Frattini et al.
Bulletin of the National Research Centre (2023) 47:78
https://doi.org/10.1186/s42269-023-01051-8

Bulletin of the National Research Centre

RESEARCH

Open Access

Effects of common fertilizers on the soil ecosystem

Natalia Frattini[1], Alcibeth Pulido Carrasquero[1], Lucía Pronsato[1], Lorena Milanesi[1] and Andrea Vasconsuelo[1*]

Abstract

Background The importance of this study lies in showing how certain usual practices of farmers such as burying stubble to fertilize soils or the routine use of fertilizers, affect soil quality. This is due to the effect that these practices have on the soil ecosystem. This study aims to reveal the importance of including bioindicators in soil analysis, showing how these activities negatively affect the soil ecosystem, which is responsible for maintaining soil quality.

Results By evasion response assays, was observed that *Eisenia foetida* avoided standing in the presence of urea 46%, phosphorous and nitrogen-based fertilizers, or crop waste; since earthworms exposed to soil-stubble (one part of ground tomato stubble, for every 5 parts of control soil), soil-fert (500 g of control soil, for each 50 g chemical fertilizer), or soil-urea (5 g/100 ml deionized water, watered over 500 g of control soil), exhibited significant avoidance responses (88.75% ± 17.3, 97.5% ± 5, and 91.25% ± 13.6 respectively. Data are means ± standard deviations *$p < 0.05$, with respect to the control). In addition, when earthworms could not escape from these stimuli, important morphological and histological changes, suggesting cell damage by apoptosis, were observed as decreased mobility.

Conclusions This work shows the importance of evaluating soil quality, with sensitive systems that allow the detection of negative effects in stages that can be reversed. That is, with parameters other than the physicochemical ones; reaching an integral assessment of the soils since it includes the entire ecosystem, thus obtaining information about the possible future state of these soils.

Keywords *E. foetida*, Fertilizers, Soil degradation, Soil ecosystem, Biomarkers

Background

Traditionally, farming or agriculture has been considered an activity unable to affect the environment. This concept changed, admitting that it causes damage to the environment at different levels (Santorufo et al. 2021). Anthropogenic changes in the use of soils, including the increase in agronomic activities to achieve food security, disturb the soil's ecosystems (Yang et al. 2020). It is estimated that near of 30% of soils are currently moderately to highly degraded due to erosion, salinization, acidification, pollution, or compaction, and 50% of cropland is moderately or severely affected by soil worsening (Kopittke et al. 2019; Escudero et al. 2023). Indeed, agriculture is practiced in increasingly degraded environments and with limited resources, while there is a constant demand for crop yields. In this context, various strategies are put into practice to obtain better responses from the soil and protect it (Hafez et al. 2021). Worldwide, the use of friendly agriculture is being applied, which finds the conservation of recourses such as soil and water (Hafez et al. 2021). This trend, in general, is based on minimum soil tillage and the use of stubble or organic waste on the soil surface. Stubble is the residue of the harvest and constitutes one of the most effective procedures to regulate soil moisture and temperature, buffer water erosion, control weeds, and provide compounds that increase soil quality (Hafez et al. 2019, 2021; Cárceles Rodríguez 2022)]. However, there are no studies on the response of the soil

*Correspondence:
Andrea Vasconsuelo
avascon@criba.edu.ar
[1] Departamento de Biologia Bioquimica y Farmacia, Universidad Nacional del Sur, 8000 Bahía Blanca, Argentina

ecosystem to this practice. Indeed, it is observed that there are not routine tools employed to detect the impact of human activities on soils, at the beginning of deterioration when it is possible to reverse it. Usually, to evaluate the state of the soil, physicochemical parameters are used, but such studies do not have an integrative character, which considers the entire soil ecosystem.

One of the criteria for soil health quality is that soil can maintain the biota (Escudero et al. 2023). Within this biota, was find earthworms, which are ecologically relevant for their role in soil maintenance and health through their abundance, function in decomposition and soil texture improvement, and their key position in terrestrial food chains (Ijomah et al. 2020). If the earthworm cannot comfortably live or survive in soil, this soil can be potentially at risk.

Currently, earthworms are used as biomarkers in ecotoxicology, evaluating soil contamination from chemical pollutants (Chao et al. 2022). There is extensive information on the use of earthworms in ecotoxicology studies. However, they are not used to assess the consequences of agricultural exploitation (or of common fertilization of gardens and farms with routine fertilizers). Of importance, there are no studies at the molecular level (oxidative stress/apoptosis) of the response of this organism to changes in its ecosystem. These studies would provide early information, when the organism detects the presence of an injury in its habitat and activates all its defense machinery.

We hypothesized that alterations in soil quality can be detected and consequently prevented, by studying changes in biological/molecular parameters of soil organisms. Thus, the aim of the present study is demonstrate that the evaluation of biologic parameters using edaphic organisms is a sensitive method of indicating early, how these practices could be detrimental to the soil in the long term.

Methods

Earthworms and soil preparation

Worms (Eisenia foetida) were from an earthworm-breeding farm (Bahía Blanca, Argentina) and kept in the laboratory under controlled conditions: in OECD soil (70% fine sand, 20% kaolin, 10% sphagnum peat with a small amount of $CaCO3$ for the adjustment of the pH and with homogeneous particle size) OECD guidelines for the testing chemicals (2016), pH 6.5, 50% of humidity with deionized water and room temperature between 21 and 22 °C in darkness.

Healthy adult earthworms (clitella formed), with an obvious annulus, of similar size (weight approx. 500–1000 mg) were selected for each experiment (OECD 2016). Worms were euthanized by icing at −20 °C (Fuller-Espie et al. 2010). The earthworms were conditioned before the assays were rinsed with distilled water, placed on a dampened Whatman paper in a Petri dish, and kept for 2 h to remove/clean their gut contents.

For controls, the soils were sieved (< 5 mm) to sift out roots and other large elements and adjusted to pH 6.5 and 50% of humidity with deionized water, thus being suitable for earthworms (control soil). Other set the experiments was performed adding to the control soils one part of ground tomato stubble (meanly leaves), for every 5 parts of soil and mixed until a homogeneous distribution was obtained (soil-stubble). Likewise, we tested, the control soil (500 g) mixed with chemical fertilizer (50 g) composed of Nitrogen 15% (nitric N 6.11%, ammoniac N 8.89%); phosphorus 15%; potassium 15%, for each part of soil (soil-fert). Also, was tested commercial prilled form urea 46% (5 gr/100 ml deionized water, watered over 500 g of control soil) used as fertilizer (soil-urea). In all the experimental conditions, the pH was controlled so that it did not vary from pH 6.5–7. The levels of each substance added to the soils were established so that if they trigger mortality, it is less than 10%.

Evasion response assay

Because organisms can avoid hostile environments, was perform this assay following the experimental protocol of evasion or avoidance behavior (ISO 2007) with modifications. This feature, useful for survival is based on the presence of sensitive receptors on the worm's body surface that can detect an ample range of contaminants or injuries (Reinecke et al. 2002). The assay was performed in three rectangular glass boxes (30 × 15 × 10 cm), divided into two equal compartments by a removable vertical partition. In the first box, one compartment was filled with control soil and the other with soil-stubble. In the second box, one compartment was filled with control soil and the second compartment with soil-fert. Likewise, in the third box, one-half was filled with control soil and the other half with soil-urea (Fig. 1A). The partition was removed and thereafter 10 adult earthworms, of similar weight, were placed onto the line of separation of each square container or box. The boxes were kept in darkness and had temperatures between 21 and 22 °C. After 2 days, the number of earthworms on both sides of each box was determined. The net avoidance response (NR) was determined with the following formula: $NR = (C - T)/N \times 100\%$, where C, T, and N represent the numbers of earthworms in the control soil (C), the conditioned soil (T), and the total number of earthworms (N), respectively.

In other sets of tests, was evaluated the migration conduct of the earthworms (5 adults healthy of similar weight specimens, in each box) when were placed

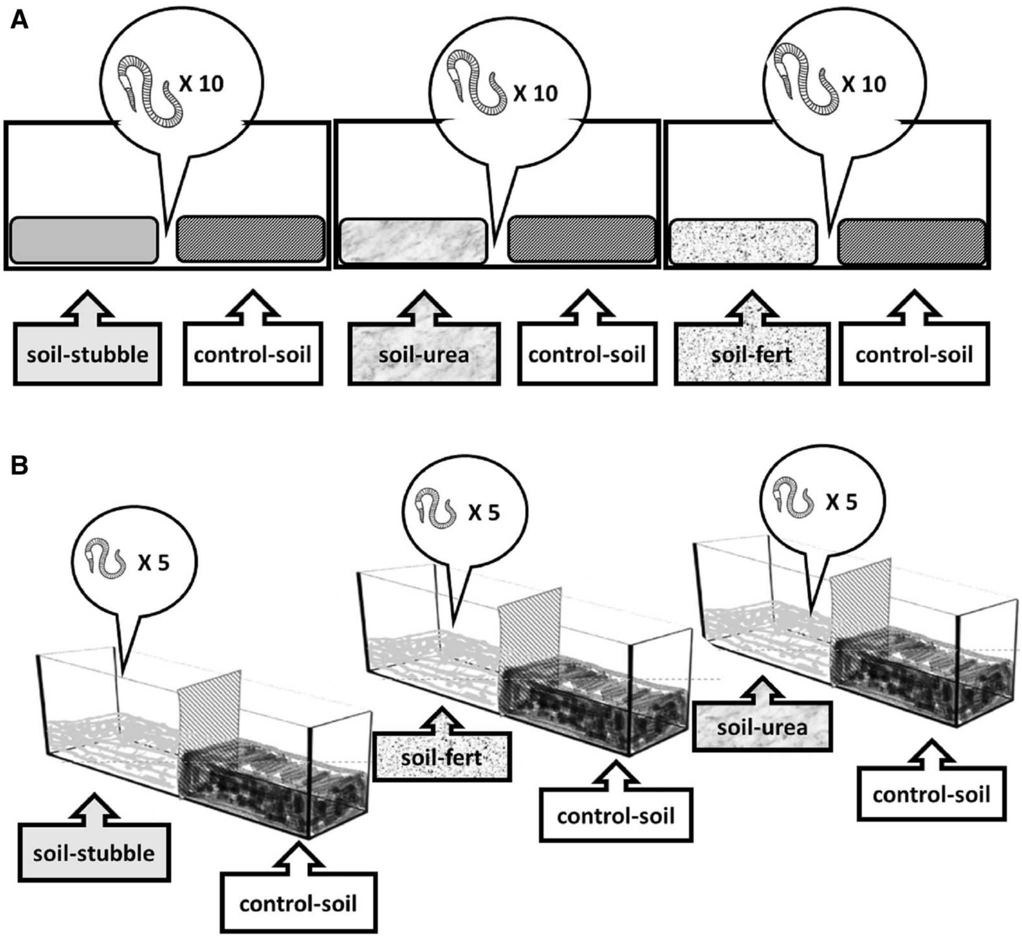

Fig. 1 Schematic representation of the procedure followed in the evasion behavior response assay. **A** Boxes with compartments containing different conditioned soils (Methods). In each box, ten similar (in size and weight) adult healthy earthworms were put in the empty areas between both soil types. After 48 h the earthworms in each half were counted. **B** Boxes with compartments separated containing different conditioned soils (Methods). Five similar (in size and weight) adult healthy earthworms were put inside each conditioned soil. After 48 h the separators in each box were removed; then mobility, localization, and morphology were analyzed (Methods). At least four replicates for each assay were performed

into the different conditioned soils for 2 days, and then the separator was removed (Fig. 1B). After another 2 days, was evaluated whether the worms had remained where they had initially been placed or whether they had migrated. The number of specimens in the different sections of each box was documented. In addition, changes in worm morphology and weight were investigated. Four replicates for each experimental condition were performed.

Histology

By analyzing the cellular structures and tissue damage the impact on earthworms of the soils with urea, tomato stubble, or with mineral fertilizer, was evaluated.

After each experiment, the earthworms were frozen, killed, and dissected employing a sterile surgical blade. The samples were transferred to a 4% formaldehyde solution (in phosphate buffer pH 7.2) and kept overnight. The tissues were dehydrated through a series of ethanol washes to remove water drops, infiltrated with wax, and then entrenched in paraffin. The fixed tissues were sliced using a microtome (1 μm films) and subjected to hematoxylin and eosin (H&E) or 4′,6-diamidino-2-fenilindol (DAPI) staining. Once the staining procedure was done, the sections were visualized under a Nikon Eclipse microscope and photographs were taken.

Statistical analysis

Data analysis was performed using standard statistical packages (InfoStat System, Córdoba, Argentina) (Balzarini et al. 2008). Values are shown as the mean ± standard deviation (S.D.) of at least four independent experiments. Statistical differences among groups were performed using ANOVA and a multiple comparison post hoc test. The data were considered statistically significant when $p < 0.05$.

Results

Effect of tomato stubble, fertilizers, and urea on earthworms: evasion behavior

First, it was observed the movement of earthworms in boxes with control soil only (dual control). The earthworms did not congregate forming clusters but distributed themselves randomly throughout the test boxes. Consequently, earthworms did not show behavior that might be mistaken for avoidance (Yeardley et al. 1996), which would invalidate avoidance assay. In addition, was verified that ≤ 10% of the earthworms died or runaway in each experimental condition.

Figure 2 shows the results of the evasion behavior test after earthworm exposure to conditioned soils of each compartment of the boxes (Methods). In dual control tests, no significant differences were found in the distribution of the worms between both compartments of the box. Earthworms exposed to soil-stubble, soil-fert, or soil-urea, exhibited significant avoidance responses (88.75% ± 17.3, 97.5% ± 5, and 91.25% ± 13.6 respectively).

When the experiment was performed putting the earthworms directly in the conditioned soils and keep there for 2 days, before removing the separator, was observed important anatomical and morphological abnormalities, with a weight decrease. The earthworms shown lifting the body, vesicles, coiling, and curling, constrictions, swelling at clitella, and fragmentation of the body, in all individuals exposed to conditioned soils with urea and organic fertilizer; and in some of the earthworms that are kept in the soil with tomato stubble (Fig. 3). These changes in morphology were accompanied by an excessive mucus secretion and, another observation was that the morphological abnormalities did not follow a pattern of distribution in the body of the worms, but were distributed rather randomly (Data not shown). No significant morphological abnormalities or excessive mucus production were observed in the worms of all the control conditions. All earthworms from controls, presented a normal pink color and healthy cuticular striation

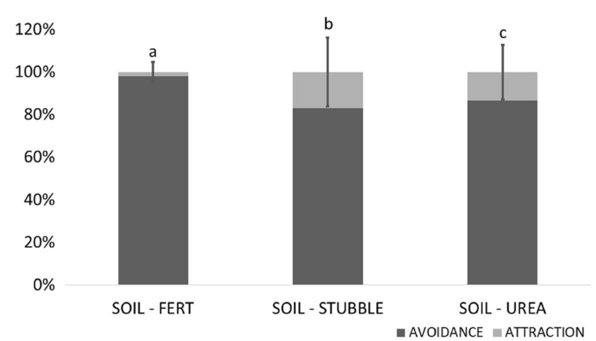

Fig. 2 Avoidance or attraction response of earthworms to conditioned soils. Net avoidance response to conditioned soils. Avoidance data are means ± standard deviations. Different letters indicate means with significant differences according to the least significant difference at $p < 0.05$, with respect to the side control of each box

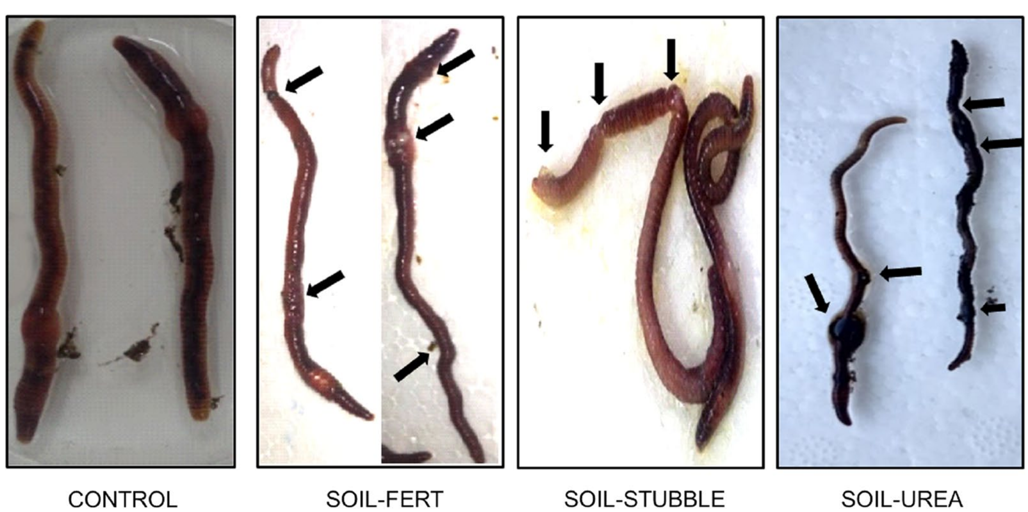

Fig. 3 Effect of soils containing Urea 46%, mineral fertilizer, or tomato stubble on the *E. foetida* morphology. After 2-day retained on conditioned soils, the earthworms present abnormal morphology (black arrows) as swelling at the clitella region, blistering of the body wall, vesicles, coiling and curling, constrictions, and fragmentation of the body. Earthworms from control soil despite normal morphology. Experiments were repeated at least three times with essentially identical results

(Fig. 3). Of importance, the mobility of the earthworm was seriously affected and in consequence, when the separator was removed, the number of worms able to migrate to control soils was insignificant (Table 1). As before, was verified that ≤ 10% of the earthworms died or runaway in each experimental condition.

Histological examinations of earthworms from conditioned soils

The earthworms from the experiments above descript, which are kept in conditioned soils for 2 days, were used to look for histological abnormalities. As was described in Methods, histological observations were performed using hematoxylin–eosin staining. As shown in Fig. 4A, an intact/normal structure of the epidermis and circular and longitudinal muscle layers was observed in the earthworms from control soils. However, morphological abnormalities have been found in earthworms from conditioned soils. It has been observed that circular and longitudinal muscles were atrophied and degraded, showing breaks, and disordered structure, and the intercellular space was further enlarged. Also, the cuticle was loosely arranged, and in some regions were degraded. All these abnormalities were more evident in earthworms from soil-fert and soil-urea (Table 1).

Interestingly, the nuclear dye DAPI showed morphological changes as nuclear fragmentation/condensation (pyknotic nuclei), suggestive of apoptosis, in the epidermis and muscle cells of earthworms from conditioned soil (Fig. 4B). Contrary to earthworms from control soil, in which the chromatin is kept relaxed and orderly.

Discussion

The present work provides information about the effects of anthropogenic activities on soil ecosystems, due to common uses and customs of agricultural actions related to the types of fertilizers used (Jastrzębska et al. 2022). As was mentioned above, the use of physicochemical parameters to evaluate the state of the soil does not represent an integrative character since do not inform about of soil ecosystem. Moreover, it is only now recognized that soil biological properties have seldom played a role in agricultural assessment (Bach et al. 2020). Due to the relevant role of earthworms in soil fertility, it is logical to assume that factors that affect these organisms will result in deteriorated and non-productive soils. A decrease in the number of worms has to severe ecological impact since the rate of decomposition of organic matter and soil aeration; vital functions for soil quality, become compromised (Zhao et al. 2022). Therefore, the earthworm biomarker itself can provide information on the environmental status of the soil (Fuller-Espie et al. 2010) and according to literature, the avoidance behavior of *E. foetida* is a very sensitive parameter to use [ISO 2007; Yeardley et al. (1996)]. In addition, given the greater sensitivity of these organisms, information on soil damage would be obtained much earlier than with routine physicochemical parameters. In agreement, it has been observed apoptosis in earthworms (Ma et al. 2016). Due apoptosis is a process that can be detected in its early stages; it could be useful for detecting affected organisms prompt and consequently, take measures to protect that soil.

Thus, in this study using the avoidance behavior of earthworms against injuries was demonstrate the negative impact of the widespread use of Urea 46% or nitrogen derivatives with phosphorus as fertilizer. Especially if it does not take into account the correct distribution to achieve a concentration in all areas that do not affect the ecosystem responsible for soil quality. Likewise, the farmers' custom of using stubble as fertilizer for cultivated soil, especially when Solanaceae are used, can also hurt the soil by affecting its ecosystem. Here observed an important avoidance behavior to the conditioned soils with stubble, urea, or fertilizer based in nitrogen and phosphorus, being more evident in this last conditioned soil. Moreover, when the earthworms could not evade these soils remaining in them for 48 h, they showed important morphological changes with loss of weight and mobility as evidenced by macroscopic observations and histological analysis. The loss of muscle tissue, may be a factor for the worsened mobility observed; this affect the function of the earthworm in the soil and its vital role in it. Also, the loss of muscle mass together with the high secretion of mucus observed, may be the causes of the

Table 1 Conditioned soils induce changes in earthworms

Soil conditions (OECD soil + aggregates) (OECD, 2016)	Average weight loss (g)	Mobility	Presence of morphological deformations	Presence of histological abnormalities
soil-control	WC	++++	–	–
soil-stubble	0.32	+++	+	+
soil-fert	0.48	++	+++	+++
soil-urea	0.86	++	+++	++++

WC Without significant changes. ++++: máximum, +: minimum, –: none

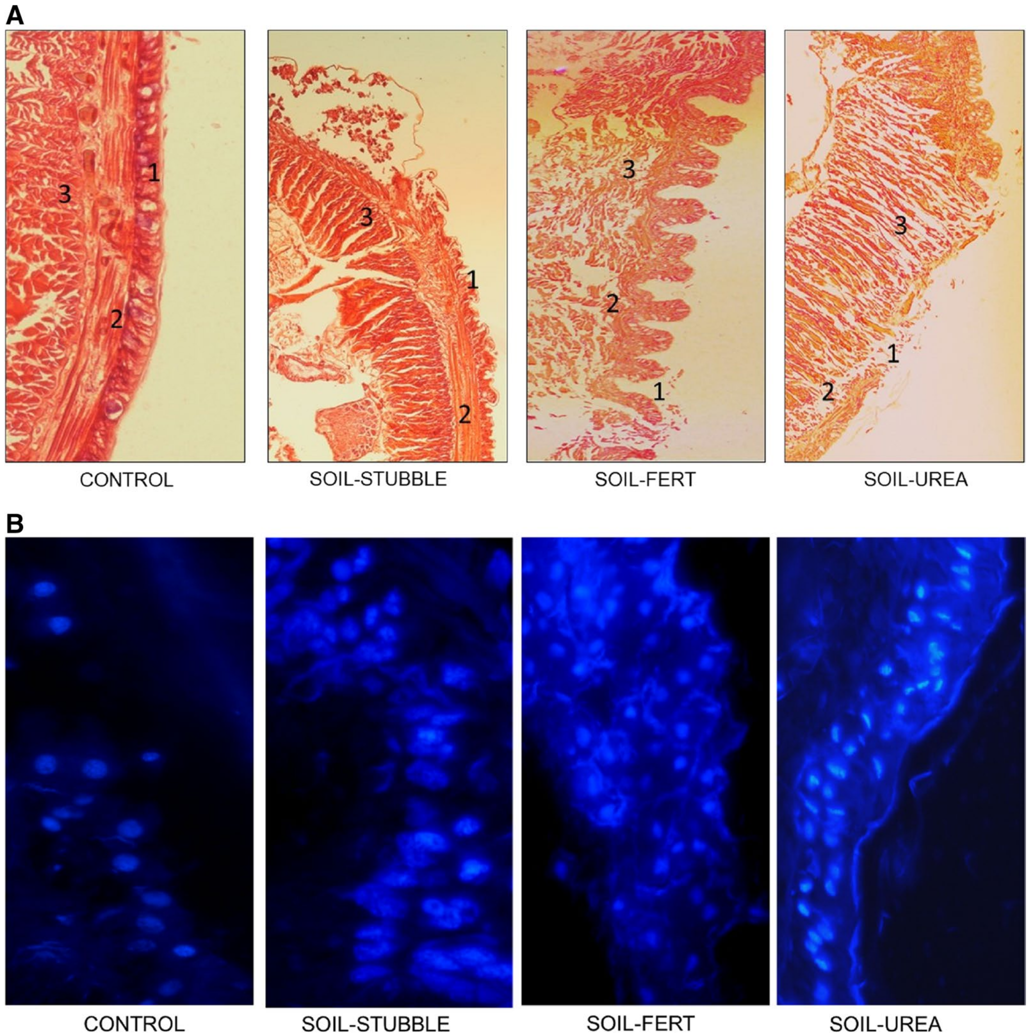

Fig. 4 Histological alterations in *E. foetida* samples from conditioned soils. **A** Earthworms from control soils present a normal appearance of the epidermis (1), circular muscle (2), and longitudinal muscle (3). In sections of earthworms from conditioned soils, loss of structural integrity and deformation in the epidermis, in the circular and longitudinal muscles are shown. Transverse sections of *E. foetida* stained with hematoxylin–eosin. **B** Nuclei of earthworms from control soils despite lax chromatin with a fine grain. Condensed, fragmented and pyknotic chromatin is observed in nuclei of earthworms from conditioned soils. Transverse sections of *E. foetida* stained with DAPI. Experiments were repeated at least three times with essentially identical results. (Magnification 40X)

decrease in weight in the earthworms that remained in the treated soils.

Of relevance, although more studies are necessary to confirm this, histological observations using DAPI stain evidenced the presence of nuclei with condensed or fragmented chromatin suggesting apoptosis. In agreement with other studies indicating that Solanaceae extracts induce apoptosis in muscle cells (Musso et al. 2019), here, the muscle cells of earthworms that are kept in soil-stubble show typical features of apoptosis.

Based on the data presented here, it would be very important that the effect that a fertilizer causes on the soil ecosystem should be specified in their commercial labels; and should indicate the appropriate dose to cause the least impact.

Conclusions

The data presented show the importance to achieve sensible controls of human activities related to soil exploitation. The use of earthworm as a sensor of soil quality represents a useful tool to avoid practices that affects ground ecosystems and drive to non-productive soil. The importance of our results lies in the fact that, in addition to observing that the worms avoided the soils with the fertilizers used here, they also showed signs of apoptosis. This information is valuable, because it allows detecting this process in its initial stages, before the organisms die. However, further research is necessary to increase the number of early parameters to be analyzed, such as antioxidant enzymes and other molecular markers of apoptosis, and establish a sensitive and robust biomarker.

Acknowledgments
Cell Signaling Technology Inc. kindly provided us with the secondary antibodies. Visuar S.A, Benito Quinquela Martín 1924, (1296), CABA, Buenos Aires, Argentina, and Fuegoservi S.R.L, Estomba 1702, B8003 AAF, de Buenos Aires for supporting our Research.

Author contributions
NF, AC, and LP perform de assays and keep the worms in the lab. They also collaborated with the writing of this work. AV performed histological sections and staining. LM and AV designed and supervised the experiments, wrote the paper. All authors read and approved the final manuscript.

Funding
This research was supported by a grant from the Universidad Nacional Del Sur; Grant number: PGI24/ZB90. LP, AV, and LM are researcher members of CONICET; NF and AC thank the CONICET for a doctoral fellowship.

Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate.
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors have no competing interests to declare relevant to this article's content.

Received: 28 February 2023 Accepted: 27 May 2023
Published online: 01 June 2023

References

Bach EM, Ramirez KS, Fraser TD, Wall DH (2020) Soil biodiversity integrates solutions for a sustainable future. Sustainability 12:2662. https://doi.org/10.3390/su12072662
Balzarini MG, Gonzalez L, Tablada M, Casanoves F, Di Rienzo JA, Robledo CW (2008) InfoStat, manual del usuario. Versión 2008. Córdoba, Argentina
Cárceles Rodríguez B, Durán-Zuazo VH, Soriano Rodríguez M, García-Tejero IF, Gálvez Ruiz B, Cuadros Tavira S (2022) Conservation agriculture as a sustainable system for soil health: a review. Soil Syst 6:87. https://doi.org/10.3390/soilsystems6040087
Chao H, Sun MM, Wu Y, Xia R, Yuan J, Hu F (2022) Quantitative relationship between earthworms' sensitivity to organic pollutants and the contaminants' degradation in soil: a meta-analysis. J Hazard Mater 429:128286. https://doi.org/10.1016/j.jhazmat.2022.128286
Escudero H, Domínguez A, Bedano J (2023) Large-scale ecologically-based farming systems foster earthworm communities and their contribution to ecosystem processes. Appl Soil Ecol 185:104800. https://doi.org/10.1016/j.apsoil.2022.104800
Fuller-Espie SL, Nacarelli T, Blake EL, Bearoff FM (2010) The effect of oxidative stress on phagocytosis and apoptosis in the earthworm *Eisenia hortensis*. Invertebr Surviv J 7:89–106
Hafez M, Popov AI, Rashad M (2019) Influence of agro-industrial wastes and azospirillum on nutrients status and grain yield under corn plant growth in arid regions. Biosci Res 16:2119–2130
Hafez M, Abo El-Ezz S, Popov AI, Rashad M (2021) Organic amendments combined with plant growth-promoting rhizobacteria (*Azospirillum brasilense*) as an eco-friendly by-product to remediate and enhance the fertility of saline sodic-soils in Egypt. Commun Soil Sci Plant Anals 52:1416–1433. https://doi.org/10.1080/00103624.2021.1885687
Ijomah OO, Adesuyi AA, Njoku KL, Ojokuku SA, Moses UD, Adesuyi O (2020) Histopathological effects and biomarker response of earthworms, *Eisenia foetida*, after exposure to crude oil contaminated soils. Environ Anal Heal-Toxicol 35:e2020021-0. https://doi.org/10.5620/eaht.2020021
Jastrzębska M, Kostrzewska M, Saeid A (2022) Conventional agrochemicals: Pros and cons. In: Chojnacka K, Saeid A (eds) Smart agrochemicals for sustainable agriculture. Academic Press, Cambridge
Kopittke PM, Menzies NW, Wang P, McKenna BA, Lombi E (2019) Soil and the intensification of agriculture for global food security. Environ Int 132:105078. https://doi.org/10.1016/j.envint.2019.105078
Ma T, Chen L, Wu L, Zhang H, Luo Y (2016) oxidative stress, cytotoxicity and genotoxicity in earthworm *Eisenia fetida* at different Di-n-butyl phthalate exposure levels. PLoS ONE 3:e0151128. https://doi.org/10.1371/journal.pone.0151128
Musso F, Lincor D, Vasconsuelo A, Pronsato L, Faraoni B, Milanesi L (2019) Adverse effects in skeletal muscle following the medicinal use of *Nicotiana glauca*. Biol Pharm Bull 42:671–679. https://doi.org/10.1248/bpb.b18-00424
OECD (2016) Test No. 222: Earthworm reproduction test (Eisenia fetida/Eisenia andrei) https://doi.org/10.1787/9789264264496-en
OECD guidelines for the testing chemicals (2016) OECD P, Paris.https://doi.org/10.1787/9789264264496-en
Reinecke AJ, Maboeta MS, Vermeulen L, Reinecke SA (2002) Assessment of lead nitrate and mancozeb toxicity in earthworms using the avoidance response. Bull Environ Contam Toxicol 68:779–786. https://doi.org/10.1007/s00128-002-0023-8
Santorufo L, Memoli V, Panico SC, Esposito F, Vitale L, Di Natale G, Trifuoggi M, Barile R, De Marco A, Maisto G (2021) Impact of anthropic activities on soil quality under different land uses. Int J Environ Res Public Health 16:8423. https://doi.org/10.3390/ijerph18168423
Yang T, Siddique KHM, Liu K (2020) Cropping systems in agriculture and their impact on soil health. Glob Ecol Conserv 23:e01118. https://doi.org/10.1016/j.gecco.2020.e01118
Yeardley RB, Gast LC, Lazorchak JM (1996) The potential of an earthworm avoidance test for the evaluation of hazardous waste sites. Environ Toxicol Chem 15:1532–1537. https://doi.org/10.1002/etc.5620150915
Zhao W, Teng M, Zhang J, Wang K, Zhang J, Xu Y, Wang C (2022) Insights into the mechanisms of organic pollutant toxicity to earthworms: advances and perspectives. Environ Pollut 303:119120. https://doi.org/10.1016/j.envpol.2022.119120

Publisher's Note