# USING IN VITRO CULTURED LUNG CELL EXPOSURE SYSTEMS TO COMPARE THE TOXICITY OF FRESH AND AGED DIESEL EXHAUST UTILIZING AN OUTDOOR SMOG CHAMBER

**Kim Maureen Lichtveld**

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Gillings School of Global Public Health, the Department of Environmental Sciences and Engineering.

Chapel Hill
2012

Approved by:

Harvey E. Jeffries

Kenneth G. Sexton

David Leith

Ilona Jaspers

Michael Madden

William Vizuete

# Abstract

KIM MAUREEN LICHTVELD: Using *In Vitro* Cultured Lung Cell Exposure Systems to Compare the Toxicity of Fresh and Aged Diesel Exhaust Utilizing an Outdoor Smog Chamber
(Under the direction of Harvey E. Jeffries and Kenneth G. Sexton)

Previous observations indicated that as urban-like atmospheres were oxidized, the modifications to their chemical composition also affected their toxicological potential. Based on these studies, I hypothesized that atmospheric oxidative processes will alter the composition of urban-like atmospheres containing diesel exhaust, resulting in a modification of biological responses from exposure.

The Electrostatic Aerosol in Vitro Exposure System (EAVES) efficiently deposits particles with no significant biological response from any internal processes and only produces a biological response when toxic PM pass through the sampler with the deposition voltage field on. Direct comparison of direct exposure EAVES with the commonly used traditional particle exposure method using a resuspension technique clearly demonstrated that the biological response in the latter was changed by extensive sample handling during the resuspension method, Furthermore, the EAVES is produces a biological effect from a much smaller sample.

This new methodology was coupled with a gas phase only exposure system to the outdoor smog chamber to further test the overall hypothesis. Two test atmospheres were generated using diesel exhaust from two vehicles mixed into urban-like gaseous

conditions and these systems were subsequently modified by either thermal (dark) or photochemical oxidation. Changes were evident in biological effect and chemical composition, but varied by source and oxidative environment. The gas phase exposures biological responses were increased only from the Mercedes thermally aged condition and in the fresh conditions for the Volkswagen. To understand the biological response from the PM exposures I examined three methods of comparison to the biological response. This indicated that aged atmospheres are significant in biological response and that the primary and secondary carbonyls present during the exposure are important.

From these studies, I have demonstrated my hypothesis that atmospheric processes do alter the chemical composition and biological effect of diesel exhaust oxidized in an urban-like environment, with the qualifier that the degree of the effect is dependent on the amount of toxic components deposited on the cells. Further, I can conclude that the source and aging environment of the test atmosphere play important roles in complex mixture toxicity.

# Dedication

To my family, for all their support through these past years.

My mother who has been a guiding light and a source of encouragement.

My father who has waited so patiently for me to finally finish school and get my papers.

My sister who always makes me laugh and keeps me grounded.

My brother whose inquisitive mind keeps me guessing bringing me laughter and thought.

To my fiancé for whom without I could not have made it. You are my rock and I know that there is no obstacle big or small that we cannot get through together, come out the other end, and through it all keep calm and most of all never stop laughing.

To IJE your sarcasm, wit, humor, and artistry pull me into your world of wonder and for that I thank you for my mini mind vacations.

To the Wandering E's I am so thankful to have you in my life. Your warm welcome and support is utterly amazing.

To my friends who have gone on this journey with me, thank you for all your support and encouragement! Thank you for the adventures, laughter, and gatherings that enabled me to escape the throws of dissertating.

Last, but definitely not least, the ones who have gone before us to guide the way and remind us how short life is and to enjoy each and every minute.

# Acknowledgements

I would like to thank Wenli Zhang, Missy Brighton, and Jonathan Ciencewicki from CEMALB, and Maryanne Boundy from the Department of Environmental Sciences and Engineering for all of their technical advice; and Beth Fowler from the Department of Environmental Sciences and Engineering for administrative assistance; Richard Kamens from Department of Environmental Sciences and Engineering and Southern States Volkswagen for the use of their vehicles.

**Chapter 2:** This work was supported in part by the US Environmental Protection Agency Cooperative Agreements R829762 and CR829522, American Chemistry Council Long Range Research Initiative, Project ID CIE-0102-02, and Exxon Mobil Foundation Basic research support gift. Although the research described in this article has been funded wholly or in part by the United States Environmental Protection Agency through cooperative agreement CR829522 with the Center for Environmental Medicine, Asthma, and Lung Biology, it has not been subjected to the Agency's required peer and policy review, and therefore does not necessarily reflect the views of the Agency and no official endorsement should be inferred. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

**Chapter 3 and 4:** This work was supported in part by the U.S. Environmental Protection Agency Cooperative Agreements R829762 and CR829522; American Chemistry Council Long Range Research Initiative, project ID CIE-0102-02; National Institute of Environmental Health Sciences T32 ES007018; the Gillings School of Global Public Health's Gillings Innovation Laboratory Program for Research and Innovations Solutions; and Exxon Mobil Foundation Basic Research support gift. Although the research described in this article has been funded in part by the U.S. Environmental Protection Agency through cooperative agreement CR829522 with the Center for Environmental Medicine, Asthma, and Lung Biology (CEMALB), it has not been subjected to the agency's required peer and policy review, and therefore does not necessarily reflect the views of the agency and no official endorsement should be inferred. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

# Table of Contents

# List of Tables

# List of Figures

# Chapter 1 : Background and Significance

## Rationale

Epidemiological studies have shown a positive relationship between measurements of ambient particulate matter (PM) and adverse health effects in susceptible human populations (patients with chronic obstructive pulmonary disease, chronic bronchitis, asthma, and cardiovascular disease) (Dockery 1994; Bates 1995; Pope 1995; Sarnat 2001; Ayres 2008; HEI Panel on the Health Effects of Traffic-Related Air Pollution 2010). Epidemiology has used the increases in hospital admissions and daily mortality reported during short term episodes of high PM in the ambient air to find a positive relationship between PM and human health effects. Even though there seems to be a positive correlation there is still no established biological mechanisms to explain the toxicity of PM to humans and the evidence of carcinogenic potential is limited because of the difficulty in eliminating the influence of confounding factors and estimating exposure (Oberdorster 1990; Salvi 1999; Bunger 2000). Despite confounding factors like cigarette smoke, Silverman et al. reported a correlation between lung cancer in miners and their exposures to diesel exhaust (Silverman 2012). This study went on to be an influential part of the decision by the International Agency for Research on Cancer (a part of the World Health Organization) to assign a Group 1 designation to diesel exhaust emissions (International Agency for Research on Cancer 2012). A Group 1 classification means that diesel exhaust is now considered to be carcinogenic to humans.

While epidemiological studies have suggested that a number of sources or source characteristics are correlated with observed health effects, subsequent laboratory-based toxicological studies often fail to confirm these as causes at levels that might occur in the ambient environment (Morgan 1997, Salvi 1999, Laks 2008, Dybdahl 2004, Carlsten 2008, Sunil 2009). Sarnet et al. suggests that the ambient measurements may be a weak indicator for the actual amount a person may be exposed to during the course of the day (Sarnat 2001).

In 2001, Mauderly requested that the scientific community take a "more integrated, or holistic, view of the air quality-health relationship" and that "epidemiology is largely limited to testing associations between health outcomes and the few pollutants that are measured routinely" (Mauderly 2001). This study took the approach of being more holistic and integrated to obtain understanding the inflammatory response of diesel exhaust in an urban-like environment.

## Diesel Exhaust Composition

Diesel exhaust (DE) is a highly complex mixture of gases and particles, continuously changing in composition as it ages in the atmosphere. PM from diesel exhaust contains an elemental carbon core surrounded by "organic compounds (such as polycyclic aromatic hydrocarbon (PAHs)), sulfate, nitrate, metals and other trace elements (manganese, sulfur, and iron)" (EPA 2002). Elemental carbon is inert to atmospheric degradation, whereas organic compounds can degrade by reactions with sunlight, ozone, and other atmospheric processes (McDow 1995, Fan 1996a, Fan 1996b, Lee 2004). The gaseous components of DE consist of oxygen, carbon dioxide, nitrogen, carbon monoxide,

water vapor, nitrogen oxides, sulfur compounds, benzene, 1-3, butadiene, nitrosamines, PAHs, nitro-PAHs, and other volatile organic compounds (VOCs) (EPA 2002). Among the gaseous components emitted from diesel engines, VOCs containing a carbonyl (aldehydes and ketones) functional group are of particular interest because some carbonyls are considered probable carcinogens that are also capable of producing non-cancer health effects (International Agency for Research on Cancer 1989).

The characteristics and composition of engine exhaust emissions from gasoline- and diesel-fueled motor vehicles differ significantly. Many factors---including engine type, fuel, engine load and after-treatment--- influence the composition of the primary atmospheric emissions. These composition changes will affect both photochemical reactivity and toxicity of the aged atmospheric mixture ultimately inhaled by humans. For example, the primary emissions from gasoline fueled engines contain fewer particulates than the equivalent volume of diesel-fueled engine emissions. Gasoline exhaust is more photochemically reactive, however, because it contains a large fraction of gaseous monocyclic aromatic compounds, which after reaction, contribute significantly to secondary particle growth (Odum 1997a, Odum 1997b, Kleindinest 2002). Diesel emissions also react photochemically, especially in the presence of other urban VOCs, and such reactions are known to modify the diesel PM surface and composition significantly (McDow 1995, Fan 1996a, Fan 1996b). While both gasoline and diesel emissions react in the atmosphere, their chemical mechanisms can be very different, making it difficult for modelers to predict the composition and toxic characteristics of the respective aged systems.

## Particulate Matter in the Lung

In the lung, particles have five major mechanisms of deposition: inertial impaction, gravitational settling, Brownian diffusion, electrostatic attraction, and interception. While these mechanisms all play a role in particle deposition in the lung, particle size mass has the greatest impact as well as the ultimate location of deposition (EPA 2002).

Deposition in the respiratory tract may occur in various regions of the lung from the extrathoracic, the tracheobronchial, and the alveolar regions (Lippmann 1977). When ~ 2.5 µm particles are inhaled, the particles' velocity are increased, and abrupt directional changes caused by airway branching makes inertial impaction in the mucus layer lining the extrathoracic and tracheobronchial regions of the lung likely. Particles trapped in extrathoracic and tracheobronchial regions are carried to the back of the throat on a pulsing ciliary membrane covered in mucus (Shaw 2005). Upon arrival, particles are swallowed and then cleared via the gastrointestinal tract. Removal of the PM in the pulmonary region does not mean that there could not be a whole body burden of exposure to PM as it passes through the body. As particle diameters decrease to less than 1 µm, the particles are more likely to behave like gases, and therefore are most likely to deposit by diffusion. This allows particles to travel deep into the alveolar region of the lung, where the particles impair gas exchange (Shaw 2005).

In the lung, particle exposure will cause injury and the recovery process begins with the recruitment of cells associated with inflammation which is regulated by lipid and protein mediators such as cytokines. Cytokines are relatively small proteins that interact and bind to specific receptors on membranes of target cells within the airway

walls and activate signal transduction mechanisms involved in intracellular communication. These cytokines are responsible for regulating cell differentiation, proliferation, and cell secretory activities. Inflammatory markers, such as, Interleukin-6, Interleukin-8, cyclooxygenase-II enzyme, and Tumor Necrosis Factor-$\alpha$ (IL-6, IL-8, Cox-2, and TNF-$\alpha$), are early mediators of the body's response to injury and are expressed rapidly after exposure to toxic agents (Salem 2006; Cao 2007; Zoran D. Ristovski 2011). These can be expressed over hours and measured either in the supernatants or through mRNA analysis. Injury of pulmonary epithelial cells results in loss of cell membrane integrity, which in turn results in the release of intercellular lactate dehydrogenase (LDH). Thus, increased release of LDH is considered to be a sign of cellular death and can be measured directly in the lung lining fluid.

## Cell Lines as a Model for the Lung

Two advantages of using *in vitro* models are that these models allow investigators: 1) to study the response of individual components of the respiratory system to inhaled toxins, 2) to examine mechanisms by which these toxicants cause inflammation, and 3) to address questions of lung toxicity in human lung cells at levels that might be unethical for human subject involvement. Although *in vitro* models do not have the ability to account for all of the interactions in the cells' natural environment, they respond to a stressful environment as they would in the body (by releasing inflammatory markers). Therefore, cellular release of these cytokines represents a reasonable measurement to estimate the epithelial cells' attempt to signal immune cells to come to the site of injury,

despite the absence of these immune response-related cells from *in vitro* cultures (Lieber 1976; ATCC 2011).

Immortalized cell lines are useful because they can be maintained under cultured conditions for an extended period of time. In addition, they are readily available allowing for optimization of replication of research without uncertainty introduced by interpersonal variability in response or interspecies extrapolation. A549 cells are a model of alveolar epithelial cells with type II cell-like characteristics, and have been extensively used to assess the toxicity of air pollutants (Doyle 2004; Sexton 2004; Bitterle 2006; Doyle 2007). Type II cells are involved in pulmonary defense mechanisms by secreting protein mediators that contribute to pulmonary inflammation (Lumb 2005). In short, Type II cells are responsible for sending out help signals. In this project, A549 cells were grown on membranous support using complete media. Upon confluence, the growth medium was replaced by serum-free media several hours before exposure to slow cellular proliferation. The cell culture inserts (Transwells or Millicells) can be used to establish an air-liquid interface, which facilitates direct exposure to pollutants without interference from media covering the cells.

The liquid under the membrane of these inserts allows cells to be adequately hydrated and supplied with nutrients while being exposed to pollutants on the cells' apical side. Cellular signaling can be measured using the supernatants or the ribonucleic acid (RNA) collected from the cells. Laboratory assays such as the LDH essay and Enzyme Linked Immuno Sorbent Assay (ELISA) use the supernatants to detect mediators re-

leased by the cells. Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) uses the RNA collected from the cells to measure intercellular production of mRNA responsible for mediator production.

## In Vitro Particulate Exposure Methods

There are many methods for particle collection: gravitational settling, thermal diffusion, thermophoresis, electrostatic precipitation, inertial impaction, and filter collection. The characteristics of each method affect their suitability for *in vitro* exposures. Each particulate exposure method was to be evaluated to assess the following: their ability to efficiently collect and deposit particulate matter for specific particles of interest (ie. 200-500nm diameter); any potential for damage to cell cultures during exposure; and how each collection method might alter the particles' primary state. A review of the suitability of candidate exposure methods with respect to these three criteria is presented below. See appendix for all calculations.

To gain an understanding of the various PM depositions onto human epithelial lung cells, assumptions need to be made to compare each methodology. The size range of DE used in this study ranges between (200-500 nm) and for the purpose of method comparisons, each method comparison will use the particle diameter of 200 nm with a PM concentration in the chamber of 1.2 $mg/m^3$. In addition, 4 µg per insert with a membrane area of 1 $cm^2$ was used as the area and amount of PM to be deposited onto the cell culture inserts. In these calculations the assumption for the density of the PM is 1.0. With these basic assumptions each method for PM method is described below.

Gravitational settling is considered acceptable for collecting super-micron particles (above 1.0 μm). Particles in the size range of DE (200-500 nm) have a slow terminal settling velocity (taking into account slip correction). This method is not appropriate for *in vitro* exposures since the time required for 4 μg of 200 nm DE particles to deposit would be $1.49 \times 10^{7}$ sec or 172.5 days. The air space above the cell culture insets would need to be 33.33 m to deposit enough material down on the each 1 $cm^2$ insert. This is obviously unacceptable for several reasons, including changes in the particles' chemistry that would occur over that time. While the particles' settling velocity range ($2.26 \times 10^{-6}$ m/sec and $9.91 \times 10^{-6}$ m/sec for 0.2 μm and 0.5 μm particles respectively) (Hinds 1999), would not damage the integrity of the cells, the time required for deposition makes its use unrealistic.

Thermal diffusion is possible when considering particles less than 100 nm in diameter, but the distance to be traveled must be short (0.1 cm), and the time that the particles have available to travel the distance must be very long (i.e. hours). In DE, only a fraction of the particles are small enough to move far by thermal diffusion, and the distance to the surface of the cells is not small enough for collection, i.e. from the air to the inserts on the collection plate. For example, for particles to diffuse 1 cm from the repeller plate to the cell culture inserts, it would take approximately 59.35 hours. Although the deposition onto the cells would be gentle, thermal diffusion is not a viable method for particle deposition onto cell culture inserts for the size range of interest, and exposure times required.

Thermophoresis occurs when the movement of a particle is altered by a temperature gradient introduced to a gas and particle mixture (particles will move in the direction of decreasing temperature). A typical thermophoresis precipitator houses a heating element with deposition plates on each side (0.12 mm from the heating element) (Hinds 1999). This method could be useful for collecting particles in the size range of DE and the deposition velocity *is* low enough that the cells would not be damaged ($1.964 \times 10^{-6}$ m/s)(Calculator 2009). However, the heating element may change the particle composition prior to deposition. In addition, the collection plate is (typically) too close to the heating element to permit the inclusion of cell culture inserts.

Inertial impaction works well for particles larger than 1 µm. When given enough lateral velocity the particles can deposit onto a wall or collection plate. Usually, particles pass through a nozzle which aims a stream of air (at high velocity) at an impaction plate. A certain size fraction of particles is deposited on each plate, with deposition of fractions with decreasing diameter on subsequent plates. This method allows cells to be simultaneously exposed to various-sized particles, allowing the researcher to determine the hazards of various sized particles dynamically. There are three significant problems with this method. First, the air flow over the cells (above 1 L/min) can cause the cell cultures to rapidly desiccate compared to their environment. Secondly, if the inserts were placed on the collection plate of the impactor, damage to the cells' integrity is likely to occur (Sillanpää 2008; Cooney 2011). For instance, the velocity of a 0.2 µm particle using the area of the nozzle at $1.05 \times 10^{-7}$ (like the Cultex system) was calculated, and found the velocity to be 158.5 m/s out of the nozzle (Hinds 1999; McDonald 2004). In addition

to the velocity the Cultex system has a 0.7 % collection efficiency for particles in the 200 nm range (Paur 2011). Finally, using inertial impaction is likely to cause loss of VOCs prior to exposure because after collection of PM out of the air stream prior to exposure the PM is no longer in equilibrium with the ambient air (Sillanpää 2008). For all of these reasons, this method is not a viable way to deposit diesel particles directly onto cell culture inserts.

Impingers pass air containing PM through a liquid in which the particles are collected (Li 2002, Madden 2003, Jaspers 2005). Compounds of interest and surface features may be altered or lost by the particles' transfer into the liquid media. Additionally, collecting particles in the liquid medium can collect portions of the gas and PM, but not all particles would be collected – further reducing the utility of impinger collection for in vitro exposures (Madden 2008). While the instillation of the collection solution should not cause structural damage to the cells, particles would be likely to agglomerate, changing the size of the individual particles originally sampled (Stewart 1995; Sillanpää 2008; Cooney 2011). In addition, air liquid interface exposures are not possible with this type of exposure method. These issues together discount this method.

Electrostatic precipitation (ESP) occurs when charged particles are subjected to an electric field, causing the particles to drift across the flow and ultimately deposit on a collection plate (Whitby 1974; Mainelis 1999). Traditionally, ESP has been used as a method to control airborne dust in residential and industrial settings (Boelter and Davidson 1997). When PM is collected with ESP, the velocity perpendicular to the collec-

tion surface is orders of magnitude lower than that of an impactor sampling at the same flow rate, resulting in a gentler deposition than found with impaction methods (Mainelis 1999). The calculation of settling velocity was determined by using the assumptions above with the additional parameters listed in the appendix and was calculated to be 0.86 cm/s. Some potential issues that might change the particle exposure using ESP could be: a charge on the particle, ozone produced by the corona wire, and the possibility of a size gradient on the collection plate. However, ESP has the ability to provide a gentle and effective method for direct particle *in vitro* exposures.

## Other ESP Exposure Devices and Ambiguities

Other researchers have used ESP-based devices as PM exposure methods. In one study, Volckens *et al.*, (2009) used a modified version of the Electrostatic in Vitro Exposure System (EAVES) exposure device (described herein) to expose concentrated ambient aerosols (PM 10) to primary human bronchial epithelial cells. Volkens found that, compared to the more conventional resuspension method, the EAVES exposures resulted in an enhanced sensitivity to the toxicity of the ambient environment (the modified EAVES needed less PM to cause a significant response than did the resuspension technique). Although there were significant responses from the primary cells exposed in the modified EAVES, the variability in these direct exposure responses was large. This variability could be the result of a number of causes, including the study's use of primary cells and that the EAVES device used was of a prototypic design. Even with that variability, however, the authors reported that effects were evident at concentrations orders of magnitude lower than were observed using traditional resuspension techniques.

Sillanpaa et.al. (2008) also used ambient exposures to test their ESP device with the addition of a particle concentrator to enrich ambient particles by making them larger. During this study they did not report any exposures to ambient PM, only to clean air to determine if the ESP was causing an effect (Sillanpää 2008).

Another ESP device described in the literature used a bi-polar Kr-85 source to charge the PM prior to deposition (Savi 2008). This system used a delivery tube to bring the charged particles directly over the cells and, as the PM flowed over the surface of the cells, an electric field below the cells attracted PM to the cellular surface. They used a cell line to reduce ambiguity from the exposures and focus on the technique, but did not include descriptions of exposures to test atmospheres. The goal of their paper was to show that this method was viable for deposition of PM, and that non-toxic particles deposited at an air-liquid interface would not cause a response from cells in their device.

## Cascaded Health Effects Models

Figure 1-1 provides a schematic of a conceptual model for relating the real world to a model system designed to study human health effects of exposure to gases and PM. This part of the model illustrates that effects begin with the source of the pollutant, followed by environmental reactions. The original source and environmentally altered source are then available for exposure to people resulting in possible health outcomes. The bottom illustrates the series of models used to implement the concept of exposure from source to response. The models represent the available pieces in our laboratory to combine forming the source to response model. This approach, based on controlled but

realistic conditions, can result in clearer causal relationships from the source to the observed health effects that are seen in epidemiological studies. In my discussion below, each component and a realistic model that can simulate parts of the real world conditions are described. With the use of these models, I hope to suggest future studies that can assist with the connection from the source, reactions with the ambient environment, and subsequent biological response from human exposures.

## Source Models

Because of the strong epidemiological findings connecting ambient PM 2.5 and detrimental health effects, much attention has focused on diesel exhaust (Bates 1995; Sarnat 2001). Ambient diesel PM has a mean aerodynamic diameter that ranges from 10 to 300 nm. These particles are readily inhaled and about 20 % are deposited into the alveolar region of the lung (Bunger 2000; Kim 2006). Current studies of diesel exhaust have used single stationary vehicles and carried out biological exposures to what is essentially freshly emitted exhaust (Li 2002; Baulig 2003; Knebel 2001; Salvi 1999; Laks 2008; Sawant 2008; Carlsten 2008; Liu 2008; Sunil 2009). In this study, I have chosen to use two diesel vehicles as primary pollution sources with different characteristics: an older vehicle representing the cars and trucks running on diesel fuel prior to the catalytic converter, and a newer vehicle representing the cars with emission controls. By using two different diesel vehicles I obtained samples from real-world sources that are on the road today, which can be more representative than other DE research that uses altered PM prior to exposure (Bauling 2003; Dybdahla 2004). My study did not account for the

role of heavy-duty trucks on the road or the heavy machinery powered by diesel, as I am initially focused on passenger cars as sources for test atmospheres.

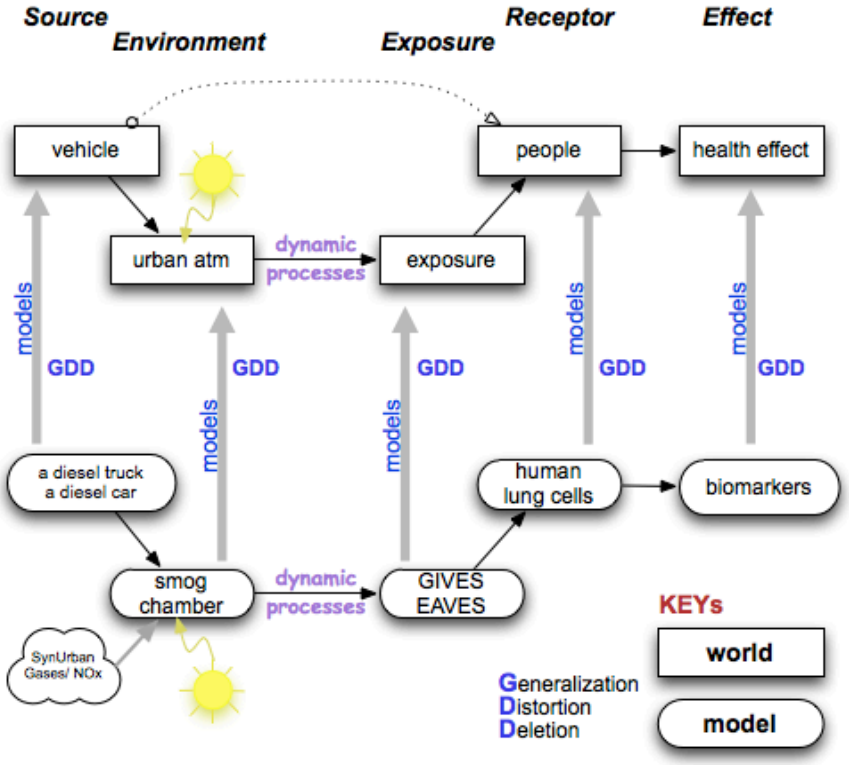


Figure 1-1. Conceptual model

## Atmosphere Models

In this study, outdoor smog chambers were used to simulate, using natural sunlight, a set of urban-like test atmospheres that allow diesel exhaust to be photochemically altered as it likely is in the real world, but under controlled and repeatable conditions (Jeffries 1976; Kamens 1981; Jeffries 1985; McDow 1995; Lee 2004; Doyle 2007; Ebersviller 2012a; Ebersviller 2012b). To provide a gaseous chemical input typical of US cities, I used a well-studied mixture of representative urban volatile organic compounds (VOC). A 54-component VOC mixture was used in these studies with oxides of nitrogen

(NO and NO2). Various amounts of diesel exhaust were added to this mixture to produce reactive urban-like atmospheres. Nevertheless, all important atmospheric chemical and chemical phase change processes did occur in the chamber [24, 27-29, 29-46], Hu 2007). This approach was critical to relevant outcomes in my study in that it was able to simulate the complex dynamic processes occurring in the sunlight that results in a large change in the diesel exhaust gases and particles as a function of time. Doing exposures in the dark just after injection in these model systems also permit the comparison of primary (fresh) emissions in contrast to the secondary (oxidized) emissions later in the simulation.

## Exposure System Model and Biological Receptor Model

In this study, I applied the methodology and findings gained from development and implementation of the Electrostatic Aerosol *in Vitro* Exposure System (EAVES), and the Gas *in Vitro* Exposure System (GIVES). These *in vitro* exposure systems are methods to elicit a response from human epithelial lung cells (A549 cells) exposed the chamber contents. In the EAVES system, cells are exposed to particles alone, without interrupting their equilibrium with the gases around them (de Bruijne 2009). Conversely, in the GIVES system, no significant amount of PM comes in contact with the epithelial lung cells, resulting in a virtually gases only exposure at the cellular surface.

These two exposure systems reside in a 37 °C incubator with sampling lines coupled to the outdoor chamber. These systems allow epithelial lung cells to be exposed to diesel particles and gases separately, facilitating comparison of the inflammatory response to each phase of the complex diesel mixture. The *in vitro* epithelial lung cells

were grown on membranous supports resulting in a monolayer of cells that were exposed across an air-liquid interface analogous to the process that occurs in the lung (Leiber 1976, Doyle 2006, Bakand 2007). In these exposure systems, cultured human epithelial lung cells were used as a targeted biological system that was capable of responding to ambient gases and particles in ways that mimic the response in the human body (Doyle 2006, Jaspers 1997). These cells represent the lower alveolar region of the lung that would be exposed to ambient air in a human (Lumb 2005, Jasper 1997, Bakand 2007). *In vitro* models have been well studied as a method to examine the mechanisms induced by exposure. These models respond as they would in the body, but may not give the true toxicological potential since they are not able to include the series of cascade systematic effects in the body. Although this system lacks this ability, inter-cellular signaling is still active despite the absence of organ-level multi-cellular complexity. The release of these inter-cellular signals served as the health effect model.

## Health Effect Model

The response at the cellular level serves as a reliable predictor of the acute adverse health effects seen in the respiratory tract (Bakland 2007). Various intercellular chemical signals occur in exposed human epithelial lung cells as a consequence of interactions with gases and particles. As these signals are expressed both within the cell and in the media supporting the cells, they can be used as markers of exposure. These same markers of inflammation and cellular death have been monitored in other diesel exposures studies using rodents and direct human exposures (Dybdahl 2004, Li 2002, Knebel

2001, Baulig 2003, Salvi 1999, Laks 2008, Sawant 2008, Carlsten 2008, Liu 2008, Sunil 2009).

### Beneficial Attributes and Limitations of Cascaded Model

The overall benefit of this cascaded modeling system is the use of various sources in a controlled, repeatable, and complex urban-like atmosphere. In addition, it has the ability to separate the inflammatory response caused by gases and PM, using two different *in vitro* exposure systems. The inclusion of photochemical aging in the outdoor chamber allowed for dynamic chemical processes to occur as they would in the real world. This, coupled to the two *in vitro* exposure systems, allowed for the separation of inflammatory response induced by the gases and PM. This model has given us better insight to explain the differences in response seen between epidemiological studies and current diesel research.

My cascade model approach allowed the research to be understandable, quantifiable, and controllable, and thus likely to contribute to new insights about processes of importance that might be obscured in the real (fully complex) world. The findings of this study are not intended to be conclusive and definitive as is required by policy makers, but to be indicative of the direction that future research needs to go.

## Hypothesis

Atmospheric processes will alter the composition of urban-like atmospheres containing diesel exhaust, which will result in a modification of biological responses from exposure. Further, an older vehicle lacking emission controls will elicit a higher biological response compared to a vehicle with EPA suggested emission controls.

## Approach

Current *in vitro* exposure methods may not be suitable for observations of particle uptake of species of interest. Widely used methods of particle collection involve steps that could result in the loss of volatile species from samples (Volckens 2002). Due to these shortcomings, a new exposure technology was developed to provide a viable method for *in vitro* PM exposures. To improve upon existing exposure methods, the new method maintains the equilibrium between gases and particles until the pollutants arrive at the cellular interface. Exposures performed in such a way minimize artifacts that affect volatile species. This new technology provides a biological response monitor to more-accurately estimate the health effects of exposure to airborne mixtures of gases and PM.

Once the new method was evaluated and optimized for PM exposure onto human epithelial lung cells, it was further investigated to determine if the results were similar to a widely used PM method. Specifically, one of the most widely used methods for *in vitro* exposures to PM is to collect PM on filters, resuspend the collected PM in a liquid medium, and subsequently add the mixture to the cell culture (Bayram 1998; Boland 1999; Abe 2000; Knebel 2002; Mazzarella 2007; Seagrave 2007). Ultimately this study evaluated the differences in handling of PM from source to exposure noting the number of steps prior to exposure. In addition, the composition of the PM delivered to the cells was evaluated, as well as the observed response from the cells at a similar exposure concentration. These comparisons permitted an evaluation to determine the relative utility of each method.

When the new direct exposure method had been tested it was used along with the gas phase exposure system to explore the effects of atmospheric aging of diesel exhaust and the resulting toxicological effects on human epithelial lung cells (Doyle 2004; Sexton 2004; Doyle 2007; Ebersviller 2012a; Ebersviller 2012b). The goal of this portion of the study was to detect any differences in the toxicological responses of the human epithelial cells through a series of experiments comparing different conditions: 1) older and newer diesel vehicle emissions, 2) fresh and aged emissions (dark and photochemically aged), and 3) observe the differences in inflammation in the gas and particle phases. This highly complex study not only used the two exposure tools but incorporated chemical analyses to attempt to explain observed biological responses.

## Objectives

My overall objective was to adopt a more integrated, holistic approach to demonstrating the features and attributes that might be causally related to human health effects. This objective was realized through a series of cascaded physical and biological modeling systems (described above).

The specific objectives listed below allow for the evaluation of the hypothesis.

1. Evaluate and validate a novel *in vitro* exposure method using electrostatic precipitation (ESP) to expose human lung cells to particulate matter (PM).

2. Compare a conventional PM *in vitro* exposure method to the *in vitro* ESP device from Objective 1 using diesel exhaust to determine the sensitivity of the respective methods.

3. Compare the toxic effects induced by exposure to fresh and aged emissions (in the sunlight and in the dark) of old and modern diesel vehicles using separate *in vitro* exposure methods developed for gases and PM.

4. Determine the specific toxicological effects independently induced by particulate and gas-phase components and their respective contribution to the biological response observed in Objective 3.

By achieving these objectives, this research project addressed a number of important questions regarding the toxicity of diesel emissions: 1) Would the new *in vitro* PM method successfully remove sampling artifacts, thereby increasing sensitivity? 2) To what extent did aging, either in the presence or absence of photochemistry, change the toxicity of vehicle emissions? 3) What were the roles of particle and gas phase composition in the toxicity induced by fresh and aged vehicle emissions?

# Chapter 2 : Design and Testing of Electrostatic Aerosol *In Vitro* Exposure System (EAVES): An Alternative Exposure System for Particles [1]

## Introduction

It has been shown that particulate matter (PM) is responsible for a significant fraction of air pollution-induced health effects, yet there remain many questions concerning mechanisms of injury and what sources and components of this complex pollution are most responsible (Pope 1995; U.S. Environmental Protection Agency 1999). Laboratory animal and human *in vivo* studies have shown that inhalation of diesel exhaust (DE) increases markers of inflammation, including inflammatory cytokine production (Salvi 2000; Singh 2004). Studies using *in vitro* models to expose epithelial cells to DE have also shown increases in the production of inflammatory cytokines, such as Interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF- $\alpha$ ), following exposure (Kim 2005; Seagrave 2007). Although *in vitro* models lack the ability to account for all intercellular interactions in the cells' natural environment, studies using *in vitro* exposure models enable investigators to examine the effects of inhaled toxins on specific cell types, and are important to determine potential cellular mechanisms mediating these responses. Over the past several years, important advances have been made concerning developing *in vitro* exposure models that closely mimic *in vivo* exposures. For example, epithelial cells grown on membrane support at air-liquid interface can be directly exposed to gas-phase

pollutants, such as ozone, similarly to the way these cells would be exposed *in vivo* (Jaspers 1997; Doyle 2004; Seagrave 2004; Sexton 2004; Doyle 2007).

Several methods to conduct *in vitro* exposures to PM have appeared in the literature (Bayram 1998; Boland 1999; Abe 2000; Aufderheide 2000; Li 2002; Madden 2003; Jaspers 2005; Seagrave 2005; Mazzarella 2007). All of these methods have known disadvantages and may, therefore, not accurately represent PM-induced health effects *in vivo*. Specifically, one of the most widely used methods for *in vitro* exposures to PM is to collect PM on filters, re-suspend the collected PM in a liquid medium, and subsequently add the mixture to the cell culture (Bayram 1998; Boland 1999; Abe 2000; Knebel 2002; Mazzarella 2007). Filters collect particulate matter efficiently, and particles are easily re-suspended in a liquid for subsequent contact with cells. Major shortcomings of filter collection, however, include the loss of VOCs (volatile organic compounds) from the PM, agglomeration of small particles during collection, and the possible alteration of the particles during the recovery process and while in the liquid medium. Impactors collect large diameter PM on plates relatively efficiently (Tsien 1997), but VOCs can again be lost during collection, and, as with filters, the collected PM needs to be transferred to a liquid medium before use with cells. In addition, impactors can only be used to sample particles of relatively large diameter due low collection efficiency for small particles (Hinds 1999; McDonald 2004). Alternatively, impingers have been used to sample air containing PM through a liquid in which the particles are collected (Li 2002; Madden 2003; Jaspers 2005). Again, compounds and surface features of interest may be altered or lost by the particles' transfer into the liquid media. In addition, once particles have

been collected in the liquid medium in this manner, it is difficult to accurately determine the concentration of PM in solution – further reducing the utility of impinger collection for *in vitro* exposures. Recently, an *in vitro* system using impaction to deposit PM directly onto cells was developed and tested (Cultex® Laboratories, Hannover, Germany) (Aufderheide 2000; Seagrave 2005). While this exposure system presents a much-improved method for *in vitro* PM exposures, there remain a number of disadvantages---including that impaction methods, while being efficient deposition methods for large particles, have a much lower utility for small particles (Knebel 2002).

Electrostatic precipitation (ESP) is a widely used method of PM collection and monitoring. Traditionally, ESP has been used as a method for aerosol collection in the control of airborne dust in residential and industrial settings (Boelter 1997). Particles are electrically charged and then subjected to a strong electric field that causes the particles to drift across the flow, and ultimately to deposit on a grounded collection plate (Whitby 1974; Knutson 1975). When PM is collected with ESP, the velocity perpendicular to the collection surface is orders of magnitude lower than that of an impactor sampling at the same flow rate. Mainelis, et al. (2002) modified a commercial ESP sampler to examine its utility as a bioaerosol sampler. Their modified ESP was used to collect test aerosols of microorganisms that were subsequently shown to be biologically viable after collection. This example demonstrated the potential for gentle collection and direct deposition onto lung cells as a viable *in vitro* exposure method. Different from the microorganisms, exposure of cultured human lung cells requires an environment similar to that in the respiratory system and epithelial cells may respond differently to the

charged particles. Our study was designed to determine if an obsolete commercial electrostatic particle collection device could be modified to both keep cells viable and to deposit different types of PM on the cells gently and efficiently. The modified device is named the Electrostatic Aerosol *in Vitro* Exposure System (EAVES) and it directly deposits PM on cells grown at an air-liquid interface, thus making it an alternative to existing methods of *in vitro* exposure for air pollution mixtures containing particulate matter that could potentially modify the state and composition of the PM.

## Materials and Methods

### Description of EAVES and its Operating Conditions

To create the EAVES, a TSI 3100 Electrostatic Aerosol Sampler (EAS) (TSI, Inc., St. Paul, MN) (TSI Inc. 2008) was modified to accommodate *in vitro* exposures of cells to PM (see Figure 2-1). First, the electronics were replaced with modern solid-state devices and cooling fans were added to help maintain the proper temperature when the device was operated. Second, a circular well was milled into the anodized aluminum collection plate to hold a titanium dish that contained tissue culture media during the exposure. This allowed the sample delivered by EAVES to directly deposit on cells maintained at air-liquid interface without significant interference from culture media, while providing nutrients from the basolateral side. The circular well is 0.6 cm deep, 3.5 cm in diameter, and is centered 3.75 cm from the corona-charging slot. A top-view and a side-view, centerline cross-section schematic of the charging and collection area of the EAVES is shown in Figure 2-2. The entire recessed well falls within the parabolic deposition pat-

tern of the particles collected, which facilitates relatively uniform particle deposition over the whole cell culture surface.

For all the experiments described here, the EAVES was housed in a tissue culture incubator held at 37˚ C. To prevent particle loss during the exposure, carbon impregnated silicon tubing was used to supply the EAVES with particle-containing air mixtures. Clean chamber air was mixed with $CO_2$ (to achieve 5 %) using a mass flow controller (Aalborg Instruments & Controls, Inc., Orangeburg, New York). The mixture was allowed to flow through EAVES for one hour at 1 L/min (including $CO_2$ at 0.05 L/min) to equilibrate the system.

Particle-containing samples from an outdoor atmospheric reaction chamber, or other test sources, also mixed with $CO_2$ (to achieve 5 %), were pulled through the device at a constant flow rate of 1 L/min; this flow was maintained by a mass flow controller (Aalborg Instruments & Controls, Inc., Orangeburg, New York).

## Growth and Exposure of Cell Cultures

A549 cells, a human epithelial lung cell line, which has retained several alveolar type II cell characteristics, were used in this study. A549 cells were grown on collagen coated membrane supports (Millicell R-CM; Millipore; Costar, Cambridge, MA) in complete media (F12K, 10 % fetal bovine serum, with antibiotics; Invitrogen, Carlsbad, CA) as described by Jaspers, *et al.* (1997). Before culturing the cells, the depth of the Millicell insert was shortened to 0.5 cm to allow for the upper edges of the tissue culture inserts to be leveled with the edge of the milled titanium dish in the EAVES. When the cells reached confluency, and several hours before exposure, the complete media were

replaced with serum-free media (F12K, 1.5 µg/mL bovine serum albumin, with antibiotics). Immediately before exposure in the EAVES, media were removed from the apical side of the membrane, while media remained in the basolateral side by contact with the porous membrane. This arrangement facilitates direct exposure of lung epithelial cells to the sample delivered by EAVES across an air-liquid interface without significant interference from the culture media, while providing the cells with nutrients from the serum free media from the basolateral side.

Four tissue culture inserts (0.69 $cm^2$ surface area each) were placed into the titanium dish with 1.5 mL of serum-free media that was then placed into the milled well in the anodized aluminum collection plate (see Figure 2-2). The titanium dish does not interfere with the media because it is a noncreative metal. The cells were exposed to various samples in the EAVES for one hour and then transferred back to individual wells of a 12-well tissue-culture plate containing 1 mL of fresh, serum-free media. In all exposures, A549 cells maintained in a regular tissue-culture incubator served as controls. Basolateral supernatants were collected nine hours post-exposure and stored at -20$^{o}$ C until analysis for cytotoxicity and inflammatory mediator production could be performed.

## Analysis of Cytotoxicity and Inflammatory Mediator Production

IL-8 proteins in the supernatant were measured using a commercially available ELISA (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. For the analysis of cytotoxicity, the basolateral supernatants were analyzed for the release of LDH, using a coupled enzymatic assay (TAKARA), according to the supplier's instructions (Takara Bio Inc., Japan).

### *Sample Stream Modification Tests*

In some experiments, samples of the test atmospheres were taken upstream and downstream of the EAVES to determine if passage through the EAVES modified the stream's reactive organic compound composition. The stream airflows were sampled through an aqueous o-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBHA) collection solution, which binds to carbonyl functional groups with high specificity. The airborne carbonyls and carbonyls on particles dissolve into the water and are derivatized by the PFBHA (Yu 1997). Samples were analyzed on a Varian CP-3800 gas chromatograph/mass spectrometer using both electron impaction (EI) and chemical ionization (CI) modes and with a flame ionization detector and using a 60 m x 0.32 mm inner diameter column with RTX-S stationary phase (Restek Corp.; Model Number 10242) with helium as the carrier gas. Methane was used as the chemical ionization reagent.

### *Sampling Efficiency Tests*

The collection efficiency of the EAVES was determined using 0.2 and 0.5 µm fluo-rescent-labeled, polystyrene latex spheres (PSL), (0.20 um Yellow-Green, YG; 0.5 um Red, polychromatic, PC, Fluoresbrite Microspheres, Polysciences, Inc., Warrington, PA). The size and concentration of these PSL particles at the inlet and the outlet of the EAVES were measured using a Scanning Mobility Particle Sizer (SMPS) (Model 3936L25, TSI Inc., St. Paul, MN). The sheath flow rate of the differential mobility analyzer (DMA) was set to 2.0 L/min, and the aerosol sample flow rate was set to 0.3 L/min. These settings allow the instrument to measure particles with midpoint diameters ranging from 19 to 882 nm. The SMPS was used to measure the air stream leaving the sample outlet of the

EAVES with the device powered on and off to evaluate the total collection efficiency of the device.

Using a setup similar to that described by Sioutas, *et al.* (Sioutas 1994; Sioutas 1999), the PSL spheres were nebulized from diluted aqueous solutions into a flask where they were mixed with clean, humidified air before entering a charge neutralizer (TSI Inc., St. Paul, MN). Once neutralized, the PSL spheres were mixed in a 20-liter glass jar to facilitate sampling.

PSL particle deposition on cell-free tissue culture membranes was visually analyzed with a black light, and quantitatively analyzed by fluorescence spectrophotometry. Samples for quantitative analysis were prepared by carefully removing all of the particles from the walls and rim of the tissue culture inserts before rinsing the inserts with 5 mL of ethyl acetate to dissolve the PSL spheres, releasing the florescent dye into solution. The ethyl acetate rinse containing fluorescent dye from the deposited PSL spheres was analyzed using a spectrophotometer (Hitachi-High Technology Corporation, F4500 Fluorescence Spectrophotometer, Tokyo, Japan) at optimized excitation and emission readings. Calibration curves were created for each particle size to be used in these tests. The fluorescence readings obtained from the cell culture inserts collections were compared to the standard curve appropriate for the particle size used in the test, resulting in a value for the total particle mass collected on each insert.

### *Tests of Diesel Exhaust Exposure*

Diesel exhaust was collected from the tailpipe of a 1980 Mercedes-Benz model 300SD operated with commercially available diesel fuel. The car was warmed up under

normal idling conditions. Without a load, the engine was throttled up to approximately 2700 rpm, with an engine temperature of 90°C, and held there throughout the duration of sampling. DE was collected from the vehicle in a large outdoor smog chamber (120 $m^3$ Teflon film chamber, see Figure 2-3) before exposure (Jeffries 1976; Jeffries 1985; Doyle 2004; Lee 2004; Doyle 2007). The vehicle's exhaust was used to pre-heat the delivery line until the temperature of the exhaust, measured at the chamber, was 125°F. DE was injected into the smog chamber until the particle mass concentration in the chamber was approximately 4 $mg/m^3$, as measured by SMPS. Size distribution and mass concentration of the aerosol entering and leaving the EAVES device were also monitored by SMPS.

### Statistical Analysis

All data are presented as the mean $\pm$ standard error from the mean (S.E.M) and expressed as fold increase over the same measurements performed on a set of cells maintained in the incubator throughout the exposure period. Data were analyzed using an unpaired Student's t-tests and differences were considered significant when $p$ was less than 0.05.

## Results

### Testing the Effect of EAVES Conditions on Cell Viability

When operated with a corona current setting of 1.5 µA at a sample rate of 1 L/min, the corona wire of the EAVES, which is used to charge the particles, produces an average of 60 ppb of ozone in the exhaust air after an hour of operation. Besides this ozone exposure there is the possibility that the electrical field applied in the sampler to

cause particles to precipitate might adversely affect cells in the device. To evaluate these potential problems, the EAVES was tested with two sets of cells using particle-free, clean air. In the first test, the electrical field was turned off for one hour and in the second, was turned on for one hour. The inflammatory response and cytotoxicity of the two sets of cells were measured following exposure. Neither LDH nor IL-8 (Figure 2-4) release was statistically different from cells maintained in the incubator for the entire exposure period, suggesting that the ozone produced and the precipitating field do not cause significant adverse effects on A549 cells.

### Effects of EAVES Conditions on Sample Stream Chemical Composition

As indicated above, the corona wire produces a small amount of ozone that could conceivably react with components of the exposure air stream and alter its chemical composition. To determine whether the chemical composition of the exposure air stream was altered by the charging mechanism and the ozone formed in the process, diesel exhaust (DE) was collected in a Teflon bag and allowed to flow through the EAVES at one liter per min. As described in Methods section, PFBHA samples were taken at the inlet and outlet of the EAVES, and were analyzed with GC/MS to determine if there were any changes in chemical composition of the air stream. The chromatograms in Figure 2-5 show the analysis of a DE sample before and after passing through the EAVES. The carbonyls readily identified by GC/MS from the DE in both samples were acetaldehyde, acetone, acrolein, methyl ethyl ketone, methyl vinyl ketone, crotonaldehyde, and benzaldehyde. As is apparent in Figure 2-5, the magnitudes of the peaks are the same in both chromatograms, suggesting that no chemical reactions that produced carbonyls or

that reacted with carbonyls occurred as the exposure air stream passed through the device.

To determine whether *de novo* particle production occurs from the ozone produced by the charging wire, an 8.9 ppbC mixture of a highly reactive VOC, d-limonene, known to produce secondary organic aerosols rapidly, was allowed to flow through the device. Measurements were taken using the SMPS at the inlet and the outlet of EAVES. The SMPS data (not shown here) indicated that there was no observable particle production due to the ozone from the corona wire reacting with the d-limonene flowing through the EAVES device.

### Total Particle Deposition in EAVES

Commercially available PSL spheres with known concentration and diameter were used to test the EAVES collection efficiency. Aerosolized PSL spheres were allowed to flow through the EAVES, either with or without the electrical field turned on. While the electrical field was turned off, no particle retention was apparent based on the SMPS data, meaning that no significant particle deposition occurred within the device. When the electrical field was turned on, however, SMPS measurements indicate that 90 % of the particles that entered the device were deposited, and 10 % exited through the outlet. The particle collection efficiency was determined to be approximately 90 % for all particles between 19 and 882 nm, representing 98 % of the total mass passing through the device. The SMPS data are shown in Figure 2-6 as two histograms of number in each size range and illustrate the collection efficiency on the total collection plate of EAVES, both with power on (light gray), and power off (black).

## Particle Deposition onto Tissue Culture Inserts

Efficiencies of particle deposition directly onto the cell culture inserts were determined by both visual and quantitative analysis of the PSL spheres. Each cell culture insert was rinsed with 5 mL of ethyl acetate to dissolve the deposited PSL spheres, releasing the fluorescent dye into solution. These were subsequently analyzed with a fluorescence spectrophotometer and the results are presented in Table 2-1. The 0.2 μm diameter PSL spheres had an average collection efficiency of 35.16 ± 9.32 percent, with an average of 1.96 μg ± 0.50 deposited per cell culture insert. The 0.5 μm PSL spheres had an average collection efficiency of 47.04 ± 9.84 percent with an average of 4.11 μg ± 0.84 deposited per tissue culture insert. To determine potential toxicological responses induced by the static charge on the particles, the PSL spheres were allowed to deposit onto A549 cells under normal operating conditions. Figure 2-7 shows that the static charge on the inert PSL spheres did not induce significant IL-8 release by the A549 cells.

## Effects of Exposure to Fresh Diesel Exhaust using EAVES

The data described thus far demonstrate that the conditions created by EAVES do not cause any pro-inflammatory or cytotoxic effects, that particles efficiently deposit on epithelial cells *in vitro*, and that the chemical composition of DE is not likely to be altered by the EAVES. In this test we determine if cells exposed to DE in EAVES produce measurable effects similar to those observed using other *in vitro* exposure methods (Steerenberg 1998; Boland 1999). With the EAVES turned on and sampling 2.3 mg/$m^3$ DE for 1 hour at a flow rate of 0.95 Lpm (total sample volume 56 L), markers of inflammation and cytotoxicity in A549 cells were significantly enhanced. The diesel particles were approximately 0.2 μm in size. From the deposition measurements conducted with

the PSL microspheres shown in **Error! Reference source not found.**, we can estimate that each tissue culture insert received approximately 2.64 ± 0.66 µg (4.18 ± 1.04 µg/cm$^2$) of diesel particles. Figure 2-8 shows that, when cells were exposed with the EAVES turned on, LDH and IL-8 production were significantly higher than when the EAVES was turned off. Exposures to DE with the EAVES turned off did not induce any significant change in IL-8 or LDH production as compared to the incubator control.

## Discussion

Traditional methods of *in vitro* particle exposure do not deposit particles in their original state directly onto cells, or they are entirely inefficient for deposition of fine and ultra-fine particles. Methods that re-suspend particles in solution may change the composition of the particles by losing the VOCs partitioned to the surface of the particles or by altering the surface characteristics. These collection methods can also alter the size distributions of the particles, leading to nonrealistic exposures.

As described here, the EAVES system overcomes many of the shortcomings of the traditional methods without introducing new ones. Deposition of the particles onto the surface of cells grown on tissue culture inserts in EAVES is based on deflection of electrically charged particles once they are subjected to an electric field. This methodology has been used extensively in the sampling and measurement of fine particles, as in the SMPS sampler used in this study, and the charging and collection mechanisms have been well studied (Hinds 1999; Mainelis 2002).

Our tests with human lung cells demonstrate that no significant cytotoxicity or inflammatory mediator production occurred to cells exposed in the EAVES with clean air sampling, with or without the electric field applied. Likewise, there were no responses to the low ozone concentration produced by the corona wire in the EAVES (60 ppb). Neither the very small electrical charges nor the low deposition velocity (0.763 cm/s) of biologically inert PSL particles deposited on the cells in EAVES caused a significant inflammatory response. Our tests demonstrate that even for a very reactive VOC like d-limonene, no detectable reaction occurred during air sample passage through the device, nor was any SOA formation apparent. Thus, there was no *de novo* production of particles. Furthermore, exposure to DE gas and particles when the EAVES charging and collection fields were turned off---and therefore no particle deposition was occurring---also did not induce significant inflammatory mediator production. The small total sample volume of only 56 L of the air stream could cause this lack of gas-only responsiveness. It could also be due to the low ozone concentration produced by the device (60 ppb). Previous studies using the smog chamber exposure system required four to five hour exposures (at 0.95 L/min) to elicit a significant response from gaseous pollutants (Doyle 2004; Sexton 2004; Doyle 2007). The short exposure time in EAVES attests to the effectiveness of EAVES' high efficiency in providing sufficient dose to induce a significant toxic response from the cells. Consequently, the inflammation and cytotoxicity measured in cells exposed to DE with the EAVES turned on can be attributed to the particulate fraction of the DE emission.

Although data shown here indicate that EAVES has the capabilities of 98 % mass collection efficiency over the entire plate, it was important to determine whether the EAVES device is capable of depositing a range of sub-micron particles over cell culture inserts and thus onto the cell monolayers. Deposition analysis using different sized PSL spheres showed that particles deposit efficiently over the cells, with about 84 – 87 % of the mass depositing directly onto the tissue culture insert, thus resulting in an efficient exposure to sub-micron particles. To evaluate how even the deposition was, each of the cell culture insert mass was calculated separately for each membranous support and shown to have similar mass deposition.

To test the EAVES with a realistic PM-containing atmospheric sample, it was necessary to measure effects induced by exposure to PM samples that had also been accessed with other toxicity measurements. There are many studies demonstrating that exposure to DE using liquid re-suspension will induce the production of inflammatory mediators, such as IL-8 (Bayram 1998; Boland 1999; Abe 2000; Knebel 2002; Mazzarella 2007). Our data here demonstrate that cells exposed to DE in the EAVES with the power turned off did not exhibit any change in inflammatory response over that of the control. Considering our measured deposition using the SMPS, which demonstrated that no significant particle mass precipitated when the device is turned off, these results were not surprising. In contrast, cells exposed to the same DE mixture with the EAVES electrostatic fields turned on produced a three-fold increase in both cytotoxicity and inflammatory mediator production as compared to the control. As indicated above, similar results have been obtained in studies using DE particles re-suspended in liquid medium

(Bayram 1998; Boland 1999; Abe 2000; Knebel 2002; Mazzarella 2007). Typically re-suspension studies, however, require between 50 and 400 μg/mL of DE particles re-suspended in a medium to detect any significant inflammatory responses (Bayram 1998; Boland 1999; Abe 2000; Knebel 2002; Mazzarella 2007). Considering the deposition efficiency assessed using the PSL spheres, the approximate mass of PM deposited onto each tissue culture insert during the DE exposure experiments with EAVES was approximately 2.64 ± 0.66 μg of DE particles (4.18 ± 1.04 μg/cm$^2$). These data indicate that exposure to DE particles using the EAVES system produces significant adverse cellular effects at the same or even lower particle concentrations and may therefore be more sensitive than traditional *in vitro* particle exposure methods.

Taken together, these data demonstrate that a well-designed, and carefully operated electrostatic particle collection device, such as the EAVES described here, is an excellent alternative to conventional exposure methods for *in vitro* exposures to air pollution mixtures containing particulate matter. This technology will allow investigators to expose cells *in vitro* to particle-containing air streams without the need to collect and re-suspend particles in a liquid before cell exposure. The EAVES approach is not only more efficient, it avoids the possibility of altering the physico-chemical characteristics of particles before exposure, thereby giving a more realistic evaluation of the possible human health effects of inhaled particulate matter.

## Figures and Tables

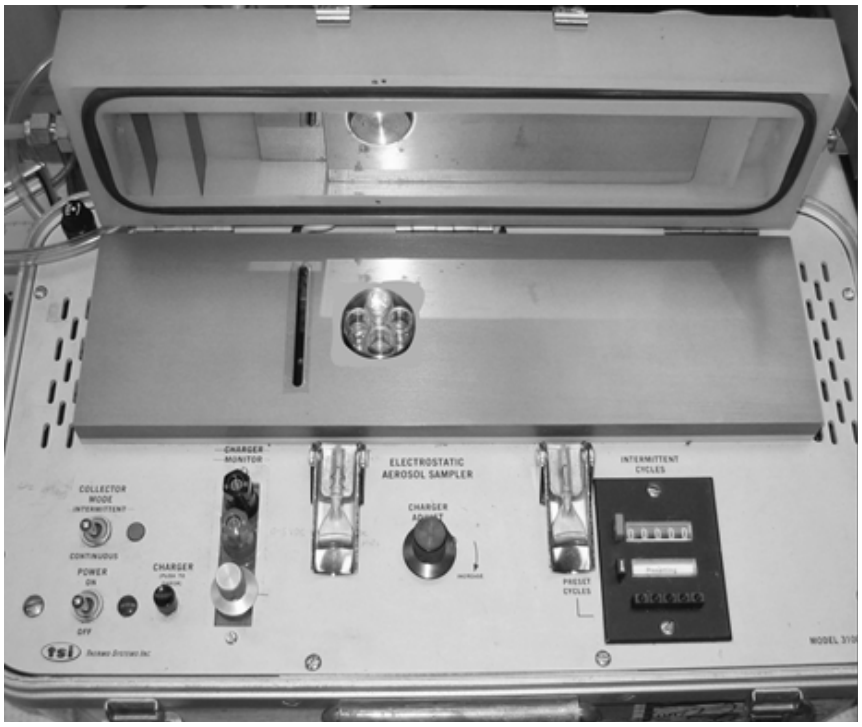


Figure 2-1. Top view image of the Electrostatic Aerosol *in Vitro* Exposure System (EAVES) holding the four Millicells

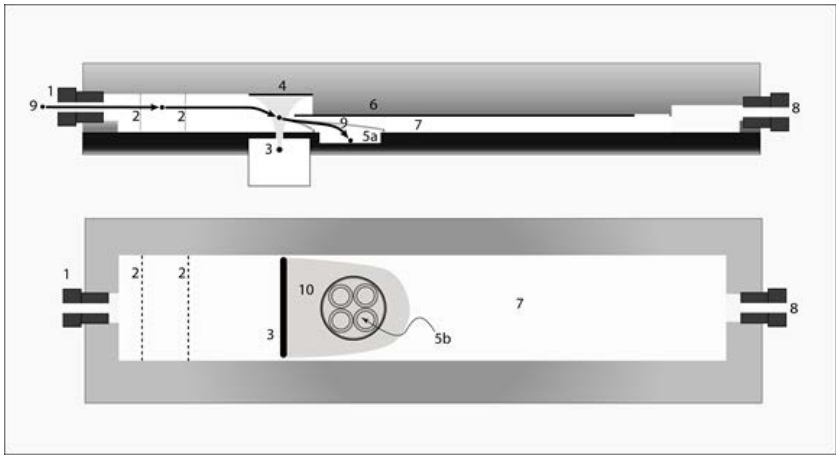


Figure 2-2. Side view schematic of the EAVES with key components labeled. (1) inlet, (2) diffusers, (3) corona wire, (4) corona power plate, (5) *in vitro* cell holding well, (6) repellant plate, (7) particle collection plate, (8) outlet, (9) particle pathway. Air flow through the system is from left to right.

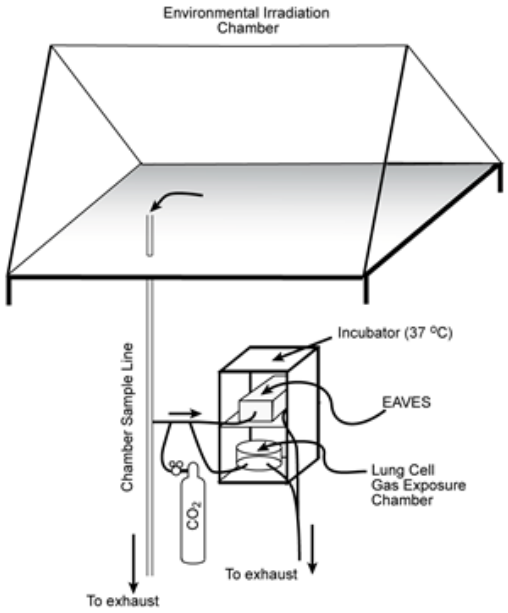


Figure 2-3. Schematic of outdoor environmental irradiation chamber, through-the-roof sample lines, in-laboratory incubator holding the *in vitro* exposure systems.
Sample air is blended with $CO_2$ to a concentration of 5 %.

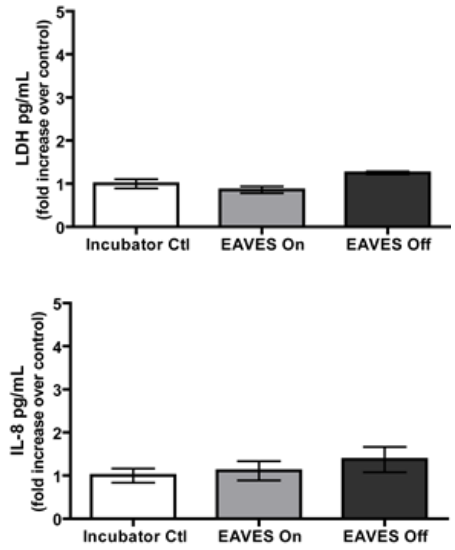


Figure 2-4. Effects of EAVES deposition field on cytotoxicity and inflammatory mediator production. A549 cells were exposed to clean air in the EAVES for one hour and tested for LDH and IL-8 release 9 hours post-exposure. Grey bars = EAVES turned on; black bars = EAVES turned off. LDH and IL-8 levels are expressed as fold increase over non-exposed incubator control measurements.

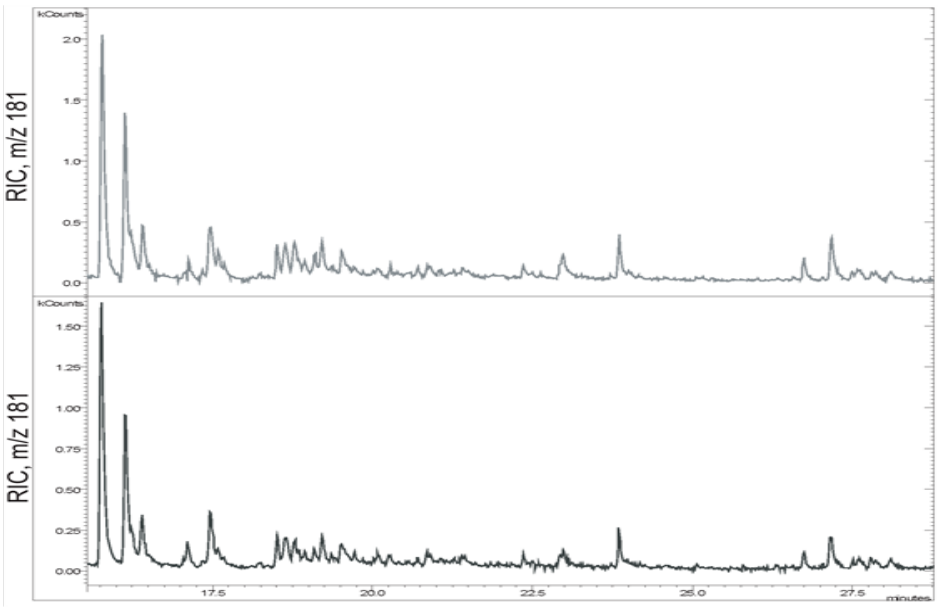


Figure 2-5. Reconstructed relative ion current of m/z 181 ion chromatograms of carbonyls detected by PFBHA-derivatization of diesel exhaust gases before (top) and after (bottom) passing through the EAVES.

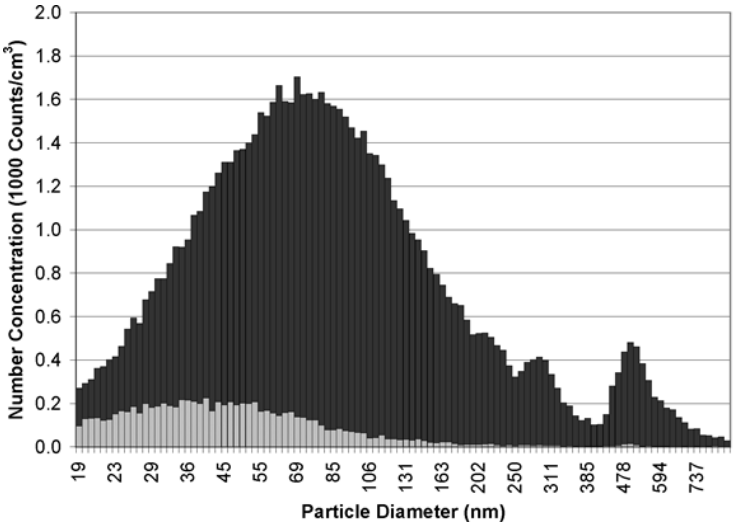


Figure 2-6. Histograms of particle number per size interval of particles entering and exiting EAVES. Black histogram = PSL spheres exiting the EAVES with the power off; Grey histogram = PSL spheres exiting the EAVES with the charging and deposition field on.

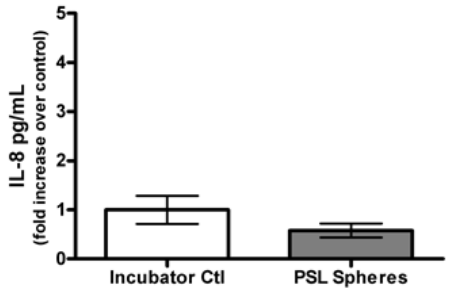


Figure 2-7. IL-8 release from A549 epithelial cells exposed to charged polystyrene latex spheres (PSL). Clear bar = incubator control; Grey bar = cells exposed to PSL spheres operating with the power applied to the charging plate. A549 cells were exposed in the EAVES for one hour and examined for IL-8 release 9 hours post-exposure. Data represent means + S.E.M.

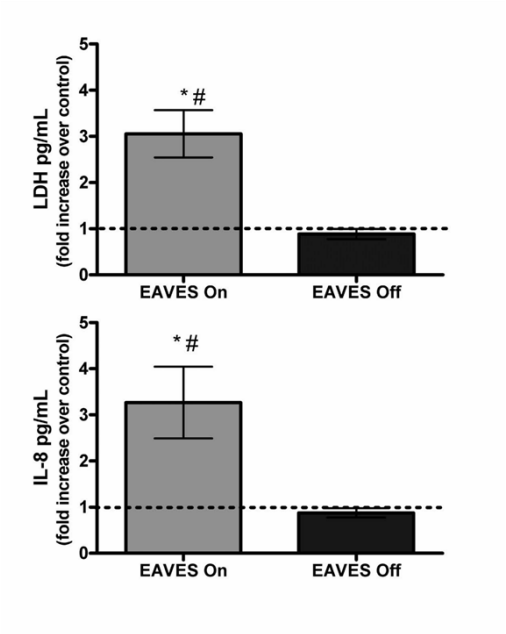


Figure 2-8. A549 epithelial cells were exposed to diesel exhaust in the EAVES for one hour and examined for LDH and IL-8 release 9 hours post-exposure. Grey bars = EAVES charging and deposition field on; black bars = EAVES power off. LDH and IL-8 levels are expressed as fold increases over non-exposed incubator control measurements. * statistically different from non-exposed incubator control; # statistically different from EAVES power off; $p < 0.05$.

Table 2-1. EAVES tests results for particle deposition using two different size fluorescent-labeled polystyrene latex spheres aerosolized in air. Sample duration was one hour.

| Particle Diameter (µm) | 0.198 | 0.513 |
|---|---|---|
| Concentration in Air ($mg/m^3$) | 1.71 | 2.36 |
| Deposition per Cell Culture Insert (µg ± 2σ) | 1.96 ± 0.50 | 4.11 ± 0.84 |
| Collection Efficiency (% ± 2s) | 35.16 ± 9.32 | 47.04 ± 9.84 |

*1. Previously published as:*

*de Bruijne, K., Ebersviller, S., Sexton, K. G., Lake, S., Leith, D., Goodman, R., Jetters, J., Walters, G. W., Doyle-Eisele, M., Woodside, R., Jeffries, H. E. and Jaspers, I .(2009) 'Design and Testing of Electrostatic Aerosol In Vitro Exposure System (EAVES): An Alternative Exposure System for Particles', Inhalation Toxicology,* ***21****:2 , 91 — 101*

# Chapter 3 : *In Vitro* Exposures in Diesel Exhaust Atmospheres: Resuspension of PM from Filters Verses Direct Deposition of PM from Air [2]

## Introduction

The purpose of this study is to illustrate potential problems with a widely used atmospheric particulate matter (PM) sampling and *in-vitro* exposure method for assessing a particular PM's ability to elicit toxicant-induced biological effects in cultured human cells. The majority of *in vitro* studies examining the effects of PM exposure have used an extractive technique based on collecting ambient PM on filters, resuspending the particles in a liquid medium, and then adding them to a cell culture (Bayram 1998; Boland 1999; Abe 2000; Knebel 2002; Bauling 2003; Dybdahla 2004; Mazzarella 2007). This method is referred to here as the "resuspended PM method". Yet, many studies of physical and thermal processes among PM and volatile and semi-volatile oxidized organic gases have shown that, in a reactive organic gas and PM system like the urban atmosphere, the exchange of mass between the gas and PM phases can be rapid and dynamic (Pankow 1997; Pankow 1998; Kamens 1999; Schwarzenbach 2003; Donahue 2006). The understanding that PM can gain mass (and acquire toxic properties) from gas-to-particle processes seems to be accepted by toxicologists and health-researchers (Mar 2000; Jang 2006; Baltensperger 2008; Franklin 2008; Gaschen 2010; McDonald 2010; Chen 2011; Godleski 2011; Zhou 2011). The concept that this mass is likely labile and can be lost

from the PM when the gaseous environment is changed is missing from these studies (Steerenberg 2006; Steenhof 2011). As an example of how fast mass may leave SOA aerosols, Kamens and Coe (Kamens 1997) measured effective rate constants for off-gassing of fluorene and phenanthrene for diesel soot particles of 0.51 $s^{-1}$ and 0.37 $s^{-1}$ (loss of 95 % of on-particle mass in 6-8 s if gas removed). For pinonaldehyde, a seven carbon product of alpha-pinene oxidation, a rate constant for off-gassing from PM of 1.8 $s^{-1}$ was used to successfully model SOA in outdoor chamber studies (loss of 95 % of on-particle mass in < 2 s) (Kamens 1999).

Previously, we have developed a direct-sampling system capable of producing PM-only-exposures to cultured human lung cells while maintaining the equilibria among gases and particles (Doyle 2004; Sexton 2004; Doyle 2007; de Bruijne 2009). This direct-sampling system has been used to demonstrate the evolution of gas-phase toxicity in photochemically aged urban-like environments (Doyle 2004; Sexton 2004; de Bruijne née Lichtveld 2007; Doyle 2007; de Bruijne  née Lichtveld 2008; Ebersviller 2008; de Bruijne 2009). and recently has been used to demonstrate the rapid transfer of gaseous toxicity to initially non-toxic PM, making this PM then toxic to exposed lung cells (Ebersviller 2012a; 2012b). Other researchers have begun to develop direct methods for PM exposures (Morin 1999; Aufderheide 2000; Seagrave 2007; Savi 2008; Sillanpää 2008; Volckens 2009; Gaschen 2010).

In this study, we compare *in vitro* responses produced by exposure to resuspend-ed PM, which was collected on a filter from a reactive gas-PM air stream with responses

produced by exposure to the PM that was directly deposited on cells, while still in equilibria, from the same reactive gas-PM air stream. We hypothesize that the separation of phases and post-treatment of filter-collected PM significantly modifies the composition and therefore the toxicity of the collected PM, resulting in a distorted view of the potential PM health effects.

## Approach

To test the hypothesis, we created controlled test environments in an outdoor sunlit chamber by combining whole diesel exhaust (gases and PM) from either of two vehicles (old and new) with an urban-like, complex volatile organic carbon compound (VOC) mixture. To modify the injected primary PM to be like urban atmospheric PM by permitting secondary organic aerosol (SOA) growth, these mixtures were exposed to natural sunlight from sunrise to sunset. Each test environment was sampled with both of our direct-exposure systems, and filter samples of PM were taken at the same time for processing by the resuspension technique and subsequent exposure of lung cells to the resuspended PM. We have shown previously that gas-phase oxidative chemistry is a major source of gas-phase toxicity in these environmental systems. We have also demonstrated that this gas-phase toxicity can and does move to the particle-phase, increasing any inherent or primary PM toxicity (Ebersviller 2012a; 2012b). This study, therefore, focuses only on the PM-only DEM to compare with a comparable exposure for the PM-only resuspension method.

## Materials and Methods

### Overview

The schematic in Figure 3-1 shows the experimental and exposure setup. The Gillings Outdoor Irradiation Chamber is located on the roof of the Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC. The triangular-cross-section chamber has a volume of 120 $m^3$ (7.4 m by 6.0 m by 5.4 m high) enclosed in Teflon film walls. The chamber is coupled to the chemistry and biology laboratory on the top floor via parallel thermally-insulated sample lines through the roof (de Bruijne 2009; Ebersviller 2012a; 2012b). In addition, provisions for filter collection and for difficult-to-sample species (such as carbonyls) are available immediately under the chamber floor (on the roof of the building). In the top-floor laboratories, analytical instruments and *in vitro* exposure system are connected to the chamber, preserving both gases and particles together as they are conveyed directly to the cells during exposure.

The rooftop chamber also has a stainless steel exhaust-transfer manifold that can be connected to the tailpipes of vehicles operated in the parking lot adjacent to the building. A venturi-driven dilution blower as described by McDonald *et al.*(2004) entrains, dilutes, and delivers hot tailpipe emissions directly into the rooftop chambers.

### Diesel Exhaust and Urban VOC Injections into Outdoor Chamber

To generate two types of PM in complex VOC-reactive atmospheres, diesel exhaust (DE) was injected into the chamber in the dark from either a 1980 Mercedes-Benz model 300SD that lacked emission controls or a 2006 Volkswagen Beetle compliant with EPA's vehicle emission standards. Both vehicles were operated with commercially avail-

able low sulfur diesel fuel. Given the differences in age and emission controls, the two vehicles produced exhausts with different compositions. These vehicles represent real on-road sources that would be encountered in the real world.

Each vehicle was allowed to idle until the vehicles' temperature gauges reached normal operating conditions. The engines were then throttled to approximately 2700 revolutions per minute and the venturi-driven dilution blower was used to deliver the DE to the roof-top chamber. DE was injected into the chamber until the particle concentration reached ca.1.2 mg/$m^3$, as measured by a scanning mobility particle sizer (SMPS - model 3936L25, TSI, Inc., St Paul, MN). During the exposure and sampling period following the generation of the urban-like complex atmosphere (described below), particles in the range of 0.02 to 1.0 µm were measured with the SMPS. Diesel-exhaust-only chemical conditions are not very reactive, and thus urban-like VOCs must be added to create a more typical oxidative environment. A VOC mixture comprised of 54 VOCs (SynUrb54; based on an average of EPA's analysis of air samples from 39 cities) was added at a total concentration of 2.0 parts per million carbon (ppmC).(Sexton 2004; Ebersviller 2012b)

Photochemically reacting the DE+VOC mixtures creates multiple generations of oxidized daughter products *in situ*, many of which are unavailable commercially. For each vehicle, the contents of the chamber were allowed to react in natural sunlight from sunrise to sunset to create oxidized urban-like test atmospheres *in situ*. All filter sampling and direct exposures described here took place in the dark, after sunset.

## Analytical Methods

The chemical and physical monitoring of the chamber was the same as that described by Ebersviller *et al.*(2012b) and will only be briefly summarized here.

Ozone was measured with a ML9811 series Ozone Photometer (Monitor Labs, Englewood, CO). Nitrogen oxides were measured with a ML9841 series $NO_x$ Oxides of Nitrogen Analyzer (Monitor Labs, Englewood, CO). All chamber data were recorded with one-minute resolution using a data acquisition system connected to a computer. Prior to each experiment, the $O_3$ and $NO_x$ meters were calibrated by gas-phase titration using a NIST standard NO tank and stable O3 source.

A Varian 3400/2000 GC/MS, with both a MS and flame ionization detector to identify and quantify the species in each mixture, was used to continually monitor gas-phase hydrocarbon compositions of the exposure atmospheres. A non-reactive tracer, carbon tetrachloride, was injected (2.5 µl) at the beginning of each experiment to permit analysis of the dilution rate of the chamber. A Varian 3800 GC, with an electron capture detector, was used to monitor the gaseous tracer concentration.

Modified mister samplers, similar to those described by Seaman *et al*.(2006), were used to determine the carbonyl content of the test atmospheres. Sampled carbonyls were detected with a O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBHA) method (Yu 1995; Yu 1997; Liu 1999; Seaman 2006). The PFBHA derivatives were analyzed by gas chromatography/mass spectrometry (GCMS) on a Varian 3800 GC/ Saturn 2200 Ion Trap MS.

## Growth of Cell Cultures and Exposure Pretreatment

For both *in vitro* methods, the A549 cell line, which is derived from a non-small-cell adenocarcinoma of the human lung, was used to assess the biological effects of the ambient PM mixture (Lieber 1976; ATCC 2011). The A549 human epithelial cells were grown on collagen coated membrane supports (Millicell R-CM; Millipore, Cambridge, MA) in complete media (F12, 10 % fetal bovine serum with antibiotics [0.01 % penicillin/streptomycin] (Invitrogen, Carlsbad, CA) as previously described (de Bruijne 2009). Several hours before exposure (after the cells reached confluency), the complete media was exchanged and replaced with serum-free medium (BSA) (F12K, 1.5 µg/ml bovine serum albumin with 0.01 % penicillin/streptomycin; Invitrogen, Carlsbad, CA). Immediately before exposure apical media was removed from all exposure and controls (Doyle 2004; Sexton 2004; Doyle 2007; de Bruijne 2009; Rager 2011; Ebersviller 2012a; Ebersviller 2012b). All exposures with the direct sampler were performed at air liquid interface. Due to the method itself, ALI exposures are never possible using resuspended PM since you have to instill the medium plus PM to conduct the exposure.

We recognize that an immortalized cell line lacks the individual-to-individual susceptibility variations of primary lines. The goal of this work was not to compare interpersonal variation in responses, but to focus on the comparison of the two exposure methods. The immortalized cell line was, therefore, ideally suited for this work.

## Resuspension Exposure Method

Chamber air was drawn through pre-weighed Teflon membrane filters (2.0 µm pore size, 47 mm diameter; Pall Corporation, Port Washington, NY) at 17.5 liters/min for

three hours to collect particles. After collection, the filters were re-weighed on a micro-balance (Mettler Toledo MX5, Columbus, OH) and then placed in a 50 mL centrifuge tube containing BSA media (Bayram 1998). The tube was vortexed and sonicated to remove PM from the filter (as determined by visual inspection). Filters were then dried in clean petri dishes at room temperature, and re-weighed. To determine the total particle mass that was resuspended in BSA, the initial mass of the filter was subtracted from the difference between the post-collection filter weight and the dry weight after extraction in BSA. Using these methods, approximately 67 % of the PM was removed from the filter surface. The mass removed from each filter was used to calculate doses for *in vitro* exposures. Resuspension controls were created by treating blank filters with the extraction procedure. All tubes containing resuspension solutions were covered with foil and stored at −20°C. All resuspended-PM exposures were conducted two months after collection.

To start the resuspension-PM *in vitro* exposures, the resuspended PM was added to the apical side of the cellular membrane and allowed to incubate for nine hours prior to isolation of RNA. All solutions were prepared to ensure that the volume instilled was the same for all resuspension exposures (274 µl; controls were exposed to the same volume of medium without the particles present). Resuspended-PM exposures were performed at 2.6 µg/cm$^2$ (the same amount delivered directly to the cells by the direct exposure sampler) (de Bruijne 2009). An additional resuspended-PM exposure was performed at 42.5 µg/cm$^2$ for a comparison with exposure concentrations used by other researchers (Bayram 1998; Boland 1999; Abe 2000; Mazzarella 2007).

## Direct Deposition Exposure Method

Direct deposition of PM (ranging from 0.02 to 10 µm in diameter) on cultured human lung cells is accomplished by applying a charge to the PM in a flowing air stream and then using a repelling electric field above the cells to rapidly, but gently, deposit the PM directly onto the air-liquid interface of the cells (de Bruijne 2009). The direct-exposure sampler used in this study is called the Electrostatic Aerosol *in Vitro* Exposure System (EAVES). This sampler operates in a tissue culture incubator maintained at 37 °C, as described previously (de Bruijne 2009). Prior testing of the direct exposure EAVES sampler has demonstrated that in an hour long exposure: (1) no significant cytotoxicity or inflammatory mediator production occurs from cellular exposure to the electrical field; (2) no cellular response was observed due to the low ozone concentration produced by the corona wire; (3) no cellular response was observed due to the electrical charges added to the PM; (4) no secondary organic aerosol formation was observed from very-reactive VOCs flowing through the sampler; (5) no cellular response was observed following exposure to toxic gases without PM present; and (6) no response was observed when mixtures of toxic gases and PM pass through the sampler without the deposition field on, while a significant response was observed from the same mixture when deposition voltage field is applied (de Bruijne 2009). Thus, the EAVES sampler can expose cells to PM while maintaining equilibria with the gaseous components of the atmosphere being sampled with no observable response caused by the operation of the device.

In this study, the EAVES sampler was operated in exactly the same manner (with no disruption of the equilibria between the gas and particle phase) as described by Ebersviller *et al.*(Ebersviller 2012a; Ebersviller 2012b). The duration of each exposure was ca. one hour.

## Analysis of mRNA levels

Total RNA was isolated from the cells using Trizol (Invitrogen, Carlsbad, CA) nine hours post-exposure and stored at -20 °C until analysis, per the manufacturer's instructions. Evaluation of inflammatory response was measured with real-time, reverse-transcriptase polymerase chain reaction (RT-PCR) for expression of Cyclooxygenase 2 (COX-2) and Interleukin 8 (IL-8) as described previously (Jaspers 2001). COX-2 and IL-8 mRNA levels were normalized against a housekeeping gene (β-actin mRNA) and reported as "fold-increase" over control. Each sample was tested for mRNA expression with three replicates.

## Statistical Analysis

All data are presented as the mean ± standard error from the mean and expressed as fold increase over the same measurements performed on a set of control cells maintained in the incubator throughout the exposure period. Data sets were analyze and compared using unpaired Student-t test with Welch's correction. Differences were considered significant when $p$ was less than 0.05.

## Results

### Chemical Characterization of Chamber Contents

As the chamber contents were irradiated by sunlight most of the primary hydrocarbons oxidized, thereby producing carbonyl-containing secondary organics. These types of atmospheric reactions have been extensively studied and described in the literature (Finlayson-Pitts 1999; Atkinson 2000; Sexton 2004). Figure 3-2 contains two chromatograms showing chamber hydrocarbon concentrations. The top chromatogram shows VOCs detected after injection (but prior to sunrise) from the mixture of emissions from the 1980 Mercedes vehicle and 2.0 ppmC of SynUrb54. The bottom chromatogram shows VOCs detected after sunset following day-long irradiation. The speciation data show that initially injected hydrocarbons have mostly been consumed by the end of the day, resulting in a lower number of peaks, and a smaller magnitude for the peaks that remain (2b). The numbers labeling the peaks correspond to the species listed in Table 3-S1.

Table 3-S1 summarizes the concentrations for each compound identified in the test atmosphere before sunrise and after sunset. This analysis is included to demonstrate that photochemical-transformation of the test atmosphere occurred. Some of the major compounds initially present included: isopentane, benzene, toluene, m-xylene, and 1,2,4-tri-methyl-benzene. By the end of the day, isopentane and m-xylene had been completely consumed, while the concentrations of benzene, toluene, and 1,2,4-tri-methyl-benzene decreased by 77.7, 70.1, and 95.0 %.

Figure 3-3 contains a selective-ion chromatogram of the derivatized carbonyl samples taken in the dark (after day-long irradiation) from the 1980 Mercedes/SynUrb54 mixture. As stated above, the samples were taken with a mister apparatus and no attempt was made to separate the gases and PM prior to collection in the derivatization solution. Therefore, the chromatograms represent a mixture of the gaseous and PM-bound carbonyls that were present in the chamber. The numbers above the peaks in Figure 3-3 are identified by compound name in Table 3-S2. Compounds that were present in the photochemically oxidized test atmosphere are formaldehyde, acetaldehyde, methyl ethyl ketone, 2-pentanone, 2-hexanone, glyoxal, and methylglyoxal.

We also determined the chemical composition of the resuspended PM material to help understand any observed differences in response between the exposure methods. Chromatograms in Figure 3-4 represent the same type of selective-ion-filtered chromatograms of derivatized carbonyls present in Figure 3-3, and demonstrate that there were almost no carbonyls detected in the PM samples. The peaks that did appear included: formaldehyde, the unreacted PFBHA reagent, and the internal standard (fluorobenzaldehyde), all of which were measured in the water and media blanks. Thus, the resuspension liquid did not contain any carbonyls from the filter-sampled PM.

## Biological Effects of Exposures

Figures 3-5 and 3-6 represent COX-2 and IL-8 expression induced by the two exposure methods (resuspension and via direct deposition). Figure 3-5 shows the response to exposures conducted using the 1980 Mercedes with SynUrb54, and Figure 3-6 shows the results from the 2006 Volkswagen with SynUrb54.

Figure 3-5 shows that direct exposure of cells to the test atmosphere using direct deposition did induce a significant increase in both COX-2 and IL-8 expression. No response was observed following exposure to the resuspended PM at the same exposure concentration. A similar pattern was observed for the tests using the 2006 Volkswagen with SynUrb54 (shown in Figure 3-6). While exposure using direct deposition induced significant increases in gene expression a small decrease for COX-2 was observed from cells exposed to the same amount of PM by the resuspension method (2.6 $\mu g/cm^2$).

To facilitate comparison to resuspension exposure levels that are often used in the literature, we performed a resuspension exposure 16 times higher than those shown in Figures 5 and 6. Comparisons of the responses from cells exposed to 2.6 and to 42.5 $\mu g/cm^2$ of resuspended PM from the Mercedes with SynUrb54 are illustrated in Figure 3-7. The COX-2 expression was not increased over the control for either resuspension exposure. For IL-8 expression, however, there was a two-fold increase in response compared to the incubator control at the 42.5 $\mu g/cm^2$ resuspension exposure level.

## Discussion

The gas and particle test atmospheres created in this study were developed to model important chemical features needed to elicit toxicant-induced biological effects. For our study to successfully mimic the variety of gases and PM present in the ambient environment, we needed to create complex, urban-like mixtures of gases and PM that contained a large number of chemical species. Prior work with SynUrb54-only systems clearly shows that urban-like, gas-only atmospheres can create a large variety of gas-phase toxicants (Sexton 2004; Ebersviller 2012b). For the purpose of the comparisons

we are making here, it is sufficient to observe that: 1) chemical analyses of chamber contents show that many of the injected VOCs reacted and that a large variety of secondary and higher generation oxygenated organics (carbonyls and multi-functional carbonyls) were formed; 2) direct sampling of the PM from the chamber onto the cells led to exposure outcomes that showed significant response thereby demonstrating that the PM had toxic properties; and 3) therefore, it is reasonable to expect a positive response from other sampling methods and subsequent exposures to same type of cells.

Results by Ebersviller, et.al.(2012a; 2012b), using the same outdoor chamber and the EAVES sampler (in combination with a second gas-only exposure system), clearly demonstrated the transfer of gas-phase-produced toxicity to a non-toxic PM. The direct sampling and exposures that occurs in the EAVES sampler maintains the equilibria between the gases and particles in the exposure system, thereby preventing the loss of volatile species from the particle phase during the sub-second time it takes to put them on the cell surface. Thus, if toxic VOCs were partitioned to the PM, direct deposition sampling would deliver this PM carrying such toxic species to the cell surface, while these volatile species are likely lost in PM extracted with a filter and suspended in a liquid media.

To challenge the *in vitro* methods using emissions from two test vehicles provided a mechanism by which we could generate distinct test atmospheres, with different gas and PM compositions. The results indicated that, for the direct deposition exposures, both vehicles induced a 2-fold expression of COX-2, and the Mercedes produced twice

the expression of IL-8 than the Volkswagen. This shows that the direct deposition sampling system can see a difference in toxicity from different vehicle emissions and that emissions derived from a vehicle with fewer emission controls in place produces more toxicity.

We have shown the photochemically reacted test atmospheres to be highly complex mixtures of multiple generations of daughter products that represent an approximation of the complexity of an ambient urban environment. Further, these *in situ* generated test atmospheres contain unidentified carbonyls and other oxygenates (e.g., epoxides), often at low-levels that are not commercially available for any other type of testing. Thus, this test environment allows us to make a more "holistic" assessment of the effects of the urban gaseous and PM environment.

The EAVES sampler does not modify the chemical composition of the sample prior to the delivery of PM to the cellular surface, as documented by de Bruijne, *et al.*(2009). Therefore, all soluble and insoluble compounds surrounding the carbon core of the DE particle are retained and available to elicit a response from exposed cells. Likewise, PM size distributions are not modified.

Filter collection and extraction techniques are popular because of their ease of use and relatively low cost. Our data indicate that extensive sample handling and modification of the PM during the resuspension method (illustrated in Figure 3-1). Furthermore, when we compare the composition of the carbonyls present during the direct deposition exposures to those measured in the resuspension media, it is apparent that

the composition has been modified (Figures 3-3 and 3-4). Not only has the carbonyl composition been modified in the resuspension media, the carbonyl-containing species have completely disappeared. As reported by Cooney *et al.*, particles can be seen to agglomerate when resuspended in liquid (Cooney 2011). Using SEM, they showed the mode particle diameter of the resuspended DE increased relative to non-suspended DE. In addition, particles in liquid settle very slowly at these particle diameters, with only the largest particles likely to reach the cellular surface. This means that during a resuspension exposure not all the particles will deposit onto the cellular surface to cause a response prior to RNA collection, making it difficult to accurately determine the amount of PM delivered to the cells.

Direct sampling deposition and exposure elicited a significant response from both test atmospheres. Based on previous experiments using photochemically-reacted SynUrb54 we expected to observe an increase in biological response relative to controls (Sexton 2004; Ebersviller 2012b). All exposures using resuspended PM obtained during the corresponding direct exposure, however, failed to induce a positive response from cells at exposure concentrations equal to the direct method for either of the biological endpoints assayed. Although there was a small, but significant, decrease for COX-2 observed from cells exposed by the resuspension method it is likely due to a slight increase of cytotoxicity making the mRNA less stable.

For the resuspended PM, there was a small but significant response observed when 16-times the amount of PM mass was used for exposure. The observed response,

however, was still only about 50 % of that induced from the EAVES-exposed cells at 2.6 $\mu g/cm^2$. As described above, when using the resuspended technique we observed that the final material used in the resuspended exposure was missing toxicants from the test atmosphere (Figure 3-4). The many carbonyls present in the direct exposure (Figure 3-3), and the lack of any in the resuspended material (Figure 3-4), strongly suggests that the response from the larger applied resuspended-PM mass is not likely to be caused by the same toxicant as the direct exposure. Rather, any observed response maybe the result of inherent, nonvolatile, non-soluble components in the treated PM (e.g., metals) that persisted through the extensive modification during filter collection and resuspension stages (Volckens 2009; Ebersviller 2012b). Furthermore, differences in biological effect between the 'dirty' and 'clean' vehicles could be detected by the direct deposition exposures, but not by the resuspended exposures. These results taken together are biological evidence that the resuspended PM has lost an important source of toxicity.

The goal of this study was to determine if the separation of phases and post-treatment of filter-collected PM from a reactive gas-and-PM airstream would significantly modify the toxicity of the collected PM compared to that detected by direct exposure of the same PM. Chemical measurements during the photochemical aging process assured that primary hydrocarbons were consumed and that a large variety of gas-phase carbonyls and other oxidized products were produced, many of which are likely to partition to the PM (Robinson 2007; Ebersviller 2012b). The same analysis of resuspended PM, however, showed that no carbonyls or other oxidized organics, other than those in the water and media blanks, were present. This is chemical evidence that filter-

collected and resuspended PM has lost an important source of cellular toxicity. As stated above, neither chemical composition nor PM size distribution was modified prior to exposure by the EAVES sampler. Also, by exposing at an air-liquid interface the EAVES sampler delivers all PM, regardless of size, to the cellular surface. Moreover, with the EAVES sampler, we are able to reliably estimate the amount of PM that actually reached the cellular surface by using collection efficiencies measured during initial development of the method (de Bruijne 2009).

Extrapolating our test conditions to the ambient environment, we conclude that it is highly likely that the separation of phases and post-collection treatment of filter-collected PM significantly modifies the toxicity of the resuspended PM. These modifications may result in a distorted view of the potential health effects compared to those elicited by direct sampling and exposure to the PM. While researchers may be attracted to the potential for high-throughput studies with resuspension techniques, we have demonstrated that sample modifications by the loss of toxicity are likely to bias the results. We recommend that resuspension techniques, therefore, be used with caution, and suggest that results obtained may represent only a lower bound on PM toxicity.

## Supporting Information

In the supporting information section of this manuscript, the identifications of peaks and concentrations are listed in the tables for Figures 3-2 and 3-3. Table 3-1 is the hydrocarbon data corresponding to Figure 3-2 and Table 3-2 is the carbonyl data corresponding to Figure 3-3. This information is available free of charge via the Internet at http://pubs.acs.org.

## Figures and Tables

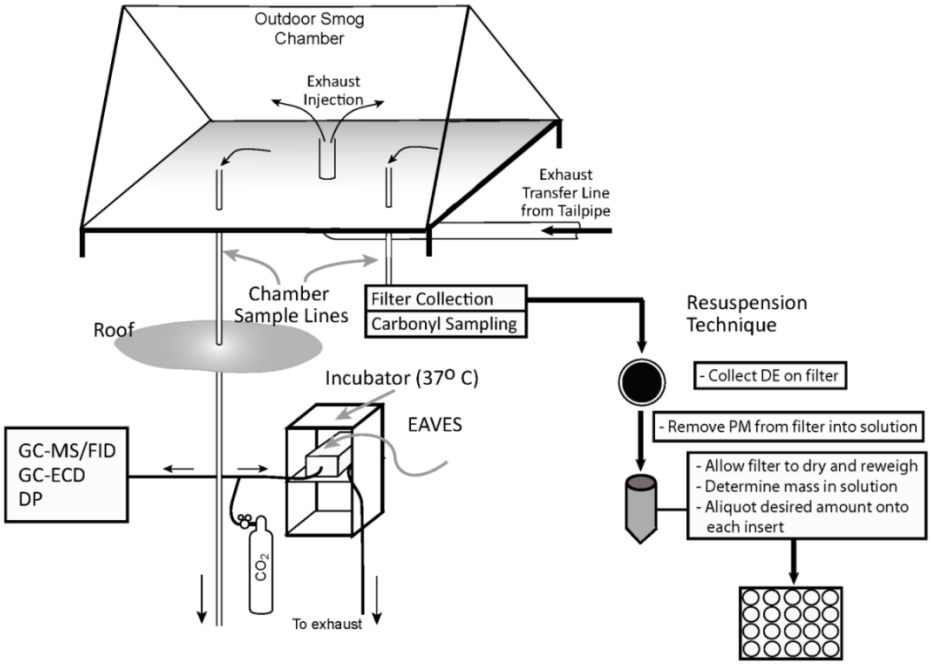


Figure 3-1. Schematic diagram of the outdoor smog chamber, the laboratory sampling systems, and the biological exposure system (EAVES). Filters were collected directly under the chamber floor and processed post-collection for the exposures to resuspended PM. The diesel exhaust sample injection lines into the chamber are also depicted.

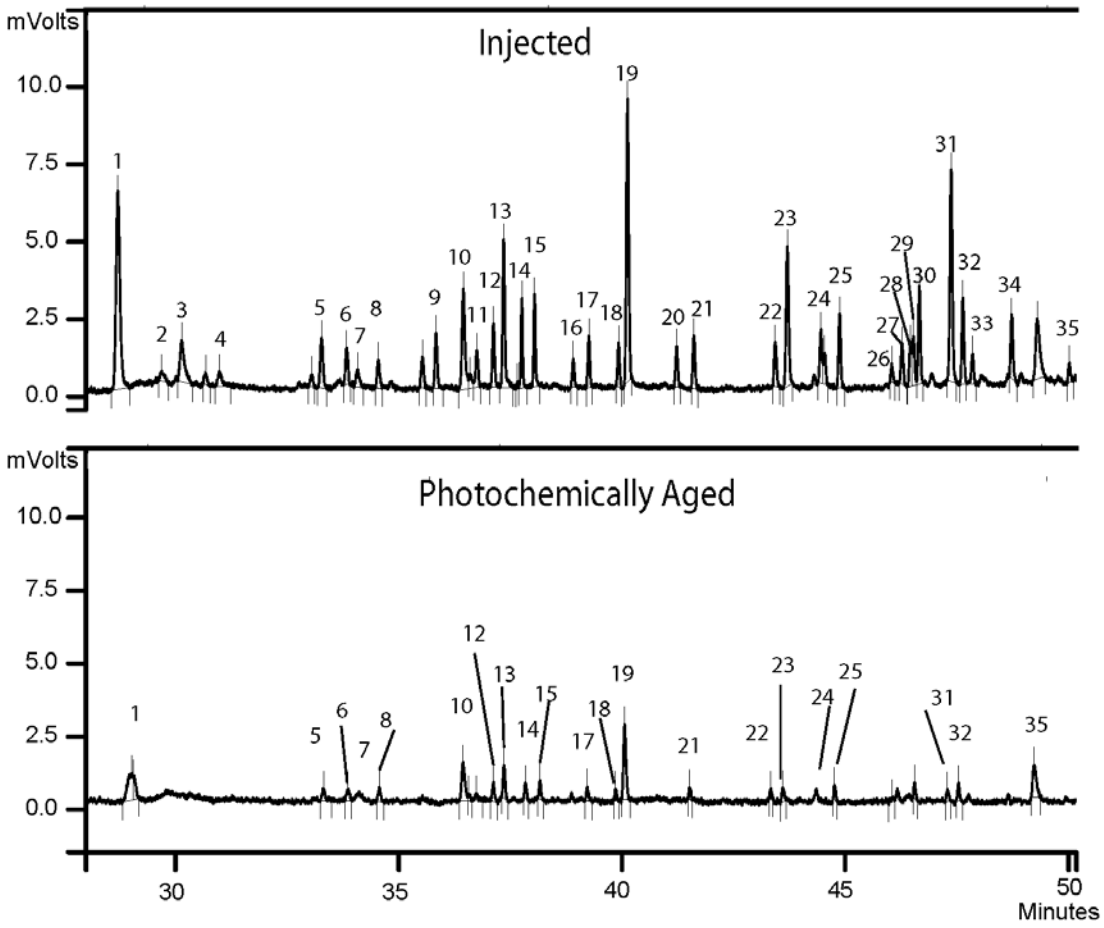


Figure 3-2. Gas chromatogram of VOC species in outdoor chamber. Top: after injection of exhaust from 1980 Mercedes (50 seconds) and 2.0 ppmC injection of SynUrb54 mixture, but before sunrise. Bottom: in dark after daylong sunlit reaction. The peak numbers correspond to the species listed in Table 1. Major compounds present in the chromatogram labeled injected were: 1 – iso-pentane, 10 – benzene, 19 – toluene, 23 – m-xylene, and 31 – 1,2,4-tri-methyl-benzene. After photochemical aging the species either completely reacted away or had significantly decreased.

Table 3-1. Peak identifications of the hydrocarbon peaks in Figure 2

| Identification # | Compound | Fresh Concentration (ppmC) | Photochemically Aged Concentration (ppmC) |
|---|---|---|---|
| 1 | isopentane | 0.149 | 0.000 |
| 2 | 1-pentene | 0.022 | 0.000 |
| 3 | n-pentane | 0.107 | 0.000 |
| 4 | c-2-pentene | 0.037 | 0.000 |
| 5 | 2-methylpentane | 0.082 | 0.023 |
| 6 | 3-methylpentane | 0.054 | 0.020 |
| 7 | 2-m-1-pentene | 0.021 | 0.000 |
| 8 | n-hexane | 0.026 | 0.014 |
| 9 | 2,3,3-tm-1-butene | 0.046 | 0.000 |
| 10 | benzene | 0.109 | 0.048 |
| 11 | cyclohexane | 0.022 | 0.000 |
| 12 | 2,3-dm-pentane | 0.034 | 0.010 |
| 13 | 3-m-hexane | 0.081 | 0.018 |
| 14 | 2,2,4-tm-pentane | 0.036 | 0.008 |
| 15 | n-heptane | 0.040 | 0.008 |
| 16 | m-cyclohexane | 0.010 | 0.000 |
| 17 | 2,5-dm-hexane | 0.018 | 0.006 |
| 18 | 2,3,4-tm-pentane | 0.015 | 0.004 |
| 19 | toluene | 0.117 | 0.035 |
| 20 | 1-octene | 0.015 | 0.000 |
| 21 | n-octane | 0.021 | 0.004 |
| 22 | ethylbenzene | 0.019 | 0.007 |
| 23 | m-xylene | 0.056 | 0.000 |
| 24 | o-xylene | 0.019 | 0.000 |
| 25 | n-nonane | 0.025 | 0.004 |
| 26 | a-pinene | 0.005 | 0.000 |
| 27 | n-propylbenzene | 0.014 | 0.000 |
| 28 | m-ethyltoluene | 0.014 | 0.000 |
| 29 | p-ethyltoluene | 0.018 | 0.004 |
| 30 | 4-methylnonane | 0.032 | 0.000 |
| 31 | 1,2,4-tm-benzene | 0.081 | 0.004 |
| 32 | n-decane | 0.029 | 0.005 |
| 33 | sec-butylbenzene | 0.011 | 0.000 |
| 34 | 1,3-diethylbenzene | 0.022 | 0.000 |
| 35 | 1,2,3,5-tetra-m-benzene | 0.009 | 0.000 |

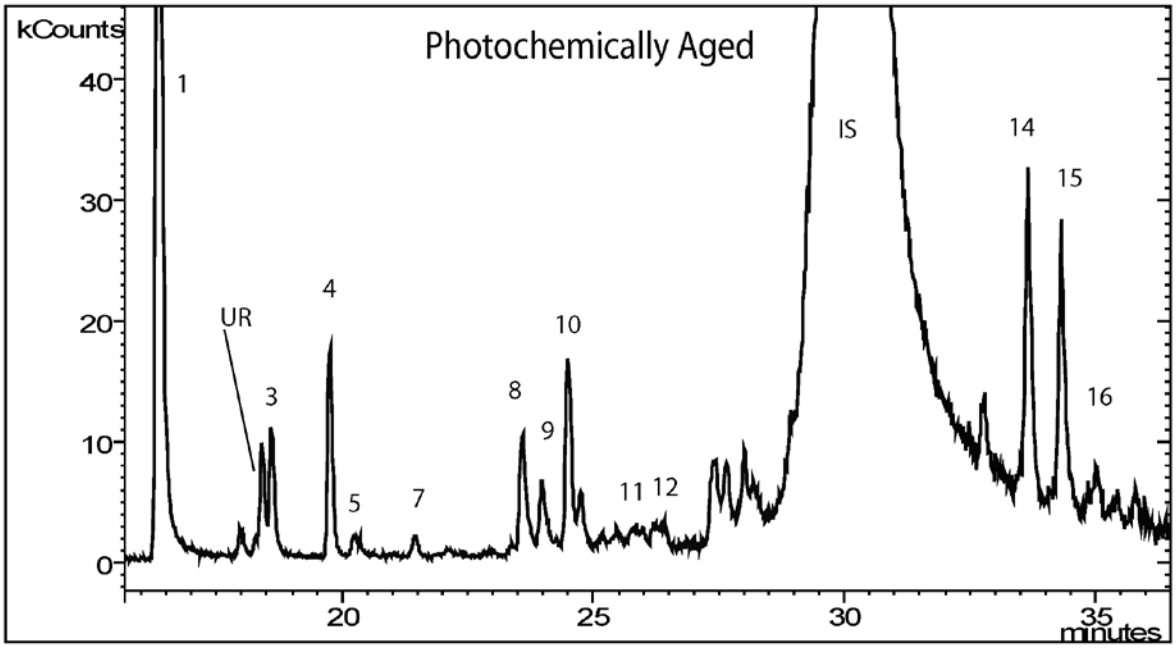


Figure 3-3. Selective-ion chromatogram for the injections from the 1980 Mercedes and 2.0 ppmC of SynUrb54 in the chamber. of carbonyls from mister samples. Samples were collected after daylong irradiation. The peak numbers correspond to the species identified in Table S2. UR – Unreacted PFBHA solution, IS – Internal Standard, and some of the major peaks are 1 – formaldehyde, 3 – acetaldehyde, 6 – methyl ethyl ketone, 8– 2-pentanone, 10 – 2-hexanone, 14 – glyoxal, and 15 – methylglyoxal.

Table 3-2. Identifications of the carbonyl peaks in Figure 3

| Identification # | Compound | Fresh Concentration (ppmC) | Photochemically Aged Concentration (ppmC) |
|---|---|---|---|
| 1 | formaldehyde | 0.878 | 0.642 |
| UR | Unreacted PFBHA | ---- | ---- |
| 3 | acetaldehyde | 0.033 | 0.017 |
| 4 | acetone | 0.027 | 0.029 |
| 5 | hydroxyacetaldehyde | 0.033 | 0.009 |
| 6 | methylethylketone | 0.007 | 0.014 |
| 7 | n-butanal | 0.015 | 0.000 |
| 8 | 2-pentanone | 0.027 | 0.082 |
| 9 | pentanal | 0.014 | 0.044 |
| 10 | 2-hexanone | 0.152 | 0.218 |
| 11 | hexanal | 0.000 | 0.069 |
| 12 | 2-heptanone | 0.000 | 0.084 |
| IS | Internal standard | ----- | ----- |
| 14 | glyoxal | 0.179 | 0.121 |
| 15 | methylglyoxal | 0.097 | 0.131 |
| 16 | biacetyl | 0.072 | 0.014 |

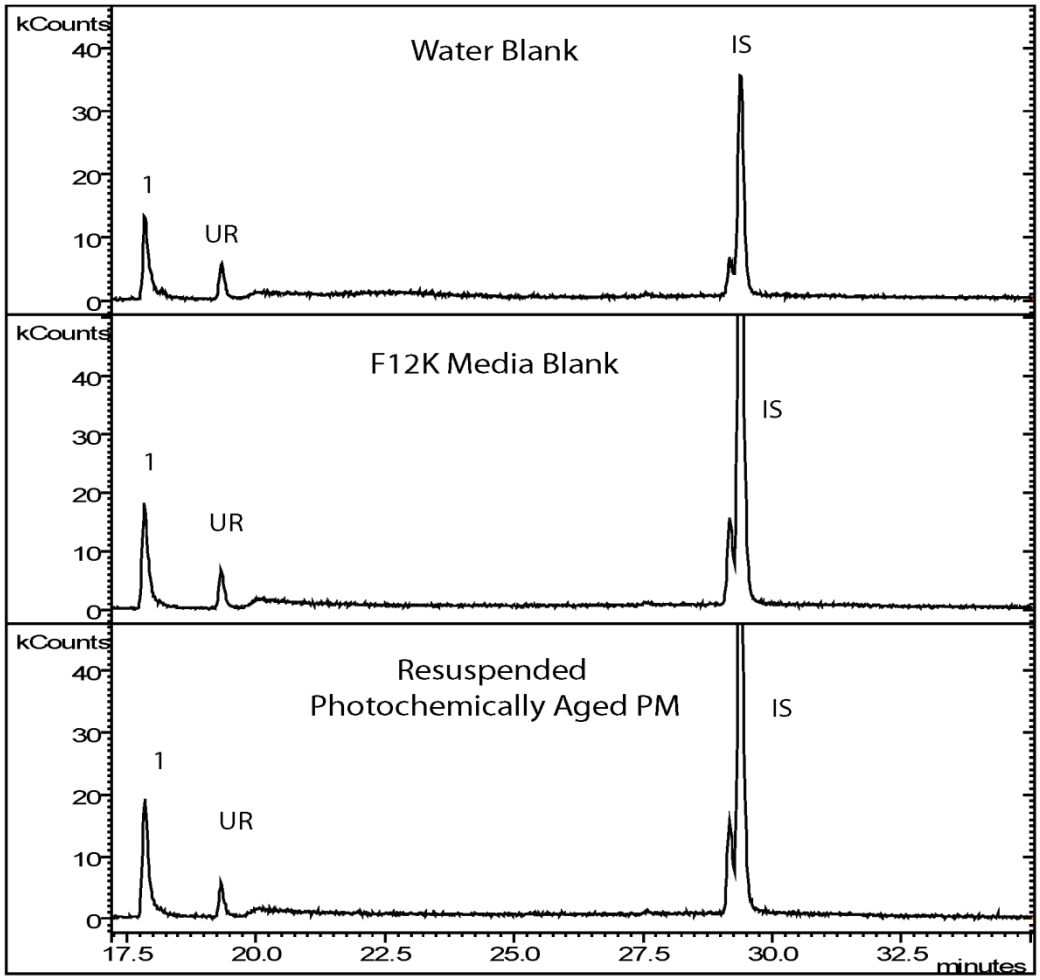


Figure 3-4. Selective-ion chromatogram of carbonyls from a water blank, a F12K media blank, and a filter sample taken after day-long irradiation of the injections from the 1980 Mercedes and 2.0 ppmC of SynUrb54 in the chamber. The peak numbers over the peaks correspond to the identities in Table 2. UR – Unreacted PFBHA reagent, IS – Internal Standard.

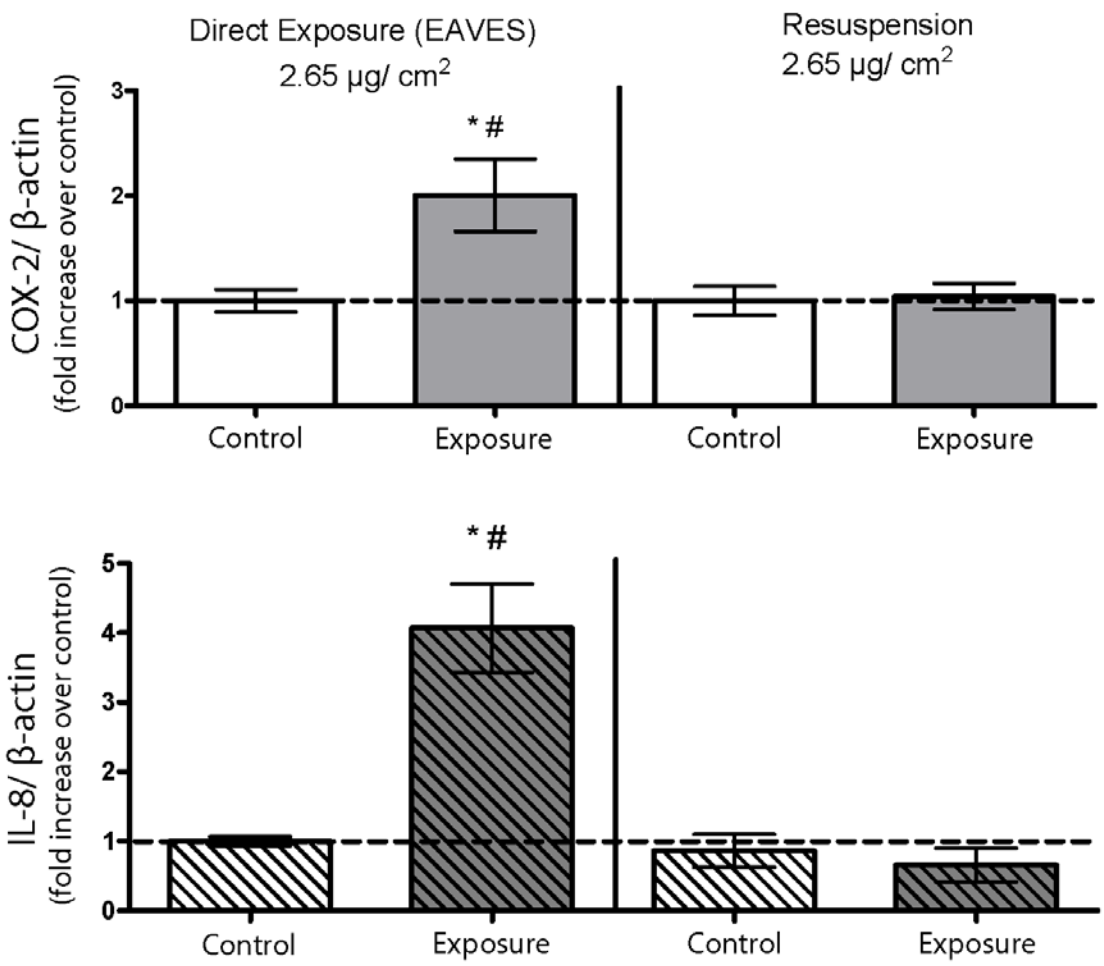


Figure 3-5. COX-2 and IL-8 m-RNA expression induced by exposure to directly deposited PM from the aged exposure in the 1980 Mercedes with SynUrb54 experiment and induced by re-suspension exposures. The symbols * indicates statistically different from non-exposed incubator control; # indicates statistically significant difference compared to resuspension exposures. The error bars represent the mean ± standard error from the mean.

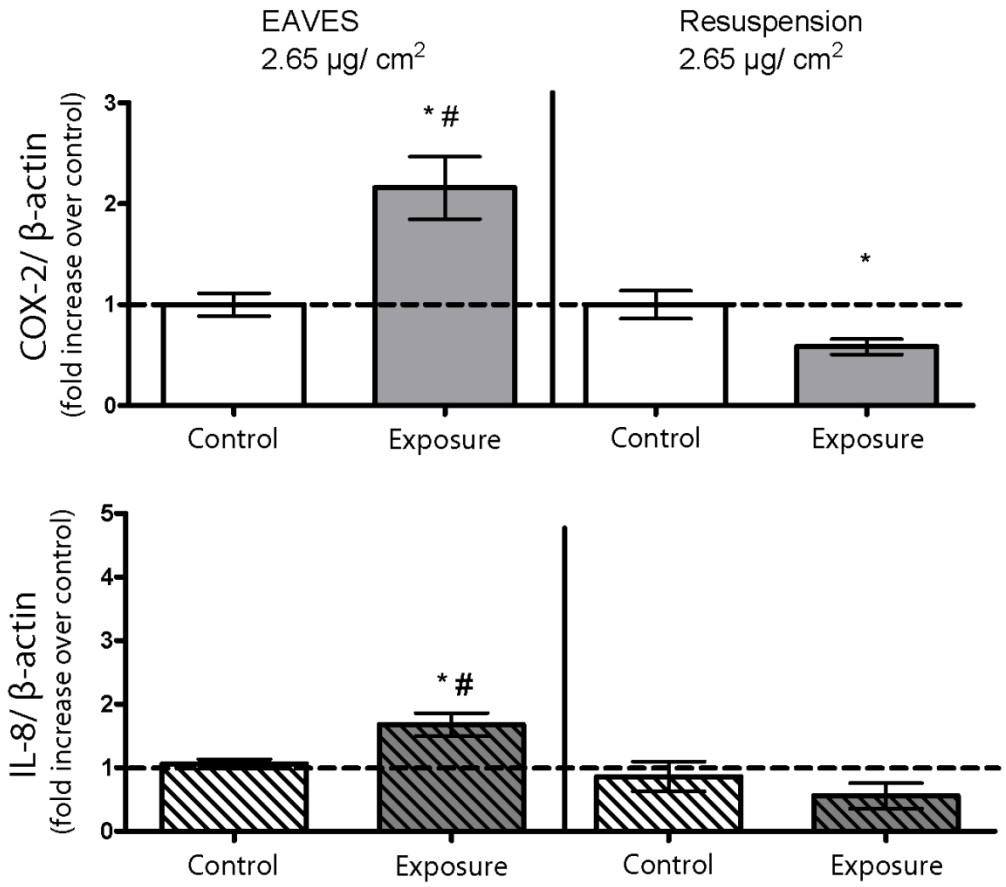


Figure 3-6. COX-2 and IL-8 m-RNA expression induced by exposure to directly deposited PM from the aged exposure in the 2006 Volkswagen with SynUrb54 experiment and induced by re-suspension exposures. The symbols * indicates statistically different from non-exposed incubator control; # indicates statistically significant difference compared to resuspension exposures. The error bars represent the mean ± standard error from the mean.

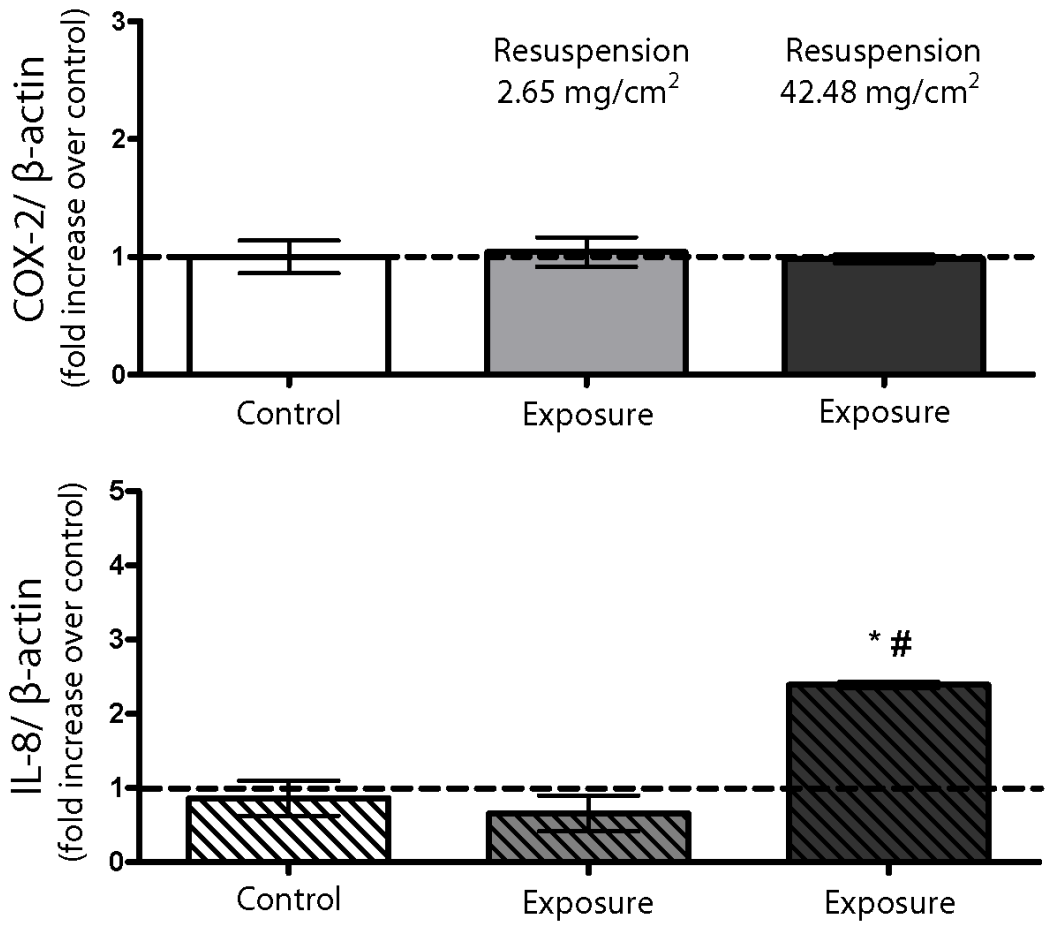


Figure 3-7. COX-2 and IL-8 m-RNA expression induced PM from the AGED Exposure in the 1980 Mercedes with SynUrb54 experiment from resuspension exposures at concentrations of 2.65 µg/cm$^2$ and 42.48 µg/cm$^2$. The symbols * indicates statistically different from non-exposed incubator control; # indicates statistically significant difference compared to resuspension exposures. The error bars represent the mean ± standard error from the mean.

*2. Previously published as:*

*Lichtveld, KM., Ebersviller, SM., Sexton, KG., Vizuete W., Jaspers I., and Jeffries, H. E. (2012) 'In Vitro Exposure in Diesel Exhaust Atmospheres: Resuspension of PM from Filters versus Direct Deposition from Air', Environmental Science and Technology, **46**:16 , 90626 — 70*

# Chapter 4 : Atmospheric Chemistry Modifies Diesel Exhaust's Biological Effects

## Introduction

Vehicle emissions are a major part of urban oxides of nitrogen, volatile organic compounds (VOCs), and particulate matter (PM) emissions and play a critical role as precursors in the photochemical generation of ozone and other oxidants (Jeffries 1995; Finlayson-Pitts 1999; U.S. Environmental Protection Agency 2002). Diesel exhaust gases consist of VOCs, aldehydes, carbon dioxide, oxides of nitrogen, water vapor, carbon monoxide, organic nitrogen compounds, and sulfur compounds. Diesel PM consists of a carbon core with a mixture of semivolatile organic species surrounding it (U.S. Environmental Protection Agency 2002). In a reactive urban environment, diesel exhaust components participate in complex gas-phase oxidation, gas-to-particle-to-gas mass transfers, and even on-particle transformation processes. Published studies of the biological effects of diesel exhaust have used single stationary vehicles with fresh emissions or standard reference materials to carry out biological exposures (Dybdahl 2004, Li 2002, Baulig 2003, Knebel 2001, Salvi 1999, Laks 2008, Sawant 2008, Carlsten 2008, Liu 2008, Sunil 2009). To achieve an understanding of the health outcomes associated with exposure to diesel exhaust and its component compounds, many toxicological studies have focused on single pollutant species or primary emission mixtures (Dreher 2000; Schlesinger 2006; Farina 2011; Steenhof 2011). Single and primary pollutant studies,

however, ignore atmospheric processes that occur in the ambient environment as well as co-pollutant health effects and, therefore, do not illustrate a holistic evaluation of health outcomes. Moreover, "such approaches are likely to oversimplify the underlying biological processes and how the mixture of air pollutants that is inhaled adversely affects health" (NAS, 2004).

Epidemiology studies have consistently found strong correlations between ambient PM and detrimental health outcomes. Diesel motor vehicles are significant sources of ambient air toxicants and particulate matter (Gertler 2002; Gertler 2002 ; Fraser 2003; Chellam 2005; Modig 2006) and hence have drawn much attention as primary contributors to air pollution risk (Bates 1995; Sarnat 2001). While the health effects from diesel exhaust identified in epidemiologic and bench top toxicology studies are largely coherent, their respective dose-response relationships differ (Dybdahla 2004; Carlsten 2008; Laks 2008; Sunil 2009). In addition to acute affects, diesel exhaust has recently been designated as a Class 1 known carcinogen with the increase risk of lung cancer (International Agency for Research on Cancer 1989; International Agency for Research on Cancer 2012). This was largely based on findings from repeat exposure to diesel exhaust (International Agency for Research on Cancer 2012).

Previous studies in our laboratory have shown that gas-phase oxidative chemistry can be a major source of gas-phase toxicity in environmental systems and that gas and particle phase interactions are dynamic and can cause toxicity to move between the phases (Ebersviller 2012a; 2012b). By excluding atmospheric processes from exposure

studies, researchers could be overlooking important modifications and secondary toxicants, for both gaseous and PM phases. It is important to understand atmospheric aging processes that likely modify the chemical composition, and thus investigate whether the observed modifications are accompanied by observed inflammatory responses induced by exposure to these atmospherically-processed gaseous and PM phases.

The rationale for this work was that studies in simple PM systems have demonstrated that atmospheric processes can modify the toxicity of gaseous pollutants *in situ* and subsequently potentially modify PM's toxicity. Based on these previous studies, I hypothesized that oxidation and partitioning processes will alter the toxicity of both the gaseous and particulate phases in real diesel and urban-like test atmospheres. The purpose of this study was, therefore, to demonstrate the role of atmospheric processes in modifying and creating gaseous and PM cellular toxicity. This study utilized real diesel vehicle exhaust mixed with an urban-like gaseous environment and allowed the mixture to react in both the dark and sunlight conditions.

## Experimental Design

To test the concept that atmospheric processes likely modify the toxicity of both gases and PM for real diesel sources, I needed to create two controlled test environments that included the types of atmospheric processes present in an ambient urban environment. Test atmospheres were created in an outdoor chamber by combining whole diesel exhaust (gases and PM) with realistic mixtures of urban hydrocarbons (i.e., SynUrb54 – described below). These experiments start in the dark and either undergo oxidation by ambient sunlight (and are retained into the dark period after sunset for ex-

posures), or undergo thermal oxidation (in the dark for the entire period). Throughout each processing period, chemical analysis of the chamber contents were performed. In addition, gas-only and PM-only biological exposures were conducted to examine whether oxidation increased or decreased the inflammatory responses observed from human epithelial respiratory cells (type A549 – described below). Cells were exposed separately to 'fresh', or 'dark-aged', or 'sunlit-aged' chamber contents. Observations made from exposure data were combined with chemical analyses to inform the discussion of my findings.

Altogether four chamber experiments, each with two cellular exposure periods were performed. Figure 4-1 shows timeline bars for each chamber experiment, illustrating significant time periods in each of the experiments: two for the Mercedes, i.e., "dark-aged" and "sunlit-aged", and similarly two for the Volkswagen. There were two biological exposures in each of the four experiments and these have labels explained below. The time line for July 04 – 05 2007 represents the dark or thermal –only oxidation experiment for the 1980 Mercedes. The green area is the duration of the Mercedes fresh exposure (M-F) that occurred right after injection of diesel and SynUrb54 components. The dark thermal oxidation period that followed the M-F exposure is represented by the grey area, with the subsequent Mercedes dark (M-D) exposure represented by the purple area. The time line for the complimentary sunlit or photochemical oxidation experiment (August 19, 2008) also begins in the dark three hours before sunrise (where M-F is represented by the green area), followed by the yellow area representing photo-oxidation by natural sunlight, and the Mercedes photochemically oxidized cellular expo-

sures window (M-P) after sunset (represented by the blue area). Similar time lines are included for the 2006 Volkswagen on the right side.

Experiments were only conducted when environmental conditions were acceptable. These conditions included suitable values for humidity, ambient temperature, and dew point. Tests were only performed when the ambient temperature was forecast to be no lower than 15$^{o}$C. In addition, experiments were not performed when the dew point in the chamber fell below 14$^{o}$C (which would result in low relative humidities in the exposure systems (described below). These lower limits are based on previous studies performed during the original method development and are needed to decrease the likelihood of an observed biological response being the result of a 'false-positive' (Doyle 2004; Sexton 2004; Doyle 2007; de Bruijne 2009).

Normalizing complex mixtures is difficult, and many variables are not independent for a given source, i.e., older diesel and newer diesel. When deciding on how to 'match' the compositions of the test atmospheres for comparison between emission sources, "identical conditions" across the four tests were impossible to achieve. Currently, there is no consensus on the 'best' method for sampling complex mixtures of airborne pollutants for biological exposures or atmospheric processing. For instance, factors that could be matched were the length of sampling time, PM load, $NO_x$ concentration. Any normalizing condition would have resulted in very different compositions and pose different challenges for analysis and exposure. In this study, I decided to emphasize matching particle mass concentration as the normalizing factor, even if this choice resulted in a

different gaseous environment. As will be shown, achieving the same PM mass in all four experiments was not obtainable. I could, however, achieve adequate and characterizable PM-dose delivered to the cells for each vehicle, as will be described below.

## Materials and Methods

### Chamber

I utilized The Gillings Outdoor Irradiation Chamber at the Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC as previously described in Lichtveld *et al.*(2012). Briefly, the chamber is coupled to the chemistry and biology laboratory on the top floor via parallel thermally-insulated sample lines through the roof (de Bruijne 2009; Ebersviller 2012a; 2012b). Along with its analytical and biological capabilities, the chamber has a stainless steel exhaust-transfer manifold that can be connected to the tailpipe of vehicles operated in the parking lot adjacent to the building.

### Diesel Exhaust and Urban VOC Injections into Outdoor Chamber

To generate two types of PM in complex VOC-reactive atmospheres, diesel exhaust (DE) was injected into the chamber in the dark from either a 1980 Mercedes-Benz model 300SD that lacked emission controls or a 2006 Volkswagen Beetle compliant with existing emission standards set by the EPA (i.e., equipped with a catalytic converter). Although diesel vehicles are no longer sold without EPA set emission standards, many older diesel vehicles are still on the road today and if well maintained can persist up to 800,000-1,600,000 km before removal from the road (Clark 2002). Both vehicles were operated with commercially available low sulfur diesel fuel, and were warmed up under normal idling conditions. Without a load, the engine was throttled up to approximately

2700 rpm, with an engine temperature of 90 °C, and held there throughout the duration of sampling. Given the differences in age and emission controls, the two vehicles produced exhausts with different compositions.

To compare the response from the two vehicles based on total PM load, DE was sampled via a venturi driven dilution blower attached to the chamber, and DE was sampled until the particle concentration reached ~2.2 mg/$m^3$, as measured by a scanning mobility particle sizer (SMPS - model 3936L25, TSI, Inc., St Paul, MN) (de Bruijne 2009; Lichtveld 2012). To achieve this concentration with emissions from the Mercedes I sampled the tailpipe for 30 and 49 seconds, while the Volkswagen was sampled for 16 and 25 minutes. These different sampling times resulted in different gas phase chamber concentrations. Methods could have been employed to remove specific components of the exhaust to match more than on particle concentration, but these methods would likely also alter the reactive organic composition. This potential alteration in composition would, therefore, not be a representative example of the true exhaust sampled from each vehicle, so I chose not to modify the exhaust and only match on particle concentration. When the desired particle concentration had been reached, 2.0 ppmC of SynUrban, a 54-component VOC mixture (SynUrb54), was added to the chamber contents to represent an urban atmosphere in which the DE could be realistically oxidized (Sexton 2004; Lichtveld 2012; Ebersviller 2012b).

For each vehicle, (M – Mercedes, V- Volkswagen) three different types of exposures were conducted: (1) Fresh exposures, using emissions with SynUrb54 without any

alteration of the contents in the chamber (labeled 'M-F' or 'V-F'); (2) dark (thermally) oxidized exposure, where the contents were allowed to age from sunset to sunrise (labeled 'M-D' or 'V-D'); and (3) photochemically oxidized exposures, where the contents of the chamber were allowed to react with natural sunlight from sunrise to sunset (labeled 'M-P' or 'V-P'). All biological exposures were performed in the dark, either before sunrise or after sunset (Ebersviller 2012b). This protocol required four outdoor chamber experiments: two experiments conducted completely in the dark, and two experiments with a sunlight period to produce the photochemically aged atmosphere for cell exposure.

## Description of Biological Exposure Systems

To explore the effects of atmospheric aging of diesel exhaust on human epithelial lung cells, two different air-liquid interface *in vitro* exposure devices were used. These were housed in an incubator and were coupled to the outdoor smog chamber.

The gaseous exposure device, Gas *in Vitro* Exposure System (GIVES), is an 8-liter modular cell-exposure chamber that housed the tissue culture plates (Billups-Rothenberg, MIC-101™, Del Mar, CA). The 8-liter cell-exposure chambers had inlet and outlet connections for continuous circulation of the chamber contents at 1 L/min (Doyle 2004; Sexton 2004). The GIVES sampler was operated at 1 L/min for three hours. All gas phase exposures were sampled for three hours to provide sufficient time for the exposures to elicit a response from cells.

The PM exposure device, Electrostatic Aerosol *in Vitro* Exposure System (EAVES), used particle charging and electrostatic precipitation to deposit particulate matter di-

rectly from an unaltered air stream sampled from the chamber(de Bruijne 2009; Lichtveld 2012). The EAVES has the capability of depositing PM, with no disruption of the equilibria between the gas and particle phase. In addition, the device does this without any biological effects from the electrical field, electrical charges added to the PM, exposure to gases without PM present, and no response when mixtures of toxic gases and PM pass through the sampler without the deposition field on. By contrast, a significant response is observed from a mixture of PM and gases when deposition voltage field is applied (de Bruijne 2009). The device was used to sample the chamber contents ca. one hour at 1 L/min, depositing ~ 2.0 µg of PM onto the cellular surface for the Mercedes and ~1.0 µg of PM for the Volkswagen. The EAVES exposure times were modified so that the mass of PM delivered to the air liquid interface was matched between all exposure periods. The duration of exposure was calculated by taking the mass of PM collected on a filter and ratio it against the mass collected from the first PM filter of the given engine i.e. the filter mass from M-P over M-F filter mass times the duration of M-F exposure.

## Analytical Methods

The chemical and physical monitoring of the chamber has been described extensively by Ebersviller *et al.* and will only be briefly summarized here (2012a).

Ozone was measured with a ML9811 series Ozone Photometer (Monitor Labs, Englewood, CO). Nitrogen oxides were measured with a ML9841 series $NO_x$ Oxides of Nitrogen Analyzer (Monitor Labs, Englewood, CO). All chamber data were recorded with one-minute resolution using a data acquisition system connected to a computer. Prior

to each experiment, the $O_3$ and $NO_x$ meters were calibrated by gas-phase titration using a NIST standard NO tank and stable $O_3$ source.

A Varian 3400/2000 GC/MS, with both a mass spectrometer (MS) and flame ionization detectors to identify and quantify the species in each mixture, was used to continually monitor gas-phase hydrocarbon compositions of the exposure atmospheres. A non-reactive tracer, carbon tetrachloride, was injected (2.5 µl) at the beginning of each experiment to monitor the dilution rate of the chamber. A Varian 3800 GC, with an electron capture detector, was used to monitor the gaseous tracer concentration.

Modified mister samplers, similar to those described by Seaman *et al*.(2006), were used to determine the carbonyl content of the test atmospheres (2006). Sampled carbonyls were detected with a O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBHA) method (Seaman 2006, Yu 1997, Yu 1995, Liu 1999).(Yu 1995; Yu 1997; Liu 1999; Seaman 2006). The PFBHA derivatives were analyzed by gas chromatography/mass spectrometry (GCMS) on a Varian 3800 GC/ Saturn 2200 Ion Trap MS operated in electron impact mode.

## Growth of Cell Cultures and Exposure Pretreatment

For both *in vitro* methods, the A549 cell line, which is derived from a non-small-cell adenocarcinoma of the human lung, was used to assess the biological effects of the ambient PM mixture (Lieber 1976; ATCC 2011). The A549 human epithelial cells were grown on collagen coated membrane supports (Millicell R-CM; Millipore, Cambridge, MA; Costar-Clear Transwell™ inserts; Costar, Cambridge MA) in complete media (F12, 10 % fetal bovine serum with antibiotics [0.01 % penicillin/streptomycin] (Invitrogen, Carls-

bad, CA) as previously described (de Bruijne 2009, Doyle 2006) . Several hours before exposure (after the cells reached confluency), the complete media was exchanged and replaced with serum-free medium (BSA) (F12K, 1.5 µg/ml bovine serum albumin with 0.01 % penicillin/streptomycin; Invitrogen, Carlsbad, CA). Immediately before exposure apical media was removed from all exposures and controls (de Bruijne 2009; Doyle 2004; Doyle 2007; Sexton 2004; Ebersviller 2012 a,b; Rager 2011).

I recognize that an immortalized cell line is not identical to primary cells, but the goal of this work was to focus on differences in biological response caused by disparate atmospheric processes rather than interpersonal variability in response. Hence, the consistence of response in this well-documented, immortalized cell line was an asset to this study.

### Analysis of post-exposure mRNA levels

Total RNA was isolated from the cells using Trizol (Invitrogen, Carlsbad, CA) nine hours post-exposure and stored at -20 °C until analysis, per the manufacturer's instructions. Evaluation of inflammatory response was measured with real-time, reverse-transcriptase polymerase chain reaction (RT-PCR) for expression of Interleukin-8 (IL-8) as described previously (Jaspers 2001). IL-8 mRNA levels were normalized against a house-keeping gene (β-actin mRNA) and reported as "fold-increase" over control. Three replicates of each sample were tested for mRNA expression.

### Statistical Analysis

All data are presented as the mean ± standard error from the mean and expressed as fold increase over the same measurements performed on a set of control cells main-

tained in the incubator throughout the exposure period. Data sets were analyze and compared using unpaired Student-t test with Welch's correction. Differences were considered significant when *p* was less than 0.05.

# Results

## Chemical Analysis and Concentrations

### *Biological Exposure Periods*

As stated earlier, Figure 4-1 shows four bars as time lines illustrating significant time periods in each of the four experiments (two for the Mercedes and two for the Volkswagen). There were two biological exposures in each of the four experiments. In addition to these experimental exposure periods, a 'control' exposure was used for each experiment, in which cells were exposed in a clean air incubator.

### *Physical Conditions During Exposure Periods*

Figure 4-2, shows average physical conditions during the five exposure periods of the experiments; the 1980 Mercedes data are on the left, and the 2006 Volkswagen data are on the right.

### *"Fresh" Exposure Conditions*

There were four "Fresh" exposure conditions to be achieved. The design was to continue vehicle injections into the chamber until a specific PM concentration (2.5 $mg/m^3$) was reached in each of the four experiments. In the case of the older Mercedes, these were very short periods, 30 seconds for the July 4, 2007 injection and 49 seconds for the August 19, 2008 experiment (see Figure 4-1). Such short duration injections are hard to control and, while the July injection was very close to the target, the August PM injection was higher by 3 %, due to an additional 19 seconds of injection die-

sel exhaust (2.22 and 2.15 mg/$m^3$ determined by filter masses). This also resulted in M-F concentrations during the dark aged exposure experiment to be 0.33 ppm of $NO_2$ and 0.71 ppm of $NO_x$, and during the photochemically oxidized experiment where M-F concentrations were 0.53 ppm $NO_2$ and 1.06 ppm $NO_x$, which was a 50 % increase. In the case of the catalyst-equipped Volkswagen, injection times of 25 minutes resulted in only 1.7 and 1.0 mg/$m^3$ as measured by filter analysis. These long injection times also influenced the $NO_2$ and $NO_x$ concentrations, which were much higher than the Mercedes during all experiments. The concentrations for the V-F exposure period were 0.43 ppm for $NO_2$ and 6.92 ppm $NO_x$. Similar initial conditions were seen during the photochemically oxidized experiment where V-F concentrations were 1.52 and 5.78 ppm for $NO_2$ and $NO_x$.

Because of variations in initial injection from each vehicle, carbonyl concentrations and compositions varied. The total carbonyl concentration present in the chamber was 1.53 ppmC for the Mercedes (M-F), with speciation data in Figure 4-3 and Table 4-1. The figure and table identify that the major carbonyls present in the fresh chamber conditions were formaldehyde, acetaldehyde, acetone, hydroxyacetaldehyde, glyoxal, and methylglyoxal. The total carbonyl concentration present in the chamber after sampling emissions from the Volkswagen was much lower at 0.5 and 0.2 ppmC (Figure 4-4 and Table 4-2). In these exposure conditions, formaldehyde, acetone, acetaldehyde, and acrolein were the major compounds present, but at lower concentrations compared to the Mercedes. Although these were present they were at much lower concentrations than the Mercedes.

### *"Dark-aged" exposure conditions*

After the dark thermal oxidation period of 3.5 hours, the particle mass concentration in the M-D exposure atmosphere was 0.92 mg/m$^3$, and $NO_2$ and $NO_x$ concentrations in the exposure atmosphere were 0.186 and 0.365 ppm (Figure 4-2). After a similar aging period, the V-D exposure atmosphere had 1.43 mg/m$^3$, and the $NO_2$ and $NO_x$ concentrations were still significantly elevated at 2.52 and 5.05 ppm (Figure 4-2). Since aging occurred in the dark, and no source of $RO_2$ radicals was available, this resulted in no ozone formation during the "dark" aging periods.

The concentrations for formaldehyde, acrolein, 2-cyclohexen-1-one, and methylglyoxal from the V-F to V-D exposure atmospheres increased, while the remaining identified carbonyl compounds showed no change (Figure 4-5). Unfortunately, the carbonyl composition of the M-D atmosphere was not able to be reported due to the loss of the samples.

### *"Photo-aged" exposure conditions*

After photochemical oxidation for 13 hours, the PM mass concentration in the M-P exposure atmosphere was 0.83 mg/m$^3$. Atmospheric processes completely consumed the NO present in the chamber, leaving 0.09 ppm of $NO_x$. Furthermore, these atmospheric processes generated 70 ppb of ozone, which was present at the end of the day for the M-P exposure (Figure 4-2). By contrast, after photochemical oxidation of the V-F chamber contents, 3.12 ppm of $NO_x$ and 1.46 ppm of $NO_2$ remained in the V-P test atmosphere. In addition, the PM concentration at the end of the day was 0.61 mg/m$^3$ (Figure 4-2).

The total carbonyl concentration in the M-P atmosphere was slightly lower than the M-F condition. Although the total carbonyl concentration was lower, the composition was significantly modified by photochemical processes, as illustrated in Figure 4-2, 4-4, and Table 4-1. As the chamber contents were irradiated by sunlight most of the primary hydrocarbons oxidized, thereby producing carbonyl-containing secondary organics. As the contents underwent further oxidation, some species present initially (M-F) were partially reduced by the end of the day (M-P) (as in formaldehyde), while others were completely consumed (such as butanal). In addition, other compounds increased in concentration due to aging processes. Such compounds were acetone, methyl ethyl ketone, 2-pentanone, and pentanal (Figure 4-4 and Table 4-1).

Despite the absence of measureable ozone production, during the V-F to V-P chamber conditions the total carbonyl concentration increased by 0.1 ppmC, resulting in a total carbonyl concentration of 3.49 ppmC. As with the M-P experiment, species were created and consumed during the aging process. Many individual species that were measured in the V-D and V-P were no longer present in the corresponding V-F atmospheric condition. In addition, some species present in the initial condition increased in concentration, while others decreased (Figure 4-5 and 4-6 and Tables 4-2 and 4-3).

## Inflammatory Response in Fresh and Aged Exposures

To facilitate the communication of my biological response measurements, I will focus on the gas-phase only exposures first and then presenting the PM-only exposure responses. Figure 4-6 contains a series of bar graphs that represent the IL-8 expression induced by exposure to emissions from the two vehicles. The data on the left side of

Figure 4-6 present the gas-phase (GIVES) and particle-phase (EAVES) exposures from the Mercedes emissions with SynUrb54, while the data on the right side show the exposures using the Volkswagen mixture with SynUrb54.

### *Gas-phase exposures*

The 1980 Mercedes gas-phase data in Figure 4-6 show that neither of the responses to the M-F chamber contents were significantly higher than the controls, and were not significantly different from one another. Once the contents of the chamber were thermally aged, I saw a significant increase in response relative to the M-F exposure for the same experiment (Figure 4-6, M-F and M-D). As described in the introduction section, previous experiments expected to see a significant increase in response from the gas-phase exposure due to photochemical oxidation processes. For the Mercedes, however, no significant increase in biological response was measured as a result of exposure to the M-P chamber conditions (Figure 4-6).

Unlike the 1980 Mercedes, exposure to both atmospheres containing fresh gaseous DE emissions from the 2006 Volkswagen Beetle (V-F) resulted in a statistically significant biological response that was four-fold higher than the control (Figure 4-6 right side set of plots). After the contents of the chamber were oxidized in the dark, the biological response elicited decreased by half, to only two-fold over the control exposure. Similar to the Mercedes, the photochemically oxidized chamber contents (V-P) elicited no significant biological response.

The top of Figure 4-6 shows that the biological responses of the gas-only (GIVES) exposures were not uniform between the vehicles. As described above, the biological

responses following the V-F exposures were significantly greater than those of the fresh exposures using the 1980 Mercedes (M-F). Both dark aged exposures (M-D and V-D) yielded biological responses that were significantly greater than the control. In addition, no biological response was observed with either M-P or V-P gas phase exposures.

### *Particle-phase exposures*

Figure 4-2 illustrates the concentration available for PM exposures using the EAVES device. As previously stated in the methods section, the duration of exposure was modified to account for differences in mass concentration during each exposure period. Assuming a similar deposition efficiency as found in (Lichtveld 2012), the delivered PM doses to the ALI was ca. 2 µg per insert for the Mercedes and ca. 1 µg per insert for the Volkswagen PM exposures. This matched dose delivered to the cellular surface, accounted for the variability in the mass concentration between experiments. By ensuring that each exposure condition received approximately the same amount of PM, a direct comparison of the results from one experiment to the next could be evaluated (within the test atmospheres created for each vehicle).

There was no significant response from either of the M-F PM-only exposures (Figure 4-6). The response observed following EAVES exposure to the M-D chamber contents indicate that, as the contents of the chamber were thermally aged the particle phase toxicity increased two-fold compared to the M-F and control. Similarly, the photochemically oxidized particle phase toxicity increased by four-fold compared to M-F exposures and control. In contrast, the Volkswagen exposures using EAVES, (V-F, V-D,

and V-P) showed no significant difference in response compared to the clean air incubator controls.

For exposures using the EAVES device, only the exposures to the Mercedes test atmospheres elicited a significant inflammatory response. Moreover, the Mercedes induced twice the response to the photochemically-oxidized DE PM than was observed from the Volkswagen.

## Discussion

I begin by discussing the inherent complexity of my test atmospheres and the difficulties that arise while conducting holistic, integrated *in vitro* exposures and chemical analysis. Next I will review the experimental conditions established to test my hypothesis that oxidation and partitioning processes will alter the toxicity of both the gaseous and particulate phases in my urban-like test atmospheres. I then discuss the extent to which I was successful in achieving all test atmosphere conditions (fresh, dark, photochemical). Next, I examine the biological responses relative to the test atmospheres and explore potential explanations for observed responses. Finally, I discuss alternate approaches I might have taken to generate the test atmospheres for this study.

### Complexity of test atmosphere creation and analysis

These experiments represent the creation of highly complex atmospheric mixtures that possess dynamic processes thereby modifying their physical composition and toxicity. These complex mixtures include vehicle emissions that can change in composition from day to day. In addition, since I used natural sunlight and ambient temperature there are some variables that I cannot directly control. Each component added to these

complex atmospheres introduces ambiguities and inter-relationships with other components of the mixtures. In addition, these experiments have many variables that are not independent of one another (i.e., sampling duration and gaseous vehicle emissions).

To characterize such complex mixtures, there was a need to rely on multiple instruments to perform optimally before, during, and after each experiment to accommodate samples with short shelf-lives. Some analytical methods operate on-line, while others require post-experimental analysis. The ability to compare results between conditions may not be apparent until well after the experiments have been completed. As a result, only in hindsight can I determine my ability to directly compare analysis outputs without ambiguity. To compensate, it was necessary to be flexible with my approach to combining and interpreting data.

In the case of this study, traditional approaches to inter-comparisons of physical composition and biological response will not stand on their own. This meant without the inclusion of the EAVES exposure methodology along with the results of previous investigations of gas and particle interactions, it would be difficult to explain the biological exposure results for the gas and particle phases (Lichtveld 2012; Ebersviller 2012a; 2012b).

In addition, to gain a clearer view of the effect of gas and particle interactions on the causal relationships between composition and effect, I have integrated the chemical analysis throughout the PM biological exposures. This holistic approach to integrated

data analysis has yielded interesting insights into the causal relationships between complex atmospheres and their associated biological responses.

## Creation and consistency of test atmospheres

To study such a complex mixture I had to have a metric by which to match each vehicle sampling period. For this study, I chose to match particle concentration in the chamber (2.2 mg/$m^3$). Although the initial conditions were not identical (i.e., initial PM mass concentration in the chamber differed by ca. 0.1 mg/$m^3$ for the M-F atmospheres (Figure 4-2), the observed biological responses indicated that neither the gas phase nor the particle phase fresh (M-F) exposures were statistically different than the controls (Figure 4-6). In this study the slight difference in PM concentration for the Mercedes did not matter for the biological response elicited from the fresh exposures.

Attempts to achieve the same particle concentration in the chamber for the experiments using the Volkswagen was prevented due to the new the new emission controls. During sampling I observed a steady state condition in which the particle concentration ceased to increase with further sampling from the tailpipe. This steady state was probably due to the catalyst beginning to operate more efficiently as the engine continued to warm up. I was able, however, to match the PM concentration between the two experiments using the Volkswagen (ca. 1.0 mg/$m^3$) (Figure 4-2). As a result of the long sampling time, there was an accumulation of gas phase components of engine exhaust, such as $NO_x$.

### *Aged conditions*

The data for the combined pollutant mixtures (vehicle emissions and SynUrb54) measured with the carbonyl method illustrate the complexity of the urban test mixtures, as shown in Figures 4-4 – 4-6 and Tables 4-1 – 4-3. These carbonyl figures along with the ozone, $NO_2$ and $NO_x$ data all provide an indication that oxidation of the chamber atmospheres occurred.

### *Dark Aged*

To achieve a dark aged atmosphere I allowed the chamber contents to interact in the dark until 3 hours prior to sunrise. Thermal oxidation processes are lower in energy and generally change slower than photochemical aging. During the Mercedes dark aged condition I was not able to analyze the carbonyl data due to the loss of the samples. Although I had lost the carbonyl data, I had indications that thermal oxidation did occur from my biological effect measurements, described in detail below.

For the Volkswagen, the data indicated that oxidation occurred for the dark aged test atmosphere. In the V-D test atmosphere I showed an increase in $NO_2$ and carbonyls. The production of carbonyls and $NO_2$ are indicators that oxidation occurred meaning that I was successful in creating a thermally aged test atmospheres.

### *Photochemically Aged*

The oxidation of NO to $NO_2$ sufficient for the production of ozone is a clear indication that oxidative processes were occurring in the chamber for the Mercedes test atmospheres (M-P). Given the amount of ozone produced (70 ppb) I expected to have seen an increase in the total carbonyls present after photochemical aging in the experi-

ment. Since this system did not produce a large amount of ozone, this would result in a smaller amount of first and second generation carbonyls. This, in turn, would result in the addition of other species to react and oxidize to smaller unreactive organics. The data presented are the measurements present at the time of the cell culture exposures and has not been corrected for dilution. If I had corrected the mixing ratio for dilution, I would have shown an increase in carbonyls after photochemical aging.

Although I did not make a measureable amount of ozone in the V-P experiment, I observed an increase in $NO_2$ and carbonyls from the V-F to the V-P chamber conditions. In addition to the increase of total carbonyls, many compounds that were not present in the fresh atmosphere (V-F) were now present at the end of the day (V-P). Of the compounds that were present most of these increased while the others decreased in concentration (see Table 4-3). The change in composition from fresh to aged atmospheres demonstrates that oxidative processes occurred during my atmospheric conditions.

## Biological exposures

### *Particle Phase Exposures*

The particle phase exposure responses would be difficult to explain by traditional methodology of exposure and response. To overcome this obstacle I attempted to follow a simpler association-type analysis of the biological response using various combinations of exposure groupings to biological response to determine relationships. First I correlated the biological response to mass exposure concentration. This method is analogous to EPA's approach to regulating ambient PM based on mass concentration (NAAQS). In Figure 4-7, the relative PM exposure in mass concentration by the duration

of exposure was plotted against the biological response. This combination illustrated a weak correlation for all PM exposure from both vehicles, with a $R^2$ value of 0.204. In addition, this result indicated that when comparing biological response to PM concentration alone the PM concentration was not the source of toxicity and other factors may be the reason for increased biological response.

The second approach grouped PM mass exposures from both vehicles by type (fresh, thermally aged, and photochemically aged). This was then plotted against biological response to determine if types of aging alone are responsible for my observed response, Figure 4-8. The three regression lines were plotted to determine if there was a correlation between the type of exposure and biological response. The fresh unreacted conditions for both vehicles indicated a poor correlation between the fresh exposures to PM and the biological response. Combining the dark aged PM exposures for both vehicles and comparing it to the biological responses indicated a strong correlation with a first order $R^2$ of 0.8905 and when a second order fit was applied this resulted in a perfect correlation. This strong correlation indicates that thermal aging of PM is likely important and that the compounds present in these aged atmospheres are significant for biological responses. Further when this method of comparison was applied to the photochemically aged exposures for both vehicles there was again a strong correlation with a first order $R^2$ of 0.8680 and again a perfect correlation for the second order fit. In addition these second order fits of the photochemical and thermally aged groups are similar varying only in curvature. This figure supports the hypothesis that aging plays an imperative role in toxicity and should not be ignored.

The third approach drew upon previous work in which it was demonstrated that carbonyls are associated with causative effects in aged atmospheres, and that they will partition onto even non-toxic PM and thereby make the PM subsequently toxic (Ebersviller *et al.* (2012)(Madden 2000). Furthermore, earlier work involving 'extracting diesel PM' and analyzing for carbonyls showed a strong correlation with IL-8 expression (Madden 2003; Anderson 2010). Thus a new exposure metric was created by weighting the PM concentration with the total carbonyl concentrations from Figure 4-2, and then subsequently grouping by exposure condition as in the second approach. This calculation was conducted by the PM mass exposure times the carbonyl to mass ratio.

The biological response was then plotted against this exposure metric to determine by adding the carbonyl concentrations, would there be an improvement of the associations between chamber conditions and biological response in the PM phase. From the analysis it was found that the ratio of carbonyls to the PM concentration and subsequently grouping the type of atmosphere present during the exposure was an important factor to understanding the biological response (Figure 4-9). From this analysis, complex mixtures when photochemically aged combined with the carbonyls present, indicate a strong correlation for an increase in biological response with an $R^2$ of 0.995. Carbonyls in a complex mixture that had been thermally aged also had a strong correlation with an $R^2$ of 0.976. This strong correlation was there, although the IL-8 response per unit dose was half that of the photochemically aged carbonyls (comparing slope or relationship of relative IL8 units as a function of dose units). Furthermore, a low correlation between the fresh exposure and inflammatory response to PM exposed *in vitro* was observed. Over-

all, aged carbonyls with PM go through dynamic changes and ultimately result in a strong correlation of aging and biological response. This finding is consistent with limited epidemiological studies, but have been considered insufficient for any conclusions (Schwarze 2006).

### *Gas Phase Exposures*

Research has shown that the polar gases found in urban mixtures tend to partition to PM (Madden 2000; Kamens 2001; Lee 2004; Donahue 2006; Hu 2007). As compounds become more oxidized, as would be the case at the end of the day, such compounds also tend to become less volatile and more likely to partition to the particle phase. As more polar compounds partition to the particle phase, the availability of those compounds for gas-phase exposures in GIVES will decrease, diminishing the response from the gas phase mixture (Ebersviller 2012a,b). By extension, gaseous compounds that partition to the particle phase would potentially increase the toxicity of the available particles. While this exposure result further support my hypothesis I cannot determine unambiguously if observed toxicity was a result of direct oxidation of the PM surface, or the partitioning of oxidized gas phase compounds to the particles. The processes described above are likely responsible for the lack of gas phase response and the increase in particle phase response observed in the M-P exposures described below.

The gas phase exposures for the atmospheres generated by the Mercedes and SynUrb54 showed that no significant biological response was seen from fresh test atmosphere. Significant increases in response were observed for the gas phase exposures

to the dark thermally-aged mixture of M-D. While this oxidation was not driven by the sun, these observations support my hypothesis that oxidation may increase toxicity.

Since previous experiments suggested photochemical oxidation causes a significant increase in toxicity in the gas phase, I expected that the gas phase response in this study would also be enhanced (Doyle 2004; Sexton 2004; Doyle 2007; Ebersviller 2012b). My data, however, indicate that the photochemical oxidation for the Mercedes emissions and the SynUrb54 did not increase the biological response in the gas phase exposure relative to the fresh emissions or control.

Due to the longer injection time required to match the PM concentration in the chamber, the 2006 Volkswagen plus SunUrb54 mixture had higher levels of $NO_x$ and NO relative to the 1980 Mercedes. From the data presented in Figure 4-2 and the high response from V-F in Figure 4-6, it would seem that the high levels NO may be responsible for the gas phase toxicity. There is not clear indication in the literature that NO alone, at the exposure levels in this study should be causing the observed biological response (Figure 4-6 V-F gas phase exposure) (Occupational Safety and Health Administration 2007). This increase in biological response from the fresh exposure should, therefore, be followed up in future studies to determine causality.

In the V-D exposure, the $NO_2$ concentration in my test atmosphere was (ca. 2.5 ppm; Figure 4-2). This concentration is comparable with the OSHA exposure limits of 1ppm-5ppm for short term 4 hour exposure periods. In addition to OSHA standards, other studies have shown that high levels of $NO_2$ cause inflammation and IL-8 produc-

tion (I. M. C. M. Rietjensa 1986; Stavert 1990; Utell 1991; Pilotto 1997; Rusznak 1998). It is possible, therefore, that the observed response in the gas phase exposure (V-D) could be caused by the high levels of $NO_2$. During the photochemically aged gas phase exposure I observed an increase in $NO_2$, but at a lower concentration than was observed in the dark aged. These levels, were near the low end of the OSHA exposure limit and at a three hour exposure duration may not have been sufficient to elicit a biological response.

During the V-D and V-P exposures, I observed an increase in carbonyl concentrations. These increases could also have resulted in the observed biological response in the V-D exposure but, the amount present may have been insufficient to elicit a response in the V-P exposure period. A likely explanation for the biological effects seen in the V-D gas phase exposure would be the combination of the increased $NO_2$ and carbonyl concentrations.

## Comparison of Emissions

One of the goals of this study was to compare a vehicle without emission controls and one with emission controls and determine if the presence of emission controls have an observable effect on toxicity.

### *Test atmosphere characterization*

Complex mixtures of this nature are comprised of known and unknown compounds mixed with PM. With the use of analytical methodologies, attempts were made to characterize this complex mixture present in each system. My findings indicated that the Mercedes had a higher concentration of carbonyls present than the Volkswagen

plus SynUrb54. This difference is of note because of the different sampling times for both vehicles, possibly resulting in higher concentrations due to sampling time. In contrast, due to the better emission controls on the Volkswagen, the catalyst rescued the PM and organics emitted. I could have expected, therefore, to see a higher concentration of carbonyls based on exhaust sampling time alone, except for the better emission control catalyst which reduces organics. If causality of biological response was due to the carbonyls present during the V-F exposure, and that the identities of the carbonyls present were the same, a greater biological response in the corresponding M-F gas phase exposures should have been elicited to complement the V-F biological responses. The response from M-F was, however, no different than the control, whereas the V-F elicited four-times the inflammatory response. I observed much higher NO and $NO_2$ concentrations in the Volkswagen chamber mixtures than in the Mercedes chamber mixtures. While the results may look like the emission controls did not improve the vehicle emissions, the large amount of $NO_x$ was due entirely to the longer injection time for the Volkswagen (discussed further below). For PM emissions, it is obvious that the Volkswagen's updated emission controls reduced the amount of PM produce by the engine. This was illustrated by Figure 4-1 and 4-2 describing the length of sampling time and PM concentrations observed in the chamber. These carbonyl measurements, however, in the Volkswagen exposure conditions were 0.5 ppmC lower compared to the Mercedes conditions.

Comparing the roles of particulate and gas-phase components in activating inflammatory responses requires more experiments to definitively answer the question of

whether one component is more responsible for the observed response than the other, or if both components play equal roles. Therefore, the modes of action need to be further evaluated to determine whether different pathways are responsible for the variability in observed response between vehicles. If exposures were conducted to unoxidized diesel plus SynUrb54 conclusions, at matched PM mass concentration, the newer Volkswagen would have been more detrimental to health than a vehicle without modern emission controls. Finally, the role of gases and particles to overall toxicity is complex, and varies with atmospheric dynamics (i.e. aging and mixing of sources).

## Comparison of emissions if duration of sampling was matched on time rather than PM concentration

I attempted to match the initial particle mass concentration between exposure sets, by sampling DE for different amounts of time. The 1980 Mercedes sampling for 30-49 seconds while the 2006 Volkswagen needed 16-25 minutes to reach a similar PM mass in the chamber (as determined by SMPS). If I had instead used time as a determinant for injection of diesel, the chamber PM concentration would not have been similar. To estimate the PM loading of the chamber air, if the experiments were matched on time, I can calculate that the 2006 Volkswagen plus SynUrb54 would have had a particle concentration of 0.04 mg/$m^3$ after a 30 second injection of diesel. Taking this number into consideration, for my hypothetical particle *in vitro* exposure I would have had to have sampled for 34 hours to deposit the same amount of particles on the cellular surface (~ 2.0 μg for all Mercedes exposures). This drastic difference in exposure duration is unreasonable, as cellular viability would likely be compromised with such a long exposure.

Continuing the hypothetical experimental protocol described above, all other chemical measurements would also have changed. For example, the NO and $NO_x$ concentrations would have been reduced from 0.44 and 5.22 to 0.22 and 0.02 ppm. Initial total carbonyl concentration would have been reduced from 0.55 to 0.01 ppmC. By reducing the emissions drastically I would expect that the biological response would also be reduced.

This hypothetical scenario is, of course, a simplification of a very complex and dynamic system. This example, however, is a useful illustration of how choosing any normalizing factor to standardize emissions sampling can result in different test atmospheres and in turn resulting in different biological responses.

## Conclusions

This study found that atmospheric processing and compositional differences can affect toxicological outcomes (as IL-8 expression). There range of variation in toxicity for a unit of PM exposure is so large, that there is no simple correlation between IL-8 and PM exposure in terms of mass, arguing against a mass based PM standard. A strong correlation was observed as a result of comparing aged atmospheres with biological response for both vehicles. While the composition did change, the resulting biological response was able to be correlated to the aged atmospheres for both vehicles. My results, therefore, have shown that atmospheric processes (thermal and photochemical) do modify the composition of atmospheres that contain diesel exhaust and this compositional modification can result in a modification of biological responses following exposures to the atmospheres, if sufficient material is actually present. Further, the evi-

dence suggests that there is an additional difference in toxicity caused by the different chemical processes involved in thermal aging and photochemical aging. The lower amount of carbonyls along with a lower PM exposure in the Volkswagen PM may explain the lack of biological response from the EAVES exposures. In addition, I can attribute this to the new emission control catalyst that had an added benefit of the reduction of initial PM and VOC's. Overall, this study and the complex analysis hopes to show that PM mass is insufficient as a basis for health standards alone. Instead PM should be considered being combined with VOC concentrations and accounted for atmospheric changes to fully understand the true potential toxicity.

These conclusions illustrate the importance of particles as a vehicle for delivering carbonyls, and the results further suggest additional experiments to discover how the distribution gas and particle toxicity shifts as the particle concentration decreases. Atmospheres may exist where the gaseous VOC's are prevalent but ambient PM concentrations may be low and in this case more toxicity may be observed in the gaseous carbonyl oxidation products that cannot partition due to insufficient PM present. Additional experiments can be designed to investigate this phenomenon and its consequences.

## Figures and Tables

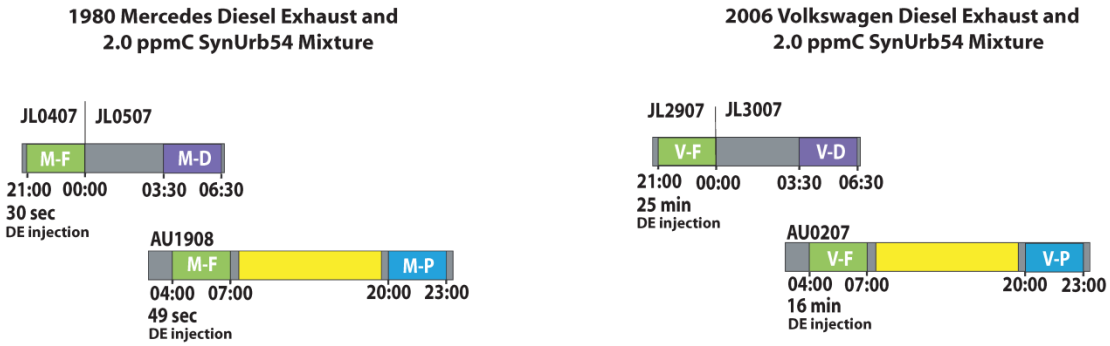


Figure 4-1. Each bar represents a full chamber experiment conducted on the date(s) and times indicated. M represents the use of the older Mercedes and V represents the use of the newer Volkswagen. Duration of diesel exhaust injections are given at the beginning of each bar. The green windows represent the exposure to the 'fresh' conditions (F); the dark gray bar represents aging in the dark; the yellow bar represents aging in sunlight; the purple windows represents the exposure to the 'dark aged' conditions; the blue windows represents exposure to the "sun aged" conditions.

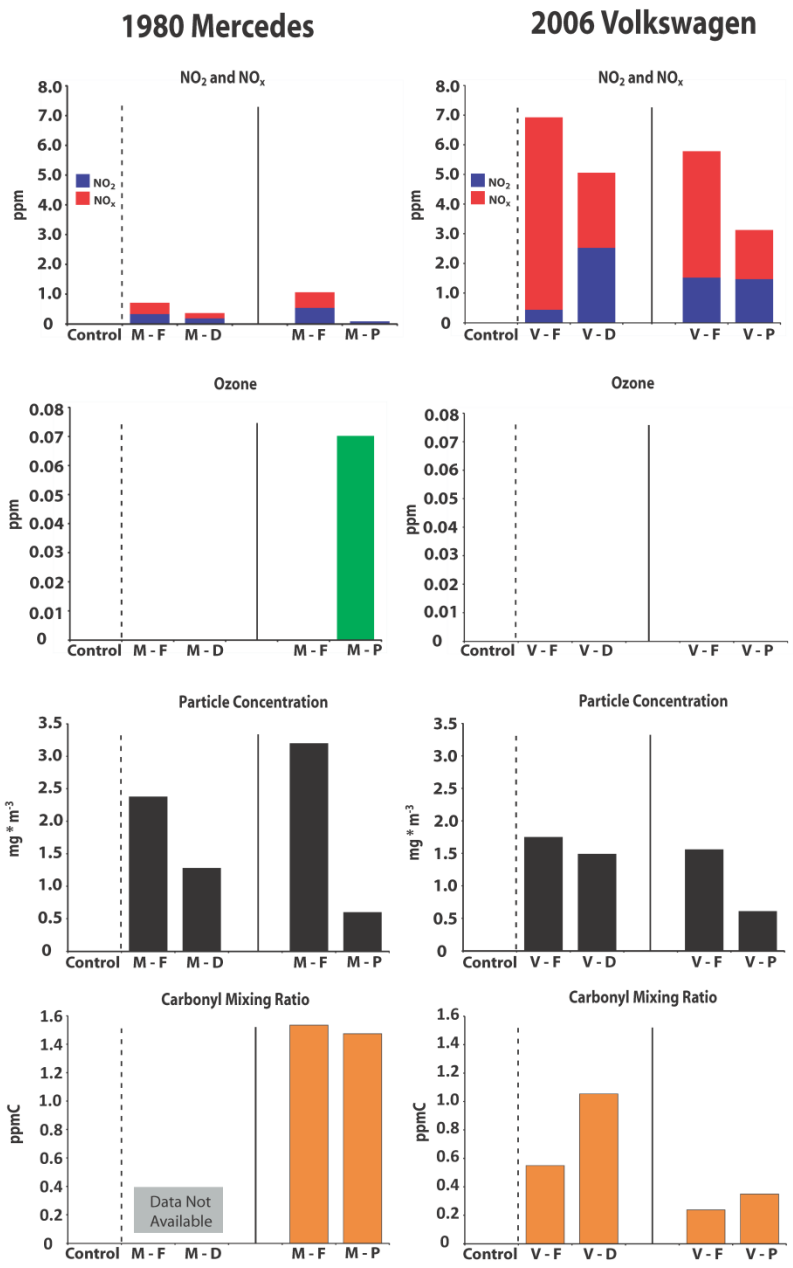


Figure 4-2.Each bar height represents average conditions during the five exposure periods labeled in Fig. 1. The left side plots are for the Mercedes emissions along with 2 ppmC of SynUrb54 VOC mixture and the right are the same for the Volkswagen with 2 ppmC of SynUrb54 injected.

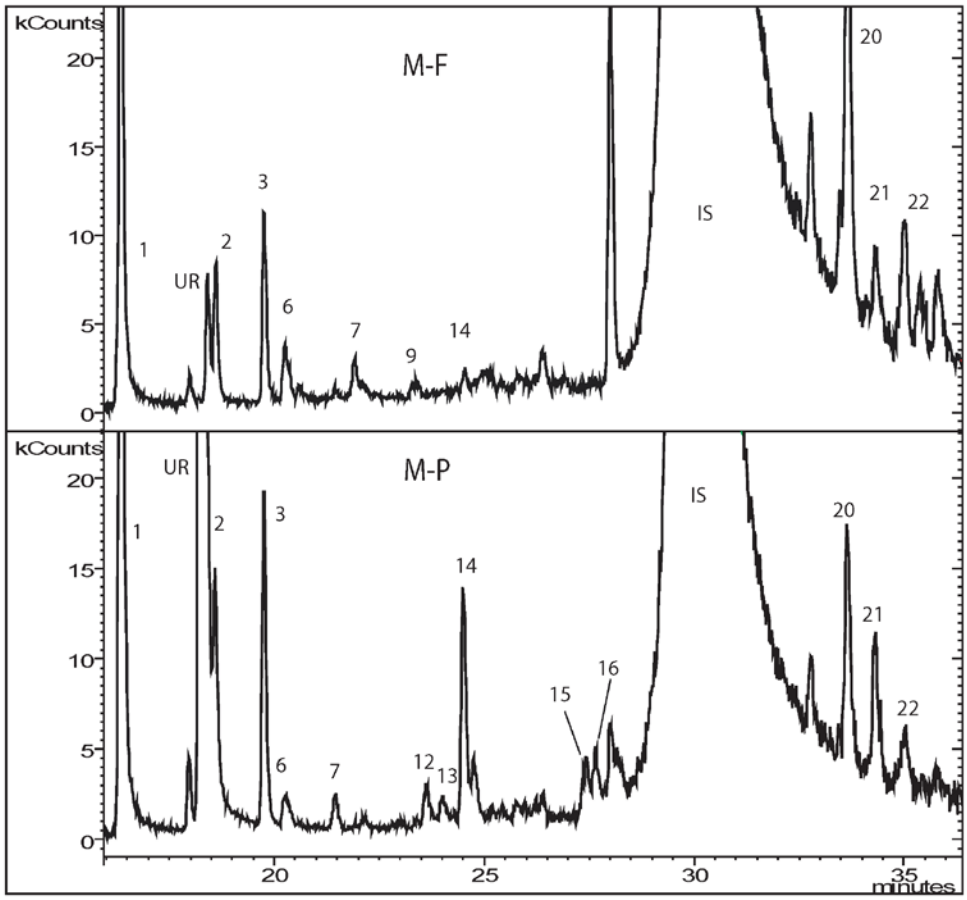


Figure 4-3. Carbonyls Detected in Photochemically Aged SynUrb54 Mixture and Exhaust of 1980 Mercedes. Selective-ion chromatogram for the injections from the 1980 Mercedes and 2.0 ppmC of SynUrb54 in the chamber. Mercedes fresh chamber contents is labeled (M-F), and Mercedes photochemically aged chamber contents is labeled (M-P). Samples were collected after daylong irradiation. The peak numbers correspond to the species identified in Table 1. UR – Unreacted PFBHA solution, IS – Internal Standard, and some of the major peaks are 1 – formaldehyde, 3 – acetaldehyde, 6 – methyl ethyl ketone, 8– 2-pentanone, 10 – 2-hexanone, 14 – glyoxal, and 15 – methylglyoxal.

Table 4-1. List of carbonyl compounds detected in the Mercedes fresh (M-F) and photochemically oxidized (M-P) exposure test atmospheres. Measurements reported in ppmC.

| Identification # | Compound | Fresh Concentration (ppmC) | Photochemically Aged Concentration (ppmC) |
|---|---|---|---|
| 1 | formaldehyde | 0.878 | 0.642 |
| UR | Unreacted PFBHA | ---- | ---- |
| 3 | acetaldehyde | 0.033 | 0.017 |
| 4 | acetone | 0.027 | 0.029 |
| 5 | hydroxyacetaldehyde | 0.033 | 0.009 |
| 6 | methylethylketone | 0.007 | 0.014 |
| 7 | n-butanal | 0.015 | 0.000 |
| 8 | 2-pentanone | 0.027 | 0.082 |
| 9 | pentanal | 0.014 | 0.044 |
| 10 | 2-hexanone | 0.152 | 0.218 |
| 11 | hexanal | 0.000 | 0.069 |
| 12 | 2-heptanone | 0.000 | 0.084 |
| IS | Internal standard | ----- | ----- |
| 14 | glyoxal | 0.179 | 0.121 |
| 15 | methylglyoxal | 0.097 | 0.131 |
| 16 | biacetyl | 0.072 | 0.014 |

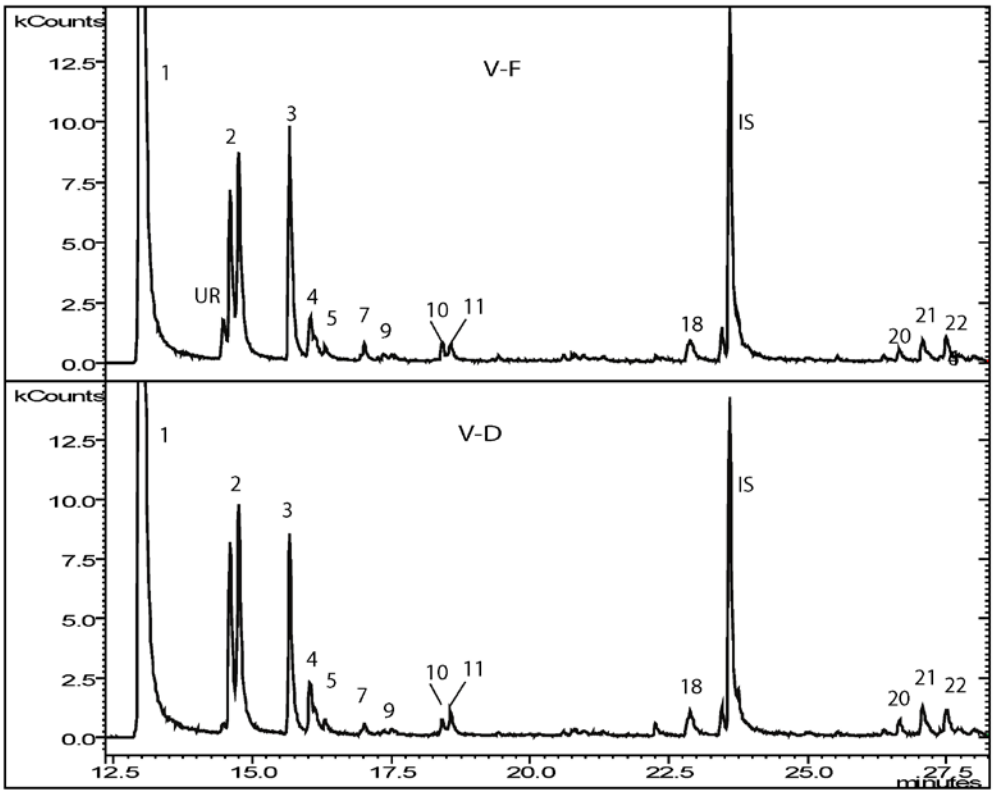


Figure 4-4. Carbonyls Detected in Dark Aged SynUrb54 Mixture and Exhaust of 2006 Volkswagen. Selective-ion chromatogram for the injections from the 2006 Volkswagen and 2.0 ppmC of SynUrb54 in the chamber. Samples were collected after thermal aging in the dark. The peak numbers correspond to the species identified in Table 2. UR – Unreacted PFBHA solution, IS – Internal Standard, and some of the major peaks are 1 – formaldehyde, 2 – acetaldehyde, 3 – acetone, 4– acrolein, 20 – glyoxal, and 21 – methylglyoxal Table2. List of carbonyl compounds detected in the Volkswagen fresh (V-F) and thermally oxidized (V-D) exposure test atmospheres. Measurements reported in ppmC.

Table 4-2. List of carbonyl compounds detected in the Volkswagen fresh (V-F) and thermally oxidized (V-D) exposure test atmospheres. Measurements reported in ppmC.

| Identification # | Compound | Fresh Concentration (ppmC) | Dark-Aged Concentration (ppmC) |
|---|---|---|---|
| 1 | formaldehyde | 0.371 | 0.696 |
| 2 | acetaldehyde | 0.024 | 0.021 |
| 3 | acetone | 0.024 | 0.018 |
| 4 | acrolein | 0.017 | 0.021 |
| 5 | propanal | 0.008 | 0.007 |
| 7 | methylethylketone | 0.006 | 0.004 |
| 8 | methylvinalketone | 0.003 | 0.004 |
| 9 | n-butanal | 0.002 | 0.003 |
| 10 | 3-methylbutanal | 0.004 | 0.005 |
| 11 | 2-but-2-enal | 0.007 | 0.012 |
| 18 | 2-cyclohexene-1one | 0.061 | 0.23 |
| 20 | glyoxal | 0.005 | 0.006 |
| 21 | methylglyoxal | 0.005 | 0.017 |
| 22 | biacetyl | 0.005 | 0.004 |

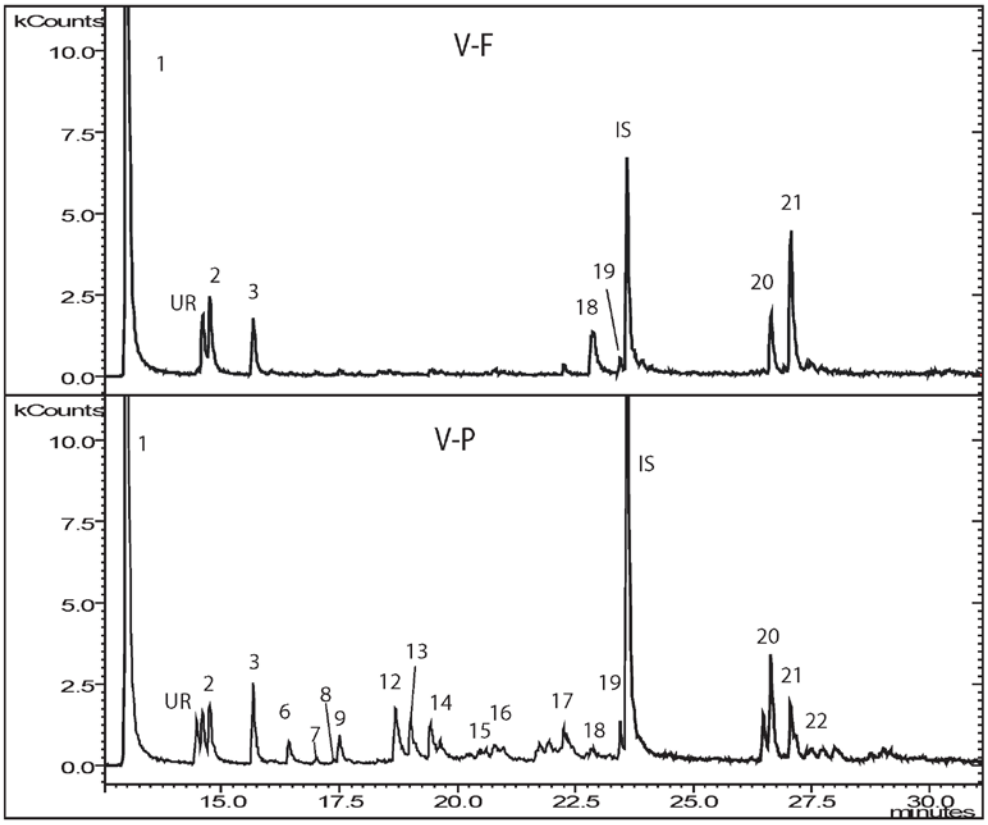


Figure 4-5. Carbonyls Detected in Photochemically Aged SynUrb54 Mixture and Exhaust of 2006 Volkswagen. Selective-ion chromatogram for the injections from the 2006 Volkswagen and 2.0 ppmC of SynUrb54 in the chamber. Samples were collected after daylong irradiation. The peak numbers correspond to the species identified in Table 3. UR – Unreacted PFBHA solution, IS – Internal Standard, and some of the major peaks are 1 – formaldehyde, 18 – 2-cyclohexene-1one, 20 – glyoxal, and 21 – methylglyoxal Table 3.

Table 4-3. List of carbonyl compounds detected in the Volkswagen fresh (V-F) and photochemically oxidized (V-P) exposure test atmospheres. Measurements reported in ppmC.

| Identification # | Compound | Fresh Concentration (ppmC) | Photochemically Aged Concentration (ppmC) |
|---|---|---|---|
| 1 | formaldehyde | 0.106 | 0.114 |
| 2 | acetaldehyde | 0.004 | 0.005 |
| 3 | acetone | 0.003 | 0.005 |
| 6 | hydroxyacetaldehyde | 0.000 | 0.004 |
| 7 | methylethylketone | 0.000 | 0.002 |
| 8 | methylvinylketone | 0.000 | 0.001 |
| 9 | n-butanal | 0.000 | 0.004 |
| 12 | 2-pentanone | 0.000 | 0.016 |
| 13 | pentanal | 0.000 | 0.016 |
| 14 | 2-hexanone | 0.000 | 0.043 |
| 15 | hexanal | 0.000 | 0.019 |
| 16 | 2-heptanone | 0.000 | 0.021 |
| 17 | 4-OH-4methyl-2-pentanone | 0.000 | 0.016 |
| 18 | 2-cyclohexene-1one | 0.090 | 0.036 |
| 19 | acenaphthenequinone | 0.002 | 0.020 |
| 20 | glyoxal | 0.008 | 0.014 |
| 21 | methylglyoxal | 0.023 | 0.008 |
| 22 | biacetyl | 0.001 | 0.005 |

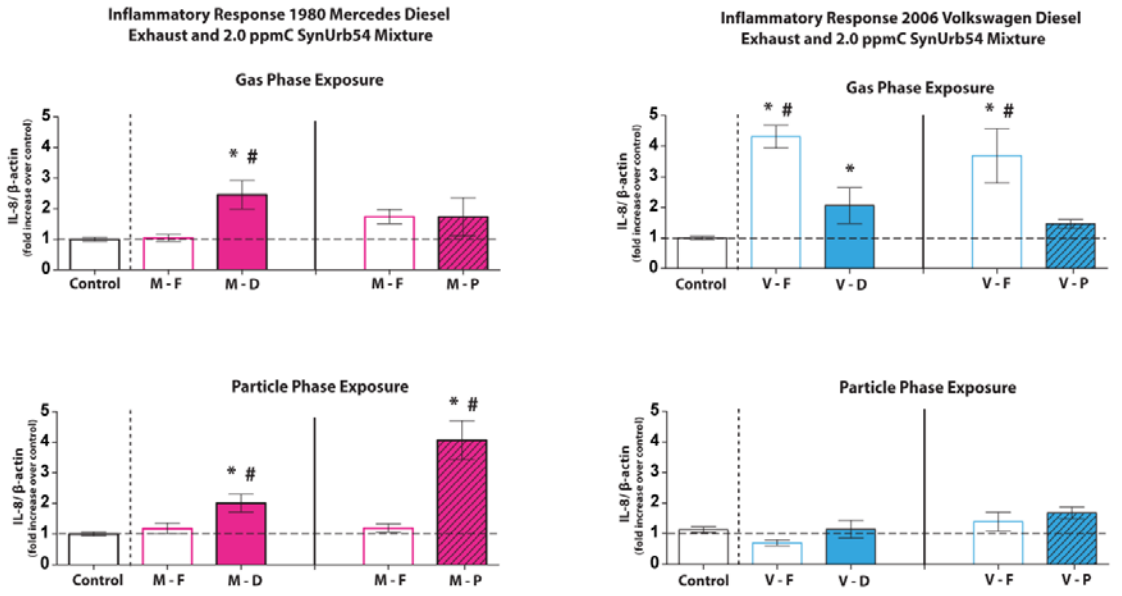


Figure 4-6. All bars are IL-8 m-RNA expression induced by exposure and controls. The exposure conditions are labeled the same as in Figure 1. In both sets, the top bar plots are responses for gas-phase only exposures and the bottom bar plots are responses for the particle-only exposures. Exposure duration for gas-only exposures were 3-h. Exposure for particle phase was ca. 2.0 µg for the Mercedes and 1µg for the Volkswagen. The symbol * indicates statistically different from non-exposed control; # indicates statistically significant difference between fresh and oxidized exposures. The error bars represent the mean ± standard error from the mean.

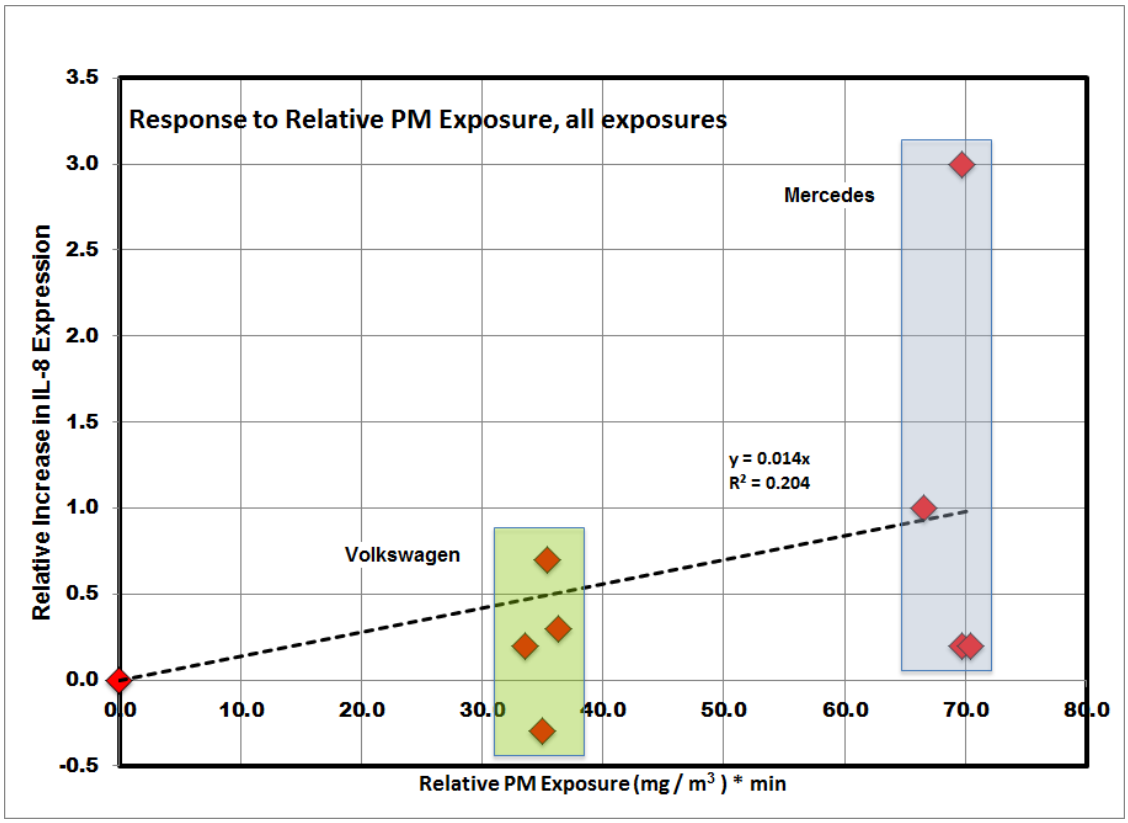


Figure 4-7. Relative PM exposure relative to the IL-8 inflammatory response using the EAVES device for the Mercedes and Volkswagen and all exposure conditions (fresh, thermally aged, and photochemically aged). The red diamonds represent the relative PM exposure to increase in response for the Mercedes exposures highlighted in the blue box, and the orange diamonds represent the Volkswagen exposures highlighted in the green box. Incubator controls are all at zero. A trend line was added to show association with an $R^2$ value of 0.204.

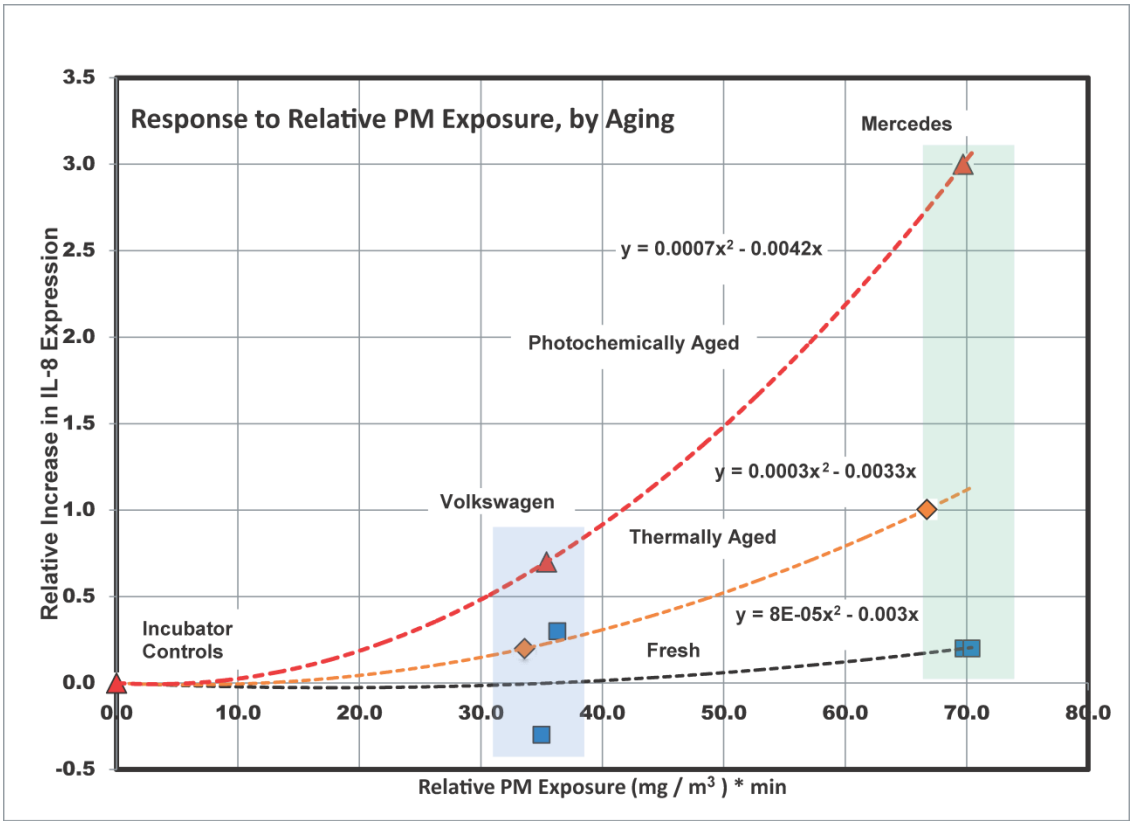


Figure 4-8. Inflammatory response relative to PM exposure that was grouped by chamber condition (fresh, thermally aged, and photochemically aged) using the EAVES device. All Volkswagen exposures are highlighted in green and Mercedes in blue. Incubator controls are all at zero. Trend lines were added to each grouping: Photochemical and thermal aging with a first order fit of $R^2$ of 0.8905 and 0.8680, and fresh exposures with an $R^2$ of 0.1462.

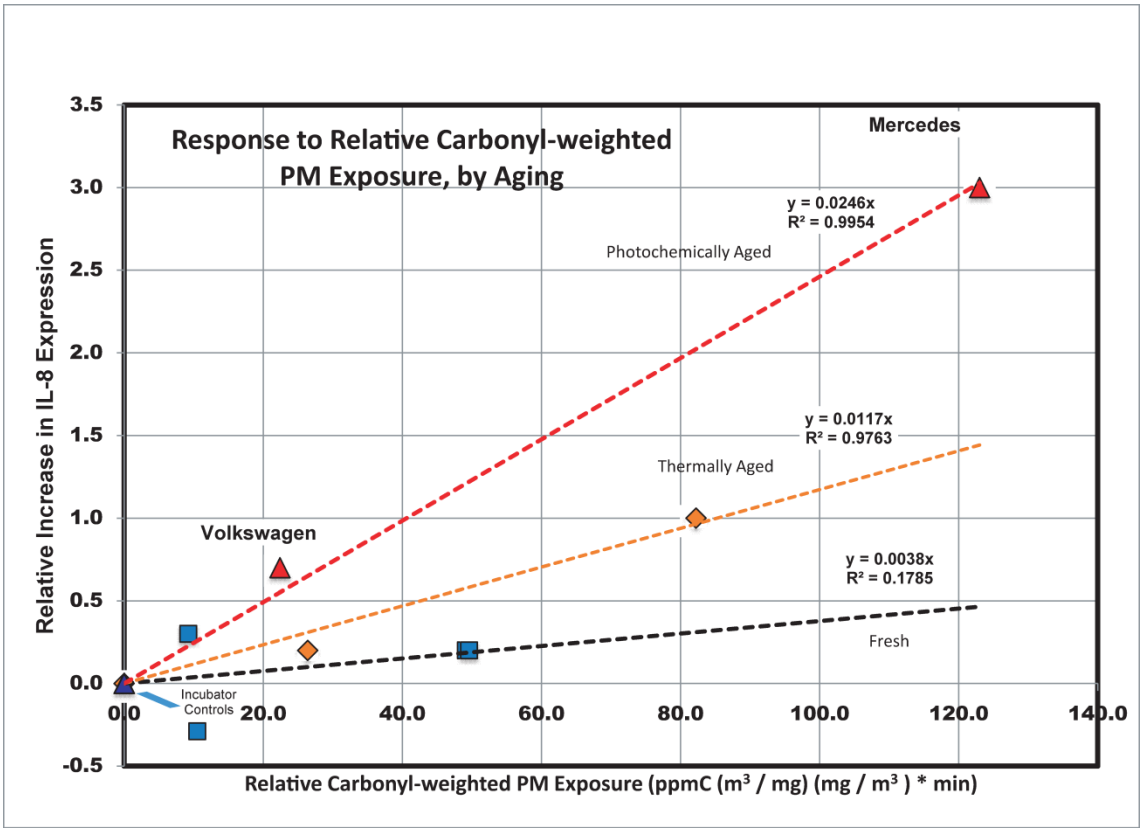


Figure 4-9. Inflammatory response relative to the carbonyl-weighted PM exposure concentration and subsequently grouped by chamber conditions (fresh, thermally aged, and photochemically aged). Trend lines were added to each grouping: Photochemical aging with an $R^2$ of 0.9954, thermal aging with an $R^2$ of 0.9763 and fresh exposures with an $R^2$ of 0.1785.

# Chapter 5 : Overall Conclusions, Limitations, Implications, and Future Work

I begin my discussion by reviewing my hypothesis and rational for this work and follow with a brief description of the findings. I proceed to describe the experimental conditions established to test my hypothesis that atmospheric processes will alter the composition of urban-like atmospheres containing diesel exhaust that will in turn result in a modification of biological responses from exposure. Next, I discuss the extent to which I was successful in achieving test atmospheres followed by a review of the biological responses relative to each experimental study. I next describe the limitations of this work and discuss whether these limit me in the conclusions about my hypothesis. Finally, I conclude with the implications of the findings and the future work that could follow as a result of this study.

## Hypothesis

My central hypothesis was that atmospheric processes will alter the composition of urban-like atmospheres containing diesel exhaust that will in turn result in a modification of biological responses from exposure.

This study used complex atmospheres of a mixture of gases and particles designed to represent a more holistic and integrated approach to examine *in vitro* inflammatory response. I attempted to minimize ambiguity by using immortalized cell lines and test

atmospheres that could be controlled and quantified. In addition, my study took quantitative measurements of the test atmospheres to understand the fundamental causes in observed differences in response from technique and aged atmospheres. Nevertheless, performing such experiments was not simple and my goals were not always achieved.

To test my hypothesis I needed a method that could be used to expose cells to PM in which the PM would not be significantly altered by the method, especially one in which partitioned organic compounds on the PM would not be lost. Fortunately, such a method, Electrostatic Aerosol *in Vitro* Exposure System (EAVES), was under development by our research team (Lake 2005; de Bruijne 2009); my task became one of evaluation of the performance of this sampling and exposure method for PM. This work was reported in Chapter 2 and was published in 2009 (de Bruijne 2009). To help demonstrate the uniqueness of this sampling/exposure method, I followed the initial characterization work with a comparison to an accepted and commonly used method, filter collection and resuspension in media, or 'resuspension method' for short. In this direct comparison study reported in Chapter 3, (i.e., simultaneous sampling of a complex gas/PM atmosphere with both methods), I hypothesized that in the resuspension method, the separation of phases and the dissolving of organic material in the media would result in loss of observed toxicity from the particles compared to the direct deposition of PM while in equilibrium in the EAVES; this was clearly demonstrated. These foundational contributions permitted me to proceed to my main work in which the combination of a 'virtually' gas-only exposure system (GIVES) and a 'virtually' PM-only exposure system (EAVES) would permit a meaningful investigation of biological effects in a com-

plex, dynamic, urban-like gas and real diesel exhaust (emitted gas and PM) system. Only when I could say that I had a viable method to expose cells to a complex mixture containing both gases and PM was I able to proceed to answer my overall hypothesis. This study exposed human epithelial lung cells to different fresh and aged test atmospheres of diesel exhaust. The capability of the exposure methods along with the physical and chemical analysis combined allowed me to conclude that atmospheric aging does alter the composition so that the biological response is affected.

## Summary of findings

Before I could begin to answer my central hypothesis it was necessary to optimize a modified electrostatic precipitator (ESP) and determine if it could be an efficient method for particle deposition onto cell culture membranes. This initial experimental design was crucial to answering the overall hypothesis, because I needed a device that could directly deposit PM on the surface of the cell culture membrane without generating adverse effects from the method itself. In Chapter 2, I report that the modified ESP or EAVES was a successful alternative to conventional exposure methods for *in vitro* exposures to particulate matter. In addition the EAVES demonstrated that: (1) no significant cytotoxicity or inflammatory mediator production occurs from cellular exposure to the electrical field; (2) no cellular response was observed due to the low ozone concentration produced by the corona wire; (3) no cellular response was observed due to the electrical charges added to the PM; (4) no secondary organic aerosol formation was observed from very-reactive VOCs flowing through the sampler; (5) no cellular response was observed following exposure to toxic gases without PM present; and (6) no re-

sponse was observed when mixtures of toxic gases and PM pass through the sampler without the deposition field on, while a significant response was observed from the same mixture when deposition voltage field is applied. These findings provided me with the necessary information to determine that the EAVES is a method that could be used to evaluate the inflammatory response to inhaled particulate matter without causing alteration in chemical composition and loss of biological response.

The next step was to determine how my new method from Chapter 2 would compare to a traditional particle exposure method using a resuspension technique. The main hypothesis of Chapter 3 was that the separation of phases and post-collection treatment of filter-collected PM significantly modifies the toxicity of the PM, resulting in a distorted view of the potential ambient PM health effects compared to that elicited by direct exposure to the PM. My data indicated that the biological response was changed by extensive sample handling and modification of the PM during the resuspension method. Furthermore, when I compare the composition of the carbonyls present during the direct deposition exposures to those measured in the resuspension media, it was apparent that the composition had been modified. Not only had the carbonyl composition been modified in the resuspension media, the carbonyl-containing species have completely disappeared. In addition, the particles had agglomerated and once resuspended in liquid they settled very slowly with only the largest particles likely to reach the cellular surface. This alone made it difficult to accurately determine the amount of PM delivered to the cells.

On the other hand, neither the chemical composition nor PM size distribution was modified prior to exposure by the EAVES sampler, as demonstrated in Chapter 2. Also, by exposing at an air-liquid interface the EAVES sampler delivers all PM, regardless of size, to the cellular surface. Moreover, with the EAVES sampler, I am able to reliably estimate the amount of PM that actually reached the cellular surface by using collection efficiencies measured during initial development of the method (Chapter 2). With all the information combined, in Chapter 3 it was concluded that separation of phases and post-collection treatment of filter-collected PM significantly modifies the ambient toxicity of the PM resulting in little to no response from the resuspension material. This went on to show that the EAVES device would be a better method to be coupled along with the GIVES exposure device that had already shown to work with gas phase exposures. Using these two exposure devices it made it possible to conduct the complex study described in Chapter 4.

Chapter 4 set out to demonstrate that photo-oxidation and partitioning processes will enhance the toxicity of both the gaseous and particulate phases in my urban-like test atmospheres. From previous studies determining the existence of gas and particle phase interactions, Ebersviller et.al used photochemically aged SynUrb54 mixture and added a nontoxic PM sourced (mineral oil). The interaction between the gas phase and particles induced increase in cellular toxicity when chamber contents were photochemically aged by directly modifying the chemical composition of the PM. In addition, that study suggested that the toxicity inherent to one phase of an air pollution mixture can affect the toxicity of another (Ebersviller 2012a; Ebersviller 2012b).

To test my hypothesis, two test atmospheres were generated using diesel exhaust (DE) from an older vehicle and newer vehicle that were subsequently modified by either thermal or photochemical oxidation. Changes were evident in biological effect and chemical composition, but varied by source and oxidative environment. I have shown that atmospheric processes (thermal and photochemical) modify the composition of atmospheres that contain diesel exhaust. Significant increases in response were observed for both the gas and particle phase exposures to the dark thermally-aged mixture of Mercedes emissions and the SynUrb54. My data, however, indicated that the photochemical oxidation for the Mercedes emissions and the SynUrb54 did not increase the toxicological response in the gas phase exposure relative to the fresh emissions or control. In contrast, the particle phase response did increase with photochemical aging four-fold over the fresh (M-F) and control exposures. Unlike the 1980 Mercedes, exposure to fresh gaseous DE emissions from the 2006 Volkswagen Beetle resulted in a four-fold higher response than the control. After the contents of the chamber were oxidized, the toxicological responses disappeared. In contrast, the Volkswagen exposures using EAVES (fresh and aged), showed no significant difference in response compared to the controls, but did show an increase. To gain further understanding of my observation the use of three comparative scatter plots in increasing complexity was used to evaluate the biological data to the chemical and physical data. From this I concluded that PM mass concentration alone is not an appropriate explanation for the PM exposure response for the endpoints measured. In actuality it is the combination of the aged atmosphere relative to the total carbonyl concentration along with the PM concentration that is associ-

ated for the biological outcome. This finding is consistent with limited epidemiological studies, but have been considered insufficient for any conclusions (Schwarze 2006). In addition the Volkswagen emission that incorporates a catalyst reduced the organic content and particle mass concentration for the exhaust. These lower concentrations resulted less reactivity than the Mercedes test atmospheres. In summary, I showed in Chapter 4 that atmospheric processes (thermal and photochemical) do modify the composition of atmospheres that contain diesel exhaust. This compositional modification can therefore result in a modification of biological responses following exposures to the atmospheres.

## Conclusions

To be able to demonstrate atmospheric aging modifies biological effects unambiguously, I first had to establish that the chamber and exposure systems would not, by themselves, induce any biological effects. Although 'Clean Air' exposures were not preformed prior to each experiment, several Clean Air test atmospheres were created in the chamber throughout this dissertation work. These tests were periodically conducted to make sure that the chamber was in fact clean, and would not cause a biological response by itself. To illustrate, in Chapter 2, I conducted exposure to clean air through the EAVES device and saw no response. In addition Ebersviller et al. (2012b) performed clean air exposures in very different and no response was measured from either control exposure. In addition these observations suggest that the response observed during the clean air exposures includes effects that might have been caused by sampling artifacts of the *in vitro* exposure systems (EAVES and GIVES). Therefore, any chance that my ob-

servations were influenced by some unknown inherent quality of the chamber itself can be dismissed. What this means for my work is that any response observed is from the test atmospheres and not the chamber itself.

There was some variability observed in the aged exposure conditions in Chapters 3 and 4. While absolute replication of exposure conditions is ideal for toxicology studies, some variability is to be expected when studying systems as complex as the ambient environment. Moreover, this variability should not be viewed as limiting to my ability to make conclusions from my data. Rather, the variability is illustrative of the complexity of the systems in question, as well as a warning against assumptions regarding the reactivity of the atmosphere and its resulting composition. To accommodate the variability in composition, and to reduce ambiguity in my exposures, speciation measurements were reported wherever available. This is enough to demonstrate that biological effects have been modified by the change in composition.

To study such a complex mixture I had to have a metric by which to match to compare each vehicle. I chose to match particle concentration in the chamber, which is easily justifiable given that there is a national air quality standard for PM. To provide an urban-like atmosphere in the chamber, I added the SynUrb54 mix. The addition of SynUrb54 requires only one syringe injection and one cylinder injection. I feel confident that the injections were performed successfully. Emissions from the two test vehicles were different, which was to be expected. Although I expected the composition to be different because of emission controls, the time required to sample the emissions from

the newer vehicle was longer than expected. Since the newer vehicle exhaust was sampled far longer than the Mercedes, the levels of NO and $NO_x$, were so high that there was never measurable ozone produced. Even though measurable ozone was not produced, I was able to show that oxidation occurred in the chamber. Moreover, the oxidation significantly modified the composition and biological response to the atmosphere sampled from the chamber.

Throughout the exposures described in this work, dilution of the chamber contents occurred as a result of removing chamber air during sampling 5 %. It has been demonstrated before that the introduction of ambient air due to dilution does not measurably affect the biological response to the test atmospheres for the endpoints measured here (Ebersviller 2012b).

This research does not attempt to directly model the atmosphere, but provides an aging environment with appropriate atmospheric processes and urban components. Frequently, I am posed with the question of "atmospheric relevance." This is an important question, but this demonstration of preliminary complex mixture exposures provides an example of the capabilities of the methods used in the laboratory study. Future research could employ and improve these methods to concentrations that are more “relevant” to the United States.

From the biological exposures, I have observed that the EAVES device is a viable and useful and probably necessary method for PM exposure containing soluble components. Resuspension exposure methods which rely on extractive processes may result in

modification of chemical composition of particles and cause agglomeration by filter collection and resuspension in solution. I have shown that the separation of phases modifies the biological effect of PM. What this means for biological exposures is that when PM is extracted the material used for exposure may not resemble the PM in the atmosphere. By extension, the observed biological response may misrepresent the actual toxicity of the ambient particles.

In Chapter 4, I showed that the fresh and aged emissions chemical and physical characteristics were very different depending on the vehicle sampled. In addition, the manner in which the test atmosphere was oxidized also determined the observed biological responses. Research has shown that the polar gases found in urban mixtures tend to partition to PM (Kamens 2001; Lee 2004; Donahue 2006; Hu 2007). As compounds become more oxidized, as would be the case at the end of the day, such compounds also tend to become less volatile, more polar, and more likely to partition to the particle phase. As more polar compounds partition to the particle phase, the availability of those compounds for gas-phase exposures in GIVES will decrease, diminishing the response from the gas phase mixture (Ebersviller 2012a,b). By extension, gaseous compounds that partition to the particle phase would potentially increase the toxicity of the available particles. While this exposure result further support my hypothesis I cannot determine unambiguously if observed toxicity is a result of direct oxidation of the PM surface, or the partitioning of oxidized gas phase compounds to the particles. The processes described above are likely responsible for the lack of gas phase response in some cases and the increase in particle phase response observed in some of the aged expo-

sures. The latter is probably dependent on the amount and type of oxidized compounds actually present in the PM.

From these studies, I can accept my hypothesis that atmospheric processes do alter the chemical composition and biological effect of diesel exhaust oxidized in an urban-like environment, with the qualifier that the degree of the effect is dependent on the amount of toxic components deposited on the cells. Further, I can conclude that the source and aging environment of the test atmosphere play important roles in complex mixture toxicity.

## Limitations

A chamber is essentially a snapshot of a parcel of air that has been isolated. Because of this isolation, the chamber simulations will not include continuous injections of mixtures of primary gases and particles. In addition, it also will not include the large dilution and mixing of 'aged' air masses from aloft. These additional dynamic processes are secondary effects for the chemical conversion of the primary initial pollutant loading and transformations. The missing emissions/species, however, do not invalidate the findings of this demonstration.

In the experimental design I did not include exposures during the midpoint of the oxidation periods. Previous studies, however, have indicated that peak toxicity in photochemically aged atmospheres may occur before sunset (Doyle, 2004; Doyle 2007). Therefore, in the future, studies evaluating the modification of test atmospheres should include *in vitro* exposures midday to accompany chemical and particle composition

measurements. Although the midday exposures were missing, it should not be viewed as an oversight in the experimental design. This work was a preliminary demonstration of the capabilities of conducting experiments in the outdoor smog chamber containing complex mixtures of gases and particles. Therefore, the addition of the midday exposure is a suggestion of how to produce a more-complete evaluation of the processes and modification of the test atmospheres.

Since this work was a preliminary attempt to find effect of atmospheric modifications on biological response to diesel exhaust, I chose to use *in vitro* exposures. These types of exposures do not account for all interactions that occur in the human body. Further, because this is not a whole body exposure, the inflammatory markers that are induced will only give an indication of the potential toxicity, not an actual health outcome, which is likely to include a cascade of systemic effects. *In vitro* techniques are, however, an accepted alternative to human exposures for pollutants whose effects might be unknown.

One of the main concerns with using an immortalized cell line is that they are not primary cells, but they were chosen as a control for inter-personal variability in response to exposure. If I had chosen primary cell cultures they would have required 28 days to become fully differentiated and I would have had limited window in which to conduct the exposures. Since this research was dependent on natural sunlight, using the A549 epithelial lung cells, which are confluent on the cell culture inserts in two days, were more advantageous.

Limited endpoints were originally selected for the initial analysis of all the diesel samples. Those selected (IL-8 and COX-2) are easily detected and associated with *in vitro* work using A549 cells, and can also be directly associated with effects found in humans. IL-8 was used in the majority of this study because it is resistant cytokine to biochemical influences, such as pH, temperature, denaturing chemicals and enzymatic reactions (Brody 1993). Once inflammation sets in, IL-8 molecules are not easily reduced inside the body, therefore are a good indicator of cellular stress. These inflammatory markers were chosen because laboratory animal and human *in vivo* studies have shown that inhalation of diesel exhaust (DE) increases markers of inflammation, including inflammatory cytokine production (Salvi 2000; Singh 2004). Studies using *in vitro* models to expose epithelial cells to DE have also shown increases in the production of inflammatory cytokines, such as Interleukin-8 (IL-8) and tumor necrosis factor-alpha (TNF-$\alpha$), following exposure (Kim 2005; Seagrave 2007).

Variability of engine emissions, complicated the interpretation of my results. Ideally I would have identical emission samples for each vehicle, used one tank of fuel for all experiments, and conducted all the experiments over a shorter period of time. Although these complications caused my data set to be imperfect, no data set is perfect, and they did not prevent me from demonstrating my hypothesis.

Filter collection and extraction techniques are popular because of their ease of use and relatively low cost. My data indicate that extensive sample handling and modification of the PM during the resuspension method (Chapter 3). Furthermore, when I

compare the composition of the carbonyls present during the direct deposition exposures to those measured in the resuspension media, it is apparent that the composition has been modified. Not only has the carbonyl composition been modified in the resuspension media, the carbonyl-containing species have completely disappeared. As reported by Cooney *et al.*, particles can be seen to agglomerate when resuspended in liquid.(Cooney 2011) Using SEM, they showed the mode particle diameter of the resuspended DE increased relative to non-suspended DE. In addition, particles in liquid settle very slowly at these particle diameters, with only the largest particles likely to reach the cellular surface. This means that during a resuspension exposure not all the particles will deposit onto the cellular surface to cause a response prior to RNA collection, making it difficult to accurately determine the amount of PM delivered to the cells.

## Implications

The findings included in this dissertation work have important implications for the air pollution field.

Laboratory based studies set out to explain and replicate the findings of epidemiological studies. To this point, this has been largely limited to complex mixtures containing diesel PM and urban VOCs, and as a result can be explained by some of the findings here. Important findings have major implications, however, in that the studies presented here attempted to close some of the gap between epidemiology and laboratory based toxicology. A primary finding in this research was that oxidation, whether photochemical or thermal, is an important factor in understanding the toxicity of air pollution. In addition, other studies conducted of complex mixtures or PM using traditional meth-

ods for PM exposure may underestimate the importance of the complex dynamic mixture on the particle surface. In addition, disrupting the equilibrium between the gas and particle phases alters the composition and possibly underestimating the dose required to observe a biological response. By omitting these factors in experimental design or the interpretation of the results, the data collected may only represent a small fraction of the exposures that occur in the ambient environment. Since all of these processes are driven by concentration factors, any modifications to the exposure stream will likely result in a misrepresentation of the actual effect seen in the ambient environment.

From my findings it is important to consider that vehicle emissions (and other PM) may not be relatively potent as they enter the atmosphere. Once the emissions are in the urban environment they are able to react quickly and their composition and biological effect changes. These changes may or may not affect the toxicity dramatically, but are dependent on the availability of organic compounds which contribute to the enhancement of PM toxicity. In addition, the decrease in toxicity from the oxidized gas phase exposure and the increased toxicity in the oxidized particle exposure implied that the movement of toxicity occurs. This movement of toxicity to the particle phase is important since it could be the carrier of highly toxic gas phase components into the deep lung where it can persist for long periods of time. Thus, the policy and risk assessment communities should consider the role of PM as a means of delivery of gas-phase toxics into regions of the lungs they may not normally reach and consider this as evidence of processes that explain the relationship of gases and particles in health risks. All of these

findings argue against maintaining a mass based PM air quality standard without incorporating the PM composition too.

Currently regulations and policies are based on emissions from a particular source or classes of sources. Although in certain circumstances this may be the right approach, complex mixtures are never alone and are not static. Because of this, they begin to change as soon as they enter the atmosphere. These dynamic changes, therefore, may make it inefficient to focus regulations on specific sources to reduce negative health outcomes caused by air pollution. These complex mixtures vary by region and will change seasonally. Therefore, it may become necessary to move from one standard for the whole country and establish regulations based on regions on or at least base standards on general classes of PM components. This is, I understand, not an easy task to overcome in a short amount of time, but I think that it is necessary to take atmospheric processes into account when setting emission standards with regards to toxicity. The findings from this study however support the understanding of this effect of PM risk as a function of composition.

The unique facility and tools used in this research program allowed me to explore the toxicity of diesel exhaust in an urban-like environment with the addition of chemical measurements to accompany the biological observations. The application of multiple parallel methods to characterize the physical composition of the test atmospheres allowed me to make more concrete conclusions about the cause of my biological findings.

Using only chemical or biological measurements as the basis for conclusions may result in misinterpretation of observations.

Since I rely on epidemiology to indicate what is regulated, I think a great benefit to epidemiological studies would be to add the EAVES and the GIVES to ambient monitoring stations. This will create the possibility of including biological receptors as a measurement to accompany traditional chemical, particle, meteorological, and epidemiological data. I believe that, the addition of the biological measurements and further exploration of the mechanisms that are driving the biological response will be a powerful tool for epidemiology and regulatory studies that include ambient air monitoring data.

## Future work

This research does not end with the work presented here. This research has led to more questions than answers, as well as more experiments utilizing the outdoor smog chamber and the actual ambient environment. One of the first things to do in the future would be to add additional end points, perhaps using a micro array. This will allow me to determine several other gene expressions at once that are effected by the DE exposures that are being conducted. An experiment with matched injection time would be another test atmosphere that should be investigated. By injecting the DE material on the bases of time and not PM I probably would get a different response from the gas and PM phases and possibly produce ozone with the Volkswagen. Also, the newest vehicle used in this study was a 2006 Volkswagen, I think that a newer vehicle with all the latest emission standards should be tested and possibly a gasoline powered vehicle as well. If new vehicles are being used then it might also be advantageous to use a dynamometer

to add a load that would be equal to on road use. In addition, the SynUrban Mixture should be updated with a new investigation on what the urban atmosphere is like now. Future studies should also include a midday exposure to account for the possibility that peak biological responses may occur prior to sunset.

Chemical analyses of the complex mixtures need to be improved. This can be done by conducting more standards in the GC so that the library of compounds is even more complete. Determining the complete composition of the PM should be considered. This may be possible by using something like an Aerodyne Aerosol Chemical Speciation Monitor (ACSM). This will allow for the chemical speciation of the particles and possibly determine the specific sources of observed responses.

Future studies should also attempt to incorporate primary cells since that is one of the major criticisms of this and other chamber work. With the use of primary cells I would be able to have a specimen that would be even closer to the lung epithelia with cilia and surfactant layers. Along with using primary cells a co-exposure to other cell lines and animals should also be done. This will allow for an easier link between the cell lines to animals and extrapolation to humans.

The most ambitious future study is developing a device that can conduct both PM and Gas phase exposures at the same time. This will be the most advantageous of all. If this is possible I could have circulating air flow containing particles but at the same time use electrostatic precipitation to deposit PM on the cellular surface. In addition, this instrument would have to allow for the cell culture inserts to be able to survive for

long periods of time since gas phase exposures, at levels seen in the chamber, take three to four hours to show significant response. Once this is thoroughly tested in the lab it should be modified to become an instrument that can be deployed out in the natural ambient environment. This deployment should happen near an air monitoring station so that chemical data can be obtained to possibly correlate to the outcome from the cell culture inserts exposed in the device. This device will give researchers a closer link between bench top and epidemiological findings.

# Appendix
# Example Calculations

Gravitational settling

Determine the time it would take enough particles to land on cell culture insert.

Diameter of particle 200nm

Cell culture insert $1cm^2$

Concentration in chamber 1.2 $mg/m^3$

Total mass on cell culture insert 4 ug

$V_{TS}$ = $2.26x10^{-6}$ m/s

$V(^{m3}) = \left(\frac{m^3}{1.2\ mg}\right)(4x10^{-3}) = 0.003\ m^3$

$cm^3 = 0.003m^3 * \left(\frac{100cm}{1m}\right)^3 = 3333.33cm^3$

$3333.33\ cm = 33.33m$ = the amount of air space above a $1cm^2$ cell culture insert to equal $3333.33cm^3$

$t = \frac{m_{of\ air\ space}}{V_{TS}} = 1.49x10^7$ s

Thermal Diffusion $\mathcal{D} = k_c T C_c$

$$\mathcal{D} = \frac{k_c T C_c}{3\pi\eta d}$$

$k_c = 1.381 \times 10^{-23}$ J/K

T = 310.7 K for high temperature in chamber

T = 288.5 K for low temperature in chamber

Cc = 1.878

$\eta = 1.8 \times 10^{-5}$ kg/ms

d = 0.2µm

$\mathcal{D} = 2.16 \times 10^{-10}$ m$^2$/s for low temperature in chamber

$\mathcal{D} = 2.34 \times 10^{-10}$ m$^2$/s for high temperature in chamber

To obtain the time it would take a particle to travel 1cm (the distance from the top of the repellant plate to the cell culture inserts), I would use the equation:

$$x = \sqrt{2\mathcal{D}t}$$

$$t = \frac{x^2}{2\mathcal{D}}$$

$x = 1$ cm

$\mathcal{D} = 2.34 \times 10^{-10}$ m$^2$/s for high temperature in chamber

t = $2.14 \times 10^5$ s or $3.56 \times 10^3$ min or 59.35 hours

Terminal Velocity in an Electric field

0.2µm diameter particle

$VTE = \frac{neECc}{3\pi\eta d}$ = 0.0086 m/s or 0.86 cm/s

Where

*n* = *n* diffusion + *n* field = 10.439

n diffusion = $\frac{d\mathfrak{k}T}{2K_E e^2} \ln\left(1 + \frac{\pi\, d\, Ci\, Ni\, t\, e^2\, K_E}{2\mathfrak{k}T}\right)$ = 8.603

n field = $\left(\frac{3\epsilon}{\epsilon+2}\right)\left(\frac{Ed^2}{4K_E e}\right)\left(\frac{\pi K_E e Z_i N_i t}{1+\pi K_E e Z_i N_i t}\right)$ = 1.836

*e* = 1.6x10$^{-19}$ C

E = 93333

Cc = 1.878

η = 1.8 x10 $^{-5}$ Pa s

d = 0.2µm

$\mathfrak{k}$ = 1.381 x 10$^{-23}$ J/K

T = 310 K

$K_E$ = 9x10$^9$ Nm$^3$ / C$^2$

$C_i$ = 240 m/s

$N_i$ = 1x10$^{13}$ m$^{-3}$

t = 2.5 s

$Z_i$ = .00015

$\epsilon = \infty$

Inertial Impaction

$Dj = \sqrt[3]{\frac{{d_{50}}^2 C_c 4 P_p Q}{9 \pi \eta \, stk_{50}}}$ = 3.651 x$10^{-4}$ m

Cc = 1.878

$P_p$ = 1.878

η = 1.8 x$10^{-5}$ kg/ms

d = 0.2μm

$stk_{50}$ = 0.24

$Q = \left(\frac{1L}{min}\right)\left(\frac{0.001\, m^3}{L}\right)\left(\frac{1\, min}{60\, sec}\right) = 1.66x10^{-5}\ \frac{m^3}{s}$

Next we need the area of the nozzle

$Area = \pi r^2$

$r = \frac{Dj}{2} = 1.83x10^{-4}$ m

$Area = 1.05x10^{-7}$

Now I can calculate the velocity of a particle

$\frac{Q}{Area} = 158.5\ \frac{m}{s}$

## References


Abe, S., H. Takizawa, I. Sugawara and S. Kudoh (2000). "Diesel Exhaust (DE)–Induced Cytokine Expression in Human Bronchial Epithelial Cells: A Study with a New Cell Exposure System to Freshly Generated DE In Vitro." Am. J. Respir. Cell Mol. Biol **22**: 296-303.

Anderson, S. E., L. G. Jackson, J. Franko and J. R. Wells (2010). "Evaluation of dicarbonyls generated in a simulated indoor air environment using an in vitro exposure system." Toxicol Sci **115**(2): 453-461.

ATCC. (2011). "Cell Biology, Designation: A549." Retrieved 10 December, 2011, from http://www.atcc.org.

Atkinson, R. (2000). "Atmospheric chemistry of VOCs and NOx." Atmos. Environ. **34**: 2063-2101.

Aufderheide, M. and U. Mohr (2000). "CULTEX--an alternative technique for cultivation and exposure of cells of the respiratory tract to airborne pollutants at the air/liquid interface." Exp. Toxicol. Pathol. **52**(3): 265-270.

Ayres, J. G., P. Borm, F. R. Cassee, V. Castranova, K. Donaldson, A. Ghio, R. M. Harrison, R. Hider, F. Kelly, I. M. Kooter, F. Marano, R. L. Maynard, I. Mudway, A. Nel, C. Sioutas, S. Smith, A. Baeza-Squiban, A. Cho, S. Duggan and J. Froines (2008). "Evaluating the Toxicity of Airborne Particulate Matter and Nanoparticles by Measuring Oxidative Stress Potential—A Workshop Report and Consensus Statement." Inhalation Toxicology **20**(1): 75-99.

Baltensperger, U., J. Dommen, M. R. Alfarra, J. Duplissy, K. Gaeggeler, A. Metzger, M. C. Facchini, S. Decesari, E. Finessi, C. Reinnig, M. Schott, J. Warnke, T. Hoffmann, B. Klatzer, H. Puxbaum, M. Geiser, M. Savi, D. Lang, M. Kalberer and T. Geiser (2008). "Combined determination of the chemical composition and of health effects of secondary organic aerosols: the POLYSOA project." J Aerosol Med Pulm Drug Deliv **21**(1): 145-154.

Bates, D. V. (1995). "The effects of air pollution on children." Environ. Health Perspect. **103**(Suppl 6): 49-53.

Bauling, A., M. Sourdeval, M. Meyer, F. Marano and A. Baeza-Squiban (2003). "Biological Effects of Atmospheric Particles on Human Bronchial Epithelial Cells. Comparison with Diesel Exhaust Particles." Toxicol. in Vitro **17**: 567-573.

Bayram, H., J. L. Devalia, R. J. Sapsford, T. Ohtoshi, Y. Miyabara, M. Sagai and R. J. Davies (1998). "The Effect of Diesel Exhaust Particles on Cell Function and Release of Inflammatory Mediators from Human Bronchial Epithelial Cells In Vitro." Am. J. Respir. Cell Mol. Biol. **18**(3): 441-448.

Bitterle, E., E. Karg, A. Schroeppel, W. G. Keryling, A. Tippe, G. A. Ferron, O. Schmid, J. Heyder, K. L. Maier and T. Hofer (2006). "Dose-controlled exposure of A549 epithelial cells at the air–liquid interface to airborne ultrafine carbonaceous particles." Chemosphere **65**(10): 1784-1790.

Boelter, K. J. and J. H. Davidson (1997). "Ozone Generation by Indoor, Electrostatic Air Cleaners." Aerosol Sci Technol **27**(6).

Boland, S., A. Baeza-Squiban, T. Fournier, O. Houcine, M. C. Gendron, M. Chévrier, G. Jouvenot, A. Coste, M. Aubier and F. Marano (1999). "Diesel exhaust particles are taken up by human airway epithelial cells in vitro and alter cytokine production." Am. J. Physiol. Lung Cell Mol. Physiol. **276**: L604-L613.

Boland, S., A. Baeza-Squiban, T. Fournier, O. Houcine, M. C. Gendron, M. Chévrier, G. Jouvenot, A. Coste, M. Aubier and F. Marano (1999). "Diesel exhaust particles are taken up by human airway epithelial cells in vitro and alter cytokine production." Am J Physiol Lung Cell Mol Physiol **276**: L604-L613.

Bunger, J., J. Krahl, K. Baum, O. Schroder, M. Muller, G. Westphal, P. Ruhnau, T. G. Schulz and E. Hallier (2000). "Cytotoxic and mutagenic effects, particle size and concentration analysis of diesel engine emissions using biodiesel and petrol diesel as feul." Arch. Toxicol **74**: 490-498.

Cao, D., P. Bromberg and J. Samet (2007). "COX-2 Expression Induced by Diesel Particles Involves Chromatin Modificaion and Degradation of HDAC1." American Thoracic Society **37**: 232-239.

Carlsten, C., J. D. Kaufman, C. A. Trenga, J. Allen, A. Peretz and J. H. Sullivan (2008). "Thrombotic Markers in Metabolic Syndrome Subjects Exposed to Diesel Exhaust." Inhalation Toxicol. **20**: 917-921.

Chellam, S., P. Kulkarni and M. P. Fraser (2005). "Emissions of organic compounds and trace metals in fine particulate matter from motor vehicles: A tunnel study in Houston, Texas." J. Air Waste Manage. Assoc.(55): 60-72.

Chen, X., P. K. Hopke and W. P. Carter (2011). "Secondary organic aerosol from ozonolysis of biogenic volatile organic compounds: chamber studies of particle and reactive oxygen species formation." Environ Sci Technol **45**(1): 276-282.

Clark, N. N., J. M. Kern, C. M. Atkinson, R. D. Nine and A. E. Blair (2002). "Factors Affecting Heavy-Duty Diesel Vehicle Emissions." Journal of the Air & Waste Managemnet Assosciation **52**(1): 84-94.

Cooney, D. J. and A. J. Hickey (2011). "Cellular response to the deposition of diesel exhaust particle aerosols onto human lung cells grown at the air-liquid interface by intertial impaction." Toxicol. in Vitro **25**: 1953-1965.

de Bruijne née Lichtveld, K., S. Ebersviller, K. G. Sexton, C. Olenick, R. Woodside, S. Casper, I. Jaspers and H. E. Jeffries (2008). "Comparing the Toxicity of Fresh and Aged Diesel Exhaust using Sparate Particle and Gaseous Exposure Systems." The Toxicologist: Supplement to Toxicological Sciences **102**(1): 1-535.

de Bruijne née Lichtveld, K., K. G. Sexton, S. Ebersviller, M. Doyle and H. E. Jeffries (2007). "Comparing the Toxicity of Fresh and Aged Diesel Exhaust using a Newly Developed in Vitro Exposure System." The Toxicologist: Supplement to Toxicological Sciences **96**(1): 1-494.

de Bruijne, K., S. Ebersviller, K. G. Sexton, S. Lake, D. Leith, R. Goodman, J. Jetters, G. Walters, M. Doyle, R. Woodside, H. E. Jeffries and I. Jaspers (2009). "Design and Testing of Electrostatic Aerosol In vitro Exposure System (EAVES): An Alternative Exposure System for Particles." Inhalation Toxicol. **21**(2): 91-101.

de Bruijne, K., K. G. Sexton, S. Ebersviller, Y.-H. Lin, I. Jaspers and H. E. Jeffries (2009). "Comparing the Toxicity of Fresh and Aged Biodiesel and Diesel Exhaust using Separate Particle and Gaseous Exposure Systems." The Toxicologist: Supplement to Toxicological Sciences **108**: 1-495.

Dockery, D. W. and C. A. P. III (1994). "Acute Repiratory Effects of Particulate Air Pollution." Annual Review of Public Health **15**: 107-132.

Donahue, N., A. Robinson, C. Stanier and S. Pandis (2006). "Coupled Partitioning, Dilution, and Chemical Aging of Semivolatile Organics." Environ. Sci. Technol. **40**(8): 2635-2643.

Doyle, M., K. G. Sexton, H. E. Jeffries, K. Bridge and I. Jaspers (2004). "Effects of 1,3-Butadiene, Isoprene, and Their Photochemical Degradation Products on Human Lung Cells." Environmental Health Perspectives **112**(15): 1488-1495.

Doyle, M., K. G. Sexton, H. E. Jeffries and I. Jaspers (2007). "Atmospheric photochemical transformations enhance 1,3-butadiene-induced inflammatory responses in human epithelial cells: The role of ozone and other photochemical degradation products." Chem. Biol. Interact.

Doyle, M., K. G. Sexton, H. E. Jeffries and I. Jaspers (2007). "Atmospheric photochemical transformations enhance 1,3-butadiene-induced inflammatory responses in human epithelial cells: The role of ozone and other photochemical degradation products." Chem. Biol. Interact. **166**(1-3): 163-169.

Dreher, K. L. (2000). "PARTICULATE MATTER PHYSICOCHEMISTRY AND TOXICOLOGY: In Search of Causality-A Critical Perspective." Inhalation Toxicol. **12**(s3): 45-57.

Dybdahla, M., L. Risom, J. Bornholdt, H. Autrup, S. Loft and H. Wallin (2004). "Inflammatory and genotoxic effects of diesel particles in vitro and in vivo." Mutat. Res. **562**: 119-131.

Ebersviller, S., K. Lichtveld, K. G. Sexton, J. Zavala, Y.-H. Lin, I. Jaspers and H. E. Jeffries (2012a). "Gaseous VOCs Rapidly Modify Particulate Matter and Its Biological Effects - Part1: Simple VOCs and Model PM." Atmos. Chem. Phys. Discussions **12**: 1-40.

Ebersviller, S., K. Lichtveld, K. G. Sexton, J. Zavala, Y.-H. Lin, I. Jaspers and H. E. Jeffries (2012b). "Gaseous VOCs Rapidly Modify Particulate Matter and Its Biological Effects: Part 2, Complex Urban VOCs and Model PM " Atmos. Chem. Phys. Discussions **12**: 7365-7418.

Ebersviller, S., K. G. Sexton, K. de Bruijne, R. Woodside, C. Olenick, I. Jaspers and H. E. Jeffries (2008). "Gas-Particle Partitioning in Urban Air Mixtures and its Effect on Human Epithelial Lung Cells." The Toxicologist: Supplement to Toxicological Sciences **102**(1): 1-535.

Farina, F., G. Sancini, P. Mantecca, D. Gallinotti, M. Camatini and P. Palestini (2011). "The acute toxic effects of particulate matter in mouse lung are related to size and season of collection." Toxicol. Lett. **202**(3): 209-217.

Finlayson-Pitts, B. J. and J. N. Pitts Jr. (1999). Chemistry of The Upper and Lower Atmosphere. New York, Academic Press.

Franklin, M. and J. Schwartz (2008). "The Impact of Secondary Particles on the Association between Ambient Ozone and Mortality." Environ Health Perspect **116**(4): 453-458.

Fraser, M. P., B. Buzcu, Z. W. Yue, G. R. McGaughey, N. R. Desai, D. T. Allen, R. L. Seila, W. A. Lonneman and R. A. Harley (2003). "Separation of fine particulate matter emitted from gasoline and diesel vehicles using chemical mass balancing techniques." Environ. Sci. Technol. **37**: 3904-3909.

Gaschen, A., D. Lang, M. Kalberer, M. Savi, T. Geiser, A. Gazdhar, C.-M. Lehr, M. Bur, J. Dommen, U. Baltensperger and M. Geiser (2010). "Cellular Reponse after Exposure of Lung Cell Cultures to Secondary Organic Aerosol Particles." Environ. Sci. and Technol. **44**: 1424-1430.

Gaschen, A., D. Lang, M. Kalberer, M. Savi, T. Geiser, A. Gazdhar, C. M. Lehr, M. Bur, J. Dommen, U. Baltensperger and M. Geiser (2010). "Cellular responses after exposure of lung cell cultures to secondary organic aerosol particles." Environ Sci Technol **44**(4): 1424-1430.

Gertler, A. W., J. A. Gillies, W. R. Pierson, C. F. Rogers, J. C. Sagebiel, M. Abu-Allaban, W. Coulombe, L. Tarnay and T. A. Cahill (2002 ). "Real-world particulate matter and gaseous emissions from motor vehicles in a highway tunnel." Res. Rep. Health Eff. Inst.: 5-56; discussion 79-92.

Gertler, A. W., D. Grosjean, E. Grosjean and Health Effects Institute (2002). "Emissions from diesel and gasoline engines measured in highway tunnels." Res. Rep. Health Eff. Inst. (107).

Godleski, J. J., A. C. Rohr, B. A. Coull, C. M. Kang, E. A. Diaz and P. Koutrakis (2011). "Toxicological evaluation of realistic emission source aerosols (TERESA): summary and conclusions." Inhal Toxicol **23 Suppl 2**: 95-103.

HEI Panel on the Health Effects of Traffic-Related Air Pollution (2010). Traffic-related air pollution: a critical review of the literature on emissions, exposure, and health effects. HEI Special Report 17. Boston, MA, Health Effects Institute**:** 1-386.

Hinds, W. C. (1999). Aerosol Technology: Properties, Behavior, and Measurement of Airborne Particles. 2nd ed. New York, John Wiley & Sons, Inc.

Hu, D. and R. Kamens (2007). "Evaluation of the UNC toluene-SOA mechanism with respect to other chamber studies and key model parameters." Atmos. Environ. **41**: 6465-6477.

I. M. C. M. Rietjensa, M. C. M. Poelena, R. A. Hempeniusa, M. J. J. Gijbelsa and G. M. Alinka (1986). "Toxicity of ozone and nitrogen dioxide to alveolar macrophages: Comparative study revealing differences in their mechanism of toxic action." Journal of Toxicology and Environmental Health **19**(4): 555-568.

International Agency for Research on Cancer (1989). "Evaluation of Carcinogenic Risks of Chemicals to Humans: Diesel and Gasoline Engine Exhausts and Some Nitroarenes." IARC Monographs **46**: 41-185.

International Agency for Research on Cancer. (2012). "Diesel Engine Exhaust Carcinogenic." Press Release No 213 Retrieved June 30, 2012.

Jang, M., A. J. Ghio and G. Cao (2006). "Exposure of BEAS-2B cells to secondary organic aerosol coated on magnetic nanoparticles." Chem Res Toxicol **19**(8): 1044-1050.

Jaspers, I., J. Ciencewicki, W. Zhang, L. Brighton, J. Carson, M. Beck and M. Madden (2005). "Diesel exhaust enhances influenza virus infections in respiratory epithelial cells." Toxicol Sci **85**: 990-1002.

Jaspers, I., E. Flescher and L. C. Chen (1997). "Ozone-induced IL-8 expression and transcription factor binding in respiratory epithelial cells." Am J Physiol Lung Cell Mol Physiol(272 (3 Pt 1)): L504-511.

Jaspers, I., W. Zhang, A. Fraser, J. M. Samet and R. W. ( 2001). "Hydrogen peroxide has opposing effects on IKK activity and IKappaBalpha breakdown in airway epithelial cells." Am. J. Respir. Cell Mol. Biol. **24**(6): 769-777.

Jeffries, H. E. (1995). Chapter 9. In: Composition, Chemistry, and Climate of the Atmosphere. Photochemical Air Pollution. H. B. Singh. New York, Van Nostand-Reinhold**:** 308-348.

Jeffries, H. E., D. L. Fox and R. M. Kamens (1976). "Outdoor Smog Chamber Studies: Light Effects Relative to Indoor Chambers." Environ. Sci. Technol.(10): 1006-1011.

Jeffries, H. E., K. G. Sexton, R. M. Kamens and M. S. Holleman (1985). Outdoor Smog Chamber Experiments: Reactivity of Methanol Exhaust. Ann Arbor, MI, Environmental Protection Agency, Office of Mobile Sources.

Kamens, R. and D. L. Coe (1997). "A Large Gas-Phase Stripping Device to Investigate Rates of PAH Evaporation from Airborne Diesel Soot Particles." Environ. Sci. and Technol. **31**(6): 1830-1833.

Kamens, R., M. Jang, C.-J. Chien and K. Leach (1999). "Aerosol Formation from the Reaction of r-Pinene and Ozone Using a Gas-Phase Kinetics-Aerosol Partitioning Model." Environ. Sci. and Technol. **33**: 1430-1438.

Kamens, R. M. and M. Jaoui (2001). "Modeling Aerosol Formation from a-pinene + NOx in the Presence of Natural Sunlight Using Gas Phase Kinetics and Gas-particle Partitioning Theory." Environ. Sci. Technol. **35**: 1394-1405.

Kamens, R. M., H. E. Jeffries, M. W. Gery, R. W. Wiener, K. G. Sexton and G. B. Howe (1981). "The Impact of a-pinene on Urban Smog Formation: An Outdoor Chamber Study." Atmos. Environ.(15): 969-981.

Kim, C. S. and S.-C. Hu (2006). "Total respiratory tract deposition of fine micrometer-sized particles in healthy adults: empirical equations for sex and breathing pattern." Journal of Applied Physiology **101**(2): 401-412.

Kim, Y.-M., W. Reed, A. G. Lenz, I. Jaspers, R. Silbajoris, H. S. Nick and J. M. Samet (2005). "Ultrafine carbon particles induce interleukin-8 gene transcription and p38 MAPK activation in normal human bronchial epithelial cells." Am. J. Respir. Cell Mol. Biol. **288**: L432-L441.

Knebel, J. W., D. Ritter and M. Aufderheide (2002). "Exposure of Human Lung Cells to Native Diesel Motor Exhaust - Development of an Optimized in Vitro Test Strategy." Toxicol. in Vitro **16**: 185-192.

Knutson, E. O. and K. T. Whitby (1975). "Aerosol Classification by Electric Mobility: Apparatus, Theory, and Applications." J. Aerosol. Sci. **6**: 443-451.

Lake, S. W. (2005). Electrostatic Precipitation as an Alternative Method for in vitro Human Lung Cell Aerosol Exposures. Department of Environmental Sciences and Engineering, School of Public Health. Chapel Hill, University of North Carolina at Chapel Hill. **Masters**.

Laks, D., R. C. de Oliveira, P. A. de Andre, M. Macchinone, M. Lemos, D. Faffe, P. H. N. Saldiva and W. A. Zin (2008). "Composition of Diesel Particles Influences Acute Pulmonary Toxicity: An Experimental Study in MICE." Inhalation Toxicol. **20**: 1037-1042.

Lee, S., M. Jang and R. M. Kamens (2004). "SOA formation from the photooxidation of a-pinene in the presence of freshly emitted diesel soot exhaust " Atmos. Environ.(38): 2597-2605.

Li, N., S. Kim, M. Wang, J. Froines, C. Sioutas and A. Nel (2002). "Use of a Stratified Oxidative Stress Model to Study the Biological Effects of Ambient Concentrated and Diesel Exhaust Particulate Matter." Inhalation Toxicology **14**(5): 459-486.

Lichtveld, K. M., S. M. Ebersviller, K. G. Sexton, W. Vizuete, I. Jaspers and H. E. Jeffries (2012). "In Vitro Exposures in Diesel Exhaust Atmospheres: Resuspension of PM from Filters versus Direct Deposition of PM from Air." Environ Sci Technol.

Lieber, M., B. Smith, A. Szakal, W. Nelson-Rees and G. Todaro (1976). "A continuous tumor-cell line from a human lung carcinoma with properties of Type II alveolar epithelial cells." Int. J. Cancer **17**(1): 62-70.

Liu, H., H. E. Jeffries and K. G. Sexton (1999). "Atmospheric photochmical degradation of 1,4-unsaturated dicarbonyls." Environ. Sci. Technol. **33**: 4212-4220.

Madden, M. C. (2008). "Complex issues with examining diesel exhaust toxicity: Is the task getting easier or harder?" Experimental and Toxicologic Pathology **60**(2–3): 135-140.

Madden, M. C., L. A. Dailey and J. G. Stonehuerner (2003). "Responses of cultured human airway epithelial cells treated with diesel exhaust extracts will vary with the engine load." J Toxicol Environ Health **66**(24): 2281-2297.

Madden, M. C., J. H. Richards, L. A. Dailey, G. E. Hatch and A. J. Ghio (2000). "Effect of Ozone on Diesel Exhasut Particle Toxicity in Rat Lung." Toxiciology and Applied Pharmacology **168**: 140-148.

Mainelis, G., S. Grinshpun, K. Willeke, T. Reponen, V. Ulevicius and P. Hintz (1999). "Collection of Airborne Microorganisms by Electrostatic Precipitation." Aerosol Science and Technology **30**(2): 127-144.

Mainelis, G. and K. Willeke (2002). "Design and Collection Efficiency of a New Electrostic Precipitator for Bioaerosol Collection." Aerosol Sci. Technol.(36): 1073-1085.

Mar, T. F., G. A. Norris, J. Q. Koenig and T. V. Larson (2000). "Associations between air pollution and mortality in Phoenix, 1995-1997." Environ Health Perspect **108**(4): 347-353.

Mauderly, J. (2001). "Forum: Diesel Emissions: Is More Health Research Still Needed?" Toxicol. Sci. **62**: 6-9.

Mazzarella, G., F. Ferraraccio, M. V. Prati, S. Annunziata, A. Bianco, A. Mezzogiorno, G. Liguori, I. F. Angelillo and M. Cazzola (2007). "Effects of diesel exhaust particles on human lung epithelial cells: An in vitro study." Respir. Med. **101**: 1155–1162.

McDonald, J., E. Barr, R. White, J. Chow, J. Schauer, B. Zielinska and E. Grosjean (2004). "Generation and characterization of four dilutions of diesel engine exhaust for a subchronic inhalation study." Environ. Sci. Technol. **38**(9): 2513-2522.

McDonald, J. D., M. Doyle-Eisele, M. J. Campen, J. Seagrave, T. Holmes, A. Lund, J. D. Surratt, J. H. Seinfeld, A. C. Rohr and E. M. Knipping (2010). "Cardiopulmonary response to inhalation of biogenic secondary organic aerosol." Inhal Toxicol **22**(3): 253-265.

McDow, S. R., M. Vartiainen, Q. Sun, Y. Hong, Y. Yao and R. M. Kamens (1995). "Combustion Aerosol Water Content and It Effect on Polycyclic Aromatic Hydrocarbon Reactivity." Atmos. Environ. **29**: 791-797.

Modig, L., B. Jarvholm, E. Ronnmark, L. Nystrom, B. Lundback, C. Andersson and B. Forsberg (2006). "Vehicle exhaust exposure in an incident case-control study of adult asthma." Eur. Respir. J. **28**: 75-81.

Morin, J.-P., F. Fouquet, C. Monteil, E. Le Prieur, E. Vaz and F. Dionnet (1999). "Development of a new in vitro system for continuous in vitro exposure of lung tissue to complex atmospheres: Application to diesel exhaust toxicology." Cell Biology and Toxicology **15**: 143-152.

Oberdorster, G. and C. P. Yu (1990). "The Carcinogenic Potential of Inhaled Diesel Exhaust: A Particle Effect?" J. Aerosol Sci. **21**(Suppl. 1): s397-s401.

Occupational Safety and Health Administration. (2007, July 12, 2007). "Nitric Oxide." Retrieved May 31, 2012, from http://www.osha.gov/dts/chemicalsampling/data/CH_256700.html.

Pankow, J. (1998). "Further discussion of the octanol/air partition coefficient Koa correlating parameter for gas/particle partitioning coefficients." Atmos. Environ. **32**(9): 1493-1497.

Pankow, J., C. Liang, J. Odum and J. Seinfeld (1997). "Gas/Particle Partitioning of Semivolatile Organic Compounds to Model Inorganic, Organic, and Ambient Smog Aerosols." Environ. Sci. Technol. **31**(11): 3086-3092.

Paur, H.-R., F. R. Cassee, J. Teeguarden, H. Fissan, S. Diabate, M. Aufderheide, W. G. Kreyling, O. Hänninen, G. Kasper, M. Riediker, B. Rothen-Rutishauser and O. Schmid (2011). "In-vitro cell exposure studies for the assessment of nanoparticle toxicity in the lung—A dialog between aerosol science and biology." Journal of Aerosol Science **42**(10): 668-692.

Pilotto, L. S., R. M. Douglas, R. G. Attewell and S. R. Wilson (1997). "Respiratory effects associated with indoor nitrogen dioxide exposure in children." Int. J. Epidemiol. **26**(4): 788-796.

Pope, C. A., M. J. Thun, M. M. Namboodiri, D. W. Dockery, J. S. Evans, F. E. Speizer and C. W. Heath (1995). "Particulate Air Pollution as a Predictor of Mortality in a Prospective Study of US Adults." Am. J. Respir. Crit. Care Med. **151**: 669-674.

Pope, C. A., M. J. Thun, M. M. Namboodiri, D. W. Dockery, J. S. Evans, F. E. Speizer and C. W. Heath (1995). "Particulate Air Pollution as a Predictor of Mortality in a Prospective Study of US Adults." American Journal of Respiratory and Critical Care Medicine **151**: 669-674.

Rager, J. E., K. Lichtveld, S. Ebersviller, L. Smeester, I. Jaspers, K. G. Sexton and R. C. Fry (2011). "A Toxicogenomic Comparison of Primary and Photochemically Altered Air Pollutant Mixtures." Environ Health Perspect **119**(11).

Robinson, A., N. Donahue, M. Shrivastava, E. Weitkamp, A. Sage, A. Grieshop, T. Lane, J. Pierce and S. Pandis (2007). "Rethinking Organic Aerosols: Semivolatile Emissions and Photochemical Aging." Science **315**: 1259-1262.

Rusznak, C., S. Jenkins, P. R. Mills, R. J. Sapsford, J. L. Devalia and R. J. Davies (1998). "Mechanisms of Pollution Induced Allergy and Asthma." Rev. fr Allergol. **38**(7S): S80-S90.

Salem, H. and S. Katz (2006). Inhalation Toxicology. New York, NY, Taylor and Francis.

Salvi, S., A. Blomberg, B. Rudell, F. Kelly, T. Sandström, S. T. Holgate and A. Frew (1999). "Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers." Am. J. Respir. Crit. Care Med. **159**(3): 702-709.

Salvi, S., C. Nordenhall, A. Blomberg, B. Rudell, J. Pourazar, F. J. Kelly, S. Wilson, T. Sandström, S.T.Holgate and A. J. Frew (2000). "Acute Exposure to Diesel Exhaust Increases IL-8 and GRO-alpha Production in Healthy Human Airways." Am. J. Respir. Crit. Care Med. **161**(2): 550-557.

Sarnat, J. A., J. Schwartz, P. J. Catalano and H. H. Suh (2001). "Gaseous pollutants in particulate matter epidemiology: confounders or surrogates?" Environ. Health Perspect. **109**(10): 1053-1061.

Savi, M., M. Kalberer, D. Lang, M. Ryser, M. Fiers, A. Gaschen, J. Ricka and M. Geiser (2008). "A Novel Exposure System for the Effcient and Contolled Deposition of Aerosol Particles onto Cell Cultures." Environ. Sci. Technol. **42**: 5667-5674.

Schlesinger, R. B., N. Kaunzli, G. Hidy, T. Gotschi and M. Jerrett (2006). "The health relevence of ambient particulate matter characteristics: coherence of toxicological and epidemiological inferences." Inhalation Toxicol. **18**(2): 95-125.

Schwarze, P. E., J. Ovrevik, M. Lag, M. Refsnes, P. Nafstad, R. B. Hetland and E. Dybing (2006). "Particulate matter properties and health effects: consistency of epidemiological and toxicological studies." Hum Exp Toxicol **25**(10): 559-579.

Schwarzenbach, R., P. Gschwend and D. Imboden (2003). Environmental Organic Chemistry. Hoboken, NJ, Wiley-Interscience.

Seagrave, J., S. Dunaway, J. D. McDonald, J. L. Mauderly, P. Hayden and C. Stidley (2007). "Responses of differentiated primary human lung

epithelial cells to exposure to diesel exhaust at an air-liquid interface." Exp. Lung. Res. **33**(1): 27-51.

Seagrave, J., J. D. McDonald and J. L. Mauderly (2005). "In vitro versus in vivo exposure to combustion emissions." Exp. Toxicol. Pathol. **57 Supp 1**.

Seagrave, J. C., C. Knall, J. D. McDonald and J. L. Mauderly (2004). "Diesel Particulate Material Binds and Concentrates a Proinflammatory Cytokine That Causes Neutrophil Migration." Inhalation Toxicology **16**(s1): 93-98.

Seaman, V. Y., M. J. Charles and T. M. Cahill (2006). "A Sensitive Method for the Quantification of Acrolein and Other Volatile Carbonyls in Ambient Air." Anal. Chem. **78**(7): 2405-2412.

Sexton, K. G., H. E. Jeffries, M. Jang, R. M. Kamens, M. Doyle, I. Voicu and I. Jaspers (2004). "Photochemical products in urban mixtures enhance inflammatory responses in lung cells." Inhalation Toxicol.(16 Suppl 1): 107-114.

Sexton, K. G., H. J. Jeffries, M. Jang, R. M. Kamens, M. Doyle, I. Voicu and I. Jaspers (2004). "Photochemical products in urban mixtures enhance inflammatory responses in lung cells." Inhalation Toxicology **16**(Suppl. 1): 107-114.

Sillanpää, M., M. D. Geller, H. C. Phuleria and C. Sioutas (2008). "High collection efficiency electrostatic precipitator for in vitro cell exposure to concentrated ambient particulate matter (PM)." Journal of Aerosol Science **39**(4): 335-347.

Silverman, D. T., C. M. Samanic, J. H. Lubin, A. E. Blair, P. A. Stewart, R. Vermeulen, J. B. Coble, N. Rothman, P. L. Schleiff, W. D. Travis, R. G. Ziegler, S. Wacholder and M. D. Attfield (2012). "The Diesel Exhaust in Miners Study: A Nested Case–Control Study of Lung Cancer and Diesel Exhaust." J Natl Cancer Inst **104**(11): 855-868.

Singh, P., D. M. DeMarini, C. A. J. Dick, D. G. Tabor, J. V. Ryan, W. P. Linak, T. Kobayashi and M. I. Gilmour (2004). "Sample Characterization of Automobile and Forklift Diesel Exhaust Particles and Comparative Pulmonary Toxicity in Mice " Environ. Health Perspect. **112**(8): 820-825.

Sioutas, C., E. Abt, J. M. Wolfson and P. Koutrakis (1999). "Evaluation of the Measurement Performance of the Scanning Mobility Particle Sizer and Aerodynamic Particle Sizer." Aerosol Sci. Technol. **30**: 84–92.

Sioutas, C., P. Koutrakis and B. A. Olson (1994). "Development and Evaluation of a Low Cutpoint Virtual Impactor." Aerosol Sci. Technol. **21**(3): 223-235.

Stavert, D. M. and B. E. Lehnert (1990). "Nitric Oxide and Nitrogen Dioxide as Inducers of Acute Pulmonary Injury when Inhaled at relatively high concentration for brief periods inhalation tox." Inhal Toxicol **2**(1): 53-67.

Steenhof, M., I. Gosens, M. Strak, K. J. Godri, G. Hoek, F. R. Cassee, I. S. Mudway, F. J. Kelly, R. M. Harrison, E. Lebret, B. Brunekreef, N. A. Janssen and R. H. Pieters (2011). "In vitro toxicity of particulate matter (PM) collected at different sites in the Netherlands is associated with PM composition, size fraction and oxidative potential--the RAPTES project." Part. Fibre. Toxicol. **8**: 26.

Steerenberg, P. A., L. van Amelsvoort, M. Lovik, R. B. Hetland, T. Alberg, T. Halatek, H. J. Bloemen, K. Rydzynski, G. Swaen, P. Schwarze, E. Dybing and F. R. Cassee (2006). "Relation between sources of particulate air pollution and biological effect parameters in samples from four European cities: an exploratory study." Inhal Toxicol **18**(5): 333-346.

Steerenberg, P. A., J. A. Zonnenberg, J. A. Dormans, P. N. Joon, I. M. Wouters, L. v. Bree, P. T. Scheepers and H. V. Loveren (1998). "Diesel exhaust particles induced release of interleukin 6 and 8 by (primed) human bronchial epithelial cells (BEAS 2B) in vitro." Exp. Lung. Res. **24**(1): 85-100.

Stewart, S. L., S. A. Grinshpun, K. Willeke, S. Terzieva, V. Ulevicius and J. Donnelly (1995). "Effect of impact stress on microbial recovery on an agar surface." Appl Environ Microbiol **61**(4): 1232-1239.

Sunil, V. R., K. J. Patel, G. Mainelis, B. J. Turpin, S. Ridgely, R. J. Laumbach, H. M. Kipen, Y. Nazarenko, M. Veleeparambil, A. J. Gow, J. D. Laskin and D. L. Laskin (2009). "Pulmonary effects of inhaled diesel exhasut in aged mice." Toxicol. Appl. Pharmacol. **241**: 283-293.

Tsien, A., D. Diaz-Sanchez, J. Ma and A. Saxon (1997). "The organic component of diesel exhaust particles and phenanthrene, a major polyaromatic hydrocarbon constituent, enhances IgE production by IgE-secreting EBV-transformed human B cells in vitro." Toxicol. Appl. Pharmacol. **142**: 256-263.

U.S. Environmental Protection Agency (1999). The Benefits and Costs of the Clean Air Act 1990 to 2010, EPA Report to Congress. Office of Air and Radiation Office of Policy.

U.S. Environmental Protection Agency (2002). Health Assessment Document for Diesel Engine Exhaust. U.S. Environmental Protection Agency and Office of Research and Development, National Center for Environmental Assessment. **67**.

Utell, M. J., M. W. Frampton, N. J. J. Roberts, J. N. Finkelstein, C. Cox and P. E. Morrow (1991). "Mechanisms of nitrogen dioxide toxicity in humans." Research Report: Health Effects Institue **43**: 1-33.

Volckens, J., L. Dailey, G. Walters and R. Devlin (2009). "Direct particle-to-cell deposition of coarse ambient particulate matter increases the production of inflammatory mediators from cultured human airway epithelial cells." Environ. Sci. Technol. **43**: 4595-4599.

Volckens, J. and D. Leith (2002). "Filter and Electrostatic Samplers for Semivolatile Aerosols: Physical Artifacts." Environ Sci Technol **36**: 4613-4617.

Whitby, K. T., R. E. Charlson, W. E. Wilson and R. K. Stevens (1974). "The size of suspended particle matter in air." Science **183**: 1098-1099.

Yu, J., H. Jeffries and K. G. Sexton (1997). "Atmospheric photo oxidation of alkylbenzenes - I. Carbonyl product analyses." Atmos. Environ. **31**(15): 2261-2280.

Yu, J., H. E. Jeffries and R. L. Lacheur (1995). "Identifying Airborne Carbonyl Compounds in Isoprene Atmospheric Photo oxidation Products by their PFBHA Oximes Using Gas Chromatography/Ion Trap Mass Spectrometry." Environ. Sci. Technol. **29**: 1923-1932.

Zhou, J., K. Ito, R. Lall, M. Lippmann and G. Thurston (2011). "Time-series analysis of mortality effects of fine particulate matter components in Detroit and Seattle." Environ Health Perspect **119**(4): 461-466.

Zoran D. Ristovski, Branka Miljevic, Nicholas C. Surawski, Lisia Morawska, Kwun M. Fong, Felica Goh and A. Y. Ian (2011). "Respiratory health effects of diesel particulate matter." Respirology **17**: 201-212.