Progress Report 1995 of

# BIOPRINT

## Biochemical Fingerprint Techniques as Versatile Tools for the Risk Assessment of Chemicals in Terrestrial Invertebrates

Third Technical Report

Report from a Workshop
held in Innsbruck, Austria
February 9-10, 1996

EC Environmental Research Programme
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## Data sheet

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Abstract: The results of the second year of the EU R&D project BIOPRINT is presented. Further results of the effects of metals (Cu, Cd and Zn) and pesticide (dimethoate) on stress proteins, metallothioneins, metalbinding proteins and esterases are given. The findings of a new metalbinding protein in earthworms are presented.

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# ENVIRONMENTAL RESEARCH PROGRAMME
# Research Area: Ecotoxicology
# Progress Report

**Contract no:** EV5V-CT94-0406

**Title:** BIOCHEMICAL FINGER-PRINT TECHNIQUES AS VERSATILE TOOLS FOR THE RISK ASSESSMENT OF CHEMICALS IN TERRESTRIAL INVERTEBRATES (BIOPRINT).

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**Key words:** Biomarkers, soil invertebrates, risk assessment.

**Reporting period:** April 1, 1995 - March 31, 1996.

# PART A. SUMMARY OF THE PROJECT

## I. GENERAL OBJECTIVES:

**The main objective** of the BIOPRINT project was the development of biochemical fingerprint techniques (biomarkers) for assessing the exposure and effect of toxicants in terrestrial invertebrates. Specific techniques have been developed for the following biomarkers: i) heat shock proteins (HSP's), ii) metallothioneins or metal binding proteins for detection of heavy metals and iii) esterases for detection of organic contaminants. In addition, advanced spectroscopic techniques (NMR, ICP-MS) were used to discover novel biomarkers in the organisms used. The applicability of these techniques was investigated for different taxonomic groups including representatives of various trophic levels: Nematoda, Collembola, Gastropoda, Isopoda and Oligochaeta.

## II. SPECIFIC OBJECTIVES FOR THE REPORTING PERIOD:

Research was focused on: 1) establishing dose-response relationships for HSP60 induction in bacterivorous nematodes after exposure to cadmium and copper, quantification of the HSP60 response and linking to sublethal effects on life-cycle variables in soil in the laboratory, 2) establishing a technique for (semi) quantifying the induction of HSP70 (and HSP60) at the protein level in the slug, *Deroceras reticulatum*, validating these biomarker tests by means of full life-cycle studies to the F1 generation with *D. reticulatum*, developing a RT-PCR technique which renders the assessment of proteotoxic conditions in the slug *(D. reticulatum)* possible at the mRNA level, 3) the suitability of metallothionein (MT) as a possible biomarker for metal exposure in the terrestrial gastropods (*Helix pomatia*, *Arianta arbustorum* and *Deroceras reticulatum*) at the protein and MT mRNA level, characterizing inducible, metal-binding proteins from terrestrial oligochaetes (*Eisenia fetida*), 4) studying the toxic response *in vitro* of the esterases in the three species; earthworm, *E. fetida*, isopod, *Porcellio scaber*, and Collembola, *Folsomia candida* using electrophoresis and densitometry and the response *in vivo* of the esterases in the three species by feeding experiments, 5) establishing links between changes in ICP-MS (Inductively-Coupled Plasma-Mass source Spectrometry) profiles and alterations in NMR (Nuclear Magnetic Resonance spectroscopy) profiles, exploring the putative copper:histidine relationship as a metabolic biomarker in earthworms, 6) optimizing a homogenisation and separation technique for analyzing metal-binding proteins in Collembola and isopods, relating the effects of cadmium and zinc on metal-binding proteins to effects on the growth of isopods and springtails.

## III. MAIN RESULTS:

1) Expression of HSP60 in the nematode *Plectus acuminatus* was dose-related with the strongest induction at the highest concentration for copper and cadmium. Cadmium appeared to be a more potent inducer of HSP60 than copper. It was shown that HSP60 is induced at concentrations which are a factor 10,000 lower than the NOEC for population growth rate in artificial soil. 2) The induction of HSP70 both at the protein and at the mRNA levels proved to be a valuable

biomarker for exposure to and toxicity assessment in both slugs and earthworms. The sensitivity of the HSP70 assay at the protein level corresponded to those metal concentrations which affected longevity and terminated reproduction when the animals were exposed chronically. Investigations on the transcription rate of the HSP70 gene(s) using the RT-PCR technique, are recommended when assessing risk for lower concentrations. 3) It was indicated that at least in the midgut gland of terrestrial gastropods, the pool of (Cd,Zn)-MTs may represent a strong biomarker for environmental cadmium exposure. In the earthworm *E. fetida*, the presence of metal-inducible and metal-binding proteins in their tissues appeared to be a promising biomarker. At the molecular level, methods of PCR amplification were developed to quantify the induction of MT mRNA in terrestrial gastropods. 4) The *in vitro* experiments revealed that the esterases in all three species were sensitive to the chemicals. All zones with esterase activity on the zymograms were inhibited by each of the chemicals. A dose-response effect was shown irrespective of the chemical used. It was observed that zinc even in the highest concentration used for the *in vitro* experiment did not show any effect on esterases of *F. candida* in the *in vivo* experiment. 5) NMR spectroscopy has shown that tissue extracts investigated contain highly complex mixtures of metabolites in earthworms. Histidine levels increased with increasing exposure to copper, and results suggest that this is a likely detoxification mechanism not previously observed. 6) It appeared that the total amount of zinc was not responsible for the effects on growth of isopods but the fluxes of zinc mainly determined the effects. The zinc accumulation was independent of the protein concentration of the food. High protein contents in the diet enabled isopods to resist higher dietary zinc concentrations. This result can be explained either by a direct use of the extra proteins for the synthesis of metal binding proteins or an indirect use as an extra energy source.

## IV. PUBLICATIONS:

Berger, B., Dallinger, R. and Thomaser, A. (1995): Quantification of metallothionein as a biomarker for cadmium exposure in terrestrial gastropods. *Environ. Toxicol. Chem.* 14: 781-791.

Berger, B., Hunziker, P.E., Hauer, C.R., Birchler, N. and Dallinger, R. (1995): Mass spectrometry and amino acid sequence of two cadmium-binding metallothionein isoforms from the terrestrial gastropod *Arianta arbustorum*. *Biochem. J.* 311: 951-957.

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Triebskorn, R. and Köhler, H.-R. (1996): The impact of metal pollution on the grey garden slug *Deroceras reticulatum* (Müller): accumulation, cellular effects and semi-quantitative evaluation of toxicity. *Environ. Pollut.* (in press)

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# PART B. DETAILED REPORT OF THE CONTRACTORS

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## I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To establish dose-response relationships for HSP60 induction in bacterivorous nematodes after exposure to cadmium and copper.

2. To quantify the HSP60 response and to link it to sublethal effects on life-cycle variables in soil in the laboratory.

3. To assess the suitability of the HSP60 response as a biomarker of chemical stress in the bacterivorous nematode *Heterocephalobus pauciannulatus*.

4. To identify metal-binding proteins and novel biomarkers in cooperation with participants 5 and 6.

## II. MAIN RESULTS OBTAINED:

### Methodology

1. Detection and quantification of HSP60 in nematodes exposed to toxicants in water:
Induction of the heat-shock protein HSP60 in the parthenogenetic nematodes *Plectus acuminatus* and *Heterocephalobus pauciannulatus* was assessed by means of 1- and 2-dimensional protein gel electrophoresis (1D- and 2D-PAGE) in two different experiments respectively. Nematodes were obtained from laboratory stock cultures. The monoclonal antibody (MAb) mouse anti-human HSP60 with secondary alkaline phosphatase conjugated rat anti-mouse was used to detect the stress protein response in adult nematodes after exposure to cadmium and copper. All experiments were run at 20°C in the dark.

Experiment 1: To gain insight into the expression of different isoforms of the HSP60, experiments were run exposing adult nematodes (3 weeks old) directly in water to copper and cadmium at the following concentrations: Cu: 0.002, 0.02, and 0.2 mg/L, Cd: 0.007, 0.07, and 0.7 mg/L. After an exposure period of 24 h at 20°C, nematodes were homogenized and analyzed for HSP60 using 2D-PAGE and subsequent MAb blotting.

Experiment 2: The following concentration ranges were used for Cu: 0.002, 0.02 and 0.2 mg/L, and Cd: 0.007, 0.07 and 0.7 mg/L. Experiments were conducted in water at 20°C for 24 h. Molecular weight markers and a commercially available HSP60 were used as calibration markers to estimate the weight of the HSP's detected. To correct for differences in total protein content among sample treatments, total muscle-filament content was used as a positive control by applying a specific MAb (most presumably directed to tropomyosin) produced by the Department of Nematology (De Boer et al. 1996). HSP60 induction was quantified using digital image analysis of subsequent immunoblots derived from 1D-PAGE gels.

2. Detection and quantification of HSP60 in *P. acuminatus* exposed to toxicants in OECD artificial soil: HSP60 induction was assessed in the nematode *P. acuminatus* exposed to copper and cadmium in OECD artificial soil using 1D-PAGE. Adult females were exposed to cadmium and copper in the following concentration range: 0.0032, 0.032 and 0.32 mg/kg dry wt. OECD soil. After 2 weeks nematodes were extracted and samples were homogenized and screened for HSP60 induction using MAb mouse anti-human HSP60.

### Results

1. Detection and quantification of HSP60 in nematodes exposed to toxicants in water:

Experiment 1: Exposure of nematodes to cadmium and copper in water resulted in a single clear spot as revealed by the 2D-PAGE blots. The location of the spot is equivalent to the ones revealed in blots from nematodes exposed to a heat-shock treatment of 60 min at 37°C. There appeared to be no different isoforms of HSP60 in either the control or the metal-exposed animals. Expression of HSP60 was dose-related with the strongest induction at the highest concentration for both metals. The results imply that cadmium is a more potent inducer of HSP60 in *P. acuminatus* compared to copper. Furthermore there was no clear evidence indicating increased isoform induction upon exposure to higher concentrations.

Experiment 2: Following the results of the 2D-PAGE experiments, HSP60 induction was assessed by means of 1D-PAGE. Exposure to cadmium resulted in an increase in HSP60 induction at higher concentrations. Compared to the control treatment induction was higher at

all concentrations. Copper however appeared to be a less strong inducer as revealed by the concentration-response relationship.

2. Detection and quantification of HSP60 in nematodes exposed to toxicants in OECD artificial soil: Nematodes exposed to copper and cadmium in OECD artificial soil for 2 weeks exhibited a strong induction of HSP60 relative to the control treatment. Induction was stronger for cadmium than for copper and for both metals induction was more pronounced in soil than in animals exposed in water for 24 h. It appeared that HSP60 was induced already at concentrations which are a factor 10,000 lower than the NOEC for population growth rate in artificial soil.

**Discussion**

It was shown by using immunoblot techniques that HSP60 in *P. acuminatus* was induced by relatively low concentrations of cadmium and copper. The results are comparable to the induction of HSP70 in the nematode *Caenorhabditis elegans* which responded sensitively to cadmium exposures. Cadmium appeared to be a stronger inducer than copper which agrees with the results obtained for sublethal life-cycle studies (Kammenga et al. 1996 and in press). The use of MAb's proved to be extremely useful for specifically identifying HSP60 in the nematode *P. acuminatus*. Apparently, a wide cross-reactivity exists for this particular commercially obtained MAb which has been raised against a different vertebrate species.

Nematodes exposed in OECD artificial soil for 2 weeks showed a strong HSP60 response for both metals tested. It appeared that concentration levels were far below the NOEC-value for population growth rate indicating the potential value of HSP60 induction for exposure assessment of heavy metals in soils. A drawback however might be that the HSP response appears to be a too sensitive biomarker to be succesfully used in the field. Therefore the benefit and value of the response has to be evaluated by field or *in-situ* bio-assay studies thus confirming its potential applicability for future exposure assessment procedures.

Densitometric image analysis of blots (grey-value analysis) provides a well suited and reproducible methodology for quantifying HSP60 response in nematodes. Standardisation of measuring grey-values in immunoblots (width, length, or square area of the spot) however is recommended to ensure reproducible results and subsequent comparison between HSP response for different toxicants and other abiotic stress factors.

Attempts were made to detect HSP60 induction in the nematode *H. pauciannulatus*. Adults were exposed to cadmium and copper in water. However, obtained results were not consistent and appeared not to be reproducible. At present, the exact reasons are still unknown and revealing the HSP response in *H. pauciannulatus* awaits further investigation.

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Kammenga J.E., Busschers M., Van Straalen N.M., Jepson P.C. and Bakker J. (1996): Stress induced fitness reduction is not determined by the most sensitive life-cycle trait. *Funct. Ecol.* 10:106-111.

Kammenga J.E. and Riksen J.A.G. (1996): Comparing differences in species sensitivity to toxicants: phenotypic plasticity vs. concentrations-response relationships. *Environ. Toxicol. Chem.* (in press).

# PART B. DETAILED REPORT OF THE CONTRACTORS

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## I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To establish a technique for (semi) quantifying the induction of heat shock protein(s) HSP70 (and HSP60) at protein level in the slug, *Deroceras reticulatum*, and to assure suitability for assessing proteotoxic stress conditions resulting from exposure to zinc, copper, and cadmium in laboratory toxicity experiments.

2. To validate these biomarker tests by means of full life-cycle studies to the F1 with *D. reticulatum* for the metal concentrations used in the stress protein studies.

3. To carry out subchronic exposure experiments with sublethal concentrations of zinc, copper and cadmium to be analyzed for metallothionein (participant 3) and by NMR and ICP-MS (participant 5). Additionally, to analyze samples of two earthworm species obtained from participant 5 for HSP70.

4. To develop and optimize a RT-PCR technique which renders the assessment of proteotoxic conditions in the slug *(D. reticulatum)* possible at the mRNA level. To quantify the production of HSP70-mRNA in response to subchronic exposure.

## II. MAIN RESULTS OBTAINED:

### Methodology

1. HSP70 and HSP60 tests at the protein level: Western-blotting techniques were used. The internal reference was the total protein concentration in the supernatant of the slug homogenate. Protein amounts (50 $\mu$g per lane, Bradford's assay) were subjected to SDS-PAGE, transferred to nitrocellulose and stained with a combination of a monoclonal mouse anti-human HSP70 (HSP60) antibody and a secondary peroxidase-coupled goat anti-mouse IgG antibody. Subsequent (semi) quantification was conducted by densitometric image analysis. As a positive control during the first developmental phase of this technique, heat-shocked individuals (20-25°C) were analyzed.

Adult laboratory reared slugs were exposed to food (carrot slices and lettuce leaves, sprinkled with $CaCO_3$ particles) soaked with either 10, 50 or 100 mg/L Cd or to 500, 1000 or 5000 mg/L Zn at 10°C during a 16 h/8 h day/night photoperiod for three weeks. The metal chloride salt was used for all exposures. Additionally, sublethal exposure experiments were conducted with lower concentrations of Cd (0.03, 0.06 or 0.15 mg/L), Zn and Cu (0.3, 1.0 or 10.0 mg/L) which were only used for contamination of the food material. In these experiments, the food was soaked for 1h in the respective metal solutions. Metal concentrations per dry weight in the substrate, the food and the total body of the animals were analyzed by AAS.

HSP70 analysis of the two earthworm species *(Aporrectodea rosea, Lumbricus rubellus)* obtained from participant 5 was conducted following the same protocol as for the slugs. Both species were exposed to soil containing either 20, 40, 80, 160, or 320 mg/kg copper. Controls were kept in uncontaminated soil (<2 mg/kg).

2. Life-cycle investigations: Juvenile *D. reticulatum* at the age of 40 d were kept on a wet filter-paper base and fed a diet of lettuce leaves and carrot slices. Both food and substrate were soaked with either 10, 50 or 100 mg/L Cd, with 100, 500 or 1000 mg/L Zn, or with a mixture of 10 mg/L Cd and 500 mg/L Zn at 10°C with a photoperiod of 16 h light/8 h darkness. Additionally, in a subsequent experiment, juveniles were exposed to contaminated food only, which were soaked for 1 h with either 0.03, 0.06 or 0.15 mg/L Cd, 0.3, 1.0 or 10.0 mg/L Zn and Cu under the same abiotic conditions as mentioned above. Controls were wetted with tap water. Chronic exposure experiments were terminated after 169 d or 275 d (subsequent experiment). Mortality, fecundity (number of egg masses, number of eggs) and offspring rate (hatched juveniles of the F1 generation) were recorded.

3. Development of the reverse transcriptase-PCR technique RT-PCR and the stress response of *D. reticulatum* on the transcriptional level (HSP70-mRNA): Based on a computer alignment of known nucleotide sequences of HSP70-mRNA and genes of a variety of vertebrate and invertebrate species (but not slugs), two highly conserved regions of circa 500 to 530 bp and 1080 to 1120 bp downstream of the end of the variable sequences of the HSP70 mRNA were chosen for the construction of 35 bp oligonucleotide primers for the PCR technique. Isolated RNA from the slugs was quantified and a constant amount reversely transcribed into cDNA from which, in turn, a 512 bp sequence corresponding to a sequence in the highly conserved region of the HSP70-mRNA was amplified by PCR. Primarily, heat shocked specimens (25°C) were analyzed to assure primer binding but, subsequently a plasmid (pUHE21-2delta12fd(dnaK$^+$)) containing the procaryotic HSP70 analogue, dnaK, was used as a positive control.

Corresponding with the studies at the protein level, adult specimens of *D. reticulatum* were

exposed to metal-enriched food (lettuce leaves and carrot soaked with either 0.03, 0.06 or 0.15 mg/L Cd, 0.3, 1.0 or 10.0 mg/L Zn and Cu). The abiotic conditions were as described above. HSP70-mRNA was transcribed reversely and amplified with the PCR technique. After amplification, the PCR products were analyzed by horizontal agarose gel electrophoresis and stained with ethidium bromide. (Semi) quantification took place by densitometric image analysis.

**Results**

1. The induction of HSP70 and HSP60 on the protein level: Heat-shocked slugs (positive controls) showed a strong induction of HSP70 and HSP60 in comparison to controls which revealed only a weak protein band at approximately 70 (60) kDa. Regarding the induction of HSP70, metal-exposed specimens of *D. reticulatum* responded to the applied metal concentrations in a dose-dependent manner. From 10 mg/L Cd and 500 mg/L Zn onwards, the HSP70 level was shown to be elevated from the controls, see Figure 1. Whilst the response to Cd remained approximately constant for concentrations >10 mg/L, an increased staining intensity of the 70 kDa band corresponded to increasing concentrations of zinc in the food/substrate indicating that the patterns of stress response of the slugs differed between different metal exposure. These general patterns of stress response did not change when image analysis data were related to the metal residues in the slug tissues. For the low concentrations of Cd (0.03 to 0.15 mg/L), Zn and Cu (0.3 to 10 mg/L), no HSP70 induction could be found at the protein level. A significant elevation of HSP60 in the supernatants even of the slugs exposed to the high metal concentrations could not be found. Contamination resulted in a far lower increase of HSP60 than of HSP70.

In principle, the earthworms *A. rosea* and *L. rubellus* responded in a similar manner to increasing metal concentrations in their environment. *A. rosea* showed an increase of the HSP70 level with increasing copper concentration up to 40 mg Cu/kg soil which decreased again in response to higher concentrations most probably due to a pathological impact of this metal on the tissues of the animals which affected the cellular protein synthesis process. This was accompanied by a high mortality in the earthworm groups exposed to the highest Cu concentrations. *L. rubellus* populations on average showed a concentration-dependent HSP70 increase in response to Cu, but in contrast to the aforementioned species, predominantly singly selected individuals appeared to respond to the stressor. The number of those specimens with an elevated HSP70 level increased with exposure to increasing copper concentrations.

2. Life-cycle investigations: The life-cycle studies revealed an adverse impact of chronic metal exposure on life-time history and reproduction, see Figure 1. Particularly the rate of offspring production was drastically diminished for *D. reticulatum*. Survival rate also appeared to be a sensitive parameter. The most relevant data are summarized in Table 1. For all three metals tested, however, effects at the lowest metal concentrations on survival, egg production and offspring rate first became obvious approximately four months after exposure had started.

**Table 1.** Life-cycle parameters affected by chronic exposure to metals in *D. reticulatum*

| Exposure [mg/L] | Time to mortality (100%) | Egg number/animal | Offspring number/animal |
|---|---|---|---|
| *Contaminated food and substrate:* | | | |
| Control | >169 d | 26.2 | 22.3 |
| 10 Cd | 125 d | 0.0 | 0.0 |
| 50 Cd | 68 d | 0.0 | 0.0 |
| 100 Cd | 36 d | 0.0 | 0.0 |
| 10 Cd + 500 Zn | 104 d | 0.5 | 0.0 |
| 100 Zn | 142 d | 1.1 | 0.4 |
| 500 Zn | 170 d | 0.8 | 0.0 |
| 1000 Zn | 57 d | 0.0 | 0.0 |
| *Contaminated food only:* | | | |
| Control | 275 d | 17.5 | 9.3 |
| 0.03 Cd | 163 d | 12.4 | 5.5 |
| 0.06 Cd | 131 d | 5.2 | 2.8 |
| 0.15 Cd | 123 d | 0.0 | 0.0 |
| 0.3 Zn | 146 d | 2.7 | 1.2 |
| 1 Zn | 113 d | 0.2 | 0.2 |
| 10 Zn | 81 d | 0.0 | 0.0 |
| 0.3 Cu | 140 d | 2.6 | 0.0 |
| 1 Cu | 154 d | 0.0 | 0.0 |
| 10 Cu | 160 d | 0.0 | 0.0 |

3. The induction of HSP70-mRNA at the transcriptional level: In both metal-exposed and control slugs as well as in the dnaK-containing plasmide (positive control) an amplification of the 512 bp fragment was possible with the RT-PCR technique. Since all relevant methodological parameters have been optimized, only a single band indicating successful selective amplification of the selected HSP70-cDNA fragment occurred in the gels. Densitometric (semi) quantification of the results revealed an elevation of HSP70 gene transcription in *D. reticulatum* as a primary response to even the lowest applied metal concentrations (0.03 mg/L Cd, 0.3 mg/L Zn, 0.3 mg/L Cu), see Figure 1. In response to cadmium or zinc, gene transcription increased with increasing metal concentration and remained constantly high (60 to 70 fold above the control level) at least at concentrations up to 0.15 mg/L Cd or 10 mg/L Zn. In response to copper, the HSP70-mRNA level raised significantly to 65 fold that of the control level, following exposure to 0.3 mg/L Cu. More elevated copper concentrations resulted in a constant HSP70-mRNA level about ten times higher than in the control.

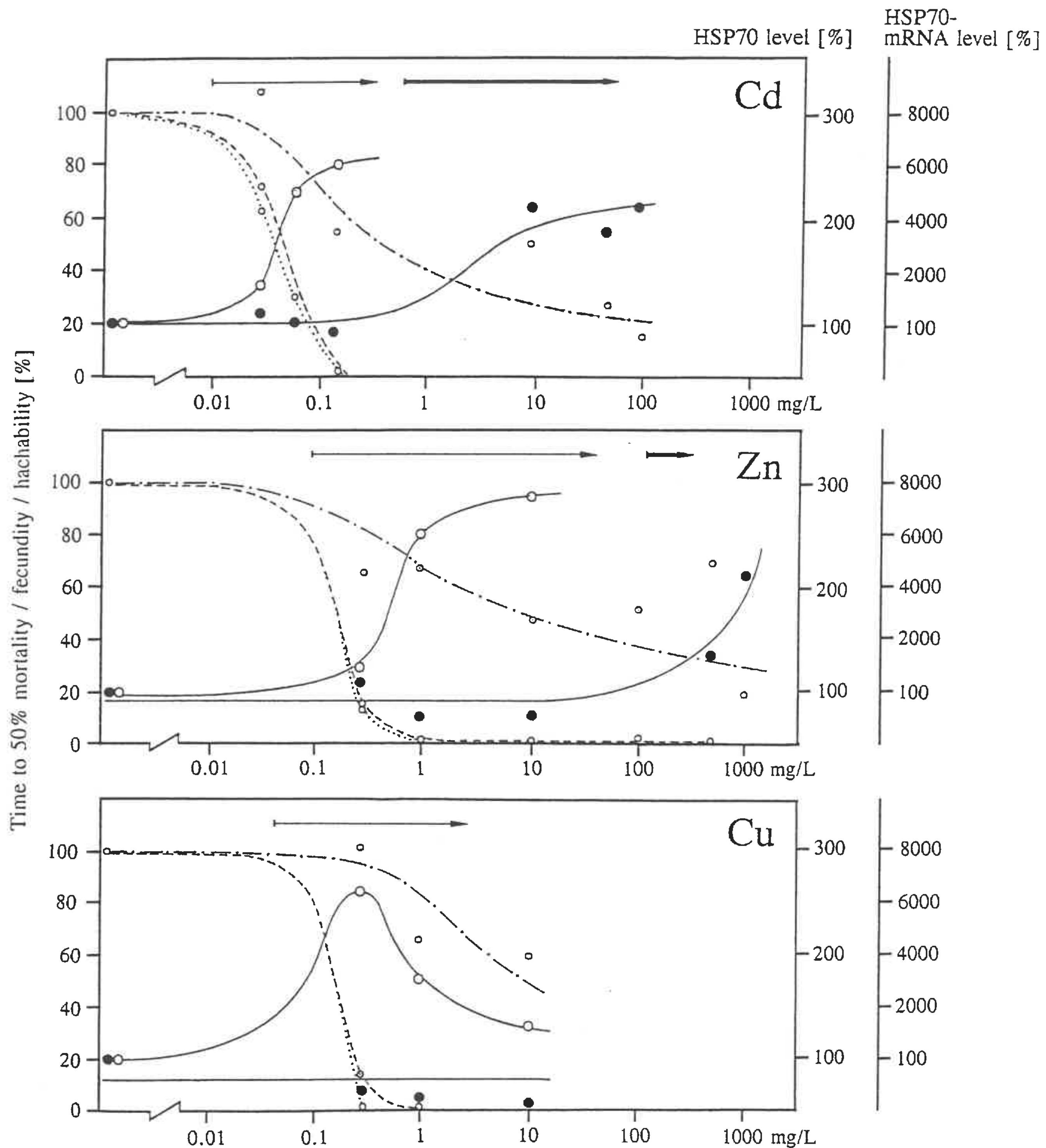


**Figure 1.** Comparative representation of biomarker reactions and alterations of life-cycle parameters in *Deroceras reticulatum* exposed to increasing Cd, Zn or Cu content in the food. The horizontal axis is represented by a logarithmic scale of the metal concentrations [mg/L] added to the food, the vertical axis refers to the percental response of the investigated parameters compared to the respective control group (100%). Different scales refer to the different investigated parameters: HSP70 induction at the protein level (black dots, ●), transcription of the HSP70 gene(s) at the mRNA level (circles, ○), time to 50% mortality (—•—•—), fecundity (number of eggs per animal, — — —) and hatchability (offspring per animal, ••••). The arrows on top of each blot symbolize the minimal metal concentration from which onwards the biomarkers are appropriate to indicate adverse effects on individual and population parameters in *Deroceras*. Thin arrow: transcription level of the HSP70 gene(s), thick arrow: HSP70 protein level.

**Discussion**

The present studies proved the induction of HSP70 both at the protein and at the mRNA levels to be a valuable biomarker for exposure to and toxicity assessment in those species examined. Threshold metal concentrations were far below the $LC_{50}$ indicating the high sensitivity of the developed assays. As shown by the comparison of the HSP70 data with life-cycle studies (Figure 1), sensitivity of the HSP70 assay at the protein level corresponded to those metal concentrations which affected longevity and terminated reproduction when the animals were exposed chronically. To detect possible risks of even lower metal concentrations, which decreased longevity and reproduction of *Deroceras* only slightly, investigations on the transcription rate of the HSP70 gene(s) using the RT-PCR technique, are recommended.

## PART B. DETAILED REPORT OF THE CONTRACTORS

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### I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To test the suitability of metallothionein (MT) as a possible biomarker for metal exposure (copper, cadmium) and for organochemicals (dimethoate) in terrestrial gastropods (*Helix pomatia*, *Arianta arbustorum* and *Deroceras reticulatum*), performing studies on the protein expression level.

2. To test the suitability of MT as a possible biomarker for metal exposure (copper, cadmium) and for organochemicals (dimethoate) in terrestrial gastropods (*H. pomatia*, *A. arbustorum*, and *D. reticulatum*), performing studies on the MT mRNA level.

3. To characterize inducible, metal-binding proteins from terrestrial oligochaetes (*Eisenia fetida*) after metal exposure (copper, cadmium), and to test their suitability as possible biomarkers for chemical exposure (copper, cadmium, dimethoate).

## II. MAIN RESULTS OBTAINED:

### Methodology

For quantification of metal- and organ-specific MT pools in the terrestrial gastropods, two assays were adopted and properly modified. The first assay - the Cadmium Chelex assay - was developed to quantitatively determine concentrations of (Cd,Zn)-MT in the midgut gland of snails. Apart from MT concentrations, this assay also allows the determination of the relative saturation of the protein with cadmium and zinc (Berger et al. 1995a). The second method - the Tetrathiomolybdate assay - was used to quantify concentrations of (Cu)-MT in midgut gland and mantle tissue of terrestrial gastropods. Both methods are based on the known structure of snail MTs (Dallinger et al. 1993; Berger et al. 1995b) and their stoichiometry of metal binding (6 metal atoms bound per mole of apo-thionein; Dallinger et al., in preparation). By combining these two assays, it becomes possible to detect different MT pools in animal tissues: the (Cd,Zn)-MT pool on the one hand; and the (Cu)-MT pool on the other.

At the level of protein biochemistry, chromatographic methods (gel permeation chromatography, ion exchange chromatography, FPLC and HPLC) were adopted to study the induction patterns of organ- and metal-specific MT isoforms in snail tissues. Moreover, different fractionation techniques including centrifugation and chromatography were used to purify and characterize inducible metal-binding proteins (MBPs) in the terrestrial earthworm species *E. fetida*. At the molecular level, methods of PCR amplification were developed to quantify the induction of MT mRNA in terrestrial gastropods.

Apart from biochemical and molecular work, physiological and toxicological experiments were performed to correlate biochemical and/or molecular parameters (MT concentrations, metal saturation of MT) with metal concentrations in animal tissues, as well as with mortality and animal growth.

### Results

a) Terrestrial gastropods: The Cadmium Chelex assay and the Tetrathiomolybdate assay were used singly or in combination to quantify parameters of MT concentration and protein metal saturation. In this way, the **dynamics** of MT induction were studied in *H. pomatia*, *A. arbustorum* and *D. reticulatum* after metal or chemical exposure. It appeared that cadmium is a potent inducer of MT, especially in the midgut gland of metal-exposed individuals. After rapid induction of MT synthesis, a **steady state level** of MT concentration is reached which persists over long periods of time. Moreover, concentrations of (Cd,Zn)-MT are highly correlated with cadmium concentrations in animal tissues. It was also shown that at very high dietary cadmium concentrations, both (Cd,Zn)-MT concentrations as well as Cd saturation levels of the protein in midgut gland reached maximal levels. These levels were clearly related to increased mortality rates of metal-burdened individuals. Moreover, it appeared that a combination of the two quantification assays allowed the detection of (Cd,Zn)-MT and (Cu)-MT concentrations separately. Finally, discrimination analyses revealed that following the exposure of snails to metals (Cd, Zn), X-ray radiation, or external stress factors (cold), the organ- and metal-specific MT pools responded to the applied stress factors to different degrees. This led to significant changes in the concentration of the respective MT pools with regard to control levels.

b) Earthworms (*E. fetida*): Different metal-binding proteins were detected in the cytosol of *E.*

*fetida*. Apart from two metal-binding components in the molecular weight range of 18 kD and 45 kD, respectively, a third protein was found with an apparent molecular weight of 5 kD. Both the 18 kD and the 5 kD component are inducible by cadmium exposure, binding relatively high amounts of this metal. They seem not to be present in control animals. Moreover, the 5 kD protein showed an increased absorbance at 254 nm. This indicates that there may be some similarities between this protein and MTs, at least as far as spectroscopic features are concerned. On the other hand, the behaviour of this protein on ion exchange chromatography suggests major differences in comparison with true MTs. In any case, the inducibility of these components may make them useful biomarkers, provided that their chemical nature can be elucidated in the near future.

## Discussion

The results of our studies indicate that at least in the midgut gland of terrestrial gastropods, the pool of (Cd,Zn)-MTs may represent a strong biomarker for environmental cadmium exposure (Dallinger 1996). This is consistent with findings in many other animal species (Hamer 1986; Kägi 1993; Dallinger 1995).

Apart from cadmium, one should expect that MT induction might also occur following the exposure of snails to organochemicals, X-ray radiation, or physiological stress factors such as cold. Such an assumption is based on the fact that in most vertebrates, an induction of MT is also achieved by non-metallic stress factors such as glucocorticoids, free radicals and organochemicals. The reason for this lies in the structure of the MT encoding gene, the promoter region of which possesses regulatory elements susceptible to non-metallic stressors as well. Our results with non-metallic stressors in snails (X-ray radiation, organochemicals) suggest that the status of organ- and metal-specific MT pools in midgut gland and mantle tissues may, at least in part, be indicative of non-metallic stress events which animals may have experienced previously. It should be pointed out, however, that in such cases it might be rather difficult to define the real "control" status of the different MT pools. Thus we conclude that MTs in gastropods may also be used as biomarkers for non-metallic stress factors, provided that further studies are undertaken in this promising area (Dallinger 1995).

With regard to earthworms (*E. fetida*), the presence of metal-inducible and metal-binding proteins in their tissues is indeed a promising aspect. However, more research is needed to characterize these proteins precisely, before they may be used as potential biomarkers in risk assessment.

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## PART B. DETAILED REPORT OF THE CONTRACTORS

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## I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To study further the response *in vitro* of the esterases in the three species, earthworm, *Eisenia fetida*, isopod, *Porcellio scaber*, and Collembola, *Folsomia candida* on the metals copper, cadmium and zinc and the insecticide dimethoate by using electrophoresis and densitometry.

2. To study the response *in vivo* of the esterases in the three species on the metals and the insecticide by feeding experiments.

## II. MAIN RESULTS OBTAINED:

### Methodology

This work was initiated in April 1994. The method for analysing the esterases of the species selected by isoelectric focusing in ultrathin agarose gels, followed by densitometry, was developed (Kammenga, 1995). Inhibition of the esterases by eserine was carried out to identify acetylcholine esterase and the first experiments for determining doses of the chemicals effecting the esterases in the species have been done. According to the objectives *in vitro* as well as *in vivo* experiments have been carried out by using each of the three metals and the pesticide dimethoate.

*In vitro* experiments for determining dose-response of the chemicals have been performed for all three species. The concentrations used of the chemicals for the different species are shown in Table 1 and based on the results found previously (Kammenga, 1995). At least 30 individuals of each species were analysed at each concentration.

**Table 1.** Concentration (M) of chemical in the staining solution for esterase for the three species.

| | *Eisenia fetida* | *Porcellio scaber* | *Folsomia candida* |
|---|---|---|---|
| Copper | 0 | 0 | 0 |
| | 0.02 | 0.02 | 0.02 |
| | 0.03 | 0.03 | 0.03 |
| | 0.035 | 0.035 | 0.035 |
| Zinc | 0 | 0 | 0 |
| | 0.02 | 0.03 | 0.03 |
| | 0.03 | 0.04 | 0.04 |
| | 0.04 | 0.05 | 0.05 |
| Cadmium | 0 | 0 | 0 |
| | 0.03 | 0.05 | 0.05 |
| | 0.04 | 0.06 | 0.06 |
| | 0.05 | 0.07 | 0.07 |
| Dimethoate | 0 | 0 | 0 |
| | 0.01 | 0.01 | 0.02 |
| | 0.02 | 0.02 | 0.03 |
| | 0.03 | 0.03 | 0.04 |

*In vivo* experiments have been carried out for two species, *P. scaber* and *F. candida*. *P. scaber* was kept in small plastic aquaria, size 40 x 20 x 20 cm, with sand at the bottom, and *F. candida* was kept in petri dishes on a layer of plaster of Paris mixed with charcoal. Both species avoided the food contaminated, hence the specimens were exposed to the chemicals by solving them in water and adding the solution to either the sand or the plaster layer. It was the intention to use the same concentration of chemicals for the *in vitro* and the *in vivo* experiments, but due to the sensitivity of the invertebrates it was necessary to adjust the concentrations, so the animals were

able to survive the treatment.

## Results

No effect of the metals was observed when applying these to the homogenate of the individual or part of it as described previously (Kammenga, 1995). Identification of acetylcholine esterase by using the inhibitor eserine was not possible, as none of the zones with esterase activity were inhibited by the chemical.

*In vitro* experiments: The esterases revealed in all three species by the method used were exposed to the chemicals according to Table 1. All zones with esterase activity on the zymograms were inhibited by each of the chemicals. For the species for which the data have been analysed, a dose-response effect was shown irrespective of the chemical used. A depiction of the result obtained is shown in Figure 1 for *F. candida* for the pesticide dimethoate. Similar dose-responses were seen for the other chemicals and for the other species, but the data analysis has yet to be finished.

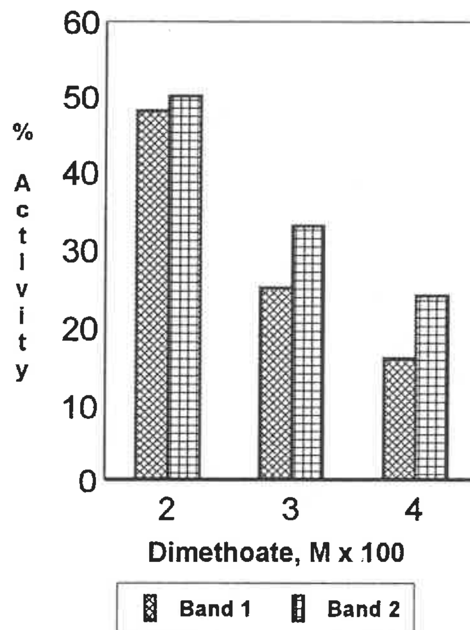


**Figure 1.** Dose-response for esterase activity of *F. candida* found in an *in vitro* experiment with dimethoate.

*In vivo* experiments: The *in vivo* experiments were performed for all the chemicals for *P. scaber* and *F. candida*, using the same concentrations as shown in Table 1 to moisture the substrate. However, it was observed that zinc even in the highest concentration used for the *in vitro* experiment did not show any effect on esterases of *F. candida* in the *in vivo* experiment. On the other hand dimethoate in the lowest concentration used for the *in vitro* experiments with *P. scaber* could not be applied to the *in vivo* experiments, as the animals died quickly after the exposure. The experiment with *E. fetida* will be proceeded in the spring of 1996. The delay of the experiment was due to a lack of specimens. An example of the results obtained in the *in vivo* experiments for *F. candida* is depicted in Figure 2.

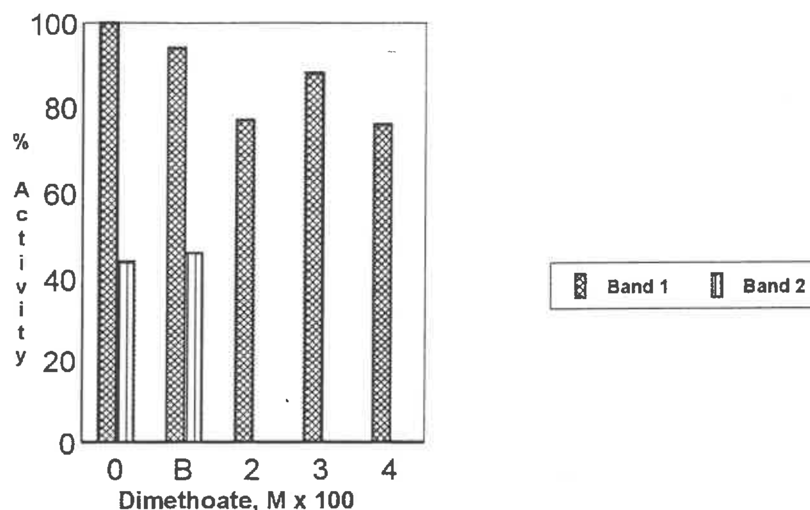


**Figure 2.** *In vivo* effect of dimethoate on the activity of esterases in *F. candida*, B = solvent without dimethoate.

## Discussion

Identification of acetylcholine esterase was not possible as the inhibition with eserine did not work. By applying dimethoate to the esterase staining solution it should be possible to distinguish between A and B esterases (Aldrich, 1953) but all the esterases observed were sensitive to dimethoate, so no classification of A and B-esterases were possible.

Applying each of the metals to the homogenate of an individual did not have any effect on the activity of the esterases. However, a reduction in the activity of the esterases was seen, when the metals were added to the staining solution for esterase. The lack of effect in the homogenate might be due to the presence of metal-binding proteins in the homogenate.

Despite that the data analysis is not finished it is obvious that the results of the *in vivo* and the *in vitro* experiments do not show the same reduction in activity of the esterases, at least not for *F. candida* and *P. scaber*. Chagnon and Guttman (1989a) demonstrated that the two allelic forms of the enzyme phosphoglucomutase responded differently to the metal copper when exposing the enzyme to copper in an *in vitro* experiment, but the authors could not confirm their results in *in vivo* experiments (Chagnon and Guttman, 1989b).

The disappearance of one of the bands in the zymogram for *F. candida* (see Figure 2), when exposing the animals to dimethoate, may lead to an erroneous conclusion of how many clones are presented in a certain area, if a patchy distribution of dimethoate exists.

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## PART B. DETAILED REPORT OF THE CONTRACTORS

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### I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To establish links between changes in ICP-MS (Inductively-Coupled Plasma-Mass source Spectrometry) profiles and alterations in NMR (Nuclear Magnetic Resonance spectroscopy) profiles. To attempt to determine pathways between sublethal exposure and metabolic dysfunction.

2. To establish ICP-MS and NMR profiles for other invertebrate species, *e.g.* slugs in cooperation with other participants of the programme.

3. To further explore the putative copper:histidine relationship as a metabolic biomarker in earthworms.

4. To link overall changes in profiles of either NMR or ICP-MS to other ecological measurements.

## II. MAIN RESULTS OBTAINED:

### Methodology

High resolution proton nuclear magnetic resonance ($^1H$ NMR) allows a rapid analysis of a wide range of metabolites and organic compounds, in complex biological fluids with very little sample pre-treatment. It has thus become a well established technique for the investigation of toxin-induced perturbations of endogenous metabolite profiles in the biological fluids of vertebrates. NMR methods have now been applied to characterise tissue extracts of a series of common European invertebrate species including the earthworms *Lumbricus rubellus* and *Eisenia andrei*, the terrestrial isopods, *Oniscus asellus* and *Porcellio scaber*, the diplopodous arthropod *Glomeris marginata* and the pulmonate gastropod *Arion subfuscus*. Tissue samples were extracted from snap-frozen, homogenized individuals, after depuration. Centrifugation and ultrafiltration of the samples was used to remove the remaining cellular components. Due to the high biochemical complexity of the resulting fluids 2-dimensional $^1H$ NMR techniques have been used (COSY and J-Resolved), to allow a further characterization of the organic components by establishing proton connectivity and spin-spin coupling patterns of partially overlapped signals. The tissue extracts gave characteristic low molecular weight metabolite fingerprints for the species studied. Endogenous metabolites identified included free amino acids, organic acids such as citrate, lactate and acetate, sugars such as glucose and organic (methylamine) bases. These NMR profiles closely reflect the metabolic status of the animal and we have applied this technology to investigate the biochemical changes associated with the toxicity of model pollutants with particular focus on earthworms following copper exposure.

Earthworms *L. rubellus* were exposed under semi-field conditions to an increasing range of soil copper concentrations *viz.* control (3 mg Cu $kg^{-1}$), 20, 40, 80, 160 and 320 mg Cu $kg^{-1}$ soil (dry weight) in purpose built mesocosms for 110 days. Each mesocosm was filled with 15.5 kg of copper-loaded forest soil, originally taken from the top 20 cm of soil at a location in Thetford forest, Norfolk, U.K. (composition; 96% sand, 4% clay, <1% organic matter, pH($H_2O$) 5.6), with a gravimetric water content of 13 - 14% (approximately 50% of its soil water-holding capacity). The elevated soil copper concentrations were prepared by mixing 13.5 kg of dry soil with 2000 ml of glass-distilled water containing the total amount of copper required for each exposure concentration, and the soil was mixed thoroughly for 10 mins in a mixer. The 36 soil filled tubes were sunk 35 cm into holes prepared in the ground in a 5 m x 5 m plot with 1 m spacings, arranged randomly in a 6 x 6 Latin square, and left to settle for 5 days, before 20 immature (100 - 250 mg wet wt.) and 5 mature (with fully developed clitellum (250 - 450 mg wet wt.)) earthworms were weighed individually and added to each cosm.

Individuals of *L. rubellus* from the cosms were depurated for a minimum of 24 h at 17°C. They were then rinsed in distilled water, blot dried and snap-frozen in liquid $N_2$. Subsequently, samples were homogenised in a 1:2 ratio of body weight to volume of physiological Ringer solution (pH 7.3). The homogenates were centrifuged (30 mins/4000 rpm) at 5°C and the supernatant was ultrafiltered (Sartorius Centristat I™) to a MW cut-off point of 10 kD. The extract was then either freeze-dried and reconstituted in $D_2O$ or 100$\mu$l of $D_2O$ was added to provide a magnetic field lock.

NMR Spectroscopy: Single pulse $^1H$ NMR spectra were obtained using a JEOL GSX500 spectrophotometer operating at 500.14 MHz observation frequency. Spectra were measured at ambient probe temperature, using 45° pulses and a 6000 Hz spectral width, with 256 free induction delays (FIDs) collected for each sample into 32,768 computer points. An acquisition time of 2.73 seconds was used with a further delay of 2.27 seconds to ensure that the spectra

were obtained under $T_1$-relaxed conditions. Where appropriate, the water $^{1}H$ signal was suppressed during acquisition by a gated secondary irradiation field at the water resonance frequency. The FIDs were multiplied by an exponential weighting function corresponding to a 0.19 Hz line broadening prior to Fourier transformation.

$^{1}H$-$^{1}H$ COrrelation SpectroscopY (COSY): Two-dimensional COSY experiments were obtained using a varian VXR600 spectrometer performed at 599.05 MHz $^{1}H$ resonance frequency. The following pulse sequence was used:

$$[D - 90^{\circ} - t_1 - 45^{\circ} - \text{collect FID}]$$

D was a 2 second relaxation delay, while $t_1$ was an incremental delay to allow modulation of the spin-spin coupling. Data were collected into 4096 computer points using 16 scans per increment and a spectral width of 6500 Hz and 512 increments, with 40 scans per FID. An acquisition time of 0.32 seconds was used.

Homonuclear $^{1}H$ 2-D J-RESolved Spectroscopy (JRES): JRES experiments were performed at 600.13 MHz using a Bruker AMX600 spectrometer. The following pulse sequence was used:

$$[D - 90^{\circ} - t_1 - 180^{\circ} - t_1 - \text{collect FID for time } t_2]$$

D was a 2 second relaxation delay, the $t_2$ acquisition time was 0.63 seconds, while $t_1$ was an incremented delay to allow modulation of the spin-spin coupling. The $F_2$ domain was collected into 8192 computer points using a spectral width of 6550Hz and the $F_1$ domain used a 30 Hz spectral width with 64 increments. Eight scans were collected for each $t_1$ increment.

**Results**

Intraspecific variation in the $^{1}H$ NMR profiles of tissue extracts was small. However, consistent differences were observed between control and copper-exposed earthworms. Two singlets were present in the downfield region of the spectra (Figure 1), and have been tentatively assigned to the amino acid histidine. An attempt was made to positively assign histidine by investigating $^{13}C$ $^{1}H$ connectivity by high field NMR. Thus, Heteronuclear Multiple Quantum Correlation (HMQC) experiments were carried out. It proved however that due to the very low concentrations of "histidine" present in the sample this system was insufficiently sensitive to obtain any information on the peaks of interest. Attempts are in hand to reduce the dynamic range and thus increase the sensitivity of the experiment.

The metal chelator EDTA was added to samples where "histidine" was not observed by $^{1}H$ NMR. It was thought that in such samples histidine may be present but bound in large metal complexes and thus invisible by NMR spectroscopy. In this case the EDTA would release histidine from the complexes by binding the metals itself, thus making the histidine appear in the NMR spectra. This, however, did not occur, adding evidence to the absence of histidine in non-copper exposed worms.

In order to investigate the relationship between copper/histidine it was necessary to integrate the two downfield histidine peaks and to compare this to another peak from an endogenous metabolite. It is necessary to compare these to another peak from the same profile. The resonance from this metabolite should be of similar intensity, in the same region of the spectra and also relatively constant between samples. The downfield doublet of tyrosine was thus used. There was, however, often interference with the downfield histidine peak and thus the more

upfield aromatic signal only was used.

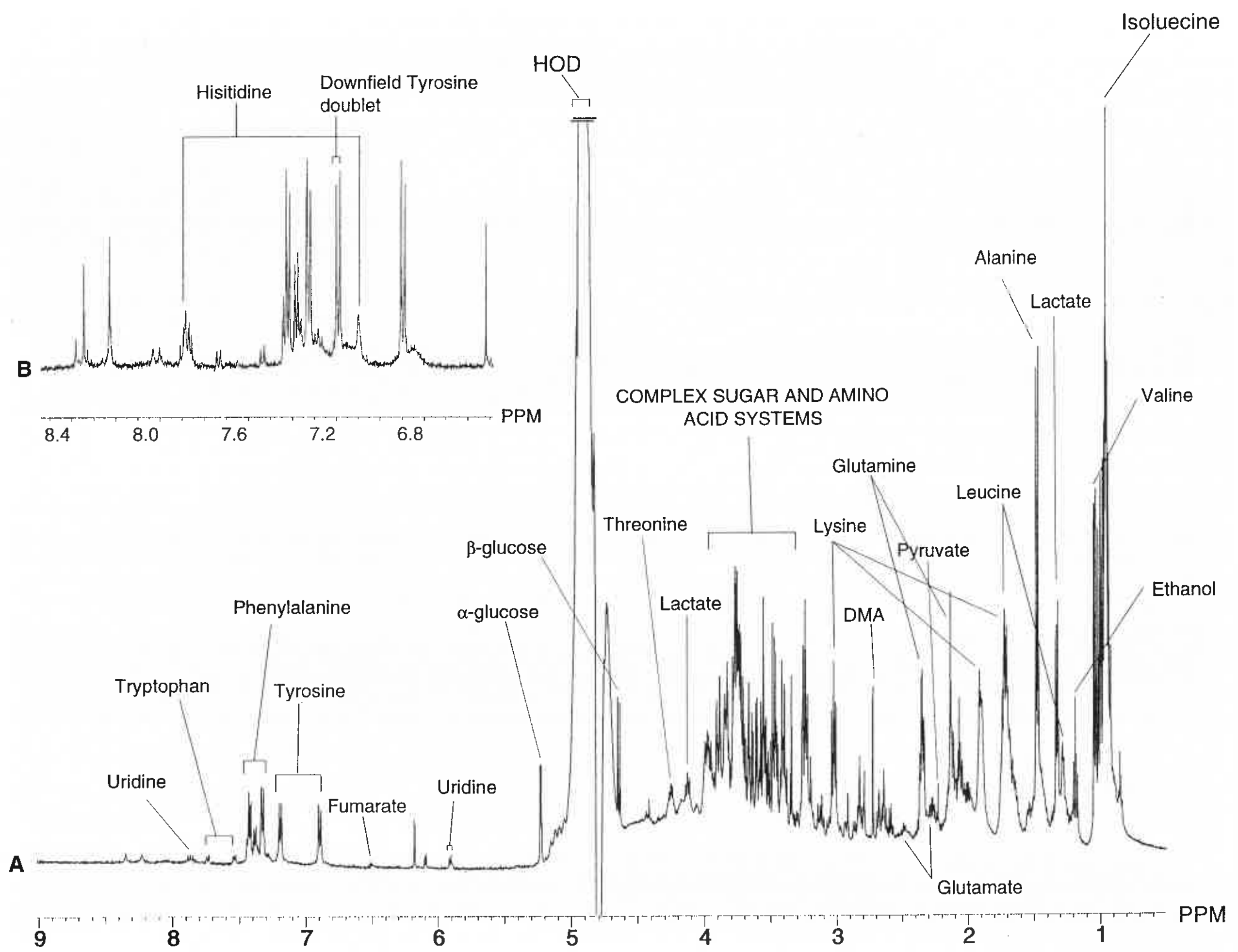


**Figure 1.** A) Single pulse 500 MHz $^{1}$H NMR spectra of the earthworm *Lumbricus rubellus* tissue extract, with some of the major metabolites assigned. B) Example of downfield region of sample containing histidine. The high degree of signal overlap is obvious, particularly with the more downfield peaks of the histidine signals.

**Discussion**

Single pulse high resolution $^1$H NMR spectroscopy has shown that tissue extracts investigated contain highly complex mixtures of metabolites (Figure 1). Despite the high degree of signal overlap observed in most of the spectra, many resonances have been assigned. The endogenous metabolites identified include most of the 20 primary amino acids, sugars such as the two glucose anomers ($\alpha$ and $\beta$), Krebs cycle intermediates such as citrate, pyruvate, fumarate and malonate, the organic bases taurine, choline and DMA and several other organic acids including lactate and acetate. Indeed, at least 200 lines can be resolved in each of the tissue extracts. Unique and characteristic fingerprints of unperturbed metabolites for the species studied have thus been produced by the application of $^1$H NMR in this study.

Two-dimensional NMR experiments have permitted even more detailed spectral assignments to be made. Correlation spectroscopy (COSY) experiments have provided information on three bond $^1$H-$^1$H connectivity, whilst homonuclear $^1$H J-resolved spectroscopy (JRES) has enabled investigations through space and scalar coupling patterns and coupling constants. Consequently, in the earthworm *L. rubellus*, more than 170 signals have been recorded, over 40% of which have been assigned hitherto. It is proposed that the NMR profiles presented reflect the metabolic status of the individual. The range of metal concentrations encountered in soils is large. Earthworms with highly permeable cuticles, are thus susceptible to toxic effects at high concentrations and deficiencies at low availabilities. The use of earthworms in pollution studies is now widespread and their ability to accumulate metals above ambient concentrations is well established. Detoxification strategies have been proposed in earthworms for a number of metals including Cd, Pb and Zn, but with the noticeable exception of Cu. We have shown that histidine levels increase with increasing exposure to copper, and suggest that this is a likely detoxification mechanism not previously observed. Thus, in addition to acting as a biomarker for copper exposure, histidine also appears to have a detoxificatory role.

Histidine can occur as either an anion or neutral molecule, and as such the stereochemistry of copper binding is known to be versatile. However, a number of copper histidine complex structures have been described. Some authors suggest that these complexes behave as a tridentate chelate and as a didentate chelate and that aromatic nitrogen may be involved in the complex, although there are exceptions. The Spartan 4.0™ computational package has been used to model possible histidine/copper complexes. Energy minimisation experiments are currently being carried out using the PM3 Hamiltonian semi-empirical Molecular Orbital method to determine the most likely complex structure.

$^1$H NMR spectroscopy has great potential in this application. Furthermore, the establishment of such biomarkers may be aided by the application of computational pattern recognition techniques which have been extensively developed for biomarker exploration in vertebrate species. Comprehensive assignment of resonances is a pre-requisite for any such $^1$H NMR investigations. It is essential that future work focuses on the links of such complex biochemical interpretation on actual effects on the species at the level of the population.

## PART B: DETAILED REPORT OF THE CONTRACTORS

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### I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To further optimize a homogenisation and separation technique for analyzing metal-binding proteins in Collembola and isopods.

2. To investigate the applicability of the developed techniques for the detection of metal-binding proteins in nematodes obtained from participant 1.

3. To relate the effects of cadmium and zinc on metal-binding proteins to effects on growth of isopods and springtails.

4. To analyze metal-binding proteins in isopods from metal-polluted and non-polluted field locations.

## II. MAIN RESULTS OBTAINED:

### Methodology

A biomarker becomes applicable if it relates the biomarker response to an adverse effect. Therefore two types of experiments were performed: dose-effect relationships were studied with zinc for the isopod *Porcellio scaber* to establish effects (1) and metal-binding proteins were characterized in both isopods and springtails as an indicator of metal exposure (2).

1. Sublethal effect of zinc on isopods: Two experiments were performed to relate the zinc concentration and distribution in the isopods to the effects of zinc on growth. For the first experiment *P. scaber* juveniles were collected from a reference and a zinc polluted site. The isopods obtained from the polluted site contained four times as much zinc as the isopods from the reference site. Both groups were exposed for 12 weeks to 4 different zinc levels in their food. Effects on growth were related to zinc accumulation and zinc distribution. Animals were stored frozen for FPLC analysis. The second experiment was performed to test the influence of food quality on zinc accumulation and susceptibility to zinc. The hypothesis was tested that animals which have more protein (energy) available will be less susceptible to zinc because they can spend more energy on active regulatory mechanisms and they can use the protein for the synthesis of metal-binding proteins. Juvenile isopods were exposed for 15 weeks to 7 different zinc concentrations. The food quality was manipulated by adding 2 or 10% peptone as a protein source. Growth, zinc, protein, and lipid concentrations in the isopods were determined at the end of the study. Some of the animal samples were stored frozen for FPLC-analysis.

2. FPLC patterns of zinc and cadmium exposed isopods and cadmium exposed Collembola:
a) Isopods (*P. scaber*) were exposed to dietary cadmium and zinc. The zinc concentrations were chosen in the range of the EC50 for growth. For cadmium two experiments were performed; in the first experiment the effect of cadmium exposure was measured at different time intervals. In the second experiment a dose-response relationship was established after three weeks of exposure. The isopods were homogenized by the method as described in the second technical report (Roesijadi and Fowler, 1991). The homogenates were analysed for protein patterns with a Superose-12 (gel filtration) column. The fractions obtained were analysed by graphite furnace AAS to find the fractions that contained Cd, Zn and Cu-binding proteins.
b) The Collembola (*Orchesella cincta*) were exposed to a cadmium concentration close to the EC50 for growth. *O. cincta* was treated in the same way as the isopods, except that the fractions were only analysed for cadmium. On the other hand, the cadmium-binding protein of *O. cincta* was further characterized with a anion exchange (Mono Q) column and 1-D electrophoresis.

### Results

1. Sublethal effects of zinc on isopods: In the first study, the isopods from the vicinity of the zinc smelter appeared as sensitive to zinc as the reference population (EC50 values for growth were 31.2 and 29.2 $\mu$mol Zn/g dry food resp.), although their initial body concentration differed by a factor 4. The accumulation of zinc did not differ between the two populations. Therefore the effect of zinc cannot be explained by the total zinc concentration, nor by the zinc concentration in the hepatopancreas, nor by the concentration in the exoskeleton. Apparently, it is not the total amount of zinc that is responsible for the effects on growth but the fluxes of

zinc that determine the effects.

In the second study, the effect of food quality was investigated. Survival of *P. scaber* was only affected at the highest zinc level at 2 % peptone (60 μmol/g dry food). As expected, with 10 % peptone growth was higher than with 2 % peptone and this was the case for all zinc concentrations. The zinc accumulation of the isopods, however, did not differ between 2 and 10% peptone. The NOEC for growth was in both cases 24 μmol/g dry food but the EC50 for growth were 35.7 and 45.6 μmol/g for 2 and 10 % peptone, respectively. Protein addition to the food reduced the sensitivity to zinc. Further FPLC studies are required to elucidate whether zinc binding proteins can explain this result.

2a. FPLC patterns of cadmium and zinc exposed isopods: The protein pattern in zinc exposed isopods did not show metal peaks in the range where commercial rabbit metallothionein eluted from the FPLC column. The patterns for protein and copper were reproducible however, the zinc patterns differed every time they were produced. It is not known if the absence of a reproducible zinc pattern is due to the technical procedure chosen or if it mirrors real variability in isopods.

The results for the two cadmium experiments were more defined. In the first experiment (time dependence) all cadmium was associated with a peak of circa 158 kDalton. Copper was located in the same peak and it is very likely that the cadmium binding peak is a dimer of haemocyanine. In the second experiment (dose-response relationship) most of the cadmium was again bound to the protein of 158 kD, however, at the two highest Cd-concentrations (48 and 100 μg/g dry food) a new protein binding cadmium was induced with a size of circa 30 kD (Figure 1).

2b. FPLC patterns of cadmium exposed Collembola: In the Second Technical Report a new homogenisation procedure was described which when employed, appeared to be succesful as most of the cadmium now remained attached to proteins. With this procedure cadmium exposure resulted in the induction of a cadmium-binding peak in the molecular weight range of metallothionein. At the moment studies are being performed to characterize the amino acid composition of this protein.

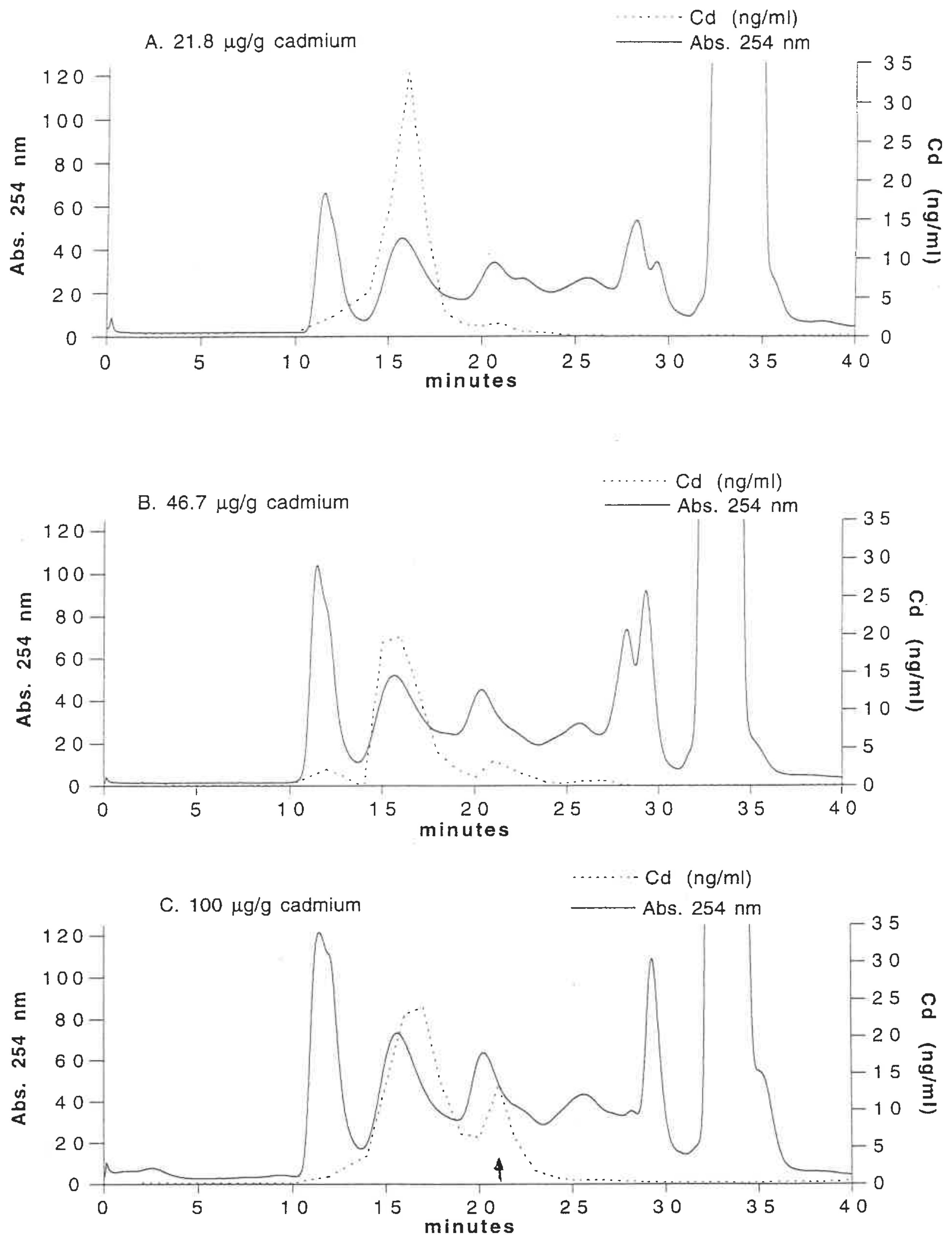


**Figure 1.** FPLC (Superos-12) patterns of isopod homogenates from isopods which were for three weeks exposed to 21.8 (A), 46.7 (B) and 100 µg/g (C) cadmium in their food. Samples were prepared from 5 isopods, homogenized in phosphate buffer (10 mM, pH 7.5), mixed with 40 % acetone (according to the method of Roesijadi and Fowler (1991)) and eluted in $NH_4CO_3$ buffer. The graphs show the distribution of cadmium as ng/ml in 1.2 ml fractions (- - - ) and the absorption pattern of the protein at 254 nm.

## Discussion

The results of the dose-effect studies in the isopod *P. scaber* showed that the effect of zinc cannot be explained by the measurement of the zinc concentration or distribution within the animal. The first experiment showed that the exposure level of zinc forms a better explanation for zinc toxicity than the internal concentration. The ultrastructural alterations found in the small cells of the woodlouse hepatopancreas following zinc exposure (Köhler et al. 1996) show cell damage at comparable zinc exposure levels. The second experiment supports this idea as it shows that zinc toxicity depends on the quality of the food offered to the isopods. High protein contents in the diet enables isopods to resist much higher dietary zinc concentrations.

This result can be explained either by a direct use of the extra proteins for the synthesis of metal binding proteins or an indirect use as an extra energy source. The results of FPLC and AAS analysis showed that it is possible to detect cadmium-binding proteins in isopods and springtails with the methods developed. It shows that isopods and springtails have different cadmium binding proteins (5 and 30 kD, respectively) which are induced at exposure levels known to interfere with growth and reproduction. The protein found in the springtail could be an MT, the protein induced in the isopod needs further analysis.

Zinc-induced metal-binding proteins yet have not been found, probably because these proteins are masked by functional zinc binding proteins or simply because zinc does not induce specific zinc binding proteins.

The role of the cadmium binding proteins of isopods and springtails now needs to be demonstrated and validated in the field. Animals need to be collected from a gradient in the vicinity of a zinc smelter in Budel. The cadmium concentrations measured in the litter from this site are known to correspond to the effect concentrations shown to occur in the laboratory.

## References

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