure: modified after (Fjellheim et al. 2016 and Pedersen, P. B., 2022)*

Designs, order, and inclusion of the different components in a RAS varies between the suppliers in the business. However, most modern intensive RASs have some form of particle removal, CO_2 removal, transformation and detoxification of nitrogenous compounds excreted by fish, oxygen supply and some kind of disinfection (Fjellheim et al. 2016). Figure 3 shows fundamental steps and a possible order of treatments processes in a modern RAS.

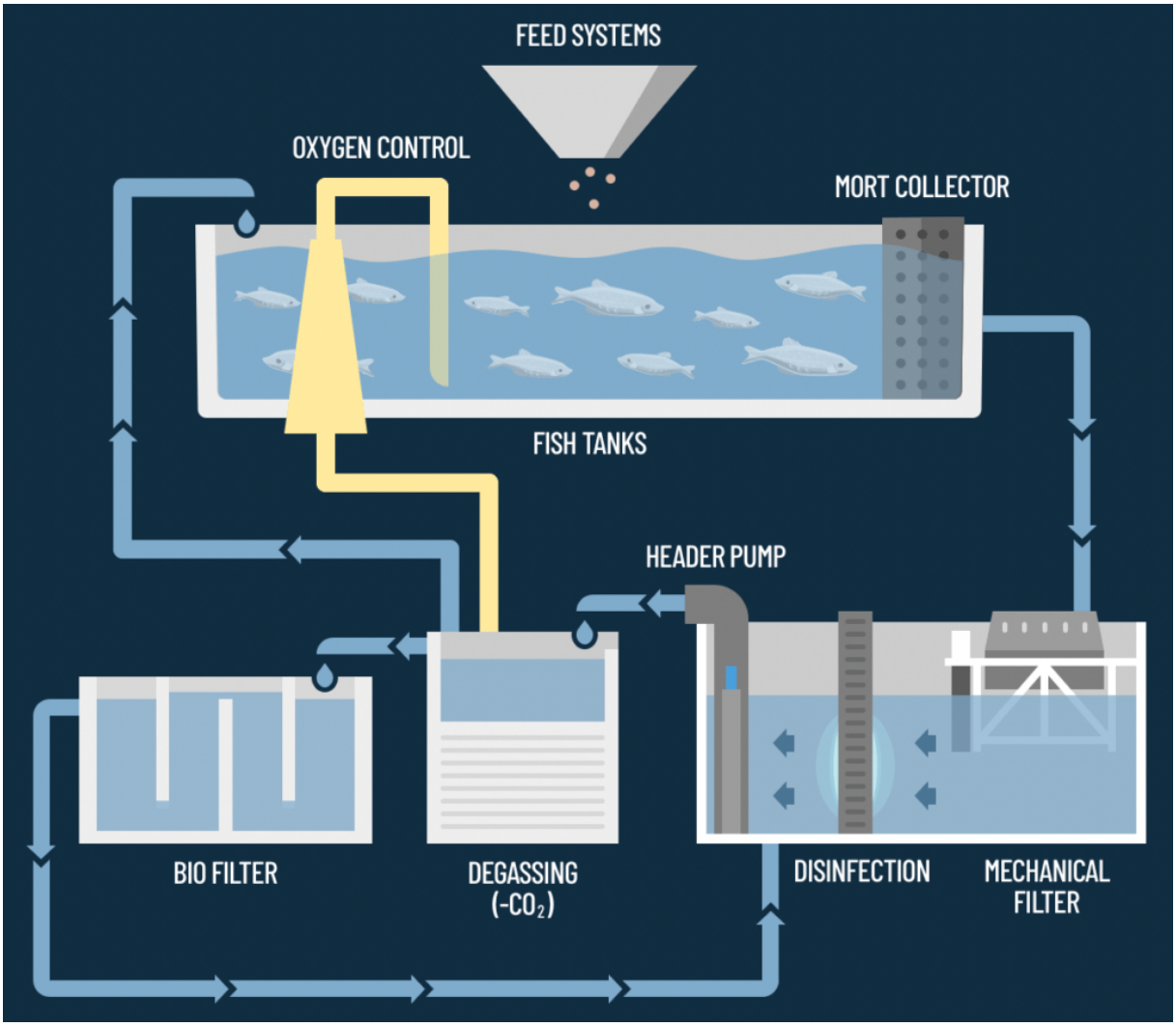

Fig 3. Sketch of a typical recirculating aquaculture system. *Water in the circulating loop is purified through several steps. The water treatment process includes mechanical filtration for particle removal and other organic matters, disinfection, biofilters (removal of nitrogen compounds, TAN and NO_2^-), degassing (removal of CO_2) and oxygen supply. Figure: Derwent Group (2019)*

Organic particles in a RAS are mainly supplied directly to the water in the fish tanks through feed with resulting feces and uneaten feed but can also originate from biofilm flocks and other slough off (von Ahnen et al., 2015, Pedersen et al., 2017). In the water, the particles occur as dissolved and particulate. If not removed, particle accumulation can lead to a poor- biofilter performance, fish health, and an increased oxygen consume and CO_2- and ammonia production (Tidwell, 2012, Pedersen et al., 2017). Larger particles (>100 µm) are often removed with a drain that takes advantage of their sinking properties. This can be done with a swirl separator, radial flow clarifier, settling basin or filters with a large mesh size (not shown in Fig.3). Smaller particles (30-100 µm) are then removed by mechanical filtration, usually drum filters or disc filters, which retain suspended solids larger than the mesh size of the screen aperture (Fig. 3).

To prevent obstruction filters are backwashed, allowing a continuous flow of water and collection of sludge (Dolan et al., 2013). Some modern facilities also include protein-skimmers and/or ozone which make it easier to filter out particles. Ideally these treatment methods are included to improve water quality by reducing color and by removing both dissolved and fine particulate materials (Chen et al., 1994, Figueiras Guilherme et al., 2020). However, this is not included in many RAS's and fine and colloidal particles tend to accumulate (Barrut et al., 2013, Pedersen et al., 2017).

Ammonia is excreted as a waste product from nitrogen metabolism in rearing fish. In a RAS, excreted ammonia exists in two states, ammonia (NH_3) and ammonium (NH_4^+), mainly dependent on pH but also salinity and temperature (Lekang, 2007). The sum of NH_3 and NH_4^+ is called Total ammonia nitrogen (TAN). In the biofilter TAN is converted to nitrate (NO_3), via the intermediate product nitrite (NO_2^-), this process of oxidation is called nitrification. In intensive RAS production, this procedure is crucial because of the toxicity of NH_3 and NO_2^- and maybe NO_3^-. The toxicity of un-ionized ammonia (NH_3) varies widely between different species but is generally toxic at very low concentrations. A common upper safety limit of NH_3-N for salmonid species in RAS is 0.0125 mg/L (Ebeling and Timmons, 2010), while Becke et al. (2019) found that concentration up to 0.05 mg/L gave minor effects on the physiology of rainbow trout. Safety limits of un-ionized ammonia for salmonid species in RAS remain a controversy (Daoust and Ferguson, 1984, Meade, 1985, Rosten et al., 2004). However, the concentration of ionized and un-ionized ammonia mainly depends on pH, pH will decide whether a given TAN ($NH_3 + NH_4^+$) value is toxic. A rule of thumb value set by Ebeling and Timmons (2010) for cold water fish is 1 mg TAN/L, while the indicative limit value from the Norwegian Food Safety Authority is set at 2 mg TAN/L (Fjellheim et al., 2016). It is crucial to know your intended operational pH value as this will determine whether a TAN value gives toxic NH_3 values.

The intermediate product from the nitrification process, nitrite, can be toxic to fish due to its active uptake over the fish's gills in competition with chloride. Nitrite in the blood affects the oxygen carrying capacity of hemoglobin by oxidizing iron in the hemoglobin molecule causing methemoglobin with a characteristic brown color (Ebeling and Timmons, 2010). Elevated levels of nitrite are caused by lack of biological oxidation to nitrate and can indicate upcoming failure of a biofilter (Lekang, 2007). Limit values for nitrite consider that there is more chloride present in salt water than fresh water and will therefore be higher in seawater.

Nitrate levels in RASs are mainly controlled by the MUW, NO_3 concentrations can therefore indicate the operational intensity of the system (Ebeling and Timmons, 2010,

Fjellheim et al., 2016). Some intensive recirculating systems may need denitrification, where nitrate is converted to nitrogen gas, as it has been recorded chronic welfare- and health problems in salmonids due to high levels of nitrate in freshwater (Davidson et al., 2014).

Both TAN- and Nitrite removal are biological processes that mainly take place in biofilms that are formed on surfaces in the biofilter (Leonard et al., 2000). This biological process depends on a range of factors including availability of oxygen, alkalinity and concentration of TAN and nitrite. Depending on the concentration of TAN and nitrite, and the half-saturation constant of the bacteria (K_s), nitrification processes in a fully active biofilm follows 0' and 1' order kinetics (Ling and Chen, 2005, Prehn et al., 2012, Kinyage et al., 2019). When an active biofilm is fully penetrated and entirely saturated due to the substrate concentration, the removal rate of TAN and Nitrite are independent of the substrate concentration. Under these conditions the nitrification process follow 0' order kinetics, meaning that the substrate removal over time is maximized and thus linear (Fig. 4). At low substrate conditions, the nitrification process in an active biofilm can be described by 1'order kinetics. Under these conditions removal of TAN and Nitrite are limited by the substrate concentration and are thus exponential (Fig. 4). Removal rates of TAN can be calculated as surface specific TAN removal (STR; g TAN/m^2/day). The STR under 0'order conditions are described by the 0'order reaction rate constant (k_{0a}), this constant can be found through linear regression of TAN removal (g TAN/m^3/day), multiplied by the water volume in the biofilter (m^3), divided by the total surface area of the biofilter (m^2). As 0'order conditions are independent of TAN concentrations the STR is constant and STR = $k_{0a.}$ The 1'order reaction rate constant (k_{1a}) can be found by exponential regression of TAN removal (/day), multiplied water volume (m^3), divided by surface area of biofilter (m^2). STR under 1'order conditions can be calculated as $k_{1a} \cdot TAN\ concentration$. The relationship between 0'order and 1'order rate constants can be described with the half saturation constant of the bacteria (K_s); $k_{0a} = k_{1a} * K_s$. The transition between 0'order and 1'order TAN removal kinetics occur at a TAN bulk concentration of $2 \cdot K_s$, this is usually around 1 mg TAN/L (Prehn et al., 2012, von Ahnen et al., 2015).

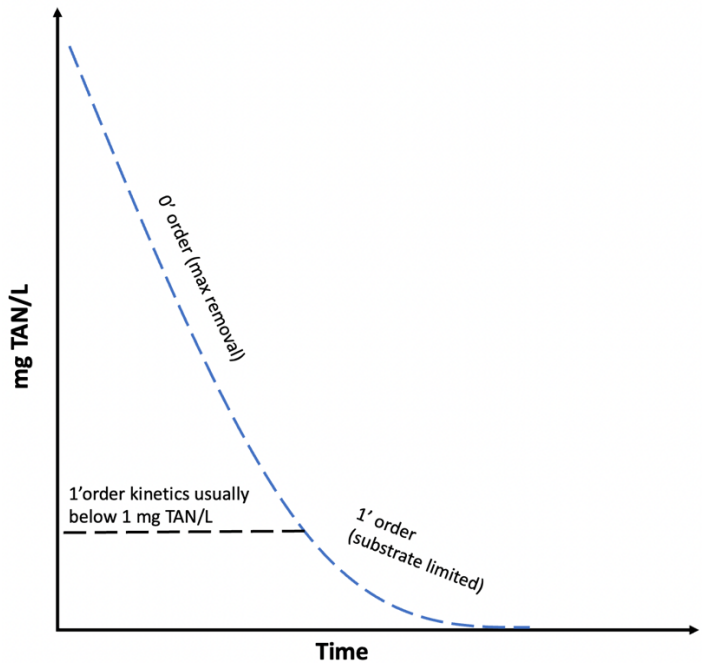

Fig 4. Nitrification kinetics in bio-media biofilm. *The figure shows TAN removal over time based on its availability in a spiking event where ammonia is added at time = 0. When the biofilm is fully saturated TAN removal follows 0'order kinetics and the removal is constant over time (linear), under these conditions' removal is maximized and not substrate dependent. At lower concentrations (usually bellow 1 mg TAN/ L) removal is dependent on the TAN concentration, substrate limited and follows 1'order kinetics (exponential). Figure: modified after (Pedersen, L-F. 2022).*

Biofilters provide a large surface area compared to volume so that nitrifying bacteria can settle and drive the nitrification process. Submerged bioreactors are widely used biofilters, the two most commonly used in production of salmon smolts are fixed bed bioreactors (FBBR) and moving bed bioreactor (MBBR) (Fjellheim et al., 2016, Pulkkinen et al., 2019). The bio-media (bio-carriers, biochips) in an MBBR is continuously upwelled by aeration through the biofilter water column. In a FBBR bio-media is static (fixed) in the reactor and water passes by. A FBBR typically requires more maintenance compared to an MBBR, as the latter is considered "self-cleaning" due to the biochips' ability to continuously scrape against each other. A disadvantage of this "self-cleaning" is that it can add more organic matter to the RAS-water as the scraping adds biofilm (organic matter) to the water (Fernandes et al., 2017).

The large surface area in the biofilter is provided by bio-media in the biofilter where nitrifying bacteria can form a thin biofilm. An ideal biofilter maximizes the specific surface area of media per unit of volume, the greater the surface area the more bacteria can grow and remove ammonia (Lekang, 2007). Dissolved nutrients and oxygen are transferred by diffusion into the biofilm, containing the two groups of nitrifying bacteria: Ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB). They are chemosynthetic autotrophic bacteria and derive their energy from ammonia and nitrite respectively, carbon dioxide is their primary

carbon source and they also require oxygen to grow (Hagopian and Riley, 1998). In biofilters, nitrifying bacteria coexist with heterotrophic bacteria, that make up 70-90% of the bacteria in RAS, which also consume oxygen (Rurangwa and Verdegem, 2015, Hüpeden et al., 2020). As a result of their significantly faster rate of growth, heterotrophic bacteria will dominate in competition for oxygen and space. Submerged biofilters will consume oxygen, due to both nitrifying- and heterotrophic bacteria, and release CO_2, produced by heterotrophic bacteria. As high concentrations of CO_2 have a negative effect on fish and oxygen is used in the fish's metabolism, water from the biofilter should be aerated for CO_2 and oxygen added before it is returned to the tanks (Ebeling and Timmons, 2010, Fjellheim et al., 2016).

Carbon dioxide is a limiting factor for production in RAS. It is introduced to the system by metabolic processes in fish and heterotrophic bacteria. In an intensive production where water exchange is limited and stocking densities are high, dissolved CO_2 will accumulate if not vented (Lekang, 2007). Degassing of CO_2 can be done in several different ways, trickling filters, waterfalls, and air-stones and surface agitation, they all have in common that they try to get the largest possible contact surface between air and water, to have the most effective venting of CO_2. Fresh air, which has a low concentration of gas phase CO_2, is pumped directly into the water mases or through the aeration system and creates a driving force where dissolved CO_2 in the water seek to create equilibrium with its concentration in the air. This driving force can transfer dissolved CO_2 in water into gas phase. The effectiveness of degassing increase with the air/water ratio and the concentration of carbon dioxide in the water which increases with decreasing pH (Fjellheim et al., 2016). Oxygenation of the water, before it is led back to the tanks, is often done by oxygenation cones. Oxygen cones add oxygen to the water by keeping oxygen bubbles in suspension until they are dissolved in the water (Ebeling and Timmons, 2010).

UV-light and ozone are commonly used as disinfection methods to maintain water quality and prevent introduction and spread of disease in RASs. UV light is typically applied through a sterilization chamber (Fig. 3) that the water passes through before being recirculated back into the system or in the intake water before it is introduced to the circulation loop. UV-light works by emitting short-wavelength radiation that damages the DNA of microorganisms (Chevrefils et al., 2006, Gullian et al., 2012). The damage the radiation causes stops replication and normal function. Ozon on the other hand breaks down organic compounds and damage cell walls of bacteria. It reacts very quickly with organic compounds or microorganisms and is then oxidized to harmless oxygen (Ramseier et al., 2011, Gonçalves and Gagnon, 2011). Both

methods have limitations and advantages (Ebeling and Timmons, 2010), that will not be discussed further.

A pump sump (not shown in Fig. 3) is a water reservoir, and this is where RAS-water is stored, often after it has been through the different treatment processes. This is a large chamber that is the lowest point in the circulation loop and where water is lifted back to the rearing tanks by pumps (Ebeling and Timmons, 2010).

1.4 Suspended solids and water quality in RAS

As mentioned in previous chapters, RAS has become increasingly prevalent in the aquaculture industry due to various factors, including its low water consumption. However, as the recirculation intensity of a RAS increases more particles tends to accumulate (Pedersen et al., 2017). The overall water quality in an aquaculture system is considered to be negatively affected by suspended particles (Ray et al., 2010, Pedersen et al., 2017). Accumulation of suspended solids in a RAS provide energy, carbon and surface area for opportunistic fast-growing heterotrophic bacteria which can have a negative effect on fish and the slower growing nitrifying bacteria's nitrification efficiency (Guerdat et al., 2011, Fjellheim et al., 2016, Pedersen et al., 2017). To create an environment where treatment processes work as they are supposed to and yields their ultimate performance, it is essential to understand how suspended particles affect a RAS. Understanding its effect can thus help to create an ideal ecosystem for aquatic animals, positively affecting fish health and economy.

The abundance of organic particles in a RAS are measured by various methods. Turbidity, total suspended solids (TSS), particle size distribution (PSD), chemical oxygen demand (COD), total and particulate organic carbon (TOC and POC) and UV transmission (UVT) are different assessments used to determine the abundance and properties of organic matter in a water sample (Pedersen et al., 2019). In the present study, TSS was used to set the operational conditions for the experiment, high and low particle load. TSS was also measured in combination with PSD analyzes of water going in to and out of the biofilter.

Measurements of PSD enables calculations of the total particle surface area (TSA) available for heterotrophic growth. Small particles represent a large surface area to volume ratio, accumulation of fine organic particles will therefore provide a substantial surface area for heterotopic bacteria to settle on (Blancheton et al., 2013). The relationship between different size classes in a PSD data set can be described by the ß-value: The ß-value express whether particles in a sample have a predominance towards smaller or larger particles (Patterson et al., 1999, Pedersen et al., 2017). A high ß-value indicates a predominance of small particles. As

larger particles are removed by mechanical treatments process in a RAS, finer particles tend to accumulate and aquaculture systems commonly have a ß-value in the range 2-5, depending on the filtration processes (Patterson et al., 1999, Fernandes et al., 2017, Pedersen et al., 2017). The ß-value only explains how the particles in a system are distributed in terms of size classes, but not the quantity. Therefore, ß-values should be seen in the context of the total number of particles, which can be estimated through the PSD analysis as particles per mL, through COD measurements as mg oxygen consumed per liter or with a TSS measurement as mg suspended solids per liter.

Assessment of microbial activity can be used to provide an insight on effects and interaction of different microbial processes in a RAS (Pedersen et al., 2019). Heterotrophic bacteria can colonize suspended particles in RAS-water because of the continuous supply of bioavailable substrate (Pedersen et al., 2017, Rojas-Tirado et al., 2018). If the particle load is high enough, the nutrients in the water can also support free-living heterotrophic bacteria (Michaud et al., 2006, Pedersen et al., 2017, Pedersen et al., 2019). Measurements of microbial activity in RAS-water may provide information whether the particle load is substantial enough for free-living heterotrophs and/or particle associated bacteria. Pedersen et al. (2017) found a strong linear correlation between TSA and bacterial activity when operating recirculating systems at intensities, 23, 3.2-4.3 and 1 m^3 MUW/ kg feed. The correlation ceased to exist at the highest operational intensity, 0.32 m^3 MUW/kg feed. Particles smaller than 5 µm in diameter, however, were not characterized and may have represented a significant surface area that supported bacterial activity. It has been shown that the distribution of particles in aquaculture systems can peak in size classes below 5 µm, these particles will therefore constitute a significant amount of the TSA (Patterson et al., 1999, Patterson and Watts, 2003). In the present study, particles were characterized down to a diameter of 0.8 µm in combination with analyzes of microbial activity.

Organic particles in water have a major impact on the microbiome and therefore also the key water treatment process that takes place in the biofilter, nitrification. The competition between chemoautotrophic nitrifying bacteria and heterotrophic bacteria for oxygen, nutrients, and space inside the biofilm on the bio-media is escalated by increasing amounts of biodegradable organic carbon (Michaud et al., 2006). Several studies have showed negative effects on nitrification caused by availability of organic carbon and the following growth conditions for heterotrophic bacteria (Ling and Chen, 2005, Michaud et al., 2006, Guerdat et al., 2011). At an organic carbon/inorganic nitrogen ratio (C/N) of 0 the nitrifying and heterotrophic bacteria seem to coexist on the outermost layers of the biofilm (Okabe et al.,

1996, Michaud et al., 2006). However, at a C/N ratio of 1.5, Okabe et al. (1996) found that nitrifiers only existed in the inner layers of the biofilm while the outer layers were dominated by heterotrophs. This suggests that the availability of oxygen and the diffusion of ammonia are reduced for the nitrifiers, causing a negative effect on the nitrifying efficiency. In an operational RAS, feeding and their waste products will always be present, a C/N ratio of zero will not exist, and heterotrophic bacteria and nitrifiers need to coexist to some degree (Barrut et al., 2013).

Knowledge of organic particles, their quantity, and characteristics in RAS, is crucial to optimize the nitrification process in biofilters and make the production method efficient and economical. This study is a part of a larger project that aims to examine the effects high particle loads have on intensive production of Atlantic salmon smolt in a RAS. The present work package focuses on water quality, particle associated microbiota and nitrification performance.

1.5 Aim and objective

To establish an optimal RAS (Recirculating Aquaculture System) environment, ensuring the effective functioning of treatment processes and maximizing their performance, it is crucial to comprehend the impact of suspended solids. This understanding plays a pivotal role in creating an ideal ecosystem for aquatic animals, ultimately enhancing fish health and economic viability. This study investigates the influence of elevated particle loads on different water quality parameters and further the effect on biofilter performance in experimental RASs. To investigate water quality, particle concentration, particle size distribution, TSS, COD, microbial activity, and nitrogen compounds (TAN, NO_2^-, and NO_3^-) were measured and analyzed. To assess particle loadings effect on biofilter nitrification performance, bio-media were spiked with ammonia to investigate the 0'order and 1'order TAN removal kinetics. By investigating these parameters, valuable insights into how particle loads influence the water quality and performance of a biofilter could be gained. Note that the present study only addresses water quality and biofilter performance. The complete project cover topics like particle loadings effect on smoltification, and fish welfare and -health (Hess-Erga, O., in preparation). Based on the aim of the thesis the following hypothesis were developed:

H01: Elevated particle load will not result in an elevated total particle surface area (TSA) in intensive RAS.

HA1: Elevated particle load will result in an elevated TSA in intensive RAS.

H02: High particle load will not affect microbial activity in an intensive RAS.
HA2: High particle load will affect microbial activity in an intensive RAS.

H03: Microbial activity at the inlet and outlet of the biofilter will show the same tendency at high and low particle loading.
HA3: Microbial activity at the inlet and outlet of the biofilter will not show the same tendency at high and low particle loading.

H04: High particle loads will not influence the concentration of inorganic compounds (nitrogen compounds) in the RAS-water.
HA4: High particle loads will influence the concentration of inorganic compounds (nitrogen compounds) in the RAS-water.

H05: Nitrification performance of the biofilter will not differ at high and low particle loadings.
HA5: Nitrification performance of the biofilter will differ at high and low particle loadings.

2. Material and methods

2.1 Experimental facilities

The project was carried out at Marineholmen RASLab (Bergen, Norway) in six of their RAS modules. Water samples from the experimental systems were brought to the laboratories of The Norwegian Institute for Water Research (NIVA) in *Merdkantilen*, (Bergen, Norway) for further analysis.

2.2 System setup

The water treatment units at RASLab consisted of Alpha- Aqua A/S's (Esbjerg, Denmark) ALPHA NanoRAS, each coupled with one fish tank of 1.26 m^3 followed by a 464 mm diameter swirl separator. The water treatment unit (1.5 m^3) comprised of a 40 µm drum filter followed by a moving bed biofilm reactor (MBBR) of 0.58 m^3, a trickling degassing chamber and a pump sump (Fig. 5).

The bio-media (KSK Saddle Chips 1.0, KSK Aqua Aps, Skive, Denmark) used in the systems had a surface area of 625 m^2/m^3. The media was matured for 2-4 weeks before they were used in the experiment in freshwater or brackish water maturation tanks fed NH_4Cl (CAS nr. 12125-02-9, Hjelle Kjemi, Bergen, Norway) and $NaHCO_3$ (CAS nr. 144-55-8, Hjelle Kjemi, Bergen Norway) daily. Each MBBR was stocked to 65%, giving a total surface area of 236 m^2. The trickling degassing chamber featured a purified grate with a shallow water column, allowing for aeration underneath the grit. The pump sump was placed under and after the degasser. Water quality parameters such as salinity, dissolved oxygen, temperature, and pH were all automatically controlled in each system (Georg Fischer AS, Rud, Norway). A multiprobe handheld device (WTW Multi 3620 IDS, Xylem, Washington, DC, USA) equipped with a salinity (Tetracon 925), temperature, dissolved oxygen (FDO 925 optical oxygen IDS sensor), and pH (VWR pH pen) was used to check the level of the control box every day. Oxygenation was done through oxygen cones before water entered back to the tanks.

Initially, each system was stocked with 39.5g Atlantic salmon to a density of 7.7 kg/m^3. The fish was fed with Skretting Nutra RC 2- or 3-mm pellets from a feeder placed on top of the tanks. Feeding was done daily and on demand with 15% more feed than what was anticipated based on RASLab feeding tables. The water flow rate to the fish tanks was maintained at 55.9 ± 1.8 L/h, corresponding to a hydraulic retention time of 18 hours. Control of water intake was automated, relying on salinity setpoints determined for each trial phase. pH and alkalinity

adjustments were achieved through the automatic dosing of a $NaHCO_3$ solution. RASs were run at 12.3 ± 0.8 °C, under 93.0 ± 5.6 % O_2 saturation and a pH ranging from 6.89 - 8.18.

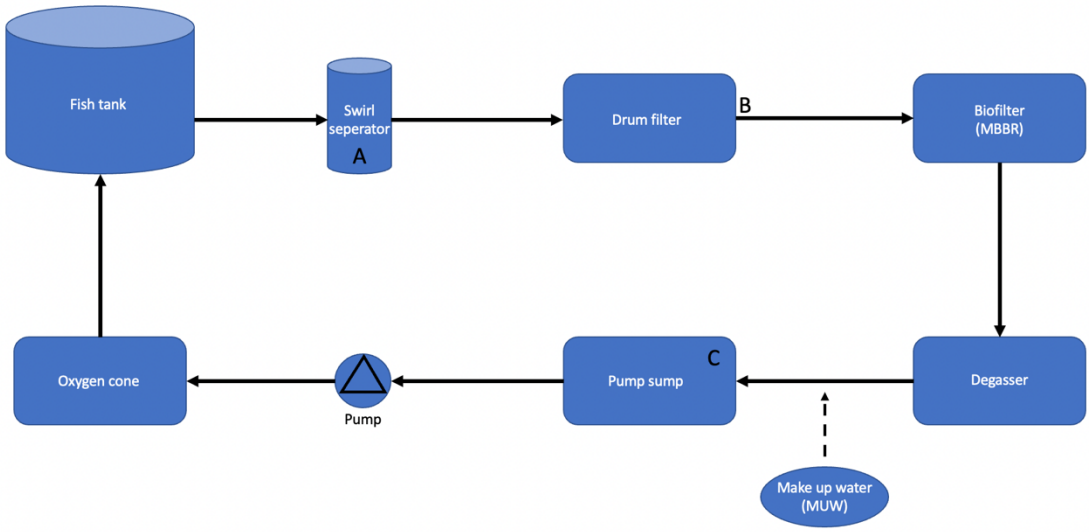

Fig 5. The process of water treatment within one RAS module at Marineholmen, RASLab (Bergen, Norway). *Make-up water (MUW) and recirculated water are purified through several different water treatment processes. The MUW is disinfected with UV-light at the inlet and added in the pump sump. The MUW is then added to the recirculated water. Purification of the recirculated water includes control of solids with a swirl separator and mechanical filtration (drum filter), removal of TAN and NO_2^- (MBBR), CO_2 removal (degassing) and oxygen supply (oxygen cones). A-C indicates where water samples were collected during the experimental period. Figure: modified after (Fjellheim et al., 2016).*

2.3 Experimental design

The experiment was carried out from August 9th, 2022, until December 6th, 2022. Two different particle loads were tested: Low concentration of total suspended solids (TSS; 1-2 mg TSS/L) and High TSS (12-15 mg TSS/L). The treatments were run in triplicate experimental-RASs (Fig. 6). Operational conditions for salinity were 1-2 ‰ the first 71 days before changing bio-media and increasing salinity to 15-18 ‰ for remaining experimental period (Fig 6).

Elevated levels of TSS were achieved by collecting waste (only feces, uneaten pellet was removed) from the swirl separators and adding it back to the pump-sump, once a day, in the high TSS treatment. Also, the backwash from the drumfilters were returned to the pump sump. The control group (Low) were operated without any organic load addition, apart from feed. Make-up water (MUW) was added after the degasser in the pump sump. All systems were generally operated with the same recirculating intensity (L MUW/kg feed) and feed loading (kg feed/ m^3 MUW), but extra MUW was added when necessary.

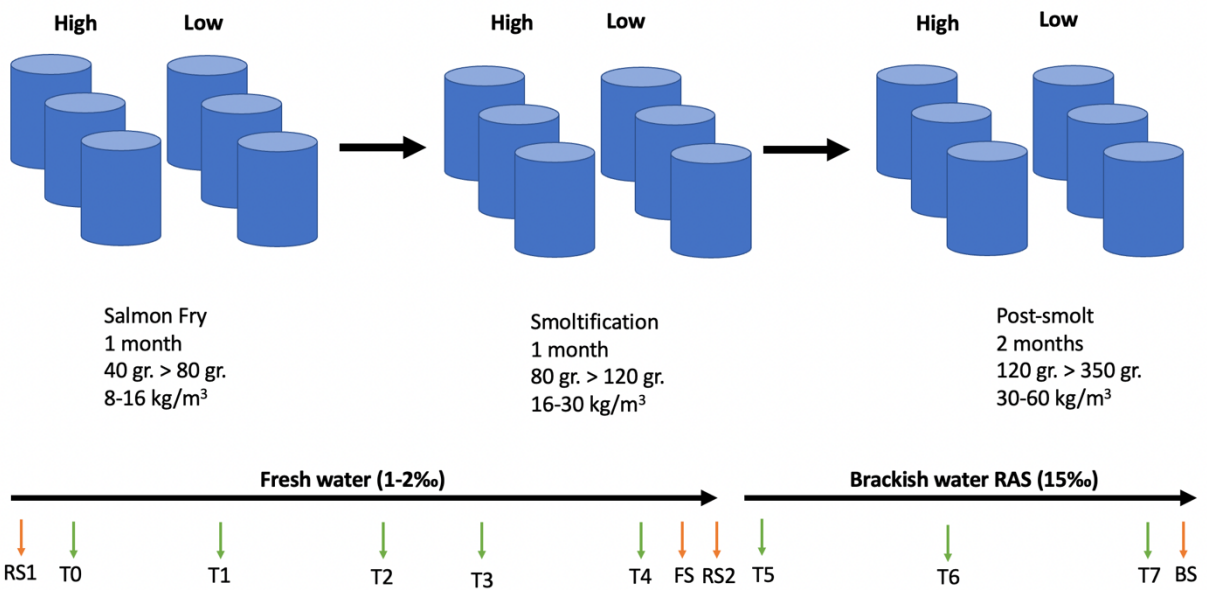

Fig 6. Experimental design. *The blue cylinders represent the triplicated rearing tanks of the six separated RASs operated under two different conditions of particle loading, high 12-15 mg TSS/L and low 1-2 mg TSS/L. T0-T7 represents sampling times through the two different levels of salinity, fresh water 1-2‰, and brackish water RAS 15‰, used during the project. RS1(Reference spiking 1), FS (Freshwater spiking), RS2 (Reference spiking 2) and BS (Brackish water spiking) shows when the different ammonia spiking experiments were conducted. Density, life stage and intended weight of the Atlantic salmon bred in the tanks is also included.*

2.4 Sampling protocol

Parameters used to monitor operational conditions and to assess water quality are summarized in Table 1, along with sampling point in the recirculation loop, their treatment, frequency and procedure.

2.4.1 Samples collected to monitor operational conditions

During the entire experimental period, total suspended solids (TSS) were regularly monitored in water samples obtained from the swirl separator (Fig. 6) in each tank. This monitoring was conducted 1-2 times per week to effectively track and assess the particle loading in the system.

2.4.2 Samples collected on sampling days

During the experimental period samples were collected at 8 different occasions, 5 samplings in the freshwater phase (T0-T4) and 3 in the brackish water phase(T5-T7) (Fig. 6). During this sampling, data on fish performance was also collected for other work packages in the project. The water quality parameters measured and calculated were: PSD, particle concentration, TSA, ß-value, microbial activity, COD, TAN, NO_2^-, and NO_3^- (Table 1). All

these parameters were measured at the inlet and the outlet of the biofilter. In addition, microbial activity was measured in bio-media from the biofilters. At the first sampling date, T0, only microbial activity in the biofilter was measured as the systems had been operated for a short time. On the rest of the sampling dates, T1-T7, all water quality parameters were measured.

2.4.3 Ammonia spiking

To test the nitrification performance of the bio-media in the RASs, bio-media from the biofilters were spiked with ammonia to investigate TAN removal kinetics. This was carried out on bio-media from the six different RASs at the end of the freshwater phase when the bio-media were changed, and at the end of the brackish water phase when the experiment was over (Fig. 6). To have a reference of the bio-media's nitrification performance before they were exposed to the treatments, two additional spiking experiments were conducted, one for freshwater and one for brackish water maturated bio-media (Fig 6).

The spiking experiments were conducted in the following manner, inspired by the experimental protocol from Kinyage et al. (2019). A volume of 0.4L bio-media and 1.8L RAS-water from each of the six systems were added to separate buckets installed with aeration stones, to provide constant upwelling of the bio-media. Before spiking with ammonia, zero samples were collected from all buckets. Initially, the buckets were spiked with ammonium chloride (NH_4Cl) to achieve a concentration of approximately 5 mg N/L. Simultaneously, Sodium bicarbonate ($NaHCO_3$) was added to achieve a concentration of approximately 100 mg HCO_3^- /L, this was done to maintain the buffer capacity of the system. Thirty seconds after NH_4Cl and $NaHCO_3$ addition, water samples were collected from the buckets with a 20 mL syringe and transferred to a test tube. Water samples were collected from each bucket at 15-, 30-, 45-, 60-, 75-, 90-, 120-, 150, 180-, 210-, 240- and 270-minutes post spiking. All samples were filtered through a 0.2 µm to avoid bacterial growth. The reference experiment was carried out in the same way except that bio-media was collected from the maturation tanks and divided in three different buckets.

Table 1 *Water quality parameters, analytical methods, procedure, treatment and processing applied.*

Parameter	Abbreviation	Units	Sample treatment and processing	Analytical method	Reference	Sampling point	Frequency of measurements
Total suspended solids	TSS	mg/L	Processed as soon as possible	Whatman™ Grade GF/C	NS 4733	Swirl separator, Inlet of biofilter and outlet of biofilter	Weekly and at sampling days
Particle size distribution	PSD	0.8- 410.3 µm divided in to 64 size classes	Processed as soon as possible	PCSS fluid lite counting system, Markus Klotz GmbH, Germany	N/A	Inlet of biofilter and outlet of biofilter	At sampling days
Particle concentration	N/A	part/mL	N/A	Calculated from PSD	N/A	Inlet of biofilter and outlet of biofilter	At sampling days
Total particle surface area	TSA	mm^2/mL	N/A	Calculated from PSD	N/A	Inlet of biofilter and outlet of biofilter	At sampling days
Beta value (ß-value)	ß	N/A	N/A	Calculated from PSD	Kavanaugh et al. (1980) and Patterson et al. (1999)	Inlet of biofilter and outlet of biofilter	At sampling days
Microbial activity (Hydrogen peroxide degradation)	HP-method	h^{-1}	Unfiltered. Possessed immediately	Colorimetry	Pedersen et al. (2019)	Inlet of biofilter, outlet of biofilter and in bio-media	At sampling days
Total chemical oxygen demand	COD_{tot}	mg O_2/L	Unfiltered + acid addition Kept at 4°C	LCK1414 (Freshwater) and LCK1814 (Brackish water), Hach Lange, Germany	N/A	Inlet of biofilter and outlet of biofilter	At sampling days
Dissolved chemical oxygen demand	COD_{diss}	mg O_2/L	Filtered 0.2 µm + acid addition Kept at 4°C	LCK1414 (Freshwater) and LCK1814 (Brackish water), Hach Lange, Germany	N/A	Inlet of biofilter and outlet of biofilter	At sampling days
Total ammonia nitrogen	TAN	mg N/L	Filtered 0.2 µm Kept at 4°C	Spectroquant1.14752.0001 Merck- Sigma-Aldrich, Germany	ISO, 1984b	Inlet of biofilter, outlet of biofilter, ammonia spiking reactors	At sampling days and samples from ammonia spiking
Nitrite	NO_2-N	mg N/L	Filtered 0.2 µm Kept at 4°C	Spectroquant1.14776.0002, Germany	ISO, 1984a	Inlet of biofilter and outlet of biofilter	At sampling days
Nitrate	NO_3-N	mg N/L	Filtered 0.2 µm Kept at 4°C	Spectroquant1.09713.0002 (Freshwater) and 1.14942.0001(Brackish water) Merck- Sigma-Aldrich, Germany	ISO,1986	Inlet of biofilter and outlet of biofilter	At sampling days

2.5 Methods description

2.5.1 TSS

TSS was analyzed based on NS 4733 (Standard-Norge, 1983). Whatman™ Grade GF/C glass microfiber filters with a pore size of 1.2 µm were used under the filtration of the water samples. Before running the water samples, the filters' initial mass (IM) was weighted on a microscale weight and logged. The filters were packed in aluminum foil to avoid contamination by dust and other air particles.

Using a filter assembly (similar to MultiVac 301 – MB – T from Rocker Scientific Co., Ltd, Kaohsiung, Taiwan, not the same model) and a vacuum hose connected to it, water was filtered through the filters into an Erlenmeyer flask. An effort was made to filter one liter of water through the filters, but in some cases the filter clogged. The amount of filtered water (WF) was logged. The filters were then dried for 3 hours at 105°C in a heating cabinet. Finally, the final mass (FM) of the filters were weighted and logged when the filters were at room temperature. The difference between the initial mass and the final mass in relation to the filtered amount of water was used to find the TSS according to **Equation (1)**:

$$TSS = \frac{(IM-FM)}{WF} \qquad \text{(Eq. 1)}$$

2.5.2 Chemical oxygen demand (COD)

Chemical oxygen demand (COD) was measured by using HACH® Lange GmbH (Düsseldorf, Germany) test kits, LCK1414 (5-60 mg/L O_2) for freshwater and LCK1814 (7-70 mg/L O_2) for brackish water. Samples for COD_{tot} had no treatment, water samples for COD_{diss} measurements were filtered through a 0.2 µm filter. The analysis consisted of taking 2 mL of water sample into a cuvette where oxidizable substances reacted with sulphuric acid and potassium dichromate solution in the presence of silver sulphate as a catalyst. Chloride was masked by mercury sulphate and reduction in yellow coloration of hexavalent chromium (Cr^{6+}) was evaluated in a spectrophotometer after the cuvette was heated at 148 °C for 2 hours. Evaluation of the yellow color reduction quantified the amount of oxygen consumed by substances in the water sample, measured in mg/L (Hach Lange, 2015).

2.5.3 Particle size distribution (PSD)

PSD was analyzed with the particle counting system PCSS fluid lite from Markus Klotz GmbH (Bad Liebenzell, Germany) and operated through the associated evaluation software, Klotz Protrend (Bad Liebenzell, Germany). Measured data were displayed on the computer and

exported to Excel v. 16.65 (Microsoft, Redmond, Whasington, US). Particles were assessed as spheres by the machine and their diameter (l) was distributed in a range from 0.8 µm to 410.3 µm, divided in to 64 size classes. The particle counter automatically did three measurements of 10 mL water sample. To determine number of particles per mL, number of particles within each size class was then divided by 10. From the three measurements, the mean value for each size class was calculated. Particle concentration (part/mL) was found by adding up the mean value from every size class. Surface area (SA) of the particles was calculated for each size class with **Equation (2)**:

$$SA = 4\pi r^2 \quad \text{(Eq. 2)}$$

Surface area per mL was determined by multiplying the SA in the size classes by the number of particles in the size classes. By adding up the SA for each size class the total surface area per mL (mm^2/mL) was determined.

Lastly, the beta value (β) was calculated. This was done by first creating new size classes based on an ascending geometric progression defined by Kavanaugh et al. (1980) and Patterson et al. (1999). The progression was based on volume where the boundaries of each size class, given as diameter (l), gave half the volume of the previous size class so that $V_{i+1}=2V_i$. The boundaries of each size class could then be defined by **Equation (4)**:

$$l_{i+1} = l_i \cdot 1.26 \quad \text{(Eq. 4)}$$

This equation described the geometric progression and gave a ratio between the variance in the size class boundaries (Δl) and the mean diameter of each size class (l^*) that is constant ($\frac{\Delta l}{l^*} = 0.23$). The 64 size classes from the PSD measurement were then divided into the new size classes. By then using **Equation (5)** from Kavanaugh et al. (1980) and Fernandes et al. (2014),:

$$\frac{dN}{dl} = A \cdot l^{-\beta} \leftrightarrow \frac{\Delta N_i}{\Delta l_i} = A \cdot l_i^{*-\beta} \quad \text{(Eq. 5)}$$

where N represents the density of particles, and A and β are empirical constants, and by further log-log transforming it with **Equation (6)** to normalize the distribution (Fernandes et al., 2014):

$$log\frac{dN}{dl} = \log A - \beta \log l \quad \text{(Eq. 6)}$$

-β is given as the slope. Information on the contribution of each size class to the particle concentration and total particle surface area of the system was easily accessible once β was calculated for a specific system (Kavanaugh et al., 1980, Fernandes et al., 2014).

2.5.4 Assessment of microbial activity based on hydrogen peroxide decomposition (HP-method)

Assessment of microbial activity in water samples and in bio-media was done by using the method from Pedersen et al. (2019). This method takes advantage of the enzymatic activity of both free living and particle associated bacteria, as well as potential contributions from other microbes.

This method used two reagents: a stock solution of hydrogen peroxide and 4A reagent that stops the breakdown of H_2O_2. The 4A regent was prepared according to Pedersen et al. (2019) by mixing 1.2 g NH_4VO_3 with 5.2 g dipicolinic acid, 60 mL Milli-Q water and 60 mL concentrated H_2SO_4. This solution was then heated until it was dissolute and then subsequently diluted to 1000 mL after cooling to room temperature. The hydrogen peroxide stock solution, 1000 mg H_2O_2/L, was made with 30% technical grade hydrogen peroxide, and diluted with Milli-Q to the final concentration.

First, 0.3 mL of 4A reagent was divided into marked cuvettes, two cuvettes for every time interval H_2O_2 decomposition was being measured. Then, 2.7 mL of water sample was added to the first two cuvettes, to measure the presence of H_2O_2 before water samples were spiked with the H_2O_2 stock solution (zero-samples). Water samples were then spiked with the H_2O_2 stock solution to reach an initial concentration of 10 mg H_2O_2/L. Immediately after spiking a stopwatch was started. Next, the water sample was flipped 10 times to ensure proper mixing. After 30 seconds, 2.7 mL of the spiked water sample was transferred to two cuvettes marked with 0.5 minutes. After 15 minutes, a new 2.7 mL of spiked water sample was transferred to the next two cuvettes marked with 15 minutes. The same was done at 30, 45 and 60 minutes. When all cuvettes had been filled with spiked sampling water, the cuvettes were inverted and given a minimum of 15 minutes to rest before the next procedure.

With a few modifications, the same method as previously described was used to assess microbial activity in bio-media. Five biochips, from each module, were placed in a plastic container with 105.4 mL of Milli-Q water. The containers were then flipped 10 times before 2.7 mL of the container water was transferred to the two zero-sample cuvettes. The containers were then spiked to an initial concentration of 10 mg H_2O_2/L, the abovementioned procedure was then followed.

Absorbance was measured in all the cuvettes in a HACH® DR6000™ UV VIS spectrophotometer. The wavelength was set to 432 nm and the values were noted. In advance, a standard curve was created from several H_2O_2 concentrations (0, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 10.0 and 25.0 mg H_2O_2 /L) with the 4A reagent. The standard curve was described by $y = 74.622x - 0.3345$, where y was the H_2O_2 concentration in mg/L and x was the absorbance measured at 432 nm. To assess decomposition of H_2O_2 in the samples the average from the two zero samples were first calculated. This average was then subtracted from the averages of all the other time intervals, to correct potential background interference by apparent H_2O_2 in the samples before spiking. The concentration at each time interval after spiking, was then determined by using the absorption averages to the equation above.

The first-order decomposition reaction rate constant (k) was then calculated by using **Equation (7)**:

$$C_t = C_0 \cdot e^{-kt} \qquad \text{(Eq. 7)}$$

where C_t indicates the concentration of H_2O_2 at time t, C_o is the initial concentration and k represents the reaction rate constant in in time^{-1}. This value was then multiplied by 60 to get the rate constant in hour^{-1}. The k-value was considered directly proportional to the total bacterial activity in the water sample (Pedersen et al., 2019).

2.5.5 Nitrogen compounds

Nitrogen compounds were assessed by using Spectroquant ammonium (1.14752.0001), nitrite (1.14776.0002), nitrate (1.09713.0002) and nitrate in sea water (1.14942.0001) -test kits from Merck – Sigma-Aldrich (Darmstadt, Germany). The ammonium, nitrite and nitrate test kits were respectively based on International Standard Organization ISO Standard - No.7150-1 (ISO, 1984b), No.6777-1 (ISO, 1984a) and No. 7890-1 (ISO,1986) and "Standard Methods for the Examination of Water and Wastewater", 22nd edition, SM 4500 (Eaton et al. 2012). The quantity of the measured nitrogen compounds was assessed with NOVA 60A Spectroquant® from Merck – Sigma-Aldrich (Darmstadt, Germany).

2.5.6 Nitrification kinetics

The following equations were inspired by calculations conducted in Ling and Chen (2005), Prehn et al. (2012) and Kinyage et al. (2019) and used to asses nitrification performance from the ammonia spiking experiments. Area-specific 0'order reaction rate constant (k_{0a}) was found with **Equation (8)**:

$$k_{0a} = k_0 \cdot V_b / SA_b \quad \text{(Eq. 8)}$$

where k_0 is the rate of TAN removal, indicated by a linear slope (mg TAN/L/day). V_b is volume of biofilter water, and SA_b is the surface area of the biofilter media. **Equation (9)** provided the surface-specific TAN removal (STR_0; g TAN/m^2/day) under 0'order conditions:

$$STR_0 = k_{0a} \quad \text{(Eq. 9)}$$

Substrate dependent 1'order TAN removal was assessed by exponential regression. **Equation (10)** was used to calculate the area-specific 1'order reaction rate constant (k_{1a}; m/d):

$$k_{1a} = k_1 \cdot V_b / SA_b \quad \text{(Eq. 10)}$$

where k_1 is the rate of TAN removal under 1'order conditions given by the exponential growth constant (day^{-1}). STRs under 1'order condition (STR_1) was given by **Equation (11):**

$$STR_1 = k_{1a} \cdot [TAN] \quad \text{(Eq. 11)}$$

where $[TAN]$ is the concentration of TAN at a specific time under 1'order conditions.

The half-saturation constant of the nitrifying bacteria (K_s) could then be found through the relationship of the 0' and 1' order rate constants given by **Equation (12):**

$$k_{0a} = k_{1a} * K_s \quad \text{(Eq. 12)}$$

The TAN bulk concentration where the transition from 0'order to 1'order kinetics (C_b) takes place was estimated from **Equation (13):**

$$C_b = 2 \cdot K_s \quad \text{(Eq. 13)}$$

2.6 Statistical analysis

Prior to statistical analysis the data from the sampling days (T0-T7) and the spiking experiment was visually inspected for outliers. Data from the sampling days were then tested for normality and homogeneity by plotting residuals vs. fitted values, and then by using the Shapiro-wilk test (Shapiro and Wilk, 1965) and Levene's test (Brown and Forsythe, 1974) in R statistical software (version 4.1.1, R Foundation for Statistical Computing, Vienna, Austria)(see Appendix III-VI and III-VII). When significance for these tests were not met, the data was log-transformed to better fit model assumptions (Appendix III-II). Excel (v. 16.65, Microsoft, Redmond, Washington, US) was then used to find the amount of datapoints from the spiking experiment which gave the highest R^2 for linear regression. This was done for all six systems

and the linear line was used to describe 0'order kinetics for each system, data that did not fit this line was used to describe 1'order kinetics.

To investigate the effect of particle load on the measured response variables, a one-way ANOVA (Zar, 1996) was performed on each response variable, treating sampling days as replicates for each system, to find if they were significantly dependent on particle loading (see Appendix III-III). Measurements from the same sampling points were compared. Following the ANOVA, a Tukey HSD (Zar, 1996) post-hoc test was conducted to assess differences in mean, and if the effect of particle load was negative or positive (See Appendix III-IV). A linear mixed effect model (LMM) was also fitted to assess random effects (replicated RASs) within the treatment groups.

To assess if the effect of particle load on the response variable was different depending on the sampling day (T) a two-way ANOVA with interaction between particle load and sampling day was performed. Following, a Tukey HSD test was performed to investigate what effect particle load had on each sampling day between high and low particle loading.

Where response variables were measured both at the inlet and the outlet of the MBBR a two-way ANOVA, with interaction between particle load and sample site (inlet or outlet) was performed to assess if either of the particle loads had a significant effect between the two sites. Then a one-way ANOVA was fitted to assess if the "delta" between inlet and outlet ($\Delta = inlet - outlet\ or\ outlet - inlet$) was significantly different between the two particle loadings. A Tukey HSD was performed on both ANOVAS.

Graphically presented data from the sampling days are the calculated means and standard error of mean (SEM) for the two operational conditions, high and low particle loading (Appendix II), unless otherwise stated.

Estimated slopes and figures visualizing 0'order nitrification kinetics were obtained through a LMM. Handling days as the predictor variable and systems as a random variable, fitted separately for the triplicates (High and Low). To estimate constants and visualize figures for the 1'order nitrification kinetics a non-linear least squares (NLS) approach was taken. R-squared (R^2) was computed to assess how well the linear- and exponential regression from the LMM and NLS fitted the data obtained from the triplicates. A LMM, with interaction between days and particle loading, was then conducted to assess particle loadings effect on nitrification. First- order nitrification kinetics were logarithmically transformed to do this.

All figures and models were fitted using RStudio (version 2022.12.0, R-Studio, Inc. Boston, MA, USA). The statistical significance was determined based on the p-values, with a significance level set at $p < 0.05$.

3. Results

3.1 Particles

3.1.1 System particle load and operational conditions

Systems with an elevated particle load (High) did not reach the operational targets of the experimental design, and the TSS was shifting throughout the experimental period. Control systems (Low) had a relatively stable TSS through the experimental period within the experimental design (Fig. 7) The effect of particle loading was consistent as there was very low random variance caused by the individual systems (Appendix III-II, Table XVII).

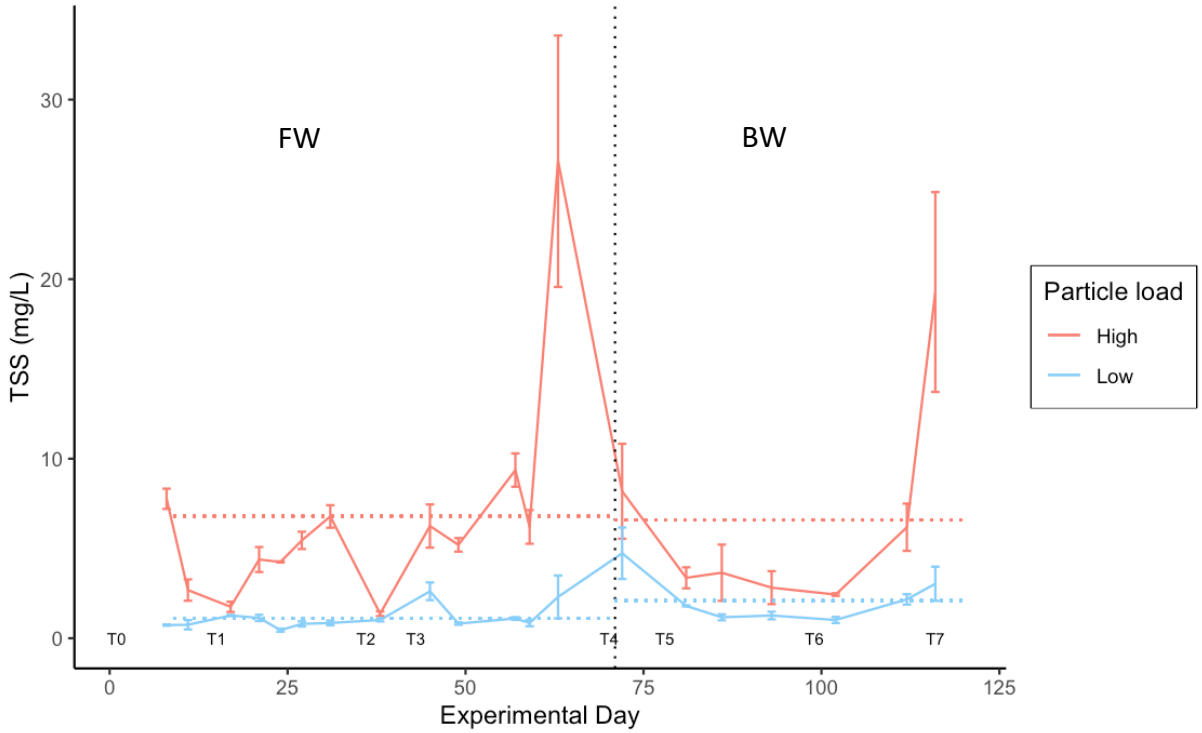

Fig 7. Total suspended solids (TSS) through experimental period. *Mean TSS (mg/L) in swirl-separator of RASs operated under two different conditions, high (red) and low particle load (blue), from experimental day 8 to 116. The numbers on the x-axis represents the experimental days and each datapoint is the calculated mean ± SEM values, n=3 for each experimental group. Vertical dotted line (experimental day 73) represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the MBBR was changed. Horizontal dotted lines represent the mean TSS through the two salinity phases, fresh-water (n=39) and brackish water (n=21), for both experimental groups, experiment sampling dates (T0-T7) are also included above the x-axis.*

Development over time for the two conditions:

High particle load systems had an average (mean ± SEM) of 6.8 ± 1.1 mg TSS/L through the freshwater phase and an average of 6.6 ± 1.5 mg TSS/L through the brackish water phase. Corresponding values for the low systems under the different salinity conditions were 1.1± 0.1

and 2.2± 0.3 mg TSS/L (Fig.7). The averages TSS for the high load systems fluctuated through the whole experimental period; They peaked at the end of the fresh- and brackish water phase with mean values of 26.6 ± 7.0 and 19.3 ± 5.6 mg TSS/L, respectively. There were also distinguishable valleys in the TSS measurements in the high particle load systems, especially right after sampling day 1 and 2 (T1 and T2) with TSS measurements of 1.8± 0.1 and 1.4± 0.3 mg TSS/L, respectively. The average TSS for the low systems were relatively stable throughout the experimental period with a peak of 4.7 ± 1.4 mg/L right after the transition to brackish water. (Fig. 7). Potential factors contributing to the volatility in the high particle load systems are discussed in Appendix I, Discussion of material and methods.

Recirculating intensity and feed loading:

Both triplicates were operated under relatively similar feed loading and recirculation intensity. The average recirculation intensity was 1057 L MUW/kg feed for the high particle load systems and 1053 L MUW/kg feed in the low, during the freshwater phase. During the brackish water phase, it was 354 L MUW/kg feed for the high particle load systems and 392 L MUW/kg feed for the low (Appendix I-II, Fig I). Feed loading (kg feed/m^3 water) was 1.6 ± 1.5 kg/m^3 (mean±SD) for the high particle load systems and 1.5± 1.1 kg/m^3 for the low, during the freshwater phase. During the brackish water phase feed loading was 3.7± 1.8 kg/m^3 for High and 4.9± 2.9 kg/m^3 for Low (Appendix I-II, Fig II). Some experimental days, 35-36 and 70-77, were not included in the calculated averages. During these days MUW was very high due to operational challenges or the fish was starved, and no feed was added. These calculations also do not consider the additional organic material that was added to the systems with a high particle load. Potential effects of high MUW during some periods of the experiment are discussed in Appendix I.

Particle concentration and distribution:

The average total amount of particles across the whole experimental period was 9833 particles/mL at the inlet and 8193 particles/mL at the outlet of the biofilter in the high particle load systems. In the low particle load systems, the averages were 4421 particles/mL at the inlet and 4163 particles/mL at the outlet of the biofilter. 92% of particles were under 5 µm in the high particle load systems, while 97% was under 5 µm in the low particle load systems. There were several observations of particles above 200 µm in the high particle load systems, while there were individual cases of observations of particles over 100 µm in the low particle load systems. A profile of the average particle distribution across all sampling days at the inlet and

at the outlet of the biofilter is presented in Appendix II-II. There was also done preliminary work to assess the percentage contribution per size class to the particle- number surface area and volume, this is presented in Appendix IV Fig. III.

3.1.2 Total suspended solids (TSS)

Particle load had a significant effect on TSS at the inlet (One-way ANOVA, $F_{1, 34} = 7.6$, $p<0.01$, Fig. 8) and the outlet (One-way ANOVA, $F_{1, 34} = 10.5$, $p<0.01$, Fig. 8) of the biofilter. The averages across all sampling days at the inlet showed that TSS was 4.2 mg/L in the high particle load systems and 1.3 mg/L in the low particle load systems. At the outlet TSS was 3.3 mg/L in the high particle load systems and 1.4 mg/L in the low particle load systems. Giving a TSS that was higher by 2.9 mg/L at the inlet and 1.9 mg/L at the outlet in the RASs that received a high particle load (Tukey HSD test, $p<0.01$, Fig. 8) (Table 2). Significance of particle loads effect on TSS was maintained when T7 was removed form analysis (Table 3). Random variance caused by the triplicated RASs was very low (Appendix III-II).

Measured mean TSS (mg/L) showed a tendency of being higher in the RASs that received a high particle load in all sampling days and at both sampling points (inlet and outlet of the biofilter). Systems that received a low particle load were relatively stable around 1.3 ±0.6 mg/L in all sampling days, while high particle load varied between 1 mg/L and 14.3 mg/L (Fig. 8, see also Appendix II). Statistical analysis indicated that the effect of particle load on TSS varied with the sampling day at the outlet of the biofilter (Two-way ANOVA, $F_{5, 24} = 4.8$, $p < 0.01$, Fig. 8), while it showed marginal dependence on sampling day at the inlet (Two-way ANOVA $F_{5, 24} = 2.4$, $p = 0.06$, Fig. 8). Isolated comparison of the effect of particle load on each sampling day, indicated that there only was an increase in TSS due to high particle load on sampling day 7 at the outlet (Tukey HSD test, $p<0.01$, Fig. 8), while there was an increase in TSS on sampling day 2 and 7 at the inlet due to high particle load (Tukey HSD test, $p<0.05$, Fig. 8).

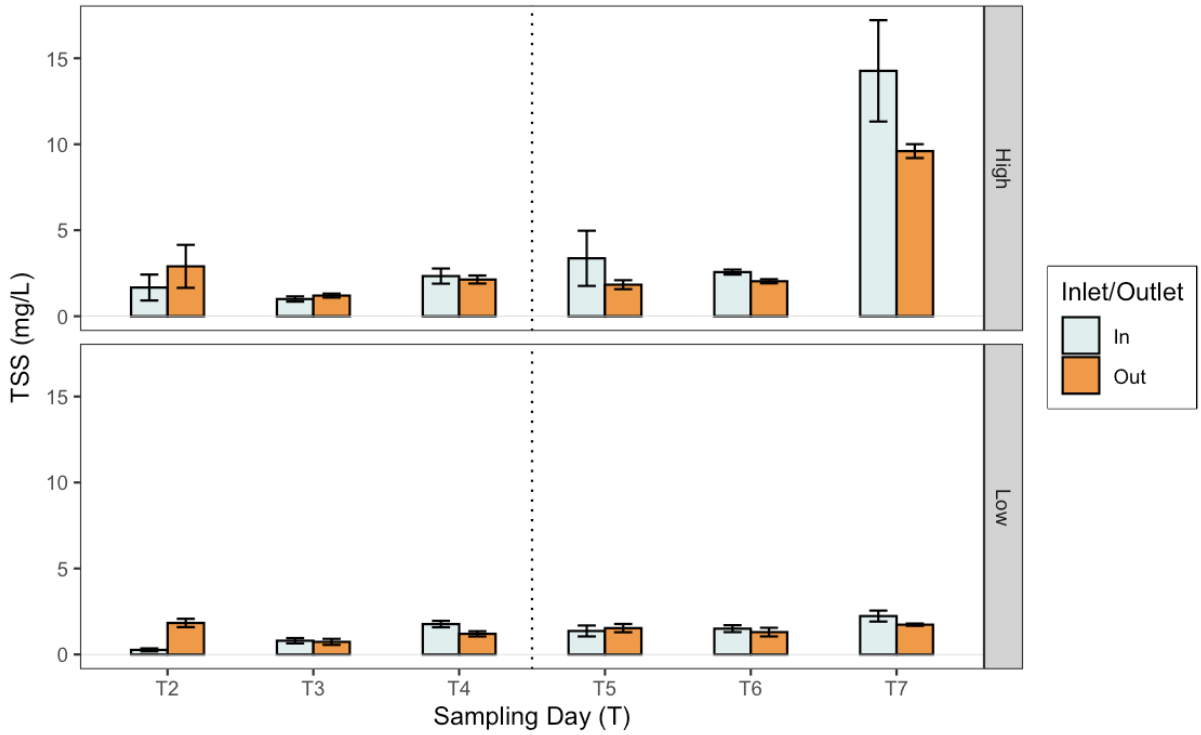

Fig 8. TSS (mg/L at the inlet and the outlet of the moving bed bioreactor (MBBR). *Mean TSS in water samples collected at the inlet and outlet of biofilters operated under different particle loadings, high and low. T2-T7, on the x-axis represents sampling days. Each bar represents the calculated mean ± SEM, n=3 for the inlet and outlet under both operational conditions. Vertical dotted line represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the MBBR was changed. Note that measurements from T1 are not included in the figure due lack of sampling water.*

The difference from outlet to inlet was neither significant for high nor low particle load. There was no significant difference between high and low particle load in measured TSS difference between the outlet and the inlet (Δ = outlet - inlet) (Fig. 8).

3.1.3 Total particle surface area (TSA)

RASs that received a high particle load had a higher mean ± SEM TSA at all sampling days at both the inlet and the outlet of the biofilter (Fig. 9, see also Appendix II). Comparison between systems that received a low and high particle load showed a significant effect on the particle surface area at the inlet (One-way ANOVA, $F_{1, 28}$ =10.6, $p<0.01$, Fig. 9) and the outlet (One-way ANOVA, $F_{1, 28}$ = 11.9, $p<0.01$, Fig. 9) of the biofilter. Comparison of averages across all sampling days showed that TSA increased with 0.58 mm^2/mL at the inlet- and 0.51 mm^2/mL at the outlet of the biofilters in the RASs that received a high particle load (Tukey HSD test, $p < 0.01$, Fig. 9) (Table 2). Significant effect of particle loading was obtained when T7 was

removed from the analysis (Table 3). There was low random variance caused by the different RASs (Appendix III-II).

Statistical analysis suggested that the effect of particle load on TSA was different depending on the sampling day at the inlet and outlet (Two-way ANOVA, $F_{4, 20} = 8.5$, $p<0.001$, Fig. 9). Comparison of high and low particle load on the different sampling days showed that high particle load had a significant positive effect on TSA on sampling day 7 (T7) at the inlet (Tukey HSD test, $p < 0.001$, Fig. 9) and 4, 6 and 7 (T4, T6 and T7) (Tukey HSD test, $p < 0.01$, Fig. 9) at the outlet.

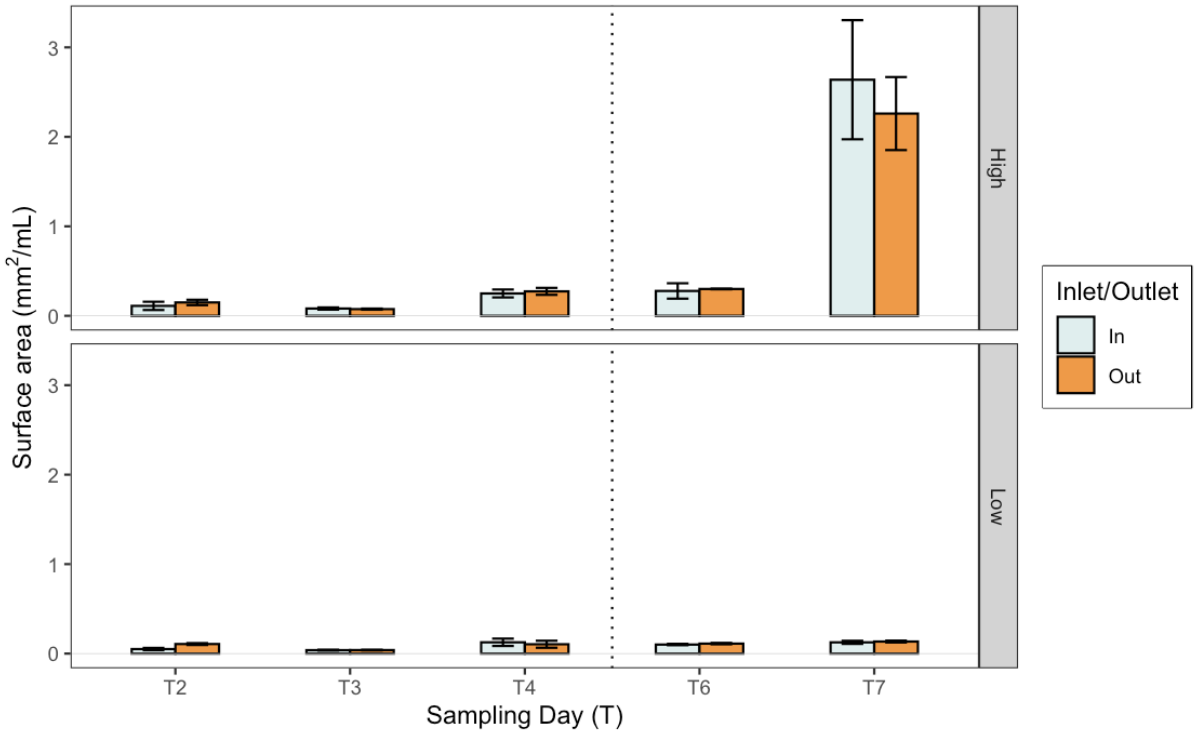

Fig 9. Total particle surface area (TSA) at the inlet and the outlet of the moving bed bioreactor (MBBR). *Mean TSA (mm^2/mL) in water samples collected at the inlet and outlet of biofilters operated under different particle loadings, high and low. T2-T7, on the x-axis represents sampling days. Each bar represents the calculated mean ± SEM, n=3 for the inlet and outlet under both operational conditions. Vertical dotted line represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the MBBR was changed. Note that measurements from T1 and T5 are not included in the figure due to their unreliable status due to algae growth in the grab samples.*

The difference from outlet to inlet was neither significant for high nor low particle load (Two-way ANOVA, $F_{1,56} = 0.01$, $p > 0.9$, Fig. 9). There was neither a significant difference between the two particle loadings in ΔTSA (outlet minus inlet) (Fig. 9).

3.1.4 Beta value (β)

Particle load had a significant effect on the β-value at both the inlet of the biofilter (One-way ANOVA, $F_{1, 28} = 5.7$, $p<0.05$, Fig. 10) and the outlet of the biofilter (One-way ANOVA, $F_{1, 28} = 13.2$, $p<0.01$, Fig. 10). The average β-value across all sampling days decreased from 3.2 to 3.0 at the inlet of the biofilter and from 3.06 to 2.78 at the outlet of the biofilters in the high particle load systems compared to the low (Tukey HSD test, $p < 0.05$, Fig. 10) (Table 2). Random variance caused by replicated RASs was low at the inlet, and 12.8% at the outlet (Appendix III-II).

During the whole trial low particle load systems showed a tendency of higher β-value than the high particle load systems at the inlet and at the outlet of the biofilter, except at sampling day 3 (T3) where β was greater in the high systems at the inlet (Fig. 10, see also Appendix II). Statistical analysis indicated that particle loads effect on β-value depended on the sampling day (T), at the inlet (Two-way ANOVA, $F_{4,20}= 8.9$, $p<0.001$, Fig. 10) but not at the outlet. There was a significant negative effect on the β-value of 0.76 at the inlet and 0.56 at the outlet on sampling day 7 (T7) in the high particle load systems (Tukey HSD test, $p<0.01$, Fig. 10). When T7 was excluded, statistical analysis indicated that that the effect of particle load on the β-value did not vary with the sampling days (Two-way ANOVA, $F_{3,16}=0.5$, $p=0.7$, Fig. 10).

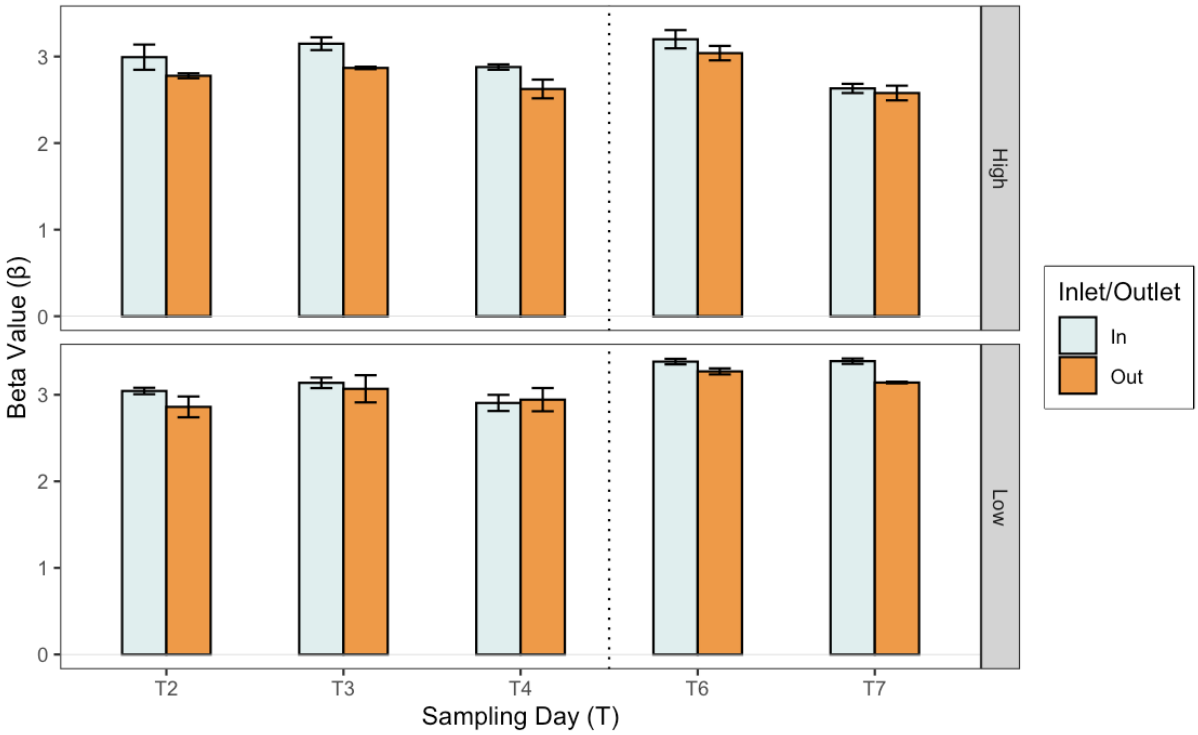

Fig 10. Beta value (β) at the inlet and the outlet of the moving bed bioreactor (MBBR). *Mean Beta Value(β) in water samples collected at the inlet and outlet of biofilters operated under different particle loadings, high and low. T2-T7, on the x-axis represents sampling days. Each bar represents the calculated mean ± SEM, n=3 for the inlet and outlet under both operational conditions. Vertical dotted line represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the MBBR was changed. Note that measurements from T1 and T5 are not included in the figure due to their unreliable status caused by algae growth in the grab samples.*

There was a significant difference in β-value between the outlet and inlet (One-way ANOVA, $F_{1,56} = 7.1$, $p = 0.01$, Fig. 10). However, there was no significant evidence suggesting that the effect was different between high and low particle loading (Two-way ANOVA, $F_{1,56} = 0.56$, $p = 0.46$). There was no significant difference in $\Delta\ \beta$-value between high and low particle loading (outlet minus inlet) (Fig. 10).

3.2 Organic compounds

3.2.1 Microbial activity

Particle load had a significant effect on the k-value at the inlet (One-way ANOVA, $F_{1, 40} = 26.3$, $p<0.001$, Fig. 11) and the outlet (One-way ANOVA, $F_{1, 40} = 9.4$, $p<0.01$, Fig. 11). High particle loading led to an increased microbial activity from 0.02 h^{-1} to 0.15 at the inlet (Tukey HSD test, $p < 0.001$, Fig. 11) and from 0.03 h^{-1} to 0.14 h^{-1} at the outlet (Tukey HSD test, $p < 0.01$, Fig. 11) (Table 2). The effect of particle loads on microbial activity were still significant when T7 was excluded from the analysis (Table 3). Random variance caused by replicated RASs was low (Appendix III-II).

RASs that received a high particle load consistently exhibited higher k-values across all sampling days (Fig. 11, see also Appendix II). The statistical analysis revealed that the impact of particle load on the k-value varied across different sampling days (T) at the at the inlet (Two-way ANOVA, $F_{6,28}= 6.3$, $p<0.001$, Fig. 11) and the outlet (Two-way ANOVA, $F_{6,28}= 12.4$, $p<0.001$, Fig. 11) of the biofilter. Isolated comparison of each sampling day between the two particle loads and sampling points showed that activity was 0.7 h^{-1} higher at sampling day 7 (T7) at the inlet of the biofilter in the high particle load systems (Tukey HSD test, $p < 0.001$, Fig. 11). In addition to increased microbial activity at the outlet of the high particle load systems on sampling day 7, there was also increased microbial activity on sampling days 5 and 6, respectively 0.06 h^{-1} and 0.05 h^{-1} (Tukey HSD test, $p < 0.05$, Fig. 11).

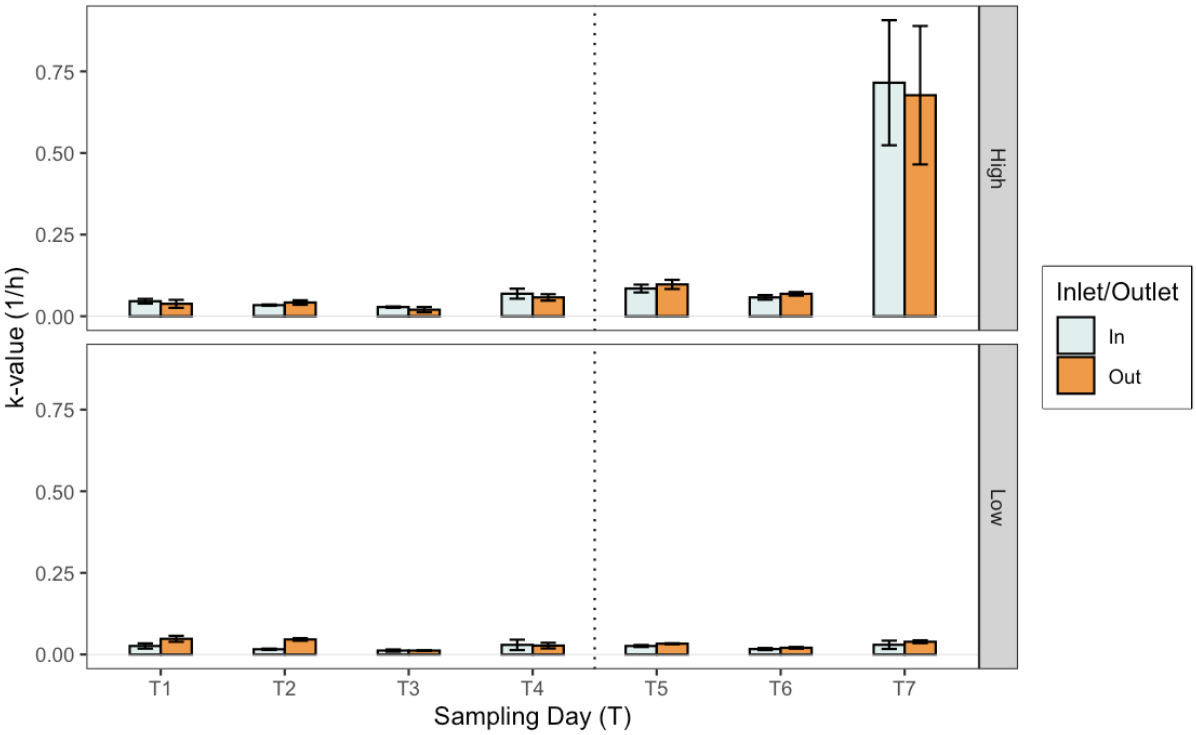

Fig 11. Microbial activity (k-value) at the inlet and the outlet of the moving bed bioreactor (MBBR). *Mean k-value in water samples collected at the inlet and outlet of biofilters operated under different particle loadings, high and low. T1-T7, on the x-axis represents sampling days. Each bar represents the calculated mean ± SEM, n=3 for the inlet and outlet under both operational conditions. Vertical dotted line represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the biofilter was changed.*

The difference from the outlet to the inlet of the biofilter was neither significant for high nor low particle load (Two-way ANOVA, $F_{1,80}$= 1.7 p=0.2, Fig. 12). The effect of particle load on the difference in Δk-value (outlet-inlet) was borderline not significant (One-way ANOVA, $F_{1, 40}$ = 3.3, p = 0.08, Fig. 12). The borderline significance ceased when T7 was excluded from the analysis, indicating notable variations in Δk-value on this sampling day compared to the others (Fig. 12)

3.2.2 Microbial activity in the bio-media

The k-value was significantly affected by particle load (One-way ANOVA, $F_{1,46}$ = 6.5, p<0.5, Fig. 12). The mean k-value across all the sampling days were 1.90 h^{-1} in the high particle load RASs and 1.55 h^{-1} in the low particle load RASs (Table 2). This gave a significant positive effect of high particle load on the k-value measured in bio-media from the MBBRs (Tukey HSD test, p<0.05, Fig. 12). Random variance caused by replicated RASs was 5.2% (Appendix III-II).

The mean k-value in bio-media collected from RASs that received a high particle load had a tendency of being higher than in the ones collected from RASs operated with a low

particle load, on every sampling day. Both operational systems hit a bottom on sampling day 6 (T6). High particle load RASs had a peak k-value of 2.95±0.3 h^{-1} on sampling day 7 (T7). Low particle load RASs had a relatively stable k-value around 1.55±0.06 h^{-1} in their bio-media (Fig. 12, Appendix II). Statistical analysis indicated that the effect of particle load on k-value varied with the sampling day (Two-way ANOVA, $F_{7,32}$= 5.2, p<0.001, Fig. 12). Isolated comparison of each sampling day between the two particle loads showed that the effect on the k-value only was significant at sampling day 7 (T7) (Tukey HSD test, p < 0.001, Fig 12). When T7 was excluded, statistical analysis indicated that that the effect of particle load on k-value did not vary with the sampling days (Two-way ANOVA, $F_{6,28}$= 0.31, p=0.93, Fig. 12).

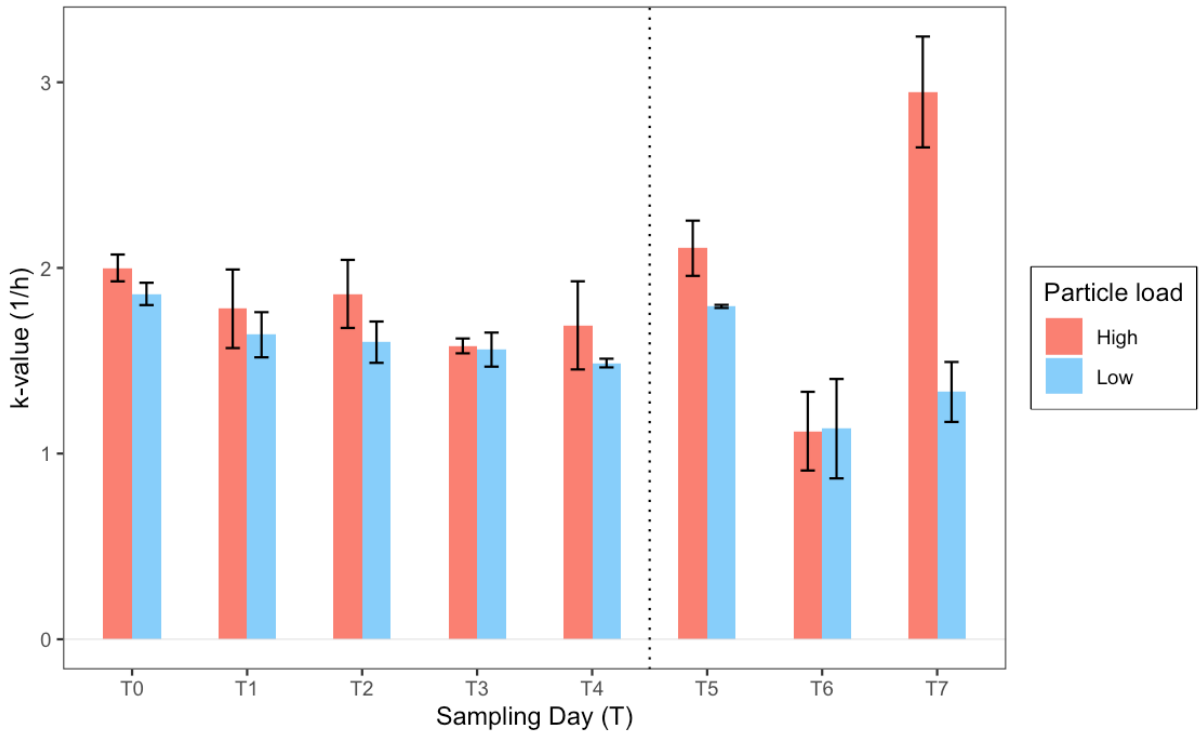

Fig 12. Microbial activity (k-value) in bio-media collected from RAS MBBR. *Mean k-value(h^{-1}) in bio-media collected from RASs operated under two different operational conditions, high- and low particle load. T0-T7 on the x-axis represents sampling days. Each bar is the calculated mean ± SEM, n = 3 for each operational condition at all sampling days. Vertical dotted line represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the biofilter was changed.*

3.2.3 Total- and dissolved Chemical oxygen demand ($COD_{tot/diss}$)

Measured values of COD_{tot} were almost identical to measured $COD_{diss,}$ at the inlet (One-way ANOVA, $F_{1,82}$ = 0.001, p = 0.98 Fig. 13) and at the outlet (One-way ANOVA, $F_{1,82}$ = 0.05, p = 0.8 Fig. 13) of the biofilter. Dissolved COD made up 99% of the COD at the inlet and 97% of the COD at the outlet.

There was a significant effect of particle loading on COD at both the inlet (One-way ANOVA, $F_{1,40} = 4.7$, $p < 0.05$, Fig. 13) and the outlet (One-way ANOVA, $F_{1,40} = 4.4$, $p < 0.05$, Fig. 13). The average measurements across all seven sampling days indicated that high particle load systems had COD measurements that were 11.2 mg/L higher at the inlet of the biofilter and 10.8 mg/L higher at the outlet of the biofilters compared to the low particle load systems (Tukey HSD test, $p < 0.05$. Fig. 13) (Table 2). The significant effect of particle load on COD was maintained when T7 was removed. Random variance caused by replicated RASs was low (Appendix III-II).

Statistical analysis revealed that the impact of particle load on COD varied across different sampling days (Two-way ANOVA, $F_{6,28}=10.7$, $p<0.001$. Fig. 13). Comparing the effect of particle load on each sampling day isolated showed that there only was a significant difference between COD measurements between high and low particle load on sampling day 7. At this sampling day COD measurements were 42.27 mg/L higher at the inlet of the biofilter and 40.17 mg/L higher at the outlet of the biofilter (Tukey HSD test, $p<0.001$, Fig 13). There were no significant differences between high particle loading and low particle loading both at the inlet- and the outlet of the biofilter at any of the other sampling days, due to the high measurements in the high particle load systems at T7.

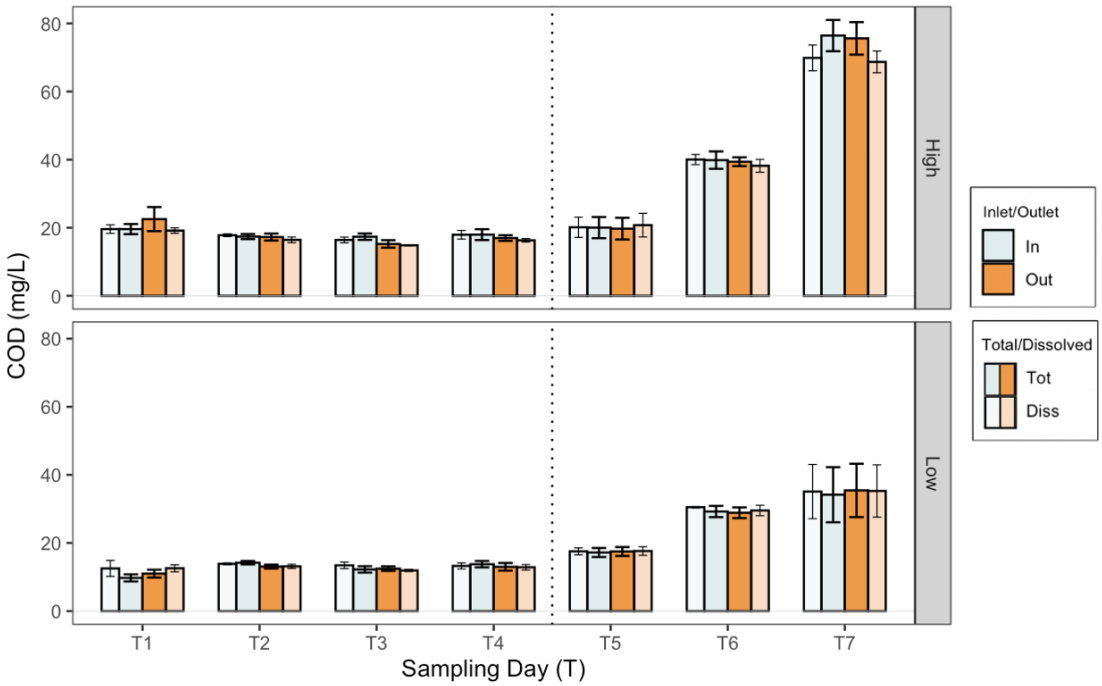

Fig 13. Total and dissolved chemical oxygen demand (COD_{tot} and COD_{diss}) at the inlet and the outlet of the moving bed bioreactor (MBBR). *Mean COD_{tot} and COD_{diss} in water samples collected at the inlet and outlet of biofilters operated under different particle loadings, high and low. Faded thin bars represent COD_{diss}, while deep thick bars represent COD_{tot}. T1-T7, on the x-axis represents sampling days. Each bar represents the calculated mean ± SEM, n=3 for the inlet and outlet under both operational conditions. Vertical dotted line represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the biofilter was changed.*

COD measurements were very similar between the outlet and the inlet of the biofilter. The difference in COD between the inlet and outlet was not significant for either high or low particle load (Two-way ANOVA, $F_{1,80}$=0.003, p=0.93, Fig. 13). ΔCOD (inlet -outlet) not significantly different between high and low particle load (One-way ANOVA, $F_{1,40}$ = 0.36, p=0.54, Fig. 13).

3.3 Inorganic compounds

3.3.1 Total ammonia nitrogen (TAN)

There was a significant difference in TAN concentration between the high and low particle load water samples at the inlet- (One-way ANOVA, $F_{1, 40} = 10.2$, $p<0.01$, Fig. 14) and at the outlet of the biofilter (One-way ANOVA, $F_{1, 40} = 5.1$, $p<0.05$, Fig. 14). Comparison of averages across all sampling days showed that TAN concentration increased from 0.32 mg/L to 0.52 mg/L at the inlet (Tukey HSD test, $p<0.01$, Fig. 14) and from 0.26 mg/L to 0.36 mg/L at the outlet (Tukey HSD test, $p<0.05$, Fig. 14) of the biofilters in the RASs that received a high particle load (Table 2). Random effect caused by replicated RASs was very low (Appendix III-II).

Measurements tended to be higher in the systems that received a high particle load at all sampling days both at the inlet and outlet of the biofilter, except sampling day 3 (T3) at the outlet. Statistical analysis indicated that the effect of particle load on TAN did not vary between the sampling days (T) (Two-way ANOVA, $F=2.39$, $p=0.054$, Fig. 14). However, there was a significant positive effect of 0.5 mg TAN/L in the high particle load system at the inlet on sampling day 1 (T1), in an isolated comparison with T1 in the low particle load systems (Tukey HSD test, $p<0.01$, Fig. 14).

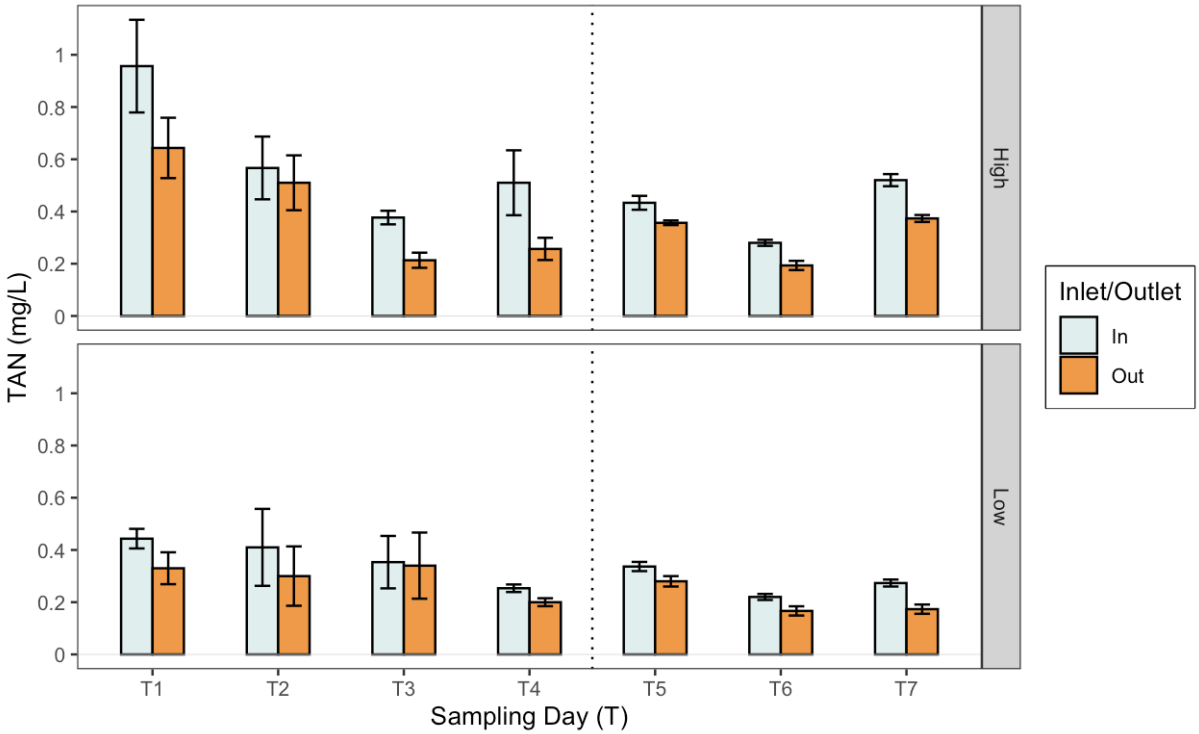

Fig 14. Total ammonia nitrogen (TAN) at the inlet and the outlet of the moving bed bioreactor (MBBR). *Mean TAN in water samples collected at the inlet and outlet of biofilters operated under different particle loadings, high and low. T1-T7, on the x-axis represents sampling days. Each bar represents the calculated mean ± SEM, n=3 for the inlet and outlet under both operational conditions. Vertical dotted line represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the biofilter was changed.*

The difference in TAN concentration between the inlet and outlet tended to be higher in the high particle load systems (Fig. 14, see also Appendix II). TAN also tended to be lower at the outlet compared to the inlet at every sampling day, under both operational conditions. High particle load had an average difference of -0.16 mg TAN/L between the inlet and the outlet of the biofilter across all sampling days (Tukey HSD test, $p<0.05$, Fig. 14). The average difference between the inlet and outlet of the biofilter across all sampling days was not significant for low particle load systems (Tukey HSD test, $p=0.56$, see also Appendix V, Fig. IV).

The change in TAN between the inlet and outlet of the biofilter (Δ= inlet-outlet) differed significantly between the high and low particle load RASs (One-way ANOVA, $F_{1, 40} = 8.7$, $p < 0.01$, Fig. 14). The average change in TAN between the inlet and outlet was 0.09 mg TAN/L higher in the high particle load systems compared to low particle load systems (Tukey HSD test, $p < 0.01$, Fig. 14, see also Appendix V, Fig. IV).

3.3.2 Nitrite (NO_2^-) inlet and outlet

Particle load had a significant effect on the nitrite concentration (mg NO_2/L) at the inlet of the biofilter (One-way ANOVA, $F_{1, 40} = 4.7$, $p < 0.05$, Fig. 15). At the outlet of the biofilter there was no significant effect of particle loading (One-way ANOVA, $F_{1, 40} = 3.1$, $p=0.09$, Fig. 15). The average NO_2^- concentration across all sampling days was 0.19 mg/L higher in the high particle load systems compared to the low particle load systems, at the inlet of the biofilter (Tukey HSD test, $p < 0.05$, Fig. 15) (Table 2). When T1 was excluded from the statistical analysis there was not significant effect of particle loading on NO_2^- concentration (One-way ANOVA, $F_{1, 34} = 1.64$, $p = 0.08$). Random variance caused by replicated RASs was low (Appendix III-II).

High particle load systems had a higher nitrite concentration than low particle load systems at most sampling days, with sampling day 3 (T3) as the only exception. Systems under both conditions reached a peak at the first sampling days, then there was a decreasing tendency in nitrite concentration the rest of the sampling days (Fig. 15, see also Appendix II-X). The effect of particle load depended on the sampling day (Two-way ANOVA, $F_{6,28} = 13.3$, $p<0.001$, Fig. 15), this applied to both the inlet and the outlet of the biofilter. Isolated comparison of each sampling day indicated that there was a significant increase in NO_2^- concentration of 0.7 mg/L on sampling day 1 (T1) in the high particle load systems compared to the low, at both the inlet and the outlet of the biofilter (Tukey HSD test, $p< 0.001$, Fig. 15). Statistical analysis suggested

that there was no significant effect of particle load on any of the other sampling days (Appendix III-IV). Further implying that increased NO_2^- concentration cause by high particle loading depended on sampling day 1.

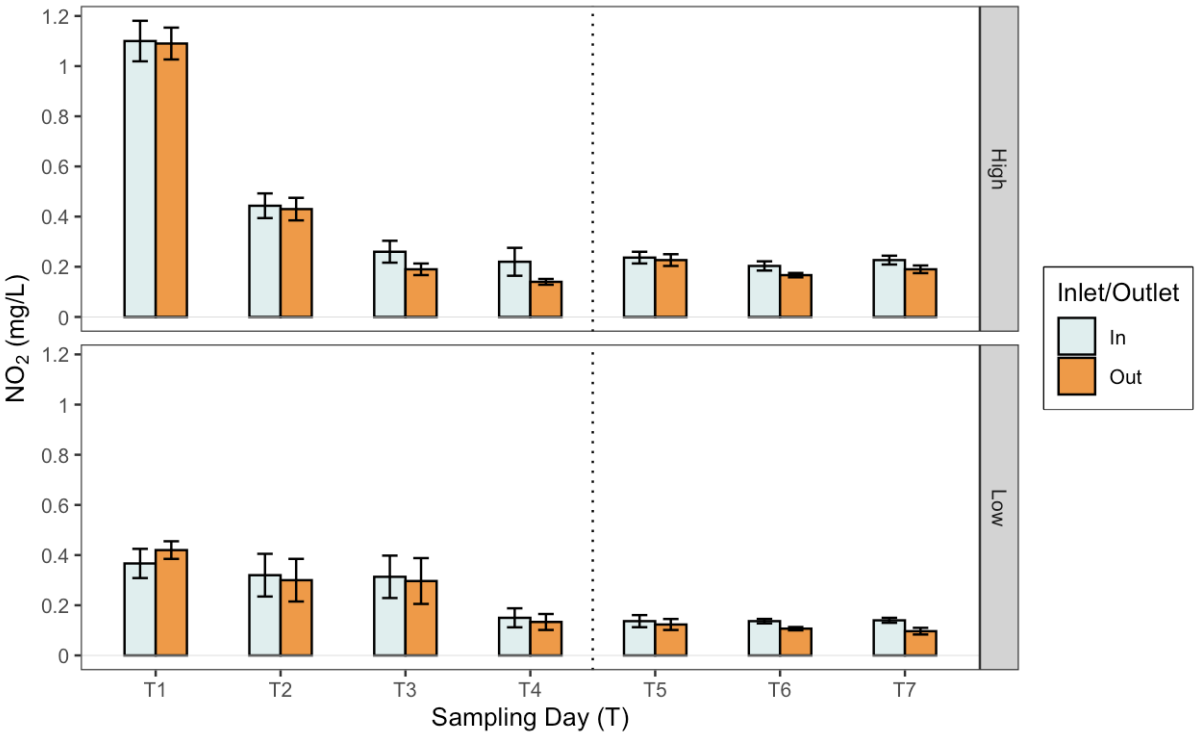

Fig 15. Nitrite concentration (mg NO_2/ L) at the inlet and the outlet of the moving bed bioreactor (MBBR). *Mean NO_2-N in water samples collected at the inlet and outlet of biofilters operated under different particle loadings, high and low. T1-T7, on the x-axis represents sampling days. Each bar represents the calculated mean ± SEM, n=3 for the inlet and outlet under both operational conditions. Vertical dotted line represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the biofilter was changed.*

Statistical analysis suggested that there was no difference in nitrite concentration at the outlet- compared to the inlet of the biofilter in both high and low particle load systems (Two-way ANOVA, $F_{1,80}$=0.05, p> 0.8, Fig. 15, see also Appendix V). There was neither a significant difference in Δ NO_2 (Δ= outlet-inlet) between high and low particle load (One-way ANOVA, $F_{1, 40}$ = 3.81, p> 0.06, Fig. 15, see also Appendix V, Fig. IV).

3.3.3 Nitrate (NO_3) inlet and outlet

There was a significant effect of particle load on nitrate concentration at the inlet (One-way ANOVA, $F_{1, 40}$ = 6.2, p < 0.5, Fig. 16) and the outlet (One-way ANOVA, $F_{1, 40}$ = 6.9, p < 0.05, Fig. 16). There was a negative effect of high particle load on nitrate concentration at both the inlet and the outlet (Tukey HSD test, p < 0.05, Fig. 16). Comparison of averages across all sampling days showed that NO_3^- concentration decreased with 12.91 mg/L at the inlet (Tukey HSD test, p<0.05, Fig. 16) and 13.95 mg/L at the outlet (Tukey HSD test, p<0.05, Fig. 16) of

the biofilters in the RASs that received a high particle load (Table 2). There was low random variance caused by the replicated RASs (Appendix III-II).

Statistical analysis revealed that the influence of particle load on NO_3^- concentration was consistent across the sampling days at both the inlet and outlet of the biofilter (Two-way ANOVA, $F_{6,28} = 2.2$, $p > 0.06$, Fig. 16). Isolated comparison of each sampling day between the two particle loads and sampling points showed that NO_3^- concentration was 42.00 mg/L lower at the inlet of the biofilter and 42.67 mg/L lower at the outlet of the biofilter in the high particle load systems on sampling day 7 (T7). The difference between high and low particle load systems were not significant at any of the other sampling days. However, when sampling day 7 (T7) was removed from the analysis NO_3^- concentration became significantly lower in the high particle load systems on sampling day 3 and 6 (T3 and T6) at both the inlet and the outlet (Tukey HSD test, $p < 0.05$, Fig. 16).

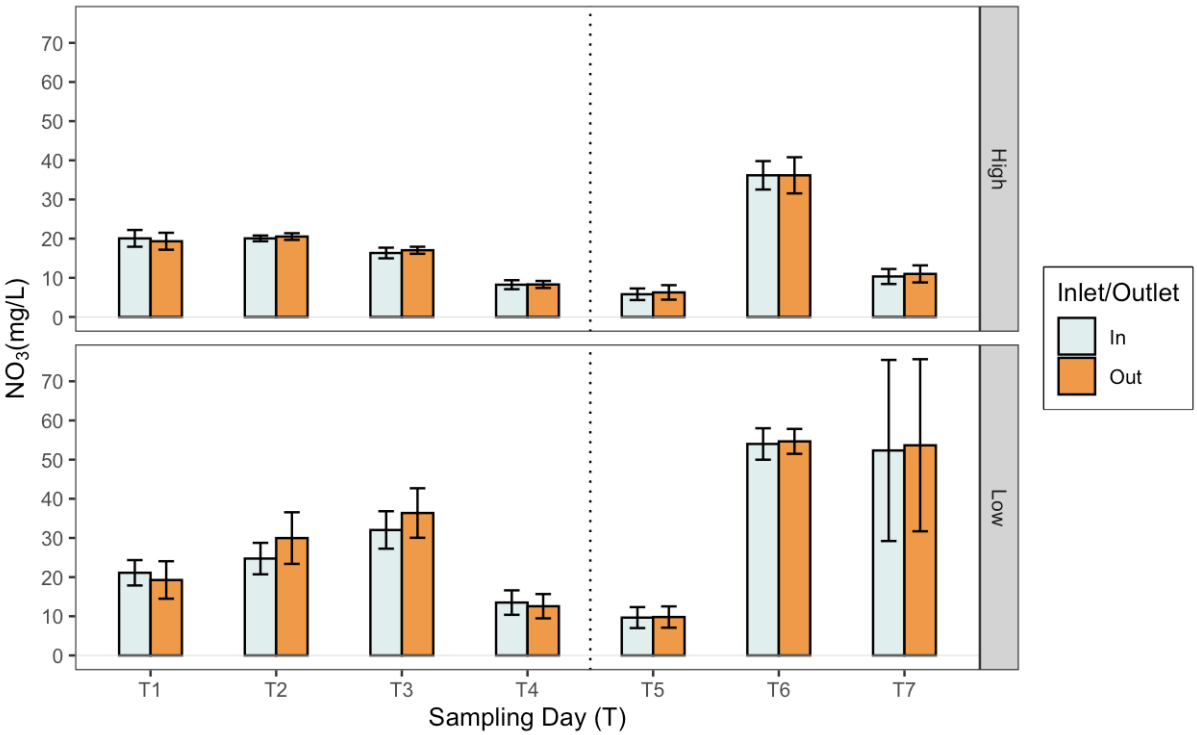

Fig 16. Nitrate concentration (mg NO_3/ L) at the inlet and the outlet of the moving bed bioreactor (MBBR). *Mean NO_3-N in water samples collected at the inlet and outlet of biofilters operated under different particle loadings, high and low. T1-T7, on the x-axis represents sampling days. Each bar represents the calculated mean ± SEM, n=3 for the inlet and outlet under both operational conditions. Vertical dotted line represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the biofilter was changed.*

Statistical analysis suggested that there was no difference in nitrite concentration at the outlet compared to the inlet for both high and low particle load systems (Two-way ANOVA, $F_{1,80} = 0.05$, $p > 0.8$). There was neither a significant difference in Δ NO_2-N (Δ= outlet-inlet) between high and low particle load (One-way ANOVA, $F_{1,40} = 1.53$, $p = 0.22$, Fig. 16, see also Appendix V, Fig. IV).

3.4 Summary of water quality parameters

Table 2. *Averages of the different water quality parameters across all sampling days throughout the experimental period. Each number represents the calculated mean± SD, n represents sampling numbers.*

	High Load		Low Load		
Parameter	**In**	**Out**	**In**	**Out**	**n**
# part/ml	9833± 2110	8193± 786	4421± 2024	4163± 726	15
TSA (mm^2/mL)	0.67± 1.01	0.61± 0.81	0.09± 0.04	0.10± 0.04	15
TSS (mg/L)	4.2± 5.1	3.3± 3.1	1.3 ±0.7	1.4± 0.5	18
Beta	3.00± 0.25	2.78± 0.20	3.20±0.21	3.06± 0.22	15
Microbial activity (h^-1)	0.15± 0.26	0.14± 0.25	0.02± 0.01	0.03± 0.01	21
Microbial activity bio-media (h^-1)	1.88± 0.57		1.55± 0.29		24
COD (mg/L)	29.8± 21.3	29.5± 21.1	18.6± 9.9	18.8± 10.1	21
TAN (mg/L)	0.52± 0.25	0.36± 0.18	0.32± 0.13	0.26± 0.12	21
NO_2^- (mg/L)	0.38± 0.31	0.35± 0.33	0.22± 0.13	0.21± 0.14	21
NO_3^- (mg/L)	16.71± 10.15	16.94± 10.13	29.62± 21.58	30.90± 22.12	21

In some water quality parameters, sampling day seven (T7) had a large impact on the calculated averages across the experimental period and resulted in large SD. However, when T7 was removed from these calculations, there were still significant differences between the calculated means. Table 3 represents the calculated means when T7 is removed.

Table 3. *Averages of different water quality parameters across sampling days excluding sampling day 7 (T7) where this particular sampling day had a large impact on the calculated means. Each number represents the calculated mean± SD, n represents sampling numbers.*

	High Load		Low Load		
Parameter	**In**	**Out**	**In**	**Out**	**n**
TSA (mm^2/mL)	0.18±0.11	0.20±0.10	0.08±0.05	0.09±0.04	12
TSS (mg/L)	2.20±1.40	2.02±1.02	1.14±0.63	1.32±0.50	15
Microbial activity (h^-1)	0.05± 0.02	0.05±0.03	0.02±0.01	0.03±0.01	18

3.5 Ammonia spiking of biofilter

3.5.1 Freshwater phase

Bio-media in the reference experiment before exposure to the operational conditions had a TAN removal rate of 23.10 mg N/L/day (k_0) (Fig. 17). Giving a STR_0 of 0.074 g N/m^2/day (Eq. 9). The removal of TAN did not reach levels below 1 mg/L and the highest R^2, for each replicated individually, was achieved when datapoints from the whole sampling period was included. Thus, 1'order TAN removal kinetics was not obtained in the freshwater reference experiment. Random variance caused by replicates was 6.1% (Appendix III-II).

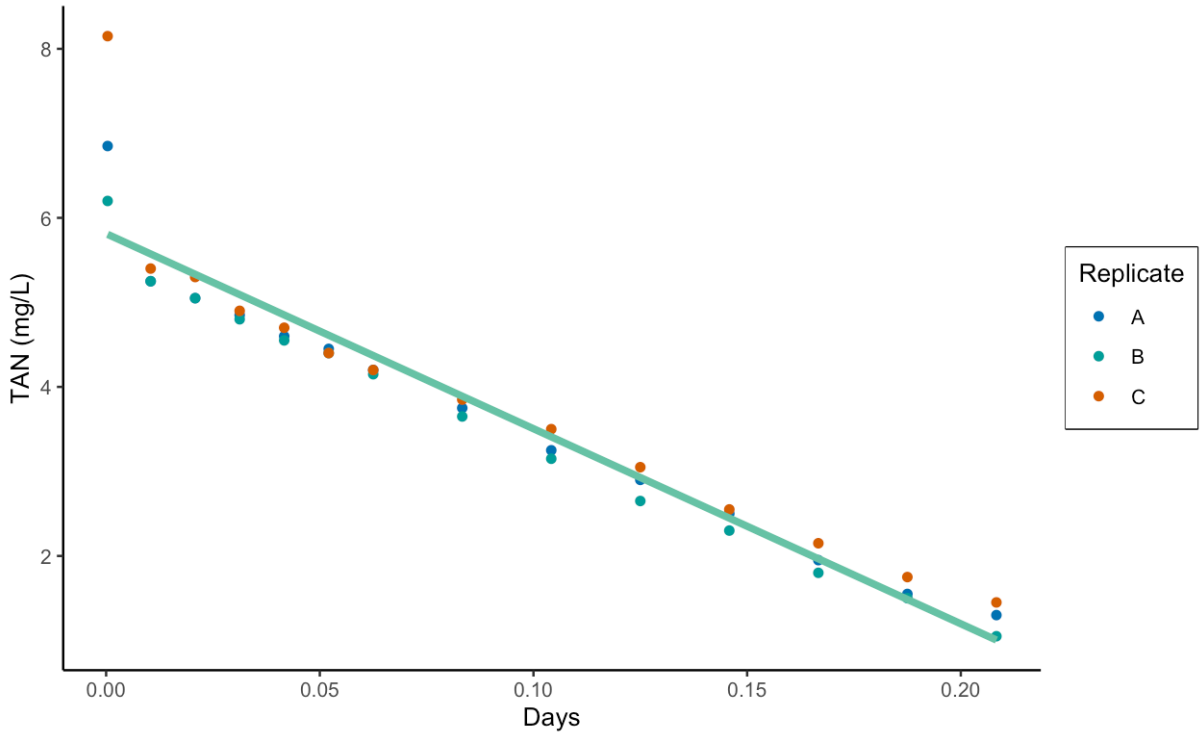

Fig 17. 0' order TAN removal in freshwater maturated bio-media spiked with ammonia prior to exposure of experimental operational condition. *The different point represents the different replicates that was spiked with ammonia separately. The green line represents the linear regression summarizing the overall removal in the three replicates. The y-axis is the concentration of TAN (mg/L), it is measured over days represented on the x-axis. R^2= 0.92*

Bio-media collected from RASs that operated at low particle load had a TAN removal rate of 22.57 mg N/L/day and the ones operated at high particle load had a removal rate of 24.27 mg N/L/day (Fig. 18). Giving a STR_0 of 0.16 g N/m^2/day and 0.17 g N/m^2/day, respectively. There was large random variance both within high and a low particle load RASs (Appendix III-II, see also Appendix VI, Fig. V).

There was no result from the statistical analysis suggesting that the 0' order TAN removal kinetics was different between the RASs that received a high and the ones that received a low particle load (LMM, $t_{64} = 1.8$, $p > 0.07$, Fig. 18).

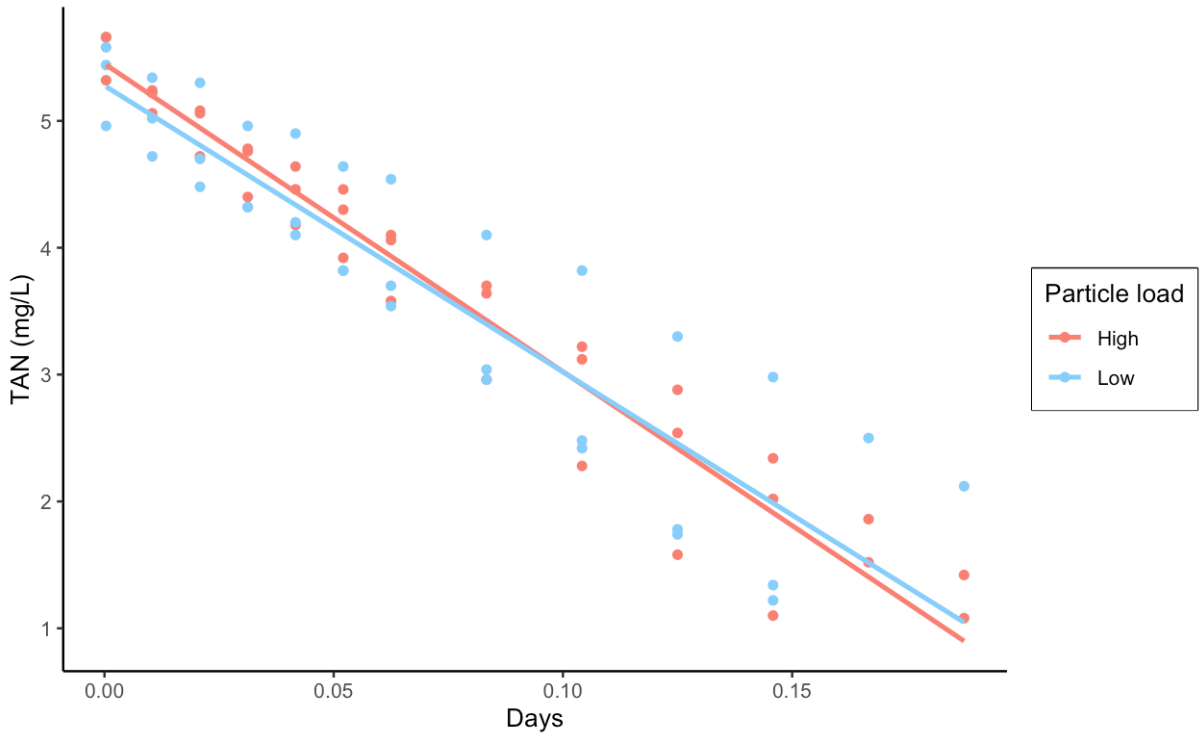

Fig 18. O' order TAN removal in freshwater bio-media spiked with ammonia after exposure to high and low particle loading. *Blue points represent the triplicate exposed to low particle load, red represents the triplicate exposed to high particle load. The blue and red line represents the linear regression summarizing the overall removal in the triplicates. The y-axis is the concentration of TAN (mg/L), it is measured over days represented on the x-axis. High particle load R^2=0.94. Low particle load R^2=0.80.*

TAN removal under 1'order conditions (k_1) was 22.94 day^{-1} for the low particle load- and 18.95 day^{-1} for the high particle load RASs (Fig.19). Giving an area-specific 1'order reaction rate constant (k_{1a}) of 0.17 m/day for the low particle load RASs and 0.14 m/day for the high particle load (Eq. 10). Only one of the RASs that received a high particle load reached the 1'order TAN removal kinetics under the sampling time, two systems exposed to low particle load reached 1'order kinetics (Fig. 18, see also Appendix VI, Fig. VI).

Results from the statistical analysis suggested that the slope for 1'order TAN removal was steeper for the RASs that received a low particle load (LMM, $t_4 = 4.9$, $p < 0.01$, Fig. 19).

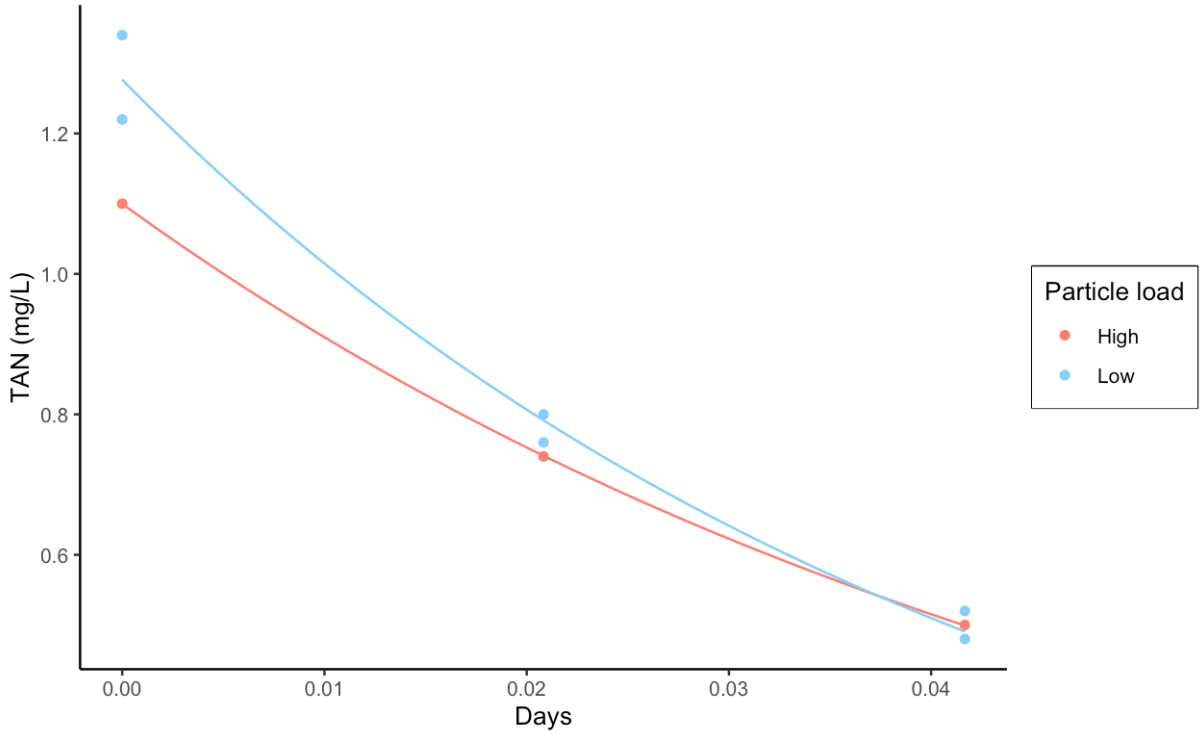

Fig 19. 1' order TAN removal in freshwater bio-media spiked with ammonia after exposure to high and low particle loading. *Blue points represent systems exposed to a low particle load, red represents a system exposed to high particle load. The blue and red line represents the exponential regression summarizing the removal of TAN in the systems. Note that only one of the RASs that was operated with a high particle load reached 1'order kinetics, the curve is therefore a representation of this RAS only. Two of the RASs operated with a low particle load reached first order kinetics. The y-axis is the concentration of TAN (mg/L), it is measured over days represented on the x-axis. Low particle load* R^2= 0.99.

3.5.2 Brackish water phase

The reference experiment conducted on brackish water maturated bio-media, before they were exposed to the operational conditions, showed a TAN removal rate of 22.23 mg N/L/day (k_0) (Fig. 20). Giving a STR_0 of 0.16 g N/m^2/day. None of the replicates in the reference experiment conducted on brackish water maturated bio-media reached concentrations below 1 mg/L and the highest R^2 was achieved by including all datapoints from all sampling times. The brackish water reference experiment did therefore not include sufficient datapoints to represent 1'order TAN removal kinetics. Random variance caused by the replicates was 5% (Appendix III-II).

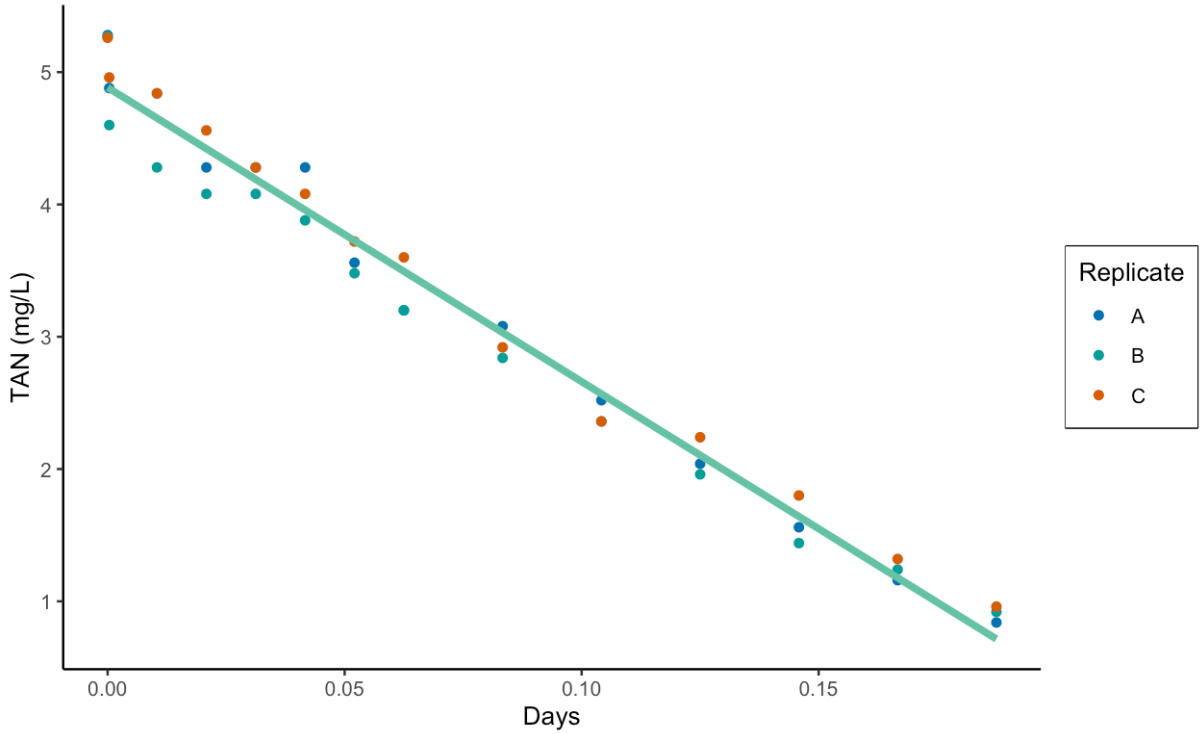

Fig 20. 0' order TAN removal in brackish-water maturated bio-media piked with ammonia prior to exposure of experimental operational condition. *The different point represents the different replicates that was spiked with ammonia separately. The green line represents the linear regression summarizing the overall removal in the three replicates. The y-axis is the concentration of TAN (mg/L), it is measured over days represented on the x-axis.* $R^2 = 0.98$

TAN removal rate after brackish water conditions was 27.53 mg N/L/day for low particle load and 32.06 mg N/L/day for high particle load RASs (Fig 21). This gave STR_0 of 0.20 g N/m^2/day for the low particle load systems and 0.23 g N/m^2/day for the high particle load systems. Random variance caused by the individual systems was also large after the brackish water phase (Appendix III-II, se also Appendix VI, Fig. VII).

The statistical analysis suggested that the slope for 0'order TAN removal was flatter for the RASs that received a low particle load (LMM, $t_{59.2} = 3.5$, $p < 0.001$, Fig. 21).

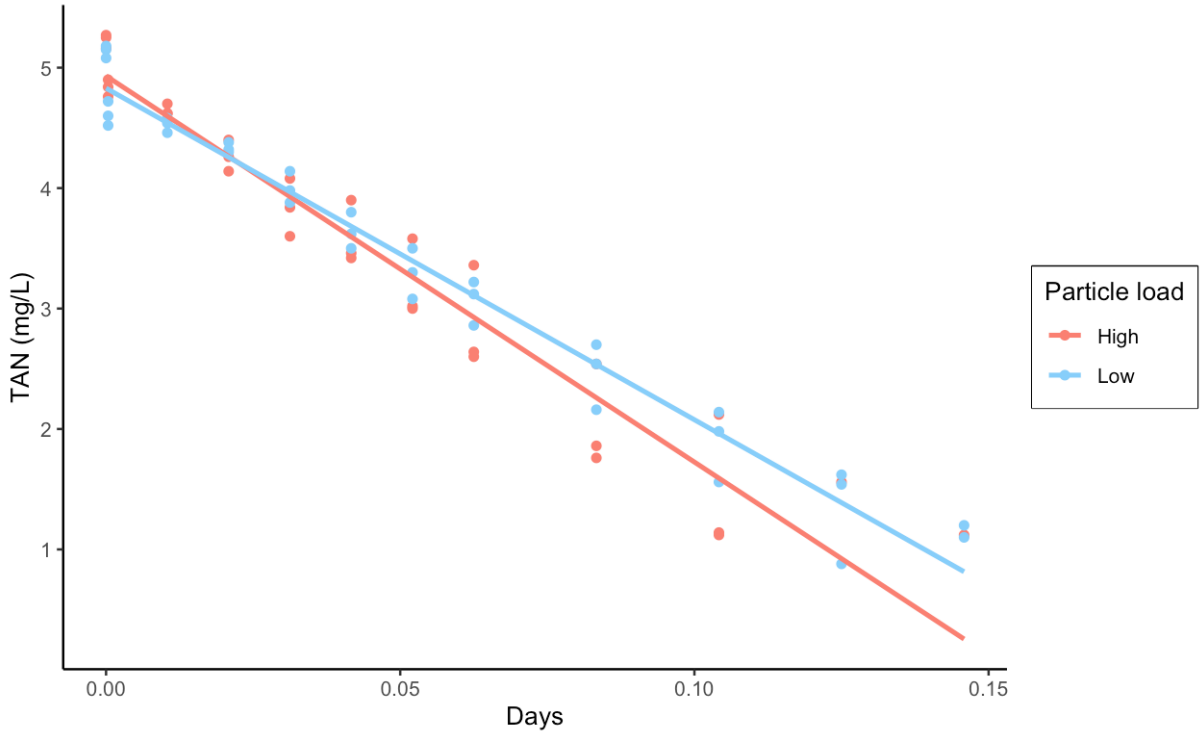

Fig 21. O' order TAN removal in brackish-water bio-media spiked with ammonia after exposure to high and low particle loading. *Blue points represent the triplicate that was exposed to a low particle load, red represents the triplicate exposed to high particle load. The blue and red line represents the linear regression summarizing the overall removal in the triplicates. The y-axis is the concentration of TAN (mg/L), it is measured over days represented on the x-axis. High particle load R^2=0.93. Low particle load R^2=0.97.*

1'order kinetics after the brackish water phase showed a TAN removal of 23.03 day^{-1} for the low particle load- and 26.38 day^{-1} for the high particle load RASs (Fig.19). This gave an area-specific 1'order reaction rate constant (k_{1a}) of 0.17 m/day for the low particle load RASs and 0.19 m/day for the high particle load (Eq. 10). There was large random variance caused by both the individual systems, especially for the low particle load triplicate (Appendix III-II, se also Appendix VI, Fig. VIII).

There was no result from the statistical analysis suggesting that the 1' order TAN removal kinetics was different between the RASs that received a high and the ones that received a low particle load, after the brackish water phase (LMM, $t_{15.2} = 0.1$, $p > 0.9$, Fig. 18).

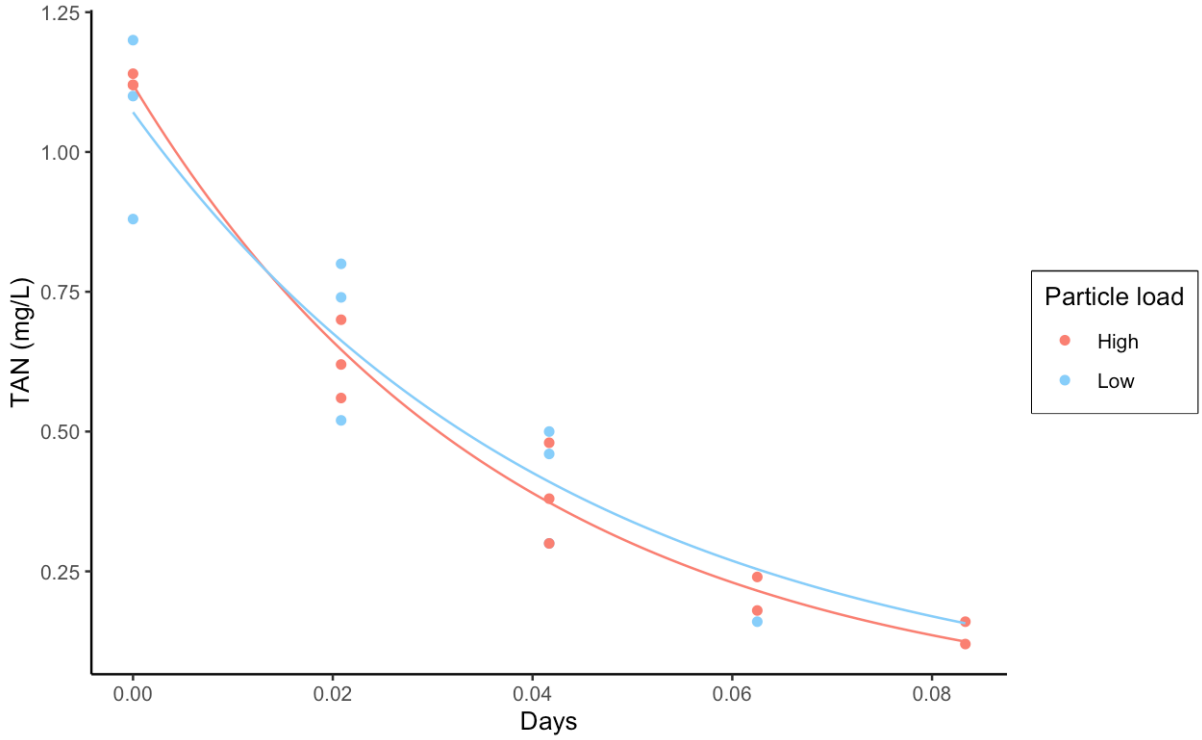

Fig 22. 1' order TAN removal in brackish-water bio-media spiked with ammonia after exposure to high and low particle loading. *Blue points represent the triplicate that was exposed to a low particle load, red represents the triplicate exposed to high particle load. The blue and red line represents the exponential regression summarizing the overall removal in the triplicates. The y-axis is the concentration of TAN (mg/L), it is measured over days represented on the x-axis. High particle load* R^2*=0.98. Low particle load* R^2*=0.87.*

4. Discussion

4.1 Operational conditions, particles and their distribution

4.1.1 Operational conditions through the experiment

The target operational condition of 12-15 mg TSS/L in the systems with high particle loading was not achieved. The operational conditions for the control systems, mean value of 1-2 mg TSS/L, were maintained throughout the experimental period. The TSS levels in the high particle load RASs fluctuated throughout the whole experimental period. This had the effect of making the differences between the two treatment groups less noticeable at some sampling days and more apparent at others. Even though the measured difference in TSS was not at the expected levels, it was possible to distinguish the difference in average TSS between the two treatments.

At several times during the experimental period the added waste in the systems with a high particle load caused challenges related to fish health and operational challenges such as clogging of pipes. When these challenges occurred MUW was increased, and accumulated particles were thus diluted. The effect on particle loading of such an event could be observed in the weekly TSS measurements just before sampling day 1 (T1), where TSS measurements in the high particle load systems reached a low point (Fig. 7). The average recirculation intensity in the high particle load right before this bottom was 2178± 570 L MUW/kg feed (See appendix I, Fig. I). Another low point could be observed in the high particle load systems right after sampling day 2 (T2). The days before this low level the fish were not fed, so there was little waste production resulting in no added waste to the tanks. This could have caused the TSS to drop in the high particle load systems during this period. Furthermore, from sampling day 6 to 7 high particle load systems were operated at relatively stable recirculating intensity around 238± 81 L MUW/kg. During this time particles had time to accumulated without dilution from MUW, indicated by high weekly TSS measurements in the high particle load systems.

The variation within the treatment group that received a high particle load tended to be greater than within the treatment group that received a low particle load, this was especially true at times when weekly TSS measurements increased. However, this did not seem to have a major impact on the variance resulting from the individual RASs (random effects). Which may come from the fact that the differences within the treatment group were less than the difference between the treatment groups. Possible reasons why some of the high particle load RASs increased more than others are further discussed in Appendix I-I and I-II.

4.1.2 Particles and their distribution

In the present study high particle load led to an increased total particle surface area (TSA) and total suspended solids (TSS) compared to systems that received a lower particle load. When examining the sampling days individually, the increased TSA was found to be statistically significant only on certain sampling days. Particularly, sampling day 7 stood out and this day coincided with the highest weekly TSS measurement. Previous literature has stated that increased intensity leads to accumulation of particles (Pedersen et al., 2017, Patterson and Watts, 2003). This indicates that the simulated increased intensity achieved through extra added particle in high systems led to the elevated TSA.

All systems were operated with mechanical filtration to continuously remove particles. Mesh size of the filters were 40 µm suggesting that the increased TSA, measured after mechanical filtration, mainly originates from particles smaller than this. Measured particle concentrations in the present study supported this, as 99% of the particle were under 40 µm in all water samples collected across all sampling days (see Appendix II-II). The calculated β-values also indicated that particles in all six systems were distributed towards smaller sizes, with β-values ranging from 2.6-3.4. Similar beta values (2.6-3.6) have previously been described in recirculating aquaculture systems operated under similar intensities (Patterson and Watts, 2003, Fernandes et al., 2014).

Further, when β-values were compared between the two treatments, results showed that low particle load systems were dominated by smaller particles to a larger degree compared to high particle load systems. This is in contrast to previously described effects of increased intensity in RAS where the predominance of smaller particles increases as intensity increases (Patterson et al., 1999, Pedersen et al., 2017). It is though important to mention that the extra added particles in this experiment were collected from the swirl separator and introduced to the system in one go. In an intensive RAS where MUW/feed is minimized, accumulation of particles is a result of continuous feeding and resulting waste that break down into smaller particles by the various treatment processes trough the circulation loop, especially in a MBBR (Pulkkinen et al., 2019, Fernandes et al., 2017). In the low particle load systems, the largest particles were removed by the swirl separator, allowing smaller particles to continuously break down. These large particles where added back to the systems once a day in the high particle load systems. The effect of this could be observed in PSD measurements. There was observed particles above 200 µm both at the inlet and at the outlet of the biofilter, in the high particle load systems at several sampling days. In the systems with a low particle load, there were individual cases of observations of particles over 100 µm on some sampling days. There were

also observed differences in the lower scale of the PSD measurements; 92% of the particles in the high particle load system and 97% of the particles in the low particle load systems constituted of particle below 5 µm. This indicates that particles in the high particle load systems were more evenly distributed compared to the low particle load systems, affecting the β-values significantly. Furthermore, this can also be visually observed in Appendix IV Fig III, where larger particles contribute to the total particle surface are to a larger degree in the high particle load RASs than in the low particle load RASs.

In high particle load systems particles removed by the drum filters were added back into the circulation loop in the pump sump, where the biofilter outlet samples were collected. This may be the reason why the β-values decreased in the samples collected at the outlet of the biofilter, compared to samples collected at the inlet of the biofilter. On the other hand, low and high particle load systems showed a trend of decreasing β-values and particle concentration between the inlet and outlet of the biofilter (Fig. 10). This implies particle loading not being a result of this particle associated difference between the inlet and outlet. This is in contrasts to findings in Fernandes et al. (2017), where MBBRs caused particles to disintegrate to smaller ones, causing higher β-values and particle concentration at the outlet of the biofilter. Contrasting results to the present study were also found in Interdonato (2012) where TSS increased from the inlet to the outlet of the biofilter, indicating that more particles are present after a moving bed biofilter. Clogging of bio-media was observed on several occasions during the experiment, in both high and low particle load systems. During these conditions the biofilter may have worked similarly to a fixed bed biofilm reactor (FBBR) removing particles from the water (Fernandes et al., 2017, Pulkkinen et al., 2019) and storing them in the biofilter. When the bio-media then started to up well, the excessive biofilm formation may have shed of (Rusten et al., 2006, Kamstra et al., 2017), causing fewer but larger particles to accumulate at the outlet of the biofilter compared to the inlet.

The highest TSA was observed in RASs operated with high particle loading. Measurements indicated that their PSD had a smaller predominance of small particles. Although this contrasts with previously described effects of increased intensity, measured β-values are comparable to those mentioned in other works of literature. Results indicate that there is a significant relationship between elevated particle load and total particle surface area (TSA). Based on this, the null hypothesis, H01, is rejected and it is concluded that elevated particle load does result in an elevated TSA in an intensive RAS, in line with the alternative hypothesis HA1.

4.2 Microbial activity

Increased TSA provide bioavailable substrate and surface area for heterotrophic bacteria to settle on (Michaud et al., 2006, Pedersen et al., 2017). In the present study RASs that received a high particle load showed a significantly higher microbial activity (k-value) compared to the systems that received a low particle load. This applied both in samples taken outside and in the biofilter itself. A biofilter is design to provide good growth conditions for nitrifying bacteria and is therefore the place in the circulation loop with the highest bacterial activity (Leonard et al., 2000). In the present study, microbial activity on bio-media exposed to a high particle load was significantly higher than the activity on bio-media exposed to a low particle load. An increase of carbon can lead to an increase of heterotrophic bacteria (Ling and Chen, 2005, Michaud et al., 2006, Guerdat et al., 2011). Okabe et al. (1996) found that an increased C/N ratio led to a four times thicker biofilm, of which heterotrophs dominated all layers of the biofilm and nitrifiers were only present in the innermost layers. Additionally, Qi et al. (2022) suggested that the lack of heterotrophic bacterial activity on bio-media was a result of removed organic matter before carriers were exposed to RAS-water. Considering the set-up of the present experiment and findings in other studies, the increased microbial activity on the bio-media of the RASs with high particle load may have been the result of a thick biofilm in which heterotrophic bacteria dominated.

In the present study low particle load systems showed close to no microbial activity in water samples, while there were detectable levels of microbial activity with mean k-values of 0.14 h^{-1} at the inlet- and 0.15 h^{-1} at the outlet of the biofilter in water samples from the high particle load RASs. Rojas-Tirado et al. (2018) showed that increased feed loading resulted in increased microbial activity in RAS water samples. Implying that increased feed loading dictates the amount of available organic matter, which again affects the microbial activity in RAS water. This is in line with findings in the present study where increased particle loading resulted in detectable microbial activity in RAS water samples. Michaud et al. (2006) showed that an increased C/N ratio, which was achieved by adding organic material from the swirl separator, increased free-living heterotrophic bacteria in the biofilter effluent. In the present study the average particle concentration across the whole experimental period was almost twice as high in the high particle load systems compared to the low particle load systems. As there were detectable levels of microbial activity in the high particle load systems, this suggests that these systems supported free-living and/or particle associated heterotrophic bacteria, which was not detected in the low particle load systems.

4.3 Organic compounds

Additional information about organic compounds were provided through COD measurements. RASs that received a high particle load had a higher COD compared to those who received a low. Interestingly, none of the water samples, taken from both high and low RASs, showed a difference between total- and dissolved COD. Previous studies have shown high values of dissolved COD compared to particulate COD ($COD_{part} = COD_{tot} - COD_{diss}$) in RASs (Rojas-Tirado et al., 2017, Fernandes et al., 2017, Rojas-Tirado et al., 2018). This is supported by findings in the present study and results implies that COD_{diss} made up most of the COD_{tot} regardless of particle loading. At the inlet, dissolved COD accounted for 99% of the total COD, while at the outlet, it comprised 97% of the COD. Under both particle loadings, the PSD measurements revealed a strong distribution of particles towards smaller sizes. Moreover, these findings provide additional evidence that dissolved matter under 0.2 µm also constitute a significant portion of the organic material.

Neither microbial activity nor COD measurements showed any differences between the inlet and outlet of the biofilter, in both the high and low particle load systems. This contrasts with previous literature where there has been observed increased bacterial activity between the inlet and the outlet of biofilters (Leonard et al., 2000, Michaud et al., 2006, Suhr and Pedersen, 2010). In Suhr and Pedersen (2010) no difference in influent and effluent COD concentrations were detected even though bacterial activity was higher at the inlet compared to the outlet. At sampling day 7 in the present study, i.e., the sampling day with one of the highest weekly TSS measurement, the same tendency of microbial activity between the inlet and outlet was found in the high particle load RASs: COD_{tot} measurements showed no difference between the inlet and outlet of the biofilter, while the average k-value decreased by 0.04 h^{-}. This could indicate that some bacteria were stored within the biofilter when water passed through. And could potentially explain the small difference in COD_{part} (1% vs. 3%) between the inlet and the outlet of the biofilters, as the increased COD_{part} could be a result of biofilm shed. However, the tendency of decreased microbial activity was not observed on other sampling days. A key factor in increasing bacterial activity in RASs is increased TSA (Pedersen et al., 2017, Michaud et al., 2006) and there was no significant difference in TSA between the outlet and the inlet of the biofilter at any other sampling days. This observation can be attributed to the absence of significant variations in microbial activity on the remaining days.

Results from the present study implies that a high particle load increases the microbial activity in a RAS, both free living and particle associated in the water, and on bio-media in the MBBR itself. Based on this the null hypothesis, H02 is rejected, and concluded that elevated

particle load does result in an elevated microbial activity, in line with the alternative hypothesis HA2. Small tendencies were observed which indicated that an increased particle load could cause differences in microbial activity between the inlet and the outlet of a biofilter. This tendency was only observed at one sampling day (T7, n=3) and other sampling days did not provide significant levels of the same tendency. Even though results regarding differences in microbial activity at the inlet and outlet contradict earlier literature, null hypothesis H03 is accepted, and conclude that there is no difference in microbial activity at the inlet and the outlet of the biofilter exposed to high and low particle loading.

4.4 Inorganic compounds (TAN, Nitrite and Nitrate concentration)

Previous literature has shown that increased TSA inhibits biofilter performance, due to good growth conditions for heterotrophic bacteria, increasing the concentration of total ammonia nitrogen (TAN) and nitrite (NO_2^-) and decreasing the amount of nitrate (NO_3^-), in intensive RAS water. In these cases, heterotrophic bacteria outcompete AOB and NOB resulting in accumulation of ammonia and nitrite and a lack of conversion of these two compounds to the less toxic end product, nitrate (Zhu and Chen, 2001, Pedersen et al., 2017, Ling and Chen, 2005). When less MUW is added per unit feeding, nitrate will accumulate as this is the end product of nitrification. Thus, a functional RAS with intensive recycling and high feed loading can be indicated by its concentration of nitrate. However, if there is not substantial removal of organic matter, TAN and nitrite can accumulate, indicating a RAS where nitrifiers get outcompeted by heterotrophic bacteria. In the present study elevated levels of TAN were observed in high particle load RASs, which implies systems where the nitrifying bacteria are constrained. This observation was strengthened by lower concentrations of the nitrification end product, nitrate, in high particle load RASs. In comparison low particle load RASs had a lower concentration of TAN and showed increased levels of nitrate, indicating biofilters where nitrifiers are less constrained. Low concentration of nitrate in the high particle load RASs can be a result of periodically large water exchange. On the other hand, low particle load systems also had days with high-water exchange and the average recirculation intensity across the experimental period was very similar in both treatment groups.

In Rojas-Tirado et al. (2017) a peak followed by stabilization of TAN and nitrite (NO_2^-) concentration were shown a few days after startup of six individual RASs. In the present study the same tendency was observed in systems that were exposed to both high and low particle loading. It is important to note that in the present study all biofilter contained maturate bio-

media before startup, in contrast to Rojas-Tirado et al. (2017). In the maturation tanks bio-media was fed with NH_4Cl once a day, upon their transfer to the RASs they were continuously exposed to ammonia from fish nitrogen metabolism. Von Ahnen et al. (2015) found indications of that biofilters operated at lower TAN loadings did not respond well to sudden increases in TAN concentration. This observation aligns with the present study, wherein both ammonia and nitrate concentrations exhibited peaks during the initial phase of the experimental period.

Nitrate concentrations increase proportionally to ammonia and nitrite during a complete nitrification process (Colt et al., 2006, Kinyage et al., 2019). In the present study the production of ammonia through fish nitrogen metabolism should be relatively similar in all systems. As the fish consumed very similar amounts of feed throughout the experimental period, and the number of fish were the same in each tank (Ebeling and Timmons, 2010, Dalsgaard and Pedersen, 2011). The average recirculation intensity was also similar between the two triplicates (Appendix I-II Fig. I). However, if one compares the measurements of the nitrogen compounds between systems with high and low particle loads, the systems with high particle loads indicate intensive RAS that have a less complete nitrification process than the systems with low load. This suggest that the nitrifying performance of the biofilters exposed to a high particle loading was poorer than those exposed to a low. This is similar to the results obtained in Ling and Chen (2005) where inhibition on nitrification by organic matter was observed due to good growth conditions for heterotrophic bacteria.

Temperature, oxygen, substrate concentration, salinity, particle loading, water flow and alkalinity are all factors that affect ammonia turnover rates in the biofilter (Rusten et al., 2006, Suhr and Pedersen, 2010, Badiola et al., 2012, Prehn et al., 2012, von Ahnen et al., 2015, Kinyage and Pedersen, 2016, Kinyage et al., 2019). In the present study, all these factors were relatively similar in all six systems, apart from particle loading and substrate concentration. In both triplicates, a consistent trend was observed where TAN concentration was higher at the inlet compared to the outlet of the biofilter. Notably, the high particle load systems exhibited a ΔTAN (inlet-outlet) that was 0.09 mg/L higher compared to the low particle load systems. This could indicate that high particle load systems removed more TAN when water passed through the biofilter. However, the average TAN concentration across all sampling days was higher in the high particle load systems. As the concentration of TAN is a limiting factor for TAN turnover (Zhu and Chen, 1999), this could explain why low systems had lower ΔTAN values than high particle load systems. On the other hand, little ammonia gets removed each time water circulates the system (Colt et al., 2006, Prehn et al., 2012). In the present study, water samples were collected from the inlet and outlet of the biofilter at approximately the same time. In Prehn

et al. (2012) it was observed that increased water velocity increases TAN transport from water to the biofilm resulting in a higher ammonia turnover. According to this, TAN levels should be relatively stable throughout the system as it is the number of times water circulates the systems that increases the TAN turnover. This contrasts with the trend observed in the present study, where TAN concentration was higher at the inlet compared to the outlet of the biofilter. On the other hand, in Suhr and Pedersen (2010) differences larger than 1 mg N/L between the inlet and outlet of biofilters were observed at several water velocities, implying that it can be relatively large variations between the inlet and the outlet of biofilters. This further suggests that the differences in ΔTAN between high and low particle load systems was a result of substrate concentration being lower in the low particle load systems.

In the present study increased concentrations of TAN were observed in the RASs that received a high particle load. In addition, lower concentrations of the end product of nitrification processes, nitrate, were observed. This indicated that high particle load systems had biofilters that were constrained compared to low particle load systems. Contrastingly high particle load systems had a significantly higher ΔTAN values than low particle load systems. However, this was likely due to the lack of substrate concentration ([TAN]) in the low particle load systems. Therefore, the null hypothesis is rejected, H04, and it is concluded that high particle loads influence the levels of nitrogen compounds (TAN, nitrite, and nitrate) in the RAS-water, HA4.

4.5 Nitrifying performance of the biofilters

Increased TSA caused by increased particle loading can promote the growth of heterotrophic bacteria, which can outcompete nitrifiers and result in accumulation of TAN and nitrite indicating poor nitrifying performance of a biofilter (Van Rijn et al., 2006, Ebeling and Timmons, 2010). Indications of these factors—increased TSA, bacterial activity, and TAN was observed in the present study, indicating that the biofilter in the RASs that were subjected to high particle loads performed poorly compared to those that were subjected to lower loads. However, when the bio-carriers were spiked with ammonia to test their nitrification performance, contrasting result were observed.

High and low particle load RASs had a higher STR_0 after both the fresh- and brackish water phase compared to the reference experiments. Additionally, within the time frame TAN removal was measured, none of the reference experiment replicates reached substrate dependent, 1'order TAN removal kinetics. These findings align with the earlier observations of

peaks in TAN and NO_2^- levels at the beginning of the experimental period. In the spiking experiment bio-media were exposed to a sudden increase to 5 mg N/L in ammonia concentration. Results indicate that bio-media constantly exposed to RAS water responded better to this increase than the bio-media that were fed with NH_4Cl once a day, in line with the findings in von Ahnen et al. (2015).

Following the freshwater phase in the current study, the STR_0 did not exhibit any significant differences between the systems with high and low particle loads. However, after the brackish water phase STR_0 was higher in the high load systems, indicating that bio-media exposed to a high particle load removed TAN faster under concentrations where the biofilm is fully penetrated and entirely saturated. These observations are not only in contrast to the indications given by TSA, microbial activity, and nitrogen compounds concentrations, but also in contrast to previous literature. In Carrera et al. (2004) and Ling and Chen (2005) there was found an exponential decrease in nitrification rate when C/N ratios were increased. Similar results have been observed in many other previous studies (Zhu and Chen, 2001, Michaud et al., 2006). In all these studies nitrification processes were strongly inhibited by heterotrophic processes. However, at C/N ratios above 1 the inhibitory effect of heterotrophs on nitrification tended to be the same. According to these studies, the greatest influence on biofilter nitrification performance will therefore be at C/N ratios between 0-1, and most RAS systems operate at C/N ratios of 1-2 (Carrera et al., 2004, Zhu and Chen, 2001, Ling and Chen, 2005). Further, in Zhu and Chen (2001) surface specific TAN removal (STR_0) was reduced form 1.5 to 0.481 $g/m^2/day$ when C/N ratios were at 1 or 2, compared to a C/N ratio of 0. In the present study, STR_0 ranged from 0.16 to 0.23 $g/m^2/day$. Considering this, organic matter could potentially have negatively affected the removal of TAN at both particles loading levels, resulting in similar nitrification rates in all six systems. Findings consistent with this were observed in von Ahnen et al. (2015) where increased feed loading did not reduce the zero order rate constant at higher feed loadings. In von Ahnen et al. (2015), it was also proposed that constant shear of biofilm within the MBBR may have counteracted the potential overgrowth of heterotrophs, thus maintaining the 0' order rate constants for nitrifiers. This could also be the case in the present study, as it aligns with the observed decrease in β-value from the inlet to the outlet in the high particle load systems.

Additional information on biofilter performance was provided by examining the removal of TAN under substrate dependent, 1'order kinetics. After the freshwater phase results indicated that the area-specific 1'order reaction rate constant (k_{1a}), was higher in the RASs that received low particle loading. On the other hand, after the brackish water phase there was no significant difference in k_{1a} between the two treatments. Datapoints that provided the difference

in k_{1a} after the freshwater phase were very limited as only half of the systems, 1 from high load triplicate and 2 from low, reached conditions where TAN removal was substrate dependent. These results should therefore be interpreted with caution. However, the fact that only one of the systems exposed to high particle load reached 1' order TAN removal kinetics, after the freshwater phase, may indicate some differences in biofilter performance. In von Ahnen et al. (2015) increased biofilter loading resulted in an elevated TAN bulk concentration. Observations in the present study after the freshwater phase could be in line with these findings. The transition between 0'order and 1'order TAN removal kinetics usually occur around a TAN bulk concentration of 1 mg N/L, but it can also be calculated by multiplying the half saturation constant of the bacteria (K_s) by two (Prehn et al., 2012). In previous literature K_s values ranging from 0.3-0.7 g N/m^3 are often used (Knowles et al., 1965, Henze, 1997). However, it can also be calculated through the relationship between 0'order and 1'order rate constants; **Equation 12**, $k_{0a} = k_{1a} * K_s$. When this calculation method of K_s was applied to the present study, an elevated transition zone from 0'order and 1'order kinetics, were discovered in both high and low particle loadings ($C_{bulk}= 2K_s$). The TAN bulk concentration was 1.9 mg N/L in the low particle load systems after the freshwater phase, and 2.4 mg N/L after the brackish water phase. In the high particle load systems, the TAN bulk concentration was also 2.4 mg N/L after both the freshwater and brackish water phases. Von Ahnen et al. (2015) suggested that an increased feed loadings resulted in a thick biofilm where it switches from being fully penetrated to partially penetrated at higher TAN concentrations compared to thinner biofilms. Results in Chen et al. (2006) also suggested that the apparent half saturation constant (K_s) varies with respect to operational conditions. In the present study this could indicate that both high and low particle load RASs had biofilms that were sufficiently thick to increase the bulk concentration of which the transition zone from 0'order to 1'order appeared. If both systems had this thick biofilm, it could further explain why the systems differed little in their nitrifying performance. On the other hand, the linear line that described 0'order kinetics in the present study gave the highest R^2, this linear regression line had its last datapoint around 1 mg/L, indicating that the best fitted regression suggests a transition zone around this TAN concentrations. However, it is also important to mention that in the present study, ½′ order kinetics was disregarded, which takes place when biofilm only is partially effective due to limited substrate penetration but the nitrification follows 0'order kinetics (Prehn et al., 2012). In von Ahnen et al. (2015) ½′ order kinetics was disregarded by excluding datapoints between 1-2 mg N/L. Thus, it is not unlikely that both systems had an elevated transition zone from 0' to 1' order kinetics, further implying that the low particle load in the present study did not differ in nitrification performance

compared to the high particle load. Furthermore, it is important to point out that a very large part of the variance in both ammonia spiking experiments were caused by the systems (random effect), indicating that the individual systems had a higher effect than the fixed effect, particle loading. This accounted for both 0'order kinetics and 1'order kinetics (see Appendix III-II).

As systems received the same amount of feed, had the same fish biomass and results indicates that they had the same nitrification performance, the concentration of nitrogenous compounds should have been relatively similar in all systems. However, this was not the case in the present study. Results suggested significantly different concentrations of nitrogenous compounds. It is known that heterotrophic bacteria can produce ammonia, and bacteria that can do this has been observed in RAS biofilters (Rodrigues and Williams, 2001, Blancheton et al., 2013, Rurangwa and Verdegem, 2015). These heterotrophs decompose proteins of fish feed and faces to ammonia by proteases and deaminases. The presence of denitrifying heterotrophs has also been observed in RAS biofilters (Schreier *et al.*, 2010, Blancheton et al., 2013). It has been reported that biofilters exposed to high C/N ratios are capable of nitrate reduction to ammonia, this is detected by decreased nitrate and simultaneous increase in ammonia (Van Rijn et al., 2006, Schreier et al., 2010). If a sufficient abundance of these heterotrophs was the case in the present study, this could have shifted the accumulation of nitrogenous compounds as seen in the high particle load systems. As the specific bacterial communities were not assessed in the present study it is impossible to determine the possibility of this being the case. However, in future studies it would be interesting to see if the presence and detection of heterotrophs could increase the ammonia concentration in RAS water trough ammonification and denitrification. This would be beneficial given that the dimensioning of a RAS facility, especially the biofilter, may need to account for a significantly increased supply of ammonia beyond what the fish produce.

If one considers previously reported findings consistency with the current study and the high random effect, it is likely that there was no difference in nitrification performance between high and low particle loading. It was considered likely that the differences in nitrification performance that were observed could be a result of how the spiking experiment was carried out, this is elaborated in Appendix I. Interestingly, results could indicate that there was production of ammonia by heterotrophic bacterial activity. Thus, the null hypothesis, H05, is accepted and it is concluded that nitrification performance of the biofilter do not differ between high and low particle loadings, under the present experimental conditions.

5. Conclusion

This study aimed to investigate the impact of elevated particle loading in Recirculating Aquaculture Systems (RAS) by conducting a comparative analysis between systems subjected to high and low particle loading. The findings of this research offer valuable insights into the consequences of heightened particle loading in RAS. By adding particles, removed by the swirl separator and backwash from the drum filters, an increased particle concentration and total particle surface area (TSA) was obtained. The increased abundance of organic matter provided good growth conditions for heterotrophic bacteria, indicated by increased- microbial activity and TAN concentrations, along with decreased nitrate concentrations, which could outcompete nitrifying bacteria and inhibit biofilter performance. Nevertheless, no difference was observed in the nitrification performance of the biofilters when bio-media from both treatments were spiked with ammonia. Previous studies have shown reduction in nitrifying performance at low loadings of organic carbon, implying that organic matter may have negatively affected the removal of TAN at both particle loading levels in the present study. This suggestion aligns with the relatively low surface-specific TAN removal (STR) rates observed, ranging from 0.16 to 0.23 g/m2/day. Calculations conducted in the present study also suggested TAN bulk concentrations higher than the often-used transition zone around 1 mg N/L, both at low and high particle loadings. This implies that even with relatively low particle loadings, there is a possibility of biofilms only being partially penetration at relatively high TAN concentrations. Furthermore, it may also appear that high particle loads can support bacterial production of ammonia, giving an addition to the ammonia excreted by fish. Overall, this study emphasizes the importance of particle removal as increased particle loading can lead to both impaired removal of ammonia and increased supply of ammonia via bacterial production.

6. Further perspective

- Even though there were observed increased concentration of TAN in the high particle load systems there was only one single observation of TAN measurements over 1 mg TAN/L, which is a low indicative limit value of TAN. Future research should therefore aim to further increase the particle loading and/or stabilize high particle loadings over time to see if this could result in elevated ammonia concentrations that would be detrimental to fish health.

- Furthermore, in future research it would also be interesting to see if increased protein content in feed and feces could lead to increased bacterial production of ammonia through ammonification.

- In addition, future research should aim to further investigate if increased particle loading can lead to an increased TAN bulk concentration where the biofilm switches from being fully penetrated to partially penetrated. Calculations in the present study indicated an increased transition zone, but measurements of TAN removal over time indicated a transition zone that occurred at levels used and found by most literature on the topic. This could give valuable insight of how thick biofilms dominated by heterotrophs can affect the concentration of which a biofilter becomes substrate independent.

7. Reference

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Appendix I. Discussion of Material and Methods

I-I Method used to elevate TSS

In the present study particle load was elevated by adding collected waste from the swirl separator back to the systems pump-sump in the high particle load RASs. This was done once a day, usually around mid-day, during the whole experimental period. This method of increasing the particle load could have been the cause of several challenges that were met in the present study.

There was observed diurnal variations in TSS measurements, where it tended to peak shortly after particle addition and then decrease until the next day's addition. Diurnal differences were handled by ensuring that all sample collections, both weekly TSS samples and sampling days, were taken at the same time of the day during the whole experimental period; Weekly TSS measurements were taken approximately 30 minutes after particle addition, while sampling days were conducted in the morning, before waste addition. Therefore, it is likely that TSS was higher when weekly TSS measurements where conducted compared to the sampling days. The reason why sampling was done in the morning before waste addition was both to have enough time to carry out analyzes and to avoid getting excessively high measurements. It is important to note that weekly TSS measurement was used to track operational conditions, while the sampling days were used to investigate the effect of the particle loading itself.

As the fish grew during the experimental period more feed was needed to meet their requirements, this also resulted in a larger production of waste. Thus, increasing the added particles back to the high particle load systems as the experiment went. Although this meant that the same level of particles was not added each time, it was a good simulation of commercial production where the load also will increase as the fish grow. However, this did not seem to affect the weekly TSS measurements (Fig. 7).

Internal difference tended to be higher in systems that received high particle loads, this was especially true at the times when TSS peaked. Comparable results can be seen in Pedersen et al. (2017) where increased feed loading gave greater differences within triplicates. In the present study, this was most likely due to extra MUW being added at some experimental days and the high particle load systems being cleaned due to operational challenges. Several times over the experimental period MUW was increased in all systems, but not necessarily on the same day. These factors most likely had a large effect on the High particle load systems. When TSS increased in the systems, it increased more in some because there was a greater degree of accumulated particles from before, this may have led to the large differences between the high

particle load RASs. This was not the same case for the low particle load systems as they did not have the same amount of accumulated particles, thus increased MUW did not lead to the same impact within the triplicate.

Future studies, investigating the current topic, should aim to increase particle loading through a continuous supply. By distributing the loading, it may be possible to avoid peaks in TSS which resulted in operational challenges. Distribution of loading could help to maintain a more stable TSS, both from day to day and within days (diurnal). This could be managed by adding particles back to the system in a similar way of how feed is introduced to the system. In the present experimental set-up this would be by conveyer belts, which can be preset so that there is no need for personnel to be present.

I-II Experimental set- up and operating the different RASs

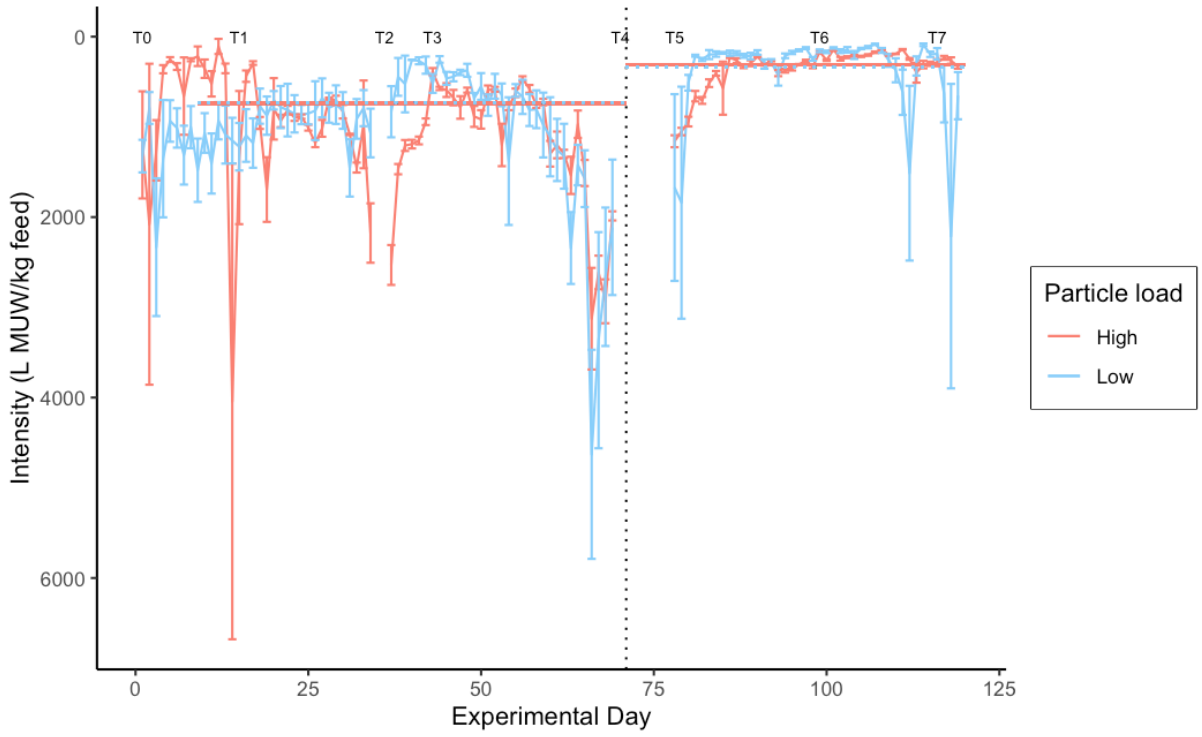
All RASs were operated with the same fish biomass in each tank, other working packages investigated how the fish were affected by the elevated particle load. As there were animals involved in the study their health and requirements were main priorities. At times, the particle loading was so high that the systems were hard to operate without compromising safety for the fish due to operational challenges. Systems used in the present study were small experimental RASs, where pipes and other RAS components are relatively small compared to commercial systems. Thus, clogging, and other challenges due to its size can become challenging at lower particle loadings. This could be detrimental for fish health, thus systems had to be cleaned and MUW had to be increased at several points during the experimental period. As a result, particles that had been accumulated over time had to be restarted again. These two factors, cleaning and increased MUW, are likely the cause of the fluctuating TSS in the high particle load RASs.

As stated in the previous chapter, increased MUW and cleaning did not necessarily happen on the same day. However, it usually happened within the same week. So, TSS in the systems with high particle load peaked and fell in a relatively similar manner, even though there were some internal differences (Fig. 7). As seen in Fig. 7 the high particle loads fluctuated during the whole experiment. However, no sampling days were placed at a peak in weekly TSS measurements, except for sampling day 7 (T7). On the other hand, it was observed that TSS could change very quickly, so measurements one day could be very different to the next. As weekly TSS was not measured at the sampling days, expect for T7, it could have peaked on other sampling days too. Nevertheless, sampling day 7 (T7) showed some interesting tendencies

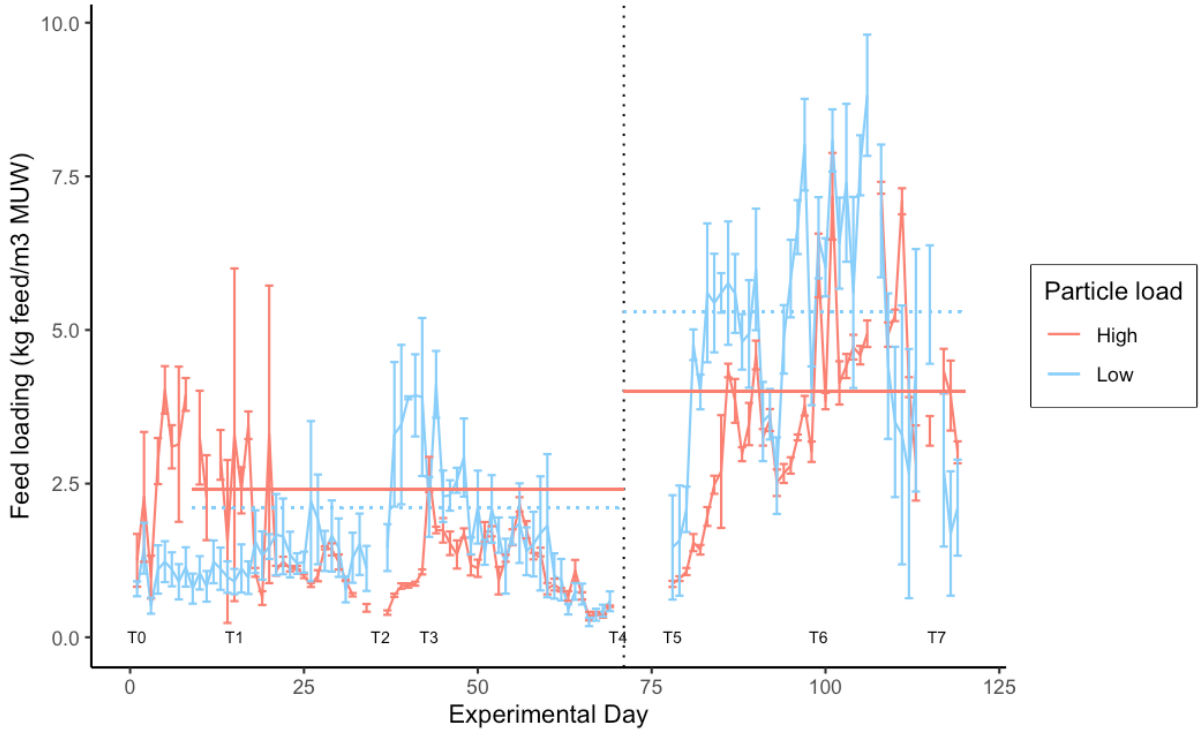
but was very high compared to the mean and the preset operational condition. The measurements were not interpreted as outliers since they corresponded to the water quality at this time and reflected the effect of particle load when sampling days was assessed as replicated measurements.

Throughout the entire experiment, the mean recirculating intensity (L MUW/kg feed) and feed loading (kg feed/m^3 MUW) were relatively similar in both triplicates (Figs. I and II). However, there were dips in feed loading and intensity over several experimental days. Before T1 (Fig. I and II), there was a valley in intensity and feed loading, and the weekly TSS measurements taken around this time were some of the lowest throughout the experimental period (Fig. 7). This supports the assumption that increased MUW has had a major impact on TSS measurements.

In the present study, bio-media in the biofilter was changed when systems were operated at brackish water salinity. During this transition, feeding was stopped, MUW increased, and particles associated with the biofilter removed. As a result, the systems had to start building up their "dirtiness" from scratch because the particles that had accumulated during the freshwater phase were zeroed out. If the bio-media was not replaced, higher TSS measurements could have been observed in the high particle load RASs towards the end of the experiment instead of the present valley that occurred after the transition (Fig. 7).



Appendix I Fig I. Recirculating intensity *Mean recirculating intensity (L MUW/kg feed) in RASs operated under two different conditions, High (red) and Low particle load (blue), from experimental day 8 to 116. The numbers on the x-axis represents the experimental days and each datapoint is the calculated mean ± SEM values, n=3 for each experimental group. Vertical dotted line (experimental day 73) represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the MBBR was changed. Horizontal lines represent the mean recirculating intensity through the two salinity phases, fresh-water (n=201) and brackish water (n=123), for both experimental groups, High (red) and low (blue). Note that some experimental days are not included, at these days the fish were starved or the amount of added MUW was very high, giving a very large impact on the means.*



Appendix I. Fig II. Feed loading *Mean feed loading (kg feed /m^3) in RASs operated under two different conditions, High (red) and Low particle load (blue), from experimental day 8 to 116. The numbers on the x-axis represents the experimental days and each datapoint is the calculated mean ± SEM values, n=3 for each experimental group. Vertical dotted line (experimental day 73) represents the shift from 1-2‰ salinity (FW) to 15‰ salinity (BW) and when bio-media in the MBBR was changed. Horizontal dotted lines represent the mean recirculating intensity through the two salinity phases, fresh-water (n=201) and brackish water (n=114), for both experimental groups, High (red) and low (blue). Note that some experimental days are not included, at these days the fish were starved or the amount of added MUW was very high, giving a very large impact on the means.*

As it was difficult to avoid operational challenges in the present experimental RASs, when particles accumulated in the systems, it became a limiting factor. This can be solved in future studies by e.g., comparing commercial RASs that have different levels of mechanical filtration. An experimental setup where one is not limited by animal welfare, as done in Qi et al. (2022), could also be an option. Ammonia could also be added artificially instead of

depending on the fish's nitrogen metabolism, similar to Zhu and Chen (1999). Future studies that have a transition in salinity can also avoid removal of accumulated particles by not replacing the bio-media as shown in Fossmark et al. (2021).

I-III Sampling- protocol (Effect of sampling day) and method

The high particle load RASs fluctuated throughout the whole experimental period. This had the effect of making the differences between the two operational conditions less noticeable at some sampling days and more apparent at others. This was especially seen on sampling day 7 (T7). In those cases where measurements at T7 was very different from previous ones (e.g., TSA and Bacterial activity at inlet and outlet of the biofilter), it was checked whether significant differences depended on this day, by removing it from the analysis. There were not any cases where significance depended on this day. However, it resulted in many cases where T7 was the only day where there were significant differences in the isolated comparison between the sampling days. If sampling day did not have such a large effect on the results, it is likely that other sampling days too would show differences between high and low particle loading in some of the measured water quality parameters.

Sampling days followed the dates where data on fish performance were collected for other work packages in the project. This was done to better understand the connection between the different work packages in the final result of the project. Due to this, some sampling days were scheduled to be close together while others were spaced widely apart. This made it difficult to investigate development over time and even more challenging when it was also shown that sampling day had an impact. It must also be added that there were relatively few sampling days, in addition to some data not being included do to its reliable status, which means that high measurements had great effects. This was handled in the present study by looking at each sampling day as repeated measurements, instead of time development, so that high but relevant measurements were less decisive. Future studies, investigating development over time, should therefore add more sampling days and spread them evenly with the same interval.

The experiment was blinded to prevent bias during measurements and assessment of data. This worked out for the packages that investigated fish performance. However, in the present part of the experiment it was not possible, as water samples, water analyses and interpretation of data was done by the same personnel. During collection and analysis, it was possible to separate the systems and samples from each other and see what treatment they had

received. To avoid this, the collection and analysis of water samples should be done by a party other than the one interpreting the data.

I-IV Methods used to analyze water quality

Many methods were used to analyze different water quality parameters in the present study, most of them were analyzes or test kits based on standards from International Organization for Standardization (ISO) or Standard Norge (NS). Hydrogen peroxide decomposition rates, the method used to measure bacterial activity in the current study, was suggested by Pedersen et al. (2019) as a fast new tool to describe microbial activity. This method reflects the microbial enzymatic activity of bacteria, but also potential contributions from other microbiota such as algae and protozoans. However, H_2O_2 decomposition is primarily governed by bacteria and RASs are known to have favorable conditions and high numbers of bacteria (Pedersen et al., 2019). Therefore, H_2O2 degradation was interpreted in the context with bacterial activity in the present study.

H_2O_2 decomposition follows exponential first order decay (Richard et al., 2007, Pedersen et al., 2019). This exponential decay is measured over 60 minutes in the present study. When H_2O_2 decomposition was analyzed in bio-media from the high particle load RASs at T7, alle decomposition was completed after 15-30 minutes. This resulted in an exponential curve that flattened out towards the end and rate constants (k) that were low and similar to the rate constants in the low particle loading systems. Other results from this day (T7) indicated that there was an increased amount of particles and microbial activity in addition to visual indications of sampling water being very turbid. Therefore, it was decided to only look at the degradation up to 30 minutes to get a rate constant that was higher and probably more in line with reality. Future studies using this method to assess microbial activity in turbid water should take this into account. A solution could be to use water samples collected directly form the biofilter instead of bio-media or by having a larger water to bio-media surface area ratio than what was used in the present study, when assessing microbial activity in a biofilter.

Challenges were also encountered in T7 when it came to PSD measurements. In two of the systems with a high particle load, water samples had to be diluted because the density of particles in water samples were too high, and the particle counter was overloaded. Dilution and following multiplication to original density may have caused some disturbance in the measurements. Water samples were processed so that the possible errors resulting from the dilution were minimal.

I-V Execution of the ammonia spiking experiment

The spiking events were executed in 2.5L measuring cups, filled with 0.4L biochips and 1.8L RAS water, giving a total surface area of 0.25 m^2, and a stoking of 22%. This stocking is way lower than in the RASs biofilters where the bio-media operated during the experiment, which was 65%. The stocking in the spiking experiment had to be this low in order to get upwelling of bio-media. The low stocking gave a low total surface area of active biofilm per volume compared to the RASs biofilters. In addition, the initial ammonia concentration was much higher in the spiking experiment than what was measured on the sampling days (5 mg N/L). This may have led to an environment where the biofilm was fully penetrated and entirely saturated over a longer time than in the RASs biofilters. If the setup in the spiking experiment better imitated the RASs biofilters, it might have provided a better understanding of the nitrification kinetics that took place there.

TAN removal was measured over 270 minutes, as similar spiking experiments used this time frame. In the present study, these 270 minutes was not enough to reach 1'order kinetics in both reference experiments and in some cases during the spiking of bio-media from the RASs. Even though this presented some interesting results, it also resulted in few data points under 1' order TAN removal kinetics. This made it difficult to describe TAN removal under substrate dependent conditions, in some cases. By planning the spiking experiments better, this could have been avoided. Firstly, it has been shown in other literature under similar conditions that it can take time before 1' order substrate dependent TAN removal is achieved (Kinyage and Pedersen, 2016, Kinyage et al., 2019). Secondly, other studies often distinguish between 0'order and 1'order kinetics by adding lower concentrations of ammonia, around 1 mg N/L, when investigating 1' order kinetics (Prehn et al., 2012, Kinyage et al., 2019). On the other hand, von Ahnen et al. (2015) showed that systems could reach 1'order kinetics within a shorter time span than what was used in the present study. To avoid this limitation, the time period over which TAN removal is observed can be increased or one can distinguish between 0' and 1' order kinetics in two separate spiking experiments. By spiking with lower concentrations (>1 mg N/L) when assessing 1' order kinetics.

I-VI Statistical analysis

To compare significance at the sampling days between the two experimental groups (High and Low) ANOVAs were used. The main reason why this was used instead of e.g., a *t*-test which is normally used when comparing two groups, was because of unequal variance.

Leven's test and Shapiro wilks test showed a tendency of unequal variance and violations of normality in some cases. In some cases, this was improved by transforming the data, but in others this wasn't done if homogeneity of variance was met. Nevertheless, a *t*-test assumes equal variance, while an ANOVA allows unequal variance (Zar, 1996). In addition, ANOVAS are generally more robust to violations of assumptions such as normality (Zar, 1996) It was therefore decided that this was the best statistical method to analyze the data of this master thesis.

Linear mixed effect models (LMM) were used as there was observed large variations within triplicates when data from the spiking experiment was interpreted in Excel. LMMs were therefore used as there were linear models with a high probability of large random effects.

Appendix II. Overview of measurements and figure data

II-I Operational conditions through experiment

Appendix II. Table I. *Mean total suspended solids (TSS; mg/L) through the experimental period, for high and low particle loading. Minimum (min), maximum (max), standard error of mean (SEM) and sample numbers from each treatment group (n).*

Date	Experimental day	Particle load	n	Mean TSS	Min TSS	Max TSS	SEM TSS
16.08.2022	8	High	3	7.8	6.8	8.7	0.6
16.08.2022	8	Low	3	0.7	0.7	0.8	0.033
19.08.2022	11	High	3	2.7	1.5	3.4	0.593
19.08.2022	11	Low	3	0.8	0.3	1.2	0.260
25.08.2022	17	High	3	1.8	1.2	2.2	0.284
25.08.2022	17	Low	3	1.3	1.2	1.3	0.033
30.08.2022	21	High	3	4.4	3.3	5.7	0.696
30.08.2022	21	Low	3	1.1	0.9	1.5	0.174
02.09.2022	24	High	3	4.3	4.2	4.3	0.029
02.09.2022	24	Low	3	0.5	0.3	0.6	0.087
05.09.2022	27	High	3	5.5	4.6	6.2	0.482
05.09.2022	27	Low	3	0.8	0.6	1.1	0.144
08.09.2022	31	High	3	6.8	5.9	8.0	0.629
08.09.2022	31	Low	3	0.9	0.6	1.1	0.132
15.09.2022	38	High	3	1.4	1.2	1.6	0.130
‘15.09.2022	38	Low	3	1.0	0.9	1.1	0.083
22.09.2022	45	High	3	6.3	4.9	8.7	1.203
22.09.2022	45	Low	3	2.6	2.1	3.6	0.492
26.09.2022	49	High	3	5.2	4.5	5.7	0.377
26.09.2022	49	Low	3	0.8	0.8	1.0	0.067
04.10.2022	57	High	3	9.4	7.6	10.6	0.928
04.10.2022	57	Low	3	1.1	1.0	1.2	0.076
06.10.2022	59	High	3	6.2	4.9	8.0	0.937
06.10.2022	59	Low	3	0.9	0.6	1.2	0.180
10.10.2022	63	High	3	26.6	14.9	39.1	7.001
10.10.2022	63	Low	3	2.3	0.8	4.7	1.195
19.10.2022	72	High	3	8.2	3.4	12.5	2.645
19.10.2022	72	Low	3	4.7	3.3	7.6	1.434
28.10.2022	81	High	3	3.4	2.6	4.5	0.585
28.10.2022	81	Low	3	1.8	1.8	1.9	0.029
02.11.2022	86	High	3	3.7	1.7	6.8	1.567
02.11.2022	86	Low	3	1.2	1.0	1.5	0.167
09.11.2022	93	High	3	2.8	1.8	4.7	0.918
09.11.2022	93	Low	3	1.3	0.9	1.6	0.213
18.11.2022	102	High	3	2.4	2.3	2.6	0.073
18.11.2022	102	Low	3	1.0	0.8	1.4	0.169
28.11.2022	112	High	3	6.2	3.6	7.6	1.317
28.11.2022	112	Low	3	2.2	1.7	2.7	0.291
06.12.2022	116	High	3	19.3	13.3	30.4	5.565
06.12.2022	116	Low	3	3.0	1.3	4.5	0.951

II-II PSD Average (0.8-410.3 µm) across all sampling days, inlet and outlet of biofilter

Appendix II. Table II. *Average particles per size class across the sampling period (n=15). Each size class represent the diameter of particles (assessed as spheres), numbers represent the mean number of particles within the size class. Total particle concentration(#part/mL) is shown in the last row.*

Size class (µm)	High In	Low In	High Out	Low Out
0.8	1526.48	726.20	1289.16	673.96
0.9	1261.13	622.19	1057.99	580.00
1	907.62	457.54	757.98	432.80
1.1	776.12	401.87	643.09	380.63
1.2	572.38	307.96	477.02	291.38
1.3	497.28	266.90	411.58	255.47
1.4	379.51	204.81	308.00	194.88
1.5	337.42	182.26	272.45	173.39
1.6	251.96	137.62	206.17	132.13
1.7	215.49	115.58	172.12	108.29
1.8	203.06	106.11	164.32	101.85
1.9	175.46	89.16	140.30	84.69
2	314.80	152.05	251.67	144.28
2.2	239.40	106.88	192.00	102.22
2.4	107.79	44.24	84.90	42.01
2.5	246.62	94.19	200.91	87.90
2.8	131.22	44.11	109.43	40.52
3	115.50	35.97	94.72	32.49
3.2	146.04	41.84	119.98	38.36
3.5	122.14	31.54	101.43	27.96
3.8	74.04	17.94	63.20	16.10
4	125.14	28.66	107.86	26.10
4.4	83.81	17.55	73.28	15.52
4.7	108.78	21.11	95.95	18.67
5.1	115.42	19.95	99.86	17.28
5.5	121.49	19.98	106.76	17.66
6	82.33	12.65	70.88	11.37
6.4	129.47	21.21	112.79	19.07
7.2	99.84	18.40	86.48	17.53
8.1	82.05	17.78	69.09	17.70
9.1	70.56	18.26	59.17	17.96
10.2	34.38	8.89	27.46	9.18
11.5	31.22	8.47	27.22	8.81
12.8	34.35	8.62	30.64	9.07
14.5	15.47	2.81	14.26	3.26
16.1	12.81	1.91	12.33	2.26
18.2	12.01	1.74	11.76	2.01
20.3	16.17	1.80	15.54	2.17
23	13.10	1.27	12.21	1.61
25.6	9.25	0.89	9.05	1.08
29	7.49	0.56	6.84	0.71
32.3	6.32	0.55	6.06	0.64
36.5	3.89	0.29	3.86	0.47
40.7	3.66	0.20	3.27	0.32
46	3.02	0.12	2.91	0.18
51.3	2.44	0.09	2.21	0.15
57.9	1.53	0.07	1.42	0.11
64.6	1.57	0.03	1.29	0.10
73	0.92	0.04	0.66	0.08
81.4	0.65	0.02	0.84	0.06
92	0.50	0.02	0.57	0.05
102.5	0.70	0.01	0.58	0.04
115.9	0.26	0.00	0.38	0.03
129.2	0.36	0.01	0.38	0.02
146	0.24	0.01	0.21	0.02
162.8	0.18	0.01	0.16	0.01
184	0.12	0.01	0.14	0.00
205.1	0.04	0.01	0.08	0.00
231.8	0.01	0.00	0.06	0.00
258.4	0.26	0.01	0.13	0.02
292	0.01	0.00	0.01	0.00
325.6	0.00	0.00	0.00	0.00
368	0.00	0.00	0.00	0.00
410.3	0.00	0.00	0.00	0.00
Particle concentration (part/ml)	9833	4421	8193	4163

II-III Total Particle surface area (TSA) inlet and outlet of biofilter

Appendix II. Table III. *Mean Total particle surface area (TSA; mm^2/mL) in water samples collected at the inlet and outlet of biofilter at the different sampling days(T), for high and low particle loading. Minimum (min), maximum (max), standard error of mean (SEM) and sample numbers from each treatment group (n).*

Date	Experimental day	T	In/Out	Particle Load	n	Mean SA	Min SA	Max SA	SEM SA
13.09.2022	36	T2	In	High	3	0.111	0.053	0.202	0.046
13.09.2022	36	T2	In	Low	3	0.051	0.038	0.076	0.013
13.09.2022	36	T2	Out	High	3	0.149	0.102	0.205	0.030
13.09.2022	36	T2	Out	Low	3	0.107	0.086	0.121	0.010
20.09.2022	43	T3	In	High	3	0.081	0.069	0.106	0.012
20.09.2022	43	T3	In	Low	3	0.038	0.029	0.045	0.005
20.09.2022	43	T3	Out	High	3	0.075	0.063	0.083	0.006
20.09.2022	43	T3	Out	Low	3	0.039	0.032	0.044	0.004
18.10.2022	71	T4	In	High	3	0.250	0.191	0.336	0.044
18.10.2022	71	T4	In	Low	3	0.126	0.071	0.207	0.041
18.10.2022	71	T4	Out	High	3	0.273	0.199	0.332	0.039
18.10.2022	71	T4	Out	Low	3	0.104	0.062	0.183	0.039
25.10.2022	78	T5	In	High	3	0.103	0.078	0.121	0.013
25.10.2022	78	T5	In	Low	3	0.064	0.000	0.141	0.041
25.10.2022	78	T5	Out	High	3	0.123	0.092	0.171	0.024
25.10.2022	78	T5	Out	Low	3	0.048	0.000	0.088	0.026
15.11.2022	99	T6	In	High	3	0.278	0.151	0.442	0.086
15.11.2022	99	T6	In	Low	3	0.101	0.089	0.115	0.007
15.11.2022	99	T6	Out	High	3	0.300	0.293	0.304	0.004
15.11.2022	99	T6	Out	Low	3	0.112	0.099	0.125	0.008
06.12.2022	116	T7	In	High	3	2.638	1.393	3.669	0.666
06.12.2022	116	T7	In	Low	3	0.126	0.102	0.159	0.017
06.12.2022	116	T7	Out	High	3	2.260	1.455	2.781	0.408
06.12.2022	116	T7	Out	Low	3	0.135	0.124	0.154	0.010

II-IV Beta value (β) inlet and outlet of biofilter

Appendix II. Table IV. *Mean beta value (β) in water samples collected at the inlet and outlet of biofilter at the different sampling days (T), for high and low particle loading. Minimum (min), maximum (max), standard error of mean (SEM) and sample numbers from each treatment group (n).*

Date	Experimental day	T	In/Out	Particle load	n	Mean beta	Min beta	Max beta	SEM beta
13.09.2022	36	T2	In	High	3	2.993	2.779	3.271	0.146
13.09.2022	36	T2	In	Low	3	3.044	3.004	3.118	0.037
13.09.2022	36	T2	Out	High	3	2.778	2.724	2.820	0.028
13.09.2022	36	T2	Out	Low	3	2.860	2.734	3.101	0.120
20.09.2022	43	T3	In	High	3	3.148	3.046	3.291	0.074
20.09.2022	43	T3	In	Low	3	3.138	3.018	3.209	0.060
20.09.2022	43	T3	Out	High	3	2.868	2.846	2.894	0.014
20.09.2022	43	T3	Out	Low	3	3.069	2.756	3.241	0.157
18.10.2022	71	T4	In	High	3	2.879	2.822	2.928	0.031
18.10.2022	71	T4	In	Low	3	2.906	2.752	3.074	0.093
18.10.2022	71	T4	Out	High	3	2.625	2.430	2.803	0.108
18.10.2022	71	T4	Out	Low	3	2.944	2.765	3.207	0.134
15.11.2022	99	T6	In	High	3	3.200	2.992	3.336	0.106
15.11.2022	99	T6	In	Low	3	3.383	3.347	3.447	0.032
15.11.2022	99	T6	Out	High	3	3.039	2.875	3.151	0.084
15.11.2022	99	T6	Out	Low	3	3.270	3.205	3.326	0.035
06.12.2022	116	T7	In	High	3	2.631	2.528	2.705	0.053
06.12.2022	116	T7	In	Low	3	3.388	3.332	3.440	0.031
06.12.2022	116	T7	Out	High	3	2.578	2.416	2.703	0.085
06.12.2022	116	T7	Out	Low	3	3.141	3.125	3.157	0.009

II-V TSS (mg/L) inlet and outlet of biofilter

Appendix II. Table V. *Mean total suspended solids (TSS; mg/L) in water samples collected at the inlet and outlet of biofilter at the different sampling days (T), for high and low particle loading. Minimum (min), maximum (max), standard error of mean (SEM) and sample numbers from each treatment group (n).*

Date	Experimental day	T	In/out	Particle load	n	Mean TSS	Min TSS	Max TSS	SEM TSS
13.09.2022	36	T2	In	High	3	1.7	0.3	2.9	0.754
13.09.2022	36	T2	In	Low	3	0.3	0.1	0.4	0.088
13.09.2022	36	T2	Out	High	3	2.9	1.6	5.4	1.250
13.09.2022	36	T2	Out	Low	3	1.8	1.5	2.3	0.240
20.09.2022	43	T3	In	High	3	1.0	0.7	1.2	0.153
20.09.2022	43	T3	In	Low	3	0.8	0.5	1.0	0.153
20.09.2022	43	T3	Out	High	3	1.2	1.0	1.4	0.115
20.09.2022	43	T3	Out	Low	3	0.7	0.4	1.0	0.176
18.10.2022	71	T4	In	High	3	2.3	1.5	3.0	0.441
18.10.2022	71	T4	In	Low	3	1.8	1.4	2.0	0.186
18.10.2022	71	T4	Out	High	3	2.1	1.7	2.5	0.233
18.10.2022	71	T4	Out	Low	3	1.2	0.9	1.4	0.153
25.10.2022	78	T5	In	High	3	3.4	1.2	6.5	1.605
25.10.2022	78	T5	In	Low	3	1.4	0.8	1.9	0.318
25.10.2022	78	T5	Out	High	3	1.8	1.4	2.3	0.260
25.10.2022	78	T5	Out	Low	3	1.5	1.2	2.0	0.240
15.11.2022	99	T6	In	High	3	2.6	2.3	2.8	0.145
15.11.2022	99	T6	In	Low	3	1.5	1.3	1.9	0.200
15.11.2022	99	T6	Out	High	3	2.0	1.8	2.2	0.120
15.11.2022	99	T6	Out	Low	3	1.3	1.0	1.8	0.252
06.12.2022	116	T7	In	High	3	14.3	9.2	19.4	2.945
06.12.2022	116	T7	In	Low	3	2.2	1.7	2.8	0.318
06.12.2022	116	T7	Out	High	3	9.6	9.1	10.4	0.404
06.12.2022	116	T7	Out	Low	3	1.7	1.6	1.8	0.067

II-VI Bacterial activity in MBBR bio-media

Appendix II. Table VI. *Mean bacterial activity (h^{-1}) in bio-media measured by H_2O_2 decomposition rates at the different sampling days (T), for high and low particle loading. Minimum (min), maximum (max), standard error of mean (SEM) and sample numbers from each treatment group (n).*

Date	Experimental day	T	Particle load	n	Mean k-value	Min k-value	Max k-value	SEM k-value
09.08.2022	1	T0	High	3	2.00	1.86	2.1	0.07
09.08.2022	1	T0	Low	3	1.86	1.74	1.92	0.06
23.08.2022	15	T1	High	3	1.78	1.38	2.1	0.21
23.08.2022	15	T1	Low	3	1.64	1.44	1.86	0.12
13.09.2022	36	T2	High	3	1.86	1.62	2.22	0.18
13.09.2022	36	T2	Low	3	1.60	1.38	1.74	0.11
20.09.2022	43	T3	High	3	1.58	1.50	1.62	0.04
20.09.2022	43	T3	Low	3	1.56	1.38	1.68	0.09
18.10.2022	71	T4	High	3	1.69	1.45	2.16	0.24
18.10.2022	71	T4	Low	3	1.49	1.46	1.53	0.02
25.10.2022	78	T5	High	3	2.11	1.90	2.39	0.14
25.10.2022	78	T5	Low	3	1.79	1.78	1.81	0.01
15.11.2022	99	T6	High	3	1.12	0.86	1.54	0.21
15.11.2022	99	T6	Low	3	1.13	0.74	1.64	0.26
06.12.2022	116	T7	High	3	2.95	2.45	3.48	0.30
06.12.2022	116	T7	Low	3	1.33	1.01	1.53	0.16

II-VII Bacterial activity inlet and outlet of biofilter

Appendix II. Table VII. Appendix II. Table VI. *Mean bacterial activity (h^{-1}) in water samples collected at the inlet and outlet of biofilter measured by H_2O_2 decomposition rates at the different sampling days (T), for high and low particle loading. Minimum (min), maximum (max), standard error of mean (SEM) and sample numbers from each treatment*

Date	Experimental day	T	In/Out	Particle load	n	Mean k-value	Min k-value	Max k-value	SEM k-value
23.08.2022	15	T1	In	High	3	0.046	0.036	0.060	0.007
23.08.2022	15	T1	In	Low	3	0.026	0.018	0.042	0.008
23.08.2022	15	T1	Out	High	3	0.038	0.018	0.060	0.012
23.08.2022	15	T1	Out	Low	3	0.048	0.030	0.060	0.009
13.09.2022	36	T2	In	High	3	0.034	0.030	0.036	0.002
13.09.2022	36	T2	In	Low	3	0.016	0.012	0.018	0.002
13.09.2022	36	T2	Out	High	3	0.042	0.030	0.054	0.007
13.09.2022	36	T2	Out	Low	3	0.046	0.042	0.054	0.004
20.09.2022	43	T3	In	High	3	0.028	0.024	0.030	0.002
20.09.2022	43	T3	In	Low	3	0.012	0.006	0.018	0.003
20.09.2022	43	T3	Out	High	3	0.020	0.012	0.036	0.008
20.09.2022	43	T3	Out	Low	3	0.012	0.012	0.012	0.000
18.10.2022	71	T4	In	High	3	0.069	0.049	0.099	0.015
18.10.2022	71	T4	In	Low	3	0.029	0.013	0.061	0.016
18.10.2022	71	T4	Out	High	3	0.058	0.040	0.074	0.010
18.10.2022	71	T4	Out	Low	3	0.027	0.017	0.044	0.009
25.10.2022	78	T5	In	High	3	0.085	0.068	0.108	0.012
25.10.2022	78	T5	In	Low	3	0.026	0.021	0.032	0.003
25.10.2022	78	T5	Out	High	3	0.097	0.071	0.119	0.014
25.10.2022	78	T5	Out	Low	3	0.033	0.030	0.035	0.002
15.11.2022	99	T6	In	High	3	0.058	0.047	0.071	0.007
15.11.2022	99	T6	In	Low	3	0.017	0.011	0.022	0.003
15.11.2022	99	T6	Out	High	3	0.069	0.061	0.079	0.006
15.11.2022	99	T6	Out	Low	3	0.020	0.017	0.025	0.002
06.12.2022	116	T7	In	High	3	0.715	0.452	1.088	0.192
06.12.2022	116	T7	In	Low	3	0.030	0.009	0.053	0.013
06.12.2022	116	T7	Out	High	3	0.677	0.456	1.101	0.212
06.12.2022	116	T7	Out	Low	3	0.039	0.031	0.045	0.004

II-VIII $COD_{tot/diss}$ inlet and outlet of biofilter

Appendix II. Table VIII. *Mean total- and dissolved chemical oxygen demand (COD; mg/L) in water samples collected at the inlet and outlet of biofilter at the different sampling days (T), for high and low particle loading. Minimum (min), maximum (max), standard error of mean (SEM) and sample numbers from each treatment group (n).*

Date	Experimental day	T	In/out	Particle load	n	Mean tot	Min tot	Max tot	SEM tot	Mean diss	Min diss	Max diss	SEM diss
23.08.2022	15	T1	In	High	3	19.6	16.9	22.0	1.480	19.6	17.1	21.1	1.258
23.08.2022	15	T1	In	Low	3	9.7	7.7	11.2	1.036	12.5	8.3	16.3	2.333
23.08.2022	15	T1	Out	High	3	22.5	17.6	29.4	3.541	19.2	18.0	20.7	0.801
23.08.2022	15	T1	Out	Low	3	11.0	9.8	13.3	1.151	12.6	11.2	14.6	1.037
13.09.2022	36	T2	In	High	3	17.4	16.6	18.9	0.751	17.8	17.1	18.5	0.404
13.09.2022	36	T2	In	Low	3	14.2	13.6	15.2	0.503	13.9	13.3	14.3	0.296
13.09.2022	36	T2	Out	High	3	17.3	16.0	19.3	1.027	16.4	14.8	17.5	0.829
13.09.2022	36	T2	Out	Low	3	13.1	12.0	13.9	0.561	13.1	12.1	14.2	0.606
20.09.2022	43	T3	In	High	3	17.4	15.5	18.5	0.940	16.4	14.8	17.7	0.850
20.09.2022	43	T3	In	Low	3	12.2	10.8	14.0	0.939	13.4	12.2	15.3	0.949
20.09.2022	43	T3	Out	High	3	15.2	13.4	17.2	1.099	14.8	14.6	15.1	0.145
20.09.2022	43	T3	Out	Low	3	12.5	11.3	13.6	0.664	11.9	11.3	12.4	0.328
18.10.2022	71	T4	In	High	3	18.0	15.6	21.0	1.594	17.9	15.7	20.1	1.271
18.10.2022	71	T4	In	Low	3	13.8	11.9	14.8	0.935	13.3	11.6	14.6	0.882
18.10.2022	71	T4	Out	High	3	17.0	15.3	17.8	0.833	16.3	15.3	16.9	0.503
18.10.2022	71	T4	Out	Low	3	13.0	10.9	14.7	1.115	12.9	11.3	14.0	0.809
25.10.2022	78	T5	In	High	3	20.0	13.8	23.3	3.118	20.1	14.2	23.3	2.969
25.10.2022	78	T5	In	Low	3	17.2	15.0	19.6	1.332	17.5	15.7	19.2	1.014
25.10.2022	78	T5	Out	High	3	19.7	13.4	23.5	3.186	20.8	13.8	24.4	3.484
25.10.2022	78	T5	Out	Low	3	17.5	15.4	19.9	1.308	17.6	15.9	20.1	1.267
15.11.2022	99	T6	In	High	3	39.9	34.8	42.8	2.544	40.0	37.7	42.8	1.488
15.11.2022	99	T6	In	Low	3	29.2	26.8	32.4	1.658	30.5	30.1	30.8	0.208
15.11.2022	99	T6	Out	High	3	39.4	37.6	41.9	1.290	38.2	35.6	41.9	1.900
15.11.2022	99	T6	Out	Low	3	28.9	25.8	31.0	1.572	29.5	26.7	32.0	1.541
06.12.2022	116	T7	In	High	3	76.4	69.3	85.0	4.589	69.9	65.5	77.4	3.783
06.12.2022	116	T7	In	Low	3	34.2	19.7	47.7	8.096	35.1	21.5	49.1	7.970
06.12.2022	116	T7	Out	High	3	75.6	69.1	84.9	4.771	68.7	64.7	75.0	3.188
06.12.2022	116	T7	Out	Low	3	35.4	22.5	49.6	7.847	35.3	22.2	48.8	7.682

II-IX TAN ($NH_4^+ + NH_3$) inlet outlet of biofilter

Appendix II. Table IX. *Mean total ammonia nitrogen concentration (TAN; mg/L) in water samples collected at the inlet and outlet of biofilter at the different sampling days (T), for high and low particle loading. Minimum (min), maximum (max), standard error of mean (SEM) and sample numbers from each treatment group (n).*

Dato	Experimental day	T	In/Out	Particle load	n	Mean TAN	Min TAN	Max TAN	SEM TAN
23.08.2022	15	T1	In	High	3	0.957	0.750	1.310	0.178
23.08.2022	15	T1	In	Low	3	0.443	0.380	0.510	0.038
23.08.2022	15	T1	Out	High	3	0.643	0.490	0.870	0.116
23.08.2022	15	T1	Out	Low	3	0.330	0.250	0.450	0.061
13.09.2022	36	T2	In	High	3	0.567	0.400	0.800	0.120
13.09.2022	36	T2	In	Low	3	0.410	0.220	0.700	0.147
13.09.2022	36	T2	Out	High	3	0.510	0.400	0.720	0.105
13.09.2022	36	T2	Out	Low	3	0.300	0.140	0.520	0.114
20.09.2022	43	T3	In	High	3	0.377	0.330	0.420	0.026
20.09.2022	43	T3	In	Low	3	0.353	0.220	0.550	0.100
20.09.2022	43	T3	Out	High	3	0.213	0.160	0.260	0.029
20.09.2022	43	T3	Out	Low	3	0.340	0.180	0.590	0.127
18.10.2022	71	T4	In	High	3	0.510	0.300	0.730	0.124
18.10.2022	71	T4	In	Low	3	0.253	0.230	0.280	0.015
18.10.2022	71	T4	Out	High	3	0.257	0.200	0.340	0.043
18.10.2022	71	T4	Out	Low	3	0.200	0.170	0.220	0.015
25.10.2022	78	T5	In	High	3	0.433	0.380	0.460	0.027
25.10.2022	78	T5	In	Low	3	0.337	0.310	0.370	0.018
25.10.2022	78	T5	Out	High	3	0.357	0.340	0.370	0.009
25.10.2022	78	T5	Out	Low	3	0.280	0.260	0.320	0.020
15.11.2022	99	T6	In	High	3	0.280	0.260	0.300	0.012
15.11.2022	99	T6	In	Low	3	0.220	0.200	0.240	0.012
15.11.2022	99	T6	Out	High	3	0.193	0.160	0.220	0.018
15.11.2022	99	T6	Out	Low	3	0.167	0.140	0.200	0.018
06.12.2022	116	T7	In	High	3	0.520	0.480	0.560	0.023
06.12.2022	116	T7	In	Low	3	0.273	0.260	0.300	0.013
06.12.2022	116	T7	Out	High	3	0.373	0.360	0.400	0.013
06.12.2022	116	T7	Out	Low	3	0.173	0.140	0.200	0.018

II-X Nitrite (NO_2^-) inlet and outlet of biofilter

Appendix II. Table X. *Mean nitrite concentration (NO_2^-; mg/L) in water samples collected at the inlet and outlet of biofilter at the different sampling days(T), for high and low particle loading. Minimum (min), maximum (max), standard error of mean (SEM) and sample numbers from each treatment group (n).*

Date	Experimental day	T	In/Out	Particle load	n	Mean NO2	Min NO2	Max NO2	SEM NO2
23.08.2022	15	T1	In	High	3	1.100	0.940	1.200	0.081
23.08.2022	15	T1	In	Low	3	0.367	0.260	0.460	0.058
23.08.2022	15	T1	Out	High	3	1.090	0.980	1.200	0.064
23.08.2022	15	T1	Out	Low	3	0.420	0.350	0.460	0.035
13.09.2022	36	T2	In	High	3	0.443	0.380	0.540	0.049
13.09.2022	36	T2	In	Low	3	0.320	0.190	0.480	0.085
13.09.2022	36	T2	Out	High	3	0.430	0.380	0.520	0.045
13.09.2022	36	T2	Out	Low	3	0.300	0.170	0.460	0.085
20.09.2022	43	T3	In	High	3	0.260	0.180	0.330	0.044
20.09.2022	43	T3	In	Low	3	0.313	0.180	0.470	0.085
20.09.2022	43	T3	Out	High	3	0.190	0.150	0.230	0.023
20.09.2022	43	T3	Out	Low	3	0.297	0.160	0.470	0.091
18.10.2022	71	T4	In	High	3	0.220	0.150	0.330	0.056
18.10.2022	71	T4	In	Low	3	0.150	0.090	0.220	0.038
18.10.2022	71	T4	Out	High	3	0.140	0.120	0.160	0.012
18.10.2022	71	T4	Out	Low	3	0.133	0.080	0.190	0.032
25.10.2022	78	T5	In	High	3	0.237	0.190	0.260	0.023
25.10.2022	78	T5	In	Low	3	0.137	0.090	0.170	0.024
25.10.2022	78	T5	Out	High	3	0.227	0.180	0.250	0.023
25.10.2022	78	T5	Out	Low	3	0.123	0.080	0.150	0.022
15.11.2022	99	T6	In	High	3	0.203	0.180	0.240	0.019
15.11.2022	99	T6	In	Low	3	0.137	0.120	0.150	0.009
15.11.2022	99	T6	Out	High	3	0.167	0.150	0.180	0.009
15.11.2022	99	T6	Out	Low	3	0.107	0.100	0.120	0.007
06.12.2022	116	T7	In	High	3	0.227	0.200	0.260	0.018
06.12.2022	116	T7	In	Low	3	0.140	0.120	0.150	0.010
06.12.2022	116	T7	Out	High	3	0.190	0.170	0.220	0.015
06.12.2022	116	T7	Out	Low	3	0.097	0.070	0.110	0.013

II-XI Nitrate (NO_3^-) inlet and outlet of biofilter

Appendix II. Table XI. *Mean nitrate concentration (NO_3^-; mg/L) in water samples collected at the inlet and outlet of biofilter at the different sampling days(T), for high and low particle loading. Minimum (min), maximum (max), standard error of mean (SEM) and sample numbers from each treatment group (n).*

Date	Experimental day	T	In/Out	Particle load	n	Mean NO3	Min NO3	Max NO3	SEM NO3
23.08.2022	15	T1	In	High	3	20.067	16.300	23.700	2.137
23.08.2022	15	T1	In	Low	3	21.100	17.000	27.500	3.242
23.08.2022	15	T1	Out	High	3	19.333	15.300	22.700	2.162
23.08.2022	15	T1	Out	Low	3	19.267	12.500	28.500	4.781
13.09.2022	36	T2	In	High	3	20.067	18.700	21.200	0.731
13.09.2022	36	T2	In	Low	3	24.733	18.300	32.100	4.011
13.09.2022	36	T2	Out	High	3	20.533	19.400	22.200	0.851
13.09.2022	36	T2	Out	Low	3	29.967	22.600	43.100	6.583
20.09.2022	43	T3	In	High	3	16.333	14.000	18.700	1.357
20.09.2022	43	T3	In	Low	3	32.033	25.100	41.200	4.780
20.09.2022	43	T3	Out	High	3	17.033	15.300	18.300	0.897
20.09.2022	43	T3	Out	Low	3	36.367	28.700	48.900	6.319
18.10.2022	71	T4	In	High	3	8.233	6.100	10.000	1.141
18.10.2022	71	T4	In	Low	3	13.500	8.000	18.800	3.119
18.10.2022	71	T4	Out	High	3	8.300	6.800	9.900	0.896
18.10.2022	71	T4	Out	Low	3	12.567	6.800	17.400	3.095
25.10.2022	78	T5	In	High	3	5.800	3.000	8.000	1.474
25.10.2022	78	T5	In	Low	3	9.667	5.800	14.800	2.674
25.10.2022	78	T5	Out	High	3	6.267	3.000	9.400	1.849
25.10.2022	78	T5	Out	Low	3	9.800	5.800	15.000	2.723
15.11.2022	99	T6	In	High	3	36.167	29.500	42.000	3.632
15.11.2022	99	T6	In	Low	3	54.000	49.500	62.000	4.010
15.11.2022	99	T6	Out	High	3	36.167	28.000	44.000	4.622
15.11.2022	99	T6	Out	Low	3	54.667	51.000	61.000	3.180
06.12.2022	116	T7	In	High	3	10.333	7.500	14.000	1.922
06.12.2022	116	T7	In	Low	3	52.333	13.500	93.500	23.123
06.12.2022	116	T7	Out	High	3	11.000	7.500	15.000	2.179
06.12.2022	116	T7	Out	Low	3	53.667	14.500	90.500	21.970

II-XII First reference spiking before freshwater phase

Appendix II. Table XII. *TAN concentration (mg/L) over time (days) in the three replicates (A-B) during the first reference ammonia spiking experiment of bio-media. Replicates were spiked with 5 mg N/L. Note that the first point is high due to insufficient mixing of the added ammonia.*

Days	A	B	C
0.00035	6.85	6.2	8.15
0.01042	5.25	5.25	5.4
0.02083	5.05	5.05	5.3
0.03125	4.85	4.8	4.9
0.04167	4.6	4.55	4.7
0.05208	4.45	4.4	4.4
0.06250	4.2	4.15	4.2
0.08333	3.75	3.65	3.85
0.10417	3.25	3.15	3.5
0.12500	2.9	2.65	3.05
0.14583	2.5	2.3	2.55
0.16667	1.95	1.8	2.15
0.18750	1.55	1.5	1.75
0.20833	1.3	1.05	1.45

II-XIII Ammonia spiking after freshwater phase

Appendix II. Table XIII. *TAN concentration (mg/L) over time (days) when bio-media exposed to high and low particle loading in freshwater were spiked with 5 mg N/L. Orange boxes represents datapoints that were used to assess 1'order TAN removal kinetics. Other data points were used to assess 0'order kinetics.*

Days	High	Low	High	Low	Low	High
0.00035	5.32	4.96	5.66	5.44	5.58	5.66
0.01042	5.06	4.72	5.24	5.02	5.34	5.22
0.02083	4.72	4.48	5.06	4.7	5.3	5.08
0.03125	4.4	4.32	4.76	4.32	4.96	4.78
0.04167	4.18	4.1	4.46	4.2	4.9	4.64
0.05208	3.92	3.82	4.3	3.82	4.64	4.46
0.06250	3.58	3.54	4.1	3.7	4.54	4.06
0.08333	2.96	2.96	3.64	3.04	4.1	3.7
0.10417	2.28	2.42	3.12	2.48	3.82	3.22
0.12500	1.58	1.74	2.54	1.78	3.3	2.88
0.14583	1.1	1.22	2.02	1.34	2.98	2.34
0.16667	0.74	0.76	1.52	0.8	2.5	1.86
0.18750	0.5	0.48	1.08	0.52	2.12	1.42

II-XIV Second reference spiking before brackish water phase

Appendix II. Table XIV. *TAN concentration (mg/L) over time (days) in the three replicates (A-B) during the second reference ammonia spiking experiment of bio-media. Replicates were spiked with 5 mg N/L.*

Days	A	B	C
0.00000	5.28	5.27	5.26
0.00035	4.88	4.6	4.96
0.01042	4.84	4.28	4.84
0.02083	4.28	4.08	4.56
0.03125	4.28	4.08	4.28
0.04167	4.28	3.88	4.08
0.05208	3.56	3.48	3.72
0.06250	3.20	3.20	3.6
0.08333	3.08	2.84	2.92
0.10417	2.52	2.36	2.36
0.12500	2.04	1.96	2.24
0.14583	1.56	1.44	1.8
0.16667	1.16	1.24	1.32
0.18750	0.84	0.92	0.96

II-XV Ammonia spiking after brackish water phase

Appendix II. Table XV. *TAN concentration (mg/L) over time (days) when bio-media exposed to high and low particle loading in brackish water were spiked with 5 mg N/L. Orange boxes represents datapoints that were used to assess 1'order TAN removal kinetics. Other data points were used to assess 0'order kinetics.*

Days	High	Low	High	Low	Low	High
0.00000	5.11	5.10	5.00	5.00	5.03	5.03
0.00035	4.84	4.6	4.76	4.72	4.52	4.9
0.01042	4.62	4.54	4.70	4.58	4.46	4.62
0.02083	4.14	4.30	4.26	4.38	4.32	4.40
0.03125	3.60	3.98	3.84	4.14	3.88	4.08
0.04167	3.42	3.50	3.46	3.80	3.62	3.90
0.05208	3.00	3.08	3.02	3.50	3.30	3.58
0.06250	2.60	2.86	2.64	3.22	3.12	3.36
0.08333	1.86	2.16	1.76	2.70	2.54	2.54
0.10417	1.14	1.56	1.12	2.14	1.98	2.12
0.12500	0.62	0.88	0.56	1.54	1.62	1.56
0.14583	0.38	0.52	0.30	1.10	1.20	1.12
0.16667	0.24	0.30	0.18	0.74	0.80	0.70
0.18750	0.16	0.16	0.12	0.46	0.50	0.48

Appendix III. Statistical analysis

III-I Mean Operational conditions through experimental period

Appendix III. Table XVI. *Mean TSS (mg/L) during the fresh- and brackish water phase, represented in fig. 7. Minimum (min), maximum (max), standard error of mean (SEM) and sample numbers from each treatment group (n).*

Fresh-/brackish water	Particle load	n	Mean TSS	Min TSS	Max TSS	SEM TSS
F	High	39	6.8	1.2	39.1	1.1
F	Low	39	1.1	0.3	4.7	0.1
B	High	21	6.6	1.7	30.4	1.5
B	Low	21	2.2	0.8	7.6	0.3

III-II Transformation and Random effects (variance in replicated RASs)

Appendix III. Table XVII. *Transformation of the data and variation within triplicates.*

Parameter	Transformation	Random variance in systems (%)
Weekly TSS meaurments	none	~0
TSA in	Log	~0
TSA out	Log	~0
β-value in	none	~0
β-value out	none	12.8
TSS in	Log	~0
TSS out	Log	~0
Bacterial activity (k-value) biomedia	none	5.2
Bacterial activity (k-value) in	Log	~0
Bacterial activity (k-value) out	Log	~0
COD_{tot}	None	~0
COD_{tot}	None	~0
TAN in	None	~0
TAN out	None	1
NO2 in	None	~0
NO2 out	None	~0
NO3 in	None	~0
NO3 out	None	~0
Spiking pre freshwater 1'order	None	6.1
Spiking pre brackish water 1'order	None	5.0
Spiking post freshwater 0'order High	none	80.2
Spiking post freshwater 1'order High	Log	NA
Spiking post freshwater 0'order Low	none	85.1
Spiking post freshwater 1'order Low	Log	NA
Spiking post brackish water 0'order High	none	56.8
Spiking post brackish water 1'order High	Log	59.3
Spiking post brackish water 0'order Low	none	35.6
Spiking post brackish water 1'order Low	Log	89.1

III-III ANOVAS

Appendix III. Table XVIII. *Results from one-way ANOVAs: effect of particle loading on the different water quality parameters. Orange row shows the ANOVA performed to investigate significance between COD_{tot} and COD_{diss} .*

Parameter	df	Sum Sq	Mean-Sq	F-value	p-value
TSA-in	1	11.08	11.08	10.55	0.003
TSA-out	1	10.07	10.07	11.93	0.002
TSA-in (excluding T7)	1	3.59	3.59	8.88	0.007
TSA-out (excluding T7)	1	3.39	3.390	10.37	0.004
Beta in	1	0.30	0.30	5.67	0.024
Beta out	1	0.58	0.58	13.17	0.001
TSS in	1	6.81	6.81	7.64	0.009
TSS out	1	3.63	3.63	10.50	0.003
TSS in (excluding T7)	1	3.445	3.445	5.525	0.026
TSS out (excluding T7)	1	1.322	1.3224	7.852	0.00911
k-value bio-media	1	1.35	1.35	6.49	0.014
k-value in	1	18.21	18.21	26.30	7.87e-06
k-value out	1	7.27	7.27	9.40	0.004
k-value in (excluding T7)	1	8.61	8.61	37.10	6.61e-07
k-value out (excluding T7)	1	2.32	2.32	6.34	0.017
COD in	1	1308	1308	4.74	0.035
COD out	1	1218	1218	4.44	0.042
COD in (excluding T7)	1	1308	1308	4.74	0.035
COD out (excluding T7)	1	310	310	5.07	0.031
TAN in	1	0.39	0.39	10.17	0.003
TAN out	1	0.12	0.12	5.13	0.029
NO2 in	1	0.27	0.27	4.69	0.036
NO2 out	1	0.20	0.20	3.09	0.087
NO2 in (excluding T1)	1	0.04	0.04	3.33	0.08
NO2 out (excluding T1)	1	0.02	0.02	1.64	0.21
NO3 in	1	1750	1750	6.15	0.017
NO3 out	1	2044	2044	6.90	0.012
$COD_{diss/tot}$ In	1	0.16	0.16	0.001	0.98
$COD_{diss/tot}$ Out	1	12	12	0.046	0.83

Appendix III. Table XIX. *Results from two-way ANOVAs with interaction between particle load and sampling day. The table shows values from the interaction, particle-loading: sampling- day.*

Parameter	df	Sum Sq	Mean-Sq	F-value	p-value
TSA-in	4	5.91	1.48	8.48	0.0004
TSA-out	4	5.52	1.38	17.60	2.43e-06
Beta in	4	0.61	0.15	8.86	0.0003
Beta out	4	0.19	0.05	1.89	0.15
Beta in (excluding T7)	3	0.03	0.01	0.53	0.66
Beta out (excluding T7)	3	0.04	0.01	0.59	0.70
TSS in	5	3.59	0.72	2.44	0.06
TSS out	5	2.26	0.45	4.84	0.003
k-value bio-media	7	2.94	0.42	5.15	0.0005
k-value bio-media (excluding T7)	6	0.13	0.021	0.31	0.925966
k-value in	6	7.69	1.28	6.26	0.0003
k-value out	6	9.60	1.60	12.43	8.52e-07
COD in	6	1781	296.80	11.88	1.31e-06
COD out	6	1637	272.80	10.68	3.55e-06
TAN in	6	0.25	0.04	1.99	0.10
TAN out	6	0.19	0.03	2.39	0.054
NO2 in	6	0.60	0.10	13.34	4.24e-07
NO2 out	6	0.55	0.09	16.51	4.83e-08
NO3 in	6	1841	306.9	2.22	0.07

NO3 out	6	1940	323.4	2.31	0.06
NO3 in (excluding T7)	5	360	72.0	2.72	0.044
NO3 out (excluding T7)	5	497	99.5	2.41	0.07

Appendix III. Table XX. *Results from two-way ANOVAs: effect of sampling point, and interaction between particle load:sampling-ponit (Inlet vs. Outlet of biofilter)*

Parameter		df	Sum Sq	Mean-Sq	F-value	p-value
TSA	In_Out	1	0.25	0.25	0.27	0.61
	Particle_load:In_Out	1	0.01	0.01	0.01	0.91
Beta	In_Out	1	0.04	0.04	7.11	0.01
	Particle_load:In_Out	1	0.003	0.003	0.56	0.46
TSS	In_Out	1	0.19	0.19	0.30	0.58
	Particle_load:In_Out	1	0.25	0.25	0.40	0.53
K-value	In_Out	1	0.62	0.62	0.85	0.36
	Particle_load:In_Out	1	1.24	1.24	1.69	0.20
COD	In_Out	1	0	0.10	0.001	0.98
	Particle_load:In_Out	1	1	0.80	0.003	0.96
TAN	In_Out	1	0.27	0.27	8.73	0.004
	Particle_load:In_Out	1	0.04	0.04	1.22	0.27
NO2	In_Out	1	0.01	0.013	0.21	0.65
	Particle_load:In_Out	1	0.003	0.003	0.05	0.82
NO3	In_Out	1	12	12	0.04	0.84
	Particle_load:In_Out	1	6	6	0.02	0.89

Appendix III. Table XXI. *Results from one-way ANOVAs: effect of particle loading on Δ (outlet-inlet or inlet-outlet).*

Parameter	df	Sum Sq	Mean-Sq	F-value	p-value
TSA	1	0.04	0.04	0.81	0.38
Beta	1	0.05	0.05	1.86	0.18
TSS	1	8.70	8.70	1.97	0.17
k-value	1	0.002	0.002	3.25	0.08
COD	1	1.59	1.59	0.37	0.55
TAN	1	0.08	0.08	8.71	0.01
NO2	1	0.006	0.006	3.81	0.06
NO3	1	11.42	11.42	1.53	0.22

III-IV Tukey-HSD

Appendix III. Table XXII. *Tukey-HSD performed on One-way ANOVAs to investigate the effect of particle load on the different water quality parameters. Diff represents the difference in mean, calculated of all sampling days, between low and high particle load, while Lwr-Upr represents the 95% confidence interval within which the true mean in difference between the two groups lie.*

Parameter	Diff (Low-High)	Lwr	Upr	p-adj
TSA-in	-1.21	-1.98	-0.45	0.003
TSA-out	-1.16	-1.85	-0.47	0.002
Beta in	0.20	0.03	0.38	0.024
Beta out	0.28	0.12	0.44	0.001
TSS in	-0.87	-1.51	-0.23	0.009
TSS out	-0.64	-1.03	-0.24	0.003
k-value bio-media	-0.33	-0.60	-0.07	0.014
k-value in	-1.32	-1.84	-0.80	7.9e-06
k-value out	-0.83	-1.38	-0.28	0.004
COD in	-11.16	-21.52	-0.80	0.035
COD out	-10.77	-21.11	-0.43	0.042
TAN in	-0.19	-0.32	-0.07	0.003
TAN out	-0.11	-0.20	-0.01	0.029

NO2 in	-0.16	-0.31	-0.01	0.036
NO2 out	-0.14	-0.29	0.02	0.087
NO3 in	12.91	2.39	23.43	0.017
NO3 out	13.95	3.22	24.68	0.012

Appendix III. Table XXIII. *Tukey-HSD performed on two-way ANOVAS to investigate effect of particle loading on the different sampling days. Diff represents the difference in mean between low and high particle load on the specific sampling day, while Lwr-Upr represents the 95% confidence interval within which the true mean in difference between the two groups lie.*

Parameter	Sampling day	Diff	Lwr	Upr	p-adj
TSA-in	Low:T2-High:T2	-0.67	-1.88	0.53	0.62
	Low:T3-High:T3	-0.75	-1.95	0.46	0.49
	Low:T4-High:T4	-0.75	-1.96	0.45	0.48
	Low:T6-High:T6	-0.92	-2.13	0.29	0.24
	Low:T7-High:T7	-2.98	-4.19	-1.78	1.1e-06
TSA-out	Low:T2-High:T2	-0.30	-1.11	0.51	0.94
	Low:T3-High:T3	-0.65	-1.45	0.16	0.19
	Low:T4-High:T4	-1.07	-1.87	-0.26	0.004
	Low:T6-High:T6	-0.99	-1.80	-0.18	0.01
	Low:T7-High:T7	-2.79	-3.59	-1.98	1.1e-06
Beta in	Low:T2-High:T2	0.05	-0.33	0.43	0.99
	Low:T3-High:T3	-0.01	-0.40	0.37	1.00
	Low:T4-High:T4	0.03	-0.35	0.41	0.99
	Low:T6-High:T6	0.18	-0.20	0.56	0.77
	Low:T7-High:T7	0.76	0.38	1.14	3e-05
Beta out	Low:T2-High:T2	0.08	-0.38	0.55	0.99
	Low:T3-High:T3	0.20	-0.26	0.66	0.86
	Low:T4-High:T4	0.32	-0.14	0.78	0.35
	Low:T6-High:T6	0.23	-0.23	0.69	0.74
	Low:T7-High:T7	0.56	0.10	1.03	0.01
TSS in	Low:T2-High:T2	-1.62	-3.22	-0.03	0.04
	Low:T3-High:T3	-0.24	-1.84	1.36	0.99
	Low:T4-High:T4	-0.25	-1.85	1.35	0.99
	Low:T5-High:T5	-0.72	-2.32	0.87	0.88
	Low:T6-High:T6	-0.55	-2.15	1.05	0.98
	Low:T7-High:T7	-1.83	-3.43	-0.23	0.01
TSS out	Low:T2-High:T2	-0.31	-1.21	0.59	0.98
	Low:T3-High:T3	-0.55	-1.45	0.35	0.55
	Low:T4-High:T4	-0.58	-1.48	0.32	0.49
	Low:T5-High:T5	-0.18	-1.08	0.72	0.99
	Low:T6-High:T6	-0.48	-1.38	0.42	0.79
	Low:T7-High:T7	-1.71	-2.61	-0.81	2e-05
k-value bio-media	Low:T0-High:T0	-0.14	-1.00	0.72	0.99
	Low:T1-High:T1	-0.14	-1.00	0.72	0.99
	Low:T2-High:T2	-0.26	-1.12	0.60	0.99
	Low:T3-High:T3	-0.02	-0.88	0.84	1.00
	Low:T4-High:T4	0.20	-1.07	0.66	0.99
	Low:T5-High:T5	0.31	-1.18	0.55	0.99
	Low:T6-High:T6	0.003	-0.85	0.88	1.00
	Low:T7-High:T7	-1.61	-2.48	-0.75	7e-06
k-value in	Low:T1-High:T1	-0.63	-1.98	0.72	0.89
	Low:T2-High:T2	-0.77	-2.12	0.58	0.71
	Low:T3-High:T3	-0.94	-2.29	0.41	0.42
	Low:T4-High:T4	-1.08	-2.44	0.27	0.22
	Low:T5-High:T5	-1.18	-2.53	0.18	0.14
	Low:T6-High:T6	-1.27	-2.62	0.08	0.08
	Low:T7-High:T7	-3.35	-4.70	-2.00	1e-07
k-value out	Low:T1-High:T1	0.31	-0.77	1.37	0.99
	Low:T2-High:T2	0.11	-0.96	1.18	1.00
	Low:T3-High:T3	-0.37	-1.44	0.71	0.99
	Low:T4-High:T4	-0.81	-1.88	0.26	0.29
	Low:T5-High:T5	-1.06	-2.13	0.01	0.05

	Low:T6-High:T6	-1.22	-2.29	-0.15	0.02
	Low:T7-High:T7	-2.78	-3.85	-1.71	0.00
COD in	Low:T1-High:T1	-9.85	-24.79	5.08	0.49
	Low:T2-High:T2	-3.20	-18.14	11.74	0.99
	Low:T3-High:T3	-5.13	-20.07	9.80	0.99
	Low:T4-High:T4	-4.20	-19.14	10.74	0.99
	Low:T5-High:T5	-2.83	-17.77	12.10	0.99
	Low:T6-High:T6	-10.63	-25.57	4.30	0.38
	Low:T7-High:T7	-42.27	-57.20	-27.33	0.00
COD out	Low:T1-High:T1	-11.53	-26.63	3.57	0.28
	Low:T2-High:T2	-4.20	-19.30	10.90	0.99
	Low:T3-High:T3	-2.77	-17.87	12.34	0.99
	Low:T4-High:T4	-3.97	-19.07	11.14	0.99
	Low:T5-High:T5	-2.23	-17.34	12.87	0.99
	Low:T6-High:T6	-10.53	-25.64	4.57	0.41
	Low:T7-High:T7	-40.17	-55.27	-25.06	0.00
TAN in	Low:T1-High:T1	-0.51	-0.95	-0.08	0.01
	Low:T2-High:T2	-0.16	-0.59	0.28	0.98
	Low:T3-High:T3	-0.02	-0.46	0.41	1.00
	Low:T4-High:T4	-0.26	-0.69	0.18	0.65
	Low:T5-High:T5	-0.10	-0.53	0.34	0.99
	Low:T6-High:T6	-0.06	-0.49	0.37	0.99
	Low:T7-High:T7	-0.25	-0.68	0.19	0.70
TAN out	Low:T1-High:T1	-0.31	-0.66	0.03	0.10
	Low:T2-High:T2	-0.21	-0.55	0.13	0.61
	Low:T3-High:T3	0.13	-0.22	0.47	0.98
	Low:T4-High:T4	-0.06	-0.40	0.29	0.99
	Low:T5-High:T5	-0.08	-0.42	0.27	0.99
	Low:T6-High:T6	-0.03	-0.37	0.32	1.00
	Low:T7-High:T7	-0.20	-0.54	0.14	0.68
NO2 in	Low:T1-High:T1	-0.73	-0.99	-0.47	0.00
	Low:T2-High:T2	-0.12	-0.38	0.14	0.88
	Low:T3-High:T3	0.05	-0.21	0.31	0.99
	Low:T4-High:T4	-0.07	-0.33	0.19	0.99
	Low:T5-High:T5	-0.10	-0.36	0.16	0.97
	Low:T6-High:T6	-0.07	-0.33	0.19	0.99
	Low:T7-High:T7	-0.09	-0.35	0.17	0.99
NO2 out	Low:T1-High:T1	-0.67	-0.89	-0.45	0.00
	Low:T2-High:T2	-0.13	-0.35	0.09	0.68
	Low:T3-High:T3	0.11	-0.12	0.33	0.88
	Low:T4-High:T4	-0.01	-0.23	0.22	1.00
	Low:T5-High:T5	-0.10	-0.33	0.12	0.90
	Low:T6-High:T6	-0.06	-0.28	0.16	0.99
	Low:T7-High:T7	-0.10	-0.32	0.13	0.95
NO3 in	Low:T1-High:T1	1.03	-34.09	36.15	1.00
	Low:T2-High:T2	4.67	-30.45	39.79	0.99
	Low:T3-High:T3	15.70	-19.42	50.82	0.92
	Low:T4-High:T4	5.23	-29.85	40.39	0.99
	Low:T5-High:T5	3.87	-31.25	38.99	1.00
	Low:T6-High:T6	17.83	-17.29	52.95	0.83
	Low:T7-High:T7	42.00	6.88	77.12	0.01
NO3 out	Low:T1-High:T1	-0.07	-35.41	35.27	1.00
	Low:T2-High:T2	9.43	-25.91	44.77	0.99
	Low:T3-High:T3	19.33	-16.01	54.67	0.75
	Low:T4-High:T4	4.27	-31.07	39.61	0.99
	Low:T5-High:T5	3.53	-31.81	38.87	1.00
	Low:T6-High:T6	18.50	-16.84	53.84	0.80
	Low:T7-High:T7	42.67	7.33	78.01	0.01
NO3 in (excluding T7)	Low:T1-High:T1	1.03	-14.12	16.19	1.00
	Low:T2-High:T2	4.67	-10.49	19.82	0.99
	Low:T3-High:T3	15.7	0.54	30.86	0.04
	Low:T4-High:T4	5.23	-9.89	20.42	0.98
	Low:T5-High:T5	3.87	-11.29	19.02	0.99

	Low:T6-High:T6	17.83	2.68	32.99	0.01
NO3 out (excluding T7)	Low:T1-High:T1	-0.07	-18.98	18.84	1.00
	Low:T2-High:T2	9.43	-9.48	28.34	0.80
	Low:T3-High:T3	19.33	0.42	38.24	0.04
	Low:T4-High:T4	4.27	-14.64	23.18	0.99
	Low:T5-High:T5	3.53	-15.38	22.44	0.99
	Low:T6-High:T6	18.50	-0.41	37.41	0.05

Appendix III. Table XXIV. *Tukey-HSD performed on Two-way ANOVA to investigate the differences between the outlet and the inlet in the different water quality parameters in the two treatment groups. Diff represents the difference in mean between the outlet and inlet of the biofilter, while Lwr-Upr represents the 95% confidence interval within which the true mean in difference between the two groups lie.*

Parameter	Particle loading	Diff (Out-In)	Lwr	Upr	p-adj
TSA	High	0.10	-0.84	1.04	0.99
	Low	0.16	-0.78	1.10	0.97
Beta	High	-0.07	-0.14	0.01	0.09
	Low	-0.04	-0.11	0.04	0.53
TSS	High	-0.02	-0.70	0.67	0.99
	Low	0.22	-0.47	0.91	0.84
k-value	High	-0.07	-0.76	0.62	0.99
	Low	0.41	-0.28	1.11	0.40
COD	High	-0.28	-13.71	13.16	0.99
	Low	0.11	-13.32	13.55	0.99
TAN	High	-0.16	-0.30	-0.01	0.03
	Low	-0.07	-0.21	0.07	0.56
NO2	High	-0.04	-0.24	0.16	0.96
	Low	-0.01	-0.21	0.19	0.99
NO3	High	0.23	-13.56	14.03	0.99
	Low	1.28	-12.52	15.07	0.99

Appendix III. Table XXV. *Tukey-HSD performed on one-way ANOVA to investigate differences in Δ between high and low particle loading (outlet-inlet or inlet-outlet). Diff represents the difference in mean between low and high particle load, while Lwr-Upr represents the 95% confidence interval within which the true mean in difference between the two groups lie.*

Parameter	Δ calc	Diff	Lwr	Upr	p-adj
TSA Δ	out-in	0.07	-0.09	0.23	0.38
Beta Δ	out-in	0.08	-0.04	0.19	0.18
TSS Δ	out-in	0.98	-0.44	2.41	0.17
k-valueΔ	out-in	0.01	-0.00	0.03	0.08
COD Δ	out-in	0.39	-0.91	1.68	0.55
TAN Δ	in-out	-0.09	-0.14	-0.03	0.01
NO2 Δ	in-out	-0.02	-0.05	0.00	0.06
NO3 Δ	in-out	1.04	-0.66	2.75	0.22

III-V Linear mixed effect model (LMM)

Appendix III. Table XXVI *Results from LMM performed to investigate differences in slopes in TAN removal rates between bio-media exposed to high and low particle loading (n =3).*

0'order kinetics post freshwater phase					
	Estimate	Std. Error	df	t-value	p-value
High intercept	5.45	0.28	4.21	19.21	2.91e-05
High Slope	-24.27	0.62	64.04	-38.90	2e-16
Low intercept diff	-0.17	0.40	4.22	-0.43	0.6885
Low slope diff	1.71	0.94	64.05	1.83	0.072
0'order kinetics post brackish water phase					
	Estimate	Std. Error	df	t-value	p-value
High intercept	4.93	0.14	4.99	34.80	3.75e-07
High Slope	-32.04	0.99	59.35	-32.37	2e-16
Low intercept diff	-0.10	0.20	4.93	-0.50	0.64
Low slope diff	4.50	1.29	59.23	3.49	0.001
1'order kinetics post freshwater phase					
	Estimate	Std. Error	df	t-value	p-value
High intercept	0.09	0.05	1.12	1.73	0.31
High Slope	-18.92	0.61	4.00	-31.08	6.39e-06
Low intercept diff	0.14	0.07	1.12	2.14	0.26
Low slope diff	-3.63	0.75	4.00	-4.87	0.01
1'order kinetics post brackish water phase					
	Estimate	Std. Error	df	t-value	p-value
High intercept	0.08	0.12	4.65	0.67	0.54
High Slope	-24.78	0.97	15.21	-25.49	6.8e-14
Low intercept diff	0.09	0.17	4.78	0.05	0.96
Low slope diff	0.17	1.80	15.15	0.09	0.93

III-VI Shapiro-wilk test

Appendix III. Table XXVII. *Results from Shapiro-Wilks test performed to check if datasets followed normal distribution*

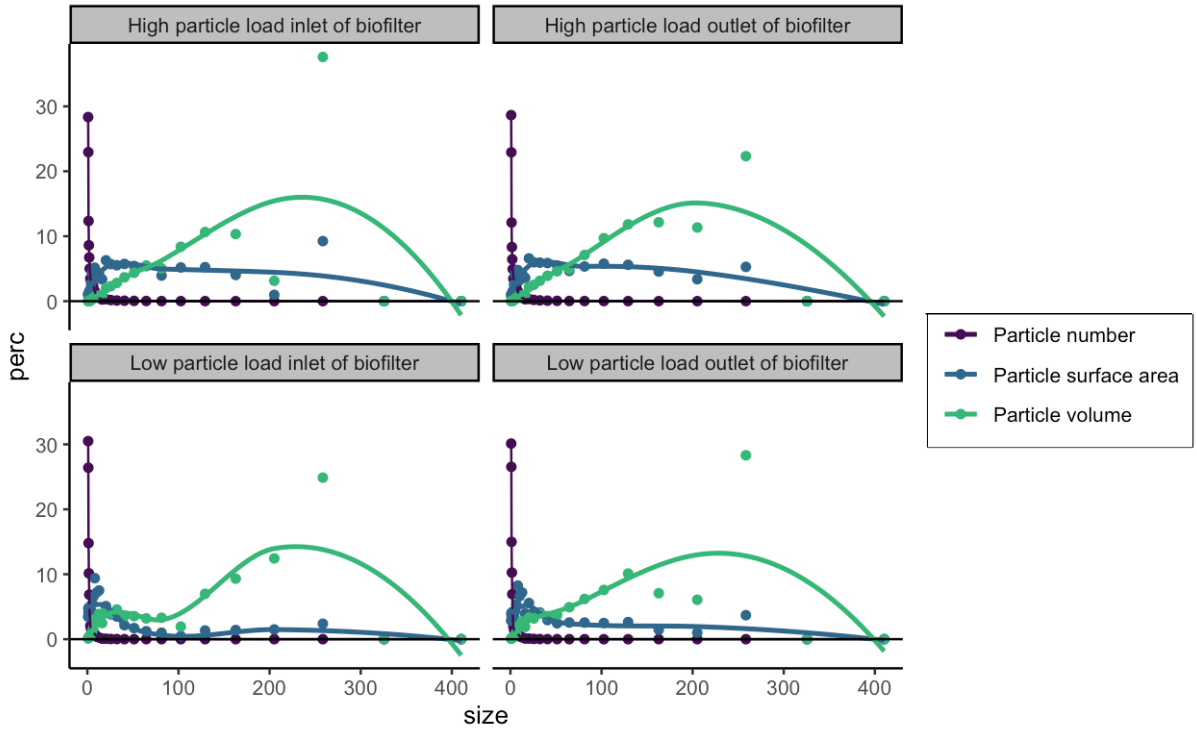
Parameter	W	p-value
TSA-in	0.93	0.05
TSA-out	0.92	0.03
Beta in	0.96	0.38
Beta out	0.94	0.08
TSS in	0.97	0.53
TSS out	0.94	0.05
k-value bio-media	0.87	0.002
k-value in	0.92	0.007
k-value out	0.94	0.02
COD in	0.77	1.082e-06
COD out	0.78	2.105e-06
TAN in	0.83	2.892e-05
TAN out	0.87	0.002
NO2 in	0.74	3.234e-07
NO2 out	0.74	2.757e-07
NO3 in	0.88	0.005
NO3 out	0.92	0.006

III-VII Levene's test

Appendix III. Table XXVIII *Results from Levene's test to examine the homogeneity of variances.*

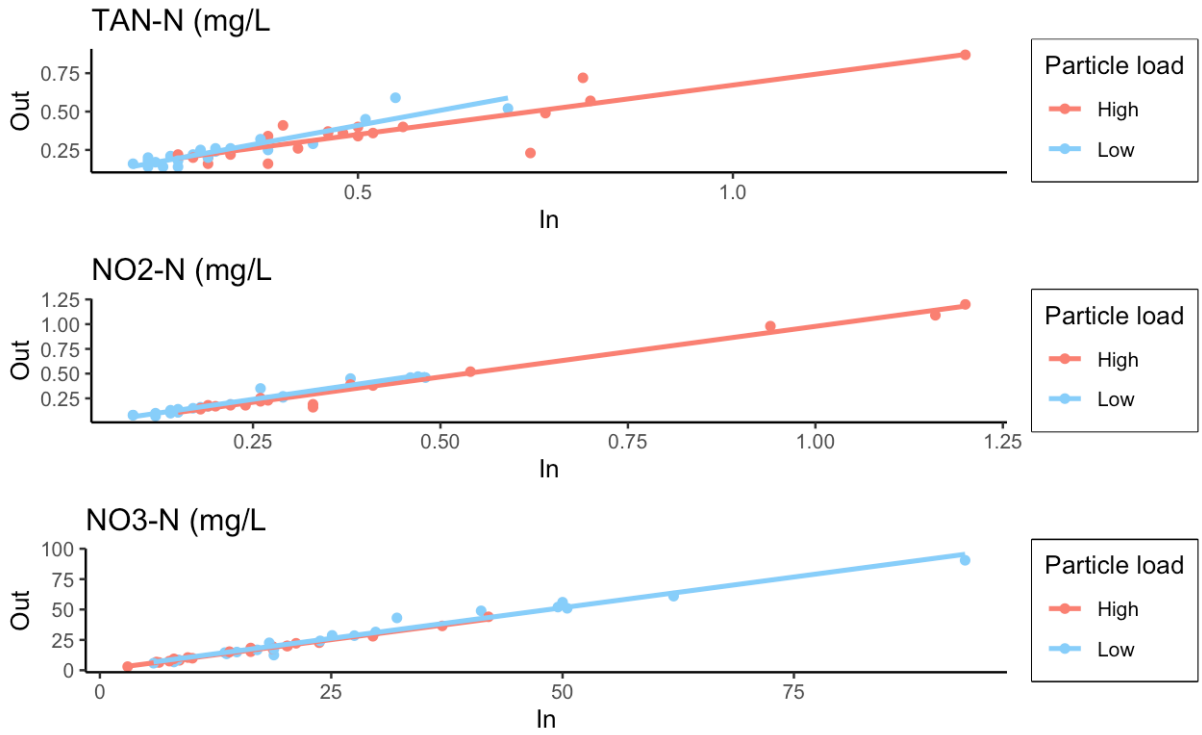
Parameter	df	F-value	p-value
TSA-in	1, 28	3.8987	0.06
TSA-out	1, 28	4.1589	0.05
Beta in	1, 28	0.2481	0.62
Beta out	1, 28	0.1549	0.70
TSS in	1, 34	0.4117	0.53
TSS out	1, 34	1.7949	0.19
k-value bio-media	1, 46	5.247	0.03
k-value in	1, 40	3.4004	0.07
k-value out	1, 40	3.2132	0.08
COD in	1, 40	1.8704	0.18
COD out	1, 40	2.0925	0.16
TAN in	1, 40	2.7362	0.11
TAN out	1, 40	1.5226	0.22
NO2 in	1, 40	1.8994	0.18
NO2 out	1, 40	1.2763	0.27
NO3 in	1, 40	4.3471	0.05
NO3 out	1, 40	6.8999	0.03

Appendix IV. PSD, contribution to particle- number, Surface area and volume per size class



Appendix IV. Fig III *Average contribution to particle- number, surface area and volume per size class across all sampling days. The x-axis represents different size classes from 0.8 µm to 410.3 µm, the y-axis represents percentage (%) of contribution. The top figures represent high particle load systems at the inlet an outlet of the biofilter, bottom figures represent low particle load systems at the inlet an outlet of the biofilter.*

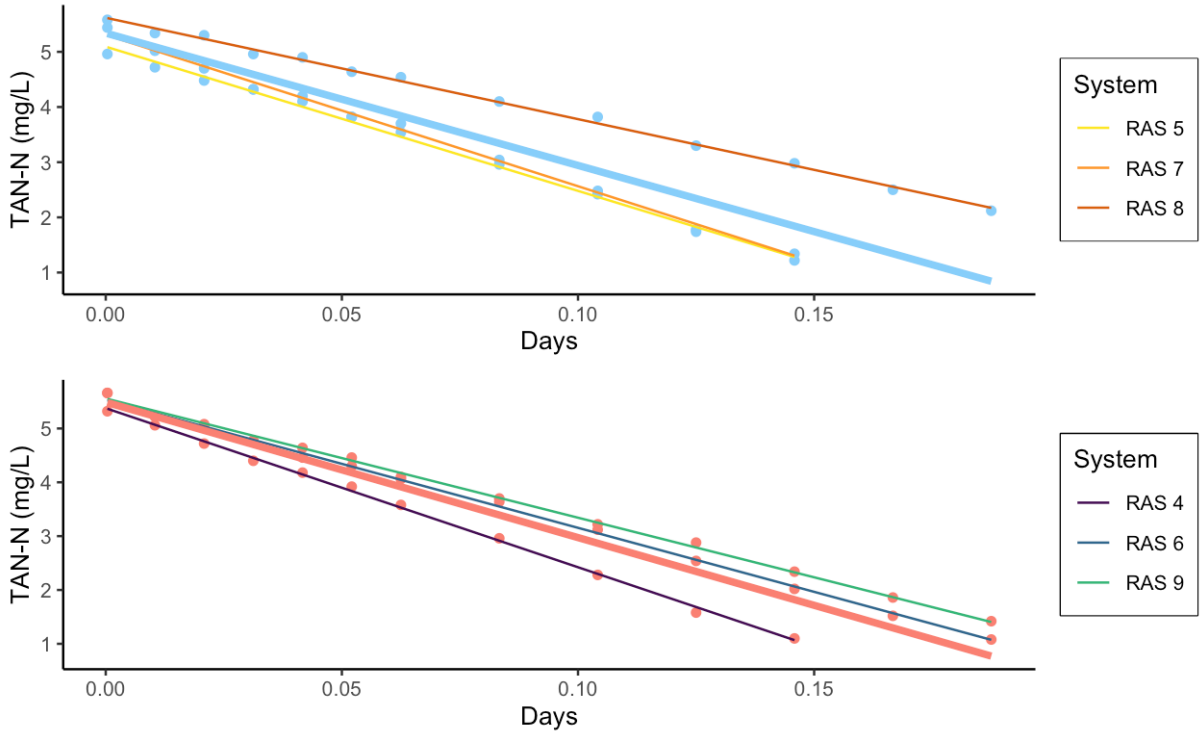
Appendix V. Nitrogen compounds Change between outlet and inlet of biofilter (Δ)



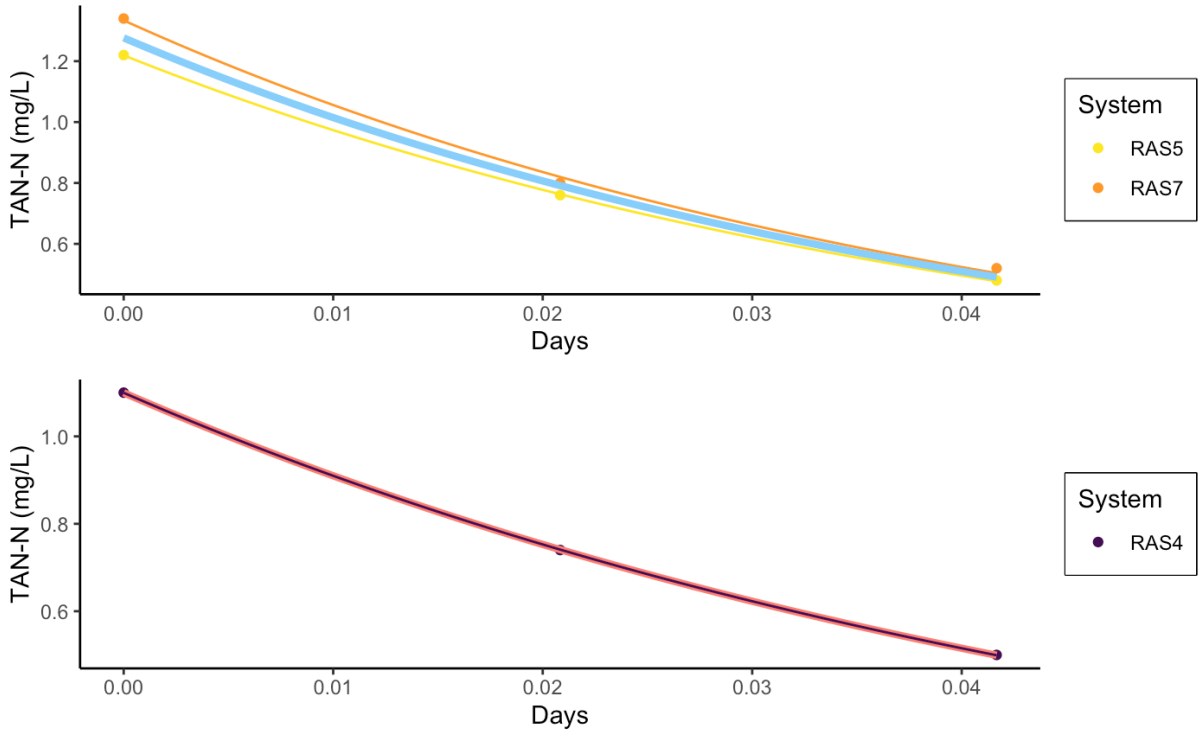
Appendix V. Fig IV *Difference in nitrogen compounds (TAN, nitrite, and nitrate) concentration between the inlet and outlet of the biofilter, for systems exposed to high and low particle loading. X-axis represents concentration (mg/L) at the inlet of the biofilter, y-axis represents concentration at the outlet of the biofilter.*

Appendix VI. Spiking experiment (TAN removal in each replicate)

V-I Fresh water phase

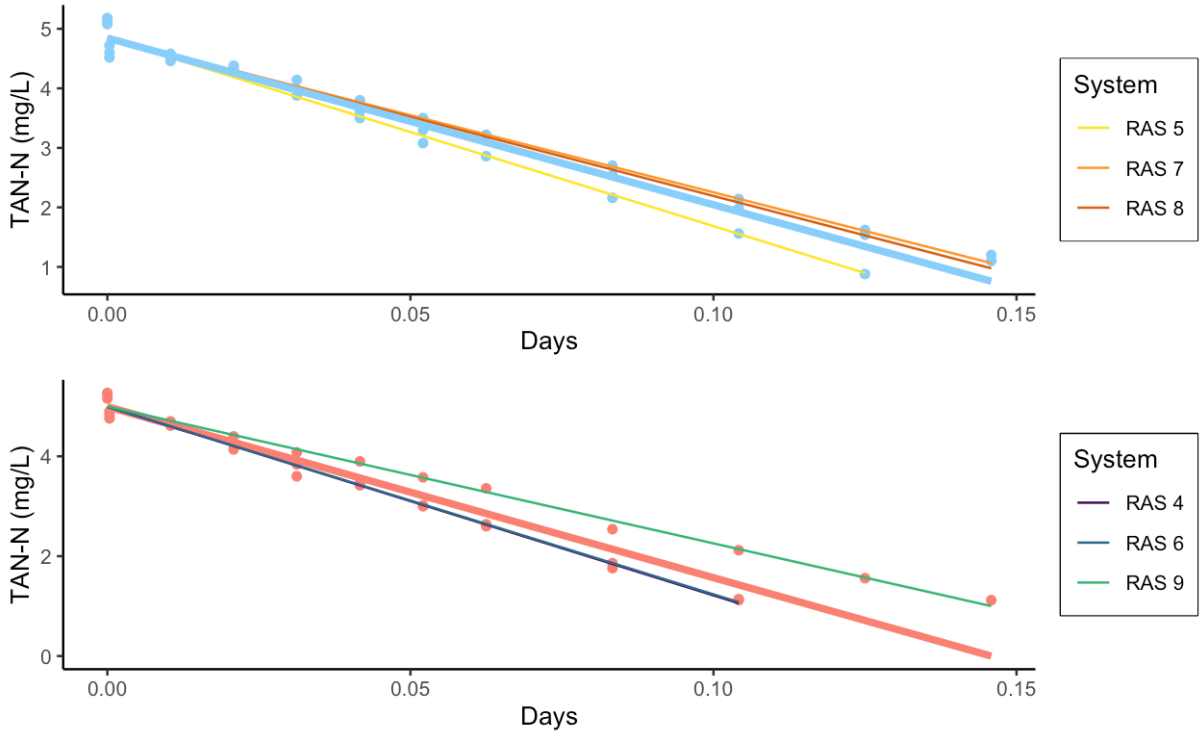


Appendix VI. Fig V. *0' order TAN removal in bio-media from each individual RAS after the freshwater phase. Thin lines represent each individual system, thick line represents the regression for all datapoints. Top figure represents RASs exposed to low particle loads, bottom represents RASs exposed to high particle loads.*

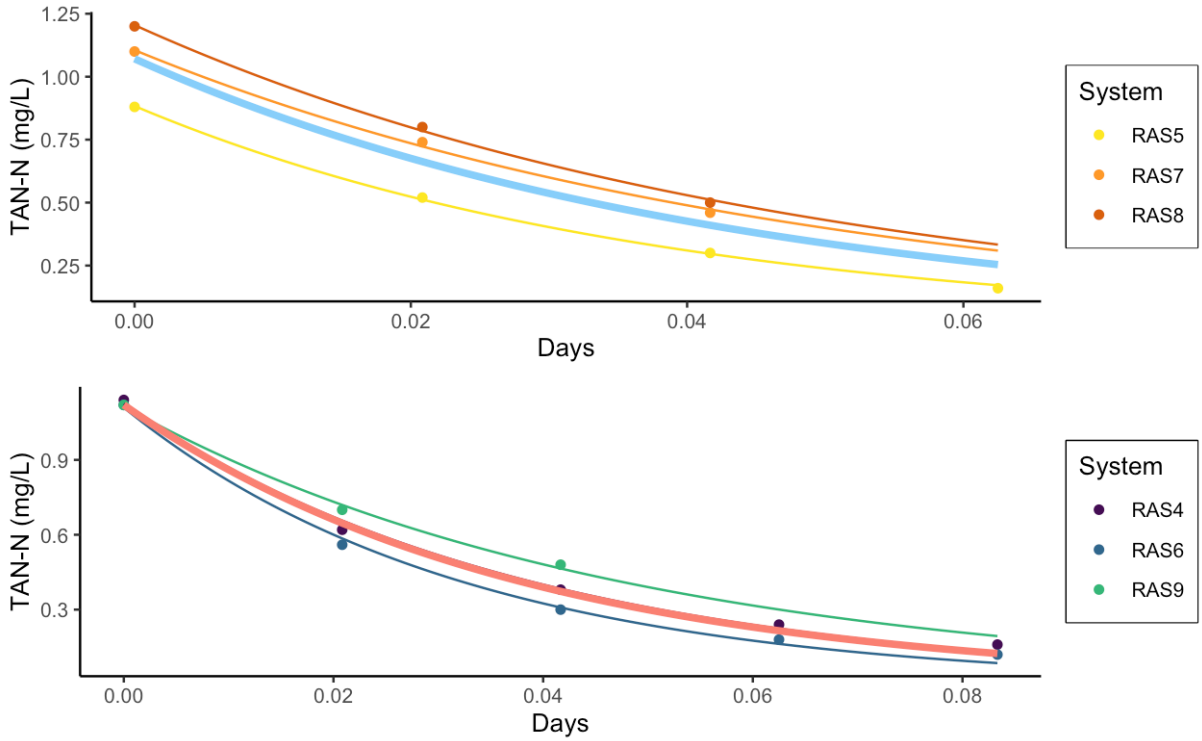


Appendix V. Fig VI. *1' order TAN removal in bio-media from each individual RAS after the freshwater phase. Thin lines represent each individual system, thick line represents the regression for all datapoints. Top figure represents RASs expoed to low particle loads, bottom represents RAS exposed to high particle loads.*

V-II Brackish water phase



Appendix V. Fig VII. *0' order TAN removal in bio-media from each individual RAS after the brackish water phase. Thin lines represent each individual system, thick line represents the regression for all datapoints. Top figure represents RASs exposed to low particle loads, bottom represents RAS exposed to high particle loads.*



Appendix V. Fig VIII. *1' order TAN removal in bio-media from each individual RAS after the brackish water phase. Thin lines represent each individual system, thick line represents the regression for all datapoints. Top figure represents RASs exposed to low particle loads, bottom represents RAS exposed to high particle loads.*