Ber. nat.-med. Verein Innsbruck	Suppl. 10	S. 257 – 267	Innsbruck, April 1992

8th International Congress of Myriapodology, Innsbruck, Austria, July 15 - 20, 1990

The Effect of Heavy Metal Stress on the Intestine of Diplopods

by

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Abstract: The intestines of several diplopod species from different families were investigated by transmission electron microscopy. Fore- and hindgut, covered by a thick cuticle, showed no signs of being sensitive to nutritional stress (food deprivation, artificial acidification of nutrients). On the other hand, the ultrastructure of the midgut and of the associated "liver cells" was affected. In the present study, these structures from specimens fed under laboratory conditions with lead contaminated food and from a control group without heavy metal influence were compared. Further specimens taken from a location contaminated with heavy metals were investigated.

In contrast to the control group, under both natural and artificial heavy metal conditions, the epithelial cells of all specimens disconnected from one another basally and often apically as well, and displayed an electron-dense cytoplasm. The protrusions of the "liver cells", which connect these cells with the epithelial layer, appeared elongated. The cytoplasm of the "liver cells" became electron-dense as well, and the surface facing the haemocoel formed numerous filiform processes of small size. However, it is suggested that these alterations are not specifically caused by heavy metals, but reflect general environmental stress.

Using atomic absorption spectrophotometry, it was shown that the metals Pb and Cd were located mainly in the intestine and to a lesser degree in the cuticle. On the other hand, Zn was deposited predominantly in the cuticle or in the tissue close to it. Within the midgut epithelium, heavy metals were only detected in the spherites, which are mineral congregations widely distributed throughout the epithelial cells of the midgut.

1. *Introduction:*

Much information has been compiled on metal polluted soils and their impact on soil animals (e.g. ERNST & JOOSSE-VAN DAMME 1983, IRELAND 1988, HOPKIN 1989, MARTIN & COUGHTREY 1986, VAN STRAALEN et al. 1987, WEIGMANN 1989). Several studies have shown that population structure may degenerate under this influence, resulting e. g. in reduced rates of decomposition of organic matter (BENGTSSON & RUNDGREN 1984, GRUTTKE et al. 1987, WALLWORK 1988). Metal levels in soil animals are often related to concentrations measured in the soils they were taken from. Such studies have occurred on phytophagous, saprophagous, as well as carnivorous species (BERGER & DALLINGER 1989, CLAUSEN 1984, HOPKIN & MARTIN 1982, IRELAND 1979, JANSSEN 1989, PROSI et al. 1983). Contaminated food has been shown to affect intestinal tissue on the fine structural level (HOPKIN 1986, HUBERT 1979, PROSI et al. 1983, STORCH 1988). Finally, certain "strategies" of animals for surviving heavy metal pollution have been discovered (DALLINGER et al. 1989, HOPKIN 1986, MOSER & WIESER 1979; WIESER & KLIMA 1969).

Diplopods feed directly on leaf litter, which may contain a heavy burden of metals. In contrast to terrestrial isopods, which have rather similar ecological requirements and have been comparatively well studied under the aspects mentioned above (see HOPKIN et al. 1986, STORCH 1988), diplopods have only received little attention. The present study thus tries:

(1) to improve our knowledge of the ultrastructure of the digestive tract,
(2) to describe the ultrastructural effects of food artificially contaminated with lead, and
(3) to compare these effects with the fine structural aspects observed in animals taken from a heavy metal polluted location.

Thus we hope that our study may contribute to a better understanding of the mechanisms involved in the various responses of soil animals to metal contaminated environments.

2. Material and Methods:

Transmission electron microscopy (TEM): Mature specimens of the species *Glomeris marginata* (VILLERS) (Glomeridae), *Craspedosoma alemannicum* VERHOEFF (Craspedosomatidae), *Mycogona germanica* (VERHOEFF) (Chordeumatidae), *Polydesmus angustus* LATZEL (Polydesmidae), *Julus scandinavius* LATZEL, *Cylindroiulus silvarum* (MEINERT), *Tachypodoiulus niger* (LEACH) and *Ommatoiulus rutilans* (C.L. KOCH) (Julidae) taken from an uncontaminated forest site near Heidelberg, F.R.G. (leaf litter contamination: 1.1 mg kg^{-1} Cd, 29.6 mg kg^{-1} Pb, 219.7 mg kg^{-1} Zn [author's data]) were fixed for TEM directly after collection. Further animals of the following species were fed a lead contaminated leaf litter agar (6 g agar, 6 g leaf litter particles, 200 ml of a 1000 mg l^{-1} Pb $(NO_3)_2$ solution [= 625.6 mg l^{-1} Pb^{2+}]) under laboratory conditions: *C. alemannicum* (for 16 days), *G. marginata, C. silvarum, T. niger* and *O. rutilans* (for 30 days each). Additionally, adults of the species *G. marginata, T. niger* and *Leptoiulus belgicus* (LATZEL) (Julidae) from the heavy metal contaminated site Braubach near Koblenz, F.R.G. (leaf litter contamination: 41.9 mg kg^{-1} Cd, 628.4 mg kg^{-1} Cu, 1658.6 mg kg^{-1} Pb, DALLINGER & PROSI 1988) were fixed for TEM.

After preparation, the intestinal tract was fixed in cacodylate buffered, 2 % glutaraldehyde (pH 7.2), rinsed repeatedly in 0.01 M cacodylate buffer, and postfixed in 1 % osmium tetroxide for 2 h. After washing in 0.01 M cacodylate and 0.05 M maleate buffer (pH 5.2), the specimens were stained *en-bloc* with 1 % uranyl acetate in maleate buffer for at least 1 h at 4° C, dehydrated in a graded ethanol series, and embedded in SPURRs medium (SPURR 1969). Ultrathin sections were stained with alkaline lead citrate for 5 min (REYNOLDS 1963). Transmission electron microscopes: Zeiss EM 9 S-2, Zeiss EM 10 CR.

Silver sulfide method: Precipitation of heavy metals in the midgut was tested in the species *L. belgicus, O. rutilans* and *T. niger* from the Braubach site according to TIMM (1958): fixation with H_2S saturated, cacodylate buffered 2 % glutaraldehyde (pH 7.4) for 2 h, rinse in 0,1 M tris-maleate buffer (pH 7.4), and postfixation in osmium tetroxide: The metal sulfides were developed with 10 % silver nitrate. After dehydration in ethanol and immersion in propylenoxide, the specimens were embedded in Araldite. The ultrathin sections were not stained and were examined with the electron microscopes mentioned above.

Atomic absorption spectrophotometry (AAS): After starving for 2 d, adults of the species *G. marginata* and *L. belgicus* from the Braubach site were dissected and divided into intestinal tract and remaining body, the latter consisting mainly of cuticle. All specimens were digested with 200 µl nitric acid (suprapure grade) at 90° C and analyzed for lead, zinc, and cadmium by graphite furnace atomic spectrophotometry (Perkin Elmer 5000, HGA 500).

3. Results:

The intestine of Diplopoda is a thin tube which passes in a straight line through the body (in *Glomeris* the tract is bent back on itself to form an "S" shape). It is divided into three main parts: fore-, mid-, and hindgut. Through transverse sections, it can be shown that the foregut and the hindgut are folded longitudinally into six folds that project into the lumen of the gut. In both cases the simple epithelial layer is covered by a thick cuticle and associated basally with longitudinal and circular muscle layers. The midgut is formed by a simple epithelial layer, longitudinal and circular muscles, and the basally associated so-called "liver cells" (Fig. 1).

Foregut: The epithelial cells of the folds are up to forty times longer than those of the narrow regions between the folds. In these, the flat cells contain numerous microtubules and form hemidesmosomes basally. The remaining "normal" epithelial cells differ only slightly from one another and exhibit three distinct regions: Basally, several invaginations of the plasmalemma form a basal labyrinth. Only few rER cisternae are detectable here. The nucleus is located centrally. Its chromatin shows only little condensation. Moreover, a well-developed Golgi apparatus close to the nucleus and many rER cisternae characterize the center of the cell. Apically, several mitochondria and small

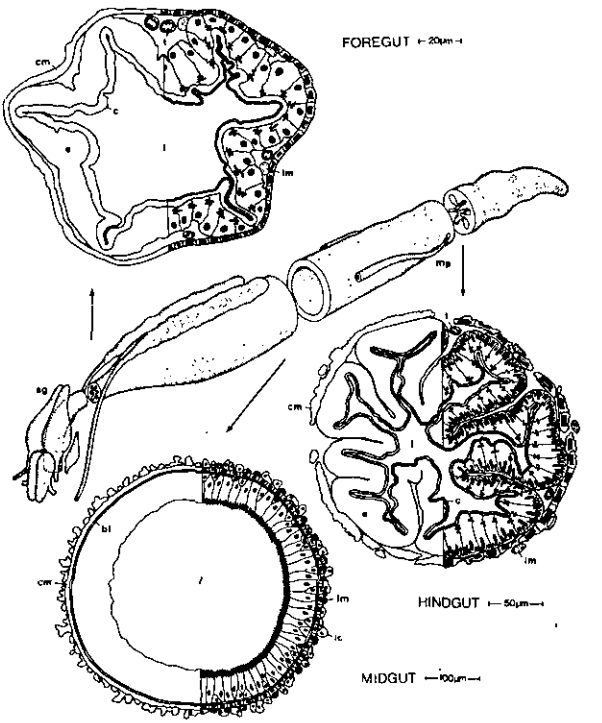

Fig. 1: Schematic representation of the intestinal tract of *C. alemannicum* and sections of fore-, mid- and hindgut.

vesicles are visible. Adjacent cells are connected by desmosomes. The epithelium of the foregut is covered by thick cuticle. The muscularis is located close to the basal lamina and composed of an inner longitudinal and an outer circular layer (Figs 2, 3).

Hindgut: In general the hindgut is similar to the foregut. The longitudinal muscle layer, however, forms the outer part of the muscularis while the circular layer is located under the basal lamina of the epithelium. Occasionally sections of tracheae pass close to the muscularis. The epithelium is covered by a cuticle and exhibits a basal labyrinth as well. In contrast to the foregut, the nucleus is often observed in the basal region. In the central part of the cell, only a few organelles (mitochondria, few ER cisternae) are located within the electron-lucent cytoplasm. Numerous mitochondria and most of the ER is found in the apical region. Septate desmosomes are similarly present (Figs 2, 4a, b).

Since fore- and hindgut were not sensitive to nutritional stress e.g. food deprivation and acidification (unpublished data), the effects of heavy metal stress were examined in the midgut and the associated "liver cells" only.

Midgut:

a) without treatment: The midgut epithelium is similarly constructed in all examined species. Within the midgut, four cell types could be distinguished: resorptive epthelial cells, regenerative cells, muscle cells, and the so-called "liver cells". The dominating cell type is the resorptive epithelial cell. In the "normal" state, the cells show an electron-lucent cytoplasm and a distinct division into five zones. The apex bears numerous microvilli. The region which follows basally is charac-

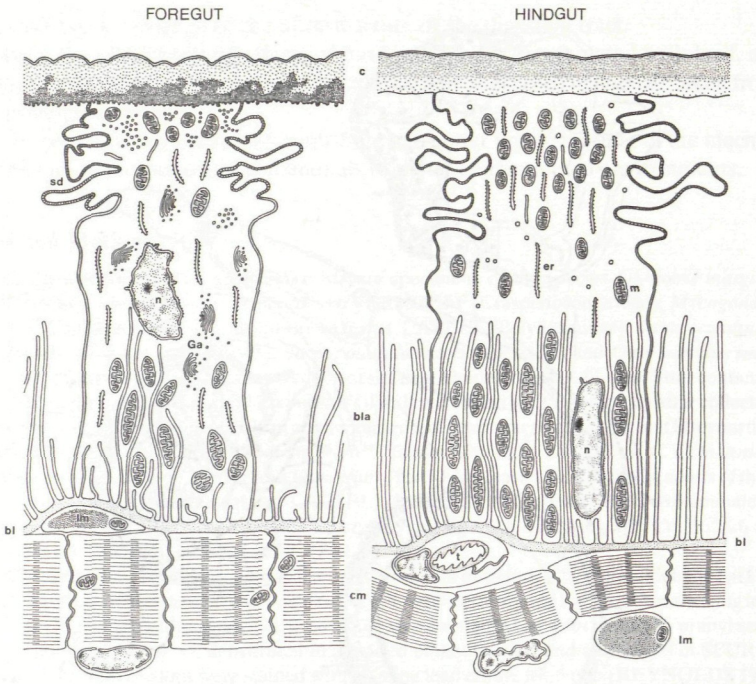

Fig. 2: Drawing of sections through the foregut (left) and the hindgut (right) of a proterandric diplopod in non-contaminated state.

terized by parallel microtubules and small pinocytic vesicles. In the following area the numerous mitochondria are conspicuous. The most extensive part of the epithelial cell then follows. The chromatin of the centrally located nucleus is only slightly condensed. The conspicuous rER is mainly arranged baso-apically. There are several cisternae of the Golgi-apparatus and only few mitochondria. The presence of mineral congregates (spherites) is another striking feature of this zone. These spherites are located within vesicles, in which myelin-like membrane structures also sometimes appear. The basal region is characterized by several invaginations, into which finger-like protrusions of the "liver cells" reach. In their apical part the epithelial cells are connected with one another by smooth septate junctions.

Between the resorptive epithelial cells, regenerative cells exist, whose apices do not reach the lumen of the gut. They are not associated with the mentioned finger-like protrusions of the "liver cells" and do not show any zonation. The electron-lucent nucleus is found in the centre of the cell. Cisternae of the Golgi-apparatus, mitochondria, and ER are evenly distributed throughout the cell. The muscularis is arranged as in the hindgut with an inner circular muscle layer and an outer longitudinal muscle layer.

In almost all examined species, the "liver cells" are loosely arranged close to the muscularis (only in *Glomeris* are they densely packed). Each cell is surrounded by its own glycocalyx. Thus the "liver cells" do not form an epithelial layer. ER-cisternae, mitochondria, and several lysosomes are distributed throughout the cell. The nucleus is located centrally. The "liver cells" form numerous finger-like structures, reaching through the intercellular clefts between the muscle cells, and penetrating through the basal lamina into the invaginations of the resorptive epithelial cells. The regenerative cells, however, are not involved in these intimate interdigitations with the "liver cells" (Figs 5 a - e, 6).

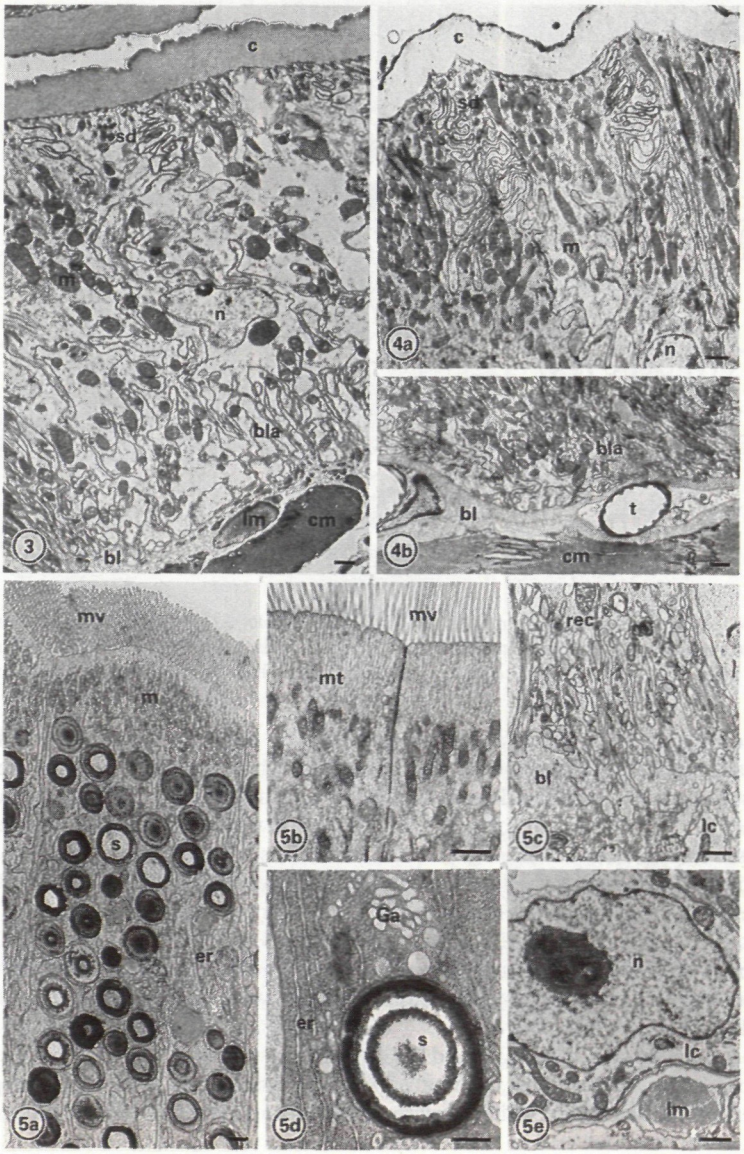

Fig. 3: Foregut of *C. alemannicum* without heavy metal stress. Scale bar = 1 μm.

Fig. 4: Hindgut of *J. scandinavius* without heavy metal treatment. a: Apical and median part, b: Basal part. Scale bar = 1μm.

Fig. 5: Midgut without heavy metal stress. a: *M. germanica,* epithelium. Scale bar = 1 μm. b: *J. scandinavius,* microvilli and apical part of the epithelium. Scale bar = 1μm. c: *P. angustus,* basal part of the epithelium, basal lamina, and finger-like protrusions of the "liver cells". Scale bar = 1μm. d: *O. rutilans,* resorptive epithelial cell. Scale bar = 500 nm. e: *M. germanica,* "liver cell" and muscularis. Scale bar = 1 μm.

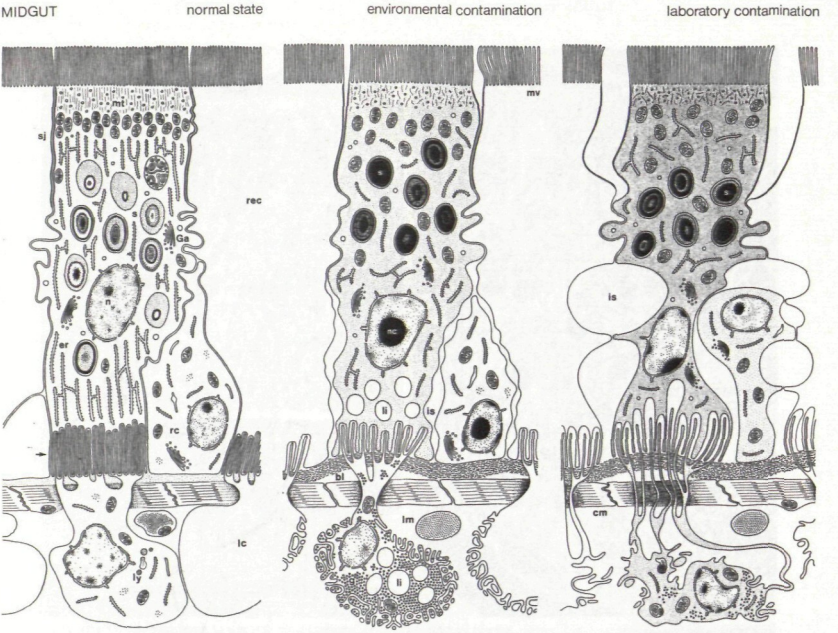

Fig. 6: Drawing of sections through the midgut of a proterandric diplopod without heavy metal treatment (left), under natural conditions in a location contaminated with heavy metals (middle), and under laboratory conditions with the application of lead (right). Arrow: zone of interaction between resorptive cell and "liver cell".

b) Specimens from polluted site: In all species taken from the location contaminated with heavy metals, the midgut cells show striking differences when compared with the control specimens. Most conspicuous is the condensation of the cytoplasm of the resorptive epithelial cells. As a consequence of this reduction in volume, both the resorptive epithelial cells and the regenerative cells are disconnected from one another in their basal and also often in their apical part, resulting in the formation of extensive intercellular spaces. The spherites appear almost completely electron-dense. The distinct zonation of the resorptive cell is no longer present: mitochondria are distributed evenly throughout the cell and the ER is no longer arranged parallel to the longitudinal axis of the cell. The microvilli, the Golgi-apparatus, the muscularis, and the regenerative cells apparently are not different from the control group. The basal lamina, however, appears thickened, folded, and condensed.

An increased storage of glycogen and lipid is sometimes observable in the "liver cells". Additionally, lipid droplets also occur in the basal part of the epithelial cells. The finger-like protrusions of the "liver cells" are elongated while the plasmalemma forms numerous filiform processes (Figs 6, 7 a - e).

c) Specimens fed with lead contaminated food: In all examined specimens, the alterations mentioned above appear more pronounced. The intercellular space is exceedingly extended and the cytoplasm of all cell types, with the exception of the muscularis, appears more condensed. As determined under environmental stress, the distribution of mitochondria and ER within the epithelial cell deviates from the "normal state". The spherites and the basal lamina also show the same appearance as described above. The finger-like protrusions of the "liver cells" are prolonged as well. However, lipid and glycogen storage is less conspicuous under laboratory conditions (Figs 6, 8 a - d).

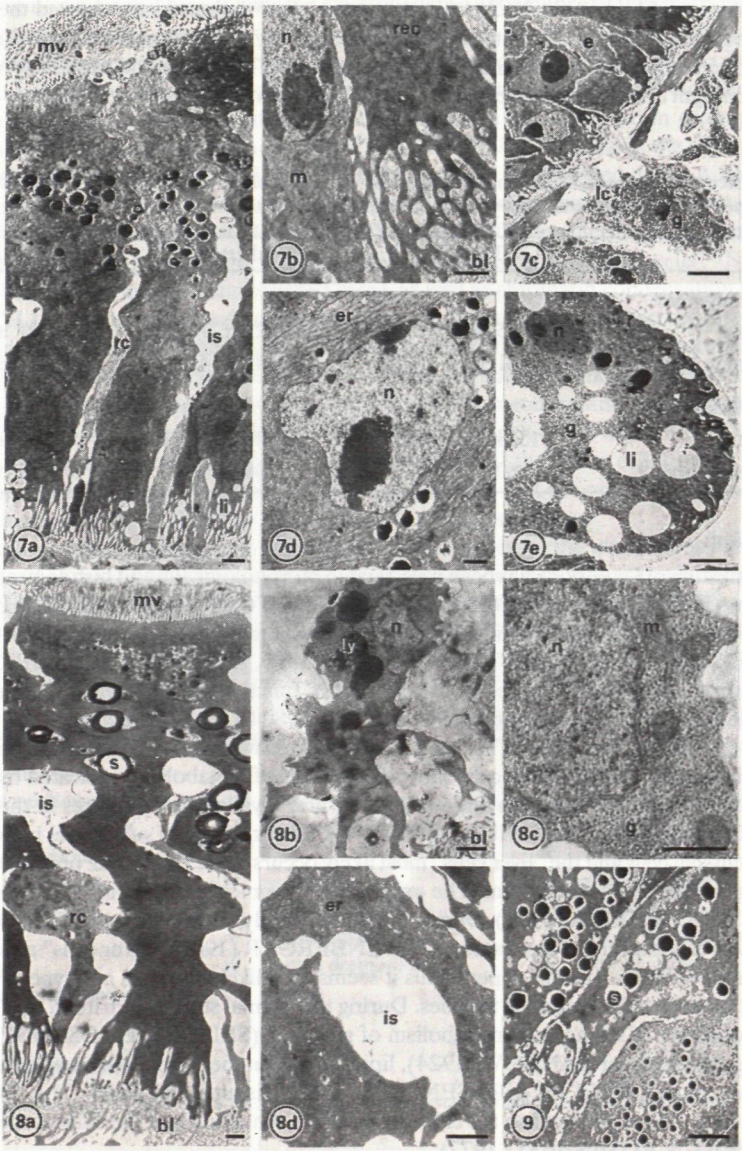

Fig. 7: Midgut under environmental heavy metal stress. a: *L. belgicus,* epithelium. Scale bar = 2 μm. b: *T. niger,* basal lamina, basal part of the epithelium, and finger-like protrusions of the "liver cells". Scale bar = 1 μm. c: *L. belgicus,* basal part of the epithelium, muscularis, and "liver cells". Scale bar = 5 μm. d: *T. niger,* resorptive epithelial cell. Scale bar = 1 μm. e: *G. marginata,* "liver cell". Scale bar = 5 μm.

Fig. 8: Midgut with laboratory heavy metal contamination. a: *O. rutilans,* epithelium and basal lamina. Scale bar = 1 μm. b: *T. niger,* "liver cell" and basal lamina. Scale bar = 1 μm. c: *G. marginata,* "liver cell". Scale bar = 500 nm. d: *G. marginata,* resorptive epithelial cell. Scale bar = 1 μm.

Fig. 9: Localization of heavy metal (deep black) in the spherites of the epithelial cells in the midgut of *T. niger* taken from a heavy metal polluted site (Braubach). Scale bar = 5 μm.

Localization of heavy metals: Atomic absorption spectrophotometry showed the metals Pb and Cd to be stored mainly in the intestine and to a lesser degree in the cuticle. Zn deposition took place predominantly in the cuticle or in the tissue close to it. The detailed data are shown in Table 1. Within the midgut epithelium, the spherites were the only heavy metal storage locations detected at all (Fig. 9).

Table 1: Concentration of lead, cadmium, and zinc in the intestine and the remaining body (mostly cuticle) of diplopods from a stand with heavy metal pollution (Braubach).

	Pb [μg/g]	Cd [μg/g]	Zn [μg/g]
Glomeris marginata			
intestine	2665,68 ± 1043,34	320,21 ± 114,00	156,91 ± 52,12
remaining body	181,35 ± 107,59	13,41 ± 4,48	622,56 ± 95,39
Leptoiulus belgicus			
intestine	1553,67 ± 794,78	23,42 ± 16,63	35,71 ± 16,04
remaining body	60,81 ± 16,35	1,06 ± 0,43	360,52 ± 101,25

4. Discussion:

The intestine of Diplopoda has been investigated morphologically and histologically in earlier publications (EFFENBERGER 1909, NUNEZ & CRAWFORD 1977, RANDOW 1924, VERHOEFF 1914, 1928/1932), including ultrastructure and functional aspects (NEUMANN 1985, SCHLÜTER 1980 a, b, c, SCHLÜTER & SEIFERT 1985, SEIFERT & ROSENBERG 1977). While transport of the food pulp seems to be the main function of the foregut, the hindgut is involved in defaecation, water resorption (SCHLÜTER 1980 b), and destruction of the peritrophic membrane (SCHLÜTER 1980 c). The best known region, however, is also the largest portion of the intestine: the midgut. Its epithelial cells show a high state of metabolism, indicated by numerous vesicles, decondensed chromatin, a large nucleolus, and myelin-like structures in the vicinity of spherites (GOURANTON 1968, HUMBERT 1974). The exchange of substances between the resorptive epithelial cells and the "liver cells" most likely occurs at the finger-like protrusions into the basal invaginations of the epithelium. The existence of fusomes in this region has been discussed (SEIFERT & ROSENBERG 1977). During the present study, such interconnections were not observed, however. Contrary to the results of EFFENBERGER (1909), "liver cells" appeared in all species examined in the present paper. Thus it seems very likely that this cell type is widely distributed, at least over the examined families. During the normal state of nutrition, the "liver cells" have the possibility of storage and metabolism of glycogen (SEIFERT & ROSENBERG 1977). Confirming the results of RANDOW (1924), lipid storage in the "liver cells" was detected in the present study, though SEIFERT & ROSENBERG (1977) disputed this ability. The "liver cells" in Diplopoda have been compared to the chloragog cells in Annelida and the cells of the vertebrate liver (SEIFERT & ROSENBERG 1977).

The appearance of spherites in the midgut epithelium (or the midgut gland) is widely distributed within the invertebrates, i.e. in Arachnida (ALBERTI & STORCH 1983, LUDWIG & ALBERTI 1988), Insecta (GOURANTON 1968, HUMBERT 1974), or Mollusca (TRIEBSKORN 1989). Though metals can be stored intracellularly in lysosomes (DALLINGER & PROSI 1988) and vacuoles (PROSI & BACK 1985), spherites are the main mineral storage organelles (BROWN 1982, HOPKIN 1986, PROSI et al. 1983, SIMKISS 1976). E.g. the elements Ca, Cu, Mg, Mn, P, and Zn have been observed in diplopod spherites (HUMBERT 1979). A detoxificative function has been ascribed to them (e.g. LUDWIG & ALBERTI 1988). Concerning the present observations, after ingestion, heavy metals are most likely deposited in the spherites (Zn mainly in

the cuticle or its vicinity). This explains the electron-dense appearance of the spherites after heavy metal treatment. The spherites are probably subsequently extruded into the intestinal lumen.

The disconnection of the epithelial cells is mainly caused by the deminuation of the cell volume, in accordance with the increasing electron-density of the cytoplasm in the mentioned cell types. The increasing appearance of intercellular space was also observed in gastropod midgut gland cells under molluscicid treatment (TRIEBSKORN 1990), and also in the diplopod midgut after starvation (SEIFERT & ROSENBERG 1977, authors' unpublished data). A further symptom of hunger, the thickened basal lamina, as observed here under heavy metal treatment, was also reported from Coleoptera (CARSTENS & STORCH 1980). Furthermore the formation and elongation of filiform processes and finger-like protrusions in the "liver cells" (SEIFERT & ROSENBERG 1977, authors' unpublished data), as well as chromatin condensation (ALBERTI & STORCH 1983), also appeared under starvation conditions. Thus the observed alterations may not be specifically caused by heavy metals. The dislocation of the mitochondria and microtubules in the resorptive epithelial cells, the deviating arrangement of the rER, and the alterations in the cell surface of the "liver cells" may indicate an effect of heavy metal treatment on the cytoskeleton. Some metabolic pathways are probably influenced as well. Even under the best nutritional conditions, the storage of glycogen and lipid in the epithelial and the "liver cells" never reached the amount found under heavy metal treatment. The possibility that the osmolarity of the cells is affected is also apparent, as indicated by the larger intercellular spaces.

Though possibly not being specifically due to heavy metals, the observations reported here demonstrate that the reaction of diplopods to environmental stress under natural conditions corresponds greatly with results obtained from laboratory studies. Further investigations should demonstrate that such impacts influence the role of diplopods in the ecosystem, e. g. in their role as decomposers.

5. Abbreviations used in the Figures:

bl	basal lamina	bla	basal labyrinth	c	cuticle
cm	circular muscle layer	e	epithelium	er	endoplasmic reticulum
g	glycogen	Ga	Golgi apparatus	is	intercellular space
l	lumen of intestine	li	lipid	lc	"liver cell"
m	longitudinal muscle layer	ly	lysosome	m	mitochondrium
mp	Malpighian tubule	mt	microtubules	mv	microvilli
n	nucleus	rc	regenerative cell	rec	resorptive epithelial cell
s	spherite	sd	septate desmosome	sg	salivary glands
sj	smooth septate junction	t	trachea		

6. Acknowledgements:

The authors are grateful to Rita Triebskorn for critical discussion and David Russell for the English correction.

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