Evaluation of cadmium toxicity to Collembola *(Proisotoma minuta)* using electron microscopy

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Abstract
Collembola are widespread decomposers in soil and are used as test animals for ecotoxicological research. The effects of cadmium (Cd) on Collembola have been studied on *Folsomia candida*, a common European species, but little information is available on Cd toxicity to *Proisotoma minuta*, a species common in Australian soils.

Laboratory studies were conducted to evaluate Cd accumulation and distribution in *P. minuta*. Adults grown in Cd contaminated soils were analysed in two ways. In the first method whole adults were removed from contaminated soil, immediately digested in acid and Cd body concentrations were determined using a graphite furnace atomic absorption spectrophotometer (GFAAS). For the second method Collembola were transferred into clean jars for skins and eggs collection for eight days. The Cd concentrations of the skins, eggs and whole animals were analysed separately. The Cd body concentrations of *P. minuta* measured immediately after removal from Cd contaminated soil showed a significant increase compared with the control at 75 and 100 μg Cd/g. Cadmium concentrations in skins and eggs were much lower than in the body indicating that Cd accumulates inside the Collembola.

The effects of Cd on the body and midgut cell structure of *P. minuta* were observed using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). SEM analysis showed that the antennae of *P. minuta* exposed to high Cd concentrations were straight and wrinkled compared to control. The TEM analysis of thin sections showed that mitochondria and the microvilli border of the digestive system were destroyed and the number of Cd-containing granules increased in Collembola exposed to Cd contaminated soils compared with the control.

Key Words
Cadmium, Collembola, *Proisotoma minuta*, electron microscopy, accumulation

Introduction

Cadmium is one of the heavy metals that has no known biological function and can be potentially toxic at low levels (Kabata-Pendias 2000). Cadmium has been reported to be responsible for altering the species composition in populations of microorganisms and some aquatic and terrestrial invertebrates (Dobson 1992). Leaf litter decomposition is significantly reduced by heavy metal pollution, and Cd has been identified as being partly responsible for this (Van Wensem *et al.* 1997; McEnroe and Helmisaari 2001). The soil biota is an important component of all terrestrial ecosystems since it acts in the primary stage of decomposition of organic matter and humus formation in food webs and provides essential nutrients to plants. The study of Cd uptake by plants and animals, in particular soil fauna, is needed in order to understand its effects and to develop a bioindicator system.

Collembola have been recognized as the most widely distributed and often highly abundant organism in most terrestrial ecosystems. The most commonly used and standardised Collembola species for ecotoxicological tests on the effects of heavy metals uses *Folsomia candida* (Hopkin 1997). Much of the published European toxicity data has been generated using this species. In Australia *F. candida* is rare and two Australian Collembola species that are more ecologically relevant to Australian soils, *Sinella communis* and *Proisotoma minuta*, have been suggested as useful alternatives (Greenslade and Vaughan 2003). *P. minuta* is found abundantly in agricultural areas such as in trash in cotton fields and also in some rehabilitated mine sites in southern Western Australia.

Bioavailability of heavy metals is related to three dynamic processes. These are the physicochemical process of desorption in the soil; physiological uptake process of living organisms; and toxicodynamic redistribution processes within the organism's body (Hamelink *et al.* 1994). There are three mechanisms

or strategies observed in Collembola related to uptake routes and accumulation of Cd when the organisms are exposed to Cd in their food (Hopkin 1989). These mechanisms that may occur together in a single collembolan species are behavioural avoidance of metal uptake, compartmentalization within organs and cells, and excretion. These mechanisms are mediated by the midgut epithelium. The study on the cellular injuries in Collembola's midgut cells due to heavy metal exposure has been done using one of the largest species, *Tetrodontophora bielanensis* (Pawert *et al.* 1996). In their study, Zn was found to localize in the midgut epithelial cells but no Cd could be detected. The induction of metal binding protein, metallothionein, has also been studied in the midgut cells of *Orchesella cincta* which was due to Cd food contamination (Hensbergen *et al.* 2000). To our knowledge, no study has been done on the effects of Cd on the body and cellular structure of *P. minuta*. The aim of this study was to evaluate Cd accumulation and distribution in *P. minuta* and to observe the effects of Cd on the body and cellular structure of *P. minuta* using SEM and TEM.

Methods

Culture of P. minuta

The establishment of *P. minuta* cultures was made according to Park and Lees (2004). The Collembola were of standard adult size with approximate length of 1.16±0.08 mm. There were no juveniles as an overcrowded population had stopped laying eggs. The population size of the overcrowded population of *P. minuta* is approximately up to 31 insects/cm ^{2} (Singh and Moore 1985). These adults laid eggs after transfer to fresh and less crowded containers (Nursita *et al.* 2004).

Cadmium exposure experiment

An acid sandy loam natural soil (Kurosol) from Arthursleigh, New South Wales was used for the experiment. Some of the physico-chemical characteristics of this soil were determined and are shown in Table 1. The soil was mechanically ground and sieved to obtain a < 2 mm fraction and 25 g samples were placed in glass jars. Dilute solutions of cadmium nitrate were added to obtain a range of Cd concentrations (50, 75, 100 and 150 μg/g). The same volume of deionised water was added to the control jars. There were three replicates for all treatments including controls. The samples were maintained at 60% moisture content of field capacity for four weeks to equilibrate and obtain a homogenous distribution of the applied metal in soil (Kula and Heimbach 1998).

Table 1. Some important physico-chemical properties of Arthursleigh soil used for the study.

Soil property	*Value*
pH (1:5 soil:water)	4.88
EC (μS/cm, 1:5 soil:water)	110.6
CEC ($mmol_c$/kg)	36.5
Organic carbon (%)	1.32
Sand (%)	76.7
Silt (%)	13.3
Clay (%)	10.0

One hundred adults were placed in the control, 50, 75 and 100 μg/g Cd contaminated soil for 28 weeks and 150 μg/g Cd soil for 19 weeks as the initial culture of *P. minuta* in soils. The difference of experimental period was due to high mortality of *P. minuta* observed in 150 μg Cd/g soil after 9 weeks of exposure. Therefore, a new set of 150 μg Cd/g sample was prepared in the 10th week (i.e. 19 weeks before transferring them into the fresh jars). After the experimental period, one hundred adults from each replicate from the control, 50, 75 and 100 μg/g Cd soils were washed once with 0.1% HCl and two times with deionised water on a vacuum filter with a 0.45 μm dark gridded membrane (Millipore). After overnight drying at 60°C to obtain the dry weight, adults were digested in 1 ml pure HNO_3 (69%, AR grade, BDH chemicals) in a 10 ml vial until all the liquid had evaporated. The residues were dissolved in 5 ml 0.1 % HNO_3 and Cd concentrations were measured using a Varian GTA 110Z GFAAS. Another one hundred adults from each replicate of the control, 50, 75 and 100 μg/g Cd soils were transferred into jars containing fresh plaster of Paris and charcoal mixture (5:1, v : v) substrate for 8 days for collecting moulted skins and eggs. Due to high mortality in 150 μg/g Cd soil culture, only 40 surviving adults were transferred into jars for skins and eggs collection. After 8 days, the body, skins and eggs concentrations of all the freshly housed *P. minuta* were analysed using GFAAS as described earlier.

ANOVA (analysis of variance, Dunnett comparison with control, and Tukey comparison of mean for all pairs) were performed using JMP version 5.0 (SAS 2002) to determine the significance of differences in the Cd accumulation of *P. minuta* between various treatments.

Scanning electron microscopy (SEM)
Adults of *P. minuta* from control and 100 μg/g Cd contaminated soil exposed for 12 weeks and 150 μg/g Cd contaminated soil exposed for 10 days were examined by SEM. The difference of experimental period was due to high mortality of *P. minuta* observed in 150 μg/g Cd treatment soil after 10 weeks of exposure. Therefore, a new set of 150 μg Cd/g sample was prepared 10 days prior to SEM examination. Three adults from each treatment were placed on the aluminium stub and killed by chloroform vapours. The samples were coated with platinum for 45 min and examined using Philips XL30 CP SEM with operating voltage ranging from 5 to 20 kV.

Transmission Electron Microscopy (TEM)
Adults of *P. minuta* from the control and 100 μg/g Cd contaminated soil exposed for 13 weeks and 150 μg/g contaminated soil exposed for 3 weeks were examined by TEM. The difference of experimental period was due to high mortality of Collembola with this treatment as described earlier. Three adults of *P. minuta* from each treatment were prepared for TEM. The sample preparation was done in a fume hood. Each adult was laterally cut using a razor blade under the dissection microscope in a petri dish with a few drops of 2.5% glutaraldehyde and divided into halves to get closer to the midgut as the target organ. The samples were fixed in 2.5% glutaraldehyde dissolved in 0.1 M phosphate buffer (pH 7.4), rinsed repeatedly in the phosphate buffer and postfixed in a mixture of 0.5 ml 1% OsO_4 solution and 0.5 ml 0.1 M phosphate buffer (pH 7.4) for 2 hr. After washing with deionised water, and dehydrating in a graded ethanol series (30-100% ethanol), the specimens were infiltrated with a graded ethanol and resin series (2:1; 1:1; 1:2; v:v, overnight for each mixture) and finally 100% resin overnight. The specimens were placed in fresh 100% resin and polymerised at 60ºC overnight. Ultrathin sections were counterstained with uranyl acetate and alkaline lead citrate for 10 min and examined using a Philips CM120 Biotwin TEM at an operating voltage of 120 kV.

Results
Cadmium body concentration
The Cd accumulation of *P. minuta* increased linearly with the increasing Cd concentration in soil (0-100 μg/g, Figure 1a). The Cd body concentration after 28 weeks of exposure was significantly higher ($P < 0.05$, Figure 1a) at Cd soil concentrations of 75 and 100 μg/g than the control. No determination of Cd body concentration could be made in *P. minuta* from 150 μg/g soil because of high mortality in these assays. The Cd concentrations in Collembola after 28 weeks Cd exposure in soil and 8 days on clean plaster of Paris/charcoal substrate were lower than their concentrations before transfer to a clean environment, but the differences were not statistically significant ($P > 0.05$, Figure 1a). A linear increase in Cd body concentration with Cd concentration of soil (0-150 μg/g) was observed in *P. minuta* 8 days after transfer to plaster of Paris/Charcoal mixture. A significantly higher Cd body concentration was observed in *P. minuta* exposed to 75, 100 and 150 μg/g at 8 days after transfer to fresh jars in comparison with the control ($P < 0.05$, Figure 1a). No significant difference was found in Cd concentrations in the moulted skins and eggs compared to controls at all Cd soil concentrations except for the moulted skins of animals exposed to 150 μg Cd/g soil (8.15 μg/g, Figure 1b). No eggs were laid at the 150 μg Cd/g soil, therefore, no Cd eggs concentration could be made at this concentration.

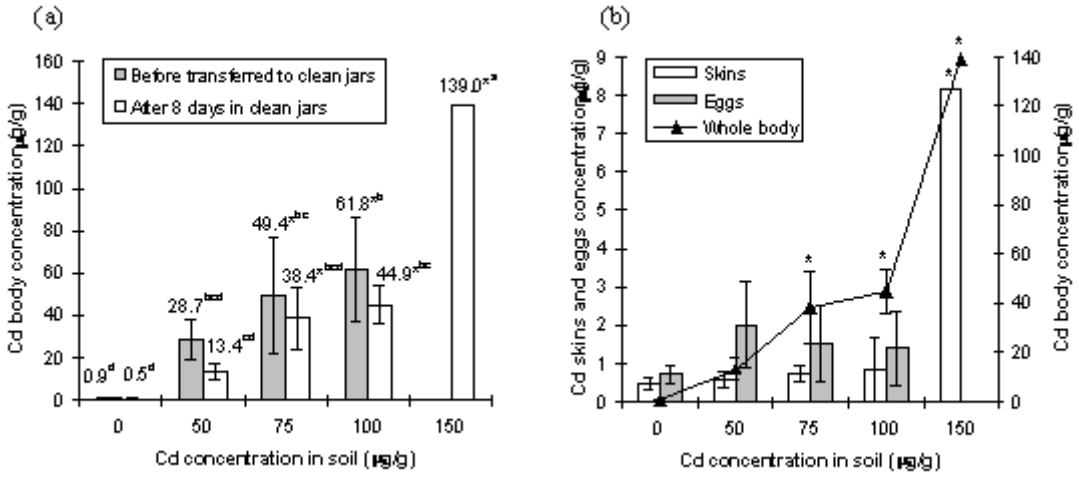

Figure 1. The Cd concentration (µg/g) of P. minuta after 28 weeks of exposure to Cd contaminated soils; (a) before and after transfer to clean jars for 8 days, (b) Cd concentrations of moulted skins, eggs and body 8 days after transfer to clean jars. Only 40 surviving adults in 150 µg Cd /g soil were transferred to clean jars for Cd skins, eggs and body concentration analyses.
*** is significantly different from control at 5% level of significance (Dunnett's test)**
a, b, c, d, Superscripts show multiple comparison of means between Cd concentrations of *P. minuta* (Tukey's test at 5% level of significance). Means followed with identical letters are not significantly different to each other.

SEM and TEM analyses

The results from SEM analysis show external damage only in animals exposed to 150 µg Cd/g soil, whilst TEM analysis showed that internal cellular damage occurred in animals exposed to 100 µg/g. The SEM results showed that adults exposed to 150 µg Cd/g soil had straight antennae (Figure 2c and f) compared with the normal antennae in the control and 100 µg/g samples (Figure 2a, b, d, e). The body and antennae skin in *P. minuta* from highly Cd contaminated soil (150 µg/g) was more wrinkled than in control and 100 µg/g treatments (Figure 2a, b, c, d, e, f).

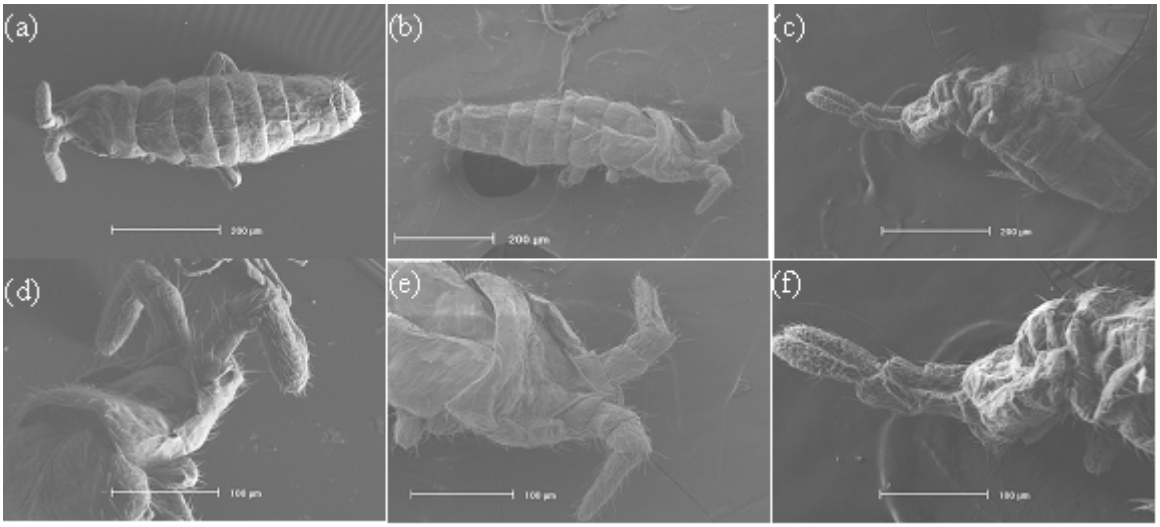

Figure 2. Body structure observation using SEM of *P. minuta* from control, 100 and 150 µg Cd/g treatments. Unaffected body structure observed after 12 weeks exposure to control (a) and 100 mg Cd/g (b) soil with normal skins and antennae compared to (c) wrinkled skins and straight antennae after 10 days exposure to 150 µg Cd/g soil. Magnified view of normal head and antennae of Collembola from control (d) and 100 µg Cd/g (e) compared to wrinkled and straight antennae at 150 µg Cd/g (f).

TEM examination of *P. minuta* thin sections of the digestive system tissues exposed to 100 and 150 µg/g Cd contaminated soils showed some cellular injuries such as fragmented microvilli border (Figure 3b, c) and degraded mitochondria (Figure 4b, c) compared with controls showing normal filament-like

microvilli and mitochondria (Fig 3a and 4a). There was an increased number of granules in *P. minuta* exposed to Cd compared to controls (Fig 3a, b, c, d).

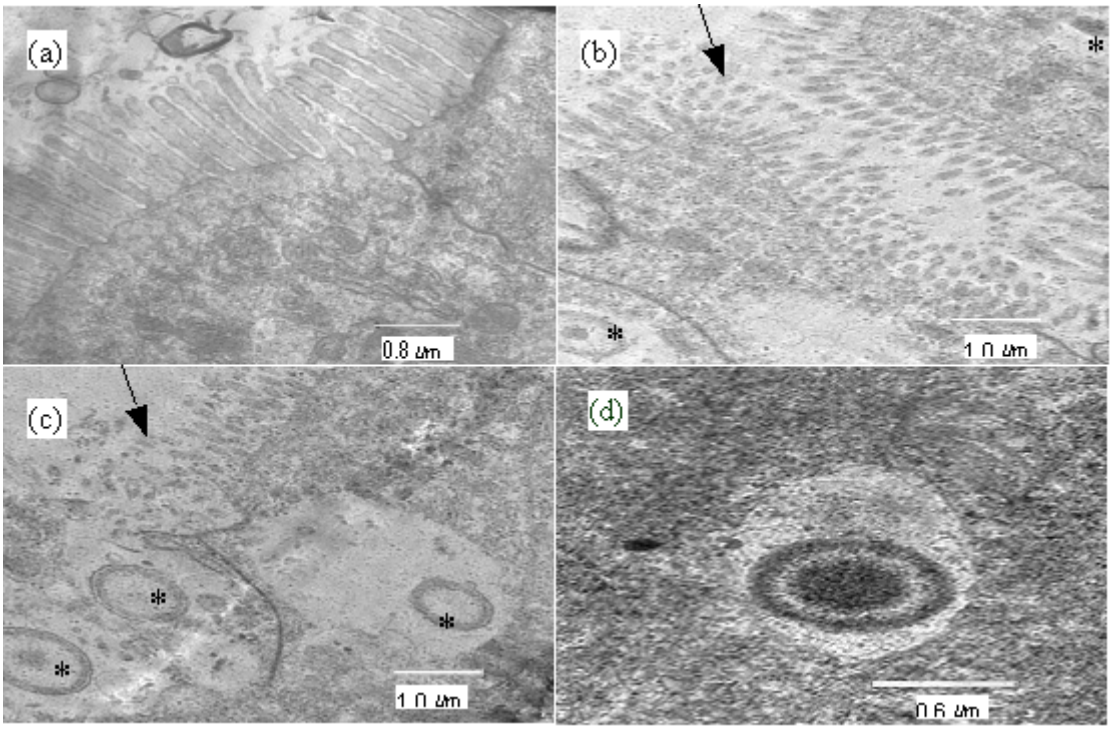

Figure 3. Cell structure of *P. minuta* observed using TEM in control, 100 and 150 µg Cd/g soil; (a) unaffected apical digestive system cells observed after 13 weeks exposure in control soil with normal filament-like microvilli, (b) dilated microvillus border (arrow) and high number of granules (*) after 13 weeks exposure to 100 µg /g Cd, (c) 3 weeks exposure to 150 µg /g Cd. (d) The magnified view of the granule found in wide areas in tissues of Collembola from 100 and 150 µg /g treatments.

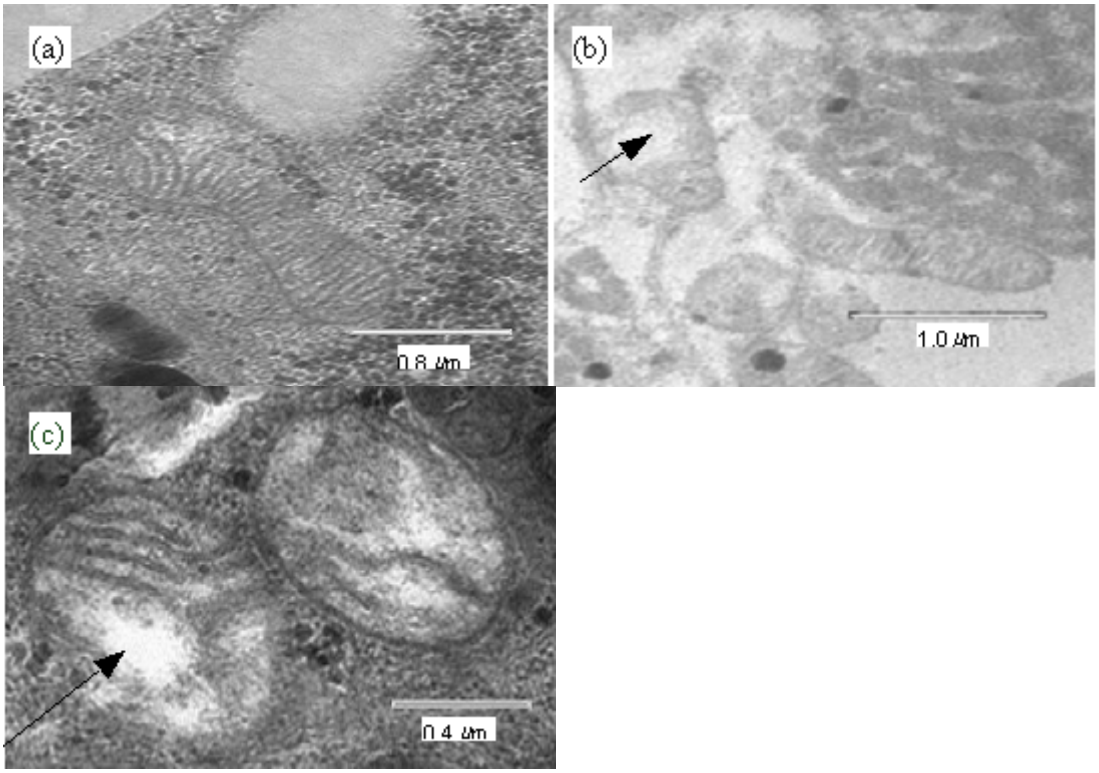

Figure 4. TEM micrographs of *P. minuta* digestive system cells from control soil with (a) normal mitochondria compared to (b) dilated mitochondria (arrow) observed in tissues of Collembola after exposed to 100 µg /g for 13 weeks and (c) 150 µg /g for 3 weeks.

Discussion

The Cd accumulation pattern of *P.minuta* in this study, which linearly increased with the increasing Cd soil concentration was similar to *T. bielanensis* and *F. candida* when exposed to Cd contaminated food (Pawert *et al.* 1996; Fountain and Hopkin 2001). Cadmium concentrations in skins and eggs were much lower indicating that Cd accumulates inside the Collembola. Cadmium body concentration was slightly decreased when animals exposed to Cd for several months were transferred to a clean environment for a few days suggesting that *P. minuta* are excreting Cd accumulated in the old gut epithelium and replacing this with the new epithelium. It has been reported that in *O. cincta* and *Tomocerus minor* the gut pellet contains about 35% of the total Cd present in the animal resulting from the cellular excretion and discharge of metal-containing granules into the gut lumen in the intestinal epithelium (Humbert 1978; Van Straalen *et al.* 1987).

Collembola's behavioural response towards toxic materials was initially observed in *F. candida* (Trublaevich and Semenova 1997). Strong responses such as seizures or shock, straight and sticking antennae, paralysis of the jumping fork, appearance of spherical fluid formation, darkening or spotting of the skins and loss of stability were observed when 5 and 10 μg/g were added to a sieved river sand as the substrate for *F. candida*. In our study, the SEM observation of adults of *P. minuta* exposed to 150 μg Cd/g soil showed a straight and sticking antennae as well as wrinkled body skin. This wrinkle may appear as dark and spotted skin when observed by eye or light microscopy. In this study the highest Cd concentration at which no observable effect (NOEC) occurred on the body structure of *P. minuta* was 100 μg /g. These big differences in Collembola's response to Cd toxicity may be due to differences in Cd availability and species used in the two experiments.

The TEM observation on *P. minuta's* midgut cells showed cellular injuries after exposure to 100 and 150 μg/g Cd in particular to microvilli borders and mitochondria in comparison with control. Similar to our result, dilated mitochondria of *T. bielanensis* midgut cells were observed at 50-100 mg Cd/l contaminated food exposure, however microvilli border did not show any significant effect (Pawert *et al.* 1996). In their study alterations of cellular structure as the result of cellular injuries in the *T. bielanensis* midgut cells were found to be dose-dependent. At 10 mg/l Cd exposure, the midgut cells showed a vesiculated rough endoplasmic reticulum and the cytoplasm was slightly condensed compared with controls (parallel cisternae endoplasmic reticulum and an electron-lucent cytoplasm). Numerous spherically-shaped type A granules occurred throughout the cells. At 50-100 mg/l Cd exposure, cytoplasm was more condensed, large vacuoles derived from rough endoplasmic reticulum dominated the central part of the cell and dilated or totally destroyed mitochondria were observed. Similarly in our study, type A granules were found in wide areas of 100 and 150 μg/g Cd exposed tissues, but no significant effect observed in endoplasmic reticulum and cytoplasm of the midgut cells in comparison with control. The differences between ultracellular effects for Cd may be due to differences between species and also the different substrate for Cd exposure. Type A granules are the most common granules observed in collembolan midgut cells when exposed to Cd, Pb and Zn contaminated food, whilst other granules could not be observed (Pawert *et al*. 1996). Previous studies using energy dispersive X-ray spectroscopy showed that the main constituents of type A granules are Ca, Mg, P, Zn, K and a trace of iron (Mason and Simkiss 1982). Group B metals such as Cd, Cu and Hg have not been detected (Hopkin 1989). There are four types of metal containing granules in terrestrial invertebrate digestive systems and hepatopancreas cells (Hopkin 1989); type A granules that consist of concentric layers of Ca and Mg phosphate that may contain group A and borderline metals, type B granules that have a more heterogeneous appearance in thin section and contain large amounts of S in association with group B and borderline metals, type C iron rich granules, and type D granules that are much larger than the first three and are composed of concentric layers of calcium carbonate. No NOEC value could be determined for *P. minuta* for the effect on cellular structure due to few Cd level tested in this study. In order to get a better understanding of the effects of Cd on *P. minuta*'s body and cellular structure, a wide range of Cd concentration in soil for sometime should be evaluated. Food as the substrate of Cd exposure may also give useful information not only on the effects of Cd on the *P. minuta* at individual and population level but also on its effects on the food chain as decomposer and prey for their predator.

Conclusions

The accumulation of Cd in *P. minuta* increased linearly with increasing Cd concentration in soil. The Cd body concentration was slightly decreased when animals exposed to Cd for several months were transferred to a clean environment for a few days suggesting that *P. minuta* may be able to excrete Cd

through shedding the epithelium of the midgut. Cadmium concentrations of moulted skins and eggs were much lower than the total body concentrations indicating that Cd accumulates inside *P. minuta's* body. The Cd body concentration was significantly higher than in control when animals were exposed to highly Cd-contaminated soil (150 μg/g) and the structure of the animal's body was affected. The antennae were straight and held in a sticking position, the whole body was wrinkled and in the digestive system cellular injuries occurred at 100 and 150 μg Cd/g soil. The mitochondria and microvilli borders were often dilated and destroyed. This damage is likely to result in insufficient nutrient absorption by the cells, disrupted metabolism and a reduction in energy supply. The increased number of granules and spherites in cells from animals from 100 and 150 μg/g Cd contaminated soil may also be involved in Cd storage and detoxification as these granules have been observed widely distributed in the digestive system cells of several invertebrates exposed to heavy metals (Hopkin 1989).

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