Ecotoxicological effects of a synthetic and a natural insecticide on earthworms and soil bacterial community

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ABSTRACT

Earthworms and microbial communities are essential non-target soil organisms that are useful to assess the collateral impact of pesticides. The present paper reports three laboratory experiments performed to investigate the effects of sub-lethal doses of two insecticides, a biologically-derived (spinosad) and a synthetic organophosphate (chlorpyrifos), on earthworm *Eisenia foetida* and microorganisms in organic soil. The effects were studied in terms of behaviour, reproduction, survival, and DNA damage (comet assay) in earthworms, and Next Generation Sequencing-Illumina was employed to detect the changes in the microbial community. In addition, the influence of earthworms on the degradation kinetics of insecticides and on microbial diversity was evaluated. The weights, reproductive activity and behaviour of earthworms were particularly compromised and followed a dose-dependent trend in chlorpyrifos trials, where the insecticide's degradation wasn't affected by the presence of *Eisenia foetida*. However, earthworms contributed to spinosad's metabolisation without significantly impacting their health. Early DNA damage was estimated in earthworms exposed to chlorpyrifos, while the impact of spinosad was significant only at the end of the toxicity test. The analysis on the microbial community indicated the buffering effect earthworms had on the bacterial communities starting from earliest sampling until the end of the trial, as well as bacterial community members' degradation response to pesticides over time.

1. Introduction

The intensive use of pesticides for crop protection against diseases led to the widespread presence of these compounds in soils (Hussain et al., 2009; Zhou et al., 2011; Stepić et al., 2013). Pesticide use may affect soil fertility and non-target organisms such as microorganisms and macroinvertebrates (Puglisi, 2012; Mincarelli et al., 2019; Vischetti et al., 2020). A broad toxicity spectrum was reported in ecosystems in correlation to pesticide exposure levels (Desneux et al., 2007; Beketov et al., 2013; Brühl et al., 2013; Wood and Goulson, 2017) and evaluating non-specific impact on soil-biota could help Regulatory Authorities to avoid underestimating the effect of pesticides (Schäfer et al., 2019). Therefore, using molecular methods and genotoxicity assays, sub-lethal dose impact evaluation is often recommended. However, data interpretation for regulatory purposes still debated (Ockleford et al., 2017; Vischetti et al., 2020).

Earthworms and microbial communities are commonly used in ecotoxicological studies because they represent a large fraction of soil living biomass and are essential in soil functioning (Pelosi et al., 2014; Delgado-Baquerizo et al., 2016; Umar et al., 2017). *Eisenia foetida* (*E. foetida*) is most commonly earthworm as exposure to pesticide-contaminated soil is almost direct due to its simple digestive system and limited tegumentary system (Sanchez-Hernandez et al., 2018b; Svobodová et al., 2018; Zhu et al., 2020). Pesticides can also affect soil microbial communities that are important to earthworms due to the enzymatic support from microbial symbionts that inhabit their gastrointestinal lumen, and at the same time, the mucilaginous secretions of earthworms usually increase exogenous microorganisms activity (Zapata et al., 2017; Gonzales-Condori et al., 2020). Aktar et al. (2009) reported that, among pesticides, insecticides caused the highest acute toxicity. Nonetheless, their comprehensive impact remains poorly investigated as leaf applied insecticides are scarcely investigated compared to herbicides. However, run-off to soil may occur at excess doses (Gil et al., 2007; Monaci et al., 2011; Cesco et al., 2021).

Chlorpyrifos, an organophosphate insecticide, is extensively used worldwide on a range of economically important crops (Thengodkar and Sivakami, 2010; Sud et al., 2020). Its toxicity on earthworms was previously investigated by avoidance, behaviour, survival and reproduction assays (Zhou et al., 2007; Yasmin and D'Souza, 2010; Hundal et al., 2016); recently, an additive and synergic toxicity effect was ascertained for chlorpyrifos mixed to other pesticides on *E. foetida* acetylcholinesterase levels and cellulose activity (Teng et al., 2022), while an effect on mortality and on the gut microbiome of the earthworm *Eudrilus euginae* was observed after exposure to chlorpyrifos at a Lethal Concentration 50 dose, i.e. the concentration expected to kill 50% of a group of test animals when administered as a single exposure (LC$_{50}$) (Krishnaswamy et al., 2021). Moreover a number of studies demonstrated its ecotoxicity versus other non target organisms, such as rainbow trout larvae (Weeks Santos et al., 2021) and Danio rerio (Mena et al., 2022) and toxicity versus humans, such as pregnant women (Taheri et al., 2022) and human brain (Miller et al., 2021). Over the past 20 years, "natural" insecticides have become increasingly adopted as an environmentally safe alternative to synthetic insecticides (Williams et al., 2003; Biondi et al., 2012; Tamez-Guerra et al., 2017). Among those, spinosad claims lower environmental toxicity due to its natural origin; a mixture of spynosins by soil actinomycete *Saccharopolyspora spinose*. Apart some paper reporting the effects of spinosad on non target organisms such as beneficial arthropods (Biondi et al., 2012) or beneficial insects (Martelli et al., 2022), very little is known about the impact of these insecticides' effect on earthworms and the soil microbial community (Badawy et al., 2016; Sekulić et al., 2020; Sparks et al., 2020) while evaluating early damage to soil organisms using DNA-based methodological tools might improve the global understanding of the stresses induced by the pesticides on the agro-ecosystems.

The present study aims to ascertain the effect of sub-lethal doses of the two insecticides mentioned above on earthworm *E. foetida* and the soil microbial community with the hypothesis that the different nature of studied insecticides could affect the functionality and health of earthworms and microbial communities differently and that the presence of earthworms favours the degradation of pesticides. Therefore we conducted; (i) avoidance and reproduction tests to investigate insecticides' harmful effects on earthworms in terms of fertility and behaviour, (ii) a genotoxicity test using the comet assay to detect the DNA damage in earthworm coelomocytes, and finally (iii) Next Generation Sequencing (NGS)-Illumina sequencing to investigate changes in the microbial community. Parallel experiments were also performed without earthworms to evaluate their influence on insecticide degradation kinetics and microbial diversity.

2. Materials and methods

2.1. Earthworms

E. foetida were supplied by the Lombricoltura Bella Farnia (Sabaudia, Italy). They were reared at $20 \pm 1°C$ in organic compost and fed with organic oats and vegetables. Adults with a well-developed clitellum (300-600 mg wet mass) were selected and acclimatised in the same substrate used in tests (Li et al., 2018; Zou et al., 2018; Zhang et al., 2019). Ten adults were used in each replicate in experiments.

2.2. Soil

Topsoil from an orchard managed with organic agricultural practices was collected from the experimental farm of the Polytechnic University of Marche (Agugliano, Italy). This Vertic Eutrodept, clay loam agricultural soil with the following properties was used across the experiments: pH 8.2; organic matter 8 %; cation exchange capacity 28.3 meq 100 g^{-1}; air dried and sieved at < 2 mm.

2.3. Insecticides and contamination

Commercial formulation Laser 480 (spinosad, g 44.2 corresponding to 480 g L^{-1}) and Dursban 75 WG (chlorpyrifos g 75.0 corresponding to 750 g L^{-1}) were supplied by Dow AgroSciences (Milano, Italy), while analytical standards of spinosad (CAS 168316-95-8, purity $\geq$ 95.0 %) and chlorpyrifos (CAS 2921-88-2, purity $\geq$ 98.0 %) were obtained from Sigma-Aldrich (Milano, Italy) and their proprieties are summarised in Supplementary Table S1. Solutions of these pesticides were freshly prepared in deionised water to adjust the soil moisture content before experiments (40 ± 10 dry mass) in chemically inert plastic containers ($18 \times 9 \times 9$ cm) with a lid that permits gaseous exchange. Two concentrations were employed for each insecticide for the avoidance and reproduction test. More in detail, Dursban 75 WG, was added at the doses of 50 % in the trials with earthworms (C$_{50}$E) and 70 % (C$_{70}$E) of the LC$_{50}$ indicated for the pesticide formulation corresponding to 340.5 mg kg^{-1} and 476.7 mg kg^{-1} of Dursban 75 WG respectively. Laser 480 was tested at the doses of 70 % in the trials with earthworms (S$_{70}$E) and 150 % (S$_{150}$E) of the LC$_{50}$ indicated for its pesticide formulation corresponding to 735 mg kg^{-1} and 1575 mg kg^{-1}, respectively. The toxicity test was conducted with a working concentration of 70 % of the LC$_{50}$ for both insecticide formulations.

2.4. Avoidance test

A "dual-control" test was run to assess that earthworms do not tend to aggregate and have a random distribution between the two sections (Yeardley et al., 1996; Hund-Rinke and Wiechering, 2001). The avoidance test was then conducted to evaluate the ability of earthworms to detect and avoid the contaminated substrate (García-Santos and Keller-Forrer, 2011; Jordaan et al., 2012; Martínez Morcillo et al., 2013) in five replicates with the two-chamber design, as described by ISO 17512-1 (2008). One-half of the box was filled with 250 grams dry weight of the contaminated substrate, the other half was filled with the same quantity of the substrate without the insecticide, and the earthworms were placed onto the separating line. After 2 days, a divider was inserted, and the earthworms on both chambers were counted. The results of the avoidance test are expressed as the net response (NR) in percentage according to ISO (2008):

$$NR = \lfloor (C - T) \div N \rfloor \times 100$$

where C and T are the numbers of worms in the control substrate and the treated substrate, respectively, N is the total number of worms in each box.

A positive NR indicates an avoidance of the contaminated substrate, whereas a negative value indicates an attraction to the pesticide tested (Xu et al., 2020)

2.5. Reproduction test

Insecticides' impact on earthworms' reproductive output (and other sub-lethal endpoints) was assessed through a reproduction test following the OECD guideline (OECD, 2016).

All trials and an uncontaminated control with earthworms (ctrlE), in three replicates, were kept under a controlled temperature ($20 \pm 1°C$) for 56 days. Adult earthworms in each replicate have been weighed and observed weekly: any unusual behaviour and morphology anomalies were recorded. After 28 days, adults were removed from the containers while substrate containing juveniles and cocoons were left for another 4 weeks-incubation. On day 56, the number of juveniles and the cocoons in each replicate were recorded. The growth rate (GR, %) was calculated as follows:

$$GR = \lfloor (W_t - W_0) \div W_0 \rfloor \times 100\%$$

where W_0 is the initial average weight of earthworms, and W_t is the

average weight of earthworms on day 28. A positive rate means the growth stimulation, while a negative rate indicates growth inhibition (Xie et al., 2013).

2.6. Toxicity test

The toxicity test was conducted to investigate the effects of the insecticides on earthworms' DNA and microbial communities with three replicates for every insecticide and control with earthworms (ctrlE). A gram of the substrate was taken at each sampling for the soil bacterial community and insecticide residues analysis, and an earthworm in each container (three earthworms for trial) was randomly collected at 1, 21 and 28 days for DNA damage analysis through the comet assay. A parallel test was conducted without adding earthworms to evaluate differences in the trend of insecticide residues and the evolution of the soil bacterial community according to the presence or absence of *E. foetida*. Specifically, the parallel test consisted of chlorpyrifos at the dose of 70 % without earthworms (C_{70}), spinosad at the dose of 70 % without earthworms (S_{70}) and an uncontaminated control without earthworms (ctrl).

2.6.1. Insecticides extraction and analysis

The extraction and analysis of chlorpyrifos followed the protocol described by Vischetti et al. (2008); spinosad was extracted and analysed following the method described by Sharma et al. (2007), and its residues were reported as a sum of spinosyn A and spinosyn D (Telesiński et al., 2015). Analyses were performed by HPLC using a Spectra SYSTEM P 4000, equipped with a Supelcosil C18 column (25 cm x 4.6 mm i.d.) and a UV-detector as in Akbar et al. (2010). Flow rate was 1 mL min^{-1} and the eluent was acetonitrile:water 70:30. Under these conditions, retention time was 6 min for chlorpyrifos and 3 and 5 min for spinosyn A and D, respectively, and the Limit of Detection was 0.67 mg L^{-1} for chlorpyrifos and 0.59 mg L^{-1} for spinosad.

2.6.2. Comet assay

Coelomocytes were collected as described in Eyambe et al. (1991) with slight modifications. Each earthworm was immersed for 4 minutes in an extrusion buffer of 5 % ethanol, 95 % PBS, 2.5 mg mL^{-1} Na_2-EDTA, and 10 mg mL^{-1} guaiacol glyceryl ether (pH 7.3). Coelomocytes were washed and collected by centrifugation (300 g, 10 min, 4°C). The washed cells were counted, resuspended in Low Melting Agarose (LMA 1 %, 37°C) and stratified on HT Trevigen slides pre-coated with Normal Melting Agarose (NMA 1 %). Each spot was produced by layering LMA containing 3000 cells; each sample was stratified in triplicate. The solidification, lysis and unwinding phases were carried out following Mincarelli et al. (2016). Electrophoresis was conducted at 11 V cm^{-1} for 20 min at 4°C. Slides were washed in H_2O, neutralised in buffer (0.4 M Tris-HCl pH 7.5), dehydrated in 75 % methanol (Valverde et al., 1999; Mincarelli et al., 2016), stained with Sybr Gold and then imaged using Lionheart FX Automated Microscope (Biotek, U.S.A.) at 200 × 200 magnification. Comet images were acquired in triplicate and processed to calculate the major DNA damage index: Tail length (TL), Tail moment (TM), and Tail intensity (TI) (Tiano et al., 2005; Orlando et al., 2018).

2.6.3. Analysis of soil bacterial community diversity

Biodiversity analysis of soil bacterial community was based on High Throughput Sequencing (HTS) of 16S rDNA amplicons. Total genomic DNA was isolated using Soil DNA Isolation Kit (NORGEN Biotek, Canada) following the manufacturer's protocol, and V3-V4 region of 16S ribosomal RNA (rRNA) gene was amplified using the universal primers 343F (5′-TACGGRAGGCAGCAG-3′) and 802R (5′-TACNVGGGTWTC-TAATCC-3′), as previously described in detail (Vasileiadis et al., 2012, 2015; Bandini et al., 2021). Thermal cycling conditions, primer concentrations and volumes are provided in Supplementary Table S2.

2.7. Statistical analysis and bioinformatics

Statistical analyses were performed in R software (R Core Team 2018, version 3.5.2) with linear mixed-effect models and pairwise significance between groups when the data respected the assumptions. Where the assumptions were not respected, the non-parametric Kruskal-Wallis test and Dunn's post hoc test were used (Bonferroni *p*-value adjustment, α = 0.05). Statistical analyses of soil bacterial community diversity were carried out as previously detailed (Vasileiadis et al., 2013; Połka et al., 2015; Cesco et al., 2021). Sequence data is available through Sequence Read Archive (NCBI-SRA) BioProject ID PRJNAXXXXXX.

3. RESULTS

3.1. Avoidance response

The results of the preliminary dual-control test showed that both validity criteria were achieved for the avoidance tests considering that no earthworms died or escaped and there was no significant preference or aggregation to one section when the same substrate was placed on each side.

The effects of the two insecticides on the avoidance behaviour are given in Fig. 1; no earthworm escaped or died during the exposure period.

All trials had a positive NR value. Only in the trial with chlorpyrifos at the upper dose ($C_{70}E$), the NR value exceeds 80 % (dotted line).

3.2. Reproduction responses

The trend of the mean weight of earthworms in each trial is reported in Fig. 2.

Significant differences between the treatments were observed starting from day 14, where the earthworm weight in chlorpyrifos trials resulted significantly lower than the control. In contrast, less marked differences with respect to the control were observed for the spinosad trials. Chlorpyrifos at the highest dose ($C_{70}E$) showed a significant loss of earthworm weight from day 21. At 21 days, the weight measured in the spinosad trial at the highest dose ($S_{150}E$) resulted lower than the control. At the end of the experiment (28 days), also the trial with spinosad at the lowest dose ($S_{70}E$) recorded a significant weight loss. A summary of the observations on the health status of earthworms during this test is reported in Table 1.

No mortality was observed in the control and spinosad trial at the lowest dose ($S_{70}E$), while 3.33 % was recorded in spinosad at the highest dose ($S_{150}E$) from 21 days. Mortality occurred from the 21 days on at the lowest concentration ($C_{50}E$) of chlorpyrifos, and increased by 10 % was recorded at 28 days. Mortality at the highest dose of chlorpyrifos ($C_{70}E$) started on the 14 days differed significantly from other treatments on 21 days (23 %) until it reached a percentage of 40 % on 28 days. Unusual behaviours were absent in control and spinosad, while started on 7 days in the chlorpyrifos treatments. Morphological anomalies were observed only in chlorpyrifos treatments from day 21 (Table 1). The production of cocoons in spinosad treatments did not differ significantly from the control. In contrast, there was an evident low production in treatments with chlorpyrifos most significant at the highest dose ($C_{70}E$). Similarly, minimal production of juveniles was recorded at the lowest dose ($C_{50}E$), and no young were counted at the highest($C_{70}E$).

### 3.3. Toxicity test responses

3.3.1. Insecticide residues

The residues of the two insecticides found in the soil during the experiment, which represent the real exposition of the soil organisms to the toxic effect, are shown in Fig. 3.

Insecticides degradation in soil proceeded with almost the same rate and half-life values, calculated applying the first-order kinetics to the
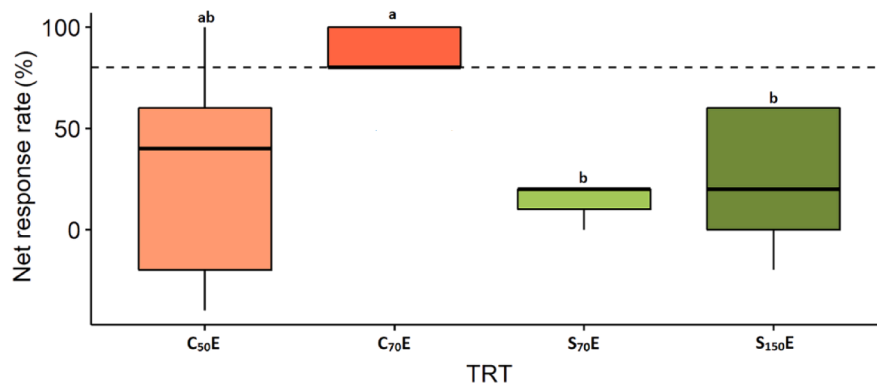
Fig. 1. Avoidance behaviour in *E. foetida* expressed in Net Response rate. Treatments (TRT): C$_{50}$E (chlorpyrifos up to 50 % of the LC$_{50}$), C$_{70}$E (chlorpyrifos up to 70 % of the LC$_{50}$), S$_{70}$E (spinosad up to 70 % of the LC$_{50}$), and S$_{150}$E (spinosad up to 150 % of the LC$_{50}$). According to Dunn's Kruskal-Wallis multiple comparisons, treatments with different lowercase letters were significantly different.
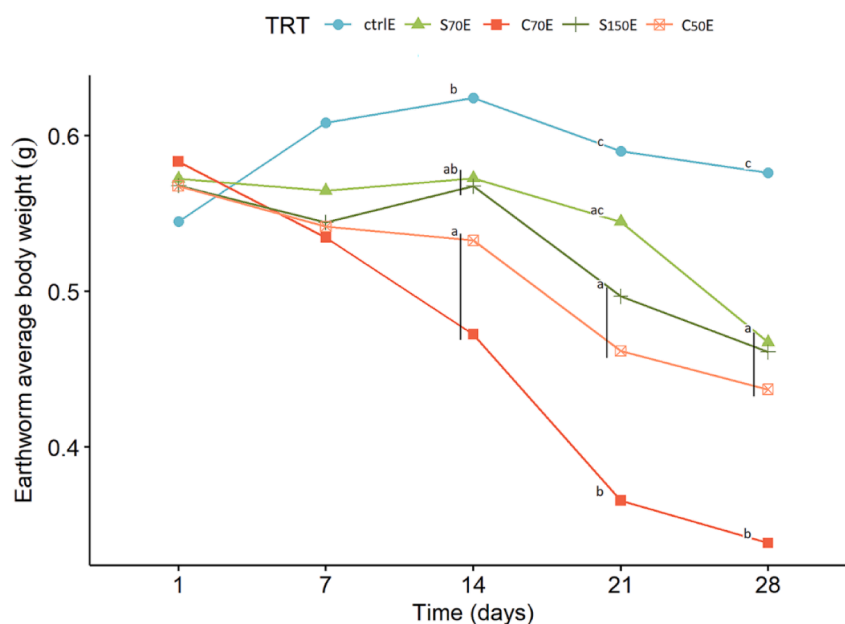
Fig. 2. Weight trend during the reproduction test. Treatments (TRT): ctrlE (negative control), S$_{70}$E (spinosad up to 70 % of the LC$_{50}$), S$_{150}$E (spinosad up to 150 % of theLC$_{50}$), C$_{50}$E (chlorpyrifos up to 50 % of the LC$_{50}$) and C$_{70}$E (chlorpyrifos up to 70 % of the LC$_{50}$). According to Dunn's Kruskal–Wallis multiple comparisons, treatments at the same time with different lowercase letters were significantly different.

degradation data, resulted in 29.6 days for C$_{70}$E, 26.5 for C$_{70}$, 19.3 for S$_{70}$E, and 28.9 for S$_{70}$, showing a slightly faster degradation for spinosad in soil with earthworms respect to the soil alone, while the presence of earthworms did not influence the degradation of chlorpyrifos. Significant differences between the recovery rate of spinosad in the presence of earthworms (S$_{70}$E) and without earthworms (S$_{70}$) were found.

3.3.2. Effect on DNA of living cells

Fig. 4 shows TM, TL, and TI indexes measured during the toxicity test. Due to their not Gaussian distribution, comet assay data are represented as box plots where the box represent 50% of the data contained between the 25th and the 75th percentile, and the bars represent the upper and the lower quartiles of distribution. The median value is reported in the box by the line that divides the box into two parts.

A significant increase in DNA damage indexes was detected in chlorpyrifos treatment after one day of exposure. On day 21, an increase in all three DNA damage indexes was confirmed; nonetheless, significant differences in the distributions were recorded only for TL. At 28 days, it was impossible to analyse the test's genotoxic damage due to the absence of surviving earthworms. In contrast, spinosad appears to have

less impact on coelomocytes. In fact, DNA damage indexes became significant only at the end of the experiment.

3.3.3. Impact on soil bacterial community diversity

Hierarchical clustering of bacterial communities at the family level across all samples in this study is presented in Fig. 5 indicate the formation of clusters mainly as a function of time. Dynamic response of bacterial communities starting from the first day with the most significant impact on 7 and 14 days was observed. Community composition stabilised after the 14 days, and the group of taxa that contributed less than a 5 % "other" was predominant in all samples. On the 7 days, the impact of Bacillaceae was found to be highly pronounced within all the samples almost regardless of treatments and clusters were mostly driven by the families of; *Microbacteriaceae, Bacillaceae, Nocardioidaceae, Cytophagaceae* and unclassified Solirubrobacterales together with Bacteroidetes. Bacteria from *Chitinophagaceae, Sphingobacteriaceae, Rhodospirillaceae, Erythrobacteraceae, Sphingomonadaceae, Xanthomonadaceae,* families were significant in the formation of the clusters observed in the final samplings.

The impact of pesticides, earthworm's presence and their

Table 1
Observations on earthworms in the reproduction test.

PARAMETERS	TRT ctrlE	S$_{70}$E	S$_{150}$E	C$_{50}$E	C$_{70}$E
NO. COCOONS/ REPLICATE (± SD; 56 DAYS)	72.67 ±11.24[b]	70.00 ±9.00[b]	59.00 ±2.00[ab]	19.67 ±7.23[a]	4.67 ±8.08[a]
NO. JUVENILES/ REPLICATE (± SD; 56 DAYS)	159.00 ±20.07[b]	138.33 ±13.01[ab]	68.00 ±37.27[ab]	3.33 ±3.06[a]	NA
HATCHABILITY (% ±RSD;56 DAYS)	2.19 ±0.05[b]	1.98 ±0.03[ab]	1.14 ±0.52[ab]	0.15 ±0.87[a]	NA
GROWTH RATE (% ±RSD; 28 DAYS)	5.63 ±2.76[b]	-18.79 ±0.82[ab]	-19.95 ±0.52[ab]	-23.96 ±0.07[ab]	-42.40 ±0.08[a]
MORTALITY (% ±RSD; 28 DAYS)	NA	NA	3.33 ±1.73	10.00 ±1.00	40.00 ±0.25
UNUSUAL BEHAVIOUR	NA	NA	NA	✓	✓
MORPHOLOGICAL ANOMALIES	NA	NA	NA	✓	✓

According to Dunn's Kruskal–Wallis multiple comparisons, treatments with different lowercase letters were significantly different.
NA: not available
✓: presence of the parameter
Unusual behaviour: low reactivity, less digging activity, compulsive movements
Morphological anomalies: injuries, miniaturisation, abnormal colouring.

combination at the beginning (1d) and the end of the experiments (28d) is shown by multivariate canonical correspondence analysis (CCA) in Fig. 6. These findings confirmed what was observed with the taxonomical clustering of bacterial communities; marked differences caused by various treatments at the beginning of the experiments and fade-out phenomenon as time passed by. Earthworms presence was of utmost importance (p=0.007) for the clustering of bacterial communities in the presence of pesticides (Fig. 6 c).

At 28 days, the impact of pesticides alone (Fig. 6 d) has become insignificant (p=0.248), while the effect of the earthworms was still significant (p=0.035) (Fig. 6 e).

The Metastats analysis results singled out several bacterial OTUs of which abundances were significantly affected by the presence of the pesticides in this experiment at the beginning (Fig. 7, Left) and at the end of experiments (Fig. 7, Right). Significantly affected OTUs are indicated with significance letters in Fig. 7.

5. Discussion

In the present study, earthworms showed a tendency to avoid contaminated soils. However, only chlorpyrifos at the highest dose resulted in a net response rate above the 80 % threshold, indicating a harmful environment (ISO, 2008; Li et al., 2015). A concentration-dependent significant weight loss in chlorpyrifos treatments is in agreement with De Silva et al. (2010). The biomass also decreased in spinosad presence, but it was still less than in chlorpyrifos. Weight loss, a physiological stress index according to Van Gestel et al. (1995) and Frampton et al. (2006), is a dose and time dependent factor, which our results are in agreement with. Zhou et al. (2007) were unable to assess the earthworms reproductive activity due to deficient cocoon production at much lower doses of chlorpyrifos. Our results confirm the toxicity of chlorpyrifos on earthworm fertility. On the contrary, in spinosad treatments, reproduction output was not disturbed in agreement with Sekulić et al. (2020), although they worked with lower doses. The hatchability values further confirmed the less impact of spinosad compared to chlorpyrifos. In general the health status of earthworms and their reproductive capacity were closely correlated as reported by Robidoux et al. (2001).

Residual levels of chlorpyrifos were not affected by earthworms, agreeing with Sanchez-Hernandez et al. (2018a). Probably its high Koc cause chlorpyrifos absorption on soil organic matter easily (Mackay et al., 2006), making it poorly bioavailable (Megharaj et al., 2011). Chlorpyrifos was toxic to the soil bacterial community, and its persistence in the soil is also related to its limited biodegradation (Singh et al., 2002). Several authors (Racke et al., 1990; Coppola et al., 2007) found that antimicrobial properties of its metabolite TCP (3,5,6-trichloro-2-pyridinol) to be the main reason. Chlorpyrifos half-life in the present experiment was lower than those reported by Pesticide Properties Data Base, where it is classified between very persistent/persistent pesticides with a typical half-life value in the soil of 386 days and a range of 19.9-1000 days for a different type of soil. This difference could be due to the good organic carbon content, which contributed to adsorption on soil colloids and efficient microbial activity. The degradation of spinosad in its natural components occurs through a combination of processes, above all by photodegradation and microbial degradation (Tamez-Guerra et al., 2017). The half-life of this natural insecticide was
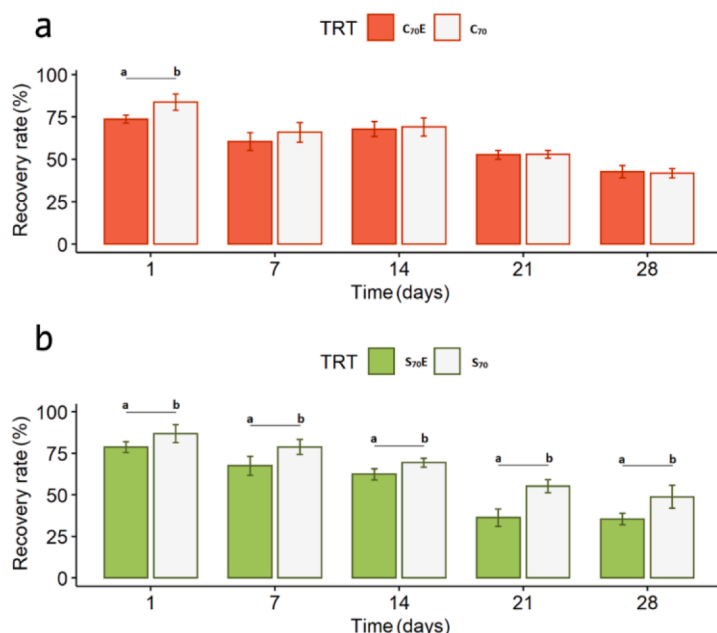
Fig. 3. Percent of insecticides residues in the soil respect to the initial concentration of 100 % **a)** Chlorpyrifos. Treatments (TRT) of chlorpyrifos up to 70 % of the LC$_{50}$ with earthworms (C$_{70}$E) and without earthworms (C$_{70}$). **b)** Spinosad. Treatments (TRT) of spinosad up to 70 % of the LC$_{50}$ with earthworms (S$_{70}$E) and without earthworms (S$_{70}$). Data were present as means ± RSD (n=3). Lowercase letters refer to significant differences between treatments at each sampling time according to Linear mixed-effects models multiple comparisons.
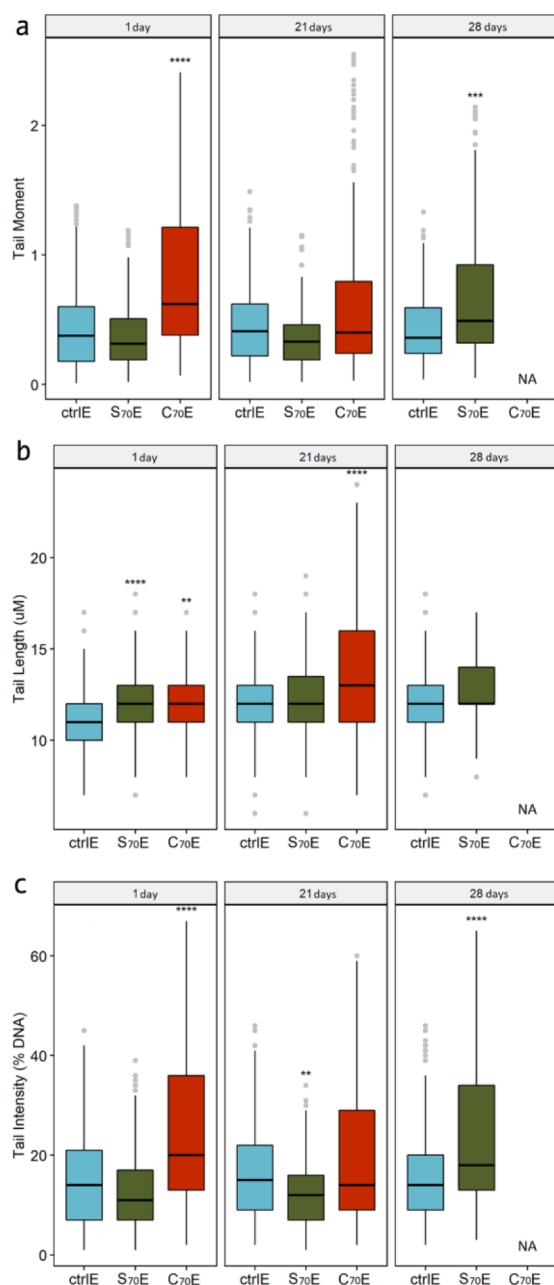
Fig. 4. Tail moment **(a)**, Tail Length **(b)** and Tail Intensity **(c)** in *E. foetida* coelomocytes. Treatments (TRT): ctrlE (negative control), $S_{70}E$ (spinosad up to 70 % of the LC_{50}), $C_{70}E$ (chlorpyrifos up to 70 % of the LC_{50}). Significance of variation was calculated versus unexposed control according to Dunn's Kruskal–Wallis multiple comparisons ($*p<0.05$; $**p<0.01$; $***p<0.001$; $****p<0.0001$).

measured between 9-10 days in case of soil photolysis or between 9-17 days in the absence of light (Thompson et al., 2000). The values found in the present experiment are in accordance with those reported in the bibliography, considering that the spinosad experiment was conducted in the dark and demonstrated its low persistence in soil with recovery values that declined consistently with time (Barden, 1998; Mandal and Singh, 2013; Telesiński et al., 2015). The presence of earthworms contributes to metabolising spinosad; probably, their digging activity permits more excellent aeration, a condition that allows a faster degradation of spinosad, according to Thompson et al. (2002).

Comet assay is a rapid and sensitive for the detection of DNA damage on the cell level and an essential biomarker in earthworm ecotoxicology

(Fourie et al., 2007; Mincarelli et al., 2019), but only a few papers worked on terrestrial habitat (Martin et al., 2005; Xiao et al., 2006). In the present study, chlorpyrifos compromised the DNA integrity of the coelomocytes immediately for all three indexes analysed (TM, TL and TI). Looking at TL, which is a better indicator of toxicity at low levels of DNA damage (Collins, 2004; Kumaravel et al., 2009), the negative effect of chlorpyrifos is confirmed at 21 days. Despite this, we observed an adaptive mechanism or a selection of resistant coelomocytes for TM and TI, leading to a lower level of toxicity, while at 28 days, a toxic effect with a dramatic decrease in viability was observed. Our data are in agreement with the limited set of studies on genotoxicity induced by chlorpyrifos on earthworms (Casabé et al., 2007; Piola et al., 2009; Curieses et al., 2018), where a significant increase in DNA damage in *E. foetida* coelomocytes treated with chlorpyrifos occurs. The data regarding the spinosad trial point to a lower and less acute toxicity in terms of organism viability and sub-lethal coelomocyte genotoxicity.

Community composition and relative abundances of soil bacteria in our study are in agreement with Liao et al. (2018), and the presence of *Cytophagaceae, Microbacteriace, Nocardioidaceae*, too, confirms the findings of the Schlatter et al. (2019) on the potential of earthworms as ecosystem engineers also affecting microbial communities. Some *Microbacteriaceae* and *Bacillaceae* are symbionts of earthworms (Tang et al., 2012; Aira et al., 2018), and the differences in abundances of these may indicate the passage from soil to the service of the earthworms. In contrast, the negative impact of chlorpyrifos on *Sphingomonas* sp was reported by Medo et al. (2015), but *Bacteroides* sp. and *Bacillus* sp. abundances were similarly lower in chlorpyrifos contaminated soil (Wang et al., 2019). These changes could be related to the fact that exposure to pesticides influences soil bacterial diversity and the gut community composition of earthworms by reducing energy resources and activating the antioxidant systems (Chang et al., 2021) and the immediate impact of the presence of spinosad and chlorpyrifos had on some of the OTUs may be related to pesticide degradation activities of; *Bacillus* sp. (Zeilinger et al., 2010; Oladipo et al., 2019; Narayanan et al., 2020; Zhang et al., 2020), Sphingomonas sp. (Kumar et al., 2021), *Pseudomonas* sp (Kumar et al., 2021), *Luteimonas* sp. (Liu et al., 2019; Elyamine and Hu, 2020; You et al., 2021). Furthermore, the omnipresent Sphingobacteriaceae, such as *Pedobacter composti/luteus* is a resource of secondary microbial metabolites without known chemistries, bio-activities and ecological roles (Figueiredo et al., 2021) in support of its important presence in Metastats. *Caulobacteraceae* sp. abundance at the 28d is in accordance with Schlatter (2019), in which earthworm presence in the soil was found to be beneficial. Our findings also agree with the only study in the literature on *Ilumatobacter* sp. by Vasileiandis *et al.* (2018) for the initial negative but transient impact of these insecticides. Overall, the effect of these insecticides at sub-lethal doses are in accordance with the recent review by Vischetti *et al.* (2020) regarding its dependence on time scale and adaptability of the microorganisms. To the best of our knowledge, the present study is the first to report the impact of spinosad and chlorpyrifos on *Intrasporangium* sp., *Saccharibacteria* sp. and *Phenylobacterium* sp.

4. Conclusions

The present study found that earthworms' behaviour, state of health and reproduction align with the damages at the DNA level. Chlorpyrifos caused a substantial morphological impairment and functional anomalies from the earliest samplings, while the impact of spinosad remained minimal. Former also negatively affected DNA integrity at early stages, and the degenerative trend led to the death of the later samplings. Whereas the latter's impact was significant only after 28 days of exposure. Time was the main factor for bacterial community changes, and then, treatments and earthworms' presence were important factors too, indicating the crucial role of *E. foetida* on the toxicity of the insecticides. The present work, by multi-technique approach, successfully identified the non-target impact of these insecticides at an early stage, reflecting
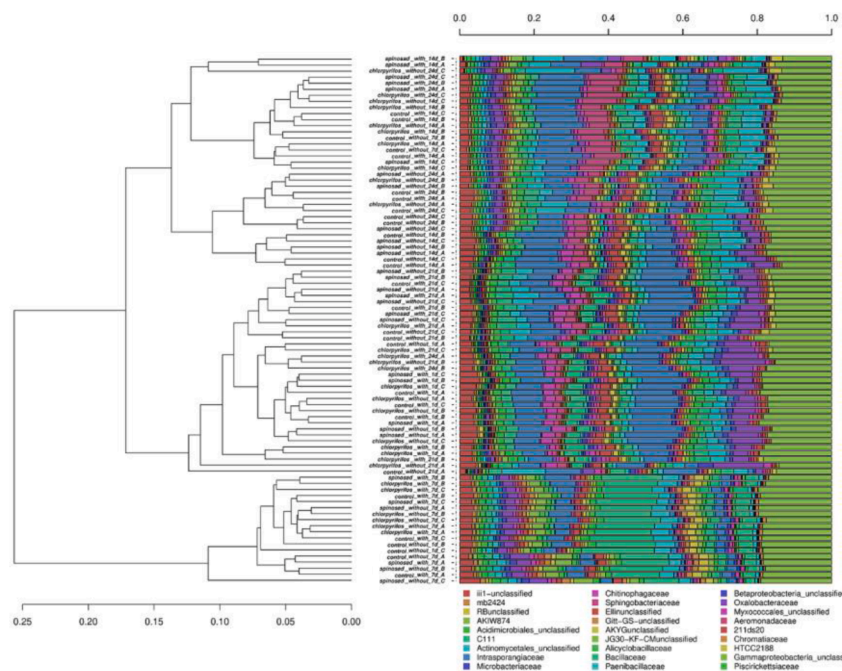
Fig. 5. Taxonomic comparison of soil bacterial communities at the family level through hierarchical clustering across all samples used in this study. Clusters were identified with the average linkage algorithm for taxa that contributed at least 5 % to a single sample. Taxa that contributed less than this threshold were added to the sequence group denoted "other."
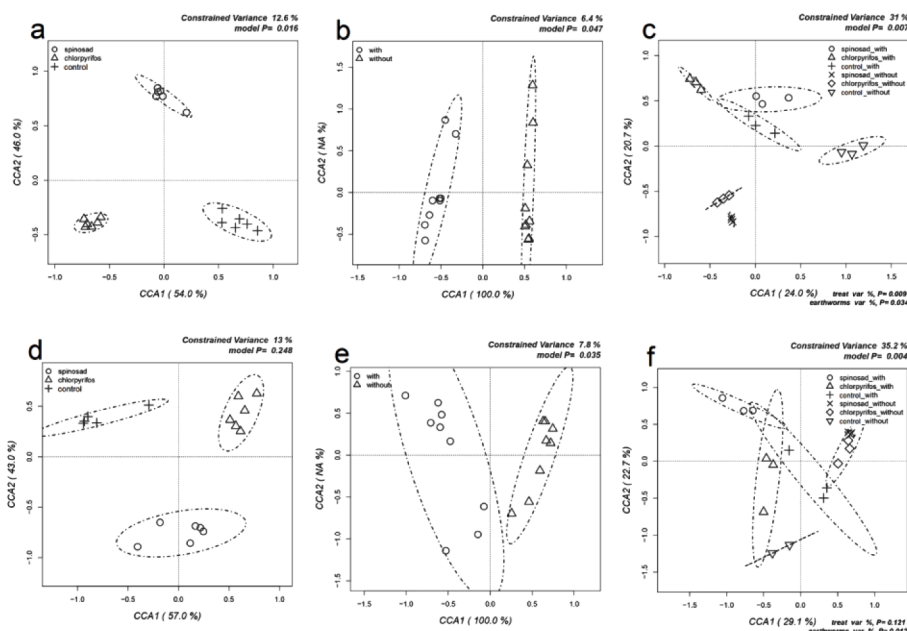
Fig. 6. Canonical correspondence analyses (CCAs) on the impact of the various factors on the structure of soil bacterial communities; pesticides **(a,d)**, earthworms **(b,e)**, pesticides and earthworms together **(c,f)** by days after treatment (**1d:** upper half, **28d:** lower half). These were determined by the relative abundances of all the OTUs obtained by Illumina sequencing of bacterial 16S amplicons.

the ecosystem's health status and therefore sets an example to future studies on how to estimate the potential real environmental impact of pesticides.

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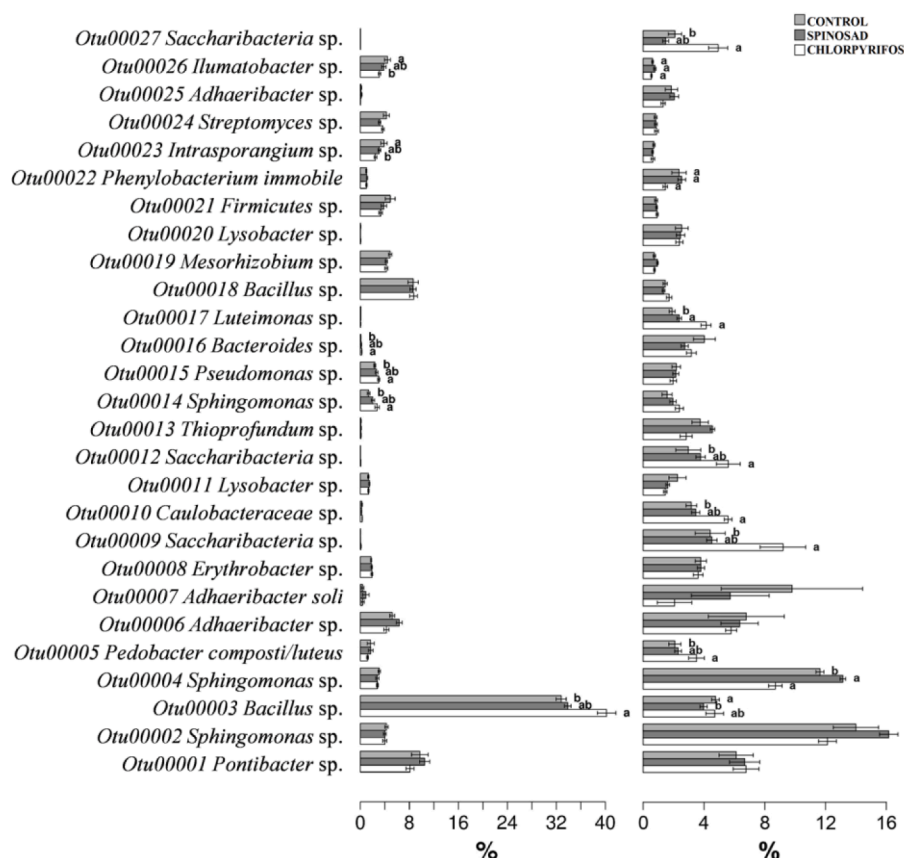
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Fig. 7. Bacterial OTUs as analysed by a Metastats aimed at identifying the ones with significant differences (letters or *, p ≤ 0.05) between pesticides treatments (Left: First sampling at 1d, Right: Final sampling at 28d).

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Declaration of Competing Interest

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

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2022.100225.

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