Invertebrate life cycle responses to PAC exposure

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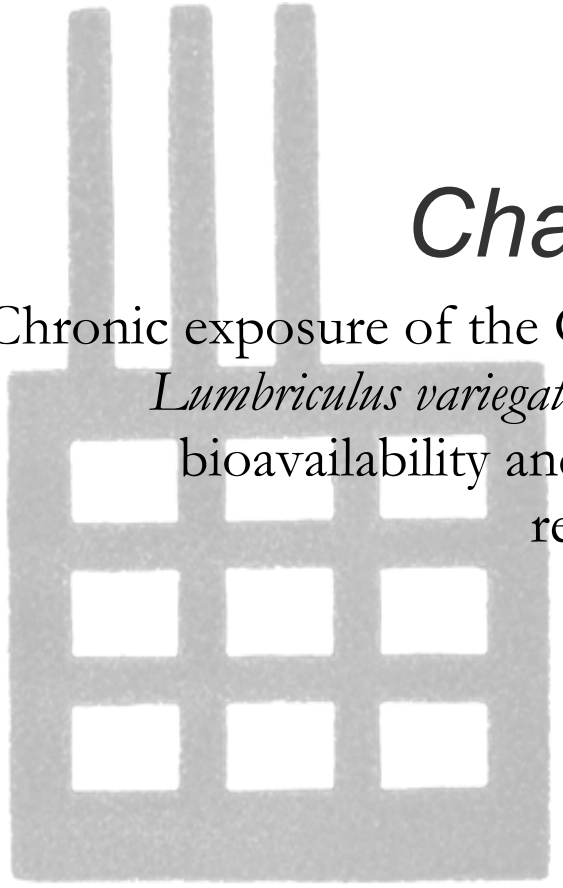
Publication date
2009
Document Version
Final published version



Chapter 4

Chronic exposure of the Oligochaete *Lumbriculus variegatus* to PACs: bioavailability and effects on reproduction

M. León Paumen, P. Stol, T.L. ter Laak, M.H.S. Kraak, C.A.M. van Gestel and Wim Admiraal, 2008. Environmental Science and Technology, 42, 3434-3440.

Abstract

This study aimed to monitor PAC availability to the oligochaete *Lumbriculus variegatus* during 28 days of exposure to spiked sediments, in order to obtain reliable chronic effect concentrations for reproduction. Sediment toxicity tests were performed using three pairs of PAC isomers: two homocyclic compounds (anthracene and phenanthrene), two azaarenes (acridine and phenanthridine) and the two main transformation products of the azaarenes (acridone and phenanthridone). During the experiment, available PAC concentrations in pore water (estimated using Solid Phase Microextraction) decreased more than total PAC concentrations in the sediment. Relating effect concentrations to PAC concentrations in pore water and in organisms showed that the two homocyclic compounds caused narcotic effects during chronic exposure, but only one of the four tested heterocyclic PACs caused narcotic effects. The transformation product phenanthridone was not toxic at the tested concentrations (up to 4000 μmol/kg dry sediment), whereas EC50 values for the parent compound phenanthridine and the isomer acridone were below the estimated limit for narcosis, suggesting a specific mode of action. These results demonstrated the unpredictable (isomer) specific toxicity of azaarenes and their transformation products, emphasizing the need of chronic toxicity testing to gain insight into the long-term effects of heterocyclic PACs, which have been overlooked in risk assessment.

Introduction

Benthic invertebrates inhabiting PAC contaminated sediments are chronically exposed to a variety of homocyclic and heterocyclic compounds (Lahr et al., 2003; Uhler et al., 2005). Risk assessment for PACs, however, is based on only a limited set of homocyclic compounds, ignoring the vast number of heterocyclic compounds (with in or on-ring substitutions) and transformation products (Crommetuijn et al., 2000). Moreover, in the past PAC toxicity has been commonly assessed in short-term high-dose experiments, in which mortality is often the only endpoint (Lee et al., 2002). During such acute exposures PACs act mainly by narcosis (Bleeker et al., 2002), but during chronic exposure the same compounds may exert sublethal effects (on e.g. growth, reproduction; Droge et al., 2006). Therefore, the present study focused on assessing chronic effects of azaarenes and their transformation products in addition to homocyclic compounds. Azaarenes are mainly formed and released into the environment by incomplete combustion of fossil fuels, in spills or effluents of several industrial activities and by pesticide use (Kuhn and Suflita, 1989). Azaarenes, containing one in-ring nitrogen substitution, can comprise up to 10% of the total PAC concentration in contaminated sites (Neilson, 1998), but toxicity data for this group of compounds are scarce, and very little is known about their chronic effects (Droge et al. 2006; Feldmannova et al. 2006).

Sorption to sediment organic matter may decrease availability of PACs to benthic invertebrates, since tissue residues and effects correlate to dissolved pore water concentrations (Moerond et al., 2007). Nevertheless, in chronic toxicity tests PAC bioavailability during exposure is rarely monitored, although changes in bioavailability could alter the outcome of the experiments (Schuler et al., 2003). Recently, several techniques have been developed to facilitate quantification of the freely dissolved (pore) water concentrations of toxicants (Mayer et al., 2000; Leppanen et al., 2006), including Solid Phase Microextraction (SPME) (King et al., 2003). The poly(dimethylsiloxane) coating on the SPME fiber mimics the structure and sorptive properties of membranes (Leslie et al., 2003), allowing a reliable estimation of the availability of organic compounds.

The aim of the present study was, therefore, to monitor PAC availability to the Oligochaete *Lumbriculus variegatus* during chronic exposure, in order to obtain reliable effect concentrations for reproduction. Asexual reproduction of *L. variegatus* is a sensitive chronic endpoint that can easily be combined with SPME and PAC accumulation measurements in the worms' body in a single test. This allowed us to express the observed effects as a function of measured PAC concentrations in

sediment, estimated PAC concentrations in pore water and PAC concentrations in the organism. Since it has been demonstrated that slight differences in chemical structure may result in substantial differences in toxicity (Kraak et al., 1997; Bleeker et al., 1999; Wiegman et al., 2001), three three-ring PAC isomer pairs were selected: two homocyclic compounds, two azaarenes and the two main stable transformation products of the azaarenes.

Materials and methods

Test organism

Lumbriculus variegatus is an endobenthic oligochaete, widely used in bioaccumulation experiments (Leppanen, 1998; Ingersoll, 2003; Schuler, 2003; Landrum, 2004). In the laboratory it reproduces via fragmentation, and a standardized protocol to determine effects on asexual reproduction of *L. variegatus* has been developed by the Organisation for Economic Co-operation and Development (OECD). This protocol, with slight modifications, was applied in the present study.

Test compounds

Six test compounds were chosen: two three-ring homocyclic compounds, anthracene and phenanthrene; two azaarene analogues, acridine and phenanthridine, and two Phase I azaarene transformation products, acridone and phenanthridone. Several properties of the compounds are listed in chapter 3. All compounds were provided by Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands).

Sediment spiking

Drontermeer sediment, a Dutch reference sediment (12-14% Organic Matter, 15.5% clay and all individual PAH concentrations below 0.01 mg/kg DW), was used for the toxicity tests. Sediment was provided by Grontmij/AquaSense (Amsterdam, the Netherlands). After sampling, it was homogenized and frozen to eliminate indigenous fauna (-20°C). Three days before the spiking procedure was started the sediment was thawed at 4°C.

The sediment was spiked to obtain the following nominal PAC concentrations: 67, 140, 280, 561 and 1122 μmol/kg DW for anthracene, 140, 280, 561, 1122 and 2244 μmol/kg DW for phenanthrene, 139, 279, 558, 1116 and 2232 μmol/kg DW for acridine and phenanthridine and 154, 318, 640, 1281 and 2561 μmol/kg DW for acridone and phenanthridone.

PACs were spiked to dry sediment (10% of the total amount). Acetone was used as carrier solvent, and for each PAC concentration series equal volumes of acetone were added to all treatments. The spiking was carried out in the dark to prevent

photodegradation of the compounds. Controls and solvent controls were included. The homocyclic compounds and the azaarenes were dissolved in 50 ml of acetone and added to 38 g of dry sediment, and the mixture was left in a closed 500 ml glass bottle for 24 h to equilibrate. The transformation products, less soluble in acetone, were dissolved in 300 ml of acetone. After the 24 h equilibration period, the bottles were opened and left for 24 h in a fume hood to allow the acetone to evaporate. Next, the spiked dry sediment was mixed with 800 ml of Dutch Standard Water (DSW, deionised water with 200 mg/l $CaCl_2.2H_2O$, 180 mg/l $MgSO_4.H_2O$, 100 mg/l $NaHCO_3$ and 20 mg/l $KHCO_3$; pH ≈ 8.2) and 432 g of wet sediment (20% water content) in 3-liter glass bottles. A 20:1 wt/wt mixture of Trouvit® and TetraPhyll®, diluted in distilled water, was used as additional food source (0.25 mg/worm/day). The glass bottles filled with the water-sediment-food mixture were placed on a roller bank (30 rpm) for 24 hours to homogenize. Next, the mixture was divided over eight replicate 200 ml glass jars and allowed to settle for 10 days. Each test replicate consisted of approximately 60 g of wet sediment and 100 ml of DSW. Four replicates per concentration were sacrificed during the experiment for PAC measurements in sediment and pore water, leaving four replicates for the toxicity test.

Toxicity tests

Four week (28-day) toxicity tests were performed in an incubator at a constant temperature of 20±1°C, with mercury lamps (light intensity approximately 50 μmol quanta/m^2/s) providing a light regime of 16 hours of light and 8 hours of darkness. UV filters were used to minimize photodegradation of the test compounds by UV-B radiation. Glass jars were covered with plastic foil and constantly aerated to keep oxygen concentrations stable. Once a week, de-ionized water was added to compensate for evaporation of the test solution. pH in the test jars ranged from 7.5 to 8.5 and oxygen concentration was always above 75% air saturation.

At the beginning of the experiment, 20 *L. variegatus* adult individuals from our laboratory culture were introduced in each of the test jars using a plastic Pasteur pipette. The worms were allowed to burrow into the sediment for 4 hours before aeration of the jars was restarted. After 28 days, the sediment was sieved to extract the worms from the sediment. The number of worms in each test jar was determined, and the average reproductive output per concentration (%) was calculated using the formula $Y=((X-20)/20)*100$, where X is the number of worms per jar at the end of the test. The worms were kept for four hours in clean tap water for gut clearance. This short gut clearance period was applied to prevent depletion of the heterocyclic compounds (Mount et al. 1999). Following, worm samples were frozen at -20°C until analysis.

PAC availability

Availability of anthracene, phenanthrene, acridine and phenanthridine in sediment pore water was measured using Solid Phase Microextraction (SPME). Due to their low hydrophobicity, SPME could not be applied to the transformation products. The fibers, obtained from the Institute for Risk Assessment Sciences (IRAS, University of Utrecht, the Netherlands), had a diameter of 110 µm and a 28.5 µm thick poly(dimethylsiloxane) (PDMS) coating, and were manufactured by Poly Micro Industries (Phoenix, AZ, USA). Three centimeter pieces of fiber were glued to a 0.4 mm Ø nylon fishing thread, washed with 70% methanol and de-ionized water and carefully introduced into the test sediment. Two replicate fibers were introduced in each test jar. Fiber measurements were performed in two jars per test concentration. The fibers remained in the sediment for three days, sufficient time for the fiber to reach equilibrium with the PCA concentrations in the pore water (Leslie et al., 2002; Ter Laak et al., 2006). Next, they were gently cleaned with a tissue, and a 2 centimeter fiber piece was cut to get rid of the glued tip of the fiber and extracted in 300 µl acetonitrile. For each toxicity test, fibers were introduced in the sediment at the beginning and at days 3, 6, 9, 13, 17, 21 and 25 of the experiment. PAC concentrations in the fibers were measured by High Performance Liquid Chromatography (HPLC), using a 1 ml/min 80% acetonitril-20% water flow rate and an injection volume of 20 µl. For the homocyclic compounds, fiber-water partition coefficients (K_f) from Ter Laak et al. (Ter Laak et al., 2006) were used to estimate concentrations in the pore water using the SPME measurements. For the azaarenes, static system experiments were performed to determine K_f values, as in (Ter Laak et al., 2005).

PAC concentrations in sediment, pore water and oligochaetes

Sediment and pore water samples were collected at the beginning, after seven and fourteen days and at the end of the experiment. For sediment samples, 40 ml was centrifuged for 15 minutes at 3000 rpm. To obtain a clear pore water sample, the supernatant was collected and centrifuged again for 15 minutes at 3000 rpm. 150 µl of acetonitrile was added to two replicated 1 ml pore water samples. Sediment and pore water samples were frozen at -20°C until analysis.

Ten grams of centrifuged sediment (water content approximately 10%) were mixed with equal amounts of anhydrous $NaSO_4$ (Merck, Darmstadt, Germany), and Soxhlet extracted for 5 hours in cellulose extraction thimbles. Hexane (Chromasolv®, Sigma-Aldrich Chemie BV) was used as extraction solvent for the homocyclic compounds and the azaarenes, acetonitrile (HPLC-grade, Biosolve BV) for the transformation products. The hexane-extracted samples were transferred into

acetonitrile by blowing off the hexane using a gentle stream of nitrogen. All extracts were analyzed by HPLC. Recovery checks were performed for each of the compounds to validate the Soxhlet extraction procedure. For this purpose, known amounts of spiking solution in the same concentration range as tested in the experiments were added to clean sediment in the extraction thimble. Extraction recoveries were 80% for anthracene, 85% for phenanthrene, 75% for acridine, 77% for phenanthridine and 88% for the transformation products. PAC concentrations in the sediment were corrected for recovery. Pore water samples were directly injected in the HPLC, to prevent overestimation of freely dissolved pore water concentrations due to exhaustive extraction of the DOM bound fraction of the compounds (Ter Laak et al., 2005).

L. variegatus tissue was sampled at the end of the experiment (28 days). Worms from the 4 replicates per concentration were pooled in order to obtain enough biomass for a reliable PAC measurement. PACs were extracted from the tissue with acetonitrile. One hundred milligrams of wet tissue were mixed with 1.5 ml of a 1% $CaCl_2$ solution and homogenized to break down the tissue. Three ml of acetonitrile were added, and the mixture was sonicated for 45 minutes in cool water (approximately 12ºC). Next, the PACs were extracted from the tissue at 4ºC for 24 hours. After this first extraction, the sample was centrifuged for 15 minutes at 3000 rpm and the supernatant was transferred to a HPLC vial. Three ml of acetonitrile was added to the pellet, the sample was homogenized and the extraction procedure was repeated. The two worm tissue extracts were pooled and measured using HPLC. PAC concentrations in the tissue were corrected for recovery. Extraction recoveries were 70% for anthracene, 72% for phenanthrene, 65% for acridine, 67% for phenanthridine, 68% for acridone and 72% for phenanthridone.

Statistical analysis

The concentration of the test compounds in sediment, pore water and worm tissue that caused 50% reduction in reproduction compared to the control was calculated according to Haanstra et al. (1985). Average measured PAC concentrations in the sediment, average estimated PAC concentrations in the pore water and PAC concentrations in the worms at the end of the 28-day exposure period were used. T-tests ($p<0.05$) were performed to compare control and acetone control reproductive outputs. Likelihood ratio tests were performed to compare effect concentrations ($X^2_1>3.84$, $p<0.05$). Statistical analysis was performed using SPSS® 11.0 for Windows.

Results

PAC concentrations in sediment, pore water and L. variegatus

Concentrations of anthracene, acridine and acridone in the sediment did not decrease substantially during the experiment (respectively 3±2, 3±3 and 0%). In contrast, concentrations of phenanthrene, phenanthridine and phenanthridone in the sediment decreased during the experiment, mainly due to microbial degradation (*27,28*). Average decreases were 19±10% for phenanthrene, 30±16% for phenanthridine and 24±12% for phenanthridone.

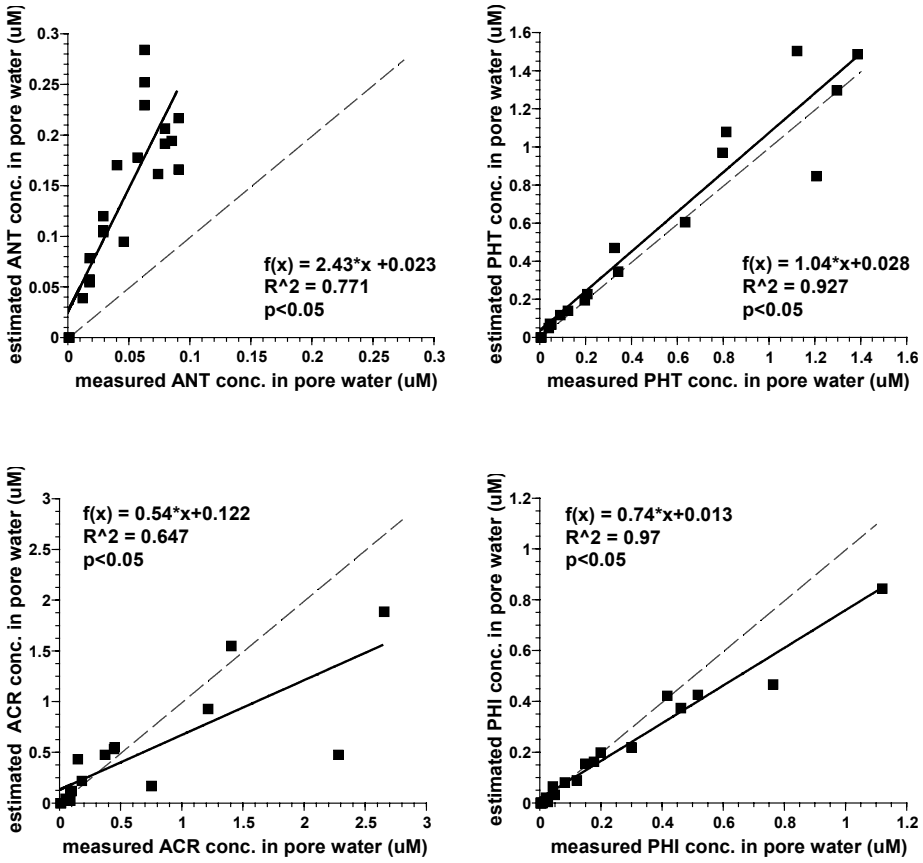


Figure 1. Relationship between Polycyclic Aromatic Compound (PAC) concentrations in pore water estimated using SPME measurements (Y-axis, µM) and measured experimentally (X-axis, µM) in spiked Drontermeer sediment during 28-day toxicity tests with *Lumbriculus variegatus*. Dashed line: 1:1 relationship.

PAC concentrations in pore water were estimated using the concentrations in SPME fibers and PDMS-water partitioning coefficients (K_f). In Figure 1, estimated concentrations (Y axis) were plotted against directly measured PAC concentrations in pore water (X axis), and a linear regression was performed in order to evaluate the performance of the SPME technique. The r^2 values of the linear regression for anthracene, phenanthrene and phenanthridine were respectively 0.77, 0.92 and 0.97, showing a consistent linear relationship between measured and estimated PAC concentrations in pore water. For acridine, the r^2 value was 0.64, due to variability in the results obtained with SPME. If direct measurements in pore water would be equivalent to concentrations in pore water estimated using SPME, the slope of the regression line should be 1. This was indeed the case for phenanthrene (1.07, 95% C.I. 0.89-1.19), but for anthracene the slope was significantly higher than 1 (2.43, 95% C.I. 1.85-3.02), meaning that the measured anthracene concentrations in the pore water were lower than estimated ones. For the two azaarenes, acridine and phenanthridine, the slope of the regression line was significantly lower than 1 (respectively 0.54 (95% C.I. 0.32-0.75) and 0.72 (95% C.I. 0.60-0.84)), meaning that estimated concentrations were somewhat lower than concentrations measured in pore water.

During exposure, estimated pore water concentrations of all PACs decreased at all concentrations in the sediment, due to increased sediment sorption in time and degradation by bacteria present in the sediment (van Herwijnen, 2003). The decrease in estimated pore water concentrations was larger in proportion than the decrease in measured concentrations in the sediment.

In general, average PAC concentrations in the worms' tissue (µmol PAC/kg wet weight) after 28 days of exposure increased with increasing PAC concentrations in the sediment, but differences in accumulation between the six tested compounds were found.

Effect concentrations

Control reproductive output after 28 days of exposure was on average 80 ± 9 %. Control and solvent control average reproductive output did not differ significantly (T-test, $p<0.05$) and therefore solvent control values were used for further analysis of the results. All tested compounds except phenanthridone affected reproduction of *L. variegatus*. In Table 1, 50 and 10% effect concentrations (EC50 and EC10s) with their 95% confidence intervals are shown. Likelihood ratio tests were performed to compare the EC50 values.

Table 1. EC50 and EC10 values (with corresponding 95% confidence intervals) for the effects of Polycyclic Aromatic Compounds (PACs) on the reproduction of *Lumbriculus variegatus* in spiked Drontermeer sediments, calculated using average measured PAC concentrations in the sediment (µmol/kg DW), pore water PAC concentrations estimated using SPME (µM) and PAC concentrations in *L. variegatus* at the end of the 28-day exposure period (µmol/kg ww).

		sediment effect concentration (µmol/kg DW) with 95% C.I.		**SPME porewater effect concentration (µM) with 95% C.I.**		**body residue effect concentration (µmol/kg ww) with 95% C.I.**	
		EC50	**EC10**	**EC50**	**EC10**	**ER50**	**ER10**
Homocyclic compounds	anthracene	532 (0-1075)	17 (0-529)	0.15 (0.064-0.246)	0.025 (0-0.08)	116 (43-210)	111 (15.16-207)
	phenanthrene	467 (285-650)	187 (17-358)	0.299 (0.15-0.45)	0.07 (0-0.4)	88 (72-105)	84 (69-99)
Azaarenes	acridine	1248 (315-2182)	197 (0-1076)	3.97 (0.47-7.46)	0.45 (0-3.9)	137 (93-181)	132 (90-173)
	phenanthridine	315 (65-565)	37 (0-272)	0.148 (0.08-0.22)	0.04 (0-0.45)	2 (0.2-2.7)	0.67 (0-2.21)
Transform. products	acridone	301 (240-363)	138 (80-196)	0.46* (0.37-0.56)	-	30 (25-36))	25 (20-30)
	phenanthridone	>4014	>4014	>5.77	>5.77	>331	>331

*EC50 for acridone calculated like in Droge et al. (2006)

EC50s based on measured PAC concentrations in sediment. From the compounds that exerted an effect, acridone was the most toxic (EC50 301 μmol/kg sediment DW), while its parent compound acridine was the least toxic (EC50 1248 μmol/kg sediment). Likelihood ratio tests showed that the sediment EC50 for acridine was significantly higher than EC50s for phenanthridine, the homocyclic compound anthracene and the transformation product acridone. In contrast, EC50s for anthracene, phenanthrene, phenanthridine and acridone did not differ from each other.

EC50s based on PAC concentrations in pore water. Pore water EC50s were calculated using estimated PAC concentrations in the pore water for the homocyclic compounds and the azaarenes. For acridone, the pore water EC50 was estimated from the sediment EC50 using its sediment-water partitioning coefficient (K_{oc}) as in Droge et al. (2006). Pore water EC50s ranged from 0.148 μM for phenanthridine to 3.97 μM for acridine, and again likelihood ratio tests showed that the EC50 for acridine was significantly higher than the EC50s for the other compounds. EC50s for anthracene, phenanthrene, phenanthridine and acridone did not differ significantly.

EC50s based on PAC concentrations in the organism. 50% effect body residues (ER50s) based on wet weight were calculated. Likelihood ratio tests showed that the ER50s for the two homocyclic compounds, anthracene and phenanthrene, did not differ significantly (116 and 88 μmol/kg WW, respectively). The ER50 for the azaarene acridine (137 μmol/kg wet weight) was similar to the homocyclic compound ER50s. In contrast, phenanthridine showed the significantly lowest ER50 (2 μmol/kg wet weight). The transformation product acridone showed a significantly lower ER50 than the homocyclic compounds and acridine (30 μmol/kg WW).

Discussion

PAC availability to L. variegatus

To our knowledge, this was the first attempt to use SPME to monitor the change in PAC availability in pore water during chronic toxicity tests. Indeed, the decrease in freely dissolved PAC concentrations in pore water during exposure time was substantial, according to previous observations on progressive sorption of PACs to particles (Kukkonen et al., 1994; Conrad et al., 2002). Even more important, the present study showed that the freely dissolved PAC concentrations in pore water decreased more than total PAC concentrations in the sediment, emphasizing the importance of monitoring availability during chronic exposure.

For the two azaarenes, estimated concentrations using SPME were somewhat lower than directly measured concentrations in the pore water. This may have been caused by an slight underestimation of PDMS-water partitioning coefficients (K_f) due to depletion in the static experimental system in which K_f values were determined, as suggested by Poerschmann et al. (2000). For anthracene, in contrast, concentrations measured directly in the pore water were 2.4 times lower than estimated pore water concentrations using SPME measurements. Due to the low water solubility of anthracene, binding of anthracene to particulate and dissolved organic carbon in the sediment pore water probably occurred during the pore water analysis (Ter Laak et al., 2005). As a result, very low concentrations were measured. Therefore, pore water concentrations estimated using SPME measurements were more reliable, and were used to calculate effect concentrations.

Effect concentrations

Effects based on measured PAC concentrations in the sediment (Figure 2A). Due to equilibrium partitioning, toxic effects of PACs will occur at lower concentrations in the (pore) water with increasing logK_{ow} of the test compounds (Lee et al., 2001). On the other hand, sorption of the test compounds to sediment organic matter increases with increasing logK_{ow} (Ter Laak et al., 2006). Because of these two counteracting influences, sediment EC50s for our test compounds were expected to be more or less constant (Van Leeuwen et al., 1992). This was indeed the case for the homocyclic compounds (anthracene and phenanthrene), the azaarenes (acridine and phenanthridine) and the transformation product acridone. The transformation product phenanthridone was the only compound that did not affect reproduction of *L. variegatus* at the tested concentrations (up to 4000 µmol/kg dry sediment). Sediment EC50s were in the range of effect concentrations determined for other benthic invertebrates exposed to PAH-contaminated sediments (Lotufo et al., 1998; Verrhiest et al., 2001; León Paumen et al., 2008).

Effects based on PAC concentrations in the pore water (Figure 2B). In previous studies (Droge et al., 2006; León Paumen et al., 2008), estimated chronic pore water effect concentrations for soil and sediment invertebrates were compared to an acute LC50-logK_{ow} relationship determined by Bleeker et al. (2002), which described the narcotic effects of several PACs to the midge *Chironomus riparius*. This way, effects of homo and heterocyclic compounds were compared, and specific effects emerging besides narcosis during chronic exposure were identified. Applying this approach to the present data set revealed that pore water EC50s for the homocyclic compounds anthracene and phenanthrene agreed well with the acute LC50-logK_{ow} relationship. From the four tested heterocyclic compounds, however, only the EC50 for acridine

was in agreement with the acute LC50-logK_{ow} relationship. The transformation product phenanthridone did not affect reproduction of *L. variegatus*. Based on its logK_{ow} value an EC50 of about 20 µM was expected, but this concentration in the pore water was not reached at the highest concentration in the sediment. In contrast, the EC50s for the azaarene phenanthridine and the transformation product acridone were clearly below the concentration corresponding to narcosis, suggesting a specific mode of action.

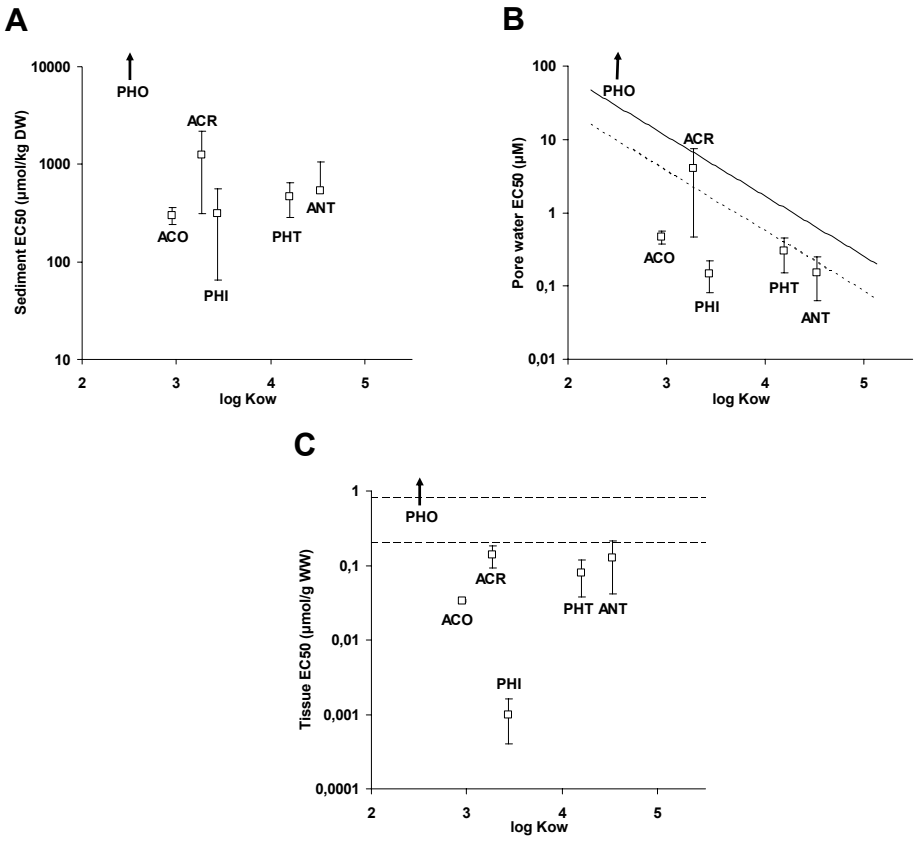


Figure 2. EC50 values for the effect of Polycyclic Aromatic Compounds (PACs) on reproduction of *Lumbriculus variegatus* in spiked Drontermeer sediment as a function of logK_{ow} (□); based on measured concentrations in sediment (µmol/kg DW, plot A), estimated concentrations in pore water (µM, plot B) and concentrations in *L. variegatus* (ER50s, µmol/g WW, plot C). Plot B: Solid line: linear logLC50-logK_{ow} relationship for *Chironomus riparius* exposed for 96 hours to similar PACs (y = -0.8162x + 3.4936, r^2=0.986, (Bleeker et al., 2002)). Dashed line: ratio LC50 from Bleeker et al. 2002 / 3, lower limit for narcosis. For acridone, the calculated pore water EC50 is shown. Plot C: dashed lines: according to CBR predictions, upper and lower limits of the tissue concentration interval after chronic exposure to narcotic chemicals (0.2 - 0.8 µmol/g WW).

The specific effects found for the azaarene phenanthridine compared to the narcotic effects of its isomer acridine were observed previously for reproduction of the soil invertebrates *Folsomia candida* and *Enchytraeus crypticus* (Droge et al., 2006). Moreover, phenanthridine was found to be more teratogenic than acridine to *Xenopus laevis* (Buryskova et al., 2006), and more genotoxic in the GFP assay (Bartos et al., 2006). Isomer specific toxicity was also found for the transformation product isomers: acridone was more toxic than expected based on logK_{ow}, as found previously in a mutagenicity study (Bleeker et al., 1999), while phenanthridone did not affect reproduction of *L. variegatus* at all at the highest concentration tested. In contrast, both transformation products affected emergence of *C. riparius* (León Paumen et al., 2008), underlining the species specific sensitivity to PACs (Droge et al., 2006).

Effects based on PAC concentrations in the organism (Figure 2C). Toxic effects can only take place if the toxicant is taken up by the organism. Therefore, we also expressed effects as a function of PAC concentrations in the organism. This allowed us to evaluate the observed deviations from narcosis in a second independent way, using the Critical Body Residue (CBR) approach. The CBR concept (McCarthy and Mackay, 1993) predicts that non polar narcotics (e.g. PACs, PCBs) cause 50% of mortality at threshold body concentrations between 2 and 8 μmol/g wet weight. This concept has been successfully applied to interpret toxicity data for sediment and soil inhabiting organisms (Fischer et al., 1999; Lee et al., 2002; Leslie et al., 2004b; Landrum et al., 2003) . Because of the unspecificity of the narcotic mode of action, it has been suggested that this threshold would be applicable to all organisms. Furthermore, analyses of chronic toxicity data suggest that threshold body concentrations for chronic exposure would be one order of magnitude lower than lethal CBRs (between 0.2 and 0.8 μmol/g wet weight; Hendriks, 1995; Lotufo et al., 1998; DiToro et al., 2000), and body concentrations far below the threshold would indicate specific modes of action of the tested compounds occurring besides narcosis (Emery and Dillon, 1996). In our study, ER50s for anthracene, phenanthrene and acridine were around one order of magnitude lower than 2-8 μmol/g wet weight, in agreement with the chronic CBR concept. Also, ER50s for the two homocyclic compounds were close (within a factor 2) to body residue predictions for chronic exposure to homocyclic PAHs (DiToro et al., 2000). In contrast, ER50s for phenanthridine and acridone were two orders of magnitude lower than the chronic CBR range, suggesting additional specific modes of action of the two compounds. Thus, deviations from narcosis found for the ER50s were in agreement with deviations from narcosis found for the pore water EC50s.

In this study, only one of the four tested heterocyclic PACs caused narcotic effects during chronic exposure, showing that (isomer) specific toxicity of azaarenes and their transformation products could not be predicted from acute effect concentrations. About two-thirds of the approximately four million known organic compounds are heterocyclic (Adrian and Suflita, 1994), and in our study toxicity of three out of the four tested heterocycles was poorly predicted by logK_{ow}. These results emphasize the need of applying chronic toxicity testing to gain insight in the long-term effects of heterocyclic PACs, which have been overlooked by the risk assessment.

Acnowledgements. This research was supported by the Technology Foundation (STW), applied science division of The Netherlands Organization for Scientific Research (NWO) (project AEB 06364).