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Impacts of Organic Matter Removal Efficiency on the Microbial Carrying Capacity and Stability of Land-Based Recirculating Aquaculture Systems

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Preface

This master's thesis concludes my master of science (M.Sc.) in Chemical Engineering and Biotechnology at the Norwegian University of Science and Technology (NTNU), and was written at the Department of Biotechnology and Food Science in the autumn of 2017. The thesis is part of the ERA–NET research project RAS–ORGMAT, which is a collaboration between SINTEF Ocean AS (Norway), the University of Copenhagen (Denmark), and CIIMAR and INESC TEC (Portugal). All experimental work presented in this thesis was carried out at NTNU's Centre of Fisheries and Aquaculture (Sealab) at Brattørkaia in Trondheim, and at the Department of Biotechnology and Food Science at NTNU Gløshaugen.

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Last but not least, I would like to thank my family and friends for all your support, great feedback and comments on my thesis. Special thanks to Mathias, and to my flatmates Cathrine and Julie, for keeping up with me. I would not have made it without any of you!

Declaration of Compliance

I declare that this is an independent work according to the exam regulations of the Norwegian University of Science and Technology (NTNU).

Trondheim, February 6, 2018
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Abstract

Accumulation of organic matter and particles generated from uneaten feed and faeces is one of the major challenges in recirculating aquaculture systems (RAS), in particular colloidal and dissolved fractions as these serve as bacterial substrate. Particles can potentially cause damage on the gills of the reared species and may reduce their resistance to pathogens. Most diseases in aquaculture of marine fish are caused by opportunistic bacteria which become pathogenic when the fish is under stressful conditions. In the present study, the consequences of different levels of organic matter removal on physicochemical and microbial water quality were investigated in replicate small-scaled RAS for production of Atlantic salmon parr (*Salmo salar*). Two RAS were run in parallel over 140 days, including one conventional system (cRAS) and one system with an implemented membrane (mRAS). In mRAS, a side-stream of 10% of the water flow was filtered through an ultrafiltration (UF) membrane prior to the bioreactor. Furthermore, the potential of the novel method for particle and bacteria monitoring GRUNDFOS BACMON was investigated by comparing with several other counting methods employed in this study, including bacteria counting with flow cytometry, counting of colony-forming units (CFU), and particle counting with a Coulter counter.

The present study demonstrated significantly lower numbers of bacteria and particles in mRAS compared to cRAS. Furthermore, the concentrations in mRAS were more stable with fewer and smaller fluctuations, and adapted to environmental changes faster than the concentrations in cRAS. No significant difference in fish survival, weight or health were observed between the systems. The results demonstrated that implementation of a UF membrane in RAS efficiently reduced the bacterial carrying capacity (CC) by removing organic matter and bacteria. Comparison of different counting methods indicated that BACMON is applicable in monitoring the temporal variations through the experiment on a daily and weekly basis, but the numbers of bacteria and particles were too high to detect correct total counts.

Sammendrag

Akkumulering av organisk materiale og partikler som genereres fra fôrrester og avføring er en av hovedutfordringene i landbaserte resirkulerende akvakultursystemer (RAS), spesielt de kolloidale og oppløste fraksjonene fordi disse fungerer som bakteriesubstrat. Partikler kan forårsake skade på gjellene til oppdrettsfisken, og kan redusere deres sykdomsresistens. De fleste sykdommer i akvakultur med marin fisk forårsakes av opportunistiske bakterier som blir patogene når fisken er under stressede forhold. I dette studiet ble konsekvensene av forskjellige nivåer av fjerning av organisk materiale på fysisk-kjemisk og mikrobiell vannkvalitet undersøkt i replikate småskala RAS for produksjon av atlanterhavslaks (*Salmo salar*). To RAS ble kjørt i parallell over 140 dager; ett konvensjonelt system (cRAS) og ett system med en implementert membran (mRAS). I mRAS ble en sidestrøm med 10% av den totale vannstrømmen filtrert gjennom en ultrafiltreringsmembran (UF) i forkant av bioreaktoren (MBR). Videre ble potensialet for en ny metode for partikkel- og bakterieovervåking, GRUNDFOS BACMON, undersøkt ved å sammenligne den med flere andre tellemetoder som ble brukt i dette studiet, inkludert bakterietelling ved flowcytometri, telling av kolonidannende enheter (CFU), og partikkeltelling ved bruk av en coulterteller.

Denne studien demonstrerte signifikant lavere antall bakterier og partikler i mRAS enn cRAS. Videre var konsentrasjonene i mRAS mer stabile med færre og mindre svingninger, og tilpasset seg til endringer i miljøet raskere enn konsentrasjonene i cRAS gjorde. Ingen signifikant forskjell i fiskeoverlevelse, vekt eller helse ble observert mellom systemene. Resultatene viste at implementering av en UF-membran i RAS effektivt reduserer den bakterielle bæreevnen ved å fjerne organisk materiale og bakterier. Sammenligning av ulike tellemetoder viste at BACMON er aktuell å bruke til å overvåke endringer over tid, mens konsentrasjonen av partikler og bakterier var for høy i vannet til at tallene som ble målt, var riktige. BACMON burde da helst ikke benyttes for å vurdere eksakte mengder av bakterier og partikler.

List of Abbreviations

ANOVA	Analysis of variance
AOB	Ammonia-oxidising bacteria
CC	Carrying capacity
CFU	Colony-forming units
cRAS	Conventional recirculating aquaculture system
DOC	Dissolved organic carbon
ESZ	Electric sensing zone
FCR	Feed conversion ratio
FSC	Forward-scattered
FTS	Flow-through system
HRT	Hydraulic retention time
LBCC	Land-based closed-containment
MBBR	Moving bed biofilm reactor
MBR	Membrane bioreactor
mRAS	Membrane-equipped recirculating aquaculture system
NOB	Nitrite-oxidising bacteria
NTNU	Norwegian University of Science and Technology
NTU	Nephelometric turbidity units
PCA	Plate count agar
POM	Particulate organic matter
RAS	Recirculating aquaculture system
SD	Standard deviation
SE	Standard error
SGR	Specific growth rate
TAN	Total ammonia nitrogen
TGC	Thermal-unit growth coefficient
TMP	Transmembrane pressure
TOC	Total organic carbon
UF	Ultrafiltration
UV	Ultraviolet

Contents

1 Introduction

Global seafood consumption has more than doubled over the last 50 years, and is expected to keep rising rapidly with global population growth and increasing amounts of fish in general population diet (FAO, 2016). Aquaculture accounts for more than half of all seafood consumed worldwide, and has recently become one of the fastest-growing animal food production sectors (NOAA, 2017). The aquaculture industry could see a paradigm shift, changing from net-based production to sea-based and land-based closed-containment aquaculture systems. At the same time, there is a growing interest in sustainable intensification of the aquaculture production. For further industry growth, there is a need for research on cost-effective production methods which minimise the environmental impact while simultaneously ensuring optimal rearing conditions for the cultivated species. One approach to this is development of recirculating aquaculture systems (RAS), which are designed to collect and remove particles, bacteria and waste products from the rearing water so it can be recycled back to the fish tanks, as opposed to the conventional single-pass flow-through systems (FTS). Production in RAS can be considered sustainable in terms of nutrient outputs and water usage, and is not seasonally or location-limited. However, RAS are energy-intensive and requires significant investment costs. Recent studies have shown that land-based RAS are commercially viable under certain conditions and reduce some of the negative environmental impacts (Liu et al., 2016), but this is still a point of intense discussion in the industry today.

Despite the environmental advantages of RAS, an entirely new set of problems crops up when employing these systems instead of FTS, as waste products that are detrimental to the fish easily accumulate in the system. One of the main concerns in RAS is accumulation of organic matter and colloidal particles originating from uneaten feed, faeces and dead and living bacteria (Chiam and Sarbatly, 2011). Particles may stress the fish and cause gill irritations. Additionally, bacterial degradation of organic matter consumes oxygen and produces waste products such as carbon dioxide and ammonia, and according to Leonard et al. (2002), the microbes can account for a considerable fraction of the total oxygen consumption and CO_2 production in RAS. To secure an optimal microbial environment for the reared fish, a low and stable carrying capacity of heterotrophic bacteria is suggested, which is typically determined by the supply of organic matter in the system (Attramadal et al., 2012a,b, 2014; Salvesen et al., 1999; Skjermo et al., 1997). Since the smallest particles have high surface-to-volume ratio, they dissolve into bacterial substrate faster than larger particles. Thus, particles should be removed from the system as rapidly as possible to prevent breakdown into smaller particles, hydrolysis and eventual production of dissolved organic matter (Chiam and Sarbatly, 2011).

The great variety in particle sizes challenges the selection of water treatment technology. Consequently, enhanced particle separation processes must be part of the solution in combination with conventional mechanical filtration. One potentially viable alternative which has been identified for particle removal as supplement to current methods is water filtration through a membrane filtration unit (Gemende et al., 2008; Holan et al., 2014b; Pulefou et al., 2008; Viadero and Noblet, 2002; Wold et al., 2014). In combination with a traditional moving bed bioreactor, this treatment technology is commonly referred to as a membrane bioreactor (MBR). The MBR technology is fully commercialised for treatment of drinking water and waste water, but only to a limited extent used for water treatment in aquaculture plants (Sharrer et al., 2007). The potential influence of an MBR as well as the mechanisms leading to fine particle accumulation in RAS are not fully understood and should be further investigated in order to improve the production efficiency and product quality of fish produced in RAS.

Aims and objectives of this study

The present master's thesis was part of the research collaboration project RAS–ORGMAT, where the aims were to develop new strategies and water treatment technologies for removal of particulate organic matter (POM) from land-based closed-containment RAS with production of Atlantic salmon parr (*Salmo salar*), and to examine how the removal efficiency of POM influenced the amount of waste products, off-flavour compounds and the carrying capacity of heterotrophic bacteria and hence the product quality and production efficiency of the fish.

For this thesis, the aim was to evaluate the effects of POM removal by membrane filtration on the physicochemical and microbial water quality in RAS. Membranes are designed to remove the smallest fractions of the particles and thereby limit the production of dissolved organic matter, and was thus hypothesised to improve the water quality. Two RAS with different particle removal efficiencies were compared; one conventional system (cRAS), and one system modified to include a membrane (mRAS). More specifically, the objectives were to:

- Evaluate and compare different methods employed for quantification of bacteria and particles in RAS.
- Characterise the consequences of enhanced removal of organic matter on bacterial abundance and particles by comparing these factors across cRAS and mRAS.
- Investigate the temporal dynamics of bacterial abundance and particles during periods with varying loads of organic matter within and between cRAS and mRAS.

2 Recirculation Aquaculture

Recirculation aquaculture systems (RAS) are land-based plants where the used production water is treated through several steps and recycled back to the rearing tanks instead of being released into a recipient water body, which is the case in traditional flow-through systems (FTS) (Fig. 2.1). In FTS, new water is continuously consumed, which requires large amounts of energy for heating and cooling, and is also constrained by requiring continuous supply of new water. As available freshwater is decreasing worldwide, reducing water consumption in aquaculture is a necessity and makes RAS technology a highly interesting and relevant alternative (Lekang, 2013). Furthermore, as RAS reuses water, the energy requirements for water heating is reduced, making the technology even more sustainable. However, RAS requires high initial investment and operational costs, and in order to assure economic viability, this needs to be balanced with a highly efficient production (Schumann et al., 2017). Aditionally, animal welfare must be safeguarded according to strict regulations. One approach to face these challenges is to optimise and control the water quality parameters in the system and thereby maximising the growth performance, ensuring good health and welfare for the fish, and enabling an even higher recirculation degree of the water.

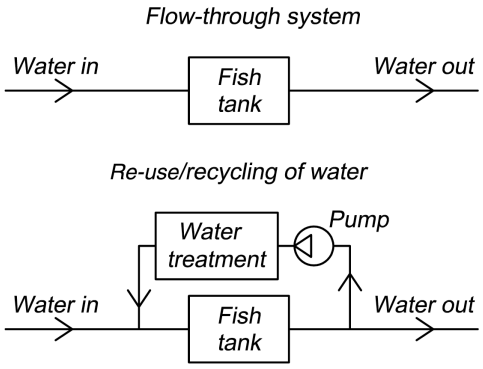

Figure 2.1. Comparison of a flow-through system (top) and a recirculated system (bottom). Figure from Lekang (2013).

High feed loading and high fish densities are typical operating conditions for RAS, and requires continuous monitoring of several physicochemical water quality parameters. However, under apparently safe conditions, sudden deviations in fish behaviour or mortality may occur. Previously, the interest in water quality has mainly focused on the physicochemical variables, but it has become more evident that microbial control is another important water quality parameter in the cultivation of marine fish (Vadstein et al., 2013).

2.1 Water quality management

One concept that greatly adds to the complexity of RAS design is the degree of water recirculation, which is based on the amount and flow-rate of the daily water supply in the system. In conventional RAS facilities, the fraction of recirculated water out of the total tank volume usually surpasses 90% (Summerfelt et al., 2001). The number of required water treatment components and the overall size of the system escalates with an increasing recirculation degree, mainly due to an increasing amount of particles in the water affecting the water quality and thus the production efficiency of the system. Water quality requirements in RAS depend on both the species and the life stage of the reared fish (Colt, 2006). Generally, healthy and fast-growing fish in aquaculture requires good water quality and require control of parameters such as pH, temperature, alkalinity, oxygen saturation and suspended solids. Additionally, control of dissolved CO_2, nitrogen, ammonia and nitrate are of particular importance in RAS, as changes in any of these may cause irreparable damages to the fish (Timmons and Ebeling, 2013). Several of these parameters depend on the bacterial populations present in the recirculated water, which makes control of the microbial environment yet another important aspect of the RAS operation (Blancheton et al., 2013). Moreover, the stability of bacterial concentrations has shown to affect the fish health positively. Applied water treatment technology makes it possible to control the most important physiochemical water quality parameters in RAS.

2.1.1 Mechanical filtration for particle removal

One of the key challenges in RAS with respect to water quality is accumulation of suspended solids, and in particular the colloidal fraction which ranges from 1 nm to 1 µm (Chiam and Sarbatly, 2011; Cripps and Bergheim, 2000; Davidson et al., 2009; Martins et al., 2010). Particles typically originate from uneaten feed, fish faeces, and biological material such as dead and living bacteria partly due to biofilm shedding from system components. Eventually, larger particles break down into smaller particles due to turbulence in the water, before they hydrolyse and produce dissolved organic compounds (Wold et al., 2014). Accumulation of particles in the rearing water of RAS has shown to be disadvantageous for the system performance and production in numerous ways. The safe limit for particle concentrations in aquaculture has been estimated to be in the range of 5–25 $\mathrm{mg\,L^{-1}}$ (*Recent Advances in Aquaculture* 1982; Timmons and Ebeling, 2013). However, this is a very wide range, and additional qualitative experiments are needed to establish a consensus on acceptable limits.

High levels of fine particles are shown to have negative impacts on RAS operation and are regarded as harmful to the fish health and the welfare with respect to both direct and indirect impacts (Chapman et al., 1987). Particles have for instance

been shown to cause damage on the gill structure and function (Bullock et al., 1994; Timmons and Ebeling, 2013), elevate stress, reduce disease resistance and induce behavioural changes (Lake and Hinch, 1999; Redding et al., 1987). Additionally, particles indirectly impact the fish welfare by reducing the water quality in various ways. Dissolved organic matter derived from solids serves as bacterial substrate for heterotrophs and enhances proliferation of opportunistic and potentially harmful bacteria (Attramadal et al., 2012c; Bullock et al., 1994), in addition to increasing the amounts of mineralised nitrogen and biological oxygen demand (Chiam and Sarbatly, 2011). Furthermore, higher loads of organic matter cause competitive inhibition of nitrifying bacteria by heterotrophs which reduces the nitrification efficiency of the biofilter (Chen et al., 2006; Michaud et al., 2006; Zhu and Chen, 2001). Particles may also protect the water from disinfection (Hess-Erga et al., 2008), clog the biofilters and increase the levels of harmful substances such as ammonia and nitrite (Eding et al., 2006), and increase biofouling of the rearing system (Holan et al., 2014a).

In order to avoid breakdown of particles into smaller ones and to prevent the aforementioned negative impacts that particle accumulation may have on the system, it is recommended to remove them as quickly as possible (Chiam and Sarbatly, 2011; Wold et al., 2014). However, the great variety in particle sizes, ranging from diameters of nm to cm, challenges the selection of water treatment technology. Moreover, particle removal efficiencies vary depending on the treatment technology used. In conventional RAS, mechanical filters such as micro-screen filters, sand filters or hydro-cyclones are employed (Wold et al., 2014). However, these treatments only manage to remove particles larger than 20–60 µm, while studies have shown that more than 95% of the particles in RAS are smaller than 20 µm, resulting in accumulation of fine suspended solids (1–35 µm), colloids (0.001–1 µm) and dissolved fractions (< 0.001 µm) of the particles (Chen et al., 1993; Holan et al., 2014a; Wold et al., 2014). Other water treatments include foam fractionators combined with ozone disinfection, which removes small particles but works out worse in freshwater than seawater systems due to larger bubbles in the water (Barrut et al., 2013; Brambilla et al., 2008). Thus, enhanced particle removal in freshwater RAS is needed.

2.1.2 Membrane filtration and biofiltration

Membrane filtration is a particle separation process which has the potential to give a more advanced and efficient removal of fine particles than conventional mechanical filtration do and thus address some of the issues previously described. In membrane filtration, a micro-porous layer provides a barrier to the finest particles and only allows dissolved components to pass through. Pressure-driven membrane processes are most commonly used in RAS, where a hydraulic pressure is used to force water molecules through the membrane while the substances being removed are retained by the membrane (Chiam and Sarbatly, 2011). The two types of

membrane filtration technology employed in RAS include ultrafiltration (UF) and microfiltration (MF) with pore sizes of 0.001–0.1 µm and 0.1–10 µm, respectively. This technology makes it possible to remove the fine and colloidal particle fractions, which may lead to reduced and more stable bacterial carrying capacity (CC) by removal of potential bacterial substrate. This may yield increased nitrification efficiency and microbial stability, and thereby healthier fish. Despite the benefits of membrane filtration, several drawbacks are associated with it, including particle fouling, reduction in water flux and frequent maintenance requirements, as well as being a costly technology due to operational costs and licensing of proprietary technology.

In addition to production of bacterial substrate, accumulation of organic matter can result in reduced conversion of total ammonia nitrogen (TAN) which is toxic to the cultivated fish. Ammonia originates from fish excretion and from decomposition of organic matter, and is converted into nitrite by ammonia-oxidising bacteria (AOB) and then into nitrate by nitrite-oxidising bacteria (NOB), which is the nitrification process. This process requires oxygen and produces H^+ ions and thus decreases the pH. Biofilters are commonly used in RAS for reduction of water exchanges by converting the toxic fish waste product ammonia into nitrate through nitrification and thereby lower the ammonia toxicity. Many different designs are used with varying benefits and drawbacks.

The combination of a membrane unit and biofilter is an increasingly employed method of treating the water in aquaculture, as the knowledge on cost effectiveness increases (Judd, 2008; Lesjean and Huisjes, 2008). Moving bed biofilm reactors (MBBR) are biofilters/bioreactors that utilise biofilm carriers. In combination with a membrane separation unit, biofilters are often referred to as membrane bioreactors (MBR). The membrane unit can be either submerged with the biofilter or be external and treating a sidestream of the water flow (sidestream MBR). Several studies have been done on the submerged type of MBR (Holan et al., 2014b; Ivanovic and Leiknes, 2008; Ivanovic et al., 2006; Leiknes et al., 2006), where the treatment had positive effects on the water quality with respect to particle counts and bacterial abundance as well as nitrification efficiency. However, there is limited knowledge on the potential benefits of using MBR as part of the water treatment in RAS with salmon.

2.2 Microbial control and stability

An important aspect of RAS is to establish microbial control in the rearing tanks by stabilising the amounts of bacterial substrate. Accumulated particles due to recirculation of the water, long hydraulic retention time and high feed loading favour microbial growth (Blancheton et al., 2013; Leonard et al., 2000; Rurangwa and Verdegem, 2015). The abundance and composition of the bacterial communities depend on the supply of organic matter and on the selective forces in the

water (Vadstein et al., 2004, 1993). Implications of changes in bacterial water quality have not been thoroughly studied. Vadstein et al. (1993) defined the term microbial maturation of the water based on the r/K-selection theory of Andrews and Harris (1986). This theory distinguishes between fast-growing bacteria (r-strategists) and slow-growing bacteria (K-strategists). The r and K terms are drawn from standard ecological algebra as illustrated in the simplified model of population dynamics (Eq. 2.1, Verhulst, 1838):

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right) \tag{2.1}$$

where N is the population, r is the maximum growth rate, K is the carrying capacity of the environment, and dN/dt denotes the derivative of N with respect to time t. Thus, the equation relates the population growth rate to the current population size, incorporating the effect of the two constant parameters r and K. According to the r/K-theory, fast-growing opportunistic bacteria are favoured in environments rich in nutrient supply that give little competition for resources, whereas the more predictable slow-growing bacteria thrive in environments scarce in nutrients. Furthermore, opportunistic bacteria typically become pathogenic to the fish when they are stressed by other factors, such as problems caused by high concentrations of particles.

The carrying capacity of the system is the maximum number of bacteria that can be sustained in the system over time, and is defined by density-dependent restrictions like availability of nutrients. Hence, the supply of organic matter is typically the limiting resource determining the carrying capacity of heterotrophic bacteria in the system. Moreover, a low and stable carrying capacity is suggested to be a strategy for securing an optimal microbial environment for the cultivated fish, which may be achieved by maintaining a low substrate availability per bacteria (Attramadal et al., 2012a,b, 2014; Salvesen et al., 1999; Skjermo et al., 1997; Vadstein et al., 1993).

Ideally, the supply of organic matter should be stable and low in order to obtain microbial numbers close to carrying capacity and a low fraction of opportunistic bacteria. Studies have shown that the number of heterotrophic bacteria in the fish tanks of a RAS is not determined by the quantity of water replaced but rather by the quantity of fish faeces reaching the biofilter (Leonard et al., 2002). Accordingly, a higher removal efficiency of organic matter between the fish tanks and the biofilter is expected to reduce the number of bacteria in the fish tanks, which can be performed by for example inserting filtration units prior to the biofilter unit. However, it is difficult to assess and control the microbial water quality in RAS, and there is a need to identify factors affecting changes in the bacterial dynamics in terms of bacterial abundance and composition in order to achieve this.

2.3 Analytic methods for cell counting in RAS

The aquatic environment in aquaculture systems contains bacterial cells, other microorganisms, and a range of abiotic particles. As the relevant particles are of microscopic sizes, typically ranging from 0.4 µm to a few 100 µm when excluding the viruses, there is a need for methods that can resolve and count them analytically. Several methods for cell counting exist, including direct and indirect counting, as well as manual and automated counting. The principles behind the methods used in the present study are described in this section.

2.3.1 Manual cell counting

Cells can be counted manually by plating and counting colonies on agar plates. Counting of colony-forming units (CFU) is a conventional method for assessing water quality in terms of bacterial counts, and a large number of studies in aquaculture have relied on this method (Attramadal et al., 2012b; Leonard et al., 2002; Michaud et al., 2009; Salvesen et al., 1999; Skjermo et al., 1997; Sugita et al., 2005). This cultivation-based method is commonly used because it requires small investment costs in equipment and is relatively easy to perform. However, the method is time-consuming as it provides a delayed response (at least 2–3 days) due to the need of cultivation, and it is incomplete in as it quantifies only a variable fraction of the viable cells present in the samples dependent on the agar medium used on the plates, and thus a substantial discrepancy between cultivable cell counts and total cell counts is often observed (Berney et al., 2008; Schreier et al., 2010; Staley and Konopka, 1985). Normally, about 99% of the microbial cells present in the environment are uncultivable. In aquaculture conditions, up to 19% of the cells have shown to be cultivable in mature water (Salvesen et al., 1999). However, this is still only a small fraction of the total amount of bacteria in the water. Furthermore, growth and distribution of colonies on the plates are not always homogeneous as the colonies may differ in diameters, densities and shapes (Corkidi et al., 1998).

2.3.2 Automated cell counting

In recent years, automated cell counting has become an attractive alternative to manual counting as it offers more reliable results in a fraction of the time, and significantly improves count reproducibility and accuracy. Automated cell counters can be based on electrical impedance, flow, or image analysis, and have varying degrees of accuracy, speed and efficiency.

The Coulter principle, invented by Wallace H. Coulter in the late 1940's, is a technology for counting and sizing particles using impedance measurements. The

principle is based on measured changes in electrical impedance produced by non-conductive particles suspended in an electrolyte (Fig. 2.2). Particles are pulled through an aperture between two electrodes, referred to as the electric sensing zone (ESZ), where they displace their own volume with electrolyte. The volume displaced is measured as a voltage pulse where the height of each pulse is proportional to the volume of the particle. In this way, the number, volume, mass and surface area size distribution of the particles are provided in one measurement. The most commonly used automated counting instrument that is based on this principle is the Coulter counter. These instruments can count several thousand particles per second, and the particle detection is unaffected by colour, shape, composition and refractive index. However, they are unable to provide cell viability information, and unable to classify the particles into bacteria and abiotic particles.

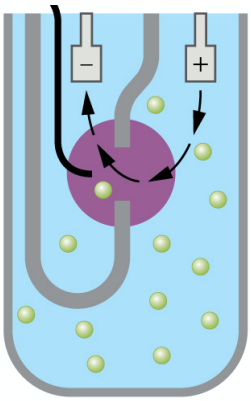

Figure 2.2. Schematic overview of the Coulter principle, showing particles (green) in electrolyte passing an aperture in the electrical sensing zone (purple).

Flow cytometry is a laser-based technology used to measure the number of individual cells and particles, in addition to an increasing number of other characteristics, and is well established in microbial studies of aquatic samples (Díaz et al., 2010; Wang et al., 2010). The method has been applied in aquaculture for cell counting in water with high precision (Attramadal et al., 2014). Cells are delivered in single file past a point of measurement where light from a powerful light source, often a laser, is scattered every time on cell passes. The light scattering depends on the size, shape and internal complexity of the cell, such as nucleus and cell membrane. The emitted light is collected by optics and directed to filters which send the light signals into detectors collecting light at particular wavelengths. Forward- and side-scattered light is detected, as well as fluorescence emitted from stained cells. Forward-scattered light is proportional to the size or the cell-surface area whereas side-scattered light reflects the internal complexity of the cell and is a measurement of refracted and reflected light. An overview of the flow cytometer is shown in Figure 2.3. Despite the high accuracy and efficiency, flow cytometry is expensive and complicated to perform and interpret under commercial aquaculture conditions (Rojas-Tirado et al., 2017).

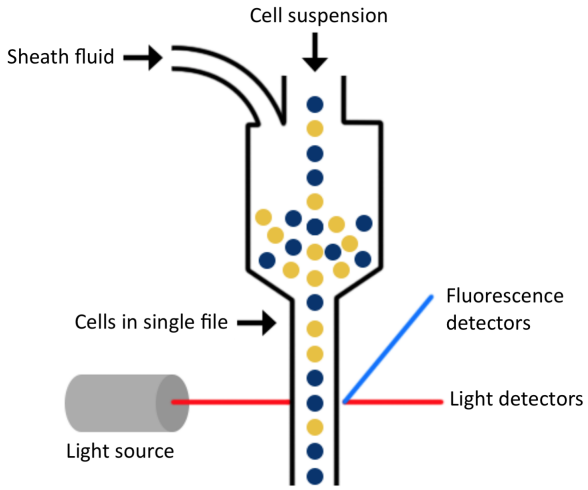

Figure 2.3. Overview of the flow cytometer. Sheath fluid focuses the cell suspension, causing cells to pass through a laser beam in single file. Forward- and side-scattered light and fluorescent signals emitted from stained cells are collected by different detectors. Modified figure from Abbexa Ltd. (2017).

Recent approaches consider the use of high-quality microscopy images where a statistical classification algorithm is used to perform automated cell detection and counting as an image-based analysis task. Image-based cell counters utilise bright-field or fluorescent microscopes coupled with digital cameras to obtain images that are analysed with an image analysis software, and are either self-contained or connected to an external computer. Samples are contained in either disposable consumables or flow cells that ensure the same volume is analysed each time, allowing for accurate volumetric counts. The consumable or flow cell is an integral part of the counting system and its performance impacts the accuracy of the results.

A novel approach in image-based cell counting is the optical on-line bacteria sensor GRUNDFOS BACMON developed by Højris et al. (2016). The sensor is based on three-dimensional scanning by a moving digital microscope which counts individual suspended particles and classifies them as either bacteria or abiotic particles by considering 59 different image parameters. The instrument measures continuously by using automated batch sampling, and delivers the results within few minutes to a computer. The method is cheap and easy to use as no reagents or chemicals are needed, which is also beneficial for the environment, and there is no need for calibration. Real-time measurements allows for control of the microbial environment with respect to total bacterial counts and gives a better knowledge for responses in the system. It is possible to react upon sudden changes or slow developments in bacteria concentrations and to work backwards to do source tracking. The method also provides high accuracy and reproducible results. Another advantage with this system is that daily fluctuations in the systems may be observed which is not the case for daily manual measuring methods.

3 Materials and Methods

The main experiment that this study is based on is part of the research project "Developing water treatment technology for land-based closed containment systems (LBCC–RAS) to increase efficiency by reducing the negative effects of organic matter" (RAS–ORGMAT, 2016–2018, ERA-Net COFASP), and took place at NTNU's Centre of Fisheries and Aquaculture (Sealab) at Brattørkaia in Trondheim. All experimental setup and work that was performed by SINTEF or by the author of this thesis in order to assess the data used in this study are presented below. The main experiment was run by SINTEF, who also performed measurements of the conventional water quality parameters, particle size analysis, and bacterial counting by CFU and BACMON. The fish health analysis was performed by Gaute Helberg (master student, NTNU). Flow cytometry was performed by the author in cooperation with Deni Košeto (researher, SINTEF). Processing of raw data and subsequent statistical testing and analysis were performed by the author.

3.1 Experimental setup and design

In an experiment with Atlantic salmon parr (*Salmo salar*), the effect of different organic removal efficiencies were investigated by comparing two recirculating aquaculture systems (RAS) with different configuration, as shown in Figure 3.1. The experimental setup included two pilot-scaled RAS: one conventional system (cRAS) and one system modified to include a membrane (mRAS). The systems consisted of six 400 L squared fish tanks with rounded corners and lightproof lids (Nofitech, Norway) for control of the photoperiod of the reared parr. Flat tank outlets with horizontal screens covering the outlet pots were used. Automatic feeders (Arvo-Tec Oy, Finland) were installed on each fish tank. Feed and faeces in the rearing water were collected in sieves and manually removed each day. The intake water was treated with ultraviolet light (UV), and the dilution water was added using a water flow meter.

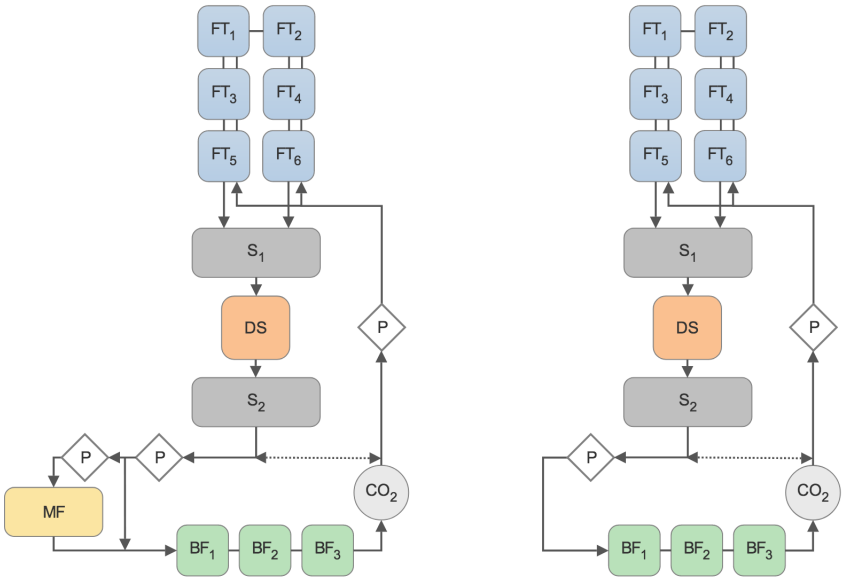

Figure 3.1. Experimental setup of the mRAS (left) and cRAS (right). The setups consisted of six fish tanks (FT), two sumps (S), a drum screen filter (DS), pumps (P), moving bed biofilters (BF) and a carbon dioxide degasser (CO_2). A membrane filtration unit (MF) filtering 10% of the water flow was implemented in mRAS.

Several water treatment components were installed for purification of the recirculated water. The water from the fish tanks was collected in a sump and passed through a mechanical drum screen filter (HEX, CM Aqua Technologies, Denmark) for particle removal before entering a second sump. The mesh openings of the mechanical filters for cRAS and mRAS were 63 µm and 26 µm, respectively. The filters were pressure driven and thus ran when a certain pressure from the particles was built up. Further, the water was driven by a frequency controlled pump (Grundfos, Denmark) into a moving bed biofilter (Nofitech, Norway) consisting of three series-connected chambers (3 × 250 L). Each chamber contained biofilm carriers (Nofitech, Norway) with a total surface area of approximately 100 m^2, and thus 300 m^2 per RAS. The biofilters were operated with upstream air and water supply from the bottom. Before the experiment was started, the biofilters were supplied with ammonium chloride (NH_4Cl) and fish feed to mature. Further, the water was aerated with a water-to-air counter-flow system and CO_2-degassed before it re-entered the fish tanks.

For the mRAS, a tubular 8 mm ultrafiltration membrane (Compact 4.0G, Pentair, Ireland) with pore sizes of 30 nm was installed prior to the biofilter. Two series-connected membranes with areas of 4 m^2 were used to filter a side-stream of 10% of the total water flow, allowing only water and dissolved matter to pass. The transmembrane pressure (TMP) was set to approximately 0.2 bar. Both systems had a hydraulic retention time (HRT) of 20 min. The total water volume of each RAS was 3500 L.

3.2 Management and operating conditions

All 12 fish tanks were each stocked with 60 salmon parr with start weights of approximately 40 g per fish, giving a fish density of 6 kg m^{-3} in each tank. The experiment lasted 140 days, counted from when the salmon parr was stocked at day 0, and can be divided into five main periods including acclimatisation and four periods with different water exchange rates and feeding conditions (Tab. 3.1). Dry feed (3 mm, Nutra Advance RC, Skretting, Norway) was automatically provided every 20 min from 8:00 to 15:00 during the entire experimental period, and the fish were exposed to light during the same time period. The fish were fed with the same amount in each tank, and uneaten pellets were collected in the outlet sieves. The feeding aimed for a much higher organic load on the system compared to large-scale commercial systems at normal operation.

The two systems were run as one complete system during the acclimatisation period (A; days 0–12) with regular feed load and water recirculation degree. Subsequently, in period 1 (days 13–52), mRAS and cRAS were run as two semi-separated systems. All hatches designed to separate the two systems were closed except for one hatch that was left open, which resulted in the same water entering the fish tanks in mRAS and cRAS, while the membrane filter in mRAS ensured that water with different organic load entered the biofilters in the two systems. Period 2 (days 53–89) started when the systems were completely separated. This period was characterised by higher organic pressure on the system due to intentional overfeeding and decreasing amounts of new water (95–90%). Period 3 (days 90–119) was characterised by normal feed load and low reuse of water (71–14%), while in period 4 (days 120–140) there was a high degree of overfeeding and a medium high water reuse (67%) resulting in high loading of organic matter on the systems.

Table 3.1. Rearing regime showing percentage of recirculated water out of total water volume of the entire systems, and feed per fish biomass for the five periods the experiment was divided into, including acclimatisation and periods 1–4. All numbers are on a daily basis.

Period	Days (d)	Recirculation degree (d^{-1})	Feed ($g\,kg^{-1}$)
Acclimatisation	0–12	94%	14.6
Period 1	13–52	94%	14.3
Period 2	53–76	95%	18.5
	77–89	90%	20.9
Period 3	90–99	71%	20.9
	100–107	43%	22.0
	108–119	14%	19.0
Period 44	120–140	67%	68.7

3.3 Water quality analysis

Physicochemical water quality parameters were measured by SINTEF for the duration of the experiment, including temperature, pH, dissolved oxygen, nitrite (NO_2–N), nitrate (NO_3–N), total ammonia nitrogen (TAN; total amount of NH_4^+ and NH_3), carbonate alkalinity (as $CaCO_3$), salinity, turbidity, total organic carbon (TOC) and dissolved organic carbon (DOC). Turbidity was measured twice a week in the sumps before and after the mechanical filter and in the outlet of the CO_2 degasser. Nitrogen compounds were measured every other day. Oxygen was measured in all fish tanks daily. The rest of the parameters were measured daily in the sumps and/or the CO_2 degasser outlet. Sodium bicarbonate ($NaHCO_3$) was added to adjust the alkalinity and maintain it at $50\,g\,L^{-1}$ as $CaCO_3$.

3.4 Quantification of bacteria and particles

Different methods were used to determine the number of bacteria and particles in the rearing water of the two RAS, including cultivation and subsequent counting of colony-forming units (CFU), flow cytometry, automated monitoring of bacteria and particles with BACMON sensors, and sizing of particles with a Coulter counter. The principle behind these technologies are described in Chapter 2, and the experimental work performed by SINTEF and/or by the author are described below.

3.4.1 Flow cytometry

For quantification of bacteria in the rearing water, the laser-based technology flow cytometry was conducted. Water samples were collected from the fish tanks, sumps and CO_2 degasser outlets and fixated with glutaraldehyde (final concentration of 0.5%) to stop further growth. Subsequently, the samples were mixed and snap frozen in liquid nitrogen and stored at $-20\,°C$. Upon further analysis, the samples were thawed in room temperature and vortexed well. Samples were then diluted at least 10 times with 0.2 µm filtrated 0.1X TE buffer to keep the cell counts below 1000 events per µL in order to assure a high degree of accuracy.

For staining of the bacterial DNA in the samples, the gel stain SYBR® Green I (Life Technologies, Thermo Fisher Scientific Inc.) was used, which has a maximum excitation wavelength of 497 nm. The fluorescence emission of DNA stained with SYBR® Green I is centred at 520 nm. The stain was diluted 50 times with 0.1X TE buffer to a working solution, of which 10 µL was added to the samples to a total of 1 mL. The samples were mixed and incubated in the dark for 15 min prior to analysis in the flow cytometer. As the stability of the dye decreases after a

certain period of time with light exposure, all samples were kept in tubes covered with aluminium foil, and only six samples were analysed each round.

The samples were analysed with a BD Accuri™ C6 Flow Cytometer. The performance of the instrument was validated using 6-peak and 8-peak bead solutions prior to each analysis to ensure that the lasers and detectors were working and that there were no bubbles, clogs or contamination in the system. A new sheath fluid was made every day, while a new cleaning solution was prepared every 14th day and a new decontamination solution was made when empty.

A medium flow rate (35 µL min^{-1}) and a 3 min collection time were set for high accuracy on the bacterial count analysis. The stained cells were excited by a blue laser (488 nm) and emitted green light. The detector for green fluorescence (FL1) collected the number of green light emissions (533±15 nm) reflecting the number of bacteria present in the sample. The threshold value of the FL1 detector was set to 2000. All samples were analysed with the same settings.

Further analysis of the results was conducted using the BD CSampler™ Software. Fluorescence signals below 2×10^3 were considered to be noise, such as viruses or tiny particles, and were excluded from the results. Signals above 10^5 in forward scatter (FSC) were considered to be cell aggregates and thus also excluded. Universal gating of the sample plots was preferably performed to ensure the same thresholds for all samples, however, as some data were lost this was not possible for all samples. Subsequently, the data were exported to Microsoft® Excel for calculation of the final bacterial concentrations.

3.4.2 Colony-forming units

The number of viable bacterial cells in the rearing water was achieved by calculating the number of colony-forming units (CFU) per volume water. The laboratory work was performed by SINTEF. Dilutions of 10^{-2} to 10^{-5} were prepared in Milli-Q® water. A volume of 100 µL of each dilution were spread on duplicate plates with Plate Count Agar (PCA) and incubated for 14 days at 10 °C. Total CFU was calculated as the average number of colonies after 14 days, where plates containing 30–300 colonies were preferably considered. For calculation of the relative fraction of fast-growing bacteria, the number of CFU that appeared after two days were counted and divided by the total CFU (Salvesen and Vadstein, 2000). Cultivability, i.e. the fraction of cultivable bacteria, was calculated by dividing numbers of CFU with total cell counts achieved from flow cytometry.

3.4.3 Automated monitoring of bacteria and particles

The concentration of bacteria and particles in the water was monitored by the in-line, automated, optical GRUNDFOS BACMON sensor (Højris et al., 2016). One BACMON sensor was applied in each RAS for continuous measurements through the whole experiment. However, due to problems with power supply and internet connection, both sensors could not be used simultaneously at all times. To evaluate the accuracy of the two sensors, they were placed in the same system and location for three days. The flow cells were changed regularly to avoid fouling. Concentrations equal to zero were assumed signal errors and thus removed from the data set.

3.4.4 Particle size analysis

Characterisation of the particle size distribution in the rearing water was analysed by SINTEF using the Multisizer 3 Coulter Counter (Beckman Coulter Inc., USA). For this study, a particle size range of 2.28–60 µm was used. Samples collected approximately once a week from the sumps and the CO_2 degasser outlet of mRAS and cRAS were analysed.

3.5 Fish growth and welfare

Fish performance assessment was performed at the beginning of the experiment (day 0), within each period, and at the end of the experiment (day 140). Growth performance was assessed by calculating the daily specific growth rate (SGR), which was calculated according to Equation 3.1 (Hopkins, 1992):

$$\mathrm{SGR}\left(\%\,\mathrm{d}^{-1}\right) = \frac{\ln W_t - \ln W_0}{t - t_0} \times 100 \tag{3.1}$$

where W_t is the average weight at time t, and W_0 is the initial individual fish biomass at time t_0. The initial weight of the fish were estimated based on the average of a sub-sample of 12 individuals from each RAS.

The thermal-unit growth coefficient (TGC) was calculated according to Equation 3.2 (Iwama and Tautz, 1981):

$$\mathrm{TGC} = \frac{\sqrt[3]{W_t} - \sqrt[3]{W_0}}{\mathrm{T}(t - t_0)} \times 1000 \tag{3.2}$$

where $\mathrm{T}(t - t_0)$ is degree days measured in °C during the period. Temperatures were averaged from the whole experimental period. Fulton's condition factor (K) is a measure of the relationship between weight and length, where a high factor is a sign of good welfare, and was calculated according to Equation 3.3 (Froese, 2006):

$$K = 100 \times \frac{W}{L^3} \tag{3.3}$$

where W and L are fish weight and length, respectively. For evaluation of the fish welfare at the end of the experiment, presence and location of any observable damage was registered.

3.6 Statistics

All data are presented as the mean ± standard error (SE) or standard deviation (SD). The SD was calculated using the STDEV function in Microsoft® Excel, and the SE was calculated by dividing the SD by the squared sample size (n). A confidence level of 95% ($p < 0.05$) was used for all statistical analysis.

Data for bacterial concentrations, particle concentrations, and physicochemical water quality parameters were tested for homogeneity of variance with Levene's test (Levene, 1960), and compared across the systems by Student's t-test, or if unequal variance, by Welch's t-test. For comparison of more than two groups of samples within each system, one-way analysis of variance (ANOVA) or the non-parametric Kruskal–Wallis test was performed. Samples collected on the same day within each RAS were treated as replicates. All statistical tests of physicochemical and microbial water quality parameters were performed with the software package PAST (Hammer et al., 2001), version 3.16.

For analysis of the correlation between the different bacteria and particle counting methods, Pearson correlation analysis was performed using the Analysis ToolPak program in Microsoft® Excel, version 16.9. Tests for significant differences in fish welfare indicators were performed using the two-sample test for equality of proportions with continuity correction.

4 Results

The results from the analysis described in Chapter 3 are given the following sections. All raw data were collected from the people who performed the physicochemical and microbial water quality analyses, and then processed and analysed by the author. The flow cytometry data were processed in cooperation with Deni Košeto (researcher, SINTEF). The fish health data were processed by Gaute Helberg (master student, NTNU).

4.1 Physicochemical water quality

Physicochemical water quality parameters monitored during the experiment included temperature, pH, oxygen saturation, nitrite (NO_2–N), nitrate (NO_3–N), total ammonia nitrogen (TAN), carbonate alkalinity (as $CaCO_3$), salinity, turbidity, total organic carbon (TOC) and dissolved organic carbon (DOC). Additionally, number of particles were monitored by BACMON sensors, and by particle size analysis with a Coulter counter. Average parameters from the whole experimental period for mRAS and cRAS are given in Table 4.1.

Table 4.1. Physiochemical water quality parameters in cRAS and mRAS averaged from the whole experimental period ± SE.

Parameter	mRAS	cRAS
Temperature (°C)	14.4 ± 0.1	13.2 ± 0.1
Oxygen saturation (%)	96.8 ± 1.1	102.3 ± 1.1
Salinity (ppt)	3.5 ± 0.2	3.6 ± 0.2
pH	7.75 ± 0.02	7.74 ± 0.01
Alkalinity ($mg\,L^{-1}$ as $CaCO_3$)	50.1 ± 1.5	50.7 ± 0.8
TAN ($mg\,L^{-1}$)	0.4 ± 0.1	0.4 ± 0.1
NO_2–N ($mg\,L^{-1}$)	0.20 ± 0.04	0.20 ± 0.05
NO_3–N ($mg\,L^{-1}$)	21.2 ± 1.3	18.8 ± 1.3
TOC ($mg\,L^{-1}$)	8.8 ± 0.7	11.4 ± 1.5
DOC ($mg\,L^{-1}$)	6.8 ± 0.5	7.2 ± 0.5
Turbidity (NTU)	1.2 ± 0.2	3.6 ± 0.5
Particles[1] ($mL^{-1}\times10^4$)	2.0 ± 0.5	3.0 ± 0.8
Particles[2] ($mL^{-1}\times10^5$)	2.18 ± 0.02	2.56 ± 0.02

[1]Monitored by Coulter counter. Size range: 2.2–20 µm.
[2]Monitored by BACMON sensors. Size range: 0–50 µm

The temperature should ideally have been the same in both systems, but due to heat production from the membrane and uneven temperature through the fish hall, the average temperature was 1.2 °C higher in mRAS than in cRAS ($p < 0.001$). Further, the oxygen saturation was lower in mRAS than in cRAS due to different oxygenation in the two systems ($p < 0.001$). No significant differences were observed in salinity, pH or alkalinity between the systems ($p = 0.681, 0.537, 0.643$, respectively). Concentrations of the nitrogenous waste compounds TAN, NO_2–N and NO_3–N did not differ between the systems ($p = 0.981, 0.914, 0.243$, respectively) and were relatively stable throughout the experiment from the beginning of period 2 (graph not included).

4.1.1 Organic carbon

Results given in Table 4.1 showed that the average concentrations of both DOC and TOC were higher in cRAS than mRAS, but the differences were not statistically significant ($p = 0.130$ and 0.528, respectively). When looking at the temporal developments, the difference in DOC concentrations was small and constant through the experiment (5–10 mg L^{-1}), whereas the TOC concentration was up to 48% higher in cRAS than mRAS in the end of period 2 (Fig. 4.1).

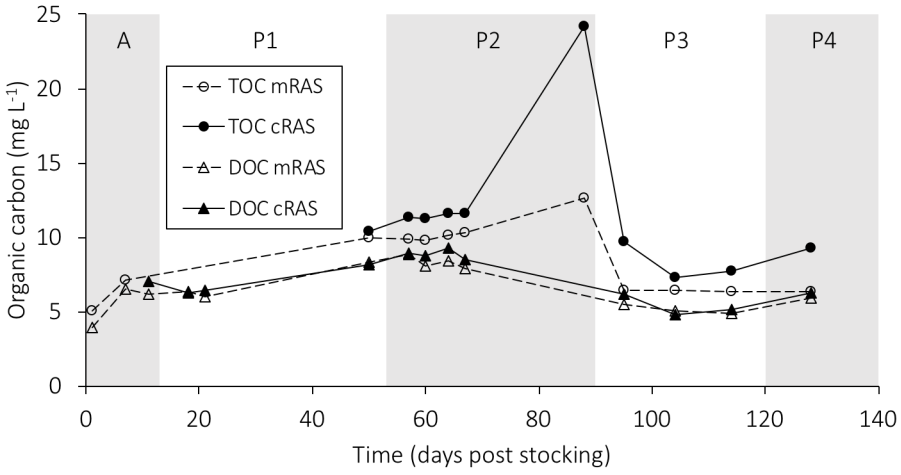

Figure 4.1. Concentrations of total organic carbon (TOC) and dissolved organic carbon (DOC) in the rearing water of mRAS and cRAS through the whole experiment. Values are given as averages, SD were negligible.

4.1.2 Particles

To investigate the effect of membrane filtration on the particle content, the number of particles and turbidity were compared across the systems. The turbidity

(Fig. 4.2a) was higher in cRAS than mRAS throughout the experiment, and significantly different in periods 3 and 4 ($p = 0.001$ and 0.010, respectively). Temporal developments of the turbidity showed both lower and more stable concentrations in mRAS than cRAS, with the largest difference being 84% on day 78 (period 2). Particle concentrations in the size range 2.2–20 µm (Fig. 4.2b) showed higher numbers in cRAS than mRAS through the whole experiment, with the largest difference of 78% on day 107. However, the difference was not statistically significant ($p = 0.190$). In contrast with the turbidity and organic matter measurements, the particles measured by the Coulter counter showed lowest densities in period 2 for both systems.

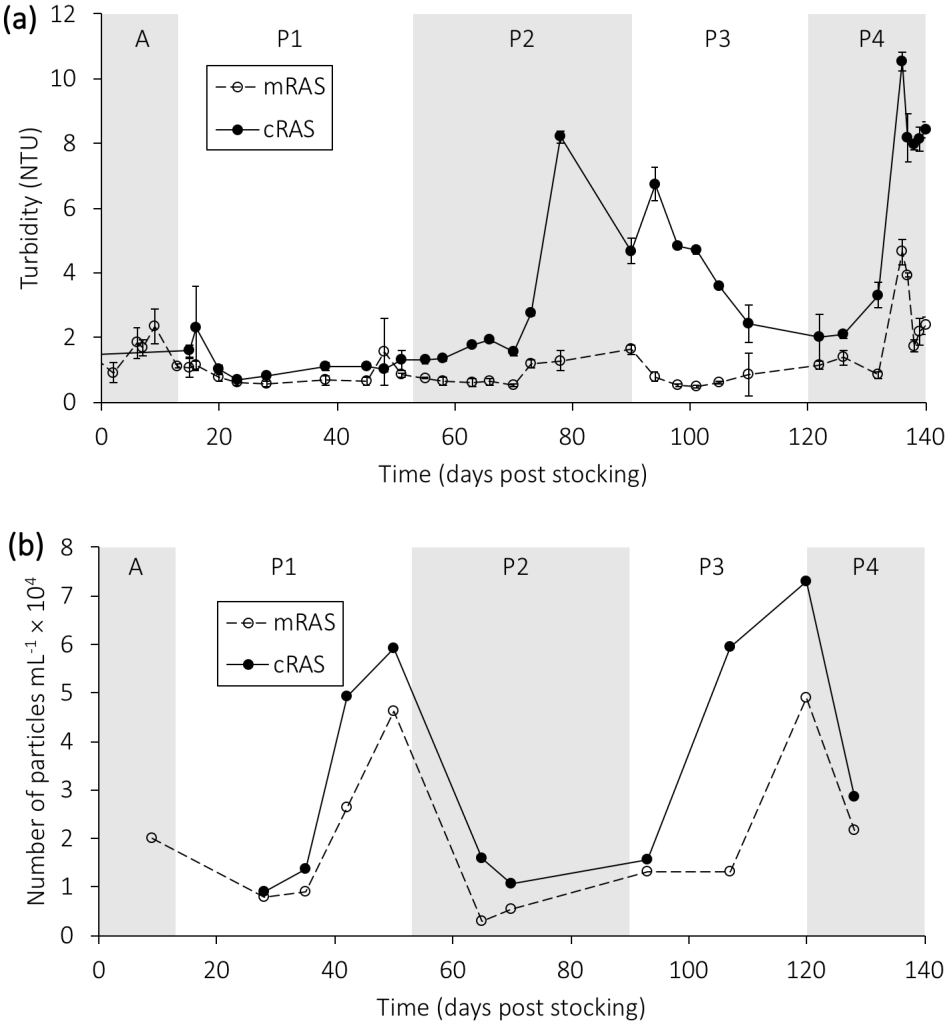

Figure 4.2. (a) Turbidity levels measured in nephelometric turbidity units (NTU), and (b) number of particles in the size range 2.2–20 µm in the rearing water of mRAS and cRAS through the whole experiment. Averages ± SE.

The particle size distribution determine that $99.6 \pm 0.1\%$ of the total number of particles in the size range of 2.2–60 µm were smaller than 20 µm in both systems, and in a subset of these particles, $98.7 \pm 0.1\%$ were smaller than 10 µm. The size distributions of the majority of the samples indicated highest frequency of the smallest particles (2–3 µm). An example of a typical particle size distribution in this experiment is given in Figure (4.3). The size distributions in cRAS and mRAS were similar throughout the experiment.

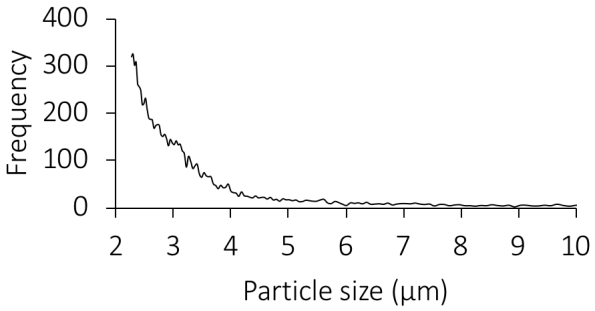

Figure 4.3. Particle size distribution in the size range of 2.2–10 µm in mRAS on day 108, measured by the Coulter counter. This distribution is representative for mRAS and cRAS for most of the experiment.

Particles monitored by BACMON did also demonstrate higher concentrations in cRAS than mRAS through most of the experiment whenever both sensors were in operation (Fig. A.1). The stability in concentration was difficult to compare but seemed higher in cRAS than mRAS certain times. The particle concentrations ranged from approximately 5×10^3 to 1.3×10^6 mL^{-1}, and were on average highest in period 2 for both systems. The largest difference between the systems was on day 87 (period 2), with about 1.1×10^6 mL^{-1} higher concentration in cRAS than mRAS. However, within the same period, the concentration varied greatly with different temporal developments across the systems.

4.2 Microbial environment

To evaluate how the enhanced particle removal efficiency by membrane filtration affected the microbial environment in the recirculating systems, the number of bacteria in the rearing water was investigated by three different methods: BACMON, flow cytometry and CFU. A summary of the numbers assessed, averaged from the whole experimental period, is given in Table 4.2, as well as the relative abundance of fast-growing bacteria to the total CFU and the fraction of cultivable bacteria out of total counts, i.e. the cultivability. All measurements showed that the membrane filtration lowered the number of bacteria, as the concentrations were

higher in cRAS than mRAS for large parts of the experiment. Further, the numbers detected by flow cytometry were higher by one order of magnitude than those quantified by BACMON and CFU. The fraction of fast-growers was on average 7% higher in cRAS than mRAS, however not significantly ($p = 0.519$), whereas the cultivability was significantly lower in cRAS than mRAS ($p = 0.019$).

Table 4.2. Bacterial abundance measured by BACMON, flow cytometry (FCM) and colony-forming units (CFU). Relative abundance of fast-growing bacteria and cultivatbility are also given. Values are given as averages from the whole experimental period ± SE. The far right column shows the reduction in numbers from cRAS and mRAS.

Method	mRAS	cRAS	Reduction
BACMON ($mL^{-1} \times 10^5$)	2.09 ± 0.01	3.36 ± 0.02	37.8%
FCM ($mL^{-1} \times 10^6$)	2.7 ± 0.2	11.0 ± 0.8	75.5%
CFU ($mL^{-1} \times 10^5$)	4.7 ± 1.8	5.1 ± 2.4	7.8%
Fast-growers (%)	18.6 ± 6.5	25.5 ± 8.3	27.1%
Cultivability (%)	13.0 ± 2.5	3.2 ± 0.6	−306.2%

4.2.1 Flow cytometry and CFU

Results of the total bacterial concentrations assessed by flow cytometry (Fig. 4.4a) demonstrated similar numbers with low variation in the two systems in period 1, with $2.2 \pm 0.5 \times 10^6$ and $2.7 \pm 0.5 \times 10^6$ mL^{-1} in mRAS and cRAS, respectively, whereas in periods 2–4, the concentrations varied more and differed significantly with numbers up to 40×10^6 mL^{-1} in cRAS ($p < 0.001$). The number of viable bacteria, i.e. total CFU, was not significantly different between cRAS and mRAS ($p = 0.891$, Fig. 4.4b) and the same trends were observed in the two systems throughout the experiment. As for the flow cytometry, an increase in bacterial numbers was observed in period 2, following a peak on day 90 in both systems with bacterial concentrations of 1.9×10^6 and 1.5×10^6 mL^{-1} in cRAS and mRAS, respectively. On day 119, there was a peak in the CFU graph, however, this was unnoticeable in the flow cytometry graph. Moreover, the variations in concentrations observed from the flow cytometry graph were more evident in cRAS than mRAS and spent longer time to adjust to changes in the feeding regime. Regression analysis showed a strong correlation between CFU and flow cytometry in mRAS ($r = 0.811$, $n = 9$), and a weak correlation in cRAS ($r = 0.418$, $n = 9$).

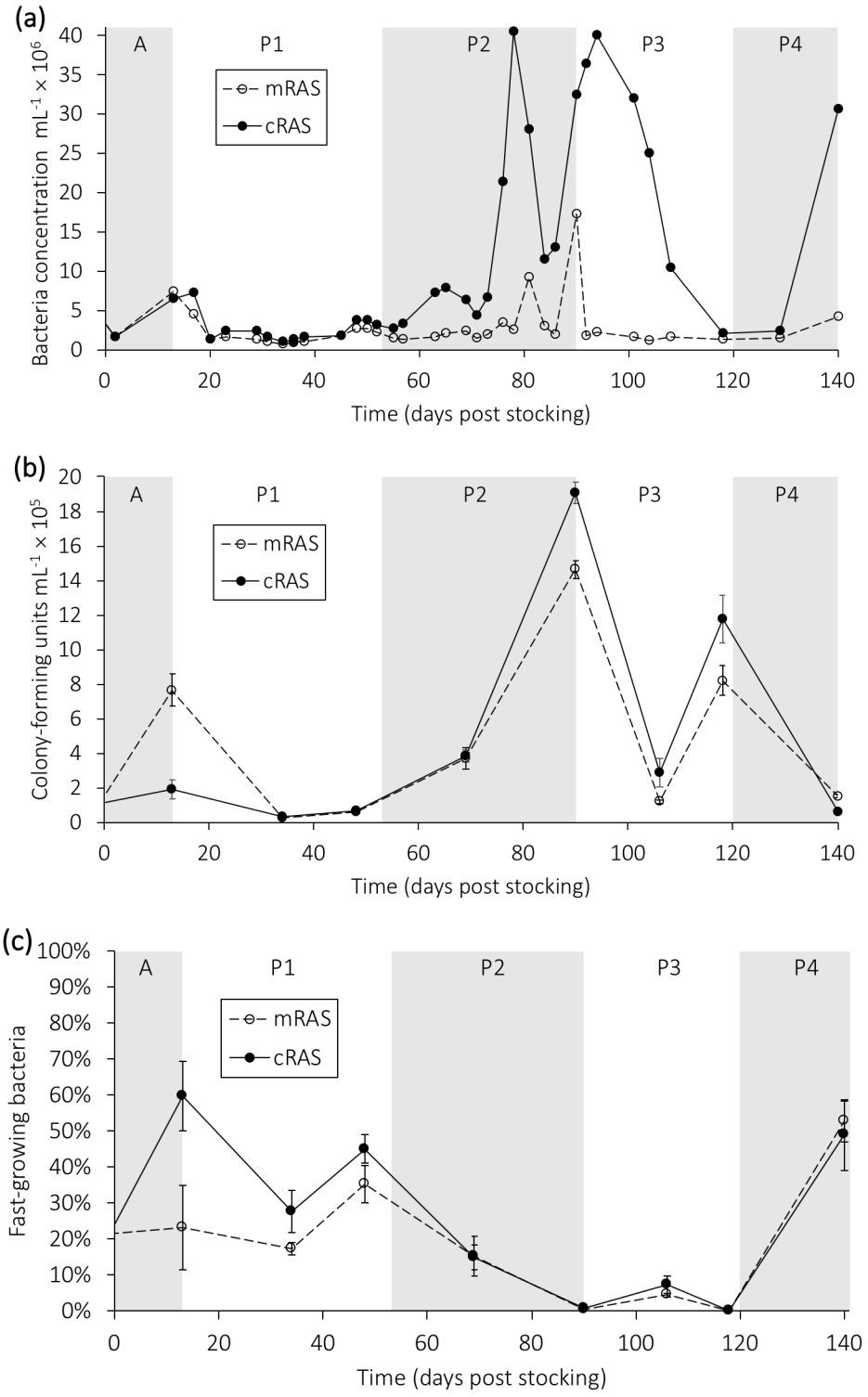

Figure 4.4. Number of bacteria assessed by (a) flow cytometry and (b) colony-forming units (CFU), and (c) the fraction of fast-growing bacteria out of total CFU in the rearing water of mRAS and cRAS through the whole experiment. Averages ± SE for CFU and fast-growers. For the flow cytometry, SE were negligible and are thus excluded from the graph.

Both total bacterial counts and CFU increased in the acclimatisation period before they decreased and stabilised in period 1 (Fig. 4.4a and b). The fraction of fast-growing colonies out of the total CFU varied in the beginning, and then showed a decrease through periods 2–3, and an increase in period 4 (Fig. 4.4c). The difference between the systems was highest in period 1, where the fraction in cRAS was 5–40% higher than in mRAS.

Cultivability, which is the fraction of cultivable bacteria out of the total bacterial counts, is given in Figure 4.5. The cultivability was higher in mRAS than cRAS throughout the experiment, and was approximately 60% on day 119 for both systems.

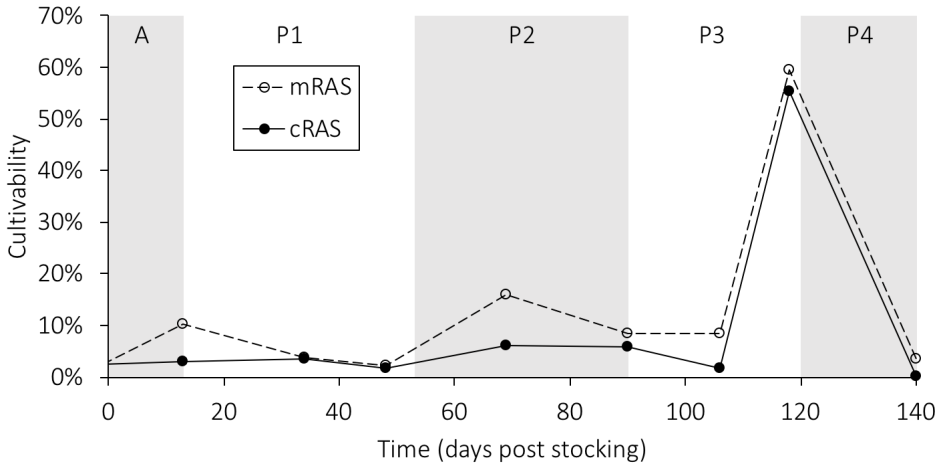

Figure 4.5. Cultivability, i.e. the total CFU divided by total bacterial counts assessed from flow cytometry.

4.2.2 BACMON

The temporal development of bacterial concentrations monitored by BACMON sensors in mRAS and cRAS is given in Figure 4.7. From day 22, the bacterial counts were higher in cRAS than mRAS through large parts of the experiment. In the period where both sensors were in operation for most of the time (period 2), the largest difference was approximately 80% on day 87. The fluctuations in the two systems followed the same trends on a daily basis in period 1 and parts of period 2. Some of the days that the flow cell was changed, sudden concentration increases were observed (e.g. on day 115). Regression analysis between BACMON and flow cytometry showed weak correlations in both mRAS and cRAS ($r = 0.478$, $n = 31$ and $r = 0.294$, $n = 21$, respectively). Comparison of BACMON and CFU showed a moderate correlation in mRAS ($r = 0.618$, $n = 8$), and no correlation in cRAS ($r = -0.168$, $n = 6$).

The BACMON sensors were placed in the same tank and system for comparison over three days (Fig. 4.6). Sensor number 1 performed 157 measurements, whereas sensor 2 performed only 144. Sensor number 1 and 2 measured numbers of 2.8 ± 0.5 and $2.4 \pm 0.5 \times 10^5$ mL^{-1}, respectively, and differed with $11 \pm 8\%$ in absolute values. Correlation analysis resulted in strong and significant correlation between the data sets ($r = 0.897$, $n = 144$).

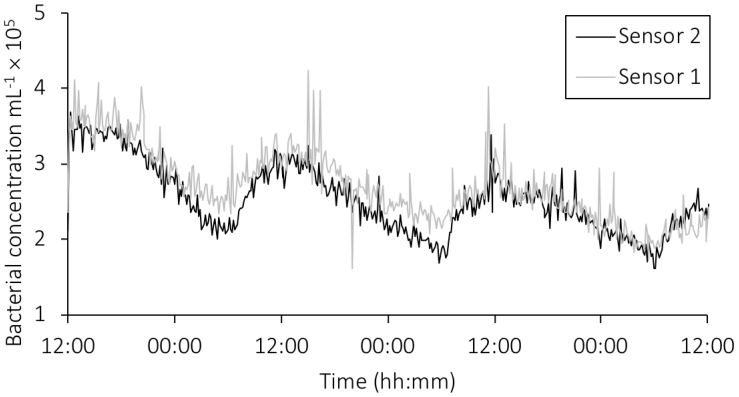

Figure 4.6. Comparison of the two BACMON sensors, both placed in sump 2 in mRAS over three days.

When the membrane was switched on (day 8), the concentrations in both systems showed high fluctuations before they dropped and stabilised somewhat at concentrations of $1\text{–}2 \times 10^3$ mL^{-1} (Fig. 4.7). After two weeks (day 21), clear changes were observed in both systems—the bacterial concentrations increased, the difference between the systems increased and the variations within each system increased. The difference between the systems became even larger a few days after the hatch, which had been left open, was closed (day 53). At the same time, the organic load increased and the variations in bacterial concentrations turned more irregular. This was especially evident in the end of period 2, when the feeding and water exchange rates were inconsistent. The concentrations decreased in period 3, and the daily fluctuations were apparently smaller but still inconsistent. For cRAS, which was only monitored in the last part of period 3, the concentrations varied highly and were at some points even below that of in mRAS. In period 4, only one sensor was in operation and measured in cRAS most of the time. During the final days, the highest detected concentrations were approximately 3×10^6 mL^{-1} (not shown in the graph).

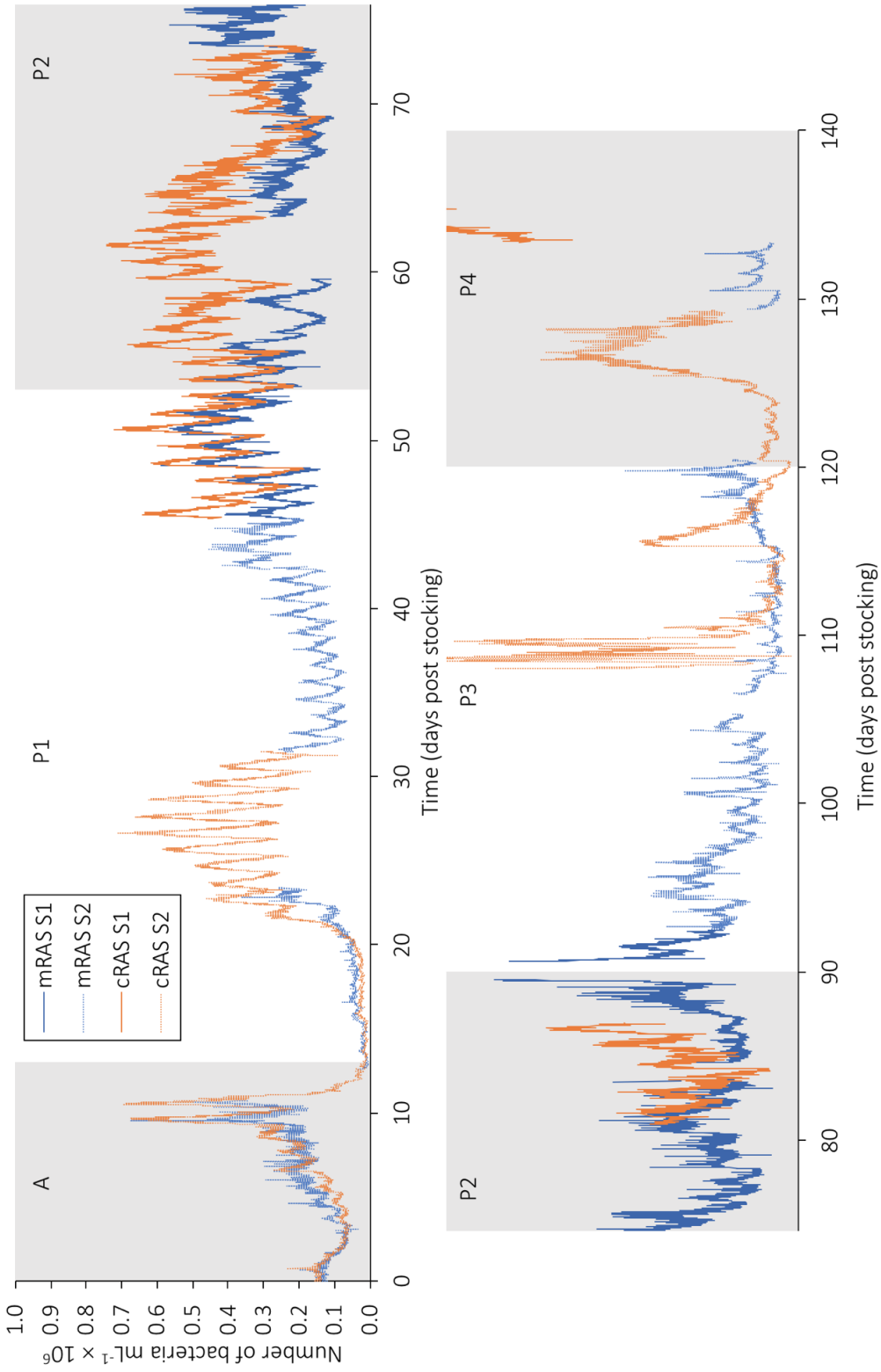

Figure 4.7. Bacterial concentrations monitored by BACMON sensors of the water in the sumps (S1 = dotted line, S2 = solid line) of mRAS and cRAS during the entire experiment.

The highly detailed measurements by BACMON, compared to the CFU and flow cytometry, allowed for continuous analysis on a daily basis. This revealed daily fluctuations following the same pattern in mRAS and cRAS, with increasing concentrations during the feeding and photoperiod (08:00–15:00) and decreasing concentrations between these periods (Fig. 4.8). The bacterial growth rates in the feeding period were higher in cRAS than mRAS, and the span in concentration within each day was larger in cRAS in period 1. However, in period 2, the fluctuations were smaller and less characteristic than period 1, even with higher feeding amounts. The absolute difference between mRAS and cRAS was typically low at the minimum values (e.g. 2% on day 48) and high at the maximum values (e.g. 27% on day 48) during each day. In period 2, however, the absolute difference was high at both minimum and maximum values during the days.

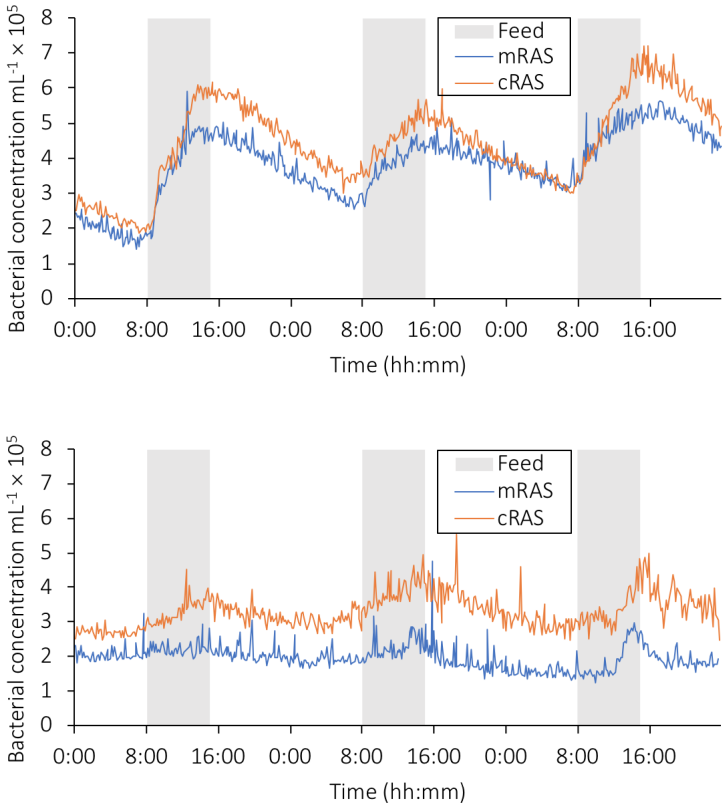

Figure 4.8. Daily fluctuations of bacterial concentration monitored by BACMON in mRAS and cRAS in (a) period 1, days 48–50, and (b) period 2, days 70–72.

In the beginning of the experiment, the bacterial concentrations were much more affected by the changes in particle concentrations than in the later stages. This is seen in period 1, where the daily fluctuations in bacteria are much higher than that of particles. The ratios between the average number of bacteria and particles within each period is given in Table 4.3, and shows that the bacteria-to-particle

ratio was lower in mRAS than cRAS within all periods. Period 3 was the period with the lowest ratios.

Table 4.3. The ratio between the average bacterial counts and particle counts monitored by the BACMON sensors in mRAS and cRAS during acclimatisation (A) and periods 1–4 (P1–P4).

Period	A	P1	P2	P3	P4
mRAS	0.8	1.6	0.7	1.1	1.3
cRAS	1.4	1.9	0.9	1.5	2.9

4.2.3 Net microbial growth potential

The average net microbial growth potential, which is the ratio of total number of bacteria incubated for 2 days out of the total number of bacteria at time zero, for mRAS and cRAS is given in Figure 4.9. The results showed high fractions in both systems, up to 5 in mRAS and 3 in cRAS. In periods 1 and 2, the growth potential was higher in mRAS than in cRAS, but the opposite was observed in period 3 and 4. Both systems showed lower net growth potential over time, and the differences between the systems decreased.

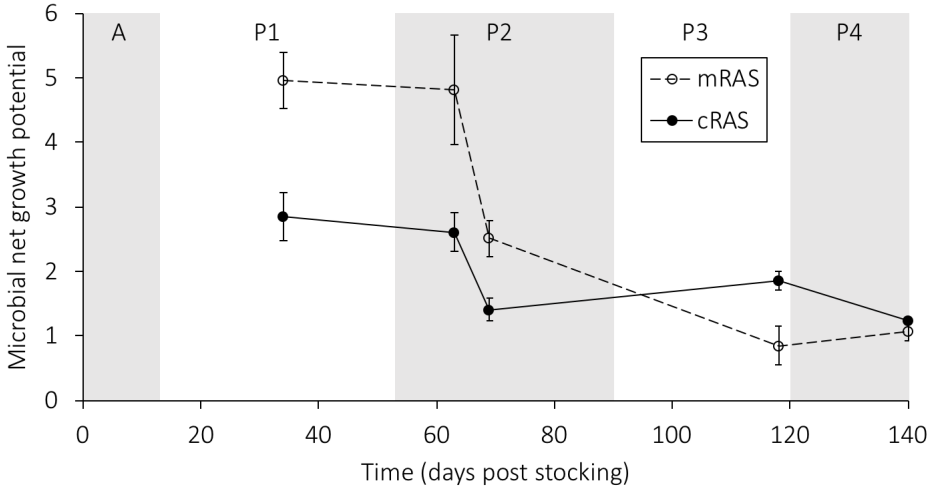

Figure 4.9. Net microbial growth potential in the rearing water from mRAS and cRAS, i.e. the ratio of total number of bacteria incubated for 2 days to the total number at time zero. Values are given as averages ± SE.

4.3 Fish health and performance

Fish performance with respect to daily SGR, TGC, and final fish weight and length (at day 140) are given in Table 4.4, averaged from the whole experimental period. The fish weight and length data are not corrected for temperature differences between the systems, and thus cannot be directly compared.

Table 4.4. Fish health data including specific growth rate (SGR), thermal-unit growth coefficient (TGC) and Foulton's condition factor (K) for the whole experimental period, and final weight and length mRAS and cRAS, given as averages ± SE.

Parameter	mRAS	cRAS
SGR (% d^{-1})	1.07 ± 0.01	0.89 ± 0.01
TGC	1.03 ± 0.01	0.93 ± 0.01
Weight (g)	140.9 ± 1.9	124.2 ± 1.6
Length (cm)	23.0 ± 0.1	22.0 ± 0.1
K	1.14 ± 0.01	1.16 ± 0.01

An overview of the number of fish with any visual signs of damage observed in the end of the experiment is given in Table 4.5. Fish welfare parameters with damage included fins, eyes, snout, operculum, head, and shape of the fish. If $p < 0.05$, the probability of damage is unequal between mRAS and cRAS.

Table 4.5. Fish welfare parameters in mRAS and cRAS showing the number of fish that had damage in each category. The p-values indicate whether the probability of damage in both systems were the same or not. The number of fish in mRAS and cRAS were 217 and 207, respectively.

Parameter	mRAS	cRAS	p-value
Fins	22	38	0.02
Eyes	14	4	0.02
Snout	2	9	0.03
Operculum	13	8	0.3
Head	12	15	0.5
Bent	2	0	0.2

Some damage was observed, but the damage was very light. For fins, eyes and snout, the probability for damage in the two systems were not the same. Fish mortality was three in each system in total, giving a survival of 99%.

5 Discussion

The present study aimed to investigate the effects of organic matter removal by membrane filtration on physicochemical and microbial water quality. This was implemented by placing a membrane filter prior to the biofilter in one out of the two RAS studied. A second objective was to evaluate different bacteria and particle counting methods. The results demonstrated that removal of organic matter had impacts on the aquatic environment in terms of number of bacteria and particles, which was reflected in turbidity, total organic carbon, number of bacteria measured by three different methods, and number of particles measured by two different methods. The different methods that were employed for quantification of bacteria and particles are evaluated and all results are further discussed in the following sections.

5.1 Evaluation of counting methods

The main reason for using several different methods for quantification of particles and bacteria in the same experiment was to compare them to see whether they correlated and gave similar and expected results, and to test if the novel counting instrument BACMON, invented by Højris et al. (2016), was applicable to this type of aquaculture. Furthermore, it is important to evaluate if easily applicable methods in the industry are reliable. As described in Chapter 2, the methods employed are based on different technologies with varying accuracy and detection size range, which must be taken into account when comparing the results.

5.1.1 Particle counting

Direct counting of particles, including the BACMON and Coulter counter, generally provide concentrations for given size ranges, whereas turbidity only provides an indication of the presence of particles based on the amount of scattered light. As dissolved organic matter and colloidal particles affect turbidity, but are too small to be detected by the particle counters, the methods may provide different results based on the particle size distribution. Moreover, settleable particles are detected by particle counters, but usually not included in turbidity. Previous studies have shown a proportional correlation between turbidity and particle counts (Højris et al., 2016), but this is dependent on the concentration of dissolved solids, the particle size distribution and the detection size range of the particle counters. The Coulter counter is an instrument with high accuracy as it corrects for

coincidence errors such as two particles passing the electrical sensing zone at the same time. The numbers detected by the Coulter counter were in the magnitude of 10^4 mL^{-1} compared to the BACMON numbers of 10^5 mL^{-1}, which indicates that a high share of the particles were smaller than the lower detection limit of the Coulter counter of 2.2 µm. However, the numbers from both these methods are probably much lower than reality, as previous studies have shown that RAS facilities with membrane filtration typically contain more than 20 million particles per mL (Holan et al., 2014a).

The accuracy of the BACMON sensor is dependent on both particle size and concentration. Particles are divided into size classes ranging from <0.62 µm to >9.5 µm. For mixed-type suspensions (i.e. bacteria and particles), the average classification certainty of particles larger than 0.77 µm has been estimated to be $78 \pm 14\%$, whereas smaller particles are likely to be classified incorrectly or even undetected (Højris et al., 2016). Furthermore, the upper detection limit of abiotic particles for BACMON is $1–5 \times 10^6$ mL^{-1}. In the present study, the number of particles probably exceeded this limit, following overloaded sensors and inaccurate results. Thus, due to high background scattering, the smallest particles may have been classified incorrectly or may not have been detected and the instrument probably underestimated the concentrations, which means that parts of the colloidal fraction may have been excluded from the BACMON measurements. Nevertheless, the instrument provided highly detailed results with more than 20,000 data points for each RAS, which allowed for monitoring of concentration movements on an hourly and daily basis, in contrast to the other counting methods with only 10–40 measurements per system. Furthermore, the ability to detect and classify all particles as either bacteria or abiotic particles is a great advantage and makes the instrument very efficient.

The turbidity seemed to be a reliable method in this experiment in terms of temporal development, as the variations corresponded to what was expected based on the feeding regime. However, as turbidity does not provide concentrations and is a result of light scattering from bacteria, particles as well as dissolved compounds, it is not a method that can be used for direct particle counting. As the smallest fraction of particles was the most interesting and relevant part of this experiment, the Coulter counter was not sufficient for counting these, as it did not provide number of particles smaller than 2.2 µm. Additionally, the temporal developments did not follow the feeding regime. However, although the Coulter counter did not provide the total particle counts in the water, the instrument provided particle size distributions that confirmed the expectation of highest frequency of the smallest particles, as particle numbers typically decrease exponentially with increasing size. In this experiment, the BACMON sensors were not used for particle size distribution analysis, but the instrument may also provide such information.

In conclusion, all the measuring methods provided interesting and relevant results for the present study, in terms of total particle counts and variations on a detailed level (BACMON), particle size distribution (Coulter counter) and temporal devel-

opment of the indirect measurements of particle concentration (turbidity). For the BACMON sensor, maintenance is required in terms of flow cell changes, where the frequency of changes is dependent on the concentration of bacteria and particles in the water. The automated solution for BACMON makes the instrument more user-friendly and efficient than the manual turbidity measurements and Coulter counter. However, automated versions of these do also exist. The greatest advantage with BACMON is that it can provide particle counts, bacterial counts, and particle size distribution in only one measurement, but the instrument is probably more applicable in monitoring of drinking water rather than in aquaculture. Further investigation should be done with respect to investment and operational costs in order to conclude on the cheapest method in the long term.

5.1.2 Bacterial counting

With respect to total bacterial counts, the order of magnitude in the different methods were 10^6 mL^{-1} for flow cytometry and 10^5 mL^{-1} for both CFU and BACMON. Flow cytometry resulted in the highest numbers which were also probably most accurate, as dilutions of the water was performed prior to analysis in order to be within the measurement range of the instrument (1×10^3–5×10^6 mL^{-1}, error $< 5\%$). As the BACMON sensors were placed directly in the rearing water, no dilution was performed prior to analysis, which was probably what caused the sensors to underestimate the number of bacteria and particles. This caused a bigger difference in concentrations between BACMON and flow cytometry for high compared to low concentrations. Furthermore, the BACMON was probably not able to detect particles in the size of most bacteria present, which also may have resulted in lower numbers. The CFU method counts only the cultivable fraction of the bacteria in the samples, whereas flow cytometry counts virtually all bacteria. In this study, the CFU method resulted in high variation in numbers between duplicate plates as well as numbers below the lower limit of 30 colonies per plate, which makes the data less reliable. Furthermore, the colonies may have been formed out of cell clumps rather than single cells, which results in lower numbers. However, the CFU generally followed the trends over time as expected, and was thus giving the information needed in this experiment.

Regression analysis showed stronger correlations between CFU and flow cytometry than any of these methods did with BACMON. Moreover, comparisons of all the methods showed stronger correlations within mRAS than cRAS, indicating that the numbers counted by the different methods were more similar at lower concentrations, as mRAS had lower bacterial concentrations than cRAS.

Both CFU and flow cytometry are methods that are susceptible to human errors to a higher extent than automated BACMON measurements are, due to many steps in the laboratory. Some maintenance in terms of flow cell change was required when employing BACMON in water with such high particle loads, but this

was considerably less time-consuming and more efficient than any of the other methods for both bacteria and particle counting. Despite several advantages with BACMON, a few downsides were also apparent in this study. Comparison between the two systems were challenging due to only one sensor operating at the time for some parts of the experiment. Additionally, the flow cells were quickly overgrown, especially in periods 3 and 4, and thus had to be changed quite often which resulted in sudden changes in concentrations. However, as this experiment included larger amounts of organic matter than what is used in large-scale RAS at normal operation, this would most likely be less problematic in such cases. The BACMON sensors were very useful in this experiment, as it was possible to monitor tendencies over time and across systems at all times when both sensors were in operation. However, BACMON could not be relied upon to provide exact numbers with respect to bacteria and particles, and should be primarily be consulted when uncovering trends in bacterial development and potential accumulation of particles, and not for exact counts.

The fastest and easiest method used for this experiment was the automated monitoring using BACMON sensors. In the aquaculture industry, this instrument would likely be more user-friendly compared to time-consuming and expensive methods such as flow cytometry and CFU. Furthermore, flow cytometry requires chemical staining solutions which makes the method limited to locations where the stains can be stored and changed regularly. On the other hand, due to the high concentration of particles and bacteria in the water in the present study, the BACMON was not able to detect exact numbers and is thus possibly not applicable for RAS facilities where thsi is desireable. Even with smaller feeding amounts and organic load in commercial plants, the number of bacteria is usually too high for BACMON to be used for exact bacterial and particle counts. However, further testing in RAS facilities should be performed for clarification in this matter. BACMON could still be used to uncover trends in bacterial development and particle monitoring.

5.2 Physicochemical water quality

Comparison of turbidity and particle concentrations between the two systems showed significantly lower levels in mRAS than cRAS, which shows that the membrane was efficient in removing particles from the water and thereby improving the water quality, as intended and expected. The concentration of total organic carbon (TOC) was also reduced by the membrane. Due to overfeeding, a higher amount of accumulated particles and organic matter in the systems compared to commercial plants were expected. The present study is in agreement with similar studies on Atlantic salmon post-smolt and on marine species such as Atlantic cod larvae which have shown that water treatment with an UF membrane in combination with a moving bed biofilm reactor (MBR) in RAS is effective in significant reduction of colloidal particles and turbidity (Holan et al., 2015, 2014a; Wold et

al., 2014).

With respect to the conventional water quality parameters, both mRAS and cRAS showed acceptable levels in the fish tanks during the whole experiment. No significant differences were observed in pH, salinity, alkalinity, or nitrogenous waste compounds including nitrite, nitrate and TAN between mRAS and cRAS, which indicates that the membrane filtration did not affect these. This gives a basis for comparison of the systems with respect to other parameters such as bacteria and particle concentrations. The temperature, however, was constantly higher in mRAS than cRAS probably due to heat production from the implemented membrane and to temperature variations throughout the room. For the fish growth, this was corrected for, but the temperature probably also affected the number and types of bacteria present in the systems. As described in Chapter 2, fast-growing heterotrophs are superior competitors for oxygen and thus tend to grow towards the surface of biofilms, covering the slower growing nitrifiers (Hagopian and Riley, 1998). Thus, a higher nitrification efficiency was expected in mRAS than cRAS as a result of lower content of organic carbon (Michaud et al., 2006). However, in the present study, the nitrification efficiency did not significantly differ between the systems. Furthermore, nitrogenous waste compounds did not exceed hazardous levels (Eddy, 2005; Timmons and Ebeling, 2013).

As expected, the difference in particle numbers between the two RAS increased significantly after the last hatch was closed and the systems were completely separated (from period 2). For number of particles measured by both BACMON and the Coulter counter, as well as the turbidity, the difference between the systems was biggest in period 3, which indicates that the membrane was most efficient when the recirculation degree was low and the organic load at normal levels. However, as the organic load was much higher than in normal operation, a higher recirculation degree could probably have been used giving the same removal efficiency in commercial situations. Turbidity and number of particles detected by the Coulter counter did not correlate, which was expected as dissolved and colloidal fractions that contributes to turbidity were not detected by the particle counter ($< 2.2\,\mu m$). Additionally, this may have resulted in a different size distribution than would have appeared if the smallest particle fractions were included.

In conclusion, the membrane filtration did not have any impact on most of the conventional water quality parameters. However, it did have a significant effect on the number of particles and turbidity. This is also reflected in Table 4.3, showing bacteria-to-particle ratios for mRAS and cRAS, and thereby indicates improved important aspects of the water quality.

5.3 Microbial environment

All methods used for bacterial counting in this study, including flow cytometry, counting of CFU and automated monitoring with BACMON sensors, showed lower bacterial concentrations in mRAS than cRAS for the majority of the experiment, which was probably due to removal of organic matter resulting in production of less bacterial substrate and thus less bacterial growth, and also removal of bacteria itself by the membrane. Lower bacterial concentrations are suggested to be associated with higher microbial stability. Further, different removal rates of organic matter probably caused different conditions for bacterial growth affecting the growth rate of fixed biofilm on surfaces and bacteria suspended in the water (Blancheton et al., 2013; Rurangwa and Verdegem, 2015). This was indicated by changes in the bacterial concentration in accordance with changes in the rearing conditions. In agreement with previous studies on membrane filtration in RAS (Holan et al., 2014a; Wold et al., 2014), the bacterial concentrations were generally more stable over time in mRAS than cRAS, with both fewer and smaller fluctuations and bacterial blooms, suggesting a more K-selected environment and thus higher microbial stability in terms of bacterial abundance in the membrane filtered water. By comparing the total counts assessed by flow cytometry, the bacteria in mRAS seemed to adapt to external changes much faster than the bacteria in cRAS did, being more insensitive to disturbances and recovering faster after a disturbance, which indicates a higher microbial stability (Shade et al., 2012).

When the amount of feed increases, the organic pressure on the system increases together with available bacterial substrate, and the bacterial growth and abundance is thus expected to increase. In the present study, the expectation of higher microbial carrying capacity (CC) during periods with high organic load was confirmed by observation of higher bacterial abundances during periods with overfeeding and/or low water exchange. According to the r/K-selection theory described in Chapter 2, the slow-growing K-strategists are favoured in environments scarce in nutrients, which means that the system with lowest levels of organic matter was expected to have the most K-selected or matured microbial environment. Moreover, very high organic load on the system is expected to give bacterial blooms of fast-growing r-strategists, especially if the load suddenly increases by e.g. changes in the feeding regiment, as these bacteria are favoured in high nutrient supplies. However, under stable conditions, with certain types of bacteria sharing the higher level of DOC-supply, the selection pressure can be the same. In stable systems, this is thought to be the case. Hence, higher amounts of organic matter will only affect the CC in some cases.

Changing and unstable conditions are suggested to favour proliferation of fast-growing opportunistic heterotrophic bacteria (r-strategists) that thrive in environments with high nutrient supply per bacteria as opposed to the slower growing K-strategists (Andrews and Harris, 1986; Attramadal et al., 2012b; De Schryver and Vadstein, 2014; Hagopian and Riley, 1998). After a certain period of time, the

bacterial growth rate will cease, leading to an equilibrium and thus a microbially matured water. During the acclimatisation period, the aquatic environments in both systems were likely unbalanced in terms of nutrients and organic matter, as these components are in constant production, degradation and accumulation, which was reflected in unstable and high concentrations of bacteria according to the numbers assessed by flow cytometry, BACMON and CFU. However, the systems seemed to mature after a short time, as the concentrations turned more dynamically robust early in period 1 and the daily fluctuations monitored by the BACMON sensors were stable and harmonic. In the present study, no microbial community analyses have yet been performed, meaning that no conclusions were made on this subject.

Low cultivability suggests a high level of specialised bacteria with a narrow niche, characterising a more stable and K-selected microbial environment (Skjermo et al., 1997). However, cultivability increases with increasing substrate load and may therefore be higher in microbially matured water (Salvesen et al., 1999). In the present study, the cultivability was highest in cRAS through the whole experiment except the acclimatisation period. As cRAS is the system with highest organic load, this was expected, and indicates that the systems had the same degree of water maturation, or that cRAS had even less matured water than mRAS. However, as these measurements are independent of each other and as the CFU were of high uncertainty due to low numbers of visible colonies, the resulting cultivability is less reliable.

As opportunistic bacteria are characterised by high growth rates, the fraction of fast-growing colonies out of the total CFU was used as an indication of the relative abundance of opportunists. In this study, the fraction of fast-growing bacteria was very similar between mRAS and cRAS which indicated that the removal rate of POM did not have any impact on the relative abundance of opportunists. Furthermore, the two systems responded similarly to changes in organic load and water exchange rates. The decrease of fast-growing bacteria in period 2 with a high organic load indicated a more mature and K-selected water than before. The rapid increase in period 4 can be explained by a high organic pressure on the system and thus a rapid increase in the carrying capacity.

The membrane treatment could potentially affect the water quality in negative ways by removing too much bacteria and substrate, leading to a lower competition for fast-growing r-strategists and thus a lower microbial stability. However, in this experiment, only 10% of the water flow was filtered through the membrane, so it was unlikely to affect the water quality negatively (Attramadal et al., 2012a).

With respect to the microbial parameters investigated in this study, the membrane seemed to have an impact on the system, by lowering the bacterial number, reducing the fluctuations in concentrations, and reducing the microbial carrying capacity. All these findings suggest a higher microbial stability in mRAS than cRAS, which is believed to increase the health and performance of the cultivated

species. However, qualitative data on the microbial community composition is needed in order to conclude on whether the populations were r- or K-selected and thus on the microbial stability. Furthermore, no difference in fish performance or mortality suggests that the water quality was more or less the same in both systems.

5.4 Fish growth performance

No significant differences in fish health, growth or survival between mRAS and cRAS indicated that the removal efficiency of particles from the water did not affect the welfare of the fish. Furthermore, no negative impacts were observed in any of the systems. The bacterial count is a measure of microbial water quality, and is a crucial factor for the reared fish as bacteria are consumed by the fish and interact with the fish, and in this way may affect their health and performance. High number of particles and bacteria in combination triggers growth of opportunistic and potentially harmful bacteria. However, no signs of disease were observed on the fish.

Colloidal particles are known to affect the gills and thereby reducing the gill performance of fish (Bullock et al., 1994; Timmons and Ebeling, 2013). Furthermore, implementation of membrane filtration in RAS have been shown to give higher growth rates for the cultivated fish (Holan et al., 2014a; Wold et al., 2014). Previous studies have shown that the content of particles in RAS significantly affected the reared fish, but many of the studies are on the larval stages which are more vulnerable than the parr life stage. However, the fish could have grown better with less particles in the water, but based on this experiment alone, this is uncertain. Furthermore, salmon is known to be a species with higher robustness against environmental stress than marine species, and highly adaptable to environmental changes. As this experiment lasted a relatively long time (140 days), a difference was expected over time. However, as the systems were run as one in period 1, the chances for development of different environments for the fish and thereby difference in fish performance decrease. Moreover, we do not know if the difference in weight performance was solely due to the temperature difference, or if it was affected by the difference in particle and bacterial numbers as well. It must also be mentioned that as long as the bacterial and particle numbers are not extreme, the composition of the microbial community is more important for fish performance than total bacterial counts (Munro et al., 1994; Salvesen et al., 1999; Salvesen and Vadstein, 2000; Verner-Jeffreys, 2003; Wold et al., 2014). Anyhow, negligible differences in fish health after temperature correction indicates that neither the bacterial or the particle concentrations exceeded the upper acceptable levels for Atlantic salmon parr, which means that the membrane filtration did not have any impact on the fish health.

6 Conclusions

In order to evaluate the effects of particle accumulation on the microbial community dynamics and fish performance in RAS, the consequences of different organic matter removal efficiencies on physicochemical and microbial water quality parameters were characterised. The system with an implemented UF membrane (mRAS) demonstrated enhanced removal efficiency of particles compared to the conventional system (cRAS), which was reflected by significantly lower turbidity and particle concentration in mRAS. The largest differences were observed in period 3, with high feed loads and low water recirculation degree. The same size distributions were observed in mRAS and cRAS, indicating that particles of all sizes were removed more efficiently in mRAS compared to cRAS.

With respect to the microbial environment in the rearing water, the enhanced organic matter removal efficiency in mRAS resulted in reduced carrying capacity and higher temporal stability of the bacterial concentration compared to cRAS, reflected by significantly lower bacterial abundance and reduced amounts of organic matter. These observations indicated a higher microbial stability in mRAS compared to cRAS. Furthermore, lower carrying capacity is suggested to select for K-strategic specialists over r-strategic opportunists. However, which bacterial species were present remains to be studied in order to analyse the microbial community composition in the systems, which will give a stronger indication on whether the communities were r- or K-selected, and thus on the microbial stability.

The health, survival and growth performance of the reared salmon parr were not significantly affected by the enhanced particle removal. This indicates that the conventional water quality parameters were within acceptable limits for the fish health, and that the parameters were possible to control independent of membrane filtration. However, further analysis of the fish data remains to be studied.

Comparison and evaluation of different counting methods indicated that the BACMON sensors were most applicable for monitoring of detailed temporal variations, whereas for total bacterial counts, flow cytometry provided the most predictable and reliable results. For total particle counts, an instrument with wider size range and/or higher upper limit is needed for accurate results.

The present study provided valuable knowledge on how a UF membrane affected the physicochemical water quality and bacterial abundance in RAS with production of Atlantic salmon parr, and on how high and varying loads of organic matter affected the bacterial abundance and fish performance. Furthermore, the study allowed for comparison and evaluation of different counting methods for bacteria and particles. Future research should focus on membrane filtration in RAS with

Atlantic salmon at different life stages, and on fish–microbe interactions, as this is not yet fully understood. Furthermore, the levels of water reuse providing stable microbial conditions, and the amount of water that should be treated by the membrane unit, are important aspects to study in order to optimise the production efficiency and sustainability of RAS. This has the potential to accommodate global seafood demands with higher production efficiency.

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A BACMON abiotic particles

Number of abiotic particles monitored by the BACMON sensors over time in mRAS and cRAS is given in Figure A.1 on the next page.

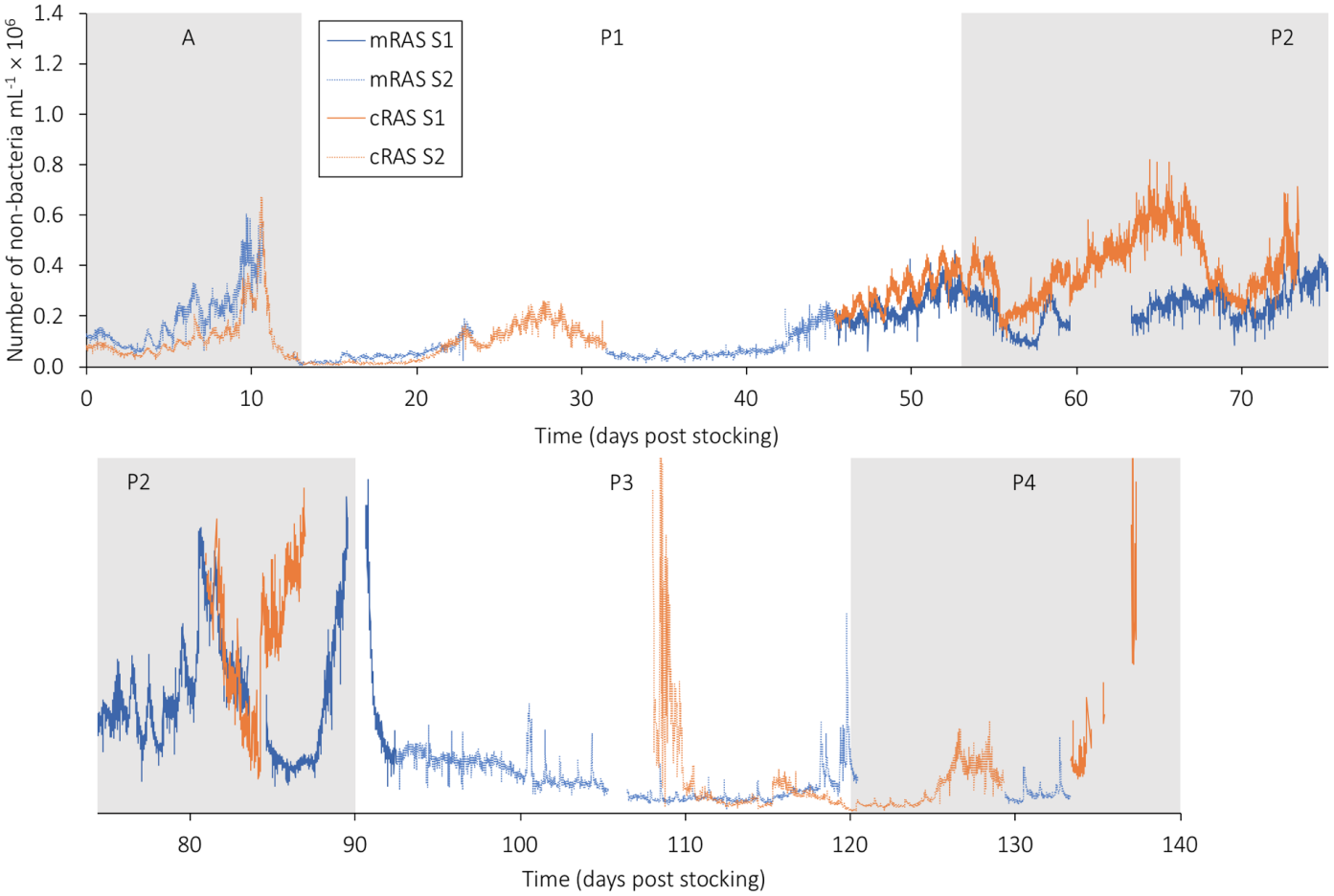

Figure A.1. Bacterial concentrations monitored by BACMON sensors of the water in the sumps (S1 = dotted line, S2 = solid line) of mRAS and cRAS during the entire experiment.