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Jani Pulkkinen

Microbiology of Biological Filters in Recirculating Aquaculture Systems

UNIVERSITY OF JYVÄSKYLÄ
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ABSTRACT

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As aquaculture production continues to increase, new technologies have been developed to minimize nutrient emissions or even recover them into other applications. Recirculating aquaculture systems (RAS) may increase aquaculture production in areas where water source is limited, and the amount of nutrient discharges affects the granting of environmental licenses. The cost–effectiveness of RAS limits the future expansion of the technology, and in particular, the management of the microbial environment has become one of the main challenges affecting the operation of RAS farms. In this dissertation, I examined the microbiology of RAS. The work focused on the nitrification process and microbial community composition in bioreactors and their connections to changes in water quality. Nitrification may be rapidly started by adding ammonia and nitrite salts to the system. I found that bioreactors had diverse microbial communities that affected several water quality parameters. The main function of microbial communities found in freshwater RAS compartments was the degradation of carbohydrates, amino acids, and lipids. When RAS was changed to use brackish water, microbial communities were seen to slowly adapt. However, the degradation of carbohydrates decreased, which was also reflected in elevated concentrations of total organic carbon and the dissolved organic matter in the water. Desinfectant (peracetic acid) addition improved water quality and did not disturb biofilter microbial communities. The fixed bed bioreactors (FBBR) trap solids and organic matter inside the reactor, but potentially also host bacterial communities specialised in the degradation of organic matter. Nitrification performance was decreased in the FBBR, but this may have been caused by the incorrect design of the reactor. The moving bed bioreactors (MBBR) may release the excess bacterial biomass into the water, but the technique kept the biofilm thin and allowed for a functionally diverse bacterial community.

Keywords: 16S rRNA gene; bacterial communities; microbiome; next-generation sequencing; nitrification; rainbow trout; quantitative PCR.

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TIIVISTELMÄ

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Vesiviljelytuotannon jatkuvan lisääntymisen myötä on kehitetty myös uusia tekniikoita, joilla ravinnepäästöt voidaan minimoida tai jopa kierrättää muihin sovelluksiin. Kalojen kiertovesikasvatuksella (RAS) vesiviljelytuotantoa voidaan lisätä alueilla, joilla on pulaa vedestä tai ravinnepäästöjen määrä vaikuttaa merkittävästi ympäristöluvan myöntämiseen. Kiertovesikasvatuksen kustannustehokkuus rajoittaa tekniikan käyttöönottoa, ja erityisesti mikrobiympäristön hallinta on noussut yhdeksi suurimmista kiertovesilaitoksen toimintaan vaikuttavista haasteista. Väitöskirjatyössäni tarkastelin kalojen kiertovesikasvatuksen mikrobiologisia prosesseja. Työssä keskityttiin nitrifikaatioprosessiin sekä muihin bioreaktoreissa tapahtuviin mikrobiologisiin toimintoihin ja niiden yhteyksiin veden laatuun liittyviin muutoksiin. Bioreaktorin käynnistysvaiheessa nitrifikaatio voidaan aloittaa nopeasti syöttämällä ammoniakki ja nitriittiä kiertoveteen. Havaitsin, että bioreaktoreissa oli monipuolisia mikrobiyhteisöjä, jotka vaikuttivat vedenlaatuun eri tavoin. Mikrobiyhteisöjen pääasiallisia toimintoja olivat hiilihydraattien, aminohappojen ja lipidien hajotus, mikä oli yhteydessä kalojen ulosteen koostumukseen kanssa. Kun kiertovesijärjestelmä vaihdettiin käyttämään murtovettä, mikrobiyhteisöt sopeutuivat hitaasti muutokseen. Sen sijaan hiilihydraattien hajotus väheni, mikä ilmeni myös veden kohonneina orgaanisen hiilen ja liuenneen orgaanisen aineen pitoisuuksina. Peretikkahapon lisäämien kiertoveteen paransi vedenlaatua mutta ei vaikuttanut mikrobiyhteisöihin. Kiinteäpetiset bioreaktorit voivat siepata kiinto- ja orgaanista ainetta kantoaineeseen, mutta niiden bakteeriyhteisöt voivat myös erikoistua orgaanisen aineen hajottajiksi. Nitrifikaation teho heikkeni kiinteäpetisessä bioreaktorissa, mutta tämä saattoi johtua reaktorin virheellisestä suunnittelusta. Liikkuvapetiset bioreaktorit vapauttavat ylimääräisen bakteeribiomassan veteen, mutta tekniikan avulla bakteeribiofilmi pysyi ohuena ja mahdollisti toiminnallisesti monipuolisen bakteeriyhteisön.

Avainsanat: 16S rRNA geeni; bakteeriyhteisöt; kirjolohi; kvantitatiivinen PCR; microbiomi; nitrifikaatio; uuden sukupolven sekvensointi.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred in the text by their Roman numerals I–IV. Contributions of authors are presented in the Table 1. In addition, all authors critically examined and revised the manuscripts.

I Pulkkinen J.T., Kiuru T., Aalto S.L., Koskela J. & Vielma J. 2018. Startup and effects of relative water renewal rate on water quality and growth of rainbow trout (Oncorhynchus mykiss) in a unique RAS research platform. *Aquacultural Engineering* 82: 38–45.

II Pulkkinen J.T., Eriksson-Kallio A.M., Aalto S.L., Tiirola M., Koskela J., Kiuru T. & Vielma J. 2019. The effects of different combinations of fixed and moving bed bioreactors on rainbow trout (Oncorhynchus mykiss) growth and health, water quality and nitrification in recirculating aquaculture systems. *Aquacultural Engineering* 85: 98–105.

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

IV Pulkkinen J.T., Aalto S.L., Suurnäkki S., Vielma J. & Tiirola M. 2020. Microbiology of different compartments of freshwater and brackish water recirculating aquaculture systems. *S*ubmitted manuscript.

TABLE 1 Contributions of authors in the original papers. In addition, all authors critically examined and revised the manuscripts. AA = All authors, AE = Anna Maria Eriksson-Kallio, JK = Juha Koskela, JP = Jani Pulkkinen, JV = Jouni Vielma, MT = Marja Tiirola, PL = Petra Lindholm-Lehto, SA = Sanni Aalto, SS = Suvi Suurnäkki, TK = Tapio Kiuru.

	I	II	III	IV
Original idea	TK, JV, JK	TK, JV	JV, JP	JP, MT
Experimental design	TK, JV, JK, JP	AA	AA	JP, JV
Data collection	JP, SA	JP, SA	SS, JP, SA	JP, SS, SA
Data analysis	JP, JK, SA	JP, AE, SA	SS, SA, JP	JP, SA, SS
Manuscript draft	JP, SA, JV	JP, AE, SA	SS, JP, SA	JP, SA, MT

ABBREVIATIONS

AOA Ammonia-oxidizing archaea
AOB Ammonia-oxidizing bacteria
C/N Carbon to nitrogen ratio
CO_2 Carbon dioxide
DNA Deoxyribonucleic acid
DOM Dissolved organic matter
FBBR Fixed bed bioreactor
FCR Feed conversion ratio
FRI Functional redundancy index
FSB Fluidized-sand bed reactor
FTS Flow through system
GSM Geosmin
HRT Hydraulic retention time
MBBR Moving bed bioreactor
MIB 2-methylisoborneol
NGS Next generation sequencing
NH_4^+ Ammonium
NO_2 Nitrite
NO_3 Nitrate
NOB Nitrite-oxidizing bacteria
OTU Operational taxonomy unit
PAA Peracetic acid
qPCR Quantitative polymerase chain reaction
RAS Recirculating aquaculture system
RBC Rotating biological contactor
RNA Ribonucleic acid
RWR Relative water renewal rate
SGR Specific growth rate
TAN Total ammonia nitrogen
TF Trickling filter
TOC Total organic carbon

1 INTRODUCTION

1.1 Current trends in aquaculture

The demand for fish for human consumption has increased from 9 kg per capita in 1961 to 20 kg in 2015, mainly due to increased wealth in developing countries and increased supply (Anon. 2018). Because fish stocks are overexploited in many places, the growth in consumption has been fulfilled by aquaculture, which has been the fastest growing food-based industry, with an annual growth rate of 6 % in the 21st century (Fig. 1). Aquaculture production has exceeded beef production and fisheries catches for human consumption, and is the most important source of aquatic-based food (Anon. 2019). However, this constant growth has raised a number of questions, mainly concerning sustainability and ethics.

The most suitable locations for aquaculture in terms of environmental impact, human conflict, and economic viability are already in use (De Silva 2001, Hersoug 2015). This has caused the industry to intensify production and seek alternative technologies to reach places where production has previously been impossible (Bergheim 2012). The two most promising technologies are offshore farming and recirculating aquaculture systems (RAS) (Bostock *et al.* 2010, Klinger and Naylor 2012), both of which are also recognised in the Finnish national strategy (Anon. 2014) and are at the centre of the European strategy in developing sustainable aquaculture (Anon. 2013, 2016). Recirculating aquaculture has numerous advantages. It can be applied in areas close to markets and with a limited water source, the production environment can be controlled throughout the year, and environmental impacts can be kept to a minimum, including nutrient discharges and fish escapees.

Recirculating aquaculture is a farming method, in which water can be reused at least 50–100 times for growth of fish or other aquatic species. This allows lower water usage rate compared to traditional net cage and flow through systems (FTS), which means lower discharges that can be treated efficiently and economically (Martins *et al.* 2010). In an RAS, the circulating water is purified

with different treatment methods to remove fish metabolites and solids that will otherwise reduce water quality (Timmons *et al.* 2018). However, problems remain to be solved if economic viability is to be achieved. System design, solids management, and biofilter operation have been acknowledged as the main issues in RAS (Martins *et al.* 2010, Badiola *et al.* 2012). In addition, high investment cost and high operating costs have discouraged investment incentives (Klinger and Naylor 2012).

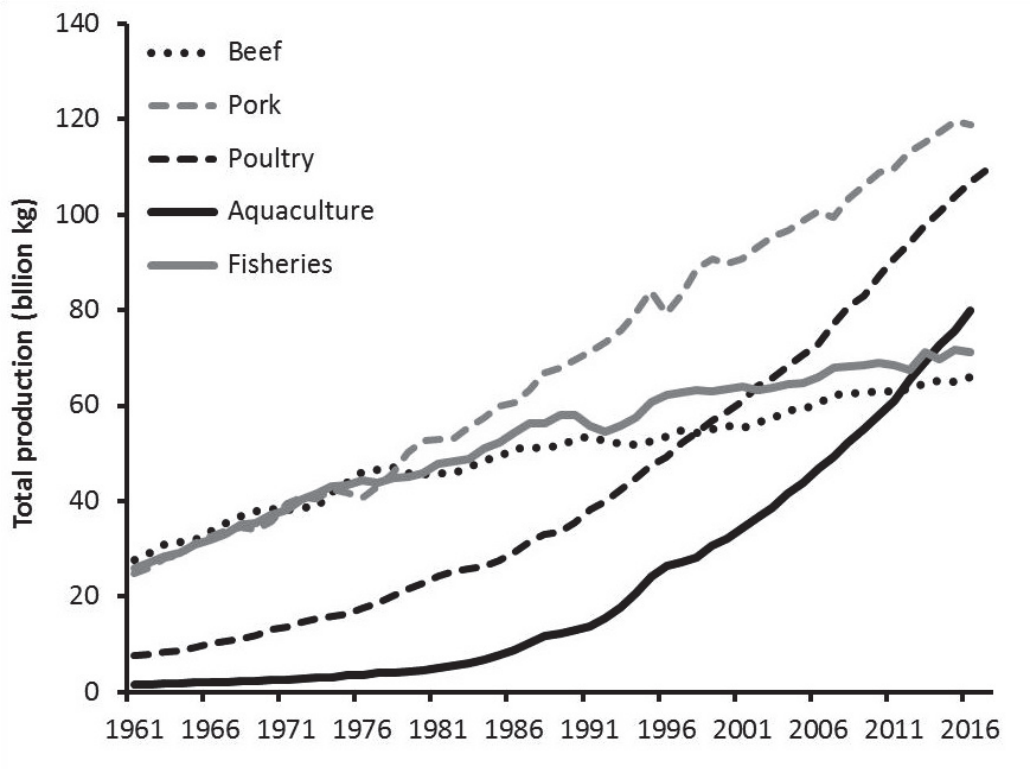

FIGURE 1 Global production of beef, pork, poultry, aquaculture and total catch from fisheries for human consumption (data from Anon. 2019).

1.2 Principles of recirculating aquaculture

Recirculating aquaculture is based on the idea that water can be reused in a fish tank if the harmful fish metabolites are removed from the water. A typical RAS consists of a solid removal system, biological filtration, and gas transfer systems (Fig. 2). An RAS can be operated at different salinities and using different water sources (i.e. ground-, lake-, seawater). As early as the late 1970s, Bohl (1977) presented the first ideas for reusing the water, yet only during the 2000s, the technology has taken a major leap, and large land-based facilities have started to appear (Bergheim *et al.* 2009, Dalsgaard *et al.* 2013, Dalsgaard *et al.* 2015b, Dalsgaard *et al.* 2017).

Fish excretes nitrogen mainly as ammonia or ammonium (Kajimura *et al.* 2004). In aqueous solution, ammonia (NH_3) is present as gaseous and cationic

ammonium (NH_4^+), and the sum of these is expressed as total ammonia nitrogen (TAN). The ratio between the gaseous and cationic form depends mainly on pH and temperature: the higher they both are, the more the gaseous form is present. In addition, the ratio depends on certain extent on salinity. The unionised fraction of TAN is extremely toxic to fish: 12 µg l^{-1} is the recommended concentration limit for salmonids (Westers and Pratt 1977, Fivelstad *et al*. 1995), which is why the ammonia needs to be removed from the rearing water. TAN production may increase if the amino acid profile in the fish feed is not balanced (Bureau and Hua 2010). Thus, fish feeds and feed load are central when dimensioning ammonia removal capacity in RAS. In addition, TAN production in fish depends on the feeding interval (Pedersen *et al.* 2012) and digestibility (Médale *et al*. 1995). Thus, it is beneficial to disperse the feed throughout the day in RAS to ensure a steady ammonia load into the bioreactors. Furthermore, TAN production may decrease if fish have faced a starvation period (Kajimura *et al.* 2004), but the feeding rate itself does not affect the TAN excretion rate (Kajimura *et al.* 2004, Dalsgaard *et al.* 2015a) or fish swimming speed (Lauff and Wood 1996).

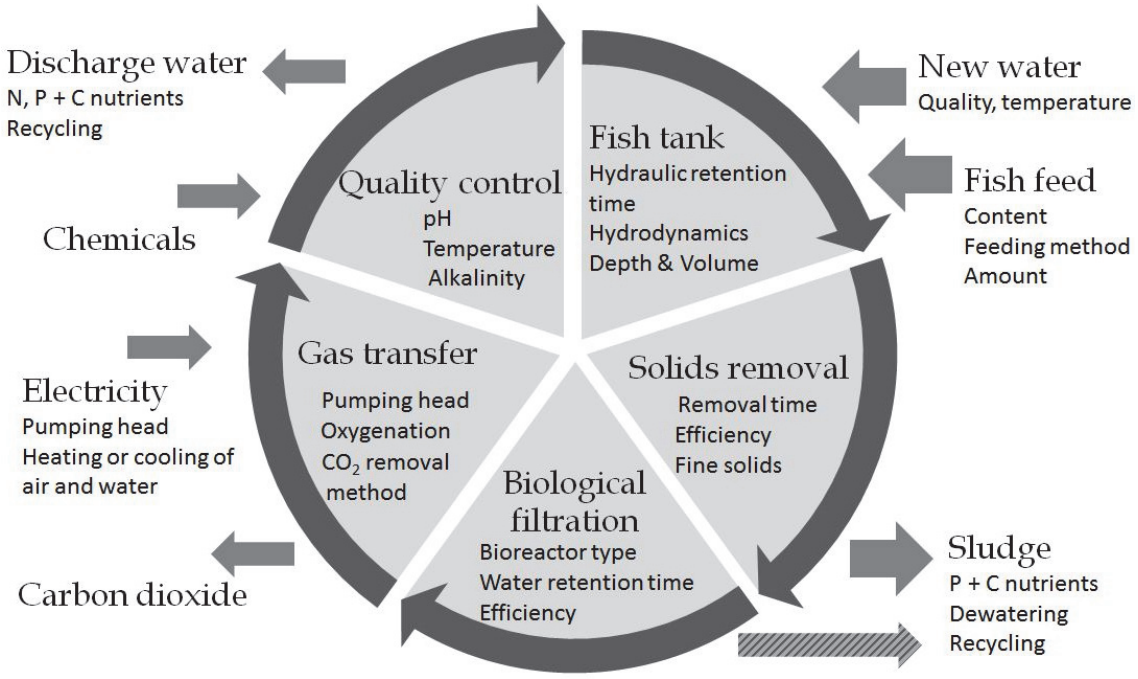

FIGURE 2 Principle of a recirculating aquaculture system and the main factors affecting water quality and system operation.

In an RAS, ammonium is removed in a nitrification process in which bacteria oxidase ammonium to nitrite (NO_2) and further to nitrate (NO_3). Both phases are performed by distinct bacteria (Wagner *et al.* 1995), archaea (Könneke *et al.* 2005) or the recently discovered one organism (comammox, van Kessel *et al.* 2015). Nitrite is highly toxic to fish (survival affected above 1 mg l^{-1}, Kroupova *et al.* 2008), but they can tolerate high nitrate concentrations. For rainbow trout (*Oncorhynchus mykiss*), 75 mg l^{-1} is the recommended upper limit for NO_3-N

(Davidson *et al.* 2014), but even higher concentrations have not affected growth or wellbeing (Davidson *et al.* 2009, Pedersen *et al.* 2012).

A typical RAS uses fixed film bioreactors, where nitrification occurs in a biofilm attached to an artificial carrier media (Malone and Pfeiffer 2006). Several different types of bioreactor operate in RAS, and it appears the moving bed bioreactors (MBBR) (Ødegaard 2006, Rusten *et al.* 2006) and submerged fixed/packed bed bioreactors (FBBR) (Kadic and Heindel 2014) are becoming more common. In addition, some farms rely on trickling filters (TF) (Greiner and Timmons 1998, Lekang and Kleppe 2000), rotating biological contactors (RBC) (Brazil 2006), or fluidized-sand biofilters (Summerfelt 2006). The nitrification rate may vary between different types of bioreactor (Table 2). However, other effects on water quality are often neglected, i.e. gas transfer (Timmons *et al.* 2018), solid accumulation (Fernandes *et al.* 2017), or solid release (Ødegaard 2006).

Nitrification is a key process in RAS performance because of the toxicity of nitrogen products, but the most important factor in recirculating aquaculture is considered to be the control of solids (Martins *et al.* 2010, Timmons *et al.* 2018). A high organic load derived from the solids may impair nitrification (Michaud *et al.* 2006). Moreover, solids may provide an attachment site for bacteria (Pedersen *et al.* 2017) and cause nutrient and organic matter leakage (Holan *et al.* 2014). In addition, solid and organic matter accumulation may create optimal conditions for sulphate–reducing bacteria that can produce hydrogen sulphide (H_2S), which is extremely toxic to fish (Nielsen and Hvitved-Jacobsen 1988, Tal *et al.* 2009). However, recent studies have shown that an accumulation of even relatively high levels of solids (above 30 mg l^{-1}) in the rearing water does not deteriorate fish health or performance, mainly because solids originate from organic matter (Becke *et al.* 2018, 2020). It thus appears that solids may have no direct effect on the fish in an RAS but are crucial for the system's overall performance. In addition, solid accumulation may affect the abiotic conditions such as decrease lighting and alter the other water quality parameters such as increase TAN and nitrite through decreasing nitrification (Micaud *et al*. 2006).

TABLE 2 Volumetric (VNR) and surface-specific (SNR) nitrification rates in different bioreactors. MBF = Microbead filter, TF = Trickling filter, RBC = Rotating biological contactor, FSB = Fluidized-sand bed reactor, FBBR = Fixed bed bioreactor, MBBR = Moving bed bioreactor.

Bioreactor type	VNR (g TAN m^{-3} d^{-1})	SNR (g TAN m^{-2} d^{-1})	Specific surface area (m^2 m^{-3})	Reference
FBBR	140	0.19	750	Pedersen *et al.* 2015
FSB	200–400	0.06–0.13	> 4 000	Summerfelt 2006
MBBR	90	0.12	750	Pedersen *et al.* 2015
MBF	224	0.57	3 936	Greiner and Timmons 1998
RBC	70	0.43	168	Brazil 2006
TF	19–84	0.12–0.36	160–234	Kamstra *et al.* 1998

A correct tank design is important for efficient solids removal. In recent years, the hydraulic retention time (HRT) in the fish tanks has almost halved from previous recommendation. This offers better solids removal, as well as more unified conditions for the fish in the tank (Summerfelt *et al.* 2016). In addition, inlet and outlet pipes, nozzle angles and fish densities affect the fish tank function (Gorle *et al.* 2018a, 2018b, 2019). The main solid removal techniques in RAS are settling and filtration (Davidson and Summerfelt 2005). Typical settlers include swirl and radial flow settlers, which provide a high removal efficiency with fairly thick sludge (4–8 % dry matter), but they need a low surface-loading rate (Twarowska *et al.* 1997, Summerfelt and Vinci 2008). Drum and disc filters are widely used filtration methods in RAS, because of high water volume treating capacity, but they remove more diluted sludge (0.1–0.4 % dry matter) compared to settlers (Twarowska *et al.* 1997, Schumann *et al.* 2017). Membrane technology can remove fine solids and colloids from the water (Holan *et al.* 2014, Davidson *et al.* 2019), but the operating costs for pumping a large quantity of water through membranes may not be economically feasible. Protein skimmers/foam fractionators are used for solids removal mainly in saline environments, where the efficiency is much higher because of the electrochemical charges (Timmons *et al.* 2018).

The third main step in recirculating aquaculture systems is gas transfer, which entails the removal of carbon dioxide and the addition of oxygen into the system. The observed effects of CO_2 on fish growth are controversial. Khan *et al.* (2018) and Mota *et al.* (2019) observed a negative linear relationship between even relatively low CO_2 concentration and Atlantic salmon (*Salmo salar*) growth. Furthermore, Good *et al.* (2018) observed no effects on growth at an elevated CO_2 level. Nevertheless, carbon dioxide needs to be removed from the system, and this is usually done by dispersing water through the air, or dispersing air through the water (Timmons *et al.* 2018). Dispersing water through the air can be done with packed columns, aeration towers, and trickling filters (TF) similar to those used for nitrification (Eding *et al.* 2006). TFs need a pumping head and are usually equipped with forced ventilation (Karimi *et al.* 2019). Dispersing air through the water can be done using air pumps and diffusors (Summerfelt *et al.* 2000), which do not need a pumping head, but the energy cost of pumping air through water is high.

In addition to the main steps, other important factors should be considered when operating an RAS farm. Nitrification produces H^+, and fish respire CO_2 into the water, which decreases the pH. Alkaline chemicals, such as sodium hydroxide, and sodium bicarbonate are used to compensate this pH drop (Timmons *et al.* 2018). Nitrifying bacteria uses inorganic carbon, thus high alkalinity levels should be maintained. If there is a low water usage rate, nitrate accumulates in the water and it may become harmful to the fish (Davidson *et al.* 2014). In such cases, denitrification is required, which is the reduction of nitrate into nitrogen gas, but this might also be used to remove nitrogen from RAS effluents (van Rijn *et al.* 2006). Sludge handling, water and hall temperature control, and energy consumption need to be considered, when designing an economically viable recirculating aquaculture system.

1.3 Microbes in RAS

1.3.1 The microbiome

The complexity of the microbial world in aquatic systems is well known but poorly understood (Miki and Jacquet 2008). Micro-organisms can have versatile interactions, including resource competition (Grover 2000), predation (Hahn and Höfle 2001), and cell–cell signalling (Waters and Bassler 2005), all of which may be affected by the environmental conditions (Hall *et al.* 2008, Kent *et al.* 2007). This complex network of microbes is called a microbiome, which includes bacteria, archaea, and viruses, as well as eukaryotic micro-organisms like fungi and algae. The recent advances in next-generation sequencing (NGS) have accelerated the inspection of microbial communities, which enables the rapid and economic characterisation of complete microbiomes from environmental samples. In RAS, micro-organisms may originate from the inlet water, air dispersion, stocked fish, fish feeds, or visitors and employees of the farm (Rurangwa and Verdegem 2014).

Based on the NGS, most bacteria in RAS have been assigned to phylum Proteobacteria (Bartelme *et al.* 2017, Rud *et al.* 2017, Lukassen *et al.* 2019), which is unsurprising given that is the largest and most diverse phylum, including members from phototrophs, heterotrophs, and chemolitotrophs (Gupta 2000). In the phylum Proteobacteria, Rhodobacterales from the Alphaproteobacteria class has been the most dominant order in the water and biofilms in RAS (Rud *et al.* 2017, Lukassen *et al.* 2019). This is also a main surface coloniser in marine environments (Dang *et al.* 2008). Other abundant phyla include Bacteroidetes, which includes species that specialise in degrading high molecular weight compounds (Fernándes-Gómez *et al.* 2013) and Actinobacteria, which can degrade plant biomass (Lewin *et al.* 2016). Based on the abundance of bacteria, the main function of the microbiome appears to be the degradation of organic material.

As the bioreactors are built to carry nitrification, nitrifying bacteria play an important role in the microbiome and the overall performance of the RAS. Typical ammonia-oxidizing bacteria (AOB) genera discovered in the bioreactors are *Nitrosomonas*, *Nitrosospira, Nitrosovibrio,* and *Nitrosococcus* (Foesel *et al.* 2008, Sakami *et al.* 2012, Brown *et al.* 2013, Navada *et al.* 2019). Besides the bacteria *Nitrosopumilus maritimus,* ammonia-oxidizing archaea (AOA) are also found in the RAS bioreactors (Sakami *et al.* 2012, Brown *et al.* 2013). Nitrite-oxidizing bacteria (NOB) genera observed in the RAS bioreactors are *Nitrospira*, *Nitrospina*, *Candidatus Nitrotoga,* and *Nitrobacter* (Brown *et al.* 2013, Ruan *et al.* 2015, Navada *et al.* 2019). Besides a nitrite-oxidizing capability, some species of *Nitrospira* are capable of oxidising ammonia and are classified as comammox-bacteria (complete ammonia-oxidiser) (van Kessel *et al.* 2015, Bartelme *et al.* 2017). Costa *et al.* (2006) posited that kinetic theory could explain the two-step nitrification process, where a short pathway (nitritation or nitratation) maximises ATP production, increasing the growth rate. Longer pathways (comammox) have more ATP-generating steps, which may increase growth yields. They stated that

higher yields were beneficial when bacteria grew in clusters as in biofilms, whereas a higher growth rate was beneficial in chemostats or other well-mixed systems. This suggests that bioreactors in RAS are suitable for comammox. The role of different nitrifying bacteria is not currently well understood but different genera have their own preferred saline conditions, pH, and substrate concentrations (Belser 1979, 1984).

1.3.2 Fish–bacteria interactions

Interactions between fish and microbes may be harmful or beneficial. The intake water volume is much less in RAS compared with the flow through systems (FTS). It is thus feasible to disinfect the intake water against pathogenic microbes (Timmons *et al.* 2018). However, pathogens may remain present in the system without causing any mortality or harm to the fish (Martins *et al.* 2013). If these opportunistic pathogens gain a competitive edge, the interaction between the fish and the microbes may have detrimental effects on the fish health (Olafsen 2001, Summerfelt 2009). In addition, the circulating water may be disinfected with UV or ozone (Summerfelt *et al.* 2009), yet the disinfection can destabilize the microbial community, which can decrease the survival and growth of the fish (Attramadal *et al.* 2012a, Dahle *et al*. 2020).

It has been shown that water microbial communities play a key role in fish larval development (Munro *et al.* 1994, Olafsen 2001, Verner-Jeffreys *et al.* 2003), and the RAS may be a potential environment for controlling these communities (Attramadal *et al.* 2012b). In general, larval RAS select microbial communities towards K-strategists, which are stable slow growing bacteria with a high affinity with resources compared to r-strategists, with a high growth rate and poor competition ability (Vadstein *et al.* 2018). The r-strategists include several known fish pathogens (Vadstein *et al.* 2018). An environment with a K-strategist community can provide a higher growth rate and greater survival in the first feeding stages (Vadstein *et al.* 2018), but the effects are unknown for older fish. Roque d'Orbcastel *et al.* (2009) observed that rainbow trout grew better in a recirculating system compared to an FTS, but in their study, this may have been because of the lower stocking capacity of the FTS.

The secondary metabolism products of certain bacteria clades from Cyanobacteria, Myxobacteria, Nocardia, and Streptomyces (Schrader and Summerfelt 2011, Lukassen *et al.* 2017) may cause an accumulation of geosmin (GSM) and 2–methylisoborneol (MIB) off-flavours in RAS water and the fish. These off-flavours are not harmful to the fish, but they create a need to purge the flavour–tainted fish, which affects the profitability of an RAS farm (Engle *et al.* 1995, Tucker 2000). Off-flavours are difficult to avoid and even more difficult to remove from the fish reared in an RAS (Lindholm-Lehto and Vielma 2019). Some bacteria can biodegrade GSM and MIB (Azaria *et al.* 2019), yet the purging of fish has been the most economic method for the removal of taints. In future, bacteriophage-based therapy (Almeida *et al.* 2019) to restrict the growth of off-flavour producing bacteria (Jonns *et al.* 2017) and reduce the need for purging the fish may be possible.

1.3.3 Bacteria–bacteria interactions

The most important and studied interaction between the different bacteria in an RAS is the relationship between the heterotrophs and autotrophic nitrifying bacteria. Heterotrophic bacteria utilise organic carbon derived from the fish feed and faeces, and are fast growers, as the generation time can be 2–3 h (Leonard *et al.* 2000). In contrast, autotrophic nitrifying bacteria use inorganic carbon, which may originate from fish respiration. Nitrifying bacteria are slow growers, as the generation time may be roughly 18–68 h (Belser 1984). The higher the C/N ratio is in the RAS, the more heterotrophic bacteria are present, which decreases nitrification efficiency (Zhu and Chen 2001, Michaud *et al.* 2006). In contrast, heterotrophic bacteria may protect the nitrifiers from detachment and grazing (Michaud *et al.* 2006), but occupy niches and prevent harmful bacterial species outbursts. They can thus maintain the balance of the microbial community (Blancheton *et al.* 2013).

Other bacterial interactions may be positive (mutualistic, syntrophic) or negative (predation, competition). At the bioreactor startup phase, more positive interactions between the bacteria occur, but after maturation, the interactions become more negative as the competition increases (Jiang *et al.* 2019). However, the increased competition may increase the ecological stability of the microbiome (Coyte *et al.* 2015, Wilson *et al.* 2017), which may lead to a more stable environment for the fish. Kandel *et al.* (2014) observed that *Bdellovibrio* and like organism (BALOs), which are bacterial predators, constituted more than 1 % of the total bacteria count and appeared to fluctuate over time. Bacterial predators may affect the microbiome by harvesting bacteria-specific preys, which may further influence the competition or cooperation with other bacteria (Welsh *et al.* 2016).

1.3.4 Microbial water quality

In recent years, the microbial water quality in aquaculture systems has received increasing interest (Rojas-Tirado 2018). The water quality in an RAS depends on the physical properties of the water treatment system, as well as the microbial processes they contain, which includes organic matter biodegradation and the metabolism of nitrogen products. Typical water quality parameters that are influenced on and by the microbes are DOC (dissolved organic carbon), COD (chemical oxygen demand), BOD (biological oxygen demand), and turbidity (Rojas-Tirado 2018). Water quality has been shown to affect bacterial community composition (Van Wyk 2006, Auffret *et al.* 2013, Michaud *et al.* 2014). However, the bacterial community composition also affects water quality. Examples include beneficial nitrification or denitrification, and harmful production of off-flavours and hydrogen sulphide (Rurangwa and Verdegem 2015). Current knowledge of biological filtration is limited to studies on nitrification and physical features, and their effect on water quality. Different types of bioreactor may have distinct physicochemical properties which create different microenvironments and further host diverse microbiomes (Bartelme *et al.* 2019).

The effects of these microbiomes on the water quality and system performance are currently not well understood.

1.4 Objectives of the study

The objective of this thesis was to investigate the processes that affect bacterial communities in recirculating aquaculture systems. The emphasis was on studying the microbiology and the nitrification efficiency of bioreactors in the different operating conditions of RAS. Four studies (I–IV) were conducted to address these objectives. The selection of the bioreactor type is traditionally based on the functionality and ease of use of the bioreactors, and the nitrification performance. However, the bioreactor's mode of operation may alter the physicochemical parameters of the water and create different microhabitats that have versatile microbiomes. I hypothesised that using different bioreactors with varying physical and chemical features will improve the system performance, which can be measured by increased fish growth and water quality. The specific research questions in the studies were:

RQ I: Can the nitrification startup be accelerated/will this startup method enhance the nitrification rate? Startup process of biological filtration was studied (I) by adding combinations of NH_4Cl, $NaNO_2$, and carbon to the RAS in comparison to starting with fish. The nitrification rate was measured during the startup process and after the fish were introduced to all the systems.

RQ II: Fixed bed and moving bed bioreactors are becoming more common in RAS. What are the physicochemical effects of these reactor types on water quality, and do they show differences in nitrification performance? A long-term experiment was performed (II) by using a FBBR, MBBR, or a combination of them in RAS, and monitored their effects on water quality and nitrification.

RQ III: Can peracetic acid be dosed safely to an RAS without disturbing the beneficial microbiome, or can it even have beneficial effects on the microbiome? PAA has become a widely used therapeutic in RAS, yet the effect on the bacterial community level is unknown. The effect of disinfectant (peracetic acid) on the bacterial communities in the different biofilms and water in RAS was inspected (III) by increasing the dose of PAA.

RQ IV: How do the microbial communities develop and differ in the different compartments of RAS? How does a salinity of 7 ppt, typical of the Finnish Baltic Sea area, affect microbial communities? Microbial communities were sampled and sequenced (IV) in fresh water and artificial brackish water RAS from the fixed bed bioreactor, moving bed bioreactor, trickling filter, tank biofilm, and circulating water during the RAS startup phase.

2 MATERIAL AND METHODS

2.1 Study system

All experiments were conducted on the Natural Resources Institute Finland (Luke) Laukaa RAS research platform. The RAS research platform serves as a research laboratory for RAS technology and development. Briefly, it consists of 10 individual RAS units, using technologies that are used at commercial RAS farms. The main water treatment steps are particle removal using a swirl separator and drum filter, biological filtration using either a fixed bed bioreactor or moving bed bioreactor, or both, and a trickling filter mainly acting as a forced–ventilated aeration column (Fig. 3). Several of these steps can be by-passed, and other adjustments can also be constructed to modify the water treatment units. The water source is the oligotrophic Lake Peurunka, without a disinfection system.

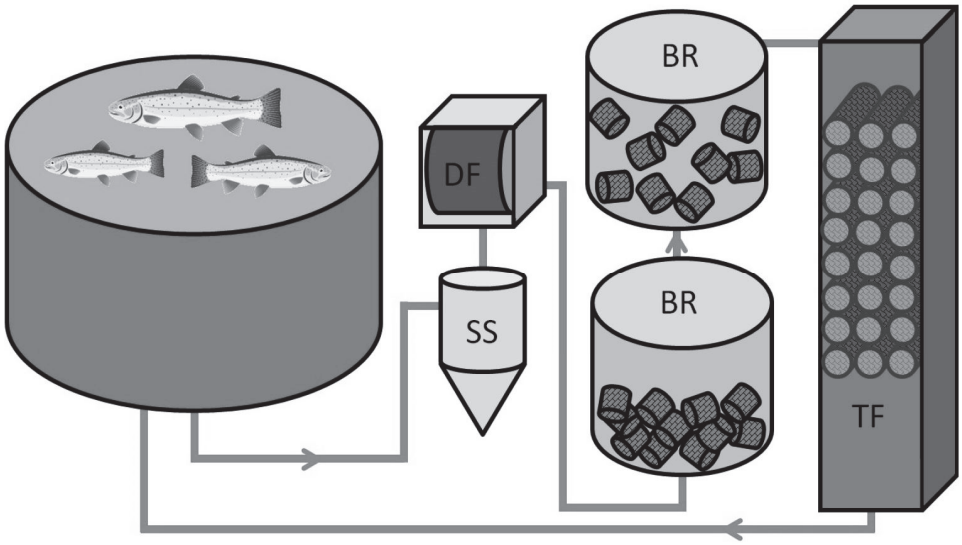

FIGURE 3 A schematic diagram of the recirculating aquaculture system used in the experiments. The bioreactor type varied between experiments. SS = swirl separator, DF = drum filter, BR = bioreactor, TF = trickling filter.

Similar plastic carrier media were used in the bioreactors (RK Bioelements, RK Plast A/S, Skive, Denmark, 750 m^2 m^{-3}), except for the fixed bed media, which was heavier (1.2 g cm^{-3}) because of the addition of $BaSO_4$, to ensure the media would lie statically at the bottom of the reactor. The moving bed media (1.0 g cm^{-3}) was constantly agitated by adding air to the bottom of the reactor at approximately 15 l min^{-1}. The trickling filters' main purpose was gas transfer, but it could contain approx. 5–17 % of the total nitrification capacity in the RAS, depending on the experimental set-up and dimensioning of the other bioreactors. The trickling filter is filled with Bio-Blok plastic media (EXPO-NET Danmark A/S, Hjørring, Denmark, 200 m^2 m^{-3}).

All experiments were carried out using rainbow trout as a test species. The rainbow trout originated from the national JALO selective breeding programme of the Natural Resources Institute Finland (Kause *et al.* 2003). Water temperature was maintained at 15–16 °C and pH at 7.2 by dosing sodium hydroxide to the systems, and. Relative water renewal rate (RWR) was set to 500 l kg^{-1} feed, except in the experiment where different RWRs were studied (I).

2.2 Water quality measurements

Each RAS had an online water quality monitoring system, which measured oxygen (OxyGuard, Farum, Denmark and oxi::lyser, s::can, Vienna, Austria), carbon dioxide (Franatech, Lüneburg, Germany), pH (ise::lyser, s::can, Vienna, Austria), and flow rates (8012, Bürkert, Ingelfingen, Germany). In addition, more novel spectrometers were installed in all systems. This measured the UV-VIS spectrum (210–420 nm), which enabled the continuous measurement of relevant RAS water quality parameters, including nitrite, nitrate, total organic carbon (TOC), total suspended solids (TSS), and turbidity (spectro::lyser, s::can, Vienna, Austria). In addition to the online monitoring system, measurements of total ammonia nitrogen, nitrite, and nitrate were made using spectrophotometry (DS 3900, Hach, Loveland, USA), as well as alkalinity with a titration method (TitraLab AT1000, Hach, Loveland, USA).

2.3 Methods for bacterial analyses

Several different bacterial analyses were conducted during the experiments (Table 3). Next-generation sequencing was done with an Ion Torrent PGM (Thermo Fisher Scientific, Waltham, USA), using 515F-Y and 806R primers (Table 5), which amplified 291 bp long sequence from the V4 region of the 16S rRNA gene (III, IV). The NGS data were analysed and classified for operational taxonomic units (OTU) using mothur (version 1.39.5, Schloss *et al.* 2009) with the latest Silva 16S v132 database.

TABLE 3 Bacterial analyses used in the experiments.

Analysis	Description	Reference
Cell counting	II	
Incubation method	I, II, III	Jäntti *et al.* 2011
16S rRNA gene-based metagenomics analysis	IV	Wemheuer *et al.* 2018
Next-generation sequencing	III, IV	Aalto *et al.* 2018
qPCR	III	Aalto *et al.* 2018

The abundance of the 16S rRNA gene, AOA, AOB, NOB and comammox were quantified with quantitative PCR (qPCR, Table 4). AOA, AOB, and comammox were targeted to quantify the gene-coding ammonia monooxygenase enzyme (*amo*A), which catalysed ammonia oxidation to hydroxylamine. NOB was targeted to quantify the gene-coding nitrite oxidoreductase enzyme (*nxr*), which catalyse nitrite oxidation to nitrate.

A modified incubation method was developed for the nitrification efficiency studies (Jäntti *et al.* 2011). Carrier media from the bioreactors were collected and incubated in a laboratory using labelled $^{15}NH_4^+$. The nitrite and nitrate thus formed were chemically converted into nitrite oxide, and the isotopic composition and concentration were measured with an Isoprime100 isotope-ratio mass spectrometer coupled to a TraceGas Pre-Concentration Unit (IsoPrime Ltd, Cheadle, England).

To inspect the total bacteria numbers in a water sample, a cell-counting method was tested with a CASY cell counter (Model TT, OLS OMNI Life Science GmbH, Basel, Switzerland). Technology was based on an electric field, which could separate dead from living cells by the electric current going through a broken cell wall or stopping at an intact cell wall.

An R-based tool (Tax4Fun2, Wemheuer *et al.* 2018) was used to predict the functional profiles of the 16S rRNA gene-sequencing data. The tool harnessed information from genetic public databases and linked this data to the 16S rRNA sequencing data. In this tool, functions are based on KEGG orthology (Kyoto Encyclopedia of Genes and Genomes) and bacterial annotations to the latest Silva 16S v132 database. This method cannot replace metagenomics shotgun sequencing, which can be used to detect genes from environmental samples, but it will provide a cost-effective insight into the potential functions present in the environmental samples.

TABLE 4 Primers used in the experiments.

Primer	Sequence	Description	Reference
515F-Y	GTGYCAGCMGCCGCGGTAA	III, IV	Parada *et al.* 2016
806R	GGACTACHVGGGTWTCTAAT	III, IV	Caporaso *et al.* 2011
amoA-1F	GGGGTTTCTACTGGTGGT	III	Rotthauwe *et al.* 1997
amoA-2R	CCCCTCKGSAAAGCCTTCTTC	III	Rotthauwe *et al.* 1998
Arch-amoAF	STAATGGTCTGGCTTAGACG	III	Francis *et al.* 2005
Arch-amoAR	GCGGCCATCCATCTGTATGT	III	Francis *et al.* 2005
comaA-244F	TAYAAYTGGGTSAAYTA	III	Pjevac *et al.* 2017
comaA-659R	ARATCATSGTGCTRTG	III	Pjevac *et al.* 2017
comaB-244F	TAYTTCTGGACRTTYTA	III	Pjevac *et al.* 2017
comaB-659R	ARATCCARACDGTGTG	III	Pjevac *et al.* 2017
F1norA	CAGACCGACGTGTGCGAAAG	III	Poly *et al.* 2008
nxrB169f	TACATGTGGTGGAACA	III	Pester *et al.* 2014
nxrB638r	CGGTTCTGGTCRATCA	III	Pester *et al.* 2014
R1norA	TCCACAAGGAACGGAAGGTC	III	Poly *et al.* 2008

2.4 Statistical analyses

Different statistical methods were used to analyse the data obtained from the experiments (Table 5).

TABLE 5 Statistical analysis used in the experiments.

Analysis	Description	Program
ANOVA	I, II, IV	SPSS
Broken-line test	I	SYSTAT
Linear regression	III	SPSS
Mann–Whitney U	II	SPSS
Mixed ANOVA	II	SPSS
PERMANOVA	III, IV	PRIMER+
SIMPER	III, IV	PRIMER+

3 RESULTS AND DISCUSSION

3.1 The microbiome in the bioreactors

3.1.1 The freshwater microbiome

The majority of the bacteria in the freshwater fixed and moving bed bioreactors belonged to the class Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, and Planctomycetes (Fig. 4, IV). When inspected in more detail, the most abundant OTUs belonged to unclassified *Rhodobacteriaceae,* unclassified *Burkholderiaceae,* and *Novosphingobium* (Table 6). The microbiome evolved continuously during the first 6 months of operation (IV).

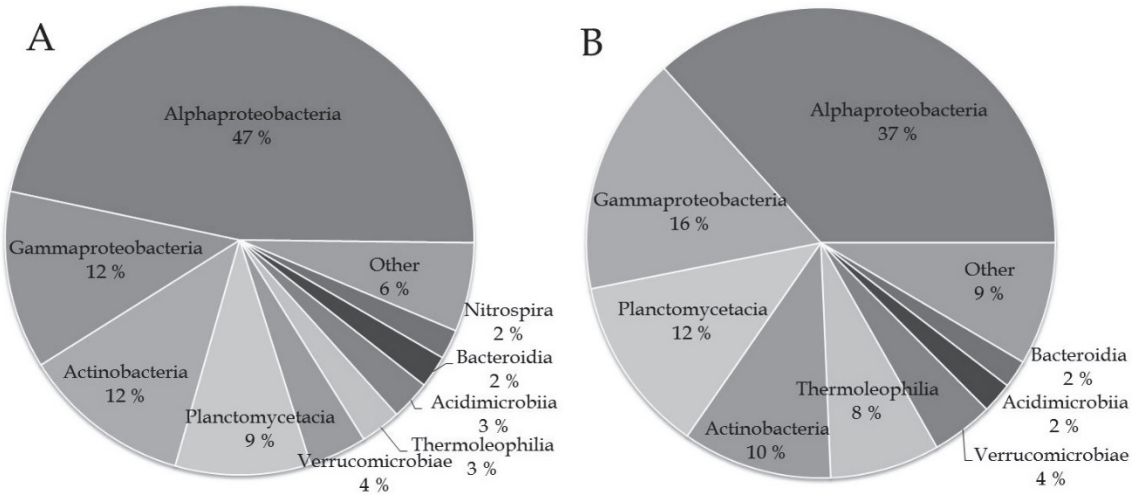

FIGURE 4 Average relative abundance of bacterial class from A) freshwater fixed bed bioreactor B) freshwater moving bed bioreactor (IV).

Bacteria in the *Rhodobacteriaceae* family can utilise organic compounds (Pohlner *et al.* 2019), and are the first major colonisers and the most abundant bacteria in marine biofilms (Dang *et al.* 2008, Elifantz *et al.* 2013). Depending on feed content, faeces may contain 48–75 % of dry matter as carbohydrates (Prabhu *et al.* 2019)

which starts immediately to leach into the water (Chen *et al.* 2003). The carbohydrates contain cellulose, hemicellulose, lignin, and starch (Meriac *et al.* 2014), which fish cannot utilise efficiently. The high abundance suggests that *Rhodobacteriaceae* are generalist heterotrophic bacteria that can support the healthy biofilm in RAS bioreactors by degrading the carbohydrates derived from fish faeces and supporting biofilm growth. The predicted functions revealed that carbohydrate degradation was the most abundant metabolic function in the bioreactors (IV).

TABLE 6 Relative abundance of bacterial genera in the freshwater fixed and moving bed bioreactors (IV). Uncl. = unclassified bacteria genera.

Fixed bed bioreactor		Moving bed bioreactor	
Genus	Relative abundance	Genus	Relative abundance
*Rhodobacteraceae*_uncl.	9.9 %	*Burkholderiaceae*_uncl.	6.0 %
Novosphingobium	6.6 %	*Solirubrobacterales* bacterium 67–14	5.9 %
*Burkholderiaceae*_uncl.	5.3 %	*Rhodobacteraceae*_uncl.	4.8 %
Mycobacterium	3.5 %	*Legionella*	3.8 %
Legionella	3.0 %	*Novosphingobium*	3.4 %
Reyranella	2.6 %	*Devosia*	3.3 %
*Xanthobacteraceae*_uncl.	2.4 %	*Galbitalea*	3.2 %
Aurantimicrobium	2.3 %	*Mycobacterium*	2.1 %
Solirubrobacterales bacterium 67–14	2.2 %	*Xanthobacteraceae*_uncl.	2.0 %
Devosia	2.2 %	*Zavarzinella*	2.0 %
Galbitalea	1.9 %	*Rhodopirellula*	2.0 %

The bioreactors in the freshwater system had a high relative abundance of the *Burkholderiaceae* family (IV). Bacteria in this family have known lipid-degrading capabilities (Matsumiya *et al.* 2007). Fish faeces can contain approx. 6–9 % of dry matter as undigested lipids (Wang *et al.* 2013). Thus, the bacteria can play an important role in maintaining good water quality by degrading these lipids. The predicted functional metabolism also revealed that lipid metabolism was one of the most important functions of the microbiome (IV).

The genera *Novosphingobium* contain versatile members, and they have been reported to degrade xenobiotic compounds, which can include chlorophenol, phenol, and other polyaromatic hydrocarbons (Kumar *et al.* 2017). These xenobiotic compounds originate from fish feeds and are typically man-made, but the concentrations are quite low (< 1 ‰) (Berntssen *et al.* 2010). In addition, *Novosphingobium* can degrade complex humic substances (Newton *et al.* 2011), which are typical in Finnish lakes. The abundance may thus also reflect the freshwater source used in the RAS. The *Mycobacterium* was also abundant and could be part of the aromatic hydrocarbon degradation (Heitkamp and Cerniglia 1989). However, the genus *Mycobacterium* contains 188 species, including several

known pathogens (Gupta *et al.* 2018), so the functional role of the genera in the bioreactors may be versatile.

The main difference between the two bioreactor types was the 10–percentage point lower abundance of Alphaproteobacteria and the 5–percentage point higher abundance of Thermoleophilia in the MBBR. Although there was a relatively large difference between bacteria classes in the bioreactors, the communities did not differ significantly (PERMANOVA, OTU-based similarity, $p = 0.4$, IV). The FBBR trapped solid particles inside the reactor, which led to a lower concentration of organic matter in the water (II). The shearing forces of the MBBR released the excess bacterial biomass into the water and grind solids, which led to higher solids and organic matter concentration in the water (II). This also led to a somewhat higher abundance of bacteria in the water samples (0.89–3.42 M ml^{-1}) compared to fixed bed bioreactors (0.51–0.83 M ml^{-1}), but the differences were not statistically significant (Mann–Whitney U test, $p = 0.2$) (II). *Rhodobacteraceae* were more abundant in the FBBR, which was probably caused by the higher biofilm thickness or organic matter accumulation in the reactor. The *Solirubrobacterales* bacterium 67–14, belonging to class Thermoleophilia was more abundant in the MBBR than in the FBBR. The *Solirubrobacterales* favours simple sugars as a carbon source (Foesel *et al.* 2016), but the full functional properties of Thermoleophilia has yet to be thoroughly studied (Hu *et al.* 2019), and it is unclear why *Solirubrobacterales* was more abundant in the MBBR.

The trickling filter harboured a different microbial community than the FBBR (PERMANOVA, pair-wise test, $p = 0.03$) (IV). The major differences were the higher abundances of *Novosphingobium* and unclassified *Burkholderiacea*, and the lower abundances of *Mycobacterium* and *Aurantimicrobium* in the TF than the FBBR (IV). The TF was not periodically backwashed compared to the interval backwash of the FBBR and the continuous wash of the MBBR, which may have affected the communities. Because there was no backwash, the biofilm was expected to be thicker, which could lead to a more stable community structure (James *et al.* 1995). However, the changes in the community composition during the first six months of operation were similar in the TF to that in the FBBR and MBBR (IV).

Peracetic acid dosages did not significantly affect the microbial communities in the fixed bed bioreactors (III). Some minor changes were observed when inspecting the individual level responses (SIMPER). It appeared that one OTU belonging to the *Rhodanobacteraceae* family was 7–18 times more abundant in the control reactors than in the reactors receiving PAA in the first part of the experiment (III). Furthermore, *Reyranella* was 4–31 times more abundant in the control reactors in the second part of the experiment (III). The *Rhodanobacteraceae* family is categorised as generalist carbohydrate degraders (Gutierrez 2019), and the *Reyranella species* have organic matter degrading capabilities (Zhang *et al.* 2019). PAA additions oxidised organic matter from the water, which led to lower organic matter load into the bioreactors, and this may have influenced the carbohydrate degrading bacteria. Liu *et al.* (2017) showed that continuous addition of PAA could lead to a high growth of heterotrophic bacteria, because commercial PAA products contained acetate and acetic acid,

which were readily available carbon sources for bacteria. Thus, the balance between water quality improvement and system imbalance was thin, as the different weekly dosing strategy of PAA caused oxidation of GSM, MIB, TAN, and organic matter but did not affected the bacteria (Lindholm-Lehto *et al.* 2019, III).

3.1.2 The brackish water microbiome

The majority of bacteria in the brackish water RAS belonged to the classes Alphaproteobacteria, Bacteroidia, and Gammaproteobacteria (Fig. 5, IV). When inspected in more detail, OTUs belonging to uncultured and unclassified *Saprospiraceae*, uncultured *Planctomycetales*, and uncultured *Gemmatimonadaceae* were the most abundant (Table 7). The water quality in the brackish water RAS decreased, as the TAN, NO_2, TOC, dissolved organic matter (DOM), and turbidity values were approx. 1.2 times higher compared to the freshwater RAS. As with the freshwater RAS, the microbiome in the brackish water RAS evolved continuously in the first six months of operation, yet the observed changes in the community composition were much slower (IV).

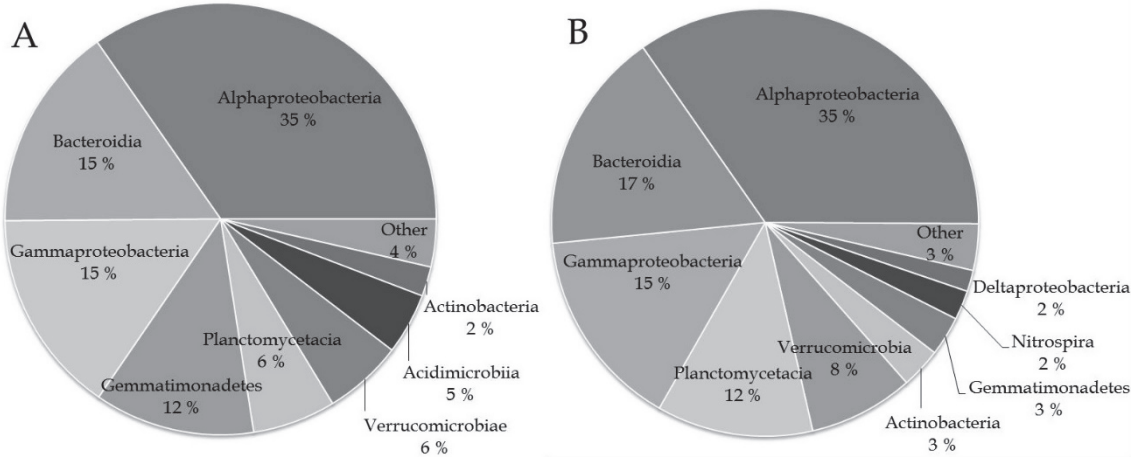

FIGURE 5 Average relative abundance of bacterial class from A) brackish water fixed bed bioreactor B) brackish water moving bed bioreactor (IV).

Saprospiraceae have been found in activated sludge systems, where they can hydrolyse amino acids (Xia *et al.* 2008). Fish faeces can contain 7–24% protein in dry matter, which depends on the source of the protein and its digestibility (Prabhu *et al.* 2019). *Saprospiraceae* were more abundant in the brackish water compared to freshwater bioreactors (IV). However, based on the functional predictions, amino acid degradation was quite similar in the freshwater and brackish water RAS, so the function was probably distributed among several bacteria species in the freshwater systems, and *Saprospiraceae* were favoured by the increased salinity (IV). This was confirmed by the functional redundancy index (FRI), which indicates the proportion of species that are capable of performing a particular function (Wemheuer *et al.* 2018). The FRI for amino acid metabolism was higher in the freshwater bioreactors compared to the brackish

water bioreactors (ANOVA, $F = 9.6$, $p < 0.01$, IV). The freshwater bioreactors thus had more species performing amino acid metabolism.

Planctomycetales belong to the phylum Planctomycetes, which include bacteria that can conduct anammox (anaerobic ammonium–oxidizing), which is direct oxidation of ammonia and nitrite into nitrogen gas (Jetten *et al.* 2009). However, *Planctomycetales* are slow-growing bacteria and cannot compete with other bacteria in high organic loads (Tal *et al.* 2006). Their high abundance may thus have been favoured by the MBBR. In addition, the abundance decreased towards the end of the experiment, because organic matter accumulated in the system (IV). Anammox-based bioreactors have been successfully operated in marine RAS (Tal *et al.* 2009), which may be a future option for removing nitrogen compounds in a saline RAS.

TABLE 7 Relative abundance of bacterial genera in the brackish water fixed and moving bed bioreactors (IV). Uncl. = unclassified bacteria genera, Uncu. = uncultured bacteria genera.

Fixed bed bioreactor		Moving bed bioreactor	
Genus	Relative abundance	Genus	Relative abundance
*Saprospiraceae*_uncu.	8.9 %	*Planctomycetales*_uncu.	6.4 %
*Gemmatimonadaceae*_uncu.	7.3 %	*Reyranella*	5.5 %
*Rhodobacteriaceae*_uncl.	5.6 %	*Saprospiraceae*_uncu.	5.1 %
*Saprospiraceae*_uncl.	4.3 %	*Saprospiraceae*_uncl.	4.8 %
Reyranella	4.0 %	*Zoogloea*	4.2 %
Luteolibacter	3.8 %	*Rhodobacteriaceae*_uncl.	3.8 %
*Burkholderiaceae*_uncl.	3.3 %	*Burkholderiaceae*_uncl.	3.3 %
Candidatus Microthrix	3.0 %	*Parvibaculum*	2.8 %
Novosphingobium	3.0 %	*Flavobacteriales*	2.6 %
Nitrosomonas	2.7 %	*Pseudohongiella*	2.4 %
Legionella	2.4 %	*Legionella*	2.3 %

The abundance of *Rhodobacteriaceae* was lower in the brackish water RAS than in the freshwater RAS. Bacteria in the genus *Reyrenella* can have similar organic matter degradation capability (Zhang *et al.* 2019), and it seemed the salinity favoured it over *Rhodobacteriaceae*.

The main differences between the brackish water FBBR and MBBR were the 9–percentage point lower abundance of Gemmatimonadetes and 6-percentage point higher relative abundance of Planctomycetacia in the MBBR. The bacterial communities were significantly different between the bioreactors (PERMANOVA, pairwise test, $p = 0.02$, IV). *Gemmatimonadaceae* were more abundant in the FBBR which contained aerobic heterotrophic bacteria, known for their ability to survive in extreme conditions and their capacity to degrade polyaromatic hydrocarbons (Cecotti *et al.* 2018). Furthermore, bacteria in the order Gemmatimonadales may be important for phosphorus removal in bioreactors (Wang *et al.* 2009), which may be beneficial features for nutrient

removal in RAS. The solid accumulation in the FBBR may have favoured the high abundance of these bacteria. The genera *Zoogloea* include bacteria with floc-forming capability and thus affect the bulking control, COD removal, and biomass formation in activated sludge (Ahn *et al.* 1996). The high abundance in the MBBR was therefore unexpected.

In contrast to the freshwater system, the trickling filter harboured a different microbiome than the MBBR (PERMANOVA, pair-wise test, $p < 0.01$) but not the FBBR (PERMANOVA, pair-wise test, $p = 0.22$) (IV). The major difference was the higher abundance of *Luteolibacter* and *Persicitalea*, and lower abundance of uncultured *Planctomycetales* and *Zoogloea* in the TF compared to the MBBR (IV). *Luteolibacter* is found in activated sludge systems and biofilms on plastics, yet its functional role is unknown (Miao *et al.* 2019). *Persicitalea* play an important role in the decomposing of organic matter (Wang *et al.* 2018), so the appearance in well-aerated TF was unexpected. However, the increased salinity caused a strong accumulation of solids in the different surfaces of the RAS, which was most pronounced in the TF (IV). Solids tend to accumulate on surfaces in saline environments because of the higher electrochemical charges (Reid *et al.* 2006). The predicted functional analysis revealed that most of the metabolic functions were supressed in the TF. In addition, species richness and FRI were lower in the TF than in the bioreactors, so the accumulation of solids in the TF decreased the microbiome's functional capacity.

Altogether, the microbiome in the bioreactors was adapted to degrade fish faeces, which contain carbon, amino acids, and lipids (Table 8). In addition, the bacteria associated to nitrogen metabolism had important role in the bioreactors.

TABLE 8 Some of the important functions of the RAS bioreactors and bacteria associated to those.

Function	Important family or genera
Ammonia-oxidation	*Nitrosomonas, Nitrospira*
Nitrite-oxidation	*Nitrospira, Candidatus Nitrotoga*
Carbohydrate degradation	*Rhodobacteriaceae, Reyranella*
Amino acids degradation	*Saprospiraceae*
Lipid degradation	*Burkholderiaceae*
Degradation of xenobiotics and complex hydrocarbons	*Novosphingobium, Mycobacterium*
Phosphorus removal	*Gemmatimonadaceae*

3.2 Nitrification

3.2.1 Nitrifying genera

The most abundant OTU from the ammonia-oxidizing bacteria belonged to the genus *Nitrosomonas,* in the Betaproteobacteria class in the FBBR (III, IV) and

MBBR (IV). However, *Nitrosomonas* and other AOB from the *Nitrosomonadacea* family accounted for only 0.46% of the total bacterial numbers in the freshwater bioreactors (IV) when estimated from the share of rRNA gene sequences. In the brackish water RAS, they consisted of 1.84 % of the total bacterial numbers (IV) in the bioreactors. *Candidatus* Nitrotoga (IV) and *Nitrospira* (III, IV) were the most abundant nitrite-oxidizing bacteria, and they consisted of 0.86 % and 1.12 % respectively of the total bacterial sequences in the freshwater RAS bioreactors (IV). *Ca* Nitrotoga and *Nitrospira* consisted only of 0.26 % and 0.85 % respectively of the total bacterial sequences in the brackish water RAS bioreactors (IV). Bacteria in *Nitrospira* genera are classified as an NOB, but they can have very versatile functions. They can degrade urea to ammonia and even grow with H_2 as a sole energy source (Daims *et al.* 2016). *Nitrospira* can play an important role in fish nitrogen waste cycling, because urea may be 12–13 % of the total dissolved nitrogen excreted by fish (Dalsgaard *et al.* 2015a). In addition, *Ca* Nitrotoga have been identified as harbouring complex functions, because they can oxidase hydrogen and sulphide (Kitzinger *et al.* 2018).

The AOB/NOB ratio was 0.2 in the freshwater RAS, and 1.7 in the brackish water RAS. According to thermodynamics and electron transfer, the AOB/NOB ratio should be 2, because the NOB generate only 2 electrons in the nitrite oxidation, whereas in AOB metabolism, 4 electrons are available in energy generation (Winkler *et al.* 2012). However, taking the detection of comammox pathway and versatile functions of the NOB into account, interpreting the AOB/NOB ratio can be complicated. Furthermore, a different expression of nitrifying genes may affect the final abundance of the nitrifying proteins. In inspecting the gene abundance and excluding the comammox gene, the *amo*A/*nxr*A+B ratio drifted from 4.7 to 1.7 during the experiment (control group, III), closer to the expected ratio at the end of the experiment (Fig. 6) in the freshwater RAS. Based on the qPCR data and lack of AOB in the bioreactors, comammox plays an important role in the freshwater RAS nitrification process. This may be beneficial for the overall process, because theoretically, nitrite would not be excreted into the water. This may be an important feature in freshwater environments where chloride ions are not present to protect the fish against nitrite poisoning (Lewis and Morris 2011). The AOB/NOB ratio was closer to what was expected, and the abundance of *Nitrospira* was low in the brackish water RAS. Thus, there the comammox may not be as important as in the freshwater RAS. However, the *coma*A gene abundance in the brackish water bioreactors was not inspected, but the other research suggests that comammox may not be abundant in saline environments (Xia *et al.* 2018).

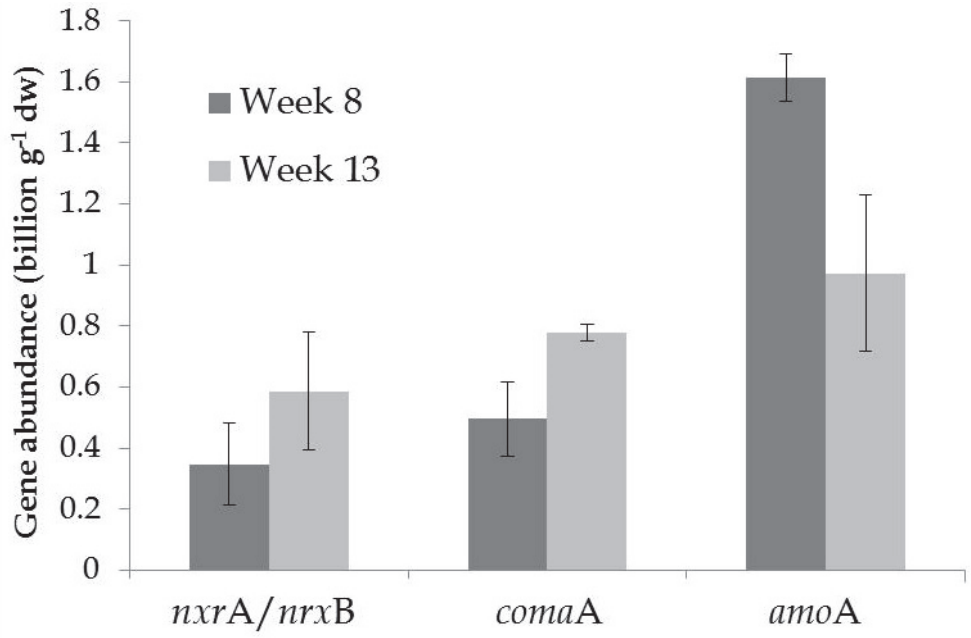

FIGURE 6 Average abundance (± SD) of the nitrite oxidoreductase (*nxr*A/*nxr*B), comammox ammonia monooxidase (*coma*A), and ammonia monooxidase (*amo*A) genes in the freshwater fixed bed bioreactors in weeks 8 and 13 (III).

The relative abundances of AOB and NOB in the brackish water FBBR and MBBR suggest some kind of compartmentalisation, because AOB were abundant in the FBBR, and NOB in the MBBR (IV). Some kind of compartmentalisation was also observed in the freshwater RAS, although the NOB or comammox (*Nitrospira*) dominated the FBBR, and NOB (*Ca* Nitrotoga) the MBBR (IV). Similar compartmentalisation was observed in a flow-through activated sludge system, where ammonia was oxidised in the first two reactors, and nitrite in the third reactor (Holben *et al.* 1998). Sakami *et al.* (2012) also observed that different ammonia-oxidizers were present in three different bioreactor tanks in the marine RAS. This compartmentalisation should be further investigated, because it may cause serious issues in a full-scale RAS when bioreactor maintenance can shut-out complete pathways, causing elevated ammonia or nitrite concentrations. Piculell *et al.* (2016) observed that a thin biofilm could disfavour NOB, so the FBBR may favour the growth of NOB. The biofilm thickness was not directly measured, but the assumption of a thicker biofilm in the FBBR is based on the dry weight of the bacterial biomass from the carrier media when undertaking DNA extraction (unpublished data).

Archaeal *amo*A was also measured with qPCR but there were no notable concentrations (III). AOA can be the major ammonia-oxidizers in marine recirculating aquaculture systems (Sakami *et al.* 2012, Brown *et al.* 2013, Huang *et al.* 2018), as well as in some freshwater systems (Bartelme *et al.* 2017). *Nitrosopumilus maritimus* can thrive in low TAN (< 0.3 mg l^{-1}) in marine RAS (Martens-Habbena *et al.* 2009, Brown *et al.* 2013), whereas in the system used in this study, TAN is usually much higher (> 0.5 mg l^{-1}) (I–IV). However, AOA *Nitrosocosmicus* spp. has been abundant in freshwater systems (Bartelme *et al.*

2017), and it is tolerant of high ammonia concentrations (Lehtovirta-Morley *et al.* 2016), so it remains unclear why AOA was not observed in the system used in this study.

3.2.2 Nitrification performance

Nitrification can be rapidly started by dosing ammonia (NH_4Cl) and nitrite ($NaNO_2$) salts into the recirculating system (I). This allows both nitrification steps (nitritation and nitratation) to start simultaneously. In dosing both of the salts 5 mg N l^{-1}, nitrification was observed to start in 23 days, compared to a simple start with fish, where nitrification started in 31 days (I). In using salts, the farmer does not need to have a high flush rate in the system to avoid ammonia toxicity in fish and fish welfare is taken account. The startup method did not improve later nitrification performance; nor did it affect the specific growth rate or feed conversion ratio of the fish (I). In addition, the hypothesis was tested that organic carbon could enhance the overall startup of the bioreactors by also activating the heterotrophic bacteria. In addition to the salts, cane sugar was dosed into the system with a C/N ratio of 5, which is a little higher than the calculated C/N ratio (2.8) of fish faeces (Wang *et al.* 2012), but lower (9.3) than in the fish feeds (Wang *et al.* 2013). It appeared that the cane sugar was too easily degrading carbon, leading to a high growth rate of heterotrophic bacteria and a total inhibition of the nitrification process. The systems were also unable to recover after the addition period. It thus caused irreversible effects to system performance (I). Cane sugar was selected, because it had a unique stable isotope profile, which was inspected in another experiment.

The nitrification rate correlated with nitrite and nitrate concentration (Fig. 7, II). The ammonia concentration mainly correlated with the fish feeding ratio. As nitrate concentration depends on the relative water renewal rate (I), nitrite concentration can be used to indirectly measure nitrification efficiency (II).

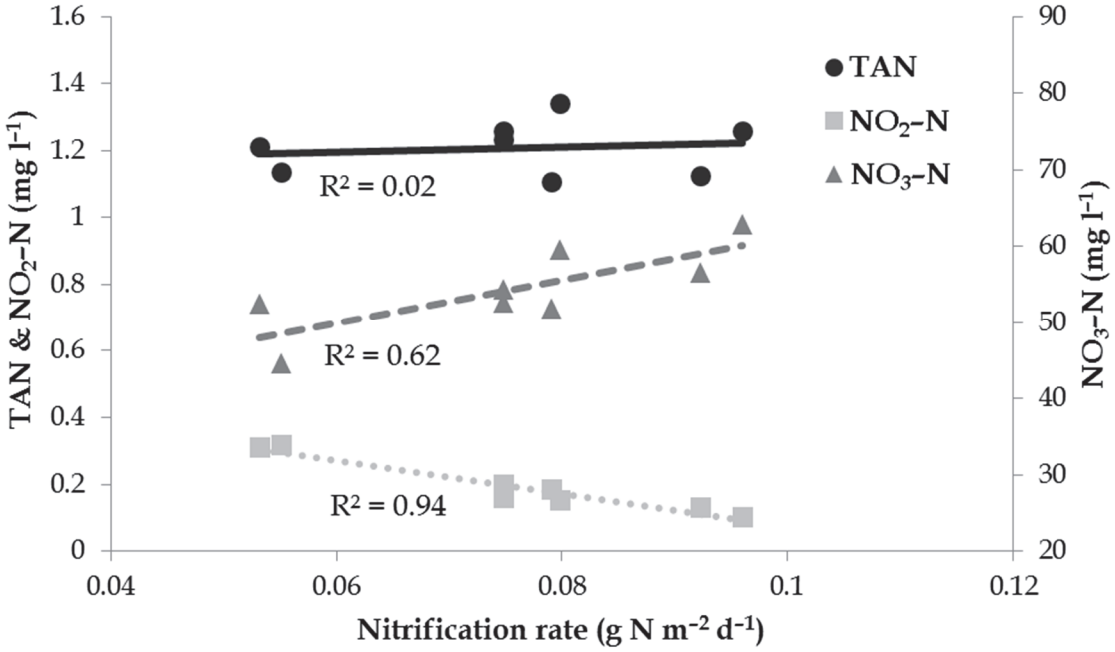

FIGURE 7 Fitted regression lines and the coefficient of determinations between the nitrification rate and total ammonia nitrogen (TAN), nitrite-nitrogen, and nitrate-nitrogen concentrations (II).

The fixed bed bioreactors had lower nitrification rates compared to the moving bed bioreactors (II), which is the opposite of what Pedersen *et al.* (2015) observed using similar carrier media. They suggested that the better nitrification in an FBBR was caused by the trapped organic material within the carrier media, which could act as activated sludge and increase the nitrification surface area. The nitrification rate was only measured from the carrier media in the laboratory, yet the elevated nitrite concentration in the water also suggested decreased nitrification in the FBBR. Prehn *et al.* (2012) observed that when water velocity through a packed bed bioreactor was increased, nitrification performance could also be increased, because the ammonia as the substrate was divided more equally within the reactor. In the system used in this study, the velocity through the carrier media was at the lower limit used by Prehn *et al.* (2012) in their experiment. Too low a velocity may therefore explain the decreased nitrification performance in this study. The design for the reactor vessel is similar in the FBBR and MBBR, but the constant mixing of the MBBR provides equal conditions inside the reactor. This highlights the importance of reactor dimensions for efficient nitrification.

The RAS intensity is determined by the feed load and flow rate of the new water, and it can be expressed as l kg^{-1} feed. The less new water is added per feed introduced, the more dissolved substances (etc. NO_3, TOC) accumulate in the system (I). The C/N ratio measured in the water should decrease when water intensity is increased, because some of the carbon is removed through the solids removal processes, whereas most of the inorganic nitrogen accumulates in the system (I). However, if only TAN production and organic carbon content in the

water are considered, the C/N ratio will increase, which may affect the nitrification rate (Michaud *et al.* 2006). In study I, the intensification of the RAS appeared to decrease the nitrification rate, based on elevated nitrite concentration (Fig. 8). Ammonia concentrations correlated with the feeding rate when the intensification increased, and the feed intake decreased (I). The elevated nitrite concentration was unexpected, but it may have been caused by the higher organic carbon content and the increased C/N ratio in the water, which decreased the nitrification rate. This phenomenon should be examined in more detail, because carbon may become a new variable for designing biofiltration capacity.

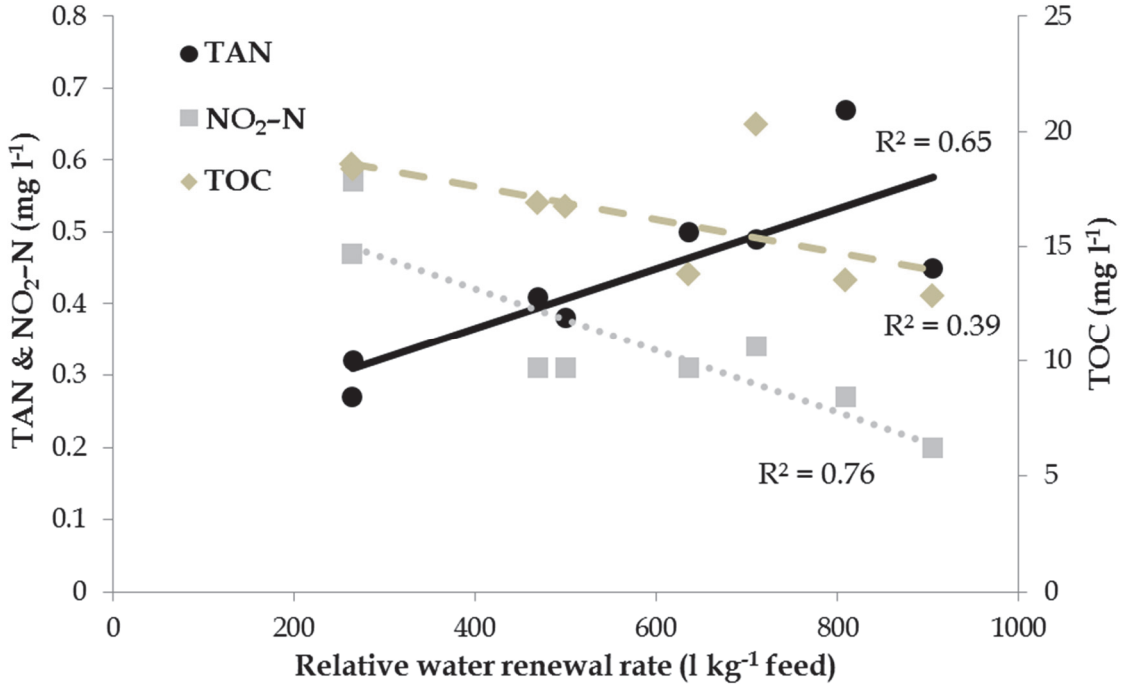

FIGURE 8 Fitted regression lines and the coefficient of determinations between the relative water renewal rate and total ammonia nitrogen (TAN), nitrite-nitrogen, and total organic carbon (TOC) (I).

4 CONCLUSIONS

As hypothesised, the use of the fixed bed bioreactor or the moving bed bioreactor influenced water quality as measured by varying concentrations of ammonia, nitrite, nitrate, organic material, and carbon dioxide. Similar observations were made with different salinity and peracetic acid applications. However, rainbow trout could adapt to different water quality, and the operating conditions did not affect fish performance. Nevertheless, operational and water quality conditions should be kept optimal for the rearing fish species in order to achieve best possible fish welfare. The following specific conclusions were made:

1. Nitrification can be rapidly started by adding ammonia and nitrite salts to the system. However, this does not increase the nitrifying performance of the bioreactors, as the bacterial communities adapt to conditions where nutrient levels become equivalent. The early adaptation of the heterotrophic bacteria community by adding organic carbon is not required, because organic load may inhibit nitrification.

2. An intensification of the recirculating system may cause an accumulation of nitrate and solids in the water, but it may also affect nitrification. Additional nitrifying capacity or organic material removal may be required if the make-up water flow decreases in relation to the feeding rate.

3. In the freshwater RAS, *Nitrospira* and *Candidatus* Nitrotoga were the most abundant nitrifying genera, while in the brackish water RAS, *Nitrosomonas* was the most abundant. *Nitrospira* with comammox potential played an important role in the nitrification in the freshwater RAS.

4. Bacteria from the family *Rhodobacteriaceae* can play a key role in organic matter degradation in bioreactors. In addition, bacteria from the family *Saprospiraceae* can degrade undigested amino acids. The family *Burkholderiaceae* is important in the bioreactors for degrading the lipids of the fish faeces.

5. Microbial communities in the freshwater and brackish water bioreactors did not stabilise in the first six months of operation, and the observed changes in the community composition were much slower in the brackish water environment.

6. The fixed bed bioreactor traps solids and organic material in the reactor, but it can also specialise the microbiome for organic matter degradation. The shearing forces of the moving bed bioreactor carrier media can release excess bacterial biomass into the water and shear solids particles. It can also keep the biofilm clean, which can also keep the microbiome functionally versatile.

7. Peracetic acid can be dosed into rearing water to chemically improve water quality, and in the long term, this will not disturb the microbiome composition, because communities are capable of adapting to operating conditions.

8. Even relatively small increase in salinity (from 0 to 7 ppt) may decrease nitrification performance, but it may also impair the potential carbohydrate, lipid, and xenobiotics metabolism.

The selection of a suitable bioreactor in the RAS is traditionally based on the potential for nitrification and the operational maintenance of the bioreactor. However, nitrification is only one of the functions bioreactors can perform. Fish faeces contains carbon, amino acids, and lipids, and the microbiome in the bioreactors is adapted to the degradation of these products. Fixed bed bioreactors enable both mechanical and biological removal of organic matter. In contrast, moving bed bioreactors release excess bacterial biomass into the water, but the lower biofilm thickness may still enable a functionally versatile microbiome. The FBBR requires periodic maintenance like backwashing, as well as more detailed reactor design, whereas the MBBR is easy to use and requires only a little maintenance. In addition, using both reactor types takes advantage of the best features. Thus, I recommend placing the fixed bed bioreactor after the moving bed bioreactor to trap the released excess biomass from the latter. Although nitrification can be started rapidly, the overall stability of the bioreactors may take longer to achieve. This should be remembered especially if saline water is going to be used in an otherwise freshwater environment. The stability of the microbiome has been shown to increase competition, which may also keep harmful bacteria under control. In addition, the biofilm should be kept operational by cleaning the excess bioaccumulation to avoid unwanted consequences.

This thesis has provided detailed information on the microbiology of the RAS bioreactors. However, the bacterial interactions were so complex that several questions remain unanswered. Recent new knowledge of complete ammonia oxidation potential of nitrite-oxidizing bacteria may bring new insights to the nitrification process in RAS, and RNA-based techniques may give better insights into the function of different bacteria. The dynamics of off-flavor producing bacteria have not yet been fully studied, although their role is important in food fish producing RAS farms. In addition, the compartmentalisation of nitrifying bacteria genera for different bioreactors may modify our understanding of nitrification and alter the design of new water treatment systems.

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"Life is like a box of chocolate, you never know what you're gonna get" said Forrest Gump. Phrase suits for this situation, because I did not either know, that I would get many sleepless nights and many frustrating hours in front of my computer, when applied in this venture. I did not either know, that the deeper I dig into this subject, the more I would fall in love with it and more happy I would be for this opportunity.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Bioreaktoreiden mikrobiologia kalojen kiertovesikasvatuksessa

Viime vuosina vesiviljelystä tuleva ravinnon määrä on ollut voimakkaasti kasvussa, ja se on jo ohittanut kalastuksesta tulevan ravinnon määrän. Se on tärkein vedestä peräisin olevan ravinnon lähde. Globaalisti vesiviljelytuotanto on myös ohittanut naudanlihan tuotannon, ja se on ollut 2000-luvulla voimakkaimmin kasvava elintarviketuotannon ala. Voimakkaalla kasvulla on myös rajansa, sillä taloudellisesti, ympäristöllisesti ja sosiaalisesti kestävät alueet kalankasvatuksessa ovat jo käytössä. Lisätuotannolle on haettu innovatiivisia tekniikoita, jotta konflikteilta vältytään kaikilla kestävän tuotannon osa-alueilla. Yksi mahdollisuus lisätä kestävästi tuotettuja kalatuotteita on kalojen kiertovesikasvatus, joka mahdollistaa ympärivuotisen kalankasvatuksen myös alueilla, joissa ei ole voinut harjoittaa perinteistä kalankasvatusta.

Kiertovesikasvatuksessa kasvatusvesi kiertää kala-altaiden ja puhdistuslaitteistojen läpi, jolloin säästyy vettä ja ravinteita voidaan kierrättää tehokkaasti. Perinteiseen kalankasvatukseen verrattuna ravinteet ovat paremmin konsentroituneina pienemmän vesimäärän takia. Vaikka kiertovesikasvatuksessa ravinteita saadaan tehokkaasti talteen, ehkäistään vieraslajien karkaamista ja kalataudit pysyvät paremmin hallinnassa, kokonaiskestävyyden kannalta energiankulutus ja tuotantotapa ovat ratkaisevassa asemassa suuren energiankulutuksen vuoksi. Kasvatusveden puhdistustekniikka koostuu pääosin mekaanisesta kiintoaineen poistosta, biologisesta typen suodatuksesta ja diffuusoreihin tai valutukseen perustuvasta kaasujen vaihdosta. Kiertovesikasvatuksen suurimpia ongelmia ovat olleet hyvän vedenlaadun ylläpito, poistoveden käsittely ja tekniikan toimivuus, jotka kaikki ovat osittain kytköksissä mikrobiologisiin prosesseihin. Mikrobiologisten prosessien parempi ymmärtäminen voi tarjota ratkaisuja prosessien kehittämiseen ja mahdollistaa entistä paremmin taloudellisen ja kestävän kalojen kiertovesikasvatuksen.

Tässä väitöskirjatyössäni tutkin kalojen kiertovesikasvatukseen liittyviä ympäristömikrobiologisia prosesseja. Työssä keskityttiin nitrifikaatioon ja muihin bioreaktoreihin liittyviin bakteeriprosesseihin. Kaikki kokeet tehtiin Luonnonvarakeskuksen Laukaan kalanviljelylaitoksen kiertovesiviljelyn kokeilu- ja oppimisympäristössä. Kiertovesilaitoksessa bioreaktoreiden tarkoitus on muuttaa kalojen aineenvaihdunnasta tuleva ammoniakki kaloille vähemmän haitalliseen nitraattiin. Tämä tapahtuu kaksivaiheisessa nitrifikaatioprosessissa, jota tekevät autotrofiset nitrifikaatiobakteerit. Vaikka bioreaktoreiden pääasiallinen tehtävä on nitrifikaatiossa, 16S rRNA -geenin sekvensoinnin perusteella havaittiin, että nitrifioivien bakteerien suhteellinen osuus oli vain noin kaksi prosenttia kaikista bioreaktoreissa olevista bakteereista. Suurin osa mikrobiyhteisöjen bakteereista kuului proteobakteereiden, bakteroidien ja aktinobakteereiden pääjaksoihin, ja näiden bakteerien toiminnallisen tarkastelun perusteella pääasialliset prosessit olivat hiilihydraattien, aminohappojen ja rasvojen hajotus.

Kun kiertovesiyksikkö ohjattiin käyttämään murtovettä, pystyivät makean veden mikrobiyhteisöt sopeutumaan uusiin olosuhteisiin, vaikka sopeutuminen oli hitaampaa verrattuna makeassa vedessä tapahtuvaan sopeutumiseen. Murtovesijärjestelmän bioreaktoreissa havaittiin hiilihydraatin ja rasvojen hajotuksen heikentyneen, mikä näkyi vedenlaadussa korkeampana orgaanisen hiilen ja orgaanisen aineen pitoisuutena. Murtovedessä myös nitrifikaatio oli heikentynyt, sillä ammonium- ja nitriittipitoisuudet olivat korkeampia verrattuna makean veden yksikköön.

Kiinteä- ja liikkuvapetisten bioreaktoreiden käyttö on yleistynyt maailmalla, joten keskityin työssäni kyseisten reaktoreiden eroavaisuuksien tarkasteluun. Kiinteäpetisissä bioreaktoreissa biofilmin alustana toimiva kantoaine on reaktorissa liikkumattomana, kun taas liikkuvapetisissä reaktoreissa kantoainetta sekoitetaan yleensä ilman avulla. Kiinteäpetiset reaktorit pystyivät sieppaamaan vedestä kiintoainetta, mutta myös biofilmin mikrobiomi oli erikoistunut orgaanisen aineksen hajottamiseen. Liikkuvapetisissä reaktoreissa ylimääräinen bakteerimassa vapautui veteen, mutta reaktori voi myös jauhaa liikkeellään vedessä olevaa kiintoainetta pienemmiksi kappaleiksi. Liikkuvapetisissä reaktoreissa kantoaineen biofilmi pysyi ohuena, mutta se pystyi silti ylläpitämään monimuotoista mikrobiyhteisöä.

Tärkeimmät nitrifioivat bakteerisuvut olivat ammoniakkia hapettava *Nitrosomonas* ja nitraattia hapettavat *Nitrospira* ja *Candidatus* Nitrotoga. Joillakin *Nitrospira*-suvun bakteereilla on havaittu olevan geenejä, jotka mahdollistavat koko nitrifikaation suorittamisen samassa bakteerisolussa, eli ne voivat hapettaa ensin ammoniakin nitriitiksi ja sen jälkeen nitriitin nitraatiksi. Tämä ominaisuus oli odotettua tärkeämpi, sillä täydellisen nitrifikaation mahdollistavan geenin runsas määrä bioreaktoreissa oli samaa tasoa perinteisen ammoniakin hapettajan ja nitriitin hapettajan geenien määrien kanssa. Tämä voi osoittautua tärkeäksi ominaisuudeksi makeassa vedessä, sillä jos ammonium hapettuu täydellisesti nitrifikaatiossa, nitriittiä ei eritetä veteen. Nitriitti on kaloille erittäin myrkyllistä, eikä makeassa vedessä ole kaloja nitriittimyrkytykseltä suojelevia kloridi-ioneja.

Nitrifikaatiotehokkuus vaihteli eri bioreaktorityyppien välillä, ja oli parempi liikkuvapetisissä bioreaktoreissa. Tällaisessa reaktorissa veden sekoittuminen mahdollistaa ammoniumina toimivan substraatin ja hapen hyvän sekoittumisen kaikkialle kantoaineeseen. Kiinteäpetisen reaktorin heikompi nitrifikaatiotehokkuus saattoi johtua reaktorin suunnitteluviasta, sillä kantoaineen läpi kulkevan veden virtausnopeus oli liian vähäinen substraatin ja hapen kulkeutumisen kannalta. Tällöin saattoi esiintyä oikovirtauksia, jotka saattoivat vähentää tehokasta reaktoritilavuutta. Reaktoreita suunniteltaessa ja mitoitettaessa on hyvä ottaa huomioon nitrifikaatiotehokkuuden lisäksi myös reaktorin mittasuhteet.

Kiinteäpetinen bioreaktori vaatii säännöllistä kiintoaineen huuhtelua ja reaktorin yksityiskohtaisempaa suunnittelua verrattuna liikkuvapetiseen reaktoriin, joka vaatii vähemmän huoltoa. Käyttämällä kumpaakin reaktorityyppiä voidaan hyödyntää molempien reaktoreiden parhaita ominaisuuksia, mutta suosittelen kiinteäpetisen reaktorin sijoittamista liikkuvapetisen reaktorin jälkeen, jotta vapautuva kiintoaine ja biomassa voidaan ottaa talteen. Nitrifikaatio voi-

daan käynnistää nopeasti eri kemikaaliannosteluilla, mutta bioreaktorien mikrobiologisen stabiiliuden saavuttaminen voi viedä pidemmän ajan. Tämä on hyvä tiedostaa kiertovesilaitoksen käynnistysvaiheessa, etenkin jos suolavettä aiotaan käyttää muuten makean veden ympäristössä. Lisäksi kantoaineiden ja reunustojen biofilmi on hyvä pitää puhtaana kiintoaineesta, joka voi haitata bakteeriyhteisöjen toimintaa. Bakteeriyhteisöjen stabiloituminen ylläpitää bakteerien välistä kilpailua, minkä on todettu pitävän haitalliset bakteerit hallinnassa. Stabiilit olosuhteet kalojen kiertovesikasvatuksessa mahdollistavat tehokkaan ja ympäristöystävällisen kalankasvatuksen.

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ORIGINAL PAPERS

I

STARTUP AND EFFECTS OF RELATIVE WATER RENEWAL RATE ON WATER QUALITY AND GROWTH OF RAINBOW TROUT (ONCORHYNCHUS MYKISS) IN A UNIQUE RAS RESEARCH PLATFORM

by

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Startup and effects of relative water renewal rate on water quality and growth of rainbow trout (*Oncorhynchus mykiss*) in a unique RAS research platform

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ABSTRACT

The aquaculture industry is growing fast but facing two major challenges: a shortage of suitable locations for growth and the need to reduce environmental impacts. One solution for both these challenges is inland production through recirculating aquaculture systems (RAS). The RAS technique is rather new, and several practical issues need to be solved. In this study, an experimental platform, consisting of ten individual RAS units, was built for small-scale testing of different RAS designs and operation methods, and two preliminary experiments were conducted. In the first experiment, the capability of different chemical additions (sodium nitrite, ammonium chloride and/or cane sugar) to fasten the startup of the nitrification bioreactor was tested. In addition, the suitability and reliability of an online water measurement system in monitoring nitrification process with was evaluated. We demonstrated that when using a combination of sodium nitrite and ammonium chloride in a concentration of 5 mg l^{-1}, nitrification started one week before than when using only ammonium chloride or a clean start with rainbow trout (*Oncorhynchus mykiss*). In the second experiment, the effect of different relative water renewal rates (RWR) on water quality, rainbow trout growth and feed conversion ratio (FCR) were examined at 16 °C. Based on the results, FCR increased when RWR went below 478 l kg^{-1}, and the specific growth rate decreased when RWR went below 514 l kg^{-1}. Furthermore, when RWR decreased, nitrate, nitrite and organic material accumulated in the circulating water. In conclusion, we showed using experimental RAS platform that online water quality monitoring is a useful tool in following the effect of different management practices. Furthermore, we demonstrated that chemical substrate additions provide the fastest biofilter startup, and that water management is still in the key role in defining the fish production in RAS.

1. Introduction

The Finnish aquaculture sector has been in decline since the beginning of the 1990 s (Official Statistics of Finland, 2017), mainly because of the strict environmental regulations concerning nutrient discharges. To meet the strict wastewater regulations regarding phosphorus and nitrogen, novel methods for increasing aquaculture production and value are needed. Recirculating aquaculture systems (RAS) uses, and thus discharges, less water, which can be treated efficiently and economically compared to traditional flow-through and net cage aquaculture systems (Martins et al., 2010).

Although the principles of RAS techniques are a few decades old (Bohl, 1977; Rogers and Klemetson, 1985), the technique has shown clear signs of expanding to a commercial scale during the last decade (Sturrock et al., 2008; Bergheim et al., 2009; Dalsgaard et al., 2013a). In Finland, recirculating systems have been used in aquaculture for more than 10 years, but companies have had economic difficulties mostly due to technical problems. However, the two largest RAS farms have a projected capacity at total of some 4500 tons, and thus RAS is developing to be a potential technology alongside more traditional forms of aquaculture.

The various challenges of RAS technology have been well covered in the recent scientific literature (Badiola et al., 2012; Klinger and Naylor, 2012) and in the symposiums (e.g. in NORDIC RAS workshops: Dalsgaard et al., 2013b, 2015/, 2017). One central problem is the difficulty to manage solids production and biofilter function to maintain a

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sufficiently high water quality in RAS (Badiola et al., 2012). Another important issue to be better understood is the RAS microbiology. For example, the interactions between beneficial and harmful bacteria are still poorly known (Rurangwa and Verdegem, 2015). To meet these challenges in the RAS sector, experimental scale units, where different operational designs can be scientifically tested, are needed. In this study, the capacity and various functions of the experimental platform with were designed in a project group consisting of Finnish RAS farmers, technology suppliers and academia. The objective was to include similar water treatment units in the experimental system as used in typical modern RAS farms. Special attention was paid to the ability to adjust water treatment for different trials, online water quality measurements and accurate make-up water flow adjustment.

This study gives an overview of the research platform, its design and system startup. The functioning of the system and online water monitoring system were tested in two experiments. The aim of the first experiment was to examine the startup of nitrification by using chemical additions, and the determination of nitrification by the online monitoring system. The startup phase of the biofilter is considered to be long, e.g. 40 days in shrimp RAS (Perfettini and Bianchi, 1990), and it can be shortened by using inoculates of nitrification bacteria (Grommen et al., 2002; Kuhn et al., 2010). However, external bacteria load might be harmful, and the community composition of the bacterial inoculates might differ from the one of the natural RAS bacterial population, thus an alternative strategies for enhancing the startup of the nitrification process are needed. Here, we tested how the addition of high concentrations of nitrification substrates (ammonia and/or nitrite) will affect biofilter startup. In addition, we tested whether organic carbon addition in the startup phase was beneficial to microbial communities, since it could facilitate the biofilter adaptation to the high organic carbon loading released from feed and faeces after the fish introduction. In the second experiment, the aim was to investigate the effect of relative water renewal rates (RWR) on water quality, fish growth and feed-conversion ratio. The intensity of RAS is often described by how much fresh water is being introduced per feed used, which in turn will influence nitrate and solid accumulation in the system (Timmons and Ebeling, 2013). We hypothesized that under lowest water renewal rates, harmful substances such as nitrate and organic load would have negative effects on fish performance. Furthermore, these two experiments provided information about the operation of the water treatment and online water quality measurement system under different operational options.

2. Material and methods

2.1. RAS design

The RAS platform was built by Arvo-Tec Oy (Joroinen, Finland) in the existing research hall at the Laukaa fish farm of Natural Resources Institute Finland (Luke) (Fig. 1). The system consists of 10 individual units, each having an individual water treatment system and water quality monitoring system. In Table 1, the dimensioning of the water treatment units to remove TAN and CO_2 is provided.

Each unit consists of a bottom-drained rearing tank, solid removal systems, biological filtration systems and an aeration system. Each water treatment step can be by-passed and, therefore, the experimental setup can be varied. Each unit consists of (in water circulating order): a 500 l square plastic rearing tank (ArvoTec, Joroinen, Finland), feed collector unit, 24 cm diameter (hydraulic loading 133-531 l min^{-1} m^{-2}) swirl separator (Eco-Trap Collector1, Pentair Aquatic Eco-Systems, Minneapolis, USA), drum filter with 60 µm filter panels (Hydrotech HDF501, Veolia, Paris, France), four separate 147 l bioreactor tanks, 0.8 m high trickling filter and 70 l pump sump (Fig. 2).

Water is pumped from the pump sump with a recirculating pump (Magna 3, Grundfos, Bjerringbro, Denmark) to the bottom-drained fish tank. Recirculating water pH is adjusted by single channel control unit (Dulcometer, ProMinent, Heidelberg, Germany) using pH-probe located in the pump sump and low-pressure metering pump (Beta b, ProMinent, Heidelberg, Germany) dosing diluted sodium hydroxide to the trickling filter prior to the pump sump.

Every fish tank has a galvanic oxygen probe (OxyGuard, Farum, Denmark) which is used with the monitoring system (Atlantic, OxyGuard, Farum, Denmark) to control the emergency oxygen diffuser located at the bottom of the tank. Emergency oxygen turns on in power failure situations and when oxygen saturation is below the adjusted set point level. Pure oxygen is added to the pump sump via ceramic diffusers to supersaturate the tank inlet water. Oxygen saturation in fish tanks is manually adjusted with constant flow regulator (Model 2851, Kytola® instruments Oy, Muurame, Finland).

The trickling filter is a forced-ventilated cascade aeration column designed to remove CO_2 from the circulating water. Filters are filled with Bio-Blok® 200 filter medium (EXPO-NET Danmark A/S, Hjørring, Denmark). Each trickling filter has a channel blower (Onnline CK 100 A, Onninen, Vantaa, Finland) on top of the filter, and the blower can be activated using a carbon dioxide set-point value. The blower is also connected to a seven-point thyristor controller for adjusting blowing speed.

Fish are fed with a commercial feeding system (T Drum 2000, Arvo-Tec, Joroinen, Finland). The tank light system is located in the tank cover. The cover is made from a special machined acrylic panel, which reflects LED light cast from the side of the panel (Aicci, Muurame, Finland). Light intensity and photoperiod can be adjusted.

Make-up water is pumped from a 600 l plastic tank with a water dosing pump (DDI-222, Grundfos, Bjerringbro, Denmark). In the RAS units, make-up water is added to the feed collector units. Make-up water alkalinity can be increased by adding sodium bicarbonate to the make-up water storage tank with an automated belt feeder. Inlet water is disinfected with UV light (Duv 01 A, Lit, Moscow, Russia) and it can be filtered with a string-wound cartridge (housing: Shelco RHS, Charlotte, USA, cartridge: MS10FP3, Shelco, Charlotte, USA) and carbon block filter (5FOS, Shelco, Charlotte, USA).

Laukaa fish farm's water source is lake Peurunkajärvi (62.44886, 25.85201), located 8 m above the farm. Farm uses water from two water depths (10 m hypolimnion and 4 m surface water) (Table 2). Water temperature can be adjusted by mixing the two inlet water sources. Water can be also heated or cooled with heat-exchangers and a heat-pump (30 HM-065, Carrier, Farmington, USA). In RAS units, water temperature is adjusted by controlling the air temperature by air conditioning system.

2.2. Data collection

Each RAS unit has a separate online water quality measurement system. Each fish tank has a spectrometer probe (spectro::lyser, s::can, Vienna, Austria), carbon dioxide sensor (Franatech, Lüneburg, Germany), pH probe (pH::lyser, s::can, Vienna, Austria) and optical oxygen probe (oxi::lyser, s::can, Vienna, Austria). The spectrometer probe measures water nitrate, nitrite, turbidity, total suspended solids, total organic carbon and UV 254 absorbance. Recirculating water flow is measured with a flow sensor (type 8012, Bürkert, Ingelfingen, Germany) located in the tank inlet water pipe. All data is collected at industrial on-line computer (con::cube, s::can, Vienna, Austria), which can be adjusted to take measurements at desired intervals. Computers can be accessed remotely over a VPN.

Drum filters are connected to a digital time counter (H7ET, Omron, Osaka, Japan), which calculates the operating time of the backwash. Time counter data is registered manually. Backwash water intake is located in the pump sump.

2.3. Nitrification bioreactor startup

Effects of different chemical additions for enhancing biofiltration

Fig. 1. RAS platform built in Natural Resources Institute Finland (Luke) Laukaa fish farm.

Table 1
Maximum capacity of RAS unit and dimensioning water treatment units.

Characteristics	Value	Unit	Reference
RAS unit (n = 10)			
Total system volume	1140	l	
Tank volume	500	l	
Rearing density	100	kg m^{-3}	
Feed loading	1	kg d^{-1}	
TAN removal needed[a]	42	g	Timmons and Ebeling (2013)
Make-up flow	0.2–150	l h^{-1}	
Carbon dioxide production[b]	622	g d^{-1}	Forsberg (1997), Summerfelt and Sharrer (2004)
Reuse flow	6–24	l min^{-1}	
Tank hydraulic retention time	21–83	min	
Bioreactor (n = 4)			
Bioreactor volume	147	l	
Carrier media volume[c]	74-110	l	
Carried media area	750	m^2 m^{-3}	RK Bioelements
Total active surface area	56-83	m^2	
TAN removal efficiency	0.3	g TAN m^{-2} d^{-1}	Supplier, Rusten et al. (2006)
Total TAN removal capacity	17-25	g	
Moving bed bioreactor air flow	15	l min^{-1}	
Trickling filter			
Packing media height	82	cm	
Packing media volume	64	l	
Packing media surface area	200	m^2 m^{-3}	Bio-Blok®200
Hydraulic loading rate	1.3-5.1	kg m^{-2} s^{-1}	
Gas/Liquid ratio[d]	0-630		

[a] Calculated with average protein content of 45% in fish feed.
[b] Biofiltration included.
[c] Maximum filling capacity for fixed bed bioreactor 75% and moving bed bioreactor 50% (Supplier).
[d] Variable speed channel blower for air supply, maximum air flow capacity 230 m^3 h^{-1} (the back pressure excluded).

activation were investigated as the first trial. Four different treatments were used: A) clean start with fish, B) addition of ammonium chloride, C) addition of ammonium chloride and sodium nitrite, and D) addition of ammonium chloride, sodium nitrite and cane sugar. The experiment was done in 8 RAS units, two replicate units per treatment. Prior to the experiment, all the systems were disinfected using a high sodium hydroxide concentration (pH 12) for two weeks.

For each RAS unit, two bioreactors were used: one fixed bed reactor (FBBR) and one moving bed reactor (MBBR), each filled with 75 litres of carrier media (RK Bioelements heavy in FBBR and medium in MBBR, Skive, Denmark). Sodium bicarbonate was added to the make-up water to maintain a pH of 7.4 in treatments B, C and D. In treatment A, the pH level was adjusted with sodium hydroxide to 7.2. Constant light was used in the fish tanks. Water temperature was adjusted to 16 °C and oxygen saturation in fish tank was kept over 80%.

During the first phase, ammonium chloride and sodium nitrite were added to the RAS system three times a week to increase water ammonia-nitrogen and nitrite-nitrogen concentrations to 5 mg l^{-1}. Cane sugar was added to the systems also three times a week to increase total organic carbon to 20 mg l^{-1}. In treatment A, 67 (average weight 596 g) 2 + rainbow trout (*Oncorhynchus mykiss*) (originating from the national JALO-selective breeding program, cultivated at Laukaa fish farm) were divided into two RAS units. The fish were fed 0.5% bw $^{-1}$ a day of commercial feed (Hercules 5 mm, Raisioagro, Raisio, Finland, chemical composition given by the manufacturer: crude protein 40%, crude fat 30%). Feeding was divided into approx. 12 feedings during 24 h and continuous illumination was used. High make-up water flow (20 l h^{-1}) was used in all systems so that the ammonium concentration in the treatment A was approx. 5 mg l^{-1}.

The second phase started five weeks after the beginning of the experiment. A total biomass of 19.2 kg of rainbow trout (average weight 580 g) was introduced in all 8 RAS units, and feeding 1.0% bw $^{-1}$ a day was started using the same feed and frequency as during the startup. The duration of the second phase was four weeks. The pH level was adjusted by sodium hydroxide to 7.2.

Nitrification startup was monitored with an online spectrometer (spectro::lyser, s::can, Vienna, Austria). Nitrite and total ammonia nitrogen (TAN) concentrations were measured three times per week prior to the addition of chemicals with a spectrophotometer (Hach method 8038, LCK342, and LCK341 with DS 3900, Hach Lange, Loveland, USA). Nitrate was measured with spectrophotometer, when nitrification was observed and in the second phase once a week (LCK 340). Nitrate values measured with spectrometer were calibrated using values measured with Hach wet chemistry.

To measure the nitrification rate (g NO_x h^{-1}), two sets of laboratory incubations were conducted, taking place after the activation period (March 16th 2016) and the second 25 days after the introduction of fish (April 5th 2016). For the incubations, water from the incoming flow

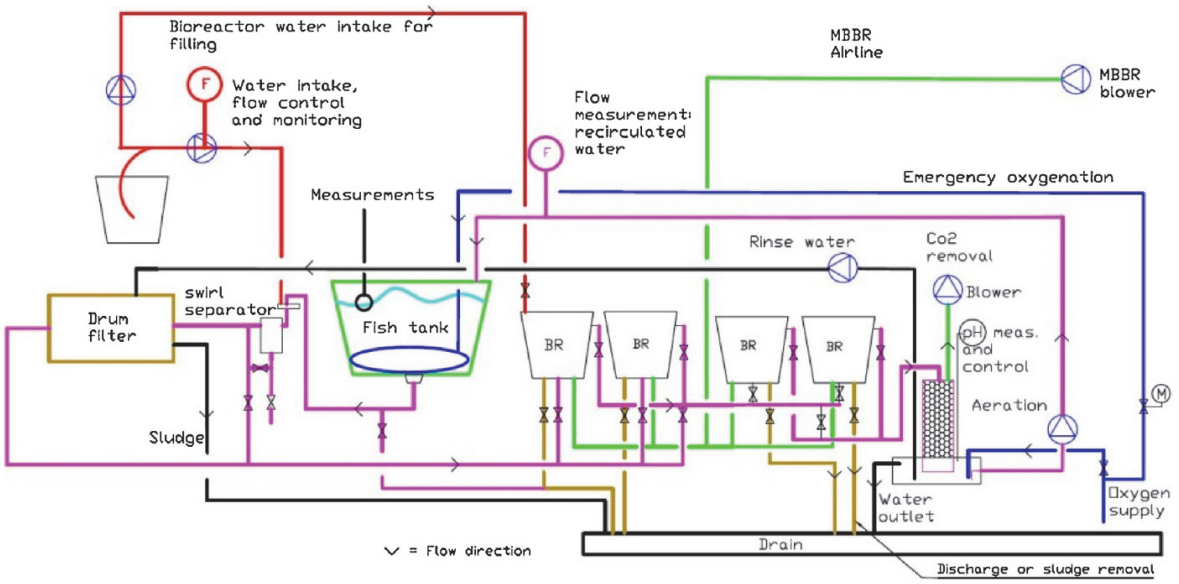

Fig. 2. Schematic diagram of one recirculating aquaculture unit in Laukaa RAS-platform.

and carrier media were collected from each moving bed tank and transferred the University of Jyväskylä. In the laboratory, carrier media were divided into experimental vials (n = 30 per vial) with 360 ml inflow water, where $^{15}NH_4^+$ was added (final concentration of 7.6 mg/L). There were two replicate vials for each moving bed pool. To ensure complete nitrification, carrier media were incubated for 3 h in the first incubation trial and for 4.5 h in the second at *in situ* temperature and under constant mixing by magnetic stirring bars. Ammonium and nitrate concentrations were followed using ion-selective electrodes (Vernier Software & Technology, Beaverton, OR, US). To measure the stable isotope composition of nitrite and nitrate, water samples were taken twice: at the beginning of the experiment, and after the ammonium peak had levelled off and concentration was close to 0 mg/L

The nitrite and nitrate were chemically converted to nitrous oxide following Miranda et al. (2001) and McIlvin and Altabet (2005), and the isotopic composition and concentration of nitrous oxide was measured with an Isoprime100 isotope-ratio mass spectrometer coupled to a TraceGas Pre-Concentration Unit (Isoprime Ltd, Cheadle, England). Differences in the nitrification activity between the treatments at the fish introduction and three weeks after the addition of the fish were studied using the Kruskal –Wallis test with pairwise comparisons, as normality assumptions were not met.

2.4. Relative water renewal rate

In the second trial, different relative water renewal rates and their effects on water quality, fish growth and feed efficiency were investigated. Four different RWR were used: 270, 490, 670 and 860 l kg^{-1} feed in duplicated RAS units.

Again, each unit had one FBBR followed by one MBBR, but there were 70 litres of carrier media in each bioreactor. Sodium bicarbonate was added 50 g per day in the make-up water tank to increase alkalinity to 20 mg l^{-1} ($CaCO_3$). The pH of the systems was adjusted by sodium hydroxide to maintain pH 7.2 in the pump sump. Constant light was used in all tanks. Water temperature was adjusted to 16 °C and oxygen saturation in fish tank was kept over 80%.

A total biomass of 29.3 kg rainbow trout (average weight 867 g, same origin as in experiment 1) was introduced in all of the 8 units. Fish were fed 1.0% bw a day with a commercial feed (Hercules 7 mm, Raisioagro, Raisio, Finland, chemical composition given by the manufacturer: crude protein 36%, crude fat 35%). Feed consumption was monitored during the weekdays and feeding was reduced if appetite decreased. The experiment lasted for 48 days and fish were weighed once in the middle of the experiment. Nitrate, nitrite and total ammonia nitrogen (TAN) concentrations were measured two times per week as in experiment 1.

Average water quality values were calculated from the last week of the experiment, when systems were considered to be on steady-state

Table 2
Water quality parameters from lake Peurunkajärvi (62.44567, 25.85161) (Water quality of surface waters, SYKE and ELY Centres).

Time	Unit	17.3.2016		5.7.2016		18.8.2016		17.10.2016	
Depth	m	5	10	5	10	5	10	5	10
Temperature	°C	2.0	2.3	17.7	9.8	16.9	10.7	8.3	7.0
Oxygen	mg/l	11.8	11.4	8.6	8.5	8.7	6.2	9.8	9.7
Oxygen saturation	%	85	83	90	75	90	56	83	82
Turbidity	FNU	0.40	0.36	0.91	0.69	1.40	2.00	0.85	0.85
Conductivity	mS/m	4.2	4.3	4.0	4.1	3.7	3.9	4.2	4.2
Alkalinity	mmol/l	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
pH		7.0	7.0	7.3	6.8	7.2	6.8	7.1	7.0
Colour	mg/l Pt	30	30	40	40	35	35	35	35
Total nitrogen	µg/l	360	380	310	360	280	370	310	310
Total phosphorus	µg/l	4.6	5.0	13.0	5.8	7.8	6.8	7.6	7.6
Iron	µg/l	57	72	110	88	86	78	83	66
CODMn	mg/l	6.8	7.2	7.4	7.4	7.0	6.8	7.4	7.4

condition. To analyse the breakpoint value of RWR, a broken-line test was performed by the SYSTAT non-linear regression procedure. The broken-line model was as follows: A + B × RWR × (RWR < BP) + B × BP × (RWR > BP), where A and B are constant, BP is the breakpoint and RWR is the relative water renewal rate l make-up water kg feed^{-1}. Specific growth rate (SGR) was calculated according to Hopkins (1992) as follows: 100 x (ln (Wt_i) – ln (Wt_0)) / (t_i-t_0), where W (t_i) is biomass at the end and W(t_0) at the beginning, t_i is time in the end of the experiment and t_0 at the beginning. The feed conversion ratio (FCR) was calculated as follows: Feed intake / (W(t_i) – W(t_0)). The amount of uneaten feed pellets was calculated on weekdays and the calculated total dry mass was subtracted from the feed intake.

3. Results and discussion

3.1. Bioreactor startup

Based on the water quality data, the fastest response in complete nitrification was observed in treatment C (added ammonium chloride and sodium nitrite) after three weeks from the start, as nitrate levels increased significantly. In treatments A (clean start with fish) and B (addition of ammonium chloride), this was seen after four weeks from the start. Treatment D (addition of external carbon with ammonium chloride and sodium nitrite) did not show any signs of nitrification (Fig. 3), as ammonium accumulated heavily after the introduction of fish and nitrate levels did not increase. Based on the stable isotope incubations, there was some nitrification activity in all treatments. Before the addition of fish, treatments A–C had a significantly higher nitrification rate than treatment D (Kruskal Wallis H-test, pairwise comparisons, P < 0.05), while treatment B was also more active than

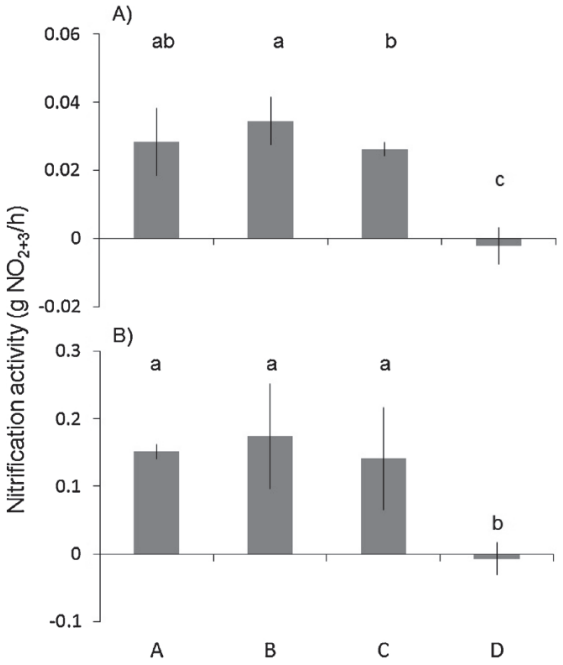

Fig. 4. The nitrification activity rates from the moving bed bioreactor (g NO_x h^{-1}) A) after activation was complete and before the fish were added and B) three weeks after addition of fish. The error bars represent the mean ± . SE. Significant differences between treatments are indicated by letters.

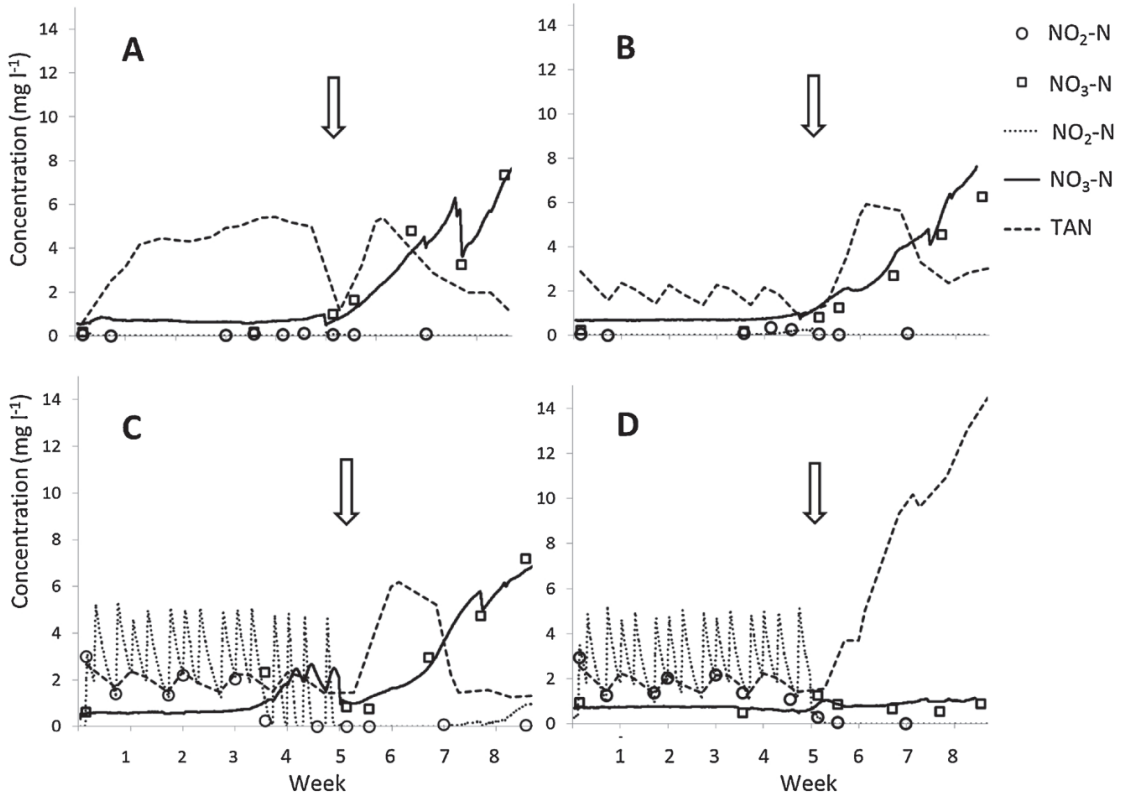

Fig. 3. Average TAN, nitrite and nitrate concentrations in four different treatment groups (n = 2 for each). A) clean start with fish, B) addition of ammonium chloride, C) addition of ammonium chloride and sodium nitrite, and D) addition of ammonium chloride, sodium nitrite and cane sugar. Arrows indicate the time when fish were introduced in all of the tanks. The lines show nitrite and nitrate concentrations from calibrated online spectrometer data and TAN concentrations measured with Hach wet chemistry three times a week (DS 3900, Hach Lange, Loveland, USA). The circles and rectangles show nitrite and nitrate data from Hach wet chemistry measurements.

treatment C (Fig. 4). However, after the introduction of fish, treatments A–C nitrified on equally high rates, while the nitrification activity was over 20 times lower, being on average negative, in treatment D (Fig. 4).

Nitrification is a two-step process, where ammonium is first oxidized to nitrite and then to nitrate (although see van Kessel et al., 2015). Both ammonia oxidizing (AOB) and nitrite oxidizing bacteria (NOB) are slow-growing, and their activity is known to be directly promoted by substrate availability (Peng and Zhu, 2006), implying that both ammonium and nitrite are needed to support fast biofilm startup. Indeed, we recorded the fastest increase in nitrate concentrations (= nitrification rates) in treatment C. Based on nitrification activity measurements, the addition of solely ammonium (treatment B) led to the highest nitrification activity. However, this could be explained by the method not distinguishing nitrate and nitrite. Treatment B probably promoted only the growth of ammonium oxiziding bacteria (AOB), and a lower biomass of the NOBs lead to nitrite accumulation, which was interpreted as higher nitrification activity. In the nitrification activity measurements done after three weeks from fish introduction, we saw equal nitrification rates and water quality in treatments A–C, suggesting that after the maturation phase, both clean start and chemical nitrogen addition were equally good startup methods for RAS. The poor nitrification performance of treatment D can be explained with high growth of heterotrophic bacteria. Nitrification is known to be decreased by water C:N, reducing by up to 70% with C:N of 4 (Michaud et al., 2006, 2014). Higher amount of organic carbon is known to support heterotrophic bacteria, which can then efficiently reduce the diffusion of nitrogenous substrate and oxygen to the autotrophic slow-growing nitrifying bacteria and outcompete them (Nogueira et al., 2002; Chen et al., 2006). Although there is some evidence that heterotrophic bacteria could protect nitrifying biofilm detachment (Furumai and Rittmann, 1994), our results strongly suggest that the addition of carbon has only deleterious effects on nitrification.

3.2. Relative water renewal rate

In week 4 and 5, the tank with RWR 500 l kg^{-1} had two breakdowns in its circulating pumps which affected water quality, feed intake and thus growth. A similar problem occurred in the tank with RWR 711 l kg^{-1} at week 6. There were differences between the treatments in some water quality parameters (Table 3), but there were considerable variance within the treatments. We noticed that nitrite and nitrate concentrations measured with spectrometer were intermingled with each other, when nitrate concentration was above 20 mg l^{-1}. Thus only Hach wet chemistry values were used. Based on the results, only nitrite and nitrate had significant differences between treatments (one-way ANOVA, $P < 0.05$). Nitrite accumulation was unexpected, and might be caused by the latter nitrification step (nitrite oxidation) being disturbed by high organic load.

Total organic carbon concentrations (TOC) measured with spectrometer was abnormally high in one tank with RWR 711 l kg^{-1} throughout the experiment. If this treatment were removed from the analysis, TOC values would have been significantly different between the treatments, being highest with the lowest RWR. Similarly, highest UV254 absorbance values were recorded with the lowest RWR. UV254 absorbance values commonly correlates with dissolved organic carbon (DOM) and especially with dissolved aromatic carbon concentration (Weishaar et al., 2003), making it a useful indicator of biological substance in water. Our results indicate that besides nitrate, also organic material was accumulating in the recirculating water when RWR was decreased. TSS and turbidity measurements were not logical between treatments. Previously, Carré et al (2017) demonstrated that a UV/VIS spectrometer is not reliable for measuring TSS or turbidity in low concentrations. Thus they calculated threshold values (TSS: 8 mg l^{-1}; turbidity: 2 NTU), above of which spectrometer can be used to estimate TSS and turbidity values. Our values were below the threshold values, indicating that there might be uncertainty when interpreted TSS and turbidity.

Based on the breakpoint analysis, the specific growth rate (SGR%) began to decline when the RWR was lower than 514 l kg^{-1} feed, and feed conversion ratio increased when RWR was lower than 478 l kg^{-1} feed. (Fig. 5). When RAS intensity is increased, the accumulation of nitrate is one limiting factor for fish growth. For example, nile tilapia (*Oreochromis niloticus*) growth was found to decline and FCR to increase at around 80–100 mg nitrate-nitrogen l^{-1} (Mota et al., 2015; Monsees et al., 2017), whereas rainbow trout has not been shown to have any responses even to high nitrate-nitrogen levels of 100-200 mg l^{-1} (Davidson et al., 2009; Pedersen et al., 2012). A maximum level of 75 mg l^{-1} nitrate has been recommended for rainbow trout (Davidson et al., 2014), at which level there is no growth effects, but some negative impacts on chronic health. Here, we found rainbow trout growth declining at much lower nitrate levels. However, reduced growth may not solely be due to accumulated nitrate, but to other water quality parameters. Recent studies with rainbow trout demonstrated the accumulation of bacteria and fine particles did not affect fish growth notably in high intensity systems (Becke et al., 2017, 2018). It might be that accumulation of organic material with combination of high nitrate load explained why growth declined in our experiment in low water change rates.

3.3. RAS platform observations

In general, the RAS research platform has proven to be a useful tool to study water treatment methods in replicated units. The overall performance and reliability of the platform has been good, but some improvement needs has been recognized during the first experiments. Problems seen in the circulating pumps resulted from air caught in the pump housing, when short-term power failure occurred. This was solved by introducing a siphon effect, so that air cannot enter into the pump housing. In small scale systems, heterotrophic growth and accumulation of solids in pipelines can change water flow and cause clogging. In our system, these problems have been partially solved by periodic pulses of pressurized air into pipelines. Also, a management protocol for the critical cleaning points has been established. Downscaling the trickling filter has proven to be difficult. Preliminary simulations with carbon dioxide control software (Timmons et al., 2002) indicated that up to 600 g of feed could be used before the tank

Table 3
Average water quality values and fish sizes (± SD) at four different water renewal rates. TAN, nitrite and nitrate values are measured with Hach wet chemistry (DS 3900, Hach Lange, Loveland, USA). UV254, TOC, TSS and turbidity are measured with spectrometer (spectro::lyser, s::can, Vienna, Austria) and CO2 with online sensor (Franatech, Lüneburg, Germany). Average water quality values are from last week of the experiment, when systems was considered to be in steady-state. Significant differences between treatments are indicated by letters.

RWR	CO_2 (mg l^{-1})	TAN (mg l^{-1})	NO_3^--N (mg l^{-1})	NO_2^--N (mg l^{-1})	UV254 (abs m^{-1})	TOC (mg l^{-1})	TSS (mg l^{-1})	Turbidity (FTU)	Start weight (kg)	End weight (kg)
270	14.5	0.30 ± 0.03	70.6 ± 9.2^{a}	0.52 ± 0.05^{a}	38.6 ± 5.6	18.5 ± 0.1	2.9 ± 1.5	1.1 ± 0.4	0.87 ± 0.01	1.26 ± 0.01
490	12.3 ± 3.5	0.40 ± 0.02	36.0 ± 10.5^{ab}	0.31 ± 0.01^{b}	31.4 ± 0.9	16.8 ± 0.1	2.9 ± 0.3	1.0 ± 0.1	0.87 ± 0.01	1.29 ± 0.2
670	14.2 ± 0.6	0.50 ± 0.01	23.8 ± 2.9^{b}	0.33 ± 0.01^{b}	27.4 ± 1.3	17.1 ± 4.6	4.9 ± 6.4	1.6 ± 1.9	0.88 ± 0.06	1.33 ± 0.04
860	16.4 ± 2.3	0.56 ± 0.11	19.2 ± 2.1^{b}	0.24 ± 0.03^{b}	25.8 ± 5.4	13.2 ± 0.5	1.1 ± 0.7	0.5 ± 0.2	0.86 ± 0.0	1.33 ± 0.0

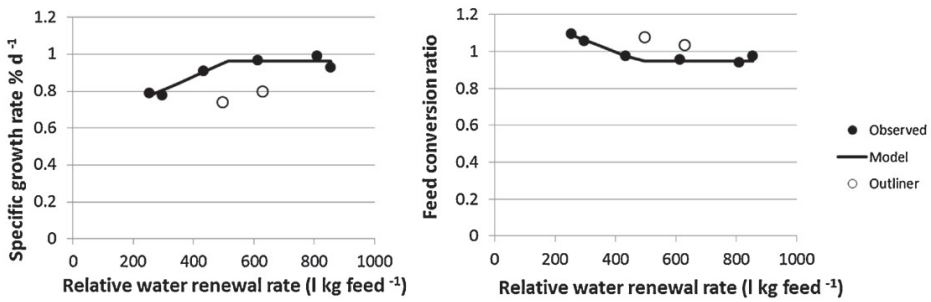

Fig. 5. The effect of relative water renewal rate on the specific growth rate, and on the feed conversion ratio of rainbow trout. The break-point value for growth rate was 514 l kg feed $^{-1}$ and for feed conversion ratio 478 l kg feed $^{-1}$. The results of individual RAS are depicting by points and the estimation of broken-line model is shown by the line. Outliners are two RAS units which had a recirculating pump failure which affected water quality and feed intake several days after the failure.

carbon dioxide concentration exceeds 15 mg L^{-1}. In practice, carbon dioxide removal has turned out to be insufficient at high feeding levels, and already 400 g per day feeding has resulted carbon dioxide levels of approx. 20 mg L^{-1}. The height of the trickling tower cannot be increased without major changes in the systems, as water flow into the trickling filter is gravity based. The first steps for carbon dioxide removal improvement will therefore be better perforated distribution plates to create uniform water distribution across the top of the column and improvement of ventilation by back pressure reduction. Also, different media in the tower may improve carbon dioxide removal. Furthermore, it has turned out that the shape of the bioreactor chambers are not optimal and low water flow velocity through filter media was found to reduce nitrification efficiency in fixed bed bioreactors.

4. Conclusions

An on-line spectrometer probe is a useful tool for continuously monitoring NO_3^-, NO_2^-, turbidity, TSS, TOC and UV transmittance in RAS. However, certain concentration thresholds for each parameter need to be achieved for reliable data. In addition, proper calibration protocols need to be introduced. To provide real-time data access and control of RAS, spectrometer data can be combined with continuous measurements using more traditional probes for dissolved oxygen, temperature, CO_2 and pH. Using RAS platform and online water quality measurements, we demonstrated that bioreactor nitrification can be rapidly started by adding a combination of ammonia and nitrite salts, and that organic carbon addition severely suppresses nitrification startup in bioreactors. Furthermore, we showed that low water renewal rates lead to nitrate and organic carbon accumulation, which can decrease rainbow trout growth, and increase feed conversion, but more research is necessary to determine precisely which parameters limit rainbow trout performance in RAS.

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II

THE EFFECTS OF DIFFERENT COMBINATIONS OF FIXED AND MOVING BED BIOREACTORS ON RAINBOW TROUT (ONCORHYNCHUS MYKISS) GROWTH AND HEALTH, WATER QUALITY AND NITRIFICATION IN RECIRCULATING AQUACULTURE SYSTEMS

by

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The effects of different combinations of fixed and moving bed bioreactors on rainbow trout (*Oncorhynchus mykiss*) growth and health, water quality and nitrification in recirculating aquaculture systems

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ABSTRACT

The effect of bioreactor design on nitrification efficiency has been well studied, but less is known about the overall impacts on water quality. Besides nitrification, submerged fixed bed bioreactors (FBBR) trap fine solid particles, whereas moving bed bioreactors (MBBR) grind solids, possibly increasing solids and particle accumulation in the system. In this experiment, the effects of different combinations of fixed bed and moving bed bioreactors on water quality, solids removal, particle size distribution, fish health based on histopathological changes and nitrification efficiency were studied in laboratory scale recirculating aquaculture systems (RAS) with rainbow trout (*Oncorhynchus mykiss*). Three set-ups with triplicate tanks were used: 1. two consecutive fixed bed bioreactors (FF); 2. a fixed bed bioreactor followed by a moving bed bioreactor (FM) and 3. two consecutive moving bed bioreactors (MM). Fish performance was not influenced by the design of the bioreactor, specific growth rate (SGR) being between 1.59 and 1.64% d^{-1} and feed conversion ratio (FCR) between 0.95 and 0.98. Water nitrite concentration was higher in the FF systems compared to FM and MM systems, whereas the average total ammonia nitrogen concentration (TAN) was not influenced by the treatments. Nitrification rate, which was measured in the laboratory, followed the water nitrite levels, indicating highest total ammonium oxidation rates in the MM systems. UV254 absorbance and total organic carbon (TOC) concentrations were higher in the groups with moving bed systems, indicating accumulation of organic substances in the circulating water. The total volume of particles was higher in the MM systems as compared to the FF systems. The total solids balance was similar in all the bioreactor groups, since the removal of solids by the FBBR backwash was compensated by the drum filter in the FM and MM systems. In general, no significant histopathological difference in gill, kidney, heart and liver tissue were observed between the RAS treatment groups and the flow-through treatment.

1. Introduction

Nitrifying bioreactor operation and management is one of the most important and complex steps in recirculating aquaculture systems (RAS) (Badiola et al., 2012; Svobodova et al., 2005). Typical RAS use so-called fixed-film bioreactors, where biofilm is formed on artificial plastic carrier media or media generated from natural substances such as sand and stones (Malone and Pfeiffer, 2006). Bacteria in the media convert toxic ammonia into less toxic nitrate in a two phase nitrification process. The nitrification process allows lower water usage rates, therefore decreasing the volume of effluents requiring the treatment before discharged into the environment. There is a wide variety of nitrifying bioreactors used in RAS, which all have particular strengths and weaknesses with no single reactor type being dominant (e.g. Timmons and Ebeling, 2013).

The nitrification capacity of the following bioreactor types has been

Abbreviations: FBBR, fixed bed bioreactor; FCR, feed conversion ratio; FF, two consecutive fixed bed bioreactors; FM, fixed bed, followed by moving bed bioreactor; LEH, lamellar epithelial cell hyperplasia; MBBR, moving bed bioreactor; MM, two consecutive moving bed bioreactors; PSD, particle size distribution; SGR, specific growth rate; TAN, total ammonia nitrogen; TGC, thermal growth coefficient; TOC, total organic carbon; TS, total solids

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widely studied: moving bed bioreactors (MBBR) (Kamstra et al., 2017), fixed bed bioreactors (FBBR) (Pedersen et al., 2015), fluidized-sand biofilters (Summerfelt, 2006), rotating biological contactor (Brazil, 2006) and trickling filters (Greiner and Timmons, 1998; Lekang and Kleppe, 2000). Besides nitrification, different bioreactor types can also have other impacts on water quality, depending on how they are designed and operated. Trickling filters, MBBRs and RBCs are constantly interacting with air, which increases the oxygen (O_2) levels and reduces carbon dioxide concentration (CO_2) (Timmons and Ebeling, 2013). However, there is very little information on how the choice of bioreactor design can affect fish health and water quality parameters.

The moving bed bioreactor was designed in Norway in the late 1980s (Rusten et al., 2006). The reactor chamber is agitated continuously with compressed air or mechanically, the carrier media being constantly moved so as to create a scrubbing effect against each other. Because of that scrubbing effect, the reactor shears solid particles, leading to the accumulation of the total amount of particles in the system (Fernandes et al., 2017). These types of reactors are easy to operate, because there is a low head loss and no need for backwashing. In addition, the constant movement enables efficient use of the whole reactor volume, and mixing with air provides oxygen for the nitrification process. Because of scrubbing, surplus microbial biomass created in the biofilm detaches from the carrier media and is later removed from the system either by outflow or in solids removal units (Ødegaard, 2006).

Fixed bed bioreactor or fixed bed biofilm reactor (FBBR) is a reactor type, where carrier media is structurally fixed in the reactor chamber (Kadic and Heindel, 2014). Depending on the fixed media type, the reactor can be susceptible to clogging and must be backwashed frequently (Schlegel and Koeser, 2007). When using small carrier media, suspended solids particles are commonly trapped in these reactors (Fernandes et al., 2017). The distribution of flow into the reactor and inside the reactor is important: turbulent flow can cause uneven distribution of substrate in the reactor and the total effective surface area for nitrification may be diminished. Turbulent flow might also create pockets, where oxygen can be depleted and hydrogen sulphide might form.

Since O_2 is added and CO_2 is removed mainly in the other compartments of RAS, the main water quality difference between FBBR and MBBR is probably the fate of solid particles in the reactor. High suspended solids loads have been reported to cause sub-lethal stress and damages to gill structure in some fish species (Au et al., 2004; Bilotta and Brazier, 2008). Thus, the amount of solid particles may influence fish health and welfare. In addition, there is a positive correlation between bacterial numbers and the surface area of particles (Pedersen et al., 2017), which may indicate that MBBR accumulates more bacteria in the circulating water than FBBR.

In this study, we compared two widely used bioreactor types: moving bed and fixed bed bioreactors. The comprehensive approach was used for comparing the effects of different bioreactor setups on ammonium removal rates, fish health in terms of histopathological lesions and growth parameters, water quality, solids accumulation and microbial dynamics. Our hypothesis was that the accumulation of solids in the circulating water causes histopathological changes and chronic stress in the fish, which affect fish growth and feed efficiency.

2. Materials and methods

2.1. Experimental setup

The experiment was carried out in the Natural Resources Institute Finland (Luke) Laukaa fish farm using an experimental RAS platform. The platform has 10 individual freshwater recirculating systems, each consisting of a 500 l bottom drained plastic rearing tank (Arvo-Tec, Joroinen, Finland), feed collector unit, 24 cm swirl separator (Eco-Trap Collector1, Pentair Aquatic Eco-Systems, Minneapolis, USA), drum filter with 60 µm filter panels (Hydrotech HDF501, Veolia, Paris, France), 2 separate 147 l bioreactor tanks (Arvo-Tec, Joroinen, Finland), trickling filter acting as a forced-ventilated cascade aeration column (Bio-Blok® 200, EXPO-NET Danmark A/S, Hjørring, Denmark) and pump sump (Fig. 1). Water pH was adjusted to 7.2 in pump sump with diluted sodium hydroxide using automated system (Prominent, Heidelberg, Germany). Sodium bicarbonate was dosed to the inlet water source to achieve an alkalinity of 50 mg l^{-1} ($CaCO_3$) in the RAS replacement water. Oxygen saturation was kept above 80% in the fish tanks. The system is described in more detail by Pulkkinen et al. (2018).

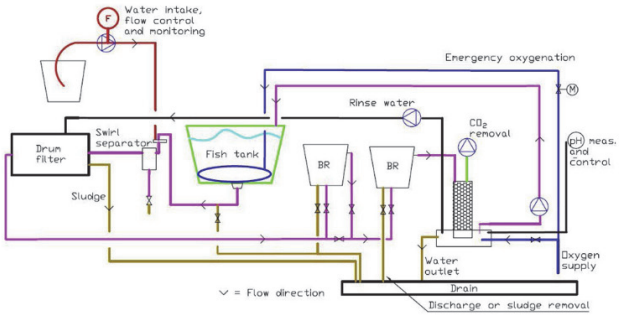

Fig. 1. Schematic diagram of one RAS unit used in this experiment. BR = bioreactor, used as a fixed bed (FBBR) or moving bed bioreactor (MBBR).

In the trial, three bioreactor setups were compared with triplicate units: Treatment 1. two consecutive fixed bed bioreactors (FF); Treatment 2. fixed bed bioreactor followed by moving bed bioreactor (FM) and Treatment 3. two consecutive moving bed bioreactors (MM). Two bioreactors per RAS unit were used, so that all units had similar amount of bioreactors. The experiment lasted 14 weeks. In one treatment group (FF), only two units existed for the second half of the experiment due to a technical failure with pH in one tank in week 8 of the experiment. A separate 500 l flow-through tank was used to grow fish of the same origin with same feed, serving as a flow-through treatment for fish histopathological sampling. Water temperature was adjusted to 16 °C by controlling the air temperature and in the flow-through group by controlling the inlet water temperature.

Similar plastic (PP) carrier media (RK Biolements heavy in fixed bed systems and medium in moving bed systems, RK Plast A/S, Skive, Denmark), tank hydraulic retention time and make-up water flow were used and measured constantly in all RAS units (Table 1). Carrier media, used in two earlier experiments, was mixed four weeks before the trial

Table 1
RAS operational design and rainbow trout (*Oncorhynchus mykiss*) rearing conditions in the trial, where different setups of fixed bed and moving bed bioreactors were studied.

Characteristics	Value	Unit
RAS unit (n = 9)		
System volume	890	l
Tank volume	500	l
Relative water renewal rate	500	l kg^{-1} feed
Recirculation flow	15	l min^{-1}
Hydraulic retention time	5–8	d
Tank hydraulic retention time	33	min
Rearing conditions		
Fish density	19–82	kg m^{-3}
Feed quantity	0.22–0.45	kg d^{-1}
Average fish size	0.11–0.53	kg
Bioreactor (n = 2)		
Bioreactor water volume	125	l
Bioreactor hydraulic retention time	8	min
Carrier media volume	66	l
Carried media area	49.5	m^2
Moving bed bioreactor air flow	15	l min^{-1}
Bioreactor hydraulic loading rate	436	l m^{-2} d^{-1}

started, and divided evenly between the bioreactors to ensure similar bacterial seed in all the RAS units. In FF and FM units, the first bioreactor was backwashed once every two weeks. In FF units the second bioreactor was backwashed once every four weeks. The FBBR backwash water amount was not taken into account in the make-up water flow calculations, because it increased the total water volume by less than 4%.

2.2. Fish and feeding

Three weeks before the trial started, a total of 820 one year old rainbow trout (*Oncorhynchus mykiss*) (average weight 99 g) originating from the National JALO-selective breeding programme (Natural Resources Institute Finland, Tervo, Finland) were divided into the 9 RAS units. When the trial started, the fish were weighed and their biomasses were equalized. The fish were weighed twice during the experiment at weeks four and eight and group weighing was used in all of the weightings. Fish were fasted one day prior to and after the weighing. Feeding was carried out with a commercial feeding system (T Drum 2000, Arvo-Tec, Joroinen, Finland) 10–14 times per day. Feed intake rate was constantly monitored using sieve in the tank outlet and uneaten feed pellets were calculated. Feed company feeding table was used for feeding rate and it was reduced by 0.1%-unit, when uneaten feed was observed. 1:1 mixture of two commercial diets was used to ensure that the results can be better generalized across various commercial feeds. Diets were produced by Raisioaqua (Circuit Red 5 mm, Raisio, Finland) and BioMar (Orbit 929 4.5 mm, Aarhus, Denmark). The crude protein and lipid contents of the diets were 43% and 42%, and 26% and 31%, respectively.

The feed conversion ratio (FCR) was calculated as: $FCR = F / G$, where F = cumulative feed intake between weightings and G = total tank biomass gain between weightings. Specific growth rate (SGR) was calculated as: $SGR = (\ln(W_{i+1}) - \ln(W_i)) / (t_{i+1} - t_i) \times 100$, where W = average fish weight at given time and $t_{i+1} - t_i$ = duration of feeding days. The thermal growth coefficient (TGC) was calculated for the whole experiment according to Jobling (2003) as: $TGC = ((W_e^{1/3}) - (W_i^{1/3})) \times (T \times t) \times 1000$, where W_e = average fish weight in the end, W_i = average fish weight at the beginning, T = average water temperature, t = duration of feeding days.

2.3. Histopathological sampling and analysis

Tissue samples (gill, kidney and liver) from 5 fish per tank were collected at the start of the experiment, twice during the experiment and again at the end of the experiment, at approximately one month intervals. The second gill arch from the right hand side was sampled and sectioned parasagittally. Kidney tissue was sampled as approximately 2 cm long sections from the distal third of the kidney and sectioned transversely. Liver tissue was sampled in approximately 1 × 1 cm sections and sectioned sagittally. The tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm and stained with haematoxylin and eosin (H&E) according to standard laboratory practice. The sections were examined using light microscopy.

The histopathological changes were reported on a scale from 0–3: minimal, mild, moderate and severe as described by Wolf et al., 2015. One section per tissue and per fish was examined.

The following parameters were studied and classified according to the severity of the lesions:

Gills: Lamellar epithelial cell hyperplasia (LEH): proliferation of the squamous epithelial cells lining the gill surface. General diffuse proliferative branchitis: filling of interlamellar spaces by a mixed population of epithelial and inflammatory cells. Focal branchitis: a local unspecific inflammatory change consisting mainly of mononuclear, lymphocytic cell types involving a smaller area, usually only a few lamellae. Lamellar fusion: one or more interlamellar sulci filled by proliferating pavement cells (with or without increased mucous cells, chloride cells, and/or leucocytes). Lamellar adhesion: the often focal attachment of adjacent lamellae with little or no evidence of cell proliferation. Lamellar thrombosis: formation of blood clots inside lamellar capillaries consisting of fragmented thrombocyte nuclei and/or pink fibrinous material within the distended capillaries.

Kidney: Tubular necrosis: Necrosis of tubular epithelial cells. Renal mineralization: mineralized material intraepithelially or intraluminally. Number of melanomacrophage centres or pigmented macrophage aggregates (PMAs): centres of mainly histiocytic macrophages that contain hemosiderin, melanin, lipofuscin, and/or ceroid pigments and that serve as repositories for end-products of cell breakdown.

Liver: Hepatocellular cytoplasmic vacuolation: intracytoplasmic vacuoles containing glycogen or lipids. Hepatitis/cholangiohepatitis: infiltration of acute or chronic inflammatory cells in liver tissue or around bile ducts.

2.4. Water sampling and analysis

Total ammonia nitrogen, nitrite and nitrate were analysed once a week from the tank outlet water using a spectrophotometer (Procedure 8038 Nessler, LCK341/342 and LCK340 respectively. DS 3900, Hach, Loveland, USA). Alkalinity was analysed once a week with a standard method of titration (ISO 9963−1:1994) (TitraLab AT1000, Hach, Loveland, USA).

Particle size distribution (PSD) was analysed from the tank water and from the water taken from top of the 2nd bioreactor at week 13 (S4031, PAMAS, Rutesheim, Germany). Optical analyses covered particle sizes from 1 µm to 200 µm. A simple comparison of PSD between treatments was made by calculating the β-values (slope of log10 frequency versus log10 particle size) according to Patterson et al. (1999). Total particle surface area and volume were calculated by using the given particle size diameter (assumed sphere) multiplied by the total number of particles.

Particle counts were also measured from tank water with a CASY cell counter with a capillary size of 45 µm (Model TT, OLS OMNI Life Science GmbH, Basel, Switzerland) at week 14. Measurement principal is based on pulse area analysis, where low voltage field is cast through the samples. Measurement range was between 0.8 µm–30 µm. The 100 ml water samples were frozen before analysis. Triplicate measurements per water sample were analysed using a sample size of 200 µl.

Total organic carbon (TOC) and UV254 (turbidity corrected) were monitored online at 6 min intervals in the fish tanks with a UV/VIS spectrometer (5 mm open path length, spectro::lyser, s::can, Vienna, Austria). Carbon dioxide concentrations were monitored in the fish tanks at 6 min intervals with a carbon dioxide sensor (Franatech, Lüneburg, Germany). Two hour average values are presented for these online measurements.

2.5. Solids sampling

Sludge was collected twice during the trial at weeks 7 and 11 for solids analysis. Sludge from swirl separators was collected using a 0.31 L tube placed at the bottom of the separators. The collection period lasted six hours. Drum filter backwash water was collected for 16 h, then weighed and mixed, after which a subsample of 1 L was collected. Fixed bed bioreactors were cleaned by vigorous agitation with air, and one litre samples were collected from the top of the reactor and from the outlet pipe. At week 11, water collected only from the top of the reactor was used because there was no difference between these sampling points. All solids samples were put into a container and oven dried (+80 °C) for two days.

Total solids (g kg^{-1}) were calculated for FBBR and drum filter as: $TS = (m_d - m_t) / S \times V / F$, where m_d = dried subsample mass (g), m_t = container mass (g), S = sample size (l), V = total outflow volume (l

d^{-1}), F = Feed intake (g d^{-1}). Total solids (g kg^{-1}) were calculated for the swirl separator as: TS = (m_d - m_t) x 4 / F, where m_d = dried subsample mass (g), m_t = container mass (g), F = feed intake (g d^{-1}).

2.6. Bioreactor nitrification rates

Bioreactor nitrification rates (g NO_x h^{-1}) were measured at the last week of the experiment, following principles described by Jäntti et al. (2011). For the incubations, inlet water and carrier media were collected from each bioreactor tank and transferred to the University of Jyväskylä. In the laboratory, carrier media were divided into experimental vials (n = 30 per vial) with 360 ml inlet water, where $^{15}NH_4^+$ was added (final concentration of 5 mg/L; 10–15 at m%). To ensure complete nitrification, the carrier media was incubated for 3 h at in situ temperature and under constant mixing by magnetic stirring bars (150 rpm). To measure ammonium and nitrate concentrations and the stable isotope composition of nitrite and nitrate, water samples were taken at the beginning of the experiment, and after 1.5 and 3 h. Water samples were filtered with 0.2 um syringe filters and frozen immediately. Later, nitrate, nitrite and ammonium concentrations were measured with a spectrophotometer (Lasa 100, Hach, Loveland, USA). The stable isotope composition of nitrite and nitrate was measured using the denitrifier method (Sigman et al., 2001). Briefly, 20 nmoles of sample NO_{2+3} were converted to N_2O by cultured denitrifying bacteria (*Pseudomonas chlororaphis* strain DSM 6698), which lack the enzyme responsible for N_2O reduction and the isotopic composition of N_2O was measured using the IsoPrime 100 CF-IRMS with a TraceGas preconcentrator interface.

2.7. Statistics

The effects of bioreactor design on nitrification efficiency, FCR, SGR, TGC, PSD and TS were analysed using one-way ANOVA, and Tukey's post hoc test was used for comparing the effects between treatments, which takes the uneven sample sizes in the end of the experiment into account (Rusticus and Lovato, 2014). A nonparametric Kruskall-Wallis test was used for total particle counts when assumptions were not met for the parametric test. Effects of bioreactor design on water quality parameters were analysed using Mixed ANOVA, where bioreactor design type (between subjects) and measurement week (within subjects) were factors. The Bonferroni post hoc test was used for comparing effects between treatments. For online measurements, daily average values were used. Statistical analyses were done with SPSS (IBM SPSS Statistics, Armonk, USA) wherein 95% confidence interval was used.

3. Results

3.1. Fish growth and histopathology

No significant differences were found between treatments for FCR and SGR during the trial or TGC for the whole experiment (Table 2). For the whole experiment, average feed loads were 30.09 kg (± 0.15 kg), 30.88 kg (± 0.35 kg) and 30.39 kg (± 0.28 kg) in the FF, FM and MM groups, respectively.

The most significant histopathological changes were noted in gill tissue (Table 1, supplementary material). The severity scores for both lamellar epithelial cell hyperplasia (LEH) and focal branchitis were slightly elevated at the beginning of the trial, for focal branchitis only in the FF group, and for LEH in all groups including the flow-through system.

In kidney and liver tissue, no notable histopathological changes were seen during the experiment. The PMAs noted during the experiment were mild to moderate, and no notable differences in their occurrence over time, or differences between treatment groups or control group, were noted. The inflammatory changes noted in this experiment were also minor and did not show any increase during the course of the experiment.

3.2. Water quality

There was no difference in the TAN values between treatment groups, whereas nitrite values decreased throughout the experiment in all groups. In the FF group, nitrite values were significantly higher than in the FM and MM groups ($P < 0.01$). Nitrate values were higher in the MM group in comparison to the FF ($P < 0.01$) and FM groups ($P < 0.01$) (Fig. 2.).

Total organic carbon, UV254 and CO_2 values were significantly different between the treatments ($P < 0.01$). TOC was lower in the FF group as compared to the FM and MM groups. The UV254 value was lowest in the FF group, and highest in the MM group. CO_2 concentration was highest in the FF group ($P < 0.01$), but there was no significant difference between the FM and MM groups (Fig. 3).

3.3. Total solids and PSD

The sum of total solids removed from the RAS units and solids removed from the swirl separators did not differ between the treatments (Fig. 4). Solids removal by the drum filters was significantly affected by the bioreactor systems ($P < 0.01$). In the RAS with two moving bed bioreactors, drum filters removed solids the most, whereas in the RAS with two fixed bed bioreactors, solids removal by drum filters was the lowest. In the FM group, drum filter solids removal was lower than in the MM group, but it was not statistically significant ($P = 0.051$).

In fish tanks, particle size distribution values (β-value, total amounts, surface area and volume) were not significantly different between the treatments, whereas differences were observed in water samples taken after biofiltration. The β-values in water sampled after the second bioreactor was significantly higher in the FF group compared to MM ($P < 0.05$), indicating that RAS with two fixed bed bioreactors has a larger share of particles in small sizes (Table 3). Total particle amounts and surface area in the biofiltered water were not affected by the treatments, whereas total particle volumes were significantly higher in the MM group compared to the FF group ($P < 0.05$). Over 80% of the particles were below 3 µm in the FF and FM group and over 90% in the FF group (Fig. 1, supplementary material).

Although treatments with moving bed bioreactors had higher

Table 2
Mean rainbow trout (*Oncorhynchus mykiss*) feed conversion ratio (FCR), specific growth rate (SGR) (% bw d^{-1}) and thermal growth coefficient (TGC) (± SD) during the trial (1 = days 0–27, 2 = days 28–55, 3 = days 56–92, 4 = 0–92) of the three RAS bioreactor designs. FF = Two consecutive fixed bed bioreactors, FM = Fixed bed bioreactor followed by moving bed bioreactor and MM = Two consecutive moving bed bioreactors (n = 3, except when marked in asterisk, where n = 2).

	FCR				SGR				TGC
Treatment	1	2	3	4	1	2	3	4	4
FF	0.85 ± 0.03	0.95 ± 0.04	*1.13 ± 0.03	*0.98 ± 0.01	2.36 ± 0.05	1.56 ± 0.02	*1.03 ± 0.02	*1.59 ± 0.00	*2.20 ± 0.01
FM	0.81 ± 0.01	0.91 ± 0.02	1.07 ± 0.03	0.95 ± 0.01	2.44 ± 0.04	1.58 ± 0.03	1.11 ± 0.03	1.64 ± 0.01	2.28 ± 0.03
MM	0.81 ± 0.02	0.95 ± 0.08	1.05 ± 0.04	0.95 ± 0.02	2.41 ± 0.03	1.54 ± 0.08	1.12 ± 0.02	1.62 ± 0.02	2.22 ± 0.05

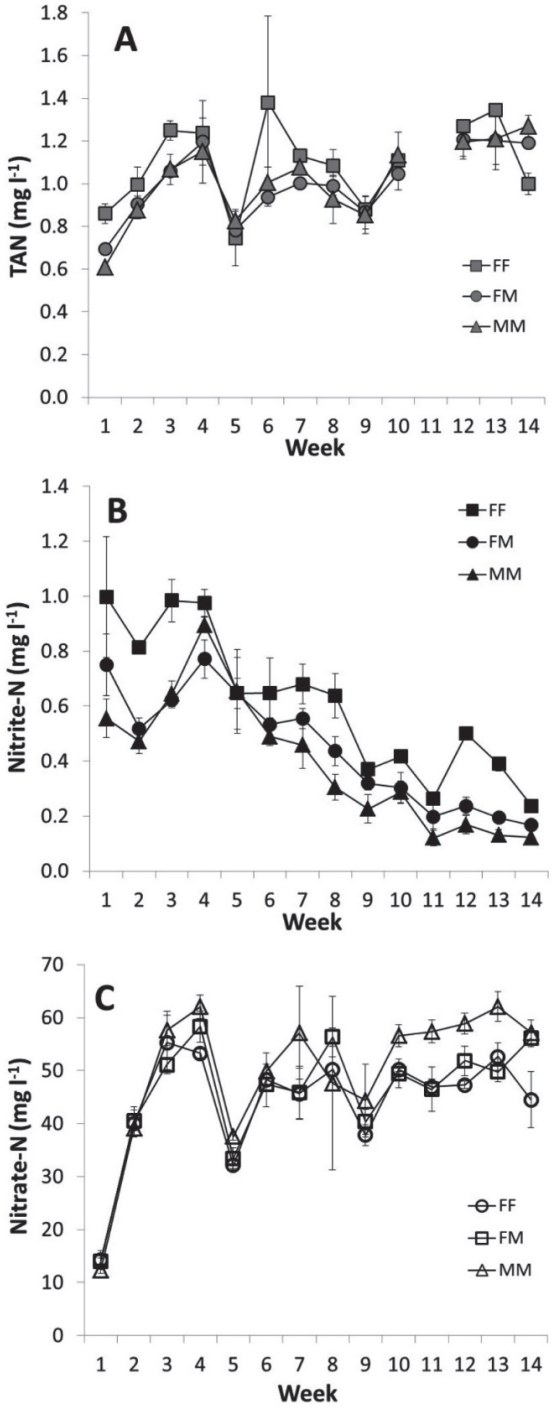

Fig. 2. Mean total ammonium nitrogen (TAN) (A), nitrite-nitrogen (B) and nitrate-nitrogen (C) values of the three RAS bioreactor designs ± SD. FF = Two consecutive fixed bed bioreactors (n = 3 at weeks 1–8 and n = 2 at weeks 9–14), FM = Fixed bed bioreactor followed by moving bed bioreactor (n = 3) and MM = Two consecutive moving bed bioreactors (n = 3).

particle counts measured with the CASY cell counter, the counts were not significantly different between the treatments due to high within-treatments variance (Kruskal-Wallis P = 0.24; Fig. 5).

3.4. Nitrification

The nitrification rate was significantly different between the treatments (P < 0.01). In the FF group, the nitrification rate was lowest, but there was no difference between the MM group and FM group (P = 0.07). The nitrification rate did not differ between the first and second moving bed bioreactor, whereas in the FF group, the second FBBR had a lower nitrification rate than the first FBBR (P < 0.01) (Fig. 6).

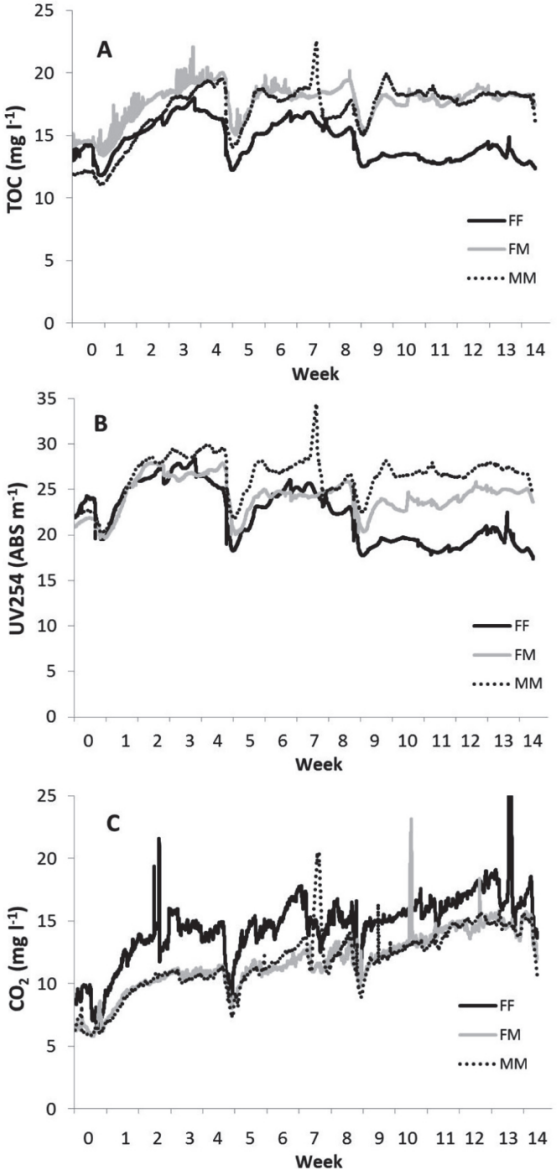

Fig. 3. Mean total organic carbon concentrations (TOC) (A), UV254 absorbance (B) and carbon dioxide concentrations (C) measured online from the fish tank with UV/VIS spectrometer and CO_2 probe of three RAS bioreactor designs. FF = Two consecutive fixed bed bioreactors (n = 3 at weeks 1–8 and n = 2 at weeks 9–14), FM = Fixed bed bioreactor followed by moving bed bioreactor (n = 3) and MM = Two consecutive moving bed bioreactors (n = 3).

4. Discussion

4.1. Fish performance

In general, fish grew well and there were minor mortalities. However, one tank was lost due to pH probe failure. We did not see any differences in fish growth between the different bioreactor

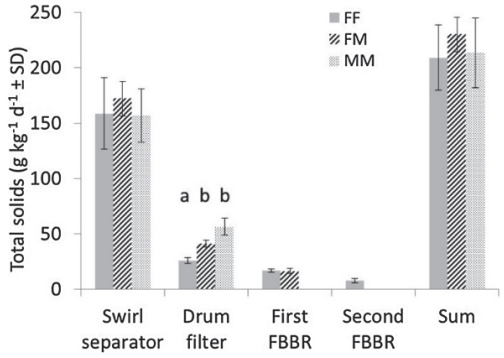

Fig. 4. Total solids removed from different water treatment steps proportioned into daily feed intake of the three RAS bioreactor designs. FF = Two consecutive fixed bed bioreactors, FM = Fixed bed bioreactor followed by moving bed bioreactor and MM = Two consecutive moving bed bioreactors (n = 3). FBBR = Fixed bed bioreactor. Mean values from two collection periods are presented (± SD). A significant difference between treatments in drum filter backwash water is marked by different letters ($p < 0.01$).

configurations, even when some difference was seen in the water quality.

We noticed higher CO_2 and NO_2-N levels in the FF group. Good et al. (2010) reported that elevated CO_2 concentration up to 24 mg l^{-1} did not affect rainbow trout growth or health. In contrast, Khan et al. (2018) noticed that CO_2 concentration in RAS water had a negative linear correlation with Atlantic salmon (*Salmo salar*) growth. This means that there is no threshold value for CO_2 where growth would decrease: the higher the concentration the more it affects the growth. In addition, elevated nitrite concentration can cause several physiological disturbances in aquatic animals, leading to decreased growth (Aggergaard and Jensen, 2001; Jensen, 2003) and even death (Svobodova et al., 2005).

In contrast to the FF group, we noticed a higher organic material load and NO_3-N levels in the MM group. Davidson et al. (2014) recommended 75 mg l^{-1} as the maximum level of nitrate for rainbow trout. This is the level where negative impacts on long term health were seen. In addition to nitrogen compounds, solids can also have detrimental effects on fish performance. Particle accumulation in fish gills has been shown to cause inflammatory responses (Lu et al., 2018) and stress (Au et al., 2004). However, Becke et al. (2018) studied the long term effect of a high suspended solids load in RAS and despite the high load, they did not find rainbow trout histopathology or growth indicators to be significantly affected.

Taken into account all of the above, all treatment groups had water quality parameters, which could have affected the growth negatively. This might be one reason, why differences between treatment groups were not observed in growth and health. In addition, rainbow trout can be tolerant to different water qualities, thus observed differences for water quality between treatments might not be biologically relevant or within treatment variability was too high to observe any differences.

For histopathological lesions in general, only minor differences between the different treatment groups or the flow-through system were seen. Most lesions were minimal to mild, and thus clinically nonsignificant. The only clinically significant moderate changes were noted in gill tissue as an increase in lamellar epithelial cell hyperplasia (LEH), which is a common, non-specific lesion seen in subacute to chronic gill damage, and in focal branchitis (Fig. 2, supplementary material). These changes were noted also in the flow-through system. Mild, clinically nonsignificant gill lesions were noted also at the start of the experiment (T0). These changes correlate partly with noted differences in water nitrogen compounds and UV254 measurements, however, no water parameters were measured for the flow-through system. The gills are structures with a large surface area in direct contact with water, and as such are often the first tissue to show changes when water quality is suboptimal. Gills show a remarkable regenerative capacity (Ferguson, 2006) and can adapt to less optimal water quality over time (Kolarevic et al., 2013). No changes in liver or kidney tissue were noted in this experiment. Melanomacrophage centres exist in normal kidney tissue of fish and they increase with age, however an excess or increase can be seen in chronically stressed fish. A lymphoid inflammatory reaction located around bile ducts, cholangiohepatitis, can be seen in connection with parasitic infections, but may also be connected with unspecific immune mediated reactions and may have a connection with water quality. None of these lesions were noted during the experiment; however, a prolonged exposure time of harming substances might be needed in order to provoke some of the studied changes in these organs.

4.2. Water quality

Online spectrometric water quality monitoring can provide useful information about short period fluctuations in the water quality, which cannot be seen in the manual water sampling. However, there are lots of substances that absorb light in the same wavelengths and affect the interpretation of the results. In addition, sensitivity can be weak, and accuracy is tolerable only above certain threshold values (Carré et al., 2017). The UV254 absorbance values correlate well with dissolved organic carbon (DOM) and dissolved aromatic carbon in particular (Weishaar et al., 2003), making it a useful indicator of biological substances in the water. When one or two MBBR were used in RAS unit, an increasing UV254 value was measured. Bacterial biomass was increasing in the bioreactors during the operation and this surplus biomass was removed from the FBBR when backwashed. In MBBR, surplus biomass was constantly removed into the circulating water, which increased the amount of organic matter in the water, as was seen in the UV254 absorbance values during the trial. TOC fluctuations followed the UV254 fluctuations, but there were no differences in the TOC values between the FM and MM groups.

Particle size distribution was measured, when systems were considered to be in their steady state. In RAS, small particles typically accumulate in the system, which is seen as high β-values (Patterson et al., 1999). In the present trial, total particle volume was highest in the MM group, which followed the overall water quality values. The

Table 3
Mean β-values, total particle counts, surfaces and volumes (± SD) at two sampling locations of the three RAS bioreactor designs. FF = Two consecutive fixed bed bioreactors (n = 3), FM = Fixed bed bioreactor followed by moving bed bioreactor (n = 3) and MM = Two consecutive moving bed bioreactors (n = 3). A significant difference between treatments is marked by different letters ($p < 0.05$).

	Fish tank				After 2nd bioreactor			
	β	Total counts (1.0×10^3 pcs ml^{-1})	Total surface area ($mm^2\,ml^{-1}$)	Total volume ($1.0 \times 10^{-3}\,mm^3\,ml^{-1}$)	β	Total surface area ($mm^2\,ml^{-1}$)	Total counts (1.0×10^3 pcs ml^{-1})	Total volume ($1.0 \times 10^{-3}\,mm^3\,ml^{-1}$)
FF	3.7 ± 0.1	39.3 ± 8.3	0.7 ± 0.1	1.4 ± 0.1	4.1 ± 0.1^{a}	0.7 ± 0.2	40.5 ± 10.7	0.7 ± 0.2^{a}
FM	3.7 ± 0.2	45.8 ± 14.1	1.2 ± 0.4	2.0 ± 0.7	3.8 ± 0.2^{ab}	0.9 ± 0.5	35.6 ± 18.9	1.6 ± 0.5^{ab}
MM	3.6 ± 0.2	33.7 ± 13.9	1.0 ± 0.4	2.4 ± 1.0	3.6 ± 0.1^{b}	1.3 ± 0.1	40.9 ± 4.2	3.2 ± 1.0^{b}

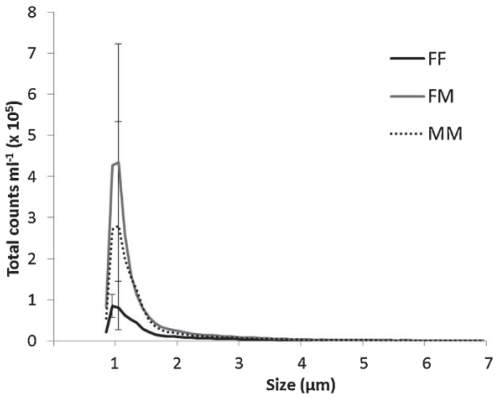

Fig. 5. Total particle counts of the three RAS bioreactor designs measured using the CASY cell counter. FF = Two consecutive fixed bed bioreactors (n = 2), FM = Fixed bed bioreactor followed by moving bed bioreactor (n = 3) and MM = Two consecutive moving bed bioreactors (n = 3) ; ± SD of the most abundant size classes, which were 1.0 µm for FF and 1.1 µm for FM and MM groups. There were no significant differences between treatments.

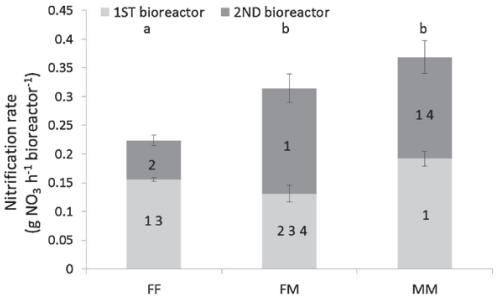

Fig. 6. Nitrification rate measured in the three RAS bioreactor designs measured using the stable isotope labelling method. FF = Two consecutive fixed bed bioreactors (n = 2), FM = Fixed bed bioreactor followed by moving bed bioreactor (n = 3) and MM = Two consecutive moving bed bioreactors (n = 3). A significant difference between treatments is marked by different letters and between different bioreactors by different numbers ($p < 0.01$).

same amount of solids was introduced into every RAS unit via fish feed and removed by water treatment units. Solids trapped and later removed by FBBR were removed by drum filter in units with MBBR. Thus, drum filters were compensating particle accumulation in the MM group, even though mesh size was 60 µm. Total particle counts measured with the CASY cell counter were between 15 and 60-fold higher than those measured with the PAMAS optic particle counter. The most likely explanation is that the cell counter is much more accurate in small size classes and there is disintegration of possible cell aggregates in the freezing period. Michaud et al. (2006) found up to 800,000 free bacterial cells per ml in biofilter effluent, which indicates that the majority of small particles are bacterial cells. Thus, total particle counts measured with the cell counter was considered to measure total bacterial counts in the water. As expected, total bacterial counts were somewhat higher in the units with moving bed bioreactors, but the difference was not significant between the groups.

In the units where MBBR was in use, CO_2 concentrations were lowest. Mixing the MBBR with compressed air was ventilating CO_2 out of the systems, but there were no differences in concentrations if one or two MBBRs were used.

4.3. Nitrification

Continuously decreasing nitrite values during the trial indicate that bioreactors were not yet fully developed at the beginning of the trial. However, this was not detected in the TAN values, which were quite stable throughout the trial. Although all carrier media were used for six months before the trial started, it is possible that mixing and transferring might have disturbed nitrite oxidizing bacteria, which are more vulnerable to changing conditions (Graham et al., 2007), and caused nitrite accumulation. This accumulation was higher in the FF group as compared to FM and MM groups, which is in contrast to other experiments that have compared similar carrier elements (Pedersen et al., 2015; Suhr and Pedersen, 2010). Fixed bed bioreactors are very susceptible to reactor dynamics, especially for the flow velocity (Kumar et al., 2011; Prehn et al., 2012). It is possible that the water velocity in our fixed bed reactors was not optimal and possibly water did not flow uniformly through all the filter media. Prehn et al. (2012) observed that when the water velocity is increased from 4.2 cm min^{-1}, which was the same velocity as in our system, to 66.7 cm min^{-1}, nitrification rates increased three-fold. In addition, the possibility of shunts in the FBBRs could have reduced the nitrification rates, because the by-pass flow may have decreased the active bioreactor surface area.

The nitrification rates measured in the laboratory were comparable to the observed nitrogen results from the water quality analyses, and both indicated that FBBR was less effective in the process. Sampling locations of the carrier media might have had some effect on the results, because MBBR has a unifying bacterial consortium throughout the reactor, while in FBBR, the bottom of the reactor can have different communities because of high substrate concentrations (Pérez et al., 2005). However, nitrification rates measured in the laboratory confirmed that nitrification in FBBR was did not work as effectively as in MBBR. Nitrification rates were very consistent in all MBBRs, demonstrating that MBBR is a very stable, reliable and maintenance-free bioreactor type to use.

5. Conclusions

Here, we demonstrated that nitrification bioreactor design affects RAS water quality, mainly through accumulation of solids and nitrification problems. When using two moving bed bioreactors, the amount of organic matter increased, while with the two fixed bed bioreactors, toxic nitrite accumulated in the circulating water. However, the drum filter compensates for the particle removal in the moving bed bioreactors. This study revealed that no single bioreactor type studied here is more beneficial than any other when rainbow trout growth and health is concerned. However, observed differences on the water quality may lead for selecting one bioreactor type over another. Solids retention capacity of FBBR may even make drum filters unnecessary, thus saving space and installations, which decreases the construction costs. On the other hand, FBBR require constant maintenance, which increases the operational costs. Maintenance free MBBR can save operational costs, but if solids accumulation is causing problems, additional solid treatment system might be needed. There can be also other aspects that can be dependent on the bioreactor type, which were not investigated in this experiment, one being formation of off-flavour compounds. In addition, when both reactor types are in use, changing sequence from FBBR followed by MBBR to MBBR followed by FBBR might highlight best features from both bioreactor types.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquaeng.2019.03.004.

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III

THE EFFECT OF PERACETIC ACID ON MICROBIAL COMMUNITY, WATER QUALITY, NITRIFICATION AND RAINBOW TROUT (ONCORHYNCHUS MYKISS) PERFORMANCE IN RECIRCULATING AQUACULTURE SYSTEMS

by

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The effect of peracetic acid on microbial community, water quality, nitrification and rainbow trout (*Oncorhynchus mykiss*) performance in recirculating aquaculture systems

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ABSTRACT

Microbial biofilters control water quality and enable the overall function of recirculation aquaculture systems (RAS). Changes in environmental conditions can affect the abundance and interactions of the diverse microbial populations of the biofilter, affecting nitrification of harmful ammonium and thus fish health. Here, we examined the effect of different application frequencies (0, 1, 2 and 4 times per week) of a common disinfectant, peracetic acid (PAA, applied 1.1 mg l^{-1} twice per day), on biofilter microbial communities, focusing especially on nitrifying microbial groups and using a high throughput sequencing of 16S rRNA gene and quantitative PCR (qPCR). In addition, we measured biofilter nitrification rates, water quality parameters, and fish performance. Although PAA additions did not significantly change the overall microbial community composition or abundance, the abundance of ammonia-oxidizing bacteria (AOB) and nitrate-oxidizing bacteria (NOB) first decreased at the beginning of the experiment but increased in numbers towards the end of the experiment with frequent PAA applications. PAA application decreased the nitrification rate, but increased the water quality in terms of reduced ammonium levels. PAA application did not significantly affect fish growth, but higher mortality was observed with the highest PAA application level of 4 times per week. These results suggest that when applied before the fish tank, PAA can be used for temporary water quality improvement without disturbing microbial communities. However, the application frequency required for persistent water quality improvement caused increased mortality.

1. Introduction

Aquaculture is one of the fastest growing food producing sectors (FAO, 2017). Recirculating aquaculture systems (RAS) have lower water consumption and lower nutrient discharge to the environment than the traditional flow-through or net pen farming, making them environmentally and economically sustainable fish-producing systems (Badiola et al., 2012). However, water quality management is crucial for successful RAS performance and fish health, where the bacterial community plays an important role (Blancheton et al., 2013; Rurangwa and Verdegem, 2015).

Biofilters play a key role in controlling water quality, hosting microbes that convert the produced ammonium into nitrate nitrogen through nitrification. Traditionally, the nitrification pathway is considered to consist of two steps driven by two different microbial groups: ammonia oxidizers (ammonia-oxidizing bacteria: AOB); and nitrite oxidizers (nitrite-oxidizing bacteria: NOB). Recently, ammonia-oxidizing archaea (AOA) have also been found in biofilters in RAS and aquariums (Bagchi et al., 2014; Bartelme et al., 2017). The nitrification function of the biofilter depends on several water quality factors, e.g. pH, oxygen concentration, and ammonium levels (Chen et al., 2006). Nitrite-oxidizing NOBs are especially sensitive (Villaverde et al., 1997; Graham et al., 2007), which can cause nitrite accumulation after changes in the RAS operating conditions. However, recently, a group of NOB capable of complete ammonia oxidation (comammox), *Nitrospira*, has been identified and found to be quite abundant in RAS biofilters (Bartelme et al., 2017). In addition to nitrifying microbes, biofilters host a diverse heterotrophic microbial community (Bartelme et al., 2017; Rud et al., 2017), that can include harmful micro-organisms (Guttman and van Rijn, 2008; Schrader and Summerfelt, 2010) or microbes producing odorous metabolites (Lukassen et al., 2017; Lindholm-Lehto et al., 2019). Typically, organic matter accumulation (high C:N) in RAS

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favors heterotrophs, limiting oxygen availability and resulting in lower nitrification rates (Michaud et al., 2006).

Peracetic acid (PAA: CH_3CO_3H) is a widely-used and efficient disinfecting agent in aquaculture and wastewater treatment plants (Kitis, 2004; Koivunen and Heinonen-Tanski, 2005; Lahnsteiner and Weismann, 2007). In aquaculture, PAA has been used for disinfection against pathogenic bacteria and other harmful micro-organisms, e.g. crayfish plague spores (Jussila et al., 2011), and for general disinfection to reduce bacterial and potentially bacterial by-product numbers (Kitis, 2004; Liu et al., 2017a). PAA has an optimal degradation time (2 mg L^{-1} to 0 mg L^{-1} in 6 h) for RAS, and it has no toxic or harmful by-products (Pedersen et al., 2009; Liu et al., 2017a). The commercial PAA products, used in this and in previous studies (Pedersen et al., 2009, 2013; Liu et al., 2017a; Davidson et al., 2019), contain microbial disinfectant PAA, and hydrogen peroxide, which degrades the organic material from fish feces and feed, and acetic acid. Previous studies have shown that regular PAA additions of 1 mg L^{-1} can decrease aerobic bacterial density in the rearing water (Liu et al., 2018), but in lower PAA concentrations of 0.1–0.3 mg L^{-1}, bacteria remained unaffected (Davidson et al., 2019). Pulse applications remove bacterial biofilm in the tank wall more efficiently than continuous PAA applications (Liu et al., 2017a). A disadvantage of PAA is its tendency to increase organic matter content in the system due to acetic acid, causing regrowth of microbes and PAA decays (Kitis, 2004; Pedersen et al., 2013). Indeed, continuous low concentration PAA applications can promote biofilm formation and microbial adaptation (Liu et al., 2017a).

Another challenge in PAA application is the potential disturbance to the crucial biofilter function. The PAA concentration cannot be too high, since a previous study has shown elevated nitrite levels, i.e. disturbed nitrification with 2 mg L^{-1} and 3 mg L^{-1} of PAA used, while this was not observed with 1 mg L^{-1} of PAA (Pedersen et al., 2009). Furthermore, semi-continuous PAA applications (0.3 mg L^{-1}) should not affect nitrification negatively (Davidson et al., 2019). In addition to nitrification, fish welfare should be considered when using PAA or any other disinfectant or substance for water quality management in RAS. Fish tolerance to PAA depends on the species (Straus et al., 2018). Rainbow trout (*Oncorhynchus mykiss*) are sensitive to PAA, as the no-observed-effect concentration (NOEC) is 2.8 mg L^{-1}, compared with the more tolerant channel catfish (*Ictalurus punctatus*) (NOEC 4.0 mg L^{-1}) and blue tilapia (*Oreochromis aureus*) (NOEC 5.8 mg L^{-1}) (Straus et al., 2018). Exposed to PAA (up to 2 mg L^{-1}), fish have shown stress responses measured by elevated cortisol levels in plasma (Gesto et al., 2018) and in rearing water (Liu et al., 2017b). In addition, increased mucus formation has been reported at similar PAA concentrations (Lindholm-Lehto et al., 2019). However, when PAA exposure has been continued, fish have been able to adapt during the treatment (Gesto et al., 2018; Liu et al., 2017b).

Although there are some previous studies of PAA and its effects in RAS, they have focused on general water quality and measured nitrification rates (Pedersen et al., 2009; Pedersen et al., 2013; Liu et al., 2017a; Davidson et al., 2019); little is known about the effects of disinfecting agents on the microbial communities in the RAS biofilter. Since PAA is widely used in RAS, this information is required to understand the stability of the biofilter function during pulse PAA applications and to improve the overall performance of RAS. In this study, the effect of three different pulse application frequencies of PAA (1.1 mg L^{-1} applied twice per day) were studied with a control group. PAA were applied 1, 2 and 4 times per week to the pump sump on a replicated laboratory-scale RAS. We studied the overall biofilter microbiological community composition, assessed with high throughput sequencing, and the total bacterial abundance and genetic nitrification potential (number of nitrification genes), assessed with quantitative PCR (qPCR). In addition, biofilter nitrification rates, water quality parameters, and the fish growth and feed conversion ratio were examined. We hypothesized that the highest application of PAA 4 times per week might have detrimental effects on microbial communities by disturbing the steady-state microbial community and thus disrupting nitrification. In addition, lower applications of PAA once a week were expected to improve water quality and decrease the microbial load in the fish tank, further leading to improved fish performance.

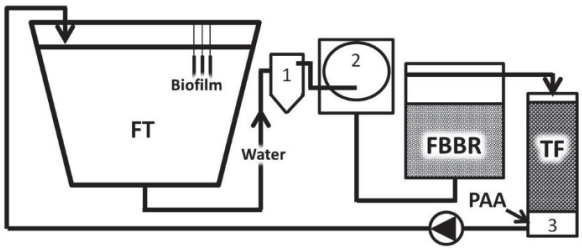

Fig. 1. Schematic diagram of one RAS unit used in the experiment. Microbial samples were taken from the fish tank biofilm, water from fish tank and from the fixed bed bioreactor. PAA was dosed into the pump sump. FT = Fish tank, 1 = Swirl separator, 2 = drum filter, FBBR = Fixed bed bioreactor, TF = Trickling filter, 3 = Pump sump.

2. Materials and methods

2.1. Experimental setup

The experiment was performed at the Natural Resources Institute Finland (Luke) Laukaa fish farm, using an experimental RAS platform. The details of the research facility are described in more depth in Pulkkinen et al. (2018). Briefly, eight individual recirculating systems were used, each consisting of a 500 L bottom-drained plastic rearing tank, a feed collector unit, a 24 cm swirl separator (Eco-Trap Collector1, Pentair Aquatic Eco-Systems, Minneapolis, USA), a drum filter with 60 µm filter panels (Hydrotech HDF501, Veolia, Paris, France), a 147 L fixed bed bioreactor (filled with 80 L of RK Biolements heavy, RK Plast A/S, Skive, Denmark), a trickling filter acting as a forced-ventilated cascade aeration column (Bio-Blok® 200, EXPO-NET Danmark A/S, Hjørring, Denmark), and a pump sump (Fig. 1). All RAS units had been used in previous experiments, and the biofilters were stabilized to full maturity. Water pH was adjusted to 7.2 in the pump sump with 20% NaOH (aq), using an automated system (Prominent, Heidelberg, Germany). Oxygen saturation was kept above 80% in the fish tanks, and water temperature around 16 °C. The relative water renewal rate was set to 500 L kg^{-1} feed, and the tank hydraulic retention time to 33 min.

In this thirteen week experiment, three different PAA application frequencies per week were used (1.1 mg PAA L^{-1} in a fish tank dosed twice per day). The experiment is described in more detail in Lindholm-Lehto et al. (2019). Briefly, control tanks and three different PAA applications per week (1, 2 and 4) applied in pump sumps were used in replicate tanks. The PAA applications were dosed by adding 4 mL of PAA solutions twice a day. A commercial PAA product was used, composed of 12–13% PAA, 19–23% hydrogen peroxide, and the remainder acetic acid (Bonsoxo 2901, Bang & Bonsomer, Helsinki, Finland).

2.2. Fish and feeding

Three weeks before PAA addition commenced, a total of 400 one-year-old rainbow trout (*Oncorhynchus mykiss*) (average weight 130 g) originating from the National JALO selective breeding programme (Natural Resources Institute Finland, Tervo, Finland) was divided into the eight RAS units. During the first two weeks after PAA addition commenced, mortality between 0-20% was observed in the RAS units, and fish were diagnosed with IPNV (Infectious Pancreatic Necrosis Virus), which may have caused the mortality. After three weeks, fish in poor condition were removed, and biomasses were equalized between the tanks (mean 12.5 kg m^{-3}). Fish were weighed once during the

experiment in week 8 by weighing the tank mass and counting the individuals. Feeding was evenly executed with a commercial feeding system (T Drum 2000; Arvo-Tec, Joroinen, Finland) 10–14 times per day in constant light. A 1:1 mixture of two commercial diets was used, Raisioaqua (Circuit Red 5 mm, Raisio, Finland) and BioMar (Orbit 929 4.5 mm, Aarhus, Denmark). The crude protein and lipid contents of the diets were 43% and 42%, and 26% and 31%, respectively, as given by the manufacturer. Feed intake was monitored using a feed collector unit, and feeding was decreased if uneaten feed was observed. At the start, the feeding rate was set to 1.5% bw d^{-1}.

2.3. Sampling for microbiome and qPCR analysis

Biofilter biofilm samples were collected for sequencing and qPCR twice after 8 and 13 weeks of the experiment. Three sets of carrier media were collected from each bioreactor unit. In addition, water and biofilm samples from the fish tank were collected for qPCR. Water samples, two per fish tank, were filtered using syringe filters (0.22 µm Millipore Express® PLUS PES membrane). The tank biofilm was collected using detachable tank material blocks (HDPE), which were placed in the tanks at the beginning of the experiment. Prior to further analysis and DNA extraction, samples were frozen (- 20 °C) for at least 24 h. To detach microbial biofilm from the biofilter and tank biofilm samples, 20 mL of water was added to the samples, and they were sonicated for 4 min (Branson 1510). They were then freeze-dried (Alpha 1–4 LD plus, Christ). DNA extraction was performed for the freeze-dried materials using the DNeasy PowerLyzer™PowerSoil DNA Isolation Kit (Qiagen) in accordance with the manufacturer's instructions. PowerLyzer Homogenizer was applied once at 3400 rpm for 45 s during the extraction. The quantity of extracted DNA was measured with Qubit™ dsDNA HS assay and Qubit 2.0 Fluorometer (Thermo Fischer Scientific). For sequencing and qPCR, three replicate samples from each biofilter unit or two water samples were pooled.

2.4. Microbial communities

The microbial community composition was studied using Ion Torrent PGM next generation sequencing, targeting the V4 region of the 16S rRNA gene with primer pair 515F–Y (GTGYCAGCMGCCGCGGTAA; Parada et al., 2016) and 806R (GGACTACHVGGGTWTCTAAT; Caporaso et al., 2011). The 25 µl PCR reaction consisted of Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Fisher Scientific), 10 ng of template DNA, and 0.4 µM of both primers. Thermal cycling consisted of 10 min initial denaturation at 95 °C, followed by 40 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s, followed by final elongation at 72 °C for 5 min. To add Ion Torrent PGM sequencing adapters and barcodes to the ends of the PCR product, one µl of the PCR product was used as a template in the second qPCR, where 10 cycles were performed using linker and fusion primers (0.04 µM of M13_515F–Y, 0.4 µM of IonA_IonXpressBarcode_M13 and P1_806R), with conditions otherwise identical to the first amplification (Mäki et al., 2016). Products were purified with the Agencourt AMPure XP purification system (Beckman Coulter Life Sciences, Indianapolis, IN, USA), quantified with Qubit™ dsDNA HS assay, and pooled in equimolar quantities for sequencing on Ion Torrent PGM using Ion PGM Hi-Q View OT2 Kit for emulsion PCR, PGM Hi-Q View Sequencing Kit for the sequencing reaction, and Ion 316 Chip v2 (all Life Sciences, Thermo Fisher Scientific). A 16S rRNA gene sequence analysis was done using mothur (version 1.39.5; Schloss et al., 2009), as in Aalto et al. (2018). The total number of sequences obtained was 294,464 and after subsampling, there were 13,245 sequences per sample. The sequences have been submitted to the NCBI Sequence Read Archive under BioProject PRJNA549384.

2.5. Gene copy numbers and nitrification rates

The abundance of 16S rRNA gene in the biofilter biofilm, tank water, and biofilm, as well as the nitrification genes in the biofilter biofilm, was quantified with qPCR. For the 16S rRNA gene, the primer pair 515F–Y and 806R was used and with the amplification protocol details mentioned above.

To quantify the abundance of the ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), and complete ammonia-oxidizing (comammox) bacteria in the biofilter samples, qPCR quantifications were performed using published primer pairs for AOA, AOB, NOB, and comammox. The abundance of AOA was quantified using $amoA_{archaea}$ primers (Francis et al., 2005), and the abundance of AOB using $amoA_{bac}$ primers (Rotthauwe et al., 1997), with the protocols described by Aalto et al. (2018). For NOB abundances, the primers used were *nxr*AF1/R2 (Poly et al., 2008; Wertz et al., 2008) and *nxr*B169F/638R (Pester et al., 2014). Comammox *Nitrospira* clade A and clade B abundances were quantified with the comaA244F/659R and comaB224F/659R primers (Pjevac et al., 2017). All qPCR reactions included 10 ng of template DNA, forward and reverse primers, and 1x Maxima SYBR Green/Fluorescein Master Mix (Thermo Fischer) in a total volume of 25 µl. The thermal conditions were as follows: initial denaturation 10 min at 95 °C, then 35 cycles at 95 °C for 30 s, 52–59 °C for 30 s, and 72 °C for 30 s. Amplification efficiencies were between 83-97% for the qPCR assays. The quantification was performed using the CFX96 qPCR thermal cycler (Bio-Rad).

Bioreactor nitrification rates (g NO_x h^{-1}) were measured following Pulkkinen et al. (2018) at the end of the experiment. Briefly, 30 biofilter carrier media pieces were incubated with $^{15}NH_4^+$ of 5 mg L^{-1} for 3 h, and the stable isotope composition of nitrite and nitrate was measured at the beginning and end of the incubation.

2.6. Water quality measurements

Water quality was monitored with an online monitoring system consisting of a spectrometer probe (spectro:lyser, s:can, Vienna, Austria), a carbon dioxide sensor (Franatech, Lüneburg, Germany), a pH probe (pH:lyser, s:can, Vienna, Austria) and an optical oxygen probe (oxi:lyser, s:can, Vienna, Austria) located in the fish tanks. The spectrometer probe measured turbidity, total suspended solids, and UV254 absorbance. Total ammonia nitrogen (TAN), nitrite, and nitrate were analyzed once a week from tank outlet water using a spectrophotometer (Procedure 8038 Nessler, LCK341/342 and LCK340 respectively, DS 3900, Hach, Loveland, USA). Alkalinity was analyzed once a week by titration with a standard method (ISO 9963–1:1994) (TitraLab AT1000, Hach, Loveland, USA).

2.7. Statistical analyses

The effects of PAA applications on water quality values, feed conversion ratio (FCR), specific growth rate (SGR), mortality and gene copy numbers and microbial community abundances were analyzed with linear regression analysis using SPSS Statistics software, version 25. Gene abundances were log-transformed before analysis to meet assumptions. The changes in the microbial community composition were assessed using the “vegan” (Oksanen et al., 2017) and “phyloseq” (McMurdie and Holmes, 2013) packages in R (R Core Team, 2018; version 3.5.1).

3. Results

3.1. Biofilter microbial community

Based on the NMDS plot, the microbial community composition changed from week 8 to week 13 in all tanks. At week 8, communities in control units and those receiving four PAA applications per week

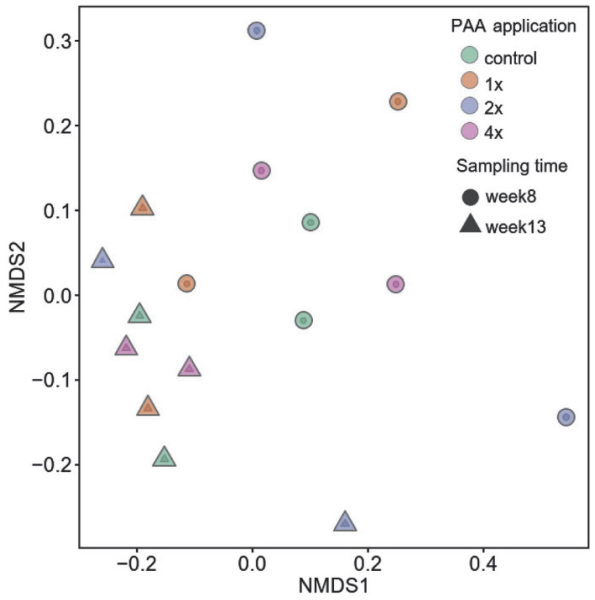

Fig. 2. Non-metric multidimensional scaling (NMDS) of bacterial communities in RAS biofilters under different PAA application frequencies (control, 1, 2, and 4 applications per week) at weeks 8 and 13.

were more similar than those with one or two PAA applications, which were more dispersed, while at week 13, the communities were quite similar in all PAA application levels (Fig. 2). The dominant phylum across all samples was Proteobacteria (28–44% of sequences) (Fig. 3). The next most abundant OTUs were from phyla Gemmatimonadetes (5–19% of sequences) and Bacteroidetes (20–45% of sequences). Although the abundance of proteobacterial classes Deltaproteobacteria (8% vs 6–7% of all sequences) and Gammaproteobacteria (12% vs 7–10% of all sequences) decreased slightly with an increased PAA application rate (Fig. 3), the overall abundance of Proteobacteria was unaffected by the PAA application frequency (linear regression, $P > 0.05$). The species richness (Chao1) increased from week 8 to week 13 except in the other one-time PAA application per week unit. The diversity generally increased from week 8 to 13, and was higher in control units than in units with PAA applications (Suppl. Fig. 1).

When only nitrifying taxonomic groups were considered (Fig. 3/ Suppl. Fig. 2), the number of sequences assigned to AOB (family Nitrosomonadaceae) appeared to decrease with the increasing PAA application frequencies at week 8 (117–235 reads, 0.7–1.3% of all sequences), but the difference was not statistically significant ($P = 0.052$). At week 13, the amount of AOB reads was very similar between PAA application frequencies (95–244 reads, 0.5–1.4% of all sequences; $P = 0.703$). The main genera were unclassified Nitrosomonadaceae (weeks 8, 13; 26–74% of AOB reads) and *Nitrosomonas* (week 13; 13–42% of AOB sequences). The number of sequences assigned to NOB/comammox (genera *Nitrospira*, *Nitrobacter*) was 0.4–3.5% of all sequences (79–623 reads) at week 8, while at week 13, it was 1.6–6.9% (284–1232 reads). The PAA application frequency did not affect the number of NOB/comammox sequences ($P > 0.05$). *Nitrospira* was the dominant genus in all samples (90–100% of NOB sequences).

3.2. Microbial abundance and nitrification rates

The total amount of bacteria (16S rRNA gene copy numbers) in tank water was equal between the control and different PAA application frequencies at both weeks ($P > 0.05$). At week 8, the total amount of bacteria was $2.7 \pm 2.2 \times 10^9$ gene copies cm^{-2} in tank biofilm and $7.8 \pm 6.3 \times 10^{11}$ gene copies g^{-1} dw in biofilters, while at week 13, there were $1.4 \pm 1.5 \times 10^9$ gene copies cm^{-2} in tank biofilm and $9.4 \pm 5.9 \times 10^{10}$ gene copies g^{-1} dw in biofilters (Fig. 4). There was

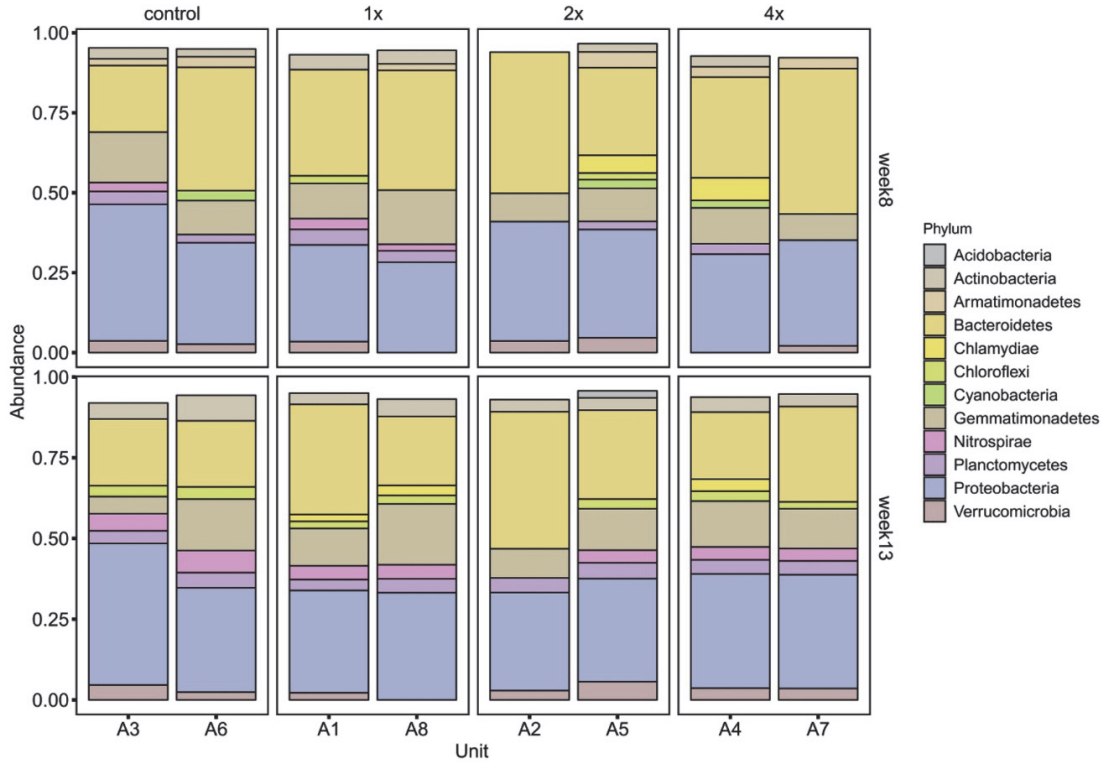

Fig. 3. The relative abundances of microbial phyla in biofilter biofilms under different PAA application frequencies (control, 1, 2, and 4 applications per week) at weeks 8 and 13.

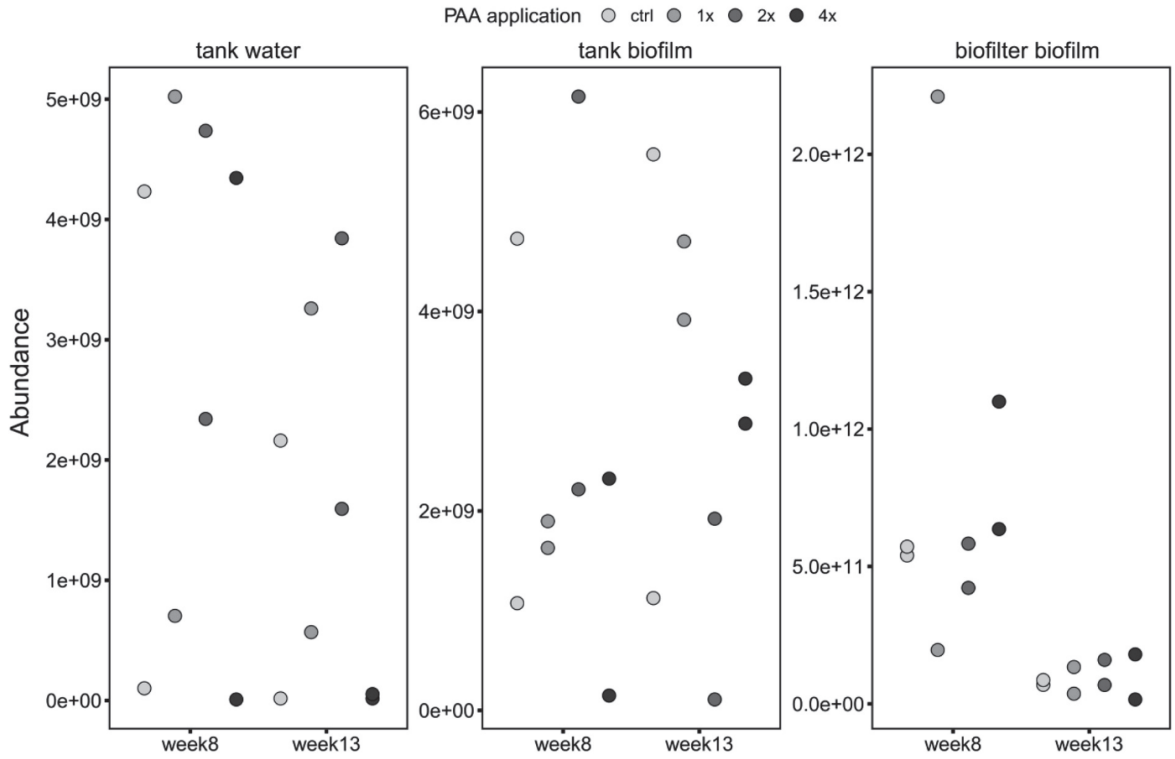

Fig. 4. The abundance of the bacterial 16S rRNA gene in tank water, in tank biofilm and in biofilter biofilm under different PAA application frequencies (control, 1, 2, and 4 applications per week). Abundance denotes gene copies ml^{-1} water for tank water samples, gene copies cm^{-2} for tank biofilm samples, and gene copies g^{-1} dw for biofilter biofilm samples.

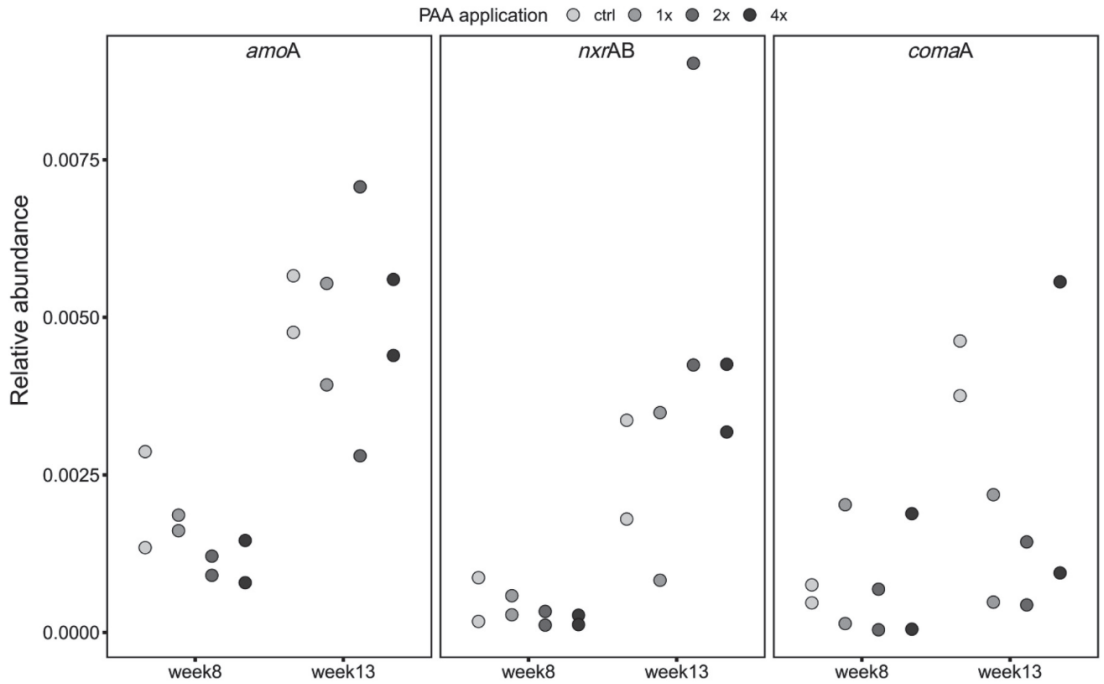

Fig. 5. The relative abundances of AOB (ammonia-oxidizing bacteria), NOB (nitrite-oxiding bacteria), and comammox (clade A) genes in biofilter under different PAA application frequencies (control, 1, 2, and 4 applications per week).

no significant relationship between total microbial abundances and PAA application frequencies neither in tank nor in biofilter biofilms. In biofilter samples, the copy numbers of nitrification genes were normalized against the total bacterial abundance. The relative abundance of the AOB gene was higher than the NOB or comammox genes, and ammonia-oxidizing archaea were not found in the samples. All the comammox *Nitrospira* were from clade A, while no comammox bacteria from clade B were observed in the samples. At week 8, the relative gene

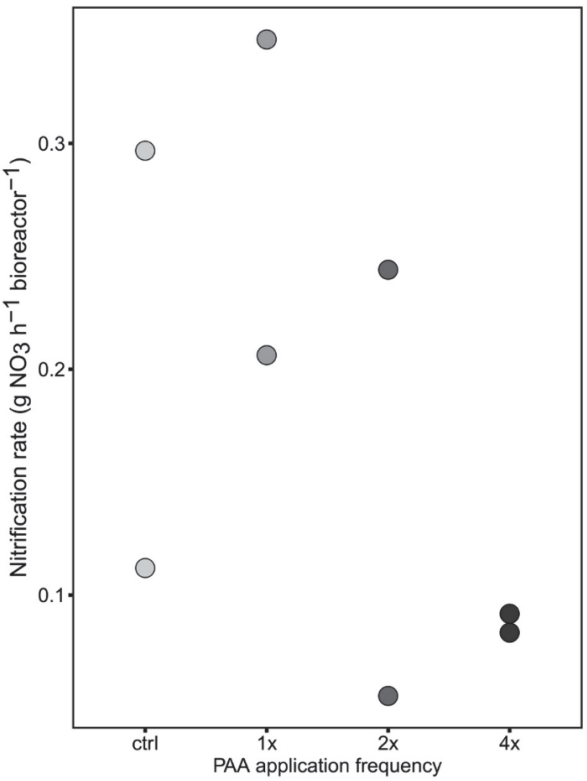

Fig. 6. The biofilter nitrification rates under different PAA application frequencies (control, 1, 2, and 4 applications per week) at week 13 at the end of the experiment.

copy numbers of AOB and NOB appeared to decrease with an increasing PAA application rate, but the relationship was not significant ($P = 0.092$) for AOB and for NOB. The abundance of comammox bacteria remained similar in all systems ($P > 0.05$). At week 13, at the end of the experiment, the relative abundance of all three nitrifying groups was higher than at week 8. The AOB copy numbers were equal between the units, while NOB abundance was highest in the units receiving biweekly PAA application, and comammox abundance in the control units and those receiving PAA four times per week (Fig. 5). Nitrification rates (g NO_3 h^{-1} bioreactor^{-1}) were highest in tanks with one PAA application per week (Fig. 6). With PAA applications of two and four times per week, the nitrification rate decreased and was lowest, at 0.09 g NO_3 h^{-1} bioreactor $^{-1}$, in tanks with four PAA applications per week (Fig. 6).

3.3. Water quality and fish performance

The turbidity and total suspended solids were lower in the 2 x PAA and 4 x PAA application rates but there was no significant relationship with PAA application frequency (Table 2, Suppl. Fig. 3; $P = 0.057$). Higher PAA application frequency significantly decreased TAN concentrations during the first part of the experiment ($P < 0.01$, weeks 3–8) and during the whole experiment ($P = 0.026$, weeks 3–13; Table 2). PAA additions did not significantly increase NO_2–N concentrations ($P > 0.05$), and NO_3–N concentrations were not affected by the PAA application frequency ($P > 0.05$, Table 1). In addition, the PAA application frequencies did not significantly affect fish performance when measured by FCR and SGR (Table 1), but the fish in the units receiving PAA four times per week had a 15% slower growth compared to fish in the control groups. However, mortality increased with increasing PAA application frequencies ($P < 0.01$, Table 1; Table 2).

Table 1
The mean water quality parameters and observed fish performance under different PAA application treatments (0, 1, 2, and 4 applications per week, PAA dosed 1.1 mg L^{-1} twice per day) ± SD. FCR = Feed conversion ratio, SGR = Specific growth rate.

	Mean of weeks 3–7 (± SD)				Mean of weeks 8–13 (± SD)				Mean of weeks 3–13 (± SD)			
Treatment	0	1	2	4	0	1	2	4	0	1	2	4
CO_2 (mg l^{-1})	8.9 ± 0.9	8.7 ± 0.4	7.4 ± 1.5	8.9 ± 0.5	11.0 ± 1.8	9.8 ± 0.6	8.9 ± 2.0	10.6 ± 0.8	9.8 ± 1.3	9.2 ± 0.5	8.0 ± 1.6	9.6 ± 0.8
UV254 (Abs m^{-1})	29.7 ± 4.2	26.5 ± 2.4	30.2 ± 1.1	24.0 ± 0.1	29.6 ± 5.7	27.4 ± 2.4	30.6 ± 0.2	26.4 ± 0.9	29.7 ± 4.8	26.9 ± 0.4	30.2 ± 0.9	25.0 ± 0.9
Turbidity (FTU)	2.0 ± 0.3	1.4 ± 0.2	1.8 ± 0.1	1.1 ± 0.3	1.8 ± 0.2	1.4 ± 0.1	1.9 ± 0.0	1.2 ± 0.1	1.9 ± 0.2	1.4 ± 0.1	1.9 ± 0.0	1.2 ± 0.1
TSS (mg l^{-1})	5.8 ± 0.4	3.9 ± 0.6	5.1 ± 0.5	3.0 ± 1.0	5.2 ± 0.3	3.9 ± 0.5	5.2 ± 0.1	3.1 ± 0.7	5.5 ± 0.4	3.9 ± 0.2	5.2 ± 0.2	3.0 ± 0.7
TAN (mg l^{-1})	0.93 ± 0.11	0.78 ± 0.08	0.78 ± 0.01	0.57 ± 0.01	0.80 ± 0.1	0.70 ± 0.15	0.80 ± 0.05	0.61 ± 0.04	0.83 ± 0.09	0.75 ± 0.05	0.78 ± 0.04	0.58 ± 0.04
NO_2–N (mg l^{-1})	0.11 ± 0.01	0.12 ± 0.02	0.10 ± 0.01	0.10 ± 0.1	0.13 ± 0.02	0.17 ± 0.04	0.13 ± 0.04	0.10 ± 0.02	0.12 ± 0.01	0.15 ± 0.02	0.12 ± 0.03	0.10 ± 0.02
NO_3–N (mg l^{-1})	54.6 ± 0.6	52.1 ± 4.2	56.8 ± 4.0	48.1 ± 4.6	60.2 ± 2.3	61.1 ± 9.7	65.5 ± 4.5	54.8 ± 3.5	55.5 ± 1.5	55.4 ± 7.4	57.5 ± 1.8	48.7 ± 3.5
Alkalinity (mg l^{-1})	62.3 ± 1.3	57.2 ± 2.3	42.7 ± 14.5	71.5 ± 2.6	63.1 ± 6.6	51.7 ± 1.6	45.9 ± 17.5	69.4 ± 3.3	62.1 ± 3.9	53.0 ± 1.4	44.3 ± 16.1	68.5 ± 3.3
pH	7.3 ± 0.0	7.3 ± 0.1	7.1 ± 0.1	7.3 ± 0.0	7.3 ± 0.1	7.3 ± 0.1	7.1 ± 0.1	7.4 ± 0.0	7.3 ± 0.0	7.2 ± 0.1	7.1 ± 0.1	7.4 ± 0.0
FCR	1.11 ± 0.1	0.92 ± 0.14	1.01 ± 0.14	1.21 ± 0.0	1.01 ± 0.05	1.12 ± 0.11	1.08 ± 0.01	1.11 ± 0.02	1.05 ± 0.02	1.02 ± 0.12	1.04 ± 0.07	1.16 ± 0.02
SGR (% d^{-1})	1.35 ± 0.18	1.32 ± 0.14	1.45 ± 0.06	1.28 ± 0.03	1.08 ± 0.05	1.04 ± 0.04	1.06 ± 0.0	1.03 ± 0.03	1.23 ± 0.07	1.19 ± 0.09	1.27 ± 0.04	1.17 ± 0.03
End weight (g)	303 ± 32	286 ± 22	308 ± 33	286 ± 9	428 ± 39	401 ± 36	431 ± 46	384 ± 12	428 ± 39	401 ± 36	431 ± 46	384 ± 12
Mortality (%)	2.9 ± 0.0	2.9 ± 2.9	2.9 ± 2.9	7.1 ± 1.4	2.9 ± 0.0	4.4 ± 1.3	4.4 ± 1.3	4.6 ± 0.0	5.7 ± 0.0	7.1 ± 1.4	7.1 ± 1.4	11.4 ± 0.0

Table 2
The linear regression models on the interactions between water quality parameters and different PAA application frequencies (0, 1, 2, and 4 applications per week). Data is shown when $p < 0.1$. TSS = total suspended solids, TAN = total ammonia nitrogen.

Variable	Week	Equation	R^2	*p* value
Turbidity	3–8	y = −2.465 x + 5.724	0.46	0.064
TSS	3–8	y = −0.800 x + 5.291	0.46	0.067
TAN	3–8	y = −8.709 x + 8.412	0.73	0.007
TSS	8–13	y = −1.008 x + 6.136	0.42	0.079
Turbidity	3–13	y = −2.900 x + 6.390	0.48	0.057
TSS	3–13	y = −0.945 x + 5.921	0.48	0.057
TAN	3–13	y = −10.370 x + 9.398	0.59	0.026
Mortality	3–13	y = 58.33 x - 2.917	0.70	0.010

4. Discussion

In this study, we demonstrated the effect of different PAA application frequencies on biofilter microbiome function and composition, especially on the key nitrification microbes. We also examined the changes in biofilter nitrification rates, water quality, and fish performance in response to PAA application frequency. We observed that the overall microbial community composition remained quite stable, and nitrification bacteria did not substantially suffer from PAA applications, increasing in abundance during the experiment. More frequent PAA application decreased biofilter nitrification rates but yet could decrease the TAN values. PAA application frequencies did not significantly affect fish growth, but a higher mortality rate and increased slime formation were observed with the highest PAA application (four times per week).

Until this study, knowledge of the response of the biofilter microbiome to PAA applications has been scarce. In a few previous studies, a focus on the effect of a bi-weekly pulsed PAA addition of 1 mg L^{-1} on RAS microbiology has been found to decrease the overall bacterial counts in the tank water and to nearly completely remove the biofilm in the fish tanks in RAS (Liu et al., 2017a, 2018), while enhanced biofilm formation has been observed under continuous PAA applications, which has been explained by the acetic acid and formed acetate feeding the heterotrophic microbial community (Liu et al., 2017a). Furthermore, when a prolonged low PAA dosage has been used, no effect on microbial counts has been observed, resulting in a minimum PAA threshold estimate of 0.30 mg L^{-1} for disinfectant purposes in RAS (Davidson et al., 2019). In agreement, we observed that the abundance of tank water microbes, either free-living or attached to particles, was unaffected by a PAA addition. However, when weeks were inspected separately, we observed that bacterial abundance was higher, yet statistically insignificant, in the units receiving a PAA application one or four times per week in both tank and biofilter biofilms at week 8. In the tanks at week 13, the PAA application rate had no overall effect on bacterial biofilm, because the bi-weekly PAA application rate was sufficient to decrease the bacterial biofilm, but the highest PAA application seemed to promote biofilm formation. In the biofilters, PAA had no clear effect on the biofilm bacterial abundance. It is likely that most of the PAA used was degraded before entering the biofilters, since it was added to the pump sump, explaining the minor beneficial and restricting effects of the PAA on the biofilm in the biofilters than in the tanks.

The dominating phylum was Proteobacteria in all RAS units throughout the experiment, while Bacteroidetes and Gemmatimonadetes were also relatively abundant. Previously, Proteobacteria and Bacteroidetes have found to be common phyla in RAS biofilter units in both freshwater and saltwater RAS, while Gemmatimonadetes were either absent or found in low abundance (Ruan et al., 2015; Gonzalez-Silva et al., 2016; Bartelme et al., 2017; Rud et al., 2017), highlighting the unique nature of microbiomes in each biofilter and RAS unit (Blancheton et al., 2013). In previous studies, *Nitrospira* has been found to be quite abundant in RAS biofilters (Bartelme et al., 2017; Keuter et al., 2017), the group including both traditional nitrite-oxidizers and comammox bacteria. Here, we found the amount of *Nitrospira* increasing from week 8 to week 13 in all units. Overall, PAA application frequency did not significantly change the biofilm bacterial community composition or decrease the diversity or species richness, agreeing with previous findings on perturbations affecting free-living microbes rather than deep biofilm layers (Wietz et al., 2009; Schreier et al., 2010).

When nitrifying microbes are the sole focus, both qPCR and sequencing results demonstrated that PAA application frequency had a slightly, yet statistically insignificant, negative effect on the nitrifier abundance at week 8. At week 13, genetic nitrification potential (gene copy numbers) and the sequences associated with NOBs/comammox increased compared to week 8 in all units. This indicates that the possibly negative effect of PAA application on the nitrification microbes decreased towards the end of the experiments, probably because the nitrification community could adapt and become less sensitive to PAA application. Previously, PAA additions have been found to only partially inhibit nitrification (Pedersen et al., 2009; Liu et al., 2017a), causing some accumulation of total ammonia nitrogen (TAN) and nitrate in RAS. Here, we measured nitrification rates using stable isotope incubations at the end of the experiment, and found higher PAA application frequency led to lower nitrification rates. However, we did not find the previously observed nitrite accumulation, suggesting that both ammonium oxidation and nitrite oxidation steps were suppressed, or that the abundant comammox bacteria were as or even more involved in ammonia oxidation than the traditional ammonia-oxidizers, releasing only nitrate as the end product.

In addition to changes in nitrification, the increased PAA application rate led to decreased turbidity and total suspended solids concentrations except in units with a PAA addition of twice a week, where values were closer to control units than other PAA application units. Decreased turbidity indicates that there were fewer large particles (Yao et al., 2014) because of the higher H_2O_2 degradation potential of organic matter. The observed decrease of total ammonia nitrogen with an increasing PAA addition was unexpected, because nitrification efficiency was lowest within the highest PAA addition. The direct oxidation of TAN is the likeliest explanation, because the slightly different daily feed consumption did not explain the difference in TAN concentrations between treatments. TAN values have previously been found to increase after a bi-weekly PAA addition due to lower nitrification rates (Liu et al., 2017a), and we also observed that the units with bi-weekly PAA applications behaved quite differently from those receiving one or four applications. There, we saw equal turbidity, TSS, TOC, TAN, and nitrate values with the control units, suggesting that this bi-weekly PAA addition promoted heterotrophic microbes more than removing them or improving water quality. However, one reason for the difference between bi-weekly and other PAA application frequencies is that the other 2x RAS unit (unit 2) showed a very different pattern in water quality and microbial results than the other systems. Furthermore, we saw that AOB and comammox microbes were less abundant in these systems, while the NOBs seemed to benefit there. In this experiment, PAA dosages were kept the same throughout the experiment, and feeding was increased from the first (mean 102 g d^{-1}) to the second (mean 127 g d^{-1}) part of the experiment. Thus, the potential PAA effect time decreased during the experiment (Pedersen et al., 2013), which explained why the water quality differences between the treatments were higher in the first part of the experiment.

The PAA additions did not significantly affect fish performance in terms of feed conversion ratio or specific growth rate. However, the total biomass growth was 14% lower in the units where PAA was added four times per week compared to other units. In addition, mortality increased with the highest PAA addition, indicating that continued applications of PAA (1.1 mg L^{-1} dosed twice per day), even below the reported no-observed-effect concentration (2.8 mg L^{-1}) (Straus et al.,

2018), were too high for the fish. High surface swimming was observed with the first additions of PAA, but this was not observed after a few weeks of additions, indicating the adaptation of fish to PAA as previously reported (Liu et al., 2017b; Gesto et al., 2018). The observed IPNV has low pathogenicity in Finland and mainly affects the first-feeding fry (Eriksson-Kallio et al., 2016). We thus concluded that it did not affect fish performance.

5. Conclusions

In conclusion, the microbial results suggest that the biofilter biofilm community is quite stable and less sensitive to PAA application, forming a "collaborome" in which heterotrophic microbes can support autotrophic nitrifiers rather than compete with them (Bartelme et al., 2017). Pulsed PAA applications disrupt nitrification, but the microbial community is capable of adapting and no long-term effect of PAA on inorganic nitrogen levels can be observed. Our study demonstrates that PAA application frequency has variable effects on microbes, water quality, and fish. Although the highest PAA application frequency ($1.1\ mg\ L^{-1}$ twice a day, four times per week) improved water quality slightly by directly oxidizing TAN, and potentially turbidity, and TSS, it led to 50% lower biofilter nitrification rates compared to the control units, and increased fish mortality. On the other hand, a bi-weekly application did not improve water quality, but fish performance was better than with other PAA application frequencies. Similarly, a one-time PAA application was too low to improve water quality, but did not interrupt nitrification or fish. The opposite chemical (i.e. direct oxidizing of TAN) and biological (i.e. decreasing nitrification thus increasing TAN) effects of PAA on water quality can complicate the interpretation of results. Based on these results, the continuous pulse applications of PAA are not a cost-efficient method for substantially improving water quality and controlling the microbial communities. However, if an accumulation of solids and/or total ammonia nitrogen is observed, PAA can be used in such cases to improve water quality.

Declaration of competing interest

The authors declare that there is no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734534.

Author contributions

All the authors planned the experiment. SS, JTP, and PL-L did the sampling and SS performed the DNA sequencing and qPCR measurements. SLA performed the statistical analyses of the microbial data, and JTP the water quality and fish performance analysis. All the authors contributed to manuscript writing.

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IV

MICROBIOLOGY OF DIFFERENT COMPARTMENTS OF FRESHWATER AND BRACKISH WATER RECIRCULATING AQUACULTURE SYSTEMS

by

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