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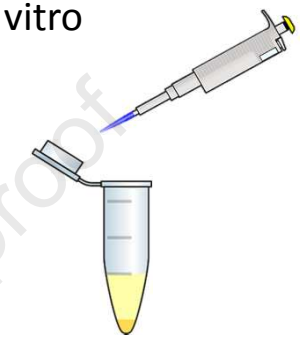
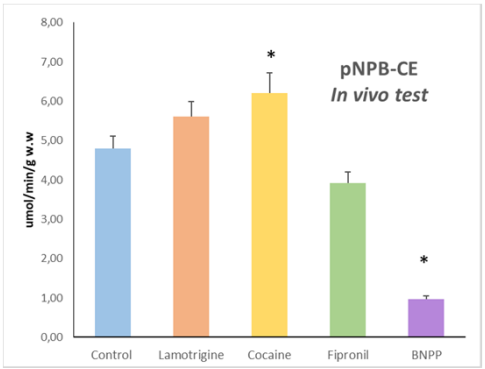
In vivo

Drugs

- Lamotrigine
- Cocaine
- Fipronil

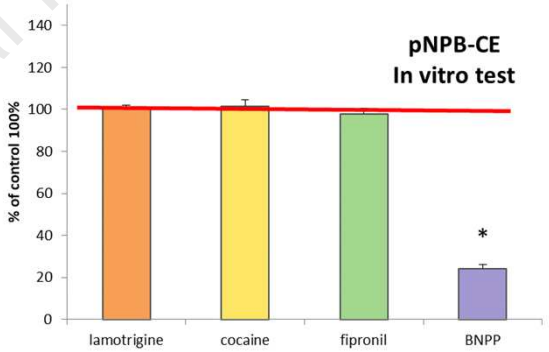
In vitro





Endpoints

- Biomarkers
- Metabolites



Biomarker responses and metabolism in *Lumbricus terrestris* exposed to drugs of environmental concern, an in vivo and in vitro approach.

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Abstract

The earthworm *Lumbricus terrestris* is an anecic species living in natural soils but it is also a sentinel in pollution monitoring. Specimens of *L. terrestris* were exposed for 48 h though the filter paper contact test at 1 mg/mL of the chemicals: Lamotrigine (LMG), Cocaine (COC), Fipronil (FIP) and the pesticide bis-4-nitrophenyl phosphate (BNPP). After that period, the activities of Acetylcholinesterase, Glutathione *S*-transferase, Carboxylesterase (CE) using different substrates, and lipid peroxidation levels were evaluated in the exposed whole tissue earthworms. The results revealed differences only in CE activity, with 4-nitrophenyl butyrate (4NPB) and 1-naphthyl butyrate (1NB) the most responsive substrates to COC. The kinetic parameters of CE were characterized, for the first time, in whole tissue of this species. The chemical analysis by LC-MS/MS, confirmed the exposure to the parent compounds, identified metabolites and evidenced biotransformation pathways in earthworms. Metabolic reactions included oxidation (LMG and FIP), hydrolysis (COC and FIP) as well as glycosylation (LMG, COC and FIP). A hitherto unknown metabolite of LMG due to the conjugation with phenylalanine glutamine was formed. The *in vivo* results on CE activity with the specific inhibitor, BNPP, were confirmed *in vitro*. Moreover, in the *in vitro* approach, the inclusion of other contaminants of environmental concern supports the potential of CE as biomarker. This study identifies the main metabolites formed by earthworms for further *in vivo* exposures under more realistic conditions and the potential use of CE measures as biomarker of emerging contaminants.

Keywords: Earthworms, Carboxylesterases, Metabolites, Lamotrigine, Cocaine, Fipronil.

1. Introduction

Water scarcity has led to the need of using water coming from sewage treatment works (STWs) for irrigation purposes. Nonetheless, this treated waste-water (TWW) may contain a large burden of chemicals of anthropogenic origin that could not be eliminated during the treatment process and end up in the effluent aimed for field irrigation of crops. Thus, soil pollution due to the use of TWW could become a problem, especially relevant when these crops are intended for human/cattle consumption but, likewise, it could also affect soil dwelling organisms. Especially relevant would be the impact on biota that play a key role in improving soil properties and considered soil engineers, such is the case of naturally occurring earthworm, with negative consequences in agriculture (Velki and Ecimovic, 2017).

The earthworm *Lumbricus terrestris*, is a common key species present in natural soils, used also as bioindicator in soil pollution assessment and bioremediation of contaminated soils (Pelosi et al., 2014). The advantage of this anecic species over other earthworm species (e.g epigeic Eisenia spp.) refers to the fact that it builds deep galleries; thus, increasing microbial proliferation and improving soil properties. It is also used in many ecotoxicological studies where its suitability has been validated (Gonzalez Vejares et al., 2010; Sanchez-Hernandez et al., 2014). Many of these toxicity studies support the use of biochemical biomarkers such as acetylcholinesterase (AChE) as an indicator of neurotoxicity, glutathione S-transferase (GST) suggestive of enhanced detoxification activities and carboxylesterases (CEs) as specific indicators of pesticide exposure but also involved in detoxification of many agrochemicals and drugs (Sanchez-Hernandez, 2006, 2020; Solé et al., 2020; Solé 2020). An adaptation of the OECD directive protocol 207 on “Earthworm, Acute Toxicity Tests” mostly based on Eisenia spp. was here adopted to test the suitability of *L. terrestris* as bioindicator of

chemicals through skin contact test, after 48h exposures. The *in vivo* and *in vitro* approach testing the suitability of whole tissue biomarker measures was based on former studies with invertebrates assessing several CE substrates and tissues (Otero and Kristoff, 2016; Sanchez-Hernandez and Wheelock, 2009). Recent *in vitro* studies with CEs suggest they could be used as a proxy of *in vivo* disturbances of a wide range of chemicals of environmental concern, including endocrine disruptors (Solé et al., 2021).

The choice of drugs was based on their demonstrated presence in TWW with potential use for water irrigation in countries (e.g. Mediterranean) where water scarcity may become a critical seasonal problem. Lamotrigine (LMG) is an antiepileptic drug used to treat epilepsy and bipolar disorders that can be found in contaminated soils and crops that have been irrigated with waste water from STWs at a mean of 5 ng/g d.w in soils (Malchi et al., 2014; Montemurro et al., 2019). LMG is also biotransformed by the oxidation action of the cytochrome P450 (CYP) to LMG N2-oxide and by conjugation to LMG N2-glucuronide in mammals and they represent the metabolites more abundant in TWW (Zonja et al., 2016). Soil microorganisms, bacteria and fungi are also involved in LMG removal, although recalcitrant conjugated metabolites can be present even after their action (Chefetz et al., 2019). Cocaine (COC) is an illicit drug that together with its main metabolite benzoylecgonine (BEG) has been consistently detected in STWs in a 5-year survey in the low μg/L range (Mastroianni et al., 2017) and in surface waters of the Mediterranean region (Postigo et al., 2010). So far, we are not aware of any studies on testing COC exposure in earthworms although the main metabolite in many species is BEG by the action of hepatic CEs (Wang et al., 2018). Fipronil (FIP) is an insecticide selectively targeting the gamma-aminobutyric acid (GABA) receptor of insects rather than mammals (Wang et al., 2016). Nonetheless, it has environmental concern in countries with high application in crop treatments, such as the USA where it was

present in STWs in the range of hundreds ng/L (Sadaria et al., 2019) or in Brazil where it has been found in most water bodies including drinking water (Montagner et al., 2019). FIP and its metabolites by CYP action (e.g. fipronil sulfone) have also been detected in all types of foodstuff, including vegetables (Li et al., 2020). In Europe, its use has been regulated but in some countries, such as Spain, it is still allowed for certain agricultural practices. Its toxicity and sublethal biomarker responses has been reviewed in many aquatic species (Wang et al., 2016) including metabolites identification (Konwick et al., 2006). The toxic action on terrestrial earthworms has only been reported for *Eisenia andrei* with survival and reproduction as endpoints (Zortea et al., 2018).

Within the context of the use of TWW for crops irrigation in countries with water scarcity problems, this study aimed to assess the suitability of *L. terrestris* as sentinel of contaminated soils. The choice of chemicals was based on their presence in TWW and the selection of common biomarkers also included carboxylesterases, using a combined *in vivo* and *in vitro* approach. The use of whole tissue homogenates for biochemical determinations was also formerly validated. Although the doses selected exceed those of environmental relevance, they were chosen for the unequivocal identification of pathways as well as metabolite identification. The obtained results could be valuable for further application under more realistic field exposures.

2. Material and Methods.

2.1 Earthworm in vivo exposure

Specimens of *Lumbricus terrestris* were obtained from a local provider and let to empty their guts and acclimatize to lab conditions for 24h before starting the 48h

exposure period. Acclimatization and exposure were carried out in a chamber at 15ºC in total darkness. Duplicate Petri dishes (13,5 cm diameter) containing a sheet of Whatman paper, were used for each drug and control conditions. The drugs were lamotrigine (LMG; CAS number 84057-84-1), cocaine hydrochloride (COC; CAS number 53-21-4), fipronil (FIP; CAS number 120068-37-3) as technical mixture regent® 80WG (80% fipronil) and bis(p-nitrophenyl) phosphate (BNPP; CAS number 645-15-8). While conditioning the earthworms to laboratory conditions for 24h, a 1 mL solution of each drug (at 1 mg/mL concentration) or 1 mL of the carrier (acetonitrile:water; 50:50) in the controls, were homogenously dispersed in the exposure and control conditions, respectively, of the Whatman paper placed in the Petri dish. These solutions were let to evaporate overnight and, just before placing the earthworms into the Petri dishes, 2 mL of MiliQ water were added to have an optimal medium for the earthworms and facilitate skin absorption. Four individual earthworms were placed in each duplicate dish at each condition (n=8) and were left in the incubator at 15^{O}C for 48 hours in total darkness. After this period, survival was checked by a soft mechanical stimulus in the front part and the specimens were weighted and, after anesthesia by placing them in a cooler for 10 min, they were frozen in N_2 liquid and stored individually at -80^{O}C for further biochemical and chemical analysis.

2. 1.1 Sample preparation

Each whole organism of frozen earthworm (n=8) was individually placed in a 50 ml stainless steel capsules and this tandem pre submerged in liquid N_2 to avoid sample defrosting. Two capsules at a time were grinded using a Mixer Mills MM400 at a frequency of 28 c/s during 1.5 min. The obtained sample was kept frozen to a

135 homogeneous powder that could be easily split for the chemical and biochemical
136 analysis, ensuring uniformity of the sample.

137

138 *2.1.2 Biomarker determinations*

139 From each specimen, 0.3 grams of frozen worm powder were homogenised in
140 ice-cold with 20 mM Tris-HCl containing 1 mM ethylenediaminetetraacetic acid
141 (EDTA) buffer (pH 7.6) at 1:5 (w:v) ratio, using a sonicator. The homogenate was then
142 centrifuged at 10,000 g (20 min, 4 °C) and the supernatant obtained (S9) was stored to -
143 80ºC until analysis.

144 *Carboxylesterase-CE-* activity was measured in the S9 fraction of the
145 homogenate using the substrates: 4-nitrophenyl acetate (4NPA), 4-nitrophenyl butyrate
146 (4NPB), 1-naphthyl acetate (1NA) and 1-naphthyl butyrate (1NB). The hydrolytic
147 activity of 4NPA and 4NPB was determined by spectrophotometric enzyme assay
148 according to Hosokawa and Satoh (2005). The kinetic assay was performed in a
149 medium of 50 mM phosphate buffer (pH=7.4), the substrate (1 mM, final
150 concentration). The formation of 4-nitrophenolate was measured at 405 nm and 25ºC.
151 An extinction coefficient of $1.8 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ was used to express the hydrolysis of
152 these nitrophenyl esters. The hydrolysis of 1NA and 1NB was measured following the
153 ultraviolet spectrophotometric method by Mastropaolo and Yourno (1981). The reaction
154 mixture consisted of the same buffer phosphate and the substrate (each at 0.25 mM,
155 final concentration). The formation of 1-naphtol was measured at 235 nm at 25ºC. An
156 extinction coefficient of $2.34 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ was used for activity calculations.

157 *Acetylcholinesterase-AChE* activity was measured using 1 mM of the substrate
158 acetylthiocholine (ATC). In each well, 180 µM dithiobisnitrobenzoate (DTNB) were

159 mixed with 25 µl of sample and after 2 min pre-incubation, ATC were added using the
160 principle of (Ellman et al., 1961). Reading was performed at 412 nm during 5 min and
161 quantified using ε =1.36 10^4 $M^{-1} \cdot cm^{-1}$. AChE measures in the whole tissue of *L.*
162 *terrestris* at the selected substrate concentration and its validation, using specific
163 inhibitors had been formerly described (Rault et al., 2007).

164 *Glutathione S-transferase-GST* activity was measured using 1 mM 1-chloro-2,4-
165 dinitrobenzene (CDNB) and 1 mM reduced glutathione (GSH) according to the protocol
166 of (Habig et al., 1974). The activity rate was measured as change in absorbance for 5
167 min at 340 nm and quantified using ε=9.6 $mM^{-1} \cdot cm^{-1}$.

168 Lipid peroxidation (LPO) was included as a measure of oxidative damage to
169 lipids in the same whole tissue S9 fraction using a colorimetric method with 1-methyl-
170 2-phenylindole. Quantification, with respect to the standard solution1,1,3,3-
171 tetramethoxypropane in acid medium to yield malondialdehyde (MDA) at 586 nm.

172 Total protein content was determined by the (Bradford, 1976), using the
173 Bradford Bio-Rad Protein Assay reagent. An external standard curve made with bovine
174 serum albumin (0.05-0.5 mg·mL-1) was used for total protein quantification.

175 All measures were carried out in triplicate at 25ºC and using a microplate reader
176 Infinite M200 TECAN.

177

178 *2.2 Earthworm in vitro exposure*

179 *2.2.1 Methodological considerations*

180 Since different homogenization buffers have been reported in the literature for
181 earthworm biomarker determinations, we contrasted a 20 mM Tris buffer at three

different pH (8.0, 7.6 and 7.2), each one with or without presence of the metal chelator EDTA at 1mM concentration (to discriminate the inclusion or A esterases). Three individual earthworms were included for these measures: AChE, GST and 4NPB-CE following the formerly described specific protocols

2.2.2 Validation of esterase measures with model inhibitors

In order to unequivocally identify B-esterase measures, three model inhibitors were tested *in vitro*. The carbamate eserine (CAS number 57-47-6), the nicotinoid BW284c51 (CAS number 402-40-4) and the organophosphorous pesticide BNPP (Hatfield and Potter, 2011) were chosen as specific inhibitors to confirm cholinesterases-ChEs, AChE and CE measures, respectively, all at a single 100 µM concentration. Briefly, *in vitro* incubation of the conveniently diluted S9 fraction (n=4) with the selected chemicals was conducted at room temperature with either buffer (control), carrier (ethanol) or the targeted chemical for 15 minutes. After this period, residual esterase (AChE and CE) activity was measured and contrasted with the activity of the control (with no drug), as described in section 2.1.2 for AChE with ATC and for CE with 4NPB as substrates (more details in Solé et al., 2021).

2.2.3 Carboxylesterase kinetics

In the particular case of CE measures, kinetic constants were formerly assayed as these parameters have not been described in whole tissue of *L. terrestris*. The apparent Vmax (in nmol/min/mg protein) and Km (in mM) values were determined in the S9 fraction (n=3) using five concentrations of the substrates (4NPA and 4NPB from 0.125 to 4 mM, and for 1NA and 1NB from 0.03 to 1 mM). Vmax and Km were calculated implementing the Michaelis-Menten equation (V=Vmax [S]/Km+[S]) with

the different concentrations and using the linearity transformation of Lineweaver-Burk plot. Since the UV method for 1NA and 1NB shows some limitations in the case of substrate saturation, a protocol with the Fast Blue RR salt (0.7 mM) was considered as an alternative photometric measure in the visible range (405 nm) as recently described (Soto-Mancera, 2020).

2.2.4 Carboxylesterase modulation by contaminants of environmental concern

The drugs: LMG, COC, FIP and BNPP (as positive control), formerly selected for *in vivo* exposures, were also considered for *in vitro* interactions at a unique 100 µM concentration (Shimizu et al., 2014). Furthermore, a wider range of chemicals of environmental concern were also included in the *in vitro* assessment of CE interactions using 4NPB as substrate. They include pharmaceutical drugs (simvastatin-SIM and fenofibrate-FENO), or plastic additives such as the antimicrobial agent triclosan (TCS), the brominated flame retardant tetrabromobisphenol A (TBBPA), the organophosphorus flame retardant Tris(1-chloropropan-2-yl) phosphate (TCPP) and several phthalates (DMP, DnBP, diBP and DEHP). In this last case, purified human recombinant CE enzymes from Sigma-Aldrich (hCE1, ref. E0162 and hCE2, ref. E0412) were also included as quality controls of the *in vitro* protocol (Solé et al., 2020; 2021). Briefly, it consisted on 15 min incubation of the diluted S9 fraction with the selected contaminants at the same 100 µM concentration and the residual CE activity measured as describe in section *2.2.2*.

2.3 Sample preparation for chemical and metabolite analysis.

To study the accumulation and metabolism of tested compounds in well-segmented worm tissues, the whole worms were freeze-dried (LyoAlfa 6, Telstar Technologies, Terrassa, Spain) for one week and kept at -20 °C until the extraction. A modified QuEChERS procedure consisting of a single extraction step was employed by adapting the protocol reported elsewhere (Montemurro et al., 2021). Briefly, 0.5 g of homogenized freeze-dried earthworm sample were transferred in a 50-mL falcon tube and hydrated by adding 8 mL of EDTA-McIIvaine buffer (pH = 4), vortexed using a BenchMixer XLQ QuEChERS Vortexer (Benchmark Scientific, Sayreville NJ, US) and rested for 30 minutes. Thereafter, 50 µL of IS mixture were added to achieve the desired concentration 20 ng/g, vortexed (2500 rpm, 2.5 min) and rested for another 30 minutes. Ten milliliters of ACN were added to the hydrated samples, vortexed for 2 minutes at 2500 rpm and the OR QuEChERS salts (4 g $MgSO_4$ + 1 g NaCl) were transferred into the falcon tube. The tubes were instantly hand shaken in order to prevent agglomeration of the salts and vortexed another time. Finally, the tubes were centrifuged (4000 rpm, 10 min, 4 ºC). A hexane-wash clean-up (Montemurro et al., 2017) was performed to remove common co-eluted matrix interferences, such as fats and phospholipids. Approximately 2 mL of supernatant and 1 mL of hexane were transferred into a test tube for a liquid-liquid extraction. After the tube has been well shaken, the two immiscible layers are expected to form again. Since lipids are apolar, they will preferably dissolve in the hexane layer. Finally, 1 mL of the bottom layer containing ACN was transferred into a 2-mL injection vial, evaporated under a gentle nitrogen flow at room temperature until total dryness and reconstituted with water/MeOH (90:10, v/v). Earthworm samples were analysed using a SCIEX X500R QTOF hybrid system (Sciex, Redwood City, CA, U.S.). More details of the LC-MS/MS analysis and chemical's

253 details are reported elsewhere (Montemurro et al., 2021) and more carefully detailed in
254 SM.

255

256 *2.4 Statistical analysis*

257 Baseline biomarker results are presented as means ± standard error of mean
258 (SEM). Non-parametric Wilcoxon test was performed for biomarker contrasts of drug
259 treated earthworms with respect to those unexposed (control) since not all the
260 parameters tested fulfil parametric requirements. Data for *in vitro* inhibition was
261 contrasted between each residual CE activity after incubations with the chemical of
262 concern (at 10^{-4} M) in respect to its control using the non-parametric Mann-Whitney test
263 (n=4). Statistical analyses were carried out using SPSS System Software v19 or R
264 software and the significance level for data analyses was set at 0.05.

265

266 **3. Results**

267 *3.1 In vivo and in vitro exposures to lamotrigine, cocaine and fipronil*

268 After a 48h exposure following the filter paper contact test assay (OECD num
269 207), none of the 3 chemicals tested including the organophosphorus pesticide BNPP
270 caused a significant effect on total protein content, the neurotoxic marker AChE
271 activity, conjugation phase II pathway measured as GST activity or LPO levels ($p>0.05$)
272 as described in Table 1. By contrast CE activity was enhanced after COC (using 4NPB
273 as substrate) but inhibited by BNPP (using the substrates 4NPB and 1NB) exposures
274 (Table 1). The same chemicals were used to test *in vitro* interactions with the enzymatic
275 pool present in the S9 fraction after 15 min incubations at room temperature. The *in*

276 *vitro* results confirmed the lack of enzyme interaction between the four tested drugs and
277 AChE activity ($p>0.05$) but its action over CE (Fig. 1). The pesticide, BNPP selected as
278 positive control for CE identification, was clearly responsible for CE inhibition in a
279 substrate dependent manner in earthworms and in the two commercial recombinant
280 human isoforms used as test control (Fig. 2). To the best of our knowledge, no
281 information is available between any of the former drugs interaction in earthworms,
282 including *L. terrestris,* and the selected biomarkers of neural transmission (AChE),
283 detoxification (GST and CE) or oxidative stress damage (LPO).

284

285 *3.2 Characterization of earthworm esterases and methodological validation*

286 The use of specific model inhibitors for AChE and CE measures was tested *in*
287 *vitro* using the earthworm S9 fraction to unequivocally validate the adopted esterase
288 protocols. First, model inhibitors of ChEs such as eserine and, in particular BW284c51
289 for AChE, and BNPP for CE-related activities, were used using the assay conditions
290 adopted. As seen in figure 3, an almost full inhibition of AChE activity was observed
291 after eserine (97%) and BW284c51 (87%) incubations while BNPP exposure did not
292 affect significantly affect this activity (1.5% inhibition) in respect to their control
293 activities. By contrast CE activity, measured with the most sensitive substrate (4NPB)
294 was not significantly affected by the ChEs inhibitors (0.3-11% inhibitions) while BNPP
295 significantly did (73.5%). These results confirm the suitability of the S9 fraction used
296 for the enzymatic determinations and the respective protocol substrates selected. In
297 earthworms, including *L. terrestris*, no full inhibitions have been described after the
298 same 100 µM dose of the well-recognized esterase inhibitor chlorpyrifos oxon (CPX)
299 on several independent tissue exposures and using several CE substrates. For instance,

remaining activity showed a resistant fraction (19–47% of control) to CPX when 4NPA was used as substrate (Sanchez-Hernandez and Wheelock, 2009).

The assays: AChE, GST and 4NPB-CE were performed using tissues homogenized at several pH and EDTA presence/absence. Since these activities were not significantly affected in any of the biomarkers tested ($p>0.05$), a 20 mM Tris buffer pH 7.6 with 1 mM EDTA as adopted as in other studies with *L. terrestris* (Sanchez-Hernandez et al., 2018; Gallego et al., 2021).

Enzymatic properties of CEs were assayed in *L. terrestris* (n=4) using a range of concentrations for each substrate. In Table 2 the kinetic parameters of Vmax (maximal velocity), Km (substrate affinity) and catalytic efficiency are indicated. Overall the naphthyl substrates showed a non-saturating reaction rate whereas those butyryl-derived showed substrate saturation behavior at the higher doses. This was reflected in a higher Vmax for the acetate derived substrate (4NPA and 4NA), higher catalytic efficiency for the naphthyl derived (1NA and 1NB) and the substrate affinity following the order 4NPA<4NPB<1NA<1NB which was not rate limiting at any of the selected protocol adopted substrate conditions of 1 mM (4NPA and 4NPB) and 0.25 mM (1NA and 1NB). The adoption of the fast blue colorimetric measure gave similar results to the UV method with comparable kinetics.

3.3 In vitro CE modulation by other chemicals of environmental concern

The chemicals of environmental concern selected were either pharmaceutical drugs (SIM and FENO), or plastic additives such as TCS, TBBPA, TCPP and the phthalates: DMP, DnBP, diBP and DEHP. Some of their characteristics and action over mammalian CEs are reported elsewhere (Solé et al., 2021). The action of these

chemicals over earthworm's S9 CEs fraction was contrasted including human purified isoforms CE1 and CE2. The present results validated the specific action of some drugs over particular human isoforms, and a less clear action over earthworm CEs, as seen for several aquatic invertebrates (Solé et al., 2021). CE measures with 4NPB were responsive to the plastic additives TCS and TBBPA in earthworm tissue homogenates (Figure 4).

3.4 Chemical characterization and metabolite identification of earthworms exposed lamotrigine, cocaine and fipronil

Qualitative and quantitative analysis were performed using SCIEX OS™ Software version 1.6 (Sciex, Redwood City, CA, U.S.). Two high resolution ions were used for confirming a positive finding for the identification in HR-QToF-MS analysis: the most abundant product ion for the quantification and the precursor ion for the confirmation (SANTE/12682/2019) (Commission, 2019). Linearity of the method was evaluated using the internal standard calibration approach with a calibration curve constructed between 0.1-6000 ng g^{-1} d.w. performed with a minimum of eight calibration points. Calibration curves were constructed using linear weighted least-squares regression (1/x as weighting factor) by plotting the ratio of the analyte signal to that of its corresponding IS and presenting coefficients of determination (R^2) above 0.99 for most compounds (ESM). Regarding sensitivity, Limit of Detection (LODs) and Limits of Quantification (LOQs) were estimated from the matrix-matched calibration curves using linear regression analysis and a signal-to-noise ratio of 3.3 and 10, respectively (Table 3).

A suspect analysis approach was used for the metabolism or degradation studies of the tested compounds taking advantage of the benefits of excellent sensitivity with accurate mass detection and rapid analysis offered by the TOF systems and SWATH acquisition. The identification of the formed metabolites was obtained by comparing the raw data obtained during the SWATH acquisition with an exhaustive list containing the information on probable metabolites for each compound obtained considering the most probable metabolic reactions (Table S1). Additionally, only for COC and FIP, a further list including common metabolites or transformation products previously reported in the literature was manually compiled by consulting the integrated SCIEX NIST-2017 spectral library included in the SCIEX OS™ software (Table S2).

The probable metabolites were tentatively confirmed using the monoisotopic masses and their isotope distributions, the mass spectra (fragment ions, for NIST list only) and the Formula Finder algorithm. Additional confidence criteria for identifying candidates were their absence in the blank and the control samples, a plausible retention time (RT), a minimum peak area ≥1000 cps, a minimum peak width of three points, and a reasonable isotopic pattern (Intensity ≥1000 cps). For high quality and accurate mass spectra, the relative error between the theoretical and observed values must have a mass accuracy threshold of ± 5 ppm on both the monoisotopic peaks and the mass of the fragments (where present).

The main parental compounds and metabolites quantified and/or identified by HR-QToF-MS analysis including the predicted molecular formulas, the expected retention time, theoretical and observed mass, mass error and formula score are indicated in Table 4.

4. Discussion

An OECD exposure protocol at a sub-lethal and acute dose of three chemicals of concern (LMG, COC and FIP), likely to be present in TWW was applied in earthworms. This protocol included biomarker measures, chemical analysis and metabolite identification. It allowed identifying the pathways of metabolism in this invertebrate, including some candidate biomarkers, as well as metabolites for more realistic field approaches.

Lamotrigine (LMG) is a phenyltriazine that inhibits voltage-gated sodium channels, decreasing release of glutamate and aspartate, and inhibits serotonin, norepinephrine and dopamine reuptake. *In vivo* exposures to this drug for 48 h using the filter paper contact test did not reveal any significant effect in the parameters selected in earthworms. To the best of our knowledge, there is no documentation of LMG effects on any of the parameters here selected in earthworms or other invertebrates. However, other triazine derivates such as the herbicide atrazine could shed some light into a similar mechanistic action. An oxidative stress condition and DNA damage caused by ROS formation was seen in *E. foetida* after 7-days exposure to atrazine (Song et al., 2009). Nonetheless, oxidative stress damage to lipids measured as LPO was not observed in exposed *L. terrestris* after the 48 h exposure. Among the effects on other common biomarkers here tested stands a sex- and concentration dependent response in GST in zebrafish after atrazine administration (Zhu et al., 2011). Confirmation of LMG uptake and metabolism was performed by the determination with a targeted LC-HRMS method for LMG and its metabolite LMG-N2-oxide in earthworms' whole tissue. The HR-MS data analysis of the tissue extracts revealed the presence of N2-methyl-LMG, a known human metabolite of this antiepileptic drug, and the 5-desamino-5-oxo-2,5-dihydro-LMG, which had been previously detected in biodegradation studies with

activated sludge from a wastewater treatment plant (Zonja et al., 2016). As far as phase II metabolites of LMG are concerned, the substrate was proposed to undergo N-glycosylation followed by malonylation of the primary hydroxyl group of the sugar (hexose) but also amino acid conjugation leading to LMG-N5-Phe-Glu formation as a result of a multi enzyme step reactions originating from the reaction with the dipeptide phenylalanine glutamic acid (Figure 5). Studies on earthworms, leaded by Bundy and co-workers on the enzyme-mediated transformation of 4-fluoroaminoaniline (Bundy et al., 2002) unequivocally identified the glutamyl conjugate by means of LC-NMR and LC-MS. While no evidence for the formation of simple conjugates could be gathered, the mass spectrometric data indicated the presence of a double conjugated phase II metabolite.

Cocaine (COC) is a well-studied drug of abuse in humans, present in STWs under specific multitudinary events (Mastroianni et al., 2017). COC is a substrate for hydrolases such as CEs (Di, 2019) and in *L. terrestris*, the use of 4NPB in CE determinations was seen as the most adequate substrate to reveal an increase on CE activity. This substrate has been repeatedly seen as more adequate in invertebrates (Dallares et al., 2019; Nos et al., 2020; Solé and Sanchez-Hernandez, 2018). Modulation of the biomarkers here selected was reported in *Daphnia magna* in a 21-day exposure study to two COC concentrations that revealed no effect on GST activity while ecological relevant endpoints such as offspring production were affected (De Felice et al., 2019). The marine mussel *Mytilus galloprovincialis* exposed to COC and BE, independently and as mixture, responded in terms of oxidative stress parameter alterations only after 96h in a tissue dependent way (De Felice and Parolini, 2020). Metabolic, antioxidant and AChE responses were also time and tissue dependent in brown mussels *Perna perna* exposed to COC (Barbosa Ortega et a., 2019). No studies

421 are available in earthworms exposed to COC although other drugs of abuse such as
422 synthetic cannabinoids interfere with human CEs *in vitro* (Thomsen et al., 2015). The
423 metabolites of COC detected with LC-HRMS were BEG, COET, and the minor
424 metabolite norcocaine (NC), all known as common human metabolites. Extracted ion
425 chromatogram, isotopic pattern, mass spectra, and library confirmation for all of them
426 are reported in Figures S1-S3. Although NC and BEG have the same monoisotopic
427 mass, it was possible to discriminate them thanks to the different retention time and the
428 use of the database (Fig. S2) as well as the use of reference standard (only for BEG).
429 Norcocaine (NC) although minority, it appears to be an active metabolite of cocaine
430 (Hawks et al., 1974). The detection of the hexose conjugate of COC (COC-Hex) was
431 rationalized as the reaction of the tertiary amine to yield a quaternary ammonium
432 compound (Figure 6). The origin for COET formation in *L. terrestris* is unknown as in
433 other invertebrate groups, such as Drosophila the presence of exogenous ethanol was a
434 requirement for this metabolite formation (Torres and Horowitz, 1999). In vertebrate
435 studies, other than in humans, dogs metabolized COC to COET when COC was
436 administered orally but not by an intravenous way (Parker and Laizure, 2010). A
437 hypothetical anaerobic metabolic pathway leading to the required ethanol necessary for
438 COET formation can only be speculated.

439 Fipronil (FIP), a controversial insecticide whose regulation is still under
440 discussion, was administered as a technical brand. Fipronil though dermal exposure did
441 not affect any of the biomarkers tested *in vivo* or *in vitro* in earthworms after 48 h
442 exposures. Former studies in fish, FIP administered via diet with the same chemical
443 formulation was responsible for an oxidative stress condition and esterase modulation
444 but the signs were not clearly manifested until 14 days food administration (Dallares et
445 al.,, 2020; Sanahuja et al., 2020). Nonetheless, larvae of the chironomid *Chironomus*

446 *riparius* responded to FIP dosage after 48 h in terms of reduced GST and catalase but
447 not AChE activity (Monteiro et al., 2019). Toxicity evaluation due to FIP exposures
448 using biomarkers is well documented in many organisms and mostly associated to
449 oxidative stress (Wang et al., 2016). Ecotoxicity by this insecticide in soil dwelling
450 earthworms has been evaluated in reproduction and survival endpoints (Zortea et al.,
451 2018). Fipronil toxicity has been seen as enantiomer dependent in *E. fetida* (Qu et al.,
452 2014). Fipronil, as the active ingredient of the commercial insecticide Standak 250 SC,
453 was revealed as no toxic to *E. andrei* in relation to other insecticides using the
454 avoidance test and loss weight as endpoints (Alves et al., 2013). Chemical analysis of
455 whole tissue identified FIP sulfone (FIP-Sulf) as the main metabolite identified in
456 exposed earthworms which confirmed uptake and phase I biotransformation of FIP.
457 Mining the HR-MS data for suspected metabolites, indicated the presence of three
458 additional biotransformation products desulfinyl (FIP-Desulfinyl), amide (FIP-Amide),
459 hexose (FIP-Hexose) and hexose-malonyl derivatives (FIP-Mal-Hex) (Figure 7).
460 Namely FIP-amide as the product of the hydrolysis of the nitrile group as well as two
461 conjugates originating from glycosylation (FIP-hexose) and subsequent malonylation
462 (FIP-Mal-hex) common reactions to those revealed after LMG exposures. The two latter
463 reactions were also observed in the metabolic pathway of a polycyclic aromatic
464 hydrocarbon, pyrene, in various invertebrate species, including *E. andrei*, (Stroomberg
465 et al., 2004) with C-hydroxylation affording the handle for conjugation. Taking into
466 account the metabolic N-glycosylation of the model compound, 4-fluoroaniline in
467 *Eisenia veneta* (Bundy et al., 2002), it is plausible to postulate that the sugar is attached
468 to the amino group of the pyrazole ring.

469 From an environmental perspective, biomarker responses in earthworms after
470 acute (short term, high concentration under lab conditions) and chronic (longer term to

low environmental exposures under field conditions) may, however, differ. This was clearly illustrated in another species, *Eisenia fetida*, when exposed to the pyretroid bifenthrin either by the filter paper contact test or under realistic soil exposures (Li et al., 2017). Moreover, metabolic rates and metabolite formation in earthworms in respect to other more studied groups (likely vertebrates) are also factors to consider in terms of toxicity performance. In particular, CEs as a confirmed biomarker to respond to a broad range of xenobiotics in mammalian systems (Di, 1019), has not been described in whole tissue of earthworms. Instead, a comprehensive characterization has been described in selected tissues of *L. terrestris* (Sanchez-Hernandez et al 2009) and of the freshwater gastropod *Planorbarius corneus* (Otero and Kristoff, 2016) using the same commercial substrates. Despite differences due to either specific or whole tissue contrasts, a common substrate preference (lower Km), hydrolysis rates (higher Vmax) and the resulting higher catalytic efficiencies are shared within the naphthyl forms in the contrasted studies. The use of model inhibitors it also allowed confirming the true nature of the measures although full inhibitions were not achieved. This lack of uncomplete inhibition, when using tissue extracts, is a common trend observed in invertebrate species including earthworms (Sanchez-Hernandez et al., 2009; Solé et al., 2021) but still supports the whole tissue earthworm CE measures.

An additional and complementary *in vitro* approach, broadening the number of chemicals of environmental concern, was adopted as formerly applied to marine invertebrates (Solé et al., 2021). Some of these chemicals are plastic additives of concern in natural soils that had been amended with sludge from STWs. This is the case for TBBPA (Abdalah et al., 2016); phthalates (Lv et al., 2018); TCS (Thomaidi et al., 2016), and TCS together with complex mixtures of pharmaceuticals (Verlicchi and Zambello, 2015). The consequences for these chemical exposures in earthworms have

been mostly evaluated under laboratory conditions (see Table 1 in Solé, 2020). In particular, TCS and TBBPA, both with endocrine disrupting properties, significantly acted on 4NPB-CE activities. Thus, we propose the inclusion of this enzymatic measure in soil-dwelling organisms likely contaminated by plastics.

5. Conclusions

In vivo and *in vitro* contrasts in earthworm's enzymatic system confirmed the suitability of the whole tissue homogenate and the use of butyrate ester as more adequate CE substrates. *L. terrestris* were little affected by chemicals of concern under the contact test protocol condition, however, they involvement in drug metabolism (e.g. COC) was demonstrated. The use of LC-TOF-MS/MS techniques allowed to unequivocally identifying, some for the first time, phase I and phase II metabolites of LMG, COC and FIP. This *in vivo* and *in vitro* approach could be further applied to other compounds of environmental concern present in soils and, in particular CE measures using 4NPB could be candidate biomarkers of plastic pollution in contaminated soils.

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Table 1. Protein content in mg/ml and enzyme activities in µmol/min/g w.w and LPO in nmol MDA/g w.w. in the S9 whole tissue fraction of *L. terrestris*. Data are mean ± SEM (n=8).* Indicates significant difference in respect to control. Acronyms as in M&M.

Treatment	Protein	AChE	GST	4NPA-CE	4NPB-CE	1NA-CE	1NB-CE	LPO
Control	6.39 ± 0.58	3.71 ± 0.46	6.37 ± 0.46	5.65 ± 0.92	4.79 ± 0.32	11.84 ± 0.73	4.91 ± 0.25	55.06 ± 3.00
Lamotrigine	6.26 ± 0.69	4.40 ± 0.67	7.32 ± 0.52	6.40 ± 1.00	5.61 ± 0.38	13.12 ± 1.09	5.62 ± 0.29	46.87 ± 2.16
Cocaine	6.85 ± 0.50	3.90 ± 0.21	5.99 ± 0.36	6.71 ± 1.09	6.20 ± 0.51*	13.45 ± 0.73	5.83 ± 0.42	48.77 ± 1.32
Fipronil	5.74 ± 0.37	3.12 ± 0.34	5.80 ± 0.27	5.19 ± 0.82	3.92 ± 0.28	11.14 ± 0.59	4.16 ± 0.31	56.57 ± 5.75
BNPP	6.58 ± 0.15	3.13 ± 0.34	6.27 ± 0.42	4.51 ± 0.98	0.97 ± 0.09*	9.40 ± 1.40	1.11 ± 0.10*	48.58 ± 2.56

Table 3. Methodological performance of LC-MS analysis of some parental (underlined) and metabolites identified in whole earthworm tissue. LOD=limit of detection and LOQ= limit of quantification.

	Linearity ($ng\ g^{-1}$)	LOD ($ng\ g^{-1}$)	LOQ ($ng\ g^{-1}$)	R^2	Accuracy (%) at 20 $ng\ g^{-1}$
Lamotrigine (LMG)	1-1000	0.00	0.01	0.99716	63.1
N2-Methyl-Lamotrigine	1-2000	0.17	0.52	0.97976	73.9
5-Desamino 5-Oxo-2,5-dihydro lamotrigine	100-4000	0.04	0.13	0.9954	57.5
Lamotrigine N2-oxide	1-2000	0.30	0.91	0.99652	80.3
Cocaine (COC)	1-6000	0.06	0.18	0.99705	80.6
Benzoylecgonine	5-6000	0.05	0.16	0.99743	56.5
Cocaethylene	2-4000	0.06	0.18	0.99764	100.4
Fipronil (FIP)	1-2000	0.001	0.002	0.99277	101.1
Fipronil Sulfone	1-2000	0.004	0.013	0.98988	94.0
Fipronil Desulfinyl	1-2000	0.004	0.012	0.98583	90.6

Table 4. LC-TOF-MS/MS characteristics of parental (in capitals) and putative metabolites/compounds detected in earthworm whole tissue.

	Expected Retention time (min)	Predicted formula of parent	Score of predicted formula	Measured mass (m/z)	Calculated mass (m/z)	ppm error	Monoisotopic mass	Confirmation	Quantified
LAMOTRIGINE	**4.29**	**C9H7Cl2N5**	**87.5**	**256.0146**	**256.0157**	**-4.2**	**255.0079**	**YES**	**YES**
N2-Methyl-Lamotrigine	4.8	C10H9Cl2N5	81.6	270.0317	270.0307	3.5	269.0235	YES	YES
5-Desamino 5-Oxo-2,5-dihydro LMG	5.69	C9H6Cl2N4O		bloq	256.9991		255.9919	YES	
Lamotrigine N2-oxide	4.03	C9H7Cl2N5O	80.3	272.0108	272.0100	2.7	271.0028	YES	YES
LMG-N5-Hex	3.17	C15H17Cl2N5O5	95.3	418.0694	418.0685	2.1	417.0607	NO	
LMG-N5-Malonyl-Hex	7.97	C18H19Cl2N5O8	71.1	504.0734	504.0689	8.8	503.0611	NO	
LMG-PheGlu	6.17		97.8	532.1198	532.1267	-12.9	531.1189	NO	
COCAINE	**4.78**	**C17H21NO4**	**92.3**	**304.1541**	**304.1549**	**-0.8**	**303.1470**	**YES**	**YES**
Benzoylecgonine	3.5	C16H19NO4	88.0	290.1386	290.1386	-0.1	289.1314	YES	YES
Cocaethylene	5.76	C18H23NO4	87.8	318.1704	318.1699	1.2	317.1627	YES	YES
Norcocaine	5.1	C16H19NO4	86.5	290.1384	290.1386	-0.9	289.1314	YES	NO
FIPRONIL	**8.91**	**C12H4Cl2F6N4OS**	**97.3**	**434.9294**	**434.9309**	**-3.3**	**435.9386**	**YES**	**YES**
Fipronil desulfinyl	9.06	C12H4Cl2F6N4	99.9	386.9635	386.9644	-2.4	387.9717	YES	YES
Fipronil sulfone	9.25	C12H4Cl2F6N4O2S	94.9	450.9260	450.9263	-0.8	451.9336	YES	YES
Fipronil amide	7.87	C12H6Cl2F6N4O2S	86.9	452.9412	452.9419	-1.6	453.9493	YES	NO
Fipronil-Hex	6.42	C18H14Cl2F6N4O6S	-	596.9814	596.9837	-3.8	597.9915	NO	
Fipronil-Malonyl-Hex	8.39	C21H16Cl2F6N4O9S	-	682.9833	682.9841	-1.1	683.9919	YES	NO

Table 2. Kinetic parameters of Vmax (maximal velocity) in nmol/min/mg prot, Km (substrate affinity) in mM and their ratio Vmax/Km (catalytic efficiency) of carboxylesterases (CE) using different substrates. Data are mean ± SD (n=3). Sample dilution (SD) of whole tissue earthworm S9 fraction. UV= ultraviolet.

	4NPA-CE	4NPB-CE	1NA-CE (UV)	1NA-CE (Fast Blue)	1NB-CE (UV)	1NB-CE (Fast Blue)
Sample dil.	1/20	1/10	1/40	1/40	1/20	1/20
Vmax	191.4 ± 2.6	63.5 ± 0.99	244.3 ± 8.6	230.7 ± 6.8	61.6 ± 1.21	47.0 ± 0.53
Km	1.09 ± 0.04	0.274 ± 0.003	0.142 ± 0.006	0.207 ± 0.007	0.033± 0.001	0.099 ± 0.001
Vmax/Km	176.3 ± 8.4	232.0 ± 3.12	1718 ± 51.8	1115 ± 4.1	1841 ± 39.7	476.3 ± 2.58

Fig 1. *In vivo* and *in vitro* effects on acetylcholinesterase (AChE) and carboxylesterase (CE) activities after exposures to selected drugs. Data are indicated as mean ± SEM (n=8). In the *in vitro* exposures, the line at 100% represents the control value and the respective bars the percentage of remaining activity in respect to the control after 15 min drug incubations. * denotes significant difference in respect to control ($p<0.05$).

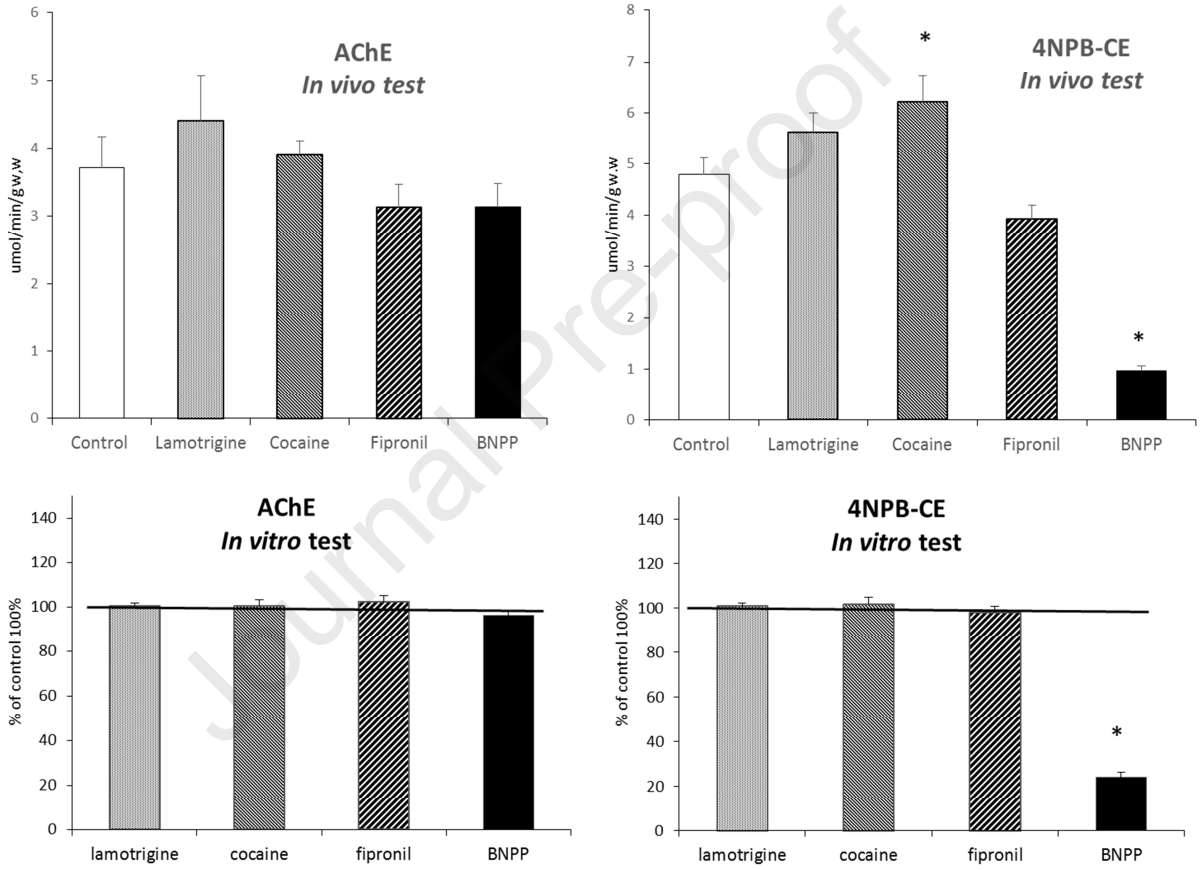


Fig 2. Percentage of residual carboxylesterase (CE) activity using 4 commercial substrates (acronym in the M&M section) in respect to control (100%) after *in vitro* incubations with 100 µM of the model pesticide BNPP (n=4). Human CE isoforms (CE1 and CE2) are included for contrasts.

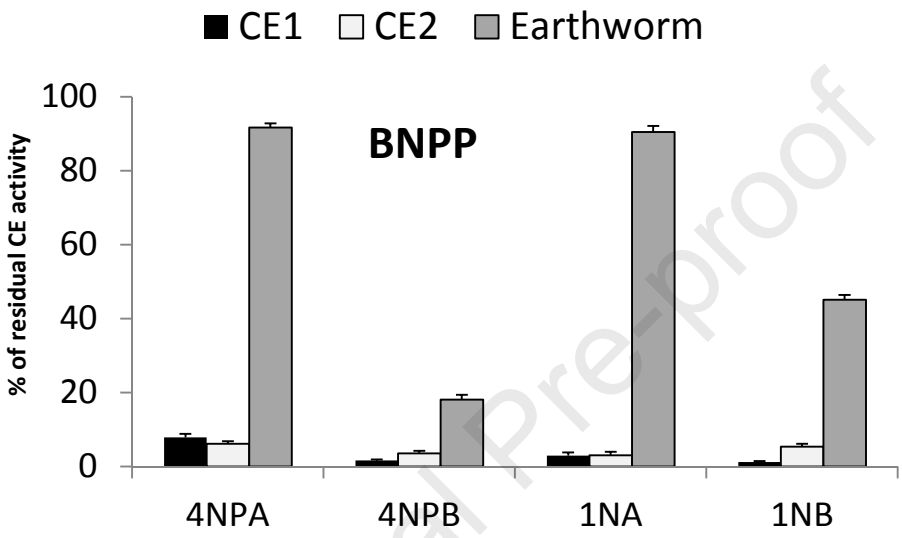


Fig. 3. Percentage of residual acetylcholinesterase (AChE) and carboxylesterase (CE) activities after *in vitro* incubations to specific model inhibitors: eserine for cholinesterases, BW284c51 for AChE and BNPP for CE (n=4). All at 100 µM concentration for 15 min.

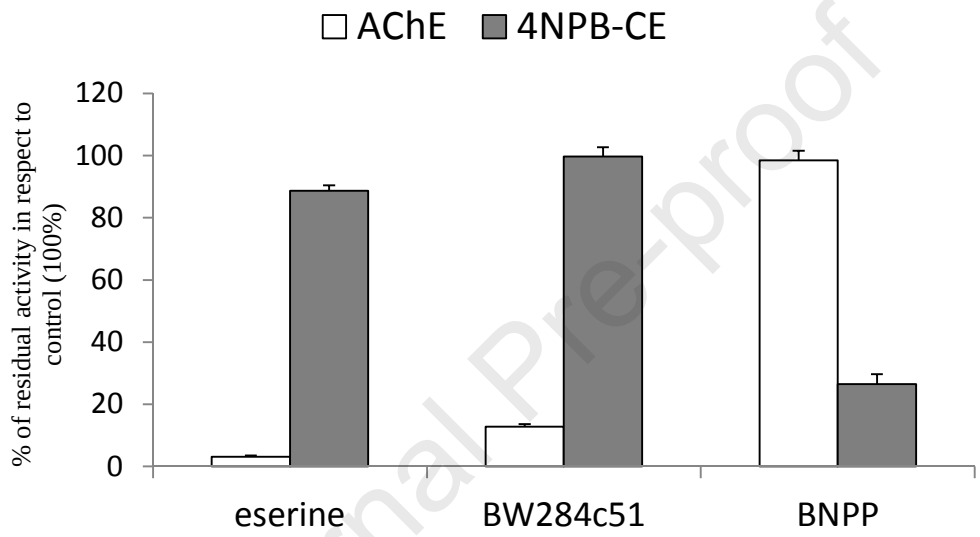


Figure 4. Percentage of residual activity carboxylesterase activity using 4NPB as substrate (4NPB-CE) after 15 min incubation with 100 µM concentration of several chemicals of environmental concern on human carboxylesterases (CE1 and CE2) and S9 tissue homogenates of whole tissue earthworms *Lumbricus terrestris* (n=4). List of full name in the M&M section. * indicates statistical difference ($p<0.05$) in respect to control (100%).

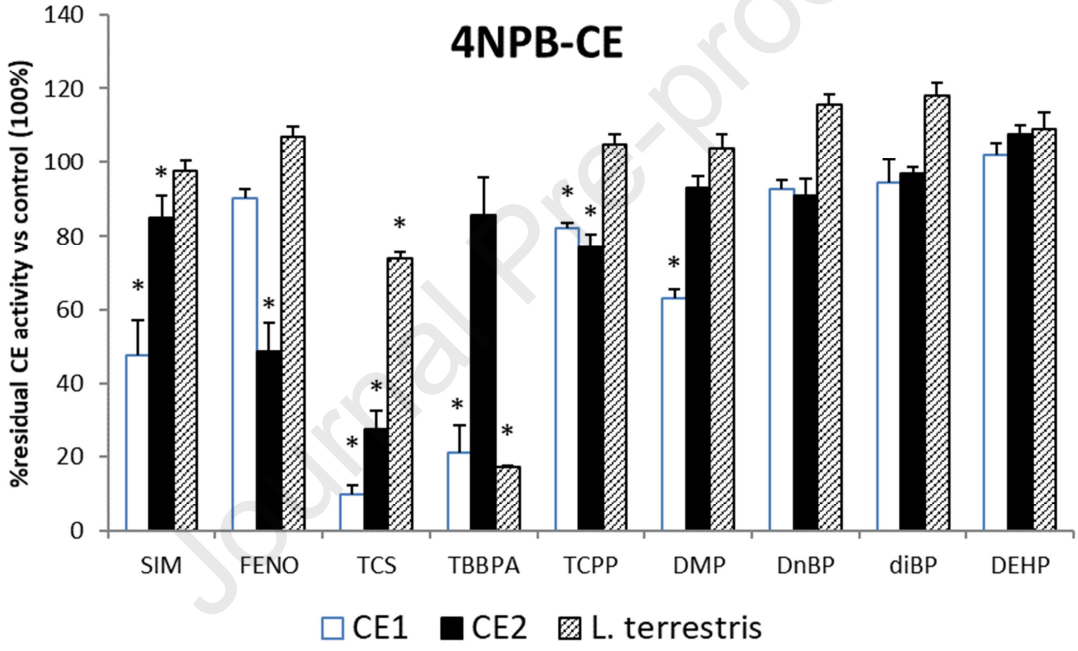


Figure 5. Metabolites identified by LC-MS in earthworm whole tissue after 48 h incubation with lamotrigine (LMG) following the paper contact OECD test protocol. On the left phase I metabolites and on the right an underlined those following phase II metabolism

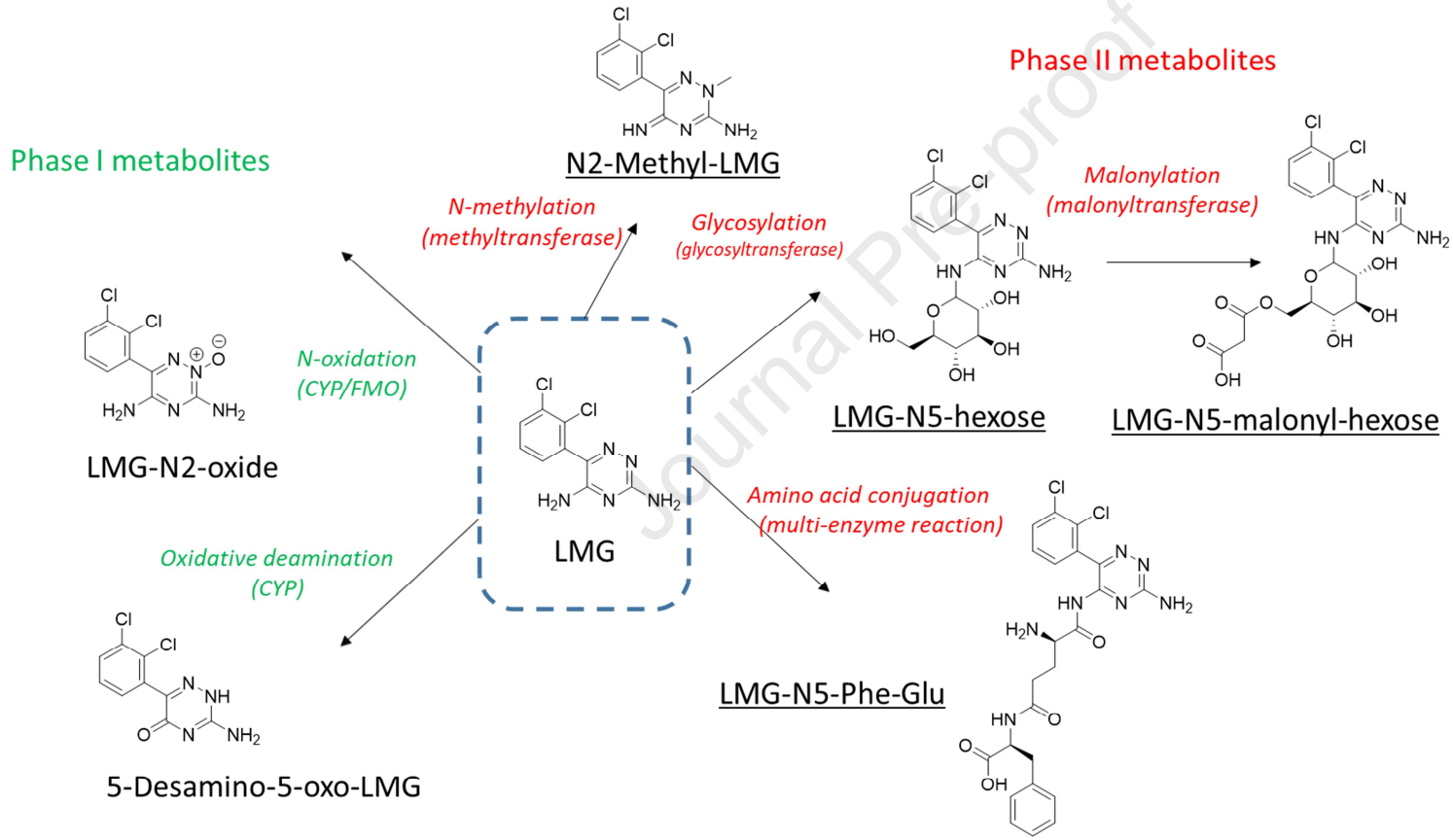


Figure 6. Metabolites identified by LC-MS in earthworm whole tissue after 48 h incubation with cocaine (COC) following the paper contact OECD test protocol. On the left phase I metabolites and on the right an underlined those following phase II metabolism

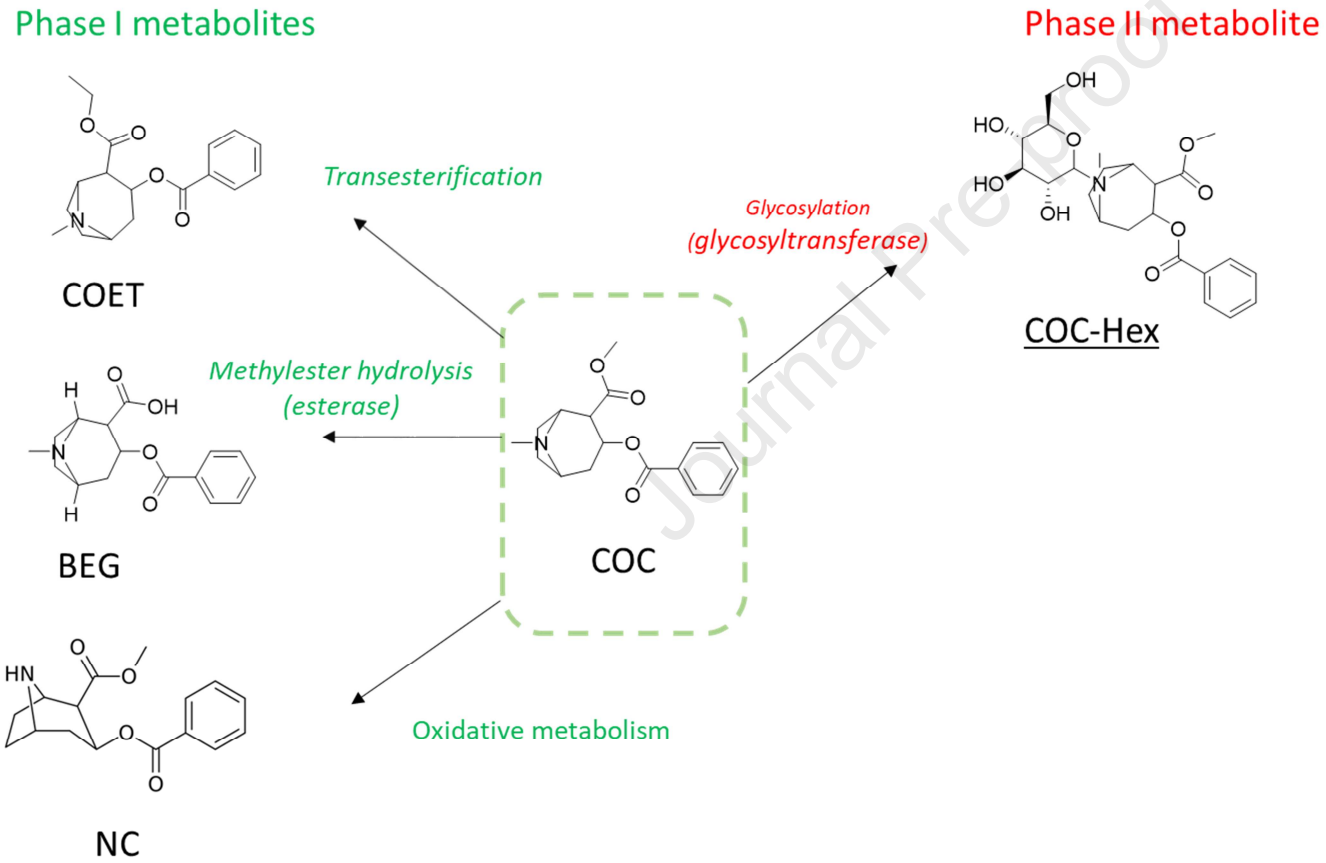
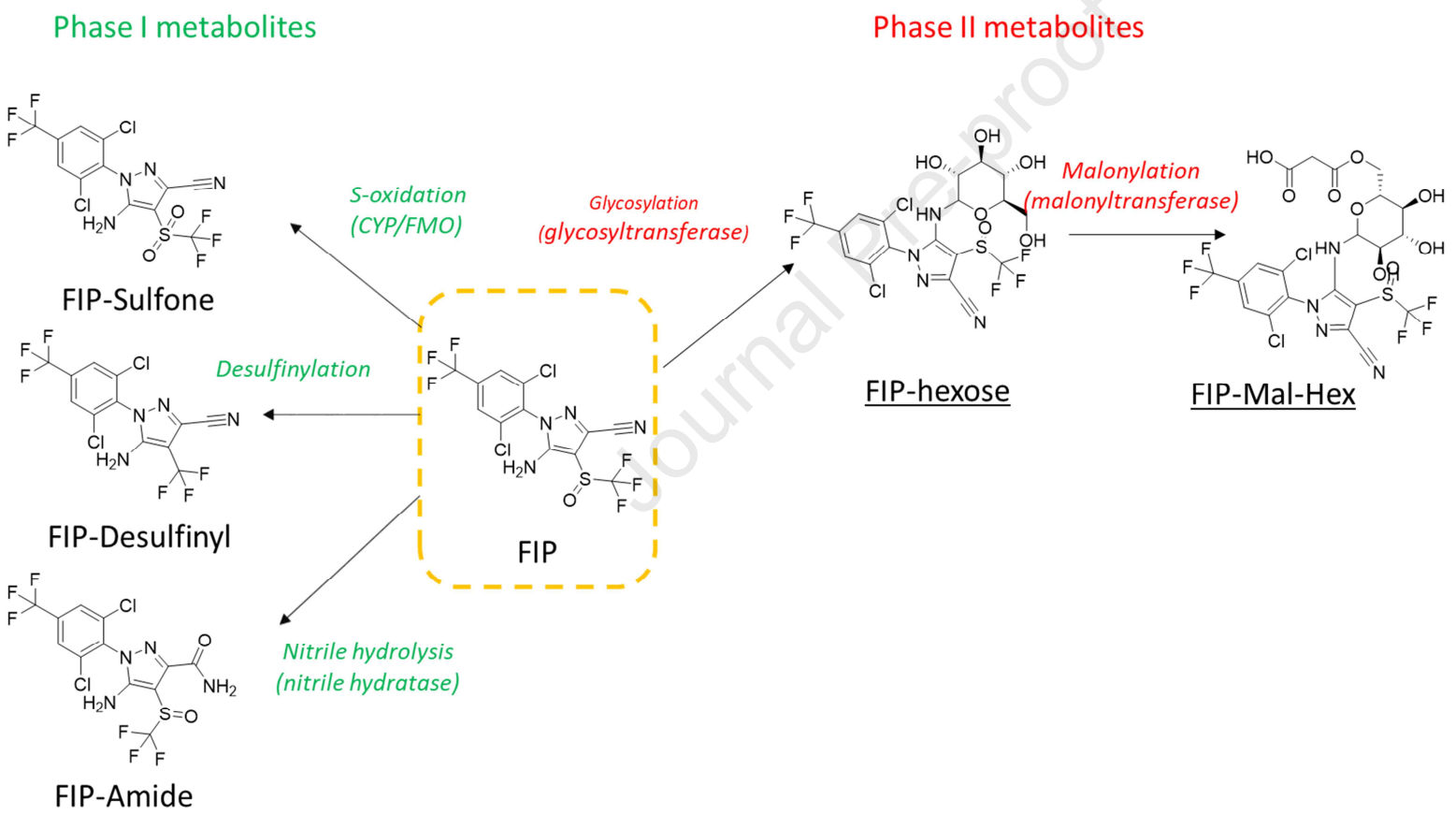


Figure 7. Metabolites identified by LC-MS in earthworm whole tissue after 48 h incubation with fipronil (FIP) following the paper contact OECD test protocol. On the left phase I metabolites and on the right an underlined those following phase II metabolism.



Highlights

- First detection of lamotrigine, cocaine and fipronil metabolites in earthworms
- New metabolite of lamotrigine from its conjugation with phenylalanine glutamine
- Carboxylesterases are involved in cocaine metabolism in earthworms
- Detection of the main cocaine human metabolites in earthworms
- Carboxylesterases are potential biomarkers of contaminated soils

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: