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Content, absorption quantities and intracellular storage sites of heavy metals in Diplopoda (Arthropoda)

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By means of atomic absorption spectrophotometry, concentrations of more than 2500 mg kg^{-1} Pb, 150 mg kg^{-1} Zn, and 320 mg kg^{-1} Cd could be detected in the intestine tissues of diplopods from a lead and silver smelter's spoil bank. While only small portions of the ingested lead and cadmium are absorbed in the midgut of these diplopods, the zinc uptake into the midgut epithelium reaches 33.8–37.5% of the zinc content in the food pulp when the animals were contaminated acutely. However, after long-term contamination with zinc, absorption and excretion of this metal balanced one another. Absorbed lead and cadmium are predominantly stored in the midgut cells of the diplopods; unspecific precipitation of heavy metal showed the spherites of the resorptive epithelial cells to be the main accumulation sites. Zinc is for the most part localized in or near the cuticle; electron energy loss spectroscopy and ESI electron spectroscopic imaging, however, showed this metal to be present also in the spherites of the midgut's resorption cells. These spherites are assigned to belong to the 'type A granule' group since (i) they are concentrically structured, (ii) they are shown to contain great amounts of calcium and (iii) copper, a class B metal, could not be detected in these deposits.

Keywords: Diplopoda, EELS, ESI, heavy metals, midgut, spherites

Introduction

Like other saprophagous soil animals, diplopods, the main ecophysiological role of which is to enhance decomposition of dead organic matter by stimulating soil microflora activity, inhabit the upper soil horizons and, therefore, they are massively influenced by either ore-borne or deposited heavy metals. Although the species number of diplopods may be greatly reduced in areas massively contaminated with heavy metals (e.g. Hopkin *et al.* 1985), in nearly every stand some species occur which are able to cope with these noxious compounds. These diplopod populations manage to survive even though food consumption rates (Hopkin *et al.* 1985), nutrient absorption efficiency (Köhler *et al.* 1992) and, in some cases, juvenile survival rates (Read & Martin 1988) decrease under heavy metal conditions.

While the importance of diplopods for the maintenance of ecophysiological processes in the intact soil has often been mentioned in literature, there are few publications on survival strategies and detoxification of heavy metals in this invertebrate group which made Hopkin (1989) to note in his detailed review on metals in terrestrial invertebrates that "it is surprising that so little work on the role of metals in their [the millipedes'] biology has been carried out". Nevertheless, some papers dealing with metal concentrations in diplopods (Siegel *et al.* 1975, Carter 1983, Beyer *et al.* 1985, Hopkin *et al.* 1985, 1989, Morgan *et al.* 1986, Hunter *et al.* 1987, Read & Martin 1988) or with metals in diplopod cells (Crane & Cowden 1968, Petit 1970, Hubert 1974, 1975, 1978a,b, 1979, Martoja *et al.* 1977) do exist, and prove that diplopods manage to metabolize and/or accumulate different kinds of heavy metal ions.

It is widely accepted that resorption of metal ions predominantly takes place in the midgut of the millipedes. The histology (Randow 1924, Verhoeff 1932) and ultrastructure (Seifert & Rosenberg 1977, Schlüter 1979a,b, 1980a–c, Neumann 1985, Schlüter & Seifert 1985, Köhler & Alberti 1992) of the intestine and its appendices is well known, and some of these studies revealed the presence of spherites in the midgut epithelium which implies—as the studies on metal distribution cited above do—the ability of biotransformation and accumulation of resorbed metals in this region.

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Based on this information, the present study thus tries (i) to quantify lead, zinc and cadmium resorption in acute as well as in chronically contaminated diplopods, (ii) to quantify accumulation of these metals in the intestine tissue, (iii) to reveal the storage sites of heavy metals in the cells responsible for resorption and (iv) to characterize the metal composition of the midgut's spherites by means of electron energy loss spectroscopy.

Material and methods

Field studies

To examine metal accumulation in diplopods from long-term contaminated sites, *Leptoiulus belgicus* (LATZEL 1884) (Julidae, $n = 5$) and *Glomeris marginata* (VILLERS 1789) (Glomeridae, $n = 10$) were collected on a spoil bank from a lead and silver smelter in Braubach near Koblenz, Germany [leaf litter contamination 41.9 mg kg^{-1} Cd, 628.4 mg kg^{-1} Cu, 1658.6 mg kg^{-1} Pb (Dallinger & Prosi 1988)]. For defecation, the animals were kept for two additional days in the laboratory; subsequently, they were dissected and divided into (i) intestine and (ii) 'remaining body' which consists mainly of cuticle. Samples were prepared for atomic absorption spectrophotometry (AAS; for lead, zinc and cadmium).

AAS

Leaf litter material and feces were oven-dried (60 °C), animal organs were freeze-dried, all samples ground, weighed, digested with nitric acid (*suprapur* grade) and topped up with distilled water (leaf litter and feces: 5 ml HNO_3, topped up to 50 ml; organs: 200 µl HNO_3, topped up to 1.2 ml). The concentrations of lead (261.4 or 283.3 nm), zinc (213.9 or 307.8 nm) and cadmium (228.8 nm) were measured with a graphite furnace atomic absorption spectrophotometer (Perkin-Elmer 5000, HGA 500).

Heavy metal absorption

Adults of the following diplopod species were kept individually for 30 days under laboratory conditions (15 °C, dark/light = 12/12 h) in plastic boxes, the bottom of which was filled with plaster of Paris on which partly decomposed leaf litter fragments were placed: *Glomeris conspersa* C.L.KOCH 1847 (Glomeridae), *Julus scandinavius* LATZEL 1884, *Allaiulus nitidus* (VERHOEFF 1891) and *Tachypodoiulus niger* (LEACH 1815) (all Julidae). Both the gypsum surface on the bottom of the boxes and the leaf litter food were kept constantly moist. Artificial contamination of both leaf litter and gypsum ground took place with a solution of 1000 mg kg^{-1} Pb^{2+} (as $Pb(NO_3)_2$); the controls were moistened with tap water.

In studies on metal adsorption and excretion, the origin of the animals has to be considered: (i) heavy metal-preconditioned animals derived from a former opencast mining area in Wiesloch near Heidelberg, Germany [leaf litter contamination 91.01 mg kg^{-1} Cd, 1962.60 mg kg^{-1} Zn, 1240.01 mg kg^{-1} Pb (Köhler *et al.* 1992)] and controls from a minor contaminated stand 10 km north of the mining area [leaf litter contamination 1.35 mg kg^{-1} Cd, 98.14 mg kg Zn^{-1} 157.35 mg kg^{-1} Pb (Köhler *et al.* 1992)].

Due to the low feces production, a species-specific investigation of the animals has not been possible. Thus, feces of all four species belonging to the same nutrition type (Köhler *et al.* 1991) have to be pooled and three animal groups were distinguished according to metal precondition and acute contamination conditions:

Group A: originally minor contaminated, no acute metal addition on the food.

Group B: originally minor contaminated, substrate artificially contaminated with 1000 mg kg^{-1} Pb^{2+}.

Group C: deriving from the former opencast mining area, substrate artificially contaminated with 1000 mg kg^{-1} Pb^{2+}.

To quantify heavy metal net absorption, (i) concentrations of lead, zinc and cadmium in either the artificially contaminated or non-contaminated (control) food ($[hm]_{food}$), (ii) mean daily food uptake (M_n), (iii) metal concentration in the feces ($[hm]_{feces}$) and (iv) mean daily feces mass (M_f) had to be examined (parameter abbreviations according to Köhler *et al.* 1992).

Daily ingested heavy metal mass ($M_{hm\ ing}$) and daily defecated heavy metal mass ($M_{hm\ def}$) are calculated by

$$M_{hm\,ing} = M_n * [hm]_{food} \quad (1)$$

$$M_{hm\,def} = M_f * [hm]_{feces} \quad (2)$$

Number of replicates: M_n and M_f $n = 25$ (group A), $n = 46$ (group B) and $n = 22$ (group C). $[hm]_{food}$ and $[hm]_{feces}$: for every group and every metal respective $n = 10$.

Transmission electron microscopy (TEM)

Adult specimens of *Cylindroiulus punctatus* (LEACH 1815) (Julidae) and *J. scandinavius* from an uncontaminated forest stand [and additionally *T. niger* from the Braubach spoil bank for electron energy loss spectroscopy (EELS)/electron spectroscopic imaging (ESI) investigations] were prepared for TEM. After dissection, the midgut was fixed in 2% glutardialdehyde dissolved in 0.01 M cacodylate buffer (pH 7.2), rinsed repeatedly in 0.01 M cacodylate buffer (pH 7.2) and postfixed in 1% reduced OsO_4 solution (1.5% $K_4[Fe[CN]_6]$) for 2 h (Karnowski 1971). After washing in 0.01 M cacodylate and 0.05 M maleate buffer (pH 5.2), the specimens were stained *en bloc* in 1% uranyl acetate in maleate buffer overnight, dehydrated in a graded ethanol series and embedded in Spurr's medium (Spurr 1969). Ultrathin sections were stained with alkaline lead citrate for 5 h (Reynolds 1963, modified) and examined in Zeiss EM 9 S-2 and Zeiss EM 10 CR transmission electron microscopes.

Cytochemistry

Precipitation of heavy metals in the midgut cells was tested in *T. niger* from the Braubach spoil bank site according to Timm (1958): tissues were fixed with H_2S saturated, cacodylate buffered 2% glutardialdehyde (pH 7.4) for 2 h, rinsed in 0.1 M Tris–maleate buffer (pH 7.4) and postfixed in OsO_4. The metal sulfides were developed with 10% silver nitrate. After dehydration in ethanol and infiltration in propylene oxide, the specimens were embedded in Araldite. The ultrathin sections were not counterstained and examined in a Zeiss EM 9 transmission electron microscope.

Scanning electron microscopy (SEM)

Sectioned specimens of soft tissue material investigated by SEM were first embedded in epoxy resin which later has to be dissolved to expose the original surface of the slice (Weissenfels 1982, modified). Four specimens of *T. niger* from an uncontaminated forest site were fixed as shown above for the TEM preparation (glutardialdehyde, reduced OsO_4, no *en bloc* staining, dehydration in ethanol). Subsequently, the specimens were transferred into a mixture of 50% ethanol/50% methacrylic acid-*n*-butylester for 2 h and infiltrated by 50% methacrylic acid-*n*-butylester/50% styrol for 48 h. Polymerization of the resin took place after addition of 4% (w/w) benzoyl peroxide (60 °C, 24 h). After sectioning, the resin was dissolved by repeated rinsing in xylol.

Samples were subsequently transferred into dimethoxi-methane, critical-point dried, mounted on stubs and coated with gold (Rosenbauer & Kegel 1978). Specimens were examined with a Philips SEM 505.

EELS and ESI

To reveal the intracellular distribution of metals, ultrathin sections of 30–40 nm from embedded midgut tissue of *T. niger* and *L. belgicus* from the Braubach spoil bank site (see above) were examined without any lead citrate counterstaining in a Zeiss CEM 902 (Castaing-Henry-Spectrometer; Zeiss, Oberkochen, Germany) energy-filtering transmission electron microscope (EFTEM) equipped with a photomultiplier for recording of electron energy loss spectra, a highly sensitive SIT-TV camera (Dage-MTI, Michigan City, IN) and an integrated digital image analyzing system (Zeiss-Kontron). EELS spectra were recorded from the spherites in the midgut's resorptive epithelial cells from 280 to 1200 eV with a slit width of 1 eV. In the spectrum regions where zinc (900–1200 eV, peak at 1050–1120 eV) and copper (800–1100 eV, peak at 930–980 eV) peaks may occur, regression analysis was performed in order to distinguish background noise from the respective element peaks.

In order to give a conventional impression of the ultrastructure structure, sensitive dark field-like images were recorded at 250 eV, directly below the edge of the carbon peak. For elemental mapping, the two windows method (Ottensmeyer & Andrew 1980, Körtje *et al.* 1990) was applied, recording images at 355 eV for calcium (background image at 335 eV), at 935 eV for copper (background image at 915 eV) and at 1068 eV for zinc (background image at 1010 eV), with an energy-selective slit width of 8 eV each. Subtraction of the respective background image from the element-sensitive image using digital image analysis displays the net calcium, copper, and zinc distribution.

Lead could not be examined since element-specific electron loss was out of range of the EFTEM detection unit; cadmium's energy loss interferes with nitrogen and is usually hidden in biological materials.

Statistics

The statistical significance of differences in metal content (comparison of two independent means) was performed with Student's *t*-test. Significance levels: **for $0.001 < P < 0.01$ (significant), ***for $P < 0.001$ (highly significant).

Results

Heavy metal accumulation

Both diplopod species, *L. belgicus* and *G. marginata*, from the smelter's spoil bank in Braubach showed increased metal contents, especially of lead and cadmium. The highest concentrations of these two metals were detected in the intestine of both species; the 'remaining body' contained comparatively small amounts of lead and cadmium. Zinc could be found predominantly in the 'remaining body'. Since this fraction consisted mainly of the cuticle and the connective tissue underlying it, zinc is supposed to be mainly located there. However, in *G. marginata*, more than 150 mg Zn kg^{-1} dry weight on the average were detected also in the intestine. Compared with *L. belgicus*, the Braubach specimens of *G. marginata* were found to contain higher concentrations of all examined metals in both body fractions. Detailed data are shown in Figure 1.

Metal absorption during gut passage

As shown in Figure 2, in uncontaminated (control) animals as well as in contaminated ones the absolute amounts of lead and cadmium ingested with the food and excreted via the feces resemble each other. This is not dose-dependent or influenced by the preconditions of the diplopods. Both metals, lead as well as cadmium, are assimilated—concerning the net metal mass which remains in the animals—only scarcely. The higher metal contents measured and calculated for the feces compared with the food are supposed to be due to measurement inaccuracies which may occur in the nanogramm range. This, however, is not in contrast to the fact that in diplopods net assimilation of lead and cadmium during the gut passage is very low and may be restricted to just a few percent of the ingested heavy metal amount.

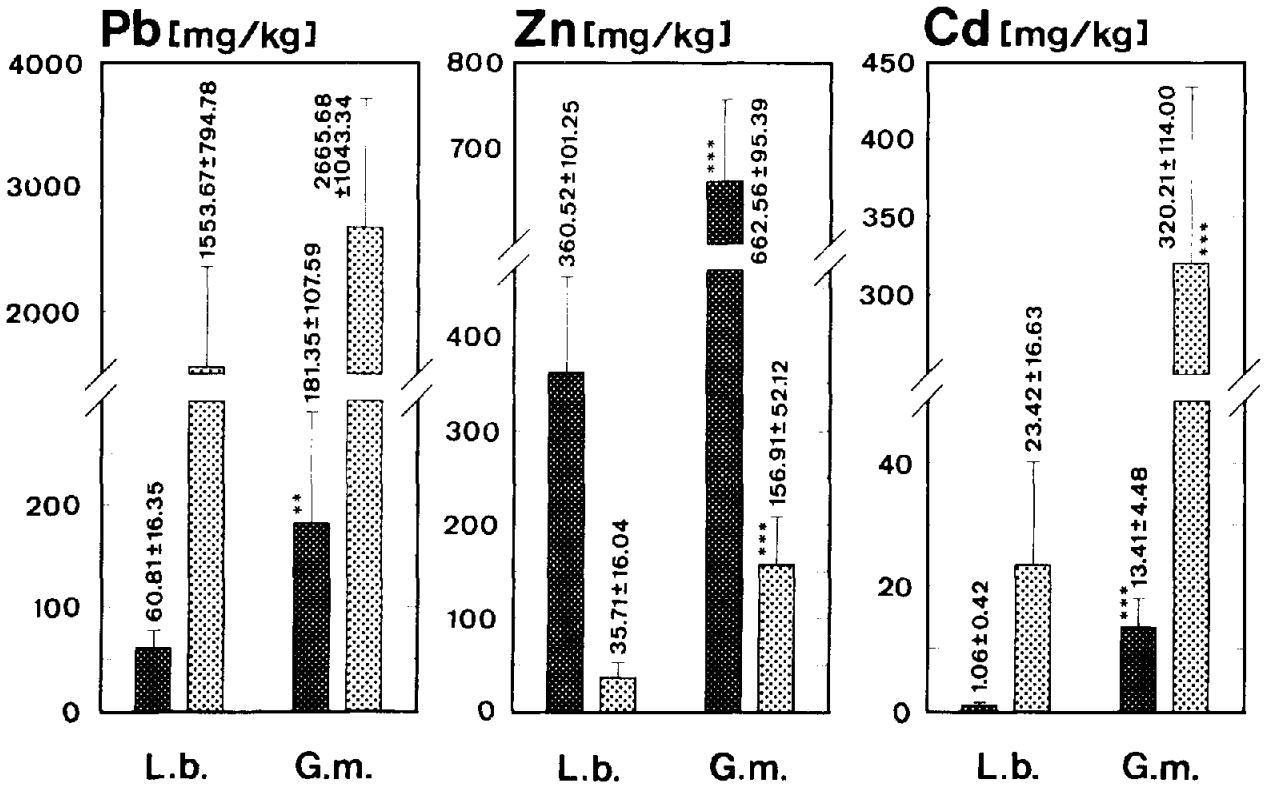

Figure 1. Concentrations of lead, zinc and cadmium in the intestine tissues and the 'remaining body' fraction in *L. belgicus* (L.b.) and *G. marginata* (G.m.). Data represent means + SD (mg kg^{-1} dry weight). Asterisks indicate significantly higher metal concentrations in the fractions of *G. marginata* compared with the respective fractions of *L. belgicus*. ⬚, intestine; ▩, 'remaining body'.

Contrary to these conditions, a net portion of 33.8–37.5% of ingested zinc was found to remain in the diplopods, but only when applied to animals taken from uncontaminated sites or under control conditions. Even under control conditions, zinc assimilation could be found since low concentrations of the essential metal zinc were also present in food material (leaf litter) which was not additionally artificially contaminated. Diplopods preconditioned in a former mining area (permanent contamination) excreted nearly the same zinc amount as ingested with the food.

Ultrastructure of the resorptive epithelium of the midgut

The diplopod's midgut is composed of a single epithelial layer of resorptive and regeneration cells surrounded by a circular muscle layer and underlaid by longitudinal muscle fibers. The resorptive epithelial cells are in close contact to isolated hepatic ('liver') cells which protrude into the hemocoel. A detailed ultrastructural description of the midgut of diplopods is given by Köhler & Alberti (1992).

As shown by SEM as well as by TEM, the apical and central part of the resorptive cells are characterized by numerous spherites, mainly anorganic deposits located in vacuoles (Figure 3a–c). These spherites are concentrically structured and consist of alternating electron-dense and electron-lucent layers. Spherite-containing vacuoles often fuse with lysosomes (Figure 3c).

In regeneration cells which appically do not reach the lumen of the gut spherites could never be detected.

Spherite composition

The unspecific sulfide precipitation indicated the presence of 'heavy metals' in the spherites of the resorptive midgut cells of *T. niger* from a long-term contaminated area (Figure 3d). Distinct elements in these spherites, however, could be proven by means of ESI and EELS. EELS spectra of single spherites showed high amounts of calcium. Additionally, zinc could be identified in the EELS spectra since the element-specific peak differed clearly from the extrapolation curve. Copper, however, could not be found by EELS (Figure 4). ESI studies also showed the presence of calcium and zinc in the spherites of *L. belgicus*, whereas the distribution of copper could not be assigned to occur specifically in these granules (Figure 5).

Discussion

The epithelium of the intestinal tract is assigned to be a layer of cells in direct contact with the external environment which must provide the first line of defense against penetration of a variety of potentially noxious substances which reside in the intestinal lumen (Walker 1976). Therefore, the most important regulatory organs for

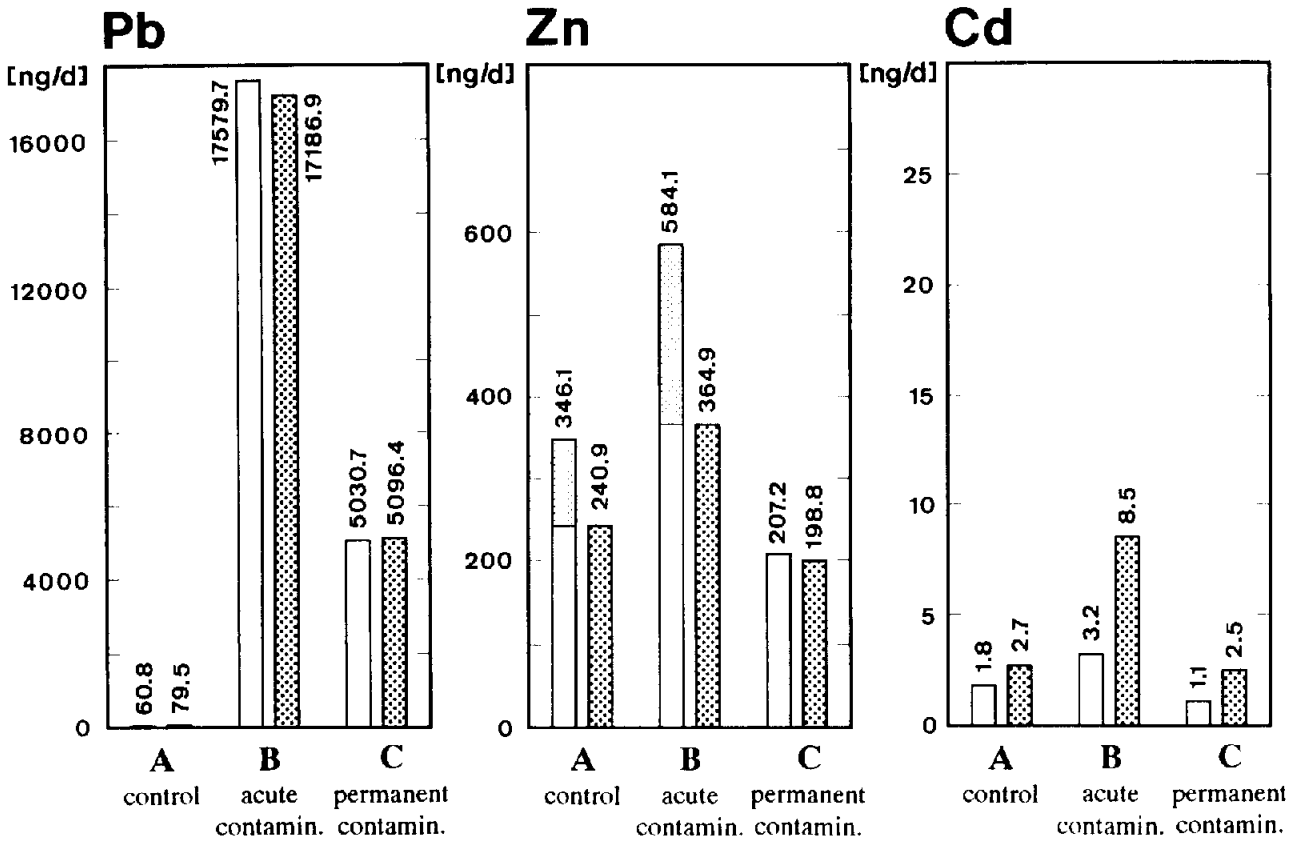

Figure 2. Daily ingested (bright bars) and by defecation excreted (dark bars) mass of lead, zinc and cadmium. A, B, and C are in accordance with the examined diplopod groups (see text). The slightly darker parts of the bright bars (in the zinc plot) represent the net portion of zinc that remained in the diplopods. Since calculated out of means, data must be given without standard deviation.

metals in terrestrial invertebrates are those associated with the digestive system (Hopkin 1989).

From all the metals present in the intestinal lumen of diplopods only a selection is preferently absorbed by the epithelium, although acid conditions in the lumen of the midgut [down to pH 5.6 (Nuñez 1975, Shukla & Shukla 1981)] guarantee considerable portions of all heavy metals being present in soluble form. Corresponding to the present results, Hopkin *et al.* (1985) estimated 38.4% of the ingested zinc being absorbed whereas cadmium (8.2%) and lead (below 0.1%) are assimilated in lower amounts. Diplopods from heavy metal contaminated sites which contain high amounts of zinc seem to manage to excrete this metal in similar amounts as ingested. Thus, this could be a possibility to maintain the animals' zinc content relatively constant. Similar mechanisms have been described for chilopods (Hopkin 1989). Although active transport systems for non-essential metals are unknown and thus lead and cadmium mainly are excluded from membrane passage, at times high concentrations of even these metals may occur in diplopods under permanent heavy metal conditions. Some exemplary data are given here: *P. angustus*: 47 mg kg^{-1} Pb, 406 mg kg^{-1} Zn (Morgan *et al.* 1986); *T. niger/Oxidus* sp.: 511–786 mg kg^{-1} Cu, 14.2–18.9 mg kg^{-1} Cd (Hunter *et al.* 1987); *G. marginata*: 32.4 mg kg^{-1} Pb, 714.8 mg kg^{-1} Zn, 71.5 mg kg^{-1} Cu, 26.5 mg kg^{-1} Cd (Read & Martin 1988). Since these studies did not distinguish between different organ fractions, the data are not completely comparable with the present results since metal concentrations in certain organs evidently can be much higher than would be apparent only from quantities in the homogenate of the animal. Considering the metal-burdened intestinal tract (just as a small portion of the animal's dry weight), however, metal concentrations of the *whole* animal in the present study may correspond to the data cited above.

As mentioned above, diplopods should possess effective mechanisms to bind and to detoxify potentially toxic (and here especially non-essential) metals in the midgut tissue in order to prevent these toxicants from being distributed throughout the whole body. Therefore, non-essential metals are predominantly stored in the intestine (and its appendices) of soil animals whereas those metals being essential for the respective animals could often also be found in other organs. Thus, for example, in the snail *Arianta arbustorum*, cadmium is deposited primarily in the hepatopancreas (Berger & Dallinger 1989). In the same species, copper, however, which is essential for the formation of hemocyanin, is distributed throughout the body with dominance in the tissues of the footsole and mantle (Berger & Dallinger 1989). Chilopods also store lead and cadmium in the midgut tissue; zinc, in contrast, is stored in the fat body (Hopkin & Martin 1983). Confirmed by the present results, in diplopods, cadmium and lead is

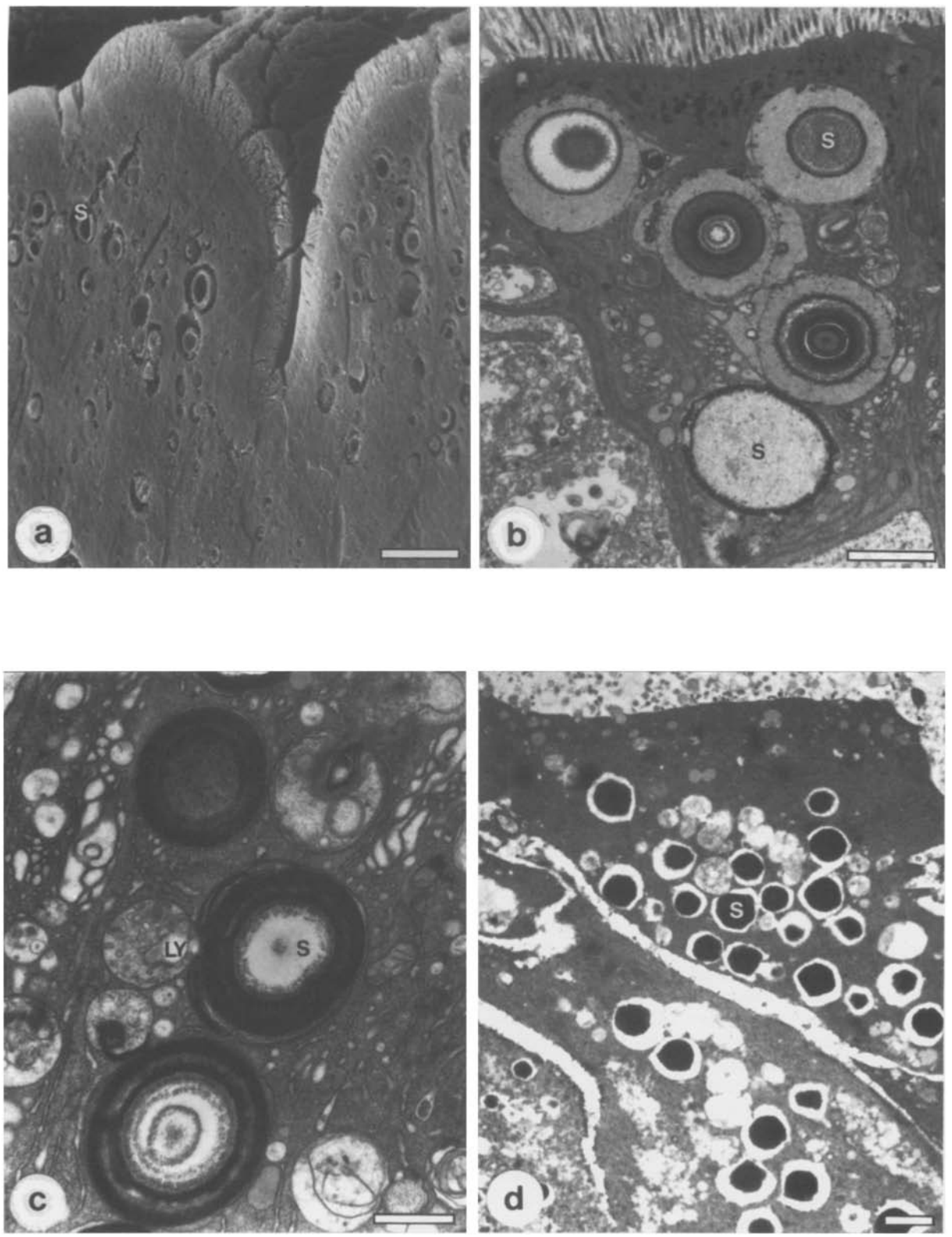

Figure 3. Sections of resorptive epithelial cells in the midgut of diplopods. S = spherites. (a) *T. niger*, control animal, SEM, apical cell region. Scale bar: 5 μm. (b) *C. punctatus*, control animal, TEM, spherites in the apical cell region. Scale bar: 2 μm. (c) *J. scandinavius*, control animal, TEM, spherites in the median cell region. Note the lysosome (LY) fusing with a spherite-containing vacuole. Scale bar: 1 μm. (d) *T. niger*, specimen from the Braubach spoil bank site, unspecific heavy metal precipitation (deep black) in the spherites. Scale bar: 2 μm.

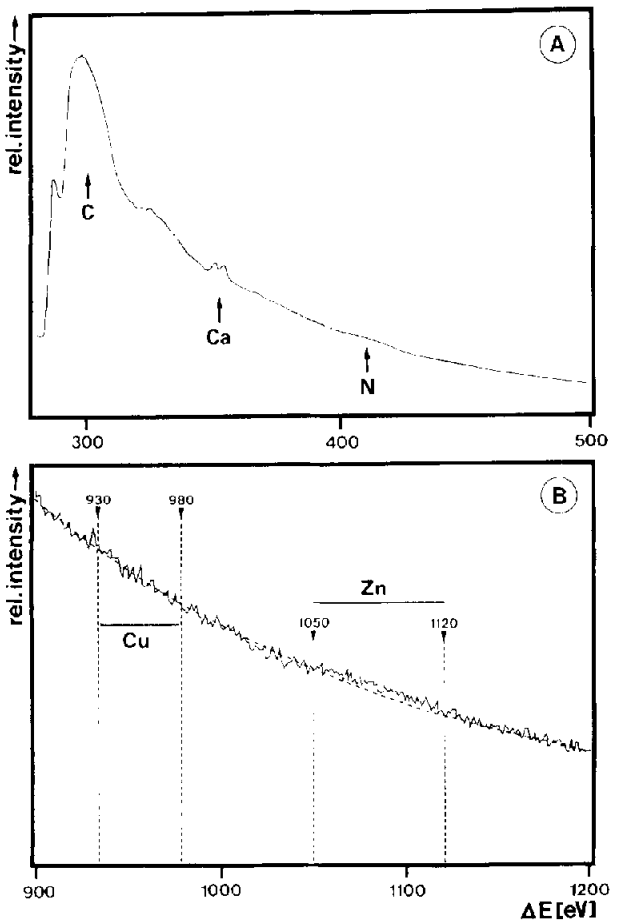

Figure 4. EELS spectra of a spherite in the median cell region of a resorptive epithelial cell in *T. niger* from the Braubach spoil bank site. (A) Spectrum region of low electron energy loss (280–500 eV) showing the presence of carbon, calcium and nitrogen. (B) Spectrum region of higher electron energy loss (900–1200 eV) in a higher magnification showing the presence of zinc. Copper could not be found in the spherites. Interrupted line: intrapolated (below 1050 eV) and extrapolated (above 1050 eV) regression line of 'background noise'.

localized predominantly in the midgut, while, iron, for example, could be found in the Malpighian tubules (Hubert 1978a). Zinc, according to Hopkin (1989), is supposed to be localized in the sub-cuticular layer, since it could be found in the cuticular fraction—including the attached epidermal tissue—whereas moulted exoskeletons did not contain any zinc. Considering pre-moult resorption of endocuticular substances, however, zinc *in situ* may be localized in as well as close to the endocuticle. This storage site seems to be very suitable for diplopods, since zinc is an essential component of carbonic anhydrase, an enzyme involved in the formation of calcium carbonate and being of extraordinary importance for organisms with a calcified cuticle or shell e.g. diplopods, isopods and snails (Maren 1967).

Uptake of metals into the midgut cells can take place in different ways: through ion channels, e.g. for manganese (Anderson 1979), via pinocytotic vesicles or, after formation of lipophilic metal complexes, directly by solution in the cell membrane (e.g. copper, zinc, cadmium or mercury) Simkiss 1983, Hopkin 1989). After uptake, metals can be intracellularly bound to proteins (e.g. metallothioneins or metallothionein-like proteins, ferritin) in order to prevent them from cellular metabolism or just to store them (e.g. Harrison 1977, Crichton 1982, Hunziger & Kägi 1985, Kägi 1987). Subsequently, metal storage can take place by intracellular precipitation (formation of insoluble salts) in spherites, which may prevent cellular metabolism for the most part from being adversely affected by these ions.

Several metals have been detected in spherites of diplopods in the past. These granules predominantly consist of calcium and phosphorous (as phosphate), but also contain zinc, manganese, copper, magnesium, iron, potassium and silicon (Hubert 1978a, 1979). Confirming these observations, in the present study, in diplopod spherites calcium and zinc but not copper were proven by EELS and ESI, which were applied on this subject for the first time. In spherites of other terrestrial invertebrates which belong to different groups of granules, numerous other metals have been proven (for a review, see Hopkin 1989). Based on chemical composition and ultrastructural appearance, Hopkin (1989, 1990) and Hopkin *et al.* (1989b) distinguish three types of *intracellular* located granules:

Type A granules. These granules are concentrically structured and are composed of concentric layers of amorphous calcium and magnesium ortho- and pyrophosphate with a small organic component (Howard *et al.* 1981, Taylor *et al.* 1986). They also contain zinc, manganese, potassium and sometimes lead, but no cadmium, copper or mercury (Mason & Simkiss 1982, Hopkin 1989, 1990). Type A spherites occur, for example, in intestinal cells of collembolans, cockroaches, and nematodes, in hepatopancreatic cells of spiders and gastropods, and in cells of the Malpighian tubules of Diptera (summarized in Hopkin 1989). Based on the results of the present study, the spherites in the resorptive epithelial cells of the midgut of diplopods also have to be integrated into this group of intracellular granules. EELS and ESI are assigned to be suitable methods for evaluating metal composition of these structures, especially since the presence of a few hundred metal atoms per section 'window' is sufficient for a positive signal. It is likely that originally type A granules are involved in calcium storage and regulation of intracellular calcium level, and are able to deposit (and possibly detoxify) other 'class A' and 'borderline' metals (Simkiss 1976, Hopkin 1989).

Type B granules. The appearance of these granules is heterogenous; however, they usually consist of electron-dense material. They contain for the most part organic substances, and always sulfur in association with cadmium, copper, mercury, zinc, lead and iron (Hopkin 1989). In cells lacking type A granules, they also contain small amounts of calcium (Hopkin & Martin 1982). This granule type usually occurs in cells of the intestine and its appendices: in midgut cells of dipterans and cockroaches,

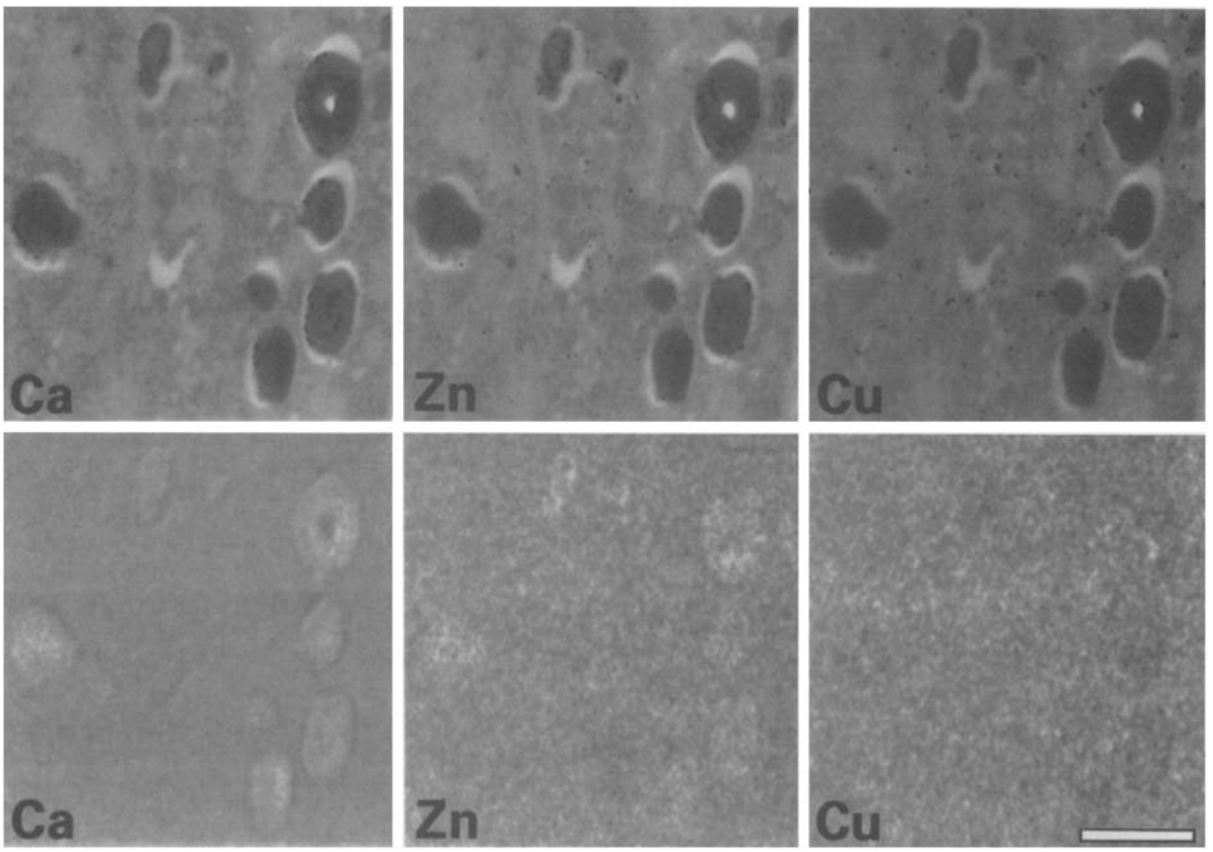

Figure 5. ESI images of spherites in the median cell region of a resorptive epithelial cell in *L. belgicus* from the Braubach spoil bank site. Localization of calcium and zinc (deep black) in the spherites. Copper could not be proven to be stored preferently in spherites. Lower row: net element distribution displayed by subtraction of the background image from the element-sensitive image. A combination of these images with the respective negatives of the structure-sensitive images at 250 eV showed the elemental distribution within the cell (upper row). Scale bar: 1 μm.

hepatopancreatic cells of spiders and isopods, in the chloragogeneous tissue of earthworms, and in the fat body cells of homopterans (summarized in Hopkin 1989).

Type C granules. These granules are of crystalline structure (Cheung & Marshall 1973) or appear as deposits of flocculent material (Hopkin 1989). The high iron content of these structures and their ultrastructural appearance led to the suggestion that they are storage sites for ferritin or its breakdown products (Cheung & Marshall 1973, Taylor & Simkiss 1984). They occur in hepatopancreatic B cells of isopods (Hopkin & Martin 1982) or in midgut cells of homopterans and lepidopteran larvae (summarized in Hopkin 1989). Granules rich in iron have also been observed in the hepatic cells of diplopods (Hubert 1979). These cells, however, were not investigated in the present study.

Since toxic metals are almost completely withdrawn from cellular metabolism, they may possibly remain in the tissues of soil invertebrates until death, they accumulate and lead to sometimes enormous metal concentrations in old specimens (see, e.g. Hames 1989). However, in the course of regeneration of spherite-containing tissues of the intestinal tract they also can be excreted together with cell debris via the lumen of the gut. This is the more important for diplopods since they are known to replace their midgut epithelium completely during every moult cycle (Hubert 1979). In some hepatopancreatic cells of isopods (B cells) and spiders this way of heavy metal excretion is common (Hames 1989, Hopkin 1989). Since these 'metal excretion processes' are—similar to metal uptake and resorption—quantitatively not identical in different animal species, the metal contents of organs can differ between sometimes even closely related species (Hames & Hopkin 1991). Thus, in the present study, average metal contents were always less in *L. belgicus* than in *G. marginata*. Similar observations have been made concerning zinc concentrations in the isopods *Oniscus asellus* and *Porcellio scaber* (Hopkin *et al.* 1989a).

Summarizing the present studies, metal uptake as well as metal accumulation could be quantified for some diplopod species. Additionally, the storage sites of some metals in the resorptive tissue were localized by means of EELS and ESI—completely new methods in this field. Other physiological and biochemical reactions of the influenced cells, e.g. induction of metal-binding proteins (possibly metallothioneins) or induction of other 'stress proteins' (possibly heat-shock-proteins) which allow the cells to survive intoxication as well as the direct adverse

affections of the free metal ions (possibly protein denaturation), are up to now (as far as we know) unknown for diplopods and form a wide field of interest for future studies.

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References

Anderson M. 1979 Mn^{2+} ions pass through Ca^{2+} channels in myoepithelial cells. *J Exp Biol* **82**, 227–238.

Berger B, Dallinger R. 1989 Accumulation of cadmium and copper by the terrestrial snail *Arianta arbustorum* L.: kinetics and budget. *Oecologia* **79**, 60–65.

Beyer WN, Pattee OH, Sileo L, Hoffman DJ, Mulhern BM. 1985 Metal contamination in wildlife living near two zinc smelters. *Environ Pollut* **38A**, 63–86.

Carter A. 1983 Cadmium, copper and zinc in soil animals and their food in a red clover system. *Can J Zool* **61**, 2751–2757.

Cheung WWK, Marshall AT. 1973 Studies on water and ion transport in homopteran insects: ultrastructure and cytochemistry of the cicapoid and cercopoid midgut. *Tiss Cell* **5**, 651–669.

Crane DF, Cowden RR. 1968 A cytochemical study of oocyte growth in four species of millipedes. *Z Zellforsch Mikrosk Anat* **90**, 414–431.

Crichton RR. 1982 Ferritin—the structure and function of an iron storage protein. In: Dunford HB, Dolphin D, Raymond KN, Sieker L, eds. *The Biological Chemistry of Iron*. New York: Reidel; 45–61.

Dallinger R, Prosi F. 1988 Heavy metals in the terrestrial isopod *Porcellio scaber* LATREILLE. II. Subcellular fraction of metal-accumulating lysosomes from hepatopancreas. *Cell Biol Toxicol* **4**, 97–109.

Hames CAC. 1989 The digestive system and metals in terrestrial isopods. *PhD Thesis*, Reading.

Hames CAC, Hopkin SP. 1991 Assimilation and loss of ^{109}Cd and ^{65}Zn by the terrestrial isopods *Oniscus asellus* and *Porecellio scaber*. *Bull Environ Cont Toxicol* **47**, 440–447.

Harrison PM. 1977 Ferritin: an iron storage molecule. *Sem Haematol* **14**, 55–70.

Hopkin SP. 1989 *Ecophysiology of Metals in Terrestrial Invertebrates*. New York: Elsevier.

Hopkin SP. 1990 Critical concentrations, pathways of detoxification and cellular ecotoxicology of metals in terrestrial arthropods. *Funct Ecol* **4**, 321–327.

Hopkin SP, Martin MH. 1982 The distribution of zinc, cadmium, lead and copper within the hepatopancreas of a woodlouse. *Tiss Cell* **14**, 703–715.

Hopkin SP, Martin MH. 1983 Heavy metals in the centipede *Lithobius variegatus* (Chilopoda). *Environ Pollut B* **6**, 309–318.

Hopkin SP, Watson K, Martin MH, Mould ML. 1985 The assimilation of heavy metals by *Lithobius variegatus* and *Glomeris marginata* (Chilopoda; Diplopoda). *Bijdr Dierk* **55**, 88–94.

Hopkin SP, Hames CAC, Bragg S. 1989a Terrestrial isopods as biological indicators of zinc pollution in the Reading area, south east England. *Monit Zool Ital (NS) Monogr.* **4**, 477–488.

Hopkin SP, Hames CAC, Dray A. 1989b X-ray microanalytical mapping of the intracellular distribution of pollutant metals. *Microsc. Anal* **14**, 23–27.

Howard B, Mitchell PCH, Ritchie A, Simkiss K, Taylor M. 1981 The composition of intracellular granules from the metal accumulating cells of the snail *Helix aspersa*. *Biochem J* **194**, 507–511.

Hubert M. 1974 Le tissu adipeux de *Cylindroiulus teutonicus* Pocock (*londinensis* C.L.K) Diplopoda, Iuloidea; étude histologique et ultrastructurale. *CR Acad Sci Paris* **278D**, 3343–3346.

Hubert M. 1975 Sur le nature des accumulations minérales et uriques chez *Cylindroiulus teutonicus* Pocock (*londinensis* C.L.K., Diplopoda, Iuloidea). *CR Acad Sci Paris* **281D**, 151–156.

Hubert M. 1978a Données histophysiologique complémentaires sur les bioaccumulations minérales et puriques chez *Cylindroiulus londinensis* (Leach, 1814) (Diplopoda, Iuloidea). *Arch Zool Exp Gen* **119**, 669–683.

Hubert M. 1978b Les cellules hépatiques de *Cylindroiulus londinensis* (Leach 1914) (Diplopoda, Iuloidea). *CR Acad Sci Paris* **286D**, 627–630.

Hubert M. 1979 Localization and identification of mineral elements and nitrogenous waste in Diplopoda. In: Camatini M, ed. *Myriapod Biology*. London: Academic Press; 127–133.

Hunter BA, Johnson MS, Thompson DJ. 1987 Ecotoxicology of copper and cadmium in a contaminated grassland ecosystem. I. Soil and vegetation contamination. *J Appl Ecol* **24**, 573–586.

Hunziger PE, Kägi JHR. 1985 Metallothionein. In: Harrison PM, ed. *Metalloproteins. Part 2, Metal Proteins with Non-redox Roles*. Basingstoke: Macmillan Press; 149–181.

Karnowski MJ. 1971 Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. *J Cell Biol* **52**, 284.

Kägi JHR. 1987 *Metallothioneins II*. Basel: Birkhäuser.

Köhler H-R, Alberti G. 1992 The effect of heavy metal stress on the intestine of diplopods. *Ber nat-med Ver Innsbruck Suppl* **10**, 257–267.

Köhler H-R, Alberti G, Storch V. 1991 The influence of the mandibles of Diplopoda on the food—a dependence of fine structure and assimilation efficiency. *Pedobiologia* **35**, 108–116.

Köhler H-R, Storch V, Alberti G. 1992 The impact of lead on the assimilation efficiency of laboratory-held Diplopoda (Arthropoda) preconditioned in different environmental situations. *Oecologia* **90**, 113–119.

Körtje K-H, Freihöfer D, Rahmann H. 1990 Cytochemical localization of calcium in the central nervous system of vertebrates. *Ultramicroscopy* **32**, 12–17.

Maren TH. 1967 Carbonic anhydrase: chemistry, physiology and inhibition. *Physiol Rev* **47**, 595–781.

Martoja R, Lhonore D, Ballan-Dufrancais C. 1977 Bioaccumulation minerale et purique chez les insectes planipennes, *Euroleon nostras*, *Myrmeleon hyalinus*, *Acanthaclisis beaticus*, *Sisyra fuscata*, *Chrysopa* sp. *Arch Zool Exp Gén* **118**, 441–455.

Mason AZ, Simkiss K. 1982 Sites of mineral deposition in metal-accumulating cells. *Exp Cell Res* **139**, 383–391.

Morgan AJ, Morris B, James N, Morgan JE, Leyshon K. 1986 Heavy metals in terrestrial macroinvertebrates: species differences within and between trophic levels. *Chem Ecol* **2**, 319–334.

Neumann W. 1985 Veränderungen am Mittledarm von *Oxidus gracilis* (C. L. Koch, 1847) während einer Häutung (Diplopoda). *Bijdr Dierk* **55**, 149–158.

Nuñez FS. 1975 The digestive tract and digestive enzymes of the desert millipede *Orthoporus ornatus* (GIRARD) (Diplopoda: Spirostreptidae). *Diss Abstr Int (B)* **36**, 1033.

Ottensberger FP, Andrew JW. 1980 High resolution microanalysis of biological specimens by electron energy loss spectroscopy and by electron spectroscopic imaging. *J Ultrastruct Res* **72**, 336–348.

Petit J. 1970 Sur la nature et l'accumulation de substances minérales dans le ovocytes de *Polydesmus complanatus* (Myriapode: Diplopode). *CR Acad Sci Paris* **270**, 2107–2110.

Randow E. 1924 Zur Morphologie und Physiologie des Darmkanals der Juliden. *Z Wiss Zool* **122**, 534–582.

Read HJ, Martin MH. 1988 A study of myriapod communities in woodlands contaminated with heavy metals. *Proc. 7th Int. Congr. Myriapodology*, Vittorio-Veneto, 1987; 289–298.

Reynolds ES. 1963 The use of lead citrate at high pH as electron opaque stain in electron microscopy. *J Cell Biol* **17**, 208–212.

Rosenbauer KA, Kegel BH. 1978 *Rasterelektronenmikroskopische Technik*. Stuttgart: Thieme.

Schlüter U. 1979a Spezialisierte Epithelzellen im Bereich des Proctodaeums von *Polydesmus* sp. (Myriapoda: Diplopoda). *Verh dtsch zool Ges (Stuttgart)* **72**, 300.

Schlüter U. 1979b The ultrastructure of an exocrine gland complex in the hind-gut of *Scaphiostreptus* sp. (Diplopoda: Spirostreptidae). In: Camatini M, ed. *Myriapod Biology*. London: Academic Press; 143–155.

Schlüter U. 1980a Die Feinstruktur der Pylorusdrüsen von *Polydesmus angustus* LATZEL und *Glomeris marginata* VILLERS (Diplopoda). *Zoomorphologie* **94**, 307–319.

Schlüter U. 1980b Plasmalemma–mitochondrial complexes involved in water transport in the hindgut of a millipede, *Scaphiostreptus* sp. *Cell Tissue Res* **205**, 333–336.

Schlüter U. 1980c Ultrastruktur der Pyloruszähnchen zweier Tasusendfüßler (*Tachypodoiulus niger*, *Polydesmus angustus*). *Acta Zool* **61**, 171–178.

Schlüter U, Seifert G. 1985 Functional morphology of the hindgut–malpighian tubule complex in *Polyxenus lagurus* (Diplopoda; Penicillata). *Bijdr Dierk* **55**, 209–218.

Seifert G, Rosenberg J. 1977 Feinstruktur der Leberzellen von *Oxidus gracilis* (C. L. KOCH, 1847) (Diplopoda, Paradoxosomatidae). *Zoomorphologie* **88**, 149–162.

Shukla GS, Shukla SC. 1981 Effect of starvation on free amino acids, pH and glycogen contents of gut in millipede *Trigoniulus lumbricinus* (Gerstacker). *Comp Physiol Ecol* **6**, 159–162.

Siegel SM, Siegel BZ, Puerner N, Speitel T, Thorarinsson F. 1975 Water and soil biotic relations in mercury distribution. *Water Air Soil Pollut* **4**, 9–18.

Simkiss K. 1976 Intracellular and extracellular routes in biomineralization. *Symp Soc Exp Biol* **30**, 423–444.

Simkiss K. 1983 Lipid solubility of heavy metals in saline solutions. *J Mar Biol Ass UK* **63**, 1–7.

Spurr AR. 1969 A low viscosity embedding medium for electron microscopy. *J Ultrastruct Res* **26**, 31–43.

Taylor MG, Simkiss K. 1984 Inorganic deposits in invertebrate tissues. *Environ Chem* **3**, 102–138.

Taylor M, Simkiss K, Greaves GN. 1986 Amorphous structure of intracellular mineral granules. *Biochem Soc Trans* **14**, 549–552.

Timm F. 1958 Zur Histochemie der Schwermetalle. Das Silbersulfidverfahren. *Dt Z gerichtl Med* **46**, 706–711.

Verhoeff KW. 1932 *Bronn's Klassen und Ordnungen des Tier-Reichs, 5, II: Gliederfüssler: Arthropoda, Klasse Diplopoda*, 2. Leipzig: Akademische Verlagsgesellschaft.

Walker WA. 1976 Host defense mechanisms in the gastrointestinal tract. *Pediatrics* **57**, 901–916.

Weissenfels N. 1982 Rasterelektronenmikroskopische Histologie von spongiösem Material. *Microscop Acta* **85**, 345–350.