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Ecotoxicological responses of *Eisenia andrei* exposed in field-contaminated soils by sanitary sewage

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ABSTRACT

The disposal of untreated sanitary sewage in the soil has several consequences for human health and leads to environmental risks; thus, it is necessary investigating, monitoring and remediating the affected sites. The aims of the current study are to evaluate ecotoxicological effects on *Eisenia andrei* earthworms exposed to soil subjected to sources of sanitary sewage discharge and to investigate whether prevention values established by the Brazilian legislation for soil quality, associated with the incidence of chemical substances in it, are satisfactory enough to assure the necessary quality for different organisms. Earthworms' behavior, reproduction, acetylcholinesterase activity, catalase, superoxide dismutase and malondialdehyde levels were evaluated. The reproduction and behavior of earthworms exposed to sanitary sewage were adversely affected. Increased superoxide dismutase and catalase activity acted as antioxidant defense mechanism. Significantly increased lipid peroxidation levels and acetylcholinesterase activity inhibition have indicated lipid peroxidation in cell membrane and neurotransmission changes, respectively. Results have confirmed that sanitary sewage induced oxidative stress in earthworms. In addition, based on biochemical data analysis, the integrated biomarker response (IBR) has evidenced different toxicity levels in earthworms between the investigated points. Finally, results have indicated that effluents released into the soil, without proper treatment, lead to contaminant accumulation due to soil saturation and it can hinder different processes and biological development taking place in the soil. In addition, the current study has shown that physical-chemical analyses alone are not enough to assess soil quality, since it is also requires adopting an ecotoxicological approach. Brazilian legislation focused on soil quality must be revised and new guiding values must be proposed.

1. Introduction

The inadequate disposal of sewage, both in raw form and simplified treatment, is related to the lack of sewage systems and leads to environmental compartments contamination (Alaswad, 2020; Mkhinini et al., 2019a). Only 60.2% of urban areas in Brazil have sewage collection systems; of these, only 73.7% have adequate treatment (Brazil, 2019).

The disposal of untreated sanitary effluents in the soil is a common practice in several places worldwide (Karimi et al., 2020; Mkhinini et al., 2019b). The main aggravating factor of inadequate sewage disposal lies on the accumulation of nutrients, pathogens, organic pollutants, emerging contaminants and heavy metals, such as copper (Cu), zinc (Zn) and manganese (Mn) in the soil (Babić et al., 2016; Dominguez-Crespo et al., 2012). The environmental risks of introducing contaminants into the soil are often assessed through physical-chemical analyses, as demanded by the Brazilian legislation (Brazil, 2009).

CONAMA Resolution n. 420 (Brazil, 2009) has set criteria and guide values for the incidence of chemical substances in different soils. According to the aforementioned Resolution, physical, chemical and

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biological analyses must be carried out to assess soil quality, but it does not mention ecotoxicological tests. However, this approach does not provide enough information about the bioavailability of these substances; thus, it is not possible assessing the toxicity of all substances, the effects of contaminants and their interaction with both the soil matrix and different organisms (Bori et al., 2017; Calisi et al., 2013; Nannoni et al., 2011; Zheng et al., 2013).

Therefore, terrestrial ecotoxicology stands out for using bioindicators to assess environmental risks, as well as for using them as biological indicators of contamination (Nannoni et al., 2011; Xu et al., 2013; Zhang et al., 2013). Earthworms stand out among the bioindicators used in terrestrial ecotoxicology tests, since they play important role in nutrient cycling, as well as regulate biological systems and account for the largest soil biomass (Bernard et al., 2015; Nahmani et al., 2007; Uwizeyimana et al., 2017; Zheng et al., 2013). In addition, earthworms belonging to species *Eisenia andrei* are often used in ecotoxicological studies, since they present high reproduction rate, short life cycle, easy maintenance in laboratory environment, as well as satisfactory sensitivity to a wide range of toxic substances (Babić et al., 2016; Dominguez-Crespo et al., 2012; Karimi et al., 2020; Ramires et al., 2020; Shi et al., 2017).

The toxicity of contaminated soils has been extensively evaluated through standard tests, which are mainly used to investigate the effects of such contamination on earthworm mortality and reproduction rates (Bori et al., 2017; Dominguez-Crespo et al., 2012; Nahmani et al., 2007). However, these tests do not provide information at subcellular level, which, in its turn, can help better understanding mechanisms associated with contaminants' toxicity (Bernard et al., 2015; Novais et al., 2011). Thus, oxidative stress biomarker responses play key role in assessing the antioxidant defense system. Among them, one finds the activity of enzymes in the first line of defense such as superoxide dismutase (SOD) and catalase (CAT), oxidative damage measurement, based on lipid peroxidation, as well as acetylcholinesterase (AChE), which is a neurotoxicity biomarker widely used in ecotoxicological studies (Calisi et al., 2013; Mkhinini et al., 2019a; Shi et al., 2017). Biomarkers have been widely used as potential tool to assess changes at the lowest biological organization level in contaminated sites, due to their sensitivity to pollution-associated stress (Uwizeyimana et al., 2017; Xu et al., 2013; Zhang et al., 2013).

However, analyzing and integrating results can be a complex task because earthworms use different biomarker response strategies depending on the contaminant they are subjected to (Beaumelle et al., 2017; Sanchez et al., 2013). Thus, indices capable of summarizing and integrating the responses of several biomarkers, such as the Integrated Biomarker Response Index (IBR), are useful tools adopted in risk assessments conducted in contaminated sites (Beliaeff and Burgeot, 2002), in order to assess earthworms' global responses to the sampled soil.

The high rate of effluent disposal without proper treatment in Brazil and changes in soil quality - mainly with respected to reference values established by the Brazilian legislation - make it necessary conducting further studies involving areas contaminated with this waste. Thus, the aims of the current study were to evaluate changes in behavior, reproduction, antioxidant defense system based on the activity of SOD and CAT enzymes, as well as on the AChE enzyme as neurotoxicity biomarker, and on the oxidative damage of lipid peroxidation in earthworms belonging to species *Eisenia andrei* who were exposed to soil subjected to sanitary sewage discharge sources for a long period-of-time; as well as to assess whether the soil quality values established by law for chemical substances are enough to protect soil organisms. Biomarkers encompassing different biological organization levels (molecular, cellular and physiological) were used to help better understanding the toxic potential of effluents and their mechanism of action in different organisms. Studies like the present one play key role in environmental preservation, as well as help identifying recurrent issues and prioritizing actions to recover areas degraded by sanitary effluents.

2. Materials and methods

2.1. Study site location and description

The study was carried out in an area of approximately 1700 m^2 in Santa Maria County, Central region of Rio Grande do Sul State, southern Brazil (29° 43' 00″ S, 53° 42' 45″ W), which has been used for continuous sanitary sewage deposition for at least 50 years.

The investigated sanitary sewage is directly discharged on the surface of an Alfisol soil, on a daily basis, at the rate of 187 tons a day^{-1}. The launch takes place through four pipes called point sources - PS-31, PS-32, PS-31A and PS-50 (Fig. 1) - and presents features similar to those of domestic sewage. The sewage is treated in a septic tank and subjected to anaerobic filter before it is released in the study site; its release points are close to a stream called Lagoão do Ouro, in Vacacaí-mirim watershed.

The sanitary sewage from point sources is released into the soil and flows through the area until it reaches a place of accumulation in the soil. Then it leaves the area saturated by the drainage channel (DCE) and heads towards the Lagoão do Ouro stream.

2.2. Soil sampling

Soil samples were collected at random, based on criterion 'proximity to the sewage source and drainage channel'. Soil sampling was carried out in August 2016 in order to evaluate the place where the sanitary sewage is dumped, infiltrate and accumulate in the soil, as well as the region where it forms a discharge flow to the drainage channel.

Soil sub-samplings were performed in the 0–15 cm layer, which were homogenized and constituted the sample for each point. The number of sampling points was defined based on equipotential lines in the area, at flow direction heading towards the nearby watercourse. In addition, these points were allocated close to sewage point sources. The first collection point (P1) was located to the North of the PS-50 source (34 m away), in the Central region of the study site. This point was saturated with sewage, presented black color and released odorous gases. The second point (P2) was close to the PS-50 source (35 m away). The third point (P3) was located to the East of the PS-50 source (65 m away). The fourth point (P4) was located shortly after the sanitary sewage discharge in PS-31 (73 m away), PS-31A (40 m away) and PS-32 (77 m away). This point is fully silted and releases odorous gases; it is surrounded by vegetation. The fifth sampled point (P5) is located at the exit of the drainage channel (DCE) (20 m away), downstream the launch points. The last collection point (P6) was close to the study site, to the South of PS-50 (82 m away from it), but it was not influenced by sanitary sewage point sources. These features enabled using the soil at point P6 as control group (Fig. 1).

2.3. Soil physical-chemical analysis

Five (5) kg of soil were collected at each point and sent to the laboratory right after collection, where they were air-dried, sieved in 2 mm mesh and subjected to physical-chemical analysis in order to determine the following traits: pH (1: 1 water: soil), organic matter (OM), cation exchange capacity (CEC), sodium (Na), potassium (K), phosphorus (P), calcium (Ca), magnesium (Mg), copper (Cu), zinc (Zn), manganese (Mn) and iron (Fe), silt, sand and clay. Organic matter (OM) was determined based on wet oxidation with potassium dichromate ($K_2Cr_2O_7$). Cation exchange capacity (CEC) at pH 7.0 (CEC 7.0) was calculated by adding exchangeable cations Ca^{2+}, Mg^{2+}, K^+ and H + Al was estimated (Tedesco et al., 1995). Exchangeable Ca^{2+} and Mg^{2+} contents were extracted using 1 mol L^{-1} potassium chloride solution and determined through GBC atomic absorption spectrophotometry (AAS) (932 AA). Available P and exchangeable K and Na contents were extracted using Mehlich 1 solution (HCl 0.05 mol L^{-1} + H_2SO_4 0.0125 mol L^{-1}) and determined through spectrophotometry and flame photometry,

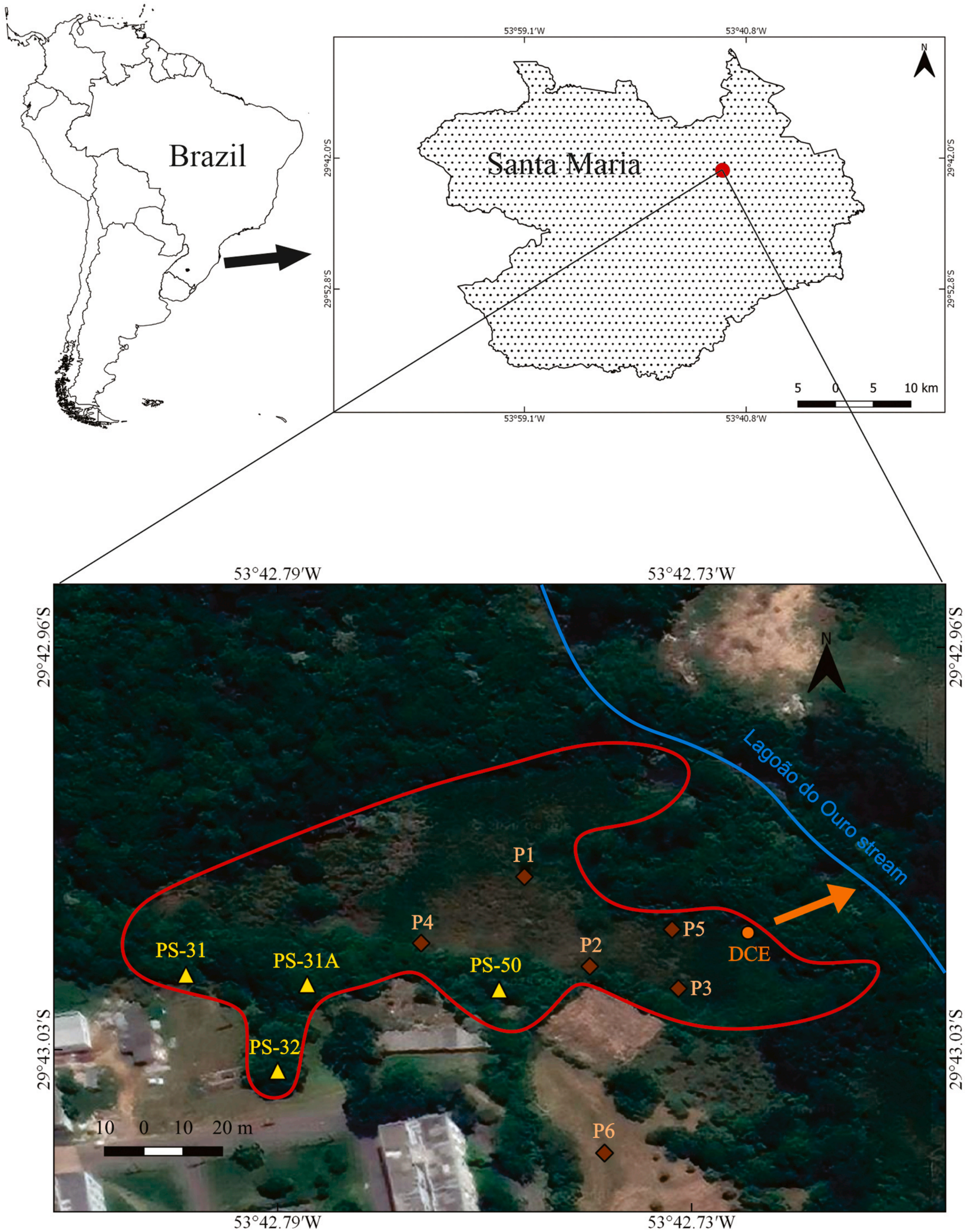

Fig. 1. Release points for soil (PS) and drainage channel (DCE) effluents influencing Lagoão do Ouro stream in Santa Maria County, RS. The delimited portion represents the area under direct influence of liquid effluents that penetrate the soil.

respectively (Tedesco et al., 1995). The H + Al value was estimated based on the equilibrium pH among soil, water and SMP buffering solution at the ratio of 2: 2: 1, m/v/v (SMP Index). The aforementioned estimate was calculated through the equation adopted by CQFS-RS/SC (2016). Particle size distribution was determined based on the pipette method, after dispersion with 0.1 mol L^{-1} sodium hydroxide and mechanical stirring (Embrapa, 1997). Available Cu, Zn, Mn and Fe levels were extracted with EDTA, based on Chaignon et al. (2003).

2.4. *Experimental conditions for ecotoxicological tests*

Ecotoxicological tests were applied to soil samples collected at six different points in the study site, which were subjected (P1, P2, P3, P4 and P5), or not (P6), to sources of sanitary sewage discharge into the soil. Tests were carried out in triplicate and the soil free from sewage source release (P6) was used as control.

2.4.1. *Avoidance test*

The avoidance tests were performed according to ISO guidelines

(ISO, 2008). The tests used 500 g of soil collected at each sampling point, arranged in 2-L experimental units to form a 5-cm soil layer. Adult *E. andrei* earthworms, whose weight ranged from 400 mg to 600 mg, were used in each experimental unit. They were placed in a container coated with filter paper, for 24 h, in order to purge their stomach contents before the experiments. Soil moisture was kept at 60% ± 5% of the maximum water holding capacity (WHC), at 20 ± 2 °C, under natural photoperiod.

Experimental units in the behavioral test were divided into two equal sections by vertically introducing a cardboard partition in the container. One section was added with 250 g of soil deriving from points under the influence of sanitary sewage (P1, P2, P3, P4 and P5), whereas the other section was added with 250 g of soil free from the pollutant (P6). The partition was then removed from the container and 10 earthworms were placed on the line separating the two soils. At the end of 48 h of exposure, the partition was reinserted and earthworms in each experimental unit section were counted (ISO, 2008). Avoidance rate was determined based on Eq. (1):

$$Avoidance = \frac{(C - T)}{N} x \quad 100 \tag{1}$$

where in:

Avoidance: earthworm escape rate (%)
C: number of earthworms in the control soil
T: number of earthworms in the test soil
N: total number of earthworms per treatment

2.4.2. Reproduction test

The treatments and soil conditions during the test are the same as avoidance tests. Each treatment was performed in 4 replicates. Ten adult earthworms (300–500 mg with a clitellum) were selected for reproduction test according to OECD 222 guideline (OECD, 2004). Ten earthworms were exposed to 500 g soils from each experimental unit in the chronical trial. Sterile cow manure at 0.50 g earthworm^{-1} wk^{-1} was supplied as food. At 28 days, ten adult earthworms were removed from each experimental unit; while the cocoons in each container were counted, measured and preserved to incubate in the container for another 28 days without food. After this period, the number of cocoons and juvenile individuals was counted to determine their reproductive success based on the ratio between these two variables (ISO, 2012).

2.5. Biomarkers' response determination

Earthworms were exposed to soil samples for 28 days in order to determine biomarkers' response. Three earthworms were collected in each unit at the 3rd, 7th, 14th and 28th experimental days. Next, they were washed in distilled water and stored in petri dishes on moist paper filter for 24 h (Cheng et al., 2020; Ramires et al., 2020; Zhao et al., 2021). Subsequently, they were frozen in liquid N and stored at −80 °C, until further analysis.

Earthworms were homogenized with sodium phosphate buffer (50 mM and pH 7.2) to determine AChE activity; potassium phosphate buffer (20 mM and pH 7.5) was used to determine SOD and CAT activities and to assess MDA level. Homogenates were centrifuged at 10.000g for 15 min. Supernatants were stored at −20 °C for further biochemical analysis.

CAT activity was measured through spectrophotometry (Nelson and Kiesow, 1972). The test mixture comprised 2 mL of potassium phosphate buffer – TFK (50 Mm), 50 μL of H_2O_2 (0.3 M) and homogenate. Changes in H_2O_2 absorbance within 60 s were measured at 240 nm. CAT activity was expressed as μmol/min/mg protein.

SOD activity was determined based on Misra and Fridovich (1972) and adapted to microplates. The activity of this enzyme is based on its ability to inhibit adrenaline oxidation caused by superoxide radical. Adrenochrome formation was measured at 480 nm. The reaction medium comprised 5, 10, 15 and 20 μL of homogenate curve, 50 mmol L^{-1} of glycine-NaOH (pH 10), at 37 °C for the final volume of 200 μL, and 5 μL of 60 mmol L^{-1} adrenaline (pH 2.0). SOD activity was expressed as IU SOD/mg protein, wherein SOD unit is the amount of enzyme capable of inhibiting oxidation speed by 50%.

Lipid peroxidation levels were estimated through malondialdehyde (MDA) reaction to 2-thiobarbituric acid (TBA) and measured through spectrophotometry (Buege and Aust, 1978). TCA (10%) and thiobarbituric acid (0.67%) were added to the homogenate up to the final volume of 1 mL. The reaction mixture was placed in microcentrifuge tube and incubated at 95 °C for 30 min. After the cooling process was over, the sample was centrifuged at 5000 g for 15 min and its optical density measured through spectrophotometry at 532 nm. Results were expressed as nmol MDA/mg protein.

Homogenate aliquots were incubated with TFK (0.1 M), H_2O and dithionitrobenzoic acid – DTNB (used as chromogen) at 30 °C, for 2 min, in order to determine AChE activity. Next, the reaction was triggered by adding acetylthiocholine to the homogenate; absorbances were determined at 412 nm for 2 min (Ellman et al., 1961). AChE activity was expressed as μmol/min/mg protein.

The protein in the samples was determined through spectrophotometry by using bovine serum albumin as standard. Sample absorbance was measured at 595 nm (Bradford, 1976).

2.6. Integrated Biomarker Response Index (IBR)

Biomarker responses recorded in each point were subjected to the "Integrated Biomarker Response Index" version 2 (IBRv2), described by Beliaeff and Burgeot (2002) and modified by Sanchez et al. (2013). This new IBR version is based on the principle of reference deviation between disturbed and undisturbed state (Sanchez et al., 2013). Parameters calculated for each point were reported in a star chart to represent the reference deviation of each investigated biomarker. The area above 0 represented biomarker induction, whereas the area below 0 indicated its inhibition (Sanchez et al., 2013). The treatment presenting the highest absolute sum of values calculated for the set of evaluated biomarkers indicated the strongest environmental interference in the evaluated biochemical parameters.

2.7. Statistical analysis

Data normality and homogeneity were checked through Shapiro-Wilk and Bartlett tests, respectively. The results were subjected to analysis of variance (ANOVA) followed by the Scott-Knott test for multiple comparisons between samples, or when necessary, to the Kruskall-Wallis test followed by Dunn's post hoc ($P < 0.05$). Data were analyzed using the *R Core Team* language and environment - Version 3.3.1 (R Core Team, 2019).

3. Results

3.1. Soil physical-chemical results

Table 1 shows values recorded for the physical-chemical attributes of the soil evaluated in the current study. P6 has shown higher pH and K concentration than places subjected to sewage influence. In addition, it recorded the lowest Fe and P concentrations. On the other hand, P5, which is located near the drainage channel, recorded the highest clay and silt contents, the highest P, Na and Ca concentrations, as well as the highest CEC. P4 was the sandiest soil, it also presented the highest Cu and Zn concentrations, as well as the lowest MO, Mn, Ca, Mg, Al concentration and CEC.

Table 1
Soil attributes at the six points sampled in the study site, which were subjected (P1, P2, P3, P4 and P5), or not (P6), to sources of sanitary sewage discharge into the soil.

Parameters	P1	P2	P3	P4	P5	P6
Clay (%)	28.3	22.8	21.7	4.6	30.0	19.5
Silt (%)	49.5	35.1	31.5	12.8	60.6	25.9
Sand (%)	22.2	42.2	46.8	82.6	9.4	54.6
pH H_2O	4.1	4.6	4.5	5.1	4.4	5.3
OM (%)	6.7	2.1	2.0	1.9	6.0	3.4
Cu (mg kg^{-1})	6.2	2.0	2.2	7.9	4.8	2.3
Zn (mg kg^{-1})	33.0	5.0	4.1	37.6	32.6	5.3
Fe (mg kg^{-1})	360.7	466.5	498.8	326.2	340.8	208.2
Mn (mg kg^{-1})	39.9	43.5	36.9	6.1	33.4	19.2
P (mg kg^{-1})	47.2	18.5	21.4	30.0	95.2	7.4
K (mg kg^{-1})	46.2	26.4	28.6	83.6	4.4	167.2
Na (mg kg^{-1})	1320.0	110.0	n.d	220.0	3410.0	440.0
Ca (cmolc kg^{-1})	10.3	7.2	7.2	6.2	18.8	14.1
Mg (cmolc kg^{-1})	1.4	1.5	1.4	0.5	2.8	1.6
Al (cmolc kg^{-1})	1.2	2.4	2.9	0.0	0.4	0.1
CEC_{pH7}	18.6	19.6	19.1	8.7	29.8	19.6

n.d: not detected.

3.2. Behavioral testing

Validation criteria set for the avoidance test were met, since there was not earthworm mortality or loss in the experimental units. Based on the avoidance test, soil samples collected at P4, P1 and P3 recorded approximately 80% earthworm evasion (Fig. 2). On the other hand, soil samples collected at P5 and P2 recorded earthworm evasion lower than 60%, whereas samples collected at P6 (control) recorded similar earthworm distribution, since an equitable distribution in both sections of the experimental control soil units was expected.

3.3. Chronical trial

The number of cocoons and juvenile earthworms has decreased in soil under sanitary sewage influence in comparison to the P6 (control) (Table 2). P2, P3, P4 and P5 presented 20 less cocoons in relation than P6, on average. In addition, eggs at point P5 have shown dark color. Earthworms exposed to the soil at P1 and P5 recorded the lowest juvenile rates, in relation to other treatments, 88.9% and 98.9% respectively. The reproductive success of earthworms found in the soil at P1 and P5 was 86% and 96% less than that recorded for the control, respectively. The weight of the cocoons did not show difference among the evaluated soil samples.

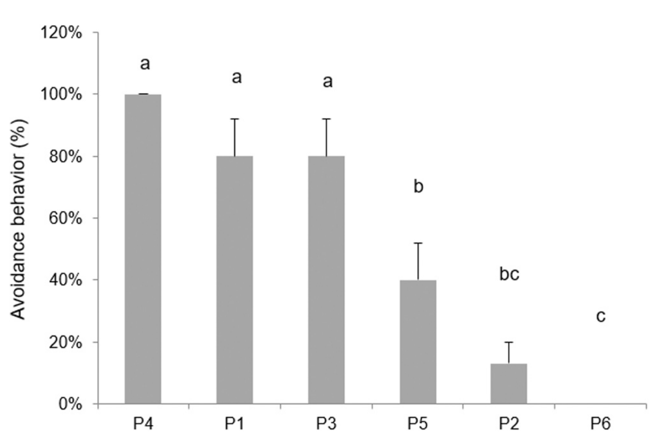

Fig. 2. Avoidance behavior of earthworms exposed to soils subjected (P1, P2, P3, P4 and P5), or not (P6), to the influence of sanitary sewage sources. Columns followed by different letters have shown statistically significant difference in avoidance behavior between points ($P < 0.05$).

Table 2
Reproductive parameters of *Eisenia andrei* exposed to soils from six sampling points subjected (P1, P2, P3, P4 and P5), or not (P6), to the influence of sanitary sewage discharge sources, after 56 days of tests under controlled conditions. Means followed by the different letter in the column show a statistically significant difference between the points ($P < 0.05$).

Point	Number of cocoons	Number of juvenile	Reproductive success	Cocoons weight (mg)
P6	36.0 ± 1.41^a	90.0 ± 8.49^a	2.50 ± 0.14^a	9.8 ± 0.57^a
P1	28.3 ± 1.53^a	9.67 ± 2.52^c	0.34 ± 0.08^b	10.2 ± 0.32^a
P2	21.0 ± 5.57^b	63.0 ± 11.27^b	3.06 ± 0.36^a	9.8 ± 0.43^a
P4	16.0 ± 4.58^b	49.3 ± 20.03^b	3.25 ± 1.36^a	9.9 ± 0.34^a
P3	14.3 ± 12.74^b	42.7 ± 19.43^b	3.76 ± 1.36^a	10.5 ± 0.58^a
P5	9.67 ± 0.58^b	1.0 ± 0.0^c	0.10 ± 0.01^b	9.7 ± 0.44^a

3.4. Biomarkers' response determination

Fig. 3 shows the superoxide dismutase (SOD) activity in earthworms exposed to different soil samples collected from sampling points subjected (P1, P2, P3, P4 and P5), or not (P6), to the influence of sanitary sewage discharge sources for 3, 7, 14 and 28 days. Results showed a significant increase in SOD activity in P1, P2 and P5 on the 3rd day of exposure, compared to control. On the 7th day of exposure, there was a significant reduction in the activity of this enzyme in P2. SOD activity increased significantly in P2 and P3 on the 14th day of exposure. On the 28th day of exposure, there was a significant increase in SOD activity in P1, P2 and P5. On the other hand, there was a significant reduction in P4, compared to the control. P2 was the only point that differed significantly from the respective control over the entire exposure period (3, 7, 14 and 28 days).

Catalase (CAT) activity decreased significantly on the 3rd day of exposure in P1 and P2, compared to the control and also to the other points. On the other hand, on the 14th day of exposure, there was a significant increase in CAT activity at these same points. In P3, there was a significant increase in CAT activity in 3rd day. There was a significant increase in the activity of this enzyme in P1 on the 28th day of exposure, and a significant reduction in P3. There was no difference between treatments on the 7th day of exposure (Fig. 4).

Lipid peroxidation increased significantly at all points (P1, P2, P3, P4, and P5), compared to control, on the 3rd day of exposure. It can also verified that in P1 and P3 there were the highest levels of lipid peroxidation among the points, in this period. There was no significant difference between the points and the control on the 7th and 14th day. On the 28th day of exposure, there was a significant reduction in the levels of lipid peroxidation only in P3 (Fig. 5).

Acetylcholinesterase activity in earthworms exposed to the investigated soils has decreased at all sampling points under the influence of sewage discharge (P1, P2, P3, P4 and P5) in 14th day exposure, as shown in Fig. 6. Also, P1, P2, P3 and P5 showed a significant reduction in AChE in relation to P4. AChE activity showed a significant reduction in P5 when compared to the control and other sampling points on the 3rd day of exposure. After seven days, there was a significant increase in the activity of this enzyme in P3 and P4 when compared to the control. In 28th days of exposure, only P2 showed no significant difference in relation to the control, whereas in P1, P3, P4 and P5 there was a significant reduction in the activity of this enzyme.

3.5. Integrated biomarker response index (IBR)

Based on the IBR approach, a star plot was generated with the scores recorded for the sampling points (Fig. 7). Points near PS-50 recorded the highest scores; P3 and P1 corresponded to the highest stress conditions.

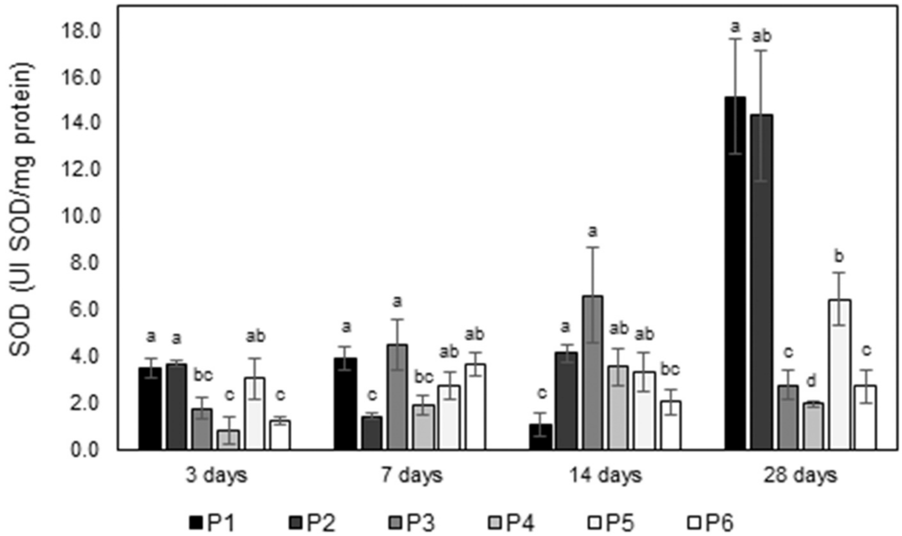

Fig. 3. Superoxide dismutase activity in Eisenia andrei exposed to soil samples collected at six different sampling points subjected (P1, P2, P3, P4 and P5), or not (P6), to the influence of sanitary sewage discharge sources, at the 3rd, 7th, 14th and 28th exposure days. Bars represent the medians, whereas vertical lines represent the standard error (n = 6). Columns followed by different letters have shown statistically significant difference between points on the same sampling day ($P < 0.05$).

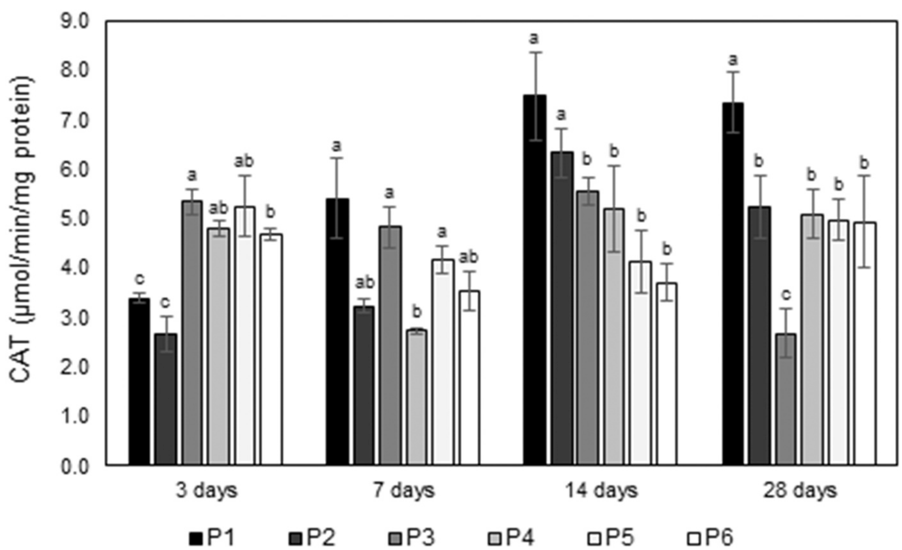

Fig. 4. Catalase activity in Eisenia andrei exposed to soil samples collected at six different sampling points subjected (P1, P2, P3, P4 and P5), or not (P6), to the influence of sanitary sewage discharge sources, at the 3rd, 7th, 14th and 28th exposure days. Bars represent the medians, whereas vertical lines represent the standard error (n = 6). Columns followed by different letters have shown statistically significant difference between points on the same sampling day ($P < 0.05$).

4. Discussion

4.1. Soil physical-chemical properties

Based on the analysis of physical and chemical attributes of the soil, P4 presented high sand content, low organic matter concentrations and therefore low CEC. Sandy soils with low OM contents have low metals adsorption capacity, and it increases the concentration of these elements in the soil solution, as well as their toxicity in organisms (Dominguez-Crespo et al., 2012; Nahmani et al., 2007; Nannoni et al., 2011; Zheng et al., 2013). Heavy metals can accumulate in different organisms and lead to loss of organ functions, as well as remove minerals from their original site and hinder their biological function. Metals can disrupt animals' reproduction, growth, survival, immune and nervous systems (Bernard et al., 2015; Mkhinini et al., 2019b; Uwizeyimana et al., 2017). Although P4 showed the highest concentrations of Cu and Zn, it was also the place with the lowest concentrations of Mn, Ca, Mg and Al. The sandy and low CEC characteristic of this soil may have contributed to the leaching of contaminants present in that location, considering that the lowest IBR value was found in P4. On the other hand, P5 recorded the highest sodium concentration and one of the lowest acidic pH values. Salinity and pH can change the physical-chemical properties and biological processes taking place in the soil, which can decrease earthworms' survival rates (Karimi, 2020; Zheng et al., 2013). The highest CEC observed for P5 was associated with lower sand fraction rate and with higher silt and clay levels, which may present 2:1 clay minerals that have permanent negative charges and high CEC.

Cu and Zn concentrations in the investigated soils were below values reported in the literature about ecotoxicological changes (Brulle et al., 2007; Nahmani et al., 2007; Xiong et al., 2013), as well as below Brazilian standards. Brazilian Resolution N. 420/2009 (Brazil, 2009) presents the guiding soil-quality values for Cu and Zn, as well as sets 60 mg Cu kg^{-1} and 300 mg Zn kg^{-1} as the prevention values (VP) for these metals. It is essential emphasizing that metal concentrations in the

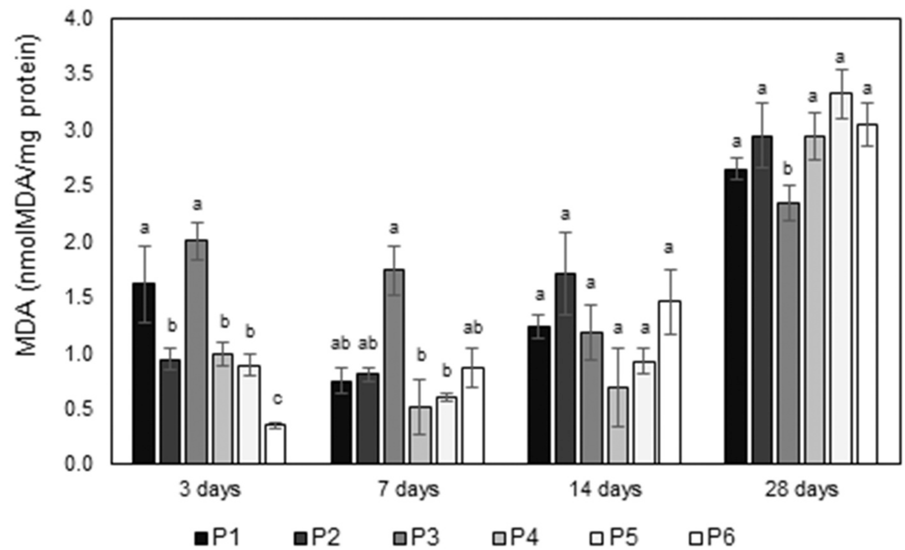

Fig. 5. Malondialdehyde accumulation in Eisenia andrei exposed to soil samples collected at six different sampling points subjected (P1, P2, P3, P4 and P5), or not (P6), to the influence of sanitary sewage discharge sources, at the 3rd, 7th, 14th and 28th exposure days. Bars represent the medians, whereas vertical lines represent the standard error (n = 6). Columns followed by different letters have shown statistically significant difference between points on the same sampling day ($P < 0.05$).

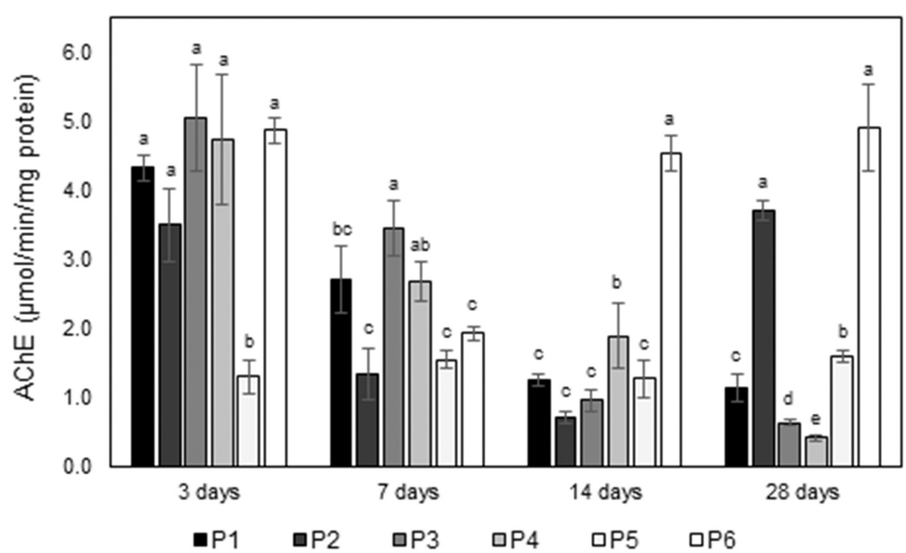

Fig. 6. Acetylcholinesterase activity in Eisenia andrei exposed to soil samples collected at six different sampling points subjected (P1, P2, P3, P4 and P5), or not (P6), to the influence of sanitary sewage discharge sources, at the 3rd, 7th, 14th and 28th exposure days. Bars represent the medians, whereas vertical lines represent the standard error (n = 6). Columns followed by different letters have shown statistically significant difference between points on the same sampling day ($P < 0.05$).

soil were evaluated based on their bioavailable forms (EDTA extractor); therefore, total metal concentrations were likely higher (USEPA method). On the other hand, the composition of sanitary sewage comprises several organic compounds, such as pharmaceuticals and personal care products (Bisognin et al., 2020; Bueno et al., 2012), which were not evaluated in this study and are not foreseen in the current legislation, but which also can cause adverse effects on the earthworm organism (Gillis et al., 2017; Vodyanitskii and Yakovlev, 2016).

4.2. Behavioral testing and chronical trial

P1, P3 and P4 have shown limited soil habitat function (Bori et al., 2017) due to earthworm evasion from contaminated soils to the control. Based on the comparison between results of the avoidance test and those of the IBR index, P1 and P3 were in compliance with the highest IBR values recorded for them; this outcome can be explained by the proximity of these points to the source of PS-50. Point P4, on the other hand, has shown impaired soil habitat function and lower IBR values than all the other analyzed points.

The smaller number of juveniles at P1 and P5 can be attributed to the assumed cost of earthworm tolerance to contaminants. Cocoon production is one of the most sensitive responses observed in chronic toxicity tests - the higher the heavy metal concentrations, the smaller the number of eggs. Reproduction is an energy-intensive process for organisms; therefore, the investment of metabolites in momentary reproduction can change their reproductive or survival potential (Arnold et al., 2008; Nahmani et al., 2007; Žaltauskait and Sodienė, 2014). In addition, earthworm eggs in the soil at P5 were black, and this finding may indicate infections. According to Arnold et al. (2008), this can be a metabolic cost from the development of tolerance to contaminants, i.e., the energy spent to allow earthworms or cocoons to survive has made them significantly susceptible to infections.

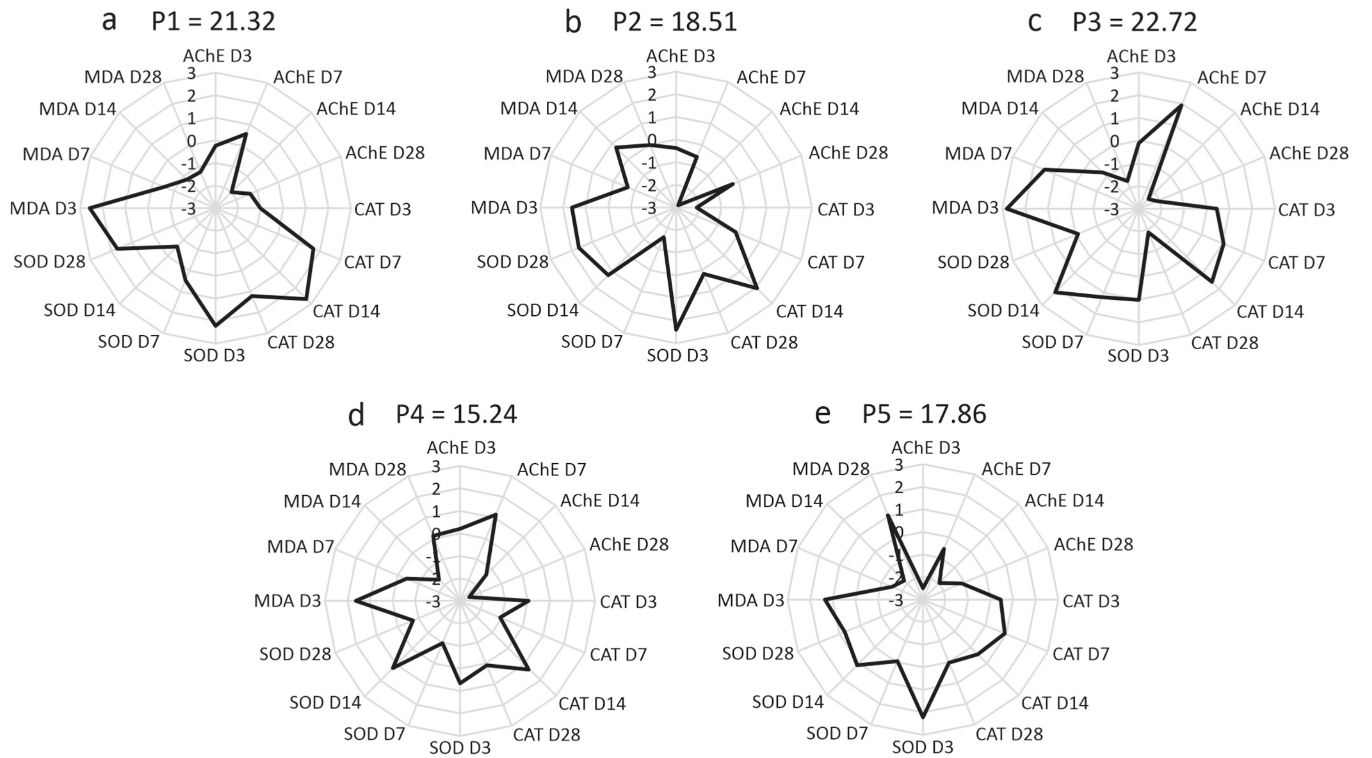

Fig. 7. Integrated biomarker response (IBR) index of sampling points after 3, 7, 14 and 28 days of exposure to domestic sewage (points above zero in the star plot mean activation or increase, whereas the ones below zero mean inhibition or decrease).

4.3. Biomarkers' response

Increased SOD activity can be attributed to enzyme biosynthesis induction by the increased number of superoxide radicals deriving from contaminants in the soil (Zhang et al., 2014). On the other hand, decreased SOD activity may have resulted from the excess of reactive oxygen species (ROS), which were not promptly eliminated and overloaded the antioxidant defense system (Xu et al., 2013; Zhang et al., 2013; Zheng et al., 2013). Antioxidant defense induction is often interpreted as organisms' adaptation to environmental disturbances, whereas the inhibition of it reflects the toxic effect of pollutants that lead to cell damage (Cossu et al., 2000). SOD activity in earthworms exposed to P2 soil was the only biomarker that showed a significant difference throughout the exposure period (3, 7, 14 and 28 days) in relation to their respective control. This fact may be related to the geographical position of P2, which is downstream from point sources and close to the sewage drainage channel, and therefore, receives all influence from the pollutants released in the area.

CAT activity decrease may have happened because its natural antioxidant defense system was saturated by large amounts of the generated superoxide anion, which may have exceeded earthworms' body defense capacity and caused gradual decrease in CAT activity (Mkhinini et al., 2019a; Zhou et al., 2013). This hypothesis corroborates what was described by Vieira et al. (2018) and Moreno et al. (2005). CAT activity is a metabolic adaptation to continuous exposure to contaminated soil, as well as a defense response to the oxidative damage generated by excess of H_2O_2 resulting from SOD activity (Novais et al., 2011; Zhang et al., 2013). In general, the activity of CAT in earthworms exposed to the soil of P1 showed greater significant differences in relation to the control among the periods analyzed. P1 is the place that presents high soil saturation due to the effluents released in the area, presenting a dark color and with bad odors, and this high pollution characteristic can be toxic and influence the enzyme activity responses.

After 3 days of exposure, the inverse relationship of SOD activity and CAT in P1 and P2 can be verified, that is, there was induction of SOD activity and inhibition of CAT activity in the period. SOD is responsible for superoxide anion radical detoxification in a non-radical H_2O_2 product, which, in turn, is eliminated by CAT. CAT degrades H_2O_2 into H_2O and O_2. SOD activity induction at these points is related to its function of detoxifying overproduced superoxide anion radicals induced by soil contamination. On the other hand, the CAT activity inhibition in these places suggests that there was a superoxide anion radicals overproduction generated due to oxidative stress caused in earthworms (Javed et al., 2016; Kono and Fridovich, 1982).

Given the complexity of the analyzed effluent, it is important taking into consideration its effects on AChE activity, since it is used as an environmental biomarker, mainly in polluted environments subjected to different contaminant types. AChE activity activation has been described in studies with earthworms exposed to heavy metals and sewage sludge (Ramires et al., 2020; Romani et al., 2003; Zheng et al., 2013). Based on the AChE activity inhibition observed in earthworms, it can be said that the continuous exposure of these organisms to contaminated soils may have exceeded the cells' capacity to withstand stress and decreased enzyme activity (Mkhinini et al., 2019b; Rodríguez-Seijo et al., 2020).

All soils under the influence of sanitary sewage have shown significant MDA produced, which indicated the incidence of lipid peroxidation in earthworms, on the 3rd day of exposure. Cell membrane peroxidation is significantly associated with uncontrolled ROS increase (Chen et al., 2017; Mkhinini et al., 2019a; Xu et al., 2013). The significant increase in MDA production occurred in first 3 days of earthworms' exposure to soil with sanitary sewage, after which there was no significant difference in relation to the control (except P3 on the 28th day). Similar results were found by Babić et al. (2016), in earthworms (*E. fetida*) exposed in soil contaminated with sewage sludge from a wastewater treatment plant (conventional activated sludge process). The authors observed an increase in lipid peroxidation levels within 96 h of exposure, after this period, there was a decline in levels until it did not differ significantly from the control, at 14 and 28 days of exposure. This fact may be related to earthworm's antioxidant defense mechanism efficiency (Han et al., 2014).

Besides changing biomarkers, oxidative stress has decreased earthworm reproduction viability, since ROS require large energy expenditure by organisms (Žaltauskait and Sodienė, 2014). This phenomenon was mainly observed for earthworms found in the soil from points with changes in the SOD (P1 and P5) and CAT (P1) activity, which also recorded the lowest reproductive success values. Based on the association with oxidative stress enzymes, and by comparing results of both the AChE activity and the behavioral test, it was possible seeing that, at the 28th exposure day, earthworms at P1, P3, P4 and P5 registered inhibition of AChE activity and the highest evasion from the exposed soil after 48 h; this outcome suggests the incidence of contaminants acting on neuromuscular transmission (Novais et al., 2011).

4.4. Integrated biomarker response index (IBR)

Based on the IBR index, sanitary sewage toxicity in the soil was higher at P3 and P1, respectively, if one takes into consideration all periods analyzed in the current study. These results are in compliance with the ones recorded for both points in the avoidance test, as well as with the number of cocoons produced at P3 and the reproductive success observed at P1. Thus, the advantage of toxicity tests based on the evaluation of the soil matrix, instead of the effluent alone, is that the result represents the integrated toxicity of all the adverse factors involved together with the dynamics of the soil matrix. Based on IBR results, toxicity was associated with sewage contaminants, rather than with the organic matter itself. P3 and P4 had similar OM content; however, according to IBR, P3 was the most toxic point, whereas P4 was the least toxic one for earthworms. The highest iron concentration was found in P3, whereas the lowest concentration of it was found in P4; however, the Brazilian legislation (Brazil, 2009) does not set reference values for this parameter. Furthermore, although P3 is not close to any sewage discharge source, it was the place that mostly influenced biomarkers' responses that have indicated that the soil in this place is saturated by contamination.

Therefore, it is extremely important and necessary developing laws focused on establishing safety values for chemical substances in the soil, as well as on addressing the likely toxic effects of these agents on different organisms. Thus, it is essential assessing the effects of sanitary sewage on different organisms living in the soil to enable ecological risk assessments, as well as to help identifying the effects of stressors and toxicity mechanisms on them (Rodríguez-Seijo et al., 2020; Uwizeyimana et al., 2017).

5. Conclusion

Earthworms exposed to contaminated soils have shown oxidative stress and damage in their lipid wall, which were evidenced by changes in CAT, SOD and AChE activity and in MDA accumulation. In addition, earthworms exposed to soils under the influence of sanitary sewage presented changes in population dynamics, which were evidenced by low reproductive success and infeasibility of new organisms resulting from such exposure. Thus, earthworms have the potential to be sensitive indicators of soil contamination by sewage.

The set of ecotoxicological responses evaluated in the present study suggests that the study site is saturated due to constant release of sanitary sewage in it, since these substances are toxic to different organisms living in the soil. IBR was useful to assess the integrated toxicity of all involved factors, since it made the analysis of biochemical results easier. The current study has shown that, although some chemical contaminants were within the limits set by the Brazilian legislation, the evaluated soil was toxic to organisms living in it. Therefore, it is necessary performing ecotoxicological assessments at the time to establish reference values for soil quality attributes. Most of all, it is essential developing laws focused on setting the maximum number possible of chemical substances in the soil.

CRediT authorship contribution statement

Roberta de Moura Lisbôa, Tamiris Rosso Storck and, Barbara Clasen: Study design, experimental execution, all laboratory analysis, data analysis and interpretation and manuscript writing. Roberta de Moura Lisbôa and Tamiris Rosso Storck: Statistical analysis and interpretation and manuscript writing. Tadeu Luis Tiecher and Gustavo Brunetto: Contaminants determination in soil samples. Andressa de Oliveira Silveira, Delmira Wolff and Barbara Clasen: Study conceptualization, data analyses and interpretation and manuscript writing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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