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Earthworm coelomocytes as a soil health assessment tool

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Abstract

Different pollutants can disrupt earthworm coelomocytes integrity and functions, and their responses can be applied as biomarkers of sublethal contaminant exposure. In this context, the aim of this study was to develop an *in vitro* protocol for coelomocyte extraction, maintenance and analysis with regard to soil health status and chemical toxicity profile assessments. The extrusion technique was first tested comparing previously depurated (purged stomach content) and non-depurated and resampled earthworms. After testing, earthworms were exposed to different 2,4D and chloroacetamide concentrations for methodology validation. The values of viability were not affected by food restriction since no statistical difference was observed between non-depurated (sample A) and depurated (sample B) organisms. Regarding to cell density, a significant ($p<0;05$) reduction of 22% was observed between non-depurated and depurated organisms, indicating that food restriction may affect cell density. However, the non-depurated resampling did not show a significant reduction, indicating that this assessment may not be affect by resampling of the same organism. For both chemical compounds, no change in cell viability was observed at all assessed concentrations and exposure times. However, for cell density, a mainly time-dependent effect was observed for organisms exposed to chloroacetamide, and concentration-dependent effect for organisms exposed to 2,4D. The proportion of immune system cells was altered, mainly after 24 h, with the increasing of granular amoebocytes proportion. The difference in the proportion of granular amoebocytes in earthworms exposed to 2,4D can be explained by the existence of recognition and elimination mechanisms for this chemical substance. Thus, assessments of pollutant responses with *in vitro* coelomocytes seem to be a powerful tool for ecotoxicological studies.

Keywords: biomarkers; cells; ecotoxicology; *Eisenia andrei*; immune system.

INTRODUCTION

Ecotoxicology, including its assays and risk assessment methodologies, has been gaining global notoriety in the last 20 years (Niva *et al*., 2016). With the main objective of presenting information on the ecological risks of chemical substances in the environment (Xiao *et al*., 2006), terrestrial ecotoxicological trials are still in the consolidation phase, mainly in the southern hemisphere (Niva *et al*., 2016). However, the complexity of the soil ecosystem and its space-time variability present a significant challenge regarding pollution assessments in this environmental compartment, as well as its impacts on edaphic biota (Lima & Brussaard, 2010).

Earthworms have made a notable contribution to terrestrial ecotoxicology (Andréa, 2010; Genázio Pereira *et al.,* 2017; Sanchez-Hernandez, 2006), and are currently widely used to asses environmental pollution impacts in standardized toxicity tests in Europe and the United States, but are still scarcely applied in Brazil when compared to other trophic level organisms (Correia & Moreira, 2010; Niva *et al*., 2016; Sisinno *et al*., 2006).

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Worms from both temperate and tropical regions are considered the most important members of soil fauna, since they are involved in decomposition regulation and nutrient cycling processes, as well as in the modification of physical soil characteristics (Bhadauria & Saxena, 2010; Ferreira, 2015). Several authors suggest that earthworms can be used to assess soil health in the same way that mussels and other molluscs are used in monitoring marine pollution (Blouin *et al.*, 2013; Ferreira, 2015; G. Brown & Domínguez, 2010). Several studies have established the capacity of *Eisenia fetida* (Lock & Janssen, 2001; Neuhauser *et al.,* 1985; Spurgeon & Hopkin, 1995), *Aporrectodea caliginosa* (Khalil *et al*., 1996) and *Rubellus Lumbricus* (Langdon *et al*., 2001) to accurately reflect soil pollutant levels.

The use of biological markers, or biomarkers, measured at the molecular or cellular level have been deemed adequate and sensitive tools for measuring biological effects in environmental quality assessments (Ferreira, 2015; G. Brown & Domínguez, 2010). The selected biomarkers should indicate that organism exposure to pollutants (biomarkers of exposure) and/or the magnitude of the body's response to the pollutant (biomarkers of effect or stress biomarkers) (Shi *et al*., 2017).

Biomarkers concerning subcellular alterations have been assessed in several studies and display adequate correlation with investigated parameters in higher physiological levels, indicating early warning signs concerning environmental pollution (Maleri *et al*., 2008; Yadav, 2016).

Earthworm coelomic fluid, a part of the hydrostatic skeleton, acts as a link between the internal and external environment, plays an important role in homeostasis maintenance, and contains an abundant population of immunocompetent cells, named coelomocytes (Kurek *et al*., 2007). Oligochaete coelomocytes are characterized by marked variability, both quantitative and qualitative, in composition, depending on organism age and physiological condition, being expelled in stress situations (Yadav, 2016). Different pollutants, both organic and inorganic, can disrupt earthworm coelomocyte integrity and functions, and these cellular responses can be applied as sublethal contaminant exposure biomarkers (Diogéne *et al*., 1997).

Earthworm coelomocyte classification is based on granule ultrastructure, color and composition differences, as well as on the behavioral traits, such as adherence and chemotaxis. However, the origin and relationships of the major coelomocyte populations, namely amoebocytes and eleocytes, are not yet fully understood (Cholewa *et al*., 2006; Kurek *et al*., 2007). These cells are involved in numerous cellular and hormonal immunity aspects, the former in nutrient transport and storage, wound healing, cellular defense, phagocytosis, encapsulation and cytotoxicity, and the latter in the secretion of antimicrobial substances (Cholewa *et al*., 2006; Kurek *et al*., 2007; Yadav, 2016).

Both *in vitro* and *in vivo* assays with coelomocytes based on the neutral red uptake (NRU) showed a binominal dose-response curve after exposure of earthworms to series of metals (Cu, Cd , Pb, Ni) (Irizar *et al*., 2015). Parelho *et al*. (2018) also showed cytotoxicity, by the reduction of NRU, on earthworms (*Amynthas gracilis*) exposed to a dairy cattle production soil contaminated with pesticides and metal. This response could be related with alterations in the relative proportion of coelomocytes subpopulations, amoebocytes and eleocytes. However, these types of studies are still incipient.

Regarding pesticides, cytotoxic effects were observed such as reduction in the density of coelomocytes of *Eisenia andrei* after 45 days of exposure to 10 mg kg^{-1} imazalil, and in viability after 15 days of exposure to regular above 0.1 mg kg^{-1}. In addition, changes in the proportion of eleocytes and amoebocytes were observed (Pereira *et al*., 2019).

In view of the above, the aims of this study were, first, to develop an in vitro protocol for extracting, maintaining and analyzing coelomocytes as promising tool for toxicity assessment chemical in a reproducible and cost-effective manner. Secondly, we aimed to understand the viability, density and coelomocytes populations dynamics (eleocytes vs. amoebocytes) after exposure to two substances (chloroacetamide and dichlorophenoxyacetic acid (2,4 D) and their implications as stress biomarkers in soil health assessments.

MATERIAL AND METHODS

Eisenia andrei

Eisenia andrei (red californian earthworm) specimens were obtained from a specialized earthworm trade (Arborium), located in Santa Cruz, Rio de Janeiro, Brazil and acclimated for 30 days in the laboratory before the experiments. The acclimatization was carried out in plastic boxes containing bovine manure as a source of organic matter. The boxes were maintained at 25 ± 2 ° C, with a 12:12 light/dark photoperiod and humidity maintained at 50% field capacity. Healthy adult organisms, weighing at least 300 mg and with a well-developed clitellum were used, as recommended by the ISO 11268-1 (ISO, 2012a) and ISO 11268-2 (ISO, 2012b) standards.

Experimental approach

In a first step, the extrusion technique based on the Eyambe *et al*. (1991) was carried out, where the conditions of depurated and non-depurated earthworms were compared. Depurated worms were maintained for 24 h on filter paper moistened with distilled water to purge their entire stomach contents prior to the cell fluid extrusion assay. Non-depurated worms were removed from the manure and immediately extruded. The possibility of fluid resampling from the same non-depurated individual 24 h after the first collection was also verified. Thus, after the first extrusion procedure, the earthworm returned to the moistened filter paper for another 24 hours and after this period the extrusion procedure was

performed again on the same individuals. This method could permit the monitoring of cellular effects in the same individual for a time period during some long exposure. Twenty replicates were used in all assays.

Coelomic fluid collection

Coelomic fluid was collected using the non-invasive extrusion technique (Eyambe *et al*., 1991). Earthworms were individually transferred to test tubes and 200 µL of the extrusion solution were added, consisting in 5.0% ethanol in brine (NaCl – 0.15 mol L^{-1}) mixed with 7 mmol L^{-1} EDTA and 50 mmol L^{-1} of the mucolytic agent guaiacol glycerol ether (GGE), adjusted to pH 7.3. After 3 min in contact with extrusion solution, 800 µL of LBSS (Lumbricus Balanced Salt Solution – 71 mmol L^{-1} NaCl; 4.7 mmol L^{-1} KCl; 0.4 mmol L^{-1} $MgSO_4.7H_2O$, 0.4 mmol L^{-1} KH_2PO_4; 0,3 mmol L^{-1} $Na_2HPO_4.7H_2O$, 4 mmol L^{-1} $NaHCO_3$ at pH 7.3) were added to each tube (Figure 1). The organisms were then removed alive from the test tubes, as described by the non-invasive method developed by Eyambe *et al*. (1991).

The solution was maintained at 4 °C for 30 min, when 700 µL of the supernatant were discarded. The remaining 300 µL were packed in ice for the cell density, viability and typing analyses.

Coelomocyte density and viability determinations

A total of 30 µL of coelomic fluid were transferred to eppendorf-type microtubes and slowly mixed with 30 µL of trypan blue dye (0.4%). Then, 20 µL were pipetted and transferred to a Neubauer chamber for cell density and viability determinations of total cells according to Kirk (1975). Viability was expressed as percentage of live cells, characterized by stained (non-viable) and non-stained (viable) cells, while (Kirk, 1975). Two Neubauer chambers per organism were prepared. Cell density and viability calculations were carried out according to equations 1 and 2, respectively. Readings were performed under an optical microscope (Olympus CX31) at 400x magnification.

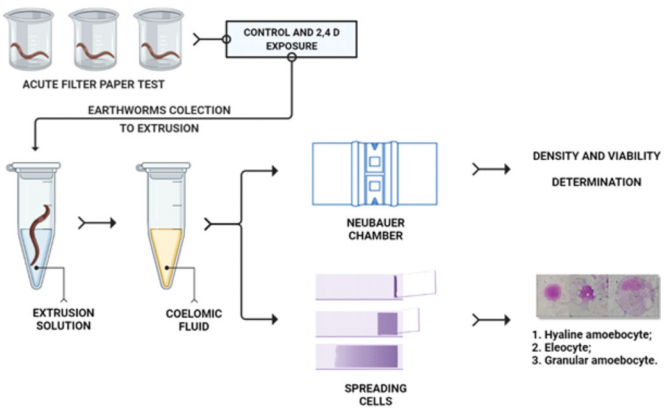

Figure 1. Graphical scheme of extrusion method with earthworms *Eisenia Andrei* and analysis of density, viability and typing of immune cell system.

$$Density = \left[\left(\frac{\sum viable\ cells}{nn.Neubauer\ chamber\ area}\right) x\ dilution\ factor\right] = cells\ mL^{-1} \quad (1)$$

$$Viability = \left[\left(\frac{\sum total\ cells - \sum stained\ cells}{\sum total\ cells}\right) x\ 100\right] = \% \quad (2)$$

Characterization of different cell types

For immune system cell typing, 20 µL of coelomocyte fluid were pipetted onto microscopic slides for analyses. The material was spread using the smear method on each slide. After 24 h drying at room temperature, the slides were fixed in methanol 99% for 10 min, followed by rinsing. Under a distilled water flow and submerged in a 10% Giemsa dye 5% solution for 10 min. Subsequently, they were rinsed again with distilled water, and finally dried at room temperature. Readings were carried out using an optical microscope (Olympus CX31) at 1,000x magnification, with immersion oil to identify eleocytes, hyaline amoebocyte and granular amoebocytes cell types. Two slides per organism were prepared.

In vitro exposure

After the technique assessment, earthworms were exposed to chloroacetamide and dichlorophenoxyacetic acid (2,4 D), in order to validate the extrusion methodology. Exposure was performed through the filter paper contact test, where earthworms are maintained in contact with the contaminants through wet filter paper, according to the OECD 207 standard (1984). Chloroacetamide was selected because it is used as a reference substance in toxicity tests (OECD, 1984) and 2,4 D was selected because it is known to be toxic to earthworms (Correia & Moreira, 2010). Beakers coated with filter paper were contaminated with 1 mL of chloroacetamide and 2,4 D at 0.001, 0.01 and 0.1 mg cm^{-2}, according to the OECD 207 standard (1984). One organism was then transferred to each beaker, using 30 replicates for each concentration, for both substances. The containers were then closed with parafilm and punctured with needles to allow oxygenation. The beakers containing the organisms were maintained in the dark for 24, 48 and 72 h, when three organisms exposed to each concentration were separated to undergo the extrusion procedure for total cell viability and density and typing determinations of immune system cells. Earthworms were classified as dead when they did not respond to gentle mechanical stimuli. Behavioral and pathological anomalies for all assays were reported.

Microscopy and statistical analyses

Microscopic analyses were performed using an Olympus CX31 microscope equipped with an image capture system and software. Results were expressed as means and standard deviation. The results were evaluated by the Shapiro-Wilk test and were compared to the control samples by an ANOVA test followed by Dunnett-test post hoc test ($p < 0.05$) when data followed a normal distribution, and by the Kruskal-Wallis test followed by Dunn's post hoc test when data followed a non-

normal distribution. All statistical tests were performed using the GraphPad Prism version 5 software.

RESULTS AND DISCUSSION

Food restriction

Figure 2 presents viability and cell density results following coelomic fluid extrusion, comparing depurated and non-depurated earthworms, the latter submitted to sequential extrusions.

The values remained on average 87 ± 5% and 7.1 ± 0.9 × 10^6 (cells mL^{-1}) for viability and density, respectively. The comparison between non-depurated (sample A) and depurated (sample B) displayed a 7% decrease, although this difference was not statistically significant. This indicates that food restriction does not have a negative effect on cytotoxicity assessments concerning cell viability and it can be used as bioindicators without compromising the result. On the other hand, the 22% reduction in cell density observed in the present study should be taken into account since a statistical difference was observed.

A new coelomocyte collection was carried out from non-depurated worms (A), 24 h after the first extrusion (sample C), and a decrease of 10 and 16% in viability and cell density, respectively, were observed. Earthworm manipulation, exposure to the extrusion solution and the short time between the collections may have caused stress and, consequently, the decrease of both viability and cell density. From a statistical point of view, the values were significantly different for cell viability, between sample A and C, indicating possible negative effects on cell viability assessments in depurated earthworms within 24 h. Therefore, longer times between collections may result in result stabilization, and should be further investigated. In addition, these data suggest the possibility of using the same earthworm in the same experiment, repeatedly, by applying the extrusion method to assess contaminant bioaccumulation effects at different times and during increased exposure periods, for example. Cellular density values were slightly lower (16%) comparing sample C with sample A, but did not compromise the effects assessment, as no statistical difference was observed between the values.

At least two cell types were identified in a Neubauer chamber, amoebocytes with a transparent cytoplasm, and eleocytes, displaying yellow and brown granules. Eleocytes, also named chloragocytes, were present at significantly

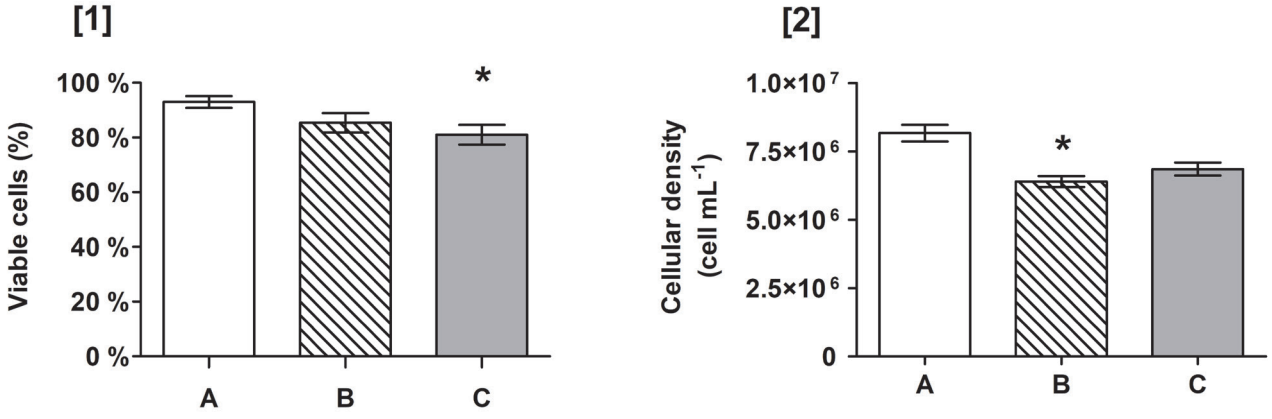

Figure 2. Viability [1] and cell density [2] of depurated earthworms (sample A), non-depurated earthworms (sample B) and depurated earthworms submitted to a new extrusion 24 h after the first coelomic fluid collection (sample C). [*] Represents statistical differences when compared to sample A (p-value <0.05). The error bar in each column represents the standard deviation of the samples.

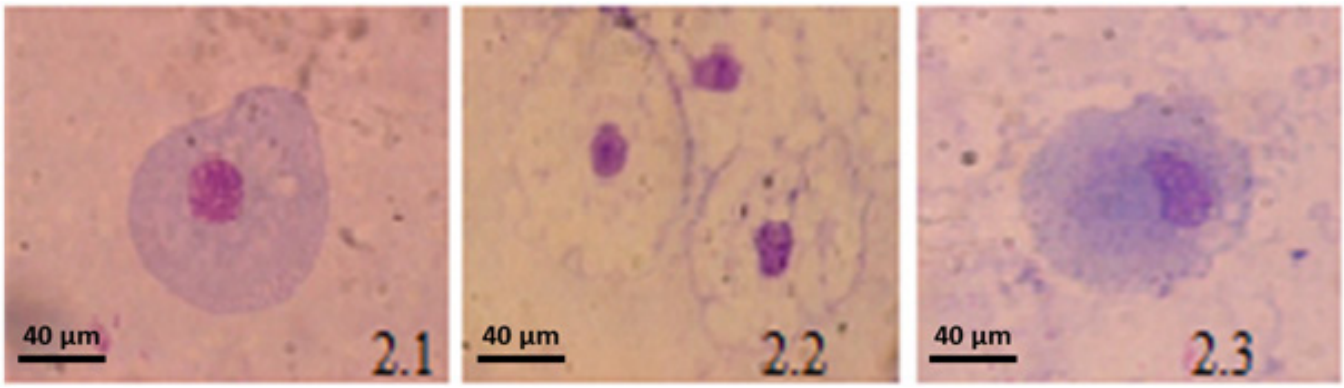

Figure 3. Different types of immune cells visualized on microscopy slides. (2.1) hyaline amoebocyte, (2.2) eleocyte and (2.3) granular amoebocyte.

higher amounts (85%) than amoebocytes in all assays. Thus, they were chosen to determine cell viability. Other cell types were observed in minute amounts, but may represent only coelomocytes in the developmental stages.

The slides containing cells fixed and stained with Giemsa contained three main cell types, namely eleocytes and two amoebocytes, classified as hyaline and granular (Figure 3).

In this method, intact cells are pigmented by the dye. The results corroborate with those reported by Cholewa *et al*. (2006), who identified different types of coelomocytes in different earthworm species by phase contrast/fluorescence microscopy and flow cytometry analyses. Microscope techniques, light microscope (LM), transmission electron microscope (TEM), scanning electron microscope (SEM) were employed to describe and classify coelomocytes of the earthworms according to Adamowicz (2005) and to Kurek *et al*. (2007). In comparison to other techniques, earthworm coelomocyte collection is, thus, a viable, easy and clean technique, making this liquid cell suspension suitable for the development of in vitro tests (Yadav, 2016).

Chloroacetamide

Figure 4 presents the viability and cell density values of organisms exposed to different chloroacetamide concentrations for different periods of time.

Viable cells were not significantly different between chloroacetamide concentrations when compared to the control, for each exposure period. The same trend was observed for cell density at 48 and 72 h. At 24 h, a statistical difference was observed for 0.001 and 0.01 mg cm^{-2} when compared to the respective controls. Oscillations in cell viability between chloroacetamide concentrations and a trend for a gradual decrease during exposure time were observed, evidencing a possible contaminant concentration-dependence. An increase in cell density was observed at the lowest concentration in comparison to the control. However, these values tended to reduce again at higher concentrations. This indicates an increase in immune system activity and possible deleterious effects on cell density caused by increasing chloroacetamide concentrations. At 48 h, the values remained high, including the controls, evidencing possible stress caused by food restriction. In addition, decreased density was also observed with increasing chloroacetamide concentrations, evidencing a concentration and time-dependence behavior. At 24 h, organism death exposed to the highest concentration (0.1 mg cm^{-2}) was observed. This was also observed at 72 h at the second highest concentration (0.01 mg cm^{-2}). The results demonstrate the lethal and acute effect of higher chloroacetamide concentrations. Concerning the cell types in earthworms exposed to chloroacetamide after 24 h, eleocytes were detected in higher amounts (81 ± 2%) compared to the other two cell types, followed by hyaline amoebocytes (17 ± 2%) and granule amoebocytes (2 ± 0.1%) (Figure 5).

The ratio between the amounts of the different cell types was similar after 48 h of exposure, while a decrease in the amount of eleocytes and an increase in the amount of hyaline amoebocytes was observed. However, eleocytes were present in higher amounts (72 ± 2%) compared to hyaline (26 ± 3%) and granular (3 ± 1%) amoebocytes.

According to Kurek *et al.* (2007), eleocytes and amoebocytes are involved distinct immune functions. Eleocytes play a role in the storage of endogenous materials, as well as metal accumulation and tissue detoxification. On the other hand, amoebocytes, according to the same author, play a role in elimination of endogenous materials by phagocytosis and encapsulation, coagulation, wound healing and granuloma formation. Because of these roles, and from the observation of increased amoebocytes compared to eleocytes, it is suggested that after 24 h of exposure, the organism perceives the presence of the contaminant, and begins its elimination process. With increasing exposure

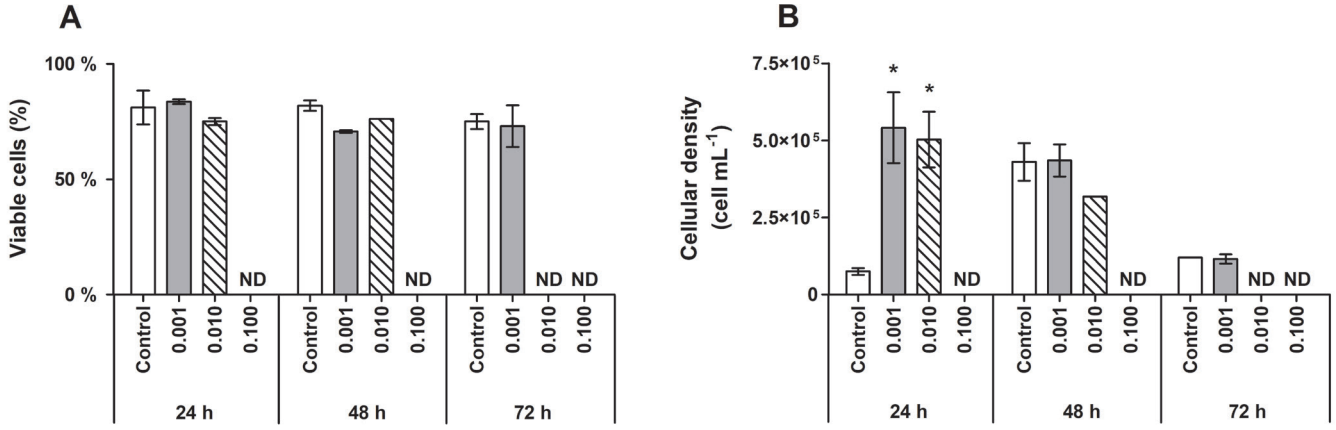

Figure 4. Cellular viability (A) and density (B) in earthworms exposed chloroacetamide at 0.001, 0.01 and 0.1 mg cm^{-2}. [*] Represents statistical differences compared to the control group (p-value <0.05). [ND] Represents non-determined data due to the death of organisms at certain times. The error bar in each column represents the standard deviation of the samples.

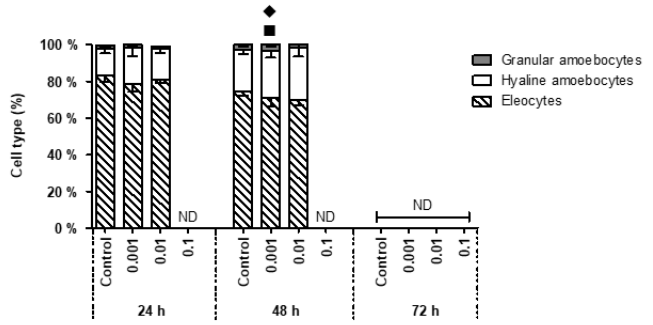

Figure 5. Eleocytes and hyaline and granular amoebocytes in organisms exposed to 0.001, 0.01 and 0.1 mg cm^{-2} chloroacetamide and controls for 72 hours during the acute contact filter paper assay. Letters indicate statistical differences between cell types (♦ - granular amoebocyte, ■ - hyaline amoebocyte and ● - eleocyte) when compared to the same types in the control group (p-value <0.05). [ND] Represents non-determined data due to the death of organisms at certain times. The error bar in each column represents the standard deviation of the samples.

times and concentrations, the intensity of this immune process increases, indicating a contaminant concentration- and time-dependence. In addition, it is possible to infer that the increased amounts of amoebocytes may lead to an inflammatory process due to granuloma formation, justifying 100% organism lethality at 72 h (Cooper *et al*., 1996).

Finally, it is important to note that no dead organisms were found in the control group during the entire assay. Thus, as no living organisms were observed at 72 h, no further extrusion was carried out with the control organisms, due to the absence of groups for comparison.

Dichlorophenoxyacetic acid (2,4 D)

Figure 6 displays cell viability and density for organisms exposed to 2,4 D. The viability values showed a marked reduction at all concentrations. However, a reestablishment of these values was observed after 48 and 72 h exposure. The highest concentration (0.1 mg cm^{-2}) resulted in the death of 100% of the organisms within 48 h of exposure, while the second highest concentration (0.01 mg cm^{-2}) did the same after 72 h of exposure. For cell density, the same pattern as for cell viability was observed during the first 24 h. At 48 h, increased cell density in the exposed organisms was observed at 0.001 and 0.01 mg cm^{-2}, which may be justified by the death of some individuals, leaving only those more resistant and, consequently, with more active immune activity. A marked reduction in cell density was observed as early as 72 h, most likely due to cell depletion of the few remaining individuals. Concerning cell density, a statistical difference was observed for exposure concentrations when compared to their respective controls. Concerning cell viability, the only statistically significant difference was observed for 0.01 mg cm^{-2} at 24 h.

Figure 7 displays the mean values of the cell types of organisms exposed to 2,4 D after 24 and 48 h. In the first 24 h, a greater amount of eleocytes was observed in all samples, with a decrease observed with increasing concentrations. An increase in the proportions of granular amoebocytes compared to eleocytes was also noted. In addition, when compared to the control, samples exposed to 2,4D presented a noteworthy decrease in the amount of hyaline amoebocytes. This pattern was also observed for 48 h, with the exception of 0.1 mg cm^{-2}, due to organism death, making it impossible to collect coelomic fluid. The same was noted for 72 h.

Comparing the results for each assessed substances, the acute lethal effect of chloroacetamide was higher than that of 2,4 D, as it led to organism death at the highest concentration (0.1 mg cm^{-2}) as early as the first exposure period. The death of individuals at the same concentration was only observed for 2,4 D at 48 h after exposure. The death of the individuals exposed to the second highest chloroacetamide concentration was observed 72 h later, in contrast with 2,4 D. For both chemical compounds, no change in cell viability was observed at all assessed concentrations and exposure times, indicating that exposure did not cause cytotoxicity levels. However, for cell density, a mainly time-dependent effect was observed for

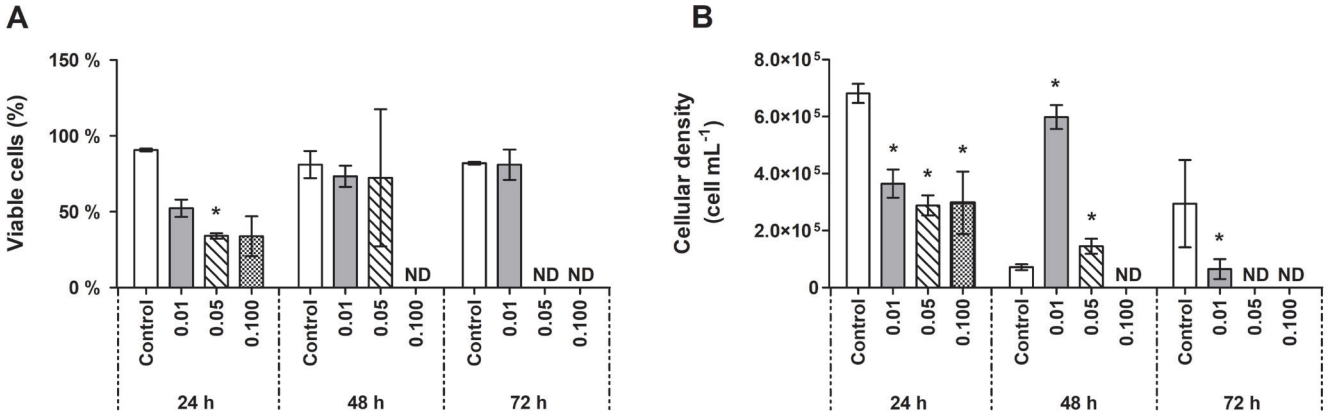

Figure 6. Cellular viability (A) and density (B) in earthworms exposed to 0.001, 0.01 and 0.1 mg cm^{-2} dichlorophenoxyacetic acid. [*] Represents statistical differences compared to the control group (p-value <0.05). [ND] Represents non-determined data due to the death of organisms at certain times. The error bar in each column represents the standard deviation of the samples.

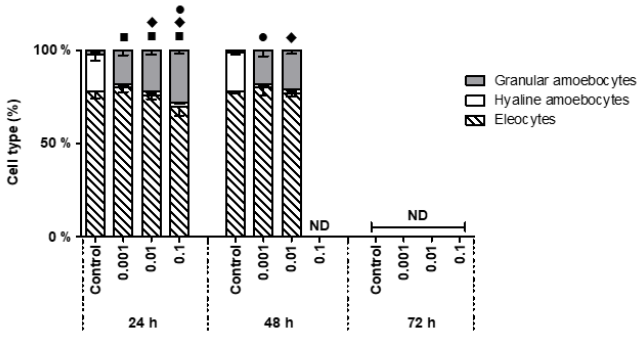

Figure 7**.** Eleocytes and hyaline and granular amoebocytes in organisms exposed to 0.001, 0.01 and 0.1 mg cm^{-2} dichlorophenoxyacetic acid and controls for 72 hours during the acute contact filter paper assay. Letters indicate statistical differences between cell (♦ - granular amoebocyte, ■ - hyaline amoebocyte and ● - eleocyte) when compared to the same types in the control group (p-value <0.05). [ND] Represents non-determined data due to the death of organisms at certain times. The error bar in each column represents the standard deviation of the samples.

organisms exposed to chloroacetamide, and concentration-dependent effect for organisms exposed to 2,4 D only in the first 24 h. In subsequent exposures to 2,4 D, a new cellular balance was established, demonstrating the adaptive capacity of these organisms.

Another striking difference observed between the two exposure tests was the ratio of hyaline and granule amoebocytes in the chloroacetamide and 2,4 D experiments, respectively. Some studies have demonstrated that the number and composition of earthworm coelomocytes are species-specific, changing during the annual cycle (Kurek & Plytycz, 2003) and in response to various adverse factors, including soil pollution (Homa *et al*., 2005). There are indications that amoebocytes derive from the mesenchymal lining (Adamowicz, 2005; Cooper & Stein, 1981), but eleocytes (chloragocytes) cover the coelomic surfaces of the alimentary tract and great blood vessels (Affar *et al*., 1998). Amoebocytes have been classified as multifunctional cells and are involved in immunity reactions, where they recognize and eliminate foreign material, mainly by phagocytosis and encapsulation (Kurek *et al*., 2007) addition, they are involved in coagulation, wound healing (Cooper & Stein, 1981; Yadav, 2016) cytotoxicity, inflammation, graft rejection, granuloma formation and coagulation of coelomic fluid (Cooper *et al*., 1996; Cooper & Stein, 1981). Eleocytes, on the other hand, contain numerous spherical granules, named chloragosomes (Affar *et al*., 1998; Cooper *et al*., 1996; Linthicum *et al*., 1977; Stein *et al*., 1977). Chloragosomes have the ability to store endogenous materials such as glycogen and lipids, as well as pigments, including riboflavin (Koziol *et al*., 2006).

While no studies to date have been published on the effects of chloroacetamide and 2,4 D on the *Eisenia andrei* coelomocytes, to the best of our knowledge, we hypothesized that, because they are storage cells, decreases in viability, whether time-dependent or concentration-dependent, may have been caused by the death process to which the organisms were being subjected to. Earthworm biomarker is indeed valuable and should be fully explored in soil pollution assessment. However, significant challenge still remains in the utilization of earthworm biomarker in real soil pollution assessment.

CONCLUSIONS

Earthworm coelomocyte extraction is, thus, a viable, easy and clean technique, making this liquid cell suspension suitable for the development of *in vitro* tests. The viability and cellular density values for both depurated, non-depurated and resampled organisms did not compromise the effects assessment. The difference in the proportion of granular amoebocytes in earthworms exposed to 2,4 D can be explained by the existence of recognition and elimination mechanisms for this chemical substance. 2,4 D presented a less toxic effect in comparison to chloroacetamide, and a rapid reestablishment in the cellular viability values of living organisms was observed. Thus, assessments of pollutant responses with *in vitro* coelomocytes can provide a powerful tool for ecotoxicological studies.

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