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Accepted Manuscript

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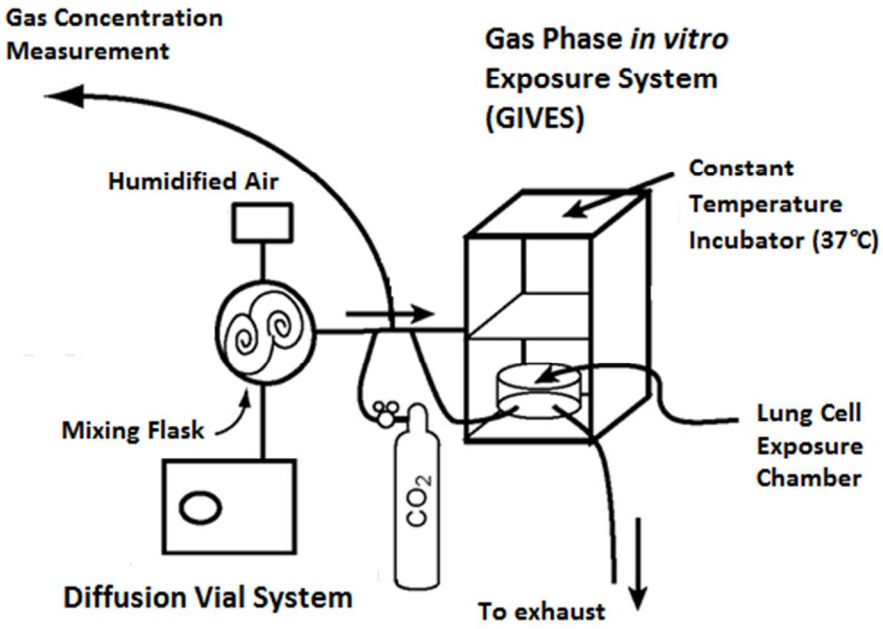

Schematic representation of the system design coupling a constant emission source from a diffusion vial to a gas phase in vitro exposure system (GIVES) for a direct air-liquid interface exposure to volatile organic compounds
153x102mm (96 x 96 DPI)

Environmental impact statement

Exposure to airborne volatile organic chemicals (VOCs) is a potential cause of various adverse health effects. Traditionally, assessments of *in vitro* toxicity of VOCs are usually performed by direct treatments of test chemicals dissolved in aqueous solutions, such as cell culture media or buffers, which may lead to significant loss of test chemicals during exposure due to evaporation or modifications of chemical composition when the test compound is unstable in water (e.g., susceptible to hydrolysis). Development of an effective and reproducible technique for *in vitro* exposure to gaseous air pollutants through an air-liquid interface as an alternative tool to represent VOCs exposures is needed to more closely represent the realistic exposure conditions.

Application of chemical vapor generation systems to deliver constant gas concentrations for *in vitro* exposure to volatile organic compounds

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For Submission to: Environmental Science: Processes & Impacts

Abstract

Exposure to volatile organic compounds from outdoor air pollution is a major public health concern; however, there is scant information about the health effects induced by inhalation exposure to photochemical transformed products of primary emissions. In this study, we present a stable and reproducible exposure method to deliver ppm-ppb levels of gaseous standards in a humidified air stream for *in vitro* cell exposure through a direct air-liquid interface. Gaseous species were generated from a diffusion vial, and coupled to a gas-phase *in vitro* exposure system. Acrolein and methacrolein, which are major first-generation photochemical transformation products of 1,3-butadiene and isoprene, respectively, are selected as model compounds. A series of vapor concentrations (0.23-2.37 ppmv for acrolein and 0.68-10.7 ppmv for methacrolein) are investigated to characterize the exposure dose-response relationships. Temperature and the inner diameter of the diffusion vials are key parameters to control the evaporation rates and diffusion rates for the delivery of target vapor concentrations. Our findings suggest that this exposure method can be used for testing a wide range of atmospheric volatile organic compounds, and permits both single compound and multiple compound sources to generate mixtures in air. The relative standard deviations (%RSD) of output concentrations were within 10% during the 4-hour exposure time. The comparative exposure-response data allow us to prioritize numerous hazardous gas phase air pollutants. These identified pollutants can be further incorporated into air quality simulation models to better characterize the environmental health risks arising from inhalation of the photochemical transformed products.

Key words: chemical vapor generation system, diffusion, volatile organic compound (VOC), air-liquid interface (ALI), *in vitro* exposure, A549, lactate dehydrogenase (LDH), interleukin-8 (IL-8)

1. Introduction

Exposure to atmospheric air pollutants has been linked to various adverse health effects in epidemiologic studies.[1] Recent evaluation by the International Agency for Research on Cancer (IARC) has also concluded that outdoor air pollution is a leading environmental cause of cancer deaths with sufficient evidence.[2] Although health risks associated with ambient air pollution have been found, the causative agents responsible for the observed health effects and the underlying toxicological mechanisms remain unclear. One of the major challenges in identifying causative agents is the fact that many components of air pollutants are modified in the atmosphere due to photochemical reactions, and hence alter the observed health effects due to compositional changes.[3] This has been illustrated by prior laboratory studies utilizing an outdoor smog chamber coupled to an *in vitro* human lung cell exposure system, showing that exposure to the mixture of photochemical transformation products from 1,3-butadiene (C_4H_6) and isoprene (2-methyl-1,3-butadiene; C_5H_8) significantly enhances the toxicological responses on cytotoxicity and proinflammatory mediator release compared to their precursor compounds.[4, 5] As a result, in addition to characterizing the health effects from primary volatile organic compound (VOC) emissions, there is a need to identify important hazardous secondary air pollutants that may be more harmful than their precursors.

Acrolein (prop-2-enal; C_3H_4O) is one of the major first-generation photochemical transformation products formed in the gas phase from 1, 3-butadiene.[6] Due to its high vapor pressure (274 mm Hg at 25 °C),[7] acrolein is highly volatile when it is produced in the atmosphere. Thus, inhalation is a major route of exposure. Inhaled acrolein is highly toxic and has been associated with asthma-like symptoms, chronic obstructive pulmonary disease, cystic fibrosis, and lung carcinogenesis.[8-10] From a chemical point of view, acrolein is a strong

electrophile possessing an unsaturated carbon-carbon double bond conjugated with an electron withdrawing carbonyl group.[11] Such reactive functional groups can rapidly attack biological nucleophiles like thiol-containing glutathione, cysteine and lysine residues in protein that lead to conformational changes and impair protein functions,[12, 13] disrupt regulation of gene expression by direct modification of the DNA-binding domain of a transcription factor,[14] and potentially bind with nucleophilic centers within DNA to form adducts and cross-links.[15] Methacrolein (2-methylprop-2-enal; C_4H_6O), as a structural analog, is one of the major first-generation photooxidation products produced from isoprene in the gas phase. The vapor pressure of methacrolein is 155 mm Hg at 25 °C.[16] In the atmosphere, methacrolein can further react with atmospheric oxidants through hydroxyl radical (OH) initiated oxidation or ozonolysis. The half-lives for these photochemical reactions are estimated to be around 11.5 hours and 10.5 days, respectively.[17, 18] Methacrolein has also been reported to cause sensory irritation after exposure through inhalation.[19] Table 1A lists some physicochemical and toxicological properties of acrolein and methacrolein.

Since acrolein and methacrolein are water soluble VOCs, current *in vitro* methods used to investigate acrolein and methacrolein toxicity often apply treatments by direct addition of chemical solutions into the cell medium, which does not maintain an air-liquid interface as found during *in vivo* exposures. This may result in significant loss of the test VOCs because of vapor evaporation from the cell medium or modification of the chemical composition (e.g., susceptible to hydrolysis) when the test compounds are dissolved in aqueous medium solutions. Therefore, an alternative method for *in vitro* gas phase exposure is needed to more closely simulate the *in vivo* exposure scenarios.[20, 21] To accomplish this goal, it requires a chemical generation system

that can produce a stable and repeatable test atmosphere that permits the air-liquid interface for *in vitro* exposures to cultured cells.

The objective of this study is to develop an effective and reproducible method for generation of gaseous air pollutants for use in *in vitro* models through an air-liquid interface to more closely represent the realistic exposure conditions to VOCs, especially for the transformation products of volatile organic air toxics. We have developed an *in vitro* gas phase exposure method by coupling a diffusion vial to a gas phase *in vitro* exposure system (GIVES) that can generate continuous sources of acrolein and methacrolein capable of ventilating *in vitro* exposure samples with sufficient volume to overcome any losses to surfaces and tissue. This system maintains a steady vapor concentration over the course of exposure time, and provides sufficient excess material needed for chemical characterization or venting. Concentrations were shown to be stable and repeatable in both magnitude and stability. In addition, this chemical generation system is humidified to prevent desiccation of the *in vitro* models, but low enough to prevent condensation in any part of the system. The concentrations generated by this device can be easily adjusted to allow for *in vitro* exposure-dose-response studies and to determine the precision of exposure and toxicological processing. We demonstrated this system by investigation of gas phase acrolein and methacrolein exposure induced cytotoxicity and proinflammatory cytokine (interleukin 8) gene expression from A549 cells.

2. Materials and methods

2.1 Design of the in vitro exposure system

2.1.1 Generation of gas-phase species from a diffusion vial

Figure 1 is a schematic showing airflows and the gas phase generator. The generator used a commercially available diffusion vial (P/N #192, 8cm tube length, 2 mm bore diameter) (VICI-

Metronics, Poulsbo, Washington) (Figure 2) to provide a continuous and constant source of chemical vapors. The emission rate was controlled by the operating temperature, the solution strength of the chemical in water, and the length and diameter of the capillary tube that are critical for evaporation and diffusion of test chemicals. The diffusion vial was housed in a temperature controlled chamber and ventilated in a controlled air stream.[22] Gas-phase acrolein and methacrolein were generated with aqueous acrolein or methacrolein solutions prepared from commercial standards (≥95.0% as anhydrous, GC grade; Fluka). Freshly prepared solutions were placed in the glass diffusion vial, and incubated in a constant temperature chamber system at 40 °C (Dynacalibrator Calibration Gas Generator model 230, VICI-Metronics, Santa Clara, CA). The chamber air flow was maintained at 0.1 L/min.

2.1.2 Addition of a humidification system to maintain cell viability during exposure

A humidification system was developed to humidify the dilution air that is mixed with the gas phase generator (Figure 1). This system permits humidified air to be used with the diffusion vial to prevent dehydration of cells that causes additional cellular stress. Clean air from an ADDCO 737-250 pure air generator (AADCO Instruments, Cleves, OH) was used for both the carrier gas and dilution air. The dilution air stream was controlled by a mass flow controller, and allowed to bubble through two thermostatically heated midget impingers (ACE Glass, Inc) in series filled with 15 milliliters of HPLC grade water (Fisher Scientific). The flow rates of the dilution air (ranging from 1.0 to 2.5 L/min) were adjusted until desired output concentration and relative humidity were achieved. The humidified air was delivered to a mixing tee using thermostatically heated lines to prevent condensation, and was blended with the dry air stream containing test chemicals from the diffusion vial source. The dew point of the final air mixture

was maintained within 16-18 °C, measured by a dew point monitor (Dew Prime I, EdgeTech, Marlborough, MA), to ensure adequate humidity for cell survival.

The output flow of the chemical generator was well mixed with the humidified dilution air using a mixing flask consisting of a simple tee and midget impinger (ACE), with the goal of preventing condensation of water or chemical agents. Condensation needs to be avoided since it can absorb some chemical agents dramatically. A water trap was inserted in line in case condensation does occur. A distribution manifold consisting of a series of "tees" allows for the mixed stream to be shared and connected to the exposure devices, dew point monitor, chemical analyzers, and a vent line to maintain atmospheric pressure. While compounds we tested are easily maintained in a gaseous state at room temperature, if higher boiling point compounds are used or higher humidities needed, then the manifold and all further distribution and sample lines can be heated.

2.1.3 Monitoring of the output vapor concentrations

To ensure stability of exposure concentrations, data were collected at 30-minute intervals using an on-line Varian CP-3800 GC equipped with flame ionization detector (FID). A dimensionless unit of mixing ratio (i.e. volume fraction) of the generated air mixtures, ppmV, was used to define concentrations in the gas phase. The responses of GC-FID to acrolein and methacrolein were calibrated externally by injecting known amount of acrolein and methacrolein standards (Sigma-Aldrich) into a 120 m^3 fixed-volume Teflon film chamber, located on the rooftop of the University of North Carolina at Chapel Hill (UNC) Gillings School of Global Public Health, for quantification of exposure concentrations. The details of chamber operation and gas-phase VOC analysis have been previously described.[23] Briefly, test compounds were incrementally added into the Teflon film chamber, vaporized, and measured as gaseous standards

without pretreatments. The gas sampling line feeding the instrument travelled from the floor of the chamber, through the roof of the building, and directly to the GC-FID in the laboratory below. The GC-FID was equipped with a packed stainless steel column (10 feet, 1/8 inch O.D., 2.1 mm I.D., 10% TCEP 100/120 Chromosorb PAW) for acrolein and methacrolein measurements. An isothermal method was used for the entire analysis. The column oven and the detector temperature temperatures were set at 70 and 200 °C, respectively. The flow rate of carrier gas (helium) was set at 20 mL/min. Calibrations were performed using the fixed standard volume (FSV) approach, as reported to be more ideal for GC-based quantification of VOCs.[24, 25] The limits of detection (LOD) for the test compounds on our GC/FID system were determined to be around 1–3 ppbV.[23] The accuracy and precision of instrumental responses were regularly checked with a commercial gas mixture cylinder of VOC standards (Ref#88-104317; prepared by National Specialty Gases and certified using National Institute of Standards and Technology traceable standards with 5% uncertainty) before and after each experiment.

It should be noted that the external calibrations conducted with the outdoor smog chamber were operated under non-photochemical active conditions (dark or overcast conditions, with very low UV and total solar radiation detected) to minimize photochemical decomposition. In addition, the half-lives for acrolein and methacrolein against photochemical oxidation (i.e., hydroxyl radical-initiated oxidation) are 15-20 hours and 11.5 hours, respectively.[17, 18] Since the calibrations were completed within 1-2 hours, the photochemical decomposition would be negligible under given conditions.

2.1.4 Gas-phase in vitro exposure system

The gas-phase *in vitro* exposure system (GIVES) consisted of an incubator cabinet to maintain a temperature of 37˚C, and an 8-liter, modular, cell-exposure chamber (Billups-

Rothenberg, MIC-101™) to enclose a cell plate system has been described previously.[4] The cell exposure chamber held an optional small dish of water to maintain humidity around the cells. The cell plates contained cell growth media in their bottom and allowed 12 removable membranous support inserts (Transwell™, as described in Jaspers, et al.)[26] to sit suspended in the cell growth media. The lung cells themselves were atop the porous bottoms of the Transwells and were exposed to air in the 8-liter chamber. The 8-liter cell-exposure chambers also had connections for flowing gas through the exposure chamber. Sample lines for GIVES were directly connected to the gas phase generator at a flow rate of 1.0 liter per minute. To maintain buffering capacity of the tissue culture media, a 5% CO_2 concentration was created in the cell exposure chambers using CO_2 gas at 0.05 liter per minute from cylinders regulated by mass flow controllers (AALBORG, Orangeburg, NY).

2.2 Cell culture

For this study, the GIVES housed A549 human alveolar type II epithelial lung cell line, derived from human alveolar cell carcinoma of the lung. The A549 cell line was used here as an *in vitro* cell model because of its human pulmonary origin. Additionally, A549 cells have been reported to be sensitive to the inhaled gases for these alveolar epithelial cells lack the mucus layer for protection against inhaled air pollutants.[4] This immortal epithelial-like cell line has been extensively used to access the toxicity of air pollutants, and it is known to produce cytokines capable of modulating immune cell activation.[26, 27] Investigation of exposure-dose-response relationships in a well-controlled bench scale exposure system facilitates testing toxicities of a number of gases to compare relative toxicity among these numerous atmospheric transformed products.

A549 cells were seeded two days prior to exposure on a 12-well culture plate with collagen-coated permeable membrane supports. A549 cells were grown at a density of 2.5×10^5 cells per well, and supplied with F12K medium plus 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA). Before exposure, the cell culture growth medium was replaced with serum-free F12K plus 1.5 μg/ml bovine serum albumin (BSA) and antibiotics. For each experiment set, six replicates of A549 cells were used for the exposure, while another six wells of A549 cells were maintained in a regular tissue-culture incubator at 37 °C with CO_2 (5%) supply, served as unexposed controls. A549 cells were allowed to grow on the permeable membrane supports, and maintained in the GIVES exposure system at 37 °C with CO_2 (5%) supply throughout the 4-hour exposure.

2.3 Toxicity endpoints

Lactate dehydrogenase (LDH) release was measured as the marker of cytotoxicity. Induced Interleukin-8 (IL-8) gene expression was measured as the indication of proinflammatory cytokine release. LDH Cytotoxicity Detection Kit (Takara Bio Inc., Japan) and Human IL-8 ELISA Set (BD Bioscience, San Diego, CA) were used to perform bioassays according to the manufacturers' protocol. For all experiments, the supernatants of the exposed cells were collected 9 hours post-exposure. Collected samples were stored at -20°C until analysis.

2.4 Statistical analysis

Statistical analysis was performed using GraphPad Prism software 4.0 (GraphPad, San Diego, CA, USA). All the cellular responses of exposure data were normalized to the incubator controls, and expressed as relative fold increases over controls, mean ± standard error of the mean (SEM), n=6 for each experiment. Student's t test was performed for data analysis to compare if the exposure responses are significantly different from unexposed controls; $p<0.05$

was considered to be statistically significant. A one-way ANOVA followed by Tukey's multiple comparison tests was used to interpret the results of concentration effects on cellular responses.

3. Results and Discussion

3.1 Quality control and assurance of the exposure method

In this study, A549 cells were exposed to gas-phase acrolein or methacrolein generated from a diffusion vial system with constant output vapor concentrations. Cells were allowed to grow post exposure for 9 hours prior to collection of the supernatants. Exposure induced cytotoxicity (LDH release) and proinflammatory cytokine (IL-8) gene expression were examined as toxicity endpoints. To ensure that the operation of exposure system would not affect the measured cellular responses, control experiments were conducted by exposing A549 cells to clean air flowing through this exposure system. Figure 3 shows that no significant differences of LDH release and IL-8 gene expression levels were detected between clean air exposure and unexposed incubator controls. These results then confirmed that the toxicological responses measured with this approach were actually induced by the target compounds.

Figure 4 shows the results of continuous GC/FID measurements over the 4-hour exposure duration. The relative standard deviation (%RSD) of the output vapor concentrations for both target compounds ranged between 1.0% and 9.5% over the 4-hour exposure time (Table 1B). The diffusion vial system is a well-developed and reliable gas generator for low-concentration calibration gases, consisting of a liquid-containing reservoir and a diffusing capillary with a uniform inner diameter. The diffusion technique has been widely used for generating standard gas sources.[28] Specifically, reliability of gaseous standard production from liquid or by headspace diffusion of aqueous standards has been reported.[29] Similar to the use of permeation tubes, the operation of the diffusion vial also experiences the saturation (dynamic), steady-state

equilibrium (kinetic), and depletion stages.[29, 30] Constant temperature and flow rates are critical for controlling precise standard gas generation.[31, 32] The sample emission rates in the steady state are very stable. Thus, gases for exposure can be generated continuously for longer periods of time, and it can be easily generated in a wide range of concentrations. This system has advantages over direct addition of chemical solutions into the cell medium for it characterizes a realistic exposure route of inhalation. The continuous and stable supply of source generation is helpful to quantify accurate levels among different exposure settings. Moreover, this approach is capable of further studying multiple compounds of interest. Nevertheless, it is worth noting that this method is not suitable for substances with extremely high or low vapor pressure or substances with decomposability, hygroscopicity or polymerizability.

3.2 Exposure induced cellular responses

In all experiments, exposure parameters and conditions were identical including the delivery flow rates (1 L/min), the exposure duration (4 hours), and the exposure surface area per well (1.12 cm^2; 12-well plate). Using the gas generator, five different concentrations of acrolein and methacrolein were created for exposures as shown in Table 1B. Details of statistical results for concentration effects on cellular responses are provided in Electronic Supplemental Information (Table S1-4).

LDH leakage is a widely used biomarker to measure chemically induced cytotoxicity of cellular membrane rupture and severe irreversible cell damage.[33] Figure 5 shows the concentration dependent cytotoxic cellular response for acrolein and methacrolein exposure. The concentration-dependent cytotoxic effects have been observed for both compounds. The potency of these chemicals, however, is very different. The cytotoxic response for acrolein exposure remains insignificant compared to unexposed controls at the low dose range, until a

277 concentration of 0.63 ppmV where LDH responses rapidly increase, and the fold change peaks at
278 15 at a dose of 2.37 ppmV. The cytotoxic response for methacrolein did not show a significant
279 increase until a concentration of 10.7 ppmV. When cells are under low dose exposure, cells
280 usually can adapt to the environment and survive. At high dose exposure when homeostasis is
281 disrupted, pathways of cell death could be triggered. The sigmoid dose-response curve of
282 acrolein exposure shows that there is a threshold at low dose level, while the biological gradient
283 increases significantly as the acrolein dose increases above the point of departure (0.63 ppmV).
284 This illustrates the value of the precision provided by these exposures systems to detect these
285 important changes in responses. Acrolein was also more toxic than methacrolein by inhalation in
286 the rat (Table 1A).

287 A second biomarker assessed in this study was IL-8, a proinflammatory mediators
288 produced by epithelial cells. Some epidemiological studies have suggested occupational asthma
289 associated with IL-8 increases.[27, 34] Figure 6 shows acrolein and methacrolein induced IL-8 gene
290 expression at different vapor concentrations. Detailed statistical outputs are provided in the
291 Electronic Supplemental Information (Table S3-4). Unlike the LDH response with acrolein, IL-8
292 levels did not increased in doses beyond 0.63 ppmV. It should be noted that cell death due to
293 exposure strongly influenced the capacity for IL-8 expression. From the acrolein LDH responses,
294 it is clear that with increasing doses there were larger fractions of the original cells no longer
295 viable and thus not capable of expressing IL-8. As a result, the low levels of IL-8 expression at
296 high dose exposures were likely only coming from a smaller population of living cells. The
297 proinflammatory effects at high dose exposures would have been more pronounced on a per cell
298 basis. On the contrary, with less cytotoxic effects and more viable cells post exposure,
299 methacrolein showed an increased response of IL-8 as exposure concentrations increased. This is

consistent with current published toxicological data showing that acrolein is a much more severe irritant. [35]

Several studies investigated the possible pathways of acrolein induced toxicity. Thompson and Burcham reported a study using microarray analysis to investigate transcriptional responses of human lung A549 cells to acrolein, and their results indicate that acrolein dysregulated a broad range of cellular pathways including those involved in apoptosis, cell cycle control, transcription, cell signaling, and protein biosynthesis.[36] Roy et.al reported a dose dependent study of A549 cells exposed to acrolein, and concluded that antiapoptosis processes dominate at low dose, shorter exposure times to acrolein, whereas proapoptotic processes dominate at high dose and longer exposure times.[10] These findings are consistent with the observations in this study that IL-8 responses were only elevated at low dose levels (0-0.63 ppmV), whereas LDH release significantly increased at high levels (0.63-2.37 ppmV) that suggest apoptosis has been triggered and dominates the cellular responses. Importantly, results from this study provide the information for a gas-phase dosimetry more relevant to the inhalation exposure route. Furthermore, this well-controlled exposure method will be capable to be used for the purposes of fast toxicity screening to prioritize numerous air toxics in a complex mixture.

4. Conclusions

Taken together, this exposure method demonstrates an alternative approach to investigate *in vitro* exposure to VOCs, especially for water-soluble secondary organic gases that are produced in a complex photochemical reaction mixture. The findings in this study indicate that acrolein significantly contributes to exposure induced cytotoxic responses, which supports the observations by Doyle at al., showing enhanced cytotoxicity from butadiene photooxidation products.[4] Methacrolein induced less cytotoxic effects on the basis of exposure concentrations,

but increased response of IL-8 at concentrations greater than 5 ppmV was observed. Other gaseous components produced in the complex mixture of isoprene photooxidation products, such as recently identified gas-phase epoxide intermediates including isoprene epoxydiols (IEPOX) and methacrylic acid epoxide (MAE), are worthy of further investigations through this approach.[37-40] Because we used short exposure times (4 hours), high concentrations are needed to achieve an adequate exposure level to observe toxic responses. Although the concentrations in this study are higher than ambient levels, the comparative exposure-response profiles will allow us to prioritize numerous air toxics in a complex mixture. These identified pollutants can be further incorporated into air quality models to characterize the environmental health risks arising from inhalation of the photochemical transformed products. Additionally, this same system and experimental protocols should be also applicable to studying indoor gaseous air pollutants in homes and workplace.

Conflict of interest statement

No conflicts of interest.

Acknowledgement

This project was supported by a Gillings Innovation Laboratory award funded by the Gillings gift.

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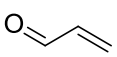
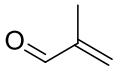
414

415 **Table 1A.** Physical and toxicological properties of compounds tested in this study.

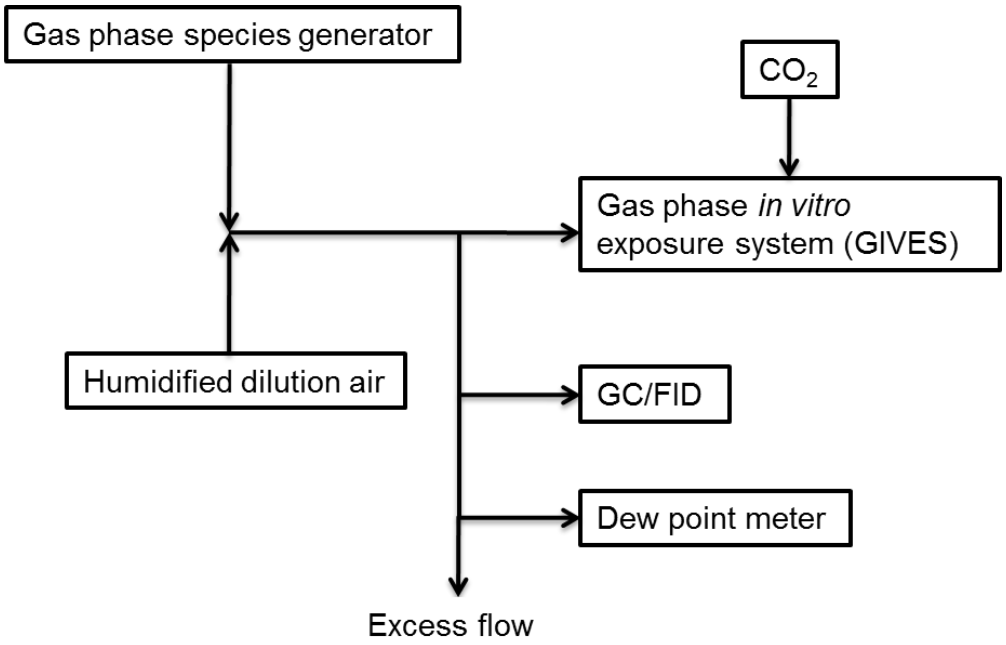
Test compound	Acrolein	Methacrolein
Molecular weight (g mol^{-1})	56.06	70.09
Formula	C_3H_4O	C_4H_6O
Structure		
Boiling point	53 °C	69 °C
Vapor pressure (at 25 °C)[7, 16]	274 mmHg	155 mmHg
Solubility in water	21.25 g/ 100 ml	6 g/ 100 ml
Henry's law constant ([M/atm])[41]	7.4	6.5
LC_{50} (rat, inhalation, 4 h)[35]	20 mg/m^3	560 mg/m^3

416

417 **Table 1B.** List of experiments and stability of output vapor concentrations for cell exposures.

#	Test Species	Measured concentrations (ppmV)	Relative standard deviation (RSD)
1	Clean air	0.00	--
2	Acrolein	0.23	9.51%
3		0.63	6.83%
4		1.00	1.02%
5		1.47	3.08%
6		2.37	4.99%
7	Methacrolein	0.68	7.99%
8		1.31	4.26%
9		2.29	6.81%
10		5.09	3.81%
11		10.7	8.49%

418



419

420 **Figure 1**. A schematic diagram of the *in vitro* exposure system showing the connection of major

421 components and the direction of air flows.

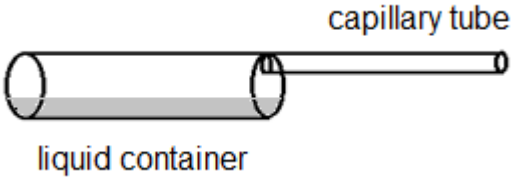
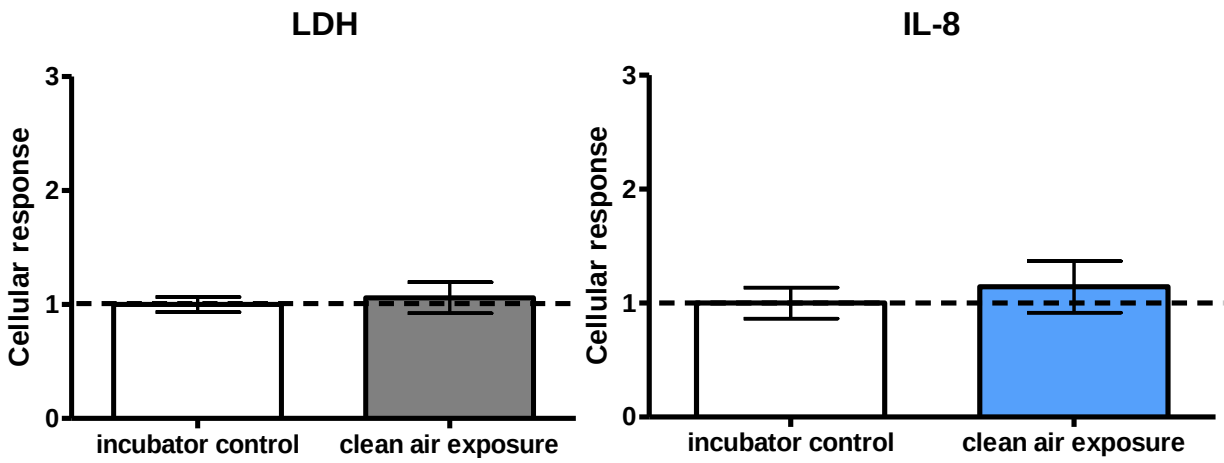


Figure 2. A schematic representation of the diffusion vial. The diffusion rate is a function of the molecular weight and the vapor pressure (which depends on temperature) of the target compound, the internal diameter and length of the capillary tube, and the operating pressure of the system.



426

427 **Figure 3.** Control experiments of the exposure system on cytotoxicity (LDH leakage) and
428 proinflammatory mediator response (IL-8 production). A549 cells were exposed to clean air in
429 the GIVES connected with the diffusion chamber for 4 hours. Results are expressed as relative
430 fold increase over control (mean ± SEM, n=6).

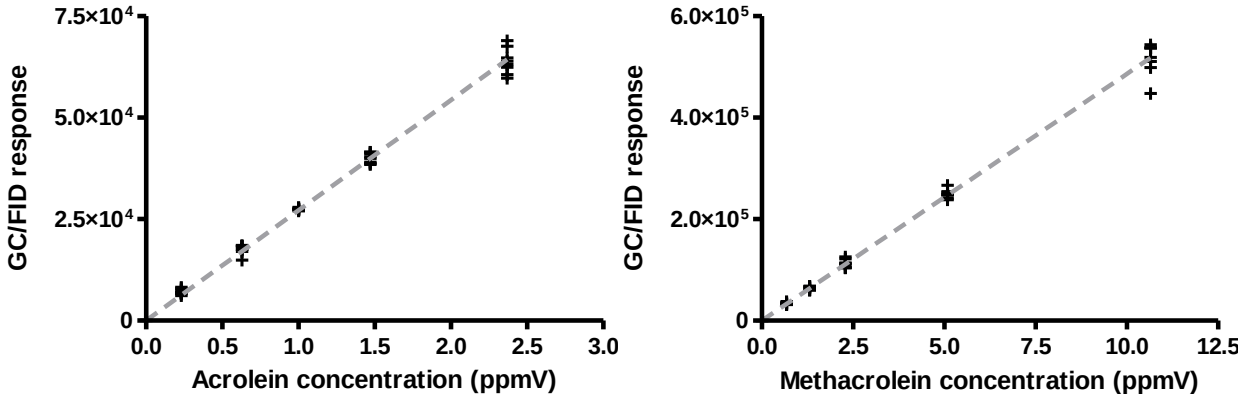
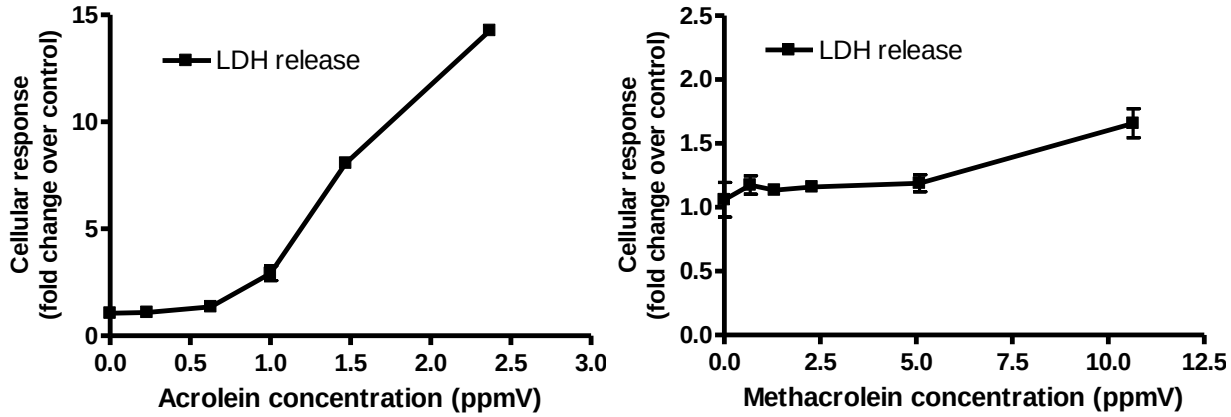


Figure 4. Measurements of the gas phase acrolein and methacrolein concentrations over the 4-hour exposure time. Vapor concentrations were monitored in a 30-minute interval throughout the experiments.



435

436 **Figure 5**. Effects of acrolein and methacrolein on LDH release of A549 human alveolar
437 epithelial lung cells. Cells were exposed for 4 hours. The supernatants of the exposed cells were
438 collected 9 hours post exposure. Results are expressed as fold change over control (mean ± SEM,
439 n=6).

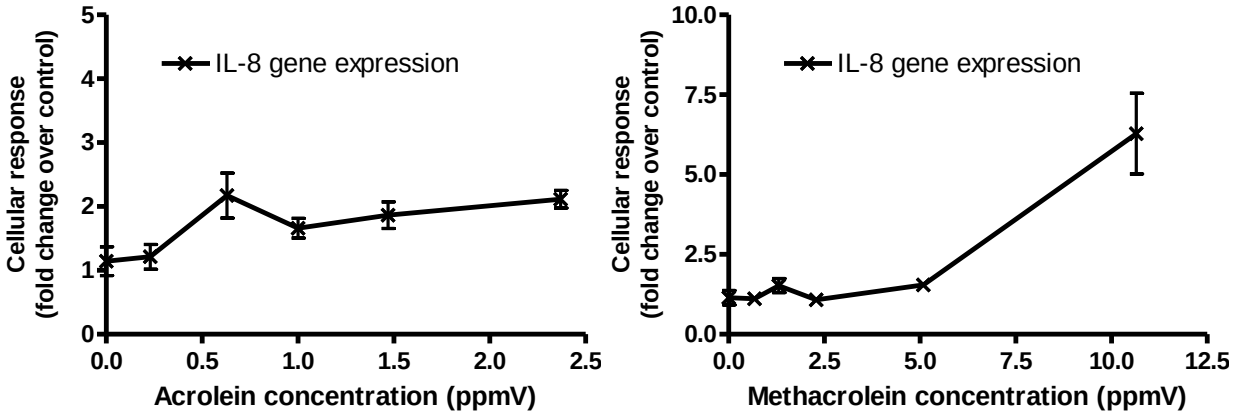


Figure 6. Effects of acrolein and methacrolein on induced IL-8 gene expression of A549 human alveolar epithelial lung cells. Cells were exposed to acrolein and methacrolein vapor for 4 hours. The supernatants of the exposed cells were collected 9 hours post exposure. Results are expressed as fold change over control (mean ± SEM, n=6). Notably, cell death due to exposure strongly influenced the capacity for IL-8 expression for acrolein exposures at high dose levels.