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Doctoral thesis

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of
Philosophiae Doctor
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ISBN 978-82-326-6772-7 (printed ver.)
ISBN 978-82-326-5529-8 (electronic ver.)
ISSN 1503-8181 (printed ver.)
ISSN 2703-8084 (online ver.)

Doctoral theses at NTNU, 2022:383

Printed by NTNU Grafisk senter

# Acknowledgements

This PhD was established in August 2018 as a collaboration between SINTEF Ocean (Department of Aquaculture) and NTNU (Department of Biotechnology and Food Science) funded by Research Council of Norway, Grant 272400. The PhD was affiliated to two projects at SINTEF, "MonMic" (Norwegian Seafood Research Fund, Grant 901392) and "Lumpus" (Regional Research Fund North, Grant 269204) and one project at NTNU (funded by a commercial facility).

First, I would like to thank my main supervisor, Professor Ingrid Bakke and co-supervisor Kari Kihle Attramadal at NTNU. I am so very thankful for your advice, all your knowledge you have shared with me, useful discussions and for the support during these four years. You both have been a huge inspiration and you are truly dedicated supervisors. I will miss you both! I am also grateful to Olav Vadstein for introducing me to microbial ecology and for editing of the second paper, to ACMS group and master students Mari Birkeland and Sunniva Gaarden for your contribution in two of the projects.

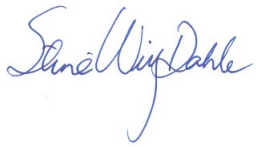
Thanks to Let Sea AS and Ecomarine Seafarm AS for the huge effort in the experiment with lumpfish. Especially Kristian Nordøy and Vebjørn Ulvang at Let Sea for your enthusiasm and for happy times shared at Dønna, and Ragnhild Olsen Fossmark for joining me at this beautiful island and cooperation in the projects. Also, to the commercial salmon smolt facilities involved, for letting us sample, for the interest in the projects and for all information you have shared.

A big thanks to my colleagues and friends at SINTEF Ocean. Especially my co-supervisor Roman Netzer and colleague Deni Ribičić, for analyses, great teamwork and cooperation in the third paper. Thanks to Marianne Aas for all practical work at the lab and for the logistics of the huge number of samples received. Thanks to Silje Forbord for sharing office with me, although covid-19 provided home office for too long. Thanks for unforgettable trips to conferences in Berlin and Montpellier, for all fun and encouragement during the years (you know who you

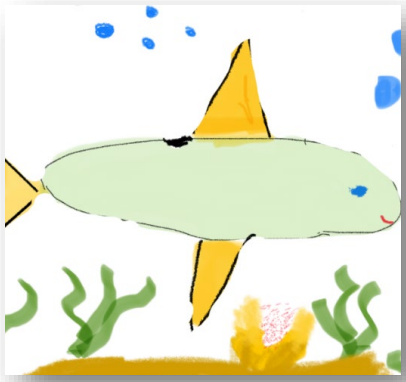
are). Thanks to Mats Mulelid for the nice illustrations used in my thesis. I also want to thank SINTEF for giving me the opportunity and for organizing my responsibilities such that I could manage both the ordinary research work at SINTEF and the PhD (Gunvor, Aleksander, Hans, Merete).

Thanks to my neighbour and designer Carl Nørstebø for the sketches of fish you made for me. Thanks to all my friends for support and for not giving up upon me during this busy period of my life.

Thanks to my mother and father, who introduced me to land-based aquaculture and aquaculture in general when I grew up in Flatanger. My mother and mother-in-law for being with my girls when time was scarce. Aleksander for being such a great dad to our girls and for all your support and love during this period. Finally, my two beautiful daughters, Agnes (7) and Signe (5) for distancing me from the PhD, with love and happiness every day, I love you!



Stine Wiborg Dahle, Trondheim September 2022



Fish by Agnes (7). The species is unknown.

# Summary

Recirculating aquaculture systems (RAS) has become a popular production system for fish, due to several benefits compared to the traditional flow-through systems. Reduced water consumption and the possibility for controlling and stabilizing the culture environment makes RAS a highly relevant technology. The microbial communities in RAS are essential for optimal physicochemical water quality and fish health. Despite progress, there is still limited knowledge on microbial community dynamics in RAS and especially in commercial systems. Characterisation of the spatial and temporal dynamics of the microbial community composition in RAS is necessary to provide knowledge on which factors influence their composition and how to ultimately steer the microbiota towards favourable conditions for good fish health. Conducting studies of both commercial systems and controlled experiments may contribute to such knowledge, but they have different advantages and disadvantages. By using small- or pilot-scale systems, studies with well-considered experimental design, including replicates and controls, can be carried out. Studies of commercial systems, may complement controlled experimental systems by providing important information at the relevant scale

In this thesis the spatial and temporal dynamics of the microbial community composition in RAS has been examined through studies of two commercial freshwater RAS producing Atlantic salmon fry and parr and an experiment with lumpfish juveniles reared in a seawater RAS with different water treatments. Microbiota of water and biofilm from rearing tanks and biofilter was characterized by using 16S rRNA gene amplicon sequencing, flow-cytometry, and culture-based methods.

The microbiota in rearing water and biofilter biofilm in one of the commercial RAS for salmon were variable over the 15 months period monitored. The organic matter load on the system significantly influenced the microbial communities of the system. Although the microbial communities changed during periods of fallowing, we observed a relatively fast return to a very similar community composition for each production period, probably as a result of a

similar selection pressure during all production batches. In the same commercial RAS, we found higher relative abundances of nitrite oxidising bacteria (NOB) than ammonium oxidising bacteria (AOB) in the nitrifying biofilter. The third most abundant *Nitrospira* OTUs were related to a previously identified complete ammonia oxidiser, comammox *Nitrospira nitrificans*. The biofilter biofilm and the water microbiota were significantly different but shared many common taxa and followed similar trends in temporal dynamics. In the second commercial RAS for salmon, fully maturated biofilters at inset of fish provided a more stable water microbiota with higher alpha diversities than the more immature and recently disinfected biofilter, indicating that K-selection acted on the suspended water microbiota, for beneficial fish-microbe interactions and a resilient system.

In the RAS experiment with lumpfish, where the HRT in fish tanks was long (60 min), we showed that in-line disinfection upstream of rearing tanks had negative effects on the microbial water quality and the fish health. In comparison, for both commercial RAS for salmon smolt production (where the HRT was 18-28 min), we found that the in-line UV treatment led to considerably lower regrowth of bacteria in the fish tanks.

In conclusion, well maturated biofilters and controlled and balanced organic loading might be characterizing a good microbial quality. In-line disinfection upstream of the rearing tanks in RAS with long HRT should be avoided, due to negative effects on microbial water quality and fish health. In RAS for salmon fry and parr production with short HRT, the negative effects of the UV treatment appeared to be reduced. To control the microbiota towards favourable conditions for good fish health, we need more knowledge about microbial dynamics and what characterizes optimal microbial water quality. This needs to be explored in the future.

# List of papers

**Paper I** **Dahle, S.W.**, Attramadal, K.J.K., Vadstein, O., Hestdahl, H.I., Bakke, I., 2022. Microbial community dynamics in a commercial RAS for production of Atlantic salmon fry (*Salmo salar*). Aquaculture, 546, 737382.

**Paper II** **Dahle, S.W.**, Gaarden, S.I., Buhaug, J.F, Netzer, R., Attramadal, K.J.K Busche, T., Aas, M., Ribičić, D., Bakke, I. Microbial community structures and dynamics in a commercial RAS during seven production batches of Atlantic salmon fry (*Salmo salar*). Submitted to Aquaculture.

**Paper III** **Dahle, S.W.**, Bakke, I., Nordøy, K., Birkeland, M., Dalum, A.S, Attramadal, K.J.K., 2020. Production of lumpfish (*Cyclopterus lumpus* L.) in RAS with distinct water treatments: Effects on fish survival, growth, gill health and microbial communities in rearing water and biofilm. Aquaculture, 522, 735097.

# Definitions

| | |
|---|---|
| Salmon fry | When the yolk sac is almost consumed, and the fish are transferred into the start-feeding department. At this point the salmon are called a fry, typically from 0.2 to 7 grams. |
| Salmon parr | After the fry stage the salmon becomes a parr, distinguished by parr marks spaced along the sides, typically from 7 to 70 grams. |
| Salmon smolt | A juvenile salmon undergone smoltification and is adapted to seawater, typically from 70-120 grams. |
| Lumpfish juvenile | In this thesis lumpfish juvenile refers to lumpfish of 0.5 grams and up to 53 grams at transfer to sea cages. |
| Salinity | Salinity is the amount of salt dissolved in a body of water. For this thesis the term is given in parts per thousand (ppt). |
| Freshwater | In this thesis the term freshwater is used for 0-3 ppt salinity. |
| Brackish water | In this thesis the term brackish water is used for 3-25 ppt salinity. |
| Seawater | In this thesis the term seawater is used for water over 25 ppt salinity. |
| Microbiota | The assemblage of living microorganisms (in this thesis bacteria) in a defined environment. |

# Contents

# Chapter 1. Introduction

## 1.1 Land-based production of salmon and lumpfish

The production of Atlantic salmon (*Salmo salar*) is one of the most profitable fish production industries worldwide (Garlock et al., 2020). The production is traditionally separated in a land-based phase (production of smolt) and a phase in open net pens in the ocean (on-growing until harvest) (Fig. 1). In hatcheries, roe, alevins and parr are reared in freshwater. At a size of 50-80 grams, the parr undergoes a physiological and morphological transition to achieve seawater tolerance, called smoltification. A salmon that has just completed this transition is defined as a smolt, typically 70-120 grams (Bergheim et al., 2009). The smolts are transported by well boats to the net pens for growth until slaughter (Fig. 1). In 2021 nearly 420 million Atlantic salmon smolts were sold for further cultivation at sea in Norway. This was 160 million more than in 2010 (Norwegian Directorate of Fisheries, 2022a), reflecting a substantially increased productivity of smolt.

In the production phase at sea, infections by the salmon louse (*Lepeophtherius salmonis*) are a major problem facing the Atlantic salmon industry. The parasite grazes on the skin and mucosal tissue of the fish and increases the vulnerability to infections and diseases (Dawson et al., 1998; Finstad et al., 2000; Denholm et al., 2002). Several methods, including mechanical, thermal, and chemical treatments, have been developed to combat sea lice (Powell et al., 2018; Guragain et al., 2021). These methods are not completely effective, are costly and require significant handling of the salmon, which stresses and affects the fish (Guragain et al., 2021). The use of chemicals has also led to the development of lice resistance against several chemicals and environmental concerns (Denholm et al., 2002; Guragain et al., 2021).

The lumpfish (*Cyclopterus lumpus* L.) is used extensible as a strategy for biological control in aquaculture due to its appetite for sea lice. The use of lumpfish to reduce sea lice infestations give environmental benefits without affecting the salmon directly (Fig. 1) (Imsland et al., 2014; Powell et al., 2018; Imsland et al., 2018). Due to these benefits, as well as increased lice resistance to chemical treatments, a new aquaculture sector emerged around 2011 in Norway (Imsland pers. Comm; Imsland et al., 2014), producing lumpfish juveniles in land-based

systems (Powell et al., 2018). Today lumpfish is the second most farmed fish in Norway after salmon, with 25 million fish produced in 2021 (Norwegian Directorate of Fisheries, 2022b).

Another strategy in salmon production to avoid exposure of lice and other environmental challenges in the sea, is to extend the land-based production phase, either as post-smolt production up to 1 kg or full life cycle. Extended production time on land is implemented for an increasing number of companies world over (Davidson et al., 2016; Bjørndal and Tusvik, 2020).

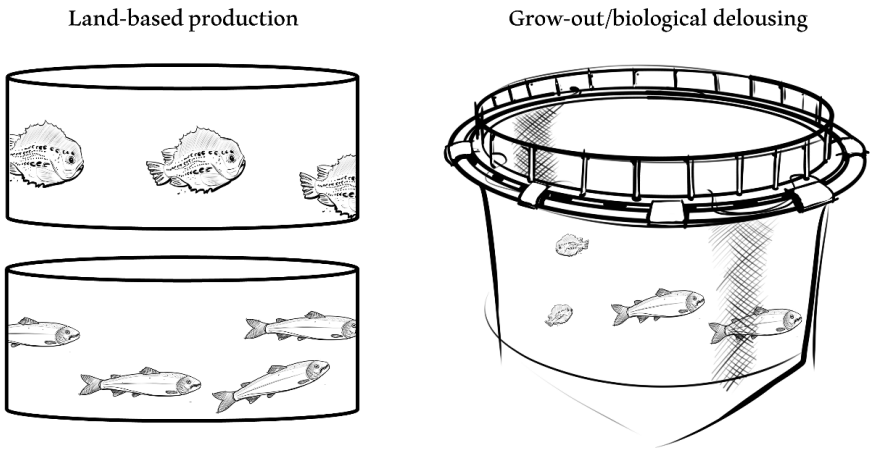



Figure 1. In the first phase, the Atlantic salmon smolts and lumpfish juveniles are produced in land-based systems. In the next phase the salmon and lumpfish are transported to sea cages for grow-out and biological delousing, respectively. Sketches: Carl Nørstebø.

## 1.2 Principles of Recirculating Aquaculture Systems

Recirculating aquaculture systems (RAS) are land-based systems for fish production where the water is reused to a high degree (typically 95-99%) (Timmons and Eberling, 2013; Lekang, 2013). RAS have become a popular technology for producing Atlantic salmon smolts globally (Bergheim et al., 2009; Badiola et al., 2012; Kolarevic et al., 2014; Davidson et al., 2017), whereas lumpfish is produced in traditional flow-through systems (FTS) in Norway (Roalkvam et al., 2019). In contrast to RAS, FTS requires supply of large amounts of new water. As available freshwater is decreasing worldwide, reducing water consumption in aquaculture is a necessity and makes RAS technology a highly relevant alternative (Martins et al., 2010; Timmons and Eberling, 2013; Kolarevic et al., 2014). In addition, RAS represent a unique

possibility for controlling and stabilizing the culture environment. In theory this means that the fish can obtain the best growth, survival and disease resistance in RAS, while maximizing the potential of a confined water resource (Blancheton et al., 2013; Dalsgaard et al., 2013; Kolarevic et al., 2014). Other advantages of RAS include siting facilities near seafood markets, and more concentrated waste streams that effectively can be treated and repurposed for value-added opportunities (van Rijn, 2013). On the other hand, RAS typically require high investment costs and the technology is highly complex (Dalsgaard et al., 2013; Timmons and Eberling, 2013).

Fish use oxygen and excrete metabolic waste products like carbon dioxide ($CO_2$) and ammonia to the surrounding water. In addition, organic matter is released to the surrounding water by the fish as faeces and excess feed. To maintain a proper water quality, oxygen must be added, and the produced waste must be treated in RAS (Timmons and Eberling, 2013; van Rijn, 2013). Therefore, RAS include biological filtration for conversion of toxic ammonia to less toxic nitrate (1.2.1), mechanical removal of particles (1.2.2), $CO_2$-degassing (1.2.4) and oxygenation (Fig. 2). RAS also need pH-regulation to make up for the buffer capacity consumed by the biofilter process (Fig. 2). Some systems also include disinfection (1.2.3). Systems that use very little new water also need to incorporate denitrification and phosphorus removal to control the accumulation of nitrate and phosphorus (van Rijn et al., 2006; Davidson et al., 2017).

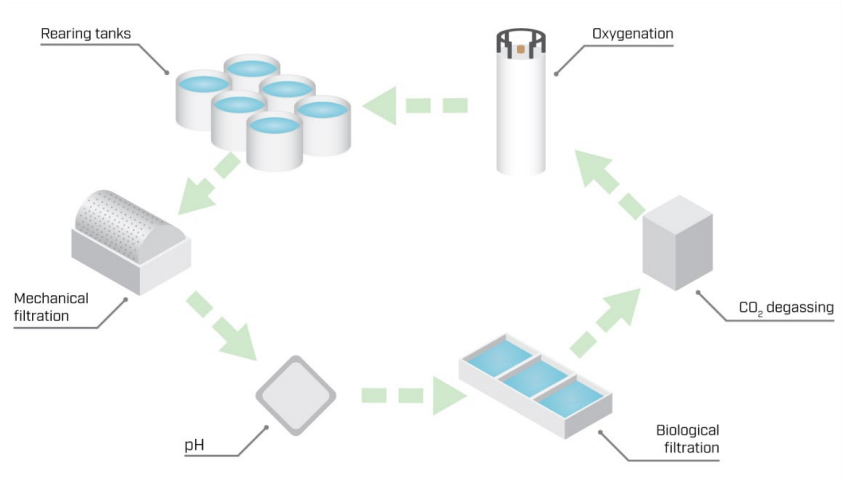



Figure 2. Schematic presentation of RAS with different treatment components. The components and the order typically vary from facility to facility. Illustration by Mats Mulelid, SINTEF.

### 1.2.1 Biological Filtration

In intensive systems with reuse of the water like RAS, it is essential to continuously remove ammonia to maintain safe concentration for the fish (Timmons and Eberling, 2013; Davidson et al., 2017). Ammonia nitrogen is present as un-ionized ammonia nitrogen ($NH_3$-N) and ammonium ion nitrogen ($NH_4^+$-N) in water, which together comprise the total ammonia nitrogen (TAN). Of the two forms of ammonia, $NH_3$ is the most toxic (Lawson, 1995). The toxicity for a certain TAN concentration increases with increasing pH as the chemical equilibrium is shifting to relatively more $NH_3$ (Timmons and Eberling, 2013). Salinity and temperature also affect the percentage of $NH_3$ (Anthonisen et al., 1976; Timmons and Eberling, 2013).

In RAS, ammonia accumulation is prevented by the biological water treatment in biofilters (Fig. 2), where nitrification is carried out by bacteria in biofilm attached to a carrier media (Malone and Pfeiffer, 2006). Biofilms are layers of bacteria that form on varios surfaces, enclosed in a polysaccharide matrix (Wietz et al., 2009). The formation of biofilm protects the microbial community from environmental stressors. In addition, biofilm formation facilitates interactions between members of the community, which gives more opportunities for

nutrient and genetic exchange, increasing chances of survival (Flemming and Wingender, 2010; Flemming et al., 2016).

The most common biofilter technology in Norway are moving bed biofilters (MBBF) and fixed bed biofilters (FBBF) (Ødegaard et al., 1994, Fjellheim et al., 2017). In FBBF the biofilter material is not moving, whereas in MBBF it is kept in constant movement during operation. FBBF filters particles, and require frequent backwashing, whereas the MBBF need less maintenance and constantly release biofilm particles to the water (Fjellheim et al., 2017). Conversion of ammonia is achieved by the autotrophic nitrifying bacteria in the biofilter (Fig. 2). Nitrification includes two steps; first ammonia is oxidized to $NO_2^-$ (Eq. 1) by ammonia oxidizing bacteria (AOB). In the second step $NO_2^-$ is oxidized to $NO_3^-$ (Eq. 2) by nitrite oxidizing bacteria (NOB). These two processes are energetically poor, leading to slow growth rates for the nitrifying bacteria (Costa et al., 2006).

$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O$ (Equation 1)

$NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$ (Equation 2)

Typical AOB genera identified in RAS biofilters are *Nitrosomonas*, *Nitrosospira, Nitrosovibrio,* and *Nitrosococcus* (Foesel et al., 2008; Schreier et al., 2010*;* Brown et al., 2013*;* Bartelme et al., 2017; Navada et al*.* 2019; Nevada et al., 2020a; 2020b; Roalkvam et al., 2020; Ma et al., 2021). Besides bacteria, ammonia oxidizing archaea (AOA) are abundant nitrifiers in RAS (Sakami et al., 2012; Brown et al., 2013; Bartelme et al., 2017; Bartelme et al., 2019; Roalkvam et al., 2020). NOB genera observed in RAS biofilters are *Nitrospira*, *Nitrospina*, *Candidatus Nitrotoga* and *Nitrobacter* (Schreier et al., 2010; Brown et al., 2013; Ruan et al., 2015; Hüpeden et al., 2016; Gonzalez-Silva et al., 2016; Bartelme et al., 2017; Navada et al*.,* 2019; 2020a; 2020b). Nitrifying bacteria belonging to the genus *Nitrospira* that perform complete ammonia oxidation (comammox), i.e., converting ammonia directly to nitrate, were recently identified in freshwater RAS (Daims et al., 2015; van Kessel et al., 2015; Bartelme et al., 2017; 2019; Hüpeden et al., 2020). Currently, the overall understanding of factors that govern the distribution and abundance of comammox is unclear. However, several studies show that comammox *Nitrospira* are more abundant in habitats with relatively low ammonia concentrations (Costa et al., 2006; Sobotka et al., 2018; Bartelme et al., 2019).

### 1.2.2 Particle removal

Particles in RAS are originating from faecal waste, uneaten feed and bacteria growing in the system (Chen et al., 1993; Becke et al., 2018). Large particles (>40-60 µm) of particulate organic carbon (POC) are removed from the culture water in RAS. The two most used technologies used for particle removal in salmon production are drum filters and belt filters (Timmons and Eberling, 2013). However, fine suspended solids (<20 µm) and dissolved organic carbon (DOC), often remains, and accumulate in the system and can constitute over 90% of all particles in RAS (Chen et al., 1993; Fernandes et al., 2014; 2015). Suspended solids are the source of most of the water quality issues in RAS, as they have an important impact on the performance of nearly all water treatment components and the fish. Fine particles can affect fish health by irritating gills (Bullock et al., 1994; Au et al., 2004) and stress fish (Lake and Hinch, 1999; Awata et al., 2011), although susceptibility varies among fish species (Lake and Hinch, 1999; Becke et al., 2018). POC and DOC provide substrate and growth of heterotrophic bacteria, and high supply increase the bacterial numbers and microbial activity in the system (Léonard et al., 2000; Pedersen et al., 2017; Rojas-Tirado et al., 2018), reduce nitrification efficiency (1.3.1) (Chen et al., 1993) as well as disinfection efficiency (1.2.3) (Hess-Erga et al., 2008; Carré et al., 2018). In addition, high levels of organic matter increase the risk of formation of anaerobic zones which can yield production of toxic hydrogen sulphide ($H_2S$) (1.3.1) (Letelier-Gordo et al., 2020).

### 1.2.3 Disinfection

Most RAS include filtration and disinfection of the intake water (Fig. 3, #1), as a biosecurity measure. In-line disinfection of water in the RAS water treatment circuit can also be used to eliminate pathogenic organisms, reduce concentration of bacteria, and to improve water quality (Summerfelt, 2003; Davidson et al., 2011; Davidson et al., 2021). There are no universal guidelines for the location of in-line disinfection. Many RAS have in-line disinfection right in front of the rearing tanks (Fig. 3, #2), others place it before the biofilter (Vadstein et al., 2018b) (Fig. 3, #4), and some do not include disinfection in the RAS loop at all. Finally, dosing of liquid disinfecting agents directly in the rearing tanks may be performed occasionally for treating the reared species or as water disinfection (Fig. 3, #3) (Pedersen and Pedersen, 2012; Pedersen et al., 2013; Mota et al., 2022).

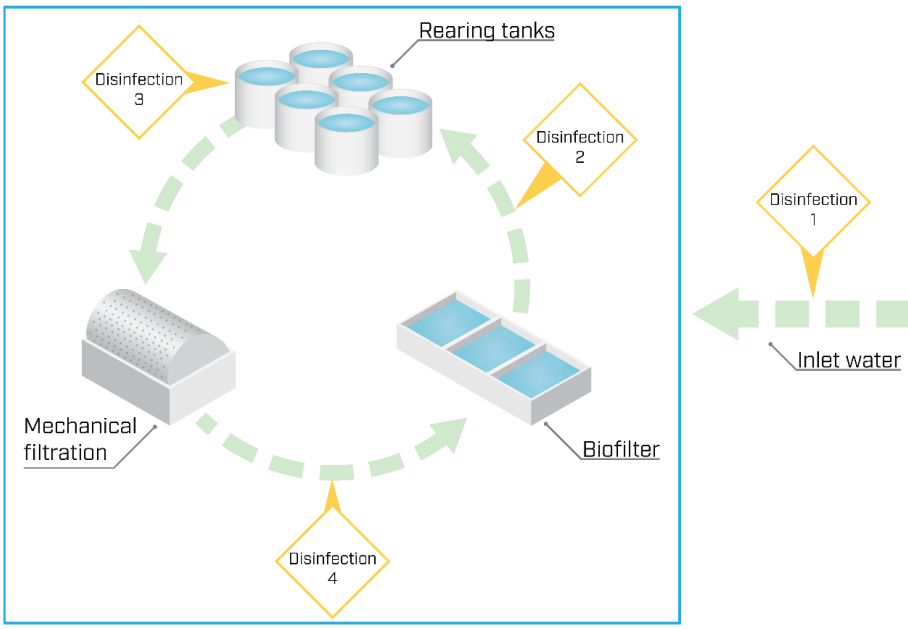



Figure 3. Water disinfection at different locations in RAS: 1) Inlet water, 2) right in front of the rearing tanks, 3) directly into the rearing tanks, 4) before the biofilter. Illustration by Mats Mulelid, SINTEF.

Biofilms develop on all surfaces in RAS and represent a reservoir of bacteria, and therefore potentially for opportunistic pathogens, that can be released to the water (King et al., 2004; Wietz et al., 2009). Thick biofilms increase also the risk of $H_2S$ production to levels that can affect the fish. Implementation of regular cleaning of biofilm is hence crucial (Lazado and Good, 2021). The biofilter is disinfected when the production struggles with suspected or identified pathogenic microorganisms in the system. Some facilites also practises regular disinfection of biofilters as a preventive measure (Tørud et al., 2019). Knowlegde on biofilms as pathogen reservoirs and the significance of disinfection of biofilters as a biosecurity practice is however lacking.

The most common disinfection methods of water in RAS involves ultraviolet light (UV) and/or ozone ($O_3$) (Summerfelt, 2003). However, for in-line disinfection in RAS, only UV is used at a disinfecting dose. Ozone is normally used at a low dose that do not represent disinfection, but for water quality improvement (Summerfelt, 2003; Summerfelt et al., 2009). Ozone or rest oxidants formed after ozonation inactivates fish pathogens by inducing changes in the cell membrane that lead to leakage of proteins and nucleic acids (Liltved et al., 1995; Summerfelt, 2003; Davidson et al., 2021). Ozone addition decrease the abundance of bacteria in water

(Liltved et al., 1995; Davidson et al., 2021; Aalto et al., 2022) and improves water quality by micro flocculation of fine particulate matter and oxidation of non-biodegradable organic molecules, nitrite, and other molecules (Summerfelt et al., 2009; Good et al., 2011; Davidson et al., 2011; Teitge et al., 2020). One of the main drawbacks of ozone addition in RAS is the reaction at high doses with bromide ions in brackish and seawater that can produce harmful by-products (Reiser et al., 2011). Ozone residuals can also be hazardous to fish and human health if the concentrations are not properly controlled (Summerfelt et al., 2009).

UV is electromagnetic radiation. UV light wavelengths of 254 nm are the most effective for inactivating microorganisms. UV damages microorganisms by altering the nucleic acids (Liltved et al., 1995). The effect can be temporal or lethal depending on the repair mechanisms (photo-reactivation or dark repair) and the degree of UV resistance of the cell (Liltved and Cripps, 1999; Timmons and Eberling, 2013). UV irradiation has been shown to inactivate microorganisms (e.g. Sharrer et al., 2005; Huyben et al., 2018), destroy dissolved ozone (Summerfelt et al., 2004) and reduce microparticle numbers by destroying bacteria (de Jesus Gregersen et al., 2020). A major advantage of using UV irradiation for disinfection in RAS is that it does not leave toxic residuals or by-products that pose a risk to fish or humans (Timmons and Eberling, 2013).

Effective disinfection depends on the dose, contact time, particle size and particle density in the water to be treated (Timmons and Eberling, 2013). Particles reduces the disinfection effect by protecting bacteria and virus from the disinfectant by shadowing or consume of the disinfectant or embedding the microorganisms in particulate matter (Liltved and Cripps, 1999; Hess-Erga et al., 2008). Hence, it can be difficult to inactivate most of the microorganisms even at an excessive dose of disinfectant at high turbidity (Sharrer et al., 2005).

### 1.2.4 $CO_2$ degassing

$CO_2$ produced by fish and bacteria can accumulate to high concentrations in the water, if not adequately removed (Fivelstad, 2013). Long-term exposure to high concentrations of $CO_2$ can negatively impact fish growth, physiology, and behaviour (Fivelstad et al., 1998; Fivelstad, 2013; Stiller et al., 2015; Khan et al., 2018; Mota et al., 2019), as $CO_2$ reduces the capacity of

the fish blood to transport oxygen due to the Bohr-Root effect (Bohr et al., 1904; Root, 1931). Safe operating levels of $CO_2$ depends on fish species, development stage and water quality, but for salmon smolts the recommended maximum limit is 15 mg/L (Hjeltnes, 2012; Fivelstad, 2013). However, studies with post smolt have showed that lower concentrations than the recommended threshold still have negative impact on growth (Mota et al., 2019). There is a debate whether high $CO_2$ concentrations in water also can cause nephrocalcinosis, a condition with accumulation of calcium and magnesium deposits in the kidney (Fivelstad et al., 2003; Good et al., 2018). High $CO_2$ levels also affect buffer consumption and the water chemistry of RAS by reducing pH, which impacts the toxicity of several important compounds in RAS such as $CO_2$, ammonia, $H_2S$, and impairs fish osmoregulation (Fivelstad et al., 1988; Fivelstad, 2013).

## 1.3 Microbiota in RAS

RAS harbour complex microbial communities that are present in the circulating water, in biofilm and in association with the fish (Rud et al., 2017; Llewellyn et al., 2014; Rojas-Tirado et al., 2018; Bartelme et al., 2019). Biofilms are found on all surfaces: in the biofilter, in rearing tanks, pipes, degassers and devices (Léonard, 2000; Michaud et al., 2009; Bartelme et al., 2019). Bacteria suspended in the RAS water interacts directly with the fish (Blancheton et al., 2013). How these microbial communities interact with each other, and the fish is not well understood.

Based on metabolic characteristics, bacteria in RAS can be divided into two main groups: heterotrophic and autotrophic bacteria. *Heterotrophic bacteria* obtain carbon and energy from degradation of suspended organic matter and produces $CO_2$ and $NH_4$ (Madigan et al., 2000; Kirchman, 2018). The heterotrophic bacteria need an electron acceptor. The most efficient electron acceptor available for the bacteria in RAS is oxygen (respiration), followed by nitrate (denitrification), and sulphate ($H_2S$-production) (Madigan et al., 2000; Kirchman, 2018). At conditions with available oxygen (in the RAS water and in the outer layer of biofilms) degradation of organic matter will consume oxygen. In anoxic conditions (inner layer of biofilm and denitrification reactor) denitrification occurs, reducing nitrate to nitrogen gas (van Rijn et al., 2006). In the absence of either oxygen and nitrate (inner layer of thick biofilm and sludge

deposits), sulphate can be used as electron acceptor, if present, in the metabolism of organic matter and production of $H_2S$ occurs. *Autotrophic bacteria* derive carbon from $CO_2$ and energy from oxidation of inorganic nitrogen, sulphur, or iron compounds in presence of oxygen (Madigan et al., 2000). One relevant example of autotrophic bacteria in RAS is nitrifying bacteria, that get energy from oxidating ammonia and nitrite (described in 1.2.1).

Bacteria compete for resources to obtain energy and space to grow. Nutrients essential for microbial growth include carbon, nitrogen, phosphorus, sulphur and metals (Madigan et al., 2000; Kirchman, 2018). Some bacteria are good at competing for resources at low substrate supply and make them successful in crowded environments, with bacterial densities close to the carrying capacity (CC), termed K-selected bacteria. On the contrary, some bacteria grow fast when resources are in excess, but have low affinity for substrate at low concentrations, and are termed r-strategists (MacArthur and Wilson, 1967; Salvesen et al., 1999; De Schryver and Vadstein, 2014; Vadstein et al. 2018b). Bacteria also have different requirement for, and response to oxygen. In addition, temperature, salinity, and pH are factors that have a huge effect on competition between bacterial populations (Madigan et al., 2000; Kirchman, 2018; Almeida et al., 2021). Therefore, seemingly small differences in conditions can make an impact on selection pressure through the system, acting on the microbial communities.

The most relevant influential factors acting on the development of the microbial community composition in RAS is external sources (make-up water, feed, the fish itself), management routines (hydraulic retention time, biofilter management, cleaning frequency), system design (particle removal, biofilter, disinfection), physicochemical water variables (pH, temperature, salinity), and grazing by eucaryotes (Schreier et al., 2010; Attramadal et al., 2012a; Blancheton et al., 2013; Rud et al., 2017; Bakke et al., 2017; Vadstein et al., 2018b; Chen et al., 2019; Duarte et al., 2019; Almeida et al., 2021) (Fig. 4). Variation in selection pressure leads to alteration in the microbial community composition in RAS, both within fish production batches (Bartelme et al., 2017; Duarte et al., 2019; Rojas-Tirado et al., 2021), between fish production batches and over time (Bakke et al., 2017; Bartelme et al., 2017; Bartelme et al., 2019; Drønen et al., 2021), but also on a daily basis (Blancheton et al., 2013). In addition, the microbial

communities that develop in the different habitats in RAS (i.e., water, fish and biofilm) in the different compartments of RAS is different from each other. Previous research has demonstrated significant differences in the community compositions between rearing water, biofilter biofilm, tank wall biofilm, fish skin, gill and gut (Michaud et al., 2009; Boutin et al., 2013; Schmidt et al., 2016; Bakke et al., 2017; Rud et al., 2017; Minniti et al., 2017; Bartelme et al., 2017; Bartelme et al., 2019; Duarte et al., 2019; Minish et al., 2020; Roalkvam et al., 2020; Drønen et al., 2021; Almeida et al., 2021).

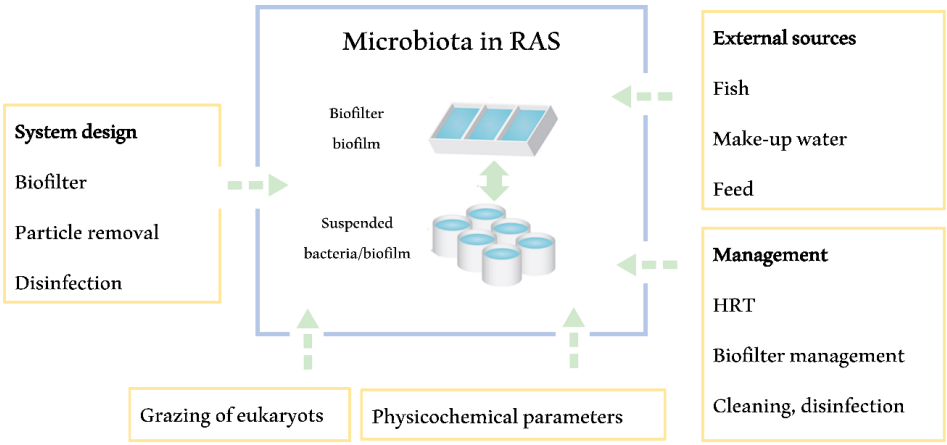



Figure 4. Influential forces acting on microbial community dynamics in RAS: System design, physicochemical water variables, management, external sources and grazing.

Microbiota is crucial for a well-functioning RAS, for two purposes: For optimal **chemical water quality (1.3.1)** and good **fish health (1.3.2)** (Blancheton et al., 2013; Bentzon-Tilia et al., 2016) (Fig. 5). The following chapter will hence focus on these two topics. The knowledge on the temporal microbial community dynamics in commercial RAS is scarce, but important, for a better understanding and ultimately to control and steer the microbiota towards a favourable state for the fish (Blancheton et al., 2013; Rurangwa and Vergedem, 2015; Bentzon-Tilia et al., 2016).

### 1.3.1 Microbial conversions for optimal chemical water quality

The nutrient and oxygen gradients in biofilter biofilms create niches that allow several microbial guilds to coexist (Flemming et al., 2016). The main role of the biofilter in RAS is to

convert toxic ammonia to less toxic nitrogen compounds, which is essential to maintain a good chemical water quality optimal for good fish health and welfare (Schreier et al., 2010) (Fig. 5). In reality there are currently no other practical options to control ammonia in RAS. Nitrifiers typically constitute a low proportion (below 20%) of the relative abundance in RAS biofilter biofilm (Fossmark et al., 2021; Hüpeden et al., 2020), but are crucial to prevent ammonia and nitrite toxicity (described in 1.2.1). Nitrifying bacteria, and therefore the efficiency of nitrification, are sensitive to rapid fluctuations in water quality variables such as pH, alkalinity, temperature, oxygen and salinity (Chen et al., 2006; Navada et al., 2019; Almeida et al., 2021). Substrate supply (ammonia, nitrite) also affect nitrification efficiency (Chen et al., 2006; Hüpeden et al., 2020).

Most of the bacteria in the biofilter biofilm are heterotrophic (Lèonard et al., 2000; Michaud et al., 2006; Michaud et al., 2009; Schreier et al., 2010). A mature biofilter has the capacity to consume the supply of organic matter it has been adapted to, and therefore efficiently keep the suspended organic matter concentrations low in the RAS water (Fig. 5). In this way, a mature biofilter stabilize the abundance and activity of heterotrophic bacteria in the water (Rojas-Tirado et al., 2019; Navada et al., 2020b). Some studies have shown that the abundance of bacteria attached to the biofilter media is correlated to the free-living bacteria in RAS water (Lèonard et al., 2000; Michaud et al., 2006; Michaud et al., 2009). However, the knowledge on interactions between the bacterial communities in biofilter and the suspended bacteria is limited (Rojas-Tirado et al., 2019).

A high supply of biodegradable organic matter in RAS water increases the activity and abundance of heterotrophic bacteria. The heterotrophs compete with the nitrifiers for oxygen and space in the biofilter (Lèonard et al., 2000; Michaud et al., 2006). Since the heterotrophic bacteria are generally fast-growing compared to the nitrifying bacteria, high levels of heterotrophs reduce the availability of oxygen to the nitrifying bacteria. A high organic carbon: ammonia nitrogen (C/N) ratio can thus inhibit nitrification and lead to low efficiency of the biofilter and elevated concentrations of ammonium and nitrite (Michaud et al., 2014; Zhang and Bishop, 1996; Nevada et al., 2020b; Navada et al., 2020c). Several studies have proposed that a C/N ratio close to one provides a stable balance between autotrophic and heterotrophic bacteria communities in the nitrifying biofilter (Zhu and Chen, 2001; Michaud et al., 2006).

High C/N ratio has shown to influence the microbial community structure and abundances in the nitrifying biofilter (Michaud et al., 2006; Michaud et al., 2014). However, a certain level of heterotrophs can be beneficial under stable conditions and high oxygen concentration, as they may protect the nitrifying bacteria in the deeper layer of the biofilm by maintaining the biofilm structure through secretion of extracellular polymeric substances (Bassin et al., 2012).

Certain species of microorganisms can produce harmful or unwanted by-products that creates problems in RAS (Fig. 5). $H_2S$ is produced by sulphate reducing bacteria (SRB) (and archaea), that uses sulphate compounds as electron acceptors to degrade organic matter, in absence of oxygen and nitrate (Fauque, 1995; Madigan et al., 2000). $H_2S$ is a toxic gas that have caused massive fish mortalities even at very low concentrations in RAS (Hjeltnes et al., 2012; Sommerset et al., 2022). SRBs can be found in different RAS environments, but availability of organic matter, nitrate and oxygen conditions define their activity. Hot spots for $H_2S$ production are sludge (Letelier-Gordo et al., 2020) and the lower levels of thick biofilm where oxygen is depleted, and in the oxic-anoxic zones (Rojas-Tirado et al., 2021). The risk of $H_2S$ production is higher in marine RAS than freshwater RAS due to higher sulphate concentrations in marine water (Letelier-Gordo et al., 2020). Typically, low levels of inactive SRBs are found in RAS (Rojas-Tirado et al., 2021) and very low concentrations of $H_2S$ (Lien et al., 2022) at normal production. The risk of poising can be controlled through preventing anaerobic conditions within the system, securing that nitrate is present and securing optimal conditions for the biofilter (Rojas-Tirado et al., 2021), and effective particle and thick biofilm removal, which avoid the SRBs becoming active.

Off-flavor is relevant for production of market size fish in RAS, where secondary metabolic products of certain bacteria (e.g. geosmin and MIB) can be produced within solids and biofilms (Guttman and van Rijn, 2008; Schrader and Summerfelt, 2010). The current solution to avoid off-flavor is to purge fasting fish for up to 15 days in clean water before slaughter (Burr et al., 2012).

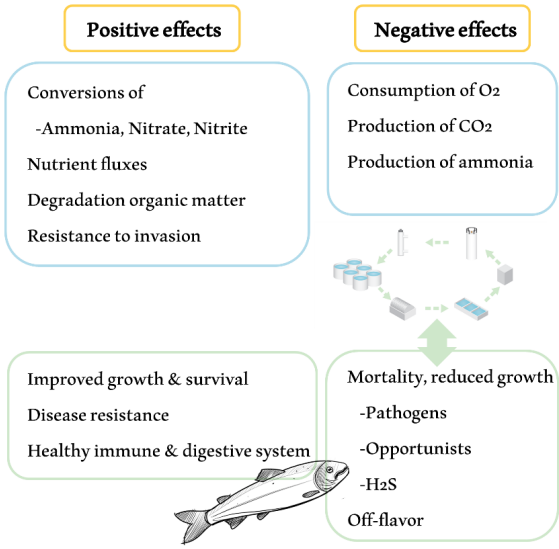



Figure 5. Positive and negative effects of bacteria in RAS, on the system and directly and indirectly on the reared fish.

### 1.3.2 Microbiota and fish health

The microbiota that the fish encounters in the surrounding water, has a significant impact on the development and health of the fish (Vadstein et al., 2013; Llewellyn et al., 2014; Derome, 2019). The relationship between the fish and the microbiota can be commensal (neither beneficial or harmful for the fish), mutualistic (both the fish and the microbiota receive some benefits) or pathogenic (harmful effects on the fish). However, most of the bacteria in RAS are either harmless or beneficial for the fish (Ringø and Birbeck, 1999; Vadstein et al., 2013; Llewellyn et al., 2014). Bacteria play important physiological roles in host development, influencing metabolic processes and regulation of fat storage (Nayak, 2010; Gomez et al., 2013). The mucosal tissue on fish skin, gills and digestive tract are colonized by different bacteria, which play a key role in the development of the host immune system and disease resistance (Nayak, 2010; Blancheton et al., 2013; Llewellyn et al., 2014) (Fig. 5). The term «microbial water quality» refers to the presence of bacteria in the rearing water that potentially may interact directly with the fish. In RAS, the microbial water quality is considered as optimal when opportunistic bacteria and specific pathogens are absent or in negligible

abundances in the water and bacteria that takes part in positive fish-microbe interactions is present (Vadstein et al., 2004; Attramadal et al., 2012a).

RAS with stable conditions and no disinfection, have in previous work been shown to promote K-selection of bacteria. This effect is hypothesized to result from the long retention time of water in the system, the large surface area available for bacterial growth, the strong competition for available organic matter in the biofilter, and the relatively stable organic matter load (and hence microbial carrying capacity) throughout the system. Environments dominated by K-strategists can provide healthy fish-microbe interactions, higher growth rates and greater survival, as shown for cod larvae (Attramadal et al., 2012a; 2014; Vestrum et al., 2018; Vestrum et al., 2020) and lobster (Drenstig and Bergheim, 2013; Attramadal et al., 2021). Also, a K-selected environment is thought to be important for disease resistance by providing effective barriers against infection and development of disease in both the system and the individual fish, by occupying niches and preventing proliferation of harmful species (Skjermo et al., 1997; Attramadal et al., 2012a; De Schryver and Vadstein, 2014; Vadstein et al., 2018b; Vestrum et al., 2018; Attramadal et al., 2021) (Fig. 5).

In contrast to K-strategists, r-strategists include both pathogens and opportunistic species that can cause secondary infections of a weakened host (Vadstein et al., 2018b). In flow-through systems, some important factors promote r-selection (Fig. 6). First, the feeding of fish and the constant dilution of bacteria with the exchange of water from the tank results in low competition for the organic matter, which favour the fast-growing r-strategists. Disinfection of incoming water reduces the number of bacteria going into the rearing tanks, which makes the effect even stronger. Disinfection of the ingoing water to the rearing tanks have been shown to give a subsequent regrowth of opportunistic bacteria in the system (Hess-Erga et al., 2010; De Schryver and Vadstein, 2014; Attramadal et al., 2012a; 2012b; Vadstein et al., 2018b). Where in the system the regrowth happens depend on the type of system and the exchange rate of the rearing tanks. In RAS with disinfection of the water directly before the rearing tanks combined with long HRT in the rearing tanks (>60 minutes), like marine hatcheries, the proliferation of opportunistic bacteria in the rearing tanks is well documented (Fig. 6). Proliferation of r-strategists results in an altered microbial community composition

that have been shown to have negative effects on larval health and survival (Vadstein et al., 2018a; 2018b; Attramadal et al., 2021) (Fig. 5, 6). The effect of opportunists on fish health is not explored for salmonids, although it can be a significant and relevant factor, especially during early developmental stages. However, the HRT of the rearing tanks is typically shorter than in marine hatcheries, ranging from 18 to 55 minutes typically (Summerfelt et al., 2016), which possibly results in lower regrowth in the rearing tanks (Bakke et al., 2017).

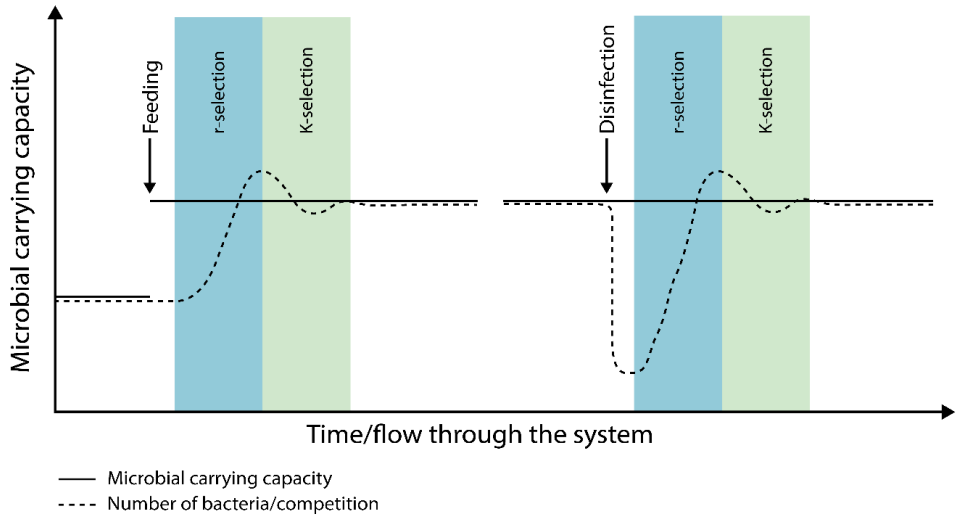



Figure 6. Two operations in RAS that promote r-selection of bacteria: Feeding (increasing the carrying capacity in the rearing tanks, thus reducing the microbe-microbe competition) and disinfection (reducing the number of bacteria and increasing the nutrients available, thus decreasing the microbe-microbe competition). Figure modified from figure by Kari J.K. Attramadal in De Schryver and Vadstein, 2014. Blue=the rearing water, green=water downstream rearing tanks.

Some bacteria are obligate or facultative pathogens that can cause disease in fish (Michaud et al., 2009; Borges et al., 2021) and represent a challenge if they are introduced and proliferate in a RAS (Fig. 5). Problematic bacterial pathogens in salmon smolt production are *Flavobacterium psychrophilum*, *Aeromonas salmonicida subsp. Salmonicida*, *Branchiomonas cisticola,* and *Yersinia ruckeri* (Sommerset et al., 2022). Occurrences of infectious bacterial diseases is not a major problem in smolt production in Norway today, but still sporadic outbreaks are reported. For lumpfish *Aeromonas salmonicida*, *Vibrio sp.*, *Pasteurella sp*. and *Pseudomonas anguilliseptica* are common problematic pathogens (Sommerset et al., 2022).

Keeping RAS free of pathogens is a difficult to impossible task. It is likely that some pathogens are present in commercial RAS at low abundances at any one time (Michaud et al., 2009; Lewin et al., 2020), but that a complex community of other bacteria contribute to naturally suppressing pathogenic proliferation (Borges et al., 2021). Effective vaccines have greatly contributed to a reduction of outbreaks from known pathogens in aquaculture in general (Sommerset et al., 2022).

High biosecurity in RAS, with the aim to reduce the risk of introducing and spreading unwanted microorganisms to and within the system, contributes to keeping pathogen outbreaks low (Sharrer et al., 2005; Hjeltnes et al., 2012; Fjellheim et al., 2017). Filtration and disinfection of intake water of good quality is the first step (Fig. 3). Also, the fact that RAS introduces low amount of new water in comparison to FTS, make RAS easier to control when it comes to introduced pathogens. Other biosecurity measures include testing of roe, fish and feed prior to stocking, in-line disinfection (Fig. 3), keeping rearing units separated and the "all-in, all-out" production approach (Hjeltnes et al., 2012; Fjellheim et al., 2017).

The main goals regarding microbiota and fish health are to provide a healthy microbial water quality and fish-microbe interactions by having effective biosecurity measures, a beneficial microbiota in the system, and efficient measures to isolate and treat tanks and systems when affected.

# Chapter 2: Aims and objectives

Research within RAS the last years have improved our understanding of the importance and function of microbes in RAS. However, there are still several knowledge gaps that needs to be filled, like how microbiota in different compartments of RAS interact with each other and with the fish, which factors influence their composition, the complex interactions between the chemical water quality and the microbial communities, and the temporal dynamics. This knowledge will contribute to a better understanding of the microbial ecology which ultimately can optimize the chemical and microbiological water quality in RAS to promote good fish health, welfare and production.

The main goal of this PhD thesis was to increase the knowledge of the spatial and temporal dynamics of the systems' microbial community composition in commercial RAS. The PhD was structured according to the following objectives:

Objective 1 Increase the knowledge of the long-term temporal microbial community dynamics of RAS water, biofilter biofilm and tank wall biofilm in commercial RAS, producing Atlantic salmon fry and parr (Paper I, II).

Objective 2 Clarify the effect of in-line UV disinfection on the microbial population of the water in the RAS loop (Paper I, II, III).

Objective 3 Identify the effects of different water treatments designs in RAS on water and biofilm microbiota, and how the resulting microbial communities affect survival, growth, and gill health of the reared fish (Paper III).

# Chapter 3: Fish culture systems and methods

The PhD work included two studies of commercial RAS producing Atlantic salmon fry and parr reared in freshwater (<3 ppt) and an experiment with lumpfish juveniles reared in a seawater RAS with different water treatments (Fig. 7).

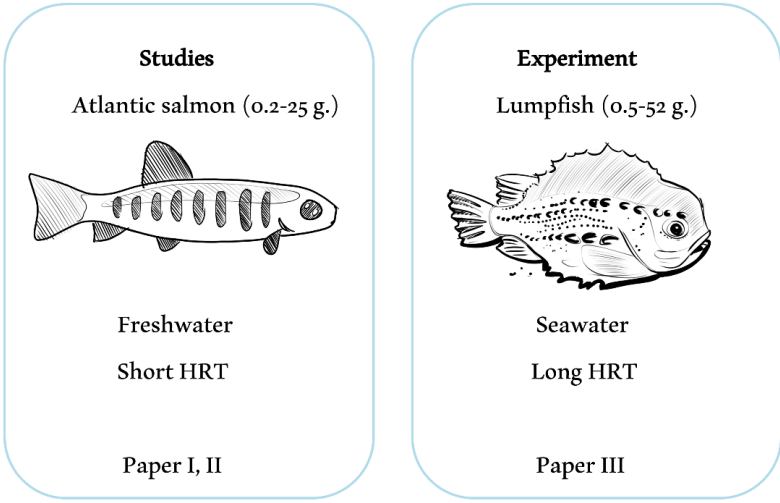



Figure 7. Two studies of commercial RAS producing Atlantic salmon fry and parr in freshwater and an experiment with lumpfish produced in seawater. HRT=hydraulic retention time in the rearing tanks. Sketches: Carl Nørstebø.

## 3.1. Study of the long-term microbial community dynamics in the water of a commercial RAS producing Atlantic salmon fry and parr (Paper I)

The first study was carried out in a commercial RAS producing Atlantic salmon fry and parr from 0.2 to 25 grams. The RAS department consisted of 18 rearing tanks (16 $m^3$) operated with freshwater (Fig. 8). The RAS included the following treatment units: a drum filter, two Moving Bed Biofilters (MBBF), two drum filters, two Fixed Bed Biofilters (FBBF), a trickling filter and a UV unit treating the full flow of water. UV dose was 100 $mJ/cm^2$ , max flow rate 450 $m^3/h$. Make-up water flow was: 3-106 $m^3$/day. The hydraulic retention time (HRT) of the rearing tanks was 23 minutes. Three different batches of fish were monitored in the same system. The spring production batches included growth of fish from 0.2 to 4 grams in 2015

(2015_spring) and 2016 (2016_spring), while the autumn 2015 production batch included growth of fish from 2.5 to 25 grams. The system was disinfected before the 2015_spring batch and between the 2015_autumn and 2016_spring batches. Before the 2015_spring batch the biofilter was restarted after disinfection with new, clean carriers and matured until the stocking of fish, whereas before the 2016_spring batch the biofilter was filled with already matured biofilm carriers from another mature biofilter. In the 2015_autumn batch the biofilter was running from the previous batch, and not disinfected before stocking of fish. Water was sampled from three rearing tanks (A, B, C), as well as directly upstream the UV (UUV) and downstream the UV (DUV). Microbiota was analysed using 16S rRNA gene amplicon sequencing of DNA and RNA extracts and by culture-based methods. In total 6 sampling events were made during a 20-months period.

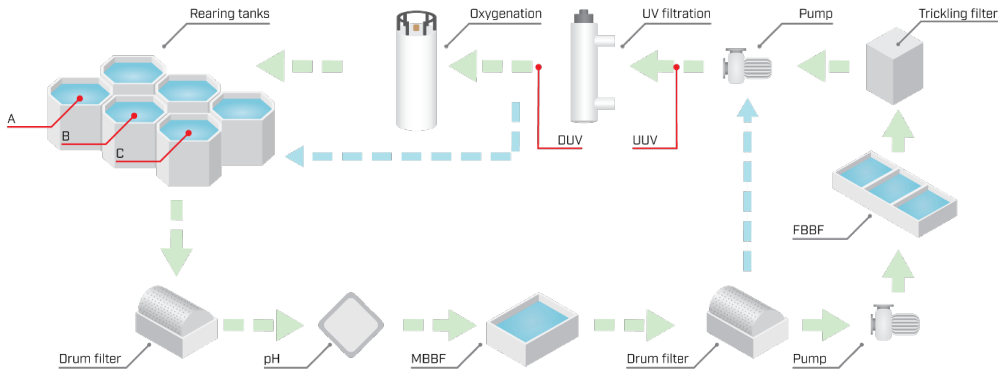



Figure 8. Schematic presentation of the RAS unit monitored. Samples for analyses of the RAS water microbiota (red lines) were taken from three rearing tanks (A, B, C) and immediately upstream (UUV) and downstream of the UV treatment unit (DUV). MBBF=Moving Bed Biofilter, FBBF=Fixed Bed Biofilter. The UV disinfection represent a full-flow disinfection. Illustration: Mats Mulelid, SINTEF.

## 3.2 Study of the long-term microbial community dynamics of water and biofilm in a commercial RAS producing Atlantic salmon fry (Paper II)

The second study was carried out in a commercial RAS producing Atlantic salmon fry from 0.2 to around 3 grams. The RAS department consisted of six rearing tanks (23-35 $m^3$) operated with freshwater (average 1.5 ppt salinity). The HRT of the rearing tanks were 18-28 minutes. The RAS included the following treatment units: a drum filter, three FBBFs, a trickling filter and a UV unit treating the full flow of water (Fig. 9). UV dose was 35 $mJ/cm^2$, max flow rate

454 m3/h. Make-up water flow was 20-180 $m^3$/day. Seven consecutive production batches were monitored, where batch 1 and 7 were only sampled for a part of the time the fish spent in the system. In production batch 6, the fish was kept in the RAS unit for a longer period, compared to the other production batches. This resulted in different number of sampling times for the different production batches. Between each production batch, there was a fallowing period for cleaning of rearing tanks, varying from 6-40 days, where the unit was maintained without fish and feed. Six positions in the RAS loop were sampled every second week for 15 months, resulting in 33 sampling times (t0-t32), including the rearing water (W-T) and biofilm of the tank wall (B-T) in two rearing tanks, as well as the biofilm of the biofilter (B-B) and the water downstream the biofilter and upstream the UV disinfection (W-S). DNA was extracted with two different kits. First, FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) was used for samples taken from t0 to t17, then ZymoBIOMICS™ DNA Miniprep kit (Zymo Research, USA) was used for samples taken from t18 to t32. This change of kits was done for economic reasons. The microbial community composition results were subsequently compared at different taxonomical levels with the same samples from both DNA extraction kits, and only small differences were found. Microbiota was analysed using 16S rRNA gene amplicon sequencing of DNA, flow-cytometry and by culture-based methods.

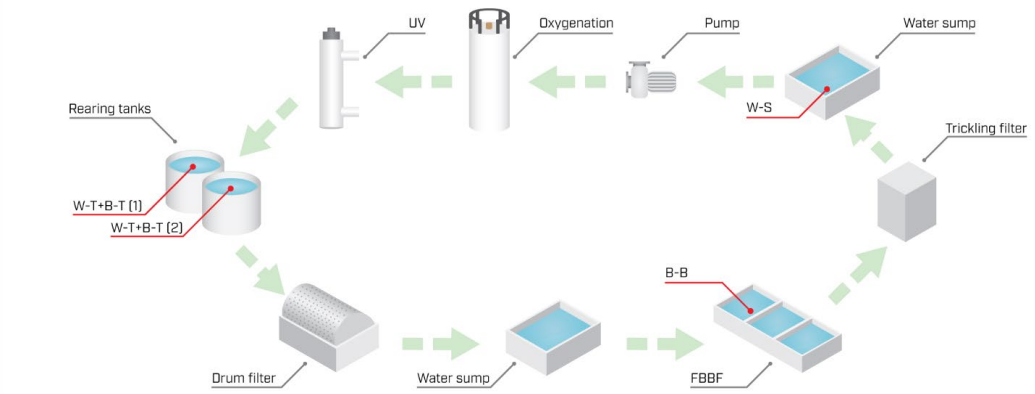



Figure 9. Schematic presentation of the RAS monitored. Sample points for RAS microbiota is presented as red lines: water samples from each of two rearing tanks (W-T 1 and 2), biofilm samples from the walls of two rearing tanks (B-T 1 and 2), biofilm (B-B) from the fixed bed biofilter (FBBF) and water from the sump downstream the biofilter and upstream the UV unit (W-S). The UV represented full-flow UV disinfection. Illustration: Mats Mulelid, SINTEF.

## 3.3 Experiment with lumpfish in one RAS with different water treatments upstream the rearing tanks (Paper III)

An experiment was conducted at Ecomarine Seafarm AS at Dønna, Norway, in cooperation with Let Sea AS. A total of 10.000 juvenile lumpfish of 0.52 grams were transferred to each of the 15 rearing tanks (0.8 $m^3$) in the on-growing system used in the experiment, operated with seawater. Four different treatments were included: 1) RAS without filtration or disinfection (RAS), 2) RAS with mechanical filtration (20 µm) (RAS-F), 3) RAS with mechanical filtration and a UV unit (RAS-F-UV), 4) RAS with mechanical filtration, UV and an ozone unit (RAS-F-UV-O). These treatments were positioned directly upstream the rearing tanks (Fig. 10). Each treatment included three rearing tanks with one inlet pr rearing tank. The outlet water from the rearing tanks, representing all treatments, were collected, and returned to the same RAS (drum filter, biofilter and degassing unit), which implies that the water from each treatment was mixed. The UV dose was 25 mJ/$cm^2$ and the flow rate 7.2 $m^3$/h. Ozone generator at 230 V was used for the ozone treatment. Make-up water flow was 50-100 $m^3$/day. In addition, a flow-through system (FTS) was included as a reference system for fish of the same group (Fig. 10). The HRT in all the rearing tanks was 60 minutes. The experiment ended on day 146, when the lumpfish had reached 52 grams, and were ready to be transferred to the net pens. Rearing water and tank wall biofilm were sampled from all tanks, at day 50 and 139. Microbiota was analysed using 16S rRNA gene amplicon sequencing, by flow-cytometry and culture-based methods. In addition, gill health was characterised by histology, and the survival and growth of fish was measured.

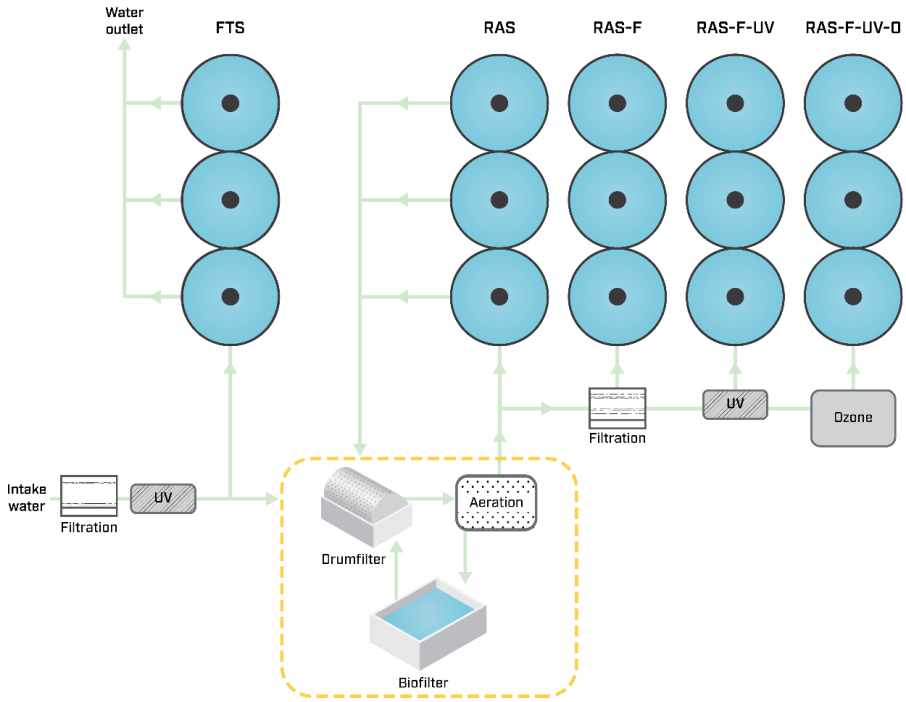



Figure 10. Schematic presentation of the systems and the different water treatments in the experiment. RAS without disinfection or filtration for removal of small particles (RAS), RAS with mechanical filtration (20 µm) (RAS-F), RAS with mechanical filtration and a UV unit (RAS-F-UV), 4) RAS with mechanical filtration, UV and an ozone unit (RAS-FU-V-O). The four different treatments were connected to the same RAS. A flow-through system (FTS) was included as a reference system, with fish from the same group. Illustration: Mats Mulelid, SINTEF.

# Chapter 4. Summary of results

## 4.1 Long-term microbial community dynamics in the water of a commercial RAS during three production batches of Atlantic salmon fry and parr (*Salmo salar*) (Paper I)

The two last production batches monitored (2015_autumn and 2016_spring) had a highly similar water microbiota despite disinfection of the system between the batches and rearing fish of different stages, different seasons, with different biomass and feeding regimes. In contrast, the first production batch (2015_spring) showed a significant different water microbiota from the other batches studied (Fig. 11). The microbial community composition of the first production batch was considerably more variable between replicate rearing tanks and over time compared to the two last production batches, even though 2016_spring batch produced fish at the same season and life stage. We suggested that differences in start-up procedures of the biofilter which resulted in different maturation status could explain these differences.

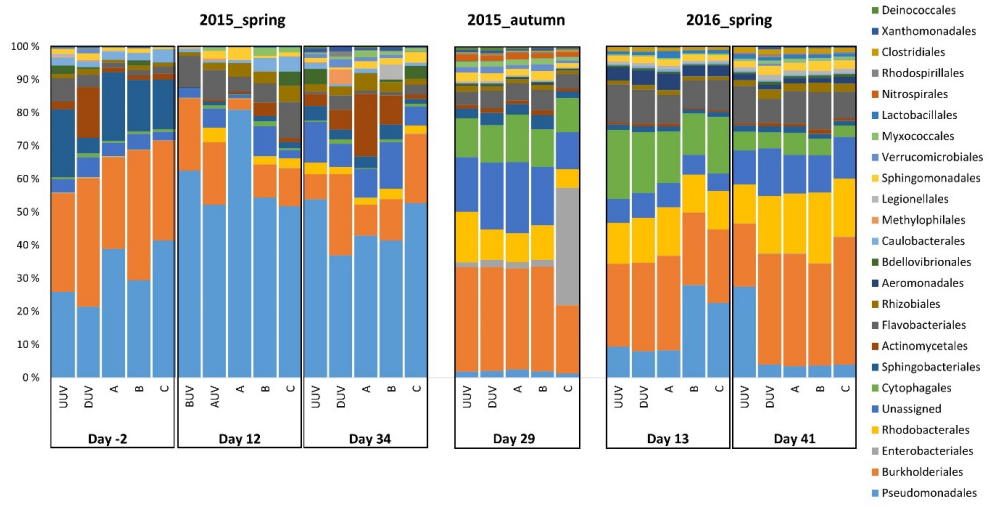



Figure 11. Relative abundances (%) of bacterial orders in water from three rearing tanks (A, B, C) of production batch 2015_spring, 2015_autumn and 2016_spring, at different sampling days, where day represent the day in production. UUV=upstream UV, DUV=downstream UV. Orders with relative maximum abundance below 1% in all samples are included in "other".

The full-flow UV treatment directly upstream of the rearing tanks had no observable effect on the community composition of the water microbiota throughout the system, for neither amplicon sequencing based on DNA nor RNA extracts. The effect of disinfection on the viable bacterial densities in the water directly downstream of the UV treatment was a 89% reduction in CFUs. For all samplings, the CFUs increased for water samples taken from the rearing tanks, compared to water samples taken directly downstream the UV treatment.

## 4.2 Long-term microbial community structures and dynamics in water and biofilm of a commercial RAS during seven production batches of Atlantic salmon fry (*Salmo salar*) (Paper II)

The microbiota composition of water and biofilm varied within and between the seven production batches studied. The fallowing periods (i.e., periods between fish production batches, without fish and feed) had a substantial effect on the microbial communities. Shifts in the composition of the water and biofilm microbiota were identified in conjunction with variations in organic matter loading both during production and fallowing. In addition, the variables oxygen saturation, biomass, and feed type, showed good correlation with variations in the water microbiota composition.

Although the microbiota changed at the fallowing periods, the water microbiota returned to a similar composition at the end of each production batch (Fig. 12). The microbial communities in the biofilter biofilm and water were significantly different but shared many abundant taxa and followed the same trends in temporal microbial dynamics.

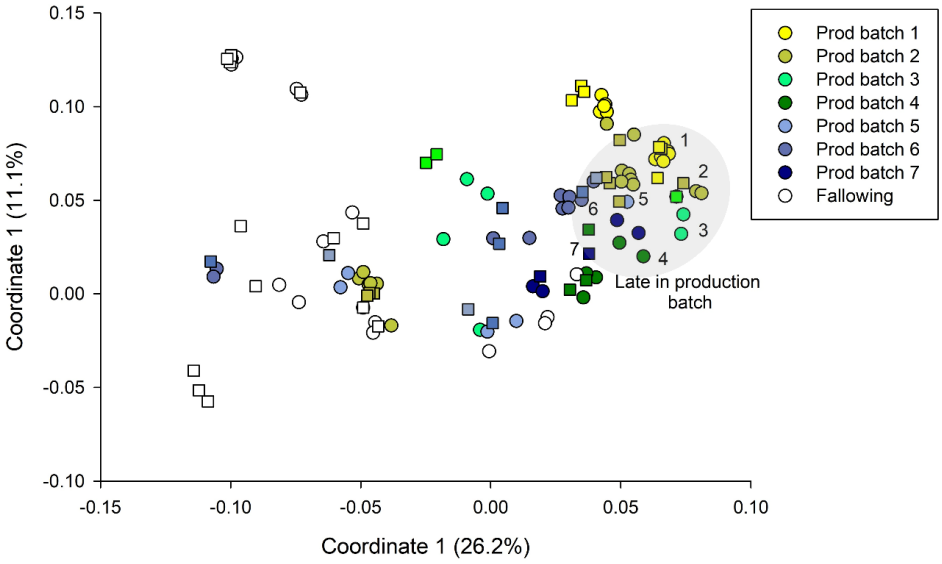



Figure 12. PCoA-plot based on Bray–Curtis similarities sorted by the seven different batches of fish and the six fallowing periods (without fish) for water samples from two rearing tanks and the water sump. Circles=rearing tanks, square=water sump. Samplings late in production batch are symbolised by numbers of a given production batch and a shaded grey area. n=76 (rearing tanks), n=43 (water sump).

OTUs representing nitrifying bacteria accounted for a relatively low proportion of the total reads in the samples of the biofilter biofilm, with maximum relative abundance of 12.5%. OTUs assigned to *Nitrospira* dominated among the OTUs classified as nitrifying bacteria, while the relative abundances of OTUs classified as ammonium oxidising bacteria (AOB) were low (*Nitrosomonas* and Nitrosomonadaceae). The relative abundance of nitrifiers tended to increase at the fallowing periods and to decrease throughout the production batches. The third most relatively abundant *Nitrospira* OTU was closely related to comammox *Nitrospira nitrificans.*

The UV treatment directly upstream of the rearing tanks had no observable effect on the community composition of the water microbiota, as both water upstream the UV and in the rearing tanks were rather similar in microbiota composition. However, with CFU analyses we found a significant higher fraction of rapid-growing bacteria in the rearing tanks compared to

the treated water upstream the UV, on one sampling day, indicating that disinfection upstream the rearing tanks allowed for growth of opportunistic bacteria.

## 4.3 The effects of different water treatments in RAS on water and biofilm microbiota, survival, growth, and gill health of lumpfish (Paper III).

The microbial community composition of the water differed significantly between all systems with different treatment designs upstream the rearing tanks, for both sampling days, except for RAS and RAS with mechanical filtration, which were similar (Fig. 13). OTUs classified as *Leucothrix* were highly abundant in the rearing water of RAS treatments with disinfection. We found no significant differences in biofilm tank wall microbiota between the systems.

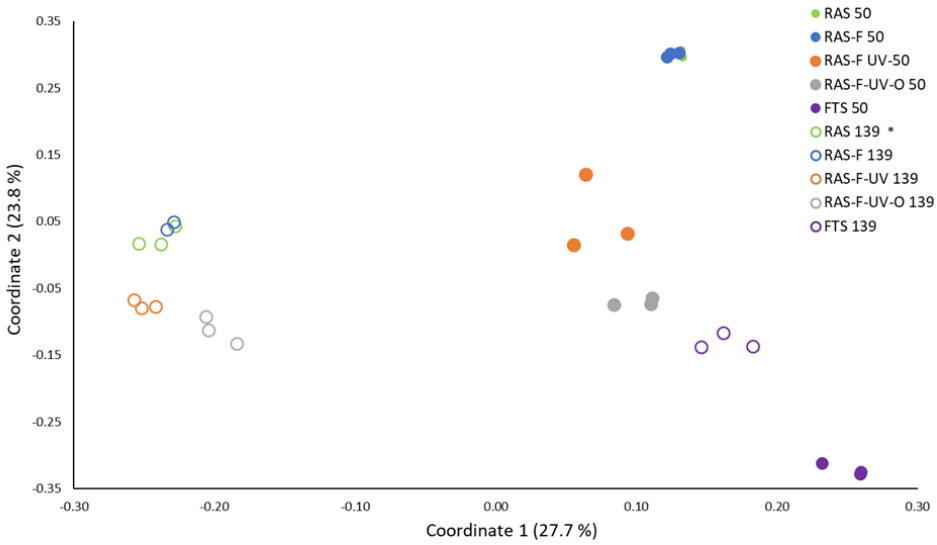



Figure 13. Principal coordinates (PCoA) plot based on Bray–Curtis similarities for water microbiota from the systems at sampling day 50 and 139 days. Filled symbols represent day 50, open symbols day 139. RAS=RAS with no additional treatment, RAS-F=RAS with a filtration unit for removal of small particles (20 µm), RAS-F-UV=RAS with filtration and disinfection with UV-irradiation, RAS-F-UV-O=RAS with particle filtration and disinfection with UV irradiation and ozone, FTS=Flow-through system. *=RAS tanks were converted to RAS-F from day 69.

The water microbiota from the tanks of the RAS without disinfection had several important characteristics, compared to RAS with disinfection and the FTS. First, the alpha diversity of the microbiota was generally higher for both sampling days in the RAS without disinfection.

Second, the Bray-Curtis similarities for comparisons of the water microbiota between replicate tanks and between the two sampling times were higher in this RAS, indicating higher stability of the water microbiota in the RAS without disinfection. Last, the RAS without disinfection had a significantly lower fraction of presumed opportunistic bacteria at the first sampling day, compared to RAS with disinfection and FTS (determined from CFU analysis).

The best gill health was identified for the fish from RAS without disinfection, where fish from the RAS with filtration had a significantly better gill health than the fish in the RAS with disinfection and FTS. Average survival and growth of lumpfish, however, were similar among the RAS systems, but higher in all RAS compared to FTS, presumably due to higher temperature.

# Chapter 5: Discussion

Bacteria have traditionally been considered as a problem in RAS and aquaculture in general, and the focus has been to eliminate bacteria within the system, to avoid pathogens and fish diseases. However, research during the last decade shows that bacteria is ubiquitous in RAS and that the high organic load makes it difficult to avoid microbial growth within the system (Vadstein et al., 2004; Attramadal et al., 2014; Vadstein et al., 2018a). We also know that bacteria are essential in RAS, for optimal fish development and health and for system performance (Nayak, 2010; Blancheton et al., 2013; Attramadal et al., 2014; Vadstein et al., 2018b). Instead of eradicating the microbes in RAS, a better strategy is therefore to aim at supporting the development of a beneficial microbial environment. Microbial ecology has been proposed as a tool for managing the microbiota in RAS for a good production, to steer the microbiota towards a favourable state for the fish (Bentzon-Tilia et al., 2010; Blancheton et al., 2013; Vadstein et al., 2018b).

Whereas land-based RAS are becoming the norm for fish production, our understanding of the complex microbial community dynamics in RAS, and how these communities impact system performance and the fish health is limited (Blancheton et al., 2013; Rurangwa and Vergedem, 2015). Most studies on water and biofilm microbiota have so far been conducted in semi-commercial RAS or in lab experiments in freshwater (Fossmark et al., 2020; Aalto et al., 2022), brackish water (Rud et al., 2017; Bakke et al., 2017) and seawater (Duarte et al., 2019; Chen et al., 2019; Roalkvam et al., 2020), whereas there have only been conducted few studies in commercial systems during state-of-the-art production in freshwater RAS, and only for short time periods (Bartelme et al., 2017; Bartelme et al., 2019; Minish et al., 2020; Hüpeden et al., 2020; Fossmark et al., 2021; Drønen et al., 2021). To obtain microbial control in RAS, we need to understand the processes governing the composition of the microbial communities suspended in water and embedded in biofilm, and how these communities impact physicochemical and microbial water quality and fish health.

## 5.1 Temporal microbial community dynamics in commercial RAS

The first objective of this thesis was to increase the knowledge on the long-term microbial community dynamics of commercial RAS for production of Atlantic salmon fry and parr. In the first commercial RAS (Paper I) we characterized the microbiota of the rearing water and water upstream/downstream the UV disinfection, in three different production batches over a total period of 20 months. This study covered only 6 sampling events and included only the suspended water microbiota, and the potential interactions between suspended and biofilm microbiota could hence not be provided. In the second study (Paper II), the long-term microbial community dynamics in another commercial RAS facility was studied, and biofilm samples from the fish tanks and biofilter were also included, in addition to water. Here, we increased the sampling times, with consecutive monitoring every second week over a 15 months' period, resulting in 33 sampling events. The high number of samples also made it possible for using supervised machine learning with the obtained data in this study. The second study showed that the microbial community composition of system microbiota (i.e., the microbial communities associated with the biofilms of surface tank and biofilter and those suspended in the water) in the commercial RAS for salmon fry production studied in Paper II was surprisingly variable over the 15-month period, compared to four other commercial RAS producing salmon smolts monitored for the same period (Dahle et al., 2020b). The fallowing periods, with no fish and feeding in the system, affected the system microbiota, when compared to production periods. The impact of feed is closely linked to the organic matter load on the system, and to the carbon to nitrogen ratio (C/N ratio). Organic matter is typically the limiting resource determining the carrying capacity of the heterotrophic bacteria (Michaud et al., 2006) and is known to influence the microbial community structure and abundances in both biofilter and water (Michaud et al., 2006; Michaud et al., 2014; Wold et al., 2014; Bartelme et al., 2017; Rojas-Tirado et al., 2018; Bakke et al., 2017; Bartelme et al., 2019; Fossmark et al., 2020; Fossmark et al., 2021). During production the organic load increase due to increased biomass, feeding and defecation. Consequently, the fraction of heterotrophic bacteria to nitrifying bacteria typically increases during production, which can impact nitrification negatively (Michaud et al., 2006; Michaud et al., 2014; Navada et al., 2020b). An increased fraction of heterotrophic bacteria to nitrifiers was apparent during production periods compared to the fallowing periods in Paper II, as the OTUs representing

nitrifying bacteria decreased in relative abundance throughout the production batches and increased during fallowing. Since the fallowing periods were rather long (average of 24 days), doses of ammonia were added to maintain the activity of the nitrifying bacteria, which also apparently resulted in an efficient biofilter. Compared to the results obtained in this thesis, we have previously observed more stable community compositions over time in the biofilter biofilm of some RAS with shorter fallowing periods or continuous production (Dahle et al., 2020b). Shorter fallowing periods, or continuous production, might result in more stable conditions for the biofilter microbiota, due to less fluctuating organic loads. However, other variables than the organic load are most likely also contributing to the differences in the stability of the biofilter microbiota between facilities, like the RAS design and management strategies, e.g., the frequency of backwash of the fixed bed biofilter. In the RAS for salmon production studied in Paper II, the biofilter was seemingly efficient for all practical purpose. However, the variations in the microbiota communities over time and the importance of a stable biofilter community for optimal biofilter function is poorly understood.

The impact of organic matter load on the microbiota composition in RAS was further investigated by using supervised machine learning (SML). SML showed that the temporal variability in microbiota composition correlated to variables closely linked to organic matter load, like fish presence, biomass of fish, oxygen saturation and feed type. The results showed that the presence of organic matter had a higher impact on the microbial communities than pH, salinity, and concentrations of TAN, nitrite, nitrate in the studied RAS. The low correlation with physicochemical parameters is most likely related to rather stable, and independently controlled, variables during the monitored period, as it is well documented that large fluctuations for instance in salinity (Gonzalez-Silva et al., 2016; Bakke et al., 2017; Rud et al., 2917; Navada et al., 2019; Fossmark et al., 2021; Almeida et al., 2021) and pH, structures the microbial communities in RAS (Hüpeden et al., 2016; Hüpeden et al., 2020; Almeida et al., 2021). The results from this thesis and previous research (e.g., Rojas-Tirado et al., 2018; Fossmark et al., 2020) show that organic matter has a high impact on the microbiota in RAS.

An interesting observation was that although the microbial communities changed when going from high to no load of organic matter in the system during fallowing, it was returning relatively fast to a very similar community composition during each production batch (Paper

II) (Fig. 12). The system thus seems to select for similar system microbiota in each production batch, most likely because of similar selection pressure caused by the system's design and operational routines for each production batch (Bakke et al., 2017). This also indicates that the most abundant taxa of the system is maintained through the changes between fallowing and production.

Although the microbial communities in the biofilter biofilm and those suspended in the water were significantly different, which was expected, due to different selective pressures (Michaud et al., 2009; Bakke et al., 2017; Bartelme et al., 2019; Almeida et al., 2021; Aalto et al., 2022), they share many of the abundant genera and show similarities in temporal microbial dynamics over time (Figure 9 in Paper II). The shared abundant taxa and the similarities in temporal dynamics of biofilter biofilm and suspended bacteria indicate that there is a relationship between the microbial communities in the biofilter and the suspended bacterial communities in the RAS. The major fraction of bacterial cells in RAS is present as biofilm (Wietz et al., 2009; Blancheton et al., 2013) and more precisely in the biofilter (Kari Attramadal, unpublished results). The biofilter biofilm communities can affect the water communities in two different ways. First, indirectly by changing the chemical water quality through nitrogen conversions and degradation of organic matter and thus changing the selection acting on the suspended bacteria and secondly, directly through dispersal of bacteria from the biofilm to the water (Lèonard et al., 2000; Schreier et al., 2010). In addition, the similarities between microbiota in biofilter and water could be a result of similar responses to a common influence from changes in operation and management. In a commercial RAS it is difficult to separate these effects, and controlled experiments would be necessary to study this further. Indications of influence of the biofilter biofilm on the water microbiota was also observed in Paper I. Production batches for Atlantic salmon fry and parr that were connected to a mature biofilter showed highly similar water microbiota despite disinfection of the system between the batches and rearing fish of different developmental stages (Fig. 11). The water microbiota of these batches had communities with high alpha diversities and a more stable microbiota composition between replicate tanks and over time, compared to the production batch with an immature biofilter that recently was disinfected, which in addition showed a significantly different water microbiota. A stable microbiota with high alpha diversity has been shown to be characteristic to K-selected communities that creates favourable fish-microbe interactions

with higher survival and growth (Attramadal et al., 2012a; Boutin et al., 2013; Attramadal et al., 2014; Vadstein et al., 2018b; Vestrum et al., 2018). We therefore hypothesized that a mature biofilter contributed to a more similar water microbiota composition over time, both within and between production batches with a K-selected community. K-selected communities can also provide the system with a more resilient microbiota against pathogen proliferation (Attramadal et al., 2012a; De Schryver and Vadstein, 2014; Attramadal et al., 2014; 2021). Another implication of these observations is that the biofilm communities in the biofilters may affect the suspended water microbiota more heavily than variables such as season, fish development stage, feeding routines and disinfection of the system. A stable and resilient microbiota in RAS, using matured biofilters and continuous production can thus be a strategy for improving the microbial water quality in RAS.

The nitrifying bacteria in the biofilter is of outmost importance for effective removal of nitrogen waste products and good chemical water quality in RAS (Reviewed in Ruiz et al., 2020). However, the knowledge of the microbial community composition and the temporal dynamics of nitrifying communities in commercial freshwater RAS, at normal production is sparse (Bartelme et al., 2017; Bartelme et al., 2019; Fossmark et al., 2021). The nitrifying bacteria constituted a small fraction of the community in the fixed bed biofilter in a RAS for commercial production of salmon fry (up to 12.5% in relative abundance) (Paper II), which is in line with other commercial freshwater RAS with good biofilter efficiency (Dahle et al., 2020b; Fossmark et al., 2021). The OTUs representing nitrifying bacteria were dominated by *Nitrospira* (NOB), while the relative abundances of OTUs representing ammonium oxidising bacteria (AOB) were low. The results corroborate previous research showing that AOB are typically in low abundance in freshwater nitrifying biofilters, while different species of *Nitrospira* are common (Hovanec and Long, 1998; Gonzalez-Silva et al., 2016; Bartelme et al., 2017; Fossmark et al., 2021; Aalto et al., 2022). The low AOB:NOB ratio can be explained by the presence of comammox *Nitrospira* (van Kessel et al., 2015; Daims et al., 2015). The third most abundant *Nitrospira* OTU was related to *Candidatus Nitrospira nitrificans*, identified as a comammox species in trickling filters in RAS (van Kessel et al. 2015), capable of complete ammonia oxidising (Costa et al., 2006; van Kessel et al., 2015; Daims et al., 2015). Currently, our understanding of factors that govern distributions and abundances of comammox is limited. However, several studies show that comammox *Nitrospira* are more abundant in

freshwater environments (Sobotka et al., 2018; Bartelme et al., 2019; Minish et al., 2020), with low ammonia concentrations and might benefit from their higher growth yield when compared to canonical ammonia oxidizers (Costa et al. 2006; Bartelme et al., 2019). Another explanation to the low relative abundance of AOB could be the presence of ammonia oxidising archaea (AOAs). AOAs has previously been identified in high abundances in marine and freshwater RAS (Brown et al., 2013; Bartelme et al., 2017; Bartelme et al., 2019) and it is likely that the AOA are competing and/or coexisting with comammox *Nitrospira* (Bartelme et al., 2019). However, the primers used in this thesis were not designed to target archaea. Although research on nitrification has made great progress in RAS, more knowledge is required to fully understand ammonia oxidation and the contribution of comammox and AOA in nitrogen conversions in the RAS biofilter.

## 5.2 Effects of in-line disinfection on water microbiota in RAS

In the commercial RAS for salmon fry and parr production (Paper I), with relatively short HRT (23 min), the in-line UV disinfection located upstream the rearing tanks reduced the concentration of culturable bacteria with 89% directly downstream the disinfection. These results corroborate previous experiments in RAS (e.g., Huyben et al., 2018). For both commercial RAS for production of salmon fry and parr examined in this thesis, which had short HRT (18-28 min) in the rearing tanks, the rearing water showed a regrowth of fast-growing, presumably opportunistic, and harmful bacteria in the rearing water (Paper I and II). The results show that the two commercial RAS studied had a potential for regrowth of opportunistic bacteria in the rearing water even though the HRT in the rearing tanks were short. However, in these systems the water microbiota was found to be relatively similar throughout the system for each sampling day for all production batches studied, both by 16S rRNA sequencing of DNA (Paper I and II) and RNA (Paper I), which supports previous findings where water microbiota was similar throughout a post-smolt RAS (Bakke et al., 2017). Also, the alpha diversity of the microbiota was not significantly different between the water going to the UV and in the rearing tanks in the two RAS studied. The short HRT limits the time for high regrowth of bacteria in the rearing tanks (Bakke et al., 2017; Vadstein et al., 2018b) and changes in microbiota composition. However, the DNA-based methods used include live, inactivated, and dead bacterial cells, which means that it is difficult to distinguish between

viable and dead bacterial cells (Li et al., 2017). A strategy to overcome this difficulty is to focus on the presence of the more rapidly degrading RNA (Li et al., 2017). However, rRNA is relatively stable, and might not have been degraded in the bacterial cells that were inactivated by UV (Paper I).

In the marine RAS for lumpfish production where different water treatment regimens were implemented, the HRT in the tanks was long (60 min), and the UV and the combined UV and ozone disinfection of the water going into the rearing tanks. This situation created niches for fast-growing bacteria in the rearing tanks (Paper III). The regrowth resulted in a significantly reduced microbial alpha diversity and a more variable microbiota composition between replicate tanks and over time in the rearing tanks, compared to RAS rearing water that was not disinfected. These characteristics of the microbial communities indicates that r-selection acted on the microbiota in the rearing tanks that had UV, with increased fraction of opportunistic bacteria. An r-selected microbial community can have negative effects on marine larval health and survival (Attramadal et al., 2012a; 2014; Vadstein et al., 2018a; 2018b; Vestrum et al., 2018; Attramadal et al., 2021). The disinfection of the water going into the rearing tanks selected for increased relative abundances of *Leucothrix* in the water, which can cause fouling of respiratory surfaces of reared species in aquaculture (Johnston et al., 1971; Dale and Bloom, 1987). The increased abundances of *Leucothrix* corresponded to the significantly more challenged gill health for the fish reared in RAS with disinfection. Gill health can be a good indicator of fish health status in relation to the farming conditions (Marshall and Bellamy, 2010). We showed a possible relationship between water treatment design, microbial water quality and fish health. The results also showed that the negative effects of poor microbial water quality are not only relevant for the viability of the first development stages of cod and lobster (Attramadal et al., 2012a; 2021), but also for later development stages of marine fish, i.e., juvenile lumpfish of 52 grams. However, the survival and growth of lumpfish were similar between the RAS treatments. Since the experiment started two months post hatch, the initial mortality had passed, and the fish may have been more robust to suboptimal microbial environments than in the early developmental stages. The disinfection and the following regrowth in the rearing tanks led to changes the microbial community structures (Fig. 13) for both the tanks with the UV and the combined UV and ozone treatments

when compared to the RAS without disinfection (Paper III). It should be stressed that these differences were significant despite the tanks of the different treatments being connected to the same RAS, and thus the water going into the tanks was a mix from all the treatments.

The results indicate that UV disinfection will have a negative effect on the microbial water quality and fish health in RAS with long HRT in the rearing tanks (Paper III), while the negative effects of the UV treatment are reduced in RAS with short HRT in rearing tanks (typically in RAS producing Atlantic salmon, Paper I and II). However, in theory, a community with considerable potential for opportunistic regrowth might be vulnerable for pathogen proliferation. Although production is state-of-the-art, which were the case for the facilities in Paper I and II, it is likely that pathogens are present in RAS at low abundances, but without causing any mortality as long as they are not able to proliferate (Michaud et al., 2009, Lewin et al., 2020; Dahle et al., 2020b). Lewin et al. (2020) detected the salmonid pathogens *Flavobacterium Psychrophilum* and *Yersinia Ruckeri* in low abundances in water samples from three individual commercial RAS for salmon with normal production. K-selected communities can in theory provide the system with a more resilient microbiota against pathogen invasion and proliferation (Attramadal et al., 2012a; 2012b; De Schryver and Vadstein, 2014; Attramadal et al., 2014; 2021), and is hence of great importance in RAS for a more robust production system (Michaud et al., 2009; Vadstein et al., 2018b; Borges et al., 2021). Absence of disinfection in the RAS loop, or alternatively, placing the disinfection upstream of the biofilter instead of directly in front of the rearing tanks, is therefore hypothesized to provide a more resilient system with lower probabilities for growth/blooms of opportunistic bacteria and pathogen proliferation. To test this hypothesis, invasion studies in RAS with and without in-line UV-disinfection could be performed. More research, including well-designed experiments are needed to provide more knowledge on the long-term effect of in-line disinfection on the microbial communities in the water.

## 5.3 Practical implications of the results

To control the microbial community composition in RAS for optimal chemical and microbial water quality, with further positive effects on fish health, it is necessary to control the

selection pressure acting on the system's microbiota. In this thesis, organic matter load, maturation state of the biofilter, and in-line disinfection were found to contribute to this selection pressure. Effective particle removal and optimal and well-balanced feeding routines should be achieved to avoid growth of heterotrophic fast-growing opportunistic bacteria and support stability of system microbiota composition in RAS. Also, this will have positive effects on the biofilter efficiency. A fully maturated biofilter at the inset of fish is of outermost importance to secure an efficient ammonia removal but might also contribute through the consumption of organic matter, and thereby increase the competition for substrates and resources. This will contribute to K-selection acting on the suspended water microbiota, for beneficial fish-microbe interactions and a resilient system.

Our results show that in-line disinfection with UV should be avoided in RAS with long HRT (typical marine hatcheries) in an otherwise well dimensioned and managed RAS, to avoid blooms of opportunistic bacteria in the rearing tanks and negative fish-microbe interactions that resulted in poorer gill health. For RAS with short HRT in rearing tanks (typical salmonid production), our results indicate that the detrimental effect of disinfection is highly reduced, but that there is a potential for regrowth of opportunistic bacteria in the rearing tanks. By locating the in-line disinfection upstream the biofilter or not having it at all in RAS with short HRT, we hypothesize that the system will be more resilient against proliferation of pathogens, that most likely are present at low abundances in most commercial RAS.

# Chapter 6: Conclusions

This PhD has provided new knowledge on the long-term microbial community dynamics of water and biofilm in commercial RAS for production of Atlantic salmon fry and parr. The system's microbial communities of the commercial RAS were highly variable over the 15 months period monitored. The organic matter load, which increased during production batches and were absent at fallowing, significantly influenced the microbial communities. Although the microbial communities changed substantially during periods of fallowing, we observed a relatively fast return to a very similar community compositions during production periods, probably because of a similar selection pressure during production.

In the same RAS, we found higher abundances of nitrite oxidising bacteria (NOB) than ammonium oxidising bacteria (AOB). One of the *Nitrospira* OTUs were related to a previously identified complete ammonia oxidiser, comammox *Nitrospira nitrificans*.

Fully maturated biofilters at the inset of fish provided a more stable water microbiota with higher alpha diversities than the more immature and recently disinfected biofilter.

We showed that in-line disinfection upstream of rearing tanks had negative effects on the microbial water quality and fish health in RAS producing lumpfish with long HRT in the rearing tanks. In comparison, we found that the in-line UV treatment led to considerably lower regrowth of bacteria in the fish tanks in the RAS for salmon smolt production with short HRT and thus the negative effects of the UV treatment appeared to be reduced.

# Chapter 7: Future perspectives

We provided new knowledge on the temporal microbial community dynamics of the system's microbiota in RAS and how different factors are affecting these microbial communities. However, there is still a way to go before we fully understand these dynamics and how to control them to favorable conditions for the reared fish. Although we have showed that K-selection creates favourable microbial conditions for fish in RAS, and that a well maturated biofilter and controlled organic loading might be characterising a good microbial quality, it is difficult to define a healthy microbiota. This is something that needs to be explored for the future, by elucidating the functionality of the microbial communities, for example with transcriptomic approaches.

Research on the nitrifying biofilters in RAS has made great progress during the last decade, to understand the complexity and to document their performance, but still there is a potential for optimising biofilter function further. Future work is needed to fully understand the different nitrifying consortia in freshwater RAS biofilters and their activity and interactions. Since the relative abundance of AOB is typically low in freshwater RAS, compared to NOB, complete ammonia oxidisers like comammox and archaea should be targeted to identify these ammonium oxidising guilds. Knowledge on their relative abundance and contributions in the nitrification process will lead to a more complete understanding of factors that control ammonia removal in RAS, and how system operations can take advantage of potentially flexible ammonia-oxidisers. This can further contribute to optimize biofilter performance.

The results indicated that the bacteria in the biofilter biofilm influences the suspended bacterial communities the RAS. We also showed that the maturation status of the biofilter affects the rearing water microbiota. The extent and nature of these interactions is however not fully understood. The microbial communities in the RAS biofilter is still difficult to control (Leonard et al. 2000, Michaud et al. 2006; 2009; Schreier et al. 2010), and more studies and experiments are required to understand the interactions between the biofilter and suspended microbiota, and how to control the communities better for optimal biofilter performance, system control and fish health.

The negative effects of in-line disinfection in RAS with short HRT in rearing tanks appeared to be highly reduced in RAS with short HRT. However, UV-disinfection favours regrowth of fast-growing, presumably opportunistic bacteria in the rearing tanks. How these communities affect Atlantic salmon is unknown but might be relevant in the early developmental stages. Also, a system with high potential for regrowth can in theory be more vulnerable for pathogen proliferation. Experiments with Atlantic salmon should be conducted, to study the long-term effects of in-line disinfection on microbial water quality and the potential influence on fish health and viability. Identical RAS with and without disinfection should be included in controlled experiments to provide a broader knowledge on the disinfection effects. Many facilities include disinfection in front of the rearing tanks, and knowledge on how this management affects the microbial communities and risks would be valuable.

For future research, technology and methods for microbial management at the ecosystem level, i.e., favourable microbial environment and optimal physicochemical water quality, has the potential to improve RAS further. Ultimately, progress within this field can lead to better fish health, a more controlled production, and a more sustainable aquaculture industry for the future.

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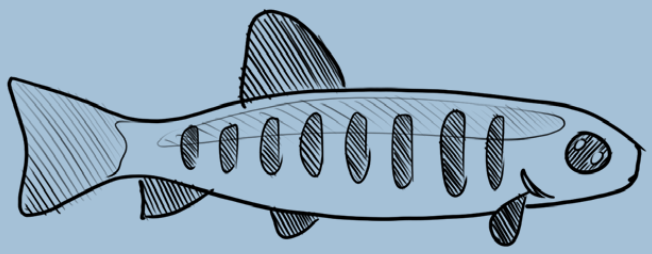
# Paper I



Aquaculture 546 (2022) 737382

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

# Microbial community dynamics in a commercial RAS for production of Atlantic salmon fry (*Salmo salar*)

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A B S T R A C T

Recirculating aquaculture systems (RAS) harbour complex microbial communities which can have an impact on the growth and development of the reared fish. This study aimed to improve our understanding of microbial community dynamics in a RAS involving three production batches of Atlantic salmon fry and parr during a period of 20 months. Water for analysis of microbiota was sampled at different positions in the RAS, and we also examined the effect of UV treatment on the water microbiota. Microbial communities were characterized by 16S rDNA amplicon sequencing of water samples taken directly upstream and downstream of the UV treatment unit and from three of the rearing tanks. In total 6 sampling events were made during a 20-month period. The study showed that: 1) Two of the production batches had a highly similar water microbiota despite disinfection of the system between the batches and rearing fish of different stages. In contrast, the first production batch showed a different water microbiota with variable composition through the system and over time. A more immature biofilter in the first batch may explain these differences. 2) The full-flow UV treatment directly upstream the rearing tanks had no observable effect on the community composition of the water microbiota in the different sampling positions in the RAS. This was likely a consequence of the low hydraulic retention time (HRT) (23 min) in rearing tanks, low bacterial regrowth in the fish tanks and community changes throughout the RAS loop. 3) The disinfection effect on viable bacterial densities in the water directly downstream of the UV treatment was around 89%, when the water was clear. Regrowth of bacteria following disinfection was low compared to those reported for marine RAS with UV disinfection and long HRT in fish tanks. The study shows that UV disinfection can be used to efficiently reduce bacterial density without compromising the microbial water quality in the fish tanks in RAS with low HRT.

## 1. Introduction

Recirculating aquaculture systems (RAS) have become a popular production system for Atlantic salmon (*Salmo salar*) (Badiola et al., 2012; Kolarevic et al., 2014; Rurangwa and Verdegem, 2015; Davidson et al., 2017). RAS provide several advantages compared to flow-through systems, like saving energy for heating, controlling and stabilizing water quality, and reducing environmental impact (Martins et al., 2010; Dalsgaard et al., 2013; Davidson et al., 2017). With a well-considered systems design, dimensioning and management strategies, RAS also have properties that can contribute to stable and mutualistic fish-microbe interactions (Skjermo et al., 1997; Attramadal et al., 2012a, 2012b, 2014; Attramadal et al., 2016; Vadstein et al., 2018; Vestrum et al., 2018; Duarte et al., 2019).

The microbial communities in RAS are complex and essential for both chemical and microbial water quality and plays a crucial role for the health of the cultured fish (Blancheton et al., 2013; Vadstein et al., 2018). Certain microbial assemblages may impact fish health positively, others may have a negative influence on the fish, and even cause mortality. The microbial communities in RAS are affected by feed and feeding regimes, the make-up water, management routines, the fish itself and selection pressure in the system (Attramadal et al., 2012a; Blancheton et al., 2013; Vadstein et al., 2018). Hence, the microbial assemblages can vary between systems and over time (Rud et al., 2017; Bakke et al., 2017; Dahle et al., 2020; Fossmark et al., 2020a, 2020b). Unstable microbial water quality with high fractions of opportunistic bacteria, is one important factor that contributes to sub-optimal conditions for the cultured fish (Bakke et al., 2017). However, the mechanisms causing these changes are fairly well understood for marine larvae (Vadstein et al., 2018), but poorly documented for salmonids. Thus, more knowledge is needed to understand and improve microbial management strategies specifically for land-based cultivation of salmonids.

Disinfection of the intake water reduces the risk of entry and spreading of pathogens into the system (Sharrer et al., 2005; Summerfelt

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https://doi.org/10.1016/j.aquaculture.2021.737382
Received 13 May 2021; Received in revised form 20 August 2021; Accepted 21 August 2021
Available online 24 August 2021

et al., 2009), and is of paramount importance for the biosecurity of land-based facilities. However, opportunistic and pathogenic bacteria may still reside among the bacteria inside the RAS, and disinfection can be used to continuously disinfect recirculated water before it returns to the rearing tanks (Summerfelt et al., 2009). Experiments with in-line UV disinfection in pilot scale RAS have shown a reduction of heterotrophic bacteria by 98% (Huyben et al., 2018) and a lower microbial activity (Huyben et al., 2018; Schumann and Brinker, 2020; de Jesus Gregersen, 2020) . UV disinfection also reduces the micro particle numbers by destroying bacteria that uses micro particles as substrate and surface area (Pedersen et al., 2017; Gregersen et al., 2020).

Disinfection kills and inactivates bacteria but does not reduce the amount of substrate available for bacterial growth. Disinfection therefore leads to a situation of low competition for the available substrate, and therefore favour r-selection and subsequent proliferation of opportunistic bacteria in the rearing water (Sharrer et al., 2005; Hess-Erga et al., 2010; Attramadal et al., 2012b; Vadstein et al., 2018; Attramadal et al., 2021). The time window between disinfection and significant bacterial regrowth is determined by the number and growth rates of bacteria surviving or seeding the water volume from biofilm following disinfection. UV disinfection within the RAS treatment loop, and especially immediately before the fish tanks, is therefore hypothesized to constitute a disadvantage for the health of fish in an otherwise well dimensioned and managed RAS (Attramadal et al., 2012b; Vadstein et al., 2018; Dahle et al., 2020; Attramadal et al., 2021). Several experiments with marine larvae have shown that UV treatment inside the RAS loop destabilise the microbial composition of the rearing with negative effects on viability and survival (Attramadal et al., 2012b; Dahle et al., 2020; Attramadal et al., 2021). However, the effects of UV irradiation in commercial freshwater RAS for salmon production on microbial community composition and blooms of opportunistic bacteria is not studied.

Here, we characterize the water microbiota in a commercial RAS for production of Atlantic salmon fry and parr by sequencing of 16S rRNA amplicons. We sampled five positions in the RAS loop and covered three distinct production batches over a 20 months' period. The aim was to map the temporal dynamics of the water microbiota in this commercial system over a long-term period, to elucidate the impact of UV disinfection on the water microbiota, and to improve the general understanding of the bacterial community dynamics in RAS.

## 2. Methods

### 2.1. Culture system

The study was based on sampling of water in a commercial RAS for production of Atlantic salmon fry and parr in Norway. The start-feeding RAS department (Inter Aqua Advance, Denmark) consisted of 18 octagonal fish tanks (16 $m^3$) operated with fresh water (Fig. 1). The intake water was from a hydroelectric power plant and was prefiltered with a coarse screen, without UV treatment. The volume of the total system was 470 $m^3$ and the system flow set to 10.8 $m^3$/min. The RAS included the following components after the fish tanks: a drum filter of 60 μm, pH-regulation, two Moving Bed Biofilters (MBBF) (Inter Aqua Advance,with Curler advance X-1 bioelement, volume: 2 × 50 $m^3$), two drum filters (60 μm), two Fixed Bed Biofilters (FBBF) (Inter Aqua Advance), Trickling filter (Inter Aqua Advance) and a UV unit (Atlantium RZ 2300–12, Teknor, Norway) with a dose of 75–100 mJ/$cm^2$, treating the full-flow right before entering the fish tanks (Fig. 1). The light regime was 24:0 with fluorescent tubes. Hydraulic retention time was 23 min in the fish tanks and in average 7–9 days for the total system. The make-up water flow varied during the production and between batches of fish, from 0.15–3.8 L $h^{-1}$ for the spring batches and 0.7–4.81 L $h^{-1}$ for the autumn batch, amounting to 300–400 L new water/kg feed. The system, included the biofilter, was disinfected before 2015_spring and between batch 2015_autumn and 2016_spring, in consecutive treatments with lye, chlorine dioxide and ozone. Before the 2015_spring batch the biofilter was started after disinfection with new, clean carriers and maturated until the stocking of fish, while before the 2016_spring batch the biofilter included already matured biofilm carriers from an already running biofilter. The 2015_autumn batch was not disinfected before stocking of fish. Production data and physicochemical water quality variables were provided by the RAS facility for the periods, including temperature, total ammonia nitrogen (TAN), nitrite, nitrate, $CO_2$, alkalinity, and pH. Normally, four to five batches of fish are produced during a year in this system.

### 2.2. Rearing regime

In this study, we monitored three different batches of fish in the same system. The spring production batches growing fish up to 3–4 g in 2015 (2015_spring) and 2016 (2016_spring) took place during the months of February, March, and April. The autumn 2015 production batch growing fish up to 25 g took place during September, October, and

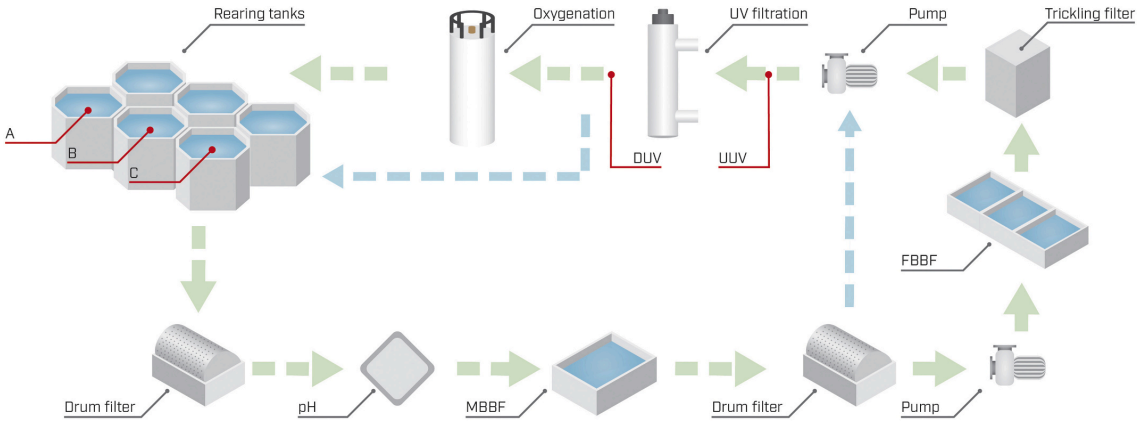


**Fig. 1.** Schematic presentation of the RAS unit monitored in the study. Samples for analyses of the RAS water microbiota (red lines) were taken from three fish tanks (A, B, C), and immediately upstream (UUV) and downstream of the UV treatment unit (DUV). MBBF = Moving Bed Biofilter, FBBF=Fixed Bed Biofilter. The UV disinfection represent a full-flow disinfection. Illustration by Mats Mulelid, SINTEF Ocean.

November (Fig. 2). The final biomass density in each tank was 37–48 kg/m$^3$ for the spring batches and 65–71 kg/m$^3$ for the autumn batch. Dead fish were removed and recorded daily to assess the daily mortality in each tank. The spring batches were fed continuously Ewos Micro 040 and 1 (Ewos, Norway) while the autumn batch was fed Ewos Micro 5 and 15, according to fish size. Feed load pr day during the period was 0.7–19 kg/tank for the spring batches and 5.6 to 25.0 kg/tank for the autumn batch.

### 2.3. Sampling for microbiological analysis

For characterization of the bacterial communities by DNA-based 16S rDNA amplicon sequencing, water directly upstream (UUV) and downstream of the UV unit (DUV) and from three rearing tanks (A, B, C) was sampled one to three times during the three production batches (Fig. 2). The 2015_spring batch was first sampled two days prior to the inset of fish (d-2) and at day 12 and 34. The 2015_autumn batch was sampled once, at day 29, and the 2016_spring batch was sampled at day 13 and 41 after inset of fish. Water samples were also collected for RNA-based 16S rRNA amplicon sequencing for 2015_autumn on day 29 and 2016_spring on day 41. The water samples were filtrated using Dynagard filters (pore size 0.2 μm, Microgon) and Omnifix® syringes. Around 150–200 mL water was filtrated for each water sample. Samples were frozen (−20 °C) immediately after sampling, transported to NTNUs laboratory and stored at −80 °C until further processing.

Water samples for quantification of colony forming units (CFU) were collected from the same positions in the RAS at selected sampling times during the production batches 2015_spring (day −2 and 34) and 2016_spring (day 13 and 41). Approximately 1 L of water was collected from each sampling position in triplicates and mixed well before 1 mL was used in CFU analysis as described below.

### 2.4. Microbial community analyses

To quantify CFU in water samples serial dilutions of the sampled water (1:10–1:1000) were prepared and streaked out on M65 agar plates with 0.1% NaCl (0.5 g/L peptone, 0.5 g/L tryptone, 0.5 g/L yeast extract, and 1 g NaCl per L water) in triplicates for each dilution. The CFU numbers were determined as the number of colonies observed after 14 days' incubation in room temperature.

For characterization of bacterial community composition, DNA was extracted using the Power Soil DNA isolation (MOBIO) as described by the manufacturer. For two sampling times, the PowerMicrobiome RNA Isolation Kit (MOBIO) was used to extract total RNA water samples, as following the protocol. cDNA was synthesized by use of Prime ScriptTM 1st strand cDNA Synthesis Kit (TaKaRa), as described by the manufacturers. Random 6 mers and approximately 1 mg total RNA was used as template in each reaction. The third and fourth variable regions (V3, V4) of the bacterial 16S rRNA gene was amplified from DNA extracts and cDNA using the primers Ill-338F (5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNCCTACGGGWGGCAGCAG) and Ill-805R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNGACTACNVGGGTATCTAAKCC) (Nordgård et al., 2017) and Phusion Hot Start DNA Polymerase (Thermo Scientific, USA). The amplicons were normalized using the SequalPrepTM Normalization Plate Kit (Invitrogen, USA) and indexed using the Nextera XT Index kit (Illumina, USA) as described in Vestrum et al. (2020). Amplicons were pooled and concentrated using the Amicon Ultra-0.5 Centrifugal Filter Device, and the resulting amplicon library was sequenced on an MiSeq run (Illumina, USA) with the MiSeq Reagent Kit v3 in the 2 × 300 bp paired-end mode at the Norwegian Sequencing Centre. The resulting sequencing data are deposited at the European Nucleotide Archive (accession numbers ERS7273454 - ERS7273493).

The Illumina sequencing data were processed using the USEARCH pipeline (version 11; https://www.drive5.com/usearch/). The command Fastq_mergepairs was used for merging of paired reads, trimming off primer sequences and filtering out reads shorter than 400 base pairs. Further processing included demultiplexing and quality trimming (the Fastq_filter command with an expected error threshold of 1). The UPARSE-OTU algorithm (Edgar, 2013) was used for chimera removal and clustering at the 97% similarity level. Taxonomy assignment was performed applying the Sintax script (Edgar, 2016) with a confidence value threshold of 0.8 and the RDP training set (version 16). The resulting OTU (operational taxonomic units) table was normalized to 37,000 number of reads (the lowest number of reads obtained among samples) per sample by determining the fraction of the OTUs for each community profile, and then multiplying with 37,000, and finally rounding off the read numbers to integers. The USEARCH commands Alpha_div and Sintax_summary was used to calculate alpha diversity indices (observed OTU richness and Shannon's diversity) and generate taxa summary tables, respectively.

### 2.5. Statistical analysis

The data for fish survival were Arcsin-transformed before statistical analysis by one-way ANOVA (SPSS version 27). For comparisons of chemical variables and microbiota, One-way ANOVA or Kruskal-Wallis were used, depending on normality and homogeneity of variance of the variables (SPSS). Statistical analyses of microbial community data, based on the OTU table, were performed using the program package PAST (version 3; Hammer et al., 2001). OTUs with a maximum abundance of less than four reads in all samples in the normalized OTU table (37,000 reads per sample) were filtered out of the OTU table prior to multivariate analyses. Principal coordinate analysis (PCoA) (Davis, 1986) was based on Bray-Curtis similarities (Bray, 1957). To test for differences in community structure between sample groups, we applied one-way PERMANOVA based on Bray-Curtis similarities (Anderson, 2001). The Similarity Percentages (SIMPER) analysis (Clarke, 1993) was used to determine the contribution from the OTUs to the Bray-Curtis dissimilarity between samples and groups.

## 3. Results

Three different batches of fish were produced in different periods in the same RAS unit during this study. The batch 2015_spring and 2016_spring both produced fry (from 0.2 to 4 g) in the same period of the year (February, March, April) in two subsequent years, whereas the 2015_autumn batch produced parr (from 2.5 to 25 g) during September,

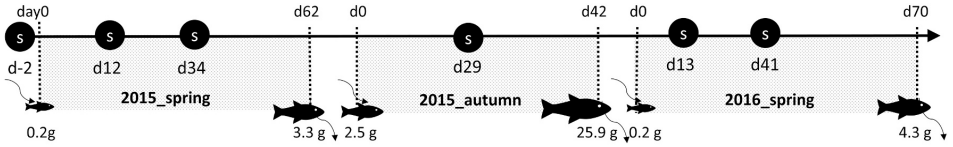


**Fig. 2.** Timeline for sampling of microbiota and production at the RAS facility with weight of fish in grams. 2015_spring, 2015_autumn and 2016_spring indicates the three production batches that were monitored. Production batches in spring were produced from February to April and production batch autumn from September to November. S = sampling, d = day of production (day 0 corresponds to the day of inset of fish in the RAS), sampling d-2 = two days before fish were put in the RAS unit.

October, and November.

### 3.1. Physicochemical water quality

The average physiochemical water quality variables were generally satisfying according to the recommended thresholds for Atlantic salmon and relatively similar among the three production batches examined (Table 1). However, the 2015_autumn production batch had higher $NO_3^-$-N concentrations than the other batches, and 2016_spring had higher total ammonia nitrogen concentration. None of these differences were statistically significant.

### 3.2. Fish performance

The average mortality of fish in batch 2015_autumn (0.01 ± 0.00%) was significantly lower compared to that in batch 2015_spring (0.05 ± 0.01%) and 2016_spring (0.06 ± 0.01%) (Kruskal-Wallis, $p = 0.001$; 0.001). The mortality was also more stable over time for batch 2015_autumn, compared to the batches from spring, which had peaks in mortality during the production (Fig. 3). 2015_spring had a maximum mortality of 0.60% at day 23, whereas for 2016_spring the highest mortality was 3.12% at day 16. The following days the mortality decreased and was relatively stable throughout the batches.

### 3.3. Microbial water quality

#### 3.3.1. Microbial community composition in the RAS water

The relative abundance of the bacterial orders differed among the production batches. The water from batch 2015_spring had a clearly different community composition compared to the other batches (Fig. 4). The most abundant bacterial order in rearing water for 2015_spring was Pseudomonadales, which was also significantly more abundant in 2015_spring than in the other two batches (Kruskal-Wallis, $p = 0.001$; 0.001). For 2015_spring samples Pseudomonadales accounted for 20–81% of the community, with an average of 46%. For the other production batches this order constituted 1–28%, with an average of 8%. In contrast, Burkholderiales was the most abundant order for production batch 2015_autumn and 2016_spring, with abundance ranging from 19 to 38%, and with an average of 28%. Rhodobacterales and Cytophagales were considerably more abundant in the water during the 2015_autumn and 2016_spring batches compared to the 2015_spring batch (Fig. 4). Ordination by Principal Coordinate Analysis (PCoA) indicated that that the bacterial community composition clustered according to batch and sampling time (Fig. 5). A PERMANOVA test confirmed that the water microbiota was significantly different between production batches ($p = 0.02$). Even though batch 2016_spring was produced in the same season as 2015_spring, with the same size of fish and operated similarly, the composition of the microbiota was more similar to production batch 2015_autumn. The autumn batch was produced during another season, with bigger size of fish (Fig. 5). Average Bray-Curtis similarities showed that the community composition for production batch 2015_spring was considerably more variable between

**Table 1**
Physicochemical water quality measured in the RAS loop (after the water treatment) during the production batch (mean ± SE)).

| | 2015_spring | 2015_autumn | 2016_spring |
|---|---|---|---|
| Temperature (°C) | 13.70 ± 0.05 | 13.20 ± 0.09 | 14.10 ± 0.05 |
| Total ammonia nitrogen (mg TAN $L^{-1}$) | 0.90 ± 0.52 | 0.73 ± 0.28 | 1.39 ± 0.33 |
| Nitrite (mg $NO_2$-N $L^{-1}$) | 0.31 ± 0.16 | 0.23 ± 0.09 | 0.26 ± 0.24 |
| Nitrate (mg $NO_3$-N $L^{-1}$) | 15.16 ± 3.74 | 25.54 ± 2.10 | 9.00 ± 7.07 |
| $CO_2$ (mg $L^{-1}$) | 13.28 ± 1.51 | 15.36 ± 1.45 | 14.47 ± 1.44 |
| Alkalinity (mg $CaCO_3$ $L^{-1}$) | 1.19 ± 0.02 | 1.41 ± 0.01 | 1.33 ± 0.01 |
| pH | 7.17 ± 0.02 | 6.97 ± 0.03 | 7.10 ± 0.01 |

Number of measurements done during production equal 42 to 70.

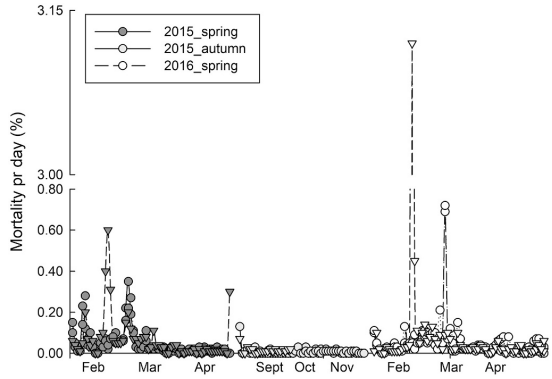


**Fig. 3.** Fish mortality during the three different batches of fish production (2015_spring, 2015_autumn and 2016_spring) from three different fish tanks (square, circle and triangle represent the three different fish tanks).

fish tanks and over time compared to batch 2015_autumn and 2016_spring (Fig. 6).

The most abundant OTU of the whole dataset, OTU_8 (*Pseudomonas*) was significantly more abundant in the 2015_spring batch than in the two other batches (Kruskal-Wallis, $p < 0.05$), with average abundances of 16% of the total reads for the 2015_spring samples, compared to only 0.005 and 0.07% for the 2015_autumn and the 2016_spring batch, respectively (Table 1, Supplementary). The genus *Acinetobacter* (Pseudomonadales) was represented by three OTUs (OTU 1, 2 and 14) that were significantly more abundant in the 2015_spring samples (Kruskal-Wallis, $p < 0.05$), with average abundances of 7% (Fig. 1, Supplementary). In the 2016_spring samples, OTU_3 (*Rhodobacteraceae*), was significantly more abundant (average abundance 10.4%) than in the 2015_spring and 2015_autumn samples (Kruskal-Wallis, $p < 0.05$). For the 2015_autumn samples, the most abundant OTU was OTU_10 (*Enterobacteriaceae*; average abundance 8%) which was considerably higher compared to the other batches (Table 1, Supplementary).

The alpha diversity of the RAS water microbiota, expressed as OTU Richness and the exponential Shannon's diversity index ($e^{Shannon's}$), was considerably higher for production batch 2015_autumn (only one sampling time) than for the water of the spring batches (Fig. 7A, B), and were significantly higher than 2015_spring (Kruskal-Wallis, $p = 0.001$). No significant differences in alpha diversity were detected between the 2015_autumn and 2016_spring batches. Generally, the diversity increased with time for the spring batches (Fig. 7A, B). The alpha diversity of the water microbiota was slightly reduced in the fish tank compared to the rest of the system (Fig. 7), which may be a consequence of regrowth. The differences between upstream/downstream the UV and the fish tanks were however not significant.

#### 3.3.2. Temporal dynamics of the water microbiota

The community composition for the water of production batch 2015_spring was substantially more variable over time, compared to the 2016-spring batch (Fig. 6). Both the PCoA ordination (Fig. 5) and the average Bray-Curtis similarities (Fig. 6) demonstrated that particularly in the 2015_spring batch, the water microbiota underwent major changes throughout the production period (Fig. 6). The relative abundance of OTUs representing *Acinetobacter* (OTU 1, 2 and 14) increased with sampling time (Supplementary, Table S1, Fig. S1), and contributed with 23.6% of the differences between timepoints (SIMPER analysis). The Bray-Curtis similarities for comparison of the tank water microbiota between sampling times were high for 2016_spring (Fig. 6) and indicated stability of the water microbiota throughout this production batch.

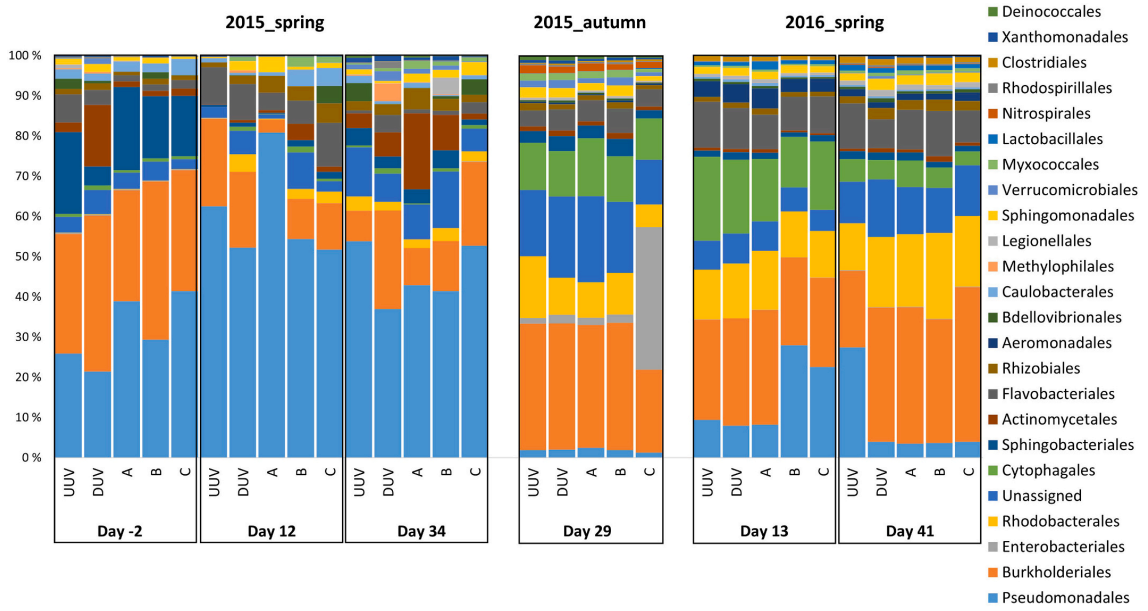


**Fig. 4.** Relative abundances (%) of bacterial orders in the rearing water of the different batches of fish (2015_spring, 2015_autumn, 2016_spring), at different sampling days, where day represent the day in production and − 2 represent two days before inset of fish. UUV = upstream UV, DUV = downstream UV. A, B and C = three different rearing tanks. Only orders that are present at abundances >1% in at least one sample are shown.

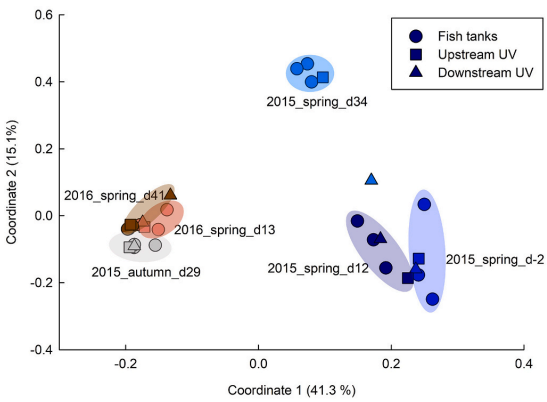


**Fig. 5.** PCoA plot based on Bray–Curtis similarities for three different batches of fish (2015_spring, 2015_autumn, 2016_spring), at different sampling days (d), where d-2 represent two days before inset of fish. Samples include tank water microbiota and water upstream and downstream the UV treatment.

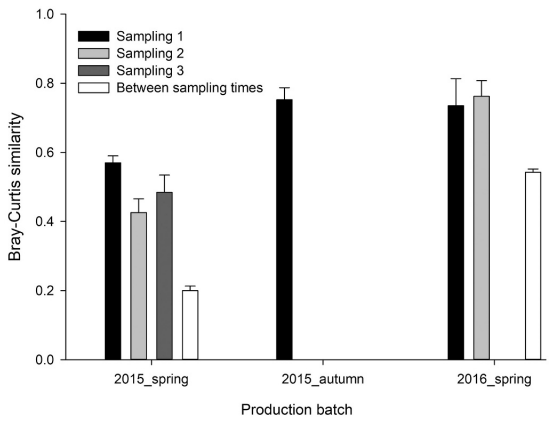


**Fig. 6.** Bray–Curtis similarities for comparing the water microbiota within each production batch at sampling 1, 2 and 3 and between sampling times. At each sampling time, the samples include upstream and downstream of the UV treatment and the three rearing tanks. Sampling days for each production batch are presented in Fig. 2. Error bars show the standard error (±SE) of all the samples, $n = 5$.

### 3.3.3. *Microbial community dynamics throughout the RAS*

The PCoA plot (Fig. 5) indicated that in general, the water microbiota was relatively similar between sampling points throughout the system on the same sampling day. This was particularly evident for Bray-Curtis similarities for the two last batches (Fig. 6). DNA-based methods include live, inactivated, and dead bacterial cells. To improve the possibility to detect changes in the active microbial communities, and to reveal potential effects of UV treatment on the water microbiota, we performed 16S rRNA amplicon sequencing based on total RNA extracts for water samples from 2015_autumn (day 29) and 2016_spring (day 41). However, neither PCoA ordinations with Bray-Curtis (Fig. 8) nor Dice-Sørensen coefficients (data not shown) indicated larger variation in community composition throughout the system for RNA-based compared to DNA-based analyses. Still, there was significant differences between the DNA and RNA based analyses (PERMANOVA; $p = 0.01$; $p = 0.008$).

We were particularly interested in the effect of the UV treatment on the water microbiota, but there were no indications this had any observable effect, judged on comparison of the DNA- and RNA-based community composition using ordination. However, a manual

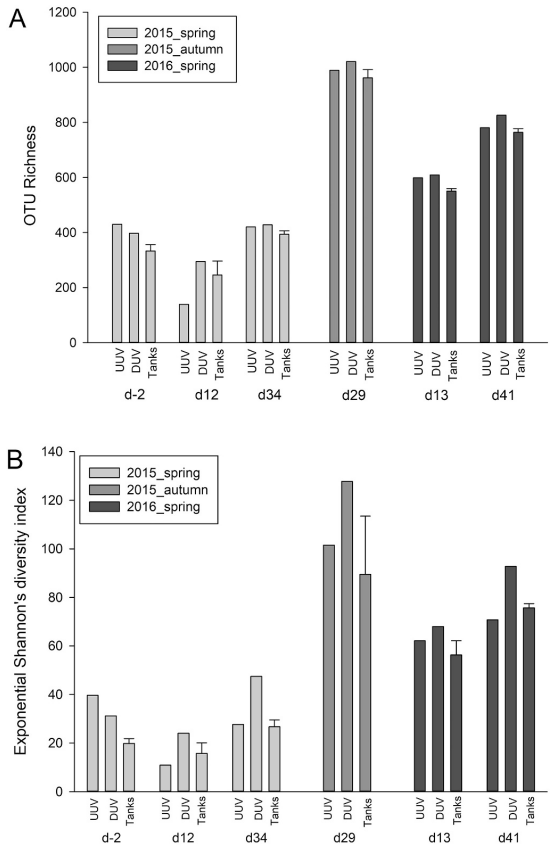


**Fig. 7.** Alpha diversity of RAS water expressed as A) OTU Richness and B) Exponential Shannon's diversity index, at different sampling days (d). Error bars show the standard error (±SE) between triplicate fish tanks. UUV = upstream UV, DUV = downstream UV. d-2 represent two days before inset of fish.

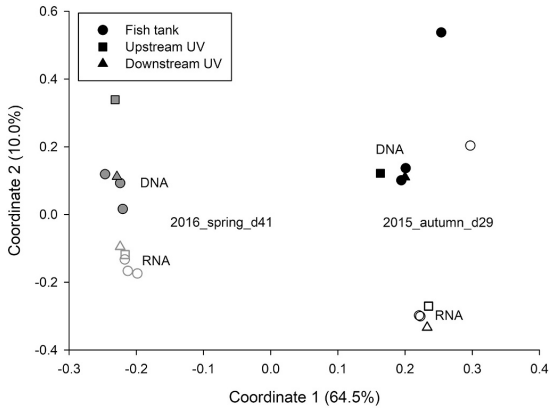


**Fig. 8.** PCoA plot based on Bray–Curtis similarities for DNA- (closed symbols) and RNA-based (open symbols) samples. Samples include microbiota from tank water and water upstream and downstream the UV treatment at two different sampling dates (2016_spring day 41 and 2015_autumn, day 29). d represent the day in production.

inspection of the OTU table revealed that three OTUs (OTU 1, 2 and 14) had a lower abundance in samples taken downstream of the UV treatment compared to upstream the UV treatment. The RNA-based data showed that these OTUs increased in the fish tanks (Supplementary Table 1, Fig. 1). These OTUs were all classified as *Acinetobacter* and were the same OTUs that were highly abundant in the 2015_spring samples (Supplementary Table 1, Fig. 1).

We also examined the effect of the full-flow UV treatment on the culturable, living bacterial cells as CFU for water samples from the two spring batches (Fig. 9). The number of CFU was lowest for the water samples taken downstream of the UV treatment for all samplings. Samples from batch 2015_spring (d-2, and d34), and the d13 sample from batch 2016_spring had a reduction of 86.7 to 91.0% in CFU after the UV treatment. In comparison, day 41 of batch 2016_spring showed a more turbid water with a higher concentration of bacteria upstream the UV (Fig. 9) and a 20.1% reduction of CFU by the UV. For all samplings, the CFU increased when the UV treated water entered the rearing tanks, indicating regrowth of bacteria (Fig. 9). The increase in CFU from the fish tanks to the UV treatment, indicates bacterial regrowth throughout the entire system. With exception of the 2016_spring_d41 samples, the CFU counts increased with a magnitude of 4 times in the fish tanks, and 3 times in the water treatment loop. This estimate includes samples from batch 2015_spring taken prior to the introduction of fish, when the regrowth was lower compared to the other sample dates batches. For day 41 of 2016_spring, however, when the UV treatment was suboptimal due to a high visually observed turbidity, the regrowth of bacteria was higher in the fish tanks than through the subsequent water treatment loop.

## 4. Discussion

Despite progress, there is still limited information available on microbial community dynamics in RAS producing salmonids, although these communities can have a large impact on the health of the fish (Blancheton et al., 2013; Vadstein et al., 2018). Increased knowledge about microbial communities in RAS is important for improved operational design, management, and a sustainable production. One approach to this knowledge is to study commercial RAS. These studies often lack an experimental control and replicates, which can be challenging in commercial systems. Also, the physicochemical parameters vary a lot during production. However, the effects of water treatment processes can be studied by sampling upstream and downstream a treatment unit. If such studies are repeated in time, the conclusions are more robust than

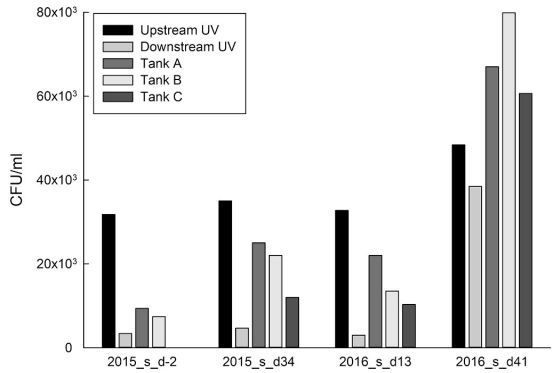


**Fig. 9.** Bacterial densities determined as CFU/ml in water upstream (UUV) and downstream the UV treatment (DUV) and in the fish tanks (Tank A, B and C) for two sampling dates for batch 2015_spring (d-2 and d34) and 2016_spring (d13 and d41). d represent the day in production, where −2 is two days before inset of fish to the RAS unit.

experiments with replication at the same sampling. Such studies also complement controlled experimental systems by extracting information at the relevant scale.

This study aimed to improve the understanding of microbial community dynamics in a commercial RAS during a period of 20 months and included three production batches of Atlantic salmon fry and parr. Water was sampled at different positions through the system, to examine the community dynamics and the effect of the full-flow UV disinfection within the loop.

### 4.1. Fish performance

The fish in the 2015_autumn batch had a significantly lower mortality (0.01%) than the spring batches (0.05; 0.06%), partly due to absence of periodic peaks in mortality. According to the RAS operators, this was normal mortality during the relevant life stages in the unit and no symptomatic fish was observed during the period. The more variable mortality of the spring batches can be related to their early stage, which is more sensitive and normally show a higher mortality than the larger fish stage produced during autumn (Tørud et al., 2019).

### 4.2. Dynamics of the RAS water microbiota over long time periods

In comparison with the two last batches, the first production batch (2015_spring) showed a different composition of the water microbiota, with a lower alpha diversity and more variable microbiota composition over time and between fish tanks (Figs. 4, 5, 6). This production batch was produced at the same time of the year as the last production batch in this study (2016_spring). The water microbiota of the two last production batches (2015_autumn and 2016_spring), on the other hand, were more similar in composition (Figs. 4, 5), even though the fish groups were produced at different seasons, with different size of fish, amounts of biomass, feeding regimes and several other parameters. Prior to the first production batch (2015_spring), the biofilter had been disinfected and started with clean carriers and then matured until the stocking with fish. The 2015_autumn batch had a matured biofilter that had been run continuously and without disinfection since the 2015_spring batch. Prior to the 2016_spring batch however, the complete system, including the biofilter was disinfected. The biofilter was then seeded from an already matured and running biofilter. Thus, the biofilter in the 2015_spring batch might have represented a more immature biofilm community, compared to the biofilters in the 2015_autumn and 2016_spring batches. Differences in start-up procedures of the biofilter for these three batches may explain the observed differences and similarities in the water microbiota between the production batches. Stable water microbiota over time and high alpha diversities have previously been proposed to characterize K-selected communities (Attramadal et al., 2012a, 2012b; Vadstein et al., 2018). Another implication of these observations is that the biofilm communities in the biofilters may affect the water microbiota more heavily than variables such as season, fish age, feeding routines and disinfection of the system. The knowledge on interactions between the biofilter biofilm community and the suspended bacteria in the water in RAS is limited. Some studies show that the abundance of free-living bacterial populations in the water can be correlated to the abundance of populations in the biofilm of the biofilter (Leonard et al., 2000; Michaud et al., 2014), and a selective exchange of bacteria is expected (Blancheton et al., 2013; Bartelme et al., 2017). The possibility for securing a stable and resilient microbiota in RAS, through the use of matured biofilters should be addressed in future studies.

### 4.3. Effects of disinfection in a RAS with a short hydraulic retention time in fish tanks

UV treatment is used as an extra hygienic barrier of the system by inactivation of potential pathogenic bacteria (Liltved et al., 1995; Sharrer et al., 2005; Hess-Erga et al., 2010). However, little is known about how efficient disinfection in the RAS loop is for preventing pathogens growing in the system. Turbid water, typical for RAS, reduce disinfection efficiency. Particles in the water are known to reduce the disinfection effect of the UV by protecting the bacteria from the UV-light (Hess-Erga et al., 2008; Huyben et al., 2018), and it can be difficult to inactivate most of the bacteria even at an excessive UV dose at high turbidity (Sharrer et al., 2005). The UV treatment kills and inactivates bacteria, but does not reduce the amount of substrate available, leading to a regrowth of opportunistic bacteria in the rearing tanks (Salvesen et al., 1999; Hess-Erga et al., 2010; Attramadal et al., 2012a, 2012b; Vadstein et al., 2018). In systems with long hydraulic retention times (HRT) in the fish tanks (60 min and longer), like marine hatcheries, significant regrowth and proliferation of opportunistic bacteria is well documented. This results in an altered microbial community composition that have negative effects on larval health and survival (Attramadal et al., 2012a; Vadstein et al., 2018; Dahle et al., 2020; Teitge et al., 2020; Attramadal et al., 2021). In the freshwater RAS for Atlantic salmon examined here, the full-flow UV treatment directly upstream the rearing tanks had no observable effect on the community composition of the water microbiota (Fig. 5). This was especially evident for the two last production batches. The similarity in community composition of the water throughout the system can be explained by the short HRT (23 min) in the fish tanks, which limits the time for regrowth in the tanks and makes the growth more likely to happen further down the line from the disinfection step, for example in the biofilter (Bakke et al., 2017; Vadstein et al., 2018). When Bray-Curtis similarities are used to compare communities, rare OTUs have little impact. Thus, rare OTUs could have been affected by the UV treatment, without effecting the Bray-Curtis similarities. However, the Sørensen-Dice index, which is based on presence – absence data, (Chao et al., 2006) and thus are more influenced by rare OTUs, did not indicate a distinct community composition through the RAS. We also used an RNA-based approach to study the active microbial response to the UV treatment. Neither this analysis indicated community changes after the UV treatment (Fig. 8). However, we did identify three *Acinetobacter* OTUs that showed a general pattern with lower abundance in samples taken downstream the UV treatment compared to those taken upstream the UV, with an average 75% reduction. This trend was especially evident for 2015_spring, which had a high abundance of *Acinetobacter* OTUs. *Acinetobacter* spp. is widespread in water ecosystems and includes both non-pathogenic, opportunistic, and fish pathogenic species (Turton et al., 2010; Hare et al., 2012). A similar strain specific reduction of *Acinetobacter* was seen by Hare et al. (2012). This indicates that *Acinetobacter* is particularly sensitive to UV treatment. Although this study included few sample events, the sampling included three production batches where all samples showed the same pattern: The full-flow UV disinfection had no observable effect on the water microbiota composition. Although immediate effects on the water microbiota were not observed, the UV disinfection may have long-term effects that influence the RAS water microbiota.

The UV treatment efficiently reduced the number of live bacteria (average 89.0% reduction of CFU), which was similar to previous studies (Huyben et al., 2018). As expected, the UV treatment efficiency was lower for more turbid water (20.1% at day 41 of 2016_spring) (Sharrer et al., 2005; Hess-Erga et al., 2008). The density of bacteria (i.e., CFU) increased 4 times in the fish tanks, indicating regrowth following the UV disinfection step. The regrowth continued through the water treatment system, reaching the highest levels of bacteria upstream of the UV treatment. The bacterial regrowth in marine hatchery rearing tanks, which have long HRT (more than one hour), can represent as much as up to a 14 time increase in bacteria numbers following disinfection, depending on the HRT (Attramadal et al., 2014; Vadstein et al., 2018). In the system studied here, with short HRT in fish tanks (23 min), the regrowth of bacteria in fish tanks was much lower (4-time increase). Moreover, the water microbiota did not change much in the fish tanks, especially for the two last production batches. This indicates that the regrowth observed in the fish tanks did not have a large impact on the

microbial community composition. It has been proposed that a fully K-selected system with a matured microbial community only can be established in RAS without UV, because point disinfection within the RAS loop will promote regrowth and selection for r-strategists in the fish tanks. Thus, UV treatment may not result in a reduction of the bacterial density of the system, but rather induce a detrimental r-selection in the fish tanks (Attramadal et al., 2012a, 2012b, 2014; Vadstein et al., 2018; Attramadal et al., 2021). The results obtained in this study indicate that these kind of negative, non-intended effects of the UV treatment is strongly reduced in RAS with short HRT in fish tanks (typically in RAS producing Atlantic salmon), and that UV disinfection can be used to restrict bacterial density without compromising the microbial water quality in the fish tanks. However, further studies should investigate the risk of successful invasion from pathogens in RAS with low HRT of tanks and UV treatment compared to systems without disinfection in the loop, as the latter is hypothesized to be more resistant to invasion.

## 5. Conclusions

In the RAS studied here, we found that the level similarity of the water microbiota between production batches could not be related to factors like season, fish age, and operational routines like for example feed loading. Two of the production batches had a highly similar water microbiota despite disinfection of the whole system between the batches and rearing fish of different stages. In contrast, the water microbiota of the first production batch differed from that of the others, was more variable; both through the system and over time and had a lower alpha diversity. A more immature biofilter in the first batch may explain these differences. Using a matured biofilter at start-up of a fish batch may contribute to establishing a more stable and resilient water microbiota in RAS compared to systems with newly started biofilters. Our results indicate that the biofilm communities in the biofilters may affect the water microbiota more heavily than season, fish age, feeding routines and disinfection. The UV directly upstream the rearing tanks had no observable effect on the community composition of the water microbiota. This was likely due to low hydraulic retention time (HRT) in rearing tanks, which limited the bacterial regrowth and community changes. The disinfection efficiency of the UV treatment was on average 89% when the water had low turbidity. This study shows that UV disinfection can be used to efficiently reduce bacterial density without compromising the microbial water quality surrounding the fish in RAS with low HRT in the fish tanks.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

*Acknowledgement*

We would like to thank Hege Brandsegg for practical assistance during the study and the RAS facility for providing data and allowing us to sample.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.737382.

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## Supplementary

Table S1. The relative abundance of the six most abundant OTUs of the dataset. All OTUs were classified at genus level, except OTU_3 and 10 that were classified at family level. Three different batches of fish (2015_spring, 2015_autumn, 2016_spring), at different sampling days (d), where d-2 represent two days before inset of fish. UUV=upstream UV, DUV=downstream UV, A, B, C= the rearing tanks.

| OTU | 8 | 1 | 2 | 14 | 3 | 10 |
|---|---|---|---|---|---|---|
| Taxa | *Pseudomonas* | *Acinetobacter* | | | *Rhodobacteraceae* | *Enterobacteriaceae* |
| **2015_spring** | | | | | | |
| 2015_d-2_UUV | 4511 | 1071 | 508 | 234 | 13 | 1 |
| 2015_d-2_DUV | 5193 | 362 | 88 | 0 | 5 | 0 |
| 2015_d-2_A | 12684 | 215 | 49 | 1 | 0 | 0 |
| 2015_d-2_B | 4917 | 6931 | 272 | 0 | 2 | 0 |
| 2015_d-2_C | 4410 | 468 | 3490 | 0 | 6 | 0 |
| 2015_d12_UUV | 10361 | 423 | 2546 | 8539 | 14 | 0 |
| 2015_d12_DUV | 14095 | 2917 | 159 | 3 | 264 | 0 |
| 2015_d12_A | 14746 | 1000 | 3738 | 0 | 24 | 0 |
| 2015_d12_B | 8869 | 1 | 10388 | 0 | 472 | 0 |
| 2015_d12_C | 2107 | 2 | 11577 | 2760 | 180 | 0 |
| 2015_d34_UUV | 60 | 10764 | 2002 | 4971 | 710 | 0 |
| 2015_d34_DUV | 6486 | 3199 | 61 | 488 | 413 | 0 |
| 2015_d34_A | 1239 | 14022 | 296 | 3000 | 529 | 1 |
| 2015_d34_B | 352 | 12941 | 290 | 1118 | 251 | 1 |
| 2015_d34_C | 99 | 12649 | 73 | 201 | 400 | 0 |
| **2015_autumn** | | | | | | |
| 2015_d29_UUV | 2 | 0 | 6 | 6 | 3624 | 515 |
| 2015_d29_DUV | 5 | 2 | 3 | 1 | 2245 | 778 |
| 2015_d29_A | 1 | 3 | 2 | 3 | 2192 | 676 |
| 2015_d29_B | 1 | 1 | 0 | 0 | 1409 | 12846 |
| 2015_d29_C | 1 | 0 | 1 | 8 | 2416 | 728 |
| **2016_spring** | | | | | | |
| 2016_d13_UUV | 13 | 328 | 1272 | 1 | 3664 | 0 |
| 2016_d13_DUV | 12 | 218 | 800 | 1 | 3996 | 1 |
| 2016_d13_A | 19 | 275 | 4002 | 15 | 3298 | 0 |
| 2016_d13_B | 20 | 179 | 870 | 0 | 4201 | 1 |
| 2016_d14_C | 30 | 2454 | 4084 | 0 | 3380 | 2 |
| 2016_d41_UUV | 71 | 4463 | 3696 | 6 | 3445 | 0 |
| 2016_d41_DUV | 80 | 37 | 253 | 6 | 770 | 0 |
| 2016_d41_A | 17 | 12 | 58 | 0 | 4942 | 0 |
| 2016_d41_B | 15 | 3 | 73 | 1 | 4910 | 0 |
| 2016_d41_C | 14 | 9 | 85 | 2 | 5831 | 0 |

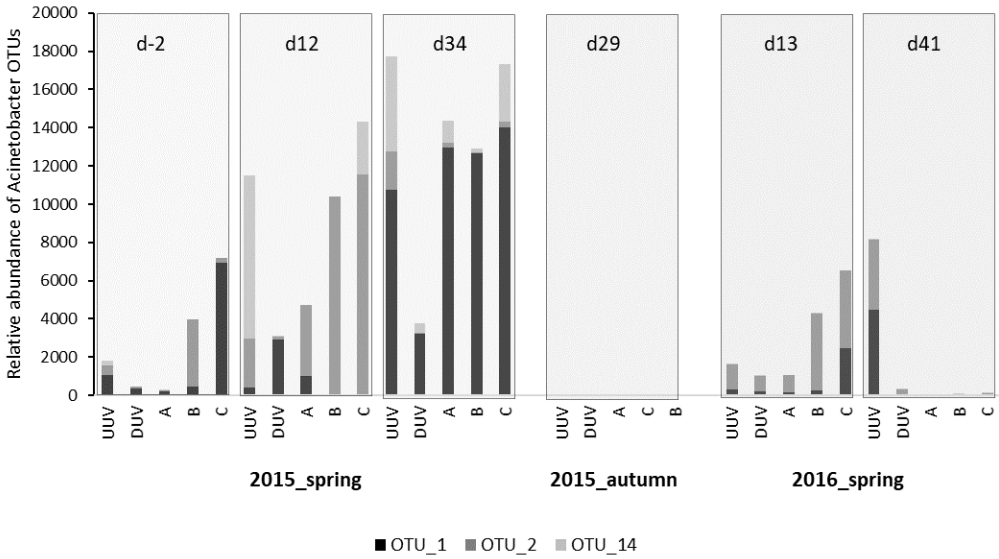
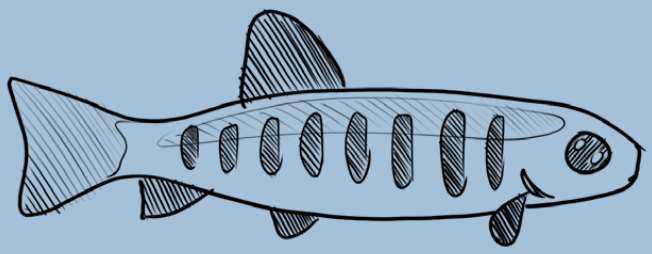



Figure S1. Relative abundance of *Acinetobacter* OTUs (OTU_1, 2 and 14) in the three different batches of fish (2015_spring, 2015_autumn, 2016_spring), at different sampling days (d), where d-2 represent two days before inset of fish. UUV=upstream UV, DUV=downstream UV, A, B, C= the rearing tanks.

# Paper II



# Long-term microbial community structures and dynamics in a commercial RAS during seven production batches of Atlantic salmon fry (*Salmo salar*)

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Key words: Recirculating aquaculture systems, microbial dynamics, Atlantic salmon, water microbiota, biofilm microbiota.

## Abstract

The microbiota of recirculating aquaculture systems (RAS) is of major importance for optimal fish health. However, the microbial communities in commercial RAS are highly complex and more knowledge is needed to potentially control and maintain beneficial microbial communities for good fish production. In this study we monitored microbial communities in a commercial RAS producing Atlantic salmon fry (*Salmo salar*) during seven consecutive production batches. The water of rearing tanks and the water sump downstream of the biofilter/upstream of the UV, as well as biofilm of the tank wall and in the biofilter were analysed using 16S rRNA gene amplicon sequencing to elucidate the spatial-temporal microbial dynamics. The results showed that the microbiota composition of water and biofilm

varied within and between the production batches, and that the fallowing periods had a substantial effect on the microbial communities. The correlation of the water and biofilm microbiota to fish presence in the system was confirmed by supervised machine learning. Shifts in the composition of the microbiota were identified in conjunction with variations in organic matter loading both during production and fallowing. In addition, variables like oxygen saturation, biomass, and feed type, showed good correlation with variations in the water microbiota composition. Although microbiota changed at fallowing, the water microbiota returned to similar compositions during the production phases. This indicates that the development of microbiota composition is strongly dictated by the similar selection pressure in the system. Nitrifying communities were dominated by *Nitrospira*, and the third most abundant *Nitrospira* OTUs were related to the comammox *Nitrospira nitrificans*. The microbial communities in the biofilter biofilm and water were significantly different but shared abundant taxa and followed the same temporal microbial dynamics and indicates an interaction between the biofilter biofilm and the suspended bacteria. CFU analysis showed that the fraction of rapid-growing bacteria was significantly higher in the rearing water than in the water sump upstream the UV disinfection, indicating that disinfection upstream the rearing tanks allowed for growth of opportunistic bacteria. A community with considerable potential for opportunistic regrowth can have consequences for the microbial water quality and the resistance against pathogen invasion The absence of an in-line disinfection step or placing the disinfection unit upstream the biofilter might provide better microbial water quality and a more resilient system against pathogen proliferation.

# 1. Introduction

Recirculating aquaculture systems (RAS) are increasingly being used for Atlantic salmon (*Salmo salar*) production (Badiola et al., 2012; Dalsgaard et al., 2013; Kolarevic et al., 2014; Davidson et al., 2017) due to the possibility of intensifying production while at the same time controlling the culture environment with minimal water usage and environmental impact (Martins et al., 2010; Dalsgaard et al., 2013; Davidson et al., 2017). The theoretical possibility of offering optimal environmental conditions means that the fish can obtain optimal growth, survival, and disease resistance in RAS, provided technology and operation are fully mastered (Blancheton et al., 2013).

Microbes are ubiquitous and represent everything from an absolute necessity to a potential threat to life in RAS production. The biofilter is a central component in RAS and typically harbours a diverse microbiota, including nitrifying bacteria. RAS operation depend on nitrifying bacteria to convert toxic nitrogenous waste products from the fish to less toxic nitrate (Martins et al., 2010; Bartelme et al., 2017). Nitrification is a two-step process performed by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) that convert ammonia to nitrite, and nitrite oxidizing bacteria (NOB) that convert nitrite to nitrate. Also, some bacteria can perform complete ammonia oxidation (comammox) in RAS (van Kessel et al., 2015). Denitrifying bacteria can be used in another treatment step to reduce water usage even further by converting nitrate into nitrogen gas that can be removed from the system (van Rijn et al., 2006). Different bacteria in nitrifying biofilters of RAS have been reviewed (Michaud et al., 2006; Schreier et al. 2010; Rurangwa and Verdegem, 2014; Ruan et al., 2015; Nevada et al., 2019; Roalkvam et al., 2020; Nevada et al., 2020a; Nevada et al., 2020b; Fossmark et al., 2021; Bartelme et al., 2017), but the knowledge on temporal dynamics in commercial RAS is still scarce (Rojas-Tirado et al., 2019).

Interaction and colonization with bacteria are essential for a normal and healthy development of the immune and digestive system of the fish (Llewellyn et al., 2014). In addition, a healthy host microbiota, as well as a beneficial and stable system microbiota, are thought to provide effective barriers against infection and development of disease (Marshall and Bellamy, 2010; Vadstein et al., 2013). On the negative side, heterotrophic bacteria degrading organic matter increase oxygen consumption and waste loading on the system. High supply of available organic matter result in heterotrophic bacteria outcompeting the nitrifying bacteria and reduces the nitrification efficiency of the biofilter (Zhu and Chen, 2001; Michaud et al., 2006; Michaud et al., 2009; Schreier et al., 2010). Under specific conditions, several different species of microorganisms can produce by-products like toxic $H_2S$ or off-flavour compounds, which can create problems in RAS (Guttman and van Rijn, 2008; Letelier-Gordo et al., 2020). In some cases, specific pathogenic species of bacteria can cause infections of the fish (Blancheton et al., 2013). However, a more common problem is the development of secondary infections of a weakened host by opportunistic bacteria (Vadstein et al., 2018).

RAS have properties that can promote microbial stability and mutualistic fish-microbe interactions (Attramadal et al., 2014; Vadstein et al., 2018). The large surface area available for bacteria, the relatively stable organic loading, and the extended total hydraulic retention

time of RAS creates strong competition between the bacteria. Strong competition for limited resources selects for a stable community dominated by slowly growing specialists at the expense of opportunists (Vadstein et al., 1993; Attramadal et al., 2012a; 2014; Vadstein et al., 2018; Vestrum et al., 2018; Attramadal et al., 2021). Also, the highly reduced amount of intake water increases the possibility of maintaining a high biosecurity into the RAS (Blancheton et al., 2013).

The microbial communities in RAS can respond rapidly to changes in the environment (Bentzon-Tilia et al., 2016) with different selection pressures acting on the microbial communities. Different forces driving the selection pressure is feed and feeding regimes, the make-up water, management routines, system design, physicochemical water quality, and the fish itself (Attramadal et al., 2012a; Blancheton et al., 2013; Bakke et al., 2017; Rud et al., 2017; Vadstein et al., 2018; Fossmark et al., 2020; Fossmark et al., 2021; Dahle et al., 2020a; Dahle et al., 2022; Almeida et al., 2021). Solutions to maintain beneficial microbial communities in RAS, which is important for system management and control, are practically lacking (Blancheton et al., 2013; Bentzon-Tilia, 2016).

In this study we characterized the microbiota of water and biofilm samples from a commercial RAS for production of Atlantic salmon fry for seven consecutive production batches. Samples were taken at six positions in the RAS loop every second week for 15 months. The six positions included the rearing water, biofilter biofilm, tank wall biofilm, as well as the treated water coming from the biofilter/upstream UV disinfection before returning to the rearing tanks. To the best of our knowledge, this is the first-time microbiota in both water and biofilm has been monitored with modern molecular methods over such a long timescale in a commercial RAS. The main objective of our study was to characterise and understand the spatial-temporal microbial community compositions and dynamics in both biofilm and water in the system, and to apply supervised machine learning demonstrating that microbiome profiles can be used for predictive and operational measures. We particularly aimed at documenting the dynamics of the general microbial community composition in contact with the salmon fry, the microbial community composition of the biofilter, and the effect of UV disinfection on the microbial population of the water in the RAS loop. This knowledge can contribute to improve the chemical and microbial water quality, to secure optimal production of Atlantic salmon in RAS for the future.

# 2. Materials and methods

## 2.1 Culture system and rearing regime

The study was based on samplings from a start-feeding department of a commercial RAS producing Atlantic salmon fry from 0.2 to around 3 g. The RAS facility was built in 2013 (Billund Aquaculture, Denmark) and is one of the largest producers of smolt in Norway. A total of seven production batches were cultivated in the monitored RAS during the period. Production batch 1 and 7 were only sampled for a part of the time the fish spent in the system. Between each production batch, there was a fallowing period for cleaning of rearing tanks with soap and hot water before a new group of fry was put in. The fallowing periods varied from 6 to 40 days, with an average of 24 days. During fallowing periods, the biofilters were fed 0.5 to 1 kg ammonium chloride ($NH_4Cl$) once a day, to maintain the nitrification activity. The ammonium chloride was added in the water sump before the biofilter (Fig. 1). The intake water from a lake (Heimsvatnet) was sand filtered and UV disinfected. The RAS consisted of six rearing tanks (dimensioned maximum biomass of 45 kg/$m^3$), with an associated water treatment loop consisting of a mechanical drum filter (60 µm mesh, Hydrotech, Veolia Water Technologies, Sweden) for particle removal, three fixed bed biofilters (FBBF) (3 x 13.5 $m^3$, RK BioElements, Denmark) for nitrification, a trickling filter (EXPO-NET BIO-BLOK®, 20 $m^3$, Denmark) for degassing of $CO_2$, and an ultraviolet irradiation treatment (MonoRay 10, UltraAqua, Denmark) of the full water flow for disinfection. Also, the RAS included oxygenation from oxygen cones and pH regulation with calcium hydroxide slurry ($Ca(OH)_2$) added in the water sump before the biofilter. Make-up water was added in the water sump before the biofilter (Fig. 1). The system was stocked with 2 kg/$m^3$ of Atlantic salmon fry and fed continuously with different commercial feeds of different pellet sizes (EWOS and Skretting, Norway). The three biofilters were backwashed with aeration every third week (one biofilter each week) to avoid clogging. The biofilters had never been disinfected throughout the seven years of operation. Final biomass at each production batch was between 20-45 kg/$m^3$. Total water flow in the start-feeding RAS was 454 $m^3$/h at all sampling times. The study resulted in 33 sampling timepoints (t0-t32). From t0 to t26 the rearing tanks had a water volume of 22.6 $m^3$, with a hydraulic retention time (HRT) of 18 minutes. However, problems with removing particles from the rearing tanks resulted in a period of reconstruction from t27 to t29 (during fallowing) where all the rearing tank walls were extended with around 30-50 cm. After the reconstruction (t30-

t32) the tanks had a volume of 35 $m^3$ and an HRT of 28 minutes. Production data and physicochemical water quality variables were provided by the RAS facility, including mortality, biomass of fish, feed type, temperature, total ammonia nitrogen (TAN), nitrite, nitrate, salinity, and pH.

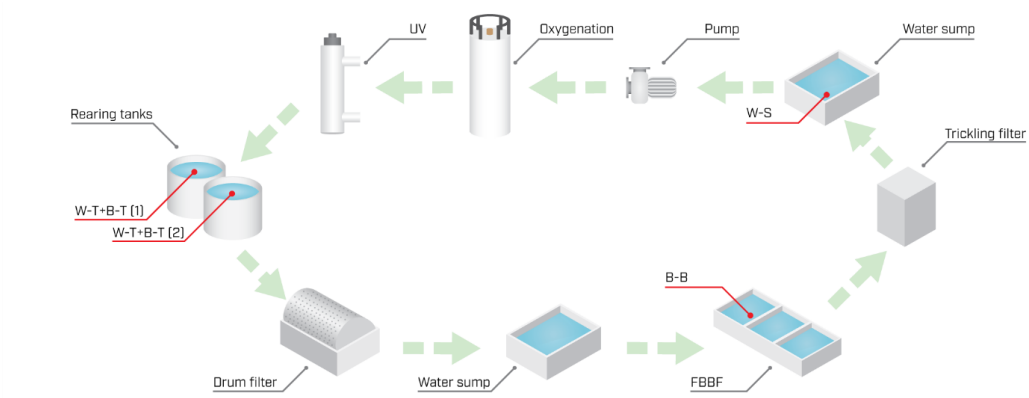



Figure 1. Schematic presentation of the RAS monitored in this study. Sample points for RAS microbiota is presented as red lines: water samples from each of two rearing tanks (W-T), biofilm samples from the surface of walls of two rearing tanks (B-T), water from the sump downstream the biofilter and degasser and upstream the UV (W-S), and biofilm (B-B) from the fixed bed biofilter (FBBF). The UV disinfection was on full-flow. Illustration by Mats Mulelid, SINTEF Ocean.

## 2.2 Sampling for microbiological analysis

Sampling for microbial community analyses was conducted biweekly over a 15-month period, from the 06th of November 2017 to the 28th of January 2019, resulting in 33 sampling timepoints (t0–t32) (Fig. 2). Samples were from four different points inside the RAS-loop: 1) water from two rearing tanks (W-T), 2) biofilm samples from tank walls (B-T) (same two rearing tanks as W-T), 3) biofilm samples from one of the fixed bed biofilters (B-B) and 4) water samples from a water sump (W-S) positioned after the biofilter, upstream the UV in the treatment loop (see Figure 1). Water samples were collected by filtering 150-200 mL water through a 0.22 µm Sterivex filter (Millipore, USA) with Omnifix® syringes. Biofilm samples were taken by swabbing (Copan Diagnostics, USA) the tank walls of the two rearing tanks and inside the fixed bed biofilter. A new area was swabbed each time. All collected samples were stored in freezers (-20 °C at the facility, -80 °C at SINTEF) until further analyses were performed. A total of 244 samples were subjected to microbial community analysis by Illumina sequencing of 16S rDNA amplicons. Water samples were also collected for analyses of flow cytometry and colony forming units (CFU) at production day 30, 34 and 40 of production batch seven.

Samples were taken from the same points as the water for microbial community analysis: the two rearing tanks and the water sump (Fig. 1).

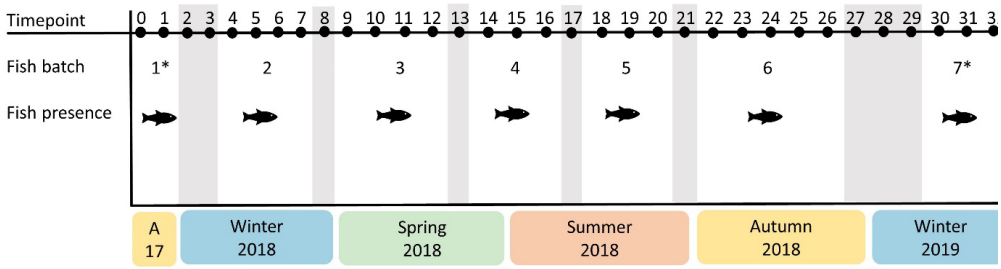



Figure 2. Timeline for sampling for microbial community analyses during seven production batches. Sampling was conducted biweekly over a 15-months period, resulting in 33 sampling timepoints, from t0 to t32 (upper numbers). Shaded areas in between production batches represent the fallowing periods where there was no fish in the department. Production batch 1* and 7* were not followed for the whole production period, as batch 1 was only monitored the last 15 days and batch 7 the first 48 days of the batch period.

## 2.3 Microbial community analyses

For DNA-extraction, two different kits were used: FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) was used for samples taken from t0 to t17, while ZymoBIOMICS™ DNA Miniprep kit (Zymo Research, USA) was used for samples taken from t18 to t32. Extraction was done as described by the manufacturers. To check if there was a difference between the two extraction kits, DNA from the same samples was extracted with each kit. The extracted DNA was sequenced, and the microbial community composition results were subsequently compared at different taxonomical levels. Only small differences were found in the microbial community composition between the two DNA-extraction kits. The Genomic DNA Clean & Concentrator™-10 kit (Zymo Research, Irvine, California) was used to purify the DNA. The extracted DNA was sent to the Centre of Biotechnology (CeBiTec), Bielefeld University (Germany) for 16S rDNA amplicon library preparation and sequencing. Library preparation was conducted after standard Illumina instructions. The variable regions 3 and 4 (v3 + v4) of the 16S rRNA gene was amplified by two PCR rounds using the 2xHiFi HotStart ReadyMix (Kapa Biosystems, USA). To cover the domains of Bacteria and Archaea, the primers 341F (5'-CCTACGGGNGGCWGCAG-'3) and 805R (5'- GACTACHVGGGTATCTAATCC-3') were used for the first PCR round (Takahashi et al., 2014). Obtained amplicons were indexed, pooled and

subsequently sequenced on an Illumina MiSeq platform (paired end sequencing; 2x300 bp). The Illumina sequencing data were processed with the USEARCH pipeline (version 9.2; https://www.drive5.com/usearch/). During merging of paired reads, primer sequences were removed and reads shorter than 380 base pairs were filtered out. The processing further included demultiplexing and quality trimming by the Fastq filter command (with an expected error threshold of 1). The UPARSE-OTU algorithm was applied for chimera removal and clustering at the 97% similarity level (Edgar, 2013). Taxonomy assignment was based on the SINTAX script (Edgar, 2016) with a confidence value threshold of 0.8 and the RDP reference data set (version 16). For identifying OTUs (Operational taxonomic units) potentially representing nitrifiers, the OTUs were also classified using the MiDAS 3.2 reference data set based on 16S rRNA gene sequences obtained from activated sludge wastewater treatment systems (Nierychlo et al., 2019). The resulting OTU table was normalised to 17 000 number of reads per sample by determining the fraction of the OTUs for each community profile, and subsequently multiplying by 17 000, and finally rounding off the read numbers to integers. A Maximum likelihood analysis was conducted to examine the phylogenetic relationships between the most abundant *Nitrospira* OTUs identified in this study and previously described *Nitrospira*, including representatives for comommox *Nitrospira*. 16S rRNA gene sequences were retrieved from the NCBI GenBank or the Ribosomal Database Project (RDP) database (Cole et al., 2014). The analysis was performed in MEGA-X software v. 10.2.4 (Kumar et al., 2018). The sequences were aligned using ClustalW with the default parameters. A maximum likelihood analysis was performed with 1 000 bootstrap replicates and the Tamura-Nei model for sequence evolution (Tamura and Nei, 1993). The resulting sequencing data are deposited at the European Nucleotide Archive (accession numbers ERS13478210-ERS13478454).

## 2.4 CFU analysis for estimating fraction of opportunistic bacteria

Agar plates was prepared by mixing 8.75 g PCA (plate count agar) (Himedia, India), 1.50 g agar powder and 500 mL Milli-Q water. Water samples were plated immediately after sampling. Samples were diluted and plated in triplicates on the petri dishes and incubated at 14 °C. Colony forming units (CFU) were registered after three and 18 days of incubation. Plates containing 30-300 colonies were used for counting. Opportunistic bacteria were defined as

the fraction of CFUs registered three days after incubation of the total number of CFUs registered after 18 days of incubation (Skjermo et al., 1997).

## 2.5 Flow cytometry and growth potential

The total number of bacterial cells in water samples was determined by flow cytometry using a BD Accuri™ C6 Flow Cytometer (BD Biosciences, USA). Six replicates pr sample were fixated with glutaraldehyde (final concentration 0.01%) and stored in refrigerator for maximum three days prior to flow cytometry analysis. Samples were diluted 1:10 with TE buffer and further stained with a 1:50 working solution of SYBR® Green II RNA Gel Stain (Life Technologies, USA). After staining, samples were incubated in the dark for 15 min. A medium flow rate (35 µL $min^{-1}$) and a 4 min collection time was used for all samples for counting of bacterial cells. The FL1 detector was set to a threshold value of 3 000. The gating that was used for all flow cytometry samples excluded fluorescent intensity signals below approximately 103.5 on the FL1 detector. Triplicate sub-samples from the same water sample were also incubated at 14 °C for three days in open 50 mL plastic tubes to determine the bacterial growth potential. After three days, samples were subjected to flow cytometry as described above. The bacterial growth potential was calculated as the fraction of total bacteria after three days incubation compared to the original number of total bacterial cells (Attramadal et al., 2016).

## 2.6 Supervised Machine Learning

The variations in microbial community composition in the biofilter biofilm and the water samples (rearing water and water sump) were examined further by using supervised machine learning (SML) models. The aim was to examine the power of measured physicochemical water quality variables and other production parameters for prediction of the total microbial community profile dynamics. The variables that were processed included: temperature, salinity, oxygen saturation, pH, nitrogen waste products (TAN, $NO_2^-$, $NO_3^-$), mortality, fish presence, biomass of fish ($kg/m^3$), and feed type (Ewos start and Skretting Nutra Sprint). SML algorithms aim at extracting information from a training dataset into a predictive model that has a potential to class labels on upcoming, unlabelled samples (Cordier et al., 2019). In this context, obtained OTU table was used as the input dataset (features), while metadata file containing physicochemical and production parameters was used as endpoint information.

The total dataset was split into a training dataset and a model evaluation dataset, contributing to 75% and 25% of total number of samples, respectively. Random forest machine learning algorithm was applied to the data, through Quantitative Insight into Microbial Ecology 2 (qiime2) pipeline v.2021.2 (Boylen et al., 2019) based on scikit-learn python machine learning package v.0.23.1. Both numerical and categorical type of predictors were used, based on the parameters used.

### 2.7 Statistical analyses

The USEARCH commands Alpha_div and Sintax_summary was used to calculate alpha diversity indices (observed OTU richness and Shannon's diversity) and generate taxa summary tables, respectively. PAST (version 4.0; Hammer et al., 2011) was used to calculate Bray-Curtis similarities. Principal Coordinate Analysis (PCoA) ordinations based on Bray-Curtis similarities (Bray and Curtis, 1957) were made to illustrate the beta-diversity (Hammer et al., 2001). One-way PERMANOVA (permutational multivariate analysis of variance) based on Bray-Curtis similarities were used to test if there was a statistically significant difference between sample-groups (Anderson, 2001), with the significance threshold set to a p-value below 0.05. When more than two groups were compared, one-way PERMANOVAs with Bonferroni-corrected p-values were used. SIMPER (Similarity Percentage) analysis based on Bray-Curtis values was performed to identify the OTUs which contributed the most to the difference in microbial community composition between selected groups (Clarke, 1993). Standard error (SE) was used to show the variation of data.

## 3. Results

### 3.1 Physicochemical water quality

The physicochemical water quality variables were generally satisfying for salmon production and relatively similar among the production batches examined. The salinity was raised occasionally when the RAS-facility encountered problems with water mold, resulting in a variation in salinity from 0.3 to 2.5 ppt during the period (Tab. 1). The oxygen saturation never fell below 91.0% and the pH was stable, varying between 6.9-7.0. The concentrations of total ammonia nitrogen (TAN), nitrite ($NO_2^-$) and nitrate ($NO_3^-$) tended to increase throughout the

production batches, as expected (Fig. 3; Fig S1, Supplementary). There were fluctuations in both $NO_2^-$ and $NO_3^-$ concentrations during the period, varying between 0.05-0.6 mg/L and 78-194 mg/L, respectively. During fallowing periods, temperature, salinity, and the concentrations of nitrogen products were lowered, while oxygen saturation increased, as expected. The pH did not change during fallowing.

Table 1. Physicochemical water quality for the seven production batches and the six fallowing periods (average ± SE). All variables were measured in the rearing tanks (Fig. 1), except from pH, which was measured in the water sump after the biofilter. Fall=fallowing, TAN=total ammonia nitrogen.

| | Temperature (°C) | Oxygen saturation (%) | pH | Salinity (ppt) | TAN (mg TAN/L) | Nitrite (mg $NO_2^-$/L) | Nitrate (mg $NO_3^-$/L) |
|---|---|---|---|---|---|---|---|
| Batch 1 | 12.9 ± 0.7 | 91.0 ± 0.9 | 6.9 | 2.0 ± 0.0 | 1.1 ± 0.2 | 0.6 ± 0.1 | 177.1 ± 7.5 |
| *Fall 1* | 12.1 ± 0.1 | 100.2 ± 0.4 | 6.9 | 1.1 ± 0.0 | 0.2 ± 0.0 | <0.05 | 85.8 ± 8.8 |
| Batch 2 | 13.8 ± 0.1 | 92.2 ± 0.3 | 7.0 | 2.5 ± 0.2 | 0.6 ± 0.2 | 0.3 ± 0.1 | 113.9 ± 13.0 |
| *Fall 2* | 12.0 ± 0.2 | 104.0 ± 0.4 | 7.0 | 1.0 ± 0.1 | 0.5 ± 0.1 | <0.05 | 78.0 ± 9.5 |
| Batch 3 | 13.7 ± 0.1 | 93.7 ± 0.9 | 6.9 | 1.9 ± 0.2 | 0.7 ± 0.1 | 0.2 ± 0.1 | 143.8 ± 22.2 |
| *Fall 3* | 12.3± 0.1 | 101.5 ± 0.9 | 6.9 | 1.0 ± 0.0 | 0.6 | <0.05 | 93.0 ± 0.0 |
| Batch 4 | 13.9 ± 0.1 | 92.8 ± 0.3 | 6.9 | 2.5 ± 0.1 | 0.5 ± 0.0 | 0.2 ± 0.0 | 179.7 ± 26.6 |
| *Fall 4* | 13.5 ± 0.3 | 99.4 ± 0.4 | 6.9 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | 97.0 ± 33.0 |
| Batch 5 | 14.0 ± 0.1 | 93.2 ± 0.2 | 6.9 | 0.9 ± 0.0 | 0.5 ± 0.0 | 0.1 ± 0.0 | 120.9 ± 17.9 |
| *Fall 5* | 13.8 ± 0.1 | 99.7 ± 0.3 | 6.9 | 0.7 ± 0.1 | 0.2 ± 0.0 | <0.05 | 140.0 ± 10.0 |
| Batch 6 | 13.7 ± 0.1 | 92.5 ± 0.5 | 7.0 | 1.1 ± 0.0 | 0.8 ± 0.1 | 0.3 ± 0.1 | 194.6 ± 21.5 |
| *Fall 6* | 12.8 ± 0.1 | 99.6 ± 0.4 | 7.0 | 0.7 ± 0.1 | - | - | - |
| Batch 7 | 13.5 ± 0.1 | 92.3 ± 0.2 | 6.9 | 1.6 ± 0.1 | 0.7 ± 0.1 | 0.2 ± 0.1 | 135.7 ± 14.0 |

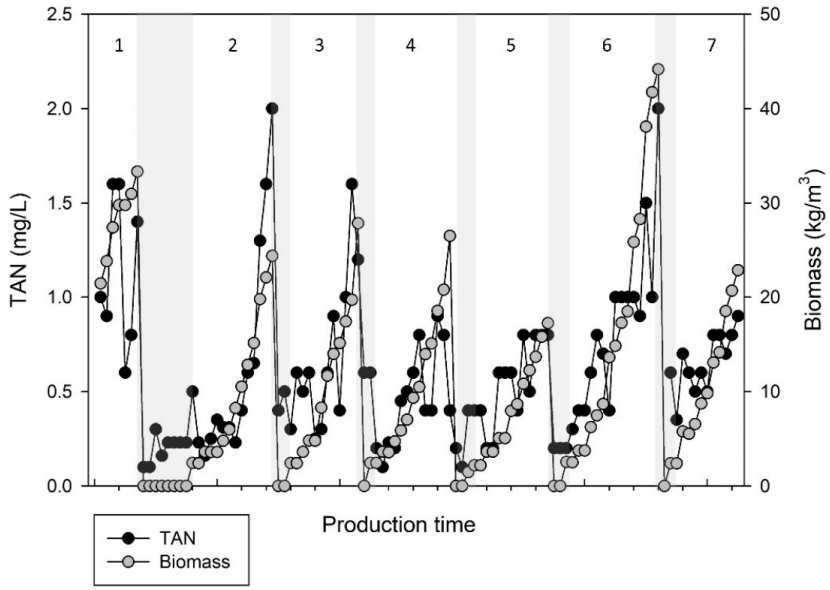



Figure 3. Total ammonia nitrogen (TAN) concentration and biomass of fish (kg/m$^3$) during the seven production batches (upper number). Shaded areas represent the fallowing periods. TAN was measured in rearing tanks (Fig. 1). The suggested threshold for TAN in Norwegian aquaculture producing Atlantic salmon in freshwater is <2 mg/L (Hjeltnes et al., 2012).

## 3.2 Fish performance

The average daily mortality was 0.11 ± 0.01% during the 15 months for all production batches. During a production batch, the daily mortality usually peaked during day 2-3 after inset of fish, after which it stabilized and decreased towards the end of the production period (Fig. 4). The exception was production batches 2 and 3, which also had an increase in daily mortality in the middle of the production period. The two rearing tanks examined in the study had approximately the same pattern of daily mortality (0.10 ± 0.01%; 0.12 ± 0.01%, respectively) throughout the period (Fig. 4). The daily mortality was significantly different between the production batches (Kruskal-Wallis, $p$ = 0.001), where production batch 7 had the highest single incident of mortality in both rearing tanks on day 13 and was the production batch with the highest average daily mortality (0.21 ± 0.06%) (Fig. 4, Tab. S1, Supplementary). Production batch 5 had the lowest average daily mortality (0.06 ± 0.01%), for the completed batches (batch 2-6) (Tab. S1, Supplementary).

The average final fish weight was similar between the batches, with 2.69 ± 0.21g, except production batch 6 with an average of 3.90g final weight. In this batch, the fish was kept in the RAS for a longer period (Tab. S1, Supplementary). Also, the specific growth rate (SGR) was similar, ranging from 5.15 to 5.29% for the completed batches (Tab. S1, Supplementary).

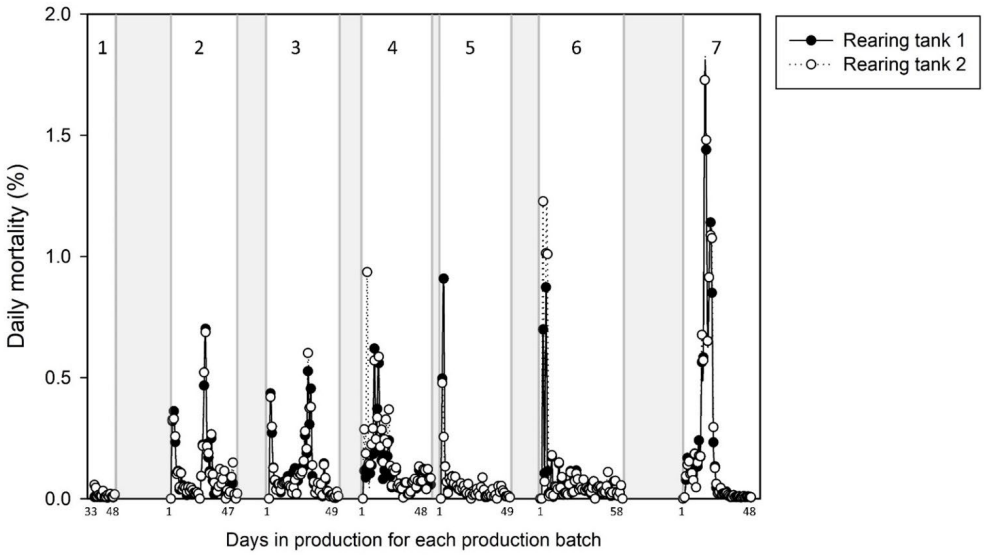



Figure 4. Daily mortality (%) during the production period for the two rearing tanks trough seven different production batches (upper numbers, 1-7). Shaded areas represent fallowing periods and the numbers on the x-axis represent the day in production for each batch (from day 1 up to 58 days).

## 3.3. Microbial community composition and dynamics in the RAS

### 3.3.1 Composition of the water and biofilm microbiota

Ordination by Principal Coordinate Analysis (PCoA) indicated differences between microbial community structures in water (rearing water, water sump downstream the biofilter/upstream the UV disinfection) and biofilm (biofilter and tank wall) samples, and biofilm from biofilter and tank wall (Fig. 5). Significance of observed differences was confirmed by one-way PERMANOVA test ($p = 1.0 \times 10^{-4}$).

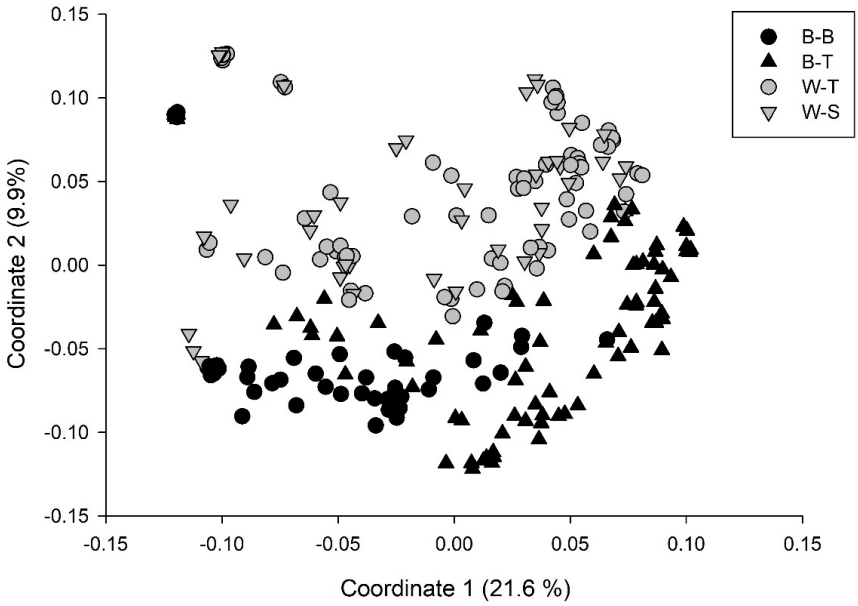



Figure 5. PCoA ordination based on Bray-Curtis similarities for water (grey symbols) and biofilm (black symbols) samples. Water samples included water from the two rearing tanks (W-T) and the water sump downstream the biofilter/upstream the UV disinfection (W-S), biofilm samples from tanks walls (B-T) and the biofilter (B-B) over a period of 15 months, total 33 timepoints. Triplicates were included from timepoint 0 to 5. n=43 (B-B), n=76 (W-T), n=43 (W-S), and n=81 (B-T).

Despite significant differences in community compositions in general, the water samples from the two rearing tanks and the water sump were similar in composition (PERMANOVA, $p > 0.24$). The biofilm and water samples showed variation in composition over time, both within and between the production batches (Fig. 6A, B).

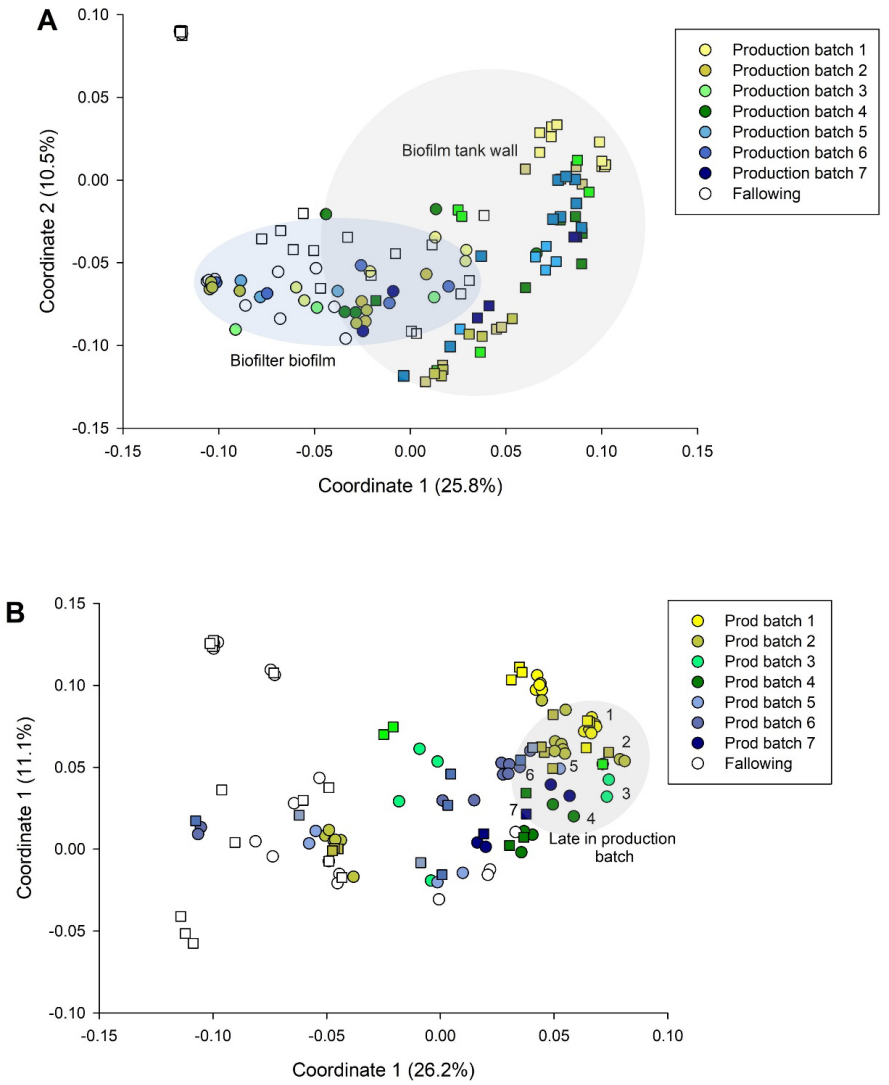



Figure 6. PCoA-plot based on Bray–Curtis similarities sorted by the seven different batches of fish and the six fallowing periods for A) biofilm samples from the biofilter and the tank walls through the seven batches of fish and the fallowing periods. Circles=Biofilter biofilm, Squares=tank wall biofilm, and B) water samples from two rearing tanks and the water sump. Circles=rearing tanks, Squares=water sump. Samplings late in production batch symbolised by numbers of a given batch and a shaded area. n=43 (Biofilter biofilm), n=81 (Biofilm tank wall), n=76 (rearing tanks), n=43 (water sump).

Alpha diversity expressed as exponential Shannon's index showed that the biofilter biofilm (B-B) had a significant higher diversity than the other sample groups (Fig. 7) (ANOVA, $p < 0.05$) except the water sump (W-S). The biofilm on the tank walls had a significant lower diversity, both in terms of OTU richness and exponential Shannon's index than the other sample groups (ANOVA, $p < 0.05$). The water from the water sump located after the biofilter/before the UV (W-S) had both higher richness and exponential Shannon's diversity compared to the rearing tanks (W-T), although not significant.

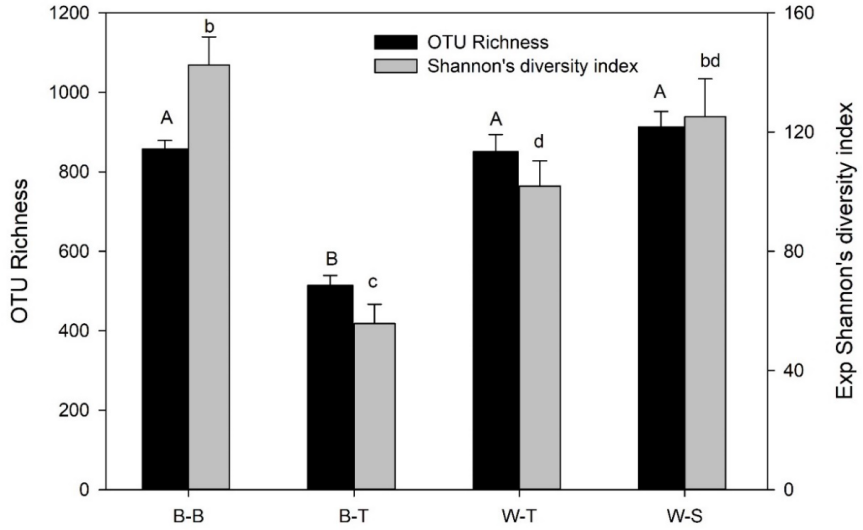
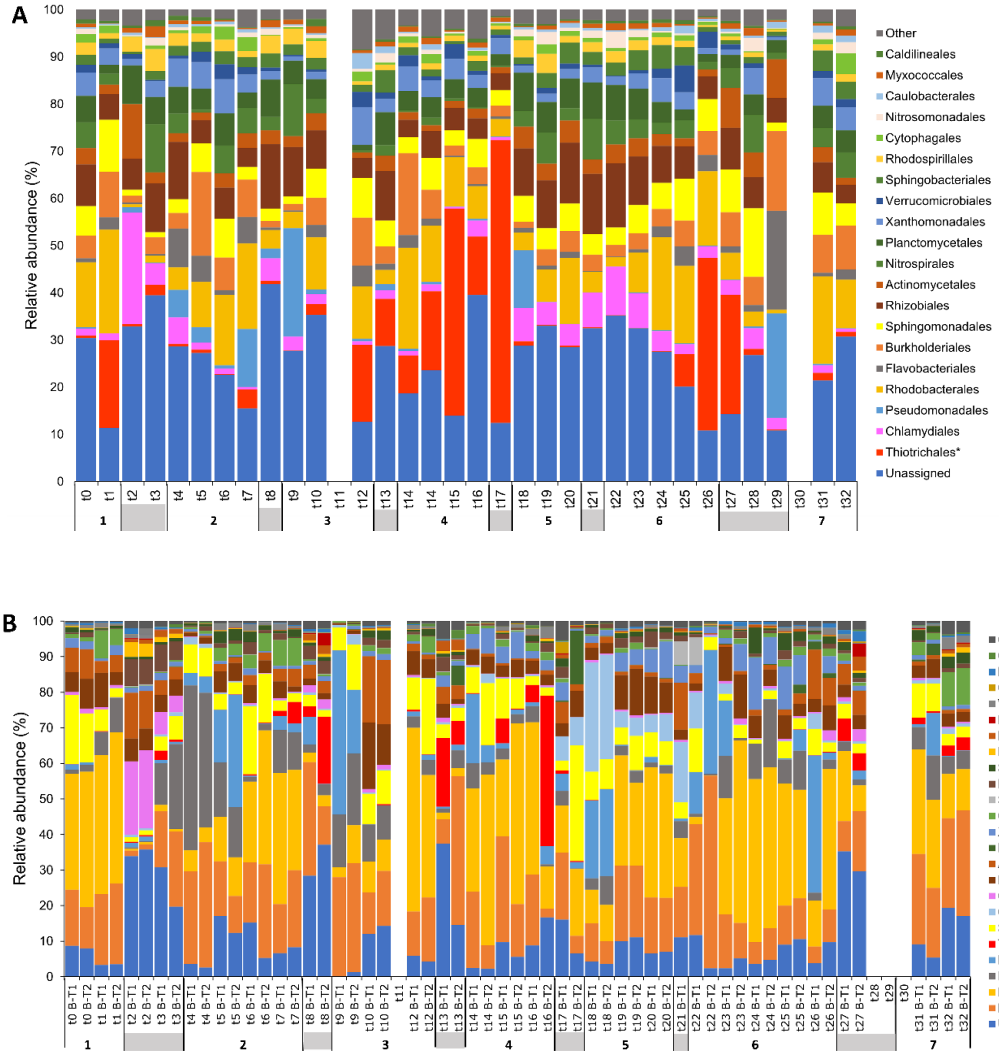



Figure 7. Alpha diversity indices expressed as the average observed OTU richness and exponential Shannon's diversity index ($e^{Shannon}$). B-B = biofilter biofilm, B-T = tank wall biofilm, W-T = water rearing tanks, W-S = water sump downstream the biofilter and upstream the UV. The indices were calculated as the mean (±SE) of all sampling times (t0-t32). N = 43 (B-B), n = 76 (W-T), n = 43 (W-S), n = 81 (B-T). Different letters indicate significant differences for OTU richness (capital letters) and exponential Shannon's diversity (lower-case letters).

The most abundant orders in the biofilter biofilm communities were Rhodobacterales (average 9.4 ± 1.4%), Thiothrichales (8.5 ± 2.6%), Rhizobiales (7.8 ± 0.6%), and Burkholderiales (5.9 ± 0.8%) (Fig. 8A). The nitrite-oxidising order Nitrospirales was the 8$^{th}$ most common order with an average relative abundance of 3.8 ± 2.8%. The ammonia oxidising Nitrosomonadales, on the other hand, had an average relative abundance of only 1.0 ± 0.9% (Fig. 8A). For the biofilm on the tank wall of the two rearing tanks, the most common order was Rhodobacterales (21.7 ± 2.7%), Burkholderiales (average 17.4 ± 2.2%), Flavobacteriales (7.4 ±

1.5%), and Sphingomonadales (7.1 ± 1.3%) (Fig. 8B). The most common orders in the water microbiota were Burkholderiales (average 17.4 ± 1.3%) and Rhodobacteriales (8.5 ± 1.1%), which were also were included in the top four orders for biofilter biofilm and biofilm tank wall. Further, Sphingomonadales (6.6 ± 0.7%) and Chlamydiales (6.3 ± 0.9%) (Fig. 8C) were abundant taxa in water samples (Fig. 8C).



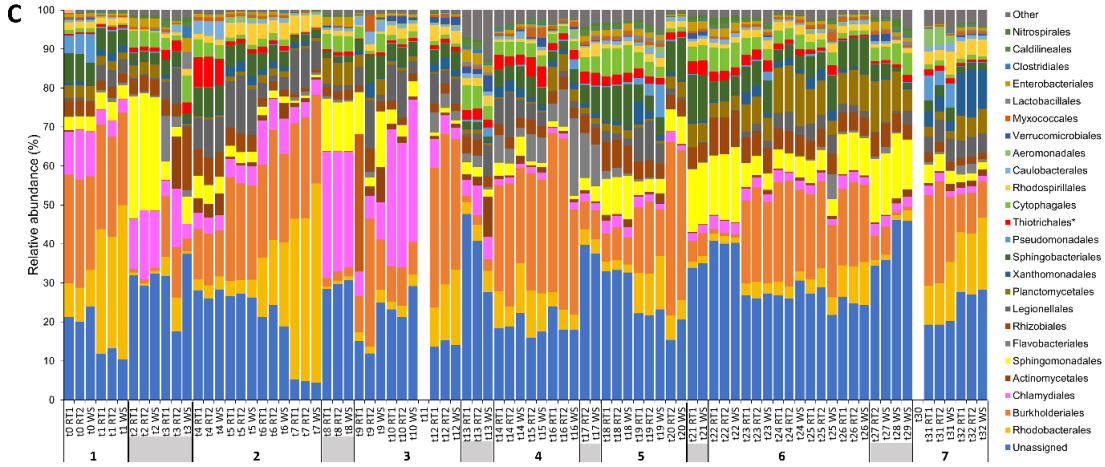



Figure 8. Microbial community composition at order level for A) biofilm samples from the fixed bed biofilter, B) biofilm samples from the tank wall of the two rearing tanks (B-T1 and B-T2) and C) water samples from the two rearing tanks (W-T1 and W-T2) and the water sump (W-S) for sampling time t0-t32. Orders with relative maximum abundance below 2% in all samples are included in "other". The numbers below the x-axis represent the seven production batches, with fallowing periods in shaded areas between. *Included in Thiothrichales is OTU_2 that was manually classified as *Thiothrix* using the RDP Classifier.

To identify which OTUs contributed most to the difference between all samples of water and biofilm, a SIMPER analysis based on Bray-Curtis similarities was conducted. Collectively, ten OTUs contributed with nearly 30% of the differences between the samples (Tab. 2). OTU_1, representing the family Rhodobacteraceae, was the most contributing OTU, singularly explaining almost 10% of the differences. OTU_1 was far more abundant in the water and in the biofilm on tank wall (16.50; 17.70%) compared to the biofilter biofilm (6.49%). This observation was also reflected in the taxa plot (Fig. 8), where Rhodobacterales was highly more abundant in tank wall biofilm (21.69%) compared to the other sample groups (8.46-9.43%), reaching maximum abundance of 51.71% at timepoint 12. OTU_2 assigned as *Thiothrix* was the second most contributing OTU, with a higher relative abundance in the biofilter biofilm (7.28%) compared to the other locations (1.50-1.87%). This OTU was dominating the order Thiotrichales which was far more abundant in the biofilter biofilm (8.51%), compared to the other sample groups (1.81 - 2.42%) (Fig. 8). OTU_4 (Burkholderiales inceartae sedis, *Sphaerotilus*) was hardly present in the rearing water or biofilm tank walls but present in biofilm biofilter (1.86%) and the water sump (6.67%). On order level however,

Burkholderiales was abundant in similar levels in tank water and tank biofilm (17.40%) while the biofilter biofilm revealed some lower abundance (5.92%). Pseudomonadales were among the top ten most abundant taxa in both biofilm types but were not detected in water. Taxa found to be abundant in biofilter biofilm and water, but not in tank wall biofilm included Nitrospirales and Caldilineales, where biofilter biofilm had the highest abundance of Nitrospirales, as expected. Chlamydiales was typically more abundant in water than biofilms.

Table 2. The ten OTUs contributing most to the difference between the microbial communities in biofilter biofilm (B-B), tank wall biofilm (B-T), rearing water (W-T) and water sump (W-S), identified by SIMPER-analysis based on Bray-Curtis similarities. The relative abundances are specified as percentages of the total reads and represent averages between all samples in the relevant sample group.

| OTU | Taxonomy | Contribution (%) | B-B | B-T | W-T | W-S |
|---|---|---|---|---|---|---|
| 1 | f:Rhodobacteraceae | 9.73 | 6.49 | 16.50 | 13.05 | 17.70 |
| 2 | f:Thiotrichaceae, g:*Thiotrix** | 3.31 | 7.28 | 1.60 | 1.52 | 1.87 |
| 4 | f: Burkholderiales_incertae_sedis, g:*Sphaerotilus* | 2.97 | 1.86 | 0.44 | 0.22 | 6.67 |
| 9 | f:Comamonadaceae, g:*Rhodoferax** | 2.80 | 1.41 | 3.49 | 3.98 | 2.48 |
| 3 | f:Mycobacteriaceae, g:*Mycobacterium* | 2.51 | 1.09 | 3.57 | 3.17 | 1.52 |
| 17 | f:Sphingomonadaceae | 1.51 | 1.08 | 1.44 | 1.66 | 2.45 |
| 5 | o:Actinomycetales* | 1.43 | 0.71 | 2.11 | 0.83 | 2.09 |
| 11 | f:Parachlamydiaceae | 1.43 | 1.83 | 1.73 | 2.15 | 0.60 |
| 12 | f:Flavobacteriaceae, g:*Chryseobacterium* | 1.36 | 0.17 | 1.66 | 0.83 | 1.84 |
| 8 | f:Moraxellaceae, g:*Acinetobacter* | 1.27 | 1.03 | 0.23 | 0.06 | 2.21 |

* OTU_2, OTU_5, and OTU_9 was classified subsequent to the Usearch data processing using the RDP Classifier tool. The taxonomy for the OTUs is given at the lowest level obtained in the classification, either at order- (o), family- (f) or genus- (g) level.

### 3.3.2 Temporal dynamics of water and biofilm communities

Both biofilter biofilm, tank wall biofilm and water showed variation in the microbial communities over time (Fig. 6, 7, 8). Bray-Curtis similarities for the whole monitored period showed that the highest variation in microbial communities was observed for the tank wall biofilm ($0.30 \pm 3.0x10^{-3}$). The rearing water ($0.35 \pm 2.0x10^{-3}$) and water sump ($0.36 \pm 2.0x10^{-3}$)

had similar variation, and biofilter biofilm the lowest variability over time (0.35 ± 0.01). A common feature was that all samples, both biofilm and water, clustered according to production batches and fallowing periods in the PCoA ordination (Fig. 6) and differed significantly between these two states (one-way PERMANOVA, water $p = 1.0 \times 10^{-4}$; biofilter biofilm $p = 2.0 \times 10^{-4}$; tank wall biofilm $p = 1.0 \times 10^{-4}$). This was also reflected in a moving window analysis, comparing the community composition at subsequent sampling times, where the lowest Bray-Curtis similarities were at the fallowing periods (Fig. 9). The fallowing periods seemed thus to affect the microbiota substantially. One of the most striking differences in microbial communities between fallowing and production periods was a strong increase in abundance of Rhodobacterales during production periods, especially for the tank wall biofilm and water (Fig. 8B, C). The microbial communities changed during fallowing, but the microbiota was developing back to the composition that was present before the fallowing, during the production batches. This was evident in the PCoA-plot where the samples from late in each production batch clustered together and was particularly evident for the water samples (Fig. 6B).

Although the community composition of the biofilm on the tank walls and in the biofilter and the water were significantly different, the samples from all locations generally followed the same temporal pattern in similarity as shown in the moving window analysis (Fig. 9). The biofilter biofilm community composition was surprisingly varying over time (Fig. 6, 8, 9). The abundance of Thiothrichales showed large variations in relative abundance over the 15 months; it increased during production batch 4 (up to 43.8%) and the subsequent fallowing period, accounting for as much as 60.0% of the total reads at the subsequent fallowing (t17) (Fig. 8A). At this timepoint Thiothrichales were dominated completely by only one OTU classified as *Thiothrix* (OTU_2). For the same production batch, *Thiothrix* also increased in relative abundance in tank wall biofilm (42.30%). Rhodobacteriales and Pseudomonadales were also predominant orders that varied highly in abundance during the monitored period. The water microbiota changed the most during the three first fallowing periods (Fig. 8, 9). At these fallowing periods the abundances of Burkholderiales decreased and Chlamydiales and Sphingomonadales increased. Chlamydiales reached maximum abundance of 37% in production batch 3, compared to 8% in the last batches (Fig. 9). Both the PCoA-plot, moving window analysis and Bray-Curtis similarities showed that the water microbiota was generally similar between samples taken from the two rearing tanks and the water sump (Fig. 8C,

PERMANOVA $p$ = 1.0) with Bray-Curtis similarity of 0.82 ± 0.03 during the period (Fig. S3, Supplementary).

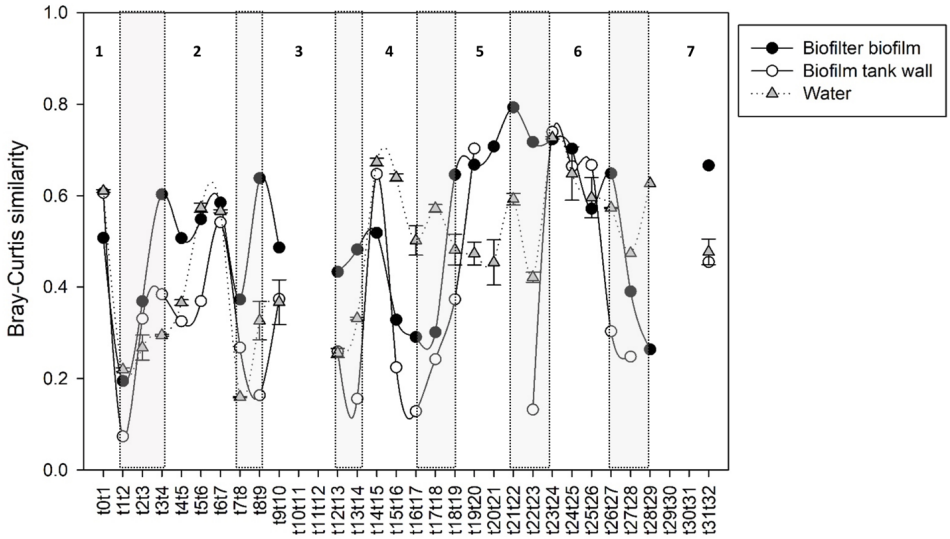



Figure 9. Moving window analysis for comparing microbial community composition of one sampling time to the following sampling time, based on average Bray-Curtis similarity for biofilter biofilm, tank wall biofilm and average of the water (the two rearing tanks and the water sump). Upper numbers represent the seven production batches and shaded timepoints the fallowing periods. Circles represent biofilm, triangle water. Error bars for water represent average SE for water in the rearing tanks and sump.

### 3.3.3 Nitrifying communities in the biofilter

OTUs potentially representing nitrifying bacteria were identified by manual inspection of the OTU table. We identified four OTUs representing the nitrite-oxidising (NOB) genus *Nitrospira*, and five OTUs represented the ammonia-oxidising (AOB) genus *Nitrosomonas* or the family Nitrosomonadaceae (Fig. 10). The four *Nitrospira* OTUs accounted for in average 77% of the total reads for the OTUs classified as nitrifiers, while the five *Nitrosomonas*/Nitrosomonadaceae OTUs comprised only on average 23%. The total abundance of the OTUs representing nitrifiers accounted for a relatively low proportion of the total reads in the samples, with maximum abundance of 12.5% (Fig. 10). Their relative abundances varied both within and between production batches. Production batch 4 had considerably lower abundance of nitrifying OTUs, than the other production batches (average

of 1.1%) and the subsequent fallowing period (0.54%). The relative abundance of nitrifiers tended to increase at the fallowing periods or immediately after the fallowing and to decrease throughout the production batches (Fig. 10). The low AOB:NOB ratio (average 0.37) for OTUs representing NOBs and AOBs could potentially be explained by some of the *Nitrospira* OTUs representing complete ammonia oxidisers (comammox). We therefore performed a phylogenetic analysis to investigate the relationships between the *Nitrospira* OTUs identified here and previously described *Nitrospira*, including both NOB and comammox *Nitrospira* members. Interestingly, maximum likelihood analysis indicated that the *Nitrospira* OTU_1771 was closely related to the comammox *Nitrospira nitrificans* (Fig. 11). OTU_1771 was on average the third most abundant OTU of all *Nitrospira* in the biofilter biofilm with an average relative abundance of 0.85% and maximum relative abundance of 2.96%.

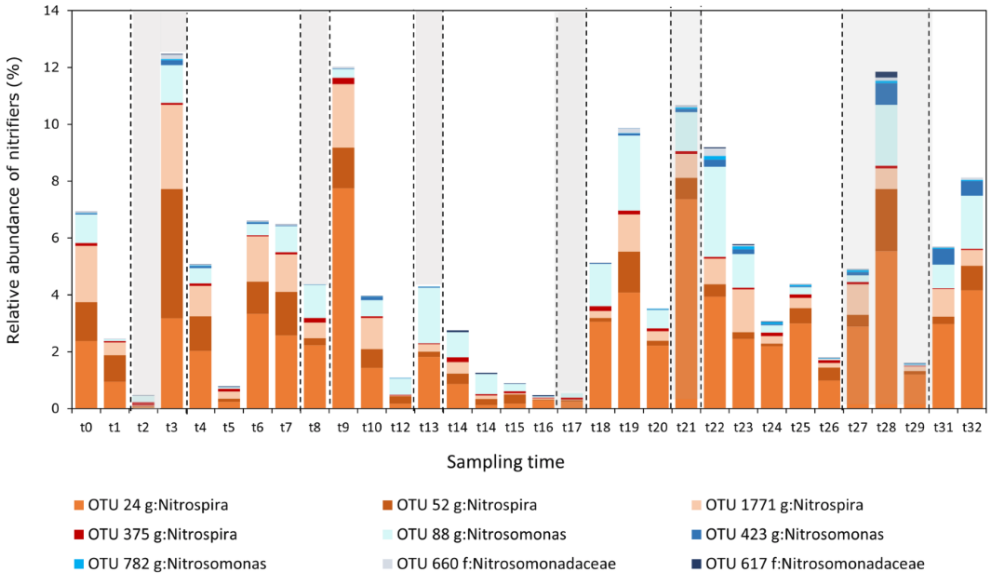



Figure 10. Relative abundance of OTUs classified as nitrifying bacteria in the biofilter biofilm samples (t0-t32) for 15 months period. The taxonomy of the OTUs is given on the lowest obtained taxonomic level, genus (g) or family (f), classified by using the Usearch Sintax script and the RDP training set v18 or MiDAS. Three replicates are included in sampling times t0-5. The upper numbers represent the seven production batches, with fallowing periods in grey shaded areas between. Red bars= *Nitrospira*, blue/green bars= *Nitrosomonas*/Nitrosomonadaceae.

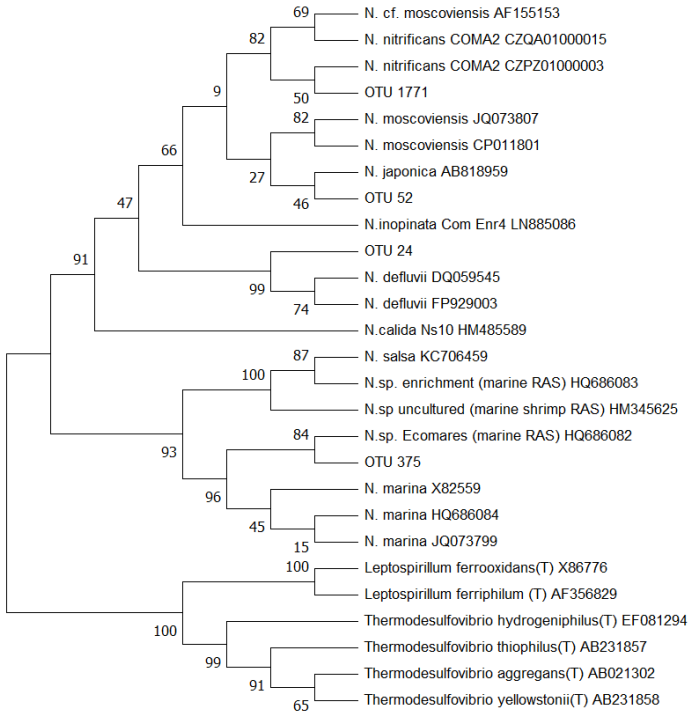



Figure 11. Maximum likelihood (ML) tree for comparing OTUs classified as *Nitrospira* to previously published *Nitrospira* 16S rRNA gene sequences. Sequences were retrieved from RDP (Cole et al., 2014) or the NCBI Genbank. Accession numbers are specified for with the species names. Sequences representing comammox candidates are denotated "comammox". Type strains are indicated by a (T). The ML analysis was performed with 1000 bootstrap replicates and the Tamura-Nei model for sequence evolution. The three was condensed with 50% cut-off value with bootstrap support values shown at the nodes. The three includes representatives for the other genera included in Nitrospiraceae familiy (*Thermodesulfovibrio* and *Leptospirillum*) and is rooted at the *Thermodesulfovibrio* node.

### 3.3.4 Factors affecting the microbial communities in RAS

We used supervised machine learning (SML) to investigate correlations between the composition of microbial communities in the water (both rearing water and water sump) and the biofilter biofilm with the measured physicochemical water quality and other rearing production parameters. Community composition in rearing tanks and water sump exhibited excellent predictability towards fish presence (100%), and good predictability towards biomass (83%) and oxygen saturation (85-88%) (Tab. 3). In addition, microbial community of rearing tanks showed to be an excellent predictor of feed type used during the production

(93%). Microbial communities of biofilter biofilm showed to predict only the fish presence (89%), amongst all the parameters tested. Lastly, mortality, salinity, pH and nitrogenous compounds (TAN, $NO_2^-$, $NO_3^-$) showed poor predictability based on microbial community dynamics of all sample types (below 80%). Finally, we examined which OTUs contribute the most to the predictability strength of sample types and parameters that display good and excellent predictions. The OTUs and corresponding taxonomy can be seen in Figure S3-S9 (Supplementary).

Table 3. The factors that were tested to be correlated to microbial community composition in the biofilter biofilm and rearing water and water sump. The chemical parameters are measured in the water sump downstream the biofilter, except oxygen that was measured in the rearing tanks. *two different feed types (Ewos and Skretting).

| **Parameter** | **Biofilter biofilm (B-B)** | **Water rearing tank (W-T)** | **Water sump (W-S)** |
|---|---|---|---|
| Fish presence | 89% | 100% | 100% |
| Biomass ($kg/m^3$) | 72% | 83% | 83% |
| Feed type* | 77% | 93% | 77% |
| Oxygen saturation | 28% | 85% | 88% |
| Mortality | 62% | 70% | 65% |
| Salinity | 17% | 64% | 30% |
| pH | 19% | 50% | 2% |
| TAN | 54% | 63% | 69% |
| $NO_2^-$ | 11% | 9% | 1% |
| $NO_3^-$ | 27% | 19% | 79% |

## 3.4 Culturable bacteria and total bacterial cell numbers in the water samples

For the last production batch, analysis of culturable bacteria (colony forming units (CFUs)) and total bacterial cell densities (flow cytometry) were included, on three different production days. On production day 30, the fraction of fast-growing, potentially opportunistic bacteria in the rearing tanks were significantly higher than in the rearing tanks compared to the water sump downstream the biofilter (t-test, $p < 0.001$) (Fig. 12). On day 34, there was no significant

difference, while on day 40 there was a higher fraction of fast-growing bacteria in the rearing tanks, compared to the water sump, although not statistically significant (Fig. 12). The rearing tanks had higher total bacterial cell densities than the water sump, although not significantly different (Tab. 4). The microbial growth potential was estimated by calculating the fraction of total bacteria after three days incubated on agar compared to the original number of total bacterial cells. The growth potential was lower in the water sump downstream from the biofilter compared to the water from the rearing tanks, although not significant. Altogether, the analyses of culturable bacteria indicated that there was a tendency of higher growth of presumptive opportunistic bacteria in the rearing tanks compared to the treated water downstream of the biofilter.

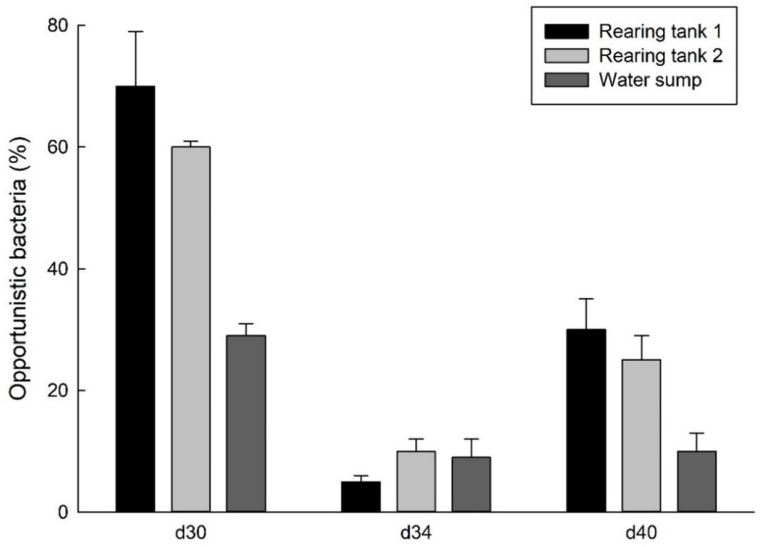



Figure 12. The average fraction of rapid growing bacteria (±SE) in water samples from three different sampling sites; rearing tank 1, rearing tank 2 and water sump downstream the biofilter, in production batch 7, day 30, 34 and 40 in the production. The averages were calculated from three replicate water samples for each sample site on each sample day ± SE.

Table 4. Average bacterial growth potential and total bacteria cells (±SE) at three different sampling sites and two different sampling dates in production batch 7, day 30 and 40. The average bacterial growth potential was calculated by dividing the increase in number of bacterial cells after three days incubation on agar by the total bacterial cells determined by flow cytometry. W-T=water rearing tank, W-S=water from sump, upstream disinfection. n=3.

| | **Day 30** | | | **Day 40** | | |
|---|---|---|---|---|---|---|
| | **W-T1** | **W-T2** | **W-S** | **W-T1** | **W-T2** | **W-S** |
| Bacterial growth potential (%) | 120 ± 11 | 131 ± 21 | 88 ± 7 | 706 ± 76 | 583 ± 33 | 488 ± 79 |
| Total bacterial cells × $10^5$ $mL^{-1}$ | 5.0 ± 0.1 | 5.4 ± 0.1 | 5.1 ± 0.1 | 3.9 ± 0.2 | 4.0 ± 0.1 | 3.6 ± 0.2 |

# 4. Discussion

This study aimed to characterise and understand the temporal dynamics of the complex microbial communities in a commercial RAS, during start-feeding of Atlantic salmon fry. To the best of our knowledge, this is the first-time microbiota of both water and biofilm has been regularly monitored over such a long timescale (15 months) in a commercial facility. The fish were healthy throughout the sampling period, and the results represent normal conditions for fry production in the studied RAS. Fish growth (SGR 5.2%) and daily mortality (0.11%) was normal during the monitored period. The physicochemical water quality variables were, in the context of commercial production, satisfying and relatively stable during the monitored period, indicating a well dimensioned RAS.

Microbial communities in water, biofilm from rearing tanks and biofilter were all significantly different from each other. The most apparent difference on OTU level was OTU_1 (Rhodobacteraceae) that was far more abundant in the tank (water and biofilm) compared to the biofilter biofilm. Our results are in accordance with a study by Rud et al. (2017) where Rhodobacteraceae was far more abundant in water compared to the biofilter biofilm. Rhodobacteraceae are well known for their metabolic versatility which contribute to nutrient cycling (Duarte et al., 2018). The second most contributing OTU, represented by *Thiothrix*, was more abundant in the biofilter biofilm than in the samples from tank (water and biofilm). *Thiothrix* have been identified previously in RAS, but at lower abundances (Rurangwa and Verdegem, 2015; Rud et al., 2017) and are capable of autotrophic denitrification (Rurangwa and Verdegem, 2015) and oxidation of inorganic sulphur compounds (Molina-Muñoz et al.

2007). The significant different community compositions between water, biofilter biofilm and tank wall biofilm are in line with previous findings and expected due to different environmental selective pressures that are shaping the microbiota in RAS (Bakke et al., 2017; Rud et al., 2017; Bartelme et al., 2017; Duarte et al., 2018; Bartelme et al., 2019; Chen et al., 2019; Minish et al., 2020). Our results corroborate previous findings that the biofilter biofilm had higher Shannon's diversity than water (Rud et al., 2017; Bartelme et al., 2019; Aalto et al., 2022). In addition, tank wall biofilm had the had the lowest alpha diversity. Differences in community composition and alpha diversity can also be explained by different frequencies and methods of cleaning of the biofilm from biofilter and tank wall. The tank wall biofilm was thoroughly cleaned and had to go through a primary succession process between each production batch. The biofilter was backwashed regularly, without disinfection, which likely removed only the outer layer of the biofilm (Michaud et al., 2014) and had probably established a more diverse and mature biofilm in the deeper layers.

The community composition of both biofilm and water was surprisingly variable over time, compared to four commercial RAS producing salmon smolts monitored for the same period (Dahle et al., 2020b). The microbiota composition of biofilm and water differed significantly between fallowing and production periods. The impact of fish presence/absence is closely linked to feeding and organic matter load on the system and the carbon to nitrogen ratio (C/N ratio). Organic matter is typically the limiting resource determining the carrying capacity of the heterotrophic bacteria (Michaud et al., 2006) and is known to perturbate the microbial community structure and abundances in both biofilter and water (Michaud et al., 2006; Wold et al., 2014; Bartelme et al., 2017; Rojas-Tirado et al., 2018; Bartelme et al., 2019; Fossmark et al., 2020). During production batches the organic load increase, and consequently, the fraction of heterotrophic bacteria to nitrifying bacteria typically increase during production, which can impact nitrification negatively (Michaud et al., 2006; Michaud et al., 2014). Increased fraction of heterotrophic bacteria to nitrifiers was apparent in this study, as the relative abundance of OTUs representing nitrifiers decreased in abundance throughout the production batches and increased during fallowing periods. The fallowing periods were rather long (up to 40 days) and the dosing of ammonia was done to maintain the nitrifying bacteria active. We have observed highly stable community compositions in biofilter biofilm of RAS with shorter fallowing periods or continuous production during Atlantic salmon smolt production (Dahle et al., 2020b). We hypothesise that shorter fallowing periods or continuous

production contributes to more stable conditions for the biofilter microbiota. A stable microbial community dominated by K-selected bacteria is suggested to indicate a more robust and resilient system against opportunistic and pathogen bacteria invasion and promote beneficial rearing conditions for the fish (Attramadal et al., 2012a; 2012b; De Schryver and Vadstein, 2014; Attramadal et al., 2014; Vadstein et al., 2018). On the contrary, alternation between production batches and fallowing can select for opportunistic bacteria that thrives under abrupt increases in organic loading (Attramadal et al. 2012b; Vadstein et al., 2018). However, importance of stable biofilter biofilm communities for optimal biofilter efficiency, microbial water quality and fish health is poorly understood and should be investigated closer in future research.

Supervised machine learning (SML) employing new learning algorithms has emerged as promising approach for data driven predictions and decision support in various disciplines (Pugliese et al., 2021). In this study we applied SML algorithms on amplicon sequencing derived OTU data and demonstrated that the composition of microbiota in both water and biofilter biofilm could predict presence of fish and fallowing periods in the system. The microbiota composition of water showed good predictability towards biomass of fish and oxygen saturation. In addition, the microbiota in the rearing tanks was a good predictor for feed type. The aforementioned variables are closely linked to each other and to fish presence and organic matter load in the system. The results shows that the presence of organic matter had a higher impact on the microbial communities than pH, salinity and nitrogen compounds in the studied RAS. However, the low correlation towards physicochemical parameters is most likely related to rather small variations during the monitored period, as it is well documented that for instance high fluctuations in salinity perturbates microbial communities in RAS (Bakke et al., 2017; Navada et al., 2019; Fossmark et al., 2021). So far there has been no published application of SML to microbial community data in RAS, but a good correlation between microbial communities and environmental impact around salmon net pens has been shown (Frühe et al., 2020). We have demonstrated here that SML models based on microbial communities could be used to predict fluctuations in RAS to a certain extent. SML has the potential to provide models that can predict instability or deteriorating conditions in RAS using microbial community dynamics.

An interesting observation was that although the microbial communities changed going from high to no load of organic matter during fallowing, it was changing back to a very similar

composition during each production batch. This was especially evident for the water samples (Fig. 6B). The system seems to select in the same way for the suspended microbiota in each production batch and is likely a result of a similar selection pressure between production batches caused by system design and operational routines. The biofilter biofilm microbiota of the biofilter may also affect the microbial communities of the water (Dahle et al., 2022), but the knowledge on these interactions is limited (Rojas-Tirado et al., 2019). A selective exchange of bacteria is expected by released bacteria from the biofilm to the water (Leonard et al., 2000; Michaud et al., 2009; Blancheton et al., 2013). Dahle et al. (2022) showed that the water microbiota developed differently in systems with immature biofilters compared to matured biofilters and suggested that the biofilm microbiota of the biofilter may affect the microbial communities of the water more heavily than season, fish size and management like disinfection. Our results along with others show that the microbial communities in the biofilter biofilm and rearing water were significantly different, but still share many abundant genera (Michaud et al., 2009; Bakke et al., 2017; Bartelme et al., 2019; Almeida et al., 2021) and generally follows similar trends of temporal dynamics (Fig. 9). The covariance in temporal dynamics and shared taxa indicate that the biofilter microbiota has a prominent role in shaping the suspended water bacterial communities in RAS. The biofilter may also act as a buffer to changes in the system where the heterotrophic populations have a high capacity to maintain the abundance of bacteria in the water in response to sudden increases of organic matter loading (Rojas-Tirado et al., 2019). The microbial composition of the water varied more over time than the biofilter biofilm, indicating that the bacterial populations in the water are more sensitive to variation in water quality and management than the more protected biofilm bacteria. This corroborates previous studies (Michaud et al., 2009; Bakke et al., 2017; Rud et al., 2017; Roalkvam et al., 2021).

Nitrifying bacteria constituted a small fraction of the biofilter community, with a maximum relative abundance of 12.5%, which is in line with other studies of RAS exhibiting good biofilter efficiency (Fossmark et al., 2021; Ribicic et al., unpublished results). The relative abundance of nitrifying bacteria varied both within and between production batches (Fig. 10). Nitrifying communities were dominated by *Nitrospira* which are commonly found in biofilters of fresh and brackish water RAS (Bartelme et al., 2017; Fossmark et al., 2021; Aalto et al., 2022; Ribicic et al., unpublished results), while the abundances of ammonium oxidising bacteria (AOB) were low. The low AOB:NOB ratio indicates the presence of comammox

*Nitropspira* bacteria, capable of complete ammonia oxidising, belonging to the *Nitrospira* genus (Costa et al., 2006; van Kessel et al., 2015). The third most abundant *Nitrospira* OTU was related to *Candidatus Nitrospira nitrificans*, identified as a comammox *Nitrospira* in trickling filters in RAS (van Kessel et al., 2015). The low abundance of OTUs classified as AOBs could also be explained by the presence of ammonia oxidising archaea (AOAs), which has been identified in high abundances in RAS (Brown et al., 2013; Bartelme et al., 2017). The primers used in this study, were however not designed to target archaea. It is likely that the AOA are competing with comammox *Nitrospira* in RAS, especially at low ammonia substrate concentrations (Bartelme et al., 2019).

The studied RAS included full-flow UV disinfection of the water directly upstream of the rearing tanks. The fraction of fast growing, potentially opportunistic, CFUs were significantly higher in the rearing tanks than in the water sump upstream the disinfection on day 30 of batch 7 and considerably higher on day 40 (Fig. 12). In addition, the alpha diversity was significantly lower in the rearing tanks compared to the water sump on the same sampling days. Also, the rearing tanks had a higher bacterial growth potential than the water sump, which indicate that higher supplies of resources are available for bacterial growth following the disinfection (Hess-Erga et al., 2010), giving favourable conditions for opportunists. Significant regrowth and proliferation of opportunistic bacteria after disinfection has been reported for systems with long hydraulic retention time (HRT) in the rearing tanks (60 minutes and longer), such as in marine hatcheries. These communities are also characterized by low alpha diversities. Significant regrowth of bacteria following UV treatment have been shown to result in an altered microbial community composition with negative effects on marine larval health and survival (Attramadal et al., 2012b; Vadstein et al., 2018; Dahle et al., 2020a; Teitge et al., 2020; Attramadal et al., 2021). However, the water microbiota composition and the total bacterial concentration was relatively similar between the rearing tanks and in the water sump in this study, as in a comparable study of a commercial RAS producing salmon fry (Dahle et al., 2022). The similarity between the two water locations can be explained by the short HRT in the rearing tanks (18-28 min), that prevented high regrowth of bacteria in the rearing tanks and therefore prevented large changes in composition through the system (Bakke et al., 2017; Dahle et al., 2022). In systems with short HRT in the rearing tanks, UV disinfection can be used to restrict bacterial density (Summerfelt et al., 2009) without compromising the microbial water quality in the rearing tanks (Dahle et al., 2022). However, in theory, a

community with considerable potential for opportunistic regrowth might be vulnerable for pathogen invasion. It is likely that pathogens are present in RAS at low abundances at normal production (Michaud et al., 2009; Dahle et al., 2020b; Lewin et al., 2020) and that a beneficial microbial communities suppress these pathogens from proliferation (Vadstein et al., 2018; Borges et al., 2021). No disinfection in the loop or disinfection before the biofilter instead of before the rearing tanks could lower the regrowth of opportunistic bacteria in the tanks, which can improve microbial water quality and provide a more resilient system against proliferation of pathogens. This is something that should be investigated in RAS with short HRT, like salmonid production, in the future.

# 5. Conclusions

Our study showed that the composition of both the water and biofilm microbiota in the commercial RAS varied over time, and that fallowing periods had a substantial effect on the microbial communities. However, the microbiota returned to similar compositions during all production periods, indicating a similar selection pressure shaped the system's microbiota during all production phases. Nitrifying communities were dominated by *Nitrospira*, and the third most abundant *Nitrospira* OTUs were related to the comammox *Nitrospira nitrificans*. Although the microbial communities in the biofilter biofilm and water were significantly different, they shared many common taxa and generally followed similar trends of temporal dynamics, which suggest an interaction between the biofilter biofilm and the suspended bacteria. CFU analysis showed that the fraction of rapid-growing bacteria was significantly higher in the rearing water than in the water sump upstream the UV disinfection, indicating that disinfection upstream the rearing tanks allowed for growth of opportunistic bacteria. The absence of an in-line disinfection step or placing the disinfection unit upstream the biofilter might provide better microbial water quality and a more resilient system against pathogen invasion.

# Acknowledgement

This research was funded by the Norwegian Seafood Research Fund, project "MonMic" (grant 901392), Research Council of Norway (grant 272400) and Norwegian salmon aquaculture

industry. We would like thank Deni Koseto (SINTEF Industry) for sample logistics and the RAS facility for providing samples and production data.

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# Supplementary

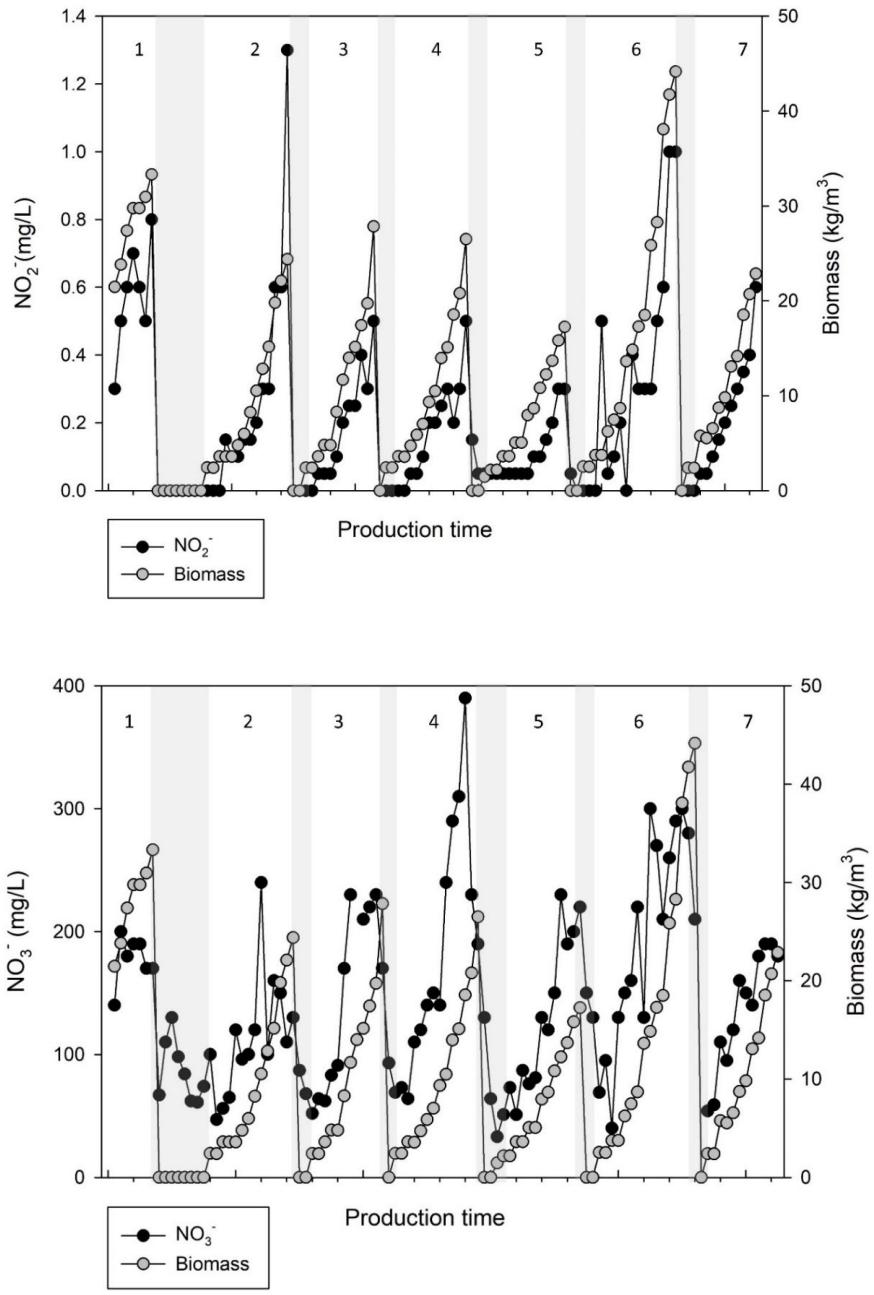



Figure S1. Nitrite ($NO_2^-$) (A) and nitrate ($NO_3^-$) (B) during the production of seven production batches (upper number) and biomass of fish (kg/m$^3$). Shaded areas represent the fallowing periods. Nitrite and nitrate were measured in the rearing tanks. The suggested threshold for nitrite in Norwegian aquaculture producing Atlantic salmon smolts is 0.1 mg/L (Hjeltnes et al., 2012), but is dependent on other factors.

Table S1. Production data for the seven production batches. Final fish weight, days in the RAS unit, specific growth rate (SGR), daily mortality and biomass for the seven production batches. *=Batch 1 and 7 were uncompleted, where batch 1 were monitored for the 15 last days and 7 for the 47 first days. The data are presented as average of the two rearing tanks ± SE.

| | **Production batch** | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1* | 2 | 3 | 4 | 5 | 6 | 7* |
| **Final weight (g)** | 2.9±0.1 | 2.4±0.3 | 2.5±0.0 | 2.5±0.1 | 2.5±0.1 | 3.9±0.3 | 2.2±0.1 |
| **Days in the RAS unit** | 15 | 47 | 49 | 48 | 49 | 58 | 47 |
| **SGR (%)** | 3.18 | 5.29 | 5.15 | 5.22 | 5.15 | 5.12 | 4.85 |
| **Daily mortality (%)** | 0.01±0.0 | 0.10±0.02 | 0.10±0.02 | 0.14±0.02 | 0.06±0.01 | 0.08±0.02 | 0.21±0.06 |
| **Final biomass (kg/m$^3$)** | 34.17 | 27.74 | 28.99 | 28.14 | 18.08 | 47.01 | 23.41 |

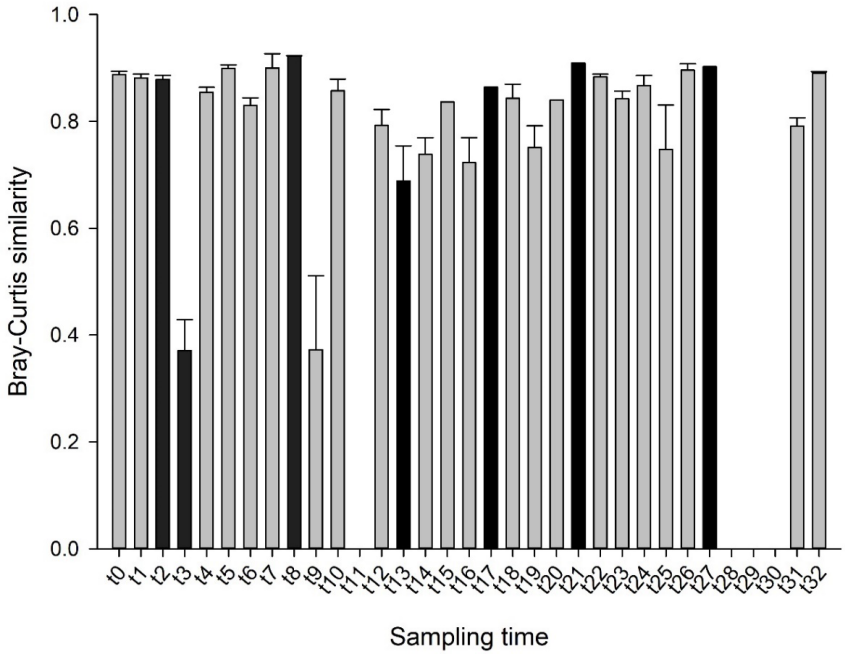



Figure S2. Average Bray-Curtis similarities (±SE) for comparisons within water samples (two rearing tanks and the water sump downstream the UV treatment) at each sample time (t0-t32). At t11 and t28-t30 there was no data available. Black bars represent the fallowing periods.

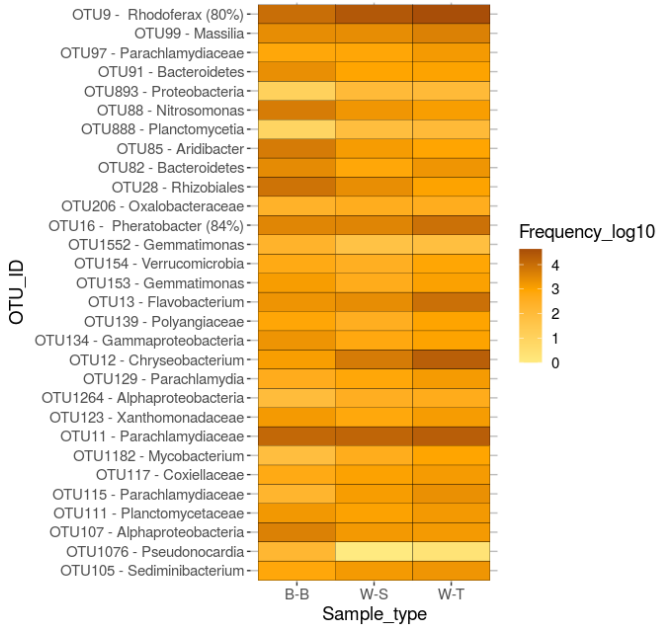



Figure S3. OTUs that contribute the most to the predictability strength of the biofilter biofilm microbial community towards fish presence in RAS. In addition, heatmap indicates sequence counts (frequency) for each OTU based on log10 scale. For reference, frequency of selected OTUs is shown also in other sample types.

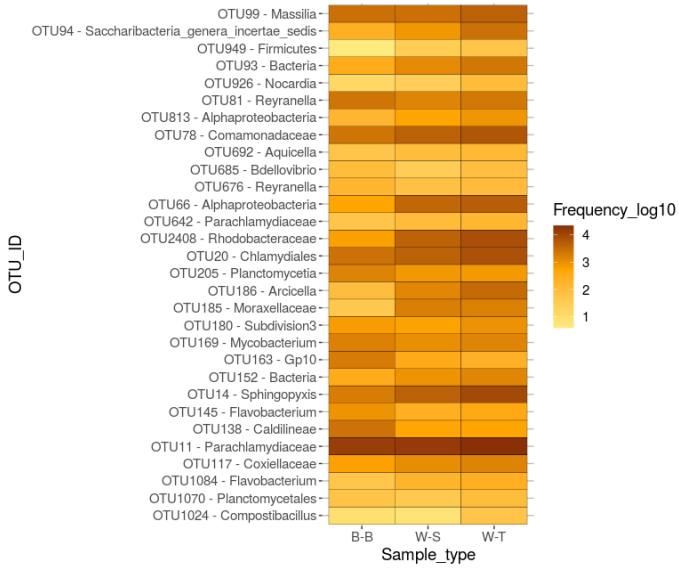



Figure S4. OTUs that contribute the most to the predictability strength of the tank water microbial community towards fish presence in RAS. In addition, heatmap indicates sequence counts (frequency) for each OTU based on log10 scale. For reference, frequency of selected OTUs is shown also in other sample types.

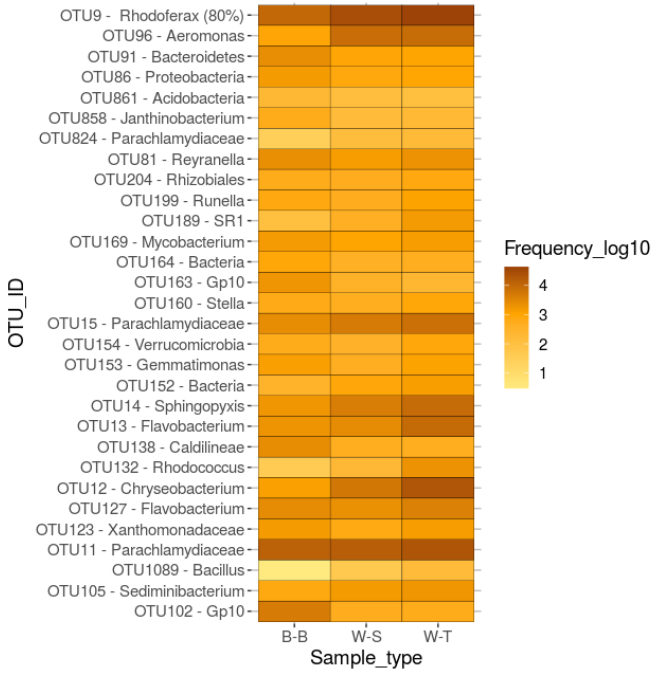



Figure S5. OTUs that contribute the most to the predictability strength of the sump water microbial community towards fish presence in RAS. In addition, heatmap indicates sequence counts (frequency) for each OTU based on log10 scale. For reference, frequency of selected OTUs is shown also in other sample types.

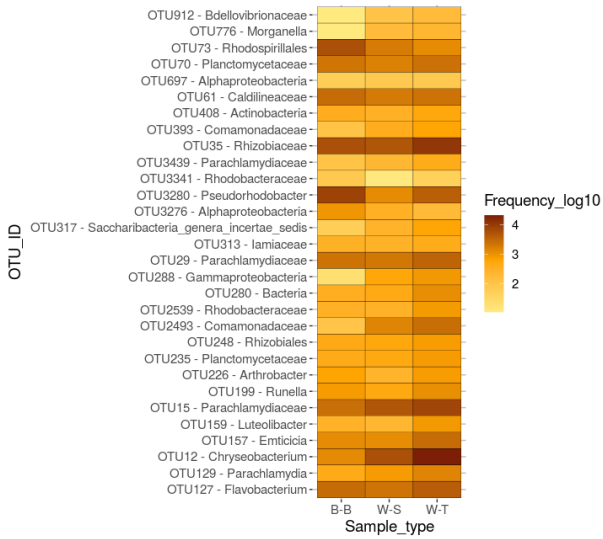



Figure S5. OTUs that contribute the most to the predictability strength of the tank water microbial community towards fish biomass in RAS. In addition, heatmap indicates sequence counts (frequency) for each OTU based on log10 scale. For reference, frequency of selected OTUs is shown also in other sample types.

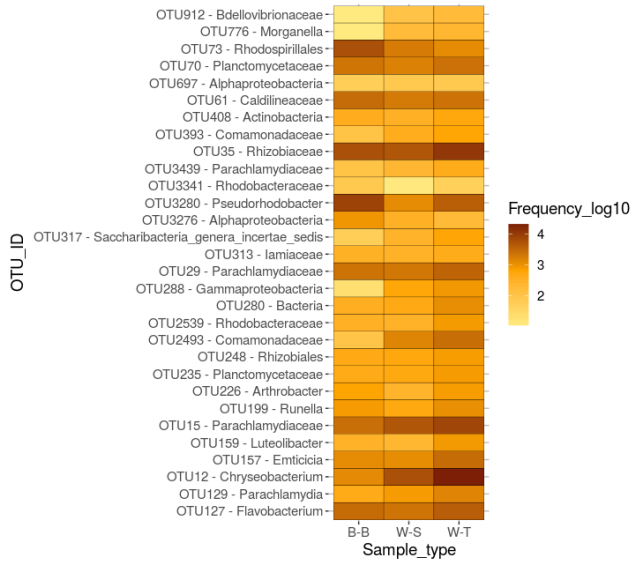



Figure S6. OTUs that contribute the most to the predictability strength of the sump water microbial community towards fish biomass in RAS. In addition, heatmap indicates sequence counts (frequency) for each OTU based on log10 scale. For reference, frequency of selected OTUs is shown also in other sample types.

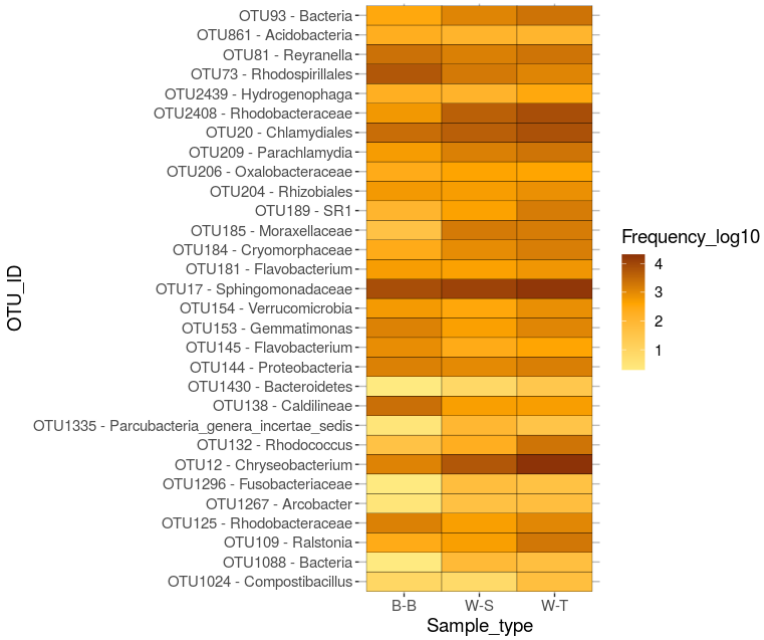



Figure S7. OTUs that contribute the most to the predictability strength of the tank water microbial community towards feed type used in RAS. In addition, heatmap indicates sequence counts (frequency) for each OTU based on log10 scale. For reference, frequency of selected OTUs is shown also in other sample types.

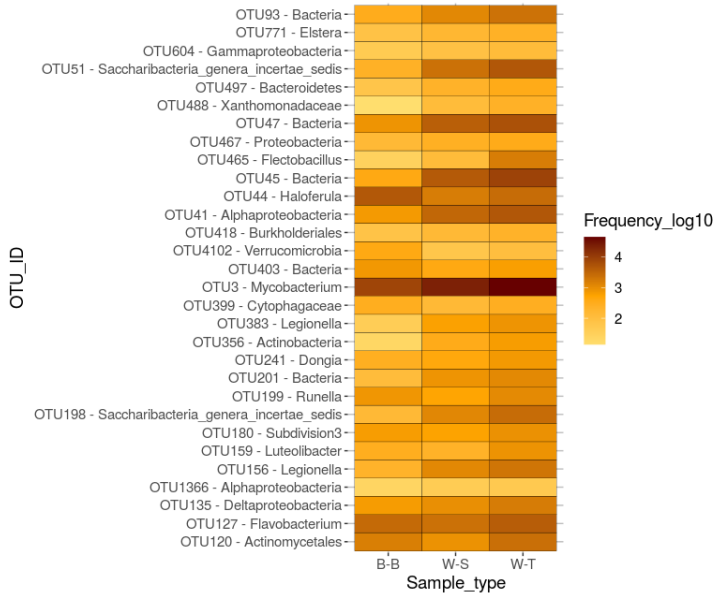



Figure S8. OTUs that contribute the most to the predictability strength of the tank water microbial community towards $O_2$ saturation of RAS. In addition, heatmap indicates sequence counts (frequency) for each OTU based on log10 scale. For reference, frequency of selected OTUs is shown also in other sample types.

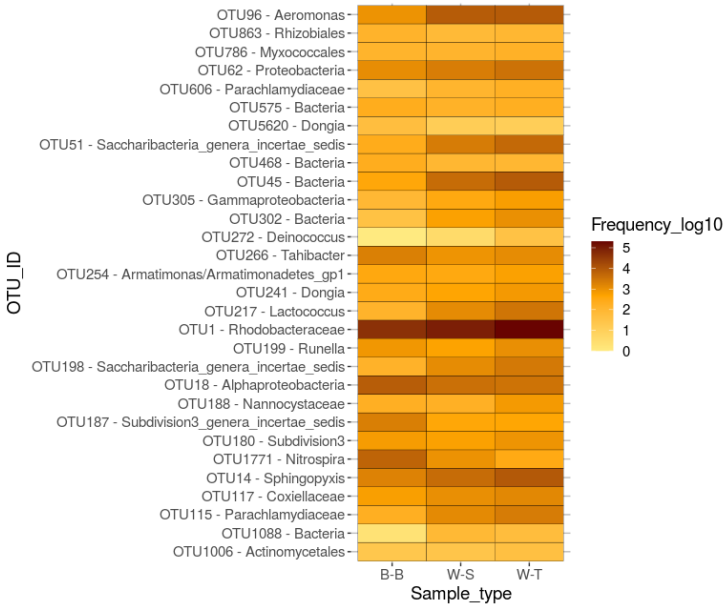
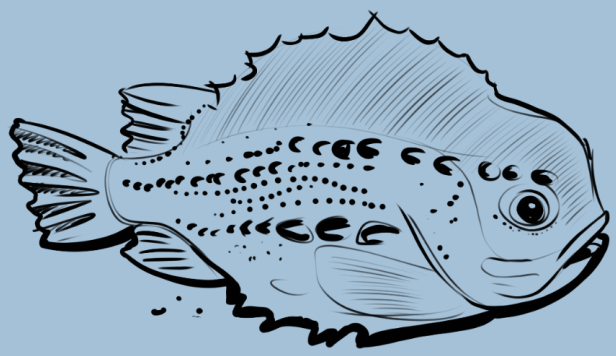



Figure S9. OTUs that contribute the most to the predictability strength of the sump water microbial community towards $O_2$ saturation of RAS. In addition, heatmap indicates sequence counts (frequency) for each OTU based on log10 scale. For reference, frequency of selected OTUs is shown also in other sample types.

# Paper III



Aquaculture 522 (2020) 735097

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

# Production of lumpfish (*Cyclopterus lumpus* L.) in RAS with distinct water treatments: Effects on fish survival, growth, gill health and microbial communities in rearing water and biofilm

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ARTICLE INFO

*Keywords:*
Recirculating aquaculture systems
Microbial communities
Lumpfish
Water quality
Disinfection
Gill health

ABSTRACT

Lumpfish (*Cyclopterus lumpus* L.) in Norway is currently produced in traditional flow-through systems (FTS). Hatcheries frequently show signs of bacterial infections, unstable microbial communities in the rearing water and varying mortality. Recirculating aquaculture systems (RAS) is proposed to create stable and healthy microbial environments, with less probabilities for blooming of opportunistic microbes. Studies have also shown that RAS increases the survival of marine fish. The aim with this study was to investigate the effect of various RAS water treatment designs on water and biofilm microbiota, survival, growth and gill health of lumpfish. An experiment with lumpfish was conducted, from 2 months post hatch to the transfer into sea cages. Five different water treatment regimens were compared: 1. RAS with no additional water treatment, 2. RAS with a filtration unit for removal of small particles, 3. RAS with filtration and disinfection with UV-irradiation, 4. RAS with filtration and disinfection with UV-irradiation and ozone and 5. FTS as a reference. The microbiota of the rearing water and tank wall biofilm were sampled and characterized by Illumina sequencing of 16S rDNA amplicons. Lumpfish juveniles reared in the RAS treatments were exposed to a more stable and diverse rearing water microbiota, with a lower share of opportunistic bacteria, a probable reason for the higher survival and better gill health of the fish compared to siblings reared in the FTS. Lumpfish reared in RAS without disinfection were exposed to a more diverse and stable water microbiota, with a lower share of opportunistic and potential harmful bacteria, compared to the lumpfish reared in RAS with disinfection and FTS. This resulted in better gill health. Fish in RAS with filtration, but no disinfection, had a better gill health than the fish in the RAS without filtration, possibly due to the reduction of small particles. The lumpfish were exposed to different microbial communities of both water and biofilm, due to the different treatments of the incoming tank water. In conclusion, our results indicate that implementation of RAS in the production of lumpfish has a potential to increase both survival, growth and gill health of the fish and that RAS with filtration of small particles, but without disinfection, result in the best fish health and performance among the investigated treatments.

## 1. Introduction

Efficient sea lice control remains one of the most important challenges for the salmon farming industry today. The lumpfish (*Cyclopterus lumpus* L.) is of great use as a strategy for biological control in aquaculture due to its appetite for the sea lice (*Lepeophtherius salmonis* Krøyer). The number of lumpfish used by the salmon farming industry has increased exponentially since 2008, and 31 million lumpfish were produced and put in sea cages in Norway during 2018. The number of cleanerfish hatcheries in Norway, most of them producing lumpfish, has increased from five to 31 in five years (Norwegian Directorate of Fisheries, 2019; Kyst.no., 2019). The first pilot trials for the commercial production of lumpfish started in 2011 (Imsland et al., 2014) and consequently research and development are still at an early stage (Powell et al., 2018). Although lumpfish appear to be fairly robust between hatching and transfer to sea cages, signs of systemic bacterial

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https://doi.org/10.1016/j.aquaculture.2020.735097
Received 26 November 2019; Received in revised form 22 January 2020; Accepted 7 February 2020
Available online 08 February 2020

infections are frequently observed in hatcheries (Alarcon et al., 2016). Research has also shown that the microbial communities in the rearing water are highly unstable (Dahle et al., 2017). In addition, the hatcheries have varying survival, ranging from 30 to 90% (producers of lumpfish, Norway, pers. comm., 2019). The most frequent bacterial diseases reported for lumpfish are caused by the pathogens *Tenacibaculum* spp., *Moritella viscosa, Aeromonas salmonicida*, *Vibrio anguillarum, Vibrio ordalii, Pseudomonas anguilliseptica* and *Pasteurella* sp. (Alarcon et al., 2016; Hjeltnes et al., 2018; Scholz et al., 2018). Currently, lumpfish are produced in flow-through systems (FTS). Knowledge on optimal husbandry and microbial water quality for rearing of lumpfish in land-based production systems is still in its infancy and research is needed.

Aquaculture is undergoing a rapid technological development and the demand for sustainability has driven the development of new aquaculture systems. There is a growing interest in the use of recirculating aquaculture systems (RAS) motivated by saving energy for cooling or heating, controlling and stabilizing physicochemical water quality and reducing environmental impact (Martins et al., 2010; Dalsgaard et al., 2013). RAS have properties that can contribute to microbial stability, which has been shown to be particularly important and successfully used in the rearing of marine fish larvae (Vadstein et al., 1993; Skjermo et al., 1997; Attramadal et al., 2012a, 2012b; Drenstig and Bergheim, 2013; Attramadal et al., 2014; Attramadal et al., 2016; Vadstein et al., 2018; Vestrum et al., 2018; Duarte et al., 2019). It has been suggested that RAS favour K-selection of bacteria and outcompete r-strategic bacteria (Attramadal et al., 2012a, 2012b; Attramadal et al., 2014; In prep.), according to the r/K-theory (McArthur and Wilson, 1967; Pianka, 1970; Vadstein et al., 1993). According to this theory, r-selection occurs in unstable environments with high availability of resources and little competition, while K-selection occurs in stable and predictable environments where the bacterial density is close to the carrying capacity (CC) of the system, and where the ability to compete for resources is favoured. Experiments have shown that RAS increases the survival of marine larvae and crustaceans compared to FTS due to K-selection of the rearing water (Attramadal et al., 2012a, 2012b, Attramadal et al., 2014).

Disinfection of the intake water reduce the entry and spreading of pathogens into the system (Sharrer et al., 2005; Wietz et al., 2009) and is of paramount importance for the biosecurity of land-based facilities. However, disinfection of rearing water in the RAS treatment loop efficiently reduces competition by killing bacteria without reducing the CC, and therefore favour r-selection and subsequent proliferation of opportunistic bacteria in the rearing water (Sharrer et al., 2005; Attramadal et al., 2012a, 2012b; Attramadal et al., 2014; Attramadal et al., 2016). For well dimensioned and managed RAS where the hydraulic retention time (HRT) of the rearing tanks is longer than the doubling time for the fastest growing planktonic bacteria, which is typical in marine juvenile production, disinfection within the RAS treatment loop is therefore hypothesized to constitute a disadvantage for the health of the fish (Attramadal et al., 2012b). Disinfection in the RAS treatment loop has been shown to change both the number and the activity of bacteria in the system and rearing tanks, as well as the microbial composition (Attramadal et al., 2012b; Interdonato, 2012). Experiments with lobster larvae showed less variable mortality and a tendency towards higher survival in RAS without disinfection compared to RAS with disinfection in front of the rearing tanks (Attramadal et al., In prep.).

While large particles are removed from RAS by mechanical filtration, smaller particles tend to remain in the system and may accumulate over time (Chen et al., 1993; Becke et al., 2018). Within a RAS, suspended solids originate from feces, uneaten feed and biofilm (Noble and Summerfelt, 1996; Summerfelt et al., 1999). The management of solids is one of the most important and challenging technical issues in RAS (Badiola et al., 2012). Particles are known to harm gill structures (Bruton, 1985) and elevate stress levels in fish (Lake and Hinch, 1999; Sutherland et al., 2008), although susceptibility varies among fish species (Becke et al., 2018). Particles in RAS also provide surface area supporting bacterial activity (Pedersen et al., 2017) and affect the CC in rearing tanks by providing organic matter. There is currently limited knowledge about how particles affect lumpfish performance.

The aim of this study was to investigate effects of RAS and various water treatment design configurations of RAS on microbial communities in water and biofilm, microbial environment, survival, growth and gill health of lumpfish. We tested four different set-ups with an increasing amount of water treatment, including: 1. RAS with no additional treatment (RAS), 2. RAS with a filtration unit for removal of small particles (20 μm) (RAS-F), 3. RAS with a unit with mechanical filtration (20 μm) and disinfection with UV-irradiation (RAS-F-UV), 4. RAS with a unit for particle filtration (20 μm) and disinfection with UV-irradiation and ozone (RAS-F-UV-O). In addition, an FTS was included as a reference system. We used these designs to address the following hypotheses: 1) Lumpfish juveniles reared in RAS will be exposed to a more stable microbial environment, dominated by K-strategists, leading to higher survival, growth and better gill health compared to siblings reared in the reference FTS. 2) Disinfection in front of the fish tanks in RAS will create r-selection in the tank water and thereby reduce microbial water quality and reduce fish performance. 3) Removal of small particles by filtration will improve gill health in addition to microbial water quality (through lowering the microbial carrying capacity). Increased knowledge of the microbial communities created by these systems will be useful for improvement of operational design and sustainable lumpfish production in the future.

## 2. Materials and methods

### 2.1. Experimental setup

A 146 days long experiment with lumpfish was conducted at Ecomarine Seafarm AS at Dønna, Norway, in cooperation with Let Sea AS. Four different treatments were included directly before the water entered rearing tanks, which were all connected to the same RAS loop: 1) RAS without disinfection or filtration for removal of small particles (RAS), 2) RAS with mechanical filtration (20 μm, mechanical filter) (RAS-F), 3) RAS-F with mechanical filtration and a UV unit (RAS-F-UV), 4) RAS-F-UV with mechanical filtration, UV and an ozone unit (RAS-F-UV-O). In addition, a traditional flow-through system (FTS) was included in the experiment as a reference system (Fig. 1). The RAS had been running for one week with the designated treatments and water in the tanks and the biofilter was mature and stable before the experiment started. Each treatment included three replicate grey fish tanks (800 L with coned bottom of 4% slope and central bottom drain). The intake water (140-m depth) was the same for all treatments and was filtrated (200 μm) and UV treated (Fig. 1). Two different UV reactors were used for the UV treatments; UV from Xylem Water Solutions (Germany) for the RAS-F-UV and Smart UV from Pentair (USA) for the RAS-F-UV-O. An Eclipse 40 Ozone generator at 230 V was used (Del, USA) for the ozone treatment. The water from the RAS tanks was in a pump sump and pumped over a drum filter of 40 μm. The RAS included a submerged fixed bed upflowing biofilter (14.0 × 3.5 × 3.0 m) with 50% filling and strong aeration. Removal of organic matter was done by flushing sediments from the biofilter once a day. Degassing was done in a pump sump with aeration. The light regime was 24:0 with led lights. Hydraulic retention time of the rearing tanks (HRT) was set to 60 min for both RAS and FTS.

### 2.2. Rearing regime

Lumpfish were hatched and fed cryopreserved live feed for the first 7 days (Planktonic AS, Norway) and thereafter fed commercial dry feed for lumpfish (Otohime, Japan) and reared in an FTS hatchery the first two months, according to commercial production procedures at

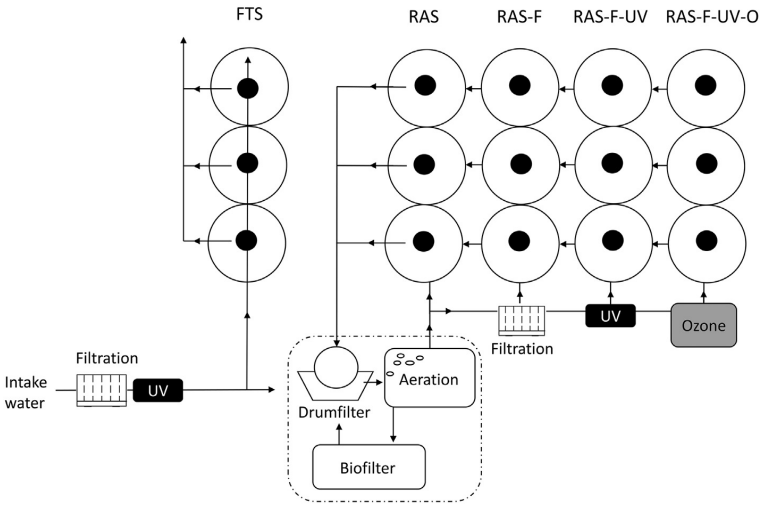


**Fig. 1.** Schematic set up of the FTS and the RAS designs.

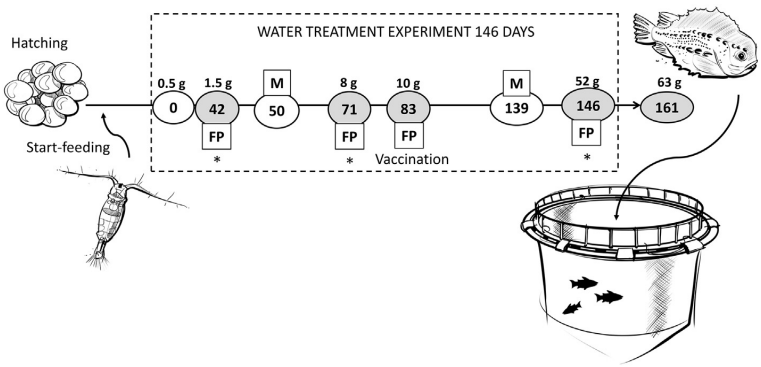


**Fig. 2.** Timeline for the experiment from hatching (2 months prior to experiment), start-feeding, start (day 0) to end of water treatment experiment (day 146), and transport to sea cages (day 161). Open circles (day 0, 50 and 139) = sampling of microbiota (M), grey circles (day 42, 71, 83 and 146) = analysis fish performance (FP), registration of fish weight and survival. Gill health was analysed at day 146. * = sorting of fish, vaccination at day 83. The total production time was 221 days (7.5 months). The weight differed between treatments and is hence an average of the total production. Sketches by Carl Nørstebø (Eggs Design, Norway).

Ecomarine Seafarm. At 0.52 g, 10.000 lumpfish juveniles were transferred to each tank (6.5 kg/m$^3$) in the on-growing systems used in the experiment (Fig. 2). The juveniles were fed continuously with an automatic belt feeder using a commercial diet (Clean Lumpfish, Skretting AS, Norway) the first two months (pellet size 0.5–0.8 mm), then the RAS treatments were fed with Lumpfish Grower (Biomar AS, Norway) with increasing pellet size (1.1–2.0 mm) for the rest of the experiment. The fish from the FTS treatment was fed Clean Lumpfish for ten days longer than the RAS treatments, due to smaller fish weight, and then Lumpfish Grower with increasing pellet size. From day 69 the water treatment for RAS was converted to a RAS-F, due to challenges with maintaining the RAS without filtration, because of the need of a heat exchanger, depending on filtration, to lower the temperature. The fish tanks were cleaned once a day by careful siphoning of the walls and bottom of the tanks. The fish were sorted at day 42 and 71 (Fig. 2), due to size differences and to maintain an optimal biomass in the tanks (15–30 kg/m$^3$). At day 83 the fish (8–11 g) were vaccinated with ALPHA MARINE micro 3.1 vaccine (Pharmaq AS, Norway) with antigens against *Aeromonas salmonicida* genotype VI, *Vibrio anguillarum* serotype O1 and *Vibrio anguillarum* serotype O2a (Fig. 2). The experiment ended at day 146 with sampling and monitoring of fish performance, and fish of 59–68 g were transported to sea cages at day 161 (Fig. 2), with a total production time of 221 days (7.5 months).

### 2.3. Water quality analyses

The pH, oxygen, salinity, total ammonia nitrogen (TAN), nitrate, nitrite and temperature were measured daily after the biofilter and before entering the tanks, and unionized ammonia was calculated from TAN, pH, salinity and temperature. $CO_2$ was measured occasionally. Temperature, salinity and oxygen saturation were measured daily in each tank. Temperature, pH, $CO_2$ and oxygen was monitored by portable electrodes (Oxyguard, Denmark). The nitrogenous waste products were measured with a palintest and a photometer (Palintest, England).

### 2.4. Fish performance

Survival and growth of larvae were calculated for four different periods, day 0–42, 43–71, 72–83 and 84–146, when the larvae were sorted or vaccinated (Fig. 2). Survival was calculated as the number of alive larvae at different time points according to number of larvae at the beginning of the period. Gills from seven fish of each treatment (totally 35 individuals) were dissected from randomly picked fish at the end of the experiment (day 146). The fish were anesthetized in advance with an overdose with Tricaine Methanesulfonate (MS222, Sigma-Aldrich, USA). Gills were fixated (4% formaldehyde) and sent to Pharmaq Analytics AS (Bergen, Norway) for analyses of gill pathology and health by histology. Formalin-fixed tissue was paraffin-embedded and

processed for histological analysis using standard procedures (Bancroft and Gamble, 2008). Gills were sectioned in the sagittal plane at 2 μm thickness before mounting on poly-L-lysine-coated slides (Superfrost Plus, Thermo scientific, Germany) and stained with haematoxylin and eosin (HE). A gill score was calculated based on the occurrence of various histopathological changes, where a score of 1–10 are considered as mild changes, 11–20 moderate changes, and 21 and up considered as comprehensive changes. The growth was calculated by measuring wet weight of fish at the same timepoints as determination of survival. Specific growth rate (SGR) was calculated according to Eq. (1) (Hopkins, 1992), with $W_f$ being the weight at time t, and $W_i$ at initial time, t = the time in days.

$$SGR = [\ln W_f - \ln W_i]/t * 100 \quad (1)$$

Thermal unit growth coefficient (TGC) were used to calculate the growth rate with consideration to temperature (Thorarensen and Farrell, 2011):

$$TGC = [W_f^{1/3} - W_i^{1/3}/T * t] * 1000 \quad (2)$$

with $W_f$ being the weight at time t, and $W_i$ at initial time. T being the average water temperature (°C) in the system for the relevant period, t = the time in days. An average of SGR and TGC for the four different periods were calculated.

### 2.5. Microbial community analyses

Bacterial concentration in the rearing water was determined by flow cytometry (BD Bioscience, USA). Tank water was sampled at two different time points, day 50 and 139 (Fig. 2), immediately fixated with glutaric dialdehyde (at a final concentration of 0.5%) and stored in darkness at 4 °C, until analysis. The Samples were diluted 1:10 with 0.1× TE-buffer, and then cells were stained with SYBR®Green I DNA Gel Stain (Life Technologies, Thermo Fisher Scientific Inc., England) for 15 min. Samples were analysed with a BD Accuri™ C6 Flow Cytometer (BD Bioscience, USA) with a flow rate 34.5 ql/min, threshold at 2000 units, and a sampling time of 3 min. The results were interpreted by using BD Accuri C6 Software. The number of colony forming units (CFU) was determined from growth on Difco Marine agar 2216 (BD, USA) (Salvesen and Vadstein, 2000). 10-fold dilutions were plated for each sample, and each dilution was plated in duplicate. Samples were incubated in darkness at 12 ± 1 °C and inspected after 2 and 14 days. Total CFU were calculated as the average of colonies after 14 days of incubation. The percentage of opportunistic bacteria/r-strategists was calculated as the fraction of fast-growing bacteria (counted on day 2 of incubation) of total CFU (Skjermo et al., 1997; Salvesen and Vadstein, 2000). The percentage of cultivable bacteria (CB) was calculated as the percentage of the total CFU counts of the total cell count with flow cytometry.

For characterization of the microbial communities in the rearing tanks, both biofilm of the tank wall and rearing water were sampled two times from each rearing tank (Fig. 2) during the experiment: 1) after 50 days, 2) after 139 days of the experiment. Three water and biofilm samples were collected from each tank at each sampling time. The water samples were filtrated using a Sterivex™ filter unit (pore size 0.22 qm, Merch Millipore, USA) and Omnifix® syringes. 150–200 mL water was filtrated for each water sample, until the filter was clogged. Biofilm from the walls of the tanks were sampled by using swabs (Copan Diagnostics, USA). Filter and swab samples were frozen (−20 °C) immediately after sampling, transported to SINTEFs laboratory and stored at −80 °C until further analyzes. DNA was extracted using FastDNA® Spin Kit for Soil (MP Biomedicals, USA) following the protocol. Genomic DNA Clean & Concentrator™-10 (Zymo Research, USA) was used to purify the DNA. To determine the concentration and pureness of DNA, a NanoDrop Spectrophotometer (Thermo Scientific Inc., England) was used. Microbial community composition of the samples collected were characterized by 16S amplicon sequencing at the Centre for Biotechnology (CeBiTec), Bielefeld University, Germany. In brief, 16S rDNA amplicons were generated from DNA-samples by two PCR rounds using the 2 × HiFi HotStart ReadyMix (Kapa Biosystems, USA). To amplify the third and fourth variable regions (V3, V4) of the 16S rRNA gene, the primers Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCT AATCC-3′) (Takahashi et al., 2014) covering the domains Bacteria and Archaea were used for the first PCR round. Sequencing adapters and multiplexing indices where added using the Nextera XT Index kit (Illumina, USA). Following each PCR round, amplicons were purified using the QIAquick PCR purification Kit (Qiagen, Germany) and finally the amplicon size and concentration was determined on a BioAnalyzer (Agilent Technologies, USA). Amplicons were pooled, and the normalized DNA libraries (4 pM DNA) were mixed with PhiX (5%) Control v3 (Illumina), denatured at 96 °C for 2 min and each library was run on a MiSeq sequencer (Illumina) lane using the MiSeq Reagent Kit v3 in the 2 × 300 bp paired-end mode. The resulting sequencing data were deposited at the European Nucleotide Archive, under Study PRJEB36184 (accession numbers ERS4260801- ERS4260856). The Illumina sequencing data were processed using the USEARCH pipeline (version 11; https://www.drive5.com/usearch/). The command Fastq_mergepairs was used for merging of paired reads, trimming off primer sequences and filtering out reads shorter than 380 base pairs. Further processing included demultiplexing and quality trimming (the Fastq_filter command with an expected error threshold of 1). Chimera removal and clustering at the 97% similarity level was performed using the UPARSE-OTU algorithm (Edgar, 2013). Taxonomy assignment was performed applying the Sintax script (Edgar, 2016) with a confidence value threshold of 0.8 and the RDP reference data set (version 16). The resulting OTU (Taxonomixal operation units) table was normalized to 20.000 number of reads per sample by determining the fraction of the OTUs for each community profile, and then multiplying with 20 000, and finally rounding off the read numbers to integers. The USEARCH commands Alpha_div and Sintax_summary was used to calculate alpha diversity indices (observed OTU richness and Shannon's diversity) and generate taxa summary tables, respectively. Sequence data was aligned (Wang et al., 2007) to the closest relative in the 16S ribosomal RNA sequences of Bacteria and Archaea in RDP (https://rdp.cme.msu.edu/).

### 2.6. Statistics

The data are presented as mean ± standard error of the mean (SE). Statistical tests were performed at the 95% confidence level ($p$ = .05). Data for larval wet weight were log10 transformed to secure a homogenous variance and tested for differences by one-way ANOVA and $t$-tests in SPSS 16.0 (SPSS Inc., USA). The data for larval survival were Arcsin-transformed before statistical comparison (one-way ANOVA) in SPSS. SPSS was also used for comparisons of the chemical variables. One-way ANOVA or Kruskal-Wallis were used, depending on the homogeneity of variance of the variables. Statistical analyses of the amplicon sequencing data were performed using the program package PAST (Hammer et al., 2001). For ordination of samples we used principal coordinate analysis (PCoA) (Davis, 1986) based on the Bray-Curtis similarities (Bray and Curtis, 1957). To test for differences in community structure between the sample groups, we applied one-way PERMANOVA based on Bray-Curtis similarities (Anderson, 2001). The null hypothesis of no difference in community profiles between groups of samples was rejected for $p$ values less than 0.05. The Similarity Percentages (SIMPER) analysis (Clarke, 1993) was used to determine the contribution from the OTUs to the Bray-Curtis dissimilarity among samples.

**Table 1**
Physicochemical water quality measured in the rearing tanks of each treatment and downstream biofilter during the experiment (mean ± SE).

| | RAS | RAS-F | RAS-F-UV | RAS-F-UV-O | FTS | Biofilter |
|---|---|---|---|---|---|---|
| Temperature (°C) | 10.3 ± 0.3 | 10.0 ± 0.3 | 10.4 ± 0.4 | 10.3 ± 0.4 | 7.5 ± 0.4 | 11.2 ± 0.2 |
| Oxygen saturation (%) | 89.9 ± 1.1 | 95.4 ± 1.10 | 101.0 ± 1.2 | 91.5 ± 1.3 | 89.0 ± 0.7 | |
| Salinity (ppt) | | | | | | 26.4 ± 0.3 |
| pH | | | | | | 7.1 ± 0.0 |
| Total ammonia N (mg TAN $L^{-1}$) | | | | | | 1.0 ± 0.1 |
| Unionized ammonia (mg $NH_3$-N $L^{-1}$) | | | | | | 1.0 ± 0.1 |
| Nitrite (mg $NO_2$-N $L^{-1}$) | | | | | | 0.2 ± 0.0 |
| Nitrate (mg $NO_3$-N $L^{-1}$) | | | | | | 16.2 ± 5.1 |
| $CO_2$ (mg/L) | | | | | | 13.4 ± 0.7 |

## 3. Results

### 3.1. Chemical water quality

The chemical water quality was generally satisfying, both downstream biofilter and in the fish tanks (Table 1). Notably, the temperature was significantly lower in the FTS (average of 7.5 °C) than in the RAS (average of 10.3 °C) (ANOVA, $p = .001$) (Table 1). During the first period of the experiment (day 1 to 41), oxygen saturation was low in all treatments (63–80%), except for RAS-F-UV. In the second period (day 42 to 70) the oxygen saturation was higher, but still unstable, and the RAS had the lowest saturation. In the third period (day 71 to 82), the oxygen saturation was stable and satisfying for all treatments (Table 1).

### 3.2. Fish performance

The survival of the lumpfish (Fig. 3) was significantly higher in the RAS treatments than in the FTS during the first and third period (Kruskal-Wallis, $p = .025$; $p = .046$). The average survival during these periods were 79.1 ± 3.8% and 97.9 ± 0.1% for FTS and the RAS treatments, respectively. At the second and last periods there were no significant differences between the survival in the different treatments, even though the RAS and RAS-F had a higher average survival, compared to the other treatments.

The growth, measured as average specific growth rate (SGR), was higher for the RAS treatments than the FTS, although it was not significant (ANOVA, $p = .58$) (Table 2). By compensating for the effect of temperature on growth, thermal growth coefficient (TGC) was calculated. No significant differences in TGC for the experimental period was identified (ANOVA, $p = .99$) (Table 2).

**Table 2**
Average specific growth rate (SGR) and thermal growth coefficient (TGC) during the experiment ± SE.

| | RAS | RAS-F | RAS-F-UV | RAS-F-UV-O | FTS |
|---|---|---|---|---|---|
| SGR | 3.4 ± 0.1 | 3.2 ± 0.3 | 3.3 ± 0.3 | 3.3 ± 0.3 | 2.4 ± 0.1 |
| TGC | 2.0 ± 0.1 | 2.1 ± 0.2 | 2.0 ± 0.1 | 2.0 ± 0.2 | 2.1 ± 0.2 |

The gill health was analysed by histology and showed that the RAS-F had a significantly lower gill score than RAS-F-UV, RAS-F-UV-O and FTS (Kruskal-Wallis, $p = .044$; $p = .006$; $p = .001$), indicating better gill health in the RAS-F system (Fig. 4). The RAS treatment had a significantly lower gill score than FTS (Kruskal-Wallis, $p = .009$) and were close to significant different from RAS-F-UV-O (Kruskal-Wallis, $p = .058$). No significant differences in gill score were found between RAS and RAS-F.

The histopathological analysis did not identify damages in the gills related to any specific agent, but several non-specific changes, like mucous cell metaplasia, degenerative changes of respiratory epithelium, lamellar- and filament epithelial hyperplasia, and focal or diffuse inflammation were observed (Fig. 5). As Fig. 4 indicates, these changes were identified more frequently in dissected gills reared in the RAS treatments that included disinfection and in the FTS.

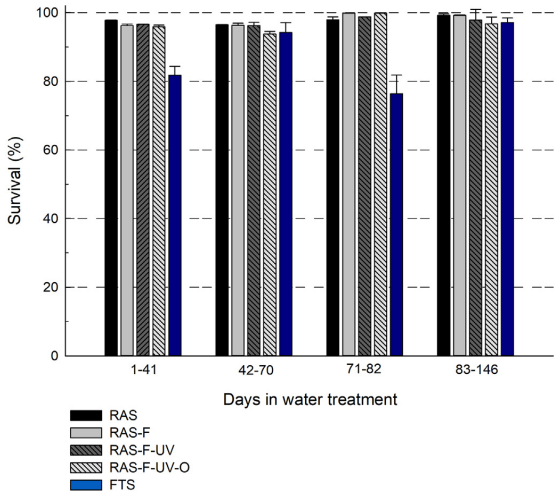


**Fig. 3.** Survival of fish during the experiment, after each of four different periods in each treatment. Average survival ± SE is given for each treatment. RAS tanks were converted to RAS-F from day 69.

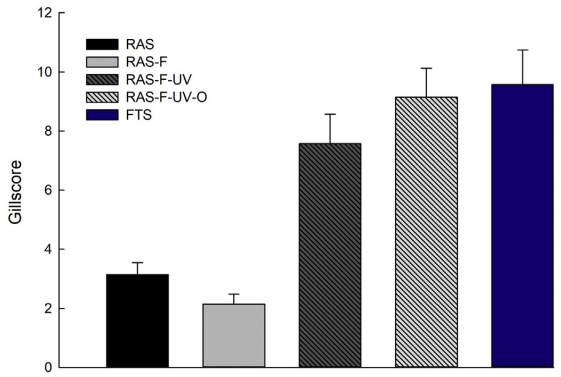


**Fig. 4.** Gill score for fish from the different treatments (average ± SE). A score of 1–10 are considered as mild changes, 11–20 moderate changes, and 21 and up are considered as comprehensive changes. RAS tanks were converted to RAS-F from day 69.

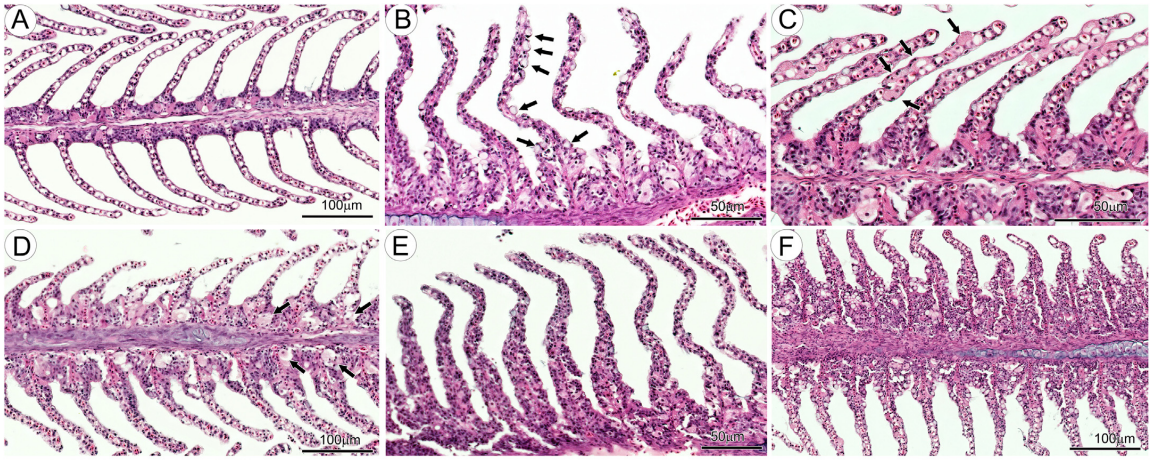


**Fig. 5.** Examples of the most frequent histopathological changes observed in gills from the experiment. A) Normal gills for comparison, B) Mucous cell metaplasia (examples of mucous cells in the respiratory epithelium along one lamella are indicated by arrows), C) Degeneration of lamellar epithelial cells seen as hypertrophic, eosinophilic cells in the respiratory epithelium (examples indicated by arrows), D) Chloride cell hyperplasia and -hypertrophy (examples indicated by arrows), E) Lamellar epithelial hyperplasia, F) Diffuse inflammation of the filament.

### *3.3. Microbiota*

#### *3.3.1. Effect of water treatment on the water microbiota*

The most abundant bacterial classes in rearing water from all systems were Gammaproteobacteria and Alphaproteobacteria (Fig. 6A). The Gammaproteobacteria was the most abundant class at day 50 and was particularly abundant in the FTS and RAS systems with disinfection, with relative abundances as high as 68%, while the Alphaproteobacteria was abundant in all systems at day 139, with relative abundance from 22 to 51%.

A PCoA plot based on Bray–Curtis similarities indicated that the rearing water microbiota differed between the systems (Fig. 7). A PERMANOVA test confirmed that the water microbiota differed significantly between all systems ($p < .5$), except between RAS and RAS-F. The PCoA plot also showed that the water microbiota changed with time for all treatments.

On day 50, The most abundant bacterial family identified in rearing water in RAS-UV and RAS-UV-O was *Thiotrichaceae* (Gammaproteobacteria) (Fig. 6B). In these systems, this family accounted for a high share of the community (up to 53%). In RAS and RAS-F this family comprised only 4% of the reads, and in the FTS the share was even lower, 2%. The same pattern was observed at day 139, at which point the RAS treatments with disinfection had the highest abundance of *Thiotrichaceae*, but the total abundance was lower than what was observed at day 50. At the genus level, the *Thiotrichaceae* was dominated mainly by *Leucothrix,* represented by three OTUs. One of these (OTU_1) was the most abundant OTU in the total data set. SIMPER analysis confirmed that OTU_1 (*Leucothrix*) accounted for most of the differences in the water microbiota between treatments at day 50, with a contribution of 32% to the Bray-Curtis dissimilarity. The *Flavobacteriaceae* was identified in samples from all treatments (Fig. 6B), but the highest abundance was identified in FTS at day 50 (32%). By comparison, the abundance was only 6% in RAS and RAS-F, and around 12% in the RAS systems with disinfection. The *Rhodobacteraceae* was abundant in the RAS and RAS-F treatments, at both sampling dates (12–28%). This family consisted mainly of the genus *Loktanella*, represented by one OTU, which was the second most abundant OTU in the entire data set (OTU_4). *Rhodobacteraceae* was also identified in the RAS treatment with disinfection and in the FTS, but at lower abundances (Fig. 6B). The abundancy of *Rhodobacteraceae* generally increased from day 50 to 139 in all treatments. The FTS showed a surprisingly high variation in the microbial community composition of the water between replicate tanks at day 139 (Fig. 6B). For example, *Oceanospirillaceae* was found in high abundance in only one of the FTS replicate tanks at day 139 (36%) and represented the genus *Oleispeira* (Fig. 6B*)*. Moreover, *Mycoplasma* was highly abundant in another of the FTS tanks (22%). In comparison, the abundances of these taxa were low (less than 1%) in the RAS treatments. *Moritella* (represented by 3 OTUs) was found in all FTS tanks (2–6%) but was in low abundance for the RAS treatments' water samples (less than 0.1%).

Both the observed OTU richness (Fig. 8A) and Shannon's diversity index (Fig. 8B) were significant higher for RAS and RAS-F water compared to the other treatments at sampling day 50. RAS and RAS-F had on average as much as 1360 ± 60 observed OTUs. In comparison, the water of RAS treatments with disinfection (-UV and -UV-O) showed a considerably lower OTU richness (693 ± 109 OTUs), and the FTS showed an even lower richness of only 281 observed OTUs. After 139 days, the bacteria species richness of the rearing water was lower than at day 50 for all RAS treatments, and the FTS had a significantly lower OTU richness compared to RAS and RAS-F (ANOVA, $p = .007$; 0.005).

The Bray-Curtis similarities of the water microbiota was high for comparisons between replicate tanks for all treatments at day 50 (Fig. 9), which indicated stability of the microbial community composition within treatments. This was still the case for three of the RAS treatments on day 139 (RAS, RAS-F, RAS-F-UV), while for the RAS-F-UV-O and FTS, there was a considerably higher variation in the water microbiota between replicate tanks (Fig. 9).

The RAS treatments had a significantly higher concentration of total bacteria in the rearing water, compared to the FTS, at both sampling days (Kruskal-Wallis, $p = .023$) (Fig. 10A). RAS had on average $4.7 \times 10^6$ cells $mL^{-1}$ while FTS had $9.4 \times 10^4$ cells $mL^{-1}$. The RAS treatments had a relatively similar total concentration of bacteria, but the fraction of opportunistic bacteria differed considerably between treatments. The RAS treatment showed only 3% of opportunistic bacteria at day 50 (Fig. 10B), which were significantly lower than the water from the RAS-F-UV and RAS-F-UV-O (ANOVA, $p = .030$; 0.014). The RAS-F had 15% opportunistic bacteria at which were significantly lower than RAS-F-UV ($p = .032$). After 139 days there were no significant differences in the fraction of opportunistic bacteria among the

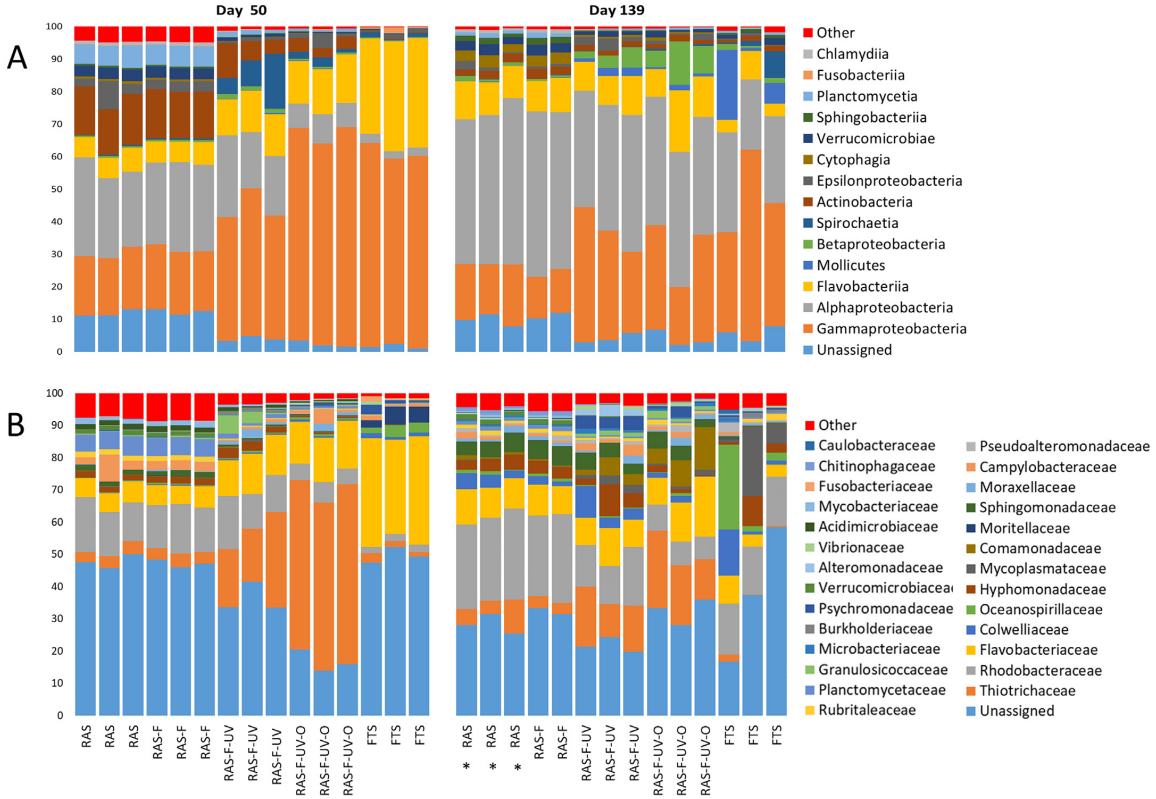


**Fig. 6.** Relative abundancies of bacterial classes (A) and families (B) in the rearing water of the different treatments, at day 50 and 139. Only classes that are present at abundances > 1% in at least one sample are shown. * = RAS tanks were converted to RAS-F from day 69.

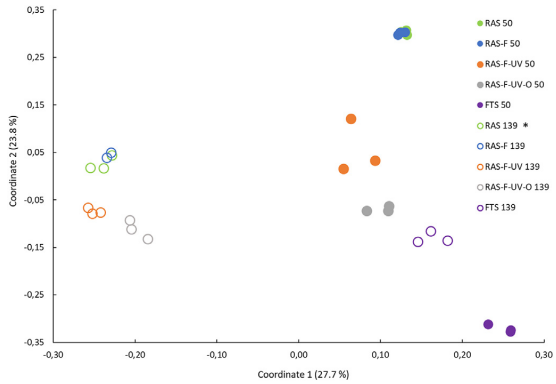


**Fig. 7.** Principal coordinates (PCoA) plot based on Bray–Curtis similarities for water microbiota from the systems at 50 and 139 days in the water treatments. Filled symbols are day 50, open symbols are day 139. * = RAS tanks were converted to RAS-F from day 69.

treatments (ANOVA, $p = .087$) (Fig. 10B).

The flow cytometry analysis showed that the bacterial density in FTS was far lower than in the RAS treatments (Fig. 10A). We further examined the fraction of culturable bacteria in the water treatment by relating the flow cytometry measures to the CFU counts. The average cultivability was considerably higher for the FTS than the RAS treatments (Fig. 11), and the difference was found to be significant on day 139 (Kruskal-Wallis, $p = .017$).

### 3.3.2. Effect of water treatment on the biofilm microbiota

One of the most abundant families identified from biofilm was *Rhodobacteraceae*, identified at the highest abundance in samples from the FTS at day 139, varying from 33 to 43% (Fig. 12). *Flavobacteriaceae* was the second most abundant family, with the highest abundance in FTS (36%) and RAS-F-UV-O (34%) at day 50. The most dominant family from water, *Thiotrichaceae* (Fig. 6B), was also relative abundant in the tank wall biofilm, particularly in RAS-UV-O, where it accounted for up to 30% of the total reads. Another pronounced family was *Hyphomonadaceae*, that was absent at day 50, but present in high abundancy at day 139, 19–23% for RAS and RAS-F, and somewhat lower abundances for the other systems (Fig. 12). As for the water microbiota, the observed OTU richness and Shannon's diversity index were lower for the FTS compared to the RAS treatments at day 50, where the RAS had an average 565 and the FTS 92 observed OTUs. At day 139 the differences in species richness and diversity between the system were not that distinctive (data now shown).

A PCoA-plot based on Bray-Curtis similarities (Fig. 13) indicated that the microbial community composition of the tank wall biofilm differed between sampling times, but the clustering of samples according to treatment system was less profound compared to what found for the water microbiota (Fig. 7). We found no significant differences in tank wall microbiota between systems (PERMANOVA, $p > .5$). Thus, the tank wall biofilm communities seemed to be less influenced by the different water treatments than the rearing water (Fig. 7). The biofilm

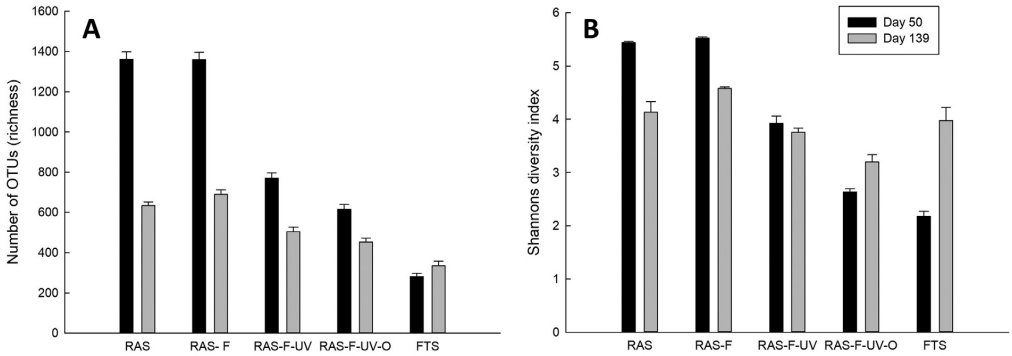


**Fig. 8.** Means of the observed OTU richness (A) and Shannon's diversity index (B) for the water microbiota at day 50 and 139. Error bars show the standard error. RAS was merged to RAS-F from day 69.

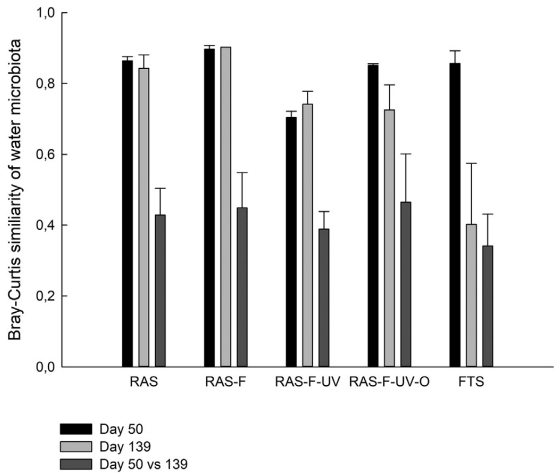


**Fig. 9.** Average Bray–Curtis similarities for comparisons of water microbiota composition within treatments at day 50 and 139 and for each treatment over time between day 50 and 139. Error bars show the standard error (SE). RAS was merged to RAS-F from day 69.

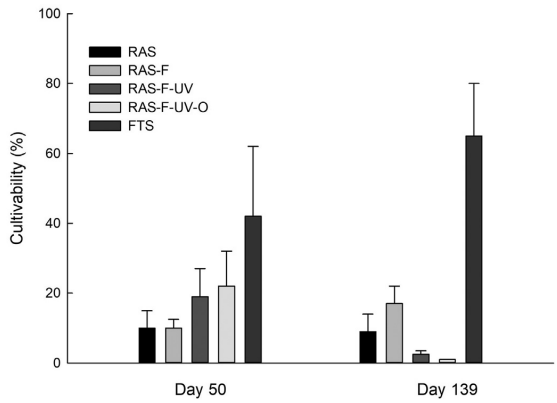


**Fig. 11.** Average Cultivability (%) at day 50 and 139 ± SE, as the percentage total CFU of the total cell count with flow cytometry.

microbiota changed over time for all treatments (Fig. 13), especially in the RAS treatments.

The tank wall biofilm and the rearing water microbiota differed within systems for all treatments at both sampling dates (Fig. 14AB), even though the differences were not statistically significant (PERMANOVA, $p > .16$). The microbial composition of the tank wall biofilm and rearing water in the FTS were more similar compared to that of the

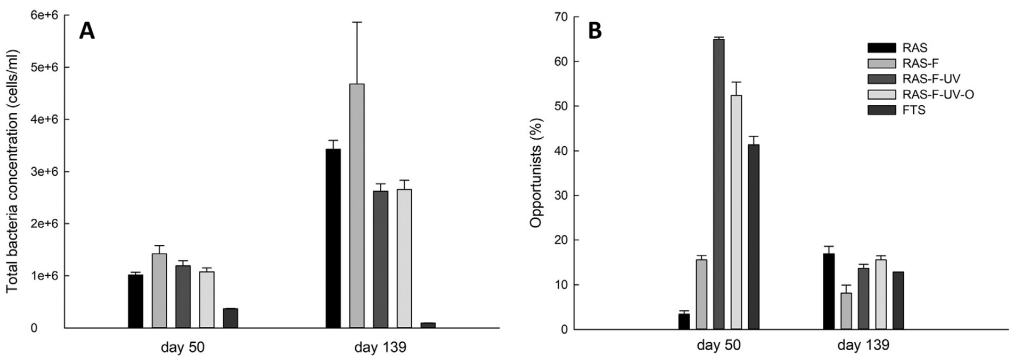


**Fig. 10.** A) Total number of bacteria (cells/ml) in the rearing water of the different treatments at day 50 and 139, analysed by flow cytometry. B) Opportunists (%), as fraction of fast-growing bacteria of total CFU $mL^{-1}$. All data presented as average ± SE. RAS tanks were converted to RAS-F from day 69.

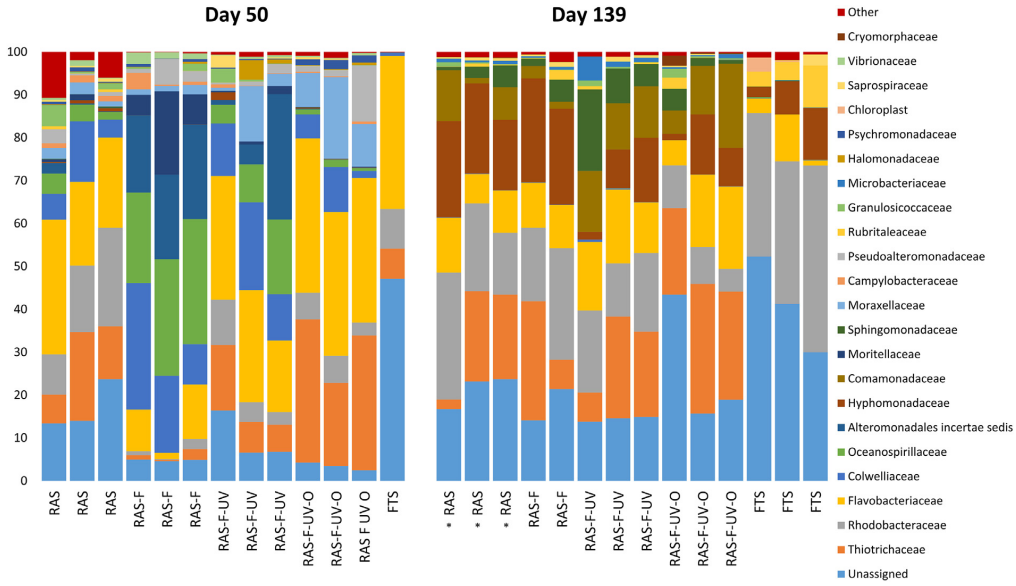


**Fig. 12.** Relative abundances of bacterial genera in tank wall biofilm samples, at day 50 and 139. Only families observed at an abundancy > 1% in at least one sample are shown. FTS at day 50 included only one sample. * = RAS tanks were converted to RAS-F from day 69.

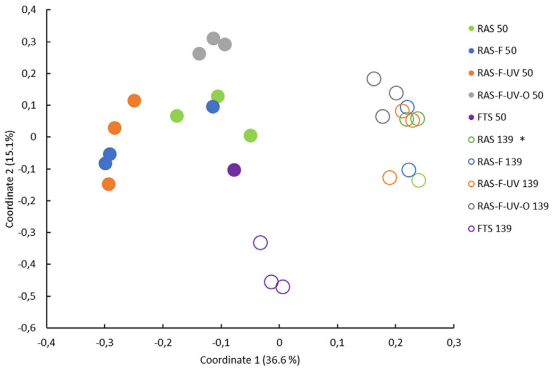


**Fig. 13.** Principal coordinates (PCoA) plot based on Bray–Curtis similarities for tank wall biofilm microbiota at day 50 (filled symbols) and day 139 (open symbols). * = RAS tanks were converted to RAS-F from day 69.

RAS treatments, on day 50 (Fig. 14A), while the RAS treatments were more similar at day 139 (Fig. 14AB).

## 4. Discussion

To the best of our knowledge this is the first study to examine the effects of RAS on growth, health, survival and microbial water quality in lumpfish rearing. In addition, it is the first study to compare the effects of different water treatment for individual tanks in the same RAS.

### 4.1. Chemical water quality

All systems had acceptable chemical water quality during the experiment, which show that the RAS was well dimensioned. However, the oxygen saturation was low in the beginning of the experiment, especially for the RAS treatment (RAS). Juvenile lumpfish is highly sensitive to reduced oxygen saturations and negative effects in terms of growth are already evident for lumpfish reared at 81% oxygen saturation (Jørgensen et al., 2017). The low oxygen saturation could therefore be the reason for the lower wet weight of fish from the RAS treatment after the first period, compared to the other RAS treatments.

### 4.2. Fish performance

The fish in the RAS treatments showed a significantly higher survival for two of the periods of the experiment, compared to the fish from the FTS. These results are in accordance with previous studies with marine fish larvae, where RAS resulted in higher survival compared to FTS (Attramadal et al., 2012a, 2012b, 2014, 2016), and support the hypothesis that lumpfish juveniles reared in RAS will show a higher survival compared to siblings reared in FTS. For the two periods with higher survival, the RAS treatments, increased survival with 19% in average compared to the FTS. This effect size would constitute a high number of fish in commercial scale, where a high density of fish can be utilized with success (Espmark et al., 2019). In general, the survival was high for all treatments in the experiment (average 76.0–99.9%), including the FTS (76.0–98.0%). Comparably, commercial production of lumpfish in Norway has a lower survival through a production cycle in FTS (Commercial producers of lumpfish in Norway, pers. comm., 2019). The higher survival of fish in FTS in this experiment can be related to the production period. The experiment started two months post hatch, at which point the initial mortality has passed and the fish may be more robust than in the early stages.

Gill health is an important indicator of fish health and welfare in relation to the farming conditions (Marshall and Bellamy, 2010). The extensive interaction between surrounding water and the thin, delicate respiratory epithelium of the gill lamellae during branchial respiration makes the gill tissue an optimal indicator on interaction between the fish and the environment (Mallat, 1985; Strzyzewska et al., 2016). Furthermore, the gills are taking care of processes like gas exchange, acid-base regulation, excretion of nitrogenous waste, ion- and osmoregulation and hormone metabolism as well as being an important immunological tissue (Evans et al., 2005). Thus, optimal function of the

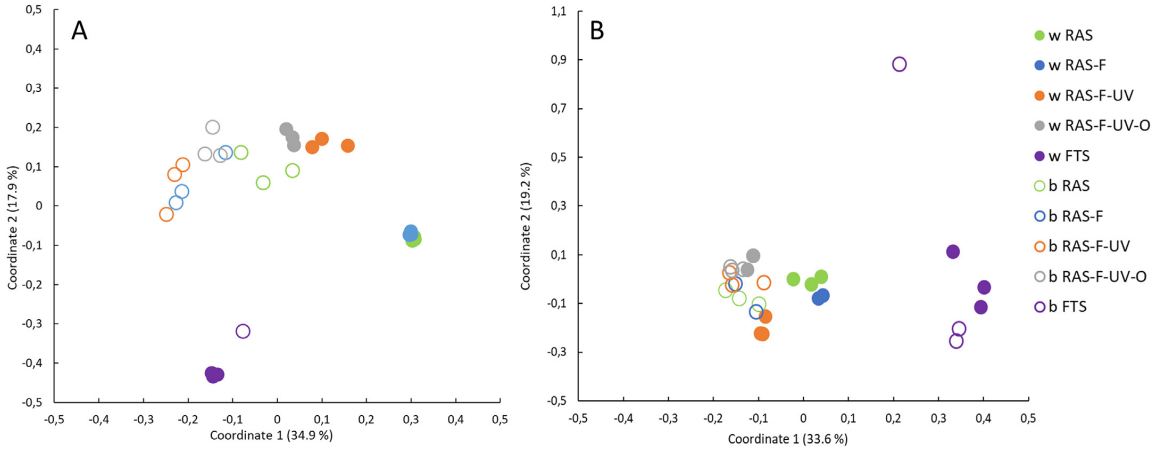


**Fig. 14.** Principal coordinates (PCoA) plot based on Bray–Curtis similarities for rearing water and tank wall biofilm microbiota from the systems at 50 days (A) and 139 days (B) in the water treatments. Filled symbols = rearing water, open symbols = tank wall biofilm. RAS tanks were converted to RAS-F from day 69.

gill is of outermost importance for fish health and -performance. The fish from the RAS treatments without disinfection (RAS and RAS-F) had a better gill health than those from FTS and the RAS treatments with disinfection (RAS-F-UV and RAS-F-UV-O). The fish of the RAS-F showed the best gill health in this experiment. This implies that the extra mechanical filtration of the incoming tank water of RAS positively affected the lumpfish.

The fish grew better in the RAS treatments than in the FTS, due to the significantly higher temperature, as shown for the Thermal-unit growth coefficient (TGC), which attempts to express growth independent of the temperature (Thorarensen and Farrell, 2011). TGC for all the treatments were rather similar during the experiment. Even though the differences are caused by temperature, this is not entirely irrelevant for system choice, since RAS is a method for maintaining a stable and optimal temperature year around, whereas FTS depends more on the sea temperature, which will vary trough the seasons. At winter, with drop in seawater temperature below 8 °C, *Moritella viscosa* thrives and is a significant problem causing winter ulcer (Einarsdottir et al., 2018; Producers of lumpfish, Norway, pers. comm., 2019). By selecting RAS, the low water temperature during winter can be avoided, and hence possibly the risk of negative interactions with *Moritella viscosa*.

The analysis of fish performance in this experiment indicates that there is a potential to increase both survival, growth and gill health by producing lumpfish in RAS, and that RAS with filtration of small particles, but no disinfection in the RAS treatment loop, seemed to result in the best fish health and performance.

### 4.3. Microbiota

#### 4.3.1. Effect of water treatment on the water microbiota

Even though the different RAS treatments were connected to the same RAS loop for the entire experiment the microbial community composition of both water and biofilm developed differently due to different treatment of the incoming tank water. These differences were clearly expressed in the rearing tanks with an HRT of only 60 min, where all treatments differed except RAS and RAS-F, at both sampling days. The extra mechanical filtration of the incoming tank water in RAS-F had possibly little influence of the rearing water microbiota or the total concentration of the bacteria. At day 69 the RAS and the RAS-F were merged to RAS-F, and hence the similar water microbiota at day 139 were expected. Since the RAS treatment was changed to RAS-F after 69 days of the experiment, we must note that differences in gill health could have been more pronounced if the different treatment of the incoming water to tanks had been continued during the whole experiment.

Disinfection had a significant influence on the bacterial community composition in this experiment. It has been shown that both UV and ozone change the microbial composition in rearing water and biofilm (Wietz et al., 2009; Interdonato, 2012). Our results indicate that both the UV and the combined UV and ozone treatment changed the microbial community structures. The most abundant family in water was *Thiotrichaceae*, with the highest abundance in the RAS-F-UV and RAS-F-UV-O treatment (21–53%). The *Thiotrichaceae* was represented by three OTUs, all classified as *Leucothrix*. The disinfection apparently selected for the *Leucothrix*. These bacteria can cause fouling of respiratory surfaces or cause internal or systemic bacterial infection in shellfish (Johnston et al., 1971). *Leucothrix mucor* has become a problem in aquaculture (Broch, 2006), especially in the cultivation of lobster at the juvenile stages (Nilson et al., 1975; Dale and Blom, 1987). Since fish in RAS-F-UV and RAS-F-UV-O also had the highest gill score among the RAS treatments, i.e. the most challenged gill health, it might be a correlation between the presence of *Leucothrix* and the poorer gill health. The rearing water in RAS and RAS-F had low abundances of *Thiotrichaceae*, and better gill health. The FTS rearing water had low abundances of *Leucothrix*, but still had the highest gill score. However, FTS was dominated by *Flavobacteriaceae* on day 50. *Flavobacteriaceae* includes important fish pathogens such as *Flavobacterium psychrophilum*, *Flavobacterium columnare* and *Tenacibaculum maritimum. The* samples from FTS contained 18 different OTUs representing *Flavobacterium*. FTS also contained high abundancies of *Mycoplasma* and *Moritella* at the genus level, which were rare in the RAS treatments. Both these genera include pathogenic species (Gudmundsdottir et al., 2006; Suhanova et al., 2011). *Moritella viscosa* has caused several incidents of mortality in the rearing of lumpfish, causing winter ulcers (Gudmundsdottir et al., 2006; Einarsdottir et al., 2018), both in hatcheries and sea cages. *Moritella* was identified in high abundance (< 82%) by Roalkvam et al. (2019) in a normal production of lumpfish in FTS. *Rhodobacteraceae* was abundant in the RAS treatments without disinfection and were increasing from day 50 to 139, with *Loktanella* as the main genus. The RAS treatments with disinfection had a very low abundance of *Loktanella*, and it was rare in the FTS. *Loktanella* include bacterial groups with potential probiotic activity (Makridis et al., 2005; Califano et al., 2017), which can have beneficial effects on fish health (Hjelm et al., 2004; Nayak, 2010). The disinfection of the water going to the RAS-F-UV, RAS-F-UV-O and FTS rearing tanks may have selected against this

potential beneficial bacterial taxon. It must be emphasized that the results from our study of a typical system for marine juvenile production are not directly transferrable to systems for other species, e.g. salmonids, where the HRT of the fish tanks is shorter (Gregersen et al., 2020). With a short HRT (15–20 min) in the fish tanks, disinfection in the RAS loop may keep the level of planktonic bacteria low in the tank water despite high loading of organic matter because the bacteria do not have the time to grow during the short time the water is in the fish tanks (Bakke et al., 2017).

RAS and RAS-F had a significantly more diverse and less variable microbial community composition compared to the other treatments at both sampling days, which might indicate a more mature and K-selected community in the RAS treatments without disinfection, as predicted. This was supported by the higher Bray-Curtis similarities for the RAS and RAS-F for comparisons both between replicate tanks and sampling times, indicating that the microbial community composition in the RAS and the RAS-F were more similar to each other and more stable over time. As hypothesized, RAS without disinfection seemed to promote K-selection.

As expected, the RAS treatments had significantly higher abundancy of total bacteria in the tank water than the FTS at both sampling points, probably due to a higher accumulation of particles in the rearing water, being a substrate for the bacteria in the system. This was measured by both flow cytometry and colony forming units (CFU). RAS had on average $5 \times 10^6$ cells $mL^{-1}$ in the rearing water while FTS had $9 \times 10^4$ cells $mL^{-1}$, which is in accordance with previous studies with marine larvae in RAS (Attramadal et al., 2012a, 2012b; Attramadal et al., 2014; Wold et al., 2014). In accordance to the hypotheses, the RAS treatments without disinfection had a lower fraction of opportunistic bacteria compared with the RAS treatments with disinfection and the FTS. In addition, the RAS treatments showed a lower cultivability of the bacteria in the rearing water compared to the other treatments, at both sampling days.

#### 4.3.2. Effect of water treatment on the biofilm microbiota

Lumpfish in aquaculture live in close contact with the biofilm on the tank walls, as they spend much of the time attached with the ventral suction disc to the tank wall and other surfaces (Hvas et al., 2018). Biofilm can represent a reservoir for opportunistic bacterial pathogens and hence the composition can be important for fish health (Wietz et al., 2009). Both the RAS treatments and the FTS had a relatively higher abundance of potential pathogens in the water compared to the biofilms. In biofilm, possible pathogenic and problematic bacteria were identified at highest abundance in the biofilm of the RAS treatments with disinfection, with 19% abundance of *Moritella* from RAS-F-UV and 33% abundance of *Leucothrix* in RAS-F-UV-O. Biofilm microbiota seemed to be less affected by the water treatments, compared to the water microbiota, as the biofilm community varied less between the RAS treatments and especially over time, than the water microbiota. This was expected, since the composition of the layered biofilm is protected against intrusion, like disinfection (Blancheton et al., 2013), and the biofilm is especially protected with surface growth over time (Wietz et al., 2009). In biofilms high competition and K-selection may generally be expected, but frequent cleaning or perturbations may open for more r-selecting conditions.

## 5. Conclusion

The lumpfish were exposed to different microbial communities of both water and biofilm, due to different treatments of the incoming tank water. Overall, the results support the hypotheses proposed for the experiment. First, lumpfish reared in the RAS treatments were exposed to a more stable microbial community, with a lower share of opportunistic bacteria, which is a probable reason for the higher survival and better gill health of the fish compared to siblings reared in the FTS. Secondly, RAS without disinfection (RAS and RAS-F) had a significantly more diverse and more stable microbial community composition compared to the tanks receiving disinfected RAS water and the FTS. In addition, these treatments had less opportunistic and potential harmful bacteria, which resulted in a better gill health of the fish compared to siblings reared in the RAS with disinfection and FTS. Thirdly, the fish in RAS-F had a better gill health than the fish in the RAS, which was operated without filtration the first 69 days, probably due to the positive effects of reduced particle load. Altogether, our results indicate that there is a potential to increase both survival, growth and gill health by producing lumpfish in RAS, and that RAS with filtration of small particles, but no disinfection, seem to result in the best fish health and performance. By selecting RAS, the industry can improve and increase the production to meet the growing demands from the salmon farming industry. The possibility that the earlier stages of lumpfish would benefit even more of being produced in RAS, from hatching and until delivery to sea cages, should be investigated further.

## Ethics statement

The experiments were conducted at a commercial producer of lumpfish, which are not under the act of animal ethic legislation in Norway. Therefore, no ethical committee was required. Sampling of fish for gill health were anesthetized as described in the manuscript.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgement

This study was funded from RFF Nord (Project number 269204). Partners in the project were SINTEF Ocean, Let Sea AS and Ecomarine Seafarm AS. We would like to thank staff at Let Sea AS and Ecomarine Seafarm AS for practical work during the experiments, Roman Netzer and Deni Ribicic (SINTEF Ocean) for coordination of sequencing.

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ISBN 978-82-326-6772-7 (printed ver.)
ISBN 978-82-326-5529-8 (electronic ver.)
ISSN 1503-8181 (printed ver.)
ISSN 2703-8084 (online ver.)





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