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1 The effects of different combinations of fixed and moving bed bioreactors on rainbow trout
2 (*Oncorhynchus mykiss*) growth and health, water quality and nitrification in recirculating aquaculture
3 systems

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14 Highlights

15 - Organic material accumulated in the two-moving-bed systems
16 - Nitrite concentrations increased in the two-fixed-bed systems
17 - Different bioreactor designs did not affect fish health or growth

18 Abstract

19 The effect of bioreactor design on nitrification efficiency has been well studied, but less is known about
20 the overall impacts on water quality. Besides nitrification, submerged fixed bed bioreactors (FBBR) trap
21 fine solid particles, whereas moving bed bioreactors (MBBR) grind solids, possibly increasing solids and
22 particle accumulation in the system. In this experiment, the effects of different combinations of fixed
23 bed and moving bed bioreactors on water quality, solids removal, particle size distribution, fish health
24 based on histopathological changes and nitrification efficiency were studied in laboratory scale
25 recirculating aquaculture systems (RAS) with rainbow trout (*Oncorhynchus mykiss*). Three set-ups with
26 triplicate tanks were used: 1. two consecutive fixed bed bioreactors (FF); 2. a fixed bed bioreactor
27 followed by a moving bed bioreactor (FM) and 3. two consecutive moving bed bioreactors (MM). Fish
28 performance was not influenced by the design of the bioreactor, specific growth rate (SGR) being
29 between 1.59 and 1.64% d^{-1} and feed conversion ratio (FCR) between 0.95 and 0.98. Water nitrite
30 concentration was higher in the FF systems compared to FM and MM systems, whereas the average
31 total ammonia nitrogen concentration (TAN) was not influenced by the treatments. Nitrification rate,
32 which was measured in the laboratory, followed the water nitrite levels, indicating highest total
33 ammonium oxidation rates in the MM systems. UV254 absorbance and total organic carbon (TOC)
34 concentrations were higher in the groups with moving bed systems, indicating accumulation of organic
35 substances in the circulating water. The total volume of particles was higher in the MM systems as
36 compared to the FF systems. The total solids balance was similar in all the bioreactor groups, since the

37 removal of solids by the FBBR backwash was compensated by the drum filter in the FM and MM
38 systems. In general, no significant histopathological difference in gill, kidney, heart and liver tissue were
39 observed between the RAS treatment groups and the flow-through treatment.

40 Keywords: biofiltration; histopathology; particle size distribution; water quality monitoring

41 List of abbreviations

42	FBBR	Fixed bed bioreactor
43	FCR	Feed conversion ratio
44	FF	Two consecutive fixed bed bioreactors
45	FM	Fixed bed, followed by moving bed bioreactor
46	LEH	Lamellar epithelial cell hyperplasia
47	MBBR	Moving bed bioreactor
48	MM	Two consecutive moving bed bioreactors
49	PSD	Particle size distribution
50	SGR	Specific growth rate
51	TAN	Total ammonia nitrogen
52	TGC	Thermal growth coefficient
53	TOC	Total organic carbon
54	TS	Total solids

55

56 1. Introduction

57 Nitrifying bioreactor operation and management is one of the most important and complex steps in
58 recirculating aquaculture systems (RAS) (Badiola et al., 2012; Svobodova et al., 2005). Typical RAS use
59 so-called fixed-film bioreactors, where biofilm is formed on artificial plastic carrier media or media
60 generated from natural substances such as sand and stones (Malone and Pfeiffer, 2006). Bacteria in the
61 media convert toxic ammonia into less toxic nitrate in a two phase nitrification process. The nitrification
62 process allows lower water usage rates, therefore decreasing the volume of effluents requiring the
63 treatment before discharged into the environment. There is a wide variety of nitrifying bioreactors used
64 in RAS, which all have particular strengths and weaknesses with no single reactor type being dominant
65 (e.g. Timmons and Ebeling, 2013).

66 The nitrification capacity of the following bioreactor types has been widely studied: moving bed
67 bioreactors (MBBR) (Kamstra et al., 2017), fixed bed bioreactors (FBBR) (Pedersen et al., 2015), fluidized-
68 sand biofilters (Summerfelt, 2006), rotating biological contactor (Brazil, 2006) and trickling filters
69 (Greiner and Timmons, 1998; Lekang and Kleppe, 2000). Besides nitrification, different bioreactor types
70 can also have other impacts on water quality, depending on how they are designed and operated.
71 Trickling filters, MBBRs and RBCs are constantly interacting with air, which increases the oxygen (O_2)
72 levels and reduces carbon dioxide concentration (CO_2) (Timmons and Ebeling, 2013). However, there is
73 very little information on how the choice of bioreactor design can affect fish health and water quality
74 parameters.

75 The moving bed bioreactor was designed in Norway in the late 1980s (Rusten et al., 2006). The reactor
76 chamber is agitated continuously with compressed air or mechanically, the carrier media being
77 constantly moved so as to create a scrubbing effect against each other. Because of that scrubbing effect,
78 the reactor shears solid particles, leading to the accumulation of the total amount of particles in the
79 system (Fernandes et al., 2017). These types of reactors are easy to operate, because there is a low head
80 loss and no need for backwashing. In addition, the constant movement enables efficient use of the
81 whole reactor volume, and mixing with air provides oxygen for the nitrification process. Because of
82 scrubbing, surplus microbial biomass created in the biofilm detaches from the carrier media and is later
83 removed from the system either by outflow or in solids removal units (Ødegaard, 2006).

84 Fixed bed bioreactor or fixed bed biofilm reactor (FBBR) is a reactor type, where carrier media is
85 structurally fixed in the reactor chamber (Kadic and Heindel, 2014). Depending on the fixed media type,
86 the reactor can be susceptible to clogging and must be backwashed frequently (Schlegel and Koeser,
87 2007). When using small carrier media, suspended solids particles are commonly trapped in these
88 reactors (Fernandes et al., 2017). The distribution of flow into the reactor and inside the reactor is
89 important: turbulent flow can cause uneven distribution of substrate in the reactor and the total
90 effective surface area for nitrification may be diminished. Turbulent flow might also create pockets,
91 where oxygen can be depleted and hydrogen sulphide might form.

92 Since O_2 is added and CO_2 is removed mainly in the other compartments of RAS, the main water quality
93 difference between FBBR and MBBR is probably the fate of solid particles in the reactor. High suspended
94 solids loads have been reported to cause sub-lethal stress and damages to gill structure in some fish
95 species (Au et al., 2004; Bilotta and Brazier, 2008). Thus, the amount of solid particles may influence fish
96 health and welfare. In addition, there is a positive correlation between bacterial numbers and the
97 surface area of particles (Pedersen et al., 2017), which may indicate that MBBR accumulates more
98 bacteria in the circulating water than FBBR.

99 In this study, we compared two widely used bioreactor types: moving bed and fixed bed bioreactors.
100 The comprehensive approach was used for comparing the effects of different bioreactor setups on
101 ammonium removal rates, fish health in terms of histopathological lesions and growth parameters,
102 water quality, solids accumulation and microbial dynamics. Our hypothesis was that the accumulation of
103 solids in the circulating water causes histopathological changes and chronic stress in the fish, which
104 affect fish growth and feed efficiency.

105 2. Materials and methods

106 2.1. Experimental setup

107 The experiment was carried out in the Natural Resources Institute Finland (Luke) Laukaa fish farm using
108 an experimental RAS platform. The platform has 10 individual freshwater recirculating systems, each
109 consisting of a 500 l bottom drained plastic rearing tank (Arvo-Tec, Joroinen, Finland), feed collector
110 unit, 24 cm swirl separator (Eco-Trap Collector1, Pentair Aquatic Eco-Systems, Minneapolis, USA), drum
111 filter with 60 µm filter panels (Hydrotech HDF501, Veolia, Paris, France), 2 separate 147 l bioreactor
112 tanks (Arvo-Tec, Joroinen, Finland), trickling filter acting as a forced-ventilated cascade aeration column

113 (Bio-Blok® 200, EXPO-NET Danmark A/S, Hjørring, Denmark) and pump sump (Fig. 1). Water pH was
114 adjusted to 7.2 in pump sump with diluted sodium hydroxide using automated system (Prominent,
115 Heidelberg, Germany). Sodium bicarbonate was dosed to the inlet water source to achieve an alkalinity
116 of 50 mg l^{-1} ($CaCO_3$) in the RAS replacement water. Oxygen saturation was kept above 80% in the fish
117 tanks. The system is described in more detail by Pulkkinen et al. (2018).

118 In the trial, three bioreactor setups were compared with triplicate units: Treatment 1. two consecutive
119 fixed bed bioreactors (FF); Treatment 2. fixed bed bioreactor followed by moving bed bioreactor (FM)
120 and Treatment 3. two consecutive moving bed bioreactors (MM). Two bioreactors per RAS unit were
121 used, so that all units had similar amount of bioreactors. The experiment lasted 14 weeks. In one
122 treatment group (FF), only two units existed for the second half of the experiment due to a technical
123 failure with pH in one tank in week 8 of the experiment. A separate 500 l flow-through tank was used to
124 grow fish of the same origin with same feed, serving as a flow-through treatment for fish
125 histopathological sampling. Water temperature was adjusted to 16 °C by controlling the air temperature
126 and in the flow-through group by controlling the inlet water temperature.

127 Similar plastic (PP) carrier media (RK Biolements heavy in fixed bed systems and medium in moving bed
128 systems, RK Plast A/S, Skive, Denmark), tank hydraulic retention time and make-up water flow were
129 used and measured constantly in all RAS units (Table 1). Carrier media, used in two earlier experiments,
130 was mixed four weeks before the trial started, and divided evenly between the bioreactors to ensure
131 similar bacterial seed in all the RAS units. In FF and FM units, the first bioreactor was backwashed once
132 every two weeks. In FF units the second bioreactor was backwashed once every four weeks. The FBBR
133 backwash water amount was not taken into account in the make-up water flow calculations, because it
134 increased the total water volume by less than 4%.

135 2.2. Fish and feeding

136 Three weeks before the trial started, a total of 820 one year old rainbow trout (*Oncorhynchus mykiss*)
137 (average weight 99 g) originating from the National JALO-selective breeding programme (Natural
138 Resources Institute Finland, Tervo, Finland) were divided into the 9 RAS units. When the trial started,
139 the fish were weighed and their biomasses were equalized. The fish were weighed twice during the
140 experiment at weeks four and eight and group weighing was used in all of the weightings. Fish were
141 fasted one day prior to and after the weighing. Feeding was carried out with a commercial feeding
142 system (T Drum 2000, Arvo-Tec, Joroinen, Finland) 10–14 times per day. Feed intake rate was constantly
143 monitored using sieve in the tank outlet and uneaten feed pellets were calculated. Feed company
144 feeding table was used for feeding rate and it was reduced by 0.1 %-unit, when uneaten feed was
145 observed. 1:1 mixture of two commercial diets was used to ensure that the results can be better
146 generalized across various commercial feeds. Diets were produced by Raisioaqua (Circuit Red 5 mm,
147 Raisio, Finland) and BioMar (Orbit 929 4.5 mm, Aarhus, Denmark). The crude protein and lipid contents
148 of the diets were 43% and 42%, and 26% and 31%, respectively.

149 The feed conversion ratio (FCR) was calculated as: FCR = F / G, where F = cumulative feed intake
150 between weightings and G = total tank biomass gain between weightings. Specific growth rate (SGR) was

151 calculated as: SGR = $(\ln(W_{i+1}) - \ln(W_i)) / (t_{i+1} - t_i) \times 100$, where W = average fish weight at given time and
152 $t_{i+1} - t_i$ = duration of feeding days. The thermal growth coefficient (TGC) was calculated for the whole
153 experiment according to Jobling (2003) as: TGC = $((W_e^{1/3}) - (W_i^{1/3})) \times (T \times t) \times 1000$, where W_e = average
154 fish weight in the end, W_i = average fish weight at the beginning, T = average water temperature, t =
155 duration of feeding days.

156 2.3. Histopathological sampling and analysis

157 Tissue samples (gill, kidney and liver) from 5 fish per tank were collected at the start of the experiment,
158 twice during the experiment and again at the end of the experiment, at approximately one month
159 intervals. The second gill arch from the right hand side was sampled and sectioned parasagittally.
160 Kidney tissue was sampled as approximately 2 cm long sections from the distal third of the kidney and
161 sectioned transversely. Liver tissue was sampled in approximately 1×1 cm sections and sectioned
162 sagittally. The tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin,
163 sectioned at 4 µm and stained with haematoxylin and eosin (H&E) according to standard laboratory
164 practice. The sections were examined using light microscopy.

165 The histopathological changes were reported on a scale from 0–3: minimal, mild, moderate and severe
166 as described by Wolf et al. 2015. One section per tissue and per fish was examined.

167 The following parameters were studied and classified according to the severity of the lesions:

168 Gills: Lamellar epithelial cell hyperplasia (LEH): proliferation of the squamous epithelial cells lining the
169 gill surface. General diffuse proliferative branchitis: filling of interlamellar spaces by a mixed population
170 of epithelial and inflammatory cells. Focal branchitis: a local unspecific inflammatory change consisting
171 mainly of mononuclear, lymphocytic cell types involving a smaller area, usually only a few lamellae.
172 Lamellar fusion: one or more interlamellar sulci filled by proliferating pavement cells (with or without
173 increased mucous cells, chloride cells, and/or leucocytes). Lamellar adhesion: the often focal attachment
174 of adjacent lamellae with little or no evidence of cell proliferation. Lamellar thrombosis: formation of
175 blood clots inside lamellar capillaries consisting of fragmented thrombocyte nuclei and/or pink fibrinous
176 material within the distended capillaries.

177 Kidney: Tubular necrosis: Necrosis of tubular epithelial cells. Renal mineralization: mineralized material
178 intraepithelially or intraluminally. Number of melanomacrophage centres or pigmented macrophage
179 aggregates (PMAs): centres of mainly histiocytic macrophages that contain hemosiderin, melanin,
180 lipofuscin, and/or ceroid pigments and that serve as repositories for end-products of cell breakdown.

181 Liver: Hepatocellular cytoplasmic vacuolation: intracytoplasmic vacuoles containing glycogen or lipids.
182 Hepatitis/cholangiohepatitis: infiltration of acute or chronic inflammatory cells in liver tissue or around
183 bile ducts.

184 2.4. Water sampling and analysis

185 Total ammonia nitrogen, nitrite and nitrate were analysed once a week from the tank outlet water using
186 a spectrophotometer (Procedure 8038 Nessler, LCK341/342 and LCK340 respectively. DS 3900, Hach,

187 Loveland, USA). Alkalinity was analysed once a week with a standard method of titration (ISO 9963-
188 1:1994) (TitraLab AT1000, Hach, Loveland, USA).

189 Particle size distribution (PSD) was analysed from the tank water and from the water taken from top of
190 the 2nd bioreactor at week 13 (S4031, PAMAS, Rutesheim, Germany). Optical analyses covered particle
191 sizes from 1 µm to 200 µm. A simple comparison of PSD between treatments was made by calculating
192 the β-values (slope of log10 frequency versus log10 particle size) according to Patterson et al. (1999).
193 Total particle surface area and volume were calculated by using the given particle size diameter
194 (assumed sphere) multiplied by the total number of particles.

195 Particle counts were also measured from tank water with a CASY cell counter with a capillary size of 45
196 µm (Model TT, OLS OMNI Life Science GmbH, Basel, Switzerland) at week 14. Measurement principal is
197 based on pulse area analysis, where low voltage field is cast through the samples. Measurement range
198 was between 0.8 µm to 30 µm. The 100 ml water samples were frozen before analysis. Triplicate
199 measurements per water sample were analysed using a sample size of 200 µl.

200 Total organic carbon (TOC) and UV254 (turbidity corrected) were monitored online at 6 minute intervals
201 in the fish tanks with a UV/VIS spectrometer (5 mm open path length, spectro::lyser, s::can, Vienna,
202 Austria). Carbon dioxide concentrations were monitored in the fish tanks at 6 minute intervals with a
203 carbon dioxide sensor (Franatech, Lüneburg, Germany). Two hour average values are presented for
204 these online measurements.

205 2.5. Solids sampling

206 Sludge was collected twice during the trial at weeks 7 and 11 for solids analysis. Sludge from swirl
207 separators was collected using a 0.31 litre tube placed at the bottom of the separators. The collection
208 period lasted six hours. Drum filter backwash water was collected for 16 hours, then weighed and
209 mixed, after which a subsample of 1 litre was collected. Fixed bed bioreactors were cleaned by vigorous
210 agitation with air, and one litre samples were collected from the top of the reactor and from the outlet
211 pipe. At week 11, water collected only from the top of the reactor was used because there was no
212 difference between these sampling points. All solids samples were put into a container and oven dried
213 (+ 80 °C) for two days.

214 Total solids ($g\ kg^{-1}$) were calculated for FBBR and drum filter as: $TS = (m_d - m_t) / S \times V / F$, where m_d =
215 dried subsample mass (g), m_t = container mass (g), S = sample size (l), V = total outflow volume ($l\ d^{-1}$), F =
216 Feed intake ($g\ d^{-1}$). Total solids ($g\ kg^{-1}$) were calculated for the swirl separator as: $TS = (m_d - m_t) \times 4 / F$,
217 where m_d = dried subsample mass (g), m_t = container mass (g), F = feed intake ($g\ d^{-1}$).

218 2.6. Bioreactor nitrification rates

219 Bioreactor nitrification rates ($g\ NO_x\ h^{-1}$) were measured at the last week of the experiment, following
220 principles described by Jäntti et al. (2011). For the incubations, inlet water and carrier media were
221 collected from each bioreactor tank and transferred to the University of Jyväskylä. In the laboratory,
222 carrier media were divided into experimental vials (n = 30 per vial) with 360 ml inlet water, where $^{15}NH_4^+$

223 was added (final concentration of 5 mg/L; 10—15 atm%). To ensure complete nitrification, the carrier
224 media was incubated for 3 hours at *in situ* temperature and under constant mixing by magnetic stirring
225 bars (150 rpm). To measure ammonium and nitrate concentrations and the stable isotope composition
226 of nitrite and nitrate, water samples were taken at the beginning of the experiment, and after 1.5 and 3
227 hours. Water samples were filtered with 0.2 um syringe filters and frozen immediately. Later, nitrate,
228 nitrite and ammonium concentrations were measured with a spectrophotometer (Lasa 100, Hach,
229 Loveland, USA). The stable isotope composition of nitrite and nitrate was measured using the denitrifier
230 method (Sigman et al., 2001). Briefly, 20 nmoles of sample NO_{2+3} were converted to N_2O by cultured
231 denitrifying bacteria (*Pseudomonas chlororaphis* strain DSM 6698), which lack the enzyme responsible
232 for N_2O reduction and the isotopic composition of N_2O was measured using the IsoPrime 100 CF-IRMS
233 with a TraceGas preconcentrator interface.

234 2.7. Statistics

235 The effects of bioreactor design on nitrification efficiency, FCR, SGR, TGC, PSD and TS were analysed
236 using one-way ANOVA, and Tukey's post hoc test was used for comparing the effects between
237 treatments, which takes the uneven sample sizes in the end of the experiment into account (Rusticus
238 and Lovato, 2014). A nonparametric Kruskall-Wallis test was used for total particle counts when
239 assumptions were not met for the parametric test. Effects of bioreactor design on water quality
240 parameters were analysed using Mixed ANOVA, where bioreactor design type (between subjects) and
241 measurement week (within subjects) were factors. The Bonferroni post hoc test was used for comparing
242 effects between treatments. For online measurements, daily average values were used. Statistical
243 analyses were done with SPSS (IBM SPSS Statistics, Armonk, USA) wherein 95 % confidence interval was
244 used.

245 3. Results

246 3.1. Fish growth and histopathology

247 No significant differences were found between treatments for FCR and SGR during the trial or TGC for
248 the whole experiment (Table 2). For the whole experiment, average feed loads were 30.09 kg (± 0.15
249 kg), 30.88 kg (± 0.35 kg) and 30.39 kg (± 0.28 kg) in the FF, FM and MM groups, respectively.

250 The most significant histopathological changes were noted in gill tissue (Table 1, supplementary
251 material). The severity scores for both lamellar epithelial cell hyperplasia (LEH) and focal branchitis were
252 slightly elevated at the beginning of the trial, for focal branchitis only in the FF group, and for LEH in all
253 groups including the flow-through system.

254 In kidney and liver tissue, no notable histopathological changes were seen during the experiment. The
255 PMAs noted during the experiment were mild to moderate, and no notable differences in their
256 occurrence over time, or differences between treatment groups or control group, were noted. The
257 inflammatory changes noted in this experiment were also minor and did not show any increase during
258 the course of the experiment.

259 3.2. Water quality

260 There was no difference in the TAN values between treatment groups, whereas nitrite values decreased
261 throughout the experiment in all groups. In the FF group, nitrite values were significantly higher than in
262 the FM and MM groups ($P < 0.01$). Nitrate values were higher in the MM group in comparison to the FF
263 ($P < 0.01$) and FM groups ($P < 0.01$) (Fig 2.).

264 Total organic carbon, UV254 and CO_2 values were significantly different between the treatments ($P <$
265 0.01). TOC was lower in the FF group as compared to the FM and MM groups. The UV254 value was
266 lowest in the FF group, and highest in the MM group. CO_2 concentration was highest in the FF group ($P <$
267 0.01), but there was no significant difference between the FM and MM groups (Fig. 3).

268 3.3. Total solids and PSD

269 The sum of total solids removed from the RAS units and solids removed from the swirl separators did
270 not differ between the treatments (Fig. 4). Solids removal by the drum filters was significantly affected
271 by the bioreactor systems ($P < 0.01$). In the RAS with two moving bed bioreactors, drum filters removed
272 solids the most, whereas in the RAS with two fixed bed bioreactors, solids removal by drum filters was
273 the lowest. In the FM group, drum filter solids removal was lower than in the MM group, but it was not
274 statistically significant ($P = 0.051$).

275 In fish tanks, particle size distribution values (β-value, total amounts, surface area and volume) were not
276 significantly different between the treatments, whereas differences were observed in water samples
277 taken after biofiltration. The β-values in water sampled after the second bioreactor was significantly
278 higher in the FF group compared to MM ($P < 0.05$), indicating that RAS with two fixed bed bioreactors
279 has a larger share of particles in small sizes (Table 3). Total particle amounts and surface area in the
280 biofiltered water were not affected by the treatments, whereas total particle volumes were significantly
281 higher in the MM group compared to the FF group ($P < 0.05$). Over 80% of the particles were below 3
282 µm in the FF and FM group and over 90% in the FF group (Fig. 1, supplementary material).

283 Although treatments with moving bed bioreactors had higher particle counts measured with the CASY
284 cell counter, the counts were not significantly different between the treatments due to high within-
285 treatments variance (Kruskal-Wallis $P = 0.24$; Fig. 5).

286 3.4. Nitrification

287 The nitrification rate was significantly different between the treatments ($P < 0.01$). In the FF group, the
288 nitrification rate was lowest, but there was no difference between the MM group and FM group ($P =$
289 0.07). The nitrification rate did not differ between the first and second moving bed bioreactor, whereas
290 in the FF group, the second FBBR had a lower nitrification rate than the first FBBR ($P <0.01$) (Fig. 6).

291 4. Discussion

292 4.1. Fish performance

293 In general, fish grew well and there were minor mortalities. However, one tank was lost due to pH probe
294 failure. We did not see any differences in fish growth between the different bioreactor configurations,
295 even when some difference was seen in the water quality.

296 We noticed higher CO_2 and NO_2-N levels in the FF group. Good et al. (2010) reported that elevated CO_2
297 concentration up to 24 mg l^{-1} did not affect rainbow trout growth or health. In contrast, Kahn et al.
298 (2018) noticed that CO_2 concentration in RAS water had a negative linear correlation with Atlantic
299 salmon (*Salmo salar*) growth. This means that there is no threshold value for CO_2 where growth would
300 decrease: the higher the concentration the more it affects the growth. In addition, elevated nitrite
301 concentration can cause several physiological disturbances in aquatic animals, leading to decreased
302 growth (Aggergaard and Jensen, 2001; Jensen, 2003) and even death (Svoboda et al., 2005).

303 In contrast to the FF group, we noticed a higher organic material load and NO_3-N levels in the MM
304 group. Davidson et al. (2014) recommended 75 mg l^{-1} as the maximum level of nitrate for rainbow trout.
305 This is the level where negative impacts on long term health were seen. In addition to nitrogen
306 compounds, solids can also have detrimental effects on fish performance. Particle accumulation in fish
307 gills has been shown to cause inflammatory responses (Lu et al., 2018) and stress (Au et al., 2004).
308 However, Becke et al. (2018) studied the long term effect of a high suspended solids load in RAS and
309 despite the high load, they did not find rainbow trout histopathology or growth indicators to be
310 significantly affected.

311 Taken into account all of the above, all treatment groups had water quality parameters, which could
312 have affected the growth negatively. This might be one reason, why differences between treatment
313 groups were not observed in growth and health. In addition, rainbow trout can be tolerant to different
314 water qualities, thus observed differences for water quality between treatments might not be
315 biologically relevant or within treatment variability was too high to observe any differences.

316 For histopathological lesions in general, only minor differences between the different treatment groups
317 or the flow-through system were seen. Most lesions were minimal to mild, and thus clinically
318 nonsignificant. The only clinically significant moderate changes were noted in gill tissue as an increase in
319 lamellar epithelial cell hyperplasia (LEH), which is a common, non-specific lesion seen in subacute to
320 chronic gill damage, and in focal branchitis (Fig. 2, supplementary material). These changes were noted
321 also in the flow-through system. Mild, clinically nonsignificant gill lesions were noted also at the start of
322 the experiment (T0). These changes correlate partly with noted differences in water nitrogen
323 compounds and UV254 measurements, however, no water parameters were measured for the flow-
324 through system. The gills are structures with a large surface area in direct contact with water, and as
325 such are often the first tissue to show changes when water quality is suboptimal. Gills show a
326 remarkable regenerative capacity (Ferguson, 2006) and can adapt to less optimal water quality over
327 time (Kolarevic et al., 2012). No changes in liver or kidney tissue were noted in this experiment.
328 Melanomacrophage centres exist in normal kidney tissue of fish and they increase with age, however an
329 excess or increase can be seen in chronically stressed fish. A lymphoid inflammatory reaction located
330 around bile ducts, cholangiohepatitis, can be seen in connection with parasitic infections, but may also
331 be connected with unspecific immune mediated reactions and may have a connection with water

332 quality. None of these lesions were noted during the experiment; however, a prolonged exposure time
333 of harming substances might be needed in order to provoke some of the studied changes in these
334 organs.

335 4.2. Water quality

336 Online spectrometric water quality monitoring can provide useful information about short period
337 fluctuations in the water quality, which cannot be seen in the manual water sampling. However, there
338 are lots of substances that absorb light in the same wavelengths and affect the interpretation of the
339 results. In addition, sensitivity can be weak, and accuracy is tolerable only above certain threshold
340 values (Carré et al., 2017). The UV254 absorbance values correlate well with dissolved organic carbon
341 (DOM) and dissolved aromatic carbon in particular (Weishaar et al., 2003), making it a useful indicator of
342 biological substances in the water. When one or two MBBR were used in RAS unit, an increasing UV254
343 value was measured. Bacterial biomass was increasing in the bioreactors during the operation and this
344 surplus biomass was removed from the FBBR when backwashed. In MBBR, surplus biomass was
345 constantly removed into the circulating water, which increased the amount of organic matter in the
346 water, as was seen in the UV254 absorbance values during the trial. TOC fluctuations followed the
347 UV254 fluctuations, but there were no differences in the TOC values between the FM and MM groups.

348 Particle size distribution was measured, when systems were considered to be in their steady state. In
349 RAS, small particles typically accumulate in the system, which is seen as high β-values (Patterson et al.,
350 1999). In the present trial, total particle volume was highest in the MM group, which followed the
351 overall water quality values. The same amount of solids was introduced into every RAS unit via fish feed
352 and removed by water treatment units. Solids trapped and later removed by FBBR were removed by
353 drum filter in units with MBBR. Thus, drum filters were compensating particle accumulation in the MM
354 group, even though mesh size was 60 µm. Total particle counts measured with the CASY cell counter
355 were between 15 and 60-fold higher than those measured with the PAMAS optic particle counter. The
356 most likely explanation is that the cell counter is much more accurate in small size classes and there is
357 disintegration of possible cell aggregates in the freezing period. Michaud et al. (2006) found up to
358 800,000 free bacterial cells per ml in biofilter effluent, which indicates that the majority of small
359 particles are bacterial cells. Thus, total particle counts measured with the cell counter was considered to
360 measure total bacterial counts in the water. As expected, total bacterial counts were somewhat higher
361 in the units with moving bed bioreactors, but the difference was not significant between the groups.

362 In the units where MBBR was in use, CO_2 concentrations were lowest. Mixing the MBBR with
363 compressed air was ventilating CO_2 out of the systems, but there were no differences in concentrations
364 if one or two MBBRs were used.

365 4.3. Nitrification

366 Continuously decreasing nitrite values during the trial indicate that bioreactors were not yet fully
367 developed at the beginning of the trial. However, this was not detected in the TAN values, which were
368 quite stable throughout the trial. Although all carrier media were used for six months before the trial
369 started, it is possible that mixing and transferring might have disturbed nitrite oxidizing bacteria, which

370 are more vulnerable to changing conditions (Graham et al., 2007), and caused nitrite accumulation. This
371 accumulation was higher in the FF group as compared to FM and MM groups, which is in contrast to
372 other experiments that have compared similar carrier elements (Pedersen et al., 2015; Suhr and
373 Pedersen, 2010). Fixed bed bioreactors are very susceptible to reactor dynamics, especially for the flow
374 velocity (Kumar et al., 2011; Prehn et al., 2012). It is possible that the water velocity in our fixed bed
375 reactors was not optimal and possibly water did not flow uniformly through all the filter media. Prehn et
376 al. (2012) observed that when the water velocity is increased from 4.2 cm min^{-1}, which was the same
377 velocity as in our system, to 66.7 cm min^{-1}, nitrification rates increased three-fold. In addition, the
378 possibility of shunts in the FBBRs could have reduced the nitrification rates, because the by-pass flow
379 may have decreased the active bioreactor surface area.

380 The nitrification rates measured in the laboratory were comparable to the observed nitrogen results
381 from the water quality analyses, and both indicated that FBBR was less effective in the process.
382 Sampling locations of the carrier media might have had some effect on the results, because MBBR has a
383 unifying bacterial consortium throughout the reactor, while in FBBR, the bottom of the reactor can have
384 different communities because of high substrate concentrations (Pérez et al., 2005). However,
385 nitrification rates measured in the laboratory confirmed that nitrification in FBBR was did not work as
386 effectively as in MBBR. Nitrification rates were very consistent in all MBBRs, demonstrating that MBBR is
387 a very stable, reliable and maintenance-free bioreactor type to use.

388 5. Conclusions

389 Here, we demonstrated that nitrification bioreactor design affects RAS water quality, mainly through
390 accumulation of solids and nitrification problems. When using two moving bed bioreactors, the amount
391 of organic matter increased, while with the two fixed bed bioreactors, toxic nitrite accumulated in the
392 circulating water. However, the drum filter compensates for the particle removal in the moving bed
393 bioreactors. This study revealed that no single bioreactor type studied here is more beneficial than any
394 other when rainbow trout growth and health is concerned. However, observed differences on the water
395 quality may lead for selecting one bioreactor type over another. Solids retention capacity of FBBR may
396 even make drum filters unnecessary, thus saving space and installations, which decreases the
397 construction costs. On the other hand, FBBR require constant maintenance, which increases the
398 operational costs. Maintenance free MBBR can save operational costs, but if solids accumulation is
399 causing problems, additional solid treatment system might be needed. There can be also other aspects
400 that can be dependent on the bioreactor type, which were not investigated in this experiment, one
401 being formation of off-flavour compounds. In addition, when both reactor types are in use, changing
402 sequence from FBBR followed by MBBR to MBBR followed by FBBR might highlight best features from
403 both bioreactor types.

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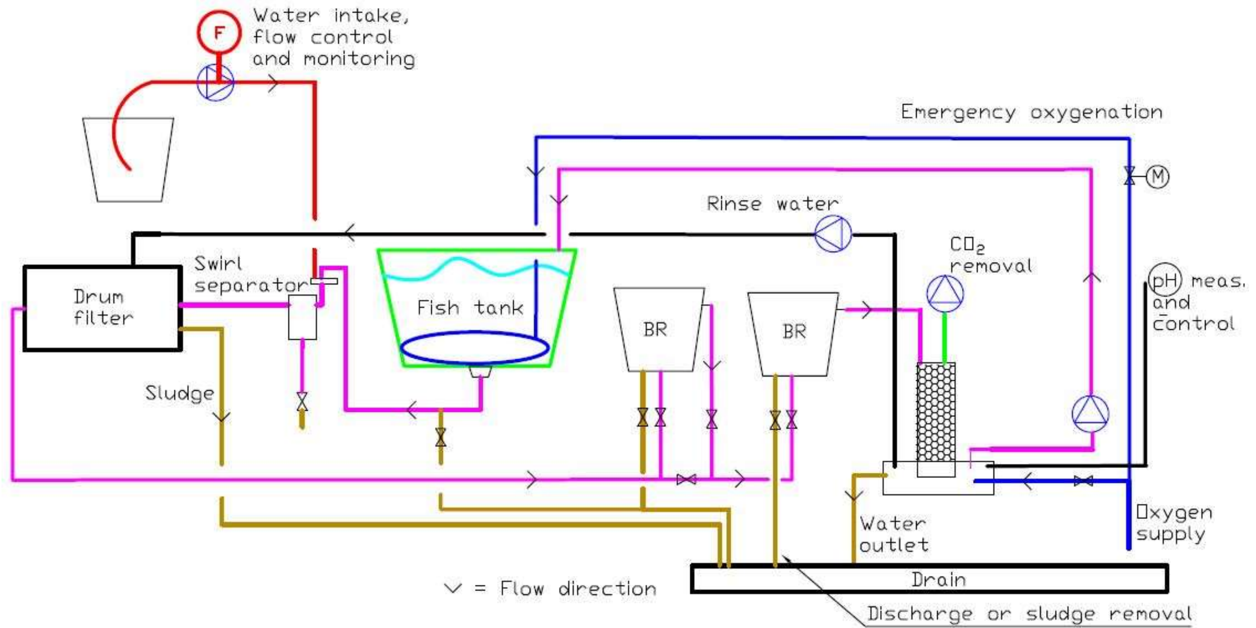
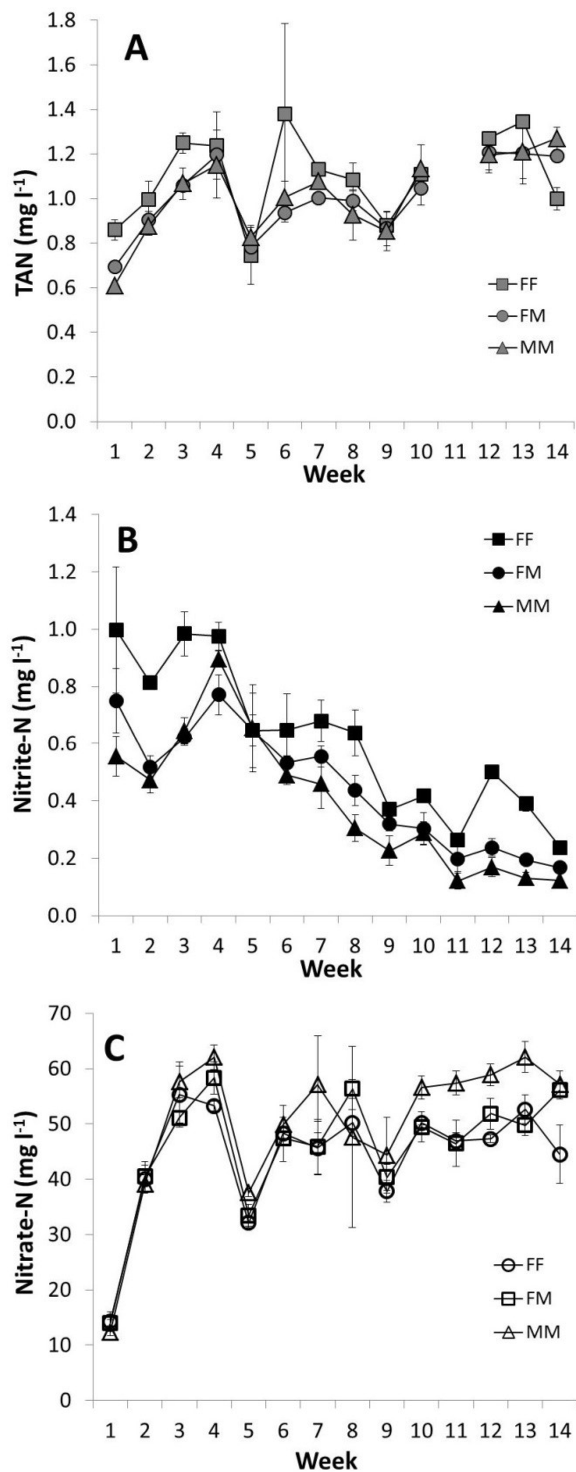


513

514 Figure 1. Schematic diagram of one RAS unit used in this experiment. BR = bioreactor, used as a fixed
515 bed (FBBR) or moving bed bioreactor (MBBR).



516

517 Figure 2. Mean total ammonium nitrogen (TAN) (A), nitrite-nitrogen (B) and nitrate-nitrogen (C) values
518 of the three RAS bioreactor designs ± SD. FF = Two consecutive fixed bed bioreactors (n=3 at weeks 1-8
519 and n=2 at weeks 9-14), FM = Fixed bed bioreactor followed by moving bed bioreactor (n=3) and MM =
520 Two consecutive moving bed bioreactors (n=3).

521 Table 1. RAS operational design and rainbow trout (*Oncorhynchus mykiss*) rearing conditions in the trial,
522 where different setups of fixed bed and moving bed bioreactors were studied.

Characteristics	**Value**	**Unit**
RAS unit (n=9)		
System volume	890	l
Tank volume	500	l
Relative water renewal rate	500	l kg^{-1} feed
Recirculation flow	15	l min^{-1}
Hydraulic retention time	5–8	d
Tank hydraulic retention time	33	min
Rearing conditions		
Fish density	19–82	kg m^{-3}
Feed quantity	0.22–0.45	kg d^{-1}
Average fish size	0.11–0.53	kg
Bioreactor (n=2)		
Bioreactor water volume	125	l
Bioreactor hydraulic retention time	8	min
Carrier media volume	66	l
Carried media area	49.5	m^2
Moving bed bioreactor air flow	15	l min^{-1}
Bioreactor hydraulic loading rate	436	l m^{-2} d^{-1}

523

524 Table 2. Mean rainbow trout (*Oncorhynchus mykiss*) feed conversion ratio (FCR), specific growth rate
525 (SGR) (% bw d^{-1}) and thermal growth coefficient (TGC) (± SD) during the trial (1 = days 0–27, 2 = days 28–
526 55, 3 = days 56–92, 4 = 0–92) of the three RAS bioreactor designs. FF = Two consecutive fixed bed
527 bioreactors, FM = Fixed bed bioreactor followed by moving bed bioreactor and MM = Two consecutive
528 moving bed bioreactors (n=3, except when marked in asterisk, where n=2).

	FCR				SGR				TGC
Treatment	1	2	3	4	1	2	3	4	4
FF	0.85 ± 0.03	0.95 ± 0.04	*1.13 ± 0.03	*0.98 ± 0.01	2.36 ± 0.05	1.56 ± 0.02	*1.03 ± 0.02	*1.59 ± 0.00	*2.20 ± 0.01
FM	0.81 ± 0.01	0.91 ± 0.02	1.07 ± 0.03	0.95 ± 0.01	2.44 ± 0.04	1.58 ± 0.03	1.11 ± 0.03	1.64 ± 0.01	2.28 ± 0.03
MM	0.81 ± 0.02	0.95 ± 0.08	1.05 ± 0.04	0.95 ± 0.02	2.41 ± 0.03	1.54 ± 0.08	1.12 ± 0.02	1.62 ± 0.02	2.22 ± 0.05

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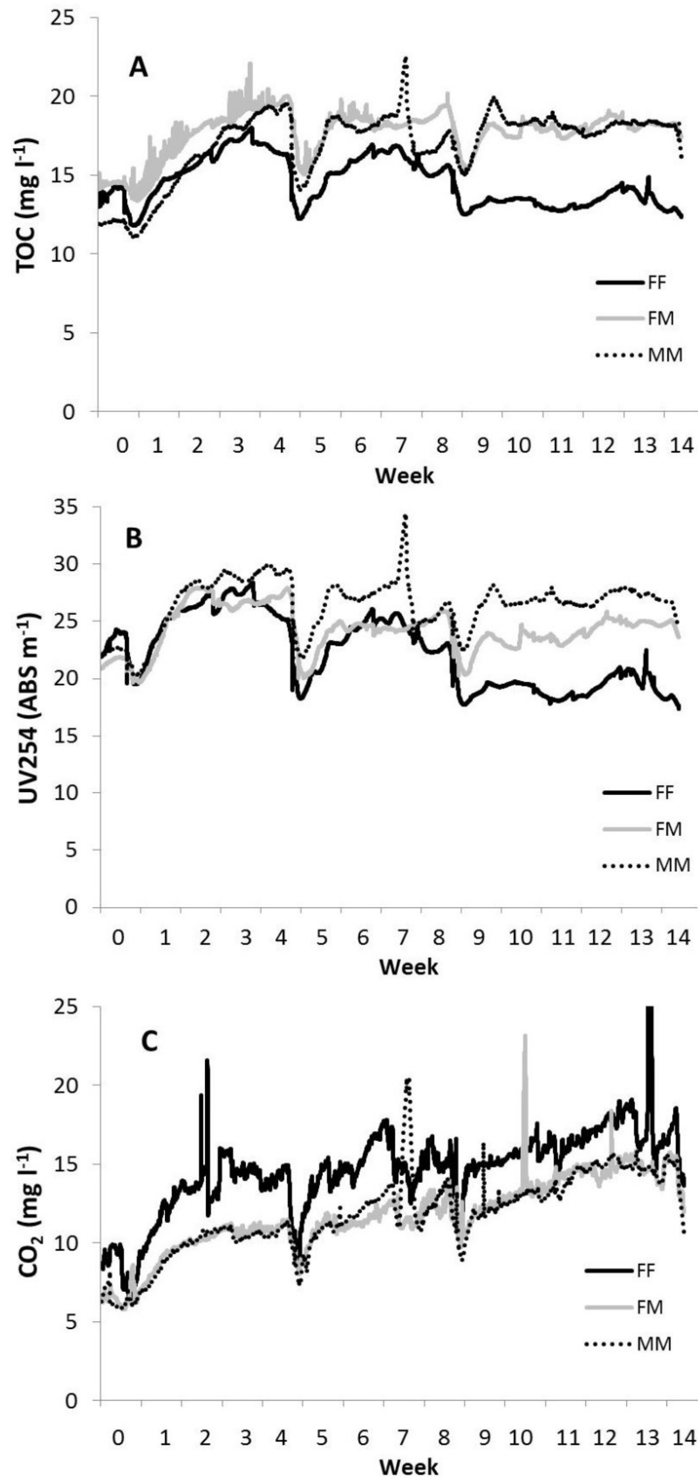


530

531 Figure 3. Mean total organic carbon concentrations (TOC) (A), UV254 absorbance (B) and carbon dioxide
532 concentrations (C) measured online from the fish tank with UV/VIS spectrometer and CO_2 probe of three
533 RAS bioreactor designs. FF = Two consecutive fixed bed bioreactors (n=3 at weeks 1-8 and n=2 at weeks
534 9-14), FM = Fixed bed bioreactor followed by moving bed bioreactor (n=3) and MM = Two consecutive
535 moving bed bioreactors (n=3).

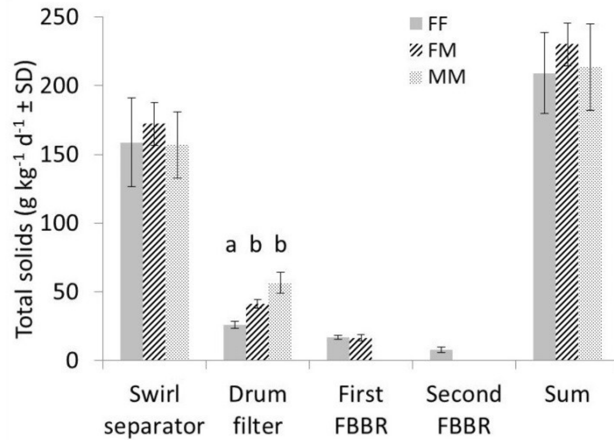
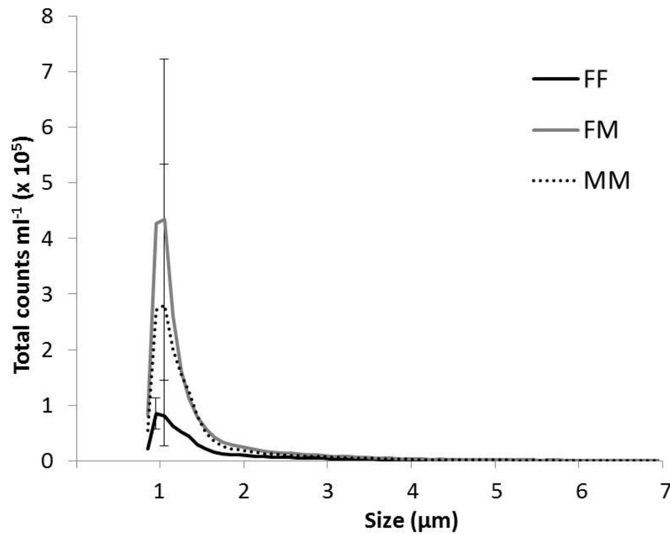


Figure 4. Total solids removed from different water treatment steps proportioned into daily feed intake of the three RAS bioreactor designs. FF = Two consecutive fixed bed bioreactors, FM = Fixed bed bioreactor followed by moving bed bioreactor and MM = Two consecutive moving bed bioreactors (n=3). FBBR = Fixed bed bioreactor. Mean values from two collection periods are presented (± SD). A significant difference between treatments in drum filter backwash water is marked by different letters ($p < 0.01$).

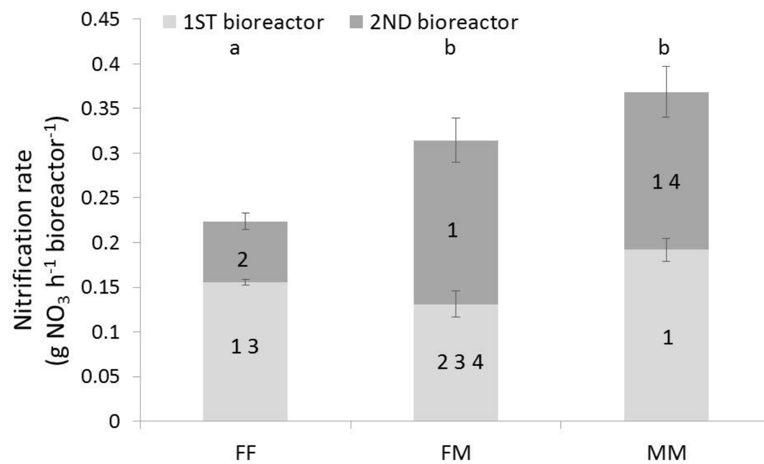
Table 3. Mean β-values, total particle counts, surfaces and volumes (± SD) at two sampling locations of the three RAS bioreactor designs. FF = Two consecutive fixed bed bioreactors (n = 3), FM = Fixed bed bioreactor followed by moving bed bioreactor (n = 3) and MM = Two consecutive moving bed bioreactors (n=3). A significant difference between treatments is marked by different letters ($p < 0.05$).

	Fish tank				After 2nd bioreactor			
	β	Total counts ($1.0x10^3$ pcs ml^{-1})	Total surface area (mm^2 ml^{-1})	Total volume ($1.0x10^{-3}$ mm^3 ml^{-1})	β	Total surface area (mm^2 ml^{-1})	Total counts ($1.0x10^3$ pcs ml^{-1})	Total volume ($1.0x10^{-3}$ mm^3 ml^{-1})
FF	3.7 ± 0.1	39.3 ± 8.3	0.7 ± 0.1	1.4 ± 0.1	4.1 ± 0.1^{a}	0.7 ± 0.2	40.5 ± 10.7	0.7 ± 0.2^{a}
FM	3.7 ± 0.2	45.8 ± 14.1	1.2 ± 0.4	2.0 ± 0.7	3.8 ± 0.2^{ab}	0.9 ± 0.5	35.6 ± 18.9	1.6 ± 0.5^{ab}
MM	3.6 ± 0.2	33.7 ± 13.9	1.0 ± 0.4	2.4 ± 1.0	3.6 ± 0.1^{b}	1.3 ± 0.1	40.9 ± 4.2	3.2 ± 1.0^{b}



549

550 Figure 5. Total particle counts of the three RAS bioreactor designs measured using the CASY cell counter.
551 FF = Two consecutive fixed bed bioreactors (n = 2), FM = Fixed bed bioreactor followed by moving bed
552 bioreactor (n = 3) and MM = Two consecutive moving bed bioreactors (n=3) ; ± SD of the most abundant
553 size classes, which were 1.0 µm for FF and 1.1 µm for FM and MM groups. There were no significant
554 differences between treatments.



555

556 Figure 6. Nitrification rate measured in the three RAS bioreactor designs measured using the stable
557 isotope labelling method. FF = Two consecutive fixed bed bioreactors (n = 2), FM = Fixed bed bioreactor
558 followed by moving bed bioreactor (n = 3) and MM = Two consecutive moving bed bioreactors (n=3). A
559 significant difference between treatments is marked by different letters and between different
560 bioreactors by different numbers ($p < 0.01$).