Ammonia oxidation and oxygen consumption rate measurements in RAS at different alkalinities

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Abstract

The need for environmentally friendly sustainable nutritious food has never been more imminent than today, with a need to increase the world´s animal-based food supply by 70% by 2050. By simply increasing the number of animals would do more harm than good. Fish are a great source of animal protein and an important source of nutrients especially for people in developmental countries however, wild capture fisheries have been stagnant for decades and today aquaculture is responsible around 50% of fish produced for human consumption. The leading method of farming has been extensive farming where fish are farmed at low densities, with low growth rates and flowthrough water however, a more sustainable way of farming and with increased production yields called recirculating aquacultural systems (RAS) is becoming more popular. Norway produces 50% of salmon sold worldwide. With salmon being of great value both financially and nutritionally it is important that their health and wellbeing are taken care of. If the RAS are not working at full potential this can result in accumulation of nitrogenous wastes like ammonia-nitrogen and nitrite-nitrogen, which are highly toxic to aquatic organisms. Biofilter within the RAS can break down these toxins through nitrification however, several environmental conditions affect the efficiency of the biofilters. One of the factors whose effects are not well understood is alkalinity. The aim of this thesis was to investigate what effects alkalinity has on nitrification and to make a protocol for measuring oxygen consumption during nitrification. Alkalinity levels of 70, 100 and 200 mg/L as $CaCO_3$ were used with no apparent effect on nitrification or oxygen consumption. The only things found to effect nitrification rates were ammonia concentrations and biofilter maturation. Oxygen consumption measurements during nitrification were successful but they can not be used as an estimate of biofilter activity.

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Abbreviations

AOB	Ammonia-oxidizing bacteria
$CaCO_3^{2-}$	Calcium carbonate
CO_2	Carbon dioxide
CO_3^{2-}	Carbonate ion
EW	Equivalent weight
H^+	Hydrogen ion/proton
HCO_3^-	Bicarbonate ion
Ka	Acid dissociation constant
M1	Module 1
M12	Module 12
MBBR	Moving bed biofilm reactor
MLR	Multiple linear regression
MW	Molecular weight
N_2	Nitrogen gas
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NH_3	unionized ammonia
NH_4^+	ionized ammonia
NH_3-N	Ammonia-nitrogen
NH_4-N	Ammonium-nitrogen
NOB	Nitrite-oxidizing bacteria
NO_2-N	Nitrite-nitrogen
NO_3-N	Nitrate-nitrogen
O_2	Oxygen
OH^-	Hydroxide ion
RAS	Recirculating Aquacultural Systems
TA	Total Alkalinity

TAN Total Ammonia Nitrogen

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Introduction

Background: Recirculating aquaculture systems (RAS) and global food security

As the world's population continues to increase, the need for sustainable nutritious food is ever-growing, as more suffer from malnutrition and lack of food (FAO, 2021). The 2019 *World Resources Report* by Searchinger *et al*. (2019) offers some potential solutions for how to improve food security, especially for people in developing countries. According to them (Searchinger *et.al.*), by 2050 the world´s animal-based food supply needs to have increased by 70%. However simply increasing the number of livestock would entail clearing more land for raising more livestock and growing additional crops for feed. As around half of the world´s vegetated land is used to produce food (Searchinger *et.al*., 2019), taking more land would mean clearing more forests, destroying more wetlands, releasing more carbon dioxide (CO_2), and disrupting an already fragile ecosystem. Instead, Searchinger and colleagues propose solving the food shortage by increasing existing (crop and livestock) yields and by increasing the fish supply. Fish are a great source of animal protein as well as being an important source of vitamins, minerals and essential fatty acids for people especially in developing countries (FAO, 2022).

With the supply of wild capture fisheries being stagnant since the 1990´s and people consuming more fish than ever (FAO, 2022) the increase in seafood supply needs to come elsewhere. With improved fisheries management and better resource utilization it is possible to increase the yield to some degree, however, as the FAO (2022) report states, most of the increase in seafood is coming from aquaculture, in fact today the aquaculture industry accounts for around 50% of the fish produced for human consumption and will likely continue to increase in coming years.

Aquaculture is the farming of aquatic species, enabling farming of everything from fish to mollusks to crustaceans and algae (Naylor *et.al*., 2021). Most aquatic species are difficult to farm and only a few dozen dominate the industry with most of them being produced in Asia, especially China. Production in developed countries is low however, Norway has managed to rank in the top 20 for fastest growth rate among the major producers and top 10 in both quantity and value (Garlock *et.al*., 2020). Norwegian farmers have mostly concentrated on monocultures, particularly Atlantic salmon (*Salmo Salar*) which has become one of the largest exports in the country as well as making Norway the biggest supplier of salmon in the world, supplying more than 50% of salmon worldwide (Iversen *et.al*., 2020). This has been made possible through focused efforts on research

and innovation on genetic breeding, procedure optimization, fish health, customized feed, cost reduction, and increasing profits (Afewerki *et.al*., 2022).

With salmon being such an important part of Norway's economy (Straume *et.al*., 2022; Sikveland *et.al.*, 2022), it is crucial that the salmon farmers are provided with as much knowledge as they can about the production, allowing them to provide the best living conditions for the fish to produce a superior product. To produce a superior salmon, it is necessary to know what optimal thriving conditions are, in order to get a healthy, sturdy, and fast-growing fish. One of the main factors influencing these characteristics is the quality of the water the fish are reared in (Austin, 1998; Zeitoun and Mehana, 2014; Zaibel *et.al*., 2016). The quality of the water depends on different factors e.g., what kind of system is being used, where is the water coming from (country, region, surface water, groundwater etc.), what is the chemical composition of the water and how it is treated.

Scientific background

RAS is used for intensive farming (Ahmad, 2021) where the organisms of interest are farmed in artificial tanks at high densities. RAS provide a high degree of environmental and productional control, increasing growth rates and yields. However, the increased work effort as well as high energy and feed requirements raise the cost of production (Timmons *et.al.*, 2018; Naylor *et.al.*, 2021) significantly above that of extensive farming where the cultured species are reared in natural ponds at low densities, with little or no feeding supplementation and very little work effort is needed. The downside of extensive farming is the low production control and unpredictability of growth rates and yield due to natural variations in the environment (Naylor *et.al.*, 2000). Extensive farming is the oldest and most widely used method of farming (FAO 2022) however, if the aquatic food supply is to enhance food security, intensive farming is the way to go. RAS and intensive farming are also behind the success of the salmon farming industry giving facilities control over the environment and the production enabling year-round production, even in locations where water shortages would otherwise be a problem (Lekang, 2007; FAO, 2022). It allows the production of more fish, on the same amount of land, over a shorter period of time, using fewer resources, increasing the farms productivity and revenue (Martins *et.al*., 2010; Timmons *et.al.*, 2018).

Salmon are an excellent source of protein, and they are filled with nutrients essential for human health and development (Lund, 2013; Colombo and Mazal, 2020; FAO 2022). Continued and increased farming of salmon is of great interest to both farmers and consumers due to its financial

profitability and nutritional values. There are however issues restricting both current productions and the future growth of the industry particularly; the production of salmon feed (Naylor *et.al.*, 2000; Shepherd *et.al.*, 2017; FAO, 2020), salmon lice (Aaen et.al., 2015) and access to freshwater and land (Searchinger, 2019).

RAS can provide a solution to the problem of both water and land shortage. By incorporating RAS into new and existing farming facilities it makes it possible to increase stocking densities, to reuse 90 - 99% of the water (Timmons et.al., 2018), to reduce the environmental impact (Bohnes *et.al.*, 2018; Ghamkhar *et.al.*, 2021) and lowering the risk of introducing pathogens into the system (Sharrer *et.al.*, 2005). However, it offers up new problems that need to be addressed for these RAS systems to work optimally, especially if companies want to move the production on land to avoid the lice problem.

RAS overview

The size and architecture of RAS systems vary considerably but the process is the same (Timmons *et.al.*, 2018; Davidson, 2020). With the different types of RAS with varying degrees of recirculation (Martins *et.al*., 2010) the quality of the water will depend on the amount of reused water. A schematic overview of the main compartments and processes of a typical RAS are shown in Figure 1. Water is pumped into the fish tanks through a vertical pipe on the side of the tank. The pipe has holes down the side where the water comes out in jets to create a (jet) flow in the tank, that can be adjusted depending on the size of the fish. For salmonoids the formula $V < 5.25/L^{0.37}$ can be used to calculate safe non-fatiguing velocities (Timmons *et.al*., 2018). The flow rate will impact the hydraulic retention time both in the fish tanks and in the biofilters. From the fish tanks the water flows through the system to the biofilters. The biofilters associated with RAS are made up of several sections and components working in conjunction (Davidson, 2020), through which the reused water is cleaned off waste and waste products to uphold the water quality. In specific compartments of the biofilter a highly diverse community of microorganisms is concentrated either in suspension or attached to and proliferating on biocarriers (Rurangwa and Verdegem, 2015; Preena *et.al*., 2018; Dahle *et.al.*, 2023), this microbial community cleans the water of waste products toxic to the fish, specifically two nitrogen containing compounds: ammonia (Randall, 2002) and its derivative nitrite (Lewis Jr. and Morris, 1986) through a process called nitrification. Before the water reaches the biofilter chambers where most of the nitrification occurs it goes through a filtration process for removal of organic material. The reason for removing the organic material, is that most of the nitrification is performed by chemo-lithoautotrophic bacteria that feed

on inorganic compounds, while other organisms in the biofilter feed on organic compounds (heterotrophs). The latter ones have a much faster growth rate and will outcompete the autotrophs if given the opportunity (Belser, 1984; Leonard *et.al*., 2000). Even though the presence of heterotrophs is needed (Blancheton *et.al.*, 2013) they are much less efficient than ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) at oxidizing ammonia-nitrogen and nitrite-nitrogen, therefore if they are allowed to take over the biofilters, the water will rapidly become toxic to the fish. By keeping a low concentration of organic matter in the biofilters it restricts the growth of the heterotrophs giving room for the autotrophs to grow. Depending on the setup of the RAS there will be one or more filtering processes where organic material like fish feces and uneaten feed are filtered out by e.g., settling tanks for solids >100 µm, drum filters for particles larger than >60 µm and flotation processes for particles <30µm (Timmons *et.al*., 2018), after this the water enters the biofilter chambers (more on this in the section "Nitrification and the players involved"). From the bio-chambers the next stage of the process is decontamination through UV-radiation and/or ozone treatment (Blancheton *et.al.*, 2013) ozone also help remove particles from the water and aids complete nitrification (Timmons *et.al*., 2018). After decontamination the water enters the last step in the cleaning process, the degassing section where gasses like CO_2 and nitrogen gas (N_2) are aerated out of the water (Martins *et.al*., 2010). CO_2 enters the water from the atmosphere but mostly by the respiration of the fish and needs to be removed as to not reach toxic levels (Hayashi *et.al*., 2004; Santos *et.al*., 2013; Good *et.al*., 2018; Mota *et.al*., 2019). N_2 is a product of the denitrification process which will be explained in the section "Nitrification and the players involved". After this the water is ready to be pumped back into the fish tanks. This is the only place where the water is actively pumped, the rest of the process is downstream of the fish tanks and is controlled by the rate of water being pumped into the rearing tanks. Before the water enters the fish tanks again it is oxygenated to make sure that there is sufficient oxygen (O_2) in the water for the fish to breathe, for salmonoids rearing water effluent should contain 6.0-8.0 mg/L dissolved oxygen (Timmons *et.al.*, 2018). If the hydraulic retention time is too high it will negatively affect the oxygen levels in the tanks and the swimming pattern of the fish, if it is too low this will also affect the fish that may not be able to swim against the current (fatiguing velocity) and the water will pass too quickly through the biofilters and efficiency of the biofilters will go down resulting in poor water quality. The nitrification process as well as many of the microorganisms in the biofilter also consume oxygen (Chen *et.al*., 2006; Preena *et.al.*, 2021), it is therefore necessary to supply the biofilter chambers with oxygen too. If the oxygen becomes too low in the biofilter the

nitrification will shut down (Timmons *et.al*., 2018) and under anoxic conditions some organisms can start to produce hydrogen sulfide (H_2S) which is extremely toxic to the fish, if large amounts are released into the water the fish will die in a matter of minutes (Navada *et.al*., 2020). No matter how much water is re-used there will always be a need for makeup water as some water will be lost to things like evaporation and flushing of the systems. The amount of makeup water used will affect environmental conditions like pH, temperature, salinity, and CO_2 all of which need to be controlled in the interest of both the fish and the biofilters (Prinčič *et.al*., 1998; Bernhard *et.al*., 2005; Chen *et.al*., 2006; Colt, 2006; Bakke *et.al*., 2017; Timmons *et.al*., 2018; Gao *et.al*., 2020).

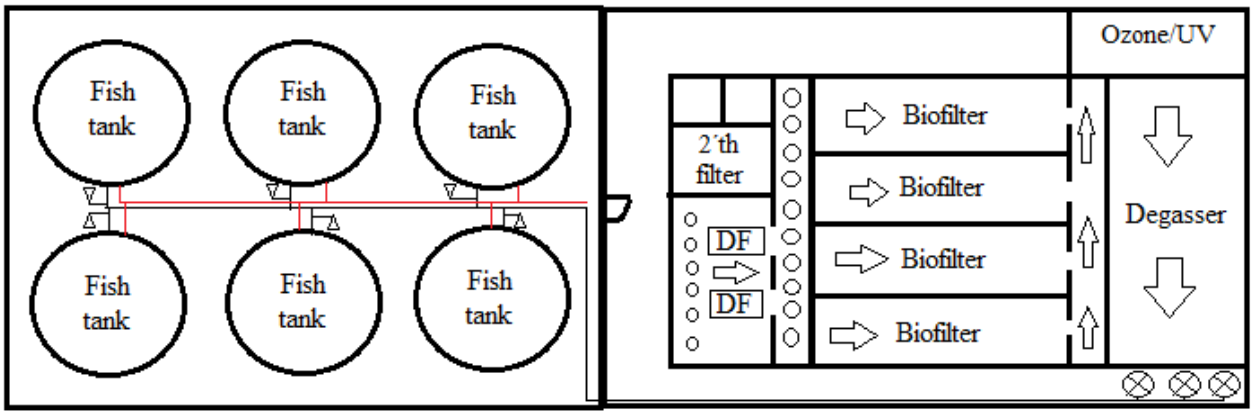


Figure 1: Schematic of a typical RAS facility. Read lines are water flowing out of the fish tanks into the biofilter (below ground). The water flows up through the holes in front of the DF (drum filters), from there it enters a ditch in front of the biofilters, from there it is suctioned down through the holes in the ditch into the biofilter. from the biofilter the water enters a new ditch leading the water to the decontamination chamber, from there the water enter the degasser and is finally pumped (circles with exes) back to the fish tanks (black thin line leading to the tanks). Before the water reaches the fish tanks it is oxygenated through oxygen cones (triangles next to the tanks). Chambers containing chemicals for pH and alkalinity control are next to the 2´th filter. Arrows indicate flow direction.

Control over production: fish and microbiota

A downside to RAS is that if not managed correctly the quality of the water will deteriorate fast, as there is an accumulation of waste materials, gasses, and potential pathogens due to the lack of water exchange. This accumulation will if not effectively counteracted affect the growth and survival of the fish (Sharrer *et.al*., 2005; Davidson *et.al.*, 2009; 2011, Martins *et.al*., 2009). The limiting factor in RAS today is the accumulation of ammonia- and nitrite-nitrogen, because even though nitrogen is an essential building block of life and an integral structural component of nucleic acids and proteins, it is only needed in small amounts and excess amounts in the water can be lethal to aquatic

organisms (Timmons *et.al.*, 2018; Preena *et.al.*, 2021). As RAS is becoming more popular and widespread with increasing hydraulic retention times and stocking densities it is especially important that the biofilters are working at full potential. Knowing what microorganisms are most efficient at removing nitrogen and under what conditions they thrive best is crucial to maintaining a healthy and well-functioning biofilter (Chen *et.al.*, 2006). Optimizing biofilter performance and ensuring good water quality also improves fish welfare and health, minimizing disease outbreaks and loss of fish (Skjermo *et.al*., 1997; Salvesen *et.al*., 1999; Attramadal *et.al*., 2014; Bakke *et.al*., 2017), insuring a steady production. The effect of pH on the biofilter has been studied for decades and still the reported optimum operational range is wide (Prinčič *et.al*., 1998; Biesterfeld et.al., 2003), but maintaining a steady pH is of great importance in aquaculture regarding both fish and biofilters (Timmons et.al., 2018). pH stability can be achieved by adding alkalinity to the water. Alkalinity is not a specific chemical, but rather a specific trait of the water (its capacity to neutralize acids) and is measured as calcium carbonate ($CaCO_3$), more in this in the section "Alkalinity". It has been suggested that keeping alkalinity levels in the biofilter above 40-50 mg/L as $CaCO_3$ is necessary for maintaining structural and functional demands of the microorganisms in the biofilter (Sharma & Ahlert, 1976; Biesterfeld *et.al*., 2003; Shanahan & Semmens, 2015; Mellbye *et.al*., 2016). Other studies recommend higher alkalinities from 100 to >200 mg/L $CaCO_3$ to ensure operational demands and stable pH (Chen *et.al*.,2006; and Shanahan & Semmems, 2015; Timmons *et.al*., 2018). Regarding the future of aquaculture and increasing salmon production, optimization of RAS and nitrogen waste disposal is detrimental. In this regard investigating how alkalinity affects nitrification is of great interest and is the focus of this thesis.

Nitrification and the players involved.

Ammonium concentrations in RAS need to be kept below concentrations that are toxic to the fish (Masser *et.al*., 1999; Colt, 2006). Ammonia-nitrogen in water is the combination of two forms of ammonia, the ionized ammonia-nitrogen (NH_4^+-N) and the unionized ammonia-nitrogen (NH_3-N), together they make up total ammonia nitrogen (TAN = NH_3-N + NH_4^+-N). The two forms of TAN: NH_3 (ammonia) and NH_4^+ (ammonium) are at equilibrium as shown in Reaction 1 (Boyd *et.al.*, 2016), with the reaction shifting towards the left with increasing pH, salinity, and temperature (Timmons *et.al*., 2018). NH_3-N is the more toxic form as it can move freely across cell membranes. It is generally accepted that NH_3-N should be held at <0.05 mg/L and TAN should be under 1.0 mg/L (Chen et.al., 2006; Timmons *et.al.*, 2018). Knowing TAN and pH it is possible to calculate

the concentration of NH_3-N in the water based on the pH dependent equilibrium between NH_4^+ and NH_3 (Equation 1)

$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-$ Reaction 1

$$NH_3\text{-}N = \frac{TAN}{1+10^{pKa-pH}}$$ Equation 1

TAN can be measured colorimetrically using a spectrophotometer. pKa is the negative log of the acid dissociation constant (Ka) for the reaction, and pH is the measured pH of the solution. (Timmons *et.al*., 2018).

$$Ka = 10^{-pKa} \rightarrow pKa = -\log(Ka)$$

Ka is a measure of the strength of an acid in solution and can be calculated with Equation 2 (Manjooran, 2020)

$Ka = [H+]\,[A-] / [HA]$ Equation 2

[H+] = concentration of H^+, [A-] = concentration of conjugate base, and [HA] = concentration of acid at reaction equilibrium.

During feeding dietary proteins are hydrolyzed into free amino acids and used for somatic growth and maintenance, however as salmon feed is high in proteins (Aas et.al., 2019) there is a large excess of amino acids and nitrogen that is not utilized. Catabolism of Glutamate to CO_2 through the removal of the α-amino group is perhaps the biggest way of removing excess amino acids (Li *et.al*., 2020). This is also a primary source of energy for the fish as the carbon backbone of Glutamate is used to generate adenosine triphosphate (ATP) (Li *et.al.*, 2020). The deamination of the amino group produces TAN, Campbell *et.al.* (1983) found that some 90% of ammonia in channel catfish came from deamination of Glutamate and glutamine. While some of the TAN in teleosts can be detoxified to urea (~10-30%) most of it is excreted as NH_3 (Wright and Land, 1998). Both urea and NH_3 are excreted over the branchial epithelia making the gills the major site for nitrogenous waste excretion. NH_3 diffuses over the gills down a favorable blood-to-water gradient (Walsh, 1997; Ip and Chew, 2010). This gradient is created as fish are able to manipulate plasma pH and gill boundary layer pH enabling them to acidify the gill water boundary layer by excreting CO_2 and H^+. CO_2 is hydrated as it enters the water and forms H^+ and bicarbonate (HCO_3^-), as NH_3 is excreted into the acidic boundary layer it is protonated to form NH_4^+ maintaining the downward partial pressure gradient of NH_3 (Weihrauch et.al., 2009). If environmental NH_3 concentrations are

increased the partial pressure gradient can be reversed so that NH_3 enters the fish from the environment and accumulates in the fish to toxic amounts. It is therefore of great importance that the biofilters are ready to receive and break down TAN as it enters the water.

Until recently nitrification was thought to be an obligate two step reaction with AOB and NOB, but in 2015 van Kessel *et.al.* (2015) discovered that some members of the bacterial Nitrospira genus are capable of complete ammonia oxidation (comammox), using the same enzymes to catalyze the reaction as the two-step reaction. Since their occurrence was confirmed the comammox bacteria have been widely studied and reported to exist in both natural and engineered systems and have been found to be quite versatile and tolerant to both heavy metals and low temperatures (Maddela *et.al.,* 2022) which is an advantage in RAS.

Ammonia oxidation (Reaction 2) is the first step of the nitrification process, where TAN is oxidized to NO_2^-, subsequently nitrite oxidation (Reaction 3) is where NO_2^- is oxidized to NO_3^- (Paredes *et.al*., 2007). NO_3^- is not considered to be highly toxic and values of 100 – 1000 mg/L have been reported (Rijn *et.al*., 2006), however new research show that values as low as 100 mg/L caused abnormal swimming behavior in rainbow trout, concentrations <75 mg/L are recommended for marine cultured fish, and as low as <25 mg/L can have sublethal effects on salmon eggs (Davidson *et.al*., 2014). RAS with low water exchange can accumulate high concentrations of NO_3 which can lead to toxicity, and it is therefore necessary to ensure the removal of this through denitrification.

$NH_4^+ + 1.5O_2 + 2HCO_3^- \rightarrow NO_2^- + 2CO_2 + 3H_2O$ Reaction 2

$NO_2^- + 0.5O_2 \rightarrow NO_3^-$ Reaction 3

The complete reaction can be written as Reaction 4 (Paredes *et.al*., 2007)

$NH_4^+ + 2O_2 + 2HCO_3^- \rightarrow NO_3^- + 2CO_2 + 3H_2O$ Reaction 4

As the water from the fish tanks enters the biofilter and reaches the biocarrier chambers the bulk of the microbial community can get to work. First ammonia is oxidized by AOB to the intermediate product hydroxylamine (NH_2OH) with the help of the enzyme ammonia monooxygenase (AMO), hydroxylamine is then further oxidized by hydroxylamine oxidoreductase (HAO) to NO_2^-. The most common genera of AOB are: *Nitrosomonas*, *Nitrosococcus*, and *Nitrosospira* (Ruiz *et.al*., 2020; Preena *et.al*., 2021). Secondly NOB employ the enzyme nitrite oxidoreductase (NOR) to oxidize NO_2^- to NO_3^-. The most common genera of NOB are: *Nitrobacter*, *Nitrococcus*, *Nitrospira*, *Nitrospina* and *Nitrotoga* (Ruiz *et.al*., 2020; Preena *et.al*., 2021). Depending on whether it is a

freshwater, brackish water or saltwater facility the activity and abundance of bacteria will change (Tal *et.al.*, 2003; Gao *et.al*., 2020; Preena *et.al*., 2021). Some AOB and NOB have been reported to have denitrifying capabilities as well making it possible to have simultaneous nitrification-denitrification (Bock *et.al*., 1995; Casciotti & Ward, 2001; Schmidt *et.al*., 2004; Füssel *et.al*., 2017) which is advantageous for the complete removal of nitrogenous wastes in RAS.

In addition to both autotrophic and heterotrophic nitrification that are carried out by bacteria, there is also archaeal nitrification, all of which are carried out under oxic conditions (Preena *et.al.*, 2021). Ammonia oxidizing archaea have been found to outnumber AOB in environments with low to intermediate ammonia concentrations (Ruiz *et.al*., 2019) and could therefore be a great fit for aquaculture.

Denitrification (Reaction 5) is the last step in the nitrogen waste removal process and although the process is predominantly performed by anaerobic heterotrophs, some aerobic autotrophs have been observed (van Rijn *et.al*., 2006). Dissimilatory nitrate removal is the most common heterotrophic denitrification, converting NO_3^- to N_2 using organic carbon compounds for biosynthesis and electron donors (Rijn et.al., 2006). Both carbon source and even small amounts of dissolved oxygen inhibit the complete reduction of NO_3^- resulting in accumulation of intermediate products such as NO_2^- (Betlach & Tiedje, 1981, Gómez *et.al*., 2002). Once the conversion of nitrogen waste to N_2 is completed, it can easily be aerated out of the water in the same way as CO_2 (Paredes *et.al*., 2007; Preena *et.al*., 2021).

$$4NO_3^- + 5C + 2H_2O \rightarrow 2N_2 + 4HCO_3^- + CO_2 \qquad \text{Reaction 5}$$

In 1995 Mulder (1995) and colleagues found bacteria in the phylum Planctomycetes that are capable of carrying out anaerobic ammonia oxidation (anammox) using NO_2^- as an electron acceptor with the production of N_2 (Reaction 6)

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad \text{Reaction 6}$$

All these organisms can be living in the biofilters at the same time but working at different times and in different niches. As the microbial community starts attaching to and maturing on the biocarriers both nutrients and oxygen become gradated by the biofilm created by the microorganisms. This gradient separates the microbes into niches allowing several microbial guilds to coexist (Navada *et.al.*, 2020). The outermost layer contains the heterotrophs as they feed on organic matter and oxygen, inside that layer come the autotrophs feeding on oxygen and inorganic

matter. The autotrophs are separated into niches as well with the AOB on the outer most part with the highest oxygen concentration and NOB towards the oxic-anoxic interface (Preena *et.al*., 2021). Having the heterotrophs on the outermost layers the autotrophs are more protected from mechanical shearing, as the biocarriers are whirled around by the aeration in the biofilter chambers, and the autotrophs can provide dead cell and soluble microbial products for the heterotrophs (Navada *et.al*., 2020; Preena *et.al*., 2021). Denitrification takes place in the innermost layers of the biofilm where conditions become anoxic. As well as being separated into niches on the biocarriers, the microorganisms can be separated into areas of the biofilter itself depending on nutrient and oxygen availability (Sich and Rijn, 1992; Leonard et.al., 2000). Some RAS have added an extra chamber designated for denitrification where oxygen levels are kept low and organic carbon is added from an external source, usually methanol, to increase denitrification (Rijn *et.al*., 2006; Paredes *et.al*., 2007), however, this should be done carefully as it is quite costly and there is a risk of NO_2^- accumulation if insufficient organic carbon is added (Hamlin *et.al*., 2008) also it may not be necessary with a mixed microbial community of nitrifiers, comammox, anammox and denitrifiers.

Aside from being produced and excreted from the fish ammonia is also released from dead and decaying matter and uneaten feed (Timmons *et.al.*, 2018; Preena *et.al*., 2021), it is therefore important that this is removed on a daily basis and overfeeding is kept to a minimum to keep the systemic nitrogen down.

Alkalinity

Total alkalinity (TA) plays an important role in maintaining stable pH and good water quality at farming facilities as it is required for continued growth and functional needs of the microorganisms as well as offsetting acidification by CO_2 addition and the nitrification process. TA can be defined as the total amount of base in the water that can accept a proton. In natural waters, carbonate alkalinity is the dominant form of alkalinity and the expression for total alkalinity in natural waters can thus be reduced to the following expression (Boyd *et.al.*, 2016).

$$TA = [HCO_3^-] + 2[CO_3^{2-}] + [OH^-] - [H^+] \qquad \text{Reaction 7}$$

As shown in Reaction 7, alkalinity can be gained by adding HCO_3^-, CO_3^{2-} or hydroxide (OH^-). The distribution of the carbonate species is pH dependent as those species are part of the carbonate system (see Reaction 8). Alkalinity is measured by titration with a strong acid until the carbonic acid endpoint is reached (pH = 4.5). At this point $[H^+]$ equals $[HCO_3^-]$ and most of the carbonate species are present in the form of CO_2 as the equilibrium of Reaction 8 shifts to the left.

CO_2 (g) ↔ CO_2 (aq) + H_2O ↔ H_2CO_3 ↔ H^+ + HCO_3^- ↔ H^+ + CO_3^{2-} Reaction 8

TA is usually measured as mg/L as $CaCO_3$ using the equivalent weight of each species and $CaCO_3$.

The use of $CaCO_3$ as a measure of alkalinity is an old measurement and was used because a major source of the alkalinity in natural waters stems from limestone where the principal component is $CaCO_3$ (Serge *et.al*., 2018). If the water supply at a farming facility has a naturally high alkalinity it might not be necessary to supply the rearing water with additional alkalinity supplements, however if it is naturally low it is important to make sure that it contains sufficient alkalinity to support microbial growth, nitrification and pH stability. According to Biesterfeld *et.al*. (2003) the origin of the alkalinity is also important, perhaps more so than pH, they found that carbonate alkalinity was superior to phosphate-, phosphate/hydroxide- and phosphate/carbonate alkalinity. Maintaining sufficient alkalinity levels can be done by adding sodium hydroxide (NaOH), hydrated lime ($Ca(OH)_2$), $NaHCO_3$ (sodium bicarbonate) or HCO_3 (Timmons *et.al*., 2018). Another reason for careful consideration of the type of base used in the system is that it can change the concentration of total inorganic carbon in the water. When adding a base to the water it increases the pH, shifting the equilibrium of Reaction 8 towards the right. If the base added is $NaHCO_3$ instead of NaOH there is an increase in total inorganic carbon that has the potential, if the pH should suddenly drop shifting the equation back towards the left, to release large amounts of CO_2. High levels of CO_2 in the water reduces the respiration efficiency of fish even at high levels of O_2 (Timmons *et.al*., 2018).

Motive, aim and significance of the Thesis.

The effects of alkalinity on the microbial community itself have not been greatly studied, and with alkalinity being such an important water variable it is something that I wanted to investigate with my thesis. The main aims of my thesis were to investigate whether alkalinity affects the nitrification rate of the biofilters and to make a protocol for measuring oxygen consumption during nitrification. Based on experiments conducted by Summerfelt *et.al.* (2015) I set up an experiment to answer these more specific research questions:

Question 1: What fraction of oxygen used in the biofilter can be ascribed to nitrification?

Question 2: Is oxygen consumption rate a good proxy for assessing nitrification rate?

Question 3: Does alkalinity affect TAN oxidation rates and oxygen consumption rates?

Question 4: Is there a difference in TAN oxidation rates and oxygen consumption rates between three compartments in the two modules and is this rate stable over time?

Providing information on the effects of alkalinity on biofilter community structure and function, contributes to the work of optimizing biofilters and providing safe clean water for salmon and other farmed species to live in. Ultimately improving the welfare and health of the culture species, giving them a better chance at survival, increasing farm productivity and ultimately resulting in better food safety and security for everyone.

Material and Methods

A 4-month long experiment at Marineholmen RASLab in Bergen, conducted by my co-supervisor Marie Aline Montjouridès, was the basis for my experiments. For the RASLab experiment two pilot scale ($2.5m^3$) RAS modules were used (module 1 and module 12) with moving bed biofilm reactors (MBBR) Figure 2. The major aim of the experiment was to see if alkalinity had any effect on the microbial composition of the biofilter, and if it had any effect on the nitrification rate. Alkalinity levels were changed between 70, 100, and 200 mg/L as $CaCO_3$ every second week in both modules (3 replicated per module at each alkalinity, 9 treatments in all). Each treatment was replicated three times in each module alternating between the treatments. Both modules operated at the same temperature ($15^oC \pm 0.23$), salinity ($15.41ppm \pm 1.7$) and stocking density ($50kg\ m^{-3}$, Atlantic salmon). Both tanks had continuous (24h) light to eliminate light as a source of influence, and a 12h feeding regime (8am – 8pm). Two peristaltic pumps adding $NaHCO_3$ and NaOH were used to set the desired alkalinity and pH levels respectively. To adjust the flow rate, alkalinity was monitored daily by titration (Thermo Scientific Orion Star T940 All-in-one Titrator, USA), and pH measurements were taken continuously with a pH meter (pH meter, Reef Factory, Poland) located after the degasser (Figure 2).

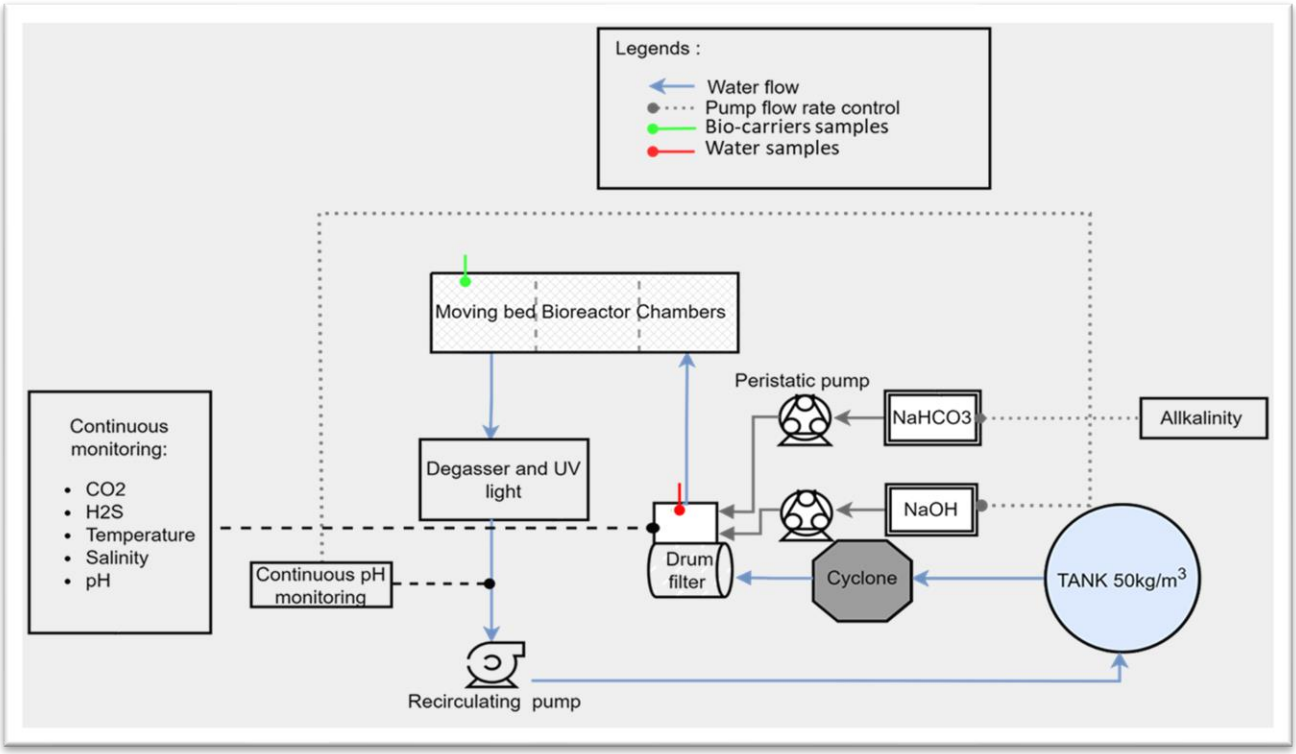


Figure 2: Schematic drawing of the RAS modules at RASLab with fish tank and pumps for maintaining pH and alkalinity. Drawing by: Marie Aline Monjouridès.

Experimental setup of laboratory scale experiments

My experiments ran alongside the RASLab experiment where I collect water and biocarriers samples from the two modules and ran my experiment in a laboratory scale reactor that Marie and I built at the Fish Immunology Laboratory. The laboratory scale bioreactor was a sealed 500ml Pyrex bottle with a contactless optical oxygen sensor on the inside of the bottle and a fiber-optic cable attached to the outside of the bottle, opposite the optical sensor (Figure 4). The fiber-optic cable along with a temperature probe were connected to a FireSting-O2 Fiber-Optic Oxygen Meter (Pyroscience) that was connected to my computer for measurement control and real-time visualization of respirometry measurements (Figure 3). The fiber-optic meter allowed us to keep the bottle sealed during the experiments, while measuring oxygen concentrations every 10 seconds. Because of the sealed bottle the temperature probe was placed in a polypropylene Griffin beaker, next to the bottle. A magnetic stirrer was used to maintain a homogenous system, at a flow where the biocarriers did not move around as to interfere with or damage the optical sensor. The bottle,

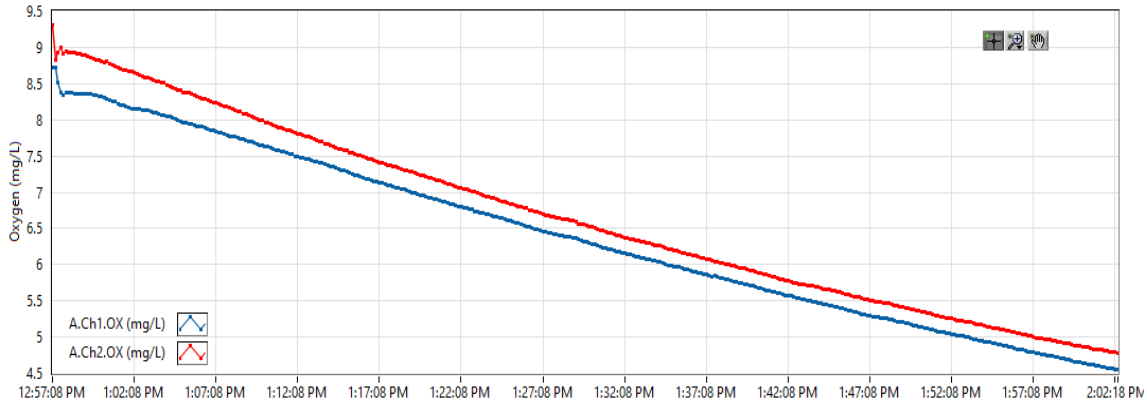


Figure 3: Real-time oxygen consumption measurements during nitrification, in a self-constructed bioreactor. The graph shows an example of oxygen consumption (mg/L) in two bioreactors running simultaneously. The two lines in the graph represent the two bioreactors.

magnetic stirrer and beaker with temperature probe were placed in an incubator (Figure 4) to keep the water temperature the same as in the RASLab systems (15°C ± 1). Setup and calibrations of the equipment were performed as instructed by the Pyro Workbench & Data Inspector PyroScience Logger Software QuickStart Manual. TAN and NO_2^- concentrations were measured before and after each experiment colorimetrically, using a Spectroquant® Pharo 300 spectrophotometer (MERCK) with a 10mm rectangular quartz cuvette (spectroquant®) and Ammonium and Nitrite Test kits (Supelco Spectroquant®), procedures were according to instructions in the kits. Alkalinity and pH were also measured before the start of each experiment, using the before mentioned titrator according to instructions by the manufacturer.

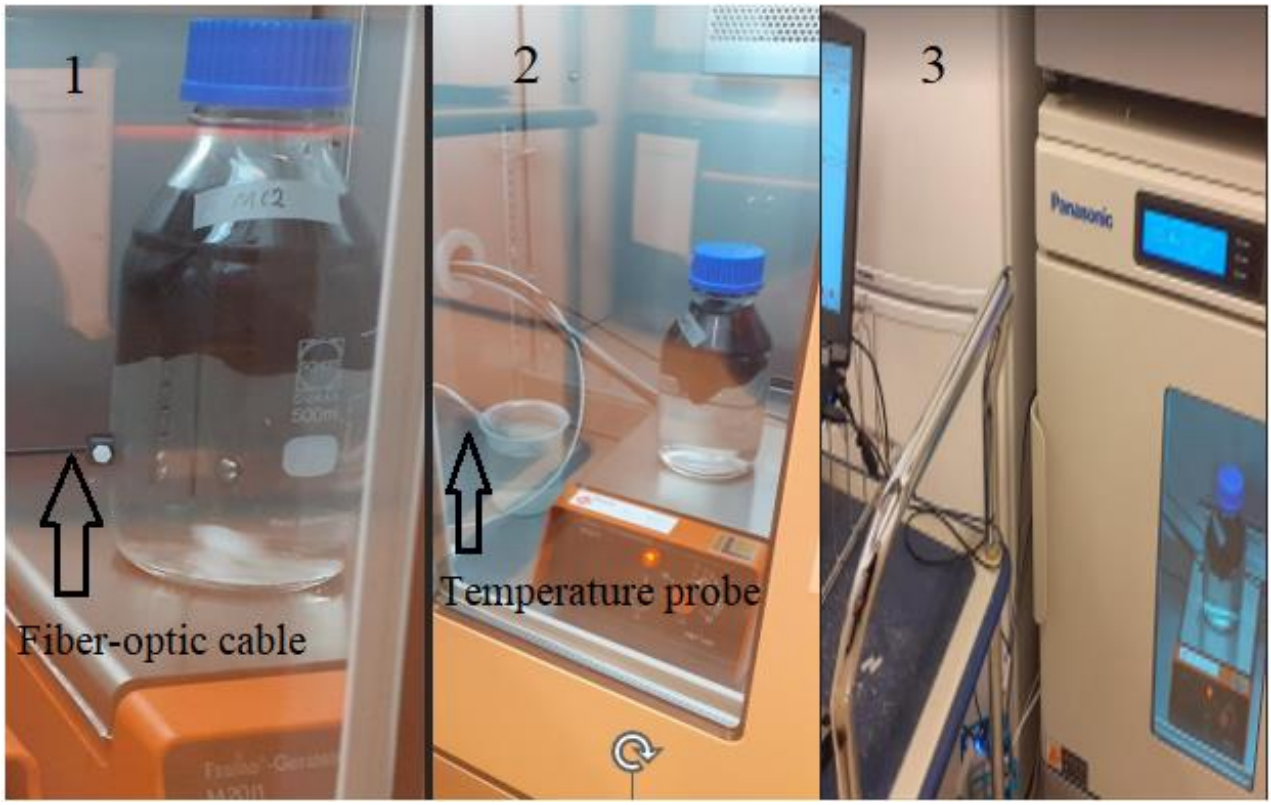


Figure 4: Pictures showing the laboratory scale bioreactor setup. Picture 1: close up on bioreactor with biocarriers and water, attached to fiber-optic cable. Picture 2: bioreactor with biocarriers and water, on magnet stirrer inside incubator, with temperature probe next to magnetic stirrer in Griffin beaker, with water from RASLab. Picture 3: laptop connected to bioreactor inside incubator.

Experimental design

I ran four different experiments throughout the testing period. All experiments had the same setup, the same measurements were taken every time, at approximately the same time, and performed in the same manner, but their objectives differed. The objective for experiment 1 was to make a protocol for measuring oxygen consumption, that could be used as a proxy for TAN oxidation. Experiment 2 was a time series test to see how ammonia oxidation and oxygen consumption rates were affected by increasing TAN concentration during the day. Experiment 3 aimed to measure background oxygen consumption, and the objective of experiment 4 was to examine TAN oxidation and oxygen consumption rates within both modules. Biocarriers for all experiments were collected randomly from compartment three (Figure 2) and water was collected after the drum filter but before the biofilter (Figure 2). Water and biocarriers were kept separate until the experiments were ready to start. First biocarriers were added to the bottles and when the water was measured for all the bottles everything was poured in at the same time and oxygen measurements were started. TAN

and NO_2^- were measured in the water before it was mixed with the biocarriers and again after one hour in the bioreactor. The biocarriers were maintained in the same water from which they were obtained before the experiment, to ensure that the observed activity during the experiment reflected the true capability of the RASLab system.

Experiment 1 – Measuring oxidation and oxygen consumption rates in the two modules.

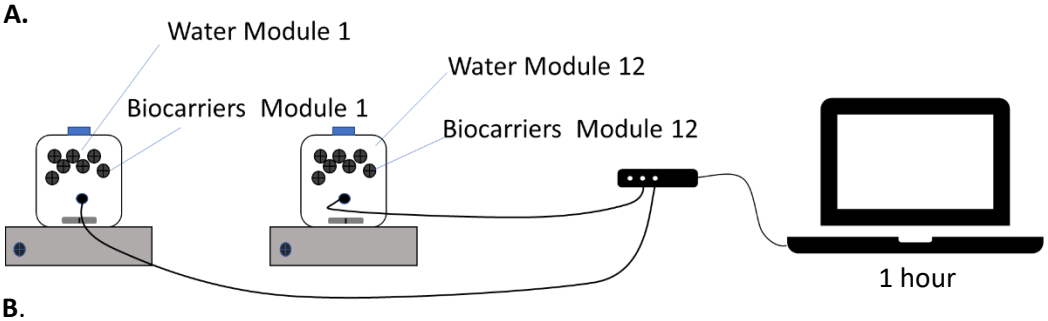
Water and biocarriers were collected from both modules at RASLab between 12pm and 1pm and brought back to the Fish Immunology Lab. This experiment was performed using two laboratory scale bioreactors consisting of two 500 ml bottles connected to the FireSting-O2 Fiber-Optic Oxygen Meter. One bottle contained biocarriers from module 1 (M1) and one contained biocarriers from module 12 (M12). Each bottle contained 560ml water from the systems and 20 biocarriers. This experiment was carried out during treatments 4-6 (Table 1).

Table 1: Overview over dates and treatments for RASLab experiment, with alkalinities kept during each treatment in both systems.

Date	Treatment	M1 alkalinity	M12 alkalinity
29.08-12.09. 2022	1	100	200
12.09-26.09. 2022	2	200	100
26.09-10.10. 2022	3	100	70
10.10-24.10. 2022	4	70	200
24.10-07.11. 2022	5	200	100
07.11-21.11. 2022	6	100	70
21.11-05.12. 2022	7	70	100
05.12-19.12. 2022	8	200	70
02.01-16.01. 2023	9	70	200

Each RASLab treatment started on a Monday (day 1) and ended two weeks later on a Monday (day 15). 4 laboratory scale experiments were performed per treatment, with each experiment consisting of 4 tests (Figure 5: A, B, C, D) with two bioreactors each (Figures 5). The experiments were run for 1 hour each consecutively on day 5, 8, 12 and 15 (Figure 5. E). Tests A and C were performed with one bottle containing water and biocarriers from M1 and one bottle containing water and biocarriers from M12 (Figure 5: A, C). Tests B and D were performed with biocarriers remaining in their respective bottles but with new water added from the other system (Figure 5: B, D).

A.



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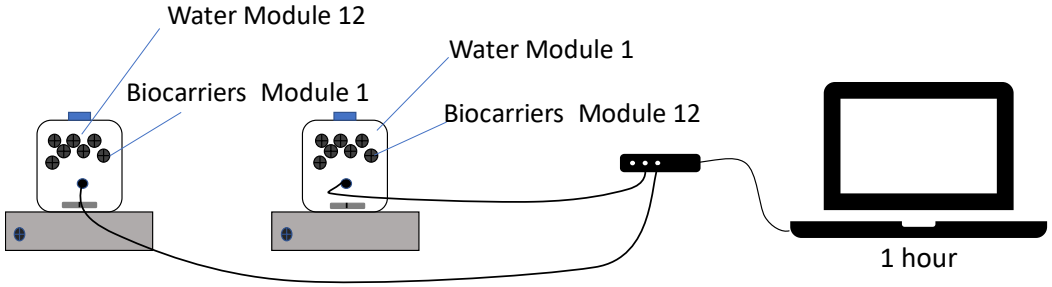

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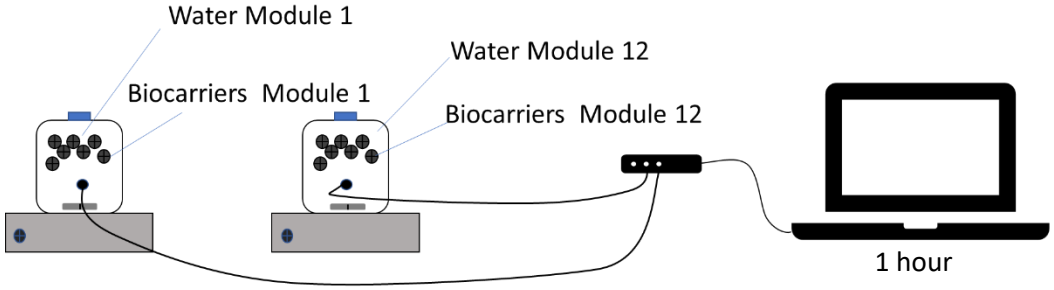
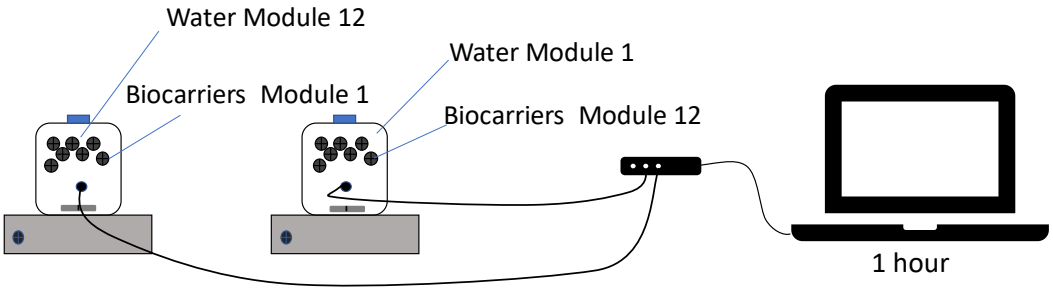


D.



E.

When ?

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15

Figure 5: Schematic of the laboratory scale bioreactor at the Fish Immunology Laboratory connected to laptop via the oxygen meter. A) Biocarriers from the RASLab experiment (Module 1 and Module 12) where incubated for 1h in water from their respective systems (Module 1 and Module 12). B) After incubation in A) water was removed and replace by water from the other system. C) After 1h incubation in B) the water was removed and biocarriers were incubated in the water from their respective systems again. D) The incubation medium of the biocarriers was changed again to the water from the other system. E) Sampling of the biocarriers and experimentation on day 5, 8, 12, and 15. Drawing by: Marie Aline Montjouridès.

Experiment 2 – Time series (TAN oxidation and oxygen consumption rates in relation to feeding).

This experiment was only conducted on M1 from treatment 4 – 9. Water and biocarriers were collected at 7am, 12pm, 4pm, and 8pm on day 11 of each treatment. The samples were brought back to the Fish Immunology Laboratory and run for 1 hour. This experiment was run using two replicate laboratory scale bioreactors containing water and biocarriers from M1.

Experiment 3 – Background oxygen measurements.

This experiment was conducted parallel to experiment 2 during treatment 7 – 9. When these tests were run three replicate laboratory scale bioreactors containing water and biocarriers from M1 were used. The two bottles from experiment 2 and one extra where a nitrification inhibitor molecule (2-chloro-6 (trichloromethyl) pyridine) was added (Figure 6).

This experiment was also conducted on M12 (only at 12pm), at four different times, treatment 5 (1), treatment 8 (2), and treatment 9 (1).

The technical name for the inhibitory molecule is N-SERVE, product name by HACH is Nitrification Inhibitor Formula 2533. N-SERVE acts on the cupper component of the cytochrome component involved in ammonia oxidation (Campbell & Aleem, 1965). It is important that the molecule is completely dissolved in the water before adding it to the biocarriers, otherwise the full effect of the molecule will be implemented right away.

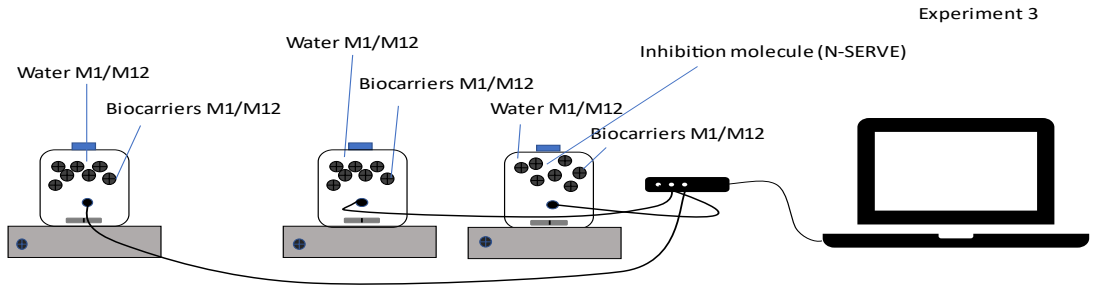


Figure 6: Schematic of the laboratory scale bioreactor at the Fish Immunology Laboratory connected to laptop via the oxygen meter. Water and biocarriers in all three bottles are from the same module. In one bottle the ammonia oxidation inhibition molecule N-SERVE is added. Drawing by: Marie Aline Montjouridès.

Experiment 4 – Internal rate measurements.

As see in Figure2 the biofilters at RASLab are separated into three compartments. The water flows from the drum filter into the first one, to the second one and to the third one. In this experiment the objective was to see if ammonia oxidation rates were the same in all three compartments. For this experiment three laboratory scale bioreactors were used (Figure 7) and biocarriers were collected from all three compartments, water was collected for all three from after the drum filter. Six samples of biocarriers + water were collected from both modules, between 12pm and 1pm on the same day, during treatments 7 – 9. One module was run at a time, while the other module was stored in an incubator, to keep the temperature the same as in RASLab. Biocarriers and water were kept separate and the biocarriers were incubated in water from where they were collected. It was alternated between the two modules which one was run first.

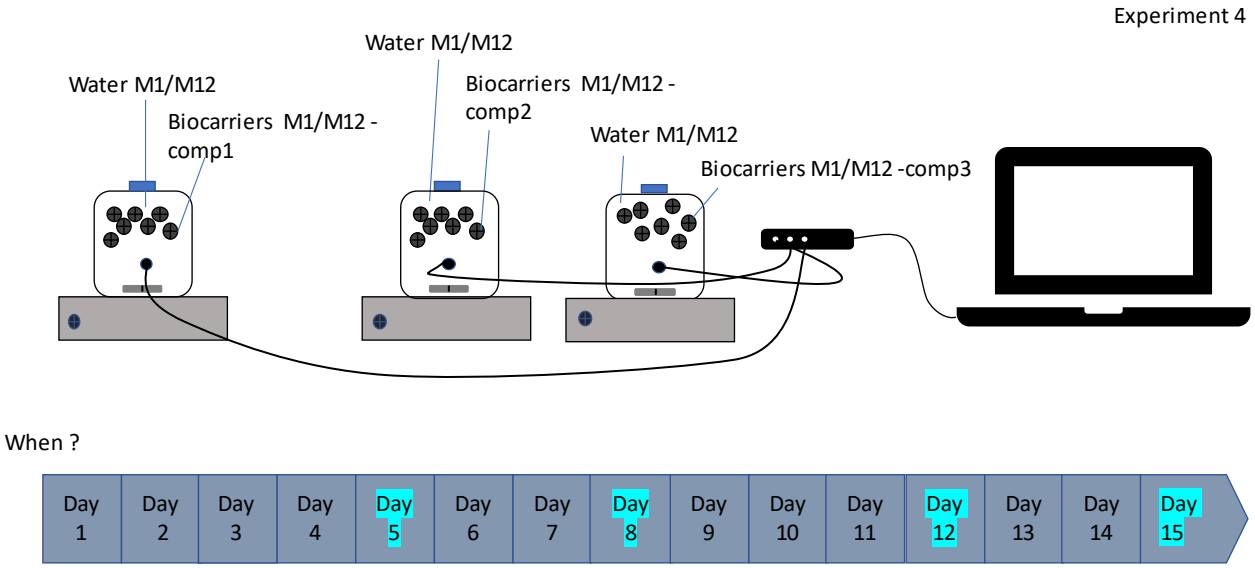


Figure 7: Schematic of the laboratory scale bioreactor at the Fish Immunology Laboratory connected to laptop via the oxygen meter. Water and biocarriers in all three bottles are from the same module, but different compartments (compartments 1,2, and 3). Drawing by: Marie Aline Montjouridès.

Statistical analysis

All statistical work was performed using Microsoft Excel Spreadsheet software (Microsoft 365) and the Analysis ToolPak add-in.

Multiple linear regression analysis (MLR) was performed using the Regression function of the Analysis ToolPak. All data were normalized before performing the MLR.

As my data was not normally distributed the Mann-Whitney U-test was used to determine if there were significant differences between groups with, a 95% confidence interval.

Results

In total 200 incubations (including replicates) were performed over a period of 4 months (Annex 1). Selected subsets of the data were used to examine to what extent oxygen consumption rates can be linked to ammonium oxidation, and how oxygen consumption rates and ammonium consumption rates vary between different environmental conditions i.e., alkalinity and initial ammonium concentration.

In order to reveal factors that influence TAN oxidation rates, total oxygen consumption rates, and the balance (the stoichiometry) between TAN and oxygen consumption rates as response variables, MLR analyses were performed. The results from MLR analysis show that TAN concentration influences all the above (TAN oxidation rates, oxygen consumption rates and the stoichiometry) while alkalinity and pH do not seem to have any influence (Figures 8 and 9). Mean oxidation rate, efficiency, oxygen consumption rate and stoichiometry were quite similar at all three alkalinity settings (Table 2). Additionally TAN oxidation rates are also influenced by time from day 0 (biofilter maturation). It was necessary to perform separate analyses for pH and alkalinity as they are strongly correlated. Data used for these analyses did not include inhibition experiments with N-SERVE nor compartments comparison experiments.

Table 2: Median pH, alkalinity, TAN concentrations, TAN oxidation rates, TAN oxidation efficiency (fraction of TAN removed during the incubation), moles TAN oxidized, moles oxygen consumed, oxygen consumption rate, and stoichiometry (balance between TAN and oxygen consumption rates) at the different alkalinity settings. Not including data from inhibition and compartment testing experiments.

	pH	Alk. (as mg/L $CaCO_3$)	T0 TAN (mg/L)	Oxidation rate (mg/L/day)	Efficiency	mol/L TAN	Mol/L O_2	Oxygen consumption rate (mg/L/day)	Stoichio-metry
70	7.063±0.05	76.86±9.6	0.660±0.27	0.44±0.16	0.646±0.11	0.0255±0.009	0.1422±0.03	4.62±1.02	5.306±5.4
100	7.242±0.10	104.9±14.14	0.663±0.21	0.46±0.16	0.731±0.09	0.0267±0.064	0.1468±0.03	4.63±0.88	5.163±1.4
200	7.465±0.10	185.2±21.37	0.570±0.30	0.50±0.18	0.720±0.14	0.0276±0.035	0.1463±0.03	4.50±1.19	5.181±5.4

Regression Statistics	
Multiple R	0.8489
R-Square	0.7207
Adjusted R Square	0.7150
Standard Error	0.5356
Observations	152

ANOVA

	df	SS	MS	F	Significance F
Regression	3	109.540	36.513	127.272	8.57614E-41
Residual	148	42.460	0.287		
Total	151	152			

	Coefficients	Standard Error	t-Stat	P-value	Lower 95%	Upper 95%
Intercept	0.9719	0.1506	6.451	1.484E-09	0.6742	1.2696
Time from day 0	-0.0138	0.0020	-7.016	7.596E-11	-0.0177	-0.0099
pH_norm	0.0281	0.0448	0.626	0.5325486	-0.0606	0.1167
T0 TAN	0.8110	0.0449	18.075	2.759E-39	0.7224	0.8997

Regression Statistics	
Multiple R	0.8495
R-Square	0.7216
Adjusted R Square	0.7159
Standard Error	0.5347
Observations	152

ANOVA

	df	SS	MS	F	Significance F
Regression	3	109.679	36.560	127.852	6.73253E-41
Residual	148	42.321	0.286		
Total	151	152			

	Coefficients	Standard Error	t-Stat	P-value	Lower 95%	Upper 95%
Intercept	0.9664	0.1506	6.4172	1.77E-09	0.6688	1.2639
Time from day 0	-0.0139	0.0020	-7.0703	5.686E-11	-0.0178	-0.0100
Alk	0.0407	0.0434	0.9374	0.3500707	-0.0451	0.1265
T0 TAN	0.8041	0.0434	18.5239	2.204E-40	0.7183	0.8899

Regression Statistics	
Multiple R	0.6181
R-Square	0.3820
Adjusted R Square	0.3652
Standard Error	0.7994
Observations	152

ANOVA

	df	SS	MS	F	Significance F
Regression	4	58.064	14.516	22.716	1.26237E-14
Residual	147	93.936	0.639		
Total	151	152			

	Coefficients	Standard Error	t-Stat	P-value	Lower 95%	Upper 95%
Intercept	0.0071	0.2545	0.0278	0.9778458	-0.4959	0.5100
Time from day 0	-0.0019	0.0034	-0.5643	0.5734232	-0.0086	0.0048
pH	0.0035	0.0670	0.0522	0.9584548	-0.1289	0.1359
T0 TAN	0.6710	0.1199	5.5946	1.049E-07	0.4340	0.9080
oxidation rate	-0.0653	0.1227	-0.5324	0.5952408	-0.3078	0.1771

Regression Statistics	
Multiple R	0.6186
R-Square	0.3827
Adjusted R Square	0.3659
Standard Error	0.7990
Observations	152

ANOVA

	df	SS	MS	F	Significance F
Regression	4	58.163	14.541	22.779	1.17021E-14
Residual	147	93.837	0.638		
Total	151	152			

	Coefficients	Standard Error	t-Stat	P-value	Lower 95%	Upper 95%
Intercept	0.0057	0.2544	0.0223	0.982256	-0.4970	0.5084
Time from day 0	-0.0020	0.0034	-0.5942	0.5532941	-0.0087	0.0047
alk	0.0258	0.0651	0.3971	0.6918483	-0.1028	0.1545
T0 TAN	0.6729	0.1181	5.6951	6.493E-08	0.4394	0.9064
oxidation rate	-0.0687	0.1228	-0.5597	0.5765639	-0.3114	0.1740

Figure 8: MLR analyses results. On the left side MLR analyses on the effects of time from day 0, pH and alkalinity and initial TAN concentrations (T0 TAN) on ammonia oxidation rate. On the right side MLR analysis on the effects of time from day 0, pH and alkalinity, initial TAN concentrations (T0 TAN) and oxidation rate on oxygen consumption rate. Separate analyses were made for the effects of pH and alkalinity due to multicollinearity.

Regression Statistics	
Multiple R	0.4288
R-Square	0.1839
Adjusted R Square	0.1673
Standard Error	0.9155
Observations	152

ANOVA

	df	SS	MS	F	Significance F
Regression	3	27.9487	9.3162	11.1148	1.27034E-06
Residual	148	124.0513	0.8382		
Total	151	152			

	Coefficients	Standard Error	t-Stat	P-value	Lower 95%	Upper 95%
Intercept	0.0519	0.2575	0.2014	0.8406586	-0.4570	0.5607
Time from day 0	0.0032	0.0034	0.9644	0.3364203	-0.0034	0.0099
pH	0.0235	0.0766	0.3072	0.7591234	-0.1279	0.1750
T0 TAN	-0.4193	0.0767	-5.4665	1.904E-07	-0.5708	-0.2677

Regression Statistics	
Multiple R	0.4332
R-Square	0.1876
Adjusted R Square	0.1712
Standard Error	0.9134
Observations	152

ANOVA

	df	SS	MS	F	Significance F
Regression	3	28.5223	9.5074	11.3956	9.10094E-07
Residual	148	123.4777	0.8343		
Total	151	152			

	Coefficients	Standard Error	t-Stat	P-value	Lower 95%	Upper 95%
Intercept	0.0411	0.2572	0.1597	0.873311	-0.4672	0.5494
Time from day 0	0.0031	0.0034	0.9192	0.3594728	-0.0035	0.0097
alk	0.0656	0.0742	0.8845	0.3778569	-0.0810	0.2122
T0 TAN	-0.4251	0.0741	-5.7326	5.367E-08	-0.5716	-0.2785

Figure 9: MLR analyses results. Effects of time from day 0, pH and alkalinity and initial TAN concentrations (T0 TAN) on the balance between TAN and oxygen consumption rates. Separate analyses were made for the effects of pH and alkalinity due to multicollinearity.

Figures 10, 11, and 12 show the relationship between TAN concentration and: TAN oxidation rates, oxygen consumption rates, and the balance between the two (the stoichiometry). From these graphs the linear relationship is not as strong in M12 as in M1.

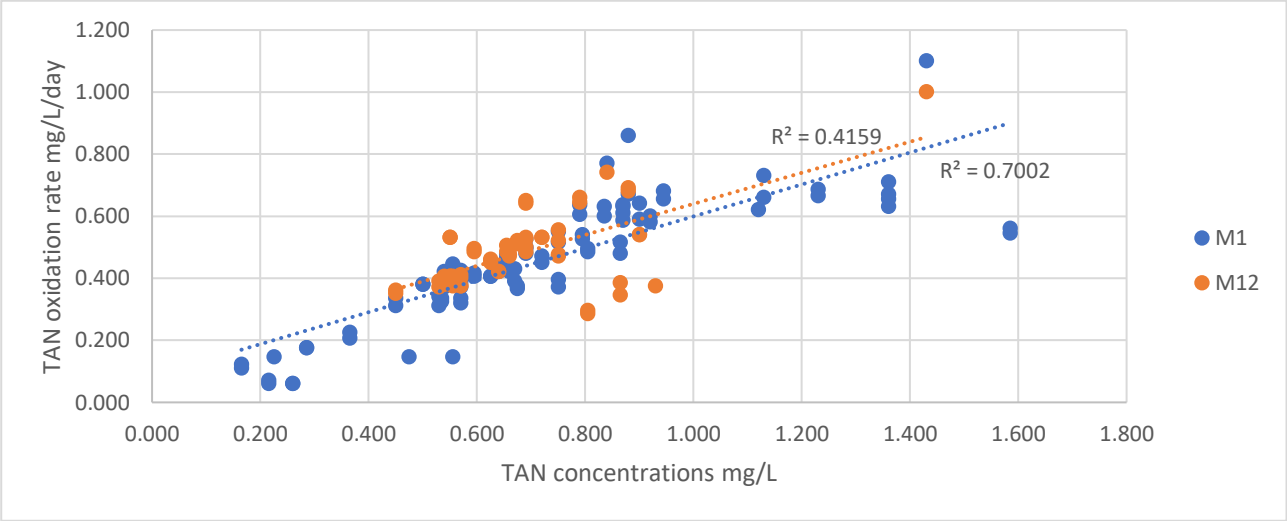


Figure 10: Relationship between TAN concentration and TAN oxidation rate. The graph shows data collected from both modules (blue: M1; orange: M12) throughout the experiments. Data not included are the inhibition experiments with N-SERVE and compartment experiments.

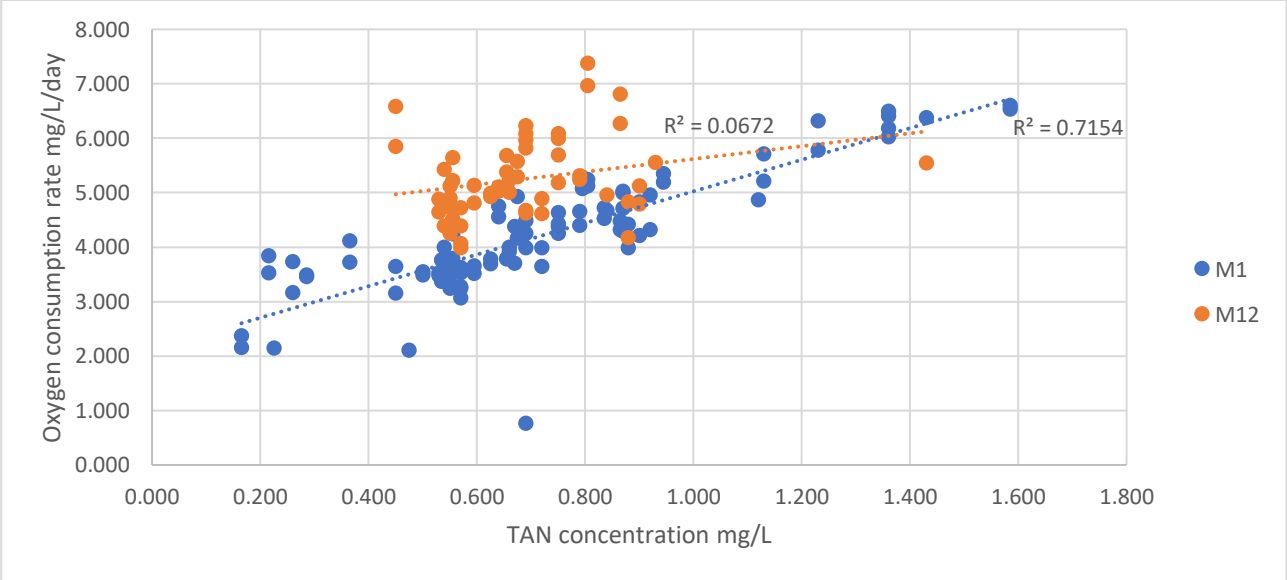


Figure 11: Relationship between TAN concentration and oxygen consumption rate. The graph shows data collected from both modules (blue: M1; orange: M12) throughout the experiments. Data not included are the inhibition experiments with N-SERVE and compartment experiments.

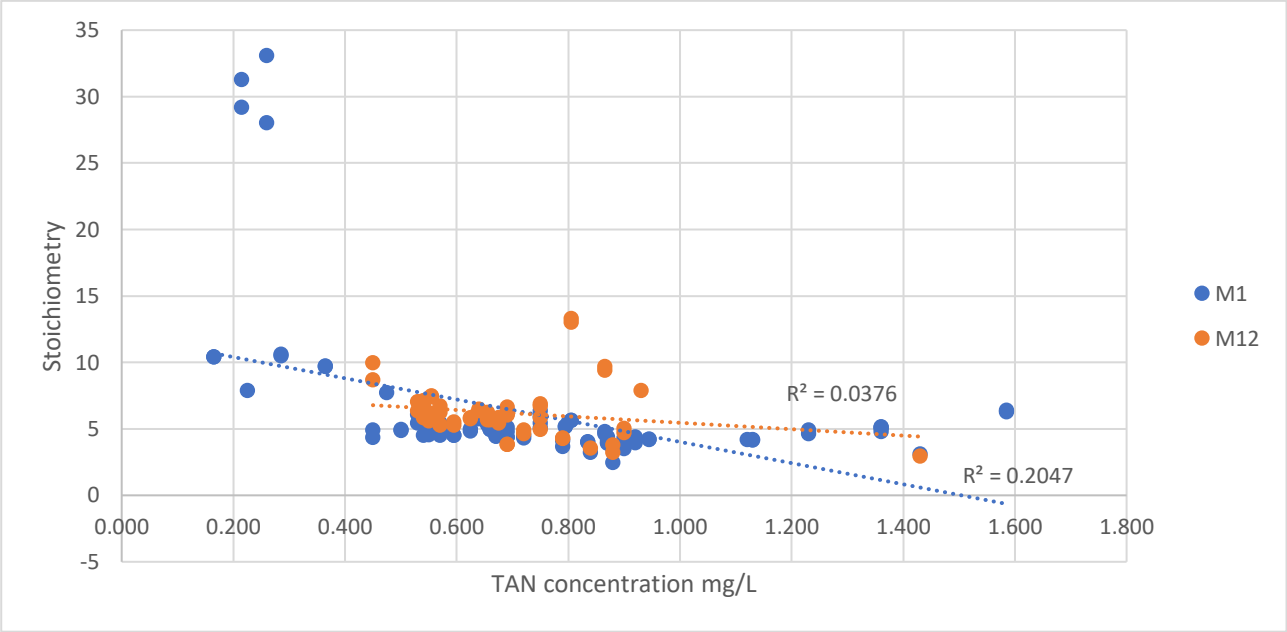


Figure 12: Relationship between TAN concentration and the balance (stoichiometry) between TAN and oxygen consumption rates. The graph shows data collected from both modules (blue: M1; orange: M12) throughout the experiments. Data not included are the inhibition experiments with N-SERVE and compartment experiments.

Looking at the efficiency of TAN oxidation, there is a negative linear relationship between time from day 0 (biofilter maturation) and the efficiency of TAN oxidation (Figure 13).

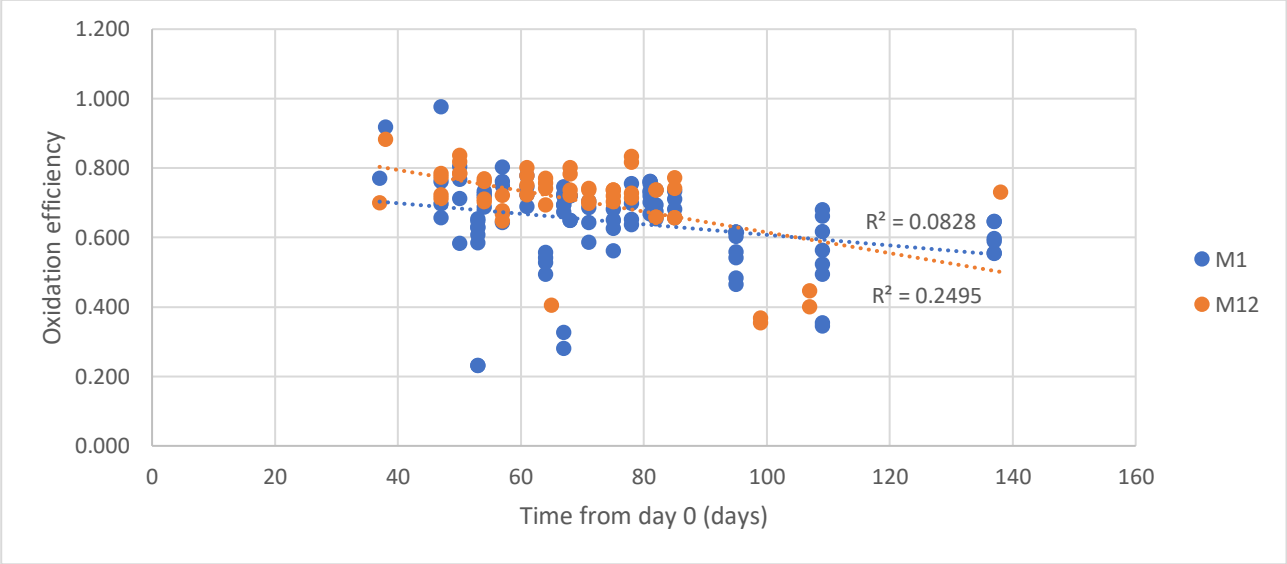


Figure 13: Relationship between time from day 0 (biofilter maturation) and TAN oxidation efficiency. The graph shows data collected from both modules (blue: M1; orange: M12) throughout the experiments. Data not included are the inhibition experiments with N-SERVE and compartment experiments.

As evident from graphs (10-13) there were some differences between the two modules. After performing the Mann-Whitney U-test it was clear that M12 had a significantly higher efficiency (z-value = 2.8) even though there were no significant difference in TAN oxidation rates (z-value = 0.2) or initial TAN concentrations (z-value = 0,05). Table 3 also shows that M12 had a higher stoichiometry ratio that M1, consuming significantly more oxygen than M1 (z-value = 4.4). Efficiency was calculated as TAN oxidation rate divided by initial TAN concentration. TAN oxidation rate = initial (T0) TAN concentration minus TAN concentration after incubation (T+1).

Table 3: Median pH, alkalinity, TAN concentrations, TAN oxidation rates, TAN oxidation efficiency (fraction of TAN removed during the incubation), moles TAN oxidized, moles oxygen consumed, oxygen consumption rate, and stoichiometry (balance between TAN and oxygen consumption rates) for the complete data set, M1 and M12.

	pH	alk. (as mg/L $CaCO_3$)	T0 TAN (mg/L)	Oxidation rate (mg/L/day)	Efficiency	mol/L TAN	mol/L O2	Oxygen consumption rate (mg/L/day)	Stoichiometry
All data	7.242±0.18	104.9±51	0.665±0.26	0.450±0.16	0.698±0.12	0.026±0.04	0.146±0.03	4.625±1.06	5.273±4.4
M1	7.259±0.19	105.5±55	0.670±0.30	0.430±0.18	0.658±0.13	0.025±0.01	0.131±0.03	4.000±1.03	4.937±5.3
M12	7.227±0.17	104.7±44	0.66±0.16	0.473±0.12	0.733±0.11	0.028±0.07	0.160±0.02	5.120±0.7	5.590±1.8

Background oxygen measurement (experiment 3) were not fully successful (Figure 14). N-SERVE is usually used on bacteria living freely in the water and it was uncertain if it would work on nitrifiers attached to biocarriers, but with some testing it finally worked during treatment 5, with 10x the amount of molecule prescribed on the bottle, achieving a 99% nitrification stop (Annex 1 – efficiency = 0.011). This amount was then used going forward and seemed to work fine. However, for the last experiments during treatment 9, the inhibition efficiency was less prominent although there was still a clear oxidation inhibition. Performing a MLR analysis showed that only time influenced the success of the inhibition (Figure 15)

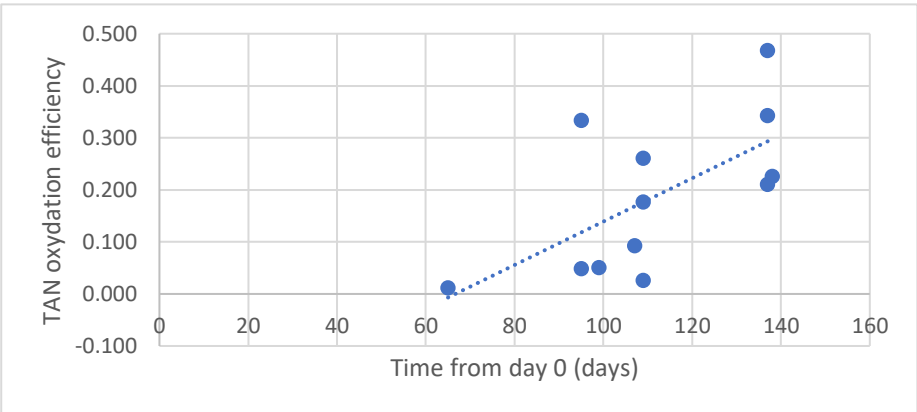


Figure 14: Inhibition efficiency by N-SERVE over time. Combined data from both modules.

Regression Statistics						
Multiple R	0.802667883					
R-Square	0.64427573					
Adjusted R Square	0.510879129					
Standard Error	0.231736834					
Observations	12					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regression	3	0.778105348	0.25937	4.82978	0.03330352	
Residual	8	0.429615681	0.0537			
Total	11	1.207721029				
	Coefficients	*Standard Error*	*t-Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-2.913571563	0.375329863	-7.7627	5.4E-05	-3.77908378	-2.048059
Time from day 0	0.011915815	0.003264332	3.65031	0.00649	0.00438825	0.0194434
pH	-0.09108937	0.088144966	-1.0334	0.33164	-0.29435203	0.1121733
T0_TAN	-0.011484553	0.053827786	-0.21336	0.83639	-0.13561165	0.1126425
Regression Statistics						
Multiple R	0.78474154					
R-Square	0.615819285					
Adjusted R Square	0.471751517					
Standard Error	0.240827513					
Observations	12					
ANOVA						
	df	*SS*	*MS*	*F*	*Significance F*	
Regression	3	0.7437379	0.24791	4.27451	0.04457315	
Residual	8	0.463983129	0.058			
Total	11	1.207721029				
	Coefficients	*Standard Error*	*t-Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	-2.836995542	0.384661843	-7.3753	7.8E-05	-3.72402734	-1.949964
Time from day 0	0.01141354	0.00334611	3.41099	0.00921	0.0036974	0.0191297
Alk	-0.038164136	0.060626995	-0.62949	0.54658	-0.17797024	0.101642
T0_TAN	0.001131484	0.055478625	0.02039	0.98423	-0.12680245	0.1290654

Figure 15: MLR analysis results. Effects of time from day 0, pH, alkalinity and initial TAN concentrations (T0 TAN) on inhibition efficiency by N-SERVE. Combined data from both modules.

Looking at the results from the internal rate measurements comparing the three compartments (experiment 4), the results differ in several ways. The rates were different between the compartments, between the two modules (Figures 16 and 17), and from the laboratory scale experiments.

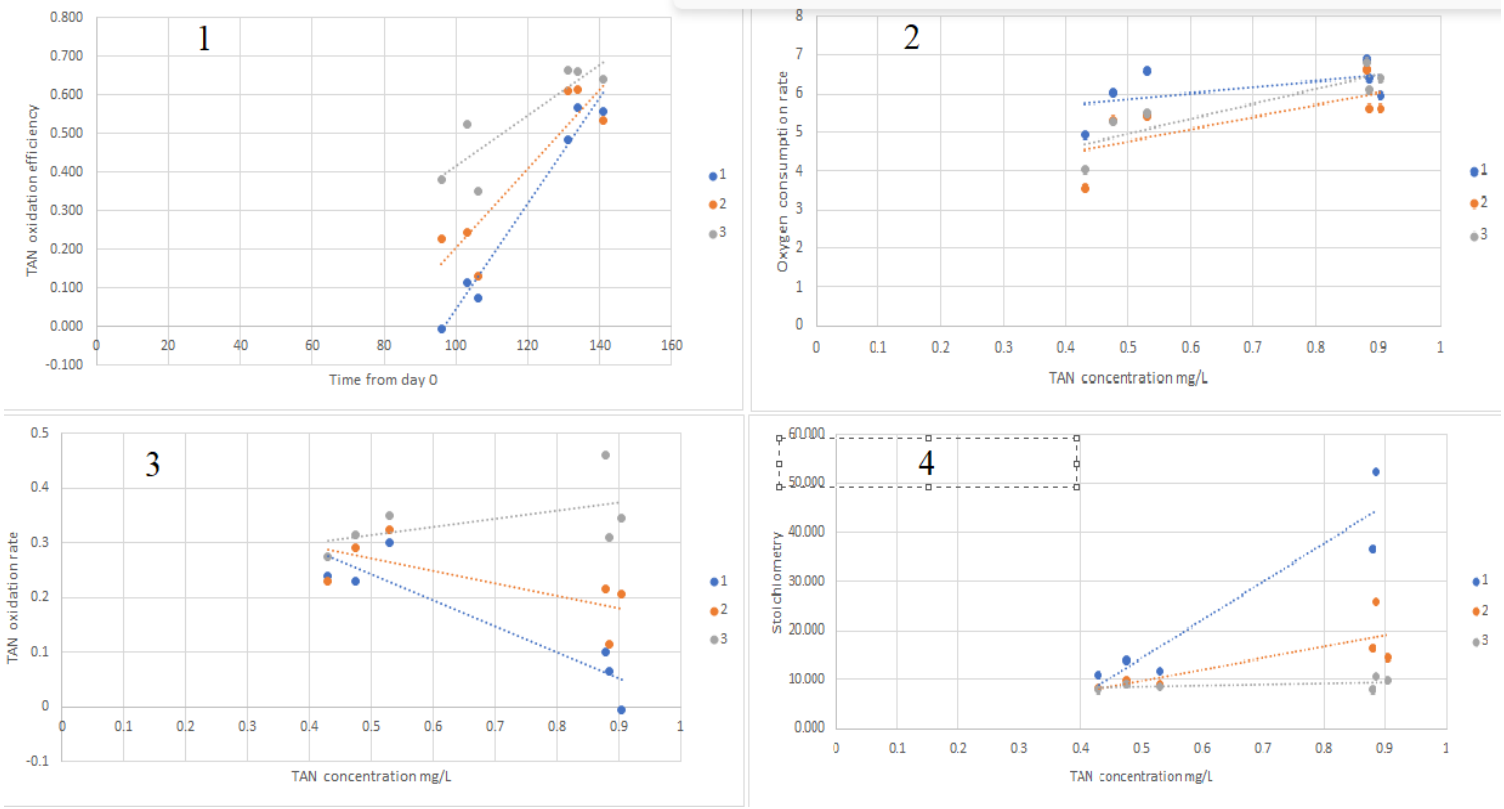


Figure 16: Graphical representations of the activity in the three compartments in module 1. Graph 1: TAN oxidation efficiencies over time (days). Graph 2: oxygen consumption rates (mg/L/day) in relation to TAN concentration (mg/L). Graph 3: TAN oxidation rates (MG/L/day) in relation to TAN concentrations (mg/L). Graph 4: stoichiometry (balance between TAN and oxygen consumption rates) in relation to TAN concentrations (mg/L). Compartment 1: blue line;

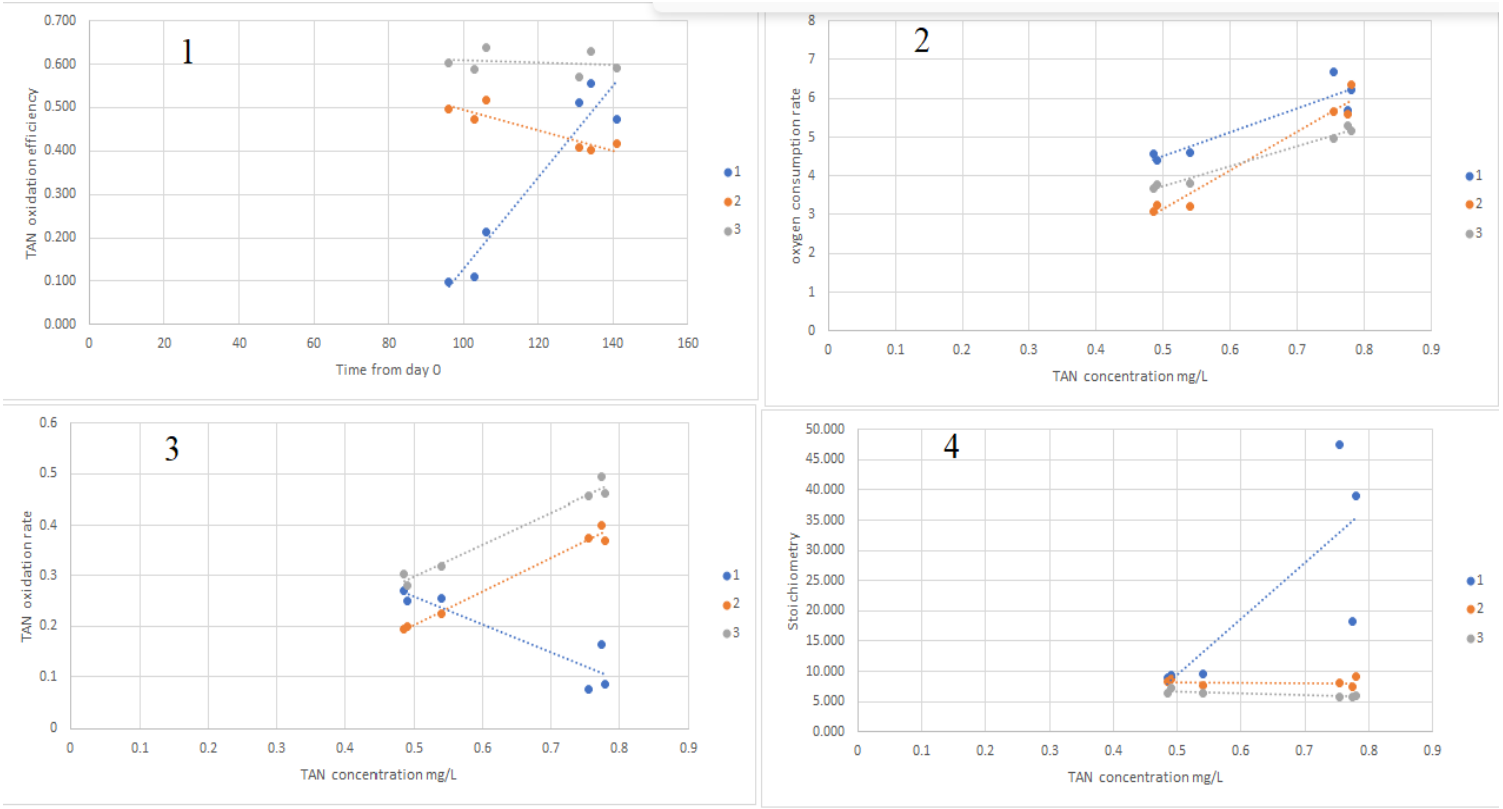


Figure 17: Graphical representations of the activity in the three compartments in module 12. Graph 1: TAN oxidation efficiencies over time (days). Graph 2: oxygen consumption rates (mg/L/day) in relation to TAN concentration (mg/L). Graph 3: TAN oxidation rates (mg/L/day) in relation to TAN concentrations (mg/L). Graph 4: stoichiometry (balance between TAN and oxygen consumption rates) in relation to TAN concentrations (mg/L). Compartment 1: blue line; compartment 2: orange line; compartment 3: grey line.

Discussion

The main aim of this thesis was to investigate the effects of alkalinity on biofilters, specifically the effects on nitrification. From my experiments I was unable to determine any differences in nitrification at the different alkalinities set forth by the project, 70, 100, and 200 mg/L as $CaCO_3$. This is in accordance with the findings in the study by Summerfelt et.al. (2015) where they tested the effects of alkalinities at 10, 70 and 200 mg/L as $CaCO_3$. Their conclusion concluded was that alkalinity did not have any effect on nitrification rates however they did find some adverse effects at alkalinity 10 like higher average TAN concentrations and less stable pH, and therefore recommended keeping the alkalinity at around 70 mg/L as $CaCO_3$. Even though I could not determine any effects of alkalinity on the nitrification rate of the biofilters, looking at Table 2 I would recommend increasing the alkalinity to around 100 mg/L as $CaCO_3$ as this seem to provide a more stable environment. Increased alkalinity also gives the farmers longer response time, if something were to happen to the alkalinity dosing mechanism. Increasing the alkalinity above 100

mg/L as $CaCO_3$ does not seem to provide any benefits and may only increase the cost of production. At low alkalinities pH is usually also low and according to a study by Shanahan and Semmens (2015) this could result in even lower pH values inside the biofilm that it inhibits nitrification.

With the results from the MLR analyses I was able to determine that both TAN oxidation rates and oxygen consumption rates were related to TAN concentration (Figure 8), with the graphical representations showing that increasing TAN concentrations result in increased TAN oxidation (Figure 10) and increased oxygen consumption (Figure 11). In a recent study by Qi et.al. (2022) it was suggested that ammonium oxidation rates in RAS biofilters can be calculated from oxygen consumption rates, given that the activity of heterotrophic oxygen consumers are low. In my experiments I observed that the ratio of consumed oxygen over consumed TAN over the course of the experiments were notoriously higher (Annex 1) than what could be expected from the stoichiometries of TAN oxidation to nitrite (Reaction 2) or completely to nitrate (Reaction 4). With stoichiometry ratios anywhere from 2.47 – 217.9 this suggests that a considerable fraction of O_2 consumption occurs by other means than TAN oxidation. Looking only at oxygen consumption as a proxy for TAN oxidation one could conclude that there was a higher AOB activity in the biofilter than there was and overestimate the nitrification rate of the biofilter. Over time as the microorganisms on the biocarriers continue to proliferate there will be more and more organisms consuming oxygen which may lead to low oxygen levels in the biofilter resulting in inhibition of nitrification. From Figure 13 we see that the TAN oxidation efficiency goes down as the biofilter matures, this may be because the outermost layer of the biofilm, the heterotrophs, is becoming so thick that the substrates are not able to get through fast enough or perhaps oxygen and/or pH are becoming to low within the biofilm that they become inhibiting. These are some of the reasons that it is so important to have trained operators working at RAS facilities that know the importance of maintaining the biofilters and know what indicators to look at.

From the time series data, we see that when TAN concentrations are low in the morning (Annex 1 – Sampling time – time after feeding began: -1) stoichiometry ratios are high, meaning that some oxygen is consumed by the nitrification process but much more is being used by other processes. As the TAN concentrations increase throughout the day the stoichiometry fits better with theoretical values but is always at least 1 - 2x higher. The stoichiometry ratio of 217.9 is from the first nitrification inhibition experiment (treatment 5) where nitrification was almost completely stopped, this means that practically all the oxygen consumed was by other organisms than nitrifiers. This also suggests that even though oxygen consumption rates can be a direct proxy for ammonium

oxidation rates in some RAS systems (e.g., the one analyzed by Qi *et.al.*), this is not always the case. However, with a steady decrease of O_2 during the laboratory scale incubations this indicates that our experimental setup was suitable for measuring oxygen consumption rates.

The question was then if I could determine how much of the oxygen consumed could be ascribe to nitrification. To answer this question, I looked at the data from the nitrification inhibition experiments with N-SERVE. From the data there was a clear inhibition each time (Annex 1), however it was also clear that the molecule did not work equally well each time, and as determined by MLR analysis (Figure 15) time from day 0 (biofilter maturation) affected the efficiency of N-SERVE. This means that as the biofilter matures and the biofilm become thicker (more microorganisms) more molecule is needed to inhibit all the AOB. Increasing the amount of N-SERVE by 15x and 20x in the last two experiments (treatment 9, 137 days from day 0, 12h after feeding started, and 138 days after day 0) respectively also increased the inhibition, but it was not enough to completely stop the nitrification. It can also be seen by looking at the mM TAN consumed each time, as TAN concentrations increase during the day, so do the mM TAN consumed, proving that there was not enough molecule to inhibit all the AOB. Knowing that increasing amounts of inhibition molecule are needed as the biofilter matures, and using the growth rate of AOB it should be possible to calculate the amount of molecule needed for complete inhibition. This would make it possible to calculate the amount of oxygen consumed during TAN oxidation. This also means that I was not able to determine the fraction of oxygen used by the AOB.

Throughout the experiments we see some differences between the two systems with M12 having a higher TAN oxidation efficiency and consuming more oxygen, and from the compartment tests we see that there are differences between the different compartments within the two RAS modules as well. In Figure 16 (M1) graph 1 we see that TAN oxidation efficiency increases in all three compartments as the biofilters mature. In Figure 17 (M12) graph 1 the same is true for compartment one however, compartments 2 and three start out with a high efficiency that slowly decreases as the biofilters mature. In graph 3 we have TAN oxidation rate as a function of TAN concentration and here is something strange, in M1 higher TAN concentrations in compartments 1 and 2 give a lower TAN oxidation rate while it increases in compartment 3. In M12 the same occurrence is seen in compartment 1 (lower TAN oxidation rate at high TAN concentrations) while the oxidation rate increases in compartments 2 and 3 the same as it does in the general trend in Figure 10. However, in Figure 10 it could look like M1 reaches a plateau at TAN concentrations above 1.2 mg/L. In graph 4 we see that the stoichiometric ratio (ratio of consumed oxygen over consumed TAN) has a steep

slope in compartment 1 in both modules, there is a small increase in compartment 2 in M1 while compartments 3 (M1) and compartments 2 and 3 in M12 have a flat rate. This could be explained by the amount of biofilm on the biocarriers, through visual assessment of the biocarriers taken from each compartment biomass richness was as follows: compartment 1> compartment 2> compartment 3 in both modules. This can be explained by the higher load of organic material in compartment 1, as the water entered this compartment first. High carbon to nitrogen (C/N) ratios have been suggested to decrease the nitrification efficiency of biofilters (Michaud *et.al*., 2006) and could explain the decline in TAN oxidation rate in compartment 1.

Whether there are differences in microbial communities (representatively, quantitively and activity level) between the two modules and between the compartments would be interesting to know. This was a part of the larger RASLab experiment where Marie also collected biocarriers samples for DNA and RNA sequencing however, the results from those tests were not available yet.

Limitations of the thesis

My experiments started at the same time as the RASLab experiments however, we were not sure how the laboratory scale bioreactor would work or if it would even work using water from the RAS modules or if it contained to much organic material that would interfere with the oxygen measurements. Through trial and error, we got the laboratory scale bioreactor to work using water from the systems, but this was not until treatment 3 was underway and we decided to start the measurements when treatment 4 started. This means I do not have any measurements from the beginning of the RASLab experiment and for many of my experiment I only have a few data points as these measurements were not planned from the start. We did not know about N-SERVE when the project started and as it was somewhat expensive, we only had limited amounts of it, which is the reason there are so few experiments with it. Although I have a lot of data and some good results, I see my experiments as more of a pilot project where I tested some things out to see what would work for a future project.

Outlook for future research

With the RASLab experiment being a small-scale experiment, it may not react the same way as a full-scale commercial RAS. Conducting the experiment over a longer time in a commercial RAS would be a good next step, as well maintaining an alkalinity level throughout the entire experiment in each system. Conducting all my experiments throughout an entire project knowing that it is possible to inhibit nitrifiers growing on biocarriers and how to measure the oxygen consumption it

may provide some more insight into how the biofilters develop and how much background oxygen in consumed. It would also be interesting to know how the different alkalinity treatments affect the fish in the system. Do the fish prefer one over another, does alkalinity have any positive/negative effects on fish health and wellbeing, does temperature have any effect on alkalinity? There are many questions to be answered.

Conclusion

Through my experiments I was unable to determine any effects of alkalinity on biofilters. The reason for this may be that we worked within optimal alkalinity range the whole time. Alkalinity of around 100 mg/L $CaCO_3$ are however, recommended as this seem to provide the most stable environment. I was able to establish a method of measuring oxygen consumption during nitrification in a laboratory scale bioreactor and show that TAN oxidation rates and oxygen consumption rates are dependent on TAN concentrations. This however does not mean that oxygen consumption can be used as a proxy for nitrification activity. As the biofilters mature heterotrophic biomass will increase (if organic material is present) increasing the oxygen consumption greatly, it will also decrease the nitrification efficiency of the nitrifiers. The nitrification inhibitory molecule N-SERVE usually used on free living bacteria in water also works on bacteria growing on biocarriers however, fare larger amounts of it are needed to induce inhibition and amount needs to be increased as the biomass of the biofilters increases. Differences in biofilter performance can be seen between biofilters but also within biofilter compartments, knowing what factors influence each section could help improve the overall biofilter performance.

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Annex

Treat-ment	Sampling time - Time after feeding began (hours)	Time from day 0 (days)	pH	Alk (as $CaCO_3$)	T0 TAN (mg/L)	Oxidation rate (mg/L/day)	Efficien	mol/L TAN	mol/L O2	oxygen consump-tion rate (mg/L/day)	Stoic.	Mole-cule	Module
3	4	37	6.910	91.17	1.430	1.100	0.769	0.0646	0.1994	6.380	3.087	no	1 (12)
3	4	37	6.910	91.17	1.430	1.000	0.699	0.0587	0.1732	5.540	2.951	no	12
3	4	38	7.184	119.20	0.840	0.770	0.917	0.0452	0.1460	4.670	3.230	no	1
3	4	38	7.184	119.20	0.840	0.740	0.881	0.4350	0.1550	4.960	3.563	no	12 (1)
4	5	47	7.042	86.32	0.690	0.480	0.696	0.0282	0.1510	3.990	4.364	no	1
4	5	47	7.315	176.90	0.880	0.859	0.976	0.0504	0.1247	3.990	2.474	no	1 (12)
4	5	47	7.042	86.32	0.900	0.590	0.656	0.0346	0.1316	4.830	3.500	no	1
4	5	47	7.315	176.90	0.880	0.670	0.761	0.0393	0.1382	4.420	3.517	no	1 (12)
4	5	47	7.315	176.90	0.880	0.690	0.784	0.0405	0.1304	4.170	3.220	no	12
4	5	47	7.042	86.32	0.690	0.650	0.722	0.0382	0.1458	4.670	3.817	no	12 (1)
4	5	47	7.315	176.90	0.880	0.680	0.773	0.0399	0.1510	4.830	3.784	no	12
4	5	47	7.042	86.32	0.690	0.640	0.711	0.0376	0.1443	4.620	3.838	no	12 (1)
4	5	50	7.126	91.18	0.670	0.390	0.582	0.0229	0.1247	3.700	4.422	no	1
4	5	50	7.412	186.80	0.790	0.635	0.804	0.0373	0.1375	4.400	3.686	no	1 (12)
4	5	50	7.126	91.18	0.900	0.640	0.711	0.0376	0.1329	4.210	4.315	no	1
4	5	50	7.412	186.80	0.790	0.605	0.766	0.0355	0.1452	4.650	4.090	no	1 (12)
4	5	50	7.412	186.80	0.790	0.645	0.816	0.0379	0.1638	5.240	4.322	no	12
4	5	50	7.126	91.18	0.900	0.540	0.783	0.0317	0.1497	4.790	4.722	no	12 (1)
4	5	50	7.412	186.80	0.790	0.660	0.835	0.0388	0.1660	5.310	4.278	no	12
4	5	50	7.126	91.18	0.900	0.540	0.783	0.0317	0.1599	5.120	5.044	no	12 (1)
4	-1	53	7.102	60.88	0.260	0.060	0.231	0.0035	0.0988	3.160	28.039	no	1
4	-1	53	7.102	60.88	0.260	0.060	0.231	0.0035	0.1166	3.730	33.097	no	1
4	4	53	7.029	62.73	0.535	0.325	0.607	0.0191	0.1053	3.368	5.517	no	1
4	4	53	7.029	62.73	0.535	0.335	0.626	0.0197	0.1175	3.760	5.975	no	1
4	8	53	7.108	63.54	0.920	0.580	0.630	0.0341	0.1349	4.315	3.961	no	1
4	8	53	7.108	63.54	0.920	0.600	0.652	0.0352	0.1549	4.955	4.397	no	1
4	12	53	7.044	68.01	1.130	0.660	0.584	0.0388	0.1629	5.210	4.203	no	1
4	12	53	7.044	68.01	1.130	0.730	0.646	0.0429	0.1786	5.715	4.168	no	1
4	5	54	7.005	60.21	0.750	0.515	0.687	0.0302	0.1157	4.250	5.052	no	1
4	5	54	7.402	180.10	0.690	0.500	0.725	0.0294	0.1404	4.490	4.776	no	1 (12)
4	5	54	7.005	60.21	0.750	0.550	0.733	0.0323	0.1370	4.630	5.437	no	1
4	5	54	7.402	180.10	0.690	0.495	0.717	0.0291	0.1488	0.760	5.113	no	1 (12)
4	5	54	7.402	180.10	0.690	0.525	0.761	0.0308	0.1861	5.960	6.042	no	12
4	5	54	7.005	60.21	0.750	0.475	0.709	0.0279	0.1872	5.990	6.710	no	12 (1)
4	5	54	7.402	180.10	0.690	0.530	0.768	0.0311	0.1899	6.080	6.106	no	12
4	5	54	7.005	60.21	0.750	0.470	0.702	0.0276	0.1901	6.080	6.888	no	12 (1)

4	4	57	7.174	93.04	0.670	0.430	0.642	0.0252	0.1447	4.380	4.480	no	1
4	4	57	7.463	165.10	0.555	0.145	0.748	0.0244	0.1184	3.790	4.852	no	1 (12)
4	4	57	7.174	93.04	0.690	0.525	0.761	0.0308	0.1329	4.250	4.401	no	1
4	4	57	7.463	165.10	0.555	0.445	0.802	0.0261	0.1324	4.240	5.073	no	1 (12)
4	4	57	7.463	165.10	0.555	0.375	0.676	0.0220	0.1630	5.220	7.409	no	12
4	4	57	7.174	93.04	0.690	0.485	0.647	0.0285	0.1818	5.820	6.379	no	12 (1)
4	4	57	7.463	165.10	0.555	0.400	0.721	0.0235	0.1761	5.640	7.494	no	12
4	4	57	7.174	93.04	0.690	0.500	0.667	0.0294	0.1948	6.230	6.626	no	12 (1)
5	4	61	7.523	176.60	0.450	0.310	0.689	0.0182	0.1013	3.150	4.366	no	1
5	4	61	7.164	92.61	0.540	0.390	0.722	0.0229	0.1250	4.000	5.459	no	1 (12)
5	4	61	7.523	176.60	0.450	0.335	0.744	0.0197	0.1136	3.640	4.897	no	1
5	4	61	7.164	92.61	0.540	0.420	0.778	0.0247	0.1116	3.570	4.518	no	1 (12)
5	4	61	7.164	92.61	0.540	0.390	0.722	0.0229	0.1500	4.780	6.550	no	12
5	4	61	7.523	176.60	0.450	0.350	0.778	0.0206	0.2057	6.580	9.985	no	12 (1)
5	4	61	7.164	92.61	0.540	0.405	0.750	0.0238	0.1697	5.430	7.130	no	12
5	4	61	7.523	176.60	0.450	0.360	0.800	0.0211	0.1828	5.850	8.664	no	12 (1)
5	4	64	7.321	111.90	0.675	0.375	0.556	0.0220	0.1147	4.930	5.143	no	1
5	4	64	7.227	96.43	0.750	0.370	0.493	0.0217	0.1383	4.430	6.373	no	1 (12)
5	4	64	7.321	111.90	0.675	0.365	0.541	0.0214	0.1155	4.160	5.323	no	1
5	4	64	7.227	96.43	0.750	0.395	0.527	0.0232	0.1369	4.380	5.901	no	1 (12)
5	4	64	7.227	96.43	0.750	0.520	0.693	0.0305	0.1779	5.690	5.833	no	12
5	4	64	7.321	111.90	0.675	0.510	0.756	0.0299	0.1741	5.570	5.823	no	12 (1)
5	4	64	7.227	96.43	0.750	0.555	0.740	0.0326	0.1618	5.180	4.963	no	12
5	4	64	7.321	111.90	0.675	0.520	0.770	0.0305	0.1652	5.290	5.416	no	12 (1)
5	4	65	7.211	96.33	0.930	0.375	0.403	0.0220	0.1737	5.556	7.888	no	12
5	4	65	7.211	96.33	0.930	0.010	0.011	0.0006	0.1279	4.092	217.852	yes	12
5	-1	67	7.645	223.10	0.215	0.060	0.279	0.0035	0.1101	3.523	31.260	no	1
5	-1	67	7.645	223.10	0.215	0.070	0.326	0.0041	0.1199	3.836	29.175	no	1
5	4	67	7.532	197.30	0.570	0.395	0.693	0.0232	0.1101	3.521	4.746	no	1
5	4	67	7.532	197.30	0.570	0.425	0.746	0.0250	0.1122	3.588	4.495	no	1
5	8	67	7.645	185.20	0.870	0.585	0.672	0.0343	0.1341	4.290	3.904	no	1
5	8	67	7.645	185.20	0.870	0.630	0.724	0.0370	0.1474	4.715	3.984	no	1
5	12	67	7.652	195.60	0.945	0.655	0.693	0.0385	0.1622	5.190	4.218	no	1
5	12	67	7.652	195.60	0.945	0.680	0.720	0.0399	0.1670	5.343	4.183	no	1
5	4	68	7.485	216.30	0.550	0.395	0.718	0.0232	0.0985	3.240	5.412	no	1
5	4	68	7.264	104.40	0.625	0.405	0.648	0.0238	0.1154	3.690	4.849	no	1 (12)
5	4	68	7.485	216.30	0.550	0.395	0.718	0.0232	0.1138	3.630	5.777	no	1
5	4	68	7.264	104.40	0.625	0.405	0.648	0.0238	0.1187	3.780	4.987	no	1 (12)
5	4	68	7.264	104.40	0.625	0.460	0.736	0.0270	0.1559	4.990	5.774	no	12
5	4	68	7.485	216.30	0.550	0.530	0.800	0.0258	0.1601	5.120	6.205	no	12 (1)

5	4	68	7.264	104.40	0.625	0.450	0.720	0.0264	0.1542	4.930	5.841	no	12
5	4	68	7.485	216.30	0.550	0.530	0.782	0.2520	0.1533	4.900	6.083	no	12 (1)
5	4	71	7.503	180.60	0.540	0.370	0.685	0.0217	0.1299	3.700	6.070	no	1
5	4	71	7.144	95.13	0.530	0.310	0.585	0.0182	0.1103	3.530	6.060	no	1 (12)
5	4	71	7.503	180.60	0.540	0.380	0.704	0.0223	0.1541	3.670	7.005	no	1
5	4	71	7.144	95.13	0.530	0.340	0.642	0.0200	0.1087	3.480	5.435	no	1 (12)
5	4	71	7.144	95.13	0.530	0.370	0.698	0.0217	0.1526	4.880	7.032	no	12
5	4	71	7.503	180.60	0.540	0.380	0.704	0.0223	0.1492	4.770	6.691	no	12 (1)
5	4	71	7.144	95.13	0.530	0.390	0.736	0.0229	0.1451	4.640	6.336	no	12
5	4	71	7.503	180.60	0.540	0.400	0.741	0.0235	0.1373	4.390	5.843	no	12 (1)
6	4	75	7.291	121.70	0.720	0.450	0.625	0.0264	0.1138	3.640	4.311	no	1
6	4	75	7.067	68.90	0.570	0.320	0.561	0.0188	0.1021	3.270	5.431	no	1 (12)
6	4	75	7.291	121.70	0.595	0.405	0.681	0.0238	0.1248	3.650	4.522	no	1
6	4	75	7.067	68.90	0.570	0.370	0.649	0.0217	0.1013	3.240	4.668	no	1 (12)
6	4	75	7.067	68.90	0.570	0.400	0.702	0.0235	0.1247	3.990	5.306	no	12
6	4	75	7.291	121.70	0.720	0.530	0.736	0.0311	0.1528	4.890	4.913	no	12 (1)
6	4	75	7.067	68.90	0.570	0.410	0.719	0.0241	0.1269	4.060	5.266	no	12
6	4	75	7.291	121.70	0.720	0.530	0.736	0.0311	0.1443	4.610	4.640	no	12 (1)
6	4	78	7.242	106.10	0.595	0.415	0.697	0.0244	0.1100	3.520	4.508	no	1
6	4	78	7.063	79.90	0.660	0.430	0.652	0.0252	0.1250	4.000	4.960	no	1 (12)
6	4	78	7.242	106.10	0.550	0.415	0.755	0.0244	0.1141	3.510	4.794	no	1
6	4	78	7.063	79.90	0.660	0.420	0.636	0.0247	0.1225	3.920	4.960	no	1 (12)
6	4	78	7.063	79.90	0.660	0.480	0.727	0.0282	0.1635	5.230	5.798	no	12
6	4	78	7.242	106.10	0.595	0.495	0.832	0.0291	0.1602	5.130	5.505	no	12 (1)
6	4	78	7.063	79.90	0.660	0.470	0.712	0.0276	0.1564	5.000	5.667	no	12
6	4	78	7.242	106.10	0.595	0.485	0.815	0.2850	0.1505	4.810	5.281	no	12 (1)
6	-1	81	7.387	124.60	0.165	0.110	0.667	0.0065	0.0673	2.152	10.415	no	1
6	-1	81	7.387	124.60	0.165	0.121	0.733	0.0071	0.0741	2.369	10.423	no	1
6	4	81	7.305	118.50	0.500	0.380	0.760	0.0223	0.1089	3.484	4.881	no	1
6	4	81	7.305	118.50	0.500	0.380	0.760	0.0223	0.1108	3.545	4.967	no	1
6	8	81	7.258	113.10	0.835	0.600	0.719	0.0352	0.1415	4.525	4.015	no	1
6	8	81	7.258	113.10	0.835	0.630	0.754	0.0370	0.1476	4.723	3.991	no	1
6	12	81	7.291	120.20	0.870	0.635	0.730	0.0373	0.1563	4.999	4.191	no	1
6	12	81	7.291	120.20	0.870	0.610	0.701	0.0358	0.1572	5.029	4.389	no	1
6	4	82	7.287	104.90	0.550	0.405	0.736	0.0238	0.1079	3.450	4.534	no	1
6	4	82	7.012	76.86	0.570	0.395	0.693	0.0232	0.1131	3.620	4.875	no	1 (12)
6	4	82	7.287	104.90	0.720	0.470	0.653	0.0276	0.1097	3.990	4.496	no	1
6	4	82	7.012	76.86	0.570	0.385	0.675	0.0226	0.1125	3.600	4.978	no	1 (12)
6	4	82	7.012	76.86	0.570	0.375	0.658	0.0220	0.1475	4.720	6.705	no	12
6	4	82	7.287	104.90	0.550	0.405	0.736	0.0238	0.1479	4.730	6.214	no	12 (1)
6	4	82	7.012	76.86	0.570	0.375	0.658	0.0220	0.1373	4.390	6.241	no	12

6	4	82	7.287	104.90	0.550	0.405	0.736	0.0238	0.1327	4.250	5.576	no	12 (1)
6	4	85	7.352	139.30	0.655	0.465	0.710	0.0274	0.1498	3.780	5.312	no	1
6	4	85	7.016	63.06	0.640	0.420	0.656	0.0247	0.1422	4.550	5.757	no	1 (12)
6	4	85	7.352	139.30	0.655	0.480	0.733	0.0282	0.1493	3.790	5.449	no	1
6	4	85	7.016	63.06	0.640	0.435	0.680	0.0255	0.1484	4.750	5.820	no	1 (12)
6	4	85	7.016	63.06	0.640	0.420	0.656	0.0247	0.1570	5.020	6.356	no	12
6	4	85	7.352	139.30	0.655	0.485	0.740	0.0285	0.1777	5.680	6.235	no	12 (1)
6	4	85	7.016	63.06	0.640	0.420	0.656	0.0247	0.1595	5.100	6.457	no	12
6	4	85	7.352	139.30	0.655	0.505	0.771	0.0297	0.1681	5.380	5.660	no	12 (1)
7	-1	95	7.095	84.97	0.285	0.175	0.614	0.0103	0.1089	3.483	10.596	no	1
7	-1	95	7.095	84.97	0.285	0.175	0.614	0.0103	0.1079	3.453	10.505	no	1
7	-1	95	7.095	84.97	0.285	0.095	0.333	0.0056	0.0914	2.923	16.381	yes	1
7	4	95	7.081	86.48	0.805	0.495	0.615	0.0291	0.1637	5.236	5.631	no	1
7	4	95	7.081	86.48	0.805	0.485	0.602	0.0285	0.1602	5.125	5.626	no	1
7	8	95	7.090	85.75	1.230	0.685	0.557	0.0402	0.1976	6.322	4.913	no	1
7	8	95	7.090	85.75	1.230	0.665	0.541	0.0390	0.1807	5.779	4.627	no	1
7	12	95	7.074	81.66	1.360	0.655	0.482	0.0385	0.1933	6.184	5.026	no	1
7	12	95	7.074	81.66	1.360	0.630	0.463	0.0370	0.1884	6.028	5.094	no	1
7	12	95	7.074	81.66	1.360	0.065	0.048	0.0038	0.1399	4.477	36.669	yes	1
7	4	96	7.119	75.40	0.755	0.075	0.099	0.0044	0.2092	6.691	47.496	no	M1 comp 1
7	4	96	7.119	75.40	0.755	0.375	0.497	0.0220	0.1766	5.648	8.018	no	M1 comp 2
7	4	96	7.119	75.40	0.755	0.455	0.603	0.0267	0.1554	4.972	5.818	no	M1 comp 3
7	4	96	7.261	123.3	0.905	-0.005	-0.006	-0.0003	0.1856	5.938	-632.261	no	M12 comp 1
7	4	96	7.261	123.3	0.905	0.205	0.227	0.0120	0.1749	5.594	14.528	no	M12 comp 2
7	4	96	7.261	123.3	0.905	0.345	0.381	0.0203	0.1999	6.394	9.867	no	M12 comp 3
8	4	99	7.150	127.70	0.805	0.295	0.366	0.0173	0.2305	7.374	13.308	no	12
8	4	99	7.150	127.70	0.805	0.285	0.354	0.0167	0.2178	6.967	13.014	no	12
8	4	99	7.150	127.70	0.805	0.040	0.050	0.0023	0.2092	6.693	89.081	yes	12
8	4	103	7.606	268.9	0.78	0.0850	0.109	0.0050	0.1943	6.215	38.927	no	M1 comp 1
8	4	103	7.606	268.9	0.78	0.3700	0.474	0.0217	0.1991	6.370	9.166	no	M1 comp 2
8	4	103	7.606	268.9	0.78	0.4600	0.590	0.0270	0.1612	5.157	5.969	no	M1 comp 3

8	4	103	6.985	87.04	0.88	0.1000	0.114	0.0059	0.2153	6.886	36.660	no	M12 comp 1
8	4	103	6.985	87.04	0.88	0.2150	0.244	0.0126	0.2065	6.605	16.355	no	M12 comp 2
8	4	103	6.985	87.04	0.88	0.4600	0.523	0.0270	0.2124	6.794	7.863	no	M12 comp 3
8	4	106	7.413	249.7	0.775	0.1650	0.213	0.0097	0.1776	5.683	18.337	no	M1 comp 1
8	4	106	7.413	249.7	0.775	0.4000	0.516	0.0235	0.1744	5.578	7.424	no	M1 comp 2
8	4	106	7.413	249.7	0.775	0.4950	0.639	0.0291	0.1658	5.305	5.706	no	M1 comp 3
8	4	106	6.841	59.95	0.885	0.0650	0.073	0.0038	0.1996	6.384	52.288	no	M12 comp 1
8	4	106	6.841	59.95	0.885	0.1150	0.130	0.0068	0.1756	5.618	26.008	no	M12 comp 2
8	4	106	6.841	59.95	0.885	0.3100	0.350	0.0182	0.1909	6.108	10.490	no	M12 comp 3
8	4	107	7.065	80.05	0.865	0.385	0.445	0.0226	0.2127	6.803	9.407	no	12
8	4	107	7.065	80.05	0.865	0.345	0.399	0.0203	0.1960	6.269	9.674	no	12
8	4	107	7.065	80.05	0.865	0.080	0.092	0.0047	0.1734	5.548	36.921	yes	12
8	-1	109	7.509	237.70	0.365	0.225	0.616	0.0132	0.1286	4.113	9.732	no	1
8	-1	109	7.509	237.70	0.365	0.205	0.562	0.0120	0.1164	3.724	9.671	no	1
8	-1	109	7.509	237.70	0.365	0.095	0.260	0.0056	0.1045	3.343	18.734	yes	1
8	4	109	7.328	225.40	0.795	0.525	0.660	0.0308	0.1586	5.073	5.144	no	1
8	4	109	7.328	225.40	0.795	0.540	0.679	0.0317	0.1650	5.278	5.204	no	1
8	4	109	7.328	225.40	0.795	0.140	0.176	0.0082	0.1209	3.867	14.705	yes	1
8	8	109	7.284	220.40	1.360	0.710	0.522	0.0417	0.2004	6.410	4.806	no	1
8	8	109	7.284	220.40	1.360	0.670	0.493	0.0393	0.2029	6.492	5.159	no	1
8	12	109	7.344	220.50	1.585	0.560	0.353	0.0329	0.2063	6.600	6.275	no	1
8	12	109	7.344	220.50	1.585	0.545	0.344	0.0320	0.2043	6.537	6.386	no	1
8	12	109	7.344	220.50	1.585	0.040	0.025	0.0023	0.1316	4.209	56.020	yes	1
9	4	131	7.148	71.15	0.49	0.250	0.510	0.0147	0.1380	4.414	9.400	no	M1 comp 1
9	4	131	7.148	71.15	0.49	0.200	0.408	0.0117	0.1018	3.258	8.673	no	M1 comp 2
9	4	131	7.148	71.15	0.49	0.280	0.571	0.0164	0.1179	3.773	7.174	no	M1 comp 3
9	4	131	7.467	170.6	0.475	0.230	0.484	0.0135	0.1879	6.01	13.911	no	M12 comp 1
9	4	131	7.467	170.6	0.475	0.290	0.611	0.0170	0.1657	5.301	9.732	no	M12 comp 2

9	4	131	7.467	170.6	0.475	0.315	0.663	0.0185	0.1643	5.256	8.883	no	M12 comp 3
9	4	134	7.166	75.51	0.485	0.270	0.557	0.0159	0.1430	4.575	9.021	no	M1 comp 1
9	4	134	7.166	75.51	0.485	0.195	0.402	0.0114	0.0962	3.077	8.401	no	M1 comp 2
9	4	134	7.166	75.51	0.485	0.305	0.629	0.0179	0.1154	3.692	6.444	no	M1 comp 3
9	4	134	7.491	185	0.53	0.300	0.566	0.0176	0.2063	6.599	11.711	no	M12 comp 1
9	4	134	7.491	185	0.53	0.325	0.613	0.0191	0.1692	5.413	8.867	no	M12 comp 2
9	4	134	7.491	185	0.53	0.350	0.660	0.0206	0.1714	5.484	8.342	no	M12 comp 3
9	-1	137	7.467	170.6	0.475	0.145	0.644	0.0085	0.0659	2.108	7.740	no	1
9	-1	137	7.260	75.67	0.225	0.145	0.644	0.0085	0.0672	2.149	7.890	no	1
9	-1	137	7.260	75.67	0.225	0.105	0.467	0.0062	0.0779	2.492	12.635	yes	1
9	4	137	7.138	73.68	0.570	0.335	0.588	0.0197	0.0959	3.067	4.874	no	1
9	4	137	7.138	73.68	0.570	0.195	0.342	0.0114	0.0931	2.978	8.130	yes	1
9	8	137	7.035	78.64	0.865	0.515	0.595	0.0302	0.1399	4.474	4.625	no	1
9	8	137	7.035	78.64	0.865	0.480	0.555	0.0282	0.1349	4.316	4.787	no	1
9	12	137	7.133	85.75	1.120	0.620	0.554	0.0364	0.1520	4.863	4.176	no	1
9	12	137	7.133	85.75	1.120	0.235	0.210	0.0138	0.1242	3.974	9.003	yes	1
9	4	138	7.441	181.70	0.555	0.405	0.730	0.0238	0.1412	4.517	5.938	no	12
9	4	138	7.441	181.70	0.555	0.125	0.225	0.0073	0.1305	4.175	17.782	yes	12
9	4	141	7.096	76.58	0.54	0.255	0.472	0.0150	0.1437	4.598	9.600	no	M1 comp 1
9	4	141	7.096	76.58	0.54	0.225	0.417	0.0132	0.1007	3.221	7.621	no	M1 comp 2
9	4	141	7.096	76.58	0.54	0.320	0.593	0.0188	0.1187	3.796	6.315	no	M1 comp 3
9	4	141	7.43	189.5	0.43	0.240	0.558	0.0141	0.1548	4.953	10.987	no	M12 comp 1
9	4	141	7.43	189.5	0.43	0.230	0.535	0.0135	0.1115	3.567	8.257	no	M12 comp 2
9	4	141	7.43	189.5	0.43	0.275	0.640	0.0161	0.1258	4.025	7.792	no	M12 comp 3

Annex 1: Table containing all laboratory scale experimental data. Denotation 1 (12) and 12 (1) were use during experiment 1, when water was switched in the bioreactors. Denotation 1 (12) means biocarriers from M1 and water from M12 mixed. Denotation 12 (1) means biocarriers from M12 and water from M1 mixed. Denotations M1 comp X and M12 comp X represent module 1 and 12 and compartments 1,2, and 3.