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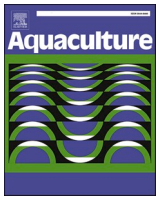

Peracetic acid mode-of-action on aquaculture microbes evaluated by dual-staining flow cytometry

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ABSTRACT

Flow cytometry with dual staining allows an exact quantification of live and dead cells. It is a potential tool for gaining new information on the mode-of-action of disinfection chemicals and supporting efficient microbial control in recirculating aquaculture systems (RAS). Here, we used a dual-staining flow cytometry to examine the disinfection efficiency of peracetic acid (PAA) against i) the fish pathogenic bacterium *Aeromonas salmonicida* and ii) on microbes present in RAS. The method allowed us to accurately quantify the *A. salmonicida* cell response to different PAA concentrations (1, 2, and 10 mg/L) in time and to calculate the PAA concentration needed to achieve 95% cell mortality (LC95 estimates). With the high PAA dose of 10 mg/L, LC95 was reached in <15 min, while with the intermediate dose of 2 mg/L, LC95 was reached only in the end of the 60 min trial and with the low 1 mg/L dose, the disinfection efficiency remained low. The addition of soluble organic matter increased the PAA decay and decreased the disinfection efficiency. In RAS water, disinfection efficiency increased with increasing PAA concentration (from 1 to 10 mg/L) but was significantly reduced with increasing organic matter concentration (TCOD from 7.6 to 30.3 mg O_2/L). The increase in the proportion of dead cells was found to be a reliable proxy for disinfection efficiency in RAS water. In the pure cell culture, both the increase in the proportion of dead cells and the fraction of reduced live cells reflected the disinfection efficiency of PAA. Altogether, the study demonstrated the PAA mode-of-action on aquaculture microbes and showed that flow cytometry with dual staining allows fast and precise examination of microbial viability under different conditions.

1. Introduction

Peracetic acid (PAA) is a strong disinfectant that is widely used in wastewater treatment plants (Kitis, 2004; Luukkonen and Pehkonen, 2017; Rossi et al., 2007) and is increasing applied to food production systems, such as horticulture (Alvaro et al., 2009) and aquaculture (Lieke et al., 2020; Pedersen et al., 2013). The antimicrobial activity is based on PAA oxidizing sulfhydryl and sulfur bonds, which will damage e.g., metabolic enzymes and cell membranes (Kitis, 2004; Wessels and Ingmer, 2013). PAA has been shown to be efficient in e.g., inactivating viruses (Schmitz et al., 2021), decreasing the culturability of bacteria (McFadden et al., 2017), inhibiting the growth of harmful microalgae (Moreno-Andrés et al., 2023), and inactivating fungal spores (Zuo et al., 2022). PAA decomposes to oxygen, water, and acetic acid, and unlike chlorine oxidants, does not form harmful disinfection by-products (Domínguez Henao et al., 2018c). In the aquaculture systems, PAA has become an efficient alternative to common disinfectants (Lazado and Good, 2021; Lieke et al., 2020), as even a relatively low PAA concentration (1 mg/L) can control microbial growth in tank walls (Liu et al., 2017) and in water (Liu et al., 2018, 2023) and eliminate both free-living stages and biofilms of pathogens (Acosta et al., 2021; Good et al., 2022), while not compromising fish welfare (Acosta et al., 2022; Davidson et al., 2019; Gesto et al., 2018; Liu et al., 2018; Straus et al., 2018) or biofilter nitrification (Lepine et al., 2023; Pedersen et al., 2009).

One major challenge in using PAA as a disinfectant is the correct dosage. Once applied, PAA decomposes rapidly, the decay rate being affected by different abiotic factors, such as pH, temperature, and salinity (Kitis, 2004; Liu et al., 2014; Pedersen and Lazado, 2020). Organic matter content in water affects the reactiveness and decomposition of PAA (Pedersen et al., 2009, 2013) and can, subsequently, affect the disinfection efficiency (Domínguez Henao et al., 2018a, 2018b). The increased total suspended solids (TSS) concentration has been found to increase the PAA decay and cause a substantial spontaneous

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consumption: an initial PAA concentration of 2 mg/L was estimated to immediately decrease by 16% in the presence of 40 mg/L of TSS, while in the presence of 160 mg/L TSS, the decrease was increased to 61% (Domínguez Henao et al., 2018a). For high PAA concentration (8 mg/L), the decrease was lower due to the higher stability of higher PAA concentration (Domínguez Henao et al., 2018b; Pedersen et al., 2009), but still increased significantly when TSS increased from 40 to 160 mg/L (4% to 29%). In addition to increasing PAA decay, organic matter directly affects the PAA disinfection efficiency, as lower bacterial inactivation is observed with higher solids concentration, independently of the size distribution (McFadden et al., 2017). This has been suggested to be due to organic matter acting as a condensation nucleus that facilitates bacterial aggregation (Domínguez Henao et al., 2018a). The organic matter levels can vary to a large extent among aquaculture facilities with different treatment technologies, feed intensities, and degree of water exchange, affecting the PAA decay rates (de Jesus Gregersen et al., 2019; Pedersen et al., 2013). This suggests that the disinfection efficiency of PAA is diminished in aquaculture systems with high organic matter concentration, which is often related to high feed loading (Pedersen et al., 2013) or insufficient solids removal. Thus, knowledge on the direct response of bacteria to PAA exposure and the threshold PAA concentration needed to achieve a sufficient microbial inactivation level under different organic matter scenarios is needed.

The importance of biofilter microbes in maintaining good water quality in RAS has been well established, while the microbial communities in the RAS water phase have gained attention only recently. The development of next-generation sequencing technique has allowed to gain large amount of data on the microbial community composition and function, which can be related to water quality, system stability, and the presence of potential pathogens (Aalto et al., 2022b; Dahle et al., 2022; Fossmark et al., 2020). However, sequencing focused on DNA gives information on the potential microbial groups rather than their activity and viability. Furthermore, it requires extensive expertise and time for sample preparation and data analysis, making it less suitable for observing sudden changes in microbial abundance and activity. Fluorescent staining coupled with flow cytometry is an established method to quantify and characterize microbes in drinking water and environmental samples (Besmer et al., 2014). In RAS environment, flow cytometry with SYBR Green, a stain which attaches to any DNA present, has been recently introduced to measure bacterial densities in water (Fossmark et al., 2020; Rojas-Tirado et al., 2018). However, a more advanced dual-staining flow cytometry (Besmer et al., 2014), where the total microbial biomass can be separated into active alive and dead cells by using a combination of SYBR Green and propidium iodide (PI), has not yet been adapted and used to study RAS water that typically has elevated levels of bacteria and organic matter, being so far used only with pure cultures of fish pathogen (Liu et al., 2023).

Here, we tested the suitability of a dual-staining flow cytometry method to examine the disinfection efficiency of PAA against *Aeromonas salmonicida*; a pathogenic, gram-negative, non-spore-forming bacterium causing furunculosis in several fish species (Noga, 2010). We also quantified microbes present in RAS water samples with different levels of organic matter (TCOD: 7.6–15.1-30.3 mg O_2/L). We used a concentration series of PAA, ranging from the low concentration of 1 mg/L, which is generally applied in RAS, to a very high concentration of 10 mg/L that exceeds the currently known LC50 values for several fish species (Straus et al., 2018) and two intermediate concentrations (2 and 5 mg/L) below LC50. We applied and compared two different measures for disinfection efficiency, the increase in the proportion of dead cells and the fraction of live cells removed and we hypothesized that dissolved or particulate organic matter decreases PAA disinfection efficiency.

2. Materials and methods

2.1. Flow cytometry sample preparation and protocol testing

Water samples were collected from two experimental pilot scale freshwater RAS at the Section for Aquaculture, Hirtshals, Denmark (Aalto et al., 2022a; Pedersen et al., 2015). Before analysis, water samples were prefiltered through a cell strainer (40 μm FisherBrand, Thermo Fisher Scientific) to avoid blocking of the sample injection port of the flow cytometer and the filtrates were diluted to a maximum density of 10,000 cells/μL with tap water filtered through 0.2 μm syringe filter (Filtropur S, Sarstedt, Germany). Following the staining protocol for environmental samples (Nescerecka et al., 2016), 500 μL of filtrated sample was labelled with 5 μL of SYBR Green I (100×, MilliporeSigma, Germany) and 5 μL of propidium iodide (600 μM, MilliporeSigma, Germany), incubated at +37 °C, and the concentrations of live and dead cells were measured with flow cytometer (Bd Accuri c6 Plus, BD Biosciences) Two potential modifications to the protocol were tested. First, the potential to replace the recommended Tris buffer (10 mM, pH = 8.1) with MQ water ($n = 3$ per treatment) was tested by incubating diluted (5 times) RAS water with 2 mg/L of PAA for 60 min, after which PAA was neutralized with 10 mg/L of sodium thiosulfate (MilliporeSigma, Germany) and the concentration of live and dead was measured following the protocol presented above. The PAA used in these trials was from a commercial product "Aqua Oxides (Sørensen, Thisted, DK), with 16% PAA and 26% hydrogen peroxide. In the second trial, the effect of incubation time at +37 °C on the proportion of dead cells was tested by incubating prefiltered and stained RAS water samples at 37 °C for 10, 15, 20, and 30 min.

2.2. Disinfection trial with Aeromonas salmonicida cells

Aeromonas salmonicida was cultured in 10 mL nutrient broth (VWR, PA, USA) under stirring (100 rpm) at room temperature and darkness overnight. The isolate was from the culture collection of the fish bacteriology laboratory at DTU Aqua and had been isolated and identified from a disease outbreak in cultured rainbow trout in 2015. The isolate had been stored at −80 degrees in veal infusion broth with an added 15 to 20% glycerol. Before the trial, the bacterial culture was diluted with filtered tap water to cell concentration corresponding RAS environment (6–10 mill/mL). The diluted bacterial culture was divided into 50 mL vials, treated with three different concentrations of PAA (1, 2, and 10 mg/L; $n = 8$ vials per treatment) and incubated at room temperature. The samples were collected after 0, 5, 10, 15, 20, 25, 30, and 60 min and PAA was neutralized with sodium thiosulfate, after which the sample was diluted with filtered tap water to a cell concentration of a maximum of 10,000 cells/μL and the amount of live and dead cells was measured with flow cytometry. To examine the effect of dissolved organic matter (soluble chemical oxygen demand; sCOD) on the disinfection efficiency of the two lowest PAA concentrations (1 and 2 mg/L), RAS water was filtered with 0.2 μm syringe filter and added instead of filtered tap water to achieve a final sCOD concentration of 9.5 mg O_2/L. Samples were then exposed to 1 mg/L or 2 mg/L of PAA and cell concentrations were measured as above. In addition, samples with diluted bacterial culture were measured as control, to ensure that there was no significant increase in the proportion of dead cells due to the experimental conditions. The disinfection efficiency was calculated in two ways: the increase in the proportion of dead cells from all the cells present (ΔProportion of dead cells; Eq. (1)) and the fraction of live cells removed (Eq. (2)) after 60 min.

$$\Delta\text{proportion of dead cells } (\%) = \text{proportion of dead cells}(\%)_{60min} - \text{proportion of dead cells}(\%)_{0min} \quad (1)$$

$$\text{the fraction of live cells removed } (\%) = \frac{\text{live cells}_{0min} - \text{live cells}_{60min}}{\text{live cells}_{0min}} \quad (2)$$

2.3. Disinfection trial with RAS water

RAS water was diluted with 0.2 μm filtered tap water to get three different levels of organic matter, measured as total COD (TCOD): 7.6, 15.1, and 30.3 mg O_2/L (1:3; 1:1, and raw RAS water). The dilutions were treated with four different concentrations of PAA (1, 2, 5, and 10 mg/L; $n = 5$ per treatment) and the disinfection exposure time was 0, 5, 15, 30, and 60 min at room temperature. The two measures of disinfection efficiency were calculated as in 2.2.

2.4. Statistical analysis

The data analysis was conducted using R (version 4.2.2; R Core Team, 2022). The concentrations of live and dead cells between the two staining buffers were compared with a *t*-test. The relationship between the proportion of dead cells (%) and exposure time was examined by linear regression analysis. The relationship between the proportion of dead *Aeromonas salmonicida* cells and exposure time of different PAA concentrations were examined with non-linear regression (function "nls") using a four-parameter log-logistic model (modified Hill equation) for 10 mg/L of PAA and exponential model for the other four treatments (1 mg/L PAA with or without sCOD, 2 mg/L PAA with or without sCOD). The time needed to reach 95% cell mortality (LC95) was estimated and considered to differ between treatments with non-overlapping confidence intervals of the estimates. The differences in the two disinfection efficiency measures between treatments was examined with one-way ANOVA and Tukey post hoc test. The relationship between the two disinfection efficiency measures to TCOD concentration of the sample between different PAA concentrations in RAS water was examined with non-linear regression using a logarithmic model.

3. Results

3.1. Evaluation of the flow cytometry protocol

TRIS buffer and Milli-Q water gave similar results in terms of the total cell concentration (t-test, $t = 0.74$, $P > 0.05$; data not shown) and the concentration of live (intact) cells and dead cells (alive: $t = 1.22$, $P > 0.05$, dead: $t = 0.51$, $P > 0.05$; Fig. 1A). Incubation time had a significant effect on the proportion of dead cells, as every 5 min extension of the incubation time increased the proportion of dead cells with 2.5% point (Fig. 1B), and consequently, a fixed incubation time of 10 min was used in the subsequent trials.

3.2. The effect of PAA on Aeromonas salmonicida with and without dissolved organic matter

When exposing diluted cell cultures of *Aeromonas salmonicida* to different concentrations of PAA for 60 min, both the increase in proportion of dead cells and the fraction of live cells removed showed a similar pattern in response to PAA (Fig. 2). The highest mortality (almost 100%) was achieved with nominal PAA concentrations of 2 and 10 mg/L (medium and high PAA concentrations), while with the low PAA concentration of 1 mg/L, the disinfection efficiency was significantly lower, either 37% (CI95%: 28–46%; dead cells: One-way ANOVA, $F_{4,10} = 174.5$, $P < 0.001$, Tukey post-hoc test, $P < 0.05$) or 38% (29–48%; live cells removed: One-way ANOVA, $F_{4,10} = 165.5$, $P < 0.001$, Tukey post-hoc test, $P < 0.05$). When adding dissolved organic matter in the form of sCOD (filtered RAS water; 9.5 mg O_2/L), the mortality was reduced as compared to the similar cell densities without sCOD addition, being 11% (11–11%) for 1 mg/L of PAA and 62% (54–70%) for 2 mg/L (Tukey post-hoc test; Fig. 2). Similarly, the removal of live cells was 14% (11–17%) for 1 mg/L of PAA and 64% (56–72%) for 2 mg/L of PAA (Tukey post-hoc test; Fig. 2).

The time to reach the threshold where 95% of cells were dead (LC95)

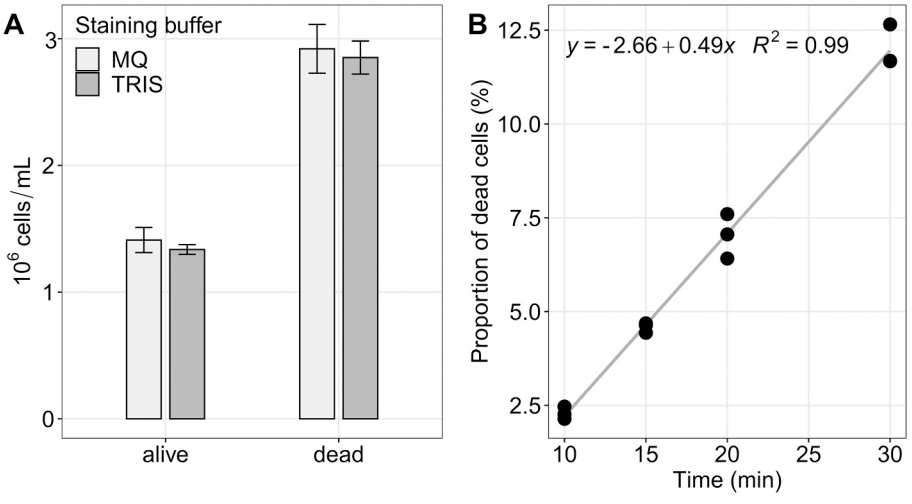

Fig. 1. A. The effect of staining buffer (TRIS = trisaminomethane, MQ = ultra-pure water on the concentration of alive and dead *A. salmonicida* cells ($n = 3$ per treatment) and B) the proportion on dead cells in relation to incubation time at 37 °C. Line indicates the linear regression between dead cells and time.

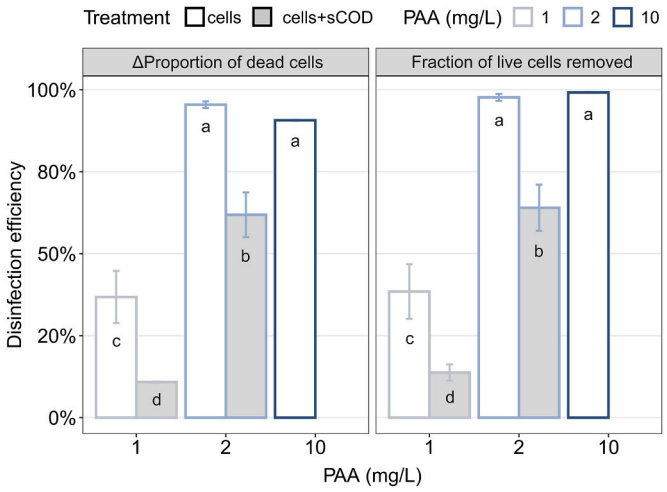

Fig. 2. The disinfection efficiency (the increase in the proportion of dead cells and fraction of live cells removed) of three peracetic acid concentrations (PAA; 1, 2, and 10 mg/L) against *Aeromonas salmonicida cells* with and without RAS soluble organic matter after 60 min exposure time. For PAA of 10 mg/L, only the effect on pure cells without the presence of added soluble organic matter (sCOD) was studied. The letters denote significant differences between treatment groups based on One-way ANOVA and Tukey post-hoc tests.

dropped significantly with increasing nominal concentrations of PAA (Fig. 3). With the high PAA dose (10 mg/L), the response time of cells was short, as the LC95 was reached only after 14 min (CI95%: 13–15 min). With the medium PAA concentration (2 mg/L), the LC95 was reached during the trial, but only after 59 min (58–60 min). With the low PAA concentration of 1 mg/L, the estimated time to reach LC95 was 78 min (77–79 min). When adding dissolved organic matter, the efficiency of PAA in inactivating cells was reduced as compared to the similar cell densities without sCOD addition. With the exposure to 2.0 mg/L of PAA, LC95 was not reached during the trial, but was estimated to occur after 67 min (66–68 min). With 1.0 mg/L of PAA, the estimated time for LC95 was 131 min (130–131 min). Without organic matter addition, LC95 was reached 24% (19 min) faster with medium than with low PAA concentration, while with organic matter, LC95 was reached 48% (64 min) faster with medium than with low PAA concentration.

3.3. *The effect of organic matter level in RAS water on the PAA disinfection efficiency*

When applying different levels of PAA on undiluted and diluted RAS water (to achieve different levels of TCOD), a higher amount of PAA led to a higher disinfection efficiency, but there was a significant decrease in the disinfection efficiency with increasing organic matter concentration (Fig. 4). When using the increase in the proportion of dead cells as a proxy of disinfection efficiency (Fig. 4A), even the highest PAA dosing of 10 mg/L led to the maximum disinfection efficiency of only 62% (59–65%), while the lowest PAA dose (1 mg/L) led to a very moderate disinfection efficiency ($\leq$ 6%). When using the fraction of live cells removed as the disinfection efficiency proxy (Fig. 4B), the values were higher than the efficiency based on dead cells, but the trend remained similar, the values increasing with increased nominal PAA concentration and decreasing with increasing TCOD.

4. Discussion

In this study, we evaluated the suitability of a dual-staining flow cytometry method to measure the biocidal effect of PAA exposure on a specific fish pathogen and on RAS water microbes under different organic matter scenarios. The flow cytometry protocol developed for environmental samples (Nescerecka et al., 2016) was modified to be simpler and faster by replacing Tris- buffer with pure water and by shortening the stain incubation time from 15+ to 10 min. None of these adjustments were found to affect the final results. When using the method for RAS water, it is also important to dilute the samples to have maximum of 10,000 cells/μL (data not shown) before stain addition to ensure that the amount of SYBR Green/PI is high enough to label all the cells present in the sample.

The dual staining method enabled us to precisely quantify the increase in the dead *Aeromonas salmonicida* cells in response to PAA treatment. In agreement with the recent study on PAA and fish pathogens (Good et al., 2022), a disinfection efficiency (measured either as the increase in the proportion of dead cells or the fraction of live cells removed) close to 100% was achieved with a high PAA dosing (10 mg/L) and already within the first 14 min. With a dosing level of 2 mg/L, 95% of cell mortality was achieved but only after 60 min after application, and with a dosing level of 1 mg/L PAA, the achieved disinfection efficiency was <40% and LC95 was estimated to be reached after only ~80 min. Previously, a similar concentration of another PAA product (E400) was found to significantly inhibit the growth of *A. salmonicida* already after 5 min (Meinelt et al., 2015), but this could be explained by initial CFUs being 2–4 times lower than the cell concentrations used in this study (6–10 mill/mL), leading to a higher concentration of disinfectant per cell.

The tested PAA exposures (concentration x exposure time) may overestimate the biostatic and biocidal effects of PAA as compared to the situation in real-life intensive RAS conditions, where the PAA half-life can be in the order of few minutes (Pedersen et al., 2013). Indeed, when we added soluble organic matter with a concentration corresponding to intermediate organic matter loading in RAS, the PAA disinfection efficiency dropped. This effect was more enhanced with the lowest PAA concentration, as time to reach LC95 was prolonged by 70% and the overall disinfection efficiency was dropped by 64–71% as compared to pure cell dilutions. For the PAA dosing of 2 mg/L, the effect of organic matter addition on disinfection efficiency was twice as small (decrease was 34–35%), as there were two times more PAA to oxidize the same amount of organic matter than in the PAA dosing of 1 mg/L.

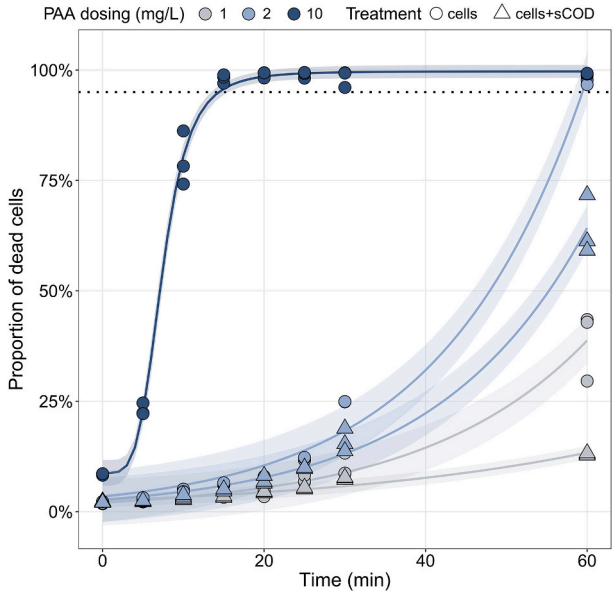

Fig. 3. The proportion of dead *Aeromonas salmonicida* cells at different exposure times with three levels of PAA dosing (1, 2, and 10 mg/L). Circles reflect diluted cell solutions and triangles cell solutions with additional soluble organic matter (sCOD). Dashed line marks for the 95% mortality (LC95) and shaded areas the 95% confidence intervals predicted by a four-parameter log-logistic model for 10 mg/L of PAA and exponential model for the other four treatments. $N = 3$ per dose and time point.

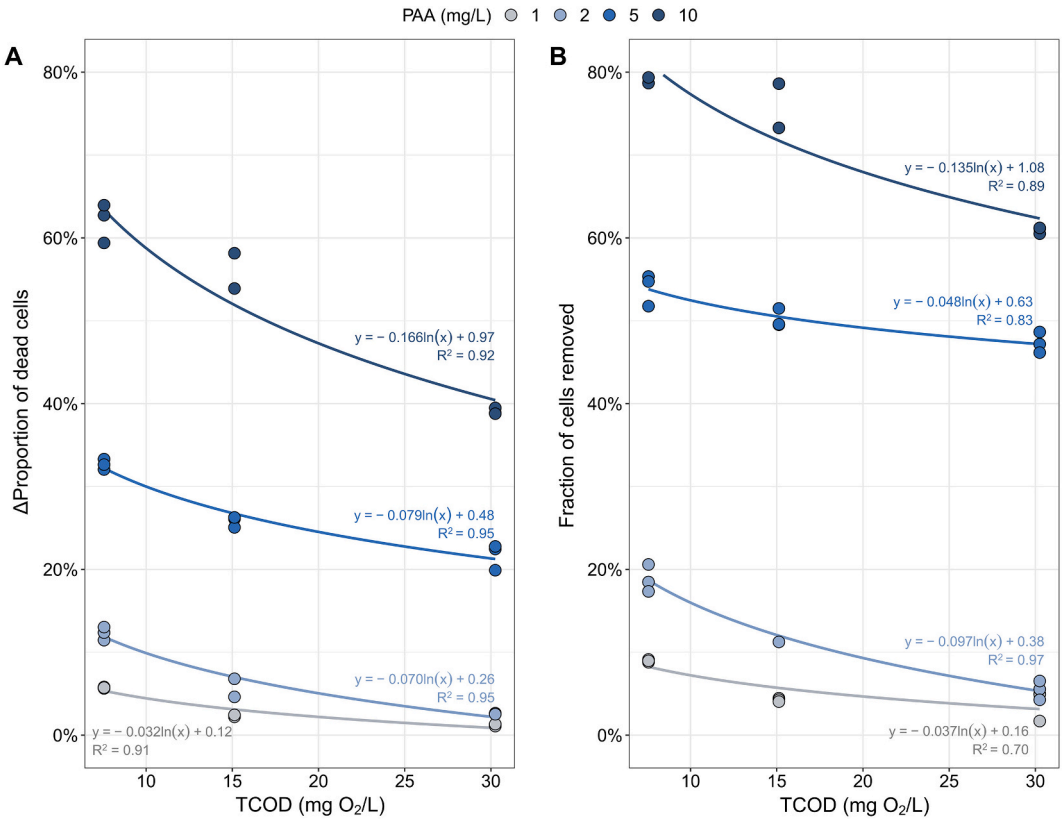

Fig. 4. The disinfection efficiency of PAA at different exposures (1, 2, 5, and 10 mg PAA/L after 60 min) expressed as A) the increase in proportion of dead cells or B) the fraction of live cells removed in RAS water in relation to organic matter (TCOD) concentration. N = 3 per dose and TCOD concentration.

The increasing organic matter is expected to reduce the disinfection efficiency of PAA by increasing the decay and by acting as a condensing nucleus for bacteria, protecting them for disinfection agent. Here, we used soluble organic matter, meaning that the decrease in the disinfection efficiency was more likely due to organic matter increasing the decay rate of PAA (Domínguez Henao et al., 2018b), which decreased the effective window of PAA as compared to the pure cell dilutions, where the only organic matter present are the *A. salmonicida* cells.

When examining the PAA disinfection in RAS water over a range of organic matter concentrations, the disinfection efficiency increased linearly with increasing nominal PAA concentration, while an increased organic matter concentration led to a logarithmic decrease in the disinfection efficiency. In RAS water, organic matter is present as microbial cells, other particulate organic matter, and dissolved organic matter. The typical proportion of soluble COD of the total COD has been found to be ≥70% in Danish trout RASs (de Jesus Gregersen et al., 2019) and in our RAS samples, the proportion was 70% (data not shown). This indicates that our result can be explained with total organic matter decreasing PAA disinfection efficiency by increasing PAA decay and/or with particulate organic matter offering a condensation nucleus to bacteria that will facilitate their aggregation and protect them from disinfection (Domínguez Henao et al., 2018a, 2018b). The drop in the disinfection efficiency (both measures) with increasing TCOD concentration was larger for the highest PAA concentration of 10 mg/L than for the intermediate-high one (5 mg/L). Already previously, the higher PAA doses have been found to lead to a more pronounced protective effect of particulate organic matter (Domínguez Henao et al., 2018a), suggesting that applying pulsed doses of high PAA concentrations in intensive RAS may enhance cell aggregation and provide opportunity for regrowth. For the lowest PAA dose of 1 mg/L, the TCOD-derived decrease in the disinfection efficiency was moderate, suggesting the dose to be low even for the lowest TCOD level.

When we compared the two disinfection efficiency measures in the RAS water trial, we found the fraction of live cells removed to be higher than the increase in the proportion of dead cells, which was not found for the *A. salmonicida* trial. This indicates either that some microbial cells were destroyed or that cells formed aggregates or flocs that were detected as one cell. The aggregation is a common microbial response to disinfection (Bohrerova and Linden, 2006; Winward et al., 2008) and was likely facilitated by the particulate organic matter present. The difference between the two disinfection measures was largest with the two highest PAA dosage levels (22%, CI95: 21–24%), as these concentrations were more stable and likely high enough to release a high amount of hydroxyl radicals that degraded the cell walls and denaturated DNA (Ao et al., 2021; Kitis, 2004). The smaller difference between the disinfection efficiency measures for the two lowest PAA dosage levels (2 mg/L: 6% (4–8%), 1 mg/L: 3% (2–3%) suggests that these concentrations were not high enough to cause complete cell degradation, as has been previously concluded for viral RNA and bacteria in sewer overflow with the correspondingly low PAA concentrations (McFadden et al., 2017; Schmitz et al., 2021). However, as there was no difference in the disinfection efficiency measures in the *A. salmonicida* trial, the more likely explanation is bacterial aggregation and removal through prefiltration with 40 μm or flow cytometer interpreting overlapping SYBR Green fluorescence signals of the cell aggregate into one or fewer cells than there was in the reality. This aggregation has previously been shown to be more pronounced in high PAA concentrations (Domínguez Henao et al., 2018a), explaining the difference between the two measures to be highest with the two highest PAA dose levels. Altogether, these results indicate that even though the proportion of the live cells removed includes both inactivated (dead) cells and degraded cells, using it as a proxy for disinfection efficiency can lead to overestimation in organic matter-rich water, and the increase in the proportion of dead cells should be used instead. When examining the effect in pure cell cultures, both disinfection measures are equally good, as there is no additional organic matter present that would act as a condensing

nucleus.

Both trials showed that the pulse addition of PAA concentration at 1 mg/L pose a minor disinfection efficiency, even against pure *A. salmonicida* cell cultures that are generally considered to be susceptible for PAA disinfection (Good et al., 2022) and in RAS water, the disinfection efficiency of 1 mg/L PAA remained under 10%. Previously, this dose has been found to be high enough to restrict biofilm formation (Liu et al., 2017) and decrease pathogen cell counts (Meinelt et al., 2015) in RAS. As this concentration is also low enough not to harm fish welfare and health, the currently known no-observed effect concentrations being 1.6 mg/L for Atlantic Salmon (Mota et al., 2022) and 2.8 mg/L for rainbow trout (Straus et al., 2018), it has become the most used PAA dose in RAS studies. Our results show that to achieve 50% disinfection efficiency in RAS water with low organic matter content (TCOD of 7.6 mg O_2/L), a pulsed addition of at least 5 mg/L of PAA is needed. This concentration exceeds the LC50 values of most fish species (Straus et al., 2018) and is known to decrease survival and cause changes in swimming behavior and skin and gill histopathology in salmon after 1 h of exposure (Mota et al., 2022). This dosing level can also impair biofilter nitrification processes (Pedersen et al., 2009). However, a very short exposure time (5–15 min) could be an option when using a high PAA concentration, as in the recent study by Acosta et al. (2022), a 5-min exposure with a PAA concentration of 4 mg/L was found not to affect seabream welfare. Addition of the suggested high PAA concentration could be used as emergency dosing e.g., to control sudden pathogen outbreaks. However, a long-term application should be avoided for the aforementioned reasons and because it could promote microbial regrowth by increasing the amount of easily degradable carbon source (acetic acid) in RAS system. Instead, using other methods such has foam fractionation and/or ozone (Aalto et al., 2022b; de Jesus Gregersen et al., 2021) to lower the bacterial abundance and the amount of biodegradable organic matter in the system would be a more beneficial long-term option.

5. Conclusions

This study demonstrates the suitability of dual-staining flow cytometry for examining microbes suspended in RAS water and their response to disinfection, in this case to PAA. We conducted a series of trials with replicated samples and measured the response of either pure *A. salmonicida* cultures or RAS water microbes during a short-term PAA exposure. This is difficult with current available methods to quantify microbial abundance, which requires living cells to be grown under specific conditions with a risk of excluding a large fraction of microbial groups (plate counting), advanced machinery and expensive chemicals (qPCR), and individual and laborious preparation and analysis of samples (dual staining microscopy). In addition to being fast and accurate, flow cytometry targets any kind of organism with DNA (SYBR Green stain) and allows the separation of dead or damaged cells from live ones. The only challenge for applying dual staining method is that the samples cannot be stored, meaning that it is most suitable for laboratory trials or requires a portable flow cytometer in the field trials. However, in case the needed information is only the total amount of cells, samples can be fixed with glutaraldehyde (Fossmark et al., 2020). Future studies are suggested to include flow cytometry fingerprinting allowing the quantification of specific microbial groups (e.g., pathogens) and their response to different conditions in RAS in real-time (Props et al., 2016).

CRediT authorship contribution statement

Sanni L. Aalto: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Lone Madsen:** Methodology, Writing – review & editing. **Lars-Flemming Pedersen:** Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Lars-Flemming Pedersen reports financial support was provided by Henrik Henriksen's Foundation.

Data availability

Data will be made available on request.

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