ASSESSING THE TOXICITY OF PERACETIC ACID TO EARLY ATLANTIC SALMON *SALMO SALAR* LIFE-STAGES

by

Natalie Redman

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Accepted:

Eric R. Annis, Ph.D. Committee Member	Susan L. Carney, Ph.D. Director, Environmental Biology Program
David L. Straus Committee Member	April M. Boulton, Ph.D. Dean of the Graduate School
Christopher Good Thesis Advisor	

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ABSTRACT

Microbial pathogens in recirculating aquaculture and egg incubation systems can cause elevated mortality and decreased production. Peracetic acid (PAA) is an effective disinfectant; however, its toxicity to early life stages of Atlantic salmon (*Salmo salar*) has not been assessed. This research determined the 24-hour LC50 value of PAA for three early life stages of Atlantic salmon: eyed eggs, fry, and fingerlings. Toxicity Relationship Analysis Program (TRAP) LC50 values for eggs treated for 5 and 10 minutes were 781.5 mg/L and 485.0 mg/L PAA, respectively. Trimmed Spearman Karber (TSK) LC50 values for eggs treated for 5 and 10 minutes were 771.1 mg/L and 462.1 mg/L PAA, respectively. TRAP LC50 values for fry and fingerlings were 4.0 mg/L and 5.3 mg/L PAA, respectively. TSK LC50 values for fry and fingerling were 4.1 mg/L and 5.3 mg/L PAA, respectively. These LC50 values provide guidance for developing safe PAA treatment protocols for Atlantic salmon eggs, fry, and/or fingerlings.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APHA (American Public Health Association): professional organization for public health professionals in the United States

ARS (Agricultural Research Service): research agency within USDA

ATU (accumulated thermal units): cumulative effect of temperature over time

DO (dissolved oxygen): measure of the free oxygen in water

DOC (dissolved organic carbon): fraction of organic carbon

H_2O_2 (hydrogen peroxide): chemical compound of hydrogen and water that can be utilized as an antimicrobial disinfectant

IACUC (Institutional Animal Care and Use Committee): provides oversight and approval for research utilizing animals

NOEC (no observed effect concentration): the concentration of toxicant that when applied does not result in a statistically significant effect

LC50 (median lethal concentration causing 50% mortality): the calculated concentration at which approximately 50% of eggs are killed by following a range of exposure concentrations

mg/L (milligrams per liter): unit of measure

NIST (National Institute of Standards and Technology): certification indicates equipment adheres to exacting requirements

PAA (peracetic acid): mixture of acetic acid, hydrogen peroxide and water that can be utilized as an antimicrobial disinfectant

PPD (potassium permanganate demand): amount of chemical required to react with all the organic matter in a water sample

RAS (recirculating aquaculture system): land-based, contained unit for rearing aquatic species, like finfish

SNARC (Stuttgart National Aquaculture Research Center): aquaculture research center located in Stuttgart, Arkansas

TCF/FI (The Conservation Fund's Freshwater Institute): aquaculture research center located in Shepherdstown, West Virginia

TOC (total organic carbon): amount of carbon found in an organic compound

TRAP (Toxicity Relationship Analysis Program): fits a symmetric, sigmoidal effects versus exposure relationship to toxicity test data

TSK (Trimmed Spearman-Karber): nonparametric method to determine LC50 from dose-response curves

TSS (total suspended solids): dry-weight of suspended particles in a water sample

USDA (United States Department of Agriculture): federal executive department

WECL (Water and Environmental Chemistry Laboratory): The Conservation Fund's Freshwater Institute water quality laboratory

INTRODUCTION

Egg incubation systems and recirculating aquaculture systems (RAS) permit the production of aquatic species, such as Atlantic salmon *Salmo salar*, in a land-based, closed-containment unit while minimizing water consumption (Summerfelt and Vinci 2008). Recirculation systems employ various water treatment technologies to clean waste-laden water from the culture tank effluent and return it back cleaned and safe for the species being grown (Summerfelt and Vinci 2008; Davidson et al. 2011a). Due to minimal water exchange within the systems and relatively high fish stocking densities, RAS provide an environment in which potentially pathogenic microorganisms can proliferate (Davidson et al. 2011a; Marchand et al. 2012; Davidson et al. 2019). Low-flow designs and high egg densities provide similar conditions in incubation systems, increasing the likelihood of pathogen transmission between eggs (Van West 2006; Marchand et al. 2012).

Diseases associated with common pathogenic bacteria and oomycetes, such as *Flavobacterium* spp. and *Saprolegnia* spp., respectively, can provide significant complications in RAS and egg incubation systems, ultimately leading to fish and egg mortalities and decreased production (Marchand et al. 2012; Good et al. 2020). Saprolegniasis, caused by the pathogenic oomycetes *Saprolegnia* spp., is estimated to kill approximately 10% of all hatched Atlantic salmon in the aquaculture industry (Phillips et al. 2008). *Flavobacterium* spp. can cause significant losses in the aquaculture industry when environmental conditions favor their proliferation and subsequent tissue damage in affected fish (Lange et al. 2019).

Disinfectants are utilized in RAS and egg incubation systems to prevent accumulation of such common pathogens (Bowker et al. 2011; Marchand et al. 2012). Ozone is commonly used in RAS due to its effectiveness in improving water quality and reducing waterborne bacterial

counts; however, it can pose health threats to humans and aquatic species if not used properly, and its implementation can be costly and complicated (Davidson et al. 2011b). Chlorine is also used for disinfection in RAS as it is relatively inexpensive and effective, but it can only be used for system disinfection once fish have been removed (King et al. 2008; Bowker et al. 2011; Ben-Asher et al. 2019). Chlorine poses a significant threat to the environment if accidentally released, and can be harmful to humans if not handled properly (Stewart et al. 1996; Bowker et al. 2011). After use, chlorine must be neutralized with sodium thiosulfate to minimize potential impacts on surrounding wildlife and aquatic species before the RAS can be properly flushed (Sharrer et al. 2005; Bowker et al. 2011). Alternative disinfectants that are economically practical, effective, and safe for the environment and culture species would be extremely valuable to the aquaculture industry.

Peracetic acid (PAA) is an antimicrobial disinfectant that has been approved for use in aquaculture in the European Union (Lehmann 1974; European Union 2014). PAA is sold commercially as a stabilized mixture of acetic acid, hydrogen peroxide, and water, and has been utilized as a bactericide, viricide and fungicide (De Swaef et al. 2016). PAA is fat soluble and a strong oxidizer, and therefore it can be extremely active against microorganisms even at a low concentration (da Silva et al. 2020; Kitis 2004; Marchand et al. 2012). Its ability to kill microorganisms has been demonstrated in a wide range of temperatures (Schliesser and Wiest 1979); however, research by Sánchez-Ruiz et al. (1995) demonstrated that alkaline pH conditions reduce its efficacy. PAA has been noted to pose minimal threat to the environment, as it rapidly degrades in water and is not known to produce toxic by-products when it reacts with organic matter (Monarca et al. 2002; Kitis 2004; Davidson et al. 2019). The toxicity of PAA to a variety

of commercial fish species in the United States has been reported (Straus et al. 2012a; Straus et al. 2018), but its toxicity to many other farmed aquatic species remains to be investigated.

Unfortunately, there is little research regarding PAA's applicability for RAS and egg incubation systems in the United States, as PAA has so far only been registered for aquaculture surface disinfection by the EPA (Straus and Meinelt 2019) when fish are not present. Although some research has been carried out in Europe regarding the application of PAA to rainbow trout (*Onchorhyncus mykiss*) and zebrafish (*Danio rerio*) (Pedersen et al. 2009; Marchand et al. 2013; Liu et al. 2016; Liu et al. 2017; Gesto et al. 2018; Lindholm-Lehto et al. 2019), further experiments are required to assess disinfection efficacy and to develop safe protocols for use in these systems containing fish or eggs. Additionally, water quality in RAS differs from that in flow-through systems due to lower water exchange in RAS, resulting in an accumulation of nutrients, dissolved metals, and nitrogen species over time. Water quality characteristics have an impact on the degradation of PAA; therefore, it is imperative that RAS operators consider their individual water chemistry in order to achieve disinfection efficacy (Liu et al. 2014). PAA could prove to be a promising disinfectant for the United States aquaculture industry, as demonstrated by its use in combating a variety of microorganisms (Brown et al. 2007; Meinelt et al. 2007; Marchand et al. 2012; Liu et al. 2016).

A first step in assessing PAA's suitability as an *in vivo* aquaculture disinfectant is to determine its toxicity to various life stages of production fish by determining its 24-hour LC50 value - the concentration at which approximately 50% mortality occurs within 24 hours. Given the increasing production of Atlantic salmon in land-based RAS facilities, and given the variability in PAA's efficacy across different water quality scenarios, it is particularly informative to assess the toxicity of PAA to Atlantic salmon in water from a "typical" RAS

environment. The objective of the present study was therefore to establish 24-hour LC50 values for three early life stages of Atlantic salmon: egg, fry, and fingerling, with fry and fingerling experiments being carried out in mature (steady-state) RAS water to support PAA's potential application in the RAS industry. Establishing early life-stage toxicity values will assist industry stakeholders and RAS operators in developing standard operating procedures for the application of PAA during Atlantic salmon incubation and early rearing production phases.

MATERIALS AND METHODS

General Procedures

Mixed-sex diploid Atlantic salmon eggs were purchased from StofnFiskur, Iceland, and shipped directly to The Conservation Fund Freshwater Institute (TCF/FI; Shepherdstown, WV, USA). The experiments outlined in this section were approved under TCF/FI's Institutional Animal Care and Use Committee (IACUC) (Protocol # 1-2020). Remaining experimental fish were humanely euthanized according to TCF/FI's IACUC-approved protocols.

A commercial formulation of PAA (15% PAA and 10% hydrogen peroxide) served as the stock solution from which PAA doses were sourced. The PAA stock solution concentration was verified using Hach method LIT2199 before individual treatments were created (Hach 2014). Individual PAA treatment concentrations were verified using CHEMetrics peracetic acid Vacu-vials® kit, a method that is adapted from APHA Standard Methods (2017) Method 4500-Cl G-2000.

Water quality analyses, with the exception of total and dissolved organic carbon determination (TOC and DOC, respectively), were conducted at TCF/FI's Water and Environmental Chemistry Laboratory. Water quality samples were collected for each trial once, directly before the administration of PAA treatments. Samples for TOC and DOC were collected at TCF/FI and shipped for analysis to the USDA-ARS Stuttgart National Aquaculture Research Center (Stuttgart, AR, USA). Temperature was monitored continuously over the course of the 24-hour period. The source water (specific for each experiment) was analyzed once before treatment for each experiment. The 15-minute potassium permanganate demand (PPD) was assessed for each experiment as a way to assist in characterization of source water across toxicity

trials (Boyd 1979; Tucker 1989). The parameters of interest, methods employed, and associated equipment for analyses are listed in Table 1.

Table 1. Water quality methods and associated equipment.

Parameter	Method[1,2]	Equipment
pH	Digital combination electrode	Hach HQ40d, Hach PHC101 probe
Dissolved Oxygen	Digital luminescent optical sensor	Hach HQ40d, Hach LDO101 probe
Dissolved Carbon Dioxide	Partial pressure detection	Portable OxyGuard CO_2 Analyzer
Temperature	Electronic thermometer	HOBO MX2203 temperature logger
Total Suspended Solids	APHA 2540 D 11	Heratherm OGS60 drying oven, Mettler Toledo MSTS304 Balance
Total Alkalinity	Hach method 8203	Accumet AB150 pH meter
Hardness	Hach method 8213	N/A
Nitrate Nitrogen	APHA 4500 NO3 D 11	YSI TruLine Nitrate ISE
Nitrite Nitrogen	Hach method 8501	Hach DR6000
Total Ammonia Nitrogen	Hach method 8038	Hach DR6000
Total Residual Chlorine	Hach method 8167	Hach DR900
Total Organic Carbon	APHA 5310 B	Teledyne Tekmar-Dohrmann Apollo 900, STS 8000 Autosampler
Dissolved Organic Carbon	APHA 5310 C	Teledyne Tekmar-Dohrmann Apollo 900, STS 8000 Autosampler
Potassium Permanganate Demand	USDA/ARS AR SOP WQ/WC 205.1	N/A

[1]APHA denotes American Public Health Association Standard Methods for the Examination of Water and Wastewater 22nd Edition

[2]USDA/ARS denotes the USDA ARS Stuttgart location in Stuttgart, AR

Atlantic salmon eyed eggs

Eyed Atlantic salmon eggs were maintained in Heath tray stacks (Figure 1) within a recirculating incubation system throughout the course of the experiment. Water for the systems was sourced from the contained freshwater spring supply located on the TCF/FI property and utilized in the incubation system at an estimated 99.8% reuse rate. Dissolved oxygen (DO), pH, and temperature were monitored using Hach HQ40d handheld probes and HOBO MX2203 TidbiT temperature loggers to ensure proper conditions (100% saturation, 7.4-8.2, and 6.5-7.5°C, respectively) for egg rearing (Timmons et al. 2018).

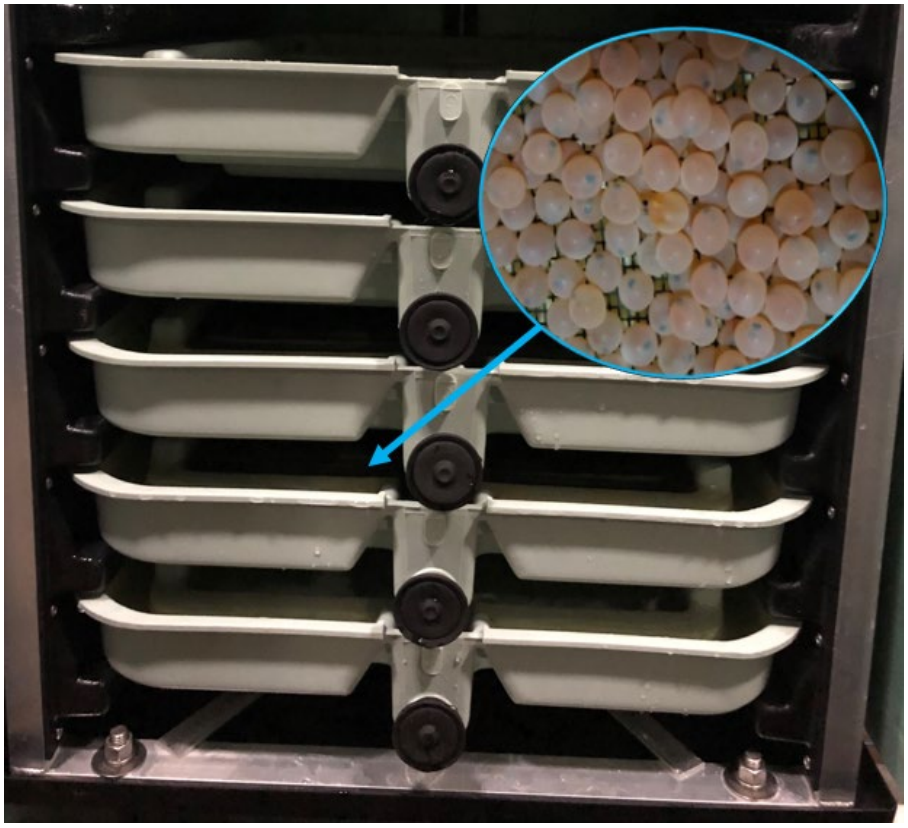

Figure 1. Heath tray incubation system.

Preliminary range-finding experiments were conducted to determine an appropriate range of PAA concentrations that would likely capture 0% and 100% egg mortality in the lowest and highest concentrations assessed, respectively, over a 24-hour period. For additional information

regarding the preliminary range-finding experiments, refer to Appendix I. Nine PAA treatments (0, 300, 400, 500, 600, 700, 800, 900 and 1,000 mg/L PAA) were administered for 5 and 10 minutes in 1 L glass jars using the verified PAA stock solution and the spring water maintaining the incubation system. PAA treatments were prepared using the verified PAA concentration, total volume of each dosing vessel (1 L acid washed glass jar) and the target concentration of each PAA treatment dose. The control treatment (0 mg/L PAA) glass jars contained spring water only. An aliquot of deionized water was administered to the control jars in order to replicate the mechanical procedure associated with PAA addition for PAA treatment jars. Twenty eggs were added to each jar containing the treatment and remained for either 5 or 10 minutes. The eggs were then rinsed with spring water before being placed into a partition within the incubation system Heath tray. Each Heath tray within the stack was partitioned into 21 sections, 9 of which contained 20 treated eggs. Three separate Heath tray stacks were employed for this experiment. Each Heath tray stack contained two trays: tray 1 contained eggs exposed to treatment for 5 minutes and tray 2 contained eggs exposed for 10 minutes (Table 2). The eggs were assessed for mortality 24 hours after the initial dose by visual observations of color change and through the use of a salt flotation solution (105 g/L NaCl) as described by Leitritz and Lewis (1980). Eggs that were opaque and white in color and/or floated in the salt solution were recorded as a mortality. Eggs that maintained the slight transparent orange color and/or sank in the salt solution were recorded as living. Eggs that were in the process of hatching but were dead were also counted as mortalities. Given that the results of the third range-finding study produced 0% and 100% mortality in the low and high dose ranges, these same doses were selected for the acute toxicity experiment but were applied in duplicate for experimental replication (following

the approach by Straus and Tucker 1993). These results assisted in determining the 24-hour LC50 value and NOEC of PAA to Atlantic salmon eggs at the two selected exposure times.

Table 2 . Egg LC50 experiment design.

Stack 1: Replicate A						
Tray #1						
T1A	T2A	T3A	T4A	T5A	T6A	T7A
T8A	0A					
Tray #2						
T1A	T2A	T3A	T4A	T5A	T6A	T7A
T8A	0A					
Stack 2: Replicate B						
Tray #1						
T1B	T2B	T3B	T4B	T5B	T6B	T7B
T8B	0B					
Tray #2						
T1B	T2B	T3B	T4B	T5B	T6B	T7B
T8B	0B					

T1 through T8 indicate the 8 PAA concentrations, 0 indicates controls, A and B denote replicates. For example: T3B is PAA target concentration 3, replicate B.

Atlantic salmon fry

Atlantic salmon fry (approximately 0.16 – 0.18 g) were maintained in challenge trough compartments, with each trough divided into nine 35 L compartments containing individual water inlets and double standpipe drains (Figure 2). The weight of individual fry was determined via wet weight; the bulk weight of the total number of fish was divided by the total number of fish. Each trough was maintained with mature RAS water from a single source. The troughs were incorporated into the loop of a 9.5 m^3 RAS containing a 5.3 m^3 culture tank stocked with Atlantic salmon. The water chemistry profile for the source water is listed in Table 1. Twenty fry that had been feeding for approximately one week were stocked into each trough compartment required for the experiment. Fry were not fed for 24 hours before treatments were administered. The troughs were maintained as flow-through systems during a 24-hour acclimation period and then adjusted to static baths during treatment periods. Dissolved oxygen (DO), pH, and temperature were monitored as described above.

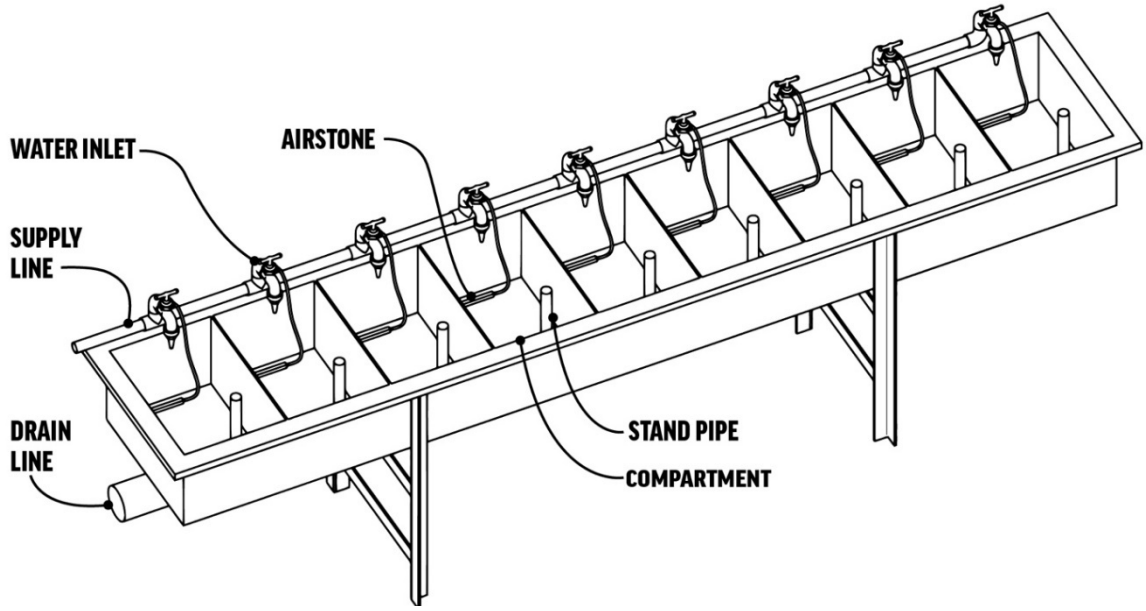

Figure 2. Illustration of challenge troughs.

Six PAA concentrations were selected based on preliminary range-finding results, as previously described; treatments were within a range where 100% survival was expected in the lowest treatment and 100% mortality was expected in the highest treatment. For additional information on the preliminary range-finding experiments, refer to Appendix II. The following treatments were administered: 0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 mg/L PAA. Each peracetic acid concentration was tested in triplicate along with triplicate control treatments that did not contain PAA (Table 3).

Table 3. Fry LC50 experiment design.

Trough 1								
T3 A	T2 A	0 A	T1 A	T5 A	T4 A	T6 A		
Trough 2								
T4 B	T1 B	T3 B	T5 B	0 B	T6 B	T2 B		
Trough 3								
T2 C	T1 C	0 C	T6 C	T4 C	T3 C	T5 C		

T1 through T6 indicate the six PAA treatments, 0 indicates controls A, B and C denote replicates. For example: T3B is PAA target treatment 3, replicate B.

The water flow to and from the challenge troughs was turned off, thereby creating a static bath with no water exchange in each compartment. The volume of PAA required for each treatment was prepared using the verified PAA concentration, total volume of each compartment and the target concentration of each PAA treatment. The control treatment was a compartment containing RAS water with no PAA. PAA treatments were assigned in random order for each compartment. Each compartment remained in a static bath state for 24 hours following the addition of the PAA treatment. The LC50 value for fry was determined 24 hours after the initial

dose using visual observations. Fry that maintained signs of life were recorded as living, whereas fry that exhibited no signs of life (movement) were recorded as mortalities.

Atlantic salmon fingerlings

Atlantic salmon fingerlings (16.3 ± 0.7 g) were assessed using the setup and procedure described above. Twenty fingerlings were stocked into each trough compartment required for the experiment. Following a range-finding experiment as described above, six PAA treatments were selected, and each treatment was tested in triplicate along with triplicate control treatments. For additional information on the preliminary range-finding experiments, refer to Appendix III. The following treatments were administered: 0, 4.5, 4.75, 5.0, 5.25, 5.5, 5.75, 6.0, and 6.25 mg/L PAA. Each treatment was tested in triplicate along with triplicate control treatments that did not contain PAA (Table 4). Each trough compartment remained in a static bath state for 24 hours following addition of the PAA treatment. The fingerlings were assessed for mortality 24 hours after the initial dose using visual observations described above to determine the LC50 value.

Table 4. Fingerling LC50 experiment design.

Trough 1								
T1 A	T2 A	T6 A	T3 A	T4 A	0 A	T5 A		
Trough 2								
T3 B	0 B	T1 B	T2 B	T5 B	T6 B	T4 B		
Trough 3								
T2 C	T6 C	T5 C	T2 C	T1 C	T3 C	0 C		

T1 through T6 indicate the six PAA treatments, 0 indicates controls A, B and C denote replicates. For example: T3B is PAA target treatment 3, replicate B.

Statistical analysis

The LC50 values were determined using the Toxicity Relationship Analysis Program (TRAP) version 1.30a and the Trimmed Spearman-Karber (Erickson 2015; TSK; Hamilton et al. 1977, 1978) method, both of which have been used extensively in previously published aquatic toxicity experiments (Besser et al. 2013; Da Costa et al. 2014; Straus et al. 2012a; Straus et al. 2018; Wang et al. 2018). The rationale for utilizing both TSK and TRAP for LC50 assessments was that while TSK is a more traditional approach, it is not widely available as a procedure within commonly used statistical software packages, whereas the relatively new TRAP approach is becoming more frequently utilized in the scientific literature and is also freely available online (Erickson 2015).

RESULTS

Water quality profiles for the egg toxicity trial using recirculated spring water, as well as the RAS water used in the fry and fingerling toxicity trials, are listed in Table 5. Mortality curves for all experiments are illustrated in Figure 3.

Table 5. Water quality profiles for water used during the Atlantic salmon egg, fry, and fingerling PAA toxicity studies.

Water Quality Parameter[†]	Egg Study	Fry Study	Fingerling Study
pH	7.7	7.2	7.1
Dissolved Carbon Dioxide	10	6	5
Dissolved Oxygen	10.4	10.1	10.2
Temperature (°C)	13.3	12.3	12.4
Total Alkalinity	264	70	55
Total Hardness	323	365	372
Total Suspended Solids	0.554	1.876	3.456
Total Organic Carbon	2.05	32.14	26.70
Dissolved Organic Carbon	1.4	31.0	27.0
Nitrate-Nitrogen	3.1	118.9	89.5
Nitrite-Nitrogen	0.001	0.017	0.025
Total Ammonia Nitrogen	0.02	0.435	0.425
Potassium Permanganate Demand[‡]	<0.25	5.5	5.5
Total Residual Chlorine	0.00	0.00	0.01

[†]Concentrations are expressed in mg/L unless otherwise noted.
[‡] (Engstrom 1971; Boyd 1979; Tucker 1989)

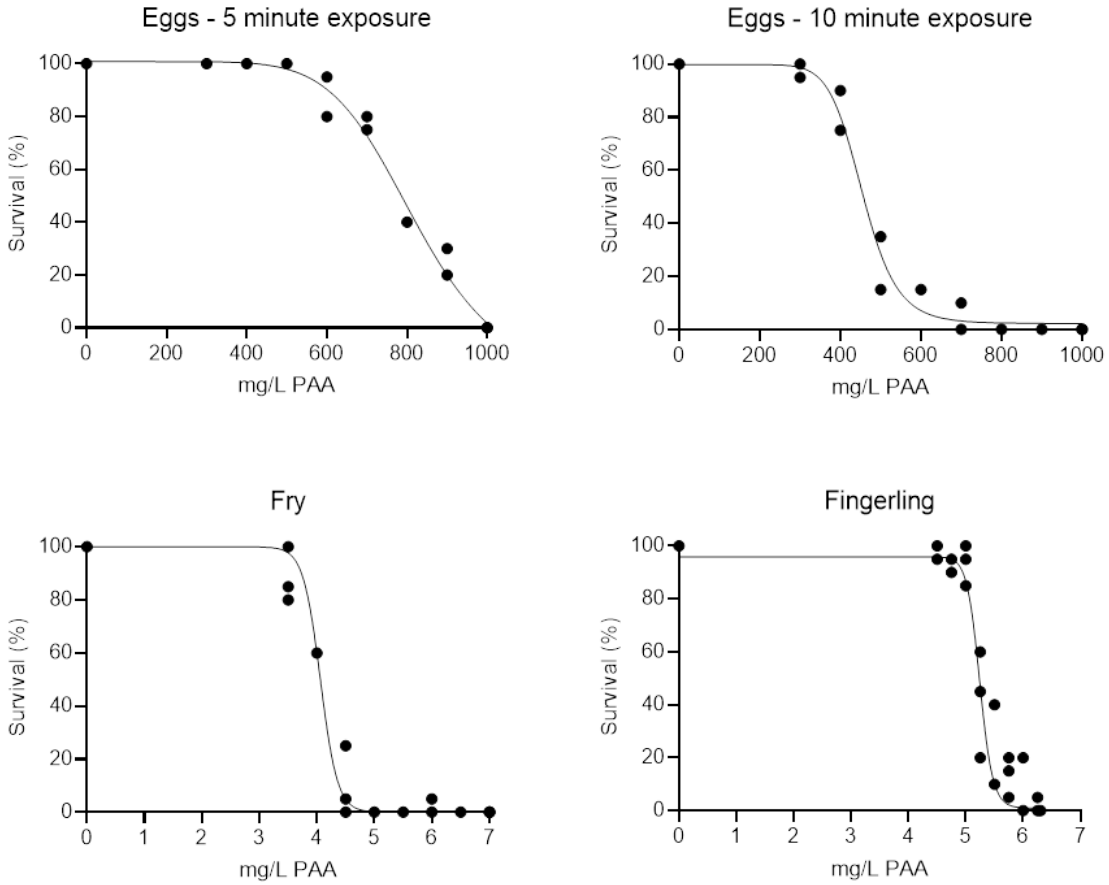

Figure 3. Early life-stage Atlantic salmon mortality curves during the acute toxicity: (i) eyed eggs, 5 min exposure (top left), (ii) eyed eggs, 10 min exposure (top right), (iii) fry stage (bottom left), and (iv) fingerling stage (bottom right).

During the preliminary egg range-finding study, average survival ranged from 100% in the control group, 300, 400, and 500 mg/L PAA treatment groups to 0% in the 1,000 mg/L PAA treatment groups subjected for 5 minutes. Average egg survival ranged from 100% in the control group to 0% in the 800, 900, and 1,000 mg/L PAA treatment groups subjected for 10 minutes. The TRAP determined LC50 values for the 5 and 10-minute treatments were 781.5 ± 12.7 (mean ± SE) mg/L and 485.0 ± 12.3 mg/L PAA, respectively. The TSK determined LC50 values for the 5- and 10-minute treatments were 771.1 ± 29.5 mg/L and 462.1 ± 23.4 mg/L PAA, respectively. The NOEC (no observed effect concentration), the concentration at which there were no discernable negative effects, was 500 mg/L and 300 mg/L PAA for the 5 and 10-minute egg treatment groups, respectively. The average fry survival rate ranged from 100% in the control groups to 0% in the 5.0 , 5.5, 6.5, and 7.0 mg/L PAA treatment groups. The LC50 values for the TRAP and TSK analyses of fry were 4.0 ± 0.04 mg/L and 4.1 ± 0.10 mg/L PAA, respectively. The average fingerling survival rate ranged from 100% in the control groups to 0% in the 6.25 mg/L PAA treatment group. The LC50 values for the TRAP and TSK analyses of fingerlings were 5.3 ± 0.03 mg/L and 5.3 ± 0.06 mg/L PAA, respectively.

DISCUSSION

Peracetic acid shows significant potential for use in aquaculture due to its broad and effective antimicrobial properties, and to its eventual degradation into harmless byproducts (Kitis et al. 2004; Pedersen et al. 2009). The primary mode of toxicity of stabilized PAA mixtures relies on the action of acetic acid, which deactivates peroxidases and catalases that would otherwise hinder the efficacy of the hydrogen peroxide within the PAA mixture (McKinney et al. 1991; Arias-Moliz et al. 2015; Liu et al. 2015). Peracetic acid is also fat soluble, making it a much more potent disinfectant compared to hydrogen peroxide alone (Block 1991; Straus et al. 2012b). Research has suggested that the mechanism of toxicity of PAA is due in part to the chemical's ability to release reactive oxygen species, leading to oxidative stress and cellular apoptosis, programmed cellular death as a result of biochemical events (Liu et al. 2016). PAA also has the ability to disrupt the chemiosmotic function of cellular cytoplasmic membranes of living organisms (Kitis 2004; Straus 2012a; Chupani et al. 2016). Once inside the cell, PAA oxidizes vital enzymes, thereby disrupting chemical pathways, active transport of substances across cellular membranes, and intracellular solute concentrations (Fraser et al. 1985).

Peracetic acid is thought to have an efficacy similar to that of ultraviolet radiation, due to its effectiveness against a wide range of microorganisms including bacteria, fungi, viruses, and protozoans (De Sanctis et al. 2016; Da Silva et al. 2020). Liu et al. (2018) reported that bacterial density decreased up to 90% in RAS (stocked with mirror carp *Cyprinus carpio*) treated with 1 mg/L PAA twice per week. Research of Jussila et al. (2011) demonstrated that PAA was effective in combating crayfish plague in European crayfish *Astacus astacus* aquaculture farms. PAA has also proven effective in controlling fungal infections caused by *Saprolegnia* spp. on channel catfish eggs *Ictalurus punctatus* (Straus et al. 2012b). Similarly, PAA has been found to

be effective in minimizing saprolegniasis related mortalities in post-vaccinated juvenile Atlantic salmon in freshwater RAS (Good et al. 2020). PAA has also been proven effective in reducing the parasitic theronts of *Ichthyophthirius multifiliis* that cause whitespot disease or "ich" in freshwater fish (Meinelt et al. 2009; Straus and Meinelt 2009). When applied at 10 mg/L for 1 minute, PAA completely inactivated *Piscirickettsia salmonis*, the bacteria known to cause salmonid rickettsial septicemia (Muniesa et al. 2019). Despite PAA's effectiveness in reducing pathogen loads, its use in RAS has proven to be safe for biofilter nitrifying bacteria colonies (Pedersen et al. 2009). Given this, the egg, fry and fingerling LC50 values and egg NOECs determined in the present study suggest that aquaculturists will likely be able to develop safe PAA application protocols to effectively reduce bacteria and oomycetes within Atlantic salmon RAS without reducing biofilter function.

Research has suggested that the degradation and disinfection efficacy of PAA is affected by water chemistry. Liu et al. (2014) observed that higher DOC increases PAA degradation. Given that the fry and fingerling toxicity trials of the present study were carried out during separate weeks, the RAS water utilized varied slightly between these studies. For example, DOC was less during the fingerling toxicity experiment (26.9 mg/L) as compared to that during the fry toxicity experiment (31.0 mg/L). The additional DOC in the RAS water during the fry study could have degraded the PAA upon addition to each trough compartment, which in turn could have resulted in a higher 24-hour LC50 value than if the DOC concentration were similar to that of the fingerling toxicity experiment. As a possible method to determine the concentration of PAA to use in production RASs for disinfection, the 15-minute PPD was used as a measure of the oxidizable organic matter in systems. In the present study, the 15-minute PPD of the source water used during the fry and fingerling experiments (5.5 mg/L) was higher than that of the

source water used during the egg experiments (<0.25 mg/L). Higher PPD indicates that more organic matter is present in the water, which is also evident given the elevated TSS, TOC and DOC concentrations of the fry and fingerling source water as compared to that of the egg source water (Lazur and Yanong 1992). Additionally, the RAS nitrate-nitrogen (NO_3-N) concentration was higher during the fry toxicity experiment (118.9 mg/L as NO_3-N) than it was during the fingerling toxicity experiment (89.5 mg/L as NO_3-N), and it is possible that this difference could have influenced LC50 values to an extent; however, further research is required to investigate the actual influence of NO_3-N on the degradation and disinfection efficacy of PAA in RAS water. Davidson et al. (2019) assessed the effects of PAA on water quality and rainbow trout *Oncorhynchus mykiss* in RAS by administering three PAA treatments (0.05, 0.10 and 0.30 mg/L PAA) semi-continuously. Their data suggested that the application of PAA did not improve RAS water quality or negatively impact trout production or biofilter performance. The research of Suurnäkki et al. (2020) demonstrated that twice-daily PAA treatments of 1.1 mg/L four times per week slightly improved water quality in RAS stocked with rainbow trout; however, biofilter nitrification rates were reduced by 50% and fish mortalities were higher compared to controls.

The eyed eggs exhibited the highest tolerance to the PAA treatments due to their membrane serving as a protective barrier against the surrounding environment. The LC50 values for the 5- and 10-minute eyed egg treatments were higher than anticipated in our spring water, given the results of previous research involving PAA's control of saprolegniasis on catfish eggs (Straus et al. 2012b) in flow-through well water. Atlantic salmon eyed eggs appear to be much more resilient to PAA than catfish eggs, which could be attributed to the thickness of the chorion of the salmon eggs. Songe et al. (2016) researched resiliency of Atlantic salmon eggs to *Saprolegnia* spp. infections in relation to egg chorion thickness; their data indicated that on

average, the chorion thickness for eggs susceptible to infections vs. eggs resistant to infections was 35.71 µm ± 2.74 µm and 42.43 µm ± 2.55 µm and (mean ± SD), respectively.

Salmon eggs in the present study were at approximately 455.9 ATU (accumulated thermal units) during the acute toxicity experiment due to shipping time requirements, and as such were close to hatching, versus the catfish eggs that were initially <24 h old and treated twice daily for 5 days until the embryos developed eyes (Straus et al. 2012a). In the present study, 50% of the unused eggs that remained in the incubation system undisturbed hatched at 511.5 ATU, approximately one week after the experiment was conducted. Following the toxicity challenge at 455.9 ATU, the control treatment groups did not contain any hatched, dead eggs; however 44% of eggs recorded as mortalities for the 5-minute treatment group were hatched and dead, and 67% of the eggs recorded as mortalities for the 10-minute treatment group were hatched and dead. It is interesting to note that in a subsequent toxicity trial at 469.9 ATU, LC50 values were much lower. The TRAP LC50 values for the 469.9 ATU eyed eggs treated for 5 and 10 minutes were 523.59 mg/L and 316.54 mg/L PAA, respectively, while TSK LC50 values for eyed eggs treated for 5 and 10 minutes were 534.64 mg/L and 318.88 mg/L PAA, respectively. There were no hatched dead eggs in control groups but with 56% of eggs recorded as mortalities for the 5-minute treatment group being hatched and dead, and with 67% of the eggs recorded as mortalities for the 10-minute treatment group being hatched and dead. The increase in hatched dead eggs during this supplemental toxicity trial can likely be attributed to the fact that the eggs utilized in the initial study were at a lower ATU range, and therefore less fragile than the eggs assessed subsequently that were closer to hatch. Eyed eggs become more fragile as they approach hatching (Yamagami et al. 1992), and therefore if younger, or lower ATU eyed eggs had been used, they would likely have been more resilient to PAA, resulting in higher 24-hour

LC50 values. Thus, the LC50 values presented for eyed eggs need to be interpreted in the context of the study's egg ATU range.

Sensitivity to PAA is related to Atlantic salmon developmental life-stage, with relatively robust fingerlings demonstrating increased PAA tolerance compared to fry. Younger fish are not as readily able to eliminate toxicants due to immature immune systems and undeveloped liver and kidneys. As younger fish continue to develop, organ systems are more sensitive to toxicants; however, as fish reach maturity, these same organ systems are likely to become more tolerant once fully developed (Bentivegna and Piatkowski 1998). Also, because of the smaller size of younger fish, toxicants can more quickly reach target organs as compared to larger, older fish (Mohammed 2013). However, as the ratio of gill surface area to body weight increases, older fish may become more sensitive to oxidants, such as PAA (Rach et al. 1997). Ozone, another oxidizing disinfectant, can also be toxic to fish; however, lethal concentrations are species-specific and depend on life stage (Summerfelt 2003).

Although some of the PAA treatments applied to the fingerlings did not cause mortality after 24 hours, a proportion of these fish appeared lethargic following PAA challenge. Seventy-eight percent of the fingerlings recorded as alive at 24 hours were lethargic; however, there was no discernable difference in the severity of lethargy between treatment groups. These lethargic fish were not recorded as mortalities, and it is unknown whether lethargic fingerlings would have fully recovered or died if they had been returned to normal culture after the experiment because all study fish remaining alive were euthanized at study's end. Chupani et al. (2014) concluded that juvenile grass carp *Ctenopharyngodon idella* exposed to 1 mg/L PAA treatment had only mild and recoverable histological alterations. This suggests that the juvenile Atlantic salmon that appeared lethargic after 24 hours of PAA exposure in the present study could have returned to

full health if given a recovery period after treatment. Additionally, Straus et al. (2012a) demonstrated that there was minimal difference between the 24-hour and 48-hour LC50 values for channel catfish sac-fry exposed to PAA, and no difference between these LC50 values for channel catfish swim-up fry. While Atlantic salmon juveniles may react similarly to the channel catfish fry given extended treatments times, future research should focus on the effects of long-term LC50 values for this species. While investigating low-dose PAA application to prevent post-vaccination saprolegniasis in juvenile Atlantic salmon in RAS, Good et al. (2020) determined that survival was significantly higher in the groups treated with PAA, and that no tissue damage associated with PAA treatment was observed through histopathological evaluation. It is interesting to note that Liu et al. (2017) reported that mirror carp exhibited an adaption of their stress response to PAA when applied in RAS over a five week period. Houshangi and Hosseini (2018) found that hemoglobin levels in rainbow trout exposed to PAA treatment of 8.9 mg/L were significantly higher than in unexposed trout; however, follow-up assessment determined that hemoglobin levels had returned to normal by two months following treatment. Similarly, Soleng et al. (2019) found that Atlantic salmon produced systematic and mucosal defenses in response to the oxidative stress caused by treatments of 0.6 and 2.4 mg/L PAA, and that the salmon were able to successfully recover from the stress induced by PAA treatment. These findings suggest that treatments less than or equal to 2.4 mg/L PAA will have a minimal impact on Atlantic salmon performance, although further research is required to determine if these findings are consistent across all Atlantic salmon life-stages and across a range of water quality profiles.

The egg, fry and fingerling LC50 values and egg NOECs determined in the present study provide a framework for aquaculturists and regulators to develop safe protocols for applying

PAA water disinfection during Atlantic salmon eyed egg, fry and fingerling life-stages. Disinfection is an integral part of fish husbandry, but there has been little research regarding PAA's applicability in RAS and egg incubation systems in the United States (Straus et al. 2012; Patrick et al. 2019), as it has only been registered for aquaculture surface disinfection by the EPA (Straus and Meinelt 2019). This research provides data to support the development of safe protocols for PAA application in RAS and egg incubation systems during early life-stage Atlantic salmon culture.

In summary, our study presents the first PAA 24-hour LC50 values for early Atlantic salmon life stages - eyed egg, fry (approximately 0.16 – 0.18 g) and fingerling (16.3 ± 0.7 g) - in reuse incubation and RAS water. These findings can support the development of PAA as a disinfectant in the Atlantic salmon RAS industry. Future research should consider the limitations of this study, and should aim to include, among other things, consistent RAS water chemistry across experiments when expanding on these findings. Results of PAA application will vary based on RAS water chemistry profiles. Aquaculturists should consider their system's specific water quality profile when developing PAA treatment regimes. Future studies with PAA should include the 15-minute PPD to help production managers determine a treatment protocol. Additional research should also focus on 48- and 96-hour LC50 values for Atlantic salmon across each life-stage as well as their recovery from high concentrations of PAA. In order to mitigate the rapid degradation of PAA associated with the presence of organic matter, it is recommended that PAA treatments are administered daily. Monitoring of PAA treated eggs post-hatch would provide valuable information as to how treatments afect hatching rates and alevin survival rates.

APPENDIX I – EGG PRELIMINARY RANGE-FINDING EXPERIMENT

A preliminary range finding toxicity experiment was conducted first to determine a narrow range of PAA treatments that resulted in at least 50% mortality among eggs over a 24-hour period. The PAA treatments were established in a 1 L glass jar using the verified PAA stock solution and the spring water maintaining the incubation system. An aliquot of deionized water was administered to the control jars in order to replicate the mechanical procedure associated with PAA addition for PAA treatment jars. PAA treatments were prepared using the verified PAA concentration, total volume of each dosing vessel (1 L acid washed glass jar) and the target concentration of each PAA treatment dose. The control treatment was a 1 L glass jar filled only with spring water. Twenty eggs were added to a jar containing the treatment and remained for either 1, 5 or 10 minutes; the eggs would then be rinsed with spring water and placed into a partition within the incubation system Heath tray. One Heath tray stack (containing three trays) was utilized for this experiment. Each Heath tray within the stack was partitioned into 21 sections, 18 of which contained 20 treated eggs (Table 6). The eggs in tray 1 of the stack were subjected to treatment for 1 minute. The eggs in tray 2 of the stack were subjected to treatment for 5 minutes. The eggs in tray 3 of the stack were subjected to the treatment for 10 minutes. The eggs were assessed for mortality 24 hours after the initial dose following the same egg sampling procedure mentioned in the methods section.

Three separate preliminary range-finding experiments were conducted. The PAA treatments (mg/L as PAA), exposure times and associated TRAP LC50 values for these experiments are listed in Table 7. LC50 values could not be calculated for the first two preliminary experiments because the average mortality rate was less than 5% at the highest PAA treatment. In the third preliminary experiment, the 1 minute treatment groups were not exposed

to the PAA treatments long enough to result in 100% mortality at the highest PAA treatment, however the 5 and 10 minute treatment groups had TRAP LC50 values of 781.47 ± 12.70 mg/L as PAA, 484.96 ± 12.32 mg/L as PAA.

Table 6. Egg preliminary range-finding experiment design.

Stack 1						
Tray #1						
0 A	0 B	5 A	5 B	10 A	10 B	20 A
20 B	30 A	30 B	40 A	40 B	50 A	50 B
60 A	60 B	70 A	70 B			
Tray #2						
0 A	0 B	5 A	5 B	10 A	10 B	20 A
20 B	30 A	30 B	40 A	40 B	50 A	50 B
60 A	60 B	70 A	70 B			
Tray #3						
0 A	0 B	5 A	5 B	10 A	10 B	20 A
20 B	30 A	30 B	40 A	40 B	50 A	50 B
60 A	60 B	70 A	70 B			

Table 7. Egg preliminary range-finding experiment details.

Preliminary Experiment #	**PAA treatments (mg/L as PAA)**	**Exposure Times (minutes)**	**TRAP LC50***
1	0, 5, 10, 20, 30, 40, 50, 60, 70	1, 5 and 10	X, X, X
2	0, 60, 90, 120, 150, 180, 210, 240, 270	1, 5 and 10	X, X, X
3	0, 300, 400, 500, 600, 700, 800, 900, 1000	1, 5 and 10	X, 781.47 ± 12.70 SE, 484.96 ± 12.33 SE

*TRAP LC50 values are in order of increasing exposure times. X denotes LC50 could not be calculated due to insufficient mortality data. SE denotes standard error.

APPENDIX II – FRY PRELIMINARY RANGE-FINDING EXPERIMENT

A range finding toxicity experiment was conducted first to determine a narrow range of PAA treatments that resulted in at least 50% mortality among fry over a 24-hour period. PAA treatments were administered in duplicate for 24 hours via the creation of a static bath along with the control treatment that did not contain PAA. An aliquot of deionized water was administered to the control trough compartments in order to replicate the mechanical procedure associated with PAA addition for PAA-treated compartments. The PAA treatments were established within each individual compartment using the verified PAA stock solution and the RAS water within the system. The water flow to and from the challenge troughs was turned off, therefore creating a static bath with no water exchange in each compartment. The volume of PAA required for each treatment was prepared using the verified PAA concentration, total volume of each trough compartment and the target concentration of each PAA treatment. The control treatment was a trough compartment filled only with RAS water. PAA treatments were assigned in random order for each trough compartment. The pre-determined amount of PAA was added to each compartment as outlined in Table 7. Each trough compartment remained in a static bath state for 24 hours following the addition of the PAA treatment. The fry were assessed for mortality 24 hours after the initial dose using visual observations as mentioned in the methods.

Table 8. Fry preliminary range-finding experiment design.

Trough 1								
8 A	6 A	0 A	2 A	1 A	2 B	5 A	10 A	0 B
Trough 2								
1 B	3 A	6 B	10 B	8 B	4 A	5 B	4 B	3 B

One preliminary range-finding experiment was conducted. The PAA treatments (mg/L as PAA) and TRAP LC50 value for this experiments are listed in Table 9.

Table 9. Fry preliminary range-finding experiment details.

Preliminary Experiment #	PAA treatments (mg/L as PAA)	TRAP LC50 value*
1	0, 1, 2, 3, 4, 5, 6, 8, 10	5.51 ± 0.07 SE

*LC50 value is estimation, inadequate partial effects data.

APPENDIX III – FINGERLING PRELIMINARY RANGE-FINDING EXPERIMENT

A range finding toxicity experiment was conducted first to determine a narrow range of PAA treatments that resulted in at least 50% mortality among fingerling over a 24-hour period. PAA treatments were administered in duplicate for 24 hours via the creation of a static bath, along with the control treatment that did not contain PAA. An aliquot of deionized water was administered to the control trough compartments in order to replicate the mechanical procedure associated with PAA addition for PAA-treated compartments. The PAA treatments were established within each individual compartment using the verified PAA stock solution and the RAS water within the system. The water flow to and from the challenge troughs was turned off, therefore creating a static bath with no water exchange in each compartment. The volume of PAA required for each treatment was prepared using the verified PAA concentration, total volume of each trough compartment and the target concentration of each PAA treatment. The control treatment was a trough compartment filled only with RAS water. PAA treatments were assigned in random order for each trough compartment. The pre-determined amount of PAA was added to each compartment as outlined in Table 10. Each trough compartment remained in a static bath state for 24 hours following the addition of the PAA treatment. The fingerlings were assessed for mortality 24 hours after the initial dose using visual observations. One preliminary range-finding experiment was conducted. The PAA treatments (mg/L as PAA) and TRAP LC50 value for this experiment are listed in Table 11.

Table 10. Fingerling preliminary range-finding experiment design.

Trough 1								
4 A	5 A	10 B	1 A	0 A	8 A	3 A	8 B	4 B
Trough 2								
1 B	0 B	6 B	3 B	5 B	10 A	6 A	2 A	2 B

Table 11. Fingerling preliminary range-finding experiment details.

Preliminary Experiment #	PAA treatments (mg/L as PAA)	TRAP LC50 value*
1	0, 1, 2, 3, 4, 5, 6, 8, 10	5.52

*LC50 value is estimation, inadequate partial effects data.

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