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TECHNICAL NOTE

Boric acid as reference substance for ecotoxicity tests in tropical artificial soil

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Abstract

Reference substances are recommended to evaluate the quality of laboratory test species and the reliability of ecotoxicity data. Boric acid (BA) has been recommended as reference substance in some standardized tests in OECD soil, but no data are available for Tropical Artificial Soil (TAS). For this purpose, avoidance tests with *Eisenia andrei*, lethality tests with *E. andrei* and *Folsomia candida*, and reproduction tests with *E. andrei, Enchytraeus crypticus* and *F. candida* were carried out in TAS (5% organic matter), following ISO guidelines, and compared between two laboratories. Collembolans were more sensitive than earthworms in lethality tests ($LC_{50} = 342$ and > 1000 mg kg^{-1}, respectively). For both laboratories, the EC_{50} values were similar for reproduction of oligochaeta species (165 mg kg^{-1} for *E. crypticus*; 242 and 281 mg kg^{-1} for *E. andrei*), but significantly different for reproduction of *F. candida* (96 and 198 mg kg^{-1}). Present results suggest that boric acid could replace the current pesticides recommended by ISO guidelines as reference substances on reproduction tests with soil invertebrates in TAS. Concerning avoidance tests, additional investigations should be performed with other substances that cause no neurotoxic effects on soil organisms.

Keywords Soil invertebrates · Soil ecotoxicology · Reproduction tests · Avoidance tests

Introduction

In order to evaluate the effects of chemicals on organisms, standardized methods are necessary (Römbke and Ahtiainen 2007). Part of the standardized methods require the use of a reference substance with known toxicity, which verifies the continuous sensitivity of cultivated organisms over time (OECD 2005, Princz et al. 2017). The selection of a reference substance should fulfill some criteria (Gourmelon and Ahtiainen 2007). A suitable reference substance should demonstrate its bioavailability during the test, general enough to affect all the interested test species and chosen endpoint in a reproducible way, must not be difficult to obtain and afford a practical and analytical method to confirm test concentrations (Römbke and Ahtiainen 2007). Ideally, a reference substance should cause effects in a reasonable range less than 1000 mg kg^{-1}, for reasons of practicality, costs and because higher concentrations are difficult to achieve in practice (Becker et al. 2011).

Currently, pesticides of high toxicity to humans and the environment are recommended as reference substances for reproduction tests with soil invertebrates in some ISO guidelines: Betanal plus (a.i. 160 g/L Phemediphan) and E 605 forte (a.i. 507,5 g/L Parathion) for reproduction test with *F. candida* (ISO 2011); and Carbendazin for reproduction tests with *E. andrei* (ISO 2012a) and *E. crypticus* (ISO 2014). Besides their toxicity, such pesticides have been banned and can disappear from the market at any time (Römbke and Ahtiainen 2007). However, boric acid (BA) has been adopted as a reference substance in other standardized tests such as avoidance for earthworms (ISO 2008) and reproduction for mites and collembolans (OECD 2008, 2009). Furthermore, its potential to replace the current adopted pesticides as reference substance in reproduction

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tests has been discussed (Römbke and Ahtiainen 2007; Becker et al. 2011; Amorim et al. 2012). A recent study (Princz et al. 2017) has demonstrated the breadth of toxicity data acquired for terrestrial species in non-tropical OECD artificial soil, but data using tropical artificial soil is scarce. Tropical artificial soil (TAS) is an adaptation of the OECD soil (OECD 1984) proposed by Garcia (2004) for ecotoxicity tests in tropical environments, replacing peat by coconut dust, which is a more readily available source of organic matter in some countries. TAS is already recognized and indicated by ISO as a type of artificial soil (ISO 2012a, b).

Given the lack of data in general, the aim of this study was to evaluate whether BA can serve as a suitable reference toxicant when used with TAS for soil species. A suite of tests were used that included earthworms, enchytraeids and collembolans, evaluating avoidance, lethality and reproduction endpoints.

Material and methods

Test chemical

An aqueous solution of 4 g/L of BA (H_3BO_3 99.8%, MERCK) was prepared for soil contamination. For each test, a range of concentrations plus a negative control (soil with only distilled water) was set up based on previous works in OECD soil (Becker et al. 2011; Amorim et al. 2012). The set of concentrations is shown in Table 1. The test chemical was spiked into premoistened soil (until 50% of the water holding capacity), homogeneously mixed, and introduced into the test vessels.

Test soil

TAS was composed by 75% fine sand (washed and dried), 20% kaolin clay and 5% coir dust (dried at 60°C). This reduced percentage of organic matter in artificial soil (from 10 to 5%) was proposed by OECD (2016) ensuring the representativeness of natural soils. The pH of the prepared soil was adjusted to 6.0 ± 0.5 adding $CaCO_3$.

Test organisms

The ecotoxicity tests with earthworms, enchytraeids and colembollans were carried out with the species *Eisenia andrei* (Annelida: Lumbricidae); *Enchytraeus crypticus* (Annelida: Enchytraeidae); and *Folsomia candida* (Collembola: Isotomidae), respectively. Tested species were maintained at 20°C ± 2°C, photoperiod 12:12 h light:dark.

Collembolans were cultured in culture vessels containing a mixture of plaster of Paris and activated charcoal (10:1), according to ISO 11267 (ISO 2011). Biological dry yeast (*Saccharomyces cerevisiae*) was provided as a food supply two times a week. Lethality tests were carried out with adults from synchronized cultures, while juveniles (10–12 d old) were used in reproduction tests.

Enchytraeids were cultured in plastic boxes filled with moistened TAS, receiving oat flour as a food supply three times a week and following the recommendations of ISO 16387 (ISO 2014). Clitellate enchytraeids were used for reproduction tests.

Earthworms were cultured in plastic boxes (about 10 L capacity) in a moistened mixture of horse manure and coconut powder, with addition of oat porridge (with water) once a week. Cultures were maintained according to ISO 11268-2 (ISO 2012a). Clitellate earthworms (2–12 months old) were used in all tests.

Ecotoxicity tests

Tests were performed in the Ecology Lab of the Federal University of Santa Catarina (UFSC), Campus of Curitibanos, and reproduced at the Soil Lab of Santa Catarina

Table 1 Summary of the tests performed indicating the test species, endpoints, concentration ranges given as active ingredient (a.i.) per kg soil (dry weight), soil type and laboratory

Organism	Tested endpoint	Concentration range (mg a.i. kg^{-1})	Soil type	Laboratory
E. andrei	Avoidance	0–125–250–500–750–1000	TAS	UDESC
	Avoidance	0–125–250–500–750–1000	TAS	UFSC
	Reproduction	0–125–250–500–750–1000	TAS	UDESC
	Reproduction	0–125–250–500–750–1000	TAS	UFSC
	Lethality	0–125–250–500–750–1000	TAS	UFSC
F. candida	Reproduction	0–12.5–25–50–100–200	TAS	UDESC
	Reproduction	0–25–50–100–200–400	TAS	UFSC
	Lethality	0–25–50–100–200–400	TAS	UFSC
E. crypticus	Reproduction	0–25–50–100–200–400	TAS	UDESC
	Reproduction	0–25–50–100–200–400	TAS	UFSC

State University (UDESC), College of Agroveterinary Sciences, with the purpose of verifying the reproducibility of such results. All tests were incubated at 20 °C ± 1 with photoperiod of 12:12 h (light:dark).

Lethality tests

Lethality tests with earthworms and collembolans were carried out only at UFSC with the main objective of defining the concentration range for reproduction tests. These tests lasted 14 days and followed the ISO guidelines 11267 (ISO 2011) for collembolans and ISO 11268-1 (ISO 2012b) for earthworms.

For collembolans, ten organisms from synchronized cultures (about 3 months old) were put into each test vessels with 30 g of contaminated or control soil. Test vessels were opened once a week for aeration. At day 7, drops of water were added to replace water loss. No food was added. At day 14, water and drops of stamp ink were added to allow the counting of the floating organisms on water surface. The number of survivals was recorded. Furthermore, this endpoint was also analyzed at the end of reproduction tests (28-day), which started with juveniles of 10–12 days old, for comparison with literature.

For lethality tests with earthworms, ten clitellate organisms were put in each test vessels containing 350 g of contaminated or control soil. Test vessels were covered with perforated plastic lids to allow aeration, and moisture was adjusted at day 7 by weighting the replicates and replacing the water loss by adding drops of distilled water. Survival was recorded at day 14 by removing and counting the living earthworms.

Avoidance tests

Avoidance tests were carried out with five replicates following ISO 17512-1 (ISO 2008) for earthworms. Test vessels were divided into two sections with a removable plastic divider, where one side received 300 g of control soil and another one received 300 g of contaminated soil. The divider was removed and 10 earthworms were placed in the centre of the test vessel. After 48 h of incubation, the divider was reinserted and the number of earthworms in each compartment was recorded. Random distribution of organisms in absence of contamination was confirmed by dual control tests, performed as described above, but receiving control soil in both sides of the test vessels.

Reproduction tests

Reproduction tests with collembolans were carried out following the ISO guideline 11267 (ISO 2011). Ten juveniles were put into test vessels containing 30 g of contaminated or control soil. Organisms were fed with approximately 2 mg of dry yeast at days 1 and 14. Twice a week the test vessels were opened allowing aeration. Drops of distilled water were added to replace water loss weekly. At day 28, test vessels were filled up with water and some drops of stamp ink, carefully stirred, and photographed after counting the floating juveniles on the water surface. Counting was carried out using the software ImageJ (Schneider et al. 2012).

Reproduction tests with earthworms were carried out following the ISO guideline 11268-2 (ISO 2012b). Ten clitellate earthworms were put into test vessels containing 350 g of contaminated or control soil. Plastic perforated lids were used to allow aeration. Cow dung free of antibiotics (5 g, dry and ground) was added weekly as food supply. Drops of distilled water were added to replace water loss weekly. At day 28, adults were removed, leaving cocoons to hatch by additional 4 weeks. At day 56, juveniles were counted using hot extraction by immersing the test vessels in water bath at 60°C, forcing the juveniles to come to soil surface.

Reproduction tests with enchytraeids were carried out following the ISO 16387 (ISO 2014). Ten clitellate organisms were put into test vessels containing 30 g of contaminated or control soil. Organisms were fed with oat flour twice a week, when the test vessels were opened allowing aeration. Drops of distilled water were added to replace water loss once a week. At day 28, alcohol (70%) was added until the soil was fully covered by the solvent, adding some drops of bengal rose to preserve and colour the organisms. After a period of 48 h, the organisms were counted using stereomicroscope (60×).

Data analysis

Results obtained in avoidance tests were analysed by Fisher exact test ($p < 0.05$) when the highest number of organisms was found in the control section. The null hypothesis assumes that 50% of test organisms stay in the test soil and that no organisms leave that section (non-avoidance) (Natal da Luz et al. 2004).

Mean number of juveniles obtained in reproduction tests was analysed by one-way Analysis of Variance (ANOVA), followed by Dunnett test ($p < 0.05$), comparing reproduction in contaminated soils versus control soil. Normal distribution of data and homogeneity of variance were verified by Shapiro Wilk's test and Bartlett test, respectively.

Median lethal concentrations (LC_{50}) for lethality tests were estimated using probit analysis with Statistica 13.0 (Dell Inc. 2015). Median effective concentrations (EC_{50}) reducing 50% of reproduction were estimated using non-linear regressions, according to Environmental Canada (2007). The best fitting model was applied (Chelinho et al. 2014).

Table 2 Median effective concentrations of Boric Acid (CI = 95% confidence interval) to *E. andrei*, *F. candida* and *E. crypticus*, in lethality, avoidance and reproduction tests in Tropical Artificial Soil (5% M.O.)

Organism	Tested endpoint	EC50/LC50 (mg kg^{-1})	Model	Laboratory
E. andrei	Lethality	>1000	–	UFSC
	Avoidance	847.4 (811.3–883.5)	Probit	UDESC
	Avoidance	>1000	–	UFSC
	Reproduction	280.9 (219.6–342.2)	Exponen	UDESC
	Reproduction	242.5 (172.3–312.7)	Exponen	UFSC
F. candida	Lethality (14d)	342.2 (335.7–348.7)	Probit	UFSC
	Lethality (28d)	225.6 (220.8–230.4)	Probit	UFSC
	Reproduction	96.8 (82.7–110.9)	Linear	UDESC
	Reproduction	198.0 (177.6–218.4)	Linear	UFSC
E. crypticus	Reproduction	165.2 (135.8–194.6)	Linear	UDESC
	Reproduction	164.8 (74.3–255.3)	Hormesis	UFSC

Results and discussion

Lethality tests

Survival of earthworms in control was 97%, fulfilling the validity criteria. Lethality in treatments with BA, even at 1000 mg kg^{-1}, was lower than 10%, indicating no acute effects of this substance to *E. andrei* in the tested concentrations, confirming the results presented by Princz et al. (2017) where LC$_{50}$ of BA was >1000 mg kg^{-1}.

Currently, chloroacetamide is suggested by ISO 11268-1 (ISO 2012b) and OECD 207 (OECD 1984) as a reference substance in lethality tests with earthworms. Results indicated that BA should not be recommended as a reference substance in lethality tests with *E. andrei* because no acute effects were observed even at 1000 mg/kg.

Collembolans showed a 14-d-LC$_{50}$ of 342.2 (335.7–348.7 mg kg^{-1}) and a 28-d-LC$_{50}$ of 225.6 (220.8–230.4). Similar values were found by Krogh (2009) in OECD soil (28-d-LC50 of 259.0 mg kg^{-1}). Such result confirms the similarity between results obtained in artificial soil with composted coco peat and sphagnum peat, also demonstrated by De Silva and van Gestel (2009) in studies with *E. andrei*. These authors found similar LC$_{50}$ for three pesticides in both soils in earthworms ecotoxicity studies.

In *F. candida* survival, LC$_{50}$ reached 342.2 (335.7–348.7), higher than the value found by Amorim et al. (2012) using LUFA 2.2, which was 139.4 (125.7–153.2). In this case, differences in sand composition of the soils, as content of carbonates or clay, could influence the bioavailability of contaminants, where TAS (5% OM) could bind more boron than LUFA 2.2 (3.9% OM).

Avoidance tests

Although BA is the recommended reference substance for avoidance tests with earthworms in OECD soil, where an avoidance behavior is expected to occur at 750 mg.kg^{-1}, the results found in TAS were controversial (Table 2). After 48 h of exposure, the earthworms from UFSC presented non-avoidance behavior, even at the highest dose (1000 mg kg^{-1}). This test was repeated and the same results were found. In contrast, earthworms from UDESC avoided BA, presenting an EC50 value of 847.4 (811.3–883.5 mg kg^{-1}, 95% CI).

Differences in EC$_{50}$ values could be related to genetic variation among laboratory cultures. Diogo et al. (2007) found differences between laboratory strains of *F. candida* in avoidance behaviour towards pesticide exposure. The authors concluded that avoidance behavior of *F. candida* and other collembolans might be influenced by the existence of highly differentiated intraspecific lineages.

Furthermore, this substance has shown limitations to be used in avoidance tests with soil invertebrates. Amorim et al. (2012) reported that BA spiked in OECD soil was not avoided by *Enchytraeus albidus* (>2000 mg kg^{-1}) neither by *F. candida* (>800 mg kg^{-1}). Bicho et al. (2015) found evidence that BA is neurotoxic to enchytraeids via the GABAergic system mechanism, where it acts as a GABA-associated protein receptor (GABAAR) antagonist possibly causing anesthetic effects. For the oribatid mite *Oppia nitens* and gamasid mite *Hypoaspis aculeifer*, the use of BA as reference substance in avoidance tests is not recommended because the organisms do not avoid it at concentrations that would inhibit reproduction or even kill the organisms (Owojori et al. 2011, 2014).

Reproduction tests

Results of BA exposure on reproduction are shown in Fig. 1. All tests fulfilled the validity criteria proposed by ISO guidelines.

Similar EC$_{50}$ values for reproduction of *E. andrei* (280.2 and 242.5 mg kg^{-1}) were found by the laboratories

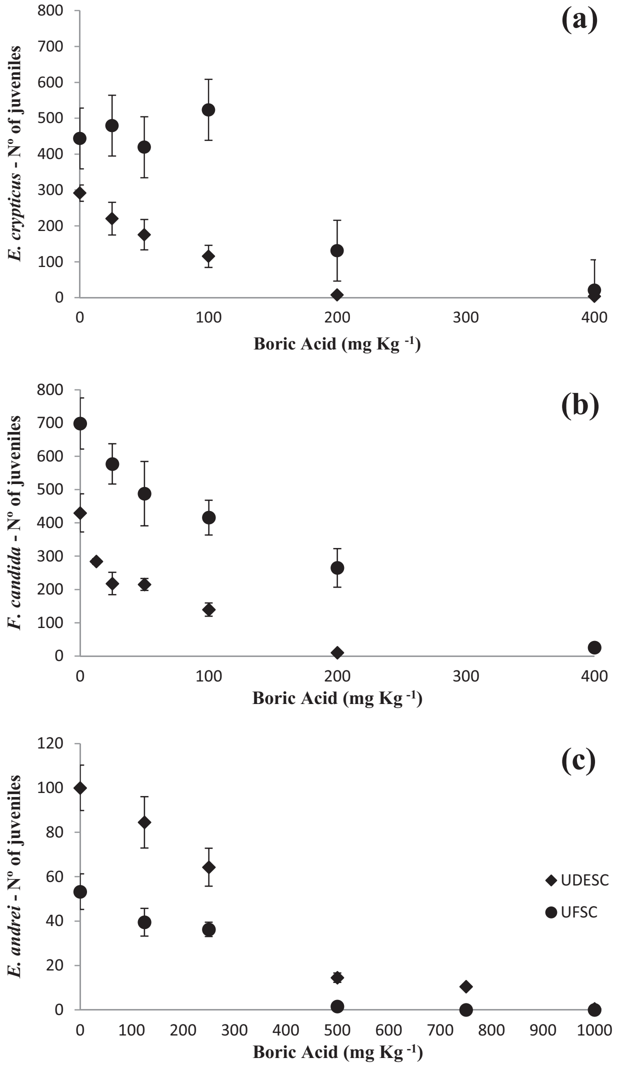

Fig. 1 Effect of BA in **a** *E. crypticus*, **b** *F. candida* and **c** *E. andrei* reproduction tests, showing the number juveniles (average ± standard deviation) for the two laboratories (◆ UDESC and ● UFSC)

(Table 2). In general, BA has shown satisfactory results to be considered as suitable reference substance for the reproduction test with *E. andrei* and *E. fetida*. Becker et al. (2011) reported the EC$_{50}$ of 484 (465–504 mg kg^{-1}, 95% CI) for reproduction of *E. fetida* in OECD soil (10% OM), and EC$_{50}$ of 433 (350–515 mg kg^{-1}, 95% CI) for *E. andrei*, in a test carried out by Stantec, Aquaterra Environmental (2004).

The lower EC$_{50}$ values found in TAS in comparison to OECD soil could be related to the lower organic matter content (5 and 10%, respectively), which represents lower capacity to adsorb and immobilize contaminants. That is the main reason why the reduction in OM content in artificial soils has been proposed (from 10 to 5%), being more realistic to assess the effects of contaminants more similar to natural soils in their possibilities for sorption of contaminants to organic carbon (EPPO 2003). Considering the type of OM, artificial soil prepared with coco peat show similar properties (e.g., water retention capacity) to OECD soil (Garcia 2004). De Silva and van Gestel (2009) found similar results in earthworm's ecotoxicity studies with three pesticides, using artificial soil with composted coco peat and OECD soil, and concluded that composted coco peat is a good substitute to sphagnum peat in artificial soil.

As well as for earthworms, EC$_{50}$ values for reproduction of *E. crypticus* were similar among the laboratories (165.2 and 164.8 mg kg^{-1}) despite data following different models (Table 2). Data fit linear model in UDESC, while a hormesis model was run in UFSC, where the organisms showed a stimulus in reproduction at lower concentrations tested (Fig. 1).

Becker et al. (2011) found EC$_{50}$ values of BA exposure for reproduction of *E. crypticus* and *Enchytraeus luxuriosus* in OECD soil of 220 (208.0–233.0 mg kg^{-1}, 95% CI) and 228 (201.0–259.0 mg kg^{-1}, 95% CI), respectively. In two tests carried out by Amorim et al. (2012), the EC$_{50}$ values for *E. albidus* were 103.5 (96.6–110.9 mg kg^{-1}, 95% CI) and 105.1 (97.4–111.8 mg kg^{-1}, 95% CI) in soil LUFA 2.2. Despite the fact that direct comparison cannot be made among different species, in general, the lower values of EC$_{50}$ found in TAS, in comparison to OECD soil, could be related to its lower OM content, leaving more boron available for the organisms. Moreover, the high organic content of OECD soil and TAS, and their high content of clay, probably bind more boron than LUFA, leaving less of this element available to soil organisms, as discussed by Amorim et al. (2012).

In spite of the similar values found by the laboratories for the reproduction of oligochaete species, reproduction of *F. candida* showed different EC$_{50}$ values (Table 2). In UFSC, EC$_{50}$ was 198.0 (177.6–218.4), more than double of presented by UDESC that was an EC$_{50}$ of 96.8 (82.7–110.9). Two hypothesis could explain such differences. The first one is related to differentiated lineages of *F. candida* between laboratories, as observed by Diogo et al. (2007). Other explanation could be related to differences in the age of exposed organisms. Crouau and Cazes (2003) pointed that one-day-old difference in starting exposure of collembolans in reproduction tests can influence the results for *F. candida*. Current ISO protocol recommends the use of juveniles of 10 to 12 days old.

Conclusions

This study helps to fulfill a gap for tropical tests in general, showing that BA has potential to be used as reference substance for soil invertebrates in tropical artificial soil replacing the current pesticides recommended by some ISO guidelines, especially in reproduction tests. Concerning avoidance tests, additional investigations should be performed with other substances that cause no neurotoxic effects on soil organisms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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