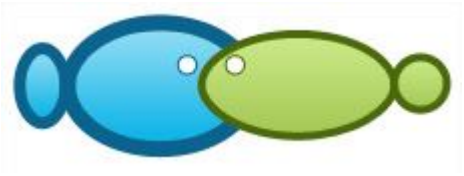

Anti-oomycetic effect of copper sulfate *in vitro* on *Achlya* spp. isolated from infected Nile tilapia (*Oreochromis niloticus*)

[1]Kwanprasert Panchai, [2]Chutima Hanjavanit, [2,3]Narumon Sangpradub, [4]Kishio Hatai

[1] Program of Biology, Faculty of Science and Technology, Nakhon Ratchasima Rajabhat University, Nakhon Ratchasima, 30000, Thailand; [2] Applied Taxonomic Research Center, Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand; [3] Centre of Excellence on Biodiversity, Bangkok 10330, Thailand; [4] Microbiology and Fish Disease Laboratory, Borneo Marine Research Institute, Universiti Malaysia Sabah, 88400, Kota Kinabalu, Malaysia. Corresponding author: C. Hanjavanit, chuhan@kku.ac.th

Abstract. The aims of the present study were to determine the oomycetecidal effect of copper sulfate on both vegetative and zoosporic stages of water molds, *Achlya* spp., in *in vitro* tests and to evaluate the efficacy toxicity on Nile tilapia (*Oreochromis niloticus*) fry. The results show that copper sulfate at 100 mgL^{-1} killed both the vegetative stage of five selected *Achlya* spp. and the zoosporic stage of *A. diffusa* BKKU1012, *A. prolifera* BKKU1125 and *Achlya* sp. BKKU1127. Additionally, 25 mgL^{-1} copper sulfate solution could kill the zoosporic stage of *A. klebsiana* BKKU1003 and *Achlya* sp. BKKU1117 and also inhibited zoospore germination of all selected *Achlya* spp. with 30 minutes treatment. In addition, 6.25 and 12.5 mgL^{-1} copper sulfate solution had no toxic effect (0% mortality) on the tilapia fry. In contrast, 25, 50 and 100 mgL^{-1} copper sulfate solutions had strong toxicity to the fish (100% mortality) with 6 hours, 2 hours and 30 minutes treatment, respectively. Thus, this study revealed that it is possible to use copper sulfate to kill the aquatic oomycetes, *Achlya* spp., if it is given 30 minutes treatment.
Key Words: achlyosis, copper sulfate, oomycetecidal effect, tilapia fry.

Introduction. *Achlya* is an important genus in the family Saprolegniaceae, with some species of the genus *Achlya* cause water mold infection to freshwater fish, especially warm water fish. These water molds can infect many kinds and stages of fish (Hussein et al 2002). Available chemicals such as malachite green, formalin, hydrogen peroxide, sodium and calcium chloride, copper, iodophore and bronopol have been recommended to prevent water mold infection (Bruno et al 2011). However, the use of malachite green is prohibited in many countries because it was reported to have teratogenic (Meyer & Jorgenson 1983), carcinogenic (Bruno et al 2011) and mutagenic properties (Clemmensen et al 1984; Fernandes et al 1991; Srivastava et al 2004) and also cause human health hazards (Adeyemo et al 2011). According to Schreier et al (1996), formalin is potentially harmful to the user's health and also remains in the ecosystem. Formalin also is a suspected carcinogen and has a potential adverse effect on the aquatic environment (Arndt et al 2001). Copper sulfate is used as an algaecide for control growth of phytoplanktonin in fish ponds, reservoirs and lakes and as a herbicide in aquatic weed control (Effler et al 1980; Carbonell & Tarazona 1993), as well as also being used as a mollusicide to kill snails and slugs in irrigation and municipal water treatment systems (Moore et al 1984). In aquaculture, copper sulfate is an effective treatment for the parasitic protozoan, *Ichthyophthirius multifiliis*, causing white spot disease in goldfish (*Carassius auratus*) (Ling et al 1993), channel catfish (*Ictalurus punctatus*) (Straus 1993, 2008; Schlenk et al 1998) and the Australian freshwater fish silver perch (*Bidyanus bidyanus*) (Rowland et al 2009). Copper sulfate is among the potential chemical candidates for control of bacteria on eggs (Straus et al 2009). Copper sulfate can inhibit

growth of bacterial coldwater disease, *Flavobacterium psychrophilum*, affecting eggs of rainbow trout (*Oncorhynchus mykiss*) *in vitro,* but it is toxic to rainbow trout eggs (Wagner & Oplinger 2013). Copper sulfate has been documented for use to inhibit hyphal growth and sporogenesis of *Lagnidium* spp. isolated from prawn, *Penaeus monodon* larvae, and crab, *Scylla serrata,* eggs (Lio-Po et al 1982). It is also the potential chemical candidate for control of water mold on eggs and fish. Miura et al (2005) found that copper fibers placed in the inflow of egg incubators led to control of zoospores of *Saprolegnia diclina*. Straus et al (2009, 2012) reported that copper sulfate is effective for anti-oomycetic activities against *Saprolegnia* spp. infection in channel catfish eggs. A recent report by Sun et al (2014) indicated that copper sulfate is used as an anti-oomycetic agent to control mycelia and zoospores of *S. parasitica in vitro*, which was isolated from infected grass carps (*Ctenopharyngodon idella*). Therefore, the aims of the present study were to determine concentrations of copper sulfate for an anti-oomycetic effect on both vegetative and zoosporic stages of water molds, *Achlya* spp., in *in vitro* tests and to evaluate the efficacy toxicity on Nile tilapia (*Oreochromis niloticus*) fry.

Material and Method. All experiments were conducted at the Department of Biology, Faculty of Science, Khon Kaen University, Thailand.

Chemical. Copper (II) sulfate ($CuSO_4$) (Merck, Germany) was used in the present study. A modification of the method described by Borisutpeth et al (2009) was used to assess the effect of copper sulfate solution against *Achlya* spp.

Sources of Achlya spp. Five randomly selected samples from *Achlya* spp. isolated from cultured Nile tilapia (Panchai et al 2015) were composed of *A. klebsiana* BKKU1003, *A. diffusa* BKKU1012, *Achlya* sp. BKKU1117, *A. prolifera* BKKU1125 and *Achlya* sp. BKKU1127. They were maintained on GY agar (0.25 g yeast extract, 1.0 g glucose, 15 g agar and 1,000 mL distilled water) (Hatai & Egusa 1979) at 25°C. The advancing edges of each 3 day growing colony agar blocks were cut out using No. 2 cork borer (5.5 mm in diameter) and used as inoculums for all experiments.

Source of fish. Nile tilapia fry (0.72±0.32 g in body weight and 3.74±0.53 cm in total length) were provided by Khon Kaen Inland Fisheries Research and Development Center, Thailand. They were maintained at room temperature and acclimatized for one week to laboratory conditions before the experiment, which were conducted during August-September 2015. The fish were fed two times daily with commercial formula food (GF Feed, Krungthai Feedmill Public Co., Ltd., Thailand). The mortality rate of the fish was monitored and kept less than 5% before the 5 days of the experiment. They were starved for one day before the experiment started. After acclimatization, the fish were randomly selected and kept in a static system of water.

Oomycetestatic effect of copper sulfate on hyphae. Copper sulfate concentration was adjusted to 100,000 mg L^{-1} using sterilized distilled water (SDW) before use. The solution was filtered through 0.2 µm millipore filter paper (Sartorius, Hannover, Germany) and serially diluted to concentrations of 10,000, 1,000 and 100 mg L^{-1} using 10% GY broth. Blocks of the growing colony were added to each 24-well tissue culture dish (Costar®, Corning Incorporated, USA) containing 2 mL of test solution, and for the control group the colony was placed in 10% GY broth without $CuSO_4$. The well tissue culture dishes were incubated at 25°C and hyphal growth was observed under the inverted microscope (Nikon Phase Contrast-2 ELWD 0.3, Japan) at 1, 2 and 5 days incubation. If no hyphal growth was observed after 5 days, the agar blocks were removed, rinsed in SDW and placed on new GY agar plates, which were incubated at 25°C. After that, the survival of the oomycete was observed again at day 2. Three replicates of each isolate were conducted.

Oomycetecidal effect of copper sulfate on hyphae. Copper sulfate solution was prepared as described above and diluted to concentrations of 100, 50, 25, 12.5 and 6.25 mg L^{-1} using 10% GY broth. The agar blocks of each isolate were placed into a plastic Petri dish containing 10 mL of various concentrations of $CuSO_4$ for 30 minutes, 1, 2, 6 and 24 hours. The blocks of control group were placed in 10% GY broth without $CuSO_4$

for the same duration as the treatment groups. Next, the mycelia were removed, rinsed with SDW, placed on new GY agar plates and incubated at 25°C. The hyphal growth of the treatment groups was compared with the control group to determine the viability within 48 hours.

Oomycetecidal effect of copper sulfate on zoospores. The minimum inhibitory concentrations (MIC) of $CuSO_4$ that inhibited the hyphal growth in the above experiment were also used to determine the proper dosage for zoospore germination. Zoospore suspensions of each isolate were prepared by the same procedure as described by Kitancharoen et al (1995) and adjusted to 1.0 x 10^3 zoospores mL^{-1}. After that, 1 mL of each concentration of $CuSO_4$ with 10 times the desired final concentration, at the same concentration as in the previous experiment, was added to 9 mL of zoospore suspension, and zoospores of the control group were inoculated into 10% GY broth without $CuSO_4$ solution. The mixture was kept at 25°C for 30 minutes, 1, 2, 6 and 24 hours. After that, 0.1 mL of the mixture was inoculated onto a GY agar plate and incubated at 25°C. The viability of the oomycetic zoospores was determined by observing the appearance of the colonies over 1, 2 and 7 day periods with the naked eye.

Effect of copper sulfate on zoospores germination. Zoospores of five *Achlya* spp. were induced as described by Kitancharoen et al (1995), and adjusted to 1.0 x 10^3 zoospores mL^{-1}. The copper sufate solutions were prepared at final concentrations of 10 times (1,000, 500, 250, 125 and 62.5 mg L^{-1}). Three mL of each 10x solution were added into 27 mL of zoospore suspension and incubated at 25°C for 30 minutes, 1, 2 and 24 hours. At the end of each incubation period, the zoospores were pelleted by centrifugation at 8,000 rpm for 5 minutes. Then, the supernatant was discarded and the zoospore pellet was rinsed 3 to 4 times with sterilized tap water (STW). The rinsed zoospores were transferred into Petri dishes containing 30 mL of 1/30 GY broth and incubated at 25°C. Zoospore germination was determined under inverted microscope to observe the presence, absence and quantity of germinating thalli at 3 hours after incubation.

Toxicity of copper sulfate to Nile tilapia fry. Two hundred live, healthy Nile tilapia fry were used for the toxicity test. The experimental design consisted of a control and five concentrations of $CuSO_4$ (6.25, 12.5, 25, 50 and 100 mgL^{-1}). Three replicates per group and ten fish in each replicate were used. The fish were not fed throughout the experiment. Numbers of dead fish were recorded after 30 minutes, 1, 2, 6 and 24 hours. The percentage of corrected mortality of fish was calculated using Abbott's formula of Barnes et al (1998).

Results

Oomycetestatic effect of copper sulfate on hyphae. It was found that all isolates were able to grow in treatment without $CuSO_4$ solution. The result of treatment with $CuSO_4$ solution showed the oomycetestatic dosage of each *Achlya* isolate was 100 mg L^{-1}.

Oomycetecidal effect of copper sulfate on hyphae. As presented in Table 1, the oomycetecidal dosage of $CuSO_4$ against the hyphal growth of *A. klebsiana* BKKU1003, *A. diffusa* BKKU1012, *Achlya* sp. BKKU1117, *A. prolifera* BKKU1125 and *Achlya* sp. BKKU1127 was 100 mg L^{-1} for 1 hour treatment, whereas for *A. klebsiana* BKKU1003 and *Achlya* sp. BKKU1117 it was 50 mg L^{-1} for 2 hours' treatment.

Table 1

Oomycetecidal effect of $CuSO_4$ concentrations and exposure times on vegetative stage of oomycetic isolates at 25°C

Isolate	*Exposure time/Concentration (mg L^{-1})*	
	1 h	*2 h*
A. klebsiana BKKU1003	100	50
A. diffusa BKKU1012	100	100
Achlya sp. BKKU1117	100	50
A. prolifera BKKU1125	100	100
Achlya sp. BKKU1127	100	100

Oomycetecidal effect of copper sulfate on zoospores. The relationships between oomycetecidal dosages of $CuSO_4$ on zoospores and exposure time are summarized in Table 2. The oomycetecidal dosage of $CuSO_4$ on zoosporic stage was 25 mg L^{-1} against both *A. klebsiana* BKKU1003 and *Achlya* sp. BKKU1117, and 100 mg L^{-1} against *A. diffusa* BKKU1012, *A. prolifera* BKKU1125 and *Achlya* sp. BKKU1127 for 30 minutes treatment. The treatment for 1 hour with 50 mg L^{-1} of $CuSO_4$ was effective against all strains used.

Table 2

Oomycetecidal effect of $CuSO_4$ concentrations and exposure times on zoosporic stage of oomycetic isolates at 25°C

Isolate	*Exposure time/Concentration (mg L^{-1})*	
	30 min	*1 h*
A. klebsiana BKKU1003	25	50
A. diffusa BKKU1012	100	50
Achlya sp. BKKU1117	25	50
A. prolifera BKKU1125	100	50
Achlya sp. BKKU1127	100	50

Effect of copper sulfate on zoospore germination. As shown in Table 3, $CuSO_4$ at 50 mg L^{-1} completely inhibited zoospore germination (100%) of *A. klebsiana* BKKU1003, *A. diffusa* BKKU1012, *Achlya* sp. BKKU1117, *A. prolifera* BKKU1125 and *Achlya* sp. BKKU1127 after 30 minutes, 1, 2 and 24 hours of exposure times. It was found that zoospores of *A. klebsiana* BKKU1003 at $CuSO_4$ concentrations of 0-25 mg L^{-1} had spherical shape and directed germination. In addition, zoospores were capable of germinating and formed a whitish colony. However, zoospores of *A. klebsiana* BKKU1003 at $CuSO_4$ concentration of 50-100 mg L^{-1} had irregularly ovoid shape and neither germination tube nor colonial formation appeared.

Table 3

Effectiveness of $CuSO_4$ on zoospore germination of *Achlya* spp.

Isolate	*Concentration (mg L^{-1})*	*Exposure time/% germination rate*			
		30 min	*1 h*	*2 h*	*24 h*
A. klebsiana BKKU1003	0	100	100	100	100
	6.25	45	50	55	43
	12.5	21	22	25	10
	25	7	15	6	1
	50	0	0	0	0
	100	0	0	0	0
A. diffusa BKKU1012	0	100	100	100	100
	6.25	58	55	64	40
	12.5	40	53	40	15
	25	5	10	5	7
	50	0	0	0	0
	100	0	0	0	0
Achlya sp. BKKU1117	0	100	100	100	100
	6.25	78	84	67	54
	12.5	58	55	50	12
	25	9	12	15	5
	50	0	0	0	0
	100	0	0	0	0
A. prolifera BKKU1125	0	100	100	100	100
	6.25	59	69	53	45
	12.5	48	45	14	12
	25	18	32	6	2
	50	0	0	0	0
	100	0	0	0	0
Achlya sp. BKKU1127	0	100	100	100	100
	6.25	76	58	45	40
	12.5	45	34	25	12
	25	21	12	7	9
	50	0	0	0	0
	100	0	0	0	0

Toxicity of copper sulfate to Nile tilapia fry. As presented in Table 4, the mortality of the fish when exposed to $CuSO_4$ concentrations of 0, 6.25 and 12.5 mg L^{-1} was 0% after 30 minutes. The highest percentage mortality (100%) was found in $CuSO_4$ concentrations of 25, 50 and 100 mg L^{-1} after 24 hours, 6 hours, 2 hours and 30 minutes exposure, respectively.

Table 4

Cumulative mortality of Nile tilapia fry exposed to $CuSO_4$ solution

$CuSO_4$ (mg L^{-1})	*Total fish*	*Exposure time/Number of dead fish (cumulative mortality)*				
		30 min	*1 h*	*2 h*	*6 h*	*24 h*
0	30	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
6.25	30	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
12.5	30	0 (0)	0 (0)	0 (0)	0 (0)	7 (23%)
25	30	2 (7%)	8 (27%)	16 (53%)	30 (100%)	30 (100%)
50	30	12 (40%)	22 (73%)	30 (100%)	30 (100%)	30 (100%)
100	30	30 (100%)	30 (100%)	30 (100%)	30 (100%)	30 (100%)

Discussion. Copper sulfate has been used in aquaculture for many years to control weeds, algae, snails (which carry catfish trematode), ecto-parasitic organisms in channel catfish production (Straus et al 2015) and it also has been recognized as a safe treatment for *Saprolegnia* spp. on the eggs of channel catfish (Straus et al 2009). In previous studies, Lio-Po et al (1982) reported that for $CuSO_4$, 5-100 mg L^{-1} were mycostatic doses which inhibited vesicle formation, and 500 mg L^{-1} was a mycocidal dose for *Lagenidium* spp. isolated from *P. monodon*. According to Miura et al (2005), copper fibers placed in the inflow of rainbow trout egg incubators led to control of zoospores of *S. diclina*, and copper fibers with 0.006 mg L^{-1} could prevent zoospores germination of *S. diclina in vitro*. Straus et al (2009) reported that daily treatment of channel catfish eggs with 10-40 mg L^{-1} $CuSO_4$ controlled growth of *Saprolegnia* spp. Sun et al (2014) reported that $CuSO_4$ at ≥ 0.5 mg L^{-1} inhibited the growth of mycelium of *S. parasitica*, and no primary zoospores were released at ≥ 1.0 mg L^{-1} *in vitro* tests. Additionally, 0.5 mg L^{-1} $CuSO_4$ could also reduce the infection rate of *S. parasitica* in the grass carp.

From the present study, $CuSO_4$ was effective at killing the vegetative stage of five selected *Achlya* isolates at 100 mg L^{-1} after 1 hour treatment. Furthermore, the killing of the zoosporic stage varied among the isolates and exposure times. Namely, lower concentrations (25 mg L^{-1} $CuSO_4$) and shorter exposure periods (30 minutes) showed a stronger effect against the zoosporic stage of *A. klebsiana* BKKU1003 and *Achlya* sp. BKKU1117, while 100 mg L^{-1} $CuSO_4$ had an effect on zoospores of *A. diffusa* BKKU1012, *A. prolifera* BKKU1125 and *Achlya* sp. BKKU1127. In addition, the inhibition effects of $CuSO_4$ against zoospore germination of all isolates were shown to be lower (50 mg L^{-1}) after 30 minutes treatment than the oomycetestatic effects (100 mg L^{-1}) on hyphal growth. This result indicates that the zoospores have higher sensitivity to $CuSO_4$ than the hyphae, which is probably due to the zoosporic stage being more sensitive to chemicals than the vegetative stage supported by Muller-Riebau et al (1995). Additionally, zoospores had an important role in the induction of oomycete infection but even if the zoospores and hyphae are not killed following chemical treatment, initial infection may be prohibited (Pickering & Willoughby 1982; Beakes et al 1994; Hatai & Hoshiai 1994; Bruno et al 2011). However, the result of oomycetecidal doses of $CuSO_4$ on the vegetative and zoosporic stages of five selected *Achlya* isolates differed from the reports of Straus et al (2009) and Sun et al (2014). This difference is expected to be due to the difference between the species of water mold, vegetative stage, zoospore formation and the exposure time to the chemicals supported by Borisuthpeth et al (2009).

In addition, the toxicity test of $CuSO_4$ with various concentrations (6.25, 12.5, 25, 50 and 100 mg L^{-1}) on Nile tilapia fry showed that 6.25 and 12.5 mg L^{-1} have no effect on mortality (0%) of the fish after 30 minutes treatment. However, the higher concentrations (25, 50 and 100 mg L^{-1}) had a harmful effect, which caused 100% mortality of the fish after 24 hours, 6 hours, 2 hours and 30 minutes exposure, respectively. This result does not agree with the study of Straus et al (2009) who

reported that 20 and 40 mg L^{-1} $CuSO_4$ could cause 59% and 51% survival rates of channel catfish eggs, respectively. According to Straus et al (2012), channel catfish fry had 71% survival rate when treated with 100 mg L^{-1} $CuSO_4$. Sun et al (2014) reported that 1.0 mgL^{-1} $CuSO_4$ caused 43.33±5.77% mortality in the grass carp infected with *S. parasitica.* Therefore, the toxicity of $CuSO_4$ may vary among species of fish, stage of fish, metabolic mechanism of copper ion, which is a specific characteristic of the individual (De Boeck et al 2004), the physiological state of the individual (Tavares-Dias et al 2011) and physico-chemical parameters of water conditions, including hardness, pH, and alkalinity (Chakoumakos et al 1979; Laurén & McDonald 1986; Straus & Tucker 1993; Wurts & Perschbacher 1994). In addition, Rand et al (1995) suggests that rates and patterns of organism metabolism and excretion as well as genetic and dietary factors and the degree of development of detoxification mechanisms can affect toxicity.

Conclusions. This study is the first report on the efficacy of $CuSO_4$ in *in vitro* tests on *Achlya* spp. isolated from Nile tilapia with achlyosis. It was suggested that 100 mg L^{-1} $CuSO_4$ were effective in killing both the vegetative stage of five selected *Achlya* spp. and the zoosporic stage of *A. diffusa* BKKU1012, *A. prolifera* BKKU1125 and *Achlya* sp. BKKU1127. However, a lower concentration of 25 mg L^{-1} killed the zoosporic stage of *A. klebsiana* BKKU1003 and *Achlya* sp. BKKU1117 and also inhibited zoospore germination of all selected *Achlya* spp. with 30 minutes treatment. In addition, 6.25 and 12.5 mg L^{-1} $CuSO_4$ had no toxic effect on the fish, resulting in 0% mortality. However, 25, 50 and 100 mg L^{-1} $CuSO_4$ are strongly toxic to the fish, which caused 100% mortality for 24 hours, 6 hours, 2 hours and 30 minutes treatment. The present study demonstrates that it is possible to use $CuSO_4$ to inhibit the growth of aquatic oomycetes *in vitro*, but this chemical was also harmful to the tilapia fry. Therefore, if the tilapia fry are often bathed with 50 mg L^{-1} $CuSO_4$ for less than 30 minutes, this may affect oomycete viability without harming the fish, which should be considered and requires further investigation.

Acknowledgements. This work was supported by the National Research University Program, Khon Kaen University, Thailand and grant No. W-2553-Ph.d-02, which is gratefully acknowledged. We would like to thank Mr. Ian Thomas for editing the English text.

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Received: 23 February 2016. Accepted: 10 April 2016. Published online: 28 April 2016.
Authors:
Kwanprasert Panchai, Program of Biology, Faculty of Science and Technology, Nakhon Ratchasima Rajabhat University, Nakhon Ratchasima, 30000, Thailand, e-mail: t_kw@hotmail.com
Chutima Hanjavanit, Applied Taxonomic Research Center, Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand, e-mail: chuhan@kku.ac.th
Narumon Sangpradub, Applied Taxonomic Research Center, Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, 40002, Thailand; Centre of Excellence on Biodiversity, Bangkok 10330, Thailand, e-mail: narumon@kku.ac.th
Kishio Hatai, Microbiology and Fish Disease Laboratory, Borneo Marine Research Institute, Universiti Malaysia Sabah, 88400, Kota Kinabalu, Malaysia, e-mail: khatai0111@nvlu.ac.jp

How to cite this article:
Panchai K., Hanjavanit C., Sangpradub N., Hatai K., 2016 Anti-oomycetic effect of copper sulfate *in vitro* on *Achlya* spp. isolated from infected Nile tilapia (*Oreochromis niloticus*). AACL Bioflux 9(2):414-421.