TP 293

Innovative Integration of LC-HRMS and In-Silico Libraries for Pharmaceutical Metabolite Annotation and MALDI-HRMS Spatial Residue Analysis in Environmental Samples

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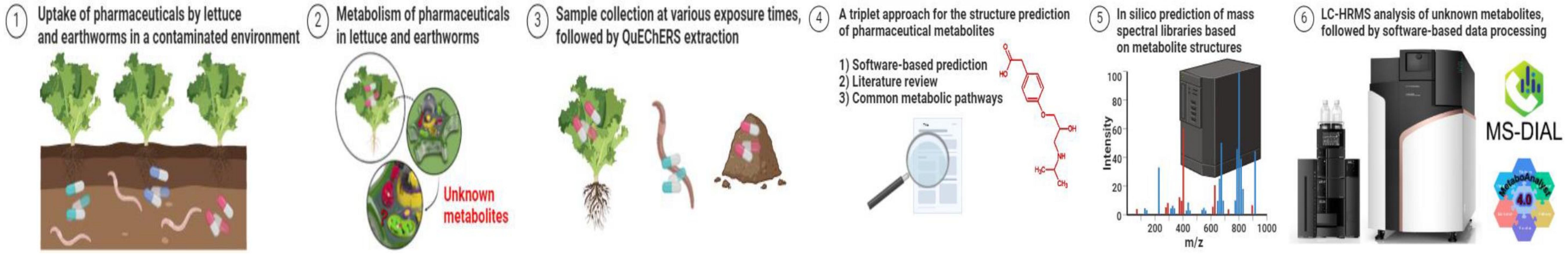
Workflow for LC-HRMS Metabolite Annotation

Steps 1–3: *Lactuca sativa* and *Eisenia fetida* were exposed to a six-pharmaceutical mix (atenolol, enrofloxacin, erythromycin, ketoprofen, sulfamethoxazole, tetracycline) in soil or water. Root and leaf samples of *Lactuca sativa*, *Eisenia fetida*, as well as soil and water samples were collected at multiple time points during the exposure experiment and analysed using a validated QuEChERS extraction method.
Step 4: Metabolite structures were predicted via literature review, software tools (BioTransformer 3.0, GLORYx, enviPath), and known metabolic pathways.
Step 5: In-silico MS/MS libraries (ESI+/−) were generated using CFM-ID 4.0, aided by a custom script developed at BUT FCH and FEEC.
Step 6: Water and lettuce extracts were analysed at Shimadzu Europa GmbH using LCMS-9050 (DDA & DIA, ESI+/−). Data were processed in MS-DIAL 4.0 and statistically evaluated with MetaboAnalyst 4.0.



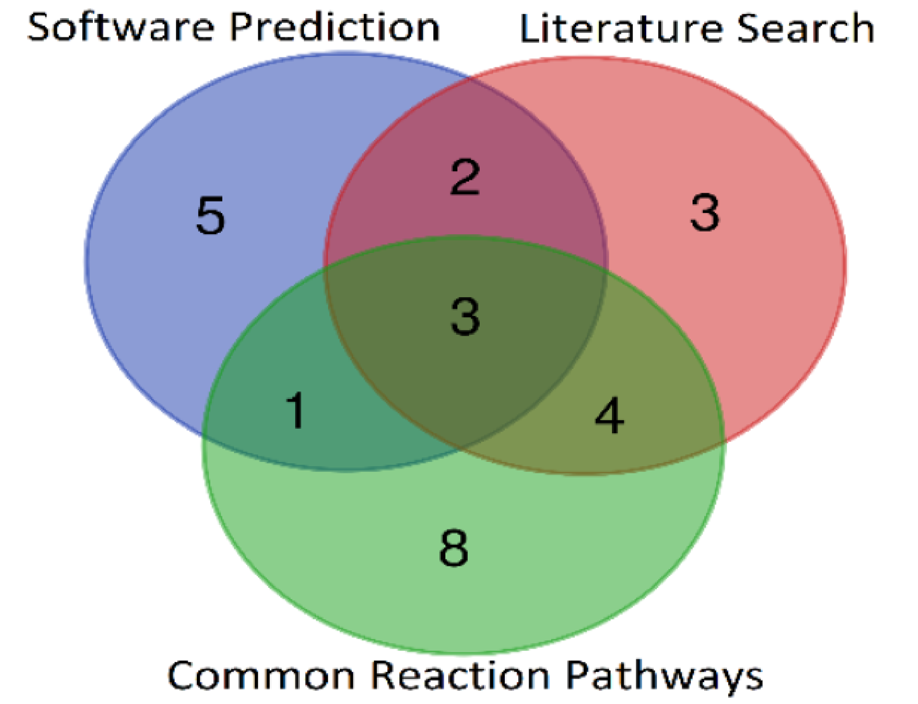


Fig. 1 Venn Diagram Comparing Metabolite Identification Efficiency Across Different Prediction Methods, Showing the Number of Metabolites Identified by Each Method.

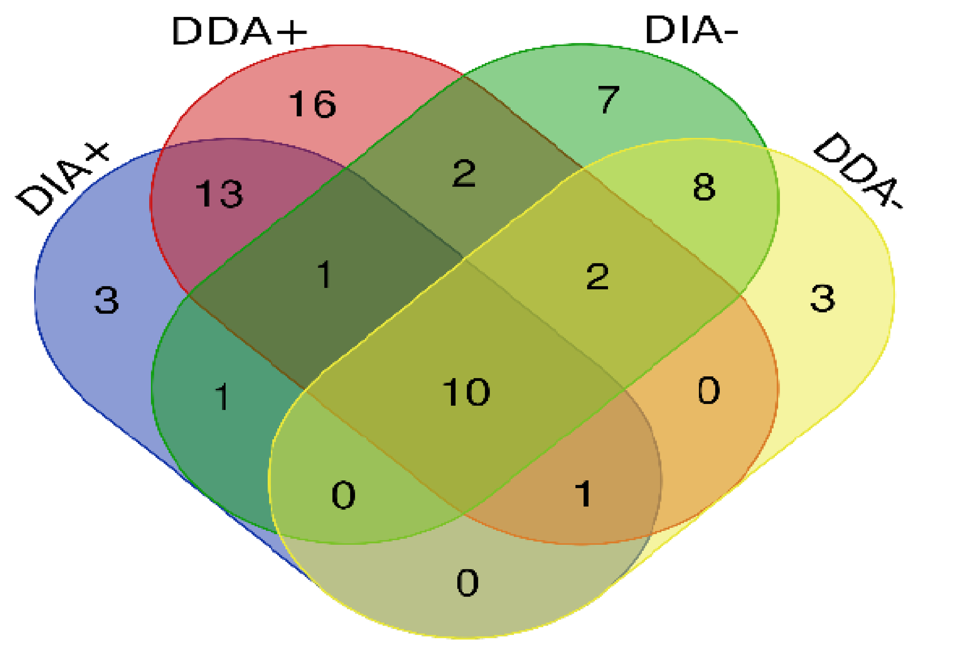


Fig. 2 Venn Diagram Comparing Metabolite Identification Efficiency Across Different MS/MS Modes, Showing the Number of Metabolites Identified by Each Mode.

Table 1 Presence of parent drugs and their metabolites in samples of earthworms and lettuce (roots and leaves) at different exposure times

	Soil Earthworms						Soil Lettuce						Hydroponics Lettuce					
	Soil	EW					Soil	Lettuce Roots			Lettuce Leaves		Water	Lettuce Roots			Lettuce Leaves	
Compound Name	21 Days	1 Day	3 Days	7 Days	14 Days	21 Days	28 Days	14 Days	21 Days	28 Days	21 Days	28 Days	28 Days	14 Days	21 Days	28 Days	21 Days	28 Days
Atenolol	✓	✓	✓	✓			✓						✓	✓	✓	✓	✓	✓
Atenolol-LS1		✓	✓	✓	✓	✓		✓	✓	✓	✓	✓	✓	✓	✓	✓		
Enrofloxacin	✓	✓	✓	✓	✓	✓	✓	✓			✓	✓	✓	✓	✓	✓	✓	✓
Enrofloxacin-R63														✓	✓	✓		
Enrofloxacin-R13														✓	✓	✓		
Ciprofloxacin														✓	✓	✓		
Enrofloxacin-R2														✓	✓	✓		
Enrofloxacin-M242														✓	✓	✓		
Erythromycin	✓	✓	✓	✓	✓	✓	✓						✓	✓	✓	✓		
Erythromycin-M1													✓	✓				
Erythromycin-M46	✓												✓	✓	✓	✓		
Erythromycin-M361													✓	✓				
Ketoprofen	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓		
Ketoprofen-R13								✓	✓	✓	✓	✓		✓	✓	✓		
Ketoprofen-R23									✓	✓				✓	✓	✓		
Ketoprofen-R63									✓	✓				✓	✓	✓		
Ketoprofen-R81								✓	✓	✓	✓	✓		✓	✓	✓		
Ketoprofen-R87									✓	✓								
Ketoprofen-R93									✓	✓								
Ketoprofen-R96										✓								
Ketoprofen-R111														✓	✓	✓		
Ketoprofen-LS24									✓	✓				✓	✓	✓		
Ketoprofen-LS25														✓	✓	✓		
Ketoprofen-M648									✓	✓								
Ketoprofen-M835								✓	✓	✓	✓	✓		✓	✓	✓		
Sulfamethoxazole	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	
Sulfamethoxazole-LS1		✓	✓	✓	✓	✓		✓	✓	✓	✓	✓		✓	✓	✓		
Sulfamethoxazole-LS6														✓	✓	✓		
Sulfamethoxazole-R63								✓	✓	✓	✓	✓		✓	✓	✓		
Tetracycline	✓	✓	✓	✓	✓	✓	✓	✓			✓		✓	✓	✓	✓		
Tetracycline-LS2														✓	✓	✓		
Tetracycline-LS3														✓	✓	✓		

MALDI-HRMS Spatial Residue Analysis

Pre-step: *Eisenia fetida* was initially exposed to a mixture of three pharmaceuticals—enrofloxacin, erythromycin, and tetracycline, each representing a distinct class. Samples were collected at 0-, 1-, 3-, and 5-days post-exposure followed by a depuration step.
Step 1: Earthworms were euthanized by freezing at −20 °C, embedded in OCT compound, and sectioned at a thickness of 10–15 µm using a cryostat maintained at −20 °C. The tissue sections were then mounted onto ITO-coated slides.
Step 2: Consequently, DHB (30 mg·mL⁻¹ in MeOH:H_2O 70:30 with 0.25% TFA) was sprayed onto the sample using the iMLayer AERO system (Shimadzu, Japan). Matrix application was carried out with the following settings: stage speed 40 mm·s⁻¹, drying time 30 s, 8 layers, wash after 8 layers, nozzle distance 5 cm, and scan pitch 1 mm.
Step 3: Optical images of earthworms were captured using an optical microscope in the iMScope™ QT (Shimadzu, Japan) before matrix application. MSI was performed with the same instrument equipped with an Nd:YAG laser (355 nm) under atmospheric pressure. Laser parameters: 80 shots, 2000 Hz repetition rate, 5 µm diameter, 20 µm × 20 µm pitch, and 77.6 laser intensity. The iMScope™ QT was coupled to an LCMS-9030 (Shimadzu, Japan) operating in positive ion mode, scanning m/z 100–1000.
Step 4: Data processing was performed using IMAGEREVEAL MS (Shimadzu, Japan).

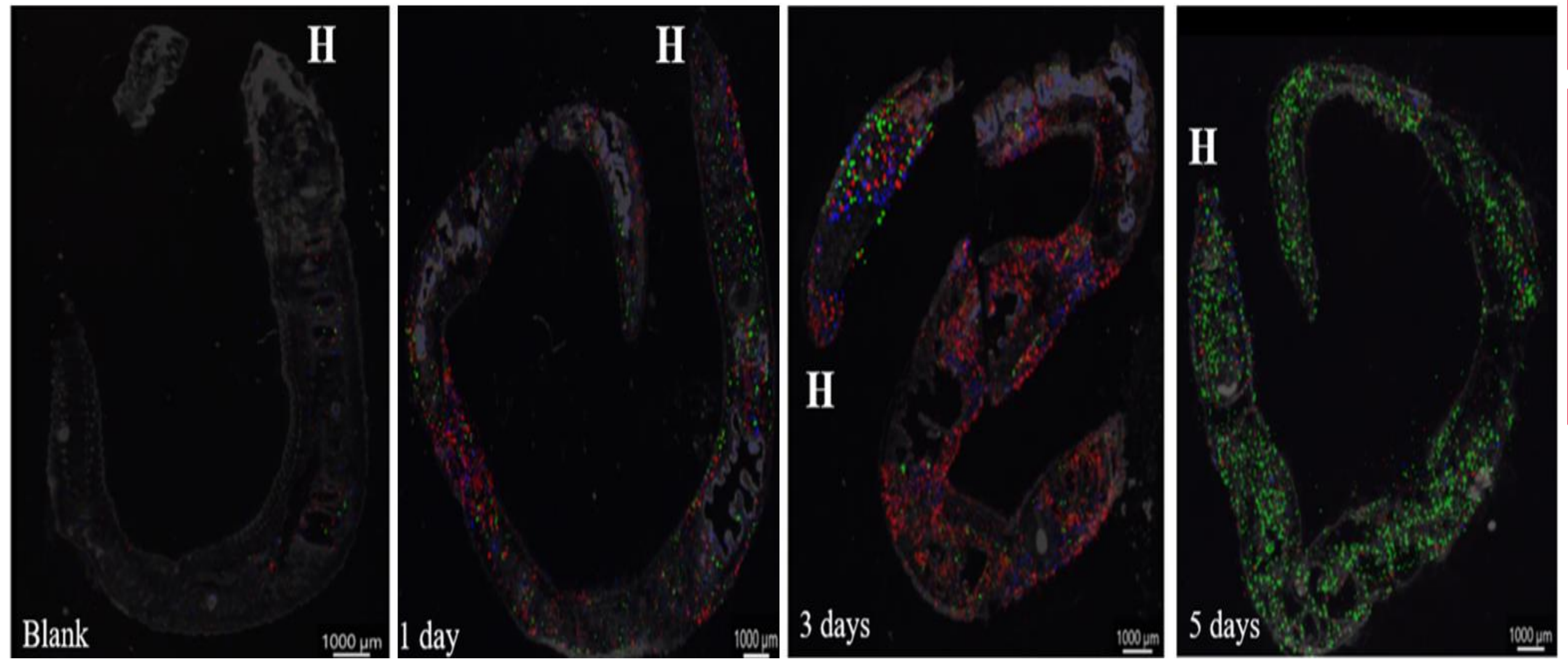


Fig 3. Spatial distribution of veterinary antibiotics in earthworm tissue visualized by blend MS images (Enrofloxacin = Red; Tetracycline = Blue and Erythromycin = Green). Time of earthworm exposure to contaminated soil is noted in bottom left, and "H" Indicates earthworm head position

Conclusion

A novel high-throughput workflow for annotating pharmaceutical metabolites in environmental samples (lettuce roots and leaves, and earthworms) was developed using open-source tools and LC-HRMS. Combining software prediction, literature review, and known pathways, 3,762 metabolites were predicted for six parent compounds (atenolol, enrofloxacin, erythromycin, ketoprofen, sulfamethoxazole, tetracycline). In silico MS/MS libraries were generated for both ionization modes and analysed using MS-DIAL for DDA and DIA data. In total, 26 statistically significant metabolites ($p < 0.05$) were annotated. Additionally, these results demonstrate that neglecting pharmaceutical metabolites leads to an underestimation of potential health and environmental risks.

Mass spectrometry imaging (Figure 3) showed uniform distribution of pharmaceuticals in *Eisenia fetida*, indicating uptake via both skin and ingestion. These findings advance understanding of bioaccumulation in soil organisms, support improved environmental risk assessment, and represent the first spatial analysis of drug uptake in *E. fetida* from a terrestrial matrix.

Acknowledgment

This work was measured within the Lab4you Shimadzu student program. Moreover, this study was financially supported by the Ministry of Education, Youth, and Sports of the Czech Republic (Project No. FCH-S-25-8807).

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