Progress Report 1994 of

BIOPRINT

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

Second Technical Report

Report from a Workshop
held in Monks Wood, U.K.
February 24-25, 1995

EC Environmental Research Programme
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ENVIRONMENTAL RESEARCH PROGRAMME
Research Area: Ecotoxicology
Progress Report

Contract no: EV5V-CT94-0406

Title: BIOCHEMICAL FINGERPRINT 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, 1994 - March 31, 1995

PART A. SUMMARY REPORT OF THE PROJECT

I. GENERAL OBJECTIVES:

The main objective of the BIOPRINT project is the development of biochemical fingerprint techniques (biomarkers) for assessing the exposure and effect of toxicants in terrestrial invertebrates. The applicability of biomarkers will be investigated for different taxonomic groups including representatives of various trophic levels: Nematoda, Collembola, Gastropoda, Isopoda and Oligochaeta.

A manageable number of biomarkers have been selected on the basis of present ecotoxicological knowledge: i) heat shock proteins (HSP's), ii) metallothioneins and metal binding proteins for detection of heavy metals and iii) esterases for detection of organic contaminants. In addition, advanced spectroscopic techniques (NMR and ICP-MS) will be used to discover novel biomarkers in the organisms used.

II. SPECIFIC OBJECTIVES FOR THE REPORTING PERIOD:

Research was focused on: 1) determination of heat-shock protein (HSP) induction in nematodes after exposure to elevated temperatures and various toxicants by means of protein electrophoresis and the application of monoclonal antibody techniques, 2) establishment of a technique to quantify HSP's in slugs and verification of the HSP test with full life cycle toxicity experiments, 3) testing the suitability of metallothionein for metal exposure in terrestrial gastropods and oligochaetes by performing studies on the protein expression level, 4) development of electrophoretic methods for analysing the enzyme esterase in earthworms, isopods and Collembola and identification by means of eserine and organophosphorus compounds, 5) developing procedures for the analysis of invertebrate fluids and/or tissues by NMR and ICP-MS and to establish a data-set of baseline profiles from earthworms prior to metal exposure, 6) a technique for analysing metal binding proteins in isopods and Collembola and the determination of the effect of heavy metals on metal binding proteins in isopods in relation to growth and reproduction.

III. MAIN RESULTS:

1,2) Progress was made by all participants in the development of suitable fingerprint techniques for different invertebrates. The application of monoclonal antibodies for the detection of specific HSP's proved to be fruitful and very specific for the species used. For all species tested, a dose-response relationship was found for various toxicants. 3) It appeared that metallothioneins are quickly induced by cadmium in different snail species. In the earthworm, however, metallothionein could not be detected, instead a myoglobin or myoerithrine-like protein was found. 4) Specific methods for analysing esterases were established for earthworms and isopods exposed to various toxicants. 5) By using the NMR profile of earthworms a novel potential biomarker, histidine, was detected which complexing activity is orders of magnitudes higher than the classic chelating agent EDTA. It appears to be actively associated with the toxicant. 6) Different homogenisation

procedures were tested and it was found that isopods contained various metal-binding proteins which are different compared to metallothionein-like proteins.

IV. OBJECTIVES FOR THE NEXT PERIOD:

The aim is to: 1) establish dose-response relationships for HSP60 induction in nematodes after exposure to various toxicants, to link the response to sublethal effects on life cycle variables and to identify metal binding proteins and novel biomarkers (NMR), 2) analyze samples from different invertebrates for HSP70 and to optimize the PCR technique to assess proteotoxicity in metal-exposed slugs on the mRNA level, 3) test the suitability of metallothioneins as possible biomarkers for chemical exposure in terrestrial gastropods by performing studies at the mRNA level, 4) study the response *in vivo* of the esterases in the three species by feeding experiments, 5) establish links between changes in ICP-MS profiles and alterations in NMR profiles and to establish ICP-MS and NMR profiles for other invertebrate species, eg. isopods, Collembola and nematodes, 6) investigate the applicability of the developed techniques for the detection of metal-binding proteins in nematodes and to analyse metal binding proteins in isopods from metal-polluted and non-polluted field locations.

V. PUBLICATIONS:

Eckwert, H., Zanger, M., Reiss, S., Musolff, Alberti, G. and Köhler, H.-R. (1994): The effect of heavy metals on the expression of hsp70 in soil invertebrates. *Verh. Dtsch. Zool. Ges. 87, 325*

Köhler, H.-R., Rahmann, B. and Rahmann, H. (1994): Assessment of stress situations in the grey garden slug, *Deroceras reticulatum*, caused by heavy metal intoxication: semi-quantification of the 70 kD stress protein (hsp70). *Verh. Dtsch. Zool. Ges. 87, 328*

Simonsen, V. and Weeks, J.M. (1994): Manual of BIOPRINT, First Technical Report. Report from a workshop held in Wageningen, The Netherlands, May 9-10, 1994.

PART B. DETAILED REPORT OF THE CONTRACTORS

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I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To determine the induction of heat-shock proteins (HSP's) in the bacterivorous nematode *Plectus acuminatus* after exposure to elevated temperatures and various toxicants by means of protein electrophoresis.

2. To apply monoclonal antibody techniques for detection of specific HSP's.

II. OBJECTIVES FOR THE NEXT PERIOD:

1. To establish dose-response relationships for HSP60 induction in bacterivorous nematodes after exposure to various toxicants.

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 the abovementioned nematode species in cooperation with participant 6 and 5 respectively.

III. MAIN RESULTS OBTAINED:

Methodology

Induction of different HSP's by temperature and toxicant stress in the parthenogenetic nematode *Plectus acuminatus* Bastian 1865 was assessed by means of 1 and 2-dimensional protein gel electrophoresis. Both classic silver staining and monoclonal antibodies (by means of immuno blotting) were used to detect HSP induction. Table 1 shows the commercially obtained mono- and polyclonal antibodies raised against different HSP's of humans, bovine, the bacterium *Synechococcus spp.* strain PCC 7492, the moth *Heliothis virescens*, the guinea pig and the rat which were tested for their suitability to detect HSP's in the nematode *P. acuminatus*.

Preliminary tests were conducted in adult nematodes after exposure to heat shock. Experiments were conducted in water at 20°C (control) and 37°C for t=60 min. Molecular weight markers were used to determine the weight of the HSP's detected.

Based on the temperature studies, suitable antibodies were selected and experiments were run to study the induction after exposure to cadmium and copper in water for 24 h. The concentrations used (Cd: 50 µM, Cu: 7.1 µM) were 50% of the LC_{50} (72 h) in water.

Table 1. Monoclonal and polyclonal antibodies used for detection of various HSP's in heat shocked and metal-stressed nematodes.

anti HSP27 kDa	monoclonal antibody raised against human HSP
anti HSP60	„ mono, human
anti HSP70	„ mono, bovine
anti HSP70	„ mono, human
anti HSP25 kDa	polyclonal antibody raised in a rat
anti HSP60	„ poly, moth
anti HSP60	„ poly, cyanobacterium
anti hsc70 kDa,	monoclonal antibody raised against constitutive HSP70 in guinea pig

Results

One-dimensional protein gel electrophoresis appeared to be unsuitable for detection of different isoforms of various HSP's. More detailed and characteristic information was obtained by using 2-dimensional electrophoresis. It appeared that classic protein silver staining of gels gave reproducible protein patterns of adult females. In addition it was found that there were no major differences in the protein composition between juveniles and adult females. However detection of HSP's required the use of antibodies thus facilitating discrimination between stressed and non-stressed nematodes. Optimal expression of proteins in 2-dimensional gel electrophoresis for detection requires approx. 100-120 adult females per gel (approx. 0.06 µg protein per female). Table 2 shows the expression of different antibodies in control and heat shocked nematodes. All antibodies showed low expression in the control except for anti HSP70 (mono, human) and anti

HSP60 (poly, cyanobacterium) which did not lead to expression. In the heat shocked nematodes all antibodies gave a strong response except for anti HSP70 (mono, human) and anti HSP60 (poly, cyanobacterium).

The results indicate a clear cross reactivity for different antibodies. The best results were obtained by using anti HSP60 (mono, human) which gave rise to a strong reponse in the heat shocked nematodes at a molecular weight of 60 kDa.

Table 2. Expression of different antibodies after exposure to 37°C for 60 min. The control treatment was 20°C (+: strong expression, ±: weak expression, -: no expression, many: many dots were observed).

	response	control
anti HSP27 mono, human	+ (50 kDa)	±
anti HSP60 mono, human	+ (60 kDa)	±
anti HSP70 mono, bovine	+ (70-50 kDa)	+
anti HSP70 mono, human	–	–
anti HSP25 poly, rat	+ (many, 50-90 kDa)	±
anti HSP60 poly, moth	+ (many, 60 kDa)	±
anti HSP60 poly, cyanobact.	± (40 and 60 kDa)	–
anti HSP70 mono, guinea pig	+ (many, 50-90 kDa)	±

Table 3 shows the expression of three different antibodies in metal stressed nematodes. Exposure to Cd and Cu resulted in a strong expression of anti HSP60 (mono, human) compared to the control treatment. Anti HSP70 (mono, guinea pig) resulted in a diffuse pattern of dots (60-80 kDa).

Table 3. Expression of different antibodies after exposure to Cd (50 µM) and Cu (7.1 µM) in water for 60 min. at 20°C (++: very strong expression, +: strong expression, ±: weak expression, -: no expression).

	treatment		
	Cd	Cu	control
anti HSP60 mono, human	++ (60 kDa)	++ (60 kDa)	±
anti HSP70 mono, bovine	+ (70 kDa)	+ (70 kDa)	+
anti HSP70 mono, guinea pig	++ (60-80 kDa)	–	±

Discussion

The application of commercially available monoclonal antibodies to detect HSP's in nematodes proved to be successful. After blotting of the gels, antibodies can be used to detect HSP induction following exposure to either heat or heavy metals.

The obtained results indicate that induction of HSP60 in *P. acuminatus* may be a promising biomarker for heavy metal stress. Results with pentachlorophenol, which are not presented here, did not show a response. These results are in accordance with the findings for mussels which were exposed to copper giving rise to HSP60 induction. Preliminary results showed dose-response relationships for HSP expresssion in *P. acuminatus* for various toxicants. Threshold concentrations appeared to be 100,000 times lower than the LC_{50} in water, indicating an early warning to chemical stress.

PART B. DETAILED REPORT OF THE CONTRACTORS

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I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To establish a technique to (semi-)quantify HSP70 (and HSP60) in the slug, *Deroceras reticulatum*.

2. To assure suitability of these biomarker tests to assess proteotoxic stress conditions due to metal contamination in laboratory toxicity experiments.

3. To validate the stress protein test using full life-cycle investigations with *D. reticulatum* for zinc and cadmium contamination.

4. To render the technique of RT-PCR accessible to slugs in order to allow investigations of the stress response on the mRNA level.

II. OBJECTIVES FOR THE NEXT PERIOD:

1. To carry out further subchronic exposure studies with further diminished concentrations of zinc, copper, cadmium, and dimethoate contamination and to analyze samples for HSP70, for metallothionein (participant 3), and by NMR and ICP-MS (participant 5).

2. To validate these data by a second set of life-cycle studies.

3. To analyze snail (participant 3), earthworm (participant 3 and 5), isopod, and Collembolan samples (participant 6) for HSP70.

4. To optimize the PCR technique to assess proteotoxicity in metal-exposed slugs on the mRNA level.

III. MAIN RESULTS OBTAINED:

1. Establishment of an HSP70 (HSP60) test for *Deroceras reticulatum.*

Methodology

To compare results quantitatively, it was necessary to measure the total protein concentration in the homogenate of individual slugs as a reference. Constant protein amount (50 µg per lane) was subjected to SDS-PAGE, transferred to nitrocellulose and HSP70 (HSP60) stained with the Western blot technique using a monoclonal mouse anti-human HSP70 (HSP60) antibody. Subsequent (semi) quantification of the results took place by densitometric image analysis. To assess the influence of the temperature on HSP production, specimens being hatched at 10°C were subjected to heat shock (20°C, 25°C) for two hours. A slug hatchery at constantly 15°C was also tested.

Results and Discussion

The distinct presence of HSP70 was detected in the animals subjected to heat shock. The immunoblot showed a single protein band at approx. 70 kDa. Only a weak band in the same region could be detected in controls (10°C). A permanent hatching temperature of 15°C resulted in increased HSP70 content similar to heat shock conditions. These observations necessitated subsequent toxicity experiments to be carried out at 10°C or lower.

2. Assessment of the toxicity of zinc and cadmium on *Deroceras reticulatum* in laboratory microcosms.

Methodology

Adult slugs derived from a laboratory hatchery at 10°C were exposed to substrate soaked with either 10, 50, or 100 mg/kg Cd^{2+} or to 500, 1,000, or 5,000 mg/kg Zn^{2+} at 10°C in a day/night cycle of 16h/8h. Specimens were kept in plastic boxes on a substrate of soil material which had been oven-dried (60°C for 2 d) and which was subsequently soaked with 10 mL of the respective metals (test animals) or with tap water (controls). Additionally, the food (lettuce leaves and carrot slices, sprinkled with $CaCO_3$ particles) was wetted with 2 mL of the same solution as the corresponding substrate material was soaked with. Metal concentrations per dry weight of substrate and food were analyzed by atomic absorption spectrophotometry.

For atomic absorption spectrophotometric investigations, complete gut clearance was achieved by maintaining the animals on uncontaminated, moist filter paper for two days after the termination of metal exposure.

Results and Discussion

From the control animals a weak band at approximately 70 kDa in the immunoblot indicated presence of proteins belonging to the HSP70 group. This protein band became much more intensively stained in slugs exposed to each metal at each of the test concentrations in a dose-dependent manner. Darkest staining occurred in specimens contaminated with the highest applied metal concentrations. Image analysis quantification of the mean relative grey value of all 70 kDa bands revealed that the patterns of stress response of *D. reticulatum* differed between different metals. A significant increase in HSP70 content could be detected in animals exposed to 10 mg/kg Cd^{2+}, although at higher tested concentrations of the respective metal ions the HSP70 content remained nearly constant. In contrast, increased zinc contamination resulted in elevated HSP70 levels even up to Zn^{2+} concentrations that were lethal to some specimens. The general patterns of stress reponse did not change when image analysis data were related to the accumulated metal concentrations in slug tissues. A significant elevation of HSP60 in the supernatant of metal-exposed slugs could not be found. Contamination resulted in a far lower increase of HSP60 than of HSP70.

The dose-dependence type of reaction and the correlation of the HSP70 induction with the concentration of the toxins revealed the HSP70 test in the present form to be a promising tool for further toxicity tests.

3. Life-cycle tests with zinc and cadmium contamination.

Methodology

Juvenile *Deroceras reticulatum* at the age of 40 days were fed a diet of lettuce and carrot on a filter paper ground both soaked with either 10, 50, or 100 mg/kg Cd^{2+}, with 100, 500, or 1,000 mg/kg Zn^{2+}, or with a mixture of 10 mg/kg Cd^{2+} and 500 mg/kg Zn^{2+} at 10°C in a day/night rhythm of 16h/8h. Exposure experiments were conducted chronically and were terminated after 169 days. Controls were wetted with tap water. Mortality, egg-laying rate and the number of hatched juveniles of the F1 generation were recorded.

Results and Discussion

In the controls, mortality of the adults occurred in relation to the deposition of eggs. According to their biology, most slugs died after egg deposition. In chronically exposed slugs, mortality was found to be increased in a dose-dependent manner. Corroborating the results of the HSP70 test, the highest applied concentrations of cadmium and zinc affected the survival rate.

Slugs exclusively exposed to cadmium showed delayed growth and did not produce any eggs. In zinc-contaminated specimens the number of egg masses produced was strongly reduced and also the fertility strictly depended on the zinc concentration: in controls 85% of the juveniles hatched from their eggs, in eggs which were produced by slugs influenced by 100 mg/kg Zn^{2+} only 36% were fertilized. Higher zinc contamination resulted in a

complete deficit of the F1 generation.

An increased production of HSP70 not only indicated proteotoxic conditions but also adverse effects on the population. The sensitivity of the HSP70 test will be characterized by subsequent 'low concentration' life-cycle studies.

4. Amplification of HSP70-cDNA by PCR corresponding to the transcriptional level of the HSP70 genes.

Methodology

Since the nucleotide sequence of the HSP70 gene(s) in *Deroceras reticulatum* is unknown, a computer alignment of the sequences of various vertebrate and invertebrate animals was carried out in order to find highly conserved regions in the gene. Two highly conserved regions about 500 to 530 bp and 1080 to 1120 downstream the end of the variable sequences of the HSP70 gene were chosen for the construction of oligonucleotide primers for the PCR technique.

To test the suitability of the method for the investigation of stress situations, total RNA of a positive control (heat shock of 25°C for 2 h) and an un-contaminated negative control slug were isolated. Total mRNA was transcribed reversely into cDNA.

Results and Discussion

In the positive control as well as in the negative control, a fragment of about 550 bp of the HSP70 gene was amplified by PCR. Agarose gel electrophoresis showed the concentration of the amplified HSP70 gene fragment much higher in the heat-shocked specimen than in the negative control. Further investigations should show whether the PCR technique is suitable to distinguish also between the HSP70 transcription rate of slugs exposed to different contaminants.

PART B. DETAILED REPORT OF THE CONTRACTORS

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I. OBJECTIVES FOR THE REPORTING PERIOD:

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

2. To look for the presence of metallothioneins (or metal-binding proteins) in terrestrial oligochaetes (*Eisenia fetida*) after metal exposure (cadmium, copper), and to characterize these proteins biochemically.

II. OBJECTIVES FOR THE NEXT PERIOD:

1. To test the suitability of metallothioneins as possible biomarkers for organochemicals (dimethoate) in terrestrial gastropods (*Helix pomatia*, *Arianta arbustorum*, and *Deroceras reticulatum*), performing studies at the protein expression level.

2. To test the suitability of metallothioneins as possible biomarkers for chemical exposure (cadmium, copper, dimethoate) in terrestrial gastropods (*Helix pomatia*, *Arianta arbustorum*, *Deroceras reticulatum*), performing studies at the mRNA level.

3. To test the suitability of metallothioneins (or metal-binding proteins) in terrestrial oligochaetes (*Eisenia fetida*) as possible biomarkers for chemical exposure (cadmium, copper, dimethoate).

III. MAIN RESULTS OBTAINED:

Methodology

A suitable method - called Cadmium Chelex assay - was adopted and modified by means of which it was possible to quantitatively determine MT concentrations (Cd-MT and Cu-MT) as well as percentage saturations of the protein with cadmium or copper in homogenates of snail tissue (Berger et al., 1995).

Moreover, methods of gel chromatography and reversed-phase HPLC were used to fractionate different MT isoforms from snail tissue. Gel and ion exchange chromatography as well as reversed-phase HPLC were also carried out to purify and characterize metal-binding proteins in earthworms (*Eisenia fetida*).

On the molecular level, methods of PCR amplification were adopted using MT-specific primers, to get the entire sequence of the MT encoding gene.

Concomitantly with this biochemical and molecular work, Cd exposure experiments were carried out to correlate biochemical or molecular data with metal concentrations in animal tissues.

Results

a) Terrestrial gastropods:

The Cd-Chelex assay was applied to study the **dynamics** of MT induction and saturation as well as **steady state levels** of these two parameters in two gastropods species: *Helix pomatia* and *Arianta arbustorum*. *Arianta arbustorum* was also included in this study, because two Cd-binding isoforms of this species are well known biochemically (Berger et al., 1995). This is a precondition for adopting the Cd-Chelex assay as a quantitative tool.

From the **dynamic studies**, it appeared that snail MTs are quickly induced within a few hours subsequent to cadmium exposure. MT concentrations increased from control levels (ca. 100 µg/g fresh wt.) to concentrations of about 1000 µg/g fresh wt. in *Helix pomatia*, and from ca. 2000 µg/g fresh wt. in controls to about 6000 µg/g fresh wt. in *Arianta arbustorum*, respectively. At the same time, the percentage Cd saturation of the protein increased from 25% in controls to 70% in exposed animals (*Helix pomatia*) and from 10% in non-exposed individuals to about 40% in Cd-treated snails (*Arianta arbustorum*), respectively. Moreover, it appeared that at the highest exposure concentrations in the diet (*Helix pomatia*: > 75 µg Cd/g, dry wt; *Arianta arbustorum*: > 200 µg Cd/g dry wt), both MT concentration and percentage cadmium saturation reached maximal levels which were not exceeded at even higher Cd concentrations in the diet.

Studies on **steady state** levels of MT concentration after long-term exposure as well as from field-collected animals demonstrated the existence of a saturation-like relationship between Cd concentrations in the midgut gland and MT tissue concentrations. The same pattern was also found for percentage Cd saturation levels of the protein. In fact, individuals showing maximal levels of both MT concentration and Cd protein saturation became moribund and died within a few days.

By means of combined gel chromatography and HPLC studies the existence of a copper-specific MT isoform in the mantle of *Helix pomatia* was demonstrated. In terms of induction or metal saturation, this isoform does not respond to cadmium exposure: All the

cadmium taken up by the animals is accumulated exclusively in the midgut gland and bound to a midgut gland-specific MT isoform. Similar results have been obtained for *Arianta arbustorum*, although in this case, the copper-binding ligand in the mantle has not yet been proven to be a true MT.

Studies at the molecular level of MT induction are not yet conclusive. This is because the primers used so far in PCR yield different amplification products which can either be proven not to be MT sequences; or which still have to be sequenced in the near future.

b) Earthworms (*Eisenia fetida*):

The presence of MT in worms has never been verified by amino acid or DNA sequencing. In fact, chromatographic studies on *Eisenia fetida* demonstrated the presence of two metal-binding proteins. However, none of these proteins proved to be a MT. After gel chromatography, the first of these proteins eluted at a molecular weight of about 18 kD. It contained, apart from cadmium and zinc, considerable amounts of iron. Preliminary results suggested that it may be a myoglobin- or myoerythrine-like protein. The second metal-binding peak mainly contained cadmium and zinc and was detected in the molecular weight range of 45 kD. Although this protein has not been sufficiently characterized yet, it is already clear that it is not a MT.

Discussion

The present studies on short-term cadmium exposure showed that steady state levels of both MT concentrations and Cd saturation levels in terrestrial snails were reached after only a few hours following exposure, at least at the highest exposure concentrations used. This indicates that acute cadmium loading exhausted the capacity of the detoxification system rapidly. In most field situations, however, animals are likely to be exposed to lower cadmium concentrations, but for longer periods of time. It has been demonstrated that in such individuals, the upper limit of detoxification capacity became exhausted, if certain upper limits of both MT concentration and Cd saturation of the protein are exceeded. It is thus concluded that in terms of concentration and Cd saturation, the MT status of an individual is likely to provide a valuable biomarker system for environmental Cd contamination.

In earthworms, the situation is more complicated due to the fact that these animals do not synthesize MTs. The likely metal-binding protein present in *Eisenia fetida*, for instance, needs first to be characterized more precisely with respect to its biochemical features, before it can be used as a potential biomarker.

References

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

PART B. DETAILED REPORT OF THE CONTRACTORS

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I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To develop electrophoretic methods for analysing the enzyme esterase in the three species: earthworm, *Eisenia fetida*, isopod, *Porcellio scaber*, and Collembola, *Folsomia candida*.

2. To identify the esterases by means of eserine and organophosphorus compounds.

3. To study the response of the esterases in the three species on the metals copper, zinc and cadmium and the insecticide dimethoate.

II. OBJECTIVES FOR THE NEXT PERIOD:

1. To study further the response *in vitro* of the esterases in the three species on the metals and the insecticide by using electrophoresis and densiometry.

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

III. MAIN RESULTS OBTAINED:

Methodology

The culture of the earthworm, provided by Dr. M. Holmstrup, Denmark, was kept in plastic boxes with a mixture of cow dung and garden soil. The three cultures of the isopod, originating from two localities in Denmark and one in The Netherlands, were kept in plastic aquaria on sand and with mainly poplar leaves as food supported with a little of carrot. The four cultures of the Collembola, one originating from France, one from The Netherlands, one from England and one provided by Dr. P. H. Krogh, Denmark, were kept on an artificial soil with a little dry yeast as food. All cultures were moistened.

Prior to analysis of the esterases single individuals of the three species were homogenised and centrifuged at 18,000 g at 5°C for 5 minutes and the supernatant was used for application to the gel.

The method used for analysing the esterases was isoelectric focusing. The electrophoresis was performed in a 1 % agarose gel containing 10 % sucrose for avoiding dryness. The size of the gel was 70 x 70 x 0.1 mm. The pH range in the gel was established by adding 80 µL ampholine 3.5-10 and 550 µL ampholine 4-6 to 10 mL of the agarose solution. The samples were applied by using 3 x 3 mm filterpaper, Whatman No. 3MM, up to 15 samples per gel. The cathode wick was soaked in sodium hydroxide or sodium phosphate and the anode wick in acetic acid, the concentration of the solutions depended on the species analysed. The conditions for the electrophoresis was 5 minutes with an upper level of 100 V, 2 mA and 5 W, and depending on the species 20 or 30 minutes with an upper level of 2000 V, 5 mA and 5W. Immediately after the electrophoresis the gel was stained with an agar overlay containing α-naphthylacetate and Fast Blue RR salt at 37°C. The metals as chlorides and the dimethoate were applied either to the staining solution or to the samples.

Results

The methods for analysing esterases of the three species were established. The zymogram of the earthworm consisted of two zones, one revealing a single band for all the individuals analysed so far, and the other showing variation with one to three bands per individual. The zymogram of the isopod revealed a complicated pattern but four zones could be identified for each individual. One zone consisted of one band, two zones of two bands and one zone of three bands. No evident variation was found. The zymogram for the Collembola revealed a zone consisting of a faint and a heavily stained band, a zone of three bands and a zone with two bands. No variance was seen.

No acetylcholinesterase activity could be identified in any of the species by applying eserine up to 10^{-2} M to the staining solution.

Application of the metals or the insecticide to the sample had less effect than application to the staining solution. Table 1 shows the concentration in the stain of the metals and of the insecticide, which have an effect on the esterases based on a visual evaluation.

Table 1. Concentrations of metals and insecticide in M having an effect on esterases in three invertebrate species.

	Copper	Zinc	Cadmium	Dimethoate
Earthworm	0.020	0.025	0.01	0.01
Isopod	0.025	0.025	0.05	0.01
Collembola	0.020	0.020	0.04	0.01

Discussion

Øien and Stenersen (1984) studied the earthworm by means of polyacrylamide electrophoresis and obtained a similar zymogram, and they observed as seen in the present study no acetylcholinesterase activity which might be seen by using the specific inhibitor eserine. However, it will be needed to check the esterases for specific activity and to determine the effect of the metals and the pesticide by using densiometry.

References

Øien, N. and Stenersen, J. (1984): Esterases of earthworms - III. Electrophoresis reveals that *Eisenia fetida* (Savigny) is two species. *Comp. Biochem. Physiol.* **78**, 277-82.

PART B. DETAILED REPORT OF THE CONTRACTORS

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I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To develop procedures for the analysis of invertebrate fluids and/or tissues by NMR. To establish a data-set of baseline profiles from earthworms prior to metal exposure.

2. To investigate alterations in the constituent NMR and ICP-MS profiles of earthworms subsequent to metal exposure.

3. To develop similar mineral baseline profiles for earthworms by the use of ICP-MS, and to realize changes in these subsequent to metal exposure.

II. OBJECTIVES FOR THE NEXT PERIOD:

1. To establish links between changes in ICP-MS profiles and alterations in NMR profiles. To attempt to determine pathways between sublethal exposure and metabolic dysfunction.

2. To establish ICP-MS and NMR profiles for other invertebrate species, eg. isopods, Collembola and nematodes in cooperation with the other participants of the programme.

3. To establish ICP-MS and NMR spectra for other soil invertebrates following exposure to dimethoate, and the other chosen inorganic elements by coordination with the other participants.

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

III. MAIN RESULTS OBTAINED:

Methodology

1. Exposure of earthworms to copper: Earthworms (*Lumbricus rubellus*) were chosen for experiments because of the need to use a species that would occur in field or semi-field conditions. These worms were exposed to an increasing range of soil copper concentrations 3 to 320 µg Cu/g in large plastic soil mesocosms under semi-field conditions for 110 days. Sub-samples were taken for profile analysis by ICP-MS and NMR (JOEL GSX500). Another sample was removed for HSP70 determination by participant 2.

2. Multi-elemental analysis by ICP-MS: Earthworms were depurated for 24h at 15°C and then frozen. Thawed samples were dried, digested in concentrated nitric acid and digested at 100°C for approximately 1 hr, subsequently these digests were allowed to cool and diluted with distilled water (10-20 mL). The semi-quantitative elemental analysis was performed by inductively-coupled plasma-mass source spectrometry (VG Elemental, UK). The instrument operating conditions used were those recommended by the manufacturer. The ICP-MS was calibrated such that a 0.1 µg/mL indium solution corresponded top 2*10(6) counts on the detector and calibrated over the mass range 5 to 247, using a mass calibration mixture of beryllium 9, cobalt 59, indium 115 and uranium 238. Scans skipped certain mass range areas 11.5-23.5; 27.4 - 41.5 and 79.5 - 80.5 to reduce different interferences. Calibration was verified by the analysis of a range of standard solutions. Samples being introduced via a Meinhart nebuliser into a spray chamber. Sample uptake was controlled by a peristaltic pump. Scan analysis using ICP-MS with internal standards provided a semi-quantitative spectrum of elements present in the sample.

3. Metabolic Profile analysis by NMR: High resolution ^{1}H NMR spectroscopy was used to investigate perturbations of endogenous metabolites resulting from copper stress in the earthworm, *Lumbricus rubellus*. Initially, coelomic fluid was extracted using a fine syringe. However, this technique failed to yield sufficient volumes of fluid (typically 10-50µL). Greater resolution of the spectra is obtained with larger initial sample volumes. As a rule 700µL of fluid is normally required for the NMR spectrometer, hence these initial low volume samples were diluted by a factor of 20. The first spectra were thus, of poor quality and insufficient in detail to enable accurate discrimination of all peaks. However, the profiles were of sufficient clarity to be able to make some general observations. Amino acids, such as alanine, lysine, glycine and glutamic acid, citric acid cycle components, such as succinate and creatine, and artifacts such as methanol and acetone, were resolved. However, for the investigation of earthworms subjected to copper stress and their subsequent metabolic profiles the samples collected were too dilute to be useful.

A second extraction method was thus developed. Whole depurated worms (48 hr) were homogenised in a 2:1 ratio of earthworm physiological Ringer. The cellular component was then centrifuged off, and the resulting fluid ultra-filtered with a molecular weight cut-off at 10 kD. Analysis of the resulting tissue fluid enabled the visualisation of many more peaks with much greater clarity and resolution. All or most of the primary amino acids could be identified, as well as the two glucose anomers and a number of the citric acid cycle components.

Results

It was observed in the up-field, aromatic region of the ^{1}H NMR spectra that two peaks were absent from the control, but present in all spectra of these worms exposed to copper stress. The peaks have been assigned to histidine, a basic, primary amino acid. Preliminary results also suggest that these concentrations of histidine, as measured by the area under the peak, increased with increasing copper exposure.

Discussion

1. ICP-MS profiles: The observed mineral profiles of the earthworms showed by semi-quantitative scanning by ICP-MS revealed little variation in elemental constituents of copper exposed worms other than a pronounced increase in copper in concordance with soil copper concentrations. There was some initial suggestion of a lowering of manganese, although this requires further investigation.
2. NMR Profile and histidine complexes: The shape of the peaks observed for histidine suggested that this molecule may be involved in a complex. The paramagnetic effects of a complexation with copper would have the effect of broadening the two peaks observed above. Histidine forms an extremely stable complex with copper. It is for example, orders of magnitude more stable than the corresponding complex with EDTA, a commonly used metal chelating agent. A number of structures for the proposed copper-histidine complex have been suggested (see above). The square planar form with tetragonal distortion and the square planar, appear to be the most likely. The significance of the presence of histidine in copper exposed worms, is that it provides an "active" biomarker of toxic exposure. That is, that the biomarker is actively associated with the toxicant in question under semi-field conditions.

PART B. DETAILED REPORT OF THE CONTRACTORS

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I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To develop and optimize a homogenisation and separation technique for analysing metal binding proteins in tissues of individual isopods and in Collembola.

2. To determine the effects of zinc on metal-binding proteins in isopods in relation to effects on growth and reproduction.

II. OBJECTIVES FOR THE NEXT 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 and reproduction of isopods.

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

III. MAIN RESULTS OBTAINED:

Methodology

1. FPLC patterns of cadmium-exposed isopods and Collembola:
Isopods (*Porcellio scaber*) and Collembola (*Orchesella cincta*) were exposed to dietary cadmium. The hepatopancreas of the isopods or complete Collembola were homogenized and analysed for protein patterns on an FPLC with a Mono Q (anion exchange) column. The FPLC fractions were analysed by graphite furnace AAS to find out which fractions contained metal-binding proteins.

2. Sublethal effects of zinc on isopods:
An experiment was performed to test the hypothesis that isopods receiving high quality food will be less susceptible to zinc because they have more energy to spend on 1. active regulatory mechanisms for zinc or 2. the production of metal-binding proteins. Juvenile individuals of *P. scaber* were exposed for 14 weeks to different concentrations of zinc in the food (chopped leaf litter). Food quality was manipulated by adding 2 or 10% peptone as a protein source. Growth, total protein content and zinc concentrations in the isopods were determined at the end of the study, and animal samples were stored for FPLC analysis.

In a second experiment, *P. scaber* juveniles were obtained from animals sampled at four different locations. Two of these locations were polluted with heavy metals, so the isopods sampled here are supposed to be adapted to metal exposure. The other two locations are non-polluted reference locations of our department. The isopods were exposed for 16 weeks to two zinc levels (21 and 38 µmol/g dry weight) sprayed on plant leaves, and effects on growth were related to zinc uptake.

Results

1. FPLC patterns of cadmium-exposed isopods and Collembola:
The protein pattern in cadmium-exposed isopods did not show metal peaks in the range where commercial rabbit metallothionein eluted from the FPLC column. The work on the detection and identification of metal-binding proteins in isopods will be continued in the second year.

In the first experiments, the Collembola were homogenized in 20 mM Tris-HCl at pH=8.0, and separated on a Mono Q column with an increasing NaCl gradient. This resulted in a cadmium peak already in the second fraction, suggesting that cadmium was released from the proteins. This was confirmed by the fact that an FPLC run with purified rabbit metallothionein showed a cadmium peak in fraction 12. Cadmium was also released from the protein (and metallothionein) when the rabbit metallothionein was added to the *O. cincta* homogenate. Apparently, the homogenisation step was responsible for this release of cadmium. Furthermore, it appeared that the cadmium analysis on the graphite furnace AAS was interfered by the high Na concentrations in the FPLC fractions.

To solve this problem, a 'new' homogenisation procedure was chosen adapted from Roesijadi and Fowler (1991), in which the Collembola are homogenised in 55% Tris-HCl at pH 8.0 with 45% acetone. After centrifugation, the supernatant is adjusted to 80%

acetone and centrifuged again. The resulting pellet is resuspended in 20 mM Tris-HCl and eluted on the Mono Q column with a NH_4Cl gradient. This procedure appeared to be more successful, as little cadmium was released from the protein. Due to the more complicated procedure, however, more protein was lost, resulting in an overall lower sensitivity of the method.

2. Sublethal effects of zinc on isopods:
In the first experiment, survival of *P. scaber* was only slightly affected at the highest zinc level (60 µmol/g dry food). With 10% peptone, growth was much higher than with 2% peptone, and this was the case with both untreated and zinc treated food. The ratio fresh/dry weight was not affected, and faeces production (which can be considered as a measure for consumption) was only reduced at the highest exposure level in the animals offered low quality food. Zinc levels in the animals appeared to be constant up to food concentrations of 43 µmol/g dry weight, suggesting regulation of the internal zinc concentration. At the highest exposure level, zinc concentrations increased and were highest in the animals offered the high protein food. The protein content of the isopods was highest in the animals offered the high quality food, and this was the case at all exposure levels.

In the second study, the animals from the vicinity of a zinc smelter appeared to be less sensitive to zinc and showed the lowest growth reduction upon exposure to the high zinc levels compared to the animals from an old lead mine and the two reference sites. Zinc concentrations were however, lower in the animals from the smelter area, especially as these animals maintain their internal concentration lower at the higher external exposure level. When total zinc uptake was considered, it appeared that this was almost similar in all animals from the four locations with on average the highest uptake in the smelter isopods and the lowest uptake in the animals from the mine area.

Discussion

The results of FPLC and AAS analysis showed that it was possible to detect metal-binding proteins in isopods and Collembola, but the method needs further improvement. It also shows that in isopods other metal-binding proteins are present, differing from the rabbit metallothionein that was used as a reference.

P. scaber offered the high quality food apparently have to spend more energy on the production of (metal-binding) proteins, as they contain higher protein levels and also higher zinc levels. The nature of these proteins still has to be established, and for that purpose animal samples of this experiment will be analysed by FPLC and AAS.

The results of the second toxicity study show that the high growth rate of the smelter isopods reduced their internal body concentration, suggesting that in isopods from metal-polluted sites adaptation has resulted in an increased tolerance to zinc. Furthermore, complexation of zinc in the body seems to play a role in metal tolerance, as this can be the only explanation why these animals are able to keep on growing on zinc-treated food. The role of metal-binding proteins in this tolerance mechanism still has to be resolved.
In a follow-up experiment of this latter study, the zinc distribution in adapted and non-adapted isopods is to be investigated to establish the role of the hepatopancreas in the reduced sensitivity of metal-adapted animals. If the hepatopancreas appears to play a

dominant role, further research should concentrate on this organ.

References

Roesijadi, G. and Fowler, B.A. (1991): Purification of invertebrate metallothioneins. In: J.F.R. Vallee & B.L. Vallee (eds.). Methods in enzymology. Volume 205. Metallochemistry. Part B. Metallothionein and related molecules. Academic Press, San Diego. pp. 263-273.