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EFFECTS OF PHENOL ON *Astyanax bifasciatus* AND *Daphnia magna*

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Abstract: In the present study, we investigated the possible toxic effects of phenol on two bioindicators: the fish *Astyanax bifasciatus* (Characiformes; Characidae) and the crustacean *Daphnia magna* (Cladocera; Daphnidae). We performed bioassays with *A. bifasciatus* (96 h and 15 days of exposure) and *D. magna* (48 and 96 h of exposure). The bioindicator organisms were exposed to the following concentrations of phenol: 0.003 mg L^{-1}, 0.01 mg L^{-1}, 0.03 mg L^{-1}, 0.06 mg L^{-1}, and 0.1 mg L^{-1}; and the control group was maintained in clear water. We assessed the rate of micronuclei in erythrocytes of fish and evaluated the immobility of *D. magna* at each concentration of the contaminant. For fish, the 15-day exposure revealed a significant difference between the tested groups ($p = 0.0002$), showing a tendency for micronuclei to increase at the highest concentrations. For the *D. magna* test, 96-hour toxicity was evident at all tested concentrations ($p < 0.05$). A better response of these organisms was observed during the longer exposure period, emphasizing the concern on the long-term effects of phenol. Effects on the lowest concentration of phenol tested (0.003 mg L^{-1}) were also observed, and this concentration is permitted by the Brazilian Legislation for Class II waters, demonstrating that the longer time of exposure to phenol can cause damage to the biota.

Keywords: aromatic compounds; ecotoxicology; micronucleus assay; mortality; pollution.

INTRODUCTION

The impact of anthropogenic development on freshwater ecosystems is of great concern to environmental managers, and such ecosystems are currently among the most threatened environments in the world (Malmqvist & Rundle 2002, Liao *et al.* 2018). Among the various toxic substances released in these environments, phenolic compounds can occur widely in aquatic ecosystems and they are considered a priority pollutant (Barber *et al.* 1995). Phenol is an aromatic chemical commonly found in domestic and industrial effluents that represents a worldwide concern in toxicology (Jiang *et al.* 2006, Michałowicz & Duda 2007, ATSDR 2008, Surkatti & El-Naas 2017).

As phenol has high water affinity and low volatility (Prpich & Daugulis 2005), exposure to untreated phenol released in the aquatic environment has been reported to be extremely harmful (Huang *et al.* 2014). These compounds have a direct effect on living species and persist

in the environment in forms that are easily assimilated by fauna and flora that inhabit or make use of these water sources (Kenaga 1982). These toxicants can then accumulate in organisms and be bio-magnified through food webs (Ahel *et al.* 1993).

Phenol contamination of water bodies has serious environmental implications due to the damaging effects it has on aquatic organisms (Maleki *et al.* 2005). Duan *et al.* (2018) found that LC_{50} values for all organisms ranged from 0.26 to 1,204.6 mg L^{-1} and at intermediate concentrations may affect metabolism (Holmberg *et al.* 1972), survival and growth (Dauble *et al.* 1983) and reproductive potential (Dauble *et al.* 1983, Mukherjee *et al.* 1991). Different endpoints may reflect different aspects of the overall toxicity exerted by the chemical; in addition, contaminants often exert more than one mode of action, which may lead to different response characteristics of biological species dependent on the metabolism and inventory of receptors (Schüürmann *et al.* 1997).

Brazilian Federal Law CONAMA nº. 357/2005 (CONAMA 2011), for water classification, determines that the disposal of the phenolic compounds in the receiving water bodies is limited to 0.003 mg L^{-1} for class II water, which is water destined for human consumption after conventional treatment, and the limit suggested by the World Health Organization is 0.001 mg L^{-1} for drinking water. The Iguaçu River is one of the most important tributaries of the Paraná River and is used for public supply, but it is considered one of the most polluted rivers in Brazil, due to large amounts of urban and industrial waste that are released without adequate treatment (Carneiro *et al.* 2014). The concentration of phenol in this river has been higher than that allowed by the resolution, with values higher than 0.003 mg L^{-1} in the river (Erbe *et al.* 2011, Kosera 2014, Teixeira 2015).

Studies using micronucleus assays with fish erythrocytes, in particular, have been carried out for genotoxic evaluations of polluted aquatic environments such as rivers (Flora *et al.* 1993, Viganò *et al.* 2002, Vaz *et al.* 2016), lakes (Grisolia & Starling 2001, Braham *et al.* 2017), and sea water (Flora *et al.* 1991, Baršienė *et al.* 2015), showing the sensitivity of these biological systems. In this study, we used *Astyanax bifasciatus* Garavello & Sampaio, 2010 (Characiformes; Characidae) because it is an abundant species with a geographic distribution that is restricted to the Iguaçu basin (Baumgartner *et al.* 2012) and also because it has been used in other studies as a test organism (Erbe *et al.* 2011, Bueno-Krawczyk *et al.* 2015).

Bioassays that are commonly used for the evaluation of water toxicity involve the toxicity factor of the static system in the laboratory for *Daphnia magna* Straus, 1820 (Cladocera; Daphnidae). Daphniids are widely used as model organisms for determining aquatic ecosystem dynamics and health (Lari *et al.* 2016). They are an ideal laboratory organism and have been used extensively in ecotoxicological studies and government standardized test method protocols; thus, we use the specimen because it is a good biological indicator of water quality and effluent toxicity (Peltier 1978, Knie & Lopes 2004, Zagatto 2008, ABNT 2016).

Considering the potential toxic effects of phenol in biota and the concentration of phenol allowed by Brazilian legislation in relation to the phenol concentrations found in the Iguaçu River, our hypothesis is that in fish exposed to higher concentrations of phenol, they will show a higher frequency of nuclear abnormalities, and the *D. magna* mortality rate will be higher at higher concentrations.

MATERIAL AND METHODS

Phenol concentrations

We prepared a synthetic medium using distilled water supplemented with nutrients (US EPA 2002). We used the following concentrations of Phenol P.A. 99 %: 0.003 mg L^{-1}, 0.01 mg L^{-1}, 0.03 mg L^{-1}, 0.06 mg L^{-1}, and 0.1 mg L^{-1}. The choice of test concentrations was based on the analysis of CONAMA resolution 357/2005 (CONAMA 2011), which establishes a value of 0.003 mg L^{-1} for total phenols in freshwater (class II) and 0.01 mg L^{-1} for total phenols in freshwater (class III), which were represented in the present study. The highest concentrations of 0.06 and 0.1 mg L^{-1} were proposed because of the amount of phenol detected in drinking water in União da Vitória, Paraná State, Brazil (Teixeira 2015). A concentration of 0.03 mg L^{-1} as an intermediate value between 0.01 and 0.06 mg L^{-1} was also proposed.

***Experimental design:* Astyanax bifasciatus**

We prepared six 30 L aquaria: one control aquarium and five experimental aquaria with their respective concentrations, no replicates. Ten specimens of the Neotropical fish *A. bifasciatus* (weight: 12.61 ± 3.65 g; length: 9.10 ± 1.05cm) - which are commonly known as "lambari" – were placed in each aquarium, totaling 60 individuals. The specimens were acquired commercially from a fish farm and acclimatized in the laboratory for one week before the start of the experiments. After acclimatization the fish in the experimental groups were subjected to exposure of 96 h and 15 days. Every 24 h, the fish were fed with feed (3 mm, 42 % protein), and 20 % of the total volume of water was exchanged, immediately restoring the phenol content to the test concentration in the aquaria. During the bioassay, we measured the following physical/chemical parameters, every 24 h: temperature (ºC), hydrogen potential (pH), electrical conductivity (μScm^{-1}), and dissolved oxygen ($mg\ L^{-1}$) were measured by using a multiparameter Asko AK88 and pHmeter MS Tecnopon. Total hardness ($mg\ L^{-1}$) and ammoniacal nitrogen ($N\text{-}NH_3$) were carried out by Alfakit.

The micronucleus test was performed according to the technique described by Heddle (1973) and Schmid (1975). For each fish collected, a glass slide was prepared using a blood sample from the heart and deposited on the slide to form a thin smear, which was dried at room temperature for 24 h then fixed in absolute ethanol for 30 min. Each slide was subsequently stained with 10 % Giemsa solution for 13 min. The frequency of micronuclei and nuclear morphologic alterations were observed at 40x magnification (Nikon optical microscope) and scored for the presence of both typical micronuclei and nuclear alterations (*Blebbed, Lobed, Notched* and *Vacuolated*) that were manifested as changes in the normal elliptic shape of the nuclei (Carrasco *et al.* 1990, Ayllon & Garcia-Vazquez 2000).

***Experimental design:* Daphnia magna**

Daphnia magna juveniles were obtained in laboratory culture with ages varying between 6 and 24 h of life. Ten specimens were distributed in recipients of 20 mL containing reconstituted water in the control and the addition of the phenol concentrations in the experimental groups, with five experimental groups and the control in triplicate, totaling 180 individuals. Subsequently, the groups were submitted to 48 h of exposure (ABNT 2016); additionally, we proposed an exposure of 96 h, in which they were fed with *Raphidocelis subcapitata* (Sphaeropleales, Selenastraceae). The immobility was evaluated after 48 and 96 h of exposure, by means of absence of movement at each concentration and control. During the bioassays, we kept the specimens in an incubator chamber for biochemical oxygen demand (BOD) to maintain a photoperiod of 12 h light/12 h dark at the optimum temperature of 24 °C (ABNT 2016). We also measured the following physical/chemical variables, every 24 h: temperature (ºC), hydrogen potential (pH), electrical conductivity (μScm^{-1}), and dissolved oxygen ($mg\ L^{-1}$), which were measured by using a multiparameterAskoAK88 and pHmeter MS Tecnopon. Total hardness ($mg\ L^{-1}$) and ammoniacal nitrogen ($N\text{-}NH_3$) were carried out by Alfakit.

The Toxicity Factor is a dimensionless number that expresses the lowest dilution of an effluent that does not have an acute deleterious effect on an organism. For the purposes of the present study, 0.1 $mg\ L^{-1}$ phenol was considered the concentration of the pure sample, with a 100 % dilution factor; 0.06 $mg\ L^{-1}$ had a 60 % dilution factor; 0.03 $mg\ L^{-1}$ had a 30 % dilution factor; 0.01 $mg\ L^{-1}$ had a 10 % dilution factor; and 0.003 $mg\ L^{-1}$ had a 3 % dilution factor. The control group had a zero-dilution factor because it only contained water.

Statistical analyses

Variables presented normal distribution among the variances by the Shapiro Wilk's test and were investigated using Analysis of Variance (ANOVA). The differences between the groups were determined by Dunnett's test. The results were considered significant at the 95 % level ($p < 0.05$). Statistical analyses were performed using the Statistica 7.1 program (StatsoftInc 2005).

RESULTS

Astyanax bifasciatus

During the whole study period, there was no statistically significant difference between the values of the physical/chemical parameters of the water in the experimental groups (Table 1).

In the piscine micronucleus test, we analyzed the frequency of nuclear abnormalities in 2,000 cells in all the treated groups. For the exposure

Table 1. Mean and standard error for physical and chemical water parameters for control and experimental groups of *Astyanax bifasciatus* (Characiformes; Characidae) measured every 24 h during the whole experiment. Control = Control group (without contaminant); mg L^{-1} = experimental group with Phenol P.A. 99% concentration of: 0.003 mg L^{-1} in water; 0.01 mg L^{-1} in water; 0.03 mg L^{-1} in water; 0.06 mg L^{-1} in water; and 0.1 mg L^{-1} in water.

Parameters	Control	0.003 mg L^{-1}	0.01 mg L^{-1}	0.03 mg L^{-1}	0.06 mg L^{-1}	0.1 mg L^{-1}	p
Temperature (°C)	18.7 ± 2.1	18.8 ± 2.1	18.6 ± 2	18.6 ± 1.9	18.6 ± 2	18.5 ± 1.9	0.98
pH	7.2 ± 0.4	7.2 ± 0.4	7.2 ± 0.4	7.2 ± 0.4	7.2 ± 0.4	7.2 ± 0.4	0.99
Ammonia (mg L^{-1})	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.04 ± 0.02	0.99
Total hardness (mg L^{-1})	194 ± 20	203 ± 10	204 ± 15	202 ± 10	198 ± 12	201 ± 8	0.84
Conductivity (μS cm^{-1})	26.5 ± 17	30.3 ± 22.5	39.6 ± 28.8	50.9 ± 31.5	30.5 ± 21.5	38 ± 30	0.72
Dissolved oxygen (mg L^{-1})	10.8 ± 1.9	11.2 ± 2.5	11.2 ± 2.8	11.2 ± 3.1	11.1 ± 2.5	11.2 ± 2.7	0.99

of 96 h, we detected no statistically significant difference among our study groups, as determined by the ANOVA ($F_{5;24}$ = 0.7893, p = 0.5677). However, the mean concentration of 0.01 mgL^{-1} was higher, presenting morphologic alterations of the *Blebbed* and *Lobed* type (Figure 1). After 15 days of exposure, we detected a statistically significant difference between the studied groups, as determined by ANOVA ($F_{5;24}$ = 7.6082, p = 0.0002). Nuclear morphological alterations of the *Blebbed* and *Notched* type were found from the concentration of 0.03 mg L^{-1} (Figure 2).

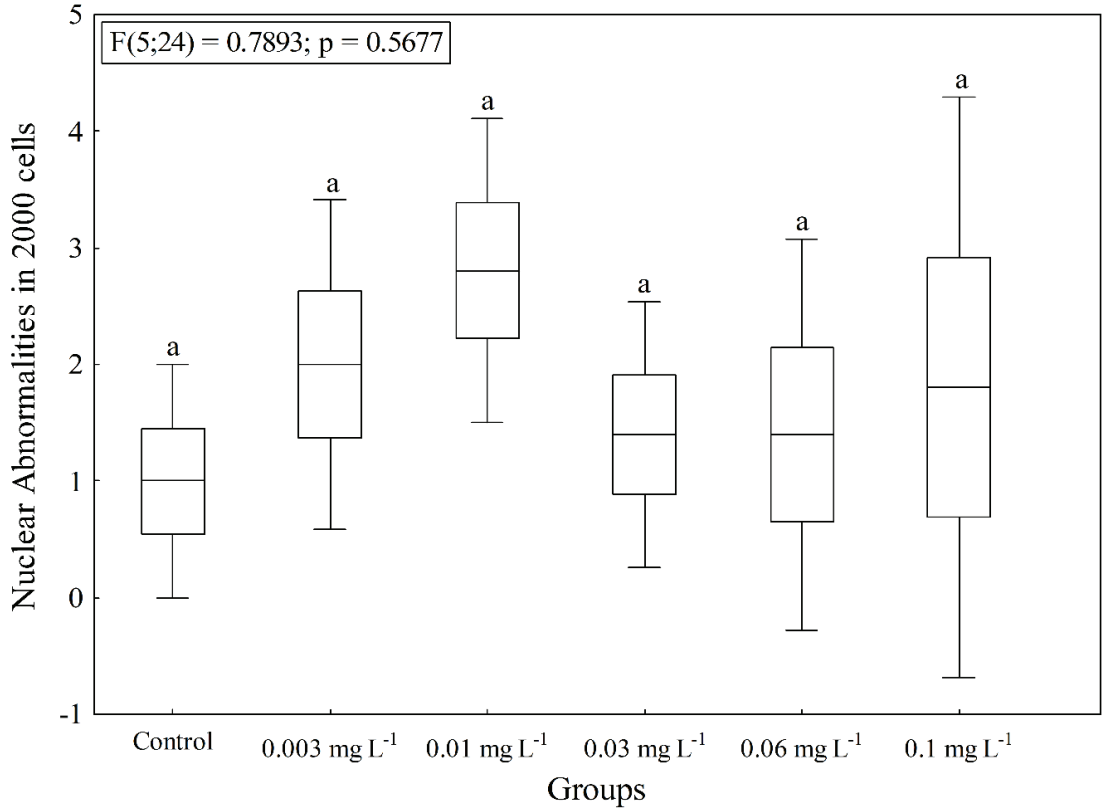

Figure 1. Comparison of nuclear abnormalities among groups subjected to the micronucleus test (96 h of exposure in phenol) for *Astyanax bifasciatus* (Characiformes; Characidae). Significant values at an alpha < 0.05. The lowercase letters indicate comparisons between groups; the same lowercase letters indicate no statistically significant difference, and different lowercase letters indicate a statistically significant difference.

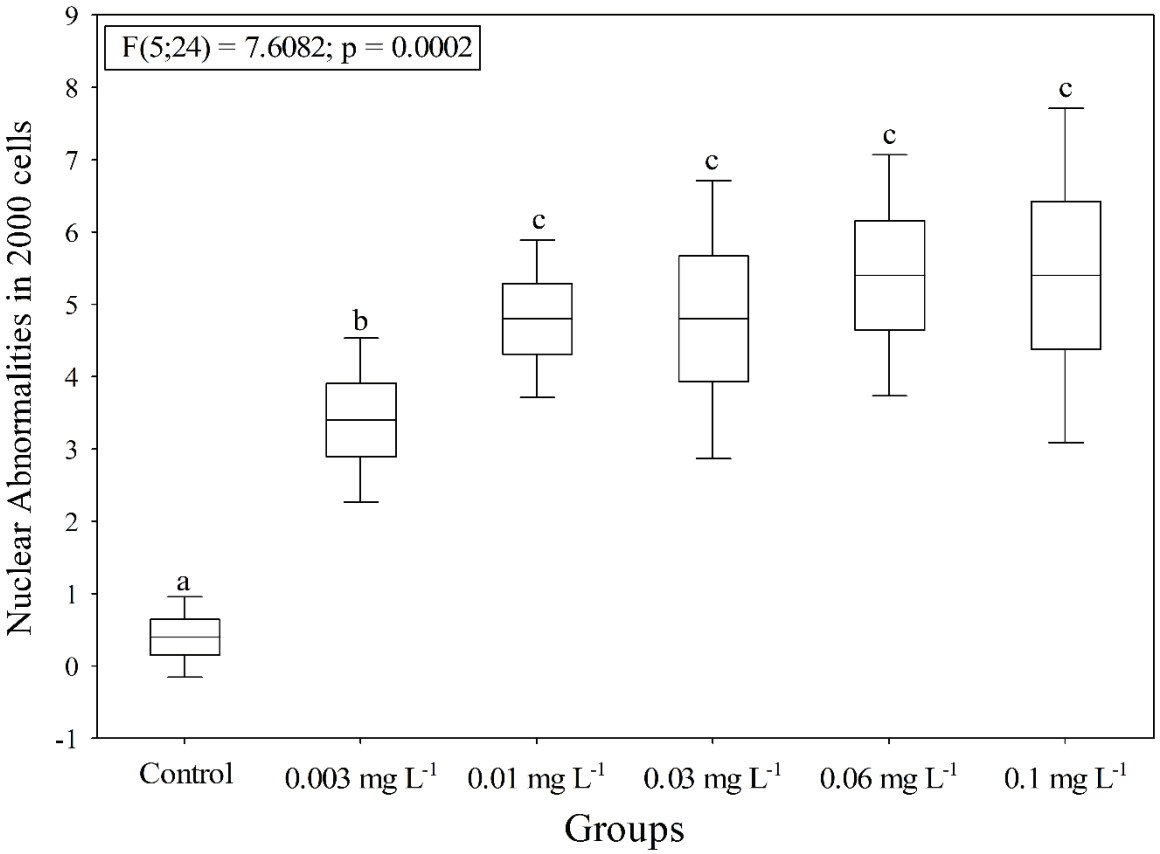

Figure 2. Comparison of nuclear abnormalities among groups subjected to the micronucleus test (15 days of exposure in phenol) for *Astyanax bifasciatus* (Characiformes; Characidae). Significant values at an alpha < 0.05. The lowercase letters indicate comparisons between groups; the same lowercase letters indicate no statistically significant difference, and different lowercase letters indicate a statistically significant difference.

Daphnia magna

There was no statistically significant difference between the values of the physical/chemical variables of the water in the experimental groups (Table 2). After 48 h of exposure, there were few immobile individuals and there was no significant difference among the tested groups (ANOVA: $F_{5;30}$ = 1, p = 0.4346) (Figure 3). In 96 h of exposure, there was a significant increase in the immobility of the individuals as the phenol concentration increased (ANOVA: $F_{5;30}$ = 11.6316, p = 0.000003). The immobility was found at a concentration of 0.003 mg L^{-1}, which killed 11.6 % of the individuals, reaching 33 % at the concentration of 0.1 mg L^{-1} (Figure 4).

DISCUSSION

For the species *A. bifasciatus,* the 15 days of exposure were more effective in detecting the formation of nuclear abnormalities, which were evident at 0.003 mg L^{-1}, becoming more frequent as the phenol concentration increased. When the DNA is damaged, or changes to the micronuclei occur and the lesion is not repaired, a series of biologically harmful reactions is initiated at the cellular level that ultimately affect the whole body of the animal (Lee & Steinert 2003). Nuclear morphological changes are induced owing to the influence of cytotoxic compounds on the integrity of the nuclear lamina, which comprises intermediate filaments and membrane-associated proteins that are responsible for conferring stability and the regular oval shape of the nucleus (Alberts *et al.* 2002). The formation of micronuclei in dividing cells is the result of chromosome breakage due to unrepaired or misrepaired DNA lesions, or chromosome mal-segregation due to mitotic malfunction. These events may be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in cell-cycle checkpoint and/or DNA repair genes, and by deficiencies in nutrients required as co-factors

Table 2. Mean and standard error for physical and chemical water parameters for control and experimental groups of *Daphnia magna* (Cladocera; Daphnidae). Control = Control group (without contaminant); mg L = experimental group with Phenol P.A. 99% concentration of: 0.003 mg L^{-1} in water; 0.01 mg L^{-1} in water; 0.03 mg L^{-1} in water; 0.06 mg L^{-1} in water; and 0.1 mg L^{-1} in water.

Parameters	Control	0.003 mg L^{-1}	0.01 mg L^{-1}	0.03 mg L^{-1}	0.06 mg L^{-1}	0.1 mg L^{-1}	p
Temperature (°C)	19 ± 1.3	19.8 ± 1.1	19.6 ± 2	18.6 ± 1.9	18.6 ± 2	18.5 ± 1.9	0.98
pH	7.7 ± 0.4	7.8 ± 0.2	7.7 ± 0.1	7.9 ± 0.1	7.7 ± 0.3	7.4 ± 0.2	0.97
Ammonia (mg L^{-1})	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02	0.99
Total hardness (mg L^{-1})	174 ± 20	190 ± 15	200 ± 5	200 ± 20	180 ± 15	210 ± 10	0.87
Conductivity (µS cm^{-1})	30.5 ± 10	29.3 ± 15	34.2 ± 25	41 ± 11	34 ± 18.8	41 ± 10	0.89
Dissolved oxygen (mg L^{-1})	10 ± 1.5	10.2 ± 2	11.1 ± 1.5	11.1 ± 1.1	10.5 ± 2.1	11.2 ± 0.7	0.97

in DNA metabolism and chromosome segregation (Iarmarcovai *et al.* 2008).

The acute toxicity of phenol is widely reported in the literature (Holcombe *et al.*1982, Duan *et al.* 2018). The mechanism of action of phenol is multifactorial (Roche & Bogé 2000). The toxic effects of phenol and its derivatives in several fish species have been reported, including hematological alterations (Roche & Bogé 2000), induction of genotoxicity (Bolognesi *et al.* 2006), carcinogenesis and mutagenesis (Tsutsui *et al.* 1997, Yin *et al.* 2006), endocrine disruption (Kumar & Mukherjee

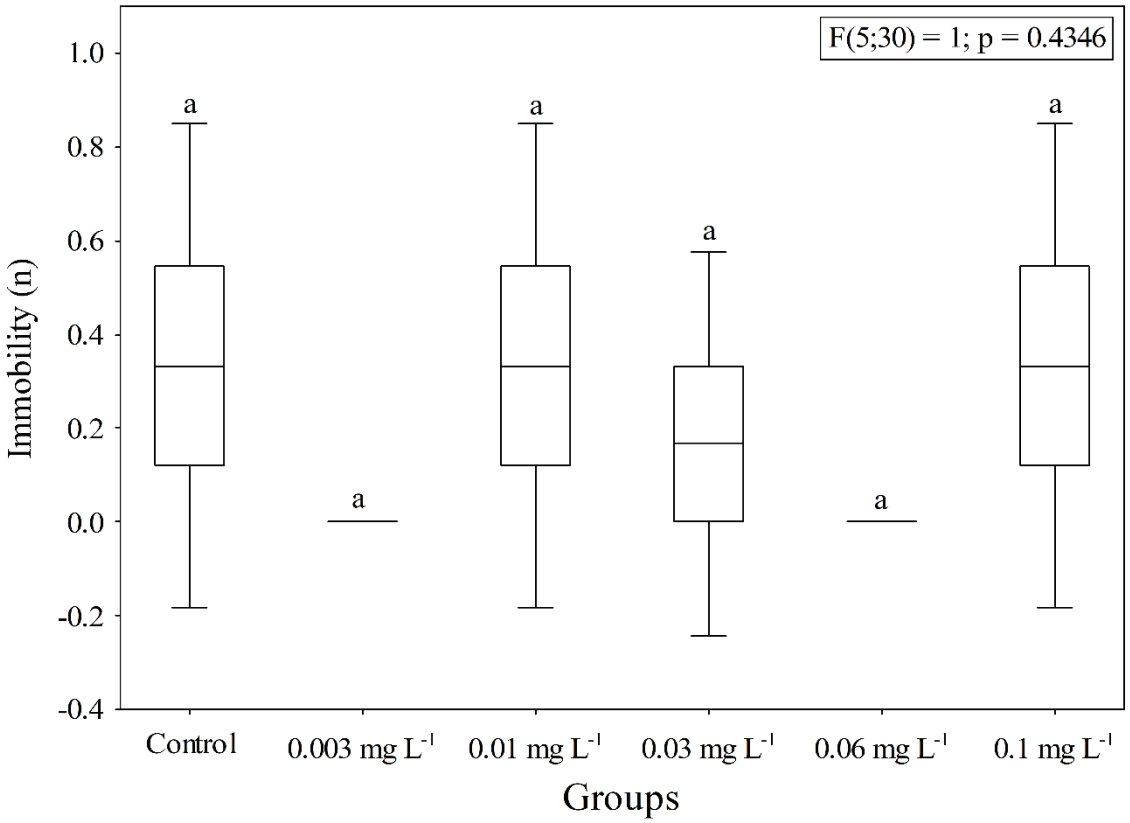

Figure 3. Number of immobile *Daphnia magna* (Cladocera; Daphnidae) after 48 h of exposure in phenol. Significant values at an alpha < 0.05. The lowercase letters indicate comparisons between groups; the same lowercase letters indicate no statistically significant difference, and different lowercase letters indicate a statistically significant difference.

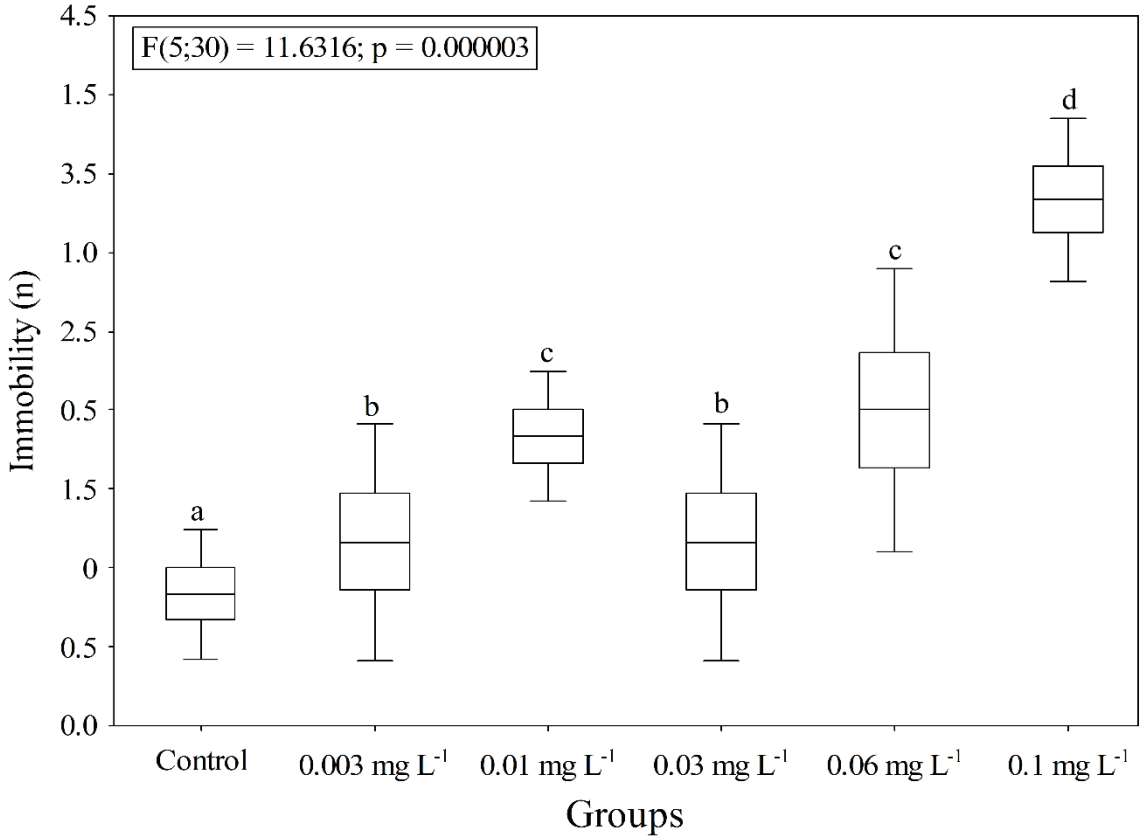

Figure 4. Number of immobile *Daphnia magna* (Cladocera; Daphnidae) after 96 h exposure in phenol. Significant values at an alpha < 0.05. The lowercase letters indicate comparisons between groups; the same lowercase letters indicate no statistically significant difference, and different lowercase letters indicate a statistically significant difference.

1988), and metabolism imbalance (Hori *et al.* 2006). Chlorinated phenol compounds had a median lethal concentration (LC_{50}) of 4.0 mg L^{-1} in the fish *Lepomis macrochirus* (Perciformes, Centrarchidae) over 24 h (Buccafusco *et al.* 1981). Adelman *et al.* (1976) demonstrated that fish exposed to pentachlorophenol exhibited an increase in swimming activity, followed by quiescence, and subsequent death. The limit suggested by the Brazilian legislation is 0.003 mg L^{-1} for class II waters, an exposure level which in this study was able to cause cellular damage, demonstrating that the concentrations found in analyses conducted in the Iguaçu River (Erbe *et al.* 2011, Kosera 2014, Teixeira 2015) may cause damage to the organisms that live in these places.

For *D. magna*, the physical/chemical variables remained adequate according to ABNT (ABNT 2016). Only the control group showed immobility in less than 10 % of subjects after chronic exposure. This shows that even at lower concentrations allowed by Brazilian legislation, which in this study was 0.003 mg L^{-1}, the death of individuals is verified. In other studies, the phenol has an LC_{50} of 0.10 mmol L^{-1} in *D. magna* (Arambasic *et al.* 1995). Colonetti (2013) demonstrated that phenol at a concentration of 12.5 mg L^{-1} caused 100% immobility in *D. magna* specimens over 48 h.

Ecotoxicity tests using bioindicators can provide insight into the potential toxic effects of hazardous chemicals in aquatic environments (Park & Choi 2007, Erbe *et al.* 2011). The Iguaçu River, which receives a large discharge of effluents, has limits of contaminants and heavy metals that are greater than those permitted by Brazilian legislation (Erbe *et al.* 2011, Melo-Silva *et al.* 2018), and may cause potential effects throughout the biota, since they are in direct contact with these contaminants.

In this study, we evaluated the response of two bioindicators in relation to phenol, which presented greater genetic and lethal effects on organisms in the higher period of exposure, even at lower concentrations allowed by Brazilian legislation. Thus, the importance of detecting the presence of

toxic substances and assessing their impact on biota is of extreme importance, because an assessment of the quality of the affected environments, which are often used by downstream human populations, is required (Kumar & Han 2010).

Once introduced into an aquatic system, pollutants can accumulate in the food chain, causing effects at higher trophic levels, and these effects are often overlooked by government agencies and public conservation policies (Hickey *et al.* 2006, Igbinosa *et al.* 2013). The health of *A. bifasciatus* following exposure to the lowest concentration of phenol tested (0.003 mg L^{-1}) and the death of *D. magna* individuals demonstrate that phenol can damage the biota, regardless of the concentration allowed by Brazilian legislation. It is shown that the values set by CONAMA for Class II waters are very high and definitely cannot provide protection for aquatic biota, so these levels should be reduced and further studies should be designed to clarify and define appropriate concentrations. This information can be used to ensure the protection of all aquatic biota and to prevent damage to humans in the future, as well as reducing the phenol concentrations present in the Iguaçu River and other water bodies.

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