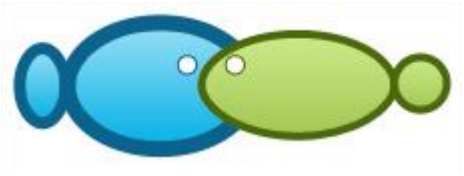

Copper sulfate as treatment for the ectoparasite *Amyloodinium ocellatum* (Dinoflagellida) on milkfish (*Chanos chanos*) fry

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Abstract. Milkfish *Chanos chanos* fry (22-day old; mean fish total length (FTL) = 13±0.6 mm) were exposed to various concentrations of copper sulfate ($CuSO_4 \cdot 5H_2O$) to determine its tolerance to the chemical. Results show that milkfish can tolerate $CuSO_4$ up to 29 mg L^{-1} for 96 h at 29.9°C with a mean survival rate of 95%. Frequent infestation by the parasitic dinoflagellate *Amyloodinium ocellatum* (Dinoflagellida) on the fins and skin of hatchery-reared milkfish with mass mortalities was observed in a commercial fish hatchery in Oton, Iloilo, Philippines. Treatment with 1, 2, and 3 mg L^{-1} $CuSO_4$ for 1 and 24 h were conducted to evaluate its efficacy against *A. ocellatum* on infected 24- to 28-day old milkfish. Although the use of 2 mg L^{-1} $CuSO_4$ for 1 h or 1 mg L^{-1} $CuSO_4$ for 24 h was effective in treating *A. ocellatum* on milkfish, a lower concentration at shorter but repeated exposures may be considered for the complete eradication of *A. ocellatum* infection.
Key Words: chemotherapy, dinoflagellate, amyloodiniosis, protozoan parasites, marine fish.

Introduction. Milkfish *Chanos chanos* (Forsskal, 1775) is an economically important species farmed in the Philippines. The increasing demand for food fish has intensified fry production leading to the emergence of parasites and disease outbreaks. One of the effective methods to control parasites during emergency situations is through chemotherapy. Copper sulfate ($CuSO_4$) is used for prevention and control of parasite infections. Several studies have shown the effectiveness of $CuSO_4$ against fish parasites (Abreu et al 2005; Straus 2008; Farmer et al 2013; Farmer et al 2014). It is usually the treatment of choice for protozoan ectoparasites of pond-reared fish (Noga 2010). Before a chemical can be used as therapeutant, the safe level to the fish should be determined. However, there is no data yet on the tolerance of milkfish to $CuSO_4$.

Recently, a commercial fish hatchery in Oton, Iloilo, Philippines (10°43´N, 122°27´E) experienced frequent infestation of an ectoparasitic dinoflagellate *Amyloodinium ocellatum* (Brown, 1931) on its cultured milkfish resulting in mass mortalities. Heavy infestation of trophonts, the parasitic stage of *A. ocellatum*, causes epithelial erosion of the skin and severe epithelial hyperplasia of the gills which can eventually lead to the death of its host (Paperna 1980; Kuperman & Matey 1999; Noga & Levy 2006). Cruz-Lacierda et al (2004) first reported the heavy infestation of *A. ocellatum* in hatchery-reared milkfish in the Philippines. The present study aims to determine the safe concentration of $CuSO_4$ on milkfish fry and to evaluate the efficacy of $CuSO_4$ in treating *A. ocellatum* on infected milkfish.

Materials and Methods

Fish safety tests. Healthy and parasite-free milkfish fry (21-22 days old, d.o., 250 pieces) were obtained from the Aquaculture Department of Southeast Asian Fisheries Development Center (SEAFDEC/AQD) in Tigbauan, Iloilo (10°43´N, 122°22´E) and

transported alive in oxygenated plastic bags to the Fish Disease Laboratory, Institute of Aquaculture (IA) of the College of Fisheries and Ocean Sciences, University of the Philippines Visayas (UPV) in Miagao, Iloilo (10°38´N, 122°14´E). The fish were transferred to a glass aquarium (30 x 30 x 45 cm) containing 20 L of sand-filtered seawater with gentle aeration and were acclimated for 24 h under laboratory conditions (salinity: 30±1 ppt, temperature: 29.9±0.2°C). No mortality was observed during the acclimation period. On May-June 2012, a series of 24-h range-finding tests were performed to select the test concentrations for the 96-h definitive test. Standard toxicity tests were done based on the procedures outlined by the American Public Health Association (APHA 2012). Fish behavior and mortality were observed at regular intervals until the termination of the experiment.

A stock solution was prepared with reagent grade copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, Scharlau), approximately (~) 25.4% copper (Cu). The concentrations used were: 0 (control, no chemical), 1, 15 and 29 mg L^{-1} with two replicates each. Ten milkfish (mean fish total length, FTL = 13±0.6 mm) were stocked at random in each glass aquarium (3 cm^3) with 1 L sand-filtered seawater (30 ppt, 30°C). All treatments were provided with gentle aeration throughout the 96 h exposure period. Experimental fish were not fed during the test.

Treatment of A. ocellatum-infected milkfish with $CuSO_4$. Based on the results of the fish safety tests, the efficacy of $CuSO_4$ in treating *A. ocellatum*-infected milkfish fry was investigated at 1-h and 24-h treatment periods. In the 1-h treatment, 150 pieces of milkfish fry (24 d.o.) naturally infected with *A. ocellatum* from a commercial hatchery in Oton, Iloilo were transported alive in oxygenated plastic bag to the laboratory of UPV on the 15th of September 2012. The infected fish were placed in a glass aquaria (30 x 30 x 45 cm) with 20 L sand-filtered seawater (33 ppt) provided with gentle aeration and were acclimated for 1 h under laboratory conditions (27°C). Afterwhich, microscopic examination (at 40-100x magnification) of fish (n = 15) was done to determine the initial mean intensity (25.4±12 trophonts/fish) and prevalence (100%) of the parasite as in Bush et al (1997). Seven milkfish fry were randomly stocked in each glass aquarium (3 cm^3) with 1 L of seawater (33 ppt, 27°C) provided with gentle aeration. The infected fish were exposed to 1, 2 and 3 mg L^{-1} $CuSO_4$ and a control (no chemical) with three replicates per treatment. The intensity and prevalence of the parasite in all treatments were computed after 1 h of exposure to the chemical. The efficacy of the treatment was evaluated by counting the trophonts on treated and untreated fish (control).

For the 24-h treatment, 500 pieces healthy, parasite-free milkfish fry (28 d.o.) from a commercial hatchery in Oton, Iloilo were stocked in a glass aquarium (30 x 30 x 45 cm) with 20 L *A. ocellatum*-infected seawater (31 ppt, 30°C) containing approximately 50 pieces *A. ocellatum*-infected milkfish fry (25 d.o.). After 14 h, fish (n = 20) were examined microscopically to determine the mean intensity (38.7±11) and prevalence (100%) of the parasite. The test concentrations used were 1, 2 and 3 mg L^{-1} $CuSO_4$ and a control (no chemical) done in three replicates per treatment (20 fish $replicate^{-1}$). The mean intensity and prevalence of the parasite, efficacy of the treatment, and survival of fish were determined 24 h after treatment. The differences on mean intensity, prevalence, survival rate and efficacy between treatments were analyzed using a one way analysis of variance and Duncan's multiple range test (DMRT) at 0.05 level of significance through SPSS software, version 16.0 (SPSS Inc., Chicago).

Results and Discussion

Fish safety tests. Copper sulfate has been documented to be effective in controlling parasite infections, however Cu in the form of cupric ion (Cu^{2+}) is toxic to fish (Cardeilhac & Whitaker 1988). The use of chemicals for disease prevention and control is based on the principle of differential toxicity, i.e. the drug or chemical must kill or eliminate the pathogen at concentrations that do not harm the host (Baticados & Paclibare 1992). The tolerance of fish to a chemical depends on the fish species and treatment conditions. Thus, the safe level of a chemical to a particular fish species must be established first

before it can be used for treatment. Table 1 shows the mean survival rate of milkfish fry exposed to different $CuSO_4$ concentrations after 96 h. A 100% survival was observed in 1 mg L^{-1} $CuSO_4$ after 96 h. High survival rate was also observed in 15 and 29 mg L^{-1} $CuSO_4$, even after 96 h of exposure.

The tolerance level of other marine fish species to $CuSO_4$ has been reported in earlier studies. Juvenile pompano *Trachinotus carolinus* (Linnaeus, 1766) (mean FTL = 25 mm) has a 96-h TL_{50} (tolerance limit corresponding to 50% survival) of 1.97 mg L^{-1} to $CuSO_4$ (30 ppt, 20-25°C) (Birdsong & Avault 1971). The reported 96-h TL_{50} of striped bass *Morone saxatilis* (Walbaum, 1792) (mean FTL = 68±1.9 mm) to $CuSO_4$ is 7.88 mg L^{-1} at 15 ppt and 26°C (Reardon & Harrell 1990). For silver sea bream *Sparus sarba* (Forsskal, 1775) fingerlings (mean body weight (BW) = 9.4±2.1 g), the 96-h TL_{50} is reported at 1.03 mg L^{-1} Cu (~4.06 mg L^{-1} $CuSO_4$) at 30-33 ppt and 28°C (Wong et al 1999). The 96-h TL_{50} of Japanese flounder *Paralichthys olivaceus* (Temminck & Schlegel, 1846) (BW = 0.3-17 g) and red sea bream *Pagrus major* (Temminck & Schlegel, 1843) (BW = 0.5-13 g) at 20°C ranged from 8.7-12.2 mg L^{-1} Cu (~34.25-48.03 mg L^{-1} $CuSO_4$) and 2.0-5.2 mg L^{-1} Cu (~7.87-20.47 mg L^{-1} $CuSO_4$), respectively (Furuta et al 2008). Based on previous records, milkfish appears to be more tolerant to $CuSO_4$ than pompano, striped bass, silver sea bream and red sea bream, but is less tolerant than Japanese flounder.

Table 1

Average survival rate of milkfish, (*Chanos chanos*), fry[a] exposed to different concentrations of copper sulfate ($CuSO_4$) at different exposure times

$CuSO_4$ (mg L^{-1})[b]	*Survival (%)/Duration (h)*[c]				
	12	*24*	*48*	*72*	*96*
0 (Control)	100	100	100	100	100
1	100	100	100	100	100
15	100	95	95	95	95
29	95	95	95	95	95

[a] 22 days old; mean total length = 13±0.6 mm; [b] 2 replicates/treatment; n = 10 fish/replicate/treatment; 29.9±0.2°C, 30±1 ppt; [c] mean of 2 replicates/treatment.

Tolerance of milkfish fry to the chemical was also evaluated at higher test concentrations (43, 57, 71, 85 and 99 mg L^{-1} $CuSO_4$), however, formation of precipitates were observed at the bottom of the aquaria. Taylor et al (1985) reported that precipitation occurred when more Cu was added to the seawater. According to Cardeilhac & Whitaker (1988), Cu ion tends to precipitate as Cu carbonate in very hard and/or alkaline water. Since seawater is alkaline, it is plausible that much of the Cu in 43-99 mg L^{-1} $CuSO_4$ could have formed insoluble Cu carbonates, thus decreasing the actual Cu concentration in the water (Cardeilhac & Whitaker 1988; Straus 2006). The results at concentrations higher than 29 mg L^{-1} were not included because of the inaccurate dissolved Cu levels in the solution.

Treatment of A. ocellatum-infected milkfish with $CuSO_4$. Several studies have reported the massive infestation of *A. ocellatum* in brackish and marine fish worldwide (Paperna 1980; Baticados & Quinitio 1984; Rigos et al 1998; Kuperman & Matey 1999; Cruz-Lacierda et al 2004; Saraiva et al 2011; Soares et al 2012; Yemmen & Bahri 2016). The primary site of infection of *A. ocellatum* is usually on the gills of the host fish (Francis-Floyd & Floyd 2011). However, in the present study, trophonts were seen all over the body surface of fish, predominantly attached on the fins and skin. The gills were not affected. Generally, larval-sized fishes are typically infested on the skin while bigger fishes are infested on the gills (Overstreet 1993). Figure 1 shows several trophonts of *A. ocellatum* attached to the caudal fin of milkfish fry.

The efficacy of $CuSO_4$ in treating *A. ocellatum* on milkfish fry for 1 and 24 h is presented in Table 2. The intensity of the parasite in $CuSO_4$-treated fish decreased significantly as compared to the control fish. The mean intensity of the parasite and the efficacy among the three $CuSO_4$ treatments in both exposure periods showed no

significant difference ($p < 0.05$). In the 1 h treatment, no mortality was observed among fish treated with 1-3 mg L^{-1} $CuSO_4$ suggesting that the treatment is safe. Although the three treatments significantly reduced the intensity of the parasite on fish, the parasite was not completely eliminated. The prevalence of the parasite was still high especially in the 1 mg L^{-1} treatment. The 24-h treatment was more efficacious in reducing the intensity and prevalence of the parasite than the 1-h treatment. However, survival of the fish after the 24 h treatment was low. At the time of the treatment, the fish were observed to be very weak that may be due to the parasite infection. The survival rate of fish among the three treatments and the control showed no significant difference suggesting that exposure to the chemical was not the main cause of mortality but could be attributed to the weak condition of the *A. ocellatum*-infected fish. The trophonts of *A. ocellatum* attach itself on the host through its rhizoids which is deeply embedded on the epithelial tissue of the attachment site (Noga & Levy 2006). High pathogenicity of *A. ocellatum* was attributed to the extensive damage caused by the rhizoids to the epithelial cells as well as feeding on host cell cytoplasm (Lom & Lawler 1973; Paperna 1980). The non-feeding of the parasitized fish during treatment could have exacerbate the condition resulting to low survival rates.

The $CuSO_4$ treatment concentrations (1-3 mg L^{-1} $CuSO_4$; ~0.25-0.76 mg L^{-1} Cu) used in the present study are slightly higher and shorter in duration than the suggested concentration in controlling *A. ocellatum* outbreaks which is 0.15-0.2 mg L^{-1} free copper ion (~0.59-0.79 mg L^{-1} $CuSO_4$) for up to three weeks (Francis-Floyd & Floyd 2011). Use of low concentration is usually employed in prolonged immersions to avoid tissue residue problems and is economically wise. Higher concentrations were used in this study since treatment duration was shorter and fish safety test showed that milkfish has a high tolerance to $CuSO_4$.

Figure 1. Trophonts of *Amyloodinium ocellatum* (arrows) attached to the caudal fin of 28-day old milkfish fry (fresh mount, scale bar = 100 µm).

Dempster (1955) reported the effective use of $CuSO_4$ (0.4-0.8 mg L^{-1} for 10 d) in treating *Oodinium* infestations on marine fishes. Paperna (1984) recommended that continuous application of 0.75 mg L^{-1} $CuSO_4$ for 2 weeks (40±1 ppt; 20°C) through continuous flow-siphon system can effectively eradicate *A. ocellatum* gilthead seabream (*Sparus aurata* L.) larvae (< 12 mm) and post-larvae (13-20 mm). In his study, flush treatment at 6 mg L^{-1} $CuSO_4$ for 2 h did not completely dislodge the trophonts from the fish. Abreu et al (2005) reported that the use of 1.5 mg L^{-1} Cu (~5.9 mg L^{-1} $CuSO_4$) for 7 d (30-33 ppt;

25-27.5°C) was effective in forcing *A. ocellatum* trophonts to detach from the infested broodstocks of Brazilian flounder *Paralichthys orbignyanus* (Valenciennes, 1839). This suggests that longer exposure is needed to completely remove the trophonts from the host fish.

Table 2

Efficacy of 1-h[a] and 24-h[b] $CuSO_4$ treatments against *Amyloodinium ocellatum* on milkfish, *Chanos chanos*, fry

Treatment duration (h)	*$CuSO_4$ (mg L^{-1})*	*A. ocellatum*		*Efficacy of treatment[+] (%)*	*Fish survival (%)[+]*
		Prevalence[+] (%)	*Mean intensity[+] ± SD*		
1	0 (Control)	100.00[a]	15.40 ± 8.08[a]	0.00[a]	100.00
	1	77.78[ab]	1.59 ± 1.09[b]	89.65[b]	100.00
	2	34.45[bc]	0.46 ± 0.47[b]	97.04[b]	100.00
	3	28.57[c]	0.45 ± 0.43[b]	97.06[b]	100.00
24	0 (Control)	100.00[a]	19.09 ± 3.78[a]	0.00[a]	35.00[a]
	1	12.30[b]	0.12 ± 0.15[b]	99.35[b]	45.00[a]
	2	2.78[b]	0.03 ± 0.05[b]	99.85[b]	53.33[a]
	3	5.34[b]	0.05 ± 0.05[b]	99.72[b]	53.33[a]

[a] 24-day old milkfish; n = 7 fish/replicate; 27°C, 33 ppt; [b] 28-day old milkfish; n = 20 fish/replicate; 30.9±0.1°C, 30 ppt; [+] Values are means of 3 replicates per treatment; values with the same superscript in a column are not significantly different ($p > 0.05$); SD, standard deviation.

The present study evaluated the effect of $CuSO_4$ to *A. ocellatum* trophonts on milkfish fry. The other stages of *A. ocellatum* such as the tomonts (encysted stage) and dinospores (free-swimming stage) may also be present in the rearing water. Exposure of *A. ocellatum*-infected flounder broodstock to $CuSO_4$ forced the trophonts to detach from the fish and fall into the bottom but the treatment was not effective to kill the tomonts (Abreu et al 2005). According to Paperna (1984), tomonts appear to be tolerant to short durations (6-12 h) of exposure to $CuSO_4$. Incubation with 0.5-10 mg L^{-1} $CuSO_4$ at 20°C did not interrupt tomont division, however, it is lethal to the sporulating tomites and the forming dinospores.

The use of chemotherapy in any disease control program is considered as a method of 'last resort'. Chemicals like $CuSO_4$ are suggested to be used only as a preventive method to avoid recurrence of the parasite and should be used responsibly. Dequito et al (2015) reported that improper hatchery management practice like poor filtration system and sanitation was observed as the possible cause of *A. ocellatum* infestations on cultured milkfish fry in a hatchery in Oton, Iloilo (same hatchery where the infected fish was obtained in this study). Thus, good husbandry practices and proper management techniques are safer, more practical and environment-friendly approach to prevent parasitic diseases.

Conclusions. Milkfish fry can tolerate copper sulfate at 1-29 mg L^{-1} for 96 h. Treatment with 2 mg L^{-1} $CuSO_4$ for 1 h is effective in the control of *A. ocellatum* trophonts on milkfish fry. However, repeated exposure may be considered to completely eradicate *A. ocellatum* from the rearing water. Regular monitoring of Cu concentration in the test water is recommended to ensure that therapeutic concentrations are maintained during the treatment period. Further studies on the efficacy of $CuSO_4$ against the other life stages of *A. ocellatum* are also recommended.

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