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Published in:
North American Journal of Aquaculture

Link to article, DOI:
10.1080/15222055.2014.976682

Publication date:
2015

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

Link back to DTU Orbit

Citation (APA):
North American Journal of Aquaculture

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Published online: 20 Feb 2015.


To link to this article: http://dx.doi.org/10.1080/15222055.2014.976682

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ARTICLE

Comparison of the Toxicity of Wofasteril Peracetic Acid Formulations E400, E250, and Lspez to *Daphnia magna*, with Emphasis on the Effect of Hydrogen Peroxide

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Abstract

Commercial peracetic acid (PAA) formulations are acidic mixtures of PAA, hydrogen peroxide (H₂O₂), acetic acid, H₂O, and stabilizers to maintain the equilibrium of the concentrations. Different PAA formulations show diverse PAA : H₂O₂ ratios, potentially leading to different toxicities at the same PAA concentration due to the different concentrations of H₂O₂ and stabilizers used. To confirm any potential differences in toxicity, we performed 24-h toxicity tests using *Daphnia magna* with three commercial PAA formulations (Wofasteril): E400, E250, and Lspez. The experiments were carried out in standard dilution water and with increased water hardness, salinity, or dissolved organic carbon to reflect various natural conditions. Results showed that the toxicity to *Daphnia* was greatest for Lspez, intermediate for E250, and lowest for E400. An E400 + H₂O₂ mixture, which possessed a composition theoretically identical to the E250 formulation, had toxic effects and 24-h LC₅₀ values similar to those of E250. This indicates an additive effect of H₂O₂ on the toxicity of PAA formulations. Moreover, a significant positive correlation was found between *Daphnia* mortality and the 3-h concentration of total peroxide (PAA and H₂O₂), with an r-value higher than that of PAA alone. A significant negative correlation between the total peroxide : PAA molar ratio and the 24-h LC₅₀ value was observed, indicating that the toxicity of PAA formulations to *Daphnia* is due to the combined effect of both PAA and H₂O₂.

Peracetic acid (PAA) formulations—mixtures of PAA, hydrogen peroxide (H₂O₂), acetic acid, H₂O, and stabilizers—are increasingly used for water treatment and pathogen control in aquaculture (Madsen et al. 2000; Pedersen et al. 2009). The PAA formulations are effective disinfectants and have limited (if any) potential impacts on fish, fish consumers, or the environment (Wagner et al. 2002; Crebelli et al. 2005; Dell’Erba et al. 2007). Investigations have revealed the effectiveness of PAA

In previous studies, commercial PAA formulations were chosen as test materials, but few studies have included comparisons of different PAA formulations. Straus and Meinelt (2009) investigated the acute toxicity of two PAA formulations—Minnfinn (4.5% PAA, 22% H₂O₂) and Wofasteril E400 (40% PAA, 12% H₂O₂)—against Ichthyophthirius multifiliis isolated from Golden Shiner Notemigonus crysoleucas and Green Swordtails Xiphophorus hellerii. The results showed that Minnfinn had significantly higher toxicity against both I. multifiliis isolates compared with Wofasteril E400 at the same PAA concentration. Despite these findings, Straus and Meinelt (2009) did not discuss the differences; their results indicated that the PAA formulation with a higher ratio of H₂O₂ to PAA was more toxic. Marchand et al. (2013) demonstrated variation in acute toxicity of different PAA formulations to Zebrafish embryos, and they hypothesized that the variation was likely due to the impact of PAA formulations on reducing the pH, potentially leading to different levels of acidosis in Zebrafish embryos. Moreover, in the Marchand et al. (2013) study, the PAA formulations with a higher H₂O₂ : PAA ratio did not demonstrate greater toxicity.

As described above, H₂O₂ may play an important role in the toxicity of PAA formulations. To understand this relationship, the results of acute toxicity tests must be correlated with the actual PAA : H₂O₂ composition in different PAA formulations. Such information has been lacking in previous studies; therefore, our objective was to fill this gap.

METHODS

Chemicals.—Three Wofasteril PAA formulations were obtained from Kesla Pharma Wolfen GmbH (Greppin, Germany): E400 (40% mass/volume [m/v] PAA, 12% m/v H₂O₂), E250 (25% m/v PAA, 30% m/v H₂O₂), and Lspez (3% m/v PAA, 40% m/v H₂O₂). The standard dilution water was prepared according to the German standard DIN EN ISO 7346-3:1997 and was composed of 294 mg of CaCl₂·2H₂O, 123.3 mg of MgSO₄·7H₂O, 63.0 mg of NaHCO₃, and 5.5 mg of KCl in 1,000 mL of H₂O. Sea salt was Tropic Marin brand (Dr. Biener GmbH, Watenberg, Germany). The main components of sea salt are NaCl and KCl and account for approximately 85% of the total salt mass; the rest is composed of SO₄²⁻, Mg²⁺, and Ca²⁺ ions. A preparation of HuminFeed (Humintech GmbH, Düsseldorf, Germany) containing about 40% organic carbon was used as a source of dissolved organic carbon (DOC). All chemicals were reagent grade.

Daphnia magna toxicity tests.—Daphnia magna were cultured in media composed of 2.4 g of CaCl₂, 60 mg of KCl, 1.23 g of MgSO₄·7H₂O, and 550 mg of NaHCO₃ in 10 L of distilled water; cultures were maintained at 26°C, with a 12-h light : 12-h dark photoperiod and continuous aeration. An algae suspension (Scenedesmus sp.) was used as feed for Daphnia. The toxicity tests were performed according to the German standard DIN 38412-11:1982-10. Daphnia that were 6–24 h old were obtained from the culture by double sieving: first with a 650-µm sieve to remove the oversized Daphnia and then with a 200-µm sieve. These Daphnia were then transferred into crystal dishes; 20 Daphnia and 40 mL of test solution were placed into each dish.

Test solutions were (1) standard dilution water, (2) standard dilution water with extra hardness (2.5-fold standard dilution water), (3) standard dilution water with extra NaCl (0.3% NaCl), (4) standard dilution water with extra sea salt (0.3% sea salt), and (5) standard dilution water with extra DOC (8 mg of DOC/L [20 mg of HuminFeed/L]). Treatment ranges were 0.5–1.5 mg/L for Wofasteril E400; 0.5–1.5 mg/L for E250; and 0.1–0.5 mg/L for Lspez. There was also a positive control containing potassium dichromate (K₂Cr₂O₇) at 1.9 mg/L. The crystal dishes (n = 3 per treatment) were incubated (without light or feed) at 20°C for 24 h in a KBW 720 Incubator (Binder GmbH, Tuttingen, Germany); the Daphnia were then observed under a dissecting microscope (Olympus SZH-ILLB). Daphnia that were unable to swim were categorized as dead.

To investigate the effect of H₂O₂ on the toxicity of PAA formulations, a separate sample was prepared by adding H₂O₂ to the E400 samples to simulate the PAA : H₂O₂ ratio of the E250 formulation.

Measurement of peracetic acid and hydrogen peroxide.—The method to determine PAA and H₂O₂ concentrations was the DPD (N,N-diethyl-p-phenylenediamine sulfate salt) photometric method. Without the peroxidase, the transparent, colorless (to light pinkish) DPD is oxidized by PAA into DPD⁺, which is pinkish and has a maximum absorption value at 550 nm (Pedersen et al. 2009). When peroxidase is added, the DPD is oxidized by both PAA and H₂O₂ (Bader et al. 1988). To measure the PAA concentration, 1 mL of PAA sample and 500 μL of buffer solution A (30.25 g of Na₂HPO₄·12H₂O; 23 g of KH₂PO₄; 0.01 g of NaCl; and 0.5 g of KI in 1,000 mL of H₂O) were mixed in a plastic cuvette. We then added 500 μL of DPD solution (1.6 g of DPD; 200 μL of 97% H₂SO₄; and 0.02 g of EDTA in 100 mL of H₂O). After 30 s, the absorption
at 550 nm (hereafter, absorption A) was measured with a DU 800 spectrophotometer (Beckman Coulter GmbH, Krefeld, Germany). For measurement of the combined concentration of PAA and H₂O₂ (i.e., total peroxide), we used the same procedure and wavelength as above except that buffer solution B was used to obtain the absorption (hereafter, absorption B). Buffer solution B was prepared by dissolving 5 mg of peroxidase (peroxidase from horseradish, Practical Grade II, A3800; Applichem GmbH, Darmstadt, Germany) in 100 mL of buffer solution A. Thus, absorption A represents the concentration of PAA, while absorption B represents the concentration of total peroxide.

**Determination of total peroxide : peracetic acid molar ratios.**—Samples of E400 and E250 at nominal PAA concentrations of 0.5, 1.0, and 3.0 mg/L and samples of Lspez at nominal PAA concentrations of 0.1, 0.2, and 0.4 mg/L were measured with buffer solutions A and B. The total peroxide : PAA molar ratios of the PAA formulations were determined through comparison of absorption A with absorption B at different nominal PAA concentrations by means of a linear relationship. Determination of total peroxide : PAA molar ratios was performed under the same conditions as the *Daphnia* toxicity tests.

**Monitoring of peracetic acid and hydrogen peroxide concentrations during testing.**—According to the German standard DIN 38412-11:1982-10, *Daphnia* toxicity tests must be performed in an incubator without light; therefore, concentrations of PAA and H₂O₂ in the samples were not measured during the toxicity tests. Additional samples of E400, E250, and Lspez were prepared according to the parameters in the toxicity tests but without adding *Daphnia*. The concentrations of PAA and H₂O₂ were measured after 3 h of static exposure.

**Statistics.**—The difference in toxicity among PAA formulations and the impacts of the various test solutions on PAA toxicity were determined via a two-tailed ANOVA test (α = 0.05) and a two-tailed *post hoc* test (Tukey’s test or Dunnett’s test, α = 0.05) applied to the data on *Daphnia* mortality at different PAA concentrations. The 24-h LC50 values (concentrations that were lethal to 50% of test organisms) were calculated via probit regression (α = 0.05) and were demonstrated in the form of mean values with 95% confidence intervals. Relationships between *Daphnia* mortality and PAA concentration, mortality and total peroxide concentration, and 24-h LC50 and the total peroxide : PAA molar ratio were determined via two-tailed Pearson’s product-moment correlation (α = 0.05). All statistical analyses were performed with IBM SPSS Statistics version 21 (IBM, Chicago).

**RESULTS**

**Toxicity of Peracetic Acid Formulations to Daphnia and Impacts of Different Test Solutions**

As shown in Figure 1, the Lspez formulation led to 100% mortality of *Daphnia* at a PAA concentration of 0.4 mg/L, indicating higher 24-h toxicity. In contrast, the E400 and E250 formulations led to 100% mortality at a PAA concentration of 1.5 mg/L. A noteworthy finding was that the 24-h toxicity of E400 was significantly lower than that of E250 and the E400 + H₂O₂ mixture (Tukey’s test: *P* = 0.000), while the 24-h toxicity of E400 + H₂O₂ was similar to that of E250 (Tukey’s test: *P* = 0.983).

Compared with the *Daphnia* exposures conducted in standard dilution water, extra sea salt significantly reduced the 24-h toxicity of all PAA formulations (Dunnett’s test: *P* = 0.000; Figure 2). Extra DOC significantly reduced the 24-h toxicity of the E400 and E250 formulations (*P* = 0.000) but did not induce a significant change in toxicity of the Lspez formulation (Dunnett’s test: *P* = 0.344). Extra NaCl significantly reduced the 24-h toxicity of Lspez (Dunnett’s test: *P* = 0.005) but had no significant effect on the 24-h toxicity of E400 (Dunnett’s test: *P* = 0.979) or E250 (Dunnett’s test: *P* = 0.998). Extra hardness significantly reduced the 24-h toxicity of E250 (Dunnett’s test: *P* = 0.000) but had no significant effect on E400 (Dunnett’s test: *P* = 0.853) or Lspez (Dunnett’s test: *P* = 0.133) toxicity.

**Twenty-Four-Hour LC50 Values**

The calculated 24-h LC50 values are listed in Table 1. In standard dilution water, the Lspez formulation had the lowest 24-h LC50 value, while the E400 formulation had the highest 24-h LC50 value. The 24-h LC50 values for E250 and the E400 + H₂O₂ mixture were nearly identical and were intermediate between those of E400 and Lspez. These results corresponded to the 24-h toxicity results described above.

The addition of extra sea salt resulted in lower 24-h LC50 values for all PAA formulations relative to the LC50 values obtained with standard dilution water. Extra DOC increased the 24-h LC50 values of E400 and E250; however, the 24-h LC50 value for E400 with extra DOC was considered an
outlier. Extra hardness increased the 24-h LC50 value for E250 but had little effect on the values for E400 and Lspez. These results also corresponded to the 24-h toxicity results. For all PAA formulations, extra NaCl had little effect on the 24-h LC50.

**Daphnia Mortality and the Three-Hour Concentrations of Peracetic Acid and Total Peroxide**

The relationship between *Daphnia* mortality and the 3-h PAA or total peroxide concentration is demonstrated in Figure 3. For E400 and Lspez, *Daphnia* mortality showed significant positive correlations with both PAA and total peroxide ($P = 0.000$). Pearson’s product-moment correlation coefficients (Pearson’s $r$) for E400 were 0.754 (PAA) and 0.728 (total peroxide); Pearson’s $r$-values for Lspez were 0.654 (PAA) and 0.90 (total peroxide). For E250, *Daphnia* mortality was not significantly correlated with either PAA (Pearson’s $r = 0.272$, $P = 0.162$) or total peroxide (Pearson’s $r = 0.286$, $P = 0.14$). When data from all PAA formulations were combined, significant positive correlations were observed between *Daphnia* mortality and both PAA and total peroxide concentration. However, Pearson’s $r$-value for *Daphnia* mortality versus total peroxide (Pearson’s $r = 0.616$, $P = 0.000$) was higher than that for *Daphnia* mortality versus PAA (Pearson’s $r = 0.256$, $P = 0.011$).

**Relationship between the 24-h LC50 and Total Peroxide : Peracetic Acid Molar Ratio**

Figure 4 shows a significant negative correlation between 24-h LC50 values and the total peroxide : PAA molar ratio under all tested conditions (Pearson’s $r = -0.692$, $P = 0.009$). The E400 and Lspez formulations with the DOC treatment were not included in the correlation calculation because the deviation in 24-h LC50 value for E400 (Table 1) and the deviation in total peroxide : PAA molar ratio for Lspez (Table 2) were too large.

**DISCUSSION**

**Effect of Additional Hydrogen Peroxide**

As depicted in Figure 1, 24-h toxicity to *Daphnia magna* differed significantly among the E400, E250, and Lspez formulations. The 24-h toxicity was lowest for E400, intermediate

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**TABLE 1.** Mean 24-h LC50 values (with 95% confidence interval in parentheses) for *Daphnia magna* exposed to Wofasteril peracetic acid formulations E400, E250, and Lspez and an E400 + hydrogen peroxide ($H_2O_2$) mixture. Exposures were conducted in five test solutions: standard dilution water alone or with extra hardness, NaCl, sea salt, or dissolved organic carbon (DOC).

<table>
<thead>
<tr>
<th>Test solution</th>
<th>E400</th>
<th>E250</th>
<th>Lspez</th>
<th>E400 + $H_2O_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard dilution water</td>
<td>0.774 (0.671–0.87)</td>
<td>0.547 (0.385–0.651)</td>
<td>0.181 (0.166–0.195)</td>
<td>0.574 (0.506–0.631)</td>
</tr>
<tr>
<td>Extra hardness</td>
<td>0.731 (0.665–0.792)</td>
<td>0.739 (0.686–0.789)</td>
<td>0.202 (0.188–0.205)</td>
<td></td>
</tr>
<tr>
<td>Extra NaCl</td>
<td>0.81 (0.756–0.862)</td>
<td>0.487 (0.325–0.593)</td>
<td>0.176 (0.153–0.198)</td>
<td></td>
</tr>
<tr>
<td>Extra sea salt</td>
<td>1.323 (1.159–1.626)</td>
<td>1.695 (1.368–2.835)</td>
<td>0.393 (0.356–0.442)</td>
<td></td>
</tr>
<tr>
<td>Extra DOC</td>
<td>2.617 (1.641–165.163)$^a$</td>
<td>0.79 (0.74–0.838)</td>
<td>0.196 (0.188–0.205)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Outlier; not considered in final statistics.
FIGURE 3. Pearson’s product-moment correlations between the mortality of *Daphnia magna* (20 individuals per replicate) exposed to Wofasteril peracetic acid (PAA) formulations E400, E250, and Lspez and the molar concentration of PAA (black shaded circles) or total peroxide (open circles) in each formulation. The correlations observed for all formulations combined are also presented. Asterisks indicate significant correlations (*$P < 0.05$; **$P < 0.01$).

Effects of Various Test Solutions on Toxicity to Daphnia

The toxicity of E400, E250, and Lspez showed differing sensitivity to the evaluated test solutions in comparison with the use of standard dilution water. This result was mainly due to different PAA degradation rates. Our previous work (Liu et al. 2014) found that salinity, hardness, and DOC induced different PAA degradation rates in E400, E250, and Lspez. Different PAA degradation rates result in different PAA residues, which in turn lead to differences in toxicity.

Daphnia Mortality versus Peracetic Acid and Total Peroxide

Since the number and size of *Daphnia* used in each replicate of the toxicity tests were the same, the impact of *Daphnia* on PAA decay was presumed to be the same among replicates.
Therefore, ignoring the impact of *Daphnia* on PAA decay, the 
3-h absorptions A and B could represent intermediate concentrations of PAA and total peroxide, respectively, during the toxicity tests and thus should be directly associated with the mortality rate.

*Daphnia* mortality was significantly and positively correlated with both PAA and total peroxide from the E400 and Lspez formulations (Figure 3). Pearson’s *r*-values for the correlations with mortality were identical between the PAA from E400 and the total peroxide from E400. However, for Lspez, total peroxide had a higher Pearson’s *r* than PAA. These findings indicate that PAA and total peroxide from E400 were equally responsible for *Daphnia* mortality, whereas total peroxide from Lspez was more likely to induce *Daphnia* mortality than PAA from Lspez. As the PAA proportion of total peroxide decreased from E400 to E250 to Lspez (Table 2), PAA appeared to contribute less to toxicity in *Daphnia*.

When results for all formulations were combined, PAA and total peroxide exhibited significant positive correlations with *Daphnia* mortality. However, values of *P* and Pearson’s *r* were much lower for PAA than for total peroxide, suggesting that for all PAA formulations, toxicity to *Daphnia* was more likely determined by the total peroxide concentration than by PAA alone.

### Twenty-Four-Hour LC50 versus Total Peroxide : Peracetic Acid Molar Ratio

The 24-h LC50 results (Table 1) differed slightly from the toxicity results (Figure 2). The only difference occurred for the toxicity of Lspez in standard dilution water treated with extra NaCl, which significantly differed from that obtained with standard dilution water alone (Figure 2). The ANOVA test indicated that the mean difference in mortality between the extra NaCl treatment and standard dilution water was 0.8667—that is, less than one dead *Daphnia*. In this case, the statistical difference does not seem to be biologically significant. Therefore, we decided that the conflicting results between Table 1 and Figure 2 were inconsequential and that the 24-h LC50 values are appropriate for use in further analysis.

The total peroxide : PAA molar ratio showed a significant negative correlation with 24-h LC50 values. Therefore, the formulation with a higher total peroxide : PAA ratio has a lower 24-h LC50 value—specifically, a higher toxicity to *Daphnia*. However, in the present study as well as in previous studies, the 24-h LC50 value was defined as the LC50 of only PAA. If the LC50 of PAA is multiplied by the total peroxide : PAA molar ratio, then theoretically the LC50 of total peroxide can be obtained. Since the changes in 24-h LC50 values lead to reversed changes in total peroxide : PAA molar ratios, it is likely that the LC50 of total peroxide could be rather stable. This indicates that even different PAA formulations can have the same LC50 of total peroxide for *Daphnia*.

### Mechanism of Toxicity

It has been demonstrated that chronic exposure of *Daphnia* to *H* 2O 2 at concentrations greater than 1.25 mg/L leads to death and/or reduced reproduction (Meinertz et al. 2008). However, *Edwardsiella tarda* is tolerant of *H* 2O 2 due to the presence of catalases (Srivivasa Rao et al. 2003). Many microorganisms can produce catalase to reduce the effect of *H* 2O 2 (Nakamura et al. 2012). For instance, Jussila et al. (2014) found no effect of *H* 2O 2 against spores of the infective crayfish plague *Aphanomyces astaci*. In that case, *H* 2O 2 may not have been able to contribute to the toxicity of PAA formulations. Peracetic acid is fat soluble and can penetrate the cell membrane (Kitis 2004). McKinney et al. (1991) described that PAA at certain concentrations can deactivate catalases, so the toxic effect of *H* 2O 2 within PAA formulations would be maintained. Such an effect was shown by Pedersen et al. (2009), who simultaneously measured PAA

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**TABLE 2.** Total peroxide : peracetic acid (PAA) molar ratio for the Wofasteril E400, E250, and Lspez formulations in each of the five test solutions (DOC = dissolved organic carbon); values were calculated from the absorption values (measurements of PAA and hydrogen peroxide [H 2O 2] concentrations). Asterisks indicate coefficient of determination values (**R** 2 > 0.99; *R* 2 > 0.95).

<table>
<thead>
<tr>
<th>Test solution</th>
<th>E400</th>
<th>E250</th>
<th>Lspez</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard dilution water</td>
<td>1.79**</td>
<td>3.36**</td>
<td>35.24*</td>
</tr>
<tr>
<td>Extra hardness</td>
<td>1.79**</td>
<td>3.41**</td>
<td>37.10*</td>
</tr>
<tr>
<td>Extra NaCl</td>
<td>1.66**</td>
<td>2.78**</td>
<td>26.34*</td>
</tr>
<tr>
<td>Extra sea salt</td>
<td>1.70**</td>
<td>3.06**</td>
<td>28.43*</td>
</tr>
<tr>
<td>Extra DOC</td>
<td>1.79**</td>
<td>3.64**</td>
<td>ND</td>
</tr>
</tbody>
</table>

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**FIGURE 4.** Pearson’s product-moment correlations between 24-h LC50 values for *Daphnia magna* from exposure to Wofasteril peracetic acid (PAA) formulations E400, E250, and Lspez and the total peroxide : PAA molar ratio for all formulations under all test conditions (E400 and Lspez treated with extra dissolved organic carbon are excluded due to high deviation). Asterisks indicate that the correlation was significant (**P < 0.01).
and H₂O₂ at different concentrations. We speculate that the mode of action for toxicity of PAA formulations is primarily the action of PAA and secondarily the action of H₂O₂. Therefore, our findings should theoretically also be valid for other aquatic organisms, but further research is needed.

**Strategy for Peracetic Acid Application in Freshwater Aquaculture**

Pedersen et al. (2013) discussed the difficulty in maintaining a safe but effective dosage of PAA while disinfecting freshwater aquaculture systems; they emphasized that the most feasible solution is to monitor PAA consumption during the process of disinfection. In addition, we recommend monitoring the combined concentration of PAA + H₂O₂ since our study indicates that the toxicity of PAA is due to the combined effect of PAA and H₂O₂. The present findings suggest that additional, combined, or simultaneous applications of H₂O₂ could be options when applying PAA for water treatment.

**Conclusions**

We demonstrated that the toxicity of PAA formulations to *Daphnia* is affected by both PAA and H₂O₂. The PAA formulation with a higher H₂O₂ : PAA ratio is more toxic to *Daphnia* because of higher H₂O₂ concentrations at the same PAA concentration. We suggest that the effect of H₂O₂ cannot be ignored in toxicity studies of PAA formulations and that the LC50 values of PAA formulations should be defined as the LC50 of total peroxide rather than only PAA.

**ACKNOWLEDGMENTS**

We thank the Schreiner Foundation for Research and Education for financial support. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the Leibniz Institute of Freshwater Ecology and Inland Fisheries, the National Institute of Aquatic Sciences at Denmark Technical University, or the U.S. Department of Agriculture (USDA). The USDA is an equal opportunity provider and employer.

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