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IMPORTANCE OF CONTAMINATION HISTORY FOR UNDERSTANDING TOXICITY OF COPPER TO EARTHWORM *EISENIA FETICA* (OLIGOCHAETA: ANNELIDA), USING NEUTRAL-RED RETENTION ASSAY

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Abstract—*Eisenia fetida* was exposed in the laboratory to a range of elevated soil copper (Cu) concentrations under two different contamination histories. An EC10 for reproduction was observed at 34 mg Cu/kg for soil spiked with Cu 1 d prior to running the experiment (newly spiked soil). Soil contaminated with Cu in the field more than 70 years previously (field-contaminated soil) caused a 10% decrease in reproduction at 248 mg Cu/kg. Survival and cocoon wet weights were not affected by soil concentrations up to 1,400 mg Cu/kg under either contamination history. Adult growth was reduced at 428 mg Cu/kg (EC10) in newly spiked soil but not in field-contaminated soil at concentrations up to 1,400 mg Cu/kg. The contamination history, as well as the toxicological parameter, was important in the interpretation of the outcome of a standard laboratory toxicity test. The lysosomal membrane stability of coelomocytes, measured as neutral-red retention time (NRR-time), was reduced at soil Cu concentrations lower than those affecting reproduction and demonstrated a dose–response relationship. The NRR-time was more severely reduced in worms exposed to newly Cu-spiked soil (EC10 = 8 mg Cu/kg) than worms exposed to field-contaminated soil (EC10 = 69 mg Cu/kg). The NRR-time reflected the bioactive Cu fraction, showing a good correlation with reproduction under both contamination histories.

Keywords—Contamination history Earthworm Lysosomal biomarker Copper Neutral red

INTRODUCTION

The effects of Cu on earthworms have frequently been measured in experiments in which the worms are exposed to newly Cu-spiked soil [1–10]. Such studies have mainly been conducted with soils spiked with a Cu salt only a few days prior to running the experiment.

In risk assessment, one of the main points of criticism when using laboratory experiments is the lack of knowledge on the relationship between laboratory experiments (using soluble metal salts for spiking) and real exposure conditions in the field. Spiking the soil with a soluble Cu salt immediately prior to running an experiment may result in a large proportion of the toxicant being present in the soil water solution compared to conditions in the field [11]. The fraction of toxicant left in the soil water, compared to the fraction bound to soil particles, varies with the metal salt added and the soil type.

Previous studies comparing laboratory-spiked soil with field-contaminated soil have dealt with field-contaminated soil containing a mixture of metals [12,13], field soil newly spiked with metals [7–10], or field soil of a different type than the soil used for laboratory spiking [7,10,13]. Comparisons between such exposure regimes rendered it difficult to elucidate the direct importance of the contamination history compared with the influences of, e.g., chemical mixtures or the soil types on the outcome of a toxicity test.

When performing laboratory studies on soil organisms with metals, it is always difficult to extrapolate these results to the field. One way to overcome these concerns is by applying a biomarker that may be assessed in both the laboratory and the field. Weeks and Svendsen [8] have recently suggested a biomarker for the effects of metals on earthworms. This biomarker measures the membrane stability of lysosomes within the coelomocytes of earthworms. Thus far, it has been applied almost entirely to studies dealing with newly spiked soil [8–10,14].

The primary aim of the present study is to evaluate differences in the lethal and sublethal toxicity of Cu to the earthworm *Eisenia fetida* under different soil contamination histories. A second aim is to test the suitability of lysosomal stability (measured as neutral-red retention) as a biomarker of Cu toxicity, to reflect Cu toxicity under different soil contamination histories, and to correlate this to effects at the population level.

MATERIALS AND METHODS

Experimental design

Two laboratory exposure regimes were performed for the exposure of *E. fetida* to Cu-contaminated soil.

The experiments were conducted in containers containing 600 g moist sandy clay soil, i.e., 500 g dry soil and 100 ml demineralized water. The water, which in the relevant cases also contained the final Cu content, was added 1 d prior to the start of the experiment. Four replicates, each with 10 earthworms per replicate (container), were used at each Cu concentration.

In the first exposure regime, *E. fetida* were exposed in the laboratory to field-collected soil, initially only containing background concentrations of Cu (15 mg Cu/kg) but spiked in the laboratory with Cu concentrations ranging from 0 to 1,400 mg Cu/kg, e.g., 0, 50, 100, 300, 700, and 1,400 mg Cu/

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kg. For all soils, the pH was adjusted to 6.5 to 7.0 by the addition of $CaCO_3$.

In the second exposure regime, *E. fetida* were exposed in the laboratory to soil collected from various points along a gradient in a Cu-contaminated field in Hygum, Denmark. The Cu gradient ranged from 15 to 1,369 mg Cu/kg, e.g., 15, 67, 211, 421, 829, and 1,369 mg Cu/kg. For all exposure concentrations, the soil pH was 6.5 to 7.0.

For both exposure regimes, other metals such as chromium, zinc, and lead were present only at background levels and no polycyclic aromatic hydrocarbons were present.

The experiments were continued for 21 d at a constant temperature of 20 ± 1°C and with a 12:12 h light:dark regime. Food in the form of 3 g horse manure (wetted with 10 ml glass-distilled water) was added to the soil surface every 7 d. Prior to rewetting, the manure had been dried at 100°C and then ground and sieved through a 2-mm sieve. Water lost from the experimental containers by evaporation was replenished every 7 d.

Earthworms

Adult *E. fetida* were incubated under conditions identical to the experimental conditions (although in the absence of toxicants) for 1 week prior to running the experiment. Adult (visible clitellum) earthworms ranged in live weight from 300 to 600 mg after 24 h depuration on moist filter paper.

Preparation and contamination of soil

The soil used (sandy clay soil) was collected from the field site, Hygum, with a known gradient of Cu contamination. No other pollutants were present (see above). Prior to the experiments, the soil was oven dried (Memmert, Schwabach, Germany, Type UL40) at 80°C overnight to kill the soil fauna and to obtain soil Cu concentrations on a dry weight basis. In the experiment using the field-contaminated soil, the soil was remoistened with distilled water after drying.

In the experiment using laboratory Cu-spiked soil, Cu was added as the chloride salt ($CuCl_2 \cdot H_2O$, Merck Pro Analysis, Merck, Darmstadt, Germany) from a stock solution (7 g Cu/l) to a soil containing background Cu concentrations (15 mg Cu/kg). In the spiked soil, the pH was adjusted between pH (H_2O) 6.5 and 7.0 by the addition of powdered $CaCO_3$ (Merck). The experimental pH was then equivalenced to the pH found at the field site.

Experimental measurements

At the end of the experiment, adult survival, growth, reproductive output, lysosomal membrane stability, and internal Cu concentrations were measured for each exposure concentration. Death was defined when the worms were totally absent (decomposed) or sluggish and totally unable to move (the latter was not observed). The surviving adult earthworms were hand collected, counted, and weighed after 24 h on moist filter paper. As a measure of the reproductive output, the number of cocoons was obtained by wet sieving the soil, and for each container, the cocoons were counted and weighed.

To measure the lysosomal membrane stability of surviving worms, coelomic fluid was extracted and stained as described by Weeks and Svendsen [8] (the viability of the extracted cells was checked with Eosin Y). For each earthworm, the lysosomal membrane stability was determined as the number of cells with fully red-stained cytosols (i.e., exhibiting dye leakage from the lysosomes to the cytosol) compared with the number of cells with unstained cytosols (no leakage); the ratio (leaked to nonleaked) was determined. For each worm, this ratio was assessed during 2 min of counting every 4 min until >50% of the cytosols were stained red or 60 min had elapsed [15]. The time required to obtain a ratio of one between the cells with leaked and nonleaked lysosomes was termed the neutral-red retention time (NRR-time).

Internal Cu concentrations were measured in depurated worms to minimize the influence of the soil-bound Cu contained in the gut on the total earthworm Cu load. Worms were then dried to constant weight, digested in concentrated nitric acid, and analyzed (see below).

Chemical analysis

Copper was assessed by flame atomic absorption spectrometry (Perkin-Elmer 4100, Perkin-Elmer, Norwalk, CT, USA). For total Cu, 1 g of soil was transferred into a graduated 100-ml Pyrex® (Corning Glass, Corning, NY, USA) conical flask to which 20 ml of concentrated Analar grade nitric acid (Merck Pro Analysis, Merck, Darmstadt, Germany) was added. The samples were allowed to stand overnight and then boiled on a hot plate. Boiling continued until brownish fumes ceased and the solution went clear. After cooling, the samples were diluted to 100 ml with glass-distilled water [16]. Ten milliliters of the solution, carefully avoiding suspended material, was poured into a test tube for analysis by atomic absorption spectrometry. Water-extractable Cu was measured by shaking 2 g dry soil with 10 ml of distilled water for 2 h at 60 rpm, followed by filtration through a 0.45-m cellulose nitrate membrane filter [17] and then analysis by atomic absorption spectrometry. The pH (H_2O) of the soil was measured at the start and end of the experiment.

Statistics

The no-observable-effect concentration (NOEC) and lowest-observable-effect concentration (LOEC) were estimated by multiple comparison using Dunnett's procedure at a 5% significance level [18]. Effect concentrations at 10% (EC10) and 50% (EC50) with bootstrapping intervals (equivalent to a 95% significance level) were estimated by fitting a logistic model to the data [18].

RESULTS

Adult survival and growth

The mean earthworm adult survival showed no change with exposure concentration. The wet weights were not affected by Cu in field-contaminated soil, but in newly spiked soil, a 10% reduction in weight was observed at soil Cu concentrations at approximately 428 mg Cu/kg (Table 1).

Cocoon production and cocoon wet weight

Under both contamination histories, cocoon production was severely affected (Fig. 1A). In laboratory-spiked soil, no cocoons were produced at concentrations of 700 and 1,400 mg Cu/kg, while in the field-contaminated soil, the reproduction was severely but not totally depressed at equivalent concentrations. For the laboratory-spiked soil, the NOEC was 100 mg Cu/kg and the LOEC was 300 mg Cu/kg. For the field-contaminated soil, the NOEC was 211 mg Cu/kg and the LOEC was 421 mg Cu/kg. The EC10 was approximately 34 mg Cu/kg in the spiked soil and approximately 248 mg Cu/kg in the field-contaminated soil (Table 1).

Mean cocoon wet weights showed no change with soil Cu

Table 1. No-observable-effect concentration (NOEC), lowest-observable-effect concentration (LOEC), and EC10/EC50 values (mg Cu/kg dry wt) for the reproductive output, wet weight, and neutral-red retention time (NRR-time) of coelomocyte lysosomes in *Eisenia fetida*; the worms were exposed for 21 d to newly Cu-spiked Hygum (Denmark) soil (newly spiked) and in Hygum Cu-contaminated soil polluted in the field more than 70 years ago (field contaminated)

Effect parameter	Exposure regime	NOEC[a]	LOEC[a]	EC10[b]	EC50[b]
Weight	Newly spiked	700	1,000	428 [−191–1,144]	—
	Field contaminated	—	—	—	—
Reproduction	Newly spiked	100	300	34 [8–181]	210 [152–258]
	Field contaminated	211	421	248 [134–317]	517 [330–617]
NRR-time	Newly spiked	0	50	8 [6–10]	39 [16–97]
	Field contaminated	67	211	69 [33–47]	163 [109–231]

[a] Based on 95% significance levels.
[b] Values enclosed are bootstrapping values (equivalent to a 95% significance level).

concentrations, the mean wet weights being between 0.00931 and 0.01103 grams (Table 2).

Lysosomal membrane stability

The NRR-times of *E. fetida* exposed to laboratory-contaminated soil were depressed at soil concentrations lower than for the worms exposed to field-contaminated soil. Earthworms exposed to laboratory-spiked soil concentrations at or above 300 mg Cu/kg had NRR-times of less than 4 min, while this was the case for worms at or above 421 mg Cu/kg in the field-contaminated soil (Fig. 1B). A 10% decrease in the NRR-time for worms exposed to newly spiked soil occurred at 8 mg Cu/kg, and the comparable level of effect for the field-contaminated soil was 69 mg Cu/kg. The equivalent NOEC and LOEC values were 0 mg Cu/kg and 50 mg Cu/kg for laboratory-spiked soil and 67 mg Cu/kg and 211 mg Cu/kg for field-contaminated soil (Table 1).

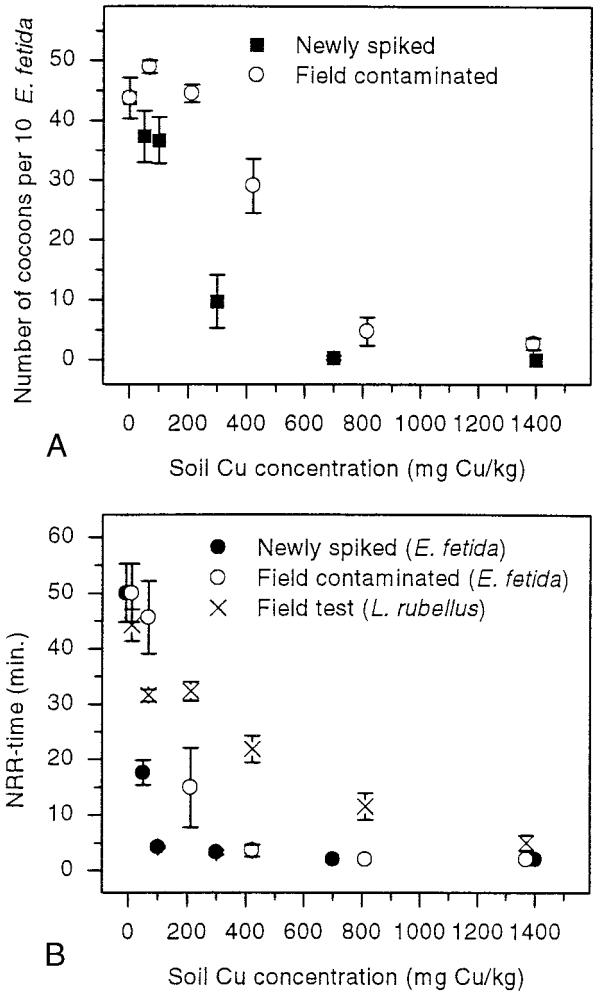

Fig. 1. (**A**) Mean number (± SEM) of cocoons per 10 adult *Eisenia fetida* and (**B**) mean (± SEM) neutral-red retention time of coelomocyte lysosomes (neutral-red retention time) following 21 d of exposure to newly Cu-spiked Hygum (Denmark) soil (newly spiked) and in Hygum Cu-contaminated soil polluted in the field more than 70 years ago (field contaminated). X represents neutral-red retention times from *Lumbricus rubellus* exposed in situ at Hygum.

Chemical analysis

The internal body Cu concentrations clearly increased with exposure to raised soil Cu concentrations. No significant differences were found between the internal Cu loads of earthworms exposed under the two contamination histories except at the highest concentrations, where the internal Cu load was highest in worms exposed to field-contaminated soil (Fig. 2).

The water-extractable Cu concentrations in the field-polluted soil showed a continuous rise with increasing total soil Cu concentrations (Fig. 3). For the newly spiked soil, the water-extractable Cu concentration also increased, but in this case, the increase flattened out around 800 to 900 mg total Cu/kg and became constant (Fig. 3). The pH of the soil remained constant during the experimental period.

DISCUSSION

This study has shown that the outcome of a single-species laboratory test with *E. fetida* was dependent on the previous contamination history of the soil in which the earthworms were exposed. Soil contaminated with Cu 70 years previously was less toxic to *E. fetida* than soil newly spiked with Cu salts. The lysosomal membrane stability of coelomocytes from exposed worms, measured as the neutral-red retention time, decreased with increasing soil Cu concentrations and the response was dose dependent on both soil contamination histories.

The soil Cu concentrations for newly spiked soil that resulted in a 50% decrease of reproductive output for *E. fetida* in the present study (EC50 = 210 mg Cu/kg) were higher than the EC50 at 53 mg Cu/kg recorded by Spurgeon et al. [3] but lower than the 716 mg Cu/kg observed by Spurgeon and Hopkin [13]. The latter experiments were all conducted in Organization for Economic Cooperation and Development soil with varying exposure duration. Adult mortality and growth were less sensitive (LC50 > 1,400 mg Cu/kg) than in previous records, e.g., LC50 at 643 mg Cu/kg [19,20] and 555 to 863

Table 2. Mean (± SD) cocoon wet weights following a 21-d exposure of *Eisenia fetida* to newly Cu-spiked Hygum (Denmark) soil (newly spiked) and in Hygum Cu-contaminated soil polluted in the field more than 70 years ago (field contaminated)

Newly spiked (mg Cu/kg)	Cocoon wet weight (g) (±SD)	Field contaminated (mg Cu/kg)	Cocoon wet weight (g) (±SD)
Control	0.01047 (±0.00172)	Control	0.01047 (±0.00172)
50	0.00931 (±0.00188)	67	0.0110 (±0.00216)
100	0.01103 (±0.0173)	211	0.01095 (±0.00186)
300	0.01065 (±0.00221)	421	0.01009 (±0.00216)
700	0.00942	829	0.00972 (±0.0022)
1,400	No cocoons	1,369	0.01044 (±0.00361)

mg Cu/kg [3] for *E. fetida* exposed to Cu in Organization for Economic Cooperation and Development soil. The internal Cu concentrations in exposed earthworms were lower than the external Cu concentrations except at the background soil Cu concentrations (Fig. 2). These observations and the general trend agree with previous observations for other species [8–10,21–23].

Exposing *E. fetida* to two different contamination histories resulted in toxicity at lower soil Cu concentrations in newly spiked soil than in soil contaminated 70 years previously. The exposure regimes with spiked soil were similar to that used in standardized tests [24–26]. This higher toxicity of Cu in newly spiked soil agrees with previous observations for the toxicity of Cu to the springtail *Folsomia fimetaria,* where newly spiked soil also was more toxic than equivalent concentrations in field-contaminated soil [27]. The lower toxicity of field-contaminated soil was similar to observations for zinc-exposed earthworms and Collembola, although in these examples, the field-contaminated soil contained elevated levels of a mixture of metals [13,17].

It may be speculated that the survivability of hatchlings in Cu-contaminated soil depends on the initial size of the hatchling. In this case, a measure of the cocoon size may provide a prediction of the survivability of the offspring, as hatchling size has been shown to depend on the cocoon size in four other earthworm species [28]. Rozen [29] observed that worms from heavily metal-contaminated areas produced cocoons with a smaller mass than worms from cleaner areas. Spurgeon and Hopkin [30] observed that *E. fetida* produced cocoons that increased in weight with increasing soil levels of a metal mixture, mainly Cu, Zn, Pb, and Cd. In the present study, in agreement with previous results for nickel-exposed *E. veneta* [15], the cocoon wet weights were independent of the Cu-exposure concentration.

The lower toxicity of the 70-year-old Cu-enriched field soil may be explained partly by differences in the bioavailability of Cu under the two test circumstances. The bioavailability of metals may be assessed by means of the water-extractable Cu fraction of the soil [31]. In the present experiment, the water-extractable soil Cu did not reflect the differences in toxicity observed between the two contamination histories, as no differences in the water-extractable concentrations were found. This may possibly be due to a rapid binding of Cu to the soil particles in the experiment with newly spiked soil. Lehmann and Harter [32] showed that, when Cu was added to soil, approximately 94% was adsorbed within 15 min, it continued to react for several hours, and after about 1 d, it reached equilibrium. In our experiment, 24 h was allowed from adding the Cu salt to introducing the animals; hence, under these assumptions, the bioavailability would be the same under the two contamination histories. Assuming that the water-extractable fraction still reliably estimates the toxic Cu level, it could be speculated that the more pronounced toxic effect in the laboratory-spiked soil was caused by an additional toxic effect

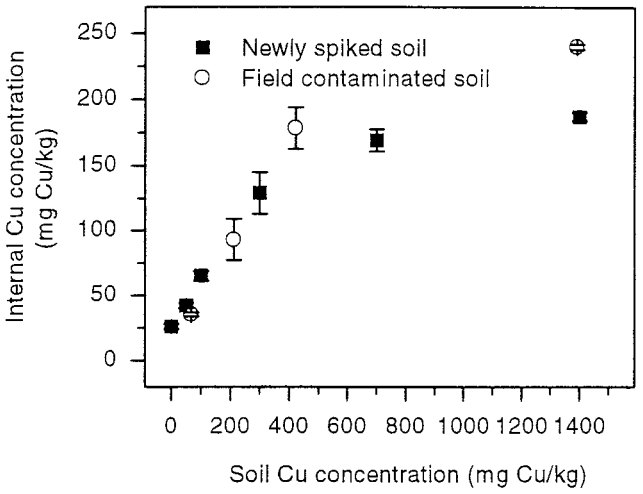

Fig. 2. Mean (± SEM) internal Cu concentration (dry wt) in *Eisenia fetida* in relation to total soil Cu concentrations (dry wt) following 21 d of exposure to newly Cu-spiked Hygum (Denmark) soil (newly spiked) and in Hygum Cu-contaminated soil polluted in the field more than 70 years ago (field contaminated).

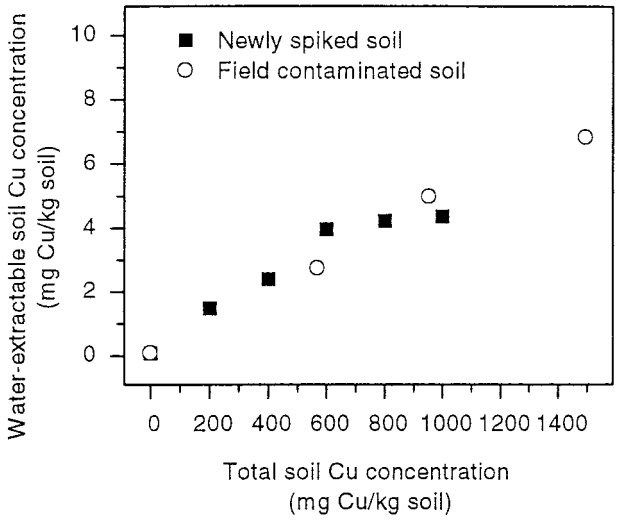

Fig. 3. Soil water-extractable Cu concentration (per dry wt) in relation to total Cu concentrations (per dry wt) in newly Cu-spiked Hygum (Denmark) soil (newly spiked) and in Hygum Cu-contaminated soil polluted in the field more than 70 years ago (field contaminated).

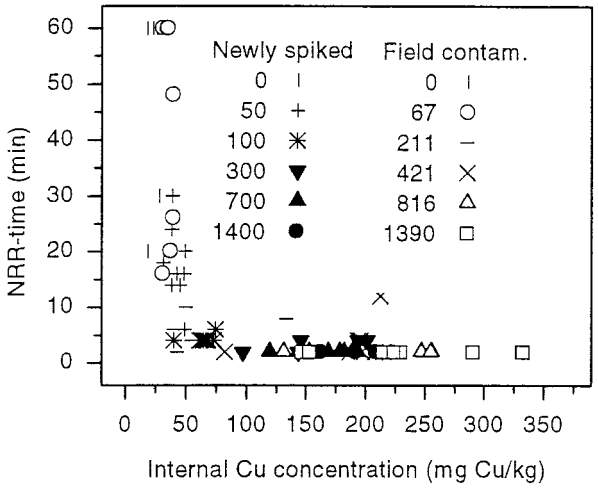

Fig. 4. Neutral-red retention time of coelomocyte lysosomes (NRR-time) in relation to internal Cu concentrations (per dry wt) of the worm *Eisenia fetida* following 21 d of exposure to newly Cu-spiked Hygum (Denmark) soil (newly spiked) and in Hygum Cu-contaminated soil polluted in the field more than 70 years ago (field contaminated).

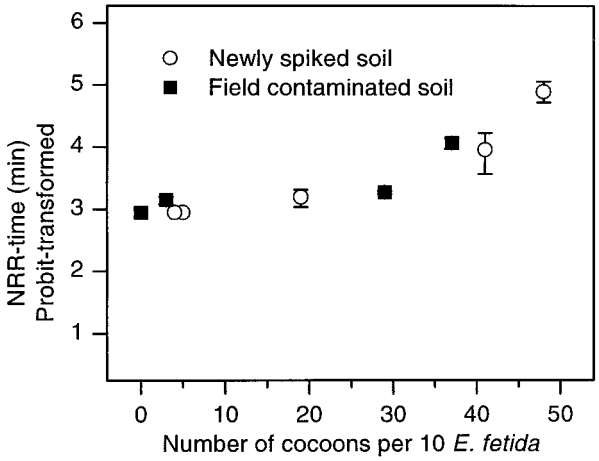

Fig. 5. Mean number of cocoons per 10 adult *Eisenia fetida* versus mean (± SEM) neutral-red retention time of coelomocyte lysosomes (neutral-red retention time) of the same earthworms following 21 d of exposure to newly Cu-spiked Hygum (Denmark) soil (newly spiked) and in Hygum Cu-contaminated soil polluted in the field more than 70 years ago (field contaminated).

of chloride, as Cu was added as a chloride salt. The possible magnitude of this chloride problem is only little studied, and research should be devoted to this area [33,34].

At concentrations above which Cu is regulated, the internal body Cu concentration of the test species could be thought to be reflecting the toxic fraction in the sense of the critical body concentration [35–37]. The continuous rise in internal Cu levels in this study indicated that the exposure levels were above the threshold level for which the worms were able to regulate the internal Cu level [38,39]. However, the internal Cu concentration in our study did not, as was also the case for the water-extractable Cu, reflect toxicity either. This may reflect the fact that the whole body Cu concentration is unlikely to be the same as the Cu concentration in individual organs and sites of toxic action within the worm.

There is an increasing interest in the use of biomarkers to estimate toxicity of compounds. One such marker is the lysosomal membrane stability test, measured as NRR-time [8]. In the present study, the NRR-time was reduced by exposure to Cu in a dose–response relationship with the external Cu concentration. This agrees with previous observations [8–10,14]. The inherent individual variability in the NRR-time in the present study was larger than in most previous studies. The NRR-time and the internal body Cu concentration showed a pattern different than that seen using NRR-time and the external Cu concentrations. Internal Cu concentrations above 50 mg Cu/kg corresponded with NRR-times of less than 4 to 6 min (Fig. 4). The totally depressed NRR-times (≤4–6 min) at internal concentrations above 50 mg Cu/kg indicate an internal threshold level and hence an impairment of the coelomocyte lysosome membrane.

Such a threshold level of approximately 50 mg Cu/kg tissue has also been observed in *Lumbricus rubellus* and *E. andrei* [8–10]. Hence, it seems that the effect level of tissue-bound Cu on the coelomocyte lysosomal membrane stability of earthworms is not species specific. Bearing this internal threshold in mind, differences in effect values among species based on external soil Cu concentration are more likely reflect differences in uptake/excretion of Cu for such species. It should be noted that, in the present experiment, as in the studies by Weeks and Svendsen [8] and Svendsen and Weeks [9,10], the internal body Cu concentration was measured following 24 h of gut depuration, which may have resulted in a loss of Cu from the tissues of the worms [39].

The correlation of the NRR-time with effects on the reproductive output agree with previous observations for nickel-exposed *E. veneta* [15]. In the present study, the ratio between the EC10 for reproduction and NRR-time (EC10 reproduction/EC10 NRR-time) was three to five while it was unity for nickel-exposed *E. veneta* [15]. The reason for the differences between these ratios is not known, and further work should be conducted in the area.

Only one study has previously compared the NRR-response of earthworms exposed to laboratory-spiked soil with field-exposed worms [8]. In [8], the response was similar under the two exposure regimes, which probably reflect the fact that, in both exposure regimes, the soils were spiked immediately before running the experiments. In the present study, the NNR-time decreased at lower soil Cu concentrations in newly spiked soil than in field-contaminated soil, a pattern also observed for cocoon production. In fact, the NRR-time seems to incorporate the difference in toxicity (expressed as cocoon production) between the two contamination histories (see Fig. 5) rather than merely the total soil Cu, the water-extractable Cu, or the internal Cu concentrations in each test.

Hence, the NRR-time reflects the bioactive Cu fraction of the soil, whereas the internal Cu concentration may reflect the bioavailable, but not necessarily toxic, Cu fraction in soil. The bioactive fraction will be reflected even at regulated levels, given the right biomarker, whereas the internal concentration possibly will express only the bioavailable concentration above regulated levels. In the reflection of the bioactive fraction of a toxicant, rather than the bioavailable fraction, lies the true potential of biomarkers. They can express the bioactive fraction that interacts with metabolic processes and omit the fraction bound in inactive sites. The latter will also be included in the measure of the internal concentration. Similarly, for organic compounds, the bioactive fraction may be a subfraction of the total body load or indeed a metabolic product of the original compound.

Table 3. The ratio between the 10% effect concentrations (EC10) for reproduction and neutral-red retention time (NRR-time) (EC10 reproduction/EC10 NRR-time) for *Eisenia fetida*; the worms were exposed for 21 d to newly Cu-spiked Hygum (Denmark) soil (newly spiked) and in Hygum Cu-contaminated soil polluted in the field more than 70 years ago (field contaminated)

	EC10 reproduction/ EC10 NRR-time	EC50 reproduction/ EC50 NRR-time
Newly spiked	4.25	5.38
Field contaminated	3.60	3.17

In the present case, ratios between the EC10 for reproduction and for NRR-time (EC10 reproduction/EC10 NRR-time) were roughly the same (Table 3), indicating that NRR-time measured in worms could predict the impact of the Cu contamination on the reproduction of the worms independently of contamination history. This trend was less convincing for the EC50 measurements.

In summary, based on the present laboratory experiment, the NRR-time of *E. fetida* coelomocytes seems a promising tool to estimate toxic effects of Cu pollution. Preliminary field tests (enclosure studies) with *L. rubellus* exposed to various concentrations of Cu-polluted soil in situ at the Hygum field site show NRR-time–Cu dose–response patterns (see Fig. 1B) resembling the patterns obtained in the present laboratory experiment with *E. fetida*. Before this biomarker is applied in large-scale field tests or as a monitoring tool, it is important to investigate the influence of confounding factors on the NRR response. Such factors may include species differences, pH, salinity, temperature, or influence of other toxins.

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