Counting *Enchytraeus crypticus* Juveniles in Chronic Exposures: An Alternative Method for Ecotoxicity Studies Using Tropical Artificial Soil

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Abstract

Soil toxicity tests are commonly applied using *Enchytraeus crypticus* to analyze reproductive outputs. However, the traditional method for counting potworms takes a long time due to the significant number of offspring. This paper compares the conventional total counting of *E. crypticus* juveniles (M1) and an alternative methodology (M2). The proposed methodology (M2) uses a simple random counting method (1/4) for the partial counting of juveniles and total estimation. Chronic bioassays (21 days of exposure) were performed in tropical artificial soil (TAS) using sugarcane vinasse as a hazardous substance. Comparing the final density of juveniles recorded in M1 and M2, no statistical differences were pointed out in either one. Applying analyses based on effective concentration (EC10 and EC50), no statistical differences were identified there either. The t-test showed that there was no statistical difference between the counting methods (M1 and M2) in each treatment (control and dilutions). Moreover, we ran the Tukey test for M1 and M2 methods separately and observed that 100% of the vinasse showed a statistical difference compared to the control treatment in both ($p \leq 0.05$), affirming that independent of the counting method, the ecotoxicological outputs were similar. Therefore, the proposed alternative is a suitable method for bioassay using *E. crypticus* in tropical artificial soil, decreasing to 1/4 the total time required for counting.

Keywords Enchytraeid · Potworms · Counting juveniles · Time optimization · Soil toxicity

Ecotoxicology is a science addressed to understand the effects of natural and anthropogenic agents on biota and its representatives (Newman 2009; Zhou et al. 2018). It is scientifically recognized that biological responses are better tools for predicting the behavior of the stressors towards ecosystems rather than physical and chemical analysis (Zaghloul et al. 2020). Earthworms are the most used organisms in terrestrial bioassessments in Latin America (Niemeyer et al. 2017). In this sense, oligochaetes have played

an important role as bioindicators of pollutant toxicity due to their sensitivity to specific toxicants and certain tolerance to pollution (Chapman 2001; Sivakumar 2015; Castro et al. 2020a; Castro et al. 2020b; Felipe et al. 2020).

Among the Oligochaeta, enchytraeids are important soil fauna components, acting as nutrient-cycling organisms and resulting in better soil porosity and fertility, either by their movement or their fecal deposition (Topoliantz et al. 2000; Jänsch et al. 2005; Rombke et al. 2017). *Enchytraeus crypticus* Westheide and Graefe (1992) showed significant applicability in acute and chronic bioassays due to its sensitivity (Hrdá et al. 2016; Lahive et al. 2019; Da Rocha et al. 2020), especially by downsizing the shorter life-cycle and higher reproduction response for chronic assessments among the enchytraeids family (Castro-Ferreira et al. 2012). The use of standard artificial soil and the inclusion of alternative substrates more abundant in the study area (e.g. coir pith and coconut fiber for tropical regions) is indicated to recover results with more validity in ecotoxicological bioassays (Abbiramy et al. 2012; ABNT 2014).

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The consolidation of ecotoxicology as a scientific subsidy for risk assessments increased the demand for bioassays and required an acceleration in the obtained responses from organisms. As broadly known by soil ecotoxicologists, the enchytraeid reproduction test takes a great investment of time by manually counting the organisms that can reach over a thousand new individuals per replicate (Droge et al. 2006; Bicho et al. 2015; Testa et al. 2020). Matějů et al. (2014) addressed this issue by proposing counting *E. crypticus* juveniles by floating worms from samples, using chemical additives, photographing them, and employing software applications for digital calculation.

Different counting methods were tested and proven in order to better approximate the piece to the whole studied object (DePatta Pillar 1998; Elphick 2008; Chao et al. 2009), and the technique selection is usually considered a cost-effective strategy (Mode et al. 1999). Simple random counting and its variants were broadly applied with reliable performance and low economic demands over the time to estimate population diversity and density (Bourdeau 1953; Goedickemeier et al. 1997; Chu et al. 2018) and have been used in ecological studies (Antoniolli et al. 2006; Henderson 2009).

This paper presents an alternative random counting procedure for soil ecotoxicological outputs obtained by the *E. crypticus* reproduction test to reduce the time spent in the manual counting of juveniles with no extra costs in devices or facilities. To validate this alternative method, we tested the statistical resemblance between the conventional total counting of *E. crypticus* juveniles suggested (M1) and an alternative methodology (M2), that consists of simple random counting (1/4) for the partial counting of juveniles and total estimation. The sugarcane vinasse was used as a contaminant because it is a commonly used subproduct of the sugarcane industry that is usually applied for fertirrigation in countries in tropical zones (Fuess and Garcia 2014; Fuess et al. 2017).

Materials and Methods

Enchytraeus crypticus (Annelida; Oligochaeta; Enchytraeidae) were obtained from the Laboratory of Ecotoxicology at the Center for Water Resources and Environmental Studies (CRHEA) and were maintained at the Aquatic Ecology Laboratory (LEAA), both in the São Carlos School of Engineering, University of São Paulo, Brazil (EESC/USP). Species cultivation was kept in substrate Bacto-Agar medium plates prepared with a sterilized mixture of salt solution containing $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (calcium chloride dihydrate – 29.4 g/L), KCl (potassium chloride – 0.74 g/L), NaHCO_3 (sodium hydrogen carbonate – 8.4 g/L), and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (magnesium sulfate

heptahydrate – 12.3 g/L). The potworms were fed 10 mg of oatmeal two times a week and maintained under $20 \pm 2^\circ\text{C}$ and a 16:8 h light-dark cycle.

Enchytraeus crypticus exposure experiments were based on OECD protocol n. 220 entitled “Enchytraeid Reproduction Test” (OECD 2016). According to the protocol, the determination of adult mortality, the estimate of the number of juveniles, the measure of soil pH, and the moisture content can be assessed over 21 days.

The assays were performed using tropical artificial soil (TAS) of NBR ISO 16,387 (ABNT 2012), also based on the soil recommended by the OECD (1984) protocol, that consists of a mixture of sand, white clay (kaolin), and coconut fiber powder (70:20:10, dry weight). As proposed by the OECD (2016), the water holding capacity was maintained at 60% by the addition of filtered water (control treatment) (USEPA 2002) or dilutions of sugarcane vinasse (liquid substance treatments). Dilutions of sugarcane vinasse in filtered water were assembled in the proportion of 0.1%, 5%, 10%, 50% and 100% (raw vinasse), named as V0.1, V5, V10, V50 and V100, respectively (Botelho et al. 2012). Due to the critical scenario of the COVID-19 pandemic in Brazil, experiments testing red oxisol soil have not been completed, but are recommended in further studies.

After the humidification, 30 g of the tested soil was transferred to cylindrical plastic vessels (6.3 cm high inner diameter 5.8 cm) in each replicate (three replicates were used for each treatment). Then, 10 enchytraeids adult organisms with visible clitellum (length size of approximately 7 mm), checked by visual observation by stereoscope microscope, were collected from cultivation and allocated in the treatments. Subsequently, 10 mg of oatmeal was distributed in all replicates. The test containers were closed, and the time of exposure started. The individuals were exposed for 21 days, and test containers were opened after 7 and 14 days for feeding and adding filtered water when necessary (maintaining the 60% humidity). The containers were kept at $20 \pm 2^\circ\text{C}$ and a 16:8 h light-dark cycle. After 3 weeks, all samples were fixed by adding 40 mL of 96% ethanol and colored with 300 μL of rose Bengal (CAS 632-69-9) solution (1% in ethanol). The coconut fiber was not colored, and therefore, only potworms were highlighted in the samples.

After the potworms had been properly colored, all samples were carefully sieved (150 μm) and washed with tap water for 40 s to separate the enchytraeids from the smaller particles of TAS. Subsequently, the samples were transferred into white trays (36 cm $\times$ 23 cm). In order to remove the remaining material from the sieve, 1 L of tap water was poured against its bottom, and the tray was positioned on a lightbox. All juveniles were visually identified, separated with Pasteur pipette, confirmed with a stereoscope microscope, and the final density recorded to obtain the total number of juveniles per replicate (OECD 2016).

The procedures for washing time, sifting, and adding 1 L of tap water on white trays (36 × 23 cm) were the same performed in the traditional counting method (OECD 2004). The white tray was divided into four fractions, previously marked on the bottom with a permanent pen into four equal quadrants (9 cm × 23 cm), to guide the counting of stained enchytraeids. The next step was based on simple random sampling (Kaur et al. 1996; Gregoire and Affleck 2018). Afterward, each sample was slightly agitated and manually spread using a Pasteur pipette to avoid possible sediment agglomeration in the tray until its whole area could be homogeneously occupied by the sample. After the withdrawal of all adults, a draw was made to select one of the four quadrants to be counted. After counting the juveniles in the selected quadrant, the total number of juveniles was estimated (Eq. 1).

$$TM_{M2} = Ic \times nQ \quad (1)$$

Td = Total density of potworm juveniles estimated;
Ic = number of individuals counted in the drawn quadrant;
nQ = number of quadrants.

To validate the application of the counting method, we applied a two-group comparison analysis (Student's t-test) for each treatment (controls and vinasse dilution), comparing the density recorded in the conventional (M1) and alternative (M2) counting methods. Also, to evaluate if the counting method influences the bioassays analysis, the ANOVA test was applied for each method, comparing the results from the control and vinasse dilution treatments. Statistical tests were performed on Past software, version 3.25 (Hammer et al. 2001) considering a significance level of 95%. Moreover, the effective concentration that affects 10% and 50% of the reproduction rate (EC10 and EC50, respectively) was calculated using R software and packages MASS and DRC (R Core Team 2014). The EC results for both methods were compared by t-test to assess the similarity of the results ($p > 0.05$).

Results and Discussion

Descriptive results from chronic ecotoxicological tests are presented in Fig. 1. In general, the number of *E. crypticus* juveniles estimated in M2 is covered by the standard deviation of the total density obtained in M1. Based on ANOVA results, the treatment that affected the reproduction rate of the individuals compared to the control was the raw sugarcane vinasse V100. Checking the correspondence between M1 and M2, the t-test showed statistical resemblance in the total number of juveniles between the two methods for all treatments (p -value > 0.05 , Table 1).

Fig. 1 Density of *E. crypticus* juveniles at the closure of chronic exposure, using a conventional counting method (M1) and alternative counting method (M2). *Statistical difference with control treatment

Moreover, the conventional statistical analysis in both methods demonstrated similar results and interpretations. As the results followed a normality distribution, ANOVA test was applied, and the results for the M1 and M2 reproduction rate indicates differences between the control and vinasse dilutions (p -value of 3.84×10^{-07} and 1.65×10^{-08} , respectively). In both methods, significant differences in the density of worms between treatments were indicated for V100 (p -value ≤ 0.05), according to the Tukey test. Furthermore, the EC10 and EC50 for M1 and M2 showed a high resemblance: EC10 of 46.35 and 48.14%; and EC50 of 80.39% and 78.25%, respectively. The t-test showed that these values are not statistically different ($p > 0.05$).

Due to the great number of juveniles generated during chronic exposure using *E. crypticus*, the traditional manual counting procedure was extensively laborious, lasting an average of 120 min per replicate. Traunspurger et al. (2009) reported difficulties in obtaining the final number of juveniles per adult in whole sediments using *Caenorhabditis elegans* and highlighted the importance of caution when counting individuals within such extensive periods. Nonetheless, such tests are dependent on the visual capability of each counter, and the size of juveniles is detectable with a naked eye. Some other approaches use stereoscopes to scan the sample (Alves et al. 2015) or a series of washing processes through a sieve to better identify the juveniles (Bicho et al. 2016). Whatever strategy is adopted, the results could be inaccurate by the exhaustion of the researcher, and tests that require a long time to count individuals often cause insecurities about the number raised.

Within the enchytraeid family, when comparing the number of juveniles reproduced per adult individual, *E. crypticus* had a higher reproduction rate than *E. albidus*

Table 1 Comparison between the total density of potworms recovered in control and vinasse dilution treatments by conventional counting method (M1) and alternative counting method (M2) using t-test, considering a confidence interval of 95%

Dilution treatment	Conventional counting method (M1)	Alternative counting method (M2)			Comparison between TD _{M1} and TD _{M2} (t-test <i>p</i> -value)
	Total density of potworms (TD _{M1})	Randomly drawn quadrant	Density of potworms counted in the drawn quadrant (Ic)	Estimated total potworms density (TD _{M2})	
Control	991	3	206	824	0.374
	1020	2	223	892	
	860	1	241	964	
V0.1	823	1	226	904	0.935
	860	2	189	758	
	793	4	206	826	
V5	940	3	210	840	0.959
	875	1	207	828	
	739	4	219	876	
V10	950	3	204	816	0.081
	977	2	193	772	
	878	2	224	894	
V50	742	4	180	720	0.454
	813	3	185	740	
	771	1	198	792	
V100	228	2	50	200	0.323
	210	4	48	194	
	352	3	59	238	

(Castro-Ferreira et al. 2012). This report helped to consolidate the reduction of the test exposure period for this species, exemplifying the necessity of continuous revisions and fair adaptations of bioassessments' demands of time. In cases of an overpopulated sample, rather than gross values, statistical results are a clearer indicator of impact since what appears to be a great number could not necessarily be interpreted as a perturbation when compared with the control situation, which could only be scaled and quantified in statistical comparisons. Applying a less tiresome approach could optimize the counting of all juveniles in the selected area and demonstrate the same statistical difference.

Domene et al. (2011) reported a growing interest in developing an alternative ecotoxicological assessment that overcomes the reproduction as the final result for potworms, as currently some extra analysis can be performed such as gene expression (Gomes et al. 2018), feeding activity (Bart et al. 2018), avoidance behavior (Pflugmacher et al. 2020), and Kinect uptaking (Mendonça et al. 2020). Although these advanced techniques are valuable in bioassessment, some of them need the basal values of effective concentrations generated by the reproduction test to deepen their analysis. Rombke (2003) pointed out the necessity of innovation with the counting method, especially by software image scan, which was accomplished by Matějů et al. (2014) with a

computational image detection of the stained worms and a time savings although high-quality equipment and handling of other chemicals are required. The alternative method proposed in this study provided a reduction in the counting time of juveniles, demanding an average of 30 min per replicate and without additional financial costs.

In conclusion, this study presented an alternative counting method for the counting of *E. crypticus* juveniles in a reproduction test. The counting method suggested by traditional protocols showed no statistical difference from the proposed alternative methodology. Therefore, focusing on obtaining a faster counting method with no additional costs, decreasing to 1/4 of the total time required in traditional counting and ensuring equivalent results, the alternative counting method is an efficient technique to support the counting of *E. crypticus* in reproduction tests, using a representative soil of ecotoxicological bioassays in tropical environments. In addition, bioassays using different types of soils are recommended as well for applying our alternative counting method, and similar results to those observed in this study are expected.

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