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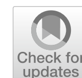
ORIGINAL PAPER



Receptor-mediated potencies of polycyclic aromatic hydrocarbons in urban sediments: comparisons of toxic equivalency risk assessment

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Abstract
The Klip River, flowing through South Africa's most populated urban area—Soweto and Lenasia—is subject to various pollution and anthropogenic influences, including great concentrations of polycyclic aromatic hydrocarbons. The aims were to determine the aryl-hydrocarbon receptor-mediated potencies of the 16 priority polycyclic aromatic hydrocarbons in sediments of the Klip River, using chemical- and bio-analytical assessments of hazard, and to compare these results with international sediment quality guidelines. Sediment samples were collected from nine sites during the dry seasons of 2013 and 2014. Two sets of toxic equivalents were calculated from analytically obtained polycyclic aromatic hydrocarbon concentrations using: (1) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalency factors and (2) relative potency factors for fish. The fraction of the sediment extracts containing polycyclic aromatic hydrocarbons was assayed with the H4IIE-*luc* reporter gene bio-assay, and the aryl-hydrocarbon receptor potency expressed as bio-assay equivalents. The bio-assay equivalents and tetrachlorodibenzo-*p*-dioxin equivalency factors were compared to Canadian sediment quality guidelines and of the three approaches, the bio-assay equivalents and the relative potency factors for fish proved the most protective. Results of this study are proof of the utility of combining biological analysis with instrumental analysis when predicting hazard. Even though there were instances where the bio-assay equivalents were orders of magnitude greater than the tetrachlorodibenzo-*p*-dioxin equivalency factors, the results still showed similar trends. It was concluded that hazard from aryl-hydrocarbon receptor-mediated potency to adversely affect aquatic organisms in the Klip River was relatively great, which indicated the need for further investigation into possible mitigations.

Keywords Hazards · Bio-assay equivalents · H4IIE-*luc* · Sediment · Toxic equivalents

Introduction

The aryl-hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates many of the biological effects of ligands, such as dioxin-like compounds, including polychlorinated dibenzo-*p*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) (Chandra Suryani et al. 2015) and polycyclic aromatic hydrocarbons (PAHs)

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(Gualtieri et al. 2011). Apart from the well-studied toxicological effects mediated by the AhR, it also mediates physiological effects from endogenous compounds such as natural ligands, including flavonoids and tryptophan derivatives and endo-3-carbinol, as well as bacterial metabolites, including phenazines and naphthoquinones (Tian et al. 2015). These physiological responses include cell differentiation of immunological cells, host defence, and homeostasis (Tian et al. 2015).

The detoxification process of exogenous compounds (ligands) through the AhR can be summarised as follows: when AhR-ligands enter the cytoplasm of cells they bind to the AhR forming a transformed ligand-AhR complex (Furue et al. 2014). This newly formed complex enters the nucleus, where it rapidly forms a heterodimeric nuclear complex with the aryl-hydrocarbon receptor nuclear translator (ARNT) protein (Hilscherova et al. 2000a, b). This dimer-complex binds onto the dioxin response element (DRE)—a specific DNA sequence in the *CYP1A1* promoter (Denison and Heath-Pagliuso 1998; Hilscherova et al. 2000a, b). Attachment to the DRE leads to transcription of adjacent responsive genes such as *CYP1A1* (Hilscherova et al. 2000a, b), which results in upregulating transcription of proteins such as the P450 enzymes, responsible for detoxification by oxidation (Baird et al. 2005; Megna et al. 2017).

PAHs are ubiquitous in the environment, produced in large volumes, and released into the atmosphere by anthropogenic processes. PAHs are known carcinogens and have adverse effects on humans and wildlife (Larsson et al. 2012). Of the 16 priority PAHs, the following congeners activate the AhR: benz(a)anthracene [BaA], chrysene [Chr], benzo(b)fluoranthene [BbF], benzo(k)fluoranthene [BkF], benzo(a)pyrene [BaP], indeno(1,2,3-cd)pyrene [InP], and dibenz(ah)anthracene [DBA] (Villeneuve et al. 2002; Zhao et al. 2014)—collectively referred to as carcinogenic PAHs (CPAHs).

Knowledge about concentrations of chemical pollutants in the environment provides only partial information on hazard or risk and/or biological effects; they might pose to local biota: knowing the concentrations of a compound does not explain risk to the environment unless the biota is negatively affected by it. The toxic equivalency (TEQ) concept was developed by the World Health Organisation (WHO), which uses the relative effects potency of individual congeners that share a common mechanism of action to calculate a predicted biological effect from the concentrations, moving a step beyond only concentrations (Van den Berg et al. 2006). Potencies relative to a reference compound are allocated to each congener. These are referred to as toxic equivalent factors (TEFs) and because the reference compound for the activation of the AhR is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)—the most potent AhR-ligand for most species and endpoints, TEF_{TCDD} is used in this text to distinguish it from systems with other reference compounds (Zhang et al. 2011). The instrumentally derived concentration of each compound is multiplied with the compound's TEF, resulting in a TEQ per compound. The TEQs per site are summed to get an expected (or estimated) prediction of the risk posed by the mixture of pollutants mediated via activation of the AhR. Such a scheme exists for a selection of the PCDD/F congeners and dioxin-like PCBs whose common mode of action is activating the AhR (Van den Berg et al. 2006).

PAH congeners that are also AhR-ligands have not been included in the WHO scheme discussed in the preceding paragraph. However, there are a variety of TEF_{TCDD} sets available in the literature for PAHs that have been derived with different assays. Barron et al. (2004), for example, derived PAH-specific fish potency factors (FPFs) that are used to evaluate the biological risk towards fish (Dong et al. 2014; Fang et al. 2014). These factors were calculated specifically using published data on *CYP1A* induction and AhR binding of PAHs in fish—these factors are expressed in terms of 2,3,7,8-TCDD (Barron et al. 2004). Additionally, Villeneuve et al. (2002) determined relative effects potencies (REPs) specially for PAHs using the in vitro luciferase assay with H4IIE-*luc* recombinant rat hepatoma cells.

The H4IIE-*luc* culture is a genetically engineered reporter gene-assay that has firefly luciferase stably transfected into rat hepatoma cells. When AhR-ligands are present, luciferase, in addition to the cytochrome enzymes, is expressed, and when the substrate luciferin is provided, light is produced. The amount of light emitted is directly proportional to the synergistic effect of the AhR agonist mix in the sample (Hilscherova et al. 2000a, b). Toxic potency of the sample is quantified in terms of a known concentration of the reference compound, TCDD. This quantification is based on the assumption that the investigated sample is a diluted form of the reference material or a mixture of chemicals behaving like TCDD (Yoo et al. 2006). The results are given as relative effect potencies (REPs) also referred to as biological equivalencies (BEQs). The H4IIE-*luc* bio-assay has been implemented as a useful tool in ecological risk assessment (Song et al. 2006; Hong et al. 2012; Xia et al. 2014). In this paper, the specific toxicity mediated via the AhR activation caused by the PAH-containing fraction of the environmental (sediment) extracts using the H4IIE-*luc* bio-assay was investigated. The BEQ values determined by the reporter gene assay take all interactions between the target compounds into consideration and a risk based on actual biological response is determined, and not merely predicted as it is for the TEQs based on different TEFs.

This study focused on the urban area of Soweto, a huge township south-west of the city of Johannesburg. This urban area is one of South Africa's most populated areas (Population Labs 2011). The upper reaches of the Klip River flow through the densely populated Soweto and Lenasia,

south-west from Johannesburg, in the most industrialised province of South Africa, the Gauteng province. The Klip River was described as a highly polluted river as early as 2006 (McCarthy and Venter 2006), and Roos et al. (2011) reported great concentrations of PAHs and other organic pollutants in Gauteng. This river flows into the second largest river of South Africa, the Vaal River, which in turn provides the majority of potable water to Gauteng province (DWAS 2004), supplying over 12 million residents. Therefore, pollutants in the Klip River system will impact an area larger than its own catchment. Concentrations of the 16 priority United States Environmental Protection Agency (USEPA) PAHs in the sediment from Soweto had been determined previously (Pheiffer et al. 2018). The aim of this study was to quantify the biological responses caused by PAHs from the sediment of Soweto (sampled in 2013 and 2014) using the H4IIE-*luc* assay (BEQs) and compare them to two separate TEQs. These were generated for the CPAHs quantified in the sediment extracts using two different sets of TEF values: (1) TEF values derived by Villeneuve et al. (2002) where they exposed H4IIE-*luc* cells to single CPAH congeners, comparing responses to 2,3,7,8-TCDD and (2) Barron et al. (2004) who published FPFs for the AhR-mediated effects of individual CPAHs on fish relative to 2,3,7,8-TCDD. The predicted risk based on the BEQs of the H4IIE-*luc* assay of the current study's sediment extracts, and the two sets of TEQs were compared to international sediment quality guidelines. The value of this research is to indicate that when risk is predicted using biological responses, the outcome seems to be more protective towards the environment than when it is based on concentration calculations only.

Materials and methods

Site selection

Sampling sites for the study area correspond to those described by Pheiffer et al. (2018): Three sites were in the Klip River and nine in a tributary, the Klip Spruit (Fig. 1). Five of these nine sites were in the rivers themselves, and the others were in dams (S2, 3, 7 and 9). The sites were chosen to represent the drainage in this urban area.

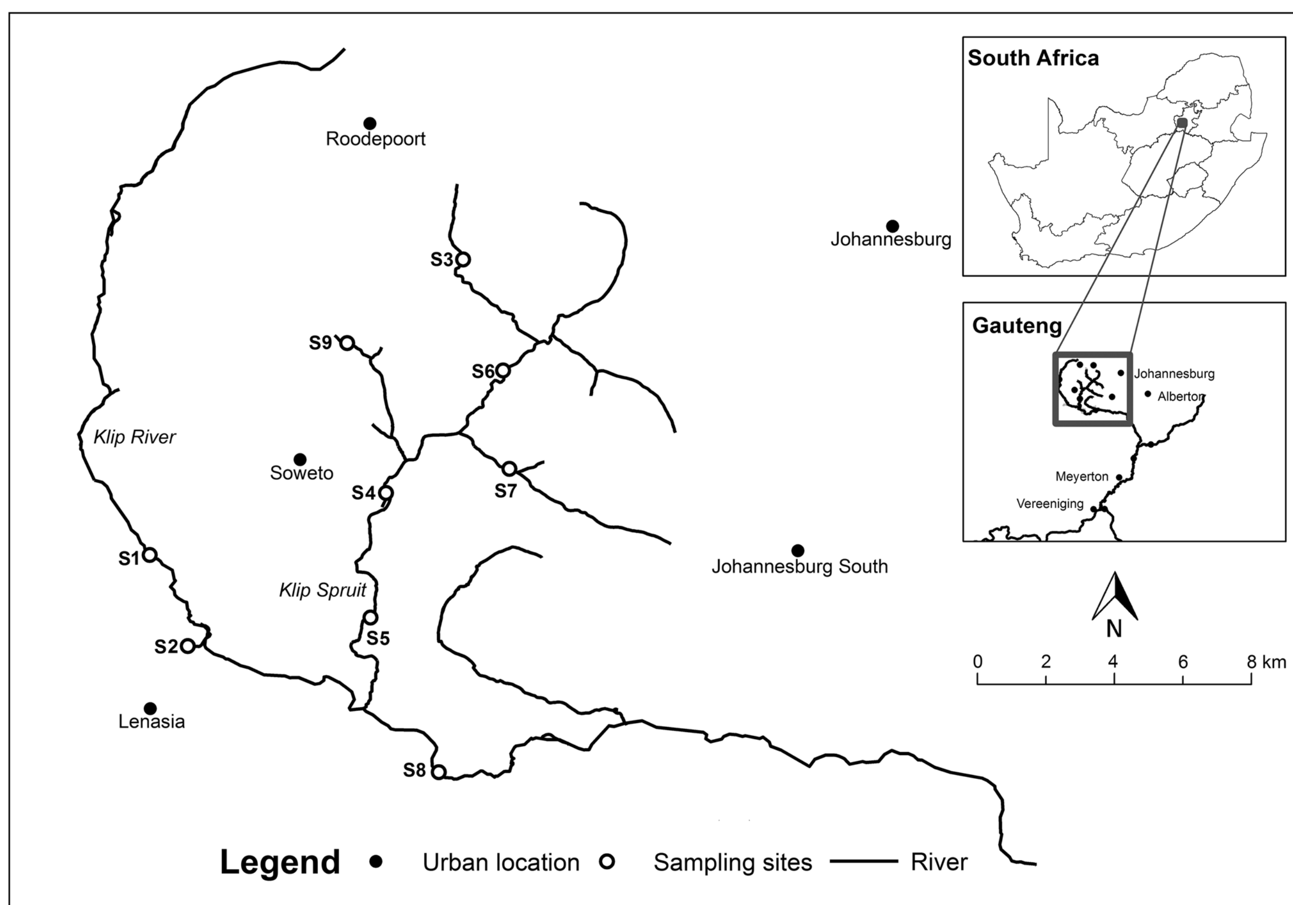

Fig. 1 Sampling sites in the upper Klip River catchment, in the urban area of Soweto

Sample collection and extraction

Sediment was collected once during the low flow conditions coinciding with the Austral winters (June/July) of 2013 and again in 2014. At each site between four to six subsamples were composited from surface sediment (top 15 cm) into a pre-cleaned metal container, mixed thoroughly, and stored in chemically resistant high-density polyethylene Nalgene® bottles (Jones 2011), which were pre-cleaned with analytical grade acetone and hexane (Honeywell-Burdick and Jackson, USA) (USEPA 1994a). The samples were kept at 4 °C in the field and stored at − 20 °C in the laboratory until extraction. The sediment samples were thawed, air-dried in the dark, ground, and sieved through a 0.5-mm mesh to create a homogenous sample (Kralik 1999) (Fig. 2).

Extraction and clean-up method was adapted from methods described previously by Vogt et al. (2019). Five grams of dried sediment were accurately weighed and extracted with 3:1 dichloromethane (DCM)/hexane (v/v) using accelerated solvent extraction (Dionex ASE 150, ThermoFischer Scientific, Austria) at 100 °C with a static time of five minutes (USEPA 1996). Thereafter, extracts were concentrated to near dryness under a gentle nitrogen gas flow (TurboVap, Caliper Life Science, USA). The extracts were resuspended into 2 mL DCM (Honeywell-Burdick and Jackson, USA) (Fig. 2) and passed through gel permeation chromatography (GPC) columns (19 × 150 mm, and 19 × 300 mm, Envirogel™) with DCM as mobile phase (USEPA 1994b), using a Waters® (USA) high-pressure liquid chromatography system to collect the size fraction containing the PAHs (Fig. 2). To ensure that the collected PAH-containing fraction does not contain cytotoxic sulphur, size-exclusion calibration standards were run to determine the elution time of elemental sulphur (USEPA 1994b), and PAH elution times were predetermined using the 16 USEPA PAHs. The PAH-containing fraction was concentrated and reconstituted into 10 mL hexane. A final solid-phase extraction clean-up step was added using Supelco 12 mL 2 g/2 g LC-Si/Florisil® cartridges (USEPA 1996, 2007). Each cartridge was conditioned with hexane before loading the sample. Elution solvents were 24 mL 1:1 DCM/hexane (v/v), followed by 8 mL DCM. The final extract was evaporated to near dryness under a gentle stream of nitrogen and reconstituted into 1 mL hexane for the bio-assay. The extractions here were the same as for the instrumental analyses (Pheiffer et al. 2018) without the addition of external standards (Fig. 2).

H4IIE-*luc* reporter gene bio-assay

The H4IIE-*luc* cells (gift by University of Saskatchewan) were maintained aseptically in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% foetal

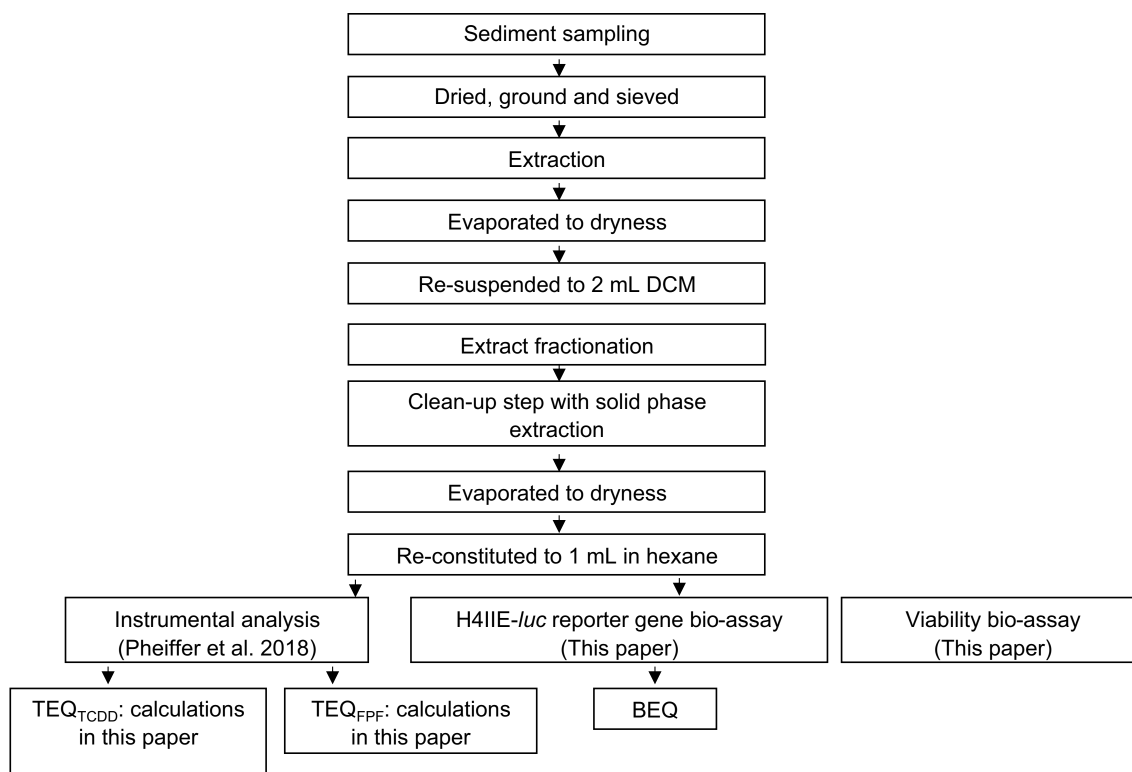

Fig. 2 Diagram summarising methods. The detail for each step is described in the text

bovine serum (Thermo Fisher Scientific) in tissue culture dishes kept in humidified air with 5% CO_2 and at 37 °C. The cells were rinsed with phosphate-buffered saline (PBS) (Sigma-Aldrich) and treated with 1.5 mL trypsin/EDTA (Gibco, Thermo Fisher Scientific) to passage them (Botha et al. 2019).

The luminescence bio-assay used is a modified version of that described by Eichbaum et al. (2018). The interior 60 wells of flat bottom 96-well plates were seeded with 250 µL H4IIE-*luc* cells at a density of 80,000 cells/mL. The external 36 wells were filled with 250 µL PBS, to create a homogenous micro-environment across each cell-containing well. The plates were incubated at standard conditions for 24 h before dosing. A threefold serial dilution of each extract was dosed in triplicate at a volume of 2.5 µL per well, to generate a dose–response curve. After 72 h incubation, the cells were inspected microscopically for viability and confluence. Cells were washed and lysed by adding 25 µL lysis buffer (Sigma-Aldrich) and flash frozen at − 80 °C for at least 10 min. Upon thawing the plate, it was placed into the luminometer (Berthold multi-mode micro-plate reader, model-LB941) where 100 µL luciferase assay reagent [20 mM tricine (Sigma-Aldrich), 1.07 mM $Mg(CO_3)_2Mg(OH)_2{\cdot}5H_2O$ (Sigma-Aldrich), 2.67 mM $MgSO_4{\cdot}7H_2O$ (Sigma-Aldrich), 0.1 mM EDTA-disodium salt (Sigma-Aldrich), 33.3 mM dithiothreitol (Sigma-Aldrich), 270 µM coenzyme A (Sigma-Aldrich), 530 µM ATP (Sigma-Aldrich) and 470 µM beetle luciferin (Malford)] (Villeneuve et al. 1999) was added automatically, and the luminescence was recorded. The digestion of luciferin by luciferase results in measurable light called relative light units (RLUs).

Quality control

Along with the sediment samples, controls were also dosed at 2.5 µL/well: a fourfold dilution series of TCDD (480, 120, 30, 7.5, 1.87, 0.468 pg/mL) (positive control); a 3-well solvent control (hexane); and a blank control (cells and nutrient media only) (Hilscherova et al. 2003).

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to test cell viability. It is a mitochondrial dehydrogenase-based assay where MTT (yellow tetrazolium salt) is reduced into purple formazan crystals (Mossman 1983; Vistica et al. 1991). The viability testing was performed parallel to the luminescence bio-assay (Fig. 2), and cells were dosed with the same series of samples and controls as in the luminescence assay, to test the cytotoxic ability of the samples.

Calculating bio-assay equivalents (BEQs)

Dose–response curves were prepared for the samples as well as the positive control by plotting the logarithm of the concentration (in the case of the control) or logarithm of the volume (in the case of the sample extract) on the *x*-axis, and the %TCDDmax on the *y*-axis. The %TCDDmax was calculated by expressing the luminescence of each sample dilution as a percentage of the maximum luminescence generated by the positive control (TCDD) (Lam et al. 2018). The REPs for the samples were calculated by dividing the effects concentration (EC_{20}, EC_{50}, EC_{80}) of the positive control by the $EC_{20–80}$ of the sample (Vogt et al. 2019). The unit of these REPs is mass TCDD-equivalents/volume extract.

Reporting all three REPs is necessary because it cannot be assumed that the AhR-ligand mixture in the environmental extracts will respond the same as 2,3,7,8-TCDD (Villeneuve et al. 2000). The REP values were back-calculated to represent the TCDD-equivalents (TCDD-eq) in terms of the sediment mass extracted (Koh et al. 2005). The limit of quantification (LOQ) for the H4IIE-*luc* bio-assay was calculated by determining the mean EC_0 for the TCDD response curves. The intercept with 95% confidence was calculated and used as the LOQ, back-calculated to a ngTCDD/g value (Villeneuve et al. 1999).

Toxic equivalence calculations and sediment quality guidelines

The toxic equivalent quotient (TEQ) for each sample was calculated (Fig. 2) (Eqs. 1 and 2).

$$\mathrm{TEQ}_{\mathrm{TCDD}} = \sum (C_i \times \mathrm{TEF}_a) \quad (1)$$

$$\mathrm{TEQ}_{\mathrm{FPF}} = \sum (C_i \times \mathrm{TEF}_b) \quad (2)$$

where TEF_a refers to values derived by Villeneuve et al. (2002) which they determined by the H4IIE-*luc* assay and TEF_b refers to values derived by Barron et al. (2004) as fish potency factors (FPF) from published data on CYP1A induction and AhR binding; and C_i represents the concentration of the respective CPAHs previously published (Pheiffer et al. 2018) (Table S1).

The Canadian TEQ guidelines for dioxin-like compounds were used in the assessment because South Africa does not have its own. This guideline was specifically created for the protection of aquatic life from dioxin-like compounds, using the TEF values for fish (CCME 2001), and since PAHs use the same mechanism of action, it was used to evaluate PAH toxicity. All equivalencies (TEQ_{TCDD}, TEQ_{FPF} and BEQ) were compared to the sediment quality guidelines. The Canadian guideline has a lower interim sediment quality guideline (ISQG) of 0.85 ngTEQ/kg and a higher probable effects level (PEL) of 21.5 ngTEQ/kg. Below the ISQG, detrimental effects to the sediment biota are rarely expected (low risk). Concentrations greater than the ISQG, yet smaller

than the PEL, are considered to cause occasional adverse effects (moderate risk), whereas levels greater than PEL are expectant to have frequent detrimental effects and pose a high risk to benthic organisms (CCME 2001).

Statistical analysis

All descriptive statistics were done with GraphPad Prism version 5 (www.graphpad.com). Statistical analysis included the Mann–Whitney *U* test, and unpaired, two-way *t* test. A difference between means or medians was considered significant when the *p* value was below 0.05. In cases where values were below the limit of quantification, half-LOQ was used. Although this method has limitations (Helsel 2005), it is a common approach when only a few values are < LOQ. Other approaches such as removing the data points, replacing the LOQ with a zero, or using the least detectable value have inherent biases (Helsel 2005). This approach was followed specifically when the instrumental data were used. Spearman's correlation was used to inspect the monotonic relationships between the datasets.

All multivariate statistics were done in Canoco for Windows Version 5. Multivariate statistical methods used in this study include principal component analysis (PCA), redundancy analysis (RDA), and multiple regression analysis. These analyses were used to investigate the nature of the differences/similarities between the various equivalencies relative to the sites (PCA), and how these equivalencies are affected by specific explanatory factors (RDA). A multiple regression analysis was applied to support the predictor variable results of the RDA.

Results and discussion

Results

The effect of the extracts on cell viability is reported in Table 1 together with the luminescence results. Luciferase induction for all samples was reproducible with coefficients of variance (CV) all less than 11%. The LOQ for the sediments was 15 ngBEQ/g (95% confidence).

Cell viability

The cell viability reported in Table 1 are the most concentrated (undiluted) extracts. Most of the 2013 extracts were cytotoxic (visually and statistically) to the cells: S1, S4, S5, S6, S7, and S9. In 2014, extracts from S1, S4, S5, and S6 were cytotoxic again (Table 1). Evidence of this cytotoxicity was also noticeable in the steep reduction in the luminescence dose–response curves caused by the undiluted (S1, S2, S4, S5, S6, S7, and S9: Fig. 3b, c), and even some of the diluted extracts (S4 and S6 (2013) Fig. 3b; and S5 (2014) Fig. 3c). Thus, the maximum dose-responses (Fig. 3) for the cytotoxic extracts are from the diluted versions of the samples (see Table 1).

Bio-luminescence assay

Although all the $REP_{20–80}$ values are reported (Table 1), responses often did not reach the 50% TCDDmax and many REP_{50} and REP_{80} values were therefore extrapolated. Only the REP_{20} will be used to compare between sites. BEQs for 2014 were generally greater than those for 2013 (Table 1)

Table 1 H4IIE-*luc* reporter gene bio-assay results showing BEQs (REP20, -50, -80) after exposure to sediment extracts (extrapolated data in italics; the greatest values per column in bold)

	2013 Sediment				2014 Sediment			
	%Cell viability	REP20 (ngBEQ/kg)	REP50 (ngBEQ/kg)	REP80 (ngBEQ/kg)	%Cell viability	REP20 (ngBEQ/kg)	REP50 (ngBEQ/kg)	REP80 (ngBEQ/kg)
S1	0*	#92 ± 12	273 ± 76	839 ± 398	0*	< LOQ		
S2	108	25 ± 2.2	30 ± 11	*37 ± 22*	84	#352 ± 76	290 ± 13	*246 ± 45*
S3	147	51.7 ± 14	49 ± 17	*50 ± 54*	116	55.9 ± 8.1	*62 ± 22*	*80 ± 65*
S4	0*	**221 ± 24**	**643 ± 236**	**1411 ± 434**	0*	**#817 ± 11**	***972 ± 302***	***1272 ± 657***
S5	7*	137 ± 18	332 ± 14	746 ± 90	5*	#679 ± 29	*447 ± 174*	*361 ± 167*
S6	25*	#122 ± 5.9	128 ± 29	*138 ± 58*	60*	79 ± 20	*58 ± 42*	*49 ± 56*
S7	0*	*<LOQ*			76	#46 ± 6.4	*32 ± 16*	*29 ± 26*
S8	90	32 ± 0.8	*25 ± 21*	*29 ± 42*	82	35 ± 2.5	*33 ± 6.0*	*32 ± 9.2*
S9	16*	< LOQ			112	6.7 ± 1.4	*1.2 ± 0.9*	*0.2 ± 0.2*

Viability results were also included as %cell viability obtained from the MTT assay

*Cytotoxicity of the raw extract, which is significantly different to the control ($p < 0.05$)

#Significantly greater than the other year ($p < 0.05$) ± standard deviation

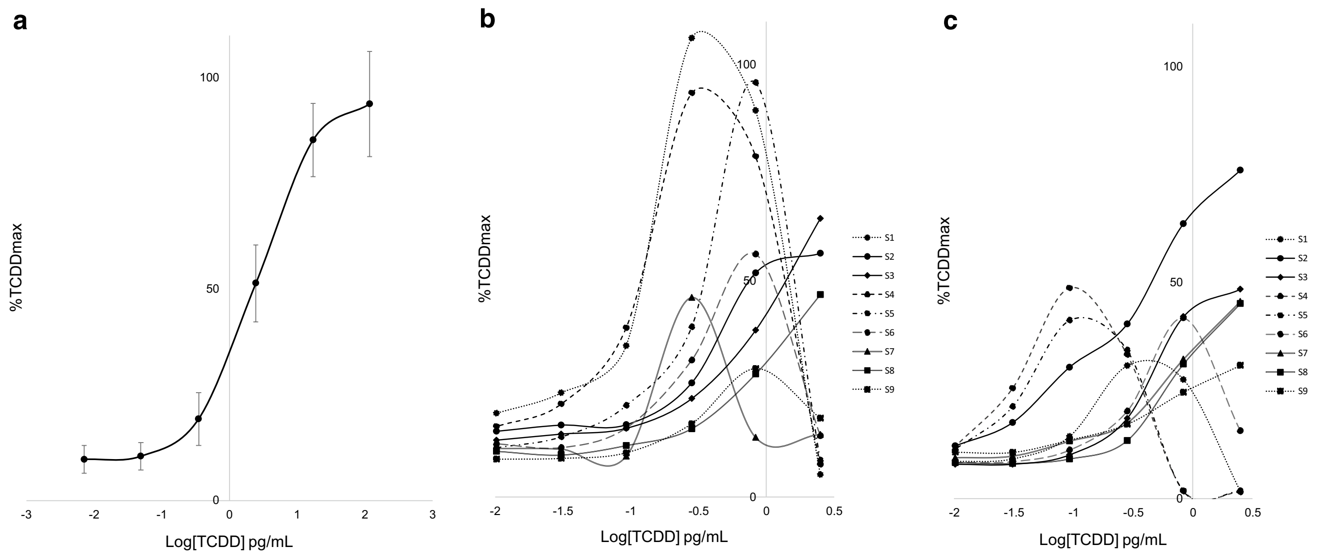

Fig. 3 Luminescence (%TCDDmax) for: **a** positive control: TCDD; **b** sediments sampled in 2013; **c** and sediments sampled in 2014. Bars in **a** is standard deviation (SD). SD bars were omitted from the other graphs for the sake of simplicity

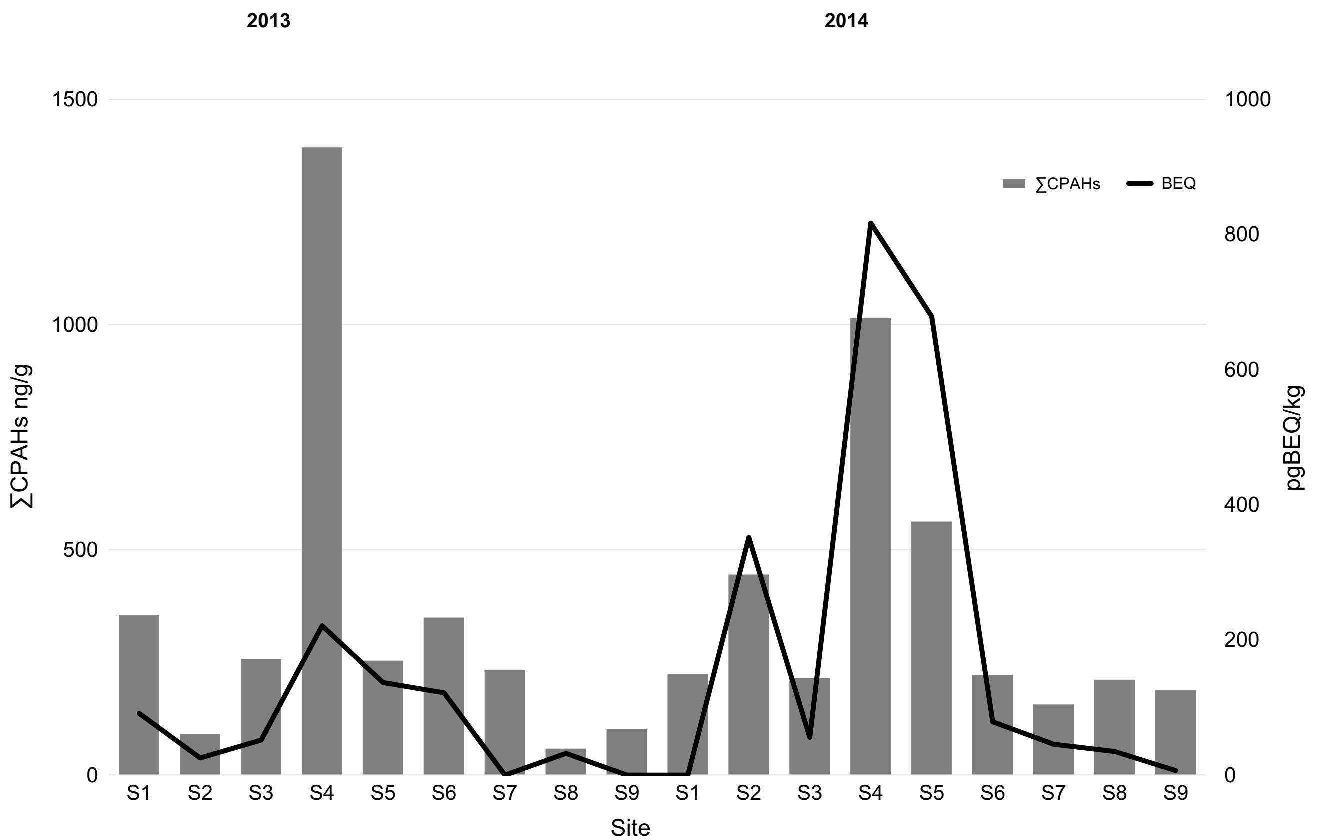

Fig. 4 BEQs compared to the ΣCPAHs for sediments of 2013 and 2014: dual axis line graph showing the relationship between ΣCPAHs (left axis; data obtained from Pheiffer et al. 2018) and BEQs (right axis)

even though the greatest concentration of ΣCPAH was measured in 2013 at S4 (Fig. 3). However, this site did not elicit the largest response from cells—most likely because of cytotoxicity (Table 1). It was site S4 of 2014 that had the greatest BEQ $_{20}$ and also the greatest concentration of ΣCPAH (Table 1, Fig. 4). The BEQ of S4 and S5 (both 2014) was fourfold greater than that of 2013 (Table 1). The only sites that had a significant ($p < 0.05$) temporal decrease were sites S1 and S6. S1 was also the only site from 2014 that was less than the LOQ (Table 1). In addition to S4, sampling sites S2, S5, and S7 exhibited a significant increase ($p < 0.05$) over the two sampling years (Table 1). S6 and S8 also had temporal increases (not significant) from < LOQ to quantifiable BEQs (Table 1). The general increase in 2014 BEQs suggests that there must have been an increase in AhR-ligands between the sampling events.

The extraction method to trap/consolidate the CPAHs into one fraction was effective to the extent that the BEQ (derived from bio-assay) and the ΣCPAH (instrumental analysis) pattern among the sites generally corresponded (Fig. 4), and the correlation between the two was positive and statistically significant (Spearman's correlation $r = 0.78$, $p = 0.0002$). The BEQs also correlated with the TEQ_{TCDD} (Spearman's correlation $r = 0.78$, $p = 0.0001$), confirming the trend. However, additional activity caused by unidentified AhR-ligands cannot be excluded.

The sites that had the greatest concentrations of BEQ, S4 and S5 for both sampling years, are situated in the middle of the study area and are the two most downstream sites of the Klip Spruit. These greater concentrations of BEQs might be from either cumulative load of pollutants upstream together with the inputs of the surrounding urban area, or a specific source of AhR-ligands located inside this heavily populated area.

Discussion

Cytotoxicity

Many of the extracts were cytotoxic. Since sulphur was excluded via GPC clean-up, these extracts must have contained other cytotoxic compounds, either AhR-ligands and/or other compounds. The consequence of this is that the maximum elicited response might very well have been higher than what was reported here if the cells' viability had not been compromised. Schirmer et al. (1998) revealed that the 2- and 3-ring PAHs, specifically naphthalene, acenaphthylene, acenaphthene, fluorene, and phenanthrene, were directly cytotoxic to a rainbow trout gill epithelial cell line, RTgill-W1. Other authors demonstrated that the toxicity of some PAHs is additive (Hongzhen and Zhang 2017). Therefore, it is probable that the observed cytotoxicity in our bioassay was due to PAH contents of the extracts, even though the concentrations of individual PAH congeners (Pheiffer et al. 2018) might not have been as great as those for which Schirmer et al. (1998) and Hongzhen and Zhang (2017) observed cytotoxicity. The results of this study therefore suggest that additive effects contributed to the observed cytotoxicity.

BEQ versus TEQ

The H4IIE-*luc* bio-assay results confirmed that there were AhR-agonists in the aquatic environment of Soweto. The luciferase activity in the bio-assay was two orders of magnitude greater than the TEQ_{TCDD} (Table 2) indicating that the bio-assay is more sensitive at predicting risk to AhR-ligands than the instrumental analysis's risk assessment. It is also possible that the fraction of the sample extract used in this study contained other, unidentified AhR-ligands. Synergism between extracted compounds can also not be excluded (Eichbaum et al. 2016; Hecker and Giesy 2011;

Table 2 Toxic equivalent quotient (TEQ_{TCDD}), bio-assay equivalent (BEQ), and Toxic equivalent quotient (TEQ_{FPF}) for dioxin-like toxicity towards fish results, calculated for the sediments of the sites from Soweto (2013 and 2014), compared to the TEQ guidelines of the CCME (2001)

	TEQ_{TCDD} (ngBEQ/kg)		BEQ (ngBEQ/kg)		TEQ_{FPF} (ngBEQ/kg)	
	2013	2014	2013	2014	2013	2014
S1	**9.7**	**6.1**	*92*	LOQ	*183.3*	*117.5*
S2	**1.7**	**6.7**	*25*	*352*	*36.4*	*269.8*
S3	**5.5**	**5.8**	*52*	*56*	*123.1*	*130.7*
S4	*39*	**9.1**	*221*	*817*	*755.1*	*525.4*
S5	**7.0**	**7.2**	*137*	*679*	*124.2*	*294.4*
S6	**8.4**	**6.4**	*122*	*79*	*184.1*	*112.8*
S7	**5.2**	**5.9**	<LOQ	*46*	*126.3*	*79.2*
S8	0.6	**5.3**	*32*	*35*	*22.7*	*107.5*
S9	**2.1**	**6.2**	<LOQ	**6.7**	*48.47*	*93.3*
	ISQG 0.85 ngTEQ/kg				**PEL** *21.5 ngTEQ/kg*	

Guideline exceedance indicated by bold (ISQG) and italics (PEL)

Hilscherova et al. 2000a, b) and Larsson et al. 2012 reported superinduction of the AhR of H4IIE-*luc* caused by PAHs. However, compared with the TEQ_{FPF} values, it was generally greater than the BEQs although the BEQs had a wider range (Table 2). Also, applying the TEQ_{FPF}, all of the sites had levels greater than the Canadian PEL, whereas the BEQs had a more varied outcome.

The difference in the severity of the toxicity predicted by the three approaches begs further investigation. The nature of the difference/similarity between the various equivalencies in relation to the sites was investigated using principal component analysis (PCA) (Fig. 5a). Factor 1 (explaining 82.8% of the variance) distinguished between those equivalencies that were 'high' (positive loading) and 'low' (negative loading). Factor 2 (16.2% of the variance) distinguished between those sites associated with the BEQ (positive loading) and those with the TEQs (negative loading). It seems as if the majority of the sites distributed in closer to the BEQ vector when compared the two TEQ vectors (Fig. 5a). This suggests that BEQ represents the toxicity prediction at the sites better—as indicated by the sites' loadings closest to the BEQ line (Šmilauer and Lepš 2014).

Effectiveness of the various toxic equivalence models was further compared by using a redundancy analysis (RDA), which determines differences and similarities within a dataset based on a specific variable. The variable chosen was the individual concentrations of the CPAHs in the sediment extract (Table S1) (Pheiffer et al. 2018). The explanatory variables accounted for 99.4% of the variation (Fig. 5b). Similar to the PCA, factor 1 (90.3%) distinguished between 'high' and 'low' equivalencies, and factor 2 (9.1%) between BEQ and TEQ. The RDA ordination (Fig. 5b) shows that BbF and BkF concentrations were associated closer to TEQ_{TCDD}, whereas BaP associated closer to BEQ, and InP, BaA, and Chr associated closer with TEQ_{FPF} (Fig. 4b). A generalised linear model was applied (Fig. S1) to further the findings of the redundancy analysis. This model predicts how the response variables (equivalencies: BEQ, TEQ_{TCDD}, and TEQ_{FPF}) will change if the predictor variables (concentrations of the individual CPAHs) changed. An *F* test was included to determine if a nonzero response was measured. The BEQs for BbF and BkF were omitted based on the significant chance of a zero response (Fig. S1C & D). This corroborates the RDA analysis (Fig. 5b) where the angle

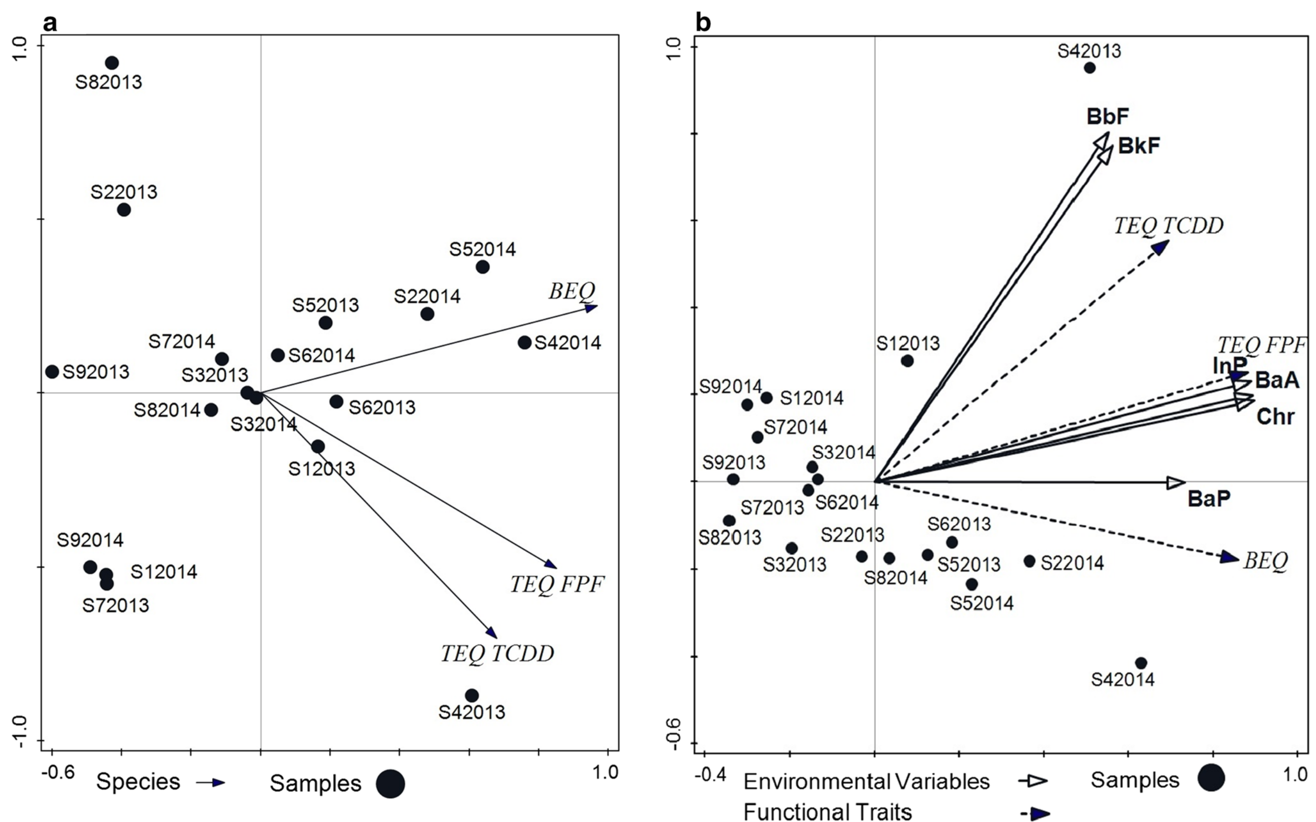

Fig. 5 Multivariate statistics. **a** Principal component analysis (PCA) biplot of toxic equivalency model results of sediments sampled in 2013 and 2014. The ordination explains 99% of the variance in the data with 82.8% by factor 1 and 16.2% by factor 2; **b** redundancy analysis (RDA) triplot of toxic equivalency model results and polycyclic aromatic hydrocarbons in sediments sampled in 2013 and 2014. The ordination explains 99.4% of the variance in the data with 90.3% by factor 1 and 9.1% by factor 2

between the vector lines of BbF and BkF, and that of BEQ were almost perpendicular, indicating no correlation of BbF and BkF with BEQ (Šmilauer and Lepš 2014). Compared with the other equivalency sets, the model was not a good fit for the influence of increasing CPAH concentrations with increasing TEQ_{TCDD} (r^2 ranged between 0.2 and 0.5, except for BbF and BkF ($r^2 = 0.99$ for both instances)). However, predictions of the afore-mentioned isomers showed low confidence (Fig. S1). The modelling of BaA, Chr, and InP responses had better fits for TEQ_{FPF} ($r^2 = 0.9$) than BEQ ($r^2 = 0.6$). In contrast, BaP had a better fit for BEQ ($r^2 = 0.72$) than TEQ_{FPF} ($r^2 = 0.6$). In both cases, the responses predicted by the multiple regression showed proportional increase in responses to increased CPAH concentrations.

The results (Fig. 5) suggest that the FPF-calculated TEQs might be a better method to use when predicting toxicity. However, this method only assumes additive effects and is inherently dependent on chemical analysis, whereas the BEQs reflect the biological reaction to the entire extract's contents and is not dependant on the chemical quantification of the separate congeners. The bio-assay reflects the additive, synergistic, and inhibitive responses of the compounds in the mixture simultaneously (Petrulis and Bunce 2000). Thus, the BEQ results represent a more realistic risk to AhR-mediated toxicity in the extracts.

Risk assessment

When concentrations of TEQ_{TCDD} were compared with Canadian sediment guidelines (CCME 2001), all the samples (except S8 of 2013) exceeded the Canadian ISQG (0.85 ngTEQ/kg) (Table 2), predicting an expected harmful effect to aquatic life. The higher PEL (21.5 ngTEQ/kg) was exceeded only once in this study, at S4 in 2013 (Table 2). These results, based on instrumental data, indicate that the benthic organisms in the study area experience high toxic risk due to CPAHs.

When concentrations of BEQs were compared with the Canadian guidelines, all except four sites exceeded the higher PEL (Table 2); S4 2014 was 38 times greater than the PEL. Also, the TEQ_{FPF}s at all the sites were greater than the PEL—in some cases up to tenfold greater (Table 2). Thus, the BEQs and TEQ_{FPF} predicted a much greater risk to benthic organisms, than when using the TEQ_{TCDD}. Exceeding the PEL implies a high risk to benthic organisms in the upper Klip River of Soweto, exposed to sediments.

Only two samples, S4 (2013) and S9 (2014), had the same risk category when using either TEQ_{TCDD} or BEQ. One sample (S8 2013) had no predicted risk when using TEQ_{TCDD} compared with the three (S7 and S9 in 2013 and S1 in 2014) when using BEQ. Even though there were distinct differences between the risk predictions when using either approach (BEQs vs TEQs), sites with contradicting risk predictions/classes (> PEL or > ISQG), both approaches indicated at least some degree of risk to benthic organisms for the majority of the samples.

Samples collected in the same location as S8 (Fig. 1) for two consecutive years (2006 and 2007) demonstrated that concentrations of BEQs decreased between the two surveys, from 161 to 86 ngBEQ/kg (Roos et al. 2011). The greater concentration of BEQ is comparable to the concentrations observed during this study (Table 1). It is important to note that the Roos et al. (2011) study analysed a different fraction of the extract. Those authors treated their extracts with sulphuric acid, before commencing the bio-assay. This step would have destroyed most of the non-persistent AhR-ligands, including the PAHs, with PCDD/Fs and dioxin-like PCBs remaining. However, the persistent compounds have a much greater affinity for the AhR, and induce larger responses. Moreover, it is probable that concentrations of BEQs would have been greater if Roos et al. (2011) had performed a bio-assay without an acid-treated extract. This is based on the PAHs present in the sample, as seen from instrumental analysis (Roos et al. 2011), which were very similar to what was present in the sediment of the current study (Table S1) (Pheiffer et al. 2018). Comparing BEQs from this study to the Canadian guidelines (CCME 2001), only one site was greater than the PEL guideline—the remaining sites with detectable concentrations were greater than the ISQG. A similar trend was seen when the TEQ_{TCDD} values were assessed, where only one site exceeded the PEL and the rest of sites exceeded the lower guideline level (CCME 2001).

In a study in and around Durban Bay, South Africa (also using the H4IIE-*luc* assay and the same fractionation technique of sediment extracts (Vogt et al. 2019), concentrations of PAHs in the mostly freshwater sites were less than those observed in the present study. The BEQs for the Umhlatuzana and Umbilo Rivers ranged from < LOD to 82 ngTEQ/kg, with a mean of 31 ngTEQ/kg. The instrumental analysis of the sediment showed corresponding low concentrations for the ΣCPAHs, varying from < LOD–87 ng/g (Vogt et al. 2018).

The Dioxin Response Chemically Activated Luciferase Expression (DR-CALUX) assay, which is the commercial version of the H4IIE-*luc* and is therefore comparable with the results reported in this study, was used to determine the AhR-agonists of sediment in the Danube River. Chemical analysis of their crude extract showed a mean ΣCPAH of 2423 ng/g (Keiter et al. 2008). The BEQs determined for the raw extracts were all greater than 1000 pgBEQ/g, and the mean BEQ of the extract with persistent ligands (acid treated) was 395 pgBEQ/g (Keiter et al. 2008). It seems that the very high PAH concentrations, relative to the present study, contributed greatly to the BEQs measured in the Danube River (Keiter et al. 2008). Sediments from Soweto had

considerably lower levels of both ΣCPAHs and BEQs when compared to the Danube. The BEQs reported by Keiter et al. (2008) were all above the Canadian PEL guideline (CCME 2001). The TEQ_{TCDD} values calculated from the PAH concentrations here reported from the Klip River only had two sites that exceeded the PEL, of which the highest exceeded the guideline tenfold. The remaining sites were higher than the ISQG level.

Concentrations of AhR-active compounds in Korean sediments were determined by use of the H4IIE-*luc* reporter gene bio-assay. Their sampling areas included inland creeks and streams that flow into Lake Shihwa—known to have moderate to high concentrations of HAHs, such as dioxins. Those samples were also acid-treated to remove the non-persistent compounds. The BEQ values Yoo et al. (2006) reported were based on the REP_{50}; therefore, the REP_{50} values of the present study were used for comparison (Table 1) even though some are extrapolated values. The REP_{50} values in the present study ranged from 1.2 to 972 pgBEQ/g (Table 1) and that of Yoo et al. (2006) between 14 and 868 pgBEQ/g. Although the BEQs by Yoo et al. (2006) were all comparable with results observed during the current study, those responses were elicited by persistent pollutants. Their responses probably would have been higher had they tested non-acid-treated extracts, even though the BEQs reported by Yoo et al. (2006) were all higher than the PEL of the Canadian guidelines (CCME 2001). No TEQ_{TCDD} was calculated since no CPAHs were reported in their study.

AhR-mediated potencies of sediments from the Yellow Sea—from the Liáoning province in China and South Korea's west coast estuaries were also determined by the same bio-assay (Hong et al. 2012). These extracts were not fractionated nor treated with acid. The mean concentration of BEQ form the Korean sediments was 4.6 pgBEQ/g (ranging < 3.4–11 pgBEQ/g) and the Chinese sediments had a mean of 4.9 pgBEQ/g (< 3.4–28 pgBEQ/g). These mean BEQs exceeded the ISQG of the Canadian guidelines (CCME 2001), whereas the maximum BEQ reported for China exceeded the PEL guideline. Although the BEQs for Soweto were predominantly greater than those reported by the Hong et al. (2012) BEQs, some were similar, e.g., S2 2013, S8 2014, and S9 (2013 and 2014) (Table 1). Hong et al. (2012) also reported instrumental analytical data for potential AhR-ligands, including PAHs. Their mean ΣCPAH was 86 ng/g (Korea) and 260 ng/g (China). The CPAHs present in these samples were calculated to contribute to 40% of the TEQs determined for the Chinese sediments and 12% for the Korean sediments. The CPAHs that were most prevalent—thus having the most potential AhR binding—were dibenz(ah)anthracene and benzo(k)fluoranthene (Hong et al. 2012). Soweto had more of the 'smaller' CPAHs, namely benz(a)anthracene and chrysene (Table S1) (Pheiffer et al. 2018). Hong et al. (2012) reported TEQ_{TCDD} values (calculated using the same TEFs) which were mostly in excess of the lower Canadian guideline (ISQG) (CCME 2001).

In a study on dioxin-activity in the Czech Republic's rivers, pollutants were separated into different fractions with a Florisil column (Hilscherova et al. 2001). One of these fractions contained the PAHs (similar to this study). The BEQs obtained with the H4IIE-*luc* cells were three orders of magnitude greater (mean of 9000 ngTCDD-eq/kg) than the Soweto BEQs. This is to be expected because the PAHs reported by Hilscherova et al. (2001) were at greater concentrations in sediments.

Using the same fractioning technique as Hilscherova et al. (2001), others (Xia et al. 2014) used the H4IIE-*luc* bio-assay to quantify AhR-activity in sediments from China. Concentrations in the Haihe and Dagu Rivers, which flow through Tianjin City and had been exposed historically to industrial and domestic waste, were comparable to the Soweto sites. The BEQs ranged between 694 and 6834 pgBEQ/g (Song et al. 2006), and the mean of these four sites was 2314 pgBEQ/g. The authors attributed the difference between the TEQ_{TCDD} they calculated (using the concentrations of PCDD/Fs and dioxin-like PCBs and their respective TEF values) and their BEQs, to PAHs, because other literature reported PAH concentrations in the area between 800 and 1200 ng/g in soil (Wang et al. 2003). Their lower BEQs were comparable to the greater BEQs of this study (S4 and S5 2014 Table 1, 2). The BEQs reported by Hilscherova et al. (2001) and Song et al. (2006) were much greater than the PEL guidelines of the CCME (2001). No TEQ_{TCDD} values were calculated since these studies did not report individual CPAHs.

AhR-activity in sediments of Lake Tai in China was studied (Xia et al. 2014). Instrumental quantification of the CPAHs reported a mean ΣCPAHs of 97 ng/g—two times less than that reported for Soweto (Table S1) (Pheiffer et al. 2018). The BEQs of the Xia et al. (2014) ranged between 17.45 and 114.5 pg/g (Xia et al. 2014), which compared well with the lesser ranges of the present study (Table 1). Moreover, the BEQs reported by Xia et al. (2014) were all greater than the CCME (2001) upper guideline (PEL), and all the TEQ_{TCDD} values calculated were greater than the lower ISQG level.

Compared with local and international studies, all of which used the H4IIE-*luc* assay, it can be concluded that the Soweto/Lenasia area is moderately to highly polluted with AhR-ligands. The instrumentally derived TEQ_{TCDD} of the present study predicted lesser risk to benthic organisms than the bio-assay derived BEQ when compared with the Canadian sediment quality guidelines for dioxin-like compounds. The BEQs were two orders of magnitude greater than the TEQ_{TCDD}, and was statistically shown to be more sensitive to PAH responses than instrumentally derived toxic

equivalence. This is corroborated by international studies, where the BEQs predicted greater risk (exceeding the PEL) in every instance compared to its corresponding TEQ_{TCDD}. These results show the usefulness of the H4IIE-*luc* bio-assay as a screening tool, which is capable of giving a good indication of possible risk of AhR-ligands to organisms in the environment, without the expense of instrumental analysis for all the compound classes capable of eliciting a response via the AhR. In a developing country like South Africa with limited resources and trained personnel, such screening tools are very useful (De Vos et al. 2013).

Conclusion

The AhR is known to regulate biochemical and toxic effects of planar aromatic hydrocarbon type environmental pollutants. The activation of the AhR results in the expression of xenobiotic metabolising enzymes. Thus, this activation indicates that the body is defending itself against exposure—even at low levels, this should be cause for alarm. In this study, three toxic equivalence methods, TEQ_{TCDD}, TEQ_{FPF} bio-assay BEQ, were used to evaluate risk prediction caused by the 16 USEPA PAHs (or the extraction fraction containing them). Of the three methods, the biological toxic equivalence (BEQs) determined with the H4IIE-*luc* tissue culture and the TEQs calculated from fish potency factors proved the most protective when compared with an international sediment quality guideline. This study is proof that biological determination of risk is more protective of the environment than those determined based on the instrumental analysis.

Although the AhR-ligands of focus in this study, PAHs, are not persistent, they are ubiquitous because of a constant release into the environment. This means the wildlife and populace are constantly exposed to PAHs. Other non-persistent AhR-ligands, as well as their persistent counterparts, may also be present leading to the overexpression of the AhR. And the overexpression of AhR has detrimental consequences such as causing cancer (Feng et al. 2013).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights This article does not contain any studies with human participants or vertebrate animals performed by any of the authors.

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Receptor mediated potencies of polycyclic aromatic hydrocarbons in urban sediments: Comparisons of toxic equivalency risk assessment

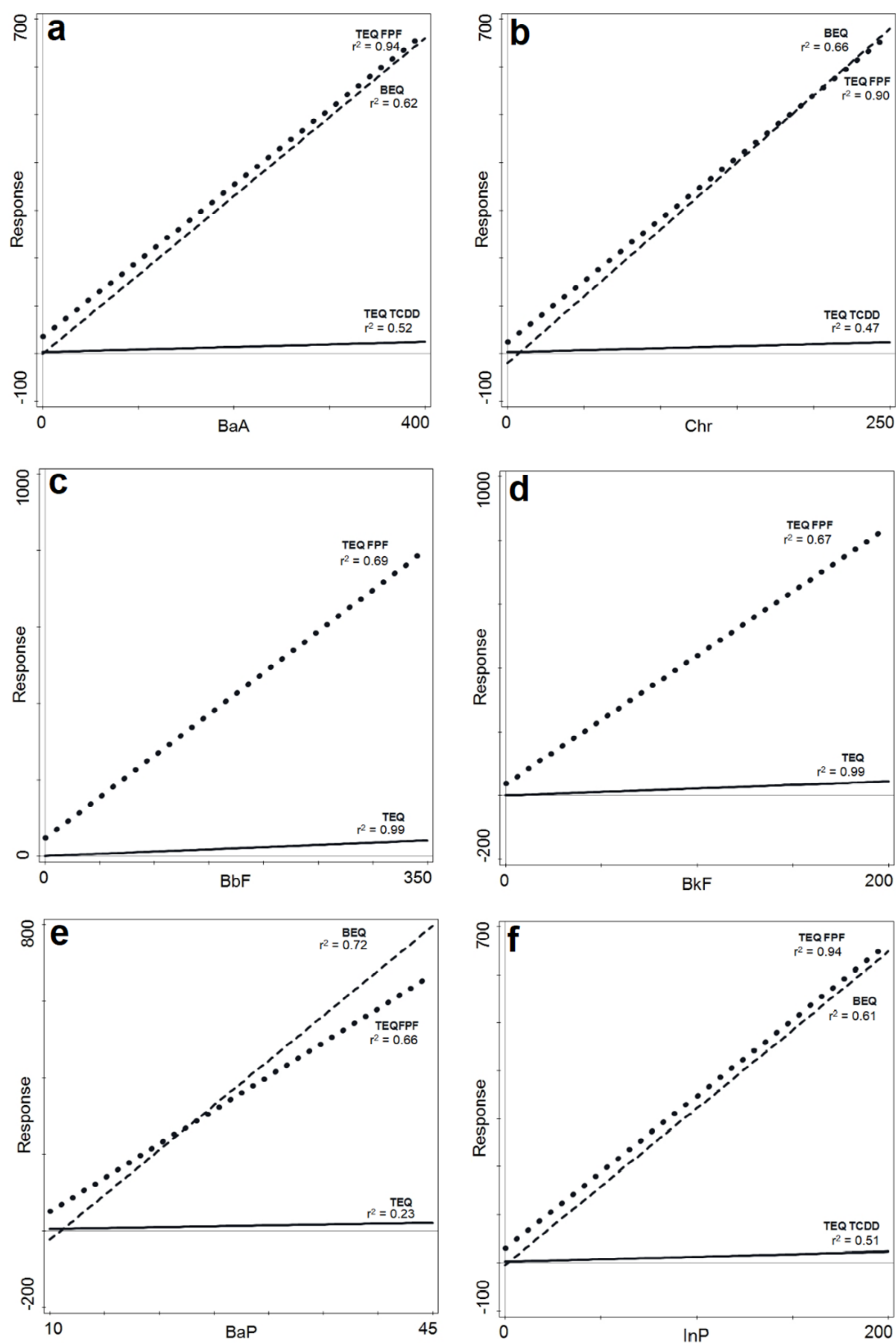

Fig. S1 Multiple regression response curves for toxic equivalency models (BEQ [dashed line], TEQ_{TCDD} [dotted line], and TEQ_{FPF} [solid line]) in terms of: A) benz(a)anthracene (BaA); B) chrysene

(Chr); C) benzo(b)fluoranthene (BbF); D) benzo(k)fluoranthene (BkF) ;E) benzo(a)pyrene (BaP); F) indeno(1,2,3-cd)pyrene (InP)

Table S1 Concentrations of individual and mean concentrations of CPAHs (ng/g dm) in sediments for both sampling years at the nine sites sampled from the study area (data from Pheiffer et al., 2018)

		BaA	**Chr**	**BbF**	**BkF**	**BaP**	**InP**	**DBA**	**ΣCPAH**
2013	**S1**	69	79	83	40	*14*	47	*23*	356
	S2	13	14	13	12	*14*	*3.2*	*23*	92
	S3	79	41	45	29	*14*	27	*23*	258
	S4	370	215	336	181	31	185	*77*	1394
	S5	56	40	60	42	*14*	19	*23*	254
	S6	96	58	71	45	*14*	43	*23*	350
	S7	55	36	43	27	17	32	*23*	233
	S8	5.2	*6.2*	*3.5*	4.0	*14*	*3.2*	*23*	59
	S9	14	14	17	9.5	*14*	11	*23*	102
2014	**S1**	43	37	52	25	*14*	30	*23*	224
	S2	141	90	53	29	*14*	95	*23*	445
	S3	40	25	49	30	*14*	34	*23*	215
	S4	373	242	60	33	45	185	*77*	1015
	S5	202	127	54	32	28	96	*23*	563
	S6	47	30	54	33	*14*	21	*23*	223
	S7	15	14	51	31	*14*	9.2	*23*	157
	S8	47	32	44	32	*14*	20	*23*	212
	S9	28	25	53	28	*14*	17	*23*	188
	%Recovery	103	98	93	97	94	91	152	
	½LOD	*9.4*	*1.9*	*1.0*	*0.9*	*4.2*	*1.0*	*23*	
	½LOQ	*32*	*6.2*	*3.5*	*3.1*	*14*	*3.2*	*77*	

½LOD and ½LOQ values are italised