Pristina Longiseta Reproduction Test: Chronic Exposure To Environmental Contaminants

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Abstract

Aquatic worms are considered a suitable group to evaluate the effects of contaminants in the environment, although one of the main challenges is using native species. Recently, *Pristina longiseta* was suggested to be used in acute bioassays for tropical regions. In this context, the aim of this study was to establish a chronic exposure for ecotoxicological bioassays utilizing the tropical native species *P. longiseta*. Firstly, we tested three exposure times (96h, 7d, and 10d) in the presence and absence of aeration. After determining the best configuration, we evaluated the effects of the chronic exposures using the standardized reference substance potassium chloride, the antibiotic sulfamethoxazole, the flame retardant tetrabromobisphenol-A, and sugarcane vinasse. Our results showed the suitability for applying the chronic exposure using *P. longiseta* and indicated the sensitivity of the offspring to KCl (EC50 = 0.51 g/L). Sulfamethoxazole and TBBPA caused a significant reduction in the offspring of *P. longiseta* (EC50 = 59.9 µg/L and 166.1 µg/L, respectively). Sugarcane vinasse showed high toxicity for the species, and a fraction of 4.26% of vinasse was calculated as EC50. Therefore, the described protocol was successfully applied as an ecotoxicological assessment to evaluate the effects of contaminants on the reproduction rate of the freshwater worm *P. longiseta*.

1. Introduction

Aquatic invertebrates have been widely utilized in ecotoxicological assays around the world, mainly due to their importance in the food chain, diversity, geographic occurrence, and adaptability to laboratory conditions (Hutchinson, 2002; Gorni et al. 2012; Corbi et al. 2015; Rosner et al. 2021). Despite the fact that these organisms are abundant in the environment, few native worm species in tropical regions have been used as bioindicators of environmental quality 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 on the ecology and biology of these species, which hinders 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 some of the species of tropical aquatic oligochaetes of the Naididae and Tubificidae families successfully used in ecotoxicological tests (Chapman et al. 1982; Smith et al. 1991; Phipps et al. 1993; Marchese and 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 (Brinkhurst and Jamieson, 1971; 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 benthic lentic and lotic waters. It has a translucent yellowish color, 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, 2010; Zattara et al. 2011; Gorni et al. 2018). According to Van Cleave (1937) and Zattara et al. (2011), the reproduction of *P. longiseta* occurs mostly in an asexual way, by paratomic fission, forming a new head and tail along the individual's body, and separating after their complete formation. The asexual reproduction ensures genetic stability and

population abundance (Timm, 2012). Sexual reproduction only occurs in nature, in stressful situations such as unfavorable environmental conditions or at specific times of the year (Van Cleave, 1937; Brinkhurst and Gelder, 2001; Rodriguez, 2004; Özpolat, 2016). Smith et al. (1991) evaluated the use of *Pristina longiseta* (as *Pristina leidyi*) in acute bioassays using cadmium and vanadium as test substances. Recently, Castro et al. (2020b) redefined the culture maintenance in the laboratory considering tropical conditions, and application of the organism in short-exposure (48h), evaluating acute effects of the reference substances potassium chloride (KCl), copper sulfate ($CuSO_4$), and zinc chloride ($ZnCl_2$). However, there is no standard protocol to perform chronic exposure using this species that shows it is suitable for acute responses.

For the feasibility of applying a species in toxicity tests, the use of reference substances and environmental contaminants is essential. The substance for sensitivity tests standardized by OECD, USEPA, and ABNT, potassium chloride (KCl), has been recommended to assess the sensitivity of oligochaetes (Corbi et al. 2015; Castro et al. 2020b; Felipe et al. 2020). 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 and Kumar, 2020; Bao et al. 2021). Several authors have identified the ecotoxicological effects of SMX on different aquatic organisms (Park and Choi, 2008; Minguez et al. 2016; Aderemi et al. 2021; Srain et al. 2020; 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 $1.88E10^{-4}$ µg/L and 38.85 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).

The brominated flame retardant Tetrabromobisphenol-A (TBBPA) is one of the most consumed compounds used to reduce the spread of flames in several products, mainly petroleum derivatives (Liu et al., 2016; Pieroni et al. 2017). Oral et al. (2021) pointed out that TBBPA may cause low acute toxicity, but in chronic exposure, can result in many consequences on reproduction, hepatic system and thyroid. Moreover, sugarcane vinasse, the main liquid waste generated in the ethanol and sugar production process, is rich in organic matter and nutrients, with an acidic pH between 4.0 and 4.5 (Fuess et al. 2018). Due to the huge 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 problems 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 already been detected in fish, microcrustaceans, marine bacteria and microalgae (Botelho et al. 2012; Marques et al. 2013; Marinho et al. 2014; Sousa, 2019; Silva et al. 2021). However, there is a body of literature that lacks answers to the vinasse effects (acute and chronic) in aquatic worms from tropical regions.

Initiatives that contribute to the use of bioassay protocols with native test organisms in tropical regions are still poorly studied due to the scarcity of these protocols when compared to those existing for organisms from temperate environments (Di Lorenzo et al. 2019; Gazonato Neto, 2019; Castaño-Sánchez,

2021). This paper presents a new protocol for performing chronic tests using *Pristina longiseta*, a tropical native species. We evaluated different test conditions to define the best exposure time, need for aeration, and the applicability of the test using a reference substance and three contaminants.

2. Material And Methods

2.1 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 x 33 cm wide x 6 cm high) were filled with dechlorinated and filtered water. On average, 20 individuals were distributed per 100 g of sterilized fine sand in a muffle (550ºC for 4 hours). Containers were kept under soft constant aeration (one bubble per second) in a light–dark cycle of 12:12 h and 25 ± 2°C. The water quality of the culture was evaluated measuring the pH (6.12), electrical conductivity (174 µS/cm), temperature (24.3 ºC), using a multiparameter device model AKLA32761. The hardness was measured using a Visocolor® ECO kit (18 mg$CaCO_3$/L). Analyses were performed according to USEPA (2002) recommendations.

2.2 Preliminary reproduction tests

Six configurations of bioassays were tested: a) presence of aeration with the offspring counting on day 4, 7 and 10; b) without aeration with the offspring counting on day 4, 7 and 10. The temperature and dark cycle were the same as the cultivation. Each configuration was evaluated in 15 replicates to determine the best condition to proceed with the *P. longiseta* chronic exposure. Each replicate received 6 organisms, length 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), 10 mg of sterilized fine sand (kept in muffle during 4 hours at 550ºC) in a glass bottle (capacity of 100 mL). Each replicate received food at the beginning (2 mg of Tetramin® macerated fish food), and only in the tests of 10d of exposure, the replicates also received food after 5 days of testing had started (2 mg of Tetramin® macerated fish food). 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 in the beginning (n = 6).

2.3 Ecotoxicological application

After defining the best test conditions in a control 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. We defined a geometric factor of 1.5 between the intervals of concentrations. 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 concentrations were obtained from a 100 g/L solution. The nominal concentrations for

Tetrabromobisphenol-A were 300, 450, 675, 1000, 1500 µg/L. The stock solution of 500 mg TBBPA/L was made in methanol. In the tests using sulfamethoxazole, the concentration range was 38, 55, 86, 128 and 192 µg/L, with the diluted stock solution 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 directly in dechlorinated tap water. The effects on the reproduction rate of *P. longiseta* were evaluated over 168 h (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 test solution and 10 g of sterilized fine sand, without aeration, under 25 ± 2ºC, 12h light: 12h dark photoperiod. The organisms were fed at the beginning of the test with 2 mg of macerated Tetramin® fish food. Offspring was defined as the total number of individuals counted disregarding the total number of incubated individuals in the beginning (n = 6).

2.4 Statistical analyses

To evaluate the best configuration of the chronic bioassay, we applied the Two-way ANOVA 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% reproduction inhibition (EC10 and EC50) using “R” software, version 3.5.0, and MASS and DRC packages. To assess a statistical difference in the number of new organisms produced in the tested concentrations, we applied the One-Way ANOVA and Kruskal-Wallis through the Past® software (Paleontological Statistics), after testing the normality of the data (Shapiro-Wilk test). All statistical analyses considered a 95% confidence interval ($p \leq 0.05$).

3. Results And Discussion

3.1 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 aeration conditions, while in the other exposure times (7d and 10d) each organism had fission at least once. During the exposure time of 7d, the mean number of new organisms was 31 and 36 in the absence and presence of aeration, respectively. A high number of new organisms in the presence of aeration was not observed in the exposure time of 10d, in which the mean number of new organisms at the replicates without aeration was 67 against 52 in the replicates with aeration (Fig. 1). No statistical differences in the number of new organisms were pointed out comparing the presence or absence of aeration (Two-way ANOVA, p-value of 0.471). On the other hand, focusing on the exposure time, the total offspring in 4d showed a significant difference compared to 7d (p-value of $7.68\ 10^{-5}$) and 10d (p-value of $1.19\ 10^{-8}$).

According to Smith et al. (1991), species from the Naididae family present new generations of individuals within a period of 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. However, the authors also state that it is possible for multiple fission zones to form after the initial fission, whereby the organism is

able to divide into more than 2 individuals. In this context, due to a good response in the simpler configuration and short time, we decided to continue the evaluation of *P. longiseta* in reproduction studies following the configuration without aeration and exposure time for 7 days, aiming to obtain the first generation.

We emphasize that the suggested duration for short-term exposure (acute testing) is 48 hours for *P. longiseta* (Smith et al. 1991; Castro et al. 2020b). Another preliminary investigation of the exposure time of 4 d 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 organisms was also considered, such as those performed for *Chironomus* sp. (10d), an aquatic invertebrate (OECD 2011), the tropical aquatic oligochaete *Allonais inaequalis* (10d) (Corbi et al. 2015; Felipe et al. 2020). Determining the configuration of chronic tests lasting 7 days, without aeration, we obtained a fast response in chronic tests for aquatic invertebrates, reducing time and costs in the application of the tests.

3.2 Ecotoxicological assessment

The reproduction bioassay (7d 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 concentration increase (Fig. 2a). Besides, the TBBPA at the lowest concentration induced a significant decrease in reproduction (mean of 3 new organisms at 300 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 the control, and a sharp drop was observed at 86 µg SMX/L (no new organism; Fig. 2c) and at 4.95% of vinasse (mean of 2 new organisms; Fig. 2d).

According to the Kruskal-Wallis test, significant differences were identified between the reproduction of organisms exposed to doses 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 450, 675, 1000 and 1500 µg/L of the TBBPA was significantly different from the reproduction registered in 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 to the control, and for the KCl, the concentrations with 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 were no statistically toxic effects identified comparing the results to control samples (TBBPA: 300 mg/L and methanol control; SMX: 38, 55 mg/L, and methanol control; KCl: 0.5, 0.3 mg/L; sugarcane vinasse: 1.5, 2.2 and 3.3%) Moreover, the classic ecotoxicological parameters EC50, NOEC, and EC10 were obtained. Among the assessed substances, sulfamethoxazole showed the highest toxicity, presenting an EC50 of 59.9 µg/L, followed by Tetrabromobisphenol-A (EC50 =166.1 µg/L). The sugarcane vinasse caused an EC50 of 4.26%, indicating that this dilution caused an inhibitory effect of 50% on reproduction. Besides, the reference substance, KCl indicated an EC50 of 0.51 g/L (Table 1).

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

Contaminants	EC50	Standard error	NOEC	EC10
KCl	0.51	0.03	0.50	0.12
TBBPA	166.1	0.70	300	5.56
SMX	59.9	1.83	55	52.1
Sugarcane vinasse	4.26	0.23	4.95	3.25

Values in g/L for KCl; in µg/L for tetrabromobisphenol-A and sulfamethoxazole; and percentage of dilution for sugarcane vinasse.

By assessing EC10, we verified that the substances did not follow the same pattern as the toxicity of the EC50. SMX caused an EC10 of 52.1 µg/L, a concentration detected that poses a risk to the organisms, with a value close to the EC50. The effect observed in 10% of the organisms for tests with TBBPA was 5.56 µg/L, indicating that the contaminant shows effects at concentrations much lower than the identified EC50. Comparing the effects of SMX and TBBPA, we observed that SMX was more toxic (EC50 59.9 µg/L) than TBBPA. However, TBBPA causes a toxicity effect in 10% of organisms (EC10 5.56 µg/L) at much lower concentrations than SMX, showing that the species of *P. longiseta* was more sensitive to the antibiotic due to a window between the effect observed in 10 and 50% of the organisms to be smaller when compared to that observed in TBBPA. KCl caused a LOEC of 0.12 g/L, and raw sugarcane vinasse also had the unobserved effect near the EC50, at 3.25%.

Pristina longiseta is known to be more sensitive to the reference substance KCl in acute bioassays when compared to *Allonais inaequalis*, another native Brazilian Oligochaeta. Castro et al. 2020a observed a LC50 of 1.36 g/L for the short exposure of *P. longiseta* to KCl, whereas Corbi et al. (2015) found a LC50 of 3.5 g/L for *A. inaequalis*. The same was observed in chronic bioassays, where the EC50 for *P. longiseta* was close to the EC10 found by Felipe et. al (2020) for *A. inaequalis* (0.50 g /L), in 10-day chronic bioassays. The concentration of the effect on 50% of *P. longiseta* offspring (EC50 0.51) is a concentration of the beginning toxic effect in *A. inaequalis*.

Regarding the flame retardant, for the crustacean *Daphnia magna*, Yang et al. (2012) observed that TBBPA changed the reproduction rate of the individuals, presenting EC10-21d of time to the first brood, the total number of spawning and number of broods of 84 µg/L; 16 µg/L and 139 µg/L. Showing that for the observation of the effect in 10% of organisms, *P. longiseta* (EC10 5.56 µg/L) was more sensitive to exposures to TBBPA than the microcrustacean *Daphnia magna*. 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 in agreement with that found in this research. 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). In addition, other authors point to TBBPA as an endocrine-disrupting agent in aquatic invertebrates, causing, in addition to impacts on reproduction, effects on species development (Yang et al. 2012; Pittinger and Pecquet, 2018; Wang et al. 2021). Corroborating the authors, our results indicated that this substance presents negative effects on the reproduction rate from concentrations below 200 µg/L. The 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, but in industrial and e-waste areas these values can be higher (e.g., 9750 ng/g dw) (Liu et al 2016). Moreover, it is known that this compound has the capacity to accumulate in different tissues of aquatic biota (Harrad et al 2009; Gong et al. 2021) and was a concern regarding its effects in long exposure.

For SMX, *P. longiseta* was more sensitive (EC10 of 52.1 µg/L) when compared to the alga *Pseudokirchneriella subcapitata* (EC10 of 150 µg/L); the microcrustacean *Ceriodaphnia dubia* (EC10 of 250 µg/L) and the cnidarian *Hydra attenuata* (EC10 of 5000 µg/L) (Straub, 2015). Comparatively, the reproduction of the naidid *P. longiseta* was more sensitive to SMX when compared to the species studied by Straub et al. (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 has chronic and sub chronic effects on *Danio rerio* zebrafish, delaying egg hatching and impacting fish body size. In addition, other studies have also observed the effects of oxidative stress in the microalgae *Raphidocelis subcapitata* (Zhang et al. 2021), and inflammatory effects on fish, *Ctenopharyngodon idella* (Wang et al. 2021), *Oreochromis niloticus* (Hu et al. 2021) and *Danio Rerio* (Qiu et al. 2020). Studies show that at relevant environmental concentrations, SMX can cause chronic effects in different aquatic organisms, corroborating the results observed for *P. longiseta* in this study. In this context, the need to investigate the effects of these contaminants on other tropical aquatic worms is evident, as the bibliography for these organisms is scarce.

Due to the potential toxicity of sugarcane vinasse and its negative effects on aquatic biota (Silva et al. 2007; Christofoletti et al. 2013), in the 1970s, restrictive laws were established prohibiting the 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, Felipe et al. 2021). Therefore, there is a body of literature lacking answers to the vinasse effects (acute and chronic) on aquatic worms from tropical regions. Verma and Dalela (1976) performed toxicity tests with 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 96h of exposure at 32 ± 2ºC. Moreover, an increase in mucus production and a reduction of proteins in liver, brain, kidneys, and muscles of *Channa punctatus* were reported in dilutions from 50% of vinasse (Kumar and Gopal, 2001). Regarding chronic studies, two species of aquatic insect had the 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% of nature vinasse can affect 50% of the offspring, showing again the high sensitivity level of this freshwater worm species.

There is a need for protocols that evaluate the potential effects of contaminants on freshwater Oligochaeta species. The fact that *Pristina longiseta* shows effects at concentrations below those of other aquatic organisms is a good indication that *P. longiseta* can be used to assess environmental contamination. As it is a benthic organism, *P. longiseta* can be an excellent indicator of contaminants not only in the water column but also into the sediment, even at low concentrations in the medium. In general, our results showed that the chronic test protocol can respond to the effects of chemical and environmental samples on the reproduction of *P. longiseta*.

4. Conclusion

This study presented a new protocol for long-term ecotoxicological assessments using the tropical worm *Pristina longiseta*, which contributes to the assessment of environmental impacts in tropical regions. We concluded that the best configuration for the reproduction test was the static system, without aeration, with an exposure time of 168 hours (7 days). Our results showed that during this exposure time it generated between 11 ± 5 new organisms in a control. *Pristina longiseta* was sensitive to different contaminants, even at low concentrations, showing an inhibition of reproduction according to the dose increase, similar to other aquatic oligochaetes used in ecotoxicological assays.

Declarations

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Authors Contributions

Tallyson Souza: Research, experiments, writing and data analysis; Gleyson Castro: Research, experiments, writing and review; Aline Bernegossi: Research, writing, and review; Mayara Felipe: Experiments, data analysis and graphing; Fernanda Pinheiro: Research, experiments and cultivation of the species in the laboratory; Vanessa Colombo-Corbi: Writing and review; Douglas Girolli: Collection and species description; Guilherme Gorni: Species description and data analysis; Juliano Corbi - Supervision, writing and final review.

Availability of data and materials

All data will be available if requested.

Ethical Approval

Not applicable for this manuscript.

Consent to Participate

Not applicable for this manuscript.

Consent to Publish

Not applicable for this manuscript.

Competing interests

The authors declare no competing interests.

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Figures

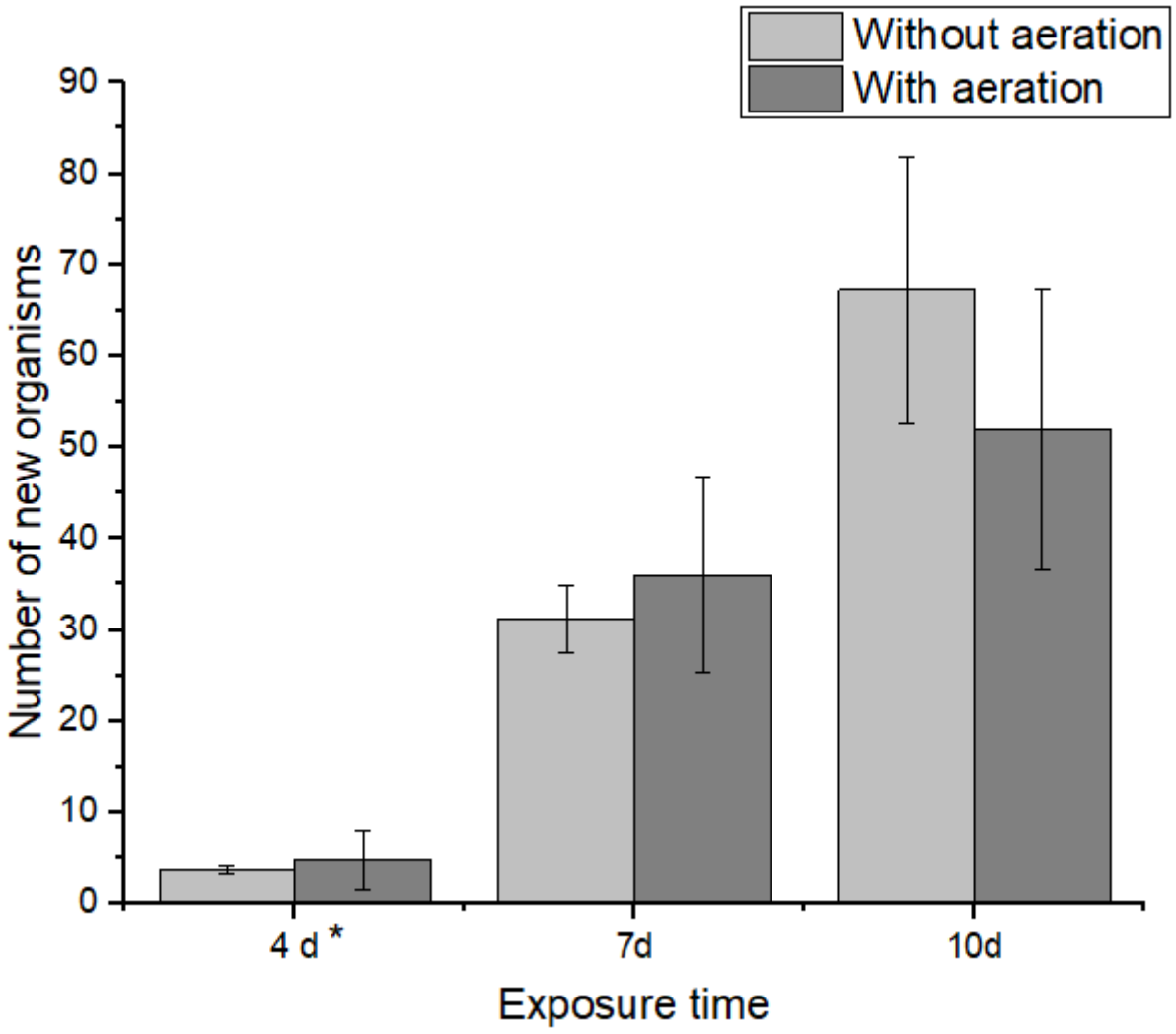

Figure 1

Offspring of *Pristina longiseta* in a control environment according to exposure time and aeration requirement

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

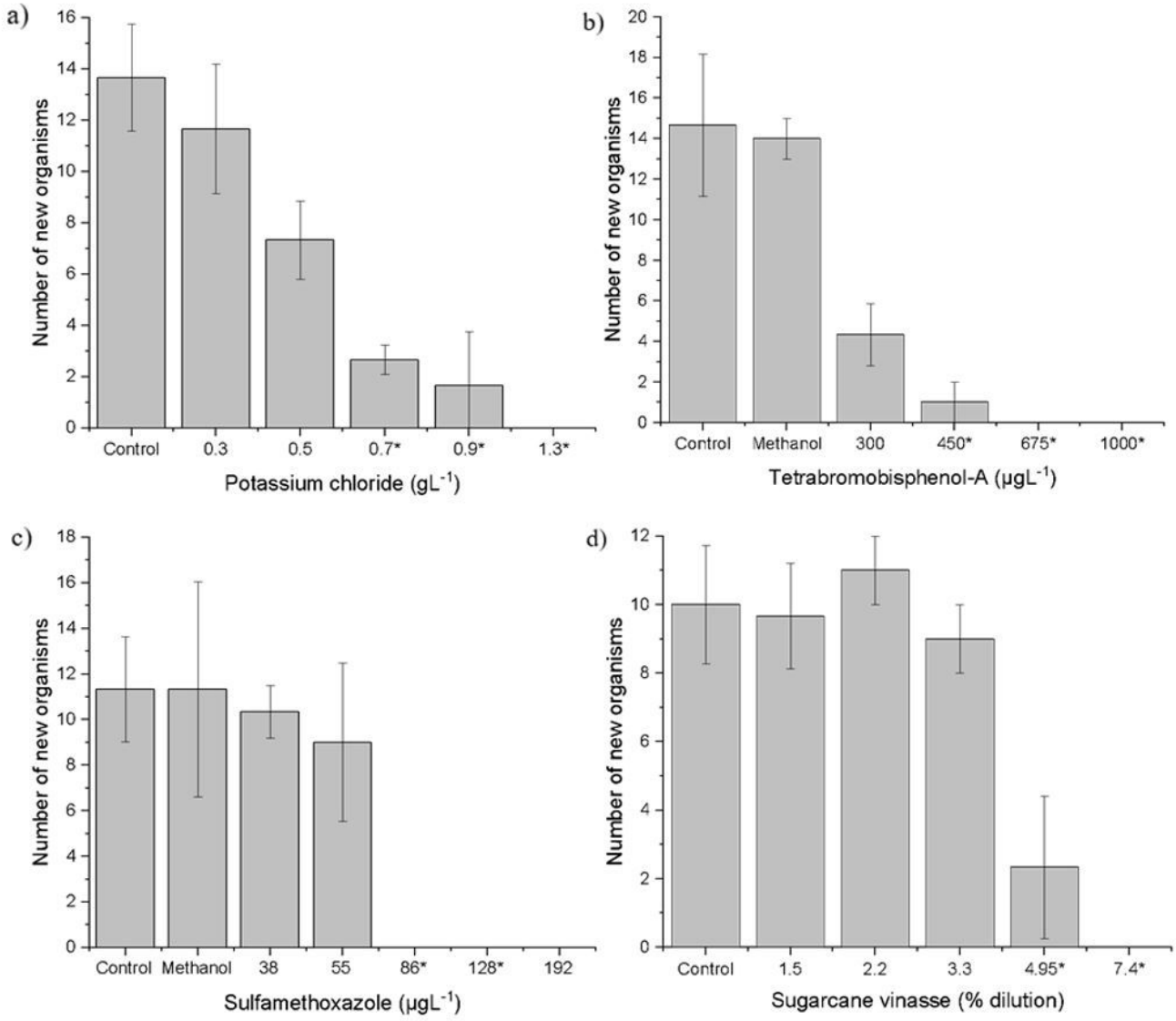

Figure 2

Effects of the reference substance (potassium chloride) and environmental contaminants (sulfamethoxazole, tetrabromobisphenol-A, and sugarcane vinasse) on the *Pristina longiseta* reproduction

* means that showed a significant difference compared to the control