Effectiveness of Copper Sulfate and Potassium Permanganate on Channel Catfish Infected with *Flavobacterium columnare*

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Abstract
Copper sulfate (CuSO₄) and potassium permanganate (KMnO₄) were evaluated for their effectiveness to curtail mortality and decrease bacterial load in fish tissues and water in channel catfish *Ictalurus punctatus* infected with *Flavobacterium columnare*, the causative agent of columnaris. Fish (average size, 16.6 g and 13.7 cm) were obtained from a single culture tank undergoing an epizootic of columnaris and were stocked at a density of 30 fish per tank in each of 12 tanks. The study consisted of three treatments (*n* = 4 replicates per treatment): an untreated control group, a CuSO₄-treated group, and a KMnO₄-treated group. Treatment rates were 2.1 mg/L CuSO₄ and 3.0 mg/L KMnO₄ and were applied on three consecutive days at 24-h intervals. Caudal fin, gill, and liver samples were taken from one fish of each replication 24 h after the last treatment for analysis by quantitative real-time PCR. A significant improvement in survival was noted among fish treated with CuSO₄ (72.7%) compared with the control fish (38.5%) and the KMnO₄-treated fish (52.2%). Survival of the KMnO₄-treated fish was not significantly different from the control. Quantitative PCR indicated a significant reduction of bacteria in the water after the first and second treatments of both therapeutics; no difference was detected in fish tissues. Histological analysis of gill samples 24 h after the last treatment indicated that CuSO₄-treated fish exhibited significantly less gill damage than untreated controls and there was no difference in KMnO₄-treated fish. Copper sulfate therapy significantly improved survival, reduced the number of bacteria in the water, and reduced gill pathologies associated with *F. columnare* infection in channel catfish.

Columnaris disease, caused by the Gram-negative bacterium *Flavobacterium columnare*, has been a significant problem in many warmwater fish species for decades and is one of the most important bacterial diseases of channel catfish *Ictalurus punctatus* commercially reared in the United States (USDA 2003). Based on the site of infection and appearance of infected tissues, the disease is commonly known as “saddleback,” “fin rot,” or “cotton wool disease.” One common clinical sign of the disease is the pronounced erosion and necrosis of external tissues, with the gills often being a major site of damage (Davis 1922). Presumptive diagnosis is based on the presence of the clinical signs mentioned above and by the cell morphology of *F. columnare* in wet mounts of scrapings from infected tissues (Noga 1996).

Because *F. columnare* primarily attacks the skin and gills, putative treatments for columnaris disease include surface-acting disinfectants such as copper sulfate (CuSO₄), potassium permanganate (KMnO₄), and hydrogen peroxide (Wakabayashi 1991; Plumb 1999). However, results from previous studies examining CuSO₄- and KMnO₄-based therapies for columnaris disease have yielded mixed results.

Jee and Plumb (1981) showed that an indefinite treatment of 4 mg/L above the 15-min potassium permanganate demand (PPD; a measure of the amount of KMnO₄ reacting with organic matter in a 15-min time frame, Boyd 1979) was necessary to control columnaris in fathead minnow *Pimephales promelas*. Potassium permanganate at 2 mg/L KMnO₄ was shown to be an effective treatment, while CuSO₄ at 1 mg/L was not effective (Thomas-Jinu and Goodwin 2004). Darwish et al. (2008) found that KMnO₄ treatments at 2 mg/L above the PPD did not reduce mortality of channel catfish owing to columnaris disease when...
treatments were initiated 24 h after challenge. In a later study, Darwish et al. (2010) demonstrated that KMnO4 was effective at 2 mg/L above the PPD when administered simultaneously with a challenge dose of *F. columnare*, but only marginal efficacy was found when the treatment was applied at 1 h after challenge.

These findings suggest that KMnO4 has clear treatment value in the early stages of infection, but limited therapeutic value after the infection has progressed (Darwish et al. 2010). Alternatively, while the use of CuSO4 is highly regarded anecdotally and is cited as a recommended treatment for columnaris disease (Plumb 1999), unequivocal efficacy has yet to be established. Despite limited research, CuSO4 can exert inhibitory activity in vitro against *F. columnare* (Schrader 2008; Darwish et al. 2012). Moreover, MacFarlane et al. (1986) reported that preexposure to CuSO4 protected against *F. columnare* challenge in striped bass *Morone saxatilis*. In a similar early intervention study, a 4-h static, 2.1-mg/L CuSO4 treatment initiated 5.5 h after exposure to *F. columnare* significantly increased survival of channel catfish (Darwish et al. 2012).

Differences in experimental design probably account for the varied outcomes of effectiveness studies on compounds such as KMnO4 and CuSO4. Differences include the variation in experimental systems and techniques used to challenge fish. For example, investigators favor treating at different time points after exposure, and other differences include the use of static or flow-through systems to carry out therapeutic studies (Thomas-Jinu and Goodwin 2004; Darwish et al. 2008, 2009, 2010). Furthermore, some studies have employed cutaneous abrasion protocols to facilitate an infection (Darwish et al. 2008) while others have used challenge vessels containing unnaturally high bacterial concentrations that can result in massive acute mortality (Thomas-Jinu and Goodwin 2004).

The objectives of the present study were two-fold: we first evaluated the effectiveness of both KMnO4 and CuSO4 on a nonchallenge-based *F. columnare* outbreak to better approximate conditions that a fish culturist would encounter. Second, in an effort to better understand the mechanisms by which CuSO4 and KMnO4 are effective, we characterized the number of bacteria before and after treatments within the water, on the surface of the fish (fin, gill, and liver), and over the course of the study.

**METHODS**

*Epizootic.*—Channel catfish fingerlings were cultured indoors at the Harry K. Dupree Stuttgart National Aquaculture Research Center (SNARC), Stuttgart, Arkansas, in a 600-L fiberglass tank and were fed and monitored daily. The turnover rate was once every 2 h and the temperature was 23 ± 1°C. There were approximately 1,000 fish being reared in the tank, at a fish density of 25 g/L. After the animal caretaker suspected that an outbreak of columnaris was causing fish mortality (10–20 fish per day) in one of the tanks, six fish were sampled and three of those had clinical signs of the disease (i.e., saddle back-type lesions, tail erosion, and loss of pigmentation). The remaining three fish selected at random exhibited no clinical signs of disease. During necropsy of the clinically ill fish, columnaris-like bacteria were observed via wet mount preparations from the areas of gill necrosis (long flexing rods congregated to form “haystacks” in areas of necrosis). Water from the stock tank in which the outbreak was occurring was sampled in 1-mL volumes in triplicate to characterize the abundance of bacteria present during an outbreak. These samples were frozen and stored at −8°C until DNA extraction. Liver, gill, and skin–fin were sampled and streaked on selective cytophaga agar (SCA; Hawke and Thune 1992). Tissue samples (∼50 mg) that included gill, liver, and caudal fin were taken from the aforementioned six fish and frozen for quantitative polymerase chain reaction (qPCR) analysis. Samples were also streaked on tryptic soy agar with 5% sheep blood. Wet-mounted tissue from a skin scrape and gill clipping of each fish were examined microscopically for ectopic parasites.

To safeguard against the spread of columnaris to surrounding tanks, water flow was halted for 24 h while awaiting diagnosis. Presumptive diagnosis was based on clinical signs, cell morphology, colony morphology, colony pigment, and growth on selective media. Identification of the bacteria was confirmed using *F. columnare*-specific primers on frozen samples at the end of the study (Panangala et al. 2007). Additional water samples from the stock tank were processed as previously described after the 24-h static period but before fish removal.

*Fish and tanks.*—The remaining fish from the epizootic were moved and 30 fish were stocked into each of twelve 20-L aquaria. Fingerlings were 16.6 ± 3.1 g (mean ± SD) and 13.7 ± 0.9 cm in size. Aquaria contained 10 L of aerated flow-through well water using the “Ultra Low-Flow System” described by Mitchell and Farmer (2010). The flow rate was set to 29 ± 1 mL/min and monitored daily; this rate allowed a natural progression of the disease in a flow-through environment (Mitchell and Farmer 2010). The slowed flow rate along with the increase in fish density was selected to be similar to previous *F. columnare* challenge studies in the system (Farmer et al. 2011). After allocation to tanks, treatments were delayed for 2 h so that fish mortality resulting from handling stress could be observed and fish replaced before the treatment application. A total of nine fish were replaced in this manner.

*Water quality.*—Water temperatures ranged from 18.9°C to 20.6°C. Temperature and dissolved oxygen were measured daily with a Wissenschaftlich-Technische Werkstätten (WTW) pH/Oxi 340i/SET meter (Weilheim, Germany). Dissolved oxygen ranged from 7.9 to 8.9 mg/L, i.e., greater than 90% saturation. Total ammonia nitrogen and nitrate were determined in each tank with a Hach DR/4000V spectrophotometer using the Nessler Method 8038 and Nitrite Low Range Method 2515 (Hach Company, Loveland, Colorado). An Accumet Basic AB15 pH meter (Fisher Scientific, Waltham, Massachusetts) was used to measure pH (7.5–8.2) at the beginning of the study. Standard titration methods (Eaton et al. 2005) were used to measure total alkalinity (213 mg/L) and total hardness (112 mg/L).
Experimental design.—The 7-d trial was composed of three treatments: (1) an untreated control, (2) 2.1 mg/L CuSO₄, and (3) 3 mg/L KMnO₄. Each treatment consisted of four replicates. Treatments applied from stock solutions of CuSO₄ and KMnO₄ were prepared at a concentration of 10 g/L. An aliquot of each stock solution was added to the respective tank, and after 1 min samples were taken for dose confirmation. The treatments were applied in 24-h intervals on three consecutive days. Treatment concentrations of copper and manganese were verified by standard methods (EPA 200.7) using inductively coupled plasma (ICP) analysis of water samples from tanks treated with CuSO₄ and KMnO₄. The study was designed to continue until the mortality associated with the outbreak had ceased; unfortunately on day 8 the supplemental aeration system failed and the study was halted.

Fish were observed daily for clinical signs associated with the disease. Fish unable to maintain neutral buoyancy were considered moribund and removed for sampling. Fish were not fed during the first 3 d of treatment, but were offered food on day 4 and throughout the rest of the study. Animal care and experimental protocols were approved by the SNARC Institutional Animal Care and Use Committee and confirmed to U.S. Department of Agriculture, Agricultural Research Service Policies and Procedures 130.4 and 635.1.

Identification and quantification of bacteria.—Dead and moribund fish were removed from the tanks daily and samples were taken from the caudal fin, gills, and liver and were cultured on SCA containing 5 µg/mL neomycin sulfate and 200 units/mL polymyxin B. This selective media can be effective in inhibiting all bacterial species tested except Flavobacterium spp. and Streptococcus spp. (Hawke and Thune 1992). If present, a maximum of three moribund or dead fish were sampled from each tank every day. Cultures were incubated at 22°C for 36 h, and then scored as being positive or negative for growth based on colony morphology; flat, yellow rhizoid colonies were scored positive.

Water samples were taken for qPCR analysis from each tank at 2 h before the first, second, and third treatments and 24 h after the third treatment. There were 48 total 1-mL samples taken, which were stored at −8°C until processing. Additionally, 24 h after the third application, one fish showing no obvious signs of disease was sampled from each tank from all treatment groups (four fish per treatment). The lower lobe of the caudal fin, a section of the left second gill arch, and a liver sample were taken (approximately 50 mg) from each fish for qPCR analysis. Samples were stored at −8°C until DNA extractions could be completed at the end of the study. The DNA extractions were performed according to the manufacturer’s directions using a DNaseasy Blood and Tissue Kit (Qiagen, Valencia, California). Water samples were extracted with the same kit following the manufacturer’s protocol for Gram-negative bacteria designed to harvest bacterial cells from a liquid suspension. This was done by the addition of a pretreatment centrifugation step of 5,000 × g for 10 min to harvest the cells prior to extraction.

The extracted template DNA was used for pathogen detection, identity confirmation, and quantification using the primers of Panangala et al. (2007), which were FcFp [5′-CCTGTACCTATTTGGGAAAAGAGG-3′], FcRp [5′-CGGTATGGCCTTGGTTATCATAGA-3′], and 6-Fam labeled probe [5′-ACAAACATGATTTTGCAGGAGTATCTGATGGG-3′] and quenched with the Black Hole Quencher-1 (BHQ-1) dye. This primer and fluorescent probe set targets at a region of the chondroitin AC lyase gene of F. columnare. Primers and hydrolysis probe were obtained from Applied Biosystems (Foster City, California). The qPCR assays were performed on a Roche Lightcycler 480 Real Time PCR system (Indianapolis, Indiana). All samples were run in duplicate to reduce cost. Reactions included 500 nM of forward (FcFp) and reverse (FcRp) primers, 250 nM labeled probe, and 1 µL template DNA. Template for tissue samples contained genomic catfish and bacterial DNA and was measured using a NanoDrop ND1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware). BioRad 2× master mix and molecular grade water were adjusted to give 20-µL total reaction volumes. The initial DNA denaturation step was 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, and then 60°C for 30 s. These data were calculated using the Roche Lightcycler 480 software release 1.5.0 SP3 macro for absolute quantification using the second derivative maximum method. A standard (1.0 × 10⁸) and a no-template control were included on each plate; the standard was used as a positive control (calibrator) and to validate the standard curve within each run (Pfaffl et al. 2002; Vandesompele et al. 2002). A standard curve using the Lightcycler 480 software was generated using bacterial samples of known quantity that had been serially diluted and covered the range of 10⁻³–10⁸. The standard curve was applied following the manufacturers protocol using the same parameters (detection format, concentrations, analysis mode, and color compensation data). The PCR amplification efficiencies were 1.974, calculated by the Lightcycler software 1.5.0 SP3 using the equation E = 1−(1/Δslope) (Pfaffl 2001) and a percent efficiency of 97.4%, which are similar to the results generated by Panangala et al. (2007). The equation describing the standard curve line was y = −3.386 x + 41.70, r² = 99.6%. The in-run positive control (calibrator) had a colony-forming units (CFU)/mL value of 1.0 × 10⁴ ± 7.1 × 10²; the mean and SD were used to calculate the coefficient of variation (CV = 100 × SD/mean), which was 2.91% between the three analysis plates with a total of six replicates on three runs. The resulting qPCR data for tissues was then divided by the amount of template (ng/µL) put in each reaction to normalize the data, and results are reported CFU/µg of template DNA. Sequence analysis of the F. columnare isolate was further characterized using primers (Darwish et al. 2004), which amplified a small section of the 16s ribosomal subunit. The resulting amplicon was cleaned up using the USB presequencing cleanup kit (Affymetrix, Santa Clara, California). The cleaned product was sequenced and analyzed with Basic Local Alignment Search Tool (BLAST) for homologous sequences in GenBank.
TABLE 1. Quantitative PCR results represented as mean colony forming unit count per nanogram of DNA (CFU/ng ± SE) from tissues of moribund and apparently normal channel catfish sampled from the stock tank during a natural epizootic of *Flavobacterium columnare*, and from survivor fish (also apparently normal) sampled from the control tanks on day 4 of the experiment after all treatments had concluded.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Caudal fin</th>
<th>Gill</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moribund fish</td>
<td>$5.99 \times 10^6 \pm 2.3 \times 10^5$</td>
<td>$7.84 \times 10^4 \pm 3.9 \times 10^4$</td>
<td>$1.88 \times 10^2 \pm 4.0 \times 10^1$</td>
</tr>
<tr>
<td>Apparently normal fish</td>
<td>$2.51 \times 10^5 \pm 1.96 \times 10^4$</td>
<td>$9.39 \times 10^3 \pm 7.8 \times 10^3$</td>
<td>$1.41 \times 10^2 \pm 6.9 \times 10^1$</td>
</tr>
<tr>
<td>Survivor control fish</td>
<td>$4.00 \times 10^4 \pm 3.3 \times 10^1$</td>
<td>$3.64 \times 10^3 \pm 2.3 \times 10^1$</td>
<td>$2.18 \times 10^2 \pm 2.0 \times 10^1$</td>
</tr>
</tbody>
</table>

Validation of the water-sampling technique was tested by adding a known amount of *F. columnare* (30 mL of a 0.72 absorbance at 550 nm $\approx 2.0 \times 10^3$) to seven tanks as previously described. Samples of 1 mL were taken 10 min after the bacteria were added. Samples were then extracted and analyzed by the qPCR method as previously described.

**Histopathology.**—Gill samples were collected and immediately fixed in Davidson’s solution at 24 h following the last treatment. After 24 h, tissues were transferred to 70% isopropanol until routine paraffin embedding. Tissues were sectioned at 5 μm thickness, mounted on slides, and stained with hematoxylin and eosin. Gills were evaluated in blinded fashion by two pathologists and were scored for hyperplasia as follows: 1 = no hyperplasia, 2 = minimal hyperplasia, 3 = severe hyperplasia, 4 = complete fusion of secondary lamellae (Soderberg et al. 1984; Muhvich et al. 1995). Gills were also examined for edema (spongiosis) and scored similarly based on the frequency and severity of edematous lesions.

**Statistical analysis.**—Survival data were analyzed with SigmaPlot 11 (San Jose, California) using Kaplan–Meier log rank survival analysis; all pairwise multiple comparisons used the Holm–Sidak method with adjusted $P$-values. Mean percent survival at the conclusion of the study was arcsine-transformed and analyzed using a generalized linear model (GLM analysis of variance [ANOVA]) and all pairwise multiple comparisons were made with the Tukey–Kramer procedure method with type III adjusted $P$-values. The qPCR data were log$_{10}$ transformed, which resulted in normally distributed data with equal variances. An ANOVA was performed on the transformed qPCR data. Differences among treatment means were separated using the Tukey–Kramer procedure for pairwise comparisons (Kramer 1956; J. W. Tukey, 1953, Princeton University, unpublished manuscript). The cumulative histology scores for both hyperplasia and edema were averaged, and the mean scores were compared using the Student’s $t$-test. Treatment effects were considered significant at $P \leq 0.05$.

**RESULTS**

**Description of a Natural *Flavobacterium columnare* Epizootic**

Because the number of *F. columnare* needed to cause an outbreak is unknown, bacterial load in the water, on the surface of the fish, and within the fish (liver) during this outbreak were characterized. The mean bacterial load in the flow-through stock tank water samples was $3.7 \times 10^2 \pm 3.8 \times 10^1$ CFU/mL during an initial sampling of the culture tank with the disease in progress. After being held static for 24 h as a result of our biosecurity procedures, the concentration of *F. columnare* had elevated 25-fold to $9.4 \times 10^3 \pm 2.9 \times 10^1$ CFU/mL; this was a statistically significant increase. The concentration of bacteria present on tissue samples from moribund and randomly selected fish from the stock tank during the outbreak as determined by qPCR are shown in Table 1. The mean template from the moribund fish caudal fin samples was $5.99 \times 10^3$ CFU/ng, which was over 16,000 times more bacteria than were present in the water at the time of sampling. Analysis of the qPCR data indicated a significant difference in the number of bacteria on fish classified as moribund when compared with fish classified as apparently normal. The analysis also indicated that significantly fewer bacteria were detected in the liver samples than in caudal fin or gill samples.

**Clinical Signs and Gross Pathology**

Moribund fish in untreated control tanks displayed signs consistent with an *F. columnare* infection; fish were lethargic and little feeding activity was noted on day 4, but this activity increased in all treatment groups over the study period. Gross pathologies were typical; the skin of moribund fish initially had discrete depigmented areas that became multifocal and diffuse, often encompassing most of the body, as the infection progressed. Necrotizing dermatitis and cutaneous sloughing exposed the underlying muscles in severe cases. The gills had focal and multifocal necrotizing branchitis with yellowish pigmentation. Severely frayed fins were common as the infection progressed. No internal gross pathology was observed.

**Survival**

Mean percent survival of the untreated control group on day 7 was 38.5% (SD = 4.2). The CuSO$_4$-treated group had a mean percent survival of 72.7% (SD = 7.1), which was significantly different from the control treatment ($P < 0.05$). Fish treated with KMnO$_4$ had a mean percent survival of 52.2% (SD = 6.9), which was not significantly different from the control treatment. There was no significant difference between CuSO$_4$ and KMnO$_4$ treatments ($P > 0.05$). Cumulative mortality of
FIGURE 1. Cumulative daily mortality of channel catfish naturally infected with *F. columnare* and not treated (control) or treated three times with copper sulfate (CuSO$_4$) or potassium permanganate (KMnO$_4$). The three treatments is represented in Figure 1. Kaplan–Meier survival curves depicting the probability of survival as functions of time are represented in Figure 2. The probability of survival was analyzed by log$_{10}$ rank survival curve which determined that the probability of survival over time for CuSO$_4$-treated fish was significantly different from the control and different from KMnO$_4$-treated fish. There was not a significant difference between KMnO$_4$-treated fish and the control. In the present study, the control group had a mean survival of 38.5%, while the increase in the survival of the CuSO$_4$-treated fish over the control fish was 34.2%, thereby resulting in an increase relative to the control of 89%, [i.e., (34.2 ÷ 38.5) × 100].

**Isolation, Identification, and Quantification of Bacteria**

Notably, *F. columnare* was cultured from at least one tissue from all fish sampled. No other bacteria were isolated, even from the six fish from the initial outbreak from which samples had been streaked on TSA, indicating this to be an exclusive *F. columnare* outbreak. The culture data included 85 necropsies of which 79 were performed after the first treatment application. Individual culture results by treatment and by tissue are...
TABLE 2. Results (number of positive cultures out of total number of sampled fish for each group) from attempted culture from tissues of moribund channel catfish infected with *Flavobacterium columnare* and treated with CuSO₄ or KMnO₄, or not treated. Samples were streaked on selective cytophaga agar, incubated at 22°C for 36 h, and read as positive if yellow rhizoid colonies were present.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>CuSO₄</th>
<th>KMnO₄</th>
<th>Control</th>
<th>Total by tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caudal fin</td>
<td>17 of 18</td>
<td>29 of 30</td>
<td>31 of 31</td>
<td>77 of 79</td>
</tr>
<tr>
<td>Gill</td>
<td>11 of 18</td>
<td>28 of 30</td>
<td>31 of 31</td>
<td>70 of 79</td>
</tr>
<tr>
<td>Liver</td>
<td>8 of 18</td>
<td>20 of 30</td>
<td>26 of 31</td>
<td>54 of 79</td>
</tr>
</tbody>
</table>

In addition to bacterial culture, qPCR data were generated from fish tissues collected 24 h after the last treatment. No samples of infected fish were found to be negative for *F. columnare* by qPCR. Mean CFU/ng template DNA by treatment and by tissue are represented in Table 3. No significant difference was detected by treatment or tissue type compared with the control. However, the mean number of bacteria (four fish per treatment, three tissues sampled per fish) showed that there were 60% fewer bacteria in the untreated control tanks, respectively.

Quantitative PCR was also used to determine the number of bacteria in the water 24 h after each of the three treatments. The water sample validation data yielded acceptable results in which the theoretical bacterial load was $6.0 \times 10^6$ CFU/mL, and the 10-min qPCR sample data in bacterial load was $3.47 \times 10^6 \pm 2.7 \times 10^5$ CFU/mL (mean ± SD). The bacterial load increased logarithmically three times during the first 24 h of the study in the untreated controls. There was a significant difference in bacterial load (CFU/mL) at 24 h after the first and second treatments of CuSO₄ and KMnO₄ compared with the untreated control samples. However, there was not a significant difference after the third treatment. Mean bacterial numbers (as CFU/mL) after each treatment and the percent reduction compared with the untreated control are shown in Table 4.

Dose Confirmation and Water Quality

The target dose for CuSO₄ was 2.1 mg/L based on 1% of the measured alkalinity of 213 mg/L; this dose rate was used because it is the recommended treatment rate for control of *Ichthyophthirius multifiliis* in catfish ponds (Wise et al. 2004). Dose verification indicated the mean CuSO₄ concentration was 2.32 ± 0.03 mg/L. The target dose for KMnO₄ was 3.0 mg/L based on the 15-min PPD plus 2 mg/L (Tucker 1989); the demand was 1 mg/L. Dose verification indicated the mean KMnO₄ concentration was 3.07 ± 0.03 mg/L. The pink color associated with the activity of KMnO₄ lasted about 45 min.

Total ammonia nitrogen and nitrite were measure daily and the mean was 1.9 ± 0.08 mg/L and 0.02 ± 0.001 mg/L, respectively. Individual treatment means for total ammonia nitrogen were 2.1, 1.6, and 1.9 mg/L for the untreated control and CuSO₄- and KMnO₄-treated tanks, respectively.

**TABLE 3.** Quantitative PCR results represented as mean colony forming unit count per nanogram of DNA (CFU/ng ± SE) from fish tissues and percent difference compared with the untreated control fish sampled 24 h after the third and final treatment (day 4) of CuSO₄ or KMnO₄, or no treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Caudal fin Mean ± SE</th>
<th>Difference from control (%)</th>
<th>Gill Mean ± SE</th>
<th>Difference from control (%)</th>
<th>Liver Mean ± SE</th>
<th>Difference from control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontreated control</td>
<td>40.00 ± 32.8</td>
<td>36.37 ± 23.2</td>
<td>21.82 ± 19.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuSO₄</td>
<td>14.95 ± 8.9</td>
<td>−62.6</td>
<td>9.08 ± 32.8</td>
<td>−75.0</td>
<td>19.96 ± 16.6</td>
<td>−8.5</td>
</tr>
<tr>
<td>KMnO₄</td>
<td>$4.23 \times 10^3$ ± 4.1 × 10³</td>
<td>+105.3</td>
<td>$1.54 \times 10^5$ ± 1.3 × 10²</td>
<td>+76.4</td>
<td>34.24 ± 32.6</td>
<td>+36.3</td>
</tr>
</tbody>
</table>
TABLE 4. Quantitative PCR results represented as mean colony forming unit count per ml of water (CFU/mL ± SE) from water samples taken 24 h after the each of three treatments of CuSO₄ or KMnO₄, or after no treatment (control) and the percent reduction compared with the untreated control. Asterisks indicate a significant difference from the untreated control ($P \leq 0.05$).

<table>
<thead>
<tr>
<th>Sample time</th>
<th>Control</th>
<th>CuSO₄</th>
<th>KMnO₄</th>
<th>CuSO₄ reduction (%)</th>
<th>KMnO₄ reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After first treatment</td>
<td>2.41E+06 ± 4.43E+05</td>
<td>9.34E+03 ± 1.87E+03</td>
<td>4.87E+05 ± 2.61E+05</td>
<td>99.99*</td>
<td>79.79*</td>
</tr>
<tr>
<td>After second treatment</td>
<td>9.23E+04 ± 1.72E+04</td>
<td>9.81E+03 ± 7.04E+03</td>
<td>2.76E+04 ± 8.14E+03</td>
<td>89.36*</td>
<td>70.10*</td>
</tr>
<tr>
<td>After third treatment</td>
<td>1.04E+05 ± 6.74E+04</td>
<td>1.20E+04 ± 5.15E+04</td>
<td>2.56E+04</td>
<td>35.19</td>
<td>50.50</td>
</tr>
</tbody>
</table>

**Histology**

Irrespective of treatment, gills sampled 24 h after the final treatment indicated some level of gill pathology (Figure 3). The most extensive gill damage was observed in untreated control fish (Figure 3A; mean histopathology score = 3.4) and was characterized by severe hyperplasia, often resulting in fusion of lamellae and complete occlusion of the lamellar space. Gills of fish treated with KMnO₄ (mean histopathology score = 3.1) had extensive hyperplasia, severe spongiosis, and occasional lamellar fusion. While not completely normal in structure, gill tissue from CuSO₄-treated fish exhibited the least amount of damage (mean histopathology score = 2.8), which consisted of

![Figure 3](image-url)

**FIGURE 3.** Representative gill sections from (A) untreated (control) fish, (B) fish treated with KMnO₄, and (C) fish treated with CuSO₄. Gills were sampled 24 h after the last treatment. Panel (D) shows the mean cumulative gill histopathology scores (means of four fish in each experimental group). The asterisk indicates a significant difference from the control ($P \leq 0.05$). The scale bars = 125 μm.
DISCUSSION

Epizootics are unpredictable, and those rare outbreaks that are exclusive to _F. columnare_ are even more unpredictable. Constructing experimental studies to emulate such outbreaks is difficult, yet those scenarios are exactly what fish farmers face daily. Generally, fish are already exposed, infected, or dying before the disease is identified and appropriate therapy is implemented. Treating a group of fish before any signs of disease or before any mortality has occurred in an efficacy trial translates poorly to field application. In the field, a fish must be observed to be either dead or moribund and at least a presumptive diagnosis must be completed before any theraputants would be warranted.

One major factor of any efficacy study is endpoint percent survival, which ultimately affects economics and profit margins. In the present study CuSO₄ treatment significantly increased survival while KMnO₄ treatment did not. However, as an unfortunate mechanical failure truncated the experiment before mortality had completely ceased, a Kaplan–Meier survival curve analysis was employed to incorporate the variable of time. This analysis relates the population survival to time and is often used in studies where resolution has not or will not be completed (Norman and Streiner 2000). The results of both the Kaplan–Meier survival curve analysis and the endpoint analysis designated the CuSO₄-treated fish significantly different from the untreated control. Neither analysis found KMnO₄ treatment to be different from the control.

Data from the present outbreak showed the _F. columnare_ concentration in water to be much less than levels used to induce disease in previous studies (Thomas-Jinu and Goodwin 2004; Darwish et al. 2008, 2009, 2010). Based on these findings, chronic exposure to a low level of bacteria that continues to attach to the fish over time until the fish’s disease threshold has been reached is possibly more characteristic of a columnaris outbreak. The data from the fish during the original outbreak revealed a large range in numbers of bacteria on the fish. This difference was based on whether the fish was moribund and displayed clinical signs or was asymptomatic, yet both classifications were exposed to the same concentration of bacteria in the water. These differences support the theory that not all fish in an outbreak are infected at the same level and that there is a large natural variation in susceptibility within a population in which a columnaris outbreak is occurring. Accordingly, challenge models in which fish are exposed to a high bacterial concentration for a relatively short period of time and then removed by way of treatment or initiating water flow are not reflective of a normal epizootic outbreak. Previous studies have examined the use of CuSO₄ and KMnO₄ for _F. columnare_; however, the limited data generated by these studies have not confirmed their theraputic value. This may be due, in part, to the artificial nature of methods used to infect fish with _F. columnare_.

In the present study, the qPCR analysis of water suggests that the most therapeutic effect for both compounds occurs during the first two treatments, with the predominant effect occurring after the first treatment. Either KMnO₄ or CuSO₄ treatments reduced the spike in bacterial load compared with the untreated control. The number of bacteria in the CuSO₄-treated tanks did not increase until after the treatment period had ceased. This reduction in bacterial load aides the understanding of how therapeutic intervention can reduce the transmission of disease and ultimately improve fish survival. Survival data for the KMnO₄ treatment was not different from the control treatment; however, the survival trend along with the initial reduction in the water suggests that it may be effective under different study conditions. A future study investigating higher single doses of KMnO₄ may produce a greater reduction of bacteria in the water column and an increased survival of the fish.

The CFU reduction on fish tissues, 30-fold lower in the CuSO₄-treated fish compared with the untreated control fish, was not significantly different statistically, but might have been biologically important and contributed to the improved survival and gill histopathology. One possible reason for the tissue data not being significant is the biological variation in the number of bacteria on different fish in an outbreak. If fish are chosen randomly with no selection based on clinical signs, the natural variability reduces the statistical power to decipher a difference. A two-way ANOVA on the initial six fish indicated a strong interaction term among the health statuses of the fish. Similar to the CFU data from the water, it is possible that the reduction of bacteria on fish tissues was greatest during and after the first two treatments, and by the third treatment the differences had diminished. However, we did not collect tissue samples during the earlier time periods, so further investigation may be warranted. It is possible that the removal of dead and moribund fish as well, as the fish removed for sampling, had decreased the bacterial load in all tanks by the third treatment. This reduction in bacterial load might have reduced the differences in the fish tissues and water samples after the third application.

The pathogen was not eliminated in treated fish at the chosen sample times and could be of concern. It is important to remember that _F. columnare_ is a ubiquitous pathogen that is often detected on healthy fish, and fish from the populations at SNARC very seldom test negative for _F. columnare_ (Farmer et al. 2011). Background levels of _F. columnare_ are often found between 1.0 × 10² and 1.0 × 10³ CFU/ng template. The results from the sampling on day 3 noted that there were over 250 times fewer bacteria on the skin and gills of the surviving apparently healthy fish when compared with the apparently healthy samples from the stock tank during the initial outbreak. Data from the present study indicate that the caudal fin and the gills are more suitable sites for detecting these bacteria than was the liver. We argue that qPCR may allow for more in-depth evaluation of
therapeutic intervention than bacterial culture techniques alone, especially in the case of ubiquitous pathogens.

The mechanism by which CuSO4 exerts a beneficial effect against *F. columnare* infection in channel catfish is probably multifaceted. Low concentrations of copper can have a stimulatory effect on the immune system of fish (Muhvich et al. 1995; Dautremepuits et al. 2004). Copper sulfate can also have a direct inhibitory effect on bacteria through the displacement of essential metals from their native binding sites or through ligand interactions; this displacement results in the alteration of the conformational structure of nucleic acids, proteins, in the oxidative phosphorylation cascade, and in the osmotic balance (Borkow and Gabbay 2005). Ongoing studies in our laboratory are examining the mechanisms by which CuSO4 affects fish physiology in addition to its direct effects on *F. columnare*.

The current recommended therapy for a columnaris outbreak is often the use of feed medicated with antibiotics. However, the overall trend in population health management is shifting away from these types of therapies to reduce the risk of antibiotic resistance. Furthermore, by the time disease outbreaks are diagnosed fish are already exhibiting clinical signs (e.g., anorexia), which severely limits the effectiveness of treating with a medicated feed. As such, alternative management practices aimed at curtailing mortality in a cost-effective manner and without antibiotic intervention are desirable. The newest practices aimed at curtailing mortality in a cost-effective manner and without antibiotic intervention are desirable.

In summary, the present study demonstrates that CuSO4 is effective in reducing mortality of channel catfish owing to columnaris disease. Copper sulfate therapy significantly reduced the number of bacteria in the water, reduced gut pathologies associated with *F. columnare* infection, and as a result, significantly improved survival. These results indicate the CuSO4 actively reduces the bacterial load not only in the water but possibly on the outer surfaces of the fish. Because of the many limitations encountered in this study, these results should be considered preliminary and warrant further investigation under more controlled and repeatable experimental conditions. Copper sulfate is an economical alternative to expensive antibiotic intervention. However, water chemistry must be taken into consideration, as CuSO4 can be toxic to fish in water with low alkalinity. Future studies should investigate the application of CuSO4 for effectiveness on outbreaks of columnaris in ponds.

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REFERENCES


