

1 **Microbial dynamics in RAS water: Effects of adding acetate as a biodegradable**
2 **carbon-source.**

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10 *aquaculture system (RAS), acetate.*

11

12 **Abstract**

13 This study evaluated the effect of an abrupt increase in easily biodegradable carbon (acetate) on bacterial
14 activity and abundance in the water of recirculating aquaculture systems (RAS). The study included a batch
15 experiment with RAS water only, and an experiment at system level where twelve pilot scale RAS were
16 used. The batch experiment was made to test how acetate concentration would influence the microbial
17 state in RAS water. Further, we wanted to observe if the selected microbial analysis tools would be able to
18 detect these changes. The second experiment was carried out in twelve identical and independent RAS that
19 had been operated under constant loading conditions (1.6 kg/m³ make-up water) for five months prior to
20 the trial. The twelve RAS were divided into four treatment groups in triplicates: i) control with biofilter
21 (Ctrl+bf); ii) control without biofilter (Ctrl-bf); iii) acetate addition in RAS with biofilter (Ac+bf); and iv)
22 acetate addition in RAS without biofilter (Ac-bf). The biofilter media from the groups without biofilter (Ac-
23 bf and Ctrl-bf) was removed just 5 h prior to the start of the trial. The two acetate treatment groups (Ac+bf
24 and Ac-bf) were spiked with 40 mg/L of acetate three consecutive times (0, 24 and 48 h). Consumption of
25 acetate, bacterial abundance and bacterial activity were followed for 72 hours after the first acetate spike
26 for both experiments. Bacterial activity was quantified by BactiQuant[®] and hydrogen peroxide (HP)
27 degradation assay. Bacterial abundance was assessed by quantifying micro-particles and free-living
28 bacteria. In the batch experiment we observed a significant increase in bacterial activity proportional to the
29 amount of acetate added, and a corresponding significant increase in microparticles (1-3 µm). In the pilot
30 scale RAS experiment, the acetate addition in RAS with biofilter did not cause an increase in bacterial
31 activity, or in the number of micro particles in the water phase but a significant increase in bacterial activity
32 and number of microparticles were observed in the RAS without biofilter (Ac-bf). These changes were
33 particularly pronounced shortly after each acetate spike.

34

35 In RAS with biofilters, the acetate was presumably consumed primarily by the bacterial community within
36 the biofilm, and consequently, only minor changes were observed in densities of free-living bacteria in the
37 water phase. The results of the study suggest that heterotrophic bacteria in the submerged biofilter have a
38 high capacity to handle fluctuation of organic matter loading in RAS, thereby stabilizing the abundance and
39 activity of bacteria in the water column.

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41

42 1. Introduction

43 In recirculating aquaculture systems (RAS), the biofilter and the water are the two major environments for
44 microbes (Blancheton *et al.*, 2013). In the biofilter, bacteria live attached to the biomedica surface, while in
45 the water, bacteria live as free-living bacteria, bacterial aggregates or attached to particles. The bacteria in
46 the water are those interacting directly with the rearing organism and other micro-particles (Blancheton *et al.*,
47 2013; Fernandes, 2015). Both environments have a delimited carrying capacity (CC), which is set by the
48 amount of substrate restricting the number of microbes that can be sustained within a system over time
49 (Vadstein *et al.*, 1993). In RAS, several factors such as feed spill, insufficient solids removal and moribund
50 fish can increase the CC and, potentially, affect the microbial water quality (Attramadal *et al.* 2012, 2016;
51 Wold *et al.*, 2014). These effects might be reflected by changes in bacterial abundancy, activity and
52 diversity.

53 The majority of the bacterial population in RAS are heterotrophic communities (Leonard *et al.*, 2000;
54 Michaud *et al.*, 2009; Michaud *et al.*, 2014; Rud *et al.*, 2017; Gonzalez-Silva *et al.*, 2016). These bacteria
55 obtain their energy by degrading dissolved and particulate organic matter (Polanco *et al.*, 2000; Bitton,
56 2011). Of the macro elements required by bacteria (carbon, nitrogen and phosphorus), carbon (organic
57 matter) is often the growth-limiting compound within RAS (Leonard *et al.*, 2000). Therefore, it can be
58 hypothesized that an increase in the supply of biodegradable organic matter in RAS water, will increase the
59 CC and consequently cause an increase in activity and abundance of heterotrophic bacteria. Concentrations
60 as low as 1 µg C/L of assimilable dissolved organic carbon have been shown to be enough to promote
61 bacterial growth of $10^3 - 10^4$ cells/ml (reviewed in Prest *et al.* 2016).

62 Several studies have proposed that an organic carbon to nitrogen ratio (C/N) close to one, provides a stable
63 balance between autotrophic and heterotrophic bacteria communities in the biofilter (Avnimelech, 1999,
64 Zhu and Chen, 2001, Nogueira *et al.*, 2002, Michaud *et al.*, 2006). The C/N ratio realized will depend on feed
65 composition, feed digestibility (Michaud *et al.*, 2014) and the efficiency of treatment units for the removal
66 of organic matter (Fernandes *et al.*, 2015). Biofilters in RAS ensure the turnover of ammonia, but also
67 interfere with the organic matter dynamics by degrading, retaining and releasing it (Hagopian and Riley,
68 1998; Rusten *et al.*, 2006; Fernandes *et al.*, 2017), which, ultimately, changes the C/N ratio and the chemical
69 composition and matrix of the water. Some studies have shown that the abundance of free-living bacteria
70 in RAS rearing water is correlated to the abundance of bacteria attached to the biofilter media (Leonard *et al.*,
71 2000; Michaud *et al.*, 2006). It has also been demonstrated that the biofilter attenuates the immediate
72 response of bacteria in the water phase when feed loading is increased (Rojas-Tirado *et al.*, 2018). Likewise,
73 it is a well-documented that mature bacterial communities in biofilters can consume additional carbon
74 sources fast (Davies, 2011; Pedersen *et al.*, 2010) and, thereby, efficiently compete for the available carbon
75 with bacteria suspended in the water. However, the dynamics between the biofilter and water column
76 communities, and the variation in the RAS water CC due to increased supply of organic matter has, to our
77 knowledge, not been studied so far.

78 The aim of the present study was to investigate the potential heterotrophic bacterial response in RAS water
79 after pulse addition of easily degradable organic matter, simulating e.g. a pulse of organic matter in a tank
80 or a system. Acetate, an easily biodegradable carbon source was added to the water in two types of
81 experiments: i) single addition of three acetate levels to RAS water in beakers, and ii) three consecutive

82 days pulse addition of acetate to RAS in equilibrium, stocked with rainbow trout, with or without biofilter.
83 Bacterial abundance and activity were assessed in the water phase with a set of quantitative monitoring
84 tools that enumerated the density of single cells, microparticles, and evaluated the enzymatic activity of
85 planktonic and particle-associated bacteria.

86 **2. Materials and Methods**

87 **2.1 System maturation and daily operation routines**

88 The experiments took place at the aquaculture facility of DTU Aqua in Hirtshals, Denmark. Twelve identical
89 pilot scale freshwater RAS of 1.7 m³ each, were used for the trial. The systems configuration is described in
90 Pedersen *et al.*, (2012), with the modification that the volume of the biofilter media was halved to match
91 the feed load operated. Each system was stocked with 12.5 kg of rainbow trout (*Oncorhynchus mykiss*) and
92 was fed a fixed amount of 125 g feed/day (EFICO Enviro 3 mm, Biomar, Denmark). The daily water
93 exchange for each RAS was 80 L/day, resulting in a feed loading of 1.56 kg/m³. Every day at 9:00 a.m., solids
94 were collected and removed from the system at the bottom of each swirl separator. Thereafter, make-up
95 water was added; water quality parameters were measured, and bicarbonate was added to compensate for
96 alkalinity loss due to nitrification. Finally, feed was added to belt feeders dosing the feed for a period of 6
97 hours. All systems were operated for five months (from December to April) to ensure stable
98 microbiological and physiochemical conditions. No feed waste was observed during the operation of the
99 systems.

100 **2.2 Experimental procedures**

101 This study was carried out in two separate experiments adding acetate to RAS water; one trial investigated
102 the bacterial response in RAS water transferred to isolated beakers followed by a second trial where twelve
103 independent and identical RAS under steady state conditions were used. The experiments were carried out
104 to: i) quantify the effect of acetate addition in different quantities on bacterial abundance and activity in
105 RAS water, and ii) evaluate the effect of a similar addition of easily biodegradable substrate to RAS with or
106 without a submerged biofilter.

107 **2.2.1 Batch trial (experiment 1)**

108 To test the effect of easily biodegradable carbon on bacterial abundance and activity in RAS water, a beaker
109 trial was carried out. Acetate was chosen as the easy biodegradable carbon source (Pedró-Alió and Brock,
110 1983; Canelhas *et al.*, 2017), and was added in three different concentrations (Table 1): low concentration
111 (LC) of 10 mg acetate/L, medium concentration (MC) of 20 mg acetate/L, a high concentration (HC) of 40
112 mg acetate/L, compared to a control without addition of acetate. These acetate quantities represent a COD
113 addition of 9.5, 19 and 38 mg O₂/L simulating a theoretical daily feed increase by 1.6, 3.2 and 6.4 times,
114 respectively, compared to a control group without acetate addition (Table 1).

115 Sixteen liters of water was taken from the pump sump in one of the 12 steady state RAS (randomly chosen),
116 it was homogenized and distributed into eight 2 L beakers. The addition of acetate was done in duplicated
117 beakers for each of the three different doses (Table 1). Two beakers were kept as the control group, and no
118 acetate was added to them. All beakers were supplied with sufficient aeration and were stirred by a
119 magnet at a velocity of 100 RPM to keep the water well mixed. The experiment was done at a fixed

120 temperature of 17 °C, corresponding to the temperature of the RAS water. The experiments lasted for 72 h,
121 and samples were taken at regular intervals to assess acetate concentration and microbial water quality.

122 **2.2.2. Pilot scale RAS trial (experiment 2)**

123 To evaluate the effect of repeated addition of acetate in the presence or absence of a biofilter, twelve
124 identical and independent RAS were used. In six of these RAS, the media in the submerged biofilter was
125 removed 5 hours before the start of the experiment to let the system stabilize from any disturbances
126 caused by removing the biofilter media. A trickling-filter with a 33 m² active surface area remained in all
127 systems mainly for degassing purposes. Four treatment groups were then established in triplicates: i)
128 control RAS with a biofilter (Ctrl+bf); ii) control RAS without a biofilter (Ctrl-bf); iii) RAS spiked with acetate
129 with a biofilter (Ac+bf); and iv) RAS spiked with acetate without a biofilter (Ac-bf). Each RAS from group
130 (Ac+bf) and (Ac-bf) were spiked three consecutive times with the highest acetate concentration (40 mg
131 acetate/L): at time 0, then a second time 24 h later, and a third time 48 h after the initial spike. The total
132 study period was 72 h. During the acetate spiking trials, RAS operation (i.e. feeding and water exchange)
133 remained unchanged.

134 **2.3 Sampling and analysis procedures**

135 For experiment 2, the water samples were collected in a standardized way (same person, time, location) by
136 siphoning gently from the pump sump of each RAS between 8:30-9:00 a.m. Water samples for experiment
137 1 and 2 were analyzed for bacterial activity and abundance. In addition, for experiment 2 ammonia, nitrite
138 and nitrate were also measured to assess performance of the submerged biofilter in the RAS that had
139 acetate added. Table 2 lists sampling procedures, treatment and processing of the microbial and
140 physiochemical water quality analysis performed. For Experiment 1, water samples from each beaker were
141 taken at time 0, 6, 24, 48 and 72 hours. For Experiment 2, water samples from each RAS were taken at time
142 0, 2, 6, 12, 24, 30, 48, 54 and 72 hours after the initial acetate spike.

143 Bacterial activity in the water was assessed with two assays: BactiQuant® and the hydrogen peroxide (HP)
144 degradation method. BactiQuant® was measured as described by Rojas-Tirado *et al.*, (2017) and Pedersen
145 *et al.* (2017), at time 0, 6, 24, 48 and 72 hours in Experiment 1, whereas for Experiment 2 samples were
146 analyzed only at the beginning (time= 0 h) and at the end (72 h). The hydrogen peroxide degradation assay
147 is based on quantification of the microbial degradation of hydrogen peroxide as described by Arvin and
148 Pedersen (2015) and Rojas-Tirado *et al.* (2018). Data for the HP removal rate in this study are presented as
149 the HP degradation rate constant (k , h⁻¹) calculated from the exponential decay equation: $C_t = C_0 * e^{-kt}$, where
150 C_t = HP concentration at time t and C_0 = the nominal HP concentration at time 0.

151 The total number of suspended bacterial cells were quantified by flow cytometry (BD Accuri™ C6 Plus)
152 using SYBR Green II (RNA gel stain in DMSO) as the fluorescent dye. Abundance of bacterial cells was
153 determined by the gating on the FL1-versus-FSC plot shown in Fig.1. Bacterial numbers were divided into
154 two groups: bacteria with high and bacteria with low relative RNA content. The RNA content in cells is
155 highly correlated to growth-rate (Bremer and Dennis, 1987), and cells with high RNA content are
156 considered to be actively growing. The water samples did not receive any treatment that could promote
157 detachment of cell from particles. Therefore, the data are referred to as bacterial cells suspended in the
158 water phase or “free-living bacteria”. Free-living cells were counted in all 12 RAS. Microparticles ranging

159 between 1 and 30 μm in diameter were quantified with a Coulter counter (Beckman coulter, Multisizer 4e).
160 This size range constitutes the dominant fraction of microparticles in RAS (Chen et al., 1993; Fernandes et
161 al., 2015).

162 Acetate was measured by ion chromatography (Metrohm; Glostrup, DK). Total ammonia nitrogen (TAN),
163 nitrite-N, nitrate-N, and phosphate (orthophosphate) were measured in each RAS at the beginning and at
164 the end of experiment 2 (analytical methods procedures in Table 2).

165 **2.4. Data Analysis**

166 The data were analyzed using MS Excel and Prism Graph Pad 5.0. Data were normalized to time zero (C/C_0)
167 to facilitate comparisons of changes between the different variables. Effects of acetate concentrations on
168 bacterial activity and micro-particle numbers were tested with a one-way analysis of variance (ANOVA). To
169 evaluate differences in bacterial activity and abundance between the different treatments in Experiment 2,
170 a one-way ANOVA was applied to the normalized data, using a probability level of 0.05. For data not
171 meeting the homoscedasticity assumption, one-way or two-way ANOVA on ranks (Kruskal-Wallis) were
172 performed. Differences in treatment means were tested by Tukey's least square test. Statistics were
173 performed using the software SigmaPlot 12.5 from Systat Software, Inc., San Jose California USA.

174 **3. Results**

175 **3.1 Batch trial (Experiment 1)**

176 *3.1.1 Acetate degradation in RAS water*

177 The dissolved fraction of COD (COD_{DISS}) in the tested RAS water before acetate addition was 35.6 $\text{mg O}_2/\text{L}$.
178 After spiking with the three different acetate concentrations, the COD_{DISS} concentrations after 6 h were:
179 42.3, 51.0 and 73.8 $\text{mg O}_2/\text{L}$ for the low (LC), medium (MC) and high (HC) acetate additions, respectively,
180 compared to 35.0 $\text{mg O}_2/\text{L}$ in the control (Table 1). Acetate was degraded at a rate of $\sim 1 \text{ mg/L}$ per hour in
181 all treatments. The low and medium concentration (10 mg/L and 20 mg/L) was completely degraded within
182 24 hours, whereas the high concentration (40 mg/L) was completely degraded within 48 hours.

183 *3.1.2. Bacterial activity*

184 The rate constant (k) of HP degradation increased 24 h after acetate addition, in a consistent manner with
185 the organic load, reaching rates of 0.5, 0.6, 0.7 and 0.8 h^{-1} for the control, LC, MC and HC groups,
186 respectively (Fig. 2a; Table 3). This concurred with significant differences ($p < 0.001$) in bacterial activity at
187 the end of the experiment for the different concentrations of acetate dosed.

188 BactiQuant[®] values ranged between 7 and 9×10^4 BQV/ml (Table 3) and no significant differences ($p > 0.05$)
189 were found between treatments at the end of the experiment (Fig. 2b).

190 *3.1.3. Microparticles number and size distribution in RAS water*

191 Obvious difference in microparticle numbers were observed between two defined size class ranges (1 - 3
192 μm and 3 - 30 μm) after addition of acetate. Microparticles between 1 and 3 μm increased according to
193 acetate addition, whereas the microparticles from 3 to 30 μm decreased in number with time in all

194 treatments (Fig. 2c). The number of microparticles_{1-3µm} after 72 h ranged from 1.8 to 3.2×10^6 particles/ml
195 for the LC to the HC (Fig. 2c; Table 3). The number of microparticles_{1-3µm} in the untreated water (control)
196 decreased linearly until the end of the experiment, from 1.85 to 1.2×10^6 (Fig. 2c; Table 3). Microparticles
197 in the size range from 1 to 3 µm constituted 95% of the total number of particles within the total range
198 from 1 to 30 µm. Significant differences ($p < 0.001$) were found in the concentration of microparticles
199 ranging 1 to 3 µm between treatments at the end of the experiment (Table 3).

200 After the acetate addition, particles increased in numbers but also in particle size (Fig. 3b, c). Figure 3a
201 shows the changes in particle size distribution (1 - 3 µm) for the different acetate additions 72 h after
202 addition. In the control beakers, the most abundant particles were approximately 1 µm, whereas in the LC
203 the most abundant particles were in the size range 1.0-1.2 µm. In the MC, the most abundant particles
204 were approximately 1.3 µm in diameter, and for the HC, the most abundant particles were approximately
205 1.8 µm in diameter. Water samples from the HC treatment were observed under a microscope, revealing
206 presence of diplococci and tetrad bacterial cell division (observations not quantified).

207 **3.2 Pilot scale RAS trial (Experiment 2)**

208 *3.2.1 Acetate degradation*

209 Acetate was rapidly degraded in the six RAS after each consecutive addition, and there was an adaptation
210 to acetate consumption as acetate was consumed at a higher rate after each addition (Fig. 4a, 4b). All RAS
211 with biofilter (Ac+bf) removed 77 ± 1.3 % of the acetate after the first spike within the first 24 hours,
212 compared to 64 - 99% for the RAS without biofilter (Ac-bf). Complete acetate degradation in all Ac + bf RAS,
213 was observed 24 h after the second and third spike. An even faster consumption of acetate was observed in
214 the RAS without biofilter media (Ac-bf). After the second and the third spike, a 70 to 100 % consumption of
215 acetate was found after 6 h (Fig. 4). A high reproducibility between replicates was observed in treatment
216 Ac+bf for acetate degradation. On the other hand, the replicates in treatment Ac-bf varied in acetate
217 consumption after the first and second acetate spike and ended up with a more even degradation rate
218 after the third spike.

219 *3.2.2 Bacterial activity*

220 Bacterial activity in the water, assessed with the BactiQuant® method, ranged five-fold from 3.4×10^4 to
221 1.76×10^5 BQV/ml between the different RAS at the beginning of the experiment (Table 4 and 5). For the
222 control group with or without biofilter, most RAS had a small increase in BQV at the end of the experiment
223 (Table 4). In the RAS where acetate was added, BQV decreased in all units with biofilter (Ac+bf) compared
224 to the start-up of the trial. The group without biofilter (Ac-bf) ended with lower bacterial activity in two
225 units (from 4.32 and 9.76×10^4 BQV/ml to 3.24 and 4.28×10^4 BQV/ml) and a 10 times higher activity (from
226 6.54×10^4 to 6.76×10^5 BQV/ml) in the third unit compared to the start-up activity (Table 5). This relatively
227 high activity caused significant differences in BQV between and within treatments ($p > 0.05$) at the end of
228 the experiment.

229 The initial and final bacterial activities quantified with the HP degradation assay are shown in Table 4 and 5
230 for control and acetate groups, respectively. For both control groups (Ctrl+bf and Ctrl-bf) and the acetate
231 group with biofilter (Ac+bf), bacterial activity was relatively stable in most of the RAS during the three days

232 of the trial (Fig. 5). The group without biofilter which had acetate added (Ac-bf) differed significantly ($p <$
233 0.001) from the other three groups over the trial period. This group had, on average, 2.3 times higher
234 bacterial activity than the control group 24 h after the first spike, and 4.6 and 5.4 higher activity after the
235 second and third spikes, respectively (Fig. 5). At the end of the experiment, bacterial activity in two of the
236 three RAS within group (Ac-bf) decreased to rates similar to those at the beginning of the experiment
237 (Table 5).

238 3.2.3 Bacterial abundance

239 The initial and final bacterial cell concentrations for total, low and high RNA are presented in Table 4 and
240 Table 5. The concentration of free-living bacteria decreased slightly in the water for both control groups
241 (Ctrl+bf and Ctrl-bf) during the three days of the experiment (Fig. 6, Table 4). The RAS group Ac+bf had an
242 increase of approximately 1.5 times more cells, 6 h after the third spike (Fig. 6). The RAS group Ac-bf
243 showed an increase of 3.4 times in free-living bacteria right after the second spike. During that sampling,
244 28% of the total cells were high RNA cells, and this prevailed until the end of the experiment. After the
245 third spike, bacteria in the RAS water declined in both acetate treatment Ac+bf and Ac-bf (Fig. 6). The
246 absence of a biofilter had a significant effect ($p < 0.05$) on the concentration of free-living bacteria in the
247 water phase after a sudden increase in organic load. The Ac-bf treatment had ~ 2.4 times higher bacterial
248 density than the Ac+bf treatment.

249 3.2.4 Microparticles

250 Particle concentration of two size classes are shown in Table 4 and Table 5. Particle concentration between
251 $1-3 \mu\text{m}$ (microparticles_{1-3 μm}) in the control RAS groups (Ctrl+bf and Ctrl-bf) remained relatively stable over
252 the experimental period. A maximum increase of 1.5 times microparticles_{1-3 μm} was observed within the
253 control groups (Fig. 7, a; Table 4) and in the Ac+bf group the highest average increase was 1.8 times the
254 initial particle concentration (Fig. 7). In the Ac-bf group, microparticles_{1-3 μm} increased significantly ($p < 0.05$)
255 compared to the other three treatment groups. A 10.8 time increase of particles was observed after the
256 second and third spike. No differences between treatments were observed for microparticles within the
257 size range $3 - 30 \mu\text{m}$ (Fig. 7b).

258 3.2.5 Concentration of inorganics N and P

259 The mean TAN concentration was 0.13 ± 0.03 mg TAN/L for the twelve RAS at the start of the trial. At the
260 end of the experiment (72 h), the TAN concentration was 0.13 ± 0.06 mg TAN/L for the two control groups
261 (Ctrl+bf and Ctrl-bf). The TAN concentration was the same for the Ac+bf treatment RAS (0.14 ± 0.04 mg
262 TAN/L), but ~ 2.7 times higher in the acetate spiked RAS without biofilter (Ac-bf) (0.37 ± 0.08 mg TAN/L; $p <$
263 0.05).

264 The mean nitrite-N concentration for the twelve RAS at the start of the trial was 0.05 ± 0.01 mg $\text{NO}_2\text{-N/L}$.
265 Similarly as TAN, the concentrations were 0.08 ± 0.05 mg $\text{NO}_2\text{-N/L}$ for both Ctrl+bf and Ctrl-bf treatments at
266 the end of the experiment, 0.05 ± 0.02 mg $\text{NO}_2\text{-N/L}$ for the Ac+bf RAS, and significantly higher ($p < 0.05$) at
267 0.19 ± 0.03 mg $\text{NO}_2\text{-N/L}$ in the Ac-bf treatment.

268 Nitrate concentrations were similar in all 4 treatment groups at the startup of the trail (64.2 ± 3 mg $\text{NO}_3\text{-}$
269 N/L). However, a significant reduction ($p < 0.05$) was observed in both treatment groups with acetate

270 addition. At the end of the trial, the nitrate-N concentration in Ctrl+bf and Ctrl-bf, was 61.8 ± 1.7 and $60.4 \pm$
271 4.8 mg NO₃-N/L, respectively, while in the Ac+bf and Ac-bf groups it was 53.5 ± 7.0 and 53.3 ± 2.2 mg NO₃-
272 N/L.

273 Phosphate concentration before the start of the experiment were similar between the twelve RAS ($3.7 \pm$
274 0.19 PO₄-P/L), ending at 3.6 ± 0.04 , 3.7 ± 0.1 , 3.0 ± 0.5 and 3.3 ± 0.2 PO₄-P/L for the Ctrl+bf, Ctrl-bf, Ac+bf
275 and Ac-bf, respectively, and without significant differences between treatments ($p > 0.05$). However,
276 significant differences ($p < 0.05$) were found when phosphate consumption was calculated (Δ PO₄-P = PO₄-
277 P_{72h} – PO₄-P_{0h}) showing RAS spiked with acetate (0.58 ± 0.14 and 0.57 ± 0.07 PO₄-P/L for Ac+bf and Ac-bf) to
278 be significant higher than both control RAS (0.06 ± 0.02 and 0.08 ± 0.06 PO₄-P /L for Ctrl+bf and Ctrl-bf).

279 4. Discussion

280 4.1. Dose-response effect of acetate addition to RAS water (Experiment 1)

281 The addition of acetate had an immediate and prolonged additive effect on the bacterial dynamics in
282 beakers with RAS water. This could be expected given the immediate bioavailability and nutritional
283 characteristics of acetate (Pedrós-Alió and Brock, 1983; Canelhas *et al.*, 2017), the inorganic nutrient
284 content and the bacterial load in RAS water (Rojas-Tirado *et al.*, 2018). The unexposed RAS water had a
285 linear reduction in bacterial activity (HP assay) of approximately 10 %/day as well as in particle numbers (1-
286 3 μm), with no changes in average size distribution over time (Fig 3b). Acetate addition caused a 50 %
287 increase in particle numbers (1-3 μm) within 24 hours, further increasing it until 48 hours for the medium
288 and high acetate concentrations. Since the size-fraction 1-3 μm includes single bacteria cells or low-number
289 bacterial aggregates (due to cell division), the results verified that biodegradable carbon was the limiting
290 factor for free-living bacteria growth in the RAS (Fig. 3a). In these beakers, temporal changes in particle size
291 distribution and concentration within the 1–3 μm range were observed over time (72 h, Fig 3c), indicating
292 an increase in bacterial size caused by cell aggregate formation presumably due to cell-division process.
293 This was confirmed by microscopic observations (*pers. comm.*). The numbers of larger micro particles (3-30
294 μm) were reduced in all four treatment groups suggesting flocculation to bigger particles due to stirring
295 and/or disintegration from degradation by particle associated bacterial activity (Pedersen *et al.*, 2017).

296 BactiQuant® did not detect any changes in bacterial activity after acetate addition as the principle of the
297 method relies on hydrolase enzyme (Reeslev *et al.*, 2011). In the presence of acetate – which is a readily
298 transportable and a low molecular weight (Chróst, 1991; Canelhas *et al.*, 2017), bacteria do not require
299 additional activation of hydrolase as acetate can be taken up by simple diffusion. Therefore, it is presumed
300 that bacterial activity assessed by BactiQuant® remained constant even though different concentrations of
301 added substrate resulted in changes in bacterial activity and cell numbers. A more realistic feed spill
302 scenario would cause formation of more complex bioavailable compounds, thereby activating bacterial
303 hydrolases and hence, detectable changes in BactiQuant®. A clear rise in BactiQuant® was observed when
304 increasing i.e. feed loading in RAS (Rojas-Tirado *et al.*, 2018). In contrast to BactiQuant®, bacterial activity
305 assessed by the HP degradation assay increased linearly according to the acetate concentrations given.

306

307

308 4.2. Effects of repeated addition of acetate to pilot-scale RAS with or without biofilter (Experiment 2)

309 Both RAS control groups (no acetate addition), reflected stability of the system in terms of microbial activity
310 and abundance during the experimental period as no changes were observed. When acetate was
311 repeatedly added at high concentrations to the RAS with biofilter (Ac+bf), both bacterial activity and
312 abundance in the water phase remained stable over time. This is somehow surprising, considering the fast
313 and easy degradation of acetate in the systems with biofilter, the findings from experiment one, and the
314 fact that bacteria were not inactivated by any disinfection treatments in the systems (Liltved and Cripps,
315 1999). This lack of change was considered to be related to the bacterial activity of the biofilter. In RAS,
316 bacterial communities will primarily reside in the biofilter due to the high surface area provided by the
317 biofilter medium, the mutualism between different functional groups, and benefits of inhabiting surfaces
318 which provide protection and keep bacteria from being flushed out (McDougald *et al.*, 2011; Madigan *et al.*,
319 2015), combined with the constant supply of nutrient-rich water. Consequently, the biofilter functioned as
320 a buffer by consuming the organic matter added and limiting the growth of bacteria in the water phase.

321 In this study, significant effects on microbial water quality parameters were only observed in the RAS where
322 biomedica had been removed from the submerged biofilter (biofilter tank and trickling filter remaining; Ac-
323 bf). In these RAS, increased bacterial activity and abundance in the water was measured by increased HP
324 degradation rates and micro particles (1-3 μm) abundance. Similar to the batch experiment (experiment 1),
325 the increase was significant after 24 h, and bacterial activity remained elevated during the three
326 consecutive acetate spikes. The flow cytometry data also showed an increase in free-living bacteria after
327 addition of acetate. Approximately 30% of the bacteria had a high RNA content suggesting that this fraction
328 was actively growing (Schaechter *et al.*, 1958; Neidhardt *et al.*, 1990). The three Ac-bf RAS displayed
329 substantial inter-system variation, stressing that RAS, despite being identical in design and operation, are
330 each unique as opposed to beaker trials. It is speculated, whether RAS-specific and localized micro
331 hydraulics, and the absence of the submerged biofilter may explain part of this variation. Although no
332 significant differences in microparticle_{3-30 μm} concentration was observed between the treatment groups,
333 the graphs in Fig. 7b shows higher particle numbers for all RAS without biofilter (Ctrl-bf and Ac-bf). This
334 indicates that the absence of the biofilter could have impacted the particle numbers at higher size range (>
335 30 μm). Since no additional parameters were measured to assess the total organic matter content during
336 experiment 2, this remains an assumption, though.

337 During the experimental period (three days), TAN and nitrite did not increase much despite of the acetate
338 addition. The small trickling filter most likely served as biofilter with nitrification during the trial period
339 (Eding *et al.*, 2006). Significant reduction of nitrate and phosphorus was measured within the RAS water
340 spiked with acetate, which can be associated to cell growth (C:N:P ratio; Prest *et al.*, 2016). Heterotrophic
341 bacteria, present in the biofilm and in the water phase, can also assimilate ammonia when easily
342 biodegradable carbon sources are added (Avnimelech, 1999; Hargreaves, 2006; Ebeling *et al.*, 2006) which
343 leads to reduced net - production of ammonia subsequently nitrite concomitantly influencing the nitrate
344 accumulation (or concentration). This was substantiated in the present study where a significant reduction
345 in nitrate concentration was found in the six acetate- treated RAS compared to the control RAS, although
346 identical make-up water addition was applied in all twelve RAS.

347

348 **4.3 Analysis of results and microbial water quality methods**

349 Before the trial start-up, the RAS were operated under constant conditions and fixed feed loading for a
350 prolonged period. During that time, bacteria stabilized according to the carrying capacity of the system, and
351 carbon limited growth was established for the heterotrophic bacteria (Leonard *et al.*, 2002; Attramadal *et*
352 *al.*, 2012; Rojas-Tirado *et al.*, 2018). It can be hypothesized that the regular and constant substrate input
353 (125 g feed/day for 5 months), was enough for bacteria in the water and the biofilter to uphold cellular
354 maintenance only and, thus, reach a low but stable bacterial growth, regulated by cellular death (Gerardi,
355 2006). When acetate was added, the consumption rate of acetate increased after each spike, in line with
356 the study by Canelhas *et al.*, (2017). When exposed to a new source of substrate or increased
357 concentrations of an already existing substrate, bacteria immediately adjust in the water phase and in the
358 biofilm by increased activity and growth (Hagopian & Riley, 1998; Pedersen *et al.*, 2010; Davies, 2011;
359 Blancheton *et al.*, 2013, Rojas-Tirado *et al.*, 2018). Higher availability of substrate allows heterotrophic
360 bacteria in the biofilter, and especially bacteria in the water phase, to allocate metabolic energy to biomass
361 production (growth), rather than cellular maintenance only (Canelhas *et al.*, 2017). Moreover, this rapid
362 change in the CC of the water probably allowed for growth of opportunistic r-strategist (fast growing
363 bacteria) bacteria (Attramadal *et al.*, 2016) as supported by the increase in the high RNA fraction observed.
364 This 3.4 times increase in bacterial abundance in RAS water had, however, no immediate impact on the fish
365 in this study (data not shown).

366 Interestingly, acetate consumption was faster in the RAS without biofilter compared to those having a
367 biofilter, perhaps illustrating the adaptive features of bacteria living in the water phase, where they can live
368 as free-living or attached to particles. This might be partly explained by the fact that the submerged
369 biofilter would trap particles coming from e.g. fish feces, but since the biofilter media was removed,
370 particles were instead distributed throughout the water column. This might have supported a fast and
371 increased development of the microbial population in the water column (free-living and particle associated
372 bacteria) in these RAS. Free-living bacteria might benefit from their larger surface to volume ratio providing
373 more surface area for the absorption of substrates (Gerardi, 2006; Pedersen *et al.*, 2017). Particle-
374 associated bacteria have been observed to have a higher frequency of cell division and are generally larger
375 than free-living bacteria which allows them a higher acetate uptake (Pedrós- Alió and Brock, 1983; Crespo
376 *et al.*, 2013). It can be assumed that these two adaptive aspects of the bacterial community in the water
377 phase and the higher number of particles, combined with an easily degradable and abundant substrate,
378 could have facilitated the faster acetate consumption observed in the RAS Ac-bf.

379 The monitoring tools used in this study detected changes in microbial water quality. However, each method
380 had its own application. The Coulter counter instrument counts numbers of particles within the 1 to 30 μm
381 range, whereas bacterial size range is generally between 0.2 and 3 μm (Gerardi, 2006). This means that
382 single cells smaller than 1 μm were not detected by the Coulter counter with the aperture used. Flow
383 cytometry can differentiate between bacteria and inert particles using staining procedures (Marie *et al.*,
384 2005) but it cannot quantify particles precisely in size groups as the Coulter counter although it managed
385 measuring bacteria down to 1 μm . The microbial activity assays with HP showed strong positive correlation
386 with acetate addition, whereas BactiQuant[®] documented stable hydrolysis activity as unaffected by acetate
387 addition. The combination of all applied methods contributed to improve the understanding of bacterial

388 dynamics in RAS in this case study. Additional information about community structure and shifts therein
389 might provide additional information in future experiments.

390 Limited knowledge exists about the interactions between the bacterial communities living as
391 biofilter/biofilm-resident on a solid carrier, or as free-living or particle-associated bacteria in the water
392 phase. This biofilm-water interaction is system specific and the buffering capacity and stabilizing effect of
393 biofilters deserved more attention. Future studies of biofilm growth dynamics in biofilters will improve the
394 understanding of microbial interactions in RAS.

395

396 **5. Conclusions**

397

398 i) Addition of easily biodegradable dissolved carbon (acetate) in beakers with RAS water
399 caused increased bacterial activity and abundance.

400 ii) Addition of acetate for three consecutive days to pilot-scale RAS with biofilters did,
401 however, not significantly affect the bacterial activity and abundance in the water phase.
402 Only in RAS where the submerged biofilter media had been removed, was a significant
403 response observed in the water phase. This confirms that the biofilm and surface-attached
404 bacteria have the capacity to consume significant pulses of organic matter, thereby
405 preventing the acute deterioration of the microbial water quality.

406 iii) The monitoring tools used in this study (flow cytometry, HP degradation assay, and particle
407 Coulter counter) complementarily detected and described the abrupt changes in bacterial
408 activity and abundance in the water due to pulse loading of organic matter.

409

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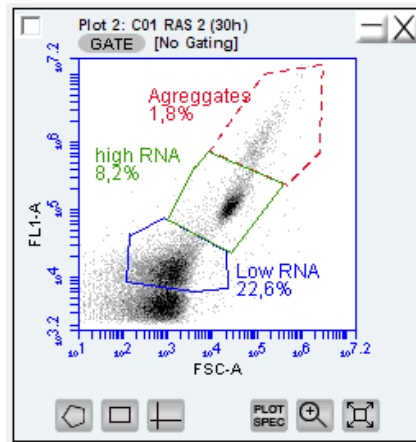
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602 **FIGURES AND TABLES**

603 **(Figure for Mat&Meth)**



604

605 **Fig. 1: Gating of cells counted with flow cytometry method. Cell gating was used to divide bacteria into two groups,**
606 **low RNA and high RNA, separating them from the aggregates. Only low and high RNA groups are taken into**
607 **consideration in the cell counting. The same gating was applied for all water samples. Bacterial cells were stained**
608 **with SYBR green II. Cells were delimited by gating using BD Accuri™ C6 Software (figure from this study).**

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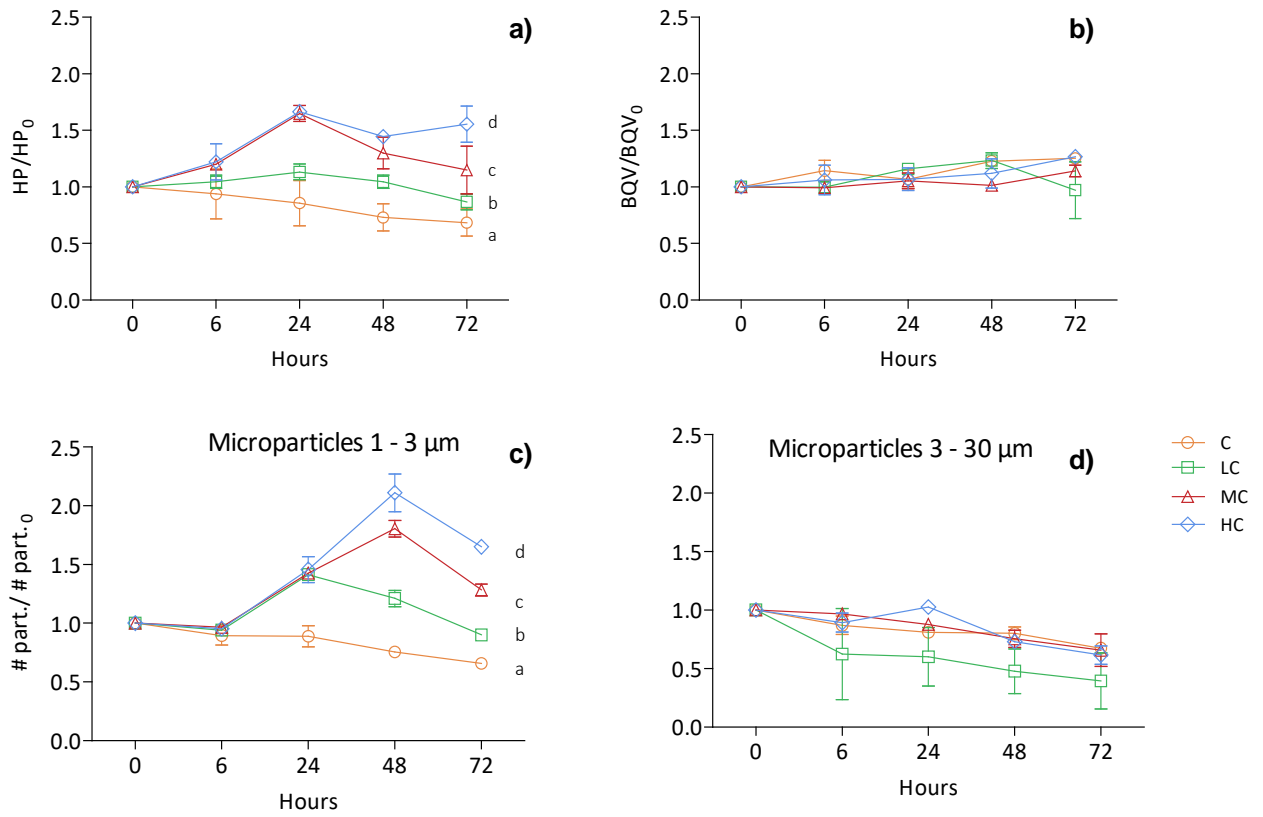
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624 (Results figures)



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 626 **Fig. 2: Effect of acetate spike on a) hydrogen peroxide removal rate constant (k), b) bactiquant value (BQV), and c)**
 627 **number of particles within the range 1 - 3 μm and within 3 - 30 μm . Data are normalized (C/C_0) and presented as**
 628 **mean \pm SD, $n = 2$ (raw data for 0 and 72 h presented in Table 3). Different superscripts within each separate figure**
 629 **indicate statistical difference between treatments ($p \leq 0.05$).**

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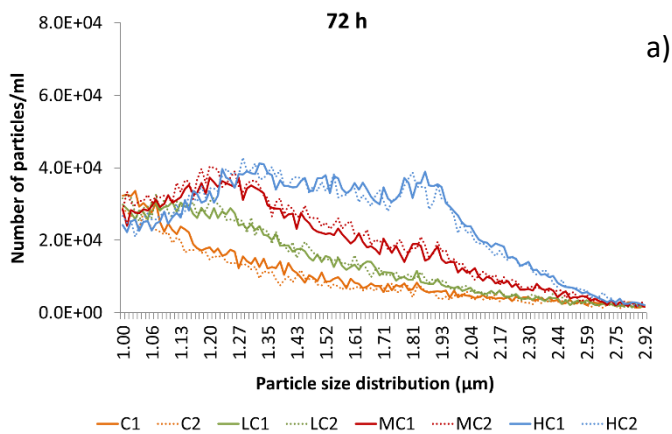
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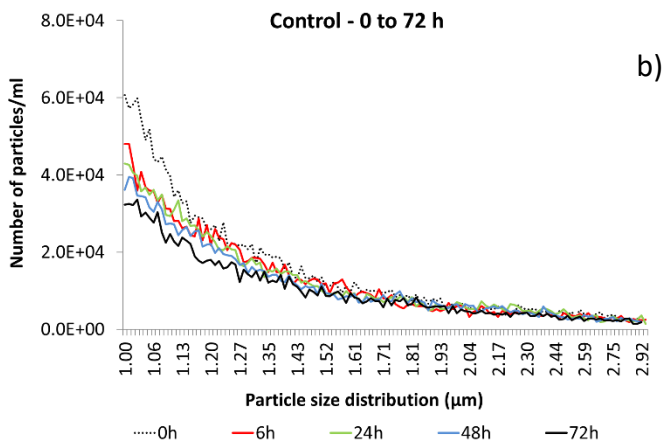
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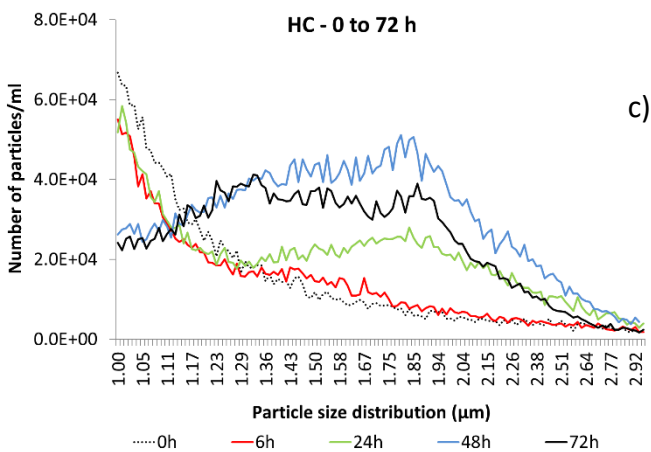
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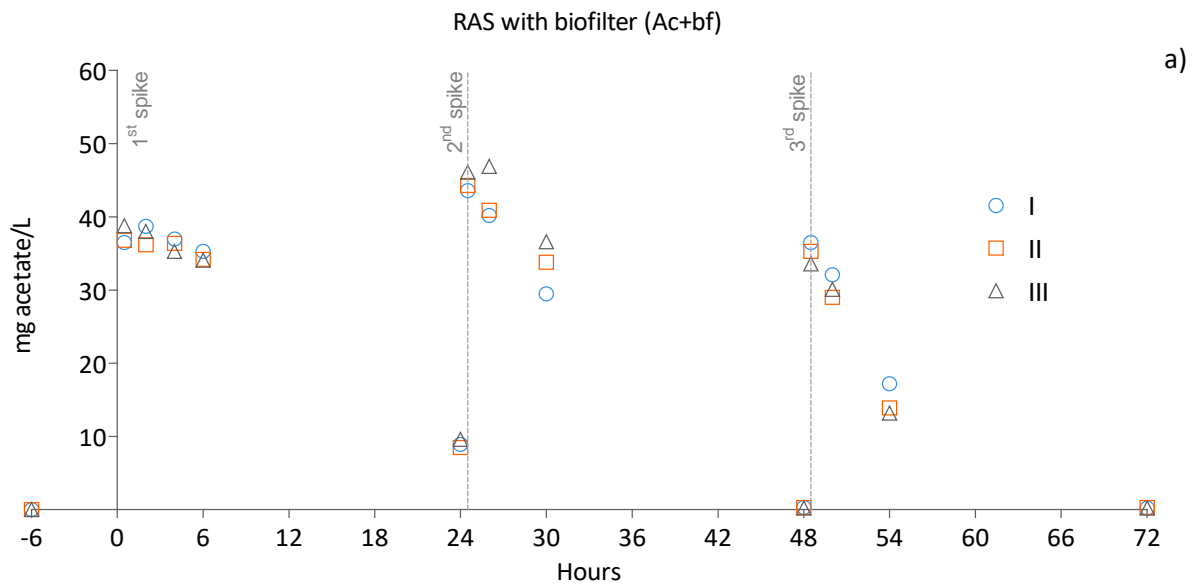


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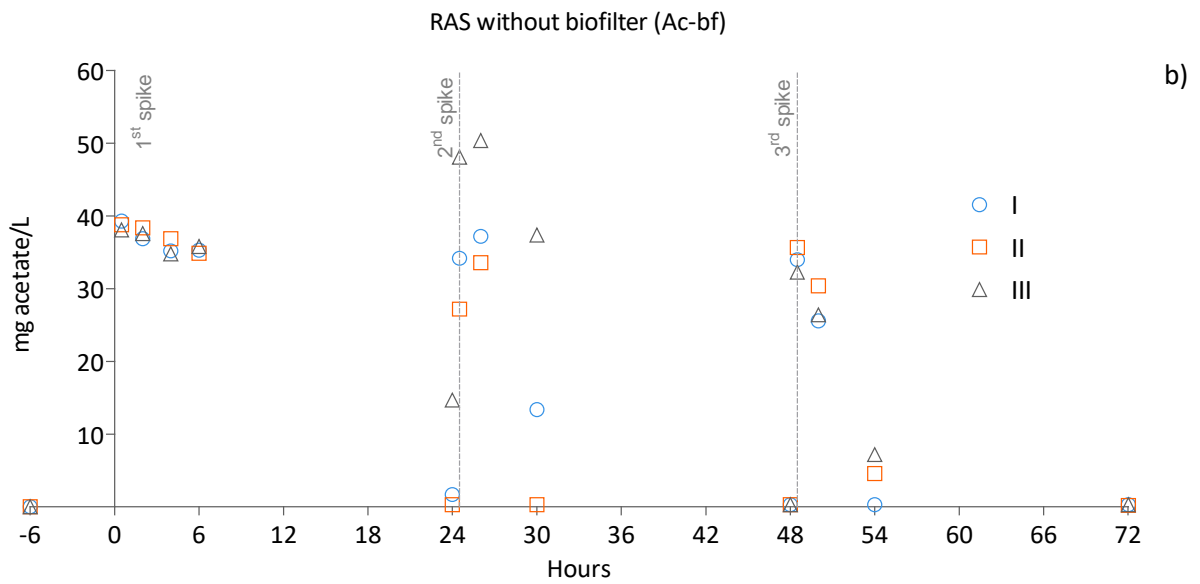


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639 Fig. 3: Particle numbers and development within the size distribution 1 - 3 µm following acetate addition to RAS water in
 640 beakers during Experiment 1: a) Changes in size distribution 72 h after different acetate addition (C1-2: no addition, LC1-2: 10
 641 mg/l added, MC1-2: 20 mg/l added, HC1-2: 40 mg/l added); b) development in particle numbers and size in one of the control
 642 beakers (C1) through 72 h; and c) development in particle numbers and size distribution in one of the beakers with high acetate
 643 concentration (40 mg/l) added.



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Fig 4: Acetate concentrations in RAS following acetate spikes at t = 0, 24 and 48 h during Experiment 2, in: a) RAS with biofilter media (Ac+bf) and b) RAS without biofilter media (Ac-bf). Roman numerals in legend indicate RAS replicates for each treatment.

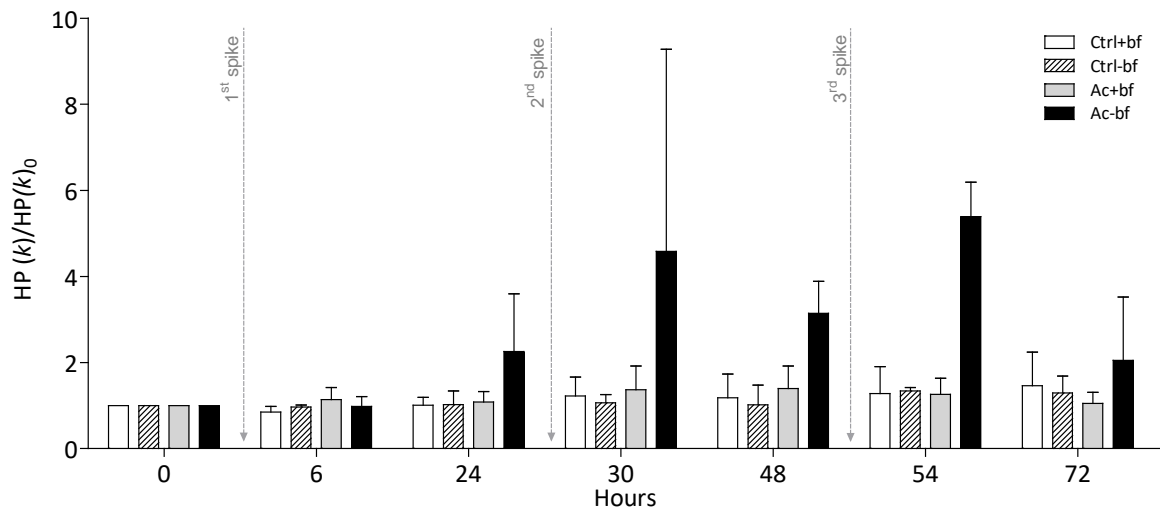
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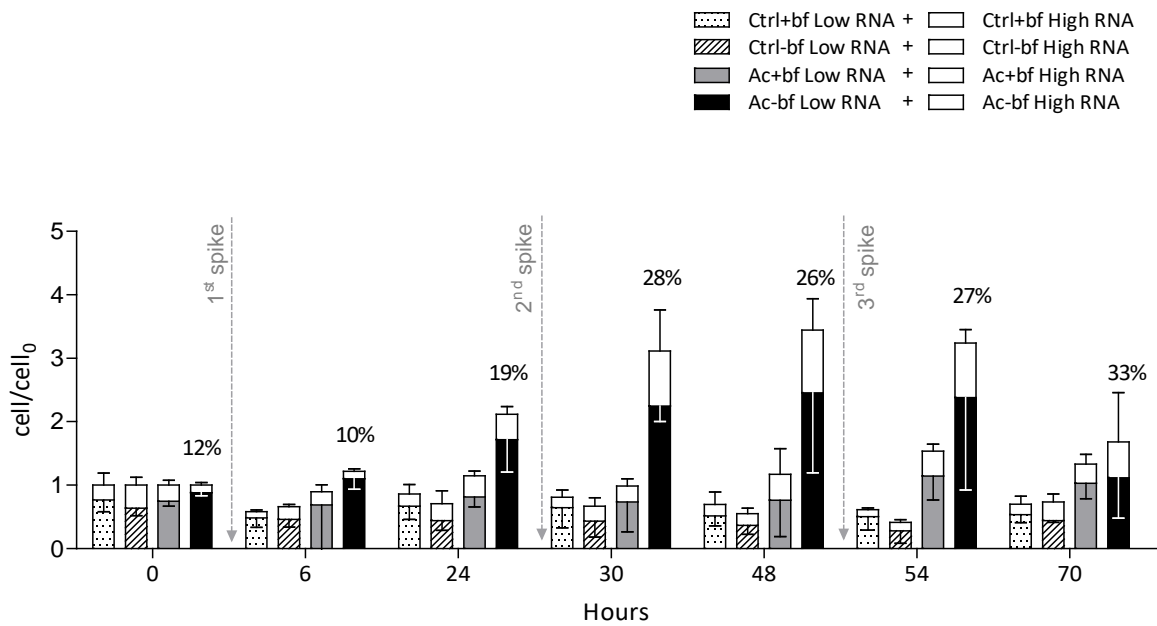
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 655 **Fig. 5: Changes in bacterial activity expressed by changes in the hydrogen peroxide (HP) removal rate constant k (h^{-1}) for the different treatment groups during 72 h in Experiment 2: control RAS with biofilter (Ctrl+bf) (white bars),**
 656 **control RAS without biofilter (Ctrl-bf) (stripe bars), RAS with biofilter (Ac+bf) spiked with acetate at $t = 0, 24, 48$ h**
 657 **(grey bars), and RAS without biofilter (Ac-bf) spiked with acetate at $t = 0, 24, 48$ h (black bars). Data are normalized**
 658 **(C/C_0) and presented as mean \pm SD, $n = 3$ (raw data for 0 and 72 h are presented in Table 4 and 5).**
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 666 **Fig. 6: Changes in bacterial abundance for the different treatment groups during 72 h in Experiment 2. For the low**
 667 **RNA cell counting: control RAS with biofilter (Ctrl+bf) (white bars), control RAS without biofilter (Ctrl-bf) (stripe**
 668 **bars), RAS with biofilter (Ac+bf) spiked with acetate at t = 0, 24, 48 h (grey bars), and RAS without biofilter (Ac-bf)**
 669 **spiked with acetate at t = 0, 24, 48 h (black bars). High RNA cells counting are shown as white upper bars and the**
 670 **percentage (%) corresponding to the high RNA fraction of the total cell count is presented only for the Ac-bf group.**
 671 **Data are normalized (C/C_0) and presented as mean \pm SD, n = 3 (raw data for 0 and 72 h are presented in Table 4 and**
 672 **5).**

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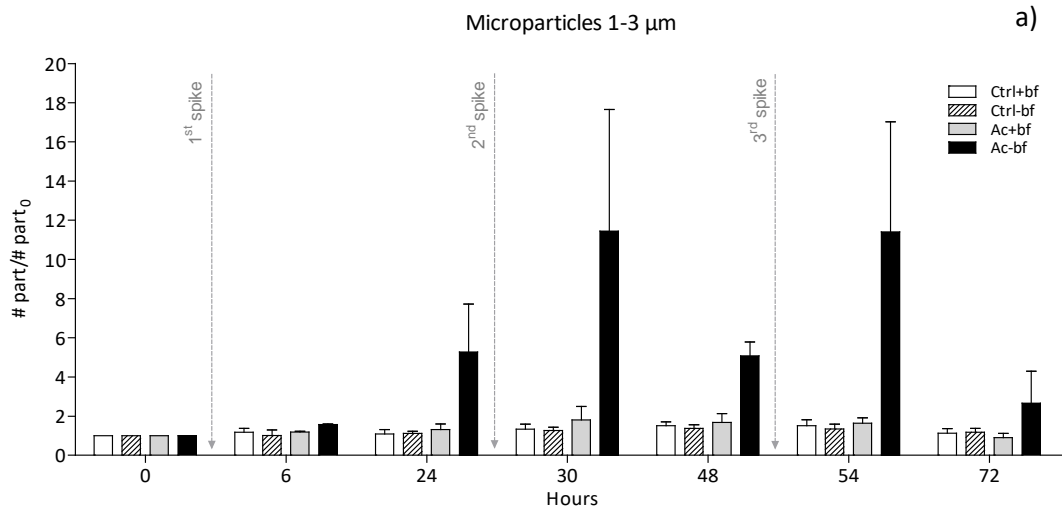
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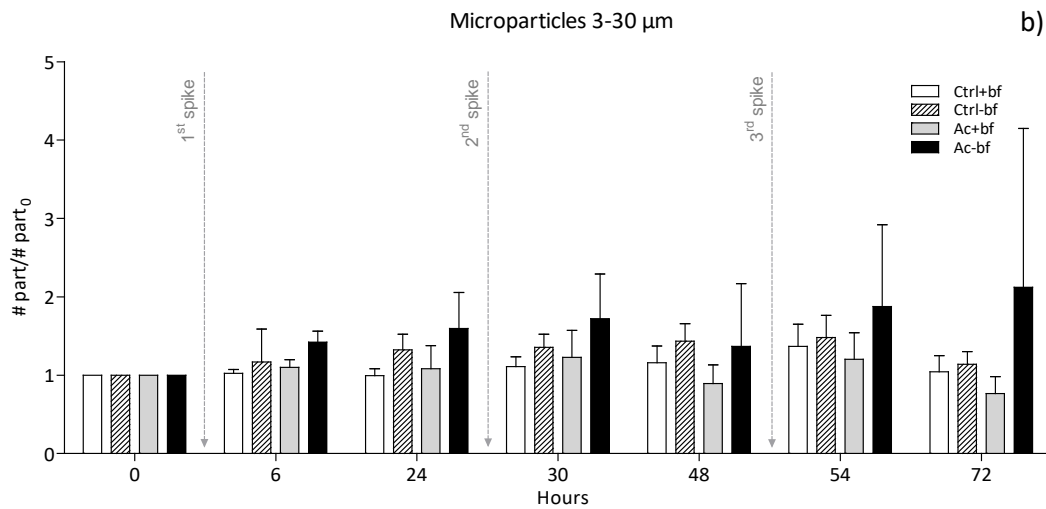
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683 **Fig 7: Number of microparticles 1 - 3 μm (a) and 3 - 30 μm (b) for the different treatment groups during 72 h in**
 684 **Experiment 2: control RAS with biofilter (Ctrl+bf) (white bars), control RAS without biofilter (Ctrl-bf) (stripe bars),**
 685 **RAS with biofilter media (Ac+bf) spiked with acetate at t = 0, 24, 48 h (grey bars), and RAS without media (Ac-bf)**
 686 **spiked with acetate at t = 0, 24, 48 h (black bars). Data are normalized (C/C₀) and presented as mean ± SD, n = 3**
 687 **(raw data for 0 and 72 h are presented in Table 4 and 5).**

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Table 1: Acetate concentrations (LC: low concentration, MC: medium concentration, HC: high concentration) used in experiment 1 and 2, and the corresponding chemical oxygen demands (COD) and theoretical equivalents of feed addition.

Parameters	Units	Daily feeding	LC	MC	HC
Acetate	mg/L	-	10	20	40
Theoretical COD	mg O ₂ /L	6 ^a	9.5	19	38
Feed (g/day)	g	125	125	125	125
Theoretical feed equivalents ^b (g/spike)		0	198	399	798

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^a Theoretical COD calculated according to Dalsgaard and Pedersen (2011): 81 mg O₂/ g feed → 81 mg O₂ * 125 g feed /1700 L (systems volume) = 6 mg O₂/L; ^b Additional feed equivalent for LC, MC and HC treatments based on the theoretical COD of 125 g feed and COD in the spiked acetate quantities.

Table 2: Microbial and physio-chemical water quality parameters and analytical methods used.

Parameter	Abbreviation /Description	Units	Sample treatment and processing	Analytical Method/Instrumentation	Reference
Bacterial Activity	BactiQuant Value	BQV	Unfiltered. Processed immediately	BactiQuant® (Mycometer, Denmark)	Manufacturers protocol
Bacterial Activity	HP degradation Assay	k (h ⁻¹)	Unfiltered. Processed immediately	Colorimetry	Arvin and Pedersen, 2015
Bacteria cell number	Cell number	cell/μl	Unfiltered. Fixed with glutaric aldehyde (1% final concentration). Frozen immediately with liquid nitrogen gas and conserved at -20°C. Processed 6 months later.	Stained with Sybr Green II and counted with Flow Cytometer (Becton Dickinson FACscan)	(Marie <i>et al.</i> , 2005; Wold <i>et al.</i> , 2014)
Particle numbers			Prefiltered with a 45 μm AA filter. Counted immediately	Multisizer 4e Coulter Counter	N/A
Temperature, pH, Dissolved Oxygen	Temp., pH, O ₂	°C, pH units, mg/L	N/A	Hach HQ40d Instruments, Hach Lange, Germany	N/A
Acetate		mg/L	Filtered 0.22 μm. Conserved at 4°C.	Ion chromatography, Metrohm, Glostrup - DK	N/A
Total ammonia nitrogen	TAN	mg/L	Filtered 0.22 μm. Conserved at 4°C.	Colorimetry	DS 224
Nitrite	NO ₂ -N	mg/L	Filtered 0.22 μm. Conserved at 4°C.	Colorimetry	DS 223
Nitrate	NO ₃ -N	mg/L	Filtered 0.22 μm. Conserved at 4°C.	Colorimetry	DS 223
Phosphate (orthophosphate)	PO ₄ -P	mg/L	Filtered 0.22 μm. Conserved at 4°C.	Colorimetry	ISO 6878:2004
Dissolved chemical oxygen demand	COD _{DISS}	mg O ₂ /L	Filtered 0.22 μm. Conserved at 4°C.	LCK 914, Hach Lange, Germany	N/A

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Table 3: Results from experiment 1 (beaker trial) for the microbial water quality parameters (mean ± SD, n = 3) in control (C), low concentration (LC), medium concentration (MC) and high acetate concentration (HC) from Experiment 1.

Parameter	Unit	Time	C	LC	MC	HC
BactiQuant	BQV × 10 ⁴ /ml	0 h	6.8 – 7	7.1	7.1 - 7.6	6.9
		72 h	8.3 – 9	5.7 - 8.1	7.9 - 9.0	8.6 - 8.9
HP	h ⁻¹	0 h	0.75 ± 0.13	0.69 ± 0.04	0.60 ± 0.00	0.54 ± 0.00
		72 h	0.51 ± 0.04	0.60 ± 0.08	0.69 ± 0.13	0.84 ± 0.08
Microparticles 1-3 μm	#part × 10 ⁶ /ml	0 h	1.8 - 1.9	1.7 - 1.9	1.8 - 1.9	1.8 - 1.9
		72 h	1.1 - 1.3	1.7 - 1.8	2.3 - 2.5	3.2
Microparticles 3-30 μm	#part × 10 ⁵ /ml	0 h	1.2 - 1.3	1.2 - 2.3	1.2	1.2
		72 h	0.8	0.6 - 0.7	0.8 - 0.9	0.6 - 0.8

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Table 4: Microbial water quality parameters for RAS without acetate.

Parameters	Units	Time	Control with biofilter (Ctrl+bf)				Control without biofilter (Ctrl-bf)			
			I	II	III	mean ± SD	I	II	III	mean ± SD
BactiQuant	BQV × 10 ⁴ /ml	0 h	3.39	5.92	4.94	4.75 ± 1.28	11.5	17.6	7.20	12.1 ± 5.20
		72 h	6.28	7.73	5.24	6.42 ± 1.25	15.3	33.3	6.86	18.5 ± 1.35
HP degradation rate constant	h ⁻¹	0 h	0.60	0.72	0.18	0.50 ± 0.28	0.30	1.38	0.84	0.84 ± 0.54
		72 h	1.38	0.54	0.24	0.72 ± 0.59	0.30	2.40	0.96	1.22 ± 1.10
Total cells	cells × 10 ⁶ /ml	0 h	5.07	8.89	13.7	9.23 ± 4.34	4.64	2.13	8.79	5.19 ± 3.36
		72 h	5.00	5.32	71.7	5.83 ± 1.17	3.13	1.87	5.83	3.61 ± 2.02
Low RNA	cells × 10 ⁶ /ml	0 h	2.80	7.80	12.0	7.56 ± 4.64	2.67	1.21	6.90	3.59 ± 2.95
		72 h	3.40	4.60	5.70	4.59 ± 1.16	1.91	0.95	4.16	2.34 ± 1.65
High RNA	cells × 10 ⁶ /ml	0 h	2.30	1.10	1.70	1.67 ± 0.62	1.97	0.92	1.90	1.60 ± 0.58
		72 h	1.60	0.71	1.40	1.25 ± 0.47	1.22	0.92	1.66	1.27 ± 0.38
Microparticles 1-3 µm	#part × 10 ⁶ /ml	0 h	3.93	1.28	0.88	2.03 ± 1.66	1.31	1.69	2.09	1.70 ± 0.39
		72 h	5.49	1.29	0.85	2.54 ± 2.56	1.52	1.69	2.90	2.03 ± 0.76
Microparticles 3-30 µm	#part × 10 ⁵ /ml	0 h	1.04	0.99	0.83	0.95 ± 0.11	1.45	1.28	1.24	1.32 ± 0.11
		72 h	1.30	0.84	0.85	0.99 ± 0.26	1.66	1.65	1.21	1.51 ± 0.26

Table 5: Microbial water quality parameters for RAS groups with added acetate.

Parameters	Units	Time	Acetate with biofilter (Ac+bf)				Acetate without biofilter (Ac-bf)			
			I	II	III	mean ± SD	I	II	III	mean ± SD
BactiQuant	BQV × 10 ⁴ /ml	0 h	3.72	3.68	12.1	6.51 ± 4.86	6.54	9.76	4.32	6.87 ± 2.73
		72 h	2.10	3.08	10.5	5.24 ± 4.61	67.6	4.28	3.24	25.0 ± 36.9
HP degradation rate constant	h ⁻¹	0 h	0.18	1.62	0.90	0.90 ± 0.72	0.48	0.30	0.3	0.36 ± 0.10
		72 h	0.24	1.32	0.90	0.82 ± 0.54	1.80	0.36	0.36	0.84 ± 0.83
Total Cells	cells × 10 ⁶ /ml	0 h	4.63	17.0	5.95	9.10 ± 6.88	6.66	6.92	4.23	5.94 ± 1.48
		72 h	4.05	24.1	9.81	1.26 ± 1.03	18.7	3.23	7.46	9.79 ± 7.98
Low RNA	cells × 10 ⁶ /ml	0 h	3.63	12.0	4.34	6.51 ± 4.38	5.67	5.96	3.92	5.18 ± 1.10
		72 h	3.50	17.0	7.68	9.38 ± 6.89	8.95	2.75	6.84	6.18 ± 3.15
High RNA	cells × 10 ⁶ /ml	0 h	0.73	5.45	1.61	2.60 ± 2.51	1.00	0.96	0.32	0.76 ± 0.38
		72 h	0.55	7.09	2.14	3.26 ± 3.41	9.73	0.48	0.62	3.61 ± 5.30
Microparticles 1-3 µm	#part × 10 ⁶ /ml	0 h	1.07	7.23	2.09	3.47 ± 3.30	1.06	1.16	0.60	0.94 ± 0.30
		72 h	7.11	7.00	2.26	3.32 ± 3.28	4.72	1.43	1.40	2.52 ± 1.91
Microparticles 3-30 µm	#part × 10 ⁵ /ml	0 h	0.97	0.88	2.05	1.30 ± 0.65	0.65	0.79	0.63	0.68 ± 0.08
		72 h	0.75	0.87	1.13	0.91 ± 0.19	0.64	0.72	2.81	1.39 ± 1.23

