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Genotoxic effect of Phenanthrene on *Chironomus sancticaroli* (Diptera: Chironomidae)

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ABSTRACT. Phenanthrene, a Polycyclic Aromatic Hydrocarbon, remains adsorbed to sedimentary particles in aquatic environments. It affects mainly benthic organisms, and is considered potentially genotoxic. In ecotoxicology, species of *Chironomus* Meigen, 1803 are widely known as bioindicators of the effects of chemicals on aquatic organisms. This study investigates the effects of phenanthrene on the size of the head capsule of *Chironomus sancticaroli* Strixino & Strixino, 1981 larvae after chronic (eight days) exposure, and DNA damage after acute (96 hours) and chronic exposure (eight days), under laboratory conditions. DNA damage, evaluated using the alkaline comet assay, detected effects for both exposure periods, indicating that phenanthrene is toxic for *C. sancticaroli*. For the acute exposure, we analyzed five concentrations of phenanthrene, between 0.16 $mg.l^{-1}$ and 1.60 $mg.l^{-1}$, detecting significant differences (Kruskall-Wallis test with $p \leqslant 0.05$) in the degree of DNA damage in all groups. These effects were not dose-dependent. For the chronic exposure, two concentrations (0.16 $mg.l^{-1}$, 0.83 $mg.l^{-1}$) were analyzed, and DNA damage was observed in both. Again, the effects were not dose-dependent. This indicates that phenanthrene is genotoxic to larvae of *C. sancticaroli* even at low concentrations. The size of the head capsule was evaluated after chronic exposure to concentrations of 0.16 $mg.l^{-1}$ and 0.83 $mg.l^{-1}$. Significant differences (ANOVA test with $p \leqslant 0.05$) were detected in the two concentrations, and a reduction in the size of the larval head capsule was observed. This suggests that phenanthrene causes delay in larval development. These results indicate that phenanthrene affects the development of and causes DNA damage in *C. sancticaroli* larvae. Therefore, we suggest that *C. sancticaroli* can be used as a biological indicator for environmental contamination with phenanthrene.

KEY WORDS. Bioassay; bioindicador; development; DNA damage; PHAs.

Polycyclic Aromatic Hydrocarbons (PAHs) are chemicals contaminants. They are considered a priority in the study of environmental contamination (Meire *et al*. 2007) because they are extensively distributed in aquatic and terrestrial environments and are toxic. Some PAHs are potential mutagens and carcinogens (WHO 1998).

Environmental contamination with PAHs results from anthropogenic activities such as fossil fuel burning, accidental spillage of oil and inadequate disposal of industrial effluents (Jacques *et al*. 2007, Meire *et al*. 2007). In the aquatic environment, these compounds remain adsorbed to sediment particles and to suspended organic matter (Netto *et al*. 2000). Therefore, benthic invertebrates are directly exposed to PAHs, which are toxic in after short and prolonged exposure (Paumen *et al*. 2008).

Phenanthrene is one of the 16 PAHs that are considered relevant for environmental monitoring by the United States Environmental Agency. It is potentially genotoxic, mutagenic and neurotoxic (USEPA 1986, Martyniuk *et al*. 2009). Even though the effects of Phenanthrene at the cellular level are still poorly understood, it inhibits protein synthesis, thereby suppressing the immune system of organisms such as earthworms, insects and mollusks (Brown *et al*. 2010, Hannam *et al*. 2010, Marinkovic *et al*. 2011).

Among benthic invertebrates, *Chironomus* larvae are used as bioindicators of the impact caused by environmental contaminants (Michailova *et al*. 2010). Toxicity can be detected at the various organization levels of these insects, for instance molecular and population level. Toxicity at the molecular level can be evaluated by observing chromosomal polytene mutations and DNA strand breaks. At the level of populations, on the other hand, toxicity is expressed as changes in size, fecundity and weight of organisms (Lee & Choi 2006, Park & Choi 2009, Nair *et al*. 2011).

Responses that occur at the molecular level determine the early effects on populations (Freire *et al*. 2008). The comet assay is an important tool for assessing genotoxicity, and has been employed in the detection and quantification of DNA damage such as breaks, alkali-labile sites and crosslinks (Cotelle & Férard 1999, Platt *et al*. 2008). The alkaline comet assay has been used to detect toxicity in Chironomidae after exposure to nonylphenol, bisphenol A and silver nitrate (Lee & Choi 2006, Park & Choi 2009, Nair *et al*. 2011, Al-Shami *et al*. 2013).

Even though phenanthrene is found in aquatic environments and chironomids are important bioindicators, the effects of phenanthrene on different levels of the biological organization of these insects are poorly understood. Such information may be important for environmental monitoring and for assessing the environmental risks of having phenanthrene in aquatic ecosystems.

The objective of the present study was to evaluate the genotoxic effect of phenanthrene on the DNA of *Chironomus sancticaroli* Strixino & Strixino, 1981 after acute (96 hours) and chronic (eight days) exposure and on the size of the head capsule, after chronic exposure (eight days), under laboratory conditions.

MATERIAL AND METHODS

Chironomus sancticaroli individuals were obtained from a colony and were maintained following the protocol of Maier & Kosalwat (1990): temperature 25 ± 2°C, photoperiod of 12 hours/12 hours, 80% humidity ± 10, aeration constant and nutrition with Dog Chow® ration. Voucher specimens of this colony are deposited in Coleção Entomológica Padre Jesus Santiago Moure of the Departamento de Zoologia, Universidade Federal do Paraná (DZUP) with accession numbers from 249269 to 249276.

The quality of the colony was evaluated with the reference compound potassium chloride (KCl). The test resulted in a range of 3.2 to 5.2 (Program Trimmed Spearman – Karber), an average LC_{50} of 4.21, and a coefficient variation equal to 11.8%. These results, within the acceptable range value ⩽30%, indicate that the colony presented ideal conditions for giving adequate responses to exposure to xenobiotics (USEPA 2000).

Toxicity tests were conducted following the Guidelines for the Testing of Chemicals 219 (OECD 2004), with the following modifications: instead of first instar larvae, second instar larvae were used in the chronic exposure experiment; photoperiod was 16:16 hours instead of 12:12 hours light: dark regimen. The experiments were conducted in 300ml glass beakers, with 95g of artificial substrate (75% fine sand, 20% clay white, 5% topsoil and 0.01% calcium carbonate) and 120 ml of reconstituted water (8 ml solution I: calcium sulfate ($CaSo_4$); 1000 ml distilled water and 4 ml solution II: potassium chloride (KCl); sodium hydrogen carbonate ($NaHCo_3$), 1.000 ml distilled water adjusted to hardness level of 16. Specimens were kept under static conditions at 25 ± 2°C and 12:12 hours light:dark regimen (Strixino & Strixino 1985). Before use, the sand was washed with tap water and sterilized in an oven for five hours at 100°C.

Water quality was assessed through the following physicochemical parameters: temperature 21.5°C, pH 7.5 ± 5, conductivity 96.8 ± 10 µS.cm, salinity 46.2 ± 2 µg.l^{-1} (ExStik II®) and hardness by titration with EDTA (dimethylethylene acetic acid) 19.5 ± 4 mg $CaCo_3$. The glassware used in the experiments was cleaned in 5% nitric acid solution for 24 hours. Phenanthrene 97.0% (Fkuka – Sigma Aldrich) was diluted in acetone and ethanol, with end concentration of phenanthrene at 4.000 mg.l^{-1}.

After the acute toxicity (96 hours) and chronic toxicity (eight days) essays we evaluated DNA damage on *C. sancticaroli* larvae exposed to phenanthrene. The objective of the acute exposure bioassay was to determine the lethal concentrations of the compound, and that of the chronic exposure was to evaluate the size of the larval head capsule.

For the acute toxicity bioassays, egg masses were removed from the colony and kept in trays with four liters of reconstituted water for seven days. The food consisted of 0.32g fish feed TetraMin® triturated every two days. Late third instar/early fourth instar larvae were exposed to phenanthrene. Each replicate contained 10 larvae. There were two control groups: one treated with a solvent (ethanol) and the other treated with reconstituted water only. In the end of the bioassay, all larvae (live and dead) were counted. Dead larvae had stretched bodies, no color and did not move.

The lethal concentrations (LC) were determined by the mortality data under the following concentrations of phenanthrene: 1.4 mg.l^{-1}, 1.5 mg.l^{-1}, 1.6 mg.l^{-1}, 1.7 mg.l^{-1} and 1.8 mg.l^{-1}. From these concentrations we were able to estipulate the concentrations used in acute exposure 0.16 mg.l^{-1} (NOEC), 0.83 mg.l^{-1} 1.06 mg.l^{-1}, 1.34 mg.l^{-1}, 1.6 mg.l^{-1} (Tab. I).

Table I. Lethal concentrations (LC) of phenanthrene for the acute exposure of *Chironomus sancticaroli*, expressed in mg.l^{-1}, with confidence interval (CI).

Lethal concentrations	Mg.l^{-1} (ppm)	CI
LC 2	0.83	0.421– 1.033
LC 10	1.06	0.694 – 1.201
LC 30	1.34	1.128 – 1.419
LC 50	1.60	1.510 – 1.655
LC 90	2.34	2.022 – 3.683
LC 99	3.20	2.503 – 7.244

The concentrations used in the chronic toxicity assays were defined based on mortality data obtained in the acute exposure experiment. Second instar larvae were more vulnerable than late third instar early fourth instar. High mortality

rates were observed in the lethal concentrations of LC_5 (0.94 $mg.l^{-1}$), LC_8 (1.01 $mg.l^{-1}$), LC_{10} (1.05 $mg.l^{-1}$), LC_{20} (1.21 $mg.l^{-1}$) with 96%, 99%, 100% and 100%, respectively. In chronic toxicity tests, second instar larvae were exposed to NOEC (No Observed Effect Concentrations calculated by $LC_{50}/10$) 0.16 $mg.l^{-1}$, 0.83 $mg.l^{-1}$ (LC_2). As in the acute toxicity test, there were two control groups, one treated with reconstituted water, and the other with a solvent (ethanol) for eight days. Twenty larvae were added to each replicate. The containers remained closed under constant aeration (OECD 2004). Every two days the water was changed by removing approximately 70% of the total volume. Together with the water we added 4.8 mg/ml fish feed, TetraMin® and phenanthrene solution for each specific concentration, due to the two-day half-life of phenanthrene in sediment (Netto *et al*. 2000).

After eight days under chronic exposure, larvae were stored in 80% alcohol. Specimens were cleared in 10% potassium hydroxide (KOH) solution for 24 hours. They were then mounted on slides (Acrilex) with the ventral region of the head capsule facing up. The length of the head capsule was measured as indicated by Strixino & Strixino (1982) using the program ZEN 2011 "blue edition", from photos taken under a dissecting scope and magnified 150x (Zeiss SteREO Discovery V20 coupled with AxioCam camera ERc5s).

DNA damage was determined by alkaline comet assay for the following concentrations of the acute exposure experiment: 0.16, 0.83, 1.06, 1.34, and 1.6 $mg.l^{-1}$; and the following concentrations for the chronic exposure: 0.16 and 0.83 $mg.l^{-1}$. We followed the method of Lee & Choi (2006), with the following modifications: 1ml PBS solution for 250 µl of the bovine serum was followed by a centrifugation phase. Ten larvae taken from the bioassay were macerated in 2 ml eppendorf tubes containing 250 ìl bovine serum and centrifuged at 1.000 rpm for five minutes. The supernatant was discarded and the cells resuspended by vortexing. Then 60 ìl of the suspension were mixed with 120 ìl low meeting point agarose (LMP) 0.5% (0.1 g, 20 ml dimethylethylene acetic acid and phosphate buffered saline (PBS)) and immediately placed on a slide coated with normal agarose 1.5% (1.5g, 100 ml dimethylethylene acetic acid and phosphate buffered saline (PBS)), covered with cover slip and kept refrigerated for 10 to 20 minutes. Subsequently, the cover slips were removed and slides were placed in lysis solution (10 mM Tris; 100 mM dimethylethylene acetic acid (EDTA), 2.5 M chloride sodium (NaCl); dimethylsulffoxide (DMSO) 10%) for 24 hours. After being removed, the slides were kept in alkaline solution (300 mM sodium hidroxide (NaOH); 1mM dimethylethylene acetic acid (EDTA)) for 30 minutes to denature the DNA. Slides were then subjected to electrophoresis (25 volts and 300 mA) for 25 minutes. When the migration ended, slides were neutralized with 0.4 M Tris (pH 7.5) and subsequently fixed with absolute ethanol for five minutes and colored with 15ìl of Ethidium Bromide (20 ìg/ml). The slides were analyzed under epifluorescence microscopy (LEICA) at 400x magnification. The degree of DNA damage was evaluated visually and classified into four categories: 0 (no damage), 1 (little damage), 2 (average damage), 3 (considerable damage) and 4 (maximum damage). A total of 50 cells corresponding to each replicate were evaluated (Kobayashi *et al*. 1995). For each slide, the degree of damage was calculated by summing up the number of cells with each kind of damage and multiplying the resulting number by the number corresponding to the category of damage. The score ranged from 0 (no damage: 50 x 0) to 200 (maximum damage: 50 x 4). All bioessays (in the control and treatment) were analyzed, 15 replicates, totaling 750 cells.

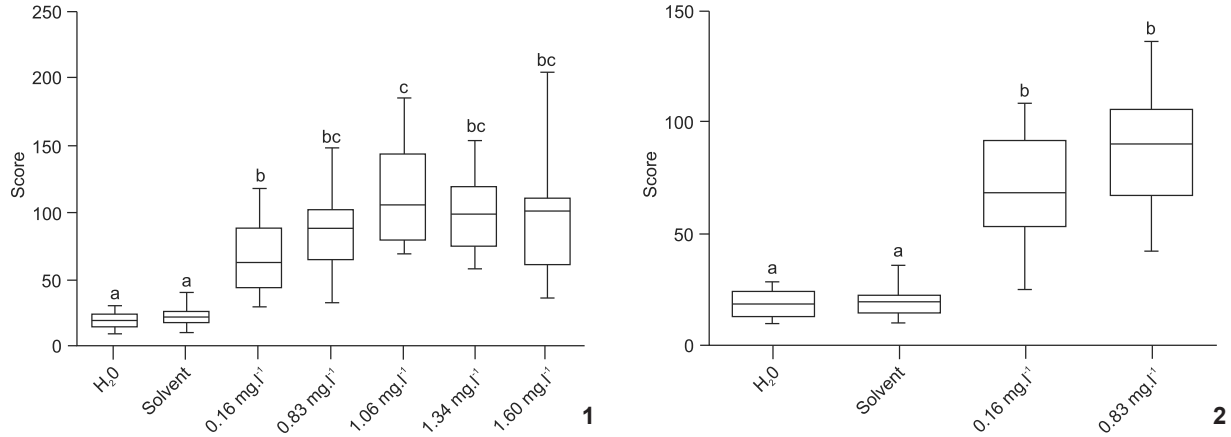
The lethal concentrations were obtained in GW-Basic Probit version 5.0. To evaluate genotoxicity the statistical analyzes were performed using the Kruskal-Wallis ($p \leqslant 0,05$) through BioEstat version 5.0 and measured head capsule data were analyzed by ANOVA ($p \leqslant 0,05$) with Tukey's ($p \leqslant 0,05$) post test using the program PAST version 1.95. The graph was produced using GraphPad Prism version 5.0.

RESULTS

In the alkaline comet assay, no significant differences among the control groups were detected ($p = 0.700$) by the Kruskall-Wallis test, with $p \leqslant 0.05$ (5%) in acute exposure (96 hours). All groups showed significant differences with respect to the control group ($p < 0.001$). However, when we compared among the different concentrations, significant differences were observed only between 0.16 $mg.l^{-1}$ and 1.06 $mg.l^{-1}$, with $p = 0.042$ (Fig. 1). A concentration-dependent response was not detected.

In the chronic exposure (eight days), no significant differences were found between the two control groups ($p = 0.960$). In the analyses evaluating concentrations (0.16 and 0.83 $mg.l^{-1}$) there were significant differences between experiment and water control, with $p < 0.0001$ for both. However, significant differences were not detected between treatments groups, with $p = 0.421$ (Fig. 2). A concentration-dependent response was not detected.

There were no significant differences in the size of the head capsule of individuals subjected to chronic exposure to phenanthrene between the control groups ($p = 0.42$ by ANOVA $p \leqslant 0.05$ (5%)), indicating that the solvent does not affect the development of *C. sancticaroli*. In the two concentrations tested, significant differences were found, with $p = 4.281$ at 0.16 $mg.l^{-1}$ and $p = 1.913$ at 0.83 $mg.l^{-1}$ (Fig. 3). However, between the two concentrations of phenanthrene in the chronic bioassay, no significant difference was detected, with $p = 0.369$, and the response was not concentration-dependent. The Tukey's test at $p \leqslant 0.05$ (5%) performed retrospectively confirmed this. There was a significant difference for both concentrations with respect to the control ($p = 7.72$ at 0.16 $mg.l^{-1}$ and 7.72 at 0.83 $mg.l^{-1}$), but no significant differences between the concentrations ($p = 0.84$).



Figures 1-2. DNA damage in *C. sancticaroli* larvae demonstrating the medium and range minimum and maximum for control groups (water and solvent) and groups exposed to phenanthrene. Different letters indicate significant differences with $p \leqslant 0.05$ (Kuskrall-Wallis test). (1) Acute exposure of 96 hours; (2) Chronic exposure of eight days. N = 15.

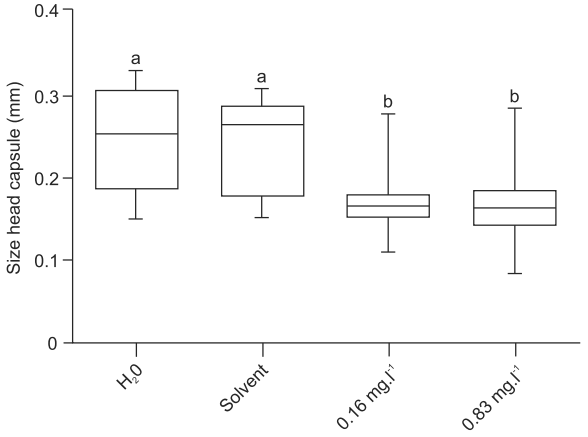


Figure 3. Size of the head capsule of *C. sancticaroli* larvae demonstrating the average, range minimum and maximum and standard deviation for control groups (water and solvent) and groups exposed to phenanthrene for eight days. Different letters indicate significant differences $p \leqslant 0.05$ (ANOVA one-way with aposteriori Tukey test).

DISCUSSION

We tested the toxic effects of phenathrene on the chironomid *C. sancticaroli* and found evidence of toxicity at both the molecular and population levels. Phenathrene was genotoxic to larvae after 96 hours and its effects continued up to eight days of exposure, causing delay in larval development.

Phenanthrene, in our data, had deleterious effects on *C. sancticaroli* at concentrations above 3.2 $mg.l^{-1}$. After both acute and chronic exposure, second instar larvae were more vulnerable to the compound than late third instar and early fourth instar larvae. The high rate of larval mortality indicates that phenanthrene primarily affects survival. This effect had been previously described by Marinkovic *et al*. (2011) and Paumen *et al*. (2008) in a study using *Chironomus riparus* Meigen, 1804.

Genotoxicity is considered an important biomarker for environmental monitoring, but little information is known about the toxicity of phenanthrene on benthic organisms. However, DNA damage has been detected in Chironomidae, indicating the genotoxicity of nonylphenol, bisphenol A and silver nitrate (Park & Choi 2009, Nair *et al*. 2011).

The results of the alkaline comet assay for both acute and chronic toxicity tests suggest that phenanthrene is genotoxic to *C. sancticaroli* larvae. Significant DNA damage was observed after acute exposure, starting with the lower concentration of 0.16 $mg.l^{-1}$ by Kruskall-Wallis test ($p \leqslant 0.05$). However, the occurrence of DNA breaks was not concentration-dependent. In chronic exposure tests we observed significant DNA damage at the two concentrations tested (0.16 and 0.83 $mg.l^{-1}$). However, DNA damage did not increase with increased concentrations. Our data indicate that phenanthrene reaches the interior of the cell, causing breaks in the genetic material.

Our results suggest that even low concentrations of phenanthrene cause DNA breaks in *C. sancticaroli* larvae after short exposure. This sensitivity may allow the identification of environmental contamination in its initial stages. The permanence of phenanthrene in the environment does not cause more serious DNA damage, even with a fivefold increased concentration, suggesting that *C. sancticaroli* larvae become resistant to the effects of the compound.

Phenanthrene is a Polycyclic Aromatic Hydrocarbon and its toxicity requires metabolic activation, which is associated with the process of detoxification. The compound can be metabolized rapidly, which reduces its toxic potential (Netto *et al*. 2000, Marinkovic *et al*. 2012). According Marinkovic *et al*. (2011), exposure to phenanthrene activates the expression of proteins that repair DNA damage, and which may also act

against the intensification of DNA breaking. DNA damage caused by phenanthrene is primarily sub lethal, and may cause long-term mutations, chromosomal aberrations and loss, altering the genetic structure of the population (Jha 2008).

Measurements of the head capsules showed significant reduction in the size of immatures under all concentrations of phenanthrene, averaging a 1.5 x reduction with respect to the control, indicating delays in the development. According to Marinkovic *et al*. (2012), phenanthrene does not change the expression of the juvenile hormone (methyl transferase acid) in *C. riparus*. Thus, it is possible that phenanthrene exposure causes changes in other essential biological functions such as feeding, causing a reduction in the size of the head capsule.

Delays in larval development following food restrictions were observed by Liber *et al*. (1996) for *Chironomus tentans* Fabricius, 1805 and by Ristola *et al*. (1999) for *C. riparus*. In those studies, a reduction in the amount of food caused a reduction in larval size and a consequent delay in the emergence of adults. In the same manner, the reduced larval size could cause delays in the life cycle of *C. sancticaroli*. However further studies are needed to test this hypothesis.

Marinkovic *et al*. (2012) did not find any changes in the development of *C. riparius* after exposing larvae to high concentrations of phenanthrene. Likewise, Paumen *et al*. (2008) and Marinkovic *et al*. (2011) observed only a short delay in the emergence of *C. riparus* adults, indicating little to no delay in larval development. The results obtained suggest that it is possible to detect DNA damage and developmental delays in *C. sancticaroli* larvae after exposure to phenanthrene.

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