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Potential ecotoxicity of biopesticides to non-target organisms of the soil

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“Whether we march with banners or without, the important thing is that we march together, all of us.”

“Good. Congratulations all of you. You’re the founder members of Lesbians and Gays supporting the miners.”

“Terrific. Let’s bring down the government.”

***in* Pride (2014)**
directed by Matthew Warchus

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Everything I've done and achieved, not only in this work but in general, has resulted from the influence of those that surround me. "We´re all useless alone. It's a good thing you're not alone." (Evelyn *in* Everything Everywhere All At Once)

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Keywords

Bioinsecticides, *Eisenia fetida*, *Folsomia candida*, Reproduction, Behaviour, Biomarkers

Abstract

In the last decades, there has been a growing effort to replace synthetic pesticides, often associated with adverse effects, with more safer and environmentally sustainable options. The application of biopesticides, obtained from natural sources, has then grown in both organic and traditional farming, aiming to achieve greener agricultural practices.

Spintor® (bioinsecticide) is a commercial formulation of the active ingredient spinosad, a naturally occurring product composed by a mixture of spynosyns A and D, derived from the fermentation of the soil actinomycete *Saccharopolyspora spinosa*. The singular and novel structure of this compound confers low resistance and efficacy against several pests such as e.g., lepidopteran and thysanoptera. Regardless of its efficacy against several agricultural pests, it is also possible for this product to be bioactive against non-target organisms, given the conservation of metabolic pathways amongst species or even due to newer pathways of toxicity. 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 testing of lower concentrations, closer to real environmental presence, and assessment of other parameters than mortality and reproduction. Moreover, previous studies have stated the toxicity of spinosad and spinosad-employed formulations to non-target organisms, from different environmental compartments.

Taking these facts into consideration, the present study aimed to evaluate the potential adverse effects of the commercial formulation Spintor® (SPIT) and its active ingredient, Spinosad (SPIN), on *Eisenia fetida*, an oligochaete, and *Folsomia candida*, an arthropod, both representatives of soil fauna, and model organisms in soil ecotoxicological studies. For that purpose, natural soil was spiked with environmentally relevant concentrations (0.00-1.49 mg of active ingredient/kg of dry soil) to assess the effects in terms of avoidance and reproduction (following ISO standard protocol). Acute exposures of 2-days were also performed (using the same concentrations). Further, *E. fetida* adults exposed for 2- and 28-days were processed to analyse different biomarkers (oxidative stress, energy pathways, and neurotransmission) and possible genotoxic effects (measured by comet assay).

A significant reduction in the juveniles production for *F. candida* was observed for both SPIT and SPIN, and although no effect was observed on *E. fetida* reproductive outputs, the oligochaeta revealed a tendency to avoid soil spiked with SPIT. The sub-individual biomarkers demonstrate significant genotoxicity upon exposure to SPIT and SPIN for 2 days. The pro-oxidant performance and energy metabolism pathways were altered in both assays for SPIT and SPIN. The 2-days exposures revealed higher activation of the defence mechanisms, and in general, SPIN exerted higher effects than SPIT on the oligochaetes. The results suggest that spynosyns based products can have an impact on arthropods and oligochaete's health, possibly affecting their essential functions in the terrestrial ecosystems.

Palavras-chave

Bioinsecticidas, *Eisenia fetida*, *Folsomia candida*, Reprodução, Comportamento, Biomarcadores

Resumo

Nas últimas décadas tem havido um crescente esforço para substituir pesticidas sintéticos, muitas vezes associados a efeitos adversos, com alternativas mais sustentáveis e seguras. A aplicação de biopesticidas obtidos de fontes naturais tem sido utilizada quer em culturas orgânicas quer tradicionais, com o objetivo de atingir práticas agrícolas mais verdes.

Spintor® (bioinsecticida) é uma formulação comercial do ingrediente ativo espinosade, um produto obtido naturalmente, composto por uma mistura de espinosinas A and D, derivadas da fermentação do actinomicete do solo *Saccharopolyspora spinosa*. A estrutura nova e singular deste composto confere baixa resistência e eficácia contra diversas pragas, e.g., lepidoptera e thysanoptera. Apesar da sua eficácia contra pestes, é também possível que este produto seja bioativo contra organismos não-alvo, dada a conservação de vias metabólicas entre espécies, ou mesmo através de novas vias de toxicidade. Apesar dos esforços das agências de regulamentação, ainda existe falta de informação relativa a exposições de curta e longa duração em organismos de solo, assim como testes com concentrações mais baixas e ambientalmente relevantes, e ainda avaliação de parâmetros para além da mortalidade e reprodução. Ainda, estudos na literatura demonstraram que o espinosade e produtos formulados com o espinosade são tóxicos para organismos não-alvo de diferentes compartimentos ambientias.

Tendo em conta estes factos, o presente estudo teve como objetivo avaliar os potenciais efeitos adversos da formulação comercial Spintor®(SPIT) e do seu ingrediente ativo Spinosad (SPIN), em *Eisenia fetida*, oligoquetas, e em *Folsomia candida*, artrópodes, ambos representantes da fauna do solo e organismos modelo em estudos ecotoxicológicos. Para tal, solo natural foi contaminado com uma gama de concentrações ambientalmente relevantes (0.00-1.49 mg de ingrediente ativo/kg de solo seco) de forma forma a avaliar os efeitos relativamente a evitamento e reprodução (seguindo protocolos padrão ISO). Exposições agudas de 2-dias foram também realizadas (usando as mesmas concentrações). Ainda, adultos de *E. fetida* após exposição por 2- e 28-dias foram processados de forma a analisar diferentes biomarcadores (stresse oxidativo, vias energéticas e neurotransmissão) e possíveis efeitos genotóxicos (ensaio do cometa).

Uma redução significativa no número de juvenies produzidos por *F. candida* foi observado após exposição a SPIT e SPIN, e ainda que não se tenha verificado efeito no balanço reprodutivo de *E. fetida*, as oligoquetas revelaram uma tendeência para evitar solo contaminado por SPIT. Os biomarcadores sub-individuais demonstraram genotoxicidade significativa após exposição a SPIT e SPIN por 2 dias. A performance prooxidativa e as vias metabólicas energéticas foram alteradas em ambos os ensaios para SPIT e SPIN. A exposição de 2-dias demonstrou uma maior ativação dos mecanismos de defesa, e em geral, SPIN exerceu maiores efeitos nas oligoquetas do que SPIT. Os resultados sugerem que os produtos formulados com base nas espinosinas possam ter impactos na saúde de artrópodes e oligoquetas, possivelmente afetando as suas funções essenciais em ecossistemas terrestres.

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Abbreviations List

ACh	Acetylcholine
AChE	Acetylcholinesterase
AU	Arbitrary Units
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
CNS	Central Nervous System
DGAV	Direção-Geral da Alimentação e Veterinária
DNA	Deoxyribonucleic Acid
DTNB	5-5'-dithio-bis-2-nitrobenzoate
DW	Dry Soil
EC	Electrical conductivity
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization
GABA	γ-Aminobutyric Acid
GLY	Glycogen
GPx	Glutathione Peroxidase
GRed	Glutathione Reductase
GSH	Glutathione
GSTs	Glutathione-S-Transferases
IBR	Integrated Biomarker Response
IPM	Integrated Pest Management
IRAC	Insecticide Resistance Action Committee
ISO	International Organization for Standardization
LDH	Lactate Dehydrogenase
LIP	Lipids
LOEC	Lowest Observed Effect Concentration
LPO	Lipid Peroxidation
MDA	Malondialdehyde
NOEC	No Observed Effect Concentration
OECD	Organization for Economic Cooperation and Development
OM	Organic Matter
PBT	Persistent/Bioaccumulative/Toxic
PCA	Principal Component Analysis
ROS	Reactive Oxygen Species
SCGE	Single-cell Gel Electrophoresis

SCOPE	Scientific Committee of Problems of the Environment
SDG	Sustainable Development Goals
SOD	Superoxide Dismutase
SPIN	Spinosad
SPIT	Spintor
TBARS	Thiobarbituric Acid Reactive Substances
WHC	Water Holding Capacity
WHO	World Health Organization

I. GENERAL INTRODUCTION AND OBJECTIVES

I.1. General Introduction

I.1.1. Plant protection products in agriculture ecosystems

The soil is an important ecosystem that plays a vital function in sustaining biodiversity and supporting agriculture practices, posing an enormous economic and social role (Abrahams, 2002; Kopittke et al., 2019). Agriculture's primary responsibility is to feed the human population and livestock. With the expected increment of the global population up to 9.7 billion by 2050, it becomes essential to ensure food security and steady production levels (Godfray et al., 2010). To attain this global demand for food, it became necessary to maximize crop yields and ensure food security, through the use of plant protection products, such as pesticides. Pesticides are chemical compounds used to manage or eradicate pests that can harm crops, such as insects, fungi, weeds, and illnesses (Gill & Garg, 2014; Kogan, 1998). Pesticides can be categorized according to the species that they affect (Casida, 2009), but more importantly, they can be divided into organic and inorganic compounds, which differ in the contamination and toxicity process (Akram et al., 2018; Devipriya & Yesodharan, 2005). In addition to the possible threat to human health (Damalas & Eleftherohorinos, 2011; Jaga & Dharmani, 2003), the intensive use of pesticides can lead to extended soil contamination, through superficial runoffs, volatilization and dispersion of the pesticide during the application (Vryzas, 2018); afterward, it can occur sorption and binding of the pesticides molecules to soil particles, which depends on the pesticide type and its Koc (soil adsorption coefficent) as well as properties of the soil (e.g., organic matter, water holding capacity, pH and microbial activity) (Akram et al., 2018; Farenhorst, 2006; Sarkar et al., 2020). This phenomenon may cause the persistence of the pesticide, or its metabolites upon degradation, in the environment, leading to prolonged toxicity (Edwards, 1975; Somasundaram et al., 1989). Additionally, pesticides can have lipofilic (hydrophobic) properties depending on its Kow (n-octanol/water partition coefficient) leading to uptake in organic matter, which can cause bioaccumulation in soil organisms (Gupta & Gupta, 2020; Mahmood et al., 2016), as is the case of plants and soil-dwellers (e.g. earthworms). Additionally, there can be contamination of water bodies through leaching from the crop fields (Munjanja et al., 2020), which can potentially disrupt aquatic ecosystems (Houdart et al., 2009). To mitigate this problem, some practices have been introduced. The European Green Deal is a set of proposals adopted by the European Commission to achieve climate neutrality by 2050 (Fetting, 2020). Several objectives are defined to improve agriculture sustainability, amongst them, the "Farm to Fork strategy" and the "Sustainable use of pesticides". The "Farm to Fork strategy" responds to the necessity of accelerating the transition to a sustainable food system that, among other objectives, aims to reverse the

loss of biodiversity. The “Sustainable use of pesticides” has the intention to reduce the use and the risk of agricultural chemicals, specially the more hazardous ones, to ensure an environmentally friendly pest control through implementation of the Integrated Pest Management (IPM) and ban the use of pesticides in sensitive areas, such as public parks and gardens (Fetting, 2020). The Integrated Pest Management (IPM) is a holistic approach that integrates diverse tactics, including biological control, crop rotation, and cultural practices, to reduce pests’ impact while lowering chemical usage (FAO, 2023). IPM aims for long-term pest management by taking into account the long-term ecological and economic repercussions of pest control technologies (Stenberg, 2017). Some of these measures include mechanical controls, monitoring, and the establishment of acceptable pest levels instead of total eradication (EPA, 2022c). Another measure is biological control, in which naturally occurring biological materials and processes, such as biopesticides, are used with assumed acceptable environmental impact (Stenberg, 2017). Therefore, a biopesticide is an agent used in farming to control pests (e.g., insects or other pathogens), derived from naturally occurring compounds. These substances are produced by bacteria, fungi, and other microorganisms, as well as some animals and plants (Parewa et al., 2021). They have risen in response to this global concern over environmental health, in substitution of synthetic compounds used in agriculture and livestock (Ayilara et al., 2023; Hole et al., 2005). Synthetic insecticides such as carbofuran, anthraquinone, paraquat, methomyl, or diuron are being replaced or even banned due to their high toxicity levels to the environment and ecosystem health (Boucaud-Maitre et al., 2019).

Most synthetic insecticides share the same toxic routes, acting on similar biochemical targets. Insecticides act on nerve targets, mainly acetylcholinesterase (AChE), AChE receptor, voltage-gated chloride channel, and GABA (γ-aminobutyric acid receptor) receptors (Casida, 2009). These similar routes of action may lead to cross-resistance of the pests by creating tolerance through mutation of genes that code for target sites, modifying them (Hemingway et al., 2004), or the upregulation of detoxification enzymes (Zhu et al., 2015). Newer modes of action of biopesticides, regarding these other synthetic insecticides commercially used, present many benefits concerning pest resistance (Fenibo et al., 2021). Biopesticides can target different genes simultaneous, leading to unlikeliness to develop cross-resistance (Dimock & Ockey, 2017). Although, they possess more restricted mechanisms of action when compared to synthetic pesticides, meaning that these natural substances affect only the pest and phylogenetic close organisms (EPA, 2022b; Usta, 2013). This high specificity can comprise a minimal toxicity to the environment and humans (Rajamani & Negi, 2021).

I.1.2. Spinosyns, Spinosad and the commercial formulation Spintor®

The above mentioned criteria are met by spinosad, which employs spinosyns, molecules presenting a singular structure (Salgado et al., 1997; Sparks et al., 2001). Spinosad is produced from a parental compound found in the bacteria *Saccharopolyspora spinosa* (Sparks et al., 2001). They are gram-positive, filamentous, and non-motile (Singh & Mazumdar, 2022). This strain was discovered in 1990 by Frederick Mertz and Raymond Yao, when isolating microorganisms from a sugar mill rum still soil (Mertz & Yao, 1990), and differentiated due to its morphological (e.g., aerial hyphae with beadlike appearance) and taxonomic properties (e.g., absence of mycolic acids), as well as new metabolites and their chemical activities (Mertz & Yao, 1990). Spinosad is composed of the macrolides spinosyn A and D (Fig. 1), molecules derived from fermentation. These lactones present a singular novel structure that is highly used against several pests (e.g., cotton bollworm *Helicoverpa armígera* and potato beetle *Leptinotarsa decemlineata*) in agriculture (Thompson, 1995).

Spinosyn A and spinosyn D are compounds with relatively high molecular weight (732 and 746, respectively). The difference between them is found at the bridging carbon of the indacene group in spinosyn D, due to the presence of an additional methyl group connected to it (Figure 1). The extra methyl group significantly affects several qualities (FAO, 2008). The spinosyns are a novel class of macrocyclic lactones (Lumaret et al., 2012). With a distinct cross-bridged macrocyclic structure, their basic structure is a tetracyclic macrolide (indacene and cyclododecane) produced from polyketides attached to two saccharides [an amino sugar (D-forosamine)] and a neutral sugar (tri-O-methyl-L-rhamnose) (Kirst et al., 1991). Spinosyn A and spinosyn D have low and pH-dependent water solubilities of 235 and 0.332 $mg.L^{-1}$, respectively, at pH 7 and 20 °C. The values of K_{ads} (adsorption coefficient) and K_{des} (desorption coefficient) for spinosyn A are determined to be 8.3-323 $mL.g^{-1}$. Spinosyns A and D have pK_{as} of 8.1 and 7.9, respectively, making them weak bases (ECHA, 2010; FAO, 2008). As a result, in both situations, the water solubility falls as the pH rises. In sterile, buffered water, both spinosyns are resistant to hydrolysis; at pH 5, there is no observable hydrolysis, and at pH 7 and 9, there is an increasing but very sluggish hydrolysis (FAO, 2008). These spinosyns underwent fast aqueous photolysis at pH 7 with a half-life of under a day (FAO, 2008). The degradation of these compounds can be attained through different pathways, which results in different sub-products (Thompson et al., 2023); the aqueous photolysis leads to the loss of the forosamine sugar and a reduction in the macrolide ring (Cleveland et al., 2002a); the degradation performed by microorganism under anaerobic conditions leads to a loss of the rhamnose ring, and under aerobic conditions there is a transformation in smaller

compounds called diketone spinosyn aglycon degradates (Paquette et al., 1998); the abiotic hydrolysis involves the loss of forosamine sugar and water, as well as, reduction on the macrolide ring (Cleveland et al., 2002a).

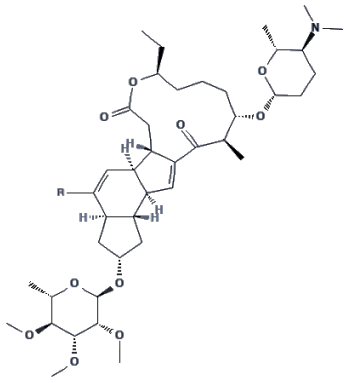

Fig.1: Chemical structure of Spinosad, a mixture of spinosyn A (R=H) and spinosyn D (R=CH_3). Image retrieved from PubChem (NCBI, 2022).

Spinosad was developed in 1996-1997 and was firstly commercialized in Korea and the United States (Thompson et al., 2023). The commercial formulation employed by farmers and available for purchase was developed by Corteva Agriscience™, trade marketing the name Spintor®. This bioinsecticide was given a selling permit in Portugal by DGAV (Direção Geral de Alimentação e Veterinária) (selling permit nº 0288) (CortevaAgriscience, 2022). Spintor® is a concentrated liquid suspension containing 480 $g.L^{-1}$ or 44.0 % of spinosad (active ingredient). It employs propylene glycol (propane-1,2-diol) as its solvent (~3.0 to 10.0 %) (CortevaAgriscience, 2022). This allows the mixture to be miscible in water to prepare the application solution (Szajewski, 2009). Furthermore, propylene glycol has not been linked to toxic effects, both in humans and animals (NTP, 2004). According to its active ingredient, it is included in group 5 of the action mode by IRAC (Insecticide Resistance Action Committee), being an allosteric modulator of the nicotinic acetylcholine receptors (nAChR) (IRAC, 2016). Spintor® is applied in several types of crops by pulverization, in greenhouses or outdoor, protecting the fields against lepidopteran larvae (e.g. *Helicoverpa armígera, Spodoptera littoralis, Agrotis ípsilon, Agrotis segetum, Autographa gamma, Pieris brassicae,* and *Pieris rapae*), Thysanoptera (*Thrips sp*), Coleoptera (e.g. *Leptinotarsa decemlineata*) (Mayes et al., 2003) as well as some Diptera and Hymenoptera (CortevaAgriscience, 2022; Mayes et al., 2003).

I.1.2.1. Action mechanism of spinosad

Spintor® (spinosad) is a contact and ingestion insecticide. It has a neurotoxic activity, as reflected by its action mechanism (Christen et al., 2019), by activating the nicotinic

acetylcholine receptors. Studies performed on the cockroaches *Periplaneta americana* have demonstrated that spinosyns affect the central nervous system (CNS) of the organism, altering nicotinic currents in neuron cell bodies, causing hyperexcitation followed by disruption of the CNS (Salgado & Saar, 2004). A study from Salgado (1997) identified the principal cause of death by spinosad in target organisms to be related with a novel mechanism of activity on the nicotinic acetylcholine receptor. Spinosad also presents activity on gamma-aminobutyric acid (GABA) receptors and GABA-gated chloride channels function is modified (Miles et al., 2002; Sparks et al., 2001). In contrast with other insecticides, or even nicotine and neonicotinoids, the binding site for spinosyn A in nicotinic acetylcholine and GABA receptors is different (Orr et al., 2009). Orr et al. (2009) suggest that the interaction is achieved with an unidentified nicotinic acetylcholine receptor subtype. Although the mechanism for GABA and nicotinic acetylcholine receptors modification is still unknown, Sparks et al. (2001) suggested that this unknown mechanism of interaction may be the base of the insecticidal properties of spinosyns and respective analogs. In fact, spinosad seems to lead to involuntary muscle contractions and paralysis, ultimaly leading to death of the pest (Salgado, 1997). Moreover, this novel mode of actions can help prevent cross-resistance with other acetylcholinesterase inhibitors, like carbamates or organophosphates.

I.1.2.2. Scientific concerns about Spinosad use as a bioinsecticide

Spinosad is classified by U.S. EPA as a reduced risk pesticide (Cleveland et al., 2002b). And even though it is considered a safer option than synthetic pesticides, some concerns have risen. Due to its biological origin and related properties, it has become largely used in organic farming, but also in traditional farming, aligned with other pesticides (Schnaars‐Uvino & Baker, 2021). However, the potential toxicity to non-target organisms should not be disregarded (Christen et al., 2019). Several metabolic pathways and even physiological functions are conserved among biologically close organisms, meaning that non-target can be affected through the same toxic route as the target pests, or even novel pathways (Diogo et al., 2023b). These toxicity mechanisms ought to be assessed in different non-target organisms to ensure environmental safety. Despite the classification as “High alert” in the BPDB (Bio-Pesticides Database) (Lewis et al., 2016), the studies performed by regulatory agencies (e.g., EFSA – European Food Safety Authority) lack assays based on short- and long-term exposure and lower concentrations for soil-dwellers and parameters other than reproductive toxicity and mortality. For *Eisenia fetida* there is a registred NOEC (No observed effect concentration) of 1.79 $mg.kg^{-1}$ for reproductive toxicity (chronic exposure). For aquatic organism there is a pletora of ecotoxicological assays (both chronic and acute)

that demonstrate a scenario of high toxicity. Moreover, there is not any registered data for non-target plants and collembola. In fact, the literature review demonstrates there is a rising concern regarding the use of spinosad and its toxicity to non-target organisms, both aquatic and terrestrial. Indeed, Monteiro et al. (2019) have shown the ecotoxicological effects of spinosad (active ingredient) on the aquatic midge *Chironomus riparius*. Concentrations of 8 and 20 $\mu g.L^{-1}$ led to a reduction in the number of emerged adults (after 28 days of exposure) and concentrations as low as 2 $\mu g.L^{-1}$ caused impact at the biochemical level through lipidic peroxidation and alterations in the energy obtention pathways, upon 2 days exposure. The disruption caused by spinosad has also been assessed for *Daphnia magna* (Duchet et al., 2010), a model ecotoxicological organism, and *Ceriodaphnia dubia* (Deardorff & Stark, 2011), in which 8 $\mu g.L^{-1}$ and 1 $\mu g.L^{-1}$ of spinosad, respectively, caused impairment of reproductive parameters. An extensive review of the effects of spinosyn products (spinosad) on beneficial arthropods has been compiled by Biondi et al. (2012), for which relevant studies regarding the lethal and side effects of spinosad on these species have been cited. For instance, the toxicity for pollinators has been evaluated due to their important ecological functions (Mayes et al., 2003). The exposure to spinosad by feeding intake in *Apis mellifera* (honey bees) for 24 hours had a LC_{50} (lethal concentration that causes the death of 50% of the individuals) of 7.34 $mg.L^{-1}$ and revealed significant decrease of AChE (acetylcholinesterase) activity in several organs of the bees in all tested concentrations (2.5, 5, 10, and 20 $mg.L^{-1}$) (Rabea et al., 2010). More recently, a study was carried out to test the effects of a commercial formulation of spinosad (Laser® 480 $g.L^{-1}$) on the oligochaete *Eisenia fetida* (De Bernardi et al., 2022). Two concentrations were tested, 735 and 1575 $mg.kg^{-1}$, and although no effects were found on the reproductive output (number of juveniles produced) but, after 48 hours there was a significant avoidance of the soil spiked with the bioinsecticide, as well as DNA damage in *E. fetida* cells upon 1 day of exposure to 735 $mg.kg^{-1}$. Despite the existing information, there is still a lack of information regarding the impact of spinosad as the active ingredient or incorporated in commercial formulation (e.g., Spintor®) on terrestrial organisms' reproductivity and behaviour, as is the case of oligochaete or soil-dwelling arthropods. Some studies have also evaluated the changes in AChE activity upon exposure in non-target organisms due to the main mechanism of action being related with neurotoxicity. However, there is no available information on the alteration of other metabolic pathways and biochemical parameters, which ought to be studied since they can deeply affect the individual's survival and wellness. As discussed above, spinosad has different degradation routes, so it can be hypothesized that the different by-products created might exert toxic effects. Spinosad, or possibly its metabolites, can sorb to sediments and organic matter, where it appears 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), having lasting effects on the ecosystem. Furthermore, the presence of excipients in commercial formulations may stabilize the active ingredient retarding the degradation and increasing the capacity of affecting other environmental compartments (EPA, 2022a). The selectivity and security of biopesticides are not absolute, as they can lead to toxicity scenarios.

I.1.3. Terrestrial organisms as indicators of soil health

Soil is a vast and rich ecosystem serving as habitat to several species, important to socioeconomic, cultural, and environmental development. This balance is endangered due to human activities, such as intensive agricultural practices and other anthropogenic pressures (e.g., deforestation, industrial contamination, mining activities), risking contamination and alteration of soil functions (e.g., carbon storage, nutrient and water cycling and storage, biomass production) (Calisi et al., 2019; Giannakis et al., 2017). Given these risks, it becomes essential to monitor the ecological and functional aspects of the soil, by developing chemical analysis methods and biological assessment techniques (Wong et al., 2018).

It is important to select appropriate methodologies and strategies to enhance the quality of the data obtained, and the reliability of the results. Chemical analysis is not enough to assess an ecological paradigm, rather the use of resident species as bioindicators can do so. These species should be representative of several habitats, the soil organisms must be relatively sensitive and be in the presence of various stressors to provide accurate information about the effects of pollutants (Rawtani et al., 2016). This is the case of terrestrial oligochaetes and arthropods, which proved to be sensitive bioindicators and easy species to work in laboratory conditions, having their whole life and food cycles in soil, thus being model organisms in ecotoxicology (Correia et al., 2017; Fountain & Hopkin, 2005; Pérès et al., 2011; Reinecke & Reinecke, 2004). A global approach is essential to promote a sustainable use of the terrestrial ecosystem and agriculture, as predicted in the Sustainable Development Goals (SDG) of the United Nations, namely the SDG 2 “No Hunger “and SDG 15 “Life on Land” (UN, 2023).

I.1.3.1. Earthworms *(Eisenia fetida)*

Earthworms belong to the class *Oligochaeta* being widespread organisms in the soil ecosystem (Schmelz, 2018) with a variety of functions from breaking down organic matter and recycling nutrients, to improving soil conditions (construction of channels for drainage, aeration, and root growth) and increase water hold capacity (WHC) (Edwards et al., 2013).

Furthermore, they establish complex symbiotic relations with soil microorganisms (McLean et al., 2006). These organisms are invertebrates with external and internal segmentation; they are hermaphrodites, with the reproductive gonads located in a swollen area of the epidermis termed clitellum, that upon fertilization passes the eggs along the body for them to be expelled as cocoons (Edwards et al., 2013). Even though asexual reproduction is possible, the reproduction usually happens between two individuals, that join the clitella and exchange sperm (Neuhauser et al., 1980). The alimentary canal has two openings, one for food intake and the other for excretion through the anus or the nephridia, and the respiration is mainly cuticular (Edwards et al., 2013). Earthworms possess a large coelomic cavity occupied with coelomocytes, protective cells that play immune functions such as secretion of a toxin, lysenin, capable of permeabilizing and provoking cellular lysis of invader pathogens (Bruhn et al., 2006), but also phagocytosis and production of several humoral factors (Tahseen, 2009).

A commonly used species of earthworms is *Eisenia fetida*, which have been distinguished as terrestrial model organisms for ecotoxicological assays regarding pollutants on soil (OECD, 1992b; Reinecke & Reinecke, 1996). They are also sensitive to anticholinesterasic compounds (Nunes, 2011; Scott-Fordsmand & Weeks, 2000), meaning they may be sensitive to spinosyns considering their main mechanism of action on target organisms. They are adjusted to decomposing organic matter, such as rotting vegetation and manure, being used commercially for vermicomposting and bait in fishing (Maboeta & Van Rensburg, 2003).

I.1.3.2. Springtails (Folsomia candida)

Springtails (Arthropods: Collembola) are a large family of hexapods and free-living soil organisms, having over 8000 described species (Hopkin, 1997). They are small organisms (up to 17 mm max.) possessing antennas and a posterior abdominal appendage, the furca, which allows them to jump (Janssens, 2007). Their body is composed of three segments, the head capsule, the thorax, from which the walking limbs extend, and the abdomen; they lack a trachea therefore they perform gaseous exchanges through porous cuticles (Davies, 1927). Springtails are sexually differentiated and can reproduce the scattering of spermatophores by male adults to be collected by the gonopore of the females (Kozlowski & Aoxiang, 2006). However, many species reproduce by parthenogenesis, a method that favors reproductive output (Simon et al., 2003). This is the case of *Folsomia candida* (Collembola: Isotomidae), whose population consists of only females (Fountain & Hopkin, 2005). At the ideal temperature (20-21 ºC) *F. candida* needs 21 to 24 days to reach the adult and sexually mature stage, to start depositing eggs; the eggs take 7 to 10 days to

hatch (Fountain & Hopkin, 2005). All life stages of *F. candida* are adapted to survive in dry soil conditions (Hilligsøe & Holmstrup, 2003). These characteristics confer the collembola *F. candida* the ability to be used in laboratory investigations (Achazi et al., 1997; Ronday & Houx, 1996), being recommended in ISO protocols (ISO, 2014). All the particularities of this species and their sensitive reaction to external factors make them a valuable bioindicator organism (Crouau & Moïa, 2006; Ganilho et al., 2022).

I.1.4. Ecotoxicological assessment

The term "ecotoxicology" was portrayed in 1969 by René Truhaut, defining it as "*the branch of toxicology which studies the toxic effects caused by natural substances or by artificial pollutants on living organisms whether animal or vegetable, terrestrial or aquatic, which constitute the biosphere. It also relates to the interaction of these substances with the physical environment in which these organisms live."* (Truhaut, 1975). This came up as a junction between ecology and toxicology; the term "ecology" was defined by Ernest Haeckel in 1866 as the science that studies the interactions between organism and their environment (McIntosh, 1980); the study of toxicology has been around since the ancient Rome, but the term was only introduced in 1799, coming from the ancient Greek "poisonous", therefore, the study of the properties, adverse effects and the mechanisms of action of toxicants on biological systems (Britannica, 2020). In 1978, John Wiley on behalf of SCOPE (Scientific Committee of Problems of the Environment) defined ecotoxicology as "*The science that studies the toxic effects of environmental stressors (chemical, physic and biological agents) on organisms, particularly on populations and communities of defined ecosystems, including the routes of entrance and transport of the agents and their interactions with the environment.*"(SCOPE, 1978). Hence, ecotoxicology is a broad branch of toxicology that combines elements of ecology with chemistry, biology, and environmental sciences to assess potential risks of toxicants to communities, develop strategies to mitigate them, and evaluate potential impacts caused by stressors (Chapman, 2006). To do so, it becomes necessary to predict the responses of exposed organisms. For that, an array of standard international tests [e.g., ISO (International Organization for Standardization) and OECD (Organization for Economic Cooperation and Development)] have been developed to assess eventual ecotoxicological damage, for example, regarding the reproduction, growth rate, and behavior, but also as a way to detect detrimental effects of chemicals to develop directives and safety measures for their application (Van der Oost et al., 2003; Walker et al., 2005).

Allied with these ecotoxicological assays it has become essential to analyse the biological response of toxicity, this is, the summatory of all alterations of the toxic causes in

the organism and the capacity of the organism to compensate for them (Walker et al., 2005). This should be performed through sensitive methods such as the evaluation of sub-individual responses to the contaminants on different metabolic pathways and physiological functions such as neurotransmission, antioxidant defences, and even energy production routes and storage. By establishing this relation between individual and sub-individual (biomarkers) paraments, a correlation is built between different levels of biological organization, which provides more ecological relevance to the study (Burton Jr, 1991; Lemos, 2021; Lemos et al., 2022; Rohr et al., 2016).

I.1.4.1. Ecotoxicological tools: bioassays

Ecotoxicological bioassays are standard tests with established and well-defined procedures, methods, and guidance. They allow the conduction, analysis, and interpretation of biological assays, capable of being repeatable intra- and interlaboratory with the implementation of clear controls (Taylor & Scroggins, 2013). The standard test's goal is to minimize factors that can be controlled, such as the inherent biological variation among organisms (OECD, 2006; Taylor & Scroggins, 2013). Several standard tests have been developed through the years.

Avoidance assays have as their objective to quickly assess (48 hours) the habitat function of the medium (e.g., soil and artificial soil) and how the test chemical can influence the behaviour of the test organism. This is a sublethal method that reflects the bioavailability of the xenobiotic in which the endpoint is the avoidance behaviour of the organism from the contaminated section (ISO, 2008).

In reproduction tests (long-term assays), the organisms are exposed to a range of concentrations of the test chemical mixed in the medium, e.g., natural soil or artificial soil, for an extended period depending on the species life cycle (e.g. 56 days for *Eisenia fetida* and 28 days for *Folsomia candida*). The concentrations range must be chosen considering sub-lethal and lethal effects over the exposure period. Mortality and growth are also endpoints evaluated in these long-term assays. The reproductive output is compared to that of the control to determine NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) which are based on ANOVA results. The EC_x (Effective Concentration) can also be calculated by the application of regression models to obtain the concentration that would cause x % reduction of the reproductive output (ISO, 2023; OECD, 2016a). The short-term exposure, has a similar experimental design to the reproduction assay but with a reduced time of exposure (48 to 96 hours), in which the effects on survival are determined (ISO, 2012; OECD, 2016a; Sheppard, 1998).

I.1.4.2. Ecotoxicological tools: biomarkers

Biomarkers are a type of sub-individual response (Ferrario et al., 2018), that is defined as any measurable parameter, that refers to the physiological, biochemical, and histological changes used as indicators of exposure to chemical contaminants (or other stressors) and/or their effects before the damage becomes irreversible (Adedeji et al., 2012). Biomarkers are classically divided into three categories: a) biomarkers of exposure – comprehend into markers of internal dose and markers of effective dose in which the former detects and measures an exogenous substance or its metabolite within the organism, and the latter detects and measures products of an interaction between a xenobiotic agent and some target molecule or cell; b) biomarkers of effect – includes both physiological and biochemical markers, e.g., body weight and determination of specific enzymes, respectively; c) biomarkers of susceptibility – the somatic or the genetic capacity of the organism to resist to the exposure of a xenobiotic, including genetic factors and alterations to susceptibility receptors (Timbrell, 1998; Van der Oost et al., 2003). Another division based on physiological parameters was suggested by Peakall (1992): biomarkers of genotoxicity (e.g., breaks on DNA strands), biomarkers of neurotoxicity (e.g., acetylcholinesterase activity), biomarkers of oxidative stress (e.g., quantification of enzyme activities from antioxidant defences), biomarkers of biotransformation (e.g., quantification of enzyme activities from biotransformation phases I and II) and biomarkers of energetic metabolism (e.g., quantification of energetic reserves like lipids and glycogen content), among others (Peakall, 1992). Biomarkers are sensitive tools that allow simple and standardized quantification (Van der Oost et al., 2003), and have been used both *in vivo* and *in vitro* to evaluate the effects of a specific xenobiotic in the organism (Binelli et al., 2006). Over recent decades, to investigate the sub-lethal chronic effects of environmental pollutants, biomarkers have been used as early warning tools in the assessment of the environmental quality of soils impacted by agriculture (Biondi et al., 2012; Reinecke & Reinecke, 1996; Soares et al., 2019).

I.1.4.2.1 Biomarkers of Genotoxicity

Genotoxicity, or genetic toxicity, can entail the toxic effects of xenobiotics and physical agents on erythrocytes and DNA (deoxyribonucleic acid), induced in different target tissues and organs, causing alteration and targeting the genome integrity, respectively (Beedanagari et al., 2014). Genetic material is an important target for stressors since it can cause disruption of cellular metabolism and modify the DNA molecule itself, bringing adverse outcomes (Jan, 2001). The outcome of the action of these genotoxic agents can

be mutations or even chromosomic damage, such as the formation of adducts, single and double-strand breaks on the DNA chain, nucleobases alteration, intra and inter-strand crosslinks and crosslinks between DNA and protein (Roos et al., 2016). There are endogenous cellular mechanisms of reparation that act on naturally occurring damage (Miller & Millere, 1966; Skipper et al., 2016). However, the presence of genotoxic agents enhances the damage causing the described outcomes, which may eventually lead to cellular death, by apoptosis or necrosis, and in extreme cases, initiate carcinogenic activities (Roos & Kaina, 2006).

To measure the genotoxic activity of various stressors there is a technique referred to as single-cell gel electrophoresis (SCGE) or simply “comet assay” due to comet-like structures formed at the end (Liao et al., 2009). This method is a quick and simple tool to assess DNA integrity, without having to perform DNA extraction and purification, and only using a small number of cells per sample (Kumaravel et al., 2009). There are two possible outcomes: 1) supercoiled DNA and no visible tail and 2) comet-like structure tail. The former means no damage in the DNA occurred, and the latter shows damage in the genetic material, such as DNA breaks and loose loops, in which the bigger the tail the bigger the damage (Lourenço et al., 2012; Reinecke & Reinecke, 2004).

This type of methodology has been widely used in environmental impact assessment studies, in model organisms from different ecosystems and trophic levels, and even successfully applied to soil invertebrates. (e.g., earthworms) (Gajski et al., 2019). In fact, focusing on the latter, the assessment of genotoxicity is used to evaluate the eventual toxic effects of pollutants and xenobiotics in genetic material (Bolognesi et al., 2019; Dhawan et al., 2009; Jha, 2008).

I.1.4.2.2 Biomarkers of Neurotoxicity

Biomarkers of neurotransmission, especially the assessment of acetylcholinesterase (AChE) activity, are one of the most used to determine neurotoxic effects in environmental settings. AChE is an enzyme that cleaves the neurotransmitter acetylcholine (ACh) into choline and acetic acid, putting an end to the nerve impulse (Nunes, 2011). This enzyme is present in most nervous tissues and neuro-muscular junctions and plays a vital role in physiological regulation (Liu et al., 2014). This enzyme’s activity can be compromised by some xenobiotics present in the environment, and its assessment was initially specific for organophosphates and carbamates, but throughout the years it was shown that AChE activity was also sensitive to other compounds (Feng et al., 2008; Guilhermino et al., 1996). The impairment of AChE causes accumulation of ACh in the synaptic fend, leading to overstimulation of the receptors. This causes a blockage of the neurotransmission leading

to systematic effects like muscle weakness, hyperactivity, loss of coordination, paralysis, convulsion, or even death (Hayden et al., 2010; Nunes, 2011). Furthermore, the activity of AChE has been used as a biomarker for the presence of different pesticides and is considered to be a reliable biomarker of exposure to non-target organisms (Nunes et al., 2005; Schiedek et al., 2006). It was found that decreases in AChE activity can be correlated with the presence of high concentrations of reactive oxygen species (ROS) and linked to lipid peroxidation (Wang et al., 2009b), and an increase in its activity was associated with neurodegeneration (Nunes, 2011). The decrease of at least 20 % of AChE's activity is considered significant and a toxicological effect of chemical exposure, as stated by US EPA (1998).

I.1.4.2.3. Biomarkers from Antioxidant defences

Several pollutants, or their by-products, can act on the organism causing toxicity through the production of ROS, leading to disturbances in oxidative homeostasis. In fact, xenobiotics can trigger the production of ROS by interfering with the redox cycling and molecular oxygen (O_2), creating species as the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), peroxyl radical (ROO·), nitric oxide (NO·) and singlet oxygen ($_1O2$) (Kaur et al., 2019). These molecular forms with unpaired electrons may cause injuries to tissues, target enzymes by deactivating them, lipid peroxidation (LPO) on cellular membranes and genotoxicity, leading to cellular disruption and apoptosis (Winston & Di Giulio, 1991). The effect of excessive production of ROS and/or the inadequacy of the antioxidant cellular defence systems and detoxification mechanisms to neutralize them is commonly referred to as oxidative stress (Ma et al., 2019). To maintain oxidative homeostasis, the organisms possess mechanistic defences to balance ROS levels. They can be divided into enzymatic defences – e.g., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GRed) – and non-enzymatic defences – e.g., glutathione (GSH) a tripeptide consisting of l-γ-glutamyl-l-cysteinyl-glycine, ascorbate (vitamin C) and β-carotene (vitamin B) (Winston & Di Giulio, 1991).

SOD acts as a first-line defence by facilitating the conversion of $O_2\bullet-$ into H_2O_2 (Fukai & Ushio-Fukai, 2011). CAT acts upon SOD in breaking down hydrogen peroxide, preventing its accumulation (Nandi et al., 2019). GPx functions as a protective agent by scavenging high levels of hydrogen peroxide, which catalyses it in oxygen and water, but in the process, glutathione is oxidized and loses its protective properties (Fanucchi, 2014). GRed enters as a restoring reducing enzyme for GSH, resulting in the production of two molecules of reduced glutathione ready to perform as a defence (Fanucchi, 2014). Besides its use as a

proton source, reduced glutathione is also a conjugation factor in a reaction catalysed by glutathione-S-transferases (GSTs). This enzyme is involved in phase II biotransformation processes, in which it conjugates GSH with electrophilic compounds (xenobiotics or its by-products) to solubilize them, but also with a wide range of organic hydroperoxides, possessing both detoxification and antioxidant protective functions (Singh & Reindl, 2021). The activity of these enzymes can be measured and assessed as biomarkers of oxidative stress.

I.1.4.2.4. Biomarkers of Cellular Damage

Even though these described defences possess strong induction upon exposure to pollutants, oxidative damage can occur leading to cellular disruption and lipoperoxidation (LPO) (Zhang et al., 2013). This is a process in which the ROS acquire electrons from membrane lipids, destabilizing the membrane and creating new lipidic radicals (L· and LOO·), which cause propagation to neighbouring molecules, and untimely leading to cell damage (Kappus, 1987). This damage can be measured through the presence of by-products, reflected in the levels of thiobarbituric acid reactive substances (TBARS) (Deng et al., 2004), being a common oxidative damage biomarker.

I.1.4.2.5. Biomarkers of Energy Metabolism

To fully understand the toxic outcomes of a given chemical for the general paradigm of the organism, the energetic status needs to be considered. Therefore, it becomes important to assess the energetic reserves and production pathways as physiological markers when testing for toxic effects, since various routes might be affected, impairing the organism's survival and physiology (Vidal et al., 2002). This is the case of lipidic reserves. If the lipid content is reduced, it can lead to problems related to energy storage, vitamin and hormone pathways, and transmission of nerve impulses (Koukouzika & Dimitriadis, 2008), ultimately affecting the normal physiology of the organism. On the other hand, an increase in the levels of lipids can lead to a dysregulation in the homeostasis through the imbalance in the endoplasmic reticulum, responsible for the correct processing of proteins (Hapala et al., 2011). These changes can affect other metabolic routes and skew the organism´s homeostasis. The other important energetic reserve that should be assessed is the glycogen content. This multibranched polysaccharide of glucose is used to store energy and is found in many organs, such as the liver and muscle, being promptly located for immediate use if necessary (Kanungo et al., 2018). Changes in the levels of this carbohydrate can be provoked by pollutants. For instance, an increase in glycogen reserves

could mean an adaptation to changes in the ecosystem such as food depletion, heat shock, or osmotic stress (Silljé et al., 1999); inversely, decreased glycogen levels can be a sign of glycogen degradation to glucose to maintain basic cellular functions, counteracting the stress posed by xenobiotics (Becker et al., 2009).

Lactate dehydrogenase (LDH) is an enzyme that reflects the metabolic rate of the exposed organisms. LDH is fundamental to respiratory and energetic physiology and is involved in anaerobic glycolysis, mainly when high levels of energy are necessary quickly or in conditions of low oxygenation (Khan et al., 2020). In addition, chemical stress seems also to be able to modulate its activity, since some studies have shown changes in their activity, namely by exposure to environmental stressors (Nunes et al., 2004; Wu & Lam, 1997), such as pesticides (Lammertyn et al., 2021). This important enzyme catalyses the reversible conversion of lactate to pyruvate with the reduction of NAD^+ to NADH and vice versa (Farhana & Lappin, 2022). In anaerobic or hypoxic conditions, cells cannot perform oxidative phosphorylation due to the lack of oxygen, the energy production shifts towards glycolysis, and LDH is upregulated to ensure this pathway (Farhana & Lappin, 2022). Hence, the *ldh* gene is upregulated by hypoxia, as well as the gluconeogenesis pathway (Pillet et al., 2016; Zhao et al., 2020a). LDH plays an important role in gluconeogenesis, this is, the transformation of pyruvate in glucose in the liver for energy; lactate produced in muscles and erythrocytes may be oxidized by LDH as a substrate for gluconeogenesis (Omlin & Weber, 2010). A recent study performed by Lammertyn and their team on the effects of an herbicide (atrazine) on *Eisenia fetida* (Lammertyn et al., 2021) demonstrated that LDH activity was increased upon exposure. The outcome of LDH activity on *E. fetida* is dependent on the type of pesticide and concentrations used (Rico et al., 2016), as well as, the feeding conditions and habitat (Diehl & Collier, 1991; Tripathi et al., 2011). In general, the feeding conditions may interfere with the parameters related with energetic reserves (Moura et al., 2021; Salvio et al., 2016); for instance, oligochaetes laboratory culture's medium contains peat and autoclaved horse manure, and they are fed weakly with oat; however, during a reproduction (long-term) assay, the organisms are exposed to natural soil and fed weakly only with autoclaved horse manure (ISO, 2023). This change in their environment/diet can lead to differences in the basal levels of some parameters (Shi et al., 2017).

The use of a multibiomarker approach has been growing in environmental and risk assessment investigations, being adopted by several authors (Adedeji et al., 2012; Van der Oost et al., 2003). It is important to assess several of the above-described parameters to obtain a broader scope of the toxicological effects of xenobiotics and perform a multivariate conjugated analysis (López-Barea, 1995; Walker, 1995). This analysis should be performed in sentinel organisms, that is, species that can be affected by the pollutant, and give

information about its toxicity, considering an actual scenario of contamination (Adams et al., 2001; Gagne, 2014).

I.2. Objectives and Dissertation Structure

The goal of the present dissertation was to obtain relevant ecotoxicological data regarding a commercial formulation of the bioinsecticide Spintor® and the corresponding active ingredient Spinosad, and their potential deleterious effects on non-target soil organisms. The existing substances' regulatory reports lack information regarding lower exposure times and environmental conditions, therefore, this work ought to bring to light new environmentally relevant information for regulation and risk assessment for soil-dwellers. For this purpose, two model organisms in terrestrial ecotoxicology were selected – earthworms *Eisenia fetida* and springtails *Folsomia candida.*

Therefore, the specific objectives for this dissertation were:

- Assess the avoidance behaviour of *E. fetida* after 2-days of exposure in soil contaminated with a range of concentrations of the active ingredient Spinosad or the commercial formulation Spintor® against non-contaminated soil.
- Evaluate the reproductive performance of *E. fetida* and *F. candida,* under contaminated soil with Spintor® and Spinosad.
- Perform short- and long-term exposures (2- and 28-days) with *E. fetida* using a range of concentrations of Spintor® and Spinosad, in order to assess sub-individual parameters. For this purpose, a battery of biomarkers regarding genotoxicity (DNA damage in coelomocytes), neurotransmission (AChE activity), antioxidant defences (SOD, CAT, GPx, GRed and GSTs activities and GSH content), lipid peroxidation (TBARS levels) and energy-related metabolism (lipidic and glycogen content and LDH activity) were analysed.
- 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®.

The dissertation is organized into five chapters. The first one corresponds to a general introduction in which the themes of the work, a literature review, the gap of knowledge, the novelty of the study, and the main objectives are presented. The Chapter II encloses the materials and methods employed in this work. In Chapter III the results of the previously described goals and developed work are presented, which are posteriorly discussed in Chapter IV. Finally, Chapter V encloses final considerations, finalizing the comparison between Spintor® and Spinosad, and presents future perspectives on this theme.

II. Materials and Methods

II.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 use, the soil was defaunated by performing one cycle of freezing (at -20 ºC) and drying (at 40 ºC) and sieved with a 4 mm mesh size for the ecotoxicological assays. The soil physico-chemical parameters are described in Table 1, and this was evaluated using a 2-mm dried soil fraction. All parameters were measured in triplicate.

The pH was determined in a soil-deionized water suspension using a 1:5 (v/v) ratio. After approximately 60 minutes of agitation, the suspension was left undisturbed for 60 minutes before pH measurement using a pre-calibrated multiparameter device (Edge, HANNA Instruments) (ISO, 2021).

Table 1: Physico-chemical properties of the natural soil (mean values ± standard deviation).

Natural Soil	pH	EC* (mS/cm)	OM* (%)	WHC_{max}* (%)
	6.25 ± 0.11	0.770 ± 0.045	5.21 ± 0.15	43.00 ± 0.15

* Data reported by Ganilho et al., 2022.

A standard artificial soil composed by a mixture of 70 % sand, 20 % kaolin, 10 % sphagnum peat, and 0.30 $g.kg^{-1}$ of $CaCO_3$ (calcium carbonate) to adjust the pH value at 6.0 ± 0.5, was prepared in the laboratory following the recommendation of the standard guideline (OECD, 1984). This soil was only used to validate the ecotoxicological assay according to the recommendations of OECD standards (OECD, 1984).

II.2. Test organisms (*Eisenia fetida* and *Folsomia candida*) and culture conditions

The earthworms species of the genus *Eisenia* (Oligochaete) are commonly used as suitable indicators of soil health due to their internal (via ingestion) and external (via dermal contact) exposure to contaminants (ISO, 2023; OECD, 2016a). These organisms are continuously breeding, producing cocoons throughout the year (Edwards et al., 2013), and can be maintained in the laboratory under controlled conditions, which makes them suitable species to be used in ecotoxicological assays. The species of soil-dwelling collembola *Folsomia candida* (Arthropod: Isotomidiae) are regularly used in ecotoxicological testing (Domene et al., 2011). These are hexapods with a thin exoskeleton that is highly permeable to water and air, representing arthropod species and possessing different exposure rates compared to earthworms (ISO, 2014; OECD, 2016b).

The earthworm *E. fetida* used in this work was obtained from laboratory cultures maintained in a controlled environment at a temperature of 20 ± 2 °C and a photoperiod of 16 hours of light and 8 hours of darkness. 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. In accordance with internationally recognized standards (ISO, 2023), only adult earthworms with well-developed clitellum and body mass ranging from 300 to 600 mg were chosen for the assays. Prior to the experiment, the organisms underwent acclimation for a minimum of 24 hours in plastic containers containing the test soil, in a controlled environment, at a temperature of 20 ± 2°C and a photoperiod of $16h^{L}$:$8h^{D}$.

The collembola *F. candida* was maintained in plastic containers filled with a culture medium consisting of moistened plaster mixed with activated charcoal in an 8:1 ratio (w:w). The cultures are provided with granulated dry yeast as their food source, twice a week, with half a teaspoon added each time. The cultures are synchronized to obtain juveniles aged between 9 and 12 days, which are used for the assays.

II.3. Insecticide and soil contamination

Spintor® (SPIT) (Qalcova™active), a liquid commercial formulation of the insecticide spinosad containing 480 $g.L^{-1}$ of the active ingredient, was acquired from Lusosem®. Spinosad (Spinosad PESTANAL®, analytical standard) (SPIN) in its active ingredient (a.i.) powder form 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 several biological compartments (Bueno & Cunha, 2020; Lefkaditis et al., 2017; Pur & Tunaz, 2022). To determine the concentrations, it was considered the area of the pots (0.011 m^2) filled with 200 g of dry soil used in plant assays, which corresponds to the application dose of 0.66 $mg.kg^{-1}$ of $soil_{dw}$ (dw = dry weight) (Soares et al., 2023). A fresh stock solution of Spintor® (480 mg/L) was prepared using deionized water to obtain the following range of concentrations: 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. These concentrations were used in avoidance, short-term, and reproduction/long-term assays. Moreover, for Spinosad the same concentrations were defined. 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 all the assays.

II.4. Ecotoxicological assessment

II.4.1. Avoidance Assay with *Eisenia fetida*

The avoidance assay was used to assess the behaviour-related reaction in an ecologically valid way by measuring organisms' ability to detect and avoid contaminated soils (ISO, 2008). For this purpose, the standardized avoidance behavioural test was used to assess the sublethal effect of Spintor® and Spinosad on the earthworms *Eisenia fetida* (ISO, 2008).

The avoidance experiments with *E. fetida* were performed in rectangular (1370.7 cm^3) plastic boxes with perforated lids in five replicates. A plastic divider was used to separate the boxes into two equal parts, one compartment was filled with 200 g of moistened contaminated soil (to test the above-described range of insecticide concentrations, 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) (Fig. 2).

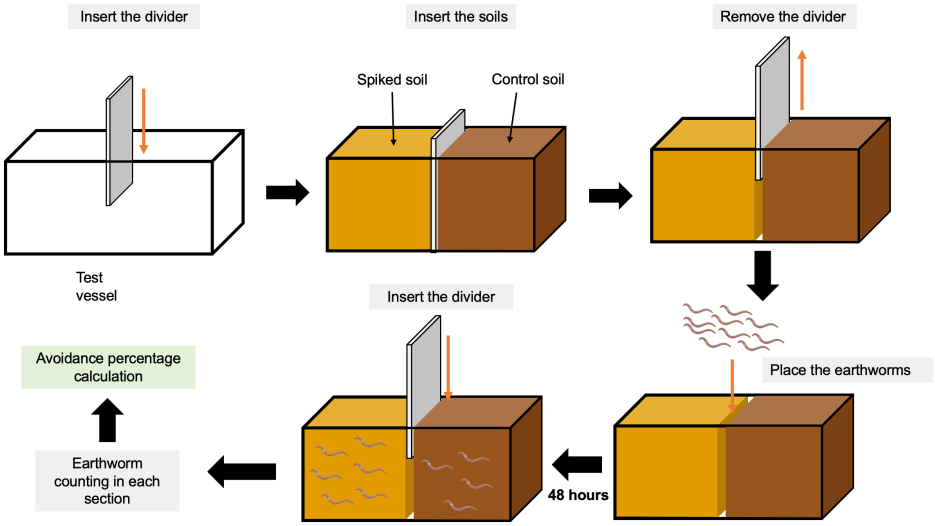

Fig. 2: Experimental design for avoidance assay with *E. fetida.*

In each of the 5 replicates per concentration, ten adult earthworms weighing between 300 and 600 mg 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 (five replicates) were conducted at the same time to confirm that earthworms in the two portions were distributed randomly. Throughout the assay period (48 h), the boxes were kept in controlled conditions

(temperature: 20 ± 2 ºC; photoperiod of $16h^L$: $8h^D$), similar to those adopted during the culture conditions.

At the end of exposure, the plastic divider was repositioned again in the center and the number of earthworms in 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 formula:

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

C - number of organisms observed in the control soil (spiked only with distilled water);
T - number of organisms observed in the test soil per concentration (spiked with an insecticide suspension);
N - total number of organisms per replicate.

II.4.2. Short-term exposure with *Eisenia fetida*

To evaluate the effects of SPIT and SPIN on several biomarkers of *E. fetida* upon a short-term exposure (2-days), the ISO protocol 11268-1 for acute toxicity was followed (ISO, 2012). The exposure was achieved in plastic test containers (1397.7 cm^3), in which three replicates were made for all treatment with contaminated 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 non-contaminated soils (CTL). They were maintained in controlled conditions (temperature: 20 ± 2 ºC; photoperiod of $16h^L$: $8h^D$). Each replicate contained 500 g of moistened soil and 10 worms with fresh weights ranging from 300 to 600 mg selected from the lab-maintained culture (Fig. 3) and acclimatized for at least 24 h (maximum of 48 h) in test soil.

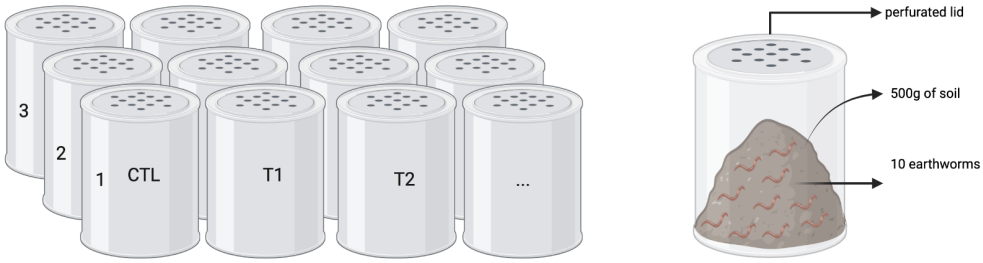

Fig. 3: Scheme of the test containers for the short-term assay with *E. fetida.*

After 48 h, the earthworms were removed from the test containers and counted (Fig. 4). Furthermore, 5 organisms per replicate were randomly chosen for genotoxicity assessment, and the remaining 5 were frozen in liquid nitrogen and kept at -80 ºC for posterior analysis of biochemical biomarkers.

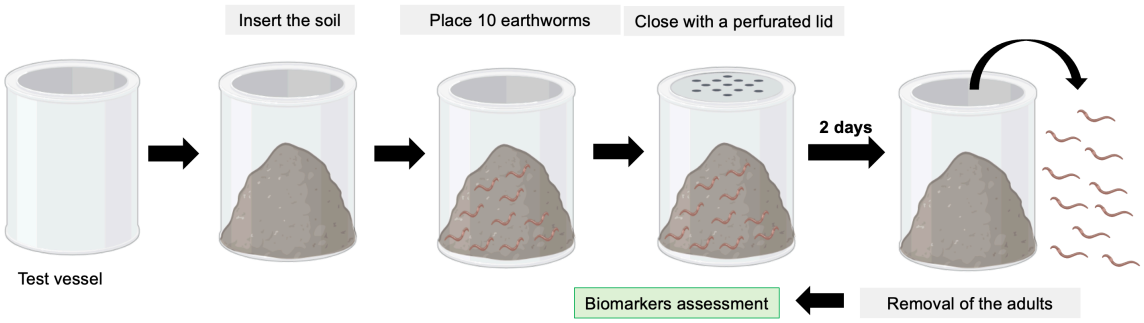

Fig. 4: Experimental design for the short-term assay with *E. fetida.*

II.4.3. Reproduction Assay with *Eisenia fetida* and *Folsomia candida*

II.4.3.1. *Eisenia fetida*

The impact of Spintor® and its active ingredient Spinosad on *E. fetida* reproductive activity was assessed following the methodology described in the standard protocol ISO 11268-2 (ISO, 2023). The exposure of *E. fetida* was performed in plastic test containers (1397.7 cm^3), filled with 500 g of moistened contaminated 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 non-contaminated soil (CTL), in five replicates and maintained in controlled conditions of temperature (20 ± 2ºC) and photoperiod ($16h^L$:$8h^D$). Ten worms with fully formed clitella and fresh weights ranging from 300 to 600 mg were selected from the lab-maintained culture and acclimatized for at least 24 h (maximum of 48 h) in test soil. An extra control with five replicates of non-contaminated moistened OECD soil was also made to validate the assay, following the recommendation of standard protocol (ISO, 2023). The assay was controlled weekly to adjust the moisture in each replicate and to feed the worms with approximately five grams of defaunated horse manure in each replicate (ISO, 2023) (Fig. 5).

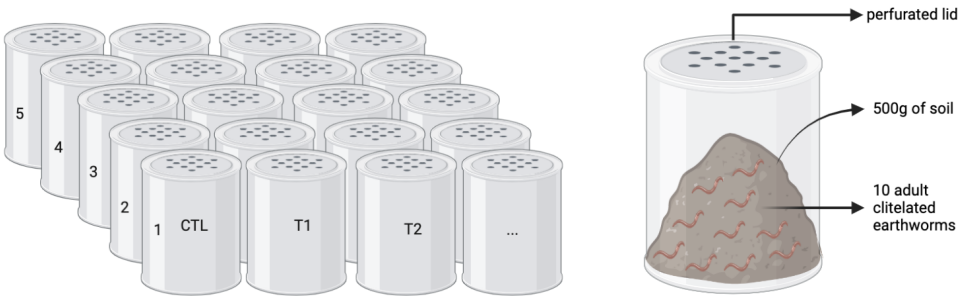

Fig. 5: Scheme of the test containers for reproduction assay with *E. fetida.*

After 28 days of exposure, the adult earthworms were removed from the test containers (ISO, 2023). Five of the adult 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 ºC for posterior biomarkers assessment. Since these earthworms were exposed to SPIT or SPIN for 28 days, the results were handled as the output of a long-term exposure to the bioinsecticide. The soil containing cocoons and juveniles was left undisturbed to complete the 56 days, according to the guideline (ISO, 2023) (Fig. 6).

On day 56, the number of juveniles in each replicate was recorded. To fulfill the validity criteria of the assay (ISO, 2023), the adult mortality could not be superior to 10%, and the number of juveniles needed to be ≥ 30 juveniles for each replicate in the controls (OECD and natural soil) by the end of the 56 days. Furthermore, a coefficient of variation of reproduction of ≤ 30% should be respected (ISO, 2023).

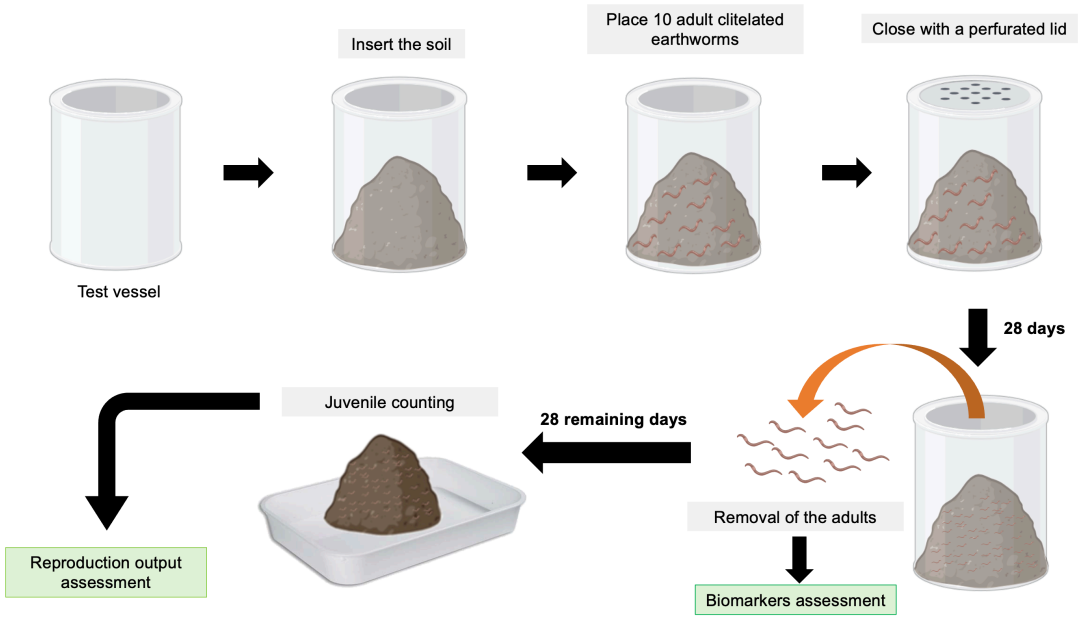

Fig. 6: Experimental design for reproduction assay with *E. fetida.*

II.4.3.2. *Folsomia candida*

The potential toxicity of SPIT and SPIN on the reproductive output of the soil arthropod *Folsomia candida* was also assessed**,** following ISO protocol 11267 (ISO, 2014). For that, organisms with ages between 9-12 days old were obtained from laboratory synchronized cultures. The experimental design included 5 replicates per concentration (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 5 replicates of non-contaminated soil as control (CTL). To each vessel, 30 grams of $soil_{dw}$ were added. 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 humidity 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 in the surface. A few drops of dark ink were added to enhance the contrast of the white individuals (Fig. 7) (Bouguerra et al., 2016). Pictures of each replicate were obtained from above, using a digital camera, for posterior counting with the ImageJ software (http://imagej.nih.gov/ij/). The exposure conditions (temperature and photoperiod) were the same as described above for *E. fetida.* To be considered a valid assay, some criteria must be met in the control. Thus, the adult's mortality must be inferior to 20%, a minimum of 100 juveniles per replicate must be checked, as well as a coefficient of variability no higher than 30 % (ISO, 2014).

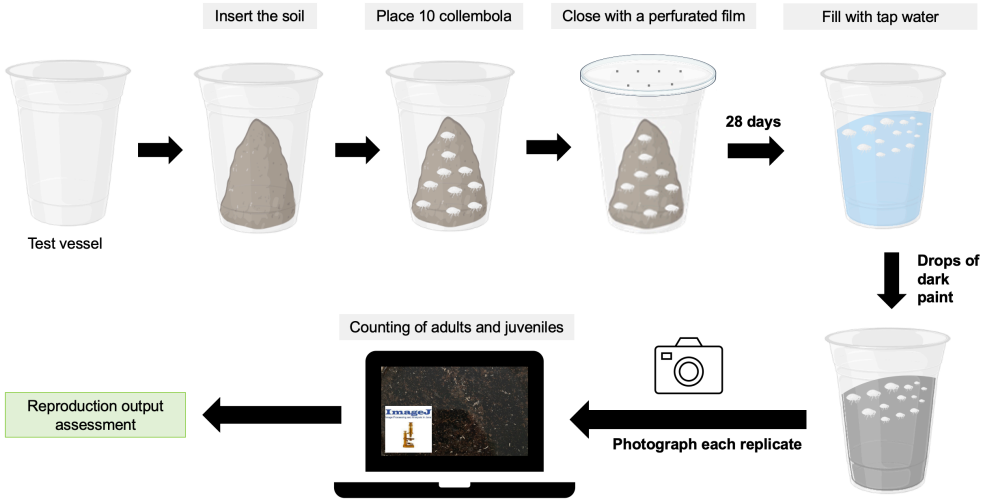

Fig. 7: Experimental design for reproduction assay with *F. candida.*

II.5. Biomarkers assessment in *Eisenia fetida*

II.5.1. 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 their digestive systems. 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* (for more detailed information see Fernandes et al. (2020), Lourenço et al. (2012), and Reinecke and Reinecke (2004)).

After letting the slides dry overnight, they were coloured using 20 µg/mL ethidium bromide and observed in a fluorescence microscope (amplification 400x). Cells were observed under a Nikon DS-Ri2 fluorescence microscope. The classification of 100 randomly chosen cells served as the basis for the visual scoring of cellular DNA on each

slide. According to DNA damage, the comet-like structures were visually categorized into five classes: 0 (no damage, round, and intact nucleus) to 4 (small nucleus and almost all DNA is fragmented forming a comet structure), according to Fig. 8. At the end of the counting, a pondered mean is calculated for each slide, in arbitrary units (AU), using the following formula:

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

Where N0, N1, N2, N3 and N4 are the numbers of comets in classes 0, 1, 2, 3 and 4, respectively. All ratings were made blind and by the same person.

Fig. 8: DNA damage classes in the coelomocytes (400x magnification): (a) class 0; (b) class 1; (c) class 2; (d) class 3; (e) class 4. Retrieved from Fernandes et al. (2020).

II.5.2. Biochemical biomarkers

II.5.2.1. *Biological samples collection and preparation*

After the short- and long-term exposure periods, the oligochaetes were euthanized by being flash-frozen in liquid nitrogen, divided in falcon tubes, and preserved at -80 ºC for posterior biochemical analysis.

For the analysis of the enzymatic activities of SOD, CAT, GPx, GRed, and GSTs, and the levels of GSH, TBARS, and 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 15000 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.

To calculate lipidic content, one individual per replicate was homogenized in 5 mL of a mixture of chloroform and methanol at 2:1 and centrifuged at 1000 g for 10 minutes. The supernatant was used to quantify the lipidic content.

For LDH activity determinations, one individual 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 minutes at 4°C. The resulting supernatants were collected and used for enzymatic analysis.

To determine AChE activity, one individual 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 minutes at 4°C. The supernatants were preserved and used for enzymatic analysis.

II.5.2.2. *Biochemical determinations*

All spectrophotometric measurements were performed in quadruplicate (except TBARS levels, made in triplicate) in a microplate reader Thermo Scientific, model Multiskan GO, version 1.00.40, with SkanIt Software 3.2.

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) as a standard. All biochemical biomarkers were then expressed per milligram of protein.

The determination of total SOD activity was performed following the Flohé methodology (Flohé & Ötting, 1984). A system of xanthine and xanthine-oxidase is used to produce superoxide radicals ($O_2^{\cdot}$-). The subsequent reduction of cytochrome c by $O_2^{\cdot -}$ was followed at 550 nm. The enzymatic activity was expressed in units 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), in which the breakdown of H_2O_2 to water and O_2 causes a drop in absorbance at a wavelength of 240 nm. 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. Cumene hydroperoxide was used as a substrate for the reaction, measured at a wavelength of 340 nm. 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. GRed mediated the oxidation of NADPH, and this reaction was followed at wavelength 340 nm. The final enzymatic activity was expressed as micromoles of NADPH oxidized per minute, per milligram of protein.

The quantification of GSH was performed based on (Diogo et al., 2023a). The GSH content was evaluated spectrophotometrically (wavelength 412 nm) in microplates, using glutathione (100 µmol/L) as standard.

According to Habig et al. (1974), GSTs activity was measured and expressed as millimoles of thioeter generated per minute per milligram of protein. GSTs catalyse the conjugation of the electrophilic center substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, resulting in the production of a thioeter, which is accompanied by an increase in absorbance at 340 nm.

The quantification of substances reactive to thiobarbituric acid (TBARS), according to Buege and Aust (1978) was a method used to determine lipid peroxidation (LPO). TBARS levels were measured at a 535 nm wavelength. The results were expressed as millimole of malondialdehyde (MDA) equivalents per milligram of protein.

The determination of glycogen content was achieved following Lo et al. (1970) methodology. This technique is based on the final measurement of the resulting glucose with ortho-toluidine at wavelength 490 nm, using glycogen (100 μg/mL) as standard.

The total lipidic content was effectuated following Folch et al. (1957). The results were expressed in % of total lipids content, based on the weight of the individual.

Determination of LDH activity was performed following the method of Vassault (1983). In the presence of pyruvate, lactate dehydrogenase causes the oxidation of the reduced form of nicotinamide adenine dinucleotide (NADH), at a wavelength of 340 nm. LDH activity followed the decrease of absorbance caused by the oxidation process and was expressed in millimoles of β-NADH oxidized per minute, per milligram of protein.

The determination of AChE activity followed a protocol established by Ellman et al. (1961). AChE hydrolyses the substrate acetylthiocholine forming thiocholine. Thiocholine reacts with 5-5'-dithio-bis-2-nitrobenzoate (DTNB), forming a complex capable of absorbing radiation at the wavelength of 412 nm. AChE activity was expressed as moles per minute per milligram of protein.

II.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 treatment, 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. 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 and the corresponding confidence limits (95%) were calculated using the software StatSoft Statistica v8. A significance level of $\alpha=0.05$ was considered.

For an integrated analysis, 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 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 the analysed biomarkers (organized in the following clockwise order: GSTs, TBARS, CAT, GSH, GLY, GPx, SOD, AChE, LDH, LIP, GRed, and Comet) to evaluate SPIT or SPIN effects upon short-term 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). To estimate the IBRv2 index, the following calculations were performed:

mean X0 – stands for the mean results of each biomarker.

Yi = log (Xi/mean X0), where Xi is the data of each biomarker in each treatment (or replicate). After that, the general mean (μ) and standard deviation (s) of Yi for each biomarker were calculated.

$$Zi = (Yi - \mu)/s.$$

$$A = \text{mean } Zi - \text{mean } Z0 \text{ (control)}$$

$$IBRv2=\sum|A|$$

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.

III. Results

III.1. Individual responses in ecotoxicological bioassays

The avoidance assay outputs for *E. fetida* are shown in Fig. 9. The assay with SPIT (Spintor®) revealed significant avoidance from the contaminated soil at the concentrations 0.44 (p = 0.0015), 0.66 (p = 0.0062) and 1.49 (p = 0.0067) mg.kg^{-1} of soil$_{dw}$ in Fisher's exact test. Therefore, a maximum of 66.0 % of avoidance of *E. fetida* to SPIT was recorded. NOEC and LOEC values for avoidance were obtained based on ANOVA analysis ($F_{[7, 32]}$ = 2.818 and p = 0.0210), respectively being, 0.29 and 0.44 mg.kg^{-1} of soil$_{dw}$. For SPIN (Spinosad), there was no significant avoidance registered.

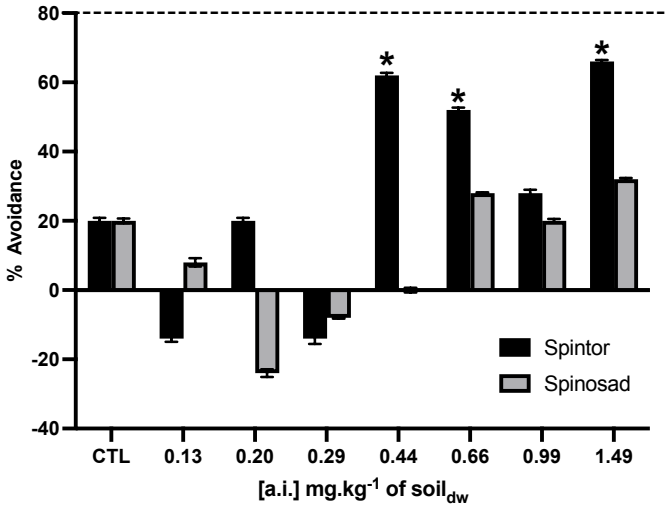

Fig. 9: Avoidance response (%) of *E. fetida*, exposed to soil spiked with different concentrations of Spintor® and Spinosad, after 2 d of exposure. Data are expressed as mean ± standard error (SE). * - Stands for significant higer percentage of organisms on the control side than on the tested side (p < 0.05).

The results from individual responses on reproduction outputs for *Eisenia fetida* and *Folsomia candida*, with Spintor® and Spinosad are shown in Fig. 10. Regarding *E. fetida*, in the reproduction assay with SPIT and SPIN, the Dunnett's test did not show any significant differences between the treatments and the control groups ($F_{[7, 32]}$ = 3.063 and p = 0.0139 for SPIT; $H_{[7]}$ = 1.114 and p = 0.9928 for SPIN).

Regarding *Folsomia candida* reproductive output, the validity criteria stipulated in the guideline (ISO, 2014) were met. Table 2 displays the values of EC_{10}, EC_{20}, and EC_{50} for SPIT®. It can be inferred that the use of SPIT® led to a significant decrease in the number of produced juveniles in the highest concentrations ($F_{[7, 32]}$ = 12.60; p < 0.0001), recording values of 0.44, 0.66 and 0.98 mg.kg^{-1} for NOEC, LOEC and EC_{50}, respectively (Fig. 10 and Table 2). For the assay with SPIN, all the treatments demonstrated a significant decrease in the production of juveniles ($F_{[7, 32]}$ = 6.559; p < 0.0001), recording a NOEC of < 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 assay with SPIN.

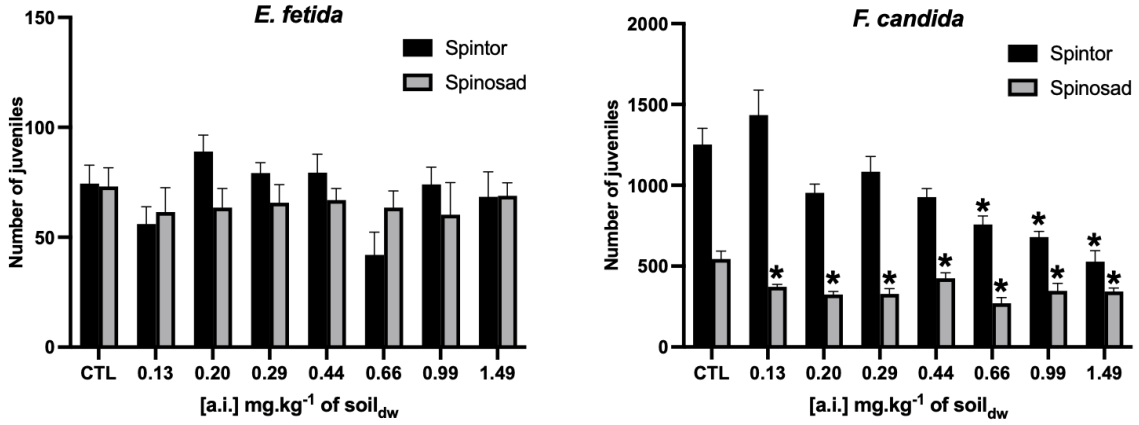

Fig. 10: Reproductive output of *E. fetida* (left) and *F. candida* (right) after exposure to 56 and 28 days of exposure, respectively, to soil spiked with different concentrations of Spintor® and Spinosad. Data are expressed as mean ± standard error (SE). * - Stands for significant differences when compared to control ($p < 0.05$).

Table 2: Effective concentrations (EC_{10}, $EC_{20,}$ and EC_{50} values) of Spintor® in mg.kg^{-1} of soil$_{dw}$ and the respective 95 % confidence intervals obtained for the reproductive output (number of juveniles) of *Folsomia candida*.

	EC_{10}	**EC_{20}**	**EC_{50}**
Spintor®	0.15	0.30	0.98
95 % confidence interval	[0.08 - 0.52]	[-0.01 - 0.30]	[0.62 - 1.35]

III.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. 11. The analysis of biochemical biomarkers after 2-days exposure of *E. fetida* showed significant differences in SOD, GPx, GRed, GSTs, LDH and AChE activities, GSH content and TBARS levels when compared to the control treatment. In addition, the results exhibited that short-term exposure to SPIT may cause DNA damage in earthworms even at low levels. In the case of 28-days exposure, despite the significant effects observed in SOD, GPx, GRed, GSTs, LDH, and AChE activities, TBARS levels and glycogen and lipids contents, the exposure to SPIT didn't cause DNA damage in the tested concentrations.

Regarding the biochemical markers of antioxidant defence, while no significant changes in catalase activity were registered in both short-term and long-term exposure of organisms, SOD activity showed significant overexpression in the concentration 0.20 $mg.kg^{-1}$ of $soil_{dw}$ in both exposure periods and at concentrations 0.66 and 1.49 $mg.kg^{-1}$ of $soil_{dw}$ for 2-days exposure (Fig. 11). The organisms exposed for 2-days revealed significant induction of GPx activity in the highest concentrations (0.99 and 1.49 $mg.kg^{-1}$ of $soil_{dw}$). Additionally, the earthworms exposed to 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. 11). On the contrary, after 28-days of exposure there was a significant decrease, but only in the concentration 0.99 $mg.kg^{-1}$ of $soil_{dw}$. 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-days exposure.

In addition, the results presented in Fig. 11 demonstrated the potency of SPIT to significantly modulate the expression of GSTs compared to control treatments in the 2-days and 28-days exposure periods (Fig. 11). For instance, 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-days exposure, demonstrated the potential occurrence of lipid peroxidation in earthworms.

Regarding the possible effects of SPIT on the energy reserves of *E. fetida* (Fig. 11), no adverse impact was observed in glycogen and lipids contents in the 2-days exposure. Contrariwise, for the 28-days 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 signs of significant induction in both exposure periods. The short-term period exposed organisms had significant induction from the concentrations 0.99 $mg.kg^{-1}$ of $soil_{dw}$. The earthworms in the long-term exposure showed a significant increase in concentrations 0.20, 0.29, 0.66, 0.99, and 1.49 $mg.kg^{-1}$ of $soil_{dw}$.

The results from the acetylcholinesterase activity (Fig. 11) inferred that there were no significant changes in AChE activity in the long-term exposed organisms when compared to the control group. However, in the 2-days exposure, there was only a significant decrease in the 0.44 $mg.kg^{-1}$ of $soil_{dw}$ concentration.

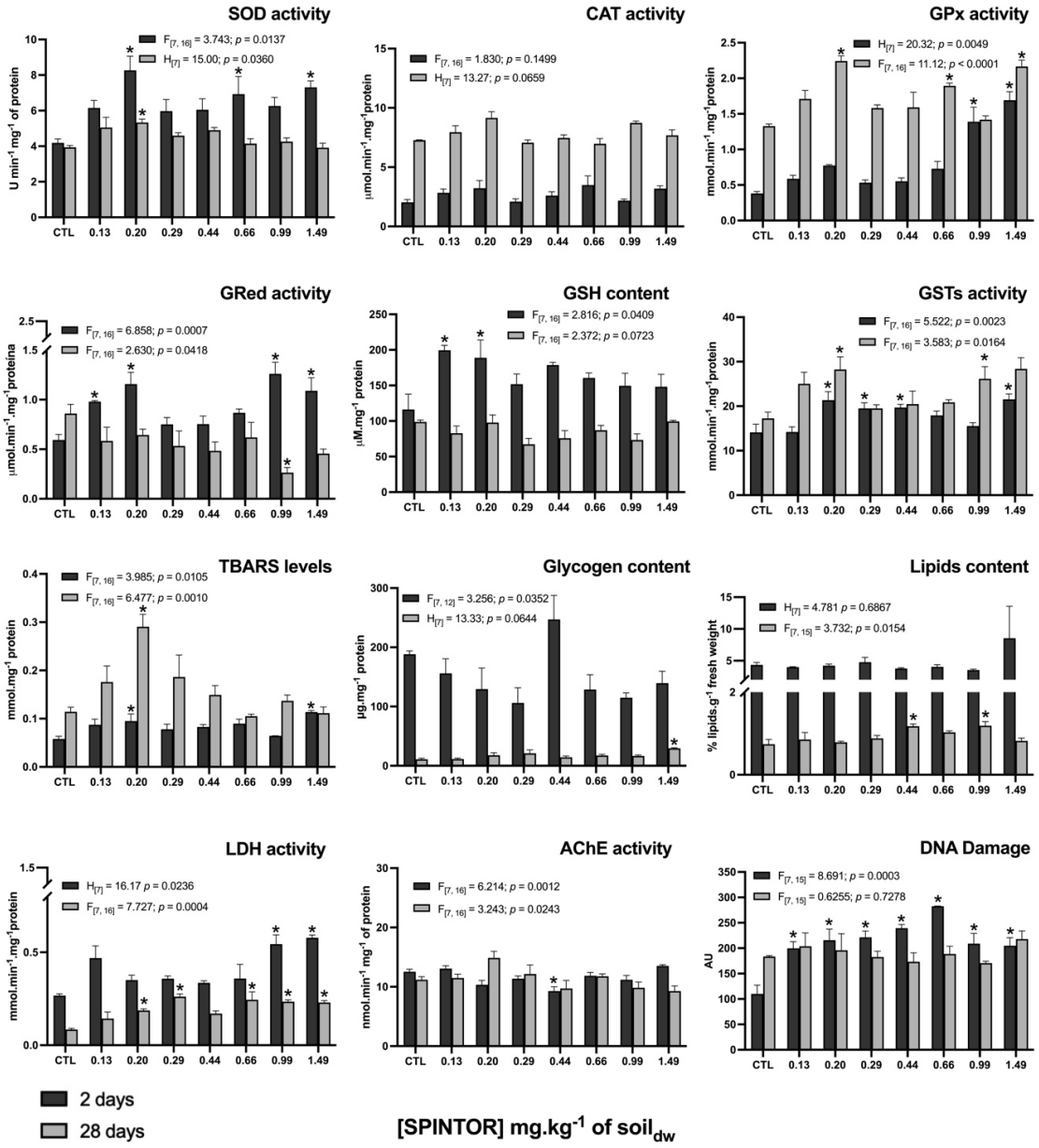

Fig. 11: Results obtained for *E. fetida* sub-individual biomarkers after short-term (2 days) and long-term (28 days) exposures to Spintor® (SPIT). Data are expressed as mean ± standard error (SE). * - Stands for significant differences when compared to control (p < 0.05).

III.3. Sub-individual responses upon short-term and long-term exposure of *E. fetida* to Spinosad

The results from the sub-individual biomarkers upon shor-term exposure (2-days) and long-term exposures (28-days) to SPIN are presented in Fig. 12. The analysis of biochemical biomarkers after 2-days exposure of *E. fetida* showed significant differences in SOD, CAT, GPx, GRed, GSTs, LDH and AChE activities, GSH content and TBARS and glycogen levels when compared to the control treatment. In addition, the data exhibited that short-term

exposure to SPIN may cause DNA damage in earthworms even at low levels. In the case of 28-days exposure, despite the significant effects observed in SOD, CAT, GPx, GRed, GSTs, LDH, and AChE activities, GSH content and TBARS levels, the exposure to SPIN didn't cause DNA damage in the tested concentrations.

Regarding the biomarkers of antioxidant defence, SOD and CAT activities both 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 activity was also increased at the middle range of concentrations (0.20, 0.29, 044 and 0.66 $mg.kg^{-1}$ of $soil_{dw}$) (Fig. 12). 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 exposure for 2-days lead to an increment of GPx activity, in the concentrations 0.20, 0.44, 0.66, and 0.99 $mg.kg^{-1}$ of $soil_{dw}$, being concomitant with the increase observed in SOD and CAT activities. GRed activity was boosted in the lowest concentration (0.13 $mg.kg^{-1}$ of $soil_{dw}$) and in the highest concentrations (0.66, 0.99, and 1.49 $mg.kg^{-1}$ of $soil_{dw}$) in the 2-days exposure, as well as, in the concentration 1.49 $mg.kg^{-1}$ of $soil_{dw}$ after 28-days (Fig. 12). The non-enzymatic antioxidant defence, GSH content (Fig. 12), demonstrated a significant boost in the 2-days exposure for the 0.66 and 0.99 $mg.kg^{-1}$ of $soil_{dw}$ concentrations. Contrarily, a significant decrease of GSH content was detected in the 28-days exposure, but only in the 0.20 $mg.kg^{-1}$ of $soil_{dw}$ concentration (Fig. 12). The activity of GSTs were found to be significantly enhanced in the 2-days exposure in the concentrations 0.44-1.49 $mg.kg^{-1}$ of $soil_{dw}$ and similarly upraised in the 28-days 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 (Fig. 12). Despite the activation of antioxidant defences, lipid peroxidation occurred as stated in the levels of TBARS that were significantly stimulated by SPIN, but only in the concentration 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. 12), which revealed no significant changes upon 28-days of exposure, for both energetic reserves. Interestingly, there was an 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. 12), 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 (0.66-1.49 $mg.kg^{-1}$ of $soil_{dw}$).

The results of AChE activity (Fig. 12) demonstrated that there was a significant enhancement in the concentration of 1.49 $mg.kg^{-1}$ of $soil_{dw}$ for both exposure periods, as well as, for the 0.66 $mg.kg^{-1}$ of $soil_{dw}$ concentrations after 28-days.

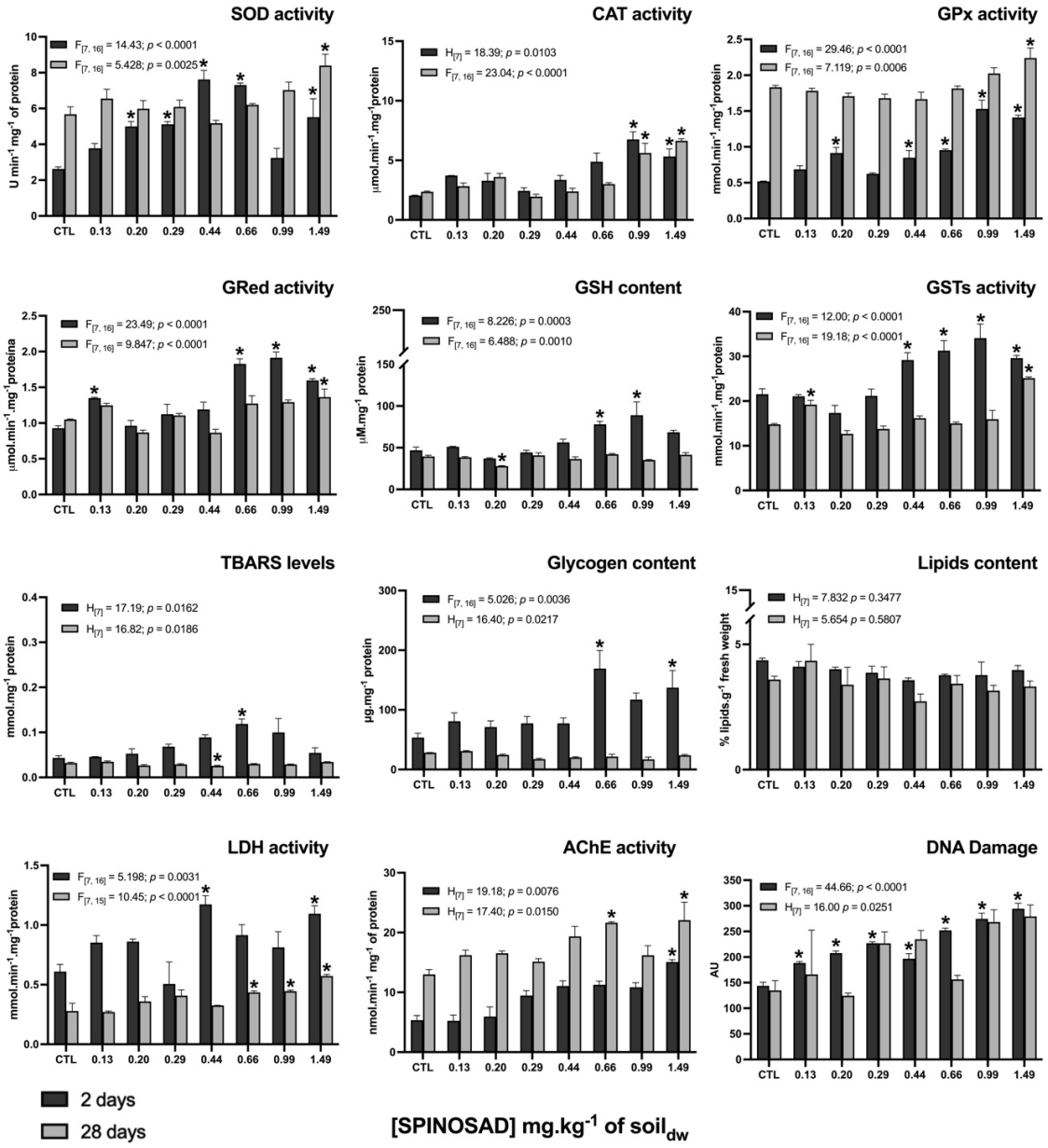

Fig. 12: Results obtained for *E. fetida* sub-individual biomarkers after short-term (2 days) and long-term (28 days) exposures to Spinosad (SPIN). Data are expressed as mean ± standard error (SE). * - Stands for significant differences when compared to control (p < 0.05).

III.4. Principal Component Analysis (PCA)

A principal component analysis (PCA) was performed (Fig. 13 (A) – (D)) to get a comprehensive view of the biomarkers' results and to understand the correlation among them and with the concentration range, depending on which variables were included. The biplot was graphed according to the time of exposure (2-days or 28-days) with the concentration ranges representing SPIT or SPIN; a second approach was graphed according to the compound (SPIT or SPIN) and the concentration range represents the

exposure times of 2- or 28-days. The percentage value presented in the axis of PC1 or PC2 corresponds to how much each principal component explains the results.

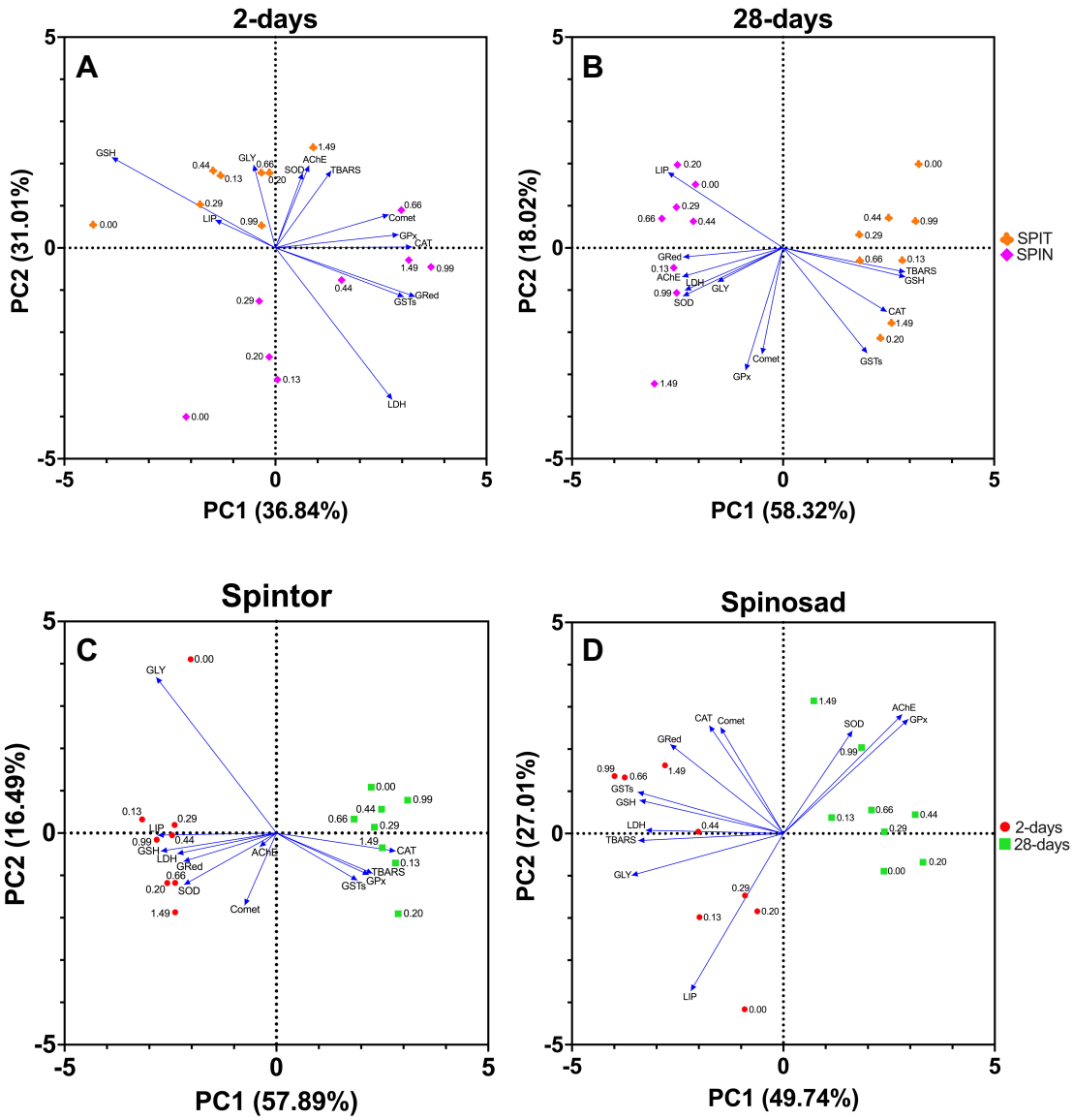

Fig. 13: Biplot of the first two components of Principal Component Analysis (PCA) including all measured sub-individual biomarkers in *Eisenia fetida* exposed to several concentrations of SPIT and SPIN during 2 and 28 days, organized by time of exposure or compound.

This multivariate approach for the different exposure times showed that biomarker response varied with exposure duration, being the main changes in Comet (DNA damage), GPx and LDH activities, and TBARS levels (as depicted by the direction, length, and position of the arrows) (Fig. 13-A and B). In fact, it is possible to observe that at 2-days, the increase of Comet (DNA damage) and GPx activity are associated with the highest concentrations (0.66-1.49 $mg.kg^{-1}$ of $soil_{dw}$) (Fig. 13-A), and at 28-days the correlation between the two biomarkers is kept but not with any concentration (Fig. 13-B). Contrarily, LDH activity was correlated with low concentrations of SPIN for 2-days (Fig. 13-A), but at

the 28-days was strongly associated with the concentration of 0.99 $mg.kg^{-1}$ of $soil_{dw}$ (Fig. 13-B). The different concentrations of the compounds formed clusters for each exposure time, and it is possible to infer that SPIT had an impact on SOD and AChE activities as well as LIP and GLY contents for 2-days (Fig. 13-A). However, for 28-days these biomarkers showed no correlation with SPIT (Fig. 13-B).

The biomarkers that most responded upon the exposure to SPIT at 2-days were SOD, GRed and LDH activity, GSH and LIP content, and Comet (DNA Damage), and for 28-days CAT, GPx, and GSTs activities and TBARS levels (Fig. 13-C). In the case of SPIN exposure, at 2-days CAT, GRed, GSTs, and LDH activities, TBARS and GSH content and Comet (DNA damage) responded to the stimulus at higher concentrations, whereas, at 28-days only SOD, GPx, and AChE activities demonstrated correlation, but at high concentrations as well (Fig. 13-D).

III.5. Integrated Biomarkers Response v2 (IBRv2)

The results for the IBRv2 index and the star plots for biomarker responses are shown in Fig. 14 (A)-(D).

Regarding the 2-days exposure with SPIT (Fig. 14-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 effects on, in descendent order, 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 relatively to control) and the decrease of AChE activity and GLY content (values below the control). Upon exposure for 28-days with SPIT (Fig. 14-B), the highest IBRv2 score stands for the second lowest concentration (0.20 $mg.kg^{-1}$ of $soil_{dw}$), and the effects on, in descending order, 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).

About the exposure to SPIN, in the 2-day scenario (Fig. 14-C), the highest index corresponded to 0.66 $mg.kg^{-1}$ of $soil_{dw}$ (real application dose), and the parameters that contributed to explain this value were, in descendent order, 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. 14-D), the highest score corresponds to the highest concentration (1.49 $mg.kg^{-1}$

of $soil_{dw}$) for each 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-days exposure with SPIN, the increase of AChE activity, Comet (DNA Damage), and LDH activity, as well as, the decrease, compared to the control, of LIP levels, GSH content, TBARS, and GLY levels were the most relevant responses.

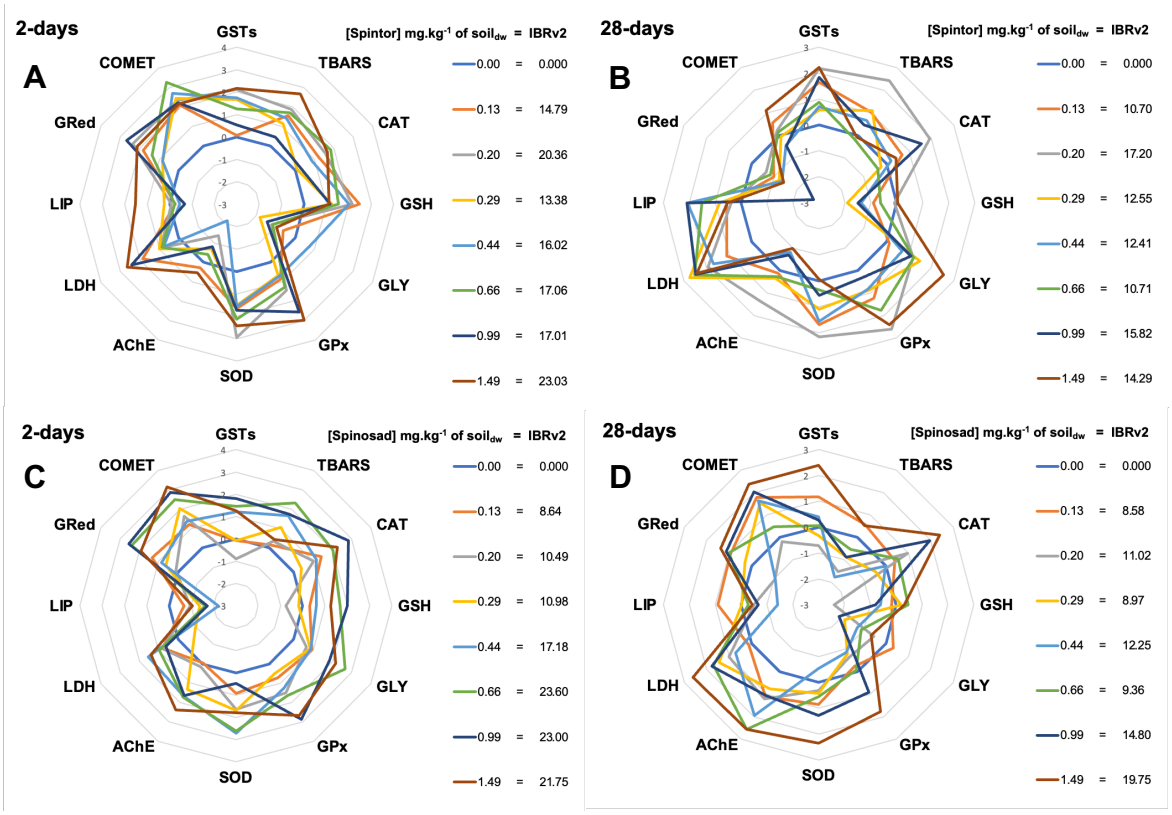

Fig. 14: The IBRv2 index results and star plot, with all the biomarkers determined on *E. fetida*, after exposure to the range of concentrations with SPIT and SPIN, for 2-days and 28-days exposures.

IV. Discussion

IV.1. Behavioural response of earthworms *E. fetida* to SPIT and SPIN

The avoidance assay is a quick, sub-lethal method to assess the potential toxicity of chemicals on living organisms and the habitat function, in the case, of terrestrial systems (ISO, 2008). Indeed, this assessment tool was considered a more accurate endpoint when compared with growth rate, mortality, and even reproduction (Syed et al., 2017). In this study, the assays aimed to evaluate the effect of SPIT and SPIN on the behavior of soil invertebrate *E. fetida* by measuring their capacity to detect and avoid these bioinsecticides. The results demonstrated that earthworms showed a tendency to avoid soil contaminated with SPIT. However, even though significant effects were registered, the maximum avoidance percentage recorded was 66%, below the 80% threshold, 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), in which a tendency of avoidance was also observed even in a much higher concentration (1575 $mg.kg^{-1}$ of $soil_{dw}$) of Laser® (commerical formulation 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 and not significant regarding to the control. Similar responses of higher avoidance behavior of *Eisenia andrei* (non-target organism) 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 & Surgan, 2006; Tsui & Chu, 2003), leading to higher avoidance for the commercial formulations. Adittionally, since this method presupposes the detection of 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 that 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 behavior of *E. fetida*.

IV.2. Effect of SPIT and SPIN on earthworms and arthropods reproduction

Although the avoidance test provided useful information on the sensitivity of earthworms, there is evidence that the reproductive output can be affected at sub-lethal concentrations, with long-term exposure times, in these non-target organisms (Aebeed et al., 2022; Ge et al., 2018). However, even in long-term design, the toxicity effects are still dependent on the

concentrations, route of exposure, and duration (Pisa et al., 2015). In the present work, the results of reproductive output for SPIT and SPIN did not show significant differences between treatments and respective controls. This outcome agrees with other authors (De Bernardi et al., 2022; Sekulić et al., 2020), who also demonstrated no effects on the reproductive performance. In fact, in the work of De Bernardi et al. (2022), the authors tested two concentrations (735 and 1575 $mg.kg^{-1}$ of $soil_{dw}$) that were calculated based on doses of 70% and 150 % of the LC_{50} indicated in the pesticide formulation (Laser® 480 $g.L^{-1}$), and although they tested much higher and not-environmentally realistic concentrations, the reproduction output of *E. fetida* was also not affected. The study by Sekulić et al. (2020) used another commercial formulation, Laser® 240 SC (240 $g.L^{-1}$) for which the recommended dose of application is 200 $mL.ha^{-1}$. They established a more realistic concentration range based on this value (0.06, 0.12, 0.24, 0.48, and 0.96 $mg.kg^{-1}$ of dry soil, after conversion to these units) that is within the frame of the concentrations tested in our work and with similar findings regarding the reproductive output. In our study, however, the chemicals may possibly have suffered degradation up until 56 days (end of the assay) and even at 28 days (removal of adults). Indeed, the degradation of spinosad has been documented to be around 9 to 17 days in soil under aerobic conditions (Hale & Portwood, 1996). De Bernardi et al. (2022) have also studied the degradation of spinosad (Laser® 480) in soil, with and without the presence of earthworms, demonstrating the decrease of the chemical in the soil throughout the exposure time, and a higher decrease when earthworms were presented. This is probably due to their digging behavior, which creates excelent 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 ingestion by the organisms. Additionally, the route of toxicity may have been different, and although no impact on reproduction (individual response) was observed under the conditions tested here, effects on other endpoints, such as sub-individual endpoints (discussed further), were found in our study. This outcome has been observed in other works, which demonstrates that spinosad-based products are generally innocuous for the reproductive performance of these earthworms, not affecting the size of communities. However, for a broader assessment of environmental health and impacts on non-target organisms, it is important to assess other ecotoxicological parameters. In De Bernardi et al. (2022) and Yang et al. (2020), *E. fetida* exposed to commercial formulation Laser 480 (spinosad, 480 $g.L^{-1}$) and ciprofloxacin (antibiotic), respectively, and they demonstrated no effects on reproduction 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 (the more sensitive) to effects at supra-individual levels.

Since there were no observed effects in the reproductive output of *E. fetida* we assesed the effect on the reproductive performance of *Folsomia candida*, another soil-dweller for which there is not any published work regarding this parameters under SPIT® or SPIN exposure. Contrariwise to earthworms, the reproduction assay with the soil springtail *F. candida* demonstrated the sensitivity of these organisms to SPIT and SPIN. The sensitivity of collembola to pesticides were already demonstrated in several studies (Fernandes et al., 2023; Ferreira et al., 2022; Simões et al., 2019). Indeed, in the present work, the exposure to SPIN revealed higher toxicity effects than SPIT, since there was a significant decrease of the number of juveniles in all concentrations of SPIN. However, the reproduction EC_{20} and EC_{50} calculated here for exposure of *F. candida* to SPIT (0.20 and 0.98 $mg.kg^{-1}$ $soil_{dw}$, respectively) demonstrate the high ecological relevance, because effects on the population of this key soil organisms can even be expected with exposure 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 also observed when the commercial formulation was applied. To the best of our knowledge, there are no previous works in which the reproductive output of *F. candida* upon exposure to spinosad (commercial formulation and active ingredient) was assessed. Even so, it was demonstrated in other studies that spinosad (commercial formulation SpinTor 2SC) reduced fertility in *H. axyridis* at the concentrations 0.055 and 0.11 $kg.ha^{-1}$ after a single direct spray in Petri dishes (Galvan et al., 2005). It also reduced the number of eggs laid and hatch ratio in *H. armigera* after oral exposure to Tracer® 480SC at concentrations 0.04 and 0.16 $mg.kg^{-1}$ exposed in climate chambers (Wang et al., 2009a). Furthermore, in the investigation of Santos et al. (2019), it was reported that spinosad (active ingredient) 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 $\mu g.L^{-1}$) compared to the recommended usage dose for field applications (500 $\mu g.L^{-1}$). Moreover, Monteiro et al. (2019) also demonstrated that spinosad (active ingredient) provoked changes in the life-history traits (larval growth, development, and emergence) of *Chironomus riparius*, significantly increasing the time of emergence at 8 $\mu g.L^{-1}$ while provoking in the same concentration, sub-individual alterations (e.g. lipid peroxidation, increase in the electron transport system and increase of LDH and GPx activities). The authors hypothesised that the energy demand could be related with the raise of antioxidant defences, which can also explain how less energy would be available for other psychological processes, delaying the emergence (Monteiro et al., 2019).

IV.3. Biochemical responses and DNA damage caused by SPIN and SPIT in *E. fetida*

Some sub-individual parameters were assessed upon exposure to SPIT and SPIN (these parameters are not a requirement for risk assessment of the ecotoxicity of active ingredients and commercial products by the EU Member States authorities). In some parameters, it is verified a difference of orders of magnitude between the controls of different assays. 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, etc..

Superoxide dismutase (SOD) is the first line of defence against ROS, namely the superoxide radical (O_2^-), transforming it in hydrogen peroxide (H_2O_2) (Ighodaro & Akinloye, 2018). The enzymes that act upon SOD to degrade H_2O_2 into water are catalase and glutathione peroxidase. GPx also catalyses the breakdown of lipid peroxides, ROOH, that could be formed upon exposure to stressors, using glutathione, in order to protect the cells from oxidative damage (Ighodaro & Akinloye, 2018). The results from both exposure periods (Fig. 11 and Fig. 12) demonstrate a higher activation of SOD, CAT, and GPx activities in the case of SPIN exposure. This entails a higher production of ROS induced by SPIN, but also in the case of SPIT, for which SOD activity was significantly increased in the 2-days exposure. It is also important to denote that there was an increase of SOD activity in the 0.66 $mg.kg^{-1}$ of $soil_{dw}$ concentration (for both SPIT and SPIN), which is the recommended application dose in the fields (and concentrations below), demonstrating effects even at ecologically and environmentally relevant scenarios. In the case of SPIT exposure, the H_2O_2 potentially produced by SOD (given its significant increase), can be related with the GPx activity, which seems to be the 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 & Sen, 2000). This conjoined effect is observed in the PCA of 2-days (Fig. 13-A), with an overlap of the loadings respective to CAT and GPx activities. The study with spinosad (a.i.) and aquatic midges by Monteiro et al. (2019) showed that GPx activity was also overexpressed through induction by spinosad, concomitant with an increase in lipid peroxidation (at the concentration of 8 $\mu g.L^{-1}$ for 48 hours), but in their case the levels of SOD and CAT activities remained unchanged compared to the control. However, another study demonstrated induction of CAT activity upon 2-days exposure of bees *A. mellifera* to spinosad (a.i.) [concentrations 2.36 and 4.71 ng a.i./bee, respectively corresponding to 1/20 and 1/10 of the LD_{50} (lethal dose at 50%)], that was explained by the induction of oxidative stress promoted by spinosad (Carvalho et al., 2013). The stimulation of SOD, CAT, and GPx activities was associated with a potential response to high ROS levels (Ighodaro &

Akinloye, 2018), representing an oxidative stress scenario promoted by SPIT and SPIN, followed by an attempt to recover the oxidative homeostasis. Contrarily to the 2-day exposure, the results for SOD activity in the 28-days 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 & Jones, 2020), indicated by the respective control. These changes may reflect the potential degradation of SPIT and SPIN in the soil, throughout the 28 days. 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 case. Since GPx is also involved in the breakdown of lipid peroxides, its activity may have increased at 28-days to allow the clearance of ROOH caused by SPIT. Hypothetically, SPIT might have lasted longer in the soil, due to the presence of stabilizing excipients, that is, less degradation than SPIN (a.i.) (Cox & Surgan, 2006; Tsui & Chu, 2003). CAT activity upon SPIN exposure does not appear to have reduced at 28-days, despite the basal levels of SOD activity; in fact, high levels of H_2O_2 might still exist in the organisms since this hydroperoxide is also produced as a sub-product by other enzymes (e.g., xanthine oxidase, glucose oxidase, and amino acid oxidase) (Andrés et al., 2022), which activities could have been disrupted by SPIN but not assessed in the present work.

Glutathione reductase is an enzyme that has a coordinated activity with other enzymes to maintain the balance between oxidized (GSSG) and reduced glutathione. GSH is a non-enzymatic defence used by GPx, as a proton donor, and by GSTs, as a conjugation co-factor. GSTs are phase II isoenzymes whose function is to solubilize electrophilic substances by GSH-binding (Valko et al., 2007; Van der Oost et al., 2003). In both cases, with SPIT and SPIN, GRed and GSTs activities showed a tendency to be stimulated at short-term exposure, as well as the levels of GSH 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 GSTs conjugation pathway and proton-donor for GPx (Pérez-Pertejo et al., 2008; Piner & Üner, 2013). In fact, the GSTs-mediated conjugation of GSH with spinosad was found in rats (FAO & WHO, 2001). The increase in antioxidant enzymes (SOD, CAT, GPx, GRed, and GSTs) and non-enzymatic antioxidants (GSH) in *E. fetida* after day 2 of exposure can reflect the initial adaptive response of these earthworms to spinosad exposure (Wu et al., 2011). Indeed, in the PCA for 2-days (Fig. 13) we can observe a positive correlation of GRed and GSTs (arrows are almost overlapped), forming a cluster with GPx and the highest concentrations of SPIN. This also infers that SPIN exerted higher toxicity upon *E. fetida* than SPIT, as stated above. Contrarily, the activity of GRed and GSH content, both for SPIT and SPIN, did not show any significant tendency to shift regarding

the control, and the PCA for 28-days (Fig. 13-B) lacks to show a positive correlation between these parameters. Hence, at the end of the 28 days, the GSH/GSSG balance may have been reestablished, as stated by Pérez-Pertejo et al. (2008) in their work, where they evaluated the alterations in the glutathione-redox balance induced by the spinosad in CHO-K1 and Vero cells. In the case of GSTs, some treatments still revealed an increase of the enzymatic activity and despite GSH still being used by GSTs, the balance may have been restored by GRed. In this case, 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 byproducts can be metabolized by this route, in *E fetida*.

TBARS levels are a biomarker for oxidative damage in cells, e.g., lipid peroxidation of cellular membranes, which can happen in situations where the oxidative stress is not compensated (Rodrigues et al., 2021). In both cases of SPIT and SPIN, at 2-days, the concentrations in which TBARS levels were significantly overexpressed correspond to concentrations for which other enzymes involved in fighting oxidative stress (e.g., SOD, GPx, and GSTs) were stimulated. In fact, in the PCA for 2-days (Fig. 13-A) there is a clear correlation of TBARS with the first line defence (e.g. SOD) and with the SPIT concentrations range. Despite the performance of these enzymes, they failed to prevent oxidative damage in these concentrations. It can also be hypothesised that the increase of GSTs is related to the scavenging of lipid peroxides, produced upon spinosad exposure (Piner & Üner, 2013). The simultaneous increase of lipid peroxidation and SOD activity is expected in a stress response (Wu et al., 2012b); the increase of GPx activity and LPO levels under spinosad exposure has also been previously described for CHO-K1 and Vero cells (Pérez-Pertejo et al., 2008) and for the liver of *Oreochromis niloticus* (exposed to 75 $mg.L^{-1}$ of commercial formulation Laser®480 for 2 days) (Piner & Üner, 2013). The levels of TBARS after the 28-days both for SPIT and SPIN were only singly increased in some concentrations, not showing any tendency of significant oxidative damage. The predisposition for a decrease of lipid peroxidation, for long-term exposures (e.g. 28-days), in *E. fetida* has been observed when exposed to decabromodiphenyl-ethane (Zhao et al., 2020b) and cyantraniliprole (Qiao et al., 2019), for which both authors explained that this decrease is probably related with the active action of antioxidant defences as well as the degradation of both products to less harmful metabolites. The PCA for 28-days (Fig. 13-B) demonstrates a change in TBARS (from the 2-days biplot), showing a high correlation of TBARS with GSH, a molecule used by GPx to catalyses the breakdown of lipid peroxides (Ighodaro & Akinloye, 2018). With this information, 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 & WHO, 2001). The re-establishment of the basal levels of SOD, GRed, and GSTs activities and GSH content may indicate, in one hand, the antioxidant pathways protected the organism's cells, and in the other, 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 pollutants (e.g. SPIT and SPIN) and efficiently combat oxidative stress.

The energetic reserves are an essential parameter to assess an organism's health status. Indeed, the glycogen and lipidic content report how certain xenobiotics affected the metabolomics of energy (Givaudan et al., 2014; Overgaard et al., 2009). Allied to these energetic reserves it is important to understand the underlying pathway of energy obtention. Regarding the content in glycogen, despite not showing a clear tendency, some occasional concentrations demonstrated a significant increase in glycogen content when the organisms were exposed to SPIN for 2 days. This can be related to two aspects: 1) other metabolic pathways, not assessed in the present work, might be disrupted by spinosad, altering the energic 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). In fact, 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 & Lappin, 2022; 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. The significant increase of LDH was also verified by Monteiro et al. (2019), in aquatic midge exposed to spinosad (at the concentration of 2 $\mu g.L^{-1}$ for 2 days), which indicates high levels of metabolic necessities and energy consumption (Rodrigues et al., 2015). Some studies found that some xenobiotics can affect the normal oxidative phosphorylation system, by altering levels of proteins and enzymes leading to tissue inflammation (Llobet et al., 2017) or even by binding its metabolites to important cofactors of the pathway, such as coenzyme A, disabling the normal function of the mitochondria (Darnell & Weidolf, 2013). This untimely 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 defenses (Cherkas et al., 2020; Monteiro et al., 2019) to combat oxidative stress. This increased activity 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; in this case, 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 (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 for spinosad (Monteiro et al., 2019); 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).

During the 28-days exposure, lipids and glycogen contents showed a clear difference in magnitude orders in comparison to the 2-days exposure (higher levels for the 2-days exposure). It can be rationalized by the fact that throughout the 28 days, the earthworms were fed only once a week with autoclaved horse manure (ISO, 2023) and had to resort to their energetic reserves. Additionally, the energetic reserves may have been used in the reproductive process. Even so, for SPIT we perceived a similar scenario to the short-term exposure, concerning the glycogen content, in which the highest concentration was significantly increased; this can be justified with the previous explanation since the activity of LDH was also increased in the highest concentration at 28-days and it is possible to obverse a positive correlation between LDH and GLY in the 28-days PCA (Fig. 13-B). The lipidic content upon the 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 of glucose necessary to counterattack 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).

Acetylcholinesterase, AChE, is the main cholinesterase present in earthworms and a valuable biomarker for neurotoxicity (Rault et al., 2007; Zhang et al., 2019a). For both exposure to SPIT and SPIN, AChE activity disruptions occurred, however, in the tested concentrations there was not an evident scenario of neurotoxicity. Nevertheless, other studies stated the neurotoxic effects of spinosad in other species, e.g., a decrease of AChE

activity in *Appis mellifera* (Abdel et al., 2013; Biondi et al., 2012; Eid et al., 2011). Typically, a decrease in AChE activity leads to neuromuscular paralysis, with uncoordinated movements, due to an overstimulation of cholinergic receptors (Hayden et al., 2010). This decrease in the organism caused by the xenobiotic has been associated with behavioural changes (Kumar et al., 2010; Xuereb et al., 2009), e.g., impossible to escape spiked soil in the avoidance assay. This inability to avoid contaminated soil has been demonstrated in Pereira et al. (2010), with the exposure of *Eisenia andrei* to methomyl. Our results demonstrated a significant decrease of AChE activity in the concentration 0.44 mg/kg for SPIT and in the same concentration a significant avoidance from the spiked soil. As stated above we are not presented with an evident scenario of neurotoxicity, but the concomitant existence of avoidance and changes in AChE activity, under SPIT exposure, ought to be studied. In fact, spinosad has a distinct mode of action that entails the disturbance of the nervous system of insects through the nicotinic acetylcholine receptors and GABA-gated ion channels (Huan et al., 2015; Kirst, 2010). Studies suggest that this action mechanism is not unique to insects and indicates a distinct nicotinic receptor subunit as a molecular alternative location for spinosyns (Orr et al., 2009; Pereira et al., 2016; Santos et al., 2019). 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 & Baldwin, 2002; Wu et al., 2012a). Furthermore, elevated levels of AChE activity can be a sign of neurodegeneration (Nunes, 2011), being a possible regulator of apoptosis and a preventer of nervous cell proliferation (Jin et al., 2004; Piner Benli & Çelik, 2021). Indeed, other metabolic pathways not assessed in the present work may be disrupted, for example, an abnormal expression of neurotoxicity-related genes stimulated by spinosad, leading to, e.g., alteration of signal transduction, ultimately causing neurotoxicity (He et al., 2023). Moreover, the PCAs for 2- and 28-days (Fig. 13-A and -B, respectively) demonstrate a positive correlation of AChE activity with the activities of antioxidant defences. This may indicate ROS-induced injury in neurotoxic-related pathways; in fact, in a study by He et al. (2023), on the effect of fluorene in the brain of *E. fetida*, it is hypothesized that oxidative stress induced by a xenobiotic can impair AChE activity. Although, more in-depth assays on neurotoxicity are necessary to assess the effect of SPIT and SPIN on *E. fetida.*

The comet assay is a molecular procedure that allows the quantification of DNA damage and has been used to assess genotoxicity upon exposure to several compounds, including pesticides (Ali et al., 2018; Cavas, 2011). In a previous work done by De Bernardi et al. (2022) using the commercial formulation Laser® 480 on *E. fetida,* it was demonstrated

significantly higher levels of genotoxic damage in the comet assay, 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 much higher (735 and 1575 $mg.kg^{-1}$) than the ones used in the present work. This means that even at lower doses, and more ecologically relevant scenarios, at the real recommended application dose (0.66 $mg.kg^{-1}$ $soil_{dw}$), the organisms are sensitive to SPIT and SPIN. Additionally, the comet assay was performed using coelomocytes, which play a major role in the innate immune system of oligochaete (Cooper & Roch, 2003; Engelmann et al., 2005). They are capable of phagocytosis and have natural killer cell capacities, mediate the lysis of pathogens, and secrete antimicrobial peptides (Cooper, 2002). 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). Despite the lack of information about the effect of SPIT (or its a.i.) on soil invertebrates, namely *E. fetida*, several past works have reported genotoxic effects of spinosad on different non-target organisms, such as in human cell lines (Yang et al., 2016; Zhang et al., 2019b), *Drosophila melanogaster*'s hemocytes (Demir, 2012), developing chick embryos (Uggini & Suresh, 2013), swiss albino male mice (Sharma & Jain, 2018) and rat bone marrow cells (Mansour et al., 2008). Since it provokes similar DNA damage in different taxonomic organisms, it can be hypothesized that the active substance, spinosad, or its metabolites, promote a genotoxic effect, which can be associated with the increased oxidative stress and reactive oxygen species (ROS) (Mendonça et al., 2019; Piner & Üner, 2013). The positive correlation of the parameter Comet in the 2-days PCA (Fig. 13-A) with antioxidant defenses (CAT, GPx, GRed, and GSTs) seems to support this hypothesis of DNA damage caused by ROS. Contrarily , the DNA damage assessed upon long-term exposure did not show any significant results for the exposures of SPI and SPIN. Even in the work of De Bernardi et al. (2022), there was a decrease to non-significant levels after 21 and 28 days, as demonstrated in this work. The lack of genotoxicity at 28-days may be hypothesized to be correlated with the ability of earthworms to recuperate from stressors (Pochron et al., 2021) aligned with the decay of the active substance in soil (De Bernardi et al., 2022; Thompson et al., 2002), throughout the 28 days.

IV.4. IBRv2 – understanding biomarkers' responses to SPIT and SPIN

The IBRv2 is a useful instrument for analysing environmental contaminants' impacts and figuring out how they affect organisms. This index has been widely utilized in field and laboratory investigations (Caliani et al., 2021; Pinto et al., 2019). In this study, the exposure

to concentrations of SPIT or SPIN led to a biomarkers' response, and the representation of this in a star plot (Fig. 14) provided a clearer visualization of which biomarkers were the most responsive/sensitive to the compounds in each exposure time.

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 (followed a similar trend). However, there was a strong induced response of some biomarkers in common for both SPIT and SPIN. Indeed, a general response of the activity of LDH, GPx, and GSTs, as well as, DNA damage (Comet), was induced by these products. This indicates that spinosad (as an active ingredient or as a part of commercial formulations) causes metabolic pathway disturbances in several pathways of *Eisenia 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*.

IV.5. Effects of Spintor® and Spinosad on non-target soil organisms

Soil organisms are at constant risk of being exposed to xenobiotics, as is the case of *Eisenia fetida* (earthworms). In order to survive, they need to possess an effective detoxification mechanism, since their skin and digestive mucous membranes are in constant direct contact with the chemicals, and any cellular disorder may lead to imbalances of the osmotic regulation, if not promptly repaired (Kılıç, 2011; Morowati, 2000). To avoid this scenario, the organisms developed a considerable regeneration ability. They possess chloragogen cells, that upon tissue damage migrate to the local and regenerate the lesion (Morgan et al., 2002; Reddy & Rao, 2008). In fact, chloragogen cells can differentiate in, e..g., eleocytes, a specific type of coelomocyte associated with balance of pyshico-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, leading to dysregulation of some metabolic and neurotransmission enzymes activities (e.g., LDH and AChE, respectively), resulting in mortality or sub-lethal effects, like avoidance (Rico et al., 2016), or other behaviors. This may be a possible route of toxicity for Spintor® and Spinosad in our study. In fact, the biotransformation of spinosad inside the organisms involves the oxidation by the system cytochrome P-450, which is a potential additional source of ROS (FAO & WHO, 2001). The registered toxicity of these compounds was higher upon short-term exposure, having more defences mechanisms disrupted and presenting significant DNA damage; it is also supported by the significant avoidance after 2-days for Spintor®, and the lack of reproductive toxicity. Despite, we observed that the organisms were still affected at 28-days in some of their sub-individual parameters; this may be the outcome of the single exposure that threw off homeostasis, and organisms had

not regained it after 28-days. 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, 1992a); this test, however, is performed in a liquid substrate and does not consider the presence of soil bacteria or other organisms. The presence of oligochaete is a contributor to the degradation of spinosyns, due to their digging activity (De Bernardi et al., 2022; Thompson et al., 2002), as well as, the presence of soil microorganisms that metabolize this compound (De Bernardi et al., 2022).

The active ingredient in commercial pesticides is combined with a variety of additional compounds, often referred to as inert ingredients, to support dilution, mixing, application, and stability (Cox & Surgan, 2006). The identities of inert components and their proportions in the formulation are rarely published because they are not expected to be hazardous. Some works have demonstrated that the supposedly inert components can modify the formulation's toxicity either on their own or in combination with the active ingredient (Krogh et al., 2003; Oakes & Pollak, 2000). Rationalizing the results, we can infer that spinosad presents higher toxicity than Spintor®, given the toxic effects on *Folsomia candida* reproductive output at lower concentrations and the higher activation of defence pathways in *Eisenia fetida*. Despite this, Spintor® also presents high toxicity towards *Folsomia candida* reproductive output and sub-individual parameters of *E. fetida*, even demonstrating higher disruption of the normal energy metabolism and the pro-oxidant state. The lowest toxicity of Spintor® may be related to the presence of excipients, comparatively to spinosad. Accordingly to the date from the safety sheet of Spintor® (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, 1992a). This study corroborates Spintor® data sheet and previous investigations, which certified propylene glycol as a safe excipient (NTP, 2004; West et al., 2014). Given the previous results, propylene glycol and the remaining excipients do not appear to provoke toxicity, in fact, they seem to reduce the toxic activity of spinosad in the commercial formulation, at least under the conditions tested here. 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 in soil particles and consequently enhance the degradation process and reduce the likelihood/period of exposure.

The analysis of the PCAs for Spintor® (SPIT) and Spinosad (SPIN) (Fig. 13-C and -D, respectively) demonstrates sub-individual parameters correlations. The DNA damage

(measured by comet assay) showed a correlation with antioxidant defences for both SPIT and SPIN (e.g., SOD or CAT activities), and for 2-days exposure, demonstrating that, possibly, the xenobiotic provoked ROS formation at early exposure staged and led to DNA damage. The same is possible to infer for LDH activity, that in both SPIT and SPIN is correlated with antioxidant defences, again demonstrating a possible route for energy metabolism and oxidative metabolism disruptions, through chemical stress of the xenobiotic. The biplots also demonstrate, for both cases, two distinct clusters for a time exposure, indicating a decrease of effect at 28-days. It is also imporant to denote that the effects assessed at 28 days in the present study were the result of an only exposure, that can be allowed the degradation of the compounds, throughout the exposure days; however, in a real case scenario, there are periodic applications in the crops, with safety intervals ranging from 1, 3, 7, 14 or 21 days (CortevaAgriscience, 2022). Nevertheless, the interpretation of all the data allows us to infer that there is an inherent toxicity of the molecule spinosad to non-target soil organisms.

V. Final Considerations

V.1. Highlights and final remarks

This study allowed the evaluation of the ecotoxicity of Spintor® (commercial formulation) and Spinosad (active ingredient) on the reproductive performance of *Folsomia candida* and several endpoints of *Eisenia fetida*. Despite being largely used worldwide (with different name brands of the commercial formulation), there is a lack of ecotoxicological information on this commercial bioinsecticide for oligochaeta and even less knowledge for soil arthropods. Therefore, the results were mainly compared with the effects of the commercial formulation and active ingredient on other species. Nevertheless, it could be inferred that there was no effect on the reproductive output of *E. fetida*, which may be related to the degradation of spinosyns on soil, but *F. candida* demonstrated a significant decrease in the number of juveniles produced, with the commercial formulation and even greatly with the active ingredient, since springtails are in general more sensible to chemicals.

It could also be inferred that *E. fetida* demonstrated a tendency to avoid Spintor®, demonstrating possible sensitivity when in immediate contact. Furthermore, the increase of enzyme's activity related to the antioxidant metabolism (SOD, CAT, GPx, GRed, and GSTs) demonstrated the activation of antioxidant defence pathways upon a scenario of oxidative stress, promoted by SPIT and SPIN, in order to avoid oxidative damage. Spinosad demonstrated a higher activation of these pathways at short-term exposure than Spintor®. Likewise, the stimulation of LDH activity demonstrates a disturbance of the energetic production pathway and metabolism, which suggests that Spintor® and Spinosad might challenge the normal respiratory pathways. Spintor® and Spinosad were found to cause DNA damage in *E. fetida* coelomocytes in a short exposure period. Contrarily, after the long-term exposure no damage was observed, suggesting a degradation of the chemicals as well as the great recuperation ability of earthworms. The results establish that the ecotoxicity of spinosyns acts upon different pathways in non-target organisms, regarding the main toxicity pathway (neurotoxicity) in target organisms. In general, the 2-days exposure revealed more toxic effects than the 28-days, which is supposed to be related to the potential degradation of the compounds through the 28 days and the inherent recovery of *E. fetida*. Likewise, the apparent slight decrease in toxicity of the commercial formulation when compared to the active ingredient may be attributed to the presence of excipients, like propylene glycol, that probably confer high mobility of this pesticide in soil.

V.2. Conclusions and prospects for future investigations

It can be concluded that there is a potential intrinsic toxicity to the molecule spinosad, present in the commercial formulation Spintor®. It has a negative impact on the health and wellness of *E. fetida* and *F. candida*, which could possibly impair their function in the ecosystem. The information taken from 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 of springtails), other non-target soil organisms such the oligochaeta *Enchytraeus crypticus,* as well as more sub-individual biomarkers of exposure (e.g., histological data and other enzymatic profiles) to fully understand the impact and risk of spinosyns application at different levels of biological organization. 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. The integration of these parameters in risk assessment analyses can help create a strong and a more complete review of the effect of this bioinsecticide and assert how safe it actually is for commercial use.

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