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Toxicidade aguda e risco ambiental do antibiótico oxitetraciclina para tilápia (*Oreochromis niloticus*), *Daphnia magna* e *Lemna minor*

[*Acute toxicity and environmental risk of oxytetracycline antibiotc for tilapia (Oreochromis niloticus), Daphnia magna, and Lemna minor*]

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RESUMO

O objetivo deste estudo foi classificar o antibiótico Terramicina® de acordo com a toxicidade aguda e o risco de intoxicação ambiental para *Oreochromis niloticus, Daphnia magna* e *Lemna minor*, com base no seu ingrediente ativo oxitetraciclina (OTC). Além disso, observou-se a ocorrência de sinais de intoxicação aguda em peixes e o efeito da diluição do antibiótico sobre as variáveis de qualidade de água. Alevinos, neonatos e frondes foram expostos a concentrações de OTC. De acordo com os resultados dos testes de toxicidade aguda, a Terramicina® foi classificada pela toxicidade aguda e pelo risco de intoxicação ambiental. Para *O. niloticus*, a CL(I)50; 48h calculada foi de 6,92 mg L^{-1}, para *D. magna* a CE(I)50; 48h foi de 0,17mg.L^{-1}, enquanto para *L. minor* a CI(I)50;7d foi de 0,68 mg L^{-1}. A Terramicina® foi classificada como muito tóxica para *O. niloticus* e extremamente tóxica para *D. magna* e *L. minor* e causa risco de intoxicação ambiental para os três organismos testados. Concentrações de 7,5 e 8,0 mg L^{-1} de OTC reduziram a concentração de oxigênio dissolvido na água. De acordo com este estudo, a Terramicina® não deve ser utilizada na aquicultura, pois é altamente tóxica e causa risco de intoxicação ambiental aos organismos teste.

Palavras-chave: peixe, antibiótico, ecotoxicidade, organismos aquáticos, macrófita

ABSTRACT

The aim of this study was to classify the antibiotic Terramycin® according to acute toxicity and the environmental risk that it poses for Oreochromis niloticus, Daphnia magna, and Lemna minor based on its active ingredient oxytetracycline (OTC). In addition, the occurrence of acute poisoning signs in fish and antibiotic dilution effect in the water quality variables were observed. For this purpose, fingerlings, neonates, and while were exposed to the concentrations of OTC. According to OTC acute toxicity test results, the Terramycin® was classified by acute toxicity and environmental poisoning risk classes. To O. niloticus, the calculated LC(I)50;48h was 6.92 mg L^{-1}*, for D. magna the EC(I)50;48h was 0.17 mg* L^{-1}*, while for L. minor, IC(I)50;7d was 0.68 mg* L^{-1}*. Terramycin® was classified as very toxic to O. niloticus, and highly toxic to D. magna and L. minor and cause risk of environmental poisoning for the three organismis tested. Concentrations of 7.5 and 8.0 mg* L^{-1} *OTC reduce the concentration of dissolved oxygen in the water. According to this study, Terramycin® should not be used in aquaculture, as it is highly toxic and causes risk of environmental toxicity test organisms.*

Keywords: fish, antibiotic, ecotoxicity, aquatic organisms, macrophyte

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INTRODUCTION

Stress is one of the major factors capable of triggering the disease process in fish reared under intensive farming systems, causing immunosuppression and increasing fish susceptibility to pathogens (Martty, 1986). Chemical control of diseases is accomplished by adding antibiotics to the fish farming water (Kinkelin *et al*., 1991). However, antibiotics and metabolites can poison non target aquatic organisms. It becomes, therefore, necessary to evaluate the acute toxicity and the environmental poisoning risk that used antibiotics concentrations pose for non-target aquatic organisms that co-habit these aquatic ecosystems (Guilherme, 2005).

A complementary environmental risk assessment starts with the calculation or estimation of risk that may exist for a given target system as a result of exposure; includes hazard identification through dose-response assessment, exposure assessment and risk characterization (Guilherme, 2005). The dose response is obtained by evaluating the acute toxicity for test organisms established by ecotoxicological tests. These tests are performed with non-target organisms that co-habit the studied ecosystems. These organisms are selected as representative of different trophic levels of aquatic biological chains. The environmental risk assessment of antibiotics used in aquaculture is essential in order to adopt measures such as adequate farming process, establishing treatment procedures, day and time of exposure to the agent.

Oxytetracycline, OTC, is one of the most widely used antibiotics in the control of gram-negative bacteria (Rigos *et al*., 2005). In Brazil, the OTC contained in Terramycin® commercial formula is an antibiotic for veterinary use, registered for the control of diarrhea, enteritis, fowl cholera, fowl typhoid, pullorosis, and respiratory diseases in cattle, sheep, goats, pigs, poultry, and rabbits.

Terramycin® is not registered for use in fish farming; however, this antibiotic is used in aquaculture without control. Kubtiza (1997) and Rach *et al*. (2008) found that a dilution of 20.0mg L^{-1} OTC in the water satisfactorily controls fish infections. Thus, because of this effectiveness in control of infections, OTC can be prospected for aquaculture use, provided it shows risk to aquatic organisms.

Toxic substances can be classified into acute toxicity classes according to Zucker (1985) and Sistema… (2013). Under Brazilian law, the class of potential environmental hazard of pesticides is printed on the label and package insert as a preventive measure to warn about immediate danger to the environment. The environmental risk assessment is an important tool for analyzing the impact of toxic substances. Risk assessment determines the highest concentration at which the resulting environmental effect is acceptable during acute exposure in certain laboratory conditions, with the estimated environmental concentration (EEC) resulting from the recommendation to use in the environment, according to the Technical… (2003).

The directive 1488/94 in the Technical… (2003) recommends that risk assessment of existing substances for immediate release to use in aquaculture should include acute toxicity tests with organisms of three different trophic levels.

This study aims to classify Terramycin® based on its active ingredient, OTC, according to acute toxicity and the environmental risk for tilapia *(O. niloticus), D. magna,* and *L. minor* while observing the occurrence of acute poisoning signs in fish and reporting the dilution effect of the antibiotic on water variables: dissolved oxygen, pH, electric conductivity and temperature.

MATERIAL AND METHODS

The acute toxicity tests using the commercial Terramycin© soluble powder with Antigerm 77 were performed according to NBR 15088 (Associação…, 2011), NBR 12713 (associação…, 2009), and LEMMA (2002) for *O. niloticus, D. magna,* and *L. minor,* respectively.

This study was approved by the Ethics Committee on Animal Use (ECAU), College of Agriculture and Veterinary Sciences, UNESP, Jaboticabal – SP (Protocol 021535/14).

Fish from the Aquaculture Center of UNESP were previously acclimated for 10 days at 27 ± 2^{0}C with a 12 hour photoperiod and fed freely on commercial diet. Assays were performed in static exposure system with three repetitions for each concentration, on all the tests, sensitivity (54 fishes) and definitive (72 fishes). Each test was repeated three times.

Initial trials were carried out with the reference substance, potassium chloride (KCl) to assess the health and sensitivity of test organism batch. The average LC(I)50;48h estimated for KCl for tilapia was 1.37 g L^{-1}, with upper (UL) and lower (LL) limits of 1.66 and 1.14 g L^{-1}**,** respectively. Thus, the fish displayed normal health and sensitivity conditions according to the control chart of other batches previously used in the laboratory. After the sensitivity test, preliminary trials with OTC were performed to determine the concentration range that causes 0 and 100% fish mortality. For this purpose, 500 fishes were used, weighing between 0.5 and 1.0 g, and were placed in the test containers at 1 g L^{-1} maximum density, including all tests: sensitivity, preliminary, and definitive.

Water quality parameters during fish acclimation were as follows: pH, between 7.0 and 8.0; electric conductivity, 170.0 and 180.0 μS cm^{-1}; dissolved oxygen, 5.0 and 6.0 mg L^{-1}; temperature, 25 and 26ºC with continuous aeration. These of water were recorded at 0, 24 and 48 hours of fish exposure to KCl (NBR 15088 Associação…, 2011).

The *D. magna* neonates were cultivated in 3 L laboratory glass containers. The culture medium used was the basic M4, consisting of reconstituted distilled water with pH 7.0. An algae suspension containing the species *Scenedesmus subspicatus* at a concentration of 5.0 x 10^6 cells/daphnia/day was provided as feeding for the organisms. The feeding in the culture containers was supplemented with fish feed and yeast solution suspension, at the ratio 1:1.

Initial tests with the sodium chloride (NaCl) reference substance were conducted to assess the health and sensitivity of the test organism batch. The average LC(I)50; 48h estimated for NaCl for the microcrustacean was 5.09 mg L^{-1}, with the upper (UP) and lower (LL) limits of 5.27 and 4.91 mg L^{-1}, respectively**.** Therefore, the organisms displayed normal health and sensitivity conditions according to the control chart of the laboratory. After the sensitivity tests, preliminary tests with the antibiotic were carried out to determine the OTC concentration range that causes 0 and 100% of organism immobility/mortality. The experimental plots consisted of five neonates aged between 6 and 24 hours. The parameters of the culture medium were: pH, 7.0; conductivity between 500.0 and 600.0 μS cm^{-1}; dissolved oxygen, 5.0 and 6.0 mg L^{-1}. The temperature of the handling room and cultivation chamber was 20 ± 1°C.

The macrophytes were grown in medium culture consisting of distilled water and reconstituted with Hoagland's solution, pH 5.8. Only healthy looking plants were selected for testing. Initial growing conditions of the acclimatized room were: light intensity 6000 lux, 12-h photoperiod, and temperature 24 ± 2ºC.

Initially, the NaCl test was performed to evaluate the sensitivity of the organisms. The average IC50(I);48h estimated for NaCl for macrophytes was 4.22 g L^{-1}, with UL and LL of 4.87 and 3.65 g L^{-1}, respectively. Therefore, the macrophytes displayed normal health and sensitivity conditions according to the control chart of the laboratory. Subsequently, OTC preliminary tests were performed to determine the antibiotic concentration range which causes 0 and 100% inhibition of fronds growth. For this, we used four macrophytes containing three fronds each that were sanitized beforehand in 10% sodium hypochlorite solution.

The OTC concentrations used for definitive tests of acute toxicity were 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 mg L^{-1} for *O. niloticus;* 0.03, 0.06, 0.09, 0.12, 0.15, 0.18, 0.21, and 0.24 mg L^{-1} for *D. magna;* and 0.1, 0.3, 0.5, 0.7, 1.0, and mg L^{-1} for *L. minor,* plus a control for each trial. The tests were conducted in static system, without power, water and syphoning. All tests were performed with three replicates, each replicates with three fishes according to NBR 15088 (Associação…, 2011), five neonates according to NBR 12713 (Associação…, 2009) and 12 macrophyte fronds according to LEMMA (2002). Each test was repeated three times. A total of 72 fishes were used, 135 neonates and 210 macrophyte fronds.

During tests with the fish water quality, variables (pH, electrical conductivity, temperature, and dissolved oxygen) were recorded at 0, 24, and 48 hours after exposure to the antibiotic.

At the end of the tests, the parameters fish mortality, *D. magna* immobility/mortality after 48 hours of exposure, and *L. minor* growth inhibition after 7 days of exposure were recorded. After the period of exposure to OTC, according to Doi and Stoskopf (2000), this antibiotic hardly degrades in water under the conditions of this study.

These records were used to calculate the values of LC(I)50;48h, EC(I)50;48h, and IC(I)50;7d for each test organism using the Trimmed Spearman Karber software (Hamilton *et al*., 1977). The OTC values obtained for non-target organisms were used to classify Terramycin® according to acute toxicity in the classes as established by Zucker (1985) and for each species as used by Sistema… (2013) to classify pesticides according to potential environmental hazard (Tab. 1).

Table 1. Values of acute toxicity and acute toxic class according to Zucker (1985) and Sistema… (2013), for non-target organisms

	Acute toxicity values (mg L^{-1})	Acute toxic class
Zucker (1985)	LC50/EC50/IC50 < 0.1	Highly toxic
	0.1 < LC50/EC50/IC50 <1	Very toxic
	1 < LC50/EC50/IC50 <10	Moderately toxic
	10 < LC50/EC50/IC50 <100	Slightly toxic
	LC50/EC50/IC50 > 100	Practically non toxic
Sistema (2013)	0 ≤ LC50/EC50/IC50 <1	Highly toxic
	1 ≤ LC50/EC50/IC50 <10	Very toxic
	10≤ LC50/EC50/IC50 <100	Moderately toxic
	100 ≤ LC50/EC50/IC50	Slightly toxic

Terramycin® was classified according to the risk of acute environmental poisoning for test organisms using the quotient method (Q) of Goktepe *et al*. (2004) and the acute environmental risk calculation (Technical…, 2003). In the Goktepe *et al*. (2004) method, the risk is calculated by dividing the estimated environmental concentration (EEC), based on the effective OTC concentration for controlling fish infections, by the LC(I)50;48h, EC(I)50;48h, and IC(I)50;7d values. According to Technical… (2003), the risk is given by dividing EEC by the predicted no effect concentration (PNEC) of the xenobiotic in acute toxicity test, that is, the highest concentration that did not cause mortality/immobility/growth inhibition of organisms.

The risk of acute environmental poisoning posed by Terramycin® was calculated with the OTC concentration of 20.0 mg L^{-1}, which is considered effective to control fish infections (Kubtiza, 1997; Rach *et al*., 2008). Risk assessment was also calculated for half and twice this recommended concentration (10.0 and 40.0 mg L^{-1}). Terramycin® was classified in acute environmental poisoning risk classes as cited by Goktepe *et al*. (2004) in which RQ > 0.5 is considered high risk, 0.05 < RQ <0.5 is considered medium risk, and RQ < 0.05 has a low risk. According to the Technical… (2003), the risk occurs when the ratio EEC/PNEC > 1 for non-target organisms belonging to all three trophic levels.

RESULTS AND DISCUSSION

For the fish *O. niloticus*, the average LC(I)50;48h OTC in Terramycin® was 6.92 ± 0.69 mg L^{-1}, with LL and UL of 6.6 ± 0.67 and 7.3 ± 0.72 mg L^{-1}, respectively. In a similar study, Carraschi *et al*. (2011) calculated the LC(I)50; 48h of 7.6mg L^{-1} OTC Terramycin® for *Piaractus mesopotamicu).* It can be seen that both values are very similar, which characterizes the sensitivity of these two similar fish farming species that are very important for Brazilian aquaculture. The value of LC(I)50; 48h OTC classifies Terramycin® as moderately toxic (Zucker, 1985) and very toxic (Sistema…, 2013) for both fish farming species.

There was no fish mortality in the control and 5.5 mg L^{-1} OTC. This result shows that there is a possibility of acute poisoning of these species with the effective concentration of 20.0 mg L^{-1} OTC recommended by Kubtiza (1997) and Rach *et al*. (2008). At OTC concentrations of 7.5 and 8.0 mg L^{-1} fishes behavior changed, the lack of ability to pitch, erratic swimming, increased opercular beat, and permanence on the surface of water were observed while in the treatments with the highest concentrations, dissolved oxygen level in the water decreased.

The comparison of acute toxicity shows that OTC is more toxic to *O. niloticus* and *P. mesopotamicus* compared to *Salvelinus namaycush,* whose LC(I)50; 48h was greater than 200.0 mg L^{-1} (Webb, 2001) and Japanese medaka *(Oryzias latipes)* of 110.1 mg L^{-1} (Kim, 2007).

For the *D. magna*, the average EC(I)50;48h was 0.17 ± 0.02 mg L^{-1}, with LL and UL of 0.13 ± 0.02 and LS 0.15 ± 0.02 mg L^{-1}, respectively. This value classifies Terramycin® as very toxic (Zucker, 1985) and highly toxic (Sistema…, 2013) for the microcrustacean. After 48h of exposure to OTC, immobility had not occurred only in the control and treatments with concentrations 0.06 and 0.03 mg L^{-1}. The effective concentration of 20.0 mg L^{-1} is lethal to *D. magna*. This information is important when selecting the best application method for Terramycin® so that the cultivation water is not contaminated thus avoiding non-target organisms.

Terramycin® based on the active ingredient OTC is more toxic to *D. magna* than the antibiotics sulfadiazine, tetracycline, tiamilin, metronidozole, oloquindox, and streptomycin, which have been reported not to cause acute effects in such aquatic species (Wollenberger *et al*., 2000). Terramycin® is also more toxic to *D. magna* than the antibacterials aminosidine, bacitracin, flumequine are for *Artemia franciscana;* whose EC(I)50;48h values were calculated as 2220.0, 21.82, and 307.7 mg L^{-1}, respectively (Migliore *et al*., 1997).

For the macrophyte *L. minor*, the average IC(I) 50;7d OTC calculated was 0.68 ± 0.04 mg L^{-1}, with LL and UL of 0.60 ± 0.02, and 0.78 ± 0.05 mg L^{-1}, respectively. This value classifies Terramycin® as very toxic (Zucker, 1985) and highly toxic (Sistema…, 2013). After seven days of exposure macrophyte growth had not been inhibited in the control and 0.1 mg L^{-1} OTC.

The macrophytes exposed to lower concentrations of OTC Terramycin® produced greater fronds than those exposed to higher concentrations as observed by Romero (2008). The author cites that OTC reduces plant buoyancy and growth. Probably, OTC active ingredient causes disruption of protein synthesis, changes chloroplast and chlorosis in the leaves of the plant. High concentrations of this active ingredient causes chlorosis in *L. minor and* in the microalgae *Prasinophyte* and reduces the number of fronds of *L. minor* (Ferreira *et al*., 2007). At concentrations above 5.3mg L^{-1} OTC reduces the growth of *Prasinophyte* microalgae (Ferreira *et al*., 2007). This concentration is higher than that tested in this study, which demonstrates that the OTC in Terramycin® may inhibit the growth of plants and algae.

The acute environmental risk of Terramycin® containing the active ingredient OTC effective for fish farming according to calculations of Goktepe *et al*. (2004) and Technical… (2003) are shown in Tab. 2 and 3, respectively.

Table 2. Environmental risk of OTC Terramycin® for *O. niloticus*, *D. magna* and *L. minor* according to Goktepe *et al*. (2004)

Test organisms	EEC (mg L^{-1})	LC/EC/IC (mg L^{-1})	RQ	Risk
O. niloticus	10.0	6.92	1.45	High risk
	20.0		2.89	
	40.0		7.23	
D. magna	10.0	0.17	58.82	High risk
	20.0		117.64	
	40.0		235.29	
L. minor	10.0	0.68	14.70	High risk
	20.0		29.41	
	40.0		58.82	

Table 3. Environmental risk of OTC Terramycin® for *O. niloticus*, *D. magna*, and *L. minor* according to Technical… (2003)

Test organisms	EEC (mg L^{-1})	PNEC (mg L^{-1})	RQ	Risk
O. niloticus	10.0	5.5	1.82	> 1 (poses risk)
	20.0		3.64	
	40.0		7.27	
D. magna	10.0	0.06	12.12	> 1 (poses risk)
	20.0		333.33	
	40.0		666.66	
L. minor	10.0	0.1	100.00	> 1 (poses risk)
	20.0		200.00	
	40.0		400.00	

Terramycin® is classified according to Goktepe *et al*. (2004) as high risk for acute environmental poisoning while according to Technical... (2003) is classified as posing risk of acute environmental poisoning for *O. niloticus, D. magna,* and *L. minor*. It is observed that the classification criterion is the predicted no effect concentration (acute PNEC), which is a value lower than the acute lethal effect of 50%. Therefore, the classification criterion of the Technical... (2003) is more accurate and provides greater environmental protection. In order to use Terramycin®, an antibiotic for veterinary use, in aquaculture, the most suitable criterion for assessing the environmental risk of acute poisoning is given by the Technical... (2003) since it is based on the PNEC.

Of the water quality parameters recorded, only the dissolved oxygen concentration was inversely proportional to the OTC concentrations in water. The oxygen concentration decreased from 4.5 ± 0.71 mg L^{-1} in the control treatment to 1.02 ± 0.4 mg L^{-1} in the treatment with 8.0 mg L^{-1} OTC. A similar result has been observed in OTC acute poisoning assessment for *Piaractus mesopotamicus* where the concentration of dissolved oxygen in the water was 4.87 mg L^{-1} in the control treatment and 1.60 mg L^{-1} at a concentration of 8.5 mg L^{-1} OTC (Carraschi *et al*., 2011). This may be caused by increased oxygen consumption by fish due to adhesion of antibiotic to the gills, which prevents oxygen absorption, and the evidence of such was the permanence of fish on the water surface.

The highest concentration tested caused 100% fish mortality while the dissolved oxygen level was 1.20 mg L^{-1}. The mortality during the trials to assess poisoning risk of OTC Terramycin® was due to the toxicity of the antibiotic for the fish and not the reduced dissolved oxygen concentration in the water. This statement is due to the fact that *O. niloticus* tolerates very low concentrations of dissolved oxygen in the water and live well with concentrations of 1.2 mg L^{-1} (Macedo, 2004).

The average water temperature was 24.0 ± 1.0ºC in the control and 23.80 ± 0.85ºC in the 8.0 mg L^{-1} of the antibiotic, the electrical conductivity was 202 ± 0.2 µS cm^{-1} in the control and 198 µS cm^{-1} ± 0.03 for concentration 8.0 mg L^{-1}, and the pH of 7.22 ± 0.05 in control and 6.80 ± 0.52 at the highest concentration tested. These water quality parameters are within the recommended by NBR 15088 (Associação…, 2011) for toxicity tests.

CONCLUSION

Terramycin® is classified as very toxic to *O. niloticus,* and highly toxic to *D. magna* and *L. minor* and cause risk of environmental poisoning for the three organismis tested. Concentrations of 7.5 and 8.0 mg L^{-1} OTC reduce the concentration of dissolved oxygen in the water.

According to this study, Terramycin® should not be used in aquaculture, as it is highly toxic and causes risk of environmental toxicity test organisms.

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