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RESEARCH ARTICLE

Pristina longiseta reproduction test: chronic exposure to environmental contaminants

Tallyson Tavares Cunha de Souza[1] · Gleyson Borges Castro[1] · Aline Christine Bernegossi[1] · Mayara Caroline Felipe[1] · Fernanda Rodrigues Pinheiro[1] · Vanessa Colombo-Corbi[2] · Douglas Aparecido Girolli[2] · Guilherme Rossi Gorni[2] · Juliano José Corbi[1]

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Abstract
Aquatic worms are considered a suitable group to evaluate the effects of contaminants on the environment, although one of the main challenges is to use the species of local occurrence. Recently, *Pristina longiseta* was suggested to be used in acute bioassays. In this context, this study aimed to establish a chronic exposure for ecotoxicological bioassays using the cosmopolitan species of occurrence in Brazilian freshwater *P. longiseta*. Firstly, we tested three exposure times (4, 7, and 10 days) under the presence or absence of aeration for reproduction outputs. After determining the best configuration (7 days without aeration), we assessed the effects of the chronic exposures using the standardized reference substance potassium chloride (KCl), the antibiotic sulfamethoxazole (SMX), the flame retardant tetrabromobisphenol A (TBBPA), and the sugarcane vinasse. Our results showed suitability for applying the chronic exposure using *P. longiseta* and indicated the sensitivity of the offspring to KCl (EC50-7d = 0.51 g/L). Sulfamethoxazole and TBBPA caused a significant decrease in the offspring of *P. longiseta* (EC50-7d = 59.9 µg/L and < 62.5 µg/L, respectively). Sugarcane vinasse showed high toxicity for the species, and 4.26% of vinasse was calculated as EC50-7d. Therefore, the described protocol was successfully applied as an ecotoxicological bioassay to evaluate the effects of environmental contaminants on the reproduction rate of the freshwater worm *P. longiseta*.

Keywords Aquatic worms · Brazilian species · Ecotoxicity · Naididae · Native species · Oligochaeta

Introduction

Aquatic invertebrates have been widely used in ecotoxicological assays worldwide, mainly due to their importance in the food chain, diversity, geographic occurrence, and adaptability to laboratory conditions (Hutchinson 2002; Gorni & Alves 2012; Corbi et al. 2015; Rosner et al. 2021). Despite these organisms being abundant in the environment, few native worm species in tropical regions have been used as bioindicators of environmental contamination or ecotoxicological assessments (Chapman 2001; Gomes et al. 2017; Gazonato Neto et al. 2019). According to Brown et al. (2013), there is a lack of knowledge of the ecology and biology of these species, which is an obstacle to the development of protocols and their application in bioassays. *Allonais inaequalis* Stephenson, 1911; *Branchiura sowerbyi* Beddard, 1892; *Pristina longiseta* Ehrenberg, 1828; *Tubifex tubifex* Müller, 1774 are species of aquatic oligochaetes of occurrence in tropical regions successfully applied in ecotoxicological tests (Chapman et al. 1982; Smith et al. 1991; Phipps et al. 1993; Marchese & Brinkhurst 1996; OECD 2008; Corbi et al. 2015; Lobo et al. 2016; Felipe et al. 2020; Castro et al. 2020a).

Pristina longiseta Ehrenberg, 1828 is a cosmopolitan freshwater species, and its occurrence was registered in Asia, Africa, Europe, and America (Harman, 1982; Yoon et al. 2000; Gorni et al. 2018; Ohtaka 2018; Castro et al. 2020b; Jaweir 2021). The species belongs to the Oligochaeta class, the Naididae family, and its habitats include the lentic and lotic benthic waters. It has a translucent yellowish color and

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✉ Tallyson Tavares Cunha de Souza
tallysontavares@usp.br

[1] Aquatic Ecology Laboratory, Department of Hydraulics and Sanitation, São Carlos School of Engineering, University of São Paulo, São Carlos, SP, Brazil

[2] Environmental Studies Center, University of Araraquara, Araraquara, SP, Brazil

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bristles on the ventral and dorsal parts, and the individuals are on average 1–5 mm in length and 0.11–0.20 mm in width (Al-Abbad & Al-Mayah 2010; Zattara & Bely 2011; Gorni et al. 2018). According to Van Cleave (1937); Zattara & Bely (2011), the reproduction of *P. longiseta* occurs mainly in an asexual way, by paratomic fission, forming a new head and tail along the individual's body and separating after their complete formation. Asexual reproduction ensures genetic stability and population abundance (Timm 2012). Sexual reproduction only occurs in nature, under stressful situations such as unfavorable environmental conditions or at specific times of the year (Van Cleave 1937; Brinkhurst and Gelder 2001; Rodriguez 2004; Özpolat et al. 2016). Smith et al. (1991) evaluated the use of *P. longiseta* (as *Pristina leidyi*) in acute bioassays using cadmium and vanadium as test substances. Recently, Castro et al. (2020b) redefined the culture maintenance at the laboratory considering tropical conditions and application of the organism in short-term exposure (48 h), evaluating acute effects of the reference substances potassium chloride (KCl), copper sulfate (CuSO4), and zinc chloride (ZnCl2). However, there is no standard protocol to perform chronic exposure using this species that was shown to be suitable for acute responses.

To ensure the reliability of the species application in toxicity tests, the use of reference substances and environmental contaminants is recommended. Thus, potassium chloride (KCl), a substance for sensitivity tests standardized internationally by the Organisation for Economic Co-operation and Development (OECD), in the USA by the Environmental Protection Agency (EPA), and by the Brazilian Association of Technical Standards (ABNT), has been recommended to assess the sensitivity of oligochaetes (Corbi et al. 2015; Castro et al. 2020b; Felipe et al. 2020). Among environmental contaminants, sulfamethoxazole (SMX) is the most prescribed sulfonamide-class antibiotic in hospitals for treating bacterial infections (Akpe et al. 2020) and has been listed by the US Geological Survey as one of the 30 most detected contaminants in effluents due to its persistence and low biodegradability (Prasannamedha & Senthil Kumar 2020; Bao et al. 2021). Several authors have identified the ecotoxicological effects of SMX on different aquatic organisms (Park & Choi 2008; Minguez et al. 2016; Srain et al. 2020; Aderemi et al. 2021; Sabino et al. 2021). However, the obtained lethal concentrations of SMX are higher than those identified in the aquatic environment of tropical countries, which can vary between 1.88×10^{-4} and 96.9 µg/L (Locatelli et al. 2011; K'oreje et al. 2016; Agramont et al. 2020; Ebele et al. 2020; Kairigo et al. 2020; Ngumba et al. 2020; Duong et al. 2021; Tokanová et al. 2021).

The brominated flame retardant tetrabromobisphenol A (TBBPA) is one of the main compounds applied to reduce the spread of flames, especially in petroleum derivatives products (Liu et al. 2016; Pieroni et al. 2017). Oral et al. (2021) found that TBBPA may cause low acute toxicity, but chronic effects can be observed even at low concentrations, impacting the reproduction, hepatic system, and thyroid of aquatic animals. Moreover, sugarcane vinasse, the leading liquid waste generated in the ethanol and sugar production processes, is rich in organic matter and nutrients, with an acidic pH between 4.0 and 4.5 (Fuess et al. 2018). Due to the large volume generated (each liter of ethanol produces 13 L of vinasse), it has been widely used in Brazil for fertigation, which in the long term can cause environmental issues such as soil acidification, seed germination inhibition, and contamination of adjacent aquatic systems and groundwater (Silva et al. 2020; Fuess et al. 2021). In aquatic organisms, lethal effects have been detected in fish, microcrustaceans, marine bacteria, and microalgae (Botelho et al. 2012; Marques et al. 2013; Marinho et al. 2014; Sousa et al. 2019; Silva et al. 2021). Nonetheless, the effects of vinasse and TBBPA (acute and chronic) on aquatic worms of occurrence in tropical regions are little known.

Studies contributing to the establishment of bioassays using test organisms occurring in tropical regions are still scarce, mainly compared to those existing for organisms from temperate environments (Di Lorenzo et al. 2019; Gazonato-Neto et al. 2019; Castaño-Sánchez et al. 2021). Therefore, this paper proposes a new protocol for performing chronic tests using *P. longiseta*, a cosmopolitan species of occurrence in Brazil. We evaluated different test conditions to define the best exposure time, aeration requirement, and test applicability using a reference substance and three environmental contaminants.

Material and methods

Species cultivation

Pristina longiseta was cultivated at the Aquatic Ecology Laboratory (LEAA), São Carlos School of Engineering, University of São Paulo (Brazil). Culture maintenance was defined by Castro et al. (2020b) as follows: 500-mL plastic containers (38 cm long × 33 cm wide × 6 cm high) were filled with dechlorinated and filtered water. The containers were kept under constant soft aeration (one bubble per second) in a light–dark cycle of 12:12 h and 25 ± 2 °C. On average, 20 individuals were distributed per 100 g of sterilized fine sand in a muffle (550 °C for 4 h). The water quality of the culture was evaluated by measuring the pH (6.5–7.0), electrical conductivity (174 µS/cm), and temperature (24.3 °C), using a multiparameter device model AKLA32761. The hardness was measured using a Visocolor® ECO kit (18 mgCaCO3/L). Analyses were performed according to the USEPA (2002) recommendations.

Preliminary reproduction tests

To evaluate the best configuration to apply the reproduction test using *P. longiseta*, we tested three exposure times and the need to keep the jars aerated. Six configurations of bioassays were tested: (a) the presence of aeration ((a.1) offspring counting on the 4th day, (a.2) offspring counting on the 7th day, and (a.3) offspring counting on the 10th day) and (b) the absence of aeration ((b.1) offspring counting on the 4th day, (b.2) offspring counting on the 7th day, and (b.3) offspring counting on the 10th day).

The temperature and dark cycle were the same as those for the cultivation. Each configuration was assessed in 15 replicates to determine the best condition to proceed with the *P. longiseta* chronic exposure to the environmental contaminants. Each replicate received 6 organisms, lengths ranging from 2 to 4 mm, with no apparent reproduction zone (fission). The organisms were exposed to 60 mL of dechlorinated tap water (pH ranging from 6.5 to 7.5) and 10 g of sterilized fine sand (kept in muffle for 4 h at 550 °C) in a glass bottle (capacity of 100 mL). Each replicates received food at the beginning (2 mg of macerated fish food TetraMin®). In the tests of 10-day exposure, in addition to providing food at the beginning, the replicates also receive food after the 5th day (2 mg of macerated fish food TetraMin®). For the tests with aeration, silicone tubes coupled to a pump (model Boyu ACQ-003 50L/M) aerated the liquid medium, following the same bubble frequency applied in the culture. At the end of the tests, the individuals of each replicate were collected individually using a Pasteur glass pipette. The offspring was defined as the total number of individuals counted, disregarding the total number of incubated individuals at the beginning ($n = 6$). No mortality was detected at this stage.

Ecotoxicological application

After defining the best test conditions in a controlled environment without any toxic substance (detailed in the Results section), we performed chronic exposures of *P. longiseta* to three environmental contaminants and a reference substance. Concentrations were initially defined according to the literature data and preliminary tests. The geometric factor of 2.0 was used to determine the TBBPA concentration ranges, while for the other samples, the factor 1.5 was applied. For the reference substance, we used the concentration range of 0.3, 0.5, 0.7, 0.9, and 1.3 g KCl/L. The test concentrations were obtained from a 100 g KCl/L solution. The concentrations for tetrabromobisphenol A were 62.5, 125, 250, 500, and 1000 µg/L. The stock solution of 500 mg TBBPA/L was made in methanol. In the sulfamethoxazole tests, the concentration range was 38, 55, 86, 128, and 192 µg/L, and the stock solution was prepared in methanol (500 mg SMX/L). For sugarcane vinasse, the dilutions were 1.5, 2.2, 3.3, 4.95, and 7.4%. All test solutions were made using dechlorinated tap water. The effects on the reproduction rate of *P. longiseta* were evaluated over 7 days. Chronic exposures were carried out in triplicate, containing 6 individuals from 2 to 4 mm in length without apparent fission zone, in beakers of 100 mL capacity, containing 60 mL of the test solution and 10 g of sterilized fine sand, without aeration, under 25 ± 2 °C, 12-h light:12-h dark photoperiod. The organisms were fed at the beginning of the test with 2 mg of macerated fish food TetraMin®, following culture feeding recommendations determined by Castro et al. (2020a, b), 1 time every 7 days. The offspring were counted from the total number of individuals, discounting the number of incubated individuals ($n = 6$).

The quantification of sulfamethoxazole (SMX) in the stock solution was performed through mass spectrometry in an LC-UHPLC-MS/MS system (Agilent Technologies 1260 Infinity HPLC) (Waters, USA), operated in 1D mode (UHPLC), and an electrospray ionization source operating in the positive mode and triple-quadrupole mass analyzer (Xevo TQD Spray, Waters). As an internal standard, sulfamethoxazole-D4 was used. The curve ranged from 0.1 to 3 mg/L, with $R^2 = 0.998$, and injection volume was 5 µL. The quantification of potassium chloride (KCl) was performed using two methods. Chloride (Cl) was quantified using the mercuric thiocyanate method (method 8113, Hach Company) (ranged from 0.1 to 25 mg/L), and potassium (K) verification was performed using the flame photometric method (3500-K B). The method used to quantify TBBPA was proposed by Macêdo et al. (2021) (LC-UHPLC-MS/MS), using tandem mass spectrometry. TBBPA extraction was performed through ultrasonic dispersive liquid–liquid microextraction with separation by high-performance liquid chromatography (Infinity-Lab Poroshell 120 EC-C18 device). The internal standard used was 13C12-TBBPA, with TBBPA showing 98% purity. The established curves ranged from concentrations of 5000, 1000, 500, 200, 10, and 1 µg/L.

Thus, the antibiotic SMX, the reference substance KCL, and the brominated flame retardant TBBPA quantified in the samples were within the maximum range of 5% difference from the actual concentrations and the nominal concentration highlighted in the ecotoxicological tests. The sugarcane vinasse characteristics were performed according to the Standard Methods for Examining Water and Wastewater (FEDERATION and APH ASSOCIATION 2005). The variables observed were the pH of 4.8; 35,370, and 28,470 mg/L of Total and Filtered Chemical Organic Demand (COD), 2376 mg/L of phenol, 6840 mg/L of carbohydrates, 3956 mg/L of glycerol, 4373 mg/L of

proteins and, 608.9 mg/L of Kjeldahl Total Nitrogen (KTN).

Statistical analyses

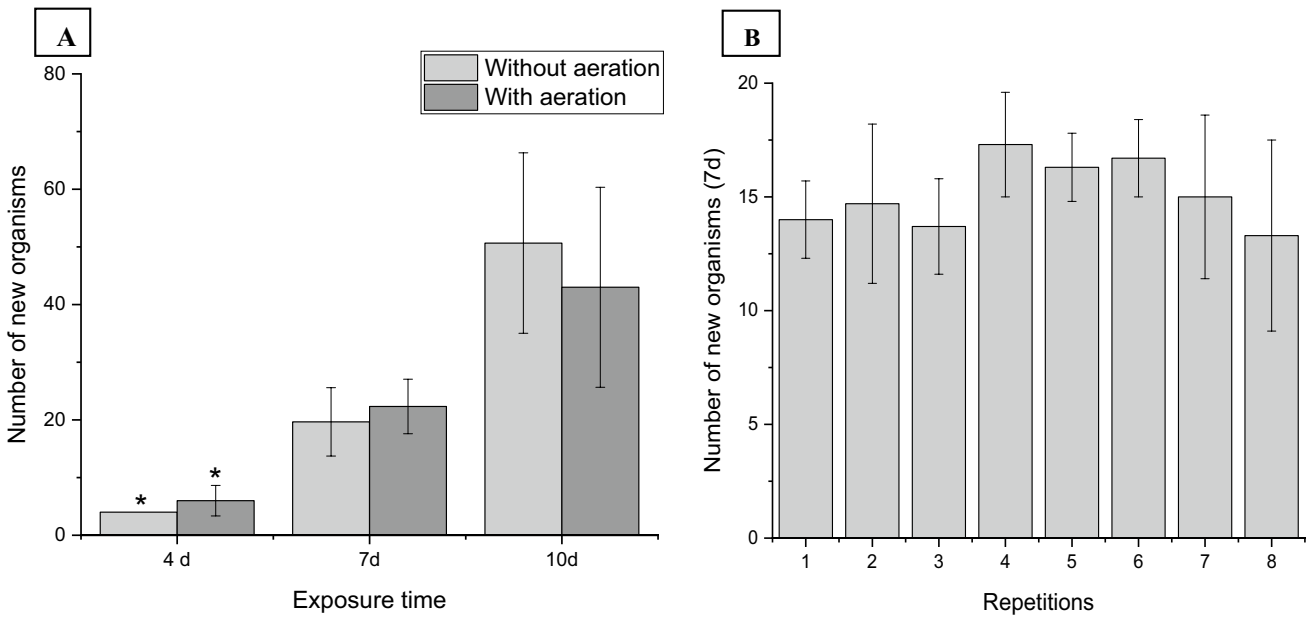
To evaluate the best configuration for the chronic bioassay, the two-way ANOVA was applied to compare the offspring production at different exposure times (4 days, 7 days, and 10 days) and in the presence and absence of aeration. To analyze the ecotoxicological application of the chosen configuration, the reproduction was evaluated as 10% and 50% inhibition (EC10 and EC50) using R *software*, version 3.5.0, and MASS and DRC packages. To evaluate a statistical difference in the number of new individuals produced at the concentrations tested, we applied the one-way ANOVA and Kruskal–Wallis variance test through the Past® *software* (Paleontological Statistics) after testing the data normality (Shapiro–Wilk test). In the case of samples that presented non-normal distribution ($p \leq 0.05$), the Kruskal–Wallis test was used to assess significant differences, followed by Dunn's post hoc test. For samples that presented normal data distribution ($p \geq 0.05$), the test used was the one-way ANOVA, followed by the Tukey post hoc test. All statistical tests and ecotoxicological responses considered a 95% confidence interval ($p \leq 0.05$).

Results and discussion

Reproduction test conditions

Evaluating the best configuration for chronic exposure in preliminary tests, we observed that in 4 days, the number of new organisms did not exceed 5 in any of the samples of aeration conditions, while in the exposure times of 7d and 10d, each organism had fission at least once. During the exposure time of 7d, the mean number of new organisms was 19 and 22 in the absence and presence of aeration, respectively. A high number of new organisms was observed in the exposure time of 10d, in which, the mean number of new organisms in the replicates without aeration was 50 against 43 in the replicates with aeration (Fig. 1A). No statistical differences were identified in the number of new organisms comparing the presence or absence of aeration, considering the results at 4, 7, and 10 days (Two-way ANOVA, $p = 0.6263$). On the other hand, focusing on the exposure time, the total offspring in 4 days showed a significant difference compared to 7 days ($p = 0.0042$) and 10 days ($p = 2.29 \times 10^{-7}$).

According to Smith et al. (1991), species from the Naididae family present new generations of individuals within 3 to 7 days. Özpolat et al. (2016) state that the *P. longiseta* (as *P. leidyi*) species takes 4 to 6 days to perform body regeneration after reproduction by paratomic fission. Additionally, the



* Significant difference in the offspring generated between the tests lasting 4d and 7d; 4d and 10d; and 7d and 10d.

Fig. 1 Reproduction assays testing *Pristina longiseta*: **A** Offspring of *P. longiseta* in the control experiments according to exposure time and aeration requirement; **B** repetitions of the condition selected (b.2) for the reproduction tests (7-day exposure time, without aeration)

authors also state that multiple fission zones can form after the initial fission, whereby the organism can divide into more than 2 individuals. In this context, associating both literature findings and the outcomes from the preliminary test in this research, the configuration without aeration and 7 days of exposure time showed to provide enough reproduction ratio to correctly identify potential toxic effects in the first generations of *P. longiseta*.

We emphasize that the suggested duration for short-term exposure (acute testing) is 48 h for *P. longiseta* (Smith et al. 1991; Castro et al. 2020b). Another preliminary investigation of the exposure time of 4 days was reported by Castro et al. (2020b); they observed the presence of new individuals after 72 h (3 days) testing the configuration of acute toxicity bioassay. The duration of chronic assays applied to other species was also considered, such as those performed for *Chironomus* sp. (10 days), an aquatic invertebrate (OECD 2011), the tropical aquatic oligochaete *Allonais inaequalis* (10 days), having these benthic organisms, life cycles longer than *P. longiseta* (Corbi et al. 2015; Felipe et al. 2020). The configuration of 7 days was chosen because no significant differences were observed comparing the reproduction values at 7 and 10 days (no aeration) ($p = 0.1141$). Furthermore, as *P. longiseta* reproduces by rapid paratomy, a faster reproduction for this species is guaranteed under optimal control conditions compared to aquatic worms of slow paratomy, such as *Dero digitata* (Van Cleave 1937; Kharin et al. 2006). In this case, in 7 days, an increase of at least 3 times the initial number of individuals is expected, obtaining a fast response in chronic tests and reducing time and costs in applying the bioassay. Beyond that, 8 repetitions of condition b.2 (without aeration and counting of new organisms in 7 days) were performed between 11 months (Fig. 1B). No statistical differences were observed between the number of new organisms in the repetitions ($p = 0.6253$, in the Kruskal–Wallis test).

Ecotoxicological assessment

The reproduction bioassay (7 days without aeration) was successfully applied for three environmental contaminants and the reference substance. The reference substance (KCl) caused a constant decrease in the number of new organisms according to the increasing concentration (Fig. 2A). Furthermore, the TBBPA at the lowest concentration induced a significant decline in reproduction (mean of 2 new organisms at 62.5 µg/L) (Fig. 2B). On the other hand, the low concentration of SMX and low percentual dilution of sugarcane vinasse induced a reproduction rate near to the control rate, and a sharp drop was observed at 86 µg SMX/L (no new organisms; Fig. 2C) and 4.95% of vinasse (mean of 2 new organisms; Fig. 2D).

According to the Kruskal–Wallis test, significant differences in reproduction were identified between treatments of all contaminants or reference substances and the control ($p \leq 0.05$). Dunn's post hoc test showed that the reproduction of *P. longiseta* at 62.5, 125, 250, 500, and 1000 µg/L of the TBBPA was significantly different from the reproduction registered in the control. The results of the methanol assays did not indicate significant differences from the control. For SMX, the concentrations that showed a significant difference compared to the control were 86, 128, and 192 µg/L. For sugarcane vinasse, only the 7.4% dilution showed a significant difference from the control, and for the KCl, the concentrations that presented a significant difference in the organism's reproduction were 0.7, 0.9, and 1.3. Thus, only at the lowest concentrations and dilutions of the samples, no toxic statistical effects were identified comparing the results to control samples (SMX, 38, 55 µg/L, and methanol control; KCl, 0.5 and 0.3 g/L; sugarcane vinasse, 1.5, 2.2, and 3.3%). On the other hand, for the assays using TBBPA, all concentrations showed similar results (no significant differences), indicating high inhibition of the species reproduction even in entirely different magnitudes (62.5 to 500 µg/L). Moreover, the classic ecotoxicological endpoints EC50-7d, NOEC, and EC10-7d were obtained. Among the assessed substances, sulfamethoxazole showed the highest toxicity, presenting an EC50-7d of 59.9 µg/L, followed by tetrabromobisphenol A (EC50-7d ≤ 62.5 µg/L). The sugarcane vinasse caused an inhibitory effect of 50% on reproduction of 4.26%. Besides, the KCl indicated an EC50-7d of 0.51 g/L (Table 1).

By assessing EC10-7d, we observed that the substances did not follow the same pattern as the toxicity of the EC50. SMX caused an EC10-7d of 52.1 µg/L; a concentration detected in the environment poses a risk to the organisms due to the value being close to the EC50-7d, with concentrations of SMX (96.9 µg/L) having already been detected in surface water at Kenya (Kairigo et al. 2020). Comparing the effects of SMX and TBBPA, we observed that SMX was more toxic (EC50-7d 59.9 µg/L) than TBBPA. Analyzing the initial toxicity (EC10), it was observed that the sulfamethoxazole (EC10-7d 52.1 µg/L) had a toxic effect at values close to the EC50-7d. In this case, SMX and sugarcane vinasse stand out as contaminants that presented the smallest interval between EC10-7d and EC50-7d. In real contamination scenario, a slight variation of these concentrations could already cause severe impairments for the Oligochaeta. In addition, the KCl caused a LOEC of 0.12 g/L, and raw sugarcane vinasse also had an unobserved effect near the EC50-7d, at 3.25%.

Pristina longiseta is known to be more sensitive to the reference substance KCl in acute bioassays (2 days) compared to *Allonais inaequalis* (4 days), another native Brazilian Oligochaeta. Castro et al. (2020a) observed an LC50-2d of 1.36 g/L for the short exposure of *P. longiseta* to KCl, whereas Corbi et al. (2015) found an LC50-4d of 3.5 g/L for *A. inaequalis*. The same was observed in

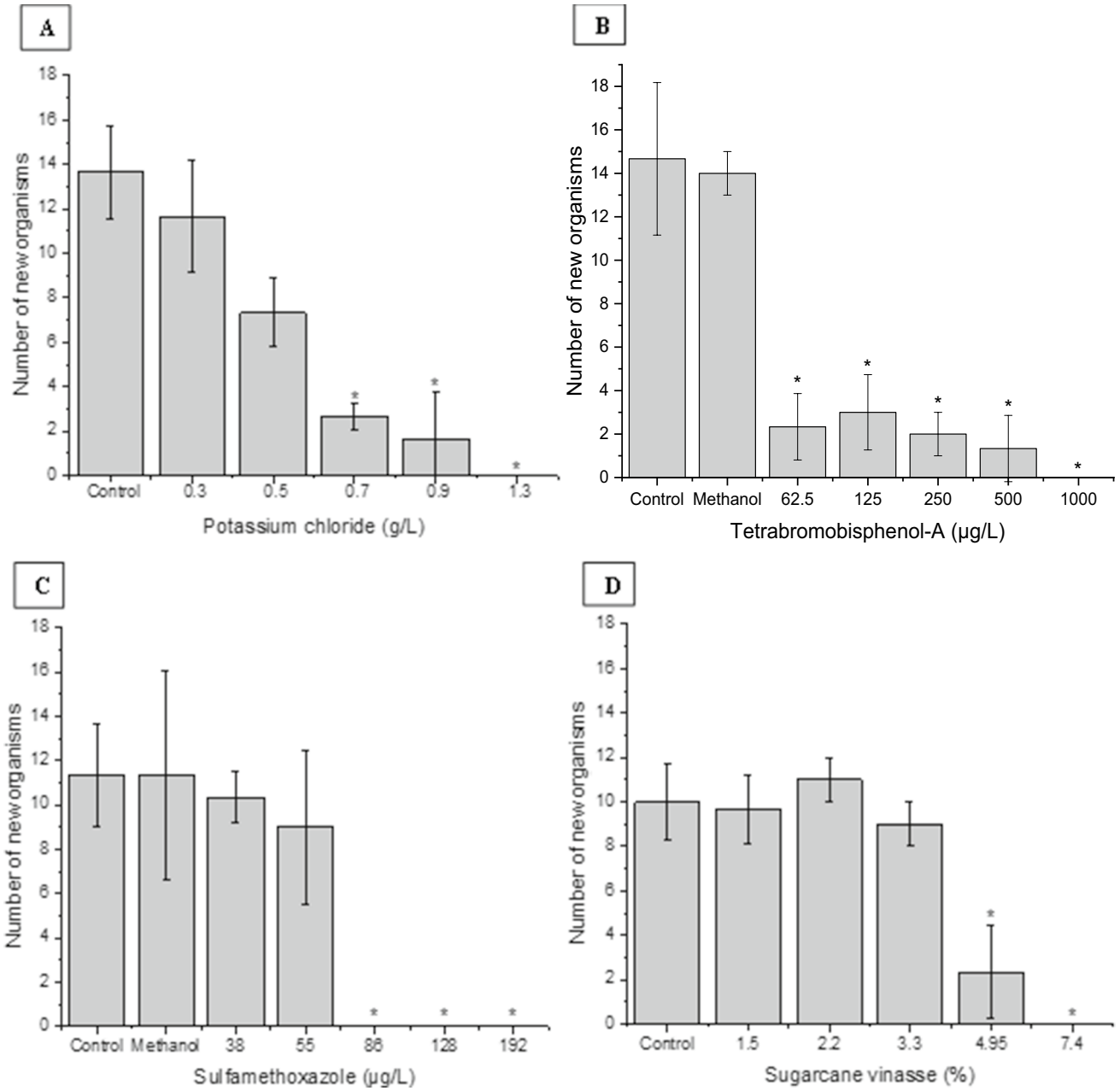


Fig. 2 Effects of the reference substance and environmental contaminants on reproduction of *P. longiseta* in 7 days: **A** potassium chloride (KCl); **B** tetrabromobisphenol A (TBBPA); **C** sulfamethoxazole (SMX); **D** sugarcane vinasse

chronic bioassays, where the EC50 for *P. longiseta* was close to the EC10-10d found by Felipe et al. (2020) for *A. inaequalis* (0.50 g/L) in 10-day chronic bioassays. The effect on 50% of *P. longiseta* offspring (EC50-7d 0.51 g/L) is a concentration of the initial toxic effect on *A. inaequalis*.

The bromate flame retardant, TBBPA, is an endocrine-disrupting agent to aquatic biota (USEPA 2015) and seems to affect the hormonal system responsible for the reproduction of the species. For the crustacean *Daphnia magna*, Yang et al. (2012) observed that TBBPA affects the reproduction of the individuals in concentrations below 150 µg/L, presenting EC10-21d of time to the first brood, a total number of spawning and number of broods of 84 µg/L, 16 µg/L, and 139 µg/L, respectively. Although EC50 and EC10 were not calculated for *P. longiseta*, the effects of the high inhibition of

Table 1 Ecotoxicological endpoints of chronic exposures of *Pristina longiseta* to KCl, TBBPA, SMX, and sugarcane vinasse, expressed as EC10, EC50, and NOEC

Test substance	EC50-7d	Standard error	EC10-7d	Standard error	NOEC
KCl	0.51 (0.43–0.59)	0.03	0.12 (0.07–0.17)	0.02	0.50
TBBPA	< 62.5	-	-	-	-
SMX	59.9 (20.8–90.9)	1.83	52.1 (29.5–74.7)	1.06	55.0
Sugarcane vinasse	4.26 (3.77–4.74)	0.23	3.25 (2.53–3.98)	0.34	4.95

Values in g/L for KCl; in µg/L for tetrabromobisphenol A and sulfamethoxazole; and percentage of dilution for sugarcane vinasse. The 95% confidence limits are indicated between brackets

Note: The EC50, EC10, and NOEC calculated for the TBBPA were not used because the values were below the range established in this study. In this case, we observed that the EC50 would be lower than the lowest concentration investigated in this research

reproduction, observed at concentrations of up to 62.5 µg/L, indicated that the species showed greater reproductive sensitivity to TBBPA than the microcrustacean *Daphnia magna*, as the concentrations that were responsible for toxic effects in 10% of the Cladocera species (EC10) caused inhibitions greater than 80% of Oligochaeta reproduction (at 62.5 µg/L). In addition, Pittinger and Pecquet (2018) reported that the effect of TBBPA on the reproduction of *D. magna* expressed as NOEC was above 300 µg/L, a value that in this research still indicated significant differences from the control for *P. longiseta*. Moreover, studies using the marine mussel *Mytilus galloprovincialis* showed that TBBPA induced the development of gametes in female and male individuals at concentrations below 375 µg/L (Wang et al. 2021). Corroborating the authors, our results indicated that this substance negatively affects the reproduction rate even at low concentrations. A review of the presence of TBBPA in different experiments showed that in freshwater environments, it remains below 4.8 µg/L and in sediment samples below 480 ng/g dw; in industrial and electronic waste areas, these values can be high, e.g., 9750 ng/g dw (Liu et al 2016), which may affect benthic species such as *P. longiseta*, more significantly in this case. Moreover, it is known that this compound can accumulate in different tissues of aquatic biota (Harrad et al 2009; Gong et al. 2021), and there is a concern regarding its effects over long exposures.

In bioassays using SMX, *P. longiseta* was more sensitive (EC10-7d of 52.1 µg/L) compared to the microalgae *Pseudokirchneriella subcapitata* (EC10-3d of 150 µg/L); the microcrustacean *Ceriodaphnia dubia* (EC10-7d of 250 µg/L); and the cnidarian *Hydra attenuata* (EC10-4d of 5000 µg/L) (Straub 2015), which may be an indication that the reproduction of species of the Oligochaeta class is more susceptible to inhibition when exposed to antibiotic SMX in an environmentally relevant concentration. Qiu et al. (2020) found that exposure to SMX caused chronic and subchronic effects on *Danio rerio* zebrafish, delaying egg hatching and impacting fish body size. In addition, other studies have described the effects of oxidative stress for microalgae *Raphidocelis subcapitata* (Zhang et al. 2021) and inflammatory effects for the fish *Ctenopharyngodon idella* (Wang et al. 2021), *Oreochromis niloticus* (Hu et al. 2021), and *Danio rerio* (Qiu et al. 2020). These studies have shown that SMX can cause chronic effects in different aquatic organisms at relevant environmental concentrations, corroborating the results observed for *P. longiseta* in this study. Thus, the need of investigating the effects of these contaminants on other tropical aquatic worms is evident, as the bibliography for these organisms is scarce.

The sugarcane vinasse is a complex residue of high concentration of organic matter, proteins, and nitrogen, which makes this residue suitable to be used as a fertilizer (Fuess et al. 2018). However, the intensive use of sugarcane vinasse can result in soil acidification and toxicity to aquatic species, even in a low percentage (Silva et al. 2007; Christofoletti et al. 2013). Due to the potential toxicity of sugarcane vinasse, in the 1970s, restrictive laws were established prohibiting vinasse disposal directly or indirectly in water bodies (Fuess & Garcia 2014; Moraes et al. 2015). Sugarcane vinasse is commonly applied in cane cultivation as fertigation, and most ecotoxicological studies related to vinasse are carried out using soil organisms (Pedrosa et al. 2005; Coelho et al. 2017; Vilar et al. 2018; Sousa et al. 2019; Fuess et al. 2021). Verma & Dalela (1976) performed toxicity tests using two species of fish, *Puntius sophore* and *Mystus vittatus*; they observed that 6.3 to 10% of vinasse caused mortality in 50% of these organisms after 4 days of exposure at 32 ± 2 °C. Moreover, an increase in mucus production and a reduction of proteins in the liver, brain, kidneys, and muscles of *Channa punctatus* were reported in dilutions from 50% of vinasse (Kumar & Gopal 2001). Regarding chronic studies, two species of aquatic insects had their reproduction analyzed. For *Drosophila melanogaster*, it was observed that 25% of vinasse decreases the egg fertility rate, and for *Chironomus* sp., 6.5% affects the emergence rate (Yesilada 1999; Nyakeya et al. 2018).

The *P. longiseta* reproduction test showed that 4.2% of raw vinasse could affect 50% of the offspring. These results showed that *P. longiseta* was more sensitive to vinasse than fish and insect species, even though the exposure time varied from each ecotoxicological bioassay. However, when compared to Cladocera, it is more resistant, as the

EC50-2d found for *C. dubia* was 0.67% (pH 4.0) and for *D. magna* was 0.8% (pH 4.0) (Botelho et al. 2012). Botelho et al. (2012) also observed the pH effect on the vinasse's toxicity. The authors showed that by increasing the pH to 7.0, the percentage of vinasse in the sample to EC50-21d also increased (2.99% and 5.62% for *C. dubia* and *D. magna*, respectively). In general, benthic organisms tend to present more significant toxicity in exposures to effluents at high rates of settleable solids compared to filtering organisms, as is the case of landfill leachates, domestic sewage, and sugarcane vinasse. In this case, the contaminants are carried to the sediment, increasing the contact zone of organisms and pollutants (Reynoldson 1987; Hayashi-Martins et al. 2017; Rigaud et al. 2019). On the other hand, for contaminants at low concentrations in the medium, Cladocera can, in many cases, present a more significant chronic toxic effect because they are non-selective filterers organisms compared to Oligochaeta (Damasceno de Oliveira et al. 2018).

There are no published data on reproductive biomarkers expressed by aquatic worms exposed to the contaminants applied in this research. However, knowledge of reproductive processes and bioaccumulation of compounds help to understand the dynamics of the contaminant's effects on the asexual reproduction of Oligochaeta. Özpolat et al. (2016), investigating the plasticity and regeneration of gonads of *Pristina leidyi*, observed that under conditions of lower food concentration (low organic load and nutrients), the aquatic worm presented a lower generation of fission zones, which could lead to a smaller number of offspring, even if the species shows rapid cell regeneration. Otherwise, Martinez et al. (2006) observed that in exposures using boric acid (BA), there was interference in the segmental regeneration of the aquatic worm *Lumbriculus variegatus*, preventing the formation of zoids, in addition to the appearance of the head and tail in the division zones, also compromising asexual reproduction of the species. The same was also observed by Sardo et al. (2011), who investigated the bioaccumulative effect of lead (Pb) in bioassays performed using *L. variegatus*, at 85.0 mg/kg concentrations. The authors pointed out that at concentrations below the lethal effect (up to 8.0 mg/kg), inhibition of growth and regeneration of the organism was observed, indicating a disruptive impact. The authors concluded that in these cases, contact and absorption through the skin for aquatic worms is a faster toxicity route than feeding (ingesting contaminated particles), with the invertebrate species being more susceptible to pollutants in the water. In conclusion, prolonged exposure to contaminants indicated that negative impacts on asexual reproduction of Oligochaeta are mainly associated with the inhibition of zoid formation and cell regeneration, caused by the sensitization of specimens to toxic exposures, which possibly occurred in this research using *P. longiseta* as well.

Therefore, protocols are needed to evaluate the potential effects of contaminants on freshwater Oligochaeta species. *Pristina longiseta* presents effects at concentrations below those of other aquatic organisms, showing it is an appropriate test organism for assessing chronic effects of environmental pollutants. As it is a benthic organism, the aquatic worm can be an excellent indicator of contaminants in the water column and the sediment, even at low concentrations in the medium. In general, our results showed that the chronic test protocol could respond effectively to the effects of chemical and environmental samples on the reproduction of *P. longiseta*.

Conclusion

This study presented a new protocol for the assessment of chronic effects on reproduction using an aquatic worm of occurrence in Brazil, which contributes to studies focused on evaluating environmental impacts in freshwaters from tropical regions. We concluded that the best and more accessible configuration for the reproduction test using *P. longiseta* was the static system, without aeration, and an exposure time of 168 h (7 days). Our results showed that during this period were generated between 15 ± 5 new organisms optimal control conditions. *Pristina longiseta* was sensitive to different contaminants, even at low concentrations, showing inhibition of reproduction according to the dose increase, similar to other aquatic oligochaetes used in ecotoxicological assays.

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Data availability All data will be available if requested.

Declarations

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