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Ecotoxicity of bioinsecticide spinosad to soil organisms: Commercial formulation *versus* active ingredient

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ABSTRACT

Spintor® (SPIT®) is a commercial formulation of a bioinsecticide with the active ingredient Spinosad (SPIN). Despite the efforts of regulatory agencies, there still is a lack of information regarding short- and long-term exposures to soil-dwellers, as well as effects at environmentally relevant concentrations. This work aimed to evaluate the effects of SPIT® and SPIN, on the oligochaete *Eisenia fetida*, and the arthropod *Folsomia candida*. For this, natural soil was spiked with environmentally relevant concentrations (0.00–1.49 mg of the active ingredient·kg^{-1} of dry soil) to assess avoidance behaviour in *E. fetida* and reproduction effects on both species. Further, in *E. fetida* adults exposed for 2- and 28-day biomarkers of oxidative stress, energetic reserves, neurotoxicity and genotoxicity were evaluated. A significant reduction in juvenile production for *F. candida* was observed for SPIT® at ≥0.66 mg kg^{-1} and SPIN at ≥0.13 mg kg^{-1}, and although no effect was observed on *E. fetida* reproduction, the oligochaeta revealed a tendency to avoid soil spiked with SPIT® at 0.44, 0.66 and 1.49 mg kg^{-1}. The sub-individual responses of *E. fetida* demonstrate genotoxicity upon exposure to SPIT® and SPIN for 2 days. The 2-day exposures of SPIT® and SPIN seem to induce defence mechanisms, and in general, SPIN exerted higher effects than SPIT® on the oligochaetes. Overall, the pro-oxidant performance and energy metabolism pathways were disrupted in both exposures to SPIT® and SPIN. The results suggest that spinosyns-based products can have an impact on soil arthropods *F. candida* and oligochaete's health, possibly affecting their essential functions in terrestrial ecosystems.

1. Introduction

The growth of the human population is a significant factor contributing to the increase in consumption per capita (Viana et al., 2022) leading to the expansion of agricultural areas and increased use of pesticides, to ensure food safety and maximize yields (Gill and Garg, 2014; UNICEF, 2020). However, the use of synthetic pesticides has raised some concerns regarding its impact under the One Health perspective (FAO, 2023), with reflection on impacts on the environment, human health, and other organisms (e.g., non-target species) (Laurent et al., 2021). In response to these concerns, there has been a growing effort to reduce the use of synthetic pesticides and replace them with more sustainable alternatives, such as biopesticides (Ayilara et al., 2023; Samada and Tambunan, 2020). Biopesticides are derived from natural sources and offer a safer alternative for crop protection and management while being more environmentally friendly (Hole et al., 2005; Hubbard et al., 2014). Partially due to their natural sources, they present newer modes of action (Dimock and Ockey, 2017; Fenibo et al., 2021; Parewa et al., 2021), more restricted action mechanisms, and higher affinity (when compared with the synthetic counterparts), affecting primarily target pest and reducing the risk of toxicity to humans and the environment (Rajamani and Negi, 2021; Usta, 2013). The global market for biopesticides use is expected to grow from 6.7 billion USD in 2023 to 13.9 billion USD in 2028 (Market Research Report, 2023), which demonstrates the increasingly high use of these products.

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A commonly used bioinsecticide, that gained interest throughout the last two decades is Spinosad (SPIN). SPIN as an active ingredient is composed of the two tetracyclic-macrolides compounds spinosyns A and D, which are naturally obtained from the fermentation of the soil actinobacterium *Saccharopolyspora spinosa* (Mertz and Yao, 1990). Due to their novel singular structure, they were implemented in crop management to control pests such as lepidopteran larvae, Thysanoptera, and Coleoptera (Mayes et al., 2003). Spinosad is an insecticide that acts both by contact and ingestion and the main mechanism of action against target species is associated with neurotoxic activity. It interacts with gamma-aminobutyric acid (GABA) receptors and activates the nicotinic acetylcholine receptors, altering the nicotinic currents in neuron cell bodies, causing hyperexcitation and disruption of the central nervous system (Christen et al., 2019; Orr et al., 2009; Salgado and Saar, 2004). The commercial formulation of SPIN, Spintor® (SPIT®), is a liquid concentrated suspension containing 480 g L^{-1} (44.0 %) of spinosad, employing propylene glycol (propane-1,2-diol) as the solvent to stabilize and solubilize the mixture in water upon the preparation of the application solution (CortevaAgriscience, 2022; Szajewski, 2009). Research has shown that the half-life of SPIN in soil is 9 to 17 days, under dark and aerobic conditions (Adak and Mukherjee, 2016; Hale and Portwood, 1996). In fact, spinosyns undergo fast degradation through direct photolysis (under neutral and alkaline conditions) in less than one day (FAO, 2008; Lewis et al., 2016). However, given the expansion of agricultural land, the use of excipients in commercial formulations to increase its potency, and the increasing use of these compounds over the years, led to a potential increase of spinosyns in soil ecosystems (Edwards, 1975; Tudi et al., 2021). Moreover, spinosyns, or possibly its metabolites, can sorb to sediments and organic matter, where they appear to be persistent due to the lack of light (half-life of up to 200 days) (Cleveland et al., 2002a; Cleveland et al., 2002b; Monteiro et al., 2019). As it occurs, non-target organisms can be affected through the same toxic route as the target pests, or even novel pathways, since several metabolic pathways and even physiological functions are evolutionarily conserved among biologically close organisms (Diogo et al., 2023b).

Despite the classification as "High alert" in the BPDB (Bio-Pesticides Database) (Lewis et al., 2016), the studies considered by regulatory agencies (e.g., EFSA – European Food Safety Authority) are minimal. Indeed, a lack of assays based on short- and long-term exposures, with relevant environmental concentrations for soil-dwellers, evaluating and reporting data from several endpoints from different levels of biological organization is recognized. For instance, there is no available information on the alteration of metabolic pathways and biochemical parameters in these organisms when exposed to spinosyns. These should be studied since the development of sub-individual changes may lead to organismal changes, deeply affecting the individual's survival and wellness (Ferrario et al., 2018; Spurgeon et al., 2004). For the oligochaeta *Eisenia fetida* exists a NOEC (no observed effect concentration) of 1.79 mg kg^{-1} for reproductive toxicity and a LC_{50} (median lethal concentration) at 14 days of above 458 mg kg^{-1} (Lewis et al., 2016). Moreover, this product has shown toxicity to aquatic species, case in point, *Chironomus riparius* with a reduction of the number of emerged adults and biochemicals alterations (Monteiro et al., 2019) and *Daphnia magna*, with impairment of reproduction parameters (Duchet et al., 2010). Other studies have shown an impact on beneficial arthropods, like *Apis mellifera*, that state a LC_{50} of 7.34 mg L^{-1} for an acute feeding assay (24 h), and a significant decrease of acetylcholinesterase activity (Biondi et al., 2012). In contrast, there is not any registered data for collembola *Folsomia candida*, despite being an important soil arthropod that contributes to nutrient mobilization, promotion of decomposition processes, and other important ecological functions (Fountain and Hopkin, 2005). The negative impact of agrochemicals on the health and wellness of soil species leads to a loss of ecosystem functions performed by these organisms (e.g., carbon transformation, nutrient cycling, and soil structure maintenance), decreasing soil health and its economic value (Baweja et al., 2020; Kibblewhite et al., 2008).

The present study aimed to: i) assess the avoidance behaviour of *E. fetida* after 2 days of exposure to the active ingredient Spinosad (SPIN) and to the commercial formulation Spintor® (SPIT®); ii) evaluate the reproductive performance of *E. fetida* and *F. candida* (springtail), under contaminated soil with SPIT® and SPIN; iii) perform short- and long-term (2 and 28 days) exposure of *E. fetida* with SPIT® and SPIN, in order to assess effects at sub-individual level – neurotransmission, antioxidant defences, lipid peroxidation, and energy-related metabolism, genotoxicity (DNA damage in coelomocytes). A principal component analysis (PCA) and biomarker response index (IBRv2) were also applied to comprehensively assess the sensitivity of earthworms; and iv) perform a comparison between the commercial formulation (SPIT®) and active ingredient (SPIN), to understand the intrinsic role of spinosyns and possible effects of the excipients added to SPIT®.

2. Material and methods

2.1. Test soil

Natural soil from the topsoil layer was collected in an open field in Vairão (Vila do Conde, Porto, Portugal). This soil has not had any prior use of agrochemicals, throughout the last thirty years (Ganilho et al., 2022). Before using for ecotoxicological assays, the natural soil was defaunated by performing one cycle of freezing (at −20 °C) and drying (at 40 °C) and sieved with a 4 mm mesh size (Martins et al., 2024). The physical and chemical parameters of natural soil were determined by Ganilho et al. (2022): $pH_{H2O} = 6.46 \pm 0.02$; $pH_{Kcl} = 5.39 \pm 0.32$; electrical conductivity (EC) = 0.77 ± 0.045; organic matter (% OM) = 5.21 ± 0.15 and the soil water holding capacity (WHC_{max}) = 43 ± 0.15.

A standard artificial soil was prepared following the recommendation of the standard guideline (OECD, 2016), composed of a mixture of 70 % sand, 20 % kaolin, 10 % sphagnum peat, and 0.30 g kg^{-1} of $CaCO_3$ to adjust the pH value at 6.0 ± 0.5. This artificial soil was only used to validate the ecotoxicological assays, according to the recommendations of OECD standards (OECD, 2016).

2.2. Test organisms (Eisenia fetida and Folsomia candida) and culture conditions

The earthworm *E. fetida* (Savigny, 1826) was used as a test organism. The earthworms were obtained from laboratory cultures maintained in controlled environmental conditions at a temperature of 20 ± 2 °C and a photoperiod of $16h^{L}$: $8h^{D}$. They were kept in plastic boxes filled with a medium consisting of a 1:1 mixture of peat and autoclaved horse manure. The medium was moistened with deionized water. Once a week, the organisms were fed with oatmeal hydrated with deionized water. Under internationally recognized standards (ISO, 2023), only adult earthworms with well-developed clitellum (only for reproduction test) and body mass ranging from 300 to 600 mg were used in the assays. Before the experiments, the organisms underwent acclimation for a minimum of 24 h in plastic containers containing the test soil, in a similar condition to the culture maintenance.

The soil arthropods *F. candida* (Willem, 1902) obtained from laboratory cultures (same conditions as described for earthworms) were maintained in plastic containers filled with a culture medium consisting of moistened plaster mixed with activated charcoal in a 10:1 ratio (w:w). The cultures were fed with granulated dry yeast and water, twice a week. Test organisms aged between 9 and 12 days were obtained from synchronized cultures.

2.3. Insecticides and soil contamination

Spintor® (SPIT®) (Qalcova™active), is a liquid commercial formulation of the insecticide spinosad containing 480 g L^{-1} of the active ingredient and was acquired from Lusosem®. Spinosad (Spinosad

PESTANAL®, analytical standard) (SPIN) active ingredient (a.i.) was obtained from Sigma-Aldrich® (CAS Number 168316-95-8) with a purity of 99.4 %. To test SPIT®'s ecotoxicity, a range of concentrations was defined, considering the maximum recommended application dose (250 mL ha^{-1}), according to the supplier's guidelines (CortevaAgriscience, 2022), as well as considering the environmental relevance and presence of this bioinsecticide in different soil compartments (Bueno and Cunha, 2020; Lefkaditis et al., 2017; Pur and Tunaz, 2022). The amount of active ingredient required to achieve the maximum recommended application dose was determined based on the total area of the pots (0.011 m^2) used in plant assays, and the amount of soil in these pots (200 g of dry soil), since the recommended application is per ha (Soares et al., 2023). Considering this, the application dose corresponds to 0.66 mg kg^{-1} of $soil_{dw}$ (dw = dry weight), and it was used to define a range of concentrations to test (factor 1.5×): 0 (non-contaminated moistened soil - CTL), 0.13, 0.20, 0.29, 0.44, 0.66, 0.99, and 1.49 mg of a.i. per kg of dry soil. All assays (avoidance, short-term, and reproduction/long-term assays) used the same concentrations for both compounds, SPIT® and SPIN, and represent nominal concentrations. The solutions were prepared in deionized water to adjust the soil moisture content to 50 ± 5 % of its water holding capacity (WHC), according to the ISO standards (ISO, 2008, 2012, 2014, 2023).

For SPIT® exposures, these concentrations were prepared from a stock solution of Spintor® (480 mg L^{-1}) diluted using deionized water, considering 480 g L^{-1} of the active ingredient spinosad (information in commercial formulation).

2.4. Ecotoxicological assessment

2.4.1. Avoidance assay with Eisenia fetida

The avoidance assays with *E. fetida* were performed in rectangular (1380 cm^3) plastic boxes with perforated lids (Antunes et al., 2008; Bouguerra et al., 2019; ISO, 2008). Five replicates, with ten adult earthworms each, per concentration, including control (CTL), were defined. A plastic divider was used to separate the boxes into two equal parts, one compartment was filled with 200 g of moistened contaminated natural soil (0.13, 0.20, 0.29, 0.44, 0.66, 0.99, and 1.49 mg of a.i. per kg of dry soil) and the second compartment was filled with the same quantity of non-contaminated moistened soil (CTL) (Antunes et al., 2008; Bouguerra et al., 2019; ISO, 2008). In each replicate adult earthworms were placed on the separating line after removing the divider, allowing them to freely move between the two compartments of the boxes. Two "dual-control" tests using non-contaminated moistened natural soil and OECD soil (artificial soil) were conducted at the same time to confirm that earthworms were distributed randomly (the ratio of worms should be within the range 40–60 %) (ISO, 2008). This intends to verify if the earthworms do not exhibit a biased behaviour. Throughout the assay period (48 h), the boxes were kept in controlled conditions, similar to those adopted during the culture conditions.

At the end of exposure, the plastic divider was placed again in the centre of the boxes and the number of earthworms on each side was counted (if the organisms were in the middle line, it was considered 0.5 for each side), and the avoidance percentage was determined using the following equation (ISO, 2008):

$$Avoidance\ (\%) = \frac{C - T}{N} * 100$$

where, C – number of organisms observed in the control soil; T – number of organisms observed in the contaminated soil; and N – total number of organisms per replicate.

2.4.2. Short-term exposure with Eisenia fetida

To evaluate the sub-lethal effects of SPIT® and SPIN on several sub-individual responses of *E. fetida* upon a short-term exposure (2 days), the ISO protocol 11268-1 for acute toxicity was followed (ISO, 2012), with some adaptations, i.e., 2 days instead of 7-day exposure to mimic the avoidance assay. We considered the 2 days' time-mark for short-term exposure since it reflects the initial adaptative responses of the organisms in a scenario in which the compounds have not suffered much degradation. For this purpose, ten organisms were exposed in plastic test containers (1470 cm^3) covered with a perforated lid and containing 500 g of moistened soil (prepared using the range of concentration previously described for SPIT® and SPIN). Three replicates, with ten organisms each, were made for all bioinsecticides concentrations and control. The assay was maintained in controlled conditions (temperature: 20 ± 2 °C; photoperiod of $16h^L$: $8h^D$). After the exposure period, the earthworms were separated: 5 organisms per replicate were randomly chosen for genotoxicity assessment and left to depurate overnight to perform comet assay, and the remaining 5 were frozen in liquid nitrogen and kept at −80 °C for posterior analysis of biochemical biomarkers.

2.4.3. Reproduction (long-term) assay with Eisenia fetida

The impact of SPIN and SPIT® on *E. fetida* reproductive activity was assessed following the methodology described in the standard protocol ISO 11268-2 (ISO, 2023), for 56 days. The exposure of *E. fetida* was performed in plastic test containers (1470 cm^3) covered with a perforated lid and filled with 500 g of moistened contaminated soil and non-contaminated soil (CTL). Five replicates with 10 earthworms in each bioinsecticide concentration and control were used and maintained in controlled conditions of temperature (20 ± 2 °C) and photoperiod ($16h^L$:$8h^D$). An extra control with five replicates of non-contaminated moistened OECD artificial soil was also made to validate the assay, following the recommendation of standard protocol (ISO, 2023). The assay was monitored weekly to adjust the soil moisture and to feed the earthworms with approximately 5 g of defaunated horse manure in each replicate (ISO, 2023).

After 28 days of exposure, the adult earthworms were removed from the test containers, as defined in the guideline (ISO, 2023). The soil containing cocoons and juveniles was left undisturbed to complete the 56 days of exposure, according to the guideline (ISO, 2023). The adult earthworms (exposed for 28 days to SPIT® or SPIN), represented a long-term exposure. Five earthworms from each replicate were left to depurate overnight to perform comet assay (genotoxicity evaluation); the remaining were frozen with liquid nitrogen and stored at −80 °C for posterior biochemical biomarkers assessment.

On day 56, the number of juveniles in each replicate was recorded. The assays fulfilled the validity criteria (ISO, 2023), meaning that the adult mortality was not superior to 10 %, and the number of juveniles was ≥30 juveniles for each replicate in the controls (OECD and natural soil) by the end of the 56 days. Furthermore, the coefficient of variation of reproduction of ≤30 % was respected (ISO, 2023).

2.4.4. Reproduction assay with Folsomia candida

The potential toxicity of SPIT® and SPIN on the reproductive output of the soil arthropod *F. candida* was also assessed, following ISO protocol 11267 (ISO, 2014). The experimental design included 5 replicates per bioinsecticide concentration and non-contaminated soil (CTL), with 10 organisms each. To each vessel was added 30 g of dry soil, and the exposure period was 28 days. During this time, the organisms were fed twice per week with approximately 2 mg of granulated dry yeast (Fermipan®, Touch - Com. Import. Export. E Representação, LDA), and moisture adjusted with dH_2O. At the end of the assay, each vessel was filled with tap water and carefully stirred to allow the collembola to float on the surface. A few drops of dark ink were added to enhance the contrast of the white individuals (Bouguerra et al., 2016). Pictures of each replicate were obtained using a digital camera, for posterior counting of organisms with the ImageJ software (http://imagej.nih.gov/ij/). The exposure conditions were the same as described above for *E. fetida* cultures. The assay was validated since the control met the required criteria – the adult's mortality was inferior to 20 % (Table S1 – supplementary material), a minimum of 100 juveniles per replicate was

verified, and the coefficient of variability was no higher than 30 % (ISO, 2014).

2.5. Biomarkers assessment in Eisenia fetida

2.5.1. Biochemical biomarkers

For the analysis of the enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRed), and glutathione-S-transferases (GSTs), and the levels of glutathione (GSH), substances reactive to thiobarbituric acid (TBARS), and glycogen (GLY), two individuals per replicate were homogenized in 4 mL of ice-cold phosphate buffer (50 mM, pH 7.0) with Triton X-100 (0.1 %), using a mechanical homogenizer (Yellow^{line} DI 18 basic). The samples were centrifuged at 15,000*g* for 10 min at 4 °C in a refrigerated centrifuge (Eppendorf 5810R), to obtain the supernatant fraction, which was divided into aliquots for further analysis of each parameter. According to the methodology described by Bradford (1976), the total protein content was quantified spectrophotometrically (wavelength 595 nm) in microplates, using ɣ-globulin (1 mg mL^{-1}) as a standard. All biochemical biomarkers were then expressed per milligram of protein. All spectrophotometric measurements were performed in a microplate reader Thermo Scientific, model Multiskan GO, version 1.00.40, with SkanIt Software 3.2.

The determination of total SOD activity was performed following the Flohé methodology (Flohe and Otting, 1984), and the enzymatic activity was expressed in units per minute per milligram of protein (a unit of SOD is defined as the quantity of necessary enzyme to inhibit the reduction rate of cytochrome *c* by 50 %). CAT activity was quantified according to (Aebi, 1984) and the number of moles of H_2O_2 consumed per minute per milligram of protein was used to express enzymatic activity. Following Flohé and Günzler (1984) methods, the total activity of GPx was quantified based on the degradation of NADPH (using cumene hydroperoxide), and the enzymatic activity was expressed as millimoles of NADPH per minute per milligram of protein. To determine GRed activity, the methodology developed by Carlberg and Mannervik (1985) was followed and GRed enzymatic activity was expressed as micromoles of NADPH oxidized per minute, per milligram of protein. The quantification of GSH content was performed based on (Diogo et al., 2023a) and expressed in μM per milligram of protein. According to Habig et al. (1974), GSTs activity was measured and expressed as millimoles of thioeter generated per minute per milligram of protein. The quantification of TBARS levels was made according to Buege and Aust (1978) and the results expressed as millimole of malondialdehyde (MDA) equivalents per milligram of protein. The determination of glycogen content was achieved following Lo et al. (1970) methodology.

To calculate lipidic content, one organism per replicate was homogenized in 5 mL of a mixture of chloroform and methanol at 2:1 and centrifuged at 1000*g* for 10 min. The supernatant was used to quantify the total lipidic content following Folch et al. (1957). The results were expressed in % of total lipids content, based on the weight of the organism.

For lactate dehydrogenase (LDH) activity determinations, one earthworm per replicate was homogenized in 2 mL of ice-cold TRIS buffer (0.1 M, pH 7.2) and centrifuged at 3300*g* for 3 min at 4 °C. The resulting supernatants were collected and used for enzymatic analysis. The determination of LDH activity was performed following the method of Vassault (1983) and was expressed in millimoles of β-NADH oxidized per minute per milligram of protein.

To determine acetylcholinesterase (AChE) activity, one organism per replicate was homogenized in 2 mL of ice-cold phosphate buffer (0.1 M, pH 7.2) and centrifuged at 3300*g* for 5 min at 4 °C. The determination of AChE activity followed a protocol established by Ellman et al. (1961) and its activity was expressed as moles per minute per milligram of protein.

2.5.2. Genotoxicity evaluation

At the end of the short-term (2 days) and long-term (28 days) exposures, five adults of *E. fetida* from each replicate were depurated overnight in plastic containers with moistened paper, to clear the digestive tube. For the evaluation of genotoxic effects, the alkaline comet assay technique was used to quantify the impacts on the DNA integrity of the coelomocytes of *E. fetida*. The extruded coelomocytes suspension was added to microscope slides prepared with agarose, and put in a basic lysis solution; upon lysis ending, slides were washed with a phosphate buffer and subjected to an electrophoresis (0.7 V/cm and 300 mA). The slides were then washed with a neutralization buffer and submerged in absolute ethanol (for more detailed information *see* Fernandes et al. (2020), Lourenco et al. (2012), and Reinecke and Reinecke (2004)). At the end of the visual scoring of DNA, DNA damage was evaluated in arbitrary units (AU), according to Fernandes et al. (2020), using the equation:

$$AU = \frac{(0*N0) + (1*N1) + (2*N2) + (3*N3) + (4*N4)}{number\ of\ analyzed\ comets} * 100$$

where *N*0, *N*1, *N*2, *N*3 and *N*4 are the numbers of comets in classes 0, 1, 2, 3 and 4, respectively (visual representation of the DNA damage classes in the coelomocytes of *E. fetida* is representing in Fernandes et al., 2020). All ratings were made blind and by the same person.

2.6. Statistical analysis

To analyse the avoidance behaviour of earthworms upon the increasing concentration of SPIT® and SPIN, the Fisher's exact test was conducted using GraphPad software (available at http://graphpad.com/quickcalcs/contingency1.cfm). A one-tailed test was employed for the treatments. The null hypothesis assumed that 50 % of the test organisms would remain in the contaminated soil, with no organisms leaving this side of the boxes, indicating no avoidance behaviour. For the analysis of the dual-controls (soil spiked with water on both sides of the boxes), a two-tailed test was used, assuming an equal distribution of individuals on both sides.

Results obtained from the reproduction assays, comet assays, and the quantification of biochemical biomarkers were checked for normality by the Shapiro-Wilk test and for homogeneity of variances by Levene's test. For all data, the analysis was done individually for each compound (SPIT® and SPIN) and per time of exposure (2 days or 28 days) using the software GraphPad Prism 9.4.1. The data that followed a parametric distribution was analysed by One-Way ANOVA followed by Dunnett's multiple comparison test; the data that followed a non-parametric distribution was analysed with the Kruskal-Wallis test followed by Dunn's multiple comparison test, to determine significant differences between the control and each tested concentration of SPIN or SPIT®. Additionally, using the nonlinear least squares regression model, the EC_X values for reproductive output and the corresponding confidence limits ($CI_{95\%}$) were calculated using the software StatSoft Statistica v8. A significance level of $\alpha = 0.05$ was considered.

For an integrated analysis of the sub-individual parameters of *E. fetida*, a Principal Component Analysis (PCA) was performed to include both exposure times with SPIT® and SPIN and explore the relationship between them; the data was log normalised and PCA was performed with the average of each biochemical parameter for each treatment, using the covariance matrix (note: using data of individual replicates led to similar results). The statistical analysis and graphs aforementioned were made using GraphPad Prism 9.4.1.

The Integrated Biomarker Response version 2 (IBRv2) index was performed to integrate the responses of all analysed biomarkers to evaluate SPIT® or SPIN effects upon short- or long-term exposure. The calculation for IBRv2 was made according to Beliaeff and Burgeot (2002), with the update described by Sanchez et al. (2013) and Diogo et al. (2023a). Star plots were used to represent the IBRv2 data,

depicting each biomarker's deviation in relation to the control (0 mg kg^{-1} of $soil_{dw}$). Biomarker induction is represented by the area up from 0, and biomarker inhibition by the area down from 0. Star plots and IBRv2 values were performed using Microsoft Excel software.

3. Results

3.1. Individual responses in ecotoxicological bioassays

The avoidance assay outputs for *E. fetida* are shown in Fig. 1. The assay with SPIT® revealed significant avoidance from the contaminated soil at the concentrations 0.44 ($p = 0.002$), 0.66 ($p = 0.006$) and 1.49 ($p = 0.007$) mg SPIT® kg^{-1} of $soil_{dw}$. Therefore, a maximum of 66.0 % of avoidance of *E. fetida* to SPIT® was recorded. No observed effect concentration (NOEC) and LOEC (lowest observed effect concentration) values for avoidance were obtained based on ANOVA results ($F_{[7, 32]} = 2.818$ and $p = 0.021$), respectively being, 0.29 and 0.44 mg SPIT® kg^{-1} of $soil_{dw}$. For SPIN, no significant avoidance was registered.

The results from individual responses on reproduction outputs for *E. fetida* and *F. candida*, with SPIT® and SPIN are shown in Fig. 2. For *E. fetida* no significant differences between the treatments and the control groups were recorded after exposure to SPIT® ($F_{[7, 32]} = 3.063$; $p = 0.014$) and SPIN ($H_{[7]} = 1.114$; $p = 0.993$). Regarding *F. candida*, there was a significant decrease in the number of produced juveniles in the highest concentrations of SPIT® ($F_{[7, 32]} = 12.60$; $p < 0.001$), recording values of 0.44, and 0.66 mg kg^{-1} for NOEC and LOEC respectively (Fig. 2). Furthermore, these findings enable us to discern the reproductive toxicity of SPIT® in *F. candida*. They presented effective concentrations value for EC_{10}, EC_{20}, and EC_{50} with 95 % confidence intervals of 0.15 [0.08–0.52], 0.30 [0–0.30], and 0.98 [0.62–1.35] mg kg^{-1} of $soil_{dw}$, respectively. For the SPIN assay, all the treatments demonstrated a significant decrease in juveniles production ($F_{[7, 32]} = 6.559$; $p < 0.001$), recording a NOEC <0.13 mg kg^{-1} of $soil_{dw}$ and a LOEC of ≤0.13 mg kg^{-1} of $soil_{dw}$. However, it was not possible to calculate the EC_X values for the *F. candida* reproduction assay with SPIN, due to the ineffectiveness to fit a dose-response curve to the data. Given the high mortality rate of the adult individuals of *F. candida* exposed to SPIT® and SPIN (Table S1 – supplementary material), at the end of the reproduction assay, it was not possible to assess sub-individual effects, due to the insufficient biological samples for quantification, taking into account the sensibility of the analysis methods.

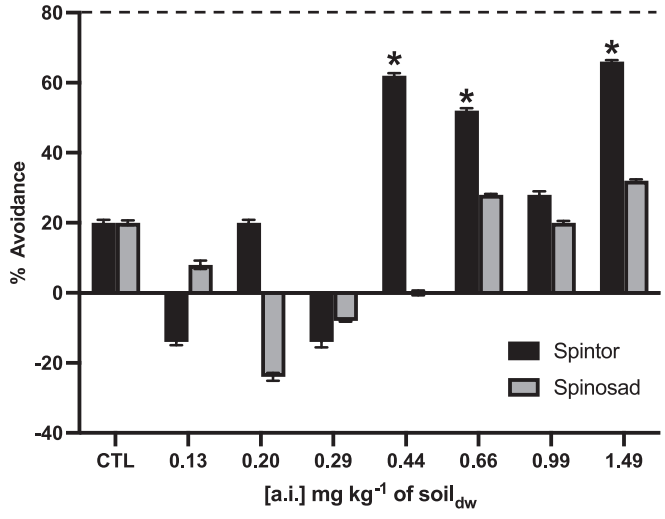

Fig. 1. Avoidance response (%) of *Eisenia fetida*, exposed to soil spiked with Spintor® (SPIT®) and Spinosad (SPIN), after 2 d of exposure. Data are expressed as mean ± standard error (SE). * Stands for a significantly higher percentage of organisms on the control side than on the tested side ($p < 0.05$).

3.2. Sub-individual responses upon short-term and long-term exposure of *E. fetida* to Spintor®

The results from the sub-individual biomarkers upon the short-term exposure (2 days) and long-term exposure (28 days) of the earthworms *E. fetida* to SPIT® are presented in Fig. 3. Significant dose-dependent alterations in the activities and levels of the cellular traits investigated were observed in *E. fetida* upon acute (2 days) exposure to SPIT®, though with some inconsistencies. In addition, the results exhibited that short-term exposure to SPIT® may cause DNA damage in earthworms even at lowest concentration tested (0.13 mg a.i. kg^{-1} $soil_{dw}$) (a.i. = active ingredient) (Fig. 3). In the 28-day exposure, despite the significant but inconsistent effects observed in SOD, GPx, GRed, GSTs, LDH, and AChE activities, TBARS levels and glycogen and lipids contents, no significant effects in DNA were recorded.

Regarding the biochemical biomarkers of antioxidant defence, no significant changes in CAT activity were registered in short-term and long-term exposed organisms, while SOD activity significantly increased in the concentration 0.20 mg kg^{-1} of $soil_{dw}$ in both exposure periods, and at 0.66 and 1.49 mg kg^{-1} of $soil_{dw}$ for the 2-day exposure (Fig. 3). The organisms exposed for 2 days revealed significant induction of GPx activity in the highest concentrations (up to 0.99 mg kg^{-1} of $soil_{dw}$). Additionally, the earthworms exposed for 28 days had significant stimulation of GPx activity in the concentrations 0.20, 0.66, and 1.49 mg kg^{-1} of $soil_{dw}$. On the other hand, the GRed activity of *E. fetida* was significantly upraised at the lowest (0.13 and 0.20 mg kg^{-1} of $soil_{dw}$) and highest (0.99 and 1.49 mg kg^{-1} of $soil_{dw}$) concentrations after 2 days of exposure (Fig. 3). Contrarily, after 28 days of exposure, GRed activity returned to baseline levels, and a significant decrease was recorded only at 0.99 mg kg^{-1} of $soil_{dw}$ dose. GSH content showed a significant increase after 2 days of exposure at the lowest concentrations (0.13 and 0.20 mg kg^{-1} of $soil_{dw}$), while no significant differences were detected in the 28-day exposure.

In addition, the organisms exposed in short-term revealed significant induction of GSTs in the concentrations 0.20, 0.29, 0.44 and 1.49 mg kg^{-1} of $soil_{dw}$. In the long-term exposure there was also significant stimulation in the 0.20 and 0.99 mg kg^{-1} of $soil_{dw}$ concentrations. Besides, the significant rise in TBARS levels in both exposure periods mainly in the 0.20 mg kg^{-1} of $soil_{dw}$ concentration and in 1.49 mg kg^{-1} of $soil_{dw}$ after 28-day exposure, demonstrated the potential occurrence of lipid peroxidation in earthworms.

Regarding the possible effects of SPIT® on the energy reserves of *E. fetida* (Fig. 3), no adverse impact was observed in glycogen and lipids contents after 2-day exposure. Contrariwise, for the 28-day exposure significant increase in the glycogen content in 1.49 mg kg^{-1} of $soil_{dw}$, and lipidic percentage in 0.44 and 0.99 mg kg^{-1} of $soil_{dw}$ concentrations were found. On the other side, the LDH activity showed significant induction in both exposure periods (0.99 and 1.49 mg kg^{-1} of $soil_{dw}$ for short-term exposure and 0.20, 0.29, 0.66, 0.99 and 1.49 mg kg^{-1} of $soil_{dw}$ for long-term exposure) (Fig. 3).

The results from the acetylcholinesterase activity (Fig. 3) inferred that in the 2-day exposure, a significant decrease was observed in 0.44 mg kg^{-1} of $soil_{dw}$ concentration. However, no significant changes in AChE activity in the long-term exposed organisms were recorded.

The exposure to SPIT® cause DNA damage in all tested concentrations, after 2 days of exposure, while at 28 days exposure DNA damage was not observed (Fig. 3; Table S2).

3.3. Sub-individual responses upon short-term and long-term exposure of *E. fetida* to spinosad

The results from the sub-individual biomarkers upon short-term and long-term exposures (28 days) to SPIN are presented in Fig. 4.

Regarding the biomarkers of antioxidant defence, SOD and CAT activities showed a significant rise in the highest concentration (1.49 mg kg^{-1} of $soil_{dw}$) in both exposure periods, and for 2-day exposure SOD

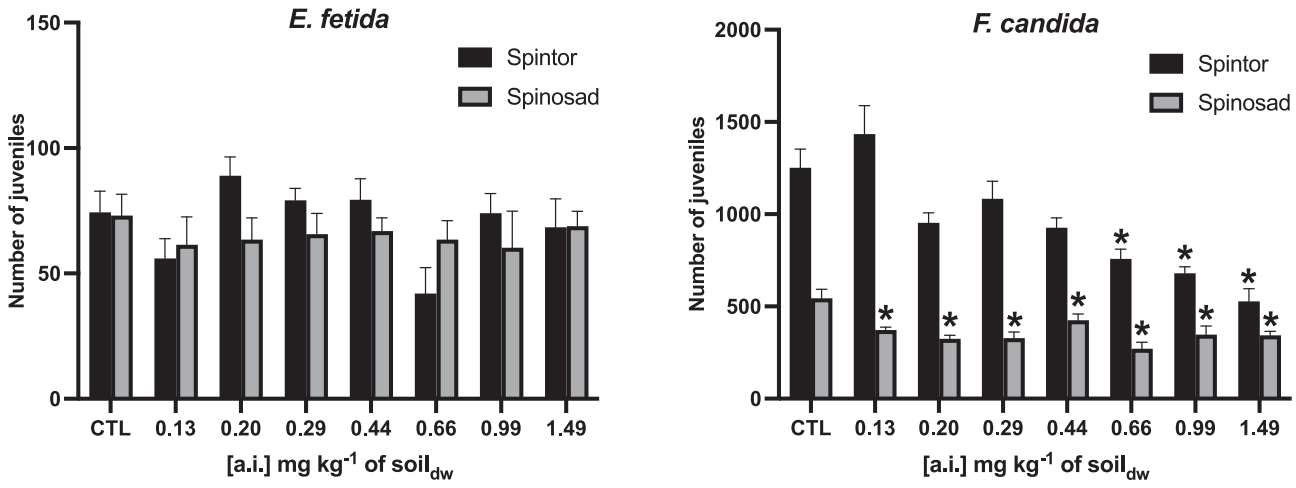

Fig. 2. Reproductive output (number of juveniles) of *Eisenia fetida* (10 organisms exposed per replicate) and *Folsomia candida* (10 organisms exposed per replicate) after exposure to 56 and 28 days of exposure, respectively, to soil spiked with Spintor® (SPIT®) and Spinosad (SPIN). Data are expressed as mean ± standard error (SE). * Stands for significant differences, when compared to control ($p < 0.05$).

activity was also increased at low concentrations tested (0.20–0.66 mg kg^{-1} of $soil_{dw}$) (Fig. 4). Additionally, CAT activity was enhanced in the concentrations 0.99 and 1.49 mg kg^{-1} of $soil_{dw}$ for both exposure periods. Similarly, GPx activity was also enhanced in concentration 1.49 mg kg^{-1} of $soil_{dw}$ for both exposure periods. More, the 2-day exposure led to an increment of GPx activity, in the concentrations 0.20–1.49 mg kg^{-1} of $soil_{dw}$. GRed activity was boosted in the lowest concentration (0.13 mg kg^{-1} of $soil_{dw}$) and in the highest concentrations (from 0.66 mg kg^{-1} of $soil_{dw}$ upwards) in the 2-day exposure, as well as, in the concentration 1.49 mg kg^{-1} of $soil_{dw}$ after 28 days (Fig. 4). The non-enzymatic antioxidant defence, GSH content (Fig. 4), demonstrated a significant increase in the 2-day exposure for the 0.66 and 0.99 mg SPIN kg^{-1} of $soil_{dw}$. Contrarily, a significant decrease of GSH content was detected in the 28-day exposure, only in the 0.20 mg kg^{-1} of $soil_{dw}$ concentration (Fig. 4). The activity of GSTs was found to be significantly enhanced in the 2-day exposure in the concentrations equal or superior to 0.44 mg kg^{-1} of $soil_{dw}$ and similarly upraised in the 28-day exposure period only in the lowest (0.13 mg kg^{-1} of $soil_{dw}$) and highest (1.49 mg kg^{-1} of $soil_{dw}$) concentrations tested (Fig. 4). Even though the antioxidant defences were activated to combat free radicals (ROS) (Fig. 4) the TBARS levels were significantly increased by SPIN, but only in the concentrations 0.44 and 0.66 mg $kg^{-1}$of $soil_{dw}$, for 28 days and 2 days exposure periods, respectively.

The potential changes in energetic reserves upon SPIN exposure were evaluated with the measurement of glycogen and lipids content (Fig. 4), which revealed no significant changes upon 28 days of exposure, for both energetic reserves. However, there was a significant overstimulation in glycogen levels after 2 days of exposure in the 0.66 and 1.49 mg kg^{-1} of $soil_{dw}$ concentrations. The anaerobic energetic metabolism was evaluated by LDH activity (Fig. 4), for which there was a significant increase at 2 days for the concentrations 0.44 and 1.49 mg kg^{-1} of $soil_{dw}$. Similarly, at 28 days there was also an increase in the highest concentrations from 0.66 mg kg^{-1} of $soil_{dw}$ upwards.

Increased AChE activity (Fig. 4) is apparent post 2-day exposure from the 0.29 mg kg^{-1} $soil_{dw}$ onward, while following 28 days of exposure significantly increased AChE activity was evident at the 0.66 and 1.49 mg kg^{-1} $soil_{dw}$ concentrations.

The SPIN exposure caused DNA damage in all tested concentrations, after 2 days of exposure, however, at 28 days no significant alterations in DNA damage were observed.

3.4. Principal component analysis (PCA)

A principal component analysis (PCA) was performed (Fig. 5) to get a comprehensive view of the biomarkers' results encompassing both exposure time and the different concentrations of the tested compounds.

Distinct differentiations in biomarker results based on exposure time were evident for both compounds. The biomarkers that most responded upon exposure to SPIT® at 2 days were SOD, GRed and LDH activity, GSH, and LIP content, while after 28 days was CAT, GPx, and GSTs activities and TBARS levels (Fig. 5-A). In the case of SPIN exposure, at 2-day CAT, GRed, GSTs, and LDH activities, TBARS, GLY, and GSH content responded to the stimulus at higher concentrations, whereas, at 28 days only SOD, GPx, and AChE activities demonstrated a positive correlation although only with the highest concentrations (at 0.99 and 1.49 mg kg^{-1} $soil_{dw}$) (Fig. 5-B).

3.5. Integrated biomarker response v2 (IBRv2)

The results for the IBRv2 index and the star plots for biomarker responses are shown in Fig. 6 (A)–(D). Regarding the 2-day exposure with SPIT® (Fig. 6-A), the highest score obtained corresponds to the highest concentration tested (1.49 mg kg^{-1} of $soil_{dw}$), and the most relevant responses to explain this were the results of GPx, TBARS, LDH, SOD, Comet, GSTs and GRed. Moreover, regardless of the concentration, the most relevant responses are based on the increase of Comet (DNA damage) and SOD, GRed, and GSH activities (highest disturbances relative to control) and the decrease of AChE activity and GLY content (values below the control). Upon exposure for 28 days with SPIT® (Fig. 6-B), the highest IBRv2 score stands for the second lowest concentration (0.20 mg kg^{-1} of $soil_{dw}$), and the results of GPx, TBARS, GSTs, and SOD were the most relevant to explain this score. In this case, the most relevant responses, no matter the concentration, were from an increase in LDH, GPx, and GSTs activities, and a decrease in AChE activity, GSH content, and GRed activity (lowest values relative to the control).

For the exposure to SPIN, in the 2-day exposure scenario (Fig. 6-C), the highest index corresponded to 0.66 mg kg^{-1} of $soil_{dw}$ (real application dose in field), and the parameters that contributed to explain this value were GLY content, SOD activity, Comet (DNA damage), GRed activity and TBARS levels. However, the most relevant responses from all concentrations were based on the rise of Comet (DNA damage), SOD, GPx, and CAT activities and, also, on the decrease of LIP content. For the exposure of 28 days (Fig. 6-D), the highest score corresponds to the highest concentration tested (1.49 mg kg^{-1} of $soil_{dw}$), and the results of the parameters LDH and AChE activities, Comet (DNA damage), CAT, GSTs, and SOD activities contributed the most to this result. Furthermore, in all concentrations for the 28-day exposure with SPIN, the

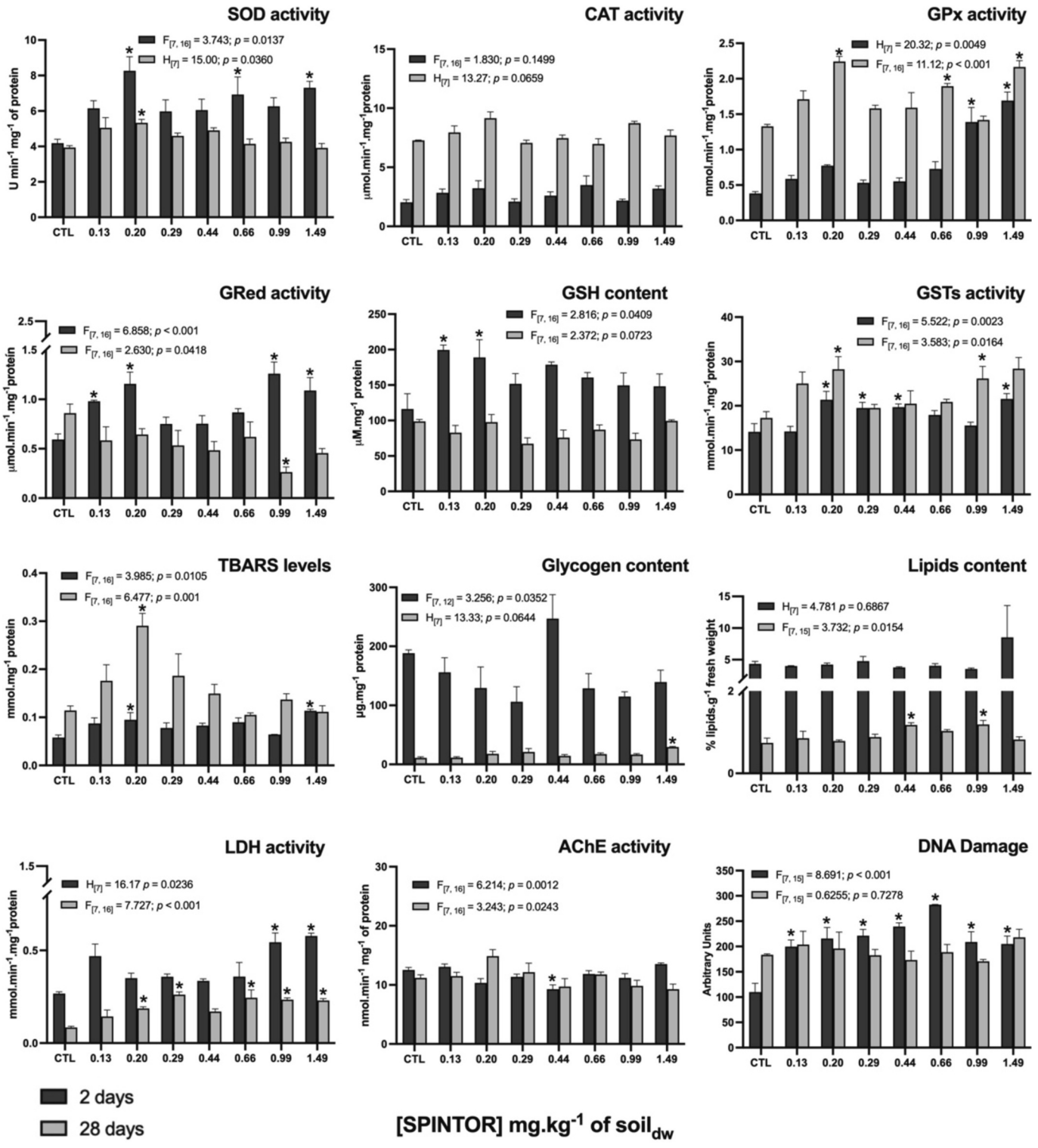

Fig. 3. *Eisenia fetida* sub-individual biomarkers after short-term (2 days) and long-term (28 days) exposures to Spintor® (SPIT®). Data are expressed as mean ± standard error (SE). * Stands for significant differences when compared to control ($p < 0.05$).

increase of AChE activity, Comet (DNA Damage), and LDH activity as well as the decrease of LIP levels, GSH content, TBARS, and GLY levels were the most relevant responses.

4. Discussion

4.1. Behavioural response of earthworms E. fetida to SPIT® and SPIN

The here-obtained results demonstrated that earthworms showed a tendency to avoid soil contaminated with SPIT® (commercial formulation). However, even though significant effects were registered, the maximum avoidance percentage recorded was 66 %, below the

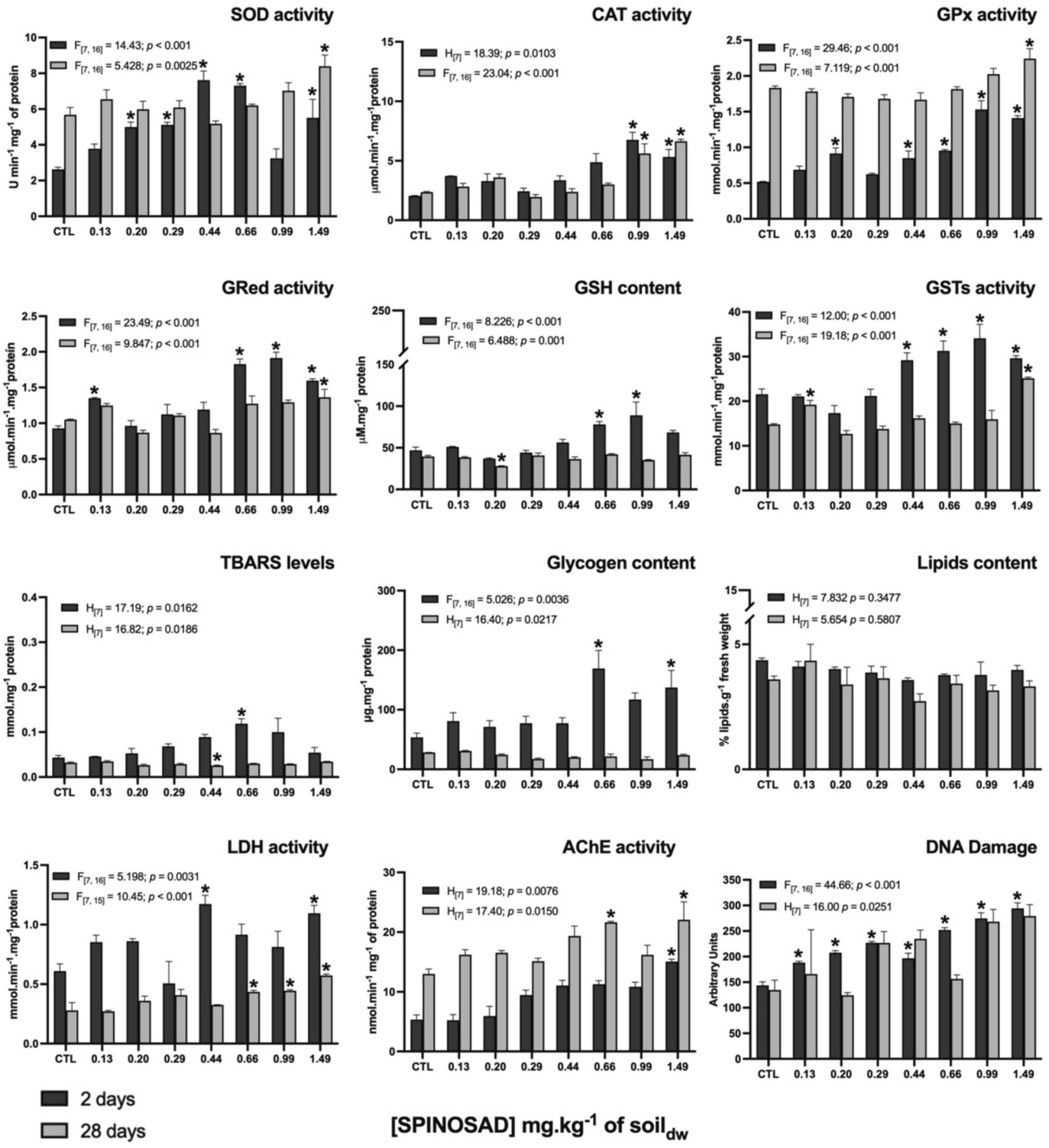

Fig. 4. *Eisenia fetida* sub-individual biomarkers after short-term (2 days) and long-term (28 days) exposures to Spinosad (SPIN). Data are expressed as mean ± standard error (SE). * Stands for significant differences when compared to control ($p < 0.05$).

threshold value of 80 %, to consider the hazardous effect on soil habitat function (Bouguerra et al., 2016). This result is in accordance with the study of De Bernardi et al. (2022), where an avoidance behaviour was also observed but in a much higher concentration (1575 mg kg^{-1} of $soil_{dw}$) of Laser® (other commercial formulation of Spinosad with 480 g L^{-1}) without compromising the habitat function. In contrast, the tendency of earthworms to avoid the active ingredient SPIN was much lower or absent compared to the commercial formulation SPIT®. Similar responses of higher avoidance behaviour of *Eisenia andrei* to commercial formulations were observed for the pesticides Viper (a.i. penoxsulam) and Mikado (a.i. sulcotrione) when comparing with their respective active ingredients (Marques et al., 2009). The authors explained that the excipients added to the commercial formulation of pesticides might increase the efficacy of the active ingredient (Cox and Surgan, 2006; Tsui and Chu, 2003), leading to higher avoidance of the commercial formulations. Additionally, since this method presupposes the detection of

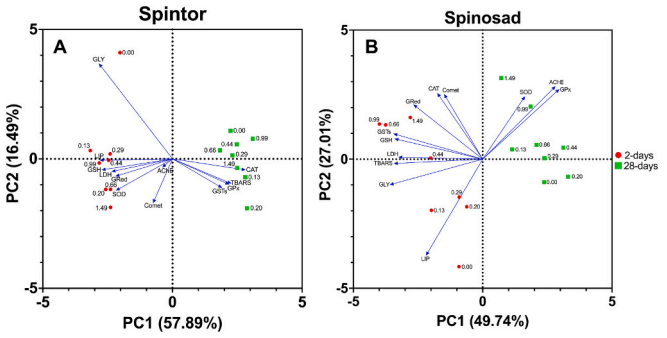

Fig. 5. Biplot of the first two components of Principal Component Analysis (PCA) including all measured sub-individual biomarkers in *Eisenia fetida* exposed to several concentrations of Spintor (SPIT®) (A) and Spinosad (SPIN) (B), during 2 or 28 days.

chemicals, the presence of the excipients may also be detected by the organisms, leading to avoidance. However, these additional chemicals are often not listed on the label; indeed, the only indication on Spintor®'s label is the presence of 44 % of spinosad and 3–10 % propylene glycol (CortevaAgriscience, 2022). Even though propylene glycol is categorized as not PBT (i.e., Persistent/Bioaccumulative/Toxic) for aquatic and terrestrial organisms (West et al., 2014), this component may enhance spinosad's activity and alter the behaviour of *E. fetida*. The greater avoidance of earthworms in soils contaminated with commercial formulations like SPIT®, compared to the active ingredient SPIN alone, highlights an important aspect of how soil organisms respond not just to active ingredients but to the entire formulation of pesticides. This behaviour has been observed in several studies across different soil organisms, suggesting that inactive ingredients or co-formulants in commercial formulations can increase their toxicity or aversiveness, leading to higher avoidance rates (e.g. Cox and Surgan, 2006; Marques et al., 2009; Tsui and Chu, 2003).

4.2. *Effect of SPIT® and SPIN on earthworms and arthropods reproduction*

In the present work, the results of reproductive output for SPIT® and SPIN did not show significant differences for *E. fetida*. This outcome agrees with other authors (De Bernardi et al., 2022; Sekulić et al., 2020), who also demonstrated no effects on the reproductive performance after exposure to pesticides. De Bernardi et al. (2022), tested two concentrations of the pesticide formulation Laser® 480 g L^{-1} (735 and 1575 mg kg^{-1} of $soil_{dw}$), and the reproduction output of *E. fetida* was not affected. Sekulić et al. (2020) used another commercial formulation of spinosad, Laser® 240 SC (240 g L^{-1}) at concentrations of 0.06–0.96 mg kg^{-1} of dry soil and recorded no effects on reproductive output. In our study, however, the chemicals may have suffered degradation. Indeed, the degradation of spinosad has been documented to be around 9 to 17 days in soil under aerobic conditions (Hale and Portwood, 1996). De Bernardi et al. (2022) also studied the degradation of spinosad (Laser® 480) in soil, and a concentration of 735 mg kg^{-1} of $soil_{dw}$ resulted in half-lives of 19.3 and 28.9 days for soil with and soil without earthworms, respectively. This is probably due to the digging behaviour,

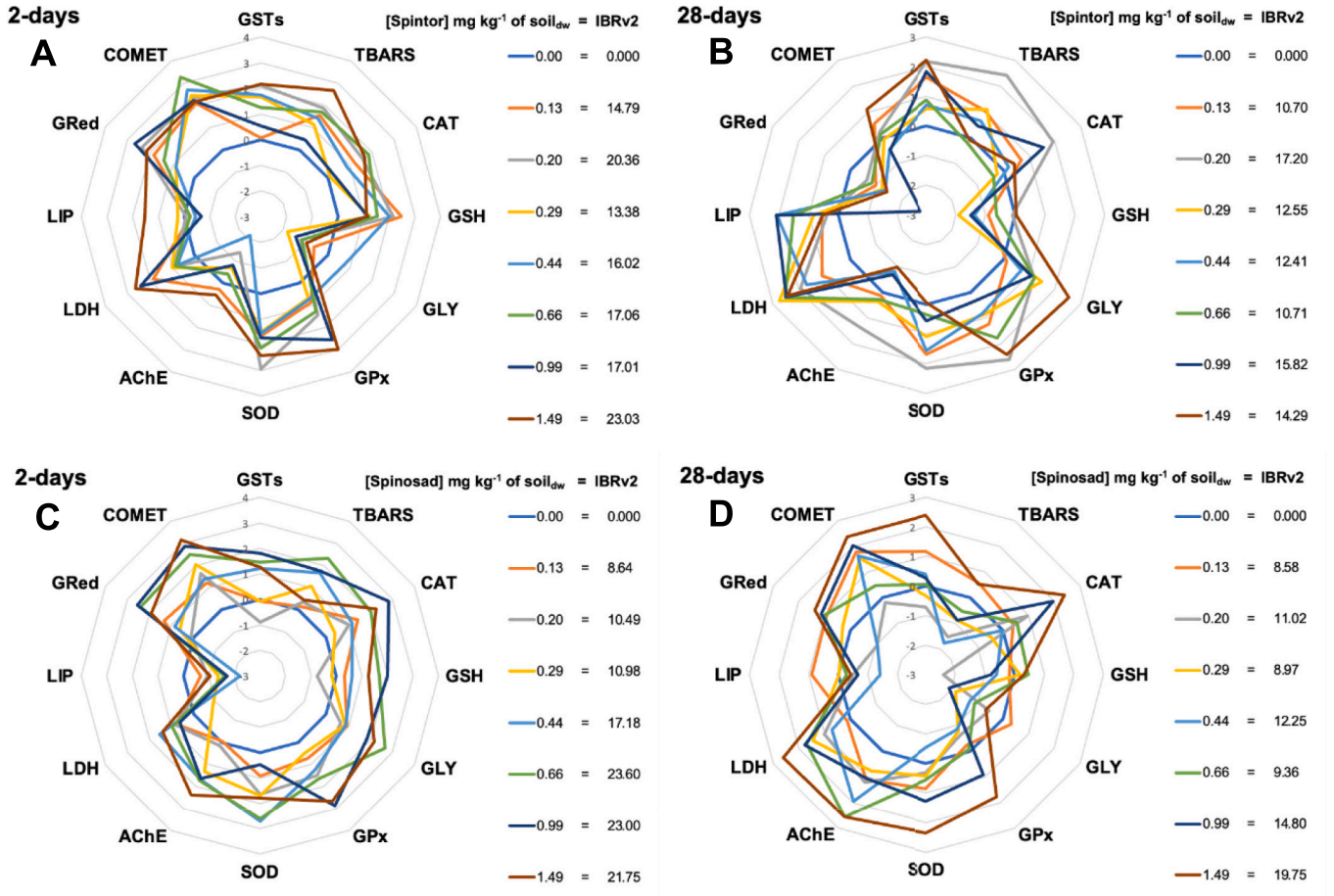

Fig. 6. IBRv2 index results and star plot, with all the biomarkers determined on *Eisenia fetida*, after exposure to: A) 2 days Spintor® (SPIT®); B) 28 days Spintor® (SPIT®); C) 2 days Spinosad (SPIN); and D) 28 days Spinosad (SPIN).

which creates excellent aeration of the soil, a condition that allows faster degradation of spinosad (De Bernardi et al., 2022; Thompson et al., 2002), or potentially due to accumulation by the organisms. This outcome has been observed in other works, which demonstrate that spinosad-based products are generally innocuous for the reproductive performance of earthworms, not affecting the size of communities. In De Bernardi et al. (2022) and Yang et al. (2020), *E. fetida* was exposed to commercial formulation Laser 480 (spinosad, 480 g L^{-1}) and ciprofloxacin (antibiotic), respectively, and no effects on reproduction were recorded but significant changes in DNA damage (both studies) and in antioxidant defences (only in Yang et al. (2020)). In this sense, it is necessary to investigate the links between responses at the sub-individual level to effects at supra-individual levels.

In this work, we also assessed the effect on the reproductive performance of *F. candida*, other soil-dweller with important soil functions for which there are no scientific studies regarding these parameters after SPIT® or SPIN exposure. Contrariwise to earthworms, the soil springtail *F. candida* demonstrated sensitivity in reproduction output, after exposure to SPIT® and SPIN. The sensitivity of collembola to pesticides was already demonstrated in several studies (Fernandes et al., 2023; Ferreira et al., 2022; Simoes et al., 2019). Indeed, in the present work, the exposure to SPIN revealed higher toxicity than SPIT®, since there was a significant decrease in the number of juveniles in all concentrations of SPIN. The lowest toxicity of SPIT® may be related to the presence of excipients, comparatively to SPIN. Accordingly to the date from the safety sheet of SPIT® (CortevaAgriscience, 2022) the biodegradability of propylene glycol, the only known excipient in the commercial formulation (between 3.0 and 10.0 % of the formulation) is 81 % after 28 days, following the same guideline test (OECD, 1992). Propylene glycol has low sorption potential and as a result, adsorption to sediment or soil particulates is not significant (Jaesche et al., 2006; Toscano et al., 2014). This indicates that propylene glycol can have a high mobility in soil (CortevaAgriscience, 2022) and since its function is to improve the solubility of spinosad in the commercial formulation (Szajewski, 2009), it can also contribute to inhibiting the adsorption of SPIT® to soil particles and consequently enhance the degradation process and reduce the likelihood/period of exposure.

However, the reproduction EC_{20} and EC_{50} calculated here for *F. candida* to SPIT® (0.20 and 0.98 mg kg^{-1} $soil_{dw}$, respectively) demonstrate the high ecological relevance, since the organism were exposed to the recommended application dose of this formulation (0.66 mg kg^{-1} $soil_{dw}$). Further, these outcomes suggest that the adverse effects are mainly attributed to the active ingredient since this decrease in the number of juveniles produced was observed only at the highest concentrations of the commercial formulation. However, no previous works in which the reproductive output of *F. candida* upon exposure to spinosad (commercial formulation and active ingredient) was found in the literature. Santos et al. (2019), reported that spinosad (commercial extended-release tablets Natular®DT used for still-water containers) revealed high toxicity (immobility effects, increase in the time of first reproduction, and decrease in the number of neonates per female) for *Daphnia magna*, even at a lower concentration (5 μg L^{-1}) compared to the recommended usage dose ~500 μg L^{-1} (i.e. 0.1 g of a.i. for 208 litter of water = 480.77 μg L^{-1}). Moreover, Monteiro et al. (2019) also demonstrated that spinosad (a.i.) provoked changes in the life-history traits (larval growth, development, and emergence) of *Chironomus riparius*, significantly increasing the time of emergence at 8 μg L^{-1} and also sub-individual alterations (e.g. lipid peroxidation, increase in the electron transport system and increase of LDH and GPx activities). The authors hypothesized that the energy demand could be related to the raise of antioxidant defences, which can also explain how less energy would be available for other physiological processes, delaying the emergence (Monteiro et al., 2019).

Eisenia fetida and *Folsomia candida* possess important ecological functions, among them, the breakdown of organic matter and nutrient cycling. Earthworms additionally contribute for the improvement of soil conditions by building channels for drainage, promoting soil aeration and root growth, as well as mediate pollution remediation (Edwards et al., 2013; Hopkin, 1997). Henceforth, negative impacts on their life cycles, like decrease of offsprings (possibly leading to community decrease and endangerment) or changes in behaviour (avoidance from contaminated sites) might impair soil health and structure (Menta, 2012). Case in point, this may reduce farming ecosystems productivity (Barrios, 2007).

4.3. Biochemical responses and DNA damage induced by SPIN and SPIT® in E. fetida

Regarding the results from SOD, CAT and GPx activities, both exposure periods showing the ability of SPIN to disturb these antioxidant enzymes (Fig. 4). It is also important to denote that there was an increase of SOD activity even at concentrations below of 0.66 mg kg^{-1} of $soil_{dw}$ concentration (for both SPIT® and SPIN), demonstrating effects even at ecologically and environmentally relevant scenarios. In the case of SPIT® exposure, the H_2O_2 potentially produced by SOD (Fig. 3), can be related to the GPx activity, which seems to be responsible to eliminate the hydrogen peroxide, probably because GPx has a higher affinity to hydrogen peroxide (Higuchi, 2014; Sies, 1993) and CAT only becomes more active at high concentrations of the hydrogen peroxide (Powers and Sen, 2000). The increase of SOD, CAT, and GPx activities indicate an oxidative stress scenario promoted by SPIT® and SPIN, followed by an attempt to recover oxidative homeostasis. Contrarily to the 2-day exposure, the results for SOD activity in the 28-day exposure, for both SPIT® and SPIN, cannot refer to an evident situation of oxidative stress. In fact, at 28 days, SOD activity appears to be close to basal levels (Deng et al., 2021; Sies and Jones, 2020), indicated by the control. These changes may reflect the potential degradation of SPIT® and SPIN in the soil, throughout the 28 days, as previously reported. In the case of SPIN exposure, GPx activity also accompanied the tendency to decrease to basal levels, but this is not verified in the SPIT® exposure. Since GPx is also involved in the breakdown of lipid peroxides, its activity may have increased at 28 days to promote the clearance of ROOH caused by SPIT®. Hypothetically, SPIT® might have persisted longer in the soil, due to the presence of stabilizing excipients, as compared to the pure active ingredient SPIN (a.i.) (Cox and Surgan, 2006; Tsui and Chu, 2003). The safety sheet for Spintor® (CortevaAgriscience, 2022) reports a biodegradability of the active ingredient spinosad inferior to 1 % in an aerobic aqueous medium after 28 days, following the guideline test OECD 301B (OECD, 1992).

Acute SPIT® and SPIN exposures initiated the increase of GRed and GSTs activities and GSH levels also increased. This may indicate that the rate of GSH synthesis through the γ-glutamyl cycle was higher than the rate of utilization by GSTs activation and GPx, and in parallel, there was an effort for re-synthesis of GSH by GRed (Maity et al., 2018). It can also be inferred that GSH was being used to detoxify spinosad, both by the GSTs conjugation pathway and proton-donor for GPx (Perez-Pertejo et al., 2008; Piner and Uner, 2013). The increase in antioxidant enzymes (SOD, CAT, GPx, GRed, and GSTs) and non-enzymatic antioxidants (GSH) in *E. fetida* after 2 days of exposure can reflect the initial adaptive response of the earthworms to SPIN exposure (Wu et al., 2011). This also infers that SPIN might exert higher toxicity upon *E. fetida* than SPIT®, as stated above, due to the higher activation of antioxidant pathways. On the other hand, the excipients present in SPIT® can alter the bioavailability of the active ingredient for this non-target organism. Furthermore, the physical and chemical characteristics of soils, such as pH, and organic matter content can influence the toxicity and viability of compounds. At the end of the 28 days, the GSH/GSSG balance may have been re-established, as stated by Perez-Pertejo et al. (2008). In the case of GSTs, some treatments still revealed an increase in enzymatic activity and despite GSH still being used by GSTs, the balance may have been restored by GRed. The intraspecies variability cannot be disregarded and some organisms might have not recuperated, since the differences were

found rather randomly distributed in the concentrations. However, the observed increase in GSTs activity may indicate that these compounds or their by-products can be metabolized by this route, in *E fetida*.

At 2 days of exposure to SPIT® and SPIN, the concentrations in TBARS levels were significantly overexpressed (0.20 and 1.49 mg kg^{-1} of $soil_{dw}$ for SPIT® and 0.66 mg kg^{-1} of $soil_{dw}$ for SPIN). In these concentrations, enzymes involved in fighting oxidative stress (e.g., SOD, GPx, and GSTs) were also stimulated. The simultaneous increase of SOD activity and lipid peroxidation is expected in a stress response (Wu et al., 2012b). Despite the performance of these enzymes, they failed to prevent oxidative damage in these concentrations. It can be hypothesized that the increase of GSTs is related to the scavenging of lipid peroxides, produced upon spinosyns exposure (Piner and Uner, 2013); more, the increase of GPx activity and LPO levels under spinosyns exposure have also been previously described for CHO-K1 and Vero cells (Perez-Pertejo et al., 2008) and *Oreochromis niloticus* (Piner and Uner, 2013). The levels of TBARS after the 28 days both for SPIT® and SPIN were increased in some concentrations, not showing any tendency of significant oxidative damage. The predisposition for less evidence of lipid peroxidation, for long-term exposures (e.g. 28 days), in *E. fetida* is probably related to the active action of antioxidant defences, as well as the degradation of both products to less harmful metabolites. It is possible to infer that SPIT® affects the antioxidant system and detoxification pathways in *E. fetida*. Indeed, the metabolism of spinosad inside the organisms entails the oxidation by the system cytochrome P-450, which is a potential additional source of ROS (FAO and WHO, 2001). The re-establishment of the basal levels of SOD, GRed, and GSTs activities and GSH content may indicate, that the antioxidant pathways protected the organism's cells, and also that the degradation of spinosad prevented the chemical from causing further oxidative damage. Based on these assumptions, earthworms seem to be able to tolerate moderate exposures to SPIT® and SPIN, and efficiently combat oxidative stress.

GLY and LIP content indicate how xenobiotics affected the energy metabolism (Givaudan et al., 2014; Overgaard et al., 2009). Allied to these energetic reserves it is important to understand the underlying pathway of energy supply. Regarding the GLY content, despite not showing a clear tendency, some transient concentrations showed a significant increase when the organisms were exposed to SPIN for 2 days (Fig. 4). This can be related to: 1) other metabolic pathways, not assessed in the present work, might be disrupted by spinosad, altering the energy reserves, e.g., by changing gut absorption of nutrients (Abouelghar et al., 2013), or 2) alteration of the clearance mechanisms of lactate produced by the induction of LDH activity (Li et al., 2022). LDH is an enzyme from glycolysis, that catalyses the reversible conversion of pyruvate in lactate, and its activity can be a bioindicator of a shift in the energy production pathways, from the oxidative phosphorylation to glycolysis upon stress (Lammertyn et al., 2021; Sahu et al., 2022). Lactate accumulation stimulates gluconeogenesis (that is, de novo glucose production), creating high levels of glucose (Emhoff et al., 2013). Moreover, the conversion to glycogen (glycogenesis) can be a protection mechanism against the accumulation of glucose, since the overload of free glucose can be also a source of oxidative stress (Cherkas et al., 2020). This is also supported by the LDH activity upraised, which serves as a gluconeogenesis checkpoint (Farhana and Lappin, 2023; Lammertyn et al., 2021; Tripathi et al., 2011). Regarding LDH activity, there was an overexpression with SPIT® and SPIN exposures, meaning that at 2 days of exposure the chemicals induced energetic stress, increasing the necessity for additional energy. Several studies found that some xenobiotics can affect the normal oxidative phosphorylation system, disabling the normal function of the mitochondria (Darnell and Weidolf, 2013). This ultimately leads to a shift towards glycolysis in the cytoplasm of the cells (Thompson et al., 2011). Furthermore, this energy demand may be related to the activation of antioxidant defences (Cherkas et al., 2020; Monteiro et al., 2019) to combat oxidative stress. This increased activity of LDH suggests that high levels of energy were necessary to respond to stress within a short period, through an alternative pathway (i.e. anaerobic through glycolysis), as also demonstrated in Monteiro et al. (2019) for spinosad, which suggests that SPIN and SPIT® might challenge the normal respiratory pathways in short-term exposure. For the activity of LDH after long-term exposure, the same scenario with SPIN and SPIT® is shown; since the antioxidant defences are still active, fast energy production is still mandatory (Cherkas et al., 2020). The high levels of LDH activity at 28 days might also confer an additional source of energy production necessary to re-establish the normal energetic metabolism, as shown in Lammertyn et al. (2021). LDH activity has been considered a reliable biomarker for the toxicity of other pesticides in *E. fetida* (Rico et al., 2016) and spinosad (Monteiro et al., 2019); and the results in this study appear to support the use of LDH activity as a plausible biomarker of exposure of *E. fetida* to spinosad (commercial formulation and active ingredient). The analysis of the PCAs for SPIT® and SPIN (Fig. 5-A and -B, respectively) demonstrates that LDH activity has a similar response to antioxidant defences, demonstrating a possible route for energy and oxidative metabolism disruptions, through chemical stress exerted by the xenobiotic.

Overall, during the 28-day exposure, for SPIT® we perceived a similar scenario in the short-term exposure, concerning the glycogen content, in which the highest concentration was significantly increased. LDH activity was also increased in the highest concentration at 28 days. The lipid content upon exposure to SPIT® was also significantly increased in some concentrations and despite not showing a clear tendency, this may be a result from the high demand for glucose, necessary to counteract the stress induced by the chemical. Possibly, even after the eventual degradation of the parental compound (e.g. spinosad), the deregulation caused could have led to abnormal energy processing, and the accumulated glucose in the cells transformed, by de novo lipid biosynthesis pathway, in fatty acids (Chen et al., 2019).

For both exposures to SPIT® and SPIN, AChE activity changes occurred, without an evident scenario of neurotoxicity. Nevertheless, other studies stated the neurotoxic effects of spinosad in other species, e.g., *Apis mellifera* (Abdel et al., 2013; Biondi et al., 2012; Eid et al., 2011). Typically, a decrease in AChE activity leads to uncoordinated movements, due to an overstimulation of cholinergic receptors (Hayden et al., 2010), leading to behavioural changes (Kumar et al., 2010; Xuereb et al., 2009), e.g., impossible to escape spiked soil in the avoidance assay. Our results demonstrated a significant decrease of AChE activity in the concentration 0.44 mg·kg^{-1} for SPIT®. The upraise of AChE activity upon 2 and 28 days exposure to SPIN has not been demonstrated in the literature, which may be the outcome of natural intraspecific differences. This may be connected to the fact that lower levels of xenobiotics may cause an overcompensation response, which may result in biphasic dose-response relationships that resemble hormetic relations (Calabrese and Baldwin, 2002; Wu et al., 2012a).

Regarding genotoxicity, De Bernardi et al. (2022) using the commercial formulation Laser® 480 on *E. fetida* demonstrated significantly higher levels of genotoxic damage, after 1 day of exposure. Our findings agree with this, since under exposure to SPIT® and SPIN for 2 days, significant DNA damage in all concentrations was observed, with an apparent dose-effect relation. Furthermore, the concentrations used in De Bernardi et al. (2022) are higher (735 and 1575 mg kg^{-1}) than the ones used in the present work. This means that even at lower doses at the real recommended application dose (0.66 mg kg^{-1} $soil_{dw}$), the organisms are sensitive to SPIT® and SPIN. The damage in coelomocytes may lead to health problems due to soil-derived pathogens, which impair the ecological functions of oligochaete (Santocki et al., 2016). Moreover, earthworms possess chloragogen cells, that upon tissue damage migrate to the lesion and regenerate it (Morgan et al., 2002; Reddy and Rao, 2008). Chloragogen cells can differentiate in a specific type of coelomocyte (e.g., eleocytes) associated with the balance of physico-chemical properties of the coelomic fluid and also immune defence (Kurek et al., 2007). However, high doses of some pesticides may disrupt the normal activity of chloragogen cells (Rico et al., 2016), ultimately resulting in

mortality or sub-lethal effects, like avoidance (Rico et al., 2016). Despite the lack of information about the effect of SPIT® (or its a.i.) on soil invertebrates, other studies reported genotoxic effects on non-target organisms from different taxonomic groups (e.g., human cell lines, *Drosophila melanogaster*'s hemocytes, developing chick embryos, Swiss albino male mice and rat bone marrow cells) (Demir, 2012; Mansour et al., 2008; Sharma and Jain, 2018; Uggini and Suresh, 2013; Yang et al., 2016; Zhang et al., 2019). Thus, spinosad, or its metabolites, can promote genotoxicity, eventually associated with increased oxidative stress and ROS content (Mendonca et al., 2019; Piner and Uner, 2013). Contrarily, the DNA damage assessed upon long-term exposure did not show significant alterations when exposed to SPIT® and SPIN, similarly to De Bernardi et al. (2022). The lack of genotoxicity effects at 28 days may be associated with the ability of earthworms to recover from stress (Pochron et al., 2021), in line with the decay of the active substance in soil (De Bernardi et al., 2022; Thompson et al., 2002). The analysis of the PCAs for SPIT® and SPIN (Fig. 5-A and -B, respectively) demonstrates that the DNA damage (measured by comet assay) showed a similar response to the antioxidant defences (e.g., SOD or CAT activities), and for 2-day exposure, demonstrating that, possibly, the xenobiotic provoked ROS formation at early exposure stage and led to DNA damage.

Results of control groups from the different assays showed difference of order of magnitude for the same parameter. This can be explained since the biomarkers response is very sensitive and, therefore, highly affected by biotic and abiotic factors (Shi et al., 2017), changing depending on the nutrient availability and the time of exposure to these conditions, or even intraspecific differences between different batches of organisms exposed.

As discussed at the end of the previous section, changes in terrestrial organisms' individual parameters might lead to impairment of soil ecological functions. However, the accumulation of repeated alterations in sub-individual parameters (measured by these biomarkers) may add-up to changes in the individual (Ferrario et al., 2018).

4.4. IBRv2 – understanding biomarkers' responses to SPIT® and SPIN

The IBRv2 is a useful tool for analysing environmental contaminants' impacts and is an index widely utilized in field and laboratory investigations (Caliani et al., 2021; Pinto et al., 2019).

A study on the effects of SPIN on *Danio rerio*, conducted by our team, has also included the IBRv2 approach (Amaral et al., 2024), obtaining interesting results for comparisons. In this study, acute (96 h) and chronic (28 days) assays were conducted, using concentration ranges of, respectively, 0.7–1.0 mg L^{-1} and 0.006–0.100 mg L^{-1}. In the acute (short-term) scenario, zebrafish demonstrated a strong induction of CAT and GRed activities and in the chronic (long-term) assay, SOD and CAT activity are induced; these findings are similar to our results, that is, an activation of antioxidant pathway upon exposure to SPIN. Moreover, our study demonstrated an alteration in sources of obtaining energy, with shifts in GLY content and LDH activity, which also agrees with Amaral et al. (2024) findings. We can then infer that SPIN not only provokes changes in the antioxidant defence mechanism, but also in the normal energy metabolism pathways in species from different taxonomic groups.

For the assays with SPIT® or SPIN, at 2 or 28 days, the IBRv2 values obtained for each concentration were similar within the respective assay. These indicate that SPIN and SPIT® cause metabolic pathway disturbances in several pathways of *E. fetida* (antioxidant defences, energetic metabolism, and genotoxicity). The IBRv2 index additionally showed that these biomarkers are sensitive and coherent to demonstrate the stress caused by spinosad on *E. fetida*.

5. Conclusions

A potential intrinsic toxicity to the molecule spinosad, present in the commercial formulation Spintor® was observed. It can have a negative impact on the health and wellness of *E. fetida* and *F. candida*, which could possibly impair their functions in the ecosystem. The information provided by this study ought to be allied to further investigations on other individual parameters of these non-target soil organisms (e.g., burrowing behaviour on oligochaete and avoidance and biomarker responses of *F. candida*), other non-target soil organisms (e.g. the oligochaeta *Enchytraeus crypticus*), as well as more sub-individual biomarkers of exposure (e.g., histopathological evaluation and other enzymatic profiles) to fully understand the impacts and risks of spinosyns application. Further, future investigations should consider the possible interaction of this pesticide with soil microbiota that can be affected and/or involved in the degradation process.

Additionally, given that changes were observed after short exposures and at concentrations lower than those that generate impacts at the organismal level, the biochemical biomarkers examined in the current study may be useful as early-warning tools in biomonitoring studies. This study reveals that commercial formulations like SPINTOR® can pose risks to soil organisms. These findings underscore the importance of considering the entire pesticide formulation in ecotoxicological assessments and regulatory frameworks. For sustainable agricultural practices, it is essential to balance the benefits of pest control with the potential impacts on soil health and non-target organisms, reinforcing the need for regulatory bodies to expand testing protocols to reflect real-world conditions and mitigate environmental risks.

Consent for publication

The paper is submitted with the mutual consent of the authors for publication.

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CRediT authorship contribution statement

Alexandre Moreira: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Verónica Nogueira:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization. **Sirine Bouguerra:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Sara C. Antunes:** Writing – review & editing, Validation, Software, Resources. **Sara Rodrigues:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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Data availability

No data was used for the research described in the article.

References

Abdel, R.M.A., Eid, K., Marei, G.I.K., 2013. Impacts of multiple applications with biofly (*Beauveria bassiana*) and spintor®(spinosad) on honey bee (*Apis mellifera*) larvae. Journal of Plant Protection and Pathology 4 (1), 49–66. https://dx.doi.org/10.21608/jppp.2013.87255.

Abouelghar, G.E., Sakr, H., Ammar, H.A., Yousef, A., Nassar, M., 2013. Sublethal effects of spinosad (Tracer®) on the cotton leafworm (Lepidoptera: Noctuidae). Journal of Plant Protection Research 53 (3). https://doi.org/10.2478/jppr-2013-0041.

Adak, T., Mukherjee, I., 2016. Investigating role of abiotic factors on spinosad dissipation. Bull. Environ. Contam. Toxicol. 96 (1), 125–129. https://doi.org/10.1007/s00128-015-1644-z.

Aebi, H., 1984. [13] Catalase in vitro. Methods Enzymol. 105, 121–126. https://doi.org/10.1016/S0076-6879(84)05016-3.

Amaral, I., Antunes, S.C., Rebelo, D., Carvalho, A.P., Rodrigues, S., 2024. Biopesticide spinosad: unraveling ecotoxicological effects on zebrafish, *Danio rerio*. Environmental Toxicology and Pharmacology 108, 104458. https://doi.org/10.1016/j.etap.2024.104458.

Antunes, S.C., Castro, B.B., Pereira, R., Gonçalves, F., 2008. Contribution for tier 1 of the ecological risk assessment of Cunha Baixa uranium mine (Central Portugal): II. Soil ecotoxicological screening. Science of the Total Environment 390 (2), 387–395. https://doi.org/10.1016/j.scitotenv.2007.07.053.

Ayilara, M.S., Adeleke, B.S., Akinola, S.A., Fayose, C.A., Adeyemi, U.T., Gbadegesin, L.A., Omole, R.K., Johnson, R.M., Uthman, Q.O., Babalola, O.O., 2023. Biopesticides as a promising alternative to synthetic pesticides: a case for microbial pesticides, phytopesticides, and nanobiopesticides [review]. Front. Microbiol. 14, 1040901. https://doi.org/10.3389/fmicb.2023.1040901.

Barrios, E., 2007. Soil biota, ecosystem services and land productivity. Ecol. Econ. 64 (2), 269–285. https://doi.org/10.1016/j.ecolecon.2007.03.004.

Baweja, P., Kumar, S., Kumar, G., 2020. Fertilizers and pesticides: their impact on soil health and environment. In: Giri, B., Varma, A. (Eds.), Soil Health. Springer International Publishing, pp. 265–285. https://doi.org/10.1007/978-3-030-44364-1_15.

Beliaeff, B., Burgeot, T., 2002. Integrated biomarker response: a useful tool for ecological risk assessment. Environ. Toxicol. Chem. 21 (6), 1316–1322. https://www.ncbi.nlm.nih.gov/pubmed/12069320.

Biondi, A., Mommaerts, V., Smagghe, G., Vinuela, E., Zappala, L., Desneux, N., 2012. The non-target impact of spinosyns on beneficial arthropods. Pest Manag. Sci. 68 (12), 1523–1536.

Bouguerra, S., Gavina, A., Ksibi, M., Rasteiro Mda, G., Rocha-Santos, T., Pereira, R., 2016. Ecotoxicity of titanium silicon oxide (TiSiO4) nanomaterial for terrestrial plants and soil invertebrate species. Ecotoxicol. Environ. Saf. 129, 291–301. https://doi.org/10.1016/j.ecoenv.2016.03.038.

Bouguerra, S., Gavina, A., da Graça Rasteiro, M., Rocha-Santos, T., Ksibi, M., Pereira, R., 2019. Effects of cobalt oxide nanomaterial on plants and soil invertebrates at different levels of biological organization. J. Soil. Sediment. 19 (7), 3018–3034. https://doi.org/10.1007/s11368-019-02285-8.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1), 248–254. https://doi.org/10.1006/abio.1976.9999.

Buege, J.A., Aust, S.D., 1978. [30] Microsomal lipid peroxidation. Methods Enzymol. 52, 302–310. https://doi.org/10.1016/S0076-6879(78)52032-6.

Bueno, M.R., Cunha, J., 2020. Environmental risk for aquatic and terrestrial organisms associated with drift from pesticides used in soybean crops. An. Acad. Bras. Cienc. 92 (Suppl. 1), e20181245. https://doi.org/10.1590/0001-3765202020181245.

Calabrese, E.J., Baldwin, L.A., 2002. Defining hormesis. Hum. Exp. Toxicol. 21 (2), 91–97. https://doi.org/10.1191/0960327102ht217oa.

Caliani, I., Campani, T., Conti, B., Cosci, F., Bedini, S., D'Agostino, A., Giovanetti, L., Di Noi, A., Casini, S., 2021. First application of an integrated biological response index to assess the ecotoxicological status of honeybees from rural and urban areas. Environ. Sci. Pollut. Res. Int. 28 (34), 47418–47428. https://doi.org/10.1007/s11356-021-14037-8.

Carlberg, I., Mannervik, B., 1985. [59] Glutathione reductase. Methods Enzymol. 113, 484–490. https://doi.org/10.1016/S0076-6879(85)13062-4.

Chen, L., Chen, X.W., Huang, X., Song, B.L., Wang, Y., Wang, Y., 2019. Regulation of glucose and lipid metabolism in health and disease. Sci. China Life Sci. 62 (11), 1420–1458. https://doi.org/10.1007/s11427-019-1563-3.

Cherkas, A., Holota, S., Mdzinarashvili, T., Gabbianelli, R., Zarkovic, N., 2020. Glucose as a major antioxidant: when, what for and why it fails? Antioxidants 9 (2), 140. https://doi.org/10.3390/antiox9020140.

Christen, V., Krebs, J., Bunter, I., Fent, K., 2019. Biopesticide spinosad induces transcriptional alterations in genes associated with energy production in honey bees (*Apis mellifera*) at sublethal concentrations. J. Hazard. Mater. 378, 120736. https://doi.org/10.1016/j.jhazmat.2019.06.013.

Cleveland, C.B., Bormett, G.A., Saunders, D.G., Powers, F.L., McGibbon, A.S., Reeves, G. L., Rutherford, L., Balcer, J.L., 2002a. Environmental fate of spinosad. 1. Dissipation and degradation in aqueous systems. J. Agric. Food Chem. 50 (11), 3244–3256. https://doi.org/10.1021/jf011663i.

Cleveland, C.B., Mayes, M.A., Cryer, S.A., 2002b. An ecological risk assessment for spinosad use on cotton. Pest Manag. Sci. 58 (1), 70–84. https://doi.org/10.1002/ps.424.

CortevaAgriscience, 2022. Spintor®. Corteva Agriscience. Retrieved 20/05/2023 from. https://www.corteva.pt/produtos-e-solucoes/protecao-de-cultivos/spintor.html#t2.

Cox, C., Surgan, M., 2006. Unidentified inert ingredients in pesticides: implications for human and environmental health. Environ. Health Perspect. 114 (12), 1803–1806. https://doi.org/10.1289/ehp.9374.

Darnell, M., Weidolf, L., 2013. Metabolism of xenobiotic carboxylic acids: focus on coenzyme A conjugation, reactivity, and interference with lipid metabolism. Chem. Res. Toxicol. 26 (8), 1139–1155. https://doi.org/10.1021/tx400183y.

De Bernardi, A., Marini, E., Casucci, C., Tiano, L., Marcheggiani, F., Ciani, M., Comitini, F., Taskin, E., Puglisi, E., Vischetti, C., 2022. Ecotoxicological effects of a synthetic and a natural insecticide on earthworms and soil bacterial community. Environmental Advances 8, 100225. https://doi.org/10.1016/j.envadv.2022.100225.

Demir, E., 2012. In vivo genotoxicity assessment of diflubenzuron and spinosad in *Drosophila melanogaster* with the comet assay using haemocytes and the SMART assay. Fresen. Environ. Bull. 21 (12a), 3894–3900.

Deng, S., Wu, Y., Duan, H., Cavanagh, J.E., Wang, X., Qiu, J., Li, Y., 2021. Toxicity assessment of earthworm exposed to arsenate using oxidative stress and burrowing behavior responses and an integrated biomarker index. Sci. Total Environ. 800, 149479. https://doi.org/10.1016/j.scitotenv.2021.149479.

Dimock, M., Ockey, S., 2017. Resistance Management: A Critical Role for Biopesticides. CAPCA Advisor, Certis USA, pp. 42–43. https://earthwiseagriculture.net/wp-content/uploads/2019/10/Resistance_Management-A_Critical_Role_for_Biopesticides.pdf.

Diogo, B.S., Antunes, S.C., Pinto, I., Amorim, J., Teixeira, C., Teles, L.O., Golovko, O., Zlabek, V., Carvalho, A.P., Rodrigues, S., 2023a. Insights into environmental caffeine contamination in ecotoxicological biomarkers and potential health effects of *Danio rerio*. Heliyon 9 (9), e19875. https://doi.org/10.1016/j.heliyon.2023.e19875.

Diogo, B.S., Antunes, S.C., Rodrigues, S., 2023b. Are biopesticides safe for the environment? Effects of pyrethrum extract on the non-target species *Daphnia magna*. Environ. Toxicol. Pharmacol. 99, 104114. https://doi.org/10.1016/j.etap.2023.104114.

Duchet, C., Coutellec, M.A., Franquet, E., Lagneau, C., Lagadic, L., 2010. Population-level effects of spinosad and Bacillus thuringiensis israelensis in Daphnia pulex and Daphnia magna: comparison of laboratory and field microcosm exposure conditions. Ecotoxicology 19 (7), 1224–1237. https://doi.org/10.1007/s10646-010-0507-y.

Edwards, C., 1975. Factors that affect the persistence of pesticides in plants and soils. Pesticide Chemistry 3, 39–56. https://doi.org/10.1016/B978-0-408-70708-4.50007-7.

Edwards, C.A., Arancon, N., Bohlen, P.J., Hendrix, P., 2013. Biology and Ecology of Earthworms.

Eid, K.S., Marei, G.I.K., Abd-Elrasol, M.A., 2011. Acute toxicity of some biopesticides and their effect on acetylcholinesterase of honey bee (*Apis Mellifera*) workers. Journal of Plant Protection and Pathology 2 (10), 805–827. https://dx.doi.org/10.21608/jppp.2011.86600.

Ellman, G.L., Courtney, K.D., Andres Jr., V., Feather-Stone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7 (2), 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

Emhoff, C.-A.W., Messonnier, L.A., Horning, M.A., Fattor, J.A., Carlson, T.J., Brooks, G. A., 2013. Gluconeogenesis and hepatic glycogenolysis during exercise at the lactate threshold. J. Appl. Physiol. 114 (3), 297–306. https://doi.org/10.1152/japplphysiol.01202.2012.

FAO, 2008. FAO Specifications and Evaluation for Agricultural Pesticides: Spinosad. https://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/Specs/Spinosad08.pdf.

FAO, 2023. Integrated Pest Management, 18/05/2023. https://www.fao.org/pest-and-pesticide-management/ipm/integrated-pest-management/en/.

FAO, WHO, 2001. Spinosad. In: Pesticide Residues in Food—2001. Part II. Toxicological Evaluations. World Health Organization. Joint FAO/WHO Meeting on Pesticide Residues. WHO/PCS/02.1. https://www.inchem.org/documents/jmpr/jmpmono/2001pr12.htm#3.0.

Farhana, A., Lappin, S.L., 2023. Biochemistry, Lactate Dehydrogenase. StatPearls. https://www.ncbi.nlm.nih.gov/books/NBK557536/.

Fenibo, E.O., Ijoma, G.N., Matambo, T., 2021. Biopesticides in sustainable agriculture: a critical sustainable development driver governed by green chemistry principles. Frontiers in Sustainable Food Systems 5, 619058. https://doi.org/10.3389/fsufs.2021.619058.

Fernandes, S., Nogueira, V., Lourenco, J., Mendo, S., Pereira, R., 2020. Inter-species bystander effect: *Eisenia fetida* and *Enchytraeus albidus* exposed to uranium and cadmium. J. Hazard. Mater. 399, 122972. https://doi.org/10.1016/j.jhazmat.2020.122972.

Fernandes, S., Buskermolen, K., Ilyaskina, D., Bakker, R., van Gestel, C.A.M., 2023. Effects of life stage on the sensitivity of *Folsomia candida* to four pesticides. Environ. Toxicol. Chem. 42 (8), 1782–1790. https://doi.org/10.1002/etc.5682.

Ferrario, C., Parolini, M., De Felice, B., Villa, S., Finizio, A., 2018. Linking sub-individual and supra-individual effects in *Daphnia magna* exposed to sub-lethal concentration of chlorpyrifos. Environ. Pollut. 235, 411–418. https://doi.org/10.1016/j.envpol.2017.12.113.

Ferreira, P., Gabriel, A., Sousa, J.P., Natal-da-Luz, T., 2022. Representativeness of *Folsomia candida* to assess toxicity of a new generation insecticide in different temperature scenarios. Sci. Total Environ. 837, 155712. https://doi.org/10.1016/j.scitotenv.2022.155712.

Flohé, L., Günzler, W.A., 1984. [12] Assays of glutathione peroxidase. Methods Enzymol. 105, 114–120. https://doi.org/10.1016/S0076-6879(84)05015-1.
Flohe, L., Otting, F., 1984. Superoxide dismutase assays. Methods Enzymol. 105, 93–104. https://doi.org/10.1016/s0076-6879(84)05013-8.
Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226 (1), 497–509.
Fountain, M.T., Hopkin, S.P., 2005. Folsomia candida (Collembola): a "standard" soil arthropod. Annu. Rev. Entomol. 50, 201–222. https://doi.org/10.1146/annurev.ento.50.071803.130331.
Ganilho, C., da Silva, M.B., Paiva, C., de Menezes, T.I., Dos Santos, M.R., Pereira, C.M., Pereira, R., Andreani, T., 2022. Environmental safety assessments of lipid nanoparticles loaded with lambda-cyhalothrin. Nanomaterials (Basel) 12 (15), 2576. https://doi.org/10.3390/nano12152576.
Gill, H.K., Garg, H., 2014. Pesticide: environmental impacts and management strategies. Pesticides-Toxic Aspects 8 (2014), 187. https://doi.org/10.5772/57399.
Givaudan, N., Binet, F., Le Bot, B., Wiegand, C., 2014. Earthworm tolerance to residual agricultural pesticide contamination: field and experimental assessment of detoxification capabilities. Environ. Pollut. 192, 9–18. https://doi.org/10.1016/j.envpol.2014.05.001.
Habig, W.H., Pabst, M.J., Fleischner, G., Gatmaitan, Z., Arias, I.M., Jakoby, W.B., 1974. The identity of glutathione S-transferase B with ligandin, a major binding protein of liver. Proc. Natl. Acad. Sci. U. S. A. 71 (10), 3879–3882. https://doi.org/10.1073/pnas.71.10.3879.
Hale, K.A., Portwood, D.E., 1996. The aerobic soil degradation of spinosad-a novel natural insect control agent. Journal of Environmental Science & Health Part B 31 (3), 477–484. https://doi.org/10.1080/03601239609373010.
Hayden, K.M., Norton, M.C., Darcey, D., Ostbye, T., Zandi, P.P., Breitner, J.C., Welsh-Bohmer, K.A., Cache County Study, I, 2010. Occupational exposure to pesticides increases the risk of incident AD: the Cache County study. Neurology 74 (19), 1524–1530. https://doi.org/10.1212/WNL.0b013e3181dd4423.
Higuchi, M., 2014. Chapter 15 - antioxidant properties of wheat bran against oxidative stress. In: Wheat and Rice in Disease Prevention and Health, pp. 181–199. https://doi.org/10.1016/B978-0-12-401716-0.00015-5.
Hole, D.G., Perkins, A.J., Wilson, J.D., Alexander, I.H., Grice, P.V., Evans, A.D., 2005. Does organic farming benefit biodiversity? Biol. Conserv. 122 (1), 113–130. https://doi.org/10.1016/j.biocon.2004.07.018.
Hopkin, S.P., 1997. Biology of the Springtails: (Insecta: Collembola).
Hubbard, M., Hynes, R.K., Erlandson, M., Bailey, K.L., 2014. The biochemistry behind biopesticide efficacy. Sustainable Chemical Processes 2, 1–8. https://doi.org/10.1186/s40508-014-0018-x.
ISO, 2008. Guideline 17512-1: Soil Quality—Avoidance Test for Determining the Quality of Soils and Effects of Chemicals on Behaviour—Part 1: Test With Earthworms (*Eisenia fetida* and *Eisenia andrei*). International Organization for Standardization.
ISO, 2012. Guideline 11268-1: Soil Quality — Effects of Pollutants on Earthworms — Part 1: Determination of Acute Toxicity to *Eisenia fetida/Eisenia andrei*. International Organization for Standardization.
ISO, 2014. Inhibition of Reproduction of Collembola (*Folsomia candida*) by Soil Contaminants. ISO Guideline 11267.. International Standardization Organization, Switzerland.
ISO, 2023. Guideline 11268-2: Soil Quality — Effects of Pollutants on Earthworms — Part 2: Determination of Effects on Reproduction of *Eisenia fetida/Eisenia andrei* and Other Earthworm Species. International Organization for Standardization.
Jaesche, P., Totsche, K.U., Kogel-Knabner, I., 2006. Transport and anaerobic biodegradation of propylene glycol in gravel-rich soil materials. J. Contam. Hydrol. 85 (3–4), 271–286. https://doi.org/10.1016/j.jconhyd.2006.02.003.
Kibblewhite, M.G., Ritz, K., Swift, M.J., 2008. Soil health in agricultural systems. Philos. Trans. R. Soc., B 363 (1492), 685–701. https://doi.org/10.1098/rstb.2007.2178.
Kumar, A., Doan, H., Barnes, M., Chapman, J.C., Kookana, R.S., 2010. Response and recovery of acetylcholinesterase activity in freshwater shrimp, Paratya australiensis (Decapoda: Atyidae) exposed to selected anti-cholinesterase insecticides. Ecotoxicol. Environ. Saf. 73 (7), 1503–1510. https://doi.org/10.1016/j.ecoenv.2010.07.016.
Kurek, A., Homa, J., Kauschke, E., Plytycz, B., 2007. Characteristics of coelomocytes of the stubby earthworm, *Allolobophora chlorotica* (Sav.). Eur. J. Soil Biol. 43, S121–S126. https://doi.org/10.1016/j.ejsobi.2007.08.051.
Lammertyn, S., Masín, C.E., Zalazar, C.S., Fernandez, M.E., 2021. Biomarkers response and population biological parameters in the earthworm *Eisenia fetida* after short term exposure to atrazine herbicide. Ecol. Indic. 121, 107–173. https://doi.org/10.1016/j.ecolind.2020.107173.
Laurent, A., Makowski, D., Aveline, N., Dupin, S., Miguez, F.E., 2021. On-farm trials reveal significant but uncertain control of *Botrytis cinerea* by *Aureobasidium pullulans* and potassium bicarbonate in organic grapevines. Front. Plant Sci. 12, 620786. https://doi.org/10.3389/fpls.2021.620786.
Lefkaditis, F.G., Arapis, G.D., Athanasiou, C.G., Kavallieratos, N.G., 2017. Spinosad and spinetoram disrupt the structure and the abundance of ground-dwelling arthropod communities in herbaceous fields. Int. J. Pest Manag. 63 (1), 54–73. https://doi.org/10.1080/09670874.2016.1219077.
Lewis, K.A., Tzilivakis, J., Warner, D.J., Green, A., 2016. An international database for pesticide risk assessments and management. Hum. Ecol. Risk Assess. Int. J. 22 (4), 1050–1064. https://doi.org/10.1080/10807039.2015.1133242.
Li, X., Yang, Y., Zhang, B., Lin, X., Fu, X., An, Y., Zou, Y., Wang, J.X., Wang, Z., Yu, T., 2022. Lactate metabolism in human health and disease. Signal Transduct. Target. Ther. 7 (1), 305. https://doi.org/10.1038/s41392-022-01151-3.
Lo, S., Russell, J.C., Taylor, A.W., 1970. Determination of glycogen in small tissue samples. J. Appl. Physiol. 28 (2), 234–236. https://doi.org/10.1152/jappl.1970.28.2.234.
Lourenco, J., Pereira, R., Silva, A., Carvalho, F., Oliveira, J., Malta, M., Paiva, A., Goncalves, F., Mendo, S., 2012. Evaluation of the sensitivity of genotoxicity and cytotoxicity endpoints in earthworms exposed in situ to uranium mining wastes. Ecotoxicol. Environ. Saf. 75 (1), 46–54. https://doi.org/10.1016/j.ecoenv.2011.08.024.
Maity, S., Banerjee, R., Goswami, P., Chakrabarti, M., Mukherjee, A., 2018. Oxidative stress responses of two different ecophysiological species of earthworms (*Eutyphoeus waltoni* and *Eisenia fetida*) exposed to Cd-contaminated soil. Chemosphere 203, 307–317. https://doi.org/10.1016/j.chemosphere.2018.03.189.
Mansour, S., Mossa, A., Heikal, T., 2008. Cytogenetic and hormonal alteration in rats exposed to recommended "safe doses" of spinosad and malathion insecticides. Int. J. Agric. Biol. 10 (1), 9–14.
Marques, C., Pereira, R., Gonçalves, F., 2009. Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and their active ingredients on natural soils. J. Soil. Sediment. 9 (2), 137–147. https://doi.org/10.1007/s11368-009-0058-0.
Martins, G.C., de Ferreira Reis, F.A., Dall'Agnol, R., Ramos, S.J., Gastauer, M., Natal-da-Luz, T., Sousa, J.P., Guilherme, L.R.G., 2024. Assessment of the reproduction of six collembolan species in tropical soils naturally rich in potentially toxic elements. Environ. Sci. Pollut. Res. Int. 31 (17), 25424–25436. https://doi.org/10.1007/s11356-024-32847-4.
Mayes, M.A., Thompson, G.D., Husband, B., Miles, M.M., 2003. Spinosad toxicity to pollinators and associated risk. Rev. Environ. Contam. Toxicol. 179, 37–71. https://doi.org/10.1007/0-387-21731-2_2.
Mendonca, T.P., Davi de Aquino, J., Junio da Silva, W., Mendes, D.R., Campos, C.F., Vieira, J.S., Barbosa, N.P., Carvalho Naves, M.P., Olegario de Campos Junior, E., Alves de Rezende, A.A., Spano, M.A., Bonetti, A.M., Vieira Santos, V.S., Pereira, B.B., Resende de Morais, C., 2019. Genotoxic and mutagenic assessment of spinosad using bioassays with *Tradescantia pallida* and *Drosophila melanogaster*. Chemosphere 222, 503–510. https://doi.org/10.1016/j.chemosphere.2019.01.182.
Menta, C., 2012. 3. Soil fauna diversity - function, soil degradation, biological indices, soil restoration. In: Biodiversity Conservation and Utilization in a Diverse World. IntechOpen. https://doi.org/10.5772/3330.
Mertz, F.K.P., Yao, R.C., 1990. *Saccharopolyspora spinosa* sp. nov. isolated from soil collected in a sugar mill rum still. Int. J. Syst. Evol. Microbiol. 40 (1), 34–39.
Monteiro, H.R., Pestana, J.L.T., Novais, S.C., Soares, A., Lemos, M.F.L., 2019. Toxicity of the insecticides spinosad and indoxacarb to the non-target aquatic midge *Chironomus riparius*. Sci. Total Environ. 666, 1283–1291. https://doi.org/10.1016/j.scitotenv.2019.02.303.
Morgan, A.J., Turner, M.P., Morgan, J.E., 2002. Morphological plasticity in metal-sequestering earthworm chloragocytes: morphometric electron microscopy provides a biomarker. of exposure in field populations. Environ Toxicol Chem 21 (3), 610–618. https://doi.org/10.1897/1551-5028(2002)021<0610:mpimse>2.0.co;2.
OECD, 1992. Test No. 301: Ready Biodegradability. https://doi.org/10.1787/9789264070349-en.
OECD, 2016. Test No. 222: Earthworm Reproduction Test (*Eisenia fetida/Eisenia andrei*). https://doi.org/10.1787/9789264264496-en.
Orr, N., Shaffner, A.J., Richey, K., Crouse, G.D., 2009. Novel mode of action of spinosad: receptor binding studies demonstrating lack of interaction with known insecticidal target sites. Pestic. Biochem. Physiol. 95 (1), 1–5. https://doi.org/10.1016/j.pestbp.2009.04.009.
Overgaard, J., Tollarova, M., Hedlund, K., Petersen, S.O., Holmstrup, M., 2009. Seasonal changes in lipid composition and glycogen storage associated with freeze-tolerance of the earthworm, *Dendrobaena octaedra*. J Comp Physiol B 179 (5), 569–577. https://doi.org/10.1007/s00360-009-0341-9.
Parewa, H.P., Joshi, N., Meena, V.S., Joshi, S., Choudhary, A., Ram, M., Meena, S.C., Jain, L.K., 2021. Role of biofertilizers and biopesticides in organic farming. Advances in Organic Farming 133–159. https://doi.org/10.1016/B978-0-12-822358-1.00009-2.
Perez-Pertejo, Y., Reguera, R.M., Ordonez, D., Balana-Fouce, R., 2008. Alterations in the glutathione-redox balance induced by the bio-insecticide Spinosad in CHO-K1 and Vero cells. Ecotoxicol. Environ. Saf. 70 (2), 251–258. https://doi.org/10.1016/j.ecoenv.2007.06.009.
Piner, P., Uner, N., 2013. Oxidative stress and apoptosis was induced by bio-insecticide spinosad in the liver of *Oreochromis niloticus*. Environ. Toxicol. Pharmacol. 36 (3), 956–963. https://doi.org/10.1016/j.etap.2013.08.009.
Pinto, J., Costa, M., Leite, C., Borges, C., Coppola, F., Henriques, B., Monteiro, R., Russo, T., Di Cosmo, A., Soares, A., Polese, G., Pereira, E., Freitas, R., 2019. Ecotoxicological effects of lanthanum in *Mytilus galloprovincialis*: biochemical and histopathological impacts. Aquat. Toxicol. 211, 181–192. https://doi.org/10.1016/j.aquatox.2019.03.017.
Pochron, S.T., Mirza, A., Mezic, M., Chung, E., Ezedum, Z., Geraci, G., Mari, J., Meiselbach, C., Shamberger, O., Smith, R., Tucker, W.J., Zafar, S., 2021. Earthworms *Eisenia fetida* recover from Roundup exposure. Appl. Soil Ecol. 158, 103793. https://doi.org/10.1016/j.apsoil.2020.103793.
Powers, S.K., Sen, C.K., 2000. Part IV • Chapter 10 - physiological antioxidants and exercise training. In: Handbook of Oxidants and Antioxidants in Exercise, pp. 221–242. https://doi.org/10.1016/B978-044482650-3/50010-9.
Pur, H.A., Tunaz, H., 2022. Determination of mortality effect of some biological larvicites on the mosquito Culex sp. Braz. Arch. Biol. Technol. 65. https://doi.org/10.1590/1678-4324-2022210017.
Rajamani, M., Negi, A., 2021. Biopesticides for pest management. In: Sustainable Bioeconomy: Pathways to Sustainable Development Goals, pp. 239–266. https://doi.org/10.1007/978-981-15-7321-7_11.

Reddy, N.C., Rao, J.V., 2008. Biological response of earthworm, *Eisenia foetida* (Savigny) to an organophosphorous pesticide, profenofos. Ecotoxicol. Environ. Saf. 71 (2), 574–582. https://doi.org/10.1016/j.ecoenv.2008.01.003.

Reinecke, S.A., Reinecke, A.J., 2004. The comet assay as biomarker of heavy metal genotoxicity in earthworms. Arch. Environ. Contam. Toxicol. 46 (2), 208–215. https://doi.org/10.1007/s00244-003-2253-0.

Report, M.R., 2023. Biopesticides Market by Type (Bioinsecticides, Biofungicides, Bionematicides), Crop Type (Cereals & Grains, Oilseeds & Pulses), Formulation (Liquid and Dry), Source (Microbials, Biochemicals) Mode of Application, Region - Global Forecast to 2028. Sep 2, 2023. https://www.marketresearch.com/MarketsandMarkets-v3719/Biopesticides-Type-Bioinsecticides-Biofungicides-Bionematicides-34495384/.

Rico, A., Sabater, C., Castillo, M.A., 2016. Lethal and sub-lethal effects of five pesticides used in rice farming on the earthworm *Eisenia fetida*. Ecotoxicol. Environ. Saf. 127, 222–229. https://doi.org/10.1016/j.ecoenv.2016.02.004.

Sahu, J.K., Behera, R.K., Baitharu, I., Naik, P.P., 2022. Biology of earthworm in the world of nanomaterials: new room, challenges, and future perspectives. Bio-Nano Interface: Applications in Food, Healthcare and Sustainability 307–328. https://doi.org/10.1007/978-981-16-2516-9_17.

Salgado, V.L., Saar, R., 2004. Desensitizing and non-desensitizing subtypes of alpha-bungarotoxin-sensitive nicotinic acetylcholine receptors in cockroach neurons. J. Insect Physiol. 50 (10), 867–879. https://doi.org/10.1016/j.jinsphys.2004.07.007.

Samada, L.H., Tambunan, U.S.F., 2020. Biopesticides as promising alternatives to chemical pesticides: a review of their current and future status. Online J. Biol. Sci 20 (2), 66–76. https://doi.org/10.3844/ojbsci.2020.66.76.

Sanchez, W., Burgeot, T., Porcher, J.-M., 2013. A novel "integrated biomarker response" calculation based on reference deviation concept. Environ. Sci. Pollut. Res. 20, 2721–2725. https://doi.org/10.1007/s11356-012-1359-1.

Santocki, M., Falniowski, A., Plytycz, B., 2016. Restoration of experimentally depleted coelomocytes in juvenile and adult composting earthworms *Eisenia andrei E. fetida* and *Dendrobaena veneta*. Applied Soil Ecology 104, 163–173. https://doi.org/10.1016/j.apsoil.2015.08.022.

Santos, V.S.V., Silva, C.E., Oliveira, C.M., de Morais, C.R., Limongi, J.E., Pereira, B.B., 2019. Evaluation of toxicity and environmental safety in use of spinosad to rationalize control strategies against *Aedes aegypti*. Chemosphere 226, 166–172. https://doi.org/10.1016/j.chemosphere.2019.03.129.

Sekulić, J., Stojanovic, M., Trakić, T., Popovic, F., Tsekova, R., 2020. Effects of the modern biorational insecticide spinosad on the earthworm *Eisenia fetida* (Savigny, 1826)(Annelida: Clitellata). Acta Zool. Bulg. 15, 71–77. https://scidar.kg.ac.rs/handle/123456789/17142.

Sharma, R., Jain, S., 2018. Spinosad: sub-acute genotoxicity studies in mice. J. Vet. Pharmacol. Toxicol. 17 (1), 30–38.

Shi, Z., Tang, Z., Wang, C., 2017. A brief review and evaluation of earthworm biomarkers in soil pollution assessment. Environ. Sci. Pollut. Res. Int. 24 (15), 13284–13294. https://doi.org/10.1007/s11356-017-8784-0.

Sies, H., 1993. Strategies of antioxidant defense. Eur. J. Biochem. 215 (2), 213–219. https://doi.org/10.1111/j.1432-1033.1993.tb18025.x.

Sies, H., Jones, D.P., 2020. Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. Nat. Rev. Mol. Cell Biol. 21 (7), 363–383. https://doi.org/10.1038/s41580-020-0230-3.

Simoes, T., Novais, S.C., Natal-da-Luz, T., Leston, S., Rosa, J., Ramos, F., Pouca, A.S.V., Freitas, A., Barbosa, J., Roelofs, D., Sousa, J.P., van Straalen, N.M., Lemos, M.F.L., 2019. Fate and effects of two pesticide formulations in the invertebrate *Folsomia candida* using a natural agricultural soil. Sci. Total Environ. 675, 90–97. https://doi.org/10.1016/j.scitotenv.2019.04.191.

Soares, C., Fernandes, B., Paiva, C., Nogueira, V., Cachada, A., Fidalgo, F., Pereira, R., 2023. Ecotoxicological relevance of glyphosate and flazasulfuron to soil habitat and retention functions - single vs combined exposures. J. Hazard. Mater. 442, 130128. https://doi.org/10.1016/j.jhazmat.2022.130128.

Spurgeon, D.J., Sturzenbaum, S.R., Svendsen, C., Hankard, P.K., Morgan, A.J., Weeks, J. M., Kille, P., 2004. Toxicological, cellular and gene expression responses in earthworms exposed to copper and cadmium. Comp Biochem Physiol C Toxicol Pharmacol 138 (1), 11–21. https://doi.org/10.1016/j.cca.2004.04.003.

Szajewski, J., 2009. Warsaw Poison Control Centre (August, 1991). Propylene Glycol (PIM 443). IPCS INChem. https://www.inchem.org/documents/pims/chemical/pim443.htm.

Thompson, D.G., Harris, B.J., Lanteigne, L.J., Buscarini, T.M., Chartrand, D.T., 2002. Fate of spinosad in litter and soils of a mixed conifer stand in the Acadian forest region of New Brunswick. J. Agric. Food Chem. 50 (4), 790–795. https://doi.org/10.1021/jf011319l.

Thompson, R.A., Isin, E.M., Li, Y., Weaver, R., Weidolf, L., Wilson, I., Claesson, A., Page, K., Dolgos, H., Kenna, J.G., 2011. Risk assessment and mitigation strategies for reactive metabolites in drug discovery and development. Chem. Biol. Interact. 192 (1–2), 65–71. https://doi.org/10.1016/j.cbi.2010.11.002.

Toscano, G., Colarieti, M.L., Anton, A., Greco, G., Biro, B., 2014. Natural and enhanced biodegradation of propylene glycol in airport soil. Environ. Sci. Pollut. Res. Int. 21 (15), 9028–9035. https://doi.org/10.1007/s11356-013-1952-y.

Tripathi, G., Kachhwaha, N., Dabi, I., Bandooni, N., 2011. Temperature-dependent alterations in metabolic enzymes and proteins of three ecophysiologically different species of earthworms. Brazilian Archives of Biology and Technology 54, 769–776. https://doi.org/10.1590/S1516-89132011000400017.

Tsui, M.T., Chu, L.M., 2003. Aquatic toxicity of glyphosate-based formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52 (7), 1189–1197. https://doi.org/10.1016/S0045-6535(03)00306-0.

Tudi, M., Daniel Ruan, H., Wang, L., Lyu, J., Sadler, R., Connell, D., Chu, C., Phung, D.T., 2021. Agriculture development, pesticide application and its impact on the environment. Int. J. Environ. Res. Public Health 18 (3), 1112. https://doi.org/10.3390/ijerph18031112.

Uggini, G.K., Suresh, B., 2013. Genotoxic effects of two different classes of insecticide in developing chick embryos. Toxicol. Environ. Chem. 95 (6), 992–1005. https://doi.org/10.1080/02772248.2013.828888.

UNICEF, 2020. The State of Food Security and Nutrition in the World 2020: Transforming Food Systems for Affordable Healthy Diets. https://doi.org/10.4060/ca9692en.

Usta, C., 2013. Microorganisms in biological pest control—a review (bacterial toxin application and effect of environmental factors). Current progress in biological research 13, 287–317. https://doi.org/10.5772/55786.

Vassault, A., 1983. Lactate dehydrogenase, UV-method with pyruvate and NADH. In: Methods in Enzymatic Analysis, 3, p. 118.

Viana, C.M., Freire, D., Abrantes, P., Rocha, J., Pereira, P., 2022. Agricultural land systems importance for supporting food security and sustainable development goals: a systematic review. Sci. Total Environ. 806, 150718. https://doi.org/10.1016/j.scitotenv.2021.150718.

West, R., Banton, M., Hu, J., Klapacz, J., 2014. The distribution, fate, and effects of propylene glycol substances in the environment. Rev. Environ. Contam. Toxicol. 232, 107–138. https://doi.org/10.1007/978-3-319-06746-9_5.

Wu, H., Xu, H., Hong, Y., Zhang, J., Wu, J., 2011. The use of biomarkers in the antioxidant responses of *Daphnia magna* to the acute and chronic exposure to no. 20 diesel oil and 2, 4-dichlorophenol. Chemical Speciation & Bioavailability 23 (2), 80–87. https://doi.org/10.3184/095422911X13027015402949.

Wu, S.J., Zhang, H., Hu, Y., Li, H.L., Chen, J.M., 2012a. Effects of 1,2,4-trichlorobenzene on the enzyme activities and ultrastructure of earthworm *Eisenia fetida*. Ecotoxicol. Environ. Saf. 76 (2), 175–181. https://doi.org/10.1016/j.ecoenv.2011.09.009.

Wu, S., Zhang, H., Zhao, S., Wang, J., Li, H., Chen, J., 2012b. Biomarker responses of earthworms (*Eisenia fetida*) exposured to phenanthrene and pyrene both singly and combined in microcosms. Chemosphere 87 (4), 285–293. https://doi.org/10.1016/j.chemosphere.2011.11.055.

Xuereb, B., Lefevre, E., Garric, J., Geffard, O., 2009. Acetylcholinesterase activity in *Gammarus fossarum* (Crustacea Amphipoda): linking AChE inhibition and behavioural alteration. Aquat. Toxicol. 94 (2), 114–122. https://doi.org/10.1016/j.aquatox.2009.06.010.

Yang, M., Xiang, G., Li, D., Zhang, Y., Xu, W., Tao, L., 2016. The insecticide spinosad induces DNA damage and apoptosis in HEK293 and HepG2 cells. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 812, 12–19. https://doi.org/10.1016/j.mrgentox.2016.11.001.

Yang, X., Li, Y., Wang, X., 2020. Effects of ciprofloxacin exposure on the earthworm *Eisenia fetida*. Environ. Pollut. 262, 114287. https://doi.org/10.1016/j.envpol.2020.114287.

Zhang, Y., Chen, H., Fan, Y., Yang, Y., Gao, J., Xu, W., Xu, Z., Li, Z., Tao, L., 2019. Cytotoxic effects of bio-pesticide spinosad on human lung A549 cells. Chemosphere 230, 182–189. https://doi.org/10.1016/j.chemosphere.2019.05.042.