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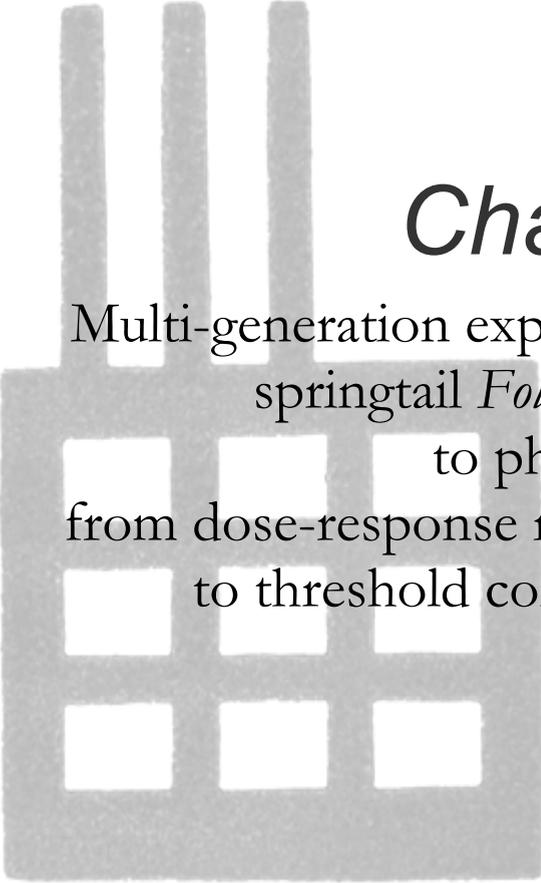
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Chapter 6

Multi-generation exposure of the
springtail *Folsomia candida*
to phenanthrene:
from dose-response relationships
to threshold concentrations

León Paumen, M., E. Steenbergen, M.H.S. Kraak, N.M. van Straalen and C.A.M. van Gestel, 2008. *Environmental Science and Technology*, 42, 6985-6990...

Abstract

Results of life-cycle toxicity experiments are supposed to be indicative for long-term population effects of 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 ten consecutive generations would progressively increase, or, alternatively, if adaptation of the test organisms to the toxicant would occur. LC50 values for the first four generations were similar (171-215 $\mu\text{mol/kg}$ dry soil), as expected for a narcotic compound. In the fourth generation, springtails exposed to the highest concentration (163 $\mu\text{mol/kg}$ dry soil, similar to the reproduction EC50 for one generation) failed to reproduce and the population went extinct. From the fifth generation onwards, survival and reproduction of *F. candida* exposed to the remaining phenanthrene concentrations (77 $\mu\text{mol/kg}$ dry soil and lower) were not affected. Thus, up to a certain threshold concentration (between 77 and 163 $\mu\text{mol/kg}$ dry soil) the springtails were able to metabolize phenanthrene and populations persisted, while above this threshold populations got extinct. The observed worsening of effects and the transition from a one generation dose-response relationship towards a ten generation threshold concentration raise 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 (Sverdrup et al. 2001; Croau and Moia 2006; Droge et al. 2006), and this could well underestimate the potential effects of a toxicant at the population level if multi-generation exposure would result in delayed reproductive failure, bioaccumulation transmitted to the offspring or accumulating DNA damage (Van Brummelen et al., 1996; Nash et al., 2004; Van Straalen and Roelofs 2006; Campiche et al. 2007). Such transgenerational effects can only be properly assessed during multi-generation 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 due to concerns about transgenerational effects of endocrine disrupting chemicals (OECD, 2006; Campiche et al., 2007). In the few available studies, acclimation and genetic adaptation has been reported for heavy metals (Mutzinger, 1990; Postma and Davids, 1995; Klerks, 1999; Bossuyt and Janssen, 2005; Wand and Wang, 2008), 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 (Street et al., 1998; Tominaga et al., 2003; Nash et al., 2004). Although too few data are available to draw general conclusions about the consequences of multi-generation 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 multi-generation effects of an organic compound, the Polycyclic Aromatic Hydrocarbon phenanthrene, which exhibited a narcotic mode of action during one-generation studies (Sverdrup et al. 2001; Droge et al. 2006).

Exposure to lipophilic organic chemicals like phenanthrene causes membrane disturbance resulting in narcosis (Bleeker et al., 2002). To neutralize their toxicity, terrestrial invertebrates biotransform PAHs (Stroomberg et al., 2003; 2004), but this process may lead to biochemical activation of the compounds and to the formation of Reactive Oxygen Species (Xue and Warshawsky, 2005). 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 multi generation exposure this accumulating damage could produce sub-lethal effects (on e.g. growth and reproduction) and the fitness of

exposed populations could be affected (Van Schooten et al., 1995; Martin et al., 2005; Van Straalen and Roelofs 2006).

In order to determine if phenanthrene exerts such long-term effects, we exposed the soil-dwelling springtail *Folsomia candida* for ten 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 five and ten 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 (Van Gestel et al., 1997; Herbert et al., 2004; Fountain and Hopkin, 2005; Croau and Moia, 2006; Domene et al., 2007; Jager et al., 2007). 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 to 50 eggs in soil pores, which hatch after approximately 10 days (Fountain and Hopkin, 2005). 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 12h/12h 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 ten consecutive generations to standard LUFA 2.2 soil (Landwirtschaftliche Untersuchungs und 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 CaCl₂) 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 $\mu\text{mol/kg}$ 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 (Droge et al., 2006), in which an EC₅₀ for reproduction of 257 $\mu\text{mol/kg}$ 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 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 hours 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 ml 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. Sub-samples 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 days old *F. candida* individuals from the synchronized culture were introduced in each replicate test jar. Exposure was conducted following the 11267 ISO guideline (ISO, 1998). The test jars were kept in a climate room at 20°C with a 12h:12h 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 ten 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 $\mu\text{mol/kg}$ dry soil). Exposure was performed for 28 days using the same protocol as described above (ISO, 1998), 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, half-way (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 grams 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 five hours using hexane. Following, the samples were concentrated approximately ten 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 LC50 and EC50 values in $\mu\text{mol/kg}$ dry soil) were calculated according to Haanstra et al. (1985), 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, LC50 and EC50 values for the different generations were compared at the 5% significance level using likelihood ratio tests (Crommentuijn et al., 1997). 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.

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 $78 \pm 11\%$ of the nominal ones. Phenanthrene concentrations in the soil decreased during exposure time, what is common for phenanthrene, and probably caused by microbial degradation (Jaget et al., 2000; Sverdrup et al., 2002c; Droge et al., 2006). The decrease in actual phenanthrene concentrations ranged from $74 \pm 16\%$ of the initial concentration for the lowest to $9 \pm 36\%$ 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 (van Herwijnen et al., 2003). 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 ten exposure generations, resulting in the following average concentrations during the entire 10-generation experiment: 18, 38, 77, 163 and 401 $\mu\text{mol/kg}$ dry soil (nominal 34, 67, 140, 281 and 561 $\mu\text{mol/kg}$ dry soil).

Multi-generation exposure of F. candida

As prescribed by the ISO guideline (1998), control survival of *F. candida* adults was always at least 80% (average $86 \pm 12\%$), 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 (0.01M CaCl₂) during the toxicity tests ranged from 5.0 to 5.5 at the beginning, half-way 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 LC50 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 $\mu\text{mol/kg}$ dry soil), complete mortality occurred during the first generation and the population got extinct. LC50 values for the first four generations ranged between 171 and 215 $\mu\text{mol/kg}$ dry soil (Table 1). LC50 values were compared using likelihood ratio tests, and did not differ significantly ($X^2_{1} < 3.841$, $p > 0.05$). 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 $\mu\text{mol/kg}$ dry soil) failed to produce juveniles and went extinct. From the fifth generation onwards, no significant effect on survival of the remaining exposure concentrations (18, 38 and 77 $\mu\text{mol/kg}$ dry soil) was observed compared to the control. Consequently, the clear concentration-response relationship observed during the first four generations completely disappeared and no LC50 values could be calculated. The highest remaining average exposure concentration is given instead (Table 1).

Table 1. LC50 and EC50 estimates ($\mu\text{mol/kg}$ dry soil) for survival and reproduction of consecutive generations of the springtail *Folsomia candida* exposed to LUFA 2.2 soil spiked with phenanthrene. LC50 and EC50 values were calculated using average actual exposure concentrations for each generation.

Generation	LC50 ($\mu\text{mol/kg}$ dry soil)	EC50 ($\mu\text{mol/kg}$ 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

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. EC50 values for the first, second, third and fourth generation (P, F1, F2, F3) were 156, 55,

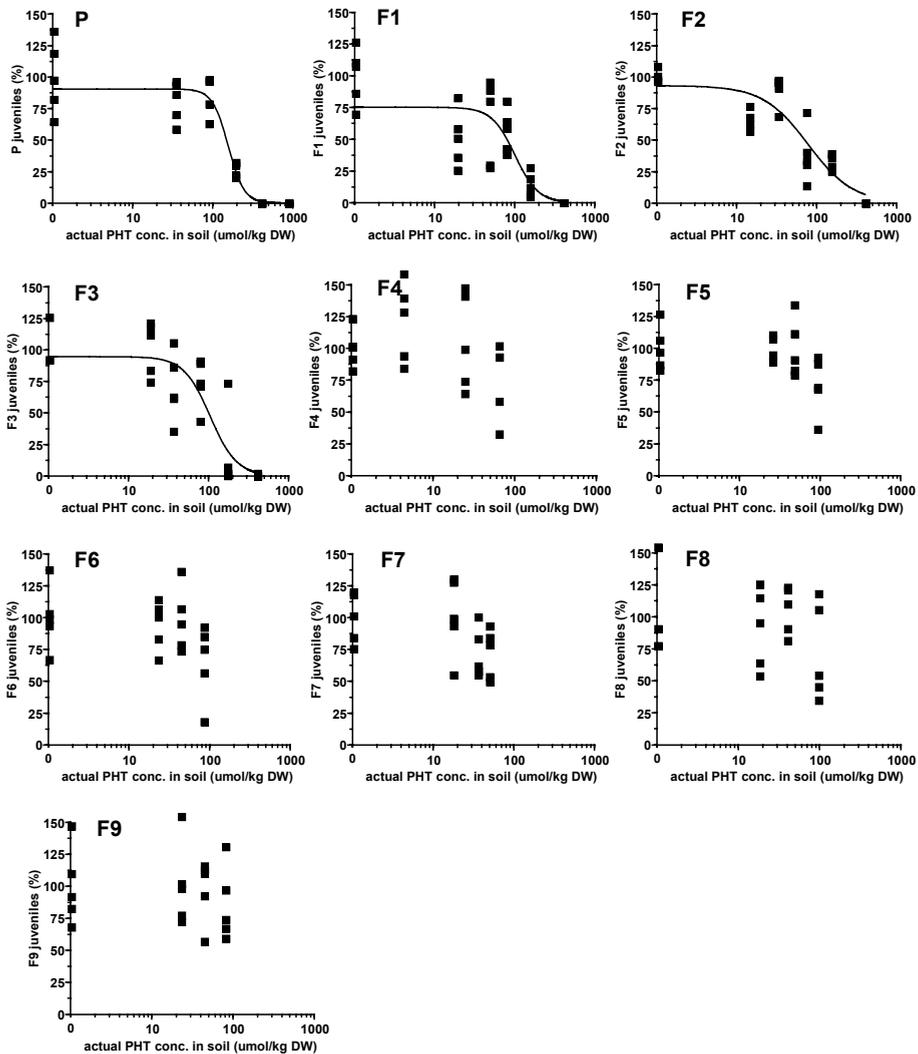


Figure 1. Concentration-response curves for reproduction of ten consecutive generations of the springtail *Folsomia candida* (Y-axis: juvenile number in % of average solvent control) exposed to LUFA 2.2 soil spiked with phenanthrene (X-axis: μmol PHT/kg dry soil; average actual concentrations for each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

78 and 102 $\mu\text{mol}/\text{kg}$ dry soil, respectively (Table 1). Likelihood ratio tests showed that EC50s for the second and third generation were significantly lower than the EC50 for the first generation ($X_{21} > 3.841$, $p < 0.05$); in contrast, the first and fourth generation EC50s did not differ. At the fourth generation (F3), no juveniles were produced at the

highest remaining phenanthrene concentration in the soil (163 $\mu\text{mol/kg}$ 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 $\mu\text{mol/kg}$ dry soil) compared to the control. Consequently, the clear concentration-response relationship observed during the first four generations completely disappeared and no EC50 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 $\mu\text{mol/kg}$ dry soil, Figure 2). At lower exposure concentrations, no significant differences in juvenile production were found between phenanthrene-exposed populations and the non-exposed control population (T-test, $p > 0.05$, Figure 2), meaning that no adaptation to phenanthrene occurred in the populations exposed for five and ten generations to the tested phenanthrene concentrations in the soil. Juvenile numbers in the adaptation experiments after ten generations were much lower than juvenile numbers after five generations, but values at the lowest exposure concentration were above the 100 juveniles set as minimal control value in the ISO guideline.

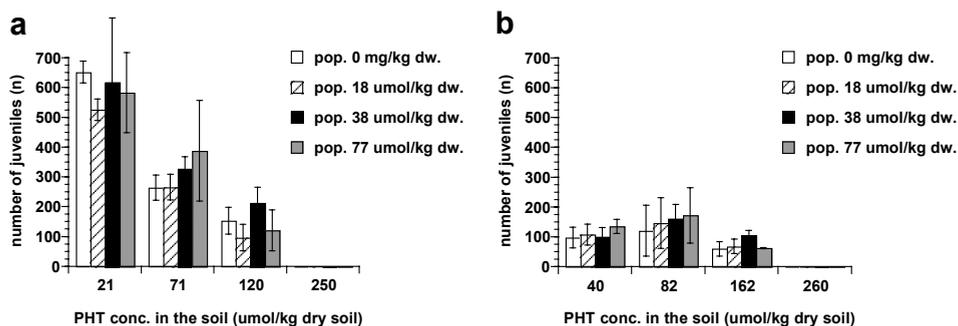


Figure 2. Effects of phenanthrene on the reproduction of populations of *Folsomia candida* exposed for 5 (a) and 10 (b) generations to different phenanthrene concentrations in LUFA 2.2 soil. X-axis shows the average actual concentrations of phenanthrene during exposure. Bars represent the number of juveniles ($n \pm \text{S.D.}$, $n=4$) for every multi-generation exposure concentration.

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. (2007)) and organic content in the LUFA 2.2 soil (2.3% OC), are shown for the first five and the tenth generation. Due to 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 phenanthrene concentration in the soil (163 $\mu\text{mol/kg}$ dry soil), which got extinct, showed the highest internal phenanthrene concentration (3.46 $\mu\text{mol PHT/g WW}$) and the highest BSAF (14). Phenanthrene concentrations and BSAFs for the tenth generation were lowest.

Table 2. Phenanthrene concentrations in *Folsomia candida* adults ($\mu\text{mol PHT/g WW}$) 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 ($\mu\text{mol PHT/kg dry soil}$). n.m.: not measured, b.d.: below detection limit ($\sim 6 \mu\text{g/l}$).

PHT conc ($\mu\text{mol/kg dry soil}$)	P		F1		F2		F3		F4		F9	
	Conc.	BSAF										
0	b.d.	b.d.	b.d.	-	0.10	-	b.d.	-	b.d.	-	b.d.	-
18	n.m.	n.m.	0.15	5.67	b.d.	-	b.d.	-	b.d.	-	b.d.	-
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				

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 $\mu\text{mol/kg}$ dry soil) went extinct after one generation. This is in agreement with the results of a previous one-generation study (Droge et al., 2006), 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, LC50 values for the first four generations were similar and in the same range as previously determined chronic LC50s for *F. candida* and *F. fimetaria* (Sverdrup et al., 2001; Droge et al., 2006). In those two studies, chronic LC50s were compared to an acute LC50- $\log K_{ow}$ relationship, which described the narcotic effects of several Polycyclic Aromatic Compounds on the midge *Chironomus riparius* (Bleeker et al., 2002) and the cladoceran *Daphnia magna* (Sverdrup et al., 2002b). This comparison revealed that the

chronic and hence also our multi-generation LC50s agreed well with the acute LC50- $\log K_{ow}$ relationship, meaning that effects of phenanthrene on survival during chronic and multi-generation 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 EC50 value was close to the LC50 value (LC50/EC50=1.4) as previously reported by Droge et al. in a one-generation study (Droge et al., 2006). This suggests a narcotic mode of action for the effect of phenanthrene on reproduction of *F. candida*. For the second and third generations, however, LC50s remained constant while EC50s decreased significantly, and as a consequence the LC50/EC50 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 (Tominaga et al., 2003; Nash et al., 2004; Brennan et al., 2006), 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 $\mu\text{mol/kg}$ dry soil), a concentration close to the first generation EC50 value (156 $\mu\text{mol/kg}$ 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 multi-generation exposure, which suggest that for organic compounds, toxic effects tend to intensify with increasing exposure generations (Street et al., 1998; Tominaga et al., 2003; Nash et al., 2004). These findings clearly demonstrate the additional value of using multi-generation exposure to tackle long-term effects of toxicants.

The population exposed to the highest remaining concentration (163 $\mu\text{mol/kg}$ dry soil) that got extinct was characterized by an extremely high BSAF and high phenanthrene concentrations in the animals: BSAF (Tominaga et al., 2003) was one 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 $\mu\text{mol/g}$ wet weight predicted by the EqP as lethal concentration for narcotic chemicals (McCarty et al., 1992; Lotufo et al., 1998). In contrast, from the fifth generation onwards, 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 (Van Brummelen et al., 1996; Neilson et al., 1998), 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 (Neilson et al., 1998; Jager et al., 2000).

The lack of effects on survival and reproduction from the fifth generation onwards at lower phenanthrene concentrations in the soil was probably due to biotransformation, previously documented for pyrene in the springtails *Orchesella cincta* and *F. candida* (Howsam et al., 2003; Stroomberg et al., 2004). On the long run, the 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 $\mu\text{mol/kg}$ dry soil), the organism was able to metabolize phenanthrene into harmless metabolites (probably hydroxy-phenanthrenes (Howsam et al., 2003; Stroomberg et al., 2004)). Above this threshold, at an exposure concentration close to the one-generation EC50 (156 $\mu\text{mol/kg}$ 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 (De Voogt et al., 1999). 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 shrimps exposed to PAH-contaminated sediments (Klerks, 1999). Results from this study can not 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 multi-generation 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 multi-generation effect levels could not be expressed as ECx values.