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Ecotoxicology of drugs used in fish disease treatment

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The fish *Hyphessobrycon eques* and *Piaractus mesopotamicus*, the snail *Pomacea canaliculata*, the aquatic plant *Lemna minor* and the microcustacean *Daphnia magna* were selected to evaluate the lethal or effective concentration (LC50/EC50) and the environmental risk of florfenicol (FLO), enrofloxacine (ENR), thiamethoxan (TH) and toltrazuril (TOL). For this, the organisms were acclimated in a bioassay room under controlled temperature and photoperiod, and then exposed to increasing drugs concentrations according to specific standard for each organism. *L. minor* is the sole organism which showed toxicity to FLO LC50; 48 h of 97.03 mg/L, which causes medium environmental risk. *P. canaliculata* was more sensible to ENR (14.64 mg/L), which causes high risk to the bioindicators. *P. mesopotamicus* was more sensible to TH toxicity (16.97 mg/L), which causes high risk also; followed by *H. eques*. TOL causes medium risk and it is more toxic for *P. mesopotamicus* (3.72 mg/L), followed by *H. eques*. *L. minor* can be used as a bioindicator for florfenicol toxicity, *P. canaliculata* for enrofloxacine and *H. eques* for TH and TOL, emphasizing that enrofloxacine and thiamethoxan cause high environmental risk.

Key words: Environmental monitoring, disease, environmental impact, chemotherapeutic products, aquaculture drugs.

INTRODUCTION

The amount of chemicals launched in the aquatic ecosystem is large due to its widespread use in almost all productive activities (Sarmah et al., 2006). Drug use raises concerns because the production systems is part of the aquatic ecosystems. The use affects in both direct and indirect ways the aquatic communities causing acute, subacute or chronic effects (Boyd and Massaut, 1999). Furthermore, the indiscriminate use of non registered drugs can result in bacteria resistance and direct toxicity for plants and non-target animals (Kolodziejska et al., 2013).

Drugs used in aquaculture belong to different chemical

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groups, among them are the beta lactans, fluoroquinolones, macrolides, sulfonamides, tetracyclines (Regitano and Leal, 2010), neonicotinoids, derivatives of urea, formaldehyde, copper sulfate, sodium chloride, malachite green and metals (Klein et al., 2004). Among the adverse effects caused by antibiotics, the development of bacteria resistance is the most important (Kumerer, 2009). The green malachite is highly toxic and teratogenic and the lethal concentration is very close to therapeutic concentration (Sudova et al., 2007); formaldehyde is found to be teratogenic and carcinogenic (Santos et al., 2012). Some drugs as florfenicol (FLO), enrofloxacine (ENR), toltrazuril (TOL) and thiamethoxan (TH) have been studied for use in fish farming disease treatment (Carraschi et al., 2014).

FLO is a derivative from tiamphenicol, inhibiting the transpeptidation of bacterial protein synthesis and is effective on gram positive and negative pathogenic and opportunistic bacteria control (Christensen et al., 2006). ENR is a quinolone that inhibits the DNA-girase activity and is effective against *A. salmonicida*, *Vibrio anguillarum*, *Y. ruckerii*, *Renibacterium salmoninarum* and *Pasteurella piscicida* (Intorre et al., 2000; della Rocca et al., 2004; Koc et al., 2009). TOL is a triazinetrione derivative that causes the reduction of an enzyme from the respiratory chain of parasites, inhibiting the nuclear division (EMEA, 2008) and is effective against *Ichthyophthirius multifiliis*, microsporidia, myxozoa, *Tricodina* spp (Mehlhorn et al., 1988) and monogeneans (Schmahl and Mehlhorn, 1988). TH is a neonicotinoid insecticide, agonist of nicotinic receptors from insects and mammals, and is effective against *Anacanthorus penilabiatus* (Carraschi et al., 2014).

Several drugs without registry are used in treatment of many pathogens. Thus, the prospection of new molecules with proved efficacy, clinic and environmental safety, is an urgent necessity in order to improve the regulations of this sector. The ecotoxicology assessment consist a fundamental point for environmental registration. Furthermore, aquaculture has a variety of drugs used simultaneously in the same area, resulting in a multicomponent in the environment (Wilson et al., 2004).

For toxicity evaluation purposes, fish are great non-target organisms, because they are exposed by direct and indirect ways to the tested drugs. The tetra-serpae, *Hyphessobrycon eques* and the pacu, *Piaractus mesopotamicus*, are neotropical fish and their sensitivity to potassium dichromate have been studied (Cruz et al., 2008). *Daphnia magna* is a microcrustacean used for toxicity evaluation due to genetic equality of the descendants (Medeiros et al., 2013). The macrophyte *Lemna minor* ,has a vegetative reproduction with new fronds (leaves) and represent the superior aquatic vegetables (OECD, 2002). The fresh water snail *Pomacea canaliculata* is not indicated in a standard for ecotoxicology assay, although its a good bioindicator since it is in direct contact with sediment and is sensible to drugs (Venturini et al., 2008).

Despite the absence of information about some drugs toxicity for non-target organisms, the aim of this research was to study the drugs effect in the aquaculture (FLO, ENR, TOL and TH) through ecotoxicological assay with organisms from different trophic levels and complexity: the fish pacu *P. mesopotamicus* and thetetra-serpae (*H. eques*), snail *P. canaliculata*, macrophyte *L. minor* and the microcrustacean *D. magna*, in order to establish the environmental safety for the use of these chemical substances in the aquatic environment.

MATERIALS AND METHODS

The active ingredients used were from the following commercial products: Aquaflor®, florfenicol (500 g/kg) from MSD©, Baytril®, enrofloxacine (10.0 g/100 ml) and Baycox®; toltrazuril (2.5 g/100mL) from Bayer©Health Care and Agita®, thiametoxan(10.0 g/100g) from Novartis©. The products were diluted in water to be used in the ecotoxicology tests. Only Aquaflor® is registered for use in Brazilian aquaculture to treat bacteria in *Oreochromis niloticus* and *Onchorhynchus mykiss*, the others are used to treat pathogens in chickens. The effective (EC50) and lethal concentration (LC50) was estimated by the software Trimmed Spearman Karber (Hamilton et al., 1977) and the ecotoxicology classification was coined from Zucker (1985): LC50 < 0.1 mg/L, extremely toxic (VHT); 0.1 < LC50 < 1.0 mg/L, highly toxic (HT); 1.0 < LC50 < 10.0 mg/L, moderately toxic (MT); 10.0 < LC50 < 100 mg/L, slightly toxic (ST) and LC50 > 100 mg/L, practically non-toxic (PNT).

Our procedures with live fish followed the protocols approved by the University's Institutional Animal Care and Use Committee under approval number 017335/10.

Acute toxicity assays with the fish (*P. mesopotamicus* and *H. eques*)

P. mesopotamicus were gotten from the Aquaculture Center of UNESP and *H. eques* from our laboratory: Study and Environmental Research Center on Weed Sciences, from the College of Agricultural and Veterinary Sciences of the UNESP, both from Jaboticabal city (Sao Paulo State), Brazil.

P. mesopotamicus that weighed between 0.5 and 1.0 g, and *H. eques* between 0.35 and 0.8 g were acclimated for 10 days under bioassay room conditions, inside 250 L tanks with water at 25.0 ± 2.0°C temperature, photoperiod of 12 h of light and fed *ad libitum* once a day (ABNT, 2011).

After the acclimatization, the fish were transferred to 3 L aquariums to evaluate the organisms sensitivity with potassium chloride (KCl,99.5%), as reference substance. The LC50;48 h was 1.54 g/L, with confidence interval of 95% between 1.28 and 1.86 g/L for pacu was 2.20 g/L, between 1.84 and 2.67 g/L, for serpae tetra.

For the drugs assay, three replicates were used with three fish per replicate with 1 g/L maximum density (Table 1). The assays were carried out in a static system, with 48 h duration, without renewal and feeding. The mortality evaluation was done daily, with removal of the dead fish (without opercular beat) from the aquarium.

Acute toxicity assays for snail (*P. canaliculata*)

The snails selected weigh between 1.0 and 2.0 g, acclimated in bioassay room for 10 days, in 60 L tanks filled with water, 25.0 ± 2.0

Table 1. Drugs concentration (mg/L) used in the assays with fish.

Drugs	*P. mesopotamicus*	*H. eques*
Enrofloxacine	0.0; 90.0; 105.0; 120.0; 135.0; 150.0	0.0; 100
Florfenicol	0.0; 100	0.0; 100
Toltrazuril	0.0; 3.0; 3.5; 4.0; 4.5; 5.0	0.0; 4.0; 6.0; 8.0; 10.0
Thiamethoxan	0.0; 10.0; 15.0; 20.0; 25.0; 30.0; 35.0	0.0; 20.0; 40.0; 60.0; 80.0

°C temperature, 12 h light/dark photoperiod, continuous aeration (standard adapted from ABNT, 2011) and fed daily with *Hydrilla verticilata*.

After the acclimatization period, the snails were transferred to 2 L aquariums to evaluate the snails sensitivity with the potassium chloride as reference substance, with 1.49 g/L effective concentration (EC50;48 h) and confidence interval between 1.14 and 1.96 g/L.

For the definitive assays, 5 snails were selected per replicate, with 48 h duration. The concentrations of TH and FLO were: 70.0, 80.0, 90.0 and 100 mg/L; of ENR, 1.0, 10.0, 25.0 e 50.0 mg/L and of TOL, 3.0, 5.0, 7.0, 10.0, 13.0, 16.0 and 19.0 mg/L and a control. There was no water renewal and feeding during the test. The snails immobility was evaluated daily, doing a pressure at the opercula with tweezers and the dead organisms were removed from the aquariums.

Acute toxicity for aquatic macrophyte *L. minor*

The plants grown were kept in crystallizers with 2 L capacity, filled with Hoagland`s medium in a bioassay room with 25.0 ± 2.0°C temperature, for 4 days (OECD 2002). First, in the sensitivity evaluation, the LC50;7d average of sodium chloride (NaCl) was 6.67 g/L, with confidence interval of 95% between 5.48 g/L and 6.85 g/L.

In the definitive assays with ENR, FLO, TOL and TH the concentrations used were 60.0, 70.0, 80.0, 90.0 and 100 mg/L, respectively. The assays were performed in a static system with three replicates. The experiments were carried out with 12 fronds per replicate during seven days, without water renewal. In the third, fifth and seventh exposure day, the increase in frond numbers and the presence of chlorosis and necrosis (death of plants) were evaluated, but the LC50; 7 days was calculated using the cumulative mortality in seven days exposure.

Acute toxicity for microcrustacean *Daphnia magna*

The microcrustacean were kept in crystallizers with M4 culture medium at 20.0 ± 2.0°C, in a bioassay room with 3.000 lux luminous intensity and photoperiod of 8 h of dark and 16 h of light. The neonates were fed with an algae suspension composed of *Scenedesmus subspicatus* ($5x10^6$ cels/individual/day) (ABNT, 2009), fermented ration solution for ornamental fish and yeast (*Saccharomyces cerevisiae*). The sensitivity was evaluated with sodium chloride and the EC50;48h was 4.31 g/L, with 3.97 and 4.69 g/L confidence interval.

The neonates aging between 4 and 24 h were selected and 5 animals were distributed per replicate. The assays were performed in tubes with M4 medium, kept in the dark for 48 h and each treatment was composed of 4 replicates, in completely randomized design, with 48 h of exposure.

In the definitive assays, the concentrations were 10.0, 25.0, 50.0, 75.0 and 100 mg/L for ENR and FLO; 10.0, 25.0, 50.0 and 75.0 mg/L for TOL, 10.0, 50.0, 100 and 200 mg/L for TH and the control.

Drugs environmental risk

The environmental risk (RQ) is the combination of exposure and drug toxicity, which was calculated using the ratio between the predicted environmental concentration (PEC), which is the concentration/dosage of drug used in the treatment and the lethal concentration LC50, found in the acute toxicity tests. The value of Q, risk quotient (RQ) (Goktepe et al., 2004), was classified as follows: RQ > 0.5 = High risk; 0.05 < RQ < 0.5 = Medium risk; RQ < 0.05 = Low risk.

The drugs PEC were: FLO: 10.0 mg/kg; ENR: 90.0 mg/kg; TOL: 1.0 mg/L and TH: 75.0 mg/L (Carraschi et al., 2014). The LC/EC50 for the drugs with no mortality until 100 mg/L was considered 100 mg/L.

RESULTS AND DISCUSSION

No mortality occurred in organisms exposed to FLO, classifying it as practically non toxic (LC50/EC50 > 100 mg/L) except for *L. minor*, the sole organism in which lethality occurred with the exposure to the drug, with 97.03 mg/L LC50;7d. No lethality occurred for fish with ENR exposition; however, it was classified as slightly toxic by the other organisms (10 < LC/EC50 < 100 mg/L). *L. minor* and *D. magna* are not affected by TH, however it classified as slightly toxic to the other organisms. *L. minor* is not affected by TOL but the tests with *D. magna* classified it as slight toxic and moderately toxic (1.0 < LC/EC < 10 mg/L) to the other organisms (Table 2).

OECD (2009) is of the opinion that acute toxicity tests must be done with concentrations until 100 mg/L, because the absence of mortality until this concentration suggests that the organism does not represent the more sensible group for the substance in a short exposure. Therefore, the assays were performed with concentrations until 100 mg/L and thus, the drugs that caused no mortality until this limit concentration was classified as practically non-toxic, according to Zucker (1985).

Most of the non-target organisms in this study were not affected by FLO (LC/EC50 > 100 mg/L), similar to *Arthemia parthenogenetica* (LC50;48h > 889.0 mg/L) (Ferreira et al., 2007). According to Carraschi et al. (2011) FLO causes no risk to *P. mesopotamicus*, because LC50 is much higher than the dosage used in the treatment. The algae *Tetraselmis chuii* (LC50;96h = 6.06 mg/L) (Ferreira et al., 2007), *T. chuii* (EC50;96h = 1.3 mg/L) and *Selenastrum capricornutum* (IC50;48h = 1.5 mg/L) (Hong-Thih et al., 2009) showed moderate

Table 2. Ecotoxicity and environmental risk of drugs for non-target organisms (mg/L).

Variables	FLO	ENR	TH	TOL
P. mesopotamicus LC50	>100	116.70	16.97	3.72
Classification	PNT	PNT	PT	MT
Environmental risk	Medium (0.1)	High (0.77)	High (4.42)	Medium (0.27)
H. eques LC50	>100	>100	49,78	6,22
Classification	PNT	PNT	PT	MT
Environmental risk	Medium (0.1)	High (0.9)	High (1.5)	Medium (0.16)
*L. minor L*C50	97.03	60.49	>100	>100
Classification	PT	PT	PNT	PNT
Environmental risk	Medium (0.1)	High (1.48)	High (0.75)	Low (0.01)
P. canaliculata EC50	>100	14.64	87.14	7.59
Classification	PNT	PT	PT	MT
Environmental risk	Medium (0.1)	High (6.14)	High (0.69)	Medium (0.13)
D. magna EC50	>100	84.39	107.18	18.57
Classification	PNT	PT	PNT	PT
Environmental risk	Medium (0.1)	High (1.06)	High (0,69)	Medium (0.05)

IC: confidence interval. MT: moderately toxic; PT: slight toxic; PNT: practically non-toxic.

toxicity to FLO, differing from this research. *L. minor* showed FLO LC50;48h 97.03 mg/L, differing of 2.96 mg/L which causes growing inhibition of the fronds according to Kolodziejska et al. (2013). This large difference between these studies is due the different kind of evaluation.

This study counted the duckweed fronds live, chlorotic and necrosis; but Kolodziejska et al. (2013) evaluated the inhibition rate determined by the frond area (mm^2) for the treated plants in relation to the untreated control. *D. magna* was not affected by FLO, similarly, as found by Kolodziejska et al. (2013). *L. minor* was more tolerant to ENR than the algae *Mycrocistis aeruginosa* (EC50 49.0 µg/L) and *Pseudokirchneriella subcapitata* (EC50 3100 µg/L) (Robinson et al., 2005).

Regarding other antibiotics, the chloroplasts from *L. minor* are more sensible to fluoroquinolones action (Robinson et al., 2005), thus, the toxicity of ENR is greater than FLO's. The ENR concentrations found in *O. niloticus* muscle and in the environment (µg/g and ng/L) (Xu et al., 2006; Pena et al., 2007) are close to those which cause toxicity for algae and aquatic plant (Robinson et al., 2005).

The antibiotics are toxic to algae and cyanobacteria. This is the main reason that the EU (EMEA/VCMP) obligates the antibiotics toxicity tests on cyanobacteria (EMEA 1998). The toxicity occurs because antibiotics were developed to affect unicellular prokaryotic organisms, which are structurally closer to unicellular microalgae than the multicellular organisms such as microcrustacean and fish (Ferreira et al., 2007).

P. mesopotamicus was a more sensible organism to TOL, with 3.72 mg/L LC50;48h, and *L. minor* was more tolerant, because it showed no phytoxicity, differently from the alga *Selenastrum capricornutum* which showed EC50% 3.16 mg/L (Rojickova et al., 1998).

D. magna was more tolerant to TH than other neonicotinoids, such as guadipyr (EC50;48h 13.01 mg/L) (Qi et al., 2013) and imidacloprid (EC50;48h 10.44 mg/L) (Song et al., 1997). The neonicotinoids show differences on its chemical structure, binding affinity, mode of action in acetylcholine receptors and different metabolites. Those features were responsible for the toxicity difference inside this group (Ford and Casida, 2006).

P. mesopotamicus and *H. eques* were more sensible to TH than *Lepomis macrochirus* and *O. mykiss* (LC50;96h > 100 mg/L). TH and organophosphates show similar mode of action in the nerve synapses. Neotropical fish (e.g. *P. mesopotamicus*) are sensible to these compounds, as verified with trichlorfon (LC50;96h 0.19 mg/L) (Mataqueiro et al., 2008).

TH has similar characteristics as some pesticides detected in groundwater, such as high polarity, high stability, low sorption coefficient, highly leachable, was detected on the soil profile at 1.8 m deep (Castro et al., 2008) and has caused high environmental risk for the organisms used in this research. Based on TH's characteristics, caution and monitoring are necessarily carried out on its use.

Although susceptible to TH and TOL, *P. mesopotamicus* had limitations as a bioindicator, because the spawning occurs once a year, then the amount of young fish available for ecotoxicological assays is low. Thus, *H. eques* displayed satisfactory sensitivity to both drugs and fit the requirements for a good bioindicator. The drugs toxicity for this study

showed safe use and less toxicity to fish than several other unregistered drugs, as the potassium permanganate (4.5 - 17.6 mg/L LC50; 96 h) on *Ictalurus punctatus* (Tucker, 1987); formaldehyde (2.02 mg/L LC50; 96 h) on *Hoplias lacerdae* (Cruz et al., 2005); green malachite (1.40 mg/L LC50; 96 h) on *Heteropneustes fossilis* (Srivastava et al., 1995) and copper sulphate (14 µg/L LC50; 48 h) on *Prochilodus scrofa* (Carvalho and Fernandes, 2006).

Therefore, the drugs ecotoxicology evaluation shows its inherent toxicity, especially for regulation purposes. The bioindicator used was based in its capacity to externalize the drugs toxicity to the environment, showing the drug safety.

Among the molecules studied on this research, FLO and TOL were safer for aquaculture. ENR and TH use requires caution, due to high toxicity levels. Thus, the wastewater treatment before disposal is a measure to avoid the negative effects.

A large amount of xenobiotics has been released into the aquatic environment, direct or indirectly, due to the expansion on the activities related to water use, such as aquatic organisms farming. Thus, the bioindicator development for environmental monitoring is essential for making a decision about the use and/or effluent discharge. For this reason the sequence of ecotoxicological assays may be executed as toxicity evaluation method using *L. minor* as florfenicol bioindicator; *P. canaliculata* for enrofloxacine and *H. eques*, for thiamethoxan and toltrazuril, suggesting that enrofloxacine and thiamethoxan causes high environmental risk for bioindicators.

Conflict of Interest

The authors declared that they have no conflict of interest.

Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted and approved by the University's Institutional Animal Care and Use Committee under approval numbers 017335/10.

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