WATER, AIR AND SOIL POLLUTION

Effects of single, binary and quinary mixtures of phenanthrene and its N-PAHs on *Eisenia fetida* in soil

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Abstract It is now acknowledged that aromatic hydrocarbons present in contaminated soils occur in mixtures. The effect of single, binary and quinary mixtures of phenanthrene and selected N-PAHs were investigated on the survival, growth and behavioural index of earthworms (*Eisenia fetida*) over a 21 d incubation in soil. The results showed that the LC_{50} values ranged from (not detected) ND - 329.3 mg kg^{-1} (single mixture), ND – 219.8 mg kg^{-1} (binary mixtures) and 148.4 mg kg^{-1} (quinary mixture), while the EC_{50} values (based on weight loss) ranged from 13.3 - 148.4 mg kg^{-1} (single mixture), 63.8 - 148.4 mg kg^{-1} (binary mixture) and 24.2 mg kg^{-1} (quinary mixture). Greater impacts were recorded where N-PAHs are present with phenanthrene. Further, behavioural index of *E. fetida* was affected after 24 h exposure to N-PAH amended soils. Among the N-PAHs however, benzo[h]quinoline recorded the greatest impact on the survival, growth and behavioural index of *E. fetida* in soil. Findings from this study showed that 3 ring-N-PAHs are more toxic than phenanthrene as expected from their physico-chemical properties. Binary and quinary mixtures of phenanthrene and N-PAHs in soil intensified toxicity, suggesting that PAHs-N-PAHs mixtures represent greater risk to soil biota.

Keywords Phenanthrene • Nitrogen-containing PAHs •Toxicity • Behavioural index • Soil • Earthworm**.**

1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a key group of contaminants present at many contaminated sites (e.g. ex-industrial sites, oil contaminated areas) (Brumley et al. 1991; Sverdrup et al. 2002; EC 2011; IARC 2012; Anyanwu and Semple 2016b). What is less well described is that their nitrogen-containing polycyclic aromatic hydrocarbons (N-PAHs) can also be present, often at high concentrations (De Voogt and Laane 2009). Many of these compounds are known to be toxic, carcinogenic, mutagenic and genotoxic, thus, are of concern to both biota and human exposure (Bleeker et al. 2002; Sverdrup et al. 2002; Kobetičová et al. 2008; Brar et al. 2010; IARC 2012; Anyanwu and Semple 2015a, b, c; 2016a, b). In addition, their toxicity cannot be determined by the number of aromatic rings alone (IARC 2012). N-PAHs contain one or more nitrogen atom(s) in place of carbon atom (Table 1). Due to the substitution of N-atom into one or more of the aromatic rings, and the resulting physico-chemical properties, they differ in terms of persistence, mobility, bioavailability and toxicity compared to analogue PAHs (Sverdrup et al. 2002; Kobetičová et al. 2008; Anyanwu et al. 2013; Anyanwu and Semple 2015a; 2016b). For example, the substitution with N-atom(s) makes N-PAHs more polar, mobile and soluble in the soil environment, and as such, putatively more toxic (Sverdrup et al. 2002; Kobetičová et al. 2008; Anyanwu and Semple 2015 c; 2016a, b).

Apart from their environmental occurrence, N-PAHs are discharged into soil from anthropogenic sources such as petroleum related activities, oil spills and combustion processes (Brumley et al. 1991; Webber 1994; Švábenský et al. 2009). Previously, N-PAH levels were reported to be 1-10 orders of magnitude lower than those of the homocyclic analogues, however, De Voogt and Laane (2009) measured concentrations ranging from similar to equal to one order of magnitude higher than their homocyclic analogues in sediments. Furthermore, studies have reported that N-PAH chemicals are phyto-toxic, inhibitors to soil microflora and have cellular autolytic impact (Willumsen et al. 2001; 2005; Anyanwu and Semple 2015b; c; 2016a, b). In addition, it has been reported that losses of PAH degradation capacity in contaminated soil may be due to the existence of N-PAHs (Meyer and Steinhart 2000). Irrespective of this, only few studies have investigated N-PAH toxicity on soil invertebrates (Sverdrup et al. 2002; Kobetičová et al. 2008; 2011) and their observed effects ranged from (LC_{50} (not detected) ND - <2000 mg kg^{-1} and EC_{50} 94 - 1033 mg k^{-1}) (Sverdrup et al. 2002; Kobetičová et al. 2008; 2011). Although there are some toxicity data for a few selected N-PAH compounds, all the investigations focused on the traditional Organization for Economic Cooperation and Development (OECD) methodologies of percentage survival / effects judgements (OECD, 1984) without considering other criteria, such as the

behavioural index (general physical condition) of the organisms during exposure. Monitoring of behavioural index of biota in contaminated soils allows assessment of possible impact of various contaminants on different invertebrate species.

Generally, contaminated soils contain complex mixtures of compounds that differ in their physico-chemical properties and toxicity to soil biota. Thus, effects of range of aromatic compounds can be intensified by the presence of other compounds. However, the effect of binary and quinary mixtures of phenanthrene and its N-PAH analogues on soil biota have not been reported in literature. Since information on toxicity is essential for effective assessment of poorly managed chemicals and/or chemical groups for environmental risk assessment, this study therefore investigated the effect of single, binary and quinary mixtures of phenanthrene and its structurally similar nitrogen-containing analogues on mature earthworms (*Eisenia fetida*) in soil using OECD (1984) guidelines (with little deviation) and behavioural index tool.

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).

2.2 Test organisms

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

2.3 Soil preparation

Soil (without contamination history) from Myerscough Agricultural College in Lancashire, UK 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 an organic matter content of 2.7% and pH 6.5 (Doick et al. 2003). The soil was air dried at room temperature, sieved with 2 mm mesh size, and rehydrated with deionised water back to original water holding capacity (WHC). Spiking procedure followed those described in Doick et al.

(2003). One third of soil (⅓; 100 g), placed in a bowl, were amended with dissolved chemical standards containing acetone (10 ml) to give concentrations of 10, 100 and 500 mg kg^{-1}. The soils were left to evaporate for 4 hours in the fume hood; after which soils were mixed with the remaining ⅔ (200 g) soil and amended to 80% WHC with deionized water. Control samples were prepared using soil amended with acetone only. After amendment, soils (50 g) were weighed into glass jars and the effects on *E. fetida* was measured for 21 d. Recoveries of tested compounds are at a range of 49.50 ± 0.55% and 104.72 ± 8.00%, except for 1,10-Phenanthroline which was not determined since it could not be detected by GC-MS

2.4 Earthworm toxicity assay

Determination of the effects of the N-PAHs on soil biota was carried out using the earthworm toxicity assay (OECD, 1984) (with little deviation). Mature *E. fetida* (0.3 – 0.4 g) were selected for the bioassay. Prior to test, the earthworms were depurated for 24 h in petri dishes containing moist filter paper, after which the earthworms were weighed. Soils (50 g) were used for the assay. Three exposure concentrations (10, 100 and 500 mg kg^{-1}) were used for each single, binary and quinary mixture, as well as control samples (0 mg kg^{-1}). Five replicates were used for each concentration. The soils were mixed thoroughly and single earthworm was added to each replicate. Samples were covered with perforated lids and incubated at 12 ± 2^{o}C. Mortality, biomass and behavioural index were the test parameters. Assessment of behavioural index (0 - 2) was carried out after 0, 1, 3, 5, 7, 14 and 21 d, with 0 = dead, 1 = moribund and 2 = healthy (Langdon et al. 1999; Anyanwu and Semple 2016b). Earthworms were presumed dead if no contractile response was observed when prodded lightly with a blunt probe; moribund when weak contractile was observed; and healthy when sharp and vigorous response was observed (Langdon et al. 1999; Anyanwu and Semple 2016b). The depurated earthworms were weighed before and after exposure to determine changes in weight (biomass). Other physical changes on different parts of the earthworms were also recorded.

2.5 Statistical analysis

The mean weight losses of the earthworms for the replicates were used as a measure of biomass change. The concentration that caused weight loss as compared to control values were calculated on the basis of initial measurement and ANOVA was used to determine the significant impact on biomass. Differences are found to be statistically significant when $p<0.05$. Further, correlations were determined between the exposure concentrations and changes in biomass using Pearsons correlation. Estimation of the concentration of the

chemicals in amended soils that caused 50% mortality (LC_{50}), and the concentration that provoked a response halfway between the baseline and maximum (EC_{50}) (i.e. weight-reduction) during the test period were determined by probit analysis in SPSS 20 software package. Graphs were plotted with SigmaPlot 10.0 version.

3 Results

3.1 Assessment of the aromatic hydrocarbons on the behavioural index of *E. fetida* in soil

Apart from the traditional OECD guidelines relating to survival / effect measurements, the study investigated the general health condition of *E. fetida* during exposure. Fig. 1 shows the behavioural indices of *E. fetida* exposed to single, binary and quinary mixtures of phenanthrene and its N-PAH analogues in soil. The 21 d assessment of behavioural index (0 - 2) showed that all the compounds negatively impacted on the health of *E. fetida* at the highest concentration of 500 mg kg^{-1} ($p<0.05$; $r^2 = 0.982$), with the exception of 1,10-Phen, 4,7-Phen (single mixture) and 1,10-Phen + Phen (binary mixture). Among the N-PAHs, benzo[h]quinoline (single mixture) exhibited greater impact on the general wellbeing of *E. fetida* in soil. For example, it was observed that *E. fetida* became moribund after few hours of exposure to B[h]Q soils and experienced mortality at 3 d in the 500 mg kg^{-1} amendments (Fig. 1).

However, 4,7-phenanthroline recorded significant effect on the behavioural index of *E. fetida* in the presence of phenanthrene (binary mixture) at the 500 mg kg^{-1} amendments ($p<0.05$) (Fig. 1). In addition, benzo[h]quinoline toxicity was slightly reduced in the presence of phenanthrene because, *E. fetida* experienced moribund phase of 5 d in health prior to mortality. Furthermore, the data showed that quinary mixtures of phenanthrene and N-PAHs significantly affected the behavioural index of the exposed earthworms even at 100 mg kg^{-1} ($p<0.05$) (Fig. 1). Other observed health effect includes; breakage of clitellum, excretion of yellowish fluid and sores.

3.2 Assessment of the aromatic hydrocarbons on the mortality (LC_{50}) of *E. fetida* in soil

During the 21 d exposure, neither mortality nor significant weight losses were observed in the control incubations. The survival (%) of *E. fetida* exposed to single, binary and quinary mixtures of phenanthrene and its nitrogen-containing analogues are shown in Fig. 2. From the result, significant mortality rates were recorded

159 in the 500 mg kg^{-1} amendments ($p<0.05$). For example, the concentration–response graph shows that survival
160 (%) of the exposed earthworms ranged from 0% – 20% in soils amended with 500 mg kg^{-1} chemicals, with the
161 exception of 1,10-Phen, 4,7-Phen (single mixture) and 1,10-Phen + phenanthrene (binary mixture) (Fig. 2). In
162 addition, *E. fetida* suffered high mortality (100%) in the 500 mg kg^{-1} B[h]Q amendment (Fig. 2a).

163 Furthermore, 100% mortality were observed in the 500 mg kg^{-1} phenanthrene + 4,7-phenantroline
164 amendment (binary mixture) ($p<0.05$). In quinary mixtures however, *E. fetida* recorded 20% – 100% mortality
165 in the 100 mg kg^{-1} and 500 mg kg^{-1} amendments, respectively (Fig. 2b). From the result, a trend of increased
166 mortality with increase in concentration was observed compared to the control soil ($p<0.05$). The toxicity data
167 (LC_{50}) of single, binary and quinary mixtures of phenanthrene and selected N-PAHs on *E. fetida* after 21 d
168 incubation in soil are summarized in Table 2. From the data, the LC_{50} ranged from ND – 329.3 mg kg^{-1}, with
169 quinary mixture recording the lowest value of 148.4 mg kg^{-1} (Table 2).

170

171 3.3 Assessment of the aromatic hydrocarbons on the biomass (EC_{50}) of *E. fetida* in soil

172 Impact of the chemicals on the biomass of the exposed earthworms was measured (Table 2). The biomass of
173 dead earthworms was not determined and therefore were assigned zero (0). Figs 3 – 4 shows the weight-changes
174 (biomass) of *E. fetida* exposed to single, binary and quinary mixtures of phenanthrene and its N-PAH analogues
175 in soil. From the result, all the aromatics showed significant biomass-effect on *E. fetida,* and there was a trend of
176 decrease in biomass with increase in chemical concentrations. The concentration–biomass–effect plots recorded
177 notable decline in the weight of the earthworms after 21 d exposure to the aromatic hydrocarbons (Fig. 3).
178 Biomass reductions were observed to be pronounced in the N-PAH amendments, especially 1,7-Phen and B[h]Q
179 (single mixture), and in the binary and quinary mixtures of phenanthrene + N-PAHs ($p<0.05$) (Figs. 3 – 4).

180 The EC_{50} measurement of single, binary and quinary mixtures of phenanthrene and its structurally
181 similar N-PAHs on the biomass of *E. fetida* after 21 d incubation are summarized in Table 2. The calculated
182 EC_{50} values ranged from 13.3 – 148.4 mg kg^{-1}. Among the chemical treatments, B[h]Q recorded the lowest EC_{50}
183 of 17.00 mg kg^{-1} (Table 2). Analysis of data using ANOVA showed statistically significant difference in the
184 earthworms biomass (weight before exposure and weight after exposure) in all the chemicals, at all the
185 concentrations ($p<0.05$). Further analysis of data showed high negative correlation between the concentrations
186 and biomass after exposure ($p<0.05$) in all the chemicals, with the exception of 1,10-Phen and 4,7-Phen which
187 showed medium but non-statistically significant relationships ($p>0.05$) (Table 2).

188

189 **4 Discussion**

190 Despite the majority of published studies having focused on PAHs and to a lesser extent their heterocyclic
191 analogues, the toxicity data relating to *Eisenia fetida* are still fragmented, with most of the studies investigating
192 single compounds and artificial soil (Sverdrup et al. 2002; Kobetičová et al. 2008; Kobetičová et al. 2011;
193 Anyanwu and Semple 2016b). This study brings to focus information on the behavioural index, survival and
194 biomass-effect of *E. fetida* following exposure to single, binary and quinary mixtures of phenanthrene and its
195 structurally similar N-PAHs.

196 The endpoint of an earthworm toxicity test is normally a measure of mortality; however, mortality is
197 unlikely to be the most sensitive parameter for risk assessment (Langdon et al. 1999; Anyanwu et al. 2013;
198 Anyanwu and Semple 2016b). This current study showed that most of the health effects, such as healthy (2) –
199 moribund (1) – mortality (0) and antagonistic interactions occurred within <24 h – 3 d of exposure in the N-
200 PAHs amended soils with particular reference to benzo[h]quinoline. This pattern of mortality and/or interaction
201 may be attributed more to uptake of the chemical(s) across the dermis rather than through ingestion. This is
202 because, earthworms added to soils amended with 500 mg kg^{-1} N-PAHs were initially healthy and very active,
203 but became moribund after a few hours and died within 3 d. This phenomenon has also been reported for the
204 highest concentration of metal and aromatic hydrocarbon amended soils (Spurgeon et al. 1994; Anyanwu and
205 Semple 2016b). Presumably, as earthworm moves through the soil, they are exposed to N-PAHs, which may
206 lead to absorption of the chemicals across the dermis. This further showed that earthworms were particularly
207 sensitive to N-PAHs compared to homocyclic phenanthrene analogue. Furthermore, other behavioral response
208 such as lack of burrowing into the amended soil and avoidance (moving away from the amended soil) was
209 observed at the highest concentration (500 mg kg^{-1}).

210 In this current study, benzo[h]quinoline was noted to be the most toxic chemical to *E. fetida*. It may be
211 that N-PAHs such as benzo[h]quinoline, having log K_{ow} of 3.43 and log K_{oc} of 4.32, may be existing more in the
212 aqueous phase (even though some may be associated with soil organic matter), hence, undergoing rapid
213 absorption and thus, triggering physical injury to the dermis of the earthworms as observed by the formation of
214 lesions, breakage of the clitellum, sores, loss of weight and eventually death (Broholm et al. 1999; Anyanwu and
215 Semple 2015b).

Variations in aromatics toxicity were observed. The variations in toxicity could be ascribed to the differences in physico-chemical properties and N-position (Anyanwu et al. 2013; Anyanwu and Semple 2015a, b, c; 2016 a, b). Furthermore, variability was measured among 1,7-phen, 1,10-phen and 4,7-phen irrespective of their similarities in physico-chemical properties. Molecular structure and/or chemical bioavailability may be attributed in this study. From the results, the recorded LC_{50} and EC_{50} values for phenanthrene are in agreement with the values reported by Sverdrup et al. (2002) (Table 3). However, the values (LC_{50} and EC_{50}) were lower than those reported by Kobetičová et al. (2008; 2011); soil physico-chemical properties and/or species differences may be attributable. Further, the absence of mortality recorded in the 1,10-Phen amendment (in the study) is in agreement with the findings of Sochová et al. (2007), who reported 1,10-Phen as the least toxic chemical to nematode (*Caenorhabditis elegans*) in soil. However, the observation was different from the reports of Kobetičová et al. (2008; 2011), who recorded high toxicity for 1,10-Phen; chemical concentrations and/or media (type of matrix) may be ascribed. In support, Anderson et al. (1999) reported that during chemical-biota-interactions, the receptor, route of entry, test duration and the matrix containing the contaminant need to be considered. Therefore, with a soil organic matter content of 2.7% and pH 6.5, the toxicity and speciation of 1,10-Phen may have been influenced (in this study). Similarly, (although not in the soil environment) the observation with 4,7-Phen (single mixture) is in agreement with Feldmannová et al. (2006), who recorded 4,7-Phen as the least toxic compound on the survival, fecundity and reproduction of *Daphnia magna*.

It has been reported that biomass of organisms (such as earthworms) can be impaired by: (i) direct toxic effects on the physiology of exposed organism, (ii) changes in the body function as the organism tries to prevent accumulation in the biological membranes and/or (iii) avoidance due to lack of feeding. Thus, the significant effect on biomass (weight) of *E. fetida* recorded in this current study may be ascribed to toxicity and/or changes in body function. However, it should be noted that the toxicological effects of N-PAHs could cause reductions in weight (Sverdrup et al. 2002; Anyanwu et al. 2013; Anyanwu and Semple 2016b). In addition, Widdows and Donkin (1989) reported changes in the individual energy budget as an organism (such as earthworm) spends energy resisting the contaminant by avoidance, elimination or reluctant to feed in a polluted environment. According to their report, the extra energy requirement will decrease the capacity for growth and development of the organism. This suggests that the effect on biomass of the exposed earthworms is as a result of changes in the energy budget as they strive to resist bioaccumulation in the site of biological response, or weight loss through lack of feeding and/or N-PAHs toxicity. Furthermore, the study showed that the biomass–

effect ratios increased with increase in N-PAHs burden on the earthworms as demonstrated by the correlation relationships; indicating greater toxicity of N-PAHs to soil biota.

5 Conclusions

In this study, the effects of single, binary and quinary mixtures of phenanthrene and its structurally similar N-PAHs were investigated since mixtures of contaminants are found in contaminated soils. The results showed that single N-PAHs possess high ecotoxicity risk as expected of their solubility and lower K_{ow} values, while mixtures of phenanthrene and N-PAHs in contaminated sites represent greater risks to soil biota. However, studies are required on the impact of N-PAHs in chronically contaminated / aged soil.

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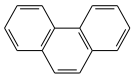
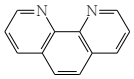
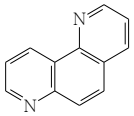
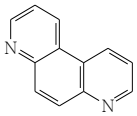
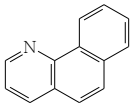
Table 1 Test chemicals and their physico-chemical properties

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

Source: www.chemspider.com/Chemical-Structure, Anyanwu and Semple (2015a, b, c ; 2016 a, b)

Table 2 Summary of the effect of single, binary and quinary mixtures of phenanthrene and its N-PAH analogues on the survival (LC_{50}) and biomass (EC_{50}) of *E. fetida* after 21 d incubation in soil

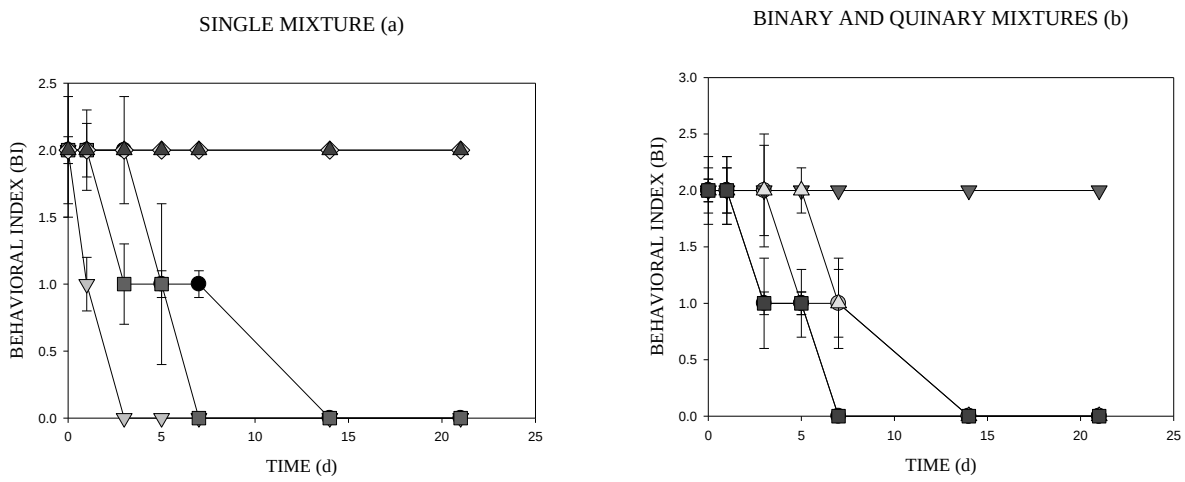
Chemical	LC_{50} (mg kg^{-1})	EC_{50} (mg kg^{-1})	R^2 (biomass)
Phen	329.30 (ND)	148.40 (ND)	-0.976
1,10-Phen	ND	102.30 (ND)	-0.686
1,7-Phen	219.80 (ND)	93.90 (ND)	-1.00
4,7-Phen	ND	13.30 (ND)	-0.690
B[h]Q	219.80 (ND)	17.00 (0-88.10)	-0.972
1,7-Phen + Phen	219.80 (ND)	63.80 (ND)	-0.979
4,7-Phen + Phen	219.80 (ND)	148.40 (ND)	-0.956
B[h]Q + Phen	219.80 (ND)	63.80 (5.5-526.70)	-0.979
Phen + N-PAHs	148.40 (ND)	24.20 (ND)	-0.961

Values show LC_{50}, EC_{50} and 95% confidence interval (in parenthesis), ND = (not determined), n = 5.

Table 3 LC_{50} and EC_{50} ranges of phenanthrene and its nitrogen-containing analogues on survival and growth of soil organisms

Chemical	Test organism	Test duration	LC_{50} (mg kg^{-1})	EC_{50} (mg kg^{-1})	Reference
Phen	*E. veneta*	28 d	134 (ND)	94 (64-125)	Sverdrup *et al*. 2002
	E. cryptius	28 d	1708 (1494-1920)	869 (627-1110)	Kobetičová *et al*. 2011
	E. fetida	21 d	329.3 (ND)	148.4 (ND)	This study
1,10-Phen	*E. fetida*	4 wks	$1500<LC_{50}<2000$	1033 (986-1097)	Kobetičová *et al*. 2008
	E.cryptius	28 d	ND	798 (653-939)	Kobetičová *et al*. 2011
	E. fetida	21 d	ND	102.3 (ND)	This study
1,7-Phen	*E. fetida*	21 d	219.8 (ND)	93.9 (ND)	This study
4,7-Phen	*E. fetida*	21 d	ND	13.3 (ND)	This study
B[h]Q	*E. fetida*	21 d	219.8 (ND)	17.0 (0-88.1)	This study

Values show LC_{50}, EC_{50} and 95% confidence interval (in parenthesis), ND = (not determined).



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401 **Fig. 1** Behavioral index of *E. fetida* exposed to 500 mg kg^{-1} of single, binary and quinary mixtures of
402 phenanthrene and its N-PAHs analogues in soil during 21 d incubation. Data shows: Phen (☻); B[h]Q (∇); 1,7-
403 Phen (■); 1,10 (◇) and 4,7-Phen (▲) (single mixture) (**Fig. 1a**). B[h]Q + Phen (●); 1,7-Phen + Phen (○); 1,10-
404 Phen + Phen (▼); 4,7-Phen + Phen (∆) and Phen + NPAHs (■) (binary and quinary mixtures) (**Fig. 1b**).

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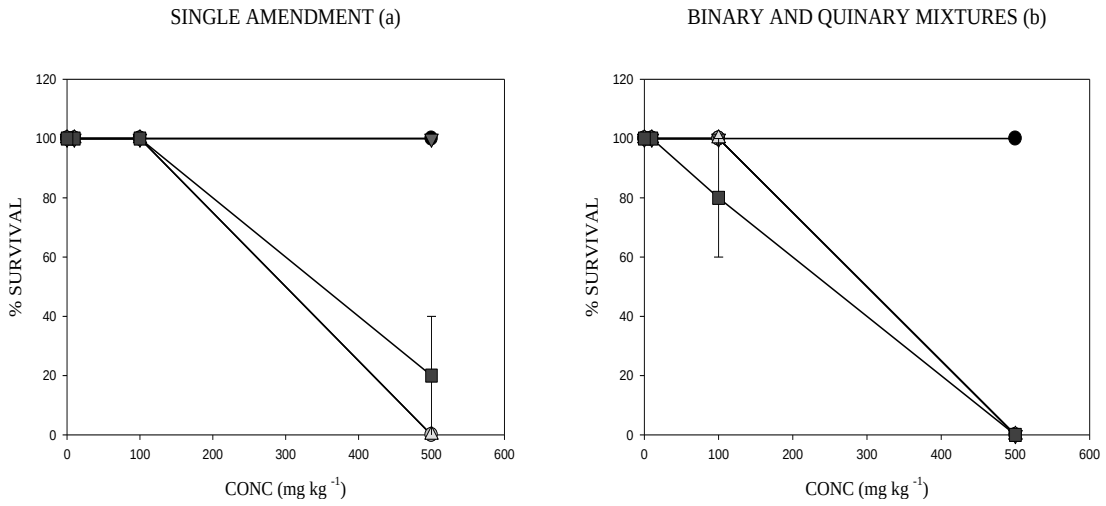

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422 **Fig. 2** Survival (%) of *E. fetida* exposed to single, binary and quinary mixtures of phenanthrene and its N-PAH
423 analogues in soil at 0 (control), 10, 100 and 500 mg kg^{-1} after 21 d incubation. Data shows: 1,10-Phen(●); 1,7-
424 Phen (○); 4,7-Phen (▼); B[h]Q (Δ) and Phen (■) (single amendment) (**Fig. 2a**). 1,10-Phen + Phen (●); 1,7-Phen
425 + Phen (○); 4,7-Phen + Phen (▼); B[h]Q + Phen (Δ) and Phen + NPAHs (■) (binary and ternary mixtures) (**Fig.**
426 **2b**).

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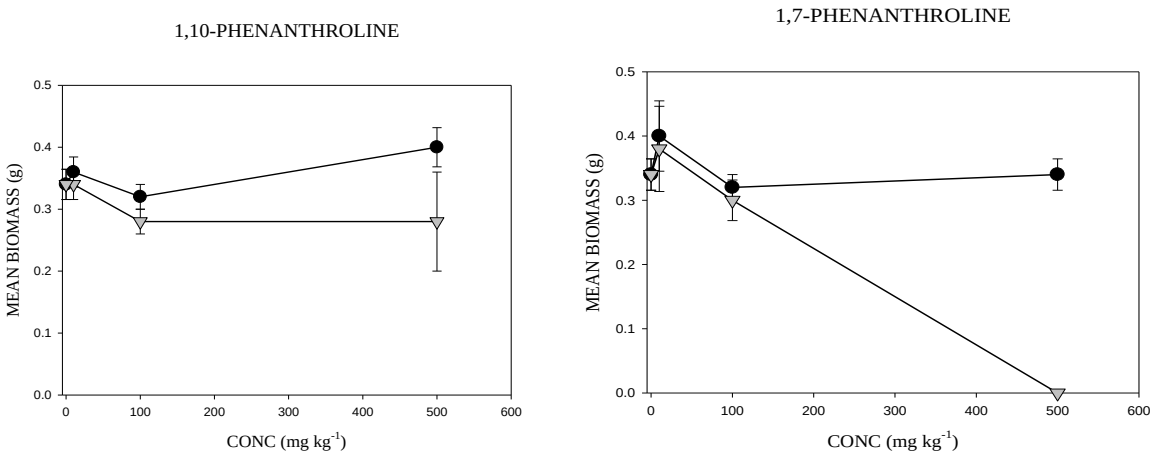

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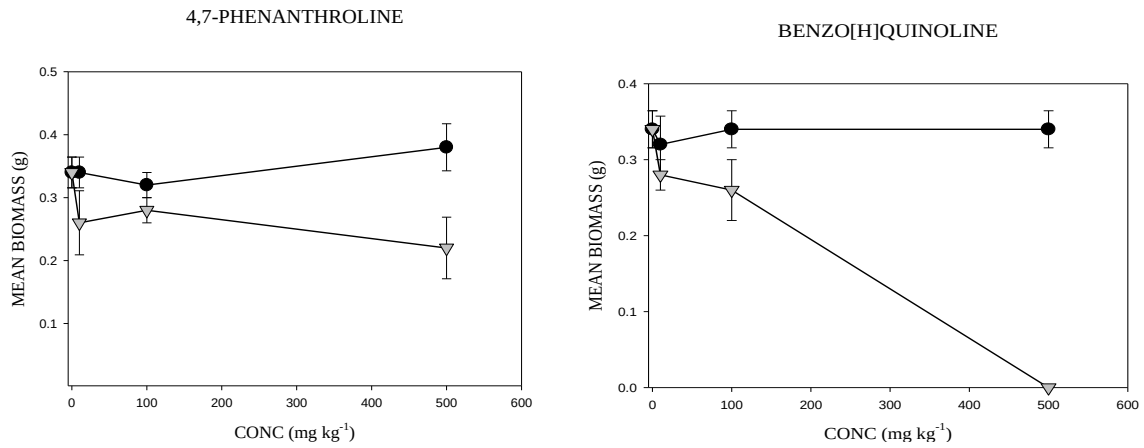

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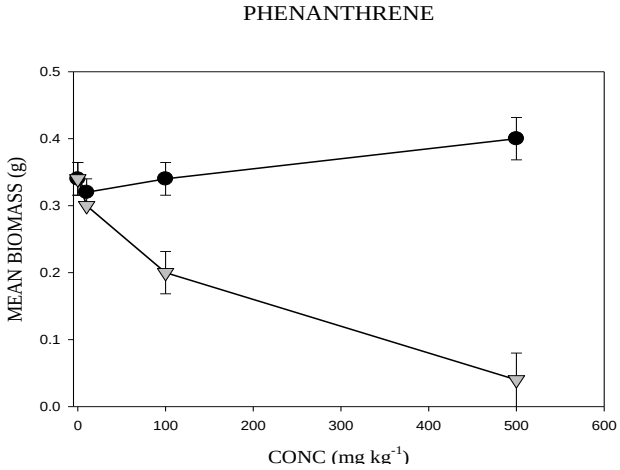

440

441 **Fig. 3** Mean biomass (g) of *E. fetida* exposed to single amendments of phenanthrene and its N-PAH analogues
442 in soil at 0 (control), 10, 100 and 500 mg kg^{-1} after 21 d incubation. Data shows: weight before exposure (●) and
443 weight after exposure (∇).

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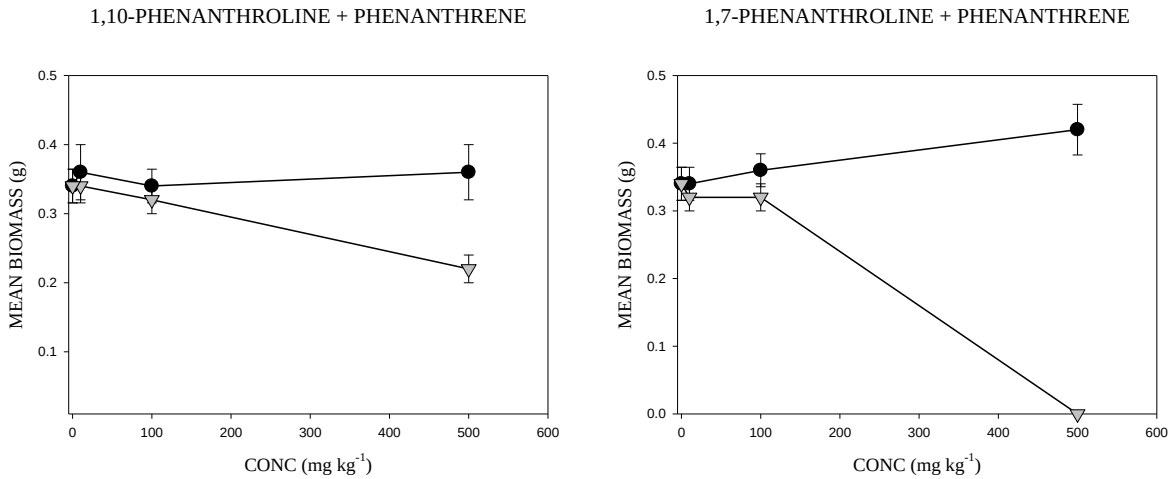

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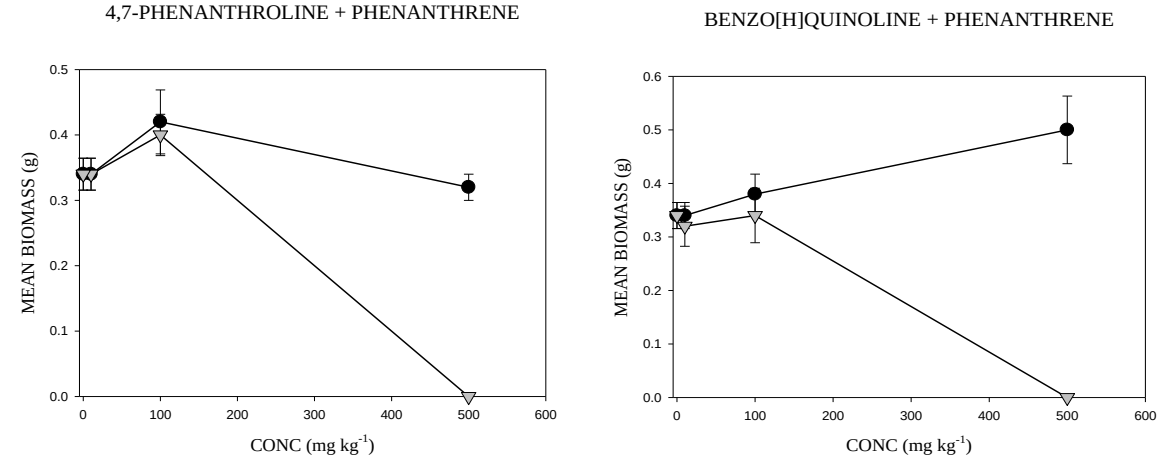

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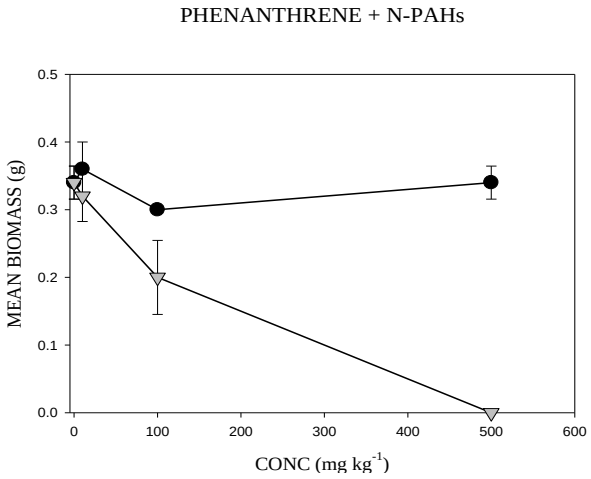

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448 **Fig. 4** Mean biomass (g) of *E. fetida* exposed to binary and quinary mixtures of phenanthrene and its N-PAH
449 analogues in soil at 0 (control), 10, 100 and 500 mg kg^{-1} after 21 d incubation. Data shows: weight before
450 exposure (●) and weight after exposure (∇).