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Physiological impacts of suspended solids in recirculating aquaculture

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Glossary

ANOVA	Analysis of Variance
AR	Aspect ratio
BCD	Bounding circle diameter
BQV	Bactiquant® value
CFU	Colony-forming units
CO_2	Carbon dioxide
C_V	Coefficient of variation
DW	Dry weight
EAR	Ellipse aspect ratio
ECAD	Equivalent circular area diameter
EEAL	Equivalent elliptical area length
EEAW	Equivalent elliptical area width
ELISA	Enzyme-linked Immunosorbent Assay
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed conversion ratio
FTS	Flow-through system
GLM	General linear model
HE	Haematoxylin and eosin stain
HSP	Heat shock protein
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
Mt	1 million tons
NH_3(-N)	Unionized ammonia (nitrogen)
NH_4(-N)	Ammonium (nitrogen)
NO_2(-N)	Nitrite (nitrogen)
NO_3(-N)	Nitrate (nitrogen)
NTU	Nephelometric Turbidity Unit
PAS	Periodic acid-Schiff

PBS	Phosphate-buffered saline
PSD	Particle size distribution
RAS	Recirculating aquaculture system
RBC	Red blood cell count
RRE	Relative removal efficiency
S.D.	Standard deviation
S.E.	Standard error
SGR	Specific growth rate
sRAS	Semi-recirculating system
TAN	Total ammonia nitrogen
TGC	Thermal growth coefficient
TSS	Total suspended solids
UV	Ultraviolet
WBC	White blood cell count

Preface

Modern aquaculture is responsible for almost half of today's global fish production. Despite this dominance, the sector continues to face several challenges, including the risks associated with escapees, increased occurrence of parasites in net cages, ever more strict legal environmental regulation of flow-through and semi-recirculating systems and pressure on water supplies, for example due to climate change. In this light, recirculating aquaculture systems (RAS) are regarded as a promising, environmentally friendly option for sustainable future fish production, independent of location and water supply. However the high investment and maintenance costs of RAS mean that further improvements are crucial if such systems are to become an economically viable solution. One of the main perceived threats for fish health and performance in RAS is the issue of solid load, but it is surprising how little is known about the nature of particles occurring in aquaculture systems, and in particular about their shape and how this may affect the physiology and performance of fish and the functioning of the system.

This thesis is intended to enhance knowledge of the suspended solids occurring in aquaculture systems and of their impacts on the physiology and performance of salmonids in RAS. Special emphasis is placed on uncoupling the effects of exposure to suspended solids from those of potentially confounding water parameters, and testing the possibility that apparently particle-related effects may be biased by autocorrelated factors such as inappropriate water quality. The introduction of this thesis reviews the current state of aquaculture, focusing on the most common production systems for salmonids and state of knowledge pertaining to suspended solids in aquaculture systems and their potential impacts on fish, then lays out the objectives of the studies undertaken. The main body of the thesis comprises four research chapters dealing with particle shape characteristics; their likely impacts as suspended solids in aquaculture systems; the potential implications of short- and long-term exposure on rainbow trout physiology and performance in RAS; and the effects of interaction between suspended solid load and elevated concentrations of unionized ammonia. In conclusion, the implications of suspended solid accumulation under experimental and

commercial conditions are discussed and promising avenues for further research into the management of particle load in aquaculture systems are identified.

Summary

Suspended solids are seen as one of the main challenges in the aquacultural production of finfish. In recirculating aquaculture systems (RAS) especially, the accumulation of fine particles is generally seen as a major threat to fish health and performance. However, previous studies in this field have not uncoupled genuinely solid-related effects from potentially confounding water parameters with which they are naturally correlated. Thus, the main goals of this thesis were to investigate possible impacts of increased particle concentration on salmonid health and performance in RAS, uncoupled from other potentially debilitating water parameters and to improve knowledge of the shapes of suspended solids occurring in aquaculture systems generally.

In the first core chapter of this thesis, the shapes and concentrations of particles occurring in commonly used salmonid production systems were characterized, revealing marked similarity in form, regardless of system type and water treatment, with the majority of particles conforming to a rather flake-like shape. Furthermore, it was shown that an assumption of ellipsoidal shape produced more accurate results in calculations to estimate the volume and surface area of particles than the more widely used standard sphere. These new findings have significant implications for the design of water treatment and for the modeling of entire aquaculture systems.

Two further studies provide an overview of the acute short-term and chronic effects of increased suspended solid concentrations on rainbow trout physiology and performance in RAS, uncoupled from other potentially confounding water parameters. In both studies, particle load in the treatment systems was increased artificially by pumping backwash water from the drum filter back into the system, causing an accumulation of fine particles, while other relevant water parameters were maintained at optimal levels. The impact of the accumulating particles on fish was examined using a wide range of physiological assays, including stress markers (Heat shock protein 70 and plasma cortisol), hematological assays (differential leukocyte count, hematocrit, blood cell indices), fin condition and histological examination of the gills. The results showed that exposure to suspended solids at concentrations of up to 30 mg L^{-1} for a

relatively short time period had no detrimental effects on rainbow trout in RAS. Most surprisingly, histological examination revealed that the gill status of rainbow trout exposed to increased particle load was in part better than that of the control fish.

The second study was a long-term experiment over a whole eighteen week growth period, set up to bring to light any chronic exposure effects of increased particle load on fish health or performance. In addition to the physiological assays carried out in the short-term experiment, further investigations were made of the bacterial burden of fish and system water. At the end, none of the investigated physiological parameters revealed any solid-related effects, thus corroborating the findings of the short-term study. Bacterial load was elevated by the increased concentration of suspended solids, but without any apparent impact on fish physiology. Thus, in contrast to previous scientific and operational assumptions, both exposure studies suggested that neither acute and nor chronic exposure to exceptionally high fine particle loads of more than 30 mg L^{-1} exerted any negatively influence on the health or performance of trout.

The fourth study provides a fully controlled insight into the combined effects of particle accumulation and increased unionized ammonia load on the physiology and performance of rainbow trout in RAS. After recognizing that of itself, exposure to suspended solids at concentrations beyond those realistically expected in commercial aquaculture had no detrimental effect on rainbow trout, fish in the final experiment were exposed to an additional burden in the form of increased levels of unionized ammonia. While minor effects of this treatment were detected on fish physiology, no relevant combined effects of elevated solid load and unionized ammonia-N at concentrations up to 0.05 mg L^{-1} were observed. Bacterial activity was strongly positively correlated to increased suspended solid load, but again no detrimental effects on fish physiology were detected.

In conclusion, the findings of this thesis show that the impacts of system-related suspended solids on salmonid health and performance are low to nonexistent, provided that accompanying water parameters are maintained within the optimal range. This suggests that suspended solids are not the key issue affecting fish welfare in RAS and that the upper safe limit for suspended solids in salmonid aquaculture production can safely be revised upwards.

Zusammenfassung

In der Aquakultur werden Schwebstoffe als eine der größten Herausforderungen für die Fischproduktion gesehen. Insbesondere in Kreislaufanlagen wird die Anreicherung von Feinstpartikeln als ein großes Risiko für die Gesundheit und Wachstumsleistung von Fischen betrachtet. Frühere Untersuchungen in diesem Kontext entkoppelten jedoch nicht die partikelbedingten Auswirkungen von anderen potenziell überlagernden Wasserparametern, so dass das beobachtete Schadenspotenzial von erhöhter Partikelkonzentration möglicherweise falsch bewertet wurde.

Ziel der vorliegenden Doktorarbeit war es, die Auswirkungen von hohen Partikelkonzentrationen in Kreislaufanlagen auf die Gesundheit und Wachstumsleistung von Salmoniden entkoppelt von potenziell störenden oder abschwächenden Wasserparametern zu untersuchen und so das Wissen über die Formfaktoren von Schwebstoffen in Aquakulturanlagen zu erweitern und zu verbessern.

Im ersten Kernkapitel dieser Doktorarbeit wurden die Form und die Konzentration von Partikeln, die in typischen Aquakulturanlagen für die Salmonidenerzeugung vorkommen, näher untersucht. Die dabei gewonnenen Ergebnisse deuten auf eine große Ähnlichkeit der Partikelgestalt hin, die weitgehend unabhängig von Anlagentyp und Wasseraufbereitung zu sein scheint. Die Mehrheit der Partikel wies dabei eine eher flockenförmige Gestalt auf. Des Weiteren wurde gezeigt, dass Volumen- und Oberflächenberechnungen von Partikeln, die auf einer ellipsoiden Form basieren, genauere Ergebnisse erbringen als bisher verwendete Berechnungen, die auf der Annahme einer kugelförmigen Gestalt basieren. Diese neuen Ergebnisse haben hohe Relevanz für die Einschätzung und Entwicklung von Verfahren zur Abwasseraufbereitung in Kreislaufanlagen als auch auf die Modellierung von Partikelprozessen in Aquakulturanlagen.

Die zwei folgenden Untersuchungen geben einen Überblick über die Auswirkungen von erhöhten Schwebstoffkonzentrationen in einem Kurzzeit- und einem Langzeitversuch auf die Physiologie und Wachstumsleistung von Regenbogenforellen. Dabei wurde die Erhöhung der Schwebstoffbelastung gezielt von anderen korrelierenden

Wasserparametern entkoppelt. Bei beiden Versuchen wurde die Partikelkonzentration im Belastungskreislauf durch das Einspeisen von Spülwasser des Trommelfilters zurück in das Kreislaufsystem künstlich erhöht und somit eine Akkumulierung von Feinstpartikeln bewirkt. Alle weiteren relevanten Wasserparameter wurden konstant im Optimalbereich gehalten. Die Auswirkungen der akkumulierenden Schwebstoffe auf die Fische wurden anhand einer Vielzahl physiologischer Untersuchungsparameter, wie Stressmarker (Hitzeschockprotein 70, Plasmacortisol), hämatologische Parameter (Differentialblutbild, Hämatokrit, Blutzellzahlen), Flossenzustand und histologischem Befund der Kiemen, untersucht. Die Ergebnisse des Kurzzeitversuchs zeigen, dass Partikelkonzentrationen von bis zu 30 mg L^{-1} in Kreislaufanlagen zumindest für die betrachtete kurze Zeitdauer keine negativen Auswirkungen auf Regenbogenforellen haben. Erstaunlicherweise offenbarte die Kiemenuntersuchung sogar geringere histologische Veränderungen bei Fischen, die den erhöhten Partikelkonzentrationen ausgesetzt waren, als bei Fischen der Kontrolle. Die Langzeitstudie über 18 Wochen wurde angeschlossen, um eventuell auftretende chronische Effekte der Partikelbelastung auf die Fischgesundheit und Wachstumsleistung zu erfassen. Dabei wurde zusätzlich zu den bereits in der Kurzzeitstudie verwendeten Untersuchungsparametern noch die bakterielle Belastung der Fische als auch des Haltungswassers untersucht. Keiner der untersuchten physiologischen Parameter ergab einen chronischen negativen Effekt durch die Partikelbelastung, so dass die Ergebnisse der Kurzzeitstudie auch im Langzeitversuch bestätigt wurden. Die erhöhte Partikelkonzentration bewirkte allerdings eine Erhöhung der bakteriellen Belastung. Diese hatte jedoch keine erkennbaren Auswirkungen auf die Physiologie der Fische. Entgegen den bisherigen praktischen und wissenschaftlichen Annahmen zeigte sowohl die Kurzzeit- als auch die Langzeitstudie, dass selbst außergewöhnlich hohe Feinstpartikel-Konzentrationen von mehr als 30 mg L^{-1} in Kreislaufanlagen keinen negativen Einfluss auf die Gesundheit und Wachstumsleistung von Regenbogenforellen haben.

Basierend auf den Ergebnissen, dass hohe Partikelkonzentrationen an sich keine negativen Auswirkungen auf Regenbogenforellen hatten, wurde zusätzlich zu der Partikelbelastung noch eine weitere Belastung in Form von erhöhter

Ammoniakkonzentration in die vierte und abschließende Untersuchung einbezogen, um unter kontrollierten Bedingungen mögliche Interaktionseffekte von Partikel- und Ammoniakbelastung auf die Physiologie und Wachstumsleistung von Regenbogenforellen in Kreislaufanlagen zu untersuchen. Es wurden jedoch keine relevanten Interaktionseffekte von Partikelbelastung und erhöhten Ammoniak-N-Konzentrationen von bis zu 0,05 mg L^{-1} festgestellt. Durch die erhöhten Ammoniakkonzentrationen wurden nur geringe physiologische Auswirkungen beobachtet. Auch in dieser Studie bewirkte die erhöhte Partikelkonzentration einen starken Anstieg der bakteriellen Aktivität, jedoch ohne sich negativ auf die Physiologie der Fische auszuwirken.

Insgesamt zeigen die Ergebnisse dieser Doktorarbeit, dass sich systemeigene Schwebstoffe in Kreislaufanlagen nur gering bzw. gar nicht auf die Gesundheit und Wachstumsleistung von Regenbogenforellen auswirken, wenn alle weiteren relevanten Wasserparameter im optimalen Bereich gehalten werden können. Somit deuten diese Ergebnisse darauf hin, dass Schwebstoffe primär nicht der ausschlaggebende Faktor für das Wohlbefinden und die Leistung von Fischen in Kreislaufanlagen sind. In der Konsequenz sollten die Grenzwerte für Schwebstoffe, die zurzeit noch in der Aquakultur verwendet werden, auf Grundlage dieser Arbeit für Salmoniden in entsprechenden Lehrbüchern angepasst werden.

1 Introduction

1.1 Aquaculture

Aquaculture is the aqueous sector of agriculture, defined as "[…] the farming of aquatic organisms, including fish, molluscs, crustaceans and aquatic plants" by the Food and Agriculture Organization of the United Nations (FAO), which goes on to say that "Farming implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection from predators, etc. Farming also implies individual or corporate ownership of the stock being cultivated […] throughout [the] rearing period […]" (FAO, 1990).

The history of aquaculture reaches far back to origins that cannot be clearly determined as there are no aquaculture-specific artifacts to guide archaeologists (Beveridge and Little, 2002). However, the cradle of aquaculture is generally considered to be in Asia and especially what is now China. In continental Europe, the key factor in the development of aquaculture is presumed to be the introduction of common carp (*Cyprinus carpio*) from the Danube by the Romans in the first or second century CE (Balon, 1995). Carp farming continued after the collapse of the Roman Empire, and was further developed in monastery ponds (Balon, 1995).

By contrast, European trout aquaculture is a relatively recent practice, only established in the mid-19th century (Beveridge and Little, 2002; Stanković et al., 2015). Following declines in natural salmonid stocks, rainbow trout (*Oncorhynchus mykiss*) were extensively introduced to Europe from the USA (Pennell and Barton, 1996). Breeding efforts were stepped up when it emerged that rainbow trout were more suitable for aquaculture production than most other freshwater salmonids (Schäperclaus and Lukowicz, 1998). Today, the species is one of the most widely introduced fish worldwide (Crawford and Muir, 2008).

Fish has always been an important food source for mankind, but in former times it was mainly provided by capture fisheries. The drastically increasing human population and rising fish consumption per capita over the last century (9 kg in 1961 to over 20 kg in

2015; FAO, 2018[1]) have driven steep increases in demand, prompting developments in fishing equipment and methods to catch what have become unsustainable quantities of fish. The result of this overexploitation of the oceans is that over 33 % of the world's marine fish stocks are currently overfished.

In 2016, total global fish production amounted to approximately 171 million tonnes (Mt) (Figure 1.1) with an estimated first sale value of USD 362 billion. Capture fisheries remain the dominant source (90.9 Mt), but aquaculture is becoming increasingly important in securing the worldwide fish supply. The sector has grown enormously from a production of less than one million tons in the early 1950s to 80 Mt in 2016, representing 46.8 % of total fish production (Figure 1.2).

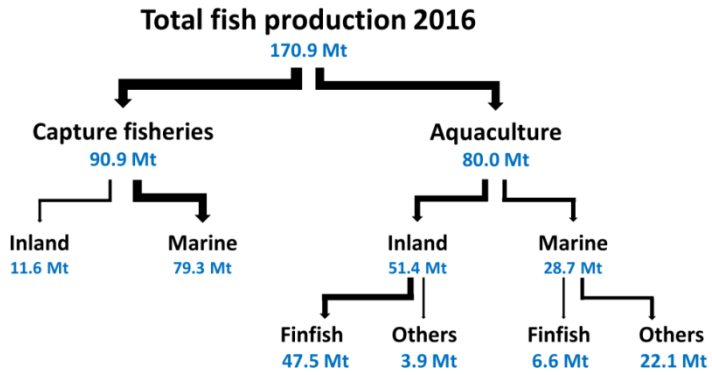


Figure 1.1: Total fish production 2016 based on FAO (2018), excluding aquatic mammals, crocodiles, alligators, caimans, seaweeds and other aquatic plants.

[1] Unless otherwise stated, information in text section 1.1 is based on FAO (2018).

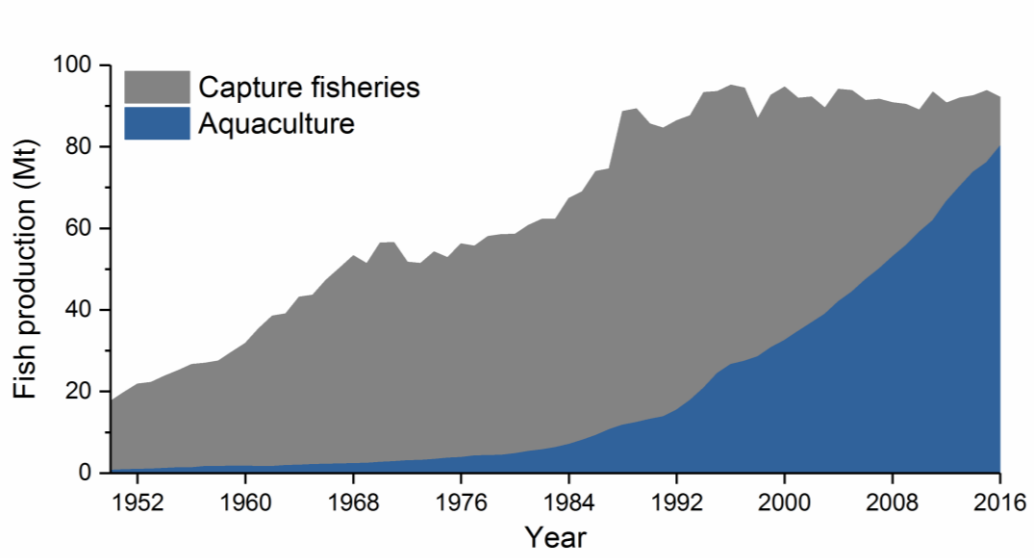


Figure 1.2: World capture fisheries and aquaculture production, excluding aquatic plants, according to FAO (www.fao.org/fishery/statistics/en).

Recognition that fish supplies from capture fisheries are limited and could no longer be substantially increased, lead to increased effort given to improving aquacultural production and rapid growth in world aquaculture in the 1980s (10.8 %) and 1990s (9.5 %). While growth has slowed since, aquaculture is still the fastest growing sector within the animal food production industry, with an average annual increase of 5.8 % (2001-2016) and accounts for much of the strong rise in fish production in recent decades. About 17 % of animal protein consumed worldwide in 2015 originated from fish. The average annual increase in fish consumption between 1961 and 2016 was 3.2 %, higher than that of meat consumption from all land animals together (2.8 %).

In 2016, global aquaculture production amounted to 110 Mt, comprising 80 Mt of fish (finfish 54.1 Mt; mollusks 17.1 Mt; crustaceans 7.9 Mt; other aquatic animals 0.9 Mt) and 30 Mt of aquatic plants. The majority of aquacultural fish production took place in Asia (89.4 %, 2016) and only 3.7 % was situated in Europe. Overall, 64.2 % of farmed fish was produced in inland aquaculture, which was heavily dominated by finfish farming (92.5 %).

As of 2016, a total of 598 species (animals and plants) were farmed worldwide, including 369 finfish species, an impressive increase in diversity since the year 2000 when the total reported was around 210 species (FAO, 2002). In 2016, the 20 most commonly produced finfish species accounted for 84.2 % of total fish produced by the

sector. Rainbow trout *Oncorhynchus mykiss* features in the top 20 most produced finfish species, but still accounts for a relatively modest total production of 0.8 Mt or 2 % of total finfish aquaculture production. Cyprinid species make up the largest portion of finfish produced worldwide, lead by grass carp (*Ctenopharyngodon idellus*), silver carp (*Hypophthalmichthys molitrix*) and common carp (*Cyprinus carpio*) which together account for 29 % of total finfish production.

1.2 Aquaculture production systems and current challenges

Aquaculture production facilities can be grouped roughly into open and closed systems. Open production systems like net cages, ponds, flow-through and semi-recirculating systems are directly connected to the surrounding environment, which bring several concomitant risks, such as pollution of recipient water bodies with nutrient-rich effluents, transmission of diseases or parasites and escapes of genetically different or non-native fishes (Bolstad et al., 2017; Edwards, 2015; Glover et al., 2012; Krkošek, 2017; Shephard and Gargan, 2017). In closed aquaculture systems on the other hand, water is recirculated, fish production units are isolated from the surrounding environment, the facility is largely independent of the external water supply and escapes of farmed fish and invasions by pathogens or parasites into the systems are wholly preventable. However, culturing fish in such highly contained conditions does also bring challenges such as pathogen control, maintenance of water quality and waste management (Badiola et al., 2012; Rurangwa and Verdegem, 2015; van Rijn, 2013).

Traditionally, the farming of rainbow trout has been carried out in ponds (Figure 1.3) which are usually operated extensively, with system water discharging untreated back into the recipient body. Modernized versions of these production systems are flow-through systems (FTS) (Figure 1.3) that allow for more intensive fish farming by using high water throughput and, in most cases, some sort of supplementary aeration. These systems generally comprise concrete raceways fed by freshwater from surface waters, springs or wells. After flowing through the raceways, the water is treated mechanically and/or biologically and then discharged back into the environment. The advantages of

these kinds of systems include low dependence on technical control. However, in the face of ongoing temperature rises and water shortages due to climate change (Barange et al., 2018) and increasingly stringent legal requirements (Nielsen, 2011), the requirement for freshwater, already a limiting factor for such systems, is set to become an even greater challenge. In the event of water scarcity, the accumulation of suspended solids and harmful substances like ammonia will become a serious problem. One means of reducing the amount of water used and making salmonid production systems more efficient is the utilization of semi-recirculating systems (sRAS) (Figure 1.3). In this kind of system, a certain amount of water is mechanically and biologically treated, aerated or oxygenated if necessary (Summerfelt et al., 2004), then reinjected into the system. This partial recycling considerably reduces the amount of freshwater used and allows the quantity of fish produced per unit freshwater to be increased (Piedrahita, 2003). Nevertheless, sRAS are still dependent to some extent on freshwater sources and are comparable to flow-through systems in terms of their pollution potential. Nutrient-rich wastewater released from FTS and sRAS is a cause for environmental concern, which has led to stricter legal environmental regulation (Dalsgaard et al., 2013; Nielsen, 2011).

Set against this background, recirculating aquaculture systems (RAS) are regarded as a potentially environmentally friendly alternative for sustainable, location-independent fish production (Martins et al., 2010). In RAS, water is processed mechanically and biologically (Figure 1.3), treated by UV or ozone and finally reoxygenated before being reinjected into the system. RAS do not require access to surface water and only minimal topping up is required to compensate for evaporation and the loss of water used to backwash the drum filter etc. Thus, compared to other forms of aquaculture production, RAS decreases the potential for local environmental impacts through maximizing the efficacy of effluent treatment and reducing the requirement for freshwater to a minimum (d'Orbcastel et al., 2009b; Piedrahita, 2003; Verdegem et al., 2006). An ability to maintain water quality, system stability, control of bacterial activity and effective organic waste management are prerequisites for animal-friendly fish production. However, the economic viability of RAS is restricted by heavy investment costs and the energy-intensive nature of production (Badiola et al., 2018). Thus the

contribution of RAS to the global fish production is still relatively small compared to open systems (d'Orbcastel et al., 2009b). Reducing energy costs per unit by increasing stocking density (Martins et al., 2005) is one means of improving the economic feasibility of RAS, but increasing fish biomass also involves increasing the quantity of used feed and thus increasing the occurrence of organic wastes (Pedersen et al., 2012). The accumulation of suspended solids is regarded as a principle cause of facility malfunctions in RAS (Badiola et al., 2012), raising further questions over their viability.

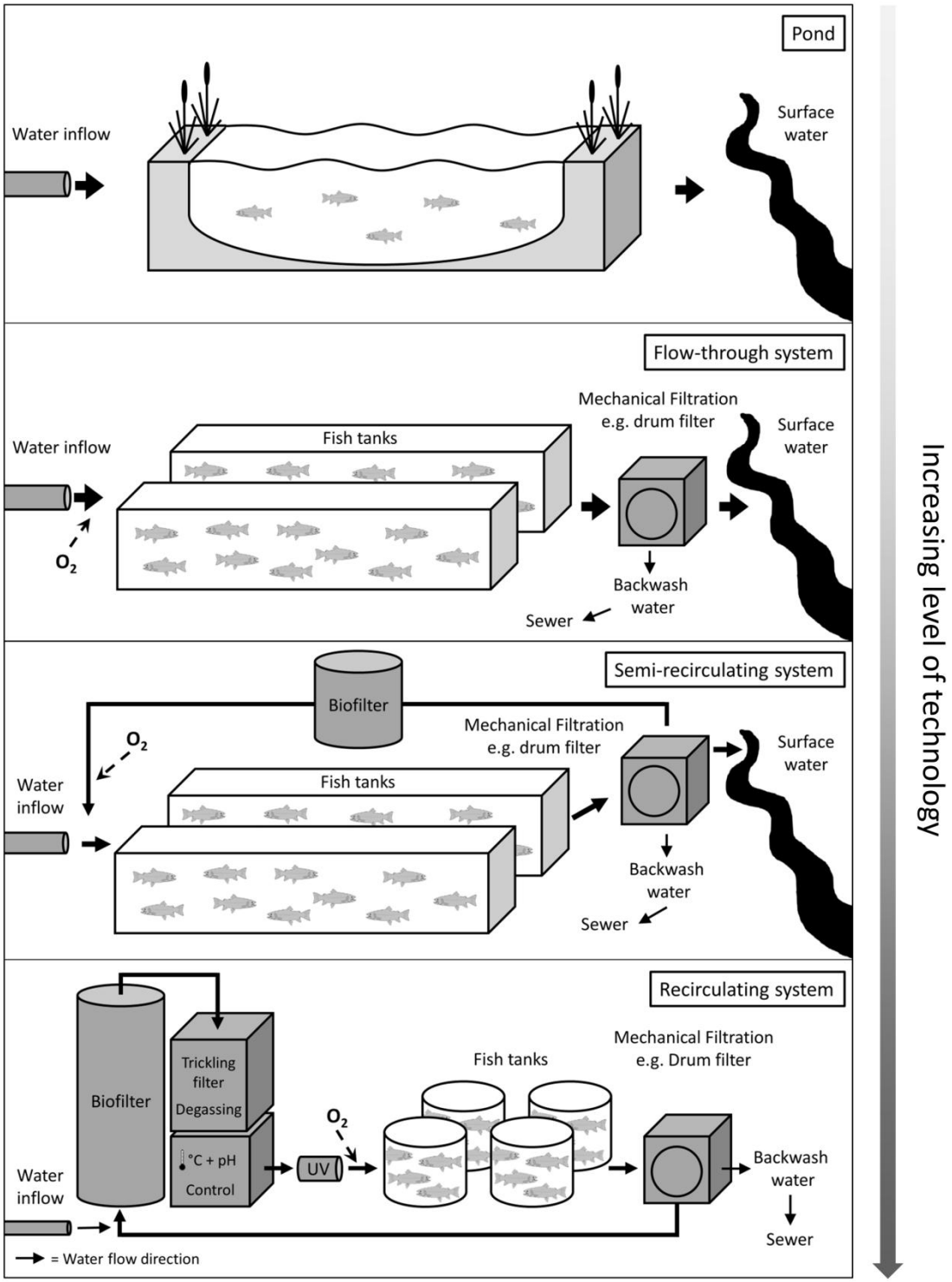


Figure 1.3: Schematic design of the most common aquaculture production systems for salmonids: pond, flow-through system, semi-recirculating and recirculating system.

1.3 Suspended solids in aquaculture systems

Suspended solids in aquaculture systems originate mainly from excreted fish feces and to a smaller extent from uneaten feed, microfauna and bacterial material from biofilters or biofilms (Bao et al., 2018; Chen and Malone, 1991; Noble and Summerfelt, 1996; Summerfelt et al., 1999; Timmons and Ebeling, 2010; Wedemeyer, 1996). The nature of feed ingredients directly impacts the stability of fecal material and thus also the quantity and quality of suspended solids occurring in aquaculture systems (Brinker et al., 2005a; Schumann et al., 2018; Unger and Brinker, 2013).

The composition of salmonid feeds has changed dramatically in recent decades, with declining fish stocks and rising prices for fish meal and fish oil (Naylor et al., 2009), driving an increasing use of plant alternatives (Glencross et al., 2007; Ytrestøyl et al., 2015). These ingredients result in less dense and more fragile fish feces (Schumann et al., 2018; Unger and Brinker, 2013) and thus in considerably increased concentrations of fine solids suspended in fish farm water (Brinker and Friedrich, 2012; Davidson et al., 2013). To combat this unwelcome side effect, commercial feed mixes have been supplemented with functional components that increase fecal stability and reduce the amount of small particles (e.g. Brinker, 2007; Brinker and Friedrich, 2012). Despite these advances, small particles are still generated by disintegrative forces such as shear (water turbulence, pumping, fish-feeding activity) and microbial activity (Brinker and Rösch, 2005; Droppo et al., 1997; McMillan et al., 2003) and may also be introduced via inputs from surface waters. Systems relying on water from natural systems are affected by events such as heavy rain, floods and snowmelt (Asselman, 1999; Langlois et al., 2005) which can result in influxes of organic and inorganic or mineral particles and other contaminants (Lenzi and Marchi, 2000; Zonta et al., 2005).

Suspended solids thus occur in a wide variety of sizes in aquaculture systems, ranging from micrometers to centimeters. The smallest fraction comprises dissolved solids, which are defined as those passing through a filter of 2 µm pore size or smaller (APHA, 2005). Particles smaller than 100 µm are non-settling particles, known as supra-colloidal solids, fine solids or fines (Timmons and Ebeling, 2010). Solids greater than 100 µm diameter are generally settlable and therefore easier to remove from systems by sedimentation techniques. While 13 µm filter gauzes are available, mesh sizes of

less than 60 µm are impractical and seldom used in aquaculture due to the non-stop backwashing and consequently higher water consumption required to keep them clear (Cripps and Bergheim, 2000; Timmons and Ebeling, 2010). The result is that fine particles accumulate in most aquaculture systems over time (Davidson et al., 2009). In RAS, up to 94 % of suspended solids are smaller than 20 µm (Chen et al., 1993; Fernandes et al., 2014).

Another important property of solids is particle shape, which is decisive for interactions with the environment (Byron et al., 2015; Cho et al., 2006; Mamane et al., 2008; Wakeman, 2007). It is therefore surprising that the shape of particles has not yet been intensively studied in the context of aquaculture systems, with just one study by Patterson and Watts (2003a) in which shapes varying from “long spicule-like particles” to “sand-like particles” and flocs consisting of loose assemblages of fine particles connected by viscid materials were identified in a commercial Atlantic salmon smolt production system. Beyond this, no information is available about the shape of suspended solids occurring in other aquaculture systems and their associated impacts on water quality, water treatment and fish welfare are unknown. Further knowledge of the shape of suspended solids in aquaculture systems is crucial to further improve performance and viability.

1.4 Solid-related effects on fish

To date, there is no consensus regarding the safe upper limits for suspended solid concentrations for farmed salmonids or cultured fish in general. The most frequently enforced value for suspended solid concentration is 25 mg L^{-1} (Alabaster and Lloyd, 1982; Timmons and Ebeling, 2010; EU Directive 2006/44/EG), but there is also evidence to suggest salmonids might tolerate much higher concentrations without suffering physiological damage (Goldes et al., 1988; Michel et al., 2013).

Suspended solids in natural waters and aquaculture systems are widely suspected to cause direct as well as indirect effects on fish physiology and performance (Figure 1.4) and fine solids have been deemed especially harmful (Chapman et al., 1987; Chen and Malone, 1991). Nevertheless, it should be noted that increases in suspended solid

concentration are often accompanied by debilitating secondary factors such as poor water quality or excessive levels of chemical contamination. Thus, there is a risk that the perceived impact of particles is biased by concomitant factors (e.g. Wong et al., 2013).

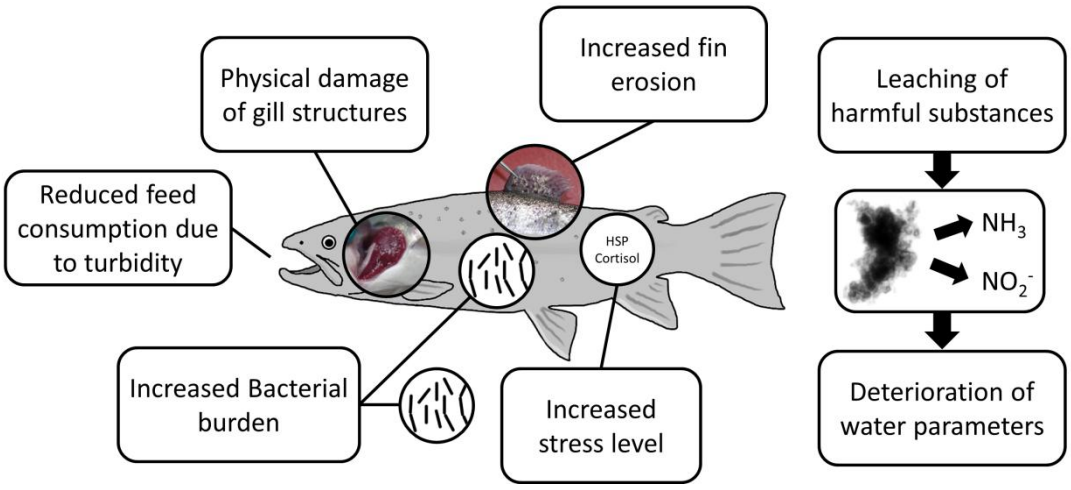


Figure 1.4: Possible direct and indirect effects of suspended solid load on rainbow trout.

The negative effects of particles on fish health include physical damage to structures like the gills (Au et al., 2004; Bash et al., 2001; Bilotta and Brazier, 2008; Bruton, 1985; Chapman et al., 1987; Humborstad et al., 2006; Wong et al., 2013); elevated stress hormone levels (Awata et al., 2011; Redding et al., 1987); impacts on the regulation of immune genes (Lu et al., 2018); and changes in hematological parameters such as hematocrit and leukocrit (Lake and Hinch, 1999). Moreover, increased concentrations of particles can impair larval development (Martins et al., 2009) and elicit behavioral changes in fish of all ages (Robertson et al., 2007). High water turbidity can alter the expression of stress-related genes (Hasenbein et al., 2016), reduce survival rates of fish (Henley et al., 2000) and directly affect feeding behavior, reaction distance, prey selection and limit the overall foraging success of salmonids as a result of reduced visibility (Barrett et al., 1992; Bash et al., 2001; Utne-Palm, 2002; Vogel and Beauchamp, 1999).

Furthermore, suspended solid load can also cause indirect harm to fish by leaching of harmful substances (e.g. ammonia) from the solid fraction. The leaching potential of

particles is known to increase with decreasing particle size (Brinker et al., 2005a; Kvåle et al., 2006) so that fine solids are in thought to pose a particular threat to water quality in aquaculture systems. Inhibition of biofilter nitrification performance by solids can lead to further deterioration in environmental or husbandry conditions (Chen et al., 2003; Ling and Chen, 2005). Increasing solid concentrations can also promote bacterial growth by providing both a food substrate and a larger surface area for colonization (Berger et al., 1996; de Jesus Gregersen et al., 2019; Pedersen et al., 2017). Both autotrophic and heterotrophic bacteria occur in RAS. The autotrophic bacteria are the driving force behind the nitrification process (Hagopian and Riley, 1998) and thus important in preventing levels of ammonia and nitrite that might be toxic to fish. Heterotrophic bacteria are more abundant (Leonard et al., 2000), and are largely responsible for the decomposition of organic matter (Rurangwa and Verdegem, 2015). Increased availability of organic carbon in the form of small suspended solids can thus lead indirectly to further deterioration in water quality by promoting the growth of heterotrophic bacteria which can outcompete nitrifying bacteria for space and oxygen, thereby reducing rates of nitrification (Michaud et al., 2014, 2006). Organic particles are thus a further important factor controlling bacterial growth and carrying capacity (Pedersen et al., 2017).

Overall, it is notable that many of the solid-related effects cited above were discovered in experiments with sediments suspended in natural waters, while few studies have focused on the effects of system-related particles in aquaculture systems. Thus questions remain regarding the true effects of such particles on fish health and performance, making clear the need for further research in this field.

1.5 Research objectives

The aim of this thesis was to close important knowledge gaps regarding solid loads in aquaculture systems and to facilitate further improvements in recirculating aquaculture systems.

Prior to these studies, almost nothing was known about the shape of particles occurring in aquaculture systems and how shape may differ depending on water treatment and production type. Furthermore, the isolation of solid-related effects from potentially damaging water parameters which accompany increases in solid concentration has often been neglected in previous studies. It is essential that the actual consequences of increased suspended solid concentrations on fish health and performance in aquaculture systems are appraised in isolation.

In the light of the above points, the main objectives of this thesis were:

i. To assess the particle shapes occurring in the three main types of production system used for rainbow trout and to evaluate potential implications for the welfare and performance of fish and for system design.

ii. To elucidate the physiological effects of short-term exposure to increased particle load on rainbow trout in RAS, uncoupled from any associated debilitating water parameters.

iii. To investigate the physiological impacts of chronically increased solid concentrations on rainbow trout in RAS over a whole growout period, uncoupled from any associated debilitating water parameters.

iv. To analyze potential interaction effects of suspended solid load and increased unionized ammonia concentration on the physiology and performance of rainbow trout.

2 General materials and methods

2.1 Experimental recirculating aquaculture systems

The experiments included in this doctoral thesis were carried out in two experimental recirculating aquaculture systems located at the Fisheries Research Station of Baden-Württemberg in Langenargen. The experimental plant consisted of two replicate RASs (Figure 2.1) with each 10 green circular fiberglass tanks (330 L) which were connected in parallel so that each tank was supplied with treated water separately.

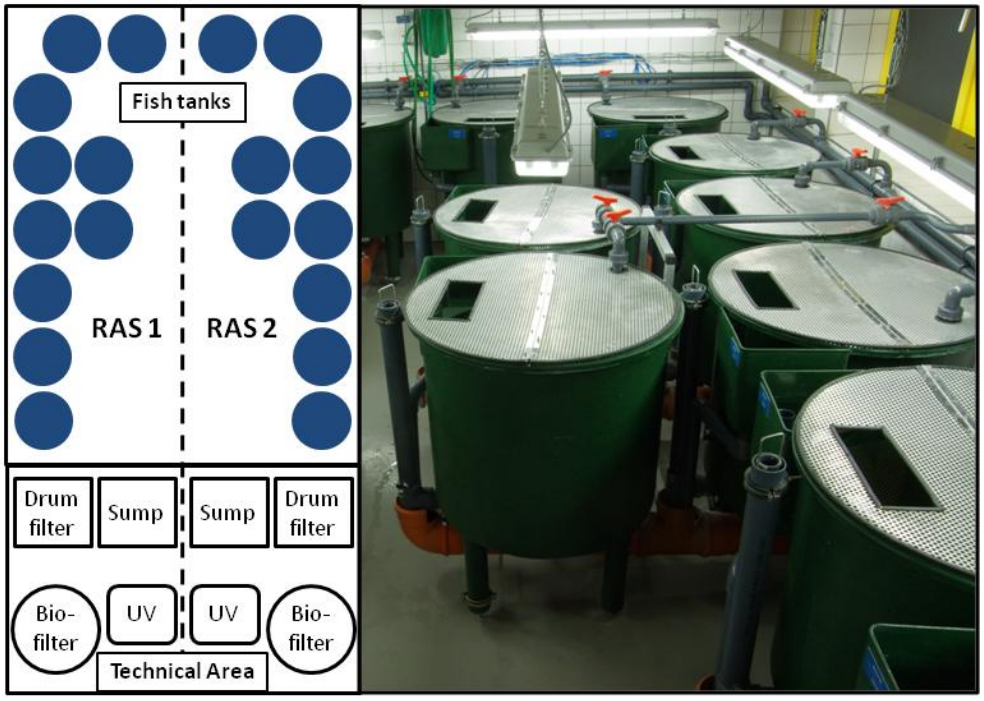


Figure 2.1: Design of the experimental recirculating aquaculture systems used in the experiments of this thesis.

Supply water was taken from the Lake Constance and UV treated before it was discharged into the systems. In both systems, drum filters (HDF801-1H, Hydrotech, Vellinge, Sweden, 100 µm filter gauze) were used for mechanical filtration. To avoid a decline in water quality, the biofilters (moving bed biofilm reactor) deployed were over-dimensioned and sufficient to remove the waste associated with 4.5 kg feed/day. The system water was UV treated (Barrier L20, Wallace & Tiernan, Günzburg, Germany; UV dose: 40 mJ/cm^2 flow volume: 6600 L/h, lamp wattage: 80 W,

measurement range UV sensor: 200 W/m^2) and oxygenated (in-house generator) before reinjected into the systems. Water consumption was reduced to a minimum and only limited to water loss due to backwashing of drum filter and evaporation. Oxygen concentrations and temperature were monitored continuously at the outlets of two tanks in each system. The room temperature was cooled to ensure a largely constant water temperature. Each two fish tanks were illuminated with a daylight lamp. The photoperiod was fixed at 12L:12D with a sigmoidal transition period of 30 minutes.

2.2 Husbandry

All exposure studies (chapter 4-6) were performed with all-female rainbow trout (Störk strain, Figure 2.2) to exclude gender-related effects. Fish were held at maximum stocking densities between 52 and 68 kg m^{-3}. The fish were fed restrictively six days a week (Sunday to Friday) using a commercial feed (Biomar EFICO Enviro 921, Aarhus C, Denmark). For more detailed information, see chapter 4-6.

Figure 2.2: All-female rainbow trout (*Oncorhynchus mykiss*) used for the experiments.

2.3 Particle accumulation procedure

Different procedures for artificial particle accumulation have been tested in pre-tests before the actual experiments to ensure a targeted and easy to control accumulation of system-related particles in the treatment RAS during the experiments. The best results were obtained by collecting backwash water from the drum filter into a tank and re-injecting it at regular intervals into the water buffer of the treatment system using a mud pump (Wilo-EMU KS 8 ES, Dortmund, Germany) (Figure 2.3). Under this process, larger particles were fragmented by shear forces and fine particles accumulated over time. Thus, no artificial particles were added to the systems and particles originated from feces and to a lower extend from uneaten feed.

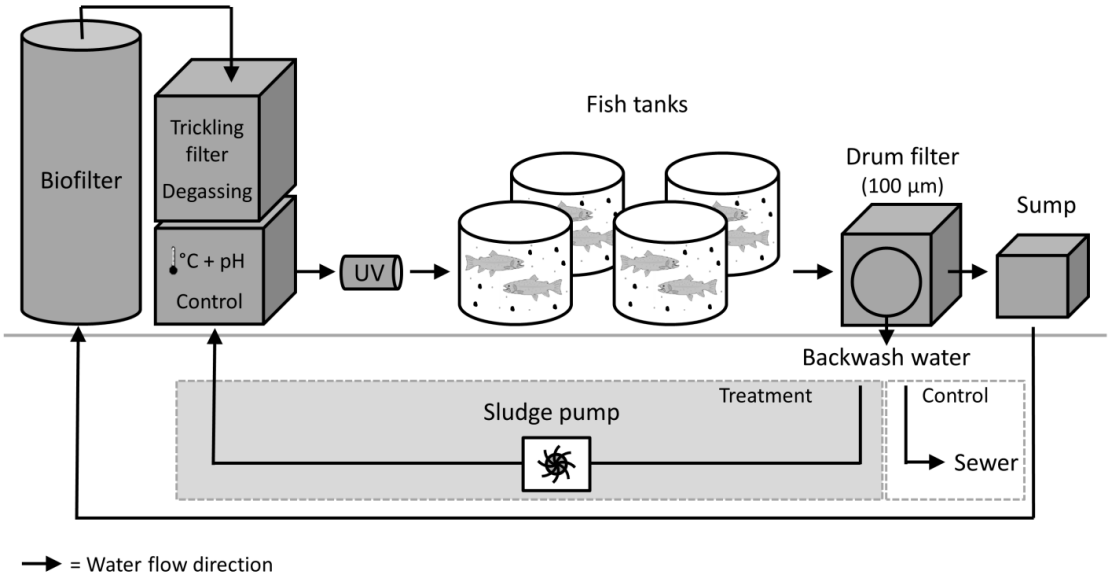


Figure 2.3: Scheme of the recirculating aquaculture systems with modification for particle accumulation in the treatment system (light grey shaded) (Becke et al., 2019).

2.4 Water analysis

A precondition of the particle exposure experiments in this thesis (chapter 4-6) was the isolation of particle accumulation effects from potentially confounding water parameters that accompany increases in solid load. Therefore, similar water parameters were maintained in control and treatment systems, within limits known to preclude impacts on fish health or performance during the whole growout period.

pH was measured daily (pH 320 with electrode Sentix41, WTW, Weilheim, Germany) in the outflow of the tanks and adjusted by the addition of sodium hydrogen carbonate. Oxygen concentrations (Oxygen Probes, OxyGuard, Farum, Denmark) and temperature (Temperature Probes, Oxyguard, Farum, Denmark) were monitored continuously at the outlets of two tanks in each system. Carbon dioxide concentrations were determined in the fish tanks using a portable dissolved CO_2 analyzer (OxyGuard CO_2 Portable, OxyGuard, Farum, Denmark).

NH_4-N concentration was measured using the Hach (Germany) analysis kit LCK 304 (0.2–2.5 mg/L). Additionally, NH_4-N concentration was measured in both RAS every 45 min using an automatic device (AMTAX SC, Hach, Germany) in the chronic and double exposure study to ensure continuity of monitoring. Nitrite and nitrate concentration was measured using the analysis kit LCK 341 (0.05–2 mg/L, Hach) and LCK 339 (1–6 mg/L, Hach) respectively. Turbidity was determined in parallel with the determination of total suspended solids using a turbidity meter (PCE-TUM 20, PCE Instruments, Germany).

2.5 Particle analysis

Total suspended solids (TSS)

The concentration of total suspended solids (Figure 2.4) was determined according to method 2540 D of the American Public Health Association (APHA, 1998), with the exception that 0.45 µm cellulose-acetate filters (diameter: 50 mm, 11106–50-N, Sartorius AG, Göttingen, Germany) were used instead of glass-fiber filters due to the smaller and better defined pore sizes. Prior to use, the filters were pretreated in boiling distilled water for 2 h, dried at 103 °C for a minimum of 2 hours, and weighed with a precision scale (0.1 mg). TSS concentration (mg/L) was ascertained at least thrice weekly in duplicate for each system using compressed air at about 0.7 MPa for filtration. Water samples of the experimental RAS were collected using a tube at a water depth of ca. 30 cm from five tanks in each system, then duplicate samples were pooled to create a representative sample for each system. Samples were collected in the early morning before feeding, in order to represent the daily minimum solid loads (best case scenario) and to ensure that the particle concentration did not fall below the aimed concentration. Additionally, daily TSS measurements were performed every 2 h from 7:00 to 19:00 and at 23:00 (CET) to show the fluctuations and maximum values of particles concentrations within the day.

Figure 2.4: Loaded cellulose-acetate filters of the control (top) and treatment RAS (down) during the short-term exposure study (chapter 4).

Particle size distribution (PSD)

Particle sizes were determined according to Brinker et al. (2005b) using a non-invasive laser particle sizer (GALAI:CIS-1, GALAI Productions Ltd. Midgal Haemak, Israel) equipped with a flow controller (GALAI:LFC- 100) and a flow-through cell (GALAI:GM-7). Particles larger than 300 µm were separated by a pre-weighed, 300 µm, even weave Polyester screen. A subsample (0.5 L) of the suspension remaining after PSD determination was filtered with a 0.45 µm cellulose-acetate filter using compressed air at about 0.7 MPa. The weight of particles above 300 µm divided by the total particle weight provided a correction for particles larger than 300 µm, which were out-of-range for laser-determination of PSDs. The PSD data were arranged into size classes (d_{i+1}=1.26 d_i, d = upper diameter of class) according to Patterson et al. (1999).

Particle Shape

Shape analysis of particles found in different types of aquaculture systems (Figure 2.5) was performed using the Particle Insight Size and Shape Analyzer (Micromeritics Instrument Corporation, Norcross, USA). The shape analyzer was able to record 28 different size and shape parameters. The most promising for describing particles in the current aquacultural context were equivalent circular area diameter (ECAD), circularity, equivalent elliptical area length (EEAL) and width (EEAW), ellipticity, ellipse aspect ratio (EAR), feret width, length and aspect ratio. For more detailed information, see chapter 3.

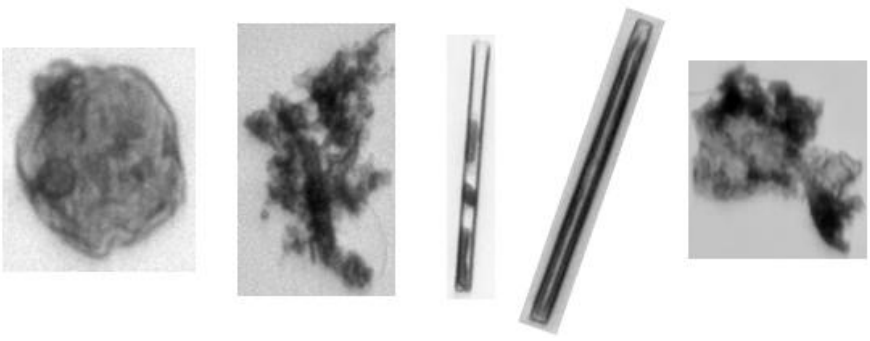


Figure 2.5: Particles with different shapes detected by the shape analyzer.

2.6 Fish performance

Following performance parameters were determined in order to investigate the effects of increased suspended solid concentrations on rainbow trout performance:

Survival rate

$$Survival\ (\%) = \frac{number\ of\ fish_{final\ day}}{number\ of\ fish_{initial\ day}} \times 100$$

Specific growth rate (SGR)

$$SGR\ (\%\ d^{-1}) = \frac{ln(mean\ final\ weight) - ln(mean\ initial\ weight)}{t(final\ day) - t(initial\ day)} \times 100$$

Feed conversion ratio (FCR)

$$FCR = \frac{Feed(kg)}{Weight\ gain(kg)}$$

Thermal growth coefficient (TGC) according to Jobling (2003)

$$TGC = \frac{(\sqrt[3]{W_t} - \sqrt[3]{W_0})}{\sum T} \times 1000,$$

where W_t and W_0 are the final and initial weights (g), respectively and ∑T is sum day-degrees Celsius (Cho, 1992; Iwama and Tautz, 1981).

Dry matter digestibility (chronic exposure study)

Dry matter digestibility was determined in the chronic exposure study using yttrium oxide (Y_2O_3). Samples were prepared as described in Brinker and Reiter (2011).

$$Digestibility\ (dry\ matter) = \frac{100 - (100 \times {Y_2O_3}_{diet})}{\frac{dry\ matter_{diet}}{100}} \times {Y_2O_3}_{feces}^{-1}$$

2.7 Hematology

Blood samples were taken from rainbow trout after anesthesia by puncturing the caudal vein. Blood smears for differential blood counts were produced with native-blood using an Undritz-Glass directly after blood sampling. After complete drying, blood smears were stained with Hemacolor (Merck, Darmstadt, Germany). Differential leukocyte counts were made by identifying 200 leukocytes (Figure 2.6) per blood smear in a meandering pattern.

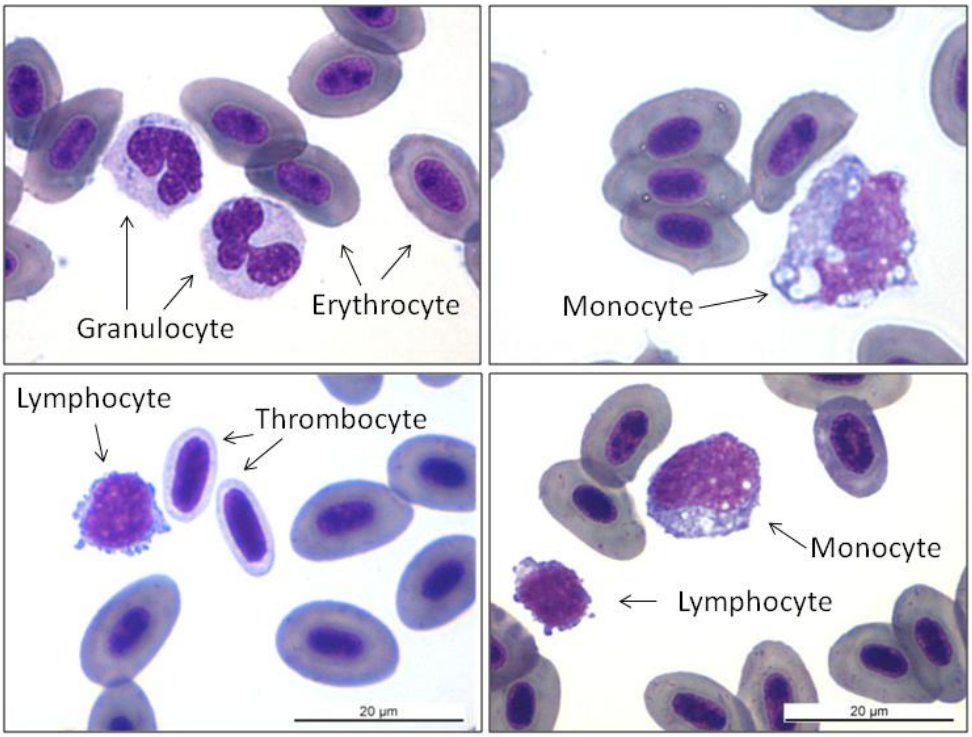


Figure 2.6: Blood cells of rainbow trout.

Glucose concentration in the blood of rainbow trout was determined using a common blood glucose meter (ACCU-CHEK Aviva, Roche, USA) as it has been shown that devices for measuring human glucose level are also suitable for use with fish blood (Bartoňkova et al., 2017; Eames et al., 2010). Hemoglobin concentration was determined by the cyanmethemoglobin method (Drabkin and Austin, 1932), with bovine hemoglobin (H2500, Sigma-Adrich, St. Louis, USA) as a standard. Cellular debris was removed from the samples with a metal eye-let before absorbance readings were taken. For hematocrit and leukocrit determination, hematocrit capillaries (sodium

heparinized) were filled with blood and centrifuged for 10 min at 14,000 × g in a hematocrit centrifuge (HAEMATOCRIT 210, Hettich, Tuttlingen, Germany). Total red and white blood cell counts were performed with a 1:200 dilution of Natt-Herrick solution (Natt and Herrick, 1952) and counted using a hemocytometer (Neubauer chamber). The blood parameters thus acquired were then used to calculate indices of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC):

$$MCV\ (fL) = \frac{Hematocrit\ (\%)}{Erythrocytes\ (Mio/\mu L)} * 10$$

$$MCH\ (pg) = \frac{Hemoglobin\ (g/dL)}{Erythrocytes\ (Mio/\mu L)} * 10$$

$$MCHC\ (g/dL) = \frac{Hemoglobin\ (g/dL)}{Hematocrit\ (\%)} * 100$$

2.8 Gill histology

Gill tissue was fixed directly upon sampling with 10 % neutrally buffered formalin for at least 24 h at room temperature, and then stored at 4 °C until further processing. The tissue was dehydrated in a series of graded ethanols, infiltrated and embedded with paraffin wax. Sections were cut at a thickness of 4–6 µm, stained with haematoxylin and eosin (chapter 4) or PAS stain (Periodic acid-Schiff stain, chapter 5 + 6) and observed under a photomicroscope (Zeiss, Oberkochen, Germany).

For each section, 5 images showing 6–7 secondary gill lamellae were taken at a magnification of 200×. The architecture of the branchial epithelium and the secondary lamellae was examined for changes, such as thickening and edematous alterations of branchial epithelium cells, lifting of the epithelium from the pavement cells, telangiectasia, hyperplasia of cells and lamellar fusion (Figure 2.7). Observed changes were scored arbitrarily from 0 to 3. Representing no change, little change, moderate change and severe change respectively. When the secondary gill lamellae were covered by a thin branchial epithelium which was intimately attached to the vascular epithelium and the interlamellar space was clearly visible, the gills were designated

"no change". Gill samples in which branchial cells exhibited a thicker appearance, a lighter coloration, a lifting of the epithelium, telangiectasia or hyperplasia or fusion of the lamellae in some places were scored as "little change". When a swelling of the gill epithelium, formation of cell edemas, separation from the vascular epithelium, telangiectasia, hyperplasia or lamellar fusion was observed in several locations of the gill sample, the changes were scored as "moderate change" and when observed changes were regular and pronounced, the gills were scored as "severely changed". Branchial epithelium thickness (µm) was measured at 10 locations in each image and a mean value was calculated and the number of goblet cells was counted per secondary lamella.

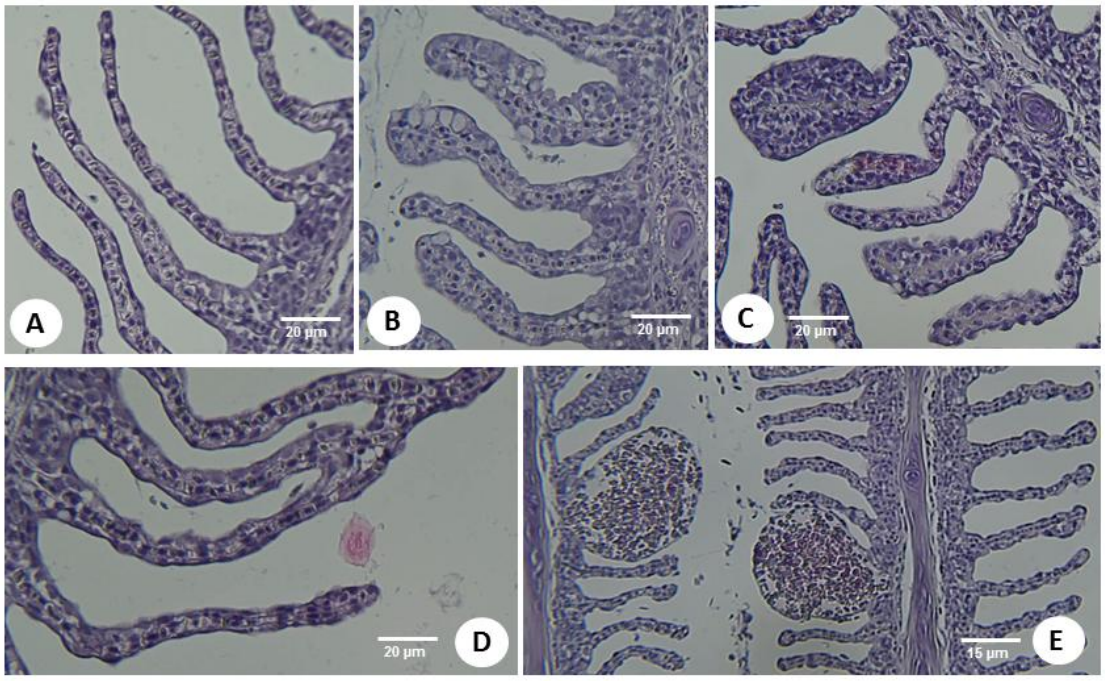


Figure 2.7: Observed histological changes of gill structures: (A) unchanged lamellar structure, (B) swelling of branchial epithelium, (C) terminal swelling, (D) lamellar fusion and telangiectasia (E) (Becke et al., 2018).

2.9 Fin condition

Fin erosion of the dorsal and both pectoral fins of rainbow trout (Figure 2.8) was assessed according to Person-Le Ruyet et al. (2007) and the fin index was determined according to Kindschi (1987), as follows:

$$Fin\ index = \frac{fin\ length}{total\ length} * 100$$

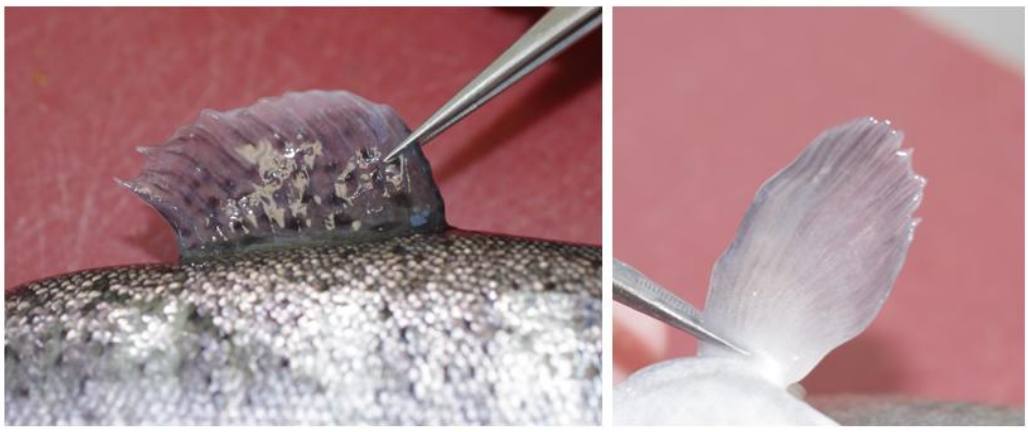


Figure 2.8: Fin erosion level 2 of the dorsal fin and erosion level 1 of the left pectoral fin according to Person-Le Ruyet et al. (2007)

2.10 Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISA) were used to determine plasma cortisol and heat shock protein 70 (HSP70) concentrations of rainbow trout.

For determination of plasma cortisol concentrations, blood samples were centrifuged for 10 min at 1000 × g (4°C, Rotina 38R). Plasma was transferred into plastic tubes and stored at −20°C until further processing. A commercial Cortisol ELISA kit (HZ-1887, Hölzel Diagnostika GmbH, Köln, Germany) was used to determine plasma cortisol concentration, after cortisol extraction with ethyl acetate.

For HSP70 determination, tissue homogenates were produced from skin, gills, liver and head kidney of rainbow trout. Concentrations of Hsp70 were determined using a commercially available ELISA kit (SEA873Hu, Cloud-Clone Corp., antibodies-online.com, Aachen, Germany). For more detailed information, see chapter 4 + 5.

2.11 Bacterial assay

Colony forming units

Counts of viable heterotrophic bacteria (recorded as colony-forming units, CFUs) were made during the chronic exposure study after 48 h of culture at 22 °C on DEV Agar (103554ZA, VWR International GmbH, Germany). Two dilutions (see Table 2.1) were prepared for each water sample with 0.9 % sterile sodium chloride solution. Plates were set up in triplicate for each dilution.

Table 2.1: Used dilutions of the system water with 0.9 % NaCl solution to determine the colony-forming units in the system water (chapter 5).

System	fish tanks	before UV	after UV
Control	1 : 100 / 1 : 1000	1 : 10 / 1 : 100	1 : 10 / 1 : 100
Treatment	1 : 100 / 1 : 1000	1 : 100 / 1 : 1000	1 : 10 / 1 : 100

Bactiquant® method

Bacterial activity in the fish tanks was assessed using a patented method called Bactiquant-water® (Mycometer A/S, Copenhagen, Denmark, Figure 2.9), which is an indirect measure of microbial enzyme activity (Reeslev et al., 2011). The Bactiquant® value (hereafter termed bacterial activity) is a dimensionless value and reflects the number of bacteria and their enzymatic activity in a given water sample under the influence of sample volume, reaction time and incubation temperature. The microbial enzymes in a water sample hydrolyze a synthetic fluorescent enzyme substrate so that fluorophores are released into the water which then could be quantified using a fluorometer. For the measurement, 10 mL water samples were filtered directly after extraction using a Millipore 0.22 µm closed filter unit (PES express). The filter was then incubated for 15 minutes with a fluorogenic enzyme substrate at room temperature and the amount of released fluorophores was quantified with a fluorometer (Mycometer A/S, Copenhagen, Denmark). Measurements were always performed in duplicate.

The Bactiquant® method has two major advantages compared to the conventional plate procedure: first, this method is significantly less time-consuming so that information on bacterial activity in water samples is available within a few minutes.

Furthermore, the Bactiquant® method also includes bacteria that are attached to particles and is therefore a much more precise method than the plate-based method where aggregates of bacteria are only counted as one colony.

Figure 2.9: Measurement of bacterial activity using the Bactiquant® method.

Analysis of the bacterial load of rainbow trout in chapter 5 and 6 was conducted by the fish health service at a governmental veterinary institute, the Staatliches Tierärztliches Untersuchungsamt (STUA) Aulendorf, Germany. Mucus smears taken from the skin and gills of sampled fish and crush preparations of spleen tissue were all analyzed for the presence of bacteria under a phase contrast microscope. Agar plates (composition see Table 2.2) were inoculated with smears taken from gills and spleen. After incubation, the number of colony forming units was assessed and arbitrarily graded as no, slight, moderate or severe bacterial load. Bacterial species were then determined by using bacteriological standard methods and confirmed by MALDI-TOF MS (Lay, 2001).

Table 2.2: Composition of agars used for analysis of the bacterial load of rainbow trout.

	Agar 1	**Agar 2**
Ingredients	980 mL demineralized water	1000 mL Aqua dest.
	2 g Bacto-Tryptone	0.5 g Bacto-Tryptone
	2 g Yeast extract	0.5 g Yeast extract
	10 mL Tween 80	0.2 g sodium acetate
	5 g NaCl	0.2 g meat extract
	0.1 g $CaCl_2$x 2 H_2O	8 g Beco-Agar
	15 g Beco-Agar	
	0.03 g Bromothymol blue	
pH	7.4 ± 0.2	7.2 - 7.4
Incubation	1 – 5 days at 20 ± 1 °C	5 – 10 days at 16 ± 1 °C
Detection	*Yersinia ruckeri*	*Flavobacterium psychrophilum*
	Agar 3	**Agar 4**
Ingredients	900 mL demineralized water	500 mL agar 3
	10 g Bacto-Tryptone	50 mL sterile, defibrinated blood
	5 g Bacto-Yeast extract	
	1 g L-Tyrosine	
	2.5 g NaCl	
	20 g Beco-Agar	
	100 mL starch	
	9 mL Phenol red (2 %)	
pH	7.2 - 7.4	7.2 - 7.4
Incubation	1 – 5 days at 20 ± 1 °C	1 – 5 days at 20 ± 1 °C
Detection	Fish pathogenic bacteria	Fish pathogenic bacteria

3 Shape characteristics of suspended solids and implications in different salmonid aquaculture production systems

A similar version of this chapter was accepted for publication by Aquaculture:
Becke, C., Schumann, M., Geist, J., Brinker, A.: Shape characteristics of suspended solids and implications in different salmonid aquaculture production systems. Aquaculture (accepted).

3.1 Abstract

In the interests of optimizing the performance and welfare of fish in aquaculture systems and informing simulated and real-world fish farm operations, the particles in two recirculating (RAS), two semi-recirculating (sRAS) and two flow-through systems (FTS) were analyzed using non-invasive digital image techniques to determine shape factors including equivalent circular area diameter (ECAD), circularity, ellipticity and feret diameter.

With the exception of feret diameter, most particle shape parameters showed little variation between systems: the majority of particles had a flake-like structure, while round or elongated particles were relatively rare. The exception was one system which yielded large numbers of elongated particles, most probably originating from intrinsic algae production. Generally, mean circularity and ellipticity of particles was about 0.5 and 0.7 respectively, indicating an ellipsoidal rather than spherical shape. Thus, in a departure from previous approaches, it was asserted that calculations based on an ellipsoidal shape will produce more accurate results than those making an assumption of sphericity, when calculating particle volume and surface area. This result has implications for system design and for theoretical calculations. Mechanical treatment did not appear to exert relevant effects on particle shape, apart from expected reductions in feret width and length.

Overall, the present results indicate that particle shape is primarily governed by feed composition and intrinsic biology rather than by production system type. Moreover, an assumption of ellipsoidal shape presents a robust modeling approach for further scientific calculations.

Candidate's contribution:

Implementation of the method for the analysis of particle shape characteristics, planning of the experimental setup, sampling of water samples and execution of particle measurements, statistical assessment and interpretation of data, writing and revision of the complete manuscript including all figures and tables.

3.2 Introduction

Management of solid wastes is one of the main issues in the aquacultural production of finfish and especially so in recirculating aquaculture systems (RAS) (Badiola et al., 2012; Bao et al., 2018). Almost all the suspended solids generated in aquaculture are derived from feeds added to the production systems, either directly in the form of uneaten food, or indirectly as feces. A smaller proportion by mass comes in the form of microorganisms, detached biofilm and/or algae (Chen and Malone, 1991; Noble and Summerfelt, 1996; Summerfelt et al., 1999; Timmons and Ebeling, 2010; Wedemeyer, 1996). Further extrinsic sources of mostly inorganic particles include the water supply. These are usually minor, but can occasionally be massive, for example during extreme weather events such as heavy rain, floods and snowmelt (Asselman, 1999; Langlois et al., 2005; Lenzi and Marchi, 2000).

For the most part, the decisive factors in the occurrence and volume of suspended solids in aquaculture systems are the quality and composition of feed, and in particular the use of ingredients that directly affect the stability of fecal material (Brinker et al., 2005a; Schumann et al., 2018; Unger and Brinker, 2013). Disintegrative forces such as shear (water turbulence, pumping, fish-feeding activity), chemical leaching or microbial activity (Brinker et al., 2005a; McMillan et al., 2003; Warrer-Hansen, 1982; Wong and Piedrahita, 2000) result in waste particles becoming smaller with time and lead to an accumulation of fine particles, especially in RAS (Becke et al., 2017, 2018, 2019; Chen et al., 1993; Davidson et al., 2009).

Until recently, the accumulation of particles and especially of fines was considered detrimental to fish health, welfare and performance (Bilotta and Brazier, 2008; Chapman et al., 1987; Chen et al., 1993; Chen and Malone, 1991; Herbert and

Merkens, 1961). However recent investigations under controlled conditions that uncouple solid load from other potentially confounding water parameters have challenged this assumption, at least for rainbow trout in RAS (Becke et al., 2017, 2018, 2019). Even so, suspended solids can, depending on size and density, contribute to deterioration in growth conditions through leaching of potentially harmful substances (e.g. ammonia, nitrite) (Chen et al., 2003; Ling and Chen, 2005) or through enhancing bacterial activity permitted by the increase in available surface area for colonization or use as a food-substrate (Becke et al., 2019; Pedersen et al., 2017).

Suspended solids are regarded as a major issue in all forms of intensive aquaculture and especially in RAS (Badiola et al., 2012). However, there is no consensus on safe concentrations beyond which fish should not be reared. Furthermore, little is known about how the shape of particles occurring in aquaculture systems might modify their interactions or impact on critical matters such as fish health and system performance. Several studies have addressed the size distributions of particles occurring in aquaculture facilities (Brinker and Rösch, 2005; Chen et al., 1993; Cripps, 1995; Patterson and Watts, 2003b; Pfeiffer et al., 2008), but without considering particle shape. Furthermore, while natural particles display a variety of shapes including spheres, rods, flakes, rectangles, fibers or a combination of these, most instruments and calculations used to assess particle size rely on an assumption of sphericity (e.g. Brinker et al., 2005b; Fernandes et al., 2014; Patterson et al., 1999). Particle size distributions and estimates of total solid volume and surface area arrived at under this assumption are all likely to be biased if the possibility of irregularities in particle shape is not addressed. A few previous studies have been made into the shape of particles in other wastewater effluents (Mamane et al., 2008; Thomas and Moore, 2004; Zahid and Ganczarczyk, 1990), but without considering any link to aquaculture. Patterson and Watts (2003a) made a qualitative shape analysis of particles from a commercial Atlantic salmon (*Salmo salar*) smolt production unit using light microscopy, but without providing exact measurements of particle shapes. To the best of our knowledge, there has been no further research on this subject in an aquaculture context, despite the potentially significant implications. For example particles which exhibit a high circularity will flow and mix better than bulkier or more irregular shapes,

which have a greater tendency to attach to other particles. A better understanding of the structure and shape of suspended particles in a given type of production system would allow a more realistic approach to evaluate system performance and the physiological effects of suspended solids on cultured fish.

The present study set out to address this important gap in understanding by investigating the shape of suspended solids in three widely used types of salmonid production system (flow-through, semi-recirculating, fully recirculating system) using digital image analysis. It was hypothesized that particle shape would differ between the three system types due to differences in technical specifications and water reuse, and that furthermore, particle shape would differ between water samples taken before and after solid drum filter treatment. It was also hypothesized that drum filtration would increase the proportion of high circularity particles due to the greater extractability of elongated and irregular particles.

3.3 Materials and methods

Study design

Flow-through systems (FTS) and semi-recirculating aquaculture systems (sRAS) are one of the most used production formats for rainbow trout (*Oncorhynchus mykiss*) at present (Lasner et al., 2016; Nielsen et al., 2016), but recirculating aquaculture systems (RAS) are expected to become a major feature of all salmonid production in the near future (Badiola et al., 2012). The current study set out to profile the particle shape distributions of these three production formats, by evaluating two separate systems of each type (Table 3.1). The different system types exhibited diverse mechanical and biological water treatment components, with all but the semi-commercial RAS farms (smaller than usual commercial enterprises, but using commercial treatment components, husbandry conditions etc.) producing trout at a commercial scale.

Table 3.1: Overview of aquaculture systems investigated in the present study.

	Flow-through systems (FTS)		Semi-recirculating systems (sRAS)		Recirculating systems (RAS)	
Name	FTS1	FTS2	sRAS1	sRAS2	RAS1	RAS2
Water volume of the investigated fish tank	300 m^3	1000 m^3	1500 m^3	190 m^3	40 m^3	6 m^3
Stocking density	60 kg m^{-3}	25 kg m^{-3}	80 kg m^{-3}	80 kg m^{-3}	40 kg m^{-3}	70 kg m^{-3}
Mechanical treatment	/	/	Drum filter (40 µm gauze)	Sedimentation tank (HRT: 0.51 h)	Sedimentation tank (HRT: n/a)	Drum filter (100 µm gauze)
Biological treatment	/	/	Moving- and fixed-bed bioreactor	Fixed-bed bioreactor	Fixed-bed bioreactor	Moving-bed bioreactor
Freshwater source	Spring	Spring	Spring + well	Spring/Lake	Brook	Lake
Water exchange	Continuous	Continuous	30 volume% day^{-1}	400 % day^{-1}	5 volume% day^{-1}	4.3 volume % day^{-1}
Feed	Skretting Optiline HE	Biomar EFICO ENVIRO 921	Biomar EFICO ENVIRO 920	Biomar EFICO ENVIRO 921	Biomar EFICO ALPHA 790	Biomar EFICO ENVIRO 921
Feeding load (m^3 freshwater)	0.04 kg m^{-3}	0.03 kg m^{-3}	6.00 kg m^{-3}	0.20 kg m^{-3}	n/a	10.4 kg m^{-3}

Sampling

Sampling took place in the autum. Water samples (3-8 L, volume increased with decreasing particle concentration) were taken in duplicate from the middle of the water column at, or directly before the outflow of the fish tanks in each system, as this is generally the area with the highest concentration of particles. Additional samples were taken after the drum filter in sRAS1 and RAS2, in order to assess the impact of mechanical treatment on particle shape distribution. Water samples were measured immediately after sampling to prevent agglomeration of particles. During sampling, special care was taken to ensure that particle concentrations in the water sample were not affected by feeding, filter flushing or other events that could have influenced the occurrence of particles. Particle shape analysis and the concentration of suspended solids (TSS) were performed on the same water sample.

Particles larger than 300 µm were separated by a pre-weighed, 300 µm, even-weave Polyester screen (Franz Eckert GmbH, Waldkirch, Germany) and not shape characterized due to a maximal measurement size of 300 µm of the used shape analyzer device. TSS concentration in the remaining suspension was determined according to APHA (1998), with the exception that 0.45 µm cellulose-acetate filters (diameter: 50 mm, 11106-50-N, Sartorius AG, Göttingen, Germany) were used instead of glass-fiber filters due to their smaller and better defined pore sizes. The final TSS concentrations comprise the TSS concentration of the measured water sample plus the weight of particles > 300 µm per liter. Filters were prepared as described by Becke et al. (2018). TSS concentration (mg L^{-1}) was ascertained in duplicate for each sampling point. Images of cellulose-acetate filters were taken after drying process using a VHX-700F digital microscope (Keyence, Neu-Isenburg, Germany) at 200× magnification. Percentage values for the relative removal efficiency (RRE) of each drum filter were calculated as follows:

$$RRE[\%] = \frac{C_1 - C_2}{C_1} \times 100,$$

where C_1 and C_2 represent TSS concentration before and after the drum filter, respectively.

Shape analysis

Shape analysis was performed using the Particle Insight Size and Shape Analyzer (Micromeritics Instrument Corporation, Norcross, USA) across a size range of 3 to 300 µm due to device restrictions. Measurements were performed on water passing from a glass vessel with a lateral outflow opening at the bottom, with gentle stirring to prevent settling throughout the measurement. Water from the vessel was pumped (Masterflex L/S, Cole-Parmer, Chicago, USA) through a thin glass measuring cuvette (500 µm flow-through diameter) at an average rate of 3 L h^{-1}, illuminated from one side so that the shapes of the particles could be recorded by a digital camera on the other side (Figure 3.1). Size and shape parameters were measured for each recorded particle using the onboard system software. The shape analyzer was able to record 28 different size and shape parameters. The most promising for describing particles in the current aquacultural context were as follows:

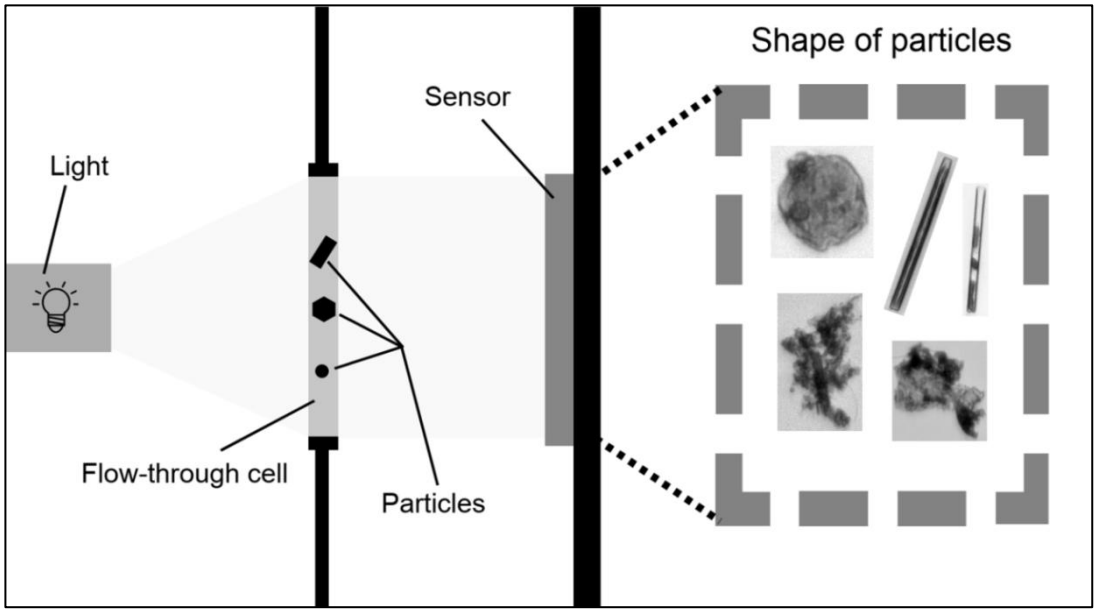


Figure 3.1: Measurement scheme of the Particle Insight Size and Shape Analyzer.

Equivalent circular area diameter (ECAD)

ECAD is defined as the area of a flat shadow or silhouette of the particle identified by the software and presented as the diameter of a circle with the same area as the silhouette (Figure 3.2). From this, the volume and surface area of an equivalent sphere can be computed as follows:

$$V_{sphere} = \frac{4}{3}\pi \times \left(\frac{ECAD}{2}\right)^3$$

$$S_{sphere} = \pi \times ECAD^2$$

Circularity

Circularity is defined as the fraction of a bounding circle's area (*A*) covered by the actual shape of a particle. It is computed from area (*A*) and bounding circle diameter (*BCD*) as follows:

$$Circularity = \frac{4A}{\pi BCD^2},$$

where BCD is the diameter of the smallest circle that encloses but does not intersect the particle (Figure 3.2). A formula outcome of 1 represents a perfect circle. Circularity is not affected by small perimeter irregularities or errors in perimeter measurement.

Ellipse model

Equivalent elliptical area length (EEAL) and width (EEAW) are defined as the length and width of an ellipse that has the same area as the silhouette of the particle (Figure 3.2), whereas ellipticity is the ratio of the shape area to the area of the bounding ellipse.

The volume of the resulting spheroid (ellipsoid of revolution) was computed as follows:

$$V_{spheroid} = \frac{4}{3} \times \pi \times a^2 \times c,$$

where *a* is the equatorial radius of the spheroid ($EEAL/2$), and *c* is the distance from center to pole along the axis of symmetry ($EEAW/2$).

The surface area of an oblate spheroid was computed as follows:

$$S_{oblate\ spheroid} = 2\pi a^2 + \pi \frac{c^2}{e} ln\left(\frac{1+e}{1-e}\right), \text{ where } e^2 = 1 - \frac{c^2}{a^2}.$$

Ellipse aspect ratio (EAR) was determined as the ratio of EEAL to EEAW. The number, volume and surface area of particles were determined according to Patterson et al. (1999), but allocated to a size class according to the narrowest dimension, EEAW, on the assumption of ellipsoidal shape.

Feret diameter

Feret width and length are measures of the smallest and largest possible distances respectively between two parallel lines that contact but do not intersect the particle (Figure 3.2). Therefore, feret length and width are not always orthogonal. Aspect ratio (AR) is determined as the ratio of feret length to feret width.

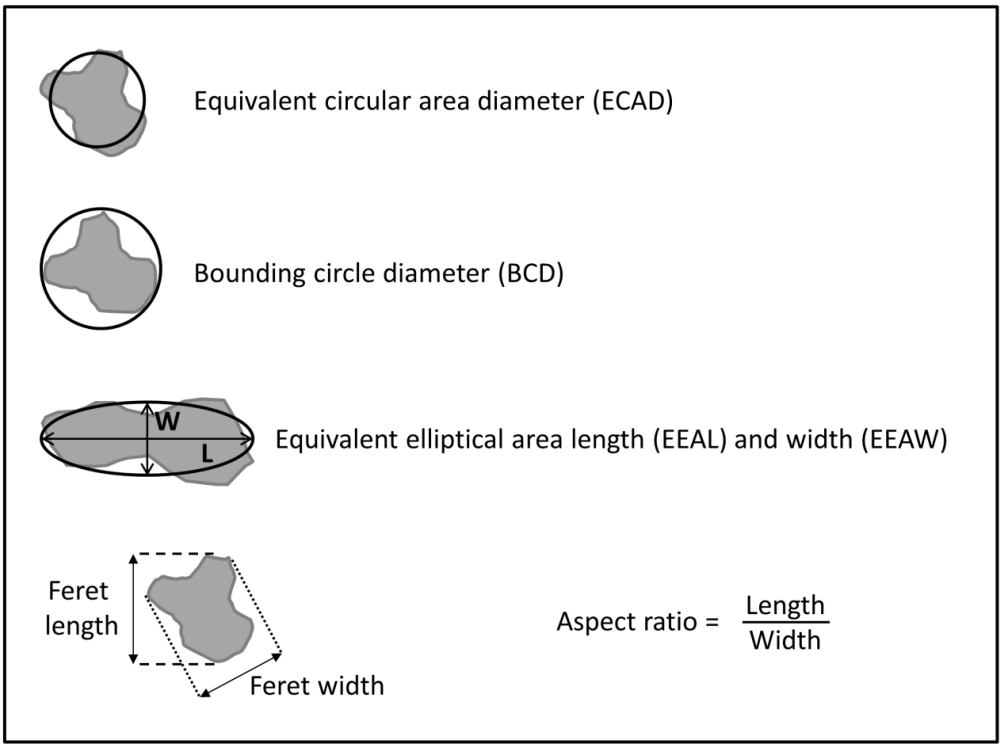


Figure 3.2: Summary of shape parameters used in the present study.

Log_{10}-Log_{10}-Fit of particle size distribution data

According to Patterson et al. (1999), particle sizes in aquaculture systems follow a near hyperbolic distribution. Log_{10}-log_{10}-transformations of observed particle diameters (in this case EEAW) and frequency were carried out to allow linear regression analysis and the resultant slope of regression (β-value) was used to make predictions about surface and volume percentages. A slope of 3 appears to be the median value for wastewater systems (Patterson et al., 1999), indicating a large number of small particles, while large particles dominate the volume distribution. Surface area contributions are shared equally among all size classes (Patterson et al., 1999). A slope of 2 indicates a dominance of larger particles in both surface area and volume distributions and thus a reduced influence of fine particles.

Data analysis

Data were checked for homoscedasticity using Levene's test (Levene, 1960) or an F-test (2-sided) in the case of mechanical treatment effects. Differences in TSS concentration, circularity, ellipticity, ellipse aspect ratio, feret width, feret length and feret aspect ratio of particles between system types were tested by analysis of variance (ANOVA). Post-hoc comparisons were made by Tukey's HSD test (Hayter, 1984). In cases of heteroscedasticity, the Kruskal-Wallis test was used and post-hoc comparisons were made by the Steel-Dwass-test. Removal efficiency predictions were analyzed using the z-test. Skewness and kurtosis were computed according to Sachs (2013). The following general linear model (GLM) was applied to investigate factors (system type, stocking density) influencing TSS concentration:

$$Y_{ijk} = \mu + a_i + b_j + \epsilon_{ijk}$$

where Y_{ijk} is the evaluated parameter, μ is the overall mean, a_i is the fixed factor *system type,* b_j is the fixed factor *stocking density* and ϵ_{ijk} is the random residual error. All data analyses were performed with JMP Pro (SAS Institute Inc.) version 14.2.0. (64-bit). Differences were considered to be significant at $P < 0.05$.

3.4 Results

Total suspended solids

TSS concentrations differed between system types ($P < 0.001$), being significantly higher in FTS than in either sRAS ($P < 0.001$) or RAS ($P < 0.01$). However, no significant difference was apparent between sRAS and RAS ($P > 0.05$).

At system level, the TSS concentration of FTS1 did not differ significantly ($P > 0.05$) from that of FTS2 or RAS1 (Figure 3.3). Furthermore, no significant difference ($P > 0.05$) was recorded between sRAS1, sRAS2 and RAS2. All other systems differed significantly from one another ($P < 0.0001$ to $P < 0.05$). FTS2 registered the highest TSS load, with 11.8 ± 0.4 mg L^{-1}, and sRAS1 the lowest at 4.5 ± 0.2 mg L^{-1}. Particles smaller than 300 µm accounted for the majority of TSS in all systems, with the exception of FTS1, where particles greater than 300 µm represented approximately 90 % of TSS. TSS measurements of the inflow water (data not shown) revealed almost no particles > 300 µm. The GLM (whole model: 12 observations, $r^2_{adjusted} = 0.914$, $P < 0.0001$) revealed a statistically significant negative correlation ($P < 0.01$) between TSS concentration and stocking density, with significantly higher values ($P < 0.01$) in FTS compared to sRAS and RAS.

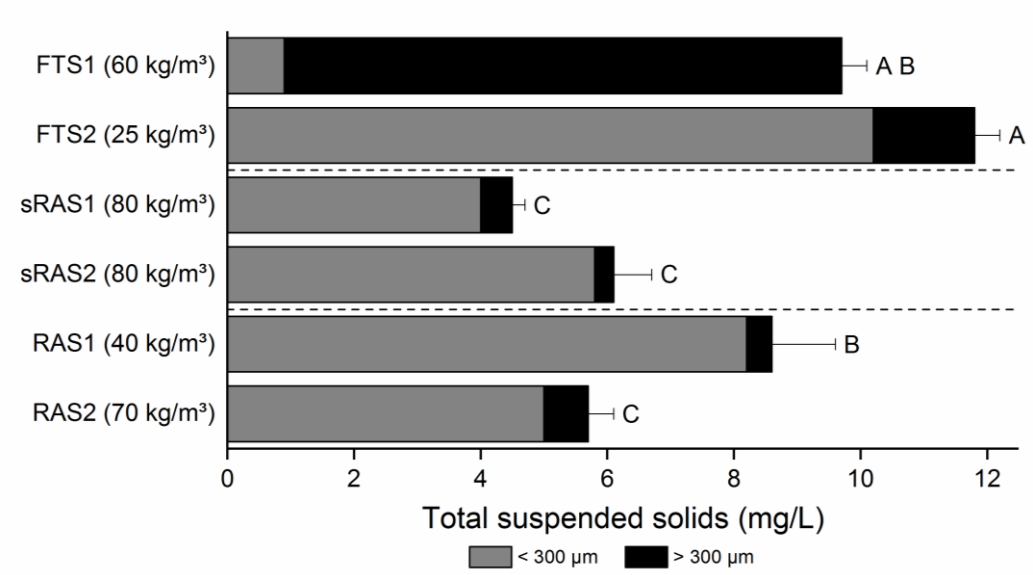


Figure 3.3: Total suspended solid concentrations (mean ± S.D.) in the different systems. Y-axis figures in brackets indicate stocking density. FTS= flow-through system, sRAS= semi-recirculating system, RAS= recirculating system. Different letters indicate statistically significant differences ($P < 0.05$).

Particle analysis

An overview of the particles from the different aquaculture systems retained on cellulose-acetate filters and used for the determination of TSS is given in Figure 3.4. At macroscopic level, it is evident that FTS2 and RAS1 were dominated by larger particles, whereas smaller particles were more prevalent in FTS1, sRAS1, sRAS2 and RAS2. There was a higher incidence of small, elongated particles in sRAS2 compared to all other systems, but more advanced assertions about the particle shape were not possible because of the likelihood that particle shape was altered by the drying process.

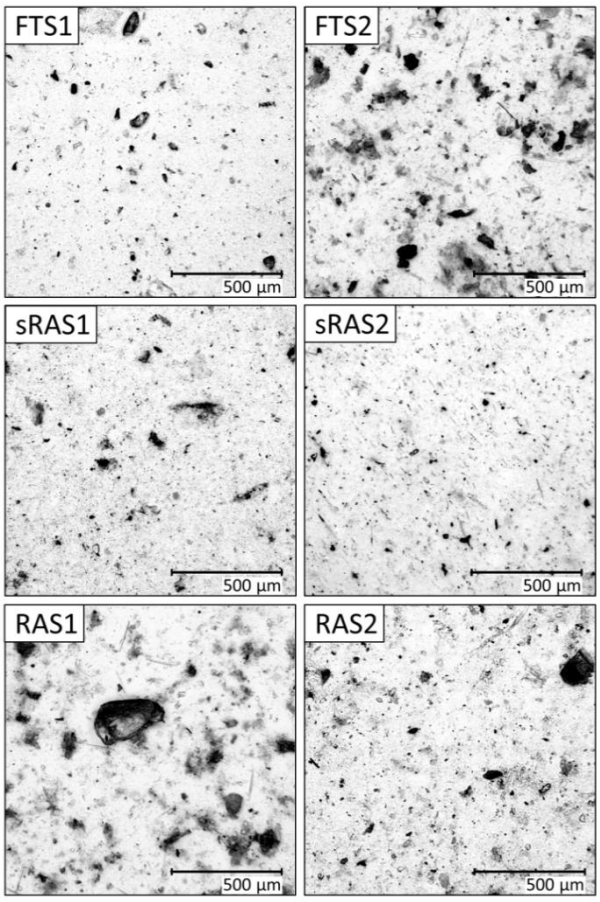
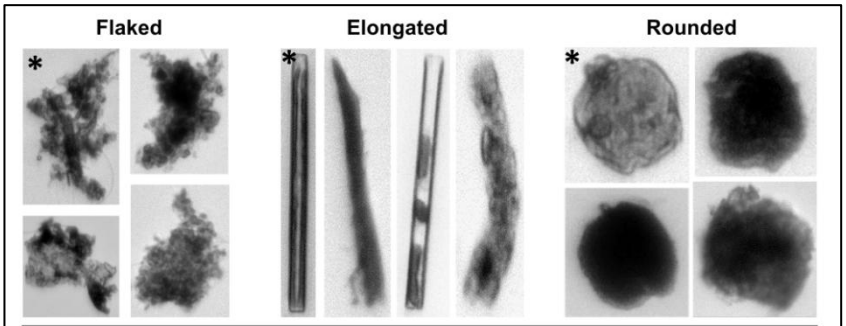


Figure 3.4: Light microscopy pictures of particles from the different aquaculture systems on filters (0.45 µm). Samples were pre-filtered using 300 µm gauze. Measuring bar represents 500 µm.

The Particle Insight Size and Shape Analyzer was used to characterize particles and build a detailed and realistic overview of their shapes. In total, between 10 936 and 77 684 particles were analyzed per system, and the typical shapes and related measurements are exemplified in Figure 3.5. The majority of particles had a flake-like form, mostly consisting of agglomerations of smaller particles, while rounded and elongated particles occurred rather infrequently. An exception was sRAS2, in which significant numbers of elongated particles were observed, mostly of algal origin (see also Figure 3.4 and Figure 3.7). Shape analysis can also be useful in deriving an overview of biological components suspended in aquacultural water samples as shown in Figure 3.6, but particles of this nature were recorded rarely during the current investigation and are thus not listed separately.



	Flaked	Elongated	Rounded
ECAD (µm)	227.4	56.0	153.5
Circularity	0.351	0.096	0.800
Ellipsicity	0.555	0.652	0.900
EEAW (µm)	183.3	22.6	147.1
EEAL (µm)	298.0	146.6	169.4
EAR	1.71	7.37	1.22
Feret length (µm)	399.0	188.3	178.5
Feret width (µm)	245.5	25.3	154.4
Feret AR	1.62	7.44	1.16

Figure 3.5: Overview of typical particle shapes found in the investigated aquaculture systems and associated shape measurements. ECAD = equivalent circular area diameter, EEAW = equivalent elliptical area width, EEAL = equivalent elliptical area length, EAR = ellipse aspect ratio. '*' indicates that shape measurements of these particles are listed as examples in the table. Images are not to scale.

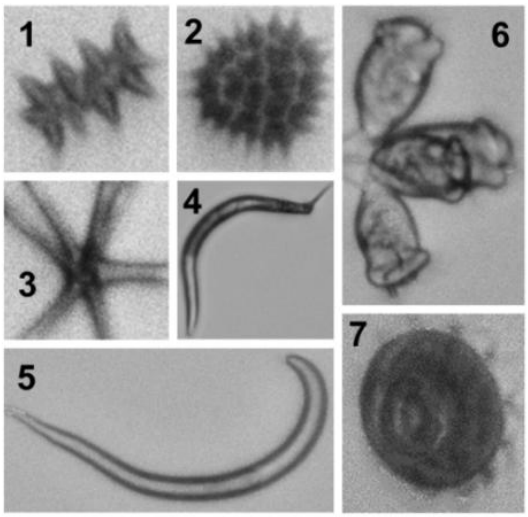


Figure 3.6: Biological components found in water samples during shape analysis: 1-3: algae, 4-5: nematodes, 6: ciliates, 7: *Trichodina* sp. (not to scale).

Circularity

Mean circularity of suspended particles did not differ significantly ($P > 0.05$) between system types and was close to 0.5 in all systems (Table 3.2). However, mean circularity of particles was lower in sRAS2 (0.475 ± 0.004) compared to the other systems due to an increased occurrence of particles with low circularity values of 0.1 to 0.4 and respectively lower proportions between 0.4 and 0.8 (Figure 3.7 A). A closer examination of these samples showed a relative high abundance of elongated algal particles. When these particles were excluded (exclusion criteria: circularity < 0.4 and feret aspect ratio > 2; data not shown), the circularity of particles in sRAS2 fell in line with that of the other systems.

Ellipticity

Mean ellipticity of particles differed significantly ($P < 0.05$) between system types, however, post-hoc comparison was not sensitive enough ($P > 0.05$) to identify the systems to which these differences belonged. Overall, the differences were small (Figure 3.7 B), and mean ellipticity was about 0.7 (Table 3.2). Furthermore, there was no statistically significant difference ($P > 0.05$) in the ellipse aspect ratio of particles between system types.

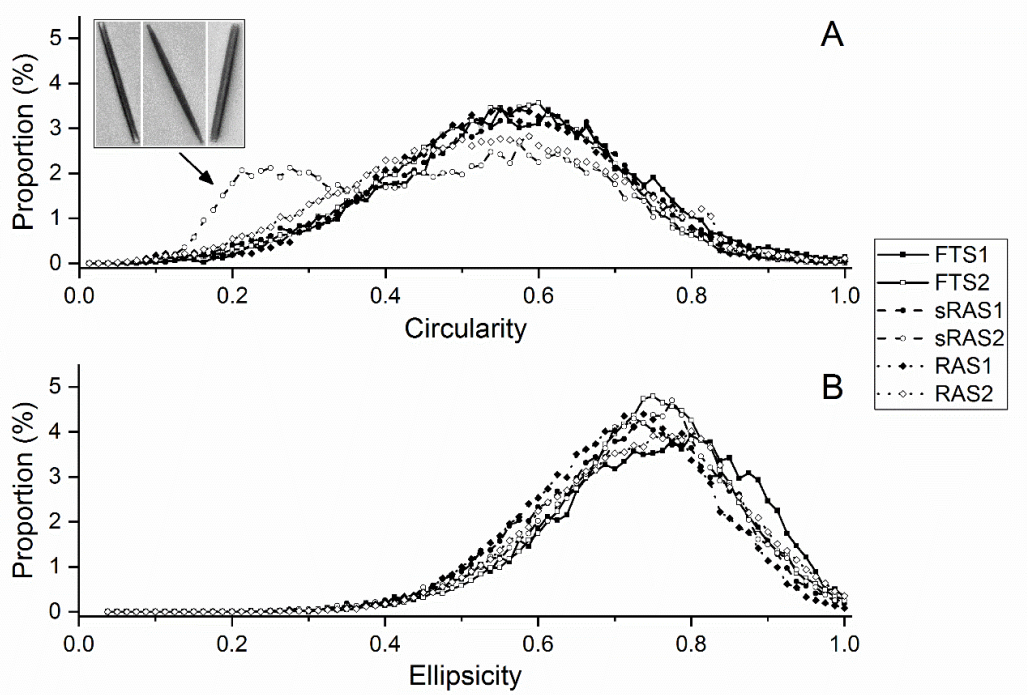


Figure 3.7: Distributions of circularity (A) and ellipticity (B) of particles in the investigated systems.

Table 3.2: Shape measurements of particles (± S.D.) found in the investigated systems.

	ECAD (µm)			Circularity		
	Mean	Skewness	Kurtosis	Mean	Skewness	Kurtosis
FTS1	12.7 ± 1.9	5.22 ± 0.75	40.94 ± 10.94	0.563 ± 0.002	-0.06 ± 0.00	-0.16 ± 0.02
FTS2	10.6 ± 0.4	5.04 ± 0.00	40.55 ± 1.91	0.536 ± 0.008	-0.26 ± 0.04	-0.04 ± 0.03
sRAS1	8.9 ± 0.1	7.29 ± 0.21	100.65 ± 10.29	0.548 ± 0.004	-0.22 ± 0.04	-0.13 ± 0.07
sRAS2	9.1 ± 0.2	4.90 ± 0.80	45.93 ± 16.50	0.475 ± 0.004	-0.02 ± 0.04	-0.92 ± 0.02
RAS1	15.0 ± 0.7	4.12 ± 0.07	24.57 ± 1.01	0.536 ± 0.008	-0.30 ± 0.04	0.14 ± 0.01
RAS2	12.5 ± 0.2	3.19 ± 0.14	16.19 ± 1.98	0.526 ± 0.004	-0.06 ± 0.01	-0.44 ± 0.01
	Ellipticity			EAR		
	Mean	Skewness	Kurtosis	Mean	Skewness	Kurtosis
FTS1	0.739 ± 0.003	-0.48 ± 0.05	0.04 ± 0.09	1.947 ± 0.089	3.14 ± 0.39	20.56 ± 6.65
FTS2	0.730 ± 0.003	-0.45 ± 0.02	0.49 ± 0.01	1.569 ± 0.015	4.37 ± 0.13	35.94 ± 1.69
sRAS1	0.711 ± 0.001	-0.35 ± 0.06	-0.05 ± 0.10	1.968 ± 0.004	3.06 ± 0.03	19.82 ± 0.16
sRAS2	0.720 ± 0.001	-0.49 ± 0.03	0.39 ± 0.16	1.767 ± 0.015	2.61 ± 0.08	9.52 ± 1.20
RAS1	0.699 ± 0.004	-0.31 ± 0.02	0.08 ± 0.05	1.936 ± 0.001	3.23 ± 0.15	20.30 ± 1.58
RAS2	0.727 ± 0.005	-0.31 ± 0.02	-0.06 ± 0.03	1.790 ± 0.009	3.75 ± 0.22	28.51 ± 5.96
	EEAL (µm)			EEAW (µm)		
	Mean	Skewness	Kurtosis	Mean	Skewness	Kurtosis
FTS1	15.55 ± 2.20	5.88 ± 0.47	53.74 ± 7.66	10.94 ± 1.77	4.80 ± 0.73	34.20 ± 9.06
FTS2	12.98 ± 0.56	5.20 ± 0.15	41.71 ± 1.38	9.25 ± 0.29	5.11 ± 0.13	43.51 ± 5.44
sRAS1	10.67 ± 0.11	5.06 ± 0.16	46.33 ± 7.07	7.51 ± 0.10	4.05 ± 0.05	23.50 ± 0.77
sRAS2	12.03 ± 0.43	4.76 ± 0.47	38.06 ± 6.17	7.55 ± 0.14	5.54 ± 1.16	61.37 ± 28.65
RAS1	18.62 ± 0.89	4.97 ± 0.18	36.35 ± 3.09	13.06 ± 0.56	4.03 ± 0.03	24.36 ± 0.44
RAS2	15.93 ± 0.26	3.61 ± 0.31	20.48 ± 4.27	10.50 ± 0.19	3.13 ± 0.07	16.49 ± 1.13
	Feret width (µm)			Feret length (µm)		
	Mean	Skewness	Kurtosis	Mean	Skewness	Kurtosis
FTS1	12.3 ± 2.0	4.95 ± 0.65	36.61 ± 6.99	18.6 ± 2.7	6.07 ± 0.42	58.37 ± 7.13
FTS2	10.9 ± 0.4	5.47 ± 0.08	46.80 ± 4.41	15.8 ± 0.7	5.52 ± 0.09	45.56 ± 0.19
sRAS1	8.6 ± 0.2	6.92 ± 0.20	91.99 ± 10.84	13.3 ± 0.2	9.53 ± 1.15	201.03 ± 61.06
sRAS2	8.8 ± 0.2	6.12 ± 1.20	71.34 ± 27.47	14.7 ± 0.6	5.10 ± 0.29	43.51 ± 2.62
RAS1	15.1 ± 0.6	4.02 ± 0.03	23.94 ± 0.45	22.6 ± 1.1	4.97 ± 0.19	35.78 ± 3.08
RAS2	12.2 ± 0.3	3.60 ± 0.11	22.11 ± 1.56	19.3 ± 0.4	3.99 ± 0.28	24.86 ± 3.77
	Feret aspect ratio					
	Mean	Skewness	Kurtosis			
FTS1	1.85 ± 0.09	3.27 ± 0.21	23.57 ± 2.39			
FTS2	1.49 ± 0.01	4.41 ± 0.18	36.50 ± 2.44			
sRAS1	1.87 ± 0.001	3.06 ± 0.03	19.82 ± 0.16			
sRAS2	1.68 ± 0.01	2.62 ± 0.09	9.52 ± 1.26			
RAS1	1.84 ± 0.004	3.23 ± 0.15	20.32 ± 1.61			
RAS2	1.70 ± 0.01	3.76 ± 0.23	28.55 ± 6.03			

Volume and surface area calculations: spheres vs. ellipsoids

The results described above prompted an inspection of the shape assumptions used in calculations of total solid volume and surface area, and specifically of the effects of assuming an ellipsoidal shape versus a classical spherical form. To this end, sRAS2 was selected as an example because of the increased occurrence of elongated (and thus non-spherical) particles.

The assumption of an ellipsoidal shape resulted in a 1.4-fold increase in the value achieved for total volume of measured particles per liter in sRAS2 (0.017 mm^3 L^{-1} vs. 0.012 mm^3 L^{-1}) and a 5.8-fold increase in the calculated total surface area of particles per liter (10.607 mm^2 L^{-1} vs. 1.831 mm^2 L^{-1}), compared to the spherical assumption. Calculations of both solid volume and surface area based on an ellipsoidal shape also show higher proportions of solids contributed by smaller particle size classes than those based on a spherical shape (Figure 3.8). Thus, applying a theoretical 40 µm filter gauze in the mechanical treatment in sRAS2, the difference in volume and surface area percentages would be 8.1% and 10.0% respectively.

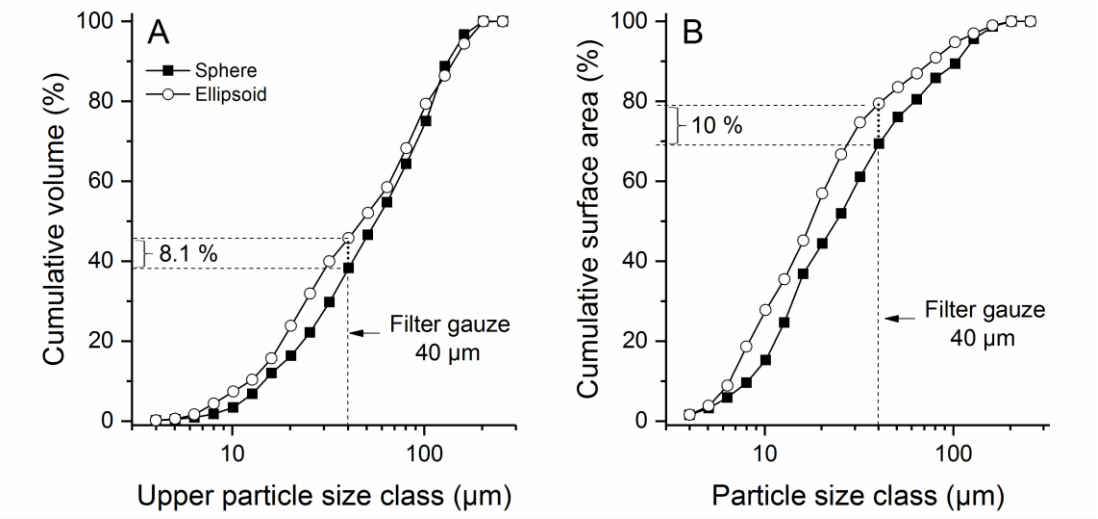


Figure 3.8: Differences in cumulative volume (A) and surface area (B) percentages of particles in sRAS2 between calculations based on assumptions of spherical and ellipsoidal shape. Horizontal dashed lines show differences between calculations at 40 µm (filter gauze size).

The theoretical removal efficiency of a 100 µm drum filter in RAS2 based on cumulative volume percentages using spherical and ellipsoidal shape assumptions are shown in Figure 3.9. The dry weight of TSS was 4.83 ± 0.04 mg L^{-1} before the drum filter and 2.26 ± 0.13 mg L^{-1} after. The calculated RRE of the drum filter with a 100 µm filter gauze was about 53.2 %. The theoretical removal efficiencies predicted using volume percentages based on spherical and ellipsoidal shape were 34.7 % and 36.7 % respectively (Figure 3.9) and both differed significantly ($P < 0.05$) from the calculated RRE assuming that volume equals mass. There was no significant difference ($P > 0.05$) between the removal efficiencies predicted using spherical and ellipsoidal shape assumptions.

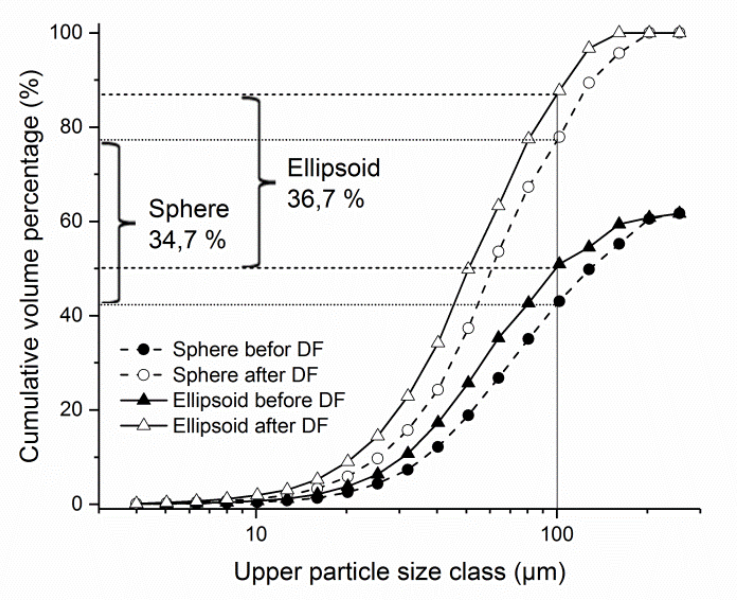


Figure 3.9: Cumulative volume percentages within size and shape classes of particles in RAS2 before and after drum filter (DF, 100 µm gauze). Dry weight of TSS was 4.83 ± 0.04 mg L^{-1} before DF and 2.26 ± 0.13 mg L^{-1} after DF. Brackets inside horizontal dashed lines reveal predicted removal efficiencies based on ellipsoidal and spherical shape assumption.

Particle number, volume and surface area by size class

The percentages of total particle numbers, total particle volume and total particle surface area contributing to each particle size class were determined based on an assumption of ellipsoidal shape (Figure 3.10). The results show no differentiation between system types (FTS, sRAS, RAS), but it should be noted that volume and surface area contributions decreased for the upper two particle size classes in all systems.

In terms of quantity, the majority of particles in all systems (from 89.0% in RAS1 to 99.4% in sRAS2) belonged to size classes < 40 µm (EEAW). Furthermore, particles between 10 µm and 100 µm (EEAW) were considerably more prevalent in both RAS than in the other systems. Meanwhile, in contrast to the other systems, sRAS2 showed a peak of particle numbers between 6 and 8 µm.

In terms of total solid volume, particles smaller than 10 µm (EEAW) were of minor importance, contributing 0.1 to 1.6% of volume in all systems except sRAS2, where the percentage was somewhat higher, at 9.1%. Systems FTS1, FTS2, sRAS1 and RAS1 were dominated by large particles (50 – 300 µm, EEAW) accounting for between 77.4% and 88.1% of particle volume. In sRAS2, solid volume was distributed more evenly between particle size classes from 20 to 100 µm (EEAW), while in RAS2, values peaked between 50 and 100 µm (EEAW) then declined with increasing particle size.

Surface area contributions were distributed relatively evenly between all size classes in sRAS1, whereas in sRAS2, 77% of total particle surface area was contributed by particle size classes between 6 and 40 µm (EEAW). In FTS1 and RAS1, the highest contributions of surface area came from particles between 40 and 200 µm (EEAW). In contrast, particles between 20 and 100 µm (EEAW) accounted for over 66% of total surface area in FTS2. In RAS2, particles between 10 and 100 µm contributed 84% of total surface area, with the greatest contribution coming from particles of around 40 µm (EEAW).

Linear regression analysis of $\log_{10}$-$\log_{10}$ fitted particle distributions (Figure 3.11) showed coefficient of determination (r^2) between 0.920 (RAS2) and 0.979 (sRAS2). With the exception of FTS1, all systems exhibited a slope near to 3, indicating a predominance of small particles in terms of total number, with large particles dominating the volume distribution and surface area contributed more or less equally

by all size classes. The slope of the linear regression analysis of FTS1 was lower than that of the other systems, pointing to domination of surface area and volume by larger particles and a smaller impact of fine particles on overall particle number than in the other systems.

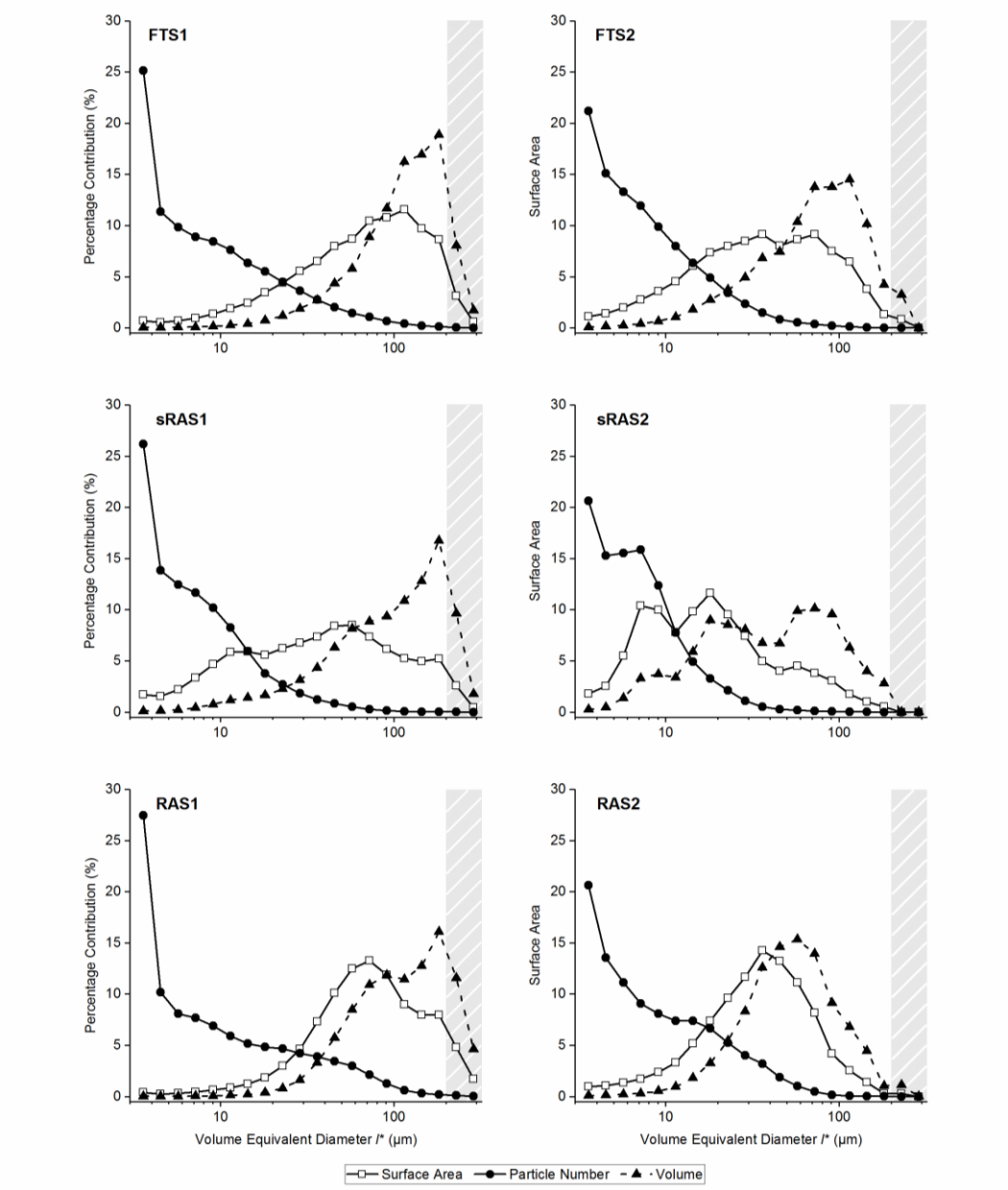


Figure 3.10: Percentage contributions of different particle size classes to surface area, particle number and volume of solids found in the different aquaculture systems, based on an ellipsoidal shape assumption. Lines between data points are only used for highlighting and should not be seen a link between size classes. Shaded area shows size range where particles are probably underrepresented due to filtration effects (300 µm gauze).

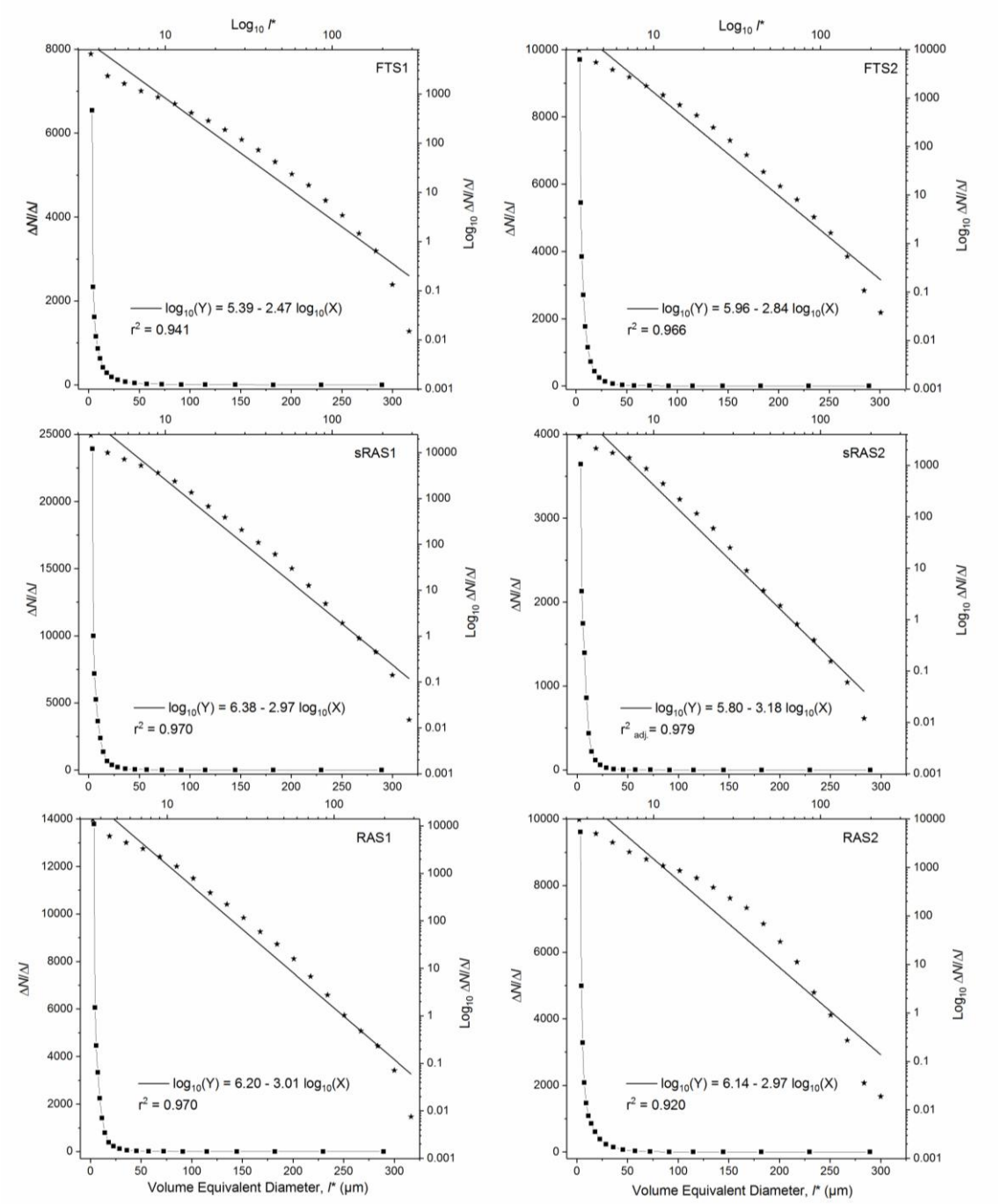


Figure 3.11: Bivariate plot of particle size distribution ($\Delta N/\Delta l$, which is the number of counts per class divided by the range of the class) versus volume equivalent diameter, l^* (median of class), (left, bottom) and its $\log_{10}$-$\log_{10}$-linear regression (right, top) for suspended solids of the different aquaculture systems according to Patterson et al. (1999).

Feret diameter

The feret widths of particles differed significantly between system types ($P < 0.05$). However, post-hoc comparisons were not sensitive enough ($P > 0.05$) to reveal which of the systems was different. Skewness of distribution was significantly higher in sRAS than in FTS ($P < 0.05$) or RAS ($P < 0.001$) and skewness of distribution was significantly lower ($P < 0.05$) in RAS than in FTS. Furthermore, the distribution of feret width in sRAS showed significantly higher kurtosis than that in FTS ($P < 0.01$) and RAS ($P < 0.001$).

The feret length of particles was significantly greater ($P < 0.01$ and $P < 0.05$) in RAS (21.0 ± 2.0 µm) than in sRAS (14.0 ± 0.9 µm) or FTS (17.2 ± 2.3 µm). There was no statistically significant difference ($P > 0.05$) between FTS and sRAS. Skewness and kurtosis of distribution differed significantly between system types ($P < 0.05$), but again, post-hoc comparison was not sufficiently powerful ($P > 0.05$) to reveal which of the systems was significantly different.

Feret aspect ratio (1.67 to 1.77) did not differ significantly between system types ($P > 0.05$). A statistically significant difference in skewness was observed between system types, but post-hoc comparison was not sensitive enough ($P > 0.05$) to reveal which of the systems was different. Furthermore, the distribution of feret AR in FTS showed significantly greater ($P < 0.05$) kurtosis than that in sRAS. The results of feret diameter measurements for each aquaculture system are shown in Table 3.2.

Impact of mechanical treatment on particle shape in sRAS1 and RAS2

Shape measurements from samples before and after the drum filters in sRAS1 and RAS2 are presented in the following. In sRAS1, circularity, ellipticity and EAR of particles did not differ significantly ($P > 0.05$) between sample points. The feret width of particles was significantly higher ($P < 0.05$) before filtration (8.55 ± 0.16 µm) than afterwards (8.01 ± 0.03 µm). Furthermore, skewness and kurtosis of feret width data was significantly higher ($P < 0.01$ and $P < 0.05$) before the filter than after. Feret length of particles was also significantly higher ($P < 0.05$) before filtration (13.25 ± 0.17 µm) than after (12.55 ± 0.01 µm), with a significantly larger skewness ($P < 0.05$) after the drum filter. However, feret AR did not differ significantly ($P > 0.05$) between sample points. In RAS2, none of the investigated shape factors differed significantly ($P > 0.05$)

between sample points, but kurtosis of data of feret width was significantly higher ($P < 0.05$) before the drum filter than after.

3.5 Discussion

The present study is a first attempt to compare the shape characteristics of suspended solids in three types of aquaculture systems with different recirculation intensity. Using digital image analysis, it expands previously limited knowledge on particle properties in aquaculture systems (Patterson and Watts, 2003a). The results reveal that the shape of particles was highly similar between the different production types, with the majority of suspended solids having a flake-like form. Contrary to previous assumptions, the present findings also suggest that calculations of particle volume and surface area should be based on an assumption of ellipsoidal shape rather than sphericity – a discovery with wide-ranging implications for the assessment of suspended solids in aquaculture systems. Furthermore, the present work suggests that while the accumulation of fines may be less relevant in RAS than previously thought, they are more important in sRAS.

The digital image analysis used here is a non-invasive approach that allows a representative and in-depth evaluation of particle size and shape factors. The advantage of this method is that large numbers of particles can be photographed while suspended within a flowing fluid, reducing the interference of possible artefacts such as changes due to drying processes or adhesion to surfaces. Furthermore, particle shape can be automatically analyzed using different defined shape factors (Blott and Pye, 2008; Hentschel and Page, 2003; Mamane et al., 2008) so that results are highly representative and observer bias can be excluded.

In contrast, while the microscopic examination of particles collected on cellulose-acetate filters allowed an overview of particles occurring in the three main salmonid aquaculture systems, it also showed the limitations of this method. First of all, microscopy is invasive, as it requires fixation of samples, e.g. on filters or in small volumes of liquid where particles adhering to surfaces may alter their shape (Mulisch and Welsch, 2015; Patterson and Watts, 2003a). Furthermore, microscopic data

analysis is labor-intensive, and the small numbers of samples that can reasonably be processed are unlikely to be statistically representative, given the extreme size and shape ranges occurring in aquaculture operations. In addition, there is a risk of observer bias (Mihlbachler et al., 2012) and true randomization is difficult to achieve (Mead et al., 2002). Finally, there is a risk that in the use of altered or fixed particle samples for microscopy, relevant special particle forms such as the biological components detected by digital image analysis in the present study, may not be discovered.

In the present study, the majority of particles recorded displayed a flake-like form and seemed to comprise dense agglomerations of smaller primary particles, regardless of system type. Patterson and Watts (2003a) also observed these kinds of particles in water from a commercial Atlantic salmon smolt production facility, but the flakes in that context seemed to be more fragile in structure. In sewage plants and industrial wastewater effluent treatments, flocculation is induced artificially to improve removal efficiency (e.g. Aguilar et al., 2003; Thomas and Moore, 2004), but this technique is rarely used in fish farms. In general, suspended flocs can play an important role within any aquatic system as they act as individual microecosystems with their own distinct physical, chemical and/or biological characteristics (Droppo et al., 1997). It has previously been shown that biofloc technology may even enhance water quality in aquaculture systems (Azim and Little, 2008; Crab et al., 2012).

Besides flake-like particles, Patterson and Watts (2003a) observed high proportions of ‘long spicule-like’ and ‘sand-like’ particles during their study. However, with the exception of sRAS2, these particle types were encountered only sporadically in the current investigation. Particles in aquacultural systems can be generally differentiated as organic (mainly smooth) (Unger and Brinker, 2013) or inorganic (mainly sharp and heavy) in origin. Special groups include biological material such as parasites, algae and other microorganisms, which often have a rather elongated shape. In case of sRAS, the elongated, rod-shaped particles observed in abundance were likely to be fragments of algae whose growth was presumably caused by a lack of shade cover over the fish ponds and the supply of water partly from a natural lake.

While the feeds used in different study systems were not all sourced from the same manufacturer, they were of similar composition, at least when it came to ingredients that impact on stability. Given the importance of feed composition on the stability of resulting fecal material (Brinker et al., 2005a; Schumann et al., 2018; Unger and Brinker, 2013), the highly convergent particle shapes observed between different investigated production types (FTS, sRAS, RAS) in the present study were largely to be expected. The dominance of this effect may partly explain the apparent lack of clear shape patterns between systems with differing degrees of water recirculation. However the shape of particles found in the present study partly did differ somewhat from those found in water from a commercial Atlantic salmon smolt production facility in 2003 (Patterson and Watts, 2003a). This is likely a result of developments in feed formulation in the intervening years. In the earlier study, the main source of particles was assumed to be wheat-related heavy cellulose content, the reduced inclusion of which in the present study could explain differences in particle shape.

The digital image analysis carried out in the present study identifies that suspended solids occurring in aquaculture systems typically exhibit an elliptical rather than round shape, with, for example, average feret aspect ratios of 1.49-1.85, regardless of production system type. This also seems to be a general rule in wastewaters, with comparable results obtained for biofilter effluent samples from a water treatment plant (Zahid and Ganczarczyk 1990).

These findings have wide-ranging implications for assessing the qualitative and quantitative impacts of suspended solids in aquaculture systems, where use of an ellipsoidal shape in modeling and other calculations is likely to result in a more realistic outcomes than the spherical shape which has guided much of the past modeling and the design of aquaculture systems (e.g. Brinker et al., 2005b; Fernandes et al., 2014; Patterson et al., 1999). The present results suggest that these previous assumptions may have led to significant underestimates of total particle volumes and surface areas, especially with regard to fine solids. The implications for system design are exemplified here for sRAS2, where removal efficiency predictions for the mechanical treatment steps display biases of 8 % and 10 % for volume and surface area calculations respectively when making an assumption of spherical shape. These findings suggest

that existing models for estimating the volume and surface area of particles in aquaculture systems should be adapted.

The surface area of particles is an especially pertinent metric in aquaculture systems, because of the associated potential for leaching of chemical contaminants (Chen et al., 2003; Ling and Chen, 2005) and bacterial colonization (Becke et al., 2019; Berger et al., 1996; Pedersen et al., 2017). Combined with knowledge gained about the mostly flake-like shape of particles in all investigated systems, optimized calculations based on assumptions of ellipsoidal shape will also enable more reliable assessments of both leaching processes and bacterial activity.

The present study shows that the solid load in RAS is dominated by particles in the size range 30 – 100 µm (EEAW). This result contradicts the widely held view that fine particles (< 20 µm) are of utmost importance in RAS (Chen et al., 1993; Davidson et al., 2009; Patterson et al., 1999). Based on the current results, the use of a 40 µm filter gauze in the RAS would increase removal efficiency 37-53 % by volume and 37-48 % by surface area. This is contradicting Fernandes et al. (2015) who measured the effects of different filter mesh sizes within the range 20-100 µm on particle load and found that similar steady state levels were reached after a time period of 6 weeks with no significant difference between the different mesh sizes.

The current results suggest that it is actually in the investigated sRAS that fine solids are of greatest importance, with regard to both surface area and volume. The low proportions of TSS contributed by larger particles chimes with the findings of Heinen et al. (1996), who determined that 96 % of TSS in a semiclosed recirculating system for rainbow trout was contributed by particles smaller than 40 µm. Presumably, the removal of large particles by mechanical filtration and shearing resulting from increased fish activity at high stocking densities prevent the accumulation of large particles in these systems (Brinker and Rösch, 2005). Thus, more emphasis should be given to the removal of fine solids in sRAS.

Notwithstanding the above, the observed decline of the two largest particle size classes in all systems in the present study is thought to be a bias caused by filtration with the 300 µm gauze and the use of EEAW (the smallest dimension of an ellipsoid) to assign size class, leading to underrepresentation in the largest two size classes.

Patterson et al. (1999) reviewed data from 11 aquaculture systems (almost all of which were RAS) indicating that fines dominated the solid load not only in terms of total particle number but also in surface area and volume, in all systems. This was reflected in slope values of the $\log_{10}$-$\log_{10}$ fitted particle size distributions greater than 4. In the present study, slopes of those $\log_{10}$-$\log_{10}$ regressions were approximately 3 in all systems, indicating much reduced emphasis on the smaller size classes. A likely explanation for the difference between this and earlier studies is the development of feeds used in salmonid production in recent decades. Feed formulations have been optimized for fecal stability, e.g. by adding guar gum (Brinker, 2007; Brinker and Friedrich, 2012), so that the potential for formation of fine particles in the system water is considerably reduced. Furthermore, biofilters may play an important role with regards to the occurrence of small particles. Fernandes *et al.* (2017) highlighted performance differences between fixed and moving bed biofilters: while the former removed fine solids, the latter tended to generate small particles, e.g. by grinding biofilm. In this respect, the different biological treatment technologies used in the investigated sRAS and RAS may have affected the concentrations and shapes of particles which could also explain the small differences in the particle size distributions. Due to the high turbulences induced by permanent aeration and mixing of moving bed biofilters, larger particles and excess biofilm may be shopped into smaller pieces and thus may lead to an accumulation of small particles (Fernandes et al., 2017; Rusten et al., 2006), whereas the use of fixed bed biofilters may lead to formation of larger particle aggregates due to low turbulences. Furthermore, it was assumed that the high turbulences in moving bed biofilters may lead to more frequent collisions of particles and thus higher circularity of particles, but the results of this study did not reveal any proof for this assumption. However, suspended solids, and especially small particles, may also be degraded by heterotrophic bacteria (Blancheton et al., 2013; Michaud et al., 2006). This means a biofilter providing relevant habitat for heterotrophs also may contribute to the removal of fine solids by bacterial action. The TSS concentrations of the investigated aquaculture systems revealed a significant negative correlation with stocking densities, indicating that increased fish densities could have an impact on particle size (Brinker and Rösch, 2005), but did not have

strong influence on TSS concentrations if an adequate solid treatment or fresh water supply is given. The high proportion of particles > 300 µm in FTS1 shows the high self-cleaning potential of this system despite a stocking density of 60 kg/m³.

In general, the removal of particles depends on the one hand on their size, and on the other hand on their shape, as the less circular the particles, the greater the chances of them being removed by filtration (Mamane et al., 2008). Thus, mechanical filtration should result in an increase in the proportion of circular particles after the filter. However, in this study, with the exception of a decrease in feret diameter, the shape of particles was not affected by mechanical treatment. A reason for this could be the development of filter cakes on the mechanical screens (Dolan et al., 2013), which reduce the importance of shape as a factor in the passage of particles by modifying the pores from two-dimensional openings into more three-dimensional network. This possibility has yet to be further investigated, but it may also explain the difference between the calculated and actual removal efficiencies presented for RAS2. As shown by Wakeman (2007), the specific resistance of particles in a filter cake increases, in one example by a factor of almost 690, with a change in shape from fiber to flake, allowing much finer material to be extracted from the system. It may be that the mainly flake-like shape of particles in all investigated aquaculture systems is a highly significant factor influencing system performance.

3.6 Conclusions

This study provides an overview of the particle shapes occurring in three main types of salmonid production system. Against expectations, the shape of particles was highly consistent between the different production types, presumably due to similarities in the feeds and treatment technologies applied. Nevertheless, the shape of particles in aquaculture systems can be affected by additional particle sources or divergent environmental factors, as seen in the abundance of elongated particles (algae) observed in one of the semi-recirculating systems studied. Furthermore, the results showed that the total volume and total surface area of suspended solids occurring in aquaculture systems should be calculated based on an assumption of ellipsoidal

particle shape rather than the classical sphere used previously. Finally, the prevalence of fine solids seems to be less than previously thought in recirculating systems, but considerably greater in semi-recirculating systems.

Thus, the main conclusions are:

- shape of particles is not determined by production type
- volume and surface area of particles should be calculated based on an assumption of ellipsoidal, rather than spherical shape
- treatment design and modeling of aquaculture systems should be adjusted in line with these new findings
- the relevance of particle size classes for aquaculture production systems should be re-evaluated using calculations based on ellipsoidal shape
- the flake-like shape of particles in aquaculture systems seems to be highly important and thus requires further investigation

4 Physiological consequences for rainbow trout (*Oncorhynchus mykiss*) of short-term exposure to increased suspended solid load

A similar version of this chapter was published as:

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4.1 Abstract

Suspended solids are an unavoidable component of fish farming, and especially so in recirculating aquaculture systems (RAS) where fine particles accumulate over time. High levels of fine particles are widely regarded as harmful to fish health and welfare. However, little is known about the direct impact of these accumulating particles on stress, performance, health or other welfare parameters of fish. In this study, the effects of solids on rainbow trout were investigated specifically, uncoupled from other potentially confounding water parameters. To this end, the suspended solid load in a replicate RAS was artificially increased by a factor of 7 to over 30 mg total suspended solids (TSS) L^{-1}, alongside a sister system operating normally, with a maximum suspended solid concentration of 5 mg L^{-1}. At these levels, the concentrations of particles smaller than 32 µm amounted to 0.3 ± 0.3 mg L^{-1} in the control system and to 8.0 ± 2.7 mg L^{-1} in the treatment system. With the exception of turbidity, all further water parameters generally considered important to salmonid welfare were kept comparable and well below harmful levels. In consequence, fish performed well in both RAS, indicating good husbandry conditions. Feeding behavior was observed to differ slightly between control and treatment RAS, but without any apparent effect on performance.

The impact of the accumulating particles on fish was examined using a wide range of physiological assays. No significant differences in stress markers (heat shock protein 70, plasma cortisol) were detected between fish in the control and treatment RAS. The same was found for hematological assays (differential leukocyte count, hematocrit, RBC indices, etc.). Fin condition was also unaffected by increased suspended solid load

and most surprisingly, histological examination not only revealed no detrimental effects of particle accumulation, but showed the gill status of fish in the solid treatment to be better than that of control fish.

Overall, this study shows that by itself, short-term exposure to suspended solids at concentrations of 30 mg L^{-1} has no detrimental effect on rainbow trout.

Candidate's contribution:

Implementation of particle accumulation procedure, husbandry of fish, sampling of blood and tissue samples, execution of physiological assays (except gill histology) and particle measurements, statistical assessment and interpretation of data, writing and revision of the complete manuscript including all figures and tables.

4.2 Introduction

Recirculating aquaculture systems (RAS) are often regarded as an environmentally friendly alternative to flow-through systems (Klinger and Naylor, 2012); Verdegem et al., 2006; (Ayer and Tyedmers, 2009). Set against a background in which global fish stocks are suffering drastic declines and water is increasingly a limiting resource, RAS technology represents an interesting alternative for sustainable, location-independent fish production. However, the relatively energy-intensive nature of RAS fish production compared to flow-through systems often leads to a comparatively poor rating in ecological assessment (d'Orbcastel et al., 2009a). Further challenges to the economic viability of RAS include high investment costs and factors reducing system stability, such as imbalances in water parameters, high bacterial load etc. (Badiola et al., 2012). The intensification of production by increasing stocking densities is one means of reducing energy costs per unit (Martins et al., 2005), but high stocking densities require optimal water quality conditions and system stability in order to ensure an economically competitive and animal-friendly fish production. Furthermore, increasing fish biomass means increasing the quantity of feed used, which in turn implies an increase in organic load (Pedersen et al., 2012).

Suspended solid load is a principle cause of facility malfunctions in RAS (Badiola et al., 2012). Within fish culture systems, suspended solids originate mainly from feces and to a lesser extent from uneaten food, bacterial material from biofilters and from microfauna (Chen and Malone, 1991; Noble and Summerfelt, 1996; Summerfelt et al., 1999; Wedemeyer, 1996). A decisive factor for the accumulation of suspended solids in recirculating aquaculture water is the stability of fecal material, which impacts directly on efficiency of mechanical cleaning systems. This texture is substantially influenced by feed composition (Brinker et al., 2005a, Unger and Brinker, 2013). Suspended solid production in aquacultural systems typically fall in the range of 30 to 60 % of feed supplied (Chen and Malone, 1991).

Large particles are removed from RAS by mechanical filtration (e.g. drum filter), but with most RAS deploying filter gauzes with mesh sizes greater than 30 µm, there is a tendency for smaller particles to remain in the system and accumulate over time (Chen et al., 1993; Davidson et al., 2009; Patterson et al., 1999). Chen et al. (1993) showed that more than 95 % of suspended solids in RAS had a diameter of less than 20 µm.

High levels of particles, especially fine particles around 5-10 µm are widely regarded as harmful to fish health and welfare (Chapman et al., 1987; Chen and Malone, 1991). For example, suspended sediment particles have been shown to cause damage to gill structure (Au et al., 2004; Bash et al., 2001; Bilotta and Brazier, 2008; Bruton, 1985; Chapman et al., 1987; Humborstad et al., 2006; Wong et al., 2013), induce behavioral changes (Robertson et al., 2007), impair larval development (Martins et al., 2009) and elevate stress (Awata et al., 2011; Lake and Hinch, 1999; Sutherland et al., 2008). Beside these direct effects, increased particle load can also indirectly impair fish welfare in RAS by worsening water quality. Excessive solid matter can clog biofilters and reduce the surface area for necessary biofilms on the carrier material (Ling and Chen, 2005) or increase the levels of harmful substances (e.g. ammonia, nitrite) leaching into the water (Chen et al., 2003).

Despite these previous investigations, little or nothing is known about the direct impact of accumulating system-related particles in RAS on fish health. There is no clear estimate of the concentration at which particles become harmful to fish, nor is there any understanding of the sizes or shape of particles that might be most detrimental.

General recommendations for safe upper limits for particle load range as widely as 25 mg L^{-1} (Alabaster and Lloyd, 1982; Timmons and Ebeling, 2010) to 80 mg L^{-1} (Wedemeyer, 1996), but the actual effects of particles in RAS have not previously been investigated. In particular, it is important to differentiate between specific effects of elevated solid load and any confounding or interaction effects that may accompany load increases, such as increased total ammonia nitrogen (TAN) levels (Chen et al., 2003). There is an urgent need for better understanding of the actual impact of suspended particles on fish health and system stability. The present study investigates size-controlled effects of accumulating particles on stress, performance and health of rainbow trout without the interference of potentially confounding or debilitating water parameters.

4.3 Materials and methods

Husbandry

In a four-week exposure trial, the effects of increased particle load on fish health were investigated. The experiment used two replicate RAS, each comprising 10 green circular fiberglass tanks, each with a capacity of 330 L. The study was performed with all-female rainbow trout (Störk strain) to exclude gender-related effects. About 650 rainbow trout were held in each system with maximum stocking densities of about 54 ± 3 kg m^{-3} (control) and 52 ± 4 kg m^{-3} (treatment). The control system was operated under regular conditions, while the particle load of the other system was artificially increased by collecting backwash water from the drum filter into a tank and re-injecting it at regular intervals into the water buffer of the system (Figure 4.1) using a mud pump (Wilo-EMU KS 8 ES, Dortmund, Germany, data see Table 4.1). Under this process, larger particles were fragmented by shear forces (McMillan et al., 2003). The rate of flow of backwash water into the system was kept constant by the flow out of the biofilter. In both systems, the drum filter (HDF801-1H, Hydrotech, Vellinge, Sweden) was equipped with 100 µm gauze. The photoperiod was fixed at 12L:12D (Lumilux daylight lamps provided around 140 lx at the water surface between 0700 h and 1900 h) with a sigmoidal transition period of 30 min. The fish were fed restrictively

6 days a week (Sunday to Friday) with a commercial feed (Biomar EFICO Enviro 921, Aarhus C, Denmark; diet composition: see Table 4.2) at 1.4 % of bodyweight at the beginning of the trial, declining to 1.1 % by the end. Bacterial growth was controlled with UV irradiation of the system water (Barrier L20, Wallace & Tiernan, Günzburg, Germany; UV dose: 40 mJ cm^{-2} flow volume: 6600 L h^{-1}, lamp wattage: 80 W, measurement range UV sensor: 200 W m^{-2}).

The most important precondition of this study was the isolation of particle accumulation effects from other potentially harmful factors. Water parameters (Table 4.3) were kept comparable between the two systems at levels deemed to have no impact on fish health or performance. pH was adjusted by adding sodium hydrogen carbonate. Oxygen concentration and temperature were monitored continuously at the outlet of two tanks in each system. Further water parameters were determined thrice weekly throughout the experiment, using water from the connecting tube from the fish tanks of each entire system. Experiments were conducted according to the German Animal Welfare Act (TierSchG) and approved by Referat Tierschutz of Regierungspräsidium Tübingen (AZ 35/9185.81-7).

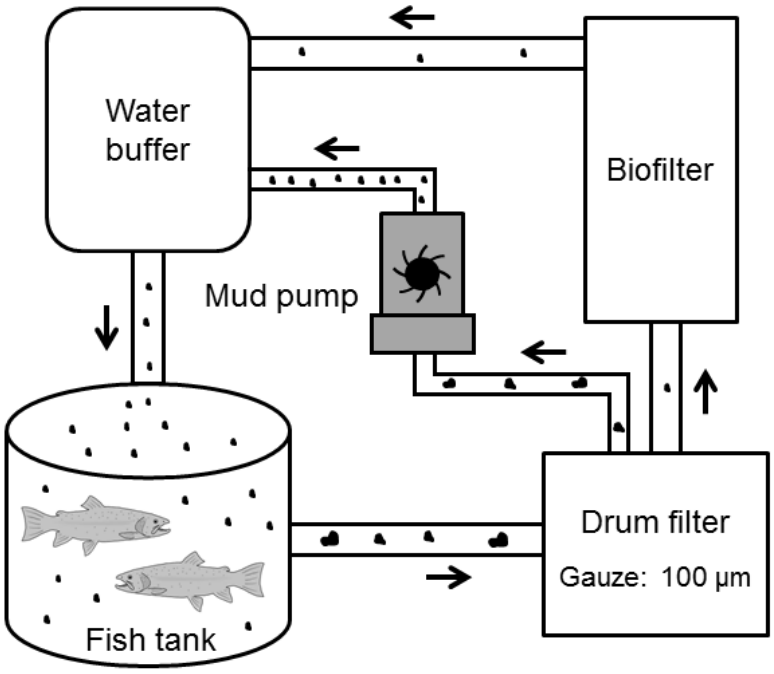

Figure 4.1: Diagram of the treatment RAS, showing the modification of the system to allow backwash water to be pumped into the water buffer.

Table 4.1: Manufacturer specifications of the mud pump used for returning backwash water to the system.

Wilo-EMU KS 8 ES	
Rated speed	2900 rpm
Free ball passage	9 mm
Maximum operating pressure	1.8 bar
Max. volume flow	23 m^3 h^{-1}
Activation	Float switch

Table 4.2: Diet composition of EFICO Enviro 921 (Biomar).

Diet composition	
Crude protein (%)	45-48
Crude lipid (%)	25-28
Carbohydrates (%)	16
Fiber (%)	1.9
Ash (%)	5.3
Total Phosphorus (%)	0.9
Nitrogen content (%)	7.4
Gross energy (MJ kg^{-1})	22-26
Digestible energy (MJ kg^{-1})	21.4

Total suspended solids

The concentration of total suspended solids (TSS) was determined according to method 2540 D of the American Public Health Association (APHA, 1998), with the exception that 0.45 µm cellulose-acetate filters (diameter: 50 mm, 11106-50-N, Sartorius AG, Göttingen, Germany) were used instead of glass-fiber filters. Prior to use, the filters were pretreated in boiling distilled water for 2 h, dried at 103°C, and weighed (0.1 mg). Water samples were collected using a tube at a water depth of ca. 30 cm from five tanks in each system, then mixed in equal parts to create a representative sample for each system. Samples were taken early in the morning prior to feeding to ensure comparability without the influence of feeding or pit cleaning procedures. TSS concentration (mg L^{-1}) was ascertained thrice weekly in duplicate for

each system using compressed air at about 0.7 MPa for filtration. The final samples were taken on the 25th day of operation.

Particle size distribution (PSD)

For particle size measurement, water samples were collected as described above. Particle sizes were then determined according to Brinker et al. (2005b) using a non-invasive laser particle sizer (GALAI: CIS-1, GALAI Productions Ltd. Midgal Haemak, Israel) equipped with a flow controller (GALAI: LFC- 100) and a flow-through cell (GALAI: GM-7). The measurements were performed in quadruplicate for each system at the end of the fourth week of experimental operation. Filters were prepared as previously described. Particles larger than 300 µm were separated by a pre-weighed, 300 µm, even weave Polyester screen. A subsample (0.5 L) of the suspension remaining after PSD determination was filtered with a 0.45 µm cellulose-acetate filter using compressed air at about 0.7 MPa. The weight of particles above 300 µm divided by the total particle weight provided a correction for particles larger than 300 µm, which were out-of-range for laser-determination of PSDs.

Fish performance

The specific growth rate (SGR) was calculated from mean weights recorded at the beginning and the end of the experiment by using the following formula:

$$SGR\ (\%\ d^{-1}) = \frac{ln(mean\ final\ weight) - ln(mean\ initial\ weight)}{t(final\ day) - t(initial\ day)} * 100$$

The feed conversion ratio (FCR) was calculated as:

$$FCR = \frac{Feed(kg)}{Weight\ gain(kg)}$$

The thermal growth coefficient (TGC) was calculated according to Jobling (2003):

$$TGC = \frac{\left(\sqrt[3]{W_t} - \sqrt[3]{W_0}\right)}{\sum T} \times 1000$$

where W_t and W_0 are the final and initial weights (g), respectively and ΣT is sum day-degrees Celsius (Cho, 1992; Iwama and Tautz, 1981).

Sampling protocol

Fish were fasted 24 h prior to each sampling. Two fish from each tank per system (n = 20) were individually sampled and anaesthetized using clove oil (concentration: 0.1 mL L^{-1}, exposure time: ca. 60 s). Directly after anesthesia, wet weight (0.1 g) and total length (0.1 cm) of each fish were measured and blood samples were taken from the caudal blood vessels and transferred to tubes containing lithium heparin (25 IU mL^{-1} blood, Sarstedt, Nümbrecht, Germany). Subsequently, fish were killed and samples of gill tissue were taken for histological examination. Further tissue samples from head kidney, liver, gills and skin were then collected for heat shock protein 70 determination.

Gill histology

Gill tissue was fixed directly upon sampling with 10 % neutrally buffered formalin for at least 24 h at room temperature, then stored at 4°C until further processing. The tissue was dehydrated in a series of graded ethanols, infiltrated and embedded with paraffin wax. Sections were cut at a thickness of 4-6 µm, stained with haematoxylin and eosin (HE) (Romeis, 1968) and observed under a photomicroscope (Zeiss, Oberkochen, Germany). For each section, 5 images showing 6-7 secondary gill lamellae were taken at a magnification of 200×. The architecture of the branchial epithelium and the secondary lamellae was examined for changes, such as thickening and edematous alterations of branchial epithelium cells, lifting of the epithelium from the pavement cells, hyperplasia of cells and lamellar fusion. Observed changes were scored arbitrarily from 0 to 3. Representing no change, little change, moderate change and severe change respectively. When the secondary gill lamellae were covered by a thin branchial epithelium which was intimately attached to the vascular epithelium and the interlamellar space was clearly visible, the gills were designated “no change”. Gill samples in which branchial cells exhibited a thicker appearance, a lighter colouration, a lifting of the epithelium or hyperplasia or fusion of the lamellae in some places were scored as “little change”. When a swelling of the gill epithelium, formation of cell edemas, separation from the vascular epithelium, hyperplasia or lamellar fusion was observed in several locations of the gill sample, the changes were scored as “moderate

change" and when observed changes were regular and pronounced, the gills were scored as "severely changed". Branchial epithelium thickness (µm) was measured at 10 locations in each image and a mean value was calculated. The gills of 20 rainbow trout from each RAS were investigated at the beginning and the end of the study.

Fin condition

Fin erosion in sampled fish was assessed according to Person-Le Ruyet et al. (2007). Additionally, the fin index was determined as follows, according to Kindschi (1987):

$$Fin\ index = \frac{fin\ length\ (cm)}{total\ length\ (cm)} \times 100$$

Heat shock protein 70

Heat shock protein 70 (Hsp70) is a widely used indicator of stress in fish (Iwama et al., 1998); (Yamashita et al., 2010) and therefore of primary interest in disease control measures in aquaculture (Sung and MacRae, 2011). Concentration of Hsp70 was determined at the end of the study by sandwich ELISA (Enzyme-linked Immunosorbent Assay) using a commercially available ELISA kit (SEA873Hu, Cloud-Clone Corp., Houston, USA, antibodies-online.com). Tissue homogenates were produced from skin, gills, liver and head kidney. In brief, 100 mg tissue was homogenized on ice and diluted with 1 ml 1xPBS. The resulting suspension was subjected to two freeze-thaw cycles to further disrupt cell membranes. After that, the homogenates were centrifuged for 10 minutes at 24 000 × *g* (Rotina 38R, Hettich, Tuttlingen, Germany). The supernatant was removed and stored at -20°C. The protein concentration of each sample was determined by using a bicinchoninic acid kit (BCA1, Sigma-Aldrich, St. Louis, USA). For the ELISA, liver samples were diluted with 1xPBS to a concentration of 1 µg of total protein ml^{-1}. All other samples were diluted with 1xPBS to a concentration of 20 µg of total protein ml^{-1}. The ELISA was then conducted according to the manufacturer's instructions.

Plasma cortisol

Blood samples were centrifuged for 10 min at 1 000 × *g* (4°C, Rotina 38R). Plasma was transferred into plastic tubes and stored at -20°C until further processing. A commercial Cortisol ELISA kit (HZ-1887, Hölzel Diagnostika GmbH, Köln, Germany) was used to determine plasma cortisol concentration, after cortisol extraction with ethyl acetate. In brief, 200 µl plasma was mixed three times with 500 µl ethyl acetate, vortexed for 30 sec each time and centrifuged for 5 min at 2 700 × *g* (Rotanta R, Hettich, Tuttlingen, Germany). The supernatant was transferred into a glass tube. After solvent evaporation, the extract was re-suspended in 200 µl 1xPBS. The ELISA was then conducted according to the manufacturer's instructions.

Hematology

Blood smears were produced from native-blood using an Undritz-Glass directly after blood sampling. Once completely dry, the smears were stained with Hemacolor (Merck, Darmstadt, Germany), and differential leukocyte counts were made by identifying 200 leukocytes in a meandering pattern from each slide. For hematocrit and leukocrit determination (McLeay and Gordon, 1977), hematocrit capillaries (sodium heparinized) were filled with blood and centrifuged for 10 min at 14 000 × *g* in a hematocrit centrifuge (HAEMATOCRIT 210, Hettich, Tuttlingen, Germany). Hemoglobin concentration was determined by the cyanmethemoglobin method (Drabkin and Austin, 1932), with bovine hemoglobin (H2500, Sigma-Adrich, St. Louis, USA) as a standard. Cellular debris was removed from the samples with a metal eyelet before absorbance readings were taken. Red blood cells were stained with toluidine blue (0.01% in 1xPBS, 1:200 dilution) and counted using a hemocytometer (Neubauer chamber). The blood parameters thus acquired were then used to calculate indices of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

Data analysis

Data were checked for homoscedasticity using Levene's test (Levene, 1960) and for normality using visual inspection of distribution. If distribution and homoscedasticity tests were passed, t-tests were performed, otherwise Wilcoxon signed-rank test were employed. The PSD data were arranged into size classes ($d_i + 1 = 1.26\ d_i$, d = upper diameter of class) according to Patterson et al. (1999). Data were converted into cumulative volume data assuming a sphere as basic shape to compare cumulative particle volume means below 102 µm, then analyzed by t-test. Fin erosion data were analyzed using Fisher´s exact test, whereas fin indices were compared by t-test. Analyses of gill histology, thickening of epithelial cells, cellular edema and tip thickening data were carried out using Fisher´s exact test. Branchial epithelium thickness was tested by t-test. All data analyses were performed with JMP Pro (SAS Institute Inc.) version 11.1.1. Differences between treatment groups were considered to be significant at $P < 0.05$.

4.4 Results

Water parameters

Except for turbidity, which necessarily differed significantly ($P < 0.01$) between control and treatment, there were no significant differences ($P > 0.05$) between water parameters of the two systems (Table 4.3). All water parameters remained within limits deemed safe for rainbow trout.

Table 4.3: Water parameters (mean ± S.D.; NO_3-N = max. value) of the control and treatment RAS.

	Control	Treatment	Statistical Significance
pH	7.38 ± 0.15	7.39 ± 0.14	n.s.
Temperature (°C)	14.1 ± 0.3	14.2 ± 0.3	n.s.
O_2 (mg L^{-1})	11.0 ± 0.8	10.8 ± 1.1	n.s.
CO_2 (mg L^{-1})	14.3 ± 0.5	15.0 ± 1.0	n.s.
NH_4-N (µg L^{-1})	467.8 ± 83.0	506.9 ± 90.4	n.s.
NO_2-N (µg L^{-1})	131.1 ± 29.4	122.8 ± 25.6	n.s.
NO_3-N (mg L^{-1})	≤ 220	≤ 192	n.s.
Water consumption (L d^{-1})	228.6 ± 72.0	223.2 ± 120.1	n.s.
Turbidity (NTU)*	2.2 ± 0.3	14.5 ± 2.3	$P < 0.01$

*NTU = Nephelometric Turbidity Unit

Fish performance

Overall, no significant differences ($P > 0.05$) were apparent in survival, final weight, FCR, SGR or TGC between fish of the control and treatment RAS (Table 4.4). Fish performed well in both systems. The exception was feeding behavior, which differed marginally between the treatment and control group by the end of the investigation period. It was observed, that fish of the treatment group showed a less aggressive and calm feeding behavior, but ingested the same amount of feed. Hence, growth and final weight were not affected.

Table 4.4: Survival, feed conversion ratio (FCR), specific growth rate per day (SGR), thermal growth coefficient (TGC) and final weight (mean ± S.D.) as determined in treatment and control RAS.

	Control	Treatment	Statistical Significance
Survival (%)	99.68	99.16	n.s.
FCR	0.88 ± 0.02	0.89 ± 0.02	n.s.
SGR (% d^{-1})	1.084 ± 0.017	1.085 ± 0.018	n.s.
TGC	1.44 ± 0.02	1.43 ± 0.30	n.s.
Final weight (g)	299.4 ± 64.9	299.2 ± 67.1	n.s.

Suspended solids analysis

TSS concentrations differed significantly ($P < 0.001$) between control and treatment systems, remaining at about 5 mg L^{-1}, in the control RAS throughout the four-week investigation period but increasing more than seven-fold in the treatment system, from about 4 mg L^{-1} to over 30 mg L^{-1} (Figure 4.2). From day 11, the TSS concentration in the treatment system exceeded 25 mg L^{-1} and the difference in TSS concentration between control and treatment was never less than 21.2 mg L^{-1}. The substantial increase of TSS in the treatment RAS after day 9 was the result of increasing discharge of backwash water into the system with a constant water inflow into the collection tank. After this, TSS concentration in the treatment system never fell below 25.5 mg L^{-1}.

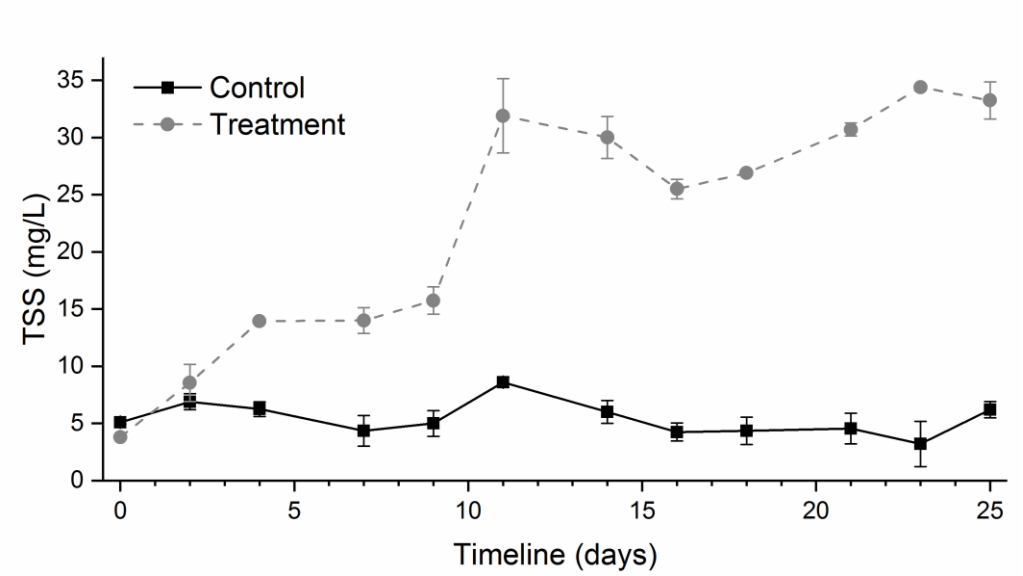

Figure 4.2: Development of total suspended solids concentration (mean ± S.D.) in control and treatment systems during the experimental period. Samples were collected in the early morning, before feeding.

Figure 4.3 shows the volume-dependent PSDs of the control and the treatment systems with average suspended particle loads (mean ± S.D.) of 4.7 ± 1.3 mg DW L^{-1} and 33.3 ± 2.4 mg DW L^{-1} in control and treatment systems respectively. The PSDs of both systems covered the whole size range with significantly (P < 0.05) higher percentages of small (<102 µm) particles in the treatment system than in the control. The cumulative volume of particles smaller than 102 µm amounted to 11.5 ± 3.4 % (mean ± S.E.) in the control system and to 41.6 ± 7.0 % (mean ± S.E.) in the treatment. The difference was particularly apparent in the particle size ranges between 25 and 51 µm, which showed substantially higher values (3.3 to 5.2 %) in the treatment system than in the control (0.8 to 1.4 %). The concentration of particles smaller than 32 µm amounted to 0.3 ± 0.3 mg L^{-1} in the control system and 8.0 ± 2.7 mg L^{-1} in the treatment system.

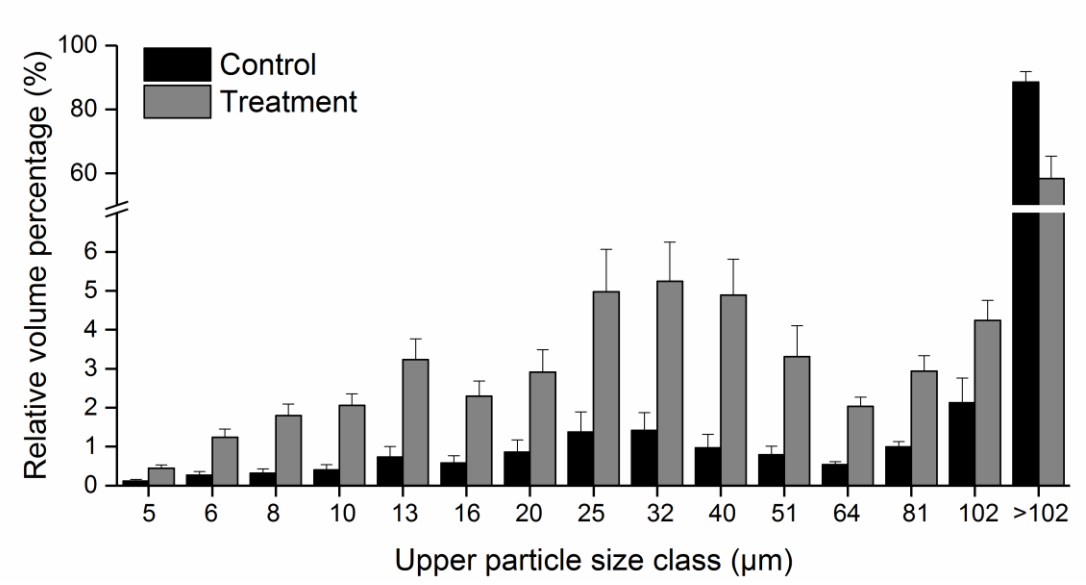

Figure 4.3: Particle size distribution from water samples (mean ± S.E.) of the control and treatment RAS. Suspended particle load was 4.7 ± 1.3 mg DW L^{-1} in the control and 33.3 ± 2.4 mg DW L^{-1} in the treatment system.

The absolute frequency of particle classes (Figure 4.4) shows a significant increase ($P < 0.05$) in particles in the treatment RAS compared to the control RAS. The total number of particles per liter was on average 2 fold greater in the treatment RAS than in the control. The increase in particles between 6-20 µm was particularly marked, being 3.4 to 4.6 fold greater in the treatment RAS. However, a high accumulation of fine particles occurred in both the control and the treatment systems, with 99.1 % and 98.7 % of all particles respectively smaller than 15 µm.

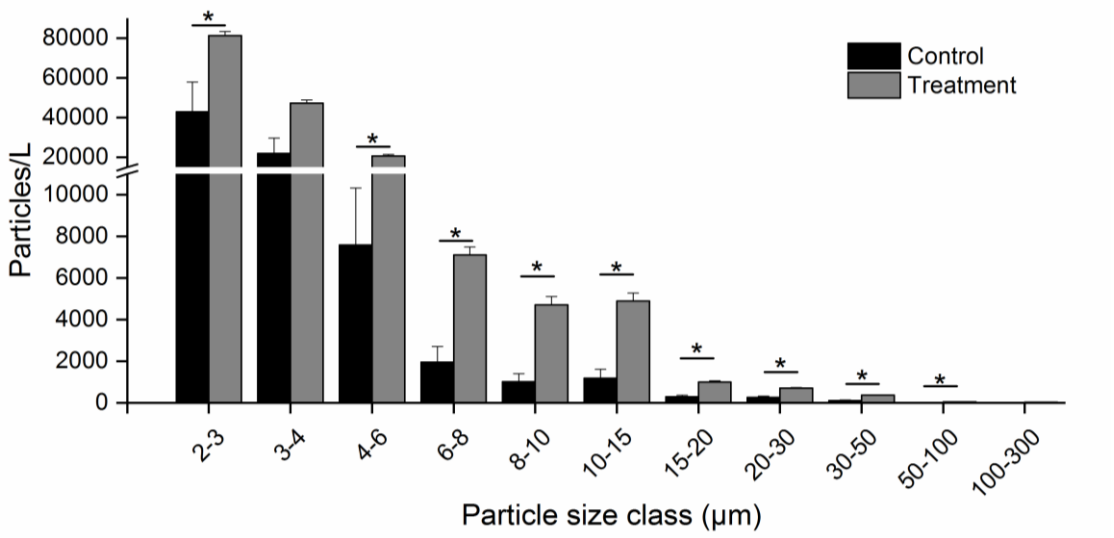

Figure 4.4: Absolute frequency within particle classes (mean ± S.E.) of the control (n = 3) and treatment RAS (n = 4). Suspended particle load was 4.7 ± 1.3 mg DW L^{-1} in the control and 33.3 ± 2.4 mg DW L^{-1} in the treatment system. *Indicates statistical difference (P < 0.05) between groups. Please note the axis break on the y-axis.

Fish health

Gill histology

Gills were examined for histological changes (Figure 4.5). Overall, no severe changes in gill structure were apparent (Figure 4.6), though some thickening of the epithelium and vacuolization and cellular edema were observed. In some cases, the thickening of the epithelium was restricted to the tip of the secondary lamellae (Figure 4.5) and thus it was recorded separately as “tip thickening”. Detachment of gill epithelium, merging of gill lamellae and infiltration of mononuclear cells into the secondary lamellae were each observed on single occasions only and therefore were not included in further evaluation because the occasional incidence is not uncommon.

There was no significant difference in occurrence of branchial epithelium thickening (Table 4.5), cellular edema or tip thickening between trout from control and treatment system at either the beginning or the end of the investigation (P > 0.05). Prevalence and intensity of epithelial thickening did not differ significantly (P > 0.05) between control and treatment at the beginning of the study, but by the end of the experiment the occurrence of thickening was significantly higher in the control group (P < 0.05). Taken together, the histological changes manifest in the treatment group were less pronounced than those in the control fish.

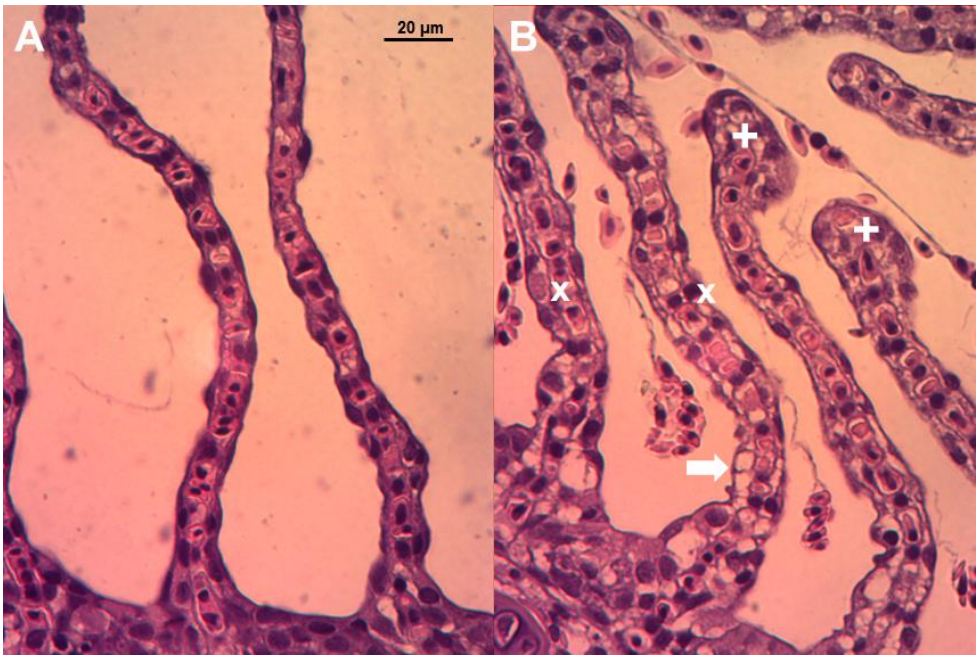

Figure 4.5: (A) Normal appearance of secondary lamellae (treatment group). (B) Secondary lamellae with histological changes (control group): + = tip thickening; x = thickening of epithelial cells; → = cellular edema. Images were taken at 400 x magnification.

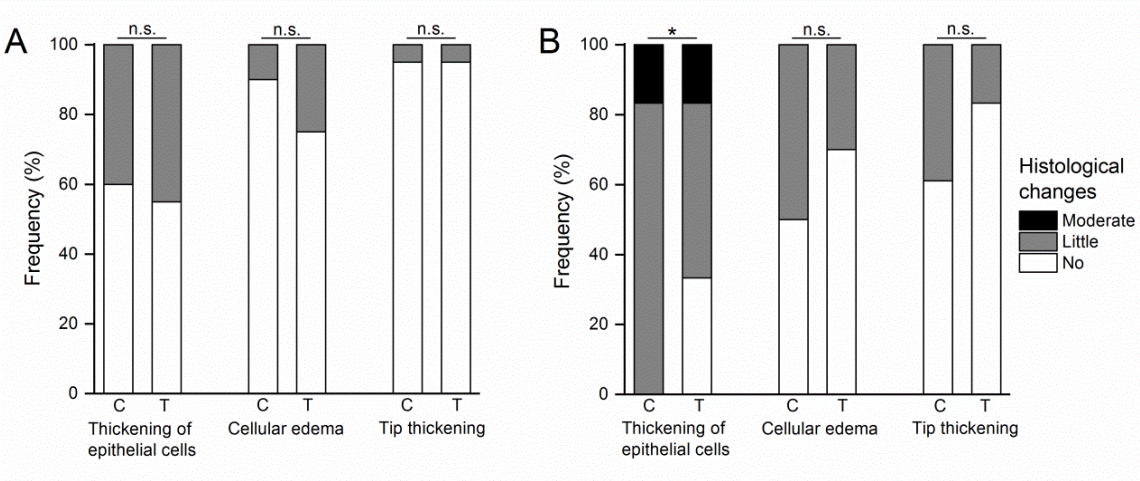

Figure 4.6: Histological changes observed (A) at the beginning and (B) at the end of the investigation period. In each case, histological changes were examined in gills of 20 rainbow trout gills per RAS. *Indicates statistical difference ($P < 0.05$) between groups. C = Control; T = Treatment.

Table 4.5: Thickness of branchial epithelium (mean ± S.D.).

	Control	Treatment	Statistical Significance
Start	4.53 ± 1.28 µm	4.31 ± 1.47 µm	n.s.
End	4.23 ± 0.82 µm	4.06 ± 1.17 µm	n.s.

Fin condition

Fin condition was rather poor at the start of the study (Figure 4.7), but the extent of fin erosion was consistent between groups, as determined according to Person-Le Ruyet et al. (2007) ($P > 0.05$). At the end of the study, however, higher levels of dorsal fin erosion were exhibited by a significantly ($P < 0.05$) higher proportion of control fish than fish from the treatment system. Erosion levels of right and left pectoral fins were not significantly different ($P > 0.05$).

Fin index (Table 4.6) reveals no significant differences ($P > 0.05$) between systems at the beginning of the experiment. However, at the end of the observation period, fin index values for dorsal fin were significantly higher ($P < 0.01$) in the treatment fish compared to the control group. No significant differences were observed in any other fin indices ($P > 0.05$).

Overall, fin erosion levels reveal a slight improvement of fin condition during the investigation period with a non-significant tendency for fin erosion to be less severe in the treatment RAS.

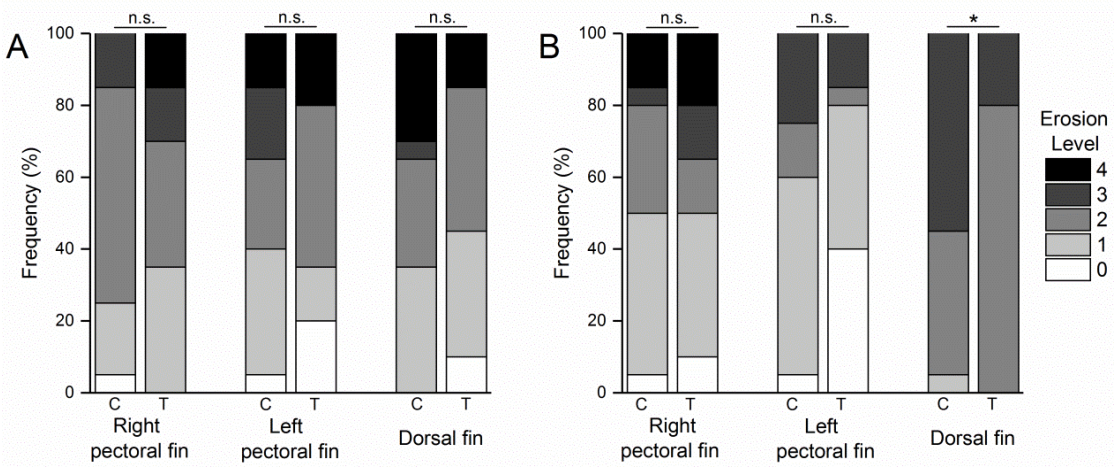

Figure 4.7: Percentages (%) of fin erosion determined according to Person-Le Ruyet et al. (2007) (A) at the beginning and (B) at the end of the investigation period. In each case, fin erosion was investigated for 20 randomly sampled rainbow trout. *Indicates statistical difference ($P < 0.05$) between groups. C = Control; T = Treatment.

Table 4.6: Fin index (mean ± S.D.) according to Kindschi (1987).

		Control (n = 20)	Treatment (n = 20)	Statistical Significance
Start	Dorsal fin	5.50 ± 1.07	5.73 ± 1.14	n.s.
	Right pectoral fin	7.83 ± 1.14	7.03 ± 1.55	n.s.
	Left pectoral fin	7.30 ± 1.62	6.99 ± 1.89	n.s.
	Caudal fin	9.09 ± 1.12	9.06 ± 1.34	n.s.
End	Dorsal fin	7.59 ± 0.98	8.43 ± 0.87	$P < 0.01$
	Right pectoral fin	8.72 ± 2.04	8.47 ± 2.19	n.s.
	Left pectoral fin	9.12 ± 1.03	9.59 ± 1.41	n.s.
	Caudal fin	9.86 ± 0.69	10.14 ± 0.69	n.s.

Hsp70 & plasma cortisol concentration

No significant differences ($P > 0.05$) in Hsp70 concentrations were found between control and treatment in all examined tissues (Figure 4.8). Hsp70 levels in skin were slightly higher for fish of the control compared to the treatment. In both groups, the occurrence of Hsp70 was distinctly lower in the head kidney than in other tissues. In contrast, Hsp70 concentrations were markedly elevated in the liver in both groups. Furthermore, plasma cortisol concentration did not differ significantly ($P > 0.05$) between fish of the control and treatment RAS (Figure 4.8).

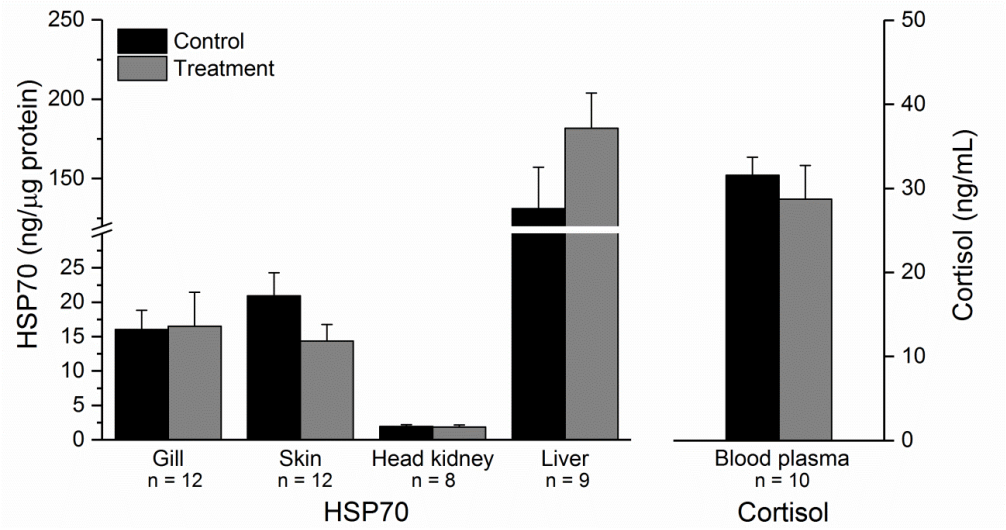

Figure 4.8: Hsp70-concentration (mean ± S.E.) in gill, skin, head kidney and liver tissues and plasma cortisol concentration of the control and treatment RAS. 'n' represents sample size of each group. No significant differences ($P > 0.05$) were found between groups.

Hematology

Overall, hematological parameters (Table 4.7) showed no significant differences ($P > 0.05$) between fish from the control and treatment RAS at either the beginning or end of the study. Differential leukocyte counts were clearly dominated by lymphocytes and no strongly increased numbers of monocytes or granulocytes were detected. Furthermore, all other hematological parameters were within normal ranges.

Table 4.7: Hematological parameters of rainbow trout determined at the beginning and in the end of the study.

	Start			End		
	n	Control*	Treatment*	n	Control*	Treatment*
Lymphocytes (%)	18[1]	96.6 ± 1.7	96.5 ± 2.5	20	95.5 ± 2.0	95.5 ± 2.2
Granulocytes (%)	18[1]	2.6 ± 1.6	3.0 ± 2.3	20	3.1 ± 1.8	2.8 ± 1.9
Monocytes (%)	18[1]	0.8 ± 0.7	0.5 ± 0.5	20	1.5 ± 0.9	1.7 ± 1.0
Erythrocytes (10^6 μL^{-1} blood)	20	1.11 ± 0.10	1.12 ± 0.16	20	0.96 ± 0.13	0.94 ± 0.09
Hematocrit (%)	20	36.0 ± 3.1	35.4 ± 5.2	20	31.8 ± 2.8	31.1 ± 2.5
Leukocrit (%)	20	1.1 ± 0.3	1.2 ± 0.3	20	1.1 ± 0.1	1.1 ± 0.2
Hemoglobin (g dL^{-1})	20	10.4 ± 1.0	10.0 ± 1.3	19[2]	9.9 ± 0.8	10.1 ± 1.0
MCH (pg)	20	93.7 ± 9.8	89.0 ± 5.0	19[2]	103.3 ± 11.8	106.6 ± 10.4
MCV (fL)	20	324.7 ± 22.8	315.6 ± 19.8	20	333.4 ± 34.8	332.6 ± 29.6
MCHC (g dL^{-1})	20	28.9 ± 2.9	28.3 ± 1.9	19[2]	31.0 ± 2.0	32.1 ± 2.6

MCH = mean corpuscular hemoglobin; MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration

*Absence of superscript indicates no significant difference between control and treatment.

[1]Each two missing values due to not analyzable blood smears.

[2]Each one missing value due to coagulation of the blood sample.

4.5 Discussion

The aim of the present study was to investigate the effect of suspended solids, especially fine particles, on fish performance and fish health, uncoupled from the potentially confounding effects of other water parameters. This precondition was entirely fulfilled, so that to the best of our knowledge the effects of suspended solids could be assessed in isolation for the first time. It was, however, expected that water parameters of the solid enriched system would be affected by increased particle load, principally by nutrient leaching (Chen et al., 2003) and maybe by biofilm reduction on the carrier material in the biofilter (Ling and Chen, 2005). In anticipation of these effects the experimental set up made use of overdimensioned biofilters (designed for 4.5 kg feed day^{-1}; maximum feed amount in the present study: 2 kg day^{-1}). During the experiment it turned out that the biofilters were so effective that all water parameters, with the exception of turbidity remained low and comparable between both systems and none of the additional measures which had been prepared (such as TAN addition) were necessary.

As expected given the experimental elevation of solid load, turbidity differed significantly between the two RAS, mainly due to the physical light absorption by the suspended solids. The increased turbidity seemed to alter the behavior of fish (Bash et al., 2001), with the observed feeding behavior in treatment system becoming calmer and less aggressive, probably indicating less social stress relating to feeding competition. However, these observed differences in feeding behavior had no effect on growth, feed intake or feed utilization.

Furthermore, feed performance and survival of both systems indicated good husbandry conditions, with no obvious debilitation resulting from solid load. According to a recently published study by Fernandes et al. (2015) the reduction of solid load using microscreens failed to yield any improvement in fish performance. This suggests that short-term exposure (4 weeks) to elevated levels of particles and especially fine particles may not have the strong impact on fish that has been previously assumed (Chapman et al., 1987; Chen and Malone, 1991).

In the present study, TSS concentrations in the treatment RAS increased over the two first weeks and by day 11 exceeded the upper safe limits of 25 mg L^{-1} proposed by

different authors (Alabaster and Lloyd, 1982; Timmons and Ebeling, 2010a) and the guideline levels for natural limnetic waters (summarized by Bilotta and Brazier, 2008), and remained consistently above the proposed upper safe limit of 25 mg L^{-1} for two weeks. It should be noted that TSS concentrations were always determined in the morning before feeding took place and thus represented the minimum level of particle load before settled material was agitated by the feeding activity of fish. Random single measurements later in the day indicated distinctly higher TSS concentrations of between 48 to 60 mg L^{-1}.

A distinct accumulation of fine particles occurred in the treatment system compared to the control. The cumulative volume of particles smaller than 102 µm amounted to a final proportion of more than 40 % in the treatment RAS. Comparable values were obtained under normal production conditions in a former study (Brinker et al., 2005a), indicating that the use of the mud pump in the experimental system established a realistic particle size distribution in the treatment RAS. The increase in articles smaller than 15 µm, widely regarded as most harmful (Chapman et al., 1987; Chen and Malone, 1991), was particularly apparent in the treatment RAS compared to the control RAS. Thus the experiment set up is in line with conditions in which previous authors have proposed detrimental effects of fine particles (Chapman et al., 1987; Chen et al., 1993; Chen and Malone, 1991).

While many studies have sought to determine the effects of sediments on fish (Bash et al., 2001; Kemp et al., 2011; Michel et al., 2013; Wong et al., 2013), the effects of system-related particles in fish farming and in RAS specifically are largely unexplored. It is important to emphasize that particles in RAS are different to those present in rivers and lakes. Suspended solids in natural systems, especially those of inorganic origin, are generally heavy, sharp and hard, and therefore carry a higher risk of mechanical damage to gills (Au et al., 2004; Bash et al., 2001; Bruton, 1985; Newcomb and Flagg, 1982). In contrast and due to their organic-based origin, solids in RAS are mainly smooth particles with a density close to that of water at approximately 1010 to 1153 kg m^{-3} (Unger and Brinker, 2013), and, mechanically at least, less harmful to fish. The size distribution of particles in RAS depends mainly on feed composition and system properties (Brinker et al., 2005a; Unger and Brinker, 2013) whereas in natural limnetic

waters it is often affected by events such as heavy rain, floods and snow melts. Thus comparing observations of solid effects on fish health from natural waters to aquaculture conditions is likely of very limited value. Furthermore, limnetic studies (Au et al., 2004; Lake and Hinch, 1999) indicate that induction of mortality in riverine fish requires concentrations of suspended sediment greater than those which occur under environmentally realistic concentrations. Michel et al. (2013) found no evidence of histological damage in gills, liver, spleen, or kidney of rainbow trout after an exposure to short-duration pulses of suspended sediments in concentrations up to 5000 mg L^{-1}, two orders of magnitude larger than maximum levels occurring in salmonid farming. Such results tally with selective pressure on fish to develop traits allowing them to survive the extreme conditions that might follow natural events such as snow melts or floods.

Overall, gill histology data gained in this study showed no detrimental effects of particle accumulation on gill structures during the four-week exposure period, despite the distinct accumulation of small particles. In fact, the histological changes observed in gill structures of rainbow trout from the tanks with elevated solid levels were less than those seen in fish from the control tanks. The complete absence of any detrimental influence of successful fine solid loading in all investigated parameters is somewhat surprising, since gills are one of the most delicate structures of the teleost body and are generally supposed to be the first organs affected by the presence of particles, at least at a subclinical level. In both the treatment and control groups, the observed changes to the branchial tissue were at such low levels that it was not possible to distinguish possible particle induced effects from naturally occurring variations in gill tissue.

Overall, our results indicate that contrary to previous assumptions, the threat to fish health of short-term exposure to RAS generated particulate solids appear to be low to non-existent. This hypothesis is further strengthened by the accompanying physiological data: not only were no significant differences observed between the treatment groups in Hsp70 expression, but no sign of elevated stress were recorded in any of the examined fish, suggesting all fish were in an unstressed state. In unstressed fish Hsps act as chaperones by assisting in the production and folding of intra-cellular

proteins and in the degradation of structurally aberrant proteins (Roberts et al., 2010; Sung and MacRae, 2011). When exposed to a stressor, Hsp production is up-regulated to maintain cellular homeostasis (Yamashita et al., 2010). The Hsp reaction to stress was initially considered to be short–lived response, but now it is presumed that Hsps play a long-term role by modulating the immune system (Roberts et al., 2010).

In the present study, high concentrations of Hsp70 occurred in liver tissues compared to the other tissues, independent of treatment. The comparatively high Hsp70 concentration in the liver might be related to the use of modern high-energy feed (Bernet et al., 2004; Brinker and Reiter, 2011; Goede and Barton, 1990) and is in line with liver alterations previously determined in one third of all investigated rainbow trout in a study by Unger and Brinker (2013) and ascribed to dietary imbalances.

Cortisol is one of the most commonly measured indicators of stress in fish (Mommsen et al., 1999) and responds to a wide variety of events and conditions deemed to be stressors, both acute and chronic (Ellis et al., 2012). The plasma cortisol concentrations of about 30 ng ml^{-1}, reported in the present study are comparable to results from commercial operation (Rance et al., 1982; Bry, 1982) and do not indicate an elevated stress due to increased suspended solid load. Hematological parameters measured in the current study showed no differences between fish from different treatments and remained within normal ranges (McCarthy et al., 1973; Řehulka et al., 2004). Also the differential leukocyte counts provide no indication of elevated stress (Davis et al., 2008).

Fin erosion is considered an interesting integrative candidate parameter for assessing fish welfare (Ellis, 2002; Turnbull et al., 2005) and should thereby also serve as a comprehensive parameter for measuring the effect of increased particle load and fin rot. However, the present results suggest that increased suspended solid load does not negatively affect fin erosion. Indeed fin condition slightly improved during the investigation.

Overall, the gained results, especially the improvements in terms of fin erosion and gill histology in the treatment system are somewhat surprising and suggest that housing conditions in the experimental system were beneficial for fish health rather than detrimental, even under suspended solid enriched conditions.

4.6 Conclusions

The results obtained in this study provide a first overview of the effects of particle accumulation on fish health in RAS, uncoupled from other potentially confounding water parameters that often accompany solid load increase. The data from the current study suggest that, at least for a relatively short period, suspended solids in concentrations up to 30 mg L^{-1} may alone have no detrimental effects on fish physiology in RAS. However, these findings have to be confirmed in long-term experiments, which should seek to clarify any chronic exposure effects in terms of fish health and performance. Furthermore, it should be noted that indirect solid-mediated effects of particle accumulation such as bacterial growth etc. will occur in regular commercial applications and should not be extenuated by the absence of direct particle-related damage.

5 Physiological consequences of chronic exposure of rainbow trout (*Oncorhynchus mykiss*) to suspended solid load in recirculating aquaculture systems

A similar version of this chapter was published as:
Becke, C., Schumann, M., Steinhagen, D., Geist, J., Brinker, A. (2018): Physiological consequences of chronic exposure of rainbow trout (*Oncorhynchus mykiss*) to suspended solid load in recirculating aquaculture systems. Aquaculture 484, 228–241.

5.1 Abstract

High levels of suspended solids, especially fines, are widely regarded as harmful to fish, particularly in recirculating aquaculture systems (RAS) where accumulation of particles is likely. However, little is known about the consequences of chronic exposure to system-related particles on the stress-levels, well-being and health of fish. In this study, the chronic effects of suspended solids on the physiology and performance of rainbow trout were investigated over the growout period, uncoupled from other potentially confounding water parameters. Compared with fish in a control system with a total suspended solid (TSS) load of 3.9 mg L^{-1}, fish in an otherwise comparable treatment system exposed to minimum particle concentrations of more than 30 mg L^{-1} exhibited no observable difference in hematological variables (differential leukocyte count, RBC and WBC counts, hematocrit), gill histology, fin condition, or heat shock protein 70 concentrations in gill, liver, skin and head kidney tissues. Slight alterations in feeding behavior and a slight increase in bacterial load on fish and in system water were observed in the treatment RAS, but without any apparent effect on fish performance or health. Furthermore, no significant difference in mortality occurred. The absence of expected effects across a wide range of physiological criteria after long-term exposure suggests that suspended solid levels over 30 mg L^{-1} are within the physiological tolerance of this species.

Candidate's contribution:

Implementation of particle accumulation procedure, husbandry of fish, sampling of blood and tissue samples, execution of physiological assays (except gill histology) and particle measurements, measurement of bacterial load in water, statistical assessment and interpretation of data, writing and revision of the complete manuscript including all figures and tables.

5.2 Introduction

Recirculating aquaculture systems (RAS) are regarded as an environmentally friendly option for sustainable fish production (Martins et al., 2010) with the potential for reduced environmental impact through facilitating effluent treatment (Piedrahita, 2003). However, despite ongoing modernization, fish production in RAS remains energy- and cost-intensive and its contribution to global production is still small relative to that of flow-through, pond- or cage-based systems (d'Orbcastel et al., 2009b). Challenges to the economic viability of RAS include high investment costs, the energy-intensive nature of production and difficulties in maintaining system stability leading to imbalances in water parameters, such as high bacterial load (Badiola et al., 2012). Whether maintaining system stability is an advantage or disadvantage of RASs is an ongoing debate. However, looking into systematic surveillance (e.g. Badiola et al., 2012; Martins et al., 2010), RASs turn out to be more vulnerable to system instability compared to open systems.

One possible solution to the energetic cost of RAS is intensification, whereby an increase in stocking densities will reduce energy costs per unit of production (Martins et al., 2005). However, high stocking densities require system stability and optimal water quality to ensure economically competitive and animal-friendly fish production. Animal welfare is becoming a decisive factor in aquaculture production (Ashley, 2007), with increasingly environmentally literate consumers pressing for more responsible methods of food production worldwide.

The management of solids is one of the most difficult technical issues in RAS (Badiola et al., 2012). Within a RAS, suspended solids originate mainly from feces and to a

lesser extent from uneaten feed, bacterial material from biofilters, and microfauna (Chen and Malone, 1991; Noble and Summerfelt, 1996; Summerfelt et al., 1999; Wedemeyer, 1996). Solids comprise mostly smooth particles with a density close to that of water (Unger and Brinker, 2013). Particles are known to harm gill structures (Bash et al., 2001; Bilotta and Brazier, 2008; Bruton, 1985; Chapman et al., 1987; Humborstad et al., 2006; Wong et al., 2013) and elevate stress levels in fish (Awata et al., 2011; Lake and Hinch, 1999; Sutherland et al., 2008), and standard recommendations commonly quote a safe upper limit of 25 mg L^{-1} in rearing water (Alabaster and Lloyd, 1982; Timmons and Ebeling, 2010a). While large particles can be easily removed from RAS by mechanical filtration, smaller particles tend to remain in the system and accumulate over time (Becke et al., 2017; Chen et al., 1993; Davidson et al., 2009). These fine particles are widely regarded as especially harmful to fish health and welfare (Chapman et al., 1987; Chen and Malone, 1991). Furthermore, nutrient leaching increases with decreasing particle size (Brinker et al., 2005a; Kvåle et al., 2006) and thus the accumulation of fines presents a high risk of deteriorating water quality. The relatively large surface/volume ratio of fine particles also offers an increased opportunity for bacterial colonization (Berger et al., 1996; Kirchman and Ducklow, 1987). However, the actual effects of accumulating system-related particles in RAS have been insufficiently studied and little is known about the primary effects of these particles on fish health. There is an urgent need for a better understanding of this issue to further optimize RAS performance and fish welfare. A recent study by Becke et al. (2017) uncoupled particle load from potentially interfering water parameters in RAS for the first time and demonstrated against all expectations that in the short term at least, particle loads up to 30 mg L^{-1} did not impart any observable negative effects on a wide array of fish health and performance parameters.

There are several studies dealing with effects of suspended sediments from natural environment on fish physiology (e. g. Humborstad et al., 2006; Wong et al., 2013). However, particle effects were only partly decoupled from potentially confounding or debilitating water parameters or chemical contaminants so that the impact of suspended sediment was amplified (Wong et al., 2013). This exemplifies the risk that

apparently particle-related effects may be biased by uncontrolled factors such as inappropriate water quality.

Based on this, the present study sought evidence of chronic effects of accumulating particles on performance and physiology of rainbow trout over a whole growout period, uncoupled from potentially confounding or debilitating water parameters. The experimental design was chosen such that the elevated solid fraction in the treatment RAS comprised mainly fine particles to increase the potential for damage. We hypothesized that chronic exposure to particle loads exceeding the recommended value of 25 mg L^{-1} would result in physiological and health effects expressed as changes in hematology, gill histology, fin condition and stress protein expression. We further hypothesized that a chronic increase in suspended solids within the RAS would increase bacterial load, creating adverse environmental conditions that would ultimately impair survival and growth performance of rainbow trout during their growout stage.

5.3 Materials and Methods

Husbandry

In an eighteen-week exposure trial, the effects of high suspended solid load on the physiology and performance of rainbow trout in two replicate RASs (each with a volume of 6 m^3) were evaluated. The experimental systems both comprised 10 green circular fiberglass tanks, each with a capacity of 330 L (Figure 5.1 A). Water exchange was reduced to a minimum and only limited to water loss due to backwashing of drum filter and evaporation. The study was performed with all-female rainbow trout (Störk strain) to exclude sex-related effects and thereby minimize variation. Approximately 600 rainbow trout were held in each system (60 fish per tank), with an average initial weight of 86.6 ± 12.0 g (control group) and 86.5 ± 10.7 g (treatment group) and maximum stocking densities of 68.4 ± 2.6 kg m^{-3} (control) and 65.2 ± 2.3 kg m^{-3} (treatment). The control RAS was operated under regular conditions, while the particle load of the treatment RAS was artificially increased by collecting backwash water from the drum filter into a tank and re-injecting it at regular intervals into the water buffer

of the system (Figure 5.1 B) using a mud pump (Wilo-EMU KS 8 ES, Dortmund, Germany). Under this process, larger particles were fragmented by shear forces (McMillan et al., 2003). The rate of flow of backwash water into the system was kept constant by the flow out of the biofilter. Thus, particles originated from feces and to a lower extend from uneaten feed. No artificial particles were added. In both systems, the drum filter (HDF801-1H, Hydrotech, Vellinge, Sweden) was equipped with a 100 µm gauze. The photoperiod was fixed at 12L : 12D (Lumilux daylight lamps provided around 140 lx at the water surface between 0700 h and 1900 h) with a sigmoidal transition period of 30 min. The fish were fed restrictively by hand, six days a week (Sunday to Friday) using a commercial feed (Biomar EFICO Enviro 921, Aarhus C, Denmark; diet composition: see Becke et al. 2017) starting at 1.6 % of body weight at the beginning of the trial and declining to 1.2 % by the end. Bacterial growth in both systems was controlled with UV irradiation of the system water (Barrier L20, Wallace & Tiernan, Günzburg, Germany; UV dose: 40 mJ cm^{-2} flow volume: 6600 L h^{-1}, lamp wattage: 80 W, measurement range UV sensor: 200W m^{-2}). Fish were put into the RAS three weeks before the beginning of the experiment to ensure acclimatization to the new environment. Experiments were conducted according to the German Animal Welfare Act (TierSchG) and approved by Referat Tierschutz of Regierungspräsidium Tübingen (AZ 35/9185.81-7).

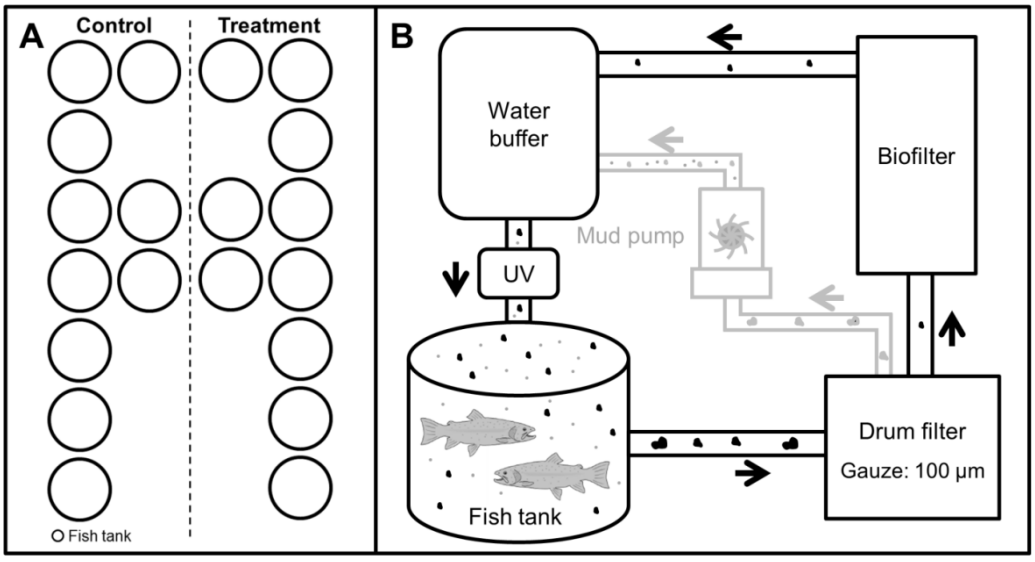

Figure 5.1: (A) Design of the recirculating aquaculture systems with (B) schematic layout of the RASs, showing the modification of the treatment system (grey) to allow backwash water to be pumped into the water buffer (modified after Becke et al. (2017)).

Water parameters

Similar water parameters (Table 5.1) were maintained in both systems, within limits known to preclude impacts on fish health or performance during the whole growout period. Thus, potential effects of particle accumulation were isolated from all measured water parameters which were considered being most relevant for fish health (Timmons and Ebeling, 2010). To avoid a decline in water quality, the biofilters deployed were over-dimensioned and sufficient to remove the waste associated with 4.5 kg feed day^{-1}, well in excess of the maximum 2.7 kg day^{-1} supplied in the present study. pH was measured daily (pH 320 with electrode Sentix41, WTW, Weilheim, Germany) in the outflow of the tanks and adjusted to approx. 7.4 by the addition of sodium hydrogen carbonate. NH_4-N concentration was measured in both RAS every 45 minutes using an automatic device (AMTAX SC, Hach, Germany) to ensure continuity of monitoring (Figure 5.2). Carbon dioxide concentrations were determined up to thrice weekly in the fish tanks using a Portable Dissolved CO_2 Analyser (OxyGuard, Farum, Denmark).

Further tests were carried out three times a week to determine levels of NH_4-N, NO_2-N and NO_3-N in water from the connecting tubes of each system, using the Hach (Germany) analysis kits LCK 304: 0.2 – 2.5 mg L^{-1}; LCK 341: 0,05 – 2 mg L^{-1}; and LCK 339: 1 – 6 mg L^{-1} respectively. Oxygen concentrations (Oxygen Probes, OxyGuard, Farum, Denmark) and temperature (Temperature Probes, Oxyguard, Farum, Denmark) were monitored continuously at the outlets of two tanks in each system. Turbidity was measured in parallel with total suspended solids (TSS) using a turbidity meter (PCE-TUM 20, PCE Instruments, Germany).

Table 5.1: Water parameters (mean ± S.D.; NO_3-N = max. value) of the control and treatment RAS and recommended limits according to Timmons and Ebeling (2010).

	Range limit	Control	Treatment	Statistical significance
pH	6.5 – 8.5	7.38 ± 0.16	7.40 ± 0.13	n.s.
Temperature (°C)	< 16	14.3 ± 0.1	14.1 ± 0.2	$P < 0.001$
O_2 (mg L^{-1})	> 6	10.5 ± 0.5	10.6 ± 0.5	$P < 0.001$
CO_2 (mg L^{-1})	< 20	14.7 ± 3.6	15.0 ± 3.3	n.s.
NH_4-N (mg L^{-1})	< 1	0.473 ± 0.142	0.463 ± 0.125	n.s.
NH_3-N (mg L^{-1})	< 0.0125	0.002 ± 0.001	0.003 ± 0.001	n.s.
NO_2-N (mg L^{-1})	< 1	0.126 ± 0.057	0.134 ± 0.059	n.s.
NO_3-N (mg L^{-1})	< 400	312	300	-
Water consumption (L d^{-1})	/	256.7	251.0	n.s.
Water exchange (volume % day^{-1})	/	4.3 ± 1.3	4.2 ± 2.1	n.s.
Turbidity (NTU)[a]	/	2.2 ± 0.6	10.0 ± 4.4	$P < 0.001$

[a]NTU = Nephelometric Turbidity Unit.

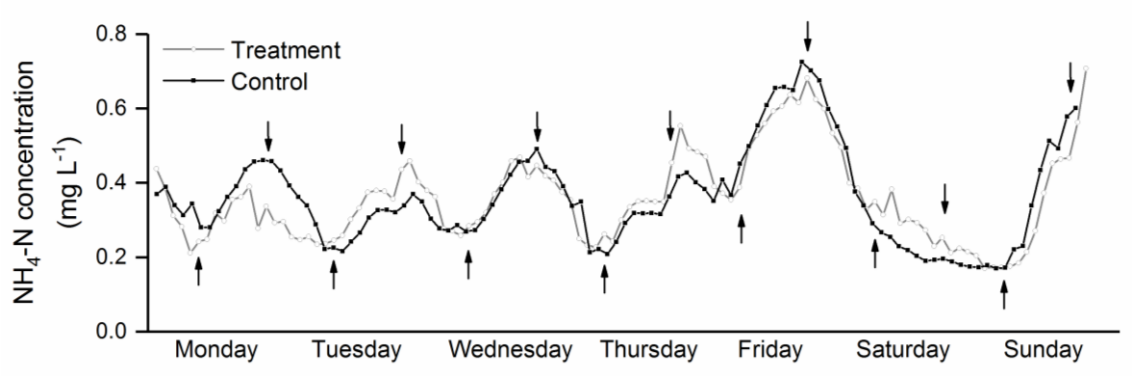

Figure 5.2: Hourly NH_4-N concentrations in the control and treatment RAS during week 10. NH_4-N levels were measured with an automatic monitoring device (AMTAX SC, Hach, Germany). Fish were fed daily from Sunday to Friday. Arrows below the line = 9 a.m., arrows above the line = 9 p.m.

Total suspended solids

The concentration of total suspended solids was determined according to method 2540 D of the American Public Health Association (APHA, 1998), with the exception that 0.45 µm cellulose-acetate filters (diameter: 50mm, 11106-50-N, Sartorius AG, Göttingen, Germany) were used instead of glass-fiber filters. Prior to use, the filters were pretreated in boiling distilled water for 2 h, dried at 103 °C, and weighed (0.1 mg). Water samples were collected using a tube at a water depth of ca. 30 cm from five tanks in each system, then mixed in equal parts to create a representative sample for each system. Samples were collected in the early morning, before feeding, in order to represent the daily minimum solid loads (best case scenario). Thus, it was ensured that the particle concentration did not fall below the aimed concentration. Daily measurements were performed to show the fluctuations and maximum values within the day. TSS concentration (mg L^{-1}) was ascertained thrice weekly in duplicate for each system using compressed air at about 0.7 MPa for filtration.

Particle size distribution (PSD)

For particle size measurement, water samples were collected as described above. Particle sizes were determined according to Brinker et al. (2005b) using a non-invasive laser particle sizer (GALAI:CIS-1, GALAI Productions Ltd. Midgal Haemak, Israel) equipped with a flow controller (GALAI:LFC- 100) and a flow-through cell (GALAI:GM-7). The measurements were performed in duplicate for each system in week 18 of experimental operation.

Fish performance

The specific growth rate (SGR) of fish in each RAS was calculated from mean weights recorded at the beginning and the end of the experiment, using the following formula:

$$SGR\ (\%\ d^{-1}) = \frac{ln(mean\ final\ weight) - ln(mean\ initial\ weight)}{t(final\ day) - t(initial\ day)} * 100$$

The feed conversion ratio (FCR) was calculated as:

$$FCR = \frac{Feed(kg)}{Weight\ gain(kg)}$$

The thermal growth coefficient (TGC) was calculated according to Jobling (2003):

$$TGC = \frac{\left(\sqrt[3]{W_t} - \sqrt[3]{W_0}\right)}{\sum T} \times 1000$$

where W_t and W_0 are the final and initial weights (g), respectively and ∑T is sum day-degrees Celsius (Cho, 1992; Iwama and Tautz, 1981).

Dry matter digestibility was determined in week 18 of the experiment using yttrium oxide. For this purpose, 1 kg of feed was coated with 1 g yttrium oxide dissolved in 5 g rapeseed oil and added in a mixing drum. After one week on this coated feed, feces were stripped from 20 rainbow trout from each system. Samples of feces and yttrium oxide-coated feed were freeze-dried and yttrium levels were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS 7700x) at the federal chemical analysis service (Chemisches und Veterinäruntersuchungsamt Sigmaringen) of Baden-

Württemberg, Germany. Samples were prepared as described in Brinker and Reiter (2011). Dry matter digestibility was determined as follows:

$$Digestibility\ (dry\ matter) = \frac{100 - (100 \times Y_2O_{3\,diet})}{\frac{dry\ matter_{diet}}{100}} \times Y_2O_{3\,feces}{}^{-1}$$

Sampling protocol

Fish were sampled at the beginning of the study, in week 10 and in week 17. Fish were fasted for 24 h prior to each sampling. Two fish from each tank per system (n = 20) were sampled individually and anaesthetized using clove oil (concentration: 0.1 mL L^{-1}, exposure time: ca. 60 s). Directly following anesthesia, wet weight (to the nearest 0.1 g) and total length (to the nearest 0.1 cm) of each fish were measured and blood samples were taken from the caudal blood vessels and transferred to tubes containing lithium heparin (25 IU mL^{-1} blood, Sarstedt, Germany). The fish were then killed by a sharp blow to the head and samples of gill tissue were taken for histological examination. Further tissue samples from head kidney, liver, gills and skin were also collected for heat shock protein 70 (Hsp70) determination and stored at -20°C until further processing.

Gill histology

Gill tissue was prepared and examined as described by Becke et al. (2017), except that gill sections were stained with PAS stain (Periodic acid-Schiff stain) (McManus, 1948). Briefly, gill tissue was fixed directly upon sampling with 10 % neutrally buffered formalin for at least 24 h at room temperature, and then stored at 4°C until further processing. The tissue was dehydrated in a series of graded ethanols, infiltrated and embedded with paraffin wax. Sections were cut at a thickness of 4–6 µm, stained and observed under a photomicroscope (Zeiss, Oberkochen, Germany). Observed changes were scored arbitrarily from 0 (no change) to 3 (severe change) including sub-steps 1 (little change) and 2 (moderate change). For each section, 5 images showing 6–7 secondary gill lamellae were inspected at a magnification of 200×. Branchial epithelium thickness (µm) was measured at 10 locations in each image and a mean value was calculated. The number of goblet cells per secondary lamella was counted and the

occurrence of telangiectasia and lamellar fusion was assessed. The gills of 20 rainbow trout from each RAS were investigated at the beginning of the investigation period, at week 10 and at week 17.

Fin condition

Fin erosion in sampled fish was assessed according to Person-Le Ruyet et al. (2007), and the fin index was determined according to Kindschi (1987), as follows:

$$Fin\ index = \frac{fin\ length\ (cm)}{total\ length(cm)} \times 100$$

Hematology

Blood smears were produced from native-blood using an Undritz-Glass directly after blood sampling. Once completely dry, the smears were stained with Hemacolor (Merck, Darmstadt, Germany), and differential leukocyte counts were made by identifying 200 leukocytes in a meandering pattern from each slide. For hematocrit and leukocrit determination (McLeay and Gordon, 1977), hematocrit capillaries (sodium heparinized) were filled with blood and centrifuged for 10 min at 14,000 × *g* in a hematocrit centrifuge (HAEMATOCRIT 210, Hettich, Tuttlingen, Germany). Hemoglobin concentration was determined by the cyanmethemoglobin method (Drabkin and Austin, 1932), with bovine hemoglobin (H2500, Sigma-Adrich, St. Louis, USA) as a standard. Cellular debris was removed from the samples with a metal eye-let before absorbance readings were taken. Total red and white blood cell counts were performed with a 1:200 dilution of Natt-Herrick solution (Natt and Herrick, 1952) and counted using a hemocytometer (Neubauer chamber). The blood parameters thus acquired were then used to calculate indices of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

Heat shock protein 70

Concentrations of Hsp70 were determined as an indicator for stress in fish (Iwama et al., 1998; Yamashita et al., 2010) at the end of the study by sandwich ELISA (Enzyme-linked Immunosorbent Assay) using a commercially available kit (SEA873Hu, Cloud-Clone Corp., antibodies-online.com, Aachen, Germany). Tissue homogenates were produced from skin, gills, liver and head kidney. In brief, 100 mg tissue was homogenized on ice and diluted with 1 mL 1xPBS. The resulting suspension was subjected to two freeze-thaw cycles to further disrupt cell membranes. After that, the homogenates were centrifuged for 10 min and 4 °C at 15,000 × *g* (UNIVERSAL 320R, Hettich, Tuttlingen, Germany). The supernatant was removed and stored at −20 °C. The protein concentration of each sample was determined by using a bicinchoninic acid kit (BCA1, Sigma-Aldrich, St. Louis, USA). For the ELISA, liver samples were diluted with 1xPBS to a concentration of 1 µg of total protein mL^{-1}. All other samples were diluted with 1xPBS to a concentration of 20 µg of total protein mL^{-1}. The Hsp70 ELISA was then conducted according to the manufacturer's instructions.

Bacterial activity

Colony forming units

Counts of viable heterotrophic bacteria (recorded as colony-forming units, CFUs) were made after 48 hours of culture at 22°C on DEV Agar (103554ZA, VWR International GmbH, Germany). Two dilutions (control: tanks 1:100/1:1000, before + after UV irradiation 1:10/1:100; treatment: tanks + before UV irradiation 1:100/1:1000, after UV irradiation 1:10/1:100) were prepared for each water sample with 0.9 % sterile sodium chloride solution. Plates were set up in triplicate for each dilution.

Bacterial load

Analysis of the bacterial load of rainbow trout was conducted for both the second (week 10; 8 rainbow trout per RAS) and final sampling (week 17; 20 rainbow trout per RAS) by the fish health service at a governmental veterinary institute, the Staatliches Tierärztliches Untersuchungsamt (STUA) Aulendorf, Germany. Mucus smears taken from the skin and gills of sampled fish and crush preparations of spleen tissue were all

analyzed for the presence of bacteria under a phase contrast microscope. Agar plates (Table 2.2) were inoculated with smears taken from gills and spleen. After incubation, the number of colony forming units was assessed and arbitrarily graded as no, slight, moderate or severe bacterial load. Bacterial species were then determined by using bacteriological standard methods and confirmed by MALDI-TOF MS (Lay, 2001).

Data analysis

Data were checked for homoscedasticity using Levene's test (Levene, 1960) and for normality by visual inspection of distributions. If distribution and homoscedasticity tests were passed, treatment effects were tested by *t*-tests, otherwise Wilcoxon tests were employed. For analysis of hematological parameters, Bonferroni corrections were used within the following groups: differential blood count (lymphocytes, granulocytes, monocytes), blood cells (erythrocytes, thrombocytes, leukocytes), red blood cell indices (MCH, MCHC, MCV) and blood composition (hematocrit, leukocrit).

Gill histology was evaluated with respect to thickening of epithelial cells, cellular edema, cell infiltration, tip thickening, detachment of the epithelium, telangiectasia and lamellar fusion and analyzed using Fisher´s exact test. Differences in branchial epithelium thickness and number of goblet cells per secondary lamella between control and treatment fish were tested by *t*-test. Fin erosion data was analyzed using Fisher´s exact test, and fin indices were compared by *t*-test. The method of least squares was used to analyze the relationship between TSS concentrations and turbidity. Bacterial load data for gill smears was analyzed using Fisher´s exact test.

All data analyses were performed with JMP Pro (SAS Institute Inc.) version 13.0.0. Differences between treatment groups were considered to be significant at $P < 0.05$.

5.4 Results

Water parameters

Turbidity differed significantly ($P < 0.001$) between the control and treatment RAS, (Table 5.1). Water temperature and oxygen concentration also showed significant differences ($P < 0.001$), but the differences in both parameters were less than 0.2 mg L^{-1} and were thus deemed unlikely to be biologically relevant. No other water parameters showed significant differences between systems ($P > 0.05$) and all remained within previously established physiological preferences for rainbow trout.

Suspended solids analysis

Total suspended solids

TSS concentration differed significantly ($P < 0.001$) between control and treatment systems, averaging 3.9 mg L^{-1} in the control RAS throughout the investigation period but increasing on average to over 30 mg L^{-1} in the treatment system (Figure 5.3). From week 10, the TSS levels in the treatment system exceeded 30 mg L^{-1} and the difference in TSS concentration between control and treatment RAS was never less than 21 mg L^{-1}. Figure 5.4 shows the daily variation in both control and treatment RAS in week 4 and week 14 of the experiment. In week 4, the TSS levels varied between 4.3 and 8.6 mg L^{-1} in the control system and between 8.6 and 19.6 mg L^{-1} in the treatment system over the course of the day. In week 14, TSS levels varied between 2.4 and 9.1 mg L^{-1} in the control system and between 32.3 and 71.0 mg L^{-1} in the treatment system during the day, with a maximum value at 11 a.m. after feeding and pit cleaning. TSS concentration exhibited a highly significant linear correlation with turbidity ($P < 0.0001$; Figure 5.5).

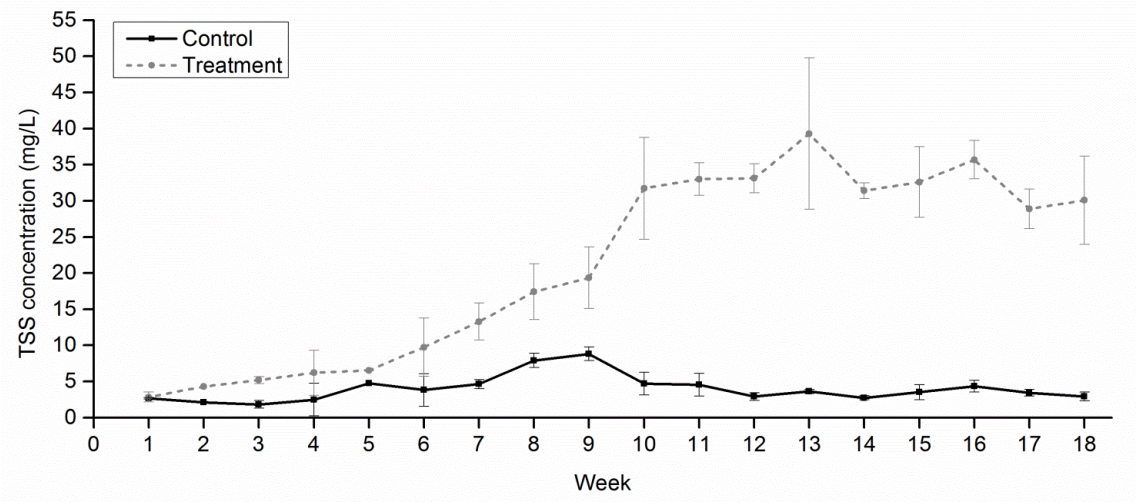

Figure 5.3: Timeline of TSS concentrations (mean ± S.D.) in control and treatment RAS over the experimental period. Samples were collected in the early morning, before feeding.

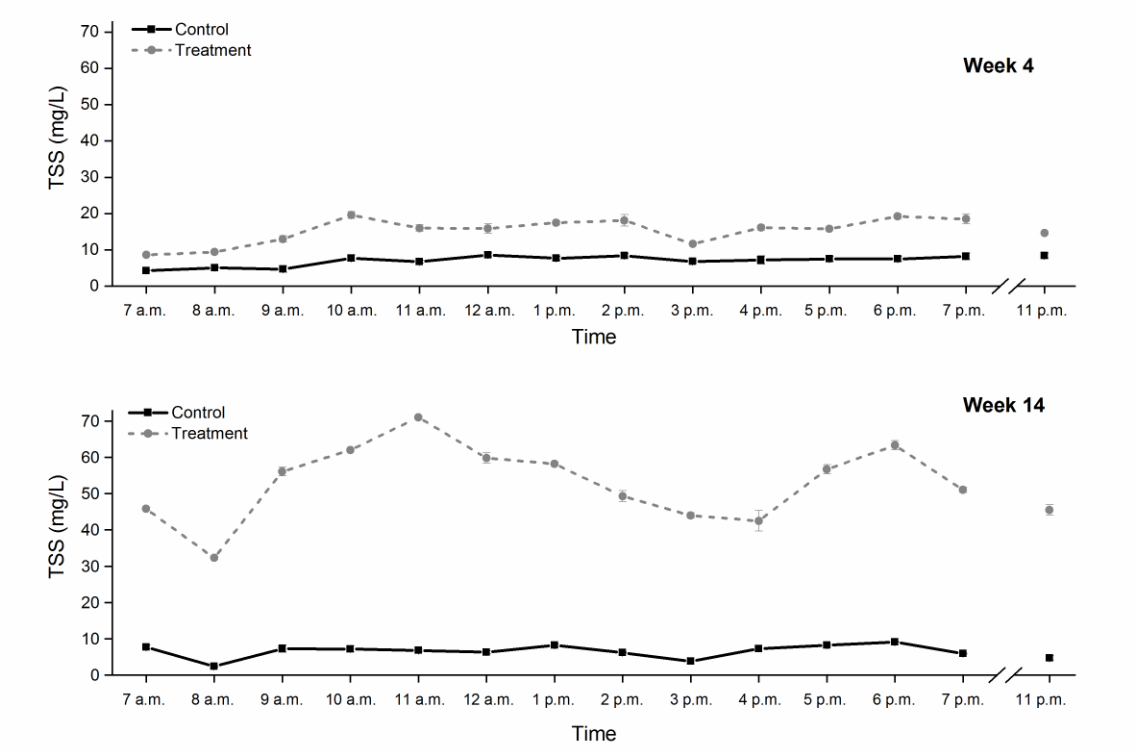

Figure 5.4: Daily variation in TSS concentration (mean ± S.D.) in control and treatment RAS in week 4 and week 14. Samples were collected every hour between 7 a.m. and 7 p.m. and at 11 p.m. Please note the axis break on the x-axis.

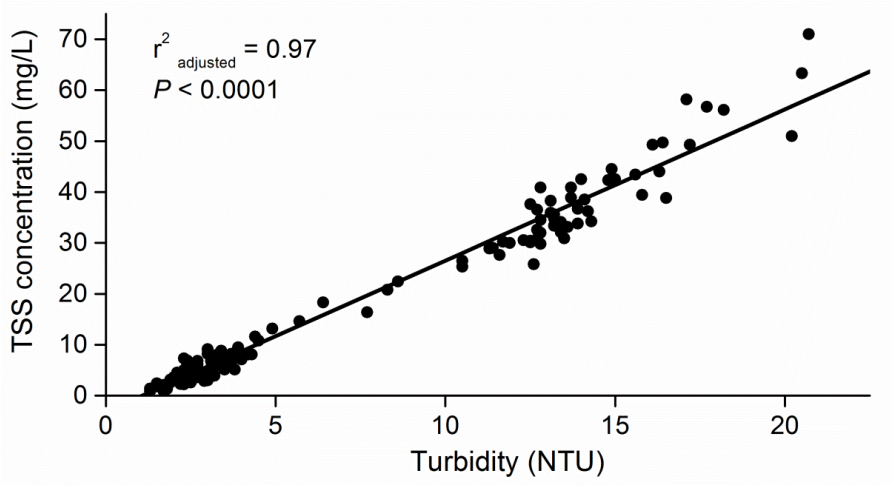

Figure 5.5: Linear relation between TSS concentration (mg L^{-1}) and turbidity (NTU).

Particle size distribution

The absolute frequencies of particle classes in the control and treatment systems are shown in Figure 5.6. The average suspended particle load was 4.3 ± 1.6 mg DW L^{-1} in the control and 42.5 ± 2.1 mg DW L^{-1} in the treatment system. On average, the total number of particles per liter was more than three-fold greater in the treatment RAS than in the control. With the exception of the 30 – 50 µm particle class ($P > 0.05$), the treatment RAS contained a significantly greater number of particles of all size classes ($P < 0.05$ and $P < 0.01$) than the control system. However, large accumulations of fine particles occurred in both systems, with 98.3 % and 97.6 % of all particles smaller than 15 µm in the treatment and control systems respectively. The concentration of particles smaller than 30 µm amounted to 0.7 ± 0.2 mg L^{-1} in the control system and 6.8 ± 0.7 mg L^{-1} in the treatment system, a differential of almost tenfold.

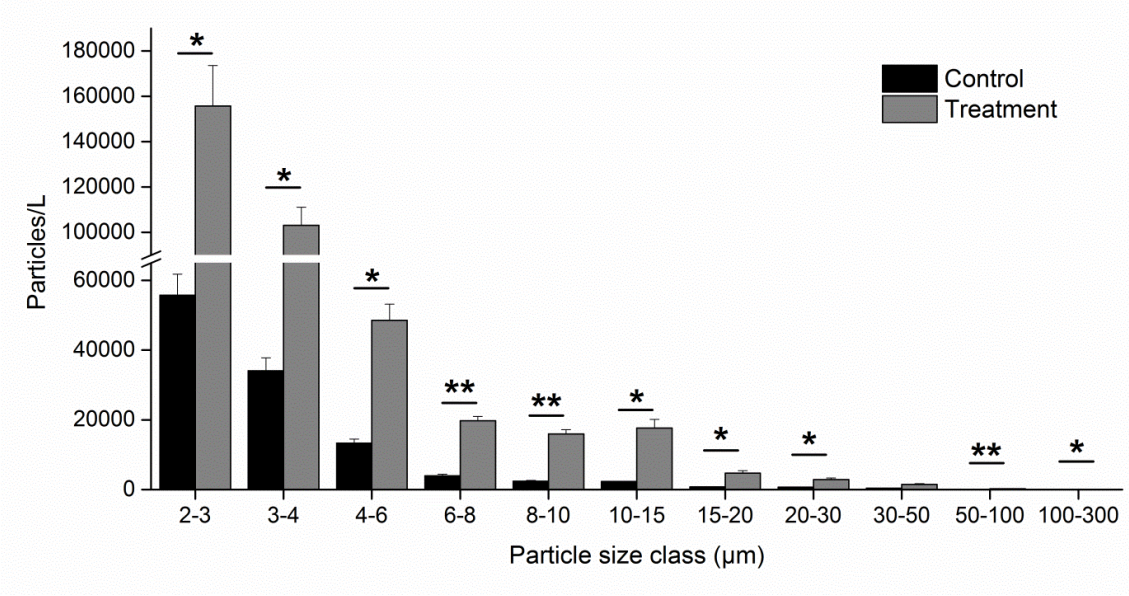

Figure 5.6: Absolute concentrations within particle classes (mean ± S.E.) of the control and treatment RAS in week 18 of the study. Suspended particle load was 4.3 ± 1.6 mg DW L^{-1} in the control and 42.5 ± 2.1 mg DW L^{-1} in the treatment system. Asterisks indicate significant differences between groups (**P < 0.01, * P < 0.05). Please note the axis break on the y-axis.

Fish performance

Overall, fish performed well in both systems as evident from low mortalities and high growth rates (Table 5.2). No significant differences (P > 0.05) were apparent in the survival, final weight, final total length, weight gain, FCR, TGC, SGR or dry matter digestibility values between the control and treatment RAS. Overall, the average mortality was 3.1 ± 1.3 and 5.6 ± 3.0 fish per tank in the control and treatment system respectively. The exception was feeding behavior, which differed marginally between groups. It was observed that fish in the treatment RAS exhibited calmer and less aggressive feeding behavior, and a noticeable delay was observed in feed intake compared with the control group.

Table 5.2: Final weight, wet weight gain, final total length, survival, feed conversion ratio (FCR), thermal growth coefficient (TGC), specific growth rate per day (SGR), and dry matter digestibility (mean ± S.D.) as determined in treatment and control RAS.

	Control	Treatment	Statistical Significance
Final weight (g)	448.9 ± 104.5	450.6 ± 98.4	n.s.
Wet weight gain (g fish^{-1})	362.0 ± 11.6	364.5 ± 13.5	n.s.
Final total length (cm)	30.8 ± 2.4	31.0 ± 2.3	n.s.
Survival (%)	94.9 ± 2.1	90.8 ± 4.9	n.s.
FCR	0.92 ± 0.02	0.91 ± 0.02	n.s.
TGC	1.81 ± 0.03	1.84 ± 0.04	n.s.
SGR (% day^{-1})	1.31 ± 0.02	1.32 ± 0.02	n.s.
Dry matter digestibility (%)	84.5 ± 0.3	83.8 ± 0.8	n.s.

3.4. Health parameters

Gill histology

Severe changes in gill structure including cellular edema, tip-thickening of secondary lamellae and telangiectasia (Figure 2.7) were observed in 2 out of 20 control fish and 1 out of 20 treatment fish by week 10 (Figure 5.7). Cases of thickening of epithelial cells, cell infiltration, lamellar fusion, detachment of the epithelium and merging of secondary lamellae were minor or moderate. Thickening of epithelial cells did not differ significantly between fish from the control and treatment RAS ($P > 0.05$) at either the beginning or at the end of the experiment. However, in the second sampling, thickening of epithelial cells appeared more pronounced ($P < 0.05$) in gills of fish from the control system. No other significant differences in gill histology were observed for any parameter ($P > 0.05$) at any time.

Thickness of the branchial epithelium did not differ significantly ($P > 0.05$) between fish from the control and treatment systems (Table 5.3) at any time, and there was no significant difference in the number of goblet cells per secondary lamella ($P > 0.05$) at the beginning of the study or at week 10. By the end of the study however, the number of goblet cells was significantly elevated ($P < 0.01$) in gills of fish from the treatment system compared to tissue from fish in the control system (Table 5.3).

Table 5.3: Thickness of branchial epithelium and number of goblet cells per secondary lamella (mean ± S.D.) as determined in fish of the control and treatment RAS. In each case, gills were examined from 20 rainbow trout per RAS.

Parameter	Time	Control	Treatment	Statistical Significance
Thickness of branchial epithelium (mean ± S.D.)	Start	6.0 ± 1.6	5.7 ± 1,3	n.s.
	Week 10	5.9 ± 2.0	5.9 ± 1.6	n.s.
	Week 17	4.7 ± 1.8	4.6 ± 1.5	n.s.
Number of goblet cells per secondary lamella (mean ± S.D.)	Start	0.8 ± 0.3	0.9 ± 0.5	n.s.
	Week 10	1.3 ± 0.5	1.3 ± 0.5	n.s.
	Week 17	1.1 ± 0.4	1.9 ± 1.1	$P < 0.01$

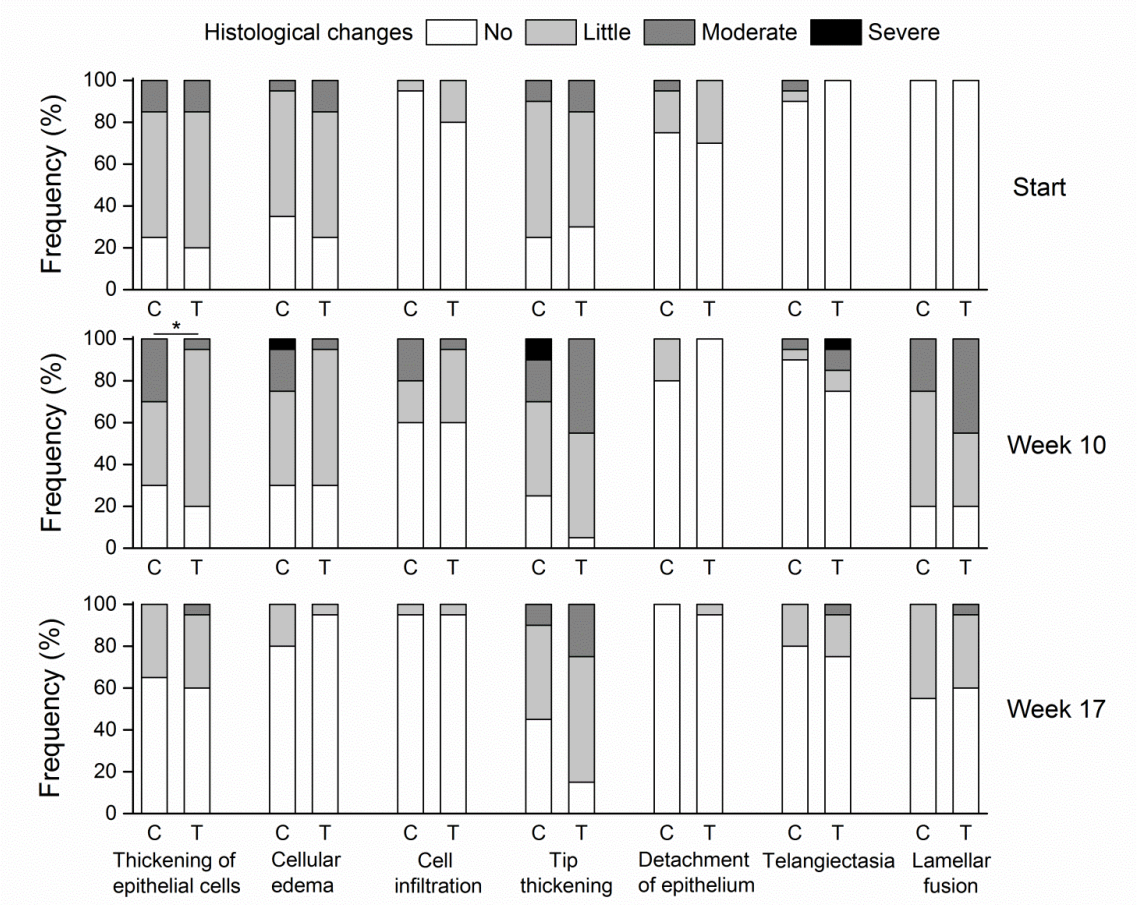

Figure 5.7: Histological changes in gill tissues observed at the beginning of the investigation period, at week 10 and at week 17. In each case, histological changes were sought in gills of 20 rainbow trout from each RAS. C = control group; T = treatment group. * Indicates statistical difference ($P < 0.05$) between groups.

Fin condition

Fin erosion (Figure 5.8) did not differ significantly ($P > 0.05$) between groups at the start of the study or in week 10. The counterclockwise current in both RAS and the resulting swimming orientation of fish lead to higher levels of fin erosion in the right pectoral fin compared to the left, in both groups. In week 17 of the study, fin erosion of the left pectoral fin was significantly higher ($P < 0.05$) in fish from the control group than for individuals from the treatment group. Erosion levels exhibited by the left pectoral and dorsal fins were not significantly different ($P > 0.05$).

No significant differences in fin index were observed between systems ($P > 0.05$) at the beginning of the study or in week 17 of the experiment (Table 5.4). In week 10, however, while no significant system differences ($P > 0.05$) were observed in the fin indices of the right or left pectoral fins the fin index of the dorsal fin was significantly higher ($P < 0.05$) for fish in the treatment RAS.

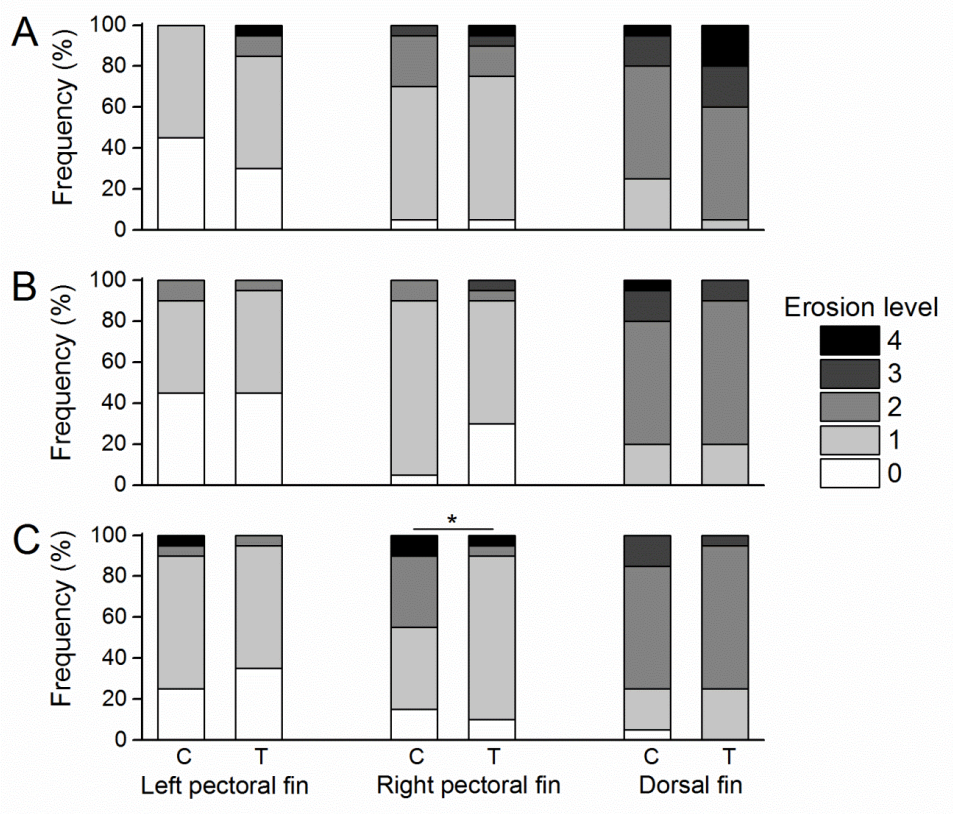

Figure 5.8: Percentages (%) of fin erosion determined according to Person-Le Ruyet et al. (2007) at the beginning of the study (A), in week 10 (B) and in week 17 (C). In each case, fin erosion was evaluated in 20 randomly sampled rainbow trout. C = control group; T = treatment group. * Indicates statistical difference ($P < 0.05$) between groups.

Table 5.4: Fin index (mean ± SD) according to Kindschi (1987)

	Start		Week 10		Week 17	
	Control	Treatment	Control	Treatment	Control	Treatment
n	20	20	20	20	20	20
Dorsal fin	7.5 ± 1.4	6.9 ± 1.4	7.7 ± 0.9*	8.4 ± 0.8*	8.5 ± 1.0	8.9 ± 0.7
Right pectoral fin	10.2 ± 1.1	10.2 ± 0.8	10.5 ± 0.7	10.6 ± 0.8	9.6 ± 1.8	10.4 ± 1.4
Left pectoral fin	11.1 ± 0.5	10.8 ± 0.9	10.9 ± 0.8	11.1 ± 0.7	10,3 ± 1.4	10.9 ± 1.0

* = significant difference ($P < 0.05$) between control and treatment group.

Hematology

At the beginning of the experiment, no significant differences ($P > 0.05$) were found in hematological parameters between fish of the control and treatment RAS (Table 5.5). In week 10 of the experiment, leukocrit and the number of leukocytes were significantly lower ($P < 0.05$ and $P < 0.001$ respectively) in fish of the treatment RAS than in fish from the control group. Furthermore, the hemoglobin concentration was significantly lower ($P < 0.05$) in the control group. No significant differences were observed in any other examined blood parameters ($P > 0.05$).

In week 17 of the experiment, MCHC was significantly higher ($P < 0.001$) in the treatment group, whereas the MCV-value was significantly higher ($P < 0.001$) in the control group. There were no significant differences in any other parameters ($P > 0.05$).

Table 5.5: Hematological parameters of rainbow trout determined at the beginning, middle and at the end stages of the study. In each case, hematological parameters were investigated for 20 randomly sampled rainbow trout from each RAS.

	Start		Week 10		Week 17	
	Control	Treatment	Control	Treatment	Control	Treatment
Erythrocytes (10^6 μL^{-1})	1.07 ± 0.16	1.04 ± 0.14	1.01 ± 0.12	1.08 ± 0.18	1.00 ± 0.18	1.04 ± 0.09
Thrombocytes (10^4 μL^{-1})	1.21 ± 0.41	1.24 ± 0.37	1.21 ± 0.39	0.97 ± 0.27	1.61 ± 0.54	1.46 ± 0.37
Leukocytes (10^4 μL^{-1})	2.18 ± 0.71	2.09 ± 0.70	2.54 ± 0.63**	1.72 ± 0.52**	2.64 ± 0.75	2.64 ± 0.97
Lymphocytes (%)	94.3 ± 4.2	94.4 ± 4.8	94.7 ± 2.5	94.7 ± 2.5	91.8 ± 4.3	93.5 ± 5.3
Granulocytes (%)	3.9 ± 3.7	3.1 ± 2.6	4.1 ± 2.2	3.5 ± 1.6	6.2 ± 2.6	4.7 ± 4.2
Monocytes (%)	1.8 ± 1.8	2.5 ± 3.3	1.3 ± 0.9	1.8 ± 1.2	2.1 ± 2.9	1.9 ± 1.6
Hematocrit	34.5 ± 3.9	31.7 ± 3.1	32.5 ± 3.3	33.0 ± 3.5	32.4 ± 4.3	30.7 ± 2.4
Leukocrit	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.2*	0.9 ± 0.1*	1.1 ± 0.2	1.1 ± 0.2
Glucose	40.6 ± 7.5	43.3 ± 8.4	55.6 ± 9.7	55.4 ± 12.0	60.9 ± 7.9	59.0 ± 6.7
Hemoglobin	9.8 ± 1.6	9.3 ± 1.0	9.3 ± 0.9*	10.0 ± 1.4*	9.6 ± 1.2	9.7 ± 0.8
MCH (pg)	93.4 ± 20.7	90.4 ± 14.7	92.3 ± 10.4	93.8 ± 12.6	97.8 ± 9.9	93.6 ± 7.4
MCHC (g dL^{-1})	28.3 ± 4.6	29.2 ± 3.4	28.6 ± 2.1	30.4 ± 2.7	29.8 ± 1.5**	31.8 ± 1.7**
MCV (fL)	338.1 ± 41.8	308.3 ± 32.0	324.2 ± 38.9	308.1± 26.5	328.7 ± 27.8**	294.9 ± 22.9**

MCH = mean corpuscular hemoglobin; MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration.

* = significant difference ($P < 0.05$) between groups.

** = significant difference ($P < 0.001$) between groups.

Heat shock protein 70

Analysis of Hsp70 concentrations (Figure 5.9) in all examined tissues revealed no significant differences ($P > 0.05$) between control and treatment fish. In both groups, Hsp70 levels were distinctly elevated in the liver compared to the other tissues.

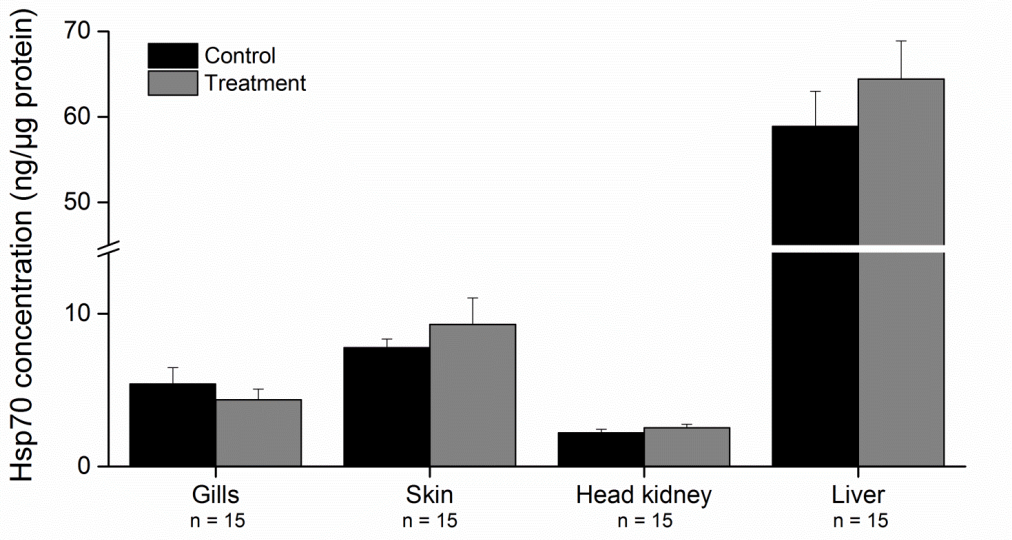

Figure 5.9: Hsp70-concentrations (mean ± S.E.) in gill, skin, head kidney and liver tissues of the control and treatment groups. 'n' represents sample size of each group. No significant differences ($P > 0.05$) were found between groups. Please note the axis break on the y-axis.

Bacterial load

Overall, the bacterial load of system water was higher in the treatment RAS than in the control system (Figure 5.10). However, the difference was only significant ($P < 0.05$) after UV irradiation. In both systems, a reduction in bacterial load was observed after both drum filtration and UV treatment.

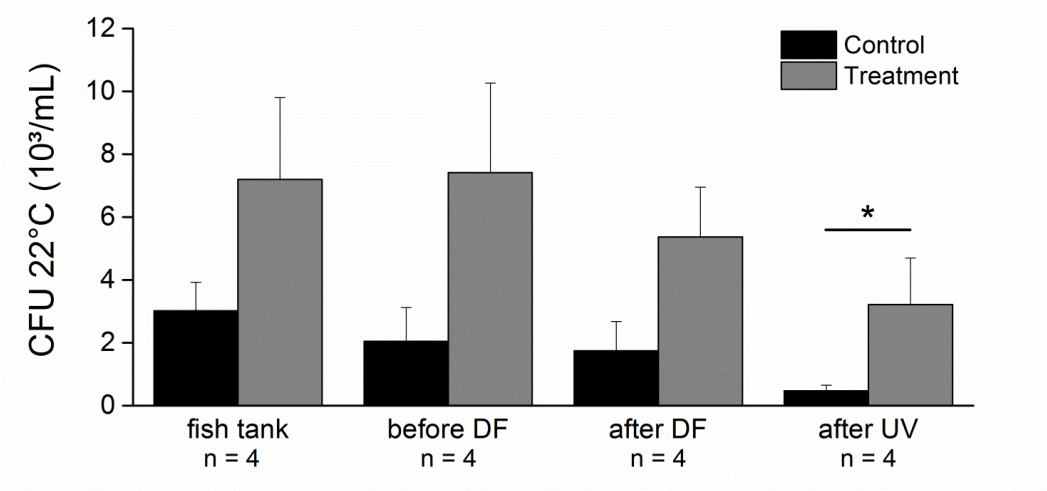

Figure 5.10: Bacterial load of the water samples at different sample points in the control and treatment RAS. DF = drum filter; UV = UV irradiation. 'n' represents sample size of each group. Asterisk indicates significant difference between groups ($P < 0.05$).

In week 10 of the experiment there was no significant difference ($P > 0.05$) in bacterial colonization observed on the gills of either group (Figure 5.11 A). The fish pathogen *Flavobacterium columnare* was directly detected on the gills of one individual in the treatment group, and smear cultures of gill tissues revealed *Flavobacterium sp.* on two fish from the control group and three fish from the treatment system. *Aeromonas sobria* was detected on the gills of one fish from the control system and *Aeromonas hydrophila* was detected on the gills of one individual from the treatment system. *F. columnare* was detected on the skin of one fish from the treatment RAS, but not on any control fish. Bacterial analysis of spleen samples revealed *Deefgea rivuli* in one fish from the control group.

In week 17 of the study, the bacterial load on the gills of trout in the treatment RAS was significantly higher ($P < 0.05$) than in the control group (Figure 5.11 B). *F. columnare* was directly detected on the gills of 30 % of fish in the treatment RAS and on 5 % of control fish, but the difference between groups for this bacterium was not significant ($P > 0.05$). *F. columnare* was also found on the skin of 20 % of the trout in the treatment system and 5 % of fish in the control system, but again, this difference was not significant ($P > 0.05$). Analyses of cultures and crush preparations of spleen

tissues revealed a slight bacterial load in one control fish and two individuals from the treatment group. No fish in either system were found to carry a severe bacterial load.

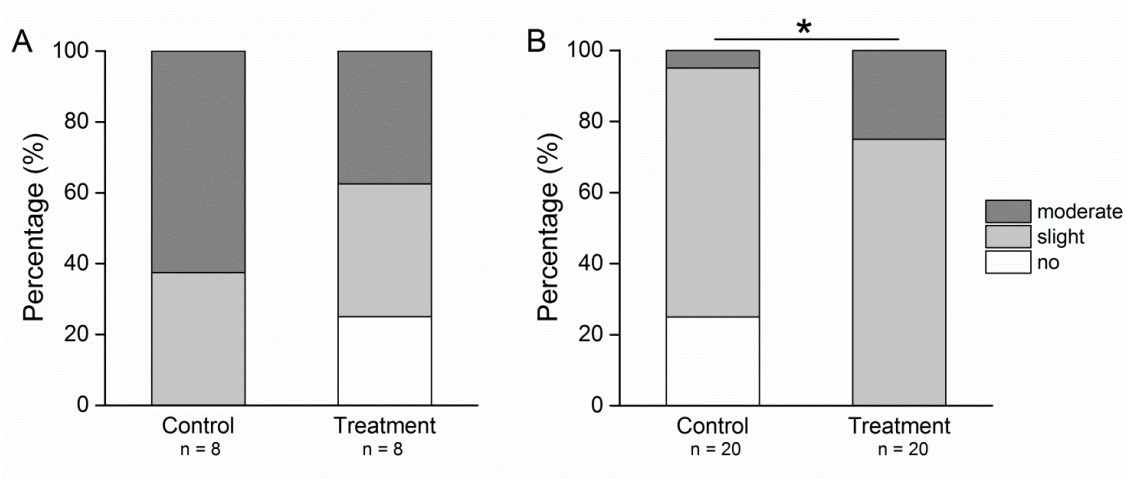

Figure 5.11: Bacterial load of rainbow trout gills of the control and treatment RAS in week 10 (A) and week 17 (B) of the experiment. Asterisk indicates significant difference ($P < 0.05$) between groups.

5.5 Discussion

This study decoupled the effects of chronic suspended solid load from relevant water quality parameters according to Timmons and Ebeling (2010), allowing a first investigation of particle-related effects in isolation. Contrary to our hypotheses and general perceptions in the literature and the aquaculture community (e.g. Bilotta and Brazier, 2008; Chen and Malone, 1991; Timmons and Ebeling, 2010), chronic exposure to elevated concentrations of fine particles in a RAS, at levels beyond those realistically expected in normal aquacultural operations, had no apparent effect on rainbow trout in the present study. Despite realized particle concentrations ranging from a minimum of 30 mg L^{-1} to repeated peaks of up to 70 mg L^{-1}, and comprising 97.6 % fines of less than 15 µm, none of the anticipated effects, including damage to gill structure (Au et al., 2004; Bash et al., 2001; Bilotta and Brazier, 2008; Bruton, 1985; Chapman et al., 1987; Wong et al., 2013) or elevated stress indicators (Lake and Hinch, 1999; Sutherland et al., 2008; Awata et al.,2011) were raised. The results suggest not only that assumptions regarding the impact of suspended solids on the health of rainbow

trout in RAS need to be revised, but furthermore, that suspended solids may not be of primary importance in fish welfare (Becke et al., 2017). Indeed, it may be that other water parameters are responsible for detrimental effects previously assigned to fine particles. Assumptions regarding the damage potential of suspended solids in RAS may have been confounded by the effects of other water quality parameters (such as TAN), which generally accompany increased solid load. Such misconceptions may partly stem from observed impairments of fish in natural waters, although the comparability of natural and aquaculture condition is limited. First of all, due to their almost exclusively organic origin, particles in RAS are mainly smooth, with a density close to that of water at approximately 1.010 to 1.153 g cm^{-3} (Unger and Brinker, 2013). In contrast, suspended solids in natural systems are mostly of inorganic origin, often sharp and with a heavy, solid composition, that poses a greater risk of mechanical damage, e.g. to gills (Au et al., 2004; Bash et al., 2001; Bruton, 1985; Kemp et al., 2011; Newcomb and Flagg, 1982). Furthermore, in natural systems, the concentration and size distribution of particles in surface waters is determined by irregular events such as heavy rain, floods and snowmelt (Asselman, 1999; Langlois et al., 2005; Lenzi and Marchi, 2000), whereas the solid load and size distribution of suspended solids in RAS is almost exclusively dependent on feeding regime, feed composition and system properties (Brinker et al., 2005a; Unger and Brinker, 2013). The suspended solids in aquaculture systems comprise mainly particles smaller than 20 µm (Becke et al., 2017; Chen et al., 1993), while the particle size of sediments suspended in rivers varies more widely, e.g. from more than 80 % smaller than 2 µm to more than 60 % greater than 63 µm (Walling and Moorehead, 1989). Clearly, the extrapolation of results concerning at least the mechanical effects of solids on fish health from natural waters to aquaculture systems is not valid.

In terms of particle load, conditions in the experimental treatment systems reflected those generally considered to be detrimental for fish health and performance in aquaculture (Bilotta and Brazier, 2008; Chapman et al., 1987; Chen and Malone, 1991; Cripps and Bergheim, 2000; Davidson et al., 2009; Masser et al., 1999). TSS in the treatment RAS consisted almost solely of fines and by week 10 exceeded the upper safe limit of 25 mg L^{-1} proposed by numerous authors (Alabaster and Lloyd, 1982;

Bilotta and Brazier, 2008; Timmons and Ebeling, 2010) and remained consistently above 30 mg L^{-1} thereafter. Repeated peak TSS concentrations of more than 70 mg L^{-1} were recorded in the treatment RAS, and the lack of any obvious effect on the experimental fish suggests that rainbow trout are very tolerant to this potential stressor. In both systems, particles smaller than 15 µm, widely regarded as most harmful (Chapman et al., 1987; Chen et al., 1993; Chen and Malone, 1991), accounted for more than 97 % of all particles and were ten times more concentrated in the treatment RAS. TSS levels were always determined in the morning and further randomly selected diurnal TSS measurements confirmed that this timeslot represented the minimum particle load on each day, before settled material was agitated by feeding activity.

As a consequence of the massive accumulation of fine particles, it was hypothesized that water quality within the RAS would decline, due to clogging of biofilters and/or leaching of harmful substances (e.g. ammonia, nitrite) from the solid fraction (Chen et al., 2003; Ling and Chen, 2005). Thus, the most important precondition of this study was the isolation of particle accumulation effects from other potentially confounding factors. This decoupling of confounding water parameters was achieved using a biofilter designed to compensate efficiently for solid-related additional load, like degradable organics (*cf*. Becke et al 2017 for details). For example, in most biofilters, heterotrophic bacteria will outcompete the nitrifying bacteria for oxygen when large amounts of organic carbon are present (Michaud et al., 2006) The high capacity biofilters deployed in the experimental setup meant that with the exception of turbidity, water parameters were maintained within previously established industry limits for fish health (MacIntyre, 2008; Timmons and Ebeling, 2010), irrespective of solid load. However, accumulation of suspended solids may lead to implications beyond fish health, for example particle deposits can clog tubes and the efficiency of biofilters can be reduced so that production of hydrogen sulfide (Masser et al., 1999; Reiffenstein et al., 1992) or off-flavor compounds (Auffret et al., 2013; Guttman and van Rijn, 2008; Houle et al., 2011) might occur.

Having controlled for the indirect effects of increased particle load, it was expected that the increased concentration of fines realized in the treatment RAS would lead

directly to decreased fish performance. Overall, however, feed performance and survival in both systems indicated good husbandry conditions. Growth was unaffected by TSS treatment and fairly good with a TGC comparable to other high-performance studies (Bureau et al., 2006; Cho, 1992). This is in line with the results of Fernandes et al. (2015) who observed no improvement in rainbow trout performance when microscreen filters were deployed in RASs to significantly reduce solid load.

Increasing solid load did generate severe increases in turbidity in the present study. Turbidity is known to affect feeding behavior by reducing feeding rates, reaction distance, prey selection and foraging success, all of which can be attributed to reduced visibility (Bash et al., 2001). In the current study, increased turbidity in the treatment RAS was associated with calmer and less aggressive feeding behavior. Furthermore, fish in the TSS enriched treatment RAS exhibited a delay in feed intake. This delay could potentially lead to a significant loss of feed in experimental or commercial settings using automatic feeders, and thus to solid-induced performance losses, when in reality, feeding was simply not able to compensate for the turbidity-changed behavior of the fish.

Increased turbidity also implies a corresponding reduction in light intensity and visibility in deeper areas of fish tanks was likely close to zero. Rainbow trout are visual predators (Barrett et al., 1992; Bash et al., 2001), so foraging success is reduced by turbidity-related reductions in light levels (Barrett et al., 1992; Utne-Palm, 2002). The same is true for lake trout (*Salvelinus namaycush*) (Vogel and Beauchamp, 1999). In the present study, however, fish were carefully hand fed so that both groups took comparably large amounts of feed, and the impact of turbidity was balanced.

In general, recirculating aquaculture systems are not considered detrimental to fish welfare when operated under optimal water conditions (Colson et al., 2015), but it was anticipated that chronic exposure to high loads of almost exclusively fine particles over a whole growout period would significantly impair physiological and health condition. However, no such adverse effects were observed at any point in the present study and even direct, long term exposure of the delicate and sensitive gill lamellae to increased particle load yielded no histologically visible negative effects. After a slight worsening of the gill structures in both control and treatment groups in the middle of the study

period, recovery was apparent towards the end of the experiment in fish from both systems. This clearly shows that far from being exhibiting cumulative negative effects of increased particle load, fish in the treatment system were able to recover from slight damage in the prevailing conditions. As already observed by Becke et al. (2017), the minor histological changes in gill structures of rainbow trout from the treatment RAS were less serious than those seen in fish from the control system. Experimental animals were euthanized by cranial percussion, which might lead to increased blood pressure in the gills, in turn potentially resulting in telangiectasia (Wolf et al., 2015). However, telangiectasia did not occur in a recent investigation (Becke et al., 2017), performing the same protocol. In the present study, this lesion type was observed in a low prevalence on fish from both treatment groups. Thus, its presence might be related to the euthanasia method rather than any factors during the husbandry of the rainbow trout.

In a previous study, Michel et al. (2013) showed that rainbow trout would tolerate short-duration pulses of suspended sediments from natural deposits in concentrations up to 5000 mg L^{-1}, (more than two orders of magnitude greater than maximum levels occurring in salmonid farming), without suffering histological damage in gills, liver, spleen or kidney. Goldes et al. (1988) did also not observe any branchial pathology in rainbow trout exposed to up to 1017 mg L^{-1} suspended clay kaolin. Gill lesions were found in fish exposed to 4887 mg L^{-1} kaolin, but this was likely due to protozoan infection (*Ichthyobodo necator*) and not directly caused by increased kaolin concentration. However, Goldes et al. (1988) assumed that the increased suspended kaolin levels were indirectly responsible for protozoan colonization by creating a favorable environment. These missing direct effects of suspended solids tally with results of the present study and suggest that the tolerance of wild trout to pulses of extremely high particle loads occurring in natural systems, e.g. following snowmelts or floods (Asselman, 1999; Langlois et al., 2005; Lenzi and Marchi, 2000) has persisted in the course of artificial selection for aquaculture. In fish gills, goblet cells produce mucus to protect delicate structures from abrasion and other external influences. Under stress, the excretion of mucus is elevated as protection against adverse environmental conditions (Shephard, 1994). Goldes et al. (1988) ascribed the recovery

of gill structures and reduction of protozoa after exposure to increased kaolin concentrations, among other things, to increased mucus secretion. In the present study, the larger numbers of goblet cells in gills from rainbow trout in the treatment RAS probably indicate an adaption to cope with the increased particle load. This adaption comes at a physiological cost, but no apparent loss of performance in a commercial context.

Fin erosion is considered a valid parameter for assessing fish welfare (Ellis, 2002; Turnbull et al., 2005) and the absence of any deterioration in fin condition in the current study despite TSS levels exceeding those generally permitted in aquacultural operations is thus remarkable. Arguably, the fin condition of rainbow trout in the solid enriched RAS was better than in the control RAS. At the end of the experiment, greater erosion was observed in the right pectoral fins of trout in the control system than in the treatment RAS, probably as a result of the calmer behavior and reduced interaction of fish under turbid conditions. This is in line with other research demonstrating altered social behavior and reduced aggression in fish in turbid water (Bash et al., 2001). In this context, and given the lack of evidence for other negative effects, increased turbidity in the treatment system could be regarded as beneficial rather than detrimental to fish welfare.

The absence of negative impacts of fine particles on trout was corroborated by Hsp70 levels, which showed no differences between fish of the control and treatment systems. The Hsp70 group is a widely distributed collection of heat shock proteins, known to be markedly elevated in response to stress (Roberts et al., 2010). Up-regulation of Hsp production serves to maintain cellular homeostasis (Yamashita et al., 2010) under conditions such as exposure to environmental contaminants (Vijayan et al., 1998; Williams et al., 1996). The Hsp70 data from the present study suggests that fish remained in an unstressed state in spite of chronic exposure to increased fine particle load. Furthermore, while the analysis of hematological parameters revealed significant differences between control and treatment RAS, the magnitudes of differences remained within the optimal range for the species, with no severe alterations. Taking all hematological and physiological data together, there is no evidence of physiological stress that can be linked to the elevated suspended solid load

in the treatment RAS. Considered alongside the recent short-term exposure study of Becke et al. (2017) these results strengthen the conclusion that the intrinsic threat to fish health from RAS-related particles is low to non-existent.

The only appreciable effect of increased suspended solid load observed in the study was on bacterial load, which was significantly higher in the treatment RAS, both in the system water and on the gills of the rainbow trout. This was expected, given that particles are an important substrate for bacterial colonization (Kirchman and Ducklow, 1987) offering both growing space and nutrient resources. Berger et al. (1996) observed that the number of attached bacteria per unit particle area decreased with increasing particle size, whereas the total number of bacteria per particle increased with increasing particle size. The increased number of particles in the treatment RAS promoted bacterial growth by offering a larger surface area and food-substrate (Pedersen et al., 2017). The UV irradiation applied in the present experiment was similar to that used in commercial operations, and easily compensated for this increased bacterial load, resulting in levels comparable to those seen in recirculating salmonid culture systems (Davidson et al., 2013; Sharrer et al., 2005) and in an experimental recirculating system used to rear sea bass (Leonard et al., 2000). Overall, the impact of the observed bacterial load in the treatment RAS can be considered small, a conclusion supported by the other physiological parameters examined in the present study, including Hsp70 concentration and differential blood count, which revealed no evidence for bacterially mediated physiological stress responses in either the control or the treatment systems.

5.6 Conclusions

This experiment was successful in uncoupling the effects of suspended particle exposure from potentially confounding water parameters that accompany increases in solid load. Thereby, the effects of chronically increased particle load on fish physiology and performance could be observed in isolation for the first time. A wide range of indicators of rainbow trout performance and physiology were examined, of which none revealed any solid-related negative effects over a whole growth period. The results corroborate previous findings relating to more acute exposure to increased

particle load (Becke et al., 2017). The results are more striking given that the solid fraction of the experimental system comprised almost exclusively fine particles at concentrations beyond any normally reached in aquacultural production, and still failed to provoke any detrimental effects on physiology and performance. Of the examined fish parameters, only bacterial load was elevated by the increase in TSS concentration, but without any apparent impact on physiology. Thus, contrary to previous assumptions, the present study provides strong evidence that even chronic exposure to exceptionally high fine particle loads does not negatively influence the health or performance of rainbow trout in RAS, provided accompanying water parameters remain within noncritical limits for salmonids. Overall, this suggests that suspended solids are not the key issue affecting fish welfare in RAS, but interaction effects, e.g. with ammonia, are not precluded.

6 Effects of unionized ammonia and suspended solids on rainbow trout (*Oncorhynchus mykiss*) in recirculating aquaculture systems

A similar version of this chapter was published as:
Becke, C., Schumann, M., Steinhagen, D., Rojas-Tirado, P., Geist, J., Brinker, A. (2019): Effects of unionized ammonia and suspended solids on rainbow trout (*Oncorhynchus mykiss*) in recirculating aquaculture systems. Aquaculture 499, 348–357.

6.1 Abstract

This study investigates the individual and combined effects of chronic exposure of rainbow trout to unionized ammonia and suspended solids in a farm-scale recirculating aquaculture system (RAS) over 13 weeks. Unionized ammonia nitrogen concentration was four times (0.05 mg L^{-1}) the generally accepted 'safe' threshold while total suspended solids (TSS) exceeded the 'safe' threshold of 25 mg L^{-1} by a factor of > 2.5. Still, rainbow trout revealed high survival rates of > 99% and no observable detrimental effects of TSS. Bacterial activity showed a close positive linear correlation with solid load and was almost exclusively explained by solid load for TSS concentration > 10 mg L^{-1}. However, bacterial activity had no apparent detrimental effect on fish health or performance. Increased unionized ammonia nitrogen concentrations had no relevant detrimental effect on rainbow trout physiology and performance at concentrations of up to 0.05 mg L^{-1}. Furthermore, the absent to minor solid-related effects across a wide range of physiological criteria combined with chronic exposure to unionized ammonia demonstrates that chemical or physical irritants are not problematic in RAS if other water and holding parameters are optimal. These findings suggest a greater than expected tolerance of rainbow trout to chronic TSS-related effects which should result in a revision of water quality threshold criteria for RAS.

Candidate's contribution:

Implementing and maintaining increased suspended solid and unionized ammonia concentrations, husbandry of fish, sampling of blood and tissue samples, execution of physiological assays (except gill histology) and particle measurements, measurement of bacterial load and bacterial activity in water, statistical assessment and interpretation of data, writing and revision of the complete manuscript including all figures and tables.

6.2 Introduction

Aquaculture is the fastest-growing sector in the animal food production industry worldwide and already accounts for more than 44 percent of global total fish production (FAO, 2016). As capture fishery production has remained relatively static since the late 1980s and the world demand for fish is increasing (FAO, 2016), aquaculture has an important role to play in ensuring a sufficient global fish supply (Naylor et al., 2000). Recirculating aquaculture systems (RAS) are often regarded as an environmentally friendly alternative to open flow-through or cage-based aquaculture systems (Ayer and Tyedmers, 2009; Klinger and Naylor, 2012; Verdegem et al., 2006), largely due to their efficient water use. However, despite ongoing development, fish production in RASs remains energy- and cost-intensive and its contribution to global production is still small (Badiola et al., 2012; d'Orbcastel et al., 2009c). One approach to optimizing the economic output of RASs is to increase stocking densities to reduce costs per unit of fish produced (Martins et al., 2005). However, more fish reared in the same volume of water leads to increased excretion loads per m^3 of water. Fish feces are the principal constituent of suspended solids in aquacultural facilities along with uneaten feed, bacterial material from biofilters and microfauna (Chen and Malone, 1991; Noble and Summerfelt, 1996; Summerfelt et al., 1999; Wedemeyer, 1996). Accumulating particles, and especially fine particles, are considered detrimental to fish health, welfare and performance (Bilotta and Brazier, 2008; Chapman et al., 1987; Chen et al., 1993; Chen and Malone, 1991; Herbert and Merkens, 1961). However, this assertion has been questioned for rainbow trout by recent investigations (Becke et al.,

2018, 2017). Nevertheless, intensification of aquacultural production resulting in an increase in suspended solid concentration, will also lead to an increase in dissolved wastes, such as unionized ammonia (NH_3) (Ip et al., 2001).

High unionized ammonia levels have a wide range of detrimental effects on fish, e.g. deterioration of gill structures, and might ultimately lead to mortality (Cameron and Heisler, 1983; Daoust and Ferguson, 1984; Ip et al., 2001; Randall and Tsui, 2002; Smart, 1976; Thurston et al., 1984; Wicks et al., 2002). The common upper safe limit of unionized ammonia-N proposed for salmonid aquaculture is 0.0125 mg L^{-1} (Timmons and Ebeling, 2010). However, there are studies reporting higher tolerance (Daoust and Ferguson, 1984; Meade, 1985). Thus, there is still controversy about the safe threshold for unionized ammonia in aquaculture operations.

A recent factor significantly influencing water quality in aquaculture is a change in feed composition. Fish meal and fish oil are increasingly being substituted by plant alternatives in salmonid diets (Glencross et al., 2007; Ytrestøyl et al., 2015). This is partly due to declining fish stocks and rising prices for fish meal and fish oil (Naylor et al., 2009). This replacement coincidently causes a less dense and more fragile composition of fish feces (Schumann et al., 2018; Unger and Brinker, 2013), considerably increasing fine suspended solids in fish farm waters (Brinker and Friedrich, 2012).

Against this background, the present study investigated the sole effect of critical unionized ammonia-N concentrations (> 0.0125 mg L^{-1}) as well as interaction effects with suspended solid load in a farm-scale RAS. It was hypothesized that chronic exposure to high unionized ammonia concentrations would cause a reduction of fish wellbeing, while the combined chronic exposure with increased suspended solid load would provoke an interactive impact. Within this context, husbandry waters were set to optimal values except for the two variables, unionized ammonia and suspended solids, being tested. The exception was bacterial activity which was held at an uncritical level (Pedersen et al., 2017; Rojas-Tirado et al., 2018), with possible covariate influences being controlled by the experimental design.

6.3 Materials and Methods

Husbandry

The experiment used two replicate RASs, each with 10 tanks (capacity of 330 L, total RAS volume 6m^3) (Figure 2.3), as described by Becke et al. (2018). The study used all-female rainbow trout (*Oncorhynchus mykiss*, Störk strain) to exclude sex-related effects. Each RAS was stocked with 785 rainbow trout with an average initial weight of 87.2 ± 8.6 g (control group) and 87.4 ± 9.2 g (treatment group). They were held at maximum stocking densities of 67.8 ± 3.0 kg m^{-3} (control) and 68.3 ± 2.6 kg m^{-3} (treatment). The control RAS was operated under regular conditions, while the particle load of the treatment RAS was artificially elevated as described in Becke et al. (2018). Briefly, a mud pump (Wilo-EMU KS 8 ES, Dortmund, Germany) was used to pump the backwash water of the drum filter back into the system. In both systems, the drum filter (HDF801-1H, Hydrotech, Vellinge, Sweden) was equipped with a 100 µm gauze, so that particles < 100 µm accumulate over time.

The photoperiod was fixed at 12L:12D with a sigmoidal transition period of 30 min (Lumilux daylight lamps) with different light intensities of 50, 100, 200, 300 and 600 lx in duplicate per system. However, without any significant effect on the results presented (unpublished data). The fish were fed restrictively according to supplier recommendations with a commercial feed (Biomar EFICO Enviro 921, Aarhus C, Denmark), by hand six days a week (Sunday to Friday) at 2.5 % of body weight at the beginning of the trial, declining to 1.3 % by the end (maximum feed amount was 2.84 kg day^{-1} per RAS). Bacterial growth was controlled with UV irradiation of the system water (Barrier L20, Wallace & Tiernan, Günzburg, Germany; UV dose: 40 mJ cm^{-2} flow volume: 6600 L h^{-1}, lamp wattage: 80 W, measurement range UV sensor: 200W m^{-2}). Fish (average weight approx. 15 g) were put into the two RASs three months before the beginning of the experiment to ensure acclimatization.

Water parameters

The experiment was subdivided into three phases (Figure 6.1): in phase 1 (week 1 – 5), water parameters in both RASs were kept at levels known to preclude negative impacts on fish health or performance (Table 6.1). In the treatment RAS, however, the

total suspended solid concentration was increased to over 35 mg L^{-1} and was subsequently held constantly above this value. The control RAS operated under commercial conditions at around 5 mg L^{-1} throughout the experiment. In phase 2 (week 6 – 10), ammonium concentration was artificially elevated in both RASs by adding ammonium chloride (A7012,9025; AppliChem, Darmstadt, Germany). Additionally, biofilter efficiency in both RASs was reduced by halving the volume of carrier material (originally designed for 4.5 kg feed day^{-1}) to attain higher NH_4-N concentrations. Ammonium nitrogen concentration was measured in both RASs every 60 minutes using an automat (AMTAX SC, Hach, Germany). In addition, pH was increased from 7.5 to around 8 in both RASs to increase the proportion of unionized ammonia nitrogen (NH_3-N) to approximately 0.0125 mg L^{-1} (Figure 3). The increase in pH was achieved by adding sodium hydrogen carbonate, dissolved in water, using a peristaltic pump (Concept 420i, Saier Dosiertechnik, Germany). The pH was constantly monitored using OxyGuard pH-probes (Farum, Denmark). The concentration of unionized ammonia-N was calculated based on actual pH and temperature according to Emerson et al. (1975).

In phase 3 (week 11 – 13), the concentration of unionized ammonia-N was further increased to an average of approximately 0.025 mg L^{-1} (Figure 6.2).

NH_4-N, NO_2-N and NO_3-N were chemically determined three times per week throughout the experiment with analysis kits (LCK 304: 0.2 – 2.5 mg L^{-1}; LCK 341: 0.05 – 2 mg L^{-1}; and LCK 339: 1 – 6 mg L^{-1}, Hach, Germany, respectively), using water from the connecting tube from the fish tanks of each RAS. Oxygen concentration (using Oxygen Probes, OxyGuard, Farum, Denmark) and temperature (using Temperature Probes, Oxyguard, Farum, Denmark) were monitored continuously at the outlets of two fish tanks in each system. Carbon dioxide concentrations were determined two times per week in the fish tanks using a portable dissolved CO_2 analyzer (OxyGuard CO_2 Portable, OxyGuard, Farum, Denmark). Turbidity was determined three times per week in parallel with the determination of total suspended solids using a turbidity meter (PCE-TUM 20, PCE Instruments, Germany).

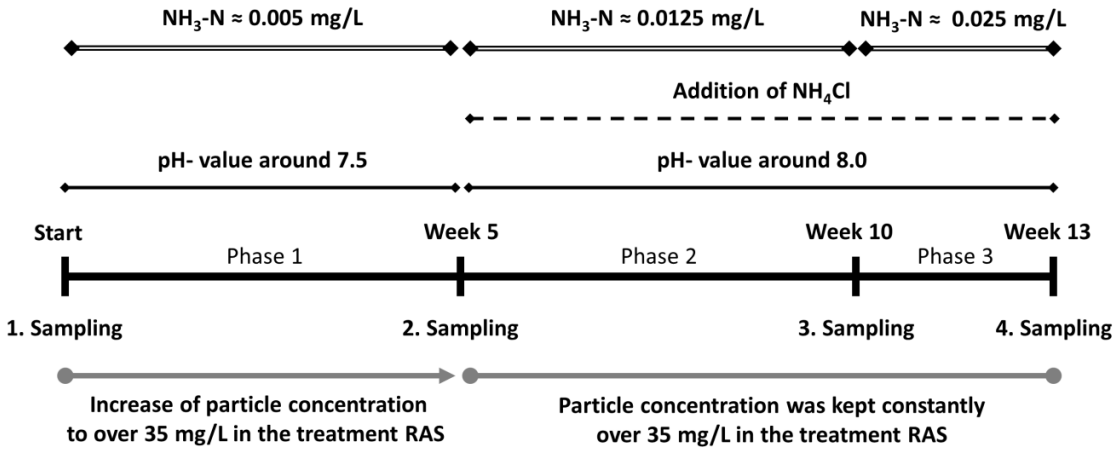

Figure 6.1: Experimental setup of the rainbow trout exposure in the RAS.

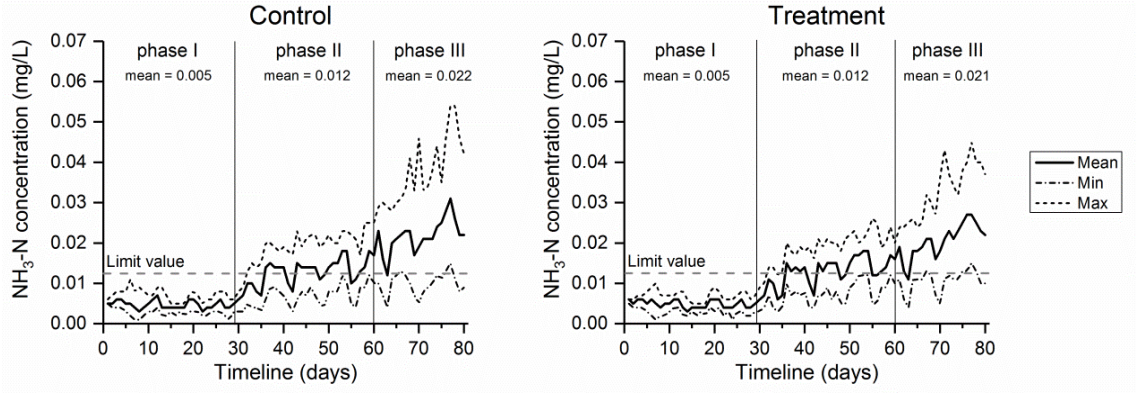

Figure 6.2: Unionized ammonia nitrogen (NH_3-N) concentration (mean, minimum (Min) and maximum values (Max)) in the control and treatment RAS during the investigation period. The dashed line shows the common limit value of NH_3-N (0.0125 mg L^{-1}) for salmonids (Timmons and Ebeling, 2010).

Analysis of suspended solids

Total suspended solids

The concentration of total suspended solids was determined three times per week in duplicate for each system according to method 2540 D of the American Public Health Association (APHA, 1998), with the exception that 0.45 µm cellulose-acetate filters (diameter: 50mm, 11106-50-N, Sartorius AG, Göttingen, Germany) were used instead of glass-fiber filters due to the smaller and better defined pore sizes. Filters were prepared as described by Becke et al. (2018). Water samples were collected using a

tube at a water depth of ca. 30 cm from five tanks in each system, then duplicate samples were pooled to create a representative sample for each system. Samples were collected in the early morning before feeding, in order to represent the daily minimum solid loads (best case scenario). To determine the within-day fluctuations and maximum values, measurements were performed every two hours on one day in week 12.

Particle size distribution (PSD)

For particle size measurement, water samples were collected as described above. Particle sizes were determined according to (Brinker et al., 2005b) using a non-invasive laser particle sizer (GALAI:CIS-1, GALAI Productions Ltd. Midgal Haemak, Israel) equipped with a flow controller (GALAI:LFC- 100) and a flowthrough cell (GALAI:GM-7). The measurements were performed in quadruplicate for each system in week 12 of experimental operation.

Fish performance

The specific growth rate (SGR) was calculated from mean weights recorded at the beginning and the end of the experiment by using the following formula:

$$SGR\ (\%\ d^{-1}) = \frac{ln(mean\ final\ weight) - ln(mean\ initial\ weight)}{t(final\ day) - t(initial\ day)} * 100$$

where *t* is time (days).

The feed conversion ratio (FCR) was calculated as:

$$FCR = \frac{Feed(kg)}{Weight\ gain(kg)}$$

The thermal growth coefficient (TGC) was calculated according to Jobling (2003):

$$TGC = \frac{\left(\sqrt[3]{W_t} - \sqrt[3]{W_0}\right)}{\sum T} \times 1000$$

where W_t andW_0 are the final and initial weights (g), respectively and ∑T is sum day-degrees Celsius (Cho, 1992; Iwama and Tautz, 1981).

Sampling protocol

Fish were sampled at the beginning, in week 5, in week 10 and in week 13 of the study. Fish were fasted 24 h prior to each sampling. Two fish from each tank per system (n = 20) were caught and anaesthetized using clove oil (concentration: 0.1 mL L^{-1}, exposure time: ca. 60 s). Directly following anesthesia, wet weight (to the nearest 0.1 g) and total length (measured from the tip of the mouth to the end of the tail fin; to the nearest 0.1 cm) of each fish were measured and blood samples were taken from the caudal blood vessels and transferred to tubes containing lithium heparin (25 IU mL^{-1} blood, Sarstedt, Nümbrecht, Germany). Subsequently, fish were killed and samples of gill tissue were collected for histological examination.

Health parameters

Gill histology

Gill tissue was prepared and examined as described by Becke et al. (2018). Briefly, observed changes were ranked rising in pathology from 0 (no change) to 3 (severe change) including sub-steps 1 (minor change) and 2 (moderate change). For each section, 5 images showing 6–7 secondary gill lamellae were inspected at a magnification of 200× using a photomicroscope (Zeiss, Oberkochen, Germany). Branchial epithelium thickness (µm) was measured at 10 locations in each image and a mean value was calculated. The number of goblet cells was counted per secondary lamella. The gills of 20 rainbow trout from each RAS were investigated at each sampling point.

Fin condition

Fin erosion as an indicator of fish welfare was assessed according to Person-Le Ruyet et al. (2007), and the fin index was determined according to Kindschi (1987), as follows:

$$Fin\ index = \frac{fin\ length\ (cm)}{total\ length\ (cm)} \times 100$$

Hematology

Hematological parameters (differential leukocyte count, hematocrit, leukocrit, hemoglobin concentration, total red and white blood cell counts) were determined as described by Becke et al. (2018). Glucose concentration was determined using a common glucose measuring device (ACCU-CHEK Aviva, Roche, Mannheim, Germany) as it has been shown that devices for measuring human glucose level are also suitable for use with fish blood (Bartoňková et al., 2017; Eames et al., 2010).

Bacterial assay

Bacterial load

Analysis of bacterial load of rainbow trout was conducted at the termination of the study (20 rainbow trout per RAS) by the fish health service at a governmental veterinary institute, the Staatliches Tierärztliches Untersuchungsamt (STUA) Aulendorf, Germany as described in Becke et al. (2018). Briefly, the number of colony forming units was assessed on skin and spleen and ranked as *no*, *sporadic*, *slight*, *moderate* or *severe* bacterial load. Bacterial species were then determined by using bacteriological standard methods and confirmed by MALDI-TOF MS (Lay, 2001).

Bacterial activity in the water

Bacterial activity in the fish tanks was assessed using a patented method called BactiQuant® (Mycometer A/S, Copenhagen, Denmark), which is an indirect measure of microbial enzyme activity. Reproducibility and repeatability of the method has been documented in a verification report by the United States Environmental Protection Agency (U.S.-EPA, 2011). Briefly, a 10 mL water sample was filtered through a Millipore 0.22 µm closed filter unit (PES express). The filter was then incubated with a fluorogenic enzyme substrate for 15 min. The synthetic fluorescent enzyme-substrate is hydrolyzed by microbial enzymes in the water sample and the amount of released fluorophores was quantified with a fluorometer (Mycometer A/S, Copenhagen, Denmark). The results were expressed in standardized Bactiquant® values (BQV; hereafter termed bacterial activity). Measurements were always performed in duplicate. During the first three weeks, bacterial activity was measured every second

day to gain a better overview of the development until the particle concentration exceeded 35 mg L^{-1} in the treatment RAS. From week 4 onwards, bacterial activity was measured twice a week.

Data analysis

Data were checked for homoscedasticity using Levene's test (Levene, 1960) and for normality using normal quantile plots. If normal distribution and homoscedasticity tests were passed, treatment effects were tested by *t*-tests, otherwise Wilcoxon tests were employed (Sokal and Rohlf, 2003). For analysis of bacterial activity, branchial epithelium thickness and number of goblet cells per secondary lamella the following linear parametric model was applied:

$$Y_{ijkl} = \mu + a_i + b_j + c_k + (ab)_{ij} + (bc)_{jk} + (ac)_{ik} + [d]_l + \epsilon_{ijkl}$$

where Y_{ijkl} is the evaluated parameter, μ is the overall mean, a_i is the fixed factor *Day of sampling*, b_j is the fixed factor *Total suspended solid concentration* and c_k is the fixed factor *Unionized ammonia concentration*, $(ab)_{ij}$ denotes the interaction between *Day of sampling* and *Total suspended solid concentration*, $(bc)_{jk}$ the interaction between *Total suspended solid concentration* and *NH_3-N concentration* and $(ac)_{ik}$ the interaction between *Day of sampling* and *NH_3-N concentration*, $[d]_l$ is the random factor *System* and ϵ_{ijkl} is the random residual error.

Fin erosion and gill histology parameters (thickening of epithelial cells, cellular edema, cell infiltration, tip thickening, detachment of the epithelium, telangiectasia and lamellar fusion) were tested using a logistic regression on ordinal data. The method of least squares was used to analyze the relation between TSS concentration and turbidity. Bacterial load data of the gills was analyzed using Fisher´s exact test. A generalized linear model (GLM) was used to analyze fin index and hematological parameters. The coefficient of variation (C_V) as a unit for the relative standard deviation was calculated in terms of bacterial activity as follows:

$$C_V(\%) = \frac{standard\ deviation\ (\sigma)}{arithmetic\ mean\ (\bar{x})} \times 100$$

All data analyses were performed with JMP Pro (SAS Institute Inc., 64-bit) version 13.2.1. Differences between treatment groups were considered to be significant at $P < 0.05$.

6.4 Results

Water parameters

Water temperature differed significantly ($P < 0.001$) between control and treatment RAS, although the absolute difference was small and below 0.6 °C (Table 6.1). In week 11 to 13, NO_2-N concentration was approximately 0.15 mg L^{-1} higher in the control RAS and differed significantly ($P < 0.05$) between RAS systems, but not in phase one and two of the experiment ($P > 0.05$). Water consumption was significantly higher ($P < 0.05$) in the control system in week 1 to 5 (approx. 40 L day^{-1}) and in week 6 to 10 (approx. 22 L day^{-1}) than in the treatment because the backwash water of the drum filter was reinjected into the treatment system. From week 11 to 13 no significant difference ($P > 0.05$) was found between systems due to adjusting the water consumption in the treatment system. However, magnitudes of differences were minimal and were thus deemed biologically not relevant. Turbidity differed significantly ($P < 0.001$) by up to 15 NTU (Nephelometric Turbidity Units) between control and treatment RAS at individual sampling time points (Table 6.1) as related to different suspended solid load. NH_4-N concentrations, pH, O_2 concentration and NO_3-N concentration did not differ significantly ($P > 0.05$) between control and treatment system. NH_4-N concentrations were increased both in the control and treatment RAS after week 5 and week 10 with concentrations peaks of up to 2.5 mg L^{-1} (control) and 2.3 mg L^{-1} (treatment) respectively, but without significant differences ($P > 0.05$) between systems. Unionized ammonia-N concentrations were also increased after week 5 from 0.005 to 0.012 mg L^{-1} and further to over 0.02 mg L^{-1} after week 10 (Figure 6.2), however, without significant differences ($P > 0.05$). Overall, with the exception of NH_4-N/NH_3-N and suspended solid load, all water parameters remained within physiological optimal range for rainbow trout (Timmons and Ebeling 2010).

Table 6.1: Water parameters (mean ± S.D.) of the control and treatment RAS and recommended limits according to Timmons and Ebeling (2010).

		Week 1 - 5		Week 6 - 10		Week 11 - 13	
	Range limit	Control	Treatment	Control	Treatment	Control	Treatment
pH	6.5 - 8.5	7.55 ± 0.10	7.53 ± 0.09	7.87 ± 0.15	7.86 ± 0.13	7.90 ± 0.10	7.88 ± 0.07
O_2 (mg L^{-1})	> 6	10.7 ± 0.2	10.6 ± 0.3	11.5 ± 0.4	11.5 ± 0.4	11.7 ± 0.2	11.6 ± 0.4
Water temperature (°C)	< 16	14.5 ± 0.3**	14.3 ± 0.3**	14.7 ± 0.2**	14.2 ± 0.3**	14.8 ± 0.2**	14.2 ± 0.2**
NH_4-N (mg L^{-1})	< 1	0.556 ± 0.121	0.601 ± 0.107	0.668 ± 0.181	0.714 ± 0.188	1.100 ± 0.239	1.140 ± 0.254
NH_3-N (mg L^{-1})	< 0.0125	0.005 ± 0.002	0.005 ± 0.002	0.012 ± 0.006	0.012 ± 0.005	0.022 ± 0.009	0.021 ± 0.008
NO_2-N (mg L^{-1})	< 1	0.408 ± 0.234	0.387 ± 0.148	0.533 ± 0.092	0.487 ± 0.081	0.682 ± 0.135*	0.529 ± 0.080*
NO_3-N (mg L^{-1})	< 400	177.0 ± 35.2	169.6 ± 32.7	221.5 ± 8.7	210.0 ± 19.3	279.8 ± 26.3	271.0 ± 23.0
Turbidity (NTU)[a]	/	2.1 ± 0.6**	11.6 ± 4.2**	2.4 ± 0.3**	16.9 ± 2.1**	2.8 ± 0.4**	18.0 ± 1.7**
Water consumption (L day^{-1})	/	191.0 ± 53.4*	150.8 ± 86.8*	257.9 ± 71.7*	236.2 ± 181.3*	284.6 ± 43.6	284.8 ± 109.3

[a] NTU = Nephelometric Turbidity Unit.

* = significant difference ($P < 0.05$) between control and treatment RAS.

** = significant difference ($P < 0.001$) between control and treatment RAS.

Suspended solids analysis

Total suspended solids

Total suspended solid (TSS) concentration differed significantly ($P < 0.0001$) between control and treatment RAS with an average concentration of 4.5 mg L^{-1} in the control and 35.2 mg L^{-1} in the treatment system (Figure 6.3 A). From week 3, the TSS concentration in the treatment system exceeded 30 mg L^{-1} and remained at an average of 40.5 mg L^{-1}. Furthermore, the difference in TSS concentration between control and treatment RAS was never less than 23.1 mg L^{-1}. Figure 6.3 B shows the within-day variation of the total suspended solids concentration in the control and treatment RAS in week 12 of the experiment with minimum values in the morning at 7:00 a.m. The highest TSS concentration on that day was 65.8 mg L^{-1} in the treatment RAS while it was 14.1 mg L^{-1} in the control RAS.

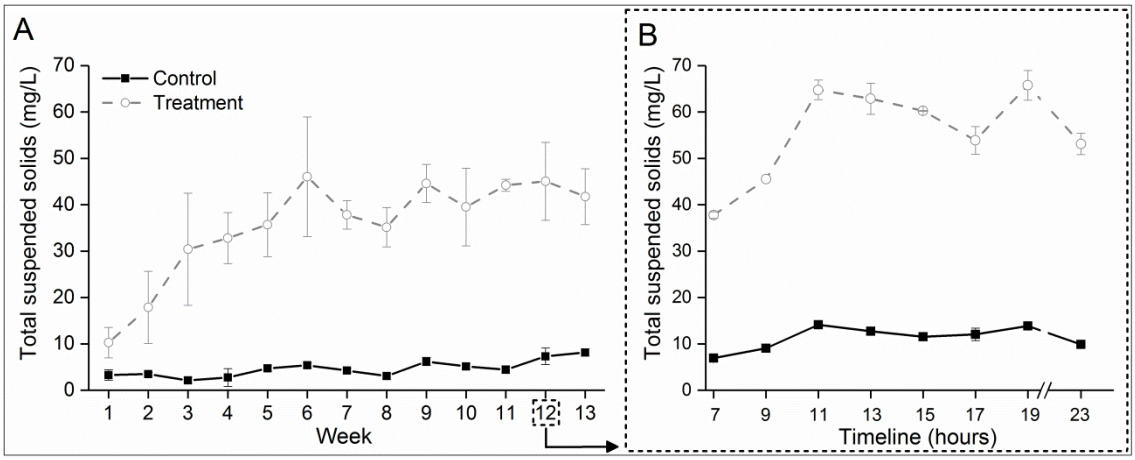

Figure 6.3: (A) Timeline of total suspended solids concentration (mean ± S.D.) in control and treatment RAS over the experimental period. (B) Representative daily variation of total suspended solids concentration (mean ± S.D.) in control and treatment RAS in week 12. Samples were collected every two hours between 7:00 and 19:00 and at 23:00 (CET). Please note the axis break on the x-axis.

Particle size distribution

At week 12, the total number of particles per liter in the treatment RAS was on average more than double that of the control. The average suspended particle load was 17.1 ± 2.1 mg dry weight L^{-1} in the control and 47.4 ± 2.7 mg dry weight L^{-1} in the treatment system. For each particle size class, the absolute frequencies differed significantly ($P < 0.001$) between control and treatment RAS (Figure 6.4). Overall, a high accumulation of fine particles occurred in both the control and the treatment RAS, with 98.6 % and 98.3 % of all particles respectively smaller than 15 µm, however, with higher quantities in the treatment RAS.

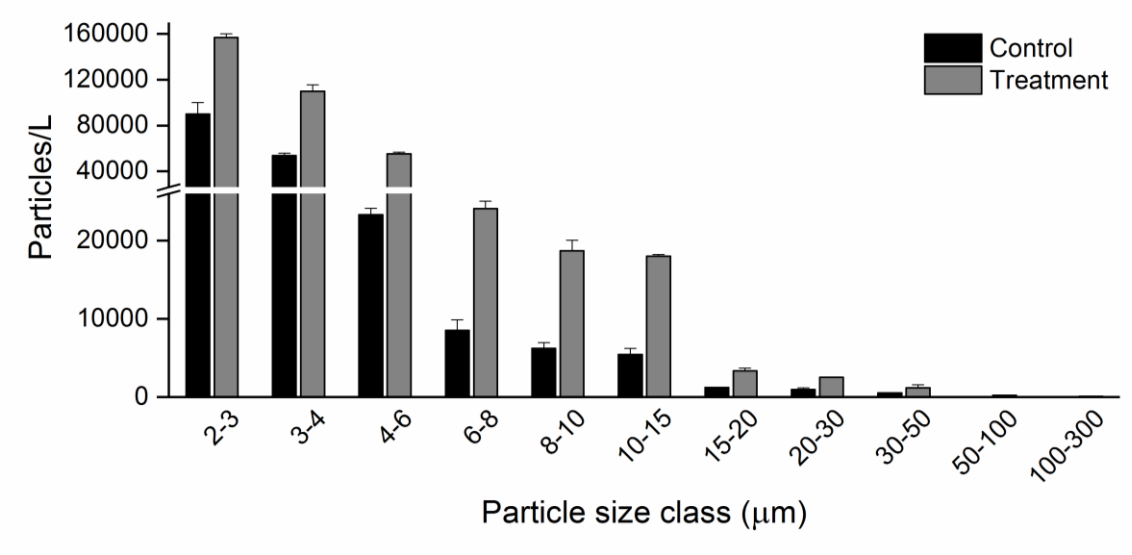

Figure 6.4: Absolute frequency within particle size classes (mean ± S.E.) of the control (n = 4) and treatment RAS (n = 4) in week 12. All particle size classes differed significantly ($P < 0.001$) between control and treatment system. Please note the axis break on the y-axis.

Fish performance

In contrast to the expectations based on recommended threshold values, fish performed very well in both systems. A slight difference in feeding behavior was observed between fish in the two systems with a less aggressive and calmer feeding behavior in the treatment RAS. Overall, no significant differences ($P > 0.05$) were apparent for final weight, survival rate, FCR, SGR and TGC between rainbow trout of the control and treatment RAS (Table 6.2).

Table 6.2: Final weight, survival, feed conversion ratio (FCR), specific growth rate per day (SGR) and thermal growth coefficient (TGC) as determined in treatment and control RAS (mean ± S.D.).

	Control	Treatment	Statistical significance
Final weight (g)	315.6 ± 61.0	317.1 ± 61.5	n.s.
Survival (%)	99.2 ± 1.1	99.6 ± 0.6	n.s.
FCR	0.89 ± 0.04	0.89 ± 0.05	n.s.
SGR (% day^{-1})	1.49 ±0.04	1.50 ± 0.05	n.s.
TGC	1.83 ± 0.06	1.88 ± 0.08	n.s.

Health parameters

Gill histology

No severe histological changes in gill structures were observed during the investigation. Cases of cellular edema, tip-thickening of secondary lamellae, telangiectasia, thickening of epithelial cells, cell infiltration, lamellar fusion, merging of secondary lamellae and detachment of the epithelium were only minor or moderate (Figure 6.5). In terms of cellular edema, all factors were significantly altered by treatment ($P < 0.05$), but magnitude of differences was small (0 – 15 %) and the observed histological change was only rated as minor. The increased TSS concentration did not have any significant effect on all further investigated histological parameters ($P > 0.05$). However, the increased unionized ammonia concentration ($P < 0.05$) and the interaction of unionized ammonia concentration and day of sampling ($P < 0.05$) led to a significantly more frequent occurrence of cell infiltrations and tip thickening of secondary lamellae. All other histological parameters were not significantly affected by the increased unionized ammonia concentration ($P > 0.05$). Furthermore, no significant interaction of increased unionized ammonia concentration and increased suspended solid load ($P > 0.05$) were found for any of the investigated histological parameters. Regarding thickness of branchial epithelium and number of goblet cells per secondary lamella (Table 6.3), no significant effects ($P > 0.05$) of increased unionized ammonia or suspended solid load were apparent at all.

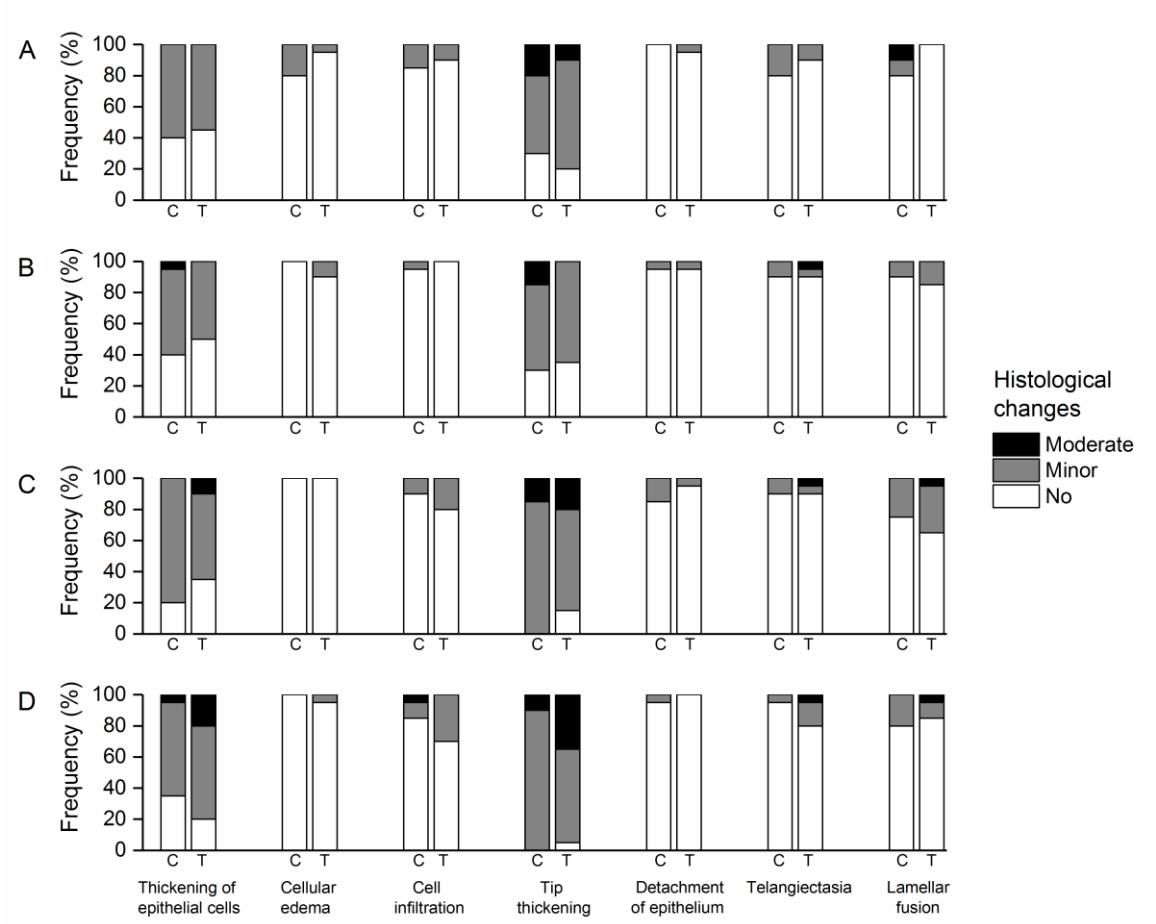

Figure 6.5: Histological changes in gill tissues observed at the start (A), at week 5 (B), at week 10 (C) and at week 13 (D) of the investigation period. In each case, histological changes were analyzed in gills of 20 rainbow trout specimens from each RAS. C = control group; T = treatment group. No significant differences ($P > 0.05$) were apparent between the control and treatments.

Table 6.3: Thickness of branchial epithelium (µm) and number of goblet cells per secondary lamella (mean ± S.D.) as determined in fish of the control and treatment RAS. In each case, gills were examined from 20 rainbow trout per RAS. There were no significant differences ($P > 0.05$) between the control and treatments.

Parameter	Time	Control	Treatment
Thickness of branchial epithelium (mean ± S.D.)	Start	3.84 ± 1.09	3.93 ± 0.83
	Week 5	4.69 ± 1.61	4.58 ± 0.98
	Week 10	4.05 ± 0.79	4.08 ± 0.94
	Week 13	4.02 ± 0.87	4.75 ± 1.37
Number of goblet cells per secondary lamella (mean ± S.D.)	Start	1.17 ± 0.39	1.22 ± 0.30
	Week 5	1.41 ± 0.41	1.65 ± 0.42
	Week 10	1.42 ± 0.39	1.43 ± 0.43
	Week 13	1.46 ± 0.38	1.38 ± 0.57

Fin condition

Neither total suspended solid concentration ($P > 0.05$) nor unionized ammonia ($P > 0.05$) had a significant effect on fish welfare measured by fin erosion in the control and treatment RAS (Figure 6.6). Furthermore, no interaction effect of increased unionized ammonia concentrations and suspended solid load was apparent ($P > 0.05$). The increased unionized ammonia concentrations caused a significantly lower fin index ($P < 0.05$) for the dorsal fin (Table 6.4). However, fin indices of the left and right pectoral fin were not affected ($P > 0.05$). Increased suspended solid load had no significant effect ($P > 0.05$) on any fin index. Furthermore, the elevated unionized ammonia concentrations did not significantly affect the impact of suspended solid load ($P > 0.05$) on fin indices.

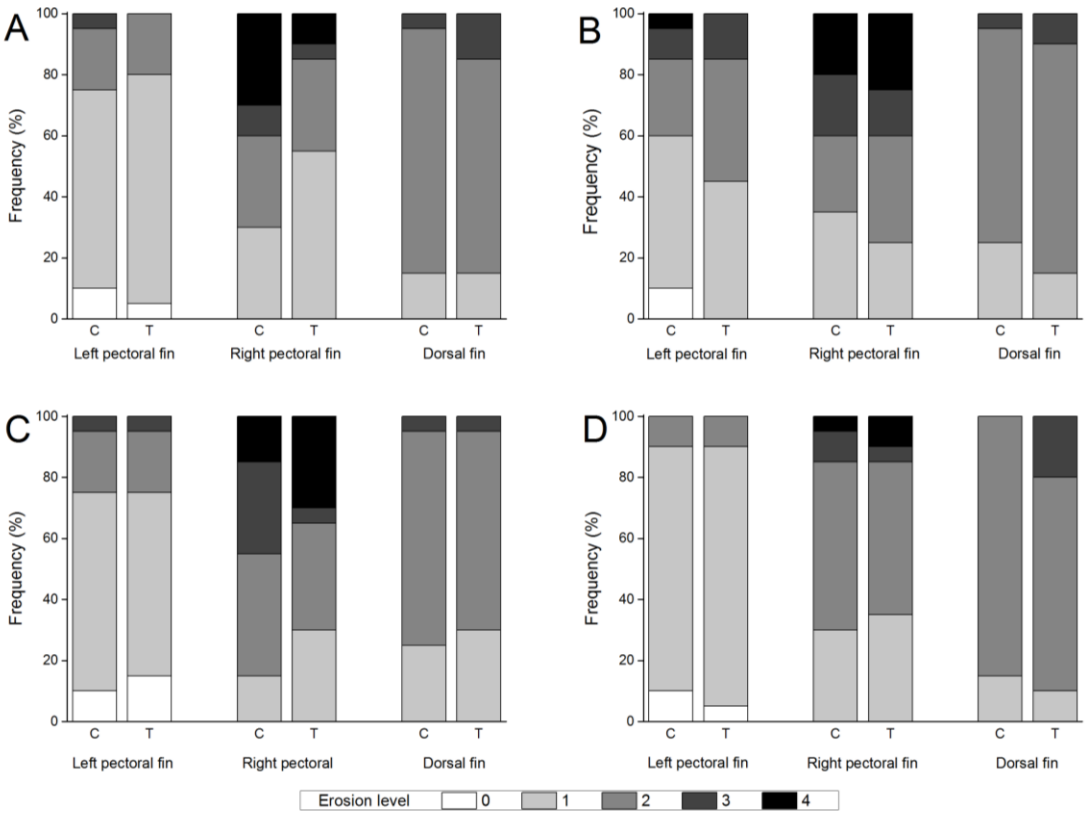

Figure 6.6: Percentages (%) of fin erosion determined according to Person-Le Ruyet et al. (2007) at the start (A), in week 5 (B), in week 10 (C) and in week 13 (D) of the study. In each case, fin erosion was investigated for 20 randomly sampled rainbow trout. C = control group; T = treatment group.

Table 6.4: Fin index (mean ± S.D.) according to Kindschi (1987) of rainbow trout from the control and treatment RAS. At each sampling, 20 fish were randomly sampled per RAS.

		Left pectoral fin	Right pectoral fin	Dorsal fin
Start	Control	9.03 ± 1.86	7.64 ± 2.34	8.63 ± 0.94
	Treatment	8.27 ± 1.29	7.15 ± 2.63	8.36 ± 0.66
Week 5	Control	8.78 ± 0.99	6.50 ± 2.00	8.47 ± 0.86
	Treatment	8.52 ± 1.31	6.52 ± 2.40	9.04 ± 0.77
Week 10	Control	9.23 ± 0.90	7.87 ± 1.89	9.02 ± 0.91
	Treatment	9.32 ± 0.69	7.83 ± 2.02	8.64 ± 0.95
Week 13	Control	8.64 ± 1.81	5.92 ± 3.20	8.52 ± 0.88
	Treatment	9.04 ± 1.57	7.96 ± 2.34	8.18 ± 1.07

Hematology

Overall, all hematological parameters (Table 6.5) were approximately within the range previously reported for salmonids (McCarthy et al., 1975, 1973; Pund, 1998; Řehulka et al., 2004). However, hematocrit was significantly decreased ($P < 0.05$) both with increasing TSS concentration and increasing body length, whereas hematocrit significantly increased ($P < 0.05$) over time. Thus, the MCV value was also significantly lower ($P < 0.01$) and the MCHC value significantly higher ($P < 0.01$) with increasing TSS concentrations. The interaction of TSS concentration and unionized ammonia concentration revealed a significant effect ($P < 0.05$) on MCHC values. The number of thrombocytes was significantly elevated ($P < 0.05$) with increasing unionized ammonia concentration and the hemoglobin concentration significantly increased ($P < 0.05$) over time. All the other parameters (glucose concentration, number of erythrocytes, MCH, number of leukocytes, leukocrit and the proportions of lymphocytes, granulocytes and monocytes) were not significantly affected ($P > 0.05$) by suspended solid load, unionized ammonia concentration or the interaction of both parameters.

Table 6.5: Hematological parameters of rainbow trout determined at the start, in week 5, 10 and 13 of the study. In each case, hematological parameters were investigated for 20 randomly sampled rainbow trout (mean ± S.D.).

	Start		Week 5		Week 10		Week 13
	Control	Treatment	Control	Treatment	Control	Treatment	Control
Erythrocytes (10^6 μL^{-1})	1.07 ±0.14	1.10 ± 0.13	1.03 ± 0.11	1.05 ± 0.10	1.03 ± 0.11	0.99 ± 0.12	0.95 ± 0.08
Hematocrit (%)	34.6 ± 2.4	33.9 ± 2.5	34.5 ± 2.9	32.3 ± 2.8	33.6 ± 3.8	31.4 ± 3.6	31.9 ± 3.3
Hemoglobin (g dL^{-1})	9.2 ± 0.7	9.5 ± 0.6	9.5 ± 0.8	9.8 ± 0.9	10.1 ± 1.0	9.7 ± 1.0	9.8 ± 0.9
MCH (pg)	87.0 ± 12.0	87.3 ± 8.9	92.7 ± 6.9	95.0 ± 10.5	98.4 ± 13.0	98.8 ± 7.5	103.2 ± 5.5
MCHC (g dL^{-1})	26.6 ± 1.5	28.2 ± 1.3	27.7 ± 1.6	30.4 ± 2.5	29.9 ± 1.3	31.0 ± 2.5	30.7 ± 2.2
MCV (fL)	326.1 ± 37.6	309.3 ± 29.8	335.3 ± 22.8	309.8 ± 25.8	328.5 ±40.6	319.0 ± 15.5	337.4 ± 24.3
Thrombocytes (10^4 μL^{-1})	1.32 ± 0.29	1.44 ± 0.36	1.26 ± 0.24	1.20 ± 0.25	1.30 ± 0.26	1.26 ± 0.30	1.36 ± 0.28
Leukocrit (%)	1.1 ± 0.3	1.2 ± 0.3	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Leukocytes (10^4 μL^{-1})	2.86 ± 0.59	2.78 ± 0.59	2.49 ± 0.62	2.76 ± 0.47	2.16 ± 0.43	2.39 ± 0.68	2.22 ± 0.55
Lymphocytes (%)	96.8 ± 1.6	97.1 ± 1.8	96.8 ± 2.2	97.6 ± 1.3	97.5 ± 1.3	97.2 ± 2.0	97.0 ± 1.7
Granulocytes (%)	2.4 ± 1.6	1.9 ± 1.5	2.4 ± 1.7	1.5 ± 1.2	1.9 ± 1.2	1.9 ± 1.6	2.2 ± 1.7
Monocytes (%)	0.8 ± 0.7	1.0 ± 0.7	0.9 ± 0.9	0.9 ± 0.8	0.6 ± 0.5	0.9 ± 1.0	0.8 ± 0.6
Glucose (mg dL^{-1})	62.6 ± 6.3	61.0 ± 7.0	58.3 ± 4.9	59.9 ± 5.3	56.7 ± 4.0	55.0 ± 5.2	56.3 ±3.2

Bacterial assay

Bacterial load

Overall, no critical bacterial load was detected in the control or treatment RAS. Bacterial load of the gills differed significantly between fish of the control and treatment RAS in terms of direct detection ($P < 0.01$) with 45 % and 10 % of the fish gills showing no bacterial load in the control and treatment RAS respectively (Figure 6.7). In contrast, 90 % of the fish gills in the treatment RAS and only 40 % of the fish gills in the control RAS revealed slight to moderate bacterial load. However, no significant difference appeared in terms of cultivation ($P > 0.05$). The bacterial load of the spleen was significantly higher ($P < 0.0001$) for rainbow trout in the suspended solids enriched RAS. In the control RAS, 95 % of the spleens revealed no to sporadic bacterial load, whereas in the treatment system 75 % of the spleens revealed slight to moderate bacterial load. The examination of the skin revealed no bacteria or ectoparasites in either RAS. The fish pathogenic bacteria *Flavobacterium columnare* was detected on two rainbow trout from the control RAS and on four rainbow trout from the treatment RAS by direct detection. The cultivation of gill smears proved the occurrence of *Aeromonas sobria* for three fish in the treatment RAS, but not in the control RAS.

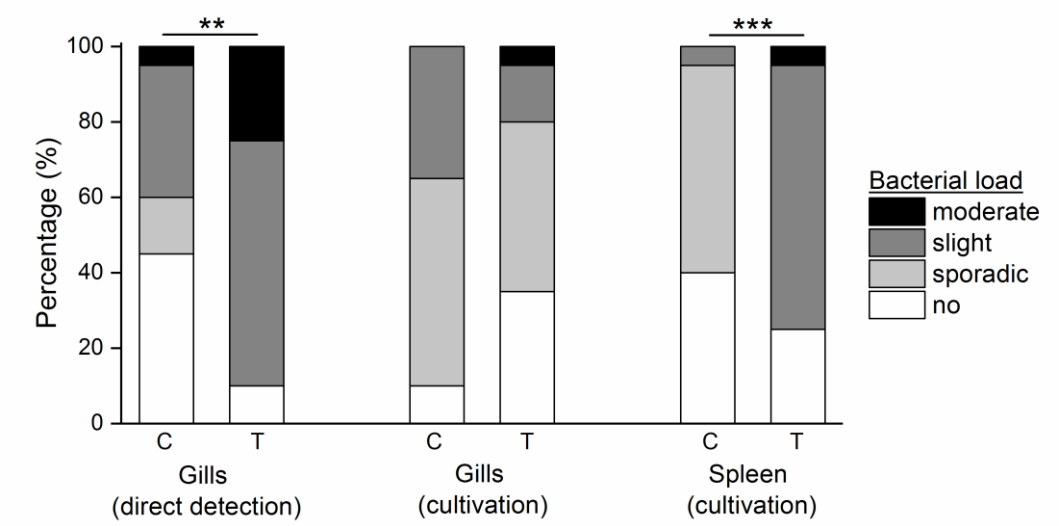

Figure 6.7: Bacteriological examination of gills (direct detection and cultivation) and spleen (cultivation) from 20 rainbow trout of the control (C) and treatment (T) RAS. ** = $P < 0.01$; *** = $P < 0.0001$.

Bacterial activity

Bacterial activity ranged between 0.12 $x10^5$ and 0.47 $x10^5$ in the control RAS (C_v = 18.0 %) and between 0.33 $x10^5$ and 3.42 $x10^5$ in the treatment RAS (C_v = 18.3 %) (Figure 6.8). Bacterial activity was only significantly affected ($P < 0.0001$) by the total suspended solid concentration. With increasing particle load in the treatment RAS, bacterial activity increased from about 0.3 $x10^5$ to over 2.6 $x10^5$ during the first three weeks. In contrast, bacterial activity in the control RAS remained roughly static between 0.2 $x10^5$ and 0.3 $x10^5$ during this time. Unionized ammonia-N concentration had no significant effect ($P > 0.05$) on bacterial activity in either RAS. Bacterial activity measured on one representative day in week 8 showed diurnal variations from 0.2 $x10^5$ to 0.4 $x10^5$ in the control RAS and from 2.3 $x10^5$ to 3.7 $x10^5$ in the treatment RAS respectively. Overall, there was a significant positive linear correlation between TSS concentration and bacterial activity ($P < 0.0001$, $r^2 = 0.98$; Figure 6.9). However, the certainty measure of the linear correlation of bacterial activity with TSS was very low in the control RAS ($r^2 = 0.10$) while it was high in the treatment RAS ($r^2 = 0.94$).

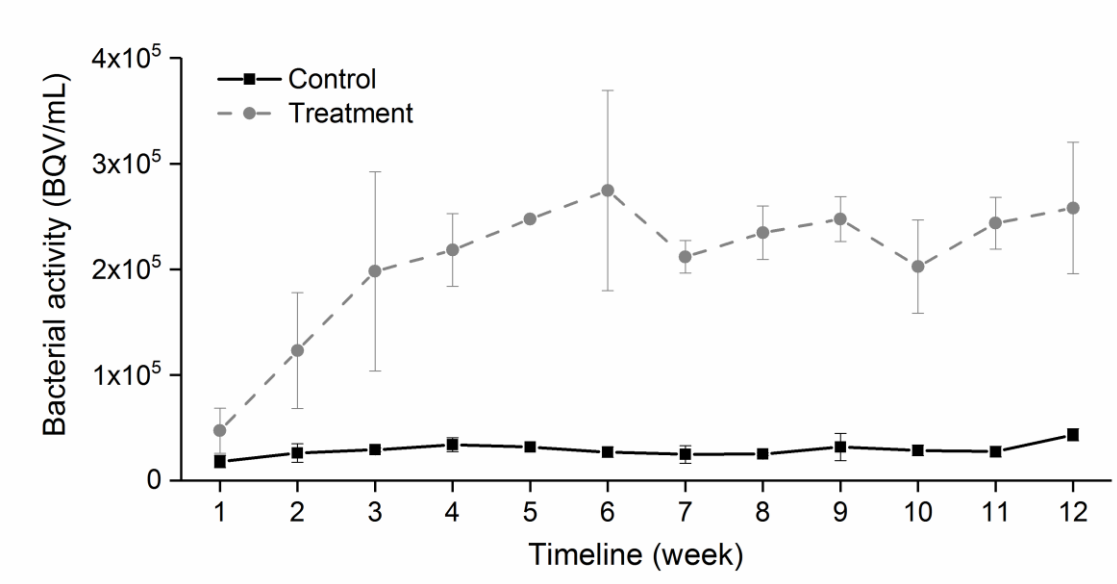

Figure 6.8: Bacterial activity (BQV mL^{-1}, mean ± S.D.) in the treatment and control RAS during the investigation period.

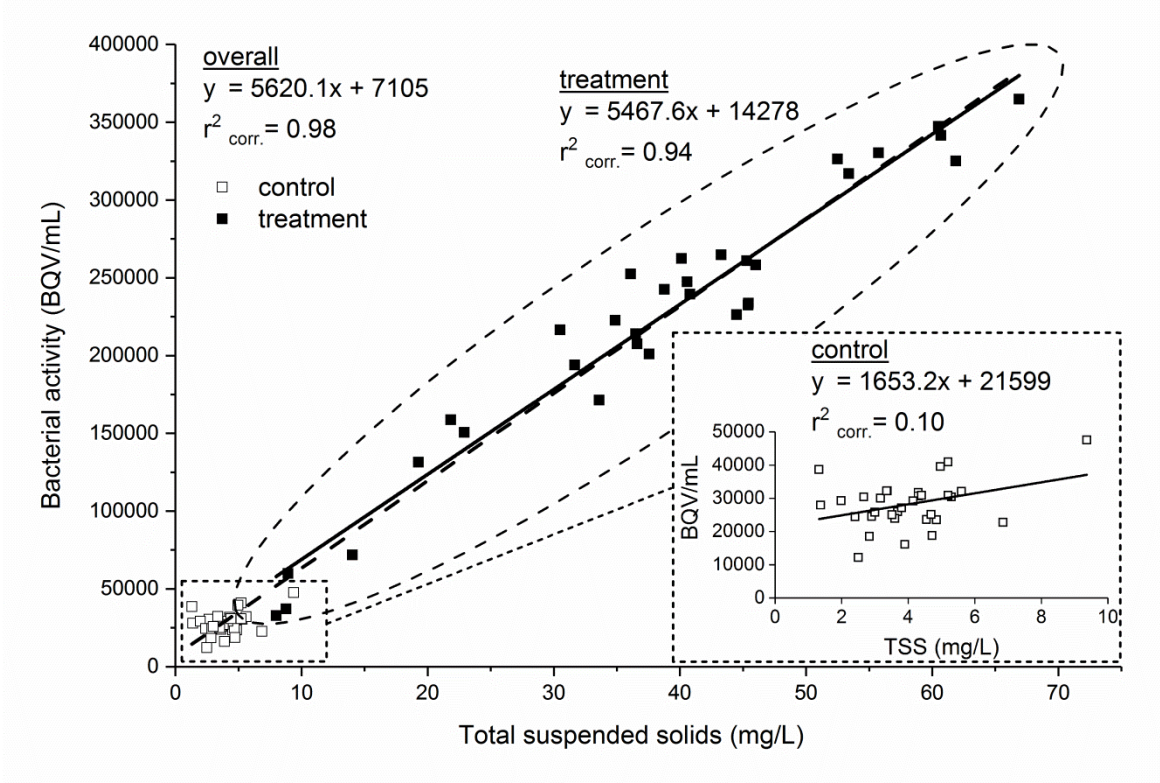

Figure 6.9: Linear relationship between bacterial activity (BQV mL^{-1}) and total suspended solid concentration (mg L^{-1}) for control (open symbol) and treatment RAS (solid symbol) and in sum. The dashed line shows the overall linear relationship, the solid lines show the linear relationship of the treatment and control system respectively.

6.5 Discussion

The experiment effectively decoupled the effects of chronic suspended solid load and elevated unionized ammonia concentrations from other relevant water quality parameters. This allowed an investigation of the sole effects of both increased unionized ammonia concentrations and suspended solid load on rainbow trout as well as their combined effects at a farm-scale.

Recent investigations (Becke et al., 2018, 2017) have shown that even massive accumulation of fine solids alone caused no detrimental effects on rainbow trout in RAS. These results were corroborated by the present findings which did not reveal relevant detrimental effects of increased suspended solid concentrations on fish at concentrations of up to almost 70 mg L^{-1}. Gills are of delicate structure and therefore highly sensitive to physical impact (Evans, 2005; Morgan and Tovell, 1973), so the

absence of any histological alteration associated with suspended solid load is of particular note. This is in line with Goldes et al. (1988) who also observed no branchial pathology in rainbow trout even when exposed to up to 1017 mg L^{-1} of suspended clay kaolin. Thus, the assumption that suspended solids alone are not a key issue affecting fish welfare in RAS is further strengthened.

However, the increased particle load caused indirect effects. It led to increased turbidity which suppressed feeding behavior of fish in the treatment RAS as previously described (Barrett et al., 1992; Becke et al., 2018, 2017; Utne-Palm, 2002). This altered feed uptake can potentially lead to a loss of feed in commercial settings using automatic feeders. To preclude this potentially disturbing effect, fish in this study were hand fed which secured the uptake of all feed pellets.

Furthermore, the increased suspended solid load induced a substantial increased bacterial load. Such a finding was expected (Becke et al., 2018) as an increased number of particles in the treatment RAS promotes bacterial growth by providing a larger surface area for bacterial colonization and food-substrate. Bacterial activity levels found in this study have been observed in other recent studies rearing rainbow trout in intensive RAS (Pedersen et al., 2017; Rojas-Tirado et al., 2018). Especially remarkable is the close linear correlation between bacterial activity and TSS, which is however quite variable at low TSS (< 5 mg L^{-1}), but nearly exclusively determined by TSS at high TSS loads. This novel outcome is of high relevance for systems with need for bacterial control. However, the physiological parameters investigated here did not reveal any evidence for bacterially mediated physiological stress response in the control or in the treatment systems. This was confirmed by the independent veterinary inspection of the rainbow trout which did not reveal any relevant pathological bacterial infestation. In contrast, Redding et al. (1987) observed a reduced tolerance to subsequent infection with *Vibrio anguillarum* for yearling steelhead when exposed to high concentrations of suspended topsoil. In the present study, however, no bacterial diseases occurred despite very high bacterial and suspended solid load in the treatment RAS. However, under different conditions, the interaction of suspended solids and bacterial occurrence might impair fish health and need to be controlled (Herbert and Merkens, 1961; Qualls et al., 1983).

Increased particle concentrations, e.g. due to increased stocking densities in RAS, are often accompanied by a decrease in water quality because of leaching of harmful substances or particle-mediated growth of heterotrophic bacteria (Chen et al., 2003; Ling and Chen, 2005). To simulate this phenomenon on a farm-scale, the concentration of unionized ammonia-N was increased to levels which exceeded the common upper safe limit of 0.0125 mg L^{-1} proposed for salmonid aquaculture (Timmons and Ebeling, 2010). It was hypothesized that the chronic exposure to increased unionized ammonia concentrations would result in a deterioration of physiology and performance of rainbow trout. However, contrary to the hypotheses and praxis as well as academic opinion (Smith and Piper, 1975; Thurston et al., 1984; Timmons and Ebeling, 2010), rainbow trout exposed to chronic unionized ammonia-N concentrations of more than four times the critical threshold did not reveal deteriorated performance in our study. Fish in both systems showed very good performance with nearly 100 % survival. Only minor physiological effects of increased unionized ammonia concentration on gill structure were observed. Nonetheless, the observed alterations of gill structures were only slight to moderate and only two (cell infiltrations, tip thickening of secondary lamellae) out of seven parameters were significantly affected by the increased unionized ammonia load. Thus, these results suggest that the rainbow trout can cope well with the given unionized ammonia concentrations. The common upper safe limit of unionized ammonia-N of 0.0125 mg L^{-1} proposed for salmonid aquaculture is based on the findings of Smith and Piper (1975). However, other authors, such as Meade (1985), Daoust and Ferguson (1984) (laboratory experiment) and Kolarevic et al. (2013) (commercial scale) previously questioned the proposed unionized ammonia limit. Nevertheless, the value of 0.0125 mg L^{-1} has been echoed widely since then and established in aquaculture textbooks (e.g. Timmons and Ebeling, 2010). However, it has to be noted that oxygen concentration was low (around 6 mg L^{-1}) in the Smith and Piper (1975) study. According to Lloyd (1961) and Brown (1968), unionized ammonia toxicity increases with decreasing oxygen levels. Thus, the interaction of low oxygen with high unionized ammonia concentration in the study of Smith and Piper (1975) might be causative for the pathological changes in the gills of rainbow trout. During the present study, however, the system water was saturated with oxygen during the whole

investigation period. Thus, in relation to oxygen, unionized ammonia toxicity was kept to a minimum which might explain the observed low impact. Overall, the presumption for a higher tolerance level of rainbow trout to unionized ammonia was confirmed by the results here showing no relevant effects on fish physiology at the given unionized ammonia concentrations.

In this context, the stress-modulated effects are important given that stressed fish are more vulnerable to external unionized ammonia toxicity than unstressed fish (Randall and Tsui, 2002). Thus, the low impact of elevated unionized ammonia concentrations while concomitantly exposed to high fine particle loads render the solid exposure harmless as well.

Regarding the impact of unionized ammonia on fin condition, only the dorsal fin was negatively affected. As fin condition is frequently consulted to assess fish welfare (Ellis, 2002; Ellis et al., 2008; Turnbull et al., 2005), the almost complete absence of any fin deterioration here is remarkable and indicates the very low impact of the unionized ammonia and solid stressors. Abbott and Dill (1985) assumed that aggressive interaction is the major cause of fin damage in hatchery salmonids. In the present study, fin condition of rainbow trout was marginally better in the solid enriched RAS than in the control RAS. This might be attributable to the calmer behavior and reduced social interaction of fish due to the turbid conditions in the treatment RAS (Bash et al., 2001).

The analysis of hematological parameters revealed significant effects for individual parameters both in terms of suspended solids and unionized ammonia. However, taking all hematological parameters together, there was no indication of pathological effects. Knoph and Thorud (1996) also did not observe any negative effect of unionized ammonia-N up to 0.112 mg L^{-1} on hematological parameters (hematocrit, RBC count) of Atlantic salmon. Furthermore, Becke et al. (2017, 2018) observed no significant effects of suspended solid load up to 70 mg L^{-1} on hematological parameters.

As a consequence of the massive accumulation of fine particles in the treatment RAS and the additionally increased unionized ammonia concentration in both systems, it was hypothesized that a multiplicative effect of these two parameters would occur in the treatment RAS, resulting in significant consequences on trout physiology. However,

none of the investigated physiology parameters revealed any relevant multiplicative effects of particle and unionized ammonia load. In contrast to our hypothesis, no synergistic impact of increased unionized ammonia concentrations and suspended solid load on fish physiology was found. These results indicate that the commonly used upper safe limits of 0.0125 mg L^{-1} for unionized ammonia-N and 25 mg L^{-1} for total suspended solids do not represent the actual critical limits for salmonid aquaculture. Fish have evolved mechanisms to counteract high unionized ammonia environments, as shown for rainbow trout (Randall and Tsui, 2002; Wicks and Randall, 2002). This suggests that rainbow trout have probably developed an improved tolerance to poor water quality in the course of artificial selection for aquaculture. Positive effects of moderately elevated ammonia concentrations have even been observed for rainbow trout when fed to satiation (Linton et al., 1999, 1997; Wood, 2004). Thus, more research that keeps track of breeding developments is needed to clarify the exact effects of water parameters on rainbow trout and fish in general both in aquacultural production and natural conditions. It might be that the upper safe limits of certain water quality parameters currently used in aquacultural production no longer correspond to the present genetic makeup of fish and that they should be revised.

6.6 Conclusions

The results from this study provide a fully controlled insight into the combined effects of particle accumulation and unionized ammonia load on physiology of rainbow trout in RAS on a farm-scale. Against expectations and widespread opinion, the solid fraction of the experimental system, comprising almost exclusively fine particles at concentrations distinctly above values normally reached in aquacultural production, failed to provoke detrimental effects on physiology and performance of rainbow trout. The same holds for unionized ammonia and the combination of both.

The results therefore indicate with respect to suspended solids and unionized ammonia that increasing fish densities to improve the economic performance of RAS beyond current limits of suspended solids and unionized ammonia is feasible, if accompanying water parameters are optimal.

Thus, the main conclusions are:

- bacterial activity was strongly affected by increased TSS concentrations, but without detrimental effects on fish physiology
- increased unionized ammonia-N concentrations up to 0.05 mg L^{-1} caused only minor effects on fish physiology
- no relevant combined effects of increased unionized ammonia-N concentrations and suspended solid load were observed
- upper safe limits of unionized ammonia-N and suspended solids need to be revised for salmonid aquaculture

7 General discussion

This thesis provides novel insights into the nature of particles occurring in commonly used salmonid aquaculture systems. Shape analysis using non-invasive digital imaging techniques revealed that the majority of particles had a flake-like form, regardless of system type and water treatment (chapter 2). Furthermore, it was shown for the first time that the volume and surface area of suspended solids occurring in aquaculture systems will be more accurately calculated using an assumption of ellipsoidal particle shape than the classical sphere used previously (e.g. Brinker et al., 2005b; Fernandes et al., 2014; Patterson et al., 1999). These findings have great implications for the design and modeling of future aquaculture systems and will enable more reliable assessment of important processes such as leaching of harmful substances and bacterial activity.
The fully controlled investigation also showed that, contrary to former scientific and practical assumptions, suspended solids at concentrations far exceeding those normally permitted in aquacultural production did not negatively influence the physiology or performance of rainbow trout in RAS (chapter 3 + 4). This was true even when levels of fish-toxic unionized ammonia were increased (chapter 5). As suspended solids were formerly considered distinctly harmful to the health and performance of farmed fish (e.g. Chapman et al., 1987; Chen and Malone, 1991), these results show the compelling necessity of uncoupling any parameter to be examined from other potentially interfering and correlating factors.

7.1 Suspended solids and shape implications

The timely removal of solid waste is deemed to be one of the most important aspects of water treatment in aquaculture (van Rijn, 2013). In this context, new knowledge of the mainly flake-like form of particles in aquaculture systems and the improvements in volume and surface area calculations achieved by assuming an ellipsoidal shape (chapter 2) will enhance understanding of particle related processes and contribute very significantly to improvements in solid treatment, especially in RAS.

Based on the new findings, the volume of particles that can be removed by mechanical filtration can be computed more accurately than before (e.g. Brinker and Rösch, 2005; Fernandes et al., 2014; Unger et al., 2015), allowing a more precise assessment of wastewater treatment efficiency and leading to further optimization of treatment. Furthermore, the adjusted ellipsoidal calculations of volume and surface area reveal that in addition to fine particles, slightly larger solids in the size range 30 – 100 µm are also dominant in RAS (chapter 2). Hitherto, fines smaller than 20 µm were regarded as of utmost relevance in RAS (e.g. Chen et al., 1993; Fernandes et al., 2014) and calculations based on the classical assumption of sphericity suggested they were the wholly dominant particle size in such systems with regard to surface area and volume (Patterson et al., 1999). Accurate modeling of particle surface area is of utmost importance in controlling the leaching of harmful substances and bacterial activity (Brinker et al., 2005a; Chen et al., 2003; Pedersen et al., 2017) and the adjusted particle size distributions, considering ellipsoidal shape, allow filtration components such as gauzes to be adjusted to maximize solid removal.

Moreover, recognizing the predominantly flake-like form of most particles in aquaculture systems (presented in chapter 2) is likely to inform more efficient removal technologies. One possibility may be the application of flocculants to promote further flake-formation and thereby further improve the removal potential of solid waste. Flocculation is often induced artificially to increase the removal efficiency of organic matter, e.g. in sewage plants and industrial wastewater effluent treatment units (Aguilar et al., 2003; Koohestanian et al., 2008; Lee et al., 2014; Thomas and Moore, 2004), and given the already flake-like characteristics of particles in aquaculture systems the technique may be highly effective in reducing fine particles loads in semi-recirculating and recirculating systems. As yet the technique is seldom used in aquaculture, but an intensive applicability study by Ebeling et al. (2003, 2004, 2005) showed that removal of up to 99 % of TSS and 92-95 % of phosphorus was possible. In this context, the widespread availability, environmental sustainability and biodegradability of chitosan-based flocculants makes them the most promising option in aquaculture production (Renault et al., 2009; Yang et al., 2016).

7.2 Fish welfare implications

In recent years, animal welfare has become an increasingly important factor in livestock farming and hence in aquaculture finfish production (Ashley, 2007; Browman et al., 2018). Environmentally literate consumers are pressing for more responsible methods of food production worldwide, including aquaculture (Stubbe Solgaard and Yang, 2011). Within aquaculture, semantic models for the assessment of welfare have already been implemented for Atlantic salmon in cage systems (Folkedal et al., 2016; Pettersen et al., 2014; Stien et al., 2013) and several scientific working groups are currently working on comparable welfare indices for other fish species (e.g. rainbow trout, pikeperch, European perch), showing the importance of addressing fish welfare in aquaculture production.

Until now, suspended solid load has been deemed detrimental for the welfare of fish in aquaculture systems (Bao et al., 2018; Timmons and Ebeling, 2010; Yavuzcan Yildiz et al., 2017). However, the findings of this thesis demonstrate unequivocally that suspended solid concentrations well above normal levels do not impair the welfare of salmonids in RAS (chapter 3 – 5). The absence of serious adverse effects is attributable to the flake-like properties of the majority of particles in the most common salmonid aquaculture systems (chapter 2). Particles in RAS are rather smooth, with a density close to that of water between 1010 and 1153 kg m^{-3} (Unger and Brinker, 2013) due to their mainly organic origin from feces and feed residues (Bao et al., 2018; Timmons and Ebeling, 2010). This may explain the absence of any histological damage to gill structures observed in the current studies (chapter 3 – 5). Other investigations have shown that the tolerance of salmonids to suspended sediments is also fairly high (Goldes et al., 1988; Michel et al., 2013), despite the high proportion of inorganic and mineral components and the sharper and more angular shape of particles. Thus, it could be assumed that the tolerance of rainbow trout to increased concentrations of suspended solids as well as suspended sediments is much higher than previously expected, presumably as an evolutionary adaption to the pulses of extremely high particle loads that occur in natural systems, for example following snowmelts or floods (Asselman, 1999; Langlois et al., 2005; Lenzi and Marchi, 2000).

In addition, suspended solid load is often correlated with deteriorating water quality, for example due to leaching of harmful substances like ammonia (Chen et al., 2003; Kvåle et al., 2006) or to reduced biofilter performance caused by increased growth of heterotrophic bacteria (Ling and Chen, 2005). During the exposure studies in this thesis (chapter 3 + 4), however, the use of high capacity biofilters ensured there was no deterioration in water quality during either the short-term (four weeks) or long-term (full grow-out) experiment. The biofilters in use were overdimensioned for the volume of feed applied (see chapter 4 for more details), suggesting that high capacity biofilters might be used in RAS to preserve water quality under more intensive fish production scenarios, thus reducing energy costs per unit of production (Martins et al., 2005) without impacting on fish welfare.

The only relevant effect of increased particle concentrations observed in these studies was a positive correlation with bacterial activity. Even so, there was no evidence from the wide range of physiological parameters examined that this increased activity led to any detrimental effect on rainbow trout (chapter 4 + 5). Nevertheless, increased particle load has been associated indirect with the deteriorating fish health by favoring the spread of pathogens (Goldes et al., 1988; Redding et al., 1987). The management of microorganisms in RAS is difficult (Rurangwa and Verdegem, 2015) and there is still no consensus about how to deal with bacterial load in aquaculture systems generally. Liu et al. (2017, 2018) suggested that the reduction of suspended bacteria density brought about by periodic disinfection of culture water in RAS was beneficial to fish health. However, there is also evidence that a stable microbial community in RAS may restrict the proliferation of opportunistic pathogens (Attramadal et al., 2012; Michaud et al., 2009) and that preventative disinfection may be counterproductive in preventing such beneficial communities from forming. It may be that elevated suspended solid loads can lead to high but stable populations of heterotrophic bacteria that actually reduce the risk of pathogen spread. This was presumably the case in the studies carried out here (chapter 4 + 5) as no deterioration in fish physiology or performance was observed despite very high bacterial activity. It may also be that the flake-like particles observed in all of the investigated aquaculture systems (chapter 2) serve as micro ecosystems (Droppo et al., 1997) that aid the establishment of stable,

beneficial bacterial populations and thus limit the spread of pathogens. There is an urgent need to further investigate the microbial communities of RAS and the effect increased concentrations of suspended solids might have on bacterial populations, including both beneficial and pathogenic forms.

Taken together, the findings of this thesis prove that suspended solid concentrations commonly found in commercial fish farms are not harmful to fish physiology and performance (chapter 3 – 5) and thus should not be deemed a threat to fish welfare in aquaculture systems. Mean suspended solid concentrations of 35 mg L^{-1} with peaks up to 70 mg L^{-1} can be regarded as noncritical for salmonids (chapter 3-5), so long as accompanying water parameters remain within safe limits. Furthermore, unionized ammonia-N levels of 0.025 mg L^{-1} with peaks up to 0.05 mg L^{-1} can also be classified as noncritical for rainbow trout in RAS (chapter 5), provided sufficient oxygen is supplied. Thus, existing limits on levels of suspended solids and unionized ammonia in salmonid aquaculture production can safely be revised upwards.

These findings have significant implications for aquaculture system designs, shifting the focus regarding fish welfare from suspended solids to other water parameters such as oxygen, CO_2, nitrite and ammonia. Oxygen content seems to be particularly important as it appears to have great impact on the toxicity of several harmful substances, including ammonia (Brown, 1968; Downing and Merkens, 1955; Lloyd, 1961; Merkens and Downing, 1957). Recently, Thorarensen et al. (2018) observed interactive effects between unionized ammonia and CO_2, resulting in reduced feed intake and growth stagnation in juvenile cod. The implications of various water parameters on fish health and performance and possible interaction effects should be investigated more closely in following studies and an overall update of thresholds for water parameters in aquaculture systems is highly desirable.

7.3 Indirect consequences of increased particle load

Suspended solid concentrations in salmonid RAS are generally kept below 12 mg L^{-1} and almost never exceed 25 mg L^{-1} (Schumann and Brinker, in prep.). This is considerably lower than the TSS concentrations used in the exposure studies described

here (chapter 3 – 5). These results suggest that stocking densities in RAS might safely be increased to compensate for the financial and energetic costs of production and thus improve the economic viability of RAS. However, increasing the density of fish also increases the occurrence of suspended solids in a system and this may have other negative impacts on production such as loss of fillet quality due to increases in off-flavor compounds in organic-rich environments (Azaria and van Rijn, 2018). Off-flavor in fish is caused mainly by the accumulation of geosmin and 2-methylisoborneol (MIB) (Guttman and van Rijn, 2008; Tucker, 2000), thought to be synthesized by the bacterial genera *Streptomyces*, *Sorangium* and *Nannocystis* (Auffret et al., 2013; Schrader et al., 2010). So far, the only reliable method for removing off-flavors is relocating the fish to freshwater tanks for a certain period (Burr et al., 2012), but this step require additional time and space and incurs costs, thereby diminishing both the productivity and the economic viability of RAS (Hanson, 2003). Furthermore, the requirement for additional freshwater damages the water-independence that otherwise makes RAS so advantageous. Geosmin and MIB can also be removed directly from husbandry water by ozonation (Antonopoulou et al., 2014), but the efficiency of this technique is considerably reduced with increasing organic matter content (Klausen and Grønborg, 2010) and is thus often not feasible in intensive aquaculture systems (Schrader et al., 2010). Further possibilities for treating off-flavor compounds in system waters include ultrasonically induced cavitation (Nam-Koong et al., 2016) and UV-TiO_2 photocatalysis (Rodriguez-Gonzalez et al., 2019), but these techniques are still at an early stage in development. More research is needed in this area in order to improve the efficiency and cost-effectiveness of fish production in RAS.

Another problem related to elevated solid levels in aquaculture systems is the occurrence of hydrogen sulphide (Reiffenstein et al., 1992; Reynolds and Haines, 1980). Spikes in this fish toxic compound released from solid deposits have been responsible for mass mortalities in commercial RAS in recent years. Thus, despite the absence of direct negative effects of suspended solids on fish health and performance in aquaculture systems, their presence can still pose a severe risk and more research into the full implications of elevated particle load is still needed.

7.4 Implications for natural systems

Despite the absence of any observable effect on fish welfare in aquaculture systems, the increased particle load associated with intensified fish production may impact on adjacent waters via nutrient-rich effluent, especially in case of flow-through and semi-recirculating systems. Under normal conditions, most particles will be extracted by drum filters and/or sedimentation basins (Bergheim and Brinker, 2003). However, intensifying aquacultural production will increase the load of particles, including difficult-to-treat fines. Phosphorus, the limiting nutrient in most freshwater ecosystems (Conley et al., 2009), is contained mainly in the solid fraction (Cripps and Bergheim, 2000; van Rijn, 2013), and thus, elevated particle content in aquaculture effluents will increase the quantity of this environmentally critical substances leaching into adjacent water (Chen et al., 2003; Kvåle et al., 2006). However, inland aquaculture systems are only point sources of pollution and are therefore more of local rather than global importance with regard to pollution of surface waters (Bergheim and Brinker, 2003).

Nevertheless, there is a need for effluent treatment of flow-through and semi-recirculating systems to be further improved in order to keep nutrient outputs low (Sindilariu, 2007; Sindilariu et al., 2009b, 2009a). RAS offer great potential in this context, as the more concentrated waste effluents they produce can be treated more easily than those of open systems (Martins et al., 2010). Furthermore, the increased occurrence of flake-like solids observed in the investigated systems (chapter 2) may also be beneficial, as the enhanced development of filter cakes on the screens may improve the efficiency of mechanical filtration (Dolan et al., 2013; Wakeman, 2007).

Another important consideration stemming from the adjusted calculations of volume and surface area of particles applied in this thesis (chapter 2) is their applicability beyond aquaculture in research fields such as sewage treatment and microplastic studies. Particles from biofilter effluents also seem to exhibit an elliptical rather than a round shape (Zahid and Ganczarczyk, 1990) and the surface area of microplastics, which often deviate greatly from a spherical shape (Zhao et al., 2014), is better approximated using an assumption of ellipticity. Surface area is a very important parameter in assessing the potential threats of microplastics in natural waters because

hydrophobic organic chemicals can be adsorbed and enriched on the surface of microplastic particles (Avio et al., 2015; Lee et al., 2014).

7.5 Future challenges for aquaculture

The most pressing challenge to which aquaculture must adapt in the future is that of climate change (Ahmed et al., 2019). The effects of climate change can already be observed in inland waters of Central Europe where droughts in the last few years led to reduced river levels and increased fish mortality due to high water temperatures and lower oxygen solubility. Water scarcity is likely to become a greater threat as droughts occur more frequently in the next decades (Barange et al., 2018; Hanjra and Qureshi, 2010; Spinoni et al., 2018). This will likely lead to increased concentrations of suspended particles in the waters supplying many fish farms, further emphasizing the relevance of the present research.

As average ocean temperatures rise (Hoegh-Guldberg and Bruno, 2010), it is likely that the coastal cages and net-pens used in marine aquaculture will be replaced by open-ocean facilities, where conditions may remain more stable (Klinger et al., 2017). Meanwhile inland, the increasing frequency and strength of floods (Handisyde et al., 2006; *IPCC*, 2014) will increase the problem of nutrient leaching from soils and lead to a deterioration in water quality that will directly affect the welfare of fish in the farms being supplied, especially flow-through and semi-recirculating systems. Floods and extreme rainfall events will also increase inputs of mineral and soil particles which constitute a higher risk of mechanical damage, e.g. to fish gills, due their density and sharp edges (Au et al., 2004; Bash et al., 2001; Bruton, 1985; Kemp et al., 2011). Furthermore, increasing water temperatures will impact negatively on oxygen solubility (Breitburg et al., 2018; Ficke et al., 2007) and become a serious threat for open systems.

The closed design of RAS offers good opportunities to cope with some of the consequences of climate change. Such systems are independent of external influences, like droughts, floods and heavy rain, and can provide optimal conditions adjusted to the specific needs of every fish species. A disadvantage of these high-tech and energy-

intensive operations is their carbon footprint (Klinger and Naylor, 2012). Overall greenhouse gas emissions from aquaculture production are currently expected to increase to 776 Mt CO_2 eq. in 2050 (Mungkung et al., 2014). In this respect, the findings obtained in this thesis will help to improve the removal of particles in RAS and thus decrease the energetic cost and carbon footprint of mechanical filtration and backwashing (Badiola et al., 2018). Additional improvements might be made through the application of renewable energy to run RAS (Badiola et al., 2018), for example electricity generated using solar, wind and wave power and use of geothermal energy or waste heat from industry to maintain suitable temperatures in warm-water systems.

In summary, it is vital that fish production in RAS is optimized to ensure the future supply of fish in a globally changing world. The studies included in this thesis make contributions towards an efficient and animal-friendly fish production in RAS.

7.6 Outlook

The findings in the present study provide a broad, sound basis for further research in the field of suspended solid analysis and its implications for aquaculture. The insights gained answer relevant open questions about the nature of suspended solids while raising other questions in relation to particles in aquaculture systems, which will have to be addressed by subsequent research.

Firstly, as suspended solids were proved to be noncritical for fish physiology and performance in RAS, more research is needed to clarify which of the other correlating water parameters may impact fish health in such systems. Oxygen seems to be a likely candidate, but interaction effects with other water parameters and chemical contaminants must also be more closely investigated in follow-up studies.

Furthermore, rainbow trout were used as representative salmonids in this thesis. It is reasonable to presume that the gained findings may also apply to other species of salmonids and other finfish, which are generally considered to share comparable requirements with regards to water quality (Alabaster and Lloyd, 1982). Nevertheless, the exact implications of suspended solid loads for other fish species should also be evaluated in further investigations.

Another area for further study is the effect of suspended solid load on microbial communities in RAS. In this thesis, it was shown that bacterial activity correlated closely with particle load, but there was no evidence of resulting physiological damage to fish. Thus, special attention should be given to the impact of increased particle concentrations on the potential spread of pathogens.

Finally, this thesis showed that the majority of particles in all investigated systems had a flake-like shape. This finding and further new knowledge about the varying particle size distributions associated with different aquaculture production types should be used to further improve waste management in aquaculture systems, to reduce the environmental impact of fish farming and to promote environment- and animal-friendly production of fish in future.

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9 Publication list

The following papers were included in this thesis:

Becke C., Schumann M., Geist J., Brinker A.: Shape characteristics of suspended solids and implications in different salmonid aquaculture production systems. Aquaculture (accepted).

Becke, C., Steinhagen, D., Schumann, M., Brinker, A. (2017): Physiological consequences for rainbow trout (*Oncorhynchus mykiss*) of short-term exposure to increased suspended solid load. Aquacultural Engineering 78, 63–74.

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

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

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11 / 2017: Becke C., Schumann M., Steinhagen D., Rojas-Tirado P.A., Geist J., Brinker A.: Auswirkungen von Schwebstoffbelastung auf Regenbogenforellen bei gleichzeitig erhöhter Ammoniumkonzentration. Fachforum Forellenzucht Baden-Württemberg, Geisingen, Germany

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Poster presentation

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10 Author contributions to the chapters

Cornelius Becke (**CB**), Mark Schumann (**MS**), Ph.D. Paula Rojas-Tirado (**PRT**), Prof. Dr. Dieter Steinhagen (**DS**), PD Dr. Alexander Brinker (**AB**), Prof. Dr. Jürgen Geist (**JG**)

Chapter 3: Shape characteristics of suspended solids and implications in different salmonid aquaculture production systems

CB, MS, AB and JG conceived the concept for the paper. Sampling and particle measurements were performed by CB. Analysis of data was done by CB with continuous input and guidance of AB. Interpretation of data was conducted by CB with support by MS and results were discussed with AB and JG. The manuscript was drafted by CB with continuous input and revision of AB and JG.

Chapter 4: Physiological consequences for rainbow trout (*Oncorhynchus mykiss*) of short-term exposure to increased suspended solid load

This study was designed by CB, MS and AB. Husbandry of fish was managed by CB with the help of MS. Particle analysis was done by CB with support by MS. Physiological assays were executed by CB. Histological examination of gills was performed by DS. Statistical analyses and interpretation of data were done by CB with support of AB. The results were critically discussed with MS and AB. The manuscript was drafted by CB under guidance and revision of AB.

Chapter 5: Physiological consequences of chronic exposure of rainbow trout (*Oncorhynchus mykiss*) to suspended solid load in recirculating aquaculture systems

The study was designed by CB, MS and AB in consultation with JG. Husbandry of fish was managed by CB with the help of MS. Particle analysis and physiological assays were done by CB. Histological examination of gills was performed by DS. Statistical analyses and interpretation of data were done by CB with support of AB. The results were critically discussed with MS, DS, AB and JG. The manuscript was drafted by CB with continuous input and revision of AB and JG.

Chapter 6: Effects of unionized ammonia and suspended solids on rainbow trout (*Oncorhynchus mykiss*) in recirculating aquaculture systems

CB, MS and AB conceived the concept of this study in consultation with JG. Husbandry of fish was managed by CB with the help of MS. Particle analysis and physiological assays were done by CB. Histological examination of gills was performed by DS. Bacterial activity measurements were performed and interpreted by CB with support by PRT. Statistical analyses and interpretation of data was done by CB with support of AB. The results were critically discussed with MS, DS, AB and JG. The manuscript was drafted by CB with continuous input and revision of AB and JG.

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