^{1}H NMR spectroscopic investigations of tissue metabolite biomarker response to Cu(II) exposure in terrestrial invertebrates: identification of free histidine as a novel biomarker of exposure to copper in earthworms

J. O. T. Gibb, C. Svendsen, J. M. Weeks and J. K. Nicholson

High resolution ^{1}H NMR spectroscopy of biofluids, cells and tissue extracts allows rapid, non-destructive analysis for a wide range of metabolites and organic compounds with minimal sample pre-treatment. We have applied high resolution ^{1}H NMR spectroscopy to investigate the biochemical effects of Cu(II) in two earthworm species *Eisenia andrei* (n=78) and *Lumbricus rubellus* (n=45) exposed under laboratory and semi-field conditions respectively. The most marked metabolic response was the elevation of endogenous whole body free histidine in animals which positively correlated with increasing copper exposure and total copper burden in the semi-field experiment. Histidine forms thermodynamically stable copper complexes under a wide range of physico-chemical conditions and we proposed that the elevation of free histidine in response to copper challenge provides an energetically 'low-cost' detoxification mechanism. Histidine elevation may also provide a novel molecular biomarker of Cu(II) exposure in environmental situations.

Keywords: *^{1}H NMR, Lumbricus rubellus,* Eisenia andrei, *histidine metabolites, copper-toxicity, tissue extracts, earthworm.*

Introduction

There is an increasing demand for the formulation of objective measures of environmental pollution and damage. This has led to the investigation of biological systems that will qualitatively and quantitatively indicate exposure to pollutants. At the simplest level this has involved the use of selected indicator species, which accumulate pollutants above environmental levels (biomarkers of exposure). However, rather than merely recording pollutant residue burdens a more diagnostic approach would be to investigate changes in endogenous metabolites which constitute biomarkers of effect. Such biomarkers are most useful if they indicate chronic, low level exposure causing sub-lethal effects. Hence there is considerable interest in the detection and use of novel biomarkers in environmentally sensitive species (Peakall 1994).

To investigate metabolic perturbations knowledge of the basal metabolic states of the pollution indicator species is a necessary pre-requisite. Metabolite characterization is conventionally achieved by applying a series of specific biochemical assays for metabolites. Such assays can be time consuming, labour intensive and may involve extensive methodological development. Alternatively ^{1}H NMR spectroscopy of biofluids and tissue extracts can be used to produce profiles of endogenous metabolites (Nicholson and Wilson 1989) and can be applied to investigate the biochemistry of potential pollution indicator species (Gibb *et al.* 1997). NMR spectroscopy has also been established as a powerful tool for the investigation of toxicological processes in mammals and when combined with the use of data pattern recognition techniques has led to the discovery of novel biomarkers of toxicity in vertebrate systems (Nicholson and Wilson 1989; Anthony *et al.* 1994).

The use of earthworms in pollutant studies is now widespread and their ability to accumulate metals above ambient concentrations is well established (Morgan and Morgan 1990). Such contaminants thus become available to the large number of predators of earthworms (Beyer and Stafford 1993). Detoxification strategies have been proposed in the earthworms for a number of metals including cadmium, lead and zinc, but with the noticeable exception of copper (Morgan and Morgan 1990). Thus the metabolic response to copper exposure has been investigated in *Lumbricus rubellus* and *Eisenia andrei* using ^{1}H NMR spectroscopy as a biochemical probe with the aim of detecting useful new biomarkers of Cu(II) exposure.

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MATERIALS AND METHODS

Earthworms were exposed to Cu(II) under two sets of conditions; semi-field conditions and laboratory conditions. In the field *Lumbricus rubellus* (Hoffmeister 1843) were exposed to the following copper concentrations for a period of 110 days; 40, 80 and 160 mg Cu kg^{-1} soil (dry weight), in outdoor mesocosms as described by Svendsen and Weeks (1997). The mesocosms consisted of 50 cm sections of medium density poly-ethylene (MDPE) piping, with a 25 cm diameter (Stewart & Lloyds Plastics, UK), enclosed at the top with nylon netting and at the bottom with a heavy-duty, water-permeable phormacil membrane (stabilized polypropylene, LBS Group, UK) and a layer of strong rigid MDPE netting (Netlon, LBS Group, UK). Copper was added, in the form of anhydrous $CuCl_2$ (BDH Ltd, UK), to 13.5 kg (dry weight) of sandy soil taken from the top 20 cm at a location in Thetford Forest, Norfolk, UK. The soil consisted of 96% sand, 4% clay and <1% organic matter, with a pH of 5.6 and was rehydrated with the appropriate amount of copper solution to give a final water content of 13%±1 (approximately 50% of the soil water-holding capacity). Once dosed, soil was mixed thoroughly for 10 min in a soil mixer (Odjob mixer, Plysu, UK). The soil was then left to stabilize for 5 days before 20 immature (100–300 mg wet weight) and five mature (250–400 mg wet weight) individuals of *L. rubellus* were introduced to each mesocosm. Each treatment was replicated three times including a control. Food in the form of 10 g of oven dried (70°C, 24 h) horse manure, rehydrated with distilled water, was added to the soil surface at 14 day intervals.

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In the laboratory *Eisenia andrei* (Bouché 1972) were exposed to the following copper concentrations for a period of 28 days; 20, 40, 80 and 160 mg Cu kg^{-1} soil (dry weight). The appropriate quantity of $CuCl_2$ solution was added to 600 g (dry weight) of soil housed in square boxes. Forest soil, as used in the field experiment and with the same final water content, was left to stabilize for 3 days prior to the addition of 10 mature worms (400–700 mg wet weight) to each container. Laboratory temperature was 15°C (± 1) with a room humidity of 75–85%. The lighting regime was 12 h on, 12 h off at 295 and 25 lux respectively. Food in the form of 3 g of oven-dried horse manure, rehydrated with distilled water, was added to the soil surface at 7 day intervals.

Upon sampling, all earthworms from both sets of conditions were rinsed with distilled water, counted and individually weighed. They were then transferred to Petri dishes and left to depurate their guts for a minimum of 24 h in the dark, at 12°C (±3) and 54% (±5) relative humidity. All worms were supplied by a commercial vermiculturist (Pell, V. R., Worm-Hive Organics, Newark, UK).

Analysis of copper by inductively coupled plasma mass spectrometry

For the metal analysis of earthworm samples from the laboratory $n = 15$, whilst in the semi-field study $n = 9$, 4, 6 and 10 for the control, 40, 80 and 160 mg Cu kg^{-1} soil treatments respectively. For both studies 1g soil samples ($n = 4$) were collected from each treatment. All samples were frozen to –20°C prior to being dried to a constant weight at 80°C. Samples were digested in 2ml concentrated nitric acid (Aristar, BDH Ltd. UK) on a heating block at 120°C until the solutions became clear. Each sample was then diluted with deionized water to give an acid concentration of 2% (v/v).

Digested samples were analysed for copper using an inductively coupled plasma mass spectrometer (VG Elemental-Plasma Quad), operated according to the manufacturer's recommendations. Analysis of standard reference material (NMS Standard Reference Material 1577a, US Dept. of Commerce) was in good agreement with certified results.

^1H NMR spectroscopy

Upon sampling four earthworms were individually analysed by ^1H NMR spectroscopy for each of the treatments with the exception of the 20 mg Cu kg^{-1} soil treatment using *E. andrei* where $n = 2$. Samples were snap-frozen in liquid nitrogen and individually homogenized in a 1:2 ratio of earthworm physiological Ringer's solution (body v/v) (Lockwood 1963). The homogenates were centrifuged, the supernatant removed, and further ultrafiltered (Sartorius Centrisart I™) to a molecular weight cut-off point of 10 kDa. The resulting extracts were freeze-dried and reconstituted in ^2H_2O.

Single pulse ^1H NMR spectra were measured on a JEOL GSX500 spectrometer operating at 500.14 MHz ^1H observation frequency. ^1H NMR spectra were measured using a 45° pulse and a 6000 Hz spectral width. The spectra, recorded at ambient probe temperature, were the result of 256 free induction decays (FIDs) collected into 32 768 computer points with an acquisition time of 2.73 s. When processing the spectra, an exponential apodization function was used (BF=0.19) prior to Fourier transformation (Ft), while one order of zerofilling was used to increase the number of FID data points to 65536. Subsequent to Ft, baseline correction was carried out on the downfield region of the spectra. Whole body histidine concentrations were quantitated in relation to the internal standard 3-trimethylsilyl-[2,2,3,3,–$^2H_4$]-1-propionate (TSP). Whole body metabolite levels were determined by paper-weights of the respective resonance peaks from printed spectra.

To investigate the dynamics within tissue extracts single-pulse ^1H NMR experiments were carried out at 298, 303, 308, 313, and 318 Kelvin on a tissue extract of *L. rubellus* exposed to 80 mg Cu kg^{-1} soil.

Statistical analysis

From measurements made prior to the experiment and at the time of sampling survival and growth of the earthworms were calculated. One-way Analysis of Variance (ANOVA) and a subsequent Tukey–Kramer multiple comparisons test were carried out on these and other parameters to investigate whether significant effects of copper exposure could be observed in either earthworm species and if so which treatments were different. The Null Hypothesis was that copper had no significant effects on any of the parameters. One-way ANOVA was also carried out on ratios of certain metabolites in tissue extracts as detected by ^1H NMR spectroscopy. The Null Hypothesis was that increased copper exposure caused no significant variation in metabolite ratios.

Results

Soil copper concentrations

At the end of each experiment soil copper concentrations were, in each case, similar to the value dosed (Table 1). Concentrations were typically higher in the laboratory, particularly at the highest concentration. This difference, however, was not great and is likely to be due to leaching of water-soluble copper in the field system.

Body copper burdens

Earthworm body copper concentrations were significantly elevated in both the field experiment, using *L. rubellus* ($P < 0.001$), and the laboratory experiment, using *E. andrei* ($P < 0.001$) following exposure to increasing soil concentrations (Figure 1(*a* and *b*)). In both studies mean copper burdens were significantly different with the exceptions of the 40 and 80 mg Cu kg^{-1} soil treatments in the field study and the 80 and 160 mg Cu kg^{-1} soil treatments in the laboratory study. Earthworm copper bioconcentration factors (body concentration/soil concentration) for the field study were 4.2, 1.5, 0.86 and 0.70 for the control, 40, 80 and 160 mg Cu kg^{-1} soil treatments respectively. Bioconcentration factors for the laboratory study were 4.0, 1.1, 0.82, 0.77 and 0.30 for the control, 20, 40, 80 and 160 mg Cu kg^{-1} soil treatments respectively.

^1H NMR spectra of tissue extracts

The ^1H NMR spectroscopic analysis of whole worm extracts showed the presence of a large number of low molecular weight metabolites, including a number of amino acids, sugars, organic acids and organic bases (Figure 2). The NMR spectra of the tissue extracts were characteristic of the two earthworm species (Figure 2). Within species individual tissue extracts showed little variability even with increasing exposure to copper. However, in the field study consistent differences were

Soil	Soil copper treatments (mg Cu kg^{-1} soil dry wt)				
	Control	20	40	80	160
Field	3±0.2	26±1.9	43.5±3.4	75.8±3.1	153±5.4
Laboratory	3.7±0.49	25±2.1	49±3.3	92±9.5	192±13.6

Table 1. Mean soil copper concentrations (mg Cu kg^{-1} soil dry wt) in the soil systems to which *Eisenia andrei* and *Lumbricus rubellus* were exposed in the laboratory and field respectively ($n = 4$) (±SE

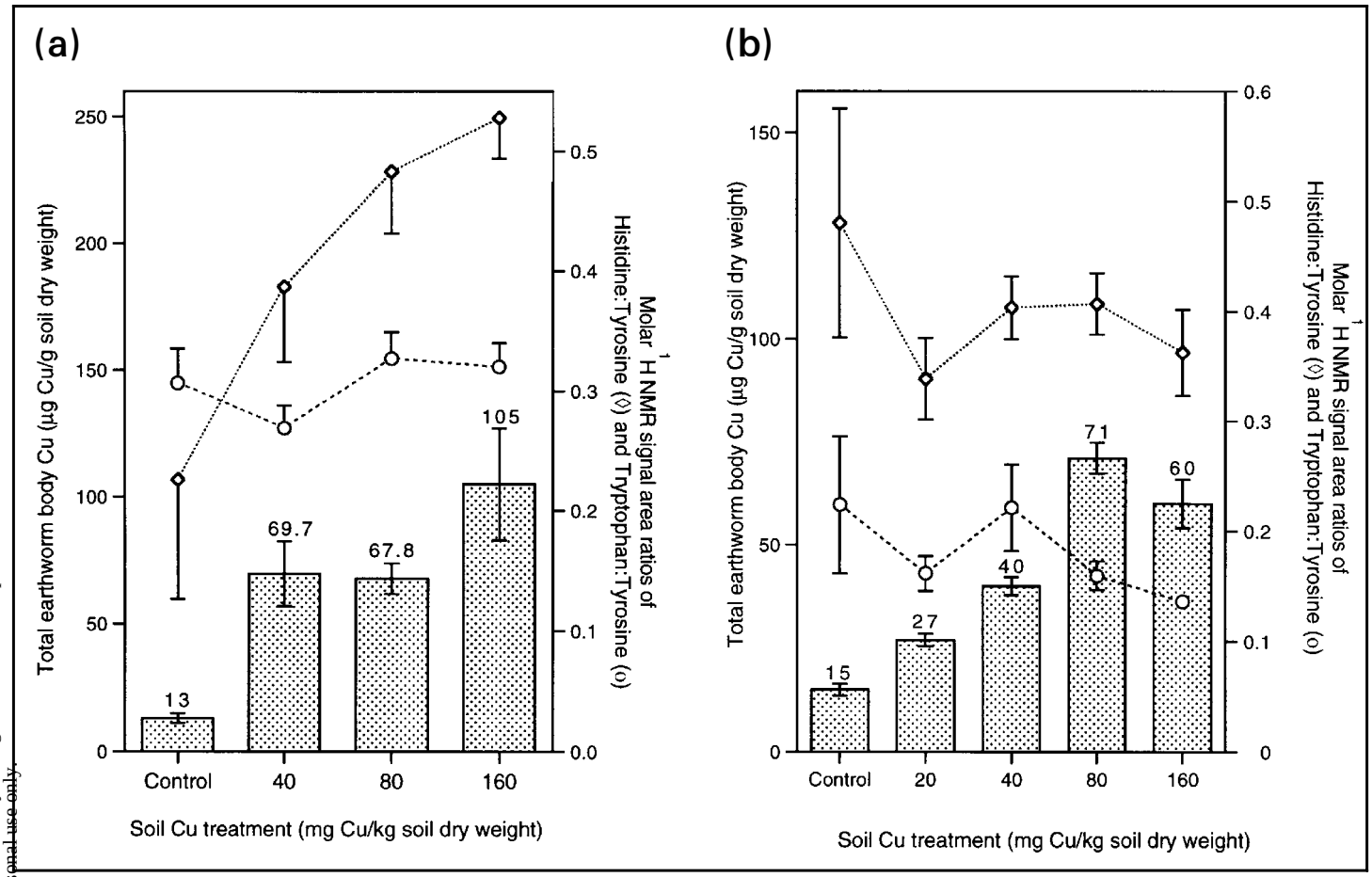

Figure 1. (A) Earthworms *Lumbricus rubellus* were exposed to four treatments of copper in field mesocosms for 110 days. (B) Earthworms *Eisenia andrei* were exposed to five treatments of copper in the laboratory for 24 days. The histidine:tyrosine ratio is indicated by (◊), whilst the tryptophan:tyrosine ratio is shown by (o). Earthworm body copper concentrations are given as dry weights (value is shown at top of each bar) and SEM bars are included. For body copper burdens in the field experiment $n = 9$, 4, 6 and 10 for the control, 40, 80 and 160 mg Cu kg^{-1} soil dry wt and for the laboratory experiment n=15 for all treatments.

seen in the aromatic region of spectra (Figure 3). Broad resonances at δ7.06 and δ7.81 and have been assigned to the simple amino acid histidine H4 and H2 protons respectively after the standard addition of authentic histidine. Histidine was present at low concentrations in control individuals of *L. rubellus*. On copper exposure, histidine levels became elevated in a dose–response fashion in comparison with other amino acids. This was illustrated with respect to tryptophan where the intensities of the peaks from the H2/H6 and H4 proton resonances from tyrosine and histidine respectively were compared (Figure 1). Statistical analysis indicated that the histidine:tyrosine ratio was significantly affected by increasing copper dosing levels ($P < 0.04$). Whole body histidine concentrations were found to reach 4.2 mM. The relative proportions (and total levels) of other amino acids remained constant with increasing copper exposure. This was illustrated when the tryptophan:tyrosine ratio was investigated, using the H7 and H2/H6 protons respectively, and shown not to vary significantly ($P = 0.320$) (Figure 1(*a*)). No such relationships are observed after the exposure of *E. andrei* to increasing concentrations of copper as neither the histidine:tyrosine ratio nor the tryptophan:tyrosine ratio differed significantly between treatments ($P = 0.635$ and 0.384, respectively) (Figure 1(*b*)).

The ^{1}H NMR signals from the histidine H2 and H4 imidazole ring protons in the spectra of worm tissue extracts showed differential line-broadening effects with respect to resonances from other amino acids; typical half-height linewidths ($\nu_{1/2}$) for histidine in the extracts being 8.0 Hz, whilst typical $\nu_{1/2}$ for other metabolites in the extract were 1–2 Hz (Figure 3). Such a broadening effect would be expected if the histidine were in dynamic chemical exchange with some other species or complexed with a paramagnetic metal ion such as Cu(II). The half-height line-widths of the histidine imidazole ring proton signals were unchanged with variation in the spectrometer probe temperature indicating that chemical exchange is an unlikely contributor to the observed line-broadening and hence tending to suggest a paramagnetic contribution.

Ecological parameters

No obvious trends were observed in the field and laboratory studies for either earthworm survival or earthworm growth (Tables 2 and 3). The only significant changes were observed for earthworm growth in the laboratory study. In this instance the ANOVA null hypothesis, that copper had no significant effects on any of the parameters, was rejected ($P = 0.015$). The Tukey–Kramer Multiple Comparisons test indicated a difference in earthworm growth betw

Parameter	Copper treatment (mg Cu kg^{-1} soil dry wt)			
	Control(12)	40(8)	80(10)	160(14)
% Survival	48	32	40	56
Mean growth	86±8.0	63±9.2	103±9.3	79±11.7

Table 2. Effect of increasing copper exposure on *Lumbricus rubellus* % survival and mean growth (% of weight at beginning of experiment) in the field (± SEM). Number of samples are given in parenthesis.

Parameter	Copper treatment (mg Cu kg^{-1} soil dry wt)				
	Control(39)	20(12)	40(25)	80(43)	160(36)
% Survival	78±8.6	60±10.0	62±2.5	86±4.0	72±7.3
Mean growth	105±4.7	114±5.85	115±5.5	122±7.5[a]	92±3.5[a]

Table 3. Effect of increasing copper exposure on *Eisenia andrei* % survival and mean growth (% of weight at beginning of experiment) in the laboratory (± SEM). Number of samples are given in parenthesis.

[a] Indicates significant differences between treatments as determined by the Tukey–Kramer Multiple Comparisons Test (where $P < 0.05$).

160 mg Cu kg^{-1} soil treatments ($P < 0.05$) in this case; earthworm growth was reduced at the higher treatment (Table 2). Over the duration of the field experiment growth of *L. rubellus* was generally lower and mortality higher than for the duration of the laboratory experiment using *E. andrei*. This is either due to the harsher and more variable environmental conditions experienced in the field, but is more likely due to the much longer duration of the field experiment.

Discussion

Single pulse high resolution ^{1}H NMR spectroscopy has shown that the tissue extracts of both earthworm species contain highly complex mixtures of metabolites (Figure 2). These give biochemical profiles shown previously to be characteristic of the species with respect to the range of low molecular metabolites identified when pollutant stress was absent (Gibb *et al.* 1997). As exposure to pollutants is known to disrupt homeostasis within an organism, resulting in an increase or decrease in particular metabolites (Nicholson and Wilson 1989), it is thought that these ^{1}H NMR spectroscopic profiles may reflect the metabolic status of the pollutant stressed individuals. Furthermore, it is at the biochemical and cellular rather than the individual level where pollutants have their effects (Moore 1985). Here biological response, or rather biochemical biomarkers, may be observed at sub-lethal exposures.

In the earthworm *L. rubellus*, exposed to copper, a response was observed in the form of elevation in the level of the endogenous amino acid histidine. Indeed a dose–response elevation of histidine was observed suggesting that endogenous histidine may be a novel biomarker of environmental exposure to copper. Timbrell *et al.* (1994) have suggested that biomarkers, in addition to giving a measure of exposure, may also provide some indication of the biochemical pathways that are perturbed in the toxic process. There are two possible pathway explanations for this elevation of free histidine. First, that copper exposure somehow disrupts histidine catabolism and excretion, leading to a subsequent elevation in tissue concentrations. Second, that histidine is actively synthesized as a response to raised ambient copper. In either case the elevation of whole body histidine can be viewed as a detoxification process as histidine forms a series of highly stable copper complexes (Dawson *et al.* 1990). Histidine-copper complexes are significantly more stable than the corresponding octahedral complex that the well known metal chelating agent EDTA forms with copper (Table 4). Indeed, the role of histidine and other common amino acids as physiological chelators of copper is well described throughout the literature for vertebrates. For example, in plasma, while the majority of copper is transported attached to albumin, a small amount is attached to amino acids or small peptides. In the albumin complex however, copper is bound by histidine at a specific tripeptide site (Asp-Ala-His) in man (Peters 1977), while canine albumin has tyrosine at position 3, rather than histidine, and hence binds copper poorly. Thus, in dogs amino acids take over the copper transport role. Furthermore, in the vertebrate liver an intermediary role for a copper:histidine complex has been suggested in the uptake of this metal (Danks 1983).

Of all the free amino acids or simple organic acids commonly found in organisms, histidine forms the most thermodynamically stable copper complexes (Dawson *et al.* 1990) (Table 3). The inference is thus, that histidine may be accumulated as part of a metal ion detoxification mechanism. This is supported by the recent work of Krämer *et al.* (1996) who observed the elevation of free histidine levels in plants tolerant of high concentrations of divalent ions following exposure to Ni(II). Of all the amino acids (with the exception of cysteine) histidine also forms the most thermodynamically stable nickel complexes (Table 4). Cysteine however, would be unsuitable as an endogenous extracellular detoxifying chelator due to its propensity to oxidize to cystine which has a much lower metal-ion affinity because of the loss of the free thiol group with its highly polarizable electrons. Krämer *et al.* (1996) also characterized a histidine–Ni(II) complex with octahedral geometry around the Ni(II) ion. Further work must be undertaken as to whether an analogous Cu(II) complex exists in invertebrate tissues and coelomic fluids.

In their study Krämer *et al.* (1996) investigated xylem sap rather than the whole organism and histidine was significantly elevated above all other amino acids following Ni(II) exposure.

Name	Log stability constant		
	Cu	Ni	Zn
Histidine	18.3	15.9	11.8
EDTA	15.5	15.3	13.1

Table 4. Stability constants of some metal complexes. For histidine the cumulative stability constants (β_2) are shown (at pH 7), whilst for ethylenediaminetetraacetic acid (EDTA) the apparent stability constants (most likely to be found at pH 7) are shown. Modified from D[illegible]

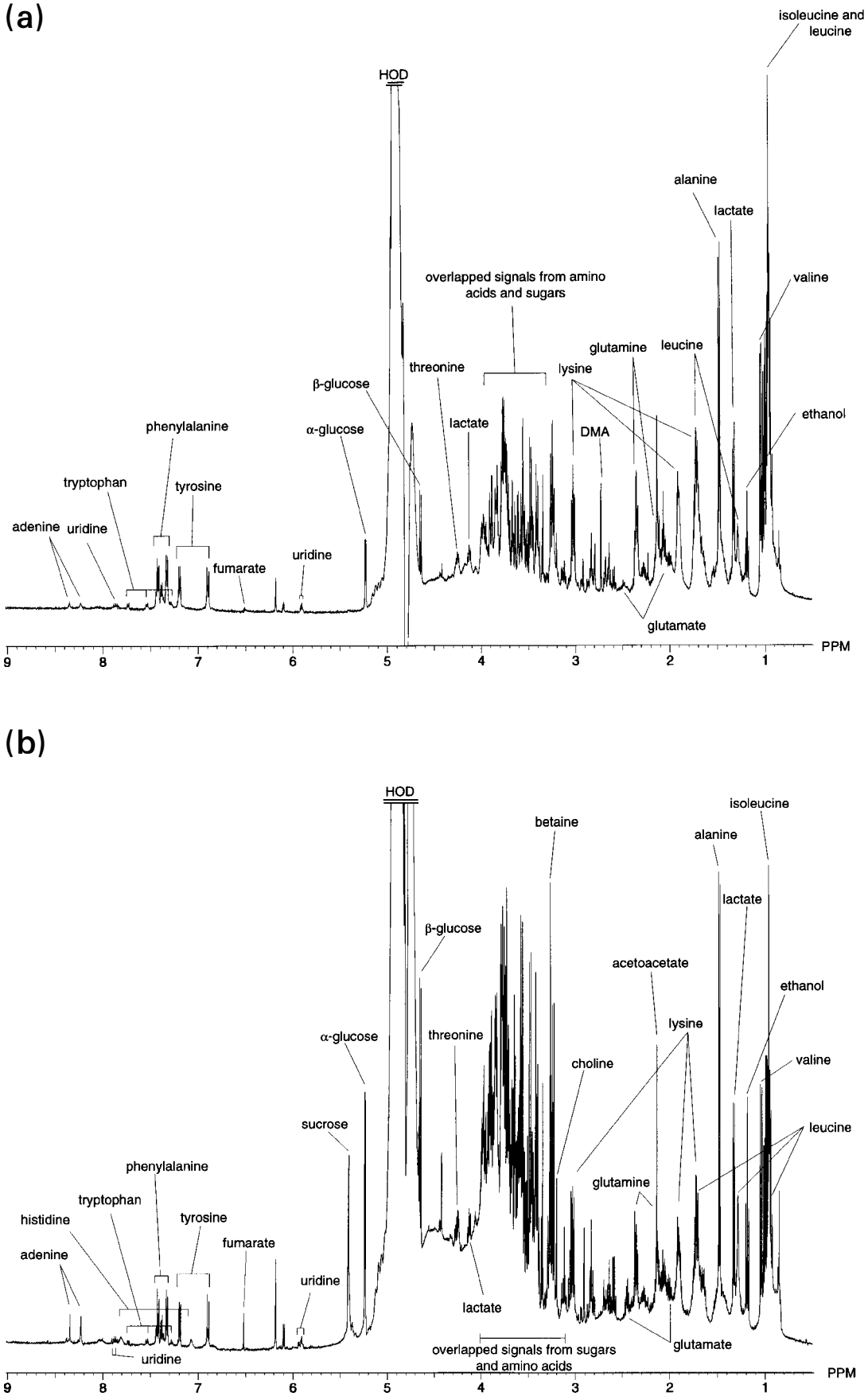

Figure 2. The 500 MHz ^{1}H NMR spectra of tissue extracts from (A) *Lumbricus rubellus* and (B) *Eisenia andrei*. Some major metabolites have been assigned. DMA, dimethylamine.

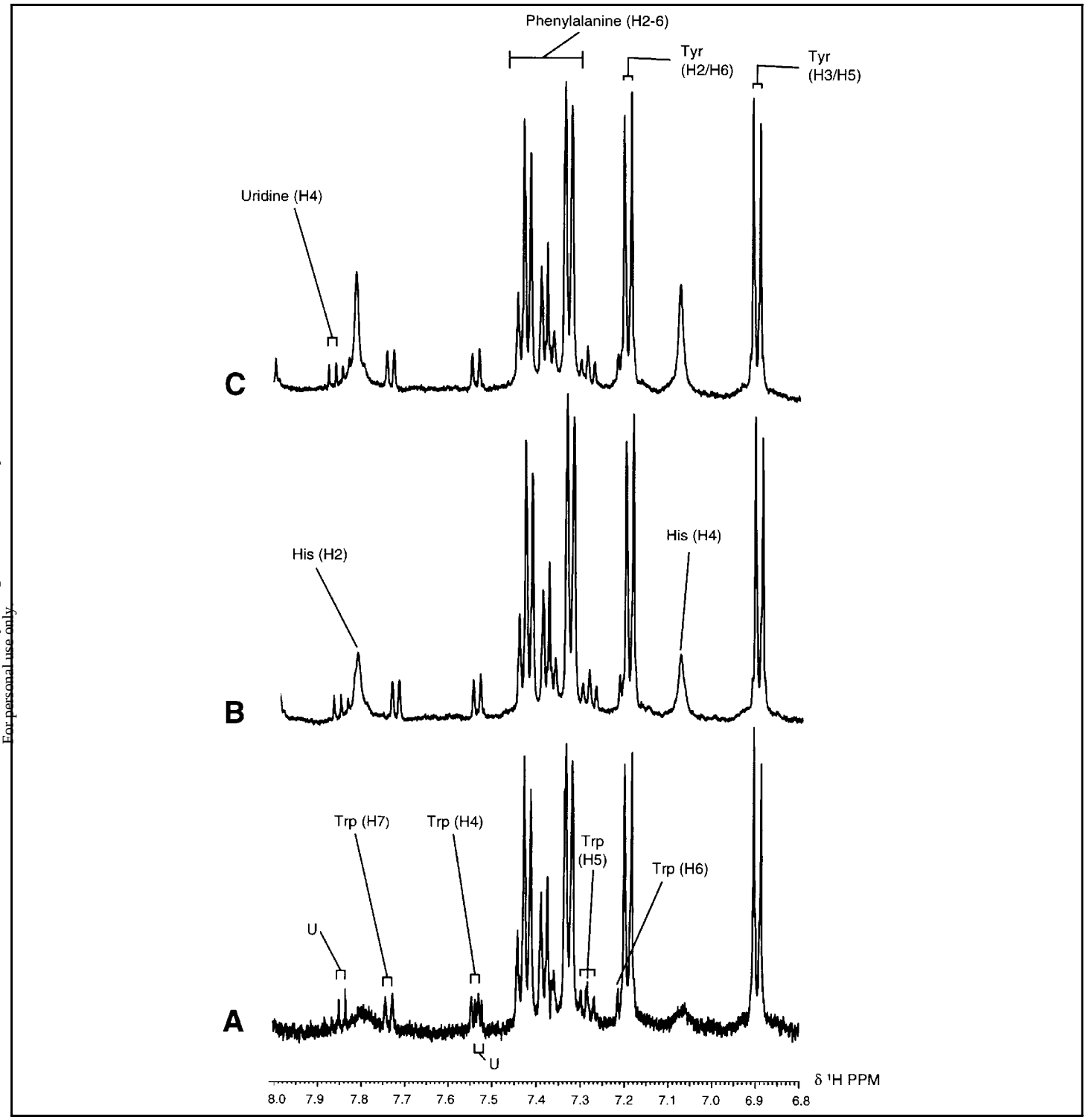

Figure 3. The 500 MHz ^{1}H NMR spectra (δ6.8 – δ8.0 region only) of earthworm (*Lumbricus rubellus*) tissue extracts from a control (labelled spectra A) and after exposure to 160 mg Cu kg^{-1} soil dw (labelled spectra B). The latter sample has been spiked with authentic histidine (labelled spectra C) to give a final concentration of approximately 1.24 mM. His, histidine; Trp, tryptophan; Tyr, tyrosine; U, unassigned. Bracketed abbreviations denote position of proton.

Our study investigated whole organism histidine levels which were in fact at significantly lower levels than other NMR-detectable amino acids (Figure 2). However, in whole earthworm tissue extracts levels of free histidine reached 4.2 mM while in the plants histidine reached 1.4 mM in response to Ni(II) exposure (Krämer *et al.* 1996). Thus histidine may be acting as an energetically 'low-cost' solution to a metal ion detoxification problem for both copper in earthworms and nickel in plants. This suggests that the elevation of free histidine in response to metal ion challenge may have a much wider occurrence and greater significance than has been previously thought.

Copper bioconcentration factors (BCF) of below 1.0, seen in most of the treatments, are consistent with literature observations (Ma *et al.* 1983, Hopkin 1989, Abdul Rida and Bouche 1994). High copper BCF were, however, seen in control organisms in the field and laboratory studies. The decrease in BCF for copper with increasing levels of this metal in the soil has also been previously observed (see Hopkin 1989 for review) although the mechanism behind this phenomena has yet to be elucidated. Copper appears to be homeostatically regulated by earthworms. Neuhauser *et al.* (1985) suggested that this may be by saturation of assimilation sites for the metal or active regulation of their assimilation. Alternatively, Streit (1984) proposed a model of copper compartmentalization in earthworms, and suggests that an excretion mechanism is activated once a critical body concentration is exceeded and despite regulation at high levels, copper concentrations continue to rise. Basic soil composition and chemistry exerts a great influence on the bioavailability of copper to earthworms (Beyer *et al.* 1982, Ma and Bodt 1993). It is thus important to be wary of comparing different soil types, although certain models have been developed to aid comparisons (Streit 1984).

The absence of a biomarker response in the earthworm *Eisenia andrei* in the laboratory experiments may possibly be explained by one of a number of reasons. The obvious differences between the two experiments were firstly period of exposure; this being much greater for *L. rubellus* (110 days compared with 28 days). This is unlikely to be highly significant as one would expect the shorter exposure time to be sufficient for a 'histidine response' to occur if it was going to happen and indeed soil copper concentrations at the end of the experiments were higher in the laboratory. Secondly, the lower growth and higher mortality in the field experiment may be due to harsher environmental conditions, thus making *L. rubellus* more sensitive to copper toxicity. However, the changes in these ecological parameters are more likely to be due to the greater length of the field study. Indeed if *L. rubellus* were sensitized to copper we would expect to see a biomarker response from *E. andrei* at least at the highest copper levels in the laboratory study. Finally, the species belong to different ecophysiological groups: *L. rubellus* is an epigeic, living at the surface of agricultural and natural soils underneath the litter layer, whilst *E. andrei* is commonly found in composts and dung heaps but rarely in natural or agricultural soils. Both species have relatively high reproductive rates and grow rapidly.

No obvious trends were observed in physiological parameters, with the exception of a significant decline in growth of *E. andrei* at the highest copper dosing levels. This highlights the weakness of using such parameters as toxicological end-points, an issue currently under much debate in the area of ecotoxicology (Weeks 1995), particularly when ecologically significant concentrations of pollutants are investigated. Other studies have shown the deleterious effects of copper at the population level but typically at higher concentrations than in this study (e.g. Ma 1984, Neuhauser *et al.* 1985). ^{1}H NMR spectroscopy thus offers a more sensitive indication of pollutant exposure than commonly used biomarkers. Furthermore it offers a number of advantages over other analytical techniques; the HPLC analysis of sap amino acid composition by Krämer *et al.* (1996), for example, necessitated extensive and time consuming co-chromatography. In comparison ^{1}H NMR spectroscopy offers rapid, non-selective analysis of a wide range of low molecular weight metabolites with no pre-selection of analytical conditions. ^{1}H NMR spectroscopy thus has great potential in its application as an ecotoxicological probe.

In summary, we propose that the use of low molecular weight chelators in metal detoxification strategies may be more widespread than previously thought. Histidine may thus act as a biomarker of such exposure. In addition, we have established links between biomarker responses and population parameters, a process fundamental in validating biomarker value.

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References

Abdul Rida, A. M. M. and Bouche M. B. (1994) A method to assess chemical biorisks in terrestrial ecosystems. In *Ecotoxicology of Soil Organisms*. M. H. Donker, H. Eijsacker and F. Heimbach, eds (CRC Press Inc., Boca Raton, Florida), pp. 373–394.

Anthony, M. L., Sweatman, B. C., Beddell, C. R., Lindon, J. C. and Nicholson, J. K. (1994) Pattern recognition classification of nephrotoxicity based on metabolic data derived from high resolution ^{1}H NMR spectra of urine. *Molecular Pharmacology*, **46**, 199–211.

Beyer, W. N. and Stafford, C. (1993) Survey and evaluation of contaminants in earthworms and in soils derived from dredged material at confined disposal facilities in the great lakes region. *Environmental Monitoring and Assessment*, **24**, 151–165.

Beyer, W. N., Chaney, R. L. and Mulhern, B. M. (1982) Heavy metal concentrations in earthworms from soil amended with sewage sludge. *Journal of Environmental Quality*, **11**, 381–385.

Danks, D. M. (1983) Hereditary disorders of copper metabolism in Wilson's Disease and Menke's Disease. In *The Metabolic Basis of Inherited Disease*, 5th edition, J. B. Stanburg, J. B. Wyngaarden, D. S. Fredrickson, J. L. Goldstein and M. S. Brown, eds (McGraw-Hill, New York), pp. 1251–1268.

Dawson, R. M. C., Elliott, D. C., Elliot, W. H. and Jones, K. M. (1990) *Data for Biochemical Research* (Clarendon Press, Oxford).

Gibb, J. O. T., Weeks, J. M. and Nicholson, J. K. (1997) Proton NMR spectroscopic studies on tissue extracts of invertebrate species with pollution indicator potential. *Comparative Biochemistry and Physiology* (in press).

Hopkin, S. P. (1989) *Ecophysiology of Metals in Terrestrial Invertebrates* (Elsevier Applied Science, London).

Krämer, U., Cotter-Howells, J. D., Charnock, J. M., Baker, A. J. M. and Smith, J. A. C. (1996) Free nickel as a metal chelator in plants that accumulate nickel. *Nature*, **379**, 635–638.

Lockwood, A. P. M. (1963) *Animal Body Fluids and Their Regulation* (Heinemann, London).

Ma, W. (1984) Sublethal toxic effects of copper on growth, reproduction and litter breakdown activity in the earthworm *Lumbricus rubellus*, with observations on the influence of temperature and soil pH. *Environmental Pollution Series A*, **33**, 207–219.

Ma, W. and Bodt, J. (1993) Differences in toxicity of the insecticide chloropyrifos to six species of earthworm (Oligochaeta, *Lumbricidae*) in standardised soil tests. *Bulletin of Environmental Contamination and Toxicology*, **50**, 864–870.

MA, W., EDELMAN, T., BEERSUM, I. V. AND JANS, Y. (1983) Uptake of cadmium, zinc, lead and copper by earthworms near a zinc-smelting complex: influence of soil pH and organic matter. *Bulletin of Environmental Contamination and Toxicology*, **30**, 424–427.

MOORE, M. N. (1985) Cellular responses to pollutants. *Marine Pollution Bulletin*, **16**, 134–139.

MORGAN, J. E. AND MORGAN, A. J. (1990) The distribution of cadmium, copper, lead, zinc and calcium in the tissues of the earthworm *Lumbricus rubellus* sampled from one uncontaminated and four polluted soils. *Oecologia*, **84**, 559–566.

NEUHAUSER, E. F., LOEHR, R. C., MILLIGAN, D. L. AND MALECKI, M. R. (1985) Toxicity of metals to the earthworm *Eisenia andrei*. *Biology and Fertility of Soil*, **1**, 149–152.

NICHOLSON, J. K. AND WILSON, I. D. (1989) High resolution proton magnetic resonance spectroscopy of biological fluids. *Progress in NMR Spectroscopy*, **21**, 449–501.

PEAKALL, D. B. (1994) The role of biomarkers in environmental assessment (1). Introduction. *Ecotoxicology*, **3**, 157–160.

PETERS, T. (1977) Serum albumin: recent progress in the understanding of its structure and biosynthesis. *Clinical Chemistry*, **23**, 5.

STREIT, B. (1984) Effects of high copper concentrations on soil invertebrates (earthworms and oribatid mites): experimental results and a model. *Oecologia*, **64**, 381–388.

SVENDSEN, C. AND WEEKS, J. M. (1997) A simple low-cost field mesocosm for ecotoxicological studies on earthworms. *Comparative Biochemistry and Physiology*, **117**, 31–40.

TIMBRELL, J. A., DRAPER, R. AND WATERFIELD, C. J. (1994) Biomarkers in toxicology: new uses for some old molecules? *Toxicology and Ecotoxicology News*, **1**, 1–11

WEEKS, J. M. (1995) The value of biomarkers for ecological risk assessment: academic toys or legislative tool? *Applied Soil Ecology*, **2**, 215–216.

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