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PHARMACOKINETIC REPORT

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Bath immersion pharmacokinetics of florfenicol in Nile tilapia (*Oreochromis niloticus*)

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Abstract

Drug administration by immersion can be a preferable method in certain conditions especially for treating small-sized, anorexic, or valuable fish. Pharmacokinetic information regarding bath treatment is considerably lacking in comparison to other common administration routes. The current study aimed to investigate if immersion can be an effective route to administer florfenicol (FF) for treatment in Nile tilapia. Nile tilapia reared at 28°C were immersed with FF solution at concentrations of 50, 100, 200, 500, and 500/200 (3 hr/117 hr) ppm for 120 hr and moved to drug-free freshwater for another 24 hr. The serum FF concentration in 100, 200, and 500/200 ppm groups reached steady-state at 12 hr with concentrations of 2.44, 3.04, and 5.26 μg/ml, respectively, which were about 2% of the bathing concentrations. The target therapeutic levels of 1–4 μg/ml were attained and maintained within 1–12 hr, depending on the immersion concentration and the target MIC. Serum FF reached the target with shorter time at higher bathing concentration. Following the 120-hr bath, the serum FF declined with the first-order half-life of approximately 10 hr. A minimum of 100 ppm FF is required for treatment purpose, and an initial high loading concentration followed by maintenance concentration is a plausible way to reach in vivo therapeutic level in short time. Greater than 99% of the residual FF in the bathing water could be removed within 15 min by 0.05% NaOCl. Our results indicated that bath immersion is a promising potential route for FF administration in Nile tilapia.

KEYWORDS

fish, florfenicol, immersion, pharmacokinetics, tilapia

1 | INTRODUCTION

Treating bacterial diseases in fish with antimicrobial agents can be performed by one of the three major methods, namely oral administration (gavage or medicated feed), injection (intraperitoneally or intramuscularly), and immersion (Noga, 2010; Treves-Brown, 2000). Each method has its own merit and limitations. Selection of the most appropriate method is based on several factors such as the value of the individual fish, the number of fish to be treated, the severity of the disease, and the rearing environment. Medicated feed is usually the only practical way to treat a whole fish population in commercial aquaculture farms provided that the majority of the fish is still feeding. Although least stressful, in-feed medication is inadvisable when the sick fish become anorexic and the actual dose intake could not be ascertained. Injection and oral gavage are commonly used to treat high-value fish (such as broodstock or valuable ornamental fish) or used in experimental works as they can guarantee the accurate dosing. However, injection and oral gavage methods are stressful to the fish, labor-intensive, require technical skill and knowledge of fish anatomy, and inapplicable for the small-sized fish. Bath treatment, on the other hand, is relatively easy to perform and applicable for small-sized fish and

anorexic fish (Francis-Floyd, 1996; Yanong, 2016). However, except for treating ectoparasites, treating systemic bacterial infection by immersion is not common because information regarding whether the blood concentration of the drug could reach therapeutic level has rarely been reported and improper treatment could induce bacterial resistance quickly. Furthermore, environmental pollution from the discharged water after the treatment is another concern unless the waste management practice is adequately implemented.

When antibacterial drugs are applied as bath treatment, it is generally advisable that they should be used daily for 5–7 days (Mashima & Lewbart, 2000). The review of antibacterial immersion and their recommended concentrations can be found in the literature (Mashima & Lewbart, 2000; Noga, 2010; Reimschuessel, Miller, & Gieseker, 2013; Wall & Wildgoose, 2005). The bathing concentrations are usually less than 50 ppm, but the concentrations of 100 ppm or higher are not uncommon for some drugs such as oxytetracycline (up to 100 ppm bath for 1–3 days), oxolinic acid (up to 200 ppm bath for 1–72 hr), flumequine (up to 500 ppm for 1–72 hr), and sulfadimidine + trimethoprim (500 + 100 ppm for 72 hr) (Mashima & Lewbart, 2000; Noga, 2010; Reimschuessel et al., 2013; Wall & Wildgoose, 2005). However, these recommended concentrations are merely general guidelines to begin with and may not be optimal for every fish species and every rearing conditions.

Florfenicol (FF) is one of the most commonly used antimicrobial drugs for ornamental fish and food fish with the recommended oral dosage of 10–15 mg/kg body weight/day for 10 days (U.S. FDA, 2020). Currently, there is no published data or recommended dose of FF as a bath therapy, probably due to the lack of evidence proving the effectiveness of this administration route. The current study aimed to investigate serum concentration and pharmacokinetic (PK) characteristics of FF in Nile tilapia (*Oreochromis niloticus*) following immersion treatment at different concentrations in order to assess the potential application of this method. Nile tilapia was used as the representative of ornamental cichlids due to its lower cost and the data availability of antibacterial PK which is usually lacking for other cichlid species.

2 | MATERIALS AND METHODS

2.1 | Chemicals

FF standard was purchased from Sigma-Aldrich. FF injectable solution (Fulicone®300, containing 300 mg/ml FF) was purchased from San Heh Pharmaceutical Corporation, Taiwan. Acetonitrile (HPLC grade) and *N*,*N*-dimethylformamide were purchased from Avantor Performance Materials. Propylene glycol was purchased from AppliChem GmbH. Sodium dihydrogen phosphate anhydrous (NaH_2PO_4) and disodium hydrogen phosphate anhydrous (Na_2HPO_4) were purchased from Panreac Química SLU. All chemicals used were of analytical grade.

2.2 | Experimental fish

A total of 16 clinically healthy Nile tilapia (*Oreochromis niloticus*) weighing between 600 and 800 g from the Gao Zheng farm, Chiayi County, Taiwan, were reared in an outdoor concrete pond at the College of Veterinary Medicine, National Chung Hsing University, Taiwan. The animal husbandry and rearing condition were essentially the same as reported in the previous publication (Rairat, Hsieh, Thongpiam, Sung, & Chou, 2019). Each individual tilapia was reared in a 70-L tank with adequate aeration to maintain optimal level of dissolved oxygen at all time. Water temperature, dissolved oxygen, and pH were 28°C, ≥5.0 mg/L, and 7.5–8.0, respectively. The animal study was approved by the Institutional Animal Care and Use Committee of National Chung Hsing University (IACUC approval No.: 107-147).

2.3 | Experimental design

In Experiment 1 (dose exploration study), two tilapia were assigned to 50 ppm group and another 2 fish were assigned to 500 ppm group. Each individual fish was acclimatized in a 70-L tank containing 60 L-freshwater at 28°C for 5–6 days before drug administration. Fish in the 50 and 500 ppm groups were immersed with FF injectable solution (Fulicone®300) at the final concentration of 50 and 500 ppm for 120 hr, without water change. It should be noted that our preliminary study indicated that FF was stable for at least 120 hr in the rearing water under the experimental condition. Following the 120 hr immersion, they were then transferred into drug-free freshwater tanks for another 24 hr to study the drug depletion kinetics. In Experiment 2 (dose confirmation study), 12 tilapia were randomly distributed into one of the three treatment groups: 100 ppm, 200 ppm, and 500/200 ppm (n = 4 for each group). The fish in the 500/200 ppm group were immersed in the 500 ppm FF for the first 3 hr before transferred to the 200 ppm tanks for another 117 hr. After drug bath for 120 hr, all tilapia were moved into drug-free freshwater for another 24 hr as in Experiment 1.

The blood samples were collected at 1, 3, 6, 12, 24, 48, 72, 96, 120, 126, 132, and 144 hr after the initiation of bath treatment. For the fish in the 500/200 ppm group, the time points were at 1, 2, 3, 6, 12, 24, 48, 84, 120, 126, 132, and 144 hr. The procedure of blood collection, sample preparation, and determination of FF concentration by HPLC-UV method was the same as described previously (Rairat et al., 2019). Briefly, 0.40–0.45 ml blood was collected from a caudal vessel, allowed to clot, and centrifuged at 3,500 rpm (2,191 × *g*; KN-70, Kubota, Japan) for 10 min. The supernatant (serum) were collected and kept at −20°C until analysis. The serum samples (200 μl) were extracted twice with 400 μl ethyl acetate and centrifuged at 3,500 rpm (2,191 × *g*) for 10 min. The ethyl acetate supernatants were evaporated to dryness. The residues were reconstituted with 200 μl mobile phase and filtered through 0.2-μm-nylon syringe filter before injected (50 μl) into the HPLC system. The HPLC system

TABLE 1 Pharmacokinetic behavior of florfenicol in Nile tilapia following 120-hr bath administration at 28°C (n = 4)

Pharmacokinetic behavior	100 ppm	200 ppm	500/200 ppm
Time to steady-state (h)	12	12	12
Time to reach 1 μg/ml (h)	6	3	1
Time to reach 2 μg/ml (h)	12	6	2
Time to reach 4 μg/ml (h)	Never	Never	6
C_{ss} (μg/ml)	2.44 ± 0.28[a]	3.04 ± 0.31[a]	5.26 ± 1.34[b]
AUC (h μg/ml)	344.2 ± 108.5[a]	388.8 ± 88.1[a]	715.5 ± 238.2[b]
λ (h^{-1})	0.055 ± 0.005[a]	0.073 ± 0.018[a]	0.065 ± 0.019[a]
$t_{1/2\,\lambda}$ (h)	12.61 ± 1.11[a]	9.96 ± 2.80[a]	11.19 ± 2.79[a]
MRT (h)	76.63 ± 2.00[a]	71.37 ± 4.24[a]	75.10 ± 3.57[a]
Ratio of C_{ss}/water concentration (%)	2.4	1.5	N/A

Note: The data were presented as mean ± *SD*.

Means with different superscripts in each row were significantly different from each other ($p < .05$).

Abbreviations: AUC, area under the serum concentration-time curve from time zero to infinity; C_{ss}, steady-state serum concentrations; MRT, mean residence time; N/A, not applicable; $t_{1/2\,\lambda}$, terminal half-life; λ, terminal rate constant.

consisted of a pump (Waters 1525, Waters), UV-visible detector (Waters 2,489, Waters), and C-18 column with 5 μm particle size, 150 × 4.6 mm (Apollo, Hichrom). The mobile phase was a mixture of acetonitrile and phosphate buffer (10 mM NaH_2PO_4-Na_2HPO_4, pH 5) at 30:70 v/v. The flow rate was 1 ml/min, and the detection wavelength was 224 nm.

2.4 | Pharmacokinetic analysis

The PK parameters, including terminal rate constant (λ), terminal half-life ($t_{1/2\,\lambda}$), area under the serum concentration-time curve from time zero to infinity (AUC), and mean residence time (MRT) were analyzed by the noncompartmental model using PKSolver 2.0 software (China Pharmaceutical University) (Zhang, Huo, Zhou, & Xie, 2010). The differences in PK parameters among different treatment groups were analyzed by Kruskal–Wallis test. Statistical analysis was performed using IBM SPSS Statistics version 22 (IBM Corporation).

2.5 | Drug degradation kinetic study

To alleviate any negative effects related to the discharge of FF-containing water into the environment, degradation kinetics of FF by sodium hypochlorite (5% NaOCl, Clorox Bleach, The Clorox Company) was investigated. Either 0.5 or 0.25% NaOCl was mixed with 100 and 500 ppm FF-containing water at the ratio of 1:9 v/v at room temperature to attain the final concentrations of 0.05 and 0.025% NaOCl, respectively. Then, the FF concentration was quantitated at 1, 15, 30, and 60 min post-treatment by HPLC-UV method. The experiment was performed in quadruplicate.

3 | RESULTS AND DISCUSSION

In Experiment 1, following the 50 ppm immersion, the FF serum concentration reached steady-state at 12 hr. The steady-state serum concentration (C_{ss}), calculated by averaging the serum concentrations from 12 hr to 120 hr, was 1.15 μg/ml (data not shown). The fish that bathed in 500 ppm FF have the average serum concentration of 2 μg/ml at 3 hr and 12 μg/ml at 24 hr (data not shown), which was considered unnecessarily high for treatment purpose (see discussion below) and may even toxic to the fish. Therefore, the 500 ppm bath experiment was canceled before completion. In both groups, there was no significant degradation of FF in the bathing water during the 120-hr immersion period.

Based on the antimicrobial susceptibility data of FF reported by epidemiological studies (with n = 74 to 100 bacterial isolates), the minimum inhibitory concentration (MIC) required to inhibit 90% of the pathogenic bacteria isolates (MIC_{90}) for tilapia are usually in the range of 1 to 4 μg/ml (Godoy et al., 2008; Lukkana, Jantrakajorn, & Wongtavatchai, 2016; De Oliveira, Queiroz, Teixeira, Figueiredo, & Leal, 2018). Therefore, the steady-state serum concentration following 50 ppm immersion (1.15 μg/ml) appeared suboptimal for most bacteria, whereas the serum concentration of the 500 ppm group (at least 12 μg/ml) can be considered an overdose. Consequently, the immersion concentrations between 50 and 500 ppm were arbitrarily selected for Experiment 2. In Experiment 2, after immersing the fish in 100 ppm FF, the serum concentration above the bacterial MIC of 1 and 2 μg/ml was attained at 6 hr (1.16 μg/ml) and 12 hr (2.07 μg/ml), respectively. In the 200 ppm group, similar concentrations were reached at 3 hr (1.07 μg/ml) and 6 hr (2.23 μg/ml), respectively (Table 1 and Figure 1). Unfortunately, the target concentration of 4 μg/ml has never been achieved in these two groups. To improve drug absorption, the loading concentration of 500 ppm bath for the first 3 hr, followed by immersion with 200 ppm for another 117 hr,

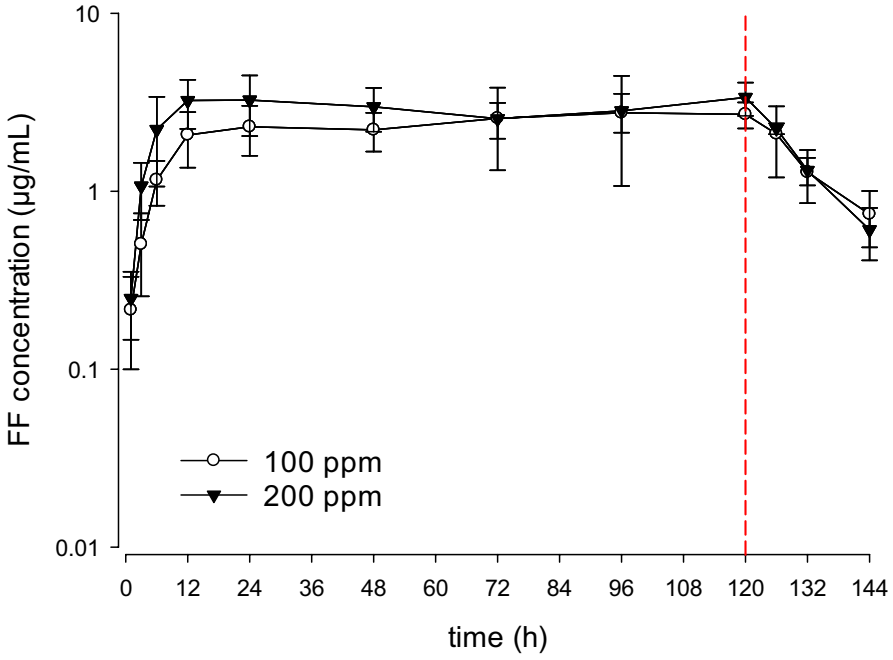

FIGURE 1 Serum concentration-time profile (mean ± *SD*) of florfenicol in Nile tilapia following 100 and 200 ppm florfenicol bath for 120 hr at 28°C

was employed. The serum concentrations reached the target MIC of 1, 2, and 4 µg/ml at 1 hr (1.31 µg/ml), 2 hr (2.86 µg/ml), and 6 hr (4.38 µg/ml), respectively (Table 1 and Figure 2), much faster than without the loading concentration. Comparing to the oral administration of the recommended dose (15 mg/kg) in Nile tilapia raised under a similar condition, the serum concentrations exceeded 4 µg/ml at 15 min (Rairat et al., 2019), much shorter than that of the immersion administration. Apparently, the drug absorption from the gastrointestinal tracts was more efficient than the gills, but the underlying mechanisms for this difference remained to be revealed. Nevertheless, applying the loading concentration of 500 ppm helps to accelerate the drug absorption through the gills significantly such that the time to reach the therapeutic target concentration would be in a reasonable period.

The C_{ss} and AUC of the 500/200 ppm group were also significantly higher than those without the loading concentration. After transferring the fish into drug-free tanks, regardless of the immersion concentration, the FF was eliminated by first-order kinetics with the $t_{1/2\lambda}$ of about 10 hr (Table 1) which was comparable to the results from the previous studies using IV and PO routes at 28°C (Rairat et al., 2019; Wang et al., 2010). The MRT following bath administration was in the range of 71–77 hr (Table 1), much longer than the IV (12 hr) and PO routes (13 hr) (Rairat et al., 2019) probably due to the long treatment period; but direct comparison of MRT among

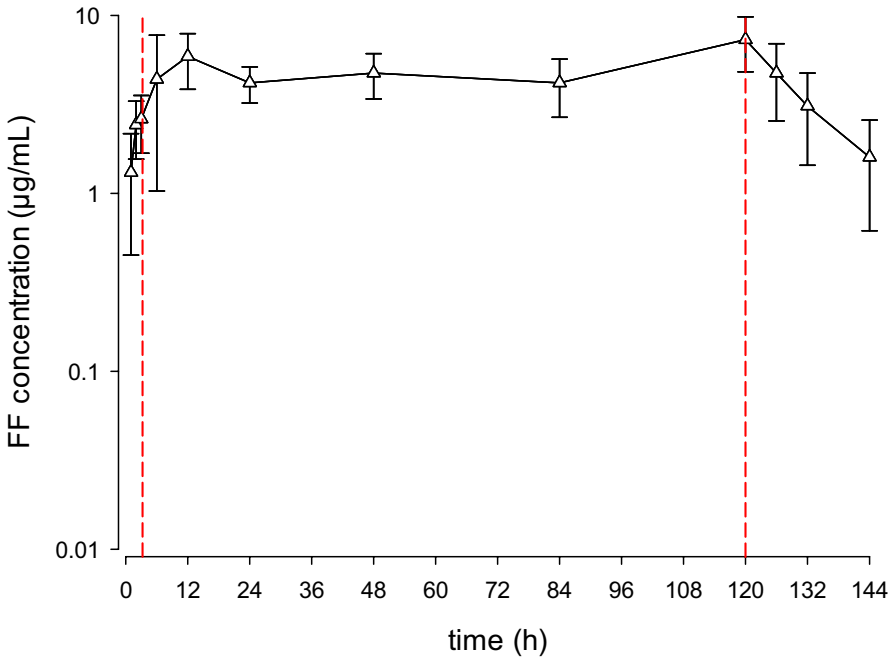

FIGURE 2 Serum concentration-time profile (mean ± *SD*) of florfenicol in Nile tilapia following 500 ppm (3 hr) and then 200 ppm (117 hr) florfenicol bath at 28°C

different route appeared problematic as the actual absorbed doses after immersion were unknown.

To the best of the authors' knowledge, serum concentrations after FF immersion treatment have been reported only in common carp (*Cyprinus carpio*) (Nejad, Peyghan, Varzi, & Shahriyari, 2017) and rainbow trout (*Oncorhynchus mykiss*) (Cobo Labarca et al., 2017). The serum FF concentrations in both studies were very low, being only 11 ng/ml (after 5 ppm bath for 10 days in the common carp) and undetectable (after 10 ppm bath for 1 hr in the rainbow trout). In the case of the rainbow trout, the low-frequency ultrasound pretreatment was attempted to increase drug absorption but without success (serum concentration only about 4 ng/ml). This information suggested that a higher immersion concentration may be required to attain the therapeutic serum concentration (about 1–4 μg/ml). The current study demonstrated that when bathing in higher FF concentration (at least 100 ppm), the target MIC of 1 μg/ml in the serum could be reached within 6 hr.

Because the exact dose uptaken by the individual fish (in "mg/kg body weight" unit) was unknown, the absolute bioavailability following bath treatment could not be pharmacokinetically calculated. Nevertheless, the extent of drug uptake by this route can be evaluated by comparing the serum concentration with the bathing water counterpart. It was revealed that at steady-state, the C_{ss} was only about 2% of the water concentrations across treatment groups (Table 1), indicating a low and likely first-order uptake kinetics. The first-order assumption was supported by the observation that higher bathing concentration resulted in higher C_{ss} and shorter time to reach the target MIC. Water-borne chemicals get into the fish body predominantly via the gills (Horsberg, 1994; Treves-Brown, 2000). For compounds with log $K_{o/w}$ value <1 including FF which has log $K_{o/w}$ of −0.04 (Switała et al., 2007), the rates of drug transport across the gill are likely low (Erickson & McKim, 1990) and limited by epithelial permeability rather than blood flow or water flow across the gill (Hayton, 1999). This might be the reason for such a low degree uptake of water-borne FF even though its physicochemical properties conform to the Lipinski's rule of five (Lipinski, Lombardo, Dominy, & Feeney, 2012). Other possible factors involved in the uptake outcome like gill metabolism or efflux pump should not be excluded but the exact mechanisms behind the gill uptake of FF are worth further investigation.

In addition to the route of administration, the extent of drug absorption also depends on drugs and fish species. Compared to oxytetracycline HCl (OTC) bath treatment at a similar concentration, FF showed greater uptake by the current study. For example, the serum concentrations after OTC immersion for rainbow trout (100 ppm for 1 hr) (Cobo Labarca et al., 2017), giant danio (*Devario aequipinnatus*) (100 ppm for 6 hr) (Vorbach, Chandasana, Derendorf, & Yanong, 2019), and gilthead seabream (*Sparus aurata*) (50 ppm for 24 hr) (Rigos, Nengas, & Alexis, 2006) were 0.050, 0.073, and 0.047 μg/ml, respectively, whereas the serum concentrations of FF in Nile tilapia after treating with similar concentrations and bathing duration were 0.22, 1.16, and 1.39 μg/ml, respectively. It should be pointed out that even the highest recommended concentration of OTC was applied (100 ppm bath) (Mashima & Lewbart, 2000; Noga, 2010; Wall & Wildgoose, 2005), the serum concentrations did not exceed 0.1 μg/ml (Cobo Labarca et al., 2017; Vorbach et al., 2019). Chelation between OTC and di-/trivalent cations in the water may be an explanation for the low OTC uptake via the gill (Rigos & Smith, 2015). This implies that the application of OTC as an immersion treatment for control of systemic bacterial infection is questionable. In contrast, the present study has proven that the therapeutic serum concentrations of FF were achievable after bath administration. Nevertheless, to the best of the authors' knowledge, drug efficacy studies (bacterial challenges) of FF bath

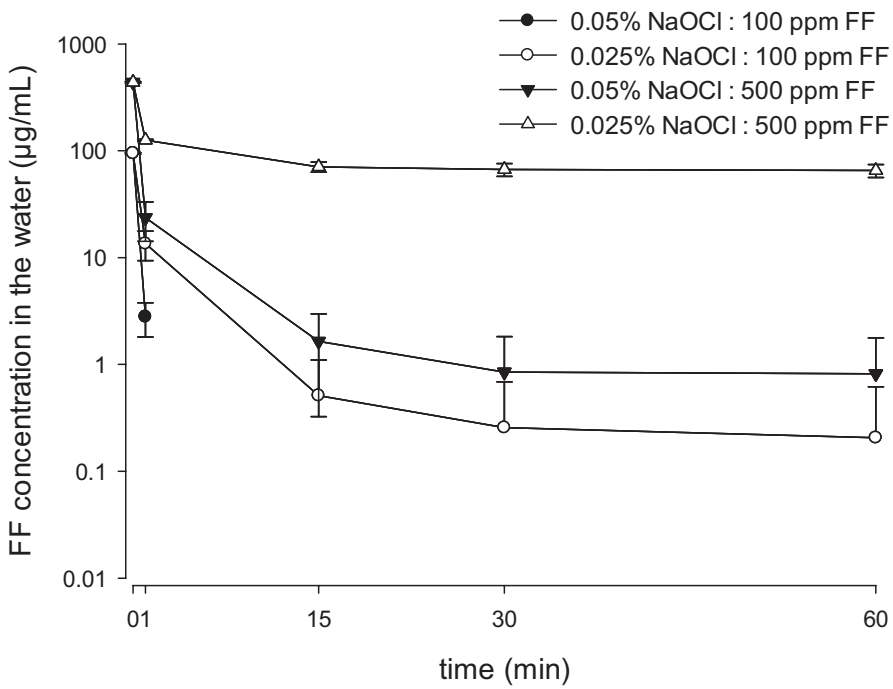

FIGURE 3 Degradation kinetics of florfenicol in water (mean ± *SD*) after treated with 0.05% and 0.025% NaOCl

treatment has not been reported so far and thus warrants further study.

In concern of environmental pollution from FF-containing discharged water, removal of FF in the water by oxidizing agents was evaluated. Our preliminary study revealed that at 60 min after mixing 0.5% NaOCl, 3% H_2O_2, and ClO_2 with 500 ppm FF in water at the ratio of 1:9 (v/v), the FF was degraded by >99%, 11%, and 10%, respectively (data not shown). Consequently, the NaOCl (household bleach), which degrades FF by chlorine oxidation (Zhang et al., 2016), was selected for the degradation kinetic study. The efficacy of NaOCl to remove water-borne FF was dependent on both NaOCl and FF concentrations (Figure 3). At FF concentration of 100 ppm, 0.05% NaOCl removed >97% FF within 1 min and the residual FF was no longer detectable since 15 min post-treatment. NaOCl at 0.025% was slightly inferior. Although it reduced FF by >99% at 15 min, the FF residue was still detectable at a low level (0.21 ppm) at 60 min. The similar results were observed when FF concentration of 500 ppm was treated with 0.05% NaOCl (but not 0.025% NaOCl) in which >99% FF could be degraded in 15 min and only 0.82 ppm remained at 60 min.

In summary, the present study demonstrated that following FF immersion at the concentrations of 100 ppm and up to 500 ppm, the therapeutic concentrations of FF in the tilapia's serum for the target MIC of 1 μg/ml could be reached within 1 to 6 hr and maintained until the end of the treatment. The residual FF in the bathing water could be removed by >99% within 15 min by 0.05% NaOCl. Therefore, FF immersion therapy was proven useful for the treatment of systemic bacterial infection in Nile tilapia and possibly other cichlids.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

C.C. Chou contributed to the conception of the study, study design, and critical review of the manuscript. Y.S.K. and C.C. Chang conducted the animal experiment and drug analysis by HPLC. T.R. involved in the study design, performed the data analysis, and prepared the draft manuscript. C.Y.H. involved in the study design and critical review of the manuscript. All authors have read and approved the manuscript.

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