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# Cadmium accumulation and metallothioneins in some members of the soil fauna

**Dedicated to Prof. Dr. LUDWIG BECK on the occasion of his 65th birthday and retirement**

**Abstract**

The oligochaetes *Dendrodrilus rubidus* (intestine/chloragog), *Cognettia sphagnetorum* (whole specimens), and the gastropod *Arion subfuscus* (midgut gland) collected in the Egge Mountains (North Rhine-Westphalia, Germany) accumulated cadmium (Cd) above the level of that soil horizon they preferably live in. Cd was also detected in the fat body and ovarioles of several carabid species (*Carabus problematicus*, *Abax parallelipipedus*, *Pterostichus oblongopunctatus*). Seasonal variations were apparently dependent on the activity and reproduction of the species investigated. In some tissues of field collected *Carabus problematicus* (intestine, fat body, ovarioles)and of experimentally Cd-stressed *Lumbricus terrestris* (intestine/chloragog), *Enchytraeus albidus* (whole specimens), *Arion subfuscus* (midgut gland), but also in control tissues metallothioneins (MTs) could be detected. These proteins had a low molecular mass (6 to 11 kDa), a high Cd-binding capacity, a considerable amount of cysteine and a higher extinction at 254 nm compared to 280 nm. Cd-stress induced an additional synthesis of these proteins, which was roughly estimated using the cysteine content of the crude MT-fraction.

**Kurzfassung**

**Cadmium-Akkumulation und Metallothioneine in einigen Bodenorganismen**

Im Eggegebirge (Nordrhein Westfalen, Deutschland) gesammelte Oligochaeten *Dendrodrilus rubidus* (Darm-Chloragog-Gewebe), *Cognettia sphagnetorum* (ganze Tiere) sowie die Nacktschnecke *Arion subfuscus* (Mitteldarmdrüse) wiesen in den untersuchten Geweben Cadmium (Cd)-Gehalte auf, die höher als der von ihnen bevorzugte Bodenhorizont waren. Auch im Fettkörper und in den Ovariolen verschiedener Carabiden-Arten (*Carabus problematicus*, *Abax parellipipedus*, *Pterostichus oblongopunctatus*) war Cd nachzuweisen. Schwankungen des Cd-Gehalts im Verlaufe eines Jahres hingen offenbar mit der Aktivität und der Fortpflanzung der jeweiligen Organismen zusammen. In den Oligochaeten *Lumbricus terrestris* (Darm-Chloragog-Gewebe), *Enchytraeus albidus* (ganze Tiere) sowie in *Arion subfuscus* (Mitteldarmdrüse) und *Carabus problematicus* (Darm, Fettkörper, Ovariolen) waren in den untersuchten Organen vor und vor allem nach experimentell verursachtem Cd-Stress Metallothioneine nachzuweisen, die sich durch relativ niedrige Molekularmassen (6 bis 11 kDa), eine hohen Fähigkeit Cd zu binden, beträchtlichen Cysteingehalt sowie durch eine Erhöhung der Extinktion bei 254 nm im Vergleich zu 280 nm auszeichneten. Cd-Stress führte zu einer deutlich erhöhten Synthese dieser Proteine, die in erster Annäherung durch die Bestimmung des Cysteingehalts abgeschätzt werden konnte.

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**Key words**

Soil fauna, ecotoxicology, cadmium, metallothionein

## 1. Introduction

The capacity of many soil organisms to take up and store heavy metals, often above substrate level, is well documented and has been used to estimate heavy metal pollution in the field and to assess ecotoxicological risk (summarised by MARTIN & COUGHTREY 1982; see also MORGAN & MORGAN 1990, WEIGMANN 1991 etc.; for a more dynamic approach considering substrate and time see PEIJNENBURG et al. 1999 a,b, CONDOR & LANNO 2000).

Especially Cadmium(Cd) stress leads to the expression of a variety of proteins with a high binding capacity for Cd and certain other heavy metals. They belong to the metallothionein (MT) protein family (for terrestrial invertebrates see reviews by POSTHUMA & VAN STRAALEN 1993, DALLINGER 1996 and DALLINGER et al. 2000). MTs are characterised by a low molecular mass (6-7 kDa), a high cysteine content (in vertebrates up to 33%), no significant amount of aromatic amino acids, and a selective capacity to bind metal ions such as copper, zinc, mercury and cadmium. Among invertebrates MTs are of considerable heterogeneity. In both, invertebrates and vertebrates MTs are expressed not only after heavy metal stress, but also under normal physiological conditions (summarised e.g. by RIORDAN & VALLEE 1991; regulation of MT gene expression is reviewed by GHOSAL & JACOB 2001).

Some years ago we studied the distribution of heavy metals (Cd, Pb, Zn) in the soil horizons and in some members of the soil macro- and mesofauna throughout the year in three beech forests along a height and deposition gradient in the Egge Mountains, North Rhine Westphalia, Germany. At that these forests were moderately polluted by Cd (RÜTHER & GREVEN 1988,1992; for a detailed characterisation of this area see GERDSMEIER & GREVEN 1991).

In the first part of the present paper we broaden knowledge of the investigation site in the Egge Moun-

tains focusing on the distribution and accumulation of cadmium in some members of its soil fauna and the soil horizons they live in. In the second more detailed part we show that the Cd content of field captured animals and those exposed experimentally to Cd is correlated with the presence of MTs.

## 2. Material and methods

### 2.1 Field data

Various soil organisms (the oligochaetes *Dendrodrilus rubidus* and *Cognettia sphagnetorum*, the arionid slug *Arion subfuscus*, and the carabids *Carabus problematicus, Abax parallelipipedus, Pterostichus oblongopunctatus*) were collected monthly from April o May to November 1986 and in March and April 1987 in three beech forests (I, II, III)in the Egge Mountains (see 3.1.)

Three to five specimens (>30 in *C. sphagnetorum*) and the specimens of *D. rubidus* were kept on moist filter paper to empty their gut. The Cd-content of the intestine/chloragog (*D. rubidus*), whole worms (C. *sphagnetorum*), the midgut gland (*A. subfuscus*) and the fat body and/or ovarioles (carabids; ovarioles were excised only in July and August) was determined by AAS. Cd-content was referred to dry mass (see 2.5.). Concentration factors were calculated for the oligochaetes and *A. subfuscus* using the total Cd-content (mean value of data collected during the entire period of investigation) of the soil horizon these animals prefer and for *C. problematicus* the preferred prey (intestine/chloragog of *D. rubidus*) as reference (see MARTIN & COUGHTRY 1992). The WILCOXON, MANN & WHITNEY U-test (SACHS 1984) was used to determine the significance of differences.

### 2.2 Experimental animals and application of Cd

*Lumbricus terrestris* were obtained from a fishing supply shop. Fifteen specimens were kept without food in aerated artificial pond water (DIETZ & ALVARADO 1970) plus 1 µg/ml Cd applied as $Cd(PO_4)_2$ for three days at 4°C. Controls were run with 15 specimens kept in water without Cd. Each of ca. 10 g *Enchytraeus albidus* obtained from a pet shop were kept similarly, but in water supplemented with only 0.25 µg/ml Cd, because pilot experiments had shown that at Cd concentrations of 1 mg/ml a considerable amount of the animals died after two days.

15 *Arion subfuscus* collected in the field were loaded with Cd by injecting 0.2 ml of a 0.6% NaCl solution containing 0.5 µg Cd/ml (applied as $Cd(PO_4)_2$ ) or without Cd into the body cavity. Injection of Cd allowed a more controlled contamination than feeding. After three days the midgut gland was dissected and prepared for Cd-determination.

Intestine/chloragog tissue and body wall (*L. terrestris*), whole *E. albidus*, the midgut gland (*A. subfuscus*) and ovarioles, intestine as well as the fat body (*C. problematicus*, captured in the Egge mountains (3.1.) and not experimentally exposed to Cd) were subjected to AAS (2.5.) and the procedures for the isolation of crude MT-fractions (2.3.).

### 2.3 Isolation of MTs

A cytosolic fraction enriched in MTs (crude MT-fraction) was prepared according to the method of BÜHLER & KÄGI (1974) and WINGE & BROUWER (1986) with some modifications. All steps of the preparation procedure were carried out on ice or at 4°C. Whole organisms or tissues were thoroughly homogenized in three volumes (mass/vol) of extraction buffer (0.1 mol/l Tris-HCl, pH 8.0, or 0.1 mol/l potassium-phosphate buffer, pH 7.0, each supplemented with 1 mmol/l PMSF and 5 mmol/l 2-mercaptoethanol). Insoluble material was removed by centrifugation at 45,000 *g* for 20 min as the first pellet (pe). The supernatant was clarified by centrifugation at 170,000 *g* for 2 h (2. pe) and then incubated at 65°C in a water bath for 8 min. The denatured and precipitated material was removed by centrifugation (3,000 *g* for 10 min; 3. pe). Non-MT-proteins were subsequently precipitated by the addition of ethanol-chloroform to the supernatant (1.2 vol ethanol and 0.1 vol chloroform, -20°C) and removed by centrifugation (3,000 *g* for 20 min; 4. pe). MT- proteins were precipitated by further addition of 2 vol ethanol (-20°C). After overnight standing the precipitate was collected by centrifugation at 3,000 *g* for 20 min and resuspended in a small volume of extraction buffer. The solution was clarified by a final 2 h centrifugation at 20,000 *g*

Separation of proteins of the crude MT-fraction was achieved by gel filtration on a calibrated Superose 12 column (Amersham-Pharmacia) in gel filtration buffer (0.1 mol/l KCl, 10 mmol/l Tris-HCl pH 8.0, 5 mmol/l $Cd(PO_4)_2$ and 5 mmol/l $CaCl_2$). The elution was monitored at 280 nm and 254 nm as MT-polypeptide-Cd complexes show a 250 nm UV absorption maximum due to Cd thiolate coordination.

### 2.4 Electrophoresis

Electrophoresis on 0.75-mm-thick polyacrylamide slab gels in the presence of sodium dodecylsulfate was performed according to LAEMMLI (1970) with minor modifications.

### 2.5 Atomic absorption spectroscopy (AAS)

For Cd measurement samples were dried (36-60 h at 105 °C), weighed, digested with conc. $HNO_3$ at 180°C for 3 to 4 h and analysed by atomic absorption spectroscopy (Perkin Elmer, model PE400 equipped with a graphite furnace, Perkin Elmer, model HGA 76). Cd and Calcium (Ca) concentrations in supernatants and fractions derived from gel filtration were measured directly.

### 2.6 Protein and cysteine determination

Protein concentrations were determined by the method of BRADFORD (1976) using bovine serum albumin as a standard.

Cysteine content of the MT-fraction was inferred from the extinction on 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) binding according to a modified method of ELLMAN (1959). DTNB to a final concentration of 10 mmol/l was added to the samples in extraction or gel filtration buffer (see above) and extinction at 412 nm read after 30 min. L-cysteine was used as a standard.

## 3. Results

### 3.1 Area of investigation

The site of investigation in the Egge Mountains included three beech forests along a height gradient and has been described repeatedly (e.g. GERDSMEIER & GREVEN 1991). Cd contamination of its soil horizons was determined bimonthly by RÜTHER & GREVEN (1992). Therefore only the most important data (forest community, height above sea level, soil, variation and mean values of total Cd-content of the $O_f$ and $O_h$ horizon during the period of investigation) will be summarized herein:

I: Luzulo-Fagetum; 320 m; loamy sand; $O_f$ 0.47 –2.04 (0.91 ±0.37, $O_h$ 0.27 – 0.67 (0.44 ±0.11) mg/kg/dm.
II: Melico-Fagetum; 360 m; silty loam; $O_f$ 0.54 – 2.12 (1.04 ±0.44), $O_h$ 0.3 – 1.05 (0.53 ±0.17) mg/kg/dm.
III: Melico-Fagetum (Asperulo-Fagetum); 410 m; stony and loamy sand; $O_f$ 0.6 – 2.27 (1.00 ±0.40), $0_h$ 0.45 – 1.05 (0.67 ±0.16) mg/kg/dm.

### 3.2 Cd content of selected animals

*Dendrodrilus rubidus* (Oligochaeta): Cd content of the intestine/chloragog varied from 3.9 to 6.56 (I), from 3.5 to 7.2 (II), and from 3.4 to 6.2 (III) µg/g/dm on average. Pooled data of the entire investigation period did not show significant differences in the Cd-content of worms of the three forests. Compared to May the Cd-content increased significantly in June (pooled data from I, II and III) (fig. 1 a). Concentration factors related to the $O_h$ horizon ranged from 4.6 to 17.0.
*Cognettia sphagnetorum* (Oligochaeta): Cd-content of whole worms varied from 6.0 to 15.3 (I), from 6.1 to 15.0 (II), and from 7.0 to 17.0 (III) µg/g/dm. Pooled data reveal that the Cd- content of worms in the forests II and III was higher than in forest I ($p<0.25$ and $p<0.05$). Monthly variations were not significant. Concentration factors related to the $O_f$ horizon ranged from 2.8 to 6.7
*Arion subfuscus* (Gastropoda): Cd-content of the midgut gland varied from 6.4 to 10.5 (I), from 5.0 to 12.0 (II), and from 7.2 to 10.25 (III) µg/g/dm. Significant differences between the specimens of the three forests could not be detected. The Cd-content decreased in June and November and increased in September ($p<0.01$) (fig. 1 b). Concentration factors related to the $O_f$ horizon ranged from 2.8 to 4.6.
*Carabus problematicus* (Coleoptera): Cd-content of the female fat body varied from 0.47 to 0.72 (I), from 0.50 to 0.84 (II), and from 0.6 to 0.87 (III) µg/g/dm. Specimens of forest III contained more Cd than those in forest I ($p<0.05$). The Cd-content decreased in July and increased in September ($p<0.01$) (fig. 1 c). Ovarioles prepared in July and August contained 0.66±0.15, the intestine 0.22±0.16, and the fat body 0.61±0.17 µg Cd/g/dm. The tentatively determined concentration factors related to the mean Cd content of the intestine/chloragog tissue of *D. rubidus* varied from 0.16 to 0.40.
*Abax parallelipipedus* (Coleoptera): Cd-content of the female fat body varied from 0.75 to 1.5 (I), from 0.9 to 1.8 (II), and from 0.9 to 1.8 (III) µg/g/dm. Compared to forest I Cd-content was higher in specimens of forest III ($p<0.05$). The Cd-content decreased in June and July ($p<0.01$ and $<0.025$) and increased in August and March ($p<0.01$) (fig. 1 d). Ovarioles analysed in July and August contained 0.38±0.11 µg Cd/g/dm.
*Pterostichus oblongopunctatus* (Coleoptera): Cd-content of the fat body (males and females) varied from 0.4 to 0.88 (I), from 0.42 to 1.1 (II), and from 0.4 to 0.9 (III) µg/g/dm. Specimens of forest II had a significantly higher Cd-content than those of forest I ($p<0.05$). The Cd-content decreased in July and October ($p<0.01$ and $<0.025$) (fig. 1 e).

### 3.3 *Lumbricus terrestris* and *Enchytraeus albidus* (Oligochaeta)

The data of the distribution of Cd in fresh tissue and in subcellular fractions of *L. terrestris* after a 3-day Cd-exposure are summarised in table 1. Despite the increase in Cd content after exposure there is only little change in the percentage distribution of Cd between the various fractions of the Cd-treated and the control samples. Values given for the different preparation steps do not reach 100% because up to 20% of Cd is lost during the procedure. The pellets of the intestine/chloragog sample after the first and second centrifugation that contained the cell debris, mitochondria and nuclei (1. pe) and the microsomal fraction (2. pe) comprised about 25% of the Cd in the starting material. Nearly 50% of the Cd was found in the crude MT-fraction. On the contrary, in the case of the body wall samples less than 30% of the Cd was in the crude MT-fraction and up to 50% in the first two pellets (particle fraction). The six fold increase in the Cd content after Cd exposure was reflected by a threefold increase in the amount of cysteine determined in the MT-fraction. In the body wall a comparable increase in the Cd-content leads only to a duplication of the measurable cysteine (tab. 1).

Table 1. Cadmium balance in % of the initial content of the different fractions obtained during isolation of MT and cysteine content of the crude fraction (MT) from the intestine/chloragog complex and the body wall of *Lumbricus terrestris* exposed 72 h to 1mg Cd/ml. 1. pe pellet after the first centrifugation (45,000 *g*), 2. pe after the second centrifugation (17,0000 g), 3. pe after heat denaturation, 4 pe after chloroform/ethanol-precipitation, MT crude fraction of water soluble proteins, cys cysteine content of the crude fraction, wm wet mass. During the preparation up to 20% of Cd is lost.

| | Initial Cd-content (100%) µg/g wm | 1. pe % | 2. pe % | 3. pe % | 4. pe % | MT % | Cys ng |
|---|---|---|---|---|---|---|---|
| intestine/chloragog control | 0.67 | 16.2 | 8.4 | 6.5 | 10.2 | 44.4 | 131 |
| intestine/chloragog cadmium | 3.9 | 14.8 | 6.7 | 6.1 | 7.7 | 48.5 | 389 |
| body wall-control | 0.58 | 31.4 | 7.6 | 8.5 | 6.9 | 29.2 | 99 |
| body wall-cadmium | 3.14 | 38.5 | 10.00 | 7.2 | 8.5 | 26.4 | 172 |

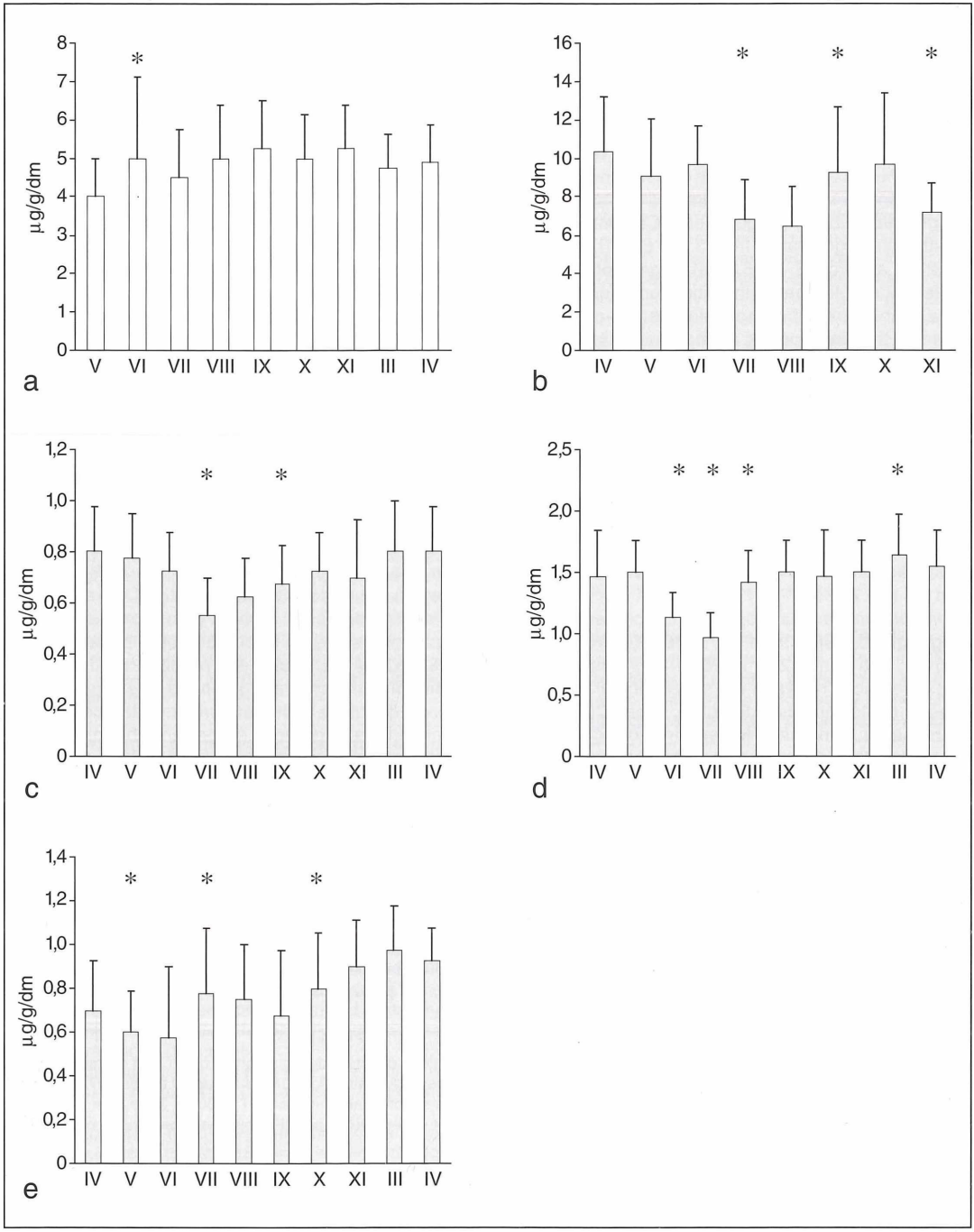


Figure 1. Mean Cd-content (µg/g/dm) of various tissues of several soil organisms during the investigation period (abscissa: months). Data were pooled from the three beech forests. a) Intestine/chloragog tissue of *Dendrodrilus rubidus*, b) midgut gland of *Arion subfuscus*, c) fat body of *Carabus problematicus*, d) *Pterostichus oblongopunctatus*, and e) *Abax parallelipipedus*.
* Significantly different.

Table 2. Cadmium balance in % of the initial content of the different fractions obtained during isolation of MT and cysteine content of the crude fraction (MT) from specimens of Enchytraeus albidus after 24 h exposure to 0.25 µg Cd/ml. For further explanation see legend of table 1.

| | Initial Cd-content (100%) µg/g wm | 1. pe % | 2. pe % | 3. pe % | 4. pe % | MT % | Cys ng |
|---|---|---|---|---|---|---|---|
| control | 0.04 | 20.4 | 19.4 | 9.7 | 2.4 | 31.6 | 260 |
| cadmium | 27.0 | 13.4 | 16.3 | 12.9 | 3.3 | 38.2 | 1141 |

Table 3. Cadmium balance in % of the initial content of the different fractions obtained during isolation of MT and cystein content of the crude fraction (MT) of the midgut gland of Arion subfuscus 48 h after injection of 0.2 ml 0.6% NaCl solution plus 0.5 µg Cd/ml. For further explanation see legend of table 1.

| | Initial Cd-content (100%) µg/g wm | 1. pe % | 2. pe % | 3. pe % | 4. pe % | MT % |
|---|---|---|---|---|---|---|
| midgut glandcontrol | 16.25 | 18.4 | 16.3 | 6.0 | 6.4 | 43.8 |
| midgut glandcadmium | 23.14 | 20.1 | 15.2 | 6.8 | 5.5 | 48.8 |

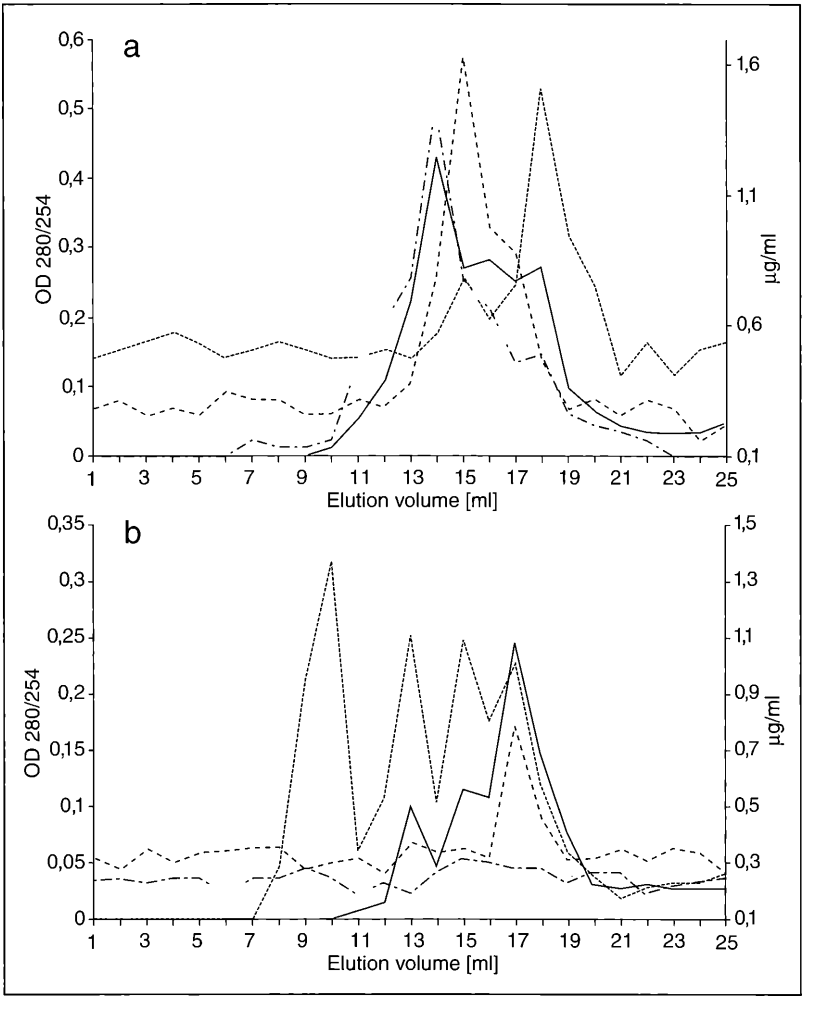


Figure 2. Gelfiltration of the MT-enriched fractions isolated a) from the body wall and b) the intestine/chloragog tissue of *Lumbricus terrestris* exposed to Cd (see table 2). For each eluted fraction of 1 ml the extinction was determined at 280 nm and 254 nm (left ordinate) as well as the Ca- and Cd-content (µg/ml; right ordinate).

——— 254 nm
280 nm
-·-·- Ca-content
Cd-content

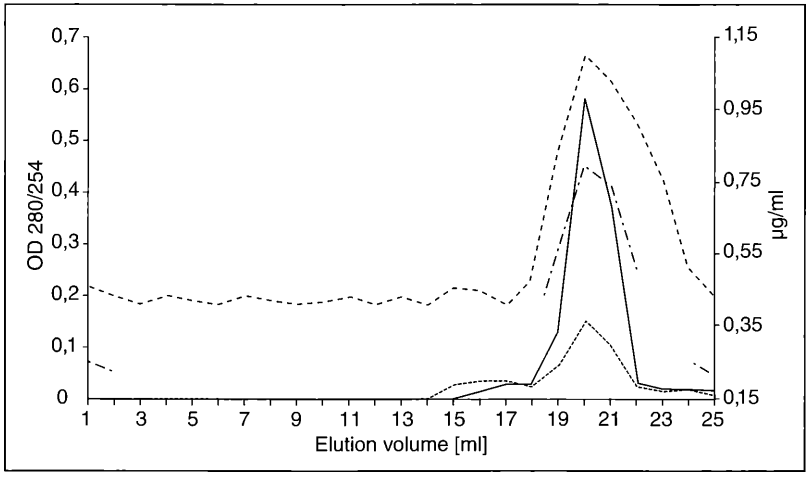


Figure 3. Gelfiltration of the MT-enriched fractions isolated from whole *Enchytraeus albidus* exposed to Cd (see table 3). For further explanation see figure 1.

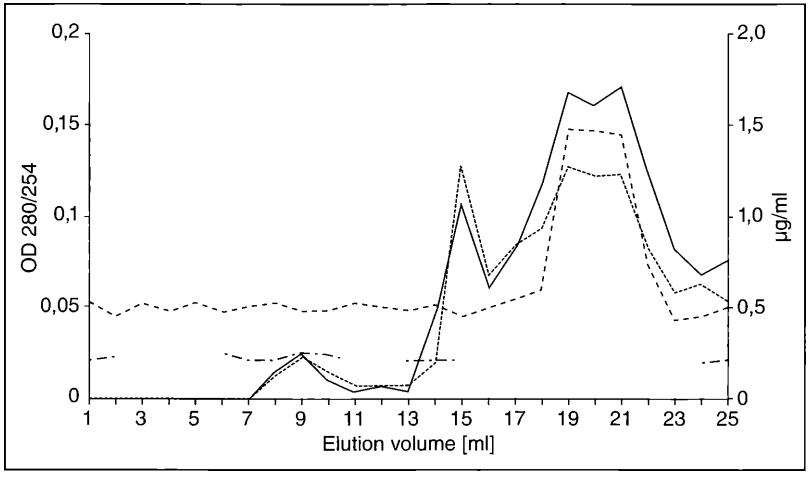


Figure 4. Gelfiltration of the MT-enriched fractions isolated from the midgut gland of *Arion subfuscus* injected with Cd containing NaCl solution (see table 4). For further explanation see figure 1.

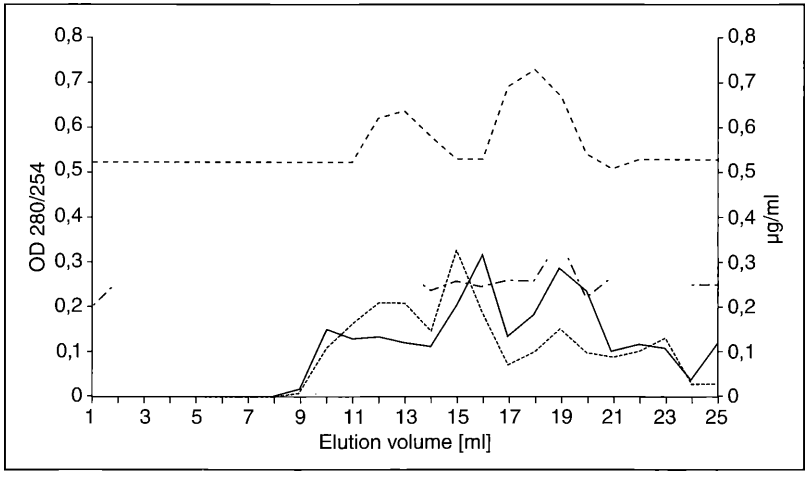


Figure 5. Gelfiltration of the MT-enriched fractions isolated from ovarioles of *Carabus problematicus* captured in the field. For further explanation see figure 1.

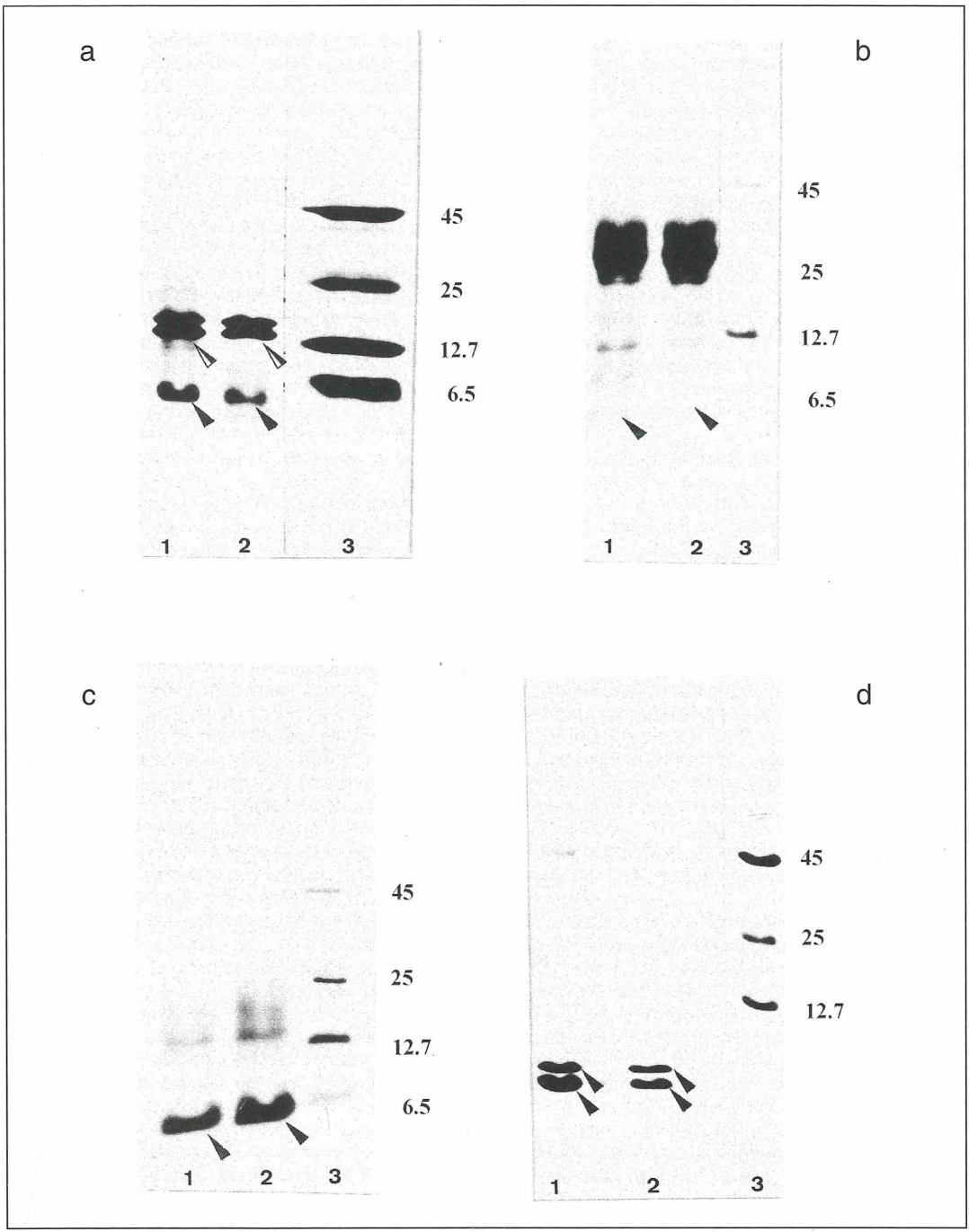


Figure 6. SDS-PAGE of the crude fractions prepared from Cd-exposed (1) and control organisms (2). Crude fraction from a) the body wall and b) the intestine/chloragog tissue of *Lumbricus terrestris*, from c) whole specimens of *Enchytraeus albidus*, and d) the midgut gland of *Arion subfuscus*. Low molecular mass metal binding proteins are marked by arrowheads, Ca-binding proteins by black and white arrowheads; 3 standard in kDa. Note increase in MT protein after Cd-exposure in a and b.

By gel filtration of the crude MT-fraction of the body wall of the Cd-exposed animals (fig. 2 a) separate peaks were obtained for proteins with high Ca- and Cd-binding. The Ca-binding fraction with a calculated binding capacity of 2.3 mol Ca/mol protein eluted first and corresponded to a molecular mass of about 20 kDa. Cd-binding was comparable low (0.8 mol/mol protein). The Cd-binding fraction contained proteins with a molecular mass of around 10 kDa according to the elution profile. 3.6 mol Cd were bound per mol of protein of this fraction; no Ca-binding could be detected. The molecular masses calculated from SDS-PAGE of the fractions were 18 and 21 kDa (two bands) for the Ca- and 6 kDa for the Cd-binding fraction (fig. 6 a). By gel filtration of the intestine/chloragog fraction a clear Cd-binding peak was obtained, but in contrast to the body wall fraction no Ca-binding peak. The Cd fraction corresponded to the body wall fraction with a binding capacity of 3.4 mol/mol protein and the same molecular mass (fig. 2 b, 6 b). The elution profile at 280 nm (fig. 2 b) revealed a considerable amount of non-MT proteins (see also fig. 6 b).
Distribution of Cd in the various fractions of the *E. albidus*-samples with and without Cd exposure is shown in table 2. The basal Cd-content of the control samples was rather low (approx. 0.04 µg/g wet mass (wm), about one tenth of that found in *L. terrestris* samples. 30-40% of the Cd was present in the sedimentable fraction as well as in the soluble crude MT-fraction. After exposure the Cd increased manifold to nearly 27 µg/g wm accompanied by a four- to fivefold increase in cysteine content of the crude Mt-fraction. Gel filtration shows a single peak with a high extinction ratio between 254 and 280 nm (fig. 3). Peak fractions revealed high binding capacities for Cd as well as for Ca. Binding capacity for Cd was 4.8 mol/mol protein and for Ca 0.6 mol/mol protein. SDS Page of the crude fractions reveals a single band (6 kDa) in both, the control and the probe (fig. 6 c).

### 3.4 *Arion subfuscus* (Gastropoda)

Distribution of Cd in the various fractions of the midgut gland is shown in table 3. The basal Cd content of the control samples collected in the field was remarkable high (16 µg/g wm). 35% of the Cd was present in the first and second pellet of Cd-loaded as well as of the control animals. In the crude MT-fraction it amounted to 45%. Only a 1.5 fold increase was seen in loaded specimens. The strong colour of the fraction prevented cysteine determination.
Gel filtration of the crude MT-fraction showed fractions with high Cd-binding and an increased extinction at 254 nm compared to 280 nm. The protein peak apparently consisted of two different proteins (fig. 3) with only slight differences in molecular mass (approx. 6.5 kDa) also seen in the SDS-PAGE (fig. 6 d). Cd-binding capacity of the combined fraction amounted to 2.7 µg/mol protein; no Ca-binding occurred.

### 3.5 *Carabus problematicus*–females (Coleoptera)

Gel filtration of the crude MT-fraction of the intestine, fat body and ovarioles of field-captured, not exposed beetles (see 3.1.2.) revealed the presence of two Cd-binding proteins in the three organs, a high molecular mass protein of about 80-100 kDa and a low molecular mass protein of about 5-6 kDa. Binding capacities for the high molecular mass protein fraction were as follows: 0.4 mol Cd and 0.2 mol Ca/mol protein in the intestine, 0.8 mol Cd in the fat body and 1.4 mol Cd in the ovarioles (fig. 5). Remarkably, the fractions from the fat body and the ovarioles did not bind any measurable Ca. For the low molecular mass protein fractions, binding capacities of 2.5 mol Cd and 0.5 mol Ca/mol protein were determined for the intestine fraction, 2 mol Cd and 0.2 mol Ca/mol protein for the fat body, and 1.2 mol Cd and 0.45 mol Ca/mol protein for the ovariole fraction. Samples were too small to allow determination of cysteine content and Cd balance.

## 4. Discussion

Cd-concentration factors estimated in this study give only a rough imagination as knowledge on habits, uptake mechanisms, exposure routes, and food of many soil organisms including variations of local edaphic factors are often not sufficiently known. For calculations we used the total Cd-content of the soil horizon the respective animals prefer and in one case the putative main prey as reference (e.g. Martin & Coughtrey 1982, Fangmeier et al. 1986, Hunter et al. 1987). However, soil quality criteria that are based on total concentrations are said to be unlikely to be predictive of adverse biological effect. Water- or neutral salt extractable metal concentrations are obviously better related to uptake and effects of metals (e.g. Janssen et al. 1996, Peijnenburg et al. 1999 a,b, Conder & Lanno 2000). Thus, our calculations based on total Cd-content demonstrate simply the capacity of the oligochaete- and gastropod-species examined herein to accumulate Cd above substrate level. Differences in the Cd-content perhaps due to the height and deposition gradient (Greven & Gerdsmeier 1991, Rüther & Greven 1992) are suggested only in the enchytraeid-species and the carabids.
Cd-concentration factors of *Dendrodrilus rubidus* are in the range of those shown for other lumbricids (e.g.Ireland 1983, Fangmeier et al. 1986, Morgan & Morgan 1990), but may be even higher (Brewer & Barrett 1995). Cd is accumulated in lumbricids and detoxified in the intestine/chloragog tissue (e.g. Ireland & Richards 1981), and here primarily within the posterior alimentary canal (Morgan & Morgan 1990, Morgan et al. 1993). As shown herein, Cd is also present in considerable amounts in the body wall. Metal uptake in earthworms and enchytraeids appears simi-

lar suggesting similarity of metal uptake routes within oligochaetes in general (PEIJNENBURG et al. 1999 b). Although single organs of enchytraeids were not examined by us, the intestine/chloragog tissue is probably the most important heavy metal binding organ as in lumbricids. The vast literature on oligochaetes approaching the problem of metal accumulation and ecotoxicological risk assessment will only be touched herein. Several soil parameters such as pH and clay determine uptake rate constants and bioaccumulation factors. Steady state concentrations of Cd appear to be predictable on the basis of the Cd concentration in the pore water and pH in *Enchytraeus crypticus* (PEIJNENBURG et al. 1999 a) and by the $CaCl_2$-extractable amount of Cd in the soil in *Eisenia andrei* (PEIJNENBURG et al. 1999 b). Steady state conditions rather than concentrations measured in a short-term bioassay should be used for interpreting test results (PEIJNENBURG et al. 1999 b) Furthermore, there is evidence that accumulation of Cd may continue for the life span of earthworms (SHEPPARD et al. 1997). It has been demonstrated that *Enchytraeus buchholzi* (species determination doubtful) exposed to different Cd-concentrations in agar and aquatic test media (e.g. RÜTHER & GREVEN 1990, WILLUHN et al. 1994 a,b, 1996) seem to accumulate Cd extremely far above substrate level. In the present study this is confirmed for field-captured *Cognettia sphagnetorum* (see 3.1.2) and cultivated *Enchytraeus albidus* (perhaps more than 500, see tab. 2), depending on the concentrations and on true bioavailability (PEIJNENBURG et al. 1999 b). Differences in concentration factors my be explained by earthworm ecology and activity (e.g. MORGAN & MORGAN 1993)

In snails and slugs the midgut gland is the main storage organ for Cd (e.g. IRELAND 1981, summarised by MARTIN & COUGHTREY 1982). Concentration factors calculated for *Arion subfuscus* lie in the range known for a variety of other slugs (MARTIN & COUGHTREY 1982). Generally large variability in metal levels have been reported, in part caused by interactions of various seasonal changes such as climate, food availability etc. (e.g. *Deroceras reticulatum*: GREVILLE & MORGAN 1989). Decrease in Cd-content in July might be connected to the relative high temperature and low rainfall (on average) measured in this month (RÜTHER & GREVEN 1988). More even climatic conditions led to a continuous increase of the wet mass of whole animals and the midgut gland that positively correlated with the Cd content (IRELAND 1984).

Carabids have been characterised as poor accumulators of heavy metal which may be due to effective detoxification and excretion (HOPKIN 1989, KRAMARZ 1999). Cd-concentrations in *Poecilus cupreus* from a laboratory culture fed with Cd-treated housefly larvae increased about thirty times (analysis of whole animals) but eliminated excess metal quickly after switching to non polluted food (KRAMARZ 1999). However, distribution and accumulation in different tissues appear poorly documented in beetles and tissues other than the fat body and the ovarioles, e.g. the cuticle (VOGEL 1988), may incorporate significant amounts of heavy metals. Furthermore, calculations of concentration factors on the basis of the Cd-content of the intestine/chloragog tissue of *D. rubidus* may be highly questionable. Fat body, intestine and ovarioles of the carabids investigated contained significant amount of Cd, but a remarkable high accumulation was not observed. A relative low accumulation was observed in other studies in which heavy metal content of whole beetles was analysed (ZÖTTL 1985, HUNTER et al. 1987).

It might be speculated that the significant decrease of Cd (and Pb and Zn, unpublished) in the fat body in females of the autumn breeder *Carabus problematicus* and in females of *Abax parallelipipedus* in June/July reflect a transport to the ovary during vitellogenesis, but more detailed studies are necessary. Differences in the Cd-content of carabids (whole individuals) could be assigned to different activities including feeding during a year (HUNTER et al. 1987).

In brief, Cd was found in all organisms investigated by us. At the time of investigation (1986/1987) the Egge Mountains apparently were moderately polluted by Cd. However, concentrations measured in the $O_h$ (and $O_l$) horizon were somewhat elevated in the uppermost beech forest (RÜTHER & GREVEN 1992). Primary and secondary decomposers (lumbricids, enchytraeids, arionids) accumulated Cd above substrate level. The carabids that are mostly second-order consumers did not significantly accumulate Cd. These findings are in agreement with the view that an accumulation of metals at a lower trophic level does not necessarily result in biomagnification in the next trophic level (e.g. VAN STRAALEN 1987, HOPKIN 1989, LASKOWSKI 1991, KRAMARZ 1999).

The Cd-binding proteins isolated and described in the present paper meet the criteria for MTs, i.e. low molecular mass, high cysteine content and strong Cd-binding. MTs of a variety of terrestrial invertebrates are well known including their primary structure (DALLINGER et al. 2000). A considerable heterogeneity apparently exists among the various invertebrate MTs, though differences may also be found as a consequence of the different methods used. It is known that the rod-shaped structure of the MTs leads to a higher apparent molecular mass in gel filtration and that the high content of cysteine can cause abnormal mobility in electrophoresis. In earthworms MTs and other metal binding proteins have been isolated and characterised (for review see SCOTT-FORDSMAND & WEEKS 2000). In this taxon MTs with molecular masses between 6 and 27 kDa have been described (e.g. *Eisenia foetida* 6-7 kDa: SUZUKI et al. 1980, YAMAMURA et al. 1981; *Lumbri-*

*cus terrestris* 6-9 kDa*:* the present paper; see also RAMSEIER et al. 1990; *Dendrodilus rubidus:* 27.5 kDa, *Lumbricus rubellus:* two isoforms 24 and 27 kDa: MORGAN et al. 1989; *Dendrobaena octaedra*: two isoforms 6.5 and 13.5 kDa: BENGTSSON et al. 1992). STÜRZENBAUM et al. (1998) isolated and sequenced the two MT isoforms of *L. rubellus* and GRUBER et al. (2000) characterised the Cd-MT in *Eisenia foetida* and discussed the creation of MT variants by posttranslational processing. A cysteine-free, Cd-binding, soluble protein with a high level of aromatic amino acids and a molecular mass of 14 kDa was isolated from *Allolobophora caliginosa* (NEYMEDDINE et al. 1992). The MT identified in the body wall of *L. terrestris* appears to be identical with that isolated from the intestine/chloragog complex as indicated by their identical mobility in the SDS-PAGE and their capacity to bind Cd. In addition, both MTs lack Ca-binding. Presence in this tissue is noteworthy, as MTs in lumbricids were detected to our knowledge to date in whole worms only, or in the intestine/chloragog complex (see literature cited above). A second protein of the body wall of 15 to 20 kDa with a weak binding capacity for Cd even in the presence of Ca is known as a soluble Ca-binding protein (HUCH et al. 1988). The toxic effect of Cd might in part be caused by its binding to Ca-binding proteins, thereby changing the Ca signal.

In *E. albidus* a large amount of the accumulated Cd was bound to a low molecular protein (6 -8 kDa). Binding of Cd (up to 4.8 mol/mol protein) was stronger than in *L. terrestris* and a strong binding of Ca could also be observed. Interestingly in *E. buchholzi* Cd specifically induces a gene (CRP-gene) that encodes a cysteine-rich, but non-MT 25-kDa protein (WILLUHN et al. 1994 a,b).

MTs have been repeatedly demonstrated in terrestrial gastropods (e.g. IRELAND 1981, DALLINGER & WIESER 1984, DALLINGER et al. 1989, 2000). IRELAND (1981) described a 9 kDa Cd-binding protein (calculated by gel filtration) and three other Zn-binding proteins in the midgut gland of the slug *Arion ater*. DALLINGER et al. (1989) showed a strictly inducible 10 kDa MT in *A. lusitanicus* which could not be detected without Cd exposure. We found two soluble, Cd-binding fractions (7 and 9 kDa, SDS-PAGE) in the midgut gland of *A. subfuscus* before and after Cd-loading. Approximately 50% of Cd were found in the particle-free cytosolic supernatant, whereas IRELAND (1981) found even >80% of the Cd in these fraction in *A. ater*. These differences cannot be explained at the moment. In the related *A. rufus* we found a MT with a molecular mass of 10 kDa, a higher Cd-binding capacity as the proteins of *A. subfuscus* and a considerable Ca-binding capacity (unpublished).

There are many studies on insect MTs (e.g. EVERAD & SWAIN 1983, KASAI et al. 1993). Accumulation of Cd was described to occur mainly by the digestive tract (95%) in the larvae of *Sarcophaga peregrina* (AOKI et al. 1984). The molecular mass was estimated by gel filtration to be about 8 kDa. Unusually low molecular mass was described for the MT from *Orchesella cincta* 3 and 4 kDa (HENSBERGER et al. 2000). As in lumbricids posttranslational processing is discussed (HENSBERGER et al. 1999). In *Drosophila melanogaster* two genes for MT exist (MOKDAD et al. 1987). Interestingly, a duplication of the MT-Gen is found in natural populations of *Drosophila melanogaster* and is accompanied by increased metal tolerance.

We could demonstrate in the field-captured *Carabus problematicus* a low molecular protein in the intestine, in the fat body and the ovarioles. Molecular masses as well as Cd- and Ca-binding capacities were similar in the three tissues and, thus, they are probably identical. The high molecular mass protein that binds Cd may belong to a group of Cd-binding non-MTs. Such proteins have been detected in several invertebrates and vertebrates (STONE & OVERNELL 1985).

It should be emphasised that MTs could be detected not only in the Cd-stressed animals, but also in the "controls", i.e. in non-exposed *E. albidus* and *L. terrestris* from a commercial dealer, as well as in *A. subfuscus* and *C. problematicus* from the field. Past of both groups of experimental animals was unknown to us. Cd-binding proteins were detected even in *Dendrobaena octaedra* of unpolluted soils (BENGTSSON et al. 1992). MTs, thus, might be induced in these specimens by stress other than heavy metal exposure or even might reflect the physiological level (e.g. TALBOT & MAGGEE 1978; see also GHOSAL & JACOB 2001). To our knowledge, this aspect has not been investigated in detail in the invertebrates examined. However, additional expression of MT is inducible by heavy metal exposure. Therefore, MTs as well as the above mentioned obviously very sensitive and specific CRP-gene (WILLUHN et al. 1994 a,b, 1996) have been used or suggested as biomarkers in the aquatic and terrestrial environment (e.g. COSSON 2000, DALLINGER et al. 2000). Nevertheless several limitations and pitfalls of these procedures were discussed such as species related variation of MTs, fluctuations of MT levels with seasons, lack of common methods to prepare and analyse the samples, possible effects of mixtures of heavy metals, etc. (e.g. COSSON 2000). A reliable standardised method based on MTs or metal binding non-MT proteins involving soil organisms apparently has not been established so far.

**Acknowledgement**

We are indebted to the "Gesamtverband des Deutschen Steinkohlenbergbaus, Essen" for financial support and Mr. M. BRENNER, Düsseldorf for preparing the figures.

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