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

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Abstract

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

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

1. Introduction

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

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

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

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

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

2. Materials and Methods

2.1 System maturation and daily operation routines

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

2.2 Experimental procedures

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

2.2.1 Batch trial (experiment 1)

To test the effect of easily biodegradable carbon on bacterial abundance and activity in RAS water, a beaker trial was carried out. Acetate was chosen as the easy biodegradable carbon source (Pedró-Alió and Brock, 1983; Canelhas *et al.*, 2017), and was added in three different concentrations (Table 1): low concentration (LC) of 10 mg acetate/L, medium concentration (MC) of 20 mg acetate/L, a high concentration (HC) of 40 mg acetate/L, compared to a control without addition of acetate. These acetate quantities represent a COD addition of 9.5, 19 and 38 mg O_2/L simulating a theoretical daily feed increase by 1.6, 3.2 and 6.4 times, respectively, compared to a control group without acetate addition (Table 1).

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

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

2.2.2. Pilot scale RAS trial (experiment 2)

To evaluate the effect of repeated addition of acetate in the presence or absence of a biofilter, twelve identical and independent RAS were used. In six of these RAS, the media in the submerged biofilter was removed 5 hours before the start of the experiment to let the system stabilize from any disturbances caused by removing the biofilter media. A trickling-filter with a 33 m^2 active surface area remained in all systems mainly for degassing purposes. Four treatment groups were then established in triplicates: i) control RAS with a biofilter (Ctrl+bf); ii) control RAS without a biofilter (Ctrl-bf); iii) RAS spiked with acetate with a biofilter (Ac+bf); and iv) RAS spiked with acetate without a biofilter (Ac-bf). Each RAS from group (Ac+bf) and (Ac-bf) were spiked three consecutive times with the highest acetate concentration (40 mg acetate/L): at time 0, then a second time 24 h later, and a third time 48 h after the initial spike. The total study period was 72 h. During the acetate spiking trials, RAS operation (i.e. feeding and water exchange) remained unchanged.

2.3 Sampling and analysis procedures

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

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

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

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

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

2.4. Data Analysis

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

3. Results

3.1 Batch trial (Experiment 1)

3.1.1 Acetate degradation in RAS water

The dissolved fraction of COD (COD_{DISS}) in the tested RAS water before acetate addition was 35.6 mg O_2/L. After spiking with the three different acetate concentrations, the COD_{DISS} concentrations after 6 h were: 42.3, 51.0 and 73.8 mg O_2/L for the low (LC), medium (MC) and high (HC) acetate additions, respectively, compared to 35.0 mg O_2/l in the control (Table 1). Acetate was degraded at a rate of ~ 1 mg/L per hour in all treatments. The low and medium concentration (10 mg/L and 20 mg/L) was completely degraded within 24 hours, whereas the high concentration (40 mg/L) was completely degraded within 48 hours.

3.1.2. Bacterial activity

The rate constant (k) of HP degradation increased 24 h after acetate addition, in a consistent manner with 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, respectively (Fig. 2a; Table 3). This concurred with significant differences ($p < 0.001$) in bacterial activity at the end of the experiment for the different concentrations of acetate dosed.

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

3.1.3. Microparticles number and size distribution in RAS water

Obvious difference in microparticle numbers where observed between two defined size class ranges (1 - 3 μm and 3 – 30 μm) after addition of acetate. Microparticles between 1 and 3 μm increased according to 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$_{1\text{-}3\mu m}$ after 72 h ranged from 1.8 to 3.2 $\times$ 10^6 particles/ml
195 for the LC to the HC (Fig. 2c; Table 3). The number of microparticles$_{1\text{-}3\mu m}$ in the untreated water (control)
196 decreased linearly until the end of the experiment, from 1.85 to 1.2 $\times$ 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 $\times$ 10^4 to
221 1.76 $\times$ 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 $\times$ 10^4 BQV/ml to 3.24 and 4.28 $\times$ 10^4 BQV/ml) and a 10 times higher activity (from
226 6.54 $\times$ 10^4 to 6.76 $\times$ 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

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

3.2.3 Bacterial abundance

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

3.2.4 Microparticles

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

3.2.5 Concentration of inorganics N and P

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

The mean nitrite-N concentration for the twelve RAS at the start of the trial was 0.05 ± 0.01 mg NO_2-N/L. Similarly as TAN, the concentrations were 0.08 ± 0.05 mg NO_2-N/L for both Ctrl+bf and Ctrl-bf treatments at the end of the experiment, 0.05± 0.02 mg NO_2-N/L for the Ac+bf RAS, and significantly higher ($p < 0.05$) at 0.19 ± 0.03 mg NO_2-N/L in the Ac-bf treatment.

Nitrate concentrations were similar in all 4 treatment groups at the startup of the trail (64.2 ± 3 mg NO_3-N/L). However, a significant reduction ($p < 0.05$) was observed in both treatment groups with acetate

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 ± 4.8 mg NO_3-N/L, respectively, while in the Ac+bf and Ac-bf groups it was 53.5 ± 7.0 and 53.3 ± 2.2 mg NO_3-N/L.

Phosphate concentration before the start of the experiment were similar between the twelve RAS (3.7 ± 0.19 PO_4-P/L), ending at 3.6 ± 0.04, 3.7 ± 0.1, 3.0 ± 0.5 and 3.3 ± 0.2 PO_4-P/L for the Ctrl+bf, Ctrl-bf, Ac+bf and Ac-bf, respectively, and without significant differences between treatments ($p > 0.05$). However, significant differences ($p < 0.05$) were found when phosphate consumption was calculated (Δ PO_4-P = PO_4-P_{72h} – PO_4-P_{0h}) showing RAS spiked with acetate (0.58 ± 0.14 and 0.57 ± 0.07 PO_4-P/L for Ac+bf and Ac-bf) to be significant higher than both control RAS (0.06 ± 0.02 and 0.08 ± 0.06 PO_4-P /L for Ctrl+bf and Ctrl-bf).

4. Discussion

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

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

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

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

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

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

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

4.3 Analysis of results and microbial water quality methods

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

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

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

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

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

5. Conclusions

i) Addition of easily biodegradable dissolved carbon (acetate) in beakers with RAS water caused increased bacterial activity and abundance.
ii) Addition of acetate for three consecutive days to pilot-scale RAS with biofilters did, however, not significantly affect the bacterial activity and abundance in the water phase. Only in RAS where the submerged biofilter media had been removed, was a significant response observed in the water phase. This confirms that the biofilm and surface-attached bacteria have the capacity to consume significant pulses of organic matter, thereby preventing the acute deterioration of the microbial water quality.
iii) The monitoring tools used in this study (flow cytometry, HP degradation assay, and particle Coulter counter) complementarily detected and described the abrupt changes in bacterial activity and abundance in the water due to pulse loading of organic matter.

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References

Arvin, E., Pedersen, L-F. 2015. Hydrogen peroxide decomposition kinetics in aquaculture water. Aquacult. Eng. 64: 1 – 7.

Attramadal, K.J.K, Salvesen, I., Xue, R., Øie, G., Størseth, T.R., Vadstein, O., Olsen, Y. 2012. Recirculation as a possible microbial control strategy in the production of marine larvae. Aquacult. Eng. 46: 27-39.

Attramadal, K. J. K., Minniti, G., Øie, G., Kjørsvik, E., Østensen, M.A., Bakke, I., Vadstein O. 2016. Microbial maturation of intake water at different carrying capacities affects microbial control in rearing tanks for marine fish larvae. Aquaculture 457: 68-72.

Avnimelech, Y. 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176: 227 – 235.

Bitton, G. 2011. Wastewater Microbiology. Fourth edition. Wiley-Blackwell, New Jersey, pp. 762.

Blancheton, J.P., Attramadal, K.J.K., Michaud, L., Roque d'Orbcastel, Vadstein, O. 2013. Insight into bacterial population in aquaculture systems and its implication. Aquacult. Eng. 53: 30 – 39.

Bremer H, Dennis P P. 1987. Modulation of chemical composition and other parameters of the cell by growth rate. In: Neidhardt F C, Ingraham J L, Low K B, Magasanik B, Schaechter M, Umbarger H E, editors. *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. Vol. 2. Washington, D.C: American Society for Microbiology, pp.1527–1542.

Canelhas, M.R., Andersson, M., Eiler, A., Lindström, E.S., Bertilsson, S. 2017. Influence of pulsed and continuous substrate inputs on freshwater bacterial community composition and functioning in bioreactors. Env. Microbiol. 19 : 5078-5087. DOI: 10.1111/1462-2920.13979.

Chen, S., Timmons, M.B., Aneshansley, D.J., Bisogni, J.J. 1993. Suspended solids characteristics from recirculating aquaculture systems and design implications. Aquaculture 112: 143-155.

Chróst, R.J. 1991. Environmental control of the synthesis and activity of aquatic microbial ectoenzymes. In : Chróst, R.J. (ed), Microbial enzymes in aquatic environments. Springer-Verlag, New York Inc., USA, pp. 29 – 59. DOI: 10.1007/978-1-4612-3090-8_3.

Crespo, B.G., Pommier, T., Fernández-Gómez, B., Pedrós-Alió, C. 2013. Taxonomic composition of the particle-attached and free-living bacterial assemblages in the Northwest Mediterranean Sea analyzed by pyrosequencing of the 16S rRNA. MicrobiologyOpen 2 (4): 541-552.

Dalsgaard, J., Pedersen, P.B. 2011. Solid and suspended/dissolved waste (N,O,P) from rainbow trout (*Oncorynchus mykiss*). Aquaculture 313: 92-99.

Davies, D. 2011. Biofilm Dispersion. In: Flemming, H-C, Wingender, J., Szewzyk, U. (eds), Biofilm highlights. Springer Series on Biofilms 5. Springer-Verlag, Berlin, pp. 243. DOI: 10.1007/978-3-642-19940-0_1.

DS 223. 1991. Water analysis – Determination of the sum of nitrite- and nitrate-nitrogen. Danish Standards Foundation, Charlottenlund, Denmark.

DS 224. 1975. Water Analysis – Determination of Ammonia-Nitrogen. Danish Standards Foundation, Charlottenlund, Denmark.

Ebeling, J.M., Timmons, M.B., Bisogni, J.J. 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. Aquaculture 257: 346-358.

Fdz-Polanco, F., Méndez, E., Urueña, M.A., Villaverde, S., García, P.A. 2000. Spatial distribution of heterotrophs and nitrifiers in a submerged biofilter for nitrification. Water Res. 34 (16): 4081 – 4089.

Fernandes, P. 2015. Interactions between micro-particles and the rearing environment in recirculating aquaculture systems. Ph.D thesis. Section for Aquaculture, DTU Aqua, Technical University of Denmark, Hirthals, Denmark, pp. 122.

Fernandes, P.M., Pedersen, L-F., Pedersen, P.B. 2015. Microscreen effects on water quality in replicated recirculating aquaculture systems. Aquacult. Eng. 65: 17-26.

Fernandes, P.M., Pedersen, L-F., Pedersen, P.B. 2017. Influence of fixed and moving bed biofilters on micro particle dynamics in a recirculating aquaculture system. Aquacult. Eng. 78: 32-41.

Gerardi, M. 2006. Wastewater Bacteria. John Wiley & Sons, Inc., New Jersey, pp. 251.

Gonzalez-Silva, B.M., Jonassen, K.R., Bakke, I., Østgaard, K., Vadstein, O. 2016. Nitrification at different salinities: Biofilm community composition and physiological plasticity. Water Res. 95: 48 – 58.

Hagopian, D.S., Riley, J.G. 1998. A closer look at the bacteriology of nitrification. Aquacult. Eng. 18: 223-244.

Hargreaves, J.A. 2006. Photosynthetic suspended-growth systems in aquaculture. Aquacult. Eng. 34: 344 – 363.

ISO 6878:2004. 2004. Water quality — determination of phosphorus — ammonium molybdate spectrometric method. International Organization for Standardization, Geneva, Switzerland, pp. 21.

Leonard, N., Blancheton, J.P., Guiraud, J.P. 2000. Populations of heterotrophic bacteria in an experimental recirculating aquaculture system. Aquacult. Eng. 22: 109 – 120.

Leonard, N., Guiraud, J.P., Gasset, E., Cailleres, J.P., Blancheton, J.P. 2002. Bacteria and nutrients – nitrogen and carbon – in a recirculating system for sea bass production. Aquacult. Eng. 26: 111 – 127.

Liltved, H., Cripps, S.J. 1999. Removal of particles-associated bacteria by prefiltration and ultraviolet irradiation. Aquaculture Research 30: 445-450.

Madigan, M.T., Martinko, J.M., Bender, K.S., Buckley, D.H., Stahl, D.A., Brock, T. 2015. Brock biology of microorganisms. 14th edition, Glenview, IL: Pearson Education, pp. 1105.

Marie, D., Simon, N., Vaulot, D. 2005. Phytoplankton cell counting by flow cytometry. In: Andersen, R. (Ed.), 488 Algal Culturing Techniques. Elsevier Academic Press, Oxford, UK: 253-285.

McDougald, D., Rice, S.A., Barraud, N., Steinberg, P.D., Kjelleberg, S. 2011. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nat. Rev. Microbiol. 10: 39-50.

Michaud, L., Blancheton, J.P., Bruni, V., Piedrahita, R. 2006. Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters. Aquacult. Eng. 34: 224-233.

Michaud, L., Lo Giudice, A., Troussellier, M., Smedile, F., Bruni, V., Blancheton, J.P. 2009. Phylogenetic characterization of the heterotrophic bacterial communities inhabiting a marine recirculating aquaculture system. J. App. Microbiol. 107: 1935 – 1946.

Michaud, L., Lo Giudice, A., Interdonato, F., Triplet, S., Ying, L., Blancheton, J.P. 2014. C/N ratio-induced structural shift of bacterial communities inside lab-scale aquaculture biofilters. Aquacult. Eng. 58: 77 – 87.

Neidhardt, F.C., Ingraham, J.L., Schaechter, M. 1990. Physiology of the bacterial cell: a molecular approach. Sinauer Associates, Inc. Sunderland, Massachussetts. pp. 507.

Nogueira, R., Melo, L.F., Purkhold, U., Wuertz, S., Wagner, M. 2002. Nitrifying and heterotrophic population dynamics in biofilm reactors: effects of hydraulic retention time and the presence of organic carbon. Water Res. 36: 469 – 481.

Pedersen, L-F., Pedersen, P.B., Nielsen, J.L., Nielsen, P.H. 2010. Long term/low dose formalin exposure to small-scale recirculation aquaculture systems. Aquacult. Eng. 42: 1-7.

Pedersen, L-F., Suhr, K., Dalsgaard, J., Pedersen, P., Arvin, E. 2012. Effect of feed loading on nitrogen balances and fish performance in replicated recirculating aquaculture systems. Aquaculture 338 – 341: 237 – 245.

Pedersen, P.B., von Ahnen, M., Fernandes, P., Naas, C.; Pedersen, L-F., Dalsgaard, J. 2017. Particle surface area and bacterial activity in recirculating aquaculture systems. Aquacult. Eng. 78: 18-23.

Pedró-Alió, C. Brock, T.D. 1983. The importance of attachment to particles for planktonic bacteria. Arch. Hydrobiol. 98 (3): 354 – 379.

Prest, E.I., Hammes, F., van Loosdrecht, M.C.M., Vrouwenvelder, J.S. 2016. Biological stability of drinking water: Controlling factors, methods and challenges. Front. Microbiol. 7: 45. DOI: 10.3389/fmicb.2016.00045.

Reeslev, M., Nielsen, J., Rogers, L. 2011. Assessment of the bacterial contamination and remediation efficacy after flooding using fluorometric detection. J. ASTM Int. 8 (10): 1-5.

Rojas-Tirado, P., Pedersen, P.B., Pedersen, L-F. 2017. Bacterial activity dynamics in the water phase during start-up of recirculating aquaculture systems. Aquacult. Eng. 78: 24-31.

Rojas-Tirado, P., Pedersen, P.B., Vadstein, O., Pedersen, L-F. 2018. Changes in microbial water quality in RAS following altered feed loading. Aquacult. Eng. 81: 80 – 88.

Rud, I., Kolarevic, J., Holan, A., Berget, I., Calabrese, S., Terjesen, B. 2017. Deep-sequencing of the bacterial microbiota in commercial-scale recirculating and semi-closed aquaculture systems for Atlantic salmon post-smolt production. Aquacult. Eng. 78: 50-62.

Rusten, B., Eikebrokk, B., Ulgenes, Y., Lyngren, E. 2006. Design and operations of the Kaldnes moving bed biofilm reactors. Aquacult. Eng. 34: 322-331.

568 Schaechter, E., Maaloe, O., Kjeldgaard, N.O. 1958. Dependency on medium and temperature of cell size and
569 chemical composition during balanced growth of *Salmonella typhimurium*. J. Gen. Microbiol. 19: 592-606.
570
571 Vadstein, O., Øie, G., Olsen, Y., Salvesen, I., Skjermo, J., Skjåk-Bræk, G. 1993. A strategy to obtain microbial
572 control during larval development of marine fish. In: Reinertsen, H., Dahle, L.A., Jørgensen, L., Tvinnereim,
573 K. (eds). Fish Farming Technology – Proceedings of the First International Conference on Fish Farming
574 Technology Balkema: Rotterdam, pp. 69–75.
575
576 van der Kooij, D., Visser, A., Hijnen, W.A.M. 1982. Determining the concentration of easily assimilable
577 organic carbon in drinking water. J. Am. Water Works Assoc. 74: 540-545.
578
579 Wold, P., Holan, A., Øie, G., Attramadal, K., Bakke, I., Vadstein, O., Leiknes, T. 2014. Effects of membrane
580 filtration on bacterial number and microbial diversity in marine recirculating aquaculture system (RAS) for
581 Atlantic cod (*Gadus morhua L.*) production. Aquaculture 422 – 423: 69 – 77.
582
583 Zhu, S., Chen, S. 2001. Effects of organic carbon on nitrification rate in fixed film biofilters. Aquacult. Eng.
584 25: 1 – 11.
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FIGURES AND TABLES

(Figure for Mat&Meth)

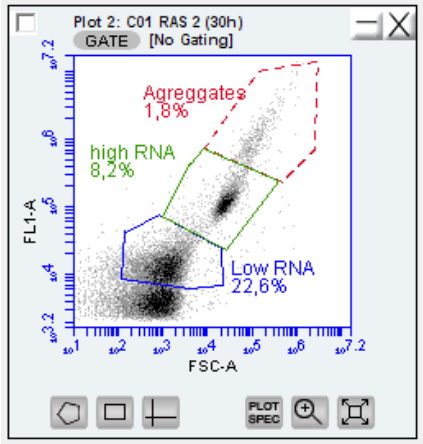
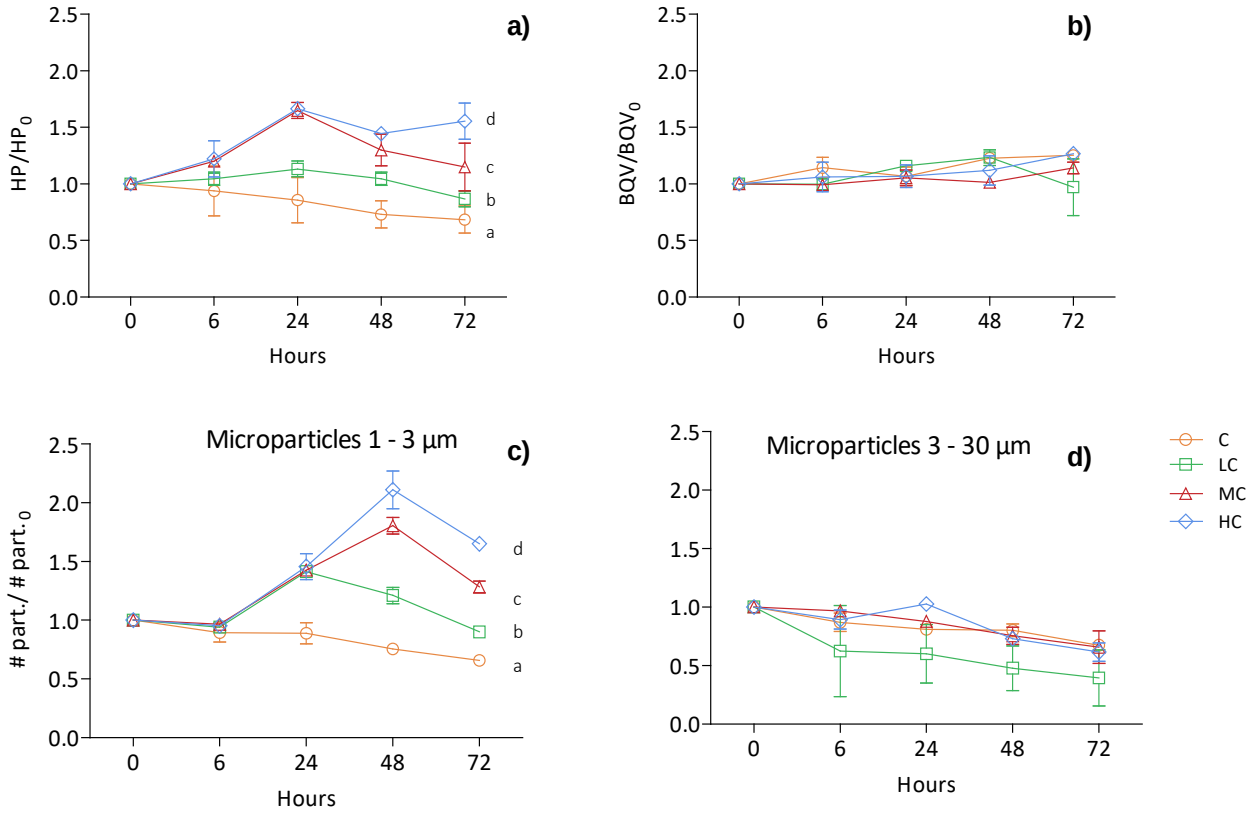


Fig. 1: Gating of cells counted with flow cytometry method. Cell gating was used to divide bacteria into two groups, low RNA and high RNA, separating them from the aggregates. Only low and high RNA groups are taken into consideration in the cell counting. The same gating was applied for all water samples.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

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 ± 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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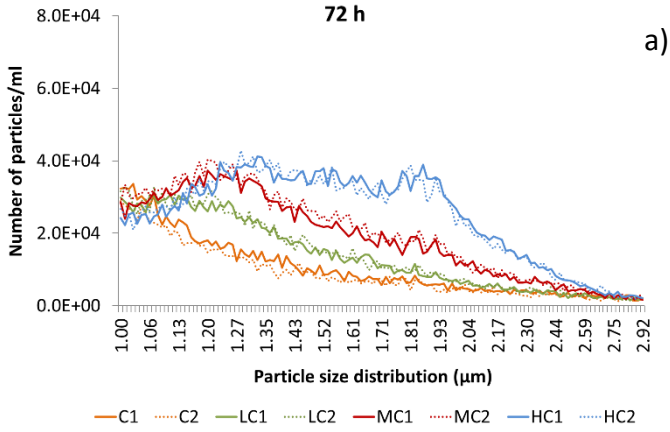

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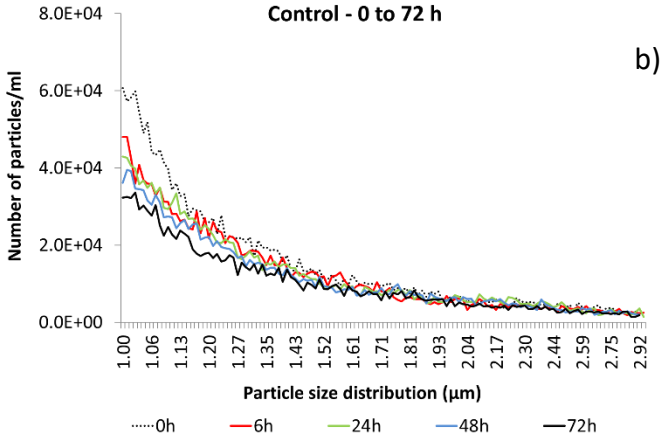

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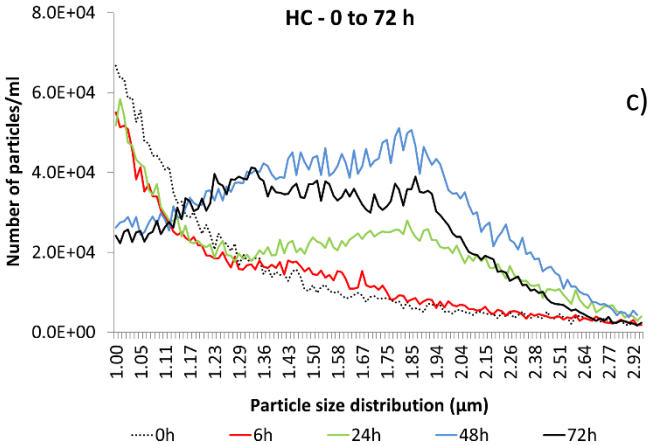

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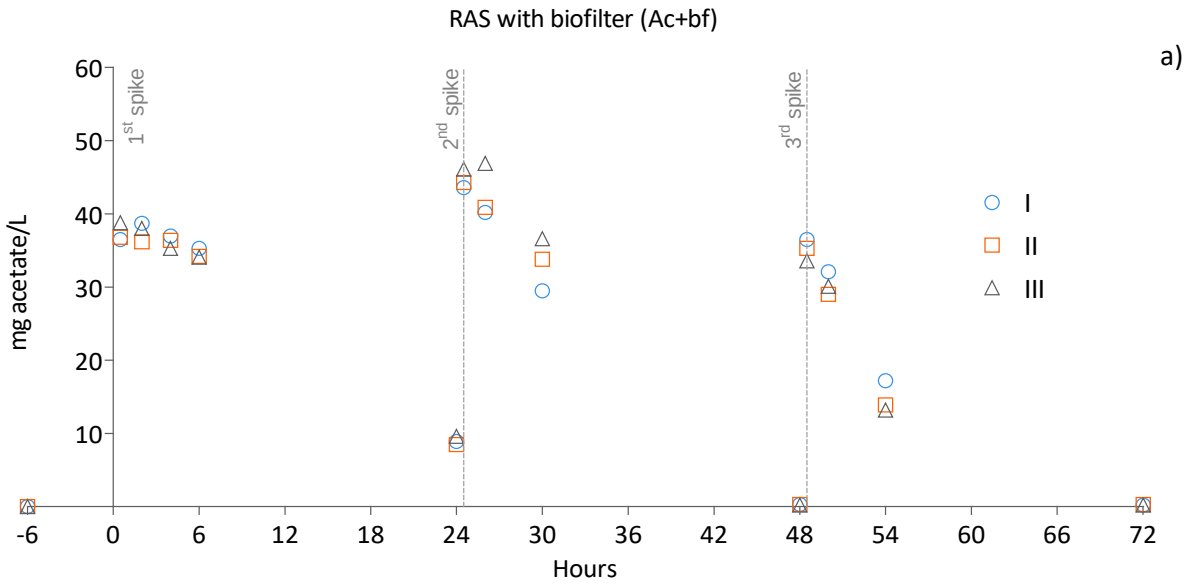
639 **Fig. 3: Particle numbers and development within the size distribution 1 - 3 µm following acetate addition to RAS water in**
640 **beakers during Experiment 1: a) Changes in size distribution 72 h after different acetate addition (C1-2: no addition, LC1-2: 10**
641 **mg/l added, MC1-2: 20 mg/l added, 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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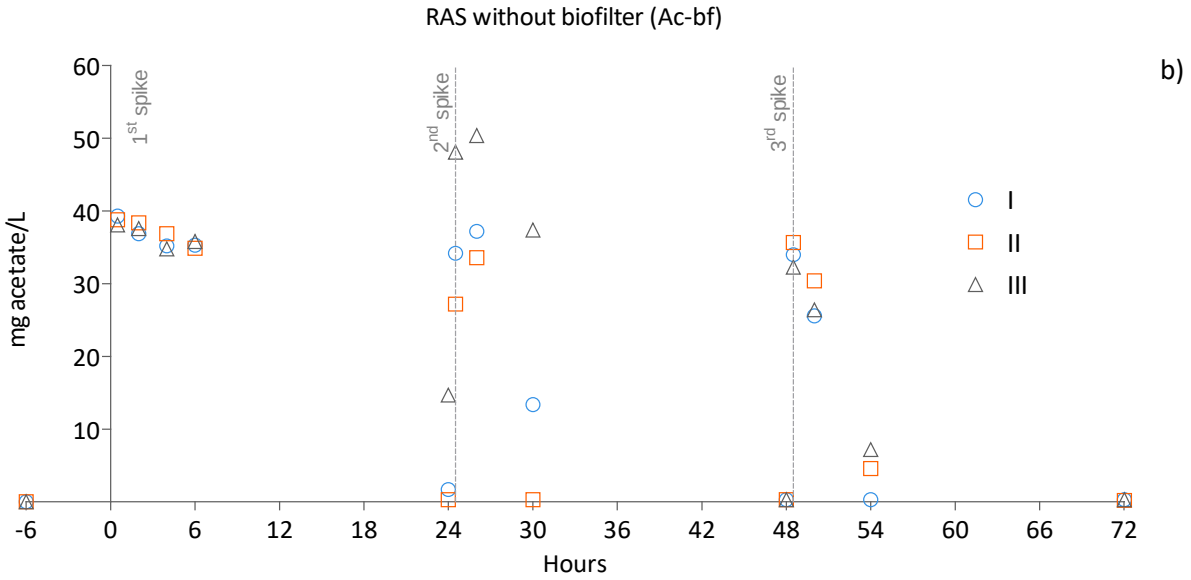

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646 **Fig 4: Acetate concentrations in RAS following acetate spikes at t = 0, 24 and 48 h during Experiment 2, in: a) RAS**
647 **with biofilter media (Ac+bf) and b) RAS without biofilter media (Ac-bf). Roman numerals in legend indicate RAS**
648 **replicates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

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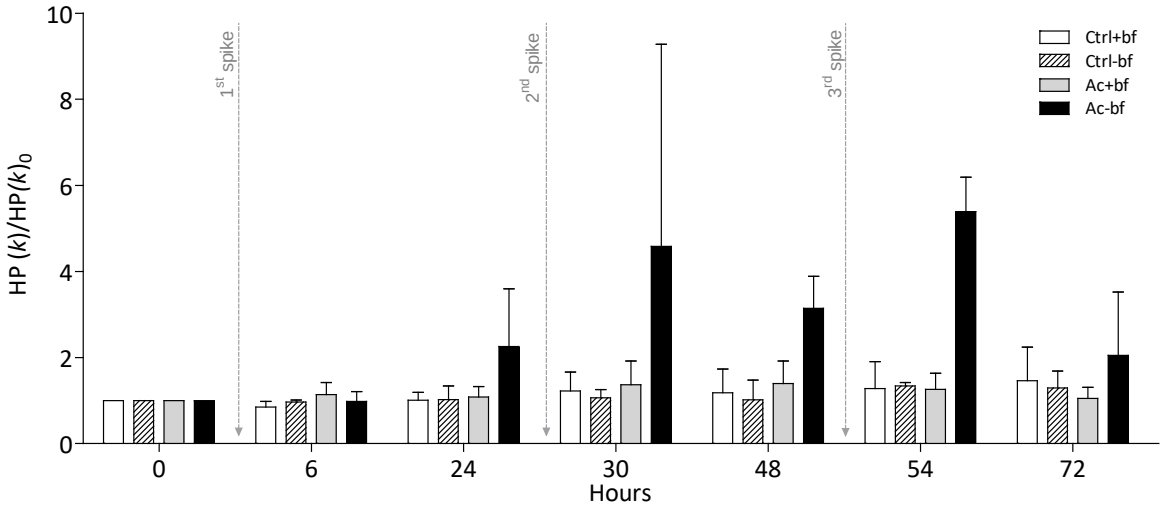

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655 **Fig. 5: Changes in bacterial activity expressed by changes in the hydrogen peroxide (HP) removal rate constant k (h^{-1}) for the different treatment groups during 72 h in Experiment 2: control RAS with biofilter (Ctrl+bf) (white bars), control RAS without biofilter (Ctrl-bf) (stripe bars), RAS with biofilter (Ac+bf) spiked with acetate at t = 0, 24, 48 h (grey bars), and RAS without biofilter (Ac-bf) spiked with acetate at t = 0, 24, 48 h (black bars). Data are normalized (C/C_0) and presented as mean ± SD, n = 3 (raw data for 0 and 72 h are presented in Table 4 and 5).**

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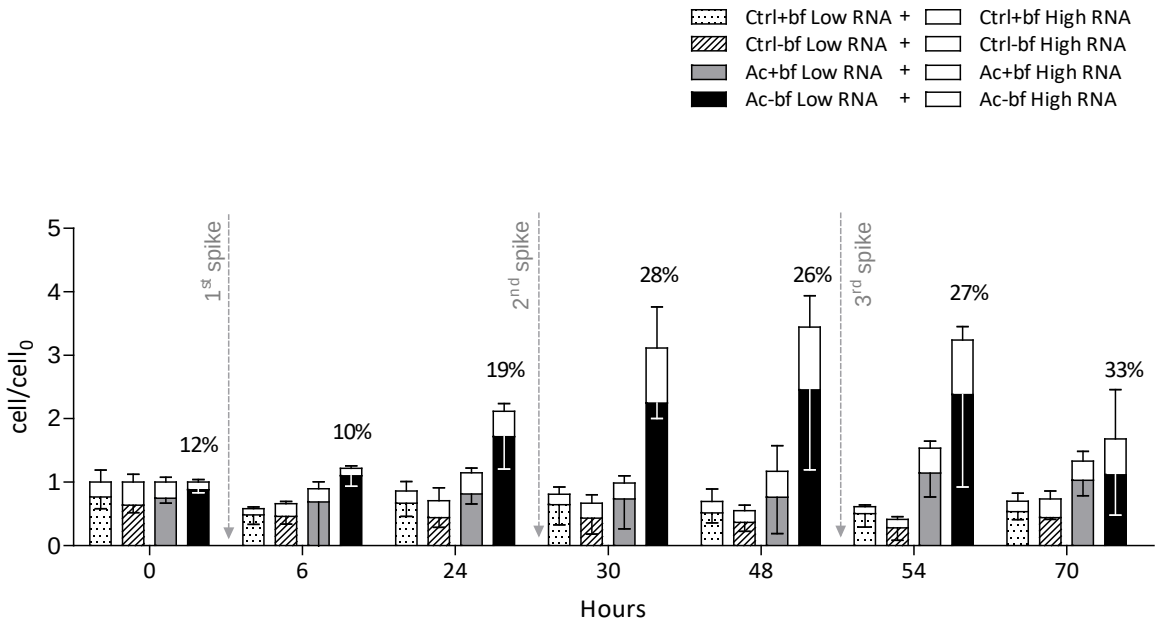

Fig. 6: Changes in bacterial abundance for the different treatment groups during 72 h in Experiment 2. For the low RNA cell counting: control RAS with biofilter (Ctrl+bf) (white bars), control RAS without biofilter (Ctrl-bf) (stripe bars), RAS with biofilter (Ac+bf) spiked with acetate at t = 0, 24, 48 h (grey bars), and RAS without biofilter (Ac-bf) spiked with acetate at t = 0, 24, 48 h (black bars). High RNA cells counting are shown as white upper bars and the percentage (%) corresponding to the high RNA fraction of the total cell count is presented only for the Ac-bf group. Data are normalized (C/C_0) and presented as mean ± SD, n = 3 (raw data for 0 and 72 h are presented in Table 4 and 5).

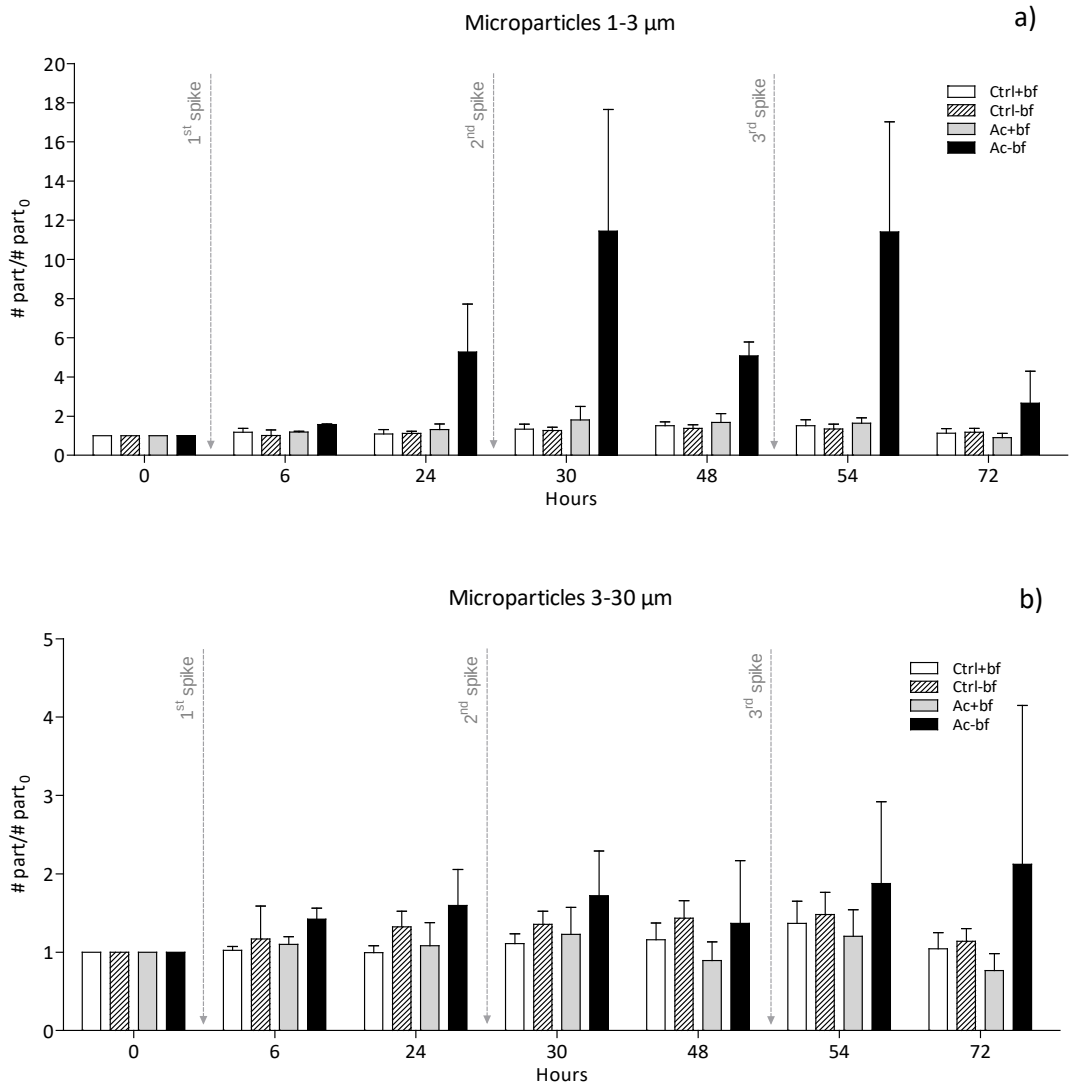

Fig 7: Number of microparticles 1 - 3 µm (a) and 3 - 30 µm (b) for the different treatment groups during 72 h in Experiment 2: control RAS with biofilter (Ctrl+bf) (white bars), control RAS without biofilter (Ctrl-bf) (stripe bars), RAS with biofilter media (Ac+bf) spiked with acetate at t = 0, 24, 48 h (grey bars), and RAS without media (Ac-bf) spiked with acetate at t = 0, 24, 48 h (black bars). Data are normalized (C/C_0) and presented as mean ± SD, n = 3 (raw data for 0 and 72 h are presented in Table 4 and 5).

694 **Table 1: Acetate concentrations (LC: low concentration, MC: medium concentration, HC: high concentration) used in experiment**
695 **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

696 [a] Theoretical COD calculated according to Dalsgaard and Pedersen (2011): 81 mg O_2/ g feed $\rightarrow$ 81 mg O_2 * 125 g feed /1700 L (systems volume) = 6
697 mg O_2/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
698 quantities.
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701 **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 *et al.*, 2005; Wold *et al.*, 2014)
Particle numbers			Prefiltered with a 45 µm AA filter. Counted immediately	Multisizer 4e Coulter Counter	N/A
Temperature, pH, Dissolved Oxygen	Temp., pH, O2	°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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704 **Table 3: Results from experiment 1 (beaker trial) for the microbial water quality parameters (mean ± SD, n = 3) in control (C), low**
705 **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