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Prevention of *Ichthyophthirius multifiliis* infestation in goldfish (*Carassius auratus*) by potassium ferrate(VI) treatment

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ABSTRACT

Ichthyophthirius multifiliis is an important freshwater teleost pathogen that often leads to significant economic losses to the aquaculture industry. The purpose of this study was to assess the acute toxicity of potassium ferrate(VI) to *I. multifiliis* theront and the concentration needed to prevent *I. multifiliis* infestation in goldfish, *Carassius auratus*. Five hundred theronts were exposed to concentrations of potassium ferrate(VI) in each well of a 96-well microtiter plate and observed for 4 h to determine the acute toxicity. Results showed that the exposure of *I. multifiliis* theronts to potassium ferrate(VI) at concentrations of 4.80 mg/L or more resulted in 100% mortality by 4 h; the LC_{50} value was estimated to be 1.71 mg/L. Aqueous static renewal 96-h bioassays were carried out to determine the acute toxicity of potassium ferrate(VI) to goldfish. The LC_{50} value for potassium ferrate(VI) in goldfish was 42.51 mg/L. Goldfish were exposed to 4000 theronts/fish in aerated tap water (a dose previously shown to result in consistent infestation) and treated with a single dose of potassium ferrate(VI) after 30 min contact with theronts. Infection level and prevalence were recorded everyday after exposure. The results revealed that potassium ferrate(VI) at the 4.80 mg/L or more concentrations can significantly reduce not only the number of trophonts on the fin of goldfish on day 3 ($P < 0.05$), but also the prevalence of ichthyophthiriasis ($P < 0.05$). Potassium ferrate(VI) at a concentration of 4.80 mg/L was considered to be the lowest effective dose to prevent infestation of *I. multifiliis* in goldfish.

1. Introduction

The ciliate, *Ichthyophthirius multifiliis*, is the main parasitic threat to freshwater fish in large parts of the world (Buchmann et al., 2001). The disease ichthyophthiriasis, commonly known as white spot disease, can result in considerable economic losses to the aquaculture industry, including the freshwater ornamental fish trade. The ciliate life cycle, which consists of three stages: an infective theront, a parasitic trophont and a reproductive tomont, is well documented (Nigrelli et al., 1976; Noe and Dickerson, 1995; Swennes et al., 2006). Free-swimming theronts enter into the epidermis of fish to feed on mucus and tissue and rapidly differentiate into trophonts, and following a period of growth and development, the trophonts leave the host actively and transform to encysted tomonts. The tomonts undergo mitosis in the cyst and release theronts, the stage infective to the fish host.

In terms of current strategies for controlling ichthyophthiriasis in aquaculture, chemical agents aimed at interrupting the life cycle by killing the free-living stages of the parasite play the major role, although in some situations water management and vaccine can also be effective (Matthews, 2005). However, the application of

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chemical treatments in aquatic systems has to face two significant problems: toxicity to fish and safety to the environment. Additionally, many effective and widely used chemotherapeutants (e.g. malachite green) against *I. multifiliis* are no longer permitted to be used by some government agencies, such as the Food and Drug Administration (FDA) of the USA. So, the search for an effective drug for ichthyophthiriasis, which is not only safe for fish and the environment, but also permitted by legislation in some areas, becomes stringent.

Potassium ferrate(VI) is an environmental friendly strong oxidant in the entire pH range, which is from −2.2 V in acid to −0.7 V in base (Wood, 1958; Ma and Liu, 2002). Because potassium ferrate(VI) combines disinfectant and coagulative functions, it can potentially be used as a dual-function chemical reagent for water and wastewater treatment (Jiang and Lloyd, 2002). Recently, some researchers have proven that potassium permanganate can be used to control ichthyophthiriasis (Straus and Griffin, 2001, 2002). Potassium ferrate(VI), is not only a stronger oxidant, but also less toxic to animals, and safer for the environment and humans. Potassium ferrate(VI)'s potential as a therapeutic agent for external protozoan parasite infections is worthy of investigation.

The purpose of this study was to assess the acute toxicity of potassium ferrate(VI) to free-living *I. multifiliis* theronts and to examine the efficacy of this chemical against *I. multifiliis* infestations in goldfish in aerated tap water. The present study also evaluated the acute toxicity of potassium ferrate(VI) to goldfish. Such information will be useful in formulating safe treatment rates for ornamental and food fish.

2. Materials and methods

2.1. Fish

Goldfish (*Carassius auratus*), weighting 4.27 ± 0.72 g, were utilized throughout the study. All fish, referred to as "naïve fish", were kept in several 300 L opaque tanks and supplied with a constant flow of aerated tap water (flow rate 1.0–1.5 L min^{-1}), at 22.0 ± 2 °C, pH 7.0 ± 0.3, with dissolved oxygen 6.0–7.8 mg/L, ammonia content (total nitrogen) 0.5–2.0 mg/L and total hardness ($CaCO_3$) 85.0–104.5 mg/L. They were fed once at 1% body weight daily with commercial fish pellet feed, produced by the Institute of Hydrobiology, Chinese Academy of Sciences.

2.2. Parasite

A local strain of *I. multifiliis* was isolated from goldfish, obtained from a pet shop and its passage was as Ling et al. (2009) described. The fish were held at 22 ± 2 °C in a static 40 L aquarium equipped with an outside biological filter and air stones to maintain enough dissolved oxygen (greater than 5 mg/L). *I. multifiliis* was collected using a method described by Clayton and Price (1988). Several heavily infected fish were placed into 300 mL of filtered aquarium water for 30 min. Mature trophonts were allowed to dislodge from the host by body movements of the fish while in close proximity. The cysts thus obtained were incubated at 23.5 ± 0.5 °C for 18–20 h, and theronts were allowed to emerge naturally. The infectious theronts were used to determine the acute toxicity of potassium ferrate(VI) to *I. multifiliis* and to challenge fish during experiments. Theront concentrations were estimated by pipetting 2-μL droplets of the theront suspension onto a glass slide and counting the organisms (40× magnification). The final concentration was extrapolated using a mean of 10 droplets from the theront suspension (Schlenk et al., 1998; Straus and Griffin, 2001).

2.3. Potassium ferrate(VI)

The potassium ferrate(VI) used in this study was supplied by the Xi'an Tian Shun Fine Chemical Plant, and the preparation of this reagent was followed by the wet oxidation method described by Jiang and Lloyd (2002). In order to obtain an accurate dosage for treatment, the concentration of potassium ferrate(VI) was measured using both chromate titration and spectroscopy methods (Jia et al., 1999; Jiang and Lloyd, 2002). This reagent containing 96% of potassium ferrate(VI) was used throughout this study.

2.4. Acute toxicity of potassium ferrate(VI) to I. multifiliis theronts

An in vitro study was designed to determine the acute toxicity of potassium ferrate(VI) to *I. multifiliis* theronts according to an immobilization method (Sin et al., 1991; Ling et al., 1993; Straus and Griffin, 2001; Buchmann et al., 2003). The theronts were placed into 96-well microtiter plates at a final concentration of 500 theronts per well with 100 μL of solution and exposed to concentrations of potassium ferrate(VI) at 0, 0.096, 0.96, 1.92, 4.80, 9.60, 14.40, 19.20, 24.00, and 48.00 mg/L, respectively. Acute toxicity was assessed directly by dissection microscopic examination (16–40× magnification) of each well at various intervals up to 4 h after treatment. The theront cells with the absence of motility and abnormal morphology were considered dead. The experiment was conducted at 23.5 ± 0.5 °C, and replicated three times using separate populations of theronts for each potassium ferrate(VI) concentration.

2.5. Determination of infective dosage

In order to achieve consistent infestation of goldfish, an experiment was conducted to determine the appropriate number of infective theronts. Sixty healthy goldfish were divided into six groups ($N = 10$), and exposed in opaque beakers to 0, 1000, 2000, 4000, 8000, and 16,000 theronts per fish, respectively. The infection protocol was referred to Ling et al. (2009). For each group, theronts were placed into an opaque 2 L beaker prior to infection and the goldfish were transferred into the beaker at a density of one fish per 100 mL of aerated tap water. After 30 min, during which time infection occurred (McCallum, 1982), all the contents of each beaker were respectively placed into six static 20 L aquaria, equipped with air stones and in which the fish had been previously acclimated for at least 1 week. The half of each aquarium water was renewed on alternate days with aerated tap water. The experiment was

Table 1
Effect of theront concentrations on the prevalence of ichthyophthiriasis for goldfish ($N = 10$) following 3-day exposure.

Theront concentration (number/fish)	Prevalence of ichthyophthiriasis[a] (%)
0	0
1000	20
2000	80
4000	100
8000	100
16,000	100

[a] Prevalence of ichthyophthiriasis: no. infected fish/total no. fish.

terminated on the third day following exposure to theronts. During each exposure, fish were examined daily for the presence of trophonts. All infections were carried out at 22.0 ± 2 °C. Table 1 shows that a theront concentration of 4000 per fish led to 100% prevalence of infestation.

2.6. Acute toxicity of potassium ferrate to goldfish

Aqueous static renewal 96-h bioassays were conducted to determine the acute toxicity of potassium ferrate(VI) to goldfish. Goldfish were placed into several 20 L aquaria (10 fish/aquarium), and potassium ferrate(VI) concentrations for goldfish aqueous exposures were 19.20, 33.60, 48.00, 62.40 and 76.80 mg/L (a preliminary study was performed to establish a mortality range of 0–100%). The test included a control without potassium ferrate(VI). The total water of each aquarium was renewed every 24-h with fresh potassium ferrate(VI) solutions or aerated tap water, and water quality parameters (dissolved oxygen, pH, and temperature) were measured in all aquaria before the media were changed (DeLorenzo et al., 2006). Mortality observations were taken from each aquarium for everyday. The fish were not fed during the exposure (Buikema et al., 1980; DeLorenzo et al., 2006).

2.7. Prevention of the infestation of I. multifiliis

An experiment was adapted from the method of Ling et al. (1993) and Straus and Griffin (2001) to estimate the effective concentrations used to prevent infestation with *I. multifiliis* theronts. This experiment consisted of three replications with 10 goldfish per aquarium. In each replicate, the dose of theronts (4000 theronts per fish) and 50 goldfish were added into five 2 L beakers containing 1000 mL aerated tap water for 30 min, respectively; then they were transferred into five static 20 L aquaria containing different concentrations of potassium ferrate(VI) (0, 1.92, 4.80, 9.60 and 19.20 mg/L). Each aquarium was equipped with air stones and in which the fish had been previously acclimated for at least 1 week, and the total water of each aquarium was renewed on alternate days with aerated tap water. Daily observation was recorded for the presence of trophonts, and on the third day following exposure to theronts the numbers of trophonts on the fish fins were scored under a dissection microscope.

2.8. Statistic analysis

All data in this study were analyzed by version 13.0 of Statistical Product and Service Solutions (SPSS). The LC_{50} with 95% confidence intervals (CI) was determined using the probit procedure of SPSS. The S–N–K (Student–Newman–Keul's test) procedure for multiple comparisons was used to determine significantly different prevalences of ichthyophthiriasis and infection levels on third day after exposure to *I. multifiliis* theronts ($\alpha = 0.05$). Because the data were not distributed normally, a natural logarithmic transformation was carried out.

3. Results

Table 2 shows the results of an in vitro study on the acute toxicity of potassium ferrate(VI) to *I. multifiliis* theronts. A concentration of 0.096 mg/L of potassium ferrate(VI) did not kill *I. multifiliis* theronts during the 4 h exposure period. Exposure of *I. multifiliis* theronts to 4.80 mg/L or more concentrations of potassium ferrate(VI) resulted in 100% mortality by 4 h. The 4-h LC_{50} value was estimated to be 1.71 mg/L (95%CI 1.33–2.49). It was observed that many theronts in concentrations of 4.8 mg/L or more assumed a spherical shape and lost motility by 1 h. Although some theronts at concentrations of 1.92 and 0.96 mg/L also had a spherical appearance by 1 h, they were able to move, albeit more slowly than

Table 2
Effect of potassium ferrate(VI) on mortality of *Ichthyophthirius multifiliis* theronts in vitro. Number of dead theronts was expressed as mean ± S.D. of three replicates.

Final concentration (mg/L)	Percent mortality (no. dead theronts)			
	30 min	1 h	2 h	4 h
Control	0 (0)	0 (0)	0 (0)	0 (0)
0.096	0 (0)	0 (0)	0 (0)	0 (0)
0.96	0 (0)	0 (0)	14 (72.0 ± 7.5)	25 (125.3 ± 11.2)
1.92	0 (0)	20 (98.0 ± 10.5)	24 (119.0 ± 10.5)	56 (282.3 ± 14.0)
4.80	38 (191.7 ± 8.6)	58 (291.3 ± 20.0)	100 (–)	100 (–)
9.60	50 (250.7 ± 19.0)	64 (322.3 ± 15.0)	100 (–)	100 (–)
14.40	59 (296.7 ± 19.0)	100 (–)	100 (–)	100 (–)
19.20	69 (344.3 ± 25.4)	100 (–)	100 (–)	100 (–)
24.00	100 (–)	100 (–)	100 (–)	100 (–)
48.00	100 (–)	100 (–)	100 (–)	100 (–)

–: No live theront was found.

Table 3
Acute toxicity of potassium ferrate(VI) to goldfish in aqueous static renewal 96-h bioassays.

Concentration (mg/L)	Total no. tested	No. dead				Survival (%)
		24 h	48 h	72 h	96 h	
Control	10	0	0	0	0	100
19.20	10	0	0	0	0	100
33.60	10	2	1	0	0	70
48.00	10	4	2	1	0	30
62.40	10	7	2	0	0	10
76.80	10	10	0	0	0	0

Table 4
Prevalence of ichthyophthiriasis and mean number of trophonts on the fins per infected goldfish on day 3.

Concentration (mg/L)	Prevalence of ichthyophthiriasis (%)	Mean number of trophont per infected fish (no. infected)
Control	100.00 ± 0^{a}	7.77 ± 2.88^{a} (30)
1.92	90.00 ± 10.00^{a}	2.18 ± 1.04^{b} (27)
4.80	43.33 ± 5.77^{b}	1.53 ± 0.66^{b} (13)
9.60	23.33 ± 5.77^{c}	1.29 ± 0.49^{b} (7)
19.20	0 ± 0	0 (0)

Each value was expressed as mean ± S.D. of three replicates, and within a column, the values followed by the different lower case letter were significantly different ($P < 0.05$).

controls. In the wells containing 0.096 mg/L concentration of potassium ferrate(VI), no abnormal morphology or loss of motility were seen after 4 h exposure.

Aqueous static renewal 96-h bioassays demonstrated that the 96-h LC_{50} value for potassium ferrate(VI) in goldfish was 42.51 mg/L (95% CI of 35.13–49.39 mg/L). Table 3 shows that no mortality occurred at a concentration of 19.20 mg/L during the 96-h study period, and most fish (at least >57%) died at concentrations >19.20 mg/L after 24 h exposure.

In the experiment of efficacy of potassium ferrate(VI) in preventing *I. multifiliis* infestation in goldfish, potassium ferrate(VI) concentrations of 4.8 mg/L or more were found to be effective. Table 4 shows that fish treated at the 19.20 mg/L concentration were free of trophonts on day 3, whereas trophonts were observed on all fish fins in the untreated control. Fish treated at the 1.92, 4.80 and 9.60 mg/L concentrations carried significantly fewer parasites than the control ($P < 0.05$) after 3-day exposure of *I. multifiliis*; and there was a significant difference in the prevalence of ichthyophthiriasis between fish treated at 4.80 mg/L concentrations and fish treated at 1.92 mg/L and the control ($P < 0.05$) (Table 4).

4. Discussion

Potassium ferrate(VI), a powerful oxidizing agent, has shown great promise as multi-function wastewater and drinking water treatment chemical for disinfection, oxidation, purification, and coagulation (Sharma, 2002). Such water treatment reagents are able to not only disinfect microorganisms, partially degrade and oxidize the organic and inorganic impurities, but also remove colloidal/suspended particulate materials and heavy metals (Jiang and Lloyd, 2002). Besides, this chemical offers significant advantages in terms of a more simplified and cheaper process, for example, use of a single chemical, single dosing and mixing system, lower equivalent chemical cost and less sludge production, and of avoiding the formation of reaction by-products of toxicological concern (Jiang and Lloyd, 2002).

Murmann and Robinson (1974) reported that ferrate(VI) was very effective in inactivating two pure laboratory cultures of bacteria. Later, Gilbert et al. (1976) and Waite (1979a,b) found that ferrate(VI) could be used as a disinfectant to kill indicator organisms and known pathogens in the Enterobacteriaceae family at the concentrations of 5×10^{-5} M. Recently, Jiang et al. (2006) showed that ferrate(VI) was able to achieve the disinfection targets (>6 $\log_{10}$ inactivation of *Escherichia coliform* (*E. coli*)) at a very low dose (6 mg/L as Fe). Additionally, some researchers have carried out studies on the ability of ferrate(VI) to inactivate viruses (Schink and Waite, 1980; Kazama, 1994, 1995). At present, though some investigators are studying the capability of ferrate(VI) to kill *Cryptosporidium* and *Giardia* (Jiang and Lloyd, 2002), no information on the effectiveness of ferrate(VI) as a potential parasiticide is available.

The disease ichthyophthiriasis, caused by an important external protozoan parasite, *I. multifiliis*, probably accounts for more damage to freshwater fish populations worldwide than any other eukaryote pathogen (Hines and Spira, 1973; Rogers and Gaines, 1975; Matthews, 2005). Straus and Griffin (2001) considered that killing infective theronts would prevent spread of the disease to other fish. It is therefore necessary to access the acute toxicity of potassium ferrate(VI) to *I. multifiliis* theronts. The present study shows that potassium ferrate(VI) at a concentration of 4.80 mg/L killed 100% *I. multifiliis* theronts by 4 h. In addition, in the 96-h bioassays of this study, no mortality and no changes in swimming activity was encountered for 96-h treatment with 19.20 mg/L concentration of potassium ferrate(VI). Potassium ferrate(VI) at a concentration of 4.80 mg/L is regarded as a safe and effective dosage for goldfish to prevent *I. multifiliis* theronts initial infection.

Also, our results demonstrate that a concentration of 4.80 mg/L concentration of potassium ferrate(VI) significantly reduced the prevalence of ichthyophthiriasis and the number of trophonts on fish fins on day 3, though this chemical was not present during initial infection (30 min). It is possible that this concentration of potassium ferrate(VI) reflects on *I. multifiliis* theronts cyclical reinfection, or directly acts on encysted trophonts and prevents the further development of *I. multifiliis* in goldfish. This founding indicates that this chemical is potentially effective against the feeding stages of *I. multifiliis* once they are associated with fish. However, the mechanism on efficacy of potassium ferrate(VI) in the treatment of the disease need to further study.

Several factors will strongly influence the effectiveness of potassium ferrate(VI) in the field. The decomposition rate of potassium ferrate(VI) highly depends on the pH of the aquatic environment. Generally, with reducing pH values, the stability of potassium ferrate(VI) reduces (Wanger et al., 1952). For example, 49% of potassium

ferrate(VI) prepared with a buffer solution at pH 7 remained after 8 h, while 71.4% of that at pH 8 remained after 10 h (Schreyer and Ockerman, 1951). Temperature should be considered in the applications, because temperature in the field also influences the decomposition rate of ferrate(VI) solution (Wanger et al., 1952; Johnson and Sharma, 1999). For a given 2-h test period, potassium ferrate(VI) solution (initial concentration: 0.01 M) was reduced by 10% at 25 °C and almost unchanged at 0.5 °C (Wanger et al., 1952). In addition, coexisting ions and easily oxidizable substances may influence effectiveness of potassium ferrate(VI) (Johnson and Sharma, 1999). Future studies will investigate the effect of different water quality parameters on preventing *I. multifiliis* theronts infestation with potassium ferrate(VI).

In summary, the results demonstrated that exposure of *I. multifiliis* theronts to 4.80 mg/L or more concentrations of potassium ferrate(VI) led to 100% mortality by 4 h; the 4-h LC_{50} value was calculated to be 1.71 mg/L (95% CI 1.33–2.49). The data of 96-h bioassays suggested that the LC_{50} value for potassium ferrate(VI) in goldfish was 42.51 mg/L. Additionally, the study validated that potassium ferrate(VI) at the concentrations of 4.80 mg/L or more appeared to be effective for treating *I. multifiliis* infestations in goldfish within aerated tap water.

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