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Short communication

Antibacterial effects of peracetic acid disinfection assessed by culturability, enzyme activity and flow cytometry

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ABSTRACT

Aquaculture water disinfection with around 1 mg/L peracetic acid (PAA) was proved in various studies to effectively impair microbial proliferation and reduce pathogen-caused fish mortality. In the present study, we monitored the antibacterial effect of biweekly water disinfection with 1 mg/L PAA at a local flow-through fish farm for 8 weeks. Results from replicate water samples showed inconsistency of antibacterial efficacy assessed with colony forming units (CFU) of total culturable bacteria and a commercial enzyme activity assay. Single PAA disinfection caused up to 90% reduction of CFU in water. In contrast, the microbial hydrolase activity was hardly affected or even enhanced. Both methods have limitations and can't truly represent viable bacteria. We further monitored the temporal response of two *Yersinia ruckeri* isolates in suspensions to single PAA disinfection via high-throughput flow cytometry. We found out that the bacterial cell membrane damage appeared not instantly but with a lag phase of at least 2 h post disinfection at 4 °C. The abundance of cell membrane damage, considered as cumulative bacterial mortality, kept rising and maximized at 48 h post disinfection. The survived cells were more likely to enter the viable but non-culturable (VBNC) state at higher PAA concentrations. One isolate was more prone to enter the VBNC state than the other. Neither *Y. ruckeri* isolate showed measurable reaction with the enzyme substrate of the commercial microbial hydrolase activity assay. Our findings refresh knowledge about the dynamics of bacterial cell mortality post PAA disinfection (probably also similar non-thermal disinfection methods). Aquaculture practice should be aware of the delayed disinfection outcomes and pay attention to the dominance of VBNC state.

1. Introduction

Peracetic acid (PAA) is a sustainable chemical disinfectant that is increasingly used in fish production systems. A great advantage of PAA is its minimal and recoverable impacts on fish health when applied at therapeutic relevant concentrations, usually below 2 mg/L PAA (Liu et al., 2017b; Liu et al., 2017a; Gesto et al., 2018; Soleng et al., 2019; Lazado et al., 2020; Liu et al., 2020; Haddeland et al., 2021; Acosta et al., 2022; Mota et al., 2022; Osório et al., 2022). This enables the fish-present water disinfection with PAA, which is authorized for organic aquaculture in EU (EU Regulation No. 1358/2014). The fish-present water disinfection with PAA was proved in previous studies to effectively reduce mortality caused by specific fish pathogens (Sudova et al., 2010; Straus et al., 2012; Pedersen and Henriksen, 2017; Lazado et al., 2019; Good et al., 2020; Abu-Elala et al., 2021) or relieve gill inflammation related to microbial-enriched water (Liu et al., 2018). In both cases, the fish-beneficial effect of PAA is realized through its strong oxidizing potency (Ao et al., 2021). At therapeutic relevant concentrations, PAA works particularly effective against bacteria (Meinelt et al., 2015; Liu et al., 2021). The antibacterial efficacy of PAA water disinfection was usually assessed by standardized agar plate cultivation of specific pathogenic bacteria or total heterotrophic/coliform bacteria (Verner-Jeffreys et al., 2009; Dominguez Henao et al., 2018; Hushangi and Hosseini Shekarabi, 2018; Liu et al., 2018; Davidson et al., 2019; Lindholm-Lehto et al., 2020; Abu-Elala et al., 2021; Acosta et al., 2021; Good et al., 2022). Recently, 16S rRNA sequencing was introduced and revealed non-selective effect of PAA water disinfection on bacterial community in recirculating aquaculture systems (Lindholm-Lehto et al., 2019; Suurnäkki et al., 2020; Teitge et al., 2020). Both methods have their limitations: CFU from the plate cultivation is an index of bacterial culturability yet not necessarily the viability dependent on specific substrate and incubation conditions (Emerson et al., 2017); 16 s rRNA

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sequencing does not distinguish live and dead bacteria (Li et al., 2017). The process of bacterial mortality or survival post PAA water disinfection remains unclear. In addition, there's increasing demand on easy-to-operate and real-time methods for assessing disinfection efficacy in aquaculture practice. Microbial enzymatic assays based on activities of catalase (Pedersen et al., 2019) or hydrolase (Pedersen et al., 2017; Rojas-Tirado et al., 2017) have great potentials. It is, however, still necessary to test whether they can be used as an indicator for aquaculture water disinfection circumstances.

2. Material and methods

2.1. Field experiment

In the present study, we performed PAA water disinfection at a local flow-through raceway fish farm for over 2 months. The fish farm consisted of one inflow channel connected with 30 replicate raceways in a comb-like layout. The raceway outflows merged into one outflow channel. Most raceways were stocked with juvenile rainbow trout or arctic char at densities of 70–80 kg/m^3. A 15%(*w/v*)-PAA product, Peraclean® 15 (Evonik), was applied via a membrane pump into the central inflow (beginning of the inflow channel) behind a turbulent mixer for 1 h every Monday and Thursday. The dosing speed was adjusted to 12 L PAA product/h based on measured central inflow rate of averagely 500 L/s to achieve 1 mg/L PAA in the inflow water. As the water temperature rose approximately from 10 °C to 17 °C, the farm was operated from flow-through mode to partial recirculation mode with progressively increasing water re-use rate (Fig. 1C). The partial recirculation was realized through pumping water from the outflow channel back to the inflow channel (behind the PAA dosing spot). Bacteria in water from non-disinfected upstream and two replicate raceways (near outflow region) were monitored during the PAA dosing scheduled on every Thursday except 5th May (Fig. 1A & B). During active PAA dosing when residual PAA in both raceways was verified to be 0.71–1.26 mg/L PAA according to Liu et al. (2016), two sets of water samples were simultaneously collected from aforementioned locations (non-disinfected upstream and disinfected two raceways). Sterile bottles were filled completely full and the lids were sealed underwater. All water samples were immediately stored on ice in darkness until lab analysis in about 3 h. One set of water samples was assessed with a commercial bacterial hydrolase activity assay, Bactiquant® Water Kit (BactiQuant A/S, Denmark). Briefly, bacteria from 50 mL water sample were retained on a 0.22-μm syringe membrane filter (Millex®-GP, Merck) and incubated at room temperature (22 °C) with the provided enzyme substrate for 10 min. The reaction between a bacterial hydrolase and the enzyme substrate resulted in release of a fluorophore, which was measured with the provided fluorometer. According to Rylander and Calo (2012), the Bactiquant measurement is targeted on bacterial beta-*N*-acetylglucosaminidase. It hydrolyzes the fluorogenic enzyme substrate 4-methylumbelliferyl *N*-acetyl-beta D-glucosaminide. The released fluorophore 4-

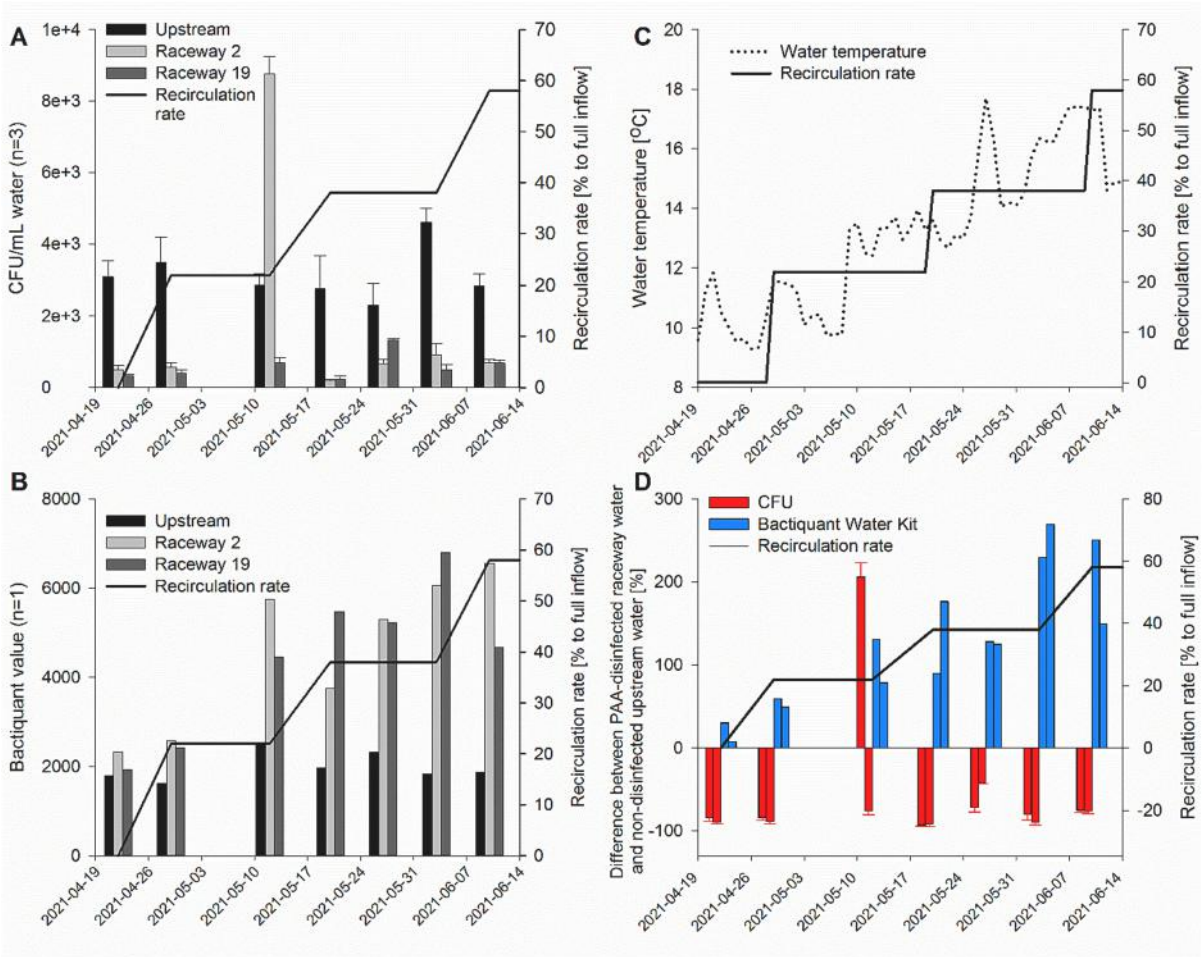

Fig. 1. The colony forming units (CFU, A) and hydrolase activity (assessed with Bactiquant® Water Kit, B) in water samples between two replicated raceways disinfected with 0.71–1.26 mg/L PAA compared to non-disinfected upstream. Recirculation rate of the farm progressively increased from 0% at the first sampling date to 58% of the full inflow (500 L/s) as the water temperature increased (C). CFU was enumerated from triplicate agar plates (Error bar: standard deviation). Bactiquant measurement was performed without replicates. Comparison between CFU and Bactiquant Water® Kit was conducted with proportional differential values between PAA-disinfected raceway water and non-disinfected upstream water (D). Date format: YYYY-MM-DD.

methylumbelliferyl can be excited at 365 nm and measured at 445 nm. The other set of water samples were serial diluted with autoclaved distilled water and cultivated on triplicate Casein peptone Soybean peptone (CASO, Carl Roth) agar plates according to Meinelt et al. (2015). Colony forming units (CFU) were enumerated after 2-week incubation at 15 °C.

To balance the interference from the partial recirculation, we repeated the test at a later time point when the recirculation rate was maximized at 290 L/s. Instead of dosing PAA at the central inflow, we dosed 1 mg/L PAA via a peristaltic pump in two replicate raceways. Water samples were simultaneously collected from the raceway inflows that was non-disinfected and at the end of the raceways when about 1 mg/L PAA was verified. Bactiquant measurement and plate cultivation were performed immediately onsite with fresh water samples.

2.2. Lab experiment

In order to observe the response of bacteria to PAA disinfection on cellular level, we further observed the temporal changes of bacterial cell viability state after single exposure to PAA via flow cytometry. Environmental water samples contain many suspended solids that may clog the nozzle of the flow cytometer and if not, result in false read. For the best accuracy, we performed the test with two cultured isolates of *Yersinia ruckeri*. It is a bacterial fish pathogen causing enteric red mouth disease in salmonids (Kumar et al., 2015), hence an important target of disinfection. Both isolates were originally isolated from infected rainbow trout and kindly provided by Dr. Elisabeth Nardy from the Chemical and Veterinary Investigation Office (Stuttgart, Germany) and had been investigated in our lab for years. Briefly, colonies from 2/3-day old cultures of both isolates were resuspended in sterile-filtered reconstituted water, respectively (one colony/10 mL water). The reconstituted water was prepared following ISO 7346–2 and ISO 15088 standards and recipe was provided in our early study (Meinelt et al., 2015; Liu et al., 2021). The resulting suspensions had densities of approximately 10^7 cells/mL and were either non-disinfected or disinfected with single dose of PAA at serial concentrations of 0.3–1.2 mg/L. The suspensions were kept at 4 °C in darkness immediately after the treatments. For flow cytometry analysis, suspensions were 100× diluted, differential stained, transferred on a cooled sterile 96-well plate and automated counted in quadruplicates with a flow cytometer (Macsquant 10, Miltenyi Biotec, Germany) at 2 h, 24 h, 48 h and 72 h post treatment. Viability staining based on membrane-integrity was performed with Sybr Green I (1× diluted from 10.000× concentrate, Invitrogen, USA) and 1 μg/mL propidium iodide (Merck, Germany) in darkness for 10 min prior to counting. The gating of 100% membrane-intact and 100% membrane-damaged bacterial cells plotted on FITC (525–550 nm) and PerCP-Vio700 (655–730 nm) filters were determined with resuspended 2/3-day old cultures and their heat-inactivated (boiling for 5 min) aliquots, respectively. After PAA disinfection, bacterial cells within the membrane-intact gating were considered viable, while those outsides were considered damaged. For detecting culturability changes, the same bacterial suspensions after 15-min disinfection were serial-diluted and cultivated on triplicate CASO agar plates as described afore. CFU enumeration was performed after 72-h incubation at 25 °C. For detecting hydrolase activity changes, 5 mL undiluted bacterial suspensions were measured with the Bactiquant® Water Kit as described afore, however, with incubation time up to 60 min at room temperature (22 °C).

2.3. Statistics

Probit-regression analysis with SPSS v22 was conducted between PAA concentrations and separately the 48-h viability and 72-h culturability of both *Y. ruckeri* isolates from the lab experiment. The regressions between isolates and between viability and culturability within each isolate were compared via relative median potency (RMP). Difference was considered significant when the 95% confidence interval of RMP did not include 1.

3. Results and discussion

When dosing PAA at the central inflow, results showed up to a 10-fold reduction of CFU in PAA-disinfected raceway water compared to non-disinfected upstream water (2.3–4.6 × 10^3 CFU/mL water) from most samples (Fig. 1A). In contrast, the hydrolase activity in PAA-disinfected raceway water was always higher than in non-disinfected upstream water (Fig. 1B & D). Noteworthy, the degree of the enhancement of hydrolase activity matched well with the recirculation rate (Fig. 1D). Instead of a seemingly enhancement of bacterial hydrolase activity by PAA, the true enhancement was more likely correlated with the recirculation rate. The recirculated water brought suspended solids back to the raceways. In nutrient-rich aquaculture systems, suspended solids are positively correlated to bacterial load because of surface colonization (Pedersen et al., 2017; Becke et al., 2019; Schumann and Brinker, 2020). Each Bactiquant measurement used 50 mL water sample, while each plate cultivation used 100 μL water samples only. Consequently, it was more likely to catch many bacterial aggregates with Bactiquant measurement than with plate cultivation. Even in case of aggregates on agar plates, they probably end up with single CFU. In contrast, the hydrolase activity of single aggregate is greatly magnified compared to single bacterial cell. Therefore, Bactiquant is more sensitive than CFU to the interference from bacterial aggregates.

When dosing PAA in individual raceways via a peristaltic pump, the proportional difference of hydrolase activity between disinfected and non-disinfected waters in each raceway was 13.2% and − 1.4%, respectively. Correspondingly, the proportional difference of CFU was −71.5% and − 91.9%, respectively. This result agreed with the earlier results when the farm was initially operated in zero-recirculation mode and PAA was applied at the central inflow (Fig. 1D, first left columns). Therefore, single water disinfection with 1 mg/L PAA resulted in negligible or slightly enhanced bacterial hydrolase activity but great reduction of bacterial culturability. Some bacterial cells may survive and enter a specific viable but non-culturable state (Schottroff et al., 2018). Bacterial enzymes may remain their activities outside cells (Baltar, 2018). In addition, real-time enzymatic measurements may miss out the bacterial mortality if it happens not immediately post disinfection.

Results from flow cytometry showed that the proportions of viable cells in non-disinfected *Yersinia ruckeri* isolates were 98.8% and 98.4%, respectively. The viable cell counts in non-disinfected suspensions of both isolates slightly increased from 2 h to 24 h, and subsequently stagnated or even slightly declined in case of isolate 2. In comparison, the viable cell counts in PAA-disinfected suspensions began to differ from the non-disinfected controls at 24 h post PAA disinfection. Higher PAA concentration resulted in greater decline of viable cell counts, while the lowest tested PAA concentration had minimal impact on viable cell counts. The strongest decline of viable cell counts occurred between 24 h and 48 h post PAA disinfection at all concentrations. From 48 h to 72 h post PAA disinfection, viable cell counts in PAA-disinfected isolate 1 stagnated, while those in isolate 2 increased, especially at higher PAA concentrations (Fig. 2). These results indicate a general lag phase from the disinfection stress till the onset of cell mortality with both bacterial isolates. The lag phase seemed to be longer when the disinfection stress was milder. After the lag phase, the mortality of stressed bacterial cells occurred continuously across a long period instead of instantly within a short period. The bacterial mortality stopped at 48 h post disinfection. Afterwards, the regrowth of the survived *Y. ruckeri* cells took effect in an isolate-dependent manner. To exclude the interference from the regrowth, data from 48 h post disinfection was selected to represent the maximal impact of PAA disinfection on bacterial viability. In contrast to flow cytometry, plate cultivation had no interference from the resumed growth of survived bacteria. The 72-h CFU, therefore, can represent the maximal impact of PAA disinfection on bacterial culturability.

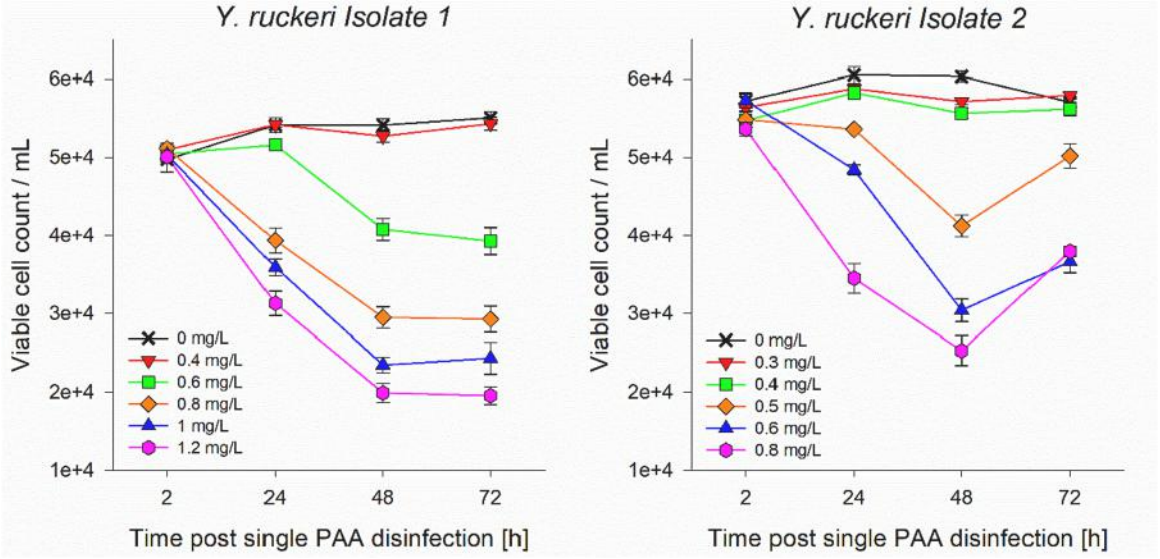

Fig. 2. Viable cell count in two *Y. ruckeri* isolates determined by flow cytometry based on cell membrane permeability at 2 h, 24 h, 48 h and 72 h post single disinfection with PAA at low concentrations or without disinfection (0 mg/L). Error bars: standard deviation ($n = 4$).

Probit regression analysis showed no significant difference between the two isolates regarding to dose-response of both viability and culturability (95% confidence interval of RMP included 1). Within each isolate though, significant difference between the dose-response of viability and culturability was detected in isolate 1 only (95% confidence interval of RMP was between 0.003 and 0.811). This difference could also be reflected by the large gap between viability and culturability curves of the isolate 1 (Fig. 3). In contrast, the viability-culturability gap of the isolate 2 was small. Within the viable cells of both isolates, the proportion of the VBNC state generally tended to increase at higher PAA concentrations. However, the VBNC state in isolate 1 became more dominant at a lower PAA concentration than in isolate 2. The isolate 1 tended to enter the VBNC state at a lower stress level than the isolate 2. In response to equal stress of 0.6 and 0.8 mg/L PAA, the resumed growth between 48 and 72 h post stress was present in isolate 2 only (Fig. 2). This was caused by a small portion of survived cells in isolate 2 that maintained their culturability (Fig. 3). This type of cells is similar to the persister cells that were found resistant to antibiotic treatment (Fisher et al., 2017). Ayrapetyan et al. (2018) suggests that the persister cell is an intermediate state within the progressive cell transition from normal to VBNC state post stress. Accordingly, the progressive transition to VBNC state of isolate 1 was shorter than isolate 2. It might be related to the genetic difference or acquisition of resistant genes from foreign plasmid (De Grandis and Stevenson, 1985; Rodgers, 2001; Huang et al., 2014). In future work, we will try to explain the observed divergence of survival strategies of the two *Y. ruckeri* isolates on genetic and gene expression levels.

The Bactiquant measurements of both bacterial isolates resulted in

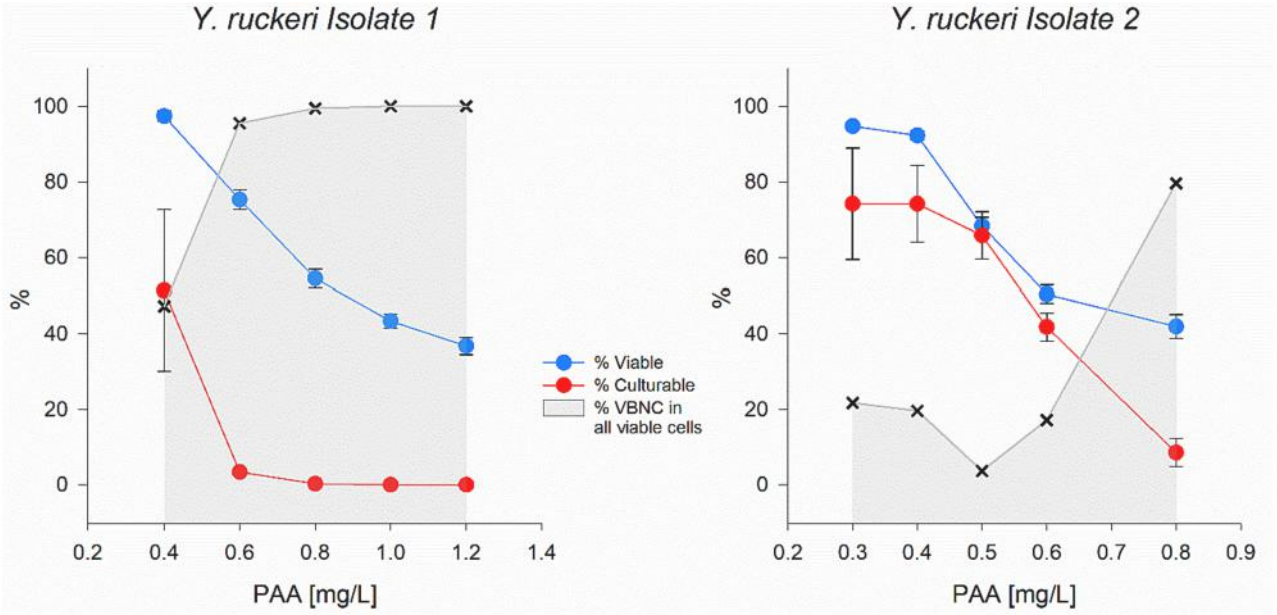

Fig. 3. The viability, culturability and the proportion of viable but non-culturable (VBNC) state in all viable cells of both *Y. ruckeri* isolates post single PAA disinfection at low concentrations compared to non-disinfected controls. Viability was determined by flow cytometry based on cell membrane permeability after 48-h dark storage at 4 °C post PAA disinfection. Culturability was determined by colony forming units after 72-h plate cultivation at 25 °C. Error bars: standard deviation (n = 4 for % Viable; $n = 3$ for % Culturable).

absorption values equal to the blank sample and hence, were considered invalid according to the manufacture's instruction. The invalidation was consistent regardless of PAA disinfection and the reaction time with the enzyme substrate. Indeed, no gene related to beta-*N*-acetylglucosaminidase in *Y. ruckeri* has been reported in NCBI database (Sayers et al., 2022). Attentions should be paid to the incompatibility of the Bactiquant Water kit with monocultures or mixed cultures dominated by beta-*N*-acetylglucosaminidase-negative bacteria.

Although water temperature in aquaculture practice is usually higher than 4 °C, the lag phase between PAA disinfection (probably also other non-thermal disinfection) and the development of bacterial mortality/inculturability should still be present although probably shortened. In case of stationary bacteria, the disinfection is expected to induce cell mortality or VBNC state progressively with a lag phase. In case of planktonic bacteria, the lag phase between disinfection and cell mortality/VBNC state is accompanied by the hydrodynamic-dependent spatial movement of cells. In both cases, assessing the disinfection efficacy should be performed multiple times with properly stored samples across a time period. Special attentions should be paid to the abundant VBNC state of survived bacteria after single PAA disinfection and risks of their potential resuscitation. Thus, reinforced PAA disinfection and other disinfection methods through periodic repetition is necessary.

CRediT authorship contribution statement

Dibo Liu: Conceptualization, Supervision, Project administration, Funding acquisition, Methodology, Investigation, Writing – original draft, Formal analysis, Visualization. **Eric Freches:** Methodology, Investigation, Writing – review & editing. **Christopher Naas:** Methodology, Investigation, Writing – review & editing. **Sascha Behrens:** Methodology, Investigation, Writing – review & editing. **Thomas Meinelt:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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