

APPLIED SOIL ECOLOGY

Effects of phenanthrene and its nitrogen-heterocyclic analogues aged in soil on the earthworm *Eisenia fetida*

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Abstract

Major sources of homocyclic polycyclic aromatic hydrocarbons (PAHs) and N-containing analogues (N-PAHs) are found in the environment after being discharged through petrogenic, pyrogenic and biogenic activities. Using a health index tool and the OECD guideline 207, the ecotoxicity of phenanthrene and its nitrogen-containing analogues to the earthworm *Eisenia fetida* were assessed in agricultural soil at different times after spiking. The effects were measured over a 21 d exposure period (over time), during which earthworms' general health condition, mortality and biomass were assessed. The LC₅₀ and EC₅₀ (based on weight loss) ranged from 404 - >500 mg kg⁻¹ dry soil and 1.2 – 500 mg kg⁻¹ dry soil, respectively. The N-PAHs were more toxic to *E. fetida* and over time more available in soil than the homocyclic-phenanthrene analogue. Benzo[h]quinoline (B[h]Q) was the most toxic and persistent of the chemicals. Furthermore, the observed physical damages to the earthworms showed that N-PAHs, especially B[h]Q, may have cellular autolytic impact on *E. fetida*. These findings offer new insight on the toxicity of aromatics in soil which might be useful in risk assessment of contaminated sites.

Keywords: N-PAHs, phenanthrene, ecotoxicity, health index, earthworms, soil.

1. Introduction

The presence of organic contaminants (such as poly-aromatic hydrocarbons) in the soil environment is of great concern due to their toxic effects on the invertebrate populations and their capacity to be taken up by biota and transferred up through the food chain. Effective protection of an ecosystem therefore requires knowledge of the ecotoxicity of chemicals to soil organisms (EC, 2012). Phenanthrene and its nitrogen-containing analogues consist of three aromatic rings (carbon and/or nitrogen) and are ubiquitous in the environment (USEPA, 2005; EC, 2011; Anyanwu and Semple, 2015a; b; c). The poly-aromatic compounds are known carcinogens, mutagens, teratogens and genotoxins (Vo-Dinh et al., 1998; EC, 2011; IARC, 2012). Irrespective of this, only the impacts of polycyclic aromatic hydrocarbons (PAHs) are well reported. However, the effects of the nitrogen-containing polycyclic aromatic hydrocarbons (N-PAHs) on soil biota and their chemical recovery (extractability) from soil at varying concentrations are less well studied. The nitrogen-containing polycyclic aromatic hydrocarbons (N-PAHs) are produced under similar circumstances as homocyclic PAHs (petrogenic, pyrogenic, biogenic) (Kaiser et al., 1996; Švábenský et al., 2009; IARC, 2012), but they display greater biological effects (Sverdrup et al., 2002; Kobeticova et al., 2008). It is now recognised that not only the homocyclic PAHs, but also heterocyclic PAHs (such as N-PAHs) contribute to the toxic effects identified in contaminated sites (Vo-Dinh et al., 1998). From both toxicological and epidemiological studies, many N-PAHs have been shown to be highly toxic (EC, 2011; Schová et al., 2007; Anyanwu and Semple, 2015a; b; c), bioaccumulative (Southworth et al., 1979), and capable of biomagnification within the food chain. As a result, their risk assessment is important for contaminated environments. Furthermore, despite the potential ecotoxicological effects of N-PAHs, there is paucity of data on their acute toxicity and/or consequences of toxicity to soil biota and LC_{50} and EC_{50} values have been determined only for a few chemicals (acridine, carbazole, phenazine, phenanthridine and 1,10-phenanthroline) (Sverdrup et al., 2002; Droge et al., 2006; Kobetičová et al., 2008; 2011).

Soil represents a major sink for organic contaminants in the environment and the extent to which chemicals are available for uptake will be relevant of their impacts on soil biota. In soil, biota (such as earthworms) is known to interact with a specific fraction of a particular contaminant (by ingestion or absorption across the body wall). This has been termed the bioavailable fraction and is dependent on the earthworm physiology, behaviour, route of exposure, time of exposure and the properties of the matrix containing the chemical (Anderson et al., 1999; Lanno et al., 2004; Semple et al., 2004).

While the impacts of PAHs on terrestrial and aquatic organisms have been extensively studied because early interest was devoted to carcinogenic activities, there is paucity of data on the effects of the N-PAHs in contaminated agricultural soil. Information on the toxicity of N-PAHs is of great importance for: (i) an effective assessment of their effects on soil biota, (ii) identification of parameters affecting the contaminant behaviour, and (iii) for risk assessment and regulation of new and existing chemicals. Moreover, assessing effects of N-PAHs based on their impacts instead of total concentration provides a more refined basis for risk management decisions. Therefore, the aims of this study were to elucidate the effects and consequences of toxicity of phenanthrene and its four nitrogen heterocyclic analogues aged in soil on the survival, biomass and health index of earthworms, using OECD guideline 207 (with little deviations) and a health indices tool (0 = dead, 1 = moribund and 2 = alive). Further, the concentrations of the chemicals were measured over the ageing period to determine their recovery / extractability in soil.

2. Materials and methods

2.1. Chemicals

Phenanthrene (Phen), 1,10-phenanthroline (1,10-Phen), 1,7-phenanthroline (1,7-Phen), 4,7-phenanthroline (4,7-Phen) and benzo[h]quinoline (B[h]Q) were purchased from Sigma-Aldrich Company Ltd, UK (Table 1). Acridine D₉ was purchased from Cambridge Isotopes Laboratories, UK.

2.2. Test organisms

Mature earthworms (*Eisenia fetida*) were purchased from Blades Biological Ltd, UK.

2.3. Soil preparation

Soil from Myerscough Agricultural College, Lancashire, UK was prepared for the test. The soil was collected from the top layer of field under pasture from a depth of approximately 5 – 20 cm. The soil was sandy-loam (19.5% clay, 60.4% sand, 20.0% silt), with organic matter content of 2.7% and pH-H₂O of 6.5 (Doick et al., 2003). The soil was air dried at room temperature, sieved with 2 mm mesh size and rehydrated back to 57% of the water holding capacity (WHC) with deionised water. Spiking procedure followed that described in Doick et al. (2003). One-third of the amount of soil (333 g) needed per treatment was placed in a bowl and spiked with

phenanthrene and N-PAH standards dissolved in acetone (10 ml) to give concentrations of 10, 100, 250 and 500 mg kg⁻¹ dry soil, respectively. The soils were left for 4 h (in a fume hood) to enable carrier solvent volatilization, after which soils were mixed with the remaining two-third (668 g) soil. Control samples (0 mg kg⁻¹) were prepared using soils amended with acetone only to serve as control (preliminary test showed no significant difference between negative control and acetone control). The spiked and un-spiked soils were incubated in half closed amber glass jars (to allow air passage, and avoid photo-degradation) and aged for 0, 30, 60 and 90 d at 21 ± 1°C (to determine chemical extractability over time). Soil moisture content was regularly checked and lost water was replenished accordingly with deionized water. After each ageing time, an earthworm bioassay was carried out, exposing the earthworms for 21 d. Soils were chemically analysed at the beginning of each earthworm test with GC-MS.

2.4. Earthworm bioassay

The effects of phenanthrene and its four nitrogen-containing analogues were determined using the guideline described in OECD 207 (1984) with little deviations on test duration, temperature and light condition (to avoid photo-degradation of chemicals). Mature earthworms (*E. fetida*) (0.3 g - 0.4 g) were selected for the study. Prior to the 21 d incubations, earthworms were left in Petri dishes with moist filter paper and allowed to depurate for 24 h, after which they were weighed. Soils (50 g) were weighed into amber glass bottles (3 cm x 3 cm) with perforated lids. Five replicates of one worm each were used for each concentration (0, 10, 100, 250 and 500 mg kg⁻¹ dry soil). Water was added to moisten the soil to 80% of the WHC; mixed thoroughly and a single earthworm was added to each replicate (without food) and incubated in the dark at 12 ± 2°C (preliminary tests showed that at 20 ± 2°C, the soil moisture content was rapidly reduced after a few hours, leading to death of the exposed organisms). Mortality, biomass and health index were the endpoints determined in the tests. Assessment of the health (condition) index (general physical condition) was carried out after 0, 1, 3, 5, 7, 14 and 21 d of exposure, with 0 = dead, 1 = moribund and 2 = alive. Earthworms were presumed dead if no contractile response was observed when prodded lightly with a blunt probe; moribund when weak contractile response was observed and alive when a sharp and vigorous response was observed (Langdon et al., 1999). The earthworms were weighed before and after exposure (after depurating for 24 h on moist filter paper) to determine changes in weight. Physical changes on different parts of the earthworms were also recorded.

2.5. Chemical analysis

Samples of each amended soil (5 g, n=3) were mixed with 5 g Na₂SO₄ in glass centrifuge tubes (35 ml) and 25 ml acetonitrile/methanol (80:20) was added to each tube. The tubes were sealed and placed horizontally on their sides on an orbital shaker (Janke and Kunkel, IKA[®]-Labortechnik KS 250) and shaken at 150 rpm for 20 h. The tubes were centrifuged at 800 rpm for 20 min (AccuSpin[™] 1, Fisher Scientific) and 5 ml of the supernatants were taken and concentrated to 1ml. The extracts were cleaned-up using a 5 mm column containing 3.5 g of 2 % water deactivated aluminium-oxide topped with 1 g of Na₂SO₄. Elution was with 30 ml ACN/MeOH (8:2). The eluents were evaporated, solvent-exchanged with ethyl-acetate and concentrated to 1 ml under nitrogen gas.

Analysis was with the ThermoQuest Trace GC Finningan Trace MS with CP-Sil 8CB operating under full scan, using 50 m x 0.25 mm, 0.12µm, Varian column. The oven temperature was programmed for 70°C (2 min); 10°C min⁻¹ to 150°C, 4°C min⁻¹ to 250°C, held for 10 min. Injector type was SSL, splitless injection of 1µl was performed with a 7 min delay period. The source temperature was 250°C, ion source EI⁺; MS interface temperature was 300°C and the electron energy was > 70 eV. Emission current was 300 µA and resolution was 1 amu. The mass range (m z⁻¹) was 120 – 300 and the scans rate was 2.5 scan sec⁻¹. Quantification was performed on the absolute calibration curve method in the range of 1 – 8 µg ml⁻¹, and a deuterated standard of acridine-D₉ (2 µg ml⁻¹) was used as internal standard. The instrument detection limits (IDLs) were estimated to be 3 x the matrix-matched calibration curve (where the peak had signal-to-noise = 3) and ranged from 0.8 – 5.0 µg ml⁻¹. Sample concentrations were calculated from the peak area ratio between the sample and internal standard. Data for standard calibration curves and their percentage recoveries were determined. All the chemicals showed linearity in the standard calibration curves (R² ranged from 0.993 – 0.997) and the average recoveries (%) were > 99%.

2.6. Statistical analysis

The weight changes of earthworms for each replicate (over time) were used as a measure of biomass-change. Concentrations that caused weight reductions as compared to the acetone control values were calculated on the basis of initial measurements and ANOVA was used to determine the significance of the effect. Estimations of the concentrations that caused 50% reduction on survival (LC₅₀) and decrease in weight (EC₅₀) were determined using probit analysis in SPSS 19 software package. Chemical recoveries were determined using the formula: $X_2 \div X_1 \times 100$. Where X₂ = final concentration and X₁ = initial concentration. Graphs were plotted using Sigma Plot 10.0 version.

3. Results

3.1. Recovery of the aromatic compounds from soil over time

Fig. 1 shows the % recoveries of phenanthrene and its N-PAH analogues aged in soil over different periods up to 90 d. The test chemicals had concentrations below the detection limits in control samples. Concentrations of 1,10-Phenanthroline unfortunately could not be determined. Increase in soil contact time (ageing time) reduced the extractability and/or % recovery of the aromatics from the test soil (Fig. 1). The concentrations of phenanthrene in the amended soils decreased more rapidly than those of the N-PAHs with increased contact time. For example, at 90 d, Phen recovery was <10% (10 mg kg^{-1}) while >30% (10 mg kg^{-1}) was measured for the N-PAHs with the exception of 4,7-Phen which recorded <20% (10 mg kg^{-1}). The N-PAHs recorded higher recovery (and/or extractability) values than phenanthrene during the 90 d soil-contact time (Fig. 1). Chemical losses were more pronounced at the lower (10 and 100 mg kg^{-1}) than at the higher concentrations (250 mg kg^{-1} and 500 mg kg^{-1}), with Phen and 4,7-Phen being readily removed or degraded (Fig 1). B[h]Q was the most persistent chemical over the course of the ageing (Fig 1).

3.2. Effects of the aromatic compounds on the health (condition) index of *E. fetida* in soil over time

The assessment of the earthworms' condition after exposure for 21 d in differentially aged (0 – 90 d) soils, spiked with chemicals at different concentrations, is shown in Fig. 2. No mortality or weight losses were observed in the control soils during a 21 d exposure period to soils aged for different periods of time. The 21 d assessments (over time) showed that health effects as a result of toxicity were significant at the highest concentration (500 mg kg^{-1}) ($p < 0.05$). The 0 – 90 d (ageing) health indices showed that N-PAHs most affected the health condition of *E. fetida* in soil over the ageing time ($p < 0.05$). *E. fetida* responded differently to individual compounds. For example, during the 21 d exposure, earthworms became moribund after a few hours of exposure and died within 3 d in the 500 mg kg^{-1} B[h]Q amended soils ($p < 0.05$), while the homocyclic PAH affected earthworm condition only at time point 0 (0 d) (Fig. 2). Among the N-PAHs, 1,7-Phen and B[h]Q significantly affected the general wellbeing of *E. fetida* during the exposures over time ($p < 0.05$). No effects were observed on the health condition of earthworms exposed to 1,10-Phen and 4,7-Phen amended soils during the 21 d assessments, over time (Fig. 2). In soils spiked with 1,7-Phen and B[h]Q physical changes, such as breakage of the clitellum, bleeding from the oral region, secretion of yellowish fluids, body lesions, body ruptures and body discolouration, were observed.

3.3. Effects of the aromatic compounds on the survival of *E. fetida* in soil over time

The survival of *E. fetida* following exposure for 21d to increasing concentrations (10, 100, 250 and 500 mg kg⁻¹) of the aromatic compounds in differentially aged (0 – 90 d) spiked soils is shown in Fig. 3. The aromatics caused 100% mortality of *E. fetida* at the highest concentration (500 mg kg⁻¹ dry soil) at time point 0 (0 d) with the exception of 1,10-Phen and 4,7-Phen (Fig. 3). In the Phen amendments, there was a noticeable effect on the survival of *E. fetida* only at time point 0 (0 d), while a dose-and time-related trend of effects were recorded in the N-PAH amended soils over time. The LC₅₀ values (over time) showed persistent N-PAHs toxicity to *E. fetida*, while phenanthrene toxicity decreased after time 0 (0 d) (Table 2). Furthermore, LC₅₀ values for the N-PAHs were quite similar over the 0 – 90 d ageing period (Table 2). Among the N-PAHs, B[h]Q amendments (500 mg kg⁻¹) resulted in 100% mortality throughout the incubations, over time and thus, was ascribed the most toxic chemical while 1,10-Phen and 4,7-Phen showed no mortality and were the least toxic chemicals.

3.4. Effects of the aromatic compounds on the biomass of *E. fetida* in soil over time

The EC₅₀ values for the effect on weight change of *E. fetida* during the 21 d exposure in differentially aged (0 – 90 d) soils spiked with polycyclic aromatic compounds at different concentrations are shown in Table 2. The weight changes of the exposed earthworms are shown in Fig. 4. The weights of dead earthworms were not measured and thus not included. *E. fetida* showed considerable reductions in weight with increasing chemical concentrations compared with the control soils. The mean weight-change values showed substantial decreases in earthworm weights over time (Fig. 4). For example, in N-PAH amended soils, *E. fetida* showed a trend of consistent decline in weight at all test concentrations over the ageing time and, weight losses were even observed for earthworms incubated at the lowest concentration (10 mg kg⁻¹ dry soil). However, in phenanthrene amended soil *E. fetida* experienced little or no weight-change with increasing soil-contact time (ageing time) (Fig. 4). Although 4,7-Phen and 1,10-Phen caused no mortality, they did induce substantial decreases in weight of the exposed earthworms over time. ANOVA showed statistically significant weight-effects of N-PAHs on *E. fetida* over time (p<0.05). Although phenanthrene amendments affected the weight of the exposed earthworms, ANOVA revealed that this effect was not statistically significant with ageing (p>0.05). Hence, the concentration-weight effects identified phenanthrene as the least toxic chemical with ageing (Fig. 4). Earthworms exposed to B[h]Q amended soils showed the greatest weight losses, mortality and health effects.

Thus, the toxicity to *E. fetida* in this study (with ageing) decreased in the order: B[h]Q > 1,7-Phen > Phen > 4,7-Phen > 1,10-Phen.

4. Discussion

The recoveries and impacts of phenanthrene and its nitrogen-containing analogues in differentially aged soils, spiked at different concentrations were measured over time. Although chemical analysis alone may not be sufficient for toxicity assessment (Fernández et al., 2005), it is still essential in toxicity assays. From the findings in this study, increase in soil contact time (ageing) resulted in chemical losses and reduction in the recoveries of the aromatics from soil, especially phenanthrene. Sorption, biodegradation, and/or losses due to ageing may be responsible for the decrease in recovery. This phenomenon is in agreement with numerous studies. For example, a rapid loss of PAHs in soil has been reported in PAH-contaminated matrices (Meyer and Steinhart, 2000; Rhodes et al., 2008; Anyanwu and Semple, 2015b). Similarly, Semple et al. (2004) and Lanno et al. (2004) reported that organic contaminants are known to interact with specific soil constituents in a dynamic manner over time, resulting in the sequestration of a portion of the chemical thereby reducing its measurable concentration. Decreases in chemical recovery over time have been reported in several earthworm toxicity studies (Johnson et al., 2002; Eom et al., 2007). These studies have attributed the decline in chemical recoveries to sequestration and/or biodegradation. Biodegradation has been described as the process by which specialist micro-organisms utilise organic contaminants as substrates for growth (Anyanwu and Semple, 2015b). Thus, the decreases in recoveries in the current study indicate degradation, sorption and/or transformation of chemicals due to ageing (USEPA, 2005). Variations in recoveries of the aromatics were recorded. Despite their structural similarities, N-PAHs showed higher recoveries than the analogue PAH (over time). Stroud et al. (2007) reported that degradation rates of different aromatic hydrocarbons are known to differ due to physico-chemical properties such as molecular size, chemical structure, hydrophobicity and solubility. In the current study, however, the decreased recoveries could be attributed to N-atom(s) substitution / position, and/or large differences in physico-chemical properties such as solubility, K_{oc} , K_{ow} values and half-life (Pereira et al., 1988; Anyanwu et al., 2013; Anyanwu and Semple, 2015a; b; c), which are suggesting a higher N-PAH availability and/or persistence in soil (van Herwijnen et al., 2004; Anyanwu and Semple, 2015b; c).

Irrespective of the time-dependent decreases in the recoveries of some of the aromatics, effects on earthworms were still obvious. This could be attributed to slow degradation of the N-PAHs, chemical availability and/or toxicity (Anyanwu and Semple, 2015b; c). Substantial toxicity was found for B[h]Q over time, and this is a clear

indication of N-PAH availability and/or persistency in the soil environment. From the data reported in this study, B[h]Q mostly affected the health condition of *E. fetida* over time. For example, in soils amended with 500 mg kg⁻¹, *E. fetida* although initially very active, became moribund after few hours of exposure and died within 3 – 5 d. This pattern of mortality suggested that the effects of N-PAHs were as a result of absorption and/or uptake of the chemicals across the body wall rather than through ingestion. This phenomenon has been reported in metal amended soils (Spurgeon et al., 1994). The study showed that the presence of N-PAHs changed the health indices of *E. fetida* in soil over time. Studies have reported that earthworms exposed to chemicals display health symptoms as a result of cellular autolysis caused by enzymatic inhibition and/or blocking of the nervous system receptors due to chemical accumulation, leading to organ paralysis and subsequently death (Kidd and James, 1991; Lou et al., 1999; Buffin, 2003; Aves et al., 2013). Thus, the physical changes observed in this study, which included breakage of the clitella, body ruptures, body lesions and discolouration, could be an indication of toxicity due to autolytic cell destruction.

This study is the first to report the effects and consequences of toxicity of selected N-PAHs aged in soil on *E. fetida*, thus, comparison with available data will only be with studies on freshly spiked soils. In this current study, phenanthrene LC₅₀ and EC₅₀ ranges are in good agreement with the values reported in spiked agricultural soils (Sverdrup et al., 2002; Droge et al., 2006; Eom et al., 2007). Similarly, the recorded N-PAHs LC₅₀ and EC₅₀ values are in range with the findings of Sverdrup et al. (2002). However, the values differed significantly from those of Kobetičová et al. (2008; 2011). Differences in the test species and soil type (matrix) may be attributable. For example, Kobetičová et al. (2008; 2011) evaluated artificial soil, which had a different organic matter content and also contained a different type of organic matter compared to the soil used in this study. LC₅₀ values are known to vary with organic matter content, pH, temperature, humidity, time of exposure and/or matrix. However, the absence of toxicity in the 1,10-Phen amendments in this study is in agreement with the findings of Schová et al. (2007), who reported 1,10-Phen as the least toxic chemical to the soil nematode *Caenorhabditis elegans*.

With greater acute toxicity attributed to more polar and lower K_{ow} value compounds (Kobetičová et al., 2008; 2011), this study showed that 3-ring N-PAHs have significant effects on the survival, growth and health indices of *E. fetida* in soil. This is largely attributed to their N-substitution(s), physico-chemical properties (such as log K_{ow}, K_{oc}, half-life), persistency and/or toxicity (Anyanwu and Semple, 2015a; b; c). However, it should be noted that the toxicity of a chemical is dependent on its availability to earthworms which in turn depends on the physico-chemical properties of soil, the characteristics of the chemical and the feeding strategies adopted by the species of earthworm (Anderson et al., 1999; NRC, 2003; Semple et al., 2004; Lanno et al., 2004). In the current

study, N-PAH concentrations in soil of 500 mg kg⁻¹ caused 100% mortality and increased weight-effects during the course of incubations. The recorded EC₅₀ values suggest that N-PAHs have considerable impacts on soil biota, especially B[h]Q. However, it should be noted that decreases in weight could be caused by changes in the earthworms' energy budget, as the organisms spend energy on coping with the chemicals by avoidance (Widdows and Donkin, 1989), lack of feeding, metabolism and handling stress from the chemicals and/or changes in the body function.

5. Conclusion

The toxicity of phenanthrene and its four nitrogen-containing analogues to the earthworm *E. fetida* were assessed in agricultural soil, directly after spiking and at different times of ageing, using the OECD guideline 207 (with little deviations) and health index judgement. The results showed that N-PAHs were more available and/or persistent in soil than the PAH analogue over the 0 – 90 d ageing period. Greater toxicity was found with N-PAHs during the course of exposures over time. N-PAHs, and especially B[h]Q caused physical damage to the earthworms (breakage of the clitella, body ruptures, body lesions, discolouration and death). Assessments of other N-PAHs are required for good understanding of their toxicity and bioavailability.

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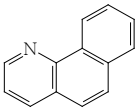
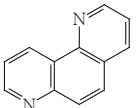
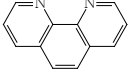
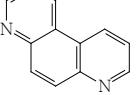
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Table 1. Test chemicals and their physico-chemical properties

Chemical	Chemical formula	Chemical structure	Molecular mass	Boiling point (°C)	Log K_{ow}	Water solubility 25°C (mg/L)	Half-life (hr)	% purity
Phenanthrene	$C_{14}H_{10}$		178.20	340.00	4.46	1.15	$2.88e+003^a$	96.00
Benzo[h]quinoline	$C_{12}H_9N$		179.20	339.00	3.43	78.70	720 ^a	97.00
1,7-Phenanthroline	$C_{12}H_8N_2$		180.21	365.10	2.51	30.64	720 ^a	99.00
1,10-Phenanthroline	$C_{12}H_8N_2$		180.21	365.10	2.51	30.64	720 ^a	99.00
4,7-Phenanthroline	$C_{12}H_8N_2$		180.21	361.20	2.40	38.04	720 ^a	98.00

^a Level III Fugacity ModelSource: www.chemspider.com/Chemical-Structure; Anyanwu and Semple (2015a; b; c)

Table 2. Summary of the toxicity of phenanthrene and its N-PAHs analogues to the survival (LC₅₀) and growth (EC₅₀) of the earthworm *Eisenia fetida* after 21 d exposure in soil, equilibrated for different periods of time after spiking with the test compounds

Chemicals	LC ₅₀ (mg kg ⁻¹ dry soil)				EC ₅₀ (mg kg ⁻¹ dry soil)			
	Days				Days			
	0	30	60	90	0	30	60	90
Phenanthrene	264 (94 -551)	>500	>500	>500	65.8 (-)	427 (-)	475 (-)	500 (-)
Benzo[h]quinoline	345 (199-672)	345 (199-672)	298 (-)	345 (199-672)	7.4 (-)	11.4 (-)	7.5 (-)	7.4 (-)
1,7-Phenanthroline	345 (199-672)	345 (199-672)	345 (199-672)	404 (-)	18.4 (0-74)	5.3 (-)	4.8 (-)	1.2 (-)
1,10-Phenanthroline	>500	>500	>500	>500	405 (-)	31.1 (-)	26.9 (-)	474 (-)
4,7-Phenanthroline	>500	>500	>500	>500	19.2 (-)	50.4 (-)	110 (-)	111 (-)

Data shows LC₅₀ (survival), EC₅₀ (weight loss), 95% confidence interval (in parentheses), over time. (-) = no 95% confidence interval could be calculated.

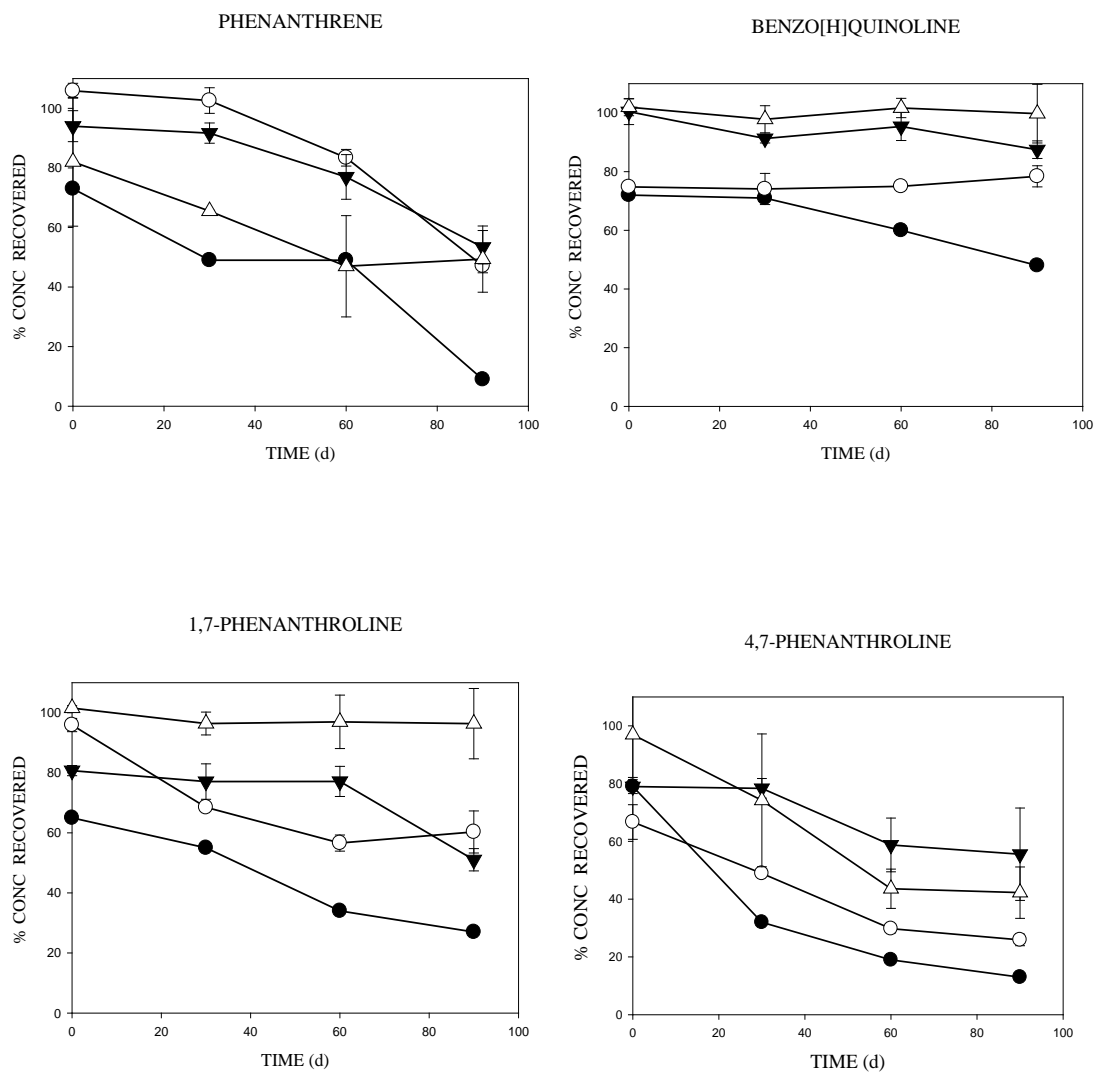


Fig. 1. Recoveries (%) of phenanthrene and its nitrogen-containing analogues from soil spiked at varying concentrations and aged over different periods of time. Data shows: 10 mg kg⁻¹ (●), 100 mg kg⁻¹ (○), 250 mg kg⁻¹ (▼) and 500 mg kg⁻¹ (Δ). Maximum Standard Error = ± 17.0

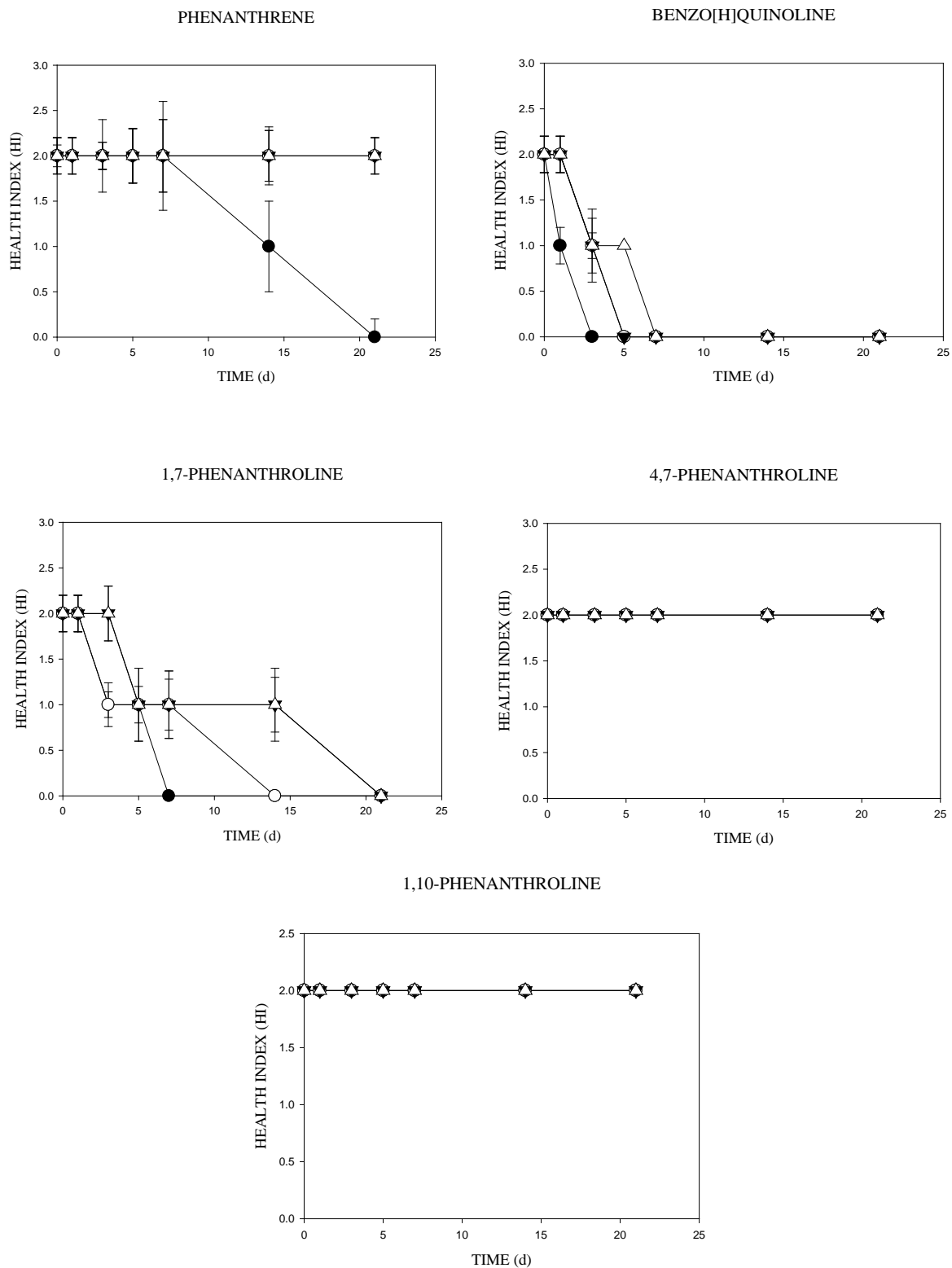


Fig. 2. Health indices of *Eisenia fetida* exposed for 21 days to soils spiked with 500 mg kg⁻¹ dry soil of phenanthrene and its nitrogen-containing analogues and aged for different periods of time. Data shows: 0 d (●), 30 d (○), 60 d (▼) and 90 d (▲), n=5. HI shows: 0 = dead; 1 = moribund; 2 = alive.

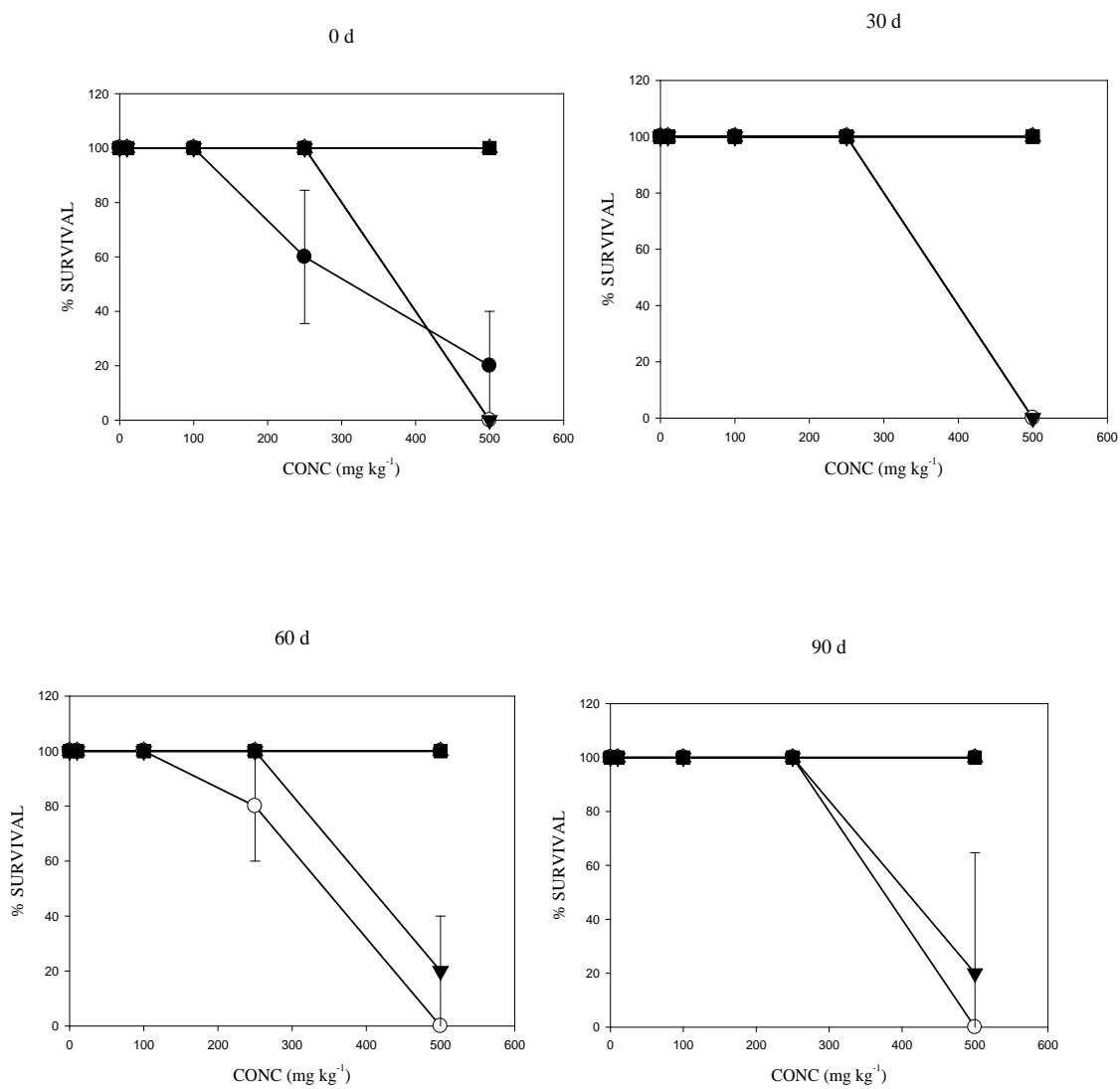


Fig. 3. Survival (%) of *Eisenia fetida* exposed for 21 days to soils spiked with phenanthrene and its nitrogen-containing analogues and aged for different periods of time. Phenanthrene (●), Benzo[h]quinoline (○), 1,7-Phenanthroline (▼), 4,7-Phenanthroline (▽) and 1,10-Phenanthroline (■), n=5. Maximum Standard Error = ± 20.1.

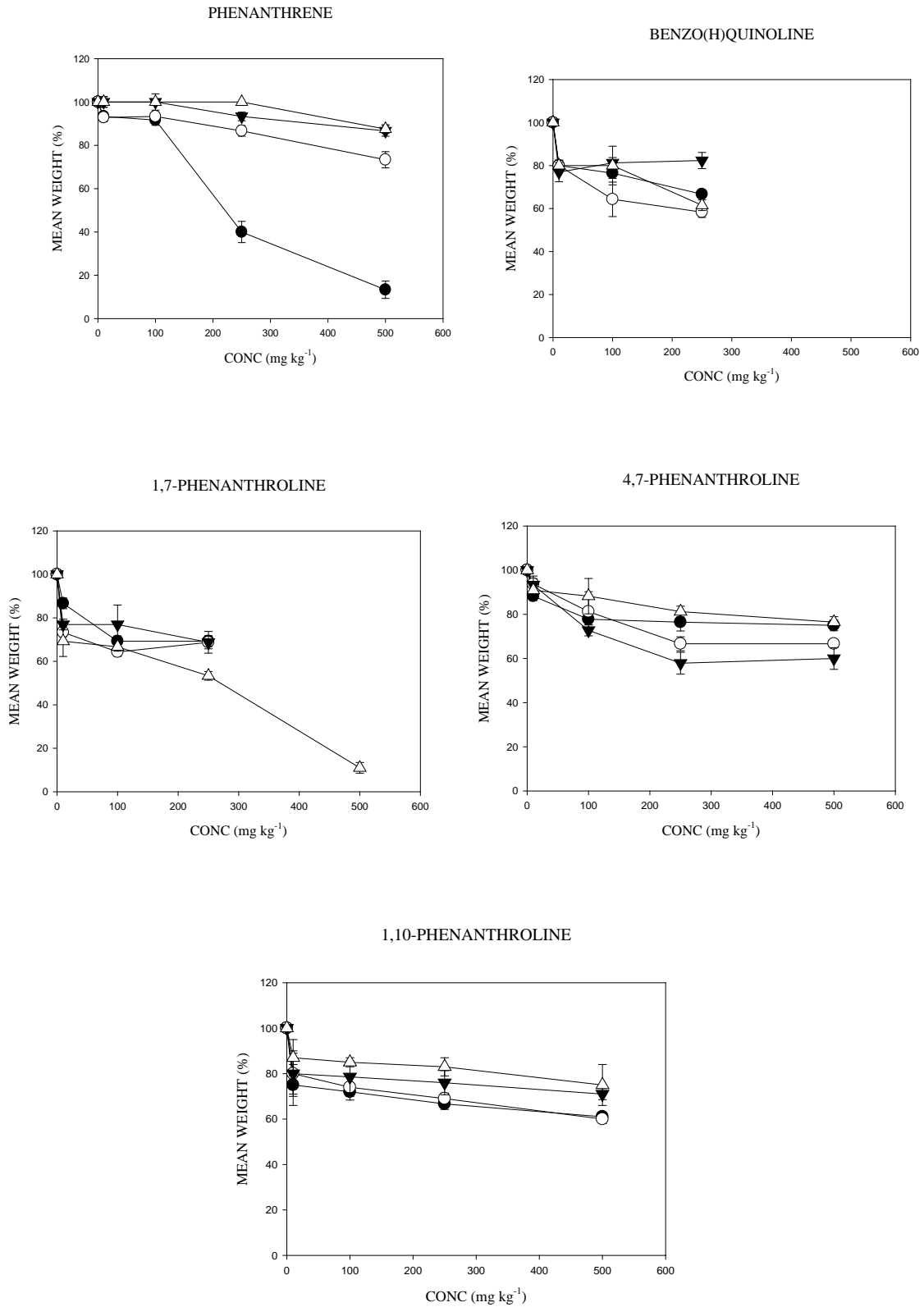


Fig. 4. Mean weight (% of control) of *Eisenia fetida* exposed for 21 d to soils spiked with different concentrations of phenanthrene and its nitrogen-containing analogues and aged for different periods of time. Data shows earthworm weights (% of control) for soils aged for: 0 d (●), 30 d (○), 60 d (▼), and 90 d (Δ), n=5.