Research Article

An *In Vitro* Air-Liquid Interface Inhalation Platform for Petroleum Substances and Constituents

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Abstract

The goal is to optimize and show the validity of an *in vitro* method for inhalation testing of petroleum substances and their constituents at the air-liquid interface (ALI). The approach is demonstrated in a pilot study with ethylbenzene (EB), a mono-constituent petroleum substance, using a human alveolar epithelial cell line model. This included the development and validation of a generation facility to obtain EB vapors and the optimization of an exposure system for a negative control (clean air, CA), positive control (nitrogen dioxide), and EB vapors. The optimal settings for the VITROCELL® 24/48 system were defined. Cytotoxicity, cell viability, inflammation, and oxidative stress were assessed in A549 after exposure to EB vapors. A concentration-dependent significant decrease in mean cell viability was observed after exposure, which was confirmed by a cytotoxicity test. The oxidative stress marker *superoxide dismutase 2* was significantly increased, but no concentration-response was observed. A concentration-dependent significant increase in pro-inflammatory markers *C-C motif chemokine ligand 2*, *interleukin (IL)6*, and *IL8* was observed for EB-exposed A549 cells compared to CA. The data demonstrated consistency between *in vivo* air concentrations at which adverse respiratory effects were observed and ALI-concentrations affecting cell viability, provided that the actual measured *in vitro* delivery efficiency of the compound was considered. It can be concluded that extrapolating *in vitro* air concentrations (adjusted for delivery efficiency and absorption characteristics and applied for testing cell viability) to simulate *in vivo* air concentrations may be a promising method to screen for acute inhalation toxicity.

1 Introduction

There is a strong demand to implement human-relevant *in vitro* testing approaches. Inhalation is one of the major routes of exposure to xenobiotics, and the lung may serve as both the target tissue and portal of entry into the systemic circulation. The *in vitro* air-liquid interface (ALI) exposure method (i.e., cells cultured on a permeable insert with the basal surface of the cells in contact with liquid culture medium and the apical surface exposed to air) is promising, as this method: (i) does not require animals, except for animal components used for cell culture such as fetal bovine serum (FBS), although efforts are made to search for alternatives (Subbiahanadar Chelladurai et al., 2021), (ii) is in total less costly and time consuming than an *in vivo* experiments; (iii) more reliably mimics human exposure compared to submerged cell culture or rodent exposures; and (iv) facilitates the evaluation of mechanistic effects of inhaled material on human lung cells and can contribute to the development of adverse outcome pathways (Lacroix et al., 2018).

The current study assesses the performance of an ALI *in vitro* system after exposure to petrochemical substances traditionally considered difficult to test in such systems. Two key hurdles had to be addressed to achieve this goal. First, an experimental system had to be designed to address the physicochemical properties that make these substances difficult to assess in cell-based systems, such as low aqueous solubility and high volatility. Secondly, substances derived from petroleum may contain many individual constituents with a range of physicochemical properties varying

Received October 21, 2020; Accepted April 8, 2021; Epub April 20, 2021; © The Authors, 2021.

ALTEX 38(4), 550-564. doi:10.14573/altex.2010211

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in relative proportion over time. Keeping in mind that individual constituents have a range of physicochemical properties, petrochemicals are often considered difficult to test due to the tendency to volatilize (e.g., gasoline constituents have a typical boiling point range of -20°C to 250°C (Nyer and Skladany, 1989)) and to partition into organic layers (e.g., octanol-water partition coefficients are greater than 6 (Reichenberg and Mayer, 2006)). Despite these issues, the chemicals must still be assessed per regulatory registration requirements that often rely on animal testing (e.g., OECD TG 403, 433, and 436), underlining the critical need for a reliable *in vitro* system for airway exposure (Arts et al., 2008).

Gohlsch et al. (2019) demonstrated that the cytotoxicity of 19 substances (tested up to a concentration of 100 µg/cm^2) in A549 cells could be used as a reliable indicator of *in vivo* toxicity. Their results on cytotoxicity in A549 cells, exposed either under submerged conditions or at the ALI, allowed to differentiate toxic from non-classified substances, and higher sensitivity was demonstrated for the ALI exposure compared to the cells exposed under submerged conditions. Compared to submerged cultures, ALI exposure allows effective dose measurements and the physicochemical fate of aerosolized or gaseous pollutants at the lung epithelial barrier is better maintained and more realistic (Upadhyay and Palmberg, 2018).

Ethylbenzene (EB), a mono-aromatic hydrocarbon constituent in the petrochemical industry, was selected as a test compound to demonstrate the performance of the ALI exposure method and predict inhalation toxicity including *in vitro*-*in vivo* dosimetry. EB can be present in a range of petroleum streams, and exposure mainly occurs in the work environment during refinery operations including, but not exclusive to, maintenance, cleaning, pipeline repairs or routine sampling and laboratory analysis of refinery products (CONCAWE report no. 97/52 Exposure profile: gasoline[1]). Based on *in vivo* experiments, EB is classified H304 (may be fatal if swallowed and enters airways) and H332 (harmful if inhaled). This hazard information can be used to calculate exposure levels at which no health risks are expected, known as derived no effect levels (DNELs) that are required by REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) (EC, 2006). DNEL values are required for inhalation, oral and dermal routes of exposure, and for both workers and the general population. DNELs are then compared to the anticipated exposures associated with each intended use to determine whether potential risks exist, and, if so, to recommend risk management measures. These DNELs are traditionally derived from *in vivo* experiments, and it therefore would be of interest to extrapolate results from *in vitro* ALI experiments to the *in vivo* situation to support traditional risk assessments.

Literature review provided only a few papers on *in vitro* studies with EB for respiratory toxicity published by Liu et al. They studied single compounds of the BTEX mixture (benzene, toluene, EB, xylenes) in comparison to the overall mixture (Liu et al., 2014) and short-term exposure of A549 cells in a submerged set-up versus a hanging drop system (air-liquid exposure in a sealed vial), demonstrating higher toxicity or lower EC50 (i.e., half maximum response) values in the latter case, which could be explained by the volatile character of the BTEX compounds (Liu et al., 2015). This points to the requirement for a more reliable ALI exposure set-up for the compounds that occur in the vapor phase upon exposure, which are easily lost in a classical cell culture set-up (submerged cells, open system).

The ultimate goal of this work is to develop an *in vitro* method to replace *in vivo* animal tests for the prediction of human *in vivo* inhalation toxicity of petroleum substances and their constituents. Steps towards accomplishing this goal are the development and validation of a generation facility to obtain vapors from petroleum substances and their constituents, and the optimization and validation of an ALI exposure system. This paper represents a pilot study on EB vapor exposure in an ALI set-up using A549 cells, a human alveolar epithelial cell line, as a model for inhalation toxicity.

2 Materials and methods

2.1 A549 cell model and culture conditions

A new vial of the human alveolar epithelial type 2-like A549 cell line was obtained from American Type Culture Collection (ATCC number: CCL-185, 80 passages, Manassas, USA), which was originally derived from lung carcinomatous tissue from a 58-year-old Caucasian male. A549 cells were grown in T-75 culture flasks and routinely maintained in Minimal Essential Medium (MEM) 1x with GlutaMAX™-1 (Brand Gibco, ThermoFisher Scientific, Waltham, USA) supplemented with 10% non-heat-inactivated FBS superior (Merck, Darmstadt, Germany) at 37°C under 5% CO_2. Before reaching 70-80% confluence, cells were subcultured using (0.05%) trypsin-EDTA solution (Brand Gibco, ThermoFisher Scientific). Medium was refreshed every 2 days, and cells were subcultured every 3 ($9x10^5$ cells in 20 mL cell culture medium (CCM)) or 4 days ($4.5x10^5$ cells in 20 mL CCM). Cells from the work cell bank were passaged at least twice before use in experiments and no more than 20 times in total. A549 cells were negative for mycoplasma.

2.2 *In vitro* ALI exposure

A549 cells were seeded at a density of 50,000 cells/insert (~151,000 cells/cm^2) on ThinCert™ polystyrene membrane inserts, pore size 0.4 µm, surface area 0.33 cm^2 (24-well format) (Greiner Bio-One, Kremsmünster, Austria, catalogue number 662641). Inserts were placed in wells of a sterile 24-well plate filled with 600 µL CCM per well, and 100 µL CCM was added apically. Plates were incubated for ± 72 hours (h) at 37°C, 5% CO_2 in a humidified incubator. Immediately before exposure, CCM was completely removed from the apical side of the membrane inserts, and the inserts were transferred into the VITROCELL® 24/48 device (VITROCELL® Systems GmbH, Waldkirch, Germany). Before positioning the inserts in the base mod-

[1] https://www.concawe.eu/wp-content/uploads/2017/01/rpt_97-52-2003-01970-01-e.pdf

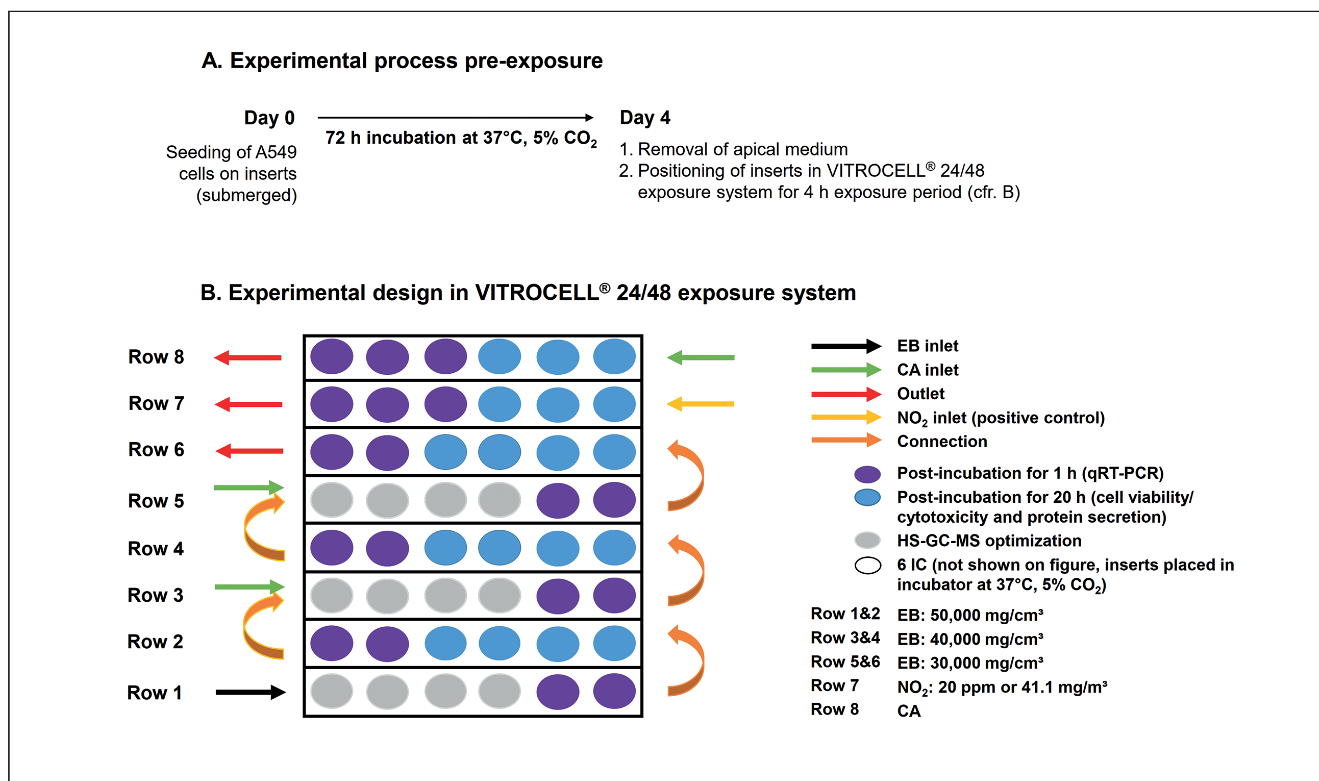

Fig. 1: Schematic drawing of experimental process (A) and design (B)

ule, each row was separately filled with CCM (20 mL), allowing cells to be nourished from below while being exposed to vapor from above. The bottom base module with inserts and CCM was positioned on a temperature-controlled heating plate set at 37°C. The base top, inlet, and exhaust module including the main distribution line were heated using a water circulation system set at 37°C. The cells were exposed to the atmosphere from the main distribution line by extraction at each of the 48 positions in the base top module using a vacuum pump. Each insert was exposed to a flow of 1.5 mL/min for 4 h. The distance between the trumpet and insert was 2 mm.

Before cells were exposed to EB vapors, the ALI system was thoroughly tested for uniformity with clean air (CA) and nitrogen dioxide (NO_2) exposure. At least 42 positions with A549 inserts in the plate were exposed to either CA (negative control) or NO_2 (in-house positive control, about 12 ppm) at a flow of 500 mL/min in the main distribution line. Experiments were repeated three times using cells of different passages.

Incubator control (IC) cells, consisting of 6 cell culture inserts without apical medium, were kept in a humidified 37°C incubator with 5% CO_2 for 24 h and served as control for CA exposure. A549 cells were exposed to 3 concentrations of EB vapors, i.e., 30,000 mg/m^3 (800 mL/min), 40,000 mg/m^3 (600 mL/min), and 50,000 mg/m^3 (490 mL/min) (in rows 6 and 5, 4 and 3, 2 and 1, respectively, 6 positions per row), CA (row 8, 6 positions), and NO_2 (20 ppm or 41.1 mg/m^3, row 7, 6 positions). After exposure, the inserts were placed in a new sterile 24-well plate (different plates for control versus exposed inserts to avoid carry over) with 600 µL CCM on the basolateral side and were allowed a recovery period of 1 or 20 h in a humidified 37°C incubator with 5% CO_2. For each biologically independent run, 12 replicate cell cultures spread over 2 rows of each EB concentration were treated in parallel in the VITROCELL® 24/48 system, of which 4 replicates per condition were incubated post-exposure (at ALI) for 1 h and analyzed for gene expression by real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR), and a further 4 replicates were incubated post-exposure (at ALI) for 20 h for further assessment of cell viability/cytotoxicity and protein secretion. In addition, 4 replicates were used for optimization of HS-GC-MS analysis (which was later replaced by dose determination in stainless steel inserts). For NO_2 and CA, only 1 row was exposed for each compound, and 3 inserts were analyzed for real-time qRT-PCR and 3 for cell viability/cytotoxicity and protein secretion. Five biologically independent runs using different cell passages were performed. Figure 1 shows a schematic drawing of the experimental process and design.

2.3 Generation and characterization of EB

Ethylbenzene (CAS 100-41-4, Sigma-Aldrich) was volatized using the following set-up (Fig. 2). A closed stainless-steel vessel (100 mL volume) with two fittings was placed on an enclosed microbalance (Mettler-Toledo AB104S, VWR, Radnor, Pennsyl-

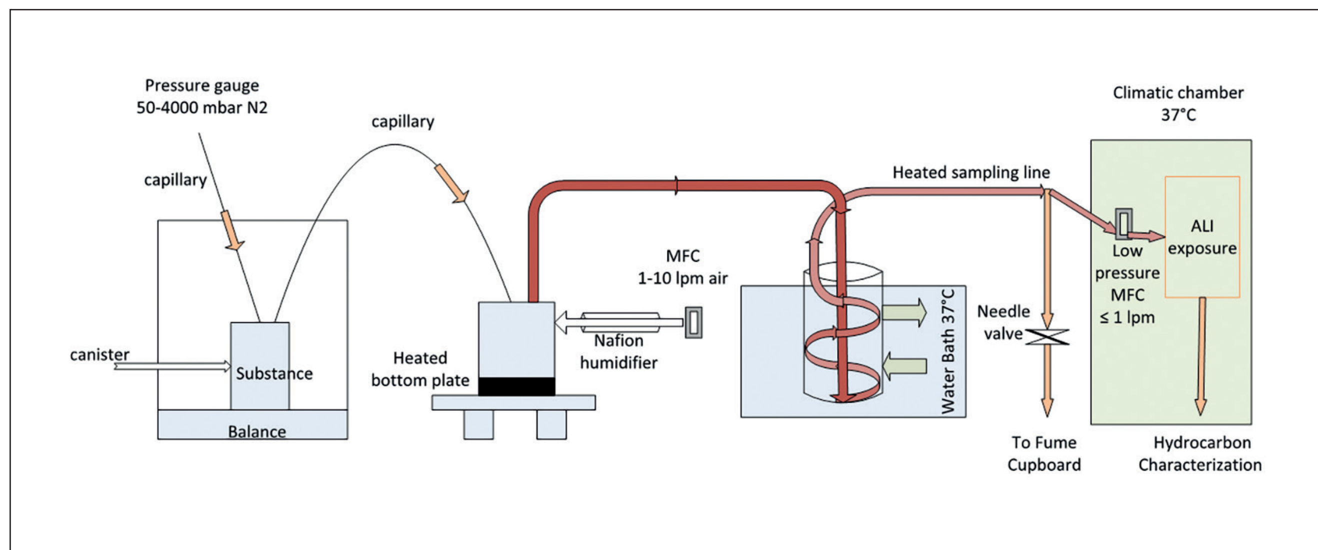

Fig. 2: Schematic drawing of EB generation setup

vania, USA). EB was injected with a pipet into the vessel, which was weighed continuously during the experiment to measure consumption. A pressure gauge was used to control the nitrogen pressure (50-4000 mbar) through the capillary into the vessel using one of the fittings. The second fitting was connected to the second capillary (outlet). By controlling the pressure, capillary size and length, the sample was precisely dosed in a tank with a heated base plate set at 195°C. The temperature of the base plate was controlled with a Variac temperature controller and monitored with a thermocouple connected to a thermometer (Fluke 52 II, Distrelec, ’s-Hertogenbosch, The Netherlands). The capillary dosage unit was based on the method described by Goelen et al. (1992) and further improved for the EB case.

When the substance dripped on the bottom plate of the tank (150-155 mg/min), it vaporized and was mixed with a controlled (Mass Flow Controller, MFC, Brooks Instrument, Hatfield, Pennsylvania, USA), humidified air flow of 3 L/min. The humidification was obtained by using two Nafion (Perma Pure, Lakewood, USA) humidifiers in parallel. A relative humidity (RH) of more than 80% is necessary to keep the A549 cells alive. Subsequently, the humidified vapor flow was guided through a double-walled glass tube positioned in a thermostatic circulating water bath (Lauda Alpha A6, VWR) set at 42°C.

The exhaust of the glass tube was connected to a glass T-piece. One side was connected to a 2-way valve to allow building up enough pressure in the flow direction of the VITROCELL® 24/48 ALI platform (VITROCELL® Systems Gmbh). The exhaust of the needle valve (overflow) was guided into the fume cupboard. The other side of the T-piece was connected through a heated sampling line (45°C) to a low-pressure MFC (Brooks Instrument) positioned inside the climatic chamber (37°C). This MFC was set at the desired flow for the first row of the ALI platform (484-492 mL/min). Before cell exposure, temperature and RH of the flow were measured (Temperature and Humidity probe 635-2135, Testo NAV/SA, Ternat, Belgium) using a T-piece.

Generated concentrations were determined by combining microbalance consumption and used airflows on the one hand and a flame ionization detector (FID) analyzer (J.U.M. Engineering 3-300A, Karlsfeld, Germany) on the other hand.

2.4 Estimation of *in vitro* test concentrations for EB

The methodology that was used to calculate *in vitro* air concentrations from concentrations of *in vivo* studies is demonstrated in Table 1. For the initial calculations, the *in vivo* absorption was considered to be equal to the *in vitro* absorption and a flow per insert of 3 mL/min; the latter was changed later during study design optimization to 1.5 mL/min (Tab. S1[2]). The *in vitro* air concentrations were calculated using the following formula:

$$C_{vitro} = \frac{C_{vivo} * t_{vivo} * V_{inh} * SA_{insert}}{SA_{lung} * F_{insert} * t_{vitro}} \qquad \text{Eq. 1}$$

C_{vitro} = *in vitro* air concentration (mg/m^3); C_{vivo} = *in vivo* exposure concentration (mg/m^3); t_{vivo} = *in vivo* exposure time (h); V_{inh} = volume inhaled air per h (m^3/h); SA_{insert} = surface area insert (cm^2); SA_{lung} = lung surface area (cm^2); F_{insert} = flow per insert (m^3/min); t_{vitro} = *in vitro* exposure time (min)

To illustrate the reasoning behind the calculations, the 4 h LC50 rat (i.e., the concentration of the chemical that will kill 50% of the test animals) is chosen as an example. Starting from the *in vivo* dose of 17,360 mg/m^3, the *in vivo* mass per cm^2 lung is calculated. Hereto, the *in vivo* exposure time, volume inhaled air per h, and lung surface area are considered. This *in vivo* mass per cm^2 lung is set equal to the mass per cm^2 insert; from here the *in vitro* concentration in air is calculated, considering *in vitro* exposure

[2] doi:10.14573/altex.2010211s

Tab. 1: Methodology for *in vivo* to *in vitro* air concentration calculations, demonstrated for the estimated calculated *in vitro* air concentrations

		Unit	**Volunteers: no effect on pulmonary function** (Moscato et al., 1987)	**Indicative occupational exposure limit (OEL), short** (EC, 2000)	**Volunteers: irritation of throat/nose and feelings of "chest congestion"** (Yant et al., 1930)	**Volunteers: no nasal irritation** (van Thriel et al., 2003)	**Volunteers: severe irritation of throat and nose (immediately)** (Yant et al., 1930)	**RD50[b], mouse, breathing rate reduction** (de Ceaurriz et al., 1981)	**RD50, mouse, breathing rate reduction** (Nielsen and Alarie 1982)	**LC50[c], rat** (Smyth et al., 1962)	**LC100, rat** (Smyth et al., 1962)	**LC100, rat** (Ivanov, 1962)
In vivo	Exposure concentration	mg/m^3	239	884	8680	425	21700	6215	17620	17360	34720	72469
	Exposure time	min	15	15	6	240	6	5	30	240	240	120
	Volume inhaled air (ECHA, 2012)	m^3/h	0.83	0.83	0.83	0.83	0.83	0.0025	0.0025	0.021	0.021	0.021
	Volume inhaled air during exposure time	m^3	0.21	0.21	0.083	3.3	0.083	0.00020	0.0013	0.084	0.084	0.042
	Total mass in the lungs	mg	49.8	184	723	1416	18083	1.30	22.0	1458	2916	3044
	Lung surface areaa	cm^2	1.00E06	1.00E06	1.00E06	1.00E06	1.00E06	82.0	82.0	4.00E3	4.00E3	4.00E3
	Concentration in the lungs	mg/cm^2	4.98E-05	1.84E-04	7.23E-04	1.42E-03	1.81E-03	1.58E-02	2.69E-01	3.65E-01	7.29E-01	7.61E-01
In vitro ALI	Concentration 24-well insert	mg/cm^2	4.98E-05	1.84E-04	7.23E-04	1.42E-03	1.81E-03	1.58E-02	2.69E-01	3.65E-01	7.29E-01	7.61E-01
	Surface area 24-well insert	cm^2	0.336	0.336	0.336	0.336	0.336	0.336	0.336	0.336	0.336	0.336
	Total mass per 24-well insert	mg	1.67E-05	6.19E-05	2.43E-4	4.76E-04	6.08E-04	5.31E-03	9.02E-02	1.22E-01	2.45E-01	2.56E-01
	Flow per insert	mL/min	3	3	3	3	3	3	3	3	3	3
	Volume aspirated air during exposure time	m^3	4.50E-05	4.50E-05	1.80E-05	7.20E-04	1.80E-05	1.50E-05	9.00E-05	7.20E-04	7.20E-04	3.60E-04
	Concentration in the presented air for	mg/m^3	0.372	1.38	13.5	0.661	33.8	354	1003	170	340	710
	x minutes	min	15	15	6	240	6	5	30	240	240	120
	Concentration in the presented air for	mg/m^3	0.023	0.0856	0.338	0.661	0.844	7.37	125	170	340	355
	x minutes	min	240	240	240	240	240	240	240	240	240	240

[a] References: human (Paur et al., 2011), mouse (Knust et al., 2009), rat (Stone et al., 1992); [b] RD50, concentration which elicits a respiratory rate decrease of 50%; [c] LC50, lethal concentration that will kill 50% of the test animals

time and flow per insert (m^3/min). *In vivo* 17,360 mg/m^3 corresponds to *in vitro* 170 mg/m^3, both for 4 h exposure and assuming *in vivo* absorption to be the same as *in vitro* absorption. The dose-range resulting from 10 *in vivo* studies considered (Tab. 1) was 0.02-355 mg/m^3 for 4 h exposure. The initially calculated ALI *in vitro* dose derived thereof was 1-625 mg/m^3 for 4 h exposure to EB, with a factor of 5 difference between consecutive concentrations. The lowest concentration (1 mg/m^3) is chosen because it is based on *in vitro* concentrations derived from acute human studies showing throat irritation (e.g., van Thriel et al., 2003); the highest concentration (625 mg/m^3) is a concentration that is above *in vitro* concentrations derived from acute lethal concentrations in animal studies (e.g., Smyth et al., 1962).

Due to lack of biological effects (data not shown) in initial ALI experiments (up to 625 mg/m^3) and during process optimization (at 7,000 mg/m^3), and based on experimental results obtained for delivery efficiency of EB (see Section 3.2), these initial *in vitro* concentrations derived through calculation from *in vivo* data had to be modified, and the high EB concentrations of 30,000-50,000 mg/m^3 were finally tested.

2.5 Chemical analysis of delivered dose

The actual delivered dose is a result of the delivery efficiency of the substance from an aerosol to the liquid lining of the cell layer (linked to its physicochemical properties) and the deposition efficiency of the exposure system.

Analysis of EB-exposed cells showed that necessary cell handling prior to headspace-gas chromatography-mass spectrometry (HS-GC-MS) analysis caused high evaporation rates that were not representative for the actual exposure conditions. Dose determination was performed using stainless steel inserts with 125 µL CCM to minimize sample handling time and thus evaporation. Inserts were placed in the VITROCELL® 24/48 module under the same conditions as the final EB experiments with cells. The mean delivered dose inside the stainless steel inserts is a proxy for the dose deposited on the cell surface and absorbed in the cells (~intracellular dose).

The samples were diluted with mineral water (Spa Reine) and doped with the isotope-labeled compound D10-EB in a sealed vial. The HS sampler heats the vial at 70°C for 30 min. During this period the EB transitions from the sample matrix into the vapor phase above. A fixed volume of the HS vapor is extracted from the vial and injected into a capillary column for GC separation. A mass spectrometer is used to detect and quantify the EB (Thermo HS-GC-MS). The MS single-ion monitoring mode of operation was used to enhance the detector sensitivity and selectivity. The internal standard method is used for the quantitative determination of EB. The quantification is based on the integrated peak areas of the most characteristic ions for EB and D10-EB.

2.6 Cell viability/cytotoxicity determination

To assess cell viability or cytotoxicity, several assays are available for application in an ALI set-up. We performed the MTT (measurement of mitochondrial activity) and lactate dehydrogenase (LDH) assay (measurement of membrane integrity).

The conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium salt into its reduced form formazan was assessed. MTT stock was prepared in Dulbecco's phosphate-buffered saline (DPBS) at a concentration of 5 mg/mL. The MTT substrate is prepared in CCM, added to the cells in culture at a final concentration of 1 mg/mL, and incubated for 2-3 h at 37°C and 5% CO_2. The formazan product of the MTT tetrazolium accumulates as an insoluble precipitate inside cells as well as being deposited near the cell surface and in the CCM. The formazan must be solubilized prior to recording absorbance readings by adding, e.g., isopropanol (2 h incubation, shaking at room temperature (RT)). The quantity of formazan (presumably directly proportional to the number of viable cells) was measured using a multi-mode microplate reader in absorbance mode (570 nm; CLARIOstar, BMG Labtech, Offenburg, Germany). Results were expressed as percentages of CA-exposed (negative) control cells.

Cytotoxicity was assessed using the LDH detection kit, CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Cytotoxicity Assay, Promega, Madison, USA) according to the manufacturer's instructions. Briefly, 20 h ALI post-exposure, 200 µL of medium was added to the apical side of the inserts and incubated at 37°C and 5% CO_2. After 20 min, medium from the apical and basolateral compartments were pooled, and 100 µL of medium was incubated for 10 min with 100 µL LDH substrate mix. The reaction was stopped by the addition of 50 µL stop solution.

As high control, cells were exposed to the lysis solution of the CytoTox-ONE™ kit. Complete CCM incubated with the quantification reagents was used as background control. Fluorescence measurements were done using a multi-mode microplate reader in fluorescence mode (ex: 530-15 nm, em: 600-20 nm; CLARIOstar, BMG Labtech).

Both assays were conducted in technical duplicates for five biologically independent runs with 4 replicate inserts per run. Changes in cell viability (MTT) and cytotoxicity (LDH) were analyzed relative to CA and were assessed by mixed models while considering the experiment ID (biological replicate) as random factor. Data were analyzed using R[2] (R version 3.5.1 (2018-07-02) – "Feather Spray") and specific packages for mixed model analyses "lme4" (Bates et al., 2015) and "lmerTest" (Kuznetsova et al., 2017). Linear mixed models were fit by REML (restricted maximum likelihood) using function lmer with default parameters. *P*-value of fixed effects smaller than 0.05 was used as cut-off for statistical significance. For gene expression and protein expression, fixed effects are presented in this manuscript together with their 95% confidence interval (CI) in bar plots. 95% CI means that there is 95% chance that the range contains the true mean or, in other words, we can interpret that if we repeat the study 100 times, we will get the same values in 95% of cases. Significance of results from CI can be assessed in the following way: If 95% CI captures the value of no effect (e.g., 0 for log2 fold change in case of gene expression), this represents a statistically non-significant result (at significance level 0.05). If 95% CI does not include the value of no effect, then this represents a statistically significant result.

[3] https://www.R-project.org/

2.7 Biomarker analysis

Based on a literature review on EB, markers for inflammation and oxidative stress should be considered. Here, *C-C motif chemokine ligand 2 (CCL2)*, *interleukin (IL)6*, *IL8*, *superoxide dismutase (SOD)*2, and *heme oxygenase (HMOX)1* were analyzed by qRT-PCRand/or enzyme-linked immunosorbent assay (ELISA).

Real-time qRT-PCR of stress response and pro-inflammatory markers

At 1 h post-exposure, total RNA was isolated from 2 pooled replicate inserts of A549 cells using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Extracted RNA was stored at -80°C until further processing. Purity and concentration of extracted RNA were measured using a Nanodrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Then, complementary DNA (cDNA) was prepared from 500 ng RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. qRT-PCR was then performed on a Lightcycler® 480 RT-PCR System (Roche, Basel, Switzerland) using the following concentrations: 5 µL 2x Lightcycler® 480 Probes Master (Roche Diagnostics), 0.5 µL 20x PrimeTime® Assay (Integrated DNA Technologies, IDT, Iowa, USA), 2 µL RNAse-free water, and 2.5 µL cDNA (10 ng). The PrimeTime® Assays are shown in Table 2. A non-template control (RNAse-free water, Probes Master, and PrimeTime® Assay) was used as negative control for each analyzed gene to exclude possible contamination from the used reagents. The thermal cycling conditions were as follows: pre-incubation for 10 min at 95°C, followed by 45 cycles of denaturation for 10 s at 95°C; annealing for 30 s at 62°C; extension for 1 s at 72°C, followed by cooling for 10 s at 40°C. All PCR reactions were carried out in duplicate using 384-well plates.

To identify crossing point (Cp) values of the PCR reactions, the "Second Derivative Maximum" method was used, which is included in the LightCycler® 480 software. This method identifies the point where the reaction's fluorescence reaches the maximum of the second derivative of the amplification curve, which corresponds to the point where the acceleration of the fluorescence signal is at its maximum. The obtained Cp values were processed using qbase^{+} (ΔΔCT method) to calculate relative gene expression of treated samples compared to negative control samples. The amplification efficiency was set to default "2".

One of the key features in qbase^{+} is that it includes a multiple reference gene normalization strategy to remove non-biological variation, which is based on the GeNorm algorithm. Therefore, 3 reference genes were included in the analysis, i.e., *actin beta (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*, and *hypoxanthine phosphoribosyltransferase 1 (HPRT1)* (Casadei et al., 2011).

Fold changes (FC) relative to CA were calculated and logarithmically transformed (log2 scale) prior to statistical analysis. Significant changes relative to CA were assessed by mixed models while considering experiment ID (biological replicate) as random factor. Data were analyzed using R and specific packages for mixed model analyses "lme4" (Bates et al., 2015) and "lmerTest" (Kuznetsova et al., 2017). *P*-value smaller than 0.05 was used as cut-off for statistical significance, and abs(mean(log2 FC)) above log2(1.5) was used to focus on biologically relevant expression changes.

Tab. 2: Overview of PrimeTime® Assays: Gene name, assay name, and RefSeqNumber

Gene name	Assay name	RefSeqNumber
ACTB	Hs.PT.42.227970.g	NM_001101
HPRT1	Hs.PT.42.3476197	NM_000194
GAPDH	Hs.PT.42.1164609	NM_002046
CCL2	Hs.PT.58.45467977	NM_002982
Il6	Hs.PT.42.3074634	NM_000600
Il8	Hs.PT.49a.755000.g	NM_000584
HMOX1	Hs.PT.42.1207236	NM_002133
SOD2	Hs.PT.58.2512947	NM_000636

Hs, species; PT, PrimeTimeAssay; 42, RefSeq build number; 1164609, assay number; g, may amplify genomic DNA

Measurement of pro-inflammatory markers

After 20 h ALI post-exposure, 200 µl medium was added on the apical side and incubated for 20 min at 37°C, 5% CO_2. Hereafter, the medium from the apical and basolateral compartment were pooled and stored at -80°C until use. Halt™ Protease Inhibitor Cocktail (ThermoFisher Scientific), 100 x diluted, was added before freezing. As positive control, 20 µg/mL lipopolysaccharide (LPS, stock 1 mg/mL in PBS) was added to the apical medium of an insert, and cells were incubated submerged in a CO_2 incubator for 24 h. The samples (2 technical repeats/sample) were assayed using human IL-6 and IL-8 uncoated ELISA assay (ThermoFisher Scientific). Briefly, 96-well plates were coated with capture antibody overnight at 4°C. After 3 washings, wells were blocked with ELISA/ELISPOT diluent and incubated at RT for 1 h. A 2-fold serial dilution was made of the IL-6 or IL-8 standard. Standard dilutions and samples (100 µL; IL-8 samples were diluted 10-fold) were added to the wells of the coated plate, and these were incubated at RT for 2 h. After 4 washes, 100 µL detection antibody was added for 1 h at RT. After 4 washes, 100 µL diluted Avidin-HRP was added for 30 min at RT. After 5 washes, 100 µL 1X 3,3',5,5'-tetramethylbenzidine solution was added for 15 min at RT. Finally, 100 µL stop solution was added, and the absorbance was measured at 450 nm (CLARIOstar, BMG Labtech). R software was used for analysis. The response curve between cytokine concentration and absorbance was analyzed using the four-parameter log-logistic function (package "drc", LL.4 method). Based on the output statistics of the model, cytokine concentrations of the samples were calculated and expressed in pg/mL. Cytokine concentrations were adjusted for cell viability (MTT) and cytotoxicity (LDH) prior to analysis. Significant changes in cytokine concentration were analyzed relative to CA using ANOVA.

Tab. 3: Measured NO_2 and ethylbenzene (EB) concentrations with mean, standard deviation (Stdev) and coefficient of variation (CV) for 5 independent experimental runs

Compound	Concentration	Run 1	Run 2	Run 3	Run 4	Run 5	Mean	Stdev	CV
NO_2	mg/m^3	41.5	41.1	39.8	42.5	40.9	41.2	1.0	2.4
EB	mg/m^3	30,952	30,911	30,749	32,598	28,533	30,749	1,449	4.7
	mg/m^3	41,783	41,544	41,291	43,773	39,227	41,524	1,617	3.9
	mg/m^3	51,209	50,900	50,598	53,640	49,933	51,256	1,414	2.8

Tab. 4: Mean concentration, standard deviation (Stdev), and coefficient of variation (CV) of ethylbenzene (EB) dose and of generated EB concentration for 3 consecutive experimental runs

EB concentration in air (mg/m^3)			EB dose in CCM (µg)		
Mean (n = 3)	**Stdev**	**CV**	**Mean** (n = 3)	**Stdev**	**CV**
51,562	229	0.4	22.7	3.50	15.4
40,990	182	0.4	14.3	4.30	30.1
30,989	137	0.4	9.10	2.90	31.9

Tab. 5: Delivery efficiency of EB measured in stainless steel inserts

Average exposure concentration in air (mg/m³)	Expected dose at 100% deposition (µg/4 h)	Average deposited dose in CCM (µg/4 h)	*In vitro* delivery efficiency (%)
51,562	18,600	22.7	0.12
40,990	14,800	14.3	0.10
30,989	11,200	9.10	0.08

2.8 Quantitative *in vitro* to *in vivo* extrapolation (QIVIVE) calculations

Air concentrations used in ALI testing can be back-calculated to *in vivo* air concentrations. The calculation method applied here is the reverse of the method (Eq. 1) used to calculate *in vitro* air concentrations from *in vivo* exposure concentrations as explained in Section 2.4 above.

3 Results

Having performed preliminary tests and several follow-up optimizations, a simultaneous 4 h ALI cell culture exposure to CA, NO_2, and EB vapors, followed by incubation under ALI conditions (20 h for cell viability/cytotoxicity and cytokine secretion, 1 h for gene expression) was established.

3.1 Generated exposure concentrations

The measured NO_2 and EB exposure concentrations for the 5 independent cell culture exposure runs can be found in Table 3. The data show a reproducible generation method with a coefficient of variation < 5% for all tested concentrations. Relative humidity and temperature of the flow directly before cell exposure was 88.6 ± 4.0% RH and 37.4 ± 0.1°C.

3.2 Chemical analysis of delivered dose, calculation of delivery efficiency, and new *in vitro* dose-range

Delivered doses were determined in three other experimental runs on the same day by exposing stainless steel inserts containing 125 µL CCM to EB. After exposure, CCM was chemically analyzed using HS-GC-MS. The generated EB concentration and deposited EB dose can be found in Table 4.

The average delivered dose to achieve about 30,000, 40,000, and 50,000 mg/m^3 exposure in stainless steel inserts was 9.10, 14.3, and 22.7 µg/4 h (Tab. 5). The average delivery efficiency calculated thereof is 0.10% (0.08-0.12%), as shown in Table 5, which was used for the new dose-range calculation below. It was expected that the delivery efficiency would be low given that the measured delivery efficiency of structurally related methylbenzene was also low (0.036%) (Steiner et al., 2018).

The *in vitro* dose-range calculated previously from experimental data was 1-625 mg/m^3 for 4 h exposure and assumed the same absorption *in vivo* as *in vitro* (see Section 2.4). This range can be recalculated from the same acute studies to a new 4 h dose-range that considers the difference between *in vivo* absorption and *in vitro* absorption (delivery efficiency by proxy, calculated in current VITROCELL® 24/48 set-up), and the modified flow from 3 to 1.5 mL/min (Tab. S1[2]). The reported *in vivo* absorption values for inhalation are 64% for humans (Chin et al., 1980) and 44% for rats (Bardodej and Bardodejova, 1970). The average measured *in vitro* delivery efficiency is 0.1% (Tab. 5).

Based on the preliminary dose-range (1-625 mg/m^3), the *in vivo* absorption, and the *in vitro* delivery efficiency, the new, lower concentration limit of the dose-range becomes 640 mg/m^3 (1*64%/0.1%) and the new upper limit becomes 275,000 mg/m^3 (625*44%/0.1%). The lower limit takes human absorption (ABS) (64%) into account, as the lower *in vivo* concentrations are for human data (Tab. S1[2]); the upper limit takes rat studies (hence 44% ABS) into account. As a result, the preliminary dose-range of 1-625 mg/m^3 changes to the current dose-range of 640-275,000 mg/m^3 during the 4 h exposure window. An overview of the preliminary and current dose-ranges is presented in Table 6.

Four tested concentrations were within the current dose-range: 7,000 mg/m^3, which resulted in no biological effects (data not

Tab. 6: *In vitro* dose ranges for 4 h exposure

Dose range (mg/m³)	Based on	mL/min	Status
1-625	*In vivo* studies in Table 1	3	preliminary
640-275,000	Preliminary dose range, *in vivo* ABS and *in vitro* delivery efficiency ("ABS")	1.5	current

shown), and the concentrations 30,000, 40,000, and 50,000 mg/m^3, which caused concentration-related biological responses as shown in this study.

3.3 Endpoint measurements

Quality control charts for cell viability (MTT) of negative (CA) and positive (NO_2) controls were set up to check uniformity in the VITROCELL 24/48® device. The lower and upper limits are 85% and 115% cell viability for CA and 40% and 69% cell viability for NO_2. The mean cell viability and standard deviation (Stdev) (MTT) for each of the five CA and NO_2 runs fitted within the limits (data not shown), so the experiments were valid for further endpoint measurement analysis.

Different biological endpoints that are relevant for acute *in vitro* ALI screening of EB were assessed, including cell viability (MTT), cytotoxicity (LDH release), oxidative stress (*HMOX1* and *SOD2* expression), and pro-inflammatory response (*CCL2*, *IL6*, and *IL8* expression and secretion). CA exposures were used as negative control. ALI cultures were exposed to NO_2 (about 20 ppm in air) as a gaseous positive control for cell viability (MTT).

Cell viability/cytotoxicity determination

The mean cell viability (MTT) for A549 cells was 94% after exposure to CA versus IC (P = 0.10) (Fig. 3). Exposure of A549 cells to NO_2 (about 20 ppm) showed a significantly decreased mean cell viability of 67% (P = 1.33E-13) compared to CA as expected. ALI exposure of A549 cells to EB induced a concentration-dependent decreased mean cell viability of 86%, 77%, and 47% for exposure to about 30,000, 40,000, and 50,000 mg/m^3, respectively, as compared to CA. The results were statistically significant for the lower to higher tested concentrations, respectively P = 3.59E-4, P = 9.64E-9, and P = 4.19E-26.

The mean cytotoxicity (LDH release) for A549 cells was 4% after exposure to CA versus IC (P = 0.09) (Fig. 4). Exposure of A549 cells to NO_2 (about 20 ppm) showed a significantly increased mean cytotoxicity of 9% (P = 2.55E-3), as compared to CA. A possible reason for a lesser effect on cells compared to MTT might be that we measured LDH release too late (after 20 h

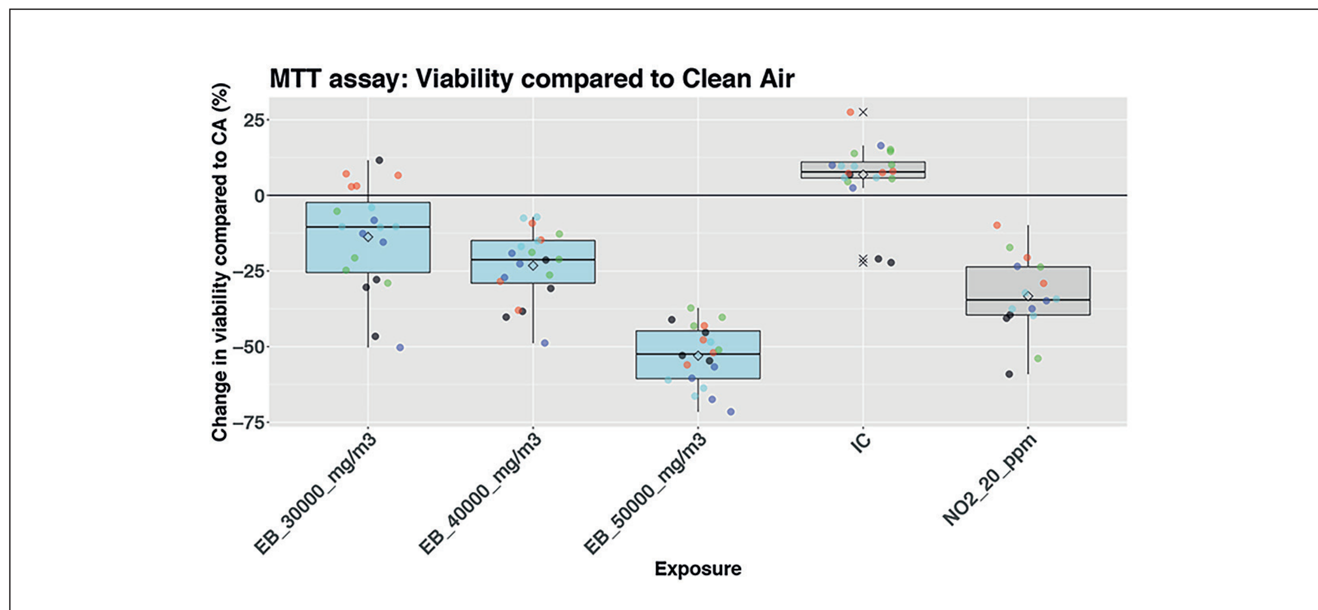

Fig. 3: Change in cell viability (as % compared to clean air (CA)) of A549 cells after 4 h exposure to ethylbenzene (EB, nominal concentration 30,000, 40,000, and 50,000 mg/m^3), NO_2 (nominal concentration 20 ppm), and incubator controls (IC) based on 5 independent biological experiments

Box and whisker plots visualizing the range of the individual data points per condition. The upper whisker extends from the hinge to the largest value no further than 1.5 x IQR from the hinge (where IQR is the inter-quartile range, or distance between the first and third quartiles). The lower whisker extends from the hinge to the smallest value at most 1.5 x IQR of the hinge. Data beyond the end of the whiskers are outlying points and are plotted with an "x"; other individual data points are overlayed and plotted with filled dots. The mean is indicated by a blue square. Different colors represent different biological experiments with their technical replicates.

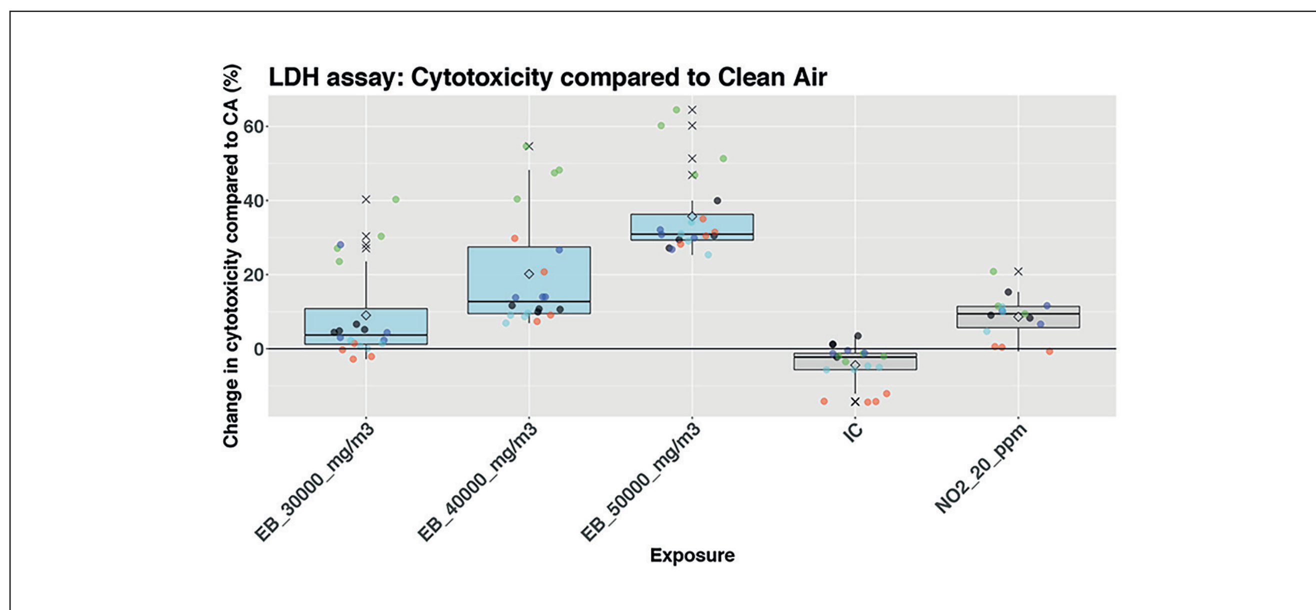

Fig. 4: Change in cytotoxicity (as % compared to clean air (CA)) of A549 cells after 4 h exposure to ethylbenzene (EB, nominal concentration 30,000, 40,000, and 50,000 mg/m^3), NO_2 (nominal concentration 20 ppm) and incubator controls (IC) based on 5 independent biological experiments

Box and whisker plots visualizing the range of the individual data points per condition. The upper whisker extends from the hinge to the largest value no further than 1.5 x IQR from the hinge (where IQR is the inter-quartile range, or distance between the first and third quartiles). The lower whisker extends from the hinge to the smallest value at most 1.5 x IQR of the hinge. Data beyond the end of the whiskers are outlying points and are plotted with an "x"; other individual data points are overlayed and plotted with filled dots. The mean is indicated by a blue square. Different colors represent different biological experiments with their technical replicates.

post-incubation), since LDH has a half-life of approximately 9 h in CCM, and NO_2 might induce cell death faster than EB. Again, EB induced a concentration-dependent increased mean cytotoxicity that was significant for all concentrations 30,000 (9%, P = 7.74E-4), 40,000 (20%, P = 8.34E-12), and 50,000 (36%, P = 1.27E-24) mg/m^3 as compared to CA.

Biomarker analysis

CA exposure significantly induced *IL6* (log2 FC = 2.9, P = 3.21E-5) and *IL8* (log2 FC = 2.5, P = 8.79E-6) gene expression as compared to IC (Fig. 5A,B), whereas at the level of cell viability/cytotoxicity, no significant difference between CA and IC was shown. No increase was observed for *CCL2* expression in CA-exposed cells (log2 FC = 0.5, P = 0.25) compared to IC (Fig. 5C). NO_2, which was used as positive control for cell viability, did not induce *CCL2*, *IL6*, and *IL8* expression as compared to CA. For IL-8 secretion (protein level), normalized for MTT cell viability, the opposite was shown (Fig. 5D). A significant release of IL-8 was observed for exposure of A549 cells to NO_2 (log2 FC = 0.94, P = 0.01), whereas CA exposure was comparable to IC. IL-6 secretion was below detection limit (data not shown), while CCL-2 secretion was not measured.

A concentration-dependent increase of pro-inflammatory markers *CCL2*, *IL6*, and *IL8* was observed for EB-exposed A549 cells compared to CA, which was significant for all concentrations (Fig. 5A-C). Exposure to about 30,000 mg/m^3 EB induced *CCL2*, *IL6*, and *IL8* expression with a log2 FC of 1.3 (P = 2.16E-3), 2.2 (P = 1.68E-4), and 2.9 (P = 1.33E-7), respectively. For exposure of A549 cells to about 40,000 mg/m^3, an increase of log2 FC of 1.4 (P = 1.01E-3), 2.9 (P = 2.51E-6), and 3.7 (P = 6.17E-10) was observed for *CCL2*, *IL6*, and *IL8*, respectively. The highest expression of *CCL2*, *IL6*, and *IL8* was observed after exposure to about 50,000 mg/m^3 with a log2 FC of 1.5 (P = 6.61E-4), 3.3 (P = 3.17E-7), and 4.5 (P = 2.88E-12), respectively.

The data for IL-8 secretion were normalized for % cell viability (MTT) (Fig. 5D). Exposure of A549 cells induced the release of IL-8 in a concentration-dependent manner that was statistically significant for about 30,000 mg/m^3 (log2 FC = 1.55, P = 1.17E-5), 40,000 mg/m^3 (log2 FC = 2.04, P = 2.86E-8), and 50,000 mg/m^3 (log2 FC = 2.62, P = 1.06E-11) compared to CA. The same was observed for IL-8 secretion normalized for % cytotoxicity (LDH) (Fig. 5E).

The oxidative stress marker *SOD2* was significantly increased for all EB exposure concentrations (log2 FC of ~ 0.8), but no concentration-response was observed (Fig. 6A). NO_2 showed a modest statistically significant increase of *HMOX1* expression compared to CA (log2 FC 0.8, P = 9.04E-3). For EB exposure, there was a concentration-dependent decreased expression that was significant for all concentrations compared to CA, but the same decrease was observed for IC (log2 FC -3.7, P = 5.95E-16) and, for that reason, this is not a relevant marker to be studied in the context of EB exposure (Fig. 6B).

Fig. 5: *Interleukin (IL)6* (A), *IL8* (B), and *C-C motif chemokine ligand 2 (CCL2)* (C) gene expression, and IL-8 secretion normalized according to cell viability (MTT) (D) and cytotoxicity (LDH) (E) as compared to clean air (CA) in A549 cells

Mean log2 fold change (FC) values based on 5 independent biological experiments after 4 h exposure to ethylbenzene (EB, nominal concentration 30,000, 40,000, and 50,000 mg/m^3), NO_2 (nominal concentration 20 ppm), and incubator controls (IC) compared to CA are shown. Error bars represent the 95% confidence limits.

3.4 QIVIVE calculations

Three *in vitro* concentrations were selected for the QIVIVE calculations and were based on the *in vitro* results for cell viability: the highest tested concentration with no observed effect (NOEC, 7,000 mg/m^3, experimental data not shown), and both the lowest tested concentration (LOEC, 86% cell viability, 30,000 mg/m^3) and the highest tested concentration (47% cell viability, 50,000 mg/m^3) with observed effect, as shown in Figure 3. Results of the reverse calculations are presented in Table S2[2] (values in italics). As no experimental value for absorption of EB in the mouse is available, the QIVIVE calculations were performed twice for the mouse: once with the value for human absorption (64%) and once with the value for absorption in rat (44%). The QIVIVE calculations were performed for different scenarios described by the *in vitro* concentration (7,000, 30,000 or 50,000 mg/m^3), the species (human, rat, or mouse), and the exposure time (15, 30, or 240 minutes); only the scenarios that are relevant for the discussion are shown in Table S2[2].

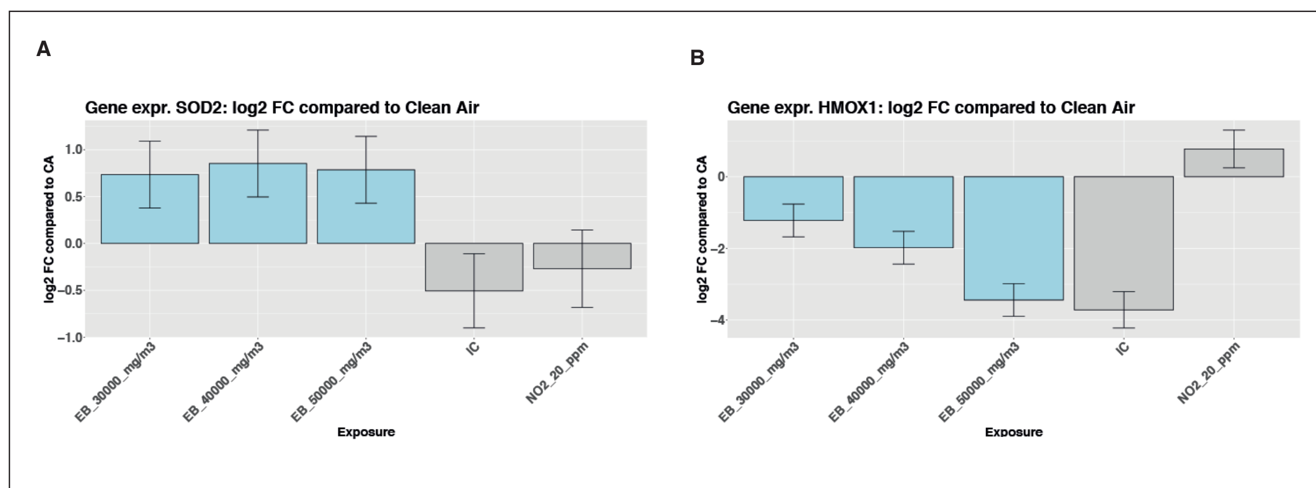

Fig. 6: Oxidative stress markers *superoxide dismutase (SOD)2* (A) and *heme oxygenase (HMOX)1* (B) gene expression as compared to clean air (CA) in A549 cells

Mean log2 fold change (FC) values based on 5 independent biological experiments after 4 h exposure to ethylbenzene (EB, about 30,000, 40,000, and 50,000 mg/m^3), NO_2 (about 20 ppm), and incubator controls (IC) compared to CA are shown. Error bars represent the 95% confidence limits.

4 Discussion

4.1 Biological responses

ALI exposure of A549 cells to EB resulted in a significant concentration-dependent decrease in mean cell viability. To compare these biological changes with previous work, only a few papers on *in vitro* studies on respiratory toxicity of EB or BTEX mixture were found (Liu et al., 2013, 2014, 2015). Short-term exposure of A549 cells in a hanging drop system, shown to be reliable for volatile compounds with high sensitivity (Liu et al., 2015), allowed the calculation of mass balances and derivation of an EC50 value for EB from the internal cellular concentration (11 mmol/kg), which was then related to the nominal air concentration of 9980 ppm (~43313 mg/m^3) for a 1 h exposure (Liu et al., 2014). This value is in the same order of magnitude as in our study. This points to the importance of a more reliable ALI exposure set-up for compounds that occur in the gaseous phase upon exposure and are easily lost in a classical cell culture set-up (e.g., submerged cells, open system).

Based on a literature review of gasoline and related compounds (Bisig et al., 2015, 2016, 2018; Kunzi et al., 2015), markers for inflammation (e.g., *tumor necrosis factor (TNF)α, IL6, IL8, CCL2*) and oxidative stress (e.g., *HMOX1*, *SOD2*) should be considered for these compounds. In this pilot study using EB, a concentration-dependent increase of pro-inflammatory markers *CCL2*, *IL6*, and *IL8* was observed for EB-exposed A549 cells compared to CA, which was significant for all concentrations. Exposure of A549 cells induced the release of IL-8 protein in a dose-dependent manner, which was significant compared to CA. The oxidative stress marker *SOD2* was significantly increased for all EB exposure concentrations, but no concentration-response was observed.

4.2 Delivery efficiency

This ALI EB vapor study calculated an average delivery efficiency of 0.10%. The delivered dose of aerosol components to cells after *in vitro* ALI exposure depends predominantly on the physicochemical properties of the substances as was demonstrated by Steiner et al. (2018). The delivery efficiency of EB was not measured by Steiner and coworkers, but those of two structurally related substances (benzene and methylbenzene) were. For comparison, relevant physicochemical properties and (low) delivery efficiencies for these compounds are listed in Table 7.

The initially calculated administered *in vitro* dose-range of 1-625 mg/m^3 for 4 h exposure to EB vapors was based on human and animal data (Tab. 1). In our calculation of the air concentrations, we assumed the same absorption *in vitro* as *in vivo*. The ALI experiments including this dose-range showed that exposure to different EB concentrations (up to 7,000 mg/m^3) induced no significant reduction in cell viability (data not shown). One of the reasons for initially showing less effect than expected could be that the *in vitro* absorption is considerably lower than the *in vivo* absorption, which was quantified as EB delivery efficiency during this pilot study (Tab. 5). The link between 4 independent acute *in vivo* studies (Tab. S1[2]), the current dose-range for ALI exposure studies (Tab. 6), and the *in vitro* biological results is discussed below.

In a first study, human volunteers who were exposed for 4 h to 425 mg EB/m^3 experienced no nasal irritation (van Thriel et al., 2003); this no-effect dose corresponds with an *in vitro* dose of 846 mg/m^3/4 h, which is just above the lower limit of the current dose-range (640 mg/m^3/4 h) and below the highest tested *in vitro* concentration (7,000 mg/m^3/4 h) with no reduction in cell viability in the current study.

Tab. 7: Physicochemical properties and delivery efficiencies of benzene, methylbenzene, and EB

Substance	Log P_{oct}	Water solubility	Polar surface area	Vapor pressure	Human *in vivo* absorption	*In vitro* delivery efficiency
Benzene	2.10	1800 mg/L	0 Å	10.00 kPa @20°C	48-80% (ATSDR)	0.05% (Steiner et al., 2018)
Methylbenzene	2.70	526 mg/L	0 Å	3.089 kPa @21°C	50-83% (ATSDR)	0.039% (Steiner et al., 2018)
EB	3.15	152 mg/L	0 Å	0.952 kPa @20°C	50-64%	0.1% (Tab. 5)

In a second study with volunteers, subjects suffered severe and immediate nose and throat irritation after a 6 min exposure to 5,000 ppm (21,700 mg/m^3) of EB (Yant et al., 1930). The corresponding recalculated *in vitro* dose of 1,080 mg/m^3/4 h is about 1.7 times above the lower limit of the current dose-range (640 mg/m^3/4 h) and below the lowest *in vitro* tested dose with effect on cell viability (30,000 mg/m^3/4 h). At a dose of 7,000 mg/m^3/4 h, which is about 7 times higher than the recalculated *in vitro* effect concentration of 1,080 mg/m^3/4 h, no reduction in cell viability was seen in the current study. Thus, cell death seems to require a higher dose than irritation. Mechanistically, it is difficult to justify a comparison between *in vitro* cell death and severe irritation; it may be more justifiable to compare irritation with stress markers. In this study, the oxidative stress marker *SOD2* was significantly increased for all EB exposure concentrations, but no concentration-dependent response was observed (Fig. 6A).

In a third study, the RD50 (17,620 mg/m^3) for breathing reduction of mice exposed for 30 min to EB (Nielsen and Alarie, 1982) corresponds with a recalculated *in vitro* dose of 160,000 mg/m^3/4 h (calculated with 64% absorption), which is above half of the upper limit of the current dose-range (275,000 mg/m^3/4 h).

The 4 h LC50 rat (17,360 mg/m^3) of a fourth study (Smyth et al., 1962) corresponds with a recalculated *in vitro* dose of 150,000 mg/m^3/4 h, which is above half of the upper limit of the current dose-range and 3 times higher than the highest tested dose in the current project (50,000 mg/m^3/4 h).

The doses tested with ALI in the current case that show effects on cell viability (30,000 to 50,000 mg/m^3/4 h) are in the lower part of the current dose-range (640-275,000 mg/m^3/4 h). The 4 h *in vitro* test concentrations of 30,000 and 50,000 mg/m^3 correspond with an insert mass load of 32.1 and 53.6 µg/cm^2 respectively (Tab. S2[2]) and a decreased mean cell viability of 86% and 47%, respectively. These findings are in line with the results of Gohlsch et al. (2019), who demonstrated that cytotoxicity in A549 cells tested up to 100 µg/cm^2 could be a reliable *in vitro* indicator for *in vivo* toxicity.

It can be concluded that the preliminary dose-range was justified as a starting point for setting the *in vitro* dosimetry, but, as demonstrated in this pilot study, differences between *in vivo* absorption rate and *in vitro* deposition and delivery efficiency should be considered before setting the final dose-range for ALI experiments.

4.3 QIVIVE

At an *in vitro* 4 h concentration of 7,000 mg/m^3, no decrease in cell viability was observed. Reverse calculation of this *in vitro* concentration to an *in vivo* concentration results in a human 4 h exposure concentration of 3,520 mg/m^3. For comparison, the acute DNEL for local effect (irritation of the respiratory tract) for workers (293 mg/m^3) is 12 times lower, and the indicative EU-OEL (occupational exposure limit) is 8 times lower (442 mg/m^3, 8 h time weighted average (TWA) for working lifetime exposure) (EC, 2000). Volunteers exposed for 4 h to 425 mg/m^3 experienced no nasal irritation (Tab. S1[2]). Furthermore, the reverse calculation of an *in vitro* 4 h concentration of 7,000 mg/m^3 for short term exposure gives a human 15 min exposure concentration of 56,300 mg/m^3. For comparison, the indicative short EU-OEL is about 60 times lower (884 mg/m^3 for 15 min (EC, 2000)), and in volunteers exposed to 239 mg/m^3 for 15 min, no effect on pulmonary function was observed (Moscato et al., 1987, Tab. 1).

At an *in vitro* 4 h concentration of 50,000 mg/m^3, the average cell viability was reduced to about 50%. Reverse calculation of this *in vitro* concentration firstly to an *in vivo* concentration in rats resulted in a 4 h exposure concentration of 5,800 mg/m^3. For comparison, the 4 h LC50 rat is 17,000 mg/m^3 (Smyth et al., 1962), showing that *in vitro* cell death may be at least a qualitative measure for *in vivo* lethality caused by EB. This is in agreement with Gohlsch et al. (2019), who demonstrated that cytotoxicity in A549 cells could be a reliable *in vitro* indicator for *in vivo* respiratory toxicity. However, the *in vivo* lethality of EB could also be a result of systemic effects caused by transport of the contaminant via the blood stream from the lungs to other organs, a process that is not simulated in the ALI exposure with lung cells, or it may be that the *in vitro* absorption is underestimated. Secondly, the IVIVE calculations of 50,000 mg/m^3/h resulted in a mouse 30 min exposure concentration of 7,990 mg/m^3 (with 44% absorption mouse = absorption rat). For comparison, the 30 min RD50 mouse for breathing rate reduction is twice as high (17,200 mg/m^3 (Nielsen and Alarie, 1982)). Thirdly, the IVIVE calculations of 50,000 mg/m^3/h resulted in a mouse 30 min exposure concentration of 5,490 mg/m^3 (with 64% absorption mouse = absorption human). For comparison, the 30 min RD50 mouse for breathing rate reduction is three times as high (17,200 mg/m^3). This may be an indication that

in vitro cell death is not a measure for non-lethal effects (in this case breathing rate reduction in mice). To our knowledge, no data on lethality for mice are available for comparison with the *in vitro* concentration causing cell death for the chemical tested in this study.

The QIVIVE calculation from *in vitro* air concentrations applied for testing cell viability to *in vivo* air concentrations appeared to be a promising method for screening for respiratory toxicity, as was shown with the LC50 rat and by Gohlsch et al. (2019). Regarding humans, no *in vitro* cell death may indicate that non-lethal effects (pulmonary function and nasal irritation) in humans will not occur. Since the mode of action is not reported, cough/irritation in humans may also occur from nerve stimulation. A549 may not be the optimal model for examining this effect. For mice, the QIVIVE concentration causing 50% breathing reduction exceeded the QIVIVE 50% cell death concentration, showing that for breathing rate reduction, *in vitro* cell death does not seem to be the right measure.

5 Conclusion

This pilot study exposed a frequently used *in vitro* model (A549 cells) at the ALI to assess inhalation toxicity of the compound EB. A facility to generate EB vapors was successfully developed. Experimental conditions using the VITROCELL® 24/48 exposure system were optimized to achieve a (low) delivery efficiency that resulted in dose-dependent biological changes. The data demonstrate consistency in effect levels when comparing cell viability in the ALI experiments with known *in vivo* non-lethal effects in humans. QIVIVE from *in vitro* air concentrations applied for testing cell viability to *in vivo* air concentrations may be a promising method for screening for acute inhalation toxicity. This pilot study with EB as a test compound demonstrated the approach of an ALI set-up, complemented with QIVIVE calculations to predict human *in vivo* inhalation toxicity, which should be further validated with other respiratory toxicants.

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Conflict of interest

The authors declare they have no conflicting interests with the content of the study.

Acknowledgements

The VITROCELL® 24/48 *in vitro* inhalation exposure system was awarded to VITO by the PETA International Science Consortium Ltd. The authors are grateful to the advisory panel Marusia A. Popovech, Katy O. Goyak, Monita Sharma, and Amy J. Clippinger for their expertise, critical review, and very fruitful discussions.