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Differential accumulation of polycyclic aromatic hydrocarbons (PAHs) in three earthworm ecotypes: Implications for exposure assessment on historically contaminated soils

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ABSTRACT

This study compared accumulation of the 16 US-EPA priority polycyclic aromatic hydrocarbons (PAHs) in three different earthworm ecotypes, *Amynthas* sp., *Eisenia fetida*, and *Lumbricus terrestris,* in four historically (> 50 years) contaminated manufactured gas plant (MGP) soils using bioassays. Epi-endogeic deep burrowing and soil ingesting *Amynthas* sp. accumulated significantly more ∑16 PAHs than any other species (upto 8.7 times more), and epigeic surface dwelling *E. fetida* showed the lowest ∑16 PAH accumulation. Results indicated the importance of earthworm habit and physiology on PAH partitioning into earthworm lipids. Exposure to soil borne PAHs via the gut, as compared with passive diffusion from pore water, was important in species tested, being most evident in burrowing species *Amynthas* sp. and *L. terrestris* and for the desorption-resistant higher molecular weight (HMW) PAHs. Biota-soil accumulation factors (BSAF) were low, as influenced by aging, sequestration and strong PAH sorption to secondary sorptive phases in the historically contaminated soils. Bioconcentration factors (BCFs) calculated from freely dissolved pore water PAH concentrations derived from polyoxymethylene solid-phase extractions (POM) were species specific, indicating stronger relationships with octanol-water partition coefficient (K_{OW}) for *Amynthas* sp. and *L. terrestris* than for *E. fetida*. Modelling demonstrated that K_{OW} values were not a reliable proxy for BCF in equilibrium partitioning theory (EqPT) to predict bioavailability for the range of earthworms tested, being less accurate for *E. fetida* compared to the burrowing species. The study showed that including burrowing and soil feeding earthworm species, such as *Amynthas* sp. and *L. terrestris*, in standard testing would benefit regulatory decisions for more accurate quantification of PAH bioavailability in ecological risk assessment on historically contaminated soils.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a widely dispersed group of pollutants in soil sourced from fossil fuel production, use, incomplete combustion and emission (Baek et al., 1991; Banger et al., 2010; Maigari and Maigari, 2015; Ogbonnaya et al., 2017; Wilson and Jones, 1993). These compounds occur at high concentrations on a multitude of industrially impacted sites (Duan et al., 2015), in particular, manufacturing gas plants (MGP) where total soil PAH concentrations up to 17000 mg kg^{-1} are reported (Kuppusamy et al., 2017), significantly greater than typical background concentrations (Liu et al., 2019; Maliszewska-Kordybach et al., 2009) and values considered safe

Abbreviations: PAH, polycyclic aromatic hydrocarbon; HOC, hydrophobic organic contaminants; C_{PW}, freely dissolved pore water concentration; DOM, dissolved organic matter; PDMS, polydimethylsiloxane; PE, polyethylene; POM, polyoxymethylene; EqPT, equilibrium partitioning theory; BCF, bioconcentration factor; BSAF, biota-soil accumulation factors; K_{OW}, octanol-water partition coefficient; MGP, manufactured gas plant; OECD, Organization for Economic and Development; f.w, fresh weight; d.w, dry weight equivalent; MDL, method detection limit; GC-MS, Gas Chromatography Mass Spectrometry; Ace-DCM, acetone/dichloromethane; SPE, solid-phase extraction; K_{POM}, POM-water partition coefficients; $C_{worm,\ lipid}$, lipid normalised PAH concentration in earthworms; OC, organic carbon; $C_{S,\ OC}$, organic carbon normalised total soil concentrations; USEPA, United States Environmental Protection Agency; RIVM, Netherlands National Institue for Public Health and Environmentt; B(a)P TEQ, benzo (a) pyrene toxic equivalence quotient; HIL, Australian health investigation levels; LC, lethal concentration; f_{OC}, organic carbon fraction; HMW, higher molecular weight; LMW, low molecular weight.

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for protection of the environment and human health (ASC NEPM, 2013). The prioritised 16 USEPA PAHs are the most well studied of this group of compounds, and exposure causes acute and chronic toxicity, including carcinogenicity and mutagenicity, and the 5-ring compound, benzo(a) pyrene, is considered one of the most toxic (ATSDR, 1995). These pollutants accumulate in soils, transfer to soil-dwelling organisms and pose a threat to terrestrial ecosystems and humans (Belfroid et al., 1995; Kraaij et al., 2003).

Soil macrofauna have intimate contact with soil (Jager et al., 2003) and accumulation in organism tissue can provide a measure of the bioavailable component of soil contaminant (Lanno et al., 2004; Pulleman et al., 2012). Therefore, earthworms are frequently used in contaminant bioaccumulation assays (Chen et al., 2018). Epigeic (surface-dwelling) *Eisenia fetida* and *Eisenia andrei* are species recommended by the Organization for Economic Co-operation and Development (OECD) (OECD, 2010) for toxicity and bioaccumulation tests (Cachada et al., 2018; Gomez-Eyles et al., 2012; Yang et al., 2013). Both species, however, are not found in agricultural soils and have been reported as less sensitive to certain contaminants than other ecologically relevant species (Bart et al., 2018; Lowe and Butt, 2007; Pelosi et al., 2013; Qiu et al., 2014; Velki and Hackenberger, 2013). For example, two species commonly found in cultivated fields that are not surface dwelling, the soil-dwelling (endogeic) *Aporrectodea caliginosa* and the deep burrowing (anecic) *Lumbricus terrestris*, are reported to be significantly more sensitive to pesticides than *E. fetida* (Pelosi et al., 2013). *E. fetida* is less sensitive to cadmium than anecic *Metaphire guillelmi* (Chen et al., 2017b) and generally less sensitive to fluoranthene and three insecticides than *A. caliginosa* and epi-endogeic *Amynthas gracilis* (Robinson et al., 2021). Regarding bioavailability, Jager et al. (2003) reported PAH body residues in *L. rubellus* (epi-endogeic species) at an average of two times lower than evident in *E. andrei* , and Wang et al. (2014) reported 5 times greater biota to soil accumulation factor (BSAF) for atrazine in *M. guillelmi* than *E. fetida*. These studies demonstrate the possible differences in contaminant accumulation and effects in different earthworm ecotypes, which is important for ecological risk assessment.

Since the 1990s, the use of simple measurements of the freely dissolved PAH pore water concentration (C_{PW}) to predict bioavailability has increased (Krauss et al., 2000; Yates et al., 2011). Measuring dissolved concentrations of hydrophobic organic compounds (HOCs) in soil pore water is complicated by the difficulty of obtaining sufficient pore water for subsequent extraction. Besides, the influence of colloid-bound HOCs must be eliminated and freely dissolved HOCs should be differentiated from those associated with dissolved organic matter (DOM), which is difficult or impossible with solvent extractions (Hawthorne et al., 2011; Hawthorne et al., 2009). Therefore, methods based on equilibrium sampling by directly inserting a “non-depletive” sorbent such as PDMS (polydimethylsiloxane), PE (polyethylene), or POM (polyoxymethylene) into the soil-water slurry to replace solvent extraction have been developed for HOCs such as PAHs and are used in bioaccumulation studies (Gomez-Eyles et al., 2012; Guo et al., 2017; Jonker et al., 2020; Silvani et al., 2019). Polyoxymethylene (POM) is a polymer frequently selected for determining C_{PW} due to well established polymer-water partition coefficient values, low cost, easily cleaned surface, and chemical stability in aqueous media and organic solvents, thus offering a simple, reproducible, sensitive and inexpensive partitioning method (Arp et al., 2015; Endo et al., 2011; Hawthorne et al., 2011; Josefsson et al., 2015). The C_{PW} can show good representation of the PAH bioavailable fraction, that is the freely available fraction with the potential to interact with a target organism at a given time (Cachada et al., 2014; Gomez-Eyles et al., 2012; Guo et al., 2017; Jonker et al., 2020). Bioaccumulation in an organism can be assessed using the C_{PW} derived from POM measurements using equilibrium partitioning theory (EqPT) (Di Toro et al., 1991; Gomez-Eyles et al., 2012; Heijden and Jonker, 2009; Ruus et al., 2013). The EqPT assumes the distribution of HOCs among the organism’s lipids, pore water, and soil organic carbon, and that partitioning is at equilibrium (Kraaij et al., 2003). Applying this theory, bioconcentration factors (BCFs) and biota-soil accumulation factors (BSAFs) can be calculated by dividing lipid normalised PAH concentrations measured in an organism by C_{PW} or organic carbon normalised total PAH concentrations measured in soil, respectively. Alternatively, concentrations in an organism are predicted from the relationship between C_{PW} and BCF (Di Toro et al., 1991; Kraaij et al., 2003), assuming the octanol-water partition coefficient (K_{OW}) is equal to the BCF (Jager, 1998).

Deviations from measured accumulation in earthworms are often evident for historically contaminated soils when EqPT is appplied for the prediction of PAH accumulation using measured C_{PW} values (Cachada et al., 2018; Jager et al., 2003; Kreitinger et al., 2007; Ruus et al., 2010). In these long term contaminated soils, sequestration and aging (Kelsey and Alexander, 1997), mass-transport limitations (Mulder et al., 2001) and strong binding to black carbon (Gustafsson et al., 1996), as well as interspecies variation are all considered to complicate prediction of bioavailability. Further, only a limited number of studies compare PAH bioaccumulation over a range of earthworm species that exist in soils and typically test no more than two species (Jager et al., 2003; Kreitinger et al., 2007; Parrish et al., 2006). Therefore, the objectives of this study were a) to compare PAH accumulation (as a measure of bioavailability) in three different earthworm ecotypes with a range in physiology and habit, exposed to historically contaminated soils, and b) to test the use of K_{OW} as a proxy for BCF using EqPT for the prediction of PAH bioaccumulation in the three different earthworms. The aim of the study was to extend and improve application of EqPT for the prediction of PAH bioavailability in exposure assessment for historically contaminated soils.

2. Methods

2.1. PAH accumulation in earthworms

2.1.1. Bioassays

Four different soils (MGP1, MGP2, MGP3, T-MGP), historically contaminated with PAHs (contaminated for more than 50 years), were used for this study. These soils were contaminated by former MGP activities in Australia. One soil was undergoing remediation treatment, including intense homogenization and fertilization (T-MGP). Soils were wet sieved using a clean 2 mm stainless steel sieve, well homogenised and stored at 4°C. Soil physical and chemical properties were determined previously by Esmaeili et al. (2021b) and are presented in Table S1.

Three earthworm species were used in bioassays; *Amynthas* sp., *L. terrestris* and *E. fetida*. *Amynthas* sp., and *L. terrestris* were obtained from a private supplier (Dun-Diggin Worm Farm, Tamworth, NSW, Australia (2017)) and *E. fetida* was obtained from a commercial supplier (Kookaburra Worm Farms, Tamworth, NSW, Australia (2017)). The three species spanned a range of biology and physiology. *E. fetida* is an epigeic earthworm; surface-dwelling, feeding on leaf litter, decaying plant matter and manure, and is not a soil ingesting burrowing species (Xing et al., 2011). *Amynthas* sp. is an epi-endogeic earthworm, deeply soil-dwelling and ingesting soil by burrowing horizontally for movement and feeding, with partial reuse of existing burrows (Görres and Melnichuk, 2012; Schult et al., 2016). *L. terrestris* is an anecic earthworm that burrows deep, but forages on the surface nocturnally with surface litter and soil organic matter making up the diet (Thorpe et al., 1996). Earthworm survival and $\sum$16 PAH bioaccumulation were evaluated in laboratory bioassays following OECD guideline, (OECD, 2010) with some modifications to exposure time,soil mass and moisture content based on relevant literature (Gomez-Eyles et al., 2012; Guo et al., 2017; Jonker et al., 2007; Kreitinger et al., 2007).

Before initiating assays, earthworms of similar weight were rinsed with deionized water, allowed to depurate on moist filter paper for 24 h, and subsequently weighed before being added to the test soil (Hickman and Reid, 2005; Liste and Alexander, 2002). Prior to weighing, excess

water was removed by gently blotting dry. For bioassays, adult earthworms with fully developed clitellum (ten worms for *E. fetida* and three worms for *Amynthas* sp. and *L. terrestris*) were exposed to 300 g (dry weight equivalent (d.w.)) of each soil in glass jars with several small air holes drilled into lids. Earthworms were maintained for 14 days (Gomez-Eyles et al., 2012; Guo et al., 2017; Kreitinger et al., 2007) under a controlled 16/8 hours light/dark cycle with light intensity of 400-800 lux at 20 ± 2°C (OECD, 2010) without the provision of feed (Jonker et al., 2007; Kreitinger et al., 2007). The soils were kept at 80 % field capacity (Jonker and van der Heijden, 2007; Khan et al., 2011; Kreitinger et al., 2007). The water content was maintained by adding deionized water on a daily basis. For each soil, four replicates were established. An uncontaminated soil was also used as a blank control (Total 16 PAHs < method detection limit (MDL)). ∑16 PAH bioaccumulation in *L. terrestris* could only be assessed in one soil, T-MGP, due to limited numbers available as a result of a severe drought in Australia at the time of the study.

Following the exposure period, earthworms in each replicate were removed from the soil, rinsed twice with deionized water, placed in glass petri dishes with moist Whatman no. 2 filter paper and kept for 24 h to allow depuration (Khan et al., 2011; OECD, 2010; Tang et al., 2002). Earthworms were again rinsed twice with deionized water, blotted dry, weighed, and placed into pre-weighed 40 mL glass vials with Teflon-lined caps (all earthworms from each replicate in one vial). Glass vials were stored frozen at -20 °C until tissue was prepared for PAH extraction and lipid determination.

2.1.2. Lipid determination in earthworms

A Soxhlet extractor was used to determine earthworm lipid content using a subsample of frozen tissue (1-2.5 g fresh weight (f.w.)). Prepared sub-samples (ground earthworms with sodium sulphate in a mass proportion of 1:7 (w w^{-1}) were weighed into soxhlet thimbles. Soxhlet extractions were performed with 100 mL of hexane/acetone (1:1 v v^{-1}) cycled at 65 °C for 16 h. Extracts were then reduced to dryness (Buchi Rotavapour RE120) and earthworm lipid content calculated by the weight difference with the initial subsample weight (Gomez-Eyles et al., 2012; Guo et al., 2017).

2.1.3. PAH extraction from earthworm tissue

The ∑16 PAH accumulation in earthworms was determined using a saponification procedure validated previously (Cachada, 2014; Gomez-Eyles et al., 2012; Rodríguez-Seijo et al., 2017). Frozen earthworms (pooled earthworms from each replicate, 1.5-3 g (f.w.)) were weighed before grinding with sodium sulfate (1:7 w w^{-1}). Homogenates were then spiked with deuterated PAH standard (40 µL from a 500 µg mL^{-1} stock solution) for recovery assessment. The extraction used 10 mL 0.5 M KOH and 10 mL acetone/hexane solution in equal volumes, sonicated at 45 °C for 1 h with agitation every 15 min. The solvent layer was decanted and the extract volume was reduced to about 1 mL using a stream of pure nitrogen before clean-up. Extraction clean up followed the method of Rodríguez-Seijo et al. (2017), with the elute mixture modified by the use of hexane:dichloromethane (DCM) (4:1 v v^{-1}; 20 mL) to enhance recovery of PAHs (Nácher-Mestre et al., 2009; Tao et al., 2009). Cleanup used glass columns with 1.5 g of alumina and 1.5 g of silica (5 and 3 % deactivated, respectively) plugged with glass wool with PAHs eluted with 20 mL of hexane:dichloromethane (4:1 v v^{-1}). Clean extracts were reduced to ~ 0.2 mL using a stream of pure nitrogen, then transferred into 2 mL amber crimp top vials. The internal standard (2-fluorobiphenyl in isooctane (30 µL from a 500 µg mL^{-1} stock solution)) was added and samples were made up to 1 mL with isooctane and sealed. Samples were stored at 4 °C for PAH quantification using Gas Chromatography Mass Spectrometry (GC-MS).

2.2. PAH extraction from soil

Exhaustive acetone/dichloromethane (Ace-DCM) extraction was used for total 16 PAH extraction from soil as previously performed and reported (Esmaeili et al., 2021b).

2.3. PAHs in pore water using POM

Freely dissolved PAH concentrations in soil were determined using POM according to the method described by Esmaeili et al. (2021b) and Arp et al. (2014). Sequential extraction (2 h) by means of sonication using n-hexane and methanol was carried out to wash pre-cut (2 × 4 cm, 100 mg) POM sheets (76 µm thick, CS Hyde Co, Lake Villa, Illinois, USA) (Hawthorne et al., 2011). POM samplers were then rinsed in ultrapure water, placed on solvent-washed aluminium foil, air-dried and stored in 40 mL glass vials, sealed with Teflon-lined caps (Thermo Fisher Scientific, Australia) (Josefsson et al., 2015). The POM equilibrations were undertaken using the method of Arp et al. (2014), with minor modification (biocide addition). In brief, soil (10 g d.w.), weighed POM, $CaCl_2$ solution (~ 35 mL, 0.01 M), and mercuric chloride (1 % w w^{-1}) were placed in a 40 mL glass vial with a headspace of 0.5 mL and sealed with a Teflon-lined cap. Vials (wrapped in aluminium foil and plastic bags) were shaken end-over-end in darkness at room temperature (20 ± 2 °C) for 28 days to reach equilibrium (Hawthorne et al., 2011; Josefsson et al., 2015). The POM samplers were removed, rinsed with Milli-Q water to remove soil particles, and dried with tissue. Dry samplers were kept frozen at -20 °C in clean 40 mL glass vials, sealed with Teflon-lined caps for extraction (Arp et al., 2014). Extraction of the POM samplers was performed using 20 mL of hexane:acetone (1:1, v v^{-1}) spiked with deuterated PAH standard (40 µL from a 500 µg mL^{-1} stock solution) for 3 h by sonication (Arp et al., 2015; Hawthorne et al., 2011). Extracts were concentrated (~ 0.5 mL) under a gentle nitrogen steam, transferred to 2 mL amber crimp-top auto-sampler vials (Kinesis, Australia), and brought up to 1 mL by the addition of the internal standard (30 µL from a 500 µg mL^{-1} stock solution) and isooctane.

2.4. PAH quantification in extracts

PAHs in all sample extracts were analysed according to Leech et al. (2020) and Esmaeili et al. (2021a) using an Agilent 7890A Gas Chromatography system with an Agilent 7693 autosampler coupled to an Agilent 5975c VL Mass Spectrometer Detector with triple axis detector. PAHs determined included the 16 US EPA PAHs using a PAH standard mixture, the deuterated PAH standards, and 2-fluro-biphenyl (internal standard), all from Trajan Scientific (Australia), as previousely reported (Esmaeili et al., 2021a; Leech et al., 2020). The MDLs for PAHs in soils and earthworm extracts are given in Table S2. Recovery values for the deuterated standards were 56-113 %, 65-117 % and 70-110 % for soil, earthworm extracts and POM respectively, with all samples being blank control and recovery adjusted.

2.5. Data analysis

Minitab (version 16, Minitab, LLC, USA) was used for all correlations, regressions and statistical analysis throughout the study. Differences between total soil PAH and pore water concentrations, or PAH accumulated in earthworms for different soils for the same PAH group size were evaluated by one-way ANOVA and t-tests. Data normality was tested with Ryan-Joiner normality test. A Tukey post hoc test with $p <$ 0.05 significance level was used after ANOVA for all parametric data. Kruskal-Wallis ANOVA and the two-sample rank test (Mann-Whitney test) were used for nonparametric data. When PAHs were assessed as ring groups this included: 2-3 ring PAHs - naphthalene; acenapthylene; acenapthene; fluorene; phenanthrene; anthracene; 4- ring PAHs - fluoranthene; pyrene; benzo(a)anthracene; chrysene; 5-6 ring PAHs - benzo (b)fluoranthene; benzo(k)fluoranthene; benzo(a)pyrene; dibenz(a,h) anthracene; benzo(ghi)perylene; indeno[1,2,3-cd]pyrene.

The C_{PW} in soil were calculated from POM extractions using the concentration of each PAH in the POM-extract with the POM sampler

weight (C_{POM}) and the POM-water partition coefficients (K_{POM}) using Eq. (1) (Josefsson et al., 2015; Mayer et al., 2003):

$$C_{PW}\left(\text{mg L}^{-1}\right) = C_{POM}\left(\text{mg kg POM}^{-1}\right)/K_{POM}\left(\text{mg kg POM}^{-1} / \text{mg L pore water}^{-1}\right) \quad (1)$$

The K_{POM} values were taken from Hawthorne et al. (2011) (Refer to Table S3).

Biota-soil accumulation factors (BSAFs) were calculated from the lipid normalised PAH concentration measured in earthworms ($C_{worm,\ lipid}$) (assumed steady-state at 14 d (Gomez-Eyles et al., 2012; Guo et al., 2017; Jonker et al., 2007; Kreitinger et al., 2007)), and the measured organic carbon (OC) normalised total soil concentrations ($C_{S,\ OC}$) using Equation (2) (Cachada et al., 2018; Muijs and Jonker, 2012; Yates et al., 2011):

$$BSAF\left(\text{kg OC kg lipid}^{-1}\right) = C_{worm,\ lipid}/C_{S,\ OC} \quad (2)$$

Bioconcentration factors (BCFs) were calculated using measured lipid normalised PAH concentrations accumulated in earthworms ($C_{worm,\ lipid}$) and calculated C_{PW} measured by POM (Eq. 1), using Eq. (3) (Arp et al., 2014; Yates et al., 2011):

$$BCF\left(\text{L kg lipid}^{-1}\right) = C_{worm,\ lipid}/C_{PW} \quad (3)$$

Predicted BSAF values ($BSAF_{predicted}$) were determined by calculating a predicted $C_{worm,\ lipid,\ predicted}$, from C_{PW} and assuming BCF is equal K_{OW} (Jonker and van der Heijden, 2007), as shown in the following equations, and compared with the BSAF values calculated from measured $C_{worm,\ lipid}$ (Cachada et al., 2018; Ruus et al., 2013):

$$C_{worm,\ lipid,\ predicted} = C_{PW} \times K_{OW} \quad (4)$$

$$BSAF_{predicted} = C_{worm,\ lipid,\ predicted}/C_{S,\ OC} \quad (5)$$

The K_{OW} values used were obtained from the United States Environmental Protection Agency (USEPA) (USEPA, 2003) and the Netherlands National Institute for Public Health and the Environment (RIVM) (Verbruggen, 2012) (refer to Table S3 for K_{OW} values).

3. Results and discussion

3.1. PAHs in soil and pore water

The four historically contaminated soils showed different characteristics and were highly contaminated (Table S1). Total 16 PAHs concentration ranged from 550 ± 31.4 (MGP3) to a maximum of 1652 ± 62.3 mg kg^{-1} d.w. in T-MGP, the soil undergoing treatment (Table 1). More than 79 % of ∑16 PAHs in soils were 4-6 ring compounds as expected in historically contaminated soils (Figure S1a). Benzo(a)Pyrene Toxic Equivalence Quotient (B(a)P TEQ) ranged from 101 to 310 mg kg^{-1}, significantly exceeding Australian health investigation levels (HIL) for all land uses (ALS, 2013; ASC NEPM, 2013). Eom et al. (2007) estimated PAH lethal concentration (LC50) values for *E. fetida* in soil ranging from 3.4 mg kg^{-1} (acenaphtylene) to 415 mg kg^{-1} (fluoranthene). In this regard, the soils used in this study show potential for toxicity to earthworms.

Calculated C_{PW} of ∑16 PAHs varied from 0.006 mg L^{-1} (MGP2) to a maximum 0.025 mg L^{-1} in MGP1. Despite the highest total soil 16 PAH

Table 1
Concentration of 16 US-EPA priority-pollutant PAHs in Manufactured Gas Plant (MGP) soils (MGP 1, MGP 2, MGP3 and T-MGP) and extracted pore water concentrations using POM. Values are the mean ± standard error ($n = 4$). Individual PAH concentration in uncontaminated blank soil was below the method detection limits (MDL).

PAH	Total PAH concentration in soil (mg kg^{-1} d.w)					Freely dissolved pore water concentration (C_{PW}, ng L^{-1})			
	Number of aromatic rings	MGP 1	MGP 2	MGP3	T-MGP	MGP 1	MGP 2	MGP3	T-MGP
Naphthalene	2	1.41 ± 0.01	6.03 ± 0.35	1.68 ± 0.08	12.80 ± 0.40	210.90 ± 1.92	201.71 ± 4.01	207.36 ± 5.91	3798.24 ± 284.52
Acenaphthylene	3	4.92 ± 0.09	15.26 ± 1.47	3.93 ± 0.48	21.46 ± 0.78	39.88 ± 0.52	39.20 ± 0.72	163.10 ± 5.85	603.88 ± 14.48
Acenaphthene	3	2.63 ± 0.07	1.74 ± 0.41	0.56 ± 0.03	5.81 ± 0.40	105.24 ± 1.37	103.44 ± 1.91	109.32 ± 3.25	505.55 ± 30.21
Fluorene	3	4.28 ± 0.08	16.67 ± 0.99	2.68 ± 0.56	11.22 ± 0.42	1681.60 ± 32.39	1071.97 ± 107.61	1286.63 ± 34.00	1540.99 ± 16.51
Phenanthrene	3	88.63 ± 5.00	83.37 ± 6.96	19.34 ± 2.75	81.42 ± 4.90	11213.47 ± 130.13	2160.50 ± 200.53	1074.78 ± 20.06	2391.82 ± 64.83
Anthracene	3	15.53 ± 1.20	27.03 ± 3.17	8.63 ± 0.74	31.12 ± 1.98	1235.36 ± 24.29	391.47 ± 0.43	497.14 ± 6.00	1185.21 ± 36.10
Fluoranthene	4	146.94 ± 7.41	111.21 ± 16.9	79.32 ± 3.15	191.06 ± 10.1	5508.88 ± 95.28	871.91 ± 55.68	1190.32 ± 16.53	1391.24 ± 13.75
Pyrene	4	141.26 ± 6.98	102.87 ± 16.12	83.65 ± 3.13	201.91 ± 9.88	4470.18 ± 72.25	598.62 ± 36.85	1049.16 ± 13.72	1121.93 ± 11.32
Benz[a]anthracene	4	62.12 ± 2.31	66.07 ± 10.00	49.15 ± 1.94	114.90 ± 4.92	235.51 ± 1.77	121.70 ± 3.23	123.70 ± 2.07	135.70 ± 0.68
Chrysene	4	74.16 ± 0.45	58.05 ± 8.55	48.91 ± 1.58	165.99 ± 6.97	251.69 ± 2.36	90.69 ± 2.49	103.58 ± 0.97	119 .22 ± 0.68
Benzo[b] fluoranthene	5	106.19 ± 2.96	69.79 ± 9.09	76.47 ± 5.98	265.48 ± 7.47	82.42 ± 0.90	49.17 ± 0.76	38.79 ± 0.97	69.29 ± 1.08
Benzo[k] fluoranthene	5	35.73 ± 0.91	28.44 ± 5.01	26.77 ± 1.26	68.50 ± 4.95	38.62 ± 0.42	31.22 ± 0.32	26.02 ± 0.71	34.70 ± 0.47
Benzo[a]pyrene	5	88.66 ± 2.47	57.86 ± 8.23	70.08 ± 5.35	203.06 ± 5.44	53.62 ± 0.66	38.30 ± 0.46	32.96 ± 0.79	48.54 ± 0.70
Dibenzo[a,h] anthracene	5	31.72 ± 1.58	22.78 ± 3.05	19.92 ± 2.09	46.94 ± 2.59	34.80 ± 0.89	40.55 ± 0.34	22.74 ± 0.66	31.32 ± 0.45
Benzo[g,h,i] perylene	6	50.25 ± 1.29	21.63 ± 2.65	30.35 ± 1.98	112.77 ± 6.42	24.13 ± 0.50	23.46 ± 0.22	20.85 ± 0.63	24.89 ± 0.35
Indeno[1,2,3-c,d] pyrene	6	49.61 ± 1.36	28.56 ± 3.36	29.31 ± 2.38	117.86 ± 5.24	10.08 ± 0.12	3.91 ± 0.07	11.65 ± 0.33	15.52 ± 0.21
Σ 16 PAHs		904.05 ± 22.68	717.35 ± 95.92	550.74 ± 31.38	1652.30 ± 62.31	25195.38 ± 303.32	5838.82 ± 452.95	5958.00 ± 74.34	13018.04 ± 304.34

concentration detected in T-MGP, C_{PW} was lower than observed for MGP1 suggesting the remediation treatment had reduced the relative freely available PAHs in T-MGP through promoting microbial activity (Table 1). High C_{PW} in MGP1 compared to MGP2 and MGP3 might be related to higher ∑16 PAH concentration in this soil combined with soil characteristics (lower clay and organic matter content, Table S1). The C_{PW} values were within the ranged observed by others for historically contaminated soils; Gomez-Eyles et al. (2012) reporting ~ 0.004 to 1×10^{-4} mg L^{-1} for 10 field contaminated soils, and Arp et al. (2014) a range of 2×10^{-5} to 0.5 mg L^{-1} for 21 historically contaminated soils with total PAH up to 2651 mg kg^{-1}. The 2-3 ring PAHs made up the largest fraction of C_{PW} in all soils (56-88%), while 5-6-ring PAHs were less than 3 % (Table 1, Figure S1b). This is explained by stronger sorption, larger organic carbon-water partition coefficient (K_{OC}) and larger POM-water partition coefficients (K_{POM}) for the strongly sorbed 5-6 ring PAHs (Hawthorne et al., 2011), and previously reported in historically contaminated soils (Arp et al., 2014; Gomez-Eyles et al., 2012; Yates et al., 2011).

3.2. Earthworm PAH concentrations

The *E. fetida* and *L. terrestris* showed 0 % mortality throughout the entire 14-day bioassay exposure period in all soils, displaying healthy and normal activity. *Amynthas* sp. also showed 0 % mortality in all soils except MGP1 (75 % mortality). Only surviving individuals for this treatment were analysed; all actively depurated gut contents but some showed symptoms of toxicity (inactivity and some black discoloration). Mean earthworm mass loss in treatment replicates (except one of the *Amynthas* sp. MGP1 replicates) was 12 %, significantly lower than the 20 % specified in the OECD guidelines for validation of the test (OECD, 2010). The cause of high mortality for *Amynthas* sp. in MGP1 was not clear but may be related to the high soil pore water PAH concentrations (Table 1) and species sensitivity. Soil pH has shown significant correlation with biotic accumulation of PAH in other studies (Jager et al., 2003). However, no significant relationship was found between soil pH and PAH accumulation in the earthworm species in our study.

The lipid normalised PAH values in different earthworm species after the 14-day bioassay differed significantly in the same soil ($p < 0.05$) (Fig. 1) and between the soils (Figure S2). Average earthworm lipids were within the range given in available literature data (2.2 % for *Amynthas* sp. and *L. terrestris,* and 3.2 % for *E. fetida* based on f.w. (Carter et al., 2014; Van der Wal et al., 2004). *Amynthas* sp. showed significantly higher ∑16 PAHs and all ring group PAHs than *E. fetida* in all four soils ($p < 0.05$) (Fig. 1). ∑16 PAHs were 2.9 to 8.7 fold greater than those in *E. fetida* over the four soils (e.g. up to 8033 mg kg $lipid^{-1}$ was the maximum concentration observed, in MGP1). *L. terrestris*, which

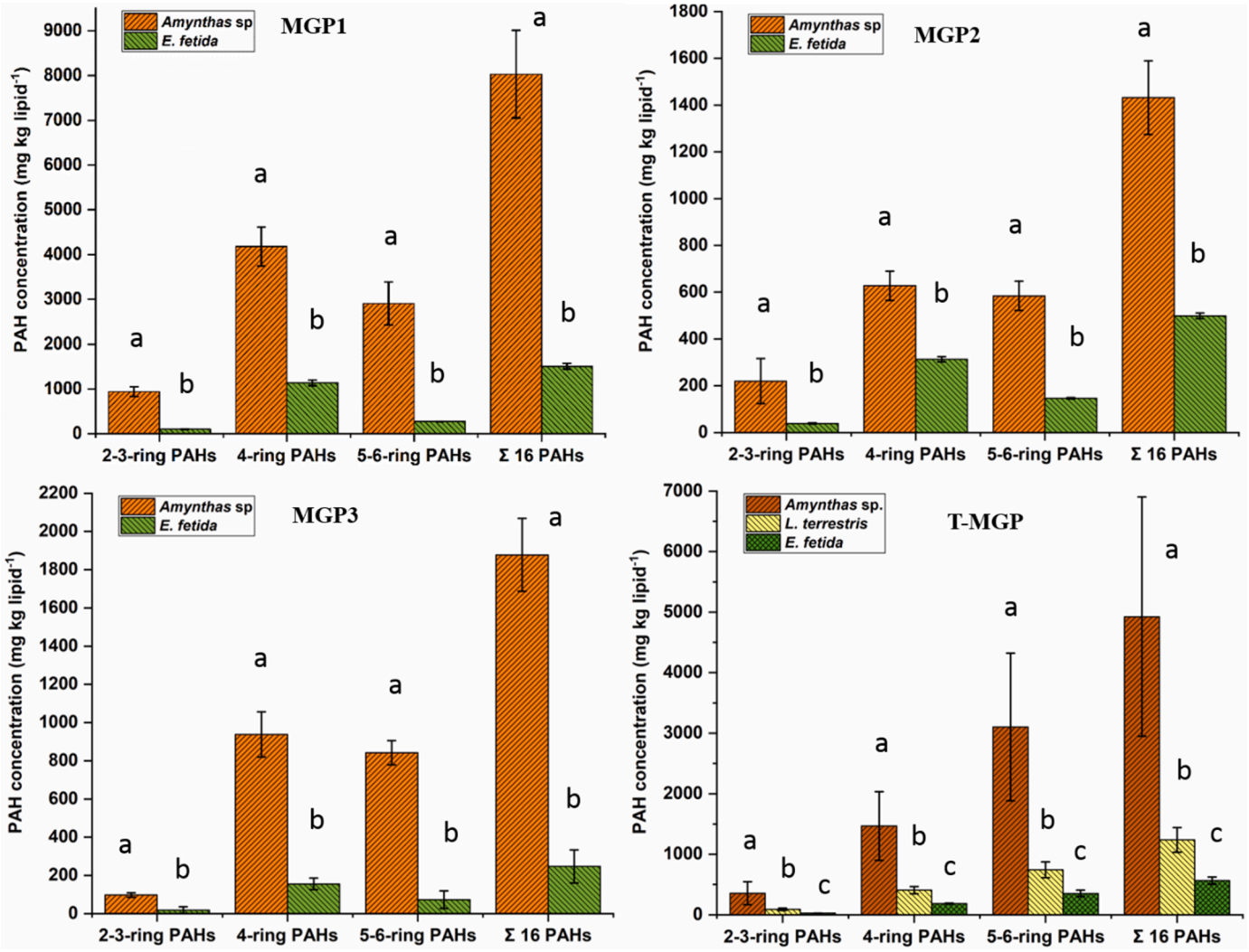

Fig. 1. Lipid normalised PAH concentration measured in earthworm tissues exposed to Manufactured Gas Plant (MGP) soils (MGP 1, MGP 2, MGP 3, T-MGP) following the 14-d bioassay (values as mean and standard deviation; $n = 4$). Letters show statistical differences between earthworm species for each PAH group size in each soil ($p = 0.05$).

was only tested in T-MGP, showed significantly smaller PAH concentrations than *Amynthas* sp., but larger than *E. fetida* for ∑16 PAHs and all ring groups ($p < 0.05$) (Fig. 1). *Amynthas* sp. and *E. fetida* both showed the maximum ∑16 PAH in MGP1 (for all ring sizes and ∑16 PAHs) ($p < 0.05$), and the lowest in MGP2 (*Amynthas* sp.) or MGP3 (*E. fetida)* (Figure S2). The MGP1 soil showed high total soil and the highest pore water PAH concentration that corresponded with the increased earthworm concentrations observed in this soil. In *Amynthas* sp., phenanthrene and anthracene showed the maximum concentrations of the 2-3 ring compounds, fluoranthene and pyrene for the 4 ring compounds, and benzo(b)fluoranthene and benzo(a)pyrene for the 5-6 ring PAHs (Figure S3). Differences in individual PAH concentration were similar but less distinct for the other earthworm species.

Earthworm PAH concentrations measured for *Amynthas* sp. far exceeded values previously reported for earthworms. For example, total PAH of 0.204 and 0.084 mg kg^{-1} f.w. have been reported for *E. fetida* and *L. terrestris* (Parrish et al., 2006), and 0.4 mg kg^{-1} f.w. ∑15 PAHs for *E. andrei* (Cachada et al. 2018) in soils with much lower PAH concentrations (in the present study on a f.w. basis: *E. fetida*: 8-48.2 mg kg^{-1} f.w. *Amynthas* sp: 32-178 mg kg^{-1} f.w. and *L. terrestris*: 28 mg kg^{-1} f.w.). On a lipid basis, Kreitinger et al. (2007) reported 335 to 1390 mg kg^{-1} lipid in *E. fetida* and 901-2556 mg kg^{-1} lipid in *A. caliginosa* in MGP soils with PAH concentrations ranging from 168-42100 mg kg^{-1}, similar to values observed in *E. fetida* and *L. terrestris* in this study, although values in *Amynthas* sp. in all 4 soils were much higher. The earthworm concentration differences observed demonstrate differences in PAH bioavailability among different contaminated soils and different earthworm ecotypes.

The HOCs are absorbed by earthworms either via passive diffusion from soil solution through the outer skin membrane, or by absorption of the compounds from soil material passing through the gut (Belfroid et al., 1995; Li et al., 2016), with the relative importance of the two exposure pathways being dependent on species physiology and feeding/burrowing habit (Chen et al., 2017a; Jager et al., 2005; Krauss et al., 2000; Wang et al., 2014). The higher PAH values observed in *Amynthas* sp. may be explained by its deep-burrowing habit and significant ingestion of soil particles (see section 2.1.1 Bioassays). The increased exposure to soil sorbed PAHs as a result of intimate soil contact and gut soil intake manifest in an increased concentration of PAHs compared to the other species tested (Chen et al., 2017a; Chen et al., 2017b; Krauss et al., 2000; Wang et al., 2014). The surface-dwelling species *E. fetida* is believed to not ingest soil and therefore has less intimate contact with soil, explaining the smaller PAH concentrations due to less exposure (Chen et al., 2017a; Chen et al., 2017b; Jager et al., 2005; Wang et al., 2014; Zhang and Schrader, 1993).

When profiling earthworm PAH concentrations in terms of aromaticity (Figure S1c), a much lower proportion of 2-3 ring PAHs was evident in earthworms (≤ 8.1 % of ∑16 PAHs on average for all earthworms in all soils) compared to 4 ring and 5-6 ring PAHs. Differences between species were most evident for these higher ring number PAHs. Excluding T-MGP, *Amynthas* sp. showed a greater proportion of 5-6 ring PAHs in all soils compared to *E.fetida* (averaging 41 and 26 % 5-6 ring in *Amynthas* and *E. fetida* respectively), while *E.fetida* showed a greater proportion of 4 ring compounds (averaging 49 and 67 % 4 ring in *Amynthas* and *E. fetida* respectively) (Figure S1c). Increased exposure via soil intake through the gut of the burrowing and soil feeding *Amynthas* sp. also explains the higher 5-6 ring PAH concentrations observed in this species because desorption-resistant high K_{OW} PAHs are released in the gut with breakdown of soil aggregates (Conrad et al., 2002; Qi and Chen, 2010; Wang et al., 2014). The combination of physical, chemical, and biological processes inside the gut such as solubilizing agents in digestive fluids, and the physical structure of the gut wall can noticeably enhance the release of contaminants from ingested soil particles compared with the passive desorption of sorbed contaminants to pore water (Edwards, 2012; Gu et al., 2016; Guo et al., 2017; Mayer et al., 1996; Mayer et al., 2007; Qi and Chen, 2010; Wang et al., 2014; Weston and Mayer, 1998). In contrast, exposure for *E. fetida* is considered to be mainly from pore water (Chen et al., 2017a; Chen et al., 2017b; Wang et al., 2014) and the 4-ring PAHs provided a significant proportion of the total pore water PAH content (Figure S1b). Concentrations of 2-3 ring PAHs were low for all species, probably due to volatilization, biodegradation and metabolism as reported by other authors (Cachada et al., 2018; Ma et al., 1998; You et al., 2006). In T-MGP, a different pattern of earthworm PAH was evident, showing a greater fraction of 5-6 ring PAHs than in the other soils. We hypothesise that the treatment process for this soil, by altering soil structure and breaking down the soil aggregates, increased accessibility of strongly bound PAHs (e.g. 5-6 ring PAHs) and made gut soil intake processes important for PAH uptake in all three species as further discussed below (Fig. S1c).

The profile similarity between PAHs in *Amynthas* sp. and PAH fractions in the three untreated MGP soils (Figures S1a & c), and a strong relationship between $C_{worm,\ lipid}$ and log $C_{S,\ OC}$ (Pearson's r = 0.94-0.99) (Fig. 2) supports soil OC as the driver for PAH uptake (PAHs associated with soil solid phase and OC) in this species. Correlations for the two other species and notably in T-MGP were also reasonable (Fig. 2), supporting that exposure via the gut was influential to total PAH observed in the tested earthworms, but especially in the burrower species that ingest soil particles. The relationship between log $C_{worm,\ lipid}$ and soil PAH concentration showed the same trends (Fig. S4a). While no significant association was evident between log $C_{worm,\ lipid}$ and log C_{PW} for any earthworm species in the untreated MGP soils ($p > 0.05$, all Pearson's $r \leq 0.56$) (Fig. S4b), this relationship was improved when developed for PAHs with log $K_{OW} < 5.5$ (2-3 and 4 ring PAHs to pyrene) in the untreated MGP soils (Figure S5) (Pearson's r = 0.60-0.84, 0.58-0.75, for *Amynthas* sp. and *E. fetida* respectively albeit the relationship was not always significant). This suggests a pore water contribution to earthworm concentrations for the low molecular weight (LMW) PAHs in these historically contaminated soils (Wilson and Jones, 1993). Relationships with C_{PW} were different in T-MGP (Figure S4b), with negative associations for all three species (Pearson's r = -0.48 for *E. fetida* and -0.55 for *Amynthas* sp. and *L. terrestris*), although the relationship was not significant for *E. fetida.* In this treated soil possibly the altered/disturbed structure of the soil (fiable due to the breakdown of soil aggregates) provided differential access to soil sorbed PAHs for all species, that facilitated gut uptake with higher concentration of HMW PAHs (Fig. S1c).

In these historically contaminated soils, earthworm PAH concentrations were closely related to soil OC normalised PAH concentrations. The results demonstrate the importance of gut uptake especially when C_{PW} is low for all earthworm ecotypes assessed particularly for the burrowing species, and support that in these soils a passive process of PAH partitioning between pore water and organism lipid phase may not always represent the major exposure (Cornelissen et al., 2006b; Ruus et al., 2010; Yates et al., 2011).

3.3. Biota-soil accumulation (BSAFs)

The median BSAF values (kg OC kg $lipid^{-1}$) for ∑16 PAHs calculated for *Amynthas* sp. ranged from 0.075 in MGP3 (ranged from 0.004 for naphthalene to 0.118 for indeno[1,2,3-c,d]pyrene) to 0.195 in T-MGP (ranged from 0.064 for acenaphthene to 0.376 for benzo[k]fluoranthene) and significantly exceeded ($p < 0.05$) corresponding values for *E. fetida* in each of the soils and *L. terrestris* in T-MGP (Table 2), confirming greater relative accumulation in this species. The median BSAF for *L. terrestris* was significantly greater than the value for *E. fetida* in T-MGP ($p < 0.05$). The BSAF values also confirm a relative increase in the accumulation of 5-6 ring PAHs compared to 4 ring compounds in *Amynthas* sp., as compared to *E. fetida* and for the 4-6 ring PAHs in all species compared to 2-3 ring PAHs, in support of earthworm PAH profiles (Fig. S1c). Median BSAFs in *Amynthas* sp. and *E. fetida* followed the order T-MGP > MGP1 > MGP2 > MGP3, (although being significant ($p < 0.05$) for *E. fetida* between T-MGP and MGP1 soils only) confirming

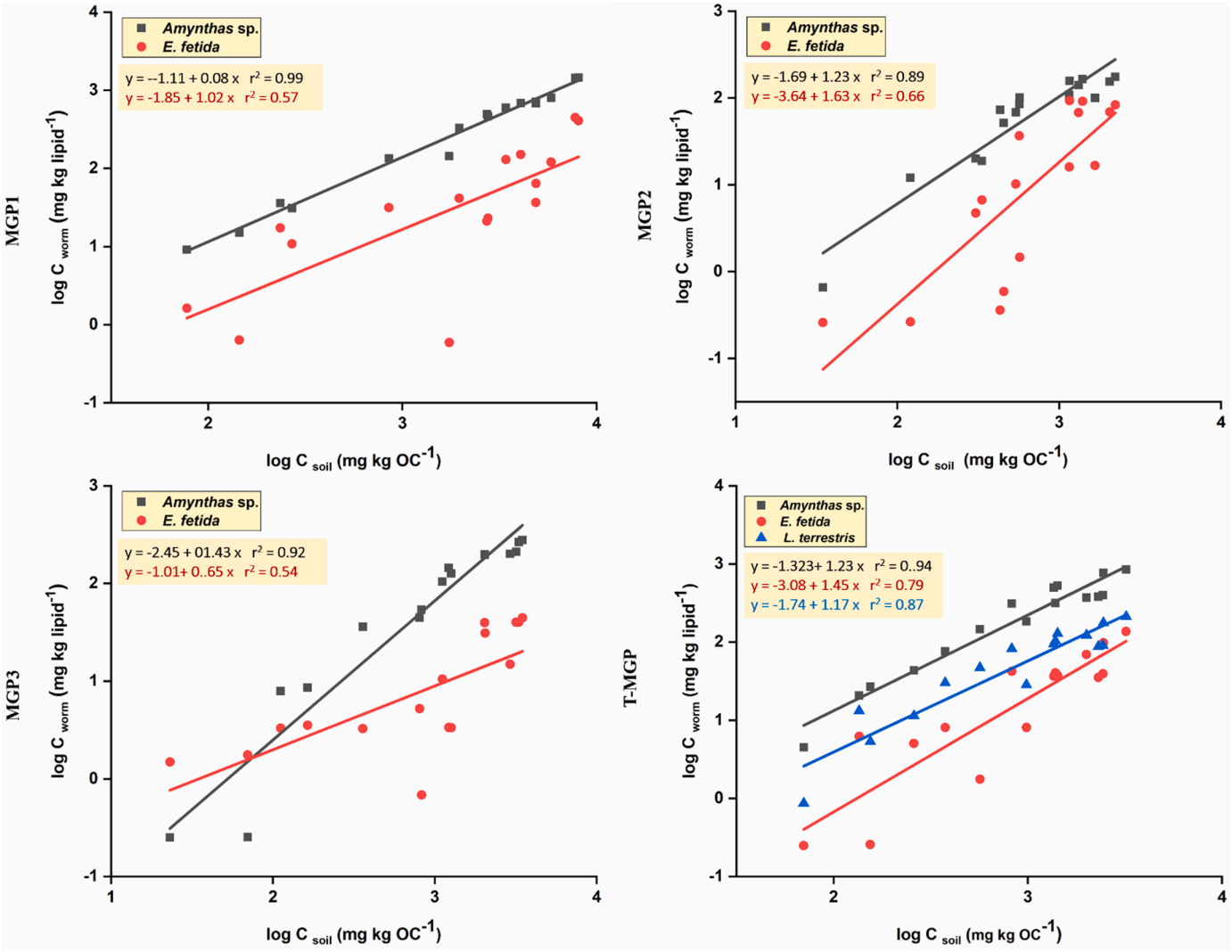

Fig. 2. Relationship between log lipid normalised PAH concentration measured in earthworm tissues and log organic carbon normalised PAH concentration in the four MGP soils (MGP 1, MGP 2, MGP 3, T-MGP).

relatively increased accumulation in T-MGP, as hypothesised above due to treatment.

The BSAF values in our study exceed those of Parrish et al. (2006), who reported PAH BSAF as 0.011 for *E. fetida* and 0.007 for *L. terrestris* in MGP soil, but are significantly lower than the average BSAF of 0.23 (∑14 PAHs) reported for epigeic worms (*E. fetida* and *E. andrei*) using field contaminated soils and soil/sediment mixtures (Jager et al., 2003). However, Ma et al. (1998) reported an average value of 0.1 (ranging from 0.03 to 0.26) for epi-endogeic *L. rubellus* in twelve contaminated field soils, which is comparable to the values for *Amynthas* sp. Further, our results are far lower than the values (0.13–0.41) reported by Krauss and Wilcke (2001) for *L. terrestris*, but similar to Cachada et al. (2018) for *E. andrei* (0.026-0.16 for ∑10 PAHs) in urban soils. Differences in the BSAF values reported are likely due to differences in PAH sequestration and aging in the different soils, different PAH congeners measured, and different feeding behaviour of the earthworm species. For example, PAH contamination in urban soils used by Krauss and Wilcke (2001) were probably less aged and sequestered than the strongly weathered MGP soils used in the present study.

The BSAF values in our study were all significantly lower (for *Amynthas* sp., *E. fetida*, and *L. terrestris* were 8.6, 57.8, and 14.7 times lower, respectively) than the EqPT-predicted BSAF theoretical value of 1 expected if PAHs were in equilibrium between soil OC and worm lipid as assumed in the BSAF partitioning model (Di Toro et al., 1991). The BSAF model assumes (*i*) minimal capacity of the organism for PAH metabolism of PAHs (a BSAF value of <1 may suggest metabolism), (*ii*) the kinetics of sorption/desorption occurs more rapidly than uptake kinetics so that PAHs are bioavailable (BSAF values <1 show that bioavailability is reduced), (*iii*) PAH affinity for organism lipids equals the affinity for the organic carbon of the soil, and (*iv*) no other sorptive phase is present in the sediment/soil other than the OC. The BSAF values are independent of soil type, PAH species and are expected to be independent of K_{OW} (Krauss et al., 2000; Ma et al., 1998; Sijm et al., 2000). Many researchers have hypothesised that BSAF values less than 1 arise from reduced PAH bioavailability (Cachada et al., 2018; Cornelissen et al., 2006a; Kreitinger et al., 2007; Moermond et al., 2005; Oen et al., 2006) and in these historically contaminated soils, the BSAF values << 1 confirm constrained equilibrium consequent of strong sequestration with low bioavailability (Thorsen et al., 2004). Possibly a secondary strong sorptive phase such as black carbon is involved in these MGP soils. Nevertheless, all earthworm species in this study showed significant PAH accumulation in lipid fractions, and a strong to moderate linear relationship with OC normalised PAH concentration in soils (Fig. 2 & S4a) confirming the importance of soil as an exposure pathway for soil borne PAHs.

Table 2
Calculated biota-soil accumulation factors (BSAFs) in earthworms (*Amynthas* sp., *E. fetida* and *L. terrestris*) after 14 days exposure to MGP soils (MGP1, MGP2, MGP3 and T-MGP). Values are the mean ($n = 4$) ± standard error.

PAH [1]	*Amynthas* sp.				*E. fetida*				*L. terrestris*
	MGP1	MGP2	MGP3	T-MGP	MGP1	MGP2	MGP3	T-MGP	T-MGP
NAP	0.117 ± 0.038	0.100 ± 0.016	0.004 ± <0.001	0.174 ± 0.059	0.021 ± 0.001	0.002 ± <0.001	0.025 ± 0.005	0.002 ± <0.001	0.034 ± 0.005
ACEY	0.115 ± 0.011	0.066 ± 0.012	0.052 ± 0.003	0.168 ± 0.052	0.040 ± 0.001	0.016 ± 0.001	0.022 ± 0.004	0.020 ± <0.001	0.044 ± 0.004
ACE	0.104 ± 0.025	0.019 ± 0.006	0.011 ± <0.001	0.064 ± 0.061	0.004 <0.001	0.007 ± <0.001	0.064 ± 0.017	0.004 ± <0.001	0.012 ± 0.003
FLU	0.154 ± 0.013	0.056 ± 0.010	0.071 ± 0.002	0.153 ± 0.049	0.073 ± 0.002	0.020 ± 0.001	0.030 ± 0.011	0.046 ± 0.001	0.098 ± 0.011
PHE	0.147 ± 0.009	0.060 ± 0.012	0.055 ± 0.002	0.187 ± 0.056	0.007 ± <0.001	0.010 ± <0.001	0.006 ± 0.004	0.008 ± <0.001	0.029 ± 0.003
ANT	0.157 ± 0.010	0.127 ± 0.035	0.100 ± 0.010	0.203 ± 0.056	0.037 ± 0.001	0.019 ± 0.001	0.009 ± 0.009	0.021 ± 0.001	0.081 ± 0.012
FLUA	0.180 ± 0.010	0.079 ± 0.009	0.080 ± 0.005	0.165 ± 0.037	0.050± 0.001	0.037 ± 0.001	0.012 ± 0.001	0.015 ± <0.001	0.038 ± 0.003
PYR	0.186 ± 0.011	0.075 ± 0.008	0.080 ± 0.005	0.164 ± 0.037	0.058 ± 0.002	0.034 ± 0.001	0.013 ± 0.001	0.016 ± <0.001	0.037 ± 0.003
BAA	0.176 ± 0.015	0.107 ± 0.008	0.096 ± 0.006	0.277 ± 0.052	0.038 ± 0.001	0.052 ± 0.001	0.015 ± 0.002	0.029 ± 0.001	0.075 ± 0.006
CHR	0.169 ± 0.015	0.135 ± 0.009	0.097 ± 0.006	0.185 ± 0.039	0.037 ± 0.001	0.080 ± 0.002	0.019 ± 0.002	0.035 ± 0.001	0.061 ± 0.004
BBF	0.137 ± 0.015	0.118 ± 0.010	0.066 ± 0.003	0.265 ± 0.063	0.021 ± <0.001	0.066 ± 0.001	0.013 ± 0.001	0.043 ± 0.003	0.066 ± 0.006
BKF	0.167 ± 0.017	0.150 ± 0.009	0.094 ± 0.004	0.376 ± 0.086	0.021 ± <0.001	0.065 ± 0.001	0.009 ± 0.005	0.051 ± 0.003	0.099 ± 0.009
BAP	0.139 ± 0.015	0.094 ± 0.010	0.069 ± 0.003	0.315 ± 0.075	0.013 ± <0.001	0.014 ± 0.001	0.005 ± 0.002	0.040 ± 0.003	0.072 ± 0.006
DAH	0.082 ± 0.007	0.133 ± 0.006	0.065 ± 0.006	0.258 ± 0.043	0.0003 ± <0.001	0.001 ± <0.001	0.001 ± <0.001	0.003 ± 0.002	0.083 ± 0.008
BGP	0.168 ± 0.014	0.169 ± 0.015	0.100 ± 0.004	0.365 ± 0.078	0.008 ± <0.001	0.001 ± <0.001	0.003 ± 0.002	0.027 ± 0.003	0.069 ± 0.006
IND	0.182 ± 0.016	0.177 ± 0.017	0.118 ± 0.003	0.371 ± 0.082	0.008 ± <0.001	0.003 ± <0.001	0.003 ± 0.001	0.026 ± 0.004	0.091 ± 0.008
Median[2]	0.155 [a]	0.104 [a]	0.075 [a]	0.195 [a]	0.021 [b]	0.017 [b]	0.012 [b]	0.024 [b]	0.068*
IQR[3]	0.132-0.170	0.073-0.129	0.063-0.096	0.167-0.277	0.008-0.039	0.006-0.041	0.006-0.020	0.014-0.036	0.038-0.081

[1] Polycyclic aromatic hydrocarbon (PAH) abbreviations: NAP: Naphthalene, ACEY: Acenaphthylene, ACE: Acenaphthene, FLU: Fluorene, PHE: Phenanthrene, ANT: Anthracene, FlUA: Fluoranthene, PYR: Pyrene, BAA: Benz[a]anthracene, CHR: Chrysene, BBF: Benzo[b]fluoranthene, BKF: Benzo[k]fluoranthene, BAP: Benzo[a]pyrene, DAH: Dibenz[a,h]anthracene, BGP: Benzo[g,h,i]perylene, IND: Indeno[1,2,3-c,d]pyrene

[2] Letters show statistical differences between *Amynthas* sp. and *E. fetida* in each soil ($p = 0.05$)

[3] Interquartile range

* The median BSAF for *L. terrestris* was significantly greater than the value for *E. fetida*, but smaller than that of *Amynthas* sp. in T-MGP soil ($p = 0.05$).

3.4. Bioconcentration factors (BCFs) and K_{OW} values

Bioconcentration factors (BCFs) can define the organism and pore water concentration relationship (Sijm et al., 2000), as represented in Eq. (3). Using passive samplers in the estimation of C_{PW} facilitates the BCF approach (Eq. (3)). Pore water is reported as a primary earthworm bioavailability phase (Di Toro et al., 1991; Kraaij et al., 2003), although the importance of soil OC for earthworm accumulation is recognised (Mayer et al., 2007; Qi and Chen, 2010; Wang et al., 2014). Median BCF values for $\sum$16 PAHs based on accumulation from pore water for *Amynthas* sp. were significantly higher than those of *E. fetida* in each of the soils ($p < 0.05$) except in MGP3 ($p > 0.05$) (Table 3), with the highest value for both the species observed in MGP1 because of high pore water concentration in this soil, consistent with results observed earlier (Fig. S2). The corresponding median value for *L. terrestris* was not statistically different from either of the other two species ($p > 0.05$). Whilst there was a significant difference in the BSAF values between earthworm species, differences in BCFs between species were much smaller indicating that uptake from pore water was less differentiated by earthworms' habit and feeding behaviour. The contribution of gut uptake to PAH accumulation is disregarded in Eq. (3), which considers pore water the main route of exposure for earthworm species. Log BCF values showed a general increase with increasing PAH molecular weight (Table 3, Figure S6). However, a plateauing/decreasing trend for the HMW PAHs, notably for *E. fetida* (Table 3, Figure S6), suggests a restriction to the partitioning between water and the earthworm which is consistent with high energy needed for cavity formation for HMW PAHs to penetrate the organism's membrane (Jonker and van der Heijden, 2007), that was less evident for the burrowing species in which soil ingestion is relatively more important.

The accumulation of PAHs between pore water and the organism lipid is typically considered a simple partitioning process driven by the chemical hydrophobicity, that can often be well described by the K_{OW} (Jonker and van der Heijden, 2007). In EqPT, K_{OW} is assumed equivalent to BCF for bioaccumulation predictions (Jager, 1998). To test whether this could be applied for the three earthworm species, relationships were developed between the derived log BCF values calculated from measured POM derived C_{PW} (Eq. 3) and log K_{OW} values sourced from the two most widely used guidelines for soils and sediments, the USEPA (USEPA, 2003) and the RIVM (Verbruggen, 2012) (Fig. 3). Positive linear relationships were evident with both sets of K_{OW} values with stronger fit and correlation (gradient and Pearson's r closer to 1) for the burrowing species *Amynthas* sp. and *L. terrestris*, and with RIVM sourced-K_{OW} for all three species, probably because the RIVM K_{OW} values consider soils and terrestrial species in derivation (Fig. 3). The linearity confirms the passive process of PAH partitioning between pore water and the earthworms' lipid (Di Toro et al., 1991; Kraaij et al., 2003) and supports that the calculated BCF was determined at equilibrium (Yates et al., 2011). Nevertheless, the calculated BCF values here were derived from measured $C_{worm,\ lipid,}$ which comprised uptake from both pore

Table 3
Average logarithms of calculated Bioconcentration Factors (BCFs) for earthworms (*Amynthas* sp., *E. fetida* and *L. terrestris*) exposed to MGP soils (MGP1, MGP2, MGP3 and T-MGP) following the 14-d bioassay.

PAH[1]	log BCF (*Amynthas* sp. (L kg $^{-1}$))				log BCF (*E. fetida* (L kg $^{-1}$))				log BCF (*L. terrestris* (L kg $^{-1}$))
	MGP1	MGP2	MGP3	T-MGP	MGP1	MGP2	MGP3	T-MGP	T-MGP
NAP	4.636	4.777	3.088	3.850	3.887	3.118	3.927	1.829	3.147
ACEY	5.890	5.708	4.721	4.859	5.433	5.082	4.336	3.923	4.274
ACE	5.154	3.802	3.361	3.950	3.782	3.399	4.134	2.691	3.234
FLU	4.332	4.244	3.789	4.129	4.012	3.795	3.410	3.605	3.934
PHE	4.805	4.666	4.618	4.886	3.513	3.888	3.686	3.527	4.075
ANT	5.036	5.243	4.860	4.808	4.407	4.417	3.817	3.832	4.408
FLUA	5.421	5.303	5.348	5.437	4.869	4.980	4.527	4.404	4.801
PYR	5.509	5.412	5.423	5.552	5.001	5.062	4.629	4.544	4.909
BAA	6.406	6.064	6.201	6.366	5.742	5.747	5.401	5.474	5.886
CHR	6.436	6.238	6.282	6.492	5.777	6.012	5.584	5.764	6.012
BBF	6.987	6.525	6.735	7.088	6.167	6.270	6.015	6.295	6.485
BKF	6.929	6.435	6.604	6.953	6.030	6.069	5.606	6.085	6.375
BAP	7.103	6.454	6.785	7.201	6.078	5.622	5.655	6.304	6.562
DAH	6.616	6.104	6.375	6.668	4.231	4.162	4.478	4.749	6.178
BGP	7.283	6.493	6.784	7.300	5.979	4.186	5.204	6.167	6.579
IND	7.692	7.412	7.094	7.532	6.321	5.573	5.459	6.382	6.922
Median[2]	6.148 [a]	5.886 [a]	5.812 [a]	5.959 [a]	5.217 [b]	5.021 [b]	4.578 [a]	4.646 [b]	5.397*
Min	4.332	3.802	3.088	3.850	3.513	3.118	3.410	1.829	3.147
Max	7.692	7.412	7.094	7.532	6.321	6.270	6.015	6.382	6.922

[1] Polycyclic aromatic hydrocarbon (PAH) abbreviations: NAP: Naphthalene, ACEY: Acenaphthylene, ACE: Acenaphthene, FLU: Fluorene, PHE: Phenanthrene, ANT: Anthracene, FlUA: Fluoranthene, PYR: Pyrene, BAA: Benz[a]anthracene, CHR: Chrysene, BBF: Benzo[b]fluoranthene, BKF: Benzo[k]fluoranthene, BAP: Benzo[a]pyrene, DAH: Dibenz[a,h]anthracene, BGP: Benzo[g,h,i]perylene, IND: Indeno[1,2,3-c,d]pyrene

[2] Letters show statistical differences between *Amynthas* sp. and *E. fetida* in each soil ($p = 0.05$)

* The median BCF for *L. terrestris* was not significantly different from either of the other two species in T-MGP soil ($p = 0.05$)

water and soil and depended on earthworm species . Therefore, when individual values were examined, the log K_{OW} and log BCF values become numerically different as shown in Figure S6. The log K_{OW} values, typically underpredicted BCF for *Amynthas* sp. in all soils but overpredicted BCF values for *E. fetida* (Figure S6) which we explain by restricted movement of HMW PAHs across the membrane of the *E. fetida*, and significant uptake via the gut for *Amynthas* sp. For *L. terrestris,* overprediction occured for LMW PAHs to pyrene, with underprediction for HMW PAHs (Figure S6). Similarly, Arp et al. (2014) noted that for earthworm *Enchytraeus crypticus* in 21 historically contaminated soils, BCF using K_{OW} for PAHs with lower molecular weight than phenanthrene were overestimated, and were underestimated for PAHs with molecular weight greater than phenanthrene. The results indicate that using K_{OW} values as a proxy of BCF for historically contaminated soils does not provide accurate prediction of earthworm bioaccumulation in these soils as further described below.

When predicted BSAF values for $\sum$16 PAHs (Eq. 5) calculated from predicted $C_{worm,lipid}$ using POM derived C_{PW} (Eq. 4) were compared with the BSAF values calculated from measured $C_{worm,\ lipid}$ (Fig. 4), *Amynthas* sp. and *L. terrestris* showed a ratio within a factor of 10 for most PAHs, although underprediction for HMW PAHs. For *E. fetida* overestimation was by over 100 fold compared to observed values especially for 5-6 ring compounds, consistent with prior results (Fig. 4 & S6). These findings demonstrate that the relationship between BCF and K_{OW} values for PAHs is complicated and depends on the target receptor and the relative importance of different exposure routes. The EqPT does not take into account differences in uptake between organisms, nor changing bioavailability through different exposure routes (e.g. gut uptake). Moreover, in historically contaminated soils with low pore water concentration, gut uptake from soil becomes relatively more important which further limits EqPT-based modelling of bioaccumulation in terresterial organisms. Our results demonstrate, therefore, that K_{OW} values may not provide a good proxy for BCF in the prediction of earthworm bioaccumulation in these historically contaminated soils. In ecological risk assessment, BCF values used require validation for relevant species and sites.

4. Conclusion

This study highlighted distinct differences between PAH concentration in three earthworm ecotypes in four historically contaminated MGP soils related to the biological process (gut uptake) and physiology (feeding/burrowing habit) of the different species. The deeply burrowing *Amynthas* sp., showed the maxiumum concentration and accumulation yet recorded in an earthworm species, the maximum concentration of the 5-6 ring PAHs, and accumulation strongly related to soil OC concentrations. The epigeic surface-dwelling *E. fetida* showed the lowest concentration. This work has clearly demonstrated the significance of soil sorbed PAHs for partitioning into earthworm lipids in all tested ecotypes especially for desorption-resistant HMW PAHs with low pore water concentrations, and in burrowing species with significant gut uptake through ingested soil particles. Low BSAF values reflected the low bioavailability in the historically contaminated soils through aging, sequestration and PAH sorption to secondary sorptive phases. The BCF values were species specific, showing stronger relationships with K_{OW} for burrowing *Amynthas* sp. and *L. terrestris* due to intimate soil/porewater contact. The EqPT-predicted BSAF values demonstrated that using K_{OW} as a proxy for BCF is complicated by differences in soils, especially for historically contaminated soils, and differences in receptor organisms. On the whole, this study has indicated that including endogeic burrowing species in bioaccumulation studies in addition to epigeic *E. fetida* for historically contaminated soils, improves prediction of bioavailability in exposure risk assessment. Future studies could include efficacy of other passive sampling techniques besides POM. Moreover, there was a lack of *L. terrestris* supply for testing in this study due to an extreme seasonal drought in Australia. Therefore, testing this species in a wider range of soils, along with other representative deep burrower species such as *A. caliginosa,* are recommended to validate and extend results here.

Authorship contribution statement

Conceived and designed the experiments: Atefeh Esmaeili, Susan Wilson, Oliver Knox,. Contributed reagents/materials/analysis tools: Atefeh Esmaeili, Susan Wilson, Oliver Knox,. Performed the

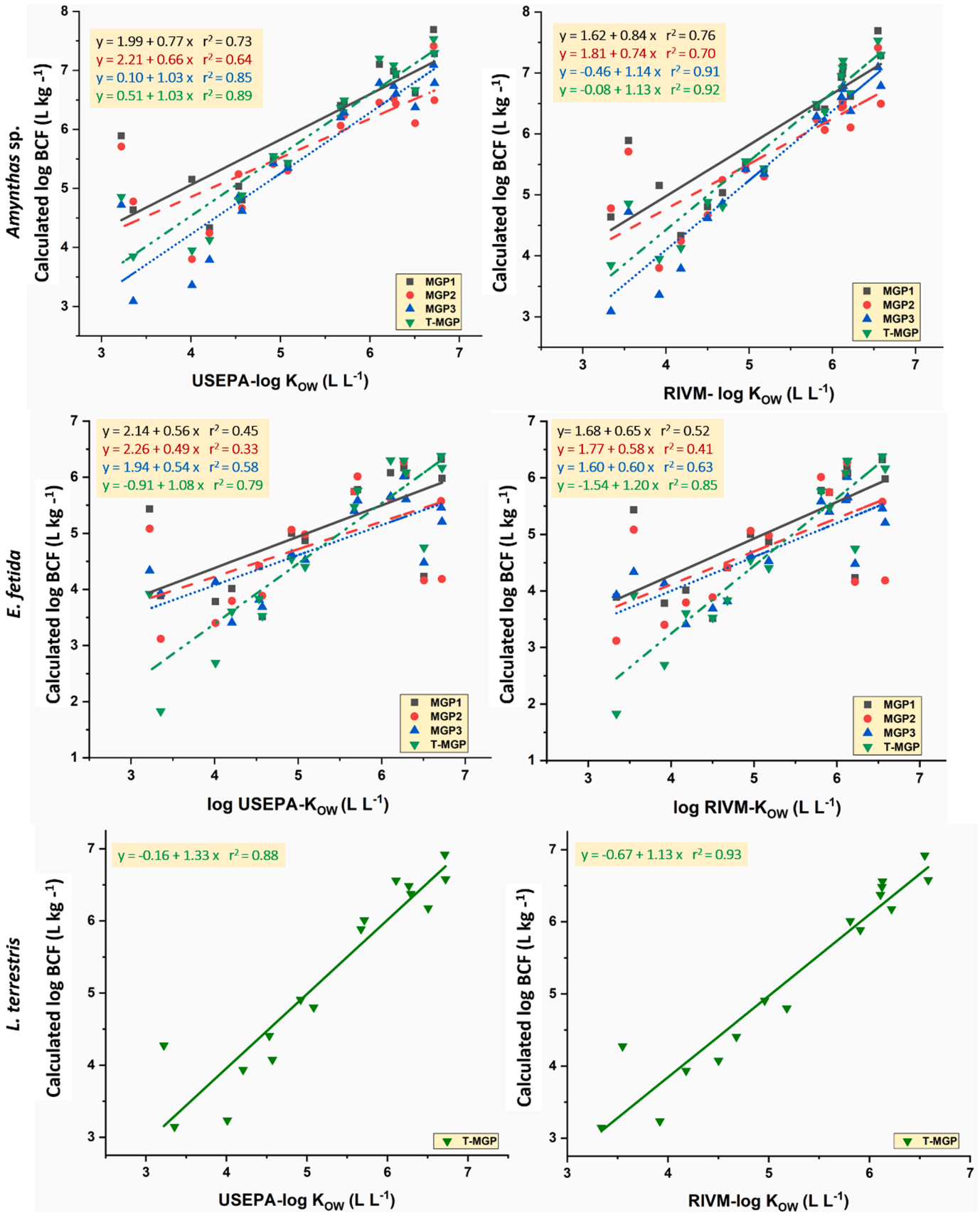

Fig. 3. Relationships between calculated log BCF values for earthworms and log K_{OW} sourced from USEPA (left) and RIVM (right) guidelines in the four MGP soils (MGP 1, MGP 2, MGP 3, T-MGP).

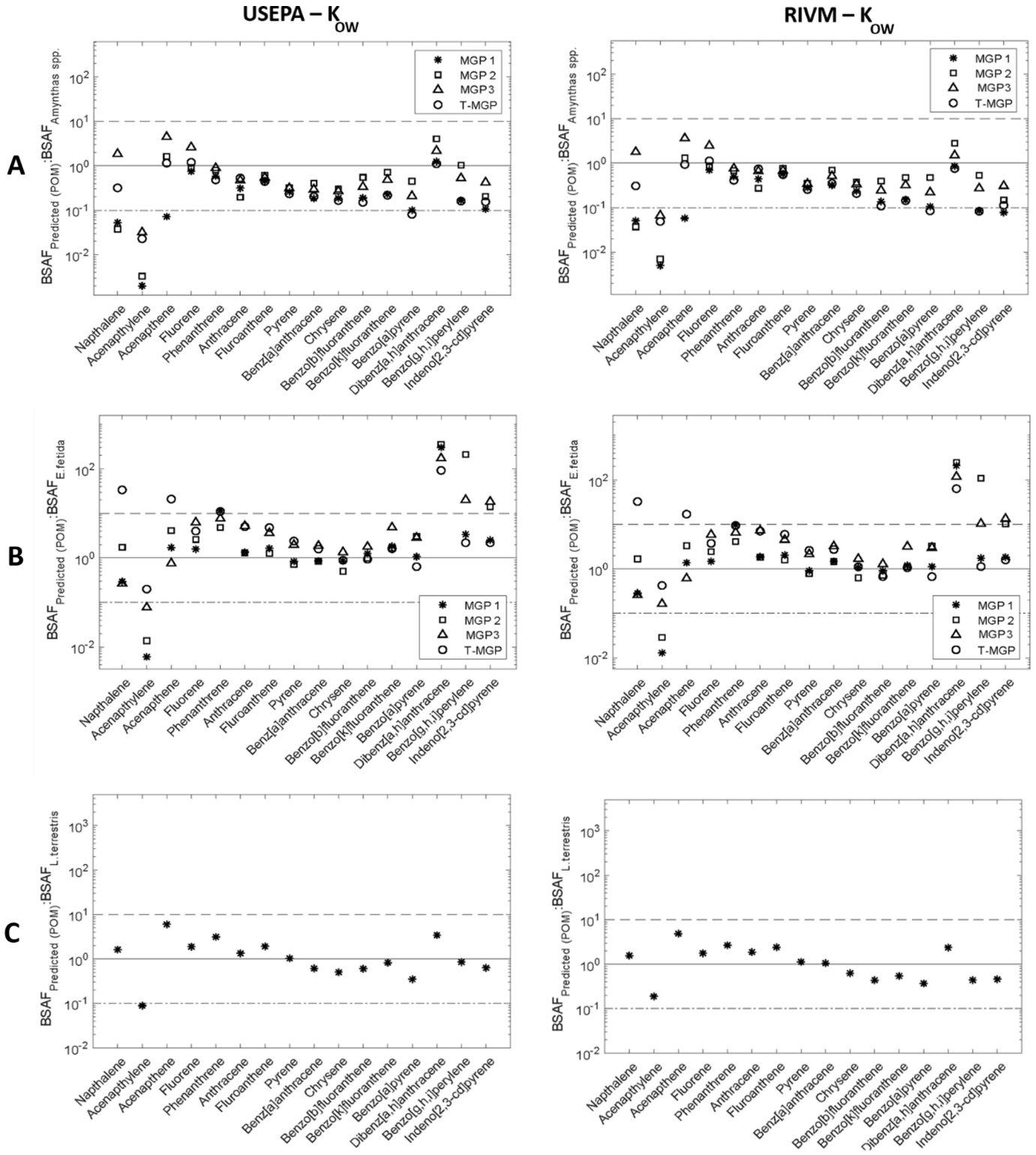

Fig. 4. Ratio between predicted biota-soil accumulation factors (BSAFs) and calculated BSAFs in soil exposed earthworms. BSAFs predicted from measurement of freely dissolved concentrations of PAHs in soil pore water, using passive samplers (POM). Solid line: 1:1 relationship ($BSAF_{predicted}$ / $BSAF_{calculated}$ = 1). Stippled lines: One order of magnitude below and above the 1:1 relationship, respectively. Left side: USEPA-K_{OW} used as a proxy for BCF; right side: RIVM-K_{OW} used as a proxy for BCF. A: *Amynthas* sp.; B: *E. fetida* and, C: *L. terrestris.*

experiments: Atefeh Esmaeili. Analyzed the data: Atefeh Esmaeili. Writing-original draft: Atefeh Esmaeili. Review, and editing: Atefeh Esmaeili, Susan Wilson, Oliver Knox, Albert Juhasz.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2022.100175.

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