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Effects of an aged copper contamination on distribution of earthworms, reproduction and cocoon hatchability

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A B S T R A C T

Contaminated soil is a problem throughout the industrialized world, and a significant proportion of these sites are polluted with heavy metals such as copper. Ecological risk assessment of contaminated sites requires ecotoxicological studies with spiked soils as well as in-situ ecological observations. Here, we report laboratory and field assessment of copper toxicity for earthworms at a Danish site (Hygum) exclusively contaminated with an increasing gradient in copper from background to highly toxic levels (> 1000 mg kg^{-1} dry soil). More specifically, we report effects on field populations, body contents of copper, hatching of earthworm cocoons and reproduction of the common species *Aporrectodea tuberculata*. Abundance of earthworms and cocoons decreased significantly from about 400–150 m^{-2} along the gradient as the soil copper concentration increased from ca. 50 to ca. 1000 mg kg^{-1}. At lower concentrations, the population was dominated by endogeic species, whereas at high concentrations the population was dominated by epigeic species. At high copper contents the internal concentration of copper was in the range 100–160 mg kg^{-1} dry tissue. Despite the high internal copper contents, hatchability of field collected cocoons was not impaired in any species. The EC50 reproduction value of *A. tuberculata* was about 220 mg copper kg^{-1} dry soil in the first two exposure periods, but nearly doubled in the third period suggesting that an acclimation response had occurred. Also in the laboratory reproduction test, cocoon hatchability was not reduced, but rather slightly stimulated by copper. Based on these results we discuss the possibility that acute exposure in laboratory experiments is more detrimental than exposure in a field situation, perhaps because increased tolerance may be acquired through natural selection and genetic adaptation through increased use of defense mechanisms such as metallothioneins. Further, we discuss that the rather high tissue copper level of earthworms from the Hygum site may have smaller effects in these free-ranging worms than it would have in acute-exposure laboratory tests because the copper is more efficiently sequestered and detoxified in the field situation where populations have been exposed for many generations.

1. Introduction

Throughout Europe past and present human activities have created a vast number of contaminated sites. A large European survey based upon voluntary national reporting estimated more than a million potentially contaminated sites in 27 European countries. On more than one third of these sites heavy metals was the main driver for concern (Panagos et al., 2013).

Ecological risk assessment of historically contaminated soils forms a specific challenge, as it has been demonstrated that classical ecotoxicological laboratory studies are suboptimal for predicting the site-specific risk to ecosystems (Jensen and Mesman, 2006). Ageing and change of bioavailability is one important factor, but not the only one. In order to improve the site-specific assessment, the so-called TRIAD approach has been suggested as a way forward (Chapman et al., 1998; Jensen and Mesman, 2006; Rutgers and Jensen, 2011). As an integrated part of the TRIAD approach information is needed for as well chemical concentration analytics, standard ecotoxicological studies with spiked soils, *ex-situ* ecotoxicological bioassays and *in-situ* ecological observations. Whereas the first two observations are relatively common, information from the latter two is typically less frequent. The present study should hence be seen in that context, as it also includes field observations and ecotoxicological studies on earthworms with soil collected from a contaminated site.

Earthworms are important organisms in soils of temperate climatic regions because of their ability to improve soil structure, their contribution to the breakdown of organic matter and release of plant nutrients (Edwards and Bohlen, 1996). Risk assessment of environmental pollutants is therefore often including studies of toxicity to earthworms (Edwards and Bohlen, 1992). In the present study we are

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concerned with the effects of copper on reproduction and hatching success of earthworm egg capsules ("cocoons").

Copper is an essential metal, but if exceeding a certain dose it can be highly toxic (Hopkin, 1989). The toxicity of copper to earthworms is portrayed at different levels of biological organization spanning from gene expression (Spurgeon et al., 2004) to membrane instability (Svendsen and Weeks, 1997), reduced reproductive rate and survival (Bengtsson et al., 1986; Van Gestel et al., 1989), and community composition (Holmstrup and Hornum, 2012).

Copper is one of the most common metal contaminants in terrestrial surface ecosystems. It can originate from smelters (Rozen, 2003), brass mills (Bengtsson et al., 1992; Bengtsson and Rundgren, 1988), from the use of copper fungicides (Helling et al., 2000) or from the use of pig slurry as fertilizer (Kerr and McGavin, 1991). Copper is rarely the only heavy metal present in contaminated land. Most often, copper contamination occurs in combination with a mixture of other different heavy metals such as zinc, lead and cadmium (Bengtsson et al., 1983; Holmstrup et al., 2011; Ma, 1988; Spurgeon and Hopkin, 1999), which precludes studying specific effects of copper under field conditions. The "Hygum site" (Jutland, Denmark), however, provides an opportunity to study the effects of copper contamination in a field setting (Pedersen et al., 1999; Strandberg et al., 2006). At this site copper is found as the single contaminant in a gradient from background levels to concentrations reaching highly toxic levels (about 3000 mg kg^{-1} dry soil). The earthworm community along this gradient has recently been described (Holmstrup and Hornum, 2012).

Effects of copper on earthworms have been addressed in several laboratory studies showing that the critical soil copper concentration is commonly above 100 mg kg^{-1} dry soil, but depending much on aging of the contamination, soil pH and texture (Bengtsson and Tranvik, 1989; Ma, 1988, 2005; Scott-Fordsmand et al., 2000). Studies of in-tissue copper concentration give a better indication of toxicity showing that negative sublethal effects in laboratory studies may appear at 30–40 µg g^{-1} dry tissue (background levels are typically below 20 µg g^{-1} dry tissue) and that lethal doses typically are 100–200 µg g^{-1} (Bindesbøl et al., 2007; Scott-Fordsmand et al., 2000; Streit, 1984; Svendsen and Weeks, 1997).

Laboratory studies most often used *Eisenia spp* (see for example Helling et al., 2000; Marinussen et al., 1997b; Owojori et al., 2009; Scott-Fordsmand et al., 2000; Spurgeon et al., 1994; Van Gestel et al., 1989) which are exclusively found in compost and organically rich soils and therefore not of particular ecological importance in agricultural or most natural soils (Edwards and Bohlen, 1996). Knowledge on copper effects in other more common and relevant species is therefore needed in order to improve risk assessment of copper. For example, species belonging to the *Aporrectodea* genus are the most widespread and abundant earthworms in temperate agricultural soils, but are rarely used in ecotoxicological tests. Species such as *A. tuberculata*, *A. rosea* and *A. longa* dominate both biomass and numbers of earthworms in grassland and agricultural soils of temperate Europe and North America (Edwards and Bohlen, 1996). Moreover, field validation of laboratory-derived critical values of copper toxicity is needed. For example, effects of copper on cocoon production and cocoon hatchability have been studied in artificially contaminated soil, but rarely in copper contaminated soil collected in the field, nor have there been any studies of the viability of cocoons collected from such soils. The aims of the present study were therefore to (i) investigate the variation of copper accumulation among several earthworm species collected along a gradient of increasing copper contamination at Hygum, Denmark, (ii) assess the abundance and hatchability of earthworm cocoons at different soil copper concentrations, and (iii) assess the effects of copper (using field collected soil) on reproduction and cocoon hatchability in the species *Aporrectodea tuberculata*.

2. Materials and methods

2.1. Study site

At the "Hygum site" (Jutland, Denmark), timber was treated from 1911 to 1924 with $CuSO_4$. Since 1924 the area had been farmed, but from 1993 it was abandoned. Through soil tillage, copper was over many years thoroughly mixed in the plowing layer (top 20 cm of soil). A transect (about 80 m long) covering copper concentrations from ca. 50 to ca 1080 mg kg^{-1} dry soil had already been defined across the area (Holmstrup and Hornum, 2012) and was used for sampling of earthworms and cocoons. Details about vegetation and soil characteristics are described elsewhere (Arthur et al., 2012; Holmstrup and Hornum, 2012; Naveed et al., 2014; Strandberg et al., 2006). The soil texture of the Hygum field was a sandy loam, with similar clay, silt and sand contents along the transect, but slightly increasing organic matter content from 0.033 kg kg^{-1} at the lowest copper concentration to 0.042 kg kg^{-1} at the highest copper concentration (Arthur et al., 2012). Soil pH-H_2O was 6.2 and did not vary significantly with copper concentration.

2.2. Sampling of earthworms in the field

Earthworms were sampled in early June 2014. Four soil cores measuring 0.25×0.25×0.25 cm were dug with a spade at each of six 1 m^2 plots along the transect (situated 8–28 m from each other) and transported to the laboratory in plastic bags. Plastic bags containing samples were kept at 5 °C until earthworms and cocoons were collected using a combination of washing and sieving (Holmstrup, 2000). Alive earthworms were subsequently identified to species where possible, otherwise to genus level, using stereomicroscope according to (Sims and Gerard, 1999). Cocoons were also determined to species and placed in separate Petri-dishes on wet filter paper.

2.3. Earthworm preparation

Earthworms collected after washing and sieving, were left in Petri dishes lined with moist filter paper at 5 °C for 24 h in order to let worms evacuate their guts from soil contents. Then, earthworms were weighed and their fresh weights were recorded to calculate the biomass of each species at each location. They were subsequently dried at 80 °C for 24 h, followed by recording of their dry weight rounded to the nearest mg.

2.4. Copper analysis of soil and earthworms

Soil samples were taken to a depth of 20 cm using a metal core sampler with diameter of 2.5 cm. Each sample consisted of nine pooled subsamples collected at random within one square meter. Since the soil has been tilled for decades we assume that the copper is evenly distributed in the top 25 cm. Soil was dried at 80 °C and sifted through a 2 mm mesh. Subsamples of ca. 2 g dry soil was digested and analyzed for total copper concentration using atomic absorption spectroscopy as described by Pedersen et al. (1999). Previous studies using soil collected at the Hygum site have shown that water-, $CaCl_2$-, and diethylenetriaminepentaacetic acid-extractable copper fractions are correlated linearly to the total copper concentration in Hygum soil (Pedersen and van Gestel, 2001; Scott-Fordsmand et al., 2000). This suggests that total soil copper gives a comparative measure of copper availability, although the bioavailability and extractability of copper from Hygum soils is markedly lower than from spiked soils (Kjær et al., 1998).

For the copper analysis of earthworm tissue, 2 mL of 65% nitric acid was added to dried earthworms in glass tubes. The tubes were put