



Body metal concentrations and glycogen reserves in earthworms (*Dendrobaena octaedra*) from contaminated and uncontaminated forest soil

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Detoxification and accumulation of cadmium and lead by earthworms carries little energetic expenses whereas strict internal regulation of aluminium and nickel has energetic costs.

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ABSTRACT

Stress originating from toxicants such as heavy metals can induce compensatory changes in the energy metabolism of organisms due to increased energy expenses associated with detoxification and excretion processes. These energy expenses may be reflected in the available energy reserves such as glycogen. In a field study the earthworm, *Dendrobaena octaedra*, was collected from polluted areas, and from unpolluted reference areas. If present in the environment, cadmium, lead and copper accumulated to high concentrations in *D. octaedra*. In contrast, other toxic metals such as aluminium, nickel and zinc appeared to be regulated and kept at low internal concentrations compared to soil concentrations. Lead, cadmium and copper accumulation did not correlate with glycogen reserves of individual worms. In contrast, aluminium, nickel and zinc were negatively correlated with glycogen reserves. These results suggest that coping with different metals in earthworms is associated with differential energy demands depending on the associated detoxification strategy.

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1. Introduction

Metal-contaminated land is found in many parts of the world due to atmospheric emissions from smelters and other metallurgical processes and this pollution has drastic effects on the impacted ecosystems (Kozlov and Zvereva, 2007). The effects of metals (especially copper (Cu), zinc (Zn), cadmium (Cd), nickel (Ni) and lead (Pb)) on soil organisms have been much studied since the early 1970s because surface litter and humus are the principal metal sinks in the soil (Bengtsson and Rundgren, 1988; Hopkin, 1989). Several field and laboratory investigations have shown that the density of soil invertebrates in many cases is severely reduced in metal polluted soils up to several km away from the emission source (Bengtsson and Rundgren, 1982, 1988; Bengtsson et al., 1983; Spurgeon et al., 1994; Spurgeon and Hopkin, 1995, 1999; Haimi and Siira-Pietikäinen, 1996). On the other hand, there is

also evidence that soil invertebrates can genetically adapt to metal stress, thus modifying metal toxicity and promote persistence in contaminated soils (Posthuma and Van Straalen, 1993; Van Straalen and Timmermans, 2002; Timmermans et al., 2005). Thus, soil invertebrates are able to detoxify and store excess metal ions in membrane enclosed cellular granules or in metallothionein complexes (Ireland and Richards, 1977; Posthuma and Van Straalen, 1993; Stürzenbaum et al., 2001; Köhler, 2002; Schill and Köhler, 2004; Vijver et al., 2004; Timmermans et al., 2005).

Earthworms are among the most sensitive soil invertebrates when it comes to metal toxicity (Bengtsson and Tranvik, 1989) and given the ecological importance of these organisms earthworms have been of particular concern in metal-contaminated areas (Edwards and Bohlen, 1996). In the present study we focus on the earthworm *Dendrobaena octaedra* which is common in forests of the northern hemisphere (Stöp-Bowitz, 1969) including forest soils contaminated with heavy metals (Bengtsson et al., 1983, 1992; Haimi et al., 2006; Rozen, 2006).

Sublethal stress from toxicants such as heavy metals can induce compensatory changes in the energy metabolism of organisms due

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to increased energy expenses associated with detoxification processes. Since most of an organism's energy consumption is used for growth, reproduction and basal metabolism, coping with toxic stress can lead to reductions in growth and reproduction and thus the scope for population growth (Sibly and Calow, 1989; Calow, 1991; Maltby, 1999). It has been shown that the difference between available energy reserves (glycogen, fat and protein) and energy consumption is indicative of an organism's overall condition, and that this correlates with higher levels of biological organization such as population growth rates (De Coen and Janssen, 2003). A decrease in the available energy reserve can therefore be used as a biomarker of metal stress (Scott-Fordsmand and Weeks, 2000). The primary energy reserve in *D. octaedra* is the extensive glycogen storage that may reach 20–30% of dry tissue weight during autumn (Holmstrup et al., 2007b; Overgaard et al., 2009). These glycogen reserves are of great importance for winter survival in *D. octaedra* since they provide glucose that is used both as a cryoprotectant (Rasmussen and Holmstrup, 2002) and as a fuel for basal metabolism during winter where food acquisition is impeded by low temperatures or outright freezing of body fluids (Calderon et al., 2009).

In the present study we collected *D. octaedra* in late autumn from areas polluted by industrial metal smelters and from unpolluted reference areas. Body concentrations of metals and glycogen contents were determined for each individual allowing for correlations with metal body burdens and hence an investigation of costs associated with toxic stress.

2. Materials and methods

2.1. Description of study sites

An overview of the study sites is given in Table S1 in Supplementary information. The study area at Øvre Årdal (61°N) is situated at the inland end of the Sognefjord in a narrow valley, surrounded by more than 1000-m high mountains. Air pollution from an aluminium smelter at Øvre Årdal has led to extensive damage to the vegetation in the vicinity of the smelter (Vike, 1999). Vegetation damage is considered to be mainly caused by the emission of gaseous fluoride compounds, but emissions of aluminium from the smelter has also been documented (Vike, 2005). Earthworms and soil were sampled close to the smelter (100–500 m from the emission source). An area outside the deposition plume in a neighbouring valley, close to the village Lærdal at a distance of about 30 km from Øvre Årdal was chosen as reference site.

Earthworms were also collected near the village of Gusum in the south-eastern part of Sweden where a brass mill had been operating since 1661. In the late 1960s, the brass mill was replaced by a new factory (New Mill) north-west of Gusum. Emissions of Zn, Cu and Pb from the factory have polluted the surrounding environment; especially downwind of the factory (the prevailing wind direction is south-east). Earthworms were sampled approximately at the same sites as reported by Bengtsson et al. (1983) and Bengtsson and Rundgren (1988). Thus, two polluted sites 600–1400 m NW of the New Mill, and three reference sites about 20 km away from the New Mill were chosen (see Table S1 in Supplementary information). The vegetation in the Gusum area is coniferous forest with the forest floor dominated by mosses, *Deschampsia flexuosa* and *Vaccinium myrtillus*.

The Olkusz research area is located in the vicinity of the zinc and lead smelter "Boleslaw" in southern Poland where a large smelter was constructed in 1969, causing serious contamination of soils over an area of a few hundred square kilometres. Although the smelter was modernised in the early 1980s and the emission of pollutants has since dropped dramatically, soils in the area remain heavily contaminated with metals. The most important contaminants are Zn, Pb, Al and Cd. The sites for earthworm collection were established about 2.5 km away from the smelter and at reference sites approximately 30 km from the pollution source. All sites were located in Scots pine forest, with grasses, mosses and *V. myrtillus* predominant on the forest floor.

2.2. Collection of soil and earthworms

At each site, soil and worms (large juveniles and adults) were collected from relatively small areas (polluted and reference) of approximately 30 by 30 m. About 2 kg soil was collected from each area by pooling many small soil samples (>20) picked at random and representing the humus layer where worms were found. The soil was transported to the laboratory and thoroughly mixed using an electric

household mixer. Approximately 300 g soil was dried at 80 °C and stored at –20 °C for subsequent analysis of heavy metals.

The worms were kept in plastic beakers with moist humus soil from the respective sites during transportation. Within 2 days of arrival at the laboratory, a number of 10-ml plastic vials were filled loosely with the homogenised soil from the respective site and one worm was added to each vial. The vials were closed with perforated lids to allow ventilation and placed at 1 ± 0.2 °C for 28 days in order to equalise the thermal conditions of worms collected at the different sites. When acclimated at this low temperature worms no longer feed, and consequently voiding of gut contents before chemical analysis was deemed unnecessary in the present study. After the acclimation period, worms for metal and glycogen measurements were quickly rinsed in demineralised water, snap frozen in liquid nitrogen and subsequently freeze dried and crushed as previously described (Holmstrup et al., 2007b).

2.3. Metal analysis

The total concentration of a range of metals in soil was measured by inductively coupled plasma–mass spectrometry (ICP–MS Agilent 7500CS) after near complete digestion in *aqua regia*. Samples of 0.5 g crushed soil/humus were extracted in closed Teflon vessels in a microwave furnace at elevated pressure and temperature. The worm tissues, approximately 20 mg dried material, were acid-digested over night in 4 ml 33% nitric acid to complete dissolution and diluted to 40 ml before analyses. Certified reference materials were analysed in parallel to the samples and gave satisfactory results. For soils the reference materials PACS-2 and BCSS-1 were used. The reference materials Tort-2, Dolt-3, and Dorm-3 are were analysed together with worm samples in order to verify the quality of the results. The uncertainty of the metal concentration in worms reported in this paper is estimated to be 20% relatively on a 95% confidence level.

2.4. Glycogen measurement

About 5 mg of dry worm tissue was transferred to 1 ml 1 M NaOH, and placed for 3 h at 80 °C which extracted all glycogen while free glucose was degraded. One hundred microliters of the glycogen extract was transferred to 900 µl acetate buffer (0.25 M, pH 4.75) containing 400 mg/l amyloglucosidase (EC 3.2.1.3, Sigma–Aldrich Denmark A/S, Copenhagen, Denmark). After 2 h at 25 °C the amyloglucosidase had cleaved all glycogen to glucose. Glycogen content was then measured in glucose units using an enzymatic method previously described (Overgaard et al., 2007). Glycogen content was calculated relative to glycogen standards (Oyster glycogen, Sigma–Aldrich Denmark A/S, Copenhagen, Denmark) that had been subjected to the same extraction procedure as tissue samples.

2.5. Statistical methods

Glycogen data were initially analysed using PROC MIXED (SAS Institute, 1999) in a nested model with collection site as a random factor nested within regions (i.e. Norway, Sweden and Poland) to test for differences between regions. Subsequently, oneway ANOVA was used to test for differences between collection sites within each region.

The association between the internal concentrations of metals and the glycogen content of *D. octaedra* was studied using graphical models (Whittaker, 1990; Lauritzen, 1996; Labouriau and Amorim, 2008). In these models the variables are represented by vertices in a graph, i.e. a collection of vertices (points) and edges connecting the vertices (lines). Two vertices (i.e. two variables) are connected by an edge (line) when the conditional correlation between them, given all the other variables, is different from zero. According to the theory of graphical models (Whittaker, 1990; Lauritzen, 1996), two variables are directly connected in the graph if, and only if, they carry new information on each other and the information they carry on each other is not already contained in the other variables present in the graph. If two variables are only connected indirectly, then they might be correlated, but this correlation is spurious, in the sense that it could be completely explained by correlations with the other variables. The graphical model with minimum BIC (Bayesian Information Criterion) was selected for the internal concentrations of metals and the glycogen content using the R package gRaphD (Abreu et al., 2009), which yields the graphical model which best represents the data (Houghton, 1988).

Bio-concentration factors (BCFs) of metals were calculated as the ratio between metal concentrations in worms and soil.

3. Results

3.1. Concentrations of metals in soil and earthworms

As expected, the levels of aluminium (Al) in soil were higher close to the aluminium factory in Øvre Årdal compared to reference sites, but not to any great extent (Table 1). Soil concentrations of Cu and Ni were also highest near the factory whereas other metals

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