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Antimicrobials in *Eisenia fetida* earthworms: A comprehensive study from method development to the assessment of uptake and degradation

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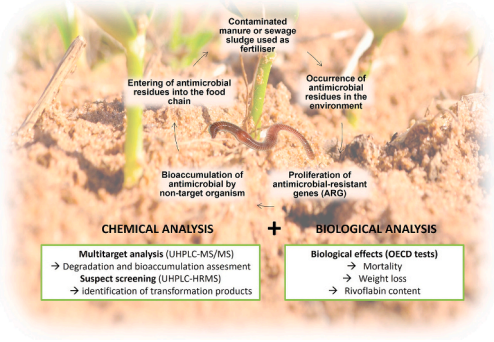
HIGHLIGHTS

- An analytical method for antimicrobial determination in earthworms was validated.
- The accumulation of sulfamethazine and tetracycline in earthworms was confirmed.
- 8 transformation products were identified in soil/earthworms for the first time.
- The formation/degradation trend over time of the transformation products was studied.
- Sulfamethazine and tetracycline disturb the immune system of earthworms.

GRAPHICAL ABSTRACT



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ABSTRACT

In this work, an accurate analytical method was developed for the simultaneous analysis of twenty-seven antimicrobials (AMs) in earthworms using liquid chromatography coupled to a triple quadrupole mass spectrometry detector (UHPLC-MS/MS). Adequate apparent recoveries (80–120 %) and limits of quantification (LOQ) (1 $\mu g \cdot kg^{-1}$ - 10 $\mu g \cdot kg^{-1}$) were obtained, with the exception of norfloxacin (34 $\mu g \cdot kg^{-1}$). The method was applied to evaluate the accumulation of sulfamethazine (SMZ) and tetracycline (TC) in earthworms after performing OECD-207 toxicity test, in which *Eisenia fetida* (*E. fetida*) organisms were exposed to soils spiked with 10 $mg \cdot kg^{-1}$, 100 $mg \cdot kg^{-1}$ or 1000 $mg \cdot kg^{-1}$ of SMZ and TC, individually. The results confirmed the bioaccumulation of both AMs in the organisms, showing a greater tendency to accumulate SMZ since higher bioconcentration factor values were obtained for this compound at the exposure concentrations tested. In addition, the degradation of both AMs

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in both matrices, soils and earthworms was studied using liquid chromatography coupled to a q-Orbitrap high resolution mass spectrometry detector. Thirteen transformation products (TPs) were successfully identified, eight of them being identified for the first time in soil/earthworm (such as 4-Amino-3-chloro-n-(4,6-dimethylpyrimidin-2-yl)benzenesulfonamide or 4-(dimethylamino)-1,11,12a-trihydroxy-6,6-dimethyl-3,7,10,12-tetraoxo-3,4,4a,5,5a,6,7,10,12,12a-decahydrotetracene-2-carboxamide, among others) and their formation/degradation trend over time was also studied. Regarding the biological effects, only SMZ caused changes in earthworm growth, evidenced by weight loss in earthworms exposed to concentrations of 100 mg·kg^{-1} and 1000 mg·kg^{-1}. Riboflavin decreased at all concentrations of SMZ, as well as at the highest concentration of TC. This indicates that these antibiotics can potentially alter the immune system of *E. fetida*. This research represents a significant advance in improving our knowledge about the contamination of soil by AM over time. It investigates the various ways in which earthworms are exposed to AMs, either by skin contact or ingestion. Furthermore, it explores how these substances accumulate in earthworms, the processes by which earthworms break them down or metabolise them, as well as the resulting TPs. Finally, it examines the potential effects of these substances on the environment.

1. Introduction

Earthworms constitute a large part of the biomass of the soil fauna and play an essential role in the proper functioning of the ecosystem. However, the permeable gut and cuticle of these organisms make them susceptible to the accumulation of contaminants in the soil. This susceptibility to chemicals, along with other biological advantages (short life cycle, the possibility to assess toxicities via ingestion or dermal exposure, the large battery of developed biomarkers…) made earthworms suitable model organisms for terrestrial ecology (Ashworth et al., 2023; Urionabarrenetxea et al., 2021; Wen et al., 2011; Yang et al., 2020).

Earthworms' immune system consists of coelomocytes, which mediate innate immunity and are mainly composed by two cell types: amebocytes and eleocytes (Plytycz et al., 2009). Amebocytes play roles in phagocytosis and cytokine production, while eleocytes are essential for the accumulation of contaminants and particles (Bilej et al., 2010). In addition, eleocytes contain autofluorescent riboflavin, which deters predators when earthworms are attacked (Pes et al., 2016) and also has antioxidant properties (Plytycz et al., 2009). Different pollutants can alter the integrity and functions earthworm coelomocytes, so measuring responses at cellular level may be useful as biomarker of sublethal exposure (Correia et al., 2021).

Antimicrobials (AMs) are molecules used to treat bacterial diseases in human and veterinary treatments (Yang et al., 2020). However, their overuse has resulted in the presence of AMs in different untargeted environmental matrices, which is of global concern because of their association with the emergence of AM-resistant strains. Despite the synthesis of the large number of new molecules during the last decades, bacteria developed many different mechanisms of AM resistance (AMR). In fact, in 2019, due to its impact on human health, the World Health Organisation (WHO) included AMR as one of the top ten global health threats (*Ten threats to global health in 2019*, s. f.).

In recent years, high levels of AMs have been detected in soils (Hu et al., 2022), with sulfonamides and tetracyclines being some of the most frequently detected families (Conde-Cid et al., 2020). AMs are highly biodegradable under different light or temperature conditions, leading to the formation of new transformation products (TPs). The main pathways by which AMs and their TPs reach soils are the application of animal excreta and discharges of inefficiently treated domestic and medical wastewaters (Carter et al., 2021; Yang et al., 2020), as well as soil fertilisation with sewage sludge from wastewater treatment plants (Golet et al., 2002).

The determination of AMs in earthworms is still a field to be further investigated. A precise and thorough extraction process is essential to accurately analyse this complex biological matrix. This, coupled with the diverse physicochemical properties of the different AM families, adds complexity to the analysis (Moreno-Bondi et al., 2009). In addition, given the presence of numerous enzymatic processes taking place in earthworm tissues, it has been reported that they are capable of transforming organic compounds into new TPs (Saranraj and Stella, 2012). However, little is known about the degradation of AM compounds in the organism, their effects at the organism level and their possible biomagnification in the food chain (Drzymała and Kalka, 2023; Kinney et al., 2008; Pino et al., 2015). This issue highlights the importance of conducting a comprehensive risk assessment. To this end, it is crucial to develop analytical methods that can detect trace amounts of AMs and monitor the formation of TPs in earthworms (Manasfi et al., 2021). By establishing this relationship with the observed biological effects, we can better understand and address the risks involved. However, most of the scientific work found in the literature is based on the latter, while those focussed on method development and validation for analytical purposes are still scarce (Bergé and Vulliet, 2015; Manasfi et al., 2021; Montemurro et al., 2021).

Within this context, the present work focused in a comprehensive study of the AMs in earthworms. To get that main goal, first, an accurate analytical method was developed for the simultaneous determination of twenty-seven AMs (i.e., five sulfonamides, four tetracyclines, four macrolides, nine (fluoro)quinolones, one imidazole and one nitroimidazole, one triazole, one diaminopyridine, one derivative of *Penicillium stoloniferum* and three antifungals) in earthworms. Extraction and clean-up steps were thoroughly optimised to get high extraction recoveries and to minimise matrix effects using liquid chromatography coupled to a low-resolution triple quadrupole mass analyser (UHPLC-MS/MS). To our knowledge, an analytical method that covers the analysis of such a variety of AMs in earthworms in a single run has never been proposed. Afterwards, the potential bioaccumulation of AMs in earthworms via dermal route or via ingestion of contaminated soil was evaluated through exposure experiments. Target AMs were monitored and we look for TPs in soils and earthworms itself using liquid chromatography high resolution mass-spectrometry (UHPLC-HRMS). The analytical results were finally correlated with the biological effects including mortality, weight loss, and changes in riboflavin content observed during the exposure experiments.

2. Experimental procedure

2.1. Reagents and materials

Table S1 collects the physicochemical properties of the target AMs and surrogate standards and the commercial supplier from which they have been acquired. Monthly prepared individual solutions (1000–3000 mg·kg^{-1}) in UHPLC-grade methanol (MeOH, 99.9 %, Scharlau, Sentmenat, Catalonia, Spain), UHPLC-grade acetonitrile (ACN, 99.9 %, Avantor Performance Materials, Gliwice, Silesia, Poland) or dimethyl sulfoxide (DMSO, Panreac AppliChem, Darmstadt, Germany) (see Table S1) were stored at −20 °C. Fluoroquinolones individual solutions required the addition of a NaOH (2 M) (99 %, Merck, Darmstadt, Hesse, Germany) (Hang et al., 2021; Wei et al., 2016). Combined dilutions of 100 mg·kg^{-1} and 5 mg·kg^{-1} were weekly prepared in ACN for sample

spiking and were both kept at 4 °C using silanised amber vials (Burhenne et al., 1999).

The extraction salts mixture consisted on NaCl (100 %), acquired from PanReac AppliChem (Castellar del Vallés, Catalonia, Spain), anhydrous citric acid H_3Cit (99.5 %) and anhydrous disodium hydrogen phosphate (Na_2HPO_4, 98 %), obtained from Scharlau, and anhydrous disodium sulphate (Na_2SO_4, 99 %) from Merck. UHPLC-grade ACN was used as extractant. Oasis HLB cartridges (500 mg, 6 cm^3, 30 μm) purchased from Waters (Milford, Massachusetts, USA) were employed in the clean-up step. Anhydrous sodium dihydrogen citrate (NaH_2Cit, 99 %) and disodium hydrogen citrate ($Na_2HCit{\cdot}1.5H_2O$, 99 %) (Honeywell Fluka, Charlotte, North Carolina, USA) were used to prepare a citrate buffer for sample dilution before loading to the SPE cartridges. For the final reconstitution of the sample oxalic acid (100 %, Merck) was used.

Along the sample treatment procedure, a Multi Reax shaker by Heidolph (Schwabach, Bavaria, Germany) and a 5840R centrifuge by Eppendorf (San Sebastián de Los Reyes, Madrid, Spain) were used.

2.2. Sample treatment

The extraction of AMs from earthworms was performed using the conditions optimised for manure samples in a previously published work by the research group (Vergara-Luis et al., 2023b) with slight modification. Briefly, 2 g of crushed and homogenised earthworms (the whole earthworm body was taken as sample) were spiked directly with a solution containing the twenty-seven AMs. Samples were extracted using 320 μL of UHPLC-water, 5 mL of ACN, 2 g of anhydrous Na_2SO_4, 0.5 g NaCl, 0.25 g anhydrous H_3Cit and 0.025 g Na_2HPO_4. A ceramic homogeniser was added, and then the mixture was manually stirred and degasified until no gas was released. Finally, all samples were vortexed (2000 cycles·min^{-1}, 8 min) and centrifuged (4000 cycles·min^{-1}, 5 min) at 10–15 °C.

The SPE technique was used to perform the sample clean-up step, using OASIS HLB 500 mg cartridges. For this procedure, the sample volume (i.e., 1 mL, 2 mL or 4 mL of the sample extract) to be loaded into the SPE cartridge, was optimised. Under optimal conditions, a 1 mL aliquot of the extraction supernatant was diluted to 20 mL with the citrate buffer (pH = 4) and loaded to 500 mg Oasis HLB cartridges, previously conditioned with 10 mL of ACN, 10 mL of Milli-Q water and 10 mL of citrate buffer (pH = 4). The cartridges were washed with 5 mL of water and dried under vacuum. Compounds were eluted using 9 mL of ACN and the extracts were evaporated to 1 mL in TurboVap LV evaporator device with a nitrogen-flow (Caliper Life Sciences, Hopkinton, Massachusetts, USA). Aliquots of 125 μL were reconstituted in 250 μL of 50:50 (v:v) ACN:oxalic acid (aq., 0.01 mol·L^{-1}, pH 2) and filtered through 0.22 μm polypropylene filters (Clarify-PP, Phenomenex, Torrance, California, USA) before UHPLC analysis.

2.3. Analysis: UHPLC-MS/MS and UHPLC-HRMS

An Agilent 1290 Infinity II UHPLC coupled to Agilent 6430 Triple Quad tandem mass-spectrometer (QqQ), from Agilent Technologies, and a Thermo Scientific-Dionex UltiMate 3000 UHPLC coupled to a Thermo Scientific-Q Exactive Focus quadrupole-Orbitrap mass spectrometer were used for multitarget analysis and suspect screening, respectively. The optimal conditions were previously studied by the research group (Vergara-Luis et al., 2023c) and are gathered in Sections 1 and 2 of the Supplementary material.

2.4. Method validation

The optimised methodology was validated in terms of instrumental and intermediate repeatability, linearity, instrumental limits of quantification (LOQ_{INS}), matrix effect at the detection, trueness, precision and procedural limits of quantification (LOQ_{PROC}) to meet the requirements set forth in the March 2021 regulation (Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 on the Performance of Analytical Methods for Residues of Pharmacologically Active Substances Used in Food-Producing Animals and on the Interpretation of Results as Well as on the Methods to Be Used for Sampling and Repealing Decisions 2002/657/EC and 98/179/EC (Text with EEA Relevance), 2021) and the Eurachem Guide (Magnusson and Örnemark, 2014).

For the assessment of instrumental parameters, a calibration curve was built in the range of 0.1–100 μg·kg^{-1}. Instrumental and intermediate repeatability were assessed by calculating the relative standard deviation (RSD %) between injection replicates (n = 3), in the same day and in different days, respectively, using the calibration solutions between 0.1 and 25 μg·kg^{-1}. LOQ_{INS} were defined as the lowest calibration point with a RSD % and a systematic error of less than the 30 %. Linearity ranges were defined considering the determination coefficients (r^2) of the calibration curves built between LOQ_{INS} and upper limit. The effect of the matrix on detection was studied by spiking matrix extracts at a concentration of 50 μg·kg^{-1} and comparing the signal obtained with that of a reference standard of the same concentration level, according to Eq. (1).

$$Matrix\ effect\ (\%) = \left(\frac{Area_{sample}}{Area_{reference}} - 1\right) \times 100 \quad (1)$$

For trueness determination, earthworm samples were spiked at concentration levels of 10 μg·kg^{-1}, 25 μg·kg^{-1} and 50 μg·kg^{-1} and absolute and apparent recoveries were calculated, using surrogate correction approach in the latter case. Precision was assessed by the calculation of the RSD % value of procedural replicates (n = 3), as for LOQ_{PROC}, the average signal of the procedural blanks plus ten times their standard deviation was considered and determined using the external calibration and the absolute recovery for each analyte in the matrix.

2.5. Uptake assays of SMZ and TC in earthworms

E. fetida earthworms were purchased from a commercial supplier (Lombricor S.C.A., Almeria, Spain) and maintained under constant temperature (20 °C) and humidity (60 %) conditions. Once a week, the earthworms were fed with AM-free horse manure, as previously proven by (Vergara-Luis et al., 2023b). Adult clitellate individuals were used in the experiments, with similar size and individual weight in the range of 300 to 400 mg.

2.5.1. OECD-207 (1984): filter paper test

To evaluate the absorption of AMs through dermal contact, the filter paper test was carried out with *E. fetida* earthworms strictly following OECD-207 (1984) standard guidelines (OECD, 1984). Briefly, a filter paper was moistened with 1 mL containing 5 mg·L^{-1}, 50 mg·L^{-1}, 500 mg·L^{-1}, 1000 mg·L^{-1} SMZ and TC, individually. Each filter paper was placed in a glass vial and a single earthworm was introduced in each vial, having a total of ten earthworms per treatment. Control sample was moistened with Milli-Q water. According to the guideline, the exposure time was initially set at 48 h, and was extended to 72 h due to the absence of observed mortality. At the end of the experiment, earthworms were collected and submitted to biological and chemical analysis.

2.5.2. OECD-207: soil toxicity test

Soil samples were prepared in accordance with OECD-207 (1984) - Guidelines for the Testing of Chemicals (OECD, 1984). The soils were composed of 70 % sand, 20 % clay, 10 % peat (sieved to 2 mm) and 0.01 % calcium carbonate adjusted to pH 7 ± 0.05. Subsequently, the soils were placed in 1 L glass containers and wetted to 40 % of their water holding capacity (WHC), to reach a wet weight of 750 g. Wetting was carried out with the respective stock solutions, containing Milli-Q water (CTL), Milli-Q water with DMSO (CTL DMSO) or the stock solutions used for spiking the soils at 10 mg·kg^{-1}, 100 mg·kg^{-1} and 1000 mg·kg^{-1} of

SMZ or TC. Establishing this concentration range, it was aimed to assess the transfer of AMs and their TPs to earthworms, as well as possible toxicity to organisms, and to elucidate whether these factors are dependent on the concentration of the AMs. Ten earthworms previously cleaned with Milli-Q water were weighed, added to each container and exposed for 14 days, using a total of forty earthworms per treatment. The experiment was conducted at 20 °C, humidity (60 %) and constant light conditions. The earthworms were not fed during the experiment. At the end of the experiment, earthworms were collected from each container and submitted to biological and chemical analysis. Regarding soils samples, aliquots were collected on days 7 and 14 of the experiment to evaluate the possible degradation of the target AMs in the soil.

2.5.3. Chemical analysis

The method validated in this work was applied for earthworms' analysis after both OECD-207 tests, using UHPLC-MS/MS for multitarget analysis and UHPLC-HRMS for the identification of TPs in the organism following the analysis criteria mentioned in Section 2.3.

Quality control samples were prepared to accurately determine the concentration of AMs in OECD soils through the experiment. For this purpose, three replicates of OECD soils spiked at 50 $\mu g \cdot kg^{-1}$ and three matrix blanks were prepared and extracted with the method validated for manure samples in a previous work (Vergara-Luis et al., 2023b) using UHPLC-MS/MS. The accuracy of the method for determining AMs in this matrix was determined in terms of trueness and precision. As for earthworms, the identification of TPs in soils was performed analysing the samples (day 7 and day 14) by UHPLC-HRMS.

2.5.4. Biological analysis

After the OECD-207 tests performance, the earthworms were counted to verify effects on survival and weighed to evaluate the loss or gain of body weight. Thus, the difference between the initial and final weight of the animals was calculated. Before weighing, the earthworms in each flask were cleaned with Milli-Q water and dried with paper- in order to avoid water excess that could interfere weighing. The results were converted into percentages relative to the mean weight of the control group.

In addition, riboflavin content was measured. For this purpose, three earthworms were selected from each replicate after the 14-day exposure, cleaned with Milli-Q water and massaged (following Asensio et al., 2007) to remove the soil present in the digestive tract. Afterwards, using a 9 v battery, the earthworm coelomocytes were extruded into a glass Petri dish, aided by 3 mL of extrusion solution (NaCl (0.23 %), EDTA (0.02 %) and PBS, pH 7 ± 0.2) (Irizar et al., 2014). Immediately afterwards, 10 μL of the solution containing coelomocytes were introduced into a Neubauer chamber to count the cells using a microscope, for subsequent adjustment of the cell concentration to 1 × 106 cells/ml. Then, 200 μL of the adjusted solution were pipetted in a 96-well plate. The fluorescence of eleocytes containing riboflavin was measured using a microplate reader (FL × 800) at 490 ± 20 nm excitation and 520 ± 20 nm emission wavelengths. Riboflavin concentration was calculated based on a standard curve, with points ranging from 0 to 32 $\mu g \cdot mL^{-1}$. The results were converted into relative percentages of the riboflavin amount detected in the control group. Riboflavin content was also expressed as a function of body weight, according to Rorat et al. (Rorat et al., 2016). Data normality was tested using the Shapiro-Wilk test and homogeneity using Bartlett. One-Way ANOVA was used followed by the Tukey test. One-way ANOVA was used to test whether any of the means of the different dosing-days are statistically different from each other. After that Tukey post hoc test was used in order to determine which specific dosing-day differed from each other. Concretely, we tested the null hypothesis of equal means of the analyte concentration in every day versus the alternative hypothesis of one or more concentration means are different from the others. Differences were considered significant at a 95 % confidence level ($p < 0.05$).

2.6. Data treatment

2.6.1. UHPLC-MS/MS analysis

Agilent MassHunter Workstation software (Quantitative Analysis for QQQ, 10.0 version) from Agilent was used for the data treatment acquired from the UHPLC-MS/MS analysis. According to the Council Directive 96/23/EC, the presence of the target compounds was confirmed by the comparison of the experimental retention time with a reference standard considering the two most specific transitions of each target compound (2002/657/EC, 2002).

2.6.2. UHPLC-HRMS analysis

Data collected by means of UHPLC-HRMS was treated using the Compound Discoverer 3.3 (Thermo-Fisher Scientific) and following a suspect screening approach. After withdrawing the baseline noise using ACN blanks as reference, the identification of candidates was carried out based on the following criteria: i) a minimum peak area equal to or >10,000; ii) a Lorentzian chromatographic peak shape; iii) exact masses with an error of less than ±5 ppm; iv) candidate presence in the suspect list, which includes AMs and TPs, generated with the BioTransformer 3.0 software. Studying different transformation reactions, nine suspect lists were prepared for SMZ with a total of 1266 TPs, as for TC seven lists with 360 TPs in total; v) a match higher than the 70 % (SFit >70 %) between the experimental and theoretical MS1 to consider the molecular formulas suggested by Compound Discoverer for annotation; vi) a ratio of three between the compounds signal in the sample respect to the blank; vii) a relative standard deviation lower than 30 % within replicates; viii) molecular formulas containing heteroatoms (O, N, Cl, Br, S and/or F); ix) a match higher than the 70 % between the experimental MS2 and the one in mzCloud database (https://www.mzcloud.org/) and/or the one *in-silico* predicted by the Mass Frontier Spectral Interpretation Software (Thermo-Fisher Scientific).

If aforementioned criteria were met, the candidates' retention times were compared to the ones estimated from the Retention Time Index (RTI) platform (http://rti.chem.uoa.gr/) and they were rejected or accepted depending on whether or not there was a statistical difference with the estimated value within the uncertainty of the built model (only box 1 and box 2 candidates were considered). The confidence level established by Schymanski et al. (Schymanski et al., 2014) was used for compounds' annotation and only those candidates annotated at a confidence level between 1 (candidate confirmed by MS1, MS2 and retention time) and 3 (candidate confirmed by MS1 and *in-silico* MS2) were reported.

3. Results and discussion

3.1. Suitability of sample treatment

In order to enhance the sensitivity of the analytical method, we have built on a previous study (Vergara-Luis et al., 2023b) to investigate the effects of loading 1 mL, 2 mL or 4 mL of sample extract into the 500 mg-SPE cartridges. The 4 mL-volume was discarded due to clogging of the cartridges by the high presence of matrix components. Regarding the other two tested volumes (i.e., 1 mL and 2 mL), no statistically significant differences were found in the absolute recoveries of the analytes ($t = 0.29 < t_{crit} = 2.01$). However, when 2 mL were loaded, a shift of the peak retention times, a broadening of chromatographic peaks or even, in some cases, a splitting of the chromatographic peaks was observed (see Fig. S1). Therefore, 1 mL was established as the optimal sample loading volume.

3.2. QA/QC parameters for the determination of AM in earthworms

3.2.1. LOQ_{INS}, linearity, repeatability and LOQ_{PROC}

The LOQ_{INS} determined for the AMs studied in this work are in the concentration range of 0.1–1.5 $\mu g \cdot kg^{-1}$. All the target AMs showed a

Table 1
LOQ_{PROC}, absolute recoveries, apparent recoveries and precision (n = 3), expressed as RSD %, of the target analytes in earthworms.

Antimicrobials	LOQ_{PROC} (μg·kg^{-1})	10 μg·kg^{-1}		25 μg·kg^{-1}		50 μg·kg^{-1}	
		R_{abs} % (RSD %)	R_{app} % (RSD %)	R_{abs} % (RSD %)	R_{app} % (RSD %)	R_{abs} % (RSD %)	R_{app} % (RSD %)
Sulfadiazine[a]	2	89 (5)	94 (3)	88 (6)	92 (4)	86 (5)	85 (4)
Sulfamethazine[a]	2	93 (8)	99 (7)	88 (8)	92 (2)	92 (11)	91 (2)
Sulfamethoxazole[b]	3	81 (5)	99 (5)	79 (5)	99 (9)	82 (6)	97 (5)
Sulfapyridine[a]	2	77 (5)	94 (1)	77 (5)	96 (6)	78 (6)	93 (5)
Sulfathiazole[a]	4	46 (3)	56 (1)	45 (5)	57 (8)	47 (9)	56 (8)
Thiabendazole[c]	3	43 (8)	85 (1)	45 (9)	106 (12)	46 (5)	108 (1)
Trimethoprim[c]	1	56 (3)	91 (6)	54 (4)	87 (3)	58 (4)	87 (2)
Ciprofloxacin[d]	4	22 (16)	90 (25)	23 (6)	95 (13)	30 (13)	102 (13)
Danofloxacin[c]	3	56 (6)	91 (3)	54 (3)	87 (4)	55 (5)	83 (5)
Enoxacin[c]	10	25 (10)	128 (16)	22 (8)	109 (5)	20 (10)	119 (5)
Enrofloxacin[c]	2	73 (12)	94 (14)	72 (6)	88 (3)	76 (4)	93 (3)
Fluconazole[c]	6	78 (2)	95 (5)	74 (10)	93 (6)	81 (5)	96 (4)
Flumequine[c]	2	91 (1)	121 (1)	91 (4)	126 (4)	97 (5)	123 (1)
Lomefloxacin[c]	3	54 (6)	87 (5)	55 (5)	88 (3)	56 (3)	84 (3)
Mycophenolic acid[b]	2	62 (4)	80 (7)	61 (5)	75 (3)	59 (4)	72 (11)
Norfloxacin[c]	34	–	–	–	–	23 (26)	124 (16)
Ofloxacin[c]	2	65 (6)	105 (5)	62 (1)	100 (3)	62 (1)	94 (4)
Pefloxacin[c]	3	63 (8)	81 (12)	58 (10)	72 (8)	71 (17)	86 (10)
Azithromycin[e]	3	10 (4)	83 (4)	10 (3)	82 (4)	10 (9)	76 (3)
Clarithromycin[e]	2	10 (4)	80 (3)	10 (4)	84 (1)	10 (8)	76 (3)
Erythromycin[e]	9	39 (19)	63 (20)	55 (6)	87 (4)	71 (13)	100 (7)
Roxithromycin[e]	10	–	–	14 (7)	91 (11)	14 (14)	92 (16)
Tetracycline[f]	2	36 (25)	105 (13)	47 (7)	114 (12)	45 (2)	107 (4)
Oxytetracycline[f]	3	23 (20)	73 (18)	23 (12)	75 (18)	24 (7)	76 (26)
Doxycycline[f]	3	47 (7)	95 (2)	53 (7)	127 (1)	47 (7)	114 (8)
Chlortetracycline[f]	3	28 (7)	73 (27)	32 (16)	95 (13)	30 (4)	70 (1)
Miconazol[c]	3	24 (5)	111 (1)	20 (13)	101 (10)	21 (9)	128 (14)

[a] [$^{2}H_4$]-Sulfamethazine.
[b] [$^{13}C_6$]-Sulfamethoxazole.
[c] [$^{2}H_5$]-Enrofloxacin.
[d] [$^{2}H_8$]-Ciprofloxacin.
[e] [$^{2}H_7$]-Roxithromycin.
[f] [$^{2}H_6$]-Tetracycline.

linear trend over the concentration range established for the calibration (LOQ_{INS}-100 μg·kg^{-1}), obtaining coefficients of determination higher than 0.9981 (Table S3). Regarding repeatability, an RSD % of 20 % between injection replicates (n = 3) analysed in the same day (instrumental repeatability) and in different days (intermediate repeatability) was set as the limit for considering adequate repeatability. Compliance with the established criteria was achieved at the concentration set as LOQ_{INS} for each AM and the following concentrations included in the calibration.

The determined LOQ_{PROC} are gathered in Table 1. The results ranged between 1 μg·kg^{-1} to 10 μg·kg^{-1}, with the exception of norfloxacin (34 μg·kg^{-1}). Comparing with the LOQ_{PROC} values reported by Bergé et al. lower values have been determined in this work for sulfamethoxazole, sulfathiazole and roxithromycin (3 μg·kg^{-1}, 4 μg·kg^{-1} and 10 μg·kg^{-1} vs 8 μg·kg^{-1}, 8 μg·kg^{-1} and 16 μg·kg^{-1}, respectively), while they obtained better results for erythromycin (9 μg·kg^{-1} vs 2 μg·kg^{-1}). Similar values were reported for sulfadiazine and sulfamethazine (Bergé and Vulliet, 2015). Montemurro et al. obtained LOQ_{PROC} values ranging from 0.7 to 1 μg·kg^{-1}. However, a direct comparison of the values is not possible because the calculation methods differ; they determined the LOQ_{PROC} using linear regression analysis on matrix-matched calibration curves (Montemurro et al., 2021).

3.2.2. Matrix effect on the detection

Fig. S2 shows the obtained matrix effect on the detection for each of the studied AMs. Overall, signal suppression was observed, although it is not significant (<26 %) for most of the studied analytes in exception of miconazole and the macrolides, which showed a strong signal suppression between −44 % and −88 %. Bergé et al. also observed an overall negative matrix effect on the detection when analysing AMs in earthworms even if they performed a dispersive clean-up step using a sorbent mixture consisting on PSA (Primary Secondary Amine) and C_{18}-bonded silica (Bergé and Vulliet, 2015). This may be due to co-elution of lipophilic matrix components, such as neutral and acidic phospholipids, which have been widely recognised as causing ion suppression under ESI conditions in biological sample extracts (Montemurro et al., 2021; Wu et al., 2013; Ye et al., 2011).

3.2.3. Trueness and precision

Absolute recoveries (Table 1) ranged from 10 % to 97 % at the three concentration levels (10 μg·kg^{-1}, 25 μg·kg^{-1} and 50 μg·kg^{-1}) set for validation, with the lowest values obtained for macrolides and miconazole (10–24 %). Overall, apparent recoveries of 80–120 % were obtained (Table 1) using isotopically labelled compounds as surrogates in accordance with the March 2021 regulation (Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 on the Performance of Analytical Methods for Residues of Pharmacologically Active Substances Used in Food-Producing Animals and on the Interpretation of Results as Well as on the Methods to Be Used for Sampling and Repealing Decisions 2002/657/EC and 98/179/EC (Text with EEA Relevance), 2021). As an exception, lower apparent recoveries (56–76 %) were obtained for two tetracyclines and sulfathiazole, and higher apparent recoveries (121–128 %) for two fluoroquinolones. Although those analytes were outside the range established by the guideline 2021/808 (Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 on the Performance of Analytical Methods for Residues of Pharmacologically Active Substances Used in Food-Producing Animals and on the Interpretation of Results as Well as on the Methods to Be Used for Sampling and Repealing Decisions 2002/657/EC and 98/179/EC (Text with EEA Relevance), 2021), precision, in terms of repeatability and expressed as RSD %, at all tested concentrations was <20 % for all AMs, with the exception of ciprofloxacin and chlortetracycline at 10 μg·kg^{-1}.

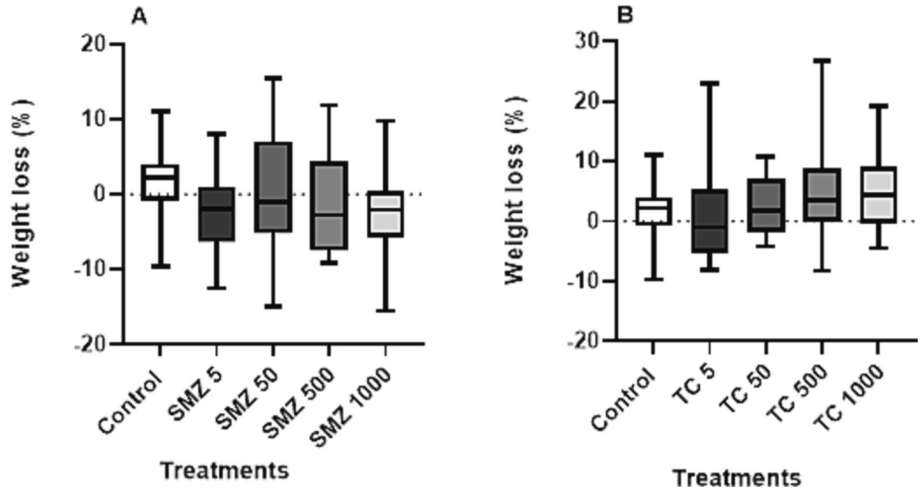

Fig. 1. Weight loss (in %) of *E. fetida* earthworms after dermal exposure to different SMZ (A) and TC (B) concentrations (0–1000 mg·kg^{-1}).

Therefore, the method was still considered reproducible and reliable for the analysis of the target AMs at the three tested concentration levels, according to SANTE/11813/2017 regulation (European Commission, directorate general for health and food safety, s. f.). However, due to the LOQ_{PROC} of roxithromycin (10 μg·kg^{-1}) and norfloxacin (34 μg·kg^{-1}), those AMs could only be validated in this work at 25 μg·kg^{-1} and 50 μg·kg^{-1} and at 50 μg·kg^{-1} levels, respectively. Comparing with the literature, this method could more accurately perform the determination of thiabendazole and trimethoprim than Kinney et al. given the recovery values they obtained after surrogate correction for these AMs, 36 % and 42 %, respectively (Kinney et al., 2008). The method attained higher recoveries for sulfonamides and macrolides compared to those obtained in the method developed by Bergé et al. (Bergé and Vulliet, 2015) at concentrations defined as 20 times LOQ_{PROC}. At the concentration they defined as LOQ_{PROC} and which is similar to the lower spiking level defined in this study, better results were still obtained in this work for roxithromycin, sulfamethoxazole and sulfathiazole. However, they could determine erythromycin with a higher accuracy at lower concentrations than the lowest recovery validated in this work, 10 μg·kg^{-1}. Montemurro et al. (2021) provided similar recovery values for sulfamethazine, sulfamethoxazole and fluconazole at 100 μg·kg^{-1} comparing with the calculated ones in this work at 50 μg·kg^{-1}. However, they obtained better results for clarithromycin, whereas in this work ciprofloxacin could be more accurately determined.

3.3. QA/QC parameters for the determination of AMs in OECD soils

This work ensured the quality of the soil AM analyses by determining key figures of merit (LOQ_{PROC}, absolute recoveries, apparent recoveries, and precision expressed as RSD %) for OECD soils spiked at a concentration level of 50 μg kg^{-1} (see Table S4). LOQ_{PROC} ranged from 1 μg·kg^{-1} to 9 μg·kg^{-1}, with the exception of the fluoroquinolones ciprofloxacin and enoxacin, for which values of 30 μg·kg^{-1} and 45 μg·kg^{-1} were calculated, respectively, and norfloxacin, which could not be validated at 50 μg·kg^{-1} since only concentrations higher than 57 μg·kg^{-1} of this compound could be detected with this method. Erythromycin was the only macrolide for which extraction from OECD soils was not feasible. Regarding the method accuracy, the apparent recoveries were in the range of 81–119 % with RSD % values lower than 21 %, agreeing with the 2021/808 regulation (Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 on the Performance of Analytical Methods for Residues of Pharmacologically Active Substances Used in Food-Producing Animals and on the Interpretation of Results as Well as on the Methods to Be Used for Sampling and Repealing Decisions 2002/657/EC and 98/179/EC (Text with EEA Relevance), 2021). Only sulfathiazole (55 %), trimethoprim (55 %), danofloxacin (53 %), pefloxacin (70 %) and chlortetracycline (126 %) were outside the established range. However, in all cases an RSD of <20 % was determined, which demonstrates the consistency of the results obtained and the suitability of the method for determining these compounds in the matrix studied. In the case of these specific AMs, the use of a more appropriate surrogate would have to be evaluated on a case-by-case basis. In the particular case of sulfathiazole, it shows a different behaviour compared to the rest of the SAs. This observation was also described in previous works carried out by the research group (Vergara-Luis et al., 2023a). This difference may be due to differences at the structural level, since sulfathiazole is the only one of these molecules that contains a thiazole ligand.

4. Earthworm exposure experiments: evaluation of SMZ and TC bioaccumulation

The methods validated in this work were applied to determine SMZ and TC concentrations in earthworms and soils after the OECD-207 tests. Both samples were subsequently analysed by UHPLC-HRMS to extend the analysis to the identification of TPs.

4.1. OECD-207: filter paper test

Only the earthworms exposed to 1000 mg·kg^{-1} of AMs were submitted to chemical analysis, while the biological effects were evaluated at all concentration levels. The analysis determined the presence of SMZ and TC in the earthworms at concentrations of 23.2 ± 0.5 mg·kg^{-1} and 88.8 ± 0.9 mg·kg^{-1}, respectively, after 48 h of exposure of the earthworms to the contaminated paper. This evidences the dermal route as a point of entry of AMs into the earthworms and their subsequent accumulation in these organisms.

After the exposure time (48 h + 24 h), no mortality was observed at different concentrations of SMZ and TC, so the lethal dose (LC) was established above 1000 mg·L^{-1} for both AMs. The absence of mortality agrees with other studies where the LC50 showed to be >2000 mg·kg^{-1} for TC (Havelkova et al., 2016; Pino et al., 2015) and higher than 4000 mg·kg^{-1} for sulfonamides (Pino et al., 2015).

Moreover, no significant differences were observed in the weight loss of the exposed organisms, with average losses below 10 % (Fig. 1). This suggests absence of a severe damage due to biomass loss. Organisms exposed to TC showed a slight trend to lose weight at higher doses; however, the high variabilities among the different exposure groups overlapped any type of statistical significance. Meanwhile, organisms exposed to SMZ showed no relationship between weight loss and

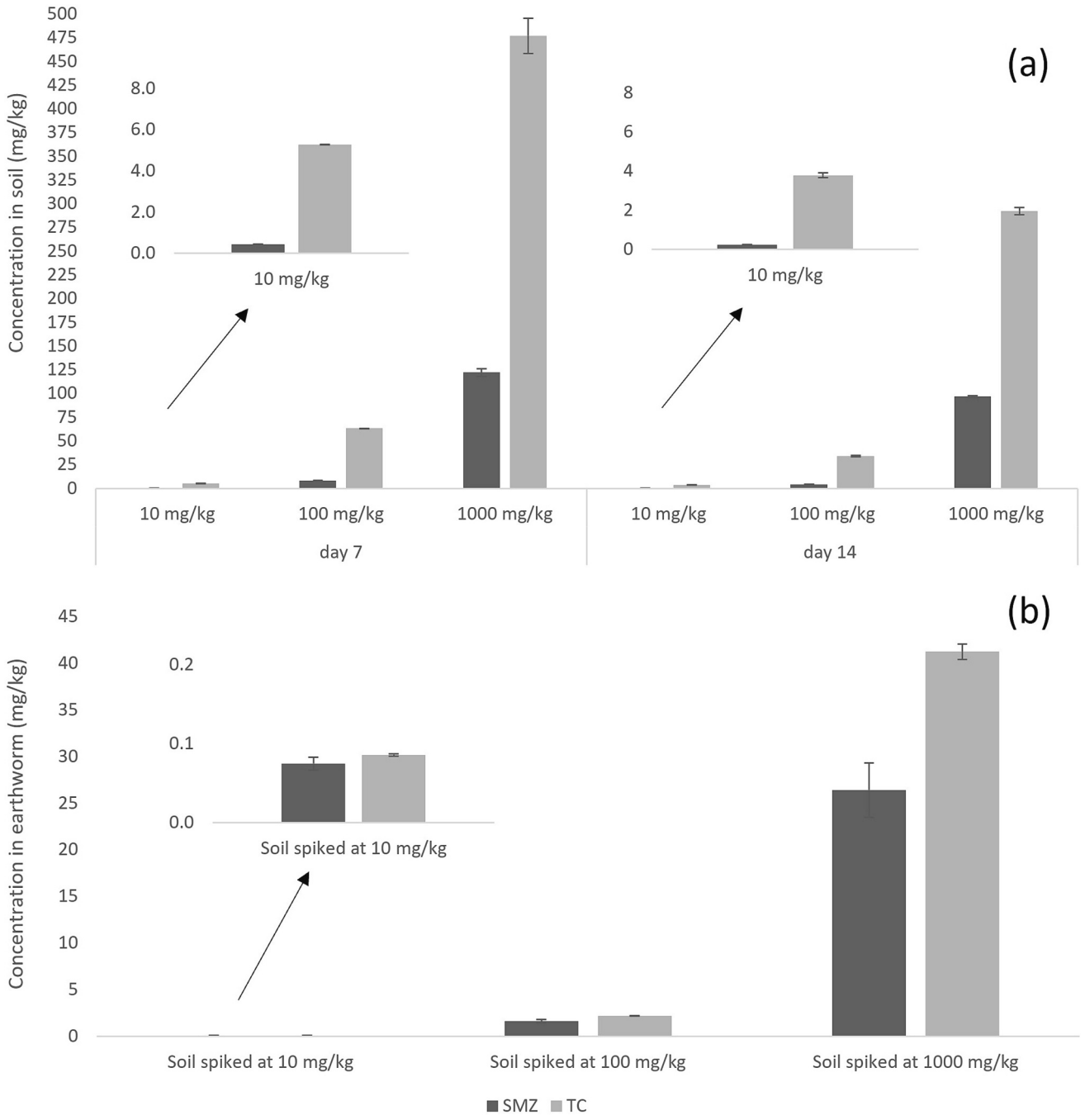

Fig. 2. Mean concentration (n = 3) of SMZ and TC detected in soils in the day 7 and day 14 of the experiment at the initial spiking levels tested (i.e., 10 mg·kg^{-1}, 100 mg·kg^{-1}, 1000 mg·kg^{-1}): (a) and mean concentration (n = 3) of SMZ and TC detected in earthworms after 14-days exposure (b).

exposure dose. pH measurements carried out to check the acidity of the dilutions ruled out a deleterious effect induced by the acidity of the TC solution.

The above results suggest that the duration of the experiment, the selected dose and dermal exposure were not sufficient to generate damage at organism level in *E. fetida* individuals.

4.2. OECD-207: soil toxicity test

4.2.1. Degradation of SMZ and TC in soils and uptake by earthworms

To evaluate the possible degradation of the target AMs in soil, soil samples were collected on days 7 and 14 of the experiment and analysed by UHPLC-MS/MS. As it can be seen in Fig. 2a, a clear decrease in AM concentrations was observed compared to the initial concentration (10 mg·kg^{-1}, 100 mg·kg^{-1} or 1000 mg·kg^{-1} in each case), suggesting a degradation process occurring in the soil during the first week of the experiment. The degradation continued over time since significantly lower concentrations were detected at the 14th day. Coincident with the degradation life of each AM, stronger degradation was observed for SMZ in soil (half-live ($t_{1/2}$) 3.4 days) compared to TC ($t_{1/2}$ 148 days) (US EPA, 2023). This result is consistent with that observed in the literature (Scaria et al., 2021).

Earthworms were analysed after the 14-day exposure to contaminated soils and the results (Fig. 2b) indicated that earthworms were able to bioaccumulate AMs either by direct ingestion of contaminated soil or by contact with it. The detection of SMZ in earthworms after different

Table 2
Identified transformation products (TPs) in soil samples spiked with 10 mg·kg^{-1}, 100 mg·kg^{-1} and 1000 mg·kg^{-1} of sulfamethazine (SMZ) or tetracycline (TC) at day 7 and day 14 of the exposure experiment, and in earthworm samples.

	Name	Formula	Smile	Exact mass	Mass error	tR (min)		Level	Soil (day 7)	Soil (day 14)	Earthworm
						Soil	Earthworm				
TP1	N4-Acetylsulfamethazine	C14H16N4O3S	CC1=CC(=NC(=N1)NS(=O)(=O)C2=CC=C(C=C2)NC(=O)C)C	320.0942	−3.92	6.48	5.93	2a	SMZ 1000 mg·kg^{-1}	SMZ 1000 mg·kg^{-1}	SMZ 10 mg·kg^{-1}, 100 mg·kg^{-1} and 1000 mg·kg^{-1}
TP2	4-Amino-3-chloro-n-(4,6-dimethylpyrimidin-2-yl) benzenesulfonamide	C12H13ClN4O2S	CC1=CC(=NC(=N1)NS(=O)(=O)C2=CC(=C(C=C2)N)Cl)C	312.0445	2.66	8.72	8.04	2b	SMZ 1000 mg·kg^{-1}	SMZ 1000 mg·kg^{-1}	SMZ 100 mg·kg^{-1} and 1000 mg·kg^{-1}
TP3	Acetylacetoneguanidine	C6H9N3	CC1=CC(=NC(=N1)N)C	123.0799	0.81	5.40	4.86	2b	SMZ 1000 mg·kg^{-1}	SMZ 1000 mg·kg^{-1}	SMZ 10 mg·kg^{-1}, 100 mg·kg^{-1} and 1000 mg·kg^{-2}
TP4	Formylsulfamethazine	C13H14N4O3S	CC1=CC(=NC(=N1)NS(=O)(=O)C2=CC=C(C=C2)NC=O)C	306.0785	−1.52	5.43	4.91	2b	SMZ 1000 mg·kg^{-1}	SMZ 1000 mg·kg^{-1}	SMZ 10 mg·kg^{-1}, 100 mg·kg^{-1} and 1000 mg·kg^{-1}
TP5	Desaminosulfamethazine	C12H13N3O2S	CC1=CC(=NC(=N1)NS(=O)(=O)C2=CC=CC=C2)C	263.0727	−1.06	9.08	8.41	2b	SMZ 1000 mg·kg^{-1}	SMZ 1000 mg·kg^{-1}	SMZ 10 mg·kg^{-1}, 100 mg·kg^{-1} and 1000 mg·kg^{-3}
TP6	(4,6-Dimethylpyrimidin-2-yl)sulfamic acid	C6H9N3O3S	CC1=CC(=NC(=N1)NS(=O)(=O)O)C	203.0365	−0.51	5.39	4.86	2b	SMZ 1000 mg·kg^{-1}	SMZ 1000 mg·kg^{-1}	SMZ 100 mg·kg^{-1} and 1000 mg·kg^{-3}
TP7	4-Amino-n-(4,6-dimethylpyrimidin-2-yl)-3-hydroxybenzenesulfonamide	C12H14N4O3S	CC1=CC(=NC(=N1)NS(=O)(=O)C2=CC(=C(C=C2)N)O)C	294.0783	−1.10	4.91	–	2b	SMZ 1000 mg·kg^{-1}	SMZ 1000 mg·kg^{-1}	–
TP8	2-[(4-Aminophenyl)sulfonylamino]-6-methylpyrimidine-4-carboxylic acid	C12H12N4O4S	CC1=CC(=NC(=N1)NS(=O)(=O)C2=CC=C(C=C2)N)C(=O)O	308.0575	2.82	5.14	–	2b	SMZ 1000 mg·kg^{-1}	SMZ 1000 mg·kg^{-1}	–
TP9	4-Hydroxydesaminosulphadimidine	C12H13N3O3S	CC1=CC(=NC(=N1)NS(=O)(=O)C2=CC=C(C=C2)O)C	279.0676	−1.06	6.06	5.81	2b	SMZ 10 mg·kg^{-1} and 100 mg·kg^{-1}	SMZ 10 mg·kg^{-1} and 100 mg·kg^{-1}	SMZ 100 mg·kg^{-1} and 1000 mg·kg^{-2}
TP10	Epitetracycline	C22H24N2O8	CC1(C2CC3C(C(=O)C(=C(C3(C(=O)C2=C(C4=C1C=CC=C4O)O)O)O)C(=O)N)N(*C*)C)O	444.1533	−0.70	3.31	3.04	2a	TC 10 mg·kg^{-1}, 100 mg·kg^{-1} and 1000 mg·kg^{-1}	TC 10 mg·kg^{-1}, 100 mg·kg^{-1} and 1000 mg·kg^{-1}	TC 10 mg·kg^{-1}, 100 mg·kg^{-1} and 1000 mg·kg^{-1}
TP11	Demecycline	C21H22N2O8	CN(*C*)C1C2CC3C(C4=C(C(=CC=C4)O)C(=C3C(=O)C2(C(=C(C1=O)C(=O)N)O)O)O)O	430.1373	−3.33	3.92	–	2b	TC 1000 mg·kg^{-1}	TC 1000 mg·kg^{-1}	–
TP12	4-(Dimethylamino)-1,11,12a-trihydroxy-6,6-dimethyl-3,7,10,12-tetraoxo-3,4,4a,5,5a,6,7,10,12,12a-decahydrotetracene-2-carboxamide	C22H22N2O9	CN(*C*)C1C2CC3C(=C(*O*)C4=C(C(=O)C=CC4=O)C3(C)C)C(=O)C2(O)C(=C(C(N)=O)C1=O)O	458.1322	4.63	8.06	–	2b	TC 1000 mg·kg^{-1}	TC 1000 mg·kg^{-1}	–
TP13	Chlortetracycline	C22H23ClN2O8	CC1(C2CC3C(C(=O)C(=C(C3(C(=O)C2=C(C4=C(C(=CC(=C41)Cl)O)O)O)O)C(=O)N)N(*C*)C)O	478.1138	1.22	6.534	–	2a	TC 100 mg·kg^{-1} and 1000 mg·kg^{-1}	TC 100 mg·kg^{-1} and 1000 mg·kg^{-1}	–

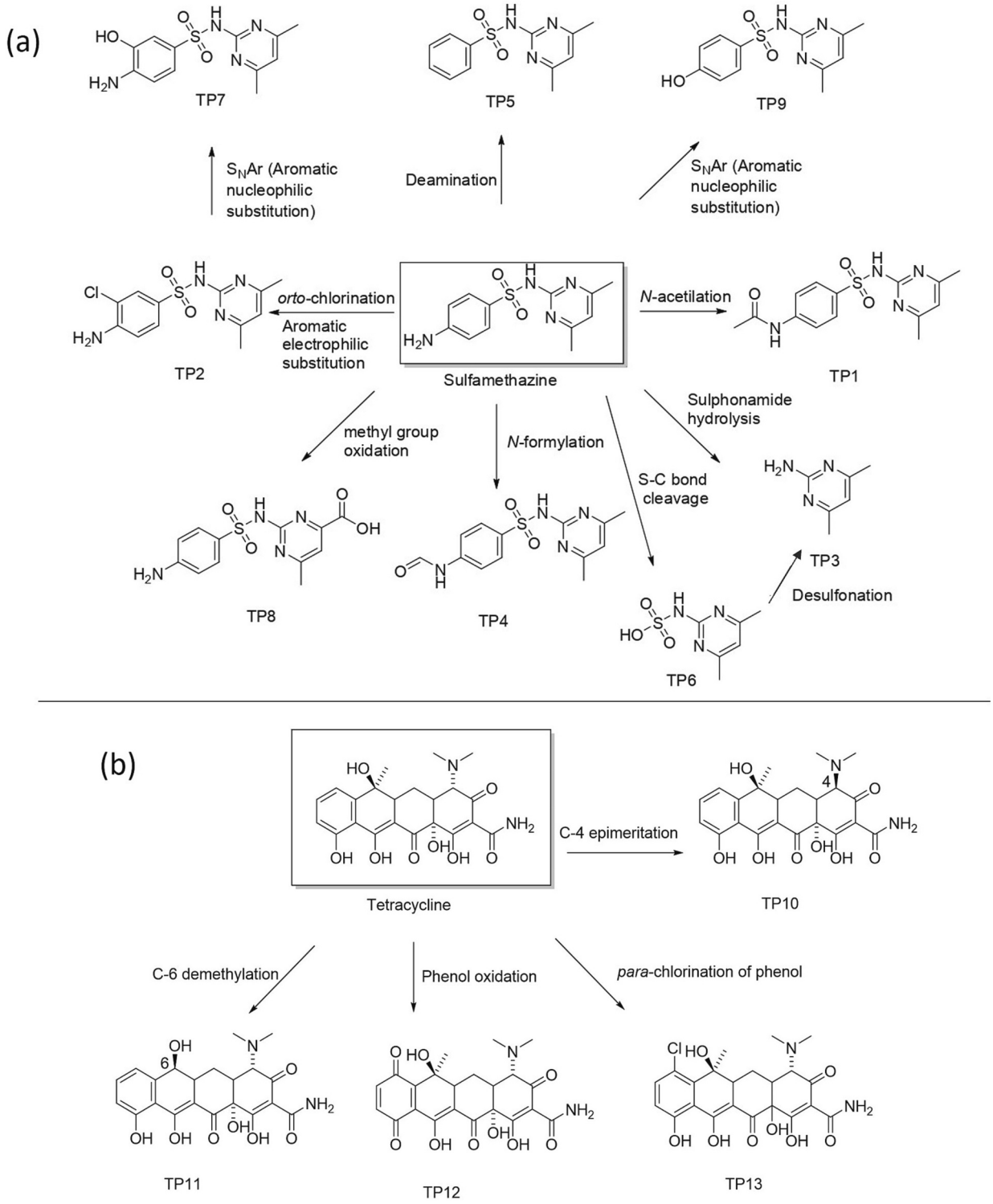

Fig. 3. Chemical structure of the SMZ-derived TPs (a) and TC-derived TPs (b), identified at level 2a or 2b, and the respective transformation reactions.

exposure experiments has also been published (Bergé and Vulliet, 2015).

The significant degradation of SMZ in soil make it less bioavailable than TC for its accumulation in the earthworm, this could explain the higher concentration of TC detected in the organism compared to that determined for SMZ. However, to understand the accumulation capacity of each AM in the earthworm, the bioconcentration factor (BCF) was calculated in each case as the ratio between the concentration detected in the earthworm and the one detected in the soil (see Table S5). According to the results, after 14-days of exposure, there was no significant differences in the BCF values between the tested concentration either for SMZ or TC. This is in agreement with the literature as it has been reported that BCF became balanced after 14–28 days in earthworms exposed to different contaminants (Zhao et al., 2023). However, the BCF values for SMZ are higher than the ones estimated for TC, which means that even if SMZ is detected at a lower concentration than TC in soil due to degradation processes, SMZ has a higher tendency to transfer and accumulate in the biota (soil adsorption coefficient (K_{oc}) 145 $L{\cdot}kg^{-1}$), whereas TC (K_{oc} 562 $L{\cdot}kg^{-1}$) shows a stronger adsorption to the soil

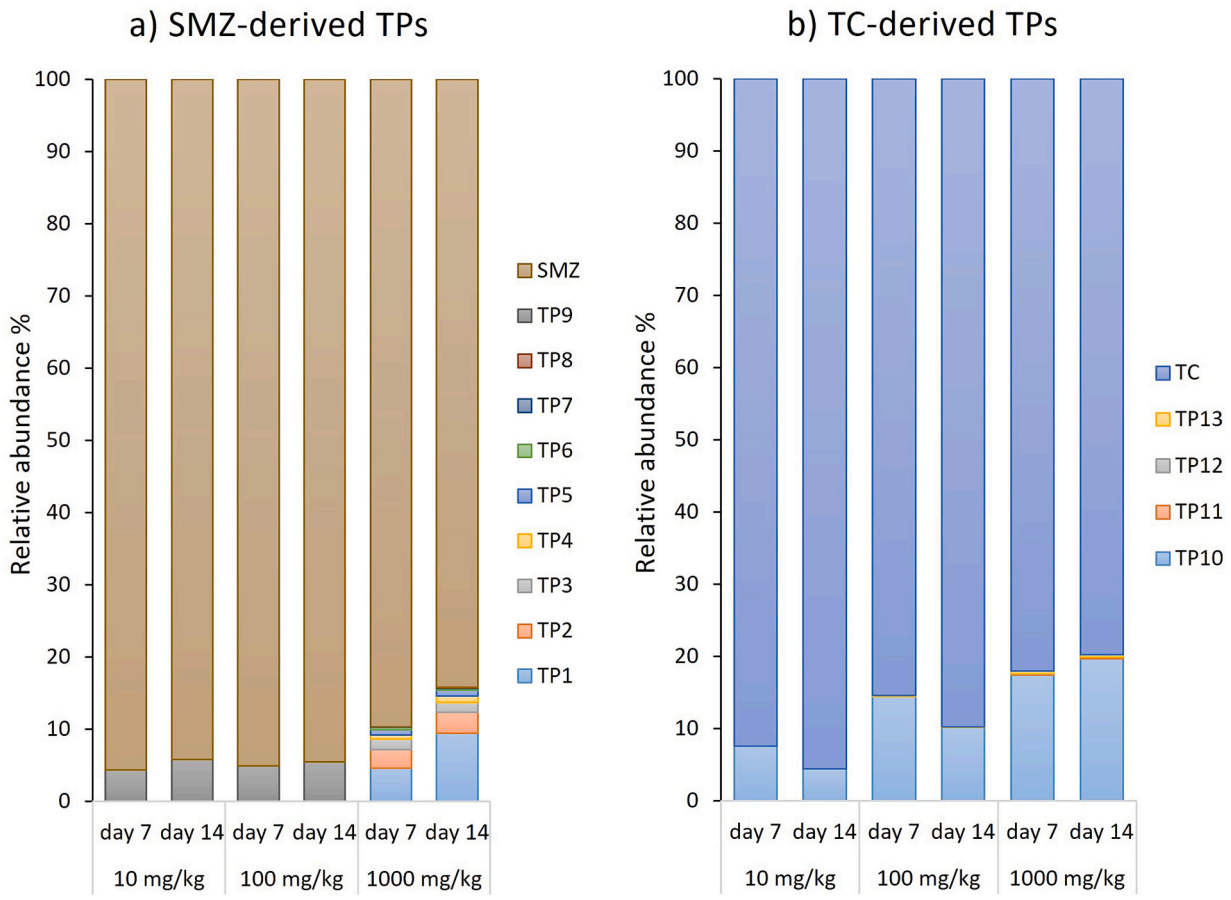

Fig. 4. SMZ-derived TPs (a) and TC-derived TPs (b) identified at levels 2a and 2b in soils (results are given as calculated as relative abundances calculated as the ratio of individual peak area with respect to the sum of all peas areas areas normalised to the highest peak area).

system (US EPA, 2023).

Furthermore, comparing the concentrations of AMs detected in earthworms after exposure to contaminated filter paper (48 h) and soil (14 days), the dermal route appears to be a faster route of entry of AMs into the organism, as in a shorter period of time earthworms absorbed the same concentration of SMZ (23.2 ± 0.5 mg·kg^{-1} vs 26.4 ± 2.9 mg·kg^{-1}) and a significantly higher concentration of TC (88.8 ± 0.9 mg·kg^{-1} vs 41.2 ± 0.8 mg·kg^{-1}).

4.2.2. TPs of AMs in soils and earthworms

TPs of SMZ and TC in earthworm and soil samples were identified by means of UHPLC-HRMS. Structural assignments based on accurate mass and fragment ion data are compiled in Table 2, while chemical structures are gathered in Fig. 3a and b. No reference standards were available for the annotated TPs so that we reached a confidence level 2a–3 according to the annotation scale proposed by Schymansky et al. (Schymanski et al., 2014), leading to the assignments of probable structures or tentative candidates. A total of nine SMZ TPs and four TC TPs were detected in earthworms and soil samples, made up of both Phase I and Phase II metabolites. To the best of our knowledge, eight of these TPs (TP 2–3, 6–9, 11–12) are reported for the first time in earthworms and/or soil samples, whereas the rest have been previously described in the literature (García-Galán et al., 2011; Grant et al., 2003; Lin et al., 2021; Montone et al., 2023; Solliec et al., 2016; Vergara-Luis et al., 2023b). Detailed information about the presence of the annotated TPs in the analysed samples along the uptake experiment is discussed herein.

In order to compare the abundance of individual TPs in the different samples/exposure days, equivalent response factors were assumed for all TPs. The conclusions should be considered qualitative due to the response factor may differ among TPs. The relative abundances of TPs (calculated as the ratio of individual peak area with respect to the sum of all peas areas) in soils/earthworms per concentration level over the exposure experiment are plotted in Figs. 4a and b and 5a and b for soil and earthworms, respectively. In order to consider the sample mass, all peak areas were corrected using sampling size.

Regarding the TPs detected in soils, in general SMZ-derived TPs (Fig. 4a) were only observed at the highest concentration level (1000 mg·kg^{-1}) and their presence increased after 14 days compared to the signal obtained in the first week. TPs 1–3 were formed to a greater extent, while TP 9 formation was observed at the two lowest concentration levels, 10 mg·kg^{-1} and 100 mg·kg^{-1}. In the latter case, the highest signal was observed on day 14 of the experiment, as for the rest of the TPs, irrespective of the concentration level. The presence of N4-Acetylsulfamethazine (TP 1) has been previously reported in different environmental samples such as soil, water and manure at low ppb quantities (Grant et al., 2003). Montone et al. confirmed that sulfonamide acetylated products are the most common TPs observed in environmental waters (Montone et al., 2023). By means of this work, their formation in the soil system has also been demonstrated, showing a higher signal compared to the rest of the detected TPs. In a previous work, formylsulfamethazine (TP 4) was also identified in soil samples amended with sheep manure (Vergara-Luis et al., 2023b). Although desaminosulfamethazine (TP 5) has been identified as TP produced by the fungus *Trametes versicolor* in in vivo experiments (García-Galán et al., 2011), no data has been found about its presence in soils, however, Montone et al. detected desaminosulfamethoxazole and desaminosulfapyridine in waste waters (Montone et al., 2023).

As for the TC-derived TPs (Fig. 4b), a trend opposite to the SMZ-derived TPs was observed. In this case, TPs were mostly formed in the

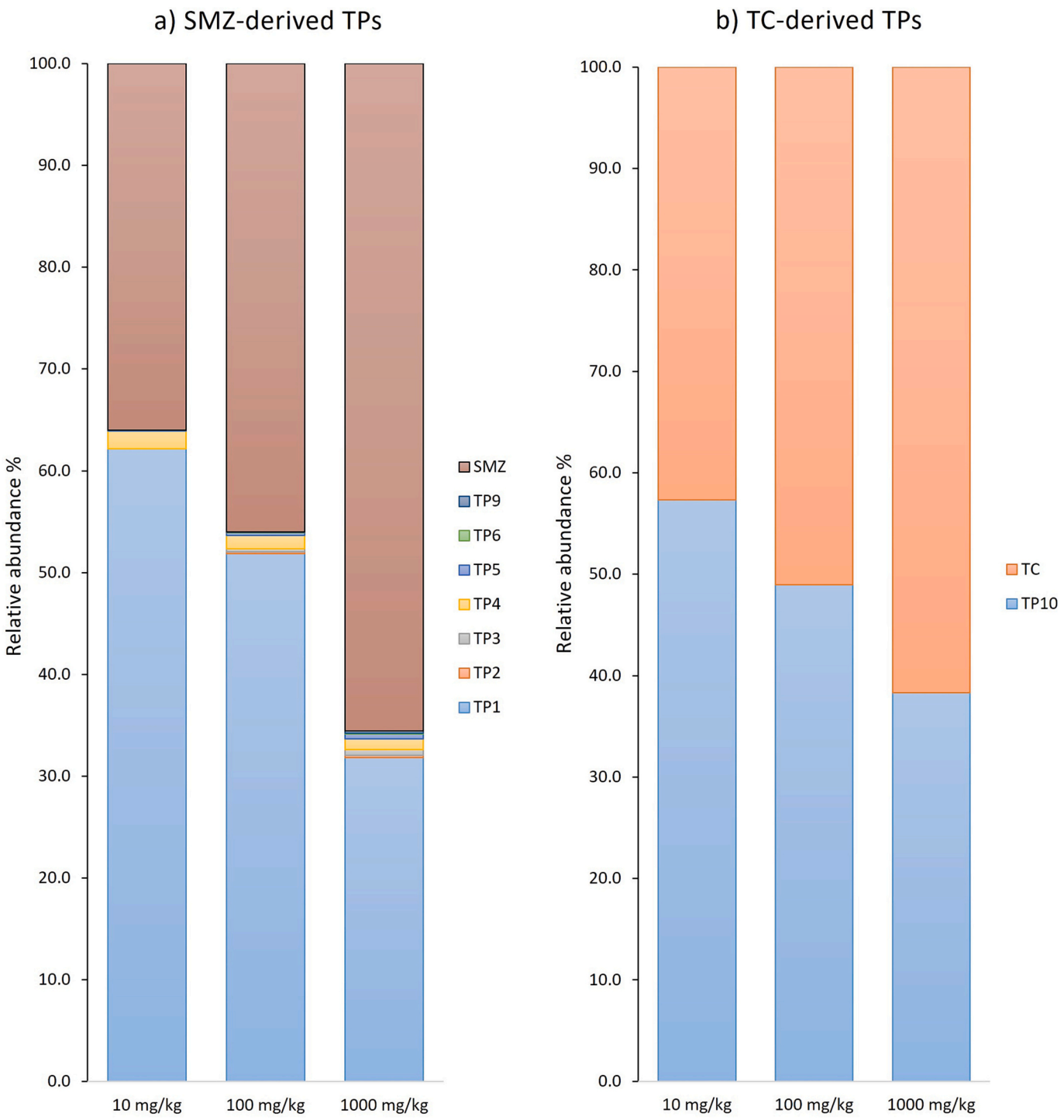

Fig. 5. SMZ-derived TPs (a) and TC-derived TPs (b) identified at levels 2a and 2b in earthworms (b) (results are given as calculated as relative abundances calculated as the ratio of individual peak area with respect to the sum of all peas areas areas normalised to the highest peak area).

first 7 days of the experiment and their presence decreased in the following days, regardless of the concentration level. Epitetracycline (TP 10) is the TP derived from TC that is formed to the highest extent in soil. This epimerisation process occurring in soil is in agreement with previously published works (Lin et al., 2021). Moreover, Lin et al. also concluded that earthworm assisted remediation could accelerate tetracycline biodegradation and epimerisation into epitetracycline (Lin et al., 2021). Solliec et al. reported the presence of epitetracycline, and chlortetracycline (TP 13) too, in soil samples amended with contaminated manure (Solliec et al., 2016). In addition to the TPs shown in Fig. 3b, other TC-derived TPs were detected and tentatively identified at level 3 of the Schymanski scale (Fig. S3) (Schymanski et al., 2014).

Regarding the earthworms, seven of the SMZ-derived TPs detected in soils were also detected in the earthworms exposed to those soils (Fig. 5a). As in soil, the product showing the highest abundance in earthworms was TP 1 and it was detected at the three concentration levels used for the exposure experiment. The rest of the identified TPs were also detected at all the exposure levels with the exception of TP 2, TP 6 and TP 9. As for the TC-derived TPs (Fig. 5b), only TP 10 was observed in the organism at all the exposure levels, which coincided

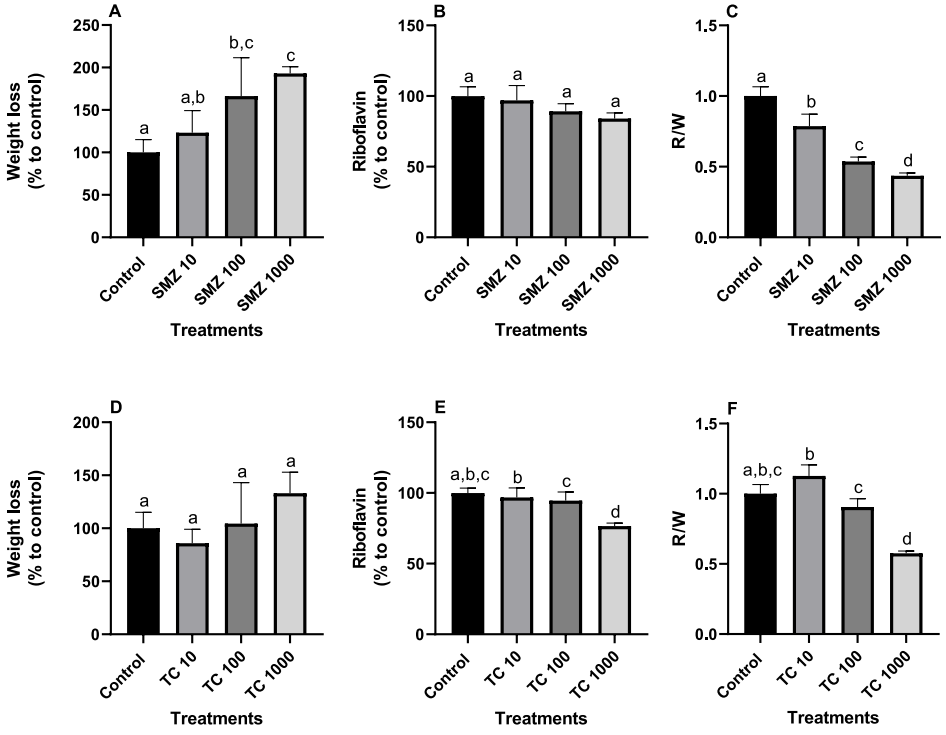

Fig. 6. Average body weight loss, riboflavin content in coelomocytes (% respect to control) and relation between riboflavin signal and body weight (R/W) in *E. fetida* earthworms exposed to concentrations of 10 mg·kg^{-1}, 100 mg·kg^{-1} and 1000 mg·kg^{-1} of SMZ and TC. The same letters do not differ from each other using the Tukey test with 95 % confidence ($p < 0.05$).

with the results obtained in soil, as it was the most abundant TP in this matrix as well. Although some work has focused on the detection of AMs in worms, confirming the accumulation of some sulfonamides and macrolides among others in the organism (Bergé and Vulliet, 2015; Montemurro et al., 2021), to our knowledge, none of these have performed a suspect analysis to identify possible TPs that may accumulate in earthworms. Therefore, this work represents an advance on currently available methodologies to understand and extend the information on the degradation/metabolisation processes of SMZ and TC by earthworms and to identify the formed TPs in earthworms.

4.2.3. Biological implications on earthworms to TC and SMZ exposure

Mortality was not observed in any of the exposure experiments. In order to assess whether a decrease on earthworms' growth can occur under the presence of AMs the loss of body weight was monitored. There were no significant body weigh alterations ($F_{3,12} = 2.988$; $p = 0.0775$) between control and earthworms exposed to TC in any of the tested concentrations, but the earthworms exposed to SMZ at high dosing levels (>100 mg·kg^{-1}) showed a significant body weight loss ($F_{3,12} = 9.253$; $p = 0.0024$) at the 95 % confidence level (Fig. 6 A and D).

Weight loss is commonly observed under stress conditions, being an indication of reduced growth in earthworms (Qiao et al., 2019; Ye et al., 2016), which may also compromise survival (Dittbrenner et al., 2012). However, considering our experimental conditions, weight loss did not affect survival in earthworms. Thus, body weight reduction may be also associated with a survival strategy to reduce the intake of toxic products (Wu et al., 2011). In fact, the highest weight losses observed in earthworms exposed to SMZ suggest higher energy demands of the animals to deal with intoxication, and so affecting their growth. Similar results were observed in *E. fetida* exposed to other contaminants, such as the insecticide cyantraniliprole (Qiao et al., 2019) and the fungicide thifluzamide (Yao et al., 2020), or other organisms (*E. andrei*) exposed to fluoroquinolone antibiotics (Parente et al., 2021).

Riboflavin levels in earthworms were also measured in order to test for any alteration in the immune system due to the presence of AMs since it is known that riboflavin levels in *E. fetida* eleocytes can be altered under certain environmental stressing conditions (Plytycz et al., 2009, 2010; Rorat et al., 2016).

The results concluded that the riboflavin content measured in the coelomocytes of earthworms exposed to SMZ showed no significant changes ($F_{3,24} = 1.077$; $p = 0.3812$) (Fig. 6B), nevertheless, when expressed as a function of body weight there was a significant reduction ($F_{3,24} = 45.92$; $p < 0.0001$) in comparison to the control (Fig. 6C). For TC exposure, significant decrease in riboflavin content ($F_{3,15} = 4.273$; $p = 0.0228$) was observed comparing to the control in earthworms exposed to the highest concentrations (Fig. 6E and F).

The decrease in riboflavin content often indicates mobilisation of the immune system (Rorat et al., 2016), being also a common response in earthworms exposed to contaminated soils (Plytycz et al., 2009, 2010; Rorat et al., 2016). Thus, it can be suggested that the AMs studied have the potential to affect the immune system of *E. fetida*. Our results corroborate other studies that verified an effect on the immune system of earthworms exposed to contaminants, such Plytycz et al. (Plytycz et al., 2010), who observed a decrease in the riboflavin content in *Dendrodrilus rubidus* earthworms exposed to metal-contaminated soils.

5. Conclusions

To the best of our knowledge, this is the first scientific study that deepens the understanding of AM contamination and its effects on the base of the food chain (earthworms), both from a chemical and biological point of view. A highly accurate analytical method has been successfully validated, which allows the simultaneous determination of a diverse set of AMs from several families, as well as the identification of different TPs. The study has demonstrated the transfer of AMs from soils to earthworms by dermal contact and direct ingestion, the former being the most rapid route of exposure. In addition, the degradation of AMs has been observed in both matrices and several TPs have been successfully identified, some of them for the first time in soil/earthworms. Regarding the biological effects, only SMZ caused changes in earthworm

growth, evidenced by weight loss in earthworms exposed to concentrations of 100 and 1000 mg·kg^{-1}. Riboflavin content decreased at all concentrations of SMZ-experiments, as well as at the highest concentration of TC-experiments, suggesting that these AMs have the potential to alter the immune system of *E. fetida*.

CRediT authorship contribution statement

I. Vergara-Luis: Investigation, Data curation, Validation, Formal analysis, Methodology, Writing - Original draft, Visualization. **C.F. Rutkoski:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **E. Urionabarrenetxea:** Writing – original draft, Supervision, Methodology, Investigation. **E.A. Almeida:** Supervision, Investigation. **E. Anakabe:** Data curation. **M. Olivares:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **M. Soto:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **A. Prieto:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.171214.

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