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Pulse versus continuous peracetic acid applications: Effects on rainbow trout performance, biofilm formation and water quality

Dibo Liu[a,b,*], David L. Straus[c], Lars-Flemming Pedersen[d], Thomas Meinelt[b]

[a] Albrecht Daniel Thaer-Institute of Agricultural and Horticultural Sciences, Faculty of Life Sciences, Humboldt-University of Berlin, Invalidenstraße 42, 10115 Berlin, Germany
[b] Department of Ecophysiology and Aquaculture, Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 310, 12587 Berlin, Germany
[c] U.S. Department of Agriculture, Agricultural Research Service, Harry K. Dupree—Stuttgart National Aquaculture Research Center, P.O. Box 1050, Stuttgart, AR 72160, USA
[d] Technical University of Denmark, DTU-Aqua, National Institute of Aquatic Sciences, Section for Aquaculture, North Sea Research Center, P.O. Box 101, DK-9850 Hirtshals, Denmark

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ABSTRACT

Peracetic acid (PAA) products are being introduced to aquaculture as sustainable disinfectants. Two strategies are used to apply PAA: high dose pulse applications, or low dose continuous application. In the present study, their impacts on fish health and water quality were investigated in triplicate flow-through tanks stocked with rainbow trout. The gentler and shorter water cortisol increase measured along twice-per-week pulse applications of 1 mg L^{-1} PAA indicated a progressive adaptation of fish. In contrast, the continuous application of 0.2 mg L^{-1} PAA caused no stress to fish. Meanwhile, no mortality and no impact on growth or innate cellular immunity were observed. The pulse applications restricted biofilm formation, and partially inhibited nitrification. Additionally, the highest oxygen concentration and stable pH were observed. In contrast, the continuous application promoted biofilm formation, and caused a pH increase and intermediate oxygen concentration. The contrast was probably due to different susceptibility of microbes to PAA-induced oxidative stress. To summarize, pulse PAA applications cause minor stress in fish, but have advantages over continuous application by ensuring better water quality.

1. Introduction

Economic and environmental sustainability is central for the development of modern aquaculture (Naylor et al., 2000). For this reason, peracetic acid (PAA) products have been introduced to aquaculture as disinfecting agents because of a high treatment efficacy (antipathogenic effect) and low environmental impacts (Kitis, 2004). In forms of mixtures of PAA and hydrogen peroxide (H_2O_2), PAA products were proven to be effective against various fish pathogens (Farmer et al., 2013; Jussila et al., 2011; Lilley and Inglis, 1997; Marchand et al., 2012; Meinelt et al., 2015; Pedersen et al., 2013; Picon-Camacho et al., 2012; Smail et al., 2004; Straus and Meinelt, 2009; Straus et al., 2012b; Sudová et al., 2010) at low concentrations that were non-lethal to fish (Kouba et al., 2012; Marchand et al., 2013; Straus et al., unpublished data; Straus et al., 2012a).

Two strategies for applying PAA products in aquaculture systems are used: pulse applications at recommended concentrations (1–2 mg PAA L^{-1}) or a continuous application (at concentrations below 0.2 mg PAA L^{-1}) according to makeup water flow. During continuous applications, the same or even higher quantities of PAA are added, or over a prolonged period of time. However, comparison of these two strategies is lacking, especially concerning their impacts on fish health, microorganisms and water conditions.

Many management practices in aquaculture can induce fish stress, and corresponding actions to minimize stress have been investigated and implemented (Zahl et al., 2012). Although disinfection with PAA products decreases the potential risk of pathogenic diseases, the exposure of the disinfectants itself may also induce stress to fish (Powell et al., 2015). Pulse or continuous PAA application strategies can lead to repeated high concentration exposure or prolonged low concentration exposure, respectively, resulting in two scenarios. One scenario is that the fish may suffer

* Corresponding author at: Albrecht Daniel Thaer-Institute of Agricultural and Horticultural Sciences, Faculty of Life Sciences, Humboldt-University of Berlin, Invalidenstraße 42, 10115 Berlin, Germany.
E-mail address: liu@igb-berlin.de (D. Liu).

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from chronic stress, exemplified by chronic or constantly elevated plasma cortisol. As a result of increased compensatory metabolic activity, the fish lose their homeostasis resulting in poor growth and suppressed immunity (Bonga, 1997; Harris and Bird, 2000; Magnadóttir, 2006). Another scenario is that the fish may adapt to repeated or prolonged PAA applications and show unaffected growth and immunity (Smith et al., 2011; Wilkie et al., 1996). Since different PAA concentrations and exposure durations are used in these two disinfection strategies, their impacts on fish health, especially stress condition, growth and immunity are likely to differ. Similarly, disinfection strategies are potentially also affecting bacterial abundance and activity (Pedersen et al., 2010), however not studied for trade PAA products. Biofilms are ubiquitous in aquaculture. They colonize surfaces (tank walls, bottom), and consist of mainly periphytic algae and bacteria (van Dam et al., 2002). Especially in RAS, biofilms are the fundamental of nitrifying biofilters (Malone and Pfeiffer, 2006). Biofilms affect various water quality parameters, such as ammonia and nitrite that are oxidized by nitrifying bacteria (Hagopian and Riley, 1998; Rurangwa and Verdegem, 2015); and hence affect oxygen and pH (Moriarty, 1997).

The aim of the present study was to compare the potential impacts of the two PAA application strategies (pulse and continuous) on fish health, microorganisms and water quality, and to determine the optimal strategy for practical use.

2. Materials and methods

2.1. Fish and experimental system

Rainbow trout (*Oncorhynchus mykiss*) weighing 115 ± 10 g and of mixed sex were purchased from BioMar Research Centre (Hirtshals, Denmark) and were acclimated to the experimental system for 3 weeks. Prior to the experiment, fish were anesthetized with KALMAGIN 20% (Laboratory Centrovet, Santiago, Chile), individually weighed and equally distributed into 9 experimental tanks (18 fish per tank); stocking density was 11.83 ± 0.14 kg m^{-3} (mean ± SD). Subsequently, fish were acclimated for another week before treatments began.

The cylindrical experimental tanks were made of Plexiglas (Volume = 180 L; ø = 0.44 m; 0.80 m high with a 0.5 m conical bottom as a modified Guelph System; see Dalsgaard and Pedersen (2011) for details). The tanks were operated as flow through systems and were further modified with in-tank aeration via an external airpump with tubing extending to a depth of 30 cm to an airstone, and an individual water pump (EHEIM Compact 1000, Deizisau, Germany) installed in each tank to create an identical radial flow. A fixed inlet flow of 20 L h^{-1} to each tank originated from a common oxygen supersaturated (dissolved oxygen: around 160%) water reservoir.

Feed (EFICO Enviro 920 4.5 mm pellets, BioMar) was offered daily at quantities equivalent to a feeding rate of 0.8% biomass to each tank from 17:30 to 17:40 via individual custom-made feeding automat (DTU-Aqua, Hirtshals, Denmark). Daily feed was progressively increased based on estimated growth using an expected feed conversion ratio of 1.0. Uneaten pellets and sediments were removed daily from the drain directly after feeding and the following morning. Temperature and dissolved oxygen concentration were measured daily immediately after feeding with a Handy Polaris portable DO meter (OxyGuard®, Farum, Denmark). In case of low oxygen in the experimental tanks, the oxygen concentration in the common water reservoir was increased.

2.2. Treatment and sampling

The nine flow-through tanks were equally and randomly divided into 3 treatment groups. One treatment group received a pulse dose of 1 mg L^{-1} PAA every Monday and Thursday, which is equivalent to a weekly dose of 360 mg PAA. The dose was administered by delivering 1.053 mL of the PAA product, Aqua Oxides (S. Sørensen, Thisted, Denmark), with an Eppendorf pipette (Hamburg, Germany), in 15 mL distilled water and subsequently adding this dilution slowly (10–15 s) into the respective tanks. The second treatment group received continuous additions of a 1:500 dilution of Aqua Oxides at the rate of 0.195 mL min^{-1}; this was equivalent to dosing with 0.2 mg L^{-1} PAA in the inflow and a weekly dose of 672 mg PAA. The continuous treatment was administered with an ISMATEC® BVP Standard peristaltic pump equipped with PharMed® Ismaprene tubing (Cole-Parmer, Wertheim, Germany). The control treatment group, without PAA exposure, received a sham treatment by adding 15 mL distilled water similar to the pulse treatment.

A series of water samples were taken from each tank during every second pulse treatment (Mondays) in the first 4 weeks. For each sampling period, water was collected immediately before, and 1, 2, 4, 8, 24, 28, 32 and 48 h after the pulse treatment. Water samples were collected from the tank outflow in 1-L glass bottles (SCHOTT, Mainz, Germany). On a non-pulse treatment day in the 5th week, fish in all tanks were additionally stressed by being gently harassed with a dipnet for 90 s. Water samples were collected immediately before, and 1, 2, 4 h after the stressor.

After 6 weeks of treatment, fish in each system were anesthetized to determine the total biomass. Two random fish in each tank were sacrificed for the determination of growth parameters and innate cellular immunity (n = 6 per treatment group). Biomass, standard length and height were measured. The whole liver was removed and weighed to calculate the liver-somatic-index. The head kidney was aseptically removed, pressed through a 70 μm EASYstrainer™ sterile mesh unit (Greiner Bio-One International, Kremsmünster, Austria) and suspended in ice-cold wash medium (RPMI-1640 with phenol red + 100 U mL^{-1} Penicillin-streptomycin +2 mM L-Glutamine + 25 mM Hepes buffer, 0.22 μm sterile filtered). All chemicals used for analyses were reagent grade.

2.3. Water parameter measurement

The pH value was measured in all treatment groups for 48 h during a pulse PAA treatment during the 1st and 4th week. The HQ40D pH probe (Hach Lange, Düsseldorf, Germany) was fixed near the water surface before the fish were acclimated. In the 6th week, water samples were collected from all tanks at the outflow shortly before feeding and 12 h post-feeding. Total ammonia-nitrogen (TAN), nitrite-N and nitrate-N were determined according to the methods described by von Ahnen et al. (2015). The PAA concentration and degradation were determined in the surface water of the pulse and continuous treatment groups according to the DPD (*N*,*N*-diethyl-*p*-phenylendiamine sulfate salt) photometric method described by Pedersen et al. (2009).

2.4. Water cortisol measurement

Cortisol in the water samples was extracted immediately after sampling. The extraction was based on the procedure described by Brüning et al. (2015) with slight modifications. Each Sep-Pak C18 Plus cartridge (Waters, Eschborn, Gemany) was activated with 5 mL methanol and rinsed with 5 mL ultrapure water before the 1 L water sample was transferred via an ISMATEC® BVP Standard peristaltic pump (Cole-Parmer, Wertheim, Germany) at a flow rate of 10 mL min^{-1}. Subsequently, the cartridges were rinsed with 5 mL ultrapure water, eluted with 5 mL ethyl-acetate and collected in 10 mL glass tubes. The eluted samples were evaporated in a water bath (40 °C) under an N_2 stream and stored at −20 °C until assayed. The evaporated cortisol samples were redissolved in

0.5 mL phosphate buffered saline solution (PBS + 5% ethanol + 0.1% bovine serum albumin, 0.22 μm sterile filtered) and measured with Cortisol ELISA test kits (IBL International, Hamburg, Germany) according to the manufacturer's instruction.

2.5. Leucocyte isolation and determination of innate cellular immunity

Respiratory burst of head kidney leucocytes was chosen as an indicator for the innate cellular immunity of rainbow trout (Ellis, 1999). The isolation of leucocytes was performed as described by Secombes (1990) with slight modifications. The cell suspensions from head kidneys were centrifuged at $400 \times g$ for 10 min in a Sigma 3-18KS centrifuge (Sigma Laborzentrifugen, Osterode, Germany). The leucocyte-enriched layer was transferred with a sterile Pasteur pipette to a new sterile centrifuge tube and re-suspended in 6 mL ice-cold culture medium (RPMI-1640 without phenol red + 100 U mL^{-1} Penicillin-streptomycin + 2 mM L-Glutamine + 25 mM Hepes buffer + 4% charcoal stripped fetal bovine serum, 0.22 μm sterile filtered). The new suspensions were washed twice with ice-cold culture medium. Subsequently, the cell density of each suspension was adjusted to 10^7 cells mL^{-1} with a Bürker-Türk hemocytometer (Brand, Wertheim, Germany) before the suspensions were seeded to sterile 96-well plates (Nunc Thermo Scientific, Waltham, Massachusetts, USA) with 100 μL cell suspension per well and 6 replicates. The plates were incubated in a humid chamber at 17 °C overnight until the non-attached cells were removed by discarding the medium.

To measure the respiratory burst (NBT assay), 100 μL of fresh culture medium at ambient temperature dissolved with 1 mg mL^{-1} nitroblue tetrazolium (NBT) was added to each well. Phorbol 12-myristate 13-acetate (PMA, 1 μg mL^{-1}) was used as a stimulant in triplicates. The plates were incubated in a humid chamber at 17 °C for 3 h. Subsequently, the medium was discarded and the cells were fixed with 100% methanol and washed twice with 70% methanol. Dried wells were mixed with 100 μL dimethyl sulfoxide (DMSO) and 100 μL 2 M KOH to dissolve the formazan. The optical density (OD) was read at 620 nm with a Tecan GENios plate reader (Tecan Group, Männedorf, Switzerland).

Viability of the attached leucocytes was simultaneously determined. To measure the cell viability (MTT assay), 100 μL of fresh culture medium at ambient temperature dissolved with 0.5 mg mL^{-1} thiazolyl blue tetrazolium bromide (MTT) was added to each well in. The plates were incubated in a humid chamber at 17 °C for 3 h. Subsequently, the medium was discarded and the cells were dried. The formazan was dissolved in 100 μL alkaline DMSO (27 μL 2 M KOH dissolved in 10 mL DMSO), mixed and the OD was read at 570 nm with the Tecan GENios plate reader. In addition, an MTT assay was performed on the attached leucocytes after incubation for 72 h without stimulants. The result was compared with the initial MTT assay and the 72-h survival rate was calculated.

2.6. Statistics

The 4 week water cortisol, growth parameters and cellular innate immunity of three treatment groups were compared via one-way ANOVA with Dunnett's post-hoc test. In case of unequal variance, a Welch's ANOVA with Dunnett's T3 post-hoc test was performed instead. The water parameters of three treatment groups were compared via one-way repeated measures ANOVA. In case of unequal variance, Friedman Repeated Measures ANOVA was performed instead. All ANOVA analyses were performed on SPSS Statistics version 21 (IBM, Chicago, Illinois, USA).

The water cortisol after the additional stressor (dipnet harassment) was analyzed with GraphPad Prism® 7 (GraphPad Software, La Jolla, California, USA). The increase of cortisol along time was interpreted as a linear regression. The slope and intercept of different groups were compared.

3. Results

3.1. Water cortisol

The water cortisol concentration in the control treatment group indicated diurnal fluctuation during the sampling period. The concentration slightly increased from 5.03 ± 0.61 ng L^{-1} in the morning, peaked at noon (5.61 ± 0.14 ng L^{-1}) and decreased in the afternoon (Fig. 1). This fluctuation was repeated throughout the 4 weeks of measurement. The water cortisol concentration from the continuous treatment group showed a similar pattern as the control group. In contrast, the water cortisol of the pulse treatment group showed a significant increase after the 1st PAA application. The water cortisol concentration remained higher than the other two groups for hours, reaching levels up to 27.88 ± 5.98 ng L^{-1}. Moreover, fish showed behavioral reactions to the pulse PAA treatments. They became more active in swimming rather than 'aligned' in the radial flow. In the following weeks, the increase of water cortisol in the pulse treatment group became progressively milder and negligible (Fig. 1). The intensity and the duration of the behavioral reaction of fish were also decreasing over time.

Noteworthy, there were a few fish that showed either aggressive or evading behavior regardless of treatment group and time. Some were chasing and biting each other, while the rest were close to the bottom of the tank with their head tilting slightly downward. In contrast, fish in other replicate tanks remained peaceful in schools. These fish introduced a prolonged higher water cortisol concentration and a different pattern than the other replicate systems within the same group (Fig. 1).

The water cortisol concentration of all groups increased after the application of the additional stressor (dipnet harassment for 90 s) in the 5th week. The proportional increase was similar for all treatment groups ($P = 0.578$). Moreover, the slope and intercept of the cortisol-time linear regression were similar in all treatment groups ($P = 0.7585$ and 0.0617, respectively; Fig. 2).

3.2. Growth

No mortality occurred during the experimental period. No uneaten pellets were observed by the daily inspections throughout the experiment. The total growth rate and feed conversion ratio were the same for all groups ($P = 0.813$ and 0.907, respectively; Table 1). The length/height ratio and liver-somatic-index of sampled fish were the same for all groups ($P = 0.771$ and 0.824, respectively; Table 1).

3.3. Innate cellular immunity

Both PAA treatment groups showed similar respiratory burst of head kidney leucocytes as the control treatment group ($df = 17$, $P = 0.663$, Fig. 3). The stimulation effect of PMA on respiratory burst was the same for all groups ($df = 17$, $P = 0.714$). The initial viability, 72-h viability and 72-h survival rate of head kidney leucocytes were the same for all groups ($df = 17$, $P = 0.597$, 0.288 and 0.187, respectively)

3.4. Water conditions

The pH showed daily fluctuations of ≤0.2 in all groups. Measurements indicated a common daily variation in pH as it slightly decreased during feeding and increased afterwards. The pulse PAA treatment induced a transient pH decrease immediately after the application, which was similar to the pH decrease during feeding

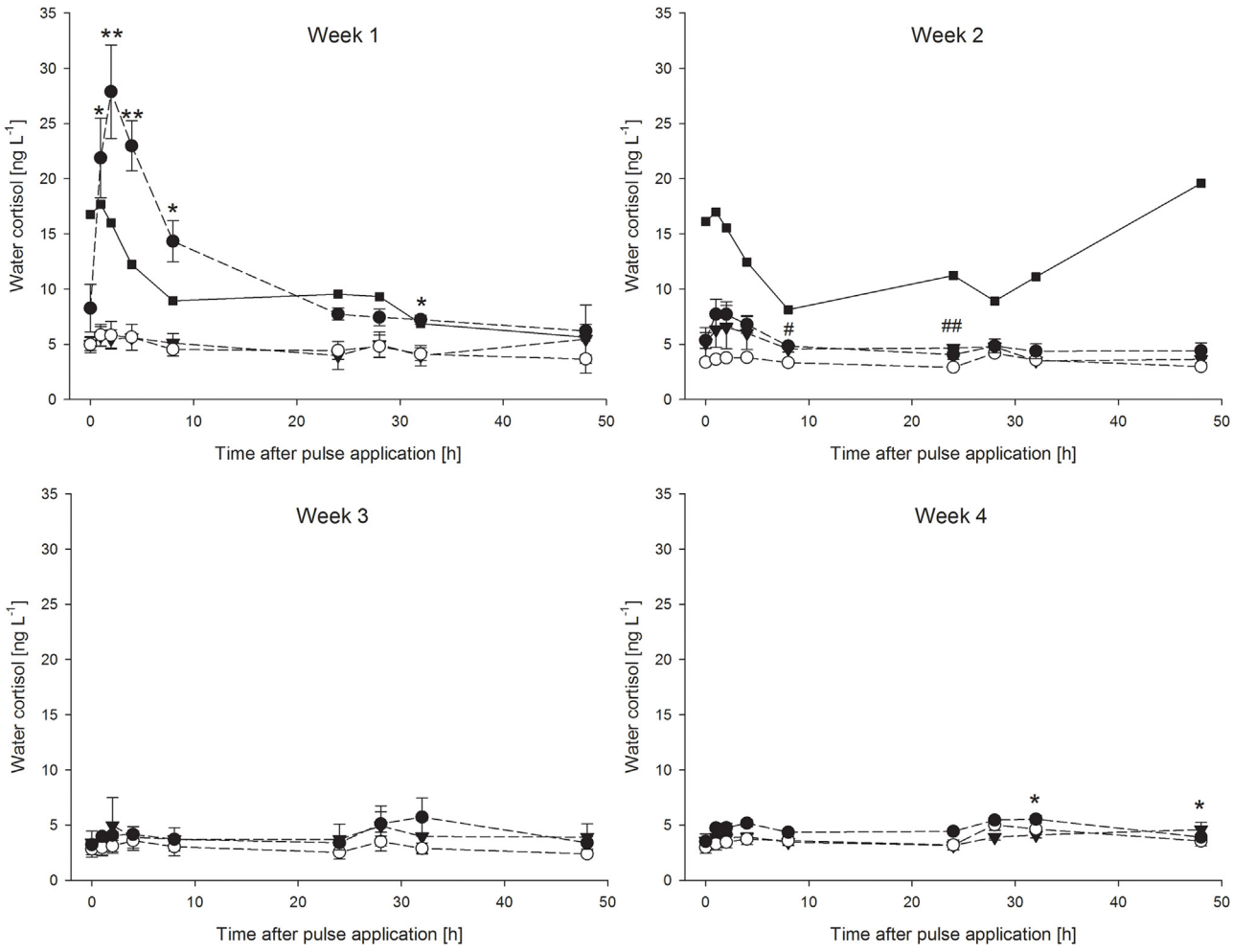

Fig. 1. The mean water cortisol concentration (n = 3) from the control group (▼), the continuous treatment group (○) and the pulse treatment group (●) based on weekly measurements during the first 4 weeks. ■: outlier tanks where fish showed aggressive or evading behavior in the control group. Error bars indicate the standard error. * and ** indicate significant difference between the control and the pulse treatment group, $P < 0.05$ and 0.01, respectively. # and ## indicate significant difference between the control and the continuous treatment group, $P < 0.05$ and 0.01, respectively.

Table 1
Growth parameters (mean ± SD) of all treatment groups after 6 weeks.

Parameters	Control	Continuous treatment	Pulse treatment	P-value
Initial biomass [kg]	2.14 ± 0.03	2.13 ± 0.02	2.12 ± 0.03	0.694
Final biomass [kg]	3.52 ± 0.02	3.51 ± 0.012	3.51 ± 0.031	0.467
Growth rate [%]	64.5 ± 1.88	64.9 ± 2.1	65.4 ± 0.9	0.813
FCR	0.70 ± 0.012	0.70 ± 0.02	0.70 ± 0.001	0.907
Liver somatic index	0.012 ± 0.001	0.012 ± 0.001	0.011 ± 0.001	0.865
Length/height ratio	3.86 ± 0.11	3.85 ± 0.20	3.79 ± 0.22	0.853

FCR = Feed conversion ratio.

(Fig. 4). The pH of the control group decreased about 0.2 from the 1st week to the 4th week, while that of the continuous treatment group slightly increased. In contrast, the pH of the pulse treatment group remained in the same range.

Compared to the control group, it was observed that the biofilm on the inner surface of the fish tank was almost completely removed by the pulse PAA treatments. Alternately, enhanced biofilm formation was observed in the tanks receiving continuous PAA treatment (Fig. 5). Temperature in each tank was equal and remained constant at 13 ± 0.2 °C. The dissolved oxygen concentration was highest in the pulse treatment group and lowest in the control group (Fig. 6, $P < 0.001$).

All groups showed a similar decrease of TAN, nitrite and nitrate within 12 h post feeding ($P = 0.544$, 0.931 and 0.841, respectively). Regardless of the sampling time, the continuous treatment group and the control group had similar concentrations of dissolved TAN, nitrite and nitrate. In contrast, the pulse treatment group had significantly elevated TAN and nitrite, and lower nitrate than the control group (Fig. 7).

The pulse treatment resulted in a mean of about 0.7 mg L^{-1} PAA when sampled 5 min after application; the PAA concentration was exponentially reduced and complete degradation of PAA was achieved within 5 h after application. In tanks receiving continuous PAA treatment, the nominal and subsequent PAA concentrations

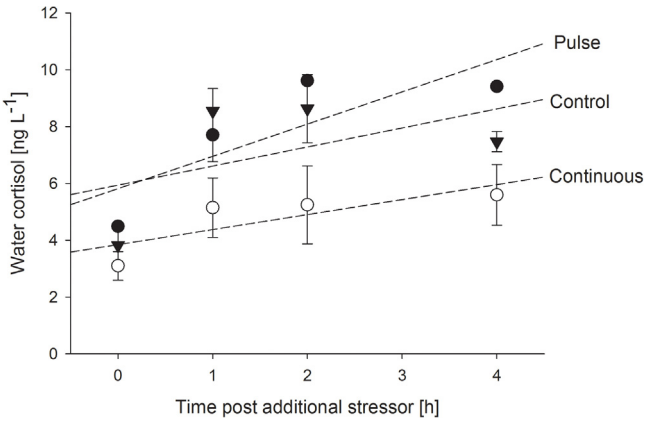

Fig. 2. The mean water cortisol concentration from the control group (▼), the continuous treatment group (○) and the pulse treatment group (●) immediately after an additional stressor (dipnet harassment for 90 s) in the 5th week. Error bars indicate the standard error. Dotted lines indicate the interpreted linear regression between time and water cortisol.

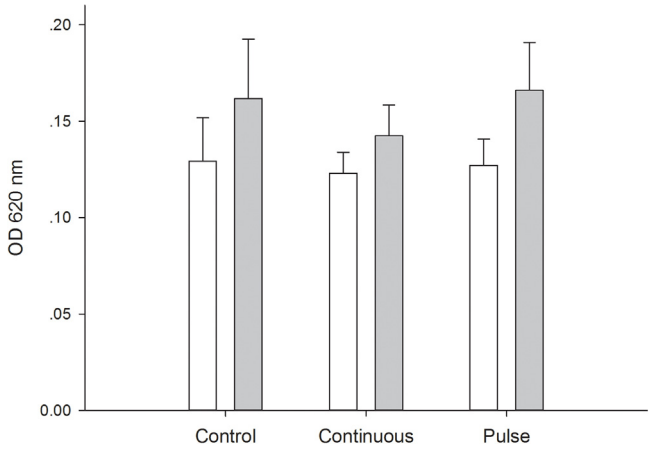

Fig. 3. The mean respiratory burst indicated as optical density (OD) at 620 nm of head kidney leucocytes in all treatment groups after 6 weeks. Error bars indicate the standard error. Open bars indicate unstimulated leucocytes. Filled bars indicate leucocytes stimulated with 1 μg mL^{-1} PMA.

remained below 0.01 mg L^{-1}, which was below the detection range of analyses.

4. Discussion

4.1. Stress adaptation of rainbow trout to PAA

Cortisol is predominantly released from the branchial blood vessels of the gill through passive diffusion (Vermeirssen and Scott, 1996). Based on this theory, measurement of water cortisol instead of plasma cortisol has been established and proven to be a reliable indicator of stress for various fish species, especially in flow-through systems (Ellis et al., 2004, 2007; Fanouraki et al., 2008; Ruane and Komen, 2003; Scott and Ellis, 2007; Sebire et al., 2007; Wong et al., 2008).

In the present study, the pulse treatment group of 1 mg L^{-1} PAA visually triggered behavioral responses of the trout indicating a stress response, and induced a strong increase of water cortisol in the 1st week. The water cortisol concentration remained higher than the control group until the second day. These results indicated that rainbow trout were stressed by the first pulse of the PAA treatment in the 1st week. In contrast, the continuous PAA treatment led to water cortisol and fish behavior similar to the control treatment

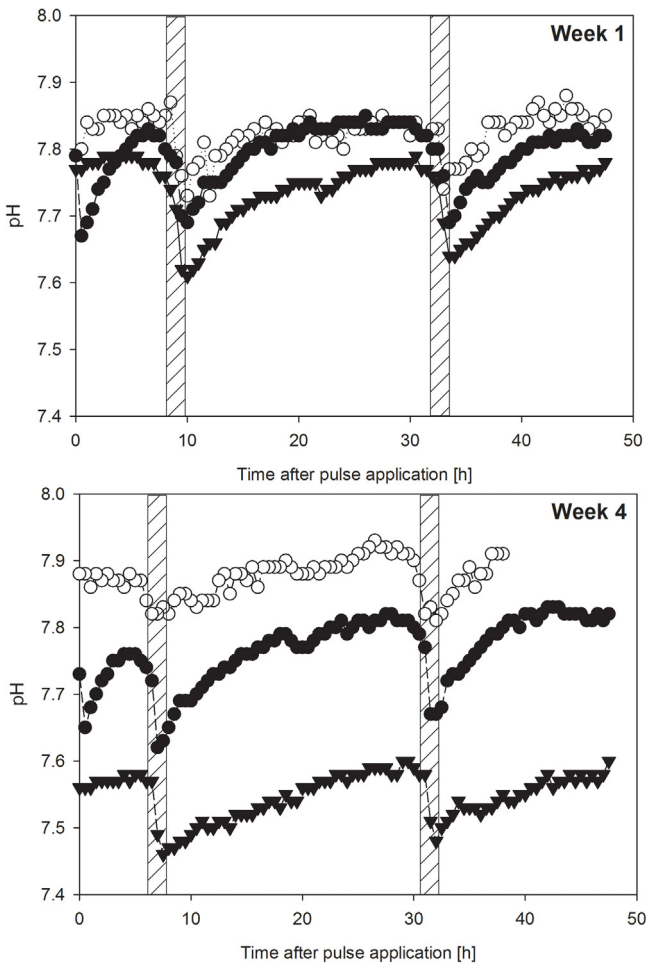

Fig. 4. The mean pH values of the control group (▼), the continuous treatment group (○) and the pulse treatment group (●) post pulse PAA application in the 1st and 4th week. Striped bars indicate the time of feeding starting at 17:30.

group throughout the experiment. This indicated that the continuous PAA treatment did not provoke stress to the rainbow trout at any time. The reason was probably that the actual PAA concentration caused by continuous application was very low and below a triggering threshold for the rainbow trout physiology to react.

From the 2nd week, the behavioral reaction of rainbow trout to the pulse treatment of PAA was still present but became shorter and less intensive. This indicated that the rainbow trout physiology could still cognitively sense the pulse treatment. Despite of this, all treatment groups had similar water cortisol, indicating that the pulse PAA treatment no longer resulted in a stress response from the rainbow trout after the 2nd week.

Therefore, results indicated a process that the pulse treatment induced a stress response in rainbow trout during the first exposure, but the stress response was down-regulated and became insignificant during successive exposures. There are two possible explanations: adaptation or exhaustion. If rainbow trout were exhausted, they were likely to suffer from chronically elevated metabolic activity and subsequently show reduced growth, suppressed immunity and reduced stress response against other stressors (Bonga, 1997; Harris and Bird, 2000; Magnadóttir, 2006). However, in the present study, rainbow trout in all groups showed similar growth, immunity and stress response to an additional stressor (dipnet harassment). Therefore, the possibility of exhaustion could be excluded and it is suggested that the observed results were due to the adaptation of rainbow trout.

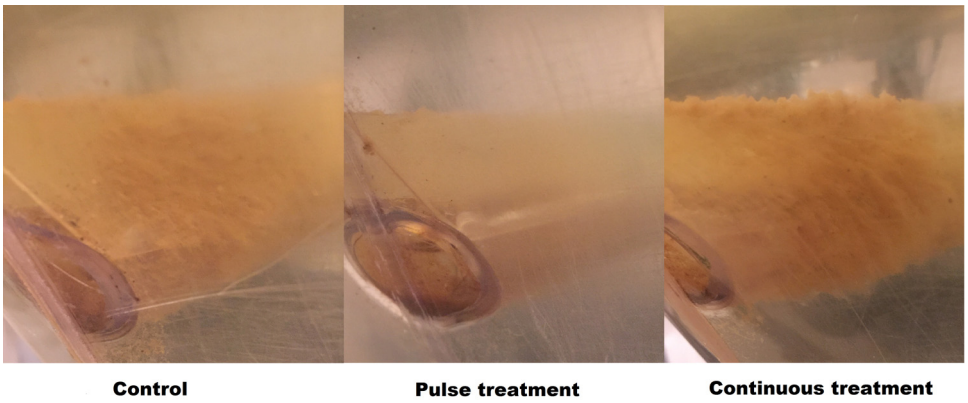

Fig. 5. Biofilm on the inner side of fish tanks in all treatment groups after 6 weeks.

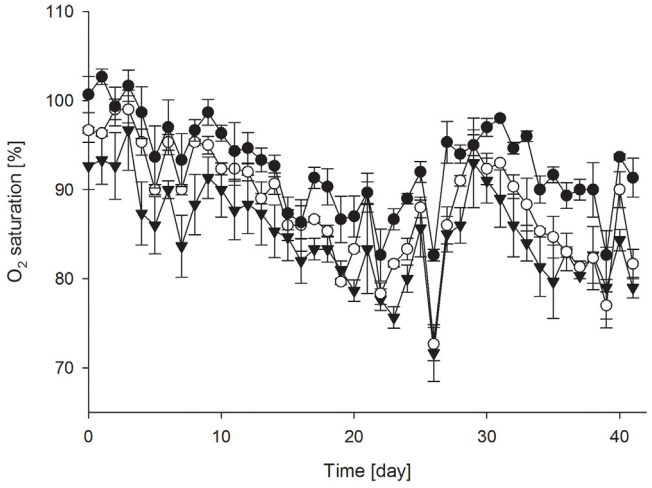

Fig. 6. The mean dissolved oxygen of the control group (▼), the continuous treatment group (○) and the pulse treatment group (●) during the 6-week experiment. Error bars indicate the standard error.

4.2. Non-treatment related variation in the water cortisol concentrations

A few apparently random fish were encountered in tanks regardless of treatment group or time that showed either aggressive or evading behavior, while those in the other replicate tanks within the same treatment group were uniform and peaceful. This was most likely the consequence of social stress, as dominant fish can induce acute or chronic stress to the subordinate fish (Jeffrey et al., 2014; Øverli et al., 1999; Sloman et al., 2001). In the present study, the elevated cortisol value in the control treatment group during weeks 1 and 2 sampling was only present for a brief period and was not repeated. In this case, the subordinate rainbow trout probably had elevated plasma cortisol which resulted in higher water cortisol concentrations. As discussed, this behavior was random, but had no effect on survival or other parameters measured other than water cortisol levels in the control treatment group.

4.3. Impact of PAA application on water quality and biofilm formation

Oxygen, pH and nitrogenous compounds (ammonium, nitrite and nitrate) are important indicators for water quality in aquaculture. Their variations are usually the combined consequence of water retention, stocking density, feed input and microbial activities. In the present study, systems in all groups had identical water retention, stocking density and feed input. Therefore, the difference of water quality among groups was mainly due to the difference on microbial activities, which, in aquaculture, are present in planktonic phase and in the biofilm. The biofilm, with similarities to periphyton, is a ubiquitous organic matrix consisting of extra polymeric substances, bacteria and periphytic algae that coevolves with their planktonic cells during conditions with nutrient load (Rao, 2010; van Dam et al., 2002). In the present study having tanks with a relatively short retention time of 9 h, the planktonic cells were constantly diluted by the flow-through design. Consequently, the microbial activities in planktonic phase were less pronounced, compared to those in the biofilm. Especially in RAS, the attached biofilm has been extensively used for the design of the nitrifying biofilter (Chen et al., 2006).

The pulse treatments of 1 mg L^{-1} PAA nearly completely removed the biofilm in the fish tank with the given exposure time. The biofilm formation begins with accumulation of organic molecules on a surface followed by bacterial colonization (Wahl, 1989). As PAA was applied shortly after fish were stocked in the systems, when the biofilm was in early development stage, the pulse PAA treatments probably inhibited the early bacterial colonization based on the reported general antibacterial effect (Kitis, 2004). Surprisingly, microbial nitrification was not completely but partly inhibited, which is in line with previous finding (Pedersen et al., 2009). Nitrification still occurred in week 5, seen as elevated but slightly lower nitrate-N levels compared to the other two treatment groups. The nitrifying bacteria, as reported by Chandran and Love (2008), showed tolerance to the batch treatment of 0.1 mM H_2O_2 with recovered nitrification activity. In the present study, a comparable concentration of PAA was used, which probably led to a similar minor inhibitory effect on nitrifying bacteria. Compared to autotrophic nitrifying bacteria, the heterotrophic bacteria, especially the aerobics, seemed to be strongly inhibited by the pulse PAA treatments. The inhibitory effect could be interpreted by the highest daily oxygen concentration measured in the pulse treatment group, which indicates the lowest microbial oxygen consumption. Moreover, the pulse PAA treatments resulted in a stable pH of 7.7–7.8 within the first 4 weeks. This suggests that the CO_2 production caused by microbial aerobic respiration was minimal and stable. The competition of heterotrophic bacteria and autotrophic nitrifying bacteria in biofilms, as reported by Wik and Breitholtz (1996), is affected by various parameters. In the present study, the nitrifying bacteria probably dominated the biofilm due to high susceptibility to PAA. This further supports the observed results that the nitrification was not completely inhibited by the pulse PAA treatments.

Despite of higher weekly PAA input than the pulse PAA treatment (360 mg PAA per week), the continuous PAA treatment (672 mg PAA per week) did not cause PAA accumulation and the

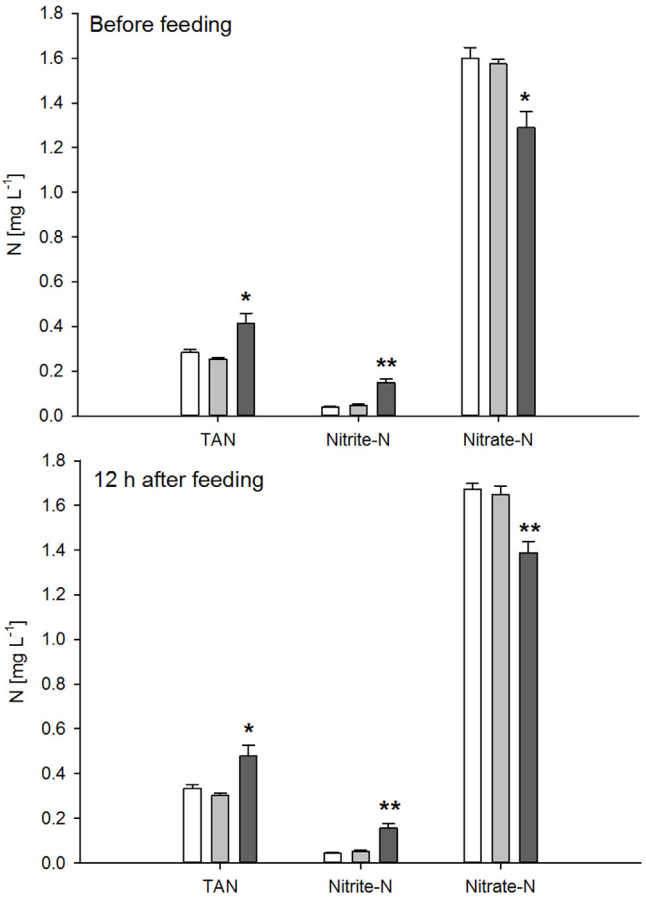

Fig. 7. The mean total ammonia-nitrogen (TAN), nitrite-N and nitrate-N in tanks of the control group (open bar), continuous treatment group (pale bar) and the pulse treatment group (black bar) before and 12 h post feeding on a non-treatment day in the 6th week. Error bars indicate the standard error. * and ** indicate significant difference from the control group ($P < 0.05$ and 0.01, respectively).

resulting concentration was close to or below detection limit. This was probably caused by fast degradation, which was potentially attenuated by microbial adaptation. Acetic acid and acetate are active ingredients in PAA trade products. Both potentially contribute to microbial growth as well-known easy degradable dissolved organic matter. The addition of organic carbon may enhance the growth of periphytic algae and heterotrophic bacteria in the biofilm (Asaduzzaman et al., 2008). This was visually confirmed by the enhanced biofilm formation in tanks receiving continuous PAA treatment, compared to the control group. Indications of biofouling on the pH probes in the control and continuous treatment groups was also observed, but the corresponding pH changes in each treatment group diverged. The pH of the control group decreased, while that of the continuous PAA treatment group slightly increased. This suggests a different composition of microbes in the biofilm. Although a diagnosis of the biofilm was not performed, based on the pH increase solely, it could be suggested that phototrophic/autotrophic algae were potentially favored over bacteria. This, in addition, could explain that the oxygen concentration measured in the continuous treatment group was higher than the untreated control group. The underlying mechanism was probably that a continuous input of trace amount of PAA, as exogenous ROS, might be still enough to induce oxidative stress to microorganisms. Bacteria, especially in growing phase, have low tolerance to the oxidative stress (Sigler et al., 1999). For survival under oxidative stress, bacteria must increase the synthesis of antioxidative enzymes, which consumes extra energy and thus inhibits their reproduction. In contrast, algae undergo photoprotective processes, which protect them from oxidative damage and increase their tolerance to oxidative stress (Niyogi, 1999). As a consequence, the continuous PAA treatment was a selective pressure to microbial composition in the biofilm, where the periphytic algae and autotrophic nitrifying bacteria were favored over heterotrophic bacteria. Despite of enhanced biofilm formation, the continuous PAA treatment did not result in enhanced nitrification. Instead, similar concentrations of TAN, nitrite-N and nitrate-N were measured in the control and continuous treatment group. The bacterial nitrification is affected by the stratified diffusion of TAN and O_2 in the biofilm. The concentrations of TAN and O_2 decrease from the surface of the biofilm to the attached medium (Chen et al., 2006). As a result, the bacterial nitrification was mostly active in the surface area of the biofilm, whereas limited in the subsurface area. Moreover, the flow-through design continuously flushed the TAN away, resulting in a TAN-limited condition. In this case, the bacterial nitrification of the control and continuous PAA treatment groups was limited by the available TAN. Therefore, a difference of nitrite/nitrate was unlikely to be present.

5. Conclusion

The application of PAA in the rearing water can induce stress to fish, depending on the concentration. The stress induced by PAA at high concentrations (1 mg L^{-1} in the present study) is adaptable for fish, indicated by less intensive behavioral reaction, down-regulated cortisol release and unaffected growth/immunity along repeated applications. Moreover, PAA pulse-applied at high concentrations (1 mg L^{-1}, twice per week in the present study) has a strong anti-microbial effect, resulting in inhibited biofilm formation, partly impaired nitrification, stable pH and highest oxygen in the rearing water. In contrast, PAA continuous-applied at low concentrations (0.2 mg L^{-1} in the present study) degrades fast, and the degradation residues can promote the biofilm formation. To avoid potential risks from opportunistic pathogens harbored in the enhanced biofilm, and to maintain a good water quality, pulse applications of PAA at high concentrations are recommended despite of minor stress in fish.

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