Science of the Total Environment 526 (2015) 222–232

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Ecotoxicological characterization of sugarcane vinasses when applied to tropical soils☆

Paulo Roger L. Alves [a,b,*], Tiago Natal-da-Luz [c], José Paulo Sousa [c], Elke J.B.N. Cardoso [a]

[a] Dep. of Soil Science, University of São Paulo, Pádua Dias Str, 11, 13416-900 Piracicaba, Brazil
[b] CAPES Foundation, Ministry of Education of Brazil, Brasília DF 70040-020, Brazil
[c] CFE, Dep. of Life Sciences, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal

HIGHLIGHTS

- In Brazil sugarcane vinasse is applied in agricultural soils to be used as fertilizer.
- Toxic potential of three vinasses was evaluated in different soils by standard tests.
- All tested vinasses showed sub-lethal effects on the test species.
- Vinasses derived from commercial distillery plants were the most toxic ones.
- Toxicity of vinasses varied with the application dose, test species and soil type.

ARTICLE INFO

Article history:
Received 7 January 2015
Received in revised form 24 March 2015
Accepted 24 March 2015
Available online xxxx

Editor: Daniel Wunderlin

Keywords:
Soil invertebrates
Oligochaeta
Arthropoda
Oxisol
Ecotoxicity

ABSTRACT

The impact of sugarcane vinasse on soil invertebrates was assessed through ecotoxicological assays. Increasing concentrations of two vinasses from different distillery plants (VA and VB), and a vinasse from a laboratory production (VC), were amended on two natural tropical Oxisols (LV and LVA) and a tropical artificial soil (TAS) to characterize the effects of the vinasses on earthworms (*Eisenia andrei*), enchytraeids (*Enchytraeus crypticus*), mites (*Hypoaspis aculeifer*) and collembolans (*Folsomia candida*). The highest concentrations of VA and VB were avoided by earthworms in all soils and by collembolans especially in the natural soils. The presence of VC in all of the tested soils did not cause avoidance behavior in these species. The reproduction of earthworms, enchytraeids and collembolans was decreased in the highest concentrations of VA and VB in the natural soils. In TAS, VB reduced the reproduction of all test species, whereas VA was toxic exclusively to *E. andrei* and *E. crypticus*. The vinasse VC only reduced the number of earthworms in TAS and enchytraeids in LVA. The reproduction of mites was reduced by VB in TAS. Vinasses from distillery plants were more toxic than the vinasse produced in laboratory. The vinasse toxicities were influenced by soil type, although this result was most likely because of the way the organisms are exposed to the contaminants in the soils. Toxicity was attributed to the vinasses' high salt content and especially the high potassium concentrations. Data obtained in this study highlights the potential risk of vinasse disposal on tropical soils to soil biota. The toxic values estimated are even more relevant when considering the usual continuous use of vinasses in crop productions.

1. Introduction

The search for energy technologies from renewable sources that have a low environmental impact, such as the production of biofuels, is a global challenge. Brazil is the only country that uses ethanol on a large scale as an alternative renewable fuel source to petroleum (Laime et al., 2011), and is currently one of the largest sugarcane ethanol producer with a production of approximately 23.6 billion liters in 2012/13 (MME, 2014). The production of this biofuel generates great volumes of vinasse, an acidic dark-brown organic wastewater with considerable amounts of organic matter (OM), potassium, and lower amounts of calcium and magnesium, among other substances (España-Gamboa et al., 2011). Each liter of ethanol produced generates about 8–15 l of vinasse (Freire and Cortez, 2000), which is mainly used in fertigation of agricultural soils (Laime et al., 2011; Christofoletti et al., 2013a).

Although fertigation with sugarcane vinasse can increase crop yields by acting as a water and nutrient source for plants and improving

☆ We declare that these experiments were conducted in accordance with EC Directive 86/609/EEC and national and institutional guidelines for the protection of human subjects and animal welfare.

* Corresponding author at: Dep. of Soil Science, Av. Pádua dias, No. 11, 13416-900, Piracicaba, São Paulo, Brazil.
E-mail address: pauloroger@usp.br (P.R.L. Alves).

http://dx.doi.org/10.1016/j.scitotenv.2015.03.150
0048-9697/

certain soil properties (Oliveira et al., 2009; Laime et al., 2011; Jiang et al., 2012; Christofoletti et al., 2013a), its environmental impacts have raised concerns because of the presence of contaminants (e.g. heavy metals, alcohol and phenolic compounds and antibiotic substances) in this type of wastewater (Almeida, 1952; Christofoletti et al., 2013a). When applied indiscriminately, vinasse may cause nutrient imbalance and salt saturation in the soil, leading to ion leaching to groundwater (Silva et al., 2007; Ribeiro et al., 2010). Furthermore, some studies have indicated that vinasse negatively affects physical soil properties, such as hydraulic conductivity and redox potential (Leal et al., 1983; Uyeda et al., 2013). In Brazil, the technical standard norm P4.231 (CETESB, 2006) establishes criteria and procedures for the application of sugarcane vinasse in agricultural soils. Aiming to prevent changes in the soil chemical properties (e.g. salt saturation), this standard has established that the maximum volume of vinasse for application should be estimated taking into account the cation-exchange capacity (CEC) of the soil, the soil potassium concentration in soil and vinasse and the amount of K_2O extracted by the sugarcane crop per hectare after each harvest (CETESB, 2006). Although these criteria are considered sufficient to comply with the objectives established in the standard norm, they do not consider the ecological effects derived from vinasse application, particularly those related to soil fauna and the associated ecological services.

The establishment of a strategy for waste application that does not compromise the presence of functionally stable communities of these organisms in the soil is of paramount importance, because the activity of soil invertebrates affects water infiltration and soil structure and has a key role in carbon, nitrogen, phosphorus and sulfur cycles through the fragmentation and decomposition of OM and regulation of microbial activity (Cortet et al., 1999; Brussaard et al., 2007; Cardoso et al., 2013). In the European Union, potential ecological risks of wastes should be assessed according the "Ecotoxic"-criterion H14, recently renamed HP 14 (Pandard and Römbke, 2013), from the European directive 2008/98/EC (that replaced the European Union Council Directive 91/689/EEC - EC, 1991, 2008). Laboratory ecotoxicological tests with standard species have been shown to be fundamental tools in the assessment of potential risks of wastes to the environment because these tests allow an integrated assessment of toxicity that considers the addictive, antagonistic and synergistic effects of contaminants. Additionally, the results obtained from ecotoxicological tests indicate the effect of the bioavailable fraction of contaminants in the waste; thus, they complement traditional chemical analyses (Pandard et al., 2006; Domene et al., 2007; Wilke et al., 2008; Natal-da-Luz et al., 2009a,b; Moser and Römbke, 2009; Cesar et al., 2014).

The toxicity of sugarcane vinasse for aquatic organisms is already known (Christofoletti et al., 2013a). However, only a very limited number of studies in terrestrial environments have evaluated the impact of this waste on soil fauna species. The presence of vinasse in soil is known to suppress certain nematode species (Pedrosa et al., 2005; Caixeta et al., 2011; Matos et al., 2011) and to cause mortality of diplopods (Christofoletti et al., 2013b). Therefore, we decided to partially fill this gap of knowledge and to contribute to the risk assessment of this residue, which is applied to tropical soils at an estimated rate of more than 200 billion liters/year (Laime et al., 2011; MME, 2014). This study aimed at characterizing the ecotoxicological potential of vinasse towards the invertebrate species *Eisenia andrei*, *Enchytraeus crypticus*, *Hypoaspis aculeifer* and *Folsomia candida*, standard species commonly used in ecotoxicological assessments. Vinasses from different origins were evaluated to determine their effects on these species on different soil types.

2. Materials and methods

2.1. Test soils and vinasses

Two Oxisols were used in the ecotoxicological tests. According to the Brazilian Soil Classification System: a Red Latosol (LV) with 33.6% of clay and a Red–Yellow Latosol (LVA) with 17.6% of clay. Both soils were collected at the 0–20 cm surface layer of sugarcane plantations in the state of São Paulo, Brazil. The sampling sites were free from application of sugarcane vinasses for more than 10 years. The soils were air-dried, sieved at 5 mm, and defaunated through three freeze-thawing cycles (48 h at −20 °C followed by 48 h at 25 °C) to eliminate the original soil fauna (Pesaro et al., 2003). Additional ecotoxicological tests were performed in tropical artificial soil (TAS) (Garcia, 2004). TAS is an adaptation of the Organisation for Economic Co-operation and Development (OECD) artificial soil (OECD, 1984) and is frequently used in laboratory ecotoxicological assays in tropical regions (Römbke et al., 2007; De Silva and Van Gestel, 2009; Alves et al., 2013, 2014). This soil consists of 70% sand (more than 50% of the particles sized between 0.05 and 0.2 mm), 20% kaolinite clay and 10% powdered coconut husks. When necessary, $CaCO_3$ was added to the mixture to obtain a TAS with a pH of 6 ± 0.5.

Physical and chemical characterization of the natural (LV and LVA) and artificial (TAS) soils was performed by measuring several parameters in the air-dried samples following the methodologies of Van Raij et al. (2001). The determination of available P, Ca, Mg and K was performed by the ion-exchange resin method. Available sulfur ($S–SO_4$) was extracted by the turbidimetric method in a 0.01 mol L^{-1} solution of Ca $(H_2PO_4)_2$. The elements Cu, Fe, Mn and Zn were extracted in diethylenetriaminepentaacetic acid–triethanolamine (DTPA–TEA; pH 7.3), whereas B was extracted in hot water/microwave. The exchangeable aluminum was determined by titration (1 mol L^{-1}), and potential acidity (H + Al) was determined by the Shoemaker–McLean–Pratt (SMP) buffer method. The soil OM was determined by colorimetry via oxidation with $Na_2Cr_2O_7.2H_2O$ and H_2SO_4 (Van Raij et al., 2001). The maximum water-holding capacity (WHC) of the soils was determined according to the International Organization for Standardization (ISO, 1996), and the pH values were measured with a 1 M KCl solution (using a proportion soil:solution of 1:5, w/v). The sand, silt and clay fractions of the soils were evaluated by the pipette method, the clay level was determined with a Bouyoucos densimeter (Dane and Topp, 2002), and sediments were measured by subtracting the estimated sand and clay volumes from the total of the sample.

Three sugarcane vinasses with different origins were used in the ecotoxicological tests. Two vinasses were collected directly from tanks of different distilleries (VA and VB). As control, a third vinasse (VC) was produced in the laboratory and this did not include additional additives and antibiotics that are usually employed during the processes of fermentation and alcohol production (Compart et al., 2013). The physical and chemical characterization of the vinasses was performed according to the methods described by Kiehl (1985), which included measuring the following parameters: pH (1 M KCl), density, electric conductivity (EC), OM content (loss on ignition at 500 °C for 6 h), concentrations of mineral residues (soluble, insoluble and total), K (K_2O), Ca, Mg, S, total C levels (TC), N, P (P_2O_5), Cu, Mn, Zn, Fe and the C:N ratio.

The total concentrations of potentially toxic elements (PTE), including As, Cd, Co, Cr, Cu, Hg, Mo, Ni, Pb and Zn, were determined for the soils and vinasses using extractions by microwave-assisted (CEM MARS Xpress™) digestion. Methods from the US Environmental Protection Agency were employed in the digestion of soils and vinasses, including EPA 3051A (0.5 g soil + 9 mL HNO_3 + 3 mL HCl) and EPA 3015A (45 mL vinasse + 4 mL HNO_3 + 1 mL HCl), respectively (USEPA, 1998, 1999). The PTE measurements in the soil and vinasse extracts were performed by the multi-element technique inductively coupled plasma optical emission spectrometry (ICP OES). The limits of quantification (LOQ) were 0.010, 0.002, 0.005, 0.005, 0.002, 0.010, 0.005, 0.001, 0.010, and 0.002 (mg L^{-1}) for As, Cd, Co, Cr, Cu, Hg, Mo, Ni, Pb and Zn, respectively. The recovery of the extraction using the EPA 3051A method was verified by the inclusion of certified soil samples (Montana Soil, NIST, SRM 2711).

2.2. Test organisms and culture conditions

The species *E. andrei* (Lumbricidae), *E. crypticus* (Enchytraeidae), *F. candida* (Isotomidae) and *H. aculeifer* (Laelapidae) were used in this study. These species are frequently used in laboratory ecotoxicological tests to assess the potential risk of chemical substances in soil (e.g., Natal-da-Luz et al., 2012; Owojori et al., 2014). The earthworms *E. andrei* were maintained in laboratory cultures using a substrate composed by horse manure (previously defaunated with freeze-thawing cycles as described above for the test soils), coconut husk powder and fine sand at a proportion of 2:1:0.15 (w:w:w), as described by Alves et al. (2013). The culture of *E. crypticus* was maintained in the laboratory in Petri dishes containing agar as described by Cesar et al. (2015). Approximately 50 mg of previously granulated oat flakes was supplied as food once per week. Every two months, the organisms were transferred to a new substrate. The mites *H. aculeifer* were maintained in plastic boxes (10.5 cm diameter and 3.5 cm height), with the bottom covered by a mixture of activated charcoal and plaster of Paris in the proportion of 1:9 (w:w). Simultaneously, cultures of *Tyrophagus putrescentiae* (cheese mites) were maintained and offered as food (prey) for *H. aculeifer* (predator) twice per week at low quantities (tip of a spatula). Cheese mites were fed weekly with dried brewer's yeast. The species *F. candida* was maintained in plastic boxes lined with a mixture of activated charcoal and plaster of Paris in the proportion of 1:11 (w:w) as described by Natal-da-Luz et al. (2009a).

Mite cultures were maintained at 20 ± 2 °C, whereas the remaining cultures were maintained at 24 ± 3 °C; all of the cultures were submitted to a photoperiod of 12:12 h light:dark.

2.3. Experimental procedures

Avoidance tests were performed with earthworms and collembolans, whereas reproduction tests were performed with all four species. For each species, a concentration gradient of each vinasse was tested (Table 1). The concentrations were first defined based on the maximum water holding capacity (WHC) of each soil and then based on the results of preliminary tests (results not shown in this study). Additionally, the definition of the test concentrations also takes into consideration some limit values based on the technical standard P4.231 (Table 1; CETESB, 2006) for the application of vinasses in agricultural soils, however, considering only the 0–20 cm of the soil layer. These values constitute the maximum dose for single applications in each agricultural soil and were estimated by the following equation:

$$V = [(0.05 \times \mathrm{CEC} - ks) \times 3744 + 185] / kvi$$

where V is the maximum dose of vinasse allowed for the treatment of sugarcane cultivation soils ($m^3\ ha^{-1}$, transformed to mL kg^{-1} DW); CEC is the soil cation-exchange capacity ($cmol_c\ dm^{-3}$); and *ks* and *kvi* are the potassium concentrations in the soil ($cmol_c\ dm^{-3}$) and in the vinasse (kg of $K_2O\ m^{-3}$), respectively.

The concentration gradients were prepared immediately before starting each test. For each concentration, a mixture of vinasse and deionized water was prepared at the adequate proportion to obtain the desired concentration of vinasse in the soil. The different vinasse dilutions were added to the soil in the volume required to reach a water content level equivalent to 60% of the WHC. Therefore, the application volumes (mL vinasse by kg of dried soil–mL kg^{-1} dry weight-DW) varied between soils depending on the WHC of each soil.

The ecotoxicological tests were performed at 24 ± 3 °C and 12:12 h light:dark (except for the avoidance test with earthworms, which was performed in complete darkness). In addition, for the tests with enchytraeids, collembolans and mites, a replicate of each treatment of each test was prepared without organisms to measure the final moisture and pH values.

Table 1
Concentrations of two vinasses originating from distilleries (VA and VB) and a vinasse produced in laboratory (VC) used in ecotoxicological tests with the species *Eisenia andrei*, *Enchytraeus crypticus*, *Folsomia candida* and *Hypoaspis aculeifer* in two natural Oxisols (Red Latosol, LV; and Red–Yellow Latosol, LVA) and a tropical artificial soil (TAS). The calculated legal limits of vinasse application for each soil are also included (see text for more details). Values are expressed in mL of vinasse kg^{-1} of soil (dry weight).

Test species	Vinasse	Soil	C1	C2	C3	C4	C5	Limit values[a]
E. andrei and *E. crypticus*	VA	LV	12.5	25	50	100	200	48
		LVA	7.5	15	30	60	120	24.6
		TAS	18.4	36.7	73.5	147	294	n.d.
	VB	LV	12.5	25	50	100	200	53.2
		LVA	7.5	15	30	60	120	27.3
		TAS	18.4	36.7	73.5	147	294	n.d.
	VC	LV	12.5	25	50	100	200	132
		LVA	7.5	15	30	60	120	67.6
		TAS	18.4	36.7	73.5	147	294	n.d.
H. aculeifer	VA	LV	19	34.3	61.7	111	200	48
		LVA	11.4	20.6	37	66.7	120	24.6
		TAS	28	50.4	90.7	163	294	n.d.
	VB	LV	19	34.3	61.7	111	200	53.2
		LVA	11.4	20.6	37	66.7	120	27.3
		TAS	28	50.4	90.7	163	294	n.d.
	VC	LV	19	34.3	61.7	111	200	132
		LVA	11.4	20.6	37	66.7	120	67.6
		TAS	28	50.4	90.7	163	294	n.d.
F. candida	VA	LV	6.2	12.5	25	50	100	48
		LVA	3.7	7.5	15	30	60	24.6
		TAS	9.2	18.4	36.7	73.5	147	n.d.
	VB	LV	1.6	3.1	6.2	12.5	25	53.2
		LVA	0.9	1.9	3.7	7.5	15	27.3
		TAS	4.6	9.2	18.4	36.7	73.5	n.d.
	VC	LV	6.2	12.5	25	50	100	132
		LVA	7.5	15	30	60	120	67.6
		TAS	18.4	36.7	73.5	147	294	n.d.

[a] Values calculated based on CETESB (2006) considering the top 20-cm soil layer; n.d. — values were not estimated for TAS.

2.4. Standard ecotoxicological tests

2.4.1. Avoidance tests with E. andrei

The avoidance tests with *E. andrei* were performed based on the ISO guideline 17512-1 (ISO, 2008a). The replicates consisted of rectangular plastic boxes (23.3 cm length, 16.7 cm width, 7.7 cm height), divided into two equal compartments by a plastic divider introduced vertically. One of the compartments had 900 g FW of soil (LV, LVA or TAS fresh weight equivalent) treated with vinasse (Table 1), and the other had the same quantity of the respective control soil. Five replicates were used for each combination. The plastic divider was removed after the introduction of the soils in each compartment and then ten adult specimens of *E. andrei* (with a developed clitellum) were placed in the middle line between compartments. The boxes were closed, and the lids were perforated to allow air circulation. The animals were not fed during the test, and the number of individuals present in each compartment was recorded after 48 h. A double-control was also prepared with control soil in both compartments to assess whether there was a random distribution of organisms in the absence of contaminants (Hund-Rinke et al., 2003).

2.4.2. Avoidance tests with F. candida

Avoidance tests with *F. candida* were performed following the procedures described in the ISO guideline 11268-2 (ISO, 2008b). In the tests with collembolans, the containers consisted of circular plastic boxes (9 cm diameter and 6 cm height) with 30 g FW of soil (LV, LVA or TAS) in each compartment (contaminated or control). As for the earthworms, soils treated with vinasses were combined with their respective controls under the same conditions used in the earthworm avoidance tests (Table 1). Five replicates were used for each combination. After the addition of soil to the compartments, the plastic divider was

removed and 20 collembolans 10 to 12 days old (originated from synchronized cultures) were placed on the middle-line of both compartments. The animals were not fed during the test. After 48 h, the content of each compartment was carefully transferred to another container, and flooded with water. Several drops of blue ink were added to the soil–water mixture to increase the contrast between the floating collembolans and the surface of the medium. Afterwards the floating collembolans (surviving adults) were counted. Similar to the tests with earthworms, double-controls were prepared for all of the soils tested.

2.4.3. Reproduction tests with E. andrei

Reproduction tests with *E. andrei* followed the procedures described in the ISO guideline 11268-2 (ISO, 1996). Circular plastic containers (12.5 cm diameter and 9.5 cm height) were filled with 500 g DW of soil (LV, LVA or TAS dry weight equivalent) treated with one of the three test vinasses (Table 1) or treated as a control. Adult earthworms with a well-developed clitellum were previously incubated in the control soil over 24 h and then 10 individuals were selected, rinsed with water, weighed and introduced to each experimental unit. The individuals used in the tests had a mean fresh body weight of 344 ± 32.1 mg (± standard deviation; $n = 2160$). The containers were closed with perforated lids, and the earthworms were fed with horse manure (≈5 g replicate^{-1}) weekly. Four replicates were used per concentration, and the test had a total duration of 56 days. After 28 days from the beginning of the tests, the adult earthworms were removed, rinsed with water and weighed. The body biomass percentage (at 28 days) relative to the initial weight was calculated. After 56 days, the containers were placed in a water bath at 60 ± 5 °C to force juveniles to move to the surface for counting.

2.4.4. Reproduction tests with E. crypticus

The reproduction tests with *E. crypticus* were based on the ISO guideline 16387 (ISO, 2004). Ten individuals of similar size with a well-developed clitellum were placed in cylindrical glass containers (9 cm diameter and 6 cm height) containing 20 g DW of soil (LV, LVA or TAS) treated with vinasse (Table 1) or the respective control soil. Finely, milled oat flakes were supplied as food (≈2 mg per replicate), and the containers were hermetically sealed. The containers were opened weekly to allow gas exchange, whereas food and water replenishment was done whenever necessary (in replicates with weight loss higher than 2%). Four replicates were used for each vinasse concentration, and eight replicates were used for the controls. After 28 days, the organisms were fixed in 80% ethanol and stained with Bengal rose (1% in ethanol) over night. Afterwards, soil was washed through a sieve (0.25 mm) with tap water, enchytraeids were transferred to a Petri dish as described by Chelinho et al. (2014), and then the total number of enchytraeids was counted under a stereoscopic microscope.

2.4.5. Reproduction tests with H. aculeifer

The effects of vinasse on the reproduction of *H. aculeifer* were evaluated based on the OECD guideline 226 (OECD, 2008). Ten females between 28 and 35 days of age (originated from synchronized cultures) were added to each container (9 cm diameter and 6 cm height) containing 20 g DW soil (LV, LVA or TAS). The animals received small portions (a tip of a spatula) of food (cheese mites) at the beginning of the test and twice per week. The containers remained hermetically sealed and were opened weekly to allow gas exchange and for water replenishment (in replicates with weight loss higher than 2%). Four replicates were performed for each vinasse concentration, and eight replicates were performed for the controls. After 14 days, the mites were removed from the soil by a MacFadyen extractor using an increasing temperature cycle for 48 h (12 h at 25 °C, 12 h at 35 °C and 24 h at 45 °C). The adults and the newly generated juveniles in each replicate were fixed in ethanol 70% and counted under a stereoscopic microscope.

2.4.6. Reproduction tests with F. candida

The reproduction tests with collembolans followed the procedures described in the ISO guideline 11267 (ISO, 1999). Cylindrical glass containers (3.5 cm diameter and 11.5 cm height) were filled with 30 g FW soil (LV, LVA and TAS) treated with vinasse (Table 1) or control soil. Ten adult collembolans 10 to 12 days old (originated from synchronized cultures) were introduced in each experimental unit and the containers were then hermetically sealed. Five replicates were performed for each vinasse concentration. At the beginning of the test and after 14 days, dried granulated yeast (≈2 mg per replicate) was supplied as food. The containers were opened weekly to allow gas exchange. After 28 days, the soil of each replicate was submerged in water to force the flotation of the individuals. Several drops of blue ink were added to the water to increase contrast and facilitate the counting of surviving adults and juveniles. The replicates were then photographed as described by Alves et al. (2014). The *F. candida* juveniles were counted in the digital images using the computer software UTHSCSA Image Tool 3.0.

2.5. Data analysis

Linear correlations between the vinasse concentrations and soils pH values were tested using Pearson's r using a level of statistical significance of $\alpha = 0.01$.

In the avoidance tests, the avoidance behavior was represented in percentage of avoidance response ([(No. control − No. test) / (No. control + No. tests) × 100]). The significance of the avoidance response (positive values) was tested with Fisher's exact test using a two-tailed test for the double-control and one-tailed test for the combination of soils contaminated with vinasse (Zar, 1999). Additionally, the AC_{50} (concentration that causes 50% of avoidance behavior) was estimated with the software PriProbit® 1.63 (Sakuma, 1998).

The significance of the differences between the percentage of initial biomass of earthworms and the number of juveniles of earthworms, enchytraeids, mites and collembolans generated in soils treated with vinasse was tested with a one-way analysis of variance (ANOVA). When differences were detected ($p \leq 0.05$), the means of the treatments were compared with their respective controls by the Dunnett post-hoc test using the software package R® (version 2.5.1.). These statistical differences were used to estimate the values of the NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) of the vinasses for each soil in the chronic toxicity tests. The EC_{20} and EC_{50} values (concentrations that reduce reproduction by 20 and 50%, respectively, when compared with the control) were estimated with non-linear regressions using the exponential, logistic, Gompertz or Hormestic models (Environment Canada, 2007) using the software Statistica 7.0®. The normality and homogeneity of the results were tested before ANOVAs with the Kolmogorov–Smirnov and Bartlett tests, respectively. In the regression analyses, these assumptions were verified a posteriori by analyzing the distribution of model residues on “normal probability” plots and “predicted values vs residues” biplots, respectively.

3. Results

3.1. Physical and chemical analysis

From the three test soils, TAS showed the highest values of OM, base saturation, pH, P, S and K. The LV soil showed higher OM, base saturation, CEC and lower pH than the LVA soil. This late had the sandiest texture and the highest Fe and Al levels (Table 2). In all of the soils, the PTE were below the limit prevention values (PV) and agricultural intervention values in a maximum protection agricultural area (IV-APMax), according to CETESB (2014) (results not shown in this study).

The VA vinasse showed the highest OM and TC levels, C/N ratios and P and S concentrations followed by the VB vinasse. The lowest Mg

Table 2
Physical and chemical characterization of the two natural Oxisols (Red Latosol, LV; and Red–Yellow Latosol, LVA), and a tropical artificial soil (TAS), and the three vinasses (VA, VB and VC) used in the laboratory ecotoxicological tests.

Parameter	Soil			Vinasse		
	LV	LVA	TAS	VA	VB	VC
pH (1 M KCl)	4.4 ± 0.1	4.9 ± 0.4	6.0 ± 0.5	4.6	4.9	5.2
OM (g dm^{-3})	25	18	75	28	17.4	13.7
TC (g dm^{-3})	–	–	–	15.6	9.7	7.6
TN (g dm^{-3})	–	–	–	0.6	0.7	0.3
C:N ratio	–	–	–	28	13	22
P (mg dm^{-3})	15	12	16	420	100	90
S (mg dm^{-3})	10	5	18	2000	1000	<4
K (mg dm^{-3})	52	80	720	9200	8300	3350
Ca (mg dm^{-3})	560	340	550	980	1270	650
Mg (mg dm^{-3})	120	84	90	1000	480	520
Al (mg dm^{-3})	<9	9	<9	–	–	–
H + Al ($mmol_c$ dm^{-3})	25	28	8	–	–	–
B (mg dm^{-3})	0.1	0.1	0.2	–	–	–
Cu (mg dm^{-3})	0.8	1	<0.4	1	1	1
Fe (mg dm^{-3})	55	116	2.5	8	33	28
Mn (mg dm^{-3})	15.4	4.3	3.1	2	7	4
Zn (mg dm^{-3})	4.9	2.4	0.9	1	1	1
CEC ($mmol_c$ dm^{-3})	63.9	54.3	58.5	–	–	–
Base saturation (%)	61	49	86	–	–	–
Sand (g kg^{-1})	502	800	725	–	–	–
Silt (g kg^{-1})	172	24	38	–	–	–
Clay (g kg^{-1})	326	176	237	–	–	–
WHC (%)	33	20	49	–	–	–
EC (mS cm^{-1})	–	–	–	20.7	20.2	10.6
Density (g cm^{-3})	–	–	–	0.9	0.9	1
Ethanol (%)	–	–	–	0.2	0	0

– Parameter not determined.

concentration was found in VB followed by VC and VA, and the lowest Fe concentration was measured in VA followed by VC and VB. The VC vinasse showed the lowest values of OM and TC content and S and K concentration. The concentrations of the remaining macronutrients and micronutrients were similar in all vinasses (Table 2). The PTE levels added by the higher concentrations of each vinasse in soils were far below the PV and the IV-APMax for agricultural soils (Supplementary Table 1; CETESB, 2014).

The soil pH increased with the increasing concentration of vinasse and a significant positive linear correlation between the tested concentrations and soil pH values ($r = 0.9$; $p < 0.01$) was found. The highest soil pH values, on average, were measured in TAS (pH = 6.28; 6.82; 5.68), followed by LVA (5.83; 6.07; 5.83) and LV (5.65; 5.85; 5.58) in the presence of the C5 concentration of VA, VB and VC (Table 1), respectively.

3.2. Avoidance tests

In the avoidance tests with earthworms (including the double-control), survival was, on average, higher than 90%, and the mean distribution of individuals in the compartments of the double-controls was 40–60% (ISO, 2008a). Therefore, the validity criteria were met. In the natural soils (LV and LVA), VA and VB were significantly avoided by the earthworms only at the highest concentrations tested. In the TAS, earthworms significantly avoided VA since the concentration 36.7 mL kg^{-1} DW and VB since 73.5 mL kg^{-1} DW. Treatments with VC vinasse did not cause any avoidance behavior (Fig. 1). The lowest AC_{50} value estimated was found in the treatments with VA in the TAS (AC_{50} = 56.7 mL kg^{-1} DW; Table 3).

The number of surviving adults in the replicates of the avoidance test with collembolans was higher than 80% for all of the tested combinations, and there was a mean distribution of 40–60% of organisms between compartments in the double-control (ISO, 2008b). Therefore, the tests complied with the validity criteria. The collembolans significantly avoided treatments of natural soils (LV and LVA) only in the highest concentrations of VA and VB, whereas for the TAS, significant avoidance occurred only at the highest concentration of VB. The VC concentrations were not avoided by the collembolans in any of the soils tested (Fig. 2). The treatments with VB in the LVA showed the lowest AC_{50} value among the treatments tested (AC_{50} = 14.7 mL kg^{-1} DW; Table 3).

3.3. Reproduction tests

In the reproduction tests with *E. andrei* and *E. crypticus*, the survival in the control soils was, on average, above 90 and 80%, and the number of juveniles generated per replicate was always higher or equal to 30 and 25 individuals, respectively. The coefficients of variation (CV) in the controls of the tests with *E. andrei* and *E. crypticus* were not higher than 30 and 50%, respectively. Therefore, the tests complied with all of the validity criteria recommended by the ISO guidelines followed (ISO, 1996, 2004).

A significant reduction in the percentage of initial body mass of the surviving adults of *E. andrei* (28-day adult growth) was observed only at the highest concentrations of VA in the LV and TAS soils (LOEC = 200 and 294 mL kg^{-1} DW, respectively; Table 3). The highest vinasse concentrations affected the reproduction of earthworms in all of the soils tested except for VC in the LV and LVA. In all of the soils, VA and VB were more toxic (showing lower EC_{50} and EC_{20}) than VC (Table 3; Fig. 3, first line of graphs). In general, the intensity of the effects of vinasses on the reproduction of earthworms was higher in the TAS, followed by the LVA and LV.

The reproduction of *E. crypticus* was significantly affected in all soils by the highest concentrations of VA and VB (Fig. 3, second line of graphs). The vinasses originated from distilleries caused higher toxicity than VC, which was not toxic to this species in the TAS. For enchytraeids, the LVA was the soil where vinasses were more toxic, followed by the LV and TAS (Table 3; Fig. 3, second line of graphs).

The reproduction tests with *H. aculeifer* complied with the validity criteria (adult survival > 80%; CV ≤ 30%; number of juveniles per control replicate ≥ 50) established by the OECD guideline (OECD, 2008). With the exception of the highest concentrations of VB in TAS (163 and 294 mL kg^{-1}), none of the vinasses caused a significant impact on the reproduction of mites in the soils tested (Fig. 3, third line of graphs; Table 3).

The reproduction tests with *F. candida* also complied with the validity criteria (adult survival > 80%; CV ≤ 30%; number of juveniles per control replicate ≥ 100) established by the ISO guideline (ISO, 1999). VA and VB significantly reduced the reproduction of collembolans in the LV soil at the highest concentration (100 and 25 mL kg^{-1}, respectively; Fig. 4). However, in the LVA and TAS soil, only VB had a significant impact in the highest concentration (15 and 73.5 mL kg^{-1}, respectively). Furthermore, VB showed the lowest EC_{50} and EC_{20} values among the vinasses studied (Table 3). VC did not have a significant impact on the reproduction of *F. candida* in any of the soils tested (Fig. 4).

4. Discussion

In general, the toxicity observed varied with the test vinasse, soil type and sensitivity of the species tested (Table 3). The reproduction of earthworms, enchytraeids and collembolans was significantly reduced by the vinasses originated from distilleries (VA and VB) in all of the soils tested except for *F. candida* in LVA and TAS, where only the highest concentrations of VB (LOEC = 15 and 73.5 mL kg^{-1} DW, respectively) significantly altered the reproduction. Moreover, only VB in TAS was toxic for *H. aculeifer*. Conversely, the vinasse produced in laboratory (VC) showed the lowest toxicity among the three vinasses in all of the tests, being significantly toxic only for the reproduction of earthworms in the TAS (LOEC = 294 mL kg^{-1} DW) and enchytraeids in the LV and LVA (LOEC = 200 and 60 mL kg^{-1} DW, respectively). Therefore, as also seen by the EC_{20} and EC_{50} values derived, the distillery vinasses were more toxic than the laboratory vinasse, regardless of the soil.

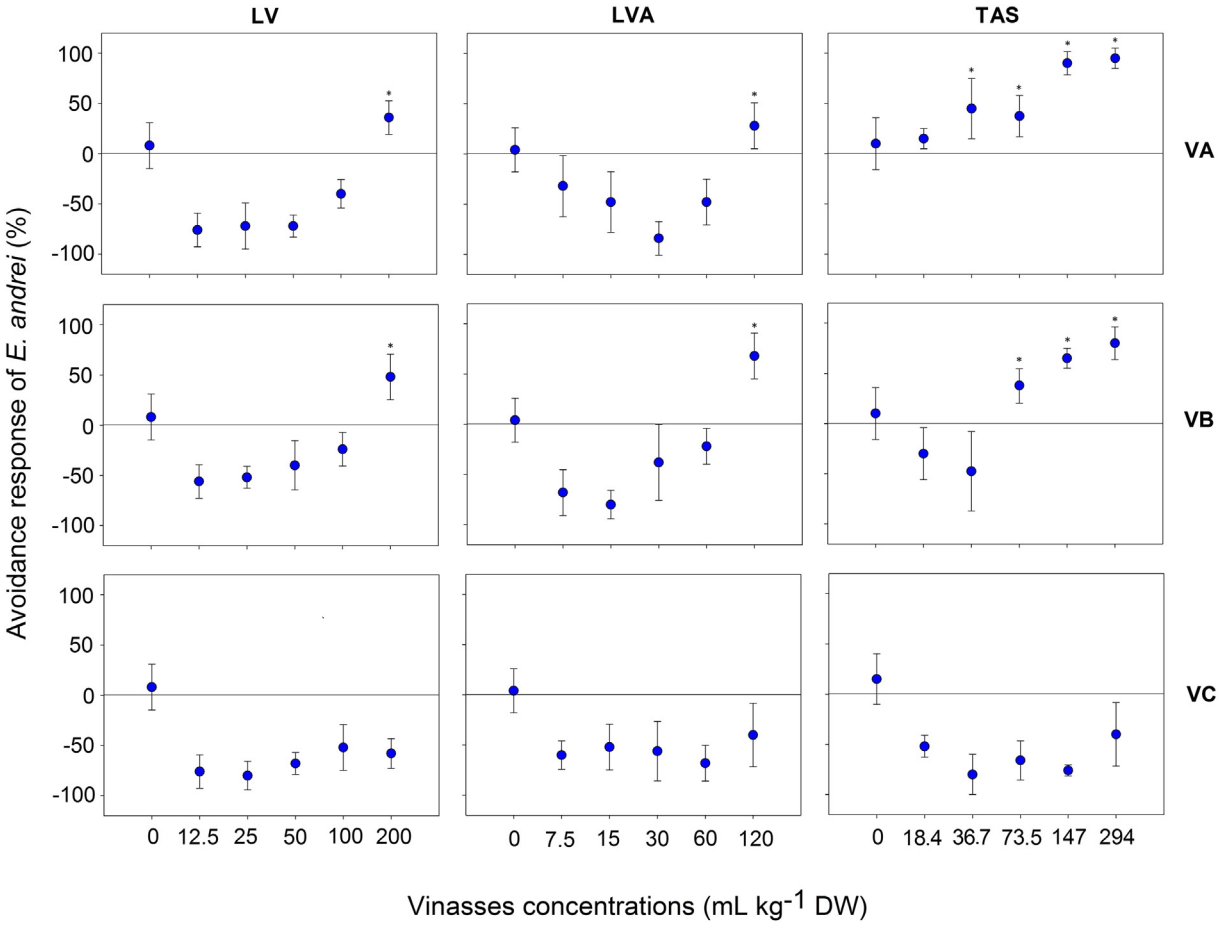

Fig. 1. Avoidance behavior of *E. andrei* in two natural Oxisols (LV and LVA) and a tropical artificial soil (TAS) in the presence of increasing concentrations of the vinasses VA, VB and VC after 48 h of exposure. Values are expressed in mean percentage of avoidance behavior ± standard deviation; n = 5. Asterisks (*) indicate significant avoidance of earthworms towards the contaminated soils (Fisher's exact test, $p \leq 0.05$).

Between the two distillery vinasses, VB showed a higher impact in most test species compared to that of VA. These differences were seen especially in the avoidance tests, except with *E. andrei* in the TAS. In the reproduction tests, VB was more toxic than VA in all of the soils only for *F. candida*, whereas for *E. crypticus* this difference only occurred in the natural soils (LV and LVA). These results also suggest that the sensitivity of the avoidance tests to the vinasses was similar to that of the reproduction tests for the same species. This fact corroborates the study of Hund-Rinke et al. (2003), who evaluated the avoidance of earthworms in the presence of soils with different contamination levels (i.e., metals, mineral oil, polycyclic aromatic hydrocarbons, trinitrotoluene, etc.). The authors indicated that earthworm avoidance tests may present a sensitivity equal or higher than that of the earthworm reproduction tests. According to Natal-da-Luz et al. (2004), the avoidance tests with *E. andrei* and *F. candida* can be used as an initial screening tool in the assessment of contaminated areas.

The differences in toxicity among vinasses are primarily attributed to differences in their composition and origin. The distinct toxicity of VA and VB was most likely due to the particular composition of the wines produced in the distilleries of each vinasse, which is dependent on the origin and composition of the raw material (Silva and Orlando, 1981). Furthermore, the system used in the preparation of the must, the method of fermentation, type of distillery equipment, distillation technique, among others, are factors that cause differences in the composition (such as in metal and salt content) of vinasses from different distilleries (España-Gamboa et al., 2011).

Christofoletti et al. (2013a) described that the ecotoxicological potential of sugarcane vinasses in aquatic and terrestrial environments is related to the presence of alcohol compounds and high concentrations of OM, K, P, S, Fe, Mn, Zn, Cu and heavy metals such as Cd, Cr, Ni, and Pb. Kannan and Upreti (2008) also suggest that the toxicity of vinasses in the soil is related to the high OM content, presence of heavy metals, and low pH of the waste. However, in the present study, the total concentrations of the potentially toxic metals (As, Cd, Co, Cr, Cu, Hg, Mo, Ni, Pb and Zn) in the soils treated with vinasses were low (Supplementary Table 1). Furthermore, the levels of alcohol compounds (ethanol) in the test vinasses were also low (Table 2), which indicates that these elements/substances have little or no influence on the observed toxicity. The presence of antibiotic compounds used in the fermentation process (Compart et al., 2013), phytotoxic substances and recalcitrant compounds (such as phenols, polyphenols, among others) often found in this type of wastes are known to contribute to the vinasse toxicity to microorganisms and plants that inhabit the disposal sites (España-Gamboa et al., 2011). However, the presence of these substances was not evaluated in this study; therefore, it is not possible to ascertain whether they were a source of toxicity. The higher toxicity observed in the soils containing VA and VB may be related to the higher concentrations of K and S in these vinasses, compared to that in VC (Table 2). According to Freitas and Rocha (2011), the cation K, which is highly present in VA and VB vinasses, may provoke toxicity to aquatic biota. The K excess in the hemolymph may interrupt the cardiac function and promote cellular disorders in aquatic invertebrates (Romano and

Table 3
No observed effect concentration (NOEC), lowest observed effect concentration (LOEC) and 20% (EC_{20}), 50% (AC_{50} and EC_{50}) effect concentrations calculated based on the results of the avoidance and reproduction tests with the species *Eisenia andrei*, *Enchytraeus crypticus*, *Hypoaspis aculeifer* and *Folsomia candida*, which were exposed to increasing concentrations of three vinasses (VA, VB and VC) in three soils (Red Latosol, LV; Red–Yellow Latosol, LVA; tropical artificial soil, TAS). Values are expressed in mL of vinasse kg^{-1} of soil (dry weight).

Test species	Endpoint	Toxic value	LV soil			LVA soil			TAS		
			VA	VB	VC	VA	VB	VC	VA	VB	VC
E. andrei	48-h avoidance	NOEC	100	100	200	60	60	120	18.4	36.7	294
		LOEC	200	200	>200	120	120	>120	36.7	73.5	>294
		AC_{50}	209[a]	201[a]	>200	129[a]	114	>120	56.7	122	>294
		Limits (95%)	(222–203)	(207–196)	(–)[b]	(134–125)	(117–111)	(–)[b]	(104–27.3)	(190–82.6)	(–)[b]
	28-h adult growth	NOEC	100	200	200	120	120	120	147	294	294
		LOEC	200	>200	>200	>120	>120	>120	294	>294	>294
		EC_{50}	303[a]	>200	>200	>120	>120	>120	>294	>294	>294
		Limits (95%)	(320–287)	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]
		EC_{20}	299[a]	>200	>200	>120	>120	>120	>294	>294	>294
		Limits (95%)	(324–273)	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]
	56-d reproduction	NOEC	100	100	200	60	60	120	73.5	73.5	147
		LOEC	200	200	>200	120	120	>120	147	147	294
		EC_{50}	150	187	>200	126[a]	120	>120	100	115	253
		Limits (95%)	(187–113)	(223–151)	(–)[b]	(150–118)	(122–118)	(–)[b]	(125–76.1)	(137–94.1)	(344–162)
		EC_{20}	96.5	123	>200	96.7	90.1	>120	85.3	89.4	121
		Limits (95%)	(133–59.7)	(167–89.7)	(–)[b]	(113–79.9)	(113–67)	(–)[b]	(107–63.8)	(113–65.7)	(191–50.9)
E. crypticus	28-d reproduction	NOEC	50	50	100	15	7.5	30	73.5	73.5	294
		LOEC	100	100	200	30	15	60	147	147	>294
		EC_{50}	110	64.8	>200	40.4	14.6	63.1	199	>294	>294
		Limits (95%)	(144–77.1)	(83.6–46)	(–)[b]	(67.4–13.4)	(21.3–7.9)	(77.9–48.3)	(233–165)	(–)[b]	(–)[b]
		EC_{20}	51.9	35.7	102	13.1	4.7	32.7	143.5	134	>294
		Limits (95%)	(83.5–20.3)	(56.2–15.3)	(149–54)	(21.7–4.3)	(6.8–2.5)	(47.6–17.8)	(177–110)	(253–15.3)	(–)[b]
H. aculeifer	14-d reproduction	NOEC	200	200	200	120	120	120	294	90.7	294
		LOEC	>200	>200	>200	>120	>120	>120	>294	163	>294
		EC_{50}	>200	>200	>200	>120	>120	>120	>294	238	>294
		Limits (95%)	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(312–164)	(–)[b]
		EC_{20}	>200	>200	>200	>120	>120	>120	>294	123	>294
		Limits (95%)	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(185–61.2)	(–)[b]
F. candida	48-h avoidance	NOEC	50	12.5	100	30	7.5	120	147	36.7	294
		LOEC	100	25	>100	60	15	>120	>147	73.5	>294
		AC_{50}	108[a]	25.9[a]	>100	63.4[a]	14.7	>120	>147	84.5[a]	>294
		Limits (95%)	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]
	28-d reproduction	NOEC	50	12.5	100	30	7.5	120	147	36.7	294
		LOEC	100	25	>100	>60	15	>120	>147	73.5	>294
		EC_{50}	63.6	20.6	>100	>60	>15	>120	>147	77.2	>294
		Limits (95%)	(77.6–49.6)	(27–14.2)	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(–)[b]	(102–52.8)	(–)[b]
		EC_{20}	42.9	11.6	>100	29.7	10.4	>120	>147	39.4	>294
		Limits (95%)	(56.9–28.9)	(17.9–5.3)	(–)[b]	(40.4–19.1)	(23.3–2.4)	(–)[b]	(–)[b]	(64.3–14.5)	(–)[b]

[a] The estimated values, overcoming the maximum concentration tested, were shown to highlight the differences between the vinasses from distillery plants (VA and VB) and that from laboratory production (VC).
[b] Data obtained did not allow for the calculation of 95% confidence intervals.

Zeng, 2007). Furthermore, the higher toxicity of VA and VB compared to VC can also result from addictive and/or synergistic effects of substance mixtures.

A significant reduction of the percentage of initial body biomass of adult earthworms was observed only at the highest concentrations of VA in the LV and TAS. Cesar et al. (2014) also observed that high doses of dredged sediments applied to tropical soils reduced the biomass of this species. However, these authors reported that the biomass of earthworms at low doses was higher than that of the control soil, an increase that they attributed to the high soil OM of the sediment (19.1%). In this study VA showed a relatively low level of OM (28 g dm^{-3} or ≈2.95%; Table 2), which apparently did not promote an increase in initial percentage of biomass even at the lowest concentrations in any of the soils tested. Studies have indicated that the reduction in biomass of earthworms in soils treated with industrial waste (with high OM content) normally occurs in the presence of high concentrations of waste when the energy benefits provided by the material overcome the impact of increased concentrations of toxic compounds in the soil. Otherwise, the energetic organic material results into an increase or at least into the maintenance of the initial biomass (Rosa et al., 2007; Natal-da-Luz et al., 2009b; Matos-Moreira et al., 2012; Cesar et al., 2014).

The toxicity of the vinasses in the different soils varied with the test organism. Considering the LOEC, AC_{50}, EC_{50} or EC_{20} values of all the vinasses in all of the tests performed (Table 3), the species *E. andrei* was most affected by the vinasses in the TAS, followed by the LVA and LV (considering reproduction and avoidance tests). VB was only toxic for mites in the TAS. Conversely, the species *E. crypticus* and *F. candida* suffered the highest impacts in the presence of the test vinasses (considering both reproduction and avoidance tests for collembolans) in the LVA, followed by the LV and TAS. The difference in the influence of the soils on the toxicity of the vinasses is most likely related to the way how the organisms are exposed to the soil contaminants. Both *E. andrei* and *E. crypticus* are soft-bodied organisms (Peijnenburg et al., 2012), and they are primarily exposed to contaminants through direct skin absorption (through contact with the soil solution) and by ingestion of soil particles (especially earthworms). However, considering that the pollutants in the vinasses are (at least partially) associated with the soil OM (these components were three times higher in TAS than in the other soils; Table 2), it was expected that earthworms would be more negatively affected in the TAS because they are exposed through contact with the soil and also by the ingestion of high quantities of contaminants from the soil (Table 3). In addition, it is known that enchytraeids are extremely sensitive to soil acidity (Amorim et al.,

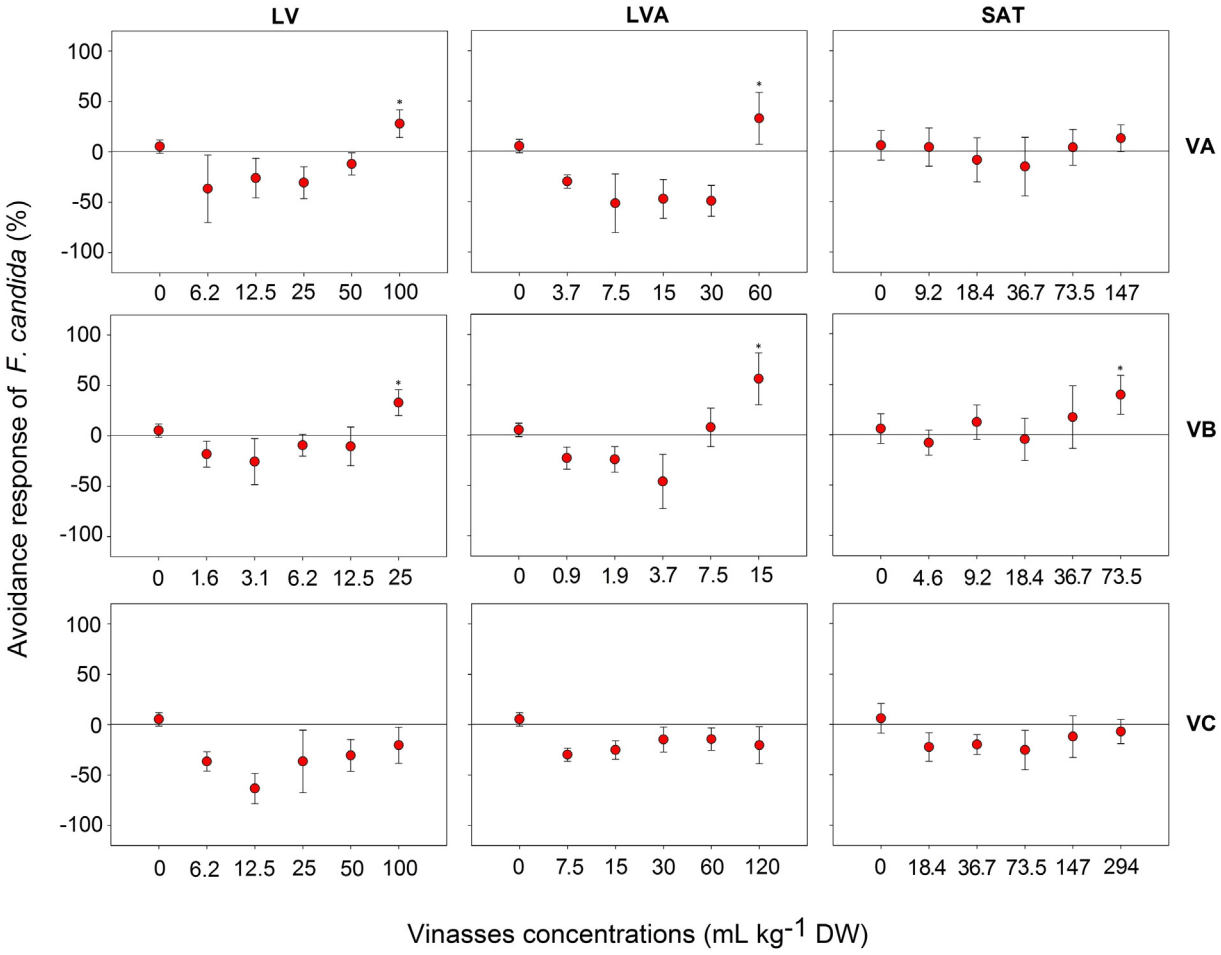

Fig. 2. Avoidance behavior of *F. candida* in two natural Oxisols (LV and LVA) and a tropical artificial soil (TAS) in the presence of increasing concentrations of the vinasses VA, VB and VC after 48 h of exposure. Values are expressed in mean percentage of avoidance behavior ± standard deviation; n = 5. Asterisks (*) indicate significant avoidance of collembolans towards the contaminated soils (Fisher's exact test, $p \leq 0.05$).

2005a; Chelinho et al., 2014) and, in some cases, toxicity might be caused by the low soil pH rather than the high concentrations of contaminants (Luo et al., 2014). Although the LVA and LV soils have lower pH than the TAS (Table 2), these properties might have contributed to increase the toxicity of vinasses to *E. crypticus* in natural soils.

The toxicity of chemicals to mites and collembolans (hard-bodied organisms that possess an exoskeleton and absorb water through specialized organs) is primarily dependent on the presence of contaminants in the soil solution because of their water uptake routes (Peijnenburg et al., 2012). Therefore, the reproduction of *H. aculeifer* and *F. candida* was assumed to be more affected in natural soils, where the low levels of OM relative to those of the TAS would result in a higher availability of contaminants in the soil solution. However, in agreement with the usually lower sensitivity of mites in comparison with other soil invertebrates (Huguier et al., 2015) this assumption was verified only for *F. candida*. Domene et al. (2012) also observed that the survival of this species of collembolans in the presence of the herbicide phenmedipham was lower in natural soils than in artificial soil (OECD soil), and they attributed this difference to the OM and silt levels of the soils. Furthermore, additional studies have shown that contaminants were more toxic for *F. candida* in natural soils than in artificial soil (Phillips et al., 2002; Amorim et al., 2005b; Crouau and Tan, 2006).

Considering only the two natural soils, the toxicity of the vinasses was generally higher in the LVA than in LV. The fact that the LVA has lower levels of clay, OM and CEC compared with that of the LV may have contributed to this difference in toxicity (Table 2). The CEC can be considered a determinant variable for toxicity in soils (Lock and Janssen, 2001) because it is a relative measure of the binding sites between contaminants (mainly metal cations and other electropositive substances) and soil. In soils with low CEC (such as LVA), there is a low capacity for contaminant adsorption and, consequently, high availability of toxic substances in soil solution. In addition, it is known that low levels of OC (Belfroid and Sijm, 1998; Zhang et al., 2010) and clay (Cesar et al., 2012, 2014) increase the toxicity of some substances in soils.

Although all of the test species were significantly affected in the chronic toxicity tests, the sensitivity of the organisms varied in at least one of the combinations tested (vinasses vs soils), regardless of the vinasse and soil sample used. According to Van Gestel (2012), a species cannot be sensitive in all situations; therefore, multiple toxicity tests with several species are required to adequately estimate the potential risk of contaminants to terrestrial ecosystems. Thus, several studies have focused on the selection of bioindicators to evaluate the specific risks of contaminants in soil (Pandard et al., 2006; Daam et al., 2011; Khan et al., 2013).

The species *E. crypticus* and *F. candida* were the only species for which the EC_{20} was lower than the legal limits of vinasse concentrations calculated for the LVA and LV, respectively (Table 1; CETESB, 2006). Although the remaining organisms (*E. andrei* and *H. aculeifer*) were also affected, the effects were mostly observed in concentrations above the legal limits (Tables 1 and 3). This fact suggests that the limit values established based on the standard P4.231 of CETESB (2006) may protect more than 80% of the individuals of *E. andrei* and *H. aculeifer* but they did not prevent significant toxic effects to 20% of

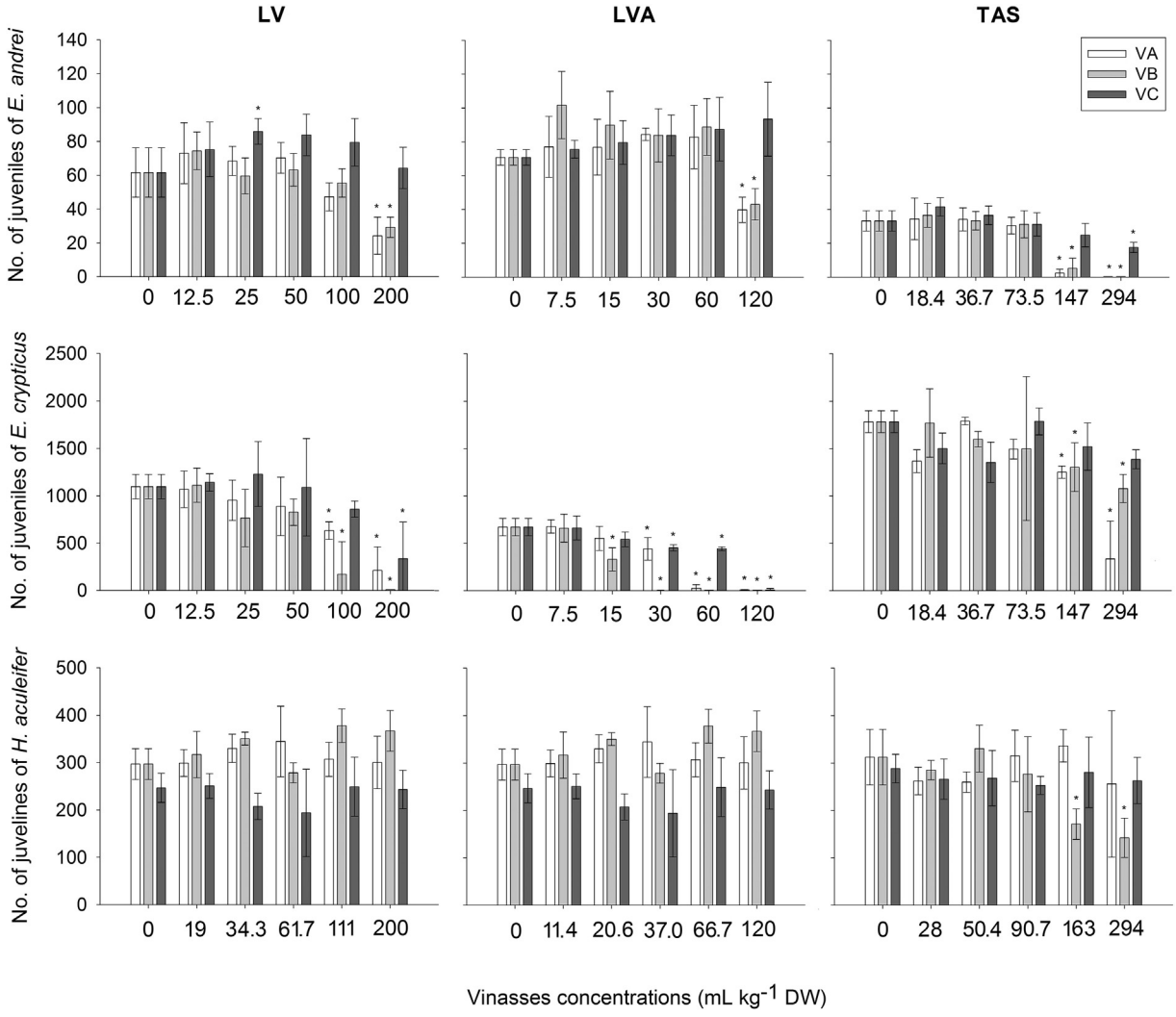

Fig. 3. Number of juveniles of *Eisenia andrei*, *Enchytraeus crypticus* and *Hypoaspis aculeifer* (mean ± standard deviation; n = 4) in two natural Oxisols (LV and LVA) and a tropical artificial soil (TAS) in the presence of increasing concentrations of the vinasses VA, VB and VC after 56, 28 and 14 days of exposure, respectively. Asterisks (*) indicate significant reduction in the number of juveniles relative to the respective control (one-way ANOVA, Dunnett's post-hoc test — $p \leq 0.05$).

individuals of *E. crypticus* and *F. candida* from the sugarcane vinasses in tropical soils. Christofoletti et al. (2013b) also observed that the maximum concentration of vinasses in the soil, which was also calculated according to CETESB (2006), can cause death in diplopods and genotoxicity in plants. In addition, considering the criteria defended by Pandard and Römbke (2013) who consider a waste as ecotoxic (the HP 14 property) when its EC_{50} occurs at a concentration lower or equal to 10%, the vinasses can be classified as hazardous especially because of their effects on *E. crypticus* (EC_{50}: 1.46% to >29.4%) and *F. candida* (EC_{50}: 2.06% to >29.4%). Because the limits for the application of vinasses in agricultural soils were established with the objective of protecting the chemical parameters of soil and based exclusively on chemical analyses (CETESB, 2006), it is understandable that certain biological parameters are not equally protected. This suggests that these limits are not adequate to preserve all of the functions of soil as an ecosystem (chemical, physical and biological parameters). However, because the volume of vinasse generated by distilleries is extremely high and its primary destination is the agricultural soils (Christofoletti et al., 2013a), it is important to adopt criteria that protect the soil fauna and associated ecosystem services.

The deleterious effects of vinasses have been reported for certain nematode species (Pedrosa et al., 2005; Caixeta et al., 2011; Matos et al., 2011). However, to our knowledge, this is the first study that reports the effects of the application of sugarcane vinasses in tropical soils to standard species of earthworms, enchytraeids, collembolans and mites, which are representatives of soil macro and mesofauna. However, additional parameters, such as microbiological parameters and plants, should also be evaluated to provide consistent guiding values, as well as a risk assessment could be performed to reduce the uncertainty about the ecological risk of vinasse in tropical soil ecosystems.

5. Conclusion

The composition and the toxic effects of vinasses on individuals of soil biota varied according to its origin. The vinasses originated in commercial plants were more toxic than that obtained through laboratory production. Only the highest concentrations of vinasses VA and VB caused avoidance behavior in earthworms and collembolans, and reduced the number of earthworm juveniles in natural soils. The biomass of earthworms was only reduced in the presence of VA. Decreases in the reproduction of enchytraeids and collembolans were observed in the presence of VA and VB concentrations lower than the estimated limits. The predatory mites were the less affected organisms by vinasses in the soils. The relationship between the vinasses' origins and different soil types resulted in specific toxic values for each species. Vinasse disposal on tropical soils should consider the sensitivity of soil invertebrates. Additionally, we recommend a comprehensive risk assessment

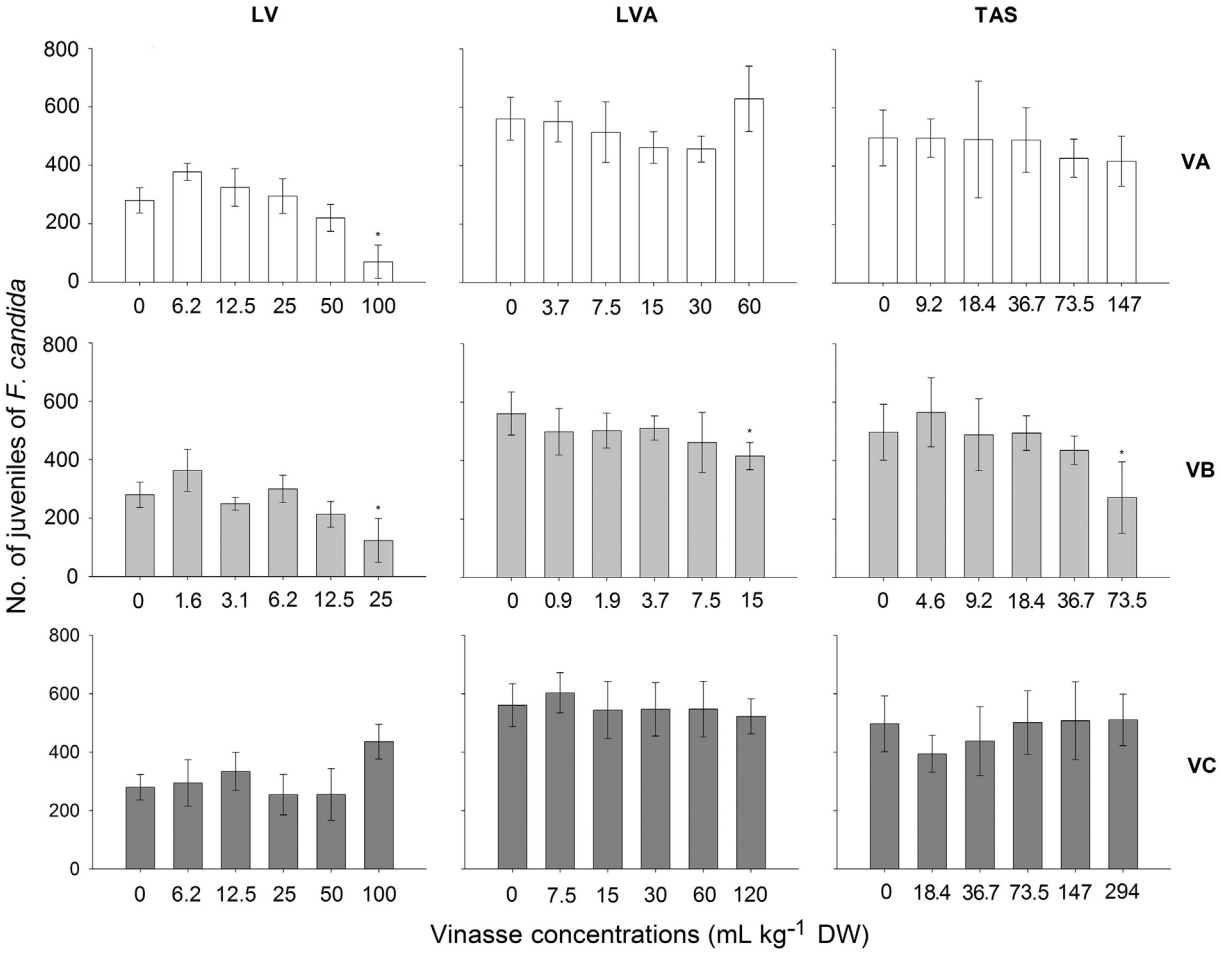

Fig. 4. Number of juveniles of *Folsomia candida* (mean ± standard deviation; n = 5) in two natural Oxisols (LV and LVA) and a tropical artificial soil (TAS) in the presence of increasing concentrations of the vinasses VA, VB and VC after 28 days. Asterisks (*) indicate significant reduction in the number of juveniles relative to the respective control (one-way ANOVA, Dunnett's post-hoc test — $p \leq 0.05$).

to reduce the uncertainties about the ecological risk involved in the use of this type of wastes in soil.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.03.150.

Acknowledgments

P.R.L.A. would like to thank the Graduate Course of Soil and Plant Nutrition of the Soil Science Department at ESALQ/USP (University of São Paulo) for the research opportunity, and wants to express his gratefulness to the CAPES Foundation for a research grant, project: 8045-13-5. E.J.B.N.C. thanks for a research grant (No. 307563/2009-0) from the National Council for Scientific and Technological Development (CNPq).

References

Almeida, J.R., 1952. O problema da vinhaça em São Paulo. No. 3. COPERSUCAR, Piracicaba.
Alves, P.R.L., Cardoso, E.J.B.N., Martines, A.M., Sousa, J.P., Pasini, A., 2013. Earthworm ecotoxicological assessments of pesticides used to treat seeds under tropical conditions. Chemosphere 90, 2674–2682.
Alves, P.R.L., Cardoso, E.J.B.N., Martines, A.M., Sousa, J.P., Pasini, A., 2014. Seed dressing pesticides on springtails in two ecotoxicological laboratory tests. Ecotoxicol. Environ. Saf. 105, 65–71.
Amorim, M.J., Römbke, J., Scheffczyk, A., Soares, A.M.V.M., 2005a. Effect of different soil types on the enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus* using the herbicide Phenmedipham. Chemosphere 61, 1102–1114.
Amorim, M.J.B., Römbke, J., Scheffczyl, A., Nogueira, A.J.A., Soares, A.M.V.M., 2005b. Effects of different soil types on the collembolans *Folsomia candida* and *Hypogastrura assimilis* using the herbicide phenmedipham. Arch. Environ. Contam. Toxicol. 49, 343–352.
Belfroid, A., Sijm, D.T.H.M., 1998. Influence of soil organic matter content on elimination rates of hydrophobic compounds in the earthworm: possible causes and consequences. Chemosphere 37, 1221–1234.
Brussaard, L., Pulleman, M.M., Ouédraogo, E., Mando, A., Six, J., 2007. Soil fauna and soil function in the fabric of the food web. Pedobiologia 50, 447–462.
Caixeta, L.B., Pedrosa, E.M.R., Guimarães, L.M.P., Barros, P.A., Rolim, M.M., 2011. Changes in soil and nematode community after sugarcane harvest and vinasse application. Nematropica 41, 271–280.
Cardoso, E.J.B.N., Vasconcellos, R.L.F., Bini, D., Miyauchi, M.Y.H., Santos, A.C., Alves, P.R.L.A., de Paula, A.M., Nakatani, A.S., Pereira, J.M., Nogueira, M.A., 2013. Soil health: looking for suitable indicators. What should be considered to assess the effects of use and management on soil health? Sci. Agric. 70, 274–289.
Cesar, R.G., Silva, M.B., Colonese, J.P., Bidone, E.D., Egler, S.G., Castilhos, Z.C., Polivanov, H., 2012. Influence of the properties of tropical soils in the toxicity and bioavailability of heavy metals in sewage sludge-amended lands. Environ. Earth Sci. 66 (2281-229).
Cesar, R.G., Natal-da-Luz, T., Sousa, J.P., Colonese, J., Bidone, E., Castilhos, Z., Egler, S., Polivanov, H., 2014. Disposal of dredged sediments in tropical soils: ecotoxicological effects on earthworms. Environ. Monit. Assess. 186, 1487–1497.
Cesar, R., Natal-da-Luz, T., Sousa, J.P., Bidone, E., Castilhos, Z., Polivanov, H., 2015. Disposal of dredged sediments in tropical soils: ecotoxicological evaluation based on bioassays with springtails and enchytraeids. Environ. Sci. Pollut. Res. 22, 2916–2924.
CETESB, 2006. Vinhaça: critérios e procedimentos para aplicação no solo agrícola. Norma técnica: P4.231. CETESB, São Paulo.
CETESB, 2014. Valores orientadores para solo e água subterrânea no Estado de São Paulo. Diário Oficial do Estado 124 (36), 1–53.
Chelinho, S., Domene, X., Campana, P., Andrés, P., Römbke, J., Sousa, J.P., 2014. Toxicity of phenmedipham and carbendazim to *Enchytraeus crypticus* and *Eisenia andrei* (Oligochaeta) in Mediterranean soils. J. Soils Sediments 14, 584–599.

Christofoletti, C.A., Escher, J.P., Correia, J.E., Marinho, J.F.U., Fontanetti, C.S., 2013a. Sugarcane vinasse: environmental implications of its use. Waste Manag. 33, 2752–2761.
Christofoletti, C.A., Escher, J.P., Fontanetti, C.S., 2013b. Assessment of the genotoxicity of two agricultural residues after processing by diplopods using the *Allium cepa* assay. Water Air Soil Pollut. 224 (4), 1523.
Compart, D.M.P., Carlson, A.M., Crawford, G.I., Fink, R.C., Diez-Gonzalez, F., DiCostanzo, A., Shurson, G.C., 2013. Presence and biological activity of antibiotics used in fuel ethanol and corn co-product production. J. Anim. Sci. 91, 2395–2404.
Cortet, J., Gomot-De Vauflery, A., Poinsot-Balaguer, N., Gomot, L., Texier, C., Cluzeau, D., 1999. The use of invertebrate soil fauna in monitoring pollutant effects. Eur. J. Soil Biol. 35, 115–134.
Crouau, Y., Tan, T.S., 2006. Importance of the nature of the dilution soils for the evaluation of toxicity of a polluted soil by a collembolan reproduction assay. Appl. Soil Ecol. 34, 280–283.
Daam, M.A., Leitão, S., Cerejeira, M.J., Sousa, J.P., 2011. Comparing the sensitivity of soil invertebrates to pesticides with that of *Eisenia fetida*. Chemosphere 85, 1040–1047.
Dane, J.H., Topp, G.C., 2002. Methods of Soil Analysis Part 4: Physical Methods. SSSA, Madison (Book Series 5).
De Silva, P.M.C.S., Van Gestel, C.A.M., 2009. Development of an alternative artificial soil for earthworm toxicity testing in tropical countries. Appl. Soil Ecol. 43, 170–174.
Domene, X., Alcañiz, J.M., Andrés, P., 2007. Ecotoxicological assessment of organic wastes using the soil collembolan *Folsomia candida*. Appl. Soil Ecol. 35, 461–472.
Domene, X., Chelinho, S., Campana, P., Alcaniz, J.M., Römbke, J., Sousa, J.P., 2012. Applying a GLM-based approach to model the influence of soil properties on the toxicity of phenmedipham to *Folsomia candida*. J. Soils Sediments 12 (6), 888–899.
EC (European Community), 1991. Council Directive 91/689/EEC of 12 December 1991 on hazardous waste. Off. J. Eur. Communities L Legis. 377, 20–27.
EC (European Community), 2008. Council Directive 2008/98/EC: directive of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain directives. Off. J. Eur. Union 312, 3–30 (549).
Environment Canada, 2007. Guidance Document on Statistical Methods for Environmental Toxicity Tests. Environmental Protection Series, EPS 1/RM/46, 2005 With 2007 Updates. Environment Canada, Ottawa.
España-Gamboa, E., Mijangos-Cortes, J., Barahona-Perez, L., Dominguez-Maldonado, J., Hernández-Zarate, G., Alzate-Gaviria, L., 2011. Vinasses: characterization and treatments. Waste Manag. Res. 29, 1235–1250.
Freire, W.J., Cortez, L.A.B., 2000. Vinhaça de cana-de-açúcar. Agropecuária, Guaíba.
Freitas, E.C., Rocha, O., 2011. Acute and chronic effects of sodium and potassium on the tropical freshwater cladoceran *Pseudosida ramose*. Ecotoxicology 20, 88–96.
Garcia, M.V.B., 2004. Effects of Pesticides on Soil Fauna: Development of Ecotoxicological Test Methods for Tropical Regions. (Thesis research work). University of Bonn.
Huguier, P., Manier, N., Owojori, O.J., Bauda, P., Pandard, P., Römbke, J., 2015. The use of soil mites in ecotoxicology: a review. Ecotoxicology 24, 1–18.
Hund-Rinke, K., Achazi, R., Römbke, J., Warnecke, D., 2003. Avoidance test with *Eisenia fetida* as indicator for the habitat function of soils: results of a laboratory comparison test. J. Soils Sediments 3, 7–12.
ISO, 1996. Soil quality: effects of pollutants on earthworms (*Eisenia fetida*)—part 2: determination of effects on reproduction. ISO, Geneva (ISO 1268-2.2).
ISO, 1999. Soil quality: inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants. ISO, Geneva (ISO 11269).
ISO, 2004. Soil quality: effects of pollutants on Enchytraeidae (*Enchytraeus* sp.)—determination of effects on reproduction and survival. ISO, Geneva (ISO 16387).
ISO, 2008a. Soil Quality: Avoidance Test for Determining the Quality of Soils and Effects of Chemicals on Behavior. Part 1: Test With Earthworms (*Eisenia fetida* and *Eisenia andrei*). ISO, Geneva (ISO 17512 1).
ISO, 2008b. Soil Quality: Avoidance Test for Determining the Quality of Soils and Effects of Chemicals on Behavior. Part 2: Test With Collembolans (*Folsomia candida*). ISO, Geneva (ISO 17512 2).
Jiang, Z.P., Li, Y.R., Wei, G.P., Liao, Q., Su, T.M., Meng, Y.C., Zhang, H.Y., Lu, C.Y., 2012. Effect of long-term vinasse application on physico-chemical properties of sugarcane field soils. Sugar Tech 14, 412–417.
Kannan, A., Upreti, R.K., 2008. Influence of distillery effluent on germination and growth of mung bean (*Vigna radiata*) seeds. J. Hazard. Mater. 153, 609–615.
Khan, M.I., Cheema, S.A., Tang, X., Hashmi, M.Z., Shen, C., Park, J., Chen, Y., 2013. A battery of bioassays for the evaluation of phenanthrene biotoxicity in soil. Arch. Environ. Contam. Toxicol. 65, 47–55.
Kiehl, E.J., 1985. Fertilizantes orgânicos. Agronômica Ceres, Piracicaba.
Laime, E.M.O., Fernandes, P.D., Oliveira, D.C.S., Freire, E.A., 2011. Possibilidades tecnológicas para a destinação da vinhaça: uma revisão. R. Trop. Ci. Agr. Biol. 5, 16–29.
Leal, J.R., Amaral, N.M.B.S., Velloso, A.C.X., Rossielo, R.O.P., 1983. Potencial redox e pH: variação em um solo tratado com vinhaça. Rev. Bras. Ciênc. Solo 7, 257–261.
Lock, K., Janssen, C.R., 2001. Effect of clay and organic matter type on the ecotoxicity of zinc and cadmium to the potworm *Enchytraeus albidus*. Chemosphere 44 (8), 1669–1672.
Luo, W., Verweij, R.A., Van Gestel, C.A.M., 2014. Contribution of soil properties of shooting fields to lead biovailability and toxicity to *Enchytraeus crypticus*. Soil Biol. Biochem. 76, 235–241.
Matos, D.S.S., Pedrosa, E.M.R., Guimarães, L.M.P., Rodrigues, C.V.M.A., Barbosa, N.M.R., 2011. Relations between nematode communities and chemical attributes of soil with vinasse. Nematropica 41, 23–38.
Matos-Moreira, M., Carral, E., Rodriguez, M.T., Lopez-Mosquera, M.E., Cunha, M., 2012. Survival, body mass change and metal bioaccumulation in *Eisenia fetida* (Oligochaeta: Lumbricidae) incubated in soil amended with organic wastes. Fresenius Environ. Bull. 21, 441–449.
MME (Ministério de Minas e Energia) Etanol - produção e consumo. Boletim mensal dos combustíveis renováveis. N° 72. Available at:, http://www.mme.gov.br/spg/menu/publicacoes.html (Accessed: September, 11, 2014).
Moser, H., Römbke, J., 2009. Ecotoxicological Characterization of Waste — Results and Experiences on an International Ring Test. Springer, Dessau.
Natal-da-Luz, T., Ribeiro, R., Sousa, J.P., 2004. Avoidance tests with collembola and earthworms as early screening tools for site-specific assessment of polluted soils. Environ. Toxicol. 23, 2188–2193.
Natal-da-Luz, T., Tidona, S., Van Gestel, C.A.M., Morais, P.V., Sousa, J.P., 2009a. The use of Collembola avoidance tests to characterize sewage sludges as soil amendments. Chemosphere 77, 1526–1533.
Natal-da-Luz, T., Tidona, S., Jesus, B., Morais, P.V., Sousa, J.P., 2009b. The use of sewage sludge as soil amendment. The need for an ecotoxicological evaluation. J. Soils Sediments 9, 246–260.
Natal-da-Luz, T., Moreira-Santos, M., Ruepert, C., Castillo, L.E., Ribeiro, R., Sousa, J.P., 2012. Ecotoxicological characterization of a tropical soil after diazinon spraying. Ecotoxicology 21, 2163–2176.
OECD, 1984. Guideline for Testing of Chemicals No. 207: Earthworm Acute Toxicity Test. OECD, Paris.
OECD, 2008. Guidelines for Testing of Chemicals No. 226: Predatory Mite (*Hypoaspis* (*Geolaelaps*) *aculeifer*) Reproduction Test in Soil. OECD, Paris.
Oliveira, E.L.D., Andrade, L.A.D.B., Faria, M.A.D., Evangelista, A.W.P., Morais, A.R.D., 2009. Uso de vinhaça de alambique e nitrogênio em cana-de-açúcar irrigada e não irrigada. Pesq. Agropec. Bras. 44, 1398–1403.
Owojori, O.J., Waszak, K., Römbke, J., 2014. Avoidance and reproduction tests with the predatory mite *Hypoaspis aculeifer*: effects of different chemical substances. Environ. Toxicol. Chem. 33, 230–237.
Pandard, P., Römbke, J., 2013. Proposal for a "Harmonized" strategy for the assessment of the HP 14 property. Integr. Environ. Assess. Manag. 9 (4), 665–672.
Pandard, P., Devillers, J., Charisson, A.M., Poulsen, V., Jourdain, M.J., Férard, J.F., Grand, C., Bispoi, A., 2006. Selecting a battery of bioassays for ecotoxicological characterization of wastes. Sci. Total Environ. 363, 114–125.
Pedrosa, E.M.R., Rolim, M.M., Albuquerque, P.H.S., Cunha, A.C., 2005. Supressividade de nematóides em cana-de-açúcar por adição de vinhaça ao solo. Rev. Bras. Eng. Agríc. Ambient. 9, 197–201.
Peijnenburg, W., Capri, E., Kula, C., Liess, M., Luttik, R., Montforts, M., Nienstedt, K., Römbke, J., Sousa, J.P., Jensen, J., 2012. Evaluation of exposure metrics for effect assessment of soil invertebrates. Crit. Rev. Environ. Sci. Technol. 42, 1862–1893.
Pesaro, M., Widmer, F., Nicollier, G., Zeyer, J., 2003. Effects of freeze–thaw stress during soil storage on microbial communities and methidathion degradation. Soil Biol. Biochem. 35, 1049–1061.
Phillips, C.T., Kuperman, R.G., Checkai, R.T., 2002. Toxicity of chemical-warfare agent HD to *Folsomia candida* in different soil types. Eur. J. Soil Biol. 38, 281–285.
Ribeiro, B.T., Lima, J.M.D., Roberto, L., Guilherme, G., Gustavo, L., Julião, F., 2010. Lead sorption and leaching from an Inceptisol sample amended with sugarcane vinasse. Sci. Agric. 67, 441–447.
Romano, N., Zeng, C., 2007. Acute toxicity of sodium nitrate, potassium nitrate, and potassium chloride and their effects on the hemolymph composition and gill structure of early juvenile blue swimmer crabs. Environ. Toxicol. Chem. 26, 1955–1962.
Römbke, J., Garcia, M.V.B., Scheffczyk, A., 2007. Effects of the fungicide benomyl on earthworms in laboratory tests under tropical and temperate conditions. Arch. Environ. Contam. Toxicol. 53, 590–598.
Rosa, E.V.C., Giuradelli, T.M., Corrêa, A.X.R., Rörig, L.R., Schwingel, P.R., Resgalla, C., Radetski, C.M., 2007. Ecotoxicological evaluation of the shortterm effects and stabilized textile sludges before application in forest soil restoration. Environ. Pollut. 146, 463–469.
Sakuma, M., 1998. Probit analysis of preference data. Appl. Entomol. Zool. 33, 339–347.
Silva, G.N., Orlando, Filho J., 1981. Concentração da composição química dos diferentes tipos de vinhaça do brasil. Bol. Tec. Planalsucar 8, 5–22.
Silva, M.A.S., Griebeler, N.P., Borges, L.C., 2007. Uso de vinhaça e impactos nas propriedades do solo e lençol freático. Rev. Bras. Eng. Agríc. Ambient. 11, 108–114.
USEPA, 1998. Method 3051a — Microwave Assisted Acid Digestion of Sediments, Sludges, Soils, and Oils. USEPA, Washington.
USEPA, 1999. Method 3015a — Microwave Assisted Acid Digestion of Aqueous Samples and Extracts. USEPA, Washington.
Uyeda, C.A., De Miranda, J.H., Duarte, S.N., De Medeiros, P.R.F., Dias, C.T.S., 2013. Influence of vinasse application in hydraulic conductivity of three soils. Eng. Agríc. 33, 689–698.
Van Gestel, C.A.M., 2012. Soil ecotoxicology: state of the art and future directions. ZooKeys 176, 275–296.
Van Raij, B., Andrade, J.C., Cantarella, H., Quaggio, J.A., 2001. Análise química para avaliação da fertilidade de solos tropicais. Editora IAC, Campinas.
Wilke, B.-M., Riepert, F., Koch, C., Kühne, T., 2008. Ecotoxicological characterization of hazardous wastes. Ecotoxicol. Environ. Saf. 70, 283–293.
Zar, J.H., 1999. Biostatistical Analysis. Prentice Hall International, London.
Zhang, R., Cui, J., Zhu, H.M., Yang, H., 2010. Effect of dissolved organic matters on napropamide availability and ecotoxicity in rapeseed (*Brassica napus*). J. Agric. Food Chem. 58 (5), 3232–3240.