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Assessment of the cytotoxic impact of heavy metals on soil invertebrates using a protocol integrating qualitative and quantitative components

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Slugs (*Deroceras reticulatum*), isopods (*Porcellio scaber*), diplopods (*Julus scandinavius*), and collembola (*Tetrodontophora bielanensis*) were exposed to cadmium, lead or zinc at differing concentrations under constant laboratory conditions. Ultrastructural alterations of monitored tissues (for diplopods and collembola, midgut epithelium; for slugs and isopods, midgut gland epithelium) were recorded qualitatively and quantitatively by transmission electron microscopy. In order to combine both qualitative and quantitative aspects of the observed responses into a proposed easy-to-handle 'impact index', we developed a protocol according to the physiological basis for the ultrastructural responses. Using this protocol, the data clearly showed the different susceptibilities (1) of organelles to the respective metal, (2) of the investigated species to particular metals, and (3) of the monitored tissue to increasing metal concentration. Moreover, the construction of matrices of impact indices for single symptoms allows the description and analysis of complex syndromes in a mathematical way. Cluster analysis of the present data on controlled laboratory experiments utilizing single contaminants revealed similar patterns of cellular responses to the tested metals in the investigated tissues of *P. scaber* and *J. scandinavius* and species-specificity for *T. bielanensis* and *D. reticulatum* monitored tissues. Some of the highest metal concentrations seemed to affect the homogeneity of these specific reponse patterns, and did not fit the respective clusters.

Keywords: biomarker, cellular pathology, electron microscopy, impact index, ultrastructure.

Introduction

The impact of the external environment on physiological changes in organs and tissues is mirrored by changes in the ultrastructure of their cells (Storch 1988). This ultrastructural reaction, however, is far from being consistent for every cell type in an organism. Nor is this reaction constant in response to every environmental perturbation. Thus, subcellular alterations depend on a variety of parameters including the organ type, the cell type and the character of the environmental stressor (e.g. temperature, food deprivation, exposure to chemicals). Moreover, the different organelles of a cell differ in their sensitivity and their response to stressors. This usually makes it difficult to interpret variation in response to environmental stressors, thus reducing such studies to the description of qualitative effects. Although much of this research has been well-conducted and given an insight into the variability of cell ultrastructure, most published studies, especially on responses of soil invertebrates to environmental factors, mainly cover qualitative changes in the ultrastructure of their cells (e.g. summarized in Storch 1984).

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In recent years, quantitative approaches to evaluate ultrastructural stress reactions have gained increasing attention in the fields of pathology, toxicology or ecotoxicology. Quantitative techniques range from gross-scan estimates (e.g. Triebskorn and Köhler 1996) to different morphometric approaches which are extremely time-consuming, particularly when absolute measures of ultrastructural change are in the centre of interest (Gundersen *et al.* 1988). Although the merits of these techniques are doubtless and although they allow the application of mathematical analysis to morphological data, these methods tend to overemphasize quantitative change and consider qualitative change only scarcely. In this paper we attempt to combine qualitative with quantitative analyses of ultrastructural changes to provide a more comprehensive method of analysis of environmental stress. The suitability of this synthetic protocol in toxicity assessment was investigated in laboratory exposure experiments through the impact of heavy metals on resorptive tissues of soil invertebrates. Since these organisms form an ecologically important group for the process of decomposition of soil organic matter, their use in such studies contributes to the understanding of the toxic potential of metals in soil. The extensive data sets usually generated with such studies, however, require methods which (1) allow investigation of numerous samples within a reasonable time and (2) offer the possibility to handle this data set adequately. Both requirements are met in our protocol.

Material and methods

Exposure conditions

Adult specimens of *Deroceras reticulatum* (Pulmonata), *Porcellio scaber* (Isopoda), *Julus scandinavius* (Diplopoda) and *Tetrodontophora bielanensis* (Collembola) were exposed to either cadmium-, lead-, or zinc-enriched food under otherwise constant conditions (table 1). Animals were housed in the laboratory for 3 weeks in plastic boxes with a moist gypsum base covered by garden soil. In all cases, metal concentrations in the food corresponded to environmentally realistic soil concentrations (Coughtrey *et al.* 1979, Dallinger and Prosi 1988, Hopkin 1989). Even the most extreme concentrations mirrored severe field conditions (e.g. Gale and Wixson 1979, Bengtsson and Rundgren 1982, 1984, Bengtsson *et al.* 1983, Tyler *et al.* 1984, Hunter *et al.* 1987, Schäfer 1987). All metal concentrations were considerably below the LC_{50} (21 days) except for the highest zinc concentration applied to *D. reticulatum*, for which the experiment was terminated after 9 days.

Metal quantification

Food, substrate, and animal samples (after gut clearance) were oven-dried (60 °C), ground, weighed, digested with 65 % nitric acid (suprapure grade) at 90 °C, and topped up with distilled water (food and substrate: 5 ml HNO_3, topped up to 50 ml; animals 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) following the protocol of Köhler *et al.* (1996). Mean total body concentrations of cadmium, lead, and zinc are also listed in table 1.

Transmission electron microscopy

After dissection, the midgut tissue (for collembolans and diplopods) or the midgut gland (for isopods and gastropods) was fixed in 2 % glutardialdehyde (GA) in 0·01 M cacodylate buffer, pH 7·2 (for diplopods: 1 % GA in 0·005 M cacodylate buffer), for 2 h, rinsed repeatedly in cacodylate buffer lacking GA (pH 7·2), and postfixed in 1 % reduced OsO_4 solution containing 1·5 % $K_4[Fe(CN)_6]$ for 2 h (Karnovsky 1971). After repeated washing in cacodylate buffer and 0·05 M maleate buffer (pH 5·2), the specimens were stained in 1 % uranyl acetate in maleate buffer overnight at 4 °C, dehydrated in a graded ethanol series and embedded either in Araldite or Spurr´s medium (Spurr 1969). Ultrathin sections were counterstained with alkaline lead citrate (Reynolds 1963, modified) and examined under a transmission electron microscope (Zeiss EM 9 S-2, EM 10 CR, or CEM 902).

Table 1. Parameters of exposure during the experiments. Metal concentrations in mg kg^{-1} dry wt.

	D. reticulatum	*P. scaber*	*J. scandinavius*	*T. bielanensis*
Source	Laboratory hatchery	Laboratory hatchery	Pristine site (near Heidelberg, Germany)	Pristine Site (Bóbr valley, Poland)
Food	Lettuce leaves/carrots	Decaying leaf litter	Decaying leaf litter	Decaying leaf litter
Mean Cd concentration in the food	0·7[a]/59/358/750	0·4[a]/57/222/419	0·4[a]/57/222/419	0·4[a]/57/222/419
Corresponding Cd conc. in the body after 21 days	3[a]/71/122/246	5[a]/108/215/195	1[a]/9/28/19	0·8[a]/3/6/18
Mean Pb concentration in the food	29[a]/405/2456/3751	7[a]/517/2778/7676	7[a]/517/2778/7676	7[a]/517/2778/7676
Corresponding Pb conc. in the body after 21 days	4[a]/30/179/1169	3[a]/74/211/264	3[a]/88/240/92	34[a]/30/143/216
Mean Zn concentration in the food	5[a]/1874/9305/14915	4[a]/1976/5730/22052	4[a]/1976/5730/22052	4[a]/1976/5730/22052
Corresponding Zn conc. in the body after 21 days	76[a]/437/393/4252[b]	171[a]/253/221/302	152[a]/229/157/650	365[a]/387/459/620
Temperature	10 °C	15 °C	8 °C	10 °C
Light/dark photoperiod	16 h/8 h	12 h/12 h	12 h/12h	12 h/12 h
Investigated tissue	Midgut gland	Midgut gland	Midgut epithelium	Midgut epithelium
References	Triebskorn and Köhler (1996)	Köhler *et al.* (1996)	Köhler and Alberti (1992) Berkus (1994)	Pawert *et al.* (1996)

[a] Control conditions.
[b] After 9 days of exposure.

Qualitative and quantitative data recording

For every species and exposure, four replicate specimens were investigated by electron microscopy. Qualitative and quantitative data derived from at least three sections of the monitored organ (midgut, midgut gland) per replicate. Ultrastructural alterations of microvilli, mitochondria, endoplasmic reticulum (ER) and nuclei were recorded in relation to control samples (not given metal-enriched food). The percentage number or percentage area of every alteration (listed in table 2) in each examined cell was scored (semi-)quantitatively in relation to the entire organelle number (mitochondria, nuclei) or to the organelle-dominated section area (ER, microvilli) by counting of mitochondrial or nuclear sections and by estimating the area occupied by altered ER structures or microvilli. Due to the semi-quantitative character of this approach, resolution was 5 % at the minimum. For each tissue section, a minimum of five cells was examined resulting in a number of at least 15 investigated cells per replicate.

Classification of symptoms into ranks

Based on previous experience with ultrastructural plasticity of the investigated tissues (e.g. Storch 1984, Triebskorn and Künast 1990, Triebskorn 1991, Triebskorn *et al.* 1991, Köhler and Alberti 1992, Triebskorn and Köhler 1992, 1996, Berkus 1994, Köhler *et al.* 1996, Pawert *et al.* 1996,) and theoretical work of Depledge *et al.* (1993), we classified the observed symptoms in three categories: (1) not altered (control status), (2) compensation (symptomatic of a cell reaction) and (3) non-compensation (symptomatic of cell destruction). Category 1 corresponded to organelle ultrastructure observed in the controls, category 3 corresponded to obviously disintegrated (ruptured or absent) organelles, and category 2 comprised all the ultrastructural alterations which did not affect morphological organelle integrity although most likely mirroring physiological changes (table 2). The parameters (investigated organelles) were chosen on the basis of known effects of metals or other toxins in invertebrate digestive organs. A selection of electron micrographs displaying typical symptoms is shown in figure 1.

Impact index determination

Observed alterations (symptoms) were classified according to the list in table 2 and their percentages of all tissue summed for each of the three categories (control state/compensation (reaction)/ non-compensation (destruction)). Thus, for every organelle type in the investigated monitor organ of the three soil invertebrate species exposed to different stressors, an average response pattern (x % control

Table 2. Classification of ultrastructural symptoms in the investigated tissues of the intestinal tract displayed by the investigated organelles.

Organelles	Control status	Compensation (= reaction)	Non-compensation (= destruction)
Microvilli	Regular shape; even size; usual length according to the cell type; smooth membrane	Shortage; uneven irregular shape without membrane rupture; wavy membrane; reduction in number	Membrane rupture; total absence
Mitochondria	Regular shape (no swelling); homogeneous matrix; cristae evenly distributed	Increase or decrease of electron density of the matrix; moderate dilation (swelling); increased formation of intramitochondrial crystals	Membrane rupture; destruction of cristae; formation of myelin-like structures; severe dilation in combination with cristae reduction
Endoplasmic reticulum	Long, slim cisternae which are usually ribosome-bestudded (rER); according to the cell type, parallel arrangement of numerous cisternae	Shortage, vesicularization, dilation or circularization of the cisternae; degranulation of rER; Increase or decrease in number of cisternae	Membrane rupture; formation of myelin-like structures
Nuclei	Regular shape and distribution of heterochromatin (according to the cell type); homogeneous karyoplasm; evenly sized perinuclear space	Structural alterations of the shape, the nucleolus and the electron density of the karyoplasm; dilation of the perinuclear space; increased mitosis number	Rupture, blebbing and destruction of nuclear membranes; total fading of the karyoplasm

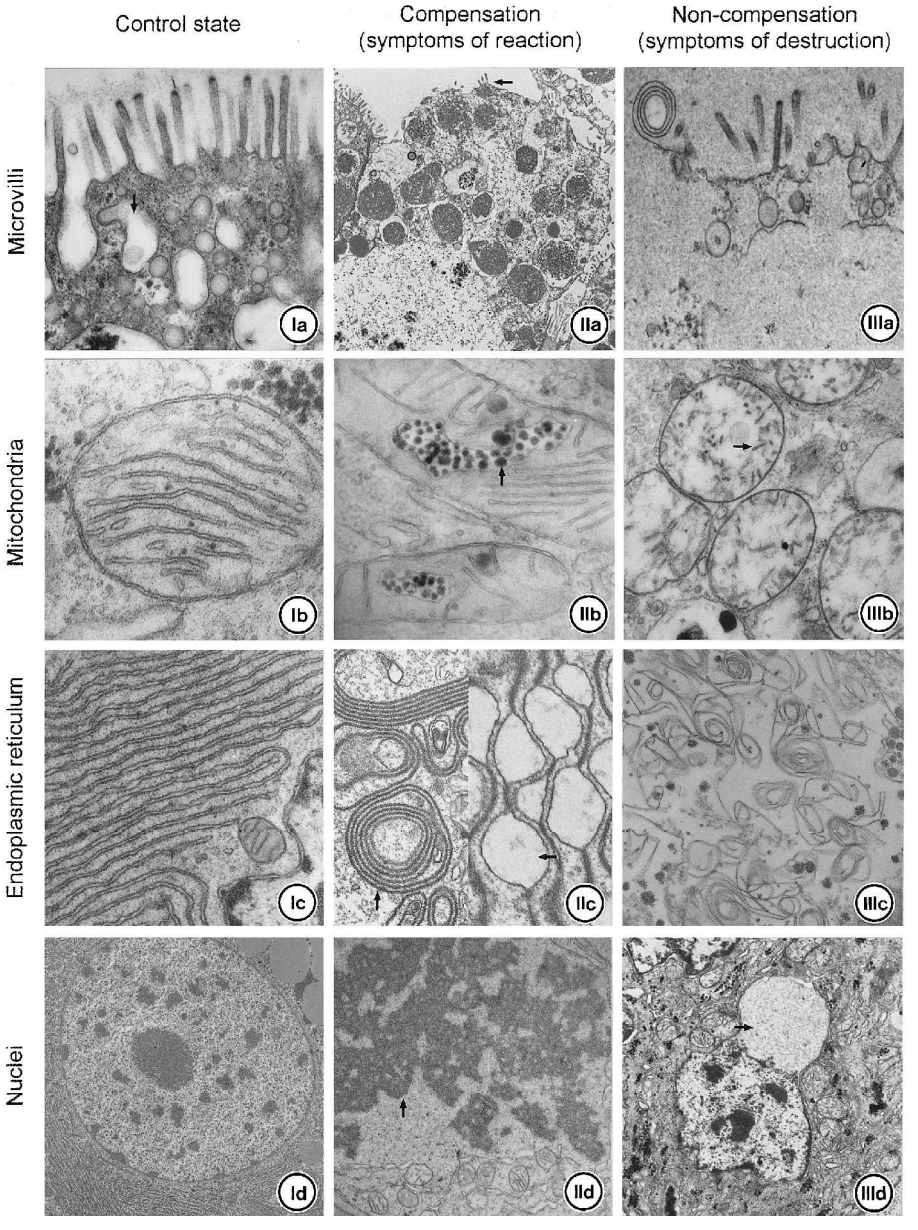

Figure 1. Transmission electron micrographs displaying typical ultrastructure of microvilli, mitochondria, ER and nuclei symptomatic for (Ia–d) the control status, (IIa–d) cell reaction and (IIIa–d) cell destruction. All micrographs were taken from sections of the midgut gland tissue of the slug, *D. reticulatum*. Control status: Ia: resorptive cell, intact microvillous border, pinocytotic vesicles (arrow); Ib: resorptive cell, intact mitochondrion; Ic: basophilic cell, regularly arranged rough ER, Id: basophilic cell, unaffected nucleus. Compensation: IIa: resorptive cell, microvilli reduced in number and size (arrow), pinocytotic vesicles are absent; IIb: resorptive cell, mitochondria with crystalline inclusions (arrow); IIc: basophilic cell, circularization of the rough ER (left, arrow), dilation and, partly, degranulation of rough ER cisternae (right, arrow); IId: basophilic cell, mitosis. Note the absence of the perinuclear cisterna and the chromatin condensation (formation of chromosomes, arrow). Non-compensation: IIIa: resorptive cell, apex completely destroyed; IIIb: resorptive cell, severely dilated mitochondria, cristae reduced in number and size (arrow); IIIc: basophilic cell, ER disintegrated into membrane fragments; IIId: basophilic cell, formation of a nuclear bleb (arrow), fading of the nucle

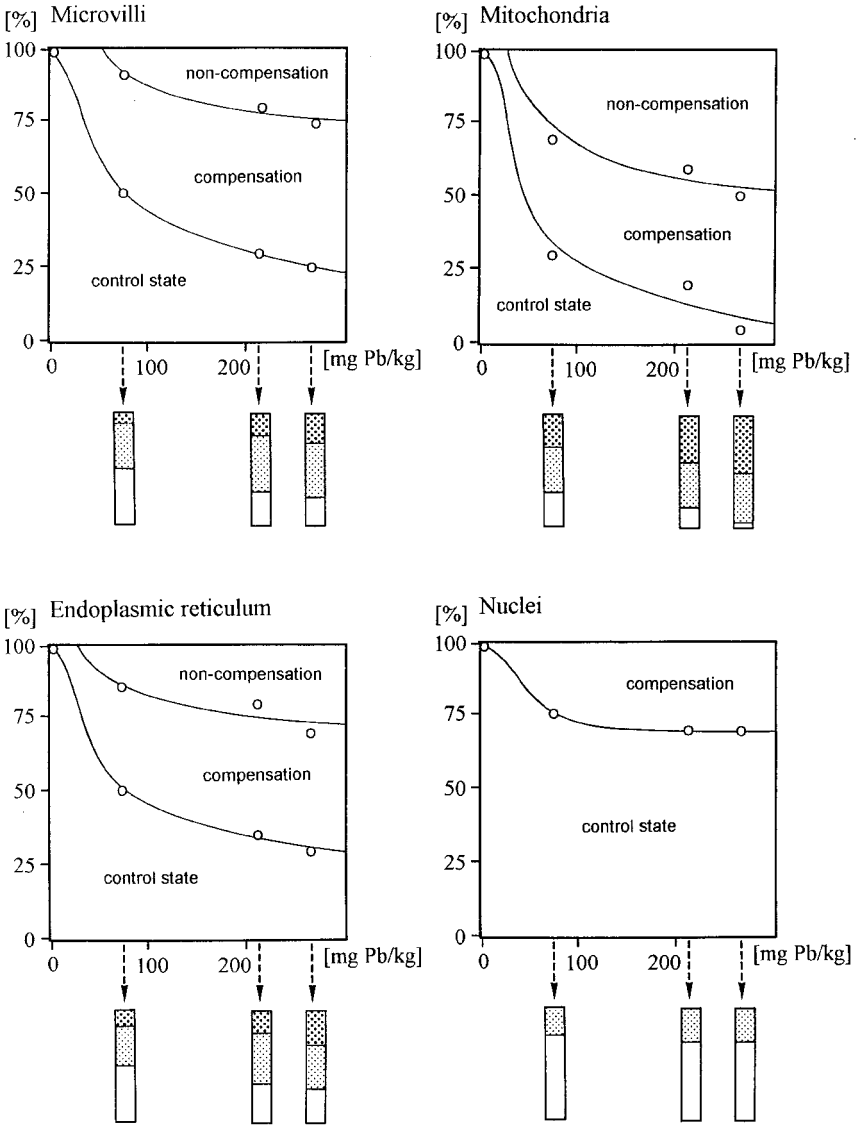

Figure 2. Concentration–effect relationships for metal exposure and the percentages obtained for the three categories of cellular response listed in table 2; exemplarily shown here for the impact of lead on the midgut gland cells of the isopod, *P. scaber*. Each of the four plots represents the reaction pattern of one organelle type. The plots form the basis for the standardized schemes in figure 3 and the columns below each plot which display the category percentages recorded for every treatment, correspond also to the columns in figure 3.

state, *y* % reaction processes, *z* % pathological destruction) was obtained. Although the ultrastructural responses of different cell types present in the investigated tissues largely resembled one another (e.g. Triebskorn and Köhler 1996), the average response patterns were calculated according to the frequency of the respective cell types within the tissues (slug midgut gland: resorptive and basophilic cells; isopod midgut gland: B and S cells; diplopod midgut: resorptive and hepatic cells; collembolan midgut: resorptive cells only). The response patterns showed distinct concentration–response relationships (exemplarily shown in figure 2) which formed the basis for the development of the schemes shown in figure 3 displaying the general course of control state, compensation and non-compensation percentages in relation to increasing toxicity. Using these schemes in figure 3, the response patterns observed for all experimental treatments were transformed into arbitrarily defined impact i

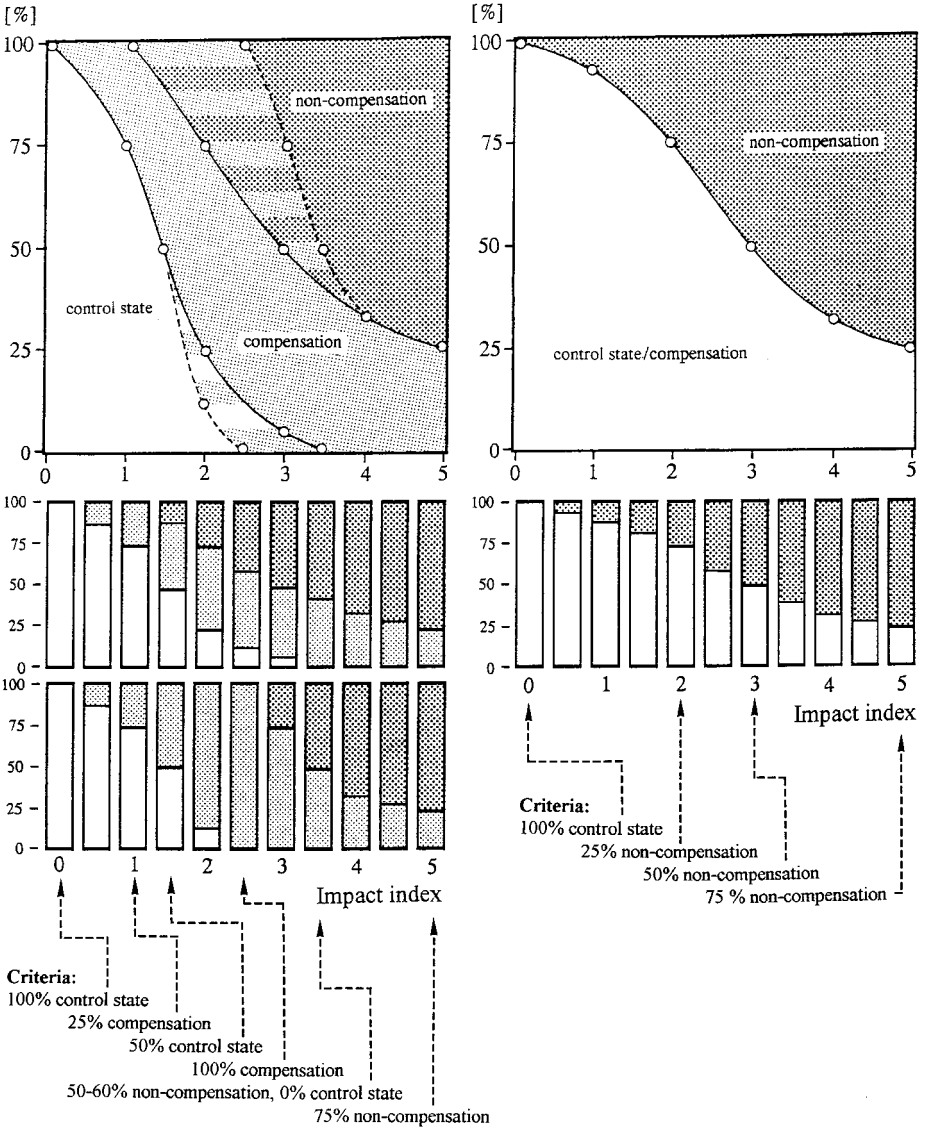

Figure 3. Schemes used to transpose ultrastructural response patterns into their respective impact index values considering the percentages of observed responses (compare with figure 2). Destruction of three quarters of the scanned organelles was arbitrarily defined as representing an impact index of 5·0 and the unaffected control status is represented by an impact index of 0·0. Left: Usually, some symptoms of compensation are detectable in organelles (top plot). Depending on the 'broadness of reactivity', either the upper or the lower column series (below the plot) was used for impact index determination. For example, the pattern '50 % destruction (= non-compensation), 40 % compensation, 10 % control status' can be expressed by an impact index of 3·0 (upper column series) as well as the pattern '25 % non-compensation, 75 % compensation' (lower column series). Right: If compensation processes cannot be ultrastructurally distinguished from the control status or if symptoms of compensation are hardly detectable (which may be sometimes the case for mitochondria), the right plot (or column series) was used (e.g. the pattern '25 % of an organelle type destroyed, 75 % 'intact' results in an impact index of 2·0). Although the displayed response patterns will not cover all theoretically possible combinations of the three response categories with one another, for all patterns observed in the present study impact index determination was possible using the schemes shown above. Selection of the suitable column series took place on an empirical basis.

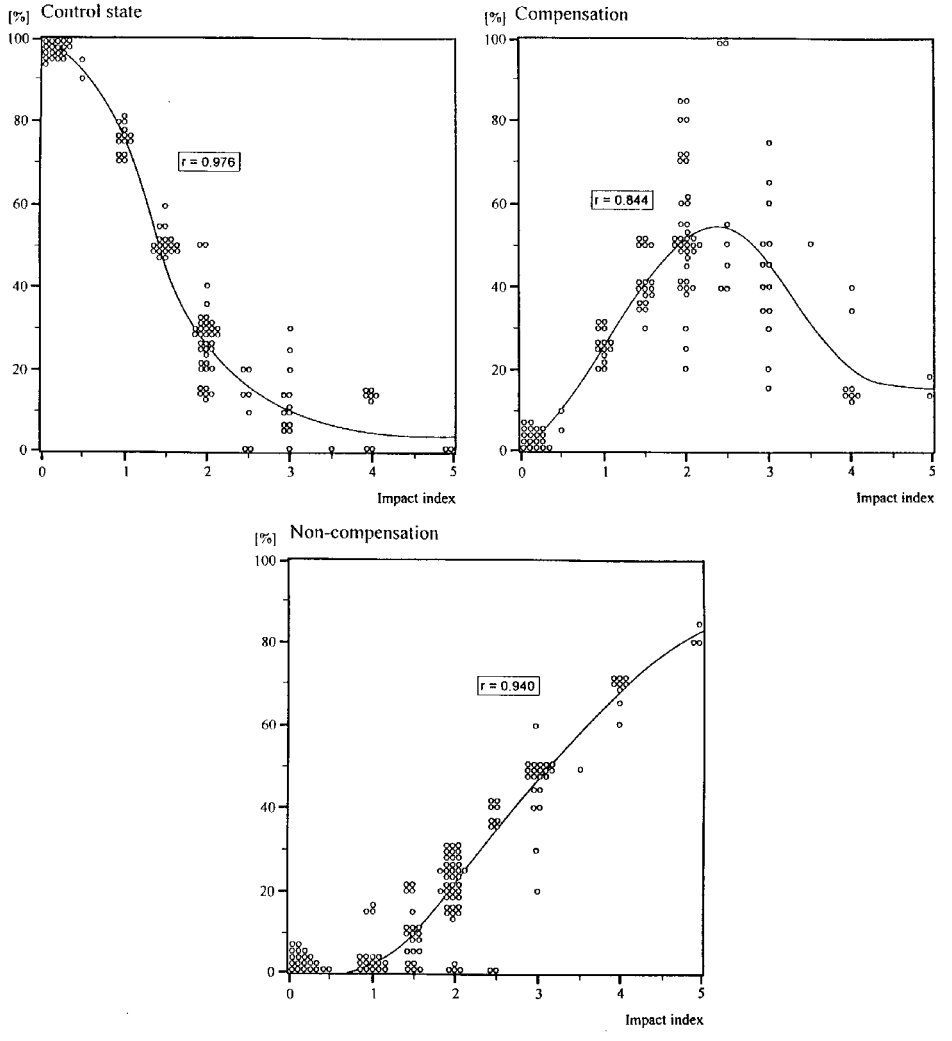

Figure 4. Impact index values generated in the present study plotted versus the respective percentages of organelles in the control state (top), symptoms of compensation (middle), and symptoms of non-compensation (bottom) to show confidence of the index in respect to ultrastructural data. All recorded data were considered to create these plots independent of exposure, species and organelle type with exception of those response patterns in which compensation could not be distinguished from control state although symptoms of non-compensation were visible. These data correspond to the right scheme in figure 3 and were omitted for the plots on compensation and control state. Regression analysis was conducted with TableCurve, Jandel Scientific. *r*: coefficient of correlation.

represent the degree of 'metal toxicity' (explanation in the legend to figure 3). To give confidence to the use of such an index, the entirety of the generated impact index values were statistically examined versus the original data on category percentages (figure 4). Finally, the impact index values for every organelle, species, and treatment were plotted versus the applied metal concentrations, allowing direct comparison of the sensitivity of different organelles and different species to each metal.

Cluster analysis of impact index data

To cluster response patterns, impact index data on microvilli, mitochondria, nuclei, and endoplasmic reticulum were listed in a matrix. For analysis, they were not weighted. Average Manhattan distance (M_{ij}) between a pair of impact index data *i, j* were calculated, and

Table 3. Percentages of ultrastructural alterations of organelles[a] summed for each of the categories control status, compensation and non-compensation. Metal concentrations are given in mg metal kg^{-1} food dry wt (in parentheses: mg metal kg^{-1} body dry wt).

Species	Metal	Concentration	Microvilli	Mitochondria	ER	Nuclei
D. reticulatum	Cd	59 (71)	15/70/15	100/00/00	15/70/15	25/60/15
		358 (122)	15/15/70	30/00/70	15/70/15	15/70/15
		750 (246)	00/20/80	20/00/80	00/35/65	15/35/50
	Pb	405 (30)	15/35/50	50/00/50	30/40/30	100/00/00
		2456 (179)	15/15/70	50/00/50	30/40/30	20/80/00
		3751 (1169)	00/15/85	50/00/50	30/40/30	20/80/00
	Zn	1874 (473)	50/25/25	75/25/00	100/00/00	100/00/00
		9305 (393)	15/15/70	00/100/00	30/50/20	15/85/00
		14915 (4252)	15/15/70	00/100/00	15/15/70	15/85/00
P. scaber	Cd	57 (108)	50/30/20	80/00/20	75/25/00	95/05/00
		222 (215)	15/50/35	80/00/20	50/40/10	90/10/00
		419 (195)	10/40/50	40/30/30	30/50/20	80/00/20
	Pb	517 (74)	50/40/10	30/40/30	50/35/15	75/25/00
		2778 (211)	30/50/20	20/40/40	35/45/20	70/30/00
		7676 (264)	25/50/25	05/45/50	30/40/30	70/30/00
	Zn	1976 (253)	20/40/40	65/00/35	55/35/10	75/25/00
		5730 (221)	10/45/45	65/00/35	25/60/15	70/30/00
		22052 (302)	05/50/45	60/00/40	20/55/25	50/35/05
J. scandinavius	Cd	57 (9)	50/50/00	50/40/10	50/40/10	75/25/00
		222 (28)	30/40/30	20/50/30	30/50/20	55/40/05
		419 (19)	20/30/50	00/50/50	05/65/30	25/50/25
	Pb	517 (88)	50/40/10	75/25/00	80/20/00	80/20/00
		2778 (240)	10/40/50	50/50/00	30/55/15	60/35/05
		7676 (92)	00/60/40	20/60/20	05/75/20	25/50/25
	Zn	1976 (229)	30/50/20	50/40/10	50/40/10	70/30/00
		5730 (157)	30/50/20	30/50/20	25/50/25	30/50/20
		22052 (650)	10/50/40	10/55/35	00/40/60	15/45/40
T. bielanensis	Cd	57 (3)	100/00/00	75/00/25	100/00/00	100/00/00
		222 (6)	100/00/00	50/00/50	75/00/25	100/00/00
		419 (18)	100/00/00	50/00/50	75/00/25	100/00/00
	Pb	517 (30)	100/00/00	75/00/25	75/25/00	100/00/00
		2778 (143)	100/00/00	50/00/50	25/50/25	100/00/00
		7676 (216)	100/00/00	50/00/50	25/15/60	100/00/00
	Zn	1976 (387)	100/00/00	85/00/15	50/50/00	100/00/00
		5730 (459)	80/20/00	85/00/15	50/20/30	50/50/00
		22052 (620)	30/20/50	85/00/15	15/15/70	50/50/00

[a] Data in the organelle columns represent: % control status/% compensation/% non-compensation.

unweighted pair group method, arithmetic average (UPGMA) using the SAHN routine of NTSYS-pc, version 1·8, cluster analysis software (Rohlf 1994):

$$M_{ij} = n^{-1} \cdot \Sigma_k \left| x_{ki} - x_{kj} \right|$$

where x_{ki} and x_{kj} = values on the criteria (organelles) k for the pair of impact index data i, j; and n = number of criteria.

Results

Confidence to the impact index

Classification of the observed symptoms into ranks resulted in a variety of different response patterns for every investigated organelle, species, metal and concentration (table 3) which were transformed into impact in

Table 4. Impact index values generated from the ultrastructural response 'patterns' (percentages of organelle control status/compensation/non-compensation) which are listed in table 3 using the schemes displayed in figure 2[a]. Metal concentrations are given in mg metal kg^{-1} food dry wt (in parentheses: mg metal kg^{-1} body dry wt).

Species	Metal	Concentration	Microvilli	Mitochondria	ER	Nuclei
D. reticulatum	Cd	59 (71)	2·0	0·0	2·0	2·0
		358 (122)	4·0	4·0	2·0	2·0
		750 (246)	5·0	5·0	4·0	3·0
	Pb	405 (30)	3·0	3·0	2·0	0·0
		2456 (179)	4·0	3·0	2·0	2·0
		3751 (1169)	5·0	3·0	2·0	2·0
	Zn	1874 (473)	2·0	1·0	0·0	0·0
		9305 (393)	4·0	2·5	2·0	2·0
		14915 (4252)	4·0	2·5	4·0	2·0
P. scaber	Cd	57 (108)	1·5	1·5	1·0	0·5
		222 (215)	2·5	1·5	1·5	0·5
		419 (195)	3·0	2·0	2·0	1·5
	Pb	517 (74)	1·5	2·0	1·5	1·0
		2778 (211)	2·0	2·5	2·0	1·0
		7676 (264)	2·0	3·0	2·0	1·0
	Zn	1976 (253)	2·5	2·5	1·5	1·0
		5730 (221)	3·0	2·5	2·0	1·0
		22052 (302)	3·0	2·5	2·0	1·5
J. scandinavius	Cd	57 (9)	1·5	1·5	1·5	1·0
		222 (28)	2·0	2·0	2·0	1·5
		419 (19)	3·0	3·5	3·0	2·0
	Pb	517 (88)	1·5	1·0	1·0	1·0
		2778 (240)	3·0	1·5	2·0	1·5
		7676 (92)	3·0	2·0	3·0	2·0
	Zn	1976 (229)	2·0	1·5	1·5	1·0
		5730 (157)	2·0	2·0	2·0	2·0
		22052 (650)	3·0	2·5	4·0	2·5
T. bielanensis	Cd	57 (3)	0·0	2·0	0·0	0·0
		222 (6)	0·0	3·0	2·0	0·0
		419 (18)	0·0	3·0	2·0	0·0
	Pb	517 (30)	0·0	2·0	1·0	0·0
		2778 (143)	0·0	3·0	2·0	0·0
		7676 (216)	0·0	3·0	3·0	0·0
	Zn	1976 (387)	0·0	1·0	1·5	0·0
		5730 (459)	1·0	1·0	2·0	1·5
		22052 (620)	3·0	1·0	4·0	1·5

[a] Control conditions per definition resulted in an impact index value of 0·0 for all parameters.

Impact index data plotted versus the percentages of each of the three categories control state, compensation and non-compensation (figure 4) revealed the index approach to be a suitable method to represent ultrastructural changes, which was confirmed by high correlation coefficient values for the respective regression curves. Increasing impact index values could be correlated with both increasing percentage of non-compensation symptoms and decreasing percentage of organelles in the control state. Symptoms of compensation showed highest frequency between impact indices of 2·0 and 3·0.

Sensitivity of organelles and species

There was a clear concentration–response relationship for all metals, organelles and species with respect to the metal concentration in

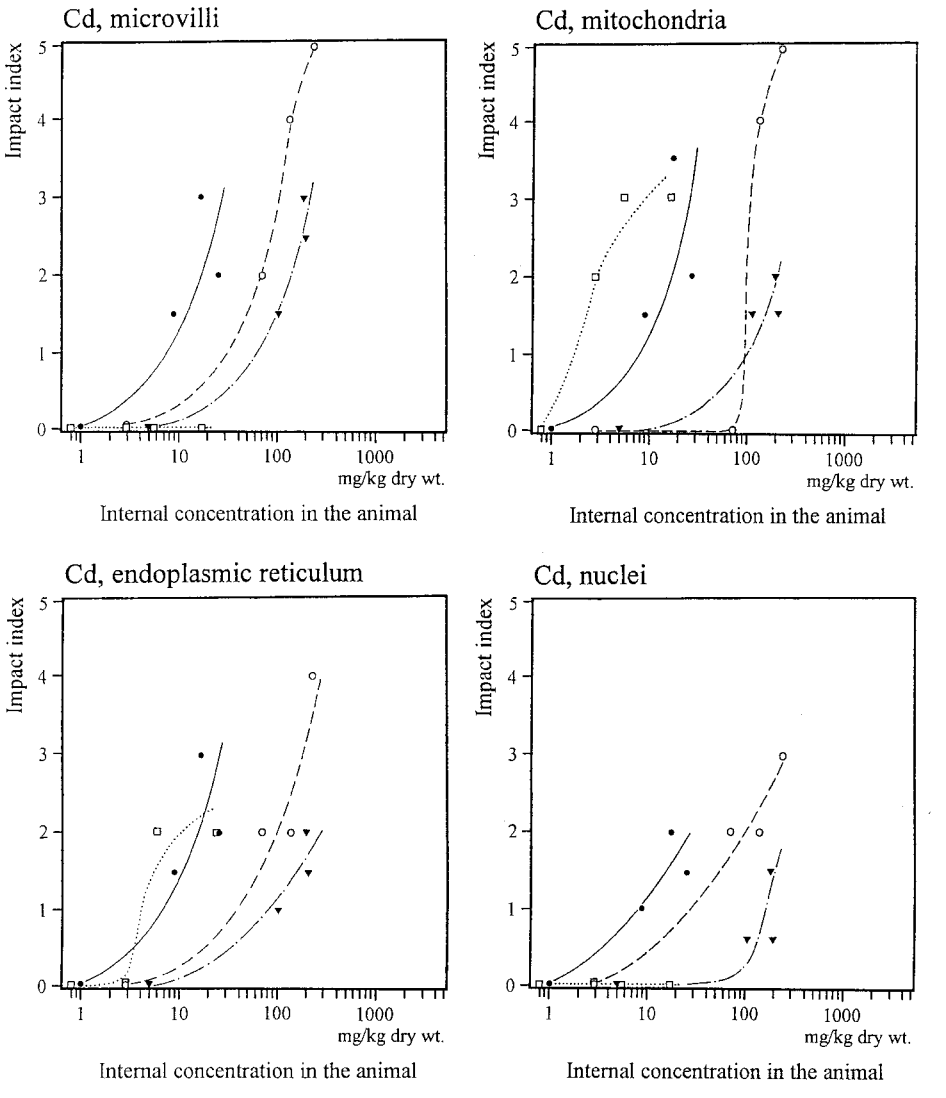

Figure 5. Relationship between internal cadmium concentrations in the animals (mg kg^{-1} dry wt) derived from cadmium exposure and the respective impact indices for microvilli, mitochondria, endoplasmic reticulum and nuclei in *D. reticulatum* (○, - - -), *J. scandinavius* (●, ——), *P. scaber* (, –·–) and *T. bielanensis* (□, ····).

relationship was confirmed with respect to the internal concentrations of metals in the animals by the fitted curves displayed in figures 5–7, although some data points did not follow the general trend.

The four investigated organelles generally indicated metal effects at similar internal threshold concentrations. Exceptions were the microvilli and nuclei in *T. bielanensis* midgut cells in response to cadmium or lead. However, organelles were not equally sensitive to elevated metal concentrations. Generally, mitochondria and particularly the nuclei of the investigated tissues were less adversely affected by elevated body concentrations than the microvilli or the ER of the same cells (compare figures 5–7).

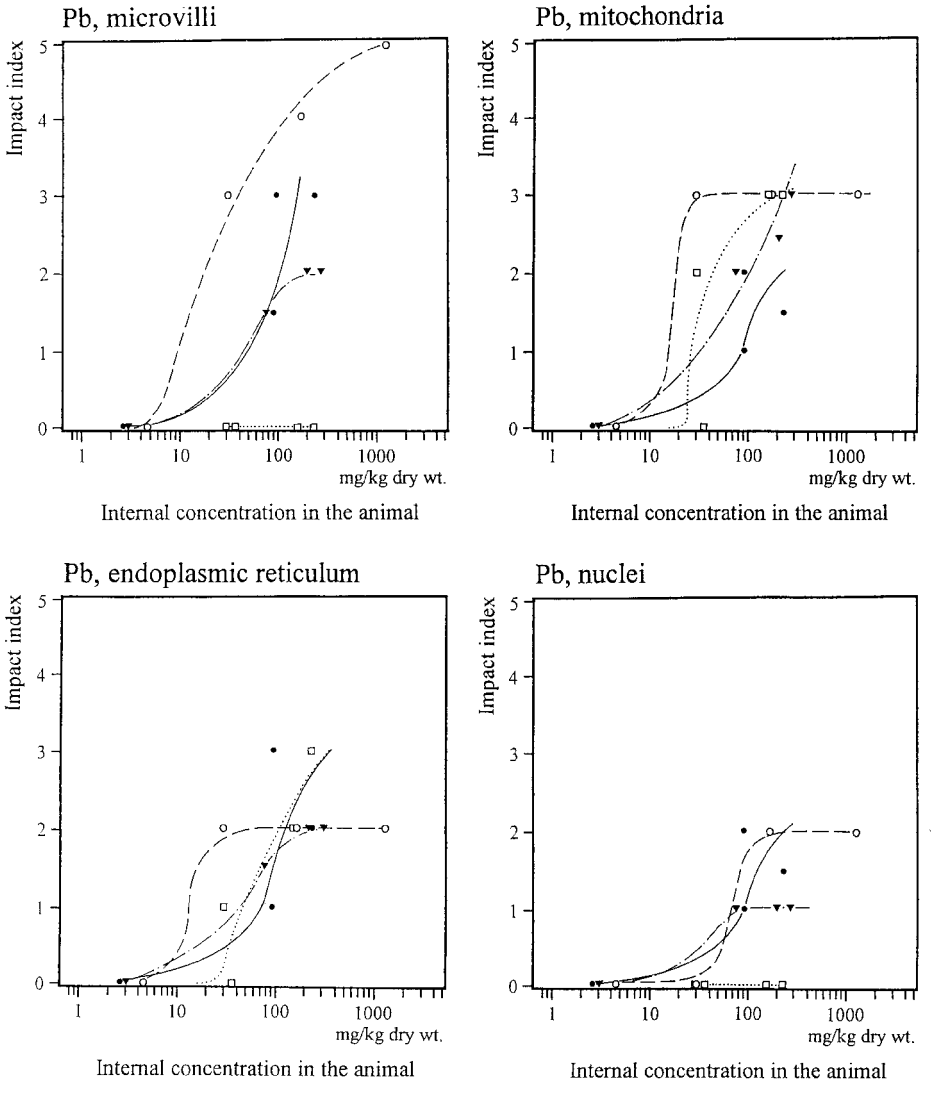

Figure 6. Relationship between internal lead concentrations in the animals (mg kg^{-1} dry wt) derived from lead exposure and the respective impact indices for microvilli, mitochondria, endoplasmic reticulum and nuclei in *D. reticulatum* (○, - - -), *J. scandinavius* (●, ——), *P. scaber* (, –·–) and *T. bielanensis* (□, ····).

Cadmium was found to induce effects in the most sensitive organelles at body concentrations of only a few mg kg^{-1} dry weight (figure 5). The minimum threshold values for the induction of ultrastructurally visible effects by cadmium varied from organelle to organelle and from species to species but, as a general trend, were up to approximately one order of magnitude lower than those for lead (figure 6) and at least two orders of magnitudes lower than those for zinc (figure 7) despite the fact that exact threshold concentrations are difficult to derive (Cairns 1992). The metal concentrations inducing a detectable response in 10–15 % of the respectively most sensitive organelles (which corresponds to an impact index of 0·5) varied according to the species from about 1 to 30 mg kg^{-1} dry wt for cadmium, from 8 to 25 mg kg^{-1} dry wt for lead, and from 100 to 350 mg kg^{-1} dry wt for zinc.

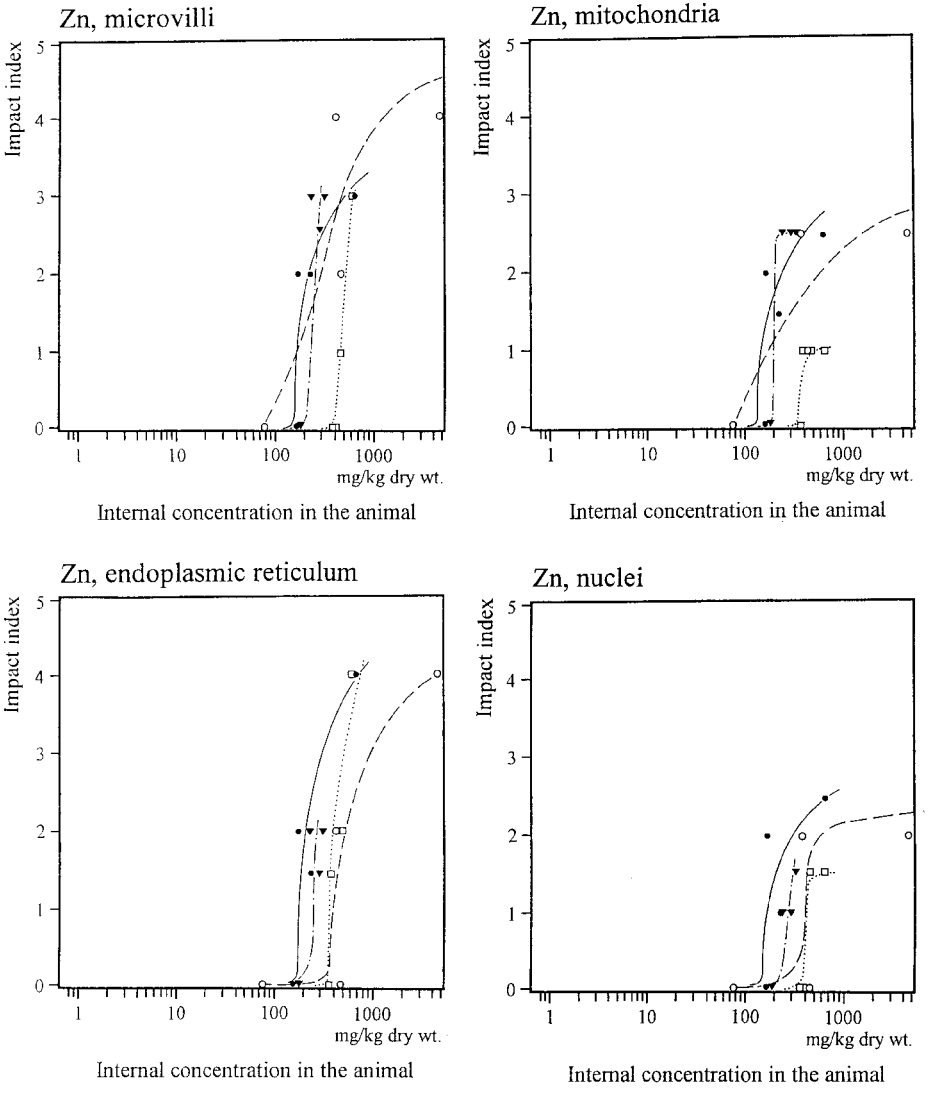

Figure 7. Relationship between internal zinc concentrations in the animals (mg kg^{-1} dry wt) derived from zinc exposure and the respective impact indices for microvilli, mitochondria, endoplasmic reticulum and nuclei in *D. reticulatum* (○, - - -), *J. scandinavius* (●, ——), *P. scaber* (, –·–) and *T. bielanensis* (□, ·····).

Sensitivity to metals also differed among the investigated species. *J. scandinavius* was more sensitive to cadmium than *D. reticulatum* and *P. scaber* (figure 5) while, in response to lead, *D. reticulatum* showed the highest sensitivity of these three species (figure 6). The lack of any effect of both the tested cadmium and lead concentrations to microvilli and nuclei in the midgut tissue of *T. bielanensis* indicates the comparatively high tolerance of this species, which was also reflected by its remarkably low mortality during exposure (Pawert *et al.* 1996). This tolerance was also verified for zinc exposure while the other three investigated species showed approximately equal sensitivity to zinc (figure

Interrelationships and patterns of organelle reactions

Independent of the species and the tissue, the degree of organelle reaction (the impact index) seemed to show a trend towards a positive linear relationship when microvilli versus nuclei, microvilli versus ER, and nuclei versus ER were compared. Reactions of mitochondria showed a similar trend with the exception of those from *T. bielanensis*. The correlation of the data sets with the regression lines, however, was poor which speaks in favour for the interpretation that specific reaction patterns occurred in different species in response to varying exposure conditions.

When comparing these particular cellular reactions, cluster analysis revealed species-specific patterns for *T. bielanensis* and *D. reticulatum* in response to low or moderate metal concentrations (figure 8). Especially in the case of zinc, however, the responses to the highest or second highest body concentration for metal did not agree with the general species-specific pattern, but rather responses clustered together with those obtained in *J. scandinavius* affected by the highest zinc concentration. Also cellular reactions to the highest cadmium level in *D. reticulatum* did not cluster closely with those of lower cadmium concentrations (figure 8). Apart from these cases, the responses observed in *P. scaber* and *J. scandinavius* were similar to one another but not species-specific and clustered together, with a trend to the formation of sub-clusters comprising responses to the lowest applied metal concentrations independent of the type of metal. Thus, generally no metal-specificity of the cellular response patterns was observed in the four investigated species.

Discussion

Our combination of multiple investigations on ultrastructural reactions to metal exposure has contributed to both basic science and applied biomarker research. Since these aspects are interwoven, basic knowledge on specificity and variability of cellular responses to toxins stands as a prerequisite for the use of ultrastructural markers in toxicity assessment. From a pharmacological point of view, it is conspicuous that quite a number of concentration–response curves in the present study appear to be parallel in their course although their response thresholds varied. This may suggest that the mechanism of action of the metals is similar in certain species (e.g. in *J. scandinavius* and *P. scaber*, according to cluster analysis) and different in others (*T. bielanensis*, *D. reticulatum*). Also when comparing the concentration–response relationships with respect to metal specificity, obvious differences among the curves could only be obtained for the threshold values' orders of magnitude but not for the general course of the curves. To interpret these similarities in metal effects, it is necessary to focus on existing data on metal action at the molecular level.

The first contact of ingested metals inside the body with cells occurs at the outer plasma membrane, where metals interact with a variety of channel proteins (e.g. Benndorf and Nilius 1988, Sheets and Hanck 1992). Although even this primary action may be sufficient for a cascade of cellular reactions, usually metals manage to permeate the plasmalemma either as cations, anions, or organic complexes (Dawson and Ballatori 1995). The uptake of the metals cadmium, zinc and lead into the tissues of the soil animals investigated here was confirmed by AAS analysis and, thus, the observed effects on organelles could be related directly to the metals.

Cadmium and zinc are able to permeate calcium channe

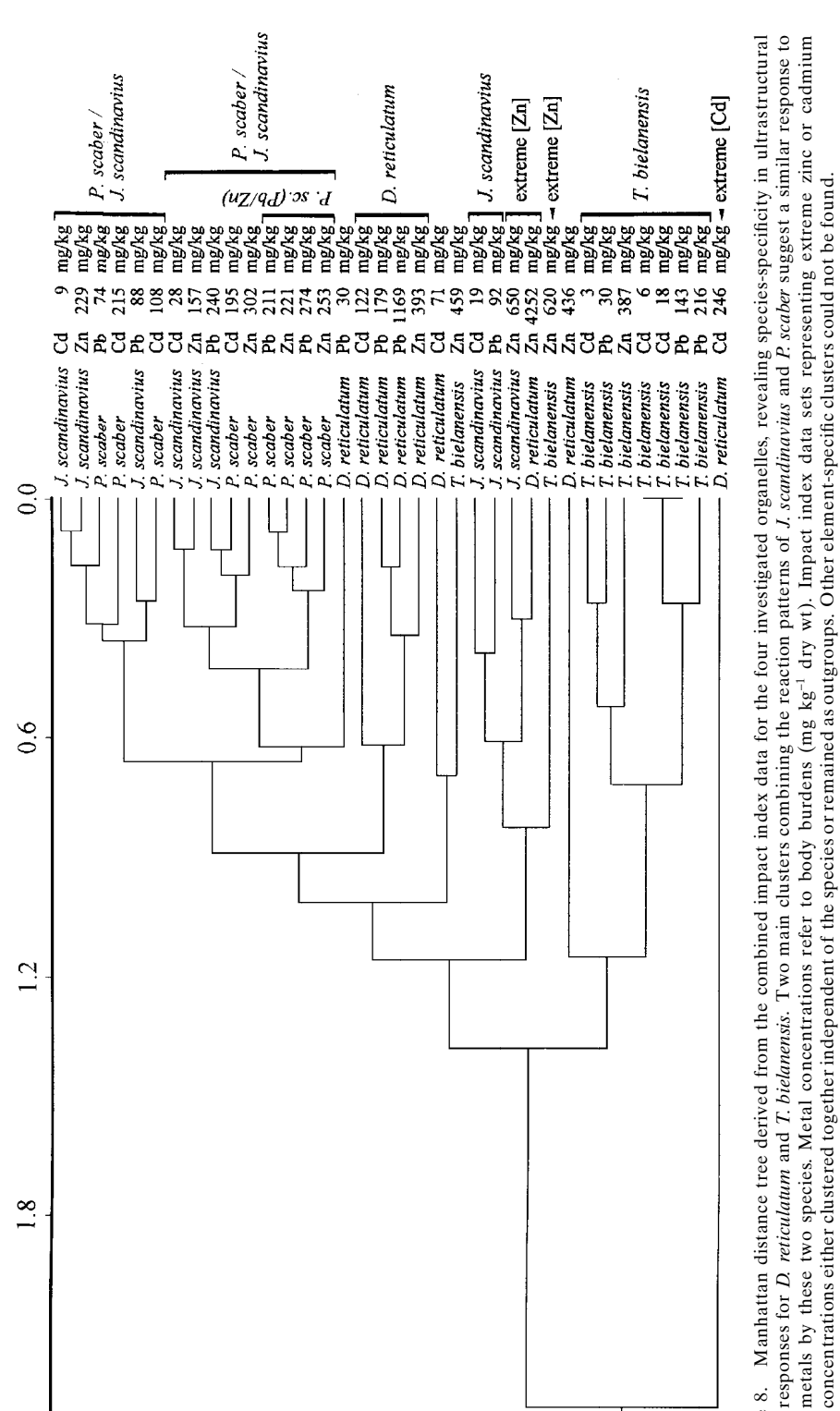

Figure 8. Manhattan distance tree derived from the combined impact index data for the four investigated organelles, revealing species-specificity in ultrastructural responses for *D. reticulatum* and *T. bielanensis*. Two main clusters combining the reaction patterns of *J. scandinavius* and *P. scaber* suggest a similar response to metals by these two species. Metal concentrations refer to body burdens (mg kg^{-1} dry wt). Impact index data sets representing extreme zinc or cadmium concentrations either clustered together independent of the species or remained as outgroups. Other element-specific clusters could not be found.

and Kawa 1977) and it has been shown that voltage-dependent channels (e.g. Hinkle *et al.* 1992) as well as receptor-activated calcium channels mediate the influx of both metals into the cytoplasm of the cell (Crofts and Barritt 1990, Blazka and Shaikh 1991). In addition, zinc is also taken up by the cell as anion complexes (Alda Torrubia and Garay 1989) or as organic complexes via amino acid and glutathione carriers (Aiken *et al.* 1992, Ballatori 1994). In turn, cadmium is transported into cells through mechanisms employed in zinc uptake (Waalkes and Poirier 1985). Divergently, the trans-membrane passage of lead seems to be restricted (< 0·1 % in isopods, Hopkin 1989), but is also facilitated by the formation of anion complexes (Simons 1986). Additionally, all types of metals are taken up by cells via receptor-mediated endocytosis, especially when they are bound to ligands (for review, see Ballatori 1991).

Independent of the divergent modes of Cd/Zn and Pb uptake, the intracellular action of toxic levels of lead, cadmium, or zinc are manifold, complex and seem to affect a set of basic metabolic pathways in all three cases. Because of the physicochemical similarities of cadmium and zinc (Goering *et al.* 1995), comparable toxic action of these two metals should be expected. One of the central processes affected by cadmium (and most likely also by zinc) is the disruption of the regulation of calcium-dependent calmodulin activation (Habermann *et al.* 1983, Suzuki *et al.* 1985) which could result in serious pathologic consequences (Goering *et al.* 1995) such as cytoskeletal damage and perturbed cell–cell communication (Trump and Berezesky 1987). However, not only the ions mimicking Ca^{2+} physicochemically but also the divalent ion Pb^{2+} may substitute for calcium in calmodulin activation causing adverse cell reactions as described above (Habermann *et al.* 1983, Magos 1991). This uniformity in intracellular action may explain the lack of metal specificity of the patterns of intracellular responses recorded in the present study. Additional corroboration for this explanation lies in the fact that all investigated metals also interfere with chromatin structure and DNA integrity (e.g. Hartwig *et al.* 1990, Waalkes *et al.* 1991, Chesters 1992). In this respect, metals may interact with calcium-regulated processes, receptor proteins, *trans*-acting gene-regulating proteins and the DNA repair mechanisms (Sunderman and Barber 1988, Hartwig *et al.* 1990, Makowski *et al.* 1991).

As shown by the present results, all organelles showed a direct concentration–response relationship, the threshold metal concentration and the response intensity of which, however, differed among the exposure conditions. Thus, the cellular reaction patterns (the syndromes) showed trends towards (1) species-specific clustering, which corresponded precisely to different metal accumulation kinetics in *D. reticulatum*, *T. bielanensis* and a group comprising both *J. scandinavius* and *P. scaber* (Gräff *et al.* 1997) and (2) concentration-dependent clustering. Both components lead to ultrastructural response patterns which may be used as biomarkers of effects as well as of exposure to metal ions. In this context, it is interesting to note the stronger correlation of the observed effects versus the applied metal concentrations in the food rather than versus the internal concentrations in the animals' tissues. The latter parameter is usually used in toxicity assessment and, thus, also in this paper, impact index data were plotted against the internal metal concentration. Although the total metal burden is not necessarily related to the effects on single organs, at least in arthropods almost the entirety of absorbed metal passes the epithelia of the digestive tract. In all the animal taxa investigated here, heavy metals are deposited in the cells of the digestive organs (summarized by Hopkin 1989). Exp

highest metal concentrations applied in the present study, however, did not always result in extremely high metal concentrations in tissues. This may have been a consequence of reduced food uptake rates (e.g. Köhler *et al.* 1996). Nevertheless, cellular ultrastructural changes reflect metal exposure also in these cases since nutrient (and subsequently energy) deprivation should be considered as a secondary effect of exposure to metals.

Species-specificity of response patterns, as we detected, should not be confused with species-specificity of qualitative ultrastructural changes, for example the formation of a network-like chromatin structure typical for nuclei of isopod hepatopancreatic cells following metal treatment (Köhler *et al.* 1996) or starvation periods (Storch 1984). In contrast to this example, ultrastructural effects of metal exposure generally should be regarded as pathological symptoms that are widely distributed in numerous cell types (summarized, for example, by Ghadially 1988). For this reason, the classification of ultrastructural alterations into three categories seems to be justified, despite some inevitable loss of qualitative and quantitative information results from this approach. In comparison to those methods which have been utilized so far to evaluate ultrastructural responses to stressors, the method presented here reduces both the qualitative description of symptoms and precise quantitative information which can be obtained by morphometrics to the level of rather broadly defined categories. Nevertheless, the advantage of our protocol, however, lies in its potential (1) to give quantitative information on pattern similarity and, consequently, on toxin-specificity, (2) to contribute to toxicity assessment when the aim is to rank various adverse conditions, and (3) to handle multiple data sets in comprehensive toxicity experiments under controlled laboratory conditions. A variety of both physiological and environmental factors as well as the presence of additional trace contaminants, however, may influence the observed parameters. Therefore, to the present state of knowledge, the applicability of the impact index method is restricted to laboratory experiments. Further research, however, may reveal reliability of our protocol also for soil invertebrate samples taken from field sites.

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