Contributions of city-specific $PM_{2.5}$ to differential *in vitro* oxidative stress and toxicity implications between Beijing and Guangzhou of China

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Abstract

Growing literature has documented varying toxic potencies of source- or site-specific fine particulate matter ($PM_{2.5}$), as opposed to the practice that treats particle toxicities as independent of composition given the incomplete understanding of the toxicity of the constituents. Quantifying component-specific contribution is the key to unlocking the geographical disparities of particle toxicity from a mixture perspective. In this study, we performed integrated mixture-toxicity experiments and modelling to quantify the contribution of metals and polycyclic aromatic hydrocarbon (PAHs), two default culprit component groups of $PM_{2.5}$ toxicity, to *in vitro* oxidative stress caused by wintertime $PM_{2.5}$ from Beijing and Guangzhou, two megacities in China. $PM_{2.5}$ from Beijing exhibited greater toxic potencies at equal mass concentrations. The targeted chemical analysis revealed higher burden of metals and PAHs per unit mass of $PM_{2.5}$ in Beijing. These chemicals together explained 38% and 24% on average of $PM_{2.5}$-induced ROS in Beijing and Guangzhou, respectively, while >60% of the effects remained to be resolved in terms of contributing chemicals. PAHs contributed approximately twice the share of the $PM_{2.5}$ mixture effects as metals. Fe, Cu, and Mn were the dominant metals, constituting >80% of the metal-shared proportion of the $PM_{2.5}$ effects. Dibenzo[a,l]pyrene alone explained >65% of the PAH-shared proportion of the $PM_{2.5}$ toxicity effects. The significant contribution from coal combustion and vehicular emissions in Beijing suggested the major source disparities of toxicologically-active PAHs between the two cities. Our study provided novel quantitative insights into the role of varying toxic component profiles in shaping the differential toxic potencies of city-specific $PM_{2.5}$ pollution.

■ INTRODUCTION

Poor air quality is among the world's leading environmental health risks.[1–3] Long-term and short-term exposure to airborne fine particulate matter ($PM_{2.5}$) have repeatedly been found to be associated with an increased risk of both morbidity and mortality in the developed world.[4] The resulting hazard ratio risk estimates (per μg m^{-3}) have been employed by authoritative organizations, such as the World Health Organization (WHO), to estimate the effects of exposure to airborne fine particulate matter on the health of populations around the world.[5,6] Ambient air pollution, mostly from $PM_{2.5}$, has been estimated to lead to 4.2 million premature deaths per year worldwide, predominantly in Asia.[7] An often used primary assumption underlying these estimations is that particle toxicities are treated as independent of composition given the incomplete understanding of the toxicity of the constituents.[7,8]

Evidence from recent epidemiological and *in vivo* studies has placed the assumption under scrutiny. For example, a nationwide study[9] spanning 272 cities in China established daily mortality risk estimates lower than those found in most studies conducted in developed countries, and observed inter-regional differences across China in the exposure-response relationship. Another *in vivo* study[10] revealed greater short-term pulmonary toxic responses in mice exposed to $PM_{2.5}$ from California than to $PM_{2.5}$ from China at equal mass concentrations; the differential toxicities appeared to be driven by a higher level of oxidized organic carbon and possibly by a greater copper content in Californian than in Chinese $PM_{2.5}$.

These epidemiological and *in vivo* findings may reflect the regionally varied sources of pollution that shape the distinct chemical compositions within a country or across the different continents.

For example, the extensive use of residential heating in wintertime in northern China leads to a higher contribution from the burning of coal than in eastern and southern China.[11,12] Particles originating from different source categories have been shown to exert differential biological effects *in vitro*.[13,14] Thus, city-specific ambient airborne PM, which is shaped by varying combinations of source categories and the prevailing meteorology, would likely have disparate toxicological properties. However, how cocktails of toxic components in ambient $PM_{2.5}$, which are the manifestation of geographical distinctions in sources of pollution, and account for the toxicity and health outcomes that have been observed is not yet understood.[3,15]

As more components have been identified, fewer gaps remain in our knowledge about the chemical mass balance of $PM_{2.5}$.[16] However, not all components contribute to the overall toxicity of $PM_{2.5}$; the relevant mixtures of toxic components and their respective contributions to the overall toxicological properties of $PM_{2.5}$ are still largely unknown.[15] Previous studies often targeted chemicals, such as metals and polycyclic aromatic hydrocarbons (PAHs), and correlated them to the total biological effects of $PM_{2.5}$.[17,18] Underlying this approach is the unproven presumption that metals and PAHs are the dominant contributors to the toxicity of $PM_{2.5}$. Without toxicological profiling of individual metals and PAHs, it remains unclear to what extent known toxic components, such as metals and PAHs, contribute to the overall toxicity of $PM_{2.5}$, or whether there is a need to identify other contributing toxic components. These critical knowledge gaps have long been pursued in previous studies, but are yet to be resolved with appropriate quantitative approaches. Therefore, mixture-toxicity experiments and modeling[19] can generate new insights into the comparative toxic component profiles of city-specific $PM_{2.5}$. Closing the toxic effect balance of $PM_{2.5}$ is more relevant to determining the health impacts of $PM_{2.5}$ than closing its

chemical mass balance.

To effectively assess chemical mixtures, a conservative approach adopting the concentration addition concept has been proposed.[20] Based on the assumption that all components in a given mixture act by a similar mode of action, doses can be added to predict the combined effects. This assumption enables the bioanalytical equivalent concentration (BEQ) approach to be used to quantitatively interpret the combined effects of environmental samples containing unresolved mixtures of chemicals on a given biological endpoint. In the BEQ, an environmental mixture is expressed as the equivalent concentration of a reference compound that causes the same biological responses. Thus, the BEQ-based mixture model serves as a pragmatic tool to determine the quantitative contributions of the identified components to the combined effects of environmental samples, particularly when assessing aquatic and terrestrial environmental quality.[21–28] While seldom attempted in toxicological studies on air pollution,[29–31] this approach can aid in identifying components associated with $PM_{2.5}$ that drive the effects of fine particles on certain health-relevant biological endpoints, such as oxidative stress.

Oxidative stress plays an essential role in air pollution-induced health effects.[32] Previous studies often assessed the chemical oxidative potential of airborne particles from acellular assays (*e.g.*, dithiothreitol (DTT) assay).[33,34] These cell-free, chemical-based assays can easily capture the intrinsically redox active components in $PM_{2.5}$, such as transition metals and quinones,[35,36] but are unable to recognize those components (*e.g.,* parent PAHs) that require metabolic activation to become reactive in humans.[37] This limitation may partially explain the controversial link between the chemical oxidative potential of ambient airborne particles and respiratory health effects.[38–42]

In vitro cell-based assays are a potential alternative to measuring intracellular reactive oxygen species (ROS),[43] a complement to DTT-based extracellular ROS generation. The BEAS-2b human bronchial epithelial cell model, for instance, largely retains the significant capability of *in vivo* pulmonary metabolism.[44] This *in vitro* metabolic competence allows the cell system to capture of all active components in $PM_{2.5}$ in an unbiased manner to induce intracellular ROS. Although they are not fully predictive of human toxicity, *in vitro* assays offer a logistically simpler platform to assess the mixture effects of $PM_{2.5}$ and contributing components, and provide first-tier evidence for further coherent investigations along the cell-animal-human continuum.

While toxic mechanisms of $PM_{2.5}$ have been extensively explored, the critical knowledge gap remains in the quantitative role of the measured components in the combined toxicity effects of $PM_{2.5}$ mixtures on the established endpoints as simple as ROS induction. The objective of this study was thus to determine component-specific contribution to *in vitro* ROS formation triggered by $PM_{2.5}$, with a focus on two metropolitan areas in China with clearly contrasting urban and pollution features. We compared the effect potencies of city-specific $PM_{2.5}$ samples at equal mass concentrations to trigger cytotoxicity and ROS in BEAS-2b human bronchial epithelial cells. Mixture-toxicity experiments and modeling were performed to test the validity of concentration-addition model in predicting the joint effects of environmentally realistic mixtures (*e.g.,* metals and PAHs) present in the studied $PM_{2.5}$ samples on ROS induction. With this premise, we then employed the BEQ concept to estimate the fractional contributions of metals and PAHs, which have conventionally been deemed to be the dominant drivers of toxicity. This study delivered a novel approach to assessing the relative importance of different components in the mixture effects of $PM_{2.5,}$ and thus shed light on the site disparities in exposure-toxicity relationship between air

pollution and human health.

■ EXPERIMENTAL SECTION

$PM_{2.5}$ sampling. For this study, we selected Beijing (North China) and Guangzhou (South China), which have distinct geographical and urban features and starkly contrasting pollution profiles (Figure S1). Details of the sampling sites are given in Table S1 of the Supporting Information (SI) section. Daily 24-h $PM_{2.5}$ samples were collected on 8 × 10 inch quartz microfiber filters (PALL, USA) using a high-volume sampler equipped with a 2.5 μm inlet at a flow rate of 1 m^3 m^{-1}. The sampling campaign was conducted in January 2014 (details are given in Table S2). During the sampling campaign in each city, the air sampler was not operated for 24 h and a filter that served as a field blank was placed inside it. Before sampling, all of the filters were pre-baked for 6 h at 500 °C to remove any contamination caused by carbonaceous materials. The filters were weighed twice, once before and once after sampling, using a balance (Sartorius Analytic, Gottingen, Germany) with a sensitivity of ±0.1 mg. After sampling, the loaded filters were covered with aluminum foil and stored at −20 °C before undergoing analysis.

Preparation of PM extracts. Each $PM_{2.5}$ filter sample (including field blanks) was extracted with Milli-Q water (pH =7) and methanol (100%) following the previously established protocol.[17] Each quartz filter (size equivalent to one-eighth of an A4 paper) was extracted in 15 mL of Milli-Q water by 30-min sonication and extracted again in 15 mL of methanol by 30-min sonication. The combined PM extracts were stored at −80°C overnight, lyophilized, and transferred into pre-weighed, sterile, amber glass vials. The amber glass vials containing the dried particle extracts were weighed again to determine the particle mass extracted from the quartz filter. The extracts

were reconstituted in cell culture medium at the concentration of 200 mg L^{-1} for exposure tests; otherwise they were stored at −80 °C until analysis.

Cell culture and bioassays. Human bronchial epithelial BEAS-2b cells were obtained from the American Type Culture Collection (ATCC) and were cultured in a DMEM medium (10% heat-inactivated fetal bovine serum and 1% penicillin-streptomycin antibiotics) at 37 °C in a humidified atmosphere with 5% CO_2. An MTT colorimetric assay was used to determine the viability of the cells. Intracellular ROS generation by $PM_{2.5}$ samples was determined using a 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay. Cells were seeded at 2×10^5 cells mL^{-1} in black 96-well plates, and grown to confluence for 24 h. After removing the medium, the cells were washed twice with PBS, and then exposed to 100 μL of $PM_{2.5}$ samples or test chemicals serially diluted in medium. *Tert*-butylhydroquinone (tBHQ), a well-known inducer of intracellular ROS,[45,46] were included as a reference chemical in each plate. After 24-h exposure, the medium was the removed and the cells were washed twice with PBS. One hundred μL of phenol red free DMEM containing 100 μM DCFH-DA was then added to the cells. After incubation for 30 minutes at 37 °C, the medium was the removed and the cells were washed twice with PBS again. Fluorescence intensity was measured at 0 h and 2 h using an automated microplate reader at excitation/emission wavelengths of 485/535 nm. ROS production was expressed as the percent increase in fluorescence intensity from 0 h to 2 h. The ROS induction ratio (IR) of the sample relative to the control was calculated using eq 1. Linear concentration–effect curves[47] with an intercept of 1 and a fitted slope (eq 2) were used to determine the effect concentration at an ROS induction ratio of 1.5 ($EC_{IR1.5}$) (eq 3).

$$\mathrm{IR} = \frac{\%_{increase\ sample\ t=2}}{\%_{increase\ control\ t=2}} \qquad (1)$$

$$\mathrm{IR} = 1 + \mathrm{slope} \cdot \mathrm{concentration} \qquad (2)$$

$$EC_{IR1.5} = \frac{0.5}{slope} \qquad (3)$$

Chemical analysis. The analysis of trace metals in the samples followed our previously established procedure.[48] An aliquot of the extracts was mixed with 70% high-purity nitric acid (HNO_3) and 65% perchloric acid ($HClO_4$). The sample was digested to dryness using a progressive heating program, and reconstituted in 5% HNO_3. Quality control was carried out by analyzing reagent blanks, replicates, and standard reference materials (NIST SRM 1648a, urban particulate matter). Concentrations of trace metals were determined using an Inductively Coupled Plasma - Mass Spectrometer (ICP-MS, Agilent 720). The concentrations of trace metals in regent blanks were <1% of the average analyte concentrations for all of the targeted metals, and the recovery rates of the metal elements in the standard reference material (NIST SRM 1648a) ranged from 96-110%.

The analysis of these organic compounds followed previously established procedures,[49] based on direct thermal desorption and derivatization from the filtered PM with subsequent gas chromatography – time-of-flight mass spectrometry (Pegasus III, Leco Inc., USA). In addition to PAHs as potential ROS inducers, we quantified hopanes as tracers of fossil fuel combustion, and anhydrosugars (levoglucosan, mannosan, and galactosan) as tracers of biomass burning. We did not measure the organic compounds in the same $PM_{2.5}$ extracts as we did for metals, due to the limited particle mass. Instead, we measured the concentrations of PAHs in the $PM_{2.5}$ that had been collected on the filter. We performed QA/QC tests using our spare $PM_{2.5}$ samples to compare the concentrations of PAHs normalized to $PM_{2.5}$ mass on the original filter and those of PAHs normalized to the particle mass in the $PM_{2.5}$ extracts. The two concentrations were similar,

qualifying the subsequent assessment of the contribution of PAHs to the ROS induction by $PM_{2.5}$ extracts.

Mixture-toxicity modeling. We selected intracellular ROS as an exemplary endpoint to quantify the contribution of the identified chemicals, including trace metals and PAHs, to the overall effect of $PM_{2.5}$. This was achieved by mixture toxicity modeling, following previously established procedures.[23,50] The effect concentrations for the tested chemicals ($EC_{IR1.5,i}$), the reference compound t-BHQ ($EC_{IR1.5,t\text{-}BHQ}$), the defined mixtures of targeted metals and PAH ($EC_{IR1.5,mix}$), and $PM_{2.5}$ sample extracts ($EC_{IR1.5,PM2.5}$) were determined in the BEAS-2b ROS assay. The relative effect potency of each active chemical (REP_i) for ROS generation can be calculated against that of t-BHQ as the reference compound (eq 4)

$$REP_i = \frac{EC_{IR1.5,t-BHQ}}{EC_{IR1.5,i}} \qquad (4)$$

$PM_{2.5}$ extracts are composed of an unresolved mixture of chemicals at unknown concentrations. The concept of bioanalytical equivalent concentrations (BEQ) can aid in the quantitative interpretation of a certain bioassay of the overall biologically active chemical burden present in a sample extract ($BEQ_{bio,PM2.5}$ in the case of $PM_{2.5}$ in the current study). $BEQ_{bio,PM2.5}$ is defined as the equivalent concentration of t-BHQ that causes the same effect (the 1.5-fold induction of ROS) as the $PM_{2.5}$ extract (eq 5).

$$BEQ_{bio,PM_{2.5}} = \frac{EC_{IR1.5,t-BHQ}}{EC_{IR1.5,PM_{2.5}}} \qquad (5)$$

To assign the quantitative contribution of each individual identified component, we tested the validity of the assumption that the sum of the effect that each individual component has on ROS generation approximates the combined effect of these chemicals mixed together, using the concentration-addition (CA) model. The model has been well validated to predict the mixture

effects of organic chemicals on non-specific endpoints, such as baseline toxicity and oxidative stress response that involve multiple mechanisms.[23,50] The validity of the mixture effects of metals and PAHs on intracellular ROS generation is yet to be confirmed. Using the concentration addition model, we predicted the concentration-effect for ROS generation through realistic mixtures of metals and PAHs present at the percent molar composition (p_i) determined in the samples using eq 6.

$$EC_{IR1.5,CA} = \frac{1}{\sum_{i=1}^{n} \frac{p_i}{EC_{IR1.5,i}}} \qquad (6)$$

An index on prediction quality (IPQ) was used to assess the deviation between the predicted and observed mixture effects.[51] An IPQ of zero means that there is a perfect agreement between model prediction and experimental observation. A positive IPQ indicates a higher CA predicted $EC_{IR1.5}$ ($EC_{IR1.5,CA}$) than an experimental one ($EC_{IR1.5,exp}$), while the opposite is true for a negative IPQ (eqs 7 and 8).

$$\text{If } EC_{IR1.5,CA} > EC_{IR1.5,exp}, \text{ then } IPQ = \frac{EC_{IR1.5,CA}}{EC_{IR1.5,exp}} - 1 \quad (7)$$

$$\text{If } EC_{IR1.5,CA} < EC_{IR1.5,exp}, \text{ then } IPQ = 1 - \frac{EC_{IR1.5,exp}}{EC_{IR1.5,CA}} \quad (8)$$

If the IPQ falls within the -1/+1 range, a good agreement can be deemed to have been reached between the experimental determination and the model prediction, which means that the joint effects of metals and PAHs was in accordance with the prediction of the concentration-addition model.

The BEQ_{chem} derived for each identified component or for their mixtures based on an instrumental analysis (eq 9) can then be used to calculate how much of an effect can be explained by the chemicals that were quantified in the samples (*i.e.,* % contribution), using eq 10.

$$BEQ_{chem} = \sum_{i=1}^{n}(C_i \cdot REP_i) \quad (9)$$

$$\%\ contribution = \frac{BEQ_{chem}}{BEQ_{bio,PM_{2.5}}} \cdot 100\% \quad (10)$$

The uncertainty analysis was performed to estimate the contribution (% contribution) by propagating the errors of all the variables involved in the calculation. The equations for error propagation are presented in Section S1 of SI.

■ RESULTS AND DISCUSSION

Differential toxic potencies of city-specific $PM_{2.5}$ at equal mass concentrations. Exposure to $PM_{2.5}$ samples from both Beijing and Guangzhou resulted in concentration-dependent cytotoxicity and ROS formation in BEAS-2b cells (Figure 1). The concentration-effect curves of the two cities diverged with different slopes, meaning that there were significant differences between the two cities in cytotoxicity and ROS formation at the same mass concentration of $PM_{2.5}$. The IC_{50} of the Guangzhou $PM_{2.5}$ for cytotoxicity (205±18 mg L^{-1}) averaged twice that of the Beijing $PM_{2.5}$ (101±15 mg L^{-1}) (Figure 1a), which means that the cytotoxic potency of Beijing $PM_{2.5}$ was nearly double that of the Guangzhou $PM_{2.5}$. Likewise, the $EC_{IR1.5}$ of the Guangzhou $PM_{2.5}$ for ROS generation (5.4±0.3 mg L^{-1}) was nearly three times that of Beijing (1.7±0.1 mg L^{-1}) (Figure 1b), meaning that the oxidative stress potency of the Beijing $PM_{2.5}$ samples was triple that of the Guangzhou $PM_{2.5}$. The average concentrations of the $PM_{2.5}$ samples in Beijing (220±102 μg m^{-3}) were approximately twice those of Guangzhou (104±32 μg m^{-3}) over the sampling period (Table S2). Should differential toxic potencies at an equal mass concentration be considered for city-specific scenarios, the exposure risks of $PM_{2.5}$ in Beijing would be more than four times that in Guangzhou. In a retrospective cohort study on 31 Canadian cities, inter-city differences in GSH-related oxidative potential were found to modify the association the risk of low birth weight and

prenatal exposure to $PM_{2.5}$ based on mass concentrations.[52] Our results together with the recent findings highlight the need to reconsider the sole use of the mass concentration as a dose metric in the risk estimate of $PM_{2.5}$ exposure, and to develop integrated toxic indicators of direct relevance to specific health outcomes for accurately adjusting the mass concentration.

Different concentrations of metals and PAHs per unit mass of city-specific $PM_{2.5}$. The question naturally follows of what components caused the differences between Beijing and Guangzhou in the biological effects that were observed at equal mass concentrations of $PM_{2.5}$. Here, we focused on metals and PAHs, which are commonly believed to be key toxic components associated with $PM_{2.5}$. The targeted metals and PAHs occurred at significantly higher levels per unit mass of $PM_{2.5}$ in Beijing than in Guangzhou (Figure 2a; Tables S4 and S5). The $PM_{2.5}$ mass-normalized concentrations of metals and PAHs in Beijing were approximately five times and an order of magnitude, respectively, higher than those in Guangzhou. In particular, the excessive cancer risk per million people due to PAHs was nearly an order of magnitude higher in Beijing than in Guangzhou, exceeding the risk value stipulated by the WHO (Figure 2b; details of the calculation methods are given in SI, Section S2 and Table S6).

Relative comparisons of the PAH congener diagnostic ratios (Figure 3) revealed a higher contribution from pyrogenic sources, such as fossil fuel combustion and vehicular emissions, in Beijing than in Guangzhou, from the overall influence of coal combustion and/or biomass burning. This is supported by significantly higher concentrations of hopanes, the tracers of fossil fuel sources (including coal combustion and vehicular emissions) in $PM_{2.5}$ from Beijing than from Guangzhou ($p < 0.0001$; Table S7). Similarities in the total concentrations of the three analyzed

297 anhydrosugars, the tracers of biomass burning, between Beijing and Guangzhou ($p = 0.2022$; Table
298 S7) suggested a similar scale of biomass burning as an emission source of PAHs. From a
299 contribution perspective, biomass burning would thus account for a much larger share in the
300 emission sources of PAHs in Guangzhou than in Beijing. Not surprisingly, a recent radiocarbon
301 analysis of carbonaceous aerosols found that the dominant source of wintertime emissions is fossil
302 fuel combustion in Beijing, and non-fossil fuel combustion in Guangzhou.[53] Source
303 apportionments of PAHs using positive matrix factorization in previous studies[54] also pointed to
304 the greater influence of coal combustion in Beijing as the key disparity in sources of pollution
305 between the two cities. For a more constrained source apportionment of toxicologically active
306 PAHs, a compound-specific radiocarbon analysis coupled with positive matrix factorization would
307 quantitatively resolve the fossil and non-fossil origins of PAHs, to prioritize the source target(s) of
308 these toxic components. Despite the limitations associated with the use of PAH congener ratios,
309 the importance of region-specific sources of emission in shaping the varying compositions of toxic
310 chemical cocktails at equal mass concentrations of $PM_{2.5}$ was reiterated in the source diagnosis. It
311 appears to echo the differences in toxic responses that were observed between the two megacities.
312
313 **Additive effects of metals and PAHs on ROS generation.** Prior to the quantitative dissection of
314 the contributions of the identified metals and PAHs to the overall $PM_{2.5}$-induced effects, we tested
315 the validity of the assumption that the sum of the effect of each individual component on ROS
316 generation approximates the combined effects of those chemicals as a mixture. We fingerprinted
317 the potency of each individual metal and PAH (Figure 4; Table S8). The $EC_{IR1.5}$ values and hence
318 the relative effect potencies of the identified metals and PAHs spanned five orders of magnitude
319 from $1.2(\pm0.4)\times10^{-9}$ M for dibenzo[a,l]pyrene (DBalP) to $8.6(\pm1.2)\times10^{-5}$ M for Cr(III). We

correlated the reported rates of DTT loss from metals and PAHs[35] with our measured $EC_{IR1.5}$ values of the corresponding chemicals (Figure S2). The relative potency ranking of metals for ROS induction in BEAS-2b cells generally followed their relative oxidative potential ranking in the DTT assay, with the only exception of Cd. However, PAHs, exemplified by pyrene (PYR) and fluoranthene (FLA), exhibited much higher potencies than their DTT-based oxidative potential suggested. Parent PAHs were generally considered to be inactive in acellular assays measuring the chemical oxidative potential of airborne particles. Our results emphasized the beneficial use of cell-based assays to incorporate toxicokinetics, which may modify inactive components in acellular assays into potent agents to induce biological effects. Therefore, acellular assays may be predictive of extracellular ROS formation in lung lining fluid, for example, through intrinsically redox-active species, such as metals and quinones. Cell-based assays may account for intracellular ROS formation by both redox-active components and those that can be metabolically activated after they enter lung cells.

We then mixed the identified metals and PAHs together at the molar compositions measured in the corresponding samples (Table S9) for a screening of their combined effects (Table S10). As the IPQs for all 25 tested mixtures of metals and PAHs fell within the range of between -1 and +1, the CA predicted ROS induction by the mixtures of active metals and PAHs that occurred in the samples agreed well with the experimentally determined ROS induction effects (Figure 5 and Table S10). Thus, the real-world mixtures of multiple metals and PAHs present in the $PM_{2.5}$ acted jointly in a concentration-additive manner on the same biological endpoint, *i.e.*, the induction of intracellular ROS in this study. Previous studies[55] have shown that synergistic or antagonistic interactions can occur in some cases that involve binary or tertiary combinations of metals and/or

343 organic compounds as designed mixtures. Such interactions may be diluted in a complex mixture
344 involving a myriad of chemicals. As predicted by the "funnel hypothesis",[56] the range of deviations
345 from concentration addition decreases with an increasing number of components in a mixture. True
346 synergism or antagonism at environmentally realistic concentrations are rare, and most mixtures
347 studied within environmental toxicology have followed concentration addition.[57] Our results
348 provided additional evidence to support the funnel hypothesis and reaffirmed that concentration
349 addition is a common mode of action by which substances in complex environmental mixtures
350 operate jointly to produce cumulative effects. Recognizing this would enable the BEQ concept to
351 be used as a relatively simple, pragmatic approach to apportioning the quantitative contribution of
352 individual components; this would not be possible if complex interactions between certain
353 components are over-emphasized.
354
355 **Contribution of metals and PAHs to $PM_{2.5}$-induced ROS generation.** The validity of the
356 concentration-addition reference model allows for $PM_{2.5}$-induced ROS generation to be
357 quantitatively attributed to individual metal and PAH components that have been identified.
358 Although metals and PAHs together accounted for a minor proportion of $PM_{2.5}$ mass
359 concentrations (6.1% for Beijing and 1.7% for Guangzhou on average; Figure 2), these minor mass
360 contributors could already explain 38% and 24% of $PM_{2.5}$-induced ROS in Beijing and Guangzhou,
361 respectively. The average fractional contribution of the measured metals to the induction of ROS
362 by $PM_{2.5}$ from Beijing (11.2±4.4%) was slightly higher than that from Guangzhou (7.3±2.0%),
363 with statistical significance (p = 0.0094) (Figure 6; Table S9). There was a significantly larger
364 difference (p = 0.0211) in the contribution of targeted PAHs to $PM_{2.5}$-induced ROS formation
365 between Beijing (26.5±10.9%) and Guangzhou (16.7±9.0%) (Figure 6; Table S9). Overall, the

366 identified metals and PAHs together contributed a 14% higher share to the mixture effect of the
367 Beijing $PM_{2.5}$ than to that of the Guangzhou $PM_{2.5}$. Of the ten metals that were analyzed as positive
368 for intracellular ROS generation, Fe, Cu, and Mn were the three dominant elements in both cities
369 (Figure 6). The three transition metals each had a similar share, amounting to >80% of the metal-
370 shared ROS induction effects. The result is consistent with previous findings indicating that these
371 transition metals dictate the oxidative potential in the DTT assay.[35] Of the 12 active PAH congeners,
372 DBalP and BaP were the two predominant drivers in both cities, explaining >80% of the total PAH-
373 induced effect, with DBalP alone contributing >65% (Figure 6). The neglect of this single congener
374 would cause 10-20% of the overall effect for Beijing and Guangzhou to remain unresolved. It is
375 stressed that the share of a component to the combined effect of a given mixture depends on both
376 the absolute concentration of the components and its relative effect potency. For example, the
377 effect potency of Fe was approximately 1.5 orders of magnitude lower than that of Cu and Mn
378 (Figure 4), but the concentration of Fe was approximately two orders of magnitude higher than Cu
379 and Mn (Table S4), which resulted in nearly equal contribution of the three transition metal;
380 Likewise, the greater effect potency of DBalP (Figure 4) compensated their lower concentrations
381 (Table S5) for its higher contribution that outcompeted the metals.
382
383 For the first time, the definitive ranking of the contribution of individual components to the total
384 toxicity of $PM_{2.5}$ was addressed in a quantitative manner through BEQ-based mixture modeling,
385 an attempt that had been pursued in many previous studies on non-air environments. Statistical
386 associations were commonly used in past investigations to link the bioactivity observed in PM
387 extracts to components such as metals and PAHs.[58–60] This approach does not resolve the toxicity
388 contribution of components at the individual chemical level, and may result in false positives. For

example, inactive PAH congeners on certain biological endpoints (*e.g.,* oxidative stress, mutagenicity) can often be found to correlate positively with PM toxicity, which may be a co-correlation with truly active congeners that originated from the same sources. Our approach can provide more definitive answers to the important questions whether commonly targeted components (*e.g.,* metals and PAHs) can fully explain the PM toxicity, and whether further identification of toxicity contributors is required.

It is worth noting that more than 60% of the total ROS induction effects remain unexplained in the current study, warranting future efforts to identify other contributing chemicals. For example, quinones and substituted PAHs (*e.g.*, hydroxylated-, alkylated-, and nitro-substituted compounds), particularly those with greater toxic potencies, can be targeted for mixture toxicity calculations. In addition to chemical contaminants, those compounds of (micro)biological origin should be included in such an exercise.[61,62] Endotoxins (*e.g.,* bacterial lipopolysaccharides), which are compounds of the outer cell membrane of Gram-negative bacteria, for instance, have been shown to induce strong oxidative stress.[63] Their potential contribution in our current samples has yet to be explored. Should the target analysis not reveal the majority of unknowns, a non-target instrumental analysis beyond that of chemical-by-chemical identification is an approach that can also be attempted.[64,65] Such approaches would help to close the gap in the effect potency balance of known and unknown toxic components acting on selected health-relevant endpoints, and shed light on those chemical mixtures that are responsible for toxicological effects in a city-specific manner.

Environmental implications. Current global exercise in ascribing mortality to outdoor $PM_{2.5}$

412 exposure relies on the practice that treats particle toxicities as independent of composition given
413 the incomplete understanding of the toxicity of the constituents. The derived guideline may
414 indicate the magnitude of mass concentration-based reduction of $PM_{2.5}$ without the consideration
415 of chemical speciation and source apportionment data. Our findings along with recent literature
416 evidence reinforce the notion that mixture effects are more realistic metrics to characterize city-
417 specific $PM_{2.5}$ exposure than their mass concentrations. As such, it is of paramount importance to
418 understand the contribution of $PM_{2.5}$-associated components to the overall mixture effects. The
419 corresponding efforts in health-oriented source apportionment can be dedicated to the major
420 toxicity contributors in $PM_{2.5}$ rather than its whole mass concentration.
421
422 The current study is well positioned to deliver a novel approach to assessing the quantitative role
423 of different components to the mixture effects of $PM_{2.5}$. Using ROS as an example, we validated
424 and applied the BEQ-based mixture-toxicity modeling approach to reveal differential toxic
425 mixtures of metals and PAHs occurring in $PM_{2.5}$ that partially account for the differential effects
426 elicited by $PM_{2.5}$ from two megacities of China. While metals and PAHs are important contributing
427 chemicals, as were quantitatively demonstrated in our study, metals may not be as dominant as
428 previously thought,[35,36] and the relative importance of PAHs may also be site and compound
429 specific. Identifying the unknown toxic components by combining (non)target analysis and
430 mixture toxicity modeling may well close the effect potency balance of known and unknown toxic
431 components acting on health-relevant endpoints. This alternative approach may overcome the
432 limitations associated with the statistical approaches that either infer the mass-dominating but
433 toxicologically irrelevant components (*e.g.,* sulphate and nitrate) or fail to resolve the contribution
434 at individual chemical level (*e.g.,* not all PAH congeners are toxicologically equal in their

contribution to the overall effects of $PM_{2.5}$). The practical implications for health-oriented emission reduction are that those toxicity-driving components of $PM_{2.5}$ become the prioritized control targets without the need for proportional mitigation of all components if based on mass concentrations only.

Revealing what toxic component mixtures cause toxicological responses addresses the chemical aspect of differential $PM_{2.5}$ toxicity. In addition, the biological aspect of differential toxicity needs to be elucidated, *i.e.,* the differential perturbations of biological pathways underlying the differential cytotoxicity and ROS formation. In this sense, system-level efforts are required, from a panel of initiating molecular markers (*e.g.,* oxidative stress, DNA damage, inflammation) to an integrated "omics" assessment,[66–68] to enhance the biological understanding of the *in vitro* exposure-toxicity relationships of city-specific $PM_{2.5}$. This can pave the way for coherence of evidence throughout cell-animal-human studies to establish a principal link from health effects to toxic components and emission sources of $PM_{2.5}$ pollution, thus facilitating the prioritization of control targets that are adaptive to city-specific scenarios to protect human health.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at XXX. It includes information about the sampling sites and collected samples, data on chemical concentrations, error propagation, dose-response curves and mathematical derivations, and a cancer risk assessment of PAHs between the two studied cities.

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Author Contributions

Ling Jin and Xiangdong Li designed the study with input from the coauthors. The manuscript was written with contributions from all of the authors. All of the authors gave their approval to the final version of the manuscript.

Notes

The authors declare that they have no competing financial interests.

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List of Figures

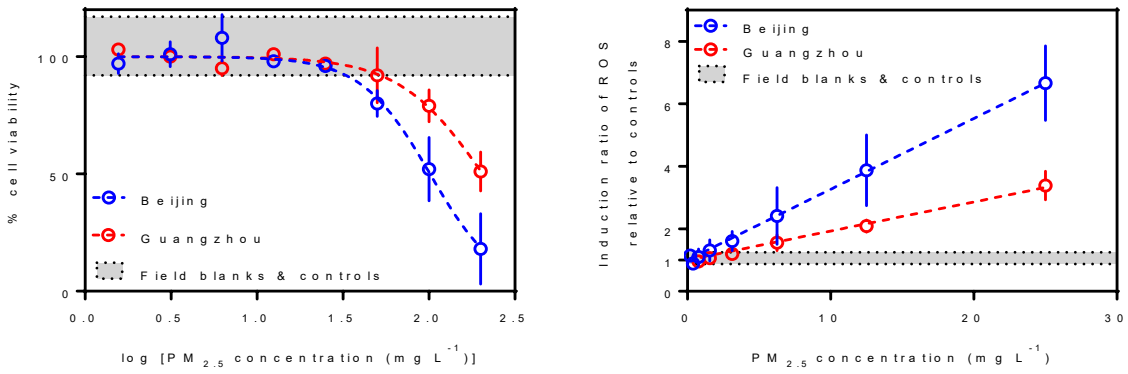
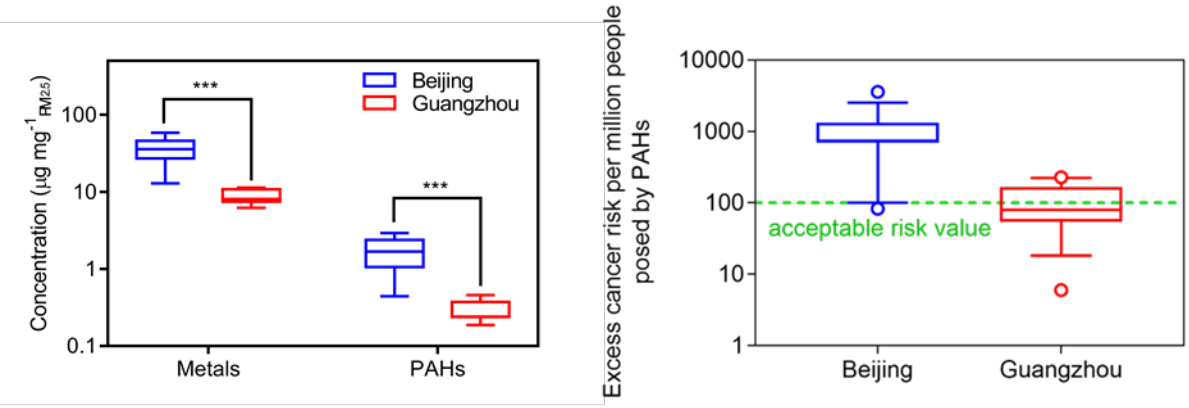


Figure 1. Combined concentration-effect curves of cytotoxicity (left) and intracellular ROS generation (right) triggered by $PM_{2.5}$ extracts from Beijing (14 samples) and Guangzhou (11 samples). The dose-response curve of each individual sample can be found in Table S3.



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775 **Figure 2.** The left panel shows the concentrations of total metals and total PAHs per unit mass of
776 $PM_{2.5}$ from Beijing and Guangzhou. Details on the concentrations of individual metal elements
777 and PAH congeners can be found in Tables S3 and S4. The right panel shows cancer risk estimates
778 from the inhalation of PAHs in $PM_{2.5}$ from Beijing and Guangzhou (detailed calculations can be
779 found in SI, Section S2).

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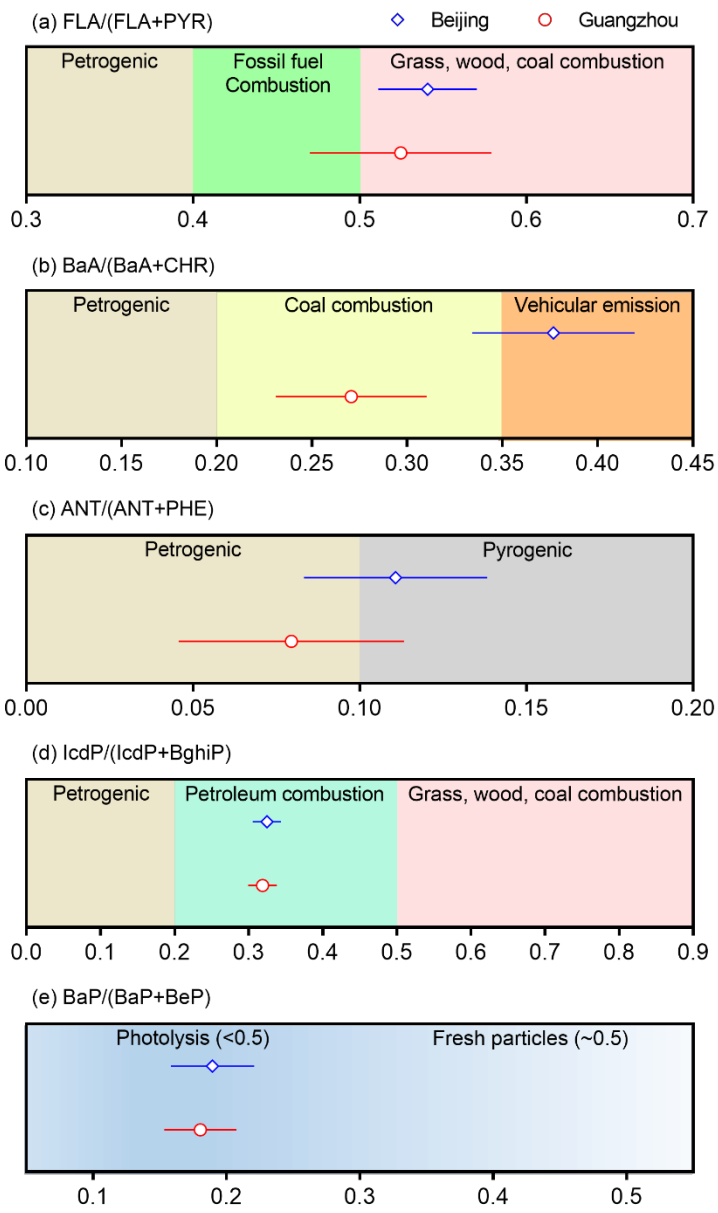

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793 **Figure 3**. PAH diagnostic ratios (mean±SD) of (a) FLA / (FLA + PYR), (b) BaA / (BaA + CHR),

794 (c) ANT / (ANT + PHE), (d) IcdP / (IcdP + BghiP), and (e) BaP / (BaP + BeP) in $PM_{2.5}$ from

795 Beijing (blue diamonds) and Guangzhou (red circles). The characteristic diagnostic ratios

796 differentiating difference sources are from Refs 69,70.

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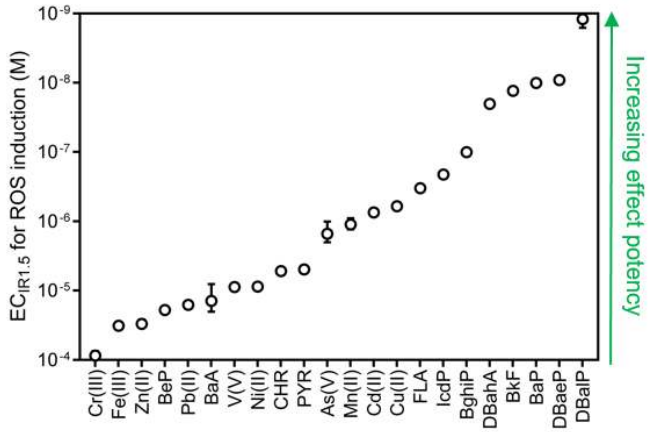

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800 **Figure 4.** Effective concentrations of each identified metal and PAH that induced 1.5-fold
801 intracellular ROS relative to controls in BEAS-2b cells ($EC_{IR1.5}$). The concentration-effect curve
802 of each chemical and related derivations are found in Table S8. Note that the *y*-axis is in a reverse
803 order for an easier readership, *i.e.*, the lower $EC_{IR1.5}$ a chemical has, the greater is its effect potency.
804 Not all error bars of $EC_{IR1.5}$ can be visually displayed because the small values are omitted on a
805 logarithmic scale. The detailed error propagation can be found in Table S8.

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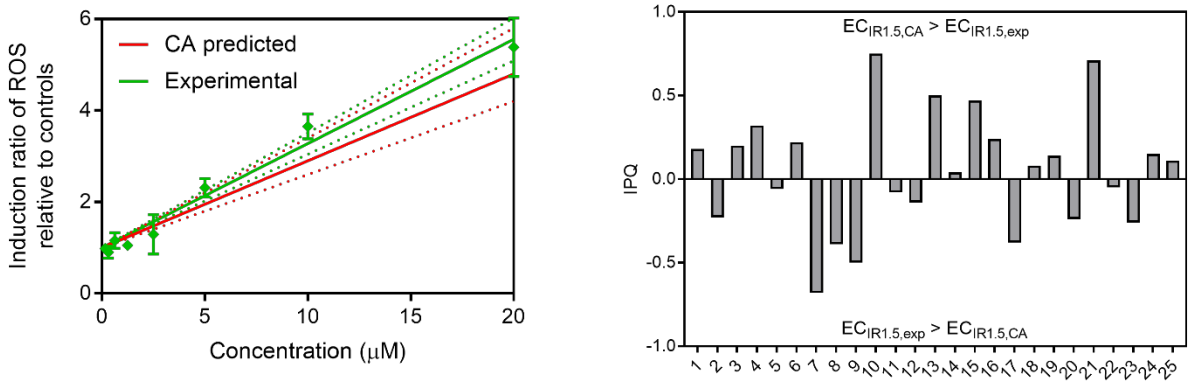

Figure 5. A comparison of the CA predicted *v.s.* experimentally determined concentration-effect curves for ROS induction by measured metals and PAHs in sample BJ-1 as an example (see the validation for the other samples in Table S8). The solid lines represent the best fit lines, and the dashed lines represent the 95% confidence intervals. The right panel shows the index on prediction quality (IPQ) for the 25 defined mixtures of metals and PAHs corresponding to the 14 Beijing (BJ-1 to BJ-14) and 11 Guangzhou (GZ-1 to GZ-11) $PM_{2.5}$ samples in order (a detailed derivation is given in Table S8).

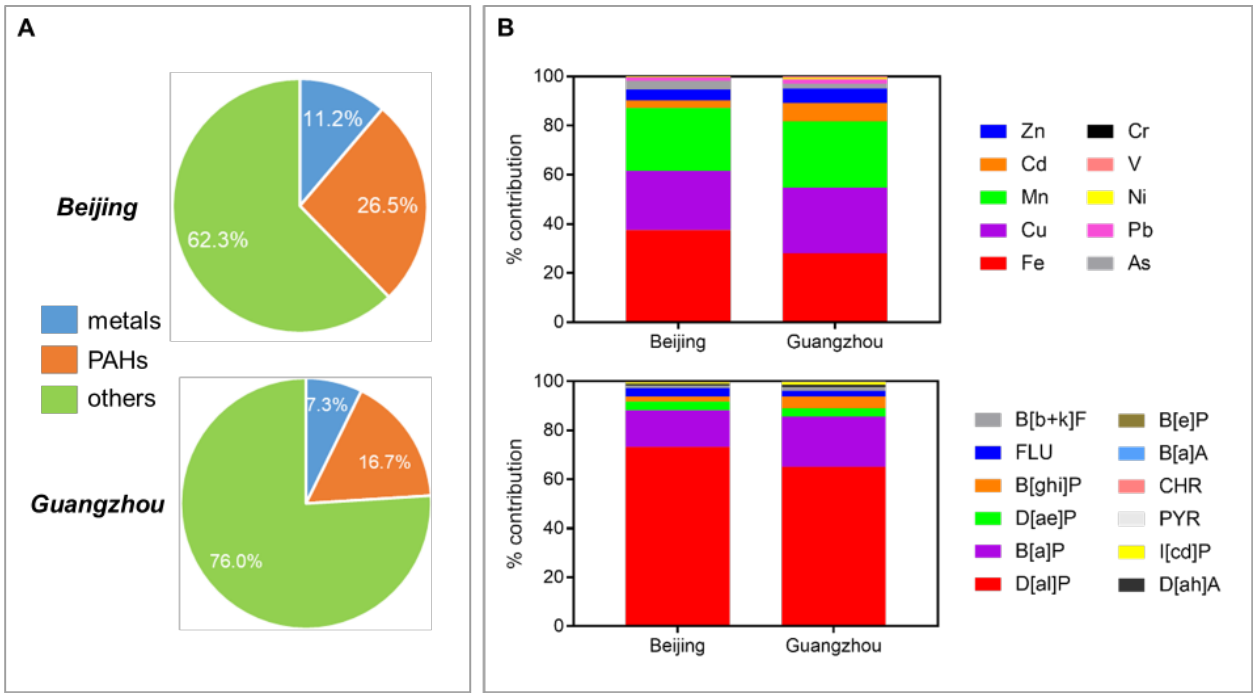

Figure 6. (A) Relative contribution of trace metals and PAHs to $PM_{2.5}$-induced intracellular ROS in Beijing (averaged from the 14 studied samples) and Guangzhou (averaged from the 11 studied samples); and (B) Individual chemical-resolved contributions to the metal- or PAH-shared ROS induction effects in Beijing (averaged from the 14 studied samples) and Guangzhou (averaged from the 11 studied samples). The detailed derivation can be found in Table S11.

851 **TOC Art**

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