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Application of an *in vitro* new approach methodology to determine relative cancer potency factors of air pollutants based on whole mixtures

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ABSTRACT

Air pollution is an example of a complex environmental mixture with different biological activities, making risk assessment challenging. Current cancer risk assessment strategies that focus on individual pollutants may overlook interactions among them, potentially underestimating health risks. Therefore, a shift towards the evaluation of whole mixtures is essential for accurate risk assessment. This study presents the application of an *in vitro* New Approach Methodology (NAM) to estimate relative cancer potency factors of whole mixtures, with a focus on organic pollutants associated with air particulate matter (PM). Using concentration-dependent activation of the DNA damage-signaling protein checkpoint kinase 1 (pChk1) as a readout, we compared two modeling approaches, the Hill equation and the benchmark dose (BMD) method, to derive Mixture Potency Factors (MPFs). MPFs were determined for five $PM_{2.5}$ samples covering sites with different land uses and our historical pChk1 data for PM_{10} samples and Standard Reference Materials. Our results showed a concentration-dependent increase in pChk1 by all samples and a higher potency compared to the reference compound benzo[*a*]pyrene. The MPFs derived from the Hill equation ranged from 128 to 9793, while those from BMD modeling ranged from 70 to 303. Despite the differences in magnitude, a consistency in the relative order of potencies was observed. Notably, $PM_{2.5}$ samples from sites strongly impacted by biomass burning had the highest MPFs. Although discrepancies were observed between the two modeling approaches for whole mixture samples, relative potency factors for individual PAHs were more consistent. We conclude that differences in the shape of the concentration–response curves and how MPFs are derived explain the observed differences in model agreement for complex mixtures and individual PAHs. This research contributes to the advancement of predictive toxicology and highlights the feasibility of transitioning from assessing individual agents to whole mixture assessment for accurate cancer risk assessment and public health protection.

1. Introduction

New approach methodologies (NAMs) have emerged as a sustainable and forward-looking approach to assess the potential hazards and risks of chemicals without using animal testing. NAMs include *in vitro*, *in silico*, and *in chemico* methods and are performed in what is considered alternative models (e.g., 2D/3D cell cultures, zebrafish, and computational biology) compared to the models used in traditional animal testing (e.g., rodents). Some of the key strengths of using NAMs are increased testing throughput and better information on mechanisms to

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support the development of adverse outcome pathways (Krewski et al., 2020; Bajard et al., 2023).

One challenge that can be addressed by NAMs is complex mixture toxicology. Mixtures pose a challenge due to potential non-additive effects, making it difficult to predict human health risks. Risk assessments categorize mixtures as either simple (e.g., consumer products) or complex (e.g., environmental pollution). When dealing with simple mixtures, the components are usually well-defined and can be assessed using a component-based approach. However, complex mixtures contain many known and unknown components, making this approach unsuitable (Luo et al., 2022; Mustafa et al., 2023). In such cases, it is preferable to assess the whole complex mixture, as this allows for the assessment of all constituents as well as possible interactions (U.S. EPA, 2000; WHO, 2000; Rager and Rider, 2023). In the assessment of complex mixture toxicity, *in vitro* NAMs have already been successfully employed to assess various mixtures of concern for human health supporting their applicability (Chen et al., 2021; Hsieh et al., 2021; Finckh et al., 2022; Manful et al., 2023). Although both NAMs and whole mixture-based testing are promoted by both academia and governmental bodies, their application in regulatory frameworks is currently limited. Therefore, their continued development and validation are necessary to promote regulatory acceptance (Rager and Rider, 2023; Schmeisser et al., 2023).

Air pollution is a complex mixture of organic and inorganic chemicals emitted as gaseous and particulate matter (PM) from natural and anthropogenic sources. Human exposure to fine particles ($PM_{2.5}$) is associated with health effects, including cardiorespiratory diseases and cancer (Holme et al., 2019; Turner et al., 2020). PM is composed of a complex mixture of inorganic elements and organic chemicals such as polycyclic aromatic hydrocarbons (PAHs). This latter group includes unsubstituted PAHs (e.g., benzo[*a*]pyrene, B[*a*]P), as well as alkyl-, oxygen- and nitrogen-substituted PAHs (alkyl-PAHs, OPAHs, and NPAHs). The relative composition of these pollutants is influenced by their emission sources, particle composition, ambient meteorological conditions, and aerosol age. Many unsubstituted and substituted PAHs are considered genotoxic, mutagenic, and tumorigenic (IARC, 2010; IARC, 2014), and therefore deemed as significant contributors to the carcinogenicity of air pollution and $PM_{2.5}$ (Holme et al., 2023; Li et al., 2023). It has been estimated that approximately 90 % of the cumulative risk of lung cancer resulting from exposure to air pollution in urban environments is attributable to PAHs present in the particulate phase. (Bartos et al., 2009). The current approach for cancer risk assessment of environmental PAHs is based on a chemical-by-chemical approach, despite evidence from *in vitro* and *in vivo* data showing that this approach cannot accurately predict the biological effects of complex PAH mixtures (Jarvis et al., 2014; Aquilina and Harrison, 2023).

We have previously proposed a whole mixture-based *in vitro* NAM to estimate the carcinogenic potencies of environmental PAH-containing air PM extracts by determining Mixture Potency Factors (MPFs) (Jarvis et al., 2013; Dreij et al., 2017; de Oliveira Galvão et al., 2022). This NAM is based on the concentration-dependent activation of DNA damage signaling proteins checkpoint kinase 1 (Chk1) and H2AX histone, both established markers of genotoxicity (Smith et al., 2010; Kopp et al., 2019). Comparing the activation of these markers induced by environmental Standard Reference Materials (SRMs) with that induced by B[*a*]P resulted in similar relative MPFs to *Salmonella* mutagenicity and *in vivo* tumorigenicity data (de Oliveira Galvão et al., 2022), supporting the applicability of *in vitro* NAMs in risk assessment of complex mixtures. Here, with the aim to further assess the applicability of this NAM, MPFs for an extended number of $PM_{2.5}$ samples were determined. Two different approaches of concentration–response modelling for deriving MPFs were compared: a four-parameter Hill equation that takes the dose resulting in 50 % of the maximal response (EC_{50}) and Top plateau values of a sigmoid concentration–response curve into account (Audebert et al., 2012), and the Benchmark Dose (BMD) method, which identifies concentrations that result in a predetermined adverse effect level (benchmark response, BMR) (Crump, 1984; Slob, 2002). Both of these approaches have been previously applied to determine relative or comparative genotoxic and/or carcinogenic potencies (Audebert et al., 2012; Wills et al., 2016a; Wills et al., 2016b; Dreij et al., 2017; Allemang et al., 2018; Wheeldon et al., 2020; de Oliveira Galvão et al., 2022) but have to the authors' knowledge not been compared before.

2. Materials and methods

2.1. Chemicals and solvents

Toluene, hexanes (isomer mixture), methyl tert-butyl ether (MTBE), 2-propanol, acetone were all HPLC grade and from Rathburn (Walkerburn, U.K.). Anhydrous n-dodecane (>99 %) and sterile Dimethyl sulfoxide (DMSO, >99.7 %) was purchased from Merck KGaA (Darmstadt, Germany). Isotope labelled internal standards and calibration standards were from NIST (Gaithersburg, MD, USA) and Chiron AS (Trondheim, Norway) detailed elsewhere in Sadiktsis et al. (2023). The organic extracts were diluted in Hybri-Max™ and sterile-filtered DMSO (>99.7 %) purchased from Merck KGaA (Darmstadt, Germany). B[*a*]P was of analytical grade and obtained from Sigma-Aldrich (Stockholm, Sweden).

2.2. Air PM sampling

The sampling campaign was conducted during the winter/dry season in five cities: Kyoto (Japan), Stockholm (Sweden), Cáceres, Limeira, and Ribeirão Preto (Brazil), during 1–7 days per sample. The studied sites are characterized by unique climatic conditions and emission sources, encompassing different land uses (i.e., urban, semi-urban/agro-industrial, and rural). Details regarding the $PM_{2.5}$ sampling campaigns, including the sampling conditions, instrumentation details, regional and long-range transport influences, and $PM_{2.5}$ emission sources were previously described in Scaramboni et al. (2024). Additional information about sampling location characteristics is listed in materials and methods of supplement.

2.3. Sample preparation and LC-GC/MS analysis

All details regarding how the organic extracts from $PM_{2.5}$ were prepared, the subsequent preparation of DMSO samples, as well as the methodologies used to quantify PAHs, alkyl-PAHs, OPAHs, and NPAHs according to de Oliveira Galvão et al. (2022) and Sadiktsis et al. (2023), are described in the supplementary materials and methods. Results of chemical characterization of samples are expressed in relative composition (%) or in mass of compound per volume of DMSO (ng/μL) used to exposure the cells.

2.4. Cell culture and exposure

Human hepatocellular carcinoma HepG2 cell line was obtained from the American Type Culture Collection (ATCC, HB-8065, Rockville, MD). HepG2 cells were used based on their sensitivity in estimating genotoxic potencies from single PAHs and PAH mixtures, and good agreement when compared with *in vivo* carcinogenicity studies (Dreij et al., 2017; de Oliveira Galvão et al., 2022). Cells were cultured in Minimum Essential Medium (MEM), supplemented with 10 % (v/v) fetal bovine serum, 100 units/mL penicillin and 0.1 mg/mL streptomycin mixture, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids all from Gibco by Life Technologies (Stockholm, Sweden), and maintained at 37 °C in 5 % CO_2. Cells were exposed to solvent control (0.1 % DMSO), field blank filter extracts (0.1 % DMSO), and organic extracts from $PM_{2.5}$ based on benzo[*a*]pyrene equivalent concentrations ($B[a]P_{eq}$) ranging from 0.0001 to 52 nM $B[a]P_{eq}$ for 48 h. $B[a]P_{eq}$ refers to the use of an extract concentration equivalent to a set concentration of B[*a*]P. The concentration range used corresponds to 0.01 to 944 μg $PM_{2.5}$/mL (see equivalences in Table S1). B[*a*]P was used as a reference compound. All

experiments were at least performed in three independent experiments.

2.5. Cell viability assays

Effects on cell viability were assessed by analyzing the relative population doubling (RPD) (OECD, 2016) and by the Alamar Blue assay (Präbst et al., 2017). For RPD, 1 x 10^5 cells/well were seeded in 24-well plates for 24 h. Trypan blue 0.4 % (Thermo Fisher Scientific, MA) was used to count the cells before and after 48 h exposures, and RPD was determined as:

$$\frac{\text{number of population doubling in treated cultures}}{\text{number of population doubling in control cultures}} x100$$

where

$$\text{population doubling} = \frac{\log\left(\frac{\text{post treatment cell number}}{\text{initial cell number}}\right)}{\log 2}$$

The Alamar blue assay was performed as previously described (de Oliveira Galvão et al., 2022). The results of both methods were expressed as percentage viability relative to the DMSO control.

2.6. Western blotting

Levels of phosphorylated Chk1 (pChk1) were measured as previously described in de Oliveira Galvão et al. (2022). HepG2 cells (3 x 10^5 cells/well) were plated in 6-well plates and incubated for 24 h before exposure. After 48 h exposures, the proteins were extracted, measured, and samples were subjected to standard SDS-PAGE. Separated proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA), and proteins were detected using antibodies against Chk1 phosphorylated at Ser-317 (#2344, Cell Signaling Technology, MA) and the endogenous control Cdk2 (M2) (sc-163, Santa Cruz Biotechnology, CA), followed by incubation with secondary antibody (Santa Cruz Biotechnology, CA). The proteins were detected using the WesternBright™ ECL chemiluminescence kit (Advansta Inc., CA) and densitometry analysis was performed using ImageJ software version 1.52 (National Institute of Health, USA). Exposure to camptothecin (10 μM) for 1 h was used as positive control.

2.7. Modeling of relative cancer potency factors

Relative cancer potency factors were determined as previously described by concentration–response analysis of the relative increase of pChk1 levels obtained from densitometric analysis of western blots (Jarvis et al., 2013; Dreij et al., 2017). In addition to the five $PM_{2.5}$ samples mentioned above, our historical data for six single PAHs (benzo[*a*]anthracene, B[*a*]A; benz[*j*]aceanthrylene, B[*j*]A; benzo[*b*]fluoranthene, B[*b*]F; B[*a*]P; dibenzo[*a,h*]pyrene, DB[*a,h*]A; dibenzo[*a,l*]pyrene, DB[*a,l*]P), and organic extracts of three Standard Reference Materials (SRMs: Coal tar SRM 1597a; Diesel PM SRM1650b, and Urban dust SRM1649b) and two urban PM_{10} samples from Stockholm were included in the modeling (Dreij et al., 2017; de Oliveira Galvão et al., 2022).

Two different approaches to concentration–response modeling, Hill equation and BMD modeling, were compared to estimate relative potencies and using B[*a*]P as the reference compound. B[*a*]P was used as the reference compound based on its classification as human carcinogen (IARC, 2010), already established use as PAH reference compound and air quality indicator to assess human cancer risks from air pollution and airborne PAHs (WHO, 2000). For the concentration–response analyses, only non-cytotoxic concentrations (cell viability ≥ 50 %) were considered, aligning with recommended guidelines (OECD, 2016).

2.7.1. Hill equation

The data were modelled using a 4-parameter logistic equation based on the Hill equation and assuming a shared slope. The formula for Y is given by:

$$Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{((\text{Hill slope})(\log EC_{50} - X))}}$$

Here, Y represents the response, starting at the Bottom and reaching the Top in a sigmoid curve. Bottom was set to 1, given that our data was normalized to DMSO control. The Hill slope describes the steepness of the curve, EC_{50} is the concentration leading to 50 % of a maximal response, and X is the logarithm of the concentration. As previously demonstrated by us and others (Audebert et al., 2012; Dreij et al., 2017), this equation can be applied to calculate relative potencies as the Hill slope-root of α-values:

$$\sqrt[Hillslope]{\frac{\alpha_{\text{PAH or PM}}}{\alpha_{\text{B[a]P}}}}$$

where,

$$\alpha = \frac{(\text{Top} - 1)}{(EC_{50}{}^{Hillslope})}$$

if a shared Hill slope can be found for the different exposures. This assumption was confirmed with an inbuilt F-test ($p < 0.05$).

2.7.2. Benchmark concentration–response analysis

This approach facilitates the interpolation between tested concentrations and calculating a benchmark dose (BMD), i.e., the dose corresponding to a prescribed change in the mean response relative to the standard deviation (Crump, 1984; WHO, 2009). Here, the exponential, Hill, inverse exponential, and lognormal models were first fitted to the data, following the European Food Safety Authority BMD guidance (Hardy et al., 2017). The benchmark response (BMR), i.e. the measured change in relation to the estimated background, was set to 50 % (equal to 1.5-fold increase of pChk1). For model selection and weighing, the Akaike Information Criterion was employed (Hardy et al., 2017). Model averaging was performed in a total of 1 000 bootstrap runs. The resulting estimates were expressed as BMD and its corresponding 90 % confidence interval composed of the benchmark dose lower and upper limits, BMDL and BMDU, respectively. Relative potencies were calculated by dividing the median BMD of 1000 iterations of model averaging for B[*a*]P with the respective BMD for the individual PAHs or extracts in question (Bosgra et al., 2009). Following the same reasoning, the confidence interval for each potency factor was obtained by the ratio of the respective BMDLs and BMDUs.

2.8. Statistical analysis

All data are presented as means ± S.E. or S.D., with $n = 3$–11. EC_{50} values for the cell viability were estimated by nonlinear regression with log transformed concentrations and normalized response function. Data were fit using least squares regression without weighting. Replicates were treated as individual data points. Differences between exposures and controls were analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison *post hoc* test. The mean differences were considered significant at $p < 0.05$. All statistical analyses of PAH content, cell viability, pChk1 levels, and the modelling of the 4-parameter logistic equation for relative potency factors were performed using GraphPad Prism 10 (GraphPad Software LLC). For BMD analyses the PROAST package (version 70.3, developed by the National Institute for Public Health and the Environment in the Netherlands, RIVM) was used in the R-software (version 4.3.1, R Development Core Team, 2023). Correlations between the two approaches of concentration–response modelling were performed by linear regression analysis followed by Spearman's

rank correlation with two-tailed *p*-values.

3. Results and discussion

3.1. Chemical characterization of samples

A summary of levels and relative composition of PAHs in the $PM_{2.5}$ samples is presented in Table 1. For most samples, highest levels were found of Σ_{20}HMW-PAHs except Cáceres that contained higher levels of Σ_{10}OPAHs. Levels of B[*a*]P, which was used to normalize the concentrations for determining relative cancer potency factors, ranged from 2.99 ng/μL (Cáceres) to 12.1 ng/μL (Ribeirão Preto) (see Table S2). The $PM_{2.5}$ filter samples from which the DMSO extracts were prepared have been previously characterized (Scaramboni et al., 2024) and showed a very similar relative composition of the 15 PAHs and 4 OPAHs that were analyzed in both studies ($R^2 = 0.75 – 0.92$, Figure S1). Here, additional analysis of alkyl-PAHs and NPAHs were included, which both showed lower contribution to the composition than the OPAHs (Table 1). Previous source apportionment showed that $PM_{2.5}$ samples from Kyoto were impacted by sources related to traffic emissions, waste burning, and long-range transport, while Stockholm samples were collected under the prevalence of vehicle exhaust emissions. For Cáceres and Ribeirão Preto, samples were characterized by higher biomass burning input than the others, presumably due to extensive wild and crop fires. Limeira samples presented emission contributions from many sources such as vehicular, industrial, and biomass burning (Scaramboni et al., 2024).

3.2. Identification of concentrations to be included in concentration response modelling

All samples caused a significant concentration-dependent reduction of cell viability at the highest concentration ($p < 0.05$, Fig. 1). Using a maximum of 50 % reduction of RPD as inclusion criteria, concentrations up to 3, 10, 15, 5, and 10 nM B[*a*]P_{eq} for Cáceres, Kyoto, Limeira, Ribeirão Preto, and Stockholm, respectively, were included in the modeling of relative cancer potency factors. In general, RPD was a more sensitive endpoint to assess cell viability compared to Alamar Blue, which showed a less evident concentration-dependent response (Fig. S2). RPD measured at approximately 1.5 × the control cell cycle time after the start of exposure has been proposed as a good method to reducing cytotoxicity-related false-positive results of genotoxicants (Greenwood et al., 2004; Fujita et al., 2016).

3.3. Determination of concentration–response relationships for pChk1 activation

In response to replication stress and DNA strand breaks, the ATM- and Rad3-related protein (ATR) activates the Chk1 protein by phosphorylation (pChk1) (Cimprich and Cortez, 2008). This results in a delayed S phase of the cell cycle and facilitates DNA repair before entering mitosis (Ciccia and Elledge, 2010; Lebrec et al., 2022). However, continuous replication stress can lead to sustained Chk1 activation, resulting in an increased number of cells that may enter mitosis prematurely, which may cause increased genomic instability (Burrell et al., 2013; Lebrec et al., 2022). Previous research has demonstrated that complex mixtures of PAHs cause sustained activation of Chk1 (Niziolek-Kierecka et al., 2012; Jarvis et al., 2013) and that the concentration–response relationship can be used to determine relative MPFs for improved cancer risk assessment of complex mixtures (Dreij et al., 2017; de Oliveira Galvão et al., 2022).

Here, a concentration-dependent increase of pChk1 at micromolar ranges of B[*a*]P and at B[*a*]P_{eq} nanomolar ranges for the five $PM_{2.5}$ samples was observed, indicating 2–3 orders of magnitude higher potency of the $PM_{2.5}$ samples than B[*a*]P (Fig. 2A-B). This high potency difference is similar to our previous studies testing various environmental complex mixtures (Dreij et al., 2017; de Oliveira Galvão et al., 2022). The samples from Limeira and Kyoto most strongly induced pChk1, up to 6-fold compared to negative control, followed by Stockholm, Cáceres and Ribeirão Preto (Fig. 2B). There were no significant differences ($p > 0.05$) in pChk1 levels between negative controls (NC) and field blanks (Blank, Fig. 2A).

3.4. Modeling of relative cancer potency factors

Next, the concentration–response data of the five $PM_{2.5}$ samples were combined with our previous pChk1 data from extracted PM_{10} samples, SRMs, and individual PAHs (Dreij et al., 2017; de Oliveira Galvão et al., 2022) in a single analysis to determine relative potency factors. The historical data were included to increase the sample size and to evaluate the outcome from two different approaches based on the Hill equation and BMD modelling. We have previously confirmed the reproducibility and validity of including our historical data in concentration–response modelling (de Oliveira Galvão et al., 2022).

Both models showed similar clusters, with the PM samples and SRMs activating pChk1 at lower concentrations, followed by the single PAHs DB[*a,h*]A and DB[*a,l*]P, and with B[*a*]A as the least potent compound (Fig. 3). The HillSlope, EC_{50} and Top parameters used in the Hill-based approach are presented in Table S4. To derive relative potency factors based on BMD modelling, individual BMDs were determined by applying a BMR of 50 %, equivalent to a 1.5-fold increase of pChkl levels compared to the negative control (BMD_{50}). This choice was supported by an end-point specific BMR of 52.8 % and similar to previous BMD analyses of *in vitro* genotoxicity datasets (White et al., 2020; Wheeldon et al., 2021). The BMD_{50} concentrations with 90 % confidence intervals (CIs) are presented in Figure S3. Several authors have promoted the use of CI (or BMDL-to-BMDU ratio) assessments as quality criteria and for grouping chemicals based on potency (Wills et al., 2016a; White et al., 2020). Such information is not available from the Hill equation model used here. All the CIs were within a 100-fold range and all but one (Söderled PM_{10}) below a 10-fold range, indicating a good quality of the underlying concentration–response data. Further analysis showed that the CIs of most whole mixture samples overlapped, although spanning 300-fold, suggesting that their potencies are not too different. For

Table 1
Summary of the amounts (ng/μL, mean ± SD) and relative composition (%) of PAHs in the $PM_{2.5}$ sample extracts. More detailed information about the individual PAH contents of the extracts is shown in Tables S2-S3 of the supplementary data.

	Σ_4LMW-PAHs*		Σ_7MMW-PAHs		Σ_{20}HMW-PAHs		Σ_{12}Alkyl-PAHs		Σ_{10}OPAHs		Σ_{11}NPAHs		Σ_{64}PAHs	
	(ng/μL)	(%)	(ng/μL)	(%)	(ng/μL)	(%)	(ng/μL)	(%)	(ng/μL)	(%)	(ng/μL)	(%)	(ng/μL)	(%)
Cáceres	2.8 ± 0.7	3	15.0 ± 1.6	15	32.6 ± 1.5	33	8.2 ± 0.4	8	37.7 ± 1.0	39	1.1 ± 0.1	1	97.3 ± 0.2	100
Kyoto	11.0 ± 3.6	4	63.4 ± 7.6	25	115 ± 6.5	46	10.3 ± 0.5	4	43.9 ± 2.1	18	6.5 ± 0.2	3	250 ± 10.8	100
Limeira	4.5 ± 1.3	2	31.1 ± 3.5	14	140 ± 7.9	64	6.2 ± 0.3	3	31.4 ± 0.7	14	5.7 ± 0.3	3	219 ± 2.7	100
Ribeirão Preto	2.9 ± 0.8	2	18.6 ± 2.0	13	81.8 ± 4.4	57	3.8 ± 0.2	3	28.4 ± 0.8	20	8.5 ± 0.2	6	144 ± 1.3	100
Stockholm	7.2 ± 2.2	5	41.2 ± 4.3	29	57.2 ± 2.9	40	8.7 ± 0.3	6	27.9 ± 2.2	19	1.2 ± 0.0	1	143 ± 6.3	100

* LMW (low-molecular weight) PAHs with 2–3 rings, MMW (medium molecular weight) PAHs with 4 rings, HMW (high molecular weight) PAHs with ≥ 5 rings (Agency for Toxic Substances and Disease Registry (ATSDR), 1995).

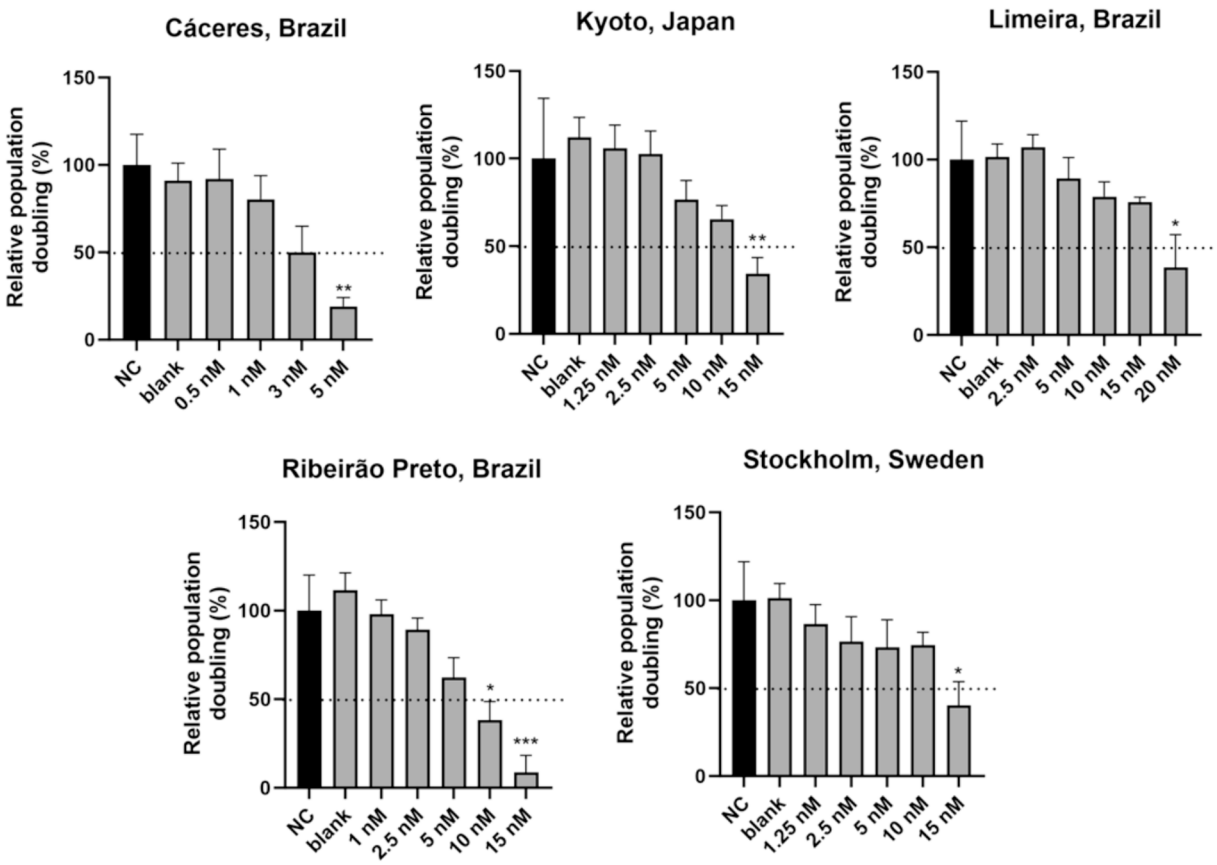

Fig. 1. Concentration-dependent decrease of relative population doubling. HepG2 cells were exposed for 48 h to $PM_{2.5}$ samples from Cáceres, Kyoto, Limeira, Ribeirão Preto, Stockholm at the indicated B[*a*]P_{eq} concentrations. Concentrations for the $PM_{2.5}$ samples are based on B[*a*]P_{eq} and refers to the use of an extract concentration equivalent to a set concentration of B[*a*]P. NC=negative control (DMSO 0.1 %), and blank = field blank filter extracts (DMSO 0.1 %). Experiments were performed at least in triplicate and data points represent mean ± S.E. *p < 0.05, **p < 0.01, ***p < 0.001 as compared with negative control by one-way ANOVA followed by Dunnett's Multiple Comparison Test. See also Fig. S2.

individual PAHs, B[*a*]A and B[*a*]P were clearly separated from the other PAHs which had overlapping CIs with BMDs in the order DB[*a,l*]P<DB[*a,h*]A<B[*j*]A<B[*b*]F (Figure S3).

The resulting relative cancer potency factors are shown in Table 2. For the $PM_{2.5}$ samples, the MPFs calculated using the Hill equation ranged between 585 and 9793, while those derived from BMD modelling ranged between 70 and 181, approximately one order of magnitude lower. Despite these differences in magnitude, the relative order of potencies was consistent for both models: the Brazilian samples from Cáceres and Ribeirão Preto displayed the highest potencies, followed by Kyoto, Limeira, and Stockholm. Notably, the samples with higher MPFs were not the samples with higher PAH concentrations (cf. Table 1), suggesting that other classes of chemicals not quantified here may be important drivers of genotoxicity in complex environmental mixtures and that non-target chemical analysis should be included in future studies to assess such relationships (Paszkiewicz et al., 2022; Hong et al., 2023). Our previous source apportionment identified biomass burning as the main emission source for Cáceres and Ribeirão Preto, and that these two sites were strongly impacted by smoke from wildfires in the Amazon and Brazilian savanna during the sampling campaign (Scaramboni et al., 2024). The IARC has classified the burning of coal and biomass fuels in households as a human carcinogen and a probable human carcinogen, respectively (IARC, 2006; IARC, 2009). Although the association between exposure to wildfire emissions and cancer risk is not well established (Korsiak et al., 2022), significant positive associations were observed between exposure to biomass burning as measured by potassium $PM_{2.5}$ and lung cancer incidence in a pooled analysis of several European cohorts (Raaschou-Nielsen et al., 2016; Hvidtfeldt et al., 2021).

Similarly to the $PM_{2.5}$ samples, the Hill equation model yielded higher MPFs compared to BMD for the PM_{10} samples and SRMs, all within one order of magnitude of each other, except for Söderled, which exhibited a two-order magnitude difference. The Urban dust and Diesel PM SRMs displayed the highest BMD MPFs among all whole mixtures at 303 and 267, respectively. Notably, the CI of the Urban dust MPF only overlapped with that of the Diesel PM MPF. In contrast, the Stockholm $PM_{2.5}$ and Söderled PM_{10} had the lowest BMD MPFs, and their CIs did not overlap with the other whole mixture samples. There were no overlaps between the Hill MPFs and the 90 % CI of the BMD MPFs, and a weak correlation was observed between the two models ($r_s = 0.29$; $p = 0.43$, Fig. S4A). In contrast, the RPFs for individual PAHs were much more similar between the two models (Table 2). A strong correlation was observed between the RPFs ($r_s = 1.00$; $p = 0.003$, Fig. S4B), with most of the Hill RPFs falling within the 90 % CI of the BMD RPFs. These findings suggest a strong agreement between the two models for individual PAHs, but not for the whole mixture samples.

The differences in MPFs between the two models, but not for RPFs, is likely due to differences in the shape of the concentration–response curves and how the two approaches model them. As depicted in Fig. 3A, the magnitude of the responses (Top variable in the Hill equation, see Table S4) induced by whole mixture samples were in general lower than those induced by individual PAHs, with a 2- to 8-fold increase of pChk1 (except for Coal tar SRM) compared to a 7- to 40-fold (B[*a*]P, off-scale). The Hill equation model includes the Top parameter, Hill slope, and EC_{50} value when calculating relative potency factors and, as a result, differences in Top and EC_{50} values will impact the outcome. In contrast,

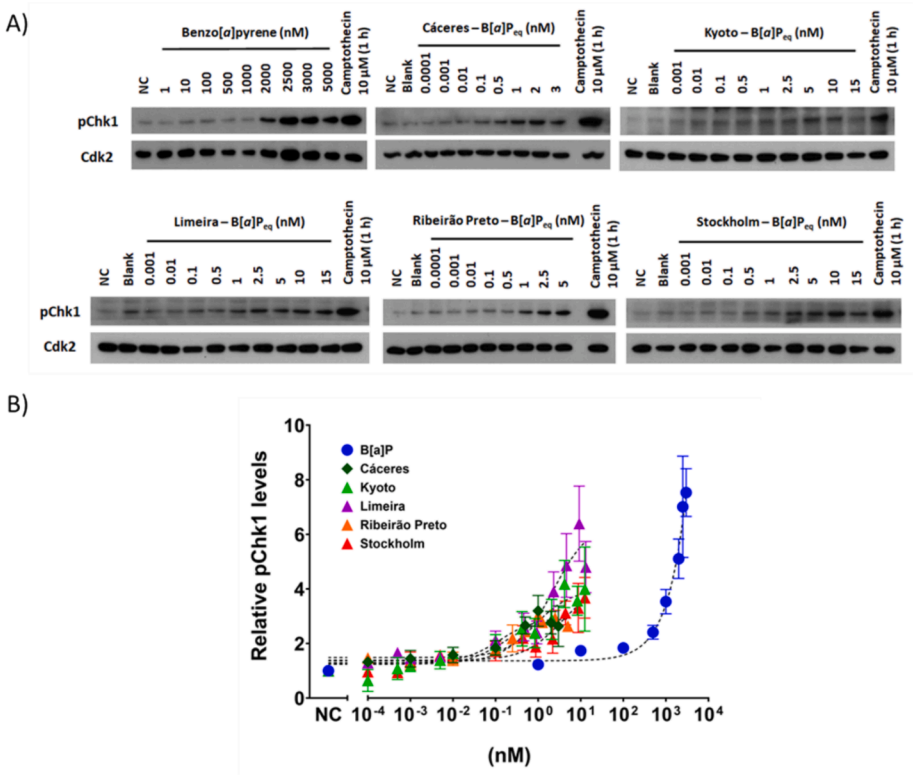

Fig. 2. Concentration-dependent induction of DNA damage signaling through pChk1 in HepG2 cells. A) Levels of pChk1 were assessed by western blot after 48 h exposure to B[*a*]P and $PM_{2.5}$ samples at the indicated concentrations. Concentrations for the $PM_{2.5}$ samples are based on B[*a*]P_{eq} and refers to the use of an extract concentration equivalent to a set concentration of B[*a*]P. Exposure to 10 µM camptothecin for 1 h was included as positive control, which induced a 13.2 ± 1.9-fold induction ($p < 0.05$). NC=negative control (DMSO 0.1 %) and Blank = field blank filters (DMSO 0.1 %). The panel shows representative blots. Cdk2 was used as loading control. B) Resulting concentration response curves of relative pChk1 levels based on densitometry of pChk1 signals normalized to loading control and relative to negative control. Data show mean ± S.E, n = 5–11.

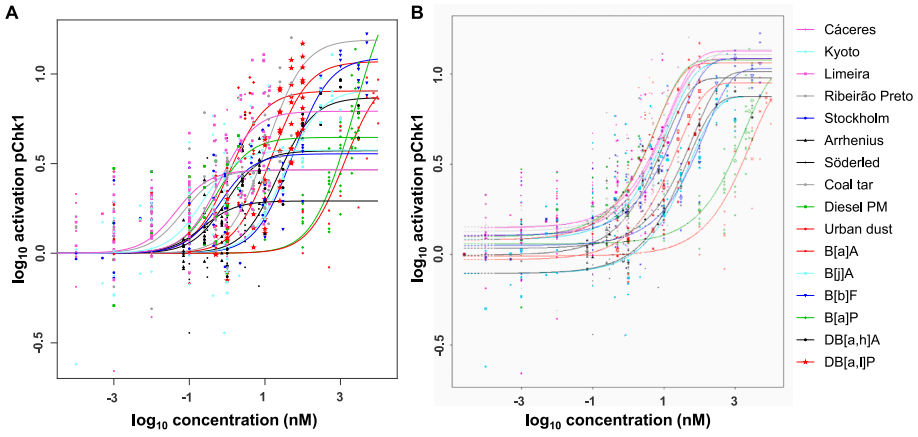

Fig. 3. Concentration-response analysis of pChk1 data based on the Hill equation (A) and the exponential model of the BMD approach (B). Data included the five $PM_{2.5}$ samples and our historical data for six single PAHs (B[*a*]A, B[*j*]A, B[*b*]F, B[*a*]P, DB[*a,h*]A, DB[*a,l*]P), three Standard Reference Materials (SRMs: Coal tar, Diesel PM, and Urban dust), and two PM_{10} samples (Arrhenius and Söderled). Concentrations for the PM and SRM samples are based on B[*a*]P_{eq} and refers to the use of an extract concentration equivalent to a set concentration of B[*a*]P. Each symbol represents individual replicates. See Table S4 and Figure S3 for modelling parameters.

the relative potency factors obtained from the BMD approach are derived from models which are parallel over the x- and y-axes, exhibiting the same magnitude of change from bottom-to-top, differing only in the estimated background for each curve, and they are not constrained to start at y = 1 (Bosgra et al., 2009). Additionally, the applied model averaging iterated the data 1 000 times, and the potency factors were determined based on the ratio between the median BMD for B[*a*]P and the exposures of interest, using a of BMR of 50 %. This explains the greater agreement between the two models for the RPFs than for the MPFs. This is further corroborated by the Coal tar SRM, which similarly to the individual PAHs, displayed a high level of pChk1 activation (15-fold, Fig. 3A) and the MPF obtained differed the least between the two models (2-fold) (Table 2).

To assess the validity of relative or comparative cancer potency factors derived from *in vitro* genotoxicity data and their applicability in human health risk assessment, they could be compared and validated against available *in vivo* mutagenicity or tumorigenicity data (Soeteman-Hernandez et al., 2015; Wills et al., 2021; Beal et al., 2023). For

Table 2
Relative cancer potency factors of the $PM_{2.5}$, PM_{10}, SRMs, and individual PAHs. Two different concentration–response modeling approaches to derive relative potency factors based on the Hill equation and BMD modeling were compared. The potency factors were also compared with available published cancer potencies.

	Relative cancer potency factors		
	Hill equation	BMD_{50} (90 % CI)[d]	Published cancer potencies[e]
Whole mixtures – MPFs			
$PM_{2.5}$ samples			
Cáceres	9793	181 (115 – 232)	–[f]
Kyoto	2461	135 (90 – 184)	–
Limeira	1299	132 (115 – 146)	–
Ribeirão Preto	6219	146 (116 – 164)	–
Stockholm	833	70 (52 – 82)	–
PM_{10} samples[a]			
Arrhenius	585	175 (161 – 185)	–
Söderled	1148	26 (3 – 53)	–
SRMs[b]			
Coal tar	128	54 (43 – 66)	553
Diesel PM	1249	267 (141 – 374)	3223
Urban dust	834	303 (274 – 325)	1606
Individual PAHs — RPFs[c]			
B[*a*]A	0.9	0.7 (0.5 – 1)	0.005 – 0.2
B[*j*]A	28	32 (29 – 35)	10 – 60
B[*b*]F	23	13 (12 – 15)	0.1 – 0.8
B[*a*]P	1	1	1
DB[*a,h*]A	41	40 (39 – 42)	1.1 – 10
DB[*a,l*]P	81	74 (70 – 79)	1 – 100

[a] PM_{10} data from (Dreij et al., 2017).
[b] Coal tar (SRM1597a); urban dust (SRM1649b); diesel PM (SRM1650b) data from (de Oliveira Galvão et al., 2022).
[c] Single PAH data from (Dreij et al., 2017; de Oliveira Galvão et al., 2022).
[d] Based on BMD_{50} with 90% lower and upper confidence limits (BMDL and BMDU).
[e] Based on *in vivo* and *in vitro* data taken from (Nisbet and LaGoy, 1992; IPCS/WHO, 1998; Larsen and Larsen, 1998; Minnesota Department of Health, 2016; de Oliveira Galvão et al., 2022).
[f] Not available.

individual PAHs, previous research has already demonstrated that *in vitro* RPFs based on activation of H2AX or Chk1 proteins are in good agreement with regulatory accepted RPFs based on *in vitro* and *in vivo* data (Audebert et al., 2012; Dreij et al., 2017). This was demonstrated here also to be true when the concentration response modelling was based on BMD modelling (Table 2).

For environmental samples, the same approach is not as applicable. Several studies have demonstrated that various environmental samples and complex mixtures are mutagenic and tumorigenic *in vivo* (Seagrave et al., 2002; Long et al., 2016; Aoki, 2017; Platel et al., 2022) some of which also involved B[*a*]P, enabling the estimation of relative potency factors (Schneider et al., 2002; Long et al., 2017). However, it is difficult to obtain these samples to include in subsequent *in vitro* genotoxicity testing. To the authors' knowledge, only four commercially available SRMs have been tested in animal bioassays alongside B[*a*]P (Marston et al., 2001; Courter et al., 2007; Courter et al., 2008; Siddens et al., 2012; de Oliveira Galvão et al., 2022). The current study found a good agreement (within one order of magnitude) between the *in vitro* MPFs for the three included SRMs and the estimated *in vivo* cancer potencies, indicating that determining cancer MPFs based on *in vitro* genotoxicity is valid (Table 2) (de Oliveira Galvão et al., 2022). However, these comparisons should be made with caution, as only one concentration of SRM was used in these *in vivo* studies. To facilitate validation *in vitro* genotoxicity assays should be performed in conjunction with new cancer bioassays that include concentration–response data and use the same environmental samples.

Alternatively, the association between *in vitro* toxicity and health endpoints could be evaluated by various modeling approaches in epidemiologic studies. Such approaches have demonstrated significant associations between the oxidative potential of PM *in vitro* and cardio-respiratory health endpoints including lung cancer (Bates et al., 2019; Gao et al., 2020). These associations are primarily due to the emissions of redox-active metals (e.g. copper and iron) and organic species (e.g., quinones) from vehicles and biomass burning, which can lead to excessive production of reactive oxygen species production in the airways, causing local and systemic inflammation and triggering various disease processes. In addition, a good agreement was demonstrated between estimated lung cancer incidence based on population exposure modelling applying *in vitro* PM MPFs and reported number of lung cancer cases in Stockholm, Sweden (Dreij et al., 2017). To apply this latter approach for the $PM_{2.5}$ samples tested here was not the aim since this was not possible for most sites and would be of limited use since the samples represented spot samples collected over a few days or weeks. Due to the short time frame during which the $PM_{2.5}$ samples were collected, and the discrepancies between the MPFs obtained from two models for the samples, it is not meaningful to try to assess what they might represent in terms of health risks for the populations living near these sites. Similarly, would assessing cancer risks based on measured levels of B[*a*]P and the WHO lifetime unit risk for inhalation be of limited relevance.

Future studies aimed at larger citywide assessments should include a year-long sampling campaign of $PM_{2.5}$ to also include seasonal changes in air quality and conducted at multiple sites to reflect local differences in emissions. In order to provide an acceptable population exposure assessment, such studies should be promoted in cities where well-developed emission inventory and dispersion models are available. Similarly, smaller site-specific studies, such as occupational environments, should combine sampling with personal exposure monitoring of $PM_{2.5}$. This would allow better validation of this *in vitro* NAM for testing environmental samples to improve human health risk assessment.

4. Conclusions

Our research focused on the application of a NAM for whole-mixture assessment that goes beyond mass measurements of PM or individual components of air pollution. As has been proposed for the oxidative potential of PM, such an approach may integrate the health-relevant fraction of air pollution and thus better reflect potential human health effects. Specifically, we applied a NAM to estimate the relative cancer potency factors of complex environmental mixtures. In doing so, we aimed to make a significant contribution to the field of predictive toxicology and cancer risk assessment. Of the two modeling approaches evaluated here, BMD is much more widely used and accepted and promoted by regulators as the state of the science for determining relative potencies based on concentration–response data. Although both models were in agreement for the individual PAHs, the discrepancy for the environmental samples needs to be further investigated as suggested here to further validate this NAM. The shift from identifying individual agents to evaluating entire mixtures is critical for accurate risk assessment.

CRediT authorship contribution statement

Marcos Felipe de Oliveira Galvão: Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Caroline Scaramboni:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Burcu Ünlü Endirlik:** Investigation, Formal analysis. **Antero Vieira Silva:** Writing – original draft, Investigation, Formal analysis. **Mattias Öberg:** Supervision, Investigation, Formal analysis. **Simone Andréa Pozza:** Investigation, Formal analysis. **Tetsushi Watanabe:** Resources, Investigation, Formal analysis. **Poliany Cristiny de Oliveira Rodrigues:** Investigation, Formal analysis. **Pérola de Castro Vasconcellos:** Resources, Investigation, Formal analysis. **Ioannis Sadiktsis:**

Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Kristian Dreij:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2024.108942.

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