An NMR-based metabonomic approach to the investigation of coelomic fluid biochemistry in earthworms under toxic stress

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Abstract The endogenous metabolites of the coelomic fluid of the earthworm *Eisenia veneta* were characterised using high-resolution one-dimensional and two-dimensional ^{1}H nuclear magnetic resonance spectroscopy. Signals from common organic acids, such as acetate, fumarate, malonate, malate, formate, and succinate, were identified together with adenosine and nicotinamide mononucleotide. The potential use of this information as a baseline dataset for future toxicological or physiological studies was demonstrated by a metabonomic analysis: a series of earthworms were dosed with the model compound 3-fluoro-4-nitrophenol, and toxic effects followed by multivariate analysis of the spectral data of the coelomic fluid. Relative concentrations of acetate and malonate were decreased in the dosed worms compared to the controls. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Metabonomics; 3-Fluoro-4-nitrophenol; Coelomic fluid; *Eisenia veneta*

1. Introduction

Many studies have investigated the composition of the coelomic fluid of earthworms at an enzymatic level, and this has demonstrated the presence of haemolytic, proteolytic, and cytotoxic enzymes that are active against foreign cells and peptides (e.g. [1–4]). However, there is currently little knowledge of the metabolite complement of the coelomic fluid of earthworms, and how this varies in response to physiological or toxic stressors.

^{1}H Nuclear magnetic resonance (NMR) spectroscopy is well suited as a technique for the analysis of metabolites in complex biological matrices, and is thus a useful probe of physiological stress and toxicity. A wide range of small organic molecules can be characterised and quantified using ^{1}H NMR spectroscopy [5], and the approach is particularly useful when, as in this case, there is no prior knowledge of the potential analytes. Only minimal sample preparation is required (typically removal of particulate matter and addition of a small percentage of deuterated solvent to provide a field-frequency lock for the spectrometer), and no further sample derivatisation or separation is required [6–8]. Consequently, there is neither preselection of target analytes nor loss of components caused by sample preparation. Analysis of biofluids, particularly urine and blood plasma, has been shown to be successful in detecting metabolic changes induced by xenobiotics in mammalian systems (e.g. [6,7,9]). The same approach has also successfully been applied to the ecotoxicity analysis of earthworms using a tissue homogenate prepared from the entire earthworm [10,11]. This use of the whole-worm homogenate possesses the obvious disadvantage that a metabolic change resulting in the relative concentration or depletion of endogenous biochemicals in a specific tissue or fluid may be obscured in the whole-worm homogenate. A secondary disadvantage is that the extra procedural steps of homogenisation and extraction are required compared to biofluid analysis. This increases sample preparation time and may discriminate against certain target groups of analytes. As a consequence, we have investigated the usefulness of worm coelomic fluid for observing pathologically induced biochemical changes.

The ^{1}H NMR spectra that are produced from such biofluids are highly complex, with a large number of resonances, many of which are overlapping. Pattern recognition (PR) techniques are necessary in order to fully interpret differences between spectra. Multivariate data analysis facilitates the detection of differences, even if they occur in minor resonances. Furthermore, this offers the potential of identifying novel biomarkers based on a combination of the levels of several different compounds simultaneously, rather than considering the responses of individual compounds in isolation. The use of PR allows useful conclusions to be drawn about metabolic changes, even when exact details of the metabolic pathway or pathways affected are unknown [12].

Here, the coelomic fluid of the earthworm *Eisenia veneta* was characterised by use of one- and two-dimensional ^{1}H NMR spectroscopy, with the aim of providing fundamental knowledge about the composition of earthworm coelomic fluid at the small-molecule level, and to provide baseline data that can be used in future toxicological or physiological studies. The xenobiotic 3-fluoro-4-nitrophenol (3F4NP) was selected as a model compound to demonstrate the potential utility of the baseline data set in ecotoxicology testing, and

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Abbreviations: TMAO, trimethylamine-*N*-oxide; NMN, nicotinamide mononucleotide; NMR, nuclear magnetic resonance; 3F4NP, 3-fluoro-4-nitrophenol

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the NMR spectra from the dosed worms were analysed using PR methods.

2. Materials and methods

2.1. Extraction of coelomic fluid

Coelomic fluid was extracted by applying a potential difference across a worm, causing it to extrude coelomocytes and coelomic fluid. The worm was placed in a Petri dish in an electrolyte solution (0.1% NaCl) that also served to collect the extruded coelomic fluid (ECF), and a potential difference of 9 V applied across the worm for 20–30 s. For the initial characterisation studies, coelomic fluid from a pool of five worms was extracted in 4 ml of 0.1% NaCl solution. For the series of 3F4NP-dosed worms, coelomic fluid from each individual was extracted in 0.5 ml of 0.1% NaCl solution, and stored at −20°C until analysis.

2.2. Dosing of earthworms

Earthworms were dosed based on a standard OECD protocol for a filter paper contact test [13]. An aqueous solution (1 ml) of 3F4NP (Fluorochem, Old Glossop, Derbyshire, UK) was added to a rectangle of filter paper (Whatman number 2) of area 52 cm^2, cut to fit exactly inside a glass vial with screwtop lid. Ten replicates were prepared at concentrations of 0, 0.25, 0.5, 0.75, 1.0, and 1.25 μg cm^{-2}. An adult individual of *E. veneta* was added to each vial, and placed in the dark at 16°C for 3 days. At the end of the 3-day period, survivors were counted and coelomic fluid extracted as described above.

2.3. Sample preparation

The ECF was cleared of coelomocytes and suspended non-dissolved matter by centrifuging at 17 000×*g* for 20 min. 450 μl of ECF was then mixed with 200 μl of 0.2 M phosphate buffer solution (pH 7.4) and 100 μl of D_2O. The phosphate buffer solution contained NaN_3, to prevent bacterial growth in the samples while waiting analysis, such that the final concentration in the prepared sample was 0.1% w/v. The D_2O contained sodium trimethylsilyl-2,2,3,3-$^2H_4$-propionate (TSP) at a concentration of 0.05% w/v. The D_2O solution provided a field-frequency lock for the spectrometer, while the TSP was used as a chemical shift reference (δ 0.0).

2.4. NMR spectroscopy

2.4.1. Initial characterisation of endogenous metabolite profiles. ^1H NMR spectra were obtained at 300 K using a Bruker Avance DRX600 spectrometer (Bruker, Coventry, UK), operating at 600.22 MHz and equipped with a 5 mm broad-band inverse probe. One-dimensional spectra were acquired using a standard pulse sequence for water suppression, with an additional T_1 relaxation delay of 2 s. 4096 free induction decays (FIDs) were collected with a spectral width of 12 019 Hz into 64 K data points. An exponential function equivalent to a line-broadening of 0.3 Hz was applied prior to Fourier transformation.

Two-dimensional COSY spectra were acquired over a spectral width of 7183 Hz in both dimensions, into 2048 data points in the F1 dimension and 256 data points in the F2 dimension. 64 FIDs were collected per data point in the F2 dimension. A T_1 relaxation delay of 2 s was included. A sine-bell apodisation function was applied to both F1 and F2 dimensions before Fourier transformation.

2.4.2. Analysis of coelomic fluid from 3F4NP-dosed worms. One-dimensional water-suppressed NMR spectra were acquired as above, except that a flow-injection probe with an autosampler was used to introduce samples into the magnet. In this case, 320 FIDs were collected into 49152 data points, and zero-filled to 64 K. A further relaxation delay of 1 s was included, giving a total pulse recycle time of 2.04 s.

2.5. Data analysis

One-dimensional ^1H NMR spectra were manually phased and baseline-corrected prior to conversion into numerical values using proprietary software (AMIX 2.1; Bruker Analytik, Rheinstetten, Germany). In this procedure, each spectrum is divided into a number of contiguous segments and the total area within each segment is integrated, to provide a set of intensity-based descriptors for each NMR spectrum. A segment width of 0.01 ppm was chosen, and the spectra quantified

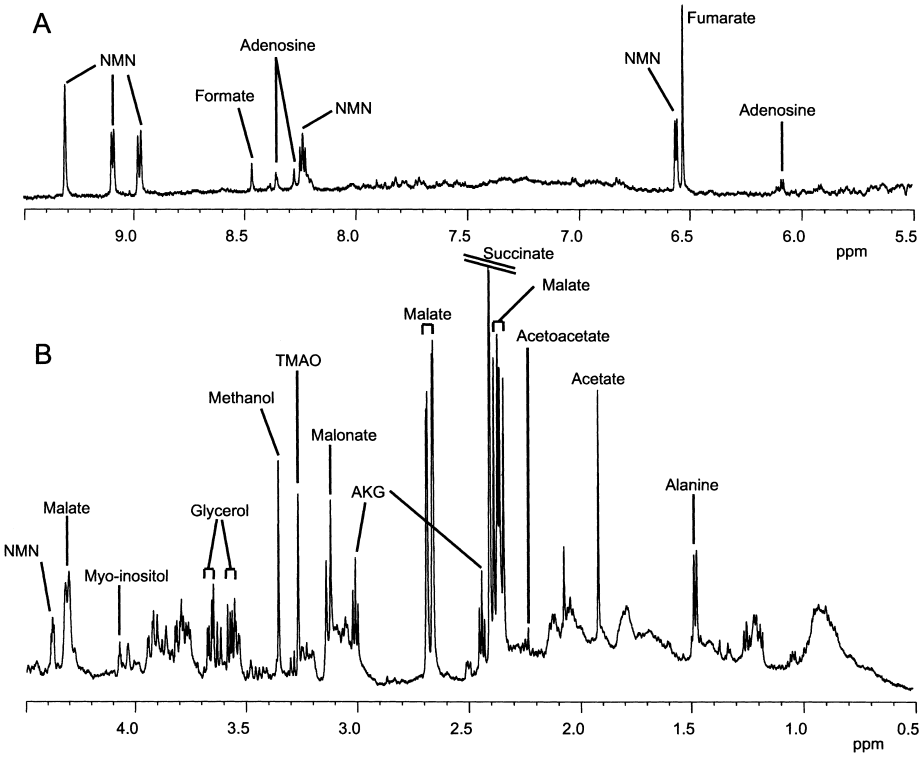

Fig. 1. One-dimensional 600 MHz ^1H NMR spectrum of coelomic fluid. A: Aromatic region (×2 compared to aliphatic region). B: Aliphatic region. AKG: α-ketoglutarate. Resonance due to succinate (δ 2.41) is not represented to its full height so as to allow clearer viewing of the other resonances.

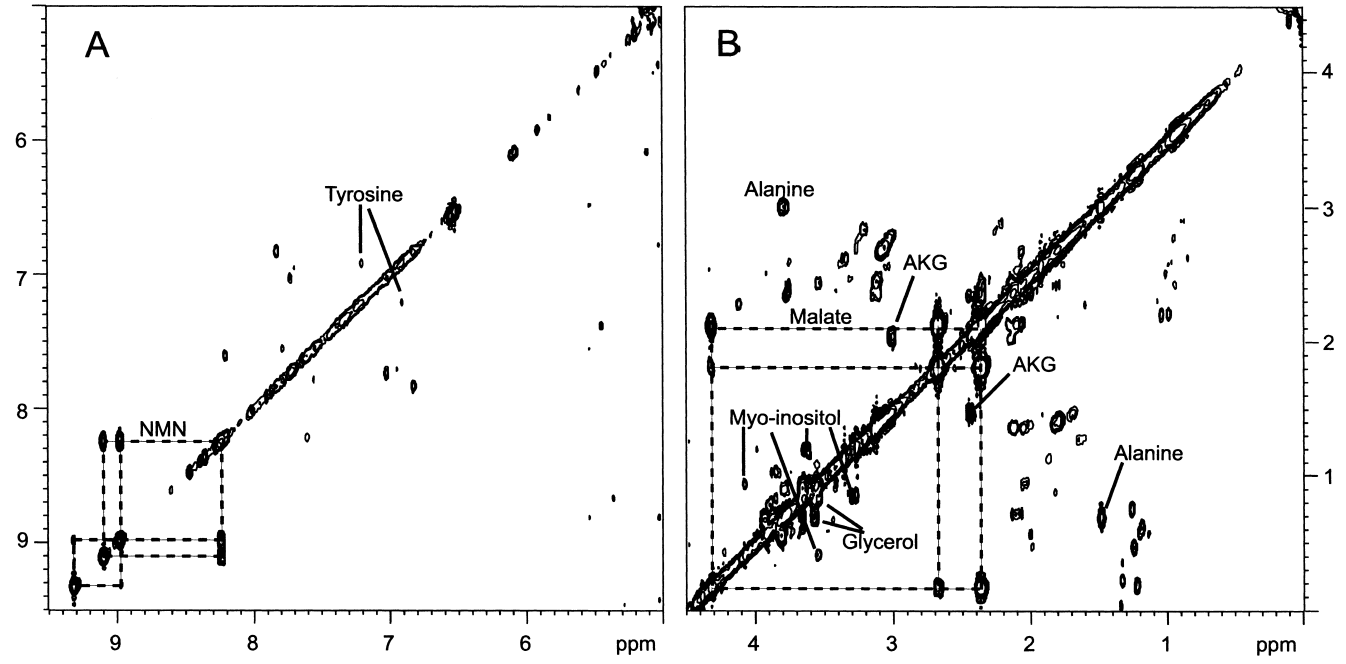

Fig. 2. Two-dimensional COSY 600 MHz ^{1}H NMR spectrum of coelomic fluid. A: Aromatic region. B: Aliphatic region. AKG: α-ketoglutarate. Correlated spin systems of NMN (A, left hand side) and malate (E, right hand side) are indicated on COSY by the dashed lines.

between δ 9.5 and 5.5 and δ 4.5 and 0.5, i.e. excluding the residual water signal (centred at δ 4.70). These data were arranged in a matrix of 34 rows by 800 columns, with each row representing an individual sample (earthworm), and each column representing the summed intensity value for a segment (chemical shift region). The data were then normalised by dividing each value by the average row value. The data columns were then mean-centred and analysed by principal components analysis (PCA) using the covariance matrix, carried out with the software package Simca-P 7.0 (Umetrics, Umeå, Sweden).

3. Results

3.1. ^{1}H NMR spectroscopic characterisation

The one-dimensional ^{1}H NMR spectrum shows a number of both broad and sharp resonances. The broad peaks arise from proteins still present in the ECF (despite an initial centrifugation of the ECF prior to analysis), and these have not been characterised using NMR spectroscopy. Superimposed on these broad lines are sharp peaks arising from small molecule metabolites. Many of the peaks can be assigned by inspection based upon comparison with tabulated literature values [8,14], and these are labelled directly on the spectrum (Fig. 1). Further confirmation of the metabolite identification was made in some cases using a two-dimensional correlation (COSY) NMR spectrum, that provides cross-peaks in a contour plot between the chemical shifts of spin-coupled nuclei. These cross-peaks are also labelled directly on the spectrum (Fig. 2).

It is apparent that the small molecule composition of the coelomic fluid is dominated by organic acids. The largest single resonance observed in the one-dimensional ^{1}H NMR spectrum is that of succinate at δ 2.41. The next most prominent resonances in the spectrum are also from organic acids: ma-

Table 1
Assignment of resonances shown in Figs. 1 and 2

δ[a]	Compound	Observed[b]
9.31s, 9.09d, 8.97d, 8.23t, 6.55d, 4.38t	nicotinamide mononucleotide	1-D, COSY
8.46s	formate	1-D
8.35s, 8.27s, 6.08d	adenosine	1-D
7.19, 6.90	tyrosine	COSY
6.52s	fumarate	1-D
4.31/2.68/2.37ABX	malate	1-D, COSY
4.07t, 3.64, 3.53, 3.28	*myo*-inositol	1-D, COSY
3.66/3.57ABX	glycerol	1-D, COSY
3.36s	methanol	1-D
3.27s	trimethylamine-*N*-oxide	1-D
3.12s	malonate	1-D
3.01t, 2.44t	α-ketoglutarate	1-D, COSY
2.41s	succinate	1-D
2.24s	acetoacetate	1-D
1.92s	acetate	1-D
1.48d	alanine	1-D, COSY

[a]Suffix indicates multiplicity of observed resonance. s: singlet, d: doublet, t: triplet, or ABX spin system.
[b]Denotes whether resonance was observed in one-dimensional NMR spectrum, COSY NMR spectrum, or both.

late and acetate. Several other organic acids can be observed within the one-dimensional ^{1}H NMR spectrum (Table 1). Other compound classes are also present: alanine was the only free amino acid to be visible in the one-dimensional spectrum, although tyrosine was detectable in the COSY spectrum. The nucleotide nicotinamide mononucleotide (NMN) was observed; the nucleoside adenosine was also present, at a much lower concentration than NMN. Finally, *myo*-inositol, glycerol, and trimethylamine-*N*-oxide (TMAO) were detected. Assignment of the resonances to NMN was made by comparison with the aromatic region resonances, in both chemical shift and multiplicity terms, to those of *N*-methyl nicotinamide [8]. In this spectrum, however, an additional doublet at δ 6.55 is observed and has been assigned to the anomeric proton of the ribose group. The integrated area of the resonance at δ 6.55 is highly correlated with the area of each of the different aromatic resonances (at δ 9.31, 9.10, 8.95, and 8.21) across many different spectra. In addition, the δ 6.55 signal shows coupling to a triplet at δ 4.95 (not shown in Fig. 1), which is in turn coupled to the resonance at δ 4.37. These data are consistent with the signals that would be expected of NMN. The signals could also arise from the closely-related compound nicotinic acid mononucleotide.

3.2. Effects of 3F4NP on earthworm coelomic fluid biochemistry

All 10 control worms were still alive at the end of the 3-day test period. The numbers of 3F4NP-dosed worms out of 10 surviving was as follows: 10 surviving at 0.25 μg cm^{-2} dose level; 9 surviving at 0.50 μg cm^{-2}; 5 surviving at 0.75 μg cm^{-2}; no survivors at 1.00 μg cm^{-2}; and no survivors at 1.25 μg cm^{-2}.

Two out of the five surviving worms at 0.75 μg cm^{-2} (the highest dose level that still had surviving worms) had changes in the NMR spectra apparent to the eye: NMN was not observed in two out of the five survivors. Multivariate data analysis (PCA) was then used to investigate this and to determine more subtle biochemical effects. The NMR spectra were reduced to intensity descriptors using AMIX, and PCA was used to investigate similarities and differences between the spectra. Six of the worms (three control worms and three at the 0.25 μg cm^{-2} dose level) had spectra which, because of problems with phasing, were not suitable for PCA with the remaining dataset. These were omitted, and PCA performed on the spectral data of the remaining 25 worms. This showed that the two worms not containing NMN were indeed extreme outliers, being completely separated from the remaining worms in both PC 1 and PC 2 (data not shown).

The PCA was repeated with the two outlying values omitted, in order to distinguish possible biochemical changes within the remaining set of worms. After examination of all the PCs, a reasonable degree of separation by dose could be observed approximately along PC 3 (Fig. 3A). The controls are significantly different from all of the dosed worms considered as a group (Student's *t*-test on PC 3 scores: $P = 0.006$), and there is also a statistically significant relationship between 3F4NP concentration and PC 3 scores ($P = 0.034$). A plot of the loadings on PC 3 against chemical shift region (Fig. 3B) showed that the dosed worms were associated with lowered levels of acetate and malonate, and increased levels of succinate and TMAO.

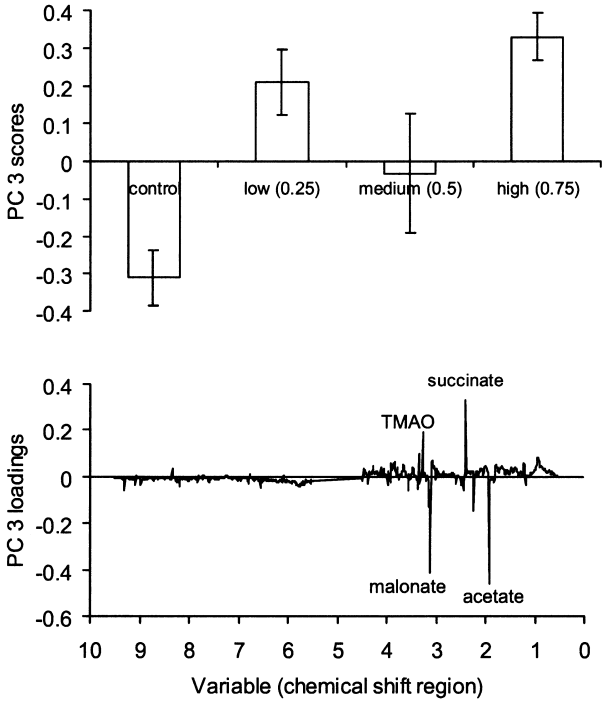

Fig. 3. PCA of NMR spectral data, two extreme points excluded. A: Change in PC 3 score (7.7% variance explained) as a function of 3F4NP concentration. Error bars represent ±S.E.M. B: Loadings plot. Ordinate represents loadings on PC 3. The abscissa shows which chemical shift region the individual variables correspond to. Variables corresponding to malonate, acetate, and succinate are labelled directly on the plot.

4. Discussion

4.1. Chemical composition of coelomic fluid

The presence of the compound NMN, which is an intermediate in NAD$^+$ biosynthesis and metabolism [15], was not expected in coelomic fluid. Possibly the presence of high relative concentrations of NMN in the coelomic fluid is indicative of occasional high enzyme activity in the coelomocytes or the cells lining the coelom: NMN could act as a reserve for rapid NAD$^+$ synthesis [15], which might be required if there was an increase in the activity of enzymes within the coelomocytes using NAD$^+$ as a cofactor.

Several of the major resonances observed in the NMR spectrum of *E. veneta* coelomic fluid can be attributed to organic acids (Table 1). Malate, succinate, fumarate, and α-ketoglutarate are citric acid cycle intermediates, and acetate, formate, and malonate are also observed. The precise biological role of these compounds in the coelomic fluid is unknown, but it seems likely that they are products of cellular metabolism: earthworms are known to follow the general biochemical pattern of carbohydrate metabolism [16–18]. The organic acids could be produced by cells lining the coelom, or could also be products of free coelomocytes.

The compounds *myo*-inositol, glycerol, and TMAO were also found in the ECF (Table 1). Previous studies have shown that adult earthworms accumulate glucose as a chemical mechanism of freeze tolerance [19], and that earthworm embryos in cocoons accumulate a polyol (probably sorbitol) as a mechanism of dehydration tolerance [20]. The compounds de-

tected in this study may act as osmolytes in the adult earthworms, to maintain the balance of the coelomic fluid and prevent cellular damage under drought conditions. Comparison of the ^{1}H NMR spectra of earthworms that had been incubated in soil held at different matric potentials could test this hypothesis.

The ^{1}H NMR spectrum (Fig. 1) contains a number of as yet unassigned resonances. Further work will be necessary to identify the compounds giving rise to these resonances; however, the assignments made in this study will serve as a baseline set for future investigations. Assignment of unknown resonances need in the future only be made after their chemometrics-based identification as potential biomarkers.

4.2. Effects of 3F4NP on earthworm biochemical profiles

The exposure of worms to 3F4NP was carried out in order to demonstrate the suitability of coelomic fluid as a matrix for NMR-based toxicology testing. PCA of the main dataset, i.e. not including the two outlying worms, showed that 3F4NP caused decreases in the relative concentrations of malonate and acetate, and increases in the relative concentrations of succinate and TMAO. Earthworms and other annelids are known to accumulate succinate as a temporary metabolic endpoint during periods of anoxia [17,21], analogous to the production of lactate by vertebrates. Possibly 3F4NP may therefore act to increase the relative amount of energy flow into the anaerobic pathway, by interference with normal carbohydrate metabolism.

This study demonstrates the advantages of using a non-selective technique such as ^{1}H NMR spectroscopy, which reports on the entire metabolite complement of a biofluid. Succinate, malonate and acetate concentrations in coelomic fluid have been demonstrated to be a potential combination biomarker of toxicity, although none of these were a priori selected as analytes, nor even known to be likely components of the coelomic fluid of *E. veneta*.

4.3. Potential for further toxicological studies

The application of multivariate data analysis techniques to the biochemical profiles of the entire small-molecule complement of biological samples, as detected by ^{1}H NMR spectroscopy, has been described as 'metabonomics' [22,23]. Metabonomics is likely to be useful in understanding the effects of changes in gene expression, in conjunction with the parallel techniques of genomics and proteomics (functional genomics) [24]. The metabonomic approach has previously [10,11] and in this study been demonstrated to be an appropriate technique for earthworm ecotoxicity analysis. NMR spectroscopy is also a valuable tool for detecting new potential biomarker compounds. The analysis and interpretation of endogenous biomarkers is likely to be of great value in the assessment of ecological risks to terrestrial invertebrates, as it can provide an 'early warning' of toxicity, as well as providing information on the integrated effects of cumulative exposures to complex mixtures that can not be gained from chemical analysis of pollutant residues [25,26]. Morgan et al. [27] stressed the importance of combining information from biomarker studies at multiple levels of organisation (e.g. a general toxic response together with effects at the genetic and protein level), ideally with mechanistic connections between levels. NMR spectroscopy-based metabonomics is well suited to providing multivariate biomarker data at a biomolecular toxicity level, which could then be combined with higher-level (for example, nucleic acid or protein level) toxicity data. The rapid nature of metabonomic analysis would enable testing to discover biomarker compounds that are relevant to different hierarchies of response, namely, general toxic insult and stress, effects of different chemical classes, and specific toxic responses to individual chemicals, together with a mechanistic understanding of the process.

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