

Invertebrate Cells as Targets for Hazardous Substances

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Keywords: Invertebrates, Cytological alterations, Hazardous Substances

Introduction

Electron microscopy is an established diagnostic method in pathology of man as well as of vertebrate animals. During the last decade, ultrastructural studies have also been performed in invertebrates to elucidate cellular injuries caused by hazardous substances (BAYNE *et al.* 1985, MOORE 1985, STORCH 1988, HOPKIN 1989). The interest in invertebrates has increased due to their suitability to monitor environmental pollution. For example field and laboratory studies have been performed using the bivalve *Mytilus edulis* or the gastropod *Littorina littorea* as indicator of chemical contamination (AUFFRET 1988, CAJARAVILLE *et al.* 1989, CAJARAVILLE *et al.* 1990, MOORE 1988). Additionally, many invertebrates, such as aphids, grasshoppers, caterpillars or slugs are of significance as pest organisms in agriculture. Others, in most cases insects like the Ichneumonidae or Planipennia, are of increasing importance in biological or integrated pest control.

In a series of laboratory tests we examined cellular reactions in a variety of invertebrates after exposure to environmentally relevant toxicants, like metals or pesticides. The following animals were selected for their environmental or commercial significance:

-the shrimp, *Penaeus monodon* (Crustacea, Decapoda), as a commercially important aquaculture species which is impaired by environmental toxicants,

- the flatworm, *Polycelis felina* (Turbellaria, Tricladida), a common inhabitant of central European brooks, which is menaced by acid rain and subsequently released aluminium,
- the millipede, *Cylindroiulus punctatus* (Diplopoda, Julida), as an important animal of the soil macrofauna, involved in the decomposition of organic material,
- the moss mite, *Nothrus silvestris* (Acari, Oribatida), as a representative of saprophagous soil animals occurring in high population densities,
- the slug, *Deroceras reticulatum* (Gastropoda, Pulmonata), which is an important pest organism in European agriculture,
- and *Chrysoperla carnea* (Insecta, Planipennia), as a beneficial species used in the biological control of aphids.

It was the aim of this study to demonstrate the suitability of electron microscopy for evaluating the effects of hazardous substances on cells of invertebrate tissues.

Materials and Methods

The animals were treated as follows:

Penaeus monodon: Prior to fixation, seven 50 days old postlarvae of the prawn *Penaeus monodon* were kept in a 50 l fibreglass tank in a static system for 10 days (temperature: 29°C, salinity: 33 ‰, oxygen saturated). Cadmium was added as CdCl₂ to the water at a concentration of 1 mg/l at the beginning of the experiment and every second day when the water was completely changed. The animals were fed with a standardized pellet food. The mortality was recorded daily. The midgut glands were sampled for electron microscopy after 5 and 10 days and processed according to routine methods (VOGT et al. 1985).

Polycelis felina: The animals were dissected five days after exposure to 800 µg/kg aluminium dissolved in soft water. They were fixed in glutaraldehyde (2% in 0.01 M cacodylate buffer; pH 7.4) and processed as described by ZAHN (1990).

Cylindroiulus punctatus: Mature specimens were fed a lead-contaminated leaf litter agar (6 g agar, 6 g leaf litter particles, 200 ml of a 1000 mg/l Pb(NO₃)₂ solution [= 625.6 mg/l Pb²⁺]) at 15°C for 30 days. The control group was held under the same conditions without lead treatment.

After dissection the midgut was fixed in cacodylate (0.01 M) buffered 2% glutaraldehyde (pH 7.4) for 2 hours and further processed following the description of KÖHLER & ALBERTI (in press).

Nothrus silvestris: The mites were fixed after oral application of 100 mg/kg cadmium (CdCl_2) for 56 days and treated according to the method of TIMM (1958), a method which enables the demonstration of heavy metals as black precipitates in ultrathin sections. The samples were treated as described by LUDWIG & ALBERTI (1988) and LUDWIG *et al.* (in press).

Deroceras reticulatum: The animals were fed a bait containing 2% or 0.1% of the carbamate molluscicide *Cloethocarb* (active ingredient: phenol-2-(2-chloro-1-methoxyethoxy)-methylcarbamate). The hepatopancreas was fixed, 1 h or 5 h after application of the substance, in a 2% glutaraldehyde solution in cacodylate buffer (0.01 M, pH 7.4) for 2 h at 4°C, after injection of the fixation medium into the body cavity. The tissues were then routinely postfixed, contrasted, dehydrated and embedded (TRIEBSKORN, 1989).

Chrysoperla carnea: Larvae of *C. carnea* were exposed to a concentration of 0.04 % of the juvenile hormone analogue Insegar (active ingredient Fenoxycarb) directly after hatching. Every 24 hours specimens were dissected and the fat body was fixed in 2.5% glutaraldehyde (dissolved in 0.05 M cacodylate buffer, pH 7.4) for at least four hours. Afterwards they were processed as described by RUMPF (1990).

Results

Penaeus monodon / cadmium

During the experimental period, 3 out of 7 prawns died, 2 at day 5 and 1 at day 8. Ultrastructural investigations of the major metabolic organ, the hepatopancreas, revealed severe damages in numerous cells after 10 days of exposition to cadmium. The most conspicuous cytopathological sign was the fading of the fibrillar component of the nucleolus which is the site of synthesis of the ribosomal RNA (Fig.1). In healthy animals, this part of the nucleolus appeared very electron dense (Fig. 2). Fading of the fibrillar component is generally considered as structural correlate of decreased rRNA synthesis (GOESSENS, 1984). Thus, the cadmium-induced fading of the

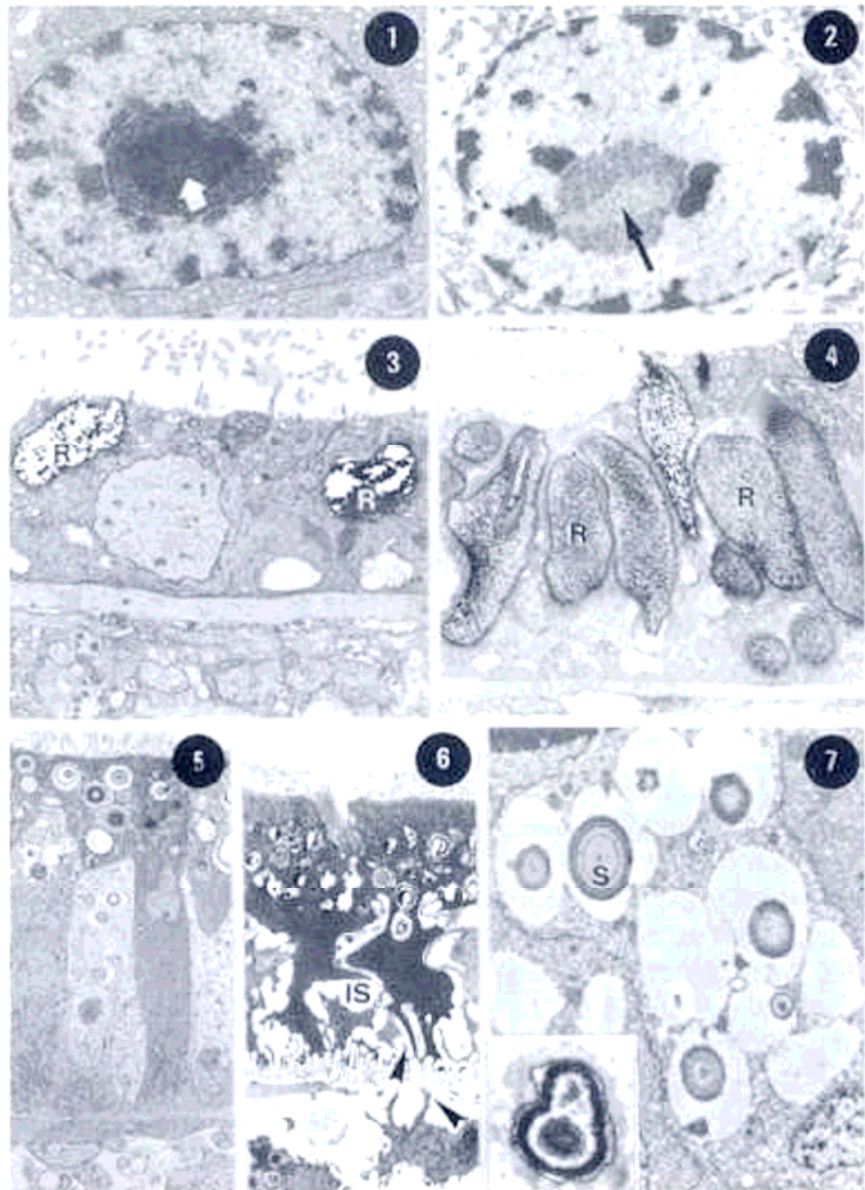
fibrillar component in *Penaeus monodon* may reflect a drastic decrease or even the stop of ribosome production.

Polycelis felina / aluminium

Five days after exposure to aluminium, in *P. felina*, the number of rhabdites, defense structures of the skin, which contain acid mucopolysaccharides, increased. At that time, the mortality had not been augmented. This reaction has already been noted under acid pH conditions and was reinforced by the presence of aluminium (Figs. 3, 4). The increased production of rhabdites and a related change in the chemical composition of the mucus (ZAHN 1990) might be explained as a defense mechanism to protect the animals against toxic injuries from the environment.

Cylindroiulus punctatus / lead

Thirty days after application of a sublethal concentration of lead, in the midgut cells of the diplopod *C. punctatus* several ultrastructural alterations were found (compare Figs. 5 and 6). The cytoplasm of the epithelial cells condenses due to a reduction of the volume of the cell. As a further consequence, the intercellular spaces dilate and the basal cell protrusions elongate. The mitochondria, normally arranged in a distinct apical zone, are evenly distributed throughout the cell. Additionally, a disarrangement of the ER cisternae and of microtubules was found. All reactions mentioned might be due to an influence of the metal on the cytoskeleton and/or the osmolar balance of the cell.



Figs. 1 -7: Heavy metal-induced ultrastructural alterations in cells of *Penaeus monodon*, *Polycelis felina*, *Cylindroiulus punctatus* and *Nothrus silvestris*.

Fig.1. Nucleus of an uncontaminated hepatopancreas cell of *Penaeus monodon* with electron-dense fibrillar component of the nucleolus (arrow). x 5625.

Fig. 2. Fading of the fibrillar component of the nucleolus (arrow) in a hepatopancreas cell of *Penaeus monodon* after application of cadmium. x 5625.

Fig. 3. Skin epithelium of untreated *Polycelis felina* with few rhabdites (R). x 3060,

Fig. 4. Increased number of rhabdites (R) in the skin of *Polycelis felina* after application of aluminium. x 5220.

Fig. 5. Midgut epithelium of a control animal of *Cylindroiulus punctatus*. x 1440.

Fig. 6. Dilatation of the intercellular spaces (IS) and elongation of basal protrusions (arrowheads) in cells of the midgut of *Cylindroiulus punctatus* after application of lead. x 2025.

Fig. 7. Midgut cell of *Nothrus silvestris* with several spherites (S) and - inset - spherite in a midgut cell of *Nothrus silvestris* after heavy metal test. x 6300 (inset x 7200).

Nothrus silvestris / cadmium

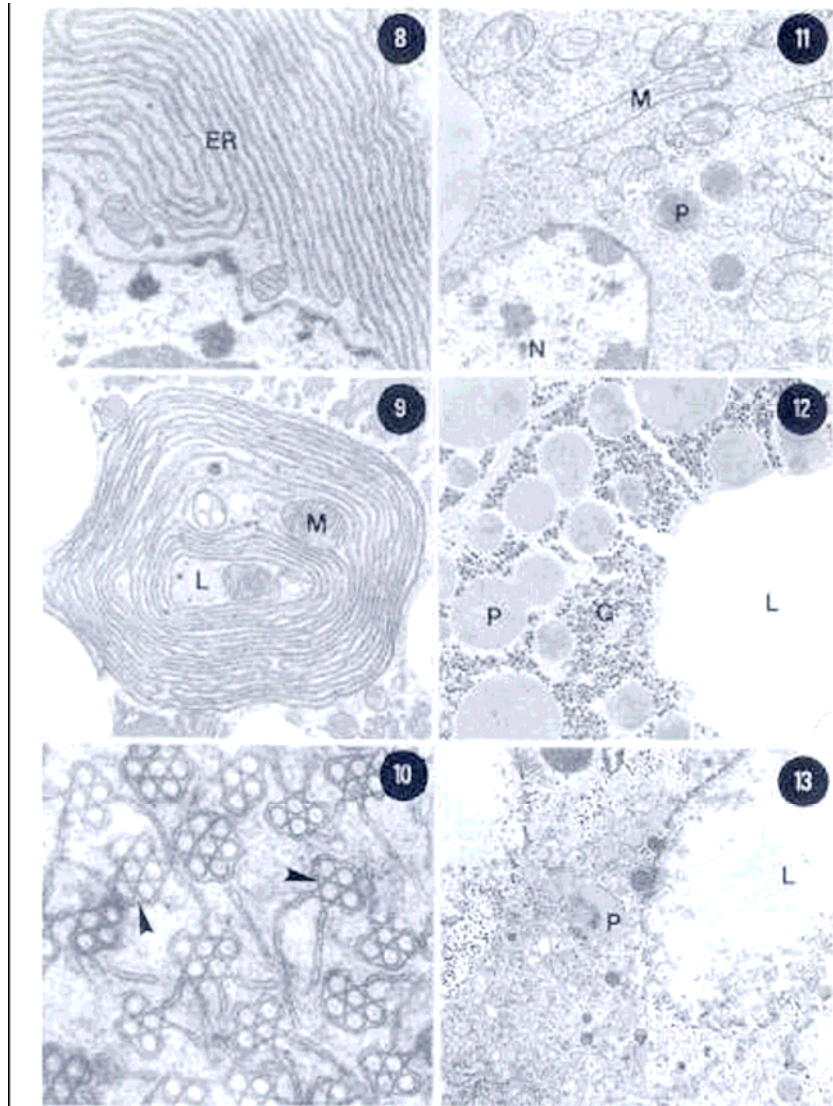
In the moss mite *N. silvestris* it was possible to detect heavy metal within spherites of the midgut cells after treatment with cadmium (Fig. 7 midgut cell with spherites before and -inset -after heavy metal test). Aside from their role in storing calcium, these spherites are thought to be involved in detoxification of metal (HOPKIN 1989, LUDWIG *et al.* in press). The metal which is bound to the organic matrix of the spherites (SIMKISS & MASON 1984), is most likely excreted via excretory vacuoles into the lumen of the midgut and finally rendered harmless.

Deraceras reticulatum / Cloethocarb

One hour after application of a bait containing 2% of the molluscicide C l o e t h o c a r b to the slug *D. reticulatum*, the cisternae of the endoplasmic reticulum of the hepatopancreatic crypt cells, which are arranged almost in parallel in controls (Fig. 8), form concentric whorls (Fig. 9). These whorls often encircle lipid droplets, mitochondria or peroxisome-like structures. Five hours after application of a bait with 0.1% of the pesticide, intracisternal cytoplasm polycylinders became obvious in the rough or degranulated ER (Fig.10). Functionally these structures might be correlated with oxidation of steroids and xenobiotics (MOJA & RALLO 1975).

Chrysoperla carnea / Fenoxycarb

After the exposure of larvae of *C. carnea* to the juvenile hormone analogue F e n o x y c a r b , high metabolic cells of the fat body characterized by many mitochondria and ribosomes (Fig.11) did not - as in control animals - convert into storage cells before pupation. In control animals, these storage cells are characterized by large amounts of protein, lipid and glycogen (Fig.12), while after treatment with F e n o x y c a r b , the energy reserves are reduced (Fig. 13). As a result, the animals are not able to pupate since the fat body cannot provide the energy needed for tissue rearrangement during metamorphosis. The electron microscopic study of cellular alterations in the fat body of *Chrysoperla carnea* was useful for obtaining additional information about the mode of action of the juvenile hormone analogue F e n o x y c a r b and to help understand the results obtained in field and laboratory tests.



Figs. 8- 13: Ultrastructural alterations in cells of *Deroceras reticulatum* and *Chrysoperla carnea* after poisoning with pesticides.

Fig. 8. Endoplasmic reticulum (ER) in a crypt cell of the hepatopancreas of an uncontaminated *Deroceras reticulatum*. x 8550.

Fig. 9. Cisternae of the ER which are arranged as a concentric whorl in a crypt cell of *Deroceras reticulatum* 1 h after application of a 2 % C l o e t h o c a r b bait. The ER surrounds lipid droplets (L) and mitochondria (M). x 7650.

Fig. 10. Cytoplasmic polycylinders in the ER of a crypt cell of the hepatopancreas in *Deroceras reticulatum* five hours after application of a bait containing 0.1% of C l o e t h o c a r b (the arrowheads indicate the lumen of the ER). x 42300.

Fig. 11. High metabolic cell in the fat body of *Chrysoperla carnea* characterized by large amounts of ribosomes and few protein (P). N: nucleus, M: mitochondrium. x 7650.

Fig. 12. Storage cell in the fat body of *Chrysoperla carnea* before pupation characterized by protein (P), lipid (L) and glycogen (G). x 2610.

Fig. 13. Storage cell of the fat body of *Chrysoperla carnea* after intoxication with F e n o x y c a r b characterized by few storage products. P: protein, L: lipid. x 4500.

Discussion

The examples presented may demonstrate that investigations of cellular reactions to environmental factors by means of electron microscopy are a valuable method of monitoring the influence of toxicants on invertebrate animals.

In our examples it is possible to distinguish between alterations of cellular structures due to detoxification and others related to cellular damage.

Concerning detoxification, the invertebrates studied utilize different strategies to react to various toxins. The flatworm *Polycelis felina*, e.g., reacts to aluminium treatment with an increase in the number of rhabdites, mucopolysaccharide-containing defense-structures of the skin. This reaction is comparable to the proliferation of mucus-producing cells in slugs poisoned by molluscicides (TRIEBSKORN & EBERT, 1989) and of fish under acid rain conditions (ZUCHELKOWSKY *et al.*, 1981; SEGNER *et al.*, 1988). In the moss mite *Nothrus silvestris*, but also in diplopods (KÖHLER & ALBERTI, in press), gastropods (SIMKISS & MASON, 1984) and many other invertebrates (HOPKIN *et al.*, 1989) heavy metals are detoxified by binding to mineral congregations in cells of the digestive system. In the shrimp, *Penaeus monodon*, such spherites are not present after cadmium application. Literature data corroborate this observation since in shrimps, cadmium bound to metallothioneins seems to be distributed throughout the cytoplasm (LYON *et al.*, 1983). Alterations of the endoplasmic reticulum, such as formation of concentric whorls or cytoplasmic polycylinders in the ER occurring in hepatopancreatic crypt cells of the slug, *Deroceras reticulatum*, have also been related to detoxification (MOJA & RALLO 1975, KLAUNIG *et al.* 1979). An increasing activity of enzymes involved in oxidative detoxification could be demonstrated in the same cells after application of Cloethocarb (TRIEBSKORN, 1991).

Generally, detoxification is only possible as long as the concentration of the toxicant in the animal does not exceed a particular threshold value. If this threshold value is exceeded, toxicity results in cellular damage, as shown for *Cylindroiulus punctatus* and *Penaeus monodon*, or in inhibition of cellular development, as in *Chrysoperla carnea*. As with the defense or detoxification mechanisms, the kind of cellular damage depends on the species involved, the tissue and the type of toxin. In hepatopancreatic cells of *Penaeus monodon*, for instance, destruction induced

by cadmium is totally different from that occurring in the midgut of *Cylindroiulus punctatus* after lead treatment.

Summarizing the results of our experiments it has become obvious that tissues of invertebrates react to hazardous substances with different ultrastructural alterations. The kind of cellular response does not only depend on the environmental factors but also on the species and tissues. Our laboratory studies indicate that electron microscopy is a valuable supplemental tool in environmental research. Cytological data obtained by controlled laboratory studies are expected to facilitate a monitoring of toxins in the field.

Acknowledgement

Thanks are due to Dr. PETNEY for the correction of the English version

Summary

In the present electron microscope study the following cytological alterations in cells of representatives of six invertebrate taxa were described and related to functional aspects.

Invertebrate Cell Targets for Hazardous Substances

Species	Xenobiotic	Ultrastructural Alteration
<i>Penaeus monodon</i> (Crustacea)	Cadmium	Fading of the fibrillar component of the nucleolus resulting in a reduction of rRNA synthesis

<i>Polycelis felina</i> (Turbellaria)	Aluminium	Increase in number of rhabdites due to a changed production of slime Alterations of several organelles due to influence on the cytoskeleton or the osmolar balance of the cell
<i>Nothrus silvestris</i> (Acari)	Cadmium	Detection of heavy metal in spherites of the midgut which are involved in detoxification
<i>Deroceras reticulatum</i> (Gastropoda)	Cloethocarb	Alterations of the ER due to detoxification
<i>Chrysoperla carnea</i> (Insecta)	Fenoxycarb	Reduced amount of storage products in fat body cells due to inhibition of the normal development of these cells

Zusammenfassung

In der vorliegenden elektronenmikroskopischen Untersuchung konnten cytologische Veränderungen in Zellen von folgenden Repräsentanten aus sechs Wirbellosen-gruppen mit funktionellen Aspekten korreliert werden:

Art	Xenobiotikum	Ultrastrukturelle Veränderung
<i>Penaeus monodon</i> (Crustacea)	Cadmium	Aufhellung der fibrillären Komponente des Nucleolus, die mit der Einstellung der rRNA Synthese einhergeht

<i>Polycelis felina</i> (Turbellaria)	Aluminium	Erhöhung der Zahl der Rhabditen verbunden mit veränderter Schleimbildung
<i>Cillindroiulus punctatus</i> (Diplopoda)	Blei	Veränderungen verschiedener Organelle in Verbindung mit Störungen im Cytoskelet und des osmotischen Gleichgewichtes der Zelle
<i>Nothrus silvestris</i> (Acari)	Cadmium	Schwermetallnachweis in Spheriten des Mitteldarmes, die an Entgiftungsprozessen beteiligt sind
<i>Deroceras reticulatum</i> (Gastropoda)	Cloethocarb	Veränderungen des ER Verbunden mit Entgiftung
<i>Chrysoperla carnea</i> (Insecta)	Fenoxycarb	Unterschiedliche Reservestoffgehalte bei Kontrollen und belasteten Tieren verbunden mit gestörter Entwicklung der Zellen

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