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Genotoxic responses of juvenile tilapia (*Oreochromis niloticus*) exposed to florfenicol and oxytetracycline

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HIGHLIGHTS

- We examine genotoxic responses of fishes exposed to florfenicol and oxytetracycline.
- Environmental concentrations of both antibiotics induce DNA damage in fishes.
- Environmental concentrations of both antibiotics are not safe for fishes.
- Florfenicol and oxytetracycline should be carefully used in aquaculture.

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ABSTRACT

Florfenicol (FLC) and oxytetracycline (OTC) are the two most commonly used antibiotics for bacterial treatment in fish farming in Brazil, and because of their intensive use, the potential harmful effects on aquatic organisms are of great concern. This study evaluated the effects of environmental concentrations of FLC and OTC on the genetic material of juvenile tilapia (*Oreochromis niloticus*) erythrocytes by using the comet assay and the occurrence of micronuclei (MN) and other erythrocytic nuclear abnormalities (ENAs) after exposure to 96 hour. The comet assay showed that fish erythrocytes exhibited significantly higher DNA damage after exposure to environmental concentrations of FLC and OTC. Although MN was not observed, ENAs were significantly higher after exposure to FLC, indicating that ENAs are a better biomarker for FLC than MN. The results showed that environmental concentrations of FLC and OTC were genotoxic to erythrocytes of *O. niloticus*; however, future studies on DNA damage recovery are needed.

1. Introduction

There has been concern in recent years about the occurrence, fate, and adverse effects of pharmaceutical residues in aquatic environments. Some of the most frequently used classes, such as antibiotics, which are used to prevent and treat bacterial infections and promote the growth of livestock (Kummerer, 2004), deserve to be highlighted due to their low biodegradability and elevated persistence in the environment, mainly in the sediment (Hektoen et al., 1995; Rico et al., 2014).

Antibiotic residues may contaminate aquatic systems through many different sources, but the main sources are wastewater and sewage discharge, veterinary use (e.g., aquaculture), run-off and leaching from agricultural cultures plowed with sludge and manure (Kummerer, 2009). Therefore, residues of antibiotics have been found in sediment (Shi et al., 2014; Chen and Zhou, 2014), surface water (Yan et al., 2013; Chen and Zhou, 2014; Bayen et al., 2014), and in aquatic animals (Li et al., 2012).

In Brazil, fish farming is an important activity for the economy of the country mainly due to the suitable climatic conditions and large availability of water resources. According to the Ministry of Fisheries and Aquaculture (MPA), Brazil currently produces approximately 2 million tons of fish, with tilapia (*Oreochromis niloticus*) as the most cultivated species (MPA, 2014). In Brazilian

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fish farming, two of the most used antibiotics are florfenicol (FLC) and oxytetracycline (OTC), which are incorporated in the fish food. This is the most convenient way to medicate farmed fish with pharmaceuticals (Shao, 2001) due to the lower amount of drugs required compared to administration through the water. Consequently, the quantity of pharmaceutical products entering the aquatic environment is also lower. However, according to Hektoen et al. (1995), 70–80% of the administered food is not eaten by fish and therefore contaminates aquatic systems.

FLC is an antibacterial fluorinated analog of thiamphenicol (Ferreira et al., 2007) that binds to the 50S and 70S subunits of ribosomes (Liu et al., 2003) and inhibits the effectiveness of the transpeptidation of bacterial protein synthesis against many gram-negative and gram-positive bacteria (Christensen et al., 2006). OTC is an antibacterial that belongs to the tetracycline group and is also commonly used in aquaculture because of its broad-spectrum efficacy in the treatment of infections caused by gram-positive and gram-negative bacteria, mycoplasma and large viruses. It inhibits protein synthesis by preventing the association of aminoacyl-tRNA with bacterial ribosomes (Reemtsma and Jekel, 2006).

Both antibiotics are biologically active, and due to their intensive use and their potential to contaminate aquatic environments, their toxicity has been evaluated for different groups of aquatic organisms, such as microalgae and crustaceans (Ferreira et al., 2007), marine bacteria (Kołodziejska et al., 2013), rotifers (Rhee et al., 2013), marine diatoms (Liu et al., 2012), and fishes (Reda et al., 2013), using different endpoints. Many studies regarding the effects of antibiotics to aquatic organisms focus on acute effects. However, concentrations of chemical agents including antibiotics are found at $\mu g\ L^{-1}$ and $ng\ L^{-1}$ in aquatic environments due to the phenomenon of dilution, and thus, the observed effects are chronic. Therefore, in toxicological evaluations is more suitable the use of environmental concentrations once the results will be more realistic and represent what is occurring in the environment. To our knowledge, studies describing the genotoxic responses of environmental concentrations of FLC and OTC for tilapia have not been conducted.

The aim of the present study is to investigate whether short-term exposure to environmental concentrations of FLC and OTC induces genotoxic responses in juvenile tilapia (*O. niloticus*) through the comet assay, MN induction and other ENAs.

2. Material and methods

2.1. Chemicals

FLC (98% purity), OTC hydrochloride (95% purity) and cyclophosphamide monohydrate, which was used in the present study as a positive control, were purchased from Sigma Aldrich (Saint Louis, MO, USA).

2.2. Determination of environmental concentrations of FLC and OTC

Environmental concentrations of FLC and OTC used in the present study for the exposure of juvenile *O. niloticus* were determined by Monteiro (2014) in the surface water of a fish farm located at Ilha Solteira Reservoir (Santa Fé do Sul, São Paulo, Brazil) through Online-Solid-Phase Extraction coupled to Liquid Chromatography-Tandem Mass Spectrometry (Online-SPE-LC/MS-MS). Ilha Solteira Reservoir has a flooded area of 1195 km^2 and volume of $210.6 \times 108\ m^3$ and is the main area producing tilapia in São Paulo State.

2.3. Acquisition and maintenance of O. niloticus

Juvenile species of *O. niloticus* (12–15 cm, $n = 200$) were purchased from a fish farm located at Santa Cruz da Conceição (São Paulo, Brazil) and were safely transported to the Laboratory of Ecotoxicology, Center for Nuclear Energy in Agriculture, University of São Paulo, in well-packed polythene bags containing oxygenated water. Prior to the exposure, fish were acclimatized for two weeks in two 300-L polyethylene tanks (hundred animals in each one) with non-chlorinated water, constant aeration and a natural photoperiod of 12 h light:12 h dark (natural photoperiod). Water (three fourths) was replaced every three days to maintain a healthy environment. Feces and food remains were aspirated during water renewal using a hose. During acclimation, the animals were fed daily *Ad libitum* with commercial food 24 h before the beginning of the exposure. To ensure good water quality, physical and chemical parameters, such as pH (7.36 ± 0.41 and 7.56 ± 0.27), conductivity ($473.70 \pm 32.30\ \mu S\ cm^{-1}$ and $444.80 \pm 25.24\ \mu S\ cm^{-1}$), dissolved oxygen ($6.81 \pm 1.85\ mg\ L^{-1}$ and $6.73 \pm 1.30\ mg\ L^{-1}$) and temperature (23.60 ± 0.29 °C and 26.49 ± 1.08 °C), for tanks 1 and 2, respectively, were measured every two days by a multi-parameter probe (Aquaread AP-1000). The physical-chemical parameters of the maintenance water were analyzed using the Excel program, and the results are expressed as the means and standard deviations.

2.4. Exposure system

The exposure system consisted of three treatment groups for each antibiotic, which were $425\ ng\ L^{-1}$ and $8000\ ng\ L^{-1}$ for the environmental concentrations of FLC and OTC, respectively, $212.5\ ng\ L^{-1}$ and $4000\ \mu ng\ L^{-1}$ (their half) and $850\ ng\ L^{-1}$ and $16000\ ng\ L^{-1}$ (their double). It is important mention that the environmental concentrations of FLC and OTC used in the present study correspond to the highest concentrations determined by Monteiro (2014) at Ilha Solteira Reservoir (São Paulo, Brazil), where fish farm activity is located. To prepare the FLC and OTC solutions, purified water was used in the appropriate amounts. A negative control (NC) (the same water used for acclimation) and a positive control (PC), the latter consisting of fish injected with the clastogenic agent cyclophosphamide at a concentration of $22\ mg\ mL^{-1}$, were also used. Cyclophosphamide was reconstituted in sterile water (autoclaved osmosis water). All treatments for both substances consisted of two replicates with 6 organisms, each one in a 40-L aquarium, which was aerated during the 96-h exposure period.

Because pH and temperature play important roles on the degradation of most antibiotics in water, they were monitored at 24 and 96 h in the aquariums that received FLC and OTC concentrations. Dissolved oxygen was also monitored for all treatments. All of the parameters were measured through a multi-parameter probe (Aquaread AP-1000), analyzed using the Excel program, and the results are expressed as the means and standard deviations.

2.5. FLC and OTC in water exposure

To confirm that the concentrations used were exposed to the fishes during the experiment, 50 mL of water from each aquarium for both antibiotics were sampled within 24 h and 96 h and was filtered in a 0.22-µm Teflon membrane. Posteriorly, the pH of the samples were adjusted to 4.0 through the addition of orthophosphoric acid (J.T Baker, Mallinckrodt Chemicals) following the extraction process, which was performed according to Monteiro (2014), as well as the analysis of antibiotics through Online-SPE-LC-MS/MS.

2.6. Comet assay

For the comet assay, peripheral blood samples were also obtained from cardiac puncture and from 3-µL aliquots diluted in