Beyond water quality: Micro particles in Recirculation Aquaculture Systems

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Publication date:
2020

Document Version
Publisher's PDF, also known as Version of record

Beyond water quality:
Micro particles in Recirculation Aquaculture Systems

Ph. D. Thesis

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Hirtshals, Denmark
2017-2020

Preface

This thesis is submitted in partial fulfilment of the requirements for obtaining a Doctor of Philosophy (*Ph.D.*) degree. The work presented within was conducted during my enrolment as a *Ph.D.* student at the Section for Aquaculture, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark, in Hirtshals, Denmark, between 2017 and 2020.

The main supervisor of this thesis was senior researcher Anne Johanne Tang Dalsgaard and the thesis was co-supervised by senior researcher Lars-Flemming Pedersen and Head of section Per Bovbjerg Pedersen.

This thesis focuses on micro particles in recirculating aquaculture systems (RAS), spanning from their origins to their removal. It is composed of a synopsis, two published papers and two manuscripts:

Paper I de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Dalsgaard, J., 2019. Micro particles and microbial activity in Danish recirculating rainbow trout (*Oncorhynchus mykiss*) farms. Aquac. Eng. 84, 60–66. doi:10.1016/j.aquaeng.2018.12.001

Paper II de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Liu, D., Dalsgaard, J., 2020. UV irradiation and micro filtration effects on micro particle development and microbial water quality in recirculation aquaculture systems. Aquaculture 518, 734785. doi:10.1016/j.aquaculture.2019.734785

Paper III de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Dalsgaard, J., 2020. Effects of storage time and temperature on microbial activity and micro particle determination in recirculating aquaculture systems water samples. *Manuscript*

Paper IV de Jesus Gregersen, K.J., Syropoulou, E., Pedersen, P.B., Pedersen, L.F., Dalsgaard, J., 2020. Foam fractionation and ozonation in fresh water recirculating aquaculture systems. *Manuscript*

Acknowledgments

To say the last few years were a rollercoaster of emotions is an understatement. Some of the most brilliant moments came thanks to all the people present in this journey and it's thanks to all of you that this journey turned out to be such a blast.

The completion of this PhD would not have been possible without the participation and support from a lot of different people. And while I may not mention all of you, you have all been incredibly important in this journey.

First and foremost a heartfelt thank you to my main supervisor, Anne Johanne. You have been a source of inspiration and support over the last 3 and a half years. This PhD would have not been possible without your guidance, support and encouragement, plus your brilliant organizational skills balancing out my ability to disorganize everything.

To Per Bovbjerg Pedersen, thank you for your support and guidance, but also for the disagreements on data interpretation or scientific planning. I believe the discussions on what size should particles be considered bacteria or whether oxygen is explosive have truly "forced" me to continue learning and forced me to reach for new "heights".

To Lars-Flemming (as Peter would put it, my in-house psychologist), thank you for endless brilliant discussions (scientific or not) and all the guidance, for all the support even before the PhD and for always taking your time to answer my millions questions. Thank you also for being one of the kindest and most thoughtful persons I have ever met.

Every PhD students gets some help from the technicians at some point, but it's fair to say no one has ever gotten so much help as I did. It's also fair to say that this PhD would not have been possible without all your help. Throughout this journey you have been a source of knowledge, laughs and inspiration.

Ole, Rasmus and Jens, thank you, not only for all the help taking care of the fish and systems, but also for listening to me rant endlessly about the wrong valves and the walls in the wrong place. You have no idea how much that helped during tough stretches of this PhD. Thank you for brainstorming and help coming with ingenious solutions for problems that should not exist.

To all the lab technicians, Brian (see, I put your name first), Ulla, Melissa, Carina, Linda and Thomas, thank you so much. Thank you for all your help in sampling and innovative ideas, and thank you for your efforts to find new solutions for new problems. All of this really improved this PhD, but also my life experience over the last 4 years. Without your help with all the samplings, this PhD would have never been possible (even running samples during a pandemic that brought the world to a standstill).

To Bodil, Sanni, Carlos, Peter, Manuel, Ivar, Dibo, Alfred, Helge, Manon and Freja. Thank you for being there for this journey, and for making the Section for Aquaculture such a brilliant place to work in. Thank you for help with samplings, discussion of results and innovative ideas. Thank you for the endless laughs, football games, BBQ and other activities that make life in Hirtshals that much sweeter.

Thank you Mathis, for the help with the synopsis, but mostly for the brilliant warm welcome when I first started here and for making me feel welcomed. Thank you for the fun moments in the office and at the house with the little one. You guys are always welcome in our home!

To Paula, Mia, Anita and Camille, while we have not spent nearly as much time together as I would have liked over the last 4 years, much of whom I am as come from our brilliant discussions, specially over some Pizzas at the Tratoria del Porto. Thank you!

To Renata and Tilo, thank you for making the office such a fun and enjoyable place. Thank you for long fun discussions and brilliant BBQ in the Hirtshals sun. Thank you for putting a smile in the little one's face every time he sees you. P.S. Sorry about the mess.

Maninha, we may only see each other once a year, and now that you have joined the "emigra" club, we will see how often we can meet. However, you have always been there as true inspiration. Thank you and good luck in this new adventure.

To Monsieur Barreau. There have been few people over the years that have had such an impact in my life as you have. I'm glad you were there, from that morning 8 years ago when you made me miss the boss, all the way to this day. Thank you for all the "driving" calls and endless discussions about pumps, engineering companies and bosses. Never change. P.S. I'm writing this while talking on the phone with you.

Primo, much of the last 8 years have been possible thanks to you and your willingness to take in Shelby and give her the best live until the end. Thank you so much.

To my Danish family, Rikke, Freja, Karen and Jens. You received us with open arms when we needed it most and have been ever present in our journey over the years. Thank you for all your support and for being there at all times.

Mãezinha, only you will know what you went through to afford me the life opportunities I have had. While we may be physically far, you are always present in my life and heart. Obrigado.

To the love of my life, you truly have been the rock holding everything together. The last few years haven't been the easiest. However they also brought us the best thing in our lives. Thank you for being there all of those years and for pushing us to new places and new challenges, for pushing me out of my comfort zone. All of this would not have been possible without that crazy idea to open a company all those years ago. Thank you for all your love. Kukitas, you are able to turn the worst day around with a simple smile and a hug. Your joy and happiness have made me happier than ever before and have been a source of strength every single day. Always continue to be the joyful, playful, happy and healthy boy you have been. Amo-vos muito ☺

To all of you and everyone else involved in this trip,
THANK YOU.

Kim João de Jesus Gregersen

List of abbreviations

Symbol	Description
A/V	Surface area to volume ratio
BOD_5	Biochemical oxygen demand after 5 days
COD	Chemical oxygen demand
DOM	Dissolved organic matter
FBB	Fixed bed biofilter
FF	Foam fractionators
HRT	Hydraulic retention time
MBB	Moving bed biofilter
MTF	Model trout farm
MUW	Make-up water
NTU	Nephelometric turbidity units
O_3	Ozone
OM	Organic matter
POM	Particulate organic matter
PSD	Particle size distribution
PS	Pilot scale
RAS	Recirculating aquaculture system
TAN	Total ammonia nitrogen
TSS	Total suspended solids
UV	Ultraviolet
WWTP	Waste water treatment plant

Abstract

Micro particles accumulate in recirculating aquaculture systems (RAS) and with the fast expansion of the RAS industry in recent years, more focus has been put on understanding the impacts of micro particles on fish and systems alike.

Micro particles are partly responsible for bacterial activity within systems due to their high surface area to volume ratio. Their small size challenges their removal, and technologies that can reduce micro particles in RAS are still lacking.

The overall aim of this PhD was to resolve the implications of micro particles on RAS operation including water quality and fish performance, and to acquire methods and technologies for controlling micro particle development in RAS.

This thesis is accompanied by four scientific manuscripts as well as unpublished data collected over the last three and a half years.

The first manuscript (**Paper I**) examines the distribution of micro particles and bacterial activity in seven Danish model trout farms (MTF). Twenty separate RAS units were sampled over a short period and water samples accessed for micro particles and bacterial activity. The results revealed large variations in micro particle loads across, as well as within, farms suggesting that system specific conditions predominate. A strong correlation ($r = 0.92$) between micro particle surface area and bacterial activity furthermore supported the hypothesis that particle surface area is important in controlling bacterial activity in lower intensity RAS.

The second manuscript (**Paper II**) assesses the potential of ultraviolet radiation (UV) and micro filtration (1 µm) for controlling micro particle levels in rainbow trout RAS. At the same time, an indirect assessment of the amount of micro particles composed by microorganisms was carried out by examining the reduction caused by UV treatment alone. A two-by-two factorial trial was conducted over a 13 week period in 12 replicated pilot scale RAS. The results showed that both micro filtration and UV had large impacts on the micro particles present in the systems, with large reductions in both numbers and volume. Micro filtration resulted in a significant reduction of particle volume (89%) and a significant reduction in particle numbers and bacterial activity (50 and 54%, respectively). Ultraviolet radiation, on the other end, lead to significant reductions in particle numbers (74%) and bacterial activity (89%). The combination of both methods reduced the presence of micro particles by approximately 88% (all metrics) and reduced bacterial activity by 95%. It was also estimated that at least 64% of all particles by numbers and 30% by volume were composed of living microorganisms.

The third manuscript (**Paper III**) tests the effects of sample storage (temperature and duration) on micro particle and bacterial activity analysis. This was done by storing samples from two different RAS at room temperature and at 4^{o}C over a total of 72 hours, and tracking changes in particle numbers, volume and surface area, as well as bacterial activity. In addition, some samples were store at -20^{o}C and subjected to similar analysis following de-frosting.

Results showed different dynamics in samples from either system, with large increases in bacterial activity and micro particles within the first 3 hours in samples stored at 4°C from one of the systems. In comparison, micro particle numbers and bacterial activity remained stable for the first 6 hours in samples obtained from the other system before starting to decline. In both cases, the results suggested that changes in micro particle numbers were bacterial driven. The results also showed that samples for bacterial activity and micro particle assessment should not be frozen.

The last manuscript (**Paper IV**) focuses on the effects of foam fractionation and ozonation in freshwater, rainbow trout RAS on the control of micro particles, bacterial activity and other water and biofilter quality parameters, with focus on the build-up of organic matter. A two-by-two factorial trial was conducted in 12 replicated pilot scale systems over 8 weeks to determine the individual and combined effects of both treatments. The results showed large reductions for the individual treatments and the highest removals for the combined treatment. Ozonation by itself reduced the number of particles by more than 83% and bacterial activity by 48%. Foam fractionation, on the other end, resulted in 54% less particles in numbers and 62% less particle volume, while it reduced bacterial activity by more than 54%.

The combination of both treatments resulted in approximately 90% reduction of particle numbers and bacterial activity, as well as 75% removal of organic matter (BOD_5). The results obtained supported previous findings on ozone's effect in RAS. Furthermore, the results showed that foam fractionation has similar removal efficiency as that typically found in saltwater, suggesting that foam fractionation could become a tool for controlling organic matter build-up in RAS, especially when combined with ozone.

The changes in physicochemical water quality parameters deriving from the treatments in **Papers II** and **IV** did not show any effect on the fish, sustaining that rainbow trout have a high degree of tolerance to micro particles.

In conclusion, the results of this thesis corroborate that rainbow trout is highly tolerant to high levels of micro particles, while the control of micro particles through different methods leads to significant improvements in different physicochemical water quality parameters in RAS.

Furthermore, micro particles in RAS are intrinsically connected to bacteria. In low intensity systems, surface area provided by micro particles seem to partly control the amount of bacterial activity in the system, while in higher intensity systems most micro particle dynamics appears to be the result of changes in bacterial populations. The main driver behind the large fluctuations in micro particles seems to be organic matter build-up.

These results highlight new possibilities for controlling micro particles in RAS, including disinfection. Together with reports from the industry, the results of **Papers II** and **IV** maintain that disinfection is an efficient way of controlling micro particles. However, while disinfection can be used to control micro particles in RAS, it does not deal with the underlying cause of bacteria, which is organic matter. As seen in **Papers II** and **IV**, control of organic matter in RAS will not only control the level of micro particles, but will also control the build-up of organic matter which is the direct cause of micro particles.

Dansk Resumé

Der sker typisk en akkumulering af mikropartikler i recirkulerede akvakulturanlæg (RAS), og betydningen af dette for selve produktionen er kommet i fokus i takt med, at industrien vokser.

Mikropartikler er kendetegnet ved, at de har en stor overflade i forhold til deres volumen, og denne overflade menes at danne grobund for bakterier. Partiklernes ringe størrelse gør, at de er svære at fjerne, og der mangler viden på området om praktisk anvendelige teknologier til formålet.

Formålet med dette Ph.d. studie var at afdække mikropartiklernes indvirkning på vandkvaliteten og fiskenes vækst i RAS, samt at tilvejebringe metoder og teknologier til at kontrollere forekomsten af mikropartikler i RAS.

Afhandlingen er en opsummering af publicerede og ikke publicerede data oparbejdet i løbet af de sidste 3½ år og inkluderer fire videnskabelige manuskripter.

Det første manuskript (**Paper I**) afdækker forekomsten af mikropartikler på danske modeldambrug samt den bakterielle aktivitet i anlæggene. Der blev over en kort periode indsamlet vandprøver fra 20 forskellige RAS enheder fordelt på syv modeldambrug, og prøverne blev analyseret for indholdet af mikropartikler og bakteriel aktivitet. Resultaterne viste, at der var en stor variation i mængden af mikropartikler både mellem modeldambrug og mellem de enkelte RAS enheder, hvilket understøtter, at forekomsten af mikropartikler er underlagt systemspecifikke forhold. En stærk korrelation ($r = 0.92$) mellem mikropartiklernes overfladeareal og den bakterielle aktivitet i vandet understøttede desuden hypotesen om, at mikropartiklernes overflade danner grobund for bakterievækst i mindre intensive RAS.

Det andet manuskript (**Paper II**) undersøger, i hvilket omfang ultraviolet stråling (UV) og mikrofiltrering (1 μm) kan kontrollere forekomsten af mikropartikler i RAS til opdræt af regnbueørreder. Desuden undersøger det hvorvidt mikropartiker i sig selv består af bakterier. Et 2^2-faktorforsøg med 3 gentagelser pr. faktorkombination blev udført i 12 ens pilotskala RAS over en periode på 13 uger. Både mikrofiltrering og UV behandling havde stor indvirkning på forekomsten af mikropartikler inklusiv en reduktion i både antal og volumen. Mikrofiltrering førte således til et signifikant fald i partikelvolumen (89%), partikelantal (50%), og bakteriel aktivitet (54%). Til sammenligning førte UV stråling til en signifikant reduktion i antallet af partikler (74%) og bakteriel aktivitet (89%). Kombinationen af de to metoder reducerede forekomsten af mikropartikler med ca. 88% samt den bakterielle aktivitet med 95%. Desuden viste studiet, at levende mikroorganismer udgjorde mindst 64% af partikerne i forhold til antal og 30% i forhold til volumen.

Det tredje manuskript (**Paper III**) undersøger indvirkningen af prøveopbevaring (temperatur og varighed) på analysen af mikropartikler og bakteriel aktivitet. Vandprøver fra to forskellige RAS blev opbevaret ved hhv. rumtemperatur og 4°C, og ændringer i partikelantal, volumen, og overfladeareal samt ændringer i bakteriel aktivitet blev fulgt i 72 timer. I tillæg blev andre prøver opbevaret ved -20°C og analyseret for mikropartikler og bakterieaktivitet efter optøning. Der var stor forskel i udviklingen i prøverne fra de to RAS. For det ene system skete der en stor stigning i bakterieaktivitet og i antallet af mikropartikler i løbet af de første 3 timer. Til sammenligning forblev antallet af mikropartikler og bakterieaktiviteten i prøverne fra det andet system stabil i de første 6 timer, hvorefter de begyndte at

falde. For begge systemer understøttede resultaterne, at ændringerne i mikropartikler er styret af bakterier. Resultaterne viste desuden, at vandprøver ikke må fryses forud for analyser af mikropartikler og bakteriel aktivitet.

Det sidste manuskript (**Paper IV**) undersøger i hvilket omfang proteinskimmere og ozonering kan kontrollere forekomsten af mikropartikler, bakterieaktivitet, samt ophobningen af organisk stof i vandfasen og i biofiltere i RAS til opdræt af regnbueørreder i ferskvand. Et 2^2-faktorforsøg blev gennemført i 12 ens pilotskala RAS over en periode på 8 uger, og effekterne af de to teknologier, anvendt hver især eller i kombination, blev bestemt. Ozonering reducerede antallet af partikler med mere end 83% og bakterieaktiviteten med 48%. Til sammenligning reducerede proteinskimmere antallet af partikler med 54%, partikelvolumenet med 62%, og bakterieaktiviteten med mere end 54%. Kombinationen af de to teknologier førte til et fald på ca. 90% i antallet af partiker og bakteriel aktivitet samt en 75% fjernelse af organisk stof (BI_5). Resultaterne understøtter tidligere undersøgelser af ozons effekt i RAS. Desuden viser de, at proteinskimmere er lige så effektive i ferskvand som det observeret i saltvand. Samlet set peger resultaterne på, at proteinskimmere - især i kombination med ozon - fremadrettet vil kunne bruges som en effektiv metode til at kontrollere indholdet af organisk stof i RAS til ferskvandsopdræt.

De resulterende fysisk-kemiske ændringer i vandkvaliteten målt i **Paper II** og **IV** havde ingen påviselig indvirkning på fiskene, hvilket understøtter, at regnbueørreder generelt har en høj tolerance i forhold til mikropartikler i vandet.

Samlet set understøtter afhandlingen, at det vha. forskellige, kendte teknologier er muligt at kontrollere forekomsten af mikropartikler i RAS og som følger deraf forbedre den fysisk-kemiske vandkvalitet signifikant. Desuden understøtter afhandlingen, at regnbueørreder har en høj tolerancetærskel i forhold til indholdet af mikropartikler i vandet.

Afhandlingen understøtter desuden, at mikropartikler og bakterier langt hen ad vejen er to sider af samme sag. Mens overfladen af mikropartikler formentlig er afgørende for bakteriedynamikken i mindre intensive RAS, er det tilsyneladende forekomsten af bakterier, der styrer dynamikken af mikropartikler i mere intensive RAS. I begge tilfælde er indholdet af organisk stof formentlig afgørende for forekomsten af mikropartikler.

I overensstemmelse med observationer i industrien, understøtter **Paper II** og **IV**, at det er muligt at kontrollere forekomsten af mikropartiker i RAS bl.a. ved hjælp af desinfektion. Desinfektion fjerner imidlertid ikke organisk stof, der er det egentlig problem i forhold til bakterievækst. Som vist i **Paper II** og **IV** vil en fjernelse af organisk stof i RAS således ikke kun reducere forekomsten af mikropartikler, men vil også fjerne selve årsagen til, at de forekommer.

Contents

1. **Introduction and Background**

Tighter environmental regulations and a need to improve fish production have led to the development of recirculation aquaculture systems (RAS). As the name implies, in RAS water is recirculated through the production loop multiple times before being discharged. RAS provide several advantages compared to traditional flow-through systems and open net sea cages, including stable productions conditions year-round, increased biosecurity, increased control over escapes and a better conditions for water treatment, resulting in lower discharge to the environment and lower water usage per kilo of fish produced (Badiola *et al.*, 2012; Dalsgaard *et al.*, 2013; Timmons and Ebeling, 2010; Verdegem *et al.*, 2006). However, it also comes with its set of new challenges.

Reduced water usage and prolonged retention time, while undoubtedly good for the environment, have inherent problems for RAS. In flow through systems, the build-up of unwanted substances is controlled by permanent water exchange. However RAS cannot rely on water exchange alone to control the build-up of substances. In order to circumvent this problem RAS are equipped with difference types of treatments to remove unwanted substances (fig. 1).

Organic matter (OM) build-up in RAS consists of both dissolved organic matter (DOM) and particulate organic matter (POM). Build-up of OM has mostly been dealt with by dilution (water exchange) and removal of POM. Removal of POM has mainly been achieved by removing particulate matter from RAS using settling and micro screen filtration. Both methods are efficient at removing large particles, resulting in effective removal of most of the particulate organic matter generated in the system (Davidson and Summerfelt, 2005; Fernandes et al., 2015).

However, as our ability to remove large particles became better, focus was put on the fractions not addressed by standard filtration technics: micro particles, here defined as particles below 100 µm.

Micro particle build-up in RAS has been known since the 90's (Cripps, 1995; Patterson *et al.*, 1999). A lack of direct addressing, coupled to a decrease in water exchange, leads to a large build-up of micro particles in RAS.

While the knowledge that micro particles are present in large numbers in RAS has been availed for a long time, their origins, implications and ways to address them has been less clear.

Previous studies have shown that several factors result in the breakage of large particles in to smaller ones (e.g. Brinker and Rösch, 2005), while internal components of RAS have been show to either trap or create micro particles (Fernandes *et al.*, 2016). Implications of micro particles for fish welfare have also long been discussed, with their small size and high surface area to volume ratio considered a potential problem for fish welfare (Bash *et al.*, 2001; Bilotta and Brazier, 2008). However, clear evidence of negative impacts of RAS generated micro particles is still lacking. In recent years, the connection between micro particles and bacterial activity has also gained attention (Pedersen et al., 2017a).

Finally, methods and equipment to deal with the build-up of micro particles are still lacking. Most filtration methods typically applied to aquaculture are either unable to deal with the smallest fractions of micro particles (e.g. drum filters) or have lacked scientific validation (e.g. foam fractionators). This typically results in systems dominated by particles below 20 µm (Chen *et al.*, 1993; Fernandes *et al.*, 2014).

With an increasing number of new RAS facilities being built, together with increased focus on higher intensity of recirculation, the risk of high levels of micro particles and potential negative impacts is high. Therefore, knowledge on the origins of micro particles, their implications and potential ways to remove them becomes essential.

2. Aim and Objectives

The main aim of this PhD was to further develop our understanding of micro particles in RAS, focusing on:

1. The origins and levels of micro particles in RAS facilities.
2. Possible implications of micro particles on RAS and on the fish within RAS.
3. Ways to control the level of micro particles in RAS.

To achieve this, four trials were carried out:

Trial 1. The levels of micro particles were measured across 20 different commercial RAS in 7 different farms across Denmark, in order to determine: 1) the actual levels present in commercial systems and 2) analyse the variation in numbers of micro particles both across farms and within the same farm. Correlations between micro particles and other water quality parameters were also studied. **Paper I**

Trial 2. Effects of using UV irradiation and micro filtration on micro particles and bacterial activity were assessed in 12 replicated pilot scale RAS. Besides assessing the potential for controlling micro particles with these technologies, an attempt was made to evaluate the proportion of micro particles that is composed of bacteria. Implications on other water quality parameters and fish heath were also included in the trial. **Paper II**

Trial 3. The implication of sample storage on micro particle analysis was tested. Samples from two RAS were collected and stored for up to 72 hours at different temperature, including freezing. Subsequently the samples were analysed for micro particles and bacterial activity. **Paper III**

Trial 4. The objective of trial 4 was to access the impact of foam fractionators and ozone (alone and combined) in the build-up of micro particles in fresh water RAS. The trial was conducted in 12 replicated pilot scale RAS. Multiple water quality and biofilter parameters were assessed to determine the implications on a system level (as opposed to only testing the water). Fish welfare was also analysed to determine potential impacts. **Paper IV**

3. RAS

RAS can be classified based on its feed loading, which is the amount of feed used per m^3 of water used (make-up water, MUW), as well as the degree of recirculation determined as the degree of water reuse in the system. According to Martins *et al.* (2010), systems can broadly be split in to four categories based on feed loading: flow through (<0.02 kg feed / m^3 MUW) , re-use (0.02 to 1 kg feed / m^3 MUW), conventional RAS (1 to 10 kg feed / m^3 MUW) and next generation RAS (>10 kg feed / m^3 MUW).

The higher the level of recirculation or feed loading, the smaller is the amount of water used in the production (Colt, 2006; Pedersen *et al.*, 2012).

In order to keep good water quality with increased levels of recirculation, multiple systems are employed to treat the water, and with increased levels of recirculation, more technology needs to be applied (fig. 1).

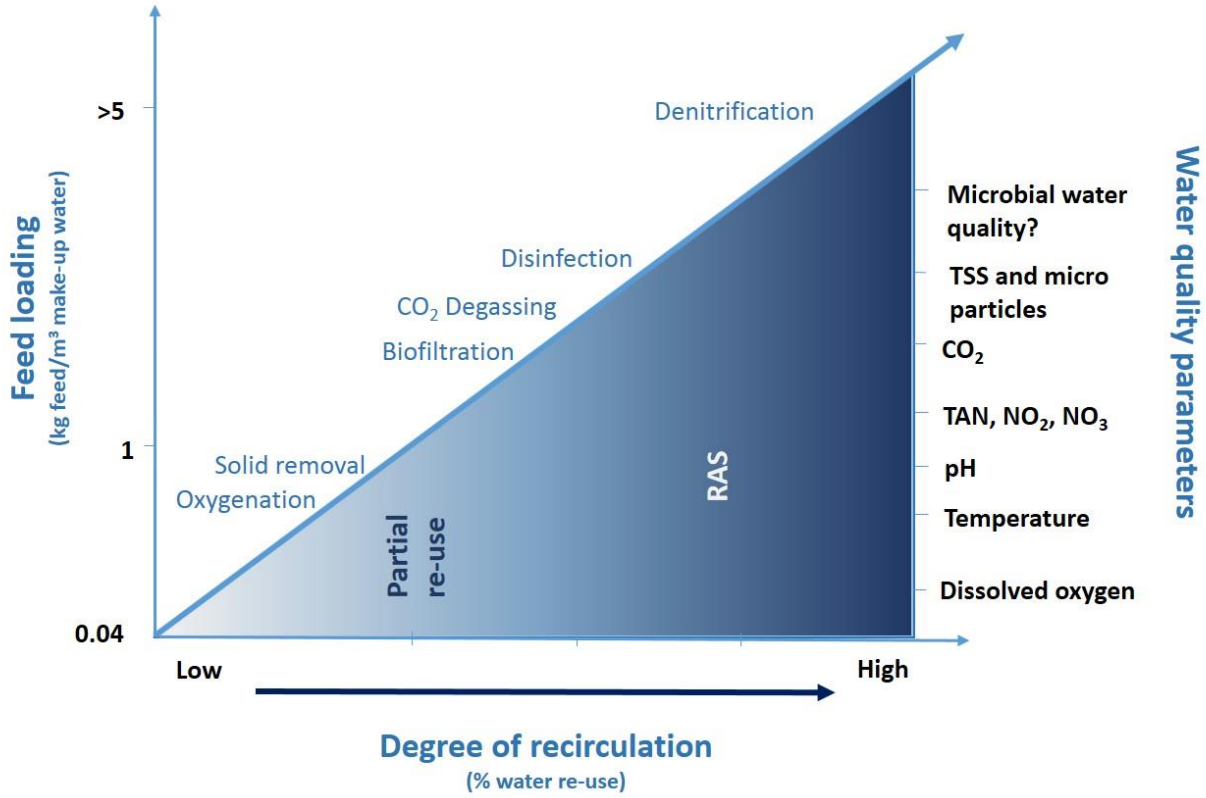

Figure 1. Degree of recirculation vs requirement for treatment units (reproduced from Rojas-Tirado *et al.*, 2018, modified from Fernandes 2015)

RAS facilities are generally composed by a few core components (fig. 2), including rearing tanks, primary solids removal, biofilters and oxygenation. Other components like degassing, disinfection or fine solids removal may also be applied. Combined, these systems keep water quality controlled, allowing for its reuse multiple times and increasing RAS overall retention time.

3.1. Organic build-up in RAS

Build-up of compounds in aquaculture, whether they are particulate or dissolved, is controlled by the amount added in to the system and the amount exported out of the system. The input side is relatively straight forward, as it is mostly a function of feed applied (Dalsgaard and Pedersen, 2011), although, as will

be discussed later, changes in this parameter can have large implications on water quality. The exportation of nutrients, on the other end, is more complex as it involves all actions in which nutrients are removed from the system. These can be, for example, primary solids removal (e.g. settlers and drum filters), micro filtration, harvesting of biomass, water exchange and backwash of biofilters.

This leads to one of the challenges of RAS: in a flow through facility or partially recirculation aquaculture, the build-up of organic and inorganic substances is kept under control by dilution in new water and exported out of the system with water changes. In high intensity RAS facilities, due to the low water exchange, this is not a possibility. This can lead to the build-up of large amounts of organic matter (both dissolved and particulate) in RAS, which will have a direct impact in the systems carrying capacity (Vadstein *et al.*, 1993).

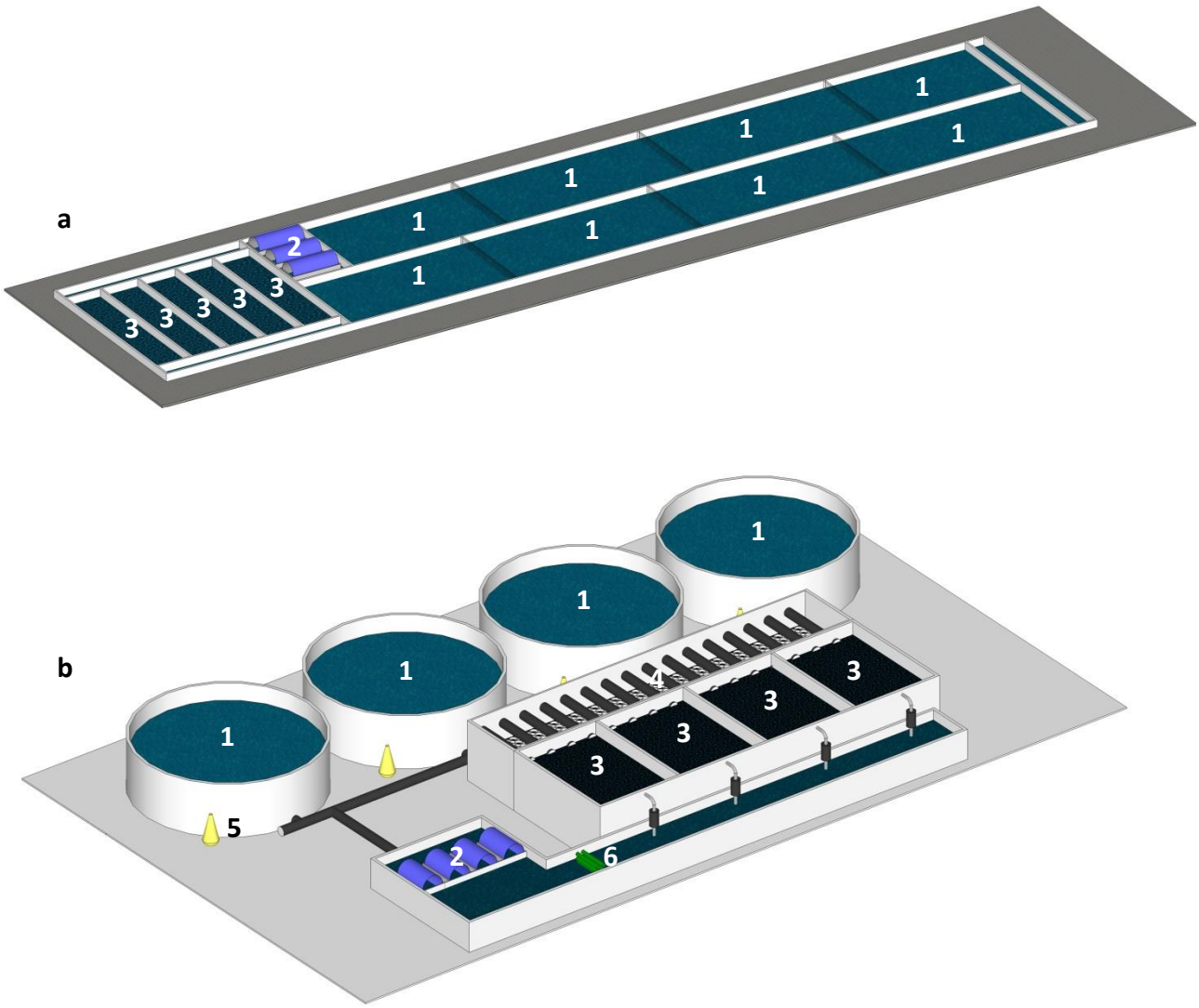

Figure 2. Typical RAS facilities in Denmark. a) Model trout farm b) Indoor RAS facility. 1 – Fish tanks; 2 – drum filters; 3 – biofilters; 4 – degassing; 5 – Oxygen cones; 6 – Ultraviolet disinfection

3.2. Carrying capacity

Central to the discussion about micro particles in RAS is the concept of system carrying capacity. Carrying capacity is generally defined as the maximum stable biomass that a system can sustain. As mentioned before, the longer retention times in RAS can lead to an increase in organic matter and thereby in the systems carrying capacity. The concept of carrying capacity was first introduced in aquaculture by Vadstein *et al.* (1993). The authors propose that RAS as a way to improve survivability of marine larvae by maintaining stable microbial communities at carrying capacity. This in turn would have a positive effect on larval development and survival, while at the same time keeping unwanted bacteria away by non-harmful bacteria occupying all available substrate. Carrying capacity is mainly affected by the input of organic matter (in both dissolved and particulate form) and the exportation of nutrients (Vadstein *et al.*, 1993).

Throughout this thesis, the concept of carrying capacity, as well as its implications on micro particles will be further discussed.

3.3. Micro particles

Perhaps the closest and most accepted definition of micro particles is given by Timmons and Ebeling (2010). Here, the authors divide particles in dissolved (<0.001 µm), colloidal (0.001 to 1 µm), super-colloidal ("fines" 1 to 100 µm) and settleable solids (>100 µm) (fig. 3). Generally, "fines" (1 to 100 µm) are the particles considered as micro particles and the fraction which is most problematic since it does not settle easily.

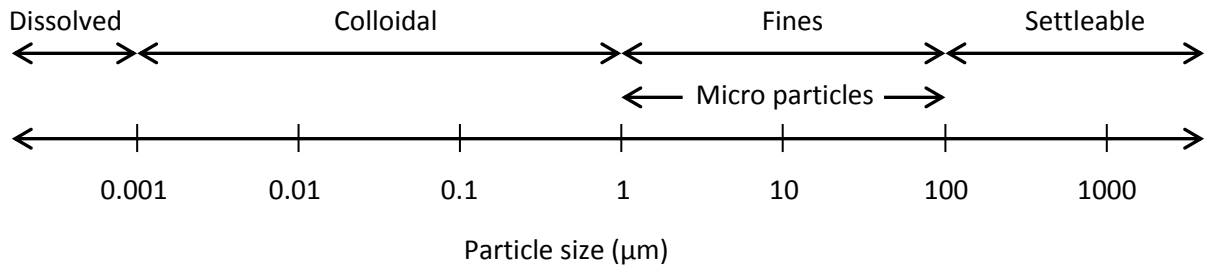

Figure 3. Solid size distribution in aquaculture waters. (Adapted from Timmons and Ebeling, 2010)

Due to their very small size, micro particles are typically not removed by normal filtration methods applied to RAS and therefore build-up, sometimes to very large amounts.

Micro particles in RAS have been studied since the 90's and early 2000's (Cripps, 1995; Patterson *et al.*, 2003, 1999; Patterson and Watts, 2003a, 2003b).

Recently micro particles has received increased attention from the industry and scientific communities as micro particles are assumed to have negative impacts on fish, both directly and indirectly (Colt, 2006; Martins *et al.*, 2010). Furthermore, recent research has shown a connection between micro particles and bacteria in RAS (Pedersen *et al.*, 2017, **Paper I**).

The lower water exchange rates used in modern RAS facilities is thought to exacerbate the build-up of micro particles in RAS, in the same way as NO_3^- build-up.

However, despite a large focus on micro particles in recent years, some large gaps remain in our knowledge about micro particles. Differences in equipment used and the different methods applied to measure particulate matter and specifically micro particles in RAS result in large variations in the published

scientific data, most of the times making comparisons and analysing trends in aquaculture systems difficult, as will be further discussed later in this thesis.

Furthermore, the very nature of micro particles makes them hard to work with, even from a sampling point of view. During the trials discussed in **Paper III**, the storage of RAS water for a period as short as 3 hours and kept in a fridge resulted in an increase of more than 35% in the number of micro particles (fig. 4).

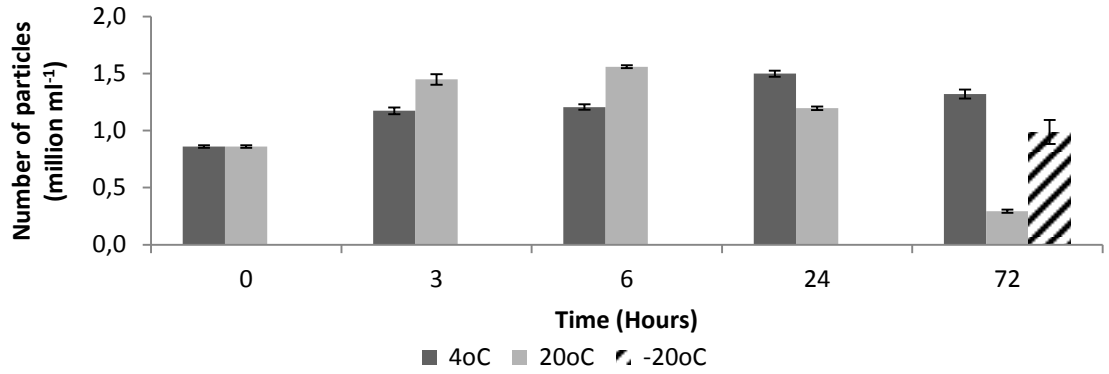

Figure 4. Changes in the number of micro particles during a 72 hour storage trial. Samples were kept at room temperature, at 4°C and frozen, and tested at different time intervals. The results showed large increases in the amounts of micro particles present in the systems, even when stored at low temperatures (Adapted from Paper III).

Such large changes in the numbers of particles in such a short period make analysing particles and their interactions even more difficult.

While both the research community and industry are aware of the build-up of micro particles in RAS, their origins, implications and ways to address this build-up are less clear and clear gaps remain in our knowledge regarding micro particles.

4. Micro particles in RAS

4.1. Particle size distribution

The first large description of the particle size distribution (PSD) in RAS was done by Patterson *et al.* (1999). Their work was based on the findings by Sheldon and Parsons (1967) and Kavanaugh *et al.* (1980) in other fields, and showed that the PSD in RAS has a near hyperbolic distribution. The near hyperbolic nature allows for the transformation of data into the β value that describes the distribution of particles in a system. In order to calculate the β value, particle data is reorganized in geometrical progressive size classes in such a way that the ratio between the difference between maximum and minimum size class boundaries (Δli) and the average diameter of the size class (Li*) is always equal (Table 1).

Table 1. Particle size class boundaries (l_i and l_{i+1}), difference between maximum and minimum size class boundaries (Δl_i), average diameter of the size class (L_{i*}) and ratio between Δ_{li} and l_{i*} (adapted from Patterson et al., 1999).

Class	l_i	l_{i+1}	Δl_i	L_{i*}	$\Delta li/li^*$
1	1	1.26	0.26	1.13	0.23
2	1.26	1.59	0.33	1.425	0.23
3	1.59	2.00	0.41	1.795	0.23
4	2.00	2.52	0.52	2.26	0.23
5	2.52	3.18	0.66	2.85	0.23
6	3.18	4.00	0.82	3.59	0.23
7	4.00	5.04	1.04	4.52	0.23
8	5.04	6.34	1.30	5.69	0.23
9	6.34	8.00	1.66	7.17	0.23
10	8.00	10.10	2.10	9.05	0.23
11	10.10	12.70	2.60	11.4	0.23
12	12.70	16.00	3.30	14.35	0.23
13	16.00	20.20	4.20	18.1	0.23
14	20.20	25.40	5.20	22.8	0.23
15	25.40	32.00	6.60	28.7	0.23
16	32.00	40.30	8.30	36.15	0.23
17	40.30	50.80	10.50	45.55	0.23
18	50.80	64.00	13.20	57.4	0.23
19	64.00	80.60	16.60	72.3	0.23
20	80.60	102.00	21.40	91.3	0.23
21	102.00	128.00	26.00	115	0.23

Based on this distribution, the β value can be calculated as $log\left(\frac{\Delta N}{\Delta l}\right) = log\ A - \beta * log\ l$, where N is the number of particles, l is the particle size, A is an empirical constant and β is the slope.

A low β value (around 2) represents a system dominated by large particles, where most volume and surface area are provided by the large particles, whilst a high β value (around 4) represents a system dominated by small particles (Patterson *et al.*, 1999) (fig. 5).

This work has been fundamental for our understanding of PSD in RAS showing a build-up of micro particles in RAS, with particles below 20 µm accounting for the majority of particles. Another parameter used to describe the PSD in RAS is the Surface Area to Volume ratio (A/V) (Fernandes et al., 2015). While scientifically less elaborated, the A/V ratio is intuitively easier to understand as it describes the relationship between surface area and volume of particles present in a system. Typically, a system dominated by large particles will have a smaller A/V ratio, while a system dominated by small particles will have a lager A/V ratio.

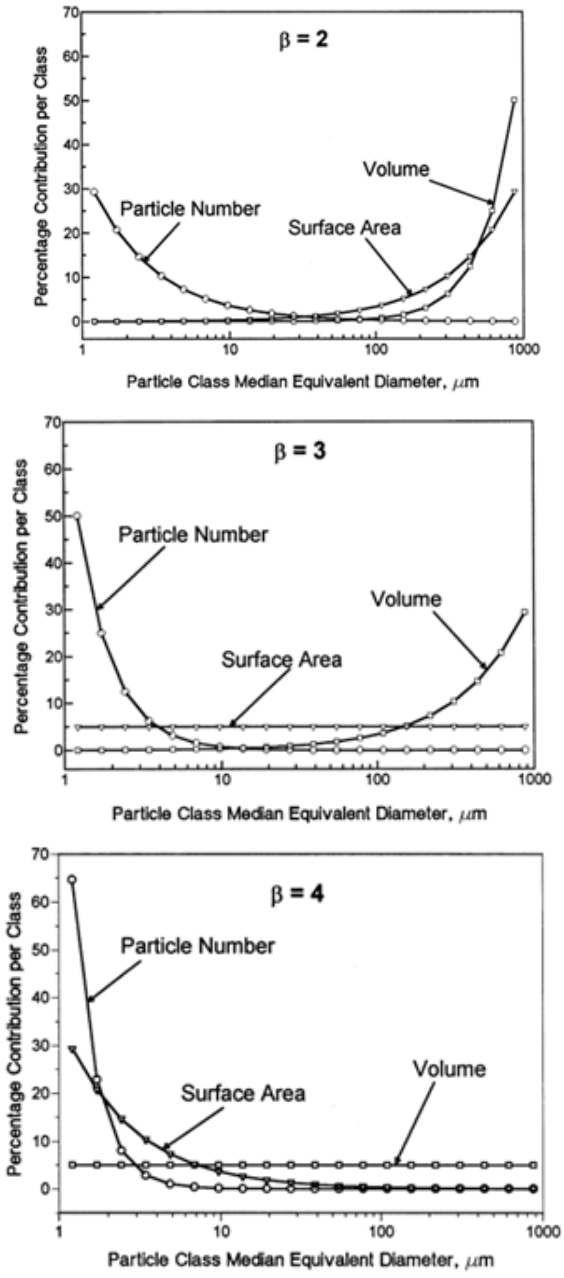

Figure 5. Hypothetical particle contribution for the different metrics (number, volume and surface area) depending on β value (reproduced from Patterson *et al.,* 1999).

Besides describing the PSD in a system, both the β value and A/V ratio are useful for determining if a treatment has had an equal impact on all particles, or if it has affected different size particles differently. To illustrate: a series of batch trials were conducted during the PhD applying foam fractionators with or without ozone, using water from different commercial and pilot scale RAS, in 400l tanks filled with 200l of water. The results indicated that foam fractionators preferential removed the larger particles, resulting in an increase in both β value (from 4.1 to 4.4 approximately) and A/V ratio (from 1.5 to 1.9). The practical implication of this was a larger removal of particle volume and almost no change in particle numbers (fig. 6).

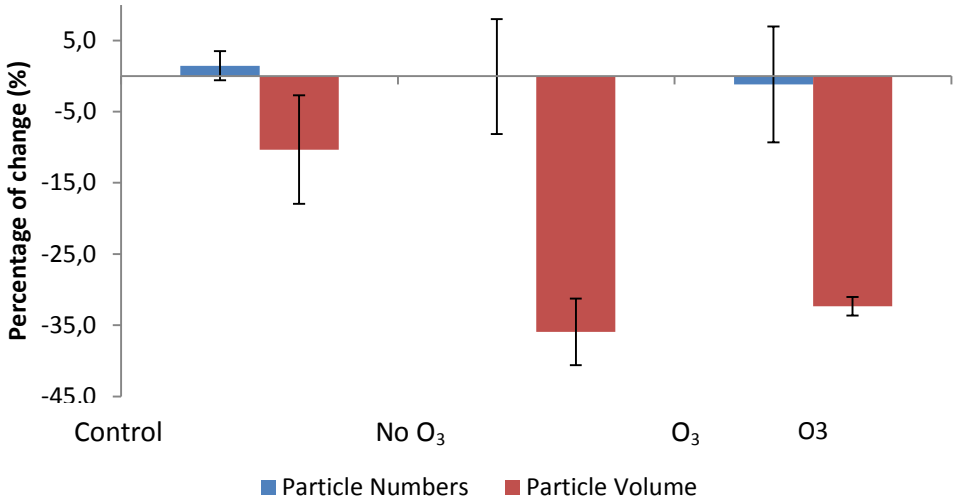

Figure 6. Changes (mean ± SD, n= 10) in the amount of micro particles (numbers and volume) in a trial with fresh water foam fractionators with or without ozone (O_3). Positive numbers indicate an increase, while negative numbers represent a removal (own unpublished data, 2019).

While PSD is important for the understanding of particulate matter dynamics within RAS, it is not enough by itself to draw conclusions on the amount of particles in a system and the potential effects of a treatment. Both the β value and the A/V ratio are relative metrics, comparing the relative abundance of each size of particles within a sample. As a conceptual example, two experimental treatments for removing particles are used in batch trials. Treatment 1 results in the removal of large particles, while treatment 2 results in the breakage of large particles in to smaller particles. Both these treatments will result in a relatively larger share of smaller particles, resulting in a higher final β value and A/V ratio. However, the practical implications are very different, since one treatment results in the removal of particles from the water, while the other results in the breakage of particles, potentially making them even more difficult to remove. For this reason it is important to report the actual amount of particles in the water.

Particles can be reported as numbers, volume and surface area, and in many scientific articles only one (or none) of these measurements is provided. This lack of information is particularly problematic in regards to numbers vs volume. Due to scaling, a 10 µm particle has the same volume of 1000 1 µm particles, while a 100 µm particle has a volume equal to 1,000,000 1 µm particles. Likewise, the effects of particle breakage can be easily seen in the surface area. For example, if a 50 µm spherical particle is broken into 8 equal spherical pieces, the volume is the same as the volume of the original particle, while the surface area doubles (Fig. 7).

Figure 7. Conceptual representation of the breakage of large particles into smaller particles. A large particle that breaks down in to 8 equal sized particles retains the same volume, but its surface area doubles.

4.2. Methods for determining particulate matter and micro particles

Reporting of micro particles is neither simple nor uniform across the scientific literature.

One of the most common ways to report the amount of solids in RAS is as total suspended solids (TSS) (Bilotta and Brazier, 2008; Schumann and Brinker, 2020). TSS expresses the mass of solids per volume of water (normally mg l^{-1}) and is obtained by filtering a water sample through a pre-weight filter (Standard DS/EN 872). While the results obtained are easily understandable (mass of particles), its use for micro particle analysis is limited. Particle volume scales up to the power of 3 with the increase in size, which means that the weight of a large particle constitutes a relatively large share of the total mass, compared to the weight of a small particle.

In order to analyse results relating to micro particles only, as defined in this dissertation, all particles above 100 µm would have to be filtered out. This would in all likelihood result in very low TSS values affecting reliability (Cripps, 1995).

While TSS is typically reported in scientific articles, very few measurements have been published on the different size fractions. One of such few studies was carried out by Cripps (1995) who analysed the distribution of TSS across different particles sizes by sequential filtration. The analysis was conducted on the effluent of a salmonid hatchery and showed that out of a total of 6.9 mg/l TSS, approximately 4.5 mg/l were composed of particles smaller than 100 µm.

TSS values from multiple aquaculture systems were compiled by Schumann and Brinker (2020), showing values of TSS below 20 mg l^{-1} across different types of production unites (fig. 8).

Turbidity is another indirect method for roughly assessing the amount of particles in the water. Like TSS this measure does not distinguish between particle sizes. Sensors like the Solitax sc line (Hach, USA) provide both a measurement of turbidity and TSS. However, importantly, both measurements read different parameters and are influenced by different factors. Turbidity meters measure reflected light of particles in the water column. This means that changes in the absorption of light in the water, e.g. due to the presence of high amounts of dissolved substances, may affect the turbidity measurements if not corrected for.

While correlations between turbidity and TSS have been found in other fields (Pavanelli and Bigi, 2005), correlations would have to be made for specific water matrixes and adjusted regularly to be of value.

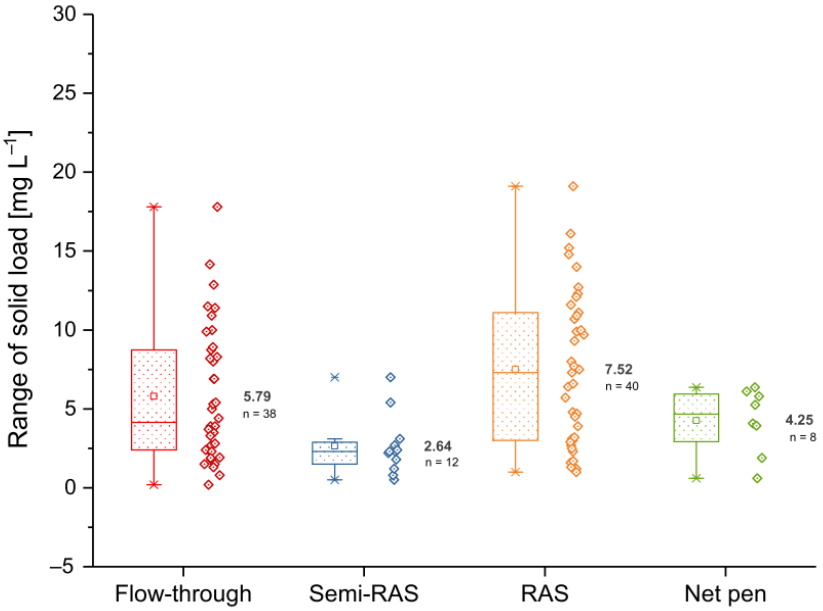

Figure 8. Range of TSS values in different salmonid aquaculture systems (reproduced from Schumann and Brinker, 2020).

A correlation coefficient (r) of 0.94 was found between the volume of micro particles and the turbidity levels in the trials described in Paper IV (fig. 9).

Perhaps the largest advantage of turbidity measurement is its simplicity and the fact that it is commercially available as an online monitoring tool that may be used to track changes in water quality in real time.

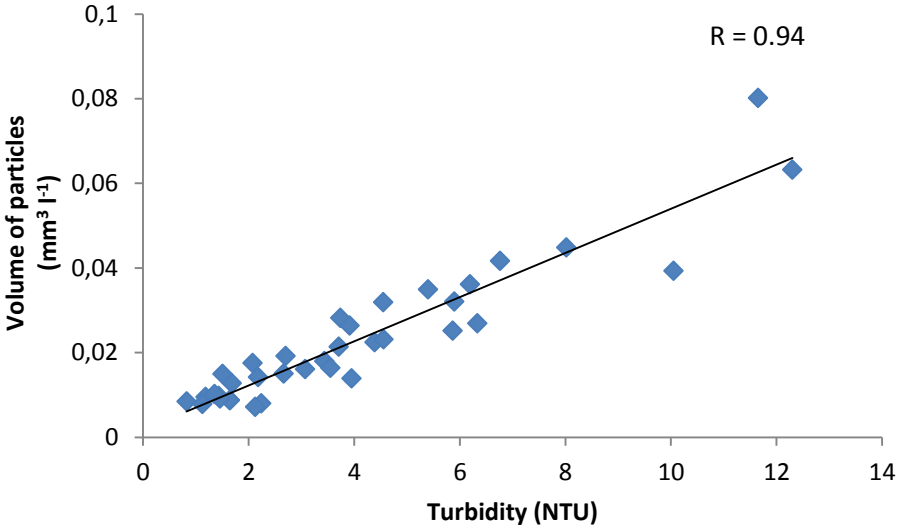

Figure 9. Correlation between turbidity (Nephelometric Turbidity Units, NTU) and particle volume of data collected during the trials described in Paper IV (adapted from Paper IV).

Many other methods can be used to estimate particulate matter, including biochemical oxygen demand after 5 days (BOD_5) and chemical oxygen demand (COD) (Dalsgaard and Pedersen, 2011; Marquet *et al.*, 2007; Summerfelt *et al.*, 1997; von Ahnen *et al.*, 2016).

In order to estimate particulate BOD_5 and COD, both total (unfiltered) samples and dissolved (filtered, typically through a 0.45 µm filter) samples are analysed (for both BOD_5 and COD). The particulate fractions are derived as the difference between total and dissolved BOD_5 and COD. Like TSS, these analyses do not differentiate between large and small particles.

Another problem with all methods mentioned is the discrepancy between size thresholds applied. While micro particles are considered to be particles between 1 and 100 um, most metrics used to analyse particulate matter use filtration sizes that do not match this size range. For example, dissolved COD and BOD_5 are derived using a 0,45 µm filter. On the other hand, TSS can apply 1.5 µm filters (Standard Methods 2540D). While the difference might seem small, PSD analyses of multiple data sets collected during this PhD have shown that the fraction between 1 and 1.6 µm can account for over 10% of particles by volume. Unfortunately, there is no ideal solution to this issue. While we could choose to use 1 µm filters in all samplings, this would mean parting with standards and would make it difficult to compare current and past literature.

The most common method to measure and analyse micro particles is trough particle measurement equipment (Brinker et al., 2005c; Fernandes et al., 2016, 2014; Patterson et al., 1999; Patterson and Watts, 2003a). The main advantage of this type of equipment is that it not only provides the numbers, volume and surface area of particles, it also measures the PSD, allowing for a proper assessment of the micro particle levels.

Three types of machines are commonly applied in aquaculture samples:

- Single Particle Optical Sensing - Light obscuration
- Laser diffraction analysis
- Volumetric disturbance - coulter principle

More recently, Becke *et al.* (2020) used a particle size and shape analyser to determine the shape of particles within aquaculture facilities. Shape analysers capture images of the particles going through the machine and use specialized software to determine the shape of the particles and respective dimensions. This technic has the obvious advantage of generating a “true” image of the particle, potentially allowing for a most accurate calculation of particle surface area.

Utilizing this technic, Becke *et al.* (2020) measured particles in 6 different aquaculture systems (two flow troughs, two semi RAS and two RAS). The results clearly pointed out that the assumption that particles are spherical is not entirely correct, but that they are more shaped as an ellipse. This can have a large impact when calculating different metrics, especially the surface area. A sphere is the shape with the smallest possible surface area given a certain volume, and if particles are assumed spherical, it is safe to say that we are always underestimating the true surface area. In the study mentioned above, using data from a system with high levels of long and narrow shaped algae (and therefore the least sphericity), the authors found that particle volume was 1.4 times higher if particles were assumed elliptical vs spherical, while the surface area was 5.8 times higher.

A shortcoming of this technic is that currently can only analyse a narrow range of particle sizes, which can exclude important fractions of micro particles, and it still relies on a two dimensional image to estimate the size of a three dimensional object.

5. Level of micro particles

Knowledge on the impact of micro particles in RAS is limited, and knowing which parameter (numbers, volume and surface area) is more important and which should be reported is challenging.

Amongst the few articles comparing multiple systems, Patterson *et al.* (1999) compiled a data set of multiple systems from multiple sources. In general, most systems analysed by the authors had a β value above 3.8 (table 2), indicating that the systems were dominated by smaller particles.

However, most of the data sets were from experimental systems, while some were measured in flow thought systems or using unknown apertures. While these data sets have been important to advance our understanding of particle distributions within RAS, the comparison between systems is still challenging. In the article by Patterson *et al.* (1999) only the size distribution is mentioned, while total numbers are not reported.

Table 2. β values obtained by Patterson *et al.* (1999) from different data sets from multiple aquaculture systems (adapted from Patterson *et al.,* 1999).

β	R	Comment	Source
2.94	0.994	Atlantic Salmon: flow-through system	Cripps
4.24	0.996	Trout: recirc.; tank 1, section 1	Timmons
4.64	0.997	Trout: recirc.; tank 1, section 3	
5.53	0.996	Trout: recirc.; tank 1, section 3; 21 days after set 2	
4.39	0.997	Trout: recirc.; tank 3, section 3	
4.31	0.998	Striped Bass: recirc.; tank 1, grab sample @ 11:00	Singh
3.52	0.998	Striped Bass: recirc.; tank 1, grab sample @ 15:00	
3.84	0.998	Striped Bass: recirc.; tank 2, grab sample 2 h after feeding	
3.94	0.993	Striped Bass: recirc.; tank 2, inflow to filter from central standpipe	
4.47	0.995	Striped Bass: recirc.; tank 2, outflow from settling basin	
3.86	0.996	Striped Bass: recirc.; tank 3: grab sample @ 15:00	
3.86	0.995	Striped Bass: recirc.; tank 4: grab sample 3 h after feeding	
3.16	0.975	Trout: recirc.; screens, foam fractionator in tank	Chen
6.29	0.998	Trout: recirc.; clarifier w/RBC	
3.04	0.977	Trout: recirc.; settling zone, sand and biofilter, O3	

The results obtained during this PhD supports the findings that RAS are generally dominated by smaller particles. This was the case for most of the 20 systems analysed in **Paper I** (fig. 10, tab. 3), with only one system having a β value below 3.5 and 16 systems having β values above 3.7.

The results revealed large variations in the particle numbers across farms, but also within the same farm. Samples were collected in systems with similar level of recirculation and feeding intensity, with the results suggesting that system specific conditions were responsible for much of the variation observed.

The numbers presented in the report were from particles between 2 and 200 µm. The full set of data is presented in table 3, which also contains the data for all commercial facilities sampled during the course of the PhD and some of the experimental set ups. The results obtained illustrate the wide spread of particles in systems, as well as the variations in β values obtained.

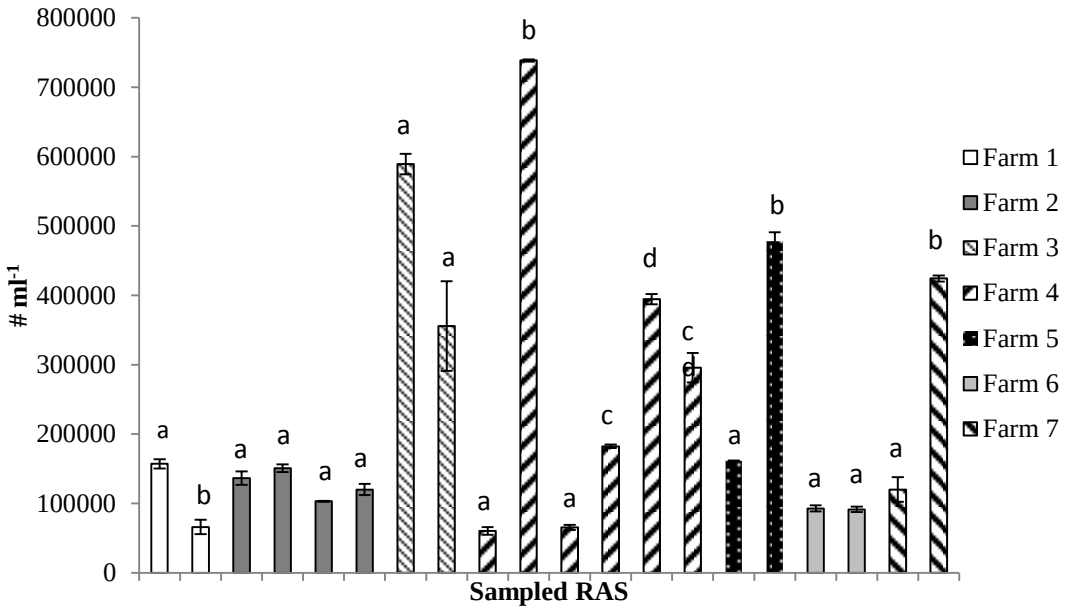

Figure 10. Micro particle numbers across 20 different RAS facilities (reproduced from Paper I).

Additionally to the 20 systems present in **Paper I**, four commercial systems and 26 pilot scale systems were sampled during this PhD (table 3) and here only the pilot scale systems treated with ozone (**Paper IV**) had β values below 3.7.

Table 3. Micro particle numbers, volume, surface area, β value and H2O2 degradation rate measured in multiple systems (commercial and research) during this PhD.

Type of farm	Number (million ml^{-1})	Volume (mm^3 ml^{-1})	Surface area (mm^2 ml^{-1})	B value	H2O2 (k^{-1})	Species	Additional treatment
PS	3.34	0.026	35.0	4.16	0.667	R. trout	
MTF	3.03	0.027	33.8	4.30	0.889	R. trout	
PS	2.58	0.010	14.9	3.88	0.240	R. trout	1 µm filter
PS	2.57	0.025	22.7	3.70	0.573	R. trout	
MTF	2.50	0.014	21.5	4.04	0.406	R. trout	
PS	2.33	0.012	22.5	3.69	0.169	R. trout	
PS	2.31	0.058	37.2	3.88	0.262	R. trout	
MTF	2.28	0.023	28.3	3.95	0.738	R. trout	
MTF	2.24	0.017	19.7	3.92	0.308	R. trout	
PS	2.23	0.063	44.8	3.90	0.392	R. trout	1 µm filter
PS	2.07	0.063	34.8	3.62	0.838	R. trout	
PS	1.91	0.032	26.8	3.77	0.527	R. trout	
MTF	1.84	0.014	13.0	3.79	0.238	R. trout	
MTF	1.82	0.022	20.5	3.86	1.410	R. trout	
PS	1.68	0.016	20.9	4.15	0.604	R. trout	Skimmer

MTF	1.67	0.008	12.8	4.15	0.080	R. trout	
PS	1.54	0.047	31.2	3.83	0.099	R. trout	UV
MTF	1.48	0.016	22.01	4.07		R. trout	
PS	1.42	0.045	20.5	3.80	0.405	R. trout	
MTF	1.25	0.014	14.9	3.98	0.590	R. trout	
MTF	1.21	0.015	15.8	3.89	0.557	R. trout	
MTF	1.15	0.006	10.5	3.46	0.312	R. trout	
Fresh water RAS	1.12	0.004	7.8	4.48		Pike perch	Inline Denitrification
PS	1.11	0.045	29.4	3.69	0.102	R. trout	UV
PS	1.10	0.014	12.6	3.70	0.413	R. trout	Skimmer
MTF	1.07	0.007	8.1	3.98	0.032	R. trout	
PS	1.05	0.042	24.8	3.67	0.075	R. trout	UV
MTF	1.05	0.010	10.7	4.16	0.226	R. trout	
MTF	0.92	0.006	8.2	4.11	0.079	R. trout	
MTF	0.91	0.013	11.8	3.85	0.597	R. trout	
Salt water RAS	0.86	0.005	7.9	3.99	0.112	Atlantic Salmon	Protein skimmer and ozone
PS	0.85	0.004	5.7	3.87	0.124	R. trout	1 µm filter
MTF	0.81	0.010	7.1	3.62	0.035	R. trout	
MTF	0.77	0.013	8.5	3.73	0.228	R. trout	
MTF	0.70	0.006	6.8	4.01	0.089	R. trout	
PS	0.67	0.018	12.1	3.65	0.203	R. trout	Skimmer
PS	0.62	0.027	12.5	3.35	1.051	R. trout	Ozone
MTF	0.52	0.006	4.9	3.74	0.149	R. trout	
MTF	0.51	0.008	4.9	3.65	0.092	R. trout	
PS	0.44	0.011	6.1	3.73	0.015	R. trout	UV and 1 µm filter
PS	0.41	0.016	7.2	3.38	0.213	R. trout	UV and 1 µm filter
PS	0.41	0.006	4.5	3.56	0.028	R. trout	Ozone
PS	0.39	0.008	6.0	3.56	0.138	R. trout	Skimmer and ozone
MTF	0.37	0.006	4.6	3.53	0.090	R. trout	
PS	0.27	0.005	3.4	3.90	0.026	R. trout	UV and 1 µm filter
PS	0.22	0.035	8.8	3.00	0.413	R. trout	Ozone
PS	0.16	0.007	3.6	3.21	0.105	R. trout	Skimmer and ozone
PS	0.15	0.009	3.2	3.19	0.098	R. trout	Skimmer and ozone

MTF – Model trout farm; PS – pilot scale RAS; R. trout – rainbow trout.

Interestingly, the type of system (pilot scale with long retention time, MTF or indoor RAS facility) had little influence on the amount of micro particles and their size distribution (β value), as can be seen in table 3. The results obtained indicate that design, treatment units and operational conditions are more important for the level of micro particles and PSD. This coincides with the observations made by Schumann and Brinker (2020) regarding the levels of TSS present in RAS, which is discussed in the next chapter.

6. Origin of micro particles

In RAS, it is accepted that most particles are the result of the breakdown of faeces, feed spill and microbial growth (Schumann and Brinker, 2020). The extent to which each of this components affects micro particles is however unknown.

Oversimplifying the process, it can be said that beside the fish in RAS, the only common and large input of organic matter to a RAS is through the addition of feed for the fish. As such, the search for the origin of micro particles should start at this point.

6.1. Faeces

In the case of salmonids, the addition of 1 kg of feed results in the production of approximately 250 g of particulate matter in the form of faeces (Chen *et al.*, 1997; Timmons and Ebeling, 2010) or approximately 200 g kg^{-1} feed in particulate COD (Dalsgaard and Pedersen, 2011).

If we couple this to the fact that systems are built similarly, it should be possible to estimate the amount of micro particles produced in a specific farm.

However, the results of **paper I** suggest that despite our knowledge regarding faeces production by the fish, systems still end up with very different amounts of micro particles, suggesting that other factors are at play when it comes to the amount of micro particles in RAS.

Assuming that the amount of faeces produced by salmonids under normal commercial operating conditions is stable, and that the biggest contributor to solids is faeces (Reid *et al.*, 2009), it seems fair to assume that the main factor determining the amount of solids in RAS would be the amount of feed supplied. However, Schumann and Brinker (2020) found no correlation between stocking density and TSS when comparing multiple salmonid RAS (fig. 11), and factors such as type of feed used, filtration applied, general practices, hydraulics and uncontrolled sedimentation seem to influence the amount of solids in RAS to a greater extent than the amount of feed provided.

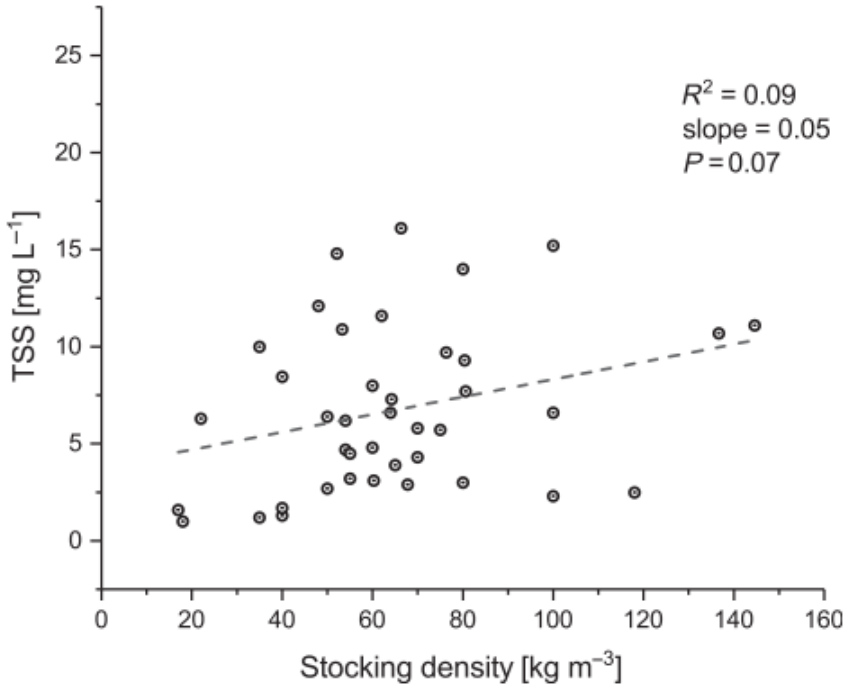

Figure 11. TSS load in function of stocking density collected from commercial, farm scale and experimental systems from multiple published studies (reproduced from Schumann and Brinker, 2020).

Faeces production is influenced by many different parameters, including species and age of fish (Clark *et al.*, 1985; Van Rijn, 2013), feed quality (Dalsgaard and Pedersen, 2011; Davidson *et al.*, 2013; Unger and Brinker, 2013) and water quality (Roque d'Orbcastel *et al.*, 2009).

As mentioned in table 4, there are many factors that affect the PSD in RAS.

Table 4. Factors affecting particles within RAS (adapted from Fernandes, 2013).

Factor	Mechanism	Effect on particles	System	Source
Time of day	Fish more active during the day	Size decreases during the day	FT RAS	Brinker and Rösch (2005) Patterson and Watts (2003)
Aeration rate	Destructive turbulence	Size decreases with increased aeration	RAS	Brambilla *et al.* (2008)
	Shear forces coupled to destructive turbulence	Size decreases after a propeller-wash bead filter	RAS	Pfeiffer *et al.* (2008)
		Size decreases and load increases with aeration rates in moving beds	WWTP WWTP WWTP WWTP WWTP RAS	Melin *et al.* (2005) Leiknes *et al.* (2006) Åhl *et al.* (2006) Ivanovic and Leiknes (2008) Ivanovic and Leiknes (2012) Fernandes *et al.* (2016)
Fish size	Constructive and suspending turbulence	Size increases with decreasing fish size	FT RAS RAS	Brinker and Rösch (2005) Franco-Nava *et al.* (2004) Merino *et al.* (2007)
Distance from raceway bottom	Differential sedimentation	Size decreases with increasing distance	FT	Brinker and Rösch (2005)
Waterfalls	Destructive fragmentation from the fall	Size decreases after a waterfall	FT	Brinker and Rösch (2005)
Tank cleaning	Biofilm scrapping from tank walls	Load increases shortly after routine cleaning	FT	Kelly *et al.* (1997)
Pumps	Centrifugal forces/ Collision with impellers	Size decreases within the pump	RAS FT RAS	McMillan *et al.* (2003) Sindilariu *et al.* (2009) Fernandes *et al.* (2014)
Biofilm	Biofilm detachment (shear/sloughing)	Load and size increase after "old" fixed bed biofilters	RAS RAS RAS RAS	Yang *et al.* (2001) Patterson and Watts (2003)

		Load and size increase after a fluidized sand filter	RAS	Franco-Nava *et al.* (2004b)
	Entrapment within the media	Fixed media decreases particle load	WWTP	Fernandes *et al.* (2014)
			WWTP	Pfeiffer *et al.* (2008)
			RAS	Bouwer (1987)
			RAS	Larsen and Harremoës (1994)
			RAS	Yang *et al.* (2001)
				Fernandes *et al.* (2016)
Quiescent zones	Differential sedimentation	Particle size decreases due to sedimentation	WWTP	Marquet *et al.* (1999)
			WWTP	Marquet *et al.* (2007)
			RAS	Merino *et al.* (2007)
			RAS	Fernandes *et al.* (2014)
Degassing units	Trickled fragmentation	Size decreases during the fall	RAS	Patterson and Watts (2003)
			RAS	Fernandes *et al.* (2014)
Ozone	Flocculation	Size increases after contact	RAS	Rueter and Johnson (1995)
			RAS	Krumins et al (2001a)
			RAS	Krumins *et al.* (2001b)
Flow rate	Turbulent vs. laminar flow	Turbulent flows disintegrate particles	WWTP	Maxey *et al.* (1996)
			RAS	Franco-Nava *et al.* (2004)
			WWTP	Khan *et al.* (2011)
Biofilter type	Fixed bed Vs moving bed	Aeration and mixing of MBB result in breakage of particles FBB result in trapping of particles	RAS	(Fernandes *et al.*, 2016)

While all of the above factors affect particles, their effects on micro particles is less clear, although the breakage of particles is likely to contribute to the increase in the numbers of micro particles.

Modern aquaculture feeds for RAS are designed and produced with faecal stability in mind, aiming at producing faecal matter composed of large particles that can be easily settled out or removed by mechanical filtration. Amongst the technics used to improve feeds in aquaculture is the use of feed binders (Brinker, 2007; Brinker *et al.*, 2005a, 2005b). Feed binders are added to the feeds in order to produce faeces with a higher stability, or in other words, faeces that break up less, remaining more compact. This in turn makes the removal of particles from RAS easier.

Under strong agitation for 8 minutes, faeces deriving from a basic trout diet were found to be composed by over 75% of particles above 100 µm in a study by Brinker (2007), and in improved diets (containing feed

binders) this proportion increased to over 84%. In general, most of these particles can be easily removed by a drum filter, suggesting that over 75% of particles generated by faeces are immediately removed.

Standard RAS design typically includes some sort of screen filter with a pore size typically ranging from 40 to 100 µm, which has been shown to remove between 70 and 90% of particulate matter (Fernandes et al., 2015).

The rest of particles are left behind, explaining the typical PSD described before, displaying an exponential increase in numbers with the decrease in particle size.

6.2. Feed spill

Another potential source of micro particles in RAS is feed spill. Research into feed spill is much less extensive than research into faeces produced, and currently there are only estimates on just how important feed spill is in regards to micro particles. In broad terms, below 5% of the feed ends up as uneaten feed (Bureau and Hua, 2010; Kokou and Fountoulaki, 2018; Reid *et al.*, 2009). So in terms of weight, for each kg of feed given to the fish, approximately 250 g of faeces are produced and less than 50 grams of feed spill is generated, suggesting that feed spill will contribute to at the most $1/6^{th}$ of the particles and could therefore be considered a secondary component in the generation of micro particles. However, as will be discussed later, it is not as simple.

6.3. Bacteria and other microorganisms

The final component that is generally considered important for the formation of micro particles is bacteria biofilm. In the past, it has been mostly referenced to as bacterial biofilm released from biofilters. However, research has shown that free swimming bacteria is an important part of RAS water quality (Rojas-Tirado *et al.*, 2018, 2016). Just how important free swimming bacteria are to micro particle numbers in RAS is unknown, but in wastewater treatment it is commonly assumed that most particles are bacteria and bacteria agglomerates, and particulate COD is commonly used to access the development of the bacterial biomass (Münch and Pollard, 1997; Contreras *et al.*, 2002).

In **paper II** an attempt was made to estimate the relative share that bacteria (and other microorganisms) make of micro particles. This was done by examining the impacts of micro filtration and UV irradiation on the development of micro particles and bacterial activity in 12 pilot scale RAS, stocked with rainbow trout, three of which fitted with UVs and a 200 µm filter, three fitted with 1 µm filters, three fitted with both UVs and 1 µm filter and three final systems fitted with 200 µm filters.

The UVs utilized produced only UVc radiation and the doses applied were not high enough to photo oxidize the organic matter. Therefore, any alteration to the number and amount of micro particles present in the system was probably the result of differences in the abundance of bacteria. At the same time the use of 1 µm filters should result in the direct removal of micro particles (both living organisms and inert particles).

The results obtained during the trial showed a very low numbers of micro particles in systems fitted with UVs compared to systems fitted with 1 µm filters (fig. 12).

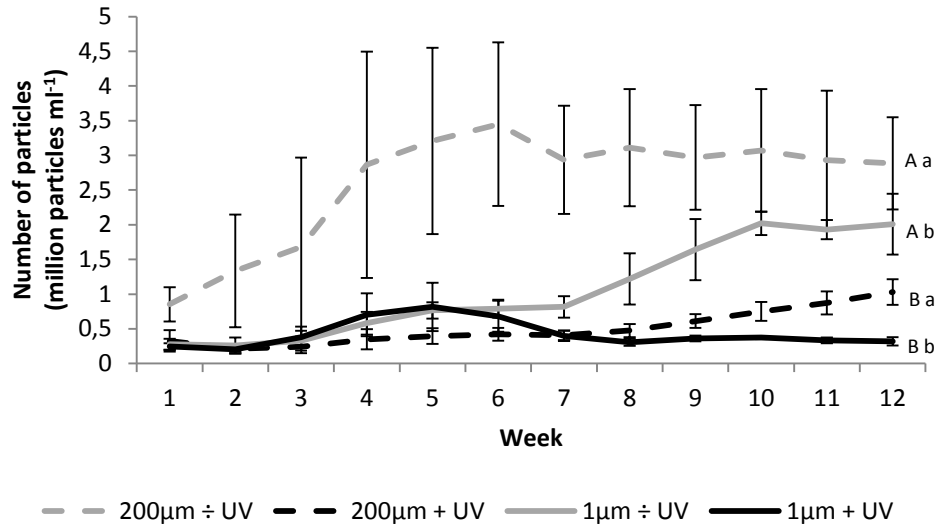

Figure 12. Effects (mean ± SD, n=9) of micro filtration, UVs and combination thereof, in the development of micro particle numbers in pilot scale RAS. 200µm ÷ UV: 200 µm filter without UV; 200µm + UV: 200 µm filter with UV; 1µm ÷ UV: 1 µm filter without UV; 1µm + UV: 1 µm filter with UV (reproduced from Paper II).

When compared within each size of filter used, the use of UVs resulted in 64% less micro particles by numbers in tanks fitted with 200 µm filters and over 84 % less were found in the tanks fitted with 1 µm filters.

The lower number of micro particles was closely followed by very low bacterial activity in the system (fig. 13).

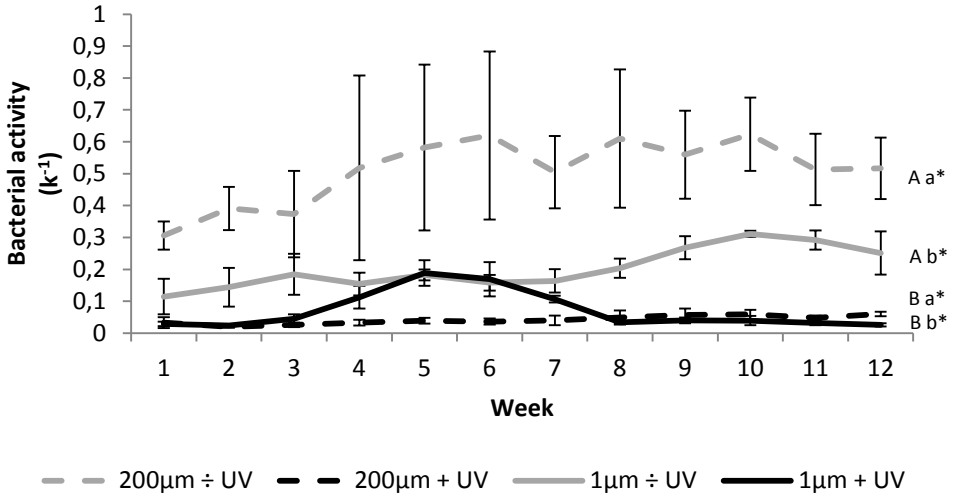

Figure 13. Changes in bacterial activity (expressed as the H_2O_2 degradation rate constant) caused by the utilization of 1µm filters, UVs and a combination of both in pilot scale RAS. 200µm ÷ UV: 200 µm filter without UV; 200µm + UV: 200 µm filter with UV; 1µm ÷ UV: 1 µm filter without UV; 1µm + UV: 1 µm filter with UV (reproduced from Paper II).

The results obtained in **paper II** suggest that potentially 64% or more of micro particles in RAS are free swimming and particle bounded bacteria present in the water (fig. 14).

Likewise, a large part of the organic matter present in the water seems to actually be bound in these bacteria, as the reduction in bacterial activity was accompanied by approximately 30% reduction in the volume of particles and approximately 25% reduction in total COD.

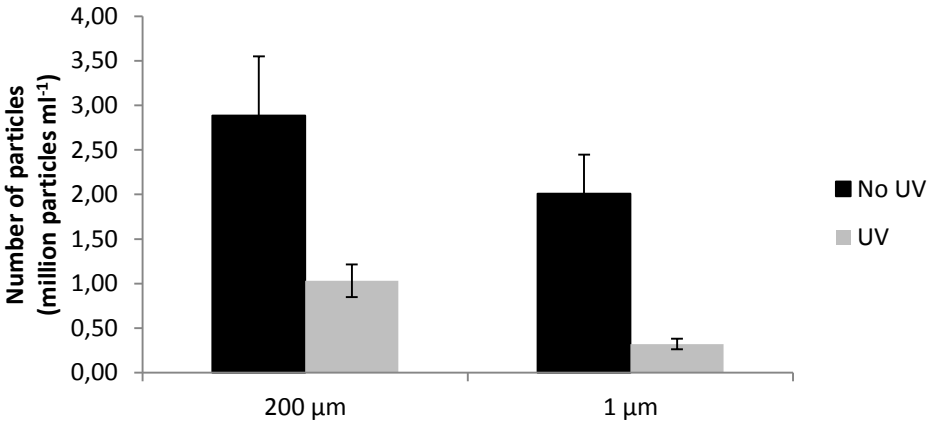

Figure 14. Differences in the number of particles caused by the use of UVs in pilot scale RAS fitted with 1µm and 200µm filters (adapted from Paper II).

These results are supported by reports from the industry. A company that operate large UV banks intermittently report that when UVs are turned off, the water transparency decreases (turbidity increases as measured by secchi depth) to as little as 0.5 m. However, once the UV banks are turned on, the turbidity clears up in less than 24 hours (personal communication). Like in **Paper II**, these observations suggest that a large portion of the particles are living organisms.

Another observation from the industry is large changes in turbidity in very short time (less than 24 hours). As previously discussed, RAS are very stable systems with control input and export of nutrients and particles. Assuming that no drastic changes in the input of feed or exportation of nutrients from the system occurred, the more likely explanation to such large variations in turbidity is bacterial blooms.

In support of this, Rojas-Tirado *et al.* (2019) showed that adding acetate as a carbon source resulted in a large increase in bacterial activity, coupled to a large increase in micro particles in the size range of 1 to 3 µm (fig . 15).

Results obtained in **Paper III** seem to further support this. Here, storing RAS water samples at either room temperature or 4^{o}C resulted in very large increases in the number of micro particles measured in the samples from RAS 2, in as little as 3 hours, even when stored at 4^{o}C (fig. 4). The large increases in micro particle numbers were also followed by large changes in bacterial activity, resulting in strong correlations between both.

These results indicate that, at least from a numbers perspective, bacteria form a large proportion of micro particles, if not the bulk.

The fact that bacteria may be responsible for a large part of micro particles in RAS may partly explain why so large variations are found within similar systems and why large variations in water quality can sometimes be observed in very short periods of time without any obvious explanations, both in commercial and research facilities.

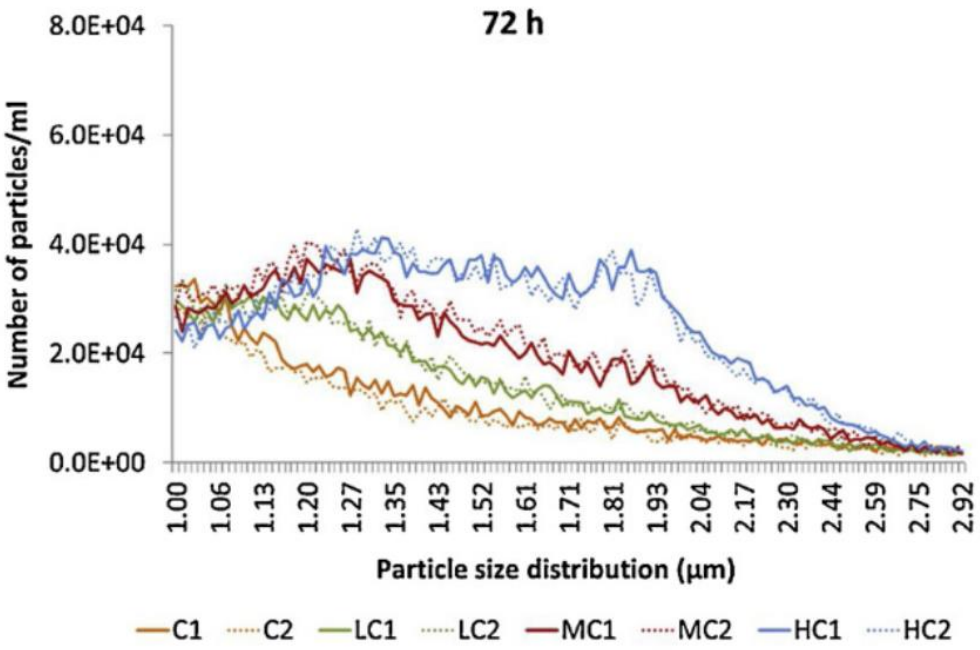

Figure 15. PSD changes in RAS water samples spiked with different levels of acetate. C: control; LC: low concentration; MC: medium concentration; HC: high concentration (reproduced from Rojas-Tirado *et al.* 2019).

Paper II also showed that particles of larger sizes are also impacted by the UVs. Despite the very large changes in the numbers of micro particles in the trial, the changes in the β values were not very large. Systems fitted with UVs had β values of 3.71 and 3.77 (respectively, fitted with 1 µm filter and 200 µm filter), while systems that did not contain UVs had β values of 3.87 and 3.86 (respectively, fitted with 1 µm filter and 200 µm filter). Figure 16 shows the cumulative distribution of particles on the control treatment and the UV treated water, and despite the large change in numbers, the relative proportion of each size fraction remained similar. This could indicate that larger particles are also either composed (bioflocs) or at least covered in bacteria that can be removed by UV filtration.

Another potential component of micro particles are microalgae. While this is not the most common occurrence of indoor facilities, outdoor farms may be affected by microalgae blooms (Becke *et al.*, 2020; Moestrup *et al.*, 2014). However, the prevalence and importance of microalgae in RAS is currently unknown.

6.4. Organic matter

However, the results discussed before lead us back to the beginning of the discussion of input of organic matter into RAS. While it is true that the majority of particles in RAS (by volume) are the result of faeces, the fact that such a large portion of micro particles may actually be composed of living organisms suggest that additional organic supplies may influence micro particle numbers trough the growth of microorganisms.

The breakdown of particles is clearly important to the generation of micro particles. In low intensity systems it has been shown to be one of the main factors controlling bacterial activity by providing organic matter and a place for bacteria to settle in. However, with the increase of recirculation intensity and hydraulic retention time (HRT), studies have shown that bacterial activity becomes independent of the amount of particulate matter (Pedersen et al., 2017a).

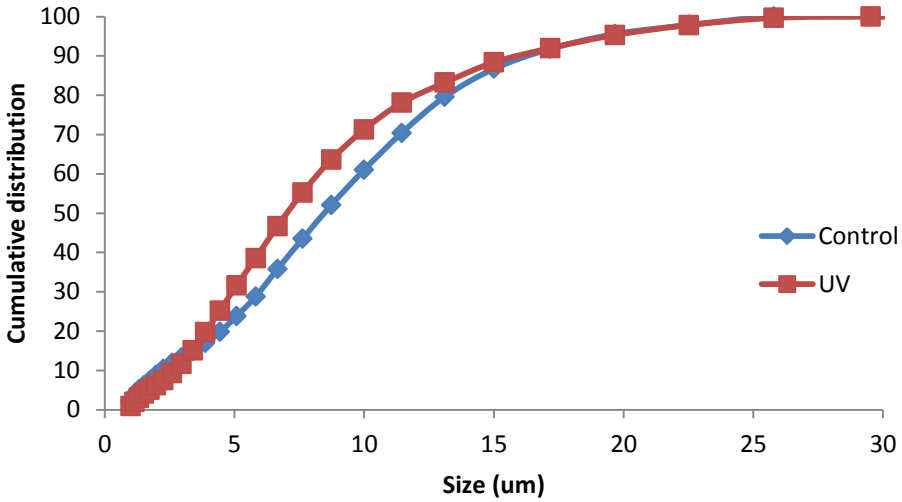

Figure 16. Cumulative distribution (volume) of micro particles. Results of control systems vs systems treated with UV (adapted from Paper II).

Most solids contained in faeces are composed of undigested starch and fibres, as well as ash from bones (Cho and Bureau, 1997). As mentioned before, 1 kg of feed produces approximately 200 g O_2 kg^{-1} feed in particulate COD on the faeces. However, the same amount of faeces only represents 32 g O_2 kg^{-1} feed in particulate BOD_5, suggesting a very low degradability (BOD / COD ratio of 0.16) (Srinivas, 2008). On the other end, the dissolved fraction produced by the fish only produces 81 g O_2 kg^{-1} feed in COD. However, due to a BOD to COD ratio of 0.5 (average biodegradability), the amount of BOD is actually higher (41 g O_2 kg^{-1} feed in BOD_5) then the particulate BOD (Dalsgaard and Pedersen, 2011).

These results suggest that, dissolved organic matter available can promote growth of bacteria and other microorganisms that compose a significant portion of micro particle (Leonard *et al.*, 2002; Rojas-Tirado *et al.*, 2019).

This leads us back to feed spill. As mentioned before, feed spill contributes at most with 50 g per kg of feed. Feed spill is normally considered as mostly uneaten pellets, which would easily be removed by settling or mechanical filtration. However, there is also broken pellets and dust created during the extrusion process, shipping, handling and feeding (e.g. feed blowers). The exact numbers for this are unfortunately unknown, but it is likely they vary depending on the type of feed, the type of storage system and feeding system, as well as how much handling is involved. Furthermore, different species of fish have different feeding habits that could increase the amount of uneaten feed.

Considering that feed pellets are designed to be highly digestible, it is likely that even small amounts of feed spill can contribute with large amounts of easily degradable material that bacteria can use to fuel growth. Unfortunately, there is very little data on just how much feed spill influences the growth of bacteria in RAS. We currently have a limited knowledge on the amount of feed spill (especially broken

down pellets and dust), as well as the impacts that they have on microbial and micro particle dynamics. However, it clearly illustrates that, at least from an organic supply for bacteria to grow, there is a potential for additional large sources and therefore, more research should be put into understanding these effects.

These results may also explain why different quality feeds can have large impacts in water quality. Not only will a lower digestibility result in higher excretion of faeces, composed of higher levels of organic matter, but the pellets themselves may break more easily. This will, in all likelihoods, result in a cumulative effect of a direct increase in particles and an increase in organic matter available for bacteria to utilize.

7. Effects of micro particles

Particles have been considered problematic for the rearing of fish in RAS for a long time and their build-up is considered one of the main issues in modern aquaculture (Badiola *et al.*, 2012).

Effects of particles in RAS can be divided in 2 categories: direct impact on the fish and impact on the system.

7.1. Direct impacts on fish

The impact of particles on fish is typically attributed to the impact particles have on the gills of fish, and it is normally assumed that smaller particles pose a bigger risk for fish health then larger particles. Previous work conducted with different species has shown negative effects of elevated TSS (Chapman *et al.*, 1987; Goldes *et al.*, 1986; Lake and Hinch, 1999; Lu *et al.*, 2018; Magor, 1988; Servizi and Martens, 1987; Wong *et al.*, 2013). However, most of these studies were conducted using sediments from natural systems like rivers and the ocean and mostly of mineral origin. In addition, most of these trials were conducted with rather high levels of TSS (up to 40 g l^{-1}), much higher than what is typically found in RAS.

Recent work conducted with rainbow trout showed no direct impacts of particles on this species (Becke *et al.*, 2019, 2018, 2017; Fernandes, 2013; Michel *et al.*, 2013, **Paper II and Paper IV**).

Michel *et al.* (2013) found no damage in the gills of rainbow trout exposed to pulse doses of mineral solids. Similarly, Fernandes (2013) found no indication of changes in the gills structure during trials with micro particles in pilot scaled RAS.

In a short-term exposure trial, Becke *et al.* (2017) found no significant impacts of increase suspended solids (up to 30 mg l^{-1}) on rainbow trout compared to a control exposure (maximum of 5 mg l^{-1}). In a subsequent trial, Becke *et al.* (2018) compared fish in a control group exposed to TSS of 3.9 mg l^{-1} against a treatment group exposed to over 30 mg l^{-1} TSS for 18 weeks. The authors analysed different health indicators including fin condition, hematologic samples, gill histology, heat shock protein 70, as well as growth parameters, and found no differences at the end of the trial, suggesting that rainbow trout are highly resilient to high suspended solid loads.

In order to test if a cumulative impact of high suspended solids in combination with a second stressor would impact rainbow trout, Becke *et al.* (2019) exposed rainbow trout to elevated levels of TSS (>35 mg l^{-1} TSS), and increased levels of ammonia (from 0.005 mg l^{-1} to 0.025mg l^{-1} NH_3-N) for a total experimental time of 13 weeks. Like in previous studies, no negative impacts of high TSS values were found.

In **Paper II**, rainbow trout were exposed to different levels of micro particles for a total period of 13 weeks (up to a 10 fold difference between the lower and highest exposers). However, no differences were found in survival or growth. A number of health parameters (not published) were also analysed in samples of gill mucus, skin mucus and in the plasma, but no differences related to the trial were found.

In **Paper IV**, rainbow trout were similarly exposed to different levels of micro particles (as a result of the different treatments) and no differences were found in growth rates or survival.

Most recent results seem to indicate that rainbow trout are generally largely tolerant to large amounts of micro particles and elevated levels of TSS without obvious negative implications. It is however possible that potential negative impact of particles is species specific, due to different tolerances and potential exposures in their natural environments, so further research should be conducted in different species.

7.2. Impacts on systems

The build-up of micro particles in RAS can lead to deterioration of water quality, including increased turbidity, decrease UVT, increased bacterial activity, increased oxygen consumption and clogging of filters.

Increase turbidity can cause visual impairment and reduce feed intake in salmonids (Ali, 1959; Barrett *et al.*, 1992; De Robertis *et al.*, 2003) as well as a delay in feed intake (Becke *et al.*, 2017). While increased turbidity has been shown to reduce the aggression between Coho salmon, the levels of turbidity required for that (30 to 60 NTU) are much higher than typically found in RAS facilities (Berg and Northcote, 1985).

The build-up of particles can also lead to a reduction in the efficiency of disinfectants, by providing protection for microorganisms. This is especially relevant when using UV (Carré *et al.*, 2018; Hess-Erga *et al.*, 2008; Mamane, 2008; Qualls *et al.*, 1983), as the increase in particle loads leads to a decrease in the ultraviolet transmittance (UVT) (**Paper IV**).

Another implication of particle build-up in RAS is the increase in available organic matter. Build-up of organic matter can lead to an increase clogging of biofilters and reduced nitrification efficiency by providing additional carbon for heterotrophic bacteria (Chen *et al.*, 2006; Guerdat *et al.*, 2011; Michaud *et al.*, 2006; Zhu and Chen, 2001). Heterotrophic bacteria grow much faster than nitrifiers and the extra availability of organic matter may lead to heterotrophic bacteria outcompeting nitrifiers in the biofilters (Hagopian and Riley, 1998). The increase in heterotrophic bacteria is also likely to increase oxygen consumption, resulting in less oxygen availability for proper biofilter performance (Wanner and Gujer, 1984; Zhang *et al.*, 1994).

Likewise, the build-up of organic matter in RAS is responsible for the production of hydrogen sulphide (H_2S) (Letelier-Gordo *et al.*, 2020), which has been linked to several large scale mortality events in commercial facilities in recent years (Dalsgaard, 2019). The importance of micro particles in this regard is still unknown. However, due to the large prevalence of this type of particles in RAS facilities, as well as the amount of organic matter contained within, it is likely that micro particles play a part in these events.

Strong correlations between micro particle surface area and bacteria activity has been found in commercial trout facilities, as well as in experimental RAS (Pedersen *et al.*, 2017; **Paper I**). Pedersen *et al.* (2017) first suggested that in low intensity RAS, particle surface area is essential for supporting bacterial activity by providing surface area that bacteria can grow on, as well as substrate to support their growth. The authors examined the correlation between the surface area of particles above 5 µm and bacterial activity in different RAS (two model trout farms, a low intensity experimental RAS and 12 high intensity RAS) and found correlations between 0,858 and 0.928 (fig. 17). However, the correlation disappeared in higher intensity systems (feed loading of 3.1 kg m^{-3} MUW). The authors suggested that this could be caused by the build-up of DOM which could potentially sustain all the bacteria in the system. Another possible explanation given by the authors was the possibility of the build-up of particles bellow 5 µm, which were not measured in the study.

In **Paper I** of this dissertation, a similar pattern was found when 20 RAS in 7 different trout farms were analysed. A correlation of 0.8 was found between micro particle surface area and bacterial activity. The correlation was substantially higher (0.92) when only surface area of particles above 10 µm was considered, and thereby excluding most free swimming bacteria, sustaining the hypothesis that the surface area provided by micro particles is important for controlling bacterial activity in lower intensity RAS (fig 18).

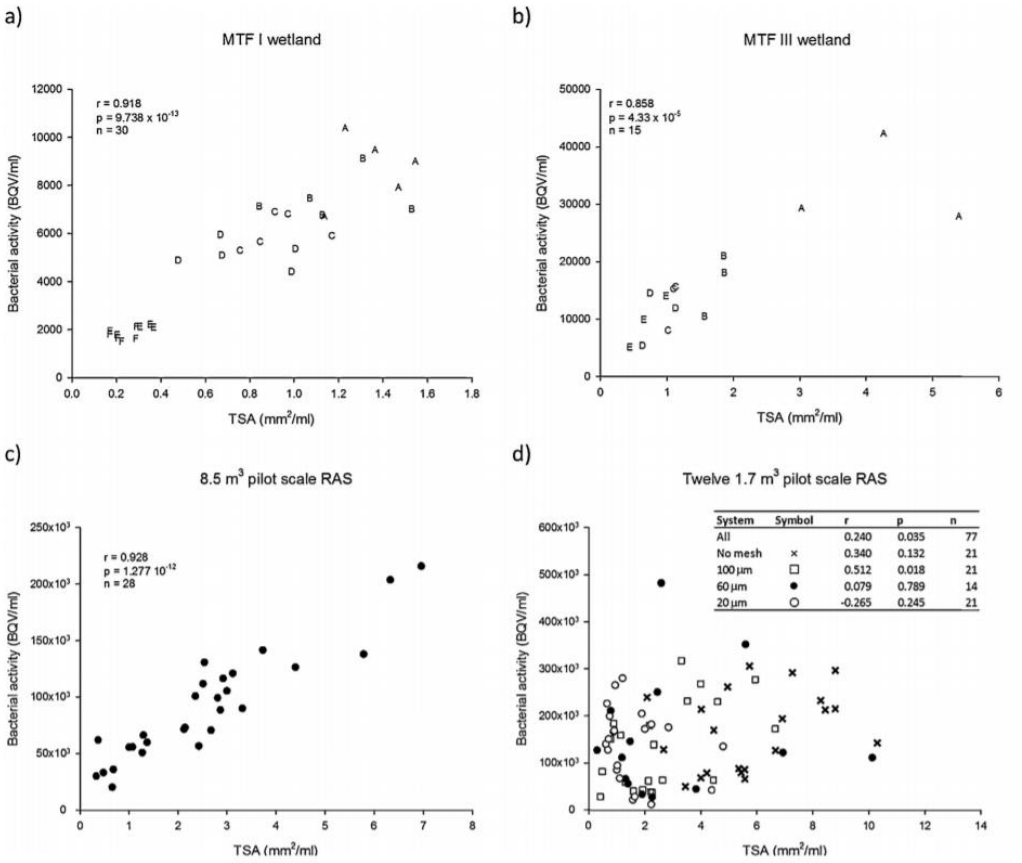

Figure 17. Correlation between bacterial activity and particle surface area in different RAS systems (reproduced from Pedersen *et al.*, 2017).

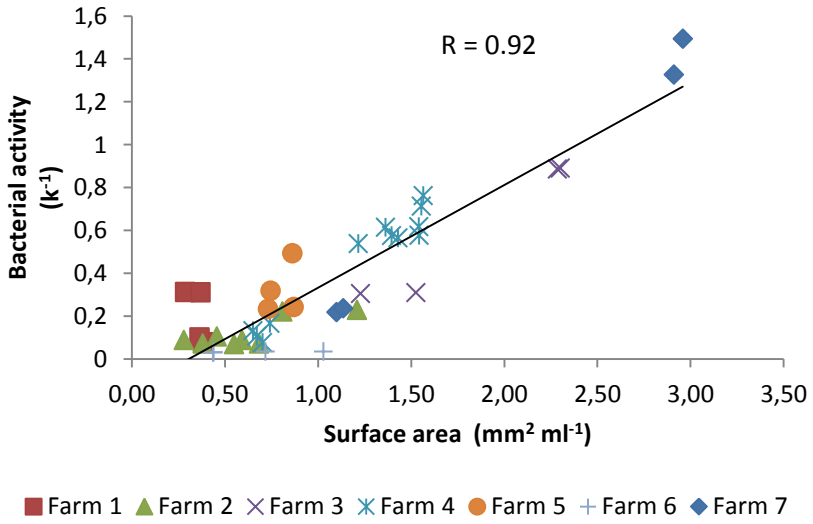

Figure 18. Relation between micro particle surface area and bacterial activity across 20 different RAS, in 7 different farms (adapted from Paper I).

8. Control of micro particles

The main reason why micro particles have been considered so problematic over time is the difficulty in removing them. While a large fraction of particles can be addressed by different types of filtration, particles below 30 µm are extremely difficult to remove.

Particle removal in aquaculture is mainly controlled via settling or mechanical filtration (fig. 19).

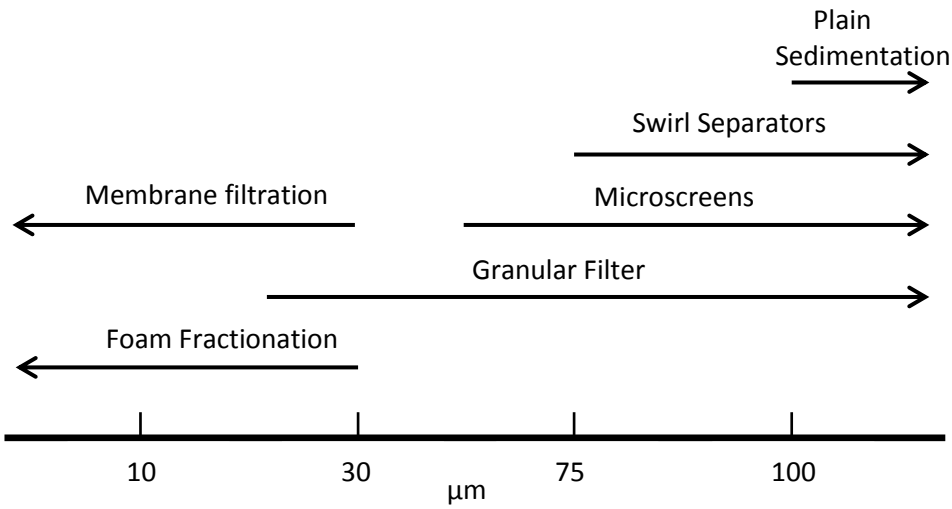

Figure 19. Particle removal devises based on particle size (adapted from Timmons and Ebeling, 2010)

8.1. Primary solids removal

In order to maintain good water quality parameters and allow for increased levels of recirculation, primary solids removal is essential (Cripps and Bergheim, 2000; Timmons and Ebeling, 2010).

The main solid waste generated in aquaculture is the faeces produced by the fish (Reid *et al.*, 2009). Primary solids removal is typically achieved using settling devices and mechanical filtration (normally mesh filters). While many new technologies are being tested and used to reduce the build-up of finer solids, it should be stated from the beginning that there is no replacement for proper primary solids removal. Solids should be removed fast and gently out of the system as soon as possible. This is essential as larger solids are much easier to remove than smaller particles (Timmons and Ebeling, 2010) and solids (both faeces and feed spill) can leach dissolved nutrients and organic matter that will build-up in the system.

Settling can be achieved in the tanks and the sludge collected in sludge cones (typical in raceways at MTF) (Jokumsen and Svendsen, 2010), or through the use of specialized settling equipment like swirl separators and radial settlers. While all this mechanisms work well, they only work on large particles (normally over 100 µm) and can take a considerable amount of space due to the necessity of a low water velocity that allows for settling of the particles (Davidson and Summerfelt, 2005).

Swirl separators and radial settlers have the advantage over settling basins as they allow for much higher hydraulic loads to be used (Lekang, 2007). However, even though they are more efficient, they will still only remove larger particles.

Drum filters can be equipped with very fine meshes. In theory this would allow for an increase removal of micro particles. However, drum filters seem to have a threshold at which point reducing the size of the mesh does not increase removal efficiency (fig. 20) (Fernandes et al., 2015). This may be caused by "cake filtration" (Dolan *et al.*, 2013). Cake filtration refers to the layer of organic matter that accumulates on the filter mesh and reduces the pore size. While cake filtration may trap smaller particles, it decreases the flow through the filter and increases backwashing frequency, resulting in higher operation costs.

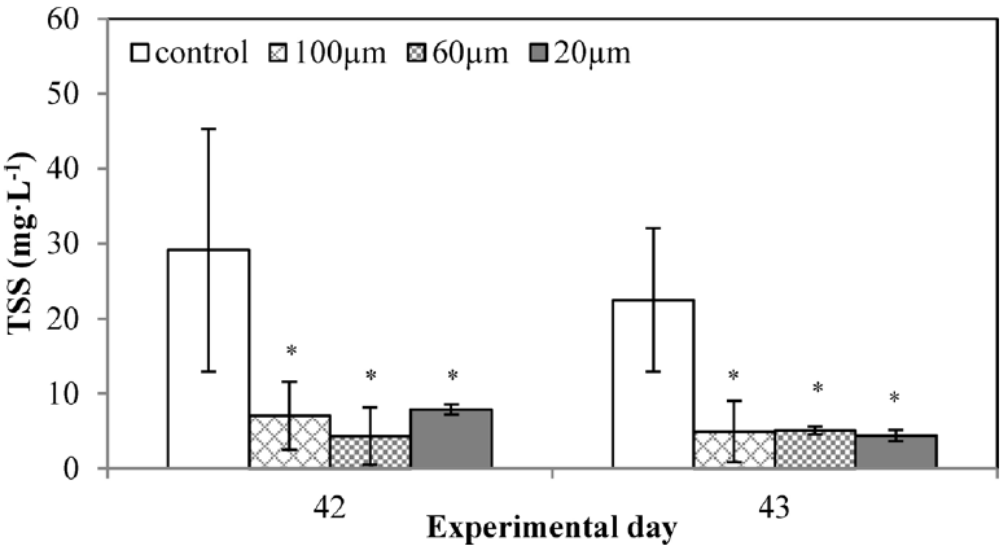

Figure 20. Mesh size effects on TSS in pilot scale RAS (reproduced from Fernandes *et al.,* 2015).

In addition, a smaller mesh size requires a larger filter to process the same amount of water. Dolan *et al.* (2013) showed that a reduction in mesh size from 100 µm to 40 µm resulted in the flow being reduced by 2/3 (Fig. 21). This would result in the necessity to increase the size of the filter to about triple the size, which is both costly in terms of acquisition, but also impractical due to potential space limitations, especially in indoor facilities. Nevertheless, some companies operate drum filters with different mesh sizes to try to reduce some of the smaller particles.

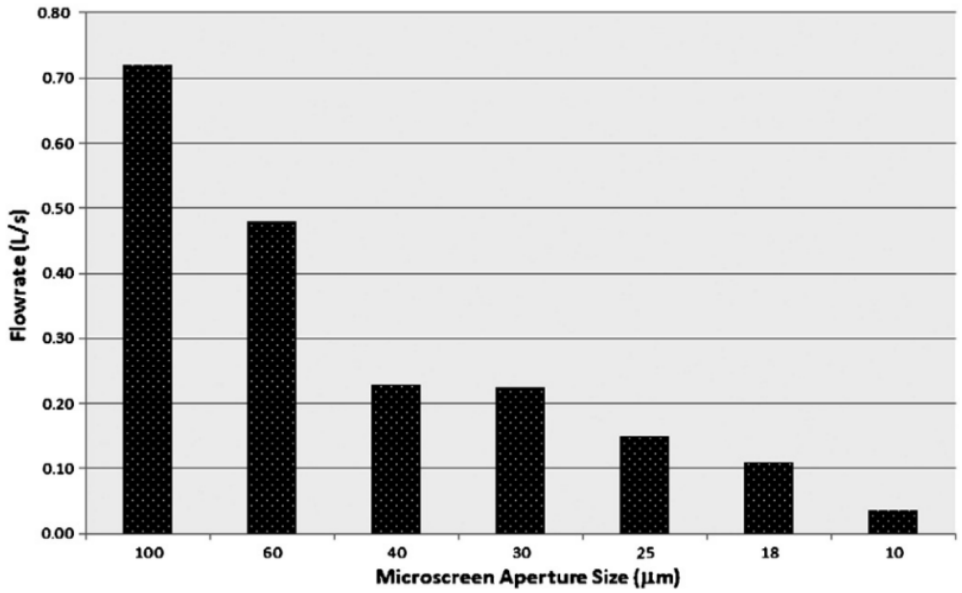

Figure 21. Micro-screen maximum flow capacity depending on mesh size, utilizing pristine distilled water (reproduced from Dolan *et al.,* 2013).

8.2. Biofilters

At the centre of a RAS there is typically a biofilter. Biofilters are used to convert ammonium (NH_4^+) to nitrite (NO_2^-) and nitrite to nitrate (NO_3^-) (Gutierrez-Wing and Malone, 2006). This process is employed as NO_3^- is not toxic compared to NH_4^+.

In RAS, biofilters are typically composed of an inert carrier (Fdz-Polanco *et al.*, 2000). This carrier provides a place for bacteria to grow and stops them from being washed out of the biofilter. Biofilters are commonly operated as fixed bed biofilters (FBB) or moving bed biofilters (MBB), or in some instances a combination of both (Fig. 22) (Gutierrez-Wing and Malone, 2006; Timmons and Ebeling, 2010).

In FBB, the media is generally fixed and undisturbed during normal operation. This leads to a build-up of bacteria and organic matter, requiring that the biofilter is backwashed at regular intervals. MBB on the other hand, are normally kept mixed by a constant supply of air. This permanent aeration enables MBB to stay relatively "clean" and to operate at a constant rate without the need for backwashing (Lekang, 2007; Timmons and Ebeling, 2010). Both types of filters offer advantages and disadvantages.

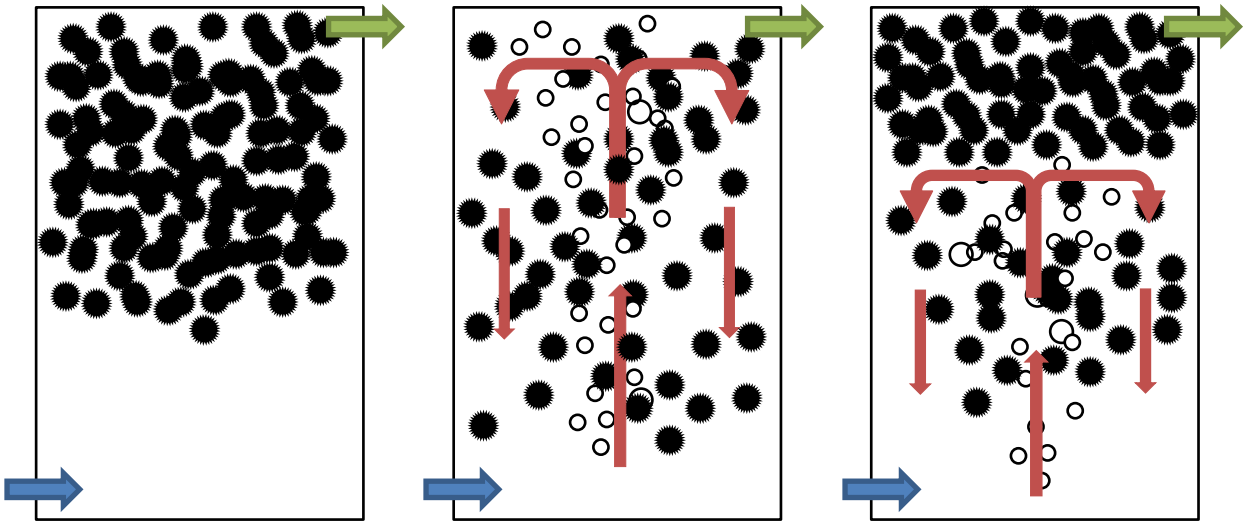

Figure 22. Different types of biofilters present in RAS facilities. Blue arrows indicate water inflow to the biofilter, green arrows indicate water outflow from the biofilter and red arrows indicate media movement. From left to right: FBB; MBB; Combined MBB in the bottom and FBB in the top.

Due to the nature of FBB, these tend to work as particle sinks where particles get trapped (fig. 23) (Fernandes *et al.*, 2016). However, in order to stop biofilters from clogging and to maintain optimal performance, FBB need to be backwashed frequently. The backwashing of biofilters works as a nutrient export mechanism.

MBB on the other end, are permanently aerated. This aeration ensures that the media is constantly mixing. Due to their operation, MBB are self-cleaning and do not require backwashing, making their operation more stable. However, due to the constant cleaning and shedding of organic material from the media carriers, moving bed biofilters tend to produce particles in RAS.

In recent years, companies have been implementing a combination of moving and fixed bed biofilters in order to obtain the benefits of both systems (stable, work "free" operation of MBB and particle entrapment of FBB). This has been achieved in two ways: different biofilter sections in series, where the last section is composed of a FBB, or up-flow biofilters using media with different densities, resulting in a mixed area at the bottom and a tightly packed section at the top of the biofilter, created by the use of low density (floating) media (fig. 23). Despite the positive results obtained by the industry utilizing these technics, scientifically we know very little about the impacts it has on water quality (micro particles, bacteria and organic matter build-up), since very few controlled studies have been conducted on the impact of different biofilter types in RAS.

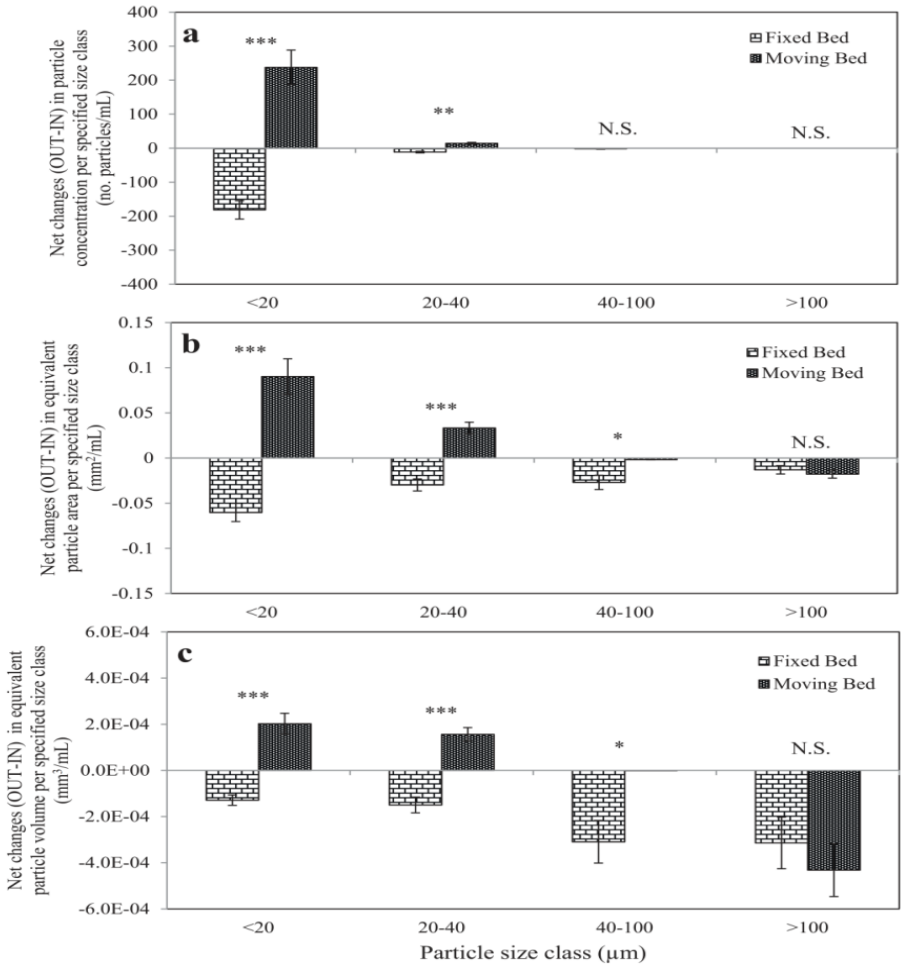

Figure 23. Net changes in particles over fixed bed and moving beds biofilters. a) number of particles b) Surface area of particles c) Volume of particles (reproduced from Fernandes *et al.,* 2016).

Recently, a replicated study was conducted on the implication of the combination of different biofilters in the removal or generation of particles in RAS (Pulkkinen *et al.*, 2019). The authors compared the use of FBB, MBB and a combination of both (FBB followed by MBB). The results obtained confirmed the previous work conducted by Fernandes *et al.* (2016), where MBB generated particles and FBB removed particles. Interestingly, the number of particles in both systems remained similar, with the largest change coming

from a large reduction in the volume of particles in the FBB, suggesting primarily a removal of large particles. Another interesting observation was that the lower removal of solids by MBB vs FBB was partially compensated by an increase in drum filter removal (fig. 24).

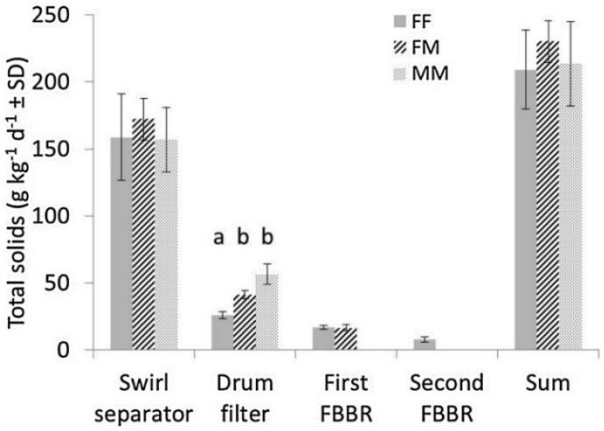

Figure 24. Total solids removed by different components in systems operated with different biofilter configurations. FF = two consecutive FBB, FM = FBB followed by MBB, and MM = two consecutive MBB (reproduced form Pulkkinen *et al.,* 2019).

8.3. Disinfection

As mentioned before, large parts of what are considered micro particles are in fact live organisms. Therefore, disinfectants can also be used for reducing the overall load of micro particles and in general for improving water quality parameters (**Paper II and Paper IV**). In **Paper II**, the use of UV resulted in large reductions in the amount of micro particles (64% by numbers and 30% by volume) (fig. 25). However, while the improvement in water quality was obvious, the fate of the organic matter reduced in the water was less obvious. It is likely that part of that organic matter was stored within the biofilter.

In **Paper IV** ozone was used as a disinfectant and large reductions in the number of micro particles were measured (75% reduction in numbers and approximately 20% reduction in volume of micro particles). Another advantage caused by the use of ozone was an increase in the clarity of the water, in line with previous studies (Davidson *et al.*, 2011; Schroeder *et al.*, 2011).

However, while it's possible to reduce the number of micro particles by disinfection, it is unlikely that the disinfectants, especially UV, will reduce the amount of organic matter in the system. At the same time bacterial loads will be far from the systems carrying capacity meaning that there will be more organic matter available that could give rise to blooms of pathogenic bacteria (Attramadal et al., 2012), if the disinfection fails.

Due to this risk, emphasis should always be placed on removing organic matter from RAS. Direct removal of organic matter will not only directly remove micro particles, but also remove substrate that can otherwise be used by bacteria to multiply from.

8.4. Membrane filtration

Membrane filtration is potential tool for micro particle removal in RAS (C. Chiam and Sarbatly, 2011; Fossmark et al., 2020; Holan et al., 2014a, 2014b, 2013; Huyben et al., 2018; Sharrer et al., 2007; Wold et

al., 2014). Membrane filtration consists on the separation of particles and macro molecules from a liquid (C. K. Chiam and Sarbatly, 2011; Hube *et al.*, 2020), and can be split in 3 categories (Sahai, 2000):

- Micro filtration (0.1 – 10 µm)
- Ultrafiltration (0.001 – 0.1 µm)
- Reverse osmosis (< 0.001 µm)

Membrane filtration is operated as dead-end or cross-flow filters (fig. 25).

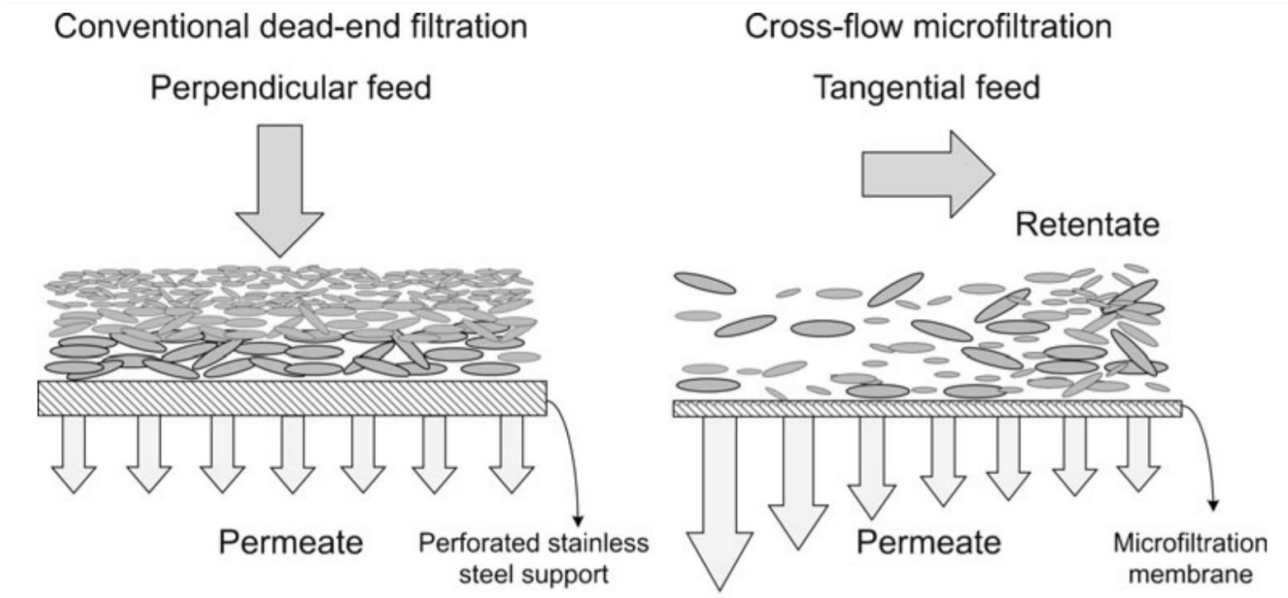

Figure 25. Dead-end and cross flow filtration comparison (reproduced from Ambrosi *et al.,* 2014).

In dead end filters all water is pressed trough the filter, similarly to drum filters. The disadvantage of such systems is that, as particles build-up, the efficiency of the filtration is greatly reduced. In cross-flow filtration the water runs parallel to the membrane. The water flow along the surface of the membrane helps to keep it clean, ensuring a longer operation time between cleaning (Ambrosi *et al.*, 2014; Sahai, 2000). Part of the water goes through the membrane (permeate) and moves back in to the system, leaving behind the particles which are too big to go through the membrane. This will cause the concentration of solids in the retentate (the water being filtered) to increase, requiring that part of it is discharged. New water is added to the loop at a rate equal to the retentate discharged. This ensures that the system is kept operating at a constant pressure. Despite the self-cleaning properties of cross-flow filtration, the membranes still need to be back washed on a regular basis. This is done when the trans-membrane pressure (TMP) (the difference between the pressure in the retentate and the permeate) is above a certain threshold. Furthermore, on regular intervals (dependent on operational conditions), the membranes may need to be clean utilizing specialized technics (e.g. chemical cleaning).

In recent years, membrane filtration has had significant attention in aquaculture as a potential method to remove fine solids and generally improve water quality. Holan *et al.* (2014b) used a membrane bioreactor (MBR) in the production of cod larvae (*Gadus morhua*) with the intent of removing colloidal particles and fine solids. The membrane had a pore size of 0.05 µm and resulted in a 77% reduction of

turbidity and up to 80% reduction in bacteria numbers. As an effect, cod larvae growth rate increased 13% by weight and survival rate increased by 3%.

More recently, Huyben *et al.* (2018) compared membrane filtration and UV in an attempt to control the bacterial load in RAS. A membrane filter with a pore size of 0.01 µm was installed in a side stream of a RAS unit, while an UV unit treated the remaining water going to the rearing tanks. The membrane filter reduced the total number of bacteria by 98.5% and TSS by 95%, while the UV reduced total bacteria by 99.6%.

Similarly to the work conducted by Holan *et al.* (2014b), Fossmark *et al.* (2020) compared the growth of Atlantic salmon parr (*Salmo salar*) in RAS with and without membrane filters. The trial resulted in better physicochemical water quality and in a more stable bacterial population.

While membrane filtration offers an excellent solution for the removal of micro particles, current cost and complexity of operating it has kept the industry from adopting it (Viadero and Noblet, 2002). Fossmark *et al.* (2020) estimated that the cost of running membrane filtration throughout the entire production cycle of Atlantic Salmon could increase the cost by as much as 27%. It is likely that with time and development of the technology the cost will be reduced.

8.5. Foam fractionation

Another option for the removal of micro particles is foam fractionation (protein skimming). Foam fractionators remove organic matter by mixing air bubbles and water and creating foam. Surfactants are the core of foam fractionation (fig. 26). Surfactants are molecules with both a hydrophobic and a hydrophilic end. The hydrophobic end wants to leave the water phase, while the hydrophilic end wants to remain in the water phase. This results in the hydrophilic end poking in to an air bubble, while the hydrophilic end remains in the water. Surfactants are normally charged (positively or negatively). This charge attracts molecules with opposite charges and, in doing so, produces foam (Brambilla *et al.*, 2008; Roy and Mohanty, 2019; Timmons and Ebeling, 2010).

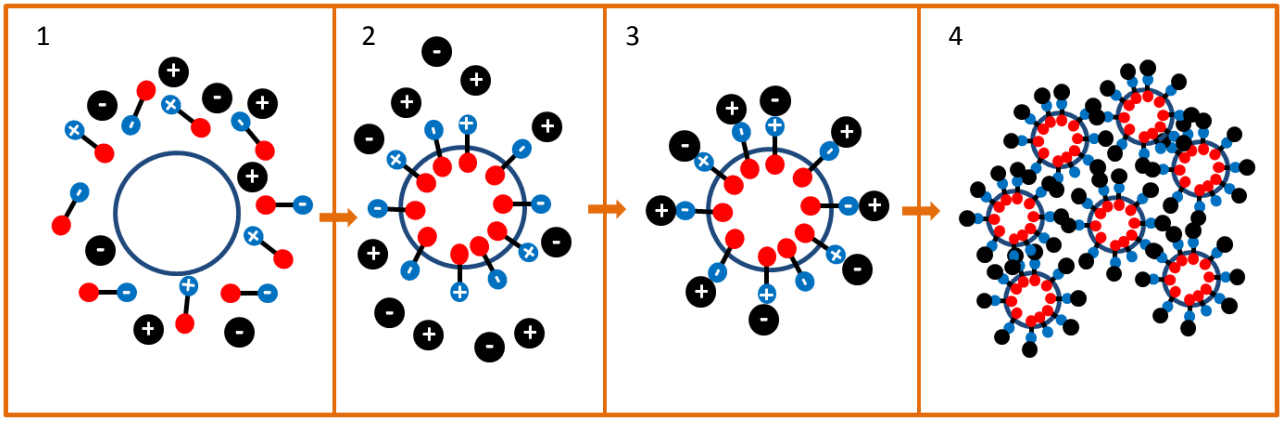

Figure 26. 1) Surfactants (blue and red molecules) are the drivers behind protein skimmers foam formation. 2) The hydrophobic end (red circle) of a surfactant pokes into an air bubble, while the hydrophilic end (blue circle) stays in the water. 3) The hydrophilic end is generally charged (positively or negatively) and attracts molecules with opposite charges. 4) Air bubbles with their attached surfactants and additional molecules join together forming foam.

Foam fractionators have been used for many years in aquaculture and their positive effects reported in previous studies (Barrut *et al.*, 2013; Brambilla *et al.*, 2008; Chen *et al.*, 1993; Shulin Chen *et al.*, 1994; Ji *et al.*, 2020; Park *et al.*, 2011; Weeks *et al.*, 1992, **Paper IV**).

Foam fractionators used in commercial operations are typically venturi or diffuser (sintered glass air stones) driven (fig. 27).

One advantage of foam fractionators is their capability of removing, not only very small particles, but also DOM (Chen *et al.*, 1994). Foam fractionators have also been shown to remove bacteria and micro algae from RAS water (Brambilla *et al.*, 2008; Figueiras Guilherme *et al.*, 2020; Park *et al.*, 2011). Due to higher surface tension in salt water, foam fractionators are typically only used in sea water and very little is known about their applicability in fresh water.

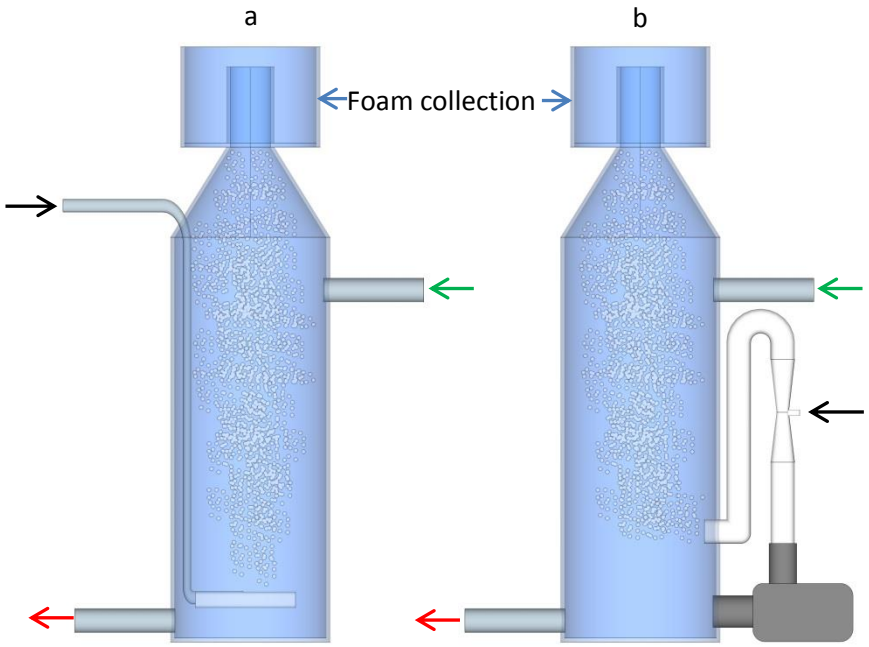

Figure 27. Foam fractionator schematic. Green arrows indicate water intake, red arrows indicate water outflow and black arrows indicate air/ozone intake. a) Diffusor design b) Venturi design

A recent study by (Ji *et al.*, 2020) is perhaps the first trial comparison the removal efficiency of foam fractionators (in saltwater) with that of more traditional micro screen filtration. Results showed that foam fractionators may have similar or higher removal rates than typical mesh sizes used in RAS (60 µm, 90 µm and 120 µm), resulting in removal rates of over 10% of solids per pass through the skimmer.

In **Paper IV**, effects of foam fractionation in fresh water RAS with or without ozone were studied in 12 replicate pilot scale systems. Foam fractionators operated in systems without ozone removed approximately 60% of all particles by number and 65% of all particles by volume (fig. 28). At the same time, bacterial activity was reduced by 55 to 60% compared to control systems without skimmers. The combination of foam fractionators and ozone resulted in the removal of approximately 90% of all particles by numbers and 75% of particle volume and improvement in multiple water quality parameters were observed. Interestingly, the use of protein skimmers also reduced the amount of DOM by approximately 20% (independently of the use of ozone).

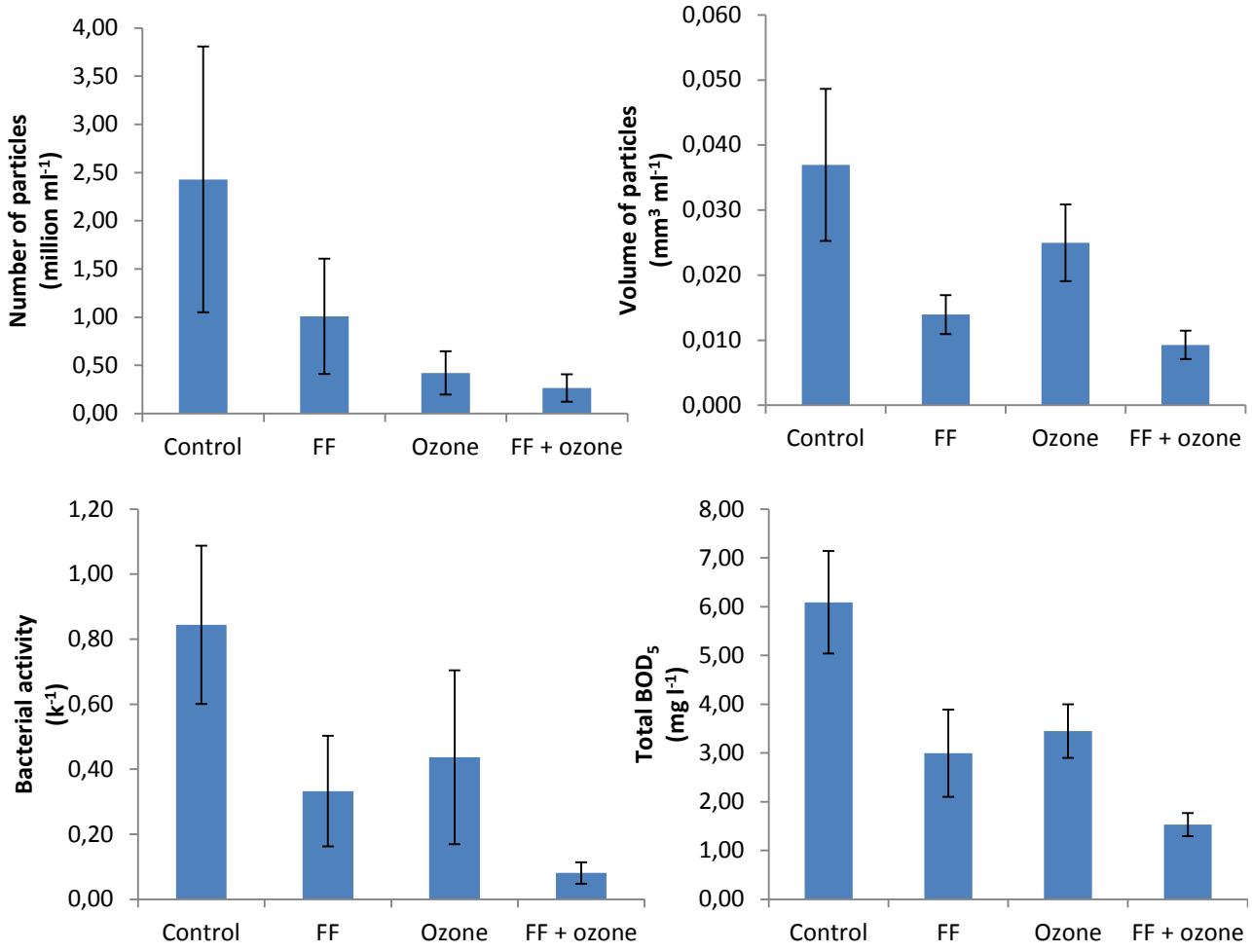

Figure 28. Effects of ozone, foam fractionators (FF) and the combined effect on different water quality parameters (adapted from Paper IV).

The results obtained using foam fractionators were similar to previous studies in saltwater (e.g. Barrut *et al.*, 2013; Brambilla *et al.*, 2008; Park *et al.*, 2011).

A downside typically attributed to foam fractionators is their inconsistency (Timmons and Ebeling, 2010), which can be caused by surface tension and presence of lipids in the water, which cause the bubbles to coalescent. However, batch trials conducted during this PhD using water from a pilot scale fresh water RAS and two commercial facilities (model trout farm and indoor pike perch facility) showed comparable removal rates of micro particle volume (fig. 29). Across a total of 10 experimental trials conducted, there was an approximately 20 to 35% removal of particle volume irrespective of the use of ozone. This suggests that there is some level of stability in foam fractionator's ability to remove organic matter from RAS.

The recent results obtained in studies utilizing foam fractionators indicate that they can be effective tools in the removal of micro particles.

Unlike the use of disinfectants for control of bacteria (and consequent reduction in the amount of micro particles), technologies like membrane filtration and foam fractionators deal with the source of the problems in RAS by directly removing large amounts of organic matter, including bacteria.

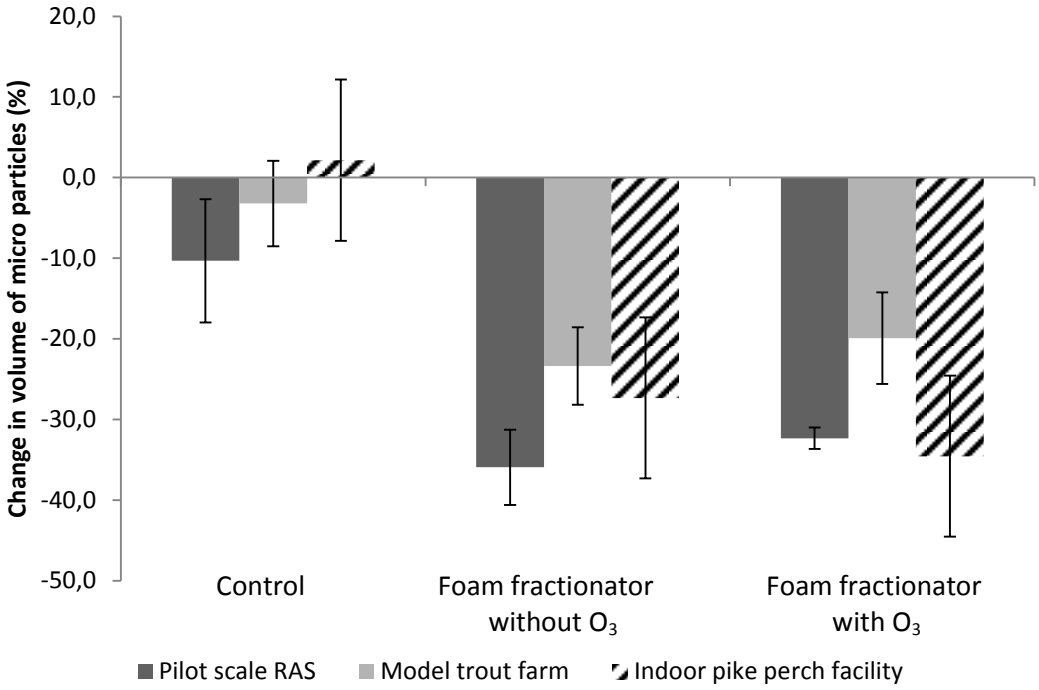

Figure 29. Changes in the amount of micro particle volume in batch trials conducted with foam fractionators (with and without ozone), using water from different freshwater RAS (pilot scale and commercial) (own unpublished data, 2019).

9. Beyond water quality

While the obvious place to look for micro particles in RAS is in the water phase, causes and implications of micro particles in RAS go far beyond the water. The results obtained during this PhD and supported by other studies, clearly indicate that, while micro particle build-up may have large direct implications on the water phase in RAS, with large changes in multiple physicochemical parameters, micro particles seem to be a symptom of a more underlying problem: organic matter build-up including both particulate and dissolved fractions.

Results obtained in **Paper II** suggest that, while micro particle build-up could be controlled in a fairly simple way using primary solids removal devices and common disinfection methods, the organic matter stays in the system. This may cause new problems (such as clogging of biofilters if organic matter accumulates inside the biofilters or potential H_2S production if organic matter deposits in areas with low circulation). Furthermore, high levels of organic matter might work as a "ticking time bomb" as bacteria are "artificially" maintained at a much lower level than the systems carrying capacity dictates. In case of a disinfection malfunction or the introduction of bacteria with a higher resistance (e.g. UV), there is a high risk of a large increase of fast growing bacteria.

Due to these factors, as well as the different interactions between all different parameters within a RAS system, it should always be attempted to investigate, not only the water, but also biofilters and other areas of a system that can accumulate organic matter, in order to analyse the true impacts caused by different experimental treatments and fully assess whether the treatment is having a positive impact in the whole system, or just transferring the "problem" to a "place out of sight".

The large individual variation shown across commercial farms and across similarly operated pilot scale research systems indicate that "each system is a system" (pers. comm. Per Bovbjerg Pedersen). Therefore, when analysing the impacts of different treatments on a system, replication is essential as large individual variations may obscure real effects of the treatments on a system wide level. Therefore, multiple systems per treatment should be used. Using only two systems (Control and treatment system), while collecting multiple samples from each system, does not constitute real replicates, but only pseudoreplicates (Heffner *et al.*, 1996). If not possible to use multiple systems, at least multiple runs of the same trial should be conducted.

Despite the challenges caused by micro particles and bacterial build-up in RAS, they may also provide opportunities for improvements in water quality.

If all micro particles in RAS were inert (non-living), there would only be two options to deal with them: stopping them from being generated in the first place or direct removal. However, as such a large portion of particles are living, there may be other avenues to explore in the quest to control micro particles.

One possibility is "environmental manipulation". Using **Paper II** as an example, the use of a disinfection system (UV) led to a large reduction in organic matter in the water phase. At face value, this offers the advantage of clear water, but may lead to an increase in the accumulation of organic matter in the biofilters, which may lead to clogging or reduced filtration performance due to heterotrophic competition. However, in a typical commercial operation of a FBB, backwashing is done regularly. By incentivising bacteria to grow in the biofilter and adjusting the backwashing frequency, it is possible that a higher exportation of nutrients would take place at each backwashing event. This may provide an additional pathway to deal with the build-up of micro particles and organic matter.

While there is sometimes a tendency to think of RAS as purely mechanical units, the reality is that at their core, RAS are a combination of engineering and biology. Fully understanding the effects that different

treatments have on the system as a whole is essential to fully understand the implications of different treatments and operational procedures. Therefore we need to move beyond water quality towards “system quality”.

10. Conclusions and future perspectives

Over the last years, micro particle accumulation in RAS has become a focus of attention in both the industry and scientific communities. The small size of micro particles make them hard to remove from RAS, while at the same time providing very large surface area. However, despite the knowledge developed over the last few years, large gaps on the knowledge regarding micro particle levels, origins, composition, implications and ways to address them remain.

While values for solids accumulation in RAS facilities are well established, the amounts of micro particles and suspended bacteria in commercial RAS have only been described on few occasions. Furthermore, due to different technics, it is not always straightforward to compare results across different farms. **Paper I** address this by sampling multiple facilities and several RAS units within each facility, in order to better understand the amounts and variations in micro particles found across commercial farms. The results of **Paper I** showed a large variation of micro particles across seemingly identical systems. Furthermore, the results sustain previous research regarding the typical PSD of RAS facilities, with most systems having β values above 3.7. This is further supported by additional data collected and presented in this dissertation. The fraction of particles below 20 µm represented by far the largest fraction of micro particles, containing over 99% of particles by number, an average of 40% of particle volume, and over 90% of particle surface area.

Solid waste in RAS is mainly derived from faeces. Micro particles have generally been considered an extension of larger solids, primarily generated by the breakage of large particles into smaller particles. However, the results obtained in **Papers II**, **III** and **IV** indicate that a large portion of micro particles are (composed of) microorganisms. While part of the faeces will eventually break down to micro particles, organic matter introduced into the system (particulate and dissolved) seems similarly important in controlling the amount of micro particles, especially in higher intensity systems with long retention times.

Due to their small size and high surface area, micro particles have been thought to cause negative impacts on fish and systems. However, the results obtained in **Papers II** and **IV** support newer research indicating that micro particles have little to no impact on rainbow trout. While these results may be species specific, the results obtained in these two papers, together with results from other studies, indicate a high tolerance of rainbow trout to micro particles. Further research should be conducted with other species.

Results of **Papers II** and **IV** demonstrate that the control of micro particles can be achieved with relative ease with the use of primary solids removal technology and disinfection. However, disinfection will not solve the underlying issue: organic matter build-up. Both micro filtration and especially foam fractionators showed good results regarding removing organic matter from systems and controlling the amount of micro particles present in freshwater RAS. The combination of direct organic matter removal (micro filters or foam fractionator) with disinfection (UV or ozone) resulted in removal rates of approximately 90% of micro particles by number, over 80% of micro particles volume and close to 90% of micro particle surface area.

Foam fractionators were particularly efficient considering ease of use, cost and a capability to remove not only particulate matter, but also dissolved, making them an effective way to partially address organic matter build-up.

Data collected along this dissertation confirm that recirculation intensity is not the main responsible for the build-up of micro particles. Rather the design, treatment units and operation of RAS are more crucial for controlling the build-up of micro particles by increasing the export of nutrients out of the system.

The results obtained during this PhD will hopefully be helpful in furthering, not only our understanding of micro particles in RAS, but also RAS systems as a whole. The large proportion of micro particles composed by microorganisms indicate that the problematic of micro particles is not only caused by accumulation of particulate matter, but by an overall build-up of organic matter (dissolved and particulate). While parts of the micro particles are simply the result of larger particles breaking down to smaller particles, the living portion does not “magically” appear in the system. Rather, it is the consequence of favourable conditions provided by high loads of organic matter. For this reason, a more holistic approach regarding RAS research needs to be taken, with all components of the loop considered, and special emphasis on the role of biofilters on the storage, release and conversion of organic matter.

11. References

Åhl, R.M., Leiknes, T., Ødegaard, H., 2006. Tracking particle size distributions in a moving bed biofilm membrane reactor for treatment of municipal wastewater. Water Sci. Technol. 53, 33–42. doi:10.2166/wst.2006.205

Ali, M.A., 1959. The ocular structure, retinomotor. Can. J. Zool. 37.

Ambrosi, A., Cardozo, N.S.M., Tessaro, I.C., 2014. Membrane Separation Processes for the Beer Industry: A Review and State of the Art. Food Bioprocess Technol. 7, 921–936. doi:10.1007/s11947-014-1275-0

Arvin, E., Pedersen, L.-F., 2015. Hydrogen peroxide decomposition kinetics in aquaculture water. Aquac. Eng. 64, 1–7. doi:10.1016/j.aquaeng.2014.12.004

Attramadal, Kari J.K., Øie, G., Størseth, T.R., Alver, M.O., Vadstein, O., Olsen, Y., 2012. The effects of moderate ozonation or high intensity UV-irradiation on the microbial environment in RAS for marine larvae. Aquaculture 330–333, 121–129. doi:10.1016/j.aquaculture.2011.11.042

Attramadal, Kari J K, Salvesen, I., Xue, R., Øie, G., Størseth, T.R., Vadstein, O., Olsen, Y., 2012. Recirculation as a possible microbial control strategy in the production of marine larvae. Aquac. Eng. 46, 27–39. doi:10.1016/j.aquaeng.2011.10.003

Badiola, M., Mendiola, D., Bostock, J., 2012. Recirculating Aquaculture Systems (RAS) analysis: Main issues on management and future challenges. Aquac. Eng. 51, 26–35. doi:10.1016/j.aquaeng.2012.07.004

Barrett, J.C., Grossman, G.D., Rosenfeld, J., 1992. Turbidity-Induced Changes in Reactive Distance of Rainbow Trout. Trans. Am. Fish. Soc. 121, 437–443. doi:10.1577/1548-8659(1992)121<0437:ticird>2.3.co;2

Barrut, B., Blancheton, J.-P., Callier, M., Champagne, J.-Y., Grasmick, A., 2013. Foam fractionation efficiency of a vacuum airlift-Application to particulate matter removal in recirculating systems. Aquac. Eng. 54, 16–21.

Bash, J., Berman, C., Bolton, S., 2001. Effects of Turbidity and Suspended Solids on Salmonids. Measurement 74.

Becke, C., Schumann, M., Geist, J., Brinker, A., 2020. Shape characteristics of suspended solids and implications in different salmonid aquaculture production systems. Aquaculture 516, 734631.

doi:10.1016/j.aquaculture.2019.734631

Becke, C., Schumann, M., Steinhagen, D., Geist, J., Brinker, A., 2018. Physiological consequences of chronic exposure of rainbow trout (Oncorhynchus mykiss) to suspended solid load in recirculating aquaculture systems. Aquaculture 484, 228–241. doi:10.1016/j.aquaculture.2017.11.030

Becke, C., Schumann, M., Steinhagen, D., Rojas-Tirado, P., Geist, J., Brinker, A., 2019. Effects of unionized ammonia and suspended solids on rainbow trout (Oncorhynchus mykiss) in recirculating aquaculture systems. Aquaculture 499, 348–357. doi:10.1016/j.aquaculture.2018.09.048

Becke, C., Steinhagen, D., Schumann, M., Brinker, A., 2017. Physiological consequences for rainbow trout (Oncorhynchus mykiss) of short-term exposure to increased suspended solid load. Aquac. Eng. doi:10.1016/j.aquaeng.2016.11.001

Berg, L., Northcote, T.G., 1985. Changes in Territoria -flaring, and Feeding Behavior in e Coho Sa mon. Can. J. Fish. Aquat. Sci. 42.

Bilotta, G.S., Brazier, R.E., 2008. Understanding the influence of suspended solids on water quality and aquatic biota. Water Res. 42, 2849–2861. doi:10.1016/j.watres.2008.03.018

Blancheton, J.P., Attramadal, K.J.K., Michaud, L., d'Orbcastel, E.R., Vadstein, O., 2013. Insight into bacterial population in aquaculture systems and its implication. Aquac. Eng. 53, 30–39. doi:10.1016/j.aquaeng.2012.11.009

Brambilla, F., Antonini, M., Ceccuzzi, P., Terova, G., Saroglia, M., 2008. Foam fractionation efficiency in particulate matter and heterotrophic bacteria removal from a recirculating seabass (Dicentrarchus labrax) system. Aquac. Eng. 39, 37–42.

Brinker, A., 2007. Guar gum in rainbow trout (Oncorhynchus mykiss) feed: The influence of quality and dose on stabilisation of faecal solids. Aquaculture 267, 315–327. doi:10.1016/j.aquaculture.2007.02.037

Brinker, A., Koppe, W., Rösch, R., 2005a. Optimizing Trout Farm Effluent Treatment by Stabilizing Trout Feces: A Field Trial. N. Am. J. Aquac. 67, 244–258. doi:10.1577/A04-078.1

Brinker, A., Koppe, W., Rösch, R., 2005b. Optimised effluent treatment by stabilised trout faeces. Aquaculture 249, 125–144. doi:10.1016/j.aquaculture.2004.12.029

Brinker, A., Rösch, R., 2005. Factors determining the size of suspended solids in a flow-through fish farm.

Aquac. Eng. 33, 1–19. doi:10.1016/j.aquaeng.2004.10.003

Brinker, A., Schröder, H.G., Rösch, R., 2005c. A high-resolution technique to size suspended solids in flow-through fish farms. Aquac. Eng. 32, 325–341. doi:10.1016/j.aquaeng.2004.07.002

Buchan, K.A.H., Martin-Robichaud, D.J., Benfey, T.J., 2005. Measurement of dissolved ozone in sea water: A comparison of methods. Aquac. Eng. 33, 225–231.

Bullock, G.L., Summerfelt, S.T., Noble, A.C., Weber, A.L., Durant, M.D., Hankins, J.A., 1997. Ozonation of a recirculating rainbow trout culture system. I. Effects on bacterial gill disease and heterotrophic bacteria. Aquaculture 158, 43–55.

Bureau, D.P., Hua, K., 2010. Towards effective nutritional management of waste outputs in aquaculture, with particular reference to salmonid aquaculture operations. Aquac. Res. 41, 777–792. doi:10.1111/j.1365-2109.2009.02431.x

Carré, E., Pérot, J., Jauzein, V., Lopez-Ferber, M., 2018. Impact of suspended particles on UV disinfection of activated-sludge effluent with the aim of reclamation. J. Water Process Eng. 22, 87–93. doi:10.1016/j.jwpe.2018.01.016

Chapman, P.M., Popham, J.D., Griffin, J., Leslie, D., Michaelson, J., 1987. Differentiation of physical from chemical toxicity in solid waste fish bioassays. Water. Air. Soil Pollut. 33, 295–308. doi:10.1007/BF00294198

Chen, S., Coffin, D.E., Malone, R.F., 1997. Sludge production and management for recirculating aquacultural systems. J. World Aquac. Soc. 28, 303–315. doi:10.1111/j.1749-7345.1997.tb00278.x

Chen, S., Ling, J., Blancheton, J.P., 2006. Nitrification kinetics of biofilm as affected by water quality factors. Aquac. Eng. 34, 179–197. doi:10.1016/j.aquaeng.2005.09.004

Chen, Shulin, Timmons, M.B., Aneshansley, D.J., Bisogni, J.J., 1993. Suspended solids characteristics from recirculating aquacultural systems and design implications. Aquaculture 112, 143–155. doi:10.1016/0044-8486(93)90440-A

Chen, S., Timmons, M.B., Bisogni, J.J., Aneshansley, D.J., 1993. Protein and its removal by foam fractionation. Progress. Fish Cult. 55, 76–82. doi:10.1577/1548

Chen, S., Timmons, M.B., Bisogni Jr, J.J., Aneshansley, D.J., 1994. Modeling surfactant removal in foam

fractionation: I - Theoretical development. Aquac. Eng. 13, 163–181.

Chiam, C., Sarbatly, R., 2011. Purification of Aquacultural Water: Conventional and New Membrane-based Techniques. Sep. Purif. Rev. 40, 126–160. doi:10.1080/15422119.2010.549766

Chiam, C.K., Sarbatly, R., 2011. Purification of aquacultural water: Conventional and new membrane-based techniques. Sep. Purif. Rev. 40, 126–160. doi:10.1080/15422119.2010.549766

Cho, C.Y., Bureau, D.P., 1997. Reduction of Waste Output from Salmonid Aquaculture through Feeds and Feeding. Progress. Fish-Culturist 59, 155–160. doi:10.1577/1548-8640(1997)059<0155:ROWOFS>2.3.CO;2

Clark, E.R., Harman, J.P., Forster, J.R.M., 1985. Production of metabolic and waste products by intensively farmed rainbow trout, Salmo gaivdnevi Richardson. J. Fish Biol. 27, 381–393. doi:10.1111/j.1095-8649.1985.tb03187.x

Colt, J., 2006. Water quality requirements for reuse systems. Aquac. Eng. 34, 143–156. doi:10.1016/j.aquaeng.2005.08.011

Cripps, S.J., 1995. Serial particle size fractionation and characterisation of an aquacultural effluent. Aquaculture 133, 323–339. doi:10.1016/0044-8486(95)00021-S

Cripps, S.J., Bergheim, A., 2000. Solids management and removal for intensive land-based aquaculture production systems. Aquac. Eng. 22, 33–56. doi:10.1016/S0144-8609(00)00031-5

Dalsgaard, J., 2019. 5th NordicRAS Workshop on Recirculating Aquaculture Systems. Berlin, Germany, 7-8 October 2019. Book of Abstracts.

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., Pedersen, P.B., 2013. Farming different species in RAS in Nordic countries: Current status and future perspectives. Aquac. Eng. 53, 2–13. doi:10.1016/j.aquaeng.2012.11.008

Dalsgaard, J., Pedersen, P.B., 2011. Solid and suspended/dissolved waste (N, P, O) from rainbow trout (Oncorynchus mykiss). Aquaculture 313, 92–99. doi:10.1016/j.aquaculture.2011.01.037

Davidson, J., Good, C., Barrows, F.T., Welsh, C., Kenney, P.B., Summerfelt, S.T., 2013. Comparing the effects of feeding a grain- or a fish meal-based diet on water quality, waste production, and rainbow trout Oncorhynchus mykiss performance within low exchange water recirculating aquaculture systems.

Aquac. Eng. 52, 45–57. doi:10.1016/j.aquaeng.2012.08.001

Davidson, J., Good, C., Welsh, C., Summerfelt, S., 2011. The effects of ozone and water exchange rates on water quality and rainbow trout Oncorhynchus mykiss performance in replicated water recirculating systems. Aquac. Eng. 44, 80–96. doi:10.1016/j.aquaeng.2011.04.001

Davidson, J., Summerfelt, S.T., 2005. Solids removal from a coldwater recirculating system - Comparison of a swirl separator and a radial-flow settler. Aquac. Eng. 33, 47–61. doi:10.1016/j.aquaeng.2004.11.002

de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.-F., Dalsgaard, J., 2018. Micro particles and microbial activity in Danish recirculating rainbow trout (Oncorhynchus mykiss) farms. Aquac. Eng. doi:10.1016/j.aquaeng.2018.12.001

de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Dalsgaard, J., 2019. Micro particles and microbial activity in Danish recirculating rainbow trout (Oncorhynchus mykiss) farms. Aquac. Eng. 84, 60–66. doi:10.1016/j.aquaeng.2018.12.001

de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Liu, D., Dalsgaard, J., 2020. UV irradiation and micro filtration effects on micro particle development and microbial water quality in recirculation aquaculture systems. Aquaculture 518. doi:10.1016/j.aquaculture.2019.734785

De Robertis, A., Ryer, C.H., Veloza, A., Brodeur, R.D., 2003. Differential effects of turbidity on prey consumption of piscivorous and planktivorous fish. Can. J. Fish. Aquat. Sci. 60, 1517–1526. doi:10.1139/f03-123

De Schryver, P., Vadstein, O., 2014. Ecological theory as a foundation to control pathogenic invasion in aquaculture. ISME J. 8, 2360–2368. doi:10.1038/ismej.2014.84

Dolan, E., Murphy, N., O'Hehir, M., 2013. Factors influencing optimal micro-screen drum filter selection for recirculating aquaculture systems. Aquac. Eng. 56, 42–50. doi:10.1016/j.aquaeng.2013.04.005

Emerick, R.W., Loge, F.J., Thompson, D., Darby, J.L., 1999. Factors Influencing Ultraviolet Disinfection Performance Part II: Association of Coliform Bacteria with Wastewater Particles. Water Environ. Res. 71, 1178–1187. doi:10.2175/106143097X122004

Fdz-Polanco, F., Méndez, E., Urueña, M.A., Villaverde, S., García, P.A., 2000. Spatial distribution of heterotrophs and nitrifiers in a submerged biofilter for nitrification. Water Res. 34, 4081–4089.

doi:10.1016/S0043-1354(00)00159-7

Fernandes, P., Pedersen, L.-F., Pedersen, P.B., 2015. Microscreen effects on water quality in replicated recirculating aquaculture systems. Aquac. Eng. 65, 17–26. doi:10.1016/j.aquaeng.2014.10.007

Fernandes, P.M., 2015a. Interactions between micro-particles and the rearing environment in reciruclating aquaculture systems. PhD Thesis. doi:10.1017/CBO9781107415324.004

Fernandes, P.M., 2015b. Interactions between micro-particles and the rearing environment in recirculation aquaculture systems. PhD Thesis.

Fernandes, P.M., Pedersen, L.-F., Pedersen, P.B., 2016. Influence of fixed and moving bed biofilters on micro particle dynamics in a recirculating aquaculture system. Aquac. Eng. doi:10.1016/j.aquaeng.2016.09.002

Fernandes, P.M., Pedersen, L.F., Pedersen, P.B., 2014. Daily micro particle distribution of an experimental recirculating aquaculture system-A case study. Aquac. Eng. 60, 28–34. doi:10.1016/j.aquaeng.2014.03.007

Figueiras Guilherme, M., de Jesus Gregersen, K.J., Pedersen, L.F., 2020. Effects of foam fractionation and chemical disinfection on the removal of different microalgae cultures. Aquac. Res. 1–10. doi:10.1111/are.14663

Fossmark, R.O., Vadstein, O., Rosten, T.W., Bakke, I., Košeto, D., Bugten, A. V., Helberg, G.A., Nesje, J., Jørgensen, N.O.G., Raspati, G., Azrague, K., Østerhus, S.W., Attramadal, K.J.K., 2020. Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (Salmo salar). Aquaculture 524, 735268. doi:10.1016/j.aquaculture.2020.735268

Franco-Nava, M.-A., Blancheton, J.-P., Deviller, G., Le-Gall, J.-Y., 2004. Particulate matter dynamics and transformations in a recirculating aquaculture system: application of stable isotope tracers in seabass rearing. Aquac. Eng. 31, 135–155. doi:10.1016/j.aquaeng.2004.01.003

Goldes, S.A., Ferguson, H.W., Daoust, P. -Y, Moccia, R.D., 1986. Phagocytosis of the inert suspended clay kaolin by the gills of rainbow trout, Salmo gairdneri Richardson. J. Fish Dis. 9, 147–151. doi:10.1111/j.1365-2761.1986.tb00996.x

Gonçalves, A.A., Gagnon, G.A., 2011. Ozone Application in Recirculating Aquaculture System: An Overview. Ozone Sci. Eng. 33, 345–367.

Guerdat, T.C., Losordo, T.M., Classen, J.J., Osborne, J.A., DeLong, D., 2011. Evaluating the effects of organic carbon on biological filtration performance in a large scale recirculating aquaculture system. Aquac. Eng. 44, 10–18. doi:10.1016/j.aquaeng.2010.10.002

Gutierrez-Wing, M.T., Malone, R.F., 2006. Biological filters in aquaculture: Trends and research directions for freshwater and marine applications. Aquac. Eng. 34, 163–171. doi:10.1016/j.aquaeng.2005.08.003

Hagopian, D.S., Riley, J.G., 1998. A closer look at the bacteriology of nitrification. Aquac. Eng. 18, 223–244. doi:10.1016/S0144-8609(98)00032-6

Hansen, K.M.S., Andersen, H.R., Ledin, A., 2010. Ozonation of estrogenic chemicals in biologically treated sewage. Water Sci. Technol. 62, 649–657. doi:10.2166/wst.2010.919

Heffner, R.A., Butler IV, M.J., Reilly, C.K., 1996. Pseudoreplication revisited. Ecol. Soc. Am. 77, 2558–2562.

Henze, M., Harremoes, P., Arvin, E., Jansen, J. la C., 1997. Wasterwater Treatment, 2nd editio. ed. Springer-Verlag Berlin Heidelberg, New York. doi:10.1007/978-3-662-22605-6

Hess-Erga, O.K., Attramadal, K.J.K., Vadstein, O., 2008. Biotic and abiotic particles protect marine heterotrophic bacteria during UV and ozone disinfection. Aquat. Biol. 4, 147–154. doi:10.3354/ab00105

Holan, A.B., Wold, P., Øie, G., Leiknes, T.O., Holan, A.B., Wold, P., Øie, G., Leiknes, T.O., 2013. Integrated Membrane Bioreactor for Water Quality Control in Marine Recirculating Aquaculture Systems Integrated Membrane Bioreactor for Water Quality Control in Marine Recirculating Aquaculture Systems. Sep. Sci. Technol. 48, 1758–1767. doi:10.1080/01496395.2013.774818

Holan, A.B., Wold, P.A., Leiknes, T.O., 2014a. Membrane performance and fouling behavior of membrane bioreactors installed in marine recirculating aquaculture systems. Aquac. Eng. 58, 45–51. doi:10.1016/j.aquaeng.2013.10.002

Holan, A.B., Wold, P.A., Leiknes, T.O., 2014b. Intensive rearing of cod larvae (Gadus morhua) in recirculating aquaculture systems (RAS) implementing a membrane bioreactor (MBR) for enhanced colloidal particle and fine suspended solids removal. Aquac. Eng. 58, 52–58.

doi:10.1016/j.aquaeng.2013.10.001

Hube, S., Eskafi, M., Hrafnkelsdóttir, K.F., Bjarnadóttir, B., Bjarnadóttir, M.Á., Axelsdóttir, S., Wu, B., 2020. Direct membrane filtration for wastewater treatment and resource recovery: A review. Sci. Total Environ. 710. doi:10.1016/j.scitotenv.2019.136375

Huyben, D., Bevan, D., Stevenson, R., Zhou, H., Moccia, R., 2018. Evaluation of membrane filtration and UV irradiation to control bacterial loads in recirculation aquaculture systems. Aquac. Int. 26, 1531–1540. doi:10.1007/s10499-018-0301-z

Iriberri, J., Undurraga, A., Muela, A., Egea, L., 1985. Heterotrophic bacterial activity in coastal waters: Functional relationship of temperature and phytoplankton population. Ecol. Modell. 28, 113–120. doi:10.1016/0304-3800(85)90016-X

Ivanovic, I., Leiknes, T.O., 2008. Impact of aeration rates on particle colloidal fraction in the biofilm membrane bioreactor (BF-MBR). Desalination 231, 182–190. doi:10.1016/j.desal.2007.11.046

Ji, M., Li, H., Li, J., Ye, Z., Zhu, S., 2020. Effect of Mesh Size on Microscreen Filtration Combined with Foam Fractionation for Solids Removal in Recirculating Aquacultural Seawater. N. Am. J. Aquac. 82, 215–223. doi:10.1002/naaq.10147

Jokumsen, A., Svendsen, L.M., 2010. Farming of freshwater rainbow trout in Denmark. DTU Aqua Reports 219, 1–47.

Kokou, F., Fountoulaki, E., 2018. Aquaculture waste production associated with antinutrient presence in common fish feed plant ingredients. Aquaculture 495, 295–310. doi:10.1016/j.aquaculture.2018.06.003

Lake, R.G., Hinch, S.G., 1999. Acute effects of suspended sediment angularity on juvenile coho salmon (Oncorhynchus kisutch). Can. J. Fish. Aquat. Sci. 56, 862–867. doi:10.1139/f99-024

Lee, C.S., Robinson, J., Chong, M.F., 2014. A review on application of flocculants in wastewater treatment. Process Saf. Environ. Prot. 92, 489–508. doi:10.1016/j.psep.2014.04.010

Lekang, O.-I., 2007. Aquaculture Engineering, Aquaculture Engineering. Blackwell Publishing.

Leonard, N., Guiraud, J.P., Gasset, E., Cailleres, J.P., Blancheton, J.P., 2002. Bacteria and nutrients - Nitrogen and carbon - In a recirculating system for sea bass production. Aquac. Eng. 26, 111–127.

doi:10.1016/S0144-8609(02)00008-0

Letelier-Gordo, C.O., Aalto, S.L., Suurnäkki, S., Pedersen, P.B., 2020. Increased sulfate availability in saline water promotes hydrogen sulfide production in fish organic waste. Aquac. Eng. 89, 102062. doi:10.1016/j.aquaeng.2020.102062

Li, T., Yan, X., Wang, D., Wang, F., 2009. Impact of preozonation on the performance of coagulated flocs. Chemosphere 75, 187–192. doi:10.1016/j.chemosphere.2008.12.014

Loge, F.J., Emerick, R.W., Thompson, D.E., Nelson, D.C., Darby, J.L., 1999. Factors Influencing Ultraviolet Disinfection Performance Part I: Light Penetration to Wastewater Particles. Water Environ. Res. 71, 377–381. doi:10.2175/106143097X122176

Lu, C., Kania, P.W., Buchmann, K., 2018. Particle effects on fish gills: An immunogenetic approach for rainbow trout and zebrafish. Aquaculture 484, 98–104. doi:10.1016/j.aquaculture.2017.11.005

Magor, B.G., 1988. Gill histopathology of juvenile Oncorhynchus kisutch exposed to suspended wood debris . Can. J. Zool. 66, 2164–2169. doi:10.1139/z88-323

Mamane, H., 2008. IMPACT OF PARTICLES ON UV DISINFECTION OF WATER AND WASTEWATER EFFLUENTS : A REVIEW Hadas Mamane School of Mechanical Engineering , Faculty of Engineering , Tel-Aviv. Rev. Chem. Eng. 24, 67–157.

Marquet, R., Muhammad, N., Vairavamoorthy, K., Wheatley, a., 2007. Particle size distribution to assess the performance of trickling filters. Process Saf. Environ. Prot. 85, 99–103. doi:10.1205/psep.05194

Martins, C.I.M., Eding, E.H., Verdegem, M.C.J., Heinsbroek, L.T.N., Schneider, O., Blancheton, J.P., Roque d'Orbcastel, E., Verreth, J.A.J., 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. Aquac. Eng. 43, 83–93.

Michaud, L., Blancheton, J.P., Bruni, V., Piedrahita, R., 2006. Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters. Aquac. Eng. 34, 224–233. doi:10.1016/j.aquaeng.2005.07.005

Michel, C., Schmidt-Posthaus, H., Burkhardt-Holm, P., Richardson, J., 2013. Suspended sediment pulse effects in rainbow trout (*Oncorhynchus mykiss*) — relating apical and systemic responses. Can. J. Fish. Aquat. Sci. 70, 630–641. doi:10.1139/cjfas-2012-0376

Moestrup, Ø., Hansen, G., Daugbjerg, N., Lundholm, N., Overton, J., Vestergård, M., Steenfeldt, S.J., Calado, A.J., Hansen, P.J., 2014. The dinoflagellates Pfiesteria shumwayae and Luciella masanensis cause fish kills in recirculation fish farms in Denmark. Harmful Algae 32, 33–39.

Park, J., Kim, P.K., Lim, T., Daniels, H. V., 2013. Ozonation in seawater recirculating systems for black seabream Acanthopagrus schlegelii (Bleeker): Effects on solids, bacteria, water clarity, and color. Aquac. Eng. 55, 1–8. doi:10.1016/j.aquaeng.2013.01.002

Park, J., Kim, Y., Kim, P.K., Daniels, H. V., 2011. Effects of two different ozone doses on seawater recirculating systems for black sea bream Acanthopagrus schlegeli (Bleeker): Removal of solids and bacteria by foam fractionation. Aquac. Eng. 44, 19–24. doi:10.1016/j.aquaeng.2010.11.001

Patterson, R.., Watts, K.., Gill, T.., 2003. Micro-particles in recirculating aquaculture systems: determination of particle density by density gradient centrifugation. Aquac. Eng. 27, 105–115. doi:10.1016/S0144-8609(02)00042-0

Patterson, R.N., Watts, K.C., 2003a. Micro-particles in recirculating aquaculture systems: Particle size analysis of culture water from a commercial Atlantic salmon site. Aquac. Eng. 28, 99–113. doi:10.1016/S0144-8609(03)00003-7

Patterson, R.N., Watts, K.C., 2003b. Micro-particles in recirculating aquaculture systems: Microscopic examination of particles. Aquac. Eng. 28, 115–130. doi:10.1016/S0144-8609(03)00028-1

Patterson, R.N., Watts, K.C., Timmons, M.B., 1999. The power law in particle size analysis for aquacultural facilities. Aquac. Eng. 19, 259–273. doi:10.1016/S0144-8609(98)00054-5

Pavanelli, D., Bigi, A., 2005. Indirect methods to estimate suspended sediment concentration: Reliability and relationship of turbidity and settleable solids. Biosyst. Eng. 90, 75–83. doi:10.1016/j.biosystemseng.2004.09.001

Pedersen, L.F., Pedersen, P.B., 2012. Hydrogen peroxide application to a commercial recirculating aquaculture system. Aquac. Eng. 46, 40–46. doi:10.1016/j.aquaeng.2011.11.001

Pedersen, L.F., Rojas-Tirado, P., Arvin, E., Pedersen, P.B., 2019. Assessment of microbial activity in water based on hydrogen peroxide decomposition rates. Aquac. Eng. 85, 9–14. doi:10.1016/j.aquaeng.2019.01.001

Pedersen, L.F., Suhr, K.I., Dalsgaard, J., Pedersen, P.B., Arvin, E., 2012. Effects of feed loading on nitrogen balances and fish performance in replicated recirculating aquaculture systems. Aquaculture 338–341, 237–245. doi:10.1016/j.aquaculture.2012.01.035

Pedersen, P.B., Grønborg, O., Svendsen, L.M., 2003. Modeldambrug: Specifikationer og godkendelseskrav: Rapport fra faglig arbejdsgruppe. Danmarks Miljøundersøgelser Arbejdsrap, 84.

Pedersen, P.B., von Ahnen, M., Fernandes, P., Naas, C., Pedersen, L.F., Dalsgaard, J., 2017a. Particle surface area and bacterial activity in recirculating aquaculture systems. Aquac. Eng. 78, 18–23. doi:10.1016/j.aquaeng.2017.04.005

Pedersen, P.B., von Ahnen, M., Fernandes, P.M., Naas, C., Pedersen, L.-F., Dalsgaard, J., 2017b. Particle surface area and bacterial activity in recirculating aquaculture systems. Aquac. Eng.

Pomeroy, L.R., Wiebe, W.J., 2001. Temperature and substrates as interactive limiting factors for marine heterotrophic bacteria. Aquat. Microb. Ecol. 23, 187–204. doi:10.3354/ame023187

Powell, A., Scolding, J.W.S., 2016. Direct application of ozone in aquaculture systems. Rev. Aquac. 10, 424–438. doi:10.1111/raq.12169

Pulkkinen, J.T., Eriksson-Kallio, A.M., Aalto, S.L., Tiirola, M., Koskela, J., Kiuru, T., Vielma, J., 2019. The effects of different combinations of fixed and moving bed bioreactors on rainbow trout (Oncorhynchus mykiss) growth and health, water quality and nitrification in recirculating aquaculture systems. Aquac. Eng. 85, 98–105. doi:10.1016/j.aquaeng.2019.03.004

Qualls, R.G., Flynn, M.P., JR, J.M.C., 1983. The role of suspended particles in ultraviolet disinfection. J. Water Pollut. Control Fed. 55, 1280–1285. doi:10.2307/25042084

Rahman, M.M., Kadowaki, S., Linn, S.M., Yamada, Y., 2012. Effects of protein skimming on water quality, bacterial abundance and abalone growth in land based recirculating aquaculture systems. J. Fish. Aquat. Sci. 7, 150–161. doi:10.3923/jfas.2012.150.161

Reid, G.K., Liutkus, M., Robinson, S.M.C., Chopin, T.R., Blair, T., Lander, T., Mullen, J., Page, F., Moccia, R.D., 2009. A review of the biophysical properties of salmonid faeces: Implications for aquaculture waste dispersal models and integrated multi-trophic aquaculture. Aquac. Res. 40, 257–273. doi:10.1111/j.1365-2109.2008.02065.x

Rojas-Tirado, P., Pedersen, P.B., Pedersen, L.-F., 2016. Bacterial activity dynamics in the water phase during start-up of recirculating aquaculture systems. Aquac. Eng. doi:10.1016/j.aquaeng.2016.09.004

Rojas-Tirado, P., Pedersen, P.B., Vadstein, O., Pedersen, L.-F., 2019. Rojas-Tirado et al. 2019.pdf.

Rojas-Tirado, P., Pedersen, P.B., Vadstein, O., Pedersen, L.F., 2018. Changes in microbial water quality in RAS following altered feed loading. Aquac. Eng. 81, 80–88. doi:10.1016/j.aquaeng.2018.03.002

Rojas-tirado, P.A., Pedersen, L. (main supervisor), Pedersen, P.B. (supervisor), 2018. Microbial water quality within recirculating aquaculture systems. PhD Thesis Natl. Inst. Aquat. Resour.

Roque d'Orbcastel, E., Person-Le Ruyet, J., Le Bayon, N., Blancheton, J.P., 2009. Comparative growth and welfare in rainbow trout reared in recirculating and flow through rearing systems. Aquac. Eng. 40, 79–86. doi:10.1016/j.aquaeng.2008.11.005

Roy, M., Mohanty, K., 2019. A comprehensive review on microalgal harvesting strategies: Current status and future prospects. Algal Res. 44, 101683. doi:10.1016/j.algal.2019.101683

Rueter, J., Johnson, R., 1995. The use of ozone to improve solids removal during disinfection. Aquac. Eng. 14, 123–141. doi:10.1016/0144-8609(94)P4431-A

Sahai, R., 2000. MEMBRANE SEPARATIONS | Filtration. Encycl. Sep. Sci. 1717–1724. doi:10.1016/b0-12-226770-2/05151-6

Schroeder, J.P., Croot, P.L., Von Dewitz, B., Waller, U., Hanel, R., 2011. Potential and limitations of ozone for the removal of ammonia, nitrite, and yellow substances in marine recirculating aquaculture systems. Aquac. Eng. 45, 35–41. doi:10.1016/j.aquaeng.2011.06.001

Schroeder, J.P., Klatt, S.F., Schlachter, M., Zablotski, Y., Keuter, S., Spieck, E., Schulz, C., 2015. Impact of ozonation and residual ozone-produced oxidants on the nitrification performance of moving-bed biofilters from marine recirculating aquaculture systems. Aquac. Eng. 65, 27–36.

Schumann, M., Brinker, A., 2020. Understanding and managing suspended solids in intensive salmonid aquaculture: a review. Rev. Aquac. 1–31. doi:10.1111/raq.12425

Schumann, M., Unger, J., Brinker, A., 2017. Floating faeces: Effects on solid removal and particle size distribution in RAS. Aquac. Eng. 78, 75–84. doi:10.1016/j.aquaeng.2016.10.007

Servizi, J., Martens, D., 1987. Some effects of suspended fraser river sediments on Sockeye Salmon (Oncorhynchus nerka).

Sharrer, M.J., Summerfelt, S.T., Bullock, G.L., Gleason, L.E., Taeuber, J., 2005. Inactivation of bacteria using ultraviolet irradiation in a recirculating salmonid culture system. Aquac. Eng. 33, 135–149. doi:10.1016/j.aquaeng.2004.12.001

Sharrer, M.J., Tal, Y., Ferrier, D., Hankins, J.A., Summerfelt, S.T., 2007. Membrane biological reactor treatment of a saline backwash flow from a recirculating aquaculture system. Aquac. Eng. 36, 159–176. doi:10.1016/j.aquaeng.2006.10.003

Skjermo, J., Saivesen, I., Øie, G., Olsen, Y., Vadstein, O., 1997. Microbially matured water : a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquac. Int. 5, 13–28. doi:10.1007/BF02764784

Srinivas, T., 2008. Environmental Biotechnology. New Age International Publisher, New Delhi.

Summerfelt, S.T., 2003. Ozonation and UV irradiation*an introduction and examples of current applications. Aquac. Eng. 28, 21–36. doi:10.1016/S0144-8609(02)00069-9

Summerfelt, S.T., Hankins, J.A., Weber, A.L., Durant, M.D., 1997. Ozonation of a recirculating rainbow trout culture system. II. Effects on microscreen filtration and water quality. Aquaculture 158, 57–67. doi:10.1016/S0044-8486(97)00064-1

Summerfelt, S.T., Sharrer, M.J., Tsukuda, S.M., Gearheart, M., 2009. Process requirements for achieving full-flow disinfection of recirculating water using ozonation and UV irradiation. Aquac. Eng. 40, 17–27. doi:10.1016/j.aquaeng.2008.10.002

Tanner, P.A., Wong, A.Y.S., 1998. Spectrophotometric determination of hydrogen peroxide in rainwater. Anal. Chim. Acta 370, 279–287. doi:10.1016/S0003-2670(98)00273-6

Timmons, M.B., Ebeling, J.M., 2010. Recirculating Aquaculture, 2nd ed. Cayuga Aqua Ventures, NY, USA.

Unger, J., Brinker, A., 2013. Floating feces: A new approach for efficient removal of solids in aquacultural management. Aquaculture 404–405, 85–94. doi:10.1016/j.aquaculture.2013.04.015

Vadstein, O., Øie, G., Salvesen, I., Skjermo, J., 1993. A strategy to obtain microbial control during larval development of marine fish. Fish Farming Technol. 69–75.

Van Rijn, J., 2013. Waste treatment in recirculating aquaculture systems. Aquac. Eng. 53, 49–56. doi:10.1016/j.aquaeng.2012.11.010

Verdegem, M.C.J., Bosma, R.H., Verreth, J.A.J., 2006. Reducing water use for animal production through aquaculture. Int. J. Water Resour. Dev. 22, 101–113. doi:10.1080/07900620500405544

Viadero, R.C., Noblet, J.A., 2002. Membrane filtration for removal of fine solids from aquaculture process water. Aquac. Eng. 26, 151–169. doi:10.1016/S0144-8609(02)00011-0

von Ahnen, M., Pedersen, P.B., Dalsgaard, J., 2016. Start-up performance of a woodchip bioreactor operated end-of-pipe at a commercial fish farm—A case study. Aquac. Eng. 74, 96–104. doi:10.1016/j.aquaeng.2016.07.002

Wallenius, K., Rita, H., Simpanen, S., Mikkonen, A., Niemi, R.M., 2010. Sample storage for soil enzyme activity and bacterial community profiles. J. Microbiol. Methods 81, 48–55. doi:10.1016/j.mimet.2010.01.021

Wanner, O., Gujer, W., 1984. Competition in biofilms. Water Sci. Technol. 17, 27–44. doi:10.2166/wst.1994.0769

Weeks, N.C., Timmons, M.B., Chen, S., 1992. Feasibility of using foam fractionation for the removal of dissolved and suspended solids from fish culture water. Aquac. Eng. 11, 251–265. doi:10.1016/0144-8609(92)90008-L

Wold, P.-A., Holan, A.B., Øie, G., Attramadal, K., Bakke, I., Vadstein, O., Leiknes, T.O., 2014. Effects of membrane filtration on bacterial number and microbial diversity in marine recirculating aquaculture system (RAS) for Atlantic cod (Gadus morhua L.) production. Aquaculture 422–423, 69–77. doi:10.1016/j.aquaculture.2013.11.019

Wong, C.K., Pak, I.A.P., Jiang Liu, X., 2013. Gill damage to juvenile orange-spotted grouper Epinephelus coioides (Hamilton, 1822) following exposure to suspended sediments. Aquac. Res. 44, 1685–1695. doi:10.1111/j.1365-2109.2012.03173.x

Wu, J., Le-Clech, P., Stuetz, R.M., Fane, A.G., Chen, V., 2008. Effects of relaxation and backwashing conditions on fouling in membrane bioreactor. J. Memb. Sci. 324, 26–32. doi:10.1016/j.memsci.2008.06.057

Zhang, T.C., Fu, Y.C., Bishop, P.L., 1994. Competition in Biofilms. Water Sci. Technol. 29, 263–270.

Zhu, S., Chen, S., 2001. Effects of organic carbon on nitrification rate in fixed film biofilters. Aquac. Eng. 25, 1–11. doi:10.1016/S0144-8609(01)00071-1

Paper I

de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Dalsgaard, J., 2019. Micro particles and microbial activity in Danish recirculating rainbow trout (Oncorhynchus mykiss) farms. Aquac. Eng. 84, 60–66. doi:10.1016/j.aquaeng.2018.12.001

Micro particles and microbial activity in Danish recirculating rainbow trout (*Oncorhynchus mykiss*) farms

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Abstract

Increasing intensities of water reuse in recirculating aquaculture systems (RAS) lead to a build-up of micro particles (< 20 µm) in the water. This build-up may have consequences for other water quality parameters and for the fish. This baseline study was carried out to determine the variation in micro particle levels (numbers, volume and surface area) and accompanying bacterial activity in commercially operated outdoor RAS, as well as the effects of different components in the recirculation loop on micro particle dynamics. Water samples were obtained during spring 2017 from 7 Danish Model Trout Farms (MTFs) producing rainbow trout (*Oncorhynchus mykiss*) in a total of 20 separate RAS units. Micro particle numbers and size distribution, bacterial activity, and inorganic and organic nutrient concentrations were analysed. Micro particle numbers ranged between $6.0 \cdot 10^4$ – $7.4 \cdot 10^5$ ml^{-1} and large variations were found between seemingly similarly operated RAS units within the same farm. There was a strong, positive correlation ($p < 0.001$) between micro particle levels and bacterial activity in the systems. Although not significant, biofilters generally seemed to trap particles whereas drum filters seemed to reduce particle volume while increasing particle numbers and surface area. The study sustains that bacterial activity in RAS is strongly associated with fine particle loading, and demonstrates for the first time the overall magnitude and level of variation in particle levels and bacterial activity that exists in commercially operated MTFs.

Keywords:

Micro particles

Bacterial activity

Recirculation aquaculture systems

Model trout farms

Water quality

1. Introduction

Recirculating aquaculture systems (RAS) are characterized by a build-up of different substances in the water phase including micro particles (particles < **20 µm;** Chen *et al.*, 1993). Micro particles derive from any faeces, feed spill, and biofilter slough off that are not removed from the system but broken down to smaller particles due to mechanical stress. Commonly installed filtration units do generally not remove micro particles that stay in suspension due to their size and low settling velocity. Previous studies have shown that an increase in the intensity of recirculation leads to an increase in the level of micro particles, and that particles tend to become smaller with higher intensity of recirculation (Shulin Chen et al., 1993; Patterson et al., 1999). Since smaller particles have a larger surface to volume ratio than larger ones, a partial break-down of particles results in an increase in surface area.

The build-up of micro particles in RAS significantly affects the water quality and may thus impact system and fish performance negatively by reducing overall water quality and fish health. Suspended organic solids acts as substrate for bacteria, increasing the biochemical oxygen demand (BOD) and CO_2 level in the water (Pedersen *et al.*, 2017). Furthermore, particles are speculated to damage fish gills directly or indirectly (Au *et al.*, 2004; Lu *et al.*, 2018). However, Becke *et al.* (2018) found no major effects on rainbow trout **(*Oncorhynchus mykiss*)** gills when exposing the fish to high suspended solids loads throughout a growout period.

Micro particles can provide surface area for bacterial growth (Attramadal *et al.*, 2012; Bullock *et al.*, 1997), and Pedersen *et al.* (2017) recently showed a correlation between micro particle surface area in RAS and bacterial activity. The findings indicated that the level of bacterial activity in semi-intensive RAS is considerably influenced by the surface area of micro particles, while the relationship seemingly breaks down in more intensive RAS presumably due to an accumulation of colloidal particles and/or free-living bacteria.

RAS are said to be microbially mature when microbial communities are close to the systems carrying capacity and dominated by slow growing bacteria that take part in nutrient fluxes (Attramadal *et al.*, 2012; Blancheton *et al.*, 2013). Unintended high loads of organic matter or unstable conditions may, on the other hand, provide space for fast growing, opportunistic bacteria that may proliferate within a short period of time (Skjermo *et al.*, 1997). A sudden development of fast growing, opportunistic, heterotrophic bacteria in RAS is of particular concern as many potentially pathogenic bacteria belong to this type of bacteria (Kari J K Attramadal et al., 2012; De Schryver and Vadstein, 2014). Consistent with these believed effects of particles, Wold *et al.* (2014) obtained a reduction in both suspended particles and bacterial activity when applying membrane filtration to an Atlantic cod (*Gadus morhua* L.) RAS unit, resulting in improved growth and survival of the cod larvae.

Most studies on micro particles have been conducted in experimental systems (e.g. Becke *et al.*, 2017; Brinker *et al.*, 2005a; Fernandes, 2015; Weeks *et al.*, 1992), and the few studies that have been conducted at commercial production facilities were conducted on a single farm only or were composed of data from different studies (Brinker *et al.*, 2005b; Pedersen *et al.*, 2017) making it difficult to assess general commercial scale conditions. The current study was carried out to assess the baseline level of micro particles and bacterial activity in outdoor Danish recirculating Model Trout Farms (MTFs; Pedersen *et al.*, 2003). Samples were obtained from multiple MTFs during the same time of year and analysed following standardized protocols allowing for a comparison of micro particle levels and bacterial activity across multiple commercial MTFs. Furthermore, samples were obtained from several positions within separate RAS units to evaluate the potential impact of different treatment components in the recirculating loop on micro particle dynamics.

2. Material and methods

2.1. Study sites and sampling

In order to minimize the impact of environmental variation on the samples, the study was conducted in a short period of time (1 month) during spring 2017. Grab samples were obtained from a total of 7 commercial MTFs distributed throughout the peninsula of Jutland, Denmark, sampling each farm once. The farms produced between 500 and 1200 t **rainbow trout per** year, and they each contained several separate RAS units. The RAS units were all composed of concrete raceways mounted with drum filters and sludge cones for particle removal, biofilters (either fixed bed, moving bed or a combination thereof), and airlifts that provided both aeration and flow of water (fig. 1).

All samplings were conducted early in the morning before feeding commenced. Due to different farm sizes, the number of RAS units sampled at each farm was different, ranging from two systems at the smallest farms to six systems at the largest farm. All in all, 20 RAS units were sampled. Water samples were in all systems obtained at the end of the production line just before the drum filters (sampling point 1 in fig. 1; n=20). This sampling point was assumed to represent the highest load of particles that the fish were exposed to. For two RAS units at each farm, additional samples were obtained before and after the biofilter as well as after the first airlift following the biofilter to assess the impact of the different treatment components in the recirculation loop on micro particle levels (sampling point 2-4 in fig 1; n=14).

Sub surface water samples were collected using a telescopic pole fitted with a 100 ml plastic beaker. Replicate samples were obtained at each sampling point, and the water samples were split in subsamples according to the component to be analysed (section 2.2).

2.2. Sample processing

Temperature, oxygen and pH were measured using a Hach HQ40d Portable Multi Meter (Hach Lange, Germany).

Aside for bacterial activity and particles above 10 µm which were measured on-site, water samples were stored or preserved as described in table 1 and transported back to the laboratory at the Section for Aquaculture, DTU Aqua in Hirtshals, Denmark. All samples were kept in a portable fridge at 4°C during transport. Here, they were analysed for the chemical oxygen demand (COD), 5 day biochemical oxygen demand (BOD_5), nitrate-N, and iron following standard methodology (summarized in table 1). Both total (unfiltered samples) and dissolved ($_{DISS}$; samples filtered through 1.6 µm glass microfiber filters (Whatman® GF/A, GE Healthcare)) BOD_5 and COD where measured, while the corresponding particulate fractions ($_{PART}$) were derived by subtracting the dissolved fraction from the total.

Micro particle numbers and size distributions were measured using two different instruments. An AccuSizer 780 SIS (Particle Sizing Systems, Santa Barbara, CA, USA) was used to measure particles between 10 and 200 µm following the procedure by Fernandes *et al.* (2014), while particles between 2 and 30 µm were measured using a Multisizer 4e Coulter Counter (Beckman Coulter, Inc, Indianapolis, USA). Both machines were calibrated utilizing the same standard solutions to ensure accurate and comparable measurements. Due to the different methodologies applied (the AccuSizer using a "Single Particle Optical Sensing technique" while the Multisizer 4e uses a "Coulter principle"), individual particle size distribution graphs were created for each sample demonstrating an overlap between the two instruments in the 10 - 20 µm size range (fig. 2). Results were therefore combined to give a complete particle size distribution between 2 - 200 µm with data from the AccuSizer used for particles between 10 and 200 µm, and data from the Multisizer used for particles below 10 µm. The complete particle size distribution was subsequently applied to calculate the β value summarizing the particle size distribution (Patterson *et al.*, 1999) of each RAS unit as described by Fernandes *et al.* (2014). The volume (V) and surface area (SA) of particles above 10 µm was calculated based on the diameter and number of particles measured by the AccuSizer assuming particles to be spherical. Similar values for particles between 1 and 10 µm were calculated automatically by the Multisizer 4e. The volume and surface area of each size class were calculated as: $V = 4/3\ \pi\ r^3\ x$ and $SA = 4\ \pi\ r^2\ x$, where V is the total volume of particles in a specific size class, SA is the total surface area of the particles in a specific size class, r is the radius of the particles, and x is the number of particles within the size class.

Bacterial activity was measured indirectly by the hydrogen peroxide (H_2O_2, HP) degradation rate (Arvin and Pedersen, 2015; Rojas-Tirado, 2018) following the protocols described by Tanner and Wong (1998) and Pedersen and Pedersen (2012). In brief, a 50 ml water sample was placed in a 100 ml plastic beaker on-site. The beaker was fixed in a thermal bath (20 °C) with a magnetic stirrer, stirring the sample at 250 rpm. 0.5

ml, 0.1% H_2O_2 (Merck KGaA, Germany) was added to the sample to obtain a nominal concentration of 10 mg l^{-1}, and the degradation of HP was measured over time by extracting a sample (1.8 ml) after 0.5, 15, 30, 45 and 60 min and placing it in a cuvette prefilled with a 0.2 ml fourfold strength HP reagent (Tanner and Wong, 1998) stopping any further degradation of H_2O_2. The residual HP concentration was subsequently derived by measuring the colour intensity on a spectrometer (Hach Lange DR 2800, Germany) at 432 nm. A first order reaction equation of the form $C_{(t)} = C_0e^{-kt}$, where $C_{(t)}$ is the concentration at time t (h), C_0 is the initial concentration, and k is the coefficient of degradation (h^{-1}), was fitted to the data using Microsoft Excel®.

2.3. Data analysis

Throughout the rest of the paper, particle data are described as: particles per ml (# ml^{-1}); volume of particles (mm^3 ml^{-1}); and surface area of particles (mm^2 ml^{-1}). All statistical analyses were performed using SigmaPlot 13.0 (Systat software Inc.), and differences were considered significant at $p < 0.05$. Data are presented as average ± standard deviation.

Particle numbers within each farm were compared using a paired t-test. Correlation analyses between micro particles and bacterial activity were conducted for the complete particle size distribution data (2 - 200 µm), as well as for particles below and above 10 µm to include and exclude bacteria (both free swimming and bioflocs), respectively (Rojas-Tirado, 2018), applying Pearson Product Moment correlation analyses.

Due to differences between systems and to allow for a comparison of the impact of different treatment components on micro particle levels, the micro particle data were normalized using the formula: x = (value after component/value before component) · 100. The results were averaged across the farms with a positive average representing an increase in the respective metric, and a negative value representing a reduction.

3. Results

Overall information of the farms sampled is presented in table 2 including stocking densities, the different BOD_5 and COD fractions, nitrate-N and iron concentrations, and the calculated β values. Because samples were obtained within a relatively short period of time (1 month), the environmental conditions were fairly stable across all farms with temperatures ranging between 8.5 - 11.5° C, pH ranging between 6.7 - 7.6, and oxygen concentrations fluctuating between 7.1 and 9.9 mg O_2 l^{-1}. No statistically significant differences were found between the farms except for the oxygen concentration at farm 5, which was higher ($p < 0.001$) than at the remaining farms (data not shown).

3.1. Micro particle levels

Micro particle levels measured with both instruments showed an overlap in the measured number of particles between 10 - 20 µm (fig. 2). The overlap was apparent for all samples with only minor variations. Micro particle numbers (2-200 µm) measured at the farms ranged between $6.0 \cdot 10^4 \pm 5.5 \cdot 10^3$ and $7.4 \cdot 10^5 \pm 5.5 \cdot 10^3$ # ml^{-1}, with an average concentration of $2.6 \cdot 10^5 \pm 1.9 \cdot 10^5$ # ml^{-1} across all systems measured (fig. 3). Except for farm 2 and 6, the number of particles differed significantly between the RAS units sampled within each farm ($p < 0.05$). There were no significant differences in β values between farms or RAS units within farms, β values ranging between 3.4 and 4.3 (table 2).

3.2. Micro particles and bacterial activity correlations

There was a strong, linear correlation between micro particle numbers, volume, and surface area, respectively and bacterial activity across all RAS units when the full set of data was used (i.e. particles between 2 - 200 µm; fig. 4), with correlation coefficients ranging between 0.74 and 0.83 ($p < 0.001$). When the correlation analysis was repeated using only data for particles above 10 µm (Fig. 4), the correlation between bacterial activity and micro particle volume decreased from 0.83 ($p < 0.001$) to 0.76 ($p < 0.001$), whereas the correlation between bacterial activity with particle numbers and with surface area increased (from 0.74 to 0.90, and from 0.80 to 0.92, respectively; $p < 0.001$). In comparison, correlations between bacterial activity and particle numbers, volume, and surface area, respectively in the smallest fraction (2 to 10 µm) were all lower compared to the two other fractions (0.73, 0.75, and 0.75, respectively; $p < 0.001$). There was no correlation between bacterial activity and $BOD_{5\text{-}DISS}$ ($r = 0.257$, $p = 0.186$, $n = 14$) or COD_{DISS} ($r = 0.116$, $p = 0.475$, $n = 20$). However, positive correlations were found between bacterial activity and $BOD_{5\text{-}PART}$ ($r = 0.553$, $p = 0.002$, $n = 14$), and between bacterial activity and COD_{PART} ($r = 0.77$, $p < 0.001$, $n = 20$). No correlation was found between iron concentrations and particles numbers in the systems sampled ($r = 0.11$, $p = 0.639$, $n = 20$).

3.3. Impact of the different components on the recirculation loop

The observed impact of the different treatment components in the recirculation loop on particle numbers, volume, and surface area was characterized by a very high variability and no significant differences could be detected across any of the components (fig. 5). There seemed, however, to be a trend of particle volume decreasing across drum filters while both particle numbers and surface area appeared to increase. In comparison, biofilters seemed to remove particles across all 3 metrics. The first air lift after the biofilter and the production unit itself had very similar impacts comprising a non-significant increase in particle volume.

4. Discussion

This study is the first to report and compare micro particle levels and bacterial activity across multiple commercial MTFs owned and managed by different operators. The results confirm that there is a general challenge of large variation between seemingly similar systems including more than one order of

magnitude differences in micro particle levels between MTFs. Nitrate-N was measured as an indicator of recirculation intensity (Pedersen *et al.*, 2012) and, along with the other measured chemical water quality parameters (table 2), sustained that the farms were relatively comparable in terms of recirculation intensity and organic matter loading. The large variation in particles between farms was therefore most likely due to operational factors such as different feed and feeding intensities, water exchange rates, stocking densities, biofilter back-wash routines, and internal hydrodynamics. Most of these parameters are, however, difficult to quantify accurately in the field. While such variation in particle numbers between farms could be expected, it was somewhat surprising that RAS units within the same farm, despite being run by the same personal and with very similar stocking densities and amounts of feed supplied, in several cases showed very high variation in micro particle levels and bacterial activity (e.g. farms 4, 5 and 7; fig. 3). This suggests that some individual system details, characteristics or operational conditions can cause considerable variations in water quality parameters.

In aquaculture systems, particle size-distributions follow an exponentially decreasing curve (the smaller the particle size the larger the number of particles) (Patterson *et al.*, 1999; Rueter and Johnson, 1995). As recirculation intensity increases so does the relative importance of smaller particles. Despite the large variation in particle numbers observed between farms and RAS units in the current study, the size distribution was fairly similar. This was confirmed by similar β values (3.89 ± 0.21; no statistical differences), sustaining that the intensity of recirculation was rather similar between systems.

Strong and positive correlations between micro particle volume as well as surface area and bacterial activity measured by H_2O_2 degradation were found in this study when the complete size-distribution of particles (i.e. 2 - 200 µm) was examined. By excluding particles below 10 µm (and thereby excluding free-swimming bacteria), a slightly stronger correlation between particle surface area and bacterial activity (r = 0.92 *versus* 0.80) was found (fig. 4).The results are consistent with those of Pedersen *et al.* (2017), examining the relationship between particle surface area and bacterial activity in aquaculture systems measured using Bactiquant®, and sustain that H_2O_2 degradation is a useful method for assessing bacterial activity in water samples. The aforementioned study suggested that particle surface area is one of the main factors affecting bacterial development in semi-intensive RAS as it provides a place for bacteria to settle, as well as a substrate on which they can grow.

One of the two RAS units sampled at farm 7 deviated substantially from the other systems by having a bacterial activity roughly 50 % above the second highest system measured. Replicate analyses all showed similar results ruling out sampling error, and we therefore speculate whether the high activity was due to a recent back-wash of the biofilter or whether the results indicate a proliferation of bacteria caused by some kind of unbalance in the system.

In the set of correlations, the number of particles correlated very strongly with bacterial activity (r = 0.90). Furthermore, positive correlations were found between bacterial activity and the particulate fractions of both BOD_5 (r =0.67) and COD (r = 0.84) indicating that most of the particles in the system were of organic nature. In contrast, none of the dissolved organic matter fractions (i.e. $BOD_{5\text{-}DISS}$ and COD_{DISS}) showed any significant correlation with bacterial activity, indicating that these parameters did not significantly influence overall bacterial activity in the systems. The dissolved organic fractions were probably composed of hard-to-degrade complex compounds, and/or free-living bacteria supposedly regulated by $BOD_{5\text{-}DISS}$ were not abundant in the systems (Rojas-Tirado, 2018).

Iron was measured as some farms were located in regions with elevated iron concentrations in the surface and ground water used as make-up water (author's pers. obs.). Iron salts are often used as a flocculent in wastewater treatment for removing suspended solids (Lee *et al.*, 2014) but had no obvious influence on particles in the current study, as no correlation between iron concentrations and micro particle levels was found.

As mentioned in the introduction, high levels of bacteria do not necessarily have adverse effects on the fish as long as conditions are stable. Rather, stable and microbially mature RAS have been proposed as a means of selecting for non-opportunistic bacteria improving the performance of marine larvae (Kari J K Attramadal et al., 2012; Skjermo et al., 1997). All farms visited in the current study applied intermittent disinfection using either hydrogen peroxide, peracetic acid, formalin, sodium chloride, or a combination thereof, and this practice most likely affected the microbial stability of the systems.

The effect of the different treatment components within the recirculation loop was difficult to assess due to the large variation observed, however, there seemed to be some general patterns (fig. 5). Drum filters generally appeared to remove particulate matter in terms of volume accompanied by a non-significant increase in particle numbers and surface area. This may indicate that while removing particulate matter, large shear forces within a drum filter cause a fragmentation of larger particles creating more and smaller particles and thereby more surface area. In comparison, biofilters appeared to work as particle traps reducing particle numbers, surface area, and volume. Some of the observed biofilter variation was probably due to different types of biofilters applied and modes of operation (fixed bed, moving bed, or a combination thereof). Under controlled conditions, Fernandes *et al.* (2015) showed that fixed bed biofilters tend to remove micro particles while moving bed biofilters tend to generate micro particles and similar effects were described by Åhl *et al.* (2006) and Ivanovic and Leiknes (2008). We were, however, unable to confirm this presumably due to the limited number of samples as well as internal system variations.

Air lifts and production units seemed to produce only minor changes in the number and surface area of particles, while both components seemed to increase the total volume of micro particles. In the case of

airlifts, an increase in particle volume might be explained by lifting of heavy/settled particles from the bottom of the raceways into the water column, while for production unit, fish excretion and potential feed spill might explain an increase in particle volume during passage. In both cases, the observations indicate that both RAS components mainly affect larger size particles resulting in an increase in particle volume, while they have only minor effect on particle numbers and surface area.

5. Conclusion

This baseline study showed that an increase in micro particle levels directly affects bacterial activity by providing surface area and substrate for bacteria. The impact of micro particles and corresponding bacterial activity on system performance and fish health, including for example different bacteria groups (slow growing strategic versus fast growing opportunistic bacteria) is, however, still not clear.

Empirical data collected from different MTFs within a short period of time demonstrated the magnitude of micro particles in RAS and underline the variation that exist between seemingly similar RAS units. Although care must to be taken regarding drawing conclusions from observations obtained under non-controlled conditions, the results on micro particle levels and bacterial activity are consistent with previous studies. Bacterial activity correlated strongly with micro particle levels within systems indicating that micro particle control may become a key management parameter in RAS.

Acknowledgement

The study was partly funded by the European Maritime and Fisheries Fund (EMFF) and the Danish Ministry of Food, Agriculture and Fisheries as part of project ”Vet-løsninger” (J.no. 33111-I-16-029).

Thanks to all fish farmers for allowing us access to their facilities and to the lab technicians Ulla Sproegel, Brian Moeller, and Melissa Lyng for all the support and help with both sampling in the field and concomitant lab work.

References

Åhl, R.M., Leiknes, T., Ødegaard, H., 2006. Tracking particle size distributions in a moving bed biofilm membrane reactor for treatment of municipal wastewater. Water Sci. Technol. 53, 33–42. doi:10.2166/wst.2006.205

Arvin, E., Pedersen, L.-F., 2015. Hydrogen peroxide decomposition kinetics in aquaculture water. Aquacult. Eng. 64, 1–7. doi:10.1016/j.aquaeng.2014.12.004

Attramadal, K.J.K., Salvesen, I., Xue, R., Øie, G., Størseth, T.R., Vadstein, O., Olsen, Y., 2012. Recirculation as a possible microbial control strategy in the production of marine larvae. Aquacult. Eng. 46, 27–39. doi:10.1016/j.aquaeng.2011.10.003

Au, D., Pollino, C., Wu, R., Shin, P., Lau, S., Tang, J., 2004. Chronic effects of suspended solids on gill structure, osmoregulation, growth, and triiodothyronine in juvenile green grouper Epinephelus coioides. Mar. Ecol. Prog. Ser. 266, 255-264. Doi: 10.3354/meps266255

Becke, C., Steinhagen, D., Schumann, M., Brinker, A., 2017. Physiological consequences for rainbow trout (Oncorhynchus mykiss) of short-term exposure to increased suspended solid load. Aquac. Eng. 78, 63–74. Doi:10.1016/j.aquaeng.2016.11.001

Becke, C., Schumann, M., Steinhagen, D., Geist, J., Brinker, A., 2018. Physiological consequences of chronic exposure of rainbow trout (*Oncorhynchus mykiss*) to suspended solid load in recirculating aquaculture systems. Aquaculture 484, 228–241. doi:10.1016/j.aquaculture.2017.11.030

Blancheton, J.P., Attramadal, K.J.K., Michaud, L., d'Orbcastel, E.R., Vadstein, O., 2013. Insight into bacterial population in aquaculture systems and its implication. Aquacult. Eng. 53, 30–39. doi:10.1016/j.aquaeng.2012.11.009

Brinker, A., Koppe, W., Rösch, R., 2005a. Optimised effluent treatment by stabilised trout faeces. Aquaculture 249, 125–144. doi:10.1016/j.aquaculture.2004.12.029

Brinker, A., Schröder, H.G., Rösch, R., 2005b. A high-resolution technique to size suspended solids in flow-through fish farms. Aquacult. Eng. 32, 325–341. doi:10.1016/j.aquaeng.2004.07.002

Bullock, G.L., Summerfelt, S.T., Noble, A.C., Weber, A.L., Durant, M.D., Hankins, J.A., 1997. Ozonation of a recirculating rainbow trout culture system. I. Effects on bacterial gill disease and heterotrophic bacteria. Aquaculture 158, 43–55.

Chen, S., Timmons, M.B., Aneshansley, D.J., Bisogni, J.J., 1993. Suspended solids characteristics from recirculating aquacultural systems and design implications. Aquaculture 112, 143–155. doi:10.1016/0044-8486(93)90440-A

De Schryver, P., Vadstein, O., 2014. Ecological theory as a foundation to control pathogenic invasion in aquaculture. ISME Journal 8, 2360-2368. doi:10.1038/ismej.2014.84

DIN 38405-D17. German standard methods for the analysis of water, waste water and sludge; Anions (Group D); Determination of borate ions (D 17). The German Institute for Standardization.

Fernandes, P.M., 2015. Interactions between micro-particles and the rearing environment in recirculation aquaculture systems. PhD Thesis. Section for Aquaculture, DTU Aqua, Technical University of Denmark, Hirtshals, Denmark.

Fernandes, P.M., Pedersen, L.F., Pedersen, P.B., 2014. Daily micro particle distribution of an experimental recirculating aquaculture system-A case study. Aquac. Eng. 60, 28–34. doi:10.1016/j.aquaeng.2014.03.007

Fernandes, P.M., Pedersen, L-F., Pedersen, P.B. 2015. Microscreen effects on water quality in replicated recirculating aquaculture systems. Aquacult. Eng. 65, 17-26. doi.org/10.1016/j.aquaeng.2014.10.007

ISO 5815-2:2003. Water quality - Determination of biochemical oxygen demand after *n* days (BOD*n*) – Part 2: Method for undiluted samples. The International Organization for Standardization.

ISO 6060:1989. Water quality - Determination of the chemical oxygen demand. The International Organization for Standardization.

ISO 7890-1:1986. Water quality - Determination of nitrate. Part 1: 2,6-Dimethylphenol spectrometric method. The International Organization for Standardization.

Ivanovic, I., Leiknes, T.O., 2008. Impact of aeration rates on particle colloidal fraction in the biofilm membrane bioreactor (BF-MBR). Desalination 231, 182–190. doi:10.1016/j.desal.2007.11.046

Lee, C.S., Robinson, J., Chong, M.F., 2014. A review on application of flocculants in wastewater treatment. Process Saf. Environ. Prot. 92, 489–508. doi:10.1016/j.psep.2014.04.010

Lu, C., Kania, P.W., Buchmann, K., 2018. Particle effects on fish gills: An immunogenetic approach for rainbow trout and zebrafish. Aquaculture 484, 98–104. doi:10.1016/j.aquaculture.2017.11.005

Patterson, R.N., Watts, K.C., Timmons, M.B., 1999. The power law in particle size analysis for aquacultural facilities. Aquacult. Eng. 19, 259–273. doi:10.1016/S0144-8609(98)00054-5

Pedersen, L.F., Pedersen, P.B., 2012. Hydrogen peroxide application to a commercial recirculating aquaculture system. Aquacult. Eng. 46, 40–46. doi:10.1016/j.aquaeng.2011.11.001

Pedersen, L.F., Suhr, K.I., Dalsgaard, J., Pedersen, P.B., Arvin, E., 2012. Effects of feed loading on nitrogen balances and fish performance in replicated recirculating aquaculture systems. Aquaculture 338–341, 237–245. doi:10.1016/j.aquaculture.2012.01.035

Pedersen, P.B., Grønborg, O., Svendsen, L.M., 2003. Modeldambrug: Specifikationer og godkendelseskrav: Rapport fra faglig arbejdsgruppe (in Danish). Danmarks Miljøundersøgelser Arbejdsrap nr 84.

Pedersen, P.B., von Ahnen, M., Fernandes, P.M., Naas, C., Pedersen, L.-F., Dalsgaard, J., 2017. Particle surface area and bacterial activity in recirculating aquaculture systems. Aquacult. Eng. 78, 18-23. doi.org/10.1016/j.aquaeng.2017.04.005

Rojas-Tirado, P.A., 2018. Microbial water quality within recirculating aquaculture systems. PhD Thesis. Section for Aquaculture, DTU Aqua, Technical University of Denmark, Hirtshals, Denmark.

Rojas-Tirado, P.A., Pedersen, P.B., Vadstein, O., Pedersen, L.-F., 2018. Changes in microbial water quality in RAS following altered feed loading. Aquacult. Eng. 81, 80-88. doi:10.1016/j.aquaeng.2018.03.002

Rueter, J., Johnson, R., 1995. The use of ozone to improve solids removal during disinfection. Aquacult. Eng. 14, 123–141. doi:10.1016/0144-8609(94)P4431-A

Skjermo, J., Saivesen, I., Øie, G., Olsen, Y., Vadstein, O., 1997. Microbially matured water: a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquacult. Int. 5, 13–28. doi:10.1007/BF02764784

Tanner, P.A., Wong, A.Y.S., 1998. Spectrophotometric determination of hydrogen peroxide in rainwater. Anal. Chim. Acta 370, 279–287. doi:10.1016/S0003-2670(98)00273-6

Weeks, N.C., Timmons, M.B., Chen, S., 1992. Feasibility of using foam fractionation for the removal of dissolved and suspended solids from fish culture water. Aquacult. Eng. 11, 251–265. doi:10.1016/0144-8609(92)90008-L

Wold, P., Holan, A. B., Øie, G., Attramadal, K., Bakke, I., Vadstein, O., Leiknes, T. O., 2014. Effects of

membrane filtration on bacterial number and microbial diversity in marine recirculating aquaculture system (RAS) for Atlantic cod (Gadus morhua L.) production, Aquaculture 422-423, 69-77. doi: 10.1016/j.aquaculture.2013.11.019

Figures

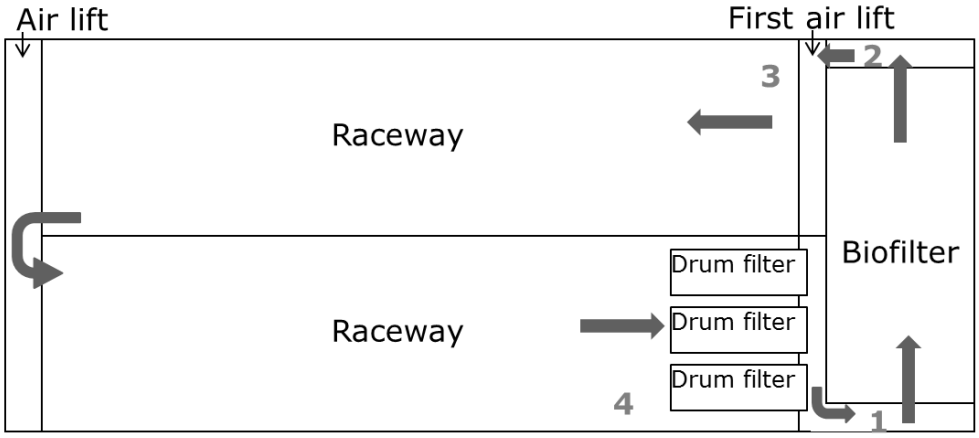

Figure 10: Simplified schematic representation of a Model Trout Farm (MTF) with the position of the different sampling stations (1 - 4). Comparison between samples collected before and after each component was used to assess its impact on micro particles. Arrows indicate the direction of the water flow.

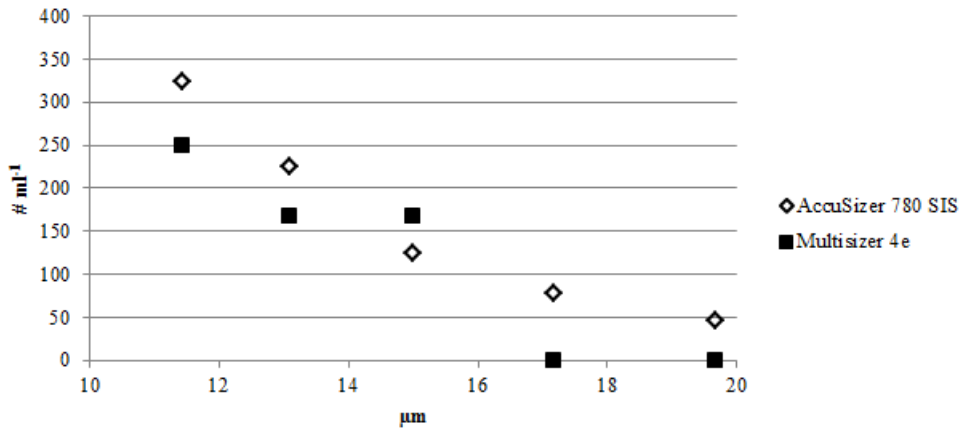

Figure 2: Example of the overlap in the 10-20 µm particle size span obtained with the Multisizer 4e and AccuSizer780 SIS

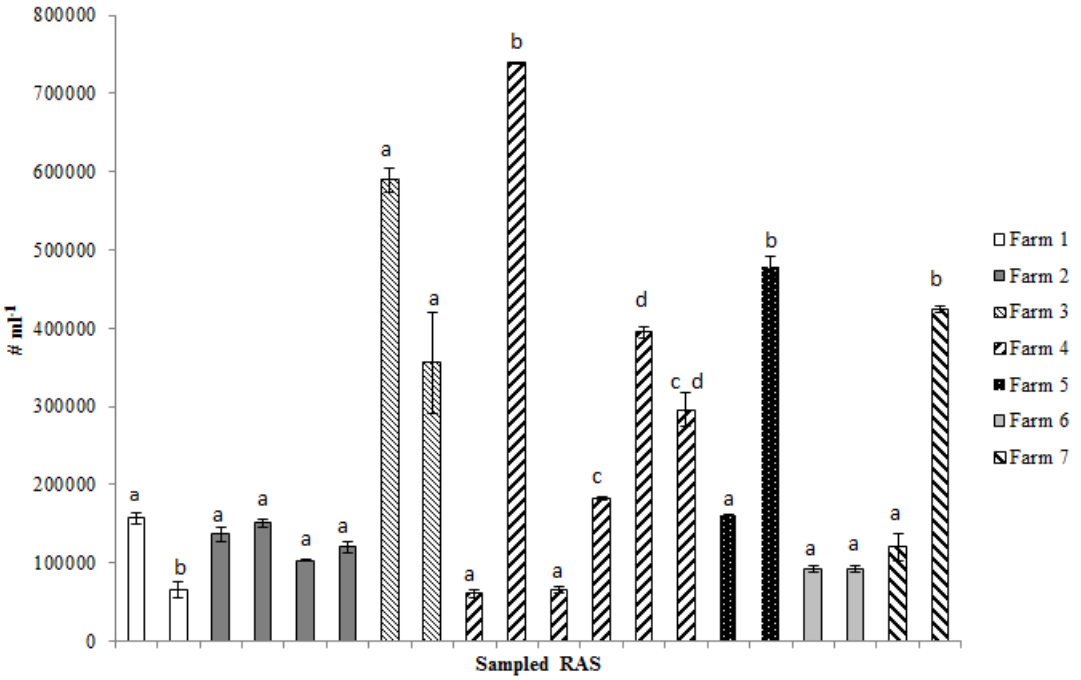

Figure 3: Number of particles (2 - 200 µm) per ml (average ± SD, n = 40) in the 20 RAS systems measured. Differently filled-in bars represent the different farms while individual bars represent a RAS unit. Different lower case letters above the bars denote significant differences between RAS units within the same farm.

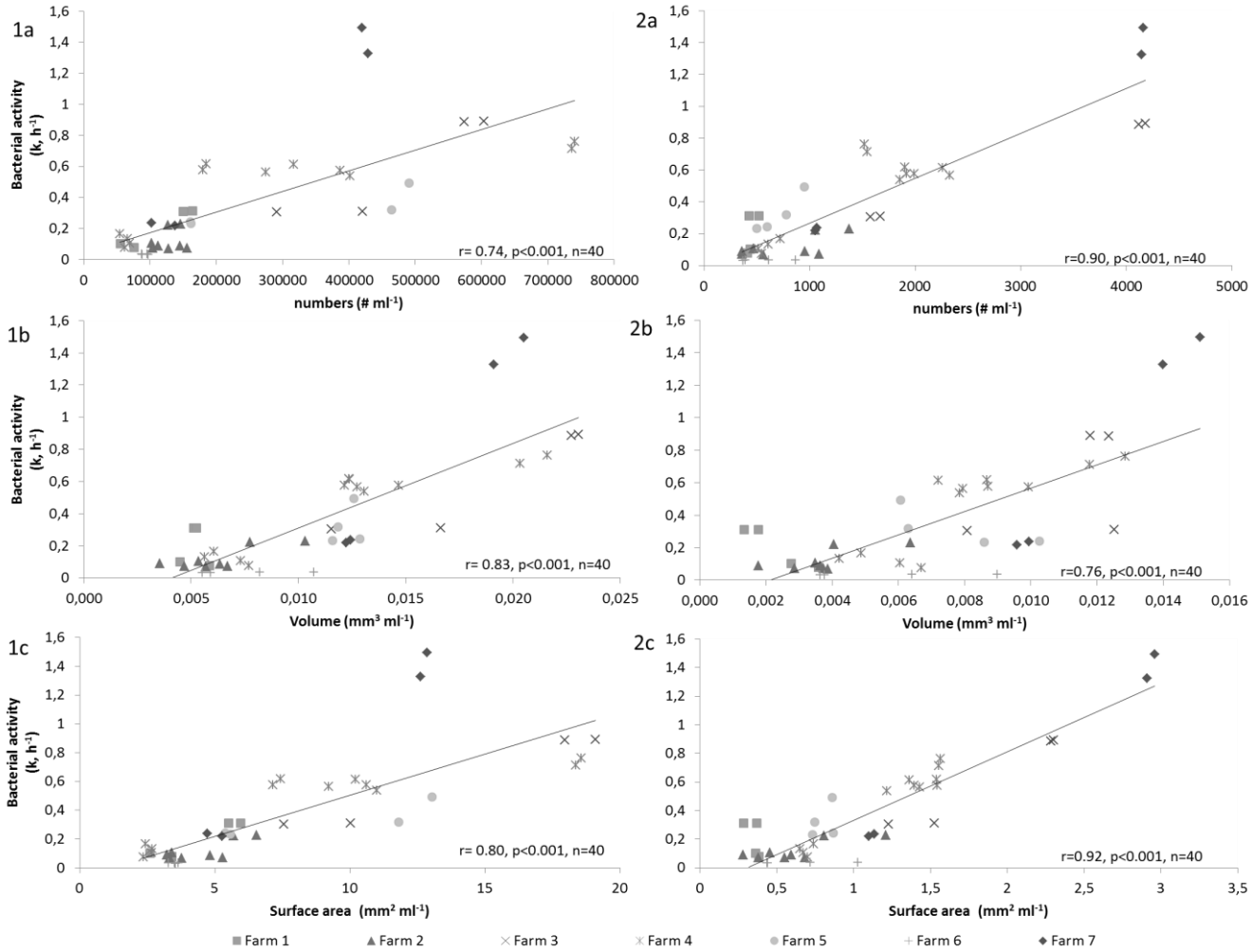

Figure 4: Correlations between bacterial activity and the different micro particle parameters measured: 1) Particles from 2 to 200 µm; 2) Particles from 10 to 200 µm. a) Number of particles; b) Volume of particles; c) Surface area of particles.

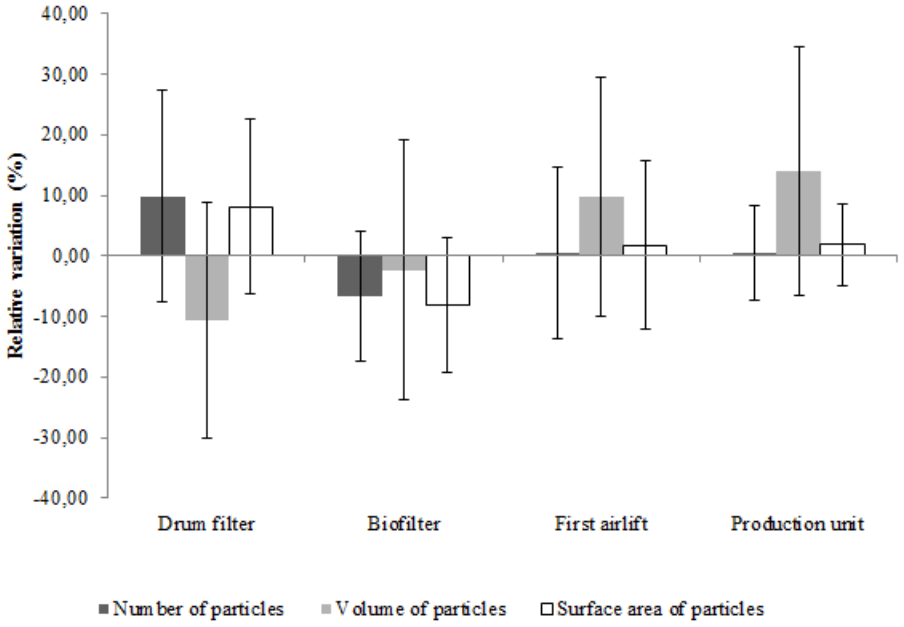

Figure 5: Relative effect (average ± SD, n = 28) of different components in the recirculation loop (drum filters, biofilters, first airlift, production unit) on micro particle levels. Positive values represent increasing values while negative values represent decreasing values. No statistical differences were found between the components.

Table 1: Water quality parameters measured and analytical methods applied

Parameter	Abbrev	Unit	Sample treatment and processing	Analytical Method	Reference
Total chemical oxygen demand	COD_{TOT}	mg O_2 l^{-1}	Unfiltered + acid addition, stored at 4°C	LCK 414 and LCK 314, Hach Lange, Germany	ISO 6060:1989
Dissolved chemical oxygen demand	COD_{DISS}	mg O_2 l^{-1}	Filtered through a 0.22 µm filter** + acid addition, stored at 4°C	LCK 414, Hach Lange, Germany	ISO 6060:1989
Particulate chemical oxygen demand	COD_{PART}	mg O_2 l^{-1}	N/A	$COD_{PART} = COD_{TOT} – COD_{DISS}$	N/A
Total biological oxygen demand after 5 days	BOD_{5-TOT}	mg O_2 l^{-1}	Unfiltered	Potientiometry/O2 probe (WTW Oxi 340i)	ISO 5815-2:2003
Dissolved biological oxygen demand after 5 days*	BOD_{5-DISS}	mg O_2 l^{-1}	Filtered through a 1.6 µm filter	Potientiometry/O2 probe (WTW Oxi 340i)	ISO 5815-2:2003
Particulate biological oxygen demand after 5 days	BOD_{5-PART}	mg O_2 l^{-1}	N/A	$BOD_{5-PART} = BOD_{5-TOT} - BOD_{5-DISS}$	N/A
Nitrate-nitrogen	NO_3-N	mg l^{-1}	Filtered through a 0.22 µm filter**, stored at 4°C	Colorimetry	ISO 7890-1:1986
Iron	Fe^{2+}	mg l^{-1}	Filtered through a 0.22 µm filter**, stored at 4°C	LCK 320, Hach Lange, Germany	DIN 38405-D17

* BOD_5 modified: samples were filtered through a 1.6 µm glass microfiber filter (Whatman® GF/A, GE Healthcare, UK) before analysis.

** 0.2 µm sterile syringe filter (Filtropur S 0.2, Sarstedt, Germany)

Table 2: Numbers of RAS units sampled on each farm and the ranges of the water quality parameters measured at the different farms including calculated ß-values

Farm	RAS units sampled	Approximate stocking density (kg_m^{-3})	COD_{DISS} ($mg\ O_2\_l^{-1}$)	COD_{PART} * ($mg\_O_2\ l^{-1}$)	$BOD_{5\text{-}DISS}$ ($mg\ O_2\ l^{-1}$)	$BOD_{5\text{-}PART}$ ($mg\ O_2\_l^{-1}$)	Nitrate ($mg\ N\_l^{-1}$)	Iron ($mg\ Fe{+}{+}\_l^{-1}$)	β value
1	2	35	14.5 – 16.4	0.7 – 1.3	2.6 – 4.1	2.0 – 2.7	9.0 - 14.0	0.04 - 0.07	3.4 - 3.8
2	4	40	18.6 – 24.5	0.4 – 1.5	2.6 – 4.3	4.7 – 5.9	5.0 - 13.5	0.24 - 0.41	4.0 - 4.1
3	2	30	29.7 – 33.0	5.0 - 6.8	4.3 – 5.6	8.7 – 9.3	6.5 - 18.5	0.30 - 0. 45	3.9 - 4.3
4	6	40	12.0 – 22.5	0.5 – 8.2	3.5 – 5.6	2.0 – 7.6	10.0 - 25.5	0.09 - 0.15	3.7 - 4.0
5	2	40	14.5 – 17.0	3.3 – 7.8	2.1 – 2.8	1.0 – 2.1	5.0 - 6.0	0.10 - 0.12	3.8 - 4.0
6	2	35	17.8 – 18.9	0.2 – 2.0	2.6 – 2.7	1.6 – 3.0	11.0 - 22.5	0.06 - 0.13	3.7 - 4.0
7	2	40	18.5 – 19.5	2.0 – 8.7	5.3 – 7.1	2.8 – 6.0	17.5 – 26.0	0.04 - 0.06	3.7 - 3.8

* COD and BOD_5 were measured in different subsamples explaining that COD_{PART} (calculated as the difference between COD_{TOT} and COD_{DISS}) was in some instances smaller than $BOD_{5\text{-}DISS}$

Paper II

de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Liu, D., Dalsgaard, J., 2020. UV irradiation and micro filtration effects on micro particle development and microbial water quality in recirculation aquaculture systems. Aquaculture 518, 734785. doi:10.1016/j.aquaculture.2019.734785

UV irradiation and micro filtration effects on micro particle development and microbial water quality in recirculation aquaculture systems

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Abstract

Recent studies have focused on micro particle build-up in recirculation aquaculture systems (RAS), and a correlation between micro particles and microbial activity has been shown. This study evaluated how micro particle build-up and microbial activity are affected by UV irradiation and micro filtration. Using 12 identical pilot scale RAS stocked with rainbow trout (*Oncorhynchus mykiss*), a two-factor factorial experiment was carried out testing in triplicate systems the effect of UV irradiation (systems with or without) in combination with cartridge filtration (1 or 200 µm pore size) on selected water quality parameters. The trial ran for 13 weeks. Water samples were obtained once a week, and the number and size distribution of micro particles was analysed. Microbial activity was derived from the hydrogen peroxide degradation rate, and concentrations of total and dissolved organic matter were measured as chemical oxygen demand (COD).

Overall, both UV and cartridge filtration had significant effect (<0.05) on micro particle distribution and microbial activity in the systems. By the end of the trial, a two-way Anova showed that UV treated RAS, independently of cartridge filtration pore size, were significantly ($p < 0.05$) lower in micro particle numbers (74% reduction), micro particle surface area (54% reduction), and dissolved COD (34% reduction) compared to systems without UV. Similarly, microbial activity was reduced up to 89% independently of cartridge filtration. UV thus appeared to reduce micro particle numbers by destroying bacteria. In addition, the effect of UV on dissolved COD suggested a possible feedback mechanism between microbial activity and substrate release in the systems. For micro filtration, a 1 vs. 200 µm pore size significantly reduced the number of micro particles (by 50%), micro particle volume (by 83%), and micro particle surface area (by 73%) independently of UV treatment. This was accompanied by a significant reduction in particulate COD (80%) and microbial activity (approximately 54% reduction independent of the use of UV). Hence, cartridge filtration appeared to reduce a build-up of micro particle by directly removing bacteria and bacteria substrate. In conclusion the study sustains that combining UV and particle removal is a potential viable tool for managing microbial water quality in RAS.

Introduction

Recent studies on micro particles in recirculating aquaculture systems (RAS) have focused on the build-up of micro particles in RAS and the correlation with microbial water quality (Becke *et al.*, 2018; de Jesus Gregersen *et al.*, 2018; Pedersen *et al.*, 2017; Fernandes *et al.*, 2016). Increasing recirculation intensity increases the level of micro particles (Patterson *et al.*, 1999), and micro particles below 20 µm may account for more than 90% of all particles in some systems (Fernandes *et al.*, 2014).

In RAS, micro particles are generated from organic waste including fish faeces and feed spill, and from biofilm released from surfaces and biofilters. Bacteria are closely linked to these micro particles and may even constitute the micro particle itself.

It is generally believed that micro particles are unwanted but the full implication of micro particles in RAS is still not well understood. Pedersen *et al.* (2017) described a strong, positive correlation between micro particle levels and microbial activity in semi-intensive RAS, suggesting that microbial activity is controlled by micro particle availability in such systems. Lu *et al.* (2018) studied the expression of different genes associated with immune response in rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) when exposed to inert styrofoam particles ranging from 0.2 to 90 µm in diameter. They found that smaller particles were more likely to build up in the gills and caused changes in gene expression. On the other hand, Becke *et al.* (2018) found no adverse effects of suspended solids on the growth of rainbow trout exposed to high levels of suspended solids in an 18 week RAS exposure trial.

To reduce the load of particulate organic matter and, consequently reduce microbial activity, a few strategies of either removing particles or avoiding their formation in the first place have been tested. Feed binders show good potential for increasing faecal stability, supposedly making them easier to remove and thereby reduce the formation of micro particles (Brinker, 2007). However, positive effects on water quality (physical, chemical, or biological changes) in RAS by adding feed binders have not yet been demonstrated. Another approach involved adding floating material to the fish feed in order to create floating faeces (Unger and Brinker, 2013). When floating faeces where tested in an experimental RAS, drum filters removal efficiency was twice as high in systems fed the experimental diet compared to a commercial control diet, however the impacts on water quality were less obvious (Schumann *et al.*, 2017).

Alternatively to modifying the feed, it may be possible to reduce the build of particle by UV irradiation or micro particle filtration. A fraction of micro particles in RAS is composed of free swimming bacteria (Franco-Nava *et al.*, 2004; Sharrer *et al.*, 2005), and the use of UV irradiation should reduce the total number of micro particles in the water by controlling bacteria development. UV irradiation degrades microbial DNA stopping bacteria from multiplying while having no direct impact on non-living / inert particles and dissolved organic matter (Timmons and Ebeling, 2010). While the use of UV irradiation can be expensive, it is a mature technology widely used in RAS and its application is fairly straight forward

(Summerfelt, 2003). Few, if any studies have demonstrated the impact of UV irradiation on microbial water quality dynamics in RAS. A recent study by Huyben *et al.* (2018) looked in to the application of UV and membrane filtration in a pilot scale RAS as a means to control bacterial load. The results obtained indicate that both technologies were capable of reducing heterotrophic bacteria by over 98% in a single pass set up, however, long term implications on the water quality were not studied.

Water quality effects of removing micro particles e.g. by microscreens or membrane filters have been widely studied. Fernandes *et al.* (2014) demonstrated significant differences in particulate parameters in RAS with and without microscreens (100, 60 and 20 µm). Albeit the time to reach equilibrium seemingly increased with increasing mesh size, particle numbers, particles surface area or particle volume in the three microscreen groups (100, 60 and 20 µm) did not significantly differ at the end of the trial. Using ultrafiltration, Wold *et al.* (2014) obtained a large reduction of micro particles in a cod larvae (*Gadus morhua*) RAS, as well as a significant reduction in the number of bacteria. However, application of this type of filtration to full scale RAS is considered complicated and costly (Viadero and Noblet, 2002; Wu *et al.*, 2008).

The objective of the currents study was to evaluate the extent to which micro particles and microbial activity in RAS could be controlled by the individual and / or combined use of UV irradiation and micro filtration. To this purpose a two-factor factorial experiment was carried out testing pilot scale RAS with or without UV irradiation and with two levels of micro filtration (1 µm and 200 µm).

2. Materials and methods

2.1. Experimental setup

The trial was conducted in twelve identical 1.7 m^3 pilot scale RAS (fig. 1). Each RAS consisted of a 500 l rearing tank, a swirl separator, a 300 l pump sump, a 800 l submerged fixed bed biofilter, and a trickling filter. The fixed bed biofilter and trickling filter were filled with BIO-BLOK 150® elements (Expo-net, Hjørring, Denmark). An Aqua Medic Ocean Runner 6500 (Aqua Medic, Bissendorf, Germany) pump provided the flow in to the biofilter. After the trickling filter, a flow of 1500 l h^{-1} was diverted in to the rearing thanks, while the remaining water overflowed back in to the pump sump, resulting in a hydraulic retention time (HRT) of approximately 20 minutes in the rearing tanks.

All systems where fitted with a cartridge filter housing treating a side stream of the pump sump flow. Six RAS were fitted with a 10” filter housing and a 1 µm cartridge filter (Ultra-Depth PP-TF 1,00 µm 10" DOE, Ultrafilter Skandinavien ApS, Denmark), while the other six RAS were fitted with an 10” filter housing and a 200 µm cartridge filter (Ultra-Pure Sleeve - Nylon 200 µm 10", Ultrafilter Skandinavien ApS, Denmark). The 200 µm cartridge filters were chosen to mimic commercial RAS conditions where drum filters are typically applied to remove large particles. Three of the RAS units fitted with either a 1 µm or a 200 µm cartridge

filter were also fitted with a 75W UV filter (Pond filter, China) placed in series after the cartridge filter. The combination of cartridge and UV filters resulted in four treatment groups in triplicate randomly distributed throughout the 12 RAS: 1 µm cartridge filter with UV (1µm + UV), 200 µm cartridge filter with UV (200µm + UV), 1 µm cartridge filter without UV (1µm ÷ UV), and 200 µm cartridge filter without UV (200µm ÷ UV). Water was pumped through the cartridge filter and UV filter using a 2200 l h^{-1} pump (Selekta 350W, Harald Nyborg, Denmark).

All systems were in operation prior to the trial and biofilters were therefore already activated. At start up, all systems were emptied, tanks cleaned, and fresh tap water was added in order to follow the temporal development in different water quality parameters. Each system was stocked with 12.5 ± 0.1 kg juvenile rainbow trout receiving 250 g feed day^{-1} (Efico, Biomar, Denmark) fed during 12 hours using automatic belt feeders. A daily water exchange (make-up water, MUW) of 80 l day^{-1} was applied, resulting in a feed loading of 3.1 kg m^{-3} (or 0.32 m^3 MUW kg^{-1} feed). Bicarbonate was added when needed to keep pH between 7.2 and 7.5. Any dead fish were removed daily and their weight noted. Swirl separators were emptied each morning and any feed spill recorded and enumerated.

The trial ran for 13 weeks and grab samples of the water in each of the 12 RAS were collected once a week. Cartridge filters and UV filter units were started after the first set of water samples were obtained, representing “system specific” time zero samples unaffected by treatments. During the first 6 weeks of the trial, all cartridge filters were swapped and cleaned once a day. However, as systems developed it became necessary to increase the capacity of the microfilters, increasing the filter housing from 10” to 20” (Ultra-Depth PP-TF 1,00 µm 20" DOE, Ultrafilter Skandinavien ApS, Denmark) while maintaining cartridge filter mesh size at 1 and 200 µm. Furthermore, the daily cleaning routine was increased from one to three times a day in systems fitted with 1 µm cartridge filters to ensure a proper water flow through the filters. The serial placement of the UV lamp after the mechanical cartridge filters was made to better simulate a typical RAS set up. However, after a few weeks, clogging of the filters caused the water flow also to the UVs to be reduced, which was reflected by in the results (larger fluctuations) experienced during weeks 5 and 6.

2.2. Water sampling and analysis

Water sampling started two days after fish were added to the systems to avoid particulate matter re-suspension related to the handling of fish. Grab samples were collected at the top of the swirl separators as indicated in figure 1. A 5 l sample was collected in each RAS and spilt into homogeneous subsamples for individual analysis. Temperature, oxygen, and pH were measured in the swirl separators in the morning before daily routines using a Hach HQ40d Portable Multi Meter (Hach Lange, Germany).

Micro particle numbers and size distribution were measured using an AccuSizer 780 SIS (Particle Sizing Systems, Santa Barbara, CA, USA) for particles between 10 and 200 µm following the procedure described

in Fernandes *et al.* (2014). Furthermore, particles between 1 and 30 µm were measured using a Multisizer 4e Coulter Counter (Bechman Coulter, Inc, Indianapolis, USA). Results from the two measurements were subsequently combined to give a complete particle size distribution in the size range 1-200 µm as explained and verified by de Jesus Gregersen *et al.* (2018).

Particles were grouped in size classes as described in Patterson *et al.* (1999). Volume (V; mm^3 ml^{-1}) and surface area (SA; mm^2 ml^{-1}) of particles within each size class was calculated (assuming spherical particles) using the equations: $V = 4/3\ \pi\ r^3\ x$, and $SA = 4\ \pi\ r^2\ x$, where V is the total volume of particles in a specific size class, SA is the total surface area of the same particles, r is particle radius within the size class, and x is the number of particles within that size class. The total particle numbers (PN), the total particle volume (PV), and the total particle surface area (PSA) for the full range measured (1-200 µm) was calculated by summing the contribution from the different size classes.

To compare systems, particle size distributions were summarized by the β value conceived by Patterson *et al.* (1999) and further described by Fernandes *et al.* (2014). In short, the β value is the slope of the log-log transformed relationship between the number of particles within size classes and the corresponding size class medians. A lower β value indicates a system dominated by larger particles whereas a higher β value indicates a system dominated by smaller particles.

Microbial activity was quantified using the hydrogen peroxide (H_2O_2) decomposition rate assay with the degradation rate constant (k_{1a}, h^{-1}) used to quantify the microbial activity (Pedersen *et al.*, 2019). This method estimates the "rate of microbial activity" based on the degradation of H_2O_2. The faster the degradation of H_2O_2, the higher the microbial activity.

Nitrate-N and chemical oxygen demand (COD) concentrations were measured spectrophotometrically following ISO 7890-1 (1986) and ISO 6060 (1989), respectively. The chemical oxygen demand was measured in non-filtered (COD_{TOT}) and filtered (COD_{DISS}) samples (0.45 µm filter, Filtropur S 0.2, Sarstedt, Germany), and particulate COD (COD_{PART}) was calculated as the difference between the two.

All samples were collected and analysed in duplicate.

2.3. Data analysis

All data were transformed into moving averages of three succeeding sampling points (e.g. data from week one corresponds to the average of the data collected at time 0, 1 and 2). Statistical analyses were performed in SigmaPlot 13.0 (Systat software Inc., USA). Results of the two main factors (UV irradiation and cartridge filtration) were compared by the end of the trial (on average values of weeks 11, 12 and 13) using two way ANOVA followed by a Holm-Sidak test in case of significant differences. In addition, Pearson Product Moment correlation analyses were carried out to test for correlation between volume of particles

and COD_{PART}, COD_{DISS} and microbial activity, COD_{TOTAL} and microbial activity, and number of particles and microbial activity. Differences were considered significant at $p < 0.05$.

All data are presented as average ± standard deviation unless otherwise stated.

3. Results

There was no cooling of the systems and the temperature therefore fluctuated between a minimum of 15.8 ± 0.5 °C to a maximum of 21.3 ± 0.5 °C. Fish mortality ranged between 0.1 and 0.3 fish day^{-1} with no significant difference between systems or treatments. Oxygen saturation fluctuated between 75 and 105%, pH fluctuated between 7.1 and 7.5, and nitrate-N ranged between 91.5 and 98.2 mg l^{-1} at the end of the trial independent of treatment groups ($p > 0.05$).

3.1. Microparticles

3.1.1. Particle numbers

The number of micro particles increased immediately after start in the 200µm ÷ UV treatment systems and stabilized after approximately 5 weeks of operation at about 3 million particles ml^{-1} (fig. 2a). In comparison, the other treatment groups remained low for the first 7 weeks before starting to diverge. The 1µm ÷ UV treatment group increased to approximately 2 million particles ml^{-1} by week 10, while the 200µm + UV and the 1µm + UV reached < 0.5 and 1 million particles ml^{-1}, respectively, after 12 weeks. By the end of the trial, both UV and cartridge filtration were found to affected particle numbers. Systems with UV treatment had significantly less particles than systems without UV irradiation and systems fitted with 1 µm cartridge filters had significantly less particles than systems using 200 µm cartridge filters (table 1).

3.1.1. Particle volume

Particle volume in systems fitted with 200 µm cartridge filters steadily increased about 3 weeks into the study and ended at 0.04 ± 0.01 and 0.05 ± 0.01 mm^3 ml^{-1}, respectively in the 200µm + UV and 200µm ÷ UV treatment systems (fig. 2b). Particle volume in systems with 1 µm cartridge filters transiently increased in week 3-6 presumably due to limited filtration capacity (section 2.1). After adjusting filtration capacity, particle volume decreased and stabilized at 0.007 ± 0.002 and 0.009 ± 0.001 mm^3 ml^{-1}, respectively in the 1µm + UV and 1µm ÷ UV systems. By the end of the trial, cartridge filtration had reduced particle volume significantly, being much lower in systems fitted with 1 vs. 200 µm cartridge filters. UV irradiation on the other hand had no significant effect on particle volume (table 1).

3.1.2. Particle surface area

Particle surface area mirrored, to some extent, the development in particle volume (fig. 2c). Treatment 200µm ÷ UV increased immediately after start and reached 38.2 ± 4.8 mm^2 ml^{-1} at the end of the trial. In the 200µm + UV group the increase were less pronounced and PSA ended at 23.5 ± 5.2 mm^2 ml^{-1}. In the 1µm ÷ UV group, PSA ended at 13.7 ± 2.8 mm^2 ml^{-1} while seemingly no increase in PSA occurred in the 1µm + UV treatment group despite a transient increase due to limited filtration capacity halfway through the

trial. A two-way Anova by the end of trial showed that both treatment factors affected PSA significantly and independently of each other, i.e., UV vs. no UV treatment and 1 µm vs. 200 µm cartridge filtration both reduced PSA in the systems (table 1).

3.1.3. β values

Particle size distributions were significantly affected by the use of UV, whereas cartridge filtration size had no effect on the distribution (table 1). Hence, β values in systems with UV were significantly lower (3.71 ± 0.12 and 3.77 ± 0.08, respectively, for 1µm + UV and 200µm + UV systems) than β values in systems without UV treatment (3.87 ± 0.10 and 3.86 ± 0.10, respectively, for 1µm ÷ UV and 200µm ÷ UV systems).

3.2. Microbial activity

Microbial activity was the only metric where there was significant interaction between UV treatment and cartridge filtration (table 1 and fig. 2d). While this interaction prevents any conclusions about main effects, the use of ultraviolet irradiation kept microbial activity low throughout the trial, except for a transient increase while the 1 µm filters were under-dimensioned, irrespective of filtration. Hence, microbial activity in UV treated systems was by the end of the trial very similar to the activity at the start of the trial (averaging 0.04 ± 0.02 vs. 0.03 ± 0.001 h^{-1}, respectively). In systems without UV irradiation, 1 µm cartridge filters reduced microbial activity by approximately 50% compared to systems with 200 µm cartridge filters (0.25 ± 0.07 vs. 0.52 ± 0.1 h^{-1}, respectively). Comparing systems with either 1 or 200 µm cartridge filters, UV filtration significantly lowered microbial activity.

Microbial activity was highly correlated with particle numbers in all treatment systems (correlation coefficients (r) ranging between 0.87 and 0.95; table 2), as well as with COD_{DISS} (r ranging between 0.61 and 0.81; table 2) and COD_{TOTAL} (r ranging between 0.65 and 0.83; table 2)

3.3. COD

Dissolved COD increased significantly slower in systems with UV compared to systems without (fig. 2e). Cartridge filtration had no impact on COD_{DISS} build-up, and there was no interaction between the two main treatment factors (table 1). All treatments, except 1µm + UV, seemed to be still increasing by the end of the trial. At this point, COD_{DISS} in 1µm + UV and 200µm + UV treated systems amounted to 18.8 ± 1.5 and 25.5 ± 2.9 mg l^{-1}, respectively, while COD_{DISS} in treatments without UV averaged 33.5 mg l^{-1}.

Particulate COD largely reflected the development in PV (fig. 2f vs. 2b). This was supported by an overall correlation coefficient of 0.96 between the two parameters (table 2). Particulate COD was only slightly affected (no statistical significance) by UV irradiation whereas 1 µm cartridge filtration reduced COD_{PART} significantly compared to 200 µm cartridge filtration (table 1 and fig. 2f). Particulate COD in treatment 200µm ÷ UV rose steadily from the beginning of the trial to a final value of 20.7 ± 6.6 mg l^{-1}, followed by 200µm + UV averaging 16.2 ± 2.8 mg l^{-1}. In comparison, treatments with 1 µm cartridge filters ended at an average of 3.7 ± 0.7 mg l^{-1}.

4. Discussion

4.1. Cartridge filtration effects

The use of 1 µm compared to 200 µm cartridge filters resulted, as expected, in a significant removal of particles by volume (~83%) accompanied by a large reduction in COD_{PART} (~80%). At the same time the microbial activity was reduced by approximately 50% in 1 µm compared to 200 µm filtered systems. However, due to the interaction between cartridge filters and UVs with respect to microbial activity, it's not possible to determine to what extent this result were caused by the cartridge filters. The reduction in microbial activity fits well with a removal of suspended solids serving both as microbial substrate and surface area for bacteria (Pedersen et al., 2017b). Besides the removal of substrate, the cartridge filters may also have resulted in a direct removal of free-living bacteria from the water. Using membrane filtration, Wold *et al.* (2014) found that water treated with a 50 nm membrane filter contained 80 % less bacteria than a control systems without filter, and that this was accompanied by increased growth and survival rate of cod larvae. In the current study, the reduction in microbial activity was less pronounced. This was probably a consequence of using 1 µm vs. 50 nm membrane filters since 1 µm filters may not remove all planktonic bacteria and colloidal particles. Additional explanations relate to different study setups and different methods of measuring bacteria / determining bacterial activity (H_2O_2 degradation assay in the present study vs. flow cytometry in the study by Wold *et al.* (2014)). Micro filtration can reduce the load of bacteria in an aquaculture system either directly or indirectly. The reduction in most metrics in the current study significantly relating to cartridge filtration (i.e., PN, PV, PSA, and COD_{PART}) indicates that 1 vs. 200 µm cartridge filtration led to a reduction in the systems microbial carrying capacity (Attramadal *et al.*, 2012a; Skjermo *et al.*, 1997). Systems are considered microbially stable when microbial activity is close to the maximum that the system can sustain based on the amount of substrate (organic matter) available. Bacterially stable systems have proven advantageous for breeding Atlantic halibut (*Hippoglossus hippoglossus*) and Atlantic cod (*Gadus morhua*) larvae (Attramadal *et al.*, 2012a; Vadstein *et al.*, 1993). Removal of particulate organic matter is also considered an advantage by, for example, reducing the scope for heterotrophic growth, as well as reducing the potential for deposition and clogging in the system.

The results obtained here indicate that the cartridge filters had both direct impacts (removal of free swimming bacteria and bacterial biofilm) and indirect impact by removing substrate for bacteria to grow on.

4.2. Effects of UV irradiation

The antimicrobial effect of UV irradiation is well documented (Emerick *et al.*, 1999; Loge *et al.*, 1999; Sharrer *et al.*, 2005; Summerfelt, 2003; Timmons and Ebeling, 2010), while the impact of UV irradiation on micro particles is less described.

UV treatment in the current study resulted in significantly fewer particles in the water compared to equivalent RAS without UV (fig. 2a) as well as in a reduction in microbial activity (comparing within same cartridge filter pore size; fig. 2d). For example, 1µm + UV treatment systems contained approximately 84% fewer particles and 70% less PSA than 1µm ÷ UV systems at the end of the trial. These reductions were accompanied by 90% less microbial activity in 1µm + UV. Similar differences were observed between 200µm + UV vs. 200µm ÷ UV systems and sustains that UV reduces the number of particles presumably by killing bacteria.

A low HRT in system rearing tanks (approximately 20 minutes) also reduced the likelihood of bacteria multiplying within the tanks when not exposed to UV disinfection. This is supported by the results as samples were obtained just after the rearing units and before the UV disinfection, as well as the fact that bacteria activity in UV treated systems by the end of the trial was similar to that in the start of the trial.

Previous studies have shown that large amounts of particles in the water reduces the efficiency of UV (Carré *et al.*, 2018; Qualls *et al.*, 1983). This was not the case in the current study, where microbial activity levels in both 1µm + UV and 200µm + UV treated systems remained low throughout the experiment despite relatively high numbers of particles. This discrepancy may relate to the use of UV from the start of the trial. In previous aquaculture related studies evaluating UV efficiency, UV has typically been applied to water samples only after they contained high levels of bacteria. Under such conditions, bacteria in biofilms attached to particle surfaces are less exposed, and this may partly protect them from the effects of UV irradiation (Emerick *et al.*, 1999; Loge *et al.*, 1999). In the current study, bacteria where subjected to constant UV dosing from the very start which probably prevented them from forming large biofilm formations on particles. Furthermore, the applied UV dose was capable of delivering up to approximately 100 mW-s cm^{-2} (62% UV transmission in the cleaner / less turbid tanks), and was thus over-dimensioned for the systems. Commercial facilities generally use UV systems producing only 30-35 mW-s cm^{-2} (Lekang, 2007). While the dose applied in the current study was high, it was still within the range applied by Sharrer *et al.* (2005) using doses of up to 1800 mW-s cm^{-2} in pilot-scale RAS, and obtaining up to 98% reduction in heterotrophic bacteria counts. In that same study, a 300 mW-s cm^{-2} dose resulted in an 81% reduction in total heterotrophic bacteria counts. This is quite similar to the reduction obtained in the current study (approximately 88% reduction when comparing within the same cartridge filter pore size) although direct comparisons are hampered by different bacteria measuring techniques. All together, the results sustain that the reduction in micro particles in systems treated with UV is mainly due to a reduction in bacteria. This hypothesis is further reinforced by the fact that the reduction in particle numbers and surface area mainly happened in the smallest size fraction, as seen from significantly smaller β values in UV treated

systems indicating that the particle size distribution changed towards relatively larger particles (Patterson *et al.*, 1999).

A further effect of UV was a reduction in both COD fractions (although only statistically significant on dissolved COD). While a reduction in particulate COD was anticipated as an effect of finer mesh cartridge filtration, a reduction in the particulate and especially in the dissolved COD fraction in UV treated systems was less expected. Microbial organic matter (organic matter which makes up the body of bacteria) constitutes part of COD_{PART} and therefore a reduction in bacterial numbers will also eventually reduce COD_{PART}. UV should on the other end have no direct impact on dissolved COD and we speculate whether the reduction relates to a lower microbial activity. Keeping bacterial activity in the water at a low level might result in a slower degradation of particulate matter and thereby, a slower transition of organic matter from the particulate to the dissolved fraction (Henze *et al.*, 1997). The positive correlation between microbial activity and COD supports this explanation (table 2). It is also likely that part of the COD was consumed and thus stored inside the biofilter due to reduced competition from free swimming bacteria making more organic matter available to bacteria in the biofilm. A slower degradation of particulate organic matter may additionally resulted in a positive feedback in a RAS by increasing the efficiency of mechanical removal devices, which could help to explain the differences in particle numbers seen between treatments. However, further research is needed to resolve this, including an assessment of the organic matter removed, as well as an assessment of the biofilter condition over the course of the trial.

The reduction in most measured parameters caused by UV irradiation, including especially the reduction in COD_{DISS}, suggests that much like cartridge filtration, UV lead to a reduction in system carrying capacity. However, while UV may have led to a reduction in the system carrying capacity of the water by reducing easily available organic matter in the water phase, it also selectively eliminated bacteria from the water phase, potentially pushing the microbiota below the carrying capacity of the systems and causing a shift in bacterial communities**.** While such changes in bacterial communities were shown to have negative effects on cod larvae (Attramadal *et al.*, 2012b), similar effects on rainbow trout remain to be studied.

Hence, while a reduced microbial activity and less particulate matter may generally be perceived as positive, it could be hazardous in case of UV failure suddenly making a part of the system carrying capacity available for fast growing bacteria. The balance between UV irradiation and microbial carrying capacity is thus important considering potential effects of a UV failure.

As discussed in the introduction, part of the total micro particles in RAS is composed of bacteria. The results obtained by the use of intensive UV reducing PN by approximately 84% suggest that, at least by numbers, the proportion of bacteria in the very small fraction of micro particles is very high in intensive RAS, since the dose of UV applied should only affect the living cells.

4.3. Combined effects

The combination of UV treatment and microfiltration had synergistic effect on microbial activity. The combined effects of UV and cartridge filtration were probably a result of direct, mechanical bacteria removal and removal of substrate, as well as inactivation of bacteria due to UV exposure.

A combination of UV and 1 µm cartridge filtration produced the best results including approximately 88% removal in all micro particle metrics (number, volume, and surface area), 86% reduction in COD_{PART}, 44% reduction in COD_{DISS}, and 95% reduction in microbial activity. These results not only suggest a large reduction in the microbial carrying capacity, but potentially also a reduction in system oxygen consumption and CO_2 accumulation. In addition, a large reduction in particulate matter could potentially reduce the risk of clogging and sludge deposition in the RAS loop.

4.4. General system performance

Fish mortality did not differ between treatment groups and were presumably primarily related to the relatively high temperatures (up to 22 ^{o}C) experienced during the trial. Similarly, nitrate levels did not vary across treatments sustaining that all systems were operated at similar recirculation intensity, and also that the use of UV and cartridge filtration had no impact on the accumulation of nitrate.

5. Conclusion

Overall, this study showed that the use of either UV treatment or 1 µm cartridge filtration significantly improved the water quality in pilot-scale RAS, with reductions across all measured water quality metrics. The use of 1 µm cartridge filtration especially reduced the amount of particulate organic matter, while UV treatment led to a large reduction in microbial activity and micro particle numbers. Furthermore, the use of UV reduced the amount of dissolved COD, suggesting a potential feedback mechanism between microbial activity and the amount of dissolved organic matter in a system. The results suggest that 1 µm filtration controlled the amount of micro particles and bacteria by direct removal thereof, but also by the removal of substrate used by bacteria. In comparison, UV appears to reduce the development of micro particles trough the inactivation of bacteria in the water and in addition, by potentially reducing the availability of dissolved substrate for microbial growth.

The use of 1 µm cartridge filtration is, however, not realistic in most commercial applications where other techniques and technologies must be applied / developed if similar micro particle removal is to be achieved. While UV may not be the first choice for addressing micro particle removal in RAS, the positive impact of the applied UV dose on micro particle levels, dissolved organic matter concentrations, and microbial activity in conjunction with their availability and ease of use, makes UV in combination with fine mechanical filtration a promising tool for managing and improving water quality in RAS.

Acknowledgement

The study was partly funded by the European Maritime and Fisheries Fund (EMFF) and the Danish Ministry of Food, Agriculture and Fisheries as part of project ”Vet-løsninger” (J.no. 33111-I-16-029).

Thanks to Ole Larsen and Rasmus Jensen for all their help taking care of the fish and systems, and to Ulla Sproegel, Brian Moeller and Melissa Lyng for all their assistance with the lab work.

References

Attramadal, K.J.K., Salvesen, I., Xue, R., Øie, G., Størseth, T.R., Vadstein, O., Olsen, Y., 2012a. Recirculation as a possible microbial control strategy in the production of marine larvae. Aquac. Eng. 46, 27–39. doi:10.1016/j.aquaeng.2011.10.003

Attramadal, K.J.K., Øie, G., Størseth, T.R., Alver, M.O., Vadstein, O., Olsen, Y., 2012b. The effects of moderate ozonation or high intensity UV-irradiation on the microbial environment in RAS for marine larvae. Aquaculture 330–333, 121–129. doi:10.1016/j.aquaculture.2011.11.042

Becke, C., Schumann, M., Steinhagen, D., Geist, J., Brinker, A., 2018. Physiological consequences of chronic exposure of rainbow trout (Oncorhynchus mykiss) to suspended solid load in recirculating aquaculture systems. Aquaculture 484, 228–241. doi:10.1016/j.aquaculture.2017.11.030

Brinker, A., 2007. Guar gum in rainbow trout (Oncorhynchus mykiss) feed: The influence of quality and dose on stabilisation of faecal solids. Aquaculture 267, 315–327. doi:10.1016/j.aquaculture.2007.02.037

Carré, E., Pérot, J., Jauzein, V., Lopez-Ferber, M., 2018. Impact of suspended particles on UV disinfection of activated-sludge effluent with the aim of reclamation. J. Water Process Eng. 22, 87–93. doi:10.1016/j.jwpe.2018.01.016

de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.-F., Dalsgaard, J., 2018. Micro particles and microbial activity in Danish recirculating rainbow trout (Oncorhynchus mykiss) farms. Aquac. Eng. doi:10.1016/j.aquaeng.2018.12.001

Emerick, R.W., Loge, F.J., Thompson, D., Darby, J.L., 1999. Factors Influencing Ultraviolet Disinfection Performance Part II: Association of Coliform Bacteria with Wastewater Particles. Water Environ. Res. 71, 1178–1187. doi:10.2175/106143097X122004

Fernandes, P.M., Pedersen, L.F., Pedersen, P.B., 2014. Daily micro particle distribution of an experimental recirculating aquaculture system-A case study. Aquac. Eng. 60, 28–34. doi:10.1016/j.aquaeng.2014.03.007

Fernandes, P.M., Pedersen, L.-F., Pedersen, P.B., 2016. Influence of fixed and moving bed biofilters on micro particle dynamics in a recirculating aquaculture system. Aquac. Eng. doi:10.1016/j.aquaeng.2016.09.002

Franco-Nava, M.-A., Blancheton, J.-P., Deviller, G., Le-Gall, J.-Y., 2004. Particulate matter dynamics and transformations in a recirculating aquaculture system: application of stable isotope tracers in seabass rearing. Aquac. Eng. 31, 135–155. doi:10.1016/j.aquaeng.2004.01.003

Henze, M., Harremoes, P., Arvin, E., Jansen, J. la C., 1997. Wasterwater Treatment, 2nd editio. ed. Springer-Verlag Berlin Heidelberg, New York. doi:10.1007/978-3-662-22605-6

Huyben, D., Bevan, D., Stevenson, R., Zhou, H., Moccia, R., 2018. Evaluation of membrane filtration and UV irradiation to control bacterial loads in recirculation aquaculture systems. Aquac. Int. 26, 1531–1540. doi:10.1007/s10499-018-0301-z

Lekang, O.-I., 2007. Aquaculture Engineering, Aquaculture Engineering. Blackwell Publishing.

Loge, F.J., Emerick, R.W., Thompson, D.E., Nelson, D.C., Darby, J.L., 1999. Factors Influencing Ultraviolet Disinfection Performance Part I: Light Penetration to Wastewater Particles. Water Environ. Res. 71, 377–381. doi:10.2175/106143097X122176

Lu, C., Kania, P.W., Buchmann, K., 2018. Particle effects on fish gills: An immunogenetic approach for rainbow trout and zebrafish. Aquaculture 484, 98–104. doi:10.1016/j.aquaculture.2017.11.005

Patterson, R.N., Watts, K.C., Timmons, M.B., 1999. The power law in particle size analysis for aquacultural facilities. Aquac. Eng. 19, 259–273. doi:10.1016/S0144-8609(98)00054-5

Pedersen, P.B., von Ahnen, M., Fernandes, P.M., Naas, C., Pedersen, L.-F., Dalsgaard, J., 2017. Particle surface area and bacterial activity in recirculating aquaculture systems. Aquac. Eng.

Pedersen, L.F., Rojas-Tirado, P., Arvin, E., Pedersen, P.B., 2019. Assessment of microbial activity in water based on hydrogen peroxide decomposition rates. Aquac. Eng. 85, 9–14. doi:10.1016/j.aquaeng.2019.01.001

Qualls, R.G., Flynn, M.P., JR, J.M.C., 1983. The role of suspended particles in ultraviolet disinfection. J. Water Pollut. Control Fed. 55, 1280–1285. doi:10.2307/25042084

Schumann, M., Unger, J., Brinker, A., 2017. Floating faeces: Effects on solid removal and particle size distribution in RAS. Aquac. Eng. 78, 75–84. doi:10.1016/j.aquaeng.2016.10.007

Sharrer, M.J., Summerfelt, S.T., Bullock, G.L., Gleason, L.E., Taeuber, J., 2005. Inactivation of bacteria using ultraviolet irradiation in a recirculating salmonid culture system. Aquac. Eng. 33, 135–149. doi:10.1016/j.aquaeng.2004.12.001

Skjermo, J., Saivesen, I., Øie, G., Olsen, Y., Vadstein, O., 1997. Microbially matured water : a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquac. Int. 5, 13–28. doi:10.1007/BF02764784

Summerfelt, S.T., 2003. Ozonation and UV irradiation. Aquac. Eng. 28, 36.

Timmons, M.B., Ebeling, J.M., 2010. Recirculating Aquaculture, 2nd ed. Cayuga Aqua Ventures, NY, USA.

Unger, J., Brinker, A., 2013. Floating feces: A new approach for efficient removal of solids in aquacultural management. Aquaculture 404–405, 85–94. doi:10.1016/j.aquaculture.2013.04.015

Vadstein, O., Øie, G., Salvesen, I., Skjermo, J., 1993. A strategy to obtain microbial control during larval development of marine fish. Fish Farming Technol. 69–75.

Viadero, R.C., Noblet, J.A., 2002. Membrane filtration for removal of fine solids from aquaculture process water. Aquac. Eng. 26, 151–169. doi:10.1016/S0144-8609(02)00011-0

Wold, P.-A., Holan, A.B., Øie, G., Attramadal, K., Bakke, I., Vadstein, O., Leiknes, T.O., 2014. Effects of membrane filtration on bacterial number and microbial diversity in marine recirculating aquaculture system (RAS) for Atlantic cod (Gadus morhua L.) production. Aquaculture 422–423, 69–77. doi:10.1016/j.aquaculture.2013.11.019

Wu, J., Le-Clech, P., Stuetz, R.M., Fane, A.G., Chen, V., 2008. Effects of relaxation and backwashing conditions on fouling in membrane bioreactor. J. Memb. Sci. 324, 26–32. doi:10.1016/j.memsci.2008.06.057

Table 1. Two-way analysis of variance (Anova) of particle data carried out on data from the end of the trial.

Treatment	*Statistical parameter*	*Particle Number (# ml^{-1})*	*Particle Volume (mm^3 ml^{-1})*	*Particle Surface Area (mm^2 ml^{-1})*	*Microbial activity (k^{-1})*	*COD_{DISS} (mg l^{-1})*	*COD_{PART} (mg l^{-1})*	*β Value*
Within UV	F	37.4	2.8	19.8	66.8	42.0	1.3	20.7
	p-value	<0.001	0.130	0.002	<0.001	<0.001	0.281	0.002
Within Filter	F	7.5	46.8	65.0	13.0	4.1	32.2	0.8
	p-value	0.025	<0.001	<0.001	0.007	0.078	<0.001	0.387
Interaction	F	0.08	1.6	0.89	7.7	2.2	0.4	1.4
	p-value	0.782	0.237	0.372	0.024	0.173	0.566	0.264

Table 2. Pearson's correlation coefficients (r) between selected water quality parameters measured throughout the trial.

Comparison	*Statistical parameter*	*1µm ÷ UV (n =12)*	*200µm ÷ UV (n =12)*	*1µm + UV (n =12)*	*200µm + UV (n =12)*	*Total data* (n =48)*
Volume of particles vs	r	0.98	0.96	0.97	0.96	0.96
COD_{PART}	p	<0.001	<0.001	<0.001	<0.001	<0.001
Microbial activity vs	r	0.66	0.81	0.78	0.61	0.78
COD_{DISS}	p	0.018	0.0013	0.0026	0.0367	<0.001
Microbial activity vs	r	0.65	0.79	0.83	0.76	0.79
COD_{TOTAL}	p	0.02	0.002	<0.001	0.003	<0.001
Microbial activity vs	r	0.93	0.95	0.93	0.87	0.95
Number of particles	p	<0.001	<0.001	<0.001	<0.001	<0.001

*Total data corresponds to correlations made with all data points from the 4 different treatments.

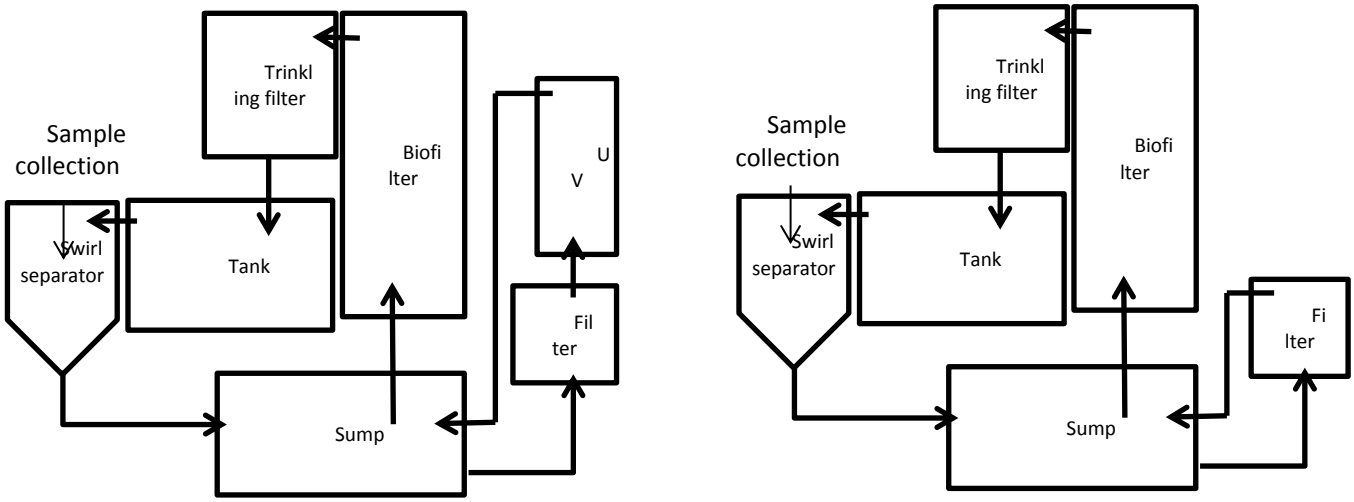
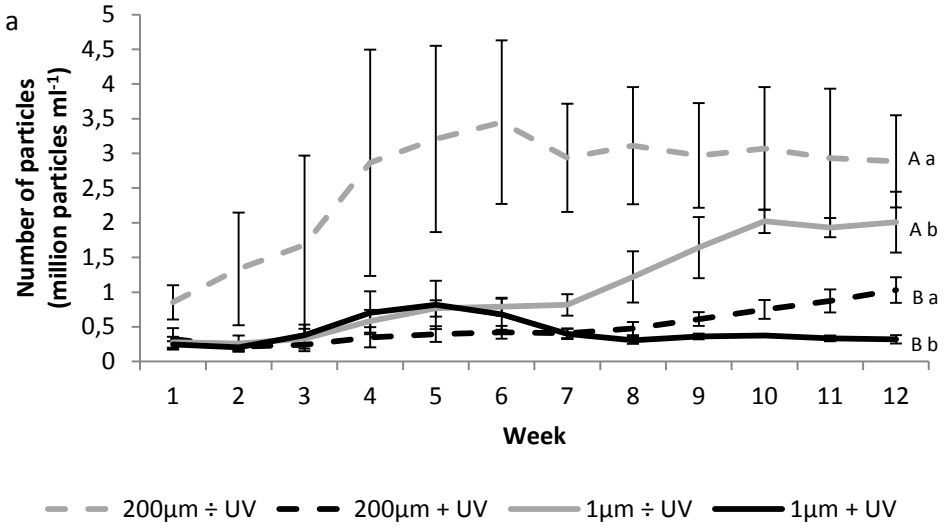
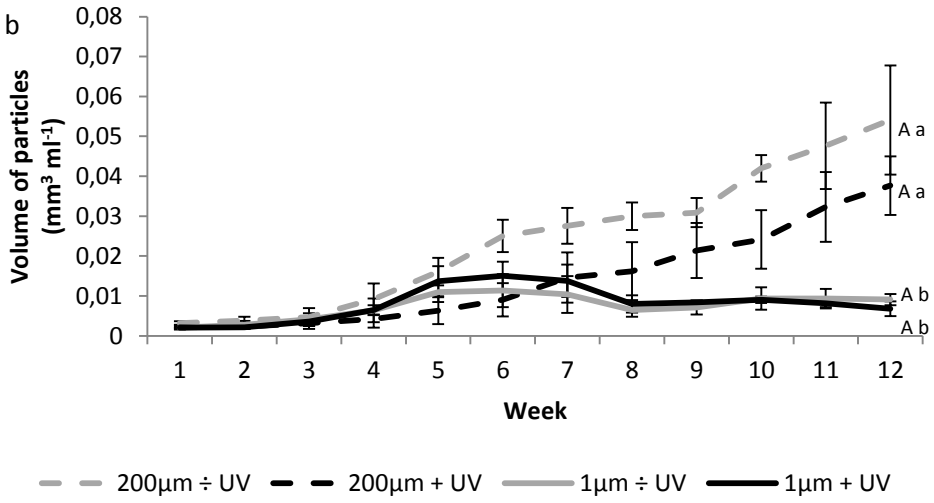
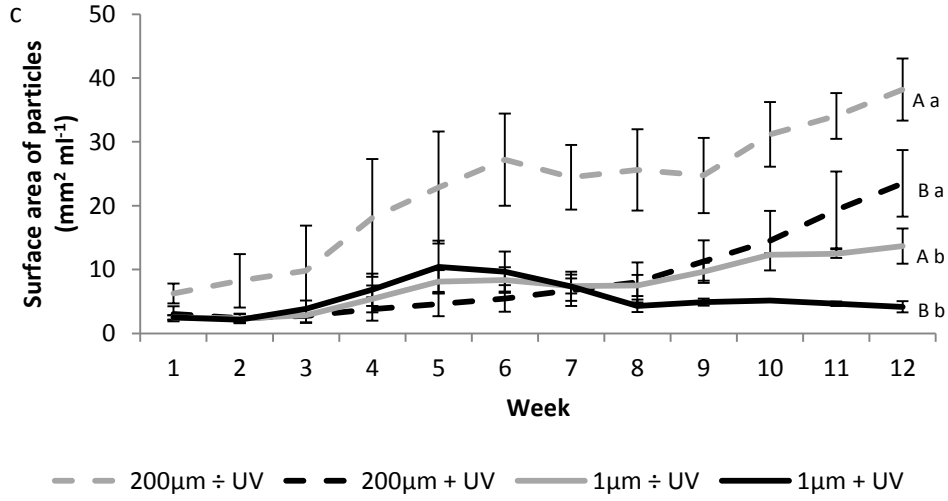


Figure 1: Simplified schematic representation of the 12 RAS units used in the trial, depicting the sampling location and components and filtration units added: a) System fitted with filter housing and UV; b) System fitted only with filter housing.

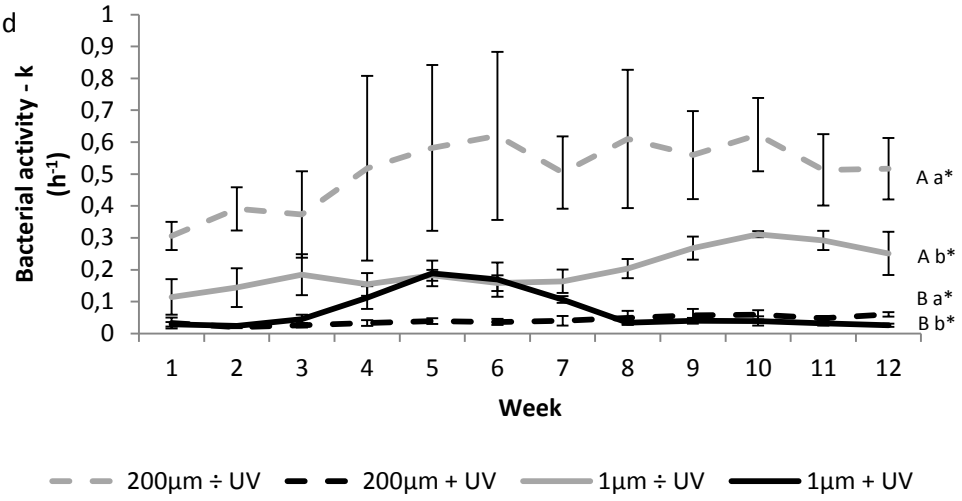
Within each UV treatment (i.e., UV or no UV) filter housings were fitted with wither a 1 µm or a 200 µm cartage filters. Arrows indicate the direction of the flow.


a
Number of particles (million particles ml-1)
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
1 2 3 4 5 6 7 8 9 10 11 12
Week
A a
A b
B a
B b
200µm ÷ UV
200µm + UV
1µm ÷ UV
1µm + UV


b
Volume of particles (mm³ ml-1)
0,08
0,07
0,06
0,05
0,04
0,03
0,02
0,01
0
1 2 3 4 5 6 7 8 9 10 11 12
Week
A a
A a
A b
A b
200µm ÷ UV
200µm + UV
1µm ÷ UV
1µm + UV

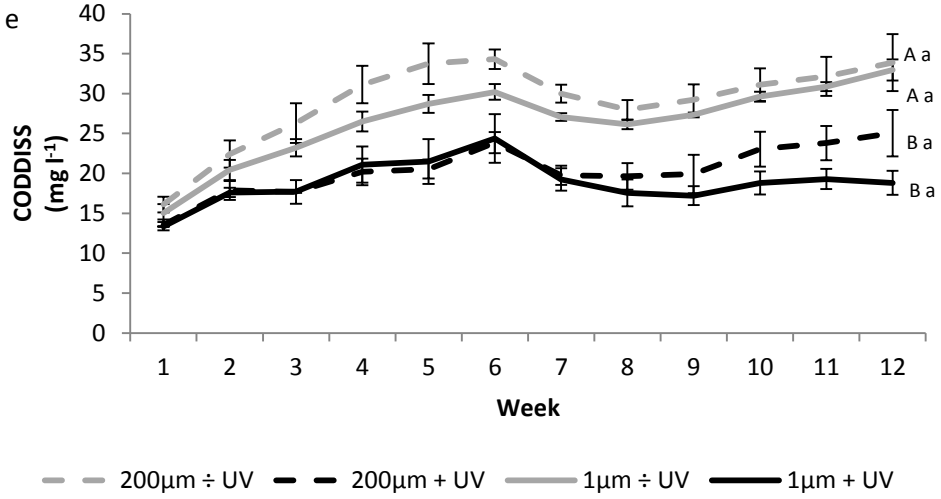

c
Surface area of particles (mm² ml-1)
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12
Week
A a
B a
A b
B b
200µm ÷ UV
200µm + UV
1µm ÷ UV
1µm + UV

d


Bacterial activity - k (h-1)
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
1 2 3 4 5 6 7 8 9 10 11 12
Week
A a*
A b*
B a*
B b*

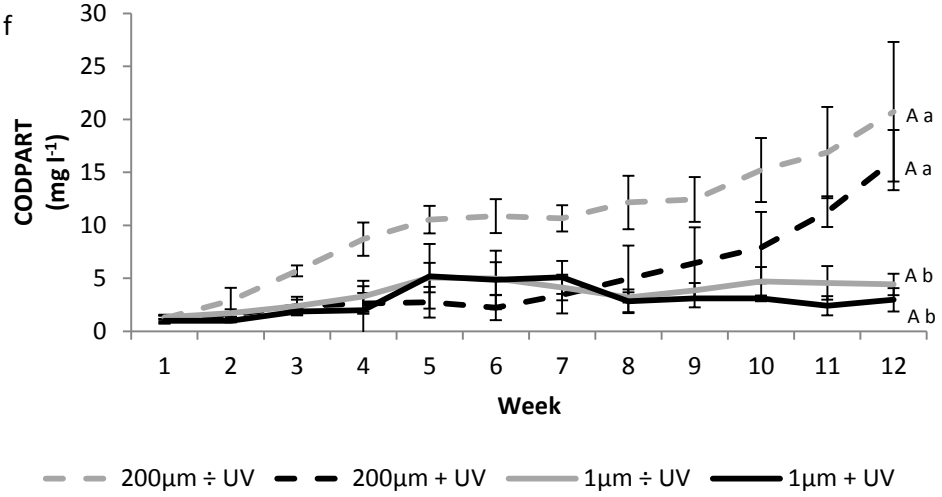
200µm ÷ UV
200µm + UV
1µm ÷ UV
1µm + UV

e


CODDISS (mg l-1)
40
35
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12
Week
A a
A a
B a
B a

200µm ÷ UV
200µm + UV
1µm ÷ UV
1µm + UV

f


CODPART (mg l-1)
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12
Week
A a
A a
A b
A b

200µm ÷ UV
200µm + UV
1µm ÷ UV
1µm + UV

Figure 2: Time series of measured water quality parameters during the trial. Results are shown as moving average of 3 data points. Statistical differences at the end of the trial are indicated in the figure and summarized in table 1. Different capital letters indicate differences within UV treatment while different small letter indicate differences within cartridge filtration treatments. a) Number of particles b) Volume of particles c) Surface area of particles d) Microbial activity e) COD particulate f) COD dissolved. *Interactions between main factors (UV and filters), in which case statistics refers to the difference within each main factor.

Paper III

de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Dalsgaard, J., 2020. Effects of storage time and temperature on microbial activity and micro particle determination in recirculating aquaculture systems water samples. *Manuscript*

Effects of storage time and temperature on microbial activity and micro particle determination in RAS water samples

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Abstract

Micro particle loads and bacterial activity in RAS have gathered a lot of attention in recent years, with their development and impacts central to the discussion about water quality. In recent years, new technics for measuring bacterial activity in RAS have also been developed. However, many of this new technics (and some of the older technics used for measuring micro particles) lack international standards regarding collection, storage and treatment, leaving a lot of this decisions up to the researchers. In order to determine the implication of sample storage in the determination of micro particles and bacterial activity in RAS water, samples from two RAS systems were collected and stored at room temperature (20^{o}C) and 4^{o}C. Samples were also frozen to test the effect of freezing the samples. Micro particles, bacterial activity (measured as H_2O_2 degradation and BactiQuant), biochemical oxygen demand after 5 days (BOD_5) and chemical oxygen demand (COD) were measured initially and then after 3, 6, 24 and 72 hours.

The results revealed opposite effects on both samples. The sample collected from RAS1 remained stable for the first 6 hours and then started to decrease, especially regarding micro particle numbers and bacterial activity. On the other end, samples collected from RAS2 show a significant increase in both bacterial activity and micro particle numbers after just 3 hours, even under storage at 4^{o}C.

The storage at 4^{o}C resulted in smaller and slower variations, probably caused by a decrease in bacterial activity. However, statistical significant differences were still observed. The frozen samples showed the largest variation, probably due to impacts on the bacterial populations.

While sample storage will always be required, the results of this trial indicate that storage of samples should be kept as short as possible and in cold conditions as storage procedure is critical for obtaining repeatable and reliable results, regarding bacterial activity and micro particle analysis.

Introduction

Monitoring and control of water quality is an important part of recirculating aquaculture system (RAS) management, research, and development. A prerequisite for this is access to accurate and reliable analytical methods. The majority of methods are standardized in terms of sampling, conservation, and storage prior to analysis in order to ensure reproducible results. Examples hereof includes biological oxygen demand after 5 days (BOD_5 - ISO 5815-2:2003), chemical oxygen demand (COD - ISO 6060:1989), total suspended solids (TSS – DS/EN 872:2005), and nitrate (ISO 7890-1:1986).

Recent attention on microbial water quality in RAS has been accompanied by use of new methods (Pedersen *et al.*, 2019; Rojas-Tirado *et al.*, 2016). Among these are methods used to estimate bacterial activity such as BactiQuant (Mycometer A/S, Denmark) and H_2O_2 degradation method (Pedersen *et al.*, 2019). These methods have been applied to quantify bacterial loads (Becke *et al.*, 2019; de Jesus Gregersen *et al.*, 2019; Pedersen *et al.*, 2017; Podduturi *et al.*, 2020; Rojas-Tirado *et al.*, 2018, 2016), while different particle counters have been used to analyse micro particles and particle size distribution (PSD), as well as enumeration and quantification of particle volume and surface area (Becke *et al.*, 2020; Brinker *et al.*, 2005a, 2005b; Chen *et al.*, 1993; Cripps, 1995; de Jesus Gregersen *et al.*, 2019; Fernandes *et al.*, 2014). However, despite becoming more commonly used, most of these methods do not have a standard protocol for sample storage. If possible, analyses are preferably made on fresh samples as post sampling conditions potentially affect measurements due to bacteria dynamics and organic matter turnover. However, on-site or real time analyses are not always an option and conservation and/or storage of water samples might be needed. Effects of this are rarely reported in scientific literature. To be able to compare results of microbial water quality obtained under different post sampling conditions, information on assay reproducibility is needed.

The objective of this study was to test effects of storage time and temperature on microbial activity and PSD in RAS water samples. This was investigated in RAS water sampled from two different facilities by storing samples at three different temperatures for up to 72 hours prior to analysis.

2. Materials and methods

2.1. Experimental setup

Water was collected from two different RAS: A pilot scale freshwater system (RAS1) and a commercial saltwater system (RAS2). The pilot scale system (RAS1) had a total volume of 8 m^3 including a 7 m^3 rearing tank with 160 kg rainbow trout (***Oncorhynchus mykiss***) fed 1 kg feed daily (Efico, Biomar, Denmark). In addition, it contained four 0.4 m^3 biofilters (2 moving beds and 2 fixed beds) ran in parallel, a trickling filter, and a drum filter. Water was collected at the outflow of the rearing tank prior to the drum filters. 1 m^3

makeup water was added daily, resulting in a feed loading of 1 kg feed m^{-3}. A detailed description of the system can be found in Fernandes *et al.* (2016).

The second system sampled (RAS2) was part of a commercial facility producing approximately 1000 tons Atlantic salmon (*Salmo salar*) per year. The specific system was composed of four grow-out tanks, four drum filters, two biofilter sections, and a degassing unit. Furthermore, the system was equipped with a UV disinfection unit and a protein skimmer with ozone. Water from this system was collected in the outflow of one of the rearing tanks before the drum filters.

2.2. Water sampling and analysis

A total volume of approximately 55 l was collected from each system. Samples were subsequently transferred to a 60 l container with a circulation pump (Tunze nanostream 6015, TUNZE® Aquarientechnik GmbH, Germany) to ensure proper mixing and homogenous sub-sampling. Ten 1.5 l sub-samples in triplicate (i.e., 30 samples in total) were obtained from each RAS. One triplicate sub-set was analysed right away while another sub-set was stored at -20°C and analysed after one month of storage. Either half of the remaining eight sub-sets were stored in climate rooms at 4 or 20°C and analysed after 3, 6, 24, and 72 hours of storage.

All samples were analysed for particle numbers (PN), particle volume (PV), total chemical oxygen demand (COD_{TOTAL}; ISO 6060, 1989), and bacterial activity. The latter was assessed by a H_2O_2 degradation assay (Pedersen *et al.*, 2019a) and BactiQuant (Mycometer A/S, Denmark) following manufacturer instructions. Samples stored for 0, 24, and 72 hours were analysed for total biological oxygen demand after 5 days ($BOD_{5\text{-}TOTAL}$; ISO 5815-2:2003) modified by adding allythiourea (ATU) to inhibit nitrification. Particles were analysed using a Multisizer 4e Coulter Counter (Bechman Coulter, Inc, Indianapolis, USA), utilizing both a 50 µm and a 250 µm aperture. All analysis were made in replicate.

In order to track sample temperature when stored at 4°C, and test a possible way to improve cooling, two extra samples were stored at 4°C. Each sample was fitted with a temperature probe connected to a logger (Hack HQ40d, Hach Company, USA), with temperature readings every 5 minutes. One sample was kept in a shelf, while the second sample was placed in a water bath with ice, to increase heat loss.

2.3. Data analysis

One way ANOVA was used to compare start values against subsequent values within each system and storage temperature. A Holm-Sidak test was performed in case of significant differences. Differences were considered significant at $p < 0.05$. No comparisons were made across different storage temperatures as the objective was to detect any changes from the original start value only.

Pearson Product Moment correlation analyses were carried out to test for correlation between particle numbers and bacterial activity. All statistical analyses were performed in SigmaPlot 13.0 (Systat software Inc., USA). Data are presented as average ± standard deviation.

3. Results

3.1. Microbial activity

3.1.1. H_2O_2 degradation

The H_2O_2 degradation rates (k, h^{-1}) in samples from RAS1 stored at 4°C remained stable after 72 hours (fig. 1a). In comparison, degradation rates in RAS1 samples stored at 20°C were significantly lower after 24 and 72 hours storage. Degradation rates in samples from RAS2 were lower than those in RAS1 and were significantly affected by both storage time and temperature. Bacterial activity in samples stored at 4°C was significantly higher at all times compared to start samples. Similarly, bacterial activity in samples stored at 20°C increased significantly until 24 h compared to start while it was significantly lower after 72 hours. Degradation rates in frozen samples from RAS1 and RAS2 were 80 and 50% lower, respectively than samples that had not been stored.

3.1.2. BactiQuant

BactiQuant results were very similar to those of H_2O_2 degradation (fig. 1b). BactiQuant values (BQVs) measured in samples from RAS1 stored at 4°C remained stable for all 72 hours. For samples stored at 20°C, BQVs were significantly lower after 24 and 72 hours storage. For RAS2 samples, BQVs increased compared to the start reaching a maximum at 24 hours in samples stored at either 4 or 20°C. As for H_2O_2, BQVs in samples stored for 72 hours at 20°C were significantly lower than in non-stored samples. Similarly, freezing samples from RAS1 and RAS2 reduced BQVs by 51 and 42%, respectively.

3.2. Micro particles

3.2.1. Particle numbers

With the exception of frozen samples from RAS2, particle numbers (PN) were significantly different from the initial samples at all storage times and temperatures (fig. 1c). For RAS1 samples, PN declined during storage, the decline more accentuated in samples stored at 20°C than at 4°C. Similarly, freezing samples from RAS1 reduced PN significantly.

In contrast, PN in samples obtained from RAS2 increased significantly during the first 24 hours of storage independently of storage temperature. However, after 72 hours, PN in samples stored at 20° were significantly lower than in non-stored samples.

Particle numbers correlated significantly with H_2O_2 degradation rates and BQV in samples from both systems (table 1).

3.2.1. Particle volume

Particle volume (PV) did not change during storage except for in frozen RAS1 where PV was significantly higher than in the initial sample (fig. 1d).

3.3. COD

Total COD remained stable throughout the length of storage for both RAS1 and RAS2 samples (fig. 1e). The only exceptions were samples from RAS1 which after 24 and 72 hours storage at 20°C, and RAS2 samples which after 72 hours storage at 20°C were slightly but significantly lower than at start.

3.4. BOD_5

There was no change in $BOD_{5\text{-}TOTAL}$ after 24 hours storage of RAS1 samples at 4°C, whereas $BOD_{5\text{-}TOTAL}$ was significantly lower after 72 hours of storage (fig. 1f). For RAS1 samples stored at 20°C, $BOD_{5\text{-}TOTAL}$ was significantly lower after 24 and 72 hours storage.

Similar trends were observed in samples from RAS2 although here, $BOD_{5\text{-}TOTAL}$ was significantly lower at all storage times and temperatures. Freezing samples from RAS1 or RAS2 similarly reduced $BOD_{5\text{-}TOTAL}$ significantly compared to non-stored samples.

3.5. Correlation

Correlation analysis between bacterial activity and particle numbers, revealed high correlation coefficients (tab. 1), with statistical significance in all comparisons ($n=27$, $p<0.001$)

3.6. Sample cooling procedure

Samples obtained from either system had an initial temperature of 15 °C. It took approximately 9 hours to cool samples to 5.0°C and 18 hours to reach 4.0°C (fig. 2) if storing them in a climate room of 4.0°C (as in the current study). In comparison, placing samples in a water bath with ice in a fridge at 4.0°C cooled them to 4.0°C in approximately 1 hour.

4. Discussion

The main focus of this study was to investigate potential implications of sample storage time prior to analysis of micro particle levels and bacterial activity. The timespan from when samples are obtained and until they are analysed in the laboratory is seldom mentioned in scientific articles despite that the distance to sampling sites may require samples to be stored for several hours at uncontrolled temperatures. Results obtained in this experiment illustrate the impact that storage time and temperature may have on analyses of micro particle levels and bacterial activity.

Water from two RAS sampled in this study showed widely different results for most metrics measured with clear implications in terms of precision of analysis and interpretation of results. Storage time and temperature had less impact on RAS1 than RAS2 samples. As seen in a previous study (de Jesus Gregersen *et al.*, 2020), the strong correlations between particle numbers and bacterial activity determinations, especially in RAS1 samples (table 1), sustain that a large share of what is typically considered micro particles in RAS samples is in fact living microorganisms. Changes in samples during storage is therefore presumably caused by changes in bacteria populations within samples. This would explain why especially

RAS1 samples stored at 4°C were less affected during storage than other samples as bacteria metabolism is slowed down at lower temperatures (Iriberri *et al.*, 1985; Pomeroy and Wiebe, 2001).

Changes in bacterial communities may also explain the concomitant increase in micro particle numbers (defacto bacteria) and bacterial activity in RAS2 samples observed just three hours after sampling but for different reasons than temperature. One reason might be a higher availability of easily degradable organic matter in RAS2 samples. However, the organic matter biodegradability index, the BOD_5/COD ratio (Srinivas, 2008), was similar in RAS1 and RAS2 samples (0.14 and 0.16, respectively) making this explanation less likely. Differences in salinity might be another explanation. However, unpublished data (supplementary material) on samples from two different pilot-scale freshwater systems and one saltwater system showed that storing these samples for 24 hours at 4°C did not affect bacterial activity or micro particle numbers.

The most likely explanation is the use of disinfectants in RAS2. Both protein skimmers with ozone and UV were applied in the commercial facility to reduce / control bacteria (Attramadal *et al.*, 2012; Bullock *et al.*, 1997; Gonçalves and Gagnon, 2011; Huyben *et al.*, 2018). Disinfection therefore likely kept bacterial activity under control and below system carrying capacity. Once water samples were collected and bacteria were no longer subjected to disinfection, they were able to utilize the organic matter available and fully exploit system carrying capacity.

In comparison, RAS1 (and unpublished-trials) were operated without disinfection. This probably allowed the systems to be close to carrying capacity (Attramadal *et al.*, 2012; Vadstein *et al.*, 1993), supporting the lack of changes in the bacterial activity during the first 3-6 hours of storage and the less pronounced change in numbers and activity over time compared to RAS2.

Changes in micro particle numbers were primarily within the 1-3 µm size range and no changes were observed in total particle volume except in frozen samples, where particle volume increased. These results reinforce the hypothesis that most changes observed were bacteria driven. This is also support by the very high correlation coefficients found between bacterial activity and bacterial numbers. The results furthermore support the hypothesis that systems operated at carrying capacity are more stable and less prone to sudden increases in bacteria populations than system kept deliberately low in bacteria numbers (De Schryver and Vadstein, 2014; Vadstein *et al.*, 1993).

In general, storing samples at 4°C appeared to dampen changes in micro particles number and bacteria activity in both systems. Still, changes were observed and this may relate to the time it took cooling samples to 4°C (fig. 2), allowing bacteria to keep multiplying especially in RAS2 samples for the reasons discussed above.

Freezing samples resulted in a decline in most metrics measured (bacteria activity and particles). While some bacteria can survive freezing for prolonged periods of time (Wallenius *et al.*, 2010), most probably

cannot. As both the H_2O_2 degradation method and BactiQuant work by measuring enzyme activity, they should not be applied to samples that have been frozen.

The results obtained in this study highlight some of the challenges faced when analysing micro particle levels and bacterial activity in RAS. As demonstrated, even short periods of storage (three hours) may have a significant impact on the results. This may potentially affect the interpretation of within system variations or it may affect system comparisons or comparisons of different treatments within an experiment.

However, while samples should clearly be analysed as soon as possible, some trials and sampling locations may require some sort of storage. A potential way to reduce the impact of sample storage could be to reduce the time taken to cool down samples. As shown in figure 2, this may for example be obtained by placing samples in a 4.0^{o}C water bath to promote a rapid loss of heat and presumably reduce further changes from happening in the samples. This was, however, not tested in the current study.

5. Conclusion

The large changes even during short term sample storage especially in samples from RAS2, are a clear indication that sampling and storage procedure are critical for obtaining repeatable and reliable results on micro particle levels and bacterial activity in RAS. While it is not always possible to analyse samples immediately after collection, the way samples are handled and stored should be considered beforehand and reported. This is especially true when comparing different treatments within a system or comparing absolute values from different systems.

Storing samples at low temperatures seems to slow down changes and increase reproducibility. Despite this, the time taken for samples to cool down may still affect results.

Acknowledgement

Thanks to Ulla Sproegel, Brian Moeller and Carina Jensen for all their assistance with the lab work, even during the Coranavirus outbreak.

References

Arvin, E., Pedersen, L.-F., 2015. Hydrogen peroxide decomposition kinetics in aquaculture water. Aquac. Eng. 64, 1–7. doi:10.1016/j.aquaeng.2014.12.004

Attramadal, Kari J.K., Øie, G., Størseth, T.R., Alver, M.O., Vadstein, O., Olsen, Y., 2012. The effects of moderate ozonation or high intensity UV-irradiation on the microbial environment in RAS for marine larvae. Aquaculture 330–333, 121–129. doi:10.1016/j.aquaculture.2011.11.042

Attramadal, Kari J K, Salvesen, I., Xue, R., Øie, G., Størseth, T.R., Vadstein, O., Olsen, Y., 2012. Recirculation as a possible microbial control strategy in the production of marine larvae. Aquac. Eng. 46, 27–39. doi:10.1016/j.aquaeng.2011.10.003

Badiola, M., Mendiola, D., Bostock, J., 2012. Recirculating Aquaculture Systems (RAS) analysis: Main issues on management and future challenges. Aquac. Eng. 51, 26–35. doi:10.1016/j.aquaeng.2012.07.004

Becke, C., Schumann, M., Geist, J., Brinker, A., 2020. Shape characteristics of suspended solids and implications in different salmonid aquaculture production systems. Aquaculture 516, 734631. doi:10.1016/j.aquaculture.2019.734631

Becke, C., Schumann, M., Steinhagen, D., Rojas-Tirado, P., Geist, J., Brinker, A., 2019. Effects of unionized ammonia and suspended solids on rainbow trout (Oncorhynchus mykiss) in recirculating aquaculture systems. Aquaculture 499, 348–357. doi:10.1016/j.aquaculture.2018.09.048

Bullock, G.L., Summerfelt, S.T., Noble, A.C., Weber, A.L., Durant, M.D., Hankins, J.A., 1997. Ozonation of a recirculating rainbow trout culture system. I. Effects on bacterial gill disease and heterotrophic bacteria. Aquaculture 158, 43–55.

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., Pedersen, P.B., 2013. Farming different species in RAS in Nordic countries: Current status and future perspectives. Aquac. Eng. 53, 2–13. doi:10.1016/j.aquaeng.2012.11.008

de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Dalsgaard, J., 2019. Micro particles and microbial activity in Danish recirculating rainbow trout (Oncorhynchus mykiss) farms. Aquac. Eng. 84, 60–66. doi:10.1016/j.aquaeng.2018.12.001

de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Liu, D., Dalsgaard, J., 2020. UV irradiation and micro filtration effects on micro particle development and microbial water quality in recirculation aquaculture systems. Aquaculture 518, 734785. doi:10.1016/j.aquaculture.2019.734785

De Schryver, P., Vadstein, O., 2014. Ecological theory as a foundation to control pathogenic invasion in aquaculture. ISME J. 8, 2360–2368. doi:10.1038/ismej.2014.84

DA/EN 872:2:2005 - Water test - Determination of the amount of suspended matter - Method of filtration through glass fiber filters. Danish Standard Foundation, Denmark

Fernandes, P., Pedersen, L.-F., Pedersen, P.B., 2015. Microscreen effects on water quality in replicated recirculating aquaculture systems. Aquac. Eng. 65, 17–26. doi:10.1016/j.aquaeng.2014.10.007

Fernandes, P.M., Pedersen, L.-F., Pedersen, P.B., 2016. Influence of fixed and moving bed biofilters on micro particle dynamics in a recirculating aquaculture system. Aquac. Eng. doi:10.1016/j.aquaeng.2016.09.002

Gonçalves, A.A., Gagnon, G.A., 2011. Ozone Application in Recirculating Aquaculture System: An Overview. Ozone Sci. Eng. 33, 345–367.

Huyben, D., Bevan, D., Stevenson, R., Zhou, H., Moccia, R., 2018. Evaluation of membrane filtration and UV irradiation to control bacterial loads in recirculation aquaculture systems. Aquac. Int. 26, 1531–1540. doi:10.1007/s10499-018-0301-z

Iriberri, J., Undurraga, A., Muela, A., Egea, L., 1985. Heterotrophic bacterial activity in coastal waters: Functional relationship of temperature and phytoplankton population. Ecol. Modell. 28, 113–120. doi:10.1016/0304-3800(85)90016-X

ISO 5815-2:2003. Water quality — Determination of biochemical oxygen demand after n days (BODn). International Organization for Standardization, Geneva, Switzerland.

ISO 6060:1989. Water quality — Determination of the chemical oxygen demand. International Organization for Standardization, Geneva, Switzerland.

ISO 7890-1:1986 Water quality — Determination of nitrate. International Organization for Standardization, Geneva, Switzerland.

Pedersen, L.F., Rojas-Tirado, P., Arvin, E., Pedersen, P.B., 2019. Assessment of microbial activity in water based on hydrogen peroxide decomposition rates. Aquac. Eng. 85, 9–14. doi:10.1016/j.aquaeng.2019.01.001

Pedersen, P.B., von Ahnen, M., Fernandes, P.M., Naas, C., Pedersen, L.-F., Dalsgaard, J., 2017. Particle surface area and bacterial activity in recirculating aquaculture systems. Aquac. Eng.

Pomeroy, L.R., Wiebe, W.J., 2001. Temperature and substrates as interactive limiting factors for marine heterotrophic bacteria. Aquat. Microb. Ecol. 23, 187–204. doi:10.3354/ame023187

Reeslev, M., Nielsen, J.C., Rogers, L., 2011. Assessment of the bacterial contamination and remediation efficacy after flooding using fluorometric detection. J. ASTM Int. 8, 290–296. doi:10.1520/JAI103482

Rojas-Tirado, P., Pedersen, P.B., Vadstein, O., Pedersen, L.-F., 2019. Rojas-Tirado *et al.* 2019.pdf.

Schumann, M., Brinker, A., 2020. Understanding and managing suspended solids in intensive salmonid aquaculture: a review. Rev. Aquac. 1–31. doi:10.1111/raq.12425

Srinivas, T., 2008. Environmental Biotechnology. New Age International Publisher, New Delhi.

Vadstein, O., Øie, G., Salvesen, I., Skjermo, J., 1993. A strategy to obtain microbial control during larval development of marine fish. Fish Farming Technol. 69–75.

Wallenius, K., Rita, H., Simpanen, S., Mikkonen, A., Niemi, R.M., 2010. Sample storage for soil enzyme activity and bacterial community profiles. J. Microbiol. Methods 81, 48–55. doi:10.1016/j.mimet.2010.01.021

Table 1. Pearson Product Moment correlation (r) between particle numbers and bacterial activity in samples from the two systems measured as BactiQuant and H_2O_2 degradation

	RAS1			**RAS2**		
	r	p	N	r	p	N
Particle n**umber**s vs. **H_2O_2** degradation rates	0.91	<0.001	27	0.67	<0.001	27
Particle n**umbers** vs. **BactiQuant** values	0.94	<0.001	27	0.89	<0.001	27

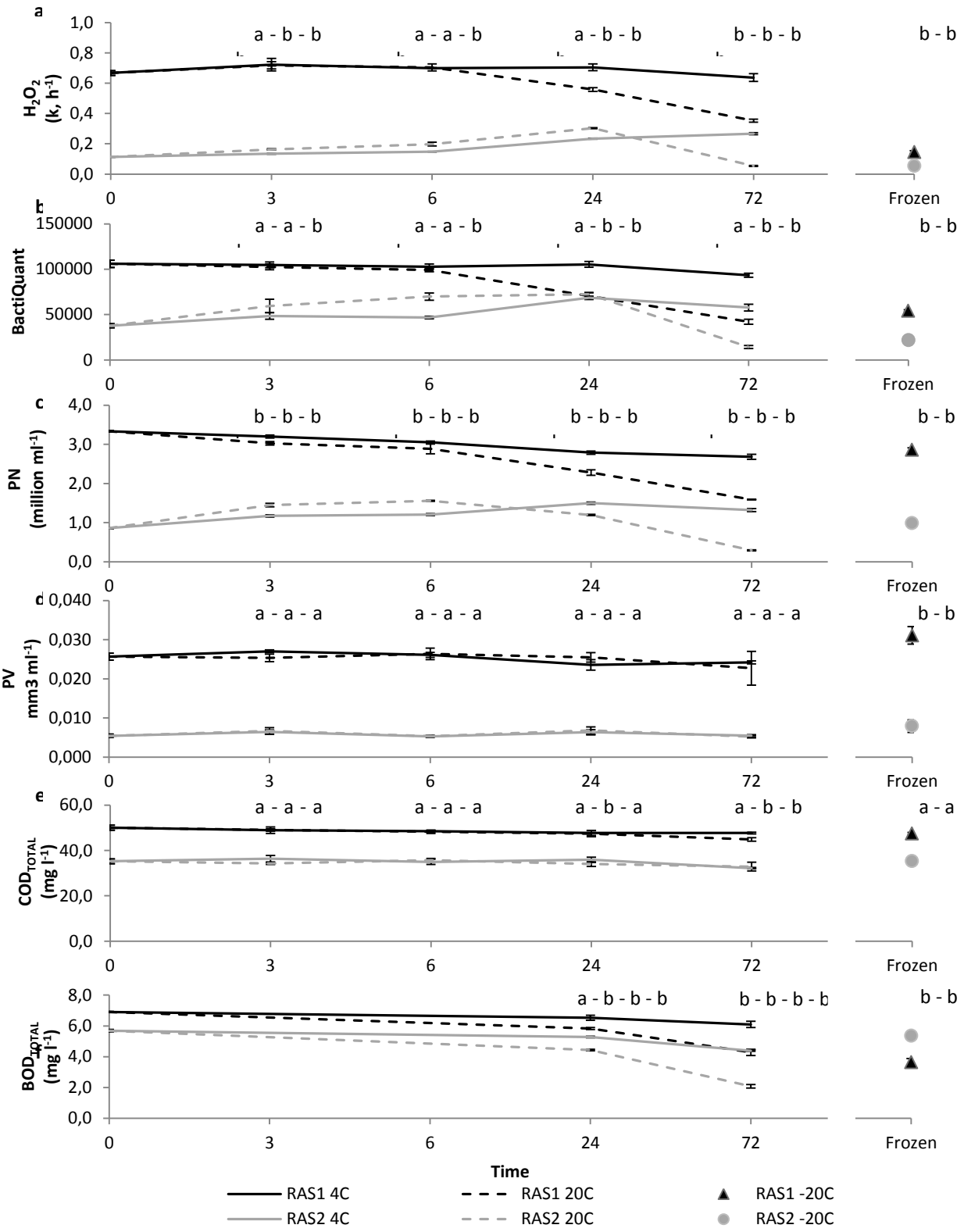
a
1,0
0,8
0,6
0,4
0,2
0,0
H_2O_2 (k, h^{-1})
a - b - b
a - a - b
a - b - b
b - b - b
b - b
0
3
6
24
72
Frozen
b
150000
100000
50000
0
BactiQuant
a - a - b
a - a - b
a - b - b
a - b - b
b - b
c
4,0
3,0
2,0
1,0
0,0
PN (million ml^{-1})
b - b - b
b - b - b
b - b - b
b - b - b
b - b
d
0,040
0,030
0,020
0,010
0,000
PV $mm3\ ml^{-1}$)
a - a - a
a - a - a
a - a - a
a - a - a
b - b
e
60,0
40,0
20,0
0,0
COD_{TOTAL} (mg l^{-1})
a - a - a
a - a - a
a - b - a
a - b - b
a - a
8,0
6,0
4,0
2,0
0,0
BOD_{TOTAL} (mg l^{-1})
a - b - b - b
b - b - b - b
b - b
Time
RAS1 4C
RAS1 20C
RAS1 -20C
RAS2 4C
RAS2 20C
RAS2 -20C

Figure 1. Changes in bacterial activity (H_2O_2 degradation rates and BactiQuant values), micro particle number and volume, and organic matter concentrations (COD_{TOTAL} and $BOD_{5\text{-}TOTAL}$) during storage of samples from RAS1 and RAS2. Different lower case letters above each sub-panel reflect statistical differences with respect to start samples, i.e., "a" indicates no difference compared to the start sample while "b" indicates statistical difference. The four letters represent each treatment in the order: RAS1 4°C, RAS1 20°C, RAS2 4°C and RAS2 20°C. Regarding frozen samples, letters represent RAS1 and RAS2 respectively. No samples were collected at time 3 and 6 for $BOD_{5\text{-}TOTAL}$. Line from start to time 24 in the $BOD_{5\text{-}TOTAL}$ graph is for visual representation only.

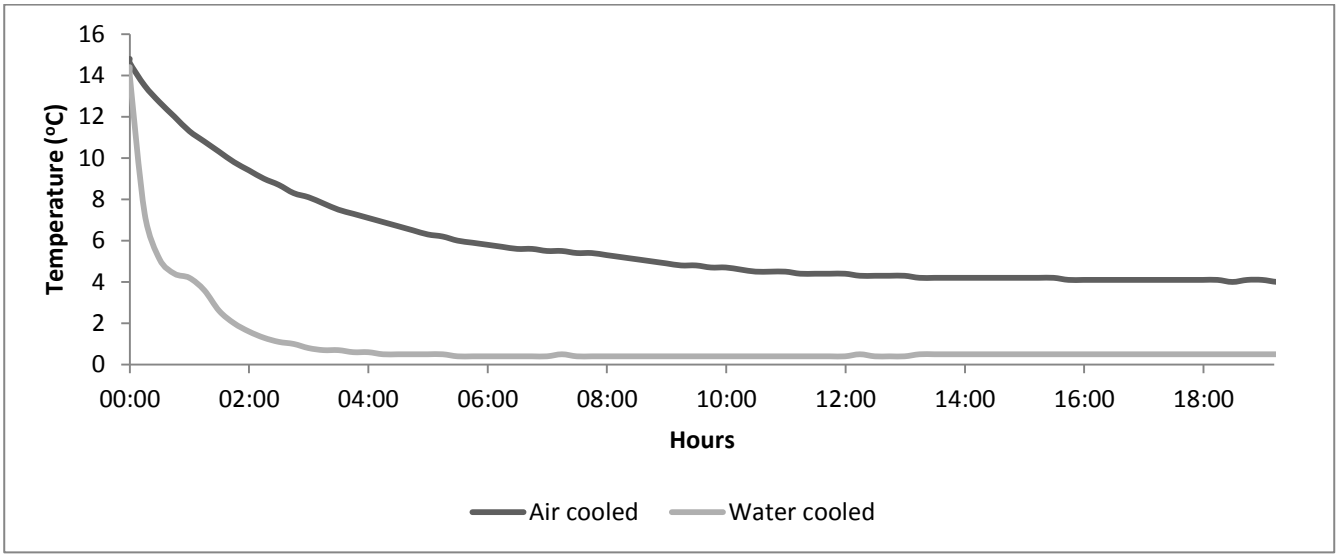

Figure 2. Temperature changes during 20 hours in samples stored at 4°C in a cooling room or in a water bath.

Paper IV

de Jesus Gregersen, K.J., Syropoulou, E., Pedersen, P.B., Pedersen, L.F., Dalsgaard, J., 2020. Foam fractionation and ozonation in fresh water recirculating aquaculture systems. *Manuscript*

Foam fractionation and ozonation in freshwater RAS

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Abstract

Foam fractionation is often considered an ineffective way of removing organic matter from freshwater due to the low surface tension. However, there is a lack of studies testing foam fractionation efficiency in freshwater aquaculture systems including recirculating aquaculture systems (RAS). Foam fractionation may be applied with or without ozone. To test its efficiency in freshwater RAS, a two-by-two factorial trial was carried with foam fractionation and ozonisation as main factors, each at two levels (applied or not applied). Each treatment combination was carried out in triplicate systems using 12 replicated pilot scale RAS with juvenile rainbow trout (*Oncorhynchus mykiss*). Besides measuring water quality parameters, potential organic matter build-up in biofilters was examined.

The trial lasted 8 weeks and samples were obtained once a week. Each system was fed 100 g feed daily and 60 L make-up water (MUW) was applied corresponding to 1.66 kg feed m^{-3}. Use of ozone by itself significantly reduced the number of particles (83 %), bacterial activity (48 %) and particulate BOD_5 (5-days biochemical oxygen demand; 54 %) while increasing ultra violet transmittance (UVT; 43 %), compared to the control treatment. Foam fractionation lead to significant reductions in particle numbers and volume (58 and 62 %, respectively), turbidity (62 %), bacterial activity (54 %) and total BOD_5 (51 %).

A combination of treatments resulted in a significant and an at least 40 % improvement in all but one metric (UVT), including a 75 % reduction in organic matter (BOD_5), 79 % reduction in turbidity, 89% reduction in particle numbers and 90 % reduction in bacterial activity compared to the control treatment.

The removal efficiencies were within the same range as those observed in previous studies conducted with foam fractionators in saltwater systems (with or without ozone), corroborating that foam fractionation may become a tool for controlling organic matter build-up and bacterial loads in freshwater RAS.

1. Introduction

The build-up of organic matter in recirculating aquaculture systems (RAS) is among the largest challenges in the industry (Martins *et al.*, 2010). Organic matter build-up in RAS derives from fish excretions and feed spill (Schumann and Brinker, 2020). Modern aquaculture facilities are typically equipped with primary solids removal technologies based on particle sedimentation (e.g. settling cones) and filtration (e.g. drum filters) (Timmons and Ebeling, 2010). As a result of prolonged retention times in RAS, together with the use of technologies which target mainly larger particles, fine solids and dissolved organic matter accumulate in the system (Shulin Chen et al., 1993; de Jesus Gregersen et al., 2019; Fernandes et al., 2014; Patterson et al., 1999).

The accumulation of fine solids is considered problematic due to their small size and large surface area to volume ratio providing food and space for bacteria growth (de Jesus Gregersen et al., 2019; Pedersen et al., 2017a). Similarly, dissolved organic provides energy for free-swimming bacteria. Increased bacterial growth in RAS in turn leads to increased oxygen consumption, clogging of biofilters and potentially reducing nitrification capacity (Chen *et al.*, 2006; Zhang *et al.*, 1994). Organic matter build-up in stagnant areas is also thought to explain recent cases of H_2S driven mortality events (Dalsgaard, 2019; Letelier-Gordo *et al.*, 2020).

A large portion of micro particles is composed of living microorganisms and can therefore be controlled by e.g. ultraviolet radiation (UV) (de Jesus Gregersen *et al.*, 2020). While UV disinfection is commercially relevant due to its technological maturity and easiness of application, it does not deal with organic matter build-up which is the underlying cause of microbial growth, causing an increase in the systems carrying capacity (Blancheton *et al.*, 2013; Vadstein *et al.*, 1993).

Direct removal of fine solids can be achieved using different strategies. Reducing drum filter mesh size is one possibility but rapidly becomes costly (Dolan *et al.*, 2013). Membrane filtration is another option shown to reduce colloidal particles in RAS by 77% and turbidity by 44% (Holan et al., 2014b). However, membrane filtration is also costly and a main reason for why it is not implemented in the industry (Viadero and Noblet, 2002). Fossmark *et al.* (2020) for example estimated that it would increase production costs by 27% to apply membrane filtration to Atlantic Salmon (*Salmo salar*) RAS.

An alternative technique for removing fine solids and even dissolved organic matter is foam fractionation (FF). Foam fractionation relies on surfactants in the water to generat foam that removes particulate and dissolved organic matter (Timmons and Ebeling, 2010). Foam fractionation has been show to concentrate TSS by 17 to 40 times in the foam condensate (Weeks *et al.*, 1992), and reduce particulate matter and bacteria in saltwater RAS (Barrut *et al.*, 2013; Brambilla *et al.*, 2008). Recently, Ji *et al.* (2020) tested the combined effects of drum filters followed by FF in saltwater RAS. The results showed similar or

better removal efficiently of FF compared to the drum filter when drum filter was equipped with mesh filters of 120 and 90 and 60 µm. Only when the drum filter was equipped with a 40 µm filter, did it had clearly superior removal efficiency.

Ozone (O_3) dosage can be coupled to FF. Ozone is a strong oxidizing agent that can be used by itself for disinfection in RAS (Gonçalves and Gagnon, 2011; Powell and Scolding, 2016).

The strong oxidizing properties of O_3 allow it to break down complex molecules in to simpler ones and in the process reduce organic matter loads (Davidson *et al.*, 2011; Summerfelt *et al.*, 2009).

As is applied with FF to improve foam fractionation efficiency not only by breaking down complex molecules so that they are more easily removed, but also by increasing coalescence of particles (Li *et al.*, 2009; Summerfelt *et al.*, 1997).

Foam fractionation has traditionally only been applied in saltwater systems due to seawaters high surface tension whereas their efficiency in freshwater RAS remains to be documented. The objective of this study was therefore to access the potential of FF and O_3 (individually and combined) for improving the water quality in freshwater RAS, including effects on organic matter build-up, micro particle accumulation and bacterial activity in the water as well as organic matter accumulation in the biofilter.

2. Materials and methods

2.1. Experimental setup

A two-by-two factorial experiment with foam fractionation and ozonisation as main factors was performed in 12 replicated, 0.8 m^3 pilot scale freshwater RAS (fig. 1) at DTU Aqua in Hirtshals, Denmark. Four treatment combinations were applied: 1) three control RAS without FF and O_3; three RAS with FF; three RAS with O_3 dosing; and three RAS with FF + O_3 dosing combined.

Each RAS was composed of a: 100 L cylindroconical biofilter filled with 40 L RK BioElements (RK BioElements, Denmark) and operated as a moving bed biofilter with an air flow of 4 L min^{-1}; a 200 L pump sump; and a 500 L cylindroconical rearing tank with a metal grid preventing fish from assessing the bottom cone (fig.1).

Two DC Runner 5.2 pumps (Aqua Medic GmbH, Bissendorf, Germany) in the pump sump pumped approximately 1500 L h^{-1} to the biofilter and 2000 l h^{-1} to the rearing tank, corresponding to a retention time in the rearing tank of approximately 15 min.

In order to test the effects of FF and O_3, six systems were fitted with foam fractionators (Sander Fresh Skim 200, Erwin Sander Elektroapparatebau GmbH, Germany), three systems were fitted with 1.8m high bubble columns (same height as the FF) where O_3 was injected and the remaining three systems were kept standard as control systems. Three of the systems fitted with FF were supplied with O_3 (injected in the

skimmer), while the remaining 3 systems were feed only air, to test the effects of FF alone. Three ozone generators (Ozonizer S 500, Erwin Sander Elektroapparatebau GmbH, Germany) were used to supply O_3. In order to mitigate small changes in O_3 production, each ozoniser supplied a system fitted with a bubble column and a system fitted with a FF.

Foam fractionators were operated with a water flow rate of 1500 l h^{-1} and an air flow rate of either 1320 l h^{-1} (air alone) or 1200 l h^{-1} (air) plus 120 l h^{-1} ozonized air. Bubble columns were supplied with 120 l h^{-1} ozonized air. Hydraulic retention time within FF and bubble columns was kept equal to ensure equal contact time in both systems. All gas intakes were controlled by flow meters (Key Instruments Variable area flow meter, Key Instruments, USA). Ozone was injected at a dosage of 20 g O_3 kg^{-1} feed. Incoming O_3 gas concentrations were measured using a UV spectrophotometer (at 254nm) and flow through cell as described in Hansen *et al.* (2010). Furthermore, to estimate the amount of O_3 that reacted in the water, O_3 gas concentrations from the foam fractionators and bubble columns outflow air were measured at regular intervals.

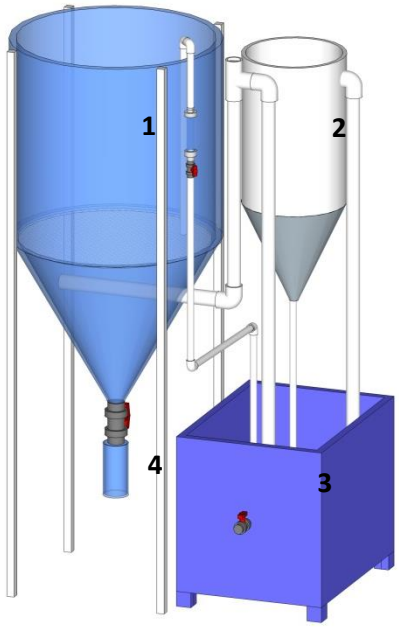

Figure 11. Pilot scale RAS including a: 1) rearing tank; 2) moving bed biofilter; 3) pump sump; and 4) sludge collector

Each system was stocked with 8.05 ± 0.03 kg juvenile rainbow trout (*Oncorhynchus mykiss*). The fish were fed a fixed ration of 100 g d^{-1} (Efico E 920, Biomar, Denmark), and 60 L of was replaced each day, resulting in a feed loading of 1.66 kg feed m^{-3}. Oxygen levels were controlled using an OxyGuard Pacific system (OxyGuard International A/S, Denmark) and ranged between 85 and 90% saturation throughout the trial. Bicarbonate was added when needed to keep pH between 7.0 and 7.3. Primary solids were collected

in settling columns at the bottom of the tanks. Each day, the conical part of the tanks were cleaned using magnetic cleaners (Tunze care magnet, TUNZE® Aquarientechnik GmbH, Germany) and the settling columns were emptied.

The trial lasted eight weeks and samples were obtained once a week. All 12 RAS had been operated without foam fractionation and ozonisation for 13 weeks prior to the trial, feed 60 grams daily and all biofilters were fully operational. Feeding was increased from 60 to 100 grams 3 days prior to the start of the trial.

2.2. Water sampling and analysis

Fish biomasses were weighed at the start and by end of the trial. Water samples were collected on day 0 prior to starting the foam fractionators and ozonisers. All water samples were collected in the morning before any daily routines. A 5 L water sample was collected from the sump of each RAS and spilt into homogeneous subsamples for individual analysis. Oxygen reduction potentials (ORP) and pH were measured daily in the sump before daily routines using a Hach HQ40d Portable Multi Meter (Hach Lange, USA), and temperature was logged automatically by the OxyGuard Pacific system (OxyGuard International A / S, Denmark).

Particles between 1 and 200 µm were measured using a Multisizer 4e Coulter Counter (Bechman Coulter, Inc, Indianapolis, USA) and both a 50µm and a 280µm apertures. Particles were grouped in size classes as described by Patterson *et al.* (1999). Assuming particles to be spherical, volume (V; $mm^3\ ml^{-1}$) and surface area (SA; $mm^2\ ml^{-1}$) of particles within each size class was calculated as: $V = 4/3 \cdot \pi \cdot r^3 \cdot x$ and $SA = 4 \cdot \pi \cdot r^2 \cdot x$, respectively, where r is particle radius within the size class and x is the number of particles within that size class. Total particle numbers (PN), total particle volume (PV), and total particle surface area (PSA) for the full range measured (1-200 µm) was calculated by summing the contribution from the different size classes.

To compare systems, particle size distributions were summarized by the β value as described by Patterson *et al.* (1999). In short, β value is the slope of the log-log transformed relationship between number of particles within size classes and the corresponding size class median diameter. A lower β value indicates a system dominated by larger particles whereas a higher β value indicates a system dominated by smaller particles.

Turbidity was measured using a Hach 2100Q (Hach Lange, USA), while UVT was measuring using a UV spectrophotometer (Beckman DU® 530 Life Science UV/Vis Spectrophotometer, Bechman Coulter, Inc, Indianapolis, USA)

Microbial activity was quantified using the hydrogen peroxide (H_2O_2) decomposition rate assay with the degradation rate constant (k, h^{-1}) quantifying microbial activity (Pedersen *et al.*, 2019). Additionally, microbial activity was measured using the BactiQuant (Mycometer A/S, Denmark) assay, expressing activity as relative BQ values.

Organic matter was measured as the 5-days biochemical oxygen (BOD_5) and chemical oxygen demand (COD). Both metrics were measured in non-filtered ($BOD_{5\text{-}Tot}$ and COD_{Tot}) and filtered ($BOD_{5\text{-}Diss}$ and COD_{Diss}) water samples using 0.45 µm filters (Advantec® membrane filter, Toyo Roshi Kaisha, Ltd, Japan). Corresponding particulate fractions (i.e., $BOD_{5\text{-}Part}$ and COD_{Part}) were calculated as the difference between the two. BOD_5 was measured following ISO 5815:1989 modified by adding allylthiourea (ATU) (Fluka Chemika).

Nitrate-N, nitrite-N and ammonium-N where measured by spectrophotometry following ISO 7890-1 (1986), DS 223 DS and DS 224, respectively.

Eight bio-elements from each biofilter were collected weekly and placed in 50 ml test tubes that were stored at -20 °C prior to COD analysis. To detach the organic matter, 20 ml Milli-Q water was added to each test tube and the tubes sonicated for 10 min using a Bransonic® ultrasonic cleaner (Branson Ultrasonics Corp, USA). The resulting water was transferred to a beaker and analysed for COD_{Tot} as described above.

Ozone concentrations in the water were measured using two methods: via the colourmetric N,N-diethyl-p-phenylenediamine (DPD) method (Buchan *et al.*, 2005; Schroeder *et al.*, 2015) and indigo method (Ozone AccuVac® Ampules, Hach Lange, USA).

2.3. Data analysis

All data are presented as average ± standard deviation. Statistical analyses were performed in SigmaPlot 13.0 (Systat software Inc., USA). Results of the two main factors (i.e., foam fractionation and ozonisation) were compared using data from the last three trial weeks (n=9), to account for system weekly variability. Data were tested for normality (Shapiro-Wilk test) and equal variance (Brown-Forsythe). Data that did not meet these requirements were log transformed prior to two-way ANOVA analysis followed by a Holm-Sidak analysis in case of significant main effects. As BactiQuant and $BOD_{5\text{-}Diss}$ results did not meet the equal variance assumption either before or after conversion they were not subjected to two-way ANOVA analysis. Differences were considered significant at $p < 0.05$.

Removal percentages were calculated relative to the control treatment based on averages of the last three trial weeks as: $\% \, removal = \frac{Treatment}{Control} * 100$.

3. Results

One fish died during the trial, and no differences were found in biomass growth rates. Oxygen saturation ranged between 85 and 90%, pH between 7.0 and 7.3, and temperature between 17 and 21°C throughout the trial. There were no differences in ammonium and nitrate levels by the end of the trial, while nitrite was significantly lower in systems fitted with foam fractionators (table 1).

3.1. Micro particles

3.1.1. Particle Numbers

Micro particle numbers declined in the first half of the trial, including control systems (fig. 2a). Systems treated with ozone displayed rapid declines within the first week (over 80 % reduction in numbers) and remained stable at a low level until the end of the trial. Systems fitted with foam fractionators showed a much slower reduction in numbers, resulting in a final reduction of 58 % compared to the control. Both factors resulted in significantly lower particle concentrations in all 3 treatment groups.

Table 1. Average water and biofilter results of the 3 last weeks of sampling (± standard deviation). * indicates statistical significant effects of the main factors (FF and O_3), while [a] indicates interactions between main factors.

Treatment	*Control*	*Foam fractionator*	*Ozone*	*Foam fractionator + Ozone*	*Units*
Num. Particles	2.43 ± 1.38	1.01 ± 1.01*	0.42 ± 0.22*	0.27 ± 0.14	million ml^{-1}
Vol. Particles	0.037 ± 0.012	0.014 ± 0.003*	0.025 ± 0.006*	0.009 ± 0.002	mm^3 ml^{-1}
S. A. particles	30.39 ± 8.77	14.32 ± 5.75*	9.84 ± 2.52*	5.23 ± 1.95	mm^2 ml^{-1}
β value	3.74 ± 0.24	3.77 ± 0.28	3.20 ± 0.22*	3.28 ± 0.26	
Turbidity	7.02 ± 2.56	2.46 ± 0.83*	4.34 ± 1.07*	1.49 ± 0.43	NTU
UVT	51.72 ± 2.59	59.37 ± 2.01[a]	73.75 ± 4.48[a]	75.94 ± 1.36	% transmission
H_2O_2	0.84 ± 0.24	0.33 ± 0.17*	0.44 ± 027*	0.08 ± 0.03	k^{-1}
Bactiquant	77011 ± 32480	35779 ± 24185	65674 ± 30563	17110 ± 6172	BQV
BOD_{5Total}	6.09 ± 1.05	2.99 ± 0.89*	3.45 ± 0.55*	1.53 ± 024	mg l^{-1}
$BOD_{5Dissol}$	0.82 ± 0.13	0.67 ± 0.10	1.01 ± 0.33	0.67 ± 0.04	mg l^{-1}
BOD_{5Part}	5.27 ± 0.98	2.33 ± 0.88*	2.44 ± 0.69*	0.86 ± 0.023	mg l^{-1}
COD_{Total}	37.64 ± 5.86	22.84 ± 2.70*	25.21 ± 2.90*	16.01 ± 1.49	mg l^{-1}
COD_{Dissol}	21.36 ± 1.71	17.84 ± 1.01*	14.83 ± 1.05*	12.78 ± 0.78	mg l^{-1}
COD_{Part}	16.29 ± 4.74	5.00 ± 2.91*	10.39 ± 2.93*	3.23 ± 1.94	mg l^{-1}
Ammonium	74.7 ± 30.0	83.8 ± 17.9	88.5 ± 36.7	82.9 ± 11.6	µg NH_4-N l^{-1}
Nitrite	119.3 ± 24.5	77.5 ± 20.6*	104.0 ± 24.3	70.5 ± 24.26	µg NO_2-N l^{-1}
Nitrate	57.5 ± 2.57	56.7 ± 2.70	57.4 ± 2.33	56.6 ± 2.65	mg NO_3-N l^{-1}
Biofilter COD	1.03 ± 0.24	0.80 ± 0.26	0.84 ± 0.21	0.80 ± 0.11	mg l^{-1}

3.1.2. Particle volume

Particle volumes increased in control systems and in systems with ozone only during the trial, albeit at different rates (fig. 1b). Systems fitted with foam fractionators declined in the start and remained stable at low levels. By the end of the trial, both the use of O_3 and FF had led to significant reductions in particle volume. Ozonisers alone resulted in a 32 % reduction, foam fractionators reduced particle volume by 62 % and the combination of both treatments resulted in a 75 % reduction compared to the control.

Table 2. Statistical results of the two way analysis of variance (ANOVA).

Treatment	*Within FF*		*Within O_3*		*Interactions*	
	F	**P**	**F**	**P**	**F**	**P**
Num. Particles	8.25	0.007	41.2	<0.001	0.8	3.660
Vol. Particles	118.5	<0.001	19.1	<0.001	0.03	0.867
S. A. particles	34.4	<0.001	72.2	<0.001	0.2	0.625
β value	0.4	0.524	33.1	<0.001	0.06	0.803
Turbidity	89.5	<0.001	17.3	<0.001	0.03	0.875
UVT	23.9	<0.001	367.8	<0.001	7.4	0.011
H_2O_2	37.0	<0.001	21.4	<0.001	1189	0.284
Bactiquant*	-	-	-	-	-	-
$BOD_{5\text{-}Tot}$	107.1	<0.001	65.0	<0.001	0.2	0.635
$BOD_{5\text{-}Diss}$*	-	-	-	-	-	-
$BOD_{5\text{-}Part}$	77.8	<0.001	56.8	<0.001	0.5	0.471
COD_{Tot}	114.1	<0.001	71.5	<0.001	0.2	0.630
COD_{Diss}	44.2	<0.001	202.9	<0.001	0.37	0.545
COD_{Part}	60.4	<0.001	10.4	0.003	3.0	0.091
Ammonium	1.7	0.203	0.12	0.731	1.9	0.173
Nitrite	39.1	<0.001	1.4	0.251	0.01	0.911
Nitrate	0.8	0.387	0.03	0.864	0.0007	0.980
Biofilter COD	0.06	3.832	1.1	0.297	1.2	0.286

*Statistical analysis not possible due to non-equal variance

3.1.3. Particle surface area

As with the previous two metrics, particle surface area was also affected by the two treatments, while it remained stable at 30.39 ± 8.77mm^2 ml^{-1} in the control group (tab. 2). Foam fractionation resulted in a 53

% reduction, O_3 treatment in a 68 % reduction and a combination of both treatments resulted in an 83 % reduction of particle surface area compared to the control, with all results being significantly significant.

3.1.4. β values

Beta values were only affected by the use of ozone. Control systems and systems with foam fractionators had similar β values by the end of the trial (3.74 and 3.77 respectively), while systems treated with O_3 displayed significantly lower β values (3.17 and 3.24 for O_3 and FF+O_3 treatments, respectively).

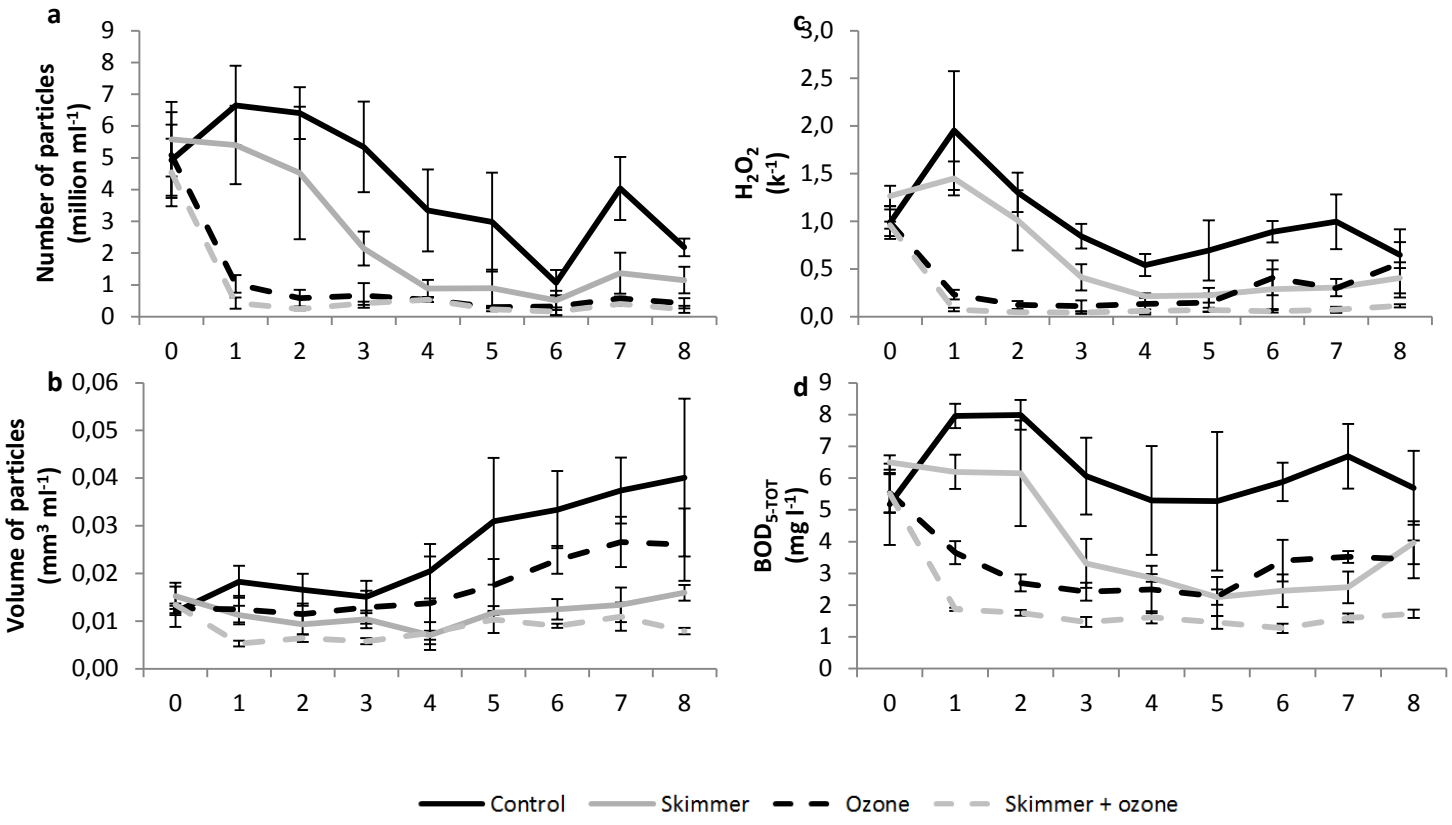

Figure 2. Variation of selected water quality parameters during the trial. a) Number of particles b) volume of particles c) bacteria activity (H_2O_2 degradation) d) BOD_{5TOTAL}

3.2. Microbial activity

3.2.1. H_2O_2 degradation

Bacterial activity, measured with the H_2O_2 degradation rate assay, was significantly affected by the two treatments (fig. 1c). Activity declined particularly rapidly in systems treated with ozone, activity after one week being reduced by 91 % in systems with ozonisers only and 96 % in systems with FF+O_3 treatments compared to the control. However, activity in systems treated with ozone only appeared to increase again and by the end of the trial was "only" 48 % lower than the control. Bacterial activity in systems with foam fractionators was reduced by 61 %, while activity in systems with both ozonisers and foam fractionators had remained low (90 % reduction) compared to the control.

3.2.2. BactiQuant

Bacterial activity measured using the BactiQuant assay varied similarly to the H_2O_2 degradation rate constants except that bacterial activity in O_3 treated systems was almost similar to the control by the end of the trial (tab. 2). Due to the lack of equal variance data were not subjected to a statistical analysis.

3.3. Turbidity and UV transmittance (UVT)

3.3.1. Turbidity

Turbidity was significantly affected by both foam fractionation and ozonation (tab. 2). By the end of the trial, a 65 % improvement in turbidity was achieved by foam fractionation compared to the control and 79 % when combining both treatments. Ozonation by itself resulted in a 38 % reduction by the end compared to the control group. However, as for bacteria activity, turbidity appeared to increase after an initial drop when applying ozone by itself.

3.3.2. UVT

Foam fractionation by itself resulted in a 15 % improvement in UVT, while ozone by itself or in combination with foam fractionation resulted in 43 and 47 % improvement, respectively. Ultraviolet transmittance was the only measurement where there was interaction between treatments (table 2), and it was therefore not possible to conclude about main effects.

3.4. BOD_5

3.4.1. $BOD_{5\text{-}Tot}$

Total BOD_5 was significantly affected by both foam fractionation and ozonation resulting in reductions of 51 %, 43 % and 75 % for FF, O_3 and FF+O_3, respectively compared to the control (fig. 1d).

3.4.2. $BOD_{5\text{-}Diss}$

In contrast to total and particulate BOD_5, the different treatments seemed to have little effect on $BOD_{5\text{-}Diss}$, (tab. 2). Lack of equal variance, however, meant that no statistical analysis was performed.

3.4.3. $BOD_{5\text{-}Part}$

The development in $BOD_{5\text{-}Part}$ was similar with that of $BOD_{5\text{-}Tot}$ for all treatment combinations (tab. 2). By the end of the trial, foam fractionation and ozonation by themselves had led to similar reductions in $BOD_{5\text{-}Part}$ compared to control of 56 and 54 %, respectively, while a combination of the two resulted in an 84 % reduction.

3.5. COD – Water and biofilter

COD was only measured in the last 3 weeks to access final values, so no considerations are made regarding trends.

3.5.1. COD_{Tot}

As with most other metrics, COD_{Tot} was significantly affected by both foam fractionation and ozonation with a combination of the two resulting in the largest decrease compared to the control (from 37.6 ± 5.9 in the control to 16.0 ± 1.5 mg L^{-1} in the FF + O_3 treatment). Foam fractionation and ozonation by themselves resulted in similar reductions of 39 and 33 %, respectively, while a combination resulted in 58 % reduction.

3.5.2. COD_{Part}

Both treatment types affected COD_{Part} significantly, declining from 16.3 ± 4.7 mg L^{-1} in the control group to 10.4 ± 2.9, 5.0 ± 2.9 and 3.2 ± 1.9 mg L^{-1}, respectively in systems with either ozonation, foam fractionation or a combination of the two (table 2).

3.5.3. COD_{Diss}

Dissolved COD was also significantly affected by the different treatments. As with every other metric, the combination of foam fractionation and ozonation had the largest effect reducing COD_{Diss} from 21.4 ± 1.7 mg L^{-1} in the control group to 12.8 ± 0.8 mg L^{-1}. Foam fractionation by itself reduced COD_{Diss} to 17.8 ± 1.0 mg L^{-1} while ozonation reduced it to 14.8 ± 1.17 mg L^{-1}.

3.5.4. Total COD in biofilters

Although seemingly lower COD_{Tot} levels that in the control group (1.03 ± 0.24 mg L^{-1}), there were no significant differences ($p>0.05$) by the end of the trial in total COD in the biofilters (approximately 17 % lower value in systems with ozonation only, and 23 % lower values in systems with foam fractionation).

4. Discussion

The different treatments had clear visual effects on the water colour and clarity as seen in figure 2. System fitted with ozone lost most of the "yellow" colour, while the overall turbidity was reduced in system fitted with FF. The loss of yellow colour was likely caused by oxidation of humic substances as seen in previous research (Davidson *et al.*, 2011; Schroeder *et al.*, 2011).

The systems were ran for 13 weeks prior to the start of the trial with a lower feed loading (1kg m^{-3}). This was changed a few days prior to the start of the trial. It is likely that this change resulted in the increase of some of the metrics, which could explain some of the initial variation in the control group (initial increase in numbers, followed by a re-stabilization).

Figure 12. Effect of the different treatments on water clarity. From left to right: Ozone, Ozone + foam fractionator, control and finally foam fractionator.

4.1. Foam fractionation

Foam fractionation has been shown to reduce organic matter loads in RAS (Barrut *et al.*, 2013; Brambilla *et al.*, 2008; Ji *et al.*, 2020; Weeks *et al.*, 1992). Previous studies were conducted in saltwater as foam fractionation is anticipated to have minimal effect in freshwater RAS due to lower surface tension

(Timmons and Ebeling, 2010). However, the current trial showed that foam fractionation also works well in freshwater with positive effects on all measured metrics.

The positive impact of foam fractionation appeared to manifest at a slower pace than that of ozonation, with a steady removal of organic matter over the course of 3 to 4 weeks (fig. 2). The use of foam fractionation seemed particularly effective at controlling particulate organic loads and particle volume (both $BOD_{5\text{-}Part}$ and COD_{Part}). These results are similar to those obtained by Barrut *et al.* (2013) using a vacuum airlift foam fractionator in seawater RAS and obtaining an approximate 80 % removal of particulate organic matter measured as dry matter. Brambilla *et al.* (2008), testing foam fractionation for removing organic matter and heterotrophic bacteria from seawater RAS with seabass (*Dicentrarchus labrax*), obtained removal rates of total suspend solids (TSS) between 12 and 40% over a single pass. The treatment in that study affected both the smallest (0.22 - 1.22 µm) and largest (> 60 µm) size fractions measured. In comparison, significant reductions in particle volume in the current study suggest that particularly the larger particles were affected / removed here.

Bacterial activity was also strongly affected by foam fractionation. The approximately 60 % reduction obtained in the current trial is similar to that obtained by Brambilla *et al.* (2008) in a seawater RAS, achieving 55 - 90 % removal depending on operational conditions, using count of viable heterotrophic bacteria in agar plates. Likewise, Rahman *et al.* (2012) achieved 2.6 times lower bacterial levels compared to a control in seawater hybrid abalone (*Haliotis discus hannai X H. sieboldii* pilot scale RAS fitted with foam fractionation.

The simultaneous reduction in both organic matter and bacterial activity observed in the current study suggests a direct removal of bacteria by foam fractionation in freshwater, similarly to that observed in seawater. In addition, the reduction in organic matter reduces a systems overall carrying capacity (Vadstein *et al.*, 1993) making it less prone to potentially harmful bacteria blooms.

4.2. Ozone

Unlike systems fitted with only foam fractionators, which showed progressive reduction in all metrics in the first half of the trial, systems dosed with ozone showed immediate responses and most metrics reached their lowest levels within the first few weeks. This development was most likely a result of ozone's oxidising effect on bacteria, corroborated by a rapid decline in bacterial activity and particle numbers compared to the control. Part of the effect was also likely caused by improved solids removal as ozone is known to improve solids removal efficiency. Park *et al.* (2013) for example found that ozone improved solids removal in a radial flow settler, while Summerfelt *et al.* (1997) found that ozone improved microscreen filtration. It is likely that ozone had similar effects in the current trial as the reduction in particle volume, $BOD_{5\text{-}Part}$ and

COD_{Part} was similar to that observed in previous trials (Davidson *et al.*, 2011; Park *et al.*, 2013; Rueter and Johnson, 1995; Summerfelt *et al.*, 1997).

Examining the effects of ozone itself in replicated seawater RAS, Davidson *et al.* (2011) found that ozonation lead to a reduction in BOD_5, total organic carbon (TOC), dissolved organic carbon (DOC), TSS, and heterotrophic bacteria abundance, while UVT increased.

During the trial, systems treated with only O_3 displayed an increase over time in most metrics. We speculate that this increase was caused by a too low realised O_3 dose. While a nominal dose of 20 g O_3 kg^{-1} feed was applied, measurements of air leaving the treatment units suggested that an ozone transfer rate to the water of approximately 35 % was achieved (in both the bubble columns and FF), corresponding to an actual dose of about 7 g kg^{-1} feed. It is possible that this lower dose allowed bacteria with higher O_3 tolerance to proliferate. The hypothesis is supported by the observed increase in bacterial activity accompanied by a similar increase in particle volume, suggesting that bacteria were forming aggregates. At the same time, particle numbers did not increase suggesting that free swimming bacteria were preferentially removed. Bacteria in biofilms and bacteria associated with particles are thus generally more resilient to disinfection, including O_3, than free living bacteria (Hess-Erga *et al.*, 2008).

One of the issues arising when using ozone in a system is its potential toxicity to the fish (Gonçalves and Gagnon, 2011; Powell and Scolding, 2016). This risk seems minimal in the current study as no ozone was detected in the water, measured both via the DPD and indigo method. Ozone presumably reacted immediately with the organic matter available as seen in a previous study on the combined use of O_3 and foam fractionators (Figueiras Guilherme *et al.*, 2020).

4.3. Combined effects

The combination of foam fractionation and ozonation resulted in the largest improvements measured during the trial. Ozone is typically applied together with foam fractionation (Kari J.K. Attramadal et al., 2012; Park et al., 2013, 2011; Schroeder et al., 2011) as it is an efficient way of transferring ozone. Similarly, ozone improves foam fractionation removal efficiency by degrading complex molecules and improving particle flocculation (Li *et al.*, 2009; Rueter and Johnson, 1995)

In the current study, ozone primary affected micro particle numbers and UVT presumably by killing free swimming bacteria (resulting in a decline in particle numbers) and oxidizing dissolved substances (e.g. humic substances) that would otherwise absorbed and refract light. On the other end, by removing solids foam fractionation led to a reduction in particulate volume, particulate COD, and turbidity. Combined, this presumably led to a reduction in system carrying capacity, aggravating the conditions for bacterial growth. Furthermore, the combined use of foam fractionation and ozonation may potentially reduce the risk of

components in the loop clogging and reduce the consumption of oxygen by heterotrophic bacteria degrading organic matter.

4.4. Effects on biofilters

Few studies have addressed the potential implications of different treatments on biofilters in RAS and their role in storing and releasing organic matter. Hence, as discussed in a previous study (de Jesus Gregersen *et al.*, 2020), a decline in organic matter in the water might be accompanied by translocation of organic matter to the biofilter. To resolve this, the current study examined the organic matter (total COD) associated with biofilter elements. Although not significant, there tended to be a lower organic matter build-up in all treated systems compared to the control, suggesting that the treatments not only improved water quality directly but also overall "system quality".

5. Conclusion

The current study showed that foam fractionation operated in freshwater RAS achieved reductions similar to foam fractionators in saltwater RAS. Furthermore, it reinforced the positive effects ozone has on RAS water quality. Foam fractionation was efficient in removing organic matter from freshwater on par with that observed in saltwater. This efficiency was further improved by simultaneously application of ozone, and the findings corroborate that combining foam fractionation and ozonation could become an efficient tool for improving the rearing conditions and overall system conditions in freshwater RAS. Furthermore, the tests conducted allowed us to understand not only the effects of FF and O_3 on the water quality, but also the implications for the biofilters.

Acknowledgement

This study was funded by Danish EPA Pesticide Research Program J.no. 667-00208 and European Maritime and Fisheries Fund (EMFF) and the Danish Ministry of Food, Agriculture and Fisheries as part of project "Lanozo" (J.no. 33111-I-17-060).

Thank you to Brian Moeller and Ulla Sproegel for their dedicated assistance and hard work in the laboratory even during a global pandemic.

References

Attramadal, K.J.K., Øie, G., Størseth, T.R., Alver, M.O., Vadstein, O., Olsen, Y., 2012. The effects of moderate ozonation or high intensity UV-irradiation on the microbial environment in RAS for marine larvae. Aquaculture 330–333, 121–129. doi:10.1016/j.aquaculture.2011.11.042

Barrut, B., Blancheton, J.-P., Callier, M., Champagne, J.-Y., Grasmick, A., 2013. Foam fractionation efficiency of a vacuum airlift-Application to particulate matter removal in recirculating systems. Aquac. Eng. 54, 16–21.

Blancheton, J.P., Attramadal, K.J.K., Michaud, L., d'Orbcastel, E.R., Vadstein, O., 2013. Insight into bacterial population in aquaculture systems and its implication. Aquac. Eng. 53, 30–39. doi:10.1016/j.aquaeng.2012.11.009

Brambilla, F., Antonini, M., Ceccuzzi, P., Terova, G., Saroglia, M., 2008. Foam fractionation efficiency in particulate matter and heterotrophic bacteria removal from a recirculating seabass (Dicentrarchus labrax) system. Aquac. Eng. 39, 37–42.

Buchan, K.A.H., Martin-Robichaud, D.J., Benfey, T.J., 2005. Measurement of dissolved ozone in sea water: A comparison of methods. Aquac. Eng. 33, 225–231.

Chen, S., Ling, J., Blancheton, J.P., 2006. Nitrification kinetics of biofilm as affected by water quality factors. Aquac. Eng. 34, 179–197. doi:10.1016/j.aquaeng.2005.09.004

Chen, S., Timmons, M.B., Aneshansley, D.J., Bisogni, J.J., 1993. Suspended solids characteristics from recirculating aquacultural systems and design implications. Aquaculture 112, 143–155. doi:10.1016/0044-8486(93)90440-A

Dalsgaard, J., 2019. 5th NordicRAS Workshop on Recirculating Aquaculture Systems. Berlin, Germany, 7-8 October 2019. Book of Abstracts.

Davidson, J., Good, C., Welsh, C., Summerfelt, S., 2011. The effects of ozone and water exchange rates on water quality and rainbow trout Oncorhynchus mykiss performance in replicated water recirculating systems. Aquac. Eng. 44, 80–96. doi:10.1016/j.aquaeng.2011.04.001

de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Dalsgaard, J., 2019. Micro particles and microbial activity in Danish recirculating rainbow trout (Oncorhynchus mykiss) farms. Aquac. Eng. 84, 60–66. doi:10.1016/j.aquaeng.2018.12.001

de Jesus Gregersen, K.J., Pedersen, P.B., Pedersen, L.F., Liu, D., Dalsgaard, J., 2020. UV irradiation and micro filtration effects on micro particle development and microbial water quality in recirculation aquaculture systems. Aquaculture 518. doi:10.1016/j.aquaculture.2019.734785

Dolan, E., Murphy, N., O'Hehir, M., 2013. Factors influencing optimal micro-screen drum filter selection for recirculating aquaculture systems. Aquac. Eng. 56, 42–50. doi:10.1016/j.aquaeng.2013.04.005

Fernandes, P.M., Pedersen, L.F., Pedersen, P.B., 2014. Daily micro particle distribution of an experimental recirculating aquaculture system-A case study. Aquac. Eng. 60, 28–34. doi:10.1016/j.aquaeng.2014.03.007

Figueiras Guilherme, M., de Jesus Gregersen, K.J., Pedersen, L.F., 2020. Effects of foam fractionation and chemical disinfection on the removal of different microalgae cultures. Aquac. Res. 1–10. doi:10.1111/are.14663

Fossmark, R.O., Vadstein, O., Rosten, T.W., Bakke, I., Košeto, D., Bugten, A. V., Helberg, G.A., Nesje, J., Jørgensen, N.O.G., Raspati, G., Azrague, K., Østerhus, S.W., Attramadal, K.J.K., 2020. Effects of reduced organic matter loading through membrane filtration on the microbial community dynamics in recirculating aquaculture systems (RAS) with Atlantic salmon parr (Salmo salar). Aquaculture 524, 735268. doi:10.1016/j.aquaculture.2020.735268

Gonçalves, A.A., Gagnon, G.A., 2011. Ozone Application in Recirculating Aquaculture System: An Overview. Ozone Sci. Eng. 33, 345–367.

Hansen, K.M.S., Andersen, H.R., Ledin, A., 2010. Ozonation of estrogenic chemicals in biologically treated sewage. Water Sci. Technol. 62, 649–657. doi:10.2166/wst.2010.919

Hess-Erga, O.K., Attramadal, K.J.K., Vadstein, O., 2008. Biotic and abiotic particles protect marine heterotrophic bacteria during UV and ozone disinfection. Aquat. Biol. 4, 147–154. doi:10.3354/ab00105

Holan, A.B., Wold, P.A., Leiknes, T.O., 2014. Intensive rearing of cod larvae (Gadus morhua) in recirculating aquaculture systems (RAS) implementing a membrane bioreactor (MBR) for enhanced

colloidal particle and fine suspended solids removal. Aquac. Eng. 58, 52–58. doi:10.1016/j.aquaeng.2013.10.001

Ji, M., Li, H., Li, J., Ye, Z., Zhu, S., 2020. Effect of Mesh Size on Microscreen Filtration Combined with Foam Fractionation for Solids Removal in Recirculating Aquacultural Seawater. N. Am. J. Aquac. 82, 215–223. doi:10.1002/naaq.10147

Letelier-Gordo, C.O., Aalto, S.L., Suurnäkki, S., Pedersen, P.B., 2020. Increased sulfate availability in saline water promotes hydrogen sulfide production in fish organic waste. Aquac. Eng. 89, 102062. doi:10.1016/j.aquaeng.2020.102062

Li, T., Yan, X., Wang, D., Wang, F., 2009. Impact of preozonation on the performance of coagulated flocs. Chemosphere 75, 187–192. doi:10.1016/j.chemosphere.2008.12.014

Martins, C.I.M., Eding, E.H., Verdegem, M.C.J., Heinsbroek, L.T.N., Schneider, O., Blancheton, J.P., Roque d'Orbcastel, E., Verreth, J.A.J., 2010. New developments in recirculating aquaculture systems in Europe: A perspective on environmental sustainability. Aquac. Eng. 43, 83–93.

Park, J., Kim, P.K., Lim, T., Daniels, H. V., 2013. Ozonation in seawater recirculating systems for black seabream Acanthopagrus schlegelii (Bleeker): Effects on solids, bacteria, water clarity, and color. Aquac. Eng. 55, 1–8. doi:10.1016/j.aquaeng.2013.01.002

Park, J., Kim, Y., Kim, P.K., Daniels, H. V., 2011. Effects of two different ozone doses on seawater recirculating systems for black sea bream Acanthopagrus schlegeli (Bleeker): Removal of solids and bacteria by foam fractionation. Aquac. Eng. 44, 19–24. doi:10.1016/j.aquaeng.2010.11.001

Patterson, R.N., Watts, K.C., Timmons, M.B., 1999. The power law in particle size analysis for aquacultural facilities. Aquac. Eng. 19, 259–273. doi:10.1016/S0144-8609(98)00054-5

Pedersen, L.F., Rojas-Tirado, P., Arvin, E., Pedersen, P.B., 2019. Assessment of microbial activity in water based on hydrogen peroxide decomposition rates. Aquac. Eng. 85, 9–14. doi:10.1016/j.aquaeng.2019.01.001

Pedersen, P.B., von Ahnen, M., Fernandes, P., Naas, C., Pedersen, L.F., Dalsgaard, J., 2017. Particle surface area and bacterial activity in recirculating aquaculture systems. Aquac. Eng. 78, 18–23. doi:10.1016/j.aquaeng.2017.04.005

Powell, A., Scolding, J.W.S., 2016. Direct application of ozone in aquaculture systems. Rev. Aquac. 10, 424–438. doi:10.1111/raq.12169

Rahman, M.M., Kadowaki, S., Linn, S.M., Yamada, Y., 2012. Effects of protein skimming on water quality, bacterial abundance and abalone growth in land based recirculating aquaculture systems. J. Fish. Aquat. Sci. 7, 150–161. doi:10.3923/jfas.2012.150.161

Rueter, J., Johnson, R., 1995. The use of ozone to improve solids removal during disinfection. Aquac. Eng. 14, 123–141. doi:10.1016/0144-8609(94)P4431-A

Schroeder, J.P., Croot, P.L., Von Dewitz, B., Waller, U., Hanel, R., 2011. Potential and limitations of ozone for the removal of ammonia, nitrite, and yellow substances in marine recirculating aquaculture systems. Aquac. Eng. 45, 35–41. doi:10.1016/j.aquaeng.2011.06.001

Schroeder, J.P., Klatt, S.F., Schlachter, M., Zablotski, Y., Keuter, S., Spieck, E., Schulz, C., 2015. Impact of ozonation and residual ozone-produced oxidants on the nitrification performance of moving-bed biofilters from marine recirculating aquaculture systems. Aquac. Eng. 65, 27–36.

Schumann, M., Brinker, A., 2020. Understanding and managing suspended solids in intensive salmonid aquaculture: a review. Rev. Aquac. 1–31. doi:10.1111/raq.12425

Summerfelt, S.T., Hankins, J.A., Weber, A.L., Durant, M.D., 1997. Ozonation of a recirculating rainbow trout culture system. II. Effects on microscreen filtration and water quality. Aquaculture 158, 57–67. doi:10.1016/S0044-8486(97)00064-1

Summerfelt, S.T., Sharrer, M.J., Tsukuda, S.M., Gearheart, M., 2009. Process requirements for achieving full-flow disinfection of recirculating water using ozonation and UV irradiation. Aquac. Eng. 40, 17–27. doi:10.1016/j.aquaeng.2008.10.002

Timmons, M.B., Ebeling, J.M., 2010. Recirculating Aquaculture, 2nd ed. Cayuga Aqua Ventures, NY, USA.

Vadstein, O., Øie, G., Salvesen, I., Skjermo, J., 1993. A strategy to obtain microbial control during larval development of marine fish. Fish Farming Technol. 69–75.

Viadero, R.C., Noblet, J.A., 2002. Membrane filtration for removal of fine solids from aquaculture process water. Aquac. Eng. 26, 151–169. doi:10.1016/S0144-8609(02)00011-0

Weeks, N.C., Timmons, M.B., Chen, S., 1992. Feasibility of using foam fractionation for the removal of dissolved and suspended solids from fish culture water. Aquac. Eng. 11, 251–265. doi:10.1016/0144-8609(92)90008-L

Zhang, T.C., Fu, Y.C., Bishop, P.L., 1994. Competition in Biofilms. Water Sci. Technol. 29, 263–270.