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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:H_2O_2 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:H_2O_2 ratios inhibited growth better than products with lower molecular PAA:H_2O_2 ratios at the same PAA concentration; this indicates that H_2O_2 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:H_2O_2 ratios should be prioritized if these pathogens are diagnosed.

KEY WORDS: PAA · Peroxide · H_2O_2 · 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 (H_2O_2), 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 H_2O_2, 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).

Freer & Novy (1902) first reported the germicidal activities of PAA, citing the 'excellent disinfection and cold sterilization actions of PAA'. The antimicrobial properties of PAA have been recognized for many years (Baldry 1983, Alasri et al. 1993, Stampi et al. 2001, Crebelli et al. 2005). Consequently, PAA has been routinely used in agriculture, food processing, wastewater treatment and hospitals (Baldry & French 1989a,b, Baldry et al. 1991, 1995, Lefevre et al. 1992, Stampi et al. 2001, Caretti & Lubello 2003, Vinnerås et al. 2003).

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:H_2O_2 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 erythrodermatitis) in cyprinids (Hiney & Olivier 1999) and motile aeromonad infection in salmonids (Noga 2010). *Yersinia ruckeri* is the causative agent of redmouth disease (enteric redmouth disease [ERM], redmouth, yersiniosis, blood spot, *Y. ruckeri* infection) in

different fish species (Tobback et al. 2007, Noga 2010). Both diseases are costly to the aquaculture industry. Verner-Jeffreys et al. (2009) tested a product containing 5% PAA against *A. salmonicida* ssp. *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:H_2O_2 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:H_2O_2 concentrations were tested (Table 1). The product with the highest molecular PAA:H_2O_2 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

Table 1. Concentrations of peracetic acid (PAA), hydrogen peroxide (H_2O_2) and the molar ratio of PAA:H_2O_2 in each commercial product used in the present study of products for reduction of bacterial growth. Suppliers: KESLA PHARMA WOLFEN GmbH (Greppin, Germany), AppliChem GmbH (Darmstadt, Germany) and Sigma-Aldrich Co. (St. Louis, Missouri, USA)

ID	Product	Supplier	PAA (%)	H_2O_2 (%)	PAA:H_2O_2
Lspez	Wofasteril L. Spez	KESLA PHARMA WOLFEN	3	40	0.034
E35	Wofasteril 035	KESLA PHARMA WOLFEN	3.5	10	0.156
AC150	Peracetic acid 15%	AppliChem	15	24	0.28
E250	Wofasteril E250	KESLA PHARMA WOLFEN	25	30	0.37
SI400	Peracetic acid solution	Sigma-Aldrich	39	6	2.91
E400	Wofasteril E400	KESLA PHARMA WOLFEN	40	12	1.49

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

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

Variable	Regression coefficient	Standard error	p-value
Concentration	−2009046	91029	<0.001
Time	−33672	5742	<0.001
Lspez	1159586	214242	<0.001
E35	1372670	214242	<0.001
AC150	3863693	214242	<0.001
SI400	187615	214242	0.382
E250	152308	214242	0.478

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).
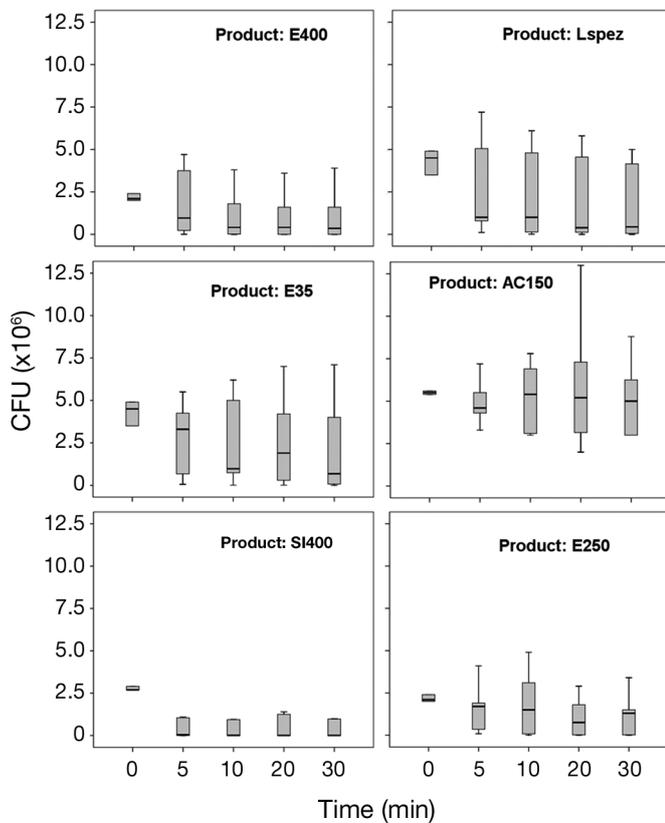
Fig. 1. Reduction of *Aeromonas salmonicida* colony-forming units (CFUs) by peracetic acid products tested over time. Line: median; box: 25th–75th percentiles (IQR); whiskers: min./max. observation within 1.5 × IQR below/above 25th/75th percentile
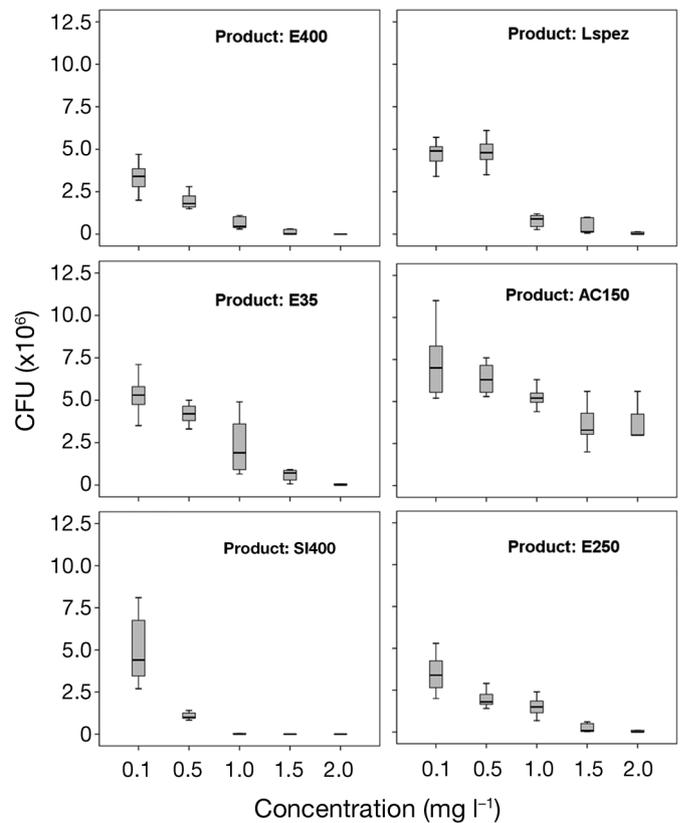

Fig. 2. Reduction of *Aeromonas salmonicida* CFUs by peracetic acid products tested over concentration. Boxplot limits as in Fig. 1

Exposure time

All products except E250 reduced the mean CFUs of *Y. ruckeri* to $<15 \times 10^{-6}$ after an exposure time of 5 min (Fig. 3). The E250 product needed a longer exposure time to reduce CFUs.

Product concentration

Following exposure to the E400, E35 and SI400 products at PAA concentrations of 0.5 mg l^{-1}, the mean *Y. ruckeri* CFUs $<15 \times 10^{-6}$ 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 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

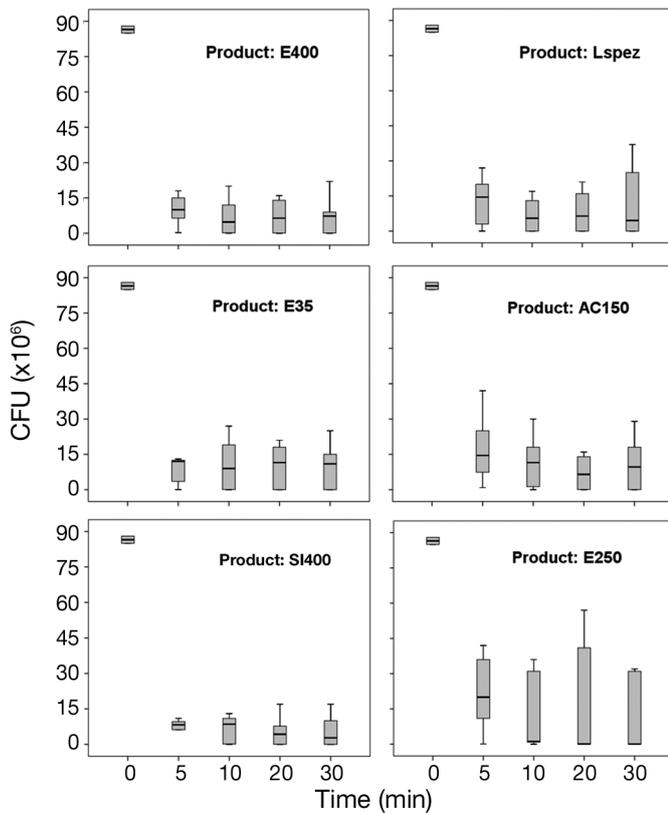| Variable	Regression coefficient	Standard error	p-value
Concentration	−10665968	2161497	<0.001
Time	−1804624	136019	<0.001
Lspez	1720500	5055315	0.734
E35	4662841	5165197	0.367
AC150	5036282	5081241	0.322
SI400	−863677	5055315	0.864
E250	7161469	5055315	0.158

Fig. 3. Reduction of *Yersinia ruckeri* CFUs by peracetic acid products tested over time. Boxplot limits as in Fig. 1
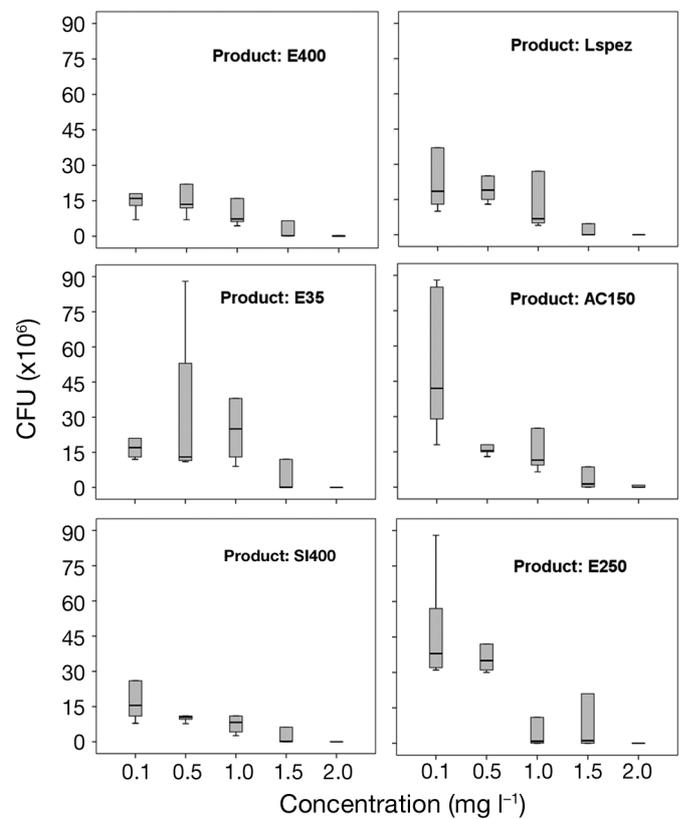

Fig. 4. Reduction of *Yersinia ruckeri* CFUs by peracetic acid products tested over concentration. Boxplot limits as in Fig. 1

Compared with concentration, time seems to be of less influence in reducing the bacteria. This implies that the effect of the PAA:H_2O_2 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:H_2O_2 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:H_2O_2 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 H_2O_2 did not cause additive toxic effects against *A. salmonicida*, and they confirmed the findings that larger doses of H_2O_2 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:H$_2$O$_2$ ratio; thus, PAA might have a higher impact in this equilibrium than H$_2$O$_2$ 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:H$_2$O$_2$ ratio inhibited growth better than products with a lower molecular PAA:H$_2$O$_2$ ratio. Therefore, H$_2$O$_2$ 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:H$_2$O$_2$ ratio should be prioritized if these pathogens are diagnosed. Future research should investigate *in vitro* toxicity to pathogens in aquatic systems.

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