Sugarcane vinasse provokes acute and chronic responses and bioaccumulation of metals in benthic macroinvertebrates

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Abstract

Due to increases in demand and global interest in bioenergy production, Brazil has stood out in the world production of sugarcane bioethanol. The intensification in the use of this alternative energy draws attention to the environmental impacts of sugarcane cultivation and industrial processes on ethanol production, highlighting the generation of a by-product with a high pollution potential called vinasse. When vinasse reaches watercourses, it may affect the biological communities such as the aquatic macroinvertebrates, which include species sensitive to environmental contamination. Thus, this study aimed to evaluate the ecotoxicological effects of sugarcane vinasse on tropical benthic macroinvertebrates (*Allonais inaequalis, Chironomus sancticaroli, Strandesia trispinosa*, and *Hyalella meinerti*). For this, the study was separated into three phases. In the first one, acute toxicity tests were carried out with the four species. The species *A. inaequalis* (average LC_{50} = 0.460% confidence interval - CI 0.380-0.540%) was more sensitive to vinasse than *C. sancticaroli* (LC_{50} 0.721%, CI 0.565-0.878%), *H. meinerti* (EC_{50} 0.781%, CI 0.637-0.925%), and *S. trispinosa* (EC_{50} 1.283%, CI 1.045-1.522%). In the second phase, the consequences of chronic exposure to vinasse were assessed in the two more sensitive species. Effects in reproduction and population growth rates for *A. inaequalis* and on development, metamorphosis, and growth of *C. sancticaroli* larvae occurred. Finally, the metal residuals in the body residue after chronic exposure to the two species were determined in the third phase. Vinasse provoked decreases in body residue of the essential metals Zn and Mn and the accumulation of Cd, Pb, and Cr with risks of biomagnification throughout the food webs. Low doses of vinasse provoked lethal and sublethal effects on benthic organisms, with several cascade effects on aquatic environments, given the ecological importance of this group in aquatic and terrestrial ecosystems.

1 Introduction

The industrial revolution increased the world's energy dependence, with emphasis on the use of fossil fuels, which are characterized by the elevated cost of transport and production, besides the high rate of carbon emission into the atmosphere, as well as the possibility of depletion of the fossil reserves (Goldemberg and Lucon, 2007). Thus, new energy alternatives began to attract attention, and global interest in bioenergy production has grown in the last decades (Martinelli; Filoso, 2008; Smeets et al., 2008; Ravindranath et al., 2009). This is the case with the production of sugarcane ethanol. Brazil is a great producer of ethanol due to this extensive territory, water availability, and favorable solar radiation, which provides the existence of large monocultures for the cultivation of sugarcane (Martinelli and Filoso, 2008). Thus, discussions regarding the use of this energy source have started, mainly about the environmental impacts arising from the large-scale production of bioethanol (Secchi et al., 2011).

During the distillation process of fermentation liquor to obtain ethanol, a liquid by-product with a high potential for pollution called vinasse is produced (Silva; Griebeler; Borges, 2007). For each liter of ethanol obtained, 8 to 18 liters of vinasse are generated (Parnaudeau et al., 2008). This compound has acidic characteristics (pH 3.5-5), with the appearance of a dark brown paste of unpleasant odor, which contains a high organic and metal load (Christofoletti et al., 2013). Consequently, ethanol-producing industries

need a destination for this residue. The fertirrigation of sugarcane fields is adopted as the main alternative in Brazil. Due to its high organic load and the presence of nutrients such as potassium, calcium, and nitrogen, fertirrigation can be beneficial for crop growth (Botelho et al., 2012). On the other hand, there are still doubts about the use of vinasse for this purpose due to impacts on soil and the possibility of contamination of water resources by leaching, percolation, and surface runoff processes (Santana and Fernandes Machado, 2008; Silva et al., 2007). In addition, the improper disposal of this waste during fertirrigation activities and accidents during storage and transport may imply a direct entry of vinasse into aquatic environments (Martinelli; Filoso, 2008). When reaching freshwater environments, vinasse may cause effects on the biological communities present there, which implies an unbalance in the aquatic environment, reducing productivity and biological diversity, causing damage to nutrient cycling and the dynamics of the food chain (McBride et al., 2011).

Benthic macroinvertebrates, such as the insect *Chironomus sancticaroli* (Diptera: Chironomidae), the Oligochaeta *Allonais inaequalis* (Haplotaxida: Naididae), the amphipod *Hyalella meinerti* (Amphipoda: Hyalellidae) and the ostracod *Strandesia trispinosa* (Podocopida: Cypridídae) can be used as bioindicators of environmental stress, mainly because their life cycles include sediment, water column and, in the case of *C. sancticaroli*, terrestrial ecosystems in the adult stage (De Castro-Català et al., 2016, Silva; Griebeler; Borges, 2007). These organisms are part of the basis of the food chain and an essential element in the transfer of energy and nutrients from primary producers to higher trophic levels, influencing the stability and resilience of ecological systems. Thus, the loss of these species, as well as changes in biomass and population growth, imply damage to ecosystem services (Chagnon et al., 2015). In addition, benthic organisms can bioaccumulate metals via different exposure routes (contaminated water and sediment) with risks of biomagnification throughout the food chain, given the importance of these organisms in the productivity of aquatic environments (Leppänen, 1995). Vinasse has several metals in its composition, and thus with the potential for bioaccumulation by exposed organisms (Christofoletti et al., 2013; Coelho et al., 2018; España-Gamboa et al., 2011).

Given the high volume of sugarcane vinasse produced, its polluting potential, and its use as a fertilizer in sugarcane crops it is necessary to evaluate the effects of exposure to this residue in aquatic organisms. Besides, there is a knowledge gap about the ecotoxicological effects of vinasse on freshwater species. Thus, the present study evaluated (i) the implications of acute exposure to vinasse on *C. sancticaroli*, *A. inaequalis, H. meinerti,* and *S. trispinosa* species, (ii) the subchronic responses on the survival, development, and growth of *C. sancticaroli* and chronic implication for the reproduction and biomass of *A. inaequalis,* and (iii) the bioaccumulation of metals presents in vinasse after subchronic and chronic exposure in both species. Thus, the results described will bring new discussions about the environmental implications of potential contamination of freshwater ecosystems caused by fertirrigation practices in sugarcane management.

2 Methodology

2.1 Test organisms

The test organisms were obtained from cultures kept at the Nucleus of Ecotoxicology and Applied Ecology (NEEA) in the Center for Water Resource and Environmental Studies. The species *C. sancticaroli* and *A. inaequalis* were maintained according to the procedure described in Pinto et al. (2021b) and Corbi et al. (2015), in plastic trays filled with culturing water (pH: 7.0-7.5, electrical conductivity: 150–160 µS cm^{-1} and hardness: 40–48 mg $CaCO_3$ L^{-1}) and artificial sediment (fine sand washed and burned at 550°C for 2h) in a proportion of 4:1 (water: sediment), with constant aeration. Food (Tetramin® suspension 5 g L^{-1}) was provided *ad libitum* each other day to *C. sancticaroli* and one time a week to *A. inaequalis*. *H. meinerti* were kept according to the Brazilian normative NBR 15470 (2013) in glass aquariums filled with 4L of culturing water (pH: 7.0-7.5, electrical conductivity: 50.5 ± 1.4 µS cm^{-1} and hardness: 12–16 mg $CaCO_3$ L^{-1}) containing the macrophytes *Egeria densa* and *Myriophyllum aquaticum* as food supply and substrate, and constant aeration. Organisms were fed *ad libitum* (Tetramin® suspension and biological yeast) each other day. The species *S. trispinosa* was obtained from outdoor cultures (see Pinto et al., 2021a) and acclimated in the laboratory ten days before experiments. For that, the water was replaced with culturing water under the same conditions as *H. meinerti.* Decomposition leaves from the outdoor cultures were provided as food resources. All cultures were kept at a temperature of 25 ± 1°C and a daily cycle of 12:12 (light: dark). For the toxicity tests with *C. sancticaroli*, the organisms were obtained from egg masses from the cultures, and *A. inaequalis* was chosen by the body size of 7.8 ± 0.37 mm (ROCHA et al., 2018) as the species reproduces by bipartition (Armendáriz, 1999). *S. trispinosa* was also chosen by the body size of 0.93 ± 0.08 mm (Rocha et al., 2018). *H. meinerti* juveniles (1–7 days) were separated from cultures and kept in a new glass aquarium until they reached the age of 7–14 days before the toxicity tests.

2.2 Acute toxicity test

Acute toxicity tests were performed under the same temperature and light conditions as the cultures using the culturing water in dilutions and laboratory control. Preliminary tests were carried out to establish the dilutions used in the acute experiments. The bioassays with *A. inaequalis* and *S. trispinosa* were made in 50 mL non-toxic plastic bottles (Copaza®) containing 10 mL of test solution, without substrate and without feeding (Rocha et al., 2018). The organisms were exposed to five dilutions of crude vinasse *A. inaequalis* (0.125, 0.25, 0.50, 1.0, and 2.0%), and *S. trispinosa* (1.0, 1.2, 1.4, 1.6 and 1.8%). The tests with *C. sancticaroli* were carried out in 400 mL non-toxic plastic bottles (Copaza®) containing 240 mL of the test solution (0.4, 0.6, 0.8, 1.0, and 1.2%) and 60 g of artificial sediment. Four replicates containing six organisms (7 to 8 days old) each were prepared in each dilution, adapting the methodology described in the OECD (2011) guideline. The tests with *H. meinerti* were carried out in 400 mL non-toxic plastic bottles (Copaza®) containing 200mL of vinasse dilutions (0.6, 0.8, 1.0, 1.2, 1.4%) and a nylon screen (4 cm x 15 cm, 0.25 mesh-size) as substrate. Four replicates by dilution containing ten organisms (7 to 14 days old) each were prepared, following the Brazilian normative NBR 15470 (2013). Food was provided at the beginning and middle of the experiments with *C. sancticaroli (*0.62 mg TetraMin® $larvae^{-1}$ day^{-1}) and *H. meinerti* (0.025 mL compound food $organism^{-1}$). The acute tests lasted 96h, except for *S. trispinosa*, which lasted 48h. All tests with the four species were repeated three

times with different populations. The endpoints analyzed were mortality (*C. sancticaroli* and *A. inaequalis*) and immobility (*H. meinerti* and *S. trispinosa*). The dissolved oxygen and temperature (YSI55-25 ft), pH (micronal B374), and electrical conductivity (Oriom 145) were measured at the beginning and end of the tests. The criteria for validity were mortality in the control group below 10% in all species (OECD, 2011; Pinto et al., 2021c).

2.3 Subchronic and chronic toxicity test

The bioassays were performed with five vinasse dilutions for *C. sancticaroli* (0.041, 0.081, 0.163, 0.325 and 0.65%) and *A. inaequalis* (0.025, 0.05, 0.10, 0.20 and 0.40%) besides of the untreated control (culturing water). The higher dilution values were selected based on the CL_{10} obtained on acute tests. Besides, these two species were selected due to their sensitivity to vinasse (see topic 3.2). The bioassays with both species were made in 400 mL non-toxic plastic bottles containing 60g of the substrate (fine sand) and 240 mL of culturing water, with eight replicates per treatment containing ten organisms each (four days old for *C. sancticaroli* and 7.8 ± 0.37 mm for *A. inaequalis*). The tests lasted eight and ten days for *C. sancticaroli* (subchronic exposure) and *A. inaequalis* (chronic exposure), respectively. Food was provided every other day (0.62 mg TetraMin® organism^{-1} ·day^{-1}). The water parameters dissolved oxygen, pH, and electrical conductivity were measured at the beginning and end of the experiments. Besides, the concentration of ammonium (Hansen and Koroleff, 2007) was determined at the experiment's end. The tests were validated for maximum mortality of 20% for *C. sancticaroli* and population doubling for *A. inaequalis* in the control treatment (Pinto et al. 2021a). Similar to the acute tests, the experiments with both species were repeated three times with different populations.

At the end of the experiment with *C. sancticaroli* (8-days), the number of alive larvae, pupae, and adults was registered. The larvae were anesthetized in phenoxyethanol and photographed with graph paper. The larval body length was measured using the free software *Kinovea* 0.8.15 (https://www.kinovea.org/) (Pinto et al., 2021). For *A. inaequalis*, the number of alive organisms was counted after ten days for reproduction determination. After that, the individuals of both species were kept in clean water for 24 h for gut cleansing and further determination of metal bioaccumulation.

2.4 Analyses of crude vinasse

Physical-chemical and biological characterization of crude vinasse used in the present study was performed. The biochemical oxygen demand (BOD) was measured by the oxygen consumption during a 5-days incubation time, while chemical oxygen demand (COD) was analyzed by the closed reflux method (APHA, 2018). Furthermore, was determined the pH (pH-meter micronal B374), electrical conductivity, salinity, total dissolved solids (conductivity meter Oriom 145), hardness (ABNT NBR 12621, 1995), total nitrogen (APHA, 2018), nitrite, nitrate, and ammonium ion (Hansen and Koroleff, 1999; Mackereth et al., 1978), total and dissolved phosphorus, and inorganic phosphate (Andersen, 1976). Besides, an aliquot was taken for metal analyses.

2.5 Chemical analyses of metals

The preserved organisms were dried (60°C) until constant weight to determine the dry biomass. The metals were extracted by acid digestion with 5 mL of HNO_3:H_2O_2 mixture (2:1 v:v) in a water bath at 80°C for four hours. Afterward, the sample volume was completed up to 10 mL with HNO_3 (2M). The blank was carried out with the acid and peroxide mixture and completed up to 10 mL (Afridi et al., 2006).

In addition, samples from the test solution were taken at the beginning of each chronic and subchronic experiment. The solution samples and crude vinasse were prepared for chemical analysis by acid digestion (US EPA, 1992). Metals were quantified by microwave plasma atomic emission spectroscopy (MP-AES 4200, Agilent Technologies). For the analytical calibration curves, multielement standard solutions (Agilent Technologies®) were utilized, except for aluminum (Sigma-Aldrich®) and potassium (SpecSol®). The limits of detection (LOD) and quantification (LOQ) were obtained according to the method described in Hage and Carr (2012).

2.6 Data analysis

In the acute tests, lethal and effect concentrations for 50%, 20%, and 10% of organisms were estimated by non-linear regression with a logistic model. The responses in the subchronic and chronic bioassays and metal residuals in the organisms were compared with the control group by one-way ANOVA, followed by Dunnett's post hoc test. Data normality and homogeneity of variances were determined using the Shapiro-Wilk and Levene tests, respectively. In the case of non-compliance with the parametric requirements, the non-parametric test of Kruskal-Wallis was used. The Ash Free Dry Weight of *C. sancticaroli* (AFDW) was estimated based on the body length of the larvae, following the method described by Pinto et al. (2021). The presented results are the average of the three repetitions of acute and chronic assays. All analyses were performed in the Statistica 7.0 software (StatSoft, 2004) with a confidence interval of 95% ($p < 0.05$).

3 Results And Discussion

3.1 Vinasse characterization

Table 1 presents the physical-chemical and biological characterization of the crude vinasse used in the experiments. Vinasse composition depends on the sugarcane harvest, the fermentation, and the distillation conditions adopted (España-Gamboa et al., 2011). Thus, this composition is variable between different studies in the literature (e.g. Coelho et al., 2018; Christofoletti et al., 2013; Correa et al., 2013; Ferreira et al., 2011; Garcia et al., 2017). The crude vinasse used in the present study had high polluting potential, denoted by the high electrical conductivity and concentration of phosphorus, nitrogen, potassium, calcium, aluminum, magnesium, iron, and other metals, in addition to a high organic load (BOD and COD) and low pH.

Table 1
Physical-chemical parameters of the crude vinasse.

Parameters	Unity	Pure vinasse
pH	-	3.91
Electrical conductivity	$\mu S\ cm^{-1}$	8,420
Salinity	%	4.5
Total dissolved solids	$mg\ L^{-1}$	4,450
Hardness	$mg\ CaCO_3\ L^{-1}$	3,500
Total nitrogen	$mg\ L^{-1}$	639
Total phosphorus	$mg\ L^{-1}$	149,670
Nitrate	$mg\ L^{-1}$	9.3
Nitrite	$mg\ L^{-1}$	0.2
Ammonium	$mg\ L^{-1}$	52.6
Inorganic phosphate	$mg\ L^{-1}$	82.9
Total dissolved phosphorus	$mg\ L^{-1}$	84.9
BOD*	$mg\ L^{-1}$	46,500
COD*	$mg\ L^{-1}$	107,000
Chromium (Cr)	$mg\ L^{-1}$	0.207
Copper (Cu)	$mg\ L^{-1}$	3
Lead (Pb)	$mg\ L^{-1}$	0.34
Manganese (Mn)	$mg\ L^{-1}$	6.24
Nickel (Ni)	$mg\ L^{-1}$	0.183
Cadmium (Cd)	$mg\ L^{-1}$	0.025
Zinc (Zn)	$mg\ L^{-1}$	1.225
Cobalt (Co)	$mg\ L^{-1}$	0.219

*BOD – Biochemical oxygen demand, COD – Chemical oxygen demand

Parameters	Unity	Pure vinasse
Strontium (Sr)	mg L^{-1}	1.518
Molybdenum (Mo)	mg L^{-1}	0.080
Lithium (Li)	mg L^{-1}	0.031
Magnesium (Mg)	mg L^{-1}	391
Antimony (Sb)	mg L^{-1}	0.53
Aluminum (Al)	mg L^{-1}	499.9
Barium (Ba)	mg L^{-1}	1.95
Calcium (Ca)	mg L^{-1}	1,140
Sodium (Na)	mg L^{-1}	60
Potassium (K)	mg L^{-1}	3,360
*BOD – Biochemical oxygen demand, COD – Chemical oxygen demand		

Table 1

3.2 Acute tests

Table 2 shows the average lethal or effect concentrations for *C. sancticaroli, A. inaequalis, S. trispinosa*, and *H. meinerti* populations. Survival of all species was higher than 90% in the control groups, and the experiments were valid. At the beginning and end of the tests, increases in the vinasse doses reduced the values of pH and dissolved oxygen (DO) and increased the electrical conductivity. At the end of acute toxicity tests, DO was below 6 mg L^{-1} (Table S1).

Table 2
Lethal and effect concentrations (mean and confidence intervals) for the acute toxicity tests with *C. sancticaroli, A. inaequalis, S. trispinosa*, and *H. meinerti*.

	C. sancticaroli (96h)	*A. inaequalis* (96h)		*S. trispinosa* (48h)	*H. meinerti* (96h)
CL_{50} (%)	0.721 (0.565–0.878)	0.460 (0.380–0.540)	CE_{50} (%)	1.283 (1.045–1.522)	0.781 (0.637–0.925)
CL_{20} (%)	0.652 (0.466–0.838)	0.395 (0.263–0.528)	CE_{20} (%)	1.085 (0.918–1.251)	0.651 (0.533–0.769)
CL_{10} (%)	0.615 (0.412–0.818)	0.363 (0.198–0.529)	CE_{10} (%)	0.982 (0.850–1.115)	0.585 (0.480–0.691)

Table 2

In the present study, *A. inaequalis* was more sensitive to vinasse than *C. sancticaroli*, followed by *H. meinerti* and *S. trispinosa*. The LC_{50}-96h value to *A. inaequalis* was also below the reported for the microcrustaceans *Ceriodaphnia dubia* (EC_{50}-48h 0.67%) and *Daphnia magna* (EC_{50}-48h 0.80%), and the fish *Danio rerio* (LC_{50}-96h 2.62%) (Botelho et al., 2012). In the same way, all species in the present study had lower lethal values than the microcrustaceans *D. magna* (EC_{50}-48h 3.6%) and *D. similis* (EC_{50}-48h 2.2%) (Ferreira et al., 2011). However, as discussed previously, the characteristics of vinasse are variable, and the results are not directly comparable.

Both cited studies used methods to treat vinasse and compared the toxicity before and after treatment. After adjusting the pH values from 4 to 7, Botelho et al. (2012) observed a reduction in toxicity with increases in the CE_{50} up to 4.5-times (final EC_{50}-48h 2.99%) for *C. dubia*, 7-times (EC_{50}-48h 5.62%) for *D. magna*, and 3.2-times (LC_{50}-96h 8.34%) for *D. rerio*. After vinasse treatment with the fungus *Pleurotus sajor-caju*, Ferreira et al. (2011) noticed a reduction of the toxicity for *D. magna* 18.4-times (final EC_{50}-48h 66%) and *D. similis* 9.5-times (EC_{50}-48h). These results show that the crude vinasse characteristics, including its acidity, influence the survival of exposed organisms. Vinasse toxicity is possibly caused by the metals and low pH (Garcia et al., 2017). Besides physiological effects, the low pH may increase the availability of metals for exposed organisms (Eggleton and Thomas, 2004). Also, acute toxicity (96h) is reported in the fish *Oreochromis niloticus* at dilutions between 5–10% (Correia et al.,2017; Marinho et al.,2014). Coelho et al. (2018) observed toxicity for the same species post-exposure to crude and leached vinasse at 2.5%.

3.3 Subchronic and chronic tests

The physical-chemical water parameters (pH, electrical conductivity, and DO) evaluated in the bioassays are presented as supplementary material (Tables S2). The pH values and electrical conductivity increased between the beginning and end of the tests. At the end of the assays, pH was higher than 6.5, and ammonium levels were lower than 2 mg L^{-1} for experiments for all species. For *C. sancticaroli*, the DO values strongly decreased from the beginning (7.2 ± 0.2 mg L^{-1}) to the end of the tests (2.8 ± 0.7 mg L^{-1}) at the higher concentration (0.65%). For the other treatments, values remained higher than 4 mg L^{-1}. Oppositely, no significant decreases in DO occurred for experiments with *A. inaequalis*, and concentrations were higher than 6 mg L^{-1}. Artificial aeration was not introduced as the decrease in oxygen levels is considered a stressor related to vinasse pollution (Gunkel et al., 2007; Pinto et al., 2021).

3.3.1 Chironomus sancticaroli

Survival of *C. sancticaroli* in the control group was 92.08 ± 6.88%, and the tests were validated. No effects on survival occurred for none of the vinasse dilutions after 8-day exposure ($p > 0.05$, Fig. 1). Regarding the

larval growth, no effects occurred for organisms exposed to low vinasse concentrations 0.041 to 0.325% ($p > 0.05$, Fig. 1). In the intermediate dilution (0.163%), organisms accelerated the development and metamorphosis by increasing the number of formed pupae (28.61 ± 20.51%) compared with the control (9.72 ± 8.67%, $p < 0.001$, Fig. 1). For the other dilutions, no differences occurred in the number of pupae formed. Sibley et al. (1997) noticed that the midge *C. tentans* start the metamorphosis when larvae achieve minimal biomass (0.5 mg), thus indicating a minimal growth that initiates the metamorphosis. In addition, studies have reported that chironomid larvae can identify contamination by metals and present different strategies for escape from these contaminants (Belowitz et al., 2014; De Haas et al., 2006; Wentsel et al., 1977). Thus, the hypothesis for the advancement of metamorphosis in the intermediate dilution (0.163%) was an avoidance response from the contamination since the larvae had sufficient growth conditions similar to the control (Fig. 1, $p > 0.05$).

Figure 1

No differences occurred in the number of adults (midges) between control and treatments from 0.041 to 0.325% ($p > 0.05$). On the other hand, exposure to the higher proportion of vinasse (0.65%) decreased the number of pupae and adults compared to the control group ($p < 0.01$), thus indicating a delay in the larval development. Similar effects occurred for *C. sancticaroli* larvae, which presented delays in development when exposed to antimony metal, the insecticide fipronil, and phenanthrene hydrocarbon (Moraes et al., 2014; Pinto et al., 2021b; Richardi et al., 2018). The body length of the fourth instar larvae of *C. sancticaroli* ranges between 9.37 to 15.1 mm, while for the third instar, the values are 5.12 to 6.60 mm (Fonseca and Rocha, 2004). The body length of the organisms from control and low concentrations (0.041 to 0.325%) is within the fourth instar ranges. In contrast, the larvae exposed to 0.65% presented a lower body length, reinforcing the hypothesis of delays in larval development in this treatment.

In the same way as for development, a decrease of 33% in the body length (7.76 ± 1.66 mm) and 50% in biomass (0.24 ± 0.13 mg, $p < 0.05$, Fig. 2) after exposure to 0.65% of vinasse occurred. In a study with four species of chironomids (*C. riparius, C. prasinus, C. tentans*, and *C. plumosus*), most of the consumed food was converted into biomass during the larvae growth phase (Péry et al., 2005). However, organisms exposed to stressors have less energy available for growth than non-stressed individuals, implying less biomass acquisition and shorter body length (Liber et al., 1996), as observed in the present study. Exposure to chemical stressors triggers severe population declines that can be inferred from the growth of the early stages of life (Liber et al., 1996). Reductions in larval growth in chironomids are associated with a proportional decline in emergence and reproduction, which leads to a smaller number of larvae in the next generation, thus reducing the population size with risks of local extinction (Sibley et al., 1997).

Figure 2

3.3.2 Allonais inaequalis

Figure 3 shows the reproduction and population growth of *A. inaequalis* post 10-days exposure. Organisms from intermediated dilutions (0.05 and 0.1%) presented increases in the final number of

organisms and population growth rate ($p < 0.05$). The increment was about 27% and 19%, respectively. Contrarily, the reproduction of organisms exposed to the highest vinasse dilution (0.4%) was 68% lower than control, with the reduction in population growth rate decreases reaching 80% ($p < 0.05$).

Figure 3

The family Naididae, which includes the *A. inaequalis* species, can alternate between sexual and asexual reproduction, distinguishing this family from most aquatic oligochaetes (Erséus et al., 2017; Parish, 1981). Asexual reproduction occurs where populations can self-perpetuate from the individual fragmentation (partition) and is predominantly among this family. Sexual reproduction mainly occurs when individuals are exposed to stressors. Thus, the asexual reproduction pattern allows an individual to generate a new offspring every few days and is associated with water quality, food availability, and temperature (Erséus et al., 2017; Parish, 1981). In this way, the increased population growth rates in the intermediate dilutions were associated with higher food availability once vinasse had a high organic matter load. On the other hand, the stress caused to *A. inaequalis* by exposure to the highest vinasse dilution (0.4%) may have triggered a reduction in the fragmentation rate and, consequently, an alteration in the reproduction of organisms. Besides, organisms exposed to this dilution presented increased biomass (3.6 times) compared with control ($p < 0.05$, Fig. 3). To other treatments, no effects occurred for the individual biomass ($p > 0.05$).

To the best of our knowledge, no effects related to vinasse exposure are described in the literature on the reproduction of aquatic oligochaetes. On the other way, Alves et al. (2015) describe the toxic effects of distinct doses of sugarcane vinasse (25 to 294 mL Kg^{-1} DW) on the terrestrials' oligochaetes *Eisenia andrei* (Lumbricidae) and *Enchytraeus crypticus* (Enchytraeidae). Exposure to vinasse impaired the reproduction of both species on natural soils, beyond the reduction of body growth and avoidance behavior of *E. andrei*, unlike the present study, where the reduced reproductive rates of *A. inaequalis* were followed by increases in biomass. However, as previously discussed, the *A. inaequalis* species presents specific reproductive strategies. Our main hypothesis was that exposure to 0.4% of vinasse inhibits the asexual reproduction of the species, which may have invested the energy in the body's growth for sexual maturation. However, post-10-days exposure, probably no sexual reproduction occurred once the naidids die soon after laying the cocoons (Learner et al, 1978). Besides, some individuals presented asexual reproduction once new organisms were found in the experiment ending at 0.4%. Further investigations can determine if the increases in body growth in these conditions are due to the sexual maturation of individuals or associated with inhibitions in asexual reproduction.

In addition, other studies have reported that the exposure of nematode *Caenorhabditis elegans* to metals zinc, aluminum, and titanium reduced its reproductive capability (Wang et al, 2009). Also, Vranken and Heip (1986) observed a significant reduction in fecundity and reproduction of a marine nematode *Diplolaimella* spec 1 (non-described species) when exposed to copper and lead. These metals are part of the chemical composition of vinasse, but because of their mixture complexity, it is not possible to exactly determine which one may be related to the effects observed in the present study.

3.4 Metals bioaccumulation

Table 3 shows the results for the metals quantified in the test solutions and the body residue of *C. sancticaroli larvae*. The concentration of Mn increases as the proportion of vinasse rises in the test solutions and decreases in the tissue residue of larvae exposed to the dilution of 0.325% compared to control larvae ($p < 0.05$). The metal Zn was quantified only in the highest vinasse doses (0.163 to 0.65%). Regarding the tissue residue, decreases occurred in the organisms exposed to dilutions from 0.081 to 0.65% ($p < 0.05$). The concentration of Zn in organisms decreased according to the vinasse concentrations rising in test solutions.

Table 3

Concentration of metals in vinasse dilutions and tissue residue of *C. sancticaroli* larvae (mean ± DP) post-8-days-exposure. Asterisks (*) and bold values denote differences in body residue from control ($p < 0.05$).

	Dilutions (%)	Mn	Zn	Cu	Ni	Cd	Pb	Cr
Test solution (µg L^{-1})	**0**	< LOQ	< LOQ	2.0 ± 0.4	1.9 ± 0.8	< LOQ	< LOQ	< LOQ
	0.041	2.8 ± 0.0	< LOQ	4.6 ± 0.9	< LOQ	< LOQ	2.9 ± 3.2	< LOQ
	0.081	4.4 ± 0.5	< LOQ	4.0 ± 0.8	< LOQ	< LOQ	1.1 ± 1.9	< LOQ
	0.163	24.6 ± 0.8	91.0 ± 3.2	47.8 ± 2.1	< LOQ	< LOQ	6.3 ± 3.2	2.3 ± 0.4
	0.325	19.2 ± 1.3	209.2 ± 4.3	36.4 ± 0.8	< LOQ	< LOQ	7.5 ± 3.2	0.7 ± 0.5
	0.65	42.8 ± 0.7	115.2 ± 2.8	56.4 ± 1.3	< LOQ	< LOQ	8.7 ± 2.8	2.1 ± 0.0
Tissue residue (µg g DW^{-1})	**0**	3.7 ± 0.6	32.5 ± 4.8	4.6 ± 1.1	0.6 ± 0.3	< LOQ	< LOQ	< LOQ
	0.041	3.3 ± 1.0	25.2 ± 1.9	6.0 ± 0.1	1.1 ± 0.1	0.1 ± 0.1	< LOQ	< LOQ
	0.081	4.0 ± 1.1	**18.6 ± 4.4***	5.8 ± 1.8	0.8 ± 0.5	< LOQ	1.1 ± 1.9	< LOQ
	0.163	2.9 ± 0.7	**15.8 ± 3.2***	4.4 ± 1.4	0.5 ± 0.2	0.1 ± 0.1	**2.5 ± 0.2***	< LOQ
	0.325	**2.4 ± 0.5***	**15.2 ± 5.8***	3.2 ± 0.8	0.7 ± 0.4	**0.1 ± 0.0***	**2.0 ± 0.4***	< LOQ
	0.65	2.5 ± 0.4	**12.8 ± 3.6***	6.6 ± 1.9	0.9 ± 0.4	**0.2 ± 0.0***	**2.5 ± 0.5***	< LOQ

LOQ = Limit of quantification of 0.001 µg L^{-1} to Mn, 0.004 µg L^{-1} to Zn, 0.0004 µg L^{-1} to Ni, 0.012 µg L^{-1} to Cd, 0.007 µg L^{-1} to Pb, and 0.002 µg L^{-1} to Cr.

The Cd concentration was above the quantification limit in control and all vinasse dilutions. Significative accumulation occurred on larvae exposed to 0.325 and 0.65% ($p < 0.05$, Table 3). Pb increased according to the vinasse proportion rises and was accumulated in larvae tissue on treatments from 0.163 to 0.65% compared with control organisms in which Pb was not detected ($p < 0.05$). Besides, Pb was quantified in organisms from 0.081%; however, due to the high variability, no statistical differences occurred relative to the control. In the same way, no differences in tissue residue of exposed larvae occurred for Cu, Ni, and Cr compared with control organisms ($p > 0.05$).

Table 3

Table 4 shows the result for the metals quantified on *A. inaequalis* tissue residue and vinasse dilutions from the beginning of the chronic bioassays. Zn was not detectable on test solutions until 0.2% and, as observed for *C. sancticaroli*, exposure to 0.1% reduced Zn tissue residue in *A. inaequalis*. Bioaccumulation of Cd and Cr in tissue residue occurred in organisms exposed to 0.1% of vinasse ($p < 0.05$). No alterations occurred for Mn, Cu, Ni, and Pb ($p > 0.05$).

Table 4

Concentration of metals in vinasse dilutions and tissue residue of *A. inaequalis* (mean ± DP) post-10-days-exposure. Asterisks (*) and bold values denote differences in body residue from control ($p < 0.05$).

	Dilutions (%)	Mn	Zn	Cu	Ni	Cd	Pb	Cr
Test solutions (µg L^{-1})	**CT**	<LOQ	<LOQ	2.0 ± 0.4	1.9 ± 0.8	<LOQ	<LOQ	<LOQ
	0.025	<LOQ	<LOQ	1.8 ± 1.3	1.1 ± 0.5	<LOQ	<LOQ	0.1 ± 0.0
	0.05	2.0 ± 0.4	<LOQ	3.0 ± 0.8	1.9 ± 0.8	<LOQ	<LOQ	0.1 ± 0.0
	0.1	7.2 ± 0.5	<LOQ	23.2 ± 2.0	13.7 ± 1.4	<LOQ	<LOQ	1.1 ± 0.0
	0.2	20.2 ± 0.5	504.4 ± 4.9	42.2 ± 1.0	4.9 ± 0.4	<LOQ	<LOQ	1.3 ± 0.4
	0.4	25.0 ± 1.5	135.0 ± 1.1	40.4 ± 0.8	3.7 ± 0.0	<LOQ	<LOQ	1.9 ± 0.4
Tissue residue (µg g DW^{-1})	**CT**	0.8 ± 0.4	34.7 ± 1.5	4.5 ± 0.8	0.8 ± 0.2	<LOQ	<LOQ	<LOQ
	0.025	1.1 ± 1.1	29.9 ± 3.5	3.9 ± 0.1	0.6 ± 0.2	<LOQ	<LOQ	<LOQ
	0.05	1.2 ± 1.6	39.7 ± 24.0	6.5 ± 4.2	2.1 ± 2.3	0.2 ± 0.2	<LOQ	<LOQ
	0.1	0.8 ± 0.6	**25.9 ± 2.5***	3.3 ± 0.2	1.0 ± 0.7	<LOQ	<LOQ	<LOQ
	0.2	0.7 ± 0.5	30.1 ± 3.8	4.0 ± 0.7	0.7 ± 0.2	**0.1 ± 0.0***	<LOQ	**0.05 ± 0.1***
	0.4	0.5 ± 0.1	23.0 ± 7.7	3.1 ± 1.0	0.6 ± 0.0	<LOQ	<LOQ	<LOQ

LOQ = Limit of quantification of 0.001 µg L^{-1} to Mn, 0.004 µg L^{-1} to Zn, 0.012 µg L^{-1} to Cd, 0.007 µg L^{-1} to Pb, and 0.002 µg L^{-1} to Cr.

Table 4

Manganese is an essential trace element that acts on biochemical and cellular functions (Santamaria; Sulsky, 2010). Ben-Shahar (2018) observed that Mn plays a direct role in multiple molecular and physiological processes mainly associated with insect development and behavior. The present study has shown decreases in Mn levels on vinasse dilution of 0.325% for *C. sancticaroli*, which may imply effects for the organisms. The reduction of Zn in *C. sancticaroli* and *A. inaequalis* was an unexpected response. It was expected that increases in this metal concentration should provoke increases in uptake rates and accumulation in tissues until a critical accumulated body concentration (Rainbow and Luoma, 2011), as observed by Lobo et al. (2021) for the aquatic oligochaetes *Branchiura sowerbyi* and *Tubifex tubifex*. However, this relationship depends on metal availability (Rainbow and Luoma, 2011). Thus, despite the increases in Zn concentration in the solutions, the metal may not be bioavailable to organisms. In this way, further studies may elucidate the mechanisms associated with these responses. Zn ions act in catalytic and structural roles in enzymes and provide a molecular basis for numerous biological functions (Maret, 2005). In a study with *Drosophila melanogaster* flies, the depletion of zinc concentrations affected female fertility (Missirlis, 2021), indicating possible risks to *C. sancticaroli* reproduction.

According to Craig et al. (1999), the metal Cd is chemically close to Zn, and its ions act on biochemical reactions and can change the activity of many hormones. However, increases in Cd may imply toxic responses in organisms. On the other hand, low doses of Pb already provoke toxicity in invertebrates (Shuhaimi-Othman et al., 2012). Exposure of the dipteran *Calliphora vicinato* to Cd and Pb induced malformation on flies and a delay of 18 and 24h, respectively, on the average emerging time (Shulman; Pakhomov; Brygadyrenko, 2017). In the same way, the present study evidenced delays in the metamorphosis of *C. sancticaroli*, marked by decreases in the number of pupae and adults. The organism accumulated both metals in the concentration (0.65%) where these effects occurred.

Levels of Zn, Cu, Pb, and Cd in collector-gatherer macroinvertebrates, such as chironomids and oligochaetes, are usually found in direct proportion to their concentrations in sediment or water (Goodyear and McNeill, 1999). Thus, these results demonstrate that the metal residues in organisms are directly influenced by environmental contamination. Besides, Chiba et al. (2011) verified that the areas with the highest concentrations of Cu, Ni, and Zn had the lowest richness and diversity of benthic macroinvertebrates, such as Chironomidae, Tubificidae, Elmidae, and Ceratopogonidae. In the same way, Bian et al. (2016) observed negative correlations between heavy metal levels on benthic communities' diversity in monitored rivers. The authors concluded that the main pollutants in the sediments were Cd, Cu, and Pb.

The maintenance of benthic macroinvertebrates is fundamental for aquatic ecosystems once they make up a large proportion of the biodiversity and are an essential part of the energy and nutrients transferred for higher trophic levels (Chagnon et al., 2015). Besides, the bioaccumulation of metals by this group provokes ecological risks to aquatic and terrestrial environments. As evidence of this process, Rubio-Franchini and Rico-Martínez (2011) and Croteau et al. (2005) reported that Pb and Cd can be biomagnified through freshwater food webs until the top predators. Because of that, the metal

contamination was not restricted to low trophic levels. The authors conclude that the lack of monitoring of this kind of pollution may increase the vulnerability of all aquatic food chains. Therefore, the results described in the present study indicate risks of biomagnification along with food webs with cascading effects on the aquatic and terrestrial ecosystems.

Several technologies are available to manage the sugarcane vinasse, which include the concentration to reduce the volume, animal food production, incineration, biological conversion to biogas production, and application in agriculture by fertirrigation (Fuess et al., 2017). The direct or indirect release of vinasse into water bodies was prohibited in Brazil at the end of the 1970s (Brazil, 1978), thus fertirrigation is the only approach used in the country (Fuess et al., 2017). Brazil does not have specific legislation that establishes safe distances from water bodies for the application of vinasse, and it is up to the states to define their requirements. The state of São Paulo, the largest producer of sugarcane in Brazil (Ogura et al., 2022), has, for example, a specific normative that establishes minimum distances from water bodies for the application of vinasse (CETESB, 2015). However, most other producing states do not have yet established these requirements (Filho and Araujo, 2016). In this way, fertirrigation practices may imply risks of indirect contamination by vinasse. Besides, illegal disposal, accidents in vinasse storage and transport, and improper disposal may provoke aquatic pollution by this compound (Martinelli and Filoso, 2008). In line with this, Gunkel et al. (2007) reported evidence of aquatic pollution by sugarcane vinasse in the Ipojuca river in the northeast region of Brazil. The authors observed decreases in dissolved oxygen and increases in BOD and COD downstream of an ethanol mill and a sugarcane crop area that receives vinasse by fertirrigation. In this way, it is possible to assume that vinasse may reach aquatic environments, and the results reported throughout this manuscript point to risks to freshwater environments.

4 Conclusions

The sugarcane vinasse was highly toxic to all test species in acute exposure with lethal effects in proportions lower than 1.5%, noting that the oligochaete *A. inaequalis* was more sensible than *C. sancticaroli, H. meinerti*, and *S. trispinosa*, respectively. In the subchronic exposure bioassays, vinasse accelerated the metamorphosis at intermediate dilution and retarded the development, and impaired the growth at the higher concentration for *C. sancticaroli*. In the same way, for *A. inaequalis*, intermediate dilutions increased the reproduction and population growth rates, and the higher vinasse concentration decreased these parameters. Sugarcane vinasse has a complex composition, including several metals, nutrients, and organic compounds. Thus, the identification of these elements by chemical analyses, as proceeded in this study, is crucial to help explain the results and expand the discussions. Essential metals were decreased in the exposed individuals, and heavy metals were accumulated. As the macroinvertebrates make up the base of the food chain, this draws attention to the risk of biomagnification through food webs with cascading effects on the ecosystems. Despite the importance of the ethanol industry and the high volume of vinasse produced, low information is available in the literature regarding the risks associated with exposure to this industrial residue poses for aquatic species.

Thus, information about the vinasse toxicity for four tropical macroinvertebrates species may contribute to the discussions about the risk assessment of fertirrigation on sugarcane crops.

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Janaina Braga do Carmo - Resources; Writing - Review & Editing.

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Figures

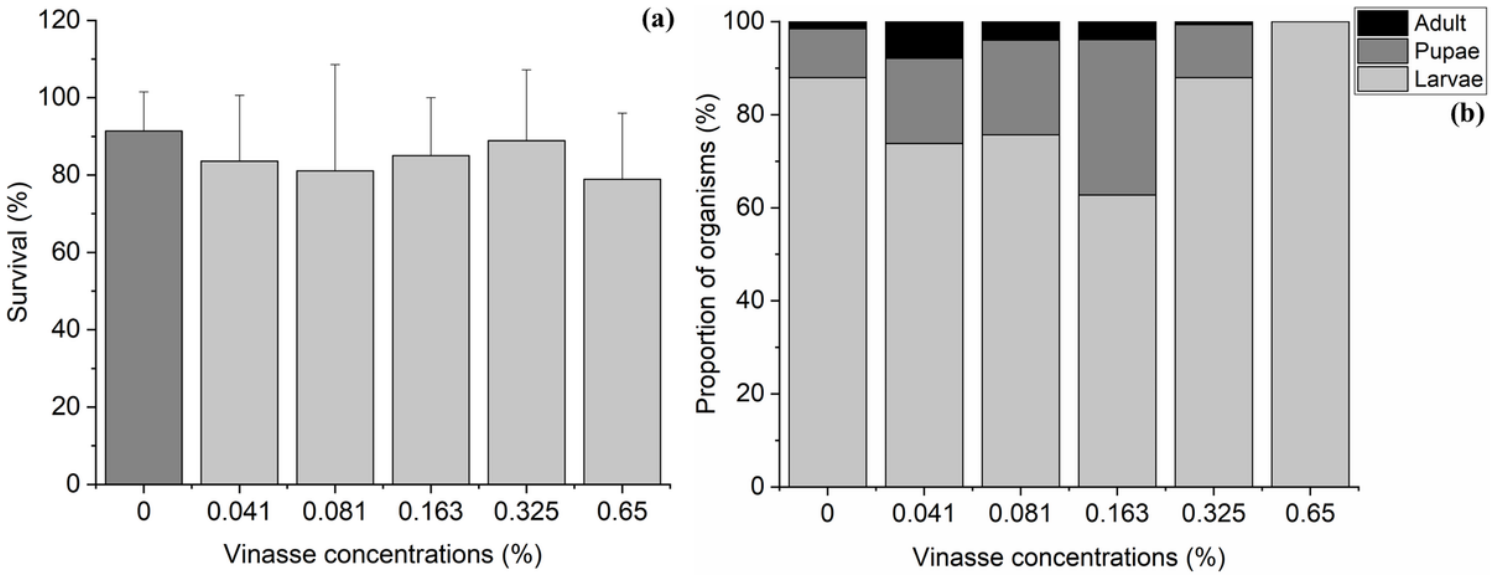

Figure 1

Survival (mean ± SD) of *C. sancticaroli* in the 8-day subchronic tests (a) and (b) percentage of larvae, pupae, and adults alive in the experiment ends (b). The number of pupae increased by 0.163% and the number of pupae and adults decreased by 0.65% ($p < 0.05$).

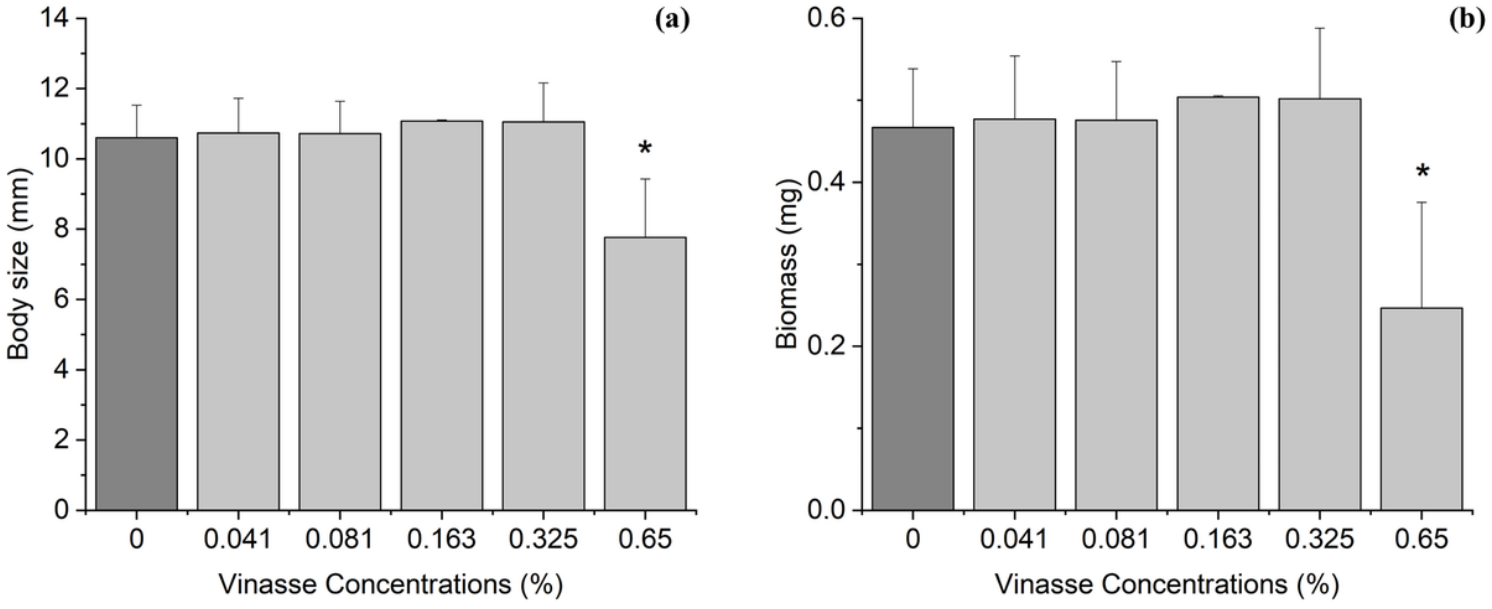

Figure 2

Body size (a) and ash-free dry weight (b) of *C. sancticaroli* larvae (mean ± SD) after 8-days of exposure to vinasse. Values statistically different from the control ($p < 0.05$) are indicated by asterisks (*).

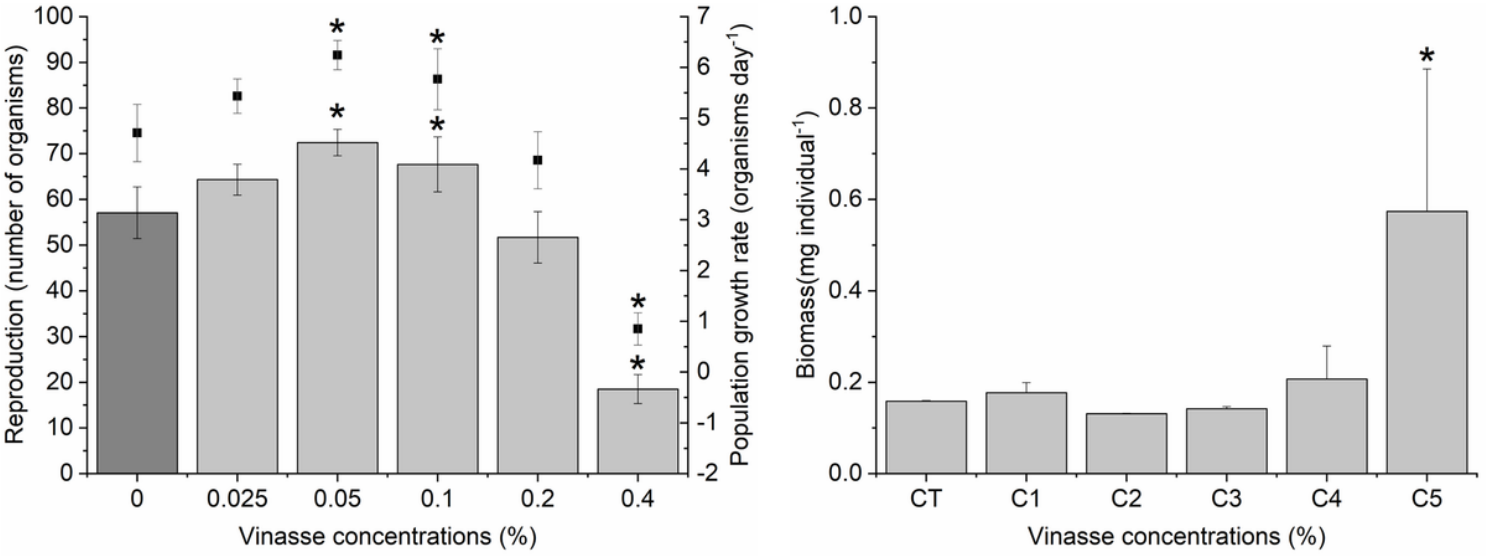

Figure 3

Reproduction and population growth rate (mean ± SD) of *A. inaequalis* post chronic exposure (a) and individual biomass (b). Values statistically different from the control ($p < 0.05$) are indicated by asterisks (*).

Supplementary Files

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