Applied Soil Ecology 214 (2025) 106353

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Research paper

Physiological stress and habitat selection in earthworms (*Amynthas gracilis*) exposed to different pesticide regimes in a tropical horticultural area

Gabriel Brenes-Bravo [a,*], Lukas Reinhard [b], Clemens Ruepert [a], Frank Solano-Campos [c], Freylan Mena [a]

[a] *Instituto Regional de Estudios en Sustancias Tóxicas/Central American Institute for Studies on Toxic Substances (IRET), Universidad Nacional, Heredia, Costa Rica*
[b] *iES Landau, Institute for Environmental Sciences, RPTU Kaiserslautern – Landau, Landau, Germany*
[c] *Escuela de Ciencias Biológicas, Universidad Nacional, Heredia, Costa Rica*

ARTICLE INFO

Keywords:
Agricultural landscapes
Soil macrofauna
biomarker
behavior
avoidance

ABSTRACT

Agricultural landscapes are constantly exposed to pesticides. Such permanent exposure threatens the ecosystem and the services that it provides to sustain agriculture. Earthworms are key components of soil macrofauna that can be susceptible to such contamination. To assess if the presence of pesticides in horticultural soils can induce physiological stress and evasive behavior in resident earthworms, we evaluated pesticide residues and soil physical and chemical characteristics across a gradient of land use: conventional farming, farming with good environmental practices, organic farming and forest. Stress was assessed by measuring biomarkers of neurotoxicity, biotransformation and oxidative stress in individuals of the earthworm *Amynthas gracilis* inhabiting sites of the mentioned gradient during dry and rainy seasons. An avoidance test was conducted where a group of *A. gracilis* was offered with soil samples from the studied gradient, and 48 h later their selection was counted.

Pesticide residues were registered in all the sites and seasons. Conventional farming site contained the highest number (43) and concentration of pesticides, with peak values during the transition and rainy season (Chlorpyrifos 38.1 ng g^{-1} dw, Boscalid 8.4 ng g^{-1} dw and Linuron 7.8 ng g^{-1} dw). However, the highest concentration of an individual pesticides was found in forest soil (Chlorpyrifos 71.9 ng g^{-1} dw). Earthworms from Conventional farming site showed over 50 % inhibition of cholinesterase activity and diminished glutathione S-transferase activity compared to the other sites, while seasonal variation was clear in GST, CAT and EROD activities in all the sites. *A. gracilis* significantly avoided (90 %) the soil from the conventional farming site and preferred good environmental practices and organic soils. Our results provide evidence that intensive pesticide use induces physiological stress in *A. gracilis* and provokes their escape from contaminated soils, potentially affecting the soil macrofauna community and ecosystem services.

1. Introduction

The use of pesticides is a widespread agricultural practice, applied to safeguard crops against insects, fungi, weeds, and other pests (World Health Organization [WHO], 2022). In the case of Costa Rica, the pesticide use reaches an average annual application of approximately 18.2 kg (kg) of active ingredient (a.i.) per hectare (ha), per year (Araya, 2015). However, recent data indicates that the actual average pesticide usage in the country exceeds this figure, reaching approximately 34.5 kg ha^{-1} yr^{-1} (Vargas, n.d). It is important to highlight that 93 % of the pesticides applied in Costa Rica are categorized as highly hazardous substances (Pomareda, 2022). In Costa Rican horticulture, pesticide use has been historically high (Galt, 2008). Reports demonstrate that larger-scale producers tend to employ higher quantities of pesticides, while smaller producers use lower amounts but a broader variety of substances (Ramírez et al., 2014). In the Zarcero region, a prominent hub for horticultural activities, more than 119 different a.i. are in use. Among these, fungicides like chlorothalonil, mancozeb or boscalid, and insecticides like acephate and cypermethrin are among the most used compounds (Ramírez et al., n.d.).

Such intensive use of pesticides poses a significant threat to soil biodiversity in agricultural landscapes (Berrocal-Montero et al., 2021; Geisen et al., 2019) especially considering the many gaps in our understanding of the environmental behavior and toxicity of currently

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* Corresponding author.
E-mail address: johnny.brenes.bravo@una.ac.cr (G. Brenes-Bravo).

https://doi.org/10.1016/j.apsoil.2025.106353
Received 29 October 2024; Received in revised form 27 July 2025; Accepted 29 July 2025

used pesticides (Fernandes et al., 2020; Silva et al., 2019). Soil biota plays a crucial role in providing various ecosystem services that support agriculture (Rodrigues et al., 2021). Accurately assessing the risks posed by pesticide pollution to these ecosystem services is a pressing need, especially in tropical regions (Daam et al., 2019).

Soil macrobiota, such as earthworms, are essential contributors to the health of agricultural ecosystems. For instance, earthworms play a key role in decomposing organic matter, leading to improved soil oxygenation, while their excretions enhance the soil microbiome and diversify enzymatic activities, thus increasing nutrient availability (Akhila and Entoori, 2022; Edwards and Arancon, 2022). Furthermore, the deposition of biogenic structures by earthworms creates assemblies of organomineral aggregates and enhances the physical properties of the soil through increasing organic matter concentration (Bhadauria and Saxena, 2009; Guhra et al., 2022). Earthworms are intricately connected with surrounding biota, relying on microorganisms as their primary nutrient source and promoting microbial activity in the soil (Brown, 1995). Similar interactions occur between earthworms and fungi, which participate in establishing mycorrhizal relationships within plant communities. Earthworms also serve as valuable indicators of soil quality, because of their ubiquity in soils, their role as a food source for various predators, and their high sensitivity to toxic chemicals (Bartz et al., 2024; Shi et al., 2017).

The impacts of pesticide use on meso- and macrofauna are evident (Aktar et al., 2009; Beaumelle et al., 2023; Gunstone et al., 2021; Gunstone and Dubey, 2021). In general, pesticide application leads to a significant reduction in soil fauna populations, affecting invertebrates and non-target microorganisms that actively participate in nutrient-cycling processes. Consequently, soil quality is directly compromised by pesticide use, with implications for crop health and productivity.

In the context of assessing the risk that pesticides pose to earthworms as soil bioindicators, Vermeulen et al. (2001) argued that the conventional evaluation of acute toxicity may not be a relevant ecological parameter for earthworms populations. Instead, a more comprehensive understanding of the health of earthworms' populations and, consequently, the soil ecosystem can be obtained by examining sub-lethal effects. To this end, the use of biomarkers represents a valuable approach (Solé, 2020). Biomarkers provide insights into biochemical and physiological responses within organisms following exposure to xenobiotics in their environment. These sensitive processes include the induction of biotransformation, oxidative stress, and antioxidant responses, which can be triggered by various xenobiotics, including pesticides. Additionally, the inhibition of cholinesterase (ChE) activity in nervous and muscular tissues is associated with the neurotoxic effects of carbamate and organophosphate insecticides (Amiard-Triquet et al., 2016). Biotransformation responses, such as the induction of phase I-related ethoxy-resorufin-O-deethylase (EROD) activity and phase II conjugation activity, glutathione S-transferase (GST), have been reported in earthworms exposed to pesticides (Rodríguez-Castellanos and Sanchez-Hernandez, 2007; Tiwari et al., 2016). Furthermore, antioxidant responses, including catalase (CAT) activity, and the assessment of cell membrane oxidative damage through lipid peroxidation (LPO), have been examined in tissues of earthworms exposed to pesticides (Tiwari et al., 2016; Wen et al., 2021). In terms of neurotoxicity, the inhibition of ChE activity has been observed in various species of earthworms exposed to different pesticides (Costa et al., 2022; Rodríguez-Castellanos and Sanchez-Hernandez, 2007; Sanchez-Hernandez et al., 2018).

Biomarkers provide early warnings about physiological effects occurring to organisms exposed to contaminants. Complementarily, behavioral changes can represent an integration of such physiological impairment, as well as responses at the individual level, with clearer ecological consequences (Shi et al., 2017). When mobility is not restricted and chemosensory functions remain intact, earthworms have the capacity to relocate to cleaner areas as an avoidance behavior (Lackmann et al., 2018; Pereira et al., 2010). However, analogous to observations in aquatic ecosystems, the rejection of polluted areas by organisms due to chemical pollutants comes with a cost, leading to the displacement of biodiversity and crucial ecosystem services (Moreira-Santos et al., 2019). Furthermore, the avoidance/preference response of earthworms towards a specific soil is influenced by various soil parameters (Fründ, Graefe and Tischer, 2010; Gainer et al., 2022). As shown in several studies (Singh et al., 2020; Subin et al., 2015), the pH, soil texture, moisture, and organic matter content collectively play a pivotal role in the habitat selection of earthworms.

For this research, we considered the characteristics of horticultural production in Zarcero, Costa Rica. This is a relatively small area with intensive production but different agricultural practices among farmers. Pesticide use in the area ranges from conventional production based on intensive use, to completely organic production; with an intermediate use in the case of conventional producers applying better agricultural practices (Ramírez et al., n.d.). In such a scenario, we anticipated higher physiological stress, reflected in biomarker responses, in *A. gracilis* inhabiting soils of farms with greater pesticide usage. Correspondingly, we expected that *A. gracilis* would prefer soils with a lower pesticide load. Considering that, this study aimed to investigate the effects of pesticide use in horticultural soils on earthworm health and habitat selection. To achieve this, we measured physiological stress signals in organisms collected from different sites representing a gradient of pesticide use, and analyzed pesticide residue levels in those sites. Additionally, we assessed the avoidance/preference behavior of earthworms when presented with the same soils.

2. Material and methods

2.1. Site selection

The town of Zarcero, in the province of Alajuela, is an area of horticultural production with a considerable use of pesticides that has presented worrying results about its negative effects on the environment (Berrocal-Montero et al., 2021; Weiss et al., 2023). Conventional production systems predominate in the area, however, there are organic production systems and farms that apply good agricultural practices (Ramírez et al., n.d.). A gradient of soils made up of forest soils (FO), organic production farm soils (OR), production farm soils with good agricultural practices (GP) and conventional (CO) production farm soils, all located in the Zarcero area (Fig. 1), was chosen for evaluation. FO represents undisturbed soils with secondary forest cover and no agricultural use, while OR consist of soils managed without agrochemicals,

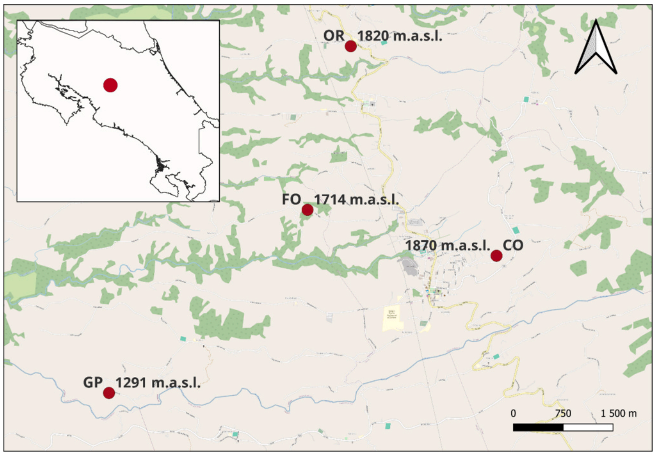

Fig. 1. Scheme of the spatial distribution of the four assessed sites. GP (10° 10′ 12.62" N, 84° 26′ 8.27" W), OR (10° 13′ 0.53" N, 84° 24′ 12.88" W), FO (10° 11′ 44.32" N, 84° 24′ 31.87" W), CO (10° 11′ 20.20" N, 84° 22′ 58.87" W) indicating altitude in meters above sea level (m a.s.l.). Areas in green correspond to forest patches.

using only biological inputs as biofertilizers. CO follows a model characterized by intensive agrochemical use and mechanical plowing. GP, in contrast, has a reduced agrochemical use, incorporating alternative strategies such as crop rotation and biological inputs, although it still maintains minimal pesticide dependence.

2.2. *Sampling*

Sampling was carried out in the dry, transition and rainy seasons, in which the selected farms were visited. Physical and chemical parameters and pesticide residues were assessed in all three seasons; biomarkers in *A. gracilis* were assessed during dry and rainy seasons; and the soil and individuals of *A. gracilis* for the behavioral test were collected during the transition season. For the assessment of biomarkers, a sampling point was defined at the edge of each assessed site. In the farms, the points were located immediately downstream from the cultivated area, in the forest, the point was on the edge near to the road. At these sites, a grid of 50 cm × 50 cm was arranged, which defined the specific sampling point. Soil was removed up to 20 cm deep, and sieved to collect the present earthworms. Different earthworm phenotypes were observed in the soil of every sampling site during a preliminary assessment. After comparing such organisms, the most abundant phenotype that was common to all sampling sites was identified as *A. gracilis* and selected for the tests. A homogenized soil sample of 2 kg was taken in the dry, transition and rainy season for pesticide residue analysis. These samples were transported to the laboratory in a cold icebox and maintained at −20 °C until extraction and analysis.

A minimum of five earthworms was collected per site. They were immediately dissected, and a sample was collected by cutting the first 3 mm of the proximal zone of each individual, this sample was identified as “head”. Subsequently, the rest of the proximal extreme, including the clitellum, was removed, and a second sample was taken by cutting approximately 5 mm of tissue in the central part, posterior to the clitellum. This sample was identified as “tract”. All collected samples were placed in 1.5 mL microcentrifuge tubes and stored in liquid nitrogen for transport to the laboratory. In the laboratory, the samples were preserved at −80 °C until further analyses.

2.3. *Soil physical and chemical parameters analysis*

Soil physical and chemical parameters (pH, texture, moisture and organic matter) were measured once per site during each of the three sampling periods. For the estimation of moisture content, a modification of the gravimetric method, as executed by Tanriverdi et al. (2016) was applied. Approximately 20 g of soil from each sample was weighed using an analytical balance (Sartorius – CPA224S). The measured quantity was placed in porcelain crucibles and introduced into a muffle furnace (Vulcan TM 3–550) at 105 °C for 24 h until the weight stabilized. The final weight of the dried sample was measured at room temperature, and the percentage of moisture content was calculated.

Regarding organic matter estimation, a modification of the loss on ignition method, as conducted by Gerenfes et al. (2022), was applied. The previously dried and weighed sample used in the moisture measurement was introduced into a muffle furnace at 550 °C for 4 h. The final weight at room temperature was measured, and the percentage of organic matter content was calculated.

For pH determination, a modification of the pH measurement method in water (H_2O) (Food and Agriculture Organization of the United Nations [FAO], 2021) was applied. Of each sample, 10 g of soil and 25 mL of ultrapure Milli-Q® water (Millipore) were added to 100 mL Erlenmeyer flasks. The content was agitated at 200 rpm for 60 min until complete homogenization. After the agitation process, the mixture was allowed to rest for an additional 60 min. The mixture was gently stirred for 10 s, before pH measurement with an electrode (Hach - HQ411D).

For texture classification, a modification of the Bouyoucos (1936) method was applied. 50 g of soil sample were added to a glass container with 20 mL of water, 5 mL of 1 M sodium hydroxide, and 5 mL of saturated sodium oxalate. It was mixed for 15 min before being transferred to a 1500 mL graduated cylinder. Water was added to reach a volume of 1000 mL (with the soil hydrometer added). The mixture was manually inverted four times, and readings were taken on the hydrometer at 40 s and 2 h after agitation. Additionally, temperature readings were taken at 40 s and 2 h after agitation, and the texture of each sample was determined.

2.4. *Pesticide residue analysis*

Pesticide residues were determined with a multi residue method by LC-MSMS according to the modified NMKL Method No 195 (NMKL, 2013). The extraction was done using 5 g of the soil sample was placed inside a 50 mL polypropylene tube. The sample was spiked with diuron-d6 and chlorpyrifos-d10 as internal standard, and 10 mL of LCMS grade ethyl acetate was added as extraction solvent. The mixture was stirred for 2 min, then placed in the ultrasonic bath (Branson-5210) for 15 min, removed, and 5 g of sodium sulfate was added as a drying agent. It was shaken again for 2 min and centrifuged for 10 min at 806 RCF. Finally, the supernatant was concentrated and filtered to arrange it in injection vials. The extracts obtained were analyzed by liquid chromatography) with tandem mass spectrometry (UPLC Acquity H Xevo TQ-S Micro, Waters) (Moschet et al., 2013).

2.5. *Biomarker analyses*

Tissue samples of *A. gracilis* were homogenized by sonication (Branson SLPt 40:0.15:4C) in a 0.1 M phosphate buffer with pH = 7.2 for head samples and pH = 7.4 for tract samples. From the homogenates of tract samples, an aliquot was separated and mixed with 0.2 mM butylated hydroxytoluene (BHT) for the LPO analysis. The rest of the homogenate was centrifuged (Eppendorf-5417 R) at 15300 RCF, 4°C for 20 min, and the supernatant was used to measure the activities of EROD, GST and CAT enzymes. The homogenates of head samples were centrifuged at 10600 RCF, 4 °C for 5 min, and the supernatant was used to measure ChE activity.

2.5.1. *Neurotoxicity*

The ChE activity was assessed with the method of Ellman et al. (1961) employing a hydrolysis reaction produced by the ChE present in the tissue analyzed on an artificially introduced substrate of acetylthiocholine 75 mM and 5′5-Dithiobis (2 nitrobenzoic acid) (DTNB) 10 mM as a chromogenic reagent. The absorbance was measured at 415 nm during 10 min with readings every 150 s, and the ChE activity was expressed as nanomoles per minute per milligram of protein.

2.5.2. *Biomarkers of oxidative stress and antioxidant response*

CAT activity was measured applying the method described by Aebi (1974), following the decomposition of the substrate hydrogen peroxide (H_2O_2) by the CAT in the sample, and the absorbance was read at 240 nm for 20 s. EROD activity was evaluated with a variation of the methodology applied by Peters et al. (1994), measuring the deethylation of 7-ethoxy-resurofin to resorufin in the presence of NADPH, and the kinetics was assessed by fluorescence, with 530 nm for excitation and 580 nm for emission, for 3 min, measuring every 20 s. GST activity was measured by the conjugation method of reduced glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) (Habig et al., 1974), and the reading was conducted at 340 nm for 3 min. LPO was evaluated as the amount of thiobarbituric acid reactive species (TBARs) according to Oakes and Van Der Kraak (2003), and the absorbance of the reaction was measured at 535 nm. All reactions were measured by spectrophotometry using a VarioSkan™ Lux multimode microplate reader (Thermo Fisher Scientific). All the biomarker responses were normalized by the protein concentration in the sample, with the method of Bradford (1976), using a protein assay kit (BioRad®) with bovine serum albumin as protein standard.

2.6. Avoidance assay

A total of 40 *A. gracilis* individuals were collected from the organic production farm during transition season and taken to the laboratory to complete an acclimatization period of 7 d in which the feeding and hydration of the medium were regulated. The avoidance test was carried out using an adaptation of the biological test method EPS 1/RM/43 (Environment Canada, 2004). The exposure wheel was devised using a cylindrical container with a diameter of 25 cm and a depth of 20 cm. A PVC tube (6 cm diameter) was placed in the center of the wheel as a central compartment with holes in the bottom to allow the entrance into all surrounding compartments. Four metallic sheets with 3 holes of 1 cm at the bottom were used as divisions to separate four test compartments around it. The central compartment had 2 holes of 1 cm connecting with each compartment of the surroundings. Each compartment of the surrounding was filled with a portion of soil to be evaluated (FO, OR, GP, and CO), the test was triplicated, and the arrangement of soils in each exposure system was randomized, ensuring that the same soil pattern was never repeated. To start the test, humidity was standardized in all compartments, then 10 worms were introduced, one by one, into the central compartment until they moved into any of the test compartments. After 48 h, the divisions between each compartment were sealed and the soil was retrieved to count the *A. gracilis* individuals per compartment.

2.7. Statistical analyses

All analyses were conducted using R version 4.3.0 (R Core Team, 2023). The outliers of each dataset were detected using the function “identify_outliers” of the package rstatix (Kassambara, 2023) then, data identified as true outliers were eliminated from de dataset manually. After that, the normality of the data was assessed with a Shapiro-Wilk test and quantile-quantile plot. As some of the variables did not meet the normality criteria, differences among biomarker responses per site and season, as well as the difference in the number of earthworms present in each soil sample during the avoidance test, were assessed with a Kruskal-Wallis test, followed by a Dunn's test as post hoc analysis, using the FSA package (Ogle et al., 2023). Biomarkers were analyzed comparing separately their responses by site for each season, and also by season for each site.

3. Results

3.1. Physical and chemical characterization

The physical and chemical parameters evaluated varied across the assessed soil types (Table 1). The soils with agricultural use (GP, OR and CO) had a loamy texture characterized by smaller particles and also showed higher moisture and content of organic matter than the FO soil which had a sandy texture, low moisture and organic matter content. This difference in moisture and organic matter was observed especially during the dry and transition seasons.

In terms of pH, values ranged from 5.47 to 7.02, generally showing an acidic profile, with GP soils registering the most acidic pH levels, and CO displaying a more neutral pH. No alterations in pH were observed in relation to seasonal changes. However, as expected, substantial variations in moisture were recorded, with a higher amount during the transitional and rainy seasons.

3.2. Presence of pesticide residues

Table 1
Soil physical and chemical parameters measured in different seasons for the four sites studied, farming with good environmental practices (GP), organic farming (OR), forest (FO) and conventional farming (CO) during the dry, transition and rainy seasons.

Season	Soil	pH	Organic matter %	Moisture %
DRY	GP	5.50	13.45	28.60
	OR	6.66	18.09	37.78
	FO	6.24	5.01	14.63
	CO	6.39	16.96	36.07
TRANSITION	GP	5.50	12.45	40.31
	OR	6.92	22.42	45.27
	FO	6.24	4.26	24.38
	CO	6.23	17.73	45.38
RAINY	GP	5.47	12.53	31.82
	OR	7.02	20.14	47.30
	FO	6.26	11.20	28.76
	CO	6.06	16.94	39.29

Pesticide residues were detected in all the evaluated soils. An increased presence of substances was detected in CO soil, as compared to the other sites (Table 2). Specifically, 43 pesticides, with 20 corresponding to fungicides, 13 to insecticides, 9 to herbicides, and 1 to a nematicide. Likewise, the concentration of pesticides detected in CO throughout the three sampling seasons was higher in comparison to the other sites, particularly during the rainy season. Interestingly, eight substances were detected in the OR soil, six in FO soils, and the lowest number of substances (5) were registered in the GP site.

It is noteworthy that certain pesticides, such as chlorpyrifos, boscalid and pyraclostrobin were found in varying treatments across different sampled seasons. Furthermore, chlorpyrifos exhibited the highest recorded concentrations, with forest soil during the dry season displaying the greatest concentration of this pesticide (71.9 ng g^{-1} dw), followed by boscalid.

Most of the detected insecticides belong to the organophosphate and carbamate groups, while fungicides identified were predominantly azoles, and herbicides were mostly triazines. However, pesticides belonging to the nicotinic, anilide and strobilurin groups, among others, were also detected, albeit in smaller quantities.

3.3. Biomarkers

The assessed biomarkers revealed differences among the status of the *A. gracilis* earthworms present in the different sites. Particularly, the organisms from CO had a significantly lower ChE activity compared to earthworms from GP and FO sites in rainy season, and earthworms from CO showed a significantly lower ChE compared to earthworms from all other sites in dry season, being the activity of CO earthworms more than 50 % lower than the other sites (Fig. 2, A). Additionally, in the GP site, the ChE activity in *A. gracilis* was significantly lower during the dry season as compared to the rainy season, a difference that was not observed on the other sites.

Consistently, the biomarkers of biotransformation and antioxidant response (EROD, GST, and CAT) showed a seasonal variation, with higher levels of activity during the dry season. This trend was very clear in EROD, with seasonal differences for all sites, which's activity during the dry season was about double as compared to the rainy season (Fig. 2, B). In the case of GST, earthworms collected during the dry season in GP and OR showed about double activity compared to their counterparts of the rainy season. Meanwhile, the animals collected from CO maintained a similar, and lower activity in both seasons, being significantly lower than FO site during the dry season (Fig. 2, C).

In the case of CAT, even though the activity in earthworms from OR and FO sites did not show significant differences, the seasonal trend was clear, being the activities close to zero in all sites during the rainy season (Fig. 2, D). Contrary to what was observed with the other biomarkers, the one related with oxidative stress (LPO) did not show any variation among sites or seasons (Fig. 2, E).

Table 2

Pesticides residues (nanogram gram dry^{-1} weight) found in the soil of four sites studied, farming with good environmental practices (GP), organic farming (OR), forest (FO), and conventional farming (CO), during the dry (DR), transition (TR) and rainy (RA) seasons. The sites are arranged from left to right from lowest to highest number of pesticides found per site, and the substances are organized by biocide classes.

SUBSTANCE	SITES											
	GP			OR			FO			CO		
	DR	TR	RA	DR	TR	RA	DR	TR	RA	DR	TR	RA
	ng g^{-1} dw											
FUNGICIDE												
Azoxystrobin			1.4			0.2				1.8	6.8	4.4
Bitertanol								1.3				2.7
Boscalid				8.0	5.9	7.8					8.4	3.6
Cyproconazole												3.0
Cyprodinil												1.8
Difenoconazole												2.3
Epoxiconazole												2.5
Fenbuconazole												1.7
Fenpropimorph								0.2				
Fluopicolide					0.3	0.4						1.7
Fluopyram												1.5
Flutolanil						0.1				1.2	2.8	2.7
Prochloraz										2.3	6.6	3.1
Propiconazole											3.7	3.6
Pyraclostrobin				0.3		0.1					0.8	1.6
Pyrimenthanil												1.8
Quinoxyfen												1.2
Tebuconazole								1.1				3.3
Triadimefon												2.1
Trifloxystrobin	0.5										0.3	1.4
HERBICIDE												
Atrazine												1.6
Clomazone												1.7
Fluazipop-p-butyl												1.1
Diuron												1.0
Linuron						4.2				2.1	7.8	5.5
Propanil												1.7
Simazine												1.4
Terbuthylazine												1.8
Terbutryn				0.6				0.5			1.0	1.5
INSECTICIDE												
Buprofezin												1.2
Cadusafos												2.0
Carbofuran			0.4									1.1
Chlorfenvinphos												1.9
Chlorpyrifos	2.5			2.2			71.9		0.4	36.1	38.1	3.1
Coumaphos												1.8
Diazinon											6.9	2.2
Ethoprophos												4.1
Fenamiphos											0.4	0.2
Imidacloprid											0.6	0.7
Phoxim	2.5											3.8
Piperonyl butoxide												1.0
Triazophos								0.5	12.7			1.9
Thiamethoxam												0.3

3.4. Avoidance assay

During the test development, no mortality occurred among the organisms, meeting the validation criteria of less than 10 % of mortality. The distribution of organisms exhibited heterogeneity, displaying a significant preference for GP and OR soils. By the end of the test, only 10 % of the earthworms were found in CO soils (Fig. 3).

4. Discussion

4.1. Pesticide residues

We hypothesized that a gradient in agricultural practices and land use would be reflected in a gradient of pesticide pollution in the soil of an area highly productive in horticulture. However, our results demonstrated that surroundings areas without the use of pesticides (OR) and secondary vegetation patches (FO) near conventional production systems also end up being contaminated. Different processes can contribute to the movement of pesticides from their source of use to other locations (Bedos et al., 2002; Cech et al., 2023). In the case of Zarcero region, the movement might be affected by being a small area with intensive production and some atmospheric variables could favor this cross-contamination. Such pesticide drift, even for long distances has been reported in Costa Rica previously (Daly et al., 2007; Shunthirasingham et al., 2011). Regarding the observed increase of pesticide residues observed during the rainy season, such seasonal variation in the pesticide content of soils has been reported for agricultural landscapes (Manjarres-López et al., 2021). The higher presence during the rainy

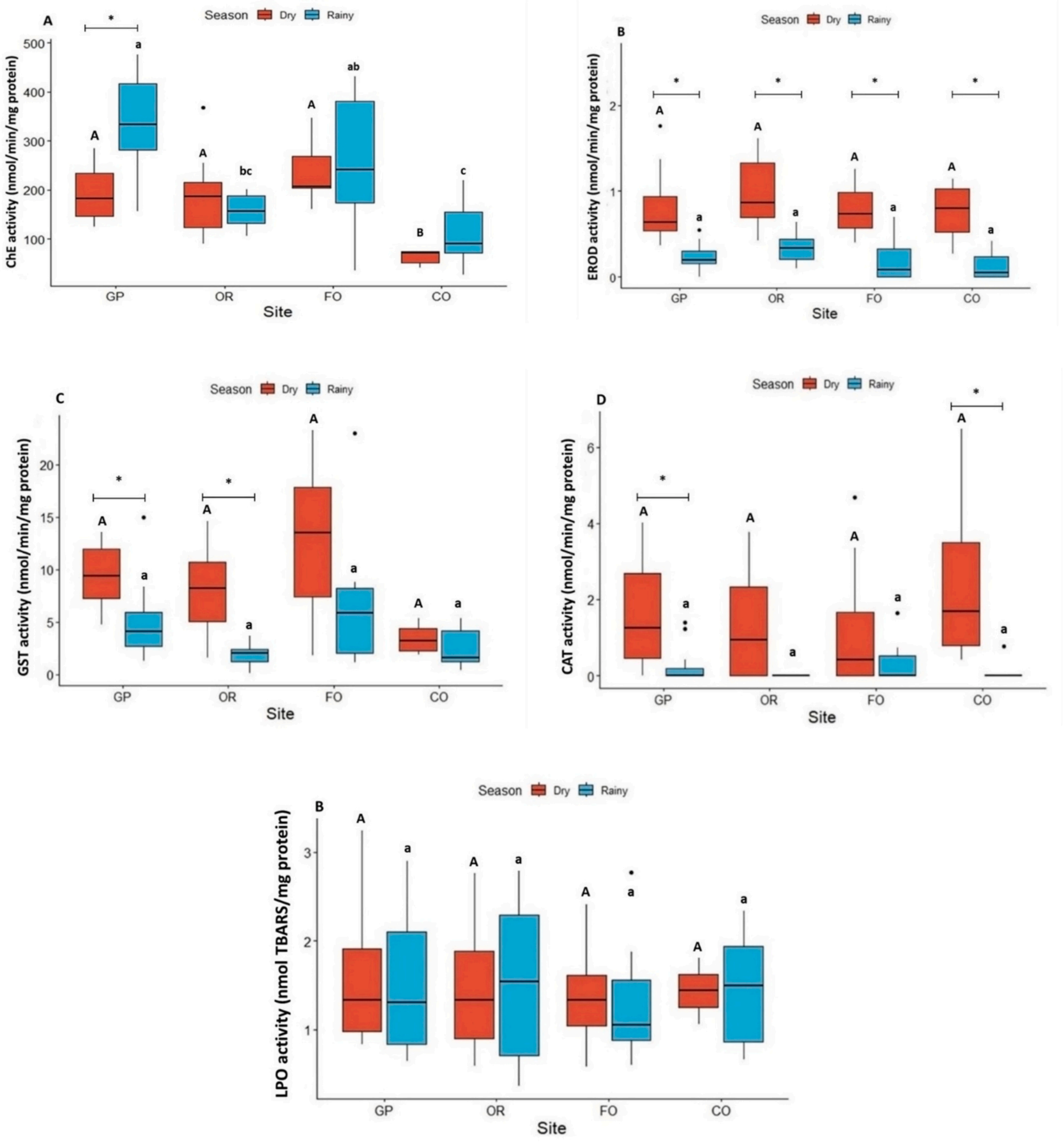

Fig. 2. Biomarkers ChE (A), EROD (B), GST (C), CAT (D) and LPO (E) measured in tissues of *Amynthas gracilis* collected from the four sites studied, forest (FO), organic farming (OR), farming with good environmental practices (GP) and conventional farming (CO) during dry and rainy seasons. Significant differences between sites and seasons are indicated with different letters ($p < 0.05$). Uppercase letters indicate significant differences ($p < 0.05$) between sites within the dry season, while lowercase letters indicate significant differences between sites within the rainy season. The asterisk denotes a significant seasonal difference within the same site.

season can be influenced by a higher use of substances during more intensive productive periods, but also by the changes in environmental conditions such as higher humidity and lower temperature and solar radiation that enhance the presence and persistence of residues in soil (Wang et al., 2025).

4.2. *Biomarkers*

Clear signs of physiological effects, including neurotoxicity and biotransformation were observed in earthworms, especially those collected at the site subjected to conventional farming (CO). The neurotoxicity, as ChE inhibition is consistent with the presence of organophosphate and carbamate compounds in that site. This response

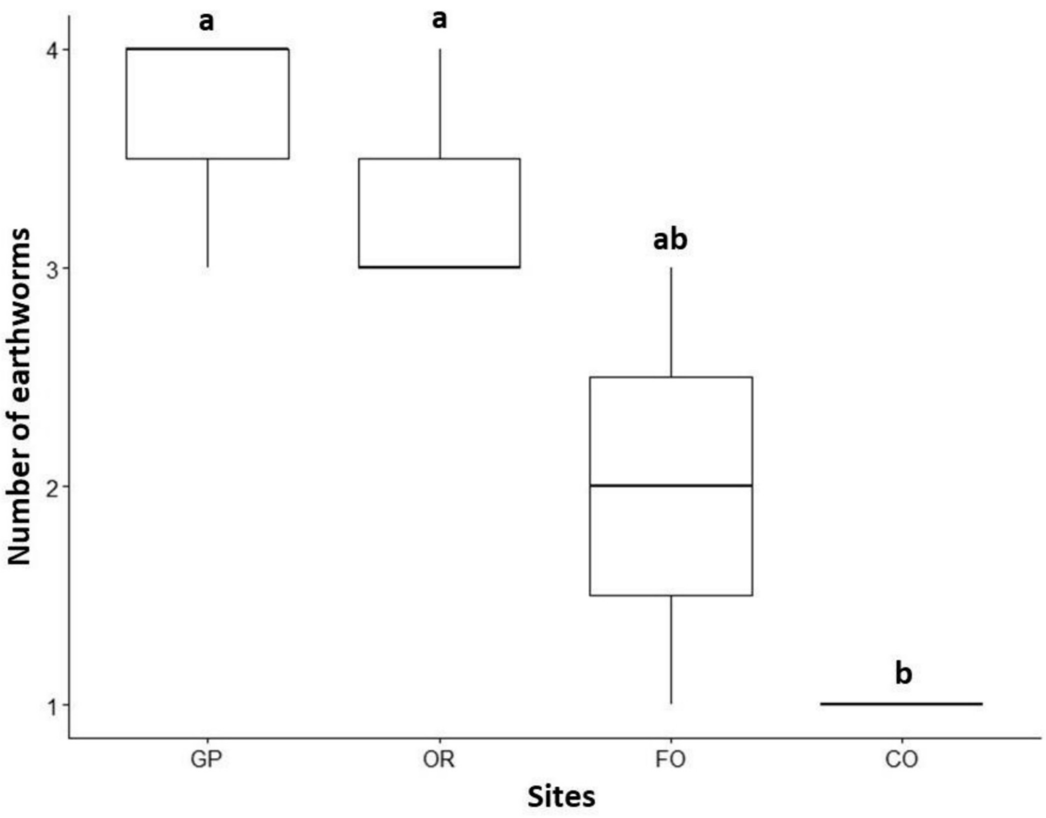

Fig. 3. Distribution of earthworms (*Amynthas gracilis*) within a test wheel with four soil types to choose, farming with good environmental practices (GP), organic farming (OR), forest (FO) and conventional farming (CO). Data from three replicates. Significant differences are indicated with different letters ($p = 0.01$).

has been previously reported in various earthworm species exposed to organophosphates (Sanchez-Hernandez et al., 2014; Sujeeth et al., 2023; Tiwari et al., 2019), such as chlorpyrifos and diazinon, found in higher amounts in CO soils. The concentrations found suggest that the decrease in ChE activity in organisms from CO soils is mainly due to the presence of chlorpyrifos in the dry season, and possibly to the sum of all the organophosphate and carbamate pesticides found in the rainy season. Furthermore, we also found the presence of a neonicotinoid insecticide (thiamethoxam) in CO soils. These pesticides, unlike organophosphates, act by blocking the postsynaptic nicotinic receptors of acetylcholine (Samson-Robert et al., 2015) however, ChE inhibition has been observed in different organisms exposed to neonicotinoids. For example, Győri et al. (2017) previously reported AChE inhibition in eels exposed to clothianidin, thiamethoxam, thiacloprid, and acetamiprid. More recently, a similar effect was observed in earthworms exposed to thiacloprid (Lackmann et al., 2023). The presence of thiamethoxam in CO soils could contribute to ChE inhibition in the earthworms collected there. On the other hand, despite the high concentration of chlorpyrifos observed in forest soil during the dry season, the ChE activity reported for earthworms in this environment is not as diminished as in CO soil during the dry season. However, no significant difference was detected between these soil types during this period.

A considerable amount of research (Chowdhary et al., 2022; Sanchez-Hernandez et al., 2014; Tiwari et al., 2019; Torabi Farsani et al., 2021) has reported an increase in CAT activity, GST activity, LPO, and EROD activity in earthworms exposed to organophosphorus and neonicotinoid insecticides. Moreover, an increase in the activity of these enzymes has been recorded in earthworms exposed to strobilurin, anilide, and benzamide fungicides (Di et al., 2016; Han et al., 2014; Wen et al., 2023). While there are no references indicating variations in the antioxidant response and oxidative stress activity of earthworms exposed to benzanilide, azole, and carboxamide fungicides, or triazine herbicides, this response has been observed by Aksakal (2020), Egaas et al. (1999), Li et al. (2010), Liu et al. (2016), Melo de Almeida et al. (2022) and Štěpánová et al. (2012), in aquatic organisms (*Danio rerio*, *Daphnia magna*, *Oncorhynchus mykiss*, *Salmo trutta*, *Carassius auratus*). Our results have not reflected significant site differences in the responses of CAT, LPO, and EROD, however, a diminished GST activity was observed in CO soils. GST is a phase II enzyme involved in the detoxification and biotransformation of endogenous and exogenous substances (Dasari, 2017). An increase in the activity of this enzyme may indicate a detoxification response in the organism; however, our results show an inhibitory pattern in CO soils. These findings are comparable to those reported by Booth and O'Halloran (2001), Lackmann et al. (2023), and Liu et al. (2017), who observed GST inhibition in the earthworms *Aporrectodea caliginosa*, *Eisenia andrei*, and *Eisenia fetida*, respectively. This may suggest that organophosphorus and neonicotinoid pesticides can inhibit GST activity. Additionally, observations in *E. fetida* (Ma et al., 2019) and *Folsomia candida* (Kovačević et al., 2023) exposed to strobilurin fungicides showed an initial increase in GST activity in the early days of exposure, followed by a long-term decrease. This could suggest a depletion of antioxidant capacity. As our organisms were collected in the field, they may have been exposed to such compounds for an extended period, impacting their antioxidant capacity.

The differences observed in biotransformation and antioxidant response biomarkers between the dry and rainy seasons have been previously observed in earthworms (Acharya and Mishra, 2020). Lovas et al. (1987) suggested that the assessment of biomarkers should be conducted within the same season due to the changes induced by environmental variation. Our results suggest that biotransformation and antioxidant responses in *A. gracilis* are higher during the dry season, however, the differences observed between sites, within the same season, should be attributed to other environmental factors, including the pesticides.

4.3. Habitat selection: assessing the avoidance

As demonstrated by Vasconcellos et al. (2013), the habitat selection of soil macrofauna is modulated by the physical and chemical properties of the soil, and the behavior of the organisms towards contaminated soils can also be influenced by such physical and chemical variables (Natal-da-Luz et al., 2008). Some of these factors specifically refer to soil texture, the amount of organic matter, and soil pH. However, concerning earthworms, some authors (De Silva and van Gestel, 2009; Hund-Rinke and Wiechering, 2001) conclude that soil abiotic factors do not affect avoidance behavior, and that it is mainly related to the presence or absence of contaminants. In this research, we have observed that both soil abiotic factors and the presence of contaminants can impact avoidance behavior. In our test, *A. gracilis* preferred OR and GP soils, with a lower preference for FO and a clear avoidance of CO soils. Regarding soil parameters, Chan and Barchia (2007) considered that soil organic carbon is a critical factor in the distribution of earthworms at a particular site. In this regard, *A. gracilis* is an epi-endogeic species, which prefers the organic matter present in the soil surface, and in the absence of such superficial organic matter, the amount of organic soil carbon in deeper layers of the soil is not a decisive factor affecting avoidance (Falco and Momo, 2010). As the soil in our avoidance test was homogenized, without a specific layer of organic matter on the surface, this factor should not have influenced the preference of *A. gracilis*. However, the FO soils, with the lower content of organic matter were less preferred than some agricultural soils richer in organic matter. In this case, a second factor could affect the attraction of the earthworms. According to Duiker (2013), earthworms prefer loamy soils instead of sandy soils where the grain size could generate an abrasive effect that damages the skin of earthworms. Then, the sandy texture of FO soil might explain why earthworms selected GP and OR soils.

On the other hand, it is well known that the presence of pesticide in the soil can elicit an evasive response in earthworms (Datta et al., 2021; Ge et al., 2018; Lackmann et al., 2023; Salvio et al., 2016). This phenomenon is attributed to the existence of a chemosensory system on the surface of their bodies, which grants them the ability to detect the presence of pesticides (Reinecke and Reinecke, 2004). However, it is important to emphasize that the manifestation of evasive behaviors is dependent upon the specific nature of the pesticide. In our research, CO soils showed a sandy loamy texture, similar to OR, and organic matter percentages intermediate between OR and GP soils, which could eliminate abiotic factors as reasons for evasive behavior. Nevertheless, the presence of many neurotoxins, and the neurotoxic effect described in resident earthworms could explain this aversion. Regarding these substances, Zhou et al. (2007) found that earthworms can avoid soils contaminated with chlorpyrifos, and Natal-da-Luz et al. (2012) reported the avoidance response in *E. andrei* exposed to soil contaminated with diazinon. Furthermore, Nkontcheu et al. (2023) estimated that chlorpyrifos and ethoprophos were among the pesticides representing higher risk to earthworms in agricultural soils. Regarding the presence of a neonicotinoid on CO soil, Chowdhary et al. (2022) identified avoidance effects of earthworms to clothianidin; however, they also observed that at low concentrations, the effect could be one of attraction. Meanwhile, Pereira et al. (2010) have highlighted the need for caution in drawing definitive conclusions regarding the evasive behavior of earthworms in systems exposed to neurotoxic xenobiotics, due to the potential influence of these compounds on organisms' decision-making processes. The clear avoidance that we observed towards CO soil where organophosphates were detected suggests a connection between the presence of these insecticides and the observed evasive behavior, consistent with the mentioned reports. In contrast, the scenario regarding fungicides and evasive behavior is more complex. On one hand, Garcia et al. (2008) observed evasive behavior of earthworms in response to the fungicides benomyl, carbendazim and lambda-cyhalothrin fungicides; however, of the fungicides reported by these authors, only carbendazim and benomyl are chemically compatible with those found in our analysis. Additionally, for most of the fungicides identified in this study, the information regarding their attractiveness or repellence for earthworms is scarce. Only Rico et al. (2016) reported an attraction behavior of *E. fetida* towards soils with low concentrations of prochloraz which is an azole fungicide. In our results, CO soils, loaded with insecticides were avoided; while, OR soils were preferred, despite the presence of several fungicides. We recognize that evasive behavior largely depends on the chemical to which organisms are exposed (Garcia et al., 2008) and the inherent sensitivity of each species (De Silva and van Gestel, 2009). This highlights the necessity of studying the effects of fungicides on non-target biota.

We observed signs of neurotoxicity and altered biotransformation in *A. gracilis* inhabiting a soil highly exposed to pesticides. Furthermore, the soil from that site was avoided when the organisms had other options to colonize, demonstrating that pesticide pollution can affect the physiology and the habitat selection of earthworms. These effects might represent a serious impairment in the functions that earthworms accomplish in the soil, and to the ecological services that this environmental compartment supplies (Datta et al., 2016; Miglani and Bisht, 2019). Incidentally, our findings support the suggestion of integrating sub-individual and individual responses in the schemes for the ecological risk assessment of pesticides in soil ecosystems (Pelosi et al., 2014). The presence and the physiological state of earthworms in agricultural soils should be assessed in order to maintain a healthy state of such ecosystems.

5. Conclusions

Earthworms of the species *A. gracilis* living in soil contaminated with pesticides from horticultural activities showed signs of neurotoxicity and reduced biotransformation. Accordingly, the soil from the most polluted source caused a clear avoidance by these earthworms. The observed effects can generate an impact in their functional role in ecosystems. Furthermore, seasonal changes and atmospheric transport can influence the presence of pesticide residues in soils, enhancing their long-term impact on ecosystems.

The combination of physiological and behavioral endpoints employed has yielded significant findings, demonstrating its potential to enhance our comprehension of the sublethal impacts of pesticides and pesticide combinations on earthworms. These outcomes are a contribution for further advancements in this integrated approach.

CRediT authorship contribution statement

Gabriel Brenes-Bravo: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Lukas Reinhard:** Writing – original draft, Methodology, Investigation, Formal analysis. **Clemens Ruepert:** Writing – original draft, Resources, Investigation, Funding acquisition, Formal analysis, Data curation. **Frank Solano-Campos:** Writing – original draft, Methodology, Investigation, Formal analysis. **Freylan Mena:** Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT, an AI language model developed by OpenAI, in order to check the language and review the writing. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Declaration of competing interest

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

Acknowledgments

This work was developed within the framework of the PROECO (SIA 0273-17) and LAREP (SIA 0293-17) programs of the Regional Institute for Toxic Substances Studies (IRET). We appreciate the contribution of PhD. Carlos Fragoso González in identifying the earthworm species evaluated in this study, as well, the three producers from Zarcero, who facilitated the selection of the sites and the collection of the samples. The access to the genetic material of the organisms was granted through the permit R-CM-UNA-009-2022-OT-CONAGEBIO.

Data availability

Data will be made available on request.

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