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Efficacy of Bath Treatments of Formalin and Copper Sulfate on Cultured White Bass, *Morone chrysops*, Concurrently Infected by *Onchocleidus mimus* and *Ichthyophthirius multifiliis*

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Concurrent ectoparasite infections on fishes are commonly encountered in the wild and in aquaculture (Rohde 1984; Hoffman 1999), for example, infections of both *Ichthyophthirius multifiliis* Fouquet, 1876 (Hymenostomatida: Ichthyopththiriidae; Colorni 2008) and gill-infecting monogenoids (Platyhelminthes: Monogenoidea; Whittington and Chisholm 2008). The decision to aggressively treat infections of either of these parasites can be prudent in aquaculture systems. Both taxa have direct life cycles and short generation times, which may result in high infection intensities that can debilitate or kill the fish host (Noga 2010). Various chemical therapies have been evaluated to prevent, reduce, or eliminate such infections. Typical treatments for killing monogenoids include: freshwater or saltwater baths, formalin, copper sulfate ($CuSO_4$), hydrogen peroxide, mebendazole, trichlorphon, and praziquantel (Whittington and Chisholm 2008). Treatments for infections of *I. multifiliis* (Ich) include: temporary exposure to high temperature, formalin, malachite green, $CuSO_4$, potassium permanganate, and sodium chloride (Colorni 2008). Despite the common occurrence of these parasites on fish, little species-specific information exists for the vast majority of fish–parasite combinations across the diversity of culture systems and captive settings. Formalin is approved by the US Food and Drug Administration (US FDA) for use as a treatment for external monogenetic trematodes in fish at up to 250 mg/L for up to 1 h (US FWS/AFS 2008). The ambiguity of this recommendation suggests a wide therapeutic range with no interpretation of repeated applications. Likewise, little information exists on the safety or efficacy of repeated formalin treatments on fish, and $CuSO_4$ has an indeterminate approval status but is widely accepted as having high efficacy.

In October 2009, white bass, *Morone chrysops* (Rafinesque, 1820; Perciformes: Moronidae), in a flow-through culture system were diagnosed with concurrent infections of the gill-infecting monogenoid *Onchocleidus mimus* (Mueller, 1936; Monogenoidea: Ancyrocephalidae) and Ich. *O. mimus* has a direct life cycle with no intermediate host required and as the intensity of infection increases can become problematic to a population. This study reports an opportunistic evaluation of the efficacy of repeated applications of formalin and $CuSO_4$ to reduce, control, or eradicate these infections in cultured white bass.

Materials and Methods

Background and Initial Assessment

Approximately 2000 white bass fingerlings weighing 10 g (±2 g; mean ± SD) each were

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held in a 2,460-L (65 × 113 × 335 cm) cement raceway with aerated well water maintained at 23 C (±0.8 C). After 30 d, it was observed that the fish collectively exhibited lethargy and loss of appetite. For diagnostic purposes, 15 white bass were randomly selected, euthanized by spinal severance and subsequently necropsied. Approximately 20 gill filaments (1.5 × 1.5 cm biopsy) from each white bass were excised from the second gill arch, wet-mounted in ambient water, and studied immediately with a compound microscope at 40–100× magnification. Microscopy revealed parasitic flatworms and ciliates, which had a horseshoe-shaped nucleus, attached to the gill. All monogeneoid and ciliate individuals within a 2.25-cm^2 area delimited by an etched microscope slide were counted to standardize and reliably assess infection intensity.

Separately, for taxonomic identification, living monogenoids attached to the gill were killed with heated freshwater (60 C) and then immediately fixed in 5% neutral-buffered formalin and prepared as whole-mounted specimens (Kritsky et al. 2011). These specimens were identified as *O. mimus* (Beverly-Burton et al. 1986) and deposited as voucher specimens in the US National Parasite Collection (Beltsville, MD, USA; USNPC No. 038473). Ciliates were fixed directly in 5% neutral-buffered formalin and identified as *I. multifiliis* (Colorni 2008). Bacterial samples from liver and kidney were cultured on Ordal's and Tryptic Soy Agar with 5% sheep blood and incubated for 48 h at 28 C (Anacker and Ordal 1955) to confirm the presence or absence of a bacterial infection.

Fish Husbandry and Water Quality Parameters

Twenty white bass fingerlings were transferred from the infected population and stocked into each of 12 ($n = 4$ for each treatment, 240 total fish) 18-L aquaria containing 10 L of 23 C filtered well water; supplemental aeration was provided via air-stones. Fish were fed daily to satiation. The stocking density was approximately 20 g/L, which simulated the original stocking density in the 2460-L cement raceway. Flow-rate was controlled by the "Ultra-Low-Flow System" developed by Mitchell and Farmer (2010); flow rate was 80 mL/min and produced a water turnover every 2 h. This rate was approximate to the turnover rate in the original raceway where the epizootic had occurred. The following water quality parameters were monitored and recorded during the experiment: water temperature, 23.3–23.8 C; dissolved oxygen, 7.4–8.1 mg/L (Wissenschaftlich-Technische Werkstätten pH/Oxi 340i/SET meter, Weilheim, Germany); total ammonia nitrogen 0.07–0.21 mg/L (Hach DR/4000V spectrophotometer, Nessler Method 8038; Hach Company, Loveland, CO, USA); total alkalinity, 210 mg/L, and total hardness, 110 mg/L, by standard titration methods (APHA 2005) prior to treatment initiation.

Chemical Exposures

Static 1 h exposures of 100 mg/L formalin (formaldehyde 37%; ACROS Organics, Geel, Belgium) and 2.1 mg/L $CuSO_4$ (Triangle Brand® Copper Sulfate Crystal; Freeport-McMoRan Sierrita Inc., Green Valley, AZ, USA) were applied every other day (five exposures over 10 d) and the control group was given distilled water. Copper sulfate treatments were added from a stock solution made by dissolving copper sulfate crystals in distilled water. After each exposure, flow was resumed at 1 L/min for 40 min (i.e., four turnovers to flush the treatments) before returning to 80 mL/min or about a 2 h turnover. Dose-confirmation samples were taken 2 min after dosing and analyzed using the Hach Formaldehyde Method (8110) for formalin and an inductively coupled plasma optical emission spectrometer (Optima 2000DV, Perkin Elmer, Waltham, MA, USA) for $CuSO_4$; the mean exposure doses for formalin and $CuSO_4$ were 110 (±9.8) mg/L and 2.1 (±0.16) mg/L, respectively.

Enumeration of Infection Intensity

Parasites were counted 20–24 h after exposure by arbitrarily removing two fish from each aquarium for sampling (2 fish/aquaria = 8 fish/treatment). On Day 10, 24 h after the fifth and final treatment the remaining fish in each treatment group were sacrificed and the parasite enumeration counts made. Infection intensity was measured as the total number of parasites counted in the sample area, as described previously. Parasite prevalence was calculated as the number of fish positive for the parasite out of the number of fish sampled for each treatment group. Percent effectiveness (US FDA/CVM 2008) was calculated using the formula below:

$$\frac{\text{PCG} - \text{PTG}}{\text{PCG}} \times 100 = \%\,\text{Effectiveness}$$

Where PCG is the mean number of parasites in the control group and PTG the mean number of parasites in the treated group.

Statistical Analyses

Statistical analysis was conducted using a one-way ANOVA (Proc ANOVA) to analyze infection intensity and treatment effect after each exposure. Treatment was the fixed effect and replication was added as random effect. Treatment effects were considered significant at $P \leq 0.05$.

Results

Mortality was minimal throughout the experiment. Two fish died in the formalin treatment group, one fish died in the $CuSO_4$ treatment group, and three fish died in the control group. Gross necropsies of the fish indicated a lack of lesions and the infected and noninfected conspecifics were morphologically indistinguishable from remaining fish in the experimental system. Pre-treatment bacteriological samples were negative for growth, and no gross disease sign indicative of a bacterial disease was observed during the study period.

Infection Intensity and Prevalence of O. mimus

Prior to treatment, the mean infection intensity and prevalence of *O. mimus* in white bass from the 2460-L raceway was 4.7 (±1.8; n = 15) and 86.7% per sample area, respectively. Figure 1 depicts the mean infection intensity of *O. mimus* in each treatment group and controls after each exposure. Over the 10-day study, formalin-treated white bass had lower infection intensity than control and $CuSO_4$-treated fish ($P < 0.05$). Copper sulfate treatments also significantly lower the infection intensity of *O. mimus* compared to the control. However $CuSO_4$-treated fish did have significantly higher infection intensity than formalin-treated fish. Infection intensity of *O. mimus* was markedly reduced after the third treatment by either chemical compared to the control ($P < 0.05$; Fig. 1); infection intensity was significantly different between the formalin-treated and $CuSO_4$-treated fish also by the third treatment and remained different through the study. Prevalence was significantly lowered only by formalin. At the conclusion of the 10-day study, mean infection intensity of infection by *O. mimus* for the formalin-treated, $CuSO_4$-treated, and control white bass was 0.5 (±0.38), 7.7 (±2.5), and 26.9 (±4.0), respectively. Percent effectiveness for the formalin and $CuSO_4$ treatments was 98.2 and 71.2%, respectively (Table 1). The prevalence on day 10 was 26% for formalin-treated white bass, whereas all $CuSO_4$-treated and control white bass were still infected.

Infection Intensity and Prevalence of I. multifiliis

Formalin and $CuSO_4$ treatments had no effect on infection intensity of Ich during the 10-day experiment. The mean infection intensity and prevalence of infection by Ich was 11.7 (±2.6; n = 15) and 86.7% prior to treatment. Infection intensity peaked after the second treatment for the $CuSO_4$ (39.9 ± 10.0) and the control groups (46.4 ± 14.1). By day 10, the mean infection intensity and prevalence in the control group had dropped to 1.13 and 37.5%, respectively. There was no statistical difference between mean infection intensity for formalin or $CuSO_4$-treated fish when compared to the control group.

Discussion

This study focused on the efficacy of formalin and $CuSO_4$ treatments against concurrent Ich and *O. mimus* infections. Formalin is

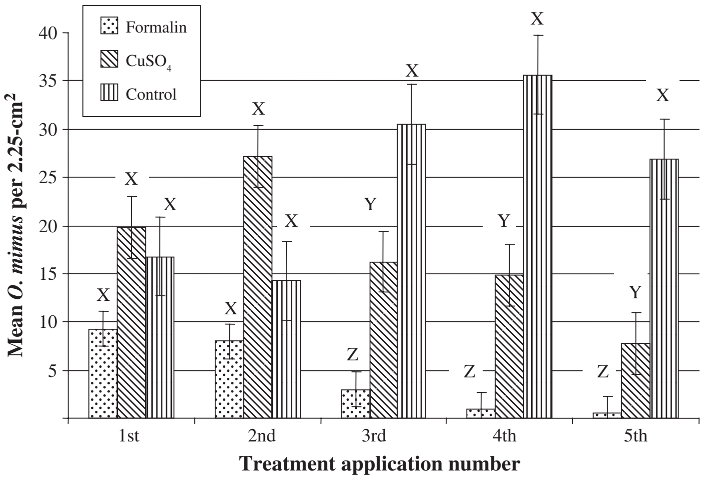

FIGURE 1. *Mean infection intensity of* Onchocleidus mimus *within a 2.25-cm^2 area on gill samples of white bass 20 h after treatment application of 100 mg/L formalin, 2.1 mg/L $CuSO_4$, or no treatment every other day for 10 d. Mean infection intensity of* O. mimus *prior to treatment was 4.7. Error bars depict standard errors and means with different letters represent significant differences between treatments at each application number.*

TABLE 1. *Percent effectiveness (P. E.). P. E. and prevalence of infection by* Onchocleidus mimus *in gill of white bass after each treatment application compared to control.*[a]

	Formalin		$CuSO_4$		Control
Treatment application	P. E.[b]	Prevalence	P. E.[b]	Prevalence	Prevalence
Pre-treatment	0	13/15	0	13/15	13/15
1st	44.8	8/8	−17.9	8/8	8/8
2nd	43.9	8/8	−89.5	8/8	8/8
3rd	**90.2***	1/8	46.7	8/8	8/8
4th	**97.5***	4/8	58.2	8/8	8/8
5th	**98.2***	10/38	71.2*	39/39	37/37

[a]Percent effectiveness was calculated as:

$$\frac{PCG - PTG}{PCG} \times 100 = \%\,\text{Effectiveness}$$

Where PCG is the mean number of parasites in the control group and PTG the mean number of parasites in the treated group.

[b]Significant differences within treatments are denoted by $*P \leq 0.05$. P. E. greater than the FDA "Minor Use Minor Species" guidelines (>90%) are in bold.

approved by the US FDA for use as a treatment for external monogenetic trematodes in fish at up to 250 mg/L for up to 1 h (US FWS/AFS 2008). The ambiguity of this recommendation suggests a wide therapeutic range with no assessment on the efficacy of repeated applications. Formalin has long been recognized as a useful treatment against monogenoid infections (Kabata 1985); however, little species-specific information exists on the safety or efficacy of repeated formalin treatments on infected fish (Schmahl 1991, 1993). Copper sulfate is not approved by the US FDA as a therapeutant for use in aquaculture, but regulatory action has been deferred pending the outcome of ongoing research to gain this approval (Bowker et al. 2011); it is used extensively in marine systems because of its high efficacy (Thoney and Hargis 1991; Colorni 2008; Whittington and Chisholm 2008).

Under the described study conditions and treatment regimen, these results indicate that *O. mimus* infection intensity and prevalence can be lowered with formalin. Static bath treatment of 1 h with formalin at 100 mg/L applied five times every other day significantly lowered the intensity of infection by *O. mimus* in the gill of white bass. The data indicated that a minimum of three applications were required to reduce infection intensity. Other treatment regimens for formalin and other chemicals have approval or exemption for use on food fish and could likewise control such infections (Post 1987; Straus 1993; Tieman and Goodwin 2001; Whittington and Chisholm 2008; Noga 2010).

A significant difference in infection intensity was also achieved in $CuSO_4$-treated fish compared to the control. However, the fact that the prevalence was not statistically reduced by $CuSO_4$ treatment and the assessed reduction of infection intensity was still significantly less than in formalin-treated fish suggests the treatment may not be optimal. Higher levels, more applications or longer treatment durations may have been more efficacious. Future studies should investigate possible treatment variables to achieve improved efficacy with $CuSO_4$.

The mean intensity of Ich infection increased through Days 5–6 before decreasing. Higher levels or longer durations of both treatments may have been efficacious or required fewer applications, but potentially could be toxic to white bass. The 24 and 96 h LC50 values of formalin for striped bass, *Morone saxatilis* was 113 and 64 mg/L (Bills et al. 1993), respectively, in water of similar alkalinity and hardness to this study.

There are drawbacks to the use of chemical treatments (e.g., cost, effects on dissolved oxygen, effects on water quality, and acute toxicity to the fish stock). Copper sulfate may require more applications, but the lower cost may make it a viable option. Formalin is an approved parasiticide for food fish but $CuSO_4$ is not. With the rates used in this study, formalin costs are 40× that of $CuSO_4$ (http://www.coppersulfatecrystals.com), and the estimated cost to treat 1000 L of water with $CuSO_4$ and formalin purchased in bulk is 1.5 and 60¢, respectively.

The reduction of *O. mimus* in formalin-treated white bass (100 mg/L for 1 h daily for 5 d) met the recommended measure of percent effectiveness according to guidelines for Minor Use in Minor Species (US FDA/CVM 2008); these guidelines state that the mean infection intensity of the treated group must be significantly less than the control group ($P < 0.05$, one-tailed test) before calculating percent effectiveness, and that at least 90% effectiveness is necessary for each parasite claim in the pivotal trials supporting approval. These criteria were fulfilled by the formalin-treated white bass subsequent to the third application. The minimum suggested requirement was not reached in the $CuSO_4$-treated white bass. The theoretical 90% reduction in mean infection intensity was not met by either treatment with regards Ich infection. Formalin treatments lowered infection intensities after three applications in this study, strongly indicating it is a more efficacious treatment to reduce potentially epizootic levels of ancyrocephalid infections.

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