Effects of Extended Feeding of Florfenicol Coated Medicated Diets on the Safety, Serum Biomarkers and Blood Cells Morphology of Nile Tilapia *Oreochromis Niloticus* (L.) Juveniles

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Abstract

Tilapia is one of the most consumed farmed fish, which requires the use of antibiotics in certain phases of its production. This study assessed the safety of 30 days of oral florfenicol (FFC)-dosing at 0-10 times the therapeutic dose (1X: 10 mg/kg biomass/day) in *Oreochromis niloticus* juveniles. Behavioural changes, feed consumption, mortality and biomass were evaluated. Besides, the levels of serum glucose, calcium, chloride, creatinine, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and blood cell morphology were determined at scheduled intervals. The 30 days of oral FFC-dosing caused 3.33% (1X) to 18.33% (10X) mortalities, reduced feed intake and biomass in a dose-dependent manner. The fish fed the therapeutic dose recorded 1.25 folds increase in biomass; while the control group recorded 1.45 folds increase in 30 days. No significant erythrocyte morphological alterations were observed in the 1X group compared to the control. However, marked morphological alterations like tear-shaped, spindle-shaped and degenerative erythrocytes in higher dosing groups indicated FFC cytotoxicity. All the serum biomarkers of *O. niloticus* increased significantly on day 10 and day 30 FFC-dosing in a dose-dependent manner, except for calcium and chloride, which reduced significantly during the dosing period. Within 2 weeks of suspension of FFC-dosing, the serum biomarker levels became normal except for alkaline phosphatase and creatinine. The recovery of biomass, feed intake, serum biomarker levels and erythrocyte morphological changes suggested that the FFC induced changes are reversible. This study has, thus, proclaimed the safety of FFC at the therapeutic dose in *O. niloticus*.

Introduction

Florfenicol (FFC) is a synthetic, broad-spectrum, fluorinated analogue of thiamphenicol with the same mechanism of action as chloramphenicol (Fukui et al. 1987). Studies have shown that its activity is quite high when compared to either chloramphenicol or thiamphenicol and more bactericidal (Gaikowski et al. 2003, 2013; Gaunt et al. 2010; Dowling 2013). In-vitro investigations with FFC have validated its potent activity against numerous fish pathogenic bacteria (Michel et al. 2003; Dowling 2013). Florfenicol is a relatively recent addition to aquaculture's approved antibiotic arsenal (USFWS 2015). Being highly lipophilic, it provides high enough concentrations to treat intracellular pathogens and cross some anatomic barriers. Florfenicol binds to the bacterial 50S ribosomal subunit and inhibits protein synthesis at the peptidyltransferase stage (Dowling 2013). The therapeutic dose of FFC is 10–15 mg/kg fish biomass/day administered as medicated feed for 10 consecutive days (USFWS 2015). In-vivo efficacy against furunculosis in *Salmo salar* and vibriosis in *Gadus morhua* have been demonstrated (Samuelsen and Bergh 2004; Higuera-Llantén et al. 2018). Florfenicol is well absorbed in all fish species tested and possesses bio-availabilities of 91 and 100% in *G. morhua* and *S. salar*, respectively (Horsberg et al. 1996; Samuelsen et al. 2003). Elimination of florfenicol from the plasma of *G. morhua* is slow compared to *S. salar* with a half-life of 43 h and 12–14 h, respectively (Samuelsen et al. 2003).

Tilapia is one of the most consumed farmed fish in the world and the most important farmed non-cyprinid fish globally, with a total production of 6.6 million tonnes in 2019. It has the potential to play a

leading role in the fight against food insecurity and malnutrition (FAO 2020). Nile tilapia *Oreochromis niloticus* contributes to 80% of the global tilapia production and has been the only cichlid species maintaining stable market prices across the globe (FAO 2020). The intensification of aquaculture has led to frequent disease outbreaks, which rely on antimicrobial therapeutics (Okocha et al. 2018). Target animal safety data are an indispensable part of the drug registration process. The recent years have seen a radical reduction in antibiotic use in some countries due to vaccination and improved husbandry practices, predominantly in Norway (Evensen 2016). Yet, antibacterial therapy remains the last stand to combat bacterial infections in aquaculture in many countries (Lulijwa et al. 2020). Florfenicol is one of the United States Food and Drug Administration (USFDA) approved and the most commonly used antibiotics in aquaculture (USFWS 2015; Evensen 2016). To curb illegal use, aquaculturally influential countries have formed safety authorities, viz., Norwegian Food Safety Authority (NFSA), EU Council Regulations (EC), USFDA and the European Medicines Agency (EMA) for proper antimicrobial monitoring, whose regulations and policies are backed up with stringent monitoring (Lulijwa et al. 2020). In intensive tilapia cultivation, the FFC has often been recommended as a therapeutic agent to control several diseases (Shiroma et al. 2020). The safety of Aquaflor®, a feed premix containing FFC at a maximum recommended dose of 15 mg/kg biomass/day in monosex *O. niloticus* (45.8 ± 10.5 g) for 20 consecutive days has been demonstrated (Gaikowski et al. 2013). Although reports are available on the use of FFC to control diseases in aquaculture (Gaikowski et al. 2003, 2013; Gaunt et al. 2010; Bowker et al. 2013), the effects of oral administration of pure FFC powder in the tropical condition is imprecisely studied. Also, safety studies are not available on the use of FFC for top-coating onto the basal feed for aquaculture. The surface coating of a drug with a palatable binder like vegetable oil increases feed consumption and its bioavailability (Ranjan et al. 2017). Since florfenicol and its residues are heat-labile (Filazi et al. 2015), top-coating is the most suitable way to medicate aquacultured fish (Ranjan et al. 2017). The present study, therefore, assessed the biosafety of top-coated FFC feeds in monosex Nile tilapia *O. niloticus* when fed at 0–10 times the lowest therapeutic dose of 10 mg/kg fish biomass/day for 30 consecutive days, i.e., 3 times the proposed 10-day dose duration.

Materials And Methods

Experimental fish and design

A single lot of healthy juvenile Nile tilapia *Oreochromis niloticus* (13.64 ± 0.52 g; 9.40 ± 0.37 cm) were procured from a grow-out farm located in Sonarpur (Lat 22°27'50.2158" N; Long 88°23'7.4004" E), West Bengal, India and transported to the laboratory in oxygen-filled bags. The fish were stocked in circular 500-L fibreglass reinforced plastic tanks at 50 numbers/tank and acclimated for 15 days. They were fed thrice daily at 2% of the bodyweight (BW) with a commercial pellet feed of 2.0 mm dia (CP Private Limited, India). The fish without any gross abnormalities from the acclimatized population were then collected and randomly allocated among 15 rectangular study tanks (L58 cm × H45 cm × W45 cm; Volume: 80 L) with 20 fish each before dosing (pre-dosing period: 7 days). The allotment was done in groups of 10, weighed and then transferred to the study tanks. This procedure was repeated twice until 20

fish were placed in each tank. The experimental fish were allotted into 5 groups, viz., group 1: 0X control, group 2: 1X FFC-diet (10 mg/kg fish biomass/day), group 3: 3X FFC-diet (30 mg), group 4: 5X FFC-diet (50 mg) and group 5: 10X FFC-diet (100 mg) in triplicate. About 50% of the water was replaced thrice weekly to avoid the accumulation of waste and excretory produces. The water quality parameters, viz., water temperature: 25.17–29.17°C; pH: 7.30–7.97; dissolved oxygen: 5.24-6.00 mg/L; nitrite: 0.23–0.65 mg/L and nitrate: 0.25–0.61 mg/L maintained optimally during the experimentation period. Water chemistry (temperature and pH) was measured daily concurrent with feeding. Dissolved oxygen, nitrite and nitrate were measured twice weekly as per APHA/AWWA/WEF (2017).

Florfenicol-diets preparation

The medicated FFC-diets were freshly prepared one week before the dosing period. The inclusion rate for the florfenicol (Tokyo Chemical Industry, CAS RN: 7321-34-2; Product Number: F0811-5g) was calculated to deliver an approximate dosage of 0-100 mg active ingredient/kg fish biomass/day for 30 consecutive days. The required amount of FFC was mixed with vegetable oil (5 mL/kg basal feed) and this emulsion was then used for top-coating as per de Oliveira et al. (2018). The top coated FFC-diets were prepared by vigorously mixing the required amounts of antimicrobial emulsion in feed to avoid antimicrobial loss by hydrosolubilization during feeding. Medicated diets were prepared in order of increasing FFC concentration. The feeds were air-dried overnight, stored in plastic sealable containers and kept in a cool place away from light. In the control feed (non-medicated feed) only 5 mL vegetable oil was added and vigorously mixed with the feed. Feeding rates and methods were identical for the control and dosing groups during the trial. The experimental protocols were approved by the Indian Council of Agricultural Research, Government of India, New Delhi under the All India Network Project on Fish Health and fulfilled the ethical guidelines including adherence to the legal requirements of India (CPCSEA, 2021).

Dose administration

The 50-day study included 7-days acclimation (pre-dosing), 30-days FFC-dosing, and 13-days post-FFC-dosing periods. During the pre-dosing and post-dosing periods, the fish groups were fed with a control diet. During the dosing period, FFC-diets were administered to the respective groups. The control group was administered the control diet. The feed ration, 2.0% of the BW, was allocated into three equivalent portions and the feed consumption was determined daily. Feeds remaining in the tank 1 h after each feeding was siphoned from the tank into a pre-weighed container, dried overnight, pooled tank-wise on daily basis and weighed. The fish biomass from each tank was determined periodically, i.e., day 10, 20, 30 FFC-dosing and day 13 post-FFC-dosing) in groups of 10, indiscriminately netted out, and calculated the difference in weight gain. The observations on fish behavioural changes, external changes, feeding behaviour and mortality were recorded daily. The feed ration was adjusted with the biomass accrual and mortalities. Behavioural responses and external observations were noted daily throughout the experimental regimen. Behaviours like swimming to the surface during feeding, aggressive feeding behaviour and distribution throughout the water column were considered normal. External bodily changes like pigmentation and gross lesions were also observed during feeding and before tank cleaning. Fish

with abnormal external changes were taken out, anaesthetized, documented and returned to the respective tanks. Feeding activity was visually assessed thrice daily.

Blood sampling

The blood sampling was done on day 0, 10, 20 and 30 FFC-dosing, and day 13 post-FFC-dosing from each group. Before the blood collection, two fish from each tank of the respective groups were arbitrarily netted out and anaesthetized using clove oil (20 µL/L water). The blood was collected by caudal vein puncture (Roberts 2012) using a 2 mL sterile plastic syringe. Instantly, 2 drops of non-heparinized blood were taken on microscopic slides followed by blood smear preparation. The fish upon blood collection were released into the respective tanks. The non-heparinized blood in the syringe was allowed to clot by keeping the syringe in a slanting position and then incubated at 4°C overnight. The serum was collected by centrifugation at 1000×g for 15 min, transferred to Eppendorf tubes and stored at -20°C for further analysis.

Determination of serum biomarkers

The serum biomarkers of stress (glucose), liver function (alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP)), kidney function (creatinine) and ionic balance (calcium and chloride) were determined using commercial kits in a Photometer (Model: 5010 v5+, Robert Riele KG, Berlin) as shown Table 1.

Statistical analyses

The data were expressed as a mean ± standard deviation. One way ANOVA followed by Tukey HSD post-hoc for the comparison of means was carried out to know the significance of differences in each of the parameters among the treatments and each of the specific treatment among the days using Statistical Package for Social Sciences (IBM-SPSS) Version: 22.0, considering a probability level of $P < 0.05$.

Results

Feeding behaviour

Belligerent feeding was observed throughout the experimental tenure in the control and 1X groups. The fish of 3X and 5X groups reduced their feeding aggressiveness with time. Increased rate of subdued behavioural responses including lounging at the tank bottom, no curiosity in feeding were noted in the 10X group. The data on feed intake during the oral FFC-dosing trial are presented in Table 2. A reduction of 1.29%, 4.25%, 13.07% and 22.88% feed intake during the 30 days of FFC-dosing was observed in the 1X, 3X, 5X and 10X groups, respectively compared to control. The decreased feed intake was also seen during the early post-FFC-dosing period. The feed intake in all the treatment groups during the FFC-dosing and post-FFC-dosing periods differed significantly ($P < 0.05$). Another noteworthy observation among the treatment groups was the increased faecal output in the 5X and 10X groups, which tend to excrete more

during the dosing period. However, no colour changes in the excreta or intestine during necropsy were observed.

Mortalities

There were no mortalities during the pre-dosing period and post-dosing period. At the end of the 30-day FFC-dosing regimen, 3.33%, 6.67%, 10.00% and 18.33% mortalities were noted in the 1X, 3X, 5X and 10X groups, respectively (Fig. 1a). The differences in mortalities between 1X and 3X groups were insignificant ($P > 0.05$). The mortalities recorded in the 10X group were high and differed significantly with 1X and 3X groups ($P < 0.05$). Significant differences existed in the mortalities among the day 10 and 30 FFC-dosing, and 13 post-FFC-dosing in the 10X group ($P < 0.05$).

Fold change in biomass

Fish of the control group showed a higher fold change in biomass than the treatment groups. The increase in biomass of the control group fluctuated between 1.22 and 1.49 folds with days of culture. The 1X group showed an increment in biomass of 1.11–1.31 folds of its original biomass. The 3X and 5X groups recorded a biomass hike of 1.129–1.289 folds and 1.126–1.286 folds, respectively. The 10X group recorded the least fold increase (1.109–1.190 folds) in biomass (Fig. 1b). The differences in biomass from day 0 to the end were 65.13 ± 23.24 g, 43.53 ± 13.37 g, 39.27 ± 15.84 g, 38.93 ± 12.73 g and 26.15 ± 1.62 g in the control, 1X, 3X, 5X and 10X groups, respectively. A significant difference existed in the biomass of the control and 10X groups ($P < 0.05$). The fold change in biomass of the 10X group on day 10 and 20 FFC-dosing, and day 13 post-FFC-dosing differed significantly ($P < 0.05$).

Abnormalities

During the feeding trial, *O. niloticus* juveniles were distributed throughout the water column of the tanks. Abnormal behaviour was not observed during the pre-dosing period. No gasping, loss of equilibrium and abnormal behaviour were observed during the dosing and post-FFC-dosing periods. Most of the fish of 5X and 10X groups exhibited dark opercular pigmentation (Figs. 2c and 2d) with hard intestine, enlarged spleen and liquefied kidney during the dosing regimen, which subsided during the post-FFC-dosing. Enlargement of the liver and gall bladder was also seen in some fish of 5X and 10X groups (Fig. 2e). Black peritoneum was observed in almost all the 5X and 10X dosed fish during sampling (Fig. 2f).

Serum biochemistry

Serum glucose

The serum glucose levels of the control group ranged between 74.00 ± 5.01 and 79.33 ± 6.11 mg/dL. All the treatment groups showed an increment in glucose levels till day 30 of FFC-dosing and subsequently, the levels reduced (Fig. 3a). The glucose levels of the 1X group differed significantly between day 10 and day 30 FFC-dosing ($P < 0.05$). The glucose levels of the 1X group increased significantly and peaked on day 30 FFC-dosing and started reducing with the termination of dosing. A similar trend in the glucose

levels was noted in other treatment groups. The glucose levels of all the treatment groups differed significantly ($P < 0.05$) on day 20 and day 30 FFC-dosing. Insignificant differences ($P > 0.05$) in glucose levels were observed between 1X and 3X as well as 5X and 10X groups on day 10 FFC-dosing. On day 13 post-FFC-dosing, the glucose levels of the 5X and 10X differed insignificantly ($P > 0.05$).

Serum calcium

The control group had a fluctuation in serum calcium levels ranging from 15.73 ± 0.12 to 15.93 ± 0.15 mg/dL. No significant differences ($P > 0.05$) in calcium levels were observed among the FFC-dosed groups during the dosing regimen. In the 1X group, the calcium levels reduced significantly in 30 days of FFC-dosing ($P < 0.05$). The fish of the 3X-10X groups also followed a similar trend. The cessation of dosing led to an increase in calcium levels in all the groups. On day 13 post-FFC-dosing, the calcium levels reached almost normal in all treatment groups (Fig. 3b).

Serum chloride

The serum chloride levels of the control were in the range of 123.00 ± 3.46-124.33 ± 2.31 mmol/L. The chloride levels of the 1X-5X groups reduced but insignificantly on day 30 FFC-dosing ($P > 0.05$). The chloride levels of the 10X group reduced significantly ($P < 0.05$) on day 10 FFC-dosing. The chloride levels of the 10X group were significantly ($P < 0.05$) lower than the control, 1X and 3X groups on day 10 FFC-dosing (Fig. 3c). Also, its levels on day 13 post-FFC-dosing differed significantly ($P < 0.05$) with day 20 and 30 FFC-dosing in the 10X group.

Serum creatinine

The serum creatinine levels of the control group fluctuated between 0.08 ± 0.02 and 0.09 ± 0.01 mg/dL. The creatinine levels of the 1X and 3X groups, though increased on day 10, differed insignificantly ($P > 0.05$). Its levels reached a peak on day 30 FFC-dosing in all the treatment groups and started reducing with the suspension of dosing. On day 30 FFC-dosing, creatinine levels of the 10X group were significantly ($P < 0.05$) higher than the control and 1X groups. On day 13 post-FFC-dosing, no significant differences in creatinine levels were observed among 1X, 3X and 5X groups. However, its levels in the 10X group were significantly ($P < 0.05$) higher than in other treatment groups (Fig. 4a).

Serum alanine aminotransferase (ALT)

The serum ALT levels of the control group fluctuated between 37.00 ± 1.73 and 38.67 ± 3.51 IU/L. On day 30 FFC-dosing, the ALT levels of the 1X group were significantly higher than the control or day 0 ($p < 0.05$). There was a significant hike in the ALT levels of the 3X group with days of FFC-dosing. The ALT levels of the 5X and 10X groups also increased significantly with a peak on day 30 FFC-dosing ($P < 0.05$). Insignificant differences were observed in ALT levels ($P > 0.05$) of control, 1X, 3X and 5X groups between day 0 and day 13 post-FFC-dosing (Fig. 4b).

Serum aspartate aminotransferase (AST)

The serum AST levels of the control group ranged from 77.33 ± 0.58 to 80.33 ± 0.58 IU/L. In the 1X group, significantly higher AST levels ($P < 0.05$) were noted on day 30 FFC-dosing, which reduced thereafter. Likewise, a significant increase in the AST levels ($P < 0.05$) was observed in the 3X-10X groups till day 30 FFC-dosing, after which their levels reduced. The AST levels of the 3X group were significantly higher ($P < 0.05$) than the 1X group on 10, 20 and 30 days of FFC-dosing. Insignificant differences ($P > 0.05$) were observed among the AST levels of 3X-10X groups both on day 20 and day 30 FFC-dosing. The AST levels of the 1X-5X groups returned to near normal on day 13 post-FFC-dosing, except for the 10X group, which had significantly higher ($P < 0.05$) AST levels (Fig. 4c).

Serum alkaline phosphatase (ALP)

The serum ALP levels of the control group fluctuated between 12.33 ± 0.58 and 13 ± 2.65 IU/L. The ALP levels of the 1X group were significantly higher than the control on all days of observation ($P < 0.05$). A significant rise in ALP levels ($P < 0.05$) was seen in the 3X group on day 30 FFC-dosing followed by a significant decrease ($P < 0.05$) on day 13 post-FFC-dosing. A similar trend was seen in the 5X and 10X groups, reaching the peak levels on day 30 FFC-dosing and on day 13 post-FFC-dosing the levels subsided. No significant differences ($P > 0.05$) were observed among the ALP levels of the 1X-10X groups on day 10, 20 FFC-dosing and 13 post-FFC-dosing (Fig. 4d).

Morphological changes in fish blood cells

Erythrocytes of the FFC-dosed *O. niloticus* showed variations in size and shape and the descriptions of the morphological changes are depicted in Figs. 5a,b. An increased incidence of immature erythrocytes at the therapeutic dose (1X) was observed. No significant morphological alterations were observed in the 1X group compared to the control. The blood smear examination of the 3X, 5X and 10X groups, however, showed erythrocytes with aberrant morphological changes at all sampling days. Erythrocytes with an increased nucleus to cytosol ratio were seen in all the treatment groups. At the higher dosing groups, the erythrocytes with rupturing cell membranes were prominent. The main deviant changes in erythrocytes were tear-shaped, spindle-shaped and degenerative erythrocytes. The tear-shaped and damaged erythrocytes increased on day 20 and day 30 FFC-dosing in the 3X, 5X and 10X groups and returned to almost normal on day 13 post-FFC-dosing. Immature erythrocytes and smudge cells were also observed in all the treatment groups. Blood smears of the 3X group showed changes in blood cells, which included peripheral nuclear erythrocytes and visibility of the micronucleus. The 5X and 10X groups showed high erythrocytic damages and an increased number of mature lymphocytes. The shapes of the erythrocytes were irregular with the nucleus at the extreme periphery. Some erythrocytes showed lysis with the nucleus bursting out. Blood smears of the 10X group also showed nuclear changes like irregular and bilobed nucleus. Although the blood cell morphological changes were higher among the treatment groups during the feeding regimen, the day 13 post-FFC-dosing blood samples showed almost normal erythrocytic and leucocytic morphology. Equally, the increased number of lymphocytes was still seen among all the treatment groups.

Discussion

Florfenicol is a palatable broad-spectrum antibiotic and has been conditionally approved by the USFDA for use in aquaculture (USFWS 2015). Florfenicol therapeutics differs from country to country but includes control of mortality due to diseases associated with the warm water bacterial pathogens like *Edwardsiella ictaluri*, *Streptococcus iniae*, *S. agalactiae*, *Flavobacterium columnare*, *Francisella asiatica* and *Aeromonas hydrophila* (Gaunt et al. 2004; 2010; Matthews et al. 2013; Soto et al. 2016; de Oliveira et al. 2018; Assane et al. 2019). The studies of Barreto et al. (2018) demonstrated the use of vegetable oil as a successful coating agent in the top-coating of FFC medicated feed. Since drug metabolism is considered temperature dependent, this study was conducted at the ideal growth temperature of 28.6 ± 1.68ºC. Our experimental results demonstrated the margin of safety of oral FFC administration to *O. niloticus* juveniles at 0–10 times the lowest therapeutic dose (10 mg/kg biomass/day) for 30 days. Our results are consistent with those found in similar studies that evaluated the safety of FFC administered in feed to other freshwater finfish (Straus et al. 2012; Gaikowski et al. 2013; Bowker et al. 2013). The feed consumption and growth of FFC-dosed *O. niloticus* were significantly reduced in a dose-related fashion, particularly in the 3X-10X groups. The fish consumed approximately most of the feed offered during the dosing period (0X: 100%, 1X: 98.71% and 3X: 95.75%), often breaking the surface of the water while feeding. The fish tend to feed by gulping the feed on the surface followed by releasing the feed inside the water column and subsequently re-gulping it. Previous works (Bowker et al. 2013; Gaikowski et al. 2013) also elucidated similar feeding behaviour among the experimental fish. Further, the extended feeding of FFC-diets beyond 10 days significantly decreased the feed consumption in the higher dosed groups. On the contrary, FFC therapy in *S. salar* (Inglis et al. 1991), *Perca flavescens* (Bowker et al. 2013) and hybrid striped bass, female *Morone chrysops* x male *M. saxatilis* (Straus et al. 2012) did not alter feed consumption. No gross or microscopic lesions were observed during the experimental tenure. The observed significant differences in feed intake among the treatment groups during the dosing and post-dosing regimen possibly related to the palatability and dose-dependent toxicity of FFC. The clinical implication of the declined feed consumption is likely of minimal importance as decreased feed consumption was only comprehended at higher levels and only after administration for longer than the proposed 10-day period. Nevertheless, the fish were able to mount biological responses during the post-dosing period and the feed intake recovered in a dose-dependent manner. The FFC-dosing at the 1X dose did not cause loss of equilibrium, gasping, flashing and hyperactivity. Contrarily, the 5X and 10X dosed *O. niloticus* had opercular pigmentation and a black peritoneal layer. Likewise, Gaikowski et al. (2013) reported body discolouration in FFC fed *O. niloticus*.

The mortalities during the first 10 days of dosing, i.e., therapeutic dosing period, were observed only in the 10X group possibly due to FFC-intoxication. The cumulative mortalities of 3.33% in 1X to 18.33% in 10X groups on day 30 of dosing corroborate the works of Gaikowski et al. (2013), who asserted the chances of FFC-intoxication due to prolonged FFC feeding. Hentschel et al. (2005) opined that any drug at a higher concentration than its permissible limit is toxic to the host organism thereby rendering several intoxication symptoms in fish. Our results on the elevated mortality with the increase in FFC-dose and dosing period supported the earlier observations (Hentschel et al. 2005; Gaikowski et al. 2013). Contrarily, Gaikowski et al. (2003) did not observe any mortality in *Ictalurus punctatus* during the experimental

period. The necropsy observation on the alterations in the internal organs such as swelled kidney and spleen in the 5X and 10X groups probably indicated the concern of FFC toxicity upon oral dosing. The therapeutic dose group (1X) revealed a slight decrease in biomass, i.e., 1.24 folds hike, on day 30 FFC-dosing compared to the control (1.45 folds). An insignificant dose-dependent decrease in fold change in biomass of the 1X, 3X and 5X groups compared to control was observed, which coincided with the decreased feed intake of the respective treatment groups. The biomass increased for all the treatment groups with the termination of FFC-dosing, thereby indicating the recovery of fish.

The mean serum glucose level of the *O. niloticus* (74.00 ± 5.00 mg/dL) was concomitant with the values recorded by Bittencourt et al. (2003). The 30 days of FFC-dosing increased the glucose levels significantly in all the treatment groups, indicating the FFC induced stress and altered carbohydrate metabolism (Sopinka et al. 2016; Julinta et al. 2019). Even at the lowest therapeutic dose, the 30 days of FFC-dosing raised the glucose levels significantly. On day 10 FFC-dosing, the glucose levels of the 1X groups were significantly high, which signified that the therapeutic FFC dose and dosing period (10 days) may be stressful to the normal *O. niloticus*. The degree of glucose increment was comparatively higher in 5X and 10X groups. Further, the elevated glucose levels in FFC doses higher than the therapeutic dose and the extended dosing period gave conclusive indications on FFC as a stress inducer. Though the glucose levels abridged significantly on day 13 post-FFC-dosing, the levels were still significantly higher than the initial levels recorded on day 0. These results suggested that the FFC-induced stress and the physiological changes persevered. Also, the fish could not recover fully even after 13 days of termination of FFC-dosing, which would influence the growth and farm production of *O. niloticus*. Possibly, more time would be required for the fish to revert to their initial conditions, which is a cause for concern for the aquaculturists.

At the therapeutic dose, the serum calcium levels reduced significantly on day 30 FFC-dosing, indicating an imbalance in osmolarity and ionic concentration. Nevertheless, within 2 weeks of cessation of FFC-dosing, the calcium levels recovered to normal. Even, the fish offered the higher doses followed a similar trend in a dose-dependent manner. Likewise, the chloride levels reduced but insignificantly on day 30 FFC-dosing in the 1X group and recovered completely on day 13 post-FFC-dosing. The higher dosed groups also followed a similar trend except for the 10X group, which failed to recover within the 2 weeks of termination of dosing. These results suggested that the effects of FFC were more on calcium than on chloride ions. Our results corroborate the observations of decreased serum calcium levels in pigs (Liu et al. 2003) and chicks (Klaudia and Alina 2015) when injected with FFC. Although aquaculture reports are not available on the relationship between serum calcium and FFC, antibiotics like tetracyclines tend to bind to calcium reducing its availability in serum (Guidi et al. 2018). The works of Zhang et al. (2016) on FFC established a relationship between FFC and free available chlorine (FAC), which according to them readily combines with FFC and transforms it for easy removal. The transformation kinetics of FAC, thus, hinted at a similar mechanism in fish blood. Noticeably, the increased output of faecal matter may have a close linkage with decreased serum calcium and chloride levels in *O. niloticus*. It has been documented that the increase in faecal output elevated the output of faecal carbonates and bicarbonates, leading to a disruption in osmotic balance and acid-base homeostasis (Wilson and Grosell 2003).

The recorded serum creatinine levels (0.08 ± 0.02–0.09 ± 0.01 mg/dL) in the control group were concomitant with the results of earlier studies (Julinta et al. 2019). The creatinine levels increased significantly in all the treatment groups with the maximum in the 5X and 10X groups. Subtle fluctuations in creatinine levels (0.20 ± 0.01–0.22 ± 0.03 mg/dL) were observed in the 1X group between day 10 and day 30 FFC-dosing. The increase in serum creatinine levels implied kidney damage and a reduction or loss of renal function (Julinta et al. 2019). Contrarily, in an earlier study by Reda et al. (2013), the FFC at the growth-promoting dose (5 mg/kg fish) significantly reduced the serum creatinine levels in *O. niloticus*. The creatinine levels in the 10X group increased by almost 4.41 folds on day 10 and 5.75 folds on day 30 FFC-dosing signifying the nephrotoxicity of FFC. Although the creatinine levels reduced on day 13 post-FFC-dosing in all the treatment groups, the levels were still significantly higher than on day 0. These observations suggested only a slight improvement in the renal functions of *O. niloticus* in 2 weeks of suspension of FFC-dosing. Perhaps, the fish would require more time to recoup.

The alterations in serum ALT and AST levels are indicative of liver tissue impairment or damage caused by drugs or stress (Julinta et al. 2019; Bojarski et al. 2020). The current study documented serum ALT levels in the range of 37.00 ± 1.73 to 38.67 ± 3.51 IU/L in the control group, which are analogous to previous studies (Julinta et al. 2019; Dawood et al. 2020). The significant increase in ALT levels in the 1X group on day 20 and day 30 FFC-dosing indicated the FFC induced liver tissue impairment or damage upon extended FFC-dosing. Nevertheless, the hike observed on day 10 FFC-dosing at the therapeutic dose (1X) was insignificant compared to control, suggesting minimal liver damage, similar to the observations of Reda et al. (2013). Contrarily, the dose-dependent elevated ALT levels as observed in the 3X-10X groups on day 10, 20 and 30 FFC-dosing hinted at the hepatotoxicity of FFC with increased dose and dosing period. The 10X group demonstrated about 5 folds increase in ALT levels on day 30 FFC-dosing. Yet, the ALT levels, more or less, recouped on day 13 post-FFC-dosing, except for the 10X group, which had significantly higher levels than on day 0. Notably, the pronounced impact of FFC on liver enlargement was noted during necropsy. The serum AST levels of control (77 ± 4.58–80.33 ± 0.58 IU/L) were concomitant with the studies of Hastuti and Subandiyono (2020). A significant increase in AST levels was observed in all the treatment groups with the highest in the 10X group on day 30 FFC-dosing, thus confirming the hepatotoxicity of FFC. The observed significant hike in AST levels in the 1X group during the FFC-dosing period also signified that the FFC even at the therapeutic dose may impair the liver tissues or cause metabolic damage. In contrast, Reda et al. (2013) observed a significant decline in serum AST levels when fed FFC at a lower dose (5 mg/kg fish) in *O. niloticus*. Although the higher doses (3X-10X) showed a significant rise in serum AST levels, there existed insignificant differences among them, thus suggesting persevering hepatotoxicity of FFC at elevated doses and portentous hepatic dysfunction in *O. niloticus*. It has been reported that amphenicols (Memik 1975) and oxytetracycline (Julinta et al. 2019) are hepatotoxic so also our study with FFC. Also, the FFC can cause an increase in the weight of the liver (Elia et al. 2016). Our results confirm the findings of Er and Dik (2014), who observed a hike in AST levels upon FFC application in *Oncorhynchus mykiss*. Except for the 10X group, all the treatment groups recuperated within 2 weeks of suspension of FFC-dosing. The serum ALP levels of control (12.00 ± 1.73–13.00 ± 2.65 IU/L) were similar to the observations of Hrubec and Smith (2000). The significant increase in ALP levels

at the therapeutic dose (1X) on day 10 FFC-dosing hinted at the possibility of FFC induced liver inflammation and hepatotoxicity (Labarrère et al. 2013; Soltanian et al. 2018). The ALP levels increased in a dose-dependent fashion with the highest in the 10X group on day 30 FFC-dosing. These results are in agreement with the observations recorded in fish during the misuse of FFC (Shiry et al. 2020) and goat (Shah et al. 2016) upon FFC injection. Although the ALP levels reduced with the cessation of FFC-dosing in all the treatment groups, their levels were still significantly higher than on day 0. These observations suggested persisting liver inflammation in FFC-dosed fish.

The oral FFC-dosing not only affected the serum biomarkers of healthy *O. niloticus* but also induced relative cytotoxicity. Our study used giemsa and safranin stains for assessing the blood cellular morphological changes upon FFC-dosing. The results showed that the safranin staining method is a good alternative to the giemsa method and has the advantage that abnormalities in other blood elements, in particular WBCs and thrombocytes, are better identified. Giemsa staining did not provide for comprehensible granulations in leucocytes. The safranin staining granted efficient visualization of smudge cells and granulations in leucocytes. However, considering the diagnosis of erythrocytes in a blood smear, the giemsa stain proved to be much superior to safranin. In a comparative study of Leishman and giemsa staining, Sathpathi et al. (2014) suggested the superiority of giemsa staining for a thick blood smear. In the present study, the giemsa staining produced distinct and evident erythrocytic morphology with subsequent alterations and abnormalities. Deformities like rupturing of nuclear membrane and vacuolation were prominent with giemsa stain. However, this did not translate into reduced sensitivity or reduced accuracy of safranin in light microscopy. This comparison, alongside cellular morphological alterations, also hinted at the effectiveness of time. Staining with safranin is shorter, which in many scenarios can be helpful.

The blood cell morphological changes were mostly restricted to the erythrocytes in our study. The predominant increase in mature and immature lymphocytes in all the treatment groups suggested a toxic or stress-related effect of FFC on the lymphoid cells and cell proliferation (Gaokowski et al. 2013). Although there was no direct evidence of hematopoietic or lymphopoietic tissue degradation, the sudden increase in lymphocytes hinted at the stress the fish endured. Likewise, Umamaheswari et al. (2019) in their studies with amoxicillin on *Labeo rohita* demonstrated a significant increase in lymphocytes. The haematopoietic tissues are normally sensitive to antibiotics. This could be the reason for the increased incidence of WBCs (phagocytic response). The works of Passantino et al. (2004) indicated the morphology of mature fish erythrocytes (more elongated) and immature erythrocytes (less elongated). Our study noted an increased incidence of immature erythrocytes in the therapeutic group throughout the dosing period. Chico et al. (2018) in their works on *O. mykiss* RBCs coined the term “shape-shifted RBCs (shRBCs)” for normal RBCs, which when exposed to certain stimuli produce apparent morphological and molecular alterations. These shape-shifting RBCs are also often observed in fish under the stressed conditions (Lewis et al. 2010; Chico et al. 2018). Such alterations in RBCs, viz., teardrop-shaped and spindle-shaped, were frequently observed in our study. The morphological changes in fish erythrocytes further confirmed the cytotoxic effect of FFC, which may result in chromosomal disparities (Ghaffar et al. 2015). The increased incidence of erythrocytes with eccentric nucleus was observed possibly due to the

higher production of caspase-activated DNAase or oxidative stress to the mitochondrion causing disruption and breakage in the cytoskeleton (Ghaffar et al. 2018). Changes like extruding nucleus, reduced cytosol density (lighter staining) and vacuolations were also observed under light microscopy. Such changes are comparable to those of Chico et al. (2018). Similar to the findings of this study with FFC, antibiotics like amikacin have been known to reduce cell size, increase deformability and osmotic fragility in erythrocytes (Lijana and Williams 1986). The increased incidence of ruptured cells at the higher doses in our study supported the results of Blaskó et al. (1986), possibly due to the attachment of antibiotics on the erythrocytic membrane and hindrance in cation transport. Smudge cells are associated with high lymphocyte counts and hence, the observations of increased incidence of smudge cells and lymphocytes are related. Cytotoxicity was prominent in the 5X and 10X groups with distinguishably irregular RBCs, ruptured cells and eccentric nuclei. Cytotoxicity of FFC at the higher concentrations has been well demonstrated in goats and reptiles (Saganuwan 2019). Nevertheless, the therapeutic dose did not show any signs of cell rupture hinting at the safety of FFC at the test dose. Upon cessation of FFC-dosing, the blood smear produced healthier cellular elements. Increased prominence of mature erythrocytes was also seen. The erythrocytic abnormalities like eccentric nuclei were not seen on day 13 post-FFC-dosing in *O. niloticus* of the higher dosed groups. The elimination of stress also terminated the formation of shRBCs. Although such erythrocytic aberrations decreased, the nucleus to cytoplasm ratio was still high among erythrocytes of all the treatment groups. Also, the increased prominence of lymphocytes persevered. The number of mature lymphocytes was still high on day 13 post-FFC-dosing in all the groups. With the observance of such blood cell morphological changes particularly in fish erythrocytes, the impact of FFC on blood cells need further studies to elucidate the mechanisms. It is believed that due to such significant deformities in blood cell morphology, the fish erythrocytes could be targeted as an efficient blood biomarker for future studies on the safety of approved aquacultural antibiotics.

Conclusion

The dietary FFC influenced the physiological state of *O. niloticus* in a dose- and time-dependent manner. This study presented a significant decrease in feed consumption and biomass at elevated doses for an extended duration (beyond 10 days). Mortalities on day 10 FFC-dosing were seen only in the 10X group. The extended 20 days of medication produced 3.33% mortalities in the 1X group. The anomalies in serum biomarker levels and blood cell morphology are more likely due to the FFC usage even at the therapeutic dose. Nevertheless, the results of the present study indicated that the FFC administered feed when consumed to deliver the lowest dose of 10 mg/kg biomass/day for the therapeutic duration would be well tolerated by *O. niloticus*.

Declarations

Ethics Approval The current study was performed in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The

experimental protocols were approved by the ICAR, Government of India, New Delhi under the All-India Network Project on Fish Health (F. No. CIBA/AINP-FH/2015-16 dated 16.7.2015)

Consent for Publication Not applicable

Availability of Data and Material The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

Competing Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors Contributions AB: Performed the wet laboratory experiments, laboratory investigation, data generation, statistical analyses, interpretation of the data and writing-original draft preparation; TJA: Conceptualization, methodology, project administration, supervision, resource mobilization, writing-reviewing and editing; JS, SS and SS: Performed the wet laboratory experiments, laboratory analysis, data generation and data curation; PKP: Conceptualization, methodology and funding. All authors agreed with the results and conclusions.

Consent to Participate Not applicable

Code Availability Not applicable.

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Tables

Table 1

The details on serum biomarkers tested and kits used

Serum biomarkers	Kits used	Reference
Glucose	Glucose test kit, GOD FS 10' (Diasys Diagnostic Systems, Germany)	Trinder 1969
Calcium	Calcium test kit, AS FS (Diasys Diagnostic Systems, Germany)	Michaylova and Ilkova 1971
Chloride	Chloride test kit, 21 FS (Diasys Diagnostic Systems, Germany)	Schoenfeld and Lewellen 1964
Creatinine	Creatinine test kit, Modified Jaffe's Reaction, Initial rate assay (Span Diagnostics Ltd., India)	Junge et al. 2004
Alanine aminotransferase (ALT)	ERBA SGPT Kit, IFCC Method, Kinetic (Erba Manheim, Germany)	Bradley et al. 1972
Aspartate aminotransferase (AST)	ERBA SGOT Kit, IFCC Method, Kinetic (Erba Manheim, Germany)	Bradley et al. 1972
Alkaline phosphatase (ALP)	Alkaline Phosphatase Kit, FS IFCC 37ºC (Diasys Diagnostic Systems, Germany)	Thomas et al., 2005

Table 2

Feed intake in florfenicol (FFC)-dosed *Oreochromis niloticus* juveniles at 0-10 times the therapeutic dose of 10 mg/kg biomass/day for 30 consecutive days

Dosing period	Quantity of feed consumed (%)				
	0 mg (0X)	10 mg (1X)	30 mg (3X)	50 mg (5X)	100 mg (10X)
Pre-dosing	100.00±0.00	100.00±0.00^{1}	100.00±0.00^{1}	100.00±0.00^{1}	100.00±0.00^{1}
FFC-dosing	100.00±0.00^{a}	98.71±0.73^{1a}	95.75±4.08^{2b}	86.93±13.07^{2c}	77.12±22.55^{2d}
Post-FFC-dosing	100.00±0.00^{a}	99.84±0.20^{1a}	96.52±2.17^{2b}	87.14±10.68^{2c}	78.70±13.92^{2d}

Florfenicol ration: X=10 mg/kg biomass/day; a-d: Values sharing common alphabetical superscripts within a row for a particular dosing period differed insignificantly (P>0.05); 1-3: Values sharing common numeral superscripts within a column for a particular treatment (dose) differed insignificantly (P>0.05).

Figures

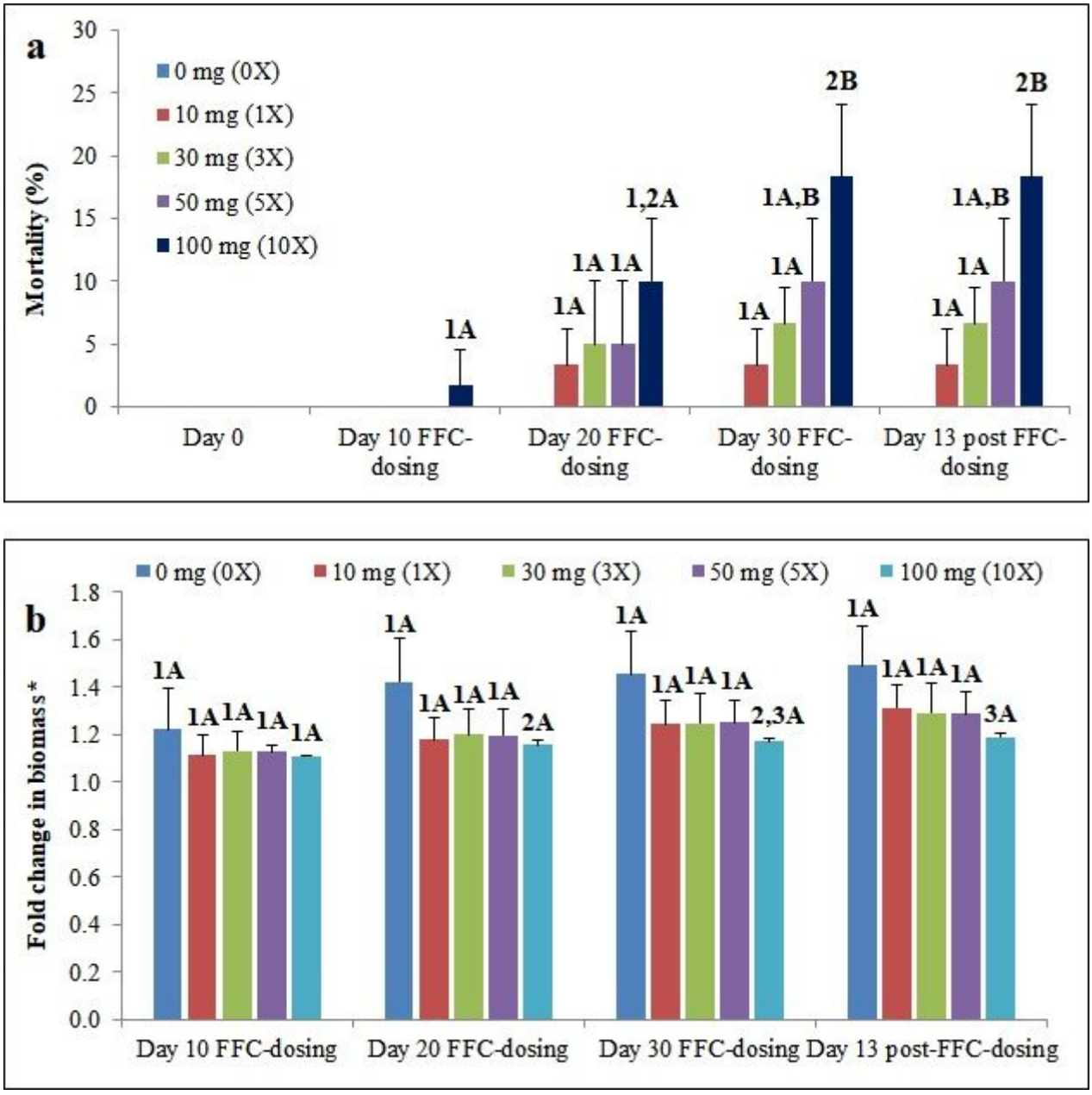


Figure 1

Effects of oral florfenicol (FFC)-dosing at 0-10 times the therapeutic dose of 10 mg/kg biomass/day for 30 consecutive days on the [a] mortality and [b] fold change in biomass of Oreochromis niloticus juveniles during the different treatment period. Pre-dosing period: 0-7 days; FFC-dosing period: 8-37 days; Post-FFC-dosing period: 38-50 days. *Biomass of 10 fish from each of the triplicate tank. A-B: Bars sharing common alphabets for a particular day differed insignificantly (P>0.05). 1-3: Bars sharing common numerals for a particular treatment (dose) differed insignificantly (P>0.05)

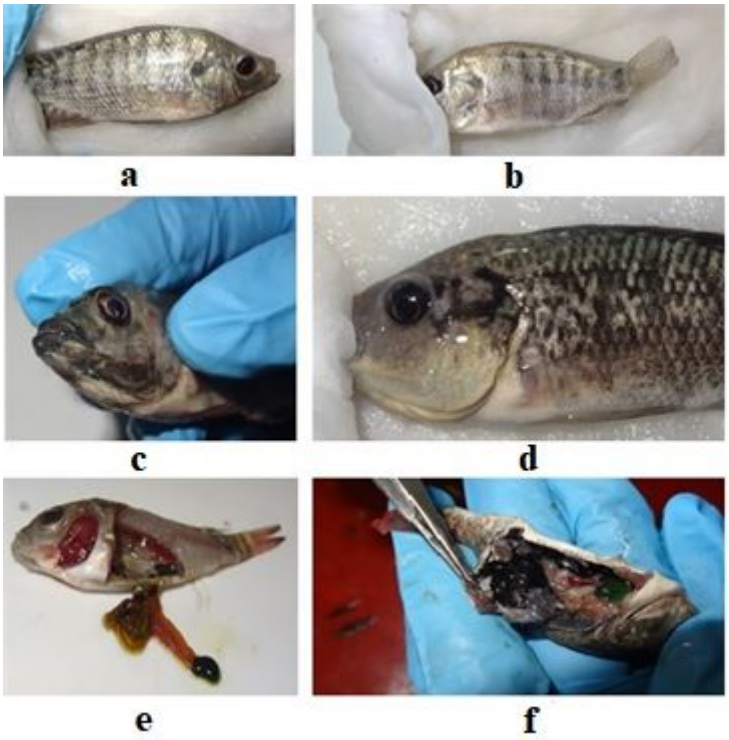
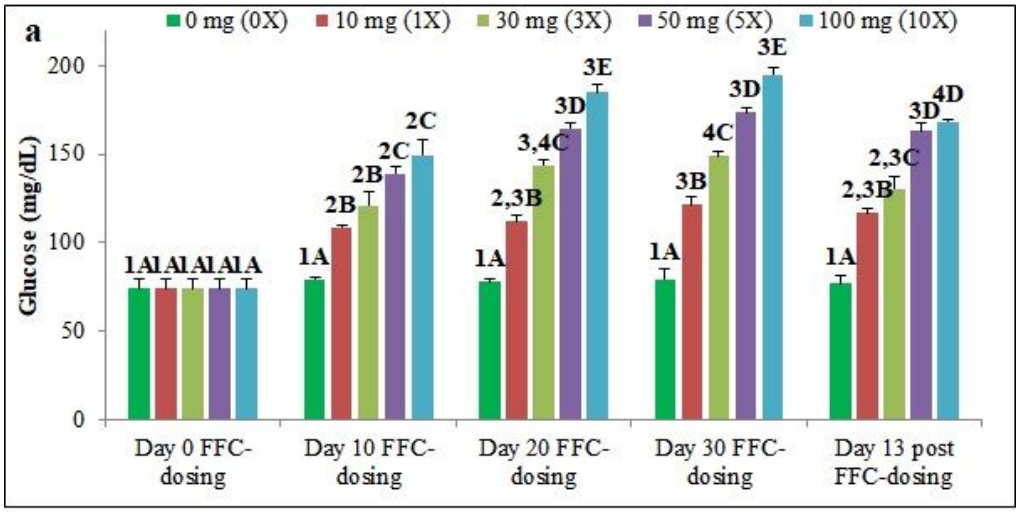
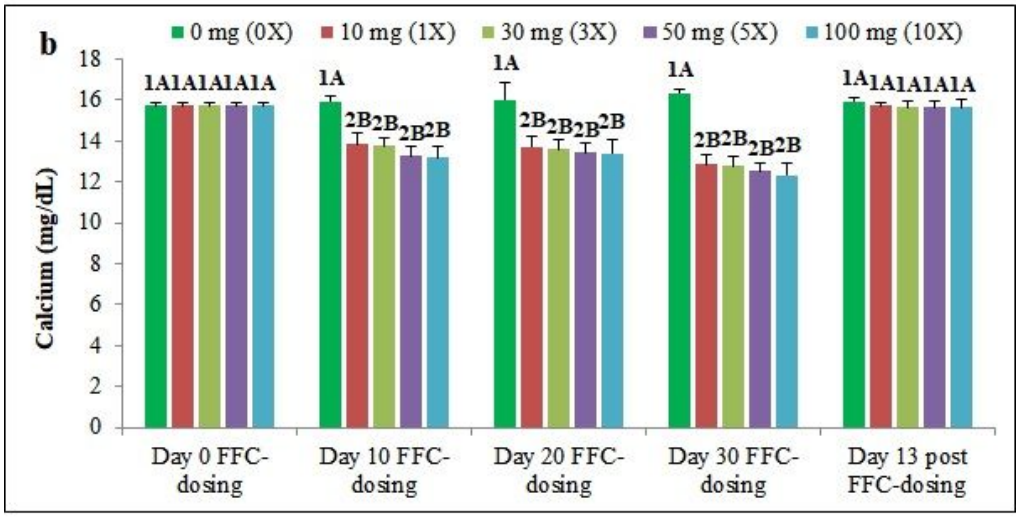


Figure 2

Effects of oral florfenicol (FFC)-dosing at 0-10 times the therapeutic dose of 10 mg/kg biomass/day for 30 consecutive days on the abnormalities of Oreochromis niloticus juveniles. [a] Control group without opercular pigmentation, [b] 1X group without opercular pigmentation, [c] 5X group with opercular pigmentation, [d] 10X group with opercular pigmentation; [e] 10X group with enlarged gall bladder and [f] 5X group with black peritoneum





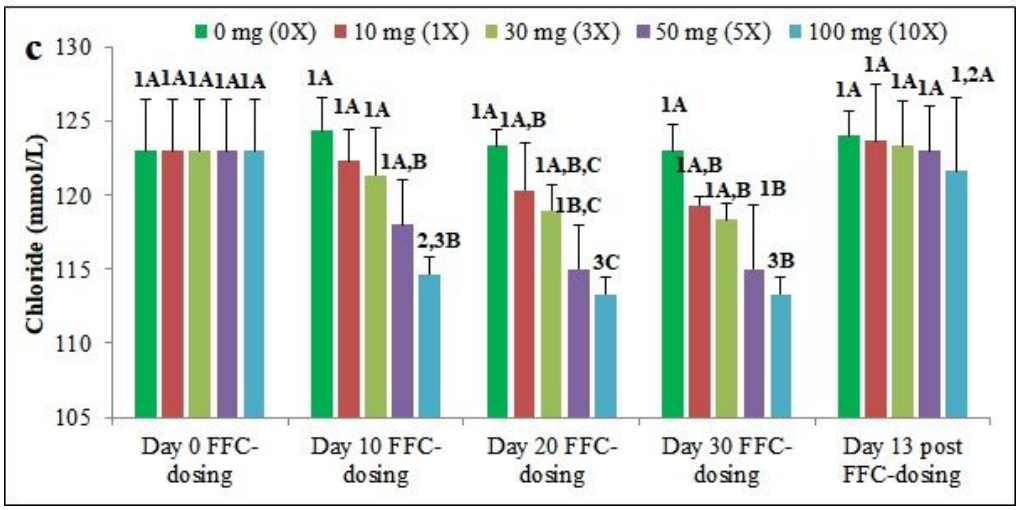


Figure 3

Effects of oral florfenicol (FFC)-dosing at 0-10 times the therapeutic dose of 10 mg/kg biomass/day for 30 consecutive days on the serum [a] glucose, [b] calcium and [c] chloride levels of Oreochromis niloticus juveniles. A-E: Bars sharing common alphabets for a particular day differed insignificantly (P>0.05). 1-4: Bars sharing common numerical for a particular treatment (dose) differed insignificantly (P>0.05)

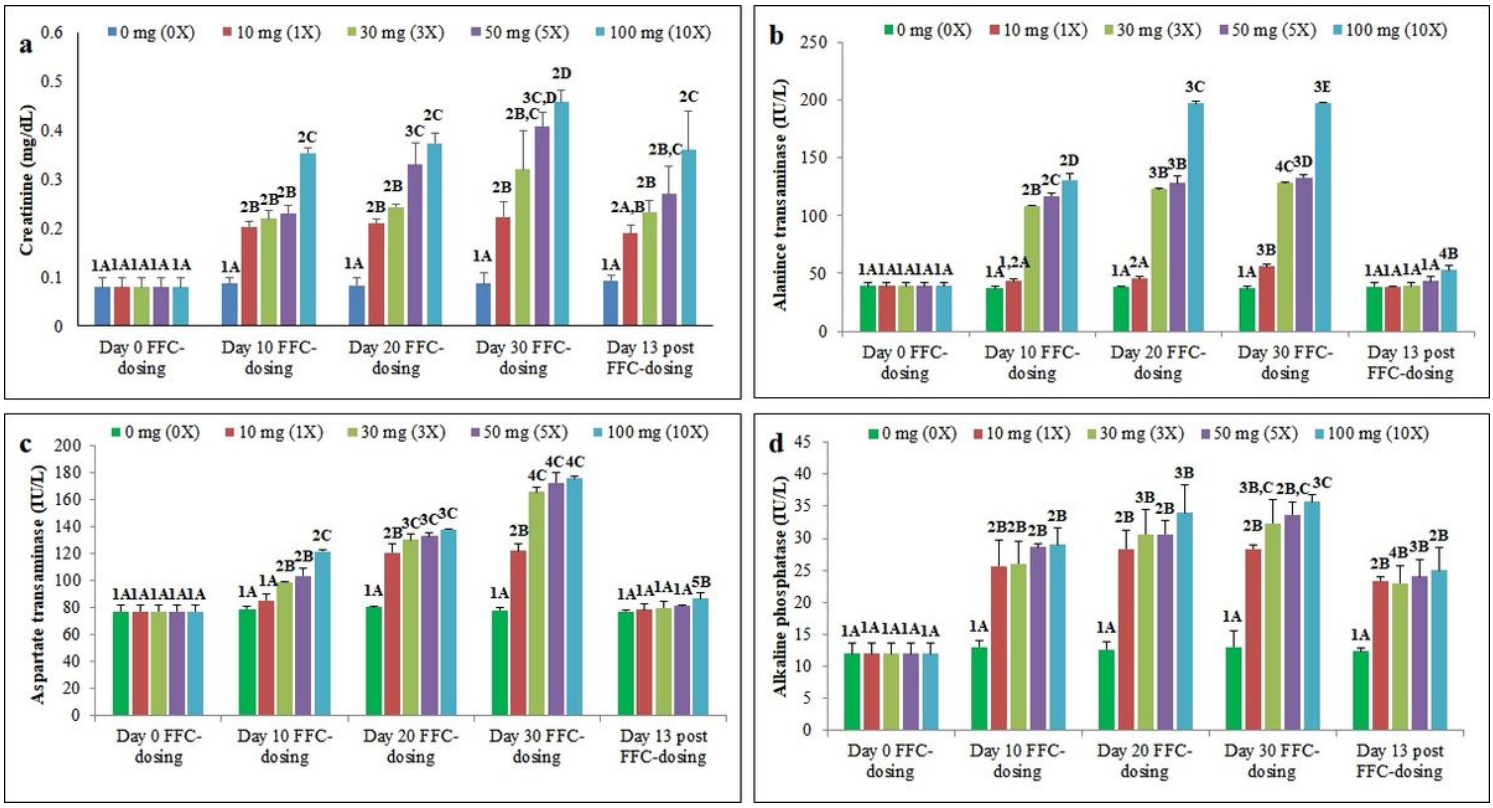


Figure 4

Effects of oral florfenicol (FFC)-dosing at 0-10 times the therapeutic dose of 10 mg/kg biomass/day for 30 consecutive days on the serum [a] creatinine [b] alanine transaminase (ALT), [c] aspartate transaminase (AST) and [d] alkaline phosphatase (ALP) levels of Oreochromis niloticus juveniles. A-E: Bars sharing common alphabets for a particular day differed insignificantly (P>0.05). 1-4: Bars sharing common numerical for a particular treatment (dose) differed insignificantly (P>0.05)

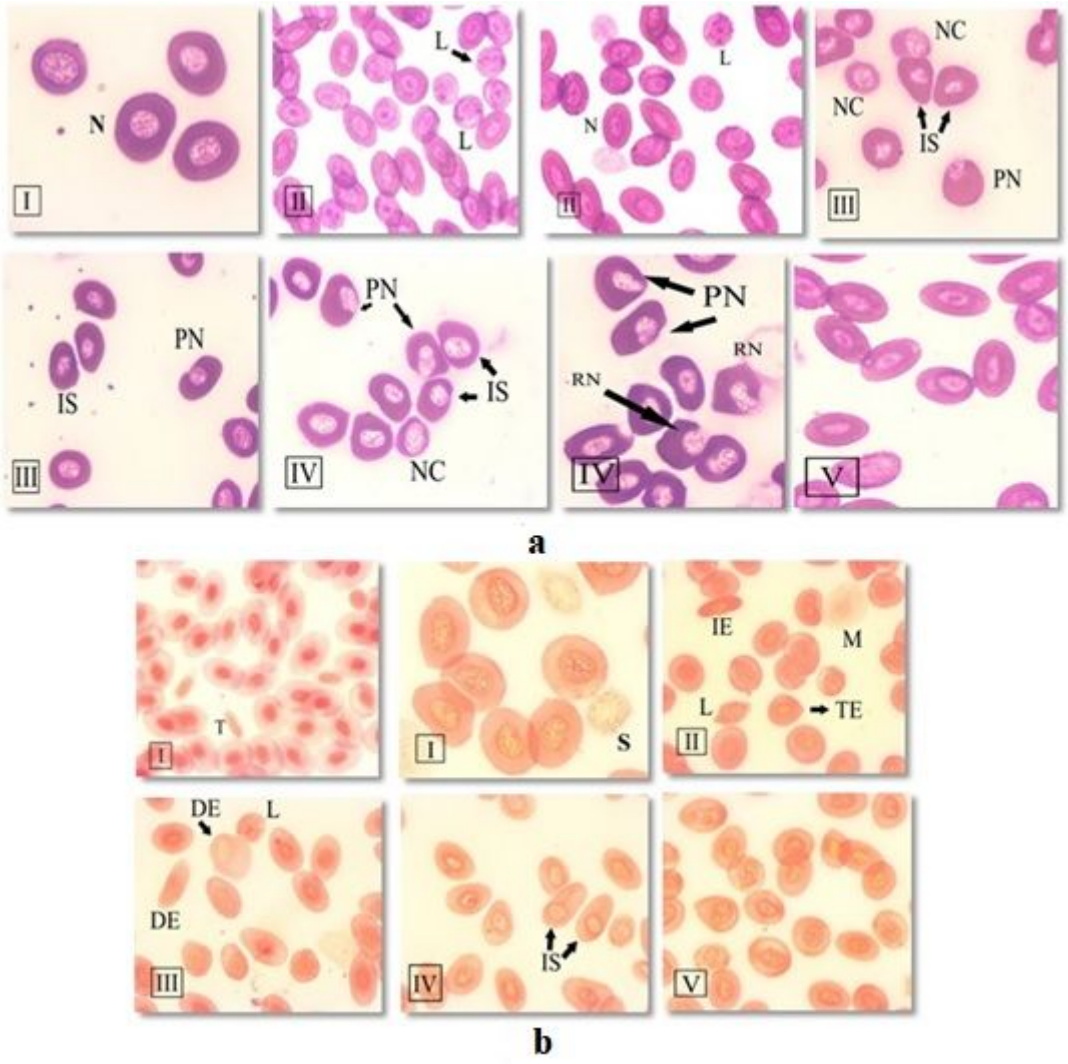


Figure 5

Effects of oral florfenicol-dosing at 0-10 times the therapeutic dose of 10 mg/kg biomass/day (1X) for 30 consecutive days on the blood morphological characters of Oreochromis niloticus juveniles. [a] Giemsa staining and [b] Safranin staining. I: 1X group, II: 3X group, III: 5X group, IV: 10X group and V: Control group. Normal erythrocyte (N), Thrombocyte (T), Smudge cell (S), Lymphocyte (L), Immature erythrocyte (IE), Tear-shaped erythrocyte (TE), Monocyte (M), Damaged erythrocyte (DE), Erythrocytes with increased nucleus-to-cytoplasm ratio (NC), Irregular shaped erythrocyte (IS), Erythrocyte with eccentric and peripheral nucleus (PN), Ruptured erythrocyte (RN), X1000