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Multigeneration Exposure of the Springtail *Folsomia candida* to Phenanthrene: From Dose–Response Relationships to Threshold Concentrations

MIRIAM LEON PAUMEN,[*,†,‡] ERIK STEENBERGEN,[‡] MICHIEL H. S. KRAAK,[†] NICO M. VAN STRAALEN,[‡] AND CORNELIS A. M. VAN GESTEL[‡]

Department of Aquatic Ecology and Ecotoxicology, IBED, University of Amsterdam, The Netherlands, and Department of Animal Ecology, IES, VU University, Amsterdam, The Netherlands

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Results of life-cycle toxicity experiments are supposed to be indicative for long-term effects of exposure to toxicants. Several studies, however, have shown that adaptation or extinction of populations exposed for several generations may occur. The aim of this study was therefore to determine if the effects of the PAH phenanthrene on survival and reproduction of the springtail *Folsomia candida* exposed for 10 consecutive generations to contaminated soil would progressively increase, or, alternatively, if adaptation of the test organisms to the toxicant would occur. LC_{50} values for the first four generations were similar (171–215 μmol/kg dry soil), as expected for a narcotic compound. In the fourth generation, springtails exposed to a concentration similar to the EC_{50} for one generation (163 μmol/kg dry soil) showed internal phenanthrene concentrations in the range known to cause mortality; no reproduction took place, and the population went extinct. From the fifth generation onwards, survival and reproduction were not affected by the remaining exposure concentrations. Apparently, up to a certain threshold concentration (above 77 and below 163 μmol/kg dry soil), the springtails were able to metabolize phenanthrene, as shown by the lack of adverse effects and the lack of adaptation. During multigeneration exposure, the graded concentration–response relationship changed into an all-or-nothing response with a defined threshold concentration. Together with the worsening of effects, this raises concerns about the use of single-generation studies to tackle long-term population effects of environmental toxicants.

Introduction

In contaminated environments, organisms are often exposed to toxicants for their entire lifetime and even for several successive generations. In contrast, chronic toxicity tests generally last for one generation or less (*1–3*), and this could well underestimate the potential effects of a toxicant at the population level if multigeneration exposure would result in delayed reproductive failure, bioaccumulation transmitted to the offspring, or accumulating DNA damage (*4–7*). Such transgenerational effects can only be properly assessed during multigeneration experiments, which are therefore urgently required, but virtually absent in the literature. Only a few attempts have been made to extend standard chronic exposure tests to more than one generation, mainly because of concerns about transgenerational effects of endocrine-disrupting chemicals (*5, 8*). In the few available studies, acclimation and genetic adaptation has been reported for heavy metals (*9–13*), while for organic compounds effects generally became more severe with increasing number of exposed generations, leading to lower genetic diversity in the exposed populations and even to extinction (*7, 14, 15*). Although too few data are available to draw general conclusions about the consequences of multigeneration exposure to toxicants, it is obvious that the ability of single-generation exposure studies to predict more ecologically relevant long-term population effects can be seriously put into question. The aim of the present study was, therefore, to determine multigeneration effects of an organic compound, the polycyclic aromatic hydrocarbon phenanthrene, which exhibited a narcotic mode of action during one-generation studies (*2, 3*).

Exposure to lipophilic organic chemicals like phenanthrene causes membrane disturbance resulting in narcosis (*16*). To neutralize their toxicity, terrestrial invertebrates biotransform PAHs (*17, 18*), but this process may lead to biochemical activation of the compounds and to the formation of reactive oxygen species (*19*). Thus, although harmful effects of PAHs are caused mainly by membrane disturbance, oxidative stress, possible toxicity of generated metabolites, and formation of protein and DNA adducts may also occur. Traditional chronic toxicity experiments are too short to observe these effects, but during multigeneration exposure this accumulating damage could produce sublethal effects (e.g., on growth and reproduction), and the fitness of exposed populations could be affected (*4, 20, 21*).

In order to determine if phenanthrene exerts such long-term effects, we exposed the soil-dwelling springtail *Folsomia candida* for 10 consecutive generations, and effects on survival and reproduction were determined every generation. To investigate if adaptation to the toxicant was taking place, additional 28-day toxicity experiments were performed after 5 and 10 generations of exposure.

Materials and Methods

Test Organism. *Folsomia candida* (Collembola, Isotomidae) is a soil-dwelling springtail, widely used in ecotoxicity testing because it has a short life cycle, is sensitive to different classes of pollutants, and can easily be cultured in the laboratory (*1, 22–26*). A population consists of parthenogenetic females, and at 20 °C sexual maturity is reached between 21 and 24 days after hatching. Each female lays batches of 30–50 eggs in soil pores, which hatch after approximately 10 days (*26*). For these experiments, individuals from a laboratory culture from the Department of Animal Ecology (VU University, Amsterdam, The Netherlands) were used. *F. candida* individuals are cultured in Perspex rings filled with humid plaster of Paris, in a climate room at 15 °C under a 12 h/12 h light/dark regime, and fed bakers' yeast. Approximately three weeks prior to the start of the experiment, a synchronized culture was set up to obtain 10–12 day old individuals to start the experiment.

Experimental Setup. *Folsomia candida* was exposed for 10 consecutive generations to standard LUFA 2.2 soil

* Corresponding author e-mail: M.LeonPaumen@uva.nl.
† University of Amsterdam.
‡ VU University.

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TABLE 1. LC_{50} and EC_{50} Estimates (μmol/kg dry soil) for Survival and Reproduction of Consecutive Generations of the Springtail *Folsomia candida* Exposed to LUFA 2.2 Soil Spiked with Phenanthrene[a]

generation	LC_{50}(μmol/ kg dry soil)	EC_{50}(μmol/ kg dry soil)
P	215 (8−422)	156 (121−191)
F1	171 (129−211)	55 (14−97)
F2	191 (129−249)	78 (49−108)
F3	196 (136−256)	102 (45−158)
F4−F9	>77	>77

[a] LC_{50} and EC_{50} values were calculated using average actual exposure concentrations for each generation.

(Landwirschafliche Untersuchungs and Forschungsanstalt (LUFA), Speyer, Germany) spiked with phenanthrene (98% purity, Sigma-Aldrich, Steinheim, Germany). LUFA 2.2 soil is characterized as a sandy loam with a total organic carbon content of 2.3 ± 0.2% and a pH (0.01 M $CaCl_2$) of 5.6 ± 0.4. The soil was excavated from a meadow where no pesticides or fertilizers were used for the last 4 years.

The soil was spiked with phenanthrene using acetone as a carrier solvent. Five nominal concentrations were used: 34, 67, 140, 281, and 561 μmol/kg dry soil (6, 12, 25, 50, and 100 mg/kg dry soil). This range was set using the results of a previous one-generation study (*3*), in which an EC_{50} for reproduction of 257 μmol/kg dry soil was determined. Controls and solvent controls were included. At the start of the spiking procedure, the required amount of phenanthrene was dissolved in 100 mL of acetone and added to one-quarter of the total amount of soil (65 g) in a 1 L glass jar, and the jar was closed. After 24 h of equilibration, the jar was opened, and the acetone was allowed to evaporate overnight. The next day, the remaining soil (195 g) was added to the soil−phenanthrene mixture and mixed thoroughly. Sixty milliliters of distilled water was added to the mixture in order to achieve a moisture content equivalent to 40−50% of the water holding capacity of the soil. The solvent control was prepared following the same procedure as for the spiked soils but without phenanthrene. Approximately 30 g of spiked soil was introduced in seven replicated 100 mL glass test jars, a few granules of dry yeast were added on top as food source for the collembolans, and the test jars were closed with a plastic lid. Subsamples were taken from the remaining 50 g of soil to determine phenanthrene concentrations, pH, and moisture content of the soil at the start of the experiment.

At the start of the experiment, ten 10−12 day old *F. candida* individuals from the synchronized culture were introduced in each replicate test jar. Exposure was conducted following the 11267 ISO guideline (*27*). The test jars were kept in a climate room at 20 °C with a 12 h:12 h light:dark regime. Once a week, all test jars were weighed and distilled water was added if necessary to compensate for evaporation losses. Also yeast granules were added to the jars if necessary. Two test jars were sacrificed after 14 days and at the end of the test to perform pH, soil moisture, and phenanthrene concentration measurements in the test soil, leaving five replicates per treatment for the toxicity test.

After 28 days, exposure of the first generation was ended, and springtails were extracted from the test jars by flotation. For this purpose, 100 mL of distilled water was added to the test soil, the mixture was gently stirred and transferred to a 400 mL glass beaker. Surviving adults were counted, and a digital picture of the floating springtails on the water surface was taken. Automatic counting and length determination of the juveniles was performed using the software package AnalySIS 5 (Soft Imaging System, Münster, Germany). Juveniles from the five replicate test jars per concentration were transferred with a flat spoon from the water surface to a single Perspex ring (Ø 15 cm), which contained an approximately 1 cm layer of humid black plaster of Paris. The next day, exposure of the second generation was started by transferring juveniles from each concentration to freshly spiked soil. Only juveniles from the first laid egg batch (largest in size), which were generally the most abundant, were chosen to be the next generation. Adults and first-batch juveniles from each generation were collected and frozen at −20 °C. Since juveniles from the first generation used to start the second generation were younger than the prescribed 10−12 days, exposure in the second and following generations was extended to 35 days. Exposure of *F. candida* was repeated for 10 consecutive generations.

After 5 and 10 generations of exposure, possible adaptation to phenanthrene in exposed *F. candida* populations was determined. For this purpose, toxicity tests were performed exposing juveniles from each phenanthrene-exposed population to a broad range of phenanthrene concentrations (nominal 56, 112, 224, and 449 μmol/kg dry soil). Exposure was performed for 28 days using the same protocol as described above (*27*), with four replicate test jars per concentration.

Phenanthrene Concentrations in Soil and *F. candida* Adults. For each generation, actual phenanthrene concentrations in the soil were determined at the beginning, halfway (day 14), and at the end of the exposure period (day 28/35) by Soxhlet extraction followed by high performance liquid chromatography (HPLC). Approximately 13 g of soil were mixed with an equal amount of anhydrous sodium sulfate and extracted for five hours using hexane (HPLC grade, Biosolve, Valkenswaard, The Netherlands). Next, the extract was collected in acetonitrile by blowing off the hexane using a gentle stream of nitrogen. Phenanthrene concentrations in the samples were measured using a HPLC system consisting of a Vydac RP 18 201TP column with a Vydac 201 GD RP-18 guard column (Alltech, Breda, The Netherlands), a Jasco FP-1520 fluorescence detector (Jasco, Essex, UK), and a Gynkotek UVD320s ultraviolet diode-array detector (Gynkotek, Germering, Germany). Recovery checks using LUFA 2.2 soil were performed to validate the efficiency of the Soxhlet extraction, and phenanthrene concentrations in the soil were corrected for recovery.

Phenanthrene concentrations in *F. candida* adults were determined at the end of each exposure period (28/35 days). For this purpose, 20−40 individuals were Soxhlet extracted for 5 h using hexane. The samples were then concentrated approximately 10 times and transferred to acetonitrile using a gentle stream of nitrogen. Samples were measured using the same HPLC system as above.

Statistical Analysis. The concentrations of the test compound in the soil that caused 50% reduction of survival (number of adults) and reproduction (number of juveniles) of the adult springtails compared to the control (respectively LC_{50} and EC_{50} values in μmol/kg dry soil) were calculated according to Haanstra et al. (*28*), using a logistic curve fitted through the raw concentration−response data. Survival and reproduction data for the control and solvent control were compared at the 5% significance level using *t* tests. Average concentrations in the soil during the experiment for each exposure generation were used to calculate effect concentrations. When possible, LC_{50} and EC_{50} values for the different generations were compared at the 5% significance level using likelihood ratio tests (*29*). Results from the adaptation experiments (reproduction of unexposed population vs pre-exposed populations) were compared at the 5% significance level using *t* tests.Statistical analysis was performed using SPSS 11.0 for Windows.

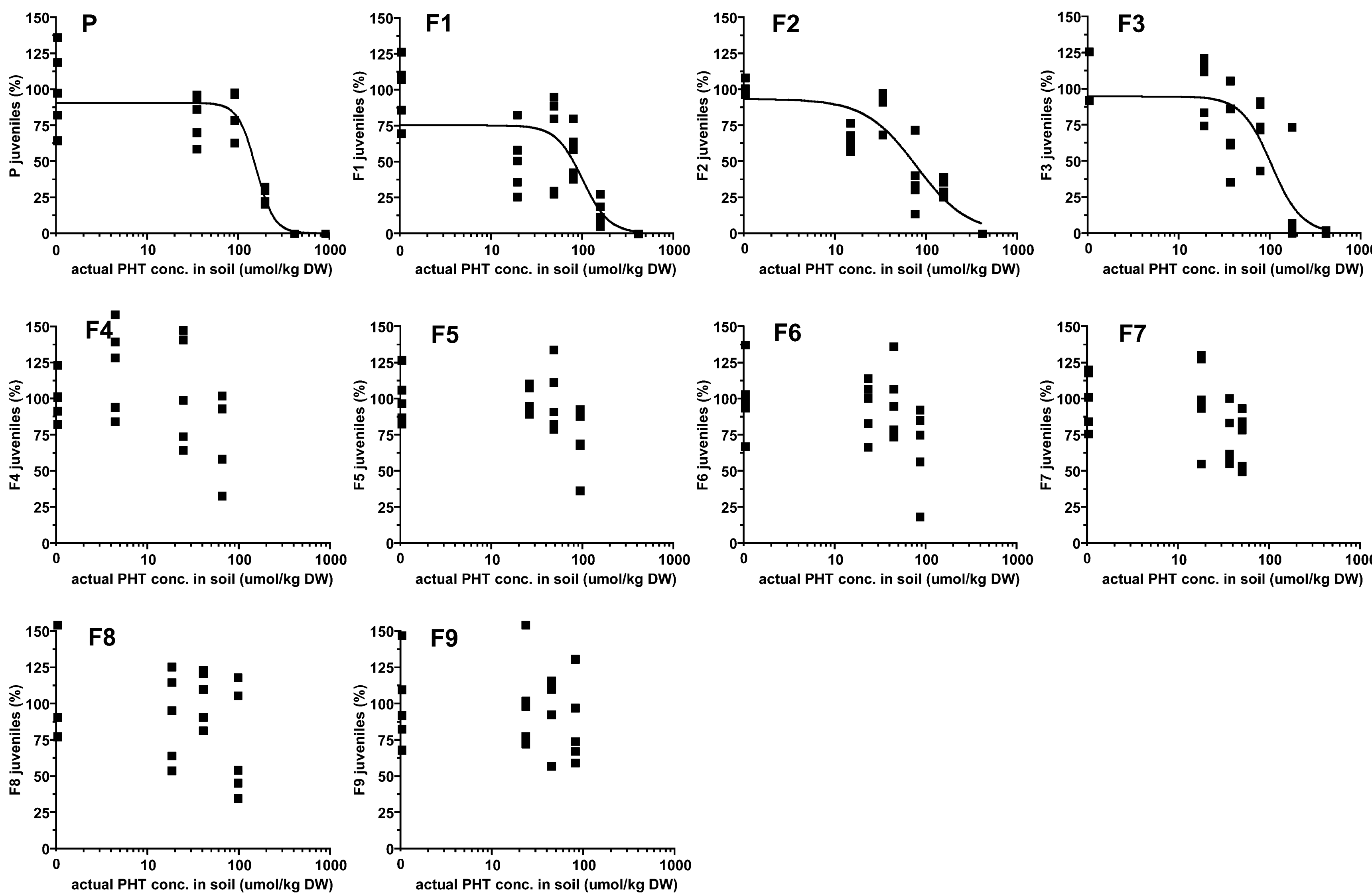

FIGURE 1. Concentration−response curves for reproduction of 10 consecutive generations of the springtail *Folsomia candida* (*Y*-axis: juvenile number in % of average solvent control) exposed to LUFA 2.2 soil spiked with phenanthrene (*X*-axis: μmol PHT/kg dry soil; average actual concentrations for each generation are used).

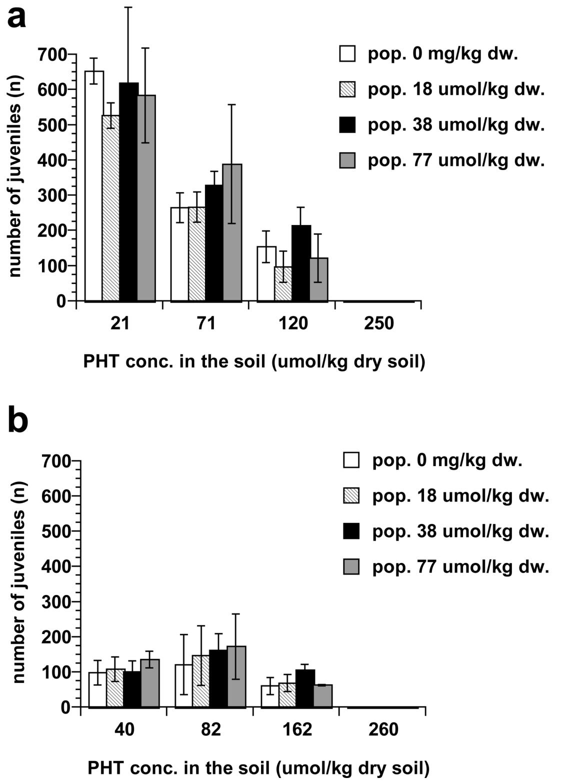

FIGURE 2. Effects of phenanthrene on the reproduction of populations of *Folsomia candida* exposed for 5 (a) and 10 (b) generations to different phenanthrene concentrations in LUFA 2.2 soil. The *X*-axis shows the average actual concentrations of phenanthrene during exposure. Bars represent the number of juveniles (n ± SD, *n* = 4) for every multigeneration exposure concentration.

Results

Phenanthrene Concentrations in Soil. Actual concentrations in the soil at the beginning of the exposure periods of the different generations, corrected for recovery (which was 90%), were on average 78 ± 11% of the nominal ones. Phenanthrene concentrations in the soil decreased during exposure time, which is common for phenanthrene and probably caused by microbial degradation (*3, 30, 31*). The decrease in actual phenanthrene concentrations ranged from 74 ± 16% of the initial concentration for the lowest to 9 ± 36% of the initial value for the highest concentration in the soil. Probably, degradation at higher concentrations in the soil was hampered by toxic effects of phenanthrene on the soil microflora (*32*). Average actual phenanthrene concentrations during exposure for each generation were used to calculate effect concentrations. To designate the exposed populations in the following text, however, actual phenanthrene concentrations per treatment level were averaged over the 10 exposure generations, resulting in the following average concentrations during the entire 10-generation experiment: 18, 38, 77, 163, and 401 μmol/kg dry soil (nominal 34, 67, 140, 281, and 561 μmol/kg dry soil).

Multigeneration Exposure of *F. candida*. As prescribed by the ISO guideline (*27*), control survival of *F. candida* adults was always at least 80% (average 86 ± 12%), there were always more than 100 juveniles in the control test jars at the end of the test (average number of juveniles ranged from 314 ± 62 at the F7 to 1994 ± 98 at the F2), and the coefficient of variation of the control reproduction over the generations did not exceed 30% (average 22%). Soil pH (0.01 M $CaCl_2$) during the toxicity tests ranged from 5.0 to 5.5 at the beginning, halfway, and after 28 days of exposure. However, when exposure time was increased to 35 days, soil pH decreased to approximately 4.5 in all treatments.

Survival. Survival of control and solvent control individuals was similar (*t* test, $p > 0.05$), and solvent control values were used for the LC_{50} calculations because the test conditions in the solvent control soil were similar to the conditions in the spiked soil. Survival of *F. candida* adults decreased with increasing phenanthrene concentrations in the soil. At the highest exposure concentration (401 μmol/kg dry soil), complete mortality occurred during the first generation and the population became extinct. LC_{50} values for the first four generations ranged between 171 and 215 μmol/kg dry soil (Table 1). LC_{50} values were compared using likelihood ratio tests and did not differ significantly ($\chi^2_1 < 3.841$, $p > 0.05$). At the fourth generation, survival was similar to that in the first three generations, but the population exposed to the highest remaining phenanthrene concentration (163 μmol/kg dry soil) failed to produce juveniles and became extinct. From the fifth generation onward, no significant effect on survival of the remaining exposure concentrations (18, 38, and 77 μmol/kg dry soil) was observed compared to the control. Consequently, the clear concentration−response relationship observed during the first four generations completely disappeared, and no LC_{50} values could be calculated. The highest remaining average exposure concentration is given instead (Table 1).

Reproduction. Juvenile numbers decreased with increasing phenanthrene concentrations in the soil for the first four generations (Figure 1), but their growth was not affected by phenanthrene (data not shown). Juvenile numbers from the solvent control were used as control values to calculate 50% effect concentrations for reproduction. EC_{50} values for the first, second, third, and fourth generation (P, F1, F2, F3) were 156, 55, 78, and 102 μmol/kg dry soil, respectively (Table 1). Likelihood ratio tests showed that EC_{50}s for the second and third generation were significantly lower than the EC_{50} for the first generation ($X^2_1 > 3.841$, $p < 0.05$); in contrast, the first and fourth generation EC_{50}s did not differ. At the fourth generation (F3), no juveniles were produced at the highest remaining phenanthrene concentration in the soil (163 μmol/kg dry soil), and the population became extinct (Figure 1). From the fifth to the tenth generation, no significant effects on reproduction of *F. candida* were observed at the remaining exposure concentrations (18, 38, and 77 μmol/kg dry soil) compared to the control. Consequently, the clear concentration−response relationship observed during the first four generations completely disappeared, and no EC_{50} values could be calculated. The highest remaining average exposure concentration is given instead (Table 1).

Adaptation. No springtails survived at the highest test concentration in the adaptation experiments (actual 250/260 μmol/kg dry soil, Figure 2). At lower exposure concentrations, no significant differences in juvenile production were found between phenanthrene-exposed populations and the nonexposed control population (*t* test, $p > 0.05$, Figure 2), meaning that no adaptation to phenanthrene occurred in the populations exposed for 5 and 10 generations to the tested phenanthrene concentrations in the soil. Juvenile numbers in the adaptation experiments after 10 generations were much lower than juvenile numbers after 5 generations, but values at the lowest exposure concentration were above the 100 juveniles set as minimal control value in the ISO guideline.

Phenanthrene Concentrations in *F. candida* Adults. In Table 2, phenanthrene concentrations in *F. candida* adults and biota to soil accumulation factors (BSAFs), normalized for lipid content of the springtails (3.6% of WW, taken from Staempfli et al. (*33*)) and organic content in the LUFA 2.2 soil (2.3% OC), are shown for the first five and the tenth generation. Because of the shorter exposure time, phenanthrene concentrations in adults from the first generation (P) were somewhat lower than concentrations in animals from the next generations (F1, F2, F3, F4). Adults from the fourth generation (F3) exposed to the highest remaining phenan-

TABLE 2. Phenanthrene Concentrations in *Folsomia candida* Adults (μmol PHT/g WW) and Biota to Soil Accumulation Factors (BSAFs, g OC/g lipid) after 1, 2, 3, 4, 5, and 10 Generations of Exposure to Phenanthrene-Contaminated LUFA 2.2 Soil (μmol PHT/kg dry soil)[a]

PHT in the soil (μmol/kg dry soil)	P		F1		F2		F3		F4		F9	
	concn	BSAF	concn	BSAF	concn	BSAF	concn	BSAF	concn	BSAF	concn	BSAF
0	b.d.l.	b.d.l.	b.d.l.	-	0.10	-	b.d.l.	-	b.d.l.	-	b.d.l.	-
18	n.m.	n.m.	0.15	5.67	b.d.l.	-	b.d.l.	-	b.d.l.	-	b.d.l.	-
38	0.03	0.62	0.43	6.60	0.07	1.65	0.10	2.02	0.05	1.5	0.01	0.21
77	0.10	0.81	1.00	9.30	0.19	1.85	0.21	2.01	0.18	2.0	0.02	0.15
163	0.30	1.13	0.97	4.56	0.75	3.57	3.46	14				

[a] n.m.: not measured, b.d.l.: below detection limit (~6 μg/L).

threne concentration in the soil (163 μmol/kg dry soil), which became extinct, showed the highest internal phenanthrene concentration (3.46 μmol PHT/g WW) and the highest BSAF (14). Phenanthrene concentrations and BSAFs for the tenth generation were lowest.

Discussion

This study aimed at determining if effects of the narcotic PAH phenanthrene on survival and reproduction of *F. candida* would worsen after several consecutive generations of exposure or, alternatively, if adaptation to the toxicant would take place. The population exposed to the highest phenanthrene concentration in the soil (401 μmol/kg dry soil) became extinct after one generation. This is in agreement with the results of a previous one-generation study (*3*), in which this concentration was causing almost 100% mortality. From the second to the fourth generation, effects on survival of *F. candida* were similar. Consequently, LC_{50} values for the first four generations were similar and in the same range as previously determined chronic LC_{50}s for *F. candida* and *F. fimetaria* (*3, 34*). In those two studies, chronic LC_{50}s were compared to an acute LC_{50}−log K_{ow} relationship, which described the narcotic effects of several polycyclic aromatic compounds on the midge *Chironomus riparius* (*3*) and the cladoceran *Daphnia magna* (*34*). This comparison revealed that the chronic and hence also our multigeneration LC_{50}s agreed well with the acute LC_{50}−log K_{ow} relationship, meaning that effects of phenanthrene on survival during chronic and multigeneration exposure of *F. candida* are mainly caused by narcosis.

Reproduction of *F. candida* during the ten consecutive exposure generations showed a different pattern than survival. For the first generation, the EC_{50} value was close to the LC_{50} value ($LC_{50}/EC_{50} = 1.4$) as previously reported by Droge et al. in a one-generation study (*3*). This suggests a narcotic mode of action for the effect of phenanthrene on reproduction of *F. candida*. For the second and third generations, however, LC_{50}s remained constant while EC_{50}s decreased significantly, and as a consequence the LC_{50}/EC_{50} ratio became larger (3 and 2.3, respectively), suggesting deviations from narcosis and specific effects of phenanthrene on reproduction of *F. candida*. These deviations could be due to cumulative damage after successive generations of exposure, as found for organic chemicals in previous long-term exposure studies (*7, 14, 35*), but also to the longer exposure time for juveniles of the second and third generations. At the fourth generation, *F. candida* failed to reproduce at the highest remaining exposure concentration (163 μmol/kg dry soil), a concentration close to the first generation EC_{50} value (156 μmol/kg dry soil). Thus, a concentration which only partially affected reproduction after one generation resulted in complete reproductive failure and subsequent extinction of the population after four generations. This is in agreement with the few available studies on multigeneration exposure, which suggest that for organic compounds, toxic effects tend to intensify with increasing exposure generations (*7, 14, 15*). These findings clearly demonstrate the additional value of using multigeneration exposure to tackle long-term effects of toxicants.

The population exposed to the highest remaining concentration (163 μmol/kg dry soil) that became extinct was characterized by an extremely high BSAF and high phenanthrene concentrations in the animals: BSAF (*14*) was 1 order of magnitude higher than equilibrium partitioning theory (EqP) predictions (BSAF = 1), and the measured concentration in the springtails was in the range of the 2−8 μmol/g wet weight predicted by the EqP as lethal concentration for narcotic chemicals (*36, 37*). In contrast, from the fifth generation onward, BSAFs were around 2 or lower, and no significant effects of phenanthrene on survival and reproduction were observed for the remaining exposure concentrations. These BSAF values are high compared to field studies on PAHs (*38, 39*) but in the range of laboratory studies on aquatic and terrestrial invertebrates exposed to several PAHs (including phenanthrene), in which PAH availability is expected to be high (*31, 39*).

The lack of effects on survival and reproduction from the fifth generation onward at lower phenanthrene concentrations in the soil was probably due to biotransformation, previously documented for pyrene in the springtails *Orchesella cincta* and *F. candida* (*17, 40*). In the long run, this constitutive ability of *F. candida* to biotransform organic compounds resulted in an all-or-nothing effect: below a certain threshold (situated between 77 and 163 μmol/kg dry soil), the organism was able to metabolize phenanthrene into harmless metabolites (probably hydroxyphenanthrenes *17, 40*). Above this threshold, at an exposure concentration close to the one-generation EC_{50} (156 μmol/kg dry soil), the biotransformation capacity was probably exceeded, but cumulative toxic effects of phenanthrene only became apparent after four consecutive generations of exposure, when reproductive failure had fatal consequences for the exposed population. In agreement, lowered biotransformation due to intoxication by the parent compound was observed for the midge *Chironomus riparius* exposed to azaarenes (*41*). The lack of adaptation also supports the all-or-nothing hypothesis: below the threshold, no adaptation took place, and above this concentration the population ultimately went extinct. In agreement with our study, lack of resistance development after prolonged exposure was reported for grass shrimp exposed to PAH-contaminated sediments (*10*). Results from this study cannot be extrapolated to the field situation, where springtail populations are formed by thousands of individuals; however, if the biotransformation potential of *F. candida* is more or less constant in a population, prolonged exposure to narcotic compounds could have dramatic consequences.

In conclusion, our experiments demonstrated that conventional one-generation effect concentrations underestimate the toxicity of phenanthrene during multigeneration exposure. Therefore, the present study underlines the concerns about the use of single generation studies to tackle long-term effects of environmental toxicants. These concerns are increased by the observation that multigeneration effect levels could not be expressed as EC_x values.

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