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METABONOMIC ASSESSMENT OF TOXICITY OF 4-FLUOROANILINE, 3,5-DIFLUOROANILINE AND 2-FLUORO-4-METHYLANILINE TO THE EARTHWORM *EISENIA VENETA* (ROSA): IDENTIFICATION OF NEW ENDOGENOUS BIOMARKERS

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Abstract—High-resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy can be used to produce a biochemical fingerprint of low-molecular-weight metabolites from complex biological mixtures such as tissue extracts and biofluids. Changes in such fingerprint profiles can be used to characterize the effects of toxic insult in in vivo systems. The technique is nonselective and requires little sample preparation or derivatization. In the present study, earthworms (*Eisenia veneta*) were exposed to three different model xenobiotics by a standard filter paper contact test, and toxicant-induced biochemical changes were then investigated by characterizing the changes in endogenous metabolites visible in 600-MHz ^{1}H NMR spectra of tissue extracts. The NMR spectral intensities were converted to discrete numerical values and tabulated in order to provide data matrices suitable for multivariate analysis. Principal component analysis showed that changes had occurred in the biochemical profiles relative to the undosed controls. The 2-fluoro-4-methylaniline–treated worms showed a decrease in a resonance from a compound identified as 2-hexyl-5-ethyl-3-furansulfonate using a combination of high-performance liquid chromatography (HPLC)–Fourier transform mass spectrometry (IonSpec, Lake Forest, CA, USA) and ^{1}H and ^{13}C NMR spectroscopy. An increase in inosine monophosphate was also observed. The 4-fluoroaniline–treated worms showed a decrease in maltose concentrations, and 3,5-difluoroaniline exerted the same effect as 2-fluoro-4-methylaniline but to a lesser extent. These changes could potentially be used as novel biomarkers of xenobiotic toxicity and could be used to determine the mechanism of action of other toxic chemicals.

Keywords—Earthworm Biomarker Nuclear magnetic resonance spectroscopy Substituted aniline

INTRODUCTION

Earthworms fill important ecological niches that contribute toward soil functionality: They play a major part in organic carbon turnover in soils, both through the comminution of large pieces of organic matter and by stimulation of microbial activity, and also help to maintain soil structure [1]. Hence, they are widely used as ecotoxicological test organisms for soil contamination, as they are considered sensitive indicator species for environmental damage [2]. Tests relying on earthworm mortality are likely to be useful only in the assessment of gross toxic effects and give no information on how the toxic effects are mediated. It is necessary to be able to measure sublethal toxic effects; this enables the detection of contamination at lower levels and can be used to provide an early warning of environmental damage before earthworm populations are affected [3].

Biomarker assays provide a sensitive method for examining sublethal toxic effects in earthworms. The term ''biomarker'' has been defined as ''a biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also, of toxic effect'' [4] and in this study will be taken to refer to individual biochemicals measured in worms. The approach can be applied to worms from both controlled tests and contaminated sites. Furthermore, analysis of xenobiotic-induced changes to the biochemistry of an organism provides a fundamental measure of toxic response [5]. The ^{1}H NMR spectroscopy of biological samples is a powerful technique for measuring biochemical profiles and consequently potentially also for environmental biomarker analysis. It provides a simultaneous, nonselective measurement of all small molecules containing nonexchangeable protons and is therefore an ideal means for searching for novel biomarker compounds [6,7]. This is likely to be particularly useful when the mechanism of toxic action of a xenobiotic on an organism is not known or is not fully understood. The word ''metabonomics'' has been coined, by analogy with genomics and proteomics, to describe the quantitative measurement of the dynamic metabolic response of a living system to a toxic or physiological insult, coupled with multivariate data analysis methods [8]. Many examples of the successful application of metabonomic toxicity analysis can be found in vertebrate models [9], and recently this approach has been extended to ecotoxicity testing in earthworms [10].

Earthworm toxicity data exist for many organic chemicals, especially pesticides, but are usually limited observations, such as lethal concentration data [11,12]. Therefore, a need exists to expand the knowledge of earthworm ecotoxicology both by

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testing a greater number of chemicals and by examining the biochemical effects. Other important ecotoxicological approaches include the assessment of sublethal indicators of toxicity, such as reproduction indices. Ultimately, it would be valuable to be able to link effects at the biochemical level to such sublethal parameters. Previous work using ^{1}H NMR spectroscopy has already shown that free histidine is a suitable biomarker for copper exposure in earthworms in a semifield situation [13] and that the toxic effects of a single model compound can be distinguished in the tissue extracts [10] of *Eisenia veneta*. Halogenated anilines are widely used industrial intermediates in chemical synthesis [14] and can cause contamination through industrial chemical wastes and as breakdown products of some pesticides [15]. For some purposes, monoaromatic compounds such as these are more appropriate model compounds than specific biocides (pesticides): They have simple chemical structures, and thus it is potentially easier to relate differences in toxicity to chemical and molecular properties [14], which is an aim of future work. This study aims to apply the NMR-based metabonomic approach to three halogenated anilines in order to determine if differences exist in the toxic effects of these three chemicals toward *E. veneta* and to identify any potential biomarkers of toxicity.

MATERIALS AND METHODS

Chemicals

The 2-fluoro-4-methylaniline (2F4MA; 99%), 3,5-difluoroaniline (35DFA; 98%), and 4-fluoroaniline (4FA; 99%) were obtained from Sigma-Aldrich (Poole, UK).

Toxicity tests

Earthworms (*E. veneta*) were taken from a culture maintained at the Centre for Ecology and Hydrology Monks Wood (Abbots Ripton, Huntingdon, UK). Fully clitellate adult individuals were selected for bioassay work and allowed to depurate on moist filter paper for 24 h before the start of the test.

The exposure method used was based on the standard Organization for Economic Cooperation and Development rapid toxicity test [16]. Medium-weight filter paper was cut to fit inside a glass vial without overlapping. The area of filter paper used was 52 cm^2. The compound under test was dissolved in hexane and applied to the filter paper to give a nominal concentration in μg/cm^2 and the hexane allowed to evaporate. The paper was then moistened with 1 ml of distilled water, a single earthworm added, and the vial sealed. The vial was then incubated in the dark for a period of 72 h. At the end of this period, the earthworm was removed, snap frozen in liquid nitrogen, and stored at -20°C until sample preparation.

Toxicity tests were performed in quintuplicate: a single set of five earthworms was used as a control set for the three compounds tested. A preliminary range-finding series of experiments were carried out on a logarithmic scale (1,000, 100, 10, 1, and 0.1 μg/cm^2) in order to determine an appropriate concentration range. Toxicity tests were then performed with five (2F4MA and 35DFA: 100, 50, 25, 10, and 1 μg/cm^2) or four (4FA: 250, 100, 50, and 25 μg/cm^2) concentration levels in addition to a control. Median lethal concentration values were calculated using a probit maximum likelihood model.

Sample preparation

Earthworms were homogenized into 1 ml of Ringer's solution [17] using an Ultra-Turrax homogenizer (Fisher Scientific, Loughborough, UK), and the homogenate was stored at -20°C until analysis. The extract was then thawed and centrifuged (9,400 *g*, 5 min) to remove suspended particulate matter. The sample was then made up to 0.75 ml in a standard 2-ml autosampler vial by combining 0.25 ml of the tissue extract supernatant with 0.25 ml sodium phosphate buffer solution (0.2 M, pH 7.4), 0.15 ml distilled water, and 0.1 ml D_2O. The buffer solution also contained 0.1% weight/volume NaN_3 to inhibit microbial growth in the samples during analysis, and the D_2O contained 0.05% weight/volume sodium trimethylsilyl-[2,2,3,3-$^2H_4$]-propionate (TSP). The TSP was used as a reference for chemical shifts, and the D_2O provided a field-frequency lock for the spectrometer.

NMR spectroscopy for toxicity analysis

Samples were analyzed on a Bruker Avance DRX600 spectrometer (Bruker UK, Coventry, UK) at a frequency of 600.13 MHz for ^{1}H observation and equipped with a flow-injection probe and a Gilson-215 robot autosampler spectrometer (Bruker UK). Samples were held at 300 K during acquisition. The ^{1}H NMR spectra were collected over a spectral width of 12 kHz into 49,152 data points, summing 160 free induction decays (FIDs), and zero filled to 64-K data points. A 2-s relaxation delay was added to the acquisition time of 2.04 s for each FID. Water resonance suppression was effected using a standard pulse sequence. An exponential apodization function equivalent to a line broadening of 0.3 Hz was applied to the raw data before Fourier transformation (FT).

Chemical shifts are reported relative to the trimethylsilyl resonance at δ 0.0. Resonances were assigned by comparison with published chemical shift data [18–20]. Two-dimensional J-resolved and J-coupling correlated spectroscopy (COSY) were also used to help confirm the assignments. The assignment of certain compounds (maltose, inosine) was also confirmed by comparison with the spectrum of an authentic standard acquired under identical conditions.

Sample preparation and analytical methods for assignment of 2-hexyl-5-ethyl-3-furansulfonate

High-pressure liquid chromatography-mass spectrometry (MS) was performed using a 150 $\times$ 4.6-mm HiChrom C18 BDS column with 5-μm packing (HiChrom, Theale, UK) and a constant flow rate of 1 ml/min with a variable wavelength ultraviolet (UV) detector, coupled to either a Bruker Daltonics Esquire MS for initial studies using low-resolution MS (Bruker Daltonics, Coventry, UK) or a Bruker Daltonics Apex III Fourier transform mass spectrometer (FTMS) equipped with a 7 Tesla magnet (Bruker) for high-resolution MS. A binary solvent system was used, with an aqueous buffer consisting of 10 mM ammonium formate solution adjusted to pH 7 and acetonitrile as the nonaqueous solvent. The solvent program was isocratic elution at 85% aqueous buffer/15% acetonitrile for 10 min, followed by a linear gradient to 50% aqueous/50% acetonitrile at 35 min.

Larger-scale sample preparation was performed using an Isolute C18 solid-phase extraction cartridge with 3 g of adsorbent (IST, Hengoed, UK). Six lyophilized earthworms were extracted in 3 ml of 50% acetonitrile/50% water solution, and the acetonitrile was removed under a stream of nitrogen. The remaining aqueous solution was loaded onto the cartridge and extracted using the same solvent system as for HPLC (vide supra). Discrete fractions were eluted with 3 ml of 0, 10, 25, 50, and 100% acetonitrile solution. The 2-hexyl-5-ethyl-3-furansulfonate (HEFS) was eluted in approximately equal quan-

tity in the 25 and 50% fraction; the 50% fraction was >95% pure as estimated by ^{1}H NMR spectroscopy and was used for all further identification. This fraction was lyophilized and then reconstituted in 300 μl water plus 300 μl acetonitrile-d_3.

The NMR spectra for identification of HEFS were acquired using a 5-mm broadband inverse probe, operating at a frequency of 600.22 MHz for ^{1}H and at 150.93 MHz for ^{13}C. The ^{1}H one-dimensional and COSY spectra were acquired as described previously. A nuclear Overhauser effect (NOESY) experiment was also acquired, using a mixing time of 500 ms, a sweep width of 4 kHz, 2-K data points in the F2 dimension, and 256 increments in the F1 dimension. In the NOESY experiment, cross peaks are observed for through-space interactions, unlike the COSY, where the interactions are through-bonds. The ^{13}C {^{1}H} NMR spectra were acquired over a spectral width of 32 kHz centered at 100 ppm using a 90° pulse angle. An acquisition time of 2.9 s was supplemented by an additional relaxation delay of 5 s between pulses. A total of 5120 FIDs were summed, and an exponential apodization function equivalent to a line broadening of 2 Hz was applied to the raw data prior to FT.

The log octanol–water partition coefficient (K_{ow}) was calculated using the ACD LogD software suite (Advanced Chemistry Development, Toronto, Canada).

Data analysis for metabonomics

The NMR spectra were first corrected for phase, and the baseline was manually adjusted. (This was performed separately for each spectrum on either side of the water suppression region.) Spectra were then converted into numeric data using the proprietary software package AMIX 2.1 (Bruker Analytik, Rheinstetten, Germany): This divides the spectrum into a number of segments and sums the total integrated signal within each segment. A segment width of 0.02 ppm was chosen, and the spectrum was quantified from 10 to 5 and from 4.5 to 0 ppm (avoiding the area around the suppressed water resonance at δ 4.69). This provided a matrix suitable for multivariate analysis in which each row represented a sample and each column contained the integrated area of a chemical shift region (segment, i.e., variable). The data rows were normalized with respect to the overall integrated NMR signal intensity by expressing the values as a percentage of the average value for each sample (row). The data values (n_i) were then transformed but not otherwise scaled prior to principal components analysis (PCA). All negative values were set to zero, and the transformation $\log(n_i + 1)$ was applied. The PCA was then carried out using Simca-P 8.0 (Umetrics, Umeå, Sweden) on mean-centered data using the covariance matrix.

RESULTS

The median lethal concentration values obtained for 4FA, 2F4MA, and 35DFA were 115, 58, and 17 μg/cm^2, respectively, classifying them as toxic, very toxic, and very toxic according to the scheme of Roberts and Dorough [11]. Each compound had, respectively, 10, 15, and 7 surviving dosed worms with no mortality in the control group. Earthworms are subject to rapid autolysis after death [21]; hence, data for dead worms were not included in further multivariate analysis, as any changes caused by toxic effects might be confounded with changes caused by tissue breakdown.

A spectrum of a tissue extract of a control earthworm is shown in Figure 1, including the assignment of resonances to individual compounds. It was difficult to pick out systematic differences between spectra of dosed and control worms by inspection. For the 4FA-dosed worms, fewer resonances were observed in the region from δ 4 to 3, which contains many complex overlapping signals from sugars and amino acids as well as from other endogenous biochemicals. For the 2F4MA- and 35DFA-dosed worms, no changes were observed in the spectra clearly visible on inspection.

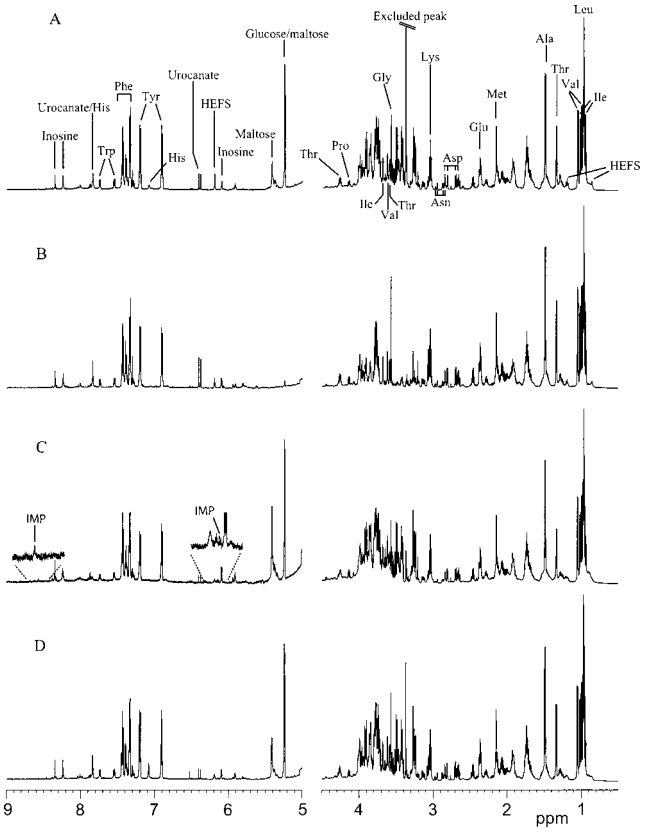

Fig. 1. Representative 600-MHz ^{1}H nuclear magnetic resonance spectra of earthworm tissue extracts. (**A**) Control. (**B**) 4-Fluoroaniline (4FA) dosed. (**C**) 2-Fluoro-4-methylaniline (2F4MA) dosed. The inset regions have been expanded to show resonances from inosine monophosphate (IMP). (**D**) 3,5-Difluoroaniline (35DFA) dosed. The left-hand side (δ 9.0–5.0) and the right-hand side (δ 4.5–0.5) do not have the same vertical scaling: The left-hand side has been expanded to permit clearer viewing of the resonances. HEFS = 2-hexyl-5-ethyl-3-furansulfonate; Trp = tryptophan; His = histidine; Phe = phenylalanine; Tyr = tyrosine; Thr = threonine; Pro = proline; Gly = glycine; Ile = isoleucine; Val = valine; Asn = asparagine; Asp = aspartate; Lys = lysine; Glu = glutamate; Met = methionine; Ala = alanine.

The level of the singlet resonance at δ 3.36 (Fig. 1) from an as yet unassigned compound was highly variable between samples and in some spectra was much larger than any of the other resonances. This resonance is thought to be either a possible contaminant (e.g., methanol would give rise to a singlet at that chemical shift) or perhaps a product of enzymatic breakdown during sample preparation. Therefore, the two variables to which this resonance made a contribution (equivalent to the chemical shift region δ 3.38–3.34) were omitted for data analysis. The PCA of the AMIX-reduced data showed significant differences in the pattern of metabolite signal intensities between treatments (Fig. 2); for instance, it can be seen that 4FA-treated worms separate from controls along principal component (PC) 1, whereas 2F4MA-treated worms separate along PC 2. No apparent separation was observed between

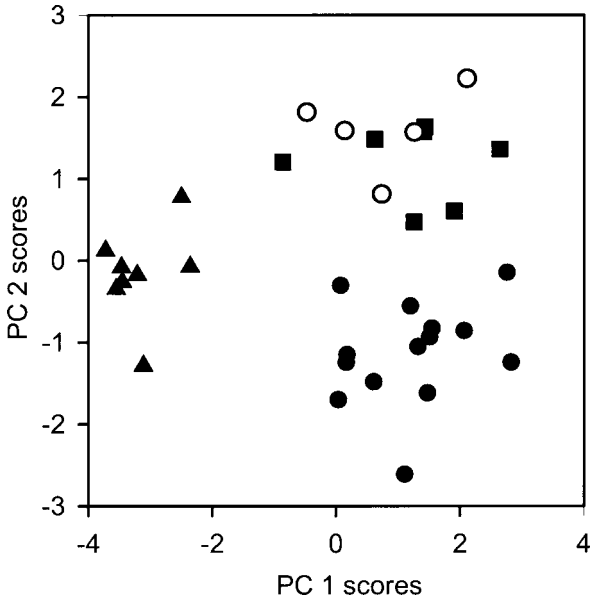

Fig. 2. Principal component (PC) analysis scores plot of integrated 600-MHz ^{1}H nuclear magnetic resonance spectral data for all dosed worms considered as a group. Principal component 1 explains 53% and PC 2 explains 18% of the variance within the data set. Symbols indicate the following: ○ control worms, ● 2-fluoro-4-methylaniline (2F4MA)-dosed worms, ■ 3,5-difluoroaniline (35DFA)-dosed worms, ▲ 4-fluoroaniline (4FA)-dosed worms.

35DFA and the control worm spectra within the first two PCs. As PCA creates axes that explain the maximum variance within a data set, it would still be possible for relatively smaller differences to exist between 35DFA and controls that simply explained less variance. To investigate this, and in order to clarify the changes occurring for each compound, PCA was also performed separately for each compound plus controls. The results are shown in Figure 3: 4FA- and 2F4MA-dosed worms both show large differences from controls, being entirely separated along PC 1. The 35DFA-treated worms are also separated from the controls within principal component space; however, the effect of 35DFA on the spectral profiles was clearly not as great as for the other two compounds, as the separation occurs along lower PCs, explaining a smaller proportion of the variance of the data. For all the different

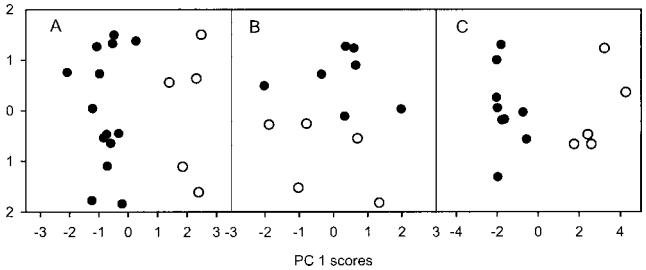

Fig. 3. Principal component (PC) analysis scores plots of integrated 600-MHz ^{1}H nuclear magnetic resonance spectral data for each compound analyzed separately. (**A**) 2-Fluoro-4-methylaniline (2F4MA), PC 1 versus PC 2. Principal component 1 explains 30% of the variance in the data. (**B**) 3,5-Difluoroaniline (35DFA), PC 1 versus rotated PC axis. Rotated PC explains 13% of the data. (**C**) 4-Fluoroaniline (4FA), PC 1 versus PC 2. Principal component 1 explains 65% of the variance in the data. The symbols indicate the following: ○ control worms, ● dosed worms. The rotated axis was formed by taking the perpendicular distance from points within the plane PC 2 versus PC 3 to a line with gradient −1 passing through the origin.

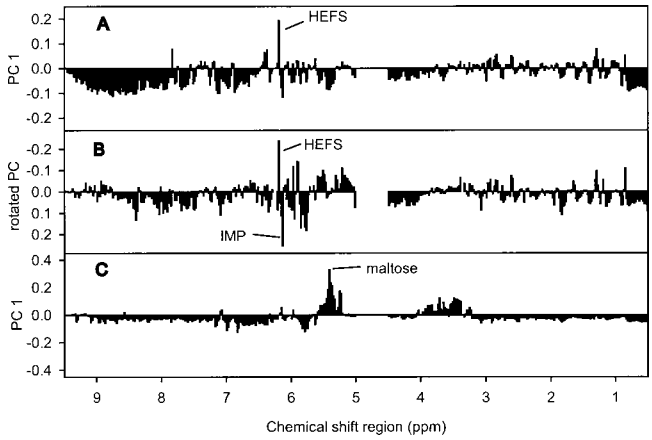

Fig. 4. Plots of principal component (PC) analysis loadings (ordinate) against chemical shift region to which the loading corresponds (abscissa). (**A**) 2-Fluoro-4-methylaniline (2F4MA), PC 1. (**B**) 3,5-Difluoroaniline (35DFA), rotated axis within PC 2 and PC 3. The scale is reversed so that bars above the axis indicate variables associated with control worms to facilitate comparison with (**A**) and (**C**). (**C**) 4-Fluoroaniline (4FA), PC 1; IMP = inosine monophosphate; HEFS = 2-hexyl-5-ethyl-3-furansulfonate.

compounds, the effect does not appear to be dose dependent at the concentrations tested; that is, all the treated worms cluster in a group, and the lowest dose caused as great an effect as the highest survived dose.

Plots of the loadings from PCA can be used in order to determine which variables contributed most to the observed separation along an axis, that is, which spectral region and hence which compounds were affected by xenobiotic treatment. This is shown for the three compounds in Figure 4. The ordinate scale for 35DFA (Fig. 4B), representing the loadings, has been reversed such that negative loadings appear above the axis while positive loadings appear below the axis. This is to facilitate comparison of the loadings plots for the three compounds: All loadings above the axis correspond to spectral regions that had a higher relative intensity in the control worms, whereas all loadings below the axis correspond to spectral regions that had a higher relative intensity in the dosed worms.

It can be seen that 4FA has a different set of loadings to the other two compounds (Fig. 4C), with the largest loading at δ 5.42 to 5.40. The loading at δ 5.24 to 5.22 is also elevated. The resonances occurring at these chemical shifts have been assigned to the protons of maltose, and the identity has been confirmed by comparison with a spectrum of authentic maltose acquired under identical conditions.

Both 35DFA and 2F4MA have a similar pattern of loadings causing separation (Fig. 4A and B). For both of these compounds, the largest loading corresponding to controls is δ 6.20 to 6.18, and the largest loading corresponding to dosed worms is δ 6.14 to 6.12; that is, the loading at δ 6.14 to 6.12 may be considered to be from a potential biomarker elevated as a consequence of exposure, whereas the loading at δ 6.20 to 6.18 may be considered to be from a biomarker decreased on exposure. The spectral region δ 6.14 to 6.12 clearly contains a doublet, although in very low concentration. This is typical of a resonance arising from a ribosyl anomeric proton for both the chemical shift (δ 6.13) and the coupling constant (6 Hz). The intensity of this doublet is correlated across different spectra to that of a singlet observed at δ 8.57. On the basis of the similarity of the chemical shifts to those reported in the lit-

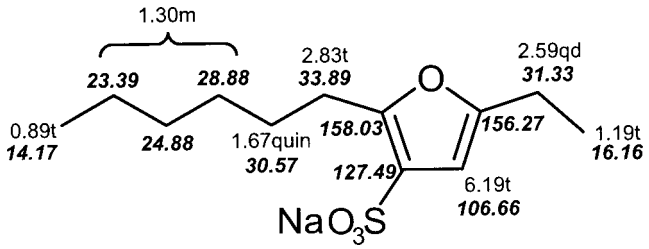

Fig. 5. Structure of 2-hexyl-5-ethyl-3-furansulfonate (HEFS) together with assigned NMR data. ^{1}H nuclear magnetic resonance (NMR) chemical shifts are shown in normal type, ^{13}C NMR chemical shifts are shown in bold italic type. The suffix indicates multiplicity: t = triplet; qd = quartet of doublets; quin = quintet; m = multiplet.

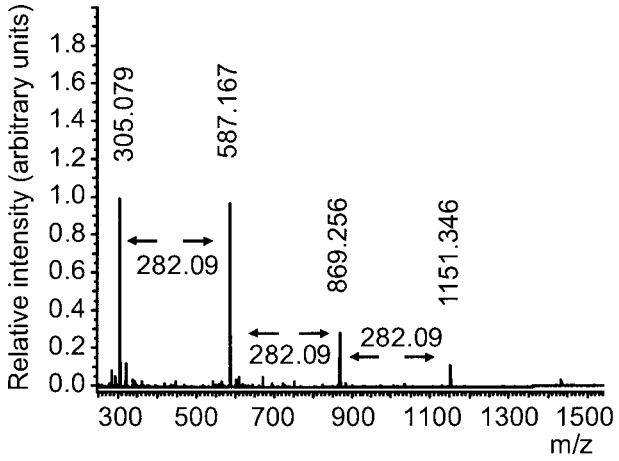

Fig. 6. Mass spectrum (from Fourier transform mass spectrometry) of 2-hexyl-5-ethyl-3-furansulfonate showing ions appearing at multiples of the molecular weight.

erature [20], these resonances are assigned to inosine monophosphate (IMP). An additional aromatic resonance would be expected at δ 8.23 but in this case is overlapped with a resonance from inosine and hence is not observed.

From examination of the original NMR spectra, the region from δ 6.20 to 6.18 clearly contains a single resonance centered at δ 6.19. This appears to be a singlet, but Gaussian resolution enhancement of the spectrum showed that the resonance is a triplet with a J coupling of 1 Hz. We utilized a combination of methods to identify this endogenous compound. Both HPLC-NMR and HPLC-MS initially showed that the resonance at δ 6.19 belonged to a compound eluting at 24 min, with a single UV maximum at 220 to 225 nm and with associated ions in the MS at 283 and 305 when operated in positive ion mode. This can be interpreted as a compound with a molecular weight of 282, giving vapor-phase ions of MH^+ at 283 and MNa^+ at 305. The HPLC-FTMS gave an ion at 283.0973, which corresponds to a calculated molecular formula of $C_{12}H_{20}SO_3Na$ (predicted value 283.0975), and confirmed that the ion at 305 was a sodium adduct with the formula $C_{12}H_{19}SO_3Na_2$; that is, both ions contained a formally neutral sodium. The ^{1}H NMR spectrum of a sample prepared using solid-phase extraction gave seven resonances, and ^{13}C NMR spectroscopy gave 12 resonances (Fig. 5). The NMR spectroscopic and MS data are consistent with a proposed chemical structure of 3-furansulfonate, with ethyl and *n*-hexyl groups in the 2 and 5 positions. With this information, a chemical structure search revealed that a compound reported to be 5-hexyl-2-ethyl-3-furansulphonate had previously been described as isolated from earthworms [22]. However, a NOESY experiment showed cross peaks between the aromatic proton and the ethyl group protons, thus proving conclusively that the structure published in Yoneda et al. [22] is incorrect, being the wrong isomer. The actual structure must be 2-hexyl-5-ethyl-3-furansulfonate and is shown in Figure 5. This compound was shown to exhibit surface-active properties by its vapor-phase behavior during FTMS: A series of ions at multiples of the molecular weight can be seen in the mass spectrum (Fig. 6). One possible interpretation is that HEFS micelles were formed in the electrospray interface and that singly charged oligomers linked by ionic salt bridges can be seen as a result of these micelles breaking down in the vapor phase. This explanation is lent credence by the behavior of sodium dodecylsulfate in the FTMS under identical conditions, which also produced a series of ions at multiples of the molecular weight (data not shown). The calculated log K_{ow} of HEFS is 3.74 ± 0.72 for the protonated form.

DISCUSSION

NMR-based metabonomic analysis of toxic effects

Two of the three compounds did not show any effects on the NMR spectra apparent to the eye, yet data analysis using PCA showed that subtle changes in spectral intensities were able to differentiate control from dosed worms. This is an indication of how multivariate pattern recognition techniques are necessary in order to extract useful information from the information-rich spectra. Log transformation of data prior to PCA using the covariance matrix was found to be the optimum method for data preparation for interpreting the results. The use of the correlation matrix in PCA scales all variables such that every variable has variance equal to one—this is required when different variables are measured in different units but may be inappropriate if the data are all measured in the same units. Thus, spectroscopic data are often analyzed using the covariance matrix, that is, without scaling [23]. However, use of the covariance matrix does bias PCA heavily toward large variables (the compounds present in highest concentration), so a preliminary log transformation was applied to reduce this effect.

The results show a clear separation of the control from dosed worms for two of the three compounds, and this separation takes place within the first two principal components (Fig. 2). The three compounds appear to exert two distinct modes of toxic action, with 4FA behaving differently from 2F4MA and 35DFA. At least three endogenous compounds can be identified as potential biomarkers. Maltose is the most important variable for 4FA and is a negative biomarker (concentrations negatively correlated with exposure). For 2F4MA and 35DFA, IMP is a positive biomarker of toxicity, and HEFS is a negative biomarker.

Maltose has not to date been observed in studies of carbohydrate metabolism in earthworms [24,25]. We confirmed its assignment by comparison to an authentic standard. Simple sugars may have secondary functions in earthworms in addition to energy metabolism. For instance, glucose has been shown to be produced as a mechanism of freeze tolerance [26]. However, the biological role of maltose in earthworms is as yet unknown.

Inosine monophosphate was observed as a biomarker of toxicity for 35DFA and 2F4MA. The concentration of free

inosine is much greater than the concentration of IMP (Fig. 1), and indeed inosine has been used as a marker compound for identification of drugs based on earthworm extracts [27]. The high concentrations of free inosine observed in earthworms imply that it is likely to play a distinct biochemical role, although again the nature of this is unknown. Inosine monophosphate is an intermediate of nucleotide synthesis and is a precursor of adenosine monophosphate [28], but it is possible that in earthworms it may have an additional role related to the presence of a relatively large store of free inosine. Possibly inosine forms a reservoir for nucleotide synthesis via an initial phosphorylation to IMP.

The final compound identified as a potential (negative) biomarker was HEFS. Although this compound has been previously described in a patent as isolated from earthworms [22], it has not been reported in the open literature. This highlights one of the major strengths of using a comprehensive analytical technique such as ^{1}H NMR spectroscopy for metabonomic analysis. It is possible to detect changes in the relative concentration of a biochemical, or metabolite, even if no a priori knowledge whatsoever exists of the chemical structure of that compound. Nor need it be known a priori whether a particular metabolite is present in the samples to be tested.

Even less biochemical understanding exists of the role played by HEFS than for the other two biomarker compounds, IMP and maltose, because comparison with the metabolism of other organisms is not possible. As far as is known, HEFS is found only in earthworms, *E. veneta*, as shown in this study, and also *Lumbricus rubellus*, *L. terrestris*, *Eisenia andrei*, and *E. fetida* (unpublished observation). We have not yet tested other earthworm species. The HEFS is an anticoagulant, blood platelet aggregation inhibitor, ulcer inhibitor, and vasoconstriction inhibitor in vertebrates [22], but this is not necessarily related to its function in earthworms. It can be present in remarkably high concentration, up to 0.1% of the total body weight of *L. rubellus*, for example [22], and thus clearly is synthesized for a specific purpose by the worm, although its route of biosynthesis is unknown. Furan compounds are common natural products; for instance, furan fatty acids are ubiquitous at low levels in plants [29] and are formed from cyclization of conjugated unsaturated fatty acids [30]. However, as no oxygen functionality exists on either of the 2,5-position alkyl chains in HEFS, it does not appear that it has been synthesized from a fatty acid. The biochemical role and synthesis of HEFS in earthworms is an interesting area for future research. Given its surface-active behavior and high concentration in earthworms, it might possibly act as a lubricant for sliding past soil particles or to increase resistance to dehydration. It would also be important to investigate that no methodological biases exist in the use of HEFS as a biomarker. For example, HEFS is moderately lipophilic, with a calculated log K_{ow} of 3.74 for the protonated form of the sulfonic acid, and thus a purely aqueous extraction method might not fully extract HEFS. Thus, it is possible that the observed changes in the NMR spectra resulted from some secondary biochemical change that affected the extractability/solubility rather than the free tissue concentration of HEFS. This will be tested in future work. However, it should be emphasized that, even if some kind of extraction bias was caused by solubility limitations, the dosed earthworms were nonetheless significantly different from the controls and that a genuine biochemical change must have taken place in the exposed worms to affect the extractability.

Endogenous metabolite profiling by high-resolution NMR spectroscopy is clearly an appropriate technique for assessing subtle toxic effects. The three compounds tested are all very similar, yet two distinctly different responses have been detected. Because all different NMR-visible compounds are measured simultaneously and the resulting data are analyzed by multivariate methods, no preselection of the biomarker molecules of interest takes place. As a result, biomarkers of both general and specific toxicity could potentially be observed. Previous studies have concluded that measurement and multivariate assessment of a range of biochemical responses in earthworms is more useful for some purposes than measuring single responses [31]. A particular strength is the potential for classifying chemicals into different groups on the basis of their multiparametric biochemical effect and hence their mechanism of toxic action. The clear differences in toxic effect between 4FA and the other two chemicals in this study were unexpected: All three compounds are anilines and do not contain substituents such as nitro groups that might be expected to cause large differences in toxicity. One possible reason is that 4FA was metabolized differently from the other two compounds. The toxicity of anilines in mammalian models is generally accepted to be caused by activation of the parent xenobiotic by initial oxidation by a cytochrome P-450 system, with the commonest resulting toxic syndromes being methemoglobinemia and nephrotoxicity [32,33]. Although the mechanisms of xenobiotic metabolism in earthworms are far from completely understood [34], they are known to possess enzymes capable of carrying out both phase I and phase II metabolism, including cytochrome P-450 mixed-function oxygenases and glutathione-*S*-transferases [35,36].

Potential field applications of earthworm biomarkers

It is difficult to extrapolate biomarker responses from laboratory tests to real soils [37]. Changes in the exposure substrate used in toxicity tests have been shown to affect the levels of a suite of biochemical markers in earthworms [38]. Thus, it cannot be assumed that the results obtained in this study using a filter paper contact test could necessarily be used in a field situation. In particular, no knowledge exists of how the levels of these potential biomarkers are affected by environmental and biological factors, for example, osmotic stress, seasonality, and stage of life cycle. However, results reported by Bundy et al. [39], using indigenous worms collected from a metal-contaminated site, indicate that HEFS also acted as a negative biomarker of exposure in *L. rubellus* (although HEFS was not identified in that study). Thus, it appears that HEFS may be useful as an indicator of nonspecific toxicity in more than one earthworm species and will potentially be useful in genuine field situations. More testing at authentic contaminated sites will be necessary in order to validate its use as a biomarker. Ideally, a biomarker response should be used only when a complete understanding exists of the biochemistry of the pathway involved, but a response based on correlation with toxic effects can still be useful in ecotoxicology [40]. Interestingly, maltose was also found to respond to metal contamination in *L. terrestris*, although the response was opposite to that in the current study; that is, relative maltose concentrations were increased as a consequence of exposure. More understanding is clearly required of the metabolic reasons for the changes in maltose levels before it could realistically be adopted as a biomarker in field conditions.

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