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Published in:
Diseases of Aquatic Organisms

Link to article, DOI:
10.3354/dao02847

Publication date:
2015

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Growth inhibition of *Aeromonas salmonicida* and *Yersinia ruckeri* by disinfectants containing peracetic acid

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ABSTRACT: Peracetic acid (PAA) is a therapeutic agent used for disinfection in aquaculture, but it must be investigated thoroughly in order to mitigate diseases without harming the fish. Successful disinfectants (like PAA) should not leave dangerous residues in the environment in order to successfully contribute to sustainable aquaculture. The aim of our study was to compare the effectiveness of 6 commercial PAA products with different molecular PAA:H2O2 ratios to reduce bacterial growth of *Aeromonas salmonicida* and *Yersinia ruckeri* and to determine effective concentrations and exposure times. All products reduced colony-forming units (CFUs) of *A. salmonicida* and *Y. ruckeri*. Products with higher molecular PAA:H2O2 ratios inhibited growth better than products with lower molecular PAA:H2O2 ratios at the same PAA concentration; this indicates that H2O2 is not the driving force in the reduction of *A. salmonicida* and *Y. ruckeri* growth by PAA in vitro. The practical application of the products with high molecular PAA:H2O2 ratios should be prioritized if these pathogens are diagnosed.

KEY WORDS: PAA · Peroxide · H2O2 · Furunculosis · Yersiniosis · Fish pathogen · Aquaculture · Bacteria · Water treatment

INTRODUCTION

For intensive aquaculture, high animal density and handling can increase the susceptibility of fish to infections. Therefore, therapeutic agents are used for effective fish farm biosecurity and to inactivate potentially pathogenic micro-organisms in aquaculture (Verner-Jeffreys et al. 2004, 2009). However, many therapeutic agents have been banned in the European Union, and elsewhere, because of harmful effects to the environment. Therefore, Schlotfeldt (1998) stated that there is a crisis in the treatment of diseases in aquaculture, since only a few therapeutic agents are allowed in Europe.

Alternative therapeutic agents, such as hydrogen peroxide (H2O2), peracetic acid (PAA) and ozone are able to mitigate diseases without harming the fish. Moreover, these agents leave no dangerous residues in the environment in order to successfully contribute to sustainable aquaculture. The oxidizing agents H2O2, PAA and ozone are low-molecular weight compounds and can pass through the bacterial membrane to react with internal cellular components, thereby causing damage to cell structure and the
release of intracellular components (Finnegan et al. 2010).


Fish pathogens such as Vibrio anguillarum, Flexibacter ovolyticus and Aeromonas salmonicida cause a significant decrease in halibut larval survival (Bergh et al. 1992, 1997). The concentrations of bacteria in halibut larvae were higher overall in recirculated water than in flow-through water (Verner-Jeffreys et al. 2004). As a promising therapeutic agent in aquaculture, 7 mg PAA l−1 decreased colony-forming units (CFUs) of Salmonella typhimurium by at least 5 log units after treatment for 1 h (Jolivet-Gougeon et al. 1996). Marchand et al. (2012) tested different PAA products at different concentrations against the fungus Saprolegnia parasitica and the bacterium Flavobacterium columnare; they found a significant reduction of bacterial growth at 1 mg l−1 for 2 of the substances tested. Other PAA products showed significant bacterial inhibition at concentrations of 2 or 4 mg l−1. Products with a lower molecular PAA:H2O2 ratio showed better growth inhibition at lower PAA concentrations in their study.

A. salmonicida is the causative agent of furunculosis (ulcer disease, goldfish ulcer disease, carp erythodermatitis) in cyprinids (Hiney & Olivier 1999) and motile aeromonad infection in salmonids (Noga 2010). Yersinia ruckeri is the causative agent of furunculosis (ulcer disease, goldfish ulcer disease, carp erythodermatitis) in cyprinids (Hiney & Olivier 1999) and motile aeromonad infection in salmonids (Noga 2010). Both diseases are costly to the aquaculture industry. Verner-Jeffreys et al. (2009) tested a product containing 5% PAA against A. salmonicida, Y. ruckeri, Carnobacterium piscicola and Lactococcus garvieae using concentrations of 0.0025, 0.005, 0.01, 0.0165 and 0.025 mg PAA l−1; they found the PAA product was toxic to the bacteria at concentrations of 0.0165 and 0.025 mg l−1.

There are very limited published data on the activity of PAA disinfectants against A. salmonicida and Y. ruckeri. The aim of the present study was to compare the effectiveness of PAA products with different molecular PAA:H2O2 ratios to reduce bacterial growth of A. salmonicida and Y. ruckeri and to determine effective concentrations and exposure times in vitro.

**MATERIALS AND METHODS**

Six commercially available PAA products with different molecular PAA:H2O2 concentrations were tested (Table 1). The product with the highest molecular PAA:H2O2 ratio (E400) was set as the reference substance. Aeromonas salmonicida and Yersinia ruckeri monocultures were obtained from the Chemisches und Veterinäruntersuchungsamt Stuttgart (Stuttgart, Germany).

The bacteria were cultured on CASO agar (Carl Roth) at 22°C in plastic petri dishes. After visual growth was observed, the plates were stored at 4°C and sub-cultured every 2 wk. The transfer of the bacteria to a new plate was done with an inoculating loop by spreading the inoculum. All plates were checked for contaminations via stereo-microscopy. At 72 h after inoculum, bacteria were transferred from an agar plate with an inoculating loop to CASO Bouillon (Carl Roth), vortexed and cultured for 24 h. A 0.1 ml bacterial broth was mixed with 10 ml of each solution and cultured for 24 h. The broth was assayed for colony-forming units (CFUs) using CASO agar (Carl Roth). The colony-forming units were counted after 24 h of incubation at 22°C.
PAA solution to give nominal concentrations of 0.1, 0.5, 1.0, 1.5 and 2.0 mg l⁻¹ in deionized water; these concentrations were chosen based on preliminary experiments. By diluting the PAA, its disinfectant effect is greatly diminished and there was no need for a neutralizer. A geometric serial dilution was prepared with each of these PAA solutions from 10⁻¹ to 10⁻⁷ (Y. ruckeri) and from 10⁻¹ to 10⁻⁶ (A. salmonicida). At exposure times of 5, 10, 20 and 30 min, CFUs were determined by using the drop-plate method (ISO 2005). For this method, 100 µl from each dilution was distributed drop-wise on a 90 mm sterile CASO agar plate containing 10 ml agar. The reduction of CFUs was determined at different concentrations and exposure times.

An unexposed initial count was a control for the time experiment. After 24 h (Y. ruckeri) and 48 h (A. salmonicida) of incubation, the CFUs were enumerated by means of a stereo-microscope. The incubation time of A. salmonicida was increased due to slower growth. All test procedures were performed under sterile conditions and at 22°C.

The influences of exposure time, concentration and product on the number of CFUs were analyzed by multiple linear regression models. Additionally, box plots were used to illustrate the influence of exposure time and concentration on the number of CFUs for each product.

### RESULTS

#### Aeromonas salmonicida

In general, A. salmonicida CFUs decreased with increasing PAA concentration for all products we tested (Table 2). A PAA concentration increase of 1 mg l⁻¹ reduced the CFUs by >2 × 10⁻⁶. The number of CFUs also decreased with increasing exposure time for all products. The increase of exposure time by 1 min decreased the CFUs by >30 000. However, the effect is negligible after 10 min exposure time. The effect of E400 in reducing CFUs was significantly different from that of the Lspez, E35 and AC150 products (p < 0.001), but similar to that of the SI400 and E250 products (Table 2).

#### Exposure time

Our reference substance, the E400 product, reduced the mean A. salmonicida CFUs to <2.5 × 10⁻⁶ after an exposure time of 5 min (Fig. 1); this was also true for the Lspez, SI400 and E250 products. The SI400 product showed the strongest effect on reducing A. salmonicida CFUs within the same exposure time. For the E35 product, mean A. salmonicida CFUs of <2.5 × 10⁻⁶ were realized after an exposure time of 10 min. In contrast, mean CFUs of <2.5 × 10⁻⁶ were not achieved at any exposure times tested with the AC150 product; exposure of AC150 up to 30 min did not lower the CFUs of A. salmonicida.

#### PAA concentration

Increasing PAA concentrations of all tested PAA products resulted in reduced A. salmonicida CFUs (Fig. 2). However, an increase of the PAA concentration from 1.5 to 2 mg l⁻¹ did not decrease CFUs further. The Lspez and E35 products effectively reduced the A. salmonicida CFUs to <2.5 × 10⁻⁶ at a PAA concentration of at least 1 mg l⁻¹. The same effect was achieved by SI400 and E250 at a PAA concentration of 0.5 mg l⁻¹. In contrast, the AC150 product did not reduce the CFUs to <2.5 × 10⁻⁶ at any tested PAA concentration.

#### Yersinia ruckeri

In general, the number of Y. ruckeri CFUs was lower with increasing concentrations for all products we tested. A PAA concentration increase of 1 mg l⁻¹ reduced the CFUs by >10 × 10⁻⁶ (Table 3). Concentrations of 2 mg PAA l⁻¹ reduced the CFUs to nearly zero. All products reduced the Y. ruckeri CFUs after 5 min exposure time. An increase of the exposure by 1 min lowered the CFUs by >1.8 × 10⁻⁶ (Table 3). The Lspez, E35, AC150, SI400 and E250 products were not significantly different from E400 (Table 3).

<table>
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Table 2. Multiple linear regression analysis of Aeromonas salmonicida colony-forming units. The E400 product has the highest peracetic acid concentration and was set as the reference substance to compare other results.
All products except E250 reduced the mean CFUs of *Y. ruckeri* to <15 × 10^{-6} after an exposure time of 5 min (Fig. 3). The E250 product needed a longer exposure time to reduce CFUs.

### Exposure time

Following exposure to the E400, E35 and SI400 products at PAA concentrations of 0.5 mg l\(^{-1}\), the mean *Y. ruckeri* CFUs <15 × 10^{-6} were achieved (Fig. 4). The Lspez, AC150 and E250 products at concentrations of 1 mg PAA l\(^{-1}\) reduced CFUs.

### DISCUSSION

**Aeromonas salmonicida**

For the experiment with *A. salmonicida*, we found that concentration, time and PAA product have significant influences on the growth of the bacteria; all products were able to reduce CFUs. Multiple linear regressions indicated that 1 concentration step reduced the CFUs by 2 × 10^{-6} and 1 time step reduced the CFUs by 33 000 (Table 2). Significant differences in CFUs were found with the E400 versus Lspez, E35 and AC150 products, while the E250 and SI400 products were not significantly different from the E400 product.

<table>
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Table 3. Multiple linear regression analysis of *Yersinia ruckeri* colony-forming units. The E400 product has the highest peracetic acid concentration and was set as the reference substance to compare other results.
Compared with concentration, time seems to be of less influence in reducing the bacteria. This implies that the effect of the PAA:H₂O₂ mixture is very rapid, and only short exposure times are necessary to reduce pathogens in the water.

Considering time as well as concentrations, the E400, E250 and SI400 products seem to be the most effective against the Gram-negative bacteria *A. salmonicida*. The minimum inhibitory concentration to affect bacterial growth ranged from 0.5 mg l⁻¹ for E400, SI400 and E250 products to 1 mg l⁻¹ and higher for Lspez, E35 and AC150 products. The minimum exposure time to inhibit bacterial growth was 5 min for all products tested except the E35 product.

Marchand et al. (2012) found that the E250 product showed less inhibition of *Saprolegnia parasitica* and *Flavobacterium columnare*. They also found that the 2 products with the lowest PAA concentrations and very low PAA:H₂O₂ ratios (Lspez and E35) to be significantly more effective on the fungus and the bacterium tested. In contrast, we found the E35, Lspez and AC150 products were less effective than SI400 or E250 products. In the present study, E250 was one of the most efficient products.

Straus & Meinelt (2009) and Meinelt et al. (2009) used *Ichthyophthirius multifiliis* to demonstrate that various PAA products affect fish pathogens differently. Mean growth reductions in our investigations indicated the products with higher concentrations of PAA (and thus a higher molar PAA:H₂O₂ ratio) were more toxic to *A. salmonicida*. This is in contrast to Marchand et al. (2012) and Straus & Meinelt (2009), but in agreement with Wagner et al. (2002). The latter authors found that an additional amount of H₂O₂ did not cause additive toxic effects against *A. salmonicida*, and they confirmed the findings that larger doses of H₂O₂ are required compared to PAA for a similar level of disinfection of this bacterium.

Additionally, there seems to be a species-dependent sensitivity to the PAA products. Whereas *F. columnare* and *S. parasitica* were sensitive to the Lspez and E35 product exposures, *A. salmonicida* and *Y. ruckeri* were more sensitive to the SI400 and E250 products.

Because PAA has a low therapeutic index and concentrations higher than 1 mg l⁻¹ become toxic to fish, the E400, SI400 and E250 products are suitable as a prophylaxis against *A. salmonicida*. For all except
the E35 product, concentrations of 1 mg l⁻¹ with a 5 min exposure could significantly reduce the CFUs of *A. salmonicida* by >10⁻⁶. In our experiments, the E35 product was not very successful. However, in contrast to the findings of Marchand et al. (2012), the SI400 product, and not the Lspez product, had the highest toxicity to *A. salmonicida*. The SI400 product is characterized by the highest PAA:H₂O₂ ratio; thus, PAA might have a higher impact in this equilibrium than H₂O₂ against *A. salmonicida*.

### Yersinia ruckeri

For *Y. ruckeri*, the exposure to PAA significantly reduced the CFUs of the bacterium by time and concentration (Table 3). No significant differences could be found between the E400 product and the other products tested.

Inhibition of *Y. ruckeri* was achieved with all PAA products dependent on concentration (Fig. 4). A concentration increase of 1 mg l⁻¹ reduced the CFUs by >10 × 10⁻⁶; prolonging the exposure by 5 min reduced the CFUs by >1.8 × 10⁻⁶ (Table 3). Compared with concentration, time seems to have less influence on reducing the bacteria and confirms the findings on *A. salmonicida*. However, the reduction of CFUs by time and concentration is much stronger than for *A. salmonicida*. Therefore, *Y. ruckeri* is more sensitive to PAA exposure than *A. salmonicida*. Exposure times longer than 5 min are necessary to reduce the growth of *Y. ruckeri*. Prolonging the exposure from 5 to 30 min does not reduce the CFUs any further.

For practical applications, a short exposure of 5 to 10 min is sufficient to reduce the population of bacteria and the stress on fish. An initial concentration of 1 mg l⁻¹ was necessary to reduce the CFUs to <15 × 10⁻⁶ for all products in this study, with the exception of the E35 product. For this product, concentrations of 1.5 mg l⁻¹ were necessary to reduce bacterial growth below 15 × 10⁻⁶. Fig. 4 shows that the efficiency of the products is very different. As found against *A. salmonicida*, the SI400 and E400 products more strongly affect bacterial growth than the other substances, and not all products are useful and manageable for the treatment of all microorganisms in aquaculture. In contrast to disinfecting waste water from sewage plants (Stampi et al. 2001, Kitis 2004) and keeping the low therapeutic index of PAA in mind, only low, non-toxic dosages are suitable in aquaculture. For *A. salmonicida* and *Y. ruckeri*, a strong product-dependent reduction of the CFUs can be realized at even lower PAA concentrations.

### SUMMARY

In conclusion, all products reduced CFUs of *A. salmonicida* and *Y. ruckeri*. Products with a higher molecular PAA:H₂O₂ ratio inhibited growth better than products with a lower molecular PAA:H₂O₂ ratio. Therefore, H₂O₂ is not the driving force in the reduction of *A. salmonicida* and *Y. ruckeri* growth by PAA in vitro. The practical application of the products with a high molecular PAA:H₂O₂ ratio should be prioritized if these pathogens are diagnosed. Future research should investigate in vitro toxicity to pathogens in aquatic systems.

### Acknowledgements

We thank the Schreiner-Foundation for Research and Education for financial support. We also thank Bradley Farmer and Cindy Ledbetter for their review of the manuscript. Mention of trade names or commercial products in this article is solely informative and does not imply recommendation or endorsement by the Leibniz Institute of Freshwater Ecology and Inland Fisheries, the DTU Aqua, or the US Department of Agriculture. The USDA is an equal opportunity provider and employer.

### LITERATURE CITED


Editorial responsibility: David Bruno, Aberdeen, UK

Submitted: September 25, 2014; Accepted: January 21, 2015
Proofs received from author(s): March 20, 2015