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ARTICLE

Safety of Copper Sulfate to Channel Catfish Eggs

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Abstract

Copper sulfate ($CuSO_4$) is used in the catfish industry to control saprolegniasis (caused by the watermolds *Achlya* spp. or *Saprolegnia* spp.) on eggs. This study was designed to establish the margin of safety of $CuSO_4$ at 10, 30, and 50 mg/L when applied for three times the normal treatment duration to hatching troughs containing channel catfish *Ictalurus punctatus* eggs in 26°C, flow-through well water. The safety of $CuSO_4$ was indicated by the percentage of fry that hatched from the eggs. Eggs were treated daily until the embryos reached the eyed stage. The least-squares means ± SE of hatch rates were 40.8 ± 9.1, 80.9 ± 6.4, 64.2 ± 8.6, and 80.3 ± 6.6% for the 0-, 10-, 30-, and 50-mg/L $CuSO_4$ treatments, respectively. The hatch rate for the 0-mg/L $CuSO_4$ treatment was significantly different from that of all other $CuSO_4$ treatments. Treating channel catfish eggs with five times the recommended dose of 10-mg/L $CuSO_4$ for three times the recommended duration did not adversely affect the hatch rates in this study. There appears to be an adequate margin of safety above the therapeutic dose of 10 mg/L for channel catfish eggs; therefore, $CuSO_4$ should be considered a safe treatment to alleviate egg losses caused by saprolegniasis.

Channel catfish *Ictalurus punctatus* are raised commercially in the United States. Adults spawn during the spring, and egg masses are brought into the hatchery for observation to estimate the amount of fry produced. Fry are then fed until they are ready to be stocked in ponds. One of the most common disease problems during the hatchery phase is saprolegniasis, which is usually caused by the watermolds *Saprolegnia* spp. and *Achlya* spp.; it can cause severe losses of channel catfish eggs held in hatcheries (Neish and Hughes 1980; Tucker and Robinson 1990). Copper sulfate ($CuSO_4$) is used in hatcheries to control saprolegniasis on eggs during hatching (Steeby and Avery 2005). Copper sulfate is not approved by the U.S. Food and Drug Administration (FDA) for therapeutic use in aquaculture, but regulatory action has been deferred pending the outcome of ongoing research.

Copper sulfate research at the Harry K. Dupree–Stuttgart National Aquaculture Research Center is designed to provide the sponsor (Freeport-McMoRan Copper & Gold, Phoenix, Arizona) with data for future FDA approval of their product to control mortalities associated with saprolegniasis on channel catfish eggs. The effectiveness of $CuSO_4$ for treating saprolegniasis on channel catfish eggs has been established (Straus et al. 2009a, 2009b, 2011); an optimum therapeutic dose of 10-mg/L $CuSO_4$ was determined and confirmed in these studies. However, the margin of safety of the 10-mg/L $CuSO_4$ treatment on channel catfish eggs has not been determined.

The purpose of this study was to determine the safety of $CuSO_4$ (with respect to hatch rates) when applied at 10 mg/L and at three and five times this rate for three times the normal treatment duration to hatching troughs containing channel

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catfish eggs in flow-through well water. The present study was required by the FDA to demonstrate the margin of safety of $CuSO_4$ to the target animal (eggs) to support a New Animal Drug Application.

METHODS

This study followed Good Laboratory Practice regulations and used the experimental hatching trough system and methods previously described (Straus et al. 2009a, 2009b, 2011). In addition, a higher temperature (26°C instead of 23.5°C) was used to reduce the possibility of saprolegniasis and a slower flow rate was used during the treatment portion of the study to lengthen the $CuSO_4$ exposure in accordance with FDA Target Animal Safety study guidelines. Four egg masses (<24 h old) were collected from channel catfish broodstock spawned at the Harry K. Dupree–Stuttgart National Aquaculture Research Center; each egg mass was the result of a single female–male pairing. Egg masses were moved to an environmentally controlled laboratory and distributed into four aluminum hatching troughs designed specifically for this type of study. Light blue, powder-coated troughs aided in concealing which tanks received $CuSO_4$ treatments. The compartments within each trough were sequentially numbered and treatments were randomly assigned. The compartments (35 L) received filtered (75-μm) well water that flowed through the troughs continuously.

Two water inlets provided well water at the normal (1.25 L/min) or reduced (0.42 L/min) flow rates in each compartment; water flowed from only one inlet at a time. The normal flow rate allowed for a 28-min volume exchange rate, which is typical in a channel catfish hatchery. The reduced flow rate (one-third the normal flow) was used during treatments to expose eggs to $CuSO_4$ for three times the normal treatment duration; the volume exchange rate was 84 min and the flow was immediately restored to the normal rate. Flow rates were monitored and adjusted daily. Tempered well water was supplied through a stainless-steel mixing valve (Intellifaucet K375SS; Hass Manufacturing, Averill Park, New York). The mixing valve used a combination of heated and unheated well water from the same source.

A rotating plastic paddle (Steeby et al. 2004) in each compartment provided water circulation, and a plastic mesh basket contained the eggs until they hatched. Each compartment drained through a polyvinyl chloride standpipe covered inside another pipe to allow for the bottom to be drained (i.e., deep-water release). Compartments were provided aeration via an air stone to maintain dissolved oxygen (DO) levels at or above 70% saturation, especially during dosing, when the flow rate was reduced. The eggs were maintained under a photoperiod of 12 h light : 12 h dark with fluorescent lighting.

The egg mass from a spawn was gently divided, and four similarly sized portions were placed into individual plastic-mesh baskets held in individual compartments of a single hatching trough. The egg portions were acclimated to environmental conditions for 1 h. There were four troughs ($n = 4$), and the mean weight ± SD of the egg portions in all compartments was 99.2 ± 4.7 g. The adhesive matrix of each egg mass portion remained intact as is typical in channel catfish hatcheries.

Three representative samples (15.8 ± 2.7 g) from the egg mass of each spawn were weighed, and the adhesive matrix was dissolved by gentle stirring in a 1.5% solution of sodium sulfite (Sigma Chemical, St. Louis, Missouri) to facilitate counting of the eggs. These counts were used to estimate the total number of eggs in each compartment.

Copper sulfate pentahydrate (25.4% Cu) used for this experiment was Triangle Brand Copper Sulfate (Freeport-McMoRan Copper & Gold). There were four different concentrations of $CuSO_4$ (0, 10, 30, and 50 mg/L). The study was blinded so that only the study director was aware of the treatment concentrations but did not count the eggs. A 70-g/L $CuSO_4$ stock solution was prepared with deionized water. Numbered amber vials were prepared for dosing with an aliquot of stock solution, deionized water, or both (total volume = 40 mL) based on the treatment assigned to each compartment. Doses were applied to each compartment by pouring the contents of the respective vial into the water opposite the egg basket to allow the rotating paddle to disperse the treatment. Doses were administered once every 24 h until any embryo within that trough developed eyes.

Saprolegniasis occurred through natural infestation. Watermolds were verified by microscopic examination for the presence of typical mycelia, hyphae, or zoosporangia. Samples from the 0-mg/L $CuSO_4$ treatment were cultured on yeast peptone sucrose media (containing 68-μg/mL streptomycin and chloramphenicol) and were identified by polymerase chain reaction amplification and sequencing of the ribosomal RNA gene internal transcribed spacer regions (White et al. 1990; Straus et al. 2009a).

At hatch, healthy yolk-sac fry swam to the bottom of the compartment and congregated in the corners. Prematurely hatched (PMH) fry typically hatched before the normal hatching began, appeared deformed, and lacked the normal mobility of healthy fry. These PMH fry were waxy yellow instead of the golden brown color associated with healthy fry and did not congregate in the corners of the compartment with healthy fry. Healthy fry were siphoned from each compartment, euthanized, preserved in a 70% solution of ethanol, and counted within 1 week. Live PMH fry were then removed and counted immediately as these accumulated in the center of the compartment along with any dead fry.

Actual $CuSO_4$ concentrations within each compartment were measured from water samples taken at midwater depth 30 s after each dosing (Straus et al. 2009b). Water samples were filtered (0.22-μm nylon syringe filter), acidified to 1% with nitric acid, and stored for later analysis (Straus et al. 2009b). Samples were analyzed with an Optima DV2000 inductively coupled plasma optical emission spectrometer (Perkin-Elmer, Waltham, Massachusetts) according to standard methods for Cu analysis (APHA et al. 2005). An Orion Research 720A meter

TABLE 1. Percent hatch of channel catfish eggs in each replicate spawn after treatment with $CuSO_4$ at zero (control), one, three, or five times the recommended concentration (10 mg/L) administered once daily on consecutive days. Treatments were applied for three times the recommended treatment duration. Least-squares (LS) means (± SE) with the same letter are not significantly different ($P > 0.10$).

	$CuSO_4$ treatment (mg/L)			
Spawn	0	10	30	50
1	57.3	93.6	74.8	94.5
2	70.9	82.3	63.5	71.4
3	22.1	77.9	61.1	70.4
4	20.8	64.5	53.1	80.9
LS mean	40.8 ± 9.1	80.9 ± 6.4 z	64.2 ± 8.6 y	80.3 ± 6.6 z

(Thermo Electron, Beverly, Massachusetts) was used to measure pH, and total alkalinity and total hardness (as $CaCO_3$) were measured by titration methods (APHA et al. 2005) at the beginning and end of the study. Dissolved oxygen and water temperature were monitored daily in each compartment with a YSI Model 95 meter (YSI Environmental, Yellow Springs, Ohio).

Safety of the treatment was assessed based on the percentage of fry that hatched from the eggs; fry were counted individually to establish the hatch rate. Treatments were assigned by using a randomized block design. There were four replicates of each treatment with one replicate per trough; an individual spawn was used in each trough. Egg hatching success was compared and analyzed by means of a generalized linear mixed model (GLIMMIX procedure in the Statistical Analysis System version 9.1.3; SAS Institute, Cary, North Carolina) to test for a group effect. A binomial error and logit link were used, and the response was entered as the proportion of hatched (normal and PMH) fry to the calculated number of eggs in the compartment. Dose ($CuSO_4$ at 0, 10, 30, or 50 mg/L) was included in the model as the fixed effect, and trough was included as the random effect. Differences were considered significant at *P*-values less than or equal to 0.10; this level of significance was used to provide a conservative estimate of $CuSO_4$ safety.

RESULTS AND DISCUSSION

During the study, the normal flow rate was 1.26 ± 0.04 L/min (mean ± SD) and the reduced flow rate was 0.42 ± 0.01 L/min. The mean temperature and DO were 26.1 ± 0.2°C and 79.4 ± 4.7% saturation, respectively, under normal flow conditions and 25.7 ± 0.4°C and 89.6 ± 3.6% saturation, respectively, under low flow conditions. The total alkalinity and total hardness (as $CaCO_3$) of the water were 214.2 ± 3.8 mg/L and 106.3 ± 0.6 mg/L, respectively; pH was 7.5 ± 0.0. In contrast to situations in which $CuSO_4$ is applied to ponds, where alkalinity and hardness are important factors in the determination of treatment rates, these conditions are not factors when treating eggs because the dose is much greater and the application is accomplished in less than 1 min (Straus et al. 2009b).

The group effect between the $CuSO_4$ treatments was statistically significant ($F = 9.44$, df = 3, $P < 0.01$); therefore, contrast statements were constructed to compare the differences between group means. The 10-, 30-, and 50-mg/L $CuSO_4$ treatments were not compared with the 0-mg/L $CuSO_4$ treatment because of the confounding effect of saprolegniasis-induced embryo mortality. There was a significant difference in hatch rate between the 10- and 30-mg/L $CuSO_4$ treatments ($P = 0.06$) but not between the 10- and 50-mg/L $CuSO_4$ treatments ($P = 0.93$). The difference between the 10- and 30-mg/L $CuSO_4$ treatments was probably not dose-related but can be attributed to random factors in allocating egg portions from each spawn to compartments or to variation between spawns, as is evident in the range of percent hatch observed among replicates in this study (Table 1). The higher egg hatch rate for the 10- and 50-mg/L $CuSO_4$ treatments in replicate 1 may explain the statistically significant difference in hatch rates between the 10- and 30-mg/L $CuSO_4$ treatments. Comparisons of the data without replicate 1 still resulted in a significant group effect, but the contrasts between the 10- and 30-mg/L $CuSO_4$ treatments were no longer different and no other significant differences were found in egg hatch rates between the $CuSO_4$ treatment groups.

A water temperature of 26°C was used in this study to minimize watermold growth on eggs in the 0-mg/L $CuSO_4$ treatment in order to compare hatch rates with those in the 10-, 30-, and 50-mg/L $CuSO_4$ treatments. Temperature in previous $CuSO_4$ effectiveness studies was 23.5°C (Straus et al. 2009a, 2009b, 2011). However, egg incubation at cooler temperatures enhances fungal growth. The higher temperature reduced the prevalence of saprolegniasis but did not completely prevent it in the present study. As expected, eggs hatched in less time due to the higher temperature. Eye pigment was first seen in the embryos during morning observations on day 5; therefore, doses were discontinued after the fourth dose. Fry started to hatch on day 6, and hatching was complete by day 8.

A small amount of watermold was detected by day 3 on eggs treated with 0-mg/L $CuSO_4$ (three replicates) and on eggs treated with 10-mg/L $CuSO_4$ (one replicate). Watermolds in the 0-mg/L $CuSO_4$ treatment grew steadily and peaked at approximately 7 cm^2 in replicates 3 and 4 (affecting survivals in Table 1)

and grew to 4 cm^2 in replicate 1. Presence of watermolds in the eggs treated with 10-mg/L $CuSO_4$ (replicate 2) was minimal and peaked at approximately 1 cm^2; although watermolds were present in this $CuSO_4$ treatment, they were absent in the 30- and 50-mg/L $CuSO_4$ treatments. Watermold samples were identified as *Saprolegnia* spp. through polymerase chain reaction and internal transcribed spacer sequence characterization. Two sequences were identified from the four cultures; these sequences were 100% and 99% homologous with the *Saprolegnia* spp. sequences UNCW286 and UNCW273, respectively, listed in GenBank (www.ncbi.nlm.nih.gov/Genbank/).

Variable hatch rates can be explained by susceptibility of an individual spawn to saprolegniasis, the number of unfertilized eggs in an individual spawn, or other pathogens affecting an individual spawn (e.g., bacteria, ciliates). The mean hatch rate in the 0-mg/L $CuSO_4$ control treatment was 41% (Table 1) due to saprolegniasis in three of the replicates. The mean hatch rate in the $CuSO_4$-treated eggs ranged from 64% to 81%. The hatch rates of each replicate (Table 1) clearly show this variability within individual spawns; such variability would be expected in commercial hatcheries. Wolters (2001) estimated the hatch rate in the channel catfish industry to be about 60% when formalin and iodine are used, as needed, to control fungal infections.

Tolerance to $CuSO_4$ appears to vary with fish age. Straus (2008) determined that the 24-h LC50 (concentration that is lethal to 50% of the test organisms) of $CuSO_4$ to channel catfish yolk-sac fry was 63 mg/L in water with 217-mg/L alkalinity and 126-mg/L hardness; Straus (2008) also demonstrated that yolk-sac fry were at least four times more tolerant of $CuSO_4$ than were swim-up fry. The apparent tolerance of sac fry to $CuSO_4$ and the observation that fungal area increased after daily treatments were stopped (Straus et al. 2011) suggest that continuing $CuSO_4$ treatments through hatch are safe and needed but are contraindicated when swim-up fry are present. Prematurely hatched fry were present in each treatment and comprised less than 1% of the total hatch; data indicated that PMH fry were arbitrarily distributed among all treatments. Torrens and Steeby (2008) suggested that low DO on the last day of incubation may account for PMH fry, and those authors found that it takes longer for these fry to reach the swim-up stage in comparison with normal fry; fry in the present study were euthanized when hatching was complete and before the swim-up stage.

Straus et al. (2009b) described the elimination rates of $CuSO_4$ from this system and the reasons for choosing the 0.22-μm nylon filter pore size and for obtaining the water sample 30 s after the treatment was applied. The 0-mg/L $CuSO_4$ treatment did not contain any dissolved Cu. The 10-, 30-, and 50-mg/L $CuSO_4$ treatments contained 1.9, 4.7, and 6.3-mg/L dissolved Cu (Table 2). The recovered amount of dissolved Cu in each $CuSO_4$ treatment ranged from 49% to 75%. The percentages of recovered Cu in the present study were similar to those reported in previous studies (Straus et al. 2009a, 2009b, 2011). The lower concentration of recovered Cu is typically due to the fact that much of the total Cu (measured in an unfiltered sample) is bound and removed by filtering to obtain the dissolved Cu concentration. Any biotic ligand, such as fish skin or eggs and many inorganic compounds, will bind to Cu (Tucker and Robinson 1990; Tessier and Turner 1995; Hargreaves and Tomasso 2004).

TABLE 2. Treatment, nominal copper concentration, measured dissolved copper (mean ± SD), and percent recovered copper after treatment of channel catfish eggs with $CuSO_4$ at zero (control), one, three, or five times the recommended concentration (10 mg/L) administered once daily on consecutive days. Treatments were applied for three times the recommended treatment duration.

$CuSO_4$ treatment (mg/L)	Nominal Cu (mg/L)[a]	Measured dissolved Cu (mg/L)[b]	Recovered Cu (%)[c]
0	0	0.00	0
10	2.54	1.92 ± 0.94	75.5
30	7.63	4.69 ± 1.06	61.5
50	12.72	6.26 ± 0.93	49.2

[a]$CuSO_4$ contains 25.4% copper.
[b]$n = 16$ (4 replicates × 4 d).
[c]Calculated as (measured Cu/nominal Cu) × 100.

In conclusion, this study established the safety of $CuSO_4$ for channel catfish eggs with respect to hatch rate. Treating eggs with five times the recommended dose of 10-mg/L $CuSO_4$ for three times the recommended duration did not adversely affect the hatch rates in this study. We found that the margin of safety exceeded five times the recommended dose of $CuSO_4$. Thus, $CuSO_4$ application appears to be a safe method for reducing saprolegniasis on channel catfish eggs and for increasing the hatch rate.

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