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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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Highlights

- The microbial community in biofilters was not affected by frequent peracetic acid (PAA) applications
- The highest PAA application frequency improved the water quality by decreasing total ammonium nitrogen values
- PAA applications disrupted nitrification, but the nitrifying community was capable of adapting
- Fish growth and feed conversion ratio were not significantly affected by the PAA application

Abstract

Microbial biofilters control water quality and enable the overall function of recirculation aquaculture systems (RAS). Changes in environmental conditions can affect the abundance and interactions of the diverse microbial populations of the biofilter, affecting nitrification of harmful ammonium and thus fish health. Here, we examined the effect of different application frequencies (0, 1, 2 and 4 times per week) of a common disinfectant, peracetic acid (PAA, applied 1.1 mg l^{-1} twice per day), on

biofilter microbial communities, focusing especially on nitrifying microbial groups and using a high throughput sequencing of 16S rRNA gene and quantitative PCR (qPCR). In addition, we measured biofilter nitrification rates, water quality parameters, and fish performance. Although PAA additions did not significantly change the overall microbial community composition or abundance, the abundance of ammonia-oxidizing bacteria (AOB) and nitrate-oxidizing bacteria (NOB) first decreased at the beginning of the experiment but increased in numbers towards the end of the experiment with frequent PAA applications. PAA application decreased the nitrification rate, but increased the water quality in terms of reduced ammonium levels. PAA application did not significantly affect fish growth, but higher mortality was observed with the highest PAA application level of 4 times per week. These results suggest that when applied before the fish tank, PAA can be used for temporary water quality improvement without disturbing microbial communities. However, the application frequency required for persistent water quality improvement caused increased mortality.

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

1. Introduction

Aquaculture is one of the fastest growing food producing sectors (FAO, 2017). Recirculating aquaculture systems (RAS) have lower water consumption and lower nutrient discharge to the environment than the traditional flow-through or net pen farming, making them environmentally and economically sustainable fish-producing systems (Badiola et al., 2012). However, water quality management is crucial for successful RAS performance and fish health, where the bacterial community plays an important role (Blancheton et al., 2013; Rurangwa and Verdegem, 2015).

Biofilters play a key role in controlling water quality, hosting microbes that convert the produced ammonium into nitrate nitrogen through nitrification. Traditionally, the nitrification pathway is considered to consist of two steps driven by two different microbial groups: ammonia oxidizers (ammonia-oxidizing bacteria: AOB); and nitrite oxidizers (nitrite-oxidizing bacteria: NOB). Recently, ammonia-oxidizing archaea (AOA) have also been found in biofilters in RAS and aquariums (Bagchi et

al., 2014; Bartelme et al., 2017). The nitrification function of the biofilter depends on several water quality factors, e.g. pH, oxygen concentration, and ammonium levels (Chen et al., 2006). Nitrite-oxidizing NOBs are especially sensitive (Villaverde et al., 1997; Graham et al., 2007), which can cause nitrite accumulation after changes in the RAS operating conditions. However, recently, a group of NOB capable of complete ammonia oxidation (comammox), *Nitrospira*, has been identified and found to be quite abundant in RAS biofilters (Bartelme et al., 2017). In addition to nitrifying microbes, biofilters host a diverse heterotrophic microbial community (Bartelme et al., 2017; Rud et al., 2017), that can include harmful micro-organisms (Guttman and van Rijn, 2008; Schrader and Summerfelt, 2010) or microbes producing odorous metabolites (Lukassen et al., 2017; Lindholm-Lehto et al., 2019). Typically, organic matter accumulation (high C:N) in RAS favors heterotrophs, limiting oxygen availability and resulting in lower nitrification rates (Michaud et al., 2006).

Peracetic acid (PAA: CH_3CO_3H) is a widely-used and efficient disinfecting agent in aquaculture and wastewater treatment plants (Kitis, 2004; Koivunen and Heinonen-Tanski, 2005; Lahnsteiner and Weismann, 2007). In aquaculture, PAA has been used for disinfection against pathogenic bacteria and other harmful micro-organisms, e.g. crayfish plague spores (Jussila et al., 2011), and for general disinfection to reduce bacterial and potentially bacterial by-product numbers (Kitis, 2004; Liu et al., 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 toxic or harmful by-products (Pedersen et al., 2009, Liu et al., 2017a). The commercial PAA products, used in this and in previous studies (Pedersen et al., 2009, Pedersen et al., 2013, Liu et al., 2017a, Davidson et al., 2019), contain microbial disinfectant PAA, and hydrogen peroxide, which degrades the organic material from fish feces and feed, and acetic acid. Previous studies have shown that regular PAA additions of 1 mg L^{-1} can decrease aerobic bacterial density in the rearing water (Liu et al., 2018), but in lower PAA concentrations of 0.1-0.3 mg L^{-1}, bacteria remained unaffected (Davidson et al., 2019). Pulse applications remove bacterial biofilm in the tank wall more efficiently than continuous PAA applications (Liu et al., 2017a). A disadvantage of PAA is its tendency to increase organic matter content in the system due to acetic acid, causing regrowth of microbes and PAA

decays (Kitis, 2004; Pedersen et al., 2013). Indeed, continuous low concentration PAA applications can promote biofilm formation and microbial adaptation (Liu et al., 2017a).

Another challenge in PAA application is the potential disturbance to the crucial biofilter function. The PAA concentration cannot be too high, since a previous study has shown elevated nitrite levels, i.e. disturbed nitrification with 2 mg L^{-1} and 3 mg L^{-1} of PAA used, while this was not observed with 1 mg L^{-1} of PAA (Pedersen et al., 2009). Furthermore, semi-continuous PAA applications (0.3 mg L^{-1}) should not affect nitrification negatively (Davidson et al., 2019). In addition to nitrification, fish welfare should be considered when using PAA or any other disinfectant or substance for water quality management in RAS. Fish tolerance to PAA depends on the species (Straus et al., 2018). Rainbow trout (*Oncorhynchus mykiss*) are sensitive to PAA, as the no-observed-effect concentration (NOEC) is 2.8 mg L^{-1}, compared with the more tolerant channel catfish (*Ictalurus punctatus*) (NOEC 4.0 mg L^{-1}) and blue tilapia (*Oreochromis aureus*) (NOEC 5.8 mg L^{-1}) (Straus et al., 2018). Exposed to PAA (up to 2 mg L^{-1}), fish have shown stress responses measured by elevated cortisol levels in plasma (Gesto et al., 2018) and in rearing water (Liu et al., 2017b). In addition, increased mucus formation has been reported at similar PAA concentrations (Lindholm-Lehto et al., 2019). However, when PAA exposure has been continued, fish have been able to adapt during the treatment (Gesto et al., 2018; Liu et al., 2017b).

Although there are some previous studies of PAA and its effects in RAS, they have focused on general water quality and measured nitrification rates (Pedersen et al., 2009; Pedersen et al., 2013; Liu et al., 2017a; Davidson et al., 2019); little is known about the effects of disinfecting agents on the microbial communities in the RAS biofilter. Since PAA is widely used in RAS, this information is required to understand the stability of the biofilter function during pulse PAA applications and to improve the overall performance of RAS. In this study, the effect of three different pulse application frequencies of PAA (1.1 mg L^{-1} applied twice per day) were studied with a control group. PAA were applied 1, 2 and 4 times per week to the pump sump on a replicated laboratory-scale RAS. We

studied the overall biofilter microbiological community composition, assessed with high throughput sequencing, and the total bacterial abundance and genetic nitrification potential (number of nitrification genes), assessed with quantitative PCR (qPCR). In addition, biofilter nitrification rates, water quality parameters, and the fish growth and feed conversion ratio were examined. We hypothesized that the highest application of PAA 4 times per week might have detrimental effects on microbial communities by disturbing the steady-state microbial community and thus disrupting nitrification. In addition, lower applications of PAA once a week were expected to improve water quality and decrease the microbial load in the fish tank, further leading to improved fish performance.

2. Materials and methods

2.1 Experimental setup

The experiment was performed at the Natural Resources Institute Finland (Luke) Laukaa fish farm, using an experimental RAS platform. The details of the research facility are described in more depth in Pulkkinen et al. (2018). Briefly, eight individual recirculating systems were used, each consisting of a 500 L bottom-drained plastic rearing tank, a feed collector unit, a 24 cm swirl separator (Eco-Trap Collector1, Pentair Aquatic Eco-Systems, Minneapolis, USA), a drum filter with 60 µm filter panels (Hydrotech HDF501, Veolia, Paris, France), a 147 L fixed bed bioreactor (filled with 80 L of RK Biolements heavy, RK Plast A/S, Skive, Denmark), a trickling filter acting as a forced-ventilated cascade aeration column (Bio-Blok® 200, EXPO-NET Danmark A/S, Hjørring, Denmark), and a pump sump (Fig. 1). All RAS units had been used in previous experiments, and the biofilters were stabilized to full maturity. Water pH was adjusted to 7.2 in the pump sump with 20% NaOH (aq), using an automated system (Prominent, Heidelberg, Germany). Oxygen saturation was kept above 80% in the fish tanks, and water temperature around 16 °C. The relative water renewal rate was set to 500 L kg^{-1} feed, and the tank hydraulic retention time to 33 min.

In this thirteen week experiment, three different PAA application frequencies per week were used (1.1 mg PAA L^{-1} in a fish tank dosed twice per day). The experiment is described in more detail in Lindholm-Lehto et al. (2019). Briefly, control tanks and three different PAA applications per week (1, 2 and 4) applied in pump sumps were used in replicate tanks. The PAA applications were dosed by adding 4 mL of PAA solutions twice a day. A commercial PAA product was used, composed of 12-13% PAA, 19-23% hydrogen peroxide, and the remainder acetic acid (Bonsoxo 2901, Bang & Bonsomer, Helsinki, Finland).

2.2 Fish and feeding

Three weeks before PAA addition commenced, a total of 400 one-year-old rainbow trout (*Oncorhynchus mykiss*) (average weight 130 g) originating from the National JALO selective breeding programme (Natural Resources Institute Finland, Tervo, Finland) was divided into the eight RAS units. During the first two weeks after PAA addition commenced, mortality between 0-20% was observed in the RAS units, and fish were diagnosed with IPNV (Infectious Pancreatic Necrosis Virus), which may have caused the mortality. After three weeks, fish in poor condition were removed, and biomasses were equalized between the tanks (mean 12.5 kg m^{-3}). Fish were weighed once during the experiment in week 8 by weighing the tank mass and counting the individuals. Feeding was evenly executed with a commercial feeding system (T Drum 2000, Arvo-Tec, Joroinen, Finland) 10-14 times per day in constant light. A 1:1 mixture of two commercial diets was used, Raisioaqua (Circuit Red 5 mm, Raisio, Finland) and BioMar (Orbit 929 4.5 mm, Aarhus, Denmark). The crude protein and lipid contents of the diets were 43% and 42%, and 26% and 31%, respectively, as given by the manufacturer. Feed intake was monitored using a feed collector unit, and feeding was decreased if uneaten feed was observed. At the start, the feeding rate was set to 1.5 % bw d^{-1}.

2.3 Sampling for microbiome and qPCR analysis

Biofilter biofilm samples were collected for sequencing and qPCR twice after 8 and 13 weeks of the experiment. Three sets of carrier media were collected from each bioreactor unit. In addition, water

and biofilm samples from the fish tank were collected for qPCR. Water samples, two per fish tank, were filtered using syringe filters (0.22 µm Millipore Express® PLUS PES membrane). The tank biofilm was collected using detachable tank material blocks (HDPE), which were placed in the tanks at the beginning of the experiment. Prior to further analysis and DNA extraction, samples were frozen (- 20 °C) for at least 24 hours. To detach microbial biofilm from the biofilter and tank biofilm samples, 20 mL of water was added to the samples, and they were sonicated for 4 min (Branson 1510). They were then freeze-dried (Alpha 1-4 LD plus, Christ). DNA extraction was performed for the freeze-dried materials using the DNeasy PowerLyzer™PowerSoil DNA Isolation Kit (Qiagen) in accordance with the manufacturer's instructions. PowerLyzer Homogenizer was applied once at 3,400 rpm for 45 s during the extraction. The quantity of extracted DNA was measured with Qubit™ dsDNA HS assay and Qubit 2.0 Fluorometer (Thermo Fischer Scientific). For sequencing and qPCR, three replicate samples from each biofilter unit or two water samples were pooled.

2.4 Microbial communities

The microbial community composition was studied using Ion Torrent PGM next generation sequencing, targeting the V4 region of the 16S rRNA gene with primer pair 515F-Y (GTGYCAGCMGCCGCGGTAA; Parada et al., 2016) and 806R (GGACTACHVGGGTWTCTAAT; Caporaso et al., 2011). The 25 µl PCR reaction consisted of Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Fisher Scientific), 10 ng of template DNA, and 0.4 µM of both primers. Thermal cycling consisted of 10 min initial denaturation at 95 °C, followed by 40 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s, followed by final elongation at 72 °C for 5 min. To add Ion Torrent PGM sequencing adapters and barcodes to the ends of the PCR product, one µl of the PCR product was used as a template in the second qPCR, where 10 cycles were performed using linker and fusion primers (0.04 µM of M13_515F-Y, 0.4 µM of IonA_IonXpressBarcode_M13 and P1_806R), with conditions otherwise identical to the first amplification (Mäki et al., 2016). Products were purified with the Agencourt AMPure XP purification system (Beckman Coulter Life Sciences, Indianapolis, IN, USA), quantified with Qubit™ dsDNA HS assay, and pooled in equimolar quantities for sequencing on

Ion Torrent PGM using Ion PGM Hi-Q View OT2 Kit for emulsion PCR, PGM Hi-Q View Sequencing Kit for the sequencing reaction, and Ion 316 Chip v2 (all Life Sciences, Thermo Fisher Scientific). A 16S rRNA gene sequence analysis was done using mothur (version 1.39.5; Schloss et al., 2009), as in Aalto et al. (2018). The total number of sequences obtained was 294,464 and after subsampling, there were 13,245 sequences per sample. The sequences have been submitted to the NCBI Sequence Read Archive under BioProject PRJNA549384.

2.5 Gene copy numbers and nitrification rates

The abundance of 16S rRNA gene in the biofilter biofilm, tank water, and biofilm, as well as the nitrification genes in the biofilter biofilm, was quantified with qPCR. For the 16S rRNA gene, the primer pair 515F-Y and 806R was used and with the amplification protocol details mentioned above.

To quantify the abundance of the ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), and complete ammonia-oxidizing (comammox) bacteria in the biofilter samples, qPCR quantifications were performed using published primer pairs for AOA, AOB, NOB, and comammox. The abundance of AOA was quantified using $amoA_{archaea}$ primers (Francis et al., 2005), and the abundance of AOB using $amoA_{bac}$ primers (Rotthauwe et al., 1997), with the protocols described by Aalto et al. (2018). For NOB abundances, the primers used were *nxr*AF1/R2 (Poly et al., 2008; Wertz et al., 2008) and *nxr*B169F/638R (Pester et al., 2014). Comammox *Nitrospira* clade A and clade B abundances were quantified with the comaA244F/659R and comaB224F/659R primers (Pjevac et al., 2017). All qPCR reactions included 10 ng of template DNA, forward and reverse primers, and 1x Maxima SYBR Green/Fluorescein Master Mix (Thermo Fischer) in a total volume of 25 µl. The thermal conditions were as follows: initial denaturation 10 min at 95 °C, then 35 cycles at 95 °C for 30 s, 52-59 °C for 30 s, and 72 °C for 30 s. Amplification efficiencies were between 83-97% for the qPCR assays. The quantification was performed using the CFX96 qPCR thermal cycler (Bio-Rad).

Bioreactor nitrification rates (g NO_x h^{-1}) were measured following Pulkkinen et al. (2018) at the end of the experiment. Briefly, 30 biofilter carrier media pieces were incubated with $^{15}NH_4^+$ of 5 mg L^{-1} for

3 hours, and the stable isotope composition of nitrite and nitrate was measured at the beginning and end of the incubation.

2.6 Water quality measurements

Water quality was monitored with an online monitoring system consisting of a spectrometer probe (spectro::lyser, s::can, Vienna, Austria), a carbon dioxide sensor (Franatech, Lüneburg, Germany), a pH probe (pH::lyser, s::can, Vienna, Austria) and an optical oxygen probe (oxi::lyser, s::can, Vienna, Austria) located in the fish tanks. The spectrometer probe measured turbidity, total suspended solids, and UV254 absorbance. Total ammonia nitrogen (TAN), nitrite, and nitrate were analyzed once a week from tank outlet water using a spectrophotometer (Procedure 8038 Nessler, LCK341/342 and LCK340 respectively, DS 3900, Hach, Loveland, USA). Alkalinity was analyzed once a week by titration with a standard method (ISO 9963-1:1994) (TitraLab AT1000, Hach, Loveland, USA).

2.7 Statistical analyses

The effects of PAA applications on water quality values, feed conversion ratio (FCR), specific growth rate (SGR), mortality and gene copy numbers and microbial community abundances were analyzed with linear regression analysis using SPSS Statistics software, version 25. Gene abundances were log-transformed before analysis to meet assumptions. The changes in the microbial community composition were assessed using the “vegan” (Oksanen et al., 2017) and “phyloseq” (McMurdie and Holmes 2013) packages in R (version 3.5.1).

3. Results

3.1 Biofilter microbial community

Based on the NMDS plot, the microbial community composition changed from week 8 to week 13 in all tanks. At week 8, communities in control units and those receiving four PAA applications per week were more similar than those with one or two PAA applications, which were more dispersed,

while at week 13, the communities were quite similar in all PAA application levels (Fig. 2). The dominant phylum across all samples was Proteobacteria (28-44% of sequences) (Fig. 3). The next most abundant OTUs were from phyla Gemmatimonadetes (5-19% of sequences) and Bacteroidetes (20-45% of sequences). Although the abundance of proteobacterial classes Deltaproteobacteria (8% vs 6-7% of all sequences) and Gammaproteobacteria (12% vs 7-10% of all sequences) decreased slightly with an increased PAA application rate (Fig. 3), the overall abundance of Proteobacteria was unaffected by the PAA application frequency (linear regression, $P>0.05$). The species richness (Chao1) increased from week 8 to week 13 except in the other one-time PAA application per week unit. The diversity generally increased from week 8 to 13, and was higher in control units than in units with PAA applications (Suppl. Fig. 1).

When only nitrifying taxonomic groups were considered (Fig. 3/Suppl. Fig. 2), the number of sequences assigned to AOB (family Nitrosomonadaceae) appeared to decrease with the increasing PAA application frequencies at week 8 (117-235 reads, 0.7-1.3% of all sequences), but the difference was not statistically significant ($P=0.052$). At week 13, the amount of AOB reads was very similar between PAA application frequencies (95-244 reads, 0.5-1.4% of all sequences; $P=0.703$). The main genera were unclassified Nitrosomonadaceae (weeks 8, 13; 26-74% of AOB reads) and *Nitrosomonas* (week 13; 13-42% of AOB sequences). The number of sequences assigned to NOB/comammox (genera *Nitrospira*, *Nitrobacter*) was 0.4-3.5% of all sequences (79-623 reads) at week 8, while at week 13, it was 1.6-6.9% (284-1,232 reads). The PAA application frequency did not affect the number of NOB/comammox sequences ($P>0.05$). *Nitrospira* was the dominant genus in all samples (90-100% of NOB sequences).

3.2 Microbial abundance and nitrification rates

The total amount of bacteria (16S rRNA gene copy numbers) in tank water was equal between the control and different PAA application frequencies at both weeks ($P>0.05$). At week 8, the total 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^{-}

^{1} dw in biofilters, while at week 13, there were 1.4±1.5×10^{9} gene copies cm^{-2} in tank biofilm and 9.4±5.9×10^{10} gene copies g^{-1} dw in biofilters (Fig. 4). There was no significant relationship between total microbial abundances and PAA application frequencies neither in tank nor in biofilter biofilms. In biofilter samples, the copy numbers of nitrification genes were normalized against the total bacterial abundance. The relative abundance of the AOB gene was higher than the NOB or comammox genes, and ammonia-oxidizing archaea were not found in the samples. All the comammox *Nitrospira* were from clade A, while no comammox bacteria from clade B were observed in the samples. At week 8, the relative gene copy numbers of AOB and NOB appeared to decrease with an increasing PAA application rate, but the relationship was not significant (P=0.092) for AOB and for NOB. The abundance of comammox bacteria remained similar in all systems (P>0.05). At week 13, at the end of the experiment, the relative abundance of all three nitrifying groups was higher than at week 8. The AOB copy numbers were equal between the units, while NOB abundance was highest in the units receiving bi-weekly PAA application, and comammox abundance in the control units and those receiving PAA four times per week (Fig. 5). Nitrification rates (g NO_3 h^{-1} $bioreactor^{-1}$) were highest in tanks with one PAA application per week (Fig. 6). With PAA applications of two and four times per week, the nitrification rate decreased and was lowest, at 0.09 g NO_3 h^{-1} $bioreactor^{-1}$, in tanks with four PAA applications per week (Fig. 6).

3.3 Water quality and fish performance

The turbidity and total suspended solids were lower in the 2 x PAA and 4 x PAA application rates but there was no significant relationship with PAA application frequency (Table 2, Suppl. Fig. 3; P=0.057). Higher PAA application frequency significantly decreased TAN concentrations during the first part of the experiment (P<0.01, weeks 3-8) and during the whole experiment (P=0.026, weeks 3-13; Table 2). PAA additions did not significantly increase NO_2-N concentrations (P>0.05), and NO_3-N concentrations were similar between the RAS units (P>0.05, Table 1). In addition, the PAA application frequencies did not significantly affect fish performance when measured by FCR and SGR

(Table 1), but the fish in the units receiving PAA four times per week had a 15% slower growth compared to fish in the control groups. However, mortality increased with increasing PAA application frequencies ($P<0.01$, Table 1; Table 2).

4. Discussion

In this study, we demonstrated the effect of different PAA application frequencies on biofilter microbiome function and composition, especially on the key nitrification microbes. We also examined the changes in biofilter nitrification rates, water quality, and fish performance in response to PAA application frequency. We observed that the overall microbial community composition remained quite stable, and nitrification bacteria did not substantially suffer from PAA applications, increasing in abundance during the experiment. More frequent PAA application decreased biofilter nitrification rates but yet could decrease the TAN values. PAA application frequencies did not significantly affect fish growth, but a higher mortality rate and increased slime formation were observed with the highest PAA application (four times per week).

Until this study, knowledge of the response of the biofilter microbiome to PAA applications has been scarce. In a few previous studies, a focus on the effect of a bi-weekly pulsed PAA addition of 1 mg L^{-1} on RAS microbiology has been found to decrease the overall bacterial counts in the tank water and to nearly completely remove the biofilm in the fish tanks in RAS (Liu et al., 2017a, 2018), while enhanced biofilm formation has been observed under continuous PAA applications, which has been explained by the acetic acid and formed acetate feeding the heterotrophic microbial community (Liu et al., 2017a). Furthermore, when a prolonged low PAA dosage has been used, no effect on microbial counts has been observed, resulting in a minimum PAA threshold estimate of 0.30 mg L^{-1} for disinfectant purposes in RAS (Davidson et al., 2019). In agreement, we observed that the abundance of tank water microbes, either free-living or attached to particles, was unaffected by a PAA addition. However, when weeks were inspected separately, we observed that bacterial abundance was higher, yet statistically insignificant, in the units receiving a PAA application one or

four times per week in both tank and biofilter biofilms at week 8. In the tanks at week 13, the PAA application rate had no overall effect on bacterial biofilm, because the bi-weekly PAA application rate was sufficient to decrease the bacterial biofilm, but the highest PAA application seemed to promote biofilm formation. In the biofilters, PAA had no clear effect on the biofilm bacterial abundance. It is likely that most of the PAA used was degraded before entering the biofilters, since it was added to the pump sump, explaining the minor beneficial and restricting effects of the PAA on the biofilm in the biofilters than in the tanks.

The dominating phylum was Proteobacteria in all RAS units throughout the experiment, while Bacteroidetes and Gemmatimonadetes were also relatively abundant. Previously, Proteobacteria and Bacteroidetes have found to be common phyla in RAS biofilter units in both freshwater and saltwater RAS, while Gemmatimonadetes were either absent or found in low abundance (Ruan et al., 2015; Gonzales-Silva et al., 2016; Bartelme et al., 2017; Rud et al., 2017), highlighting the unique nature of microbiomes in each biofilter and RAS unit (Blancheton et al., 2013). In previous studies, *Nitrospira* has been found to be quite abundant in RAS biofilters (Bartelme et al., 2017; Keuter et al., 2017), the group including both traditional nitrite-oxidizers and comammox bacteria. Here, we found the amount of *Nitrospira* increasing from week 8 to week 13 in all units. Overall, PAA application frequency did not significantly change the biofilm bacterial community composition or decrease the diversity or species richness, agreeing with previous findings on perturbations affecting free-living microbes rather than deep biofilm layers (Wietz et al., 2009; Schreier et al., 2010).

When nitrifying microbes are the sole focus, both qPCR and sequencing results demonstrated that PAA application frequency had a slightly, yet statistically insignificant, negative effect on the nitrifier abundance at week 8. At week 13, genetic nitrification potential (gene copy numbers) and the sequences associated with NOBs/comammox increased compared to week 8 in all units. This indicates that the possibly negative effect of PAA application on the nitrification microbes decreased towards the end of the experiments, probably because the nitrification community could adapt and

become less sensitive to PAA application. Previously, PAA additions have been found to only partially inhibit nitrification (Pedersen et al., 2009, Liu et al., 2017a), causing some accumulation of total ammonia nitrogen (TAN) and nitrate in RAS. Here, we measured nitrification rates using stable isotope incubations at the end of the experiment, and found higher PAA application frequency led to lower nitrification rates. However, we did not find the previously observed nitrite accumulation, suggesting that both ammonium oxidation and nitrite oxidation steps were suppressed, or that the abundant comammox bacteria were as or even more involved in ammonia oxidation than the traditional ammonia-oxidizers, releasing only nitrate as the end product.

In addition to changes in nitrification, the increased PAA application rate led to decreased turbidity and total suspended solids concentrations except in units with a PAA addition of twice a week, where values were closer to control units than other PAA application units. Decreased turbidity indicates that there were fewer large particles (Yao et al., 2014) because of the higher H_2O_2 degradation potential of organic matter. The observed decrease of total ammonia nitrogen with an increasing PAA addition was unexpected, because nitrification efficiency was lowest within the highest PAA addition. The direct oxidation of TAN is the likeliest explanation, because the slightly different daily feed consumption did not explain the difference in TAN concentrations between treatments. TAN values have previously been found to increase after a bi-weekly PAA addition due to lower nitrification rates (Liu et al., 2017a), and we also observed that the units with bi-weekly PAA applications behaved quite differently from those receiving one or four applications. There, we saw equal turbidity, TSS, TOC, TAN, and nitrate values with the control units, suggesting that this bi-weekly PAA addition promoted heterotrophic microbes more than removing them or improving water quality. However, one reason for the difference between bi-weekly and other PAA application frequencies is that the other 2x RAS unit (unit 2) showed a very different pattern in water quality and microbial results than the other systems. Furthermore, we saw that AOB and comammox microbes were less abundant in these systems, while the NOBs seemed to benefit there. In this experiment, PAA dosages were kept the same throughout the experiment, and feeding was

increased from the first (mean 102 g d^{-1}) to the second (mean 127 g d^{-1}) part of the experiment. Thus, the potential PAA effect time decreased during the experiment (Pedersen et al., 2013), which explained why the water quality differences between the treatments were higher in the first part of the experiment.

The PAA additions did not significantly affect fish performance in terms of feed conversion ratio or specific growth rate. However, the total biomass growth was 14% lower in the units where PAA was added four times per week compared to other units. In addition, mortality increased with the highest PAA addition, indicating that continued applications of PAA (1.1 mg L^{-1} dosed twice per day), even below the reported no-observed-effect concentration (2.8 mg L^{-1}) (Straus et al., 2018), were too high for the fish. High surface swimming was observed with the first additions of PAA, but this was not observed after a few weeks of additions, indicating the adaptation of fish to PAA as previously reported (Liu et al., 2017b; Gesto et al., 2018). The observed IPNV has low pathogenicity in Finland and mainly affects the first-feeding fry (Eriksson-Kallio et al., 2016). We thus concluded that it did not affect fish performance.

5. Conclusions

In conclusion, the microbial results suggest that the biofilter biofilm community is quite stable and less sensitive to PAA application, forming a “collaborome” in which heterotrophic microbes can support autotrophic nitrifiers rather than compete with them (Bartelme et al., 2017). Pulsed PAA applications disrupt nitrification, but the microbial community is capable of adapting and no long-term effect of PAA on inorganic nitrogen levels can be observed. Our study demonstrates that PAA application frequency has variable effects on microbes, water quality, and fish. Although the highest PAA application frequency (1.1 mg L^{-1} twice a day, four times per week) improved water quality slightly by directly oxidizing TAN, and potentially turbidity, and TSS, it led to 50% lower biofilter nitrification rates compared to the control units, and increased fish mortality. On the other hand, a bi-weekly application did not improve water quality, but fish performance was better than with

other PAA application frequencies. Similarly, a one-time PAA application was too low to improve water quality, but did not interrupt nitrification or fish. The opposite chemical (i.e. direct oxidizing of TAN) and biological (i.e. decreasing nitrification thus increasing TAN) effects of PAA on water quality can complicate the interpretation of results. Based on these results, the continuous pulse applications of PAA are not a cost-efficient method for substantially improving water quality and controlling the microbial communities. However, if an accumulation of solids and/or total ammonia nitrogen is observed, PAA can be used in such cases to improve water quality.

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Author contributions

All the authors planned the experiment. SS, JTP, and PL-L did the sampling and SS performed the DNA sequencing and qPCR measurements. SLA performed the statistical analyses of the microbial data, and JTP the water quality and fish performance analysis. All the authors contributed to manuscript writing.

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Figures and tables

Figure legends

Figure 1. Schematic diagram of one RAS unit used in the experiment. Microbial samples were taken from the fish tank biofilm, water from fish tank and from the fixed bed bioreactor. PAA was dosed into the pump sump. FT = Fish tank, 1 = Swirl separator, 2 = drum filter, FBBR = Fixed bed bioreactor, TF = Trickling filter, 3 = Pump sump.

Figure 2. Non-metric multidimensional scaling (NMDS) of bacterial communities in RAS biofilters under different PAA application frequencies (control, 1, 2, and 4 applications per week) at weeks 8 and 13.

Figure 3. The relative abundances of microbial phyla in biofilter biofilms under different PAA application frequencies (control, 1, 2, and 4 applications per week) at weeks 8 and 13.

Figure 4. The abundance of 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.

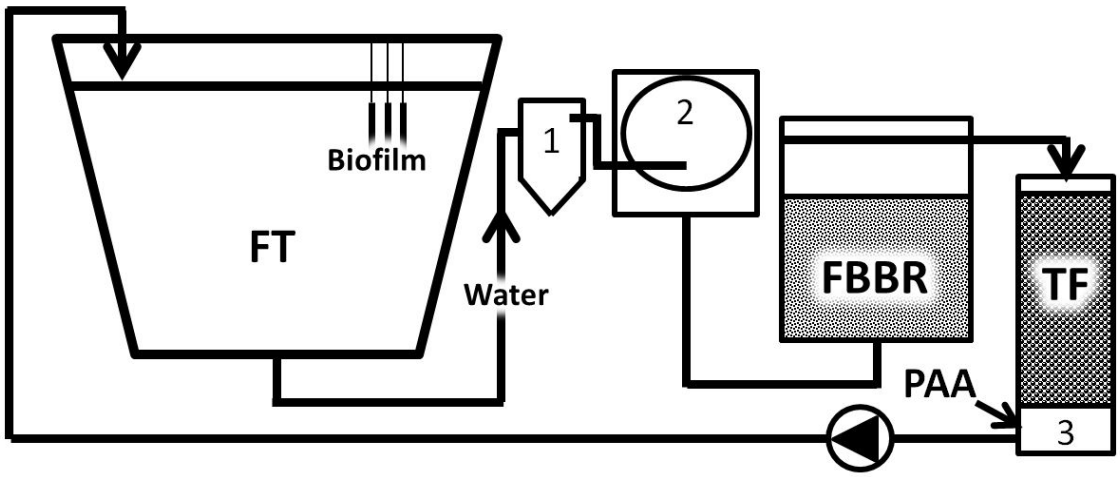
Figure 5. The relative abundances of AOB (ammonia-oxidizing bacteria), NOB (nitrite-oxiding bacteria) and comammox clade A genes in biofilter under different PAA application frequencies (control, 1, 2, and 4 applications per week).

Figure 6. The biofilter nitrification rates under different PAA application frequencies (control, 1, 2, and 4 applications per week) at week 13 at the end of the experiment.

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.



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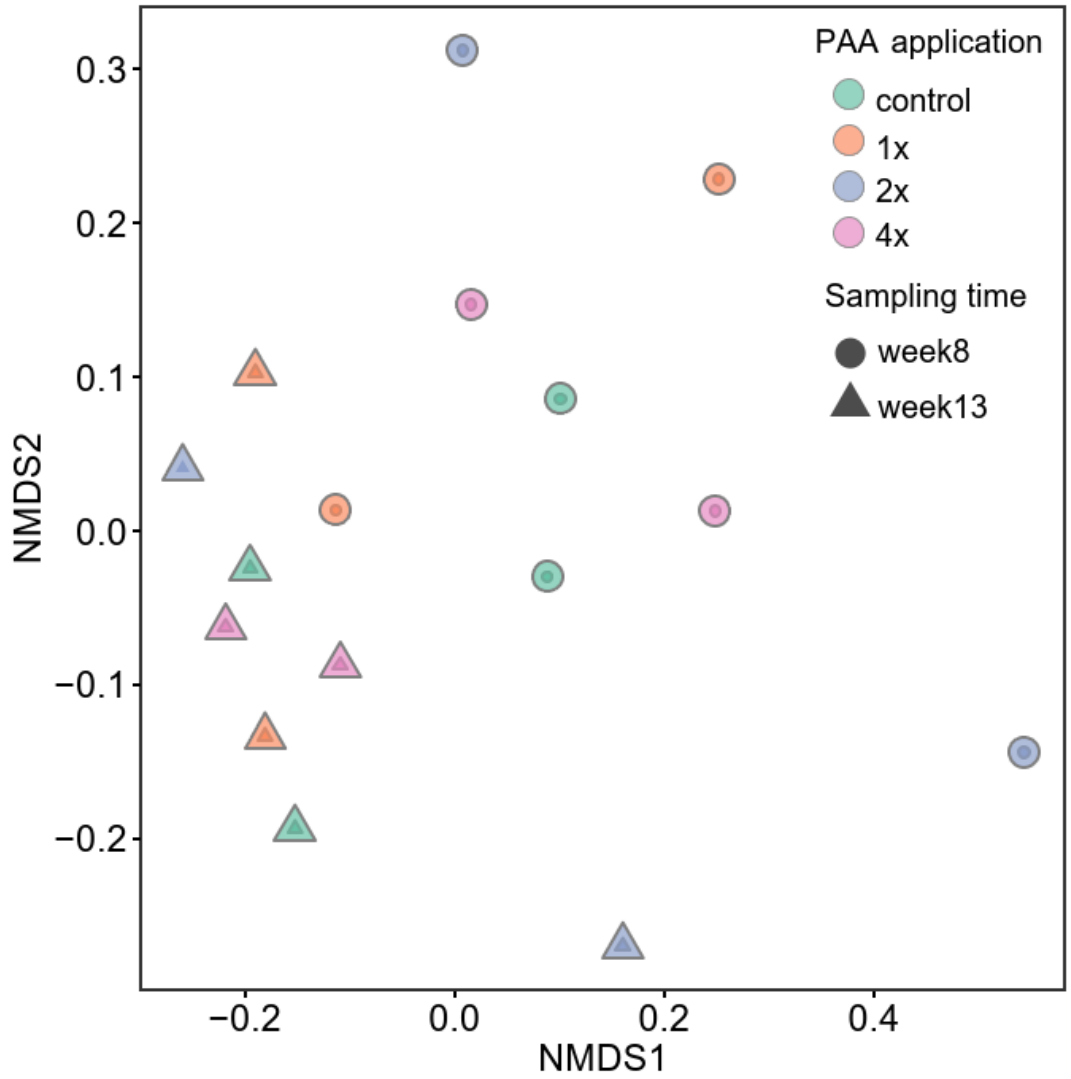

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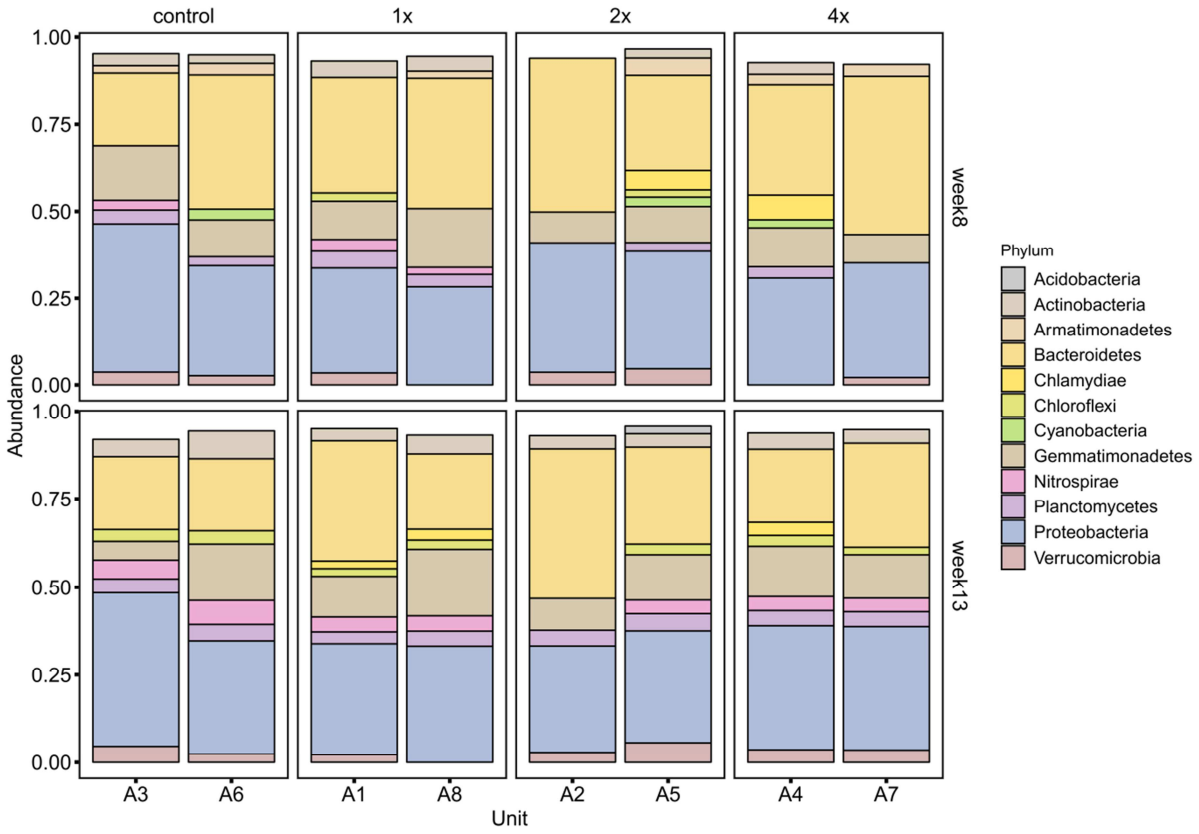

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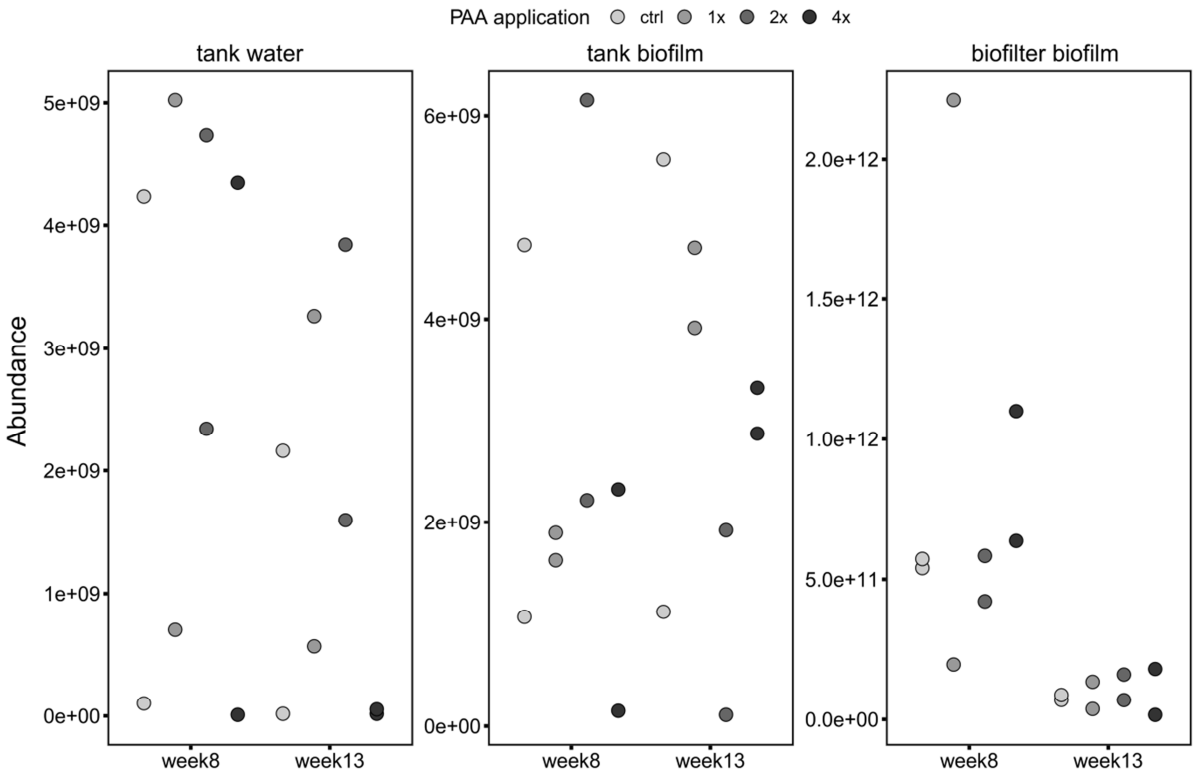

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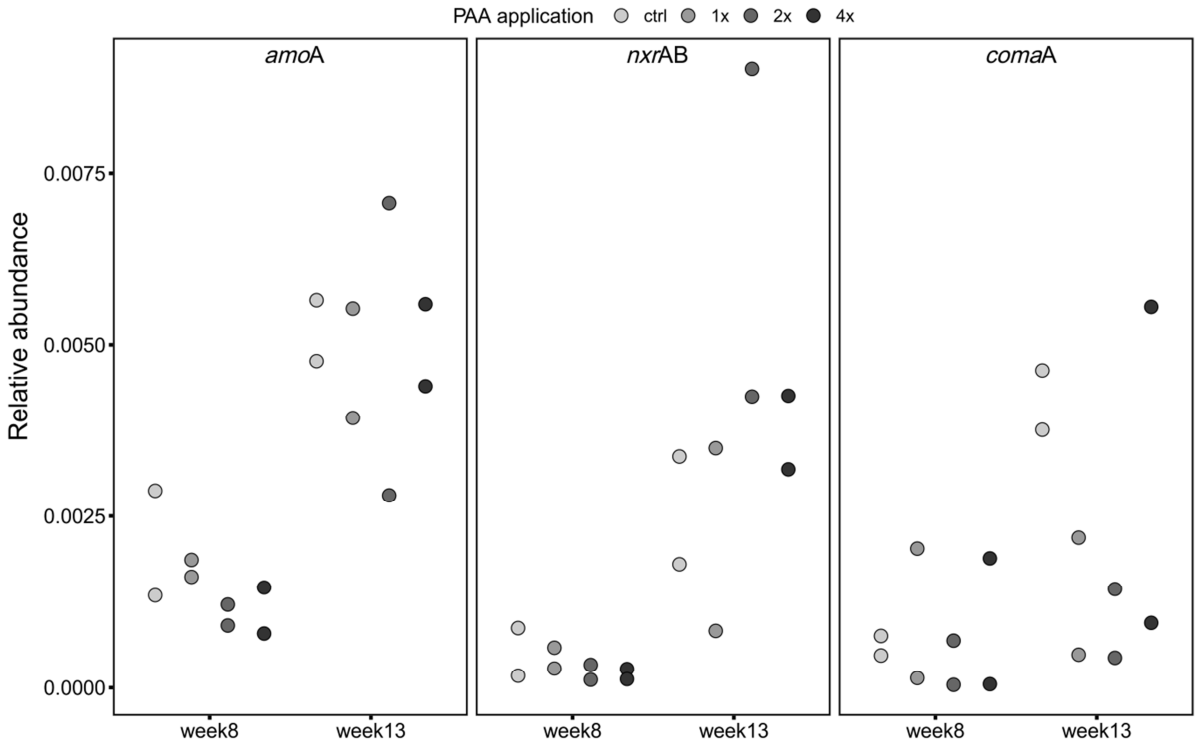

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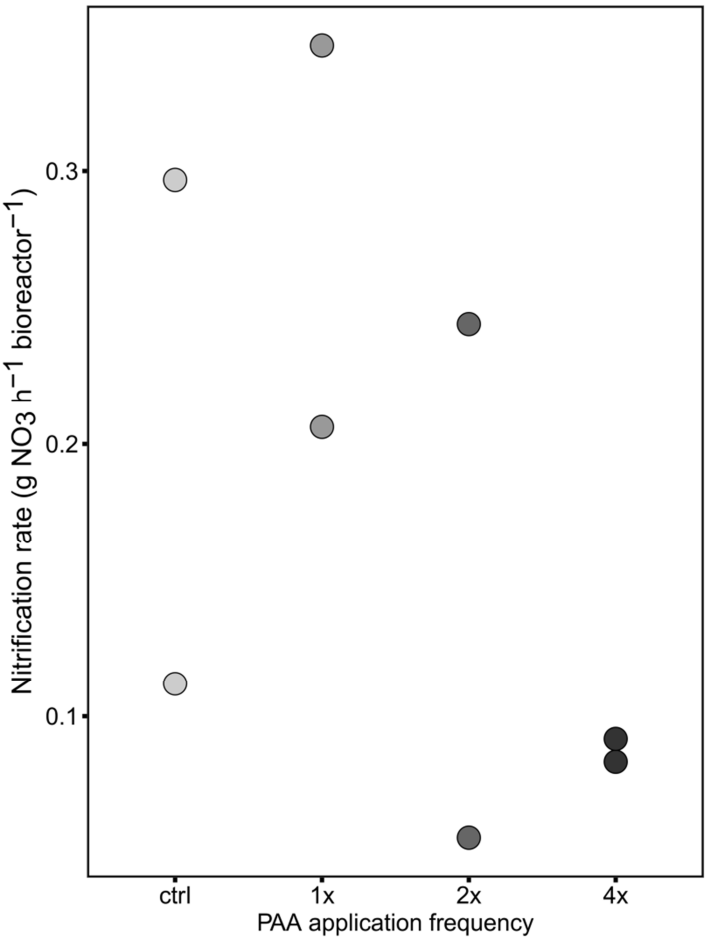

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587 Table 1. The mean water quality parameters and observed fish performance under different PAA application treatments (0, 1, 2, and 4 applications per

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

589

	Mean of weeks 3-7 (±SD)				Mean of weeks 8-13 (±SD)				Mean of weeks 3-13 (±SD)			
Treatment	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

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