Biomarker responses in *Lumbricus terrestris* exposed to model drugs

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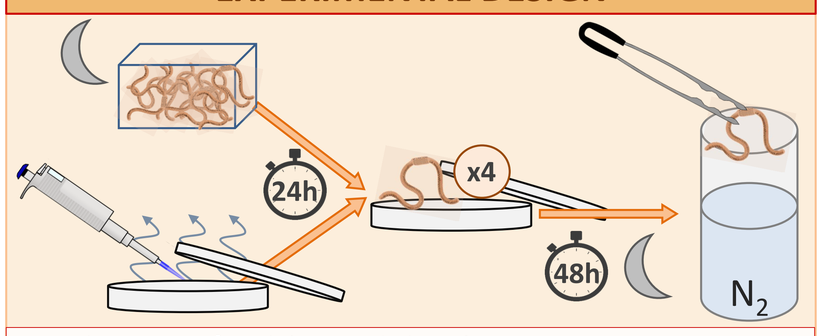
ABSTRACT

The earthworm *Lumbricus terrestris*, is a common anecic key species in natural soils used also as bioindicator in pollution monitoring. Specimens of *L. terrestris* were exposed in Petri dishes to filter paper embedded with 1 mg/mL to different compounds for 48 h. The chemicals were: Lamotrigine, Cocaine, Fipronil (as Regent®800WG) and the organophosphorus pesticide bis-4-nitrophenyl phosphate (BNPP). At the end of this period, earthworms were immediately frozen in liquid N_2 and their tissues, homogenized by cryogrinding, aimed to determine chemical exposure and metabolite formation as well as to biomarker determinations. The biomarkers analyzed were the activities of the enzymes Acetylcholinesterase (AChE), Glutathione-S-transferase (GST) and Carboxylesterase (CES) using different substrates. The results obtained revealed differences in enzymatic activity among substrates, being CE activities with the substrates 4-nitrophenyl butyrate (4-NPB) and 1-naphthyl butyrate (1-NB) the most responsive to cocaine and BNPP. The results for BNPP, corroborate data obtained after *in vitro* exposure. This study highlights the utility of longer chain butyrate esters in CE measures in invertebrate and the agreement between *in vivo* and *in vitro* responses in this species. Chemical analysis and metabolite formation should confirm the extent of the exposure and other range of concentrations should be used to assess any dose-response effects.

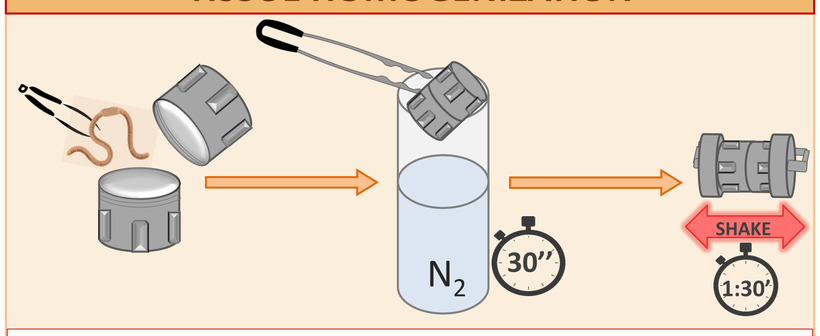
MATERIAL AND METHODS

EXPERIMENTAL DESIGN



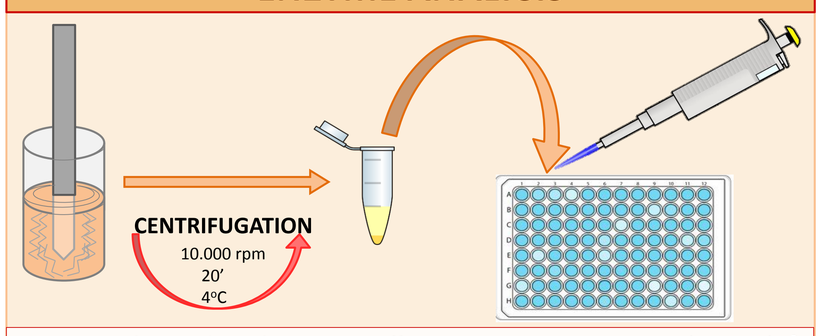
- Acclimatization→ 24 h (15ºC in darkness).
- Petri dishes with 1mg/mL pollutant (let evaporate) x 2.
- Rehydrate→ 2mL of MiliQ water and place 4 individuals/plate.
- Incubate 48h at 15ºC in total darkness.
- Check survival and freeze with liquid N_2.
- Store them at -80ºC individually for further analysis.

TISSUE HOMOGENIZATION



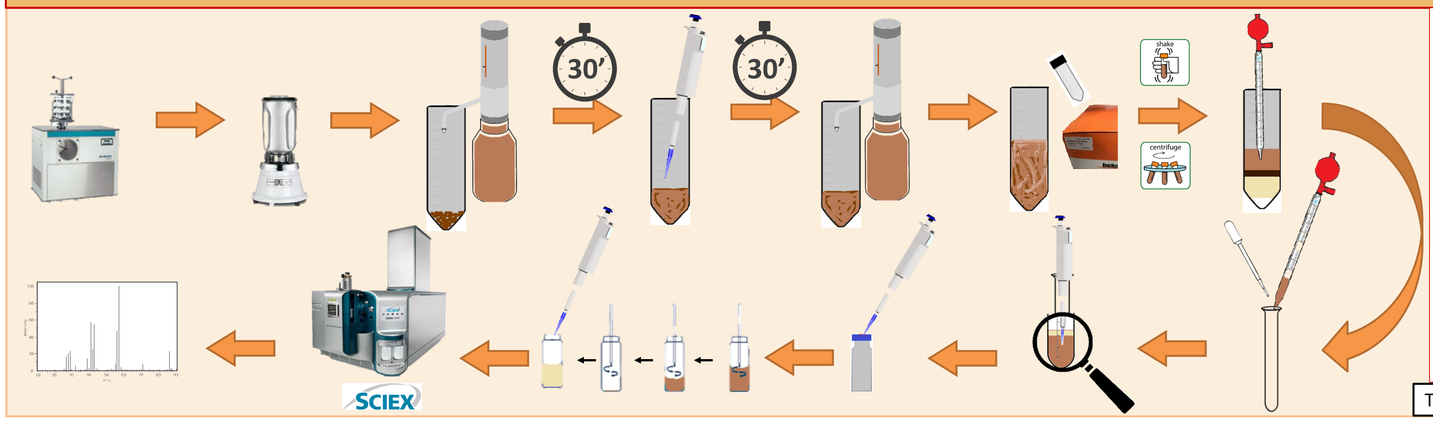
- Place frozen earthworm into steel capsule.
- Immersed capsule in liquid N_2 for 30 seconds.
- Place capsule in a mixer and grinded (MM400) for 1:30' (frequency: 28 repeats/second).
- Collect homogenous powder in a Falcon 15mL and store at -80ºC.

ENZYME ANALYSIS



- 0.3 g pulverized earthworm + Buffer Tris 20mM + 1mM EDTA (pH=7.6) → Ratio 1:5.
- Sonicate (cycle = 0.5, amplitude = 60, during 5 cycles x 5) and centrifuge (20 min, 10.000 rpm at 4ºC) → Frozen supernatant.
- Enzymatic analysis → 25µl sample + 200µl RM (buffer-P 50 mM pH 7.4 and substrate). Read spectrophotometer.

CHEMICAL METHOD DEVELOPMENT



- Earthworm samples get lyophilized and homogenized.
- 0.5 g pulverized earthworm is hydrated with 8 mL EDTA-McIlvaine buffer pH=4, afterwards vortexed (2:30 min, 2500 rpm) and left for 30 min.
- Sample is spiked with internal standard, vortexed and left for 30 minutes.
- 10 mL ACN (solvent) is added to the falcon and again vortexed.
- The original QuEChERS salts (4g $MgSO_4$ + 1g NaCl) are added. The falcon is shaken by hand and vortexed. Subsequently the falcon is centrifuged (10 min, 4000 rpm, 4°C)
- 2 mL supernatant is transferred into a test tube, where 1 mL hexane is added. Afterwards vortexed again at 1500 rpm for 1:30 min.
- 1 mL of the underlying layer is transferred into injection vials, to get evaporated and finally reconstituted in 1 mL H_2O/MeOH (90:10).
- Finally sample is analyzed and quantified by LC/HRMS QTOF X500R.

The modified QuEChERS methodology is currently being applied to analyze the uptake of 50 PCs and metabolites in earthworms

RESULTS AND DISCUSSION

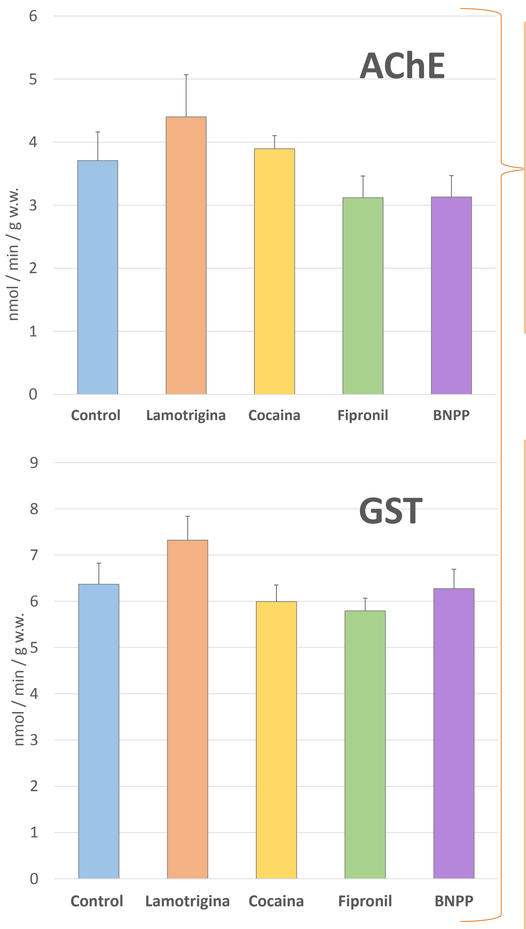


Figure 1. GST and AChE activity in earthworm exposed to different compounds of environmental concern

AChE and GST activities were not affected by in vivo drug exposures of earthworms. In vitro inhibition test with whole tissue homogenate confirmed the lack of enzymatic interaction with AChE activity.

Lamotrigine is a drug used to treat epilepsy and different mental disorders, it causes a significant potentiation in neuromuscular synaptic transmission and reduces action potential firing[1].
Cocaine is a drug of abuse that can be found in WWTPs.
Fipronil is an insecticide acting as GABA channel antagonist, highly selective for arthropods (500 times more affinity than in mammals)[2].
BNPP is an organophosphorus pesticide used as positive control for CE identification.

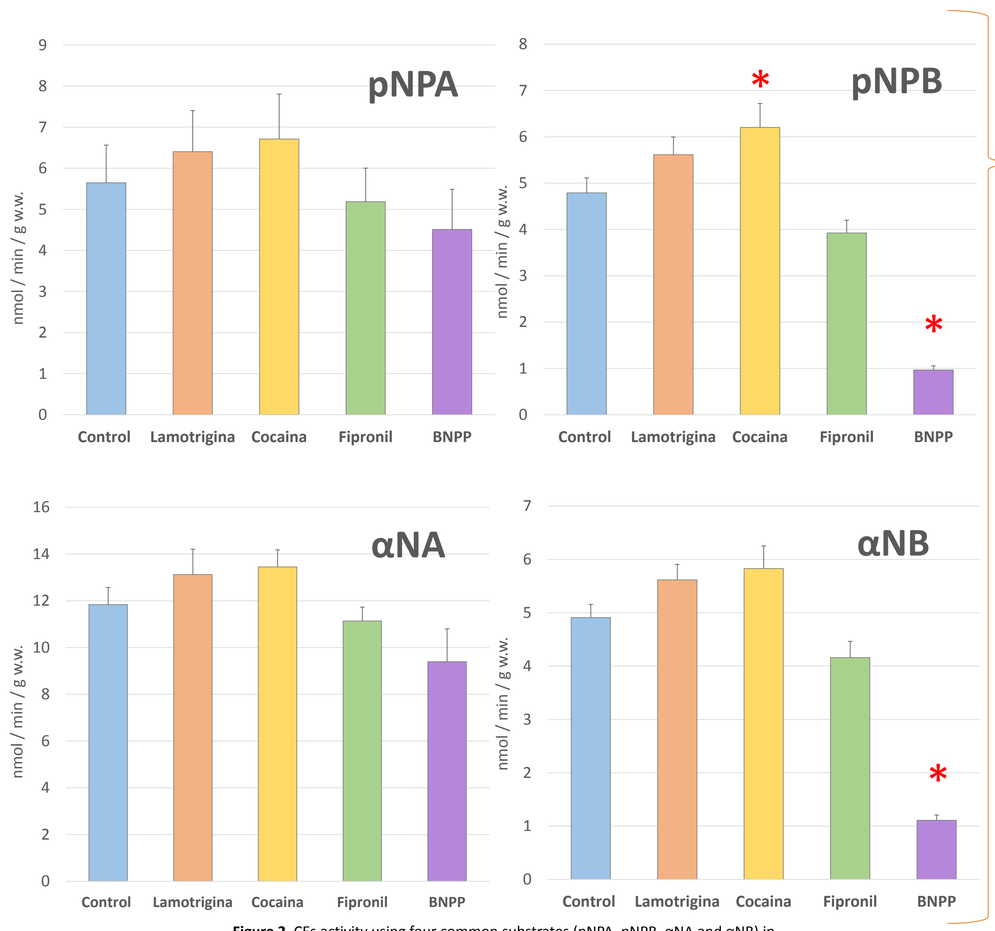


Figure 2. CEs activity using four common substrates (pNPA, pNPB, αNA and αNB) in earthworms exposed to different compounds. Significant differences to control are indicated by an asterisk (*).

Four different substrates that have been used to determinate carboxylesterases. pNPB and αNB are the most adequate substrates.

BNPP inhibits carboxylesterases in invertebrates, and was used as test control[3].
Cocaine caused an induction of the activity mostly seen with pNPB as substrate. Other studies in invertebrates, benzoylecgonine (cocaine residue) also caused alterations in different biomarkers[4].
Lamotrigina and **Fipronil** did not alter carboxylesterase activity.
These *in vivo* results were confirmed *in vitro* mainly for BNPP exposures.

CONCLUSIONS

- BNPP is a suitable compound for the diagnose of carboxylesterases.
- Butyrate substrates (pNPB and αNB) are more adequate for carboxylesterase activity measures in invertebrates.
- pNPB may be an optimal substrate for carboxylesterase measures as an indicator of exposure to cocaine and potential derivatives for invertebrates.
- Chemical analysis will allow us to know the exposure and identify metabolites.

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