Toxicity of Copper Sulfate to Flavobacterium psychrophilum and Rainbow Trout Eggs

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Abstract

Tests were conducted to determine the concentrations of copper sulfate needed to kill *Flavobacterium psychrophilum*, the cause of bacterial coldwater disease, either in vitro or on Rainbow Trout *Oncorhynchus mykiss* eggs. For the in vitro test, a plastic strip dipped in a solution of *F. psychrophilum* was exposed for 15 min to copper sulfate solutions of 0, 1, 5, 10, 20, 35, 50, 75, or 100 mg/L. Bacteria were "too numerous to count" at concentrations ≤ 10 mg/L $CuSO_4$; significant reductions in prevalence relative to untreated controls were noted for concentrations ≥ 35 mg/L. However, CFUs were still observed at 50 and 75 mg/L (20% of plates with tryptone yeast extract salts media). No yellow-pigmented CFUs typical of *F. psychrophilum* were observed at 100 mg/L $CuSO_4$. For the in vivo test, eggs were exposed for 15 min to 100, 300, 500, and 700 mg/L $CuSO_4$ or 100 mg/L iodine (control). Survival to hatch was significantly lower at 500 (44.3 ± 15.2%, mean ± SD) or 700 mg/L $CuSO_4$ (1.7 ± 0.8%) than for controls treated with 100 mg/L iodine (93.6 ± 0.9%) or at copper sulfate concentrations ≤ 300 mg/L. The 15-min LD_{50} and LD_{10} for copper sulfate were 461 mg/L (95% confidence interval: 457–466 mg/L) and 259 mg/L (251–266 mg/L). The prevalence of yellow CFUs at 100 mg/L $CuSO_4$ (40.0%) was significantly higher than in untreated controls. Significant reductions in yellow CFUs were achieved using 300, 500, or 700 mg/L $CuSO_4$ (7.5, 2.5, or 0.0% of plates with CFUs, respectively) or 100 mg/L iodine (2.5%), relative to untreated control eggs. Overall, since the concentrations of copper sulfate required to eliminate *F. psychrophilum* were toxic to the eggs, copper sulfate is not recommended for coldwater disease control in Rainbow Trout eggs based on conditions and parameters in this study.

Fungal and bacterial growth on fertile fish eggs can compromise egg survival and lead to disease outbreaks after hatching (Rangdale et al. 1997; Hussein et al. 2001; Barnes et al. 2005). Transported eggs may be vectors of disease when moved among hatcheries and wild sources, especially for vertically transmitted (i.e., intra-ovum) diseases such bacterial kidney disease *Renibacterium salmoninarum* and bacterial cold-water disease *Flavobacterium psychrophilum* (Bruno and Munro 1986; Elliott et al. 1995; Kumagai et al. 1998). Control of fungal and bacterial growth on fish eggs was traditionally accomplished using malachite green (Foster and Woodbury 1936; Cline and Post 1972), but concerns about its teratogenicity (Meyer and Jorgenson 1983) led to the exploration of alternatives (Bailey 1984; Bailey and Jeffrey 1989). Povidone iodine and formalin have subsequently been the disinfectants most often used in fish

culture (Alderman 1984; Fowler and Banks 1991; Rach et al. 1997; Barnes et al. 2002). Alternatives to these disinfectants have been the focus of recent research due to concerns about *F. psychrophilum*, the bacterium responsible for cold-water disease and Rainbow Trout *Oncorhynchus mykiss* fry syndrome (Cipriano 2005; Wagner et al. 2008, 2010).

Copper sulfate is among the potential chemical candidates for control of bacteria on eggs (Straus et al. 2009). Copper sulfate is currently used in aquaculture as algicide and as a treatment for protozoan parasites (Schäperclaus 1991; Straus 2006). While the effects of copper have been studied in fish from a chronic toxicology viewpoint (Sorensen 1991) and fish physiological and behavioral impacts have been studied in detail (Sorensen 1991; Heath 1995), few studies have evaluated copper as an egg disinfectant. Cline and Post (1972) evaluated Cutrine, a complex

of metallic copper and copper triethanolamine, and found that concentrations that were high enough to control fungal growth were toxic to eggs of Rainbow Trout and Brown Trout *Salmo trutta*. However, Straus et al. (2009) found that daily treatment of Channel Catfish *Ictalurus punctatus* eggs with 10–40 mg/L CuSO$_4$ controlled fungal growth. Miura et al. (2005) discovered that copper fiber placed in the inflow of egg incubators led to control of fungal growth. A recent report by Dwyer (2010) indicated that over 120 fish farms in Japan are using the copper fiber method now. The bactericidal effects of copper for fish pathogens have received little attention, though copper and silver ions have been used synergistically in hospital hot water lines to kill *Legionella* spp. (Lin et al. 2002). To our knowledge, no one has examined copper sulfate for controlling *F. psychrophilum* or other bacteria on fish eggs. Consequently, the objective of this study was to determine the lethal concentrations of copper sulfate for control of *F. psychrophilum* in in vitro tests and evaluate the efficacy and safety of relevant concentrations in subsequent tests on Rainbow Trout eggs.

METHODS

In vitro toxicity to F. psychrophilum.—Copper sulfate solutions of 0, 1, 5, 10, 20, 35, 50, 75, and 100 mg/L were made using sterile hatchery well water (222 mg/L total hardness, 222 mg/L total alkalinity, pH 7.6). Chemical exposure duration was 15 min, and each concentration had five replicate test tubes (10 × 75 mm). For the test, a sterile plastic test strip (50 × 5 mm with a 5 mm × 5 mm paper square affixed to the end of one side of the strip; EM Quant; EMD Chemicals, Gibbstown, New Jersey) was dipped into a solution of *F. psychrophilum* for 10 s. The bacteria solution was made by transferring 1 mL of MAT broth (tryptone yeast extract salts [TYES] supplemented with 1% maltose and 0.02 sodium acetate; Crump et al. 2001) from a 3-d-old culture of *F. psychrophilum* to 2 mL of sterile well water in a test tube and mixing. Using sterile forceps, the test strip was immediately transferred after the bacteria dip to a sterile test tube containing 4 mL of the copper sulfate solution. After 15 min in the copper sulfate, the strip was dipped in two tubes with sterile well water in succession, for 2 s in each. After this rinse, the strip tip was rubbed along the center axis of a petri dish with TYES agar (Buller 2004), rubbing one side in one direction and rubbing the other side of the strip to make another path going back across the plate. The plate was then wrapped with laboratory film and incubated at 15°C. The plates were monitored for bacterial growth, noting colony numbers and type. Gram stains were made of yellow colony isolates to ensure they were Gram-negative, long, thin rods typical of *F. psychrophilum*.

Egg toxicity and disinfection efficacy.—Treatments for this test were 100, 300, 500, and 700 mg/L CuSO$_4$, with a 100 mg/L iodine treatment as a control. The duration of exposure was 15 min for each chemical treatment. There were four replicates per treatment, with 100 mL of eggs per replicate. Eggs at the eyed stage from Rainbow Trout of the Gunnison River–

Harrison Lake strain were used for the experiment. Copper sulfate and iodine solutions were prepared using hatchery well water (222 mg/L total hardness, 222 mg/L total alkalinity, pH 7.6, 13.4°C) in 1-L plastic beakers. Separate plastic beakers with prepared solution were created for each replicate. Eggs were held in nets during the exposure and after treatment were rinsed once by dipping in fresh hatchery well water, followed by immediate transfer to vertical stack incubation trays (one tray per replicate). As soon as the chemical treatments were completed, 10 eggs from each replicate were transferred by sterile forceps to sterile test tubes (one egg added per tube) with 2 mL peptone diluent solution (Barnes et al. 2005) at 15°C. The eggs were kept in an incubator overnight (about 15 h) at 15°C, and samples were plated the following morning on TYEST (tryptone yeast extract salts with tobramycin added to suppress competing bacteria; Kumagai et al. 2004). To plate the samples, the test tubes with eggs were agitated for 2 min using a vortex mixer. Then, 100 μL of solution was added to a petri dish containing TYEST media. The solution was then distributed using a sterilized plastic spreader, and the petri dish was wrapped with laboratory film and incubated at 15°C. Plates were monitored for bacterial growth over a 7-d period; total and yellow CFUs were enumerated and colony descriptions were made. Gram stains of any yellow CFU were made to determine if the bacteria were Gram-negative long, thin rods typical of *F. psychrophilum*. After being placed in the incubation trays, the eggs were monitored until shortly after hatch. The number of dead eggs and fry and crippled fry were noted for each replicate.

Statistical analysis.—SPSS version 13.0 (Chicago, Illinois) and NCSS (Number Cruncher Statistical System, Kaysville, Utah) were used for the analyses. Analysis of variance (ANOVA) of arcsine-transformed data (Kirk 1982) was used for comparing egg survival and deformity data. Sheffé's test was used for subsequent mean comparisons. For CFU data, the percentage of plates with yellow CFUs was compared among concentrations using the Kruskal–Wallis test (one-way ANOVA of ranked data). The concentration at which no *F. psychrophilum* survived was also noted. For the in vitro copper sulfate toxicity tests, prevalence of yellow CFUs was compared among chemical treatments and replicates using a hierarchical log-linear model. Replicate effects were not significant for either experiment, so data were pooled for chi-square analysis of treatment effects using binomial data (yellow CFUs present or absent). Total CFUs per plate in the egg toxicity test were classified into three categories of abundance: 0 (no CFUs), 1–100 CFUs, or >100 CFUs. These data were analyzed with a hierarchical linear model using replicate, CFU category, and chemical treatment as variables; Partial tables of collapsed data (across replicates) subsequently were analyzed with chi-square tests. Replicates effects were not significant ($P > 0.05$) for each concentration, so data were pooled across replicates for comparing CFU distributions among concentrations. Probit analysis was used to determine the copper sulfate dose at which 50% and 10% egg mortality is expected (LD$_{50}$, LD$_{10}$).
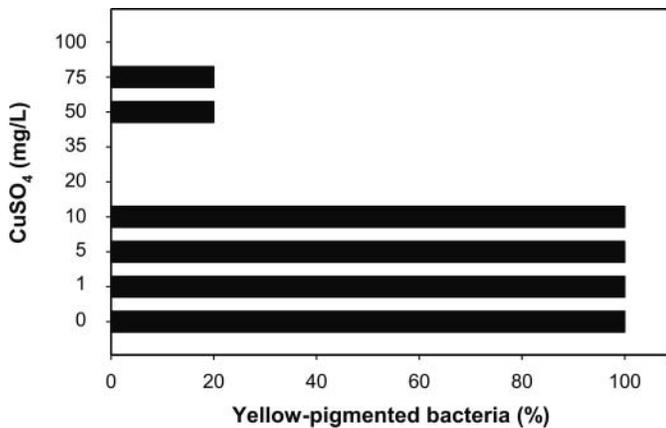
FIGURE 1. Comparison of the percentage of bacterial plates with growth of yellow-pigmented bacteria on TYES media after wiping the surface with a plastic strip that had been dipped in a solution of *F. psychrophilum*, then dipped in one of various concentrations of copper sulfate for 15 min and rinsed twice.

RESULTS

In vitro Toxicity to *F. psychrophilum*

Concentrations of ≥ 20 mg/L $CuSO_4$ led to significant reductions in the prevalence of yellow-pigmented bacteria compared with untreated controls ($P < 0.001$, $\chi^2 = 38.6$, df $= 8$; Figure 1). At 10 mg/L $CuSO_4$, the number of yellow CFUs were reduced (≤ 36 per plate) compared with lower concentrations or untreated controls (all plates were classified as "too numerous to count"), but 100% of plates still had colonies typical of *F. psychrophilum*. For both 50 and 75 mg/L $CuSO_4$, 20% of plates (one of five) had one CFU typical of *F. psychrophilum*. No yellow-pigmented bacteria were observed at 100 mg/L $CuSO_4$.

Egg Toxicity and Disinfection Efficacy

The toxicity test indicated that concentrations of copper sulfate >300 mg/L for 15 min compromised egg survival (Figure 2). Survival to hatch was significantly lower at 500 ($44.3 \pm 15.2\%$ SD) or 700 mg/L $CuSO_4$ ($1.7 \pm 0.8\%$) than for controls treated with 100 mg/L iodine ($93.6 \pm 0.9\%$) or at copper sulfate concentrations ≤ 300 mg/L ($P < 0.01$, $F = 178.2$, df $= 19$). Survival to hatch for eggs exposed to 100 or 300 mg/L $CuSO_4$ (88.8–93.2%) did not significantly differ from controls ($P > 0.68$). The 15-min LD_{50} and LD_{10} for copper sulfate were 461 mg/L (95% confidence interval: 457–466 mg/L) and 259 mg/L (251–266 mg/L), respectively. Eggs exposed to 700 mg/L $CuSO_4$ had a significantly lower deformity prevalence than the other treatments ($P < 0.01$, $F = 19.7$, df $= 19$), which did not significantly differ from each other.

The bacteriology indicated that copper sulfate concentrations that were efficacious for bacteria control were lethal to the eggs. The hierarchical log-linear model indicated that replicates and interaction terms were not significant, but treatment effects were significant for both the yellow-pigmented bacteria ($P < 0.001$, $\chi^2 = 48.5$, df $= 5$) and total CFUs of all other bacteria
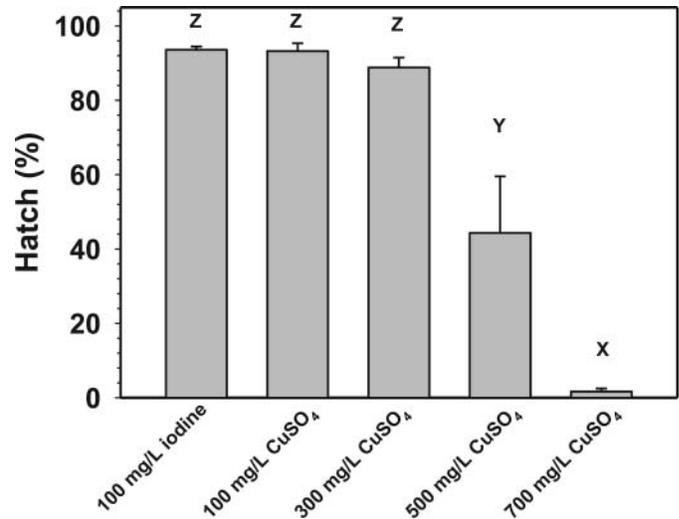

FIGURE 2. Comparison of survival to hatch (mean $\pm$ SD) for eyed Rainbow Trout eggs exposed to various concentrations of copper sulfate or 100 mg/L iodine (control) for 15 min.

($P < 0.001$, $\chi^2 = 92.2$, df $= 15$). Relative to untreated control eggs (17.5% prevalence), chemical treatments significantly ($P < 0.001$) reduced the prevalence of yellow CFUs at concentrations of ≥ 500 mg/L $CuSO_4$ (prevalence $\leq 2.5\%$) or 100 mg/L iodine (0.0%; Figure 3). At 100 mg/L $CuSO_4$, there was a significantly higher prevalence of yellow CFUs (40.0%; $P = 0.03$, $\chi^2 = 4.94$, df $= 1$) than in untreated controls (Figure 3). The abundance of other bacteria followed a similar trend, where significant reductions were achieved using 300, 500, or 700 mg/L $CuSO_4$ (7.5, 2.5, or 0.0% of plates with CFUs, respectively) or 100 mg/L iodine (2.5%), relative to untreated control eggs (45.0%; $P < 0.001$; Figure 4). The prevalence of other bacteria from eggs treated with 100 mg/L $CuSO_4$ did not significantly differ from untreated controls ($P = 0.116$, $\chi^2 = 2.5$, df $= 1$).
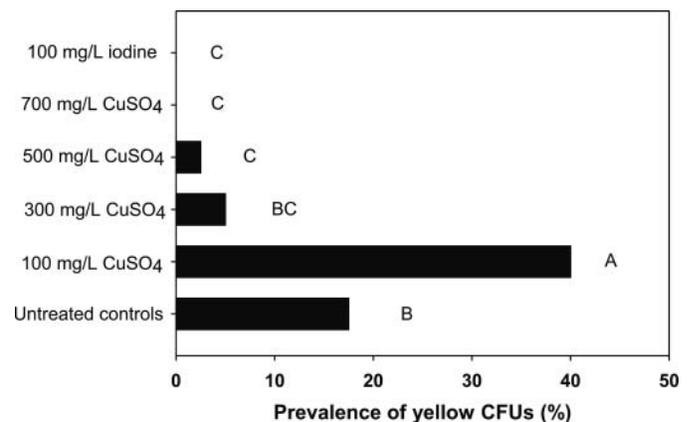

FIGURE 3. Prevalence of yellow CFUs ($N = 40$) among eyed Rainbow Trout eggs exposed to various concentrations of copper sulfate or iodine. Means that are not significantly different share a common letters to the right of the bar ($P > 0.05$, χ^2 test).
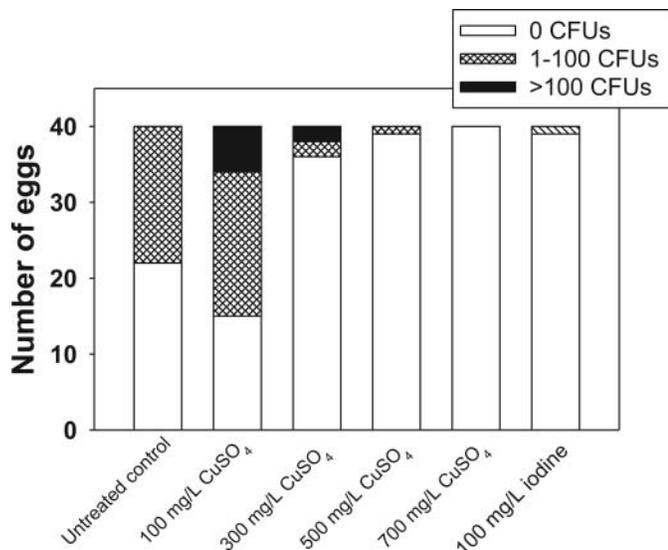
FIGURE 4.   Frequency distribution of CFUs in three categories of abundance for eyed Rainbow Trout eggs exposed to various concentrations of copper sulfate or iodine.

DISCUSSION

No fungus was observed on any of the eggs in the various treatments of this study. Copper sulfate concentrations of 10–60 mg/L appear to be fungicidal in other studies with Channel Catfish (Straus et al. 2009) and Northern Leatherside Chub *Lepidomeda copei* (Wagner et al. 2012). Miura et al. (2005) noted that copper concentrations of 0.006–0.020 mg/L prevented fungal development in Rainbow Trout eggs. Separate tests showed that copper nitrate concentrations of 0.006 mg/L prevented the zoospore germination of *Saprolegnia declina* (Miura et al. 2005).

The effects of copper on bacteria have been studied extensively, primarily from an environmental contamination perspective (Jonas 1989; Díaz-Raviña and Bååth 1996; Nwuche and Ugoji 2008), a bioremediation perspective (Markwiese et al. 1998; Markwiese and Colberg 2000; Andreazza et al. 2011), or related to human health and surface disinfection (Lin et al. 2002; Santo et al. 2011). Use of copper to control bacterial plant pathogens has also been extensively studied (Lukens 1977; Menkissoglu and Lindow 1991). However, data concerning the bactericidal effects of copper sulfate on fish pathogens are few. However, MacFarlane et al. (1986) reported that Striped Bass *Morone saxatilis* fingerlings were protected from exposure to the bacterial fish pathogen *Flavobacterium columnare* (formerly *Flexibacter columnaris*) if the fish were treated with copper ion (10 μg/L) for 5 d immediately after exposure to the bacterium; however, *F. columnare* survived the copper dosage, indicating the protection was related to factors other than bacteria mortality. Schreiber et al. (1985) noted that the mean toxic concentration of copper to the marine fish pathogen *Vibrio alginolyticus* was lower for anaerobic isolates (TC_{50} = 2.1 μM) than aerobic cultures (TC_{50} = 6.4 μM). Gordon et al. (1994) found

that *V. alginolyticus, V. parahaemolyticus*, *Escherichia coli*, and *Pseudomonas aeruginosa* tolerated 50 μM (3.18 mg/L) copper when added to log phase cultures. To control the bacterial fish pathogen *F. psychrophilum,* the in vitro tests of this study indicated that copper sulfate concentrations needed to exceed 75 mg/L, and the in vivo test showed that concentrations of >300 mg/L were needed when eggs were present. In the in vitro test, it is not clear why growth of *F. psychrophilum* was observed at 50 and 75 mg/L but not at 20 and 35 mg/L. As a control, the sterilized well water that was used for diluting the MAT broth and for strip rinsing was plated on TYEST media. The results from the control show that the growth observed at 50 and 75 mg/L cannot be attributed to contamination. It is likely that concentrations of 20 and 35 mg/L kill most, but not all, *F. psychrophilum* bacteria, and the lack of growth observed at these concentrations can be attributed as random chance caused by having five replicates of each concentration. Future experiments should test these concentrations with additional replication.

Unfortunately, copper sulfate concentrations that controlled *F. psychrophilum* were toxic to Rainbow Trout eggs. Cline and Post (1972) similarly found that Cutrine, a copper-based chemical, was toxic to eyed fish eggs at the effective bactericidal concentration (425 mg/L for 1 h/d for 5 d). The effective concentration was similar to that observed in this study, indicating that the copper ion was likely the active ingredient. Gee and Sarles (1942) observed that bactericidal in vivo concentrations were up to 20 times the concentration needed in vitro to control bacterial fish pathogens. Bacterial biofilms, which are a collection of bacterial microcolonies and extracellular polymers that develop over time, are notoriously difficult to control, requiring more effort (i.e., higher concentrations and longer exposures) to kill than fresh bacteria suspensions (Characklis and Marshall 1990; Presterl et al. 2007; Sundell and Wiklund 2011). Bacteria also have the ability to adapt to sublethal concentrations (Trevors and Cotter 1990; Gordon et al. 1994). Copper toxicity is also known to be reduced by 30 times or more in the presence of organic solutes (Menkissoglu and Lindow 1991).

The toxic concentration of copper sulfate to Rainbow Trout eggs was higher than that reported for Rainbow Trout fingerlings in a 96-h test (20 μg/L in soft acid water to 520 μg/L in hard alkaline water; Howarth and Sprague 1978). In 48-h exposures with Rainbow Trout fingerlings, LC50 values for total copper were 110 μg/L at pH 7.5 and 120 μg/L at pH 6.5 (Shaw and Brown 1974). Similar concentrations have been reported for fingerling Rainbow Trout in other studies (Campbell and Stokes 1985). Lethal concentrations of copper sulfate for fingerling Channel Catfish, sunshine bass *Morone chrysops* × *M. saxatilis*, or Golden Shiner *Notemigonus crysoleucas* were ≤5 mg/L, though the exposures occurred over a 46–96-h period (Lewis and Lewis 1971; Straus 2006). Duration of exposure is a critical variable for toxicity (Shaw and Brown 1974). For example, most Threespine Sticklebacks *Gasterosteus aculeatus* tolerated copper nitrate concentrations of 0.3 mg/L for 24 h, but continued exposure for 1 week reduced

the threshold tolerance concentration to 0.02 mg/L (Doudoroff and Katz 1953). The toxicity of copper varies with salinity, pH, and total hardness and alkalinity (Howarth and Sprague 1978; Cusimano et al. 1986; Heath 1995), so egg toxicity will likely vary with changes in these parameters as well.

In summary, the results of this study indicate that copper sulfate concentrations needed to control *F. psychrophilum* were toxic to Rainbow Trout eggs. Further evaluation of copper sulfate might include evaluation of lower, nontoxic doses applied daily or every other day. For now, although copper sulfate can be useful to control fungal growth, it is not recommended as a Rainbow Trout egg disinfectant to control bacterial cold-water disease.

ACKNOWLEDGMENTS

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