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Continuous Monitoring of *Folsomia candida* (Insecta: Collembola) in a Metal Exposure Test

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Current recommended ecotoxicological tests with the parthenogenetic springtail *Folsomia candida* using standard OECD soil do not allow for continuous monitoring during the exposure period. Effects of chemicals cannot be determined until the end of the experiment (typically after 4 weeks), since the animals stay below the soil surface. In this study, *F. candida* were maintained on a plaster of Paris/graphite substrate for 7 weeks and were supplied with an aqueous suspension of yeast contaminated with Cd, Cu, Pb, and Zn as nitrate salts. Growth rate, time to first batch of eggs, quantity of food consumed, and the presence of graphite in the gut (a sign of avoidance of yeast) were all affected by metal contaminated diets. The relative toxicities of Cd:Cu:Pb:Zn in the yeast were 1.0:1.07:12.0:4.3, respectively (on a weight basis) with Cd being the most toxic. Internal body concentrations increased, and the concentration factor (metal concentration in *F. candida*/metal concentration in yeast) decreased with increasing metal exposure. In general, metals are much less toxic when added to the food of *F. candida* than when incorporated into soil in standard tests. It is suggested that Collembola have a greater tolerance of metals in the diet since they avoid contaminated food, and are able to excrete assimilated metals at moulting via exfoliation of the midgut epithelium where the elements are retained as part of a storage–detoxification system. The methodology described in this article allows effects on growth to be observed as early as 7 days after the beginning of the experiment. © 2001 Academic Press

***Key Words: Folsomia candida*; ecotoxicology; cadmium; copper; lead; zinc; Isotomidae; Collembola; springtails.**

INTRODUCTION

The widespread interest in developing soil invertebrate tests to assess ecotoxicological effects of chemicals has resulted in several proposals to standardize experiments with springtails (Collembola). Procedures using *Folsomia candida* to survey soil toxicity have been explored (ISO, 1994; Moore and DeRuiter, 1993; Riepert, 1996; Trublaevich and Semenova, 1997; Wiles and Krogh, 1998). *F. candida* belongs to the Family Isotomidae and is euedaphic (soil dwelling; see Hopkin, 1997). It is unpigmented, eyeless, parthenogenetic, reaches an adult length of between 1.5 and 3 mm, and is easy to breed, with low expenditure on time and equipment. The development period is short (2–3 weeks at 20°C) and the reproductive rate is high (Spahr, 1981; Usher and Stoneman, 1977). Springtails are also suitable for ecotoxicological testing due to their presence in all types of soil and their importance to soil biology. They are affected by human-induced changes both mechanical and chemical (Stork and Eggleton, 1992) and are exposed to toxins via the epidermis, ventral tube (water uptake), or gut via food; however, it is not clear which uptake routes are the most important (Bruus Pedersen *et al*., 1997).

The recent ban on dumping sewage sludge at sea has meant an increase in the disposal of sludge onto arable fields. Consequently there is concern regarding the effects of toxicants including metals on soil fauna (Bruce *et al*., 1997, 1999). In metal-contaminated soils a decrease in the density and sometimes diversity of Collembola is known to occur, concomitant with a decrease in the rate of decomposition of the litter layer (Bengtsson *et al*., 1988; Bengtsson and Rundgren, 1988; Hopkin, 1994). While some authors have reported changes in species assemblages as the main effect of metals on Collembola in soil (Bruce *et al*., 1997; Filser and Hölscher 1997; Hågvar and Abrahamsen, 1990; Lübben, 1989; Nüss, 1994; Williamson and Evans, 1973), other workers have found differences in the abundances of Collembola at metal-contaminated sites, with fewer springtails occurring closer to the source of pollution (Haimi and Siirapietikainen, 1996; Kuznetsova and Potapov, 1997; Strojan, 1978). A major component of the diet of most springtails is fungi (Van Straalen and Van Meerendonk, 1987). This is an important exposure route for toxicants as most fungal species accumulate metals in their hyphae (Bengtsson *et al*., 1983, 1985b; Hopkin, 1989, 1994).

The ISO (1994) draft describes the recommended protocol for conducting a standard soil exposure test for

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F. candida. This involves the addition of 10 *F. candida* to each of at least four replicates of an artificial soil made from a mixture of *Sphagnum* peat (10% by weight), kaolinite clay (20%), and industrial quartz sand (70%) adjusted to a pH of 6 ± 0.5 using calcium carbonate. Collembola are then incubated for 4 weeks at 20°C. At the end of the experiment the animals are separated from the soil by flooding with water and adults and offspring are counted. The main disadvantage of this test is that the effects of the toxic agent are not known until the Collembola are extracted from the soil at the end of the exposure period (e.g., Sandifer and Hopkin, 1996, 1997). Hence parameters such as growth, oviposition, and hatching times cannot be monitored (Scott-Fordsmand *et al*., 1997). Only two studies have exposed *F. candida* to metals in yeast, with Cd and Zn being tested individually (Crommentuijn *et al*., 1995; 1997b; Smit, 1997, respectively).

The aims of this study were to develop a test system that allows control of exposure to chemicals through ingestion, and constant monitoring throughout the exposure period. In addition, the experiments were designed to *simultaneously* examine the *relative toxicities* of Cd, Cu, Pb, and Zn, which are often found together at elevated concentrations in metal-contaminated sites. Two experiments were run, the first using 10 Collembola in each replicate and the second with individuals to avoid density-dependent effects.

MATERIALS AND METHODS

Experiment 1 was carried out using 4 ± 1-day-old and Experiment 2, 14 ± 1-day-old *F. candida*, obtained from synchronous cultures according to ISO (1994) and Wiles and Krogh (1998) in four, 6-cm height × 17-cm length × 11.5-cm width, clear plastic boxes. Collembola were cultured on a moist substrate of 8:1 plaster of Paris: graphite powder by weight (depth approx. 0.5 cm) under a light: dark regime of 16:8 h. After 3 days, adults were removed and cultures maintained at 20°C, to allow eggs laid by the females to hatch.

Both experiments were carried out in 60-ml plastic containers (6 cm high, 3.7 cm in diameter) with plastic screw-top lids (Sterilin (Bibby), Merck Laboratory Supplies, Cat. No. 275/0460/11). The plaster of Paris and graphite mixture was made up in small quantities (80 g plaster, 10 g graphite powder, and 70 ml distilled water) and poured into the containers to approximately 0.5-cm depth. Containers were then left for at least 2 days before use.

Before setting up the experiments, test containers were washed out with distilled water and then left to soak in distilled water overnight. The following day, excess water was removed and one 18 × 18-mm glass coverslip was placed on the substrate in the center of each container.

Yeast was prepared by making up stock solutions of 1 g of dried active baker's yeast to 2 ml of double-distilled water (control), or 2 ml solution of the metal nitrate salt under test (BDH, Chemicals) to give the desired concentration of Cd, Cu, Pb, and Zn on a wet weight basis. Actual concentrations of metals in yeast were determined in nitric acid digests by atomic absorption spectrometry (Varian Spectra-30 Flame AAS with automatic background correction), using the methodology of Hopkin (1989).

Experiment 1 (*10 F. candida in Each Replicate*)

Actual concentrations of metals in the yeast suspensions (μg metal g^{-1} wet weight) were 28, 95, 287, 1360, and 5020 (Cd); 10, 54, 195, 994, and 2900 (Cu); 115, 406, 2170, 9710, and 49,200 (Pb); and 337, 996, 3090, 9490, and 37,100 (Zn). Concentrations of metals in the control were < 0.1 μg metal g^{-1} wet weight for Cd and Pb, but 1.0 and 34 μg metal g^{-1} wet weight for Cu and Zn, respectively. Five replicates were prepared for each exposure concentration and the control. Sixty microliters (equivalent to approx. 34.0 μg dry weight) of the appropriate yeast mixture was placed on the center of each coverslip using a micropipette, ensuring that the mixture did not spread onto the plaster of Paris. Ten *F. candida* were added to each container using a fine paintbrush and the lids were replaced. Relative humidity was maintained at 100% during the experiment by spraying the inside of the lids with distilled water every 48 h. The four metal treatments and the control were run simultaneously and managed in the same way. All experiments were conducted at 20°C in a 16:8-h light:dark regime.

Mortality. Every 48 h, dead springtails were removed from the containers. A few of the replicates at the highest metal concentrations became overgrown with fungal hyphae and these were excluded from the final analysis.

Growth. At Days 0, 21 28, 35, 42, 49, growth was estimated by randomly selecting five springtails from each replicate and measuring their length individually from the end of the posterior abdominal segment to the anterior margin of the head, using an Olympus MVZ 1×–4× microscope and graticule at ×40 magnification. The body length of springtails could be measured to within 0.1 mm. The mean length (± SE) of 4-day-old springtails at Day 0 was 0.5 ± 0.02 mm. A calibration line of length against dry weight was prepared using 50 specimens of the full size range (0.1–2.2 mm) from the stock culture. This gave an exponential curve ($y = 0.8317e^{1.8626}$, $R = 0.922$). The weight of the springtails was then calculated from their length (while alive) using the graph. EC_{50} and $EC_{10\,growth}$ values (the concentration of metal at which growth was reduced to 50 and 10% of that of the control) were calculated for 21, 28, 35, 42, and 49 days.

Feeding. At the end of the experiment, the coverslips were removed from the containers, oven-dried for 24 h at 60°C, and weighed to estimate dry weight of yeast

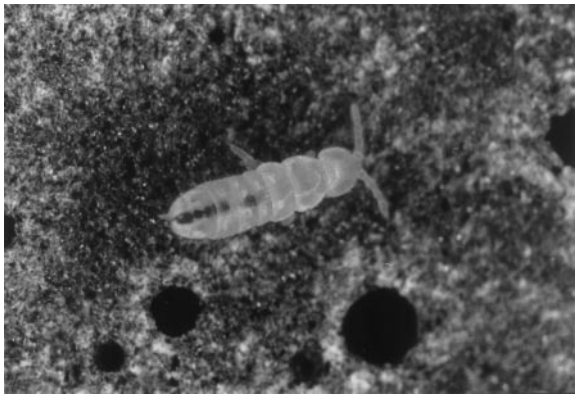

FIG. 1. *F. candida* (1.5 mm in length) from the group exposed to the highest concentration of Zn. The gut contains graphite (visible through the body wall) which has been ingested in preference to contaminated yeast.

remaining. Due to the lack of pigment in the cuticle of *F. candida* it is possible to observe the gut contents of individuals (Fig. 1) and at the end of the experiment the percentage of Collembola that contained graphite (ingested from the substrate) could be calculated.

Reproduction. The containers were examined every 48 h for the presence of eggs and juveniles. The total number of juveniles that had hatched by the end of the experiment was recorded also.

Internal body concentration (*IBC*). Surviving adult Collembola were removed from the containers at the end of the experiment and deep frozen at -20°C prior to analysis of total metal concentration. Animals from each exposure concentration were then pooled, dried at 60°C overnight, weighed, and digested in 1 ml of boiling Aristar nitric acid (BDH, Chemicals). Once cooled, digests were made up to 5 ml with double-distilled water. The digests were analyzed by flame atomic absorption spectrometry initially (as for yeast) and then carbon furnace atomic absorption spectrometry (Varian Spectra-30 AAS with automatic background correction) if metal concentrations were below the detection limits for flame AAS (Hopkin, 1989).

Experiment 2 (*One F. candida* in Each Replicate)

This experiment was conducted in a similar way to Experiment 1 except for the following details. Actual concentrations (μg metal g^{-1} wet weight) of metals in the yeast were 28, 98, 290, 1200, and 5300 (Cd); 4, 67, 220, 940, and 2690 (Cu); 134, 363, 2140, 8980, and 28,700 (Pb); and 330, 820, 3300, 10,000, and 37,000 (Zn). A small amount of the suspension of yeast (20 μl) was placed in each test container on the glass coverslips. One *F. candida* was added to each replicate using a fine paintbrush. The four metal treatments and the control were run simultaneously and managed in the same way. Experimental conditions were the same as for Experiment 1.

Growth. At Days 0, 7, 14, 21, 28, 35, 42, and 49, growth was estimated by measuring each springtail in each replicate and converting these values to weight using the length : weight regression curve determined previously. The mean length ($\pm$ SE) of 14-day-old springtails at Day 0 was 1.5 ± 0.04 mm. EC_{50} and $EC_{10\,growth}$ values were calculated for each of the above days.

Reproduction. In each container, a line was scored with a needle to produce a furrow in the substrate, approximately 2 cm long and 1 mm deep. This was to encourage springtails to lay their eggs in the furrow rather than underneath the glass coverslip. After females had laid their first batch of eggs they were removed to new test containers with the same conditions, and observations continued. Parameters tested were the number of eggs in the first and second batch and total number of eggs, time taken for eggs to hatch, number of juveniles successfully hatching from first batch, and total number of juveniles.

Internal body concentration. Surviving adult Collembola were removed from the containers at the end of the experiment and frozen prior to analysis of their total metal concentrations. Animals were analyzed individually by graphite furnace AAS using the same methods as in Experiment 1.

Statistics. Between-concentration differences were analyzed using two sample *t* tests and Mann–Whitney tests depending on whether the data were normally distributed or not (Minitab 10.51Xtra). EC_{50} and $EC_{10\,growth}$ values were calculated using a linear interpolation technique based on the inhibition concentration (ICp) approach on the ICp 2.0 software available from the USEPA (www.EPA.gov/nerleerd/stat2.htm).

RESULTS

Experiment 1

Mortality. A significant increase in mortality compared to the control was seen for Cd (21, 28, and 35 days), Cu (28 and 49 days), and Zn (14, 21, 28, 35, 42, and 49 days), but only in the springtails exposed to the highest metal concentration in each case (two-sample *t* test $P < 0.05$). Collembola feeding on yeast contaminated with Pb exhibited no significant change in mortality, even at the highest exposure concentrations. Thus mortality is a relatively insensitive parameter and the data are not presented in detail here.

Growth. On Days 21, 28, 35, 42, and 49 there was a reduction in growth rates compared to the control, which was significant for all four metals at the highest exposure concentrations, and also for Zn at 9490 μg g^{-1}. In

TABLE 1
$EC_{50\,growth}$ Values in μg g^{-1} (Standard Deviation) of *F. candida* Fed Yeast Contaminated with Metals

Time (days)	Cadmium	Copper	Lead	Zinc
21	1406 (721)	8750 (2490)	5580 (1020)	5580 (1010)
28	2290 (689)	2440 (178)	27500 (2580)	9850 (2320)
35	2540 (373)	2690 (264)	33000 (2960)	8520 (2750)
42	4180 (163)	Pooled means < 50% control	35500 (2160)	7960 (812)
49	4090 (369)	Pooled means < 50% control	34700 (3610)	9150 (2510)

addition at lower concentrations, growth was reduced for Cd at 1360 μg g^{-1} (21, 28, and 35 days), Cu at 195 and 994 μg g^{-1} (35 days), Pb at 9710 μg g^{-1} (21, 35, and 49 days), and Zn at 3090 μg g^{-1} (21, 35, and 49 days; Mann–Whitney test $P < 0.05$).

As time proceeds the level of metal required to reduce growth by 50% compared to the control ($EC_{50\,growth}$) demonstrated a tendency to increase (except for Cu—see below, Table 1). This phenomenon results from the slower growth rate of springtails at the highest exposure concentrations that consume less food than those at lower concentrations and the control (see later, Fig. 3). Thus they take longer to reach the maximum adult weight. $EC_{50\,growth}$ values for Cu at 42 and 49 days could not be calculated, as the tested concentrations were not sufficiently high to reduce growth by 50%. $EC_{50\,growth}$ values were less consistent and are not presented here. Hence growth is a more sensitive measure of the effects of dietary additions of metals on *F. candida* than adult mortality.

Relative toxicities. In contaminated field sites the four metals used in these experiments would not be expected to occur at the same concentrations. At Avonmouth, SW England, the ratios of Cd, Cu, Pb, and Zn in surface soils 3 km downwind of a primary smelting works are near 1:5:50:100 (Table 2). The tests on growth described in this article allow the determination of the relative toxicities of metals. However, to give field-relevance to the data, the ratios of metals in the field soils must be considered. This can be done by calculating the Relative Toxicity Factor (T_F), introduced by Hopkin and Spurgeon (2001; see Table 3).

Although Cd was the most toxic element in the laboratory test, it is Zn that is most likely to reduce the growth of Collembola at Avonmouth because it possesses by far the highest T_F (23.3) in comparison to Cd (1.0), Cu (4.7), and Pb (4.2). The T_F was also highest for Zn in experiments on reproduction in Collembola exposed via OECD soil (Sandifer and Hopkin, 1997). Pb and Zn are more toxic in soil (Pb $T_F = 10.0$, Zn $T_F = 66.6$) than when introduced into the diet (Pb $T_F = 4.2$, Zn $T_F = 23.3$, Table 2).

TABLE 2
Relative to Cd Toxicities (T_{Cd}) and Relative Toxicity Factors (T_F) for Cd, Cu, Pb, and Zn in *F. candida* Derived from 28-Day $EC_{50\,growth}$ Values from Experiment 1 (This Study, Exposed via Food) and 28-Day $EC_{50\,reproduction}$ Values (Exposure via Soil) from Sandifer and Hopkin (1997)

	Cadmium	Copper	Lead	Zinc
Approximate concentrations of metals in surface soil at Avonmouth μg g^{-1}	10	50	500	1000
Ratio of metals in Avonmouth soil relative to Cd (C_{Cd}).	1	5	50	100
28-day $EC_{50\,growth}$ values in Experiment 1 (this study)	2290	2440	27500	9850
T_{Cd}	1.0	1.07	12.0	4.3
T_F	1	4.7	4.2	23.3
28-day $EC_{50\,reproduction}$ values (Sandifer and Hopkin, 1997)	590	700	2970	900
T_{Cd}	1.0	1.2	5.0	1.5
T_F	1.0	4.2	10.0	66.6

Note. See Table 3 for the method used to calculate T_{Cd} and T_F.

Feeding. The percentage of animals with graphite visible in their guts after 49 days (Fig. 1) was significantly greater at the highest exposure concentrations (Fig. 2, two-sample t test) $P < 0.05$).

The quantity of yeast consumed by *F. candida* during Experiment 1 generally decreased as the concentration of

TABLE 3
Procedure for Determining the Relative Toxicity Factor (T_F) in *F. candida* for Cd, Cu, Pb, and Zn in Contaminated Field Soils, Adapted from Hopkin and Spurgeon (2001)

1. Determine concentration of each metal in field soils (Cd, Cu, Pb, Zn).
2. Calculate concentration of each metal relative to Cd (C_{Cd}):

$$C_{Cd} = \frac{\text{Concentration of metal in soil (μg g}^{-1}\text{ dry weight)}}{\text{Concentration of Cd in soil (μg g}^{-1}\text{ dry weight)}}.$$

3. Determine toxic concentration of each metal in food or soil affecting $EC_{50\,growth}$ or $EC_{50\,reproduction}$ for *F. candida* using laboratory tests.
4. Calculate toxicity of each metal relative to Cd (T_{Cd}):

$$T_{Cd} = \frac{EC_{50}\text{ value for metal μg g}^{-1}}{EC_{50}\text{ value for Cd μg g}^{-1}}.$$

5. Calculate relative toxicity factor (T_F) for each metal relative to its concentration in field soils:

$$T_F = \frac{C_{Cd}}{T_{Cd}}.$$

6. The metal with the highest T_F value is the one most likely to be causing toxic effects in the field.

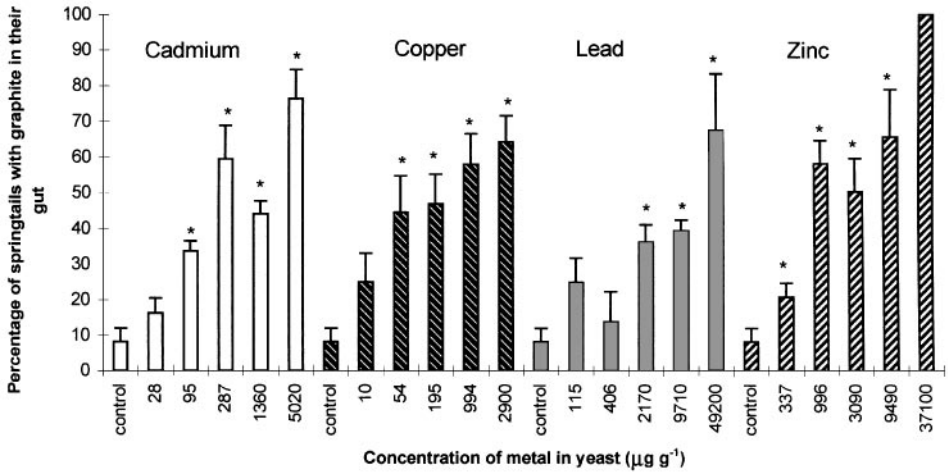

FIG. 2. Percentage of *F. candida* which contained graphite in their gut after 49 days at different exposure concentrations of Cd, Cu, Pb, and Zn in the food. (*) significantly different from the control (two-sample t test $P < 0.05$). Only three animals survived the highest exposure concentration of Zn in one replicate; all had graphite in their guts after 49 days.

metal increased (Fig. 3). At 54 μg Cu g^{-1} a significantly greater quantity of yeast was consumed than in the control. Significantly less yeast was consumed at 5020 μg Cd g^{-1} than at lower exposure concentrations of the metal including the control. At the two highest concentrations of Pb (9710 μg g^{-1} and 49,200 μg g^{-1}) significantly less food was consumed than at all lower concentrations and the control. The springtails in the Zn test consumed more food in the control than at all other concentrations of Zn. However, note that since only three animals were alive (all in one replicate) at the end of the test for Zn 37,200 μg g^{-1}, no statistical analysis was possible (although the mean weight of yeast consumed was very close to exposure concentration 9490 μg g^{-1}).

Reproduction. Due to low hatchability of eggs (thought to be caused by cannibalism and degradation of the eggs themselves), insufficient data were available for the time of juvenile emergence and number of juveniles at the end of the experiment to be assessed. Although in a few replicates no eggs appeared during the duration of the test, replicates in which females did lay eggs had small standard errors for the mean times for the first batch of eggs to appear (see Fig. 4, two-sample t test $P < 0.05$). It is apparent that the appearance of eggs is delayed at the highest exposure concentrations.

Internal body concentration. IBCs of adult *F. candida* revealed a dose-dependent relationship, increasing as the

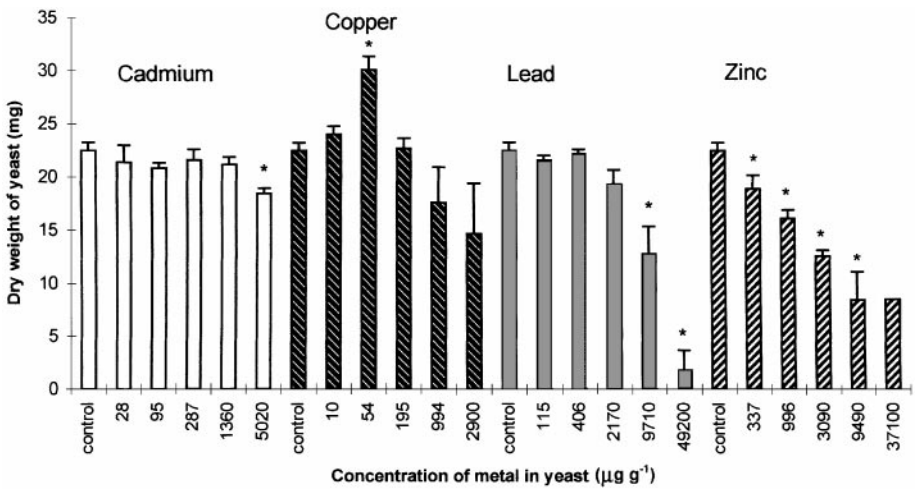

FIG. 3. Quantity of yeast consumed by *F. candida* after 49 days at increasing concentrations of metals. (*) significantly different from the control (two-sample t test $P < 0.05$). Only three animals survived the highest concentration of Zn; all in one replicate.

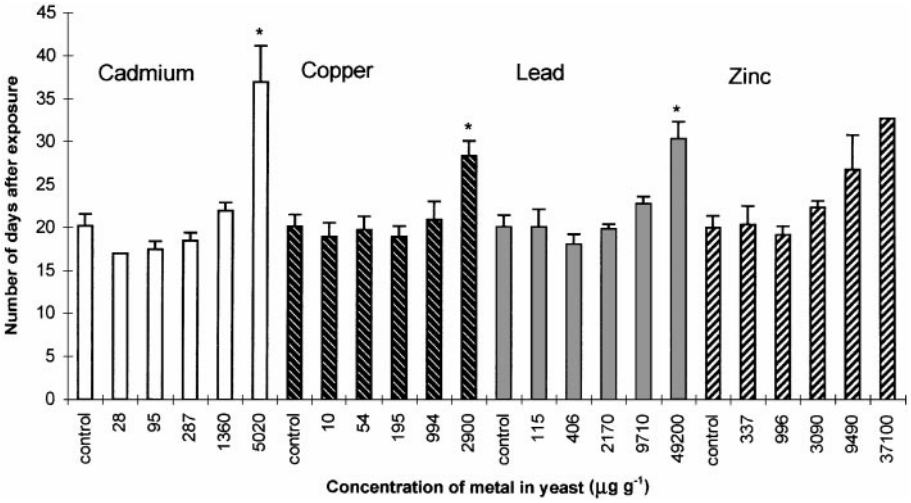

FIG. 4. Mean time of first appearance of eggs laid by *F. candida* during Experiment 1 (note: replicates in which no eggs were laid were omitted from the analysis). (*) significantly different from the control (two-sample t test $P < 0.05$). Only three animals survived the highest concentration of Zn (all in one replicate).

exposure concentration of metals increased, except for Cu where animals exposed to the highest concentration (2900 µg g^{-1}) did not accumulate as much Cu as the two next lowest concentrations (195 and 994 µg g^{-1}, Table 4).

As the metal exposure concentration increases for Cd, Cu, and Zn the concentration factor in the animal decreases. For Pb, however, the concentration factors are extremely low at all concentrations (Table 4).

Experiment 2

Growth. Reduced growth rates were observed for all four metals at Days 7, 14, 21, 28, 35, 42, and 49 at the highest exposure concentrations (except Cu, 14 and 42 days). Growth was also retarded at 10,000 µg g^{-1} in all weeks for Zn, from 7 to 42 days in Cd at 1200 µg g^{-1} and 7 to 35 days at 8980 µg g^{-1} for Pb. Growth was significantly reduced in Zn at concentrations of 3300 µg g^{-1} from 14 to 35 days (Fig. 5, two-sample t test $P < 0.05$). At the highest exposure concentrations, springtails never reached the weight of control individuals.

In this experiment the level of metal required to reduce the growth by 50% compared to the control ($EC_{50\,growth}$) again demonstrated a tendency to increase for Cd and Zn with time. Results for Pb were less clear. In Cu from 7 to 49 days, an $EC_{50\,growth}$ value could not be obtained, probably due to the negligible effect of Cu concentrations and age of *F. candida* at the onset of exposure. $EC_{10\,growth}$ values also had a tendency to increase for Cd, Pb, and Zn but were less reliable for Cu (data not provided).

Reproduction. Of all of the parameters tested (the number of eggs in the first and second batch and total number of eggs, time taken for eggs to hatch, number of juveniles from first batch, and total number of juveniles), none indicated any significant differences between exposure concentrations.

TABLE 4
Exposure Concentration (EC, µg g^{-1}) and Internal Body Concentration (IBC, µg g^{-1}) of *F. candida*, and Concentration Factor (CF) of the Four Metals (CF = IBC/EC)

Cadmium			Copper			Lead			Zinc		
EC	IBC	CF	EC	IBC	CF	EC	IBC	CF	EC	IBC	CF
0	0	0	1	18	18.1	0	0	0	34	129	3.79
28	8	0.29	10	19	1.67	115	0	0	337	148	0.44
95	17	0.18	54	66	1.23	406	19	0.05	996	113	0.12
287	47	0.16	195	274	1.40	2170	78	0.04	3090	341	0.11
1360	128	0.09	994	411	0.41	9710	463	0.05	9490	671	0.07
5020	261	0.05	2900	146	0.05	49200	3077	0.06	37100	3506	0.09

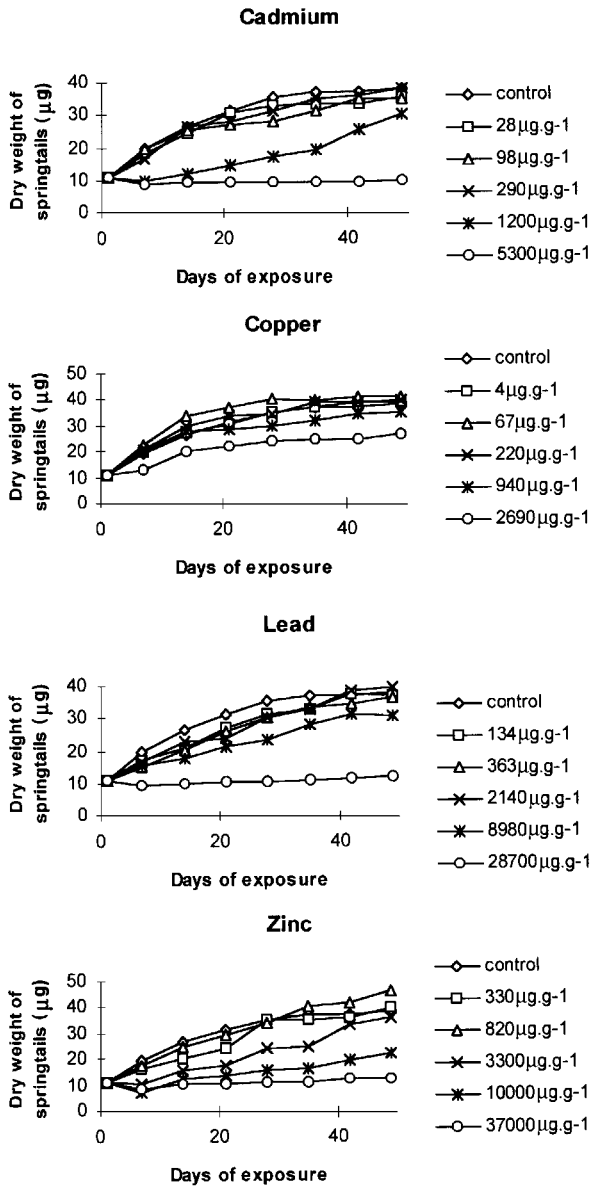

FIG. 5. Growth (mean of five individuals dry weight in µg) of *F. candida* measured over 49 days during exposure to metals in food. Collembola at high metal exposures have reduced growth (standard error bars are omitted for clarity, see text).

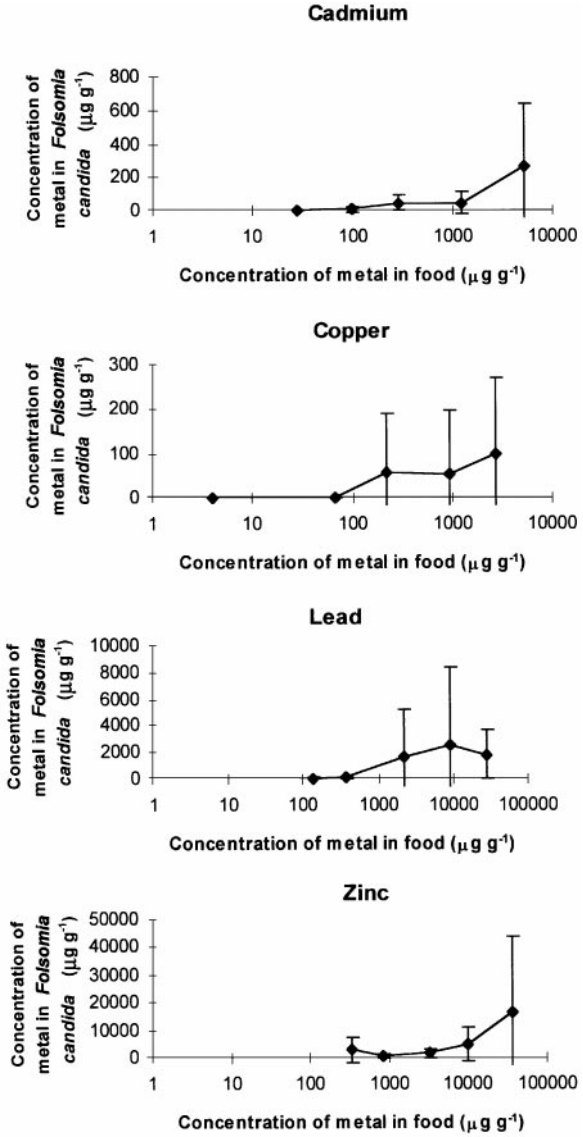

FIG. 6. Relationship between internal body concentrations of metals (± standard error bars) in *F. candida* and exposure concentration of metals in the food. Pb follows the general trend except for the highest exposure concentration, in which the animals may have stopped feeding.

This was probably due to high variation between replicates, and so no conclusions could be drawn from the results of reproduction in this experiment.

Internal body concentrations. A dose-dependent relationship was once again apparent in animals analyzed individually (Fig. 6). As the exposure concentration increases so does the IBC, except for Pb at the highest exposure concentration (28,700 µg g^{-1}), in which the metal was not accumulated as much as the next lowest exposure concentration (8980 µg g^{-1}). There was high variation between the replicates manifested by the large standard errors (Fig. 6). Significant differences (Mann–Whitney test $P < 0.05$) existed between Cd at 98 µg g^{-1} and both 290 and 5300 µg g^{-1}, and between Pb control and 8980 and 28,700 µg g^{-1}. The latter exposure concentration also had a significantly higher IBC than Pb at 134 and 363 µg g^{-1}. For Zn differences from the control were seen at 3300, 10,000, and 37,000 µg g^{-1}. Collembola exposed to 820 µg Zn g^{-1} had a significantly lower IBC than at 10,000 and 37,000 µg Zn g^{-1}, the latter of which was higher than at 3300 µg Zn g^{-1} (Mann–Whitney test $P < 0.05$). There were no statistical differences between the IBCs for Cu at the levels tested here (Mann–Whitney test $P > 0.05$). Statistical differences between the exposure

concentrations and the control for Cd and Cu are not apparent due to metal concentrations in the control being below the detection limits of the analytical equipment.

DISCUSSION

At high metal concentrations a greater percentage of Collembola contained graphite powder from the substrate in their digestive tract (Figs. 1 and 2). Presumably the springtails were using the substrate as an alternative food source and were attempting to acquire nutrition from the graphite. The consequence of avoiding yeast and choosing to feed on a poor-quality food source is retarded growth seen at the highest metal concentrations as early as the 7th day from the beginning of exposure to the end of the experiment (49 days). Decreased growth has also been observed in *Orchesella cincta* fed Fe-enriched algae (Nottrot *et al*., 1987), *F. candida* fed Zn-contaminated yeast (Smit, 1997), and *Folsomia fimetaria* exposed to Ni in soil (Scott-Fordsmand *et al*., 1999). Growth is a primary life-history characteristic and hence reduced growth is indicative of reduced fitness.

Other studies on Collembola have found that several species have the ability to detect and avoid metal contamination (e.g., Bengtsson *et al*., 1994, with *Folsomia fimetarioides* and *Isotomiella minor* (Zn and Cu); Filser and Hölscher, 1997, with *Folsomia candida* (Cu); Joosse and Verhoef, 1983, with *Orchesella cincta* (Pb)). If contamination of food is severe, starvation may result. Evidence for avoidance of a contaminated diet was demonstrated in Experiment 1 at the highest metal concentrations, where significantly less yeast was consumed (Fig. 3).

However, Collembola are tolerant of moderate metal contamination due to their ability to shed the epithelium of the midgut at each moult. Metals deposited in intracellular mineral concretions and/or vacuoles of the midgut cells are lost by apocrine extrusion and holocrine excretion (Humbert, 1978; Joosse and Buker, 1979; Pawert *et al*., 1996). This waste material is then voided in the feces.

In natural populations, avoidance behavior and an increased excretion efficiency may evolve through natural selection to give tolerant populations (Posthuma *et al*., 1992; Posthuma and Van Straalen, 1993). Increased moulting frequency consumes more energy and may result in reduced growth (Posthuma *et al*., 1993a).

Reproduction

Although eggs were laid in most of the experimental containers, hatching success and juvenile numbers were low and insufficient for statistical analysis in both experiments. It was observed that some batches of eggs in Experiment 1 had "disappeared." Cannibalism of eggs has been observed at high populations densities, and Green (1964) and Usher *et al*. (1971) found that reproduction was inhibited at more than one adult per 0.1 and 0.05 cm^2, respectively. The area available for individuals in Experiment 1 and Experiment 2 was 0.34 cm^2 and 3.4 cm^2, respectively, so high density is unlikely to be the cause of the relatively low reproduction rate.

The time for the first egg to appear in the containers (Experiment 1) was significantly later in the highest metal-contaminated yeast treatments, probably due to slower growth and, presumably, a longer time to reach reproductive capability. The individuals at high concentrations of all four metals never reached the weight of the control *F. candida* by Day 49, but they were capable of laying eggs, if somewhat later. In experiments of 4 weeks duration (starting with similarly aged individuals) this evidence may never be obtained (ISO, 1994; Riepert, 1996; Sandifer and Hopkin, 1996, 1997; Trublaevich and Semenova, 1997; Wiles and Krogh, 1998). However, due to the reduced survival and later reproductive development it can be speculated that population growth of metal-exposed *F. candida* will be somewhat slower than in noncontaminated conditions. Crommentuijn *et al*. (1997b) calculated that the intrinsic rate of natural increase (r) declines in *F. candida* populations that are exposed to higher concentrations of Cd in the food. In field conditions it was suggested that although the average adult size in polluted sites becomes smaller, Collembola can survive in the environment because the metal-tolerant fungi which thrive there are protein-rich (Bengtsson *et al*., 1985a). However, even though some species at a polluted site may be tolerant of metal contamination, this is not true for all species, and so a change in community structure occurs (Hågvar and Abrahamsen, 1990).

Growth and Mortality

In acute toxicity tests with Cd-spiked sand, *F. candida* exhibited paralysis at 10 and 20 μg Cd g^{-1} (Trublaevich and Semenova, 1997). $EC_{50\,growth}$ values found in Experiment 1, in which *F. candida* were exposed to Cd-contaminated yeast, were almost four times higher than $EC_{50\,growth}$ values for *F. candida* in Cd-contaminated soil (Table 5). Crommentuijn *et al*. (1995) also found effective concentration values to be higher for food rather than soil exposure.

Mortality was not significantly higher than in the control (Experiment 1) until levels of Cu exceeded 994 μg g^{-1} yeast. This agrees with the findings of Scott-Fordsmand *et al*. (1997), who found no mortality in *F. fimetaria* up to 1000 μg Cu g^{-1} in a soil exposure test. $EC_{50\,growth}$ results for Cu could not be calculated for 42 and 49 days (Experiment 1) and 7–49 days (Experiment 2) due to the mean value being less than 50% of the control. Hence Cu at the levels tested here does not reduce growth by more than 50% of the control. One difference between the experiments was the age at the beginning of exposure. If *F. candida* are exposed to Cu

TABLE 5
28-Day $EC_{50growth}$ of *F. candida* for Cd, Cu, Pb, and Zn μg g^{-1} in Yeast (±SE) and Soil

Exposure via	Cadmium	Copper	Lead	Zinc
Food (yeast)	2290 (689)	2440 (178)	27,500 (2580)	9850 (2320)
Soil	252–566[a,f]	No data available	No data available	500–1228[b,c,d,e,f]

Note. Soil data from Crommentuijn *et al.* (1993)[a]; Smit (1997)[b]; Smit *et al.* (1998)[c]; Smit and Van Gestel (1997)[d]; Smit and Van Gestel (1998)[e]; Van Gestel and Hensbergen (1997)[f].

contamination at an early age (4 days) they are more susceptible to growth retardation than if exposed when more mature (14 days).

Trublaevich and Semenova (1997) found in a 24-h exposure test that Pb had no effect on *F. candida* up to 10 μg g^{-1} dry sand. This is not surprising since Pb exposure had no significant mortality effects at the end of 49 days exposure at levels of 49,200 μg Pb g^{-1} yeast. Joosse and Verhoef (1983) observed that *Orchesella cincta* exhibited slower growth and a shorter moult interval when fed on Pb-contaminated food. Retarded growth of *F. candida* in Experiment 2 was seen particularly at the highest Pb exposure concentration (Fig. 5).

The 28-day $EC_{50\,growth}$ value for *F. candida* in Experiment 1 (9850 μg Zn g^{-1} yeast) was almost 10 times the $EC_{50\,growth}$ value found in soil toxicity tests (526 μg Zn g^{-1} at 19°C) by Smit and Van Gestel (1997). This is in good agreement with Smith (1997), who calculated an $EC_{50\,growth}$ value of 13,600 μg Zn g^{-1} on *F. candida* fed Zn-contaminated yeast. Decreased growth at 3300, 10,000, and 37,000 μg Zn g^{-1} yeast was evident in Experiment 2. Lower rates of growth occur also in *O. cinta* at a concentration of 4000 μg Zn g^{-1} in food (Posthuma, 1990).

Relative Toxicities

Cd is clearly the most toxic of the four metals (Table 2). However, in field sites contaminated with these elements (e.g., Avonmouth, SW England), effects on soil fauna are most likely to be due to Zn, as it is present in such high concentrations in the soil (Tables 2 and 3, Sandifer and Hopkin, 1997; Spurgeon and Hopkin, 1995). In other laboratory experiments the order of toxicity of metals effecting growth was found to be the same as for soil exposure affecting reproduction, i.e., Cd > Cu > Zn > Pb (Sandifer and Hopkin, 1997).

Internal Body Concentrations

F. candida exhibited a dose-dependent accumulation of Cd, Cu, Pb, and Zn except at the highest concentration of Cu (Experiment 1) and Pb (Experiment 2). Dirven-van Breemen and Posthuma (1999) found a decrease in body concentrations of Cd at levels above 227 μg g^{-1} exposure. This is probably due to reduced feeding at high levels of contamination. Other workers have also calculated dose-dependent assimilation of metals, but suggested that accumulation depends on the specific metal and species of springtail (Gräff *et al.*, 1997; Smit, 1997; Van Straalen *et al.*, 1989). This was reflected in the field where Collembola collected from around a brass mill in SE Sweden were found to have much greater IBCs closer to the source of contamination (Bengtsson and Rundgren, 1988). The concentration factor (Table 4) of metal (except Pb) in the animals decreased as exposure concentrations increased. This is further evidence for higher elimination rates of metals and/or avoidance of contaminated food at high metal concentrations. A high variation in IBCs of metals between individuals is evident from Experiment 2 (see error bars, Fig. 6). This is most likely to be related to the stages in the moult cycle of the springtails. Animals that have just moulted would be expected to have much lower amounts of metal in the body than animals at intermoult. Collembola exposed to high Cd, Pb, and Zn concentrations have significantly higher IBCs than those at control or lower concentrations (Table 4). For Cu, however, there is no statistically significant increase in IBCs, which may be due to high variation between individuals and the low effect of Cu exposure concentrations tested in this experiment.

Lethal body concentrations in *O. cincta* and *Tomocerus minor* fed Cd in algal paste were 37 and 75 μg Cd g^{-1}, respectively (Crommentuijn *et al.*, 1994). IBCs in *F. candida* reached 26 μg g^{-1} at exposure concentrations of 5020 μg Cd g^{-1} (Experiment 1). Mortality was significantly different from the control at an IBC of 261 μg g^{-1} dry body weight (exposure concentrations of 5020 μg Cd g^{-1}). Van Gestel and Van Diepen (1997) concluded that the lethal body concentration (LBC) for Cd in *F. candida* is 200–300 μg g^{-1} dry body weight, which is in good agreement with these results.

Cu levels of between 1 and 994 μg g^{-1} in this study were found to have little effect on *F. candida* when added to food. Similarly values between 11 and 122 μg g^{-1} had no effect on *F. candida* when added to soil, even though individuals have been known to accumulate Cu with increasing exposure concentrations (Bruus Pedersen *et al.*, 1997).

As with other metals, Pb is accumulated linearly with increasing exposure concentrations; however, the concentration factor remains low probably due to the low bioavailability of Pb. *O. cincta* sampled from around a lead/zinc smelter reached IBCs of 24,600 μg Pb g^{-1} although individuals of *Tomocerus longicornis* contained only half this concentration (Rabitsch, 1995). IBCs in *O. cincta* are almost eight times the concentration found in *F. candida* exposed to the highest Pb concentrations in this study. At moulting, 48% of Pb and 30% of Cd can be lost via intestinal exfoliation (Van Straalen *et al.*, 1987; Van Straalen and Van

Meerendonk, 1987). Van Straalen (1987) found that concentration factors for Pb decreased with increasing exposure in soil tests.

In contaminated soil (100–800 μg Zn g^{-1}) *F. candida* can regulate internal Zn concentrations at levels of between 30 and 80 μg g^{-1} (Van Gestel and Hensbergen, 1997). Internal Zn concentrations in *F. candida* were found to be significantly raised above exposure concentrations of 484 μg g^{-1} in soil, but ranged from 110 to 250 μg g^{-1} dry weight at exposure levels below this (Smit and Van Gestel, 1995). IBCs in Experiment 1 reached 3507 μg g^{-1} with exposure concentrations up to 37,100 μg g^{-1}.

Collembola have a high excretion efficiency compared to other soil arthropods, and consequently lower internal body concentrations of most metals (Janssen *et al*., 1991; Janssen and Hogervorst, 1993; Van Straalen *et al*., 1987; Van Straalen, 1987, 1996; Van Straalen and Van Wensem, 1986). Because of this, life-history patterns differ between populations exposed to uncontaminated conditions and populations of Collembola from metal-contaminated sites (depending on duration and intensity of exposure (Posthuma and Van Straalen, 1993; Posthuma *et al*., 1993b)). Selection for metals appears to favor individuals that grow fast, mature early, and have a high excretion efficiency (Posthuma and Janssen, 1995; Van Straalen *et al*., 1986).

Many workers have found reproduction to be a more sensitive measure of metal toxicity (in *F. fimetaria* three times more sensitive) than growth (Scott-Fordsmand, 1998; Scott-Fordsmand *et al*., 1997; Smith, 1997; Van Gestel and Hensbergen, 1997; Van Straalen *et al*., 1989). Metals affect reproduction indirectly by reducing growth (Smit, 1997), and the effects on growth are seen well before reproductive effects can be detected. The consequence of metal contamination on growth in this experiment can be demonstrated earlier than effects on reproduction, as early as the 7th day.

Van Straalen (1993) highlighted the need for standardization of soil ecotoxicology tests. Standard test procedures begin with 10- to 12-day-old *F. candida* that are at approximately their fifth instar (sub-adults; Crommentuijn *et al*., 1993; ISO, 1994; Riepert, 1996). It is apparent, however, that juveniles are more vulnerable than adults to the effects of chemicals. Thus the use of newly hatched individuals may be more representative of field conditions.

The laying of eggs in Experiment 1 and hatching time in experiments by Riepert (1996) have been found to require longer than a 4-week test to be reliable. Sandifer and Hopkin (1996) found no reproduction in *F. candida* at high concentrations of Pb and this may simply be because 4 weeks was insufficient time for reproduction to occur.

CONCLUSIONS

F. candida exhibits reduced growth at high metal concentrations in food due in part to lowered ingestion rates of the contaminated diet. Evidence for avoidance resides in the greater quantity of uneaten food remaining at the end of the experiment and the presence of graphite in the digestive tract. Increased mortality at high metal exposure concentrations may be due partly to increased accumulation of metals, and increased susceptibility to metals due to starvation. Reproduction (egg laying time) was delayed, due to retarded growth at high metal concentrations. $EC_{50\,growth}$ values are higher in food contaminated with metals compared to metal-contaminated soil, because Collembola have the ability to excrete metals from the body by intestinal exfoliation. Exposure routes other than ingestion, e.g., cuticle and/or ventral tube, are probably more important in metal-contaminated field soils. Lower concentration factors of metals in *F. candida* at increased exposures (except for Pb) may be due to decreased ingestion, increased excretion, and possibly lower relative assimilation rates at the highest concentrations. The test used in this study is inexpensive, easy to conduct, and provides much more information on the effects of chemicals on Collembola than the ISO "standard test."

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