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Mixture Effects of Benzene, Toluene, Ethylbenzene, and Xylenes (BTEX) on Lung Carcinoma Cells via a Hanging Drop Air Exposure System

Faye F. Liu,[†,‡] Beate I. Escher,[†] Stephen Were,[§] Lesley Duffy,[∥] and Jack C. Ng*[,†,‡]

[†]National Research Centre for Environmental Toxicology (Entox), The University of Queensland, 39 Kessels Road, Coopers Plains, Brisbane, Queensland 4108, Australia

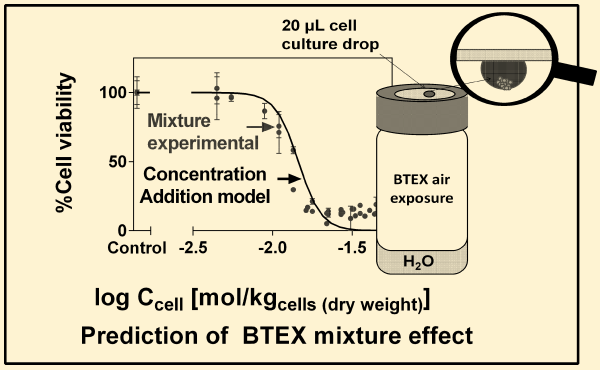
[‡]CRC for Contamination Assessment and Remediation of the Environment, Mawson Lakes, Adelaide, South Australia 5095, Australia

[§]Department of Agriculture, Fisheries and Forestry, Queensland, Health and Food Sciences Precinct (DAFF), 39 Kessels Road, Brisbane, Queensland 4108, Australia

[∥]CSIRO Food and Nutritional Sciences, 39 Kessels Road, Coopers Plains, Brisbane, Queensland 4108, Australia

Supporting Information

ABSTRACT: A recently developed hanging drop air exposure system for toxicity studies of volatile chemicals was applied to evaluate the cell viability of lung carcinoma A549 cells after 1 and 24 h of exposure to benzene, toluene, ethylbenzene, and xylenes (BTEX) as individual compounds and as mixtures of four or six components. The cellular chemical concentrations causing 50% reduction of cell viability (EC_{50}) were calculated using a mass balance model and came to 17, 12, 11, 9, 4, and 4 mmol/kg cell dry weight for benzene, toluene, ethylbenzene, *m*-xylene, *o*-xylene, and *p*-xylene, respectively, after 1 h of exposure. The EC_{50} decreased by a factor of 4 after 24 h of exposure. All mixture effects were best described by the mixture toxicity model of concentration addition, which is valid for chemicals with the same mode of action. Good agreement with the model predictions was found for benzene, toluene, ethylbenzene, and *m*-xylene at four different representative fixed concentration ratios after 1 h of exposure, but lower agreement with mixture prediction was obtained after 24 h of exposure. A recreated car exhaust mixture, which involved the contribution of the more toxic *p*-xylene and *o*-xylene, yielded an acceptable, but lower quality, prediction as well.



1. INTRODUCTION

Benzene, toluene, ethylbenzene, and xylenes (BTEX) are important constituents in petroleum products such as gasoline (petrol). They are also used in the manufacturing of paint, rubber products, and adhesives.[1,2] BTEX components are single-ring aromatic compounds, and their toxicities to different organs vary.[3−8] Acute exposure to BTEX as found typically in gasoline has been associated with skin and sensory organ irritation (e.g., eye and nose irritation), central nervous system (CNS) problems (e.g., tiredness, dizziness, headache, and loss of coordination), and effects on the respiratory system.[9] Chronic exposures are associated with problems in the kidney, liver, and blood systems.[3−6] Chronic exposure to small amounts of BTEX compounds in the ambient (outdoor) air, at work, and at home is associated with various adverse health effects.[10]

BTEX mixture toxicity studies have been focused on binary interactions such as benzene with toluene,[11] ethylbenzene,[12] and xylenes.[12] Sporadic ternary and quaternary mixture studies have been reported for occupational exposure to benzene, toluene, and xylene over unspecified durations based on measurements of serum immunoglobulin,[13] rat embryonic development assay for benzene, toluene, and xylene exposure,[14] and rat and human exposure to toluene, ethylbenzene, and *m*-xylene.[15] Data on the BTEX mixture are incomplete, and especially lacking is information on how the dose and the percentage of dose proportion of each component may influence a joint action.[9]

While BTEX *in vivo* toxicity might be more complex,[16] their cytotoxicity to cell lines is most likely caused by nonspecific baseline toxicity or narcosis.[17] Because all BTEX compounds are likely to act according to the same mode of action in cell lines, their mixture effect is hypothesized to follow the mixture toxicity concept of concentration addition.[18,19]

A549 is a lung carcinoma cell line that has been used widely in toxicity studies of the lung.[20] BTEX is known to be metabolically activated by cytochrome P450 enzymes (CYPs). P450s, particularly CYP2 family enzymes, catalyze phase I oxidation.[3−6] Although CYPs are expressed primarily in the liver, all of the CYPs that are involved in BTEX metabolism are

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Table 1. EC_{50} and Index on Prediction Quality (IPQ) Values for Concentration Addition (CA) and Independent Action (IA) for BTEX Compounds

BTEX mixture	constant concentrate ratio mixture[a] (B/T/E/*m*X/*o*X/*p*X) (%)	EC_{50} mmol/$kg_{dry\ weight}$ experimental	EC_{50} (CA) mmol/$kg_{dry\ weight}$ 95% CI	EC_{50} (IA) mmol/$kg_{dry\ weight}$ 95% CI	IPQ[c] CA	IPQ[c] IA
equipotent mixtures	25/25/25/25	14.6 ± 1.9	14.5 ± 0.1	26.5 ± 8.3	0	0.8
double toluene	17/50/17/17	11.0 ± 2.3	13.7 ± 0.1	25.5 ± 0.2	0.2	1.3
double ethylbenzene	17/17/17/50	12.8 ± 1.5	14.1 ± 0.1	30.3 ± 0.01	0.1	1.4
double *m*-xylene	17/17/50/17	13.0 ± 1.4	13.8 ± 0.1	29.1 ± 0.1	0.1	1.2
exhaust mixture[b]	4/18/7/14/21/36	11.2 ± 1.4	7.1 ± 0.1	13.7 ± 0.4	−0.6	0.2
24 h equipotent	25/25/25/25	3.4 ± 0.4	4.4 ± 0.1	2.9 ± 0.1	0.2	−0.2

[a]Ratios according to the EC_{50} at 1 h HD air exposure. [b]Exhaust for reported ratio of motor vehicles.[27] [c]Index on prediction quality for concentration addition calculated according to Escher et al.[31]

expressed in the lung.[21] CYP2E1 and CYP2B6 are expressed in A549 cells, with the former being involved in the metabolism of all BTEX components and the latter not involved in benzene metabolism.[4,22,23]

Using benzene as a candidate air toxicant and A549 cells as an *in vitro* cell model, we have previously developed and validated a hanging drop (HD) air exposure system that mimics liquid interface exposure via the air phase in the lung.[24] This HD system is more sensitive, more reliable, and cheaper to use compared to other classically used air exposure systems.[24]

In this article, we performed a concentration−response assessment of cell viability of A549 cells exposed to individual and mixtures of benzene, toluene, ethylbenzene, and xylenes (BTEX) using a fixed concentration ratio mixture design.[25,26] With the knowledge of mass balance in this cell system from a previous study and established mixture toxicity models, we quantitatively describe and predict the effects of mixtures. This study further validates the hanging drop procedure as an *in vitro* air exposure system and provides more insight for BTEX mixture effect in terms of changes in mixture ratios and specific concentrations in relation to cell dry weight. It also is advantageous for comparing toxicity across different taxa.

2. EXPERIMENTAL PROCEDURES

2.1. Chemical Compounds. Benzene [CAS 71-43-2], toluene [CAS 108-88-3], ethylbenzene [CAS 100-41-4], *m*-xylene [CAS 108-38-3], *p*-xylene [CAS 106-42-3], and *o*-xylene [95-47-6] of purity >99.7% were obtained from Merck Pty Ltd., Australia.

2.2. Exposure Chambers. Volatile organic analysis (VOA) vials with Teflon-coated septum of 20 mL (LECO no. 21799, USA) and 40 mL (LECO no. 21796) volumes were used as static exposure chambers for air exposures. Because of the fact that the septa do not withstand autoclaving, clean VOA vials were uncapped, and both vials and septa were laid out with the Teflon side facing up, inside a biohazard hood, with the UV light turned on overnight before the caps were placed back on aseptically the next day. Sterilization was achieved through UV radiation and exposure to induced ozone. No visible cloudiness in the culture medium was found, as an indication of microbe contamination, through naked eye inspection, and no unexpected decrease of cell viability in the negative control was found throughout the study period.

2.3. Cell Culture Conditions. *2.3.1. Flask Culture.* Human lung epithelial carcinoma cell line A549 cell culture (ATCC CCL-185, USA) was maintained under 5% CO_2 in a humidified incubator at 37 °C or directly in a 37 °C constant temperature room. A549 cells were subcultured in DMEM medium with 10% fetal calf serum (Sigma, USA), 100 μg/mL penicillin and streptomycin (Invitrogen, USA), and 25 mM HEPES (Invitrogen no. 15630080, USA) for up to 20 passages.

2.3.2. Hanging Drop Culture. The hanging drop air exposure system was previously developed and validated.[24] Briefly, it consists of the following steps: (1) Inoculation of 20 μL cell culture drops onto the septum that fit the screw-on lid of a 20 or 40 mL VOA vial, (2) inversion of the lid to hang the culture drop as a hanging drop (HD), and (3) sealing the drop in a VOA vial for air exposure. After 24 h incubation at 37 °C, designated aliquots of BTEX as individual compounds or mixtures were injected into the vials through the septum using a 10 μL Hamilton airtight syringe as described in Liu et al.[24] The HD exposure chambers were then incubated at 37 °C for 1 or 24 h before cell viability was assessed in the HD cells (Section 2.5).

2.4. BTEX Mixture Design. In order to study the mixture effect, mixtures of benzene, toluene, ethylbenzene, and *m*-xylene (BTE*m*X) were created according to their toxic equivalence by mixing the components in ratios equivalent to their EC_{50} values, which is a constant concentration ratio mixture design. The mixture components are shown in Table 1. Mixture 1 was the equipotent mixture, and mixtures 2−4 contained double toluene, double ethylbenzene, and double *m*-xylene, respectively. In Table 1, the concentration ratios are listed in constant concentration ratio mixtures, and these ratios were converted to molar ratios according to their densities at room temperature and to their molar concentrations for the convenience of mixture modeling. A composition similar to a car exhaust mixture (Table 1) was made according to the reported BTEX ratio from a study involving 80 Australian vehicles.[27] The mixtures involving double concentrations of toluene, ethylbenzene, or *m*-xylene were designed to identify the component that would the most influence the BTEX mixture effect.

2.5. Cell Viability Assay. Cell exposures were done according to our previous study.[24] Briefly, upon completion of incubation, the HD exposure chambers were opened in a laminar flow cabinet with the lid facing up to expose the 20 μL culture drop on the septum surface. Twenty microliters of CellTiter-Glo reagent was then transferred onto the culture drop before each lid was covered with a clear cover and incubated under light on a platform shaker at approximately 12 rotations/s. This was the maximum speed that did not disturb the drops. After 13 min of shaking, which was the maximum allowable light exposure time according to the manufacture's instructions, the drops were transferred to the 384-well plate (Corning no. 3712) for luminescence measurement in an Infinite M200 plate reader (TECAN, Switzerland).

2.6. Chemical Analysis. BTEX evaporation time and the stability of concentration in the HD exposure chambers were assessed by measuring changes of benzene concentration according to our previously described method.[24] Briefly, five replicates of 40 mL HD chambers were set up, and into each chamber was added 2 mL of sterile distilled water to maintain the chamber humidity before being tightly capped. Before analysis, 10 μL of benzene was injected into each chamber through the septum. The headspace benzene concentrations were quantified using GC-MS after 10, 40, 60, 90, 100, and 120 min incubation at 37 °C. Another five replicates of the HD chambers were also set up, and their benzene concentrations were monitored after 2, 24, 28, and 48 h incubations.

For GC-MS analysis, 10 μL of head space sample was taken at each incubation time point with a 50 μL Hamilton syringe and directly injected into a Trace GC Ultra with DSQ single quadruple GC-MS

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from Thermo Scientific equipped with a Phenomenex ZB-624 capillary column (20 m × 0.18 mm i.d., 1 μm film) as previously described.[24] More details on GC-MS parameters are shown in Table S1 in the Supporting Information.

2.7. BTEX Bioavailability in the Hanging Drop Air Exposure System. Each exposure consisted of 11−22 doses of three replicates, and doses were converted into intracellular concentration using a mass balance model.[24] As illustrated in Figure S1 in the Supporting Information, bioavailability of BTEX, represented as freely dissolved concentrations and concentrations taken up into the cells, was calculated from published physicochemical properties using a four-component mass balance model, which was adapted from our previous paper and included air, water, and medium components as well as cells.[24] Equations 1−3 were used to calculate the fraction in the air (f_a), the aqueous phase (f_w), and the cells (f_{cell})

$$f_{cell} = \frac{1}{1 + \frac{1}{K_{cellw}}\frac{V_w}{m_{cell}} + \frac{K_{aw}}{K_{cellw}}\frac{V_a}{m_{cell}} + \frac{K_{FBSw}}{K_{cellw}}\frac{m_{FBS}}{m_{cell}}} \quad (1)$$

$$f_a = \frac{1}{1 + \frac{K_{FBSw}}{K_{aw}}\frac{m_{FBS}}{V_a} + \frac{K_{cellw}}{K_{aw}}\frac{m_{cell}}{V_a} + \frac{V_w}{K_{aw}V_a}} \quad (2)$$

$$f_w = \frac{1}{1 + K_{aw}\frac{V_a}{V_w} + K_{cellw}\frac{m_{cell}}{V_w} + K_{FBSW}\frac{m_{FBS}}{V_w}} \quad (3)$$

where K_{aw}, K_{FBSw}, and K_{cellw} are partition coefficients between air and water, fetal bovine serum (FBS) and water, and cell and water, respectively; V_a and V_w are volumes of air and water; and m_{FBS} and m_{cell} are the dry masses of FBS and cells. K_{FBSw} and K_{cellw} were predicted from protein− and lipid−water partition coefficients as described previously.[24] K_{aw} values were taken from recent publications,[28,29] with temperature adjustments applied when necessary,[29] and dry weight fraction values of FBS and rat hepatoma H4IIE cells were measured gravimetrically after freeze-drying.[24]

2.8. Data Analysis, Statistics, and Mixture Effect Modeling. GraphPad Prism 5 (GraphPad Software, USA) was used to fit the concentration response curve using a log−logistic model (eq 4). The BTEX concentrations were the modeled concentrations in the cells ($mol/kg_{dry\ weight}$); the $logEC_{50}$ and the slope were the adjustable fit parameters.

$$\%\ \text{cell viability} = \frac{100}{1 + 10^{(\log EC_{50} - \text{logconcentration}) \times \text{slope}}} \quad (4)$$

The mixture effect of BTEX was modeled with the concentration addition model (eq 5) and independent action (eq 6)[18,30]

$$E_{CA} = \frac{1}{\sum_{i=1}^{n} \frac{P_i}{E_i}} \quad (5)$$

$$E_{IA} = 1 - \prod_{n}^{i=1} (1 - E_i) \quad (6)$$

p_i (p_1, p_2, ..., p_n) is the fraction of compound i used in the mixtures. E_i (E_1, E_2, ..., E_n) is the effect of compound i as used in the mixtures, E_{CA} represents the predicted mixture effect for concentration addition, and E_{IA} is the effect predicted for IA; EC_{IA} was derived from running eq 6 for a series of concentrations.

The index on prediction quality (IPQ), which is a measure of the deviation between the observed and predicted mixture effects, was calculated with eqs 7 and 8 according to previous studies.[30,31]

If $EC_{50,CA} > EC_{50}$ mixture, then

$$IPQ = \frac{EC_{50,CA}}{EC_{50,mixture}} - 1 \quad (7)$$

If $EC_{50,CA} >$ EC50 mixture, then

$$IPQ = 1 - \frac{EC_{50,CA}}{EC_{50,mixture}} \quad (8)$$

Errors for EC_{CA} and EC_{IA} were calculated with error propagation using eqs 9 and 10 with the experimental errors of the ECs of the individual compounds and assuming negligible errors in p_i

$$\delta\log EC_{CA} = \left(\left(\frac{\delta\log EC_{CA}}{\delta\log EC_1}\right)^2 \times \delta\log EC_1{}^2 + \left(\frac{\delta\log EC_{CA}}{\delta\log EC_2}\right)^2 \times \delta\log EC_2{}^2 + \ldots + \left(\frac{\delta\log EC_{CA}}{\delta\log EC_n}\right)^2 \times \delta\log EC_n{}^2\right)^{1/2} \quad (9)$$

For $n = 3$, the error for the independent action (IA) prediction is

$$\delta E_{IA} = \sqrt{\begin{array}{l}((1 - EC_2)(1 - EC_3))^2 \times \delta EC_1{}^2 \\ + ((1 - EC_1)(1 - EC_3))^2 \times \delta EC_2{}^2 \\ + ((1 - EC_1)(1 - EC_2))^2 \times \delta EC_3{}^2\end{array}} \quad (10)$$

As effect concentrations were log-normally distributed, the differences between treatments were analyzed using one-way ANOVA or Student's t test with log-transformed cellular concentration.

2.9. Caution: *These chemicals are hazardous and should be handled carefully.*

All components of BTEX are flammable and are irritants through skin and eye contact. Benzene has been classified as a group 1 carcinogen by the IARC.[10] Ethylbenzene is a class 2B compound,[32] and toluene and xylenes are group III.[16,33] Storage of BTEX should be in an approved area in sealed containers, segregated in a cool and dry place with ventilation. BTEX exposure should be carried out in a fume cupboard with appropriate personal protective equipment to avoid inhalation, skin contact, or splash to the eye.

3. RESULTS

3.1. Concentrations in the Headspace. Concentrations in the HD chamber were monitored only for benzene, assuming that all other BTEX compounds behave similarly, which is justified by the similarity in their physicochemical properties. The benzene concentration in the HD chambers was plotted against the time of incubation for under 2 h and for between 2 and 48 h (Figure 1). The benzene concentration reached its maximum at a minimum detection time of 10 min, and the injected liquid visually disappeared almost instantly.

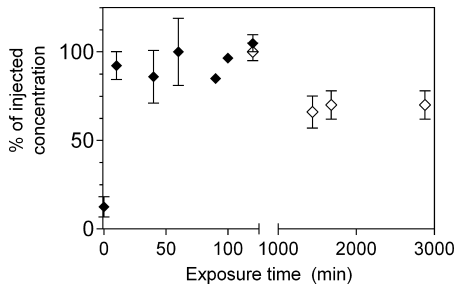

Figure 1. Benzene air concentrations at 37 °C in the 40 mL hanging drop air exposure chamber. The dose was injected at $t = 0$; closed diamonds, 2 h exposure; open diamonds, 48 h exposure. Error bars represent the standard deviations of five replicates; data with no error bars indicates only one data point.

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This observation indicates a very quick evaporation and distribution in the headspace of the HD exposure chamber.

There was approximately a 30% decrease in the headspace benzene concentration from 2 to 24 h, and that concentration remained unchanged for up to 48 h. The small initial loss was likely caused by sorption to some surfaces (glass vial, septum) because leakage through the septum would have incurred a continuous loss over time. Overall, the loss is small enough that the starting air concentration after injection can be used as an input parameter for modeling the cellular concentration. Hence, the amount of benzene depletion was noted, but it not used to adjust the EC_{50} values for the 24 h exposures.

3.2. Bioavailability. According to the mass balance model, illustrated in Figure 2, more than 99% of BTEX remained in air and less than 0.3% partitioned to the aqueous phase. This represents the amount that was available to the cells, and it was extremely low because of the low volume mass contribution of this compartment. However, concentrations in cells were relatively high (i.e., the chemicals were accumulating in the cells). The fraction in cells varied by a factor of 10 between the least bioaccumulative (benzene) and the most bioaccumulative (ethylbenzene) BTEX component. This indicated that even if the same amounts of all BTEX components were dosed, their respective biologically active cellular concentrations will vary among the compounds.

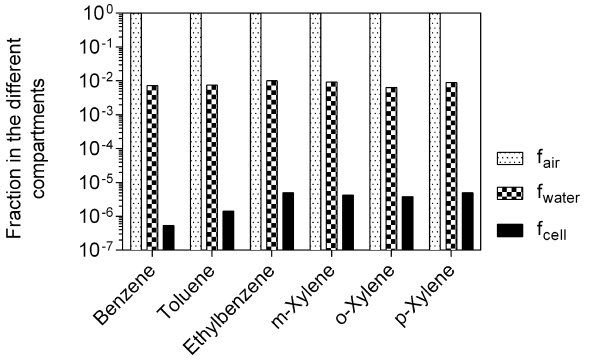

Figure 2. Mass balance of BTEX components in a 20 mL hanging drop air exposure system. More than 99% of BTEX remained in the air phase, approximately 1% is in the aqueous phase, and a negligible amount is in the cells. Benzene fraction values were reported from Liu et al.[24] Reproduced with permission from ref 24. Copyright 2013 Elsevier.

3.3. EC_{50} of BTEX Individual Components at 1 and 24 h. Repeatable concentration–response curves were obtained for all individual BTEX exposures for both 1 and 24 h (Figure 3). As the dose metric, we used the cell concentrations calculated with the mass balance model. Injections at volumes below 1 μL, using 10 μL Hamilton syringes, caused larger standard deviations for *o*-xylene and *p*-xylene exposures as well as all BTEX components for the 24 h exposures. As shown in Figure 3A, the toxic potency of BTEX in A549 cells decreased (i.e., the EC_{50} value increased) at 1 h of exposure in the following sequence: *o*-xylene, *p*-xylene, *m*-xylene, ethylbenzene, toluene, and benzene. Benzene was the least toxic, and *p*-xylene and *o*-xylene were approximately equipotent. Significant differences among the exposure effect of BTEX components were found through one-way ANOVA ($p = 0.01$). As shown in Figure 3B, after 24 h exposure, the sequence of toxicity after *p*-xylene and *o*-xylene is benzene, ethylbenzene, *m*-xylene, and toluene ($p < 0.01$, one-way ANOVA).

While the nominal EC_{50} varied by a factor of 46 between the most potent (*m*-xylene) and the least potent compound (benzene), the modeled EC_{50} normalized to cell dry weight of BTEX ranged only from 4–17 $mmol/kg_{cell\ dry\ weight}$ (i.e., it varied only by a factor of 4) (Table 2). The $EC_{50,cell}$ corresponded to the expected internal effect concentration (IEC) of baseline toxicants in various aquatic species.[34] The relative potencies after 1 h of exposure decreased in the order *p*-xylene, ethylbenzene, *m*-xylene, *o*-xylene, toluene, and benzene (Table 2).

3.4. Mixture Effect Modeling at 1 and 24 h. Concentration–response curves for all four mixtures with different fixed concentration ratios (Table 1) agreed well with the predicted curves for concentration addition models for 1 h exposure (Figure 4). This is also demonstrated by the low IPQ values of 0.0–0.2 (Table 1). The 24 h exposure modeling demonstrated an acceptable but lower prediction value using the concentration addition model (CA IPQ = 0.2) and a similar prediction value using the independent action model of (IA IPQ = −0.2). The EC_{50} value of the equipotent mixture (mixture 1) dropped by a factor of 4 (indicating 4 times higher toxicity) from 1 to 24 h of exposure, which agreed with exposures of BTEX single compounds (Figure S5 in the Supporting Information). There consistently seemed to be 10% of the cells alive after 1 h of exposure even at very high BTEX mixture dosages, but at 24 h, these doses killed all cells. The same phenomenon appeared, although it was not as evident, in the BTEX single compound 1 h exposure, in which only about 5% of cells were alive after 1 h of exposure (Figure 3A). These are likely to be false positive results because of the background ATP levels in the cell lysate after 1 h of exposure; the amount of ATP would have been hydrolyzed completely after 24 h of exposure. These false positive results, however, did not affect

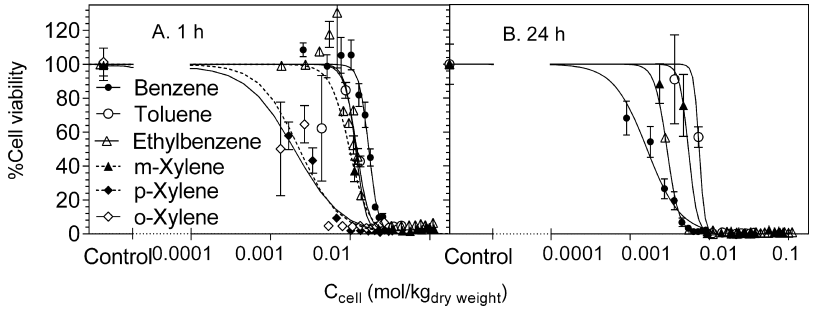

Figure 3. Concentration response of A549 cells exposed individually to benzene, toluene, ethylbenzene, *m*-xylene, *p*-xylene, and *o*-xylene (BTEX) for (A) 1 h and (B) 24 h. Data for benzene were from Liu et al.[24] Reproduced with permission from ref 24. Copyright 2013 Elsevier.

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Table 2. BTEX Toxicity and Bioavailability in 20 and 40 mL HD Air Exposure Systems

	cell sorbed fraction		1 h EC_{50} (% cell viability)		relative potency to benzene (95% CI)	
	20 mL HD	40 mL HD	EC_{50} nominal[a] (ppm)	EC_{50}[b] (mmol/kg)	nominal	IEC[b]
benzene[c]	5.31×10^{-7}	2.65×10^{-7}	92 185 ± 205	17 ± 0[e]	1.0 (1.0−1.0)[e]	1.0 (1.0−1.0)[e]
toluene	1.41×10^{-6}	7.03×10^{-7}	35 638 ± 2595	12 ± 3	2.6 (2.4−2.8)	1.4 (1.1−1.9)
ethylbenzene	4.95×10^{-6}	2.47×10^{-6}	9980 ± 998	11 ± 1	10.4 (9.4−11.7)	1.5 (1.4−1.7)
m-xylene	4.20×10^{-6}	2.10×10^{-6}	9957 ± 0[e]	9 ± 0[e]	9.3 (9.3−9.3)[e]	1.9 (1.9−1.9)[e]
o-xylene	3.80×10^{-6}	1.90×10^{-6}	3982 ± 2489	4 ± 1[d]	23.2 (14.3−61.9)	4.3 (3.4−5.7)
p-xylene	4.94×10^{-6}	2.47×10^{-6}	1991 ± 9957	4 ± 4[d]	46.3 (23.4−2714)	4.3 (2.1−17.0)

[a]Calculation of ppm in air is given in the Supporting Information, and the high air BTEX concentration provides positive kinetic partition to the cells. [b]Cellular concentration in cell dry weight. [c]Data from Liu et al.[24] [d]High standard deviation resulting from the unavoidable injection volume limitation of the 10 μL Hamilton syringe. [e]Errors were negligible.

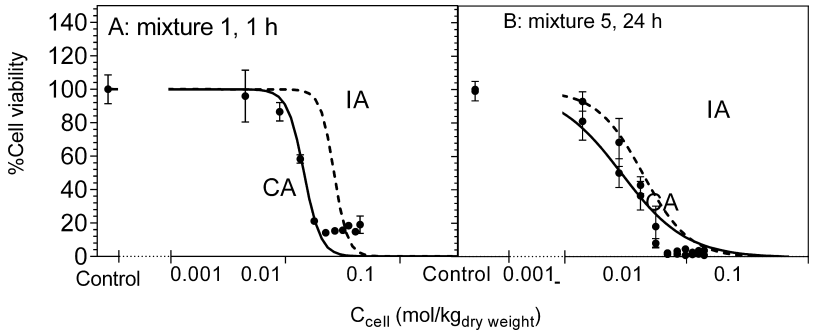

Figure 4. Example concentration−response curves for the equipotent mixture 1 (Table 1) at (A) 1 h and (B) 24 h for the corresponding concentration addition (CA) and independent action (IA) models.

the dose−response regression, as the regression line was forced through zero. Experimental data and model fits of the remaining mixture effect modeling are presented in the Supporting Information, Figures S2−S4.

3.5. Mixture Effect Modeling of the Recreated Exhaust. Mixture effects of the recreated BTEX ratio simulating exhaust fumes generated from motor vehicles[27] contained *p*-xylene and *o*-xylene in addition to the previously mentioned benzene, toluene, ethylbenzene, and *m*-xylene, and it also produced repeatable concentration−response curves. The log−logistic concentration−response curve of the recreated exhaust mixture aligned with the modeled concentration addition effect (CA IPQ = −0.6). However, the independent action model had an even greater prediction quality (IA IPQ = 0.2) (Figure 5). For this particular mixture as well as the 24 h BTE*m*X exposure, the injection volumes of *p*-xylene and *o*-xylene for the 1 h exposure and of the benzene, toluene, ethylbenzene, and *m*-xylene (BTE*m*X) components for the 24 h exposure were very low (below 1 μL) and resulted in higher uncertainty. The reason for this is that the EC_{50} determination of *p*-xylene and *o*-xylene involved injections of less than 1 μL using a Hamilton syringe. This incurred much larger dosing variation and has yielded less accurate EC_{50} values. Therefore, the predictions of the CA and IA models were further from the true value. Larger exposure chambers are recommended in order to elucidate EC_{50}'s closer to the true value, which would facilitate better prediction quality, as indicated by a higher IPQ. Because exhaust mixtures in real life are much more complex and their BTEX ratio varies from country to country, this mixture study is not representative; it is shown simply for illustrative purposes.

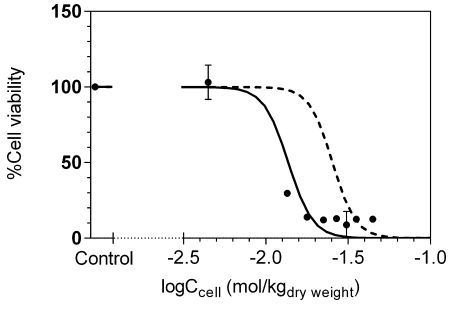

Figure 5. Effects of the recreated exhaust mixture on the corresponding concentration addition (CA) and independent action (IA) models. The BTEX exhaust mixture ratio simulated the ratio found in a study involving Australian motor vehicles[27] (Table 1). Error bars are for three replicates; for data with no error bars shown, the error is two small to be visible. Regression curve was fit using GraphPad Prism 5.

4. DISCUSSION

4.1. Exposure Concentrations and Cellular Concentration. Although sparse, BTEX effect studies in humans have been reported in occupational exposures.[7,13] Animal studies for BTEX exposures are also limited.[14,15] As has been reviewed previously,[24] *in vitro* air exposure via culture media has been criticized as being unrealistic because of the physicochemical characteristics of the culture media, which may act as barrier or accumulating sink for the air toxicants.

Since McCarty and Mackay's proposal[35] of the importance of critical body residue in environmental exposure, body burden modeling has been gaining momentum in environmental studies.[36−38] In pharmacokinetic studies, cellular concentration has also been monitored and modeled to guide anticancer drug dosage application,[39] to explain toxicological effects of antifungal agents,[40] and to determine cryoprotection ability for mouse zygotes.[41] With regard to *in vitro* air exposure

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studies,[42−46] the concept of chemical distribution among different matrices according to their physicochemical properties has been overlooked until the rebirth of the use of the hanging drop air exposure system.[24] With the aid of mass balance calculations, we were able to present, for the first time, the cellular EC_{50} for toluene, ethylbenzene, and xylenes at 1 h of HD air exposure. As shown in Table 2, compared to the constant concentration ratio mixture for BTEX of 46-fold, there is only a 4-fold difference from the most potent (*p*-xylene) to the least potent (benzene) in cellular concentration. BTEX acute toxicity falls into a category of baseline toxicity or narcosis.[36,47−49]

4.2. Mixture Effects. To date, the CAS registry contains more than 72 million unique organic and inorganic chemical substances, and risk assessment for exposure effects is no doubt a huge task. Attempts to model the chemical complexity of environmental pollution and predicted cytotoxic effects using mathematical and statistical algorithms would not only bring logical insight to the principles of chemical interactions but also greatly speed risk evaluation and risk assessment in environmental and occupational exposures.

As proven in many aquatic species and microorganisms, the mixture effect of baseline toxicity exhibits a concentration addition effect.[36,50−55] BTE*m*X exposure of A549 cells at different concentration ratios in this article confirmed concentration addition. The confirmed concentration additive mixture effect was not only in agreement with published binary interactions but also with ternary and quaternary mixtures of BTEX compounds studied in humans and animals.[14,15]

4.3. Role of Metabolism. BTEX components are known to be bioactivated by CYP2 enzymes.[23,56,57] Although CYP2E1 and CYP2B6 are expressed in human lung carcinoma A549 cells, their capability to metabolize BTEX components has not yet been investigated in these cells. Although the K_m value of benzene toward CYP2E1 has not been reported, nor has that of all BTEX components toward CYP2B6, the log K_m values for toluene, ethylbenzene, *m*-xylene, *o*-xylene, and *p*-xylene in liver microsomes have been reported to be 1.49, 1.27, 1.69, 1.67, and 1.66,[58] indicating very similar metabolic activation of all BTEX compounds. While we cannot be sure that all BTEX chemicals act according to the same mode of action, the good agreement between the model of concentration addition and the experimental data gives ample evidence that despite some modulation by metabolism the mixture effect can be essentially considered concentration additive.

Thus, we expect that metabolism is a relevant toxicokinetic process for BTEX compounds in A549 cells, and the observed toxicity was caused by a combination of effects of parents and metabolites, with the metabolites having similar potency as the corresponding parent. Metabolism cannot be implemented explicitly in the mass balance model, as no quantitative information on metabolism is available.

Therefore, the internal exposure concentrations were expressed solely as concentrations of the parent compound per dry weight of cells. It is expected that these concentrations are realistic and are not impaired by metabolism, providing that the uptake kinetics are faster than the metabolism and elimination kinetics. No depletion was expected because the fraction of mass in the cells was negligible compared to the mass in water and air and can be replenished efficiently.

4.4. Implications of Hanging Drop Air Exposure Model. Bioavailability determines the fraction of the toxicant that enters the cells and results in subsequent toxicity. Previous studies on air exposure of *in vitro* systems[42,46,59] have focused on achieving a direct air−cell contact to avoid the "media barrier effect" and have focused less on quantitative exposure concentrations. In a sealed environment, compounds will distribute themselves until they reach equilibrium according to their physicochemical properties.[29] Hence, any changes of volume/quantity in the system such as that of air, water, medium, and cell will influence the mass balance in an *in vitro* exposure system.[29]

In the events of toxic exposures of an *in vitro* study, cellular concentrations would provide a true indication of exposure dose. Compared with other classically used air exposure systems, this newly developed HD air exposure model has the advantage that by using the mass balance model it is possible to predict exposure concentrations and even internal concentrations in the cells.[24]

4.5. Exposure Limitations. The largest commercially available VOA vials are only 40 mL in volume, which limits their applicability to lower concentration effects. However, at 1 h exposure, BTE*m*X mixture effects at various concentration ratios gave highly reproducible concentration−response curves.

Lung carcinoma A549 cells were used as a sample cell model to prove that the concept of the HD air exposure model and mass balance model can be applied to mixture studies. Although the HD is a good representation for inhalation exposure, other exposure routes, such as skin absorption, and other cell types cannot be represented by the HD model.

Measurement of cellular BTEX and the concentration of its metabolites involves harvest and lysis of the exposed cells. While their water-soluble metabolic products may fall into a detectable range for HPLC, volatile parent compounds in the cells would be lost as soon as the HD chamber is opened. Hence, the rate of metabolism, which describes the metabolic capabilities of A549 cells, could not be determined in this work.

Additional research is needed to explore coculture and multiculture systems and their metabolic capabilities. The HD air exposure model allows estimation of the associated internal effect concentrations, which is a very useful tool in relating exposure effects between different taxa. Nevertheless, we acknowledge that the potential limitation of our current air−liquid interface is the culture medium, which differs in composition from that of real lung fluid.

5. CONCLUSIONS

In summary, the HD air exposure system is an easy and economical option for mixture air exposure effect studies. With the HD air exposure system being so easy to set up, sensitive, reliable, and cheap to run,[24] it provides huge potential for relating toxic responses across different taxa and for bridging information between the dosage response of *in vitro* cell studies to animal and human exposure responses.

With the advantage of ease of dosing by injection to the headspace, the hanging drop as an *in vitro* air exposure model enabled us to characterize the mixture effect of volatile chemicals and relate exposure to cellular concentrations (mol per kilogram of cell in dry weight) by mass balance modeling. These advances provide not only a new direction for *in vitro* air exposure studies but also a handy tool in environmental risk assessment of volatile chemicals. Although we performed a proof of principle for the cytotoxicity end point, the panel of biological end points can be expanded to sublethal end points such as the induction of oxidative stress or genotoxicity.

■ ASSOCIATED CONTENT

Supporting Information

Schematic diagram of the distribution of volatile organic particles in the HD system, individual 1 h BTEX mixture effects with their respective modeling, and changes in EC_{50} values of A549 cells exposed to BTE*m*X for 1 and 24 h. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +61 7 32749020; Fax: + 67 32749003; E-mail: j.ng@uq.edu.au.

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■ ABBREVIATIONS

BTEX, benzene, toluene, ethylbenzene, *m*-xylene, *o*-xylene, and *p*-xylene; BTE*m*X, benzene, toluene, ethylbenzene, and *m*-xylenes; HD, hanging drop; VOC, volatile organic compound; EC_{50}, effect concentration causing 50% of maximum effect; VOA vials, volatile organic analysis vials; IPQ, index of prediction quality

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