



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

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## Accepted Manuscript

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

3

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22 Abstract

23 This study investigates the individual and combined effects of chronic exposure of rainbow  
24 trout to **unionized ammonia** and suspended solids in a farm-scale recirculating aquaculture  
25 system (RAS) over 13 weeks. **Unionized ammonia nitrogen** concentration was four times  
26 (0.05 mg/L) the generally accepted 'safe' threshold while total suspended solids (TSS)  
27 exceeded the 'safe' threshold of 25 mg/L by a factor of > 2.5. Still, rainbow trout revealed  
28 high survival rates of > 99% and no observable detrimental effects of TSS. Bacterial  
29 activity showed a close positive linear correlation with solid load and was almost  
30 exclusively explained by solid load for TSS concentration > 10 mg/L. However, bacterial  
31 activity had no apparent detrimental effect on fish health or performance. Increased  
32 **unionized ammonia nitrogen** concentrations had no relevant detrimental effect on rainbow  
33 trout physiology and performance at concentrations of up to 0.05 mg/L. Furthermore, the  
34 absent to minor solid-related effects across a wide range of physiological criteria combined  
35 with chronic exposure to **unionized ammonia** demonstrates that chemical or physical  
36 irritants are not problematic in RAS if other water and holding parameters are optimal.  
37 These findings suggest a greater than expected tolerance of rainbow trout to chronic TSS-  
38 related effects which should result in a revision of water quality threshold criteria for RAS.

39

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

42

43 Highlights:

- 44 • Study of combined chronic effects of critical solid and unionized ammonia exposure
- 45 • Full control of water parameters except turbidity in replicated RAS
- 46 • Only minimal effects of NH<sub>3</sub>-N up to 0.05 mg/L on fish physiology
- 47 • No interaction effects between unionized ammonia and suspended solid load
- 48 • Close linear correlation of suspended solid load and bacterial activity

49

50 1 Introduction

51 Aquaculture is the fastest-growing sector in the animal food production industry worldwide  
52 and already accounts for more than 44 percent of global total fish production (FAO, 2016).  
53 As capture fishery production has remained relatively static since the late 1980s and the  
54 world demand for fish is increasing (FAO, 2016), aquaculture has an important role to play  
55 in ensuring a sufficient global fish supply (Naylor et al., 2000). Recirculating aquaculture  
56 systems (RAS) are often regarded as an environmentally friendly alternative to open flow-  
57 through or cage-based aquaculture systems (Ayer and Tyedmers, 2009; Klinger and  
58 Naylor, 2012; Verdegem et al., 2006), largely due to their efficient water use. However,  
59 despite ongoing development, fish production in RASs remains energy- and cost-intensive  
60 and its contribution to global production is still small (Badiola et al., 2012; Roque  
61 d'Orbcastel et al., 2009). One approach to optimizing the economic output of RASs is to  
62 increase stocking densities to reduce costs per unit of fish produced (Martins et al., 2005).  
63 However, more fish reared in the same volume of water leads to increased excretion loads  
64 per m<sup>3</sup> of water. Fish feces are the principal constituent of suspended solids in  
65 aquacultural facilities along with uneaten feed, bacterial material from biofilters and  
66 microfauna (Chen and Malone, 1991; Noble and Summerfelt, 1996; Summerfelt et al.,  
67 1999; Wedemeyer, 1996). Accumulating particles, and especially fine particles, are  
68 considered detrimental to fish health, welfare and performance (Bilotta and Brazier, 2008;

69 Chapman et al., 1987; Chen et al., 1993; Chen and Malone, 1991; Herbert and Merkens,  
70 1961). However, this assertion has been questioned for rainbow trout by recent  
71 investigations (Becke et al., 2017, 2018). Nevertheless, intensification of aquacultural  
72 production resulting in an increase in suspended solid concentration, will also lead to an  
73 increase in dissolved wastes, such as **unionized ammonia** (NH<sub>3</sub>) (Ip et al., 2001).  
74 High **unionized ammonia** levels have a wide range of detrimental effects on fish, e.g.  
75 deterioration of gill structures, and might ultimately lead to mortality (Cameron and Heisler,  
76 1983; Daoust and Ferguson, 1984; Ip et al., 2001; Randall and Tsui, 2002; Smart, 1976;  
77 Thurston et al., 1984; Wicks et al., 2002). The common upper safe limit of **unionized**  
78 **ammonia-N** proposed for salmonid aquaculture is 0.0125 mg/L (Timmons and Ebeling,  
79 2010). However, there are studies reporting higher tolerance (Daoust and Ferguson, 1984;  
80 Meade, 1985). Thus, there is still controversy about the safe threshold for **unionized**  
81 **ammonia** in aquaculture operations.

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

89 Against this background, the present study investigated the sole effect of critical **unionized**  
90 **ammonia-N** concentrations (> 0.0125 mg/L) as well as interaction effects with suspended  
91 solid load in a farm-scale RAS. It was hypothesized that chronic exposure to high  
92 **unionized ammonia** concentrations would cause a reduction of fish wellbeing, while the  
93 combined chronic exposure with increased suspended solid load would provoke an  
94 interactive impact. Within this context, husbandry waters were set to optimal values except

95 for the two variables, unionized ammonia and suspended solids, being tested. The  
96 exception was bacterial activity which was held at an uncritical level (Pedersen et al.,  
97 2017; Rojas-Tirado et al., 2018), with possible covariate influences being controlled by the  
98 experimental design.

99

## 100 2 Materials and Methods

### 101 2.1. Husbandry

102 The experiment used two replicate RASs, each with 10 tanks (capacity of 330 L, total RAS  
103 volume 6m<sup>3</sup>) (Figure 1), as described by Becke et al. (2018). The study used all-female  
104 rainbow trout (*Oncorhynchus mykiss*, Störk strain) to exclude sex-related effects. Each  
105 RAS was stocked with 785 rainbow trout with an average initial weight of 87.2 ± 8.6 g  
106 (control group) and 87.4 ± 9.2 g (treatment group). They were held at maximum stocking  
107 densities of 67.8 ± 3.0 kg m<sup>-3</sup> (control) and 68.3 ± 2.6 kg m<sup>-3</sup> (treatment). The control RAS  
108 was operated under regular conditions, while the particle load of the treatment RAS was  
109 artificially elevated as described in Becke et al. (2018). Briefly, a mud pump (Wilco-EMU KS  
110 8 ES, Dortmund, Germany) was used to pump the backwash water of the drum filter back  
111 into the system. In both systems, the drum filter (HDF801-1H, Hydrotech, Vellinge,  
112 Sweden) was equipped with a 100 µm gauze, so that particles < 100 µm accumulate over  
113 time.

114 The photoperiod was fixed at 12L:12D with a sigmoidal transition period of 30 min (Lumilux  
115 daylight lamps) with different light intensities of 50, 100, 200, 300 and 600 lx in duplicate  
116 per system. However, without any significant effect on the results presented (unpublished  
117 data). The fish were fed restrictively according to supplier recommendations with a  
118 commercial feed (Biomar EFICO Enviro 921, Aarhus C, Denmark), by hand six days a  
119 week (Sunday to Friday) at 2.5 % of body weight at the beginning of the trial, declining to  
120 1.3 % by the end (maximum feed amount was 2.84 kg/day per RAS). Bacterial growth was

121 controlled with UV irradiation of the system water (Barrier L20, Wallace & Tiernan,  
122 Günzburg, Germany; UV dose: 40 mJ/cm<sup>2</sup> flow volume: 6600 L/h, lamp wattage: 80 W,  
123 measurement range UV sensor: 200W/m<sup>2</sup>). Fish (average weight approx. 15 g) were put  
124 into the two RASs three months before the beginning of the experiment to ensure  
125 acclimatization.

126

## 127 2.2. Water parameters

128 The experiment was subdivided into three phases (Figure 2): in phase 1 (week 1 – 5),  
129 water parameters in both RASs were kept at levels known to preclude negative impacts on  
130 fish health or performance (Table 1). In the treatment RAS, however, the total suspended  
131 solid concentration was increased to over 35 mg/L and was subsequently held constantly  
132 above this value. The control RAS operated under commercial conditions at around 5  
133 mg/L throughout the experiment. In phase 2 (week 6 – 10), ammonium concentration was  
134 artificially elevated in both RASs by adding ammonium chloride (A7012,9025; AppliChem,  
135 Darmstadt, Germany). Additionally, biofilter efficiency in both RASs was reduced by  
136 halving the volume of carrier material (originally designed for 4.5 kg feed/day) to attain  
137 higher NH<sub>4</sub>-N concentrations. Ammonium nitrogen concentration was measured in both  
138 RASs every 60 minutes using an automat (AMTAX SC, Hach, Germany). In addition, pH  
139 was increased from 7.5 to around 8 in both RASs to increase the proportion of **unionized**  
140 **ammonia nitrogen** (NH<sub>3</sub>-N) to approximately 0.0125 mg/L (Figure 3). The increase in pH  
141 was achieved by adding sodium hydrogen carbonate, dissolved in water, using a peristaltic  
142 pump (Concept 420i, Saier Dosiertechnik, Germany). The pH was constantly monitored  
143 using OxyGuard pH-probes (Farum, Denmark). **The concentration of unionized ammonia-**  
144 **N was calculated based on actual pH and temperature according to Emerson et al. (1975).**  
145 In phase 3 (week 11 – 13), the concentration of **unionized ammonia-N** was further  
146 increased to an average of approximately 0.025 mg/L (Figure 3).



147 NH<sub>4</sub>-N, NO<sub>2</sub>-N and NO<sub>3</sub>-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<sub>2</sub> analyzer (OxyGuard CO<sub>2</sub> 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

173 2.3.2. Particle size distribution (PSD)

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

179

180 2.4. Fish performance

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

$$183 \text{ SGR } (\%d^{-1}) = (\ln(\text{mean final weight}) - \ln(\text{mean initial weight})) / (t(\text{final day}) - t(\text{initial day})) \times 100;$$

184 where  $t$  is time (days).

185 The feed conversion ratio (FCR) was calculated as:

$$186 \text{ FCR} = \text{Feed (kg)} / \text{Weight gain (kg)}$$

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

$$188 \text{ TGC} = (W_t^{1/3} - W_0^{1/3}) \times (\sum T)^{-1} \times 1000;$$

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

191

192 2.5. Sampling protocol

193 Fish were sampled at the beginning, in week 5, in week 10 and in week 13 of the study.

194 Fish were fasted 24 h prior to each sampling. Two fish from each tank per system ( $n = 20$ )  
 195 were caught and anaesthetized using clove oil (concentration: 0.1 mL/L, exposure time:

196 ca. 60 s). Directly following anesthesia, wet weight (to the nearest 0.1 g) and total length

197 (measured from the tip of the mouth to the end of the tail fin; to the nearest 0.1 cm) of each

198 fish were measured and blood samples were taken from the caudal blood vessels and

199 transferred to tubes containing lithium heparin (25 IU/mL blood, Sarstedt, Nümbrecht,  
200 Germany). Subsequently, fish were killed and samples of gill tissue were collected for  
201 histological examination.

202

## 203 2.6. Health parameters

### 204 2.6.1. Gill histology

205 Gill tissue was prepared and examined as described by Becke et al. (2018). Briefly,  
206 observed changes were ranked rising in pathology from 0 (no change) to 3 (severe  
207 change) including sub-steps 1 (minor change) and 2 (moderate change). For each section,  
208 5 images showing 6–7 secondary gill lamellae were inspected at a magnification of 200×  
209 using a photomicroscope (Zeiss, Oberkochen, Germany). Branchial epithelium thickness  
210 (µm) was measured at 10 locations in each image and a mean value was calculated. The  
211 number of goblet cells was counted per secondary lamella. The gills of 20 rainbow trout  
212 from each RAS were investigated at each sampling point.

213

### 214 2.6.2. Fin condition

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

$$217 \textit{Fin index} = (\textit{fin length} / \textit{total length}) * 100$$

218

### 219 2.6.3. Hematology

220 Hematological parameters (differential leukocyte count, hematocrit, leukocrit, hemoglobin  
221 concentration, total red and white blood cell counts) were determined as described by  
222 Becke et al. (2018). Glucose concentration was determined using a common glucose  
223 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<sup>®</sup> (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<sup>®</sup> 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

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

252

## 253 2.8. Data analysis

254 Data were checked for homoscedasticity using Levene's test (Levene, 1960) and for  
255 normality using normal quantile plots. If normal distribution and homoscedasticity tests  
256 were passed, treatment effects were tested by *t*-tests, otherwise Wilcoxon tests were  
257 employed (Sokal and Rohlf, 2003). For analysis of bacterial activity, branchial epithelium  
258 thickness and number of goblet cells per secondary lamella a linear parametric model was  
259 applied (Supplement 1).

260 Fin erosion and gill histology parameters (thickening of epithelial cells, cellular edema, cell  
261 infiltration, tip thickening, detachment of the epithelium, telangiectasia and lamellar fusion)  
262 were tested using a logistic regression on ordinal data. The method of least squares was  
263 used to analyze the relation between TSS concentration and turbidity. Bacterial load data  
264 of the gills was analyzed using Fisher's exact test. A generalized linear model (GLM) was  
265 used to analyze fin index and hematological parameters.

266 The coefficient of variation ( $C_v$ ) as a unit for the relative standard deviation was calculated  
267 in terms of bacterial activity as follows:

$$268 \quad C_v (\%) = (\text{standard deviation } (\sigma) / \text{arithmetic mean } (\bar{x})) \times 100$$

269 All data analyses were performed with JMP Pro (SAS Institute Inc.) version 13.2.1. (64-bit)

270 Differences between treatment groups were considered to be significant at  $P < 0.05$ .

271

## 272 3 Results

### 273 3.1. Water parameters

274 Water temperature differed significantly ( $P < 0.001$ ) between control and treatment RAS,  
275 although the absolute difference was small and below 0.6 °C (Table 1). In week 11 to 13,

276 NO<sub>2</sub>-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<sub>4</sub>-N concentrations, pH, O<sub>2</sub>  
287 concentration and NO<sub>3</sub>-N concentration did not differ significantly ( $P > 0.05$ ) between  
288 control and treatment system. NH<sub>4</sub>-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<sub>4</sub>-  
294 N/NH<sub>3</sub>-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

302 the treatment system exceeded 30 mg/L and remained at an average of 40.5 mg/L.  
303 Furthermore, the difference in TSS concentration between control and treatment RAS was  
304 never less than 23.1 mg/L. Figure 4 B shows the within-day variation of the total  
305 suspended solids concentration in the control and treatment RAS in week 12 of the  
306 experiment with minimum values in the morning at 7:00 a.m. The highest TSS  
307 concentration on that day was 65.8 mg/L in the treatment RAS while it was 14.1 mg/L in  
308 the control RAS.

309

### 310 3.2.2. Particle size distribution

311 At week 12, the total number of particles per liter in the treatment RAS was on average  
312 more than double that of the control. The average suspended particle load was  $17.1 \pm 2.1$   
313 mg dry weight/L in the control and  $47.4 \pm 2.7$  mg dry weight/L in the treatment system. For  
314 each particle size class, the absolute frequencies differed significantly ( $P < 0.001$ ) between  
315 control and treatment RAS (Figure 5). Overall, a high accumulation of fine particles  
316 occurred in both the control and the treatment RAS, with 98.6 % and 98.3 % of all particles  
317 respectively smaller than 15  $\mu\text{m}$ , however, with higher quantities in the treatment RAS.

318

### 319 3.3. Fish performance

320 In contrast to the expectations based on recommended threshold values, fish performed  
321 very well in both systems. A slight difference in feeding behavior was observed between  
322 fish in the two systems with a less aggressive and calmer feeding behavior in the  
323 treatment RAS. Overall, no significant differences ( $P > 0.05$ ) were apparent for final  
324 weight, survival rate, FCR, SGR and TGC between rainbow trout of the control and  
325 treatment RAS (Table 2).

326

### 327 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

378 Overall, no critical bacterial load was detected in the control or treatment RAS. Bacterial  
379 load of the gills differed significantly between fish of the control and treatment RAS in  
380 terms of direct detection ( $P < 0.01$ ) with 45 % and 10 % of the fish gills showing no  
381 bacterial load in the control and treatment RAS respectively (Figure 6). In contrast, 90 % of  
382 the fish gills in the treatment RAS and only 40 % of the fish gills in the control RAS  
383 revealed slight to moderate bacterial load. However, no significant difference appeared in  
384 terms of cultivation ( $P > 0.05$ ). The bacterial load of the spleen was significantly higher ( $P$   
385  $< 0.0001$ ) for rainbow trout in the suspended solids enriched RAS. In the control RAS, 95  
386 % of the spleens revealed no to sporadic bacterial load, whereas in the treatment system  
387 75 % of the spleens revealed slight to moderate bacterial load. The examination of the skin  
388 revealed no bacteria or ectoparasites in either RAS. The fish pathogenic bacteria  
389 *Flavobacterium columnare* was detected on two rainbow trout from the control RAS and on  
390 four rainbow trout from the treatment RAS by direct detection. The cultivation of gill smears  
391 proved the occurrence of *Aeromonas sobria* for three fish in the treatment RAS, but not in  
392 the control RAS.

393

### 394 3.5.2. Bacterial activity

395 Bacterial activity ranged between  $0.12 \times 10^5$  and  $0.47 \times 10^5$  in the control RAS ( $C_v = 18.0$  %)  
396 and between  $0.33 \times 10^5$  and  $3.42 \times 10^5$  in the treatment RAS ( $C_v = 18.3$  %) (Figure 7).

397 Bacterial activity was only significantly affected ( $P < 0.0001$ ) by the total suspended solid  
398 concentration. With increasing particle load in the treatment RAS, bacterial activity  
399 increased from about  $0.3 \times 10^5$  to over  $2.6 \times 10^5$  during the first three weeks. In contrast,  
400 bacterial activity in the control RAS remained roughly static between  $0.2 \times 10^5$  and  $0.3 \times 10^5$   
401 during this time. **Unionized ammonia-N** concentration had no significant effect ( $P > 0.05$ )  
402 on bacterial activity in either RAS. Bacterial activity measured on one representative day in  
403 week 8 showed diurnal variations from  $0.2 \times 10^5$  to  $0.4 \times 10^5$  in the control RAS and from

404  $2.3 \times 10^5$  to  $3.7 \times 10^5$  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

430 can potentially lead to a loss of feed in commercial settings using automatic feeders. To  
431 preclude this potentially disturbing effect, fish in this study were hand fed which secured  
432 the uptake of all feed pellets.

433 Furthermore, the increased suspended solid load induced a substantial increased bacterial  
434 load. Such a finding was expected (Becke et al., 2018) as an increased number of  
435 particles in the treatment RAS promotes bacterial growth by providing a larger surface  
436 area for bacterial colonization and food-substrate. Bacterial activity levels found in this  
437 study have been observed in other recent studies rearing rainbow trout in intensive RAS  
438 (Pedersen et al., 2017; Rojas-Tirado et al., 2018). Especially remarkable is the close linear  
439 correlation between bacterial activity and TSS, which is however quite variable at low TSS  
440 (< 5 mg/L), but nearly exclusively determined by TSS at high TSS loads. This novel  
441 outcome is of high relevance for systems with need for bacterial control. However, the  
442 physiological parameters investigated here did not reveal any evidence for bacterially  
443 mediated physiological stress response in the control or in the treatment systems. This  
444 was confirmed by the independent veterinary inspection of the rainbow trout which did not  
445 reveal any relevant pathological bacterial infestation. In contrast, Redding et al. (1987)  
446 observed a reduced tolerance to subsequent infection with *Vibrio anguillarum* for yearling  
447 steelhead when exposed to high concentrations of suspended topsoil. In the present  
448 study, however, no bacterial diseases occurred despite very high bacterial and suspended  
449 solid load in the treatment RAS. However, under different conditions, the interaction of  
450 suspended solids and bacterial occurrence might impair fish health and need to be  
451 controlled (Herbert and Merkens, 1961; Qualls et al., 1983).

452 Increased particle concentrations, e.g. due to increased stocking densities in RAS, are  
453 often accompanied by a decrease in water quality because of leaching of harmful  
454 substances or particle-mediated growth of heterotrophic bacteria (Chen et al., 2003; Ling  
455 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

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

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

492 Regarding the impact of **unionized ammonia** on fin condition, only the dorsal fin was  
493 negatively affected. As fin condition is frequently consulted to assess fish welfare (Ellis,  
494 2002; Ellis et al., 2008; Turnbull et al., 2005), the almost complete absence of any fin  
495 deterioration here is remarkable and indicates the very low impact of the **unionized**  
496 **ammonia** and solid stressors. Abbott and Dill (1985) assumed that aggressive interaction  
497 is the major cause of fin damage in hatchery salmonids. In the present study, fin condition  
498 of rainbow trout was marginally better in the solid enriched RAS than in the control RAS.  
499 This might be attributable to the calmer behavior and reduced social interaction of fish due  
500 to the turbid conditions in the treatment RAS (Bash et al., 2001).

501 The analysis of hematological parameters revealed significant effects for individual  
502 parameters both in terms of suspended solids and **unionized ammonia**. However, taking all  
503 hematological parameters together, there was no indication of pathological effects. Knoph  
504 and Thorud (1996) also did not observe any negative effect of **unionized ammonia-N** up to  
505 0.112 mg/L on hematological parameters (hematocrit, RBC count) of Atlantic salmon.  
506 Furthermore, Becke et al. (2017, 2018) observed no significant effects of suspended solid  
507 load up to 70 mg/L on hematological parameters.

508 As a consequence of the massive accumulation of fine particles in the treatment RAS and  
509 the additionally increased **unionized ammonia** concentration in both systems, it was  
510 hypothesized that a multiplicative effect of these two parameters would occur in the  
511 treatment RAS, resulting in significant consequences on trout physiology. However, none  
512 of the investigated physiology parameters revealed any relevant multiplicative effects of  
513 particle and **unionized ammonia** load. **In contrast to our hypothesis, no synergistic impact**  
514 **of increased unionized ammonia concentrations and suspended solid load on fish**  
515 **physiology was found.** These results indicate that the commonly used upper safe limits of  
516 0.0125 mg/L for **unionized ammonia-N** and 25 mg/L for total suspended solids do not  
517 represent the actual critical limits for salmonid aquaculture. Fish have evolved  
518 mechanisms to counteract high **unionized ammonia** environments, as shown for rainbow  
519 trout (Randall and Tsui, 2002; Wicks and Randall, 2002). This suggests that rainbow trout  
520 have probably developed an improved tolerance to poor water quality in the course of  
521 artificial selection for aquaculture. Positive effects of moderately elevated ammonia  
522 concentrations have even been observed for rainbow trout when fed to satiation (Linton et  
523 al., 1997, 1999; Wood, 2004). Thus, more research that keeps track of breeding  
524 developments is needed to clarify the exact effects of water parameters on rainbow trout  
525 and fish in general both in aquacultural production and natural conditions. It might be that  
526 the upper safe limits of certain water quality parameters currently used in aquacultural  
527 production no longer correspond to the present genetic makeup of fish and that they  
528 should be revised.

529

## 530 5 Conclusions

531 The results from this study provide a fully controlled insight into the combined effects of  
532 particle accumulation and **unionized ammonia** load on physiology of rainbow trout in RAS  
533 on a farm-scale. Against expectations and widespread opinion, the solid fraction of the

534 experimental system, comprising almost exclusively fine particles at concentrations  
535 distinctly above values normally reached in aquacultural production, failed to provoke  
536 detrimental effects on physiology and performance of rainbow trout. The same holds for  
537 **unionized ammonia** and the combination of both.

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

542

543 Thus, the main conclusions are:

- 544 • Bacterial activity was strongly affected by increased TSS concentrations, but  
545 without detrimental effects on fish physiology
- 546 • increased **unionized ammonia-N** concentrations up to 0.05 mg/L caused only minor  
547 effects on fish physiology
- 548 • no relevant combined effects of increased **unionized ammonia-N** concentrations  
549 and suspended solid load were observed
- 550 • upper safe limits of **unionized ammonia-N** and suspended solids need to be revised  
551 for salmonid aquaculture

552

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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 ( $\text{NH}_3\text{-N}$ ) 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  $\text{NH}_3\text{-N}$  (0.0125 mg/L) for salmonids  
770 (Timmons and Ebeling, 2010).

771

772 Figure 4: (A) Timeline of total suspended solids concentration (mean  $\pm$  S.D.) in control and  
773 treatment RAS over the experimental period. (B) Representative daily variation of total  
774 suspended solids concentration (mean  $\pm$  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  $\pm$  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  $\pm$  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  $\text{NH}_3\text{-N}$  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

ACCEPTED MANUSCRIPT

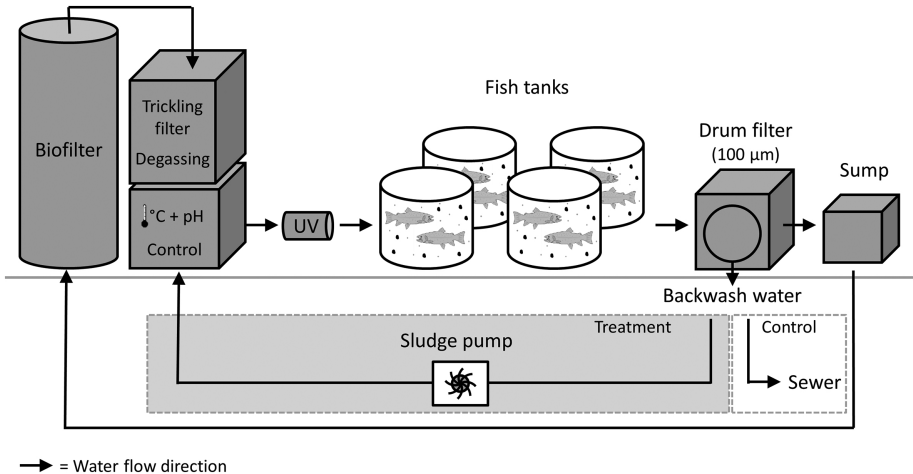


Figure 1

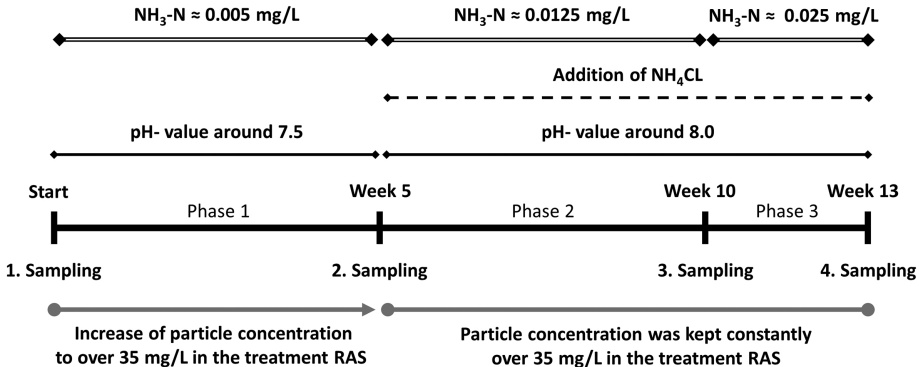


Figure 2



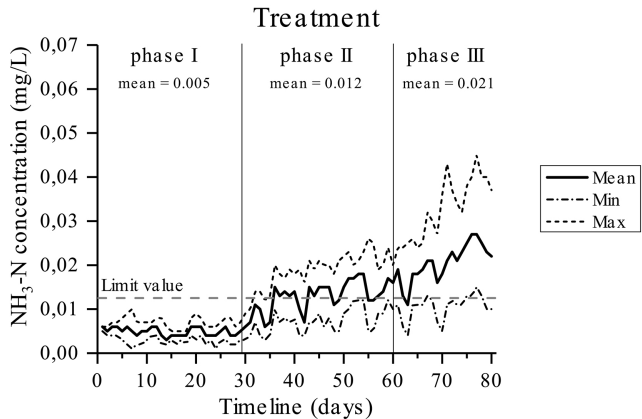
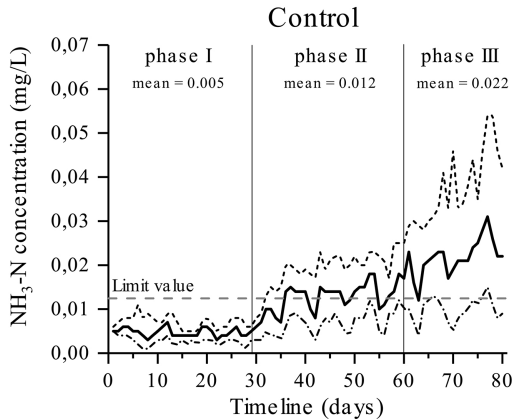


Figure 3

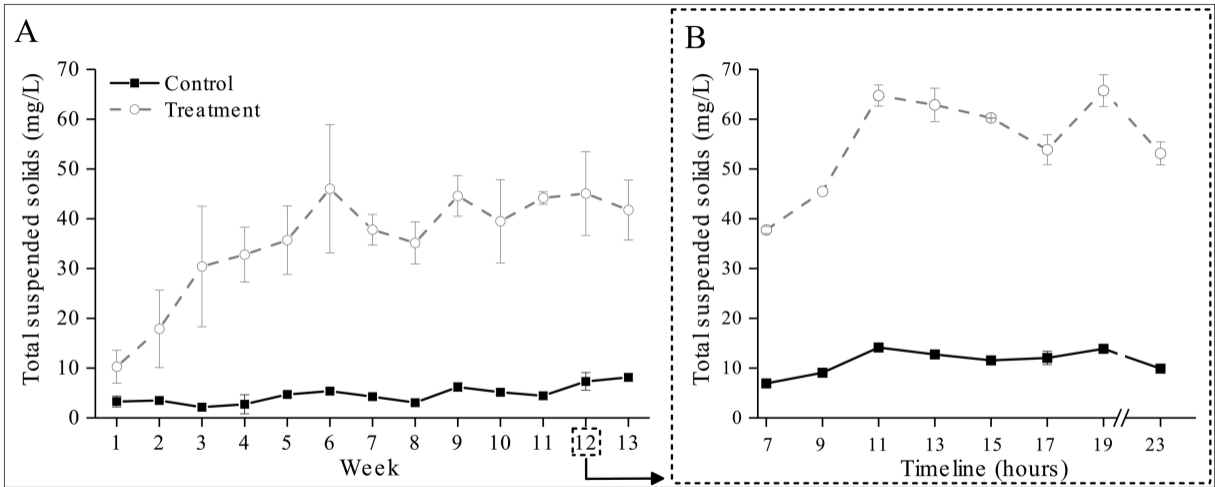


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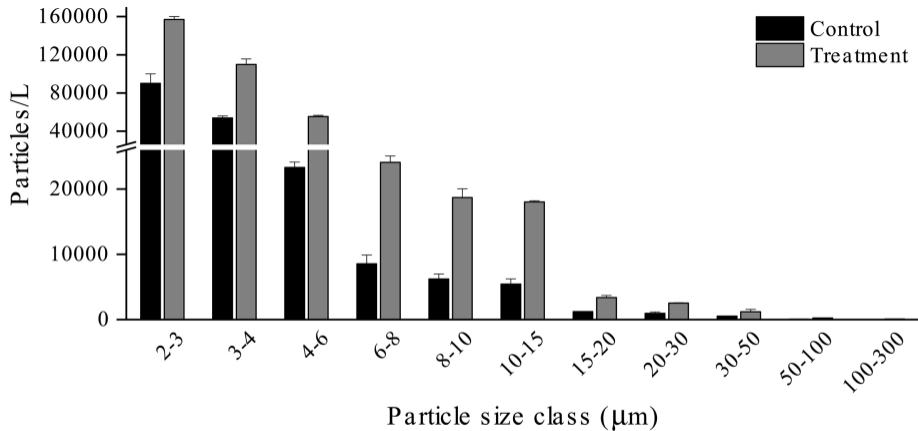


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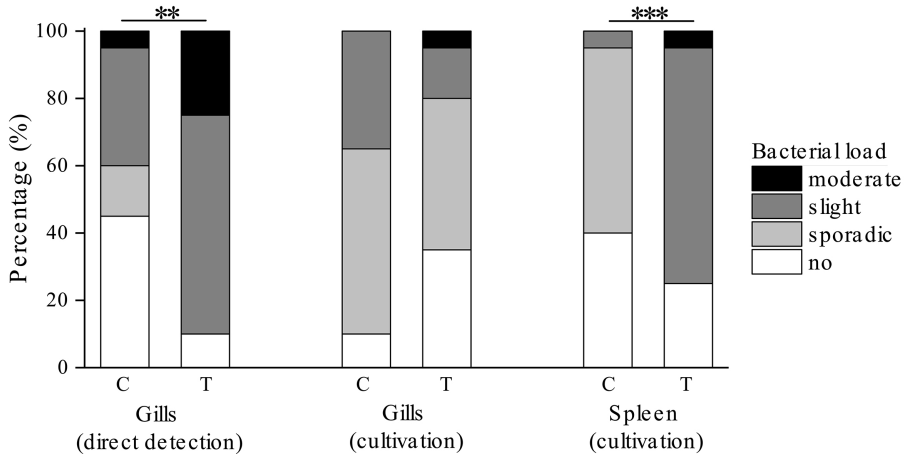


Figure 6

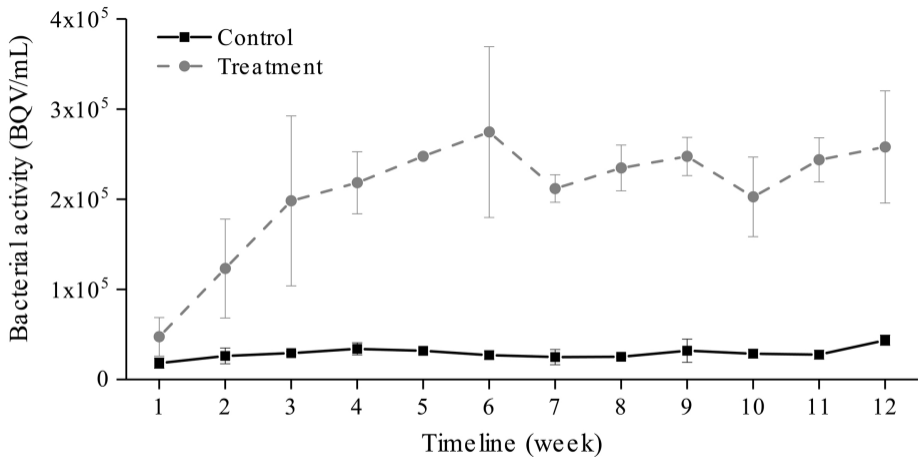


Figure 7

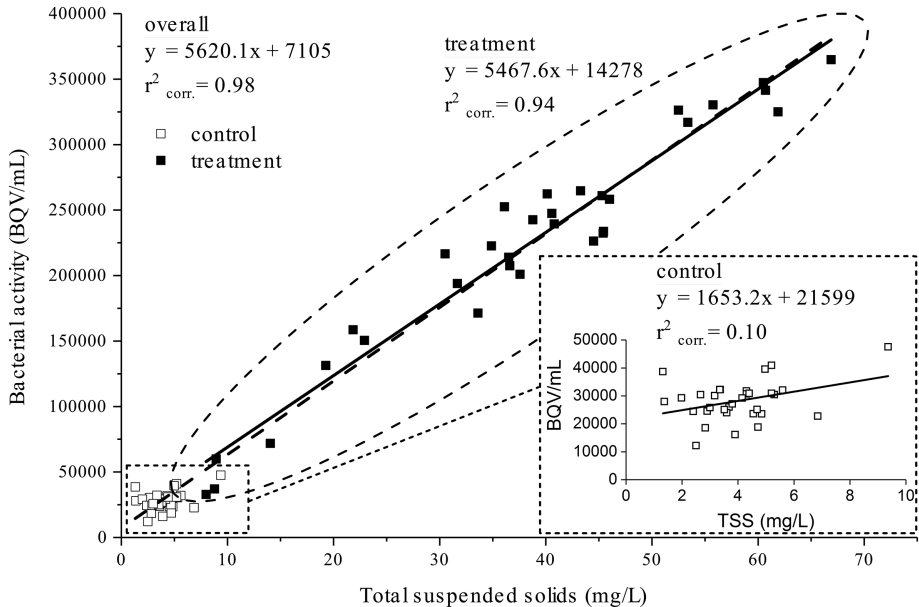


Figure 8