Progress Report 1998 of

BIOPRINT-II

Application and Standardisation of Biochemical Fingerprint Techniques in View of the Risk Assessment of Toxicants in Soil Ecosystems

Third Technical Report

EU Environment and Climate Research Programme
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Editor:
Jan E. Kammenga, project-coordinator
Wageningen Agricultural University,
The Netherlands

Contributions from:
H.-R. Köhler
R. Dallinger
V. Simonsen and J. Scott-Fordsmand
J.M. Weeks
M.H. Donker

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ENVIRONMENT & CLIMATE RESEARCH PROGRAMME (1994-1998)

Research Area: Ecotoxicology

Summary Progress Report

1. Contract no: ENV4-CT96-0222

2. Title: APPLICATION AND STANDARDISATION OF BIOCHEMICAL FINGER-PRINT TECHNIQUES IN VIEW OF THE RISK ASSESSMENT OF TOXICANTS IN SOIL ECOSYSTEMS (BIOPRINT-II).

3. Reporting period: August 1, 1997 - July 31, 1998.

4. Scientific coordinator: Dr. J.E. Kammenga

5. Project participants:

1. Dr. J.E. Kammenga, Department of Nematology, Wageningen Agricultural University, Binnenhaven 10, 6709 PD Wageningen, The Netherlands.

2. Dr. H.R. Köhler, Department of Cell Biology, University of Tübingen, Auf der Morgenstelle 28, D-72076, Tübingen, Federal Republic of Germany.

3. Dr. R. Dallinger, Institut für Zoologie, Abteilung Zoophysiologie, Technikerstraße 25, Universität Innsbruck, A 6020 Innsbruck, Austria.

4. Dr. V. Simonsen, National Environmental Research Institute, Department of Terrestrial Ecology, PO Box 314, Vejlsovej 25, DK-8600 Silkeborg, Denmark.

5. Dr. J.M. Weeks, Institute of Terrestrial Ecology, Monks Wood, Abbots Ripton, Huntingdon PE17 2LS, England.

6. Dr. M.H. Donker, Department of Ecology and Ecotoxicology, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands.

Key words: Biomarkers, soil invertebrates, risk assessment.

PART A. SUMMARY PROGRESS REPORT OF THE PROJECT

I. GENERAL OBJECTIVES:

The main objective of the BIOPRINT-II project is the deployment of biochemical fingerprint techniques, which have already been developed within the BIOPRINT project (EV5V-CT94-0406), for assessing the exposure and effect of toxicants on soil invertebrates in the field. The research programme can be divided into 4 topics:

1) To investigate the impact of confounding factors (temperature and pH) on the biomarker response.

2) To study the biomarker response to combined mixtures of toxicants.

3) To study the transient response and persistence.

4) To apply biochemical fingerprint techniques in the field.

5) To standardize biomarker assays for risk assessment procedures.

The research programme includes organisms of different taxonomic and ecological groups which play a vital role in soil ecosystem processes, e.g. nematodes, diplopods, isopods, Collembola, gastropods and oligochaetes. In accordance to the BIOPRINT project the following biomarkers will be studied: i) heat shock proteins, ii) metallothioneins and metal binding proteins for detection of heavy metals, iii) esterases for detection of organic contaminants, iv) histidine complexing and lysosome integrity. This project will explicitly focus on the applicability of these biomarkers in soil invertebrates in a chronically polluted fieldsite near Avonmouth (UK).

II. SPECIFIC OBJECTIVES FOR THE REPORTING PERIOD:

1) To further study the use of a mini-container test kit as a potential bio-assay using nematodes in experimental field sites and the laboratory and to apply a field tool kit as an *in situ* bio-assay for assessing HSP60 induction in nematodes in a contaminated field near Avonmouth (UK). 2) To study the influence of the confounding factor acidity (pH) on the HSP70 induction by metal exposure in slugs (*Deroceras reticulatum*) and to quantify the stress reaction (HSP70) in a variety of soil animal species collected in the well-established metal gradient around the smelting works at Avonmouth, UK. 3) To develop a quantification assay and to relate the response of MT-pools to other sub-lethal endpoints and to assess the suitability of MT-pools in field experiments. 4) To continue the study of the transciency of the response of esterases in laboratory and field populations from Avonmouth of the Collembolan *Folsomia candida* and to perform field experiments with *Lumbricus rubellus* at the copper-polluted site at Hygum, Vejle. 5) To develop multivariate statistical techniques enabling interpretation of data generated from ICP-MS elemental profiles of soil invertebrates collected from the field in the vicinity of the Avonmouth smelter site, and to link biomarker measurements from these deployed animals with ecologically significant effects upon the structure or function of a soil invertebrate community. 6) To relate the

amount of metallothionein in the springtail *Orchesella cincta* to cadmium exposure level, duration of exposure and temperature of exposure and to analyze metallothionein in different springtail species exposed under laboratory and field conditions.

III. SPECIFIC OBJECTIVES FOR THE NEXT PERIOD:

1) To semi-quantify the RT-PCR method to measure HSP60 induction in the nematode *Plectus acuminatus* and to evaluate the applicability of measuring HSP60 induction in nematodes in an *in situ* bio-assay to assess exposure to toxicants in the field. Evaluation of the transiency of the HSP60 response in *P. acuminatus*. Furthermore the exposure assessment of toxicants in relation to temperature and pH using HSP60 induction in the nematode *P. acuminatus* will be carried out. 2) To study the influence of metal cocktails (mixture toxicity) on the HSP70 induction in slugs (*Deroceras reticulatum*) and to repeat the analyses for HSP70 in isopods (*Oniscus asellus, Porcellio scaber*) taken in 1998 from field sites in the metal gradient near the smelting works at Avonmouth, UK. Additionally, model predictability of real HSP70 results will be obtained in the field using metal concentration and confounding factors as variables. 3) To develop a metal saturation assay for quantitative detection of earthworm MTs and a quantitative approach for measuring of earthworm MT mRNA in response to metal exposure. To modify the metal saturation assay for quantification of the novel Cu binding MT isoform in the snail's midgut gland, and to complete our studies on quantitative measurements of snail MT mRNA. 4) To determine the activity of esterases from various invertebrates by densitometry and to do further analyses on invertebrates from Avonmouth with the emphasis on the earthworm, *Lumbricus rubellus*. Finally a protocol will be written for the use of esterase, determined by electrophoresis and densitometry, as a tool for detecting contaminated areas. 5) To further develop multivariate statistical techniques enabling interpretation of data generated from both ICP-MS elemental profiles of soil invertebrates, earthworms, and for locomotory activity measurements for woodlice collected from the field in the vicinity of the Avonmouth smelter site. To consider the ecological relevance of any metabolic changes in earthworm biofluid collected along the contamination profile at Avonmouth using NMR and pattern recognition techniques. Further attempts will be undertaken to link biomarker measurements from these deployed animals with more ecologically significant effects upon the structure or function of the soil invertebrate community using techniques such as soil respiration, microbial decomposition and measurements of feeding activity (bait lamina). 6) To analyze the metallothionein MT expression in springtails collected in spring 1998 at Avonmouth and to compare these values with the data of animals collected in autumn 1997. Laboratory raised springtails will be exposed to soil and litter samples from the Avonmouth gradient and the difference in MT expression between field and laboratory exposed animals will be determined. Furthermore quantitative PCR techniques will be developed to measure the MT mRNA as an even more susceptible biomarker for metal exposure.

IV. MAIN ACTIVITIES UNDERTAKEN:

Methodology

1) Initially tests were performed in the laboratory and in the field (using mini-containers) to investigate the possibilities of the biomarker system for transplanting nematodes. For assessing

HSP60 induction in nematodes we exploited a RT-PCR with specific primers for nematode HSP60. 2) Both field obtained and adult laboratory reared slugs were exposed to contaminated food at either 5°C, 10°C or 15°C, or at pH 5 or pH 8.5, respectively, to reveal the confounding effect of different temperature and pH. Samples (whole animals) were frozen in liquid nitrogen and analyzed for HSP70 using a standardized one-dimensional Western blotting technique. 3) For characterisation and isolation of MT isoforms in *Eisenia fetida*, methods of gel permeation, ion exchange chromatography and Reversed-Phase HPLC were applied. In *Helix pomatia*, an additional Cu-binding MT isoform was isolated and characterized by means of chromatographic techniques from the midgut gland of copper-loaded snails. After removal of copper from the protein with ammonium-tetrathiomolybdate, the apo-MT was S-methylated and characterised by collision-induced tandem and electrospray mass spectrometry. We are currently testing a metal saturation assay to also quantitatively detect this Cu-specific isoform from the midgut gland. 4) The experiments with *Folsomia candida* as a test organism was continued. Experiments with copper and zinc were conducted as the previous described experiments (Kammenga 1997). Due to problems with the software for a scanning device, only the electrophoretic analyses were performed and the determination of the activity of esterases, the densitometric measurements, will be done during the autumn 1998. *E. fetida* was applied for the laboratory experiments in soil from Hygum (DK), in the autumn 1997 and in the spring 1998. Two types of experiments were conducted, one in control soil spiked with $CuCl_2$ and the other in soil with the natural contamination. 5) Neutral red retention and immune-system activity in the laboratory, including the effects (non-recorded) of both biotic and abiotic confounding factors, studies were undertaken *in situ* using purposely constructed mesh containment bags. *Eisenia veneta* was placed directly into soil cubes (cut using a stainless steel box corer) at each site using the mesh containment bags. Three replicate bags were deployed (with 20 worms in each) for each location. After a 4-week deployment immune-system activity of the earthworms was determined by incubating earthworm coelomocytes with rabbit red blood cells. Lysosome membrane stability was measured (on the same earthworms) as the ability of the lysosomes in coelomocytes to retain neutral red dye. 6) For quantifying the presence and the distribution of metallothionein (MT) in the springtail *Orchesella cincta*, two routes have been followed and modified; the biochemical and the molecular route. In the first route metal binding proteins were purified from cadmium exposed springtails. In the second route we identified the MT-gene. We were able to isolate two cadmium binding peptides from *O. cincta*. Mass spectromic analysis revealed that the molecular masses of these peptides were 2989 and 4139 Dalton (MALDI-TOF analysis in cooperation with R. van der Schors and K. Li of the Department of Molecular Neurobiology). Amino acid sequencing of the smallest peptide results in a sequence common for MTs. Using different PCR techniques with primers based on the identified amino sequence the cDNA was characterized.

Results and discussion

1) The results of the first transplantation experiments in Avonmouth were very disappointing: the mini-containers contained many other nematode species. The system was leak. In new experiments a nylon membrane was fixed to the container with 2-component glue. This glue sustained incubation conditions (both in soil and in water) and nematodes did not escape anymore nor entered the containers from the surrounding environment. The transplantation experiments in Avonmouth succeeded. So nematodes could successfully be transplanted using the mini-container test kit if suitable conditions were provided. HSP60 mRNA concentrations in *P. acuminatus* could be measured exploiting RT-PCR by using primers that were specific for *P. acuminatus*

HSP60. In the laboratory experiments a dose-response relationship could be observed. 2) The results obtained from the laboratory experiments showed that there was little or no influence of temperature between 5 and 10°C on the HSP70 response in soil invertebrates. The experiments on persistence of the stress response in time made evident that the HSP70 level integrates over weeks, possibly due to the longevity of "active" metal species which have been taken up by the animals. There was an influence of the confounding factor "acidity" (low pH), which was known to lead to increased mobility (water-solubility) of the applied cadmium which, subsequently, may have caused a significant elevation of the HSP70 level in response to an otherwise irrelevant cadmium concentration. 3) Three MT isoforms were characterized from *E. fetida* by their primary sequence. The synthesis of this protein is inducible by cadmium exposure, as shown by semi-quantitative chromatographic induction studies. Due to the primary structure, the *E. fetida* MT can be regarded as a class II MT. A Cu-binding MT isoform was isolated and characterized from the midgut gland of copper-loaded individuals of *Helix pomatia*. Results were also obtained by applying our biomarker approach with terrestrial snails to field conditions. So far, we have tested our method in individuals of *H. pomatia* and *Arianta arbustorum* from differently polluted sites in Austria. These sites include uncontaminated areas, moderately contaminated localities, as well as highly polluted sites. The data which are presently available suggest that the biomarker model with MT isoforms might also work under field conditions. 4) Like the observations done on *L. rubellus*, exposed to contaminated soil from Hygum (DK), also *E. fetida* showed some reduction in the esterase activity, but at the high concentration very little reduction was observed. However, also for these experiments a more specific densitometric analysis will be carried out. Polymorphisms were detected in the esterase from *L. rubellus*, which was not seen in any previous experiment with *L. rubellus*. The genetic interpretation of the esterase was that the esterase was determined by one locus with two codominant alleles. A linear regression on the frequency of the overall most common allele and the content of zinc in the earthworms was found. 5) The conclusion reached in most immunotoxicological studies is that heavy metals act to suppress immunocompetence. Immunotoxicity of metals may occur via direct effects upon a specific immune system component or, alternatively, via inhibition of immunoregulation, which can result in immunosuppresion, hypersensitivity or autoimmune disorders. Both the neutral red retention assay and the immune-system activity assay for the earthworms in this study responded significantly to the ambient pollution levels recorded in the soils for where the earthworms were deployed. The neutral red retention assay results showed a clearer dose-response relationship and were more sensitive over those for the immuno-assay, however, immunocompetence was clearly reduced. 6) The MT concentration of *O. cincta* collected from the Avonmouth pollution gradient showed a good relationship with the amount of exchangeable cadmium in the soil. In a second experiment the effect of exposure time on the MT expression was studied. It was shown that the MT expression increased after week 1 to week 4 exposure and then stabilized. It was shown that the cadmium concentration of the animals is not temperature dependent although the MT concentration decreased at higher temperature.

The presence of cadmium bound MT was demonstrated in springtails collected in Avonmouth. The MT concentration was well related to the $CaCl_2$ exchangeable cadmium concentration of the soil.

Conclusions

The application of biochemical finger-print techniques for assessing effect of toxicants on soil invertebrates is well underway. Combined field experiments by all participants were and will be carried out in Avonmouth (UK) where both field sampled animals as well as *in situ* bioassays will be tested for their suitability in risk assessment procedures.

V. JOINT PUBLICATIONS:

J.E. Kammenga (ed.) (1997) Progress Report of BIOPRINT-II. Application and Standardsation of Biochemical Fingerprint Techniques in View of the Risk Assessment of Toxicants in Soil Ecosystems. National Environmental Research Institute, Denmark, 36 pp.

VI. CHANGES IN STATUS:

None.

VII. PROJECT SCHEDULE:

At present the project is on schedule. All participants are preparing for a joint field experiment starting in spring 1999.

VIII. SUMMARY OF PROGRESS ACHIEVED:

The nematode transplantation experiments in Avonmouth succeeded. So nematodes could successfully be transplanted using the mini-container test kit if suitable conditions were provided. HSP60 mRNA concentrations in *P. acuminatus* could be measured exploiting RT-PCR by using primers that were specific for *P. acuminatus* HSP60 and a dose-response relationship could be observed. For other soil species laboratory experiments showed that there is little or no influence of temperature between 5 and 10°C on the HSP70 response. The experiments on persistence of the stress response in time made evident that the HSP70 level integrated over weeks, possibly due to the longevity of "active" metal species which have been taken up by the animals. There was an influence of the confounding factor "acidity" (low pH), which is known to lead to increased mobility (water-solubility) of the applied cadmium which, subsequently, may have caused a significant elevation of the HSP70 level in response to an otherwise irrelevant cadmium concentration. Three MT isoforms were characterized from the earthworm *E. fetida* by their primary sequence. The synthesis of this protein was inducible by cadmium exposure, as shown by semi-quantitative chromatographic induction studies. A Cu-binding MT isoform was isolated and characterized from the midgut gland of copper-loaded individuals of *H. pomatia*. Results were also obtained by applying this biomarker approach with terrestrial snails to field conditions. The data suggest that the biomarker model with MT isoforms might also work under field conditions. A correlation between allelic frequency of esterase from *L. rubellus* and Zn concentration was found. The earthworm *E. fetida* showed some reduction in the esterase activity, but at the high

soil contaminated levels very little reduction was observed. The conclusion reached in most immuno-toxicological studies in earthworms is that heavy metals act to suppress immunocompetence. Both the Neutral red retention assay and the immune-system activity assay for the earthworms in this study responded significantly to the ambient pollution levels recorded in the soils for where the earthworms were deployed. The Neutral red retention assay results showed a clearer dose-response relationship and were more sensitive over those for the immuno-assay. 6) The MT concentration of the springtail *O. cincta* collected from the Avonmouth pollution gradient showed a good relationship with the amount of exchangeable cadmium in the soil. It was shown that the MT expression increased after week 1 to week 4 exposure and then stabilized and that the cadmium concentration of the animals was not temperature dependent although the MT concentration decreased at higher temperature. The presence of cadmium bound MT was demonstrated in springtails collected in Avonmouth. The MT concentration was well related to the $CaCl_2$ exchangeable cadmium concentration of the soil.

PART B. DETAILED REPORT OF THE CONTRACTORS

Contractor: Wageningen Agricultural University

Leading scientist: Dr. J.E. Kammenga
Scientific staff: Dr. J.E. Kammenga, Drs. M.S.J. Arts

Address: Department of Nematology, Wageningen Agricultural University, Binnenhaven 10, 6709 PD Wageningen, The Netherlands.

Telephone: +31 317 482 998/197
Fax: +31 317 484 254
E-mail: Jan.Kammenga@medew.nema.wau.nl

I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To further study the use of the mini-container test kit as a potential bio-assay using nematodes in experimental field sites and the laboratory.

2. To apply a field tool kit as an *in situ* bio-assay for assessing HSP60 induction in nematodes (1D-PAGE and RT-PCR) in a contaminated field near Avonmouth (UK).

II. OBJECTIVES FOR THE NEXT PERIOD:

1. To semi-quantify the RT-PCR method to measure HSP60 induction in the nematode *Plectus acuminatus*.

2. To evaluate the applicability of measuring HSP60 induction in nematodes in an *in situ* bio-assay to assess exposure to toxicants in the field. Evaluation of the transiency of the HSP60 response in *P. acuminatus*.

3. Exposure assessment of toxicants in relation to temperature and pH using HSP60 induction in the nematode *P. acuminatus*.

4. Application of AFLP technology for detection of potential biomarker responses in populations of *P. acuminatus* exposed to cadmium.

III. DEVIATIONS FROM THE WORK PLAN:

None.

IV. MAIN RESULTS OBTAINED:

Methodology

1. The use of the mini-container test kit for transplanting nematodes: Initially tests were performed in the laboratory and in the field in Wageningen to investigate the possibilities of the system for transplanting nematodes. The soil for filling the mini-containers, the same as the one in which the containers were to be buried, was taken into the laboratory, loosened up and irregularities (stones, roots, animals) were removed. After the soil was weighed (wet weight), it was heated in the microwave at full power for 1 minute to eliminate living organisms and dried in an oven at 80°C. After reaching the dry weight, the soil was homogeneously mixed with a bacterial extract (2x108 bacteria (*Acinetobacter johnsonion*) in yeast extract (4g/L) per gram soil dry weight) on a roller bank during the night. The nematode *P. acuminatus* was reared on 1% proteose pepton agar with *A. johnsonion* as the food-source. The nematodes were collected in tap water by letting them crawl through a cotton filter during the night and were concentrated by precipitation. The concentration was determined by counting and adjusted if necessary. The containers were filled for three-quarter with the soil, subsequently the nematodes were added and covered with the remaining quarter of soil. The final moisture content of the soil with the nematodes in the container approached the original wet weight of the soil (the volume of yeast extract was adapted to achieve this). The containers were closed with gauze discs with a mesh size of 20 µm. Because in the field test in Wageningen a few other nematode species were recovered as well, gauze discs with a mesh size of 10 µm were applied in the first transplantation experiments in Avonmouth. The container holders were loosely wrapped into plastic foil to prevent dehydration and kept at 15-20°C until use.

2. For assessing HSP60 induction in nematodes first suitable methods had to be selected and evaluated. With gel electrophoresis techniques (2D-PAGE) the expression of HSP60 in *P. acuminatus* could be measured (Kammenga et al. 1998). However, the results with these techniques were not very reproducible and HSP60 antibodies were not specific for nematodes. These problems might be solved by exploiting a RT-PCR with specific primers for nematode HSP60. Therefore, first specific primers had to be designed. Genomic DNA of *P. acuminatus* was multiplied using PCR with degenerated primer-sets based on conserved parts in known HSP60 sequences of a number of organisms. The resulting fragments were cloned and sequenced. One fragment matched with a very high probability to a HSP60 nucleotide sequence of the nematode *Caenorhabditis elegans* and other eukaryotic organisms using BLAST (Altschul et al. 1990). Subsequently, specific primers were designed based on the sequence of that fragment and checked on genomic DNA of *P. acuminatus* and *A. johnsonion*. To isolate mRNA, a Dynabeads kit (ITK Diagnostics) was applied and its protocol adjusted. Frozen nematodes (in 20 µl tap water at -80°C or liquid nitrogen) were grounded in a specially designed glass potter in lysis buffer. After centrifugation, the pellet was kept at -20°C for protein determination to estimate the relative number of nematodes present in the sample. To serve the same purpose the supernatant was kept

at -20°C after mRNA extraction. cDNA was synthesized by Superscript-II (Life Technologies) following the normal procedures and multiplied with PCR using the specific HSP60 primers.

Results and discussion

1. The results of the first transplantation experiments in Avonmouth were very disappointing: the mini-containers contained many other nematode species. The system was leak, although this had not appeared in the pilot studies in the Netherlands. New experiments were performed applying membranes instead of gauze. If the containers were filled with nematodes and tap water, closed with the membrane and incubated in tap water during the night, nematodes still escaped. Since these nematodes could not pass straight through the membrane itself, they must have passed through the edge where the membrane touches the container. Therefore, in new experiments the membrane was fixed to the container with 2-component glue. This glue sustained incubation conditions (both in soil and in water) and nematodes did not escape anymore nor entered the containers from the surrounding environment. The type of membrane used was also very important in the transplantation experiments. Dialysis membrane appeared to be unsuitable for longer incubation periods in Avonmouth, probably due to microbiological breakdown of this membrane. Nylon membranes (e.g. Nytran 0,45 µm (Schleicher & Schuell)) were suitable for all incubation periods. Therefore it was decided to work only with nylon membranes for transplantation experiments.

The second transplantation experiments in Avonmouth succeeded. So nematodes could successfully be transplanted using the mini-container test kit if suitable conditions were provided. Recovery of the nematodes from these containers was under normal circumstances approximately 50% of the original nematode concentration by direct recovery and 25% by letting them crawl through a filter during the night. The experiments in Avonmouth showed that the number of recovered nematodes greatly varied between the different containers within one field site and between the different field sites. The number of nematodes recovered from the most polluted site was significantly lower than from the other sites.

2. HSP60 mRNA concentrations in *P. acuminatus* could be measured exploiting RT-PCR by using primers that are specific for *P. acuminatus* HSP60 and the method described before. No reaction was observed in samples containing everything except *P. acuminatus*, in samples containing *P. acuminatus* that had not been ground and samples containing only *A. johnsonion* DNA. In samples containing *P. acuminatus* that had been ground, a reaction was always observed. Even nematodes that had not been exposed (controls) often reacted although to a lesser extent than the exposed ones. This could be explained by the fact that HSP60 is always present in cells, but the concentration rises under stress conditions. In the experiments a kind of dose-response relationship could be observed (Figure 1).

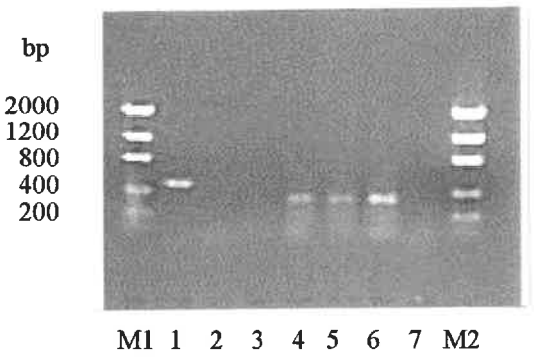


Figure 1. PCR results using specific primers for HSP60 in *P. acuminatus*. M1 = Mass ladder (2 µL); 1 = *P. acuminatus* DNA; 2 = *A. johnsonion* DNA; 3, 4, 5, 6, 7 = *P. acuminatus* (± 500) RT-PCR after 2 hours of exposure to $CdCl_2$: 3= 0 mg $CdCl_2$ /L; 4= 0.07 mg CdCl2 /L; 5= 0.7 mg $CdCl_2$ /L; 6= 7 mg $CdCl_2$ /L; 7= 7 mg $CdCl_2$ /L without grinding; M2= Mass ladder (4 µL).

For a reliable interpretation of the results, the method should at least be semi-quantified.

References

Altschul S.F., Gish W., Miller W., Myers E.W. and Lipman D.J. (1990) Basic local alignment search tool. *J. Molec. Biol.* 215: 403-410.

Kammenga J.E., Arts M.S.J. and Oude-Breuil W.J.M. (1998) HSP60 as a potential biomarker of toxic stress in the nematode *Plectus acuminatus*. *Arch. Environ. Contam. Toxicol.* 34: 253-258.

PART B. DETAILED REPORT OF THE CONTRACTORS

Contractor: Eberhard-Karls-University, Department of Cell Biology, Zoological Institute.

Leading scientist: Dr. H.-R. Köhler
Scientific staff: Dr. H. Eckwert, Dipl. Biol. I. Einfeldt, Drs T. Knigge, Dr. H.-R. Köhler.

Address: Auf der Morgenstelle 28 D-72076 Tübingen, Germany

Telephone: +49 7071 297 8864
Fax: +49 7071 294 634
E-mail: Heinz-r.koehler@uni-tuebingen.de

I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To check the influence of the confounding factors temperature and pH on the HSP70 induction by metal exposure in slugs (*Deroceras reticulatum*).

2. To check the transiency of this response to elevated metal concentrations by continuous exposure and recovery experiments.

3. To quantify the stress reaction (HSP70) in diplopod (*Julus scandinavius*) and isopod (*Oniscus asellus*, *Porcellio scaber*) samples taken from several woodland sites in Southern Germany and a metal gradient near Avonmouth, UK.

II. OBJECTIVES FOR THE NEXT PERIOD:

1. To study the influence of metal cocktails (mixture toxicity) on the HSP70 induction in slugs (*Deroceras reticulatum*).

2. To repeat the analyses for HSP70 in isopods (*Oniscus asellus*, *Porcellio scaber*) taken in 1998 from field sites in the metal gradient near the smelting works at Avonmouth, UK.

3. To model predictability of real HSP70 results obtained in the field using metal concentration and confounding factors as variables.

III. MAIN RESULTS OBTAINED:

Methodology

1. Laboratory experiments: Adult laboratory reared slugs were exposed to food (carrot slices and lettuce leaves, sprinkled with $CaCO_3$ particles) soaked with a solution of either 0.15 mg Cd/L (resulting in 3.3 mg Cd/kg dry wt.) or 1.5 mg Cd/L (resulting in 33.0 mg Cd/kg dry wt.) both as $CdCl_2$ for 1 hour. The exposure experiments lasted from 10 to 25 days which were followed by a recovery period of up to 28 days where the animals were fed non-contaminated food. Experiments were conducted at either 5°C, 10°C or 15°C, or at pH 5 or pH 8.5, respectively, to reveal the confounding effect of different temperature and pH.

Samples (whole animals) were frozen in liquid nitrogen and analyzed for HSP70 using a standardized one-dimensional Western blotting technique. The internal reference was the total protein concentration in the supernatant of the homogenate. Protein amounts (50 µg per lane, determined by Bradford's assay) were subjected to SDS-PAGE, transferred to nitro-cellulose and stained with a combination of a monoclonal mouse anti-human HSP70 antibody and a secondary peroxidase-coupled goat anti-mouse IgG antibody. Subsequent quantification of the grey values of the Western-blot bands was conducted by densitometric image analysis. The mean grey value of control specimens was set arbitrarily to 1.00 as a standard reference. Based on this, relative grey values were calculated for all other treatments.

2. Field studies: Adults of the diplopod *Julus scandinavius* and the woodlice *Oniscus asellus* and *Porcellio scaber* were sampled in (a) a metal gradient near the smelter works at Avonmouth, UK, and (b) differently polluted field sites in Southern Germany. Highest metal pollution (zinc, lead, cadmium) was revealed for a former opencast mining area near Wiesloch south of Heidelberg. Specimens were frozen in liquid nitrogen directly in the field and analyzed for HSP70 as described above.

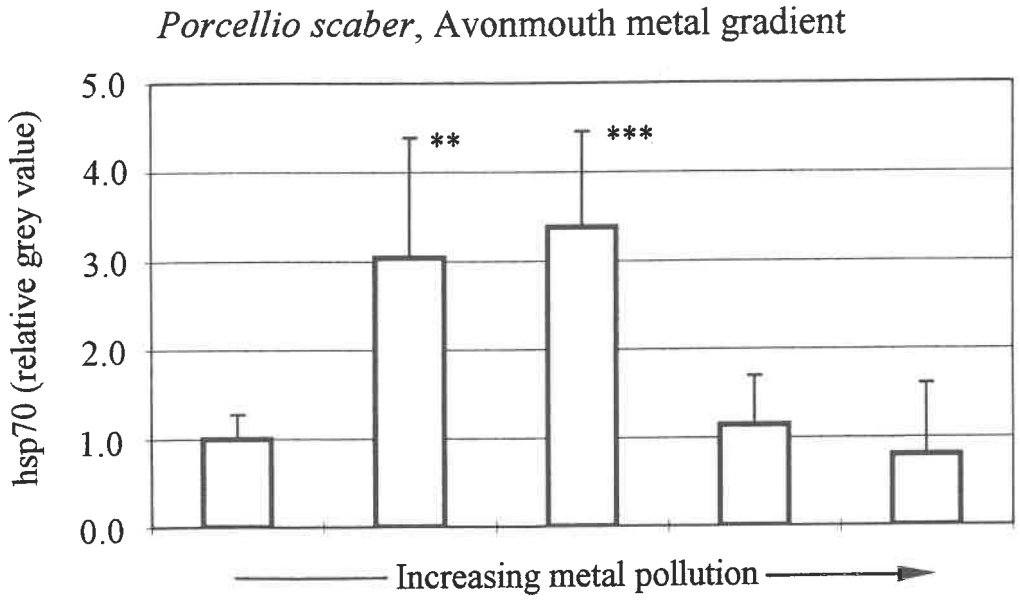
Results and discussion

1. Temperature and pH as confounding factors and transiency of the stress response: A concentration of 3.3 mg Cd/kg did not induce the HSP70 level in *Deroceras reticulatum* after 20 days of exposure at 5°C. Also 10°C did not influence the HSP70 level, either applied in combination with 3.3 mg Cd/kg or without. In contrast, constant maintenance of the slugs at 15°C was found to induce HSP70 by the 1.7 fold compared to the HSP70 level at 10°C. A pH of 8.5 did not induce HSP70 neither alone nor in combination with 3.3 mg Cd/kg. In contrast, pH 5 elevated the HSP70 level significantly to 155% of the control level after 10 days of exposure when combined with 3.3 mg Cd/kg in the food. A pH of 5 alone did not have any significant effect.

A concentration of 33.0 mg Cd/L in the food's soaking solution resulted in an elevated HSP70 level of about 2.5 fold of the control after 25 days exposure. Although after 10 days of exposure, a significant induction of HSP70 was not evident, the stress protein level raised in the subsequent 15 days independent from maintenance of the toxic conditions: a slug group which was fed non-contaminated food subsequent to 10 days of cadmium exposure reached the peak of HSP70 induction 15 days after cadmium removal. After longer recovery periods, the HSP70 level

decreased again whereby this decrease could be gradually related to the intensity of the preceded exposure.

2. Field studies: The HSP70 level in isopods taken from the Avonmouth metal gradient increased with decreasing distance to the smelter works with the exception of the stands which were closest to the mine. In "resident" animals from these stands, the HSP70 level was not significantly elevated in comparison to the controls which can be taken as to be indicative for the development of metal tolerance in these populations (Figure 1). Also the different metal burdens in the soils of the investigated stands in Southern Germany mirrored the results obtained for woodlice taken from the metal gradient near Avonmouth. A correlation of the lead and zinc concentrations in soil versus the HSP70 level could be found for all stands except for a military area (which presumably is influenced by pollutants other than the analyzed heavy metals) and several long-term polluted sites, e.g. in the vicinity of a former opencast mine (Figure 2). There was a significant dependence of the HSP70 level and (a) soil pH, and (b) the concentration of water-soluble lead and zinc in soil for all investigated sites (except for the military area).



mean soluble					
zinc [mg/L],	0.02	18.10	71.52	238.84	380.82
cadmium [mg/L]	0.00	0.51	1.99	5.72	9.71
lead [mg/L]	0.01	0.00	1.84	2.16	2.36

Figure 1. Mean HSP70 level ± sd in *Porcellio scaber* collected in the Avonmouth metal gradient. The control (set to 1.0) derived from an unpolluted stand in Germany. Data on metal concentrations were provided by M. Donker, Vrije Universiteit, Amsterdam (participant 6).

The results obtained from the laboratory experiments so far showed that there is little or no influence of temperature between 5 and 10°C on the HSP70 response in soil invertebrates. Since these animals tend to migrate into deeper soil horizons when air temperature rises in summer or around noon, they are mostly exposed to the average temperature in Central European soils of 7-8°C. The experiments on persistence of the stress response in time made evident that the HSP70

level integrates over weeks, possibly due to the longevity of "active" metal species which have been taken up by the animals. There was an influence of the confounding factor "acidity" (low pH), which is known to lead to increased mobility (water-solubility) of the applied cadmium which, subsequently, may have caused a significant elevation of the HSP70 level in response to an otherwise irrelevant cadmium concentration. These results were corroborated by the field investigations. The feasibility of the marker HSP70 to provide information on toxic conditions was proven by the concentration-response relationship obtained for numerous diplopod and isopod populations. Results from long-term exposed populations, however, deviated from this trend both in German and UK stands and led to the suggestion that adaptation mechanisms have evolved in these areas. Additional findings on survival, food selection and uptake, and on stress protein induction in presumably "metal-tolerant" soil invertebrate populations under artificial metal exposure support this interpretation.

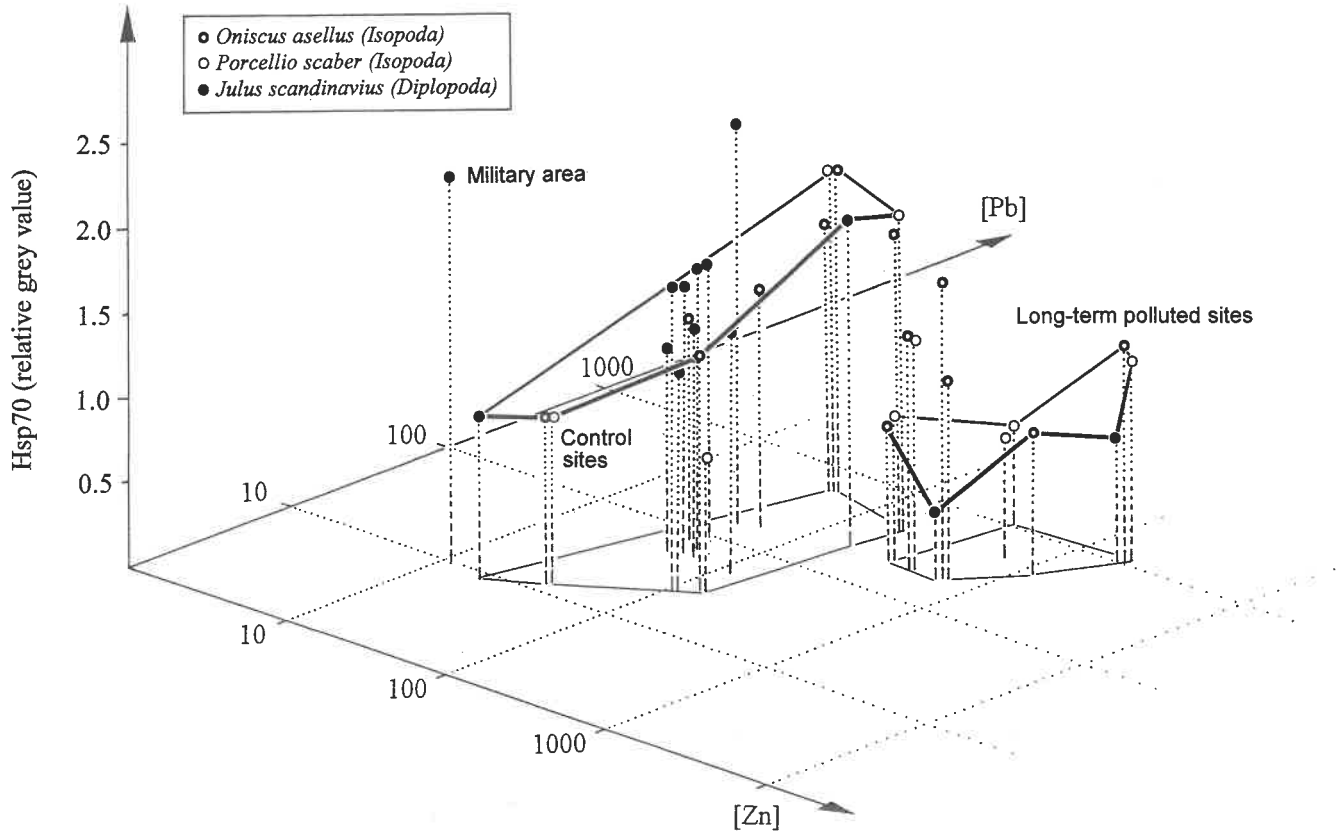


Figure 2. Mean HSP70 level in different populations of *Julus scandinavius*, *Porcellio scaber* and *Oniscus asellus* taken from field sites in Southern Germany. Stress response was related to the concentration of lead and zinc only; it should be mentioned that also high concentrations of cadmium are present in the long-term polluted mining sites.

PARTNERSHIP

1. Dr J.E. Kammenga
Department of Nematology
Wageningen Agricultural University
Binnenhaven 10
6709 PD Wageningen, The Netherlands
Tel. +31 317 482998/482197
Fax +31 317 484254
E-mail Jan.Kammenga@medew.nema.wau.nl

2. Dr H.-R. Köhler
University of Tübingen
Zoological Institute, Department of Cell Biology
Auf der Morgenstelle 28
D-72076 Tübingen, Federal Republic of Germany
Tel. +49 7071 296949
Fax +49 7071 294634
E-mail Heinz-r.koehler@uni-tuebingen.de

3. Dr R. Dallinger
Universität Innsbruck
Institut für Zoologie, Abteilung Zoophysiologie
Technikerstraße 25,
A 6020 Innsbruck, Austria
Tel. + 43 512 507 6182
Fax + 43 512 507 2930
E-mail Reinhard.Dallinger@uibk.ac.at

4. Dr V. Simonsen
National Environmental Research Institute
Department of Terrestrial Ecology
Vejlsovej 25, PO Box 314,
DK-8600 Silkeborg, Denmark
Tel. +45 8920 1400
Fax +45 8920 1413
E-mail: vs@dmu.dk

5. Dr J.M. Weeks
Institute of Terrestrial Ecology, NERC
Monks Wood, Abbots Ripton,
Huntingdon PE17 2LS, United Kingdom
Tel. +44 1487 773 381
Fax +44 1487 773 467
E-mail: J.weeks@ite.ac.uk

6. Dr M.H. Donker
Department of Ecology and Ecotoxicology
Vrije Universiteit
De Boelelaan 1087
1081 HV Amsterdam, The Netherlands
Tel. +31 20 444 7078
Fax +31 20 444 7123
E-mail: Donker@bio.vu.nl

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In a second experiment the effect of exposure time on the MT expression was studied. It is known that the Cd concentration of *O. cincta* reaches its equilibrium within three weeks of exposure. Therefore we expect that the amount of MT will also reach its equilibrium after three weeks. Springtails were exposed to cadmium for a three months period and after 1, 2, 4, 6, 8 and 12 weeks animals were collected to measure the amount of cadmium bound MT. It seems that the amount increases from week 1 to week 4 and then stabilizes. The only exception is the MT concentration of the animals after 12 weeks, at this point we again find an increase of the MT concentration.

In a third experiment springtails were exposed at 4 different temperatures 10, 13, 16 and 18 degrees Celsius. All animals were exposed for three moulting intervals. We calculated physiological time t for the different temperatures and exposed all animals for the same physiological time. The data show that the cadmium concentration of the animals is not temperature dependent although the MT concentration decreases at higher temperature.

The presence of cadmium bound MT is demonstrated in springtails collected near Avonmouth. The MT concentration is well related to the $CaCl_2$ exchangeable cadmium concentration of the soil. The experiments running at this moment will show if the expression found in field animals is comparable to the expression found in laboratory cultured animals placed on Avonmouth litter and soil.

It will remain difficult to establish the real exposure of the animals in the field because of few reasons. The bioavailability of metals is not known although we tried to estimate it by determining water soluble and $CaCl_2$ exchangeable metal concentrations in the soil samples. Secondly it is difficult to establish the role of the different uptake routes for the springtails. Thirdly we do not know if the animals are genetically or physiologically adapted in the field and therefore may have a different expression level than animals without a history of metal exposure. Although there are some questions left to be answered at this moment we think that the MT expression of this springtail is a promising biomarker.

The results of our laboratory studies proved that the MT expression of the springtail *O. cincta* is linearly related to its exposure level until the lethal level is reached. The same has been found in the gastropod *Helix pomatia* (dr. R. Dallinger, participant 3) and in the nematode *Caenorhabditis elegans*. Since the amount of MT was positively related to the exposure level of the animal, the amount of Cd bound MT may be a strong biomarker for environmental cadmium exposure. This result is in good agreement with findings in other animals (Hamer 1986).

The quantitative expression of the MT bound cadmium was further examined. Apart from cadmium, one should expect that MT induction may also occur following exposure to other metals or changing physiological conditions. The temperature dependent expression was studied and we found that the MT concentration decreases at higher temperatures, probably due to an higher turn-over rate of the protein. These results can be used to "recalculate" the expression level for a known field temperature. From our studies on time dependent MT expression it appears that after three weeks of metal exposure the MT expression reaches its equilibrium. The only exception to this rule is the increased MT level after 12 weeks of cadmium exposure but this exception may be explained by the presence of juvenile springtails.

We think that the data collected show that the MT concentration in springtails may be a promising biomarker because the transiency of its expression and the good dose-response relationship. The disadvantage of the measurement of MT in springtails is that you need at least 100 individuals for one sample but this problem may be solved by the development of quantitative PCR techniques to quantify the amount of mRNA instead.

PART B. DETAILED REPORT OF THE CONTRACTORS

Contractor: Institut für Zoologie und Limnologie, Universität Innsbruck

Leading scientist: Dr. R. Dallinger
Scientific staff: Dr. R. Dallinger, B. Lagg, Mag. C. Gruber, Mag. C. Martin

Address: Institut für Zoologie und Limnologie der Universität Innsbruck, Technikerstraße 25, A-6020 Innsbruck

Telephone: +43 512 507/6182
Fax: +43 512 507/2930
E-mail: Reinhard.dallinger@uibk.ac.at

I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To completely characterize the new MT from *Eisenia fetida*, to develop an appropriate quantification assay, and to test the suitability of the protein as a biomarker for environmental exposure.

2. To find out biological and toxicological parameters to be related to the ratio of MT pools (Cd-MT pool and Cu-MT pool) in *Helix pomatia*.

3. To complete our studies on the suitability of snail and earthworm MTs as biomarkers under field conditions.

II. OBJECTIVES FOR THE NEXT PERIOD:

1. To develop a metal saturation assay for quantitative detection of earthworm MTs.

2. To develop a quantitative approach for measuring of earthworm MT mRNA in response to metal exposure.

3. To modify our metal saturation assay for quantification of the novel Cu binding MT isoform in the snail's midgut gland.

4. To complete our studies on quantitative measurements of snail MT mRNA.

5. To validate our results on snail and earthworm MT quantification under field conditions.

III. MAIN RESULTS OBTAINED:

Methodology

1. Earthworms (*Eisenia fetida*)
For characterisation and isolation of MT isoforms in *E. fetida*, methods of gel permeation, ion exchange chromatography and Reversed-Phase HPLC were applied. The isolated MT was S-methylated and desalted, and peptide maps were obtained by cleaving the whole protein with trypsine, as well as with endoproteinases Glu-C and Asp-N. Sequences of produced peptides were resolved by automated Edman degradation and collision-induced tandem mass spectrometry, and masses of whole protein and peptides were detected by electrospray mass spectrometry. A saturation assay is currently being developed and adopted to detect *Eisenia* MT isoforms quantitatively under both laboratory and field conditions.

2. Terrestrial snails (*Helix pomatia* and *Arianta arbustorum*)
In *H. pomatia*, an additional Cu-binding MT isoform was recently isolated and characterized by means of chromatographic techniques from the midgut gland of copper-loaded snails. After removal of copper from the protein with ammonium-tetrathiomolybdate, the apo-MT was S-methylated and characterised by collision-induced tandem and electrospray mass spectrometry. We are currently testing a metal saturation assay also to detect this Cu-specific isoform quantitatively from the midgut gland.

Apart from this, we have continued our studies on toxicological parameters related to the ratio of different MT pools, namely the Cd-MT pool in the midgut gland and the Cu-MT pool in the mantle of snails (*H. pomatia* and *A. arbustorum*). We are now able to quantify these different metal- and organ-specific MT pools by applying metal-specific saturation assays.

We have also carried out a series of field studies with individuals of *H. pomatia* and *A. arbustorum* from differently contaminated sites in Austria to relate the concentrations of metal-specific MT pools in the snail organs to the degree of environmental pollution. It appeared that the field tests were in agreement with the previously found metal-MT relationships established in the laboratory, although interpretation of data is still difficult.

Results and discussion

1. Earthworms (*Eisenia fetida*)
Three MT isoforms were characterized from *E. fetida* by their primary sequence (Gruber et al. 1998). The main MT variant, which is found at highest concentrations in exposed earthworm tissues, predominantly binds cadmium and minor amounts of copper. The synthesis of this protein is inducible by cadmium exposure, as shown by semi-quantitative chromatographic induction studies. The isoform is composed of 41 amino acids and shows the following amino acid sequence:

```
1                 10                  20                  30                  40
D T Q C C G K S T C Q R E G S T C C C T N C R C L K S E C L P G C K K L C C A D A
```

Due to this primary structure, the *E. fetida* MT can be regarded as a class II MT (Kojima 1991). The metal-free protein has a molecular mass of 4602 Da and contains 12 cysteine residues

forming two double and one triple Cys motifs. The MT binds 4 cadmium atoms per molecule, which corresponds to a co-ordination stoichiometry of 4 sulphur atoms per metal ion, assuming a tetrahedral co-ordination with probably four of the involved sulphur atoms bridging between the four cadmium ions. Two additional MT isoforms found in *E. fetida* show a high degree of homology in comparison to the major variant, showing only a few exchanged amino acid positions.

Apart from these cadmium-binding MT isoforms, one additional component was found which predominantly binds copper. Its amino acid sequence has not been determined yet.

2) Terrestrial snails (*H. pomatia* and *A. arbustorum*)

It has been stated in our last report (Kammenga et al. 1997) that the high versatility of the MT biomarker system in terrestrial snails is based on the metal and organ specificity of different isoforms (Dallinger et al. 1998a). These isoforms are apparently devoted to different tasks: a cadmium-binding variant in the snail's midgut gland is responsible for the detoxification of cadmium, whereas a copper-binding isoform in the mantle tissue is involved in the regulation of copper, possibly in connection with hemocyanin synthesis (Dallinger et al. 1997). The different MT isoforms can specifically be detected and quantified by metal saturation assays (Dallinger et al. 1998b).

Only very recently, an additional Cu-binding MT isoform was isolated and characterized from the midgut gland of copper-loaded individuals of *H. pomatia*. This latter isoform is identical to the above-mentioned Cu-binding isoform from the mantle tissue of the same species. In contrast to the mantle protein, however, the midgut gland copper-isoform is highly inducible by copper, binding all of the metal present in this organ after copper loading. This means that we have, to date, isolated and characterized three organ- and metal-specific MT isoforms from *H. pomatia*: One cadmium-specific isoform from the midgut gland (Dallinger et al. 1993); one copper-specific isoform from the mantle (Berger et al. 1997); and recently, one copper-specific isoform from the midgut gland of copper-loaded Roman snails. Cd-binding MT isoforms were also characterized from the midgut gland of another helicid species, *A. arbustorum* (Berger et al. 1995).

We are currently developing a method to quantitatively detect the novel copper-binding MT isoform from the midgut gland by a modified metal saturation assay, in order to test whether quantification of this protein can be used as a specific tool for copper pollution in our biomarker studies.

We have also obtained interesting results by applying our biomarker approach with terrestrial snails to field conditions. So far, we have tested our method in individuals of *H. pomatia* and *A. arbustorum* from differently polluted sites in Austria. These sites include uncontaminated areas (Thaur and Rum near Innsbruck, Tyrol), moderately contaminated localities (Innsbruck city and Matrei near the Brennerpass, Tyrol), as well as highly polluted sites (Arndoldstein, Carinthia, and Brixlegg, Tyrol). The data which are presently available suggest that the biomarker model with MT isoforms might also work under field conditions, although the results obtained in this case are not easily available to correct interpretation. It will be interesting in the future to test whether the biomarker approach can also be applied to the novel copper-specific isoform discovered in the snail's midgut gland.

The elucidation of the primary structure of *E. fetida* MTs (see above) demonstrates that at least some earthworm species do indeed possess true MTs. However, due to their low molecular size and their primary structure with only 12 cysteine residues present, earthworm MTs must be regarded as class II MTs (Kojima 1991). This is also suggested by the primary structure of

another earthworm MT, which has very recently been resolved by a molecular approach in *Lumbricus rubellus* (Stürzenbaum et al. 1998). In both species, MTs are inducible by cadmium exposure. This indicates that the MT system in earthworms is highly responsive to environmental pollution by metals. Hence, earthworm MTs can be regarded as true candidates for a biomarker approach in these animals.

At least for *E. fetida* MTs, the metal stoichiometry is well known (see above). This allows to apply metal saturation assays for their quantification, as already shown before in terrestrial gastropods (Berger et al. 1995a). In order to work, however, each metal saturation assay has to be modified for quantification in homogenate preparations of a given animal species. This will be the task of our future work with *E. fetida* MTs during the next period of this project.

It was stressed in our last report (Kammenga et al. 1997) that the strength of the biomarker approach with MTs in terrestrial gastropods lies in the organ- and metal-specific response and quantification of different isoforms. This isoform-specific approach can now also be applied to the newly discovered copper-binding isoform from the snail's midgut gland. In contrast to the copper-MT present in the mantle tissue, the midgut gland isoform is highly responsive to copper exposure. This fact makes the new isoform a biomarker candidate for environmental pollution with copper. Thus, our future investigations will focus on the question whether and to which degree the copper-binding MT isoform from the midgut gland of Roman snails can be used as biomarker molecules for copper exposure. The same question holds for the second species under investigation, *A. arbustorum*: does this helicid species also possess a copper-responsive MT isoform in its midgut gland, and, if so, can it be used too as a biomarker molecule?

Goals which have not been achieved

Our work on quantification of earthworm MT mRNA is only at the beginning, but we hope to obtain reliable results with this method until the end of the project period. Quantitative measurements of MT mRNA in snails are also still not conclusive.

Our work of testing the established biomarkers of snails and earthworms under field conditions is on a good way. However, we expect to be able to correctly interpret our results only towards the end of this project period. The reason for this lies in the variability and complexity of interfering factors which are encountered under field conditions.

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PART B. DETAILED REPORT OF THE CONTRACTORS

Contractor: National Environmental Research Institute, Terrestrial Ecology

Leading scientist: Dr. V. Simonsen
Scientific staff: Dr. V. Simonsen, Dr. J. Scott-Fordsmand, technician A. Christiansen

Address: Vejlsøvej 25, P. O. Box 314, DK-8600 Silkeborg, Denmark

Telephone: + 45 89 20 14 00
Fax: + 45 89 20 14 13
E-mail: vs@dmu.dk

I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To continue the study of the transciency of the response of esterases in laboratory cultures of Collembola (*Folsomia candida*).

2. To do electrophoresis on esterases from the earthworm, *Eisenia fetida*, kept in copper contaminated soil from Hygum at Vejle, Denmark, and in spiked control soil from the same location.

3. To do electrophoresis on esterases of various invertebrates from Avonmouth.

4. To determine copper and zinc content in the earthworm, *Lumbricus rubellus*, from Avonmouth.

II. OBJECTIVES FOR THE NEXT PERIOD:

1. To determine the activity of esterases from various invertebrates by densitometry.

2. To do further analyses on invertebrates from Avonmouth with the emphasis on the earthworm, *Lumbricus rubellus*.

3. To write up a protocol for the use of esterase, determined by electrophoresis and densitometry, as a tool for detecting contaminated areas.

III. MAIN RESULTS OBTAINED:

Methodology

1. The experiments with *F. candida* as a test organism were continued. Experiments with copper and zinc were conducted as the previous described experiments (Simonsen 1997). Due to problems with the software for the scanning device, only the electrophoretic analyses were performed and the determination of the activity of esterases, the densitometric measurements, will be done during the autumn 1998.

2. Due to problems with the culture of *L. rubellus*, *E. fetida* was applied for the laboratory experiments in soil from Hygum, in the autumn 1997 and in the spring 1998. Two types of experiments were conducted, one in control soil spiked with $CuCl_2$ and the other in soil with the natural contamination. The worms were exposed to the soil for 3 weeks at 20°C with 12 hours of light and 12 hours in dark. In total 118 individuals were analysed. The electrophoretic procedure was as previously described (Simonsen 1995) and the densitometry will be performed during the autumn 1998.

3. Two collecting trips to Avonmouth, one in the autumn 1997 and one in the spring 1998, were undertaken. The earthworm *L. rubellus* and the isopods *Oniscus asellus* and *Porcellio scaber* were collected in the autumn 1997, see Table 1, and again in the spring 1998, number of individuals not shown.

Table 1. Invertebrates collected at Avonmouth, autumn 1997. Values are numbers of individuals analysed.

Location	Species		
	L. rubellus	*O. asellus*	*P. scaber*
1	8	15	
2	10	8	
2A	8	16	
3	10	11	3
4	10	5	10
5	5		10

The determination of esterases from *L. rubellus* and *P. scaber* were analysed according to previous described method (Simonsen 1995) and a corresponding method was developed for *O. asellus*. As the other experiments the densitometry will be done in the autumn 1998.

4. Copper and zinc content in *L. rubellus* were determined by AAS according to Hopkin (1989).

Results and discussion

1. The results of the experiments with *F. candida* are summarized in Table 2 and so far only visually evaluation of the activity of esterases has been performed.

Table 2. List of results from *F. candida*.

Treatment	Yeast eaten	Dead animals among 30	Eggs	Activity of esterases
0.08 M $CuCl_2$ and 10°C	All	1	Eggs	Reduced
0.08 M $CuCl_2$ and 15°C	All	0	Eggs	Reduced
0.08 M $CuCl_2$ and 20°C	All	0	Eggs	Slightly reduced
0.08 M $CuCl_2$ and 26°C	All	2	Eggs	Reduced
0.08 M $CuCl_2$ and pH = 4	All	2	Eggs	Reduced
0.08 M $CuCl_2$ and pH = 5	All	2	Eggs	Reduced
0.08 M $CuCl_2$ and pH = 6	Some left	0	Eggs	Slightly reduced
0.08 M $CuCl_2$ and pH = 7	All	1	Eggs	Normal
0.05 M $ZnCl_2$ and 10°C	All	3	Few eggs	Reduced
0.05 M $ZnCl_2$ and 15°C	All	2	Eggs	Reduced
0.05 M $ZnCl_2$ and 20°C	All	1	Eggs	Normal
0.05 M $ZnCl_2$ and 26°C	All	0	Eggs	Slightly reduced
0.05 M $ZnCl_2$ and pH = 4	All	0	Eggs	Reduced
0.05 M $ZnCl_2$ and pH = 5	All	0	Eggs	Reduced
0.05 M $ZnCl_2$ and pH = 6	All	0	Eggs	Slightly reduced
0.05 M $ZnCl_2$ and pH = 7	All	0	Eggs	Slightly reduced

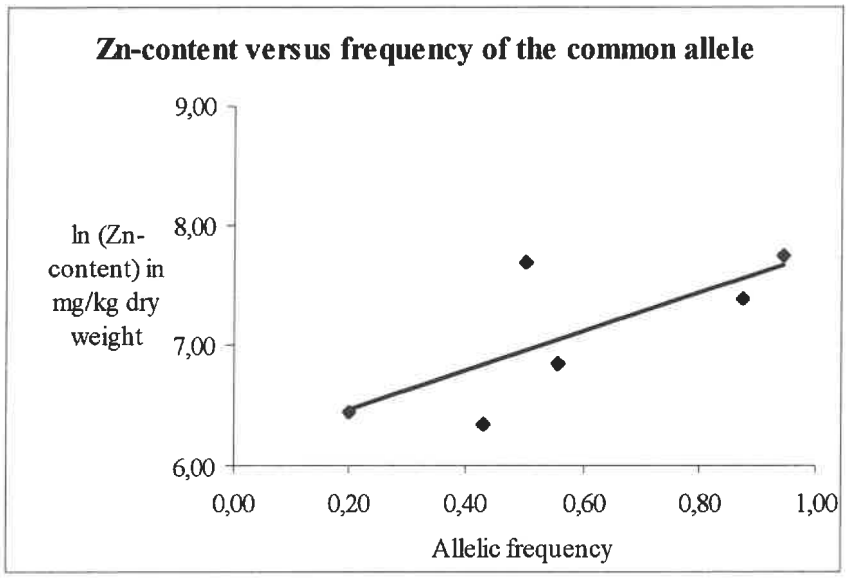
When the densitometry has been performed, it will be possible to quantitatively detect if a combined effect of temperature or pH and a metal causes a greater reduction of the activity than each of them.

2. Like the observations done on *L. rubellus*, exposed to soil from Hygum, also *E. fetida* showed some reduction in the activity, but at the high concentration very little reduction. However, also for these experiments the densitometric analysis has to be carried out.

3. A polymorphism was detected in the esterase from *L. rubellus*, which was not seen in the previous experiment with *L. rubellus* (Simonsen 1997). The genetic interpretation of the esterase was that the esterase was determined by one locus with two codominant alleles. A linear regression on the frequency of the overall most common allele and the content of zinc in the earthworms was found, see Figure 1.

4. The content of copper and zinc in the worms was as expected, due to accumulation, significantly higher than in the soil, but no correlation between the content in worms and soil was found.

Figure 1. Zinc content in earthworms as a function of allelic frequency.



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PART B. DETAILED REPORT OF THE CONTRACTORS

Contractor: Institute of Terrestrial Ecology, NERC, Monks wood

Leading scientist: Dr J.M. Weeks
Scientific staff: Dr J.M. Weeks, C. Svendsen (MSc.), Dr E. Lenz, D. Foster, Dr D. Spurgeon, T. Olesen (MSc.), P. Hankard (BSc.)

Address: Institute of Terrestrial Ecology, NERC, Monks Wood, Abbots Ripton, Huntingdon, Cambridgeshire, PE17 2LS, UK

Telephone: +44 1487 773381
Fax: +44 1487 773467
E-mail: j.weeks@ite.ac.uk

I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To deploy purposely designed mesh bags containing earthworms (*Eisenia veneta*) along a metal-pollution gradient in the vicinity of the Avonmouth Metal Smelter (Avon, UK) and measure changes in lysosomal stability and immuno-activity in coelomocyte cells taken from whole earthworms.

2. To measure the significance of any changes in the locomotory behaviour of woodlice collected along the same contamination gradient as described above.

II. OBJECTIVES FOR THE NEXT PERIOD:

1. To further develop multivariate statistical techniques enabling interpretation of data generated from both ICP-MS elemental profiles of soil invertebrates, earthworms, and for locomotory activity measurements for woodlice collected from the field in the vicinity of the Avonmouth smelter site.

2. To consider the ecological relevance of any metabolic changes in earthworm biofluid collected along the contamination profile at Avonmouth using NMR and pattern recognition techniques.

3. Further attempt to link biomarker measurements from these deployed animals with more ecologically significant effects upon the structure or function of the soil invertebrate community using techniques such as soil respiration, microbial decomposition and measurements of feeding activity (bait lamina).

4. To consider and measure the influence of other abiotic and biotic influences on the lysosomal membrane of earthworm coelomocytes.

III. MAIN RESULTS OBTAINED:

Methodology

1. After establishing dose-response relationships and the immediate applicability under field conditions of two earthworm coelomocyte based biomarkers; neutral red retention and immuno-system activity in the laboratory, including the effects (non-recorded) of both biotic and abiotic confounding factors, studies were undertaken *in situ* using purposely constructed mesh containment bags. *Eisenia veneta* was placed directly into soil cubes (cut using a stainless steel box corer) at each site using the mesh containment bags. Three replicate bags were deployed (with 20 worms in each) for each location. After a 4-week deployment immune-system activity of the earthworms was determined by incubating earthworm coelomocytes with rabbit red blood cells. Lysosome membrane stability was measured (on the same earthworms) as the ability of the lysosomes in coelomocytes to retain neutral red dye. Concomitant metal body burdens will be measured in affected earthworms by ICP-MS from each of the collecting points to measure any elevation in body metal contents during the time of earthworm deployment.

2.Adults of the terrestrial isopod, *Oniscus asellus* were collected from five sites along the Avonmouth contamination profile, and twelve individuals from each site were tracked for four hours using a computer automated video tracking system (see Sorensen et al. (1997) for details of the system and tracking conditions). Initial examination of this data has failed to reveal any obvious changes in locomotory activity patterns. Thirty adult *O. asellus* were collected from the same sites during May 1998 for behavioural analysis. Data from this and the previous collection will be subjected to advanced statistical analysis in due course.

Results and discussion

1. Although effects of metals were dependent upon such variables as species, exposure parameters, including dose, route and duration, the conclusion reached in most immunotoxicological studies is that heavy metals act to suppress immunocompetence. Immunotoxicity of metals may occur via direct effects upon a specific immune system component or, alternatively, via inhibition of immunoregulation, which can result in immunosuppresion, hypersensitivity or autoimmune disorders. It has been postulated that metal toxicity may, at least in part, be due to autoimmunity, since autoimmune disorders exist for all of the major target tissues affected by heavy metals (Zelikoff and Thomas 1998). Both the neutral red retention assay (NRR) and the immune-system activity assay for the earthworms in this study responded significantly to the ambient pollution levels recorded in the soils for where the earthworms were deployed. The NRR assay results showed a clearer dose-response relationship and were more sensitive over those for the immuno-assay, however, immuno-competence was clearly reduced.

Lysosomes in general are susceptible to a wide range of physical and chemical stressors. In particular the integrity of the membrane is important to maintain an internally acidic pH (to preserve optimal enzyme activity) as well as to reduce the risk of cellular autodigestion, which could lead to severe cell damage or death. Today, in part due to our studies lysosomal membrane stability or fragility is widely accepted to be indicative of environmental quality. Recent advances in the field of cellular biomarkers have made use of the knowledge on lysosomal membrane fragility to develop the NRR assay. NRR monitors the ability of the lysosomes to retain the dye

(neutral red), and any premature diffusion is correlated to cellular stress. The NRR assay has been successfully applied in the toxicological assessment of marine mussels, freshwater snails and terrestrial worms. Although the structure and function of the lysosomal system is well described, little is known about the lysosomal membrane. Given that a leaky (*i.e.* stressed) will lead to an elevation of the crucial internal low pH, it can be hypothesised that a homeostatic mechanism will try to counteract this process by the up-regulation of lysosomal glycoproteins. Thus the lysosomal membrane is susceptible to pollutants in general, *i.e.* shows a non-specific response. Therefore it may be hypothesised that exposure to heavy metals results in lysosomal detoxification pathways being switched on. Hence increased detoxification requires increased quantities of lysosomal components, in particular those responsible for the maintenance of the lysosomal membrane, such as the membrane bound glycoproteins (Stürzenbaum 1997). Such studies will inevitably enable us to deploy biomarkers more reliably, as we become more aware of the limitation or likely confounding features that would occlude correct interpretation of such a response measured in the field.

2. There was considerable variation in locomotory parameters measured for woodlice collected from the same location, with a trend for greater inter-population differences at the different locations.

References

Stürzenbaum S. (1997) Molecular genetic responses of earthworms to heavy metals. *PhD thesis*, University of Wales, Cardiff.

Zelikoff J.T. and Thomas P.T. (1998) Immunotoxicology of environmental and occupational metals. Taylor and Francis, 361 pp.

Sorensen F.F., Weeks J.M. and Baatrup E. (1997) Altered locomotry behaviour in woodlice (*Oniscus asellus* (L.) collected at a polluted site. *Environ. Toxicol. Chem.* 16(4): 685-690.

PART B: DETAILED REPORT OF THE CONTRACTORS

Contractor: Vrije Universiteit Amsterdam

Leading scientist: Dr. M.H. Donker
Scientific staff: Prof. dr. N.M. van Straalen, Dr. C.A.M. van Gestel, drs. P.J. Hensbergen, M.J.M. van Velzen

Address: De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands

Telephone: +31 20 444 7078
Fax: +31 20 444 7123
E-mail: Donker@bio.vu.nl

I. OBJECTIVES FOR THE REPORTING PERIOD:

1. To relate the amount of metallothionein in *Orchesella cincta* to cadmium exposure level, duration of exposure and temperature of exposure.

2. To analyze metallothionein in different springtail species exposed under laboratory conditions.

3. To analyze metallothionein in different springtail species from metal polluted and non polluted locations.

II. OBJECTIVES FOR THE NEXT PERIOD:

1. To analyze the metallothionein MT expression in springtails collected in spring 1998 at Avonmouth and to compare these values with the data of animals collected in autumn 1997.

2. To expose laboratory raised springtails to soil and litter samples from the Avonmouth gradient and to determine the difference in MT expression between field and laboratory exposed animals.

3. To analyze metallothionein in different springtail species exposed under laboratory conditions. We started with the comparison of *Orchesella cincta* and *Orchesella villosa*, two common species both found at the Avonmouth gradient.

4. To develop quantitative PCR techniques to measure the MT mRNA as an even more susceptible biomarker for metal exposure.

III. MAIN RESULTS OBTAINED

Methodology

For quantifying the presence and the distribution of metallothionein (MT) in the springtail *Orchesella cincta*, two routes have been followed and modified; the biochemical and the molecular route. In the first route metal binding proteins were purified from cadmium exposed springtails. In the second route we identified the MT-gene. We were able to isolate two cadmium binding peptides from *O. cincta*. Mass spectromic analysis revealed that the molecular masses of these peptides were 2989 and 4139 Dalton (MALDI-TOF analysis in cooperation with R. van der Schors and K. Li of the Department of Molecular Neurobiology). Amino acid sequencing of the smallest peptide results in a sequence common for MT's (Figure 1). Using different PCR techniques with primers based on the identified amino sequence the cDNA was characterized (Figure 1). Surprisingly, within the open reading frame of the cDNA there were an additional 47 amino acids. From these results we conclude that the cadmium bound proteins we measure with our chromatographical techniques are two parts of a real MT although we do not know at what moment the protein degrades into two pieces. Since we use MT bound cadmium as a biomarker for metal exposure the instability of the protein forms no problem for quantifying the amount of MT present. Therefore we conclude that our isolation procedure as evolved *i.e.* two chromatographical separation steps (FPLC and HPLC) is sufficient to quantify the amount of cadmium bound MT.

5' ACTCTAAAAACTCAAGCAGCCCCAGAAAAAACTGTTTCATTTCAATCTCGCAGTTCT

TCAAATTCTCTAACGAAAACC ATG TCG TCA ACT CAA GCA TCC GCA TCT
Met Ser Ser Thr Gln Gly Ser Ala Ser

GAA GCC ATC AGA AAT TGC TTA TGT TGT GGC GAA AAT TGC AAA TGC
Glu Ala Ile Arg Asn **Cys** Leu **Cys** **Cys** Gly Glu Asn **Cys** Lys **Cys**

GGG GGT GCT GAA GGC AAA TCG CCC ACT TGT TGT AAA GAG AAA AAG
Gly Gly Ala Glu Gly Lys Ser Pro Thr **Cys** **Cys** Lys Glu Lys Lys

TGT TGT GGA GGA GGA GCA ACT CAA ACT GCA TCT TGT TGC ACT TGC
Cys **Cys** Gly Gly Gly Ala Thr Gln Thr Ala Ser **Cys** **Cys** Thr **Cys**

TGT GGT CCA GAT TGT GTC TGC AAG GAT GGA GCC AGC CTT CCT TGT
Cys Gly Pro Asp **Cys** Val **Cys** Lys Asp Gly Ala Ser Leu Pro **Cys**

TGT GCA AAC AAA ACA TGT TGT AAA TGA
Cys Ala Asn Lys Thr **Cys** **Cys** Lys

ACTTCCGTTAAACATTCTTACCTTATAAACCTTATAACCTTATAAACAATATTCGCCT

TCCTTCATCTAAAATAATTTTTTATGAATAAAATAATATAGACTTGATATTGCTACAT

GAATAACCGTTTCGTAAAACC$(A)_n$ 3'

Figure 1. cDNA and amino acid sequence of *Orchesella cincta* metallothionein. Outlined is the protein sequence of the 2989 D protein that has been determined at the protein level. Several starts of the poly-A tails were found marked by vertical lines.

Results and discussion

The MT concentration of *O. cincta* collected from the Avonmouth gradient showed a good relationship with the amount of exchangeable cadmium in the soil (Figure 2). The only exception is the MT concentration at site 4 but this site is exceptional in all animals investigated in this project. Therefore we think that some other factors dominate the expression of the stress proteins at site 4.

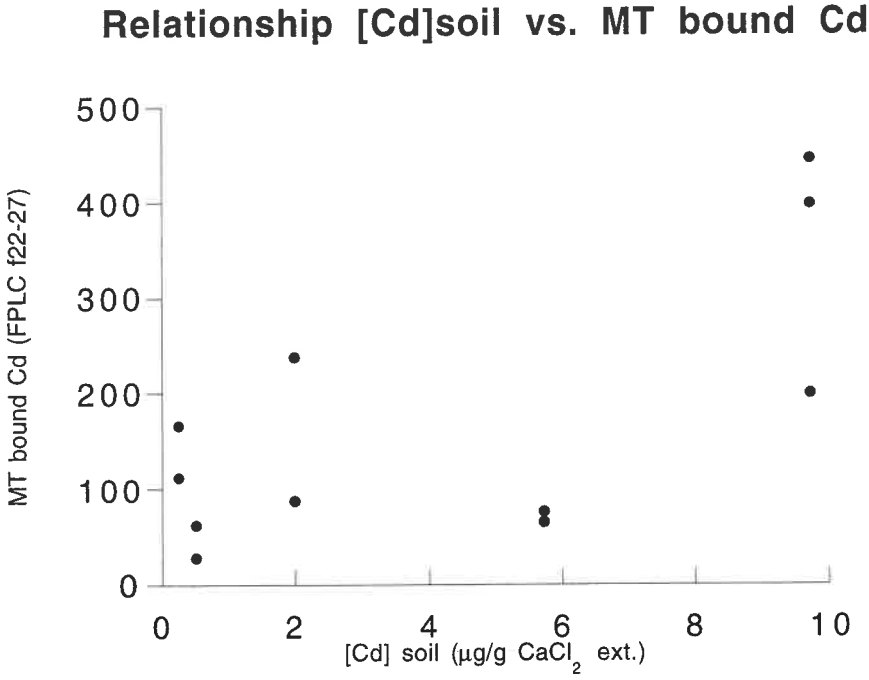

Figure 2. Relationship between the cadmium concentration of the soil and the amount of cadmium bound MT present in fraction 22 to 27 of the gel-permeation chromatography of *O. cincta* homogenates from animals collected along the Avonmouth gradient. On the horizontal axis the $CaCl_2$-exchangeable Cd concentration of the different Avonmouth sites is presented.

When the animals collected in spring 1998 are measured, more information will be obtained about the transiency of the found MT expression.

In the laboratory we investigated the applicability of MT in *O. cincta* as a biomarker for metal exposure by analyzing its dependence on time, temperature and concentration; a biomarker becomes applicable if:

1. the expression is concentration dependent
2. the MT expression is insensitive to other confounding factors such as time or temperature.
3. the biomarker response is related to a known adverse effect.

Therefore, apart from the biochemical and molecular work, physiological and toxicological experiments were performed to correlate the factors mentioned to the MT expression of *O. cincta*.

In a first experiment the concentration dependence of the MT-concentration in *O. cincta* was studied. This study showed that the MT expression is well related to cadmium exposure until the LC50 is reached. Knowing that this relationship is linear and that the dose effect relationship for cadmium in *O. cincta* is well known (Crommentuijn et al. 1994) we are able to relate MT levels to effect levels.