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Published in:
Aquaculture

Link to article, DOI:
10.1016/j.aquaculture.2018.09.048

Publication date:
2018

Document Version
Peer reviewed version

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PII: S0044-8486(18)31503-5
DOI: doi:10.1016/j.aquaculture.2018.09.048
Reference: AQUA 633572

To appear in: *aquaculture*

Received date: 11 July 2018
Revised date: 24 September 2018
Accepted date: 25 September 2018

Please cite this article as: Cornelius Becke, Mark Schumann, Dieter Steinhagen, Paula Rojas-Tirado, Juergen Geist, Alexander Brinker , Effects of unionized ammonia and suspended solids on rainbow trout (Oncorhynchus mykiss) in recirculating aquaculture systems. Aqua (2018), doi:10.1016/j.aquaculture.2018.09.048

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

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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) the generally accepted 'safe' threshold while total suspended solids (TSS) exceeded the 'safe' threshold of 25 mg/L 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. 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. 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.

Keywords: Fish health, Water quality, Particle accumulation, Turbidity, Salmonid, Bacterial activity

Highlights:

- Study of combined chronic effects of critical solid and unionized ammonia exposure
- Full control of water parameters except turbidity in replicated RAS
- Only minimal effects of NH_3-N up to 0.05 mg/L on fish physiology
- No interaction effects between unionized ammonia and suspended solid load
- Close linear correlation of suspended solid load and bacterial activity

1 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; Roque d'Orbcastel et al., 2009). 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., 2017, 2018). 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 (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) 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.

2 Materials and Methods

2.1. Husbandry

The experiment used two replicate RASs, each with 10 tanks (capacity of 330 L, total RAS volume 6m^3) (Figure 1), 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 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, 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.

2.2. Water parameters

The experiment was subdivided into three phases (Figure 2): 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 1). In the treatment RAS, however, the total suspended solid concentration was increased to over 35 mg/L and was subsequently held constantly above this value. The control RAS operated under commercial conditions at around 5 mg/L 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) 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 (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 (Figure 3).

147 NH_4-N, NO_2-N and NO_3-N were chemically determined three times per week throughout
148 the experiment with analysis kits (LCK 304: 0.2 – 2.5 mg/L; LCK 341: 0,05 – 2 mg/L; and
149 LCK 339: 1 – 6 mg/L, Hach, Germany, respectively), using water from the connecting tube
150 from the fish tanks of each RAS. Oxygen concentration (using Oxygen Probes, OxyGuard,
151 Farum, Denmark) and temperature (using Temperature Probes, Oxyguard, Farum,
152 Denmark) were monitored continuously at the outlets of two fish tanks in each system.
153 Carbon dioxide concentrations were determined two times per week in the fish tanks using
154 a portable dissolved CO_2 analyzer (OxyGuard CO_2 Portable, OxyGuard, Farum,
155 Denmark). Turbidity was determined three times per week in parallel with the
156 determination of total suspended solids using a turbidity meter (PCE-TUM 20, PCE
157 Instruments, Germany).
158
159 2.3. Analysis of suspended solids
160 2.3.1. Total suspended solids
161 The concentration of total suspended solids was determined three times per week in
162 duplicate for each system according to method 2540 D of the American Public Health
163 Association (APHA, 1998), with the exception that 0.45 µm cellulose-acetate filters
164 (diameter: 50mm, 11106-50-N, Sartorius AG, Göttingen, Germany) were used instead of
165 glass-fiber filters due to the smaller and better defined pore sizes. Filters were prepared as
166 described by Becke et al. (2018). Water samples were collected using a tube at a water
167 depth of ca. 30 cm from five tanks in each system, then duplicate samples were pooled to
168 create a representative sample for each system. Samples were collected in the early
169 morning before feeding, in order to represent the daily minimum solid loads (best case
170 scenario). To determine the within-day fluctuations and maximum values, measurements
171 were performed every two hours on one day in week 12.
172

2.3.2. Particle size distribution (PSD)

For particle size measurement, water samples were collected as described above. Particle sizes were determined according to Brinker et al. (2005) 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.

2.4. 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:

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

where *t* is time (days).

The feed conversion ratio (FCR) was calculated as:

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

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

$TGC = (W_t^{1/3} - W_0^{1/3}) \times (\sum T)^{-1} \times 1000;$

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

2.5. 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, 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 blood, Sarstedt, Nümbrecht, Germany). Subsequently, fish were killed and samples of gill tissue were collected for histological examination.

2.6. Health parameters

2.6.1. 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.

2.6.2. 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 = (fin length/total length)*100*

2.6.3. 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

224 shown that devices for measuring human glucose level are also suitable for use with fish
225 blood (Bartoňková et al., 2017; Eames et al., 2010).
226
227 2.7. Bacterial assay
228 2.7.1. Bacterial load
229 Analysis of bacterial load of rainbow trout was conducted at the termination of the study
230 (20 rainbow trout per RAS) by the fish health service at a governmental veterinary institute,
231 the Staatliches Tierärztliches Untersuchungsamt (STUA) Aulendorf, Germany as
232 described in Becke et al. (2018). Briefly, the number of colony forming units was assessed
233 on skin and spleen and ranked as *no*, *sporadic*, *slight*, *moderate* or *severe* bacterial load.
234 Bacterial species were then determined by using bacteriological standard methods and
235 confirmed by MALDI-TOF MS (Lay, 2001).
236
237 2.7.2. Bacterial activity in the water
238 Bacterial activity in the fish tanks was assessed using a patented method called
239 BactiQuant® (Mycometer A/S, Copenhagen, Denmark), which is an indirect measure of
240 microbial enzyme activity. Reproducibility and repeatability of the method has been
241 documented in a verification report by the United States Environmental Protection Agency
242 (U.S.-EPA, 2011). Briefly, a 10 mL water sample was filtered through a Millipore 0.22 µm
243 closed filter unit (PES express). The filter was then incubated with a fluorogenic enzyme
244 substrate for 15 min. The synthetic fluorescent enzyme-substrate is hydrolyzed by
245 microbial enzymes in the water sample and the amount of released fluorophores was
246 quantified with a fluorometer (Mycometer A/S, Copenhagen, Denmark). The results were
247 expressed in standardized Bactiquant® values (BQV; hereafter termed bacterial activity).
248 Measurements were always performed in duplicate. During the first three weeks, bacterial
249 activity was measured every second day to gain a better overview of the development until

the particle concentration exceeded 35 mg/L in the treatment RAS. From week 4 onwards, bacterial activity was measured twice a week.

2.8. 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 a linear parametric model was applied (Supplement 1).

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 *(%) = (standard deviation (σ) / arithmetic mean ($\bar{x}$)) x 100*

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

3 Results

3.1. 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 1). In week 11 to 13,

276 NO_2-N concentration was approximately 0.15 mg/L higher in the control RAS and differed
277 significantly ($P < 0.05$) between RAS systems, but not in phase one and two of the
278 experiment ($P > 0.05$). Water consumption was significantly higher ($P < 0.05$) in the control
279 system in week 1 to 5 (approx. 40 L/day) and in week 6 to 10 (approx. 22 L/day) than in
280 the treatment because the backwash water of the drum filter was reinjected into the
281 treatment system. From week 11 to 13 no significant difference ($P > 0.05$) was found
282 between systems due to adjusting the water consumption in the treatment system.
283 However, magnitudes of differences were minimal and were thus deemed biologically not
284 relevant. Turbidity differed significantly ($P < 0.001$) by up to 15 NTU (Nephelometric
285 Turbidity Units) between control and treatment RAS at individual sampling time points
286 (Table 1) as related to different suspended solid load. NH_4-N concentrations, pH, O_2
287 concentration and NO_3-N concentration did not differ significantly ($P > 0.05$) between
288 control and treatment system. NH_4-N concentrations were increased both in the control
289 and treatment RAS after week 5 and week 10 with concentrations peaks of up to 2.5 mg/L
290 (control) and 2.3 mg/L (treatment) respectively, but without significant differences ($P >$
291 0.05) between systems. Unionized ammonia-N concentrations were also increased after
292 week 5 from 0.005 to 0.012 mg/L and further to over 0.02 mg/L after week 10 (Figure 3),
293 however, without significant differences ($P > 0.05$). Overall, with the exception of NH_4-
294 N/NH_3-N and suspended solid load, all water parameters remained within physiological
295 optimal range for rainbow trout (Timmons and Ebeling 2010).
296
297 3.2. Suspended solids analysis
298 3.2.1 Total suspended solids
299 Total suspended solid (TSS) concentration differed significantly ($P < 0.0001$) between
300 control and treatment RAS with an average concentration of 4.5 mg/L in the control and
301 35.2 mg/L in the treatment system (Figure 4 A). From week 3, the TSS concentration in

the treatment system exceeded 30 mg/L and remained at an average of 40.5 mg/L. Furthermore, the difference in TSS concentration between control and treatment RAS was never less than 23.1 mg/L. Figure 4 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 in the treatment RAS while it was 14.1 mg/L in the control RAS.

3.2.2. 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 in the control and 47.4 ± 2.7 mg dry weight/L in the treatment system. For each particle size class, the absolute frequencies differed significantly ($P < 0.001$) between control and treatment RAS (Figure 5). 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.

3.3. 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 2).

3.4. Health parameters

328 3.4.1. Gill histology

329 No severe histological changes in gill structures were observed during the investigation.
330 Cases of cellular edema, tip-thickening of secondary lamellae, telangiectasia, thickening of
331 epithelial cells, cell infiltration, lamellar fusion, merging of secondary lamellae and
332 detachment of the epithelium were only minor or moderate (Supplement 2). In terms of
333 cellular edema, all factors were significantly altered by treatment ($P < 0.05$), but magnitude
334 of differences was small (0 – 15 %) and the observed histological change was only rated
335 as minor. The increased TSS concentration did not have any significant effect on all further
336 investigated histological parameters ($P > 0.05$). However, the increased unionized
337 ammonia concentration ($P < 0.05$) and the interaction of unionized ammonia concentration
338 and day of sampling ($P < 0.05$) led to a significantly more frequent occurrence of cell
339 infiltrations and tip thickening of secondary lamellae. All other histological parameters were
340 not significantly affected by the increased unionized ammonia concentration ($P > 0.05$).
341 Furthermore, no significant interaction of increased unionized ammonia concentration and
342 increased suspended solid load ($P > 0.05$) were found for any of the investigated
343 histological parameters.
344 Regarding thickness of branchial epithelium and number of goblet cells per secondary
345 lamella (Supplement 3), no significant effects ($P > 0.05$) of increased unionized ammonia
346 or suspended solid load were apparent at all.
347
348 3.4.2. Fin condition
349 Neither total suspended solid concentration ($P > 0.05$) or unionized ammonia ($P > 0.05$)
350 had a significant effect on fish welfare measured by fin erosion in the control and treatment
351 RAS (Supplement 4). Furthermore, no interaction effect of increased unionized ammonia
352 concentrations and suspended solid load was apparent ($P > 0.05$).

353 The increased unionized ammonia concentrations caused a significantly lower fin index
354 ($P < 0.05$) for the dorsal fin (Supplement 5). However, fin indices of the left and right
355 pectoral fin were not affected ($P > 0.05$). Increased suspended solid load had no
356 significant effect ($P > 0.05$) on any fin index. Furthermore, the elevated unionized
357 ammonia concentrations did not significantly affect the impact of suspended solid load
358 ($P > 0.05$) on fin indices.
359
360 3.4.3. Hematology
361 Overall, all hematological parameters (Table 3) were approximately within the range
362 previously reported for salmonids (McCarthy et al., 1973, 1975; Pund, 1998; Řehulka et
363 al., 2004). However, hematocrit was significantly decreased ($P < 0.05$) both with
364 increasing TSS concentration and increasing body length, whereas hematocrit significantly
365 increased ($P < 0.05$) over time. Thus, the MCV value was also significantly lower ($P <$
366 0.01) and the MCHC value significantly higher ($P < 0.01$) with increasing TSS
367 concentrations. The interaction of TSS concentration and unionized ammonia
368 concentration revealed a significant effect ($P < 0.05$) on MCHC values. The number of
369 thrombocytes was significantly elevated ($P < 0.05$) with increasing unionized ammonia
370 concentration and the hemoglobin concentration significantly increased ($P < 0.05$) over
371 time. All the other parameters (glucose concentration, number of erythrocytes, MCH,
372 number of leukocytes, leukocrit and the proportions of lymphocytes, granulocytes and
373 monocytes) were not significantly affected ($P > 0.05$) by suspended solid load, unionized
374 ammonia concentration or the interaction of both parameters.
375
376 3.5. Bacterial assay
377 3.5.1. 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). 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.

3.5.2. 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 7). 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

404 2.3 x10^5 to 3.7 x10^5 in the treatment RAS respectively. Overall, there was a significant
405 positive linear correlation between TSS concentration and bacterial activity ($P < 0.0001$, r^2
406 = 0.98; Figure 8). However, the certainty measure of the linear correlation of bacterial
407 activity with TSS was very low in the control RAS ($r^2 = 0.10$) while it was high in the
408 treatment RAS ($r^2 = 0.94$).
409
410 4 Discussion
411 The experiment effectively decoupled the effects of chronic suspended solid load and
412 elevated unionized ammonia concentrations from other relevant water quality parameters.
413 This allowed an investigation of the sole effects of both increased unionized ammonia
414 concentrations and suspended solid load on rainbow trout as well as their combined
415 effects at a farm-scale.
416 Recent investigations (Becke et al., 2017, 2018) have shown that even massive
417 accumulation of fine solids alone caused no detrimental effects on rainbow trout in RAS.
418 These results were corroborated by the present findings which did not reveal relevant
419 detrimental effects of increased suspended solid concentrations on fish at concentrations
420 of up to almost 70 mg/L. Gills are of delicate structure and therefore highly sensitive to
421 physical impact (Evans, 2005; Morgan and Tovell, 1973), so the absence of any
422 histological alteration associated with suspended solid load is of particular note. This is in
423 line with Goldes et al. (1988) who also observed no branchial pathology in rainbow trout
424 even when exposed to up to 1017 mg/L of suspended clay kaolin. Thus, the assumption
425 that suspended solids alone are not a key issue affecting fish welfare in RAS is further
426 strengthened.
427 However, the increased particle load caused indirect effects. It led to increased turbidity
428 which suppressed feeding behavior of fish in the treatment RAS as previously described
429 (Barrett et al., 1992; Becke et al., 2017, 2018; 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), 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

456 unionized ammonia-N was increased to levels which exceeded the common upper safe
457 limit of 0.0125 mg/L proposed for salmonid aquaculture (Timmons and Ebeling, 2010). It
458 was hypothesized that the chronic exposure to increased unionized ammonia
459 concentrations would result in a deterioration of physiology and performance of rainbow
460 trout. However, contrary to the hypotheses and praxis as well as academic opinion (Smith
461 and Piper, 1975; Thurston et al., 1984; Timmons and Ebeling, 2010), rainbow trout
462 exposed to chronic unionized ammonia-N concentrations of more than four times the
463 critical threshold did not reveal deteriorated performance in our study. Fish in both systems
464 showed very good performance with nearly 100 % survival. Only minor physiological
465 effects of increased unionized ammonia concentration on gill structure were observed.
466 Nonetheless, the observed alterations of gill structures were only slight to moderate and
467 only two (cell infiltrations, tip thickening of secondary lamellae) out of seven parameters
468 were significantly affected by the increased unionized ammonia load. Thus, these results
469 suggest that the rainbow trout can cope well with the given unionized ammonia
470 concentrations. The common upper safe limit of unionized ammonia-N of 0.0125 mg/L
471 proposed for salmonid aquaculture is based on the findings of Smith and Piper (1975).
472 However, other authors, such as Meade (1985), Daoust and Ferguson (1984) (laboratory
473 experiment) and Kolarevic et al. (2013) (commercial scale) previously questioned the
474 proposed unionized ammonia limit. Nevertheless, the value of 0.0125 mg/L has been
475 echoed widely since then and established in aquaculture textbooks (e.g. Timmons and
476 Ebeling, 2010). However, it has to be noted that oxygen concentration was low (around 6
477 mg/L) in the Smith and Piper (1975) study. According to Lloyd (1961) and Brown (1968),
478 unionized ammonia toxicity increases with decreasing oxygen levels. Thus, the interaction
479 of low oxygen with high unionized ammonia concentration in the study of Smith and Piper
480 (1975) might be causative for the pathological changes in the gills of rainbow trout. During
481 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 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 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 for unionized ammonia-N and 25 mg/L 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., 1997, 1999; 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.

5 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 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

Acknowledgements

We thank Helga Bentele and HP Billmann for their excellent technical assistance, especially with fish husbandry and system maintenance. We also thank the state veterinary institute of Aulendorf for the bacteriological examination of the fish. We also acknowledge the scientific language editing by Erica Bower as well as the valuable input of three anonymous reviewers. This research was funded by the Deutsche Bundesstiftung Umwelt (Az 30996).

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758
759

760 Figure captions:
761
762 Figure 1: Scheme of the recirculating aquaculture systems with modification for particle
763 accumulation in the treatment system (light grey shaded).
764
765 Figure 2: Experimental setup of the rainbow trout exposure in the RAS.
766
767 Figure 3: Unionized ammonia nitrogen (NH_3-N) concentration (mean, minimum (Min) and
768 maximum values (Max)) in the control and treatment RAS during the investigation period.
769 The dashed line shows the common limit value of NH_3-N (0.0125 mg/L) for salmonids
770 (Timmons and Ebeling, 2010).
771
772 Figure 4: (A) Timeline of total suspended solids concentration (mean ± S.D.) in control and
773 treatment RAS over the experimental period. (B) Representative daily variation of total
774 suspended solids concentration (mean ± S.D.) in control and treatment RAS in week 12.
775 Samples were collected every two hours between 7:00 and 19:00 and at 23:00 (CET).
776 Please note the axis break on the x-axis.
777
778 Figure 5: Absolute frequency within particle size classes (mean ± S.E.) of the control (n =
779 4) and treatment RAS (n = 4) in week 12. All particle size classes differed significantly ($P <$
780 0.001) between control and treatment system. Please note the axis break on the y-axis.
781
782 Figure 6: Bacteriological examination of gills (direct detection and cultivation) and spleen
783 (cultivation) from 20 rainbow trout of the control (C) and treatment (T) RAS. ** = $P < 0.01$;
784 *** = $P < 0.0001$
785
786 Figure 7: Bacterial activity (BQV/mL, mean ± S.D.) in the treatment and control RAS during
787 the investigation period.
788
789 Figure 8: Linear relationship between bacterial activity (BQV/mL) and total suspended
790 solid concentration (mg/L) for control (open symbol) and treatment RAS (solid symbol) and
791 in sum. The dashed line shows the overall linear relationship, the solid lines show the
792 linear relationship of the treatment and control system respectively.
793

794 Highlights:

795 • Study of combined chronic effects of critical solid and unionized ammonia exposure

796 • Full control of water parameters except turbidity in replicated RAS

797 • Only minimal effects of NH_3-N up to 0.05 mg/L on fish physiology

798 • No interaction effects between ammonia and suspended solid load

799 • Close linear correlation of suspended solid load and bacterial activity

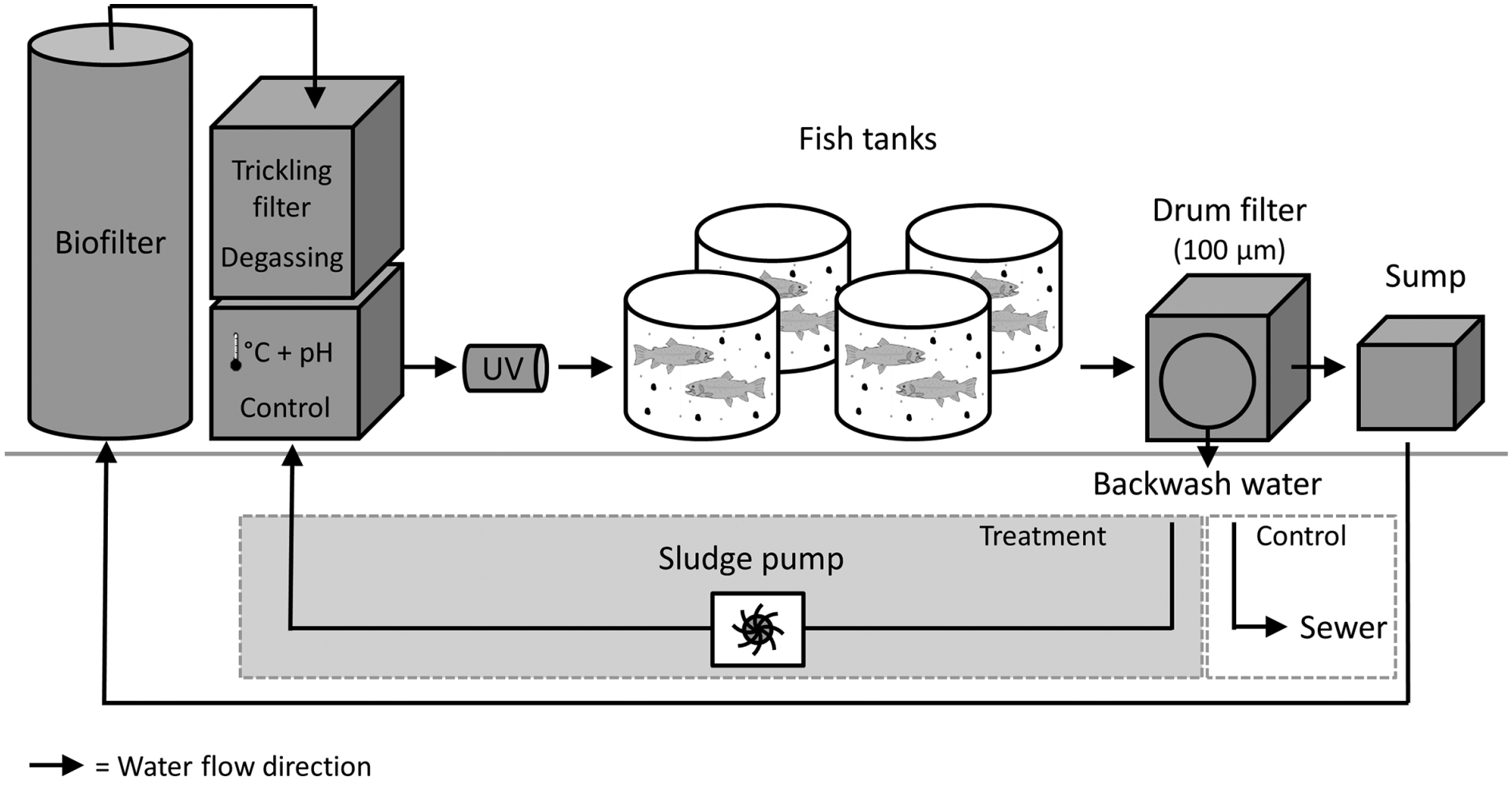
Biofilter
Trickling filter
Degassing
°C + pH
Control
UV
Fish tanks
Drum filter
(100 µm)
Sump
Backwash water
Treatment
Sludge pump
Control
Sewer
= Water flow direction

Figure 1

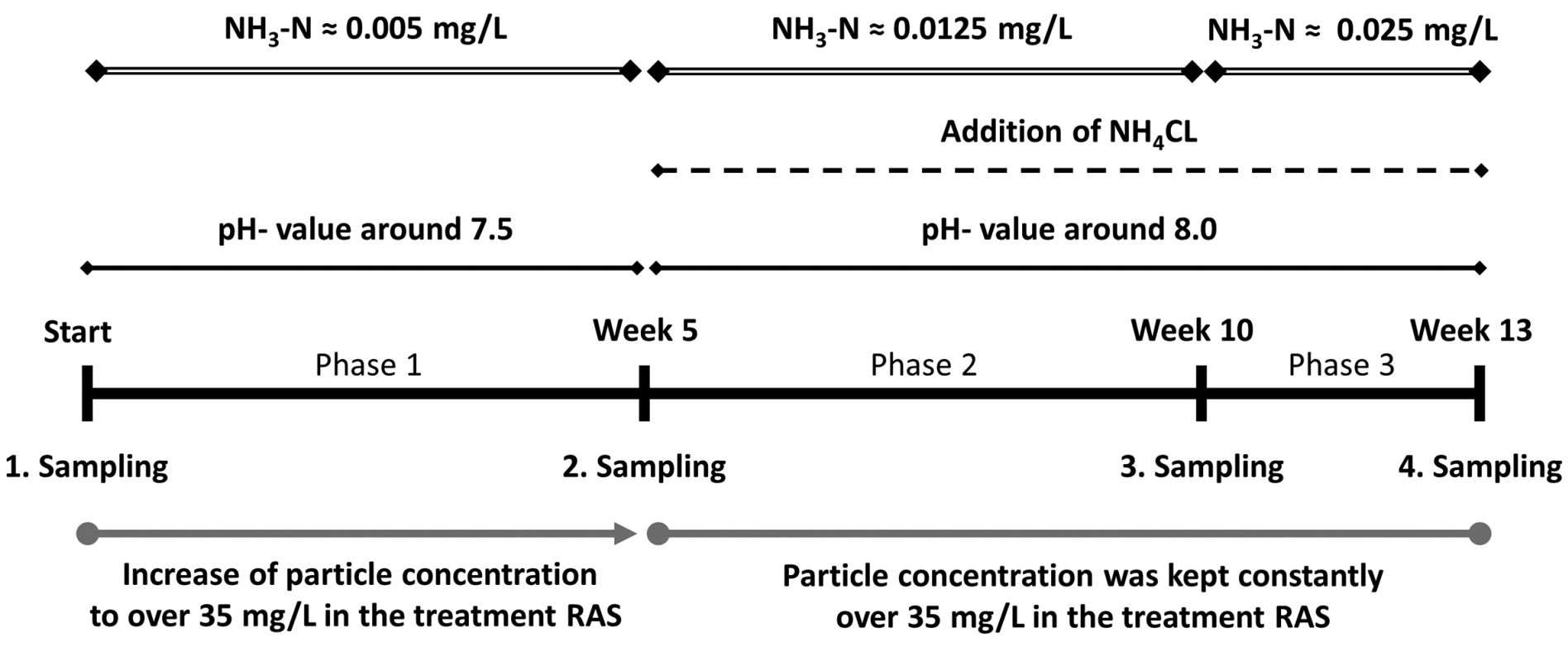
NH3-N ≈ 0.005 mg/L
NH3-N ≈ 0.0125 mg/L
NH3-N ≈ 0.025 mg/L
Addition of NH4CL
pH- value around 7.5
pH- value around 8.0
Start
Week 5
Week 10
Week 13
Phase 1
Phase 2
Phase 3
1. Sampling
2. Sampling
3. Sampling
4. Sampling
Increase of particle concentration to over 35 mg/L in the treatment RAS
Particle concentration was kept constantly over 35 mg/L in the treatment RAS

Figure 2

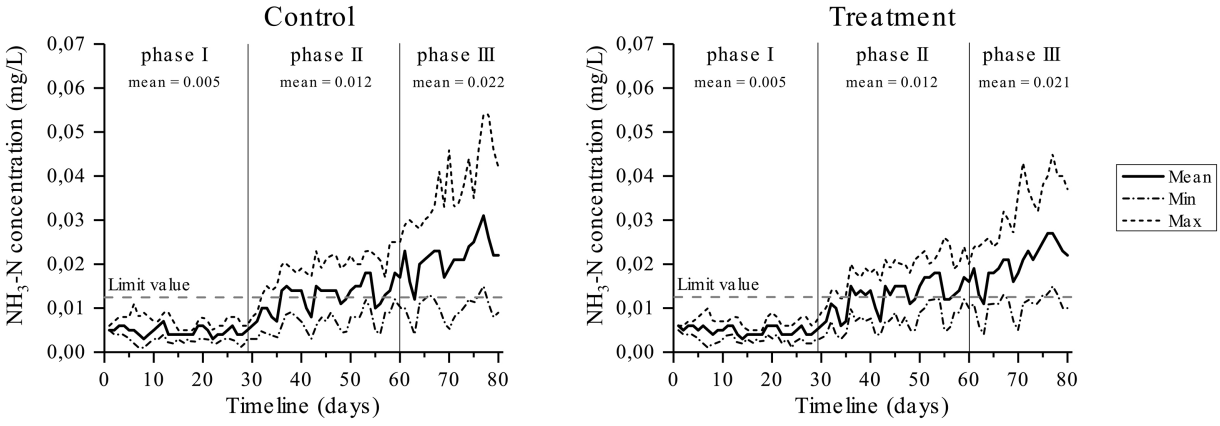
Control
Treatment
phase I
phase II
phase III
mean = 0.005
mean = 0.012
mean = 0.022
mean = 0.021
Limit value
NH3-N concentration (mg/L)
Timeline (days)
0,00
0,01
0,02
0,03
0,04
0,05
0,06
0,07
0
10
20
30
40
50
60
70
80
Mean
Min
Max

Figure 3

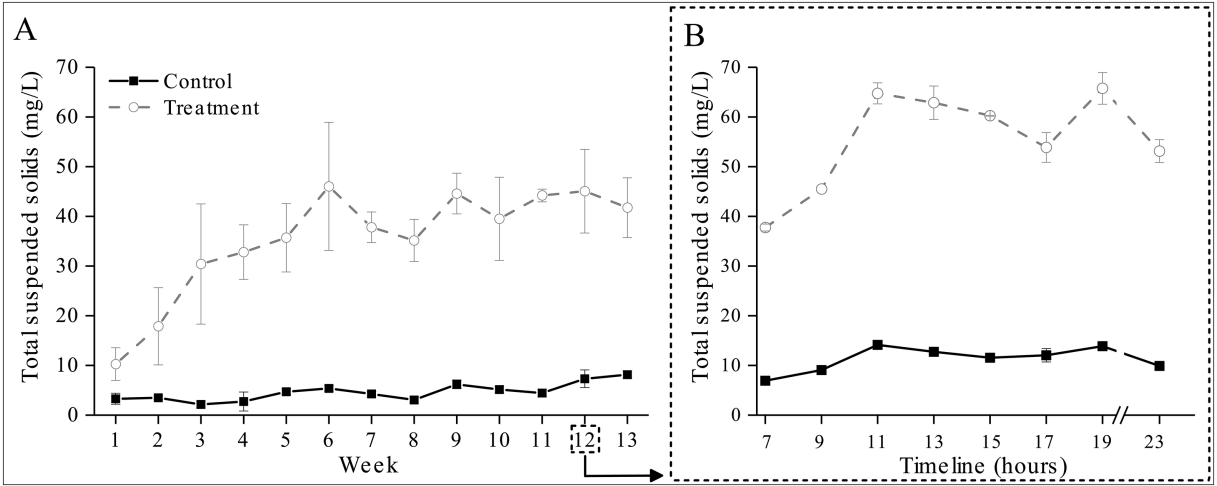
A
Control
Treatment
Total suspended solids (mg/L)
0
10
20
30
40
50
60
70
1
2
3
4
5
6
7
8
9
10
11
12
13
Week
B
Total suspended solids (mg/L)
0
10
20
30
40
50
60
70
7
9
11
13
15
17
19
23
Timeline (hours)

Figure 4

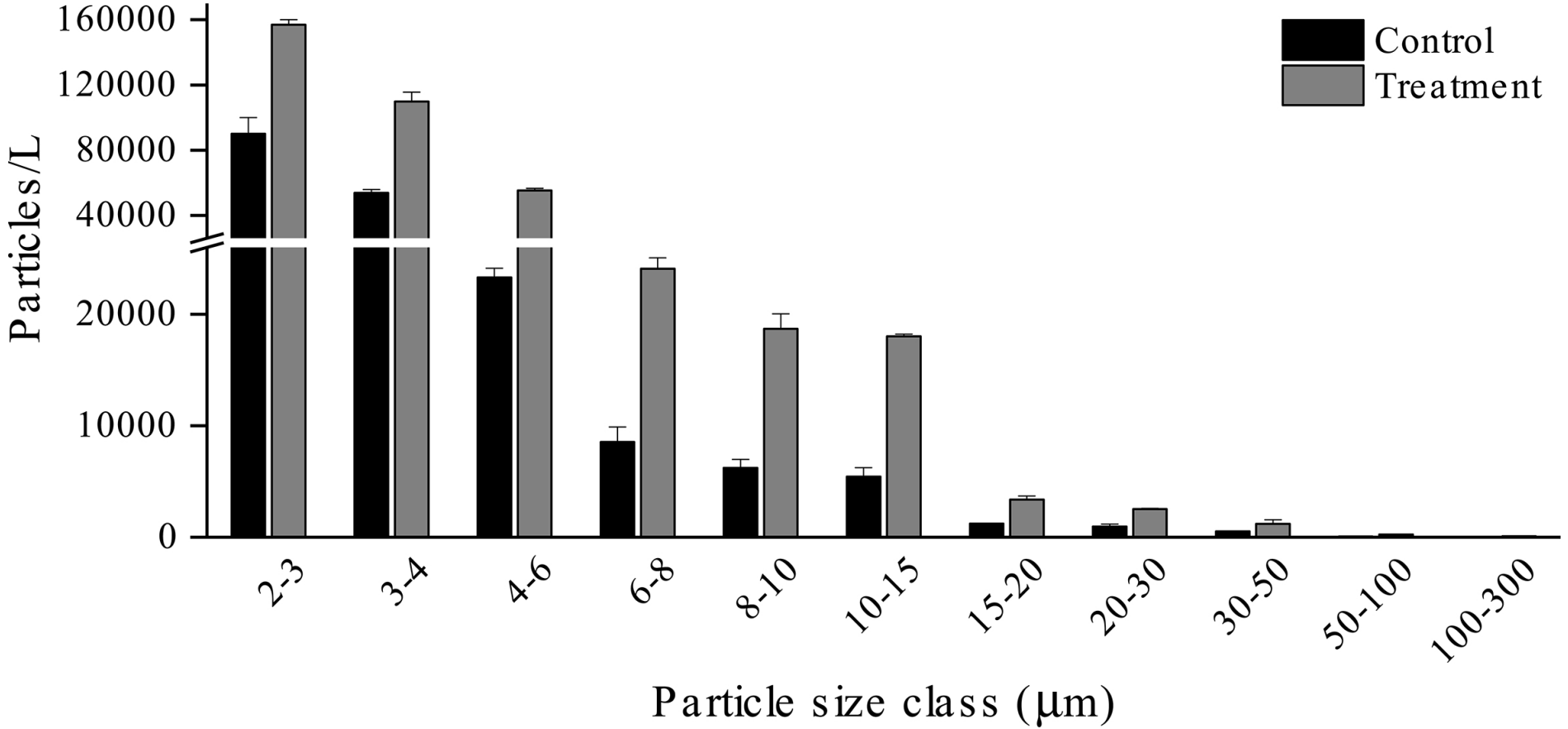
160000
120000
80000
40000
20000
10000
0
Particles/L
Control
Treatment
2-3
3-4
4-6
6-8
8-10
10-15
15-20
20-30
30-50
50-100
100-300
Particle size class (μm)

Figure 5

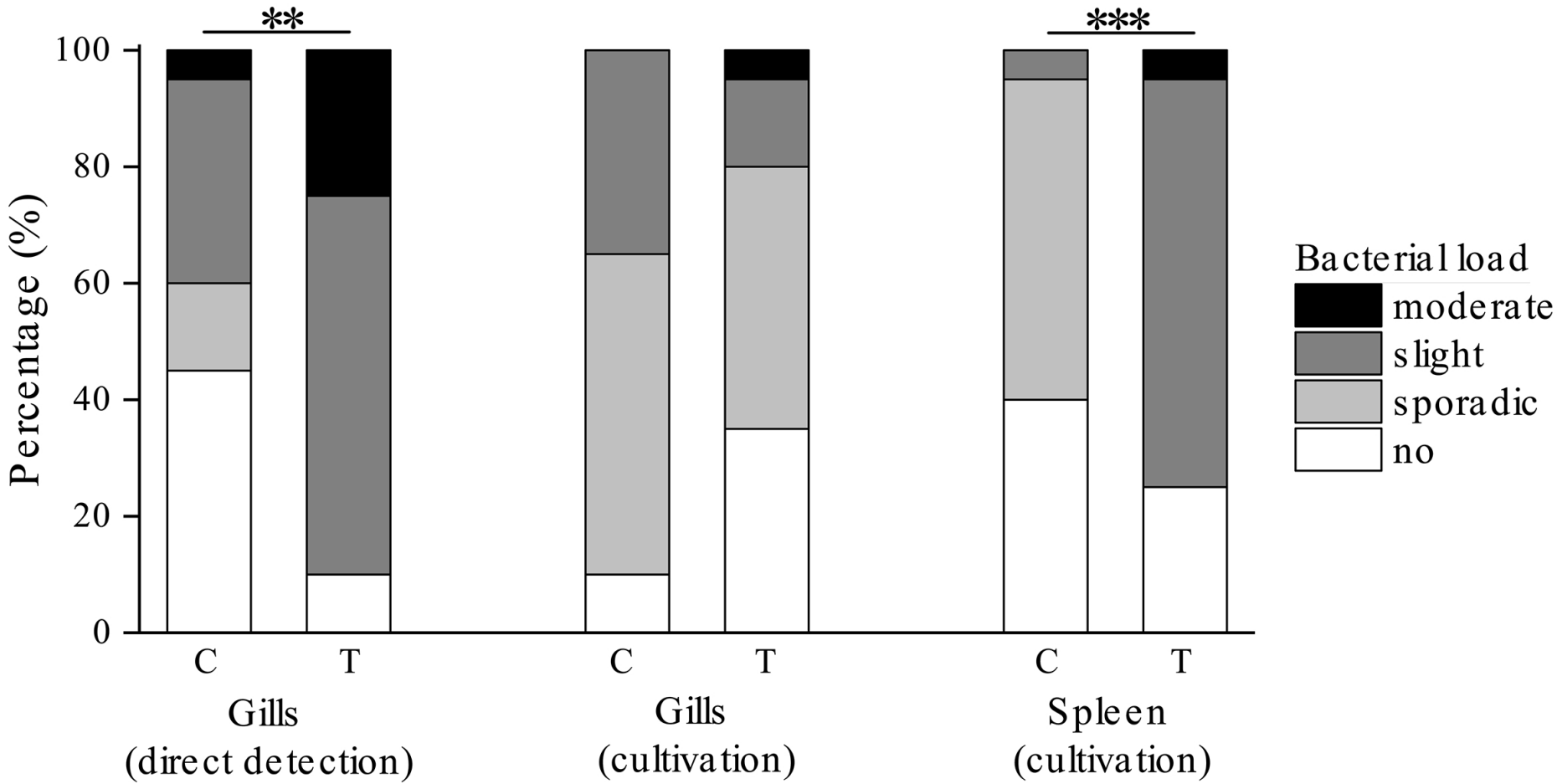
**
***
100
80
60
40
20
0
Percentage (%)
C
T
C
T
C
T
Gills
(direct detection)
Gills
(cultivation)
Spleen
(cultivation)
Bacterial load
moderate
slight
sporadic
no

Figure 6

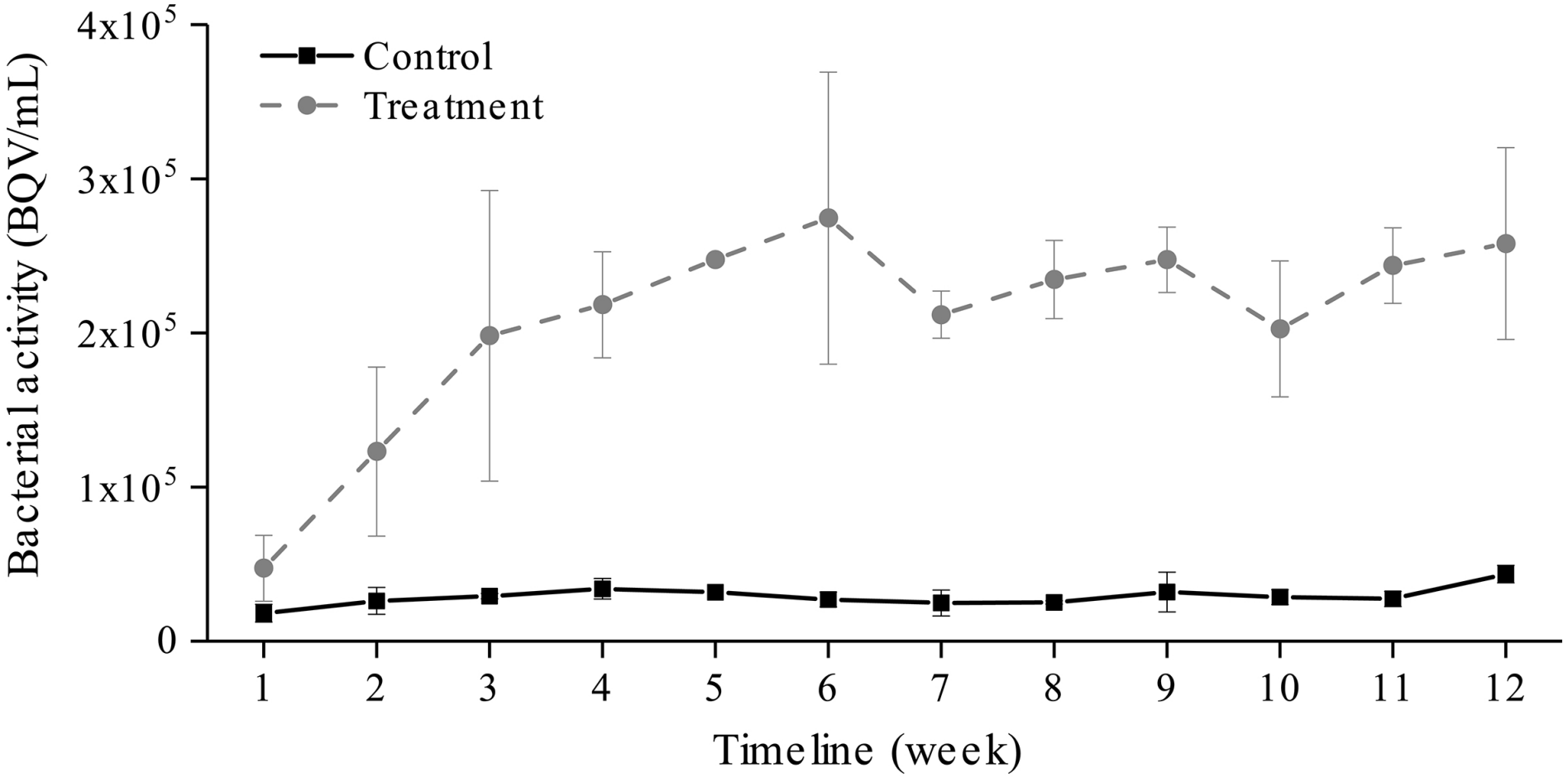
Control
Treatment
Bacterial activity (BQV/mL)
4x10^5
3x10^5
2x10^5
1x10^5
0
1
2
3
4
5
6
7
8
9
10
11
12
Timeline (week)

Figure 7

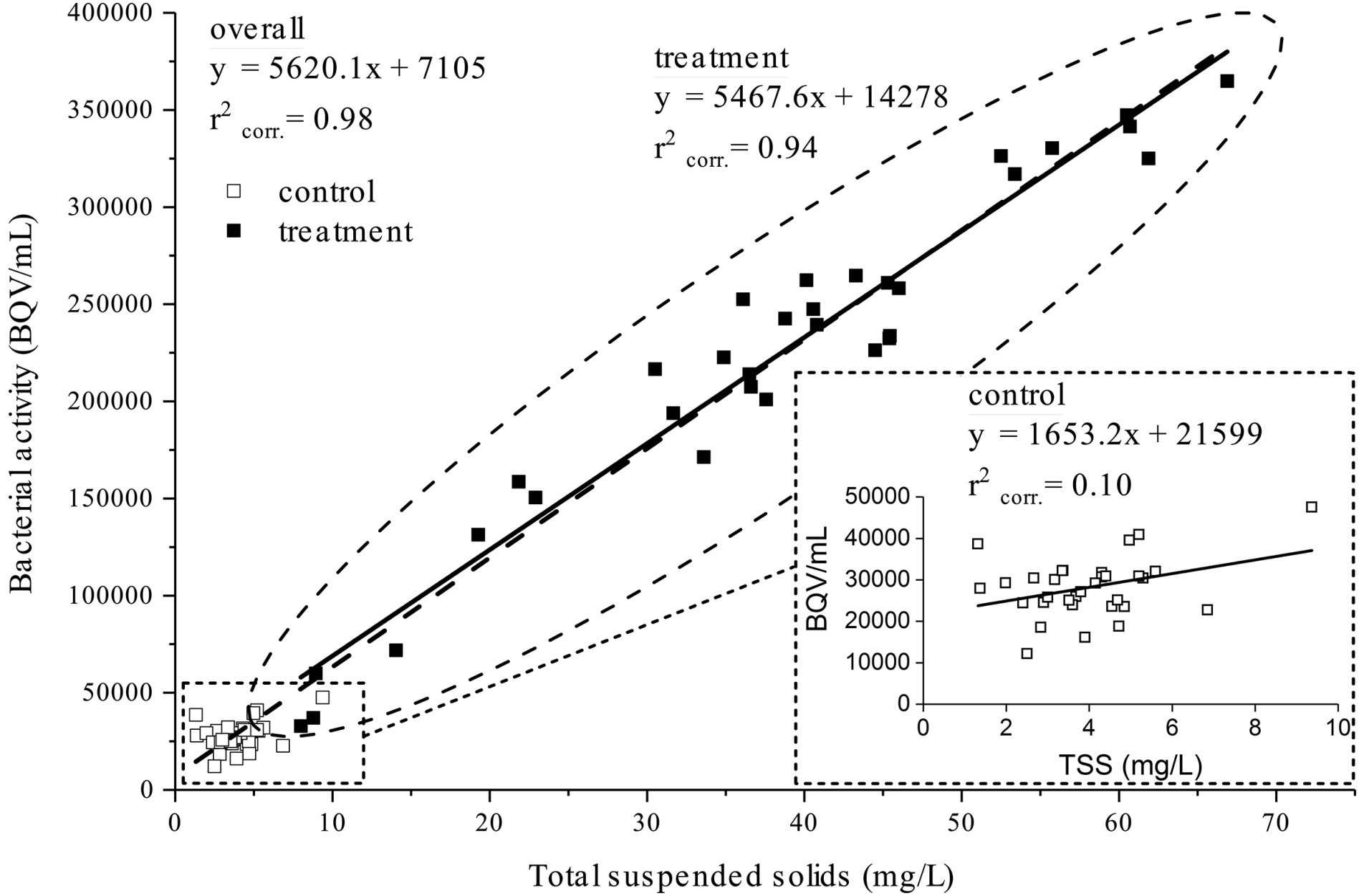
overall
y = 5620.1x + 7105
r2 corr.= 0.98
control
treatment
treatment
y = 5467.6x + 14278
r2 corr.= 0.94
control
y = 1653.2x + 21599
r2 corr.= 0.10
Bacterial activity (BQV/mL)
Total suspended solids (mg/L)
BQV/mL
TSS (mg/L)
0
50000
100000
150000
200000
250000
300000
350000
400000
0
10
20
30
40
50
60
70
10000
20000
30000
40000
2
4
6
8

Figure 8