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The effect of peracetic acid on microbial community, water quality, nitrification and rainbow trout (*Oncorhynchus mykiss*) performance in recirculating aquaculture systems

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1 **The effect of peracetic acid on microbial community, water quality, nitrification and rainbow trout**

2 **(*Oncorhynchus mykiss*) performance in recirculating aquaculture systems**

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10 **Highlights**

11 - The microbial community in biofilters was not affected by frequent peracetic acid (PAA)

12 applications

13 - The highest PAA application frequency improved the water quality by decreasing total ammonium

14 nitrogen values

15 - PAA applications disrupted nitrification, but the nitrifying community was capable of adapting

16 - Fish growth and feed conversion ratio were not significantly affected by the PAA application

17 **Abstract**

18 Microbial biofilters control water quality and enable the overall function of recirculation aquaculture

19 systems (RAS). Changes in environmental conditions can affect the abundance and interactions of

20 the diverse microbial populations of the biofilter, affecting nitrification of harmful ammonium and

21 thus fish health. Here, we examined the effect of different application frequencies (0, 1, 2 and 4

22 times per week) of a common disinfectant, peracetic acid (PAA, applied 1.1 mg l^{-1} twice per day), on

23 biofilter microbial communities, focusing especially on nitrifying microbial groups and using a high

24 throughput sequencing of 16S rRNA gene and quantitative PCR (qPCR). In addition, we measured

25 biofilter nitrification rates, water quality parameters, and fish performance. Although PAA additions

26 did not significantly change the overall microbial community composition or abundance, the

27 abundance of ammonia-oxidizing bacteria (AOB) and nitrate-oxidizing bacteria (NOB) first decreased

28 at the beginning of the experiment but increased in numbers towards the end of the experiment

29 with frequent PAA applications. PAA application decreased the nitrification rate, but increased the

30 water quality in terms of reduced ammonium levels. PAA application did not significantly affect fish

31 growth, but higher mortality was observed with the highest PAA application level of 4 times per

32 week. These results suggest that when applied before the fish tank, PAA can be used for temporary

33 water quality improvement without disturbing microbial communities. However, the application

34 frequency required for persistent water quality improvement caused increased mortality.

35 *Keywords*: 16S rRNA; biofiltration; comammox; nitrification genes; peracetic acid

36 **1. Introduction**

37 Aquaculture is one of the fastest growing food producing sectors (FAO, 2017). Recirculating

38 aquaculture systems (RAS) have lower water consumption and lower nutrient discharge to the

39 environment than the traditional flow-through or net pen farming, making them environmentally

40 and economically sustainable fish-producing systems (Badiola et al., 2012). However, water quality

41 management is crucial for successful RAS performance and fish health, where the bacterial

42 community plays an important role (Blancheton et al., 2013; Rurangwa and Verdegem, 2015).

43 Biofilters play a key role in controlling water quality, hosting microbes that convert the produced

44 ammonium into nitrate nitrogen through nitrification. Traditionally, the nitrification pathway is

45 considered to consist of two steps driven by two different microbial groups: ammonia oxidizers

46 (ammonia-oxidizing bacteria: AOB); and nitrite oxidizers (nitrite-oxidizing bacteria: NOB). Recently,

47 ammonia-oxidizing archaea (AOA) have also been found in biofilters in RAS and aquariums (Bagchi et

48 al., 2014; Bartelme et al., 2017). The nitrification function of the biofilter depends on several water

49 quality factors, e.g. pH, oxygen concentration, and ammonium levels (Chen et al., 2006). Nitrite-

50 oxidizing NOBs are especially sensitive (Villaverde et al., 1997; Graham et al., 2007), which can cause

51 nitrite accumulation after changes in the RAS operating conditions. However, recently, a group of

52 NOB capable of complete ammonia oxidation (comammox), *Nitrospira*, has been identified and

53 found to be quite abundant in RAS biofilters (Bartelme et al., 2017). In addition to nitrifying

54 microbes, biofilters host a diverse heterotrophic microbial community (Bartelme et al., 2017; Rud et

55 al., 2017), that can include harmful micro-organisms (Guttman and van Rijn, 2008; Schrader and

56 Summerfelt, 2010) or microbes producing odorous metabolites (Lukassen et al., 2017; Lindholm-

57 Lehto et al., 2019). Typically, organic matter accumulation (high C:N) in RAS favors heterotrophs,

58 limiting oxygen availability and resulting in lower nitrification rates (Michaud et al., 2006).

59 Peracetic acid (PAA: CH_3CO_3H) is a widely-used and efficient disinfecting agent in aquaculture and

60 wastewater treatment plants (Kitis, 2004; Koivunen and Heinonen-Tanski, 2005; Lahnsteiner and

61 Weismann, 2007). In aquaculture, PAA has been used for disinfection against pathogenic bacteria

62 and other harmful micro-organisms, e.g. crayfish plague spores (Jussila et al., 2011), and for general

63 disinfection to reduce bacterial and potentially bacterial by-product numbers (Kitis, 2004; Liu et al.,

64 2017a). PAA has an optimal degradation time (2 mg L^{-1} to 0 mg L^{-1} in 6 hours) for RAS, and it has no

65 toxic or harmful by-products (Pedersen et al., 2009, Liu et al., 2017a). The commercial PAA products,

66 used in this and in previous studies (Pedersen et al., 2009, Pedersen et al., 2013, Liu et al., 2017a,

67 Davidson et al., 2019), contain microbial disinfectant PAA, and hydrogen peroxide, which degrades

68 the organic material from fish feces and feed, and acetic acid. Previous studies have shown that

69 regular PAA additions of 1 mg L^{-1} can decrease aerobic bacterial density in the rearing water (Liu et

70 al., 2018), but in lower PAA concentrations of 0.1-0.3 mg L^{-1}, bacteria remained unaffected (Davidson

71 et al., 2019). Pulse applications remove bacterial biofilm in the tank wall more efficiently than

72 continuous PAA applications (Liu et al., 2017a). A disadvantage of PAA is its tendency to increase

73 organic matter content in the system due to acetic acid, causing regrowth of microbes and PAA

74   decays (Kitis, 2004; Pedersen et al., 2013). Indeed, continuous low concentration PAA applications

75   can promote biofilm formation and microbial adaptation (Liu et al., 2017a).

76   Another challenge in PAA application is the potential disturbance to the crucial biofilter function.

77   The PAA concentration cannot be too high, since a previous study has shown elevated nitrite levels,

78   i.e. disturbed nitrification with 2 mg L^{-1} and 3 mg L^{-1} of PAA used, while this was not observed with 1

79   mg L^{-1} of PAA (Pedersen et al., 2009). Furthermore, semi-continuous PAA applications (0.3 mg L^{-1})

80   should not affect nitrification negatively (Davidson et al., 2019). In addition to nitrification, fish

81   welfare should be considered when using PAA or any other disinfectant or substance for water

82   quality management in RAS. Fish tolerance to PAA depends on the species (Straus et al., 2018).

83   Rainbow trout (*Oncorhynchus mykiss*) are sensitive to PAA, as the no-observed-effect concentration

84   (NOEC) is 2.8 mg L^{-1}, compared with the more tolerant channel catfish (*Ictalurus punctatus*) (NOEC

85   4.0 mg L^{-1}) and blue tilapia (*Oreochromis aureus*) (NOEC 5.8 mg L^{-1}) (Straus et al., 2018). Exposed to

86   PAA (up to 2 mg L^{-1}), fish have shown stress responses measured by elevated cortisol levels in

87   plasma (Gesto et al., 2018) and in rearing water (Liu et al., 2017b). In addition, increased mucus

88   formation has been reported at similar PAA concentrations (Lindholm-Lehto et al., 2019). However,

89   when PAA exposure has been continued, fish have been able to adapt during the treatment (Gesto

90   et al., 2018; Liu et al., 2017b).

91   Although there are some previous studies of PAA and its effects in RAS, they have focused on

92   general water quality and measured nitrification rates (Pedersen et al., 2009; Pedersen et al., 2013;

93   Liu et al., 2017a; Davidson et al., 2019); little is known about the effects of disinfecting agents on the

94   microbial communities in the RAS biofilter. Since PAA is widely used in RAS, this information is

95   required to understand the stability of the biofilter function during pulse PAA applications and to

96   improve the overall performance of RAS. In this study, the effect of three different pulse application

97   frequencies of PAA (1.1 mg L^{-1} applied twice per day) were studied with a control group. PAA were

98   applied 1, 2 and 4 times per week to the pump sump on a replicated laboratory-scale RAS. We

99   studied the overall biofilter microbiological community composition, assessed with high throughput

100   sequencing, and the total bacterial abundance and genetic nitrification potential (number of

101   nitrification genes), assessed with quantitative PCR (qPCR). In addition, biofilter nitrification rates,

102   water quality parameters, and the fish growth and feed conversion ratio were examined. We

103   hypothesized that the highest application of PAA 4 times per week might have detrimental effects

104   on microbial communities by disturbing the steady-state microbial community and thus disrupting

105   nitrification. In addition, lower applications of PAA once a week were expected to improve water

106   quality and decrease the microbial load in the fish tank, further leading to improved fish

107   performance.

108   **2. Materials and methods**

109   **2.1 Experimental setup**

110   The experiment was performed at the Natural Resources Institute Finland (Luke) Laukaa fish farm,

111   using an experimental RAS platform. The details of the research facility are described in more depth

112   in Pulkkinen et al. (2018). Briefly, eight individual recirculating systems were used, each consisting of

113   a 500 L bottom-drained plastic rearing tank, a feed collector unit, a 24 cm swirl separator (Eco-Trap

114   Collector1, Pentair Aquatic Eco-Systems, Minneapolis, USA), a drum filter with 60 μm filter panels

115   (Hydrotech HDF501, Veolia, Paris, France), a 147 L fixed bed bioreactor (filled with 80 L of RK

116   Biolements heavy, RK Plast A/S, Skive, Denmark), a trickling filter acting as a forced-ventilated

117   cascade aeration column (Bio-Blok® 200, EXPO-NET Danmark A/S, Hjørring, Denmark), and a pump

118   sump (Fig. 1). All RAS units had been used in previous experiments, and the biofilters were stabilized

119   to full maturity. Water pH was adjusted to 7.2 in the pump sump with 20% NaOH (aq), using an

120   automated system (Prominent, Heidelberg, Germany). Oxygen saturation was kept above 80% in the

121   fish tanks, and water temperature around 16 °C. The relative water renewal rate was set to 500 L kg$^-$

122   ^{1} feed, and the tank hydraulic retention time to 33 min.

123   In this thirteen week experiment, three different PAA application frequencies per week were used

124   (1.1 mg PAA L^{-1} in a fish tank dosed twice per day). The experiment is described in more detail in

125   Lindholm-Lehto et al. (2019). Briefly, control tanks and three different PAA applications per week (1,

126   2 and 4) applied in pump sumps were used in replicate tanks. The PAA applications were dosed by

127   adding 4 mL of PAA solutions twice a day. A commercial PAA product was used, composed of 12-13%

128   PAA, 19-23% hydrogen peroxide, and the remainder acetic acid (Bonsoxo 2901, Bang & Bonsomer,

129   Helsinki, Finland).

130   **2.2 Fish and feeding**

131   Three weeks before PAA addition commenced, a total of 400 one-year-old rainbow trout

132   (*Oncorhynchus mykiss*) (average weight 130 g) originating from the National JALO selective breeding

133   programme (Natural Resources Institute Finland, Tervo, Finland) was divided into the eight RAS

134   units. During the first two weeks after PAA addition commenced, mortality between 0-20% was

135   observed in the RAS units, and fish were diagnosed with IPNV (Infectious Pancreatic Necrosis Virus),

136   which may have caused the mortality. After three weeks, fish in poor condition were removed, and

137   biomasses were equalized between the tanks (mean 12.5 kg m^{-3}). Fish were weighed once during the

138   experiment in week 8 by weighing the tank mass and counting the individuals. Feeding was evenly

139   executed with a commercial feeding system (T Drum 2000, Arvo-Tec, Joroinen, Finland) 10-14 times

140   per day in constant light. A 1:1 mixture of two commercial diets was used, Raisioaqua (Circuit Red 5

141   mm, Raisio, Finland) and BioMar (Orbit 929 4.5 mm, Aarhus, Denmark). The crude protein and lipid

142   contents of the diets were 43% and 42%, and 26% and 31%, respectively, as given by the

143   manufacturer. Feed intake was monitored using a feed collector unit, and feeding was decreased if

144   uneaten feed was observed. At the start, the feeding rate was set to 1.5 % bw d^{-1}.

145   **2.3 Sampling for microbiome and qPCR analysis**

146   Biofilter biofilm samples were collected for sequencing and qPCR twice after 8 and 13 weeks of the

147   experiment. Three sets of carrier media were collected from each bioreactor unit. In addition, water

6

148   and biofilm samples from the fish tank were collected for qPCR. Water samples, two per fish tank,

149   were filtered using syringe filters (0.22 µm Millipore Express® PLUS PES membrane). The tank biofilm

150   was collected using detachable tank material blocks (HDPE), which were placed in the tanks at the

151   beginning of the experiment. Prior to further analysis and DNA extraction, samples were frozen (- 20

152   °C) for at least 24 hours. To detach microbial biofilm from the biofilter and tank biofilm samples, 20

153   mL of water was added to the samples, and they were sonicated for 4 min (Branson 1510). They

154   were then freeze-dried (Alpha 1-4 LD plus, Christ). DNA extraction was performed for the freeze-

155   dried materials using the DNeasy PowerLyzer™PowerSoil DNA Isolation Kit (Qiagen) in accordance

156   with the manufacturer's instructions. PowerLyzer Homogenizer was applied once at 3,400 rpm for

157   45 s during the extraction. The quantity of extracted DNA was measured with Qubit™ dsDNA HS

158   assay and Qubit 2.0 Fluorometer (Thermo Fischer Scientific). For sequencing and qPCR, three

159   replicate samples from each biofilter unit or two water samples were pooled.

160   **2.4 Microbial communities**

161   The microbial community composition was studied using Ion Torrent PGM next generation

162   sequencing, targeting the V4 region of the 16S rRNA gene with primer pair 515F-Y

163   (GTGYCAGCMGCCGCGGTAA; Parada et al., 2016) and 806R (GGACTACHVGGGTWTCTAAT; Caporaso

164   et al., 2011). The 25 µl PCR reaction consisted of Maxima SYBR Green/Fluorescein qPCR Master Mix

165   (Thermo Fisher Scientific), 10 ng of template DNA, and 0.4 µM of both primers. Thermal cycling

166   consisted of 10 min initial denaturation at 95 °C, followed by 40 cycles at 95 °C for 30 s, 50 °C for 30 s

167   and 72 °C for 60 s, followed by final elongation at 72 °C for 5 min. To add Ion Torrent PGM

168   sequencing adapters and barcodes to the ends of the PCR product, one µl of the PCR product was

169   used as a template in the second qPCR, where 10 cycles were performed using linker and fusion

170   primers (0.04 µM of M13_515F-Y, 0.4 µM of IonA_IonXpressBarcode_M13 and P1_806R), with

171   conditions otherwise identical to the first amplification (Mäki et al., 2016). Products were purified

172   with the Agencourt AMPure XP purification system (Beckman Coulter Life Sciences, Indianapolis, IN,

173   USA), quantified with Qubit™ dsDNA HS assay, and pooled in equimolar quantities for sequencing on

7

174   Ion Torrent PGM using Ion PGM Hi-Q View OT2 Kit for emulsion PCR, PGM Hi-Q View Sequencing Kit

175   for the sequencing reaction, and Ion 316 Chip v2 (all Life Sciences, Thermo Fisher Scientific). A 16S

176   rRNA gene sequence analysis was done using mothur (version 1.39.5; Schloss et al., 2009), as in

177   Aalto et al. (2018). The total number of sequences obtained was 294,464 and after subsampling,

178   there were 13,245 sequences per sample. The sequences have been submitted to the NCBI

179   Sequence Read Archive under BioProject PRJNA549384.

180   **2.5 Gene copy numbers and nitrification rates**

181   The abundance of 16S rRNA gene in the biofilter biofilm, tank water, and biofilm, as well as the

182   nitrification genes in the biofilter biofilm, was quantified with qPCR. For the 16S rRNA gene, the

183   primer pair 515F-Y and 806R was used and with the amplification protocol details mentioned above.

184   To quantify the abundance of the ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB),

185   and complete ammonia-oxidizing (comammox) bacteria in the biofilter samples, qPCR

186   quantifications were performed using published primer pairs for AOA, AOB, NOB, and comammox.

187   The abundance of AOA was quantified using $amoA_{archaea}$ primers (Francis et al., 2005), and the

188   abundance of AOB using $amoA_{bac}$ primers (Rotthauwe et al., 1997), with the protocols described by

189   Aalto et al. (2018). For NOB abundances, the primers used were *nxr*AF1/R2 (Poly et al., 2008; Wertz

190   et al., 2008) and *nxr*B169F/638R (Pester et al., 2014). Comammox *Nitrospira* clade A and clade B

191   abundances were quantified with the comaA244F/659R and comaB224F/659R primers (Pjevac et al.,

192   2017). All qPCR reactions included 10 ng of template DNA, forward and reverse primers, and 1x

193   Maxima SYBR Green/Fluorescein Master Mix (Thermo Fischer) in a total volume of 25 µl. The

194   thermal conditions were as follows: initial denaturation 10 min at 95 °C, then 35 cycles at 95 °C for

195   30 s, 52-59 °C for 30 s, and 72 °C for 30 s. Amplification efficiencies were between 83-97% for the

196   qPCR assays. The quantification was performed using the CFX96 qPCR thermal cycler (Bio-Rad).

197   Bioreactor nitrification rates (g NO_x h^{-1}) were measured following Pulkkinen et al. (2018) at the end

198   of the experiment. Briefly, 30 biofilter carrier media pieces were incubated with $^{15}NH_4^+$ of 5 mg L^{-1} for

8

199   3 hours, and the stable isotope composition of nitrite and nitrate was measured at the beginning

200   and end of the incubation.

201   **2.6 Water quality measurements**

202   Water quality was monitored with an online monitoring system consisting of a spectrometer probe

203   (spectro::lyser, s::can, Vienna, Austria), a carbon dioxide sensor (Franatech, Lüneburg, Germany), a

204   pH probe (pH::lyser, s::can, Vienna, Austria) and an optical oxygen probe (oxi::lyser, s::can, Vienna,

205   Austria) located in the fish tanks. The spectrometer probe measured turbidity, total suspended

206   solids, and UV254 absorbance. Total ammonia nitrogen (TAN), nitrite, and nitrate were analyzed

207   once a week from tank outlet water using a spectrophotometer (Procedure 8038 Nessler,

208   LCK341/342 and LCK340 respectively, DS 3900, Hach, Loveland, USA). Alkalinity was analyzed once a

209   week by titration with a standard method (ISO 9963-1:1994) (TitraLab AT1000, Hach, Loveland,

210   USA).

211   **2.7 Statistical analyses**

212   The effects of PAA applications on water quality values, feed conversion ratio (FCR), specific growth

213   rate (SGR), mortality and gene copy numbers and microbial community abundances were analyzed

214   with linear regression analysis using SPSS Statistics software, version 25. Gene abundances were log-

215   transformed before analysis to meet assumptions. The changes in the microbial community

216   composition were assessed using the "vegan" (Oksanen et al., 2017) and "phyloseq" (McMurdie and

217   Holmes 2013) packages in R (version 3.5.1).

218   **3. Results**

219   **3.1 Biofilter microbial community**

220   Based on the NMDS plot, the microbial community composition changed from week 8 to week 13 in

221   all tanks. At week 8, communities in control units and those receiving four PAA applications per

222   week were more similar than those with one or two PAA applications, which were more dispersed,

223   while at week 13, the communities were quite similar in all PAA application levels (Fig. 2). The

224   dominant phylum across all samples was Proteobacteria (28-44% of sequences) (Fig. 3). The next

225   most abundant OTUs were from phyla Gemmatimonadetes (5-19% of sequences) and Bacteroidetes

226   (20-45% of sequences). Although the abundance of proteobacterial classes Deltaproteobacteria (8%

227   vs 6-7% of all sequences) and Gammaproteobacteria (12% vs 7-10% of all sequences) decreased

228   slightly with an increased PAA application rate (Fig. 3), the overall abundance of Proteobacteria was

229   unaffected by the PAA application frequency (linear regression, $P>0.05$). The species richness

230   (Chao1) increased from week 8 to week 13 except in the other one-time PAA application per week

231   unit. The diversity generally increased from week 8 to 13, and was higher in control units than in

232   units with PAA applications (Suppl. Fig. 1).

233   When only nitrifying taxonomic groups were considered (Fig. 3/Suppl. Fig. 2), the number of

234   sequences assigned to AOB (family Nitrosomonadaceae) appeared to decrease with the increasing

235   PAA application frequencies at week 8 (117-235 reads, 0.7-1.3% of all sequences), but the difference

236   was not statistically significant ($P=0.052$). At week 13, the amount of AOB reads was very similar

237   between PAA application frequencies (95-244 reads, 0.5-1.4% of all sequences; $P=0.703$). The main

238   genera were unclassified Nitrosomonadaceae (weeks 8, 13; 26-74% of AOB reads) and *Nitrosomonas*

239   (week 13; 13-42% of AOB sequences). The number of sequences assigned to NOB/comammox

240   (genera *Nitrospira*, *Nitrobacter*) was 0.4-3.5% of all sequences (79-623 reads) at week 8, while at

241   week 13, it was 1.6-6.9% (284-1,232 reads). The PAA application frequency did not affect the

242   number of NOB/comammox sequences ($P>0.05$). *Nitrospira* was the dominant genus in all samples

243   (90-100% of NOB sequences).

244   **3.2 Microbial abundance and nitrification rates**

245   The total amount of bacteria (16S rRNA gene copy numbers) in tank water was equal between the

246   control and different PAA application frequencies at both weeks ($P>0.05$). At week 8, the total

247   amount of bacteria was $2.7\pm2.2\times10^{9}$ gene copies cm^{-2} in tank biofilm and $7.8\pm6.3\times10^{11}$ gene copies g^{-}

10

248   ^{1} dw in biofilters, while at week 13, there were $1.4\pm1.5\times10^9$ gene copies cm^{-2} in tank biofilm and

249     $9.4\pm5.9\times10^{10}$ gene copies g^{-1} dw in biofilters (Fig. 4). There was no significant relationship between

250   total microbial abundances and PAA application frequencies neither in tank nor in biofilter biofilms.

251   In biofilter samples, the copy numbers of nitrification genes were normalized against the total

252   bacterial abundance. The relative abundance of the AOB gene was higher than the NOB or

253   comammox genes, and ammonia-oxidizing archaea were not found in the samples. All the

254   comammox *Nitrospira* were from clade A, while no comammox bacteria from clade B were observed

255   in the samples. At week 8, the relative gene copy numbers of AOB and NOB appeared to decrease

256   with an increasing PAA application rate, but the relationship was not significant (P=0.092) for AOB

257   and for NOB. The abundance of comammox bacteria remained similar in all systems (P>0.05). At

258   week 13, at the end of the experiment, the relative abundance of all three nitrifying groups was

259   higher than at week 8. The AOB copy numbers were equal between the units, while NOB abundance

260   was highest in the units receiving bi-weekly PAA application, and comammox abundance in the

261   control units and those receiving PAA four times per week (Fig. 5). Nitrification rates (g NO$_3$ h^{-1}

262   bioreactor^{-1}) were highest in tanks with one PAA application per week (Fig. 6). With PAA applications

263   of two and four times per week, the nitrification rate decreased and was lowest, at 0.09 g NO$_3$ h^{-1}

264   bioreactor $^{-1}$, in tanks with four PAA applications per week (Fig. 6).

265   **3.3 Water quality and fish performance**

266   The turbidity and total suspended solids were lower in the 2 x PAA and 4 x PAA application rates but

267   there was no significant relationship with PAA application frequency (Table 2, Suppl. Fig. 3; P=0.057).

268   Higher PAA application frequency significantly decreased TAN concentrations during the first part of

269   the experiment (P<0.01, weeks 3-8) and during the whole experiment (P=0.026, weeks 3-13; Table

270   2). PAA additions did not significantly increase NO$_2$-N concentrations (P>0.05), and NO$_3$-N

271   concentrations were similar between the RAS units (P>0.05, Table 1). In addition, the PAA

272   application frequencies did not significantly affect fish performance when measured by FCR and SGR

11

273 (Table 1), but the fish in the units receiving PAA four times per week had a 15% slower growth

274 compared to fish in the control groups. However, mortality increased with increasing PAA

275 application frequencies (P<0.01, Table 1; Table 2).

4. Discussion

277 In this study, we demonstrated the effect of different PAA application frequencies on biofilter

278 microbiome function and composition, especially on the key nitrification microbes. We also

279 examined the changes in biofilter nitrification rates, water quality, and fish performance in response

280 to PAA application frequency. We observed that the overall microbial community composition

281 remained quite stable, and nitrification bacteria did not substantially suffer from PAA applications,

282 increasing in abundance during the experiment. More frequent PAA application decreased biofilter

283 nitrification rates but yet could decrease the TAN values. PAA application frequencies did not

284 significantly affect fish growth, but a higher mortality rate and increased slime formation were

285 observed with the highest PAA application (four times per week).

286 Until this study, knowledge of the response of the biofilter microbiome to PAA applications has been

287 scarce. In a few previous studies, a focus on the effect of a bi-weekly pulsed PAA addition of 1 mg L^{-1}

288 on RAS microbiology has been found to decrease the overall bacterial counts in the tank water and

289 to nearly completely remove the biofilm in the fish tanks in RAS (Liu et al., 2017a, 2018), while

290 enhanced biofilm formation has been observed under continuous PAA applications, which has been

291 explained by the acetic acid and formed acetate feeding the heterotrophic microbial community (Liu

292 et al., 2017a). Furthermore, when a prolonged low PAA dosage has been used, no effect on

293 microbial counts has been observed, resulting in a minimum PAA threshold estimate of 0.30 mg L^{-1}

294 for disinfectant purposes in RAS (Davidson et al., 2019). In agreement, we observed that the

295 abundance of tank water microbes, either free-living or attached to particles, was unaffected by a

296 PAA addition. However, when weeks were inspected separately, we observed that bacterial

297 abundance was higher, yet statistically insignificant, in the units receiving a PAA application one or

298 four times per week in both tank and biofilter biofilms at week 8. In the tanks at week 13, the PAA

299 application rate had no overall effect on bacterial biofilm, because the bi-weekly PAA application

300 rate was sufficient to decrease the bacterial biofilm, but the highest PAA application seemed to

301 promote biofilm formation. In the biofilters, PAA had no clear effect on the biofilm bacterial

302 abundance. It is likely that most of the PAA used was degraded before entering the biofilters, since it

303 was added to the pump sump, explaining the minor beneficial and restricting effects of the PAA on

304 the biofilm in the biofilters than in the tanks.

305 The dominating phylum was Proteobacteria in all RAS units throughout the experiment, while

306 Bacteroidetes and Gemmatimonadetes were also relatively abundant. Previously, Proteobacteria

307 and Bacteroidetes have found to be common phyla in RAS biofilter units in both freshwater and

308 saltwater RAS, while Gemmatimonadetes were either absent or found in low abundance (Ruan et

309 al., 2015; Gonzales-Silva et al., 2016; Bartelme et al., 2017; Rud et al., 2017), highlighting the unique

310 nature of microbiomes in each biofilter and RAS unit (Blancheton et al., 2013). In previous studies,

311 *Nitrospira* has been found to be quite abundant in RAS biofilters (Bartelme et al., 2017; Keuter et al.,

312 2017), the group including both traditional nitrite-oxidizers and comammox bacteria. Here, we found

313 the amount of *Nitrospira* increasing from week 8 to week 13 in all units. Overall, PAA application

314 frequency did not significantly change the biofilm bacterial community composition or decrease the

315 diversity or species richness, agreeing with previous findings on perturbations affecting free-living

316 microbes rather than deep biofilm layers (Wietz et al., 2009; Schreier et al., 2010).

317 When nitrifying microbes are the sole focus, both qPCR and sequencing results demonstrated that

318 PAA application frequency had a slightly, yet statistically insignificant, negative effect on the nitrifier

319 abundance at week 8. At week 13, genetic nitrification potential (gene copy numbers) and the

320 sequences associated with NOBs/comammox increased compared to week 8 in all units. This

321 indicates that the possibly negative effect of PAA application on the nitrification microbes decreased

322 towards the end of the experiments, probably because the nitrification community could adapt and

323 become less sensitive to PAA application. Previously, PAA additions have been found to only partially

324 inhibit nitrification (Pedersen et al., 2009, Liu et al., 2017a), causing some accumulation of total

325 ammonia nitrogen (TAN) and nitrate in RAS. Here, we measured nitrification rates using stable

326 isotope incubations at the end of the experiment, and found higher PAA application frequency led to

327 lower nitrification rates. However, we did not find the previously observed nitrite accumulation,

328 suggesting that both ammonium oxidation and nitrite oxidation steps were suppressed, or that the

329 abundant comammox bacteria were as or even more involved in ammonia oxidation than the

330 traditional ammonia-oxidizers, releasing only nitrate as the end product.

331 In addition to changes in nitrification, the increased PAA application rate led to decreased turbidity

332 and total suspended solids concentrations except in units with a PAA addition of twice a week,

333 where values were closer to control units than other PAA application units. Decreased turbidity

334 indicates that there were fewer large particles (Yao et al., 2014) because of the higher H_2O_2

335 degradation potential of organic matter. The observed decrease of total ammonia nitrogen with an

336 increasing PAA addition was unexpected, because nitrification efficiency was lowest within the

337 highest PAA addition. The direct oxidation of TAN is the likeliest explanation, because the slightly

338 different daily feed consumption did not explain the difference in TAN concentrations between

339 treatments. TAN values have previously been found to increase after a bi-weekly PAA addition due

340 to lower nitrification rates (Liu et al., 2017a), and we also observed that the units with bi-weekly PAA

341 applications behaved quite differently from those receiving one or four applications. There, we saw

342 equal turbidity, TSS, TOC, TAN, and nitrate values with the control units, suggesting that this bi-

343 weekly PAA addition promoted heterotrophic microbes more than removing them or improving

344 water quality. However, one reason for the difference between bi-weekly and other PAA application

345 frequencies is that the other 2x RAS unit (unit 2) showed a very different pattern in water quality

346 and microbial results than the other systems. Furthermore, we saw that AOB and comammox

347 microbes were less abundant in these systems, while the NOBs seemed to benefit there. In this

348 experiment, PAA dosages were kept the same throughout the experiment, and feeding was

349 increased from the first (mean 102 g d^{-1}) to the second (mean 127 g d^{-1}) part of the experiment.

350 Thus, the potential PAA effect time decreased during the experiment (Pedersen et al., 2013), which

351 explained why the water quality differences between the treatments were higher in the first part of

352 the experiment.

353 The PAA additions did not significantly affect fish performance in terms of feed conversion ratio or

354 specific growth rate. However, the total biomass growth was 14% lower in the units where PAA was

355 added four times per week compared to other units. In addition, mortality increased with the

356 highest PAA addition, indicating that continued applications of PAA (1.1 mg L^{-1} dosed twice per day),

357 even below the reported no-observed-effect concentration (2.8 mg L^{-1}) (Straus et al., 2018), were

358 too high for the fish. High surface swimming was observed with the first additions of PAA, but this

359 was not observed after a few weeks of additions, indicating the adaptation of fish to PAA as

360 previously reported (Liu et al., 2017b; Gesto et al., 2018). The observed IPNV has low pathogenicity

361 in Finland and mainly affects the first-feeding fry (Eriksson-Kallio et al., 2016). We thus concluded

362 that it did not affect fish performance.

363 **5. Conclusions**

364 In conclusion, the microbial results suggest that the biofilter biofilm community is quite stable and

365 less sensitive to PAA application, forming a "collaborome" in which heterotrophic microbes can

366 support autotrophic nitrifiers rather than compete with them (Bartelme et al., 2017). Pulsed PAA

367 applications disrupt nitrification, but the microbial community is capable of adapting and no long-

368 term effect of PAA on inorganic nitrogen levels can be observed. Our study demonstrates that PAA

369 application frequency has variable effects on microbes, water quality, and fish. Although the highest

370 PAA application frequency (1.1 mg L^{-1} twice a day, four times per week) improved water quality

371 slightly by directly oxidizing TAN, and potentially turbidity, and TSS, it led to 50% lower biofilter

372 nitrification rates compared to the control units, and increased fish mortality. On the other hand, a

373 bi-weekly application did not improve water quality, but fish performance was better than with

374   other PAA application frequencies. Similarly, a one-time PAA application was too low to improve

375   water quality, but did not interrupt nitrification or fish. The opposite chemical (i.e. direct oxidizing of

376   TAN) and biological (i.e. decreasing nitrification thus increasing TAN) effects of PAA on water quality

377   can complicate the interpretation of results. Based on these results, the continuous pulse

378   applications of PAA are not a cost-efficient method for substantially improving water quality and

379   controlling the microbial communities. However, if an accumulation of solids and/or total ammonia

380   nitrogen is observed, PAA can be used in such cases to improve water quality.

387   **Author contributions**

388   All the authors planned the experiment. SS, JTP, and PL-L did the sampling and SS performed the

389   DNA sequencing and qPCR measurements. SLA performed the statistical analyses of the microbial

390   data, and JTP the water quality and fish performance analysis. All the authors contributed to

391   manuscript writing.

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536   Figures and tables

537   Figure legends

538   Figure 1. Schematic diagram of one RAS unit used in the experiment. Microbial samples were taken

539   from the fish tank biofilm, water from fish tank and from the fixed bed bioreactor. PAA was dosed

540   into the pump sump. FT = Fish tank, 1 = Swirl separator, 2 = drum filter, FBBR = Fixed bed bioreactor,

541   TF = Trickling filter, 3 = Pump sump.

542   Figure 2. Non-metric multidimensional scaling (NMDS) of bacterial communities in RAS biofilters

543   under different PAA application frequencies (control, 1, 2, and 4 applications per week) at weeks 8

544   and 13.

545   Figure 3. The relative abundances of microbial phyla in biofilter biofilms under different PAA

546   application frequencies (control, 1, 2, and 4 applications per week) at weeks 8 and 13.

547   Figure 4. The abundance of bacterial 16S rRNA gene in tank water, in tank biofilm and in biofilter

548   biofilm under different PAA application frequencies (control, 1, 2, and 4 applications per week).

549   Abundance denotes gene copies ml^{-1} water for tank water samples, gene copies cm^{-2} for tank biofilm

550   samples, and gene copies g^{-1} dw for biofilter biofilm samples.

551   Figure 5. The relative abundances of AOB (ammonia-oxidizing bacteria), NOB (nitrite-oxiding

552   bacteria) and comammox clade A genes in biofilter under different PAA application frequencies

553   (control, 1, 2, and 4 applications per week).

554   Figure 6. The biofilter nitrification rates under different PAA application frequencies (control, 1, 2,

555   and 4 applications per week) at week 13 at the end of the experiment.

556   Table legends

557   Table 1. The mean water quality parameters and observed fish performance under different PAA

558   application treatments (0, 1, 2, and 4 applications per week, PAA dosed 1.1 mg L^{-1} twice per day) ±

559   SD. FCR = Feed conversion ratio, SGR = Specific growth rate.

560   Table 2. The linear regression models on the interactions between water quality parameters and

561   different PAA application frequencies (0, 1, 2 and 4 applications per week). Data is shown, when p <

562   0.1. TSS = total suspended solids, TAN = total ammonia nitrogen.
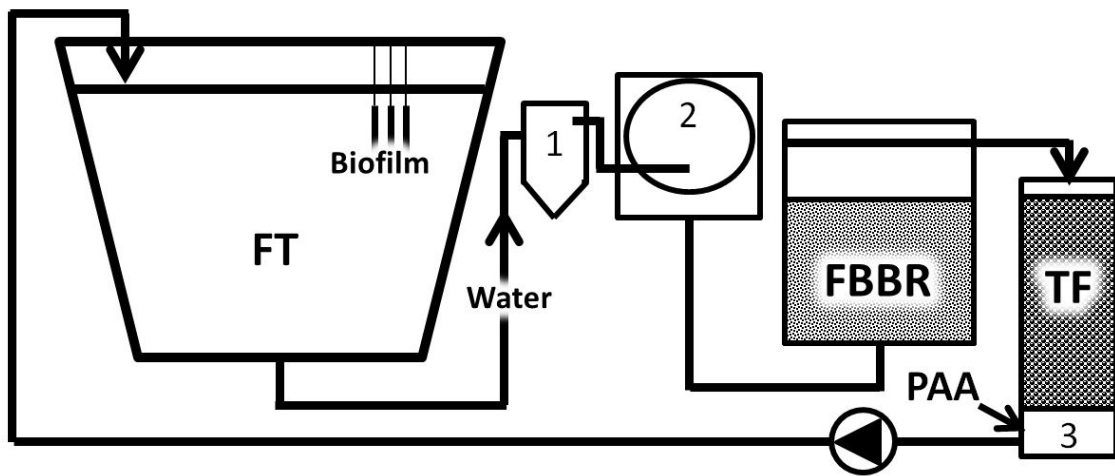
563

564   Figure 1. Schematic diagram of one RAS unit used in the experiment. Microbial samples were taken

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566   into the pump sump. FT = Fish tank, 1 = Swirl separator, 2 = drum filter, FBBR = Fixed bed bioreactor,

567   TF = Trickling filter, 3 = Pump sump.
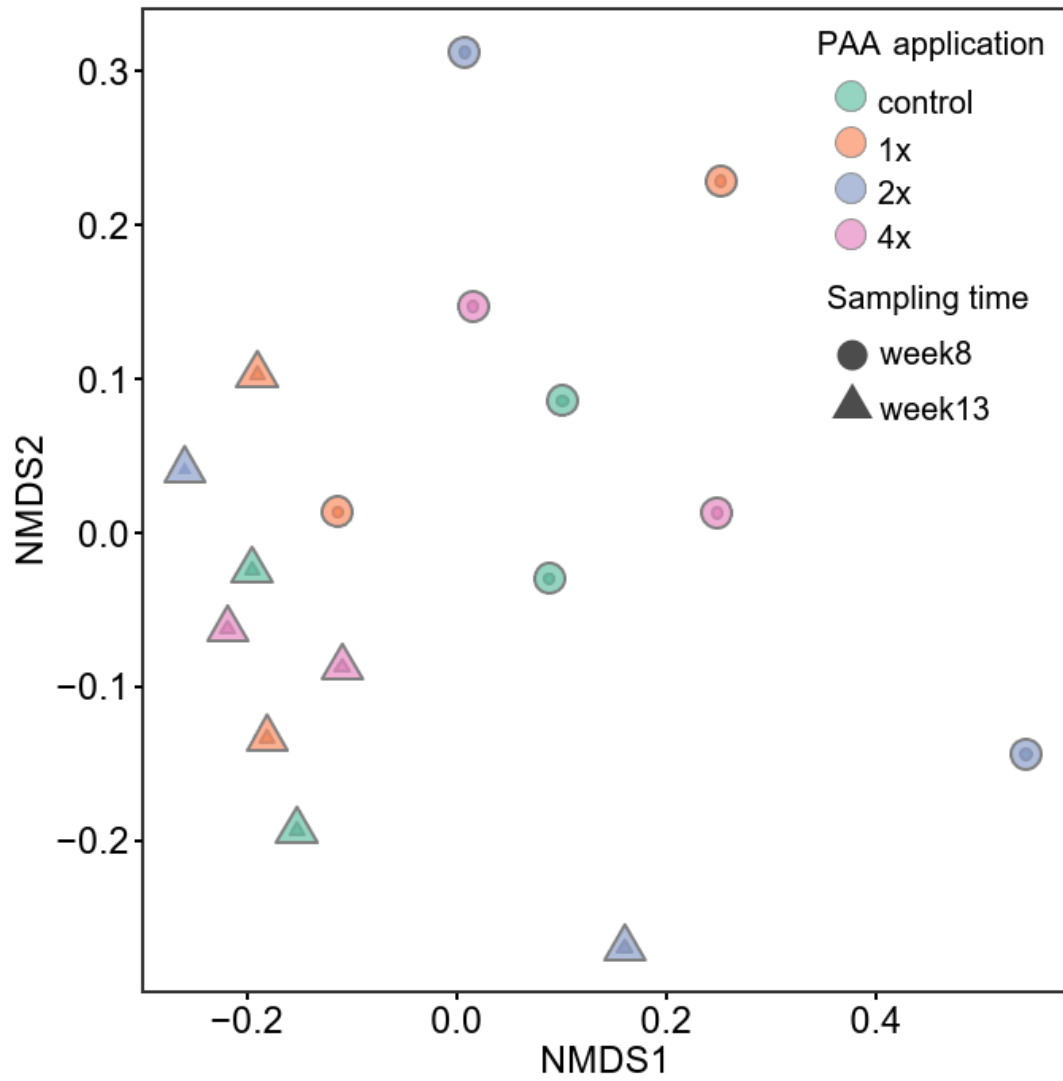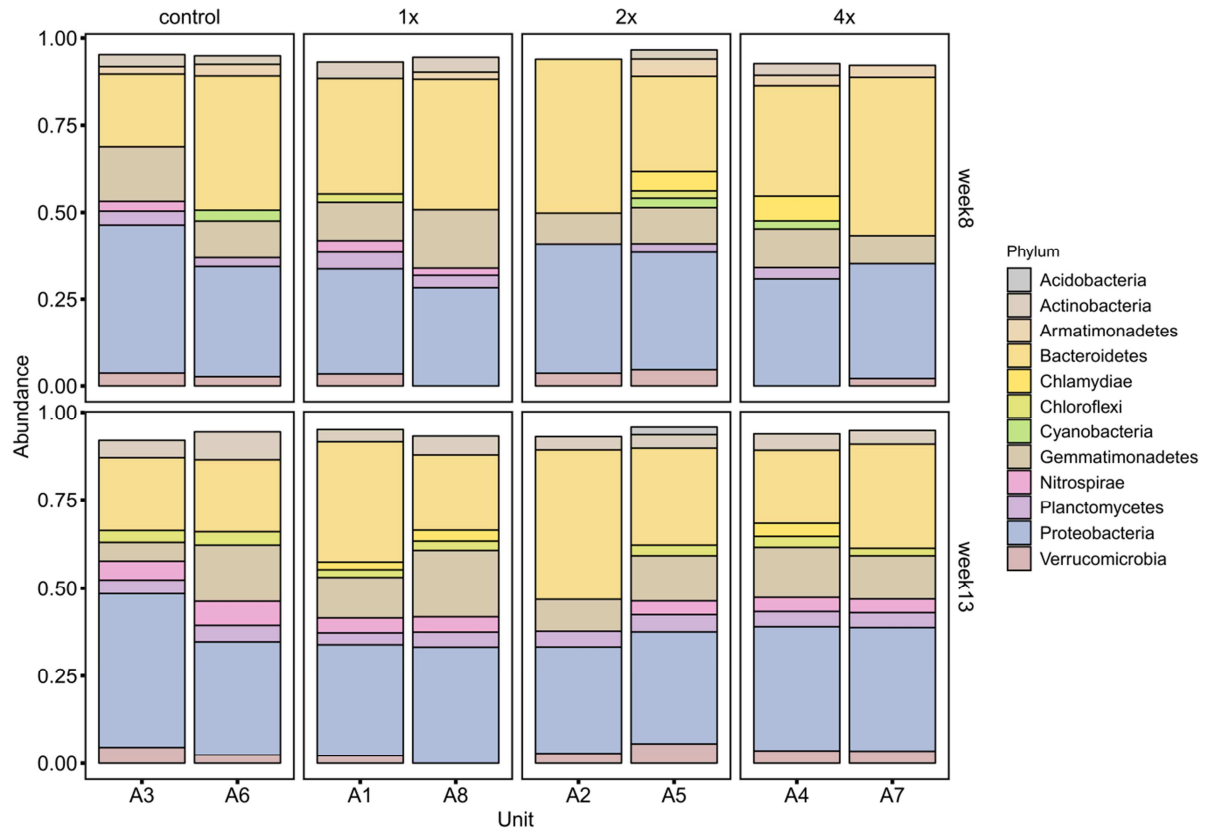
568

569   Figure 2. Non-metric multidimensional scaling (NMDS) of bacterial communities in RAS biofilters

570   under different PAA application frequencies (control, 1, 2, and 4 applications per week) at weeks 8
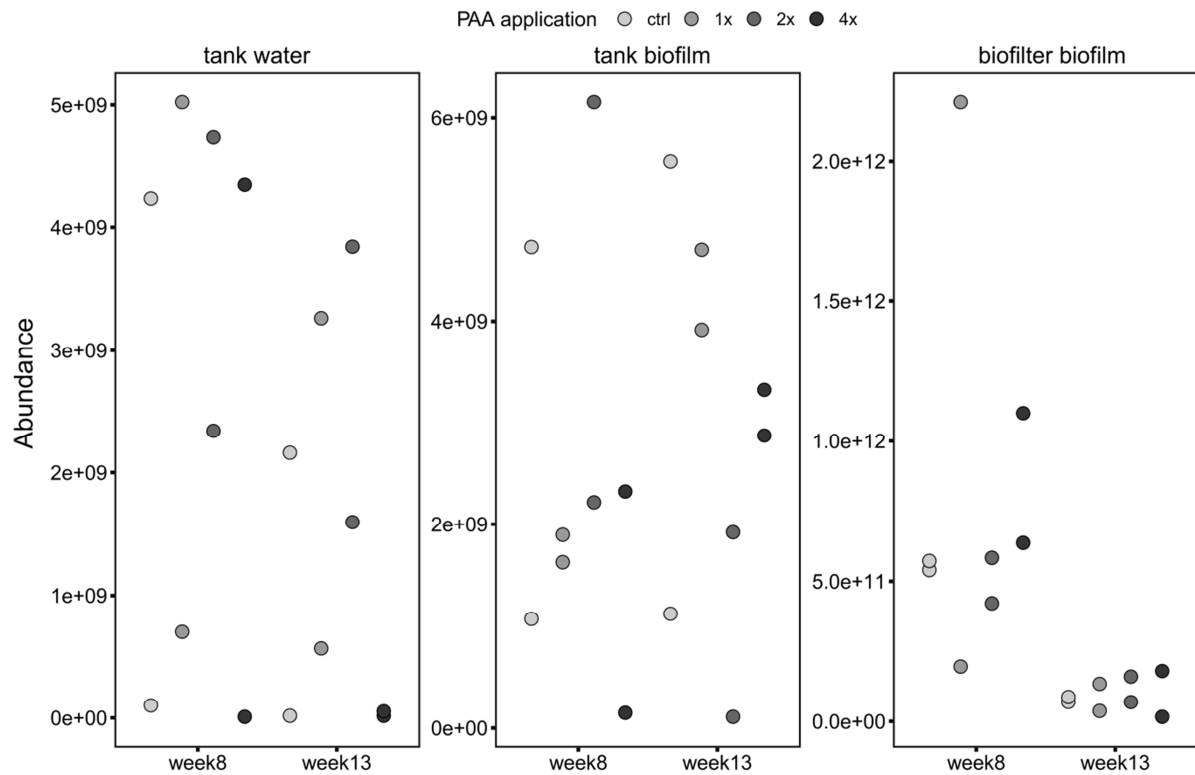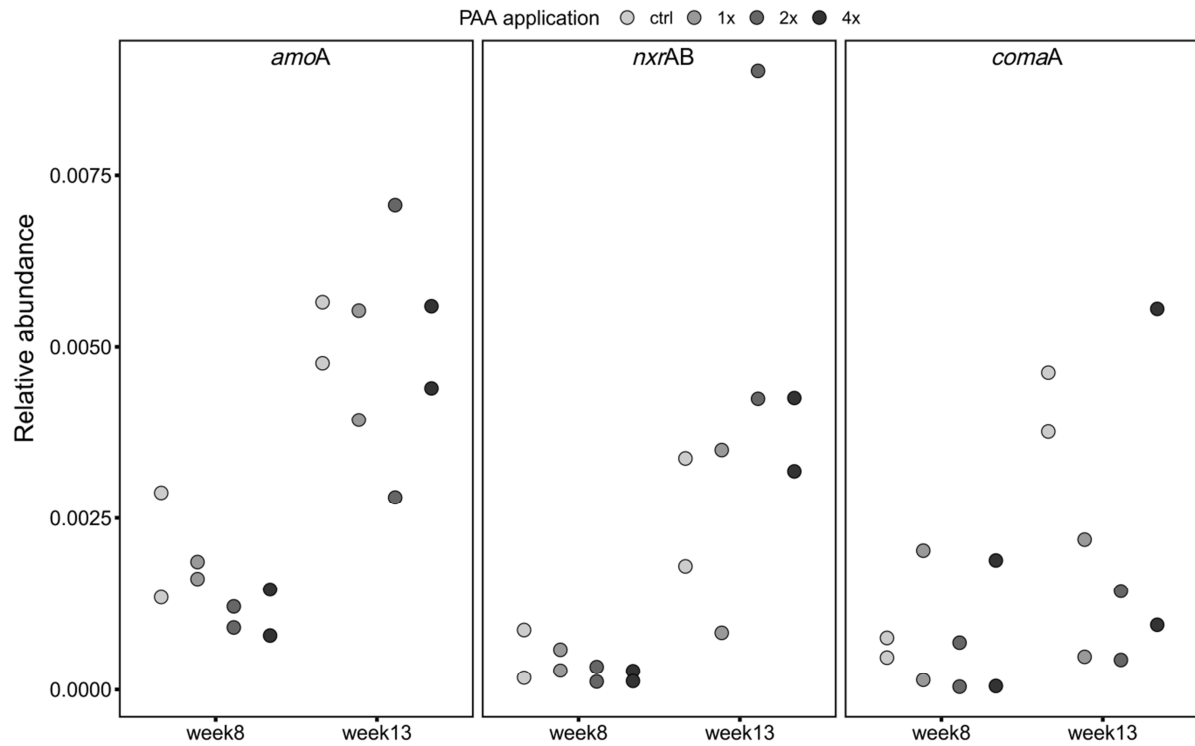
571   and 13.

572

573   Figure 3. The relative abundances of microbial phyla in biofilter biofilms under different PAA

574   application frequencies (control, 1, 2, and 4 applications per week) at weeks 8 and 13.

575

Figure 4. The abundance of the bacterial 16S rRNA gene in tank water, in tank biofilm and in biofilter

biofilm under different PAA application frequencies (control, 1, 2, and 4 applications per week).

Abundance denotes gene copies ml^{-1} water for tank water samples, gene copies cm^{-2} for tank biofilm

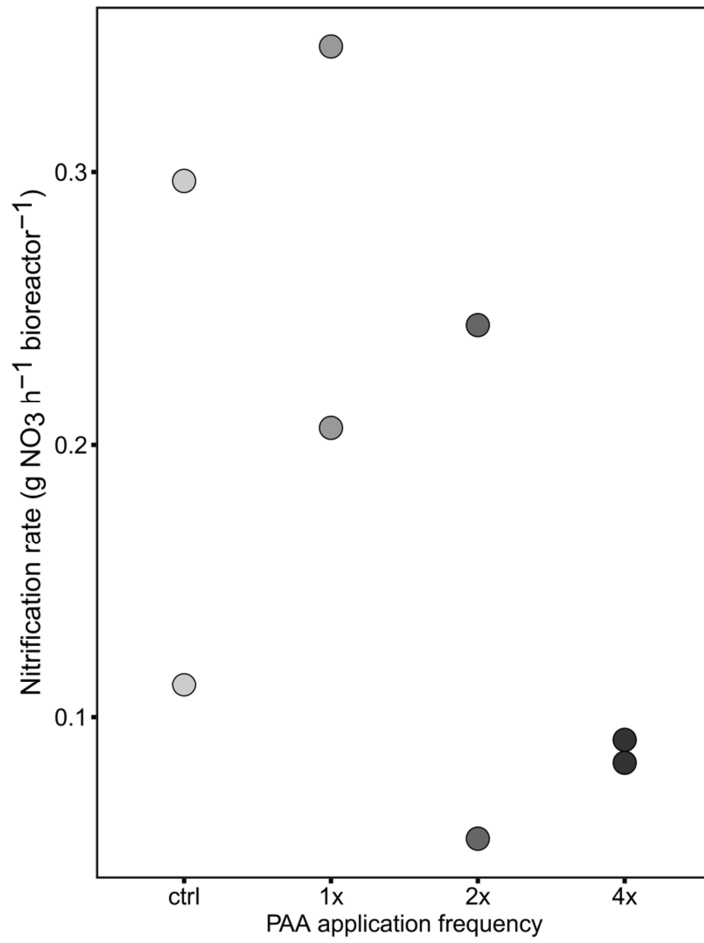samples, and gene copies g^{-1} dw for biofilter biofilm samples.

580
581   Figure 5. The relative abundances of AOB (ammonia-oxidizing bacteria), NOB (nitrite-oxiding

582   bacteria), and comammox (clade A) genes in biofilter under different PAA application frequencies

583   (control, 1, 2, and 4 applications per week).

584

585 Figure 6. The biofilter nitrification rates under different PAA application frequencies (control, 1, 2,

586 and 4 applications per week) at week 13 at the end of the experiment.

587 Table 1. The mean water quality parameters and observed fish performance under different PAA application treatments (0, 1, 2, and 4 applications per

588 week, PAA dosed 1.1 mg L^{-1} twice per day) ± SD. FCR = Feed conversion ratio, SGR = Specific growth rate.

589

Treatment	Mean of weeks 3-7 (±SD)				Mean of weeks 8-13 (±SD)				Mean of weeks 3-13 (±SD)			
	0	1	2	4	0	1	2	4	0	1	2	4
CO_2 (mg l^{-1})	8.9 ± 0.9	8.7 ± 0.4	7.4 ± 1.5	8.9 ± 0.5	11.0 ± 1.8	9.8 ± 0.6	8.9 ± 2.0	10.6 ± 0.8	9.8 ±1.3	9.2 ± 0.5	8.0 ± 1.6	9.6 ± 0.8
UV254 (Abs m^{-1})	29.7 ± 4.2	26.5 ± 2.4	30.2 ± 1.1	24.0 ± 0.1	29.6 ± 5.7	27.4 ± 2.4	30.6 ± 0.2	26.4 ± 0.9	29.7 ± 4.8	26.9 ± 0.4	30.2 ± 0.9	25.0 ± 0.9
Turbidity (FTU)	2.0 ± 0.3	1.4 ± 0.2	1.8 ± 0.1	1.1 ± 0.3	1.8 ± 0.2	1.4 ± 0.1	1.9 ± 0.0	1.2 ± 0.1	1.9 ± 0.2	1.4 ± 0.1	1.9 ± 0.0	1.2 ± 0.1
TSS (mg l^{-1})	5.8 ± 0.4	3.9 ± 0.6	5.1 ± 0.5	3.0 ± 1.0	5.2 ± 0.3	3.9 ± 0.5	5.2 ± 0.1	3.1 ± 0.7	5.5 ± 0.4	3.9 ± 0.2	5.2 ± 0.2	3.0 ± 0.7
TAN (mg l^{-1})	0.93 ± 0.11	0.78 ± 0.08	0.78 ± 0.01	0.57 ± 0.01	0.80 ± 0.1	0.70 ± 0.15	0.80 ± 0.05	0.61 ± 0.04	0.83 ± 0.09	0.75 ± 0.05	0.78 ± 0.04	0.58 ± 0.04
NO_2-N (mg l^{-1})	0.11 ± 0.01	0.12 ± 0.02	0.10 ± 0.01	0.10 ± 0.1	0.13 ± 0.02	0.17 ± 0.04	0.13 ± 0.04	0.10 ± 0.02	0.12 ± 0.01	0.15 ± 0.02	0.12 ± 0.03	0.10 ± 0.02
NO_3-N (mg l^{-1})	54.6 ± 0.6	52.1 ± 4.2	56.8 ± 4.0	48.1 ± 4.6	60.2 ± 2.3	61.1 ± 9.7	65.5 ± 4.5	54.8 ± 3.5	55.5 ± 1.5	55.4 ± 7.4	57.5 ± 1.8	48.7 ± 3.5
Alkalinity (mg l^{-1})	62.3 ± 1.3	57.2 ± 2.3	42.7 ± 14.5	71.5 ± 2.6	63.1 ± 6.6	51.7 ± 1.6	45.9 ± 17.5	69.4 ± 3.3	62.1 ± 3.9	53.0 ± 1.4	44.3 ± 16.1	68.5 ± 3.3
pH	7.3 ± 0.0	7.3 ± 0.1	7.1 ± 0.1	7.3 ± 0.0	7.3 ± 0.1	7.3 ± 0.1	7.1 ± 0.1	7.4 ± 0.0	7.3 ± 0.0	7.2 ± 0.1	7.1 ± 0.1	7.4 ± 0.0
FCR	1.11 ± 0.1	0.92 ±0.14	1.01 ± 0.14	1.21 ±0.0	1.01 ± 0.05	1.12 ± 0.11	1.08 ±0.01	1.11 ± 0.02	1.05 ± 0.02	1.02 ± 0.12	1.04 ±0.07	1.16 ±0.02
SGR (% d^{-1})	1.35 ± 0.18	1.32 ± 0.14	1.45 ± 0.06	1.28 ± 0.03	1.08 ± 0.05	1.04 ± 0.04	1.06 ± 0.0	1.03 ± 0.03	1.23 ± 0.07	1.19 ± 0.09	1.27 ± 0.04	1.17 ± 0.03
End weight (g)	303 ± 32	286 ± 22	308 ± 33	286 ± 9	428 ± 39	401 ± 36	431 ± 46	384 ± 12	428 ±39	401 ± 36	431 ± 46	384 ± 12
Mortality (%)	2.9 ± 0.0	2.9 ± 2.9	2.9 ± 2.9	7.1 ± 1.4	2.9 ± 0.0	4.4 ± 1.3	4.4 ± 1.3	4.6 ± 0.0	5.7 ±0.0	7.1 ± 1.4	7.1 ± 1.4	11.4 ± 0.0

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590 Table 2. The linear regression models on the interactions between water quality parameters and

591 different PAA application frequencies (0, 1, 2, and 4 applications per week). Data is shown when p <

592 0.1. TSS = total suspended solids, TAN = total ammonia nitrogen.

Variable	Week	Equation	R^2	p value
Turbidity	3-8	y = -2.465 x + 5.724	0.46	0.064
TSS	3-8	y = -0.800 x + 5.291	0.46	0.067
TAN	3-8	y = -8.709 x + 8.412	0.73	0.007
TSS	8-13	y = -1.008 x + 6.136	0.42	0.079
Turbidity	3-13	y = -2.900 x + 6.390	0.48	0.057
TSS	3-13	y = -0.945 x + 5.921	0.48	0.057
TAN	3-13	y = -10.370 x + 9.398	0.59	0.026
Mortality	3-13	y = 58.33 x - 2.917	0.70	0.010

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Conflict of Interest

The authors declare that there is no conflict of interest