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EVALUACIÓN DE ESTRÉS FISIOLÓGICO Y VARIACIONES EN EL MICROBIOMA
INTESTINAL DE LOMBRICES DE TIERRA (*Amyntas gracilis*) EXPUESTAS A
SUELOS HORTÍCOLAS CON DIFERENTES REGÍMENES DE APLICACIÓN DE
PLAGUICIDAS EN ZARCERO, COSTA RICA.

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IV. Resumen

Los suelos agrícolas están constantemente expuestos a plaguicidas. Esta exposición permanente amenaza el ecosistema y los servicios que proporciona para sostener la agricultura. Las lombrices de tierra son componentes clave de la macrofauna del suelo que pueden ser susceptibles a dicha contaminación. Para evaluar si la presencia de plaguicidas en suelos hortícolas puede inducir estrés fisiológico, comportamiento evasivo, o modificar el microbioma intestinal en las lombrices de tierra residentes, analizamos residuos de plaguicidas y características físico-químicas en sitios con diferentes prácticas de manejo: agricultura convencional (CO), la agricultura con buenas prácticas ambientales (GP), la agricultura orgánica (OR) y el bosque (FO). El estrés fisiológico se evaluó midiendo biomarcadores de neurotoxicidad (actividad colinesterasa - ChE), biotransformación (actividades glutatión S-transferasa - GST y Etoxi-resorufina-O-deetilasa - EROD) y estrés oxidativo (actividad catalasa - CAT y peroxidación lipídica - LPO) en individuos de la lombriz de tierra *Amyntas gracilis* que habitan en los sitios del gradiente de estudio durante las estaciones seca y lluviosa. También, se realizó una prueba de evitación en la cual un grupo de *A. gracilis* fue introducido a muestras de suelo del gradiente estudiado, y 48 horas después se contó su selección. Para evaluar si la exposición al suelo con plaguicidas afectaría la composición del microbioma intestinal de la especie estudiada se realizó un análisis de las variaciones en la estructura de la comunidad bacteriana, la cual se obtuvo mediante una asignación taxonómica a partir de la amplificación de la región V4 del gen 16S rRNA del ADN extraído del contenido intestinal de 5 individuos por cada tipo de suelo en temporada seca y lluviosa. Residuos de plaguicidas fueron detectados en todos los sitios muestreados, pero se observaron niveles más altos de contaminación en las muestras de suelo bajo

agricultura convencional, con presencia de las 42 sustancias detectadas durante la temporada lluviosa, incluyendo muchos insecticidas de alta toxicidad para los invertebrados del suelo. Se observó inhibición de ChE y disminución de las actividades de GST en *A. gracilis* que habitaban el sitio de agricultura convencional, además de una clara variación estacional en las actividades de GST, CAT y EROD en todos los sitios. *A. gracilis* evitó significativamente el suelo del sitio de agricultura convencional y prefirió suelos con características físico-químicas similares, pero con niveles más bajos de plaguicidas. Además, se registró una estructura de la comunidad bacteriana muy similar en el contenido intestinal de organismos en todos los suelos evaluados en temporada seca, y se observó una mayor diversidad bacteriana en los contenidos intestinales de los organismos de los suelos de OR y GP, principalmente y con menor medida en FO en temporada lluviosa. Este cambio estacional no fue observado en el microbioma de las lombrices provenientes de suelos de CO. Nuestros resultados evidencian que el uso intensivo de plaguicidas puede inducir estrés fisiológico en *A. gracilis* y provocar su escape de suelos contaminados, además de alterar la estructura de la comunidad bacteriana del contenido intestinal. Estas respuestas afectarían a la comunidad de macrofauna del suelo y agotarían los servicios que proporcionan.

Abstract

Agricultural landscapes are constantly exposed to pesticides. This permanent exposure threatens the ecosystem and the services 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, evasive behavior and alter the gut microbiome in resident earthworms, we evaluated pesticide residues and soil physical-chemical parameters in sites with different management practices:

conventional farming (CO), farming with good environmental practices (GP), organic farming (OR), and forest (FO). Physiological stress was assessed by measuring biomarkers of neurotoxicity (cholinesterase activity – ChE), biotransformation (glutathione S-transferase – GST and ethoxy-resorufin-O-deethylase – EROD activities), and oxidative stress (catalase activity – CAT and lipid peroxidation – LPO) in individuals of the earthworm *Amyntas gracilis* inhabiting sites within the mentioned gradient during dry and rainy seasons. Additionally, an avoidance test was conducted where a group of *A. gracilis* was introduced to soil samples from the studied gradient, and their selection was counted 48 hours later. To evaluate if pesticide exposure in soil would affect the composition of the gut microbiome of the studied species, an analysis of variations in bacterial community structure was performed. This was achieved through taxonomic assignment from the amplification of the V4 region of the 16S rRNA gene from DNA extracted from the gut contents of 5 individuals per soil type during dry and rainy seasons. Pesticide residues were detected at all sampled sites, with higher levels of contamination observed in soil samples from conventional farming, including the presence of all 42 analyzed substances during the rainy season, many of which are highly toxic insecticides to soil invertebrates. Inhibition of ChE and reduced GST activities were observed in *A. gracilis* inhabiting the conventional farming site, alongside clear seasonal variation in GST, CAT, and EROD activities at all sites. *A. gracilis* significantly avoided soil from the conventional farming site and preferred soils with similar physical-chemical characteristics but lower pesticide levels. Furthermore, a very similar bacterial community structure was recorded in the gut contents of organisms from all soils during the dry season, and a higher bacterial diversity was observed in the gut contents of organisms from OR and GP soils mainly, and to a lesser extent in FO soils during the rainy season; this seasonal change was not observed in gut microbiome of earthworms from CO

soils. Our results indicate that intensive pesticide use can induce physiological stress in *A. gracilis*, and provoke their escape from contaminated soils, and also alter the bacterial community structure of the gut contents. Such responses would affect the soil macrofauna community and deplete the services they provide.

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“Si he logrado ver más lejos ha sido porque he subido a hombros de gigantes”

– Isaac Newton-

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IX. Lista de abreviaturas

AChE: Acetilcolinesterasa

ASV: Variante de Secuencia de Amplicón

CAT: Catalasa

CDNB: 1-cloro-2, 4-dinitrobenceno

ChE: Colinesterasa

CO: Finca de Producción Convencional

CONAGEBIO: Comisión Nacional para Gestión de la Biodiversidad

DNA: Ácido Desoxirribonucleico

ECOTOX: Laboratorio de Estudios Ecotoxicológicos

EROD: 7-etoxy-resorufin-O-destilase

FAO: Food and Agriculture Organization of the United Nations (español: Organización de las Naciones Unidas para la Alimentación y la Agricultura)

FO: Bosque

GP: Finca de Producción con Buenas Prácticas Agrícolas

GST: Glutación S-transferasa

IRET: Instituto Regional en Estudios de Sustancias Tóxicas

LAREP: Laboratorio de Análisis de Residuos de Plaguicidas

LCMS: Cromatografía Líquida- acoplada a Espectrometría de Masas en tandem

LPO: Peroxidación Lipídica

NAPDH: Nicotinamida Adenina Dinucleótido Fosfato Reducido

OR: Finca de Producción Orgánica

PCR: Reacción en Cadena de la Polimerasa

RCF: Fuerza Centrífuga Relativa

rRNA: Ácido Ribonucleico Ribosomal

TBARS: Especies Reactivas del Ácido Tiobarbitúrico

UNA: Universidad Nacional

UPCL: Cromatografía Líquida de Alta Eficiencia

OMS: Organización Mundial de la Salud

X. Descriptores

Agricultural landscapes

Soil macrofauna

Metabarcoding

Biomarker

Gut microbiome

Behavior

Avoidance

XI. Introducción general

El uso extendido de plaguicidas es una práctica agrícola común alrededor del mundo. Estos insumos pretenden la protección de los cultivos contra insectos, hongos, malas hierbas y otras plagas (Organización Mundial de la Salud [OMS], 2022). En este contexto Costa Rica registra un alto uso de plaguicidas con valores promedio cercanos a los 18.2 kg (Araya, 2015). Sin embargo, datos recientes demuestran que el promedio real de uso aparente de plaguicidas en el país ronda los 34.45 kg de ingrediente activo por hectárea al año (Vargas, 2022), de los cuales el 93% puede ser considerados plaguicidas de alta peligrosidad (Pomareda, 2022).

La horticultura es una de las actividades que ha presentado un aumento en el uso de plaguicidas en el país en los últimos años (Galt, 2008). Se ha encontrado que los productores con grandes áreas de cultivo utilizan mayor cantidad de plaguicida y en áreas menores se aplica menor cantidad de plaguicida, pero con mayor número de productos (Ramírez et al. 2014). Se ha reportado que en la zona de Zarcero se utiliza más de 119 ingredientes activos (i.a.) diferentes, determinando que el plaguicida de mayor uso en la zona es el fungicida clorotalonil, seguido por cipermetrina, mancozeb, acefato y boscalid (Ramírez et al., 2017).

El uso intensivo de plaguicidas representa una amenaza significativa para la biodiversidad del suelo en los paisajes agrícolas (Geisen et al., 2019), especialmente considerando los grandes vacíos en nuestra comprensión del comportamiento ambiental y la toxicidad de los plaguicidas actualmente utilizados (Fernandes et al., 2020; Silva et al., 2019). Sabemos que la biota del suelo desempeña un papel crucial en la provisión de varios servicios ecosistémicos que apoyan la agricultura. Evaluar con precisión los riesgos que la

contaminación por plaguicidas representa para estos servicios ecosistémicos es una necesidad urgente, especialmente en las regiones tropicales (Daam et al., 2019).

Existe gran variedad de investigaciones que describe los impactos del uso de plaguicidas sobre la meso fauna edáfica (Gunstone & Dubey, 2021; Aktar et al., 2009; Gunstone et al., 2021). En general, el uso de plaguicidas se traduce en una reducción significativa de las poblaciones en los suelos, invertebrados y microorganismos no objetivo, que participan activamente en el ciclo de nutrientes. En consecuencia, la fertilidad de los suelos se ve directamente comprometida por el uso de plaguicidas; esto implica en consecuencia un impacto a los cultivos, con implicaciones para la salud y productividad de los cultivos.

Las lombrices, por ejemplo, son uno de los más importantes descomponedores de materia orgánica. Su actividad genera estructuras que mejoran la oxigenación de los suelos (Edwards and Aracon, 2022) y sus deposiciones enriquecen el microbioma, diversificando la actividad enzimática que aumenta la disponibilidad de nutrientes en los suelos y la capacidad de los suelos para degradar contaminantes orgánicos (Sun et al., 2020). Además, la deposición de estructuras biogénicas por parte de las lombrices de tierra crea ensamblajes de agregados organominerales y mejoran las propiedades físicas del suelo al aumentar la concentración de materia orgánica (Bhaduria & Saxena, 2009). También, las lombrices de tierra sirven como valiosos indicadores de la calidad del suelo debido a su alta presencia en este compartimento ambiental, su papel como fuente de alimento para varios depredadores y su alta sensibilidad a los contaminantes químicos (Bartz et al., 2024; Shi et al., 2017).

Para Vermeulen et al. (2001), en el contexto de la evaluación del riesgo que implica la aplicación de plaguicidas para las lombrices como bioindicador, la evaluación convencional de toxicidad aguda no es un parámetro ecológico relevante. En ese sentido, el desarrollo y aplicación de metodologías de identificación de efectos subletales pueden ofrecer un mejor panorama de la salud de poblaciones de oligoquetos y, por lo tanto, también del suelo. Metodologías basadas en el cambio del comportamiento en el que se exponen a los organismos a diferentes tipos de suelos contaminados con la posibilidad de escapar, son generalmente consideradas relevantes para evaluar la sensibilidad de los organismos a los contaminantes presentes (Pereira et al. 2010), ya que pueden representar una integración de los deterioros fisiológicos, así como respuestas a nivel individual con consecuencias ecológicas más claras (Shi et al., 2017). Además, el análisis de biomarcadores, que incluye procesos de biotransformación, estrés oxidativo y respuestas antioxidantes que pueden ser desencadenados por xenobióticos, representan un valioso acercamiento ya que proporcionan alertas tempranas sobre los efectos fisiológicos que ocurren en los organismos expuestos a contaminantes (Solé. 2020). Por otra parte, el microbioma intestinal de las lombrices se ha convertido en un buen bioindicador relacionado con el desempeño y las funciones de su huésped y responde a diferentes contaminantes químicos ambientales (Sun et al., 2020). Al respecto, varios estudios han demostrado que el análisis molecular mediante secuenciación del gen ARNr 16S de la microbiota intestinal permite caracterizar cambios en el microbioma de diversas especies de oligoquetos, inducidos por variaciones ambientales del medio en el que el organismo se desenvuelve (Berg et al. 2016; Egert et al. 2004; Kim et al. 2021). Por esta razón, identificar variaciones en la microbiota intestinal de las lombrices de tierra puede ser un buen método para determinar la salud de los organismos y del suelo.

Considerando que la zona de Zarcero es un área de producción agrícola con alta presión de aplicación de plaguicidas y comprendiendo el papel fundamental de las lombrices de tierra para la salud de los suelos y el agroecosistema, se desarrolló este proyecto con el fin de investigar los efectos de las variaciones de la aplicación de plaguicidas en suelos con diferentes usos (producción orgánica, producción buenas práctica, producción convencional, suelo de bosque) en temporada seca y lluviosa, sobre el estado (marcadores de estrés fisiológico, comportamiento, variaciones de la microbiota intestinal) de la especie de lombriz de tierra *Amyntas gracilis*, un oligoqueto ampliamente distribuido en ecosistemas agrícolas.

Para esto, el primer artículo de esta investigación tiene como objetivo evaluar la respuesta de estrés fisiológico mediante biomarcadores bioquímicos y del comportamiento evitativo en *A. gracilis* expuestas a suelos de sitios con diferentes prácticas de manejo, compuesto por suelos de producción orgánica, producción con buenas prácticas agrícolas, producción convencional y suelos de bosque. También, se analizaron las características físico-químicas del gradiente de suelos anteriormente expuestos con el fin de obtener un análisis integral de las condiciones.

El objetivo del segundo artículo se enfoca en evaluar cómo las características del suelo y la presencia de plaguicidas, en función de los sitios estudiados, afecta la abundancia y diversidad del microbioma intestinal de las lombrices de tierra *A. gracilis* expuestas a ese gradiente.

XII. Conclusiones generales

En esta investigación se estudiaron los efectos producidos en individuos de *A. gracilis* a nivel molecular e individual, los cuales fueron expuestos a un gradiente de aplicación de plaguicidas, compuesto por suelos de producción orgánica, producción con buenas prácticas agrícolas, producción convencional y bosque, tanto en temporada seca, como transición y lluviosa, durante el año 2022.

Los resultados muestran claros efectos de neurotoxicidad y reducción de la capacidad de biotransformación en los organismos de los suelos con mayor carga de plaguicidas (producción convencional). Consecuentemente, también se observó una evitación casi total de estos suelos en el ensayo de comportamiento, a pesar de contar con parámetros físico-químicos similares a los suelos preferidos por los organismos (orgánico y buenas prácticas).

También se observó que los suelos de bosque, aunque presentaban una baja carga de plaguicidas, tenían baja cantidad de materia orgánica y humedad, además de una textura arenosa. Estas condiciones produjeron una evitación media de las lombrices de tierra a estos suelos, indicando que las características físico-químicas y la textura del suelo también influyen considerablemente en la preferencia de hábitat de *A. gracilis*.

Además, nuestro análisis de la estructura de la comunidad microbiana en el contenido intestinal reveló cambios significativos en la microbiota intestinal de las lombrices de tierra entre las temporadas seca y lluviosa. También, los resultados sugieren que las altas cargas de plaguicidas pueden suprimir la diversidad en las comunidades microbianas y que estas pueden interferir con la capacidad del microbioma del contenido intestinal de las lombrices

de tierra para adaptarse a los cambios estacionales, afectando potencialmente su rol ecológico en los suelos.

XVIII. Recomendaciones

A partir de las experiencias de investigación del trabajo realizado, se indican las siguientes recomendaciones con el objetivo de robustecer los resultados obtenidos y sugerir áreas para futuras investigaciones.

Se recomienda realizar más estudios de este tipo en una variedad de organismos del ecosistema suelo. Esto permitirá analizar las relaciones ecológicas, la interacción de las mezclas de sustancias y sus efectos sobre la fauna edáfica. Este tipo de estudios pueden ser cruciales para establecer lineamientos científicos sólidos para la recuperación y protección de los suelos afectados por plaguicidas.

Se sugiere realizar un estudio de laboratorio con cargas de plaguicidas bien caracterizadas y controles negativos para evaluar la relación causa-efecto, analizando en un entorno más controlado las respuestas observadas en nuestra investigación (fisiológica, comportamiento, comunidad microbiana).

Se recomienda realizar un análisis de la presencia residual de plaguicidas en el tejido de las lombrices, lo que podría brindar una ventana para evaluar el nivel de bioacumulación y de exposición de los organismos a los plaguicidas, así como estudiar cómo estos fenómenos se pueden vincular a los resultados observados en este estudio.

Como recomendación final, en complemento a esta investigación, se sugiere realizar un análisis de biodiversidad evaluando macroinvertebrados edáficos por valoración taxonómica, así como también aplicar métodos de evaluación de la actividad y diversidad como “bait

lámina”. Esto permitirá analizar más ampliamente las variaciones y comportamientos ecológicos en el suelo y establecer relaciones con lo observado en las lombrices.

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**XV. ARTÍCULO I: Physiological stress and habitat selection in earthworms
(*Amyntas gracilis*) exposed to different pesticide regimes in a tropical horticultural
area**

Sustentante

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PHYSIOLOGICAL STRESS AND HABITAT SELECTION IN EARTHWORMS
(*AMYNTHAS GRACILIS*) EXPOSED TO DIFFERENT PESTICIDE REGIMES IN A
TROPICAL HORTICULTURAL AREA

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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 other physical-chemical characteristics in sites influenced by a gradient of soil and agricultural management, including conventional farming, farming with good environmental practices, organic farming and forest. Stress was assessed by measuring biomarkers of neurotoxicity (cholinesterase activity-ChE), biotransformation (glutathione S-transferase-GST and ethoxy-resorufin-O-deethylase-EROD activities) and oxidative stress (catalase activity-CAT and lipid peroxidation-LPO) in individuals of the earthworm *Amyntas gracilis* inhabiting sites of the mentioned gradient during dry and rainy seasons. To evaluate if the soil exposure to pesticides would affect the habitat selection by earthworms, 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 sampled but higher levels of contamination were observed in the soil samples under conventional farming, with presence of all 43 substances analyzed during the rainy season including many insecticides of high toxicity to soil invertebrates. Inhibition of ChE and diminished GST activities were observed in *A. gracilis* inhabiting the conventional farming site, while seasonal variation was clear in GST, CAT and EROD activities in all the sites. *A. gracilis* significantly avoided the soil from the conventional farming site and preferred soils with similar physical-chemical properties but with lower levels of pesticides. Our results evidence that intensive pesticide use can induce physiological stress in *A. gracilis* and provoke their escape from contaminated soils. Such responses would affect the soil macrofauna community and deplete the services they provide.

Keywords: Agricultural landscapes, soil macrofauna, biomarker, behavior, avoidance

1 Introduction

The use of pesticides is a widespread agricultural practice, applied with 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 kilograms (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/yr (Vargas, 2022). It is noteworthy that 93% of this pesticide inventory is 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 less 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., 2017).

Such intensive use of pesticides poses a significant threat to soil biodiversity in agricultural landscapes (Geisen et al., 2019; Montero et al., 2021) especially considering the many gaps in our understanding of the environmental behavior and toxicity of currently 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 (Edwards and Aracon, 2022; Labenz, n.d.). 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 (Bhadoria 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. 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; 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 fertility 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. 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 et al., 2011; 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., 2017). 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. Materials 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., 2017). 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, was chosen for evaluation.

2.2 Sampling

Sampling was carried out in the dry, transition and rainy seasons, in which the selected farms were visited. Physical-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 site was defined at the edges of the cultivation area. At these sites, a grid of 50 cm x 50 cm was arranged, which defined the specific sampling plot. Soil was extracted up to 20 cm deep, it was homogenized and sieved to collect the earthworms found in that volume of soil. 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-chemical parameters analysis

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 H₂O (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 (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'-Dithiobis (2 nitrobenzoic acid) (DTNB) 10mM 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 normality of the data was assessed with a Shapiro-Wilks test and quantile-quantile plot. As some of the variables did not meet the normality criteria, differences among biomarker responses, or

preferences 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).

3 Results

3.1 Physical-chemical characterization

The physical-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.

Table 1. Soil physical-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

3.2 Presence of pesticide residues

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.

Table 2. Pesticides residues (nanogram/gram dry 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 dw											
<i>FUNGICIDE</i>												
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
Prochloraz						2.3	6.6
Propiconazole							3.7
Pyraclostrobin		0.3		0.1		0.8	1.6
Pyrimethanil							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
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
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

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 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 Biomarker analyses

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 GP in dry season (Fig. 1, 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 behavior that was not observed on the other sites.

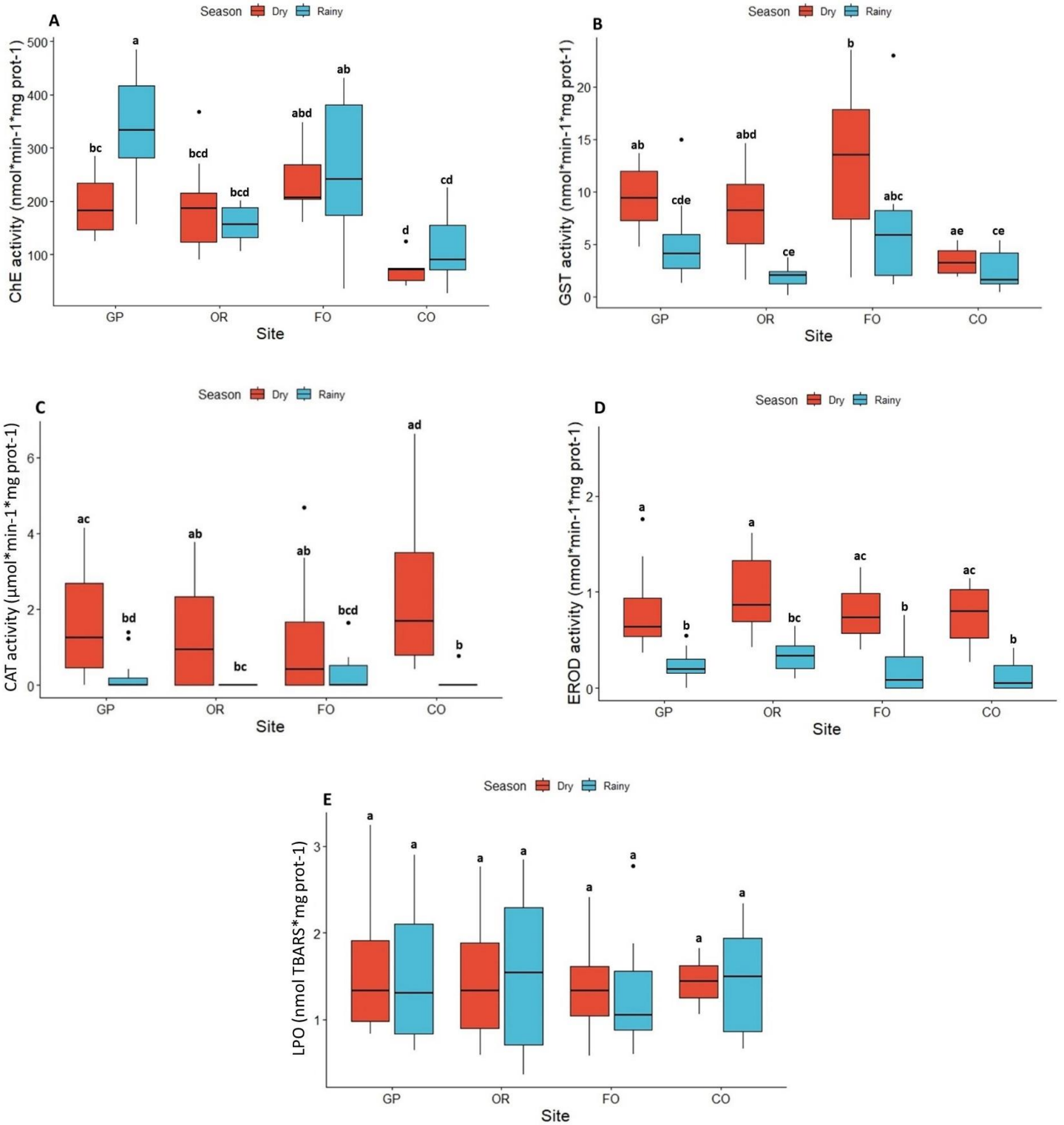


Figure 1. Biomarkers measured in tissues of *Amynthus gracilis* collected from the four sites studied, forest (FO), organic farming (OR), farming with good environmental practices (GP)

and conventional farming (CO) during dry and rainy seasons. Significant differences between sites and seasons are indicated with different letters ($p < 0.05$).

Consistently, the biomarkers of biotransformation and antioxidant response (GST, CAT, and EROD) showed a seasonal variation (Fig. 1, B-D), with higher levels of activity during the dry season; except in the case of GST in earthworms from CO site, which maintained a similar, and lower activity in both seasons, being significantly lower than FO site during the dry season (Fig. 1, B).

In the case of CAT, even though the activity in earthworms from OR and FO sites did not show significant differences, this seasonal trend was clear (Fig. 1, C). 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. 1, E).

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. 2).

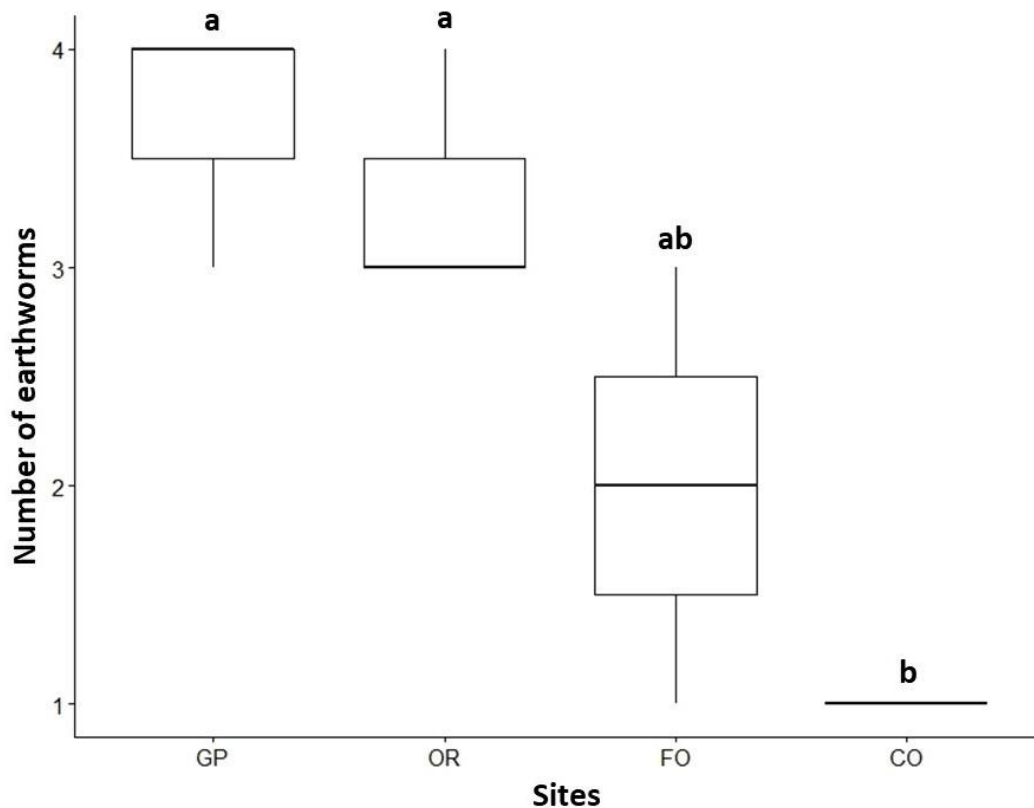


Figure 2. Distribution of earthworms (*Amyntas gracilis*) within a test wheel with four soil types to choose, farming with good environmental practices (GP), organic farming (OR), forest (FO) and conventional farming (CO). Data from three replicates. Significant differences are indicated with different letters ($p = 0.01$).

4 Discussion

Evidence suggests that agricultural practices, such as tillage and pesticide application, directly impact soil organisms (Nabel et al., 2021), resulting in a loss of biodiversity. Additionally, extensive research has shown that pesticides can affect soil organisms across various levels of biological organization (Beaumelle et al., 2023), and that the ecological significance of these effects is closely linked to the specific level of biological organization at which organisms are affected (Carriquiriborde et al., 2021). Furthermore, numerous studies (Datta et al., 2021; Lammertyn et al., 2021; Miglani and Bisht 2019; Panico et al.,

2022) have highlighted the adverse effects of pesticides to soil biota, ranging from subcellular to population levels. In this study, we investigated the impact of pesticide presence in soils with varying degrees of pesticide use on *A. gracilis*.

4.1 Pesticide residues

We hypothesized that a gradient in agricultural practices and soil use would be reflected in a gradient of pesticide pollution in the soil of an area highly productive in horticulture. However, the results demonstrated that a farm where no pesticide use is declared (OR), or a site with no agricultural use (FO) can be exposed to pesticides. 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).

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 has been previously reported in various earthworm species exposed to organophosphates (Tiwari et al., 2019; Sanchez-Hernandez et al., 2014; Sujeeth et al., 2023), 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 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 Egaas et al. (1999), Li et al. (2010), Štěpánová et al. (2012), Melo de Almeida et al. (2022), Aksakal (2020), Liu et al. (2016), 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-chemical properties of the soil, and the behavior of the organisms towards contaminated soils can also be influenced by such physical-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 epigeic-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. gacillis*. 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 and Stehouwer (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 horticulture 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.

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.

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

During the preparation of this work the author(s) 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 author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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XVI. ARTÍCULO II: Seasonal dynamics and pesticide impact on gut microbiome in *Amyntas gracilis* earthworms: A comparative study across agricultural landscapes assessed by 16S rRNA amplicon-based sequencing

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SEASONAL DYNAMICS AND PESTICIDE IMPACT ON GUT MICROBIOME IN
AMYNTHAS GRACILIS EARTHWORMS: A COMPARATIVE STUDY ACROSS
AGRICULTURAL LANDSCAPES ASSESSED BY 16S rRNA AMPLICON-BASED
SEQUENCING

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Abstract: The use of pesticides in agricultural soils can affect the biodiversity of these ecosystems and interfere with the ecological services they provide. The gut microbiome of earthworms serves as a relevant bioindicator for changes in diversity within one of the most representative groups of soil macrofauna. In this study, we defined a gradient of soil and pesticide use regimes within a horticultural area, including conventional management, good agricultural practices, organic production and forest. Earthworms (*A. gracilis*) from these four conditions were sampled, and their microbiomes were assessed by sequencing the V4 region from the 16S rRNA gene. Comparison of ASVs indicated the presence of 142 bacterial genera among all the samples, with Actinobacteriota, Proteobacteria, Firmicutes and Bacteroidota being dominant. Simpson's diversity index revealed diminished biodiversity in the gut microbiomes of earthworms from conventionally managed soil, with a negative correlation observed with the presence of insecticide chlorpyrifos. Furthermore, a seasonal shift in the dominance of bacterial taxa was observed between dry and rainy seasons. These shifts were evident in the gut microbiome of organisms from organically managed and good practices soils but not in the conventional site. Our results demonstrate that earthworm gut microbiome serves as a responsive bioindicator for biodiversity changes in agricultural landscapes, suggesting that key features of the microbial community can be altered by pesticide exposure.

Keywords: Earthworms, gut microbiome, pesticides, seasonal changes, metabarcoding.

1 Introduction

Biodiversity and its associated ecosystem services are essential for agricultural landscapes, providing ecological stability and soil and water improvement (Erisman et al., 2016). Despite the acknowledged effectiveness of pesticides in pest management and crop protection (Özkara et al., 2016), the use of such substances represents a risk for soil ecosystems under agricultural use, or even remote areas receiving residues from crop applications (Brühl et al., 2024; Gunstone et al., 2021). There is ample research indicating loss of biodiversity in agricultural areas (Beketov et al., 2013; Emmerson et al., 2016; Kumar et al., 2021) due to the effects of pesticides, which could have significant impacts on such ecosystems.

As key elements of soil biodiversity, earthworms improve soil conditions, contributing to nutrient cycling and soil structure enhancement. Furthermore, earthworm activity leads to an increase in the availability of vital nutrients such as nitrogen and phosphorus, crucial for sustaining plant growth (Chauhan, 2014) and their presence fosters microbial diversity, thereby augmenting soil fertility (Bhadauria and Saxena, 2009).

Concerning the gut microbiome of earthworms, it is well-documented that the abundance of both anaerobic and aerobic bacteria is significantly higher in their gut as compared to surrounding soils (Horn et al., 2003). This may explain the pivotal role that gut microbiome plays in organic matter decomposition dynamics and nutrient cycling (Medina-Sauza et al., 2019), as well as in the degradation of organic pollutants, heavy metals, and microplastics (Sun et al., 2020). It is well known that environmental characteristics and food sources can affect the composition of earthworm's gut microbiota (Sapkota et al., 2020), and exposure to pesticide-contaminated soil has been shown to disrupt the structure and abundance of earthworm's gut microbiomes (Astaykina et al., 2022). Furthermore, the gut microbiota forms a complex relationship with earthworms and plays a crucial role in their physiological

functions, immune system and ability to digest food (Liu et al., 2018), as well as their ability to adapt to new environments (Y. Yang et al., 2023). For this reason, exposure to pesticides could have a negative effect on worm populations, impairing their ecological function.

Consequently, earthworm's gut microbiome has become a good bioindicator, related to the fitness and functions of their host, and responding to different chemical environmental pollutants (Sun et al., 2020). Currently the application of metagenomic analysis is used to assess the microbial community composition in earthworm's gut and their changes due to external stressors (Owagboriaye et al., 2021; Xia et al., 2022). Such approach has demonstrated how exposure to pesticides with different biocide actions, including herbicides, fungicides and insecticides, can affect the abundance and diversity of bacteria in the gut of different earthworm species (Chang et al., 2021; Song et al., 2022; G. Zhu et al., 2021).

As a case to study effects of pesticide pollution in soil biota, Costa Rican agriculture is recognized for its high use of pesticides (Vargas, 2022). In the Northern part of the country, the area of Zarcero sustains an intensive horticultural production, including conventional, organic, and good agricultural practices models (Ramírez et al., 2017). In this setting, considerable pesticide use has been diagnosed; as well as their residues in the environment, and their adverse environmental effects (Ramírez et al., 2017; Weiss et al., 2023). In this regard, recent evidence demonstrated the vulnerability of earthworms to pesticide exposure in the Zarcero area (Brenes et al., in prep). Evasive behavior and alterations in biomarkers indicative of biotransformation and neurotoxic processes were observed in resident *Amyntas gracilis* individuals as response to soils highly exposed to pesticides. Such responses evidence how earthworms are sensitive to this type of pollution, in a real scenario.

Considering the responsiveness of earthworm's gut microbiomes as a bioindicators and the intensive pesticide use in Zarcero's horticulture, in this study we utilized DNA

metabarcoding to investigate the gut microbiome of *A. gracilis* earthworms inhabiting four sites characterized by varying levels of pesticide contamination. Our main goal was to ascertain whether the presence of pesticides in soil correlates with structural changes the intestinal microbiome of these organisms. We hypothesized that any discernible correlation between soil pesticide concentrations and shifts in the earthworms' gut microbiome could potentially influence the health of the organisms and thereby impact their ecological functionality.

2 Materials and methods

2.1 Sample collection

Sampling was conducted in Zarcero, Alajuela, Costa Rica, once during the dry season and once during the rainy season of 2022. A gradient in pesticide application regime was defined: forest soil (FO) with no agricultural management, organic farming soil (OR) with no pesticide use, farming soil with good practices (GP), and conventional farming soil (CO). At each sampling site, a sampling point was defined within a 50 cm x 50 cm grid, from which, 20 cm of soil depth was removed and sieved to obtain the earthworms. On site, the proximal region of the earthworms was dissected up to 5 mm after the clitellum, subsequently, the gut contents of each individual were extracted and placed into a 1.5 mL microcentrifuge tube, which was stored in liquid nitrogen during transportation to the laboratory. Samples were kept at -80 C until further analysis.

2.2 Soil pesticide residues and physical - chemical parameters

Pesticide residues were determined with a multi residue method by LC-MSMS according to the modified NMKL Method No 195 (NMKL 2013), the extracts were analyzed by high-

resolution liquid chromatography tandem mass spectrometry (LC-HR MS/MS) (Moschet et al., 2013). The moisture content of the samples was estimated using the gravimetric method, and the organic matter content was analyzed applying the loss on ignition method. In addition, the texture of the soils was estimated from a modification of the Bouyoucos (1936) method, and the pH of the samples was analyzed using the H₂O measurement method. All analyzes were carried out as indicated in Brenes et al. (in prep).

2.3 Total DNA extraction

The DNeasy PowerLyzer PowerSoil Kit (Qiagen Corp.) was used for extracting total microbial DNA from the gut content, following the manufacturer's specifications. The DNA concentration, quality and integrity were verified through gel electrophoresis and NanoDrop 2000 (Thermo Fisher Scientific). Additionally, a PCR was performed on all the samples to assure the integrity of the 16S ribosomal RNA (rRNA) gene, using the universal primers 342F (5'-CTACGGGGGGCAGCAG-3') and 806R (5'-GGACTACCGGGGTATCT-3'). DNA was stored at -20 °C until sequencing was performed.

2.4 Sequencing by metagenome amplicon and bioinformatics analysis

For this study, 40 samples were analyzed: forest soil (n = 10), organic farming soil (n = 10), farming soil with good practices (n = 10) and conventional farming soil (n = 10) distributed in different culture systems on rainy (n = 5) and dry seasons (n = 5). The V4 region from the 16S rRNA gene was PCR amplified using the primers 515F (5'-GTGCCAGCMGCCGCGTAA-3') y 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The amplicons were used for library preparation and sequencing with the Illumina Novaseq 6000 system 2 x 250 bp (Novogene Inc.). Cutadapt 3.5 (Martin, 2011) was used to remove primer

sequences, and DADA2 (Callahan et al., 2016) was used to infer amplicon sequence variants (ASV) in the R environment v4.3.2 (R Core Team, 2024). A naïve Bayesian classifier (Q. Wang et al., 2007) and the SILVA 138.1 database (Quast et al., 2013) were employed for taxonomic assignment. ASVs assigned to eukaryote, chloroplast, and mitochondria were removed. PICRUSt2 v2.5.2 (Douglas et al., 2020), HMMER (Eddy, 2011), EPA-ng (Barbera et al., 2019) and GAPP (Czech et al., 2020) were used for the phylogenetic placement of the ASVs in a reference phylogenetic tree from the Integrated Microbial Genomes (IMG) database (Markowitz et al., 2012). ASVs that aligned poorly to the reference alignment were also removed from the analysis. Raw data files are available under BioProject ID PRJNA1134209 from the National Center for Biotechnology (NCBI).

2.5 Statistical analyses

Diversity indices, such as Shannon (Shannon, 1948), Chao1 (Chao, 1987) and Simpson (Simpson, 1949) were obtained with phyloseq v1.42.0 (McMurdie & Holmes, 2013), and Faith's phylogenetic diversity (Faith, 1992) with picante v1.8 (Kembel et al., 2010) after normalizing the count table by scaling with ranked subsampling to the lowest number of reads in a sample (Beule & Karlovsky, 2020). Alpha diversity metrics were summarized as box plots made with ggplot2 version 3.4.3. Normal distribution was assessed with Shapiro-Wilk test, and differences in diversity indices between sites were evaluated with Kruskal-Wallis and post-hoc Dunn test with the Benjamini-Hochberg false discovery rate (FDR) adjustment for multiple pairwise comparisons. In addition, to investigate the relationship between continuous explanatory variables and diversity indices, a Spearman correlation analysis was performed to quantify the strength of the linear association and generalized linear models were applied with a quasi-Poisson distribution.

For beta diversity analysis, uninformative ASVs were removed with an abundance and prevalence filter step (Dhariwal et al., 2017; Motiei et al., 2020). Weighted UniFrac distance between samples was calculated and the results were presented as 2D ordination plots based on principal coordinates analysis (PCoA) (Lozupone et al., 2011) using phyloseq v1.42.0. Permutational multivariate analysis of variance (PERMANOVA), pairwise PERMANOVA and ANOSIM tests were conducted using the vegan package v2.6-4 (Oksanen et al., 2024), to assess the statistical significance of the patterns in the microbial community composition. Physicochemical variables were plotted as fitted arrows onto the PCoA using the envfit function to assess the association between the variables and the structure of the bacterial community.

ANOVA-like differential expression (ALDEx2) analysis for compositional data (Fernandes et al, 2013) was employed to identify specific bacterial taxa with varying relative abundance between sites. In addition, aldex.corr from the package ALDEx2 was used to calculate Pearson, Spearman and Kendall correlations between the taxa and continuous variables. PICRUSt2 (Douglas et al., 2020) predicted microbiome functions at the KEGG ortholog level were converted to KEGG pathway abundances with ggpicrust2 v1.7.3 (Yang et al., 2023), and linear models for differential abundance (LinDA) analysis (Zhou et al., 2022) was used to test for differences between sites. Finally, heatmaps of relative abundance profiles at phylum, class and genus levels were generated using ampvis2 v2.8.7 (Andersen et al., 2018).

3 Results

3.1 Soil parameters and pesticide presence

Our soil analysis (Brenes et al., in prep) indicated that soils from CO, GP and OR possess a predominantly loamy texture, while the FO soil exhibited a sandy texture. This characterization was similar for moisture and organic matter, with these parameters showing a better condition in CO, GP and OR, while FO soil showed lower amounts of organic matter and moisture. These differences were observed in both dry and rainy season. The pH in all soils was slightly acidic, with GP soil showing the lowest pH value in both seasons.

Regarding the presence of pesticides, the CO soils recorded the highest number of pesticides in the dry season and specially in the rainy season where the quantity was considerably high (42 pesticides detected), mainly azole group fungicides and organophosphate insecticides. It is also noteworthy that chlorpyrifos was the only pesticide detected in all soils during the dry season, with the FO soils showing the highest value (71.9 ng/g dw).

3.2 Sequencing

Our research determined, by sequencing 16S rRNA gene V4 region amplicons, the microbial community structure of earthworms at four studied sites.

A total of 4,755,498 raw paired-end reads were produced from the 38 earthworm intestinal samples. Following PCR primer trimming with Cutadapt, and quality trimming, denoising, read merging and chimera filtering with DADA2, 1,921,779 high-quality sequences were kept, with an average of 50573 reads per sample. DADA2 also yielded a total of 7862 ASVs, of which 4178, 3707, 2593 and 2025 ASVs were present in the OR, GP, FO, and CO samples,

respectively. In total, 802 bacterial genera from 111 classes that belong to 49 prokaryotic phyla were identified in the gut of *A. gracilis*.

3.3 Bacterial community composition

After applying abundance and prevalence filters (>4 reads in 10% of the samples), the gut microbiome of *A. gracilis* consisted of 14 phyla, 22 classes and 142 genera with assigned taxonomy. Most of the ASVs belonged to the phyla Actinobacteriota (369 ASVs), Proteobacteria (250) and Firmicutes (212). Actinobacteriota was also the most abundant phyla in the gut microbiome of *A. gracilis* in all four sites (Figure 1A). The phyla Proteobacteria and Firmicutes were also abundant in all four sites, with relative abundances ranging from 15.8 to 43.5%. The classes Actinobacteria, Gammaproteobacteria and Bacilli were the most abundant (Fig 1B), while the genera *Pseudarthrobacter*, *Burkholderia-Caballeronia-Paraburkholderia*, *Bacillus* and *Solibacillus* were the most abundant (Fig. 1C)

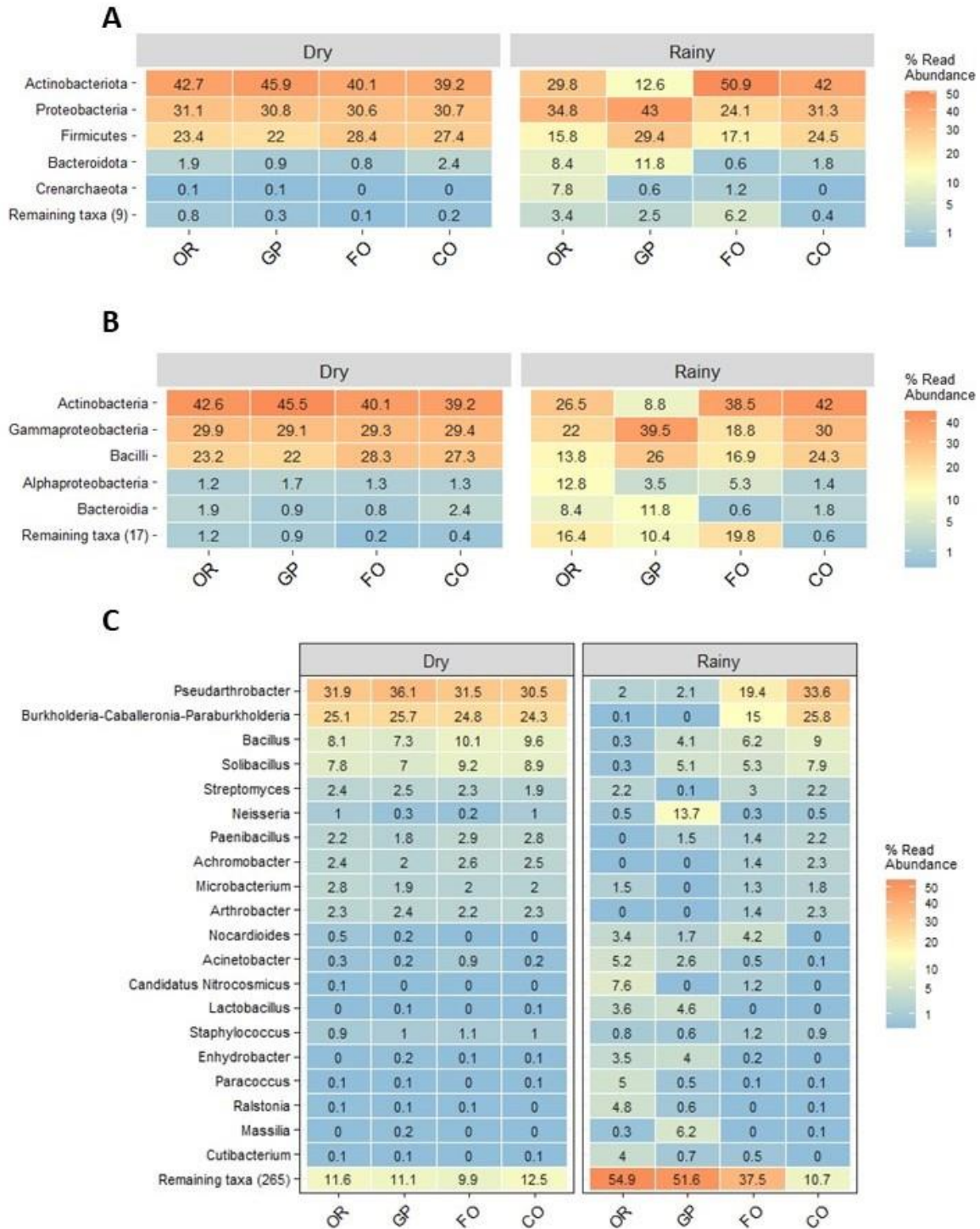


Figure 1. Heatmaps of the relative abundance of the 5 phyla (A), top 5 classes (B) and top 20 genera (C) for the microbial community in the 4 sampling sites within the dry and rainy

seasons. Organic management (OR), good management (GP), conventional management (CO) and forest (FO).

According to ALDEx2 analysis, in the dry season 13 genera presented differential abundance between the sites ($p < 0.05$), including some of the more abundant genera, such as *Paenibacillus*, *Solibacillus*, *Achromobacter*, and *Bacillus*. *Achromobacter* and *Sphingobacterium* relative abundance was the lowest in the the GP samples, while *Paenibacillus*, *Solibacillus*, *Bacillus*, *Fictibacillus* and *Planococcus* relative abundance was lower in OR and GP samples when compared to FO and CO samples. Interestingly, the genus *Leptotrichia* was significantly more abundant in the OR samples than in the other sites.

In the rainy season, 19 genera presented differential abundance between the sites ($p < 0.05$), including some of the most abundant genera, such as *Microbacterium*, *Arthrobacter*, *Streptomyces*, *Achromobacter*, *Burkholderia-Caballeronia-Paraburkholderia*, *Pseudarthrobacter* and *Paenibacillus*. The relative abundance of these 7 genera was significantly higher in the FO and CO samples than in OR and GP samples.

A succession in the dominant genera was observed between the dry and the rainy seasons according to relative abundances (Fig. 1C). This succession was drastic in OR and GP, where the higher relative abundances for the rainy season shifted to genera that were not dominant during the dry season. This was evident in a decrease in the abundance of *Pseudarthrobacter*, *Burkholderia* complex, *Bacillus*, *Solibacillus*, and *Streptomyces*, and an increase in the abundance of genera that are less represented in the dry season, such as *Nocardioides*, *Acinetobacter*, *Lactobacillus* and *Candidatus Nitrocosmicus*. This seasonal effect was not

drastic as OR or GP soil in the gut microbiome of the worms from FO soils, and most interesting, it did not occur at all in the animals from the CO site.

3.4 Correlations between taxa and continuous variables

The only pesticide present in soil samples that showed significant correlation with the relative abundance of bacterial taxa was chlorpyrifos. According to aldex.corr analysis, the phyla Firmicutes, Actinobacteriota and Proteobacteria positively correlated with chlorpyrifos concentration. Contrarily, Crenarchaeota and Chloroflexi negatively correlated with chlorpyrifos. Similarly, the relative abundance of 5 classes (Actinobacteria, Bacilli, Gammaproteobacteria, Alphaproteobacteria, and Bacteroidia) was positively correlated with chlorpyrifos concentration. Otherwise, the relative abundance of Acidimicrobiia and Thermoleophilia was negatively correlated with chlorpyrifos.

In addition, the relative abundance of 22 genera (*Rhodococcus*, *Brevibacterium*, *Arthrobacter*, *Pseudarthrobacter*, *Promicromonospora*, *Sphingobacterium*, *Fictibacillus*, *Planococcus*, *Planomicrobium*, *Paenibacillus*, *Ensifer*, *Phyllobacterium*, *Achromobacter*, *Burkholderia-Caballeronia-Paraburkholderia*, *Bacillus*, *Solibacillus*, *Stenotrophomonas*, *Microbacterium*, *Salirhabdus*, *Streptomyces*, *Micromonospora*, and *Staphylococcus*) was positively correlated with chlorpyrifos concentration. Conversely, the relative abundance of 8 genera (*Nocardioides*, *Mycobacterium*, *Gaiella*, *Solirubrobacter*, *Cutibacterium*, *Candidatus Nitrocosmicus*, *Phenylobacterium* and *Hyphomicrobium*) was negatively correlated with chlorpyrifos. No correlation between the taxa relative abundance with soil pH, OM, and moisture, as well as with the concentration of other pesticides was found.

3.5 Alpha diversity

Alpha diversity indexes were obtained for each of the samples. A Kruskal-Wallis H test showed that there was no statistically significant difference in Shannon index between samples from the different sites ($\chi^2(3) = 5.82, p = 0.121$). A similar result was obtained for observed Chao1 indices. However, a Kruskal-Wallis H test showed that there was a statistically significant difference in Simpson index between samples from the different sites within the rainy season ($\chi^2(3) = 8.74, p = 0.03$). A post-hoc Dunn's test identified a significant reduction in Simpson index in samples from CO when compared to OR and GP (BH-adjusted $p = 0.04$, Figure 2).

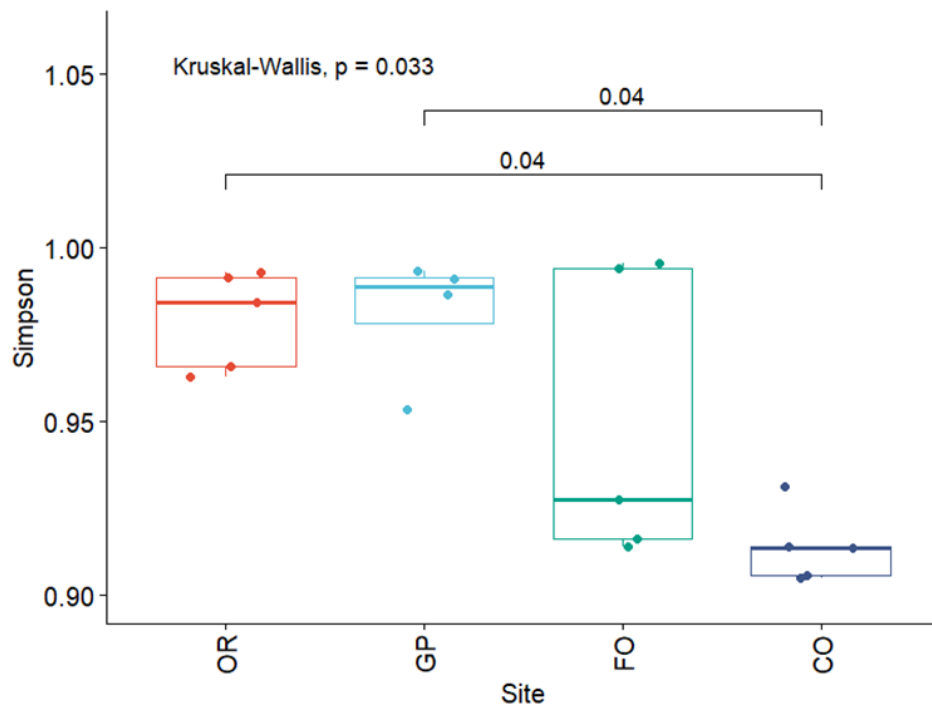


Figure 2. Bacterial alpha diversity represented as Simpson index of the microbial communities in the 4 sampling sites within the rainy season. Organic management (OR), good management (GP), conventional management (CO) and forest (FO).

No strong correlations were found between Chao1, Simpson and Shannon and pH, OM or moisture, both in dry and rainy seasons. Generalized linear models (GLM) analysis confirmed the lack of effect of pH, OM and moisture over the Shannon index in the dry season, however in the rainy season, OM and moisture had a significant effect over Shannon index ($p < 0.05$). In addition, in the rainy season GLM detected significant interactions between pH and OM ($p < 0.05$), as well as between pH and moisture ($p < 0.05$) over the Shannon index, however no interaction was detected for OM and moisture. GLM analysis indicated that OM and moisture had an effect over the Simpson index, both in the dry and rainy season. GLM detected significant interactions between pH and OM ($p < 0.05$), as well as between pH and moisture ($p < 0.05$) over the Simpson index, both in the dry and rainy seasons, yet again no interaction was detected for OM and moisture.

Shannon index was negatively correlated with chlorpyrifos ($r = -0.55$, $p < 0.05$) but only in the rainy season, and a similar trend was observed for Simpson. In addition, in the rainy season, Simpson also presented a negative correlation with linuron ($r = -0.52$, $p < 0.05$), flutolanil ($r = -0.52$, $p < 0.05$), and pyraclostrobin ($r = -0.52$, $p < 0.05$), and in the dry season presented a positive correlation with azoxystrobin, flutolanil and linuron ($r = 0.63$, $p < 0.01$).

GLM analysis detected that Shannon was only affected by chlorpyrifos ($p < 0.05$), and that Simpson was only affected by azoxystrobin ($p < 0.05$) in the dry season. Similarly, Simpson was affected by chlorpyrifos and flutolanil ($p < 0.05$) in the rainy season.

3.6 Beta diversity

According to the PCoA analysis based on weighted UniFrac distances (Figure 4), the samples from OR and GP formed distinct clusters along the first axis according to the season, and within the rainy season, most samples from OR clustered tightly together with samples from GP along the second axis. No clear separation of samples within the dry season was detected along the first and second axes. Regarding the microbiome of earthworms from CO, the absence of seasonal variation was clear, agreeing the observation regarding relative abundance described earlier. An ANOSIM analysis detected significant differences in the structure of the microbial community at different sites (ANOSIM $R=0.126$, $p < 0.01$) and seasons (ANOSIM $R=0.142$, $p < 0.01$).

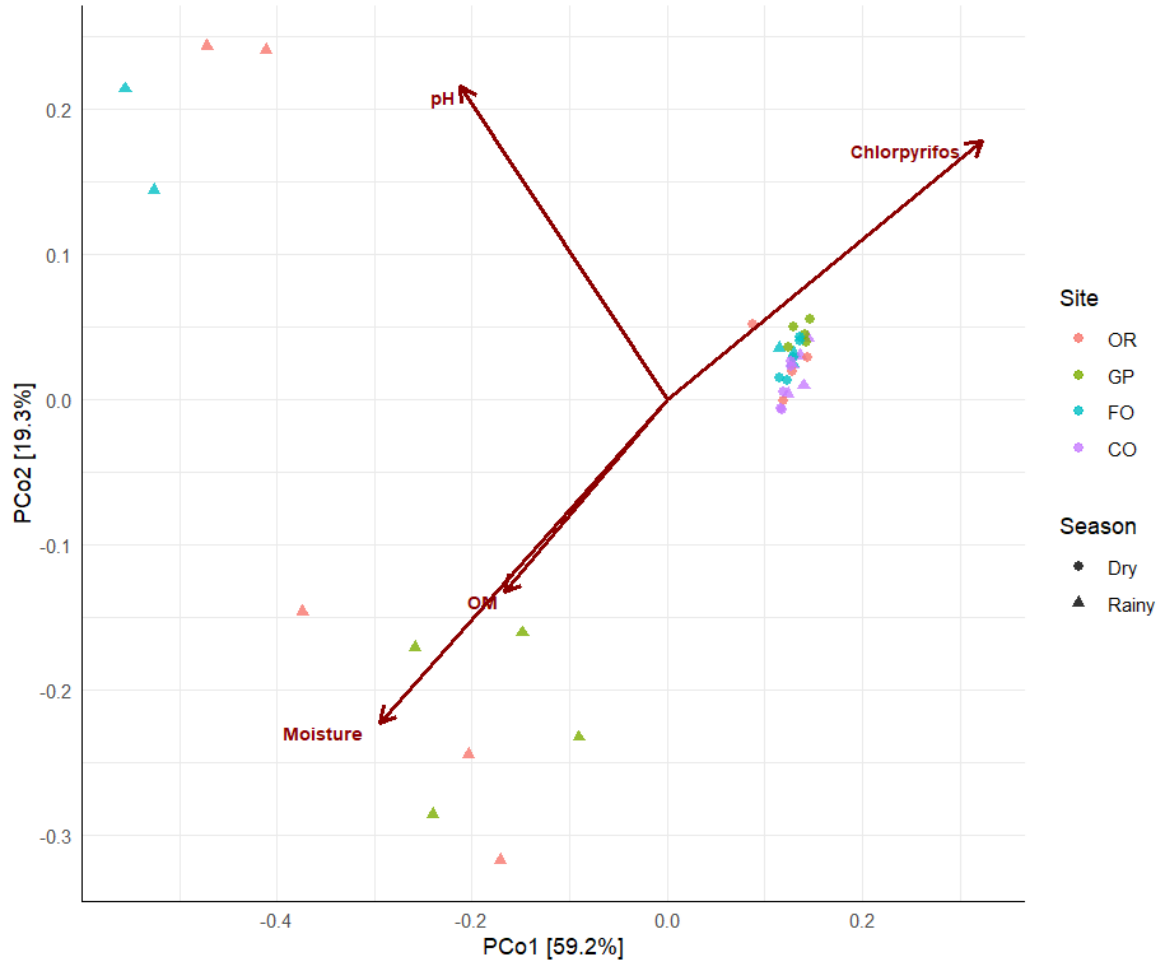


Figure 3. Principal coordinate analysis (PCoA) based on weighted UniFrac distances from ASV relative abundance data. Distribution of the samples from the organic (OR), good practices (GP), forest (FO) and conventional (CO) sites is shown in the first two axes of the PCoA.

4 Discussion

As we know, earthworms make up the biggest component of the animal biomass found in the soil (Yang et al., 2023) and their feeding is mainly related to microorganism. Given that their gut microbiota is richer than that of the soil's itself (Bhadauria & Saxena, 2009; Egert et al., 2004). Earthworms exert an enriching effect through the deposition of decomposed organic

matter with a balanced microbial population (Govindarajan & Prabakaran, 2015). In this regard, gut microbiota communities' changes can negatively affect their hosts' fitness (behavior, feeding, adaptation), as well as their ecological role by limiting their ability to improve soil conditions (Berg et al., 2016).

Changes in the composition of gut microbiome structure in earthworms can be mediated by the physicochemical conditions of the areas they inhabit, as well as by the availability of different food sources (Liu et al., 2018; Y. Yang et al., 2023). Additionally, the presence of contaminants can play an important role in the specialization of microbiomes, as has been previously observed in soils (Walder et al., 2022). In our research, very similar conditions of pH, organic matter, moisture and texture were observed among the different sites evaluated under agricultural soil conditions (CO, GP and OR), with the exception of forest soil (FO), which reported low amounts of organic matter and moisture. Considering these conditions, our results indicate that the gut bacterial community in *A. gracilis* is primarily composed of Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes in both dry and rainy seasons. This demonstrates high consistency with other studies that have observed this same composition of the microbiome in *E. fetida* and *E. andrei* (Aira et al., 2022; Liu et al., 2018; Ma et al., 2017; Singh et al., 2015). This suggest that the observed microbial community structure in *A. gracilis* could correspond to a common characteristic core, which may be shared among different species.

At the genus level, considerable changes were observed in the gut microbiome, mainly mediated by the effect of seasonal change. Changes in the microbial community composition based on season have been reported before on earthworms (Hao et al., 2024), and it is well known that the activity of earthworms is closely related to seasonal changes (Singh et al.,

2021). Such changes in the microbiome could be linked to seasonal associated variations in environmental conditions (moisture and organic matter). Furthermore, as we know, among microbiome ecological interactions in soil (mutualism, parasitism, commensalism, etc.), competition for resources dominates these interactions (Wang & Kuzyakov, 2024). In this regard, the change in the microbiome structure in the rainy season suggests that the changes in moisture, pH and organic matter content improved the conditions for other species that eventually could compete for resources enhancing the diversity of gut microbiome in worms from GP, OR and FO. Furthermore, seasonal shifts in gut microbiome have been described in other organisms, including insects (Ferguson et al., 2018), fish (Savard et al., 2023), or mammals (Baniel et al., 2021; Fan et al., 2022). These studies have associated the plasticity of gut microbiome with the adaptation of the host organisms to changing environmental variables and food sources (Baniel et al., 2021). In the case of earthworms, Drake and Horn (2007) referred to their guts as an anoxic microenvironment which sustains a transient microbial community that benefits from the available ingested organic matter and participate in its fermentation. However, ours is the first report of seasonal shift in gut microbial community in this species. But even more interesting was the absence of a community shift in the guts of earthworms from the conventional agriculture site (CO). Considering that other key variables such as humidity and organic matter content were similar to the other sites where community shifted, we suggest that the higher pesticide pollution present in CO should be associated with this impairment.

Changes in the earthworm gut microbiome due to pesticides

Regarding the relationships between the pesticides detected in the studied sites and the gut microbiome of their earthworms, there was a clear influence of chlorpyrifos on reduced

diversity and the absence of a seasonal change observed in the organisms from CO. Krishnaswamy et al. (2021) described how this organophosphate is capable of reducing diversity and changing dominance in the gut microbiome of an earthworm, which aligns with our observations. Furthermore, some authors have reported a considerable decrease in the microbial diversity in earthworms exposed to organophosphate, carbamate and organochlorine pesticides (Chang et al., 2021; Kavitha et al., 2020). This behavior has been observed even with the application of pesticides under recommended rate (Astaykina et al., 2022). Explaining this behavior can be complex; however, we know that CO soils had the highest pesticide number detected, especially high in the rainy season. Furthermore, it is known that bacterial groups from the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria have demonstrated a high capacity for degrading carbamate, organophosphate, organochlorine and pyrethroid pesticides (Manish et al., 2021). These results are consistent with our findings and may suggest a specialization of the microbiome in CO caused by the high presence of pesticides.

There are other factors related with the fitness of the *A. gracilis* from the CO site that might explain this outcome. In previous studies (Brenes et al., in prep), we observed inhibition of cholinesterase activity (ChE) and a trend towards a decrease in glutathione S-transferase activity (GST) in individuals of *A. gracilis* sampled from CO soil in the rainy season. We know that the gut microbiota and the host organism's fitness are directly related. Additionally, it is known that neurotoxicity affects their behavior (feeding) (Nofyan et al., 2017; Pavlov et al., 1992). Thus, the organism's feeding activity could be affected by the observed ChE inhibition, indirectly affecting the composition of the organism gut microbiome. On the other hand, a mechanism proposed by Chang et al. (2021) associates

disruption in antioxidant capacity and energy resources with a lag in the organism's growth and metabolism, which could reflect a greater sensitivity of the gut microbiota to soil contaminants. Similar effects have been observed before (Yu et al., 2022) with microplastic exposure, where a relationship between bacterial diversity and variations in antioxidant capacity in *E. fetida* has been demonstrated.

Functions of the microbiome

Our results have demonstrated a higher relative abundance of the genera *Pseudarthrobacter*, *Burkholderia* complex, *Bacillus*, *Solibacillus*, and *Streptomyces* during the dry season in the gut microbiome of *A. gracilis* from all the assessed sites, maintaining a very stable core in terms of abundance and genus.

It is known that *Pseudarthrobacter* (Actinobacteria) are denitrifying bacteria (Su et al., 2019) so their presence in the earthworm gut microbiome could play an important role in the nitrogen cycle, being involved in the degradation of nitrogenous compounds. Additionally, organisms identified from the *Burkholderia* complex (Proteobacteria) have been associated with biocontrol functions, bioremediation, and plant growth promotion in soils, as well as pathogenic effects in humans (Rojas-Rojas et al., 2018). While the presence of these organisms has been previously observed in the gut content of *Aporrectodea caliginosa* (Aira et al., 2022), their role in the earthworm gut is still not well understood.

On the other hand, bacteria of the genus *Bacillus* (Firmicutes) have been observed to play an important role in improving cellulose digestion capacity (Yang et al., 2023) and are crucial in nutrient cycling and the decomposition of organic matter in the earthworm gut (Chang et al., 2021). Additionally, *Bacillus* is known to produce phosphoesterase, an enzyme capable

of degrading organophosphorus pesticides (Krishnaswamy et al., 2021), which may be related to a degradation function in the organisms sampled from all soils during the dry season, primarily due to the presence of chlorpyrifos in those soils. Furthermore, *Solibacillus* (Firmicutes) has been previously reported in the gut microbiome of *E. fetida* (Pérez-Pérez et al., 2018). Although its intestinal function has not been described, this bacterium has been observed to play important roles in the decomposition of organic matter and the formation of humic substances (Zhu et al., 2020). As we know, humic substances improve soil fertility and nutrient absorption (Trevisan et al., 2010), and earthworms are considered “hot spots” for the transformation of these substances (Tikhonov et al., 2016). Likewise, *Streptomyces* (Actinobacteria) is a bacterium enriched in the gut microbiome of earthworms and is closely related to carbon and nitrogen metabolism (Zhang et al., 2022). An increase in the abundance of this bacterium has been observed in the intestines of earthworms exposed to pesticides, which could suggest a link between its increase and the degradation of these compounds (Astaykina et al., 2022).

Regarding the shift observed in the rainy season, *Nocardioides* (Actinobacteria) is well-known for its functions which include chemoheterotrophic, aerobic chemoheterotrophic, and degradation of aromatic organic compounds, with high presence in the early phases of composting (Tortosa et al., 2021). However, it has also been attributed with important characteristics in the rapid degradation of contaminants, utilizing them as sources of carbon and nitrogen (Ma et al., 2023). Additionally, it is known that strains of *Acinetobacter* (Proteobacteria) can utilize a wide range of carbon sources as food (Ren & Palmer, 2023). An increase in their numbers in the gut microbiome in earthworms exposed to cadmium has also been reported, with authors suggesting that this increase is a response to cope with the

stress caused by this contaminant (Zhou et al., 2021). A similar behavior has been observed in earthworms exposed to tetracycline (Yin et al., 2021). However, the presence of pesticides in the rainy season in the soils of GP, OR and FO is low. Therefore, it is possible that the observed increase in organic matter in our analysis created good conditions to conducive the proliferation of these bacteria.

In the case of *Lactobacillus* (Firmicutes), its wide distribution in horticultural soils (Raman et al., 2022), such as those analyzed in this study, has been observed, showing biocontrol functions against pathogens harmful to crops. Additionally, it is known that bacteria like *Lactobacillus*, adapted to acidic or alkaline pH, are used as probiotics to improve gastrointestinal health (Msimbira et al., 2022). In earthworms fed with *Lactobacillus* probiotics, a considerable increase in size and weight has been observed compared to controls (Sakthika & Anbumalar, 2022). This suggests that their presence has a positive impact on the health of the earthworms.

Finally, we know that *Candidatus Nitrocosmicus* (Thaumarchaeota) plays a role in nitrification process, degrading ammonium compounds into nitrite (Sauder et al., 2017). This suggests that the role of *Candidatus Nitrocosmicus* in the earthworms gut its related to nitrification of ammonium from decomposed organic matter or organic fertilizers in soils, into a bioavailable form of nitrogen, which can be good for improvement of plant growth.

Our results should contribute to the knowledge of the earthworm's gut microbiome, its importance and behavior as a bioindicator. Furthermore, our data describes key features of the response of this microbial community to pesticide pollution, *in situ*. We also noticed that gut microbiome studies have exclusively focused on bacteria, at this point we must suggest

that future assessments include eukaryotes, like fungi, protists and even small metazoans being part of the earthworm diet (Sapkota et al., 2020).

5 Conclusions

To our knowledge, this is the first study to analyze the composition of earthworm's gut across different pesticide regimes and seasons in the Central America region. Our results demonstrated significant changes in the gut microbiota of earthworms between seasons, and suggest that high pesticide loads can reduce the diversity in these microbial communities. High pesticide exposure can also interfere with the capacity of earthworm's gut microbiome to adapt to seasonal changes, potentially affecting their ecological role in soils.

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