PM Biological Effect Modification by Gases in Urban Air

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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

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Abstract

SETH MORRILL EBERSVILLER: PM Biological Effects Modification by Gases in Urban Air
(Under the direction of Harvey Jeffries and Ken Sexton)

In its most-recent document summarizing the state of the science related to PM, the National Research Council (NRC) stated that "a finding that the effect of particles depends on the concentration of another pollutant—that is, 'effect modification'— would have implications for setting NAAQS independently for the various criteria pollutants". This dissertation contains a series of manuscripts describing new methods and approaches to evaluate the dynamic entanglements of the composition and toxic effects of gases and particles described by the NRC.

The first study develops a new *in vitro* exposure method that is able to expose cultured human lung cells to the particulate matter (PM) present in air pollution mixtures while they are still in equilibrium with the gases surrounding them. When coupled with a gas-only *in vitro* exposure method already in use, and the analytical equipment in our laboratory, we have a uniquely suited set of tools to investigate 'entanglement' phenomena. The remaining three manuscripts describe a three-part study designed to demonstrate dynamic entanglements among gaseous organic compounds (VOCs), PM, and their subsequent biological effects. We studied these entanglements in increasingly complex VOC and PM mixtures in urban-like conditions in a large outdoor chamber.

First, we demonstrate the existence of 'effect modification' for the case of single gas-phase toxicants and an inherently non-toxic PM (mineral oil aerosol, MOA). In the presence of the single gas-phase toxicant in the dark, the initially non-toxic PM became toxic to lung cells in the PM-only *in vitro* exposure system. Next, we used sunlight to create secondary gas-phase toxicants from a complex mixture of 54 VOCs similar to those in urban air, with the MOA used in part one. Finally, testing was further applied to systems with primary diesel exhaust (gas and PM) from a single vehicle operated with different types of fuel. In almost every case, we observed increases in cellular toxicity when chamber contents were photochemically aged.

These studies prove unambiguously that the toxicity inherent to one phase of an air pollution mixture can affect the toxicity of another – precisely the 'effect modification' described by the NRC.

Dedication

To my Parents; this couldn't have happened without you

To Mooker and O; for keeping my feet on the ground and reminding me to laugh

And most of all to the Monkey and my Papillon;

For their patience, strength, and for never letting me forget what is truly important

Acknowledgements

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Chapter 1:
Background and Significance

"A finding that the effect of particles depends on the concentration of another pollutant – that is, 'effect modification' – would have implications for setting NAAQS independently for the various criteria pollutants"

Research Priorities for Airborne Particulate Matter, NAS, 2004, p. 99

Disentanglement of Gases and Particles

The National Research Council has called repeatedly for efforts to 'disentangle'

the contributions of gases and particles to adverse health outcomes (NRC 2004).   Re-

searchers have long known that gases and particles interact in important and dynamic

processes during the aging of particulate matter (PM) in the atmosphere. Volatile or-

ganic compounds (VOCs) are constantly shifting between the gas and particle phases of

ambient air while at the same time some VOCs are being modified while in each phase

(Kamens, Jeffries et al. 1981; Pankow, Liang et al. 1997; Kamens and Jaoui 2001; Lee,

Jang et al. 2004; Donahue, Robinson et al. 2006; Hu and Kamens 2007; Robinson,

Donahue et al. 2007). What has been unclear to this point, however, is if – and how –

these interactions affect the actual toxicity of each phase.

Currently, studies of health implications of PM exposures focus primarily on the

toxicity of PM itself, ignoring the complexity of the air surrounding PM in the ambient

environment. While these studies attempt to discover the inherent toxicity of PM, it is

recognized that "such approaches are likely to over simplify the underlying biological

processes and how the mixture of air pollutants that is inhaled adversely affects health"

(NRC 2004). Developing a clear understanding of how these processes affect the toxicity

of ambient PM will, therefore, be a significant contribution toward understanding the intricacies of the entanglement of the phases.

Decades of work has already been done by epidemiologists, toxicologists, and physical chemists to disentangle the processes involved, and to characterize those that are thought to be the most important for protecting human health. Each field brings its own strengths to bear on breaking down barriers to understanding the complexity of the system. The tendency of each of the disciplines involved to isolate themselves from the rest, however, adds to the already imposing difficulty of disentangling the effects of particles from those of the mixture as a whole.

Epidemiology Viewpoint

Limited ambient observations from epidemiology studies across many cities have given definite insights into this issue. Epidemiological studies continually show positive associations between measurements of ambient PM and adverse health outcomes, especially in susceptible populations (asthmatics, children, patients with COPD, etc.)(Bates 1995; Pope, Thun et al. 1995; Sarnat, Schwartz et al. 2001). Typically, epidemiologists use increases in hospital admissions and daily mortality, reported during short term episodes of high PM in the ambient air, to associate a positive relationship between PM and human health effects. Despite the positive correlations there are still *"no established biological mechanisms to explain the toxicity of PM to humans"* and evidence of carcinogenic potential is limited by the ability to eliminate confounding factors and estimate exposure (Oberdorster and Yu 1990; Steerenberg, Zonnenberg et al. 1998; Salvi, Blomberg et al. 1999; Bunger, Krahl et al. 2000; Dreher 2000; Takigawa, Wang et al.

2009). Epidemiological studies have correlated a number of sources or source charac-

teristics with observed health effects. There has been, however, a continuing inability

of epidemiologists to agree on specific sets of causal constituent and co-pollutant condi-

tions. One possible explanation for the ambiguity is inconsistency in the species being

measured during field campaigns. Technological progress has made the measurement

of many species more feasible for current studies than were previously available.   Un-

fortunately, a combination of cost and the sheer volume of data make it impossible for

researchers in field campaigns to monitor all components of the ambient environment.

In addition, just because a species *can* be measured does not mean that it will have sig-

nificant influence on the manifestation of adverse health effects. In general, therefore,

epidemiology looks to toxicological studies to evaluate their findings of association with

a given source. If such studies can verify the association with a measured effect in a

controlled laboratory environment, it indicates species of interest that merit monitoring

during epidemiology field campaigns. In general, however, laboratory-based toxicologi-

cal studies fail to confirm the effects at concentrations observed in the ambient envi-

ronment (Morgan, Reger et al. 1997; Salvi, Blomberg et al. 1999; Dybdahla, Risom et al.

2004; Carlsten, Kaufman et al. 2008; Laks, Oliveira et al. 2008; Sunil, Patel et al. 2009).

The most striking examples of these discrepancies are the associations found with sul-

fate in PM and diesel exhaust particles.

Toxicology Viewpoint
     Traditional toxicological studies typically focus on individual pollutants, or very

simple mixtures of pollutants. Understandably, toxicologists focus their attention on

reproducible sources of toxicity, such as 'standard' diesel exhaust particles from NIST (NIST 1991), that are well-characterized and available over long periods of time. In evaluations of the effects of diesel exhaust using 'real world' sources, the vast majority of the current studies *not* using particles from NIST use single, stationary vehicles or motors, and carry out biological exposures with what is, essentially, freshly emitted exhaust (Salvi, Blomberg et al. 1999; Knebel, Ritter et al. 2002; Li, Kim et al. 2002; Bauling, Sourdeval et al. 2003; Dybdahla, Risom et al. 2004; Carlsten, Kaufman et al. 2008; Laks, Oliveira et al. 2008; Liu, Ballaney et al. 2008; Sawant, Cocker et al. 2008; Sunil, Patel et al. 2009). Most of the studies listed above used filter-collected particle samples that were resuspended and instilled onto *in vitro* cells or into mammals for *in vivo* exposures. Epidemiologists have found size-dependent associations between ambient PM and adverse health effects, which led to the institution of NAAQS for both PM_{10} and $PM_{2.5}$. The use of suspension and instillation of particles for toxicity testing, however, will alter the size distribution of the PM so much as to be an unrealistic representation of the original source (Figure 1) (Kamens 2007; Cooney and Hickey 2011). In addition, the processing and storage of these particles dramatically compromises their utility as surrogates for PM from the ambient environment.
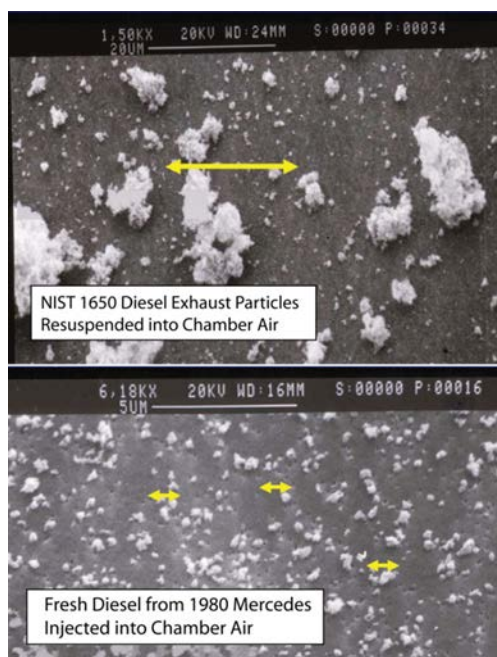
Figure 1: Electron micrographs of two diesel exhaust samples taken from chamber air.
The top image is of a 'standard' DEP, the bottom is from exhaust injected directly from the tailpipe into the Kamens Aerosol Chamber (Kamens 2007)

A few studies have used various methods involving chambers, all of which used diluted fresh exhaust as their PM source. Despite the extent of the characterization and speciation that can be done on these PM sources, whether 'real world' or 'standard', almost all completely miss the importance of the *atmosphere as a source* (Arnold 2009). Traditional studies completely omit atmospheric processes that alter the composition of *both* phases an air pollution mixture. These processes result in precisely the 'effect modification' mentioned in the NRC report.

While simplifications involving 'standard' materials and fresh vehicle exhaust are attractive to laboratory researchers, they ignore the entanglement of components of urban airsheds and are, therefore, representative of only a small portion of the overall picture. Considering these distortions of reality, it is hardly surprising that toxicology studies in a controlled laboratory setting have difficulties in replicating the findings of epidemiology.

Physical Chemistry and Aerosol Science Viewpoints

Extensive work by physical chemists and aerosol scientists has helped characterize the sources, and explain the environmental fate of ambient PM. The majority of the recent effort has gone into the development of atmospheric models for the generation of

secondary organic aerosols (SOA) and 'source apportionment' studies (Kleindienst, Corse et al. 2002; Fraser, Buzcu et al. 2003; Lee, Jang et al. 2004; Chellam, Kulkarni et al. 2005; Ito, Christensen et al. 2005; Mar, Ito et al. 2005; Donahue, Robinson et al. 2006; Hopke, Ito et al. 2006; Hu and Kamens 2007; Healy, Wenger et al. 2008). The impetus for the focus on SOA formation is the EPA, and its regulatory approach to PM. As mentioned above, epidemiology studies continually find associations between PM exposure and negative health outcomes, and it is these studies that the EPA uses as the basis for its policy decisions. Since the epidemiology studies being referenced by the EPA focused on mass measurements as their characterization of ambient PM, the EPA has based their NAAQS for PM_{10} and $PM_{2.5}$ on mass concentration measurements. It became quickly obvious to aerosol scientists and atmospheric chemists that primary emissions alone could not account for all of the PM mass observed in field studies. As such, being able to predict the behavior of ambient PM on a mass basis has become of particular interest to those attempting to successfully represent PM in airshed models.

The advent and subsequent refinement of partitioning theory has greatly advanced the understanding of atmospheric processes that affect the composition of ambient PM (Jang, Kamens et al. 1997; Pankow, Liang et al. 1997; Harner and Bidleman 1998; Kamens and Jaoui 2001; Healy, Wenger et al. 2008). With this additional information, researchers have been able to determine that atmospheric reactions between gases create species that contribute to SOA. Further, these can be a significant factor in ambient PM mass loading (Kamens, Jeffries et al. 1981; Kamens and Jaoui 2001;

Kleindienst, Corse et al. 2002; Lee, Jang et al. 2004; Donahue, Robinson et al. 2006; Hu

and Kamens 2007; Healy, Wenger et al. 2008).

Ambient Measurements and Source Apportionment Viewpoints

As measurement techniques have become more refined, a second focus has

emerged for those investigating atmospheric PM – source apportionment. The founda-

tions of source apportionment lie, again, in the findings of epidemiological studies.

Some studies found associations between adverse health effects and specific compo-

nents of ambient PM that could be attributed to a select few sources (Gauderman, Avol

et al. 2005; Thurston, Ito et al. 2005; Hopke, Ito et al. 2006). This provides the EPA with

an attractive regulatory approach that allows the Agency to attempt to address the del-

eterious effects of air pollution without being able to account for all of the entangle-

ment of the processes involved. The Agency already has in place mechanisms by which

it can mitigate the emissions of specific point sources, easing the creation of new regula-

tions (Jeffries 2010). Nevertheless, this *again* ignores the influence of the atmosphere as

a *source* of toxicity. Emissions targeted by source apportionment studies might be per-

fectly benign as they enter the atmosphere. It is possible for such sources that atmos-

pheric processes impart toxicity to them as they age in the atmosphere (Doyle, Sexton

et al. 2004; Sexton, Jeffries et al. 2004; Donahue, Robinson et al. 2006; Doyle, Sexton et

al. 2007). The influence of atmospheric processes cannot, therefore, be ignored when

determining causation of effects, if abatement strategies are to be effective.

In addition, associations can only be found with species that can be measured. The

treatment and storage of ambient PM samples likely bias the findings of epidemiology

studies toward species that were not easily lost from their filter samples (such as metals and elemental carbon) (Singh, DeMarini et al. 2004; Lipsky and Robinson 2006). That is not to say that the species measured are *not* those responsible for the documented adverse health outcomes, but without the inclusion of *all* members of a complex mixture in the statistical treatment of the data, no definitive answer can be reached. Speciation of samples and sampling artifacts, therefore, remain the primary obstacles to successful disentanglement of sources and their contribution to observed health outcomes.

Hampering the assessment of some health implications is the tendency, again, of the members of each of the disciplines involved to sequester themselves from the members of the other disciplines. It is natural for physical scientists to focus on improvements to measurement technologies and mechanism development, rather than performing direct assessments of the toxicity associated with the species being generated in their experiments. Recently, for instance, atmospheric chemists have determined through attempts to verify their model mechanisms against field data, that PM may be acting as a sink for both 'reactivity' and specific species not predicted to be found in the condensed phase, such as glyoxal (Volkamer, San Martini et al. 2007; Williams, Goldstein et al. 2010). While this has definite implications for a number of regulatory and policy reasons concerned with airshed models and state implementation plans (SIPS), it also has important implications for the toxicology and risk assessment communities.

Recently, there have been limited sponsored studies that have attempted to disentangle some of the gas and particle interactions that may impact the toxicity of PM (McDonald 2007; McDonald, Doyle-Eisele et al. 2010). The primary focus of these studies was explaining the association of sulfate with adverse health outcomes found by several epidemiology studies. Despite their attempts to induce a toxic response with sulfate alone and sulfate mixed with gaseous hazardous air pollutants (HAPs), they were only able to exhibit an increase in toxicity of the particle phase when they introduced oxidative reactions to their systems (specifically, O_3 mixed with α-pinene). While these reaction pathways may play important roles in the toxicity of ambient PM, these studies failed to isolate the physical processes by which air toxics are taken up by PM. A clear understanding of the physical processes involved, and how they directly affect the delivery and/or sequestering of species from the gas phase is vital to understanding the entanglement of gases and PM, and how it truly impacts human health.

Embracing Entanglement

The recent trend toward multidisciplinary collaboration has brought together researchers with sufficiently varied expertise to begin to fill the gaps in our understanding of the entanglements of the phases. While focused, isolated research studies often yield valuable knowledge, researchers need to embrace the entanglement of the environment, rather than attempting to split apart the environment to suit research needs. The idea that the environment can be separated into parts and then added back together again to regain the whole is unrealistic in this case. The atmosphere itself is a 'source',

and apparently a very important one, for affecting the toxicity of the material in the condensed phase (NRC 2004).

Gases vs. Particles as VOC Delivery Mechanisms to Lung Cells

Overview

For a pollutant to elicit a response from a biological system, it must have proximity to that system. What this means is that, for our cell cultures to exhibit an observable response, they must be able to absorb VOCs from the air above them. If the cells are to absorb VOCs from the airstream above them, the VOC must be transported to the air-liquid interface, where diffusion processes limit transfer of the VOC to the liquid layer. The absorbed dose of the VOC from the air is, therefore, determined by both its lipo-philicity and its ability to diffuse through water (McClellan and Henderson 1995; Schwarzenbach, Gschwend et al. 2003; Salem and Katz 2006). In this way, particulate matter that collects VOCs from the air around it can act as both a VOC concentrator and a delivery mechanism to bring the VOCs directly to the surface of the cells, mitigating the effects of diffusion processes on a VOC's uptake. Also, time is the single greatest non-toxicant-specific factor in determining toxicity. The longer residence time of PM in the deep lung (relative to gases) will allow protracted exposure to volatile species that would likely exit the respiratory system quickly if they were in the gas phase and the source of the toxicant was removed. Therefore, I believe that – rather than altering the actual mechanisms that cause toxicity, inclusion of a VOC in the organic layer of PM simply facilitates delivery to, and uptake by, airway epithelial cells.

Illustrative Example

Using partitioning theory and a targeted concentration for these experiments, an estimate of the relative dose delivered to the cellular membrane can be calculated for each exposure mixture. First, 1 ppmV of gas-phase acrolein is delivered to the air-liquid interface of our in vitro cell cultures (at 37 °C and 1 atm). The amount of acrolein that will partition to the extracellular liquid layer can be estimated with the octanol-air partitioning coefficient (K_{ioa})(Harner and Bidleman 1998; Pankow 1998; Schwarzenbach, Gschwend et al. 2003). As a literature value of K_{ioa} is not available for acrolein, it can be calculated from the air-water (K_{iaw}) and octanol-water (K_{iow}) partitioning coefficients (Schwarzenbach, Gschwend et al. 2003; Montgomery 2007):

$$K_{ioa} = \frac{K_{iow}}{K_{iaw}} = \frac{0.98}{0.00499} = 196$$

which means that, qualitatively, the acrolein would much rather be in the surfactant covering the alveolar membrane than in the air above it. This assessment is supported by Schwarzenbach, who states that we expect that most compounds of interest in the environment to partition 'favorably' from air to a condensed organic phase (2003). Quantitatively, this means that, at equilibrium and with a steady supply of acrolein in the gas phase, we expect that roughly 200 times more acrolein will be in the extracellular liquid layer than the air above it. Therefore, at equilibrium, ~0.5 mg of acrolein could potentially enter each liter of the liquid layer covering the cells.

This is, of course, an abstraction from reality, as neither the boundary layer above the cells nor the surface area of exposure factor into this calculation, and the surfactant

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layer above the alveolar cells isn't likely to be able to accommodate that amount of so-lute. Assuming an 'average' American man (5' 9.5"; 191 lbs/86.8 kg, CDC 2002), there should be 8.7 – 43 mL of liquid covering the internal surfaces of the alveolar region of the lung (0.1 – 0.5 mL/kg body weight; Notter, 2000). This means that in an 'average' American man, up to 4.4 – 21.5 µg of acrolein would be absorbed by the liquid lining of the lungs (calculated with K_{ioa}).

Moreover, to enter the cell itself, the acrolein has to cross an aqueous layer that lies between the surfactant layer and the cellular membrane(Becker, Kleinsmith et al. 2003). Even though acrolein is a polar compound, the aqueous layer contains ionic salts which are very effective at excluding organics from solution. The result is that diffusion of organics across the aqueous layer is greatly slowed (Schwarzenbach, Gschwend et al. 2003). This allows the aqueous layer to act as a barrier, protecting the cells from organ-ics that might otherwise be able to penetrate their membranes.

It is possible for a small amount of organic to diffuse across the aqueous layer, though it is thermodynamically unfavorable. As stated above, when an organic com-pound is delivered directly to the cellular membrane by PM, not only does it bypass the diffusion-limited transfer across the protective aqueous layer, the exposure to the cellu-lar membrane is effectively extended until a clearance process (such as consumption by macrophages) can remove the particle from contact with the membrane.

If organics *do* penetrate the aqueous layer, they should immediately partition to the cellular membrane. Once apolar and low-polarity organics become infused in the

lipid layer of the cell membrane, they can resist cellular clearance processes, and as such remain in the cell for an extended period of time. Moreover, when organics enter the cell in this way, they have free access to all cellular compartments, including the nucleus. This allows potential carcinogens (such as acrolein) to interact with DNA and protein synthesis mechanisms (Schwarzenbach, Gschwend et al. 2003; Salem and Katz 2006).

Originally, researchers developed octanol-water partitioning values as a means to estimate a compound's affinity for natural organic matter (Harner and Bidleman 1998; Schwarzenbach, Gschwend et al. 2003). Therefore, when mineral oil aerosol is introduced to the air stream, the affinity of the VOCs for the mineral oil should be about the same as for octanol (K_{ioa}). When an aerosol particle deposits on the surface of the airway epithelium, it can penetrate the aqueous layer, thereby bypassing the cell's defensive screen, and placing the surface of the aerosol droplet in direct contact with the cellular membrane. Researchers have also found that octanol has slightly different affinities for organic compounds than various types of biota, and have produced correction factors for various types of organic phases commonly found in animals (Schwarzenbach, Gschwend et al. 2003). If the correction factors for cellular membranes are applied to the octanol-water constant, we find that VOCs will actually preferentially partition from the mineral oil to the cellular membrane (the octanol-liposome constant K_{olip} = 0.51; from equations in Schwarzenbach). Simple diffusion will, therefore, push VOCs from the particle into the cellular membrane, where it can reside until it is able to diffuse into the cell body. If VOCs enter the cell in this manner, they should be free of endosomal mem-

branes – giving them free access to all compartments of the cell, including the nucleus (McClellan and Henderson 1995; Becker, Kleinsmith et al. 2003). If the VOCs are metabolized by cellular processes, it will shift the partitioning equilibrium to cause even more of the toxicant to diffuse into the cell's interior (Foster and Costa 2005). In the lung, diffusion along concentration gradients that cross the capillary membrane can cause an equivalent shift in equilibrium (Becker, Kleinsmith et al. 2003).

In the absence of particles, if the exposure stream is removed, equilibria between gas-phase toxicants and the air-liquid interface will shift, thereby causing VOCs within the liquid layer and the cells themselves to off-gas back to the air above the cells. This shift back to the gas phase will decrease the effective dose that remains in contact with the biological system (Schwarzenbach, Gschwend et al. 2003). An analogous real-world situation is the recommendation to move a person to 'fresh air' if they receive a high level of exposure to VOCs (this recommendation is common to MSDS and retail product warning labels).

If particulate with VOCs dissolved in it is deposited on the surface of the cells, however, it may effectively maintain exposure for the duration of the rest period. Even if equilibrium favors the VOC re-volatilizing into the air above the membrane rather than entering the cell itself, the local concentration of the VOC in the diffusive boundary layer above the cells will be elevated. This elevated concentration should serve to distribute the dose more uniformly across the surface of the membrane or, at least, minimize the

off-gassing of the VOC from the interior of the cells by maintaining the concentration gradient from the apical air to the cell interior.

Effects on Dosimetry

There are a number of ways that the partitioning of a VOC into particulate might affect the dosimetry of that compound. First, PM has multiple routes of entry into the body. The nasal and bronchial airways have very effective mechanisms for removing very large and very small particles from the air stream. Once removed, these particles are cleared from the airway via the GI tract. While this represents a clearance mechanism for the airway, the whole-body burden of the substance is not decreased (Salem and Katz 2006). Moreover, some substances that are not biologically active in the neutral pH of the airway (such as weak acids) will become activated by the higher H^+-ion concentration in the digestive system (McClellan and Henderson 1995). Another route of entry for particle-bound VOCs that may not be available to gas-phase VOCs is the lymphatic system.   Alveolar macrophages and interstitial fluid are both capable of transporting PM to the lymph nodes, which can act as reservoirs for 'translocated' PM. When retained particulate leaves the lymph nodes, it enters the circulatory system, by which it can be distributed across the organ systems of the body (McClellan and Henderson 1995).

Bearing all of these factors in mind, I feel that particle uptake will likely have the largest effect on the dosimetry of VOCs based on their solubility. Both the aqueous and lipid solubility of a compound will factor into the overall effect on its dose, but dosimetry will likely be most affected for highly water-soluble and highly water-insoluble com-

pounds. Compounds with high aqueous solubility will usually be taken out of the airway early. Conversely, compounds with low aqueous solubility may have very low absorption by the airway, and the bulk of the inhaled gas will be exhaled immediately. Dissolution into PM will, therefore, not only deliver both of these classes of compounds to a region of the lung they would not normally enter, it will also hold them at an elevated concentration for an extended period of time, thereby increasing their overall dose to that region of the lung (and possibly to the body as a whole).

Cellular Response

The most common injury that occurs in the lung from inhalation of air pollution is the induction of inflammation at the site of exposure. Inflammation is best defined as "localized and protective response, serving to eliminate or isolate injurious agents and facilitate the repair and regeneration of damaged tissue" (Huether and McCance 2004). This recovery is achieved by the recruitment of cells associated with inflammation, and 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.

Cytokines are thereby responsible for regulating cell differentiation, proliferation, and cell secretory activities. Therefore, investigation of inflammation caused by chemical exposure can proceed by detection and quantification of various cytokines involved in inflammatory processes. Inflammatory events induced in A549 cells by pollutants are signaled by an increase in expression of various inflammatory proteins, just as they

would be in the lung. Measuring these markers of inflammation and cytotoxicity can quantitatively assess the degree of inflammation or injury. Epithelial cells and macrophages release pro-inflammatory mediators such as the interleukins (IL-1, IL-6, IL-8, etc.), tumor necrosis factor-alpha (TNF-α), and macrophage inflammatory proteins (MIPs) which signal the body to recruit additional immune and inflammatory cells to clear the particle. These cytokines are early mediators of the body's response to injury and are expressed rapidly after exposure to toxic agents (Salem and Katz 2006).

Alternatives to direct biological exposures exist in the literature (e.g., DTT measurements of reactive oxygenated species (Rattanavaraha, Rosen et al. 2011)). While assays such as the DTT method are useful surrogates for direct biological exposures, the availability of methods to perform direct biological exposure in this study makes a surrogate measure unnecessary. Moreover, methods such as these are not able to differentiate between gas- and PM-phase species and as such will not be employed here.

Choice of *In Vitro* Model
As the primary gas-exchange region, the alveolar region is the primary target for HAPs. Most commonly, the respiratory epithelium is defined as the lining of the gas-exchange region. Therefore, epithelial cells are well-suited to be used in examinations of the effects of HAPs in the lung. In the gas-exchange region, the lining is covered by both type I pneumocytes (~96% of the surface area) and cuboidal type II cells (4% of the surface area). Type I cells most readily facilitate gas-exchange, while type II cells secrete surfactant that keeps the alveolar walls from sticking to one another. Once type I cells are damaged, type II cells proliferate and differentiate to repopulate the injured epithe-

lium (Huether and McCance 2004; Gardner 2006). A549 cells are a model of respiratory epithelial cells, with some type II cell-like characteristics, that have been used extensively to assess the toxicity of air pollutants. In addition, one of the major targets for ozone-induced toxicity is the proximal alveolar region and epithelial cells (Dungworth 1989), thus further justifying the use of an alveolar epithelial cell model.

Motivation

The motivation for this research program began with two observations. The first is that cells exposed with the PM sampler (EAVES) do not respond to air pollution mixtures when there are no particles present. The second is the disappearance of the response from cells exposed to aged SynUrb gases when diesel exhaust is present.

First Motivation – Monitors for Gas and Particle Phase Toxicities

The first observation that started this program occurred during the extensive initial testing of the Electrostatic Aerosol *in Vitro* Exposure System (EAVES). As an accident of the device design, cells exposed in the EAVES device are *insensitive* to the composition of the gas-phase. What this means is that, when there are no particles present in the air stream, exposure in the EAVES device does not induce a response from the cells (de Bruijne, Ebersviller et al. 2009). Whether this lack of response to gas-phase insult is due to the relatively short period of exposure in the device or some other factor is still unclear. Even without completely understanding why the cells don't respond to gases alone, we can use the effect to our benefit.

In addition, it has also been observed that, although the particles are never removed from the airstream prior to its entry into the gaseous cell-exposure chamber, ex-

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posures carried out in the Gas *In Vitro* Exposure System (GIVES) do not show effects caused by PM. The relatively long settling time of particles typically used in our experiments (100 – 800 nm mode diameter) prevent the deposition of sufficient PM to elicit a response in the GIVES. The settling velocity of a 500 nm particle is 9.91 x 10^{-6} m s^{-1}, while the deposition velocity in the EAVES device is 7.63 x 10^{-4} m s^{-1}, meaning that PM is removed from the airstream in EAVES much more efficiently than in the GIVES device (Hinds 1999).

The net result is a pair of *in vitro* exposure systems that are uniquely suited to investigations of gas-particle interactions. The EAVES and GIVES can monitor the effects of the gases and particles separately, but without disturbing the equilibrium between them (see Chapter 3, Part 1 for further description of the systems). This advance in particular will allow us to investigate these phenomena in ways not previously available to the research community.
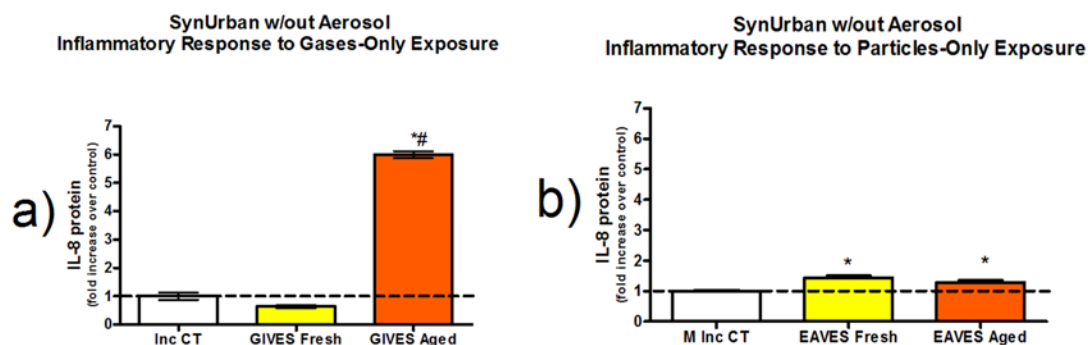
Figure 2: Inflammatory response of cells to a) gas-only exposure and b) particle-only exposure for Fresh and Aged SynUrb with no PM present in the chamber. These results are presented and discussed more fully in Chapter 3, Part 2.
* Indicates a significant change relative to the incubator control; # Indicates a significant change between the 'Fresh' and 'Aged' exposures.

Second Motivation – Gas Phase Toxicity was Missing When PM was Present

When SynUrban gases are photochemically aged (without seed particles present), there is a strong increase in the toxic response to the gases after irradiation (Figure 2). Furthermore, there is no increase in response from particle-only exposures in this system (no SOA forms, so there is no response from cells exposed in EAVES). This is due mainly to the lack of SOA nucleation in the system without the presence of seed particles. This is somewhat counter-intuitive, because SynUrb contains about 27% carbon that is aromatic (Bloss, Wagner et al. 2005; Hu and Kamens 2007).

When SynUrb is aged in the presence of biodiesel, however, there is no increase in response from the gas-only exposures (this system is discussed further in Chapter 3, Part 3). This is contrary to one of the major approaches to addressing the toxicity of mixtures. Typically, the approach is to assume an 'additive' effect. That is, when toxic species are added together, the net toxicity is the sum of the individual toxicities of each of the species present in the mixture. In the biodiesel with SynUrb system, not only does
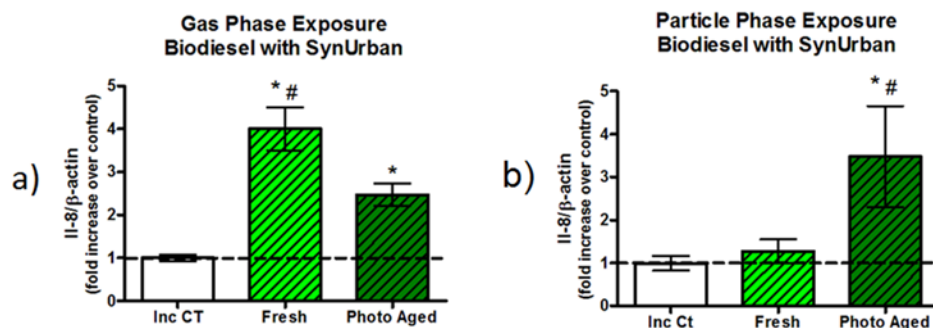
Figure 3: The effects of photochemical aging on biodiesel emissions mixed with SynUrb Mix. Inflammatory response of cells to a) gas-only exposure and b) particle-only exposure. These results are presented and discussed more fully in Chapter 3, Part 3.
Cells were exposed to incubator air, fresh emissions, and emissions that have been irradiated for one whole day. * Indicates a significant change relative to the incubator control; # Indicates a significant change between the 'fresh' and 'aged' exposures.

the toxicity seen in the 'Fresh' gaseous emissions *not* increase, it *decreases* significantly with aging (Figure 3a). This unanticipated result led to the question: where could the toxicity have gone?

Examination of the particle-only (EAVES) exposure provides a likely candidate. While current theories on PM toxicity would expect a response from exposure to DE particles, the 'Fresh' exhaust particles did not induce a significant response from the cells. After a full day of irradiation, however, the 'Aged' exhaust particles elicited a four-fold increase in response relative to both the incubator controls and the 'Fresh' exposure. Though this result was unexpected when first observed, it has been noted in several systems since (Lichtveld 2008). For brevity sake, these results will not be discussed here.

For aerosol scientists and atmospheric chemists, the concept of species moving between phases is not a new one (as detailed above). It is possible, therefore, that toxic

species in the Fresh emissions were taken up by the particles during aging. This conclusion, however, completely ignores the evolution and consumption of species that occurs with atmospheric aging. Another explanation is that the gaseous components of the Fresh system responsible for the observed Inherent toxicity were rendered biologically inert with aging, and that the PM, conversely, acquired toxicity with aging. There are many iterations of these (and other) processes that might result in the observed outcome.

Modeling the Systems as States with Processes that Alter the States

Table 1: Conceptual Gas and Particle States, Processes, and Properties in the Atmosphere

State	Rate of Change	Processes	Toxicity
Fresh	None	• Only dilution	• Inherent
Thermally Transforming	Low Low Energy System	• Particle uptake and off-gassing • On-particle reactions • Depend on composition of both phases	• Transformed Inherent • Acquired • Redistributed
Photo chemically Transforming	Fast High Energy System	• All processes from Thermally Aging • Secondary reactions: $\cdot$OH, O_3, VOC oxidation etc.	• Transformed Inherent • Acquired • Transformed Acquired • Redistributed
Aged	Low Low Energy System	• Thermal processes • Depend upon composition • Lingering secondary reactions from Photo-Aging	• Transformed Inherent • Acquired • Transformed Acquired • Redistributed

Evolution of State

Therefore, conclusions drawn from these data are complicated by the question of whether DE particles possess some characteristic that imparts an inherent toxicity to them. In addition, recent evidence has been found for the presence of species in PM that were previously thought too volatile for particle uptake (Volkamer, San Martini et al. 2007; Williams, Goldstein et al. 2010). We already have, therefore, conceptual explanations for our observations. What we require is clear proof of the explanations that are most feasible.

To create a conceptual framework in which this research program can take place, a series of evolving *States* and associated *Processes* that act on the States will be described. Each State describes a type of atmospheric system that will be encountered in the research program. The States are not intended to represent reality, but rather to define a set of vocabulary that can be used to describe phenomena of interest. Each State has characteristic Processes associated with it that act on both the gas and particle components of the system to evolve the State from its original composition. The Processes outlined below represent possible explanations for observed effects, but are not intended to explicitly represent all possible chemical and thermodynamic phenomena that may occur in the ambient environment. Rather, they are broad characterizations of those phenomena.

Table 1 provides the names and principal characteristics of these States.

Fresh

Characteristics

The 'Fresh' state is an entirely conceptual construct. Many researchers, myself included, refer to 'fresh' emissions as if they are a static state. In the real world, emissions – whether they are gaseous emissions from a refinery or exhaust from a diesel engine – begin to change as soon as they enter the atmosphere. As such, there is no such thing as a static Fresh state in the ambient environment. As experimentalists, however, we need a 'zero' to which we can compare our experimental results. The Fresh state fulfills that need.

For the sake of having a baseline for experimental work, we define the artificial Fresh state as having a static composition over the time periods necessary for our sample collection and exposures. If these measurements are carried out in an efficient manner, and at night, the composition of the mixtures should change slowly enough that an average composition over several hours is a sufficient approximation for our needs.

Toxicity

The Fresh state has, by definition, only Inherent toxicity in both the gas and particle phases. In reality, the semi-volatile and volatile components of each phase begin to intermingle (becoming entangled) as soon as the emissions dilute into the atmosphere.

Thermally Transforming

Characteristics

The 'Thermally Aging' state can be thought of as representing 'dark aging' in the chamber, or emissions mingling freely in the atmosphere at night, with no external oxi-

dative processes working on the system. This state involves relatively low energy processes, such as particle uptake and off-gassing of VOCs and water. Also included in this state are on-particle processes that can modify the chemical composition of the system as a whole. The specific processes involved are dependent upon the compositions of both the gas and particle phases.

Toxicity

Already at this state, the toxicities are fully entangled. When dealing with a PM material that possesses some degree of Inherent toxicity, it is impossible to separate its Inherent toxicity from toxicity acquired from the atmosphere. Any attempt to isolate the two will result in a fundamental change in both the gas and particle phases of the mixture, thereby altering the result of the attempted analysis.

Further complicating the separation of effects is the inability to determine the degree to which the Inherent toxicity of the PM is, itself, being modified. In this system, the processes that modify the chemical composition of the primary emissions will happen predominantly in the condensed phase. In some systems, Inherent toxicity may diminish with consumption of some species by aging processes in the atmosphere. In another system, atmospheric processing may enhance the Inherent toxicity itself. In all cases, species acquired from the atmosphere may contribute significantly to the overall toxicity of the PM, or their contribution might be negligible.

Conversely, species originating in the particle phase may be driven into the gas phase by atmospheric processes. In these cases, the gas phase is 'Acquiring' toxicity from the particle phase. Furthermore, species that originate in the gas phase might par-

tition to the particle phase, thereby decreasing the Inherent toxicity of the gas phase. The partitioned species might then undergo on-particle processes that transform them to one of many 'daughter' species. These daughter species may then re-enter the gas phase, imparting Acquired toxicity to the gas phase.

It is not currently possible to determine the individual contributions of the Inherent and Acquired toxicity to the overall toxicity observed following exposure. It is only possible to determine whether there has been a net change in the observed toxicity due to atmospheric modification. Any attempt to ascertain the contribution of one process by isolation from the rest risks the fundamental modification of the system. As such, it is easy to see why the disentanglement of gases and particles has given the research community, as a whole, so much difficulty.

Photochemically Transforming

Characteristics

All of the processes present in the Thermally Aging state still occur in the Photochemically Transforming state. The Photochemically Transforming state, however, includes much higher-energy processes as well. In addition to the on-particle processes present in the Thermally Transforming state, energy from the irradiation of the system imparts energy directly to reactions that transform primary pollutants to their daughter products. The result of these reactions is an even greater entanglement in the phases.

Due to the increased thermal energy during the day, on-particle processes have more energy with which to transform components. Additionally, species are more likely to off-gas from the condensed phase into the gas phase. Individual species (regardless

of the phase in which they originated) can undergo reaction by photochemically-generated radicals. In general, these reactions increase the polarity of the species involved. Typically, this increase in polarity makes species more likely to be taken up by the PM in the system, where they can undergo further modification before they start the off-gassing/uptake cycle again. It is these cycles of processes that result in the atmosphere acting as a source of acquired toxicity for both phases.

Toxicity

The toxicity of the Photochemically Transforming state can be rapidly-evolving. Both phases can be modified directly by either irradiation or reactions with photochemically-generated species. Species can be generated *in situ* and then consumed in a few hours. This can result is a peak in toxicity at any point in the day. In addition to oxidized daughter products of species present in the Fresh state, secondary oxidizing species (such as O_3) may be present in this system. Such species add a new dimension to the acquired toxicity of the gas phase, and can react directly with the particle phase as well. Moreover, Fresh species emitted into a Photochemically Transforming system can be modified very quickly, resulting in the types of entanglements that continue to confound studies of near-roadway health effects.

Aged

Characteristics

This is, again, an experimental simplification of a real-world condition. In reality, the 'Aged' state is not static, nor is it a return to the Thermal Aging state. In addition to all of the processes involved in the Thermally Transforming state, some secondary (oxi-

dative) species may linger from the Photochemically Transforming state. For the purposes of this research program, an 'Aged' state will be defined as one in which the high-energy aging processes have ceased (such as after sundown). The Aged state will continue to evolve, but at a much slower rate than the Photochemically Transforming state, and with lower-energy reactions and processes. The Aged state serves as our experimental stopping point and, as with the Fresh state, if our measurements and exposures are performed efficiently the composition of the Aged state should be changing slowly enough that we can assume it to be static to suit our needs.

Toxicity

As mentioned above, secondary species may that might result in either direct toxicity or an evolving toxicity in both phases persist in this state but are, generally, reacting at a relatively slow rate. The remaining entanglements are similar to those in the Thermally Aging state. While this simple statement is accurate, the complexity of the system and processes involved should be re-emphasized. Interactions between an even broader range of polar, oxygenated species and particulate present in the system results in an environment that is hopelessly entangled.

Hypothesis

The lung cell toxicity of urban-like atmospheric mixtures can initially be due to either the inherent toxicity of gases present, the inherent toxicity of particulate matter present, or both. Furthermore, under both dark and irradiated conditions the inherent toxicity can be enhanced or decreased by atmospheric processes in either phase, and total toxicity can be acquired by production of new toxic species. Both the inherent and

acquired toxicity can be dynamically re-distributed by movement of toxic species be-tween the phases such that the total toxicity may: a) increase (be acquired) or b) de-crease (be diminished) or c) be preserved (be sheltered from loss). Such changes can strongly affect the delivered dose upon exposure. This entanglement of species implies that the phases might not be separable without distorting their toxicities. The result would likely be a misestimation of the source and total toxicity for the atmospheric sys-tem.

The purpose of this thesis work is to demonstrate (quantitatively and incremental-ly) the changes in lung cell responses that occur upon exposure to increasingly complex gas and particle systems. These model systems will be capable of revealing (as unam-biguously as possible) the existence of inherent and acquired gas and particulate toxici-ties, and their dynamic entanglement, under dark and photochemical conditions.

The primary goal is to provide both chemical and toxicological data that demon-strates and defends the existence of 'effect modification' as called for by the National Research Council. This finding would have an impact on the EPA's policy approach of relying on individual NAAQS verses a "mixture" approach.

Aims
The research program will pursue the following aims:

Aim 1: Improve the ability of the community to assess the health effects of

exposure to PM by developing a new *in vitro* exposure methodolo-

gy that removes sampling and handling artifacts inherent to current exposure methods

Aim 2: Design and implement a simple system that will demonstrate the ability of VOCs known to be in aged mixtures of air pollutants to increase the toxicity of particulate matter through a limited number of thermal processes alone (when there is no chemistry occurring in the system)

Aim 3: Use a synthetic gas-phase system that is adequately representative of the complexity of the gaseous components of urban air to determine the behavior of the acquired toxicity of the mixture as it photochemically ages during the day

Aim 4: Using the same complex system, determine the degree to which a non-toxic PM can acquire toxicity from aged, gaseous pollutants, and the effect of PM on *in vitro* exposures to both phases.

Aim 5: Determine the effect of using an alternative fuel (biodiesel) on the composition and toxicity of emissions from a diesel engine

Aim 6: Determine the manner in which the chemical composition and toxicity of photochemically-aged emissions compare to those of fresh emissions, using diesel and biodiesel, both with and without the presence of a representative, reactive urban atmosphere

Research Strategy

If processes affecting the toxicity of PM are to be successfully investigated, a research program needs to be developed that can address the entanglement of the states and processes involved. An experimental system must be designed that removes most of the complexity of the ambient environment so that individual processes can be targeted in a very simple (but focused) system. As the program progresses and processes have been characterized, complexity can be added back in until the experimental system is adequately complex to represent the ambient environment. The following strategies will be used to meet these criteria:

Study 1: Current *in vitro* exposure technology may not be suitable for observations of particle uptake of species of interest. Most current methods involve some sort of collection and then equilibration or heating step that will result in the loss of volatile species from samples (Singh, DeMarini et al. 2004; Swanson, Kado et al. 2009; Williams, Goldstein et al. 2010).

Due to these shortcomings, a new exposure technology will be introduced to improve likelihood of observing phenomena of interest. To improve upon existing exposure methods, the new method should maintain the equilibrium between gases and particles until the pollutants arrive at the cellular interface. The exposure should be performed in such a way that minimizes artifacts that affect volatile species. This new technology will provide a biological response monitor to more-accurately estimate the health effects of exposure to airborne mixtures of gases and PM.

Study 2: Devise methods to isolate the effects that the uptake of gases by air-borne PM has on the observed toxicity of the PM. Initially, start with atmospheres containing only daughter species commonly found in photochemically-aged, more-complex mixtures. Create a highly-designed, completely synthetic atmosphere that simplifies the system by removing the chemistry that transforms the parent compounds into their daughter species. This study is to investigate the manner in which acquired toxicity of PM is influenced by particle uptake of oxygenated compounds. Species in the volatile to semi-volatile range will be selected for investigation.

To reduce the number of processes that must be investigated to explain observed effects on PM toxicity, a synthetic PM material will be selected. To be suitable for this study, the material should produce a particle of sufficient complexity to be representative of atmospheric PM, but lack any inherent toxicity of its own. This is not an attempt to simulate the actual atmosphere, but an investigation of phenomena that occur in the environment, and their contribution to the acquired toxicity of both the gas and particle phases.

This study will be the first clear-cut proof of direct modification of PM toxicity by gaseous organic air pollutants.

Study 3: The complexity of the system will be increased both by the number of species present in the system, and by the utilization of photochemistry to generate oxygenated species *in situ*. Synthetic Urban Mix (SynUrb) will be irradiated in an outdoor

smog chamber both with and without SynUrb to generate an Aged system. The toxic response will be evaluated both with and without a non-toxic PM present.

In this study, the chemistry involved will be made more complete, but the particle components will be kept simple. Carrying out the program in this way makes one aspect of the pollution mixture complex at a time to decrease uncertainty in the source of observed effects. Therefore, the synthetic PM material selected in Study 2 will be propagated to this study. While this approach misses aging processes that occur on-particle and inherent toxicity in the PM used, it will greatly simplify the analysis of each phase, thereby allowing more-concrete conclusions to be drawn about the processes involved.

This study will demonstrate that the phenomena observed in Study 2 can be extended to mixtures with complexity representative of the ambient environment. This study also introduces the atmosphere as a source of acquired toxicity for both phases.

Study 4: The final phase of this program will attempt to incorporate the complexity of real-world sources with the already complex reactive environment from Study 3. The resulting system will adequately represent the complexity of the species and processes present in the ambient environment. The Inherent toxicity of DE particles and on-particle aging processes that occur in urban settings will be added back to the system, so that the relevant processes are in balance with one another as they would be in the ambient environment. In addition, the system will be tested with DE alone, to determine the influence of the gaseous background components (SynUrb) on any observed toxicity.

This study, when incorporated with the results of studies above, will result in a better understanding of permissible explanations for the evolution of toxicity in the system. This study also provides a demonstration of the influence of atmospheric processes as a source of toxicity. The outcome could have serious ramifications for the manner in which ambient PM, and all independently regulated air pollutants, are regulated (NRC 2004).

Conclusion

The realization of this research program will provide researchers and policy makers with information vital to their ability to focus their efforts on the species and processes responsible for observed health outcomes. In addition, it will provide insight into the relationship between gases and particles in terms of chemistry, exposure, and potential health effects. This work will also provide direction for development of risk assessment models by indicating processes that must be represented, and the amount of detail the processes must contain in future models. A clearer understanding of the processes and species important to observed responses to pollution exposures will diminish the gap between the findings of epidemiology studies and toxicology studies done in controlled laboratory environments. While the fundamental contribution being made to each discipline will be different, the overall contribution to the air pollution research community will be significant.

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

This chapter encompasses Aim 1 and Study 1, and has been published in Inhalation Toxicology as a stand-alone manuscript of the same title[1].

Introduction

Materials and Methods

Results

Discussion

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, Thun et al. 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, Nordenhall et al. 2000; Singh, DeMarini et al. 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-α), following exposure (Kim, Reed et al. 2005; Seagrave, Dunaway et al. 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,

Flescher et al. 1997; Doyle, Sexton et al. 2004; Seagrave, Knall et al. 2004; Sexton, Jeffries et al. 2004; Doyle, Sexton et al. 2007).

Several methods to conduct *in vitro* exposures to PM have appeared in the literature (Bayram, Devalia et al. 1998; Boland, Baeza-Squiban et al. 1999; Abe, Takizawa et al. 2000; Aufderheide and Mohr 2000; Li, Kim et al. 2002; Madden 2003; Jaspers, Ciencewicki et al. 2005; Seagrave, McDonald et al. 2005; Mazzarella, Ferraraccio et al. 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, Devalia et al. 1998; Boland, Baeza-Squiban et al. 1999; Abe, Takizawa et al. 2000; Knebel, Ritter et al. 2002; Mazzarella, Ferraraccio et al. 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, Diaz-Sanchez et al. 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, Barr et al. 2004). Alternatively, impingers have been used to sample air con-

taining PM through a liquid in which the particles are collected (Li, Kim et al. 2002; Madden 2003; Jaspers, Ciencewicki et al. 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 and Mohr 2000; Seagrave, McDonald et al. 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, Ritter et al. 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 and Davidson 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, Charlson et al. 1974; Knutson and Whitby 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 sub-

sequently 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.

Figure 4: Top view image of the Electrostatic Aerosol *In Vitro* Exposure System (EAVES) holding the four Millicells

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 4). 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 nu-

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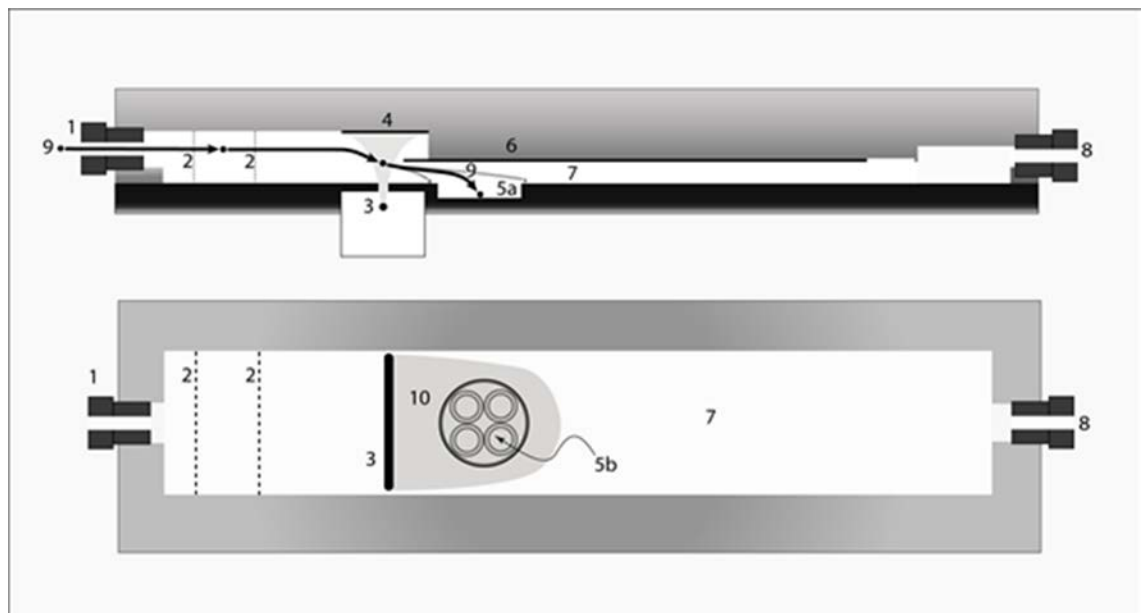
Figure 5: Side view schematic of the EAVES with key components labeled.
(1) inlet, (2) diffusers, (3) corona wire, (4) corona power plate, (5) in vitro cell holding well, (6) repellant plate, (7) particle collection plate, (8) outlet, (9) particle pathway. Air flow through the system is from left to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

brate 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 5). 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° 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, Jeffries et al. 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 fluorescent-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, Koutrakis et al. 1994; Sioutas, Abt et al. 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 6) before exposure (Jeffries, Fox et al. 1976; Jeffries, Sexton et al. 1985; Doyle, Sexton et al. 2004; Lee, Jang et al. 2004; Doyle, Sexton et al. 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.

Figure 6: 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%.

Statistical Analysis

All data are presented as the mean ± 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
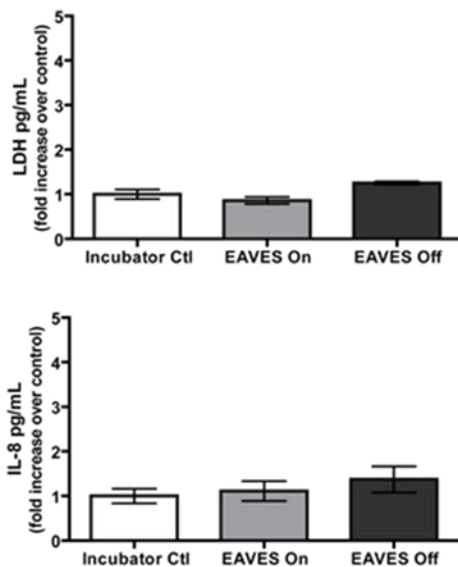
Figure 7: Effects of EAVES deposition field on cytotoxicity and inflammatory mediator production.
A549 cells were exposed to clean air in the EAVES for one hour and tested for LDH and IL-8 release 9 hours post-exposure. Grey bars = EAVES turned on; black bars = EAVES turned off. LDH and IL-8 levels are expressed as fold increase over non-exposed incubator

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 7) re-lease was statistically different from cells maintained in the incubator for the entire ex-

posure 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
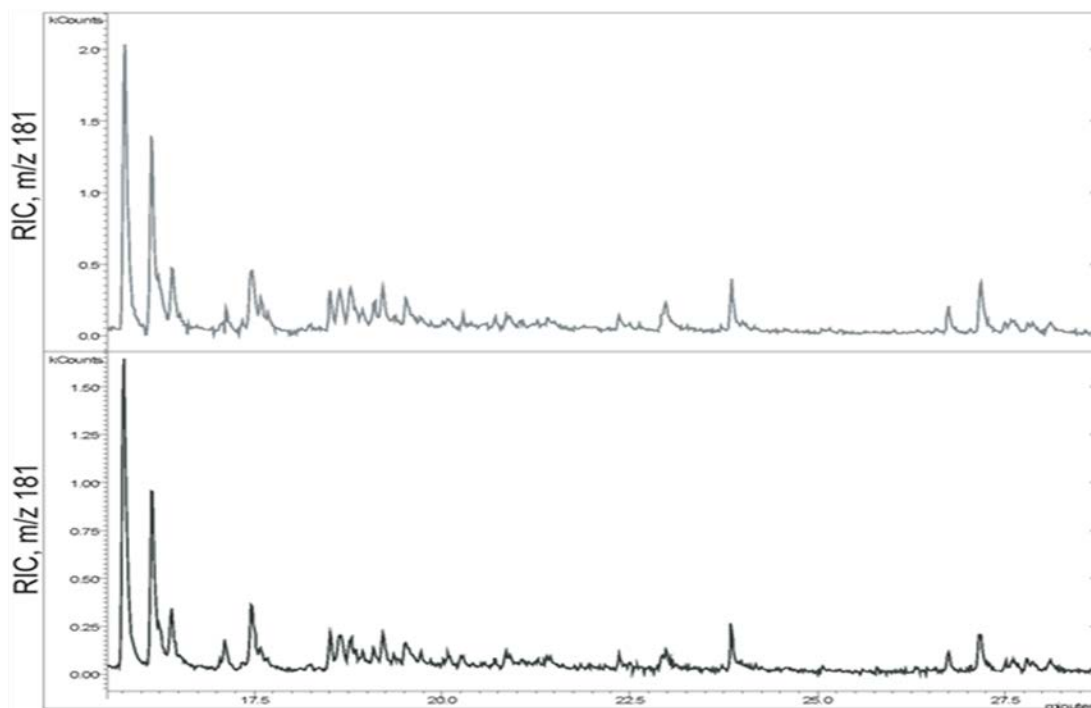
Figure 8: Reconstructed relative ion current of m/z 181 ion chromatograms of carbonyls detected by PFBHA-derivatization of diesel exhaust gases before (top) and after (bottom) passing through the EAVES.

any changes in chemical composition of the air stream. The chromatograms in Figure 8 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 8, 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 9 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).
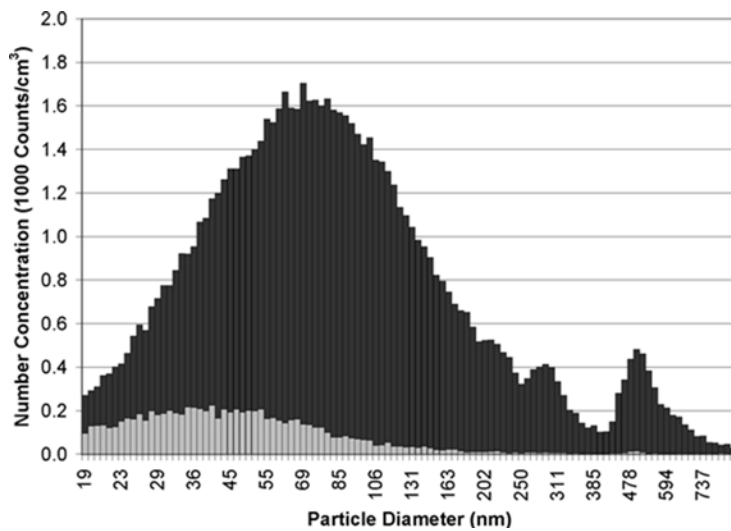
Figure 9: Histograms of particle number per size interval of particles entering and exiting EAVES.
Black histogram = PSL spheres exiting the EAVES with the power off; Grey histogram = PSL spheres exiting the EAVES with the charging and deposition field on.

Particle Deposition onto Tissue Culture Inserts

Table 2: 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

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. 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 parti-
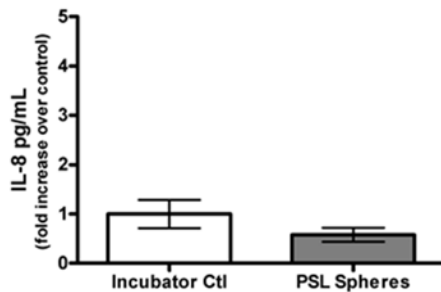
Figure 10: IL-8 release from A549 epithelial cells exposed to charged polystyrene latex spheres (PSL).
Clear bar = incubator control; Grey bar = cells exposed to PSL spheres operating with the power applied to the charging plate. A549 cells were exposed in the EAVES for one hour and examined for IL-8 release 9 hours post-exposure. Data represent means + S.E.M.

cles, the PSL spheres were allowed to deposit onto A549 cells under normal operating conditions. Figure 10 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, Zonnenberg et al. 1998; Boland, Baeza-Squiban et al. 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

Figure 11: 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.

diesel particles were approximately 0.2 µm in size. From the deposition measurements conducted with the PSL microspheres shown in Table 2, we can estimate that each tis-

sue culture insert received approximately 2.64 ± 0.66 µg (4.18 ± 1.04 µg/cm^2) of diesel particles. Figure 11 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 and Willeke 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, Sexton et al. 2004; Sexton, Jeffries et al. 2004; Doyle, Sexton et al. 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, Devalia et al. 1998; Boland, Baeza-Squiban et al. 1999; Abe, Takizawa et al. 2000; Knebel, Ritter et al. 2002; Mazzarella, Ferraraccio et al. 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, Devalia et al. 1998; Boland, Baeza-Squiban et al. 1999; Abe, Takizawa et al. 2000; Knebel, Ritter et al. 2002; Mazzarella, Ferraraccio et al. 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, Devalia et al. 1998; Boland, Baeza-Squiban et al. 1999; Abe, Takizawa et al. 2000; Knebel, Ritter et al. 2002; Mazzarella, Ferraraccio et al. 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.

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:
Gaseous VOCs Rapidly Modify Particulate Matter and Its Biological Effects

This chapter encompasses Aims 2-6 and Studies 2-4, and is being submitted to Atmospheric Chemistry and Physics as a set of three companion manuscripts.

Part 1 – Simple VOCs and Model PM[1]

Part 2 – Complex Urban VOCs and Model PM[2]

Part 3 – Petroleum Diesel and Biodiesel Oxidations[3]

Each Part contains the following sections:

Introduction

Methods

Results

Discussion

Conclusions

Chapter 3:
Gaseous VOCs Rapidly Modify Particulate Matter and Its Biological Effects
Part 1, Simple VOCs and Model PM

Introduction

In its most-recent document summarizing the state of the science related to PM, the US National Research Council (NRC) stated that "a finding that the effect of particles depends on the concentration of another pollutant—that is, 'effect modification'— would have implications for setting NAAQS [National Ambient Air Quality Standards] independently for the various criteria pollutants" (NRC 2004).

This study was designed to demonstrate the existence of "effect modification" in as simple a system as possible, and to increase the recognition of the atmosphere itself as a significant source of detrimental biological effects that are typically associated with PM. Researchers have long known that gases and particles interact in important and dynamic processes during the aging of particulate matter (PM) in the atmosphere. Volatile organic compounds (VOCs) are constantly shifting between the gas and particle phases of ambient air and at the same time can be modified by chemical reactions in each phase. Partitioning theory has evolved over decades, and has more recently been coupled to atmospheric chemistry models in an attempt to capture and characterize these interactions in a quantitative way (Kamens, Jeffries et al. 1981; Pankow, Liang et al. 1997; Kamens and Jaoui 2001; Donahue, Robinson et al. 2006; Hu and Kamens 2007; Kamens 2007). What has remained uncharacterized to this point is if—and how—these

interactions affect the actual toxicity of each phase. While toxicological co-pollutant effects such as synergism, antagonism and the effects of pre-exposures have been investigated, much less is known about the direct effects of gas/particle interactions on the resulting toxicological risks resulting from changes in composition and dosimetry of both phases. Inclusion of these processes is necessary if the contribution of the atmosphere as a source of toxicity is to be modeled accurately in a laboratory setting.

Currently, most studies of health implications of PM exposures focus primarily on the toxicity of extracted PM itself or direct primary PM (Farina, Sancini et al. 2011; Steenhof, Gosens et al. 2011). This approach, while attractive to laboratory researchers, ignores the complexity of the air surrounding PM in the ambient environment, thereby missing the contribution of the atmosphere itself as a source of toxicity. The NRC states that "such approaches are likely to over-simplify the underlying biological processes and how the mixture of air pollutants that is inhaled adversely affects health" (NRC 2004). The absorbed dose of a VOC from the air is determined by both its lipophilicity and its ability to diffuse through water (McClellan and Henderson 1995; Schwarzenbach, Gschwend et al. 2003; Salem and Katz 2006). If airway cells are to absorb VOCs, the VOC must be transported to the air-liquid inter- face in the lung, where diffusion processes limit transfer of the VOC to the liquid layer. Compounds with high aqueous solubility will usually be taken out of the airway early. Conversely, compounds with low aqueous solubility may have very low absorption by the airway, and the bulk of the inhaled gas will be exhaled immediately. Dissolution into PM will not only deliver both of these classes of compounds to a region of the lung they would not normally enter, it will also hold

them at an elevated concentration for an extended period of time, thereby increasing their overall dose to that region of the lung (and possibly to the body as a whole).

PM is able to bypass the diffusion-limited transfer of species across the aqueous layer above the surface of the cells in the airway. Bypassing this transfer brings partitioned VOCs into direct contact with the cellular membrane, thereby altering the dosimetry of the VOCs. When species are delivered to the cell surface in this manner, simple diffusion will push the VOCs from the particle into the cellular membrane, where they can reside until they are able to diffuse into the cell body (Becker, Kleinsmith et al. 2003; Schwarzenbach, Gschwend et al. 2003). Once apolar and low-polarity organics become infused in the lipid layer of the cell membrane, they can resist cellular clearance processes. When VOCs enter the cell in this manner, they will most likely be free of endosomal membranes—giving them free access to all compartments of the cell, including the nucleus (McClellan and Henderson 1995; Becker, Kleinsmith et al. 2003). If the VOCs are metabolized by cellular processes or diffuse across the capillary membrane into the bloodstream, it will simply shift the partitioning equilibrium to cause more of the toxicant to diffuse into the cell's interior, thereby maintaining an elevated dose (Foster and Costa 2005).

The purpose of this work is to demonstrate that physical/thermal processes among gases and particles can alter the delivery of air toxics to lung cells. This and subsequent studies (Parts 2 and 3) describe an integrated, step-wise approach to move from very simple systems to systems that approach the complexity of the ambient at-

mosphere. Altogether, these studies reveal the existence of inherent and acquired gas and particulate cellular toxic effects, and their dynamic entanglement, that is, they provide both chemical and biological effects data that demonstrate and defend the existence of PM "effect modification" as called for by the NRC.

Methods

Experimental Design

Our total experimental design is divided into "simple" systems (systems in which no photochemistry occurs) and "complex" systems (in which species were photochemically oxidized in situ). The latter also include chamber injections of real emissions from a diesel engine operating with different fuels. The results presented in Part 1 are based on the simplest system in which particle uptake of VOCs can be investigated. This simplicity is by design, and the main motivation behind creating such a highly simplified system is the desire to assign causality as unambiguously as possible. Thus, as many competing processes as possible have been deliberately removed to allow observations of the detrimental effects responses to PM exposure to be assigned to a very limited number of chemical and physical processes.

To reduce the number of factors that had to be investigated to explain observed effects on PM effects properties, a model PM material was selected. To be suitable for this study, the material had to produce a particle of sufficient complexity to be representative of atmospheric PM, but lack any inherent biological effects properties of its own. This approach is not an attempt to simulate the actual atmosphere, but to create a model with its inherent generalizations, distortions, and deletions, but still suitable to

illustrate contributions to the acquired biological effects properties of both the gas and particle phases.

Our experiments are only performed when the minimum outdoor temperature is forecast to be at least 15.0 °C. In the work presented here, the measured temperature range that occurred across all experiments was 16.5-28.0 °C. For experiments in which the chamber needed to be humidified, the chamber was first flushed with the output of a clean air generator to purge background VOCs, and then humidified with HPLC-grade water to a dew point (DP) of at least 16.0 °C, but less than 19.0 °C. While this narrow range of DP may not represent the entire range of relative humidities that may be en-countered in the ambient environment, it does limit variability in the system caused by humidity effects while still avoiding false-positives from biological effects endpoints caused by desiccation of the cells. To remove the possibility that either the VOCs or the PM used in these experiments might be modified by photochemical processes, all exper-iments were performed in the large outdoor chamber at night.

In vitro cell cultures were exposed to six types of air mixtures: clean air, clean air containing mineral oil aerosol (MOA), a single volatile organic compound (VOC) only (with no aerosol present), and a single VOC mixed with MOA. To explore the degree to which volatility alters the uptake of VOCs by PM, single VOCs in both the volatile and semi-volatile range were selected (bringing the total number of air mixtures to six). No cells were exposed to both VOCs at one time. The goal of this work is not to compare

the toxicity of specific VOCs and, as such, the biological responses of the lung cells will not be cross-compared between VOCs.

Chamber

UNC's atmospheric chemistry groups use environmental irradiation chambers (smog chambers) to study the dynamics of the chemical and physical processes that occur naturally in the atmosphere (Jeffries, Fox et al. 1976; Kamens, Jeffries et al. 1981; Jeffries, Sexton et al. 1985; Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004). The chambers allow complex atmospheres to be repeatedly generated in a controlled environment, but still aged in a more-realistic manner than has previously been available to researchers. More recently, the chambers have been coupled directly to two in vitro exposure systems capable of evaluating the relative toxicities of both the gas and particle phases of air pollution mixtures while maintaining the equilibria between the gas and particle phases throughout exposure (Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004; Doyle, Sexton et al. 2007; de Bruijne, Ebersviller et al. 2009). The retention of these equilibria is vital to an accurate estimate of the effects of exposure to airborne pollutants, due to the dynamic nature of the atmospheric processes involved. These characteristics, when combined, lend themselves to a very simple approach for investigating the direct modification of particle toxicity by the gas phase. The UNC smog chambers are, therefore, uniquely suited to explore the processes of interest here.
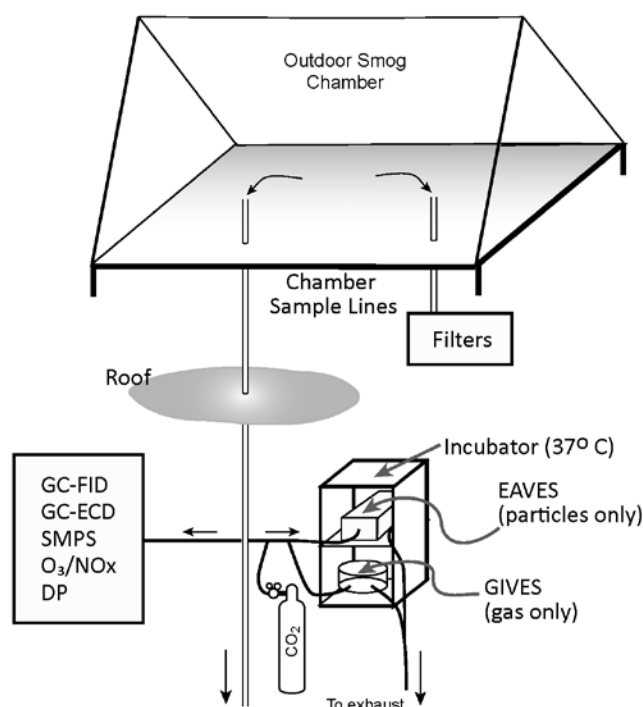
Figure 12: Chamber schematic.
Sample lines pass through the floor of the chamber, the roof, and then directly into the lab below. Filter sampling is performed on the roof of the building to minimize loss to the sample lines.

The studies described here used the Gillings Innovation Laboratory 120 m^3 chamber on the roof of the School of Global Public Health at UNC and the sampling and biological analysis laboratories are located directly below it (Fig. 12). The chamber is equipped with a high-volume vent fan connected to a HEPA filter that removes PM from ambient air during large-volume flushing of the chamber. In addition, the chamber is connected to a clean air generator that scrubs moisture, VOCs and PM from air before it enters the chamber. Pollutants to be studied (VOCs, PM, etc.) can be injected into the chamber through its floor. Chamber contents are monitored continuously via sample lines that pass through the floor of the chamber, the roof of the building, and into the laboratory below. The sample lines are made of a variety of materials to accommodate both the

analytical and toxicological needs of the laboratory. The laboratory is equipped with a suite of analytical instruments that sample the chamber directly, allowing detailed analysis of its contents to be performed in real-time. For analytes that may be lost in long transfer lines, samples are collected directly below the chamber on the roof of the building.

Choice of Gases and Particles to Test

Several volatile organic compounds were considered for inclusion in this study of gas/particle interactions. The VOC species chosen have been detected in our own chamber experiments involving complex VOC systems, and were also reported by Grosjean, et al. in a study of carbonyl compounds measured at the inlet and outlet of a highway tunnel (2001). Candidate species that were reported in multiple studies, or known to be of particular interest to air pollution researchers, were given preference in selection (Grosjean, Grosjean et al. 2001; US EPA 2008).

We selected p-tolualdehyde (TOLALD) to represent a semi-volatile species likely to be in both the gas and particle phases in the ambient environment (96%; Sigma-Aldrich, St. Louis, MO). While p-tolualdehyde may not seem an obvious choice, it has several qualities to recommend it for this study. Along with its tendency to be present in both phases, it is a first generation oxidation product of vinyl-aromatic compounds and has an aldehyde function while retaining its aromatic function. We selected acrolein (ACRO) to represent a more-volatile species generally considered unlikely to be in the particle phase (95%; Sigma-Aldrich, St. Louis, MO). In addition to ACRO being on the US EPA's

"Dirty Thirty" list of toxicants, we have extensive experience using acrolein for both chemical and toxicological tests.

Mineral oil aerosol (MOA) was used as a surrogate for organic-containing ambient PM (pharmaceutical grade, 100%). Exposure to mineral oil elicits no acute biological effects from lung cells, making mineral oil an ideal PM material for this study. While most ambient PM has a mixture of elemental and organic carbon (EC/ OC), the EC fraction is at the core of the particle and is (generally) completely surrounded by an OC layer. What this means is that any interactions between the PM and the gas phase happens via the OC fraction. In addition, mineral oil aerosolized with a large Collison nebulizer (May 1973) has a size distribution similar to diesel exhaust particles observed in other chamber studies. For this study, MOA is a convenient and sufficiently complex surrogate for ambient PM that contains organic material.

Prior to use, all mineral oil was steri-filtered in the lab to remove any particulate or biological contaminants. In addition, the very large negative pressure required to pull the mineral oil through the small pores of the filter apparatus served as a mechanism by which to remove any dissolved VOCs which may alter the toxicity of the aerosol. The steri-filtered mineral oil was kept sealed and stored in a sterile lab to minimize the possibility that the filtered stock would absorb gaseous VOCs from laboratory air in our chemistry/exposure laboratory. For experiments in which particles were needed, a large-volume Collison nebulizer was used to aerosolize mineral oil directly into the

chamber, to a concentration of ca. 1.4 mg m^{-3}, as determined by SMPS (described below).

Gas Phase Compositional Analysis

The gas-phase composition and dilution rate of the chamber were monitored continuously by two Varian 3800 gas chromatographs (GCs), one with an electron capture detector (ECD) and the other with a flame ionization detector (FID). As stated above, the sample lines feeding the GCs travel from the floor of the chamber, through the roof of the building, and directly to the GCs in the lab below. The GC-FID has dual packed columns (one for light hydrocarbons, the other for heavy and aromatic hydrocarbons) and uses helium as a carrier gas. The GC-ECD has a single packed column, and is used for measurement of electron-accepting species. The GC-ECD uses a blend of argon and methane as a carrier gas. Each GC was equipped with an automated valve system that sampled the chamber contents every 30 minutes, and was run at a static temperature throughout sampling.

A Varian 3800 GC with Saturn 2200 mass spectrometer (MS) was used to analyze condensed-phase samples (such as function-specific or filter samples). The GCMS is also equipped with an FID, uses an RTX-5 fused-silica capillary column (60 m long, 0.32 mm ID, 0.5 μm film thickness; Restek US, Bellefonte, PA) and was operated under a temperature-optimized elution program with helium as the carrier gas. Initially, the MS was operated in EI mode, and the ion trap was set to collect all mass fragments from 40–650 mass units. When increased sensitivity was desired, the ion trap was programmed to retain fragments with a more-focused range of sizes.

Particle Phase Analysis

Scanning Mobility Particle Sizer

During all experiments, the size distribution and mass concentration of the aerosols in the test atmospheres were monitored with a Scanning Mobility Particle Sizer (SMPS - TSI 3081 Differential Mobility Analyzer with TSI 3022A Condensation Particle Counter; TSI Inc., St. Paul, MN). The instrument quantified particles from 19 to 882 nm in diameter. The SMPS was set to scan continually on a 3 minute-per-sample cycle (150 s up-scan, 30 s down-scan). The particle size distributions, as well as the number and mass concentrations, were logged continually for each experiment.

Teflon Membrane Filters

Teflon membrane filters (2 μm pore size, 47 mm diameter: Pall Corporation, New York) were used to collect particles at an average flow rate of 17.5 Lpm for 1–4 hours. Filters were collected concurrently with exposure periods. Static was removed from the filters prior to weighing both before and after sampling. These procedures were carried out efficiently so that the filter was exposed to laboratory air as little as possible to minimize the loss of VOCs during weighing. After each filter was weighed, it was placed in a certified-clean sample jar, and spiked with Storage Internal Standard (IS) to characterize the loss of VOC with storage. Then each jar was sealed with Parafilm and placed immediately into a -20 °C freezer for storage until extraction and analysis. The aerosol mass concentration in the air was calculated by dividing the mass collected on the filter by the total volume of air sampled during collection. This value was used as a check against the value calculated from SMPS data.

Species-specific Analysis of Carbonyls

Filter samples were analyzed by a modified protocol using o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine chloride (PFBHA). PFBHA is used to selectively sample for carbonyl-containing organic compounds (Yu, Jeffries et al. 1995; Yu, Jeffries et al. 1997; Liu, Jeffries et al. 1999; Liu, Jeffries et al. 1999; Healy, Wenger et al. 2008). PFBHA selectively derivatizes carbonyl-containing functional groups of organic species, with preference given to aldehydes and ketones over organic acids and esters. Mass spectra of PFBHA-derivatized carbonyl-containing compounds display a signature peak at $m/z =$ 181 that is usually the base peak of the spectrum. In this study, filter samples were in-cubated under a PFBHA solution (after sample collection) to collect carbonyl-containing species from the condensed phase. Sample processing and analysis were then carried out as described before (Yu, Jeffries et al. 1995; Yu, Jeffries et al. 1997; Liu, Jeffries et al. 1999; Liu, Jeffries et al. 1999; Healy, Wenger et al. 2008). All filter samples from this study were analyzed by GCMS (as stated above), using a large-volume injection liner to increase the sensitivity of the instrument (Termonia, Lacomblez et al. 1988).

Biological Effects Model and Measurements of Response

Cultured human epithelial lung cells, type A549, were used as the biological recep-tor model in this study. A549 human respiratory cells are a commercially available cell line that has been shown to re- act in a manner that is representative of the human air-way in vivo (Lieber, Smith et al. 1976; ATCC 2011). While we concede that, in certain cir-cumstances, primary lung cells may be a more desirable model than an immortalized cell line, A549 cells (which lack the individual-to-individual susceptibility variations of the primary lines) are used worldwide, allowing for easier replication of experiments,

and are perfectly suited for this demonstration-of-principle work. The A549 cells were cultured and prepared for exposure as explained previously (Jaspers, Flescher et al. 1997; Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004; de Bruijne, Ebersviller et al. 2009).

The Gas In Vitro Exposure System (GIVES) and Electrostatic Aerosol in Vitro Exposure System (EAVES), described in more detail below, allow the cellular toxicity of the gaseous and particle phase components of the same air pollution mixture to be evaluated independently of one another, and with no substantive modification to the sample prior to exposure. This allows cells to be exposed to pollutants as they might be found in the ambient environment. These exposure systems, as models of the human deep lung, have their own generalizations, distortions, and deletions. These models are intended to be biosensors–a means by which to further characterize the distribution of species among the gas and particle phases, and to indicate which systems warrant further evaluation for harmful biological effects. This is measured by gauging cellular responses to treatment air exposures relative to clean air exposures.

Following exposure, all cell culture samples were allowed to express their responses for 9 hours in clean air in an incubator. After the 9 hours of expression, basolateral supernatants (media below the cells) were collected. All liquid samples were stored at -40 °C for later analysis of toxicological endpoints (see below).

Gas In Vitro Exposure System (GIVES)
Gas exposures from the chamber occur in air-tight, eight liter, modular cell-exposure chamber (Billups- Rothenberg, MIC-101TM, Del Mar, CA), as described before

(Jaspers, Flescher et al. 1997; Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004). Briefly, the modular chamber holds tissue culture plates containing Transwells (Costar, Cambridge, MA) for concurrent exposures. The inlet of the exposure chamber is connected to a sampling manifold coupled directly to the smog chamber on the roof (Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004; Doyle, Sexton et al. 2007). As there is no mechanism to force particles from the air stream onto the surface of the cells, no response from the cells is ever observed as a result of exposure to PM – even though the particles are never removed from the air stream prior to entry into the cell-exposure chamber. Exposure of cell cultures in the GIVES lasted 4 hours.

Electrostatic Aerosol In Vitro Exposure System (EAVES)

The Electrostatic Aerosol in Vitro Exposure System (EAVES) is a necessary and sufficient sampler to test the basic hypothesis in this study. The EAVES is designed to expose human lung cells directly to particles without prior collection in media, thereby providing an efficient and effective alternative to more conventional particle in vitro exposure methods (de Bruijne, Ebersviller et al. 2009; Lichtveld, Ebersviller et al. 2011). Thus, although the air stream is not altered prior to entry into the EAVES, exposure to air pollution mixtures containing gas-phase toxic species, but that lack PM, do not elicit a response from cell cultures in this sampler. When PM that has cellular toxic properties is present in the air stream, however, there is a significant increase in response from exposed cells (relative to clean air controls)(de Bruijne, Ebersviller et al. 2009). Cells exposed in the EAVES device are grown on Millicell membranes (Costar, Cambridge, MA) as described before (de Bruijne, Ebersviller et al. 2009). The durations of exposure for

membranes in the EAVES device were determined by the particle mass concentration in the air for each experiment, and lasted between 30 minutes and one hour. The exposure duration was varied to ensure equal deposition of PM on the cellular surface, while still providing ample time for a dose of PM to be delivered to the cells that was sufficient to elicit a response. For experiments in which no PM was present, the duration of exposure in the device was matched to the corresponding experiment with PM (i.e., TOLALD only was matched to the duration of exposure for TOLALD + PM).

Control Exposures

In addition to each pollutant mixture listed above, cells were exposed to clean chamber air with each exposure system, as well as to incubator air. The response of the clean air exposure was compared to incubator controls to ensure that they were equal (as determined by a t-test, $p = 0.05$). The response of cells to clean air exposure was then used as a normalizing factor for the response of cells to each of the pollution mixtures.

Biological Effects Endpoints (Cytotoxicity and Inflammation)

Cytotoxicity is expressed as increase of lactate dehydrogenase (LDH) levels over clean air controls. LDH is only released by cells when the integrity of their cellular membranes have been compromised through either damage or failure (apoptosis). LDH levels were measured using a Cytotoxicity Detection Kit (TaKaRa Bio Inc., Tokyo, Japan). Interleukin 8 (IL-8) cytokine is an inter-cellular signaling protein that is released by cells in response to stimulus by an irritant. IL-8 release was evaluated using an ELISA kit (BD Biosciences, San Diego, CA). Though it has been noted previously that the presence of

PM interferes with the proper functioning of ELISA kits (Seagrave, Knall et al. 2004), it was determined that this interference was not an issue with the PM used in this study. Inflammatory response is reported as increase of IL-8 levels over clean air controls.

Tables of response ratios accompany bar charts of endpoint measurements. Response ratios were calculated by dividing the cellular response to the exposure listed in the column heading of the table by the response to the exposure listed in the first column. For example, in Table 5, the gas-only response to TOLALD by itself was 3.21 times higher than the clean air exposure. The gas-only response to TOLALD + MOA, however, was 0.83. Therefore, in Table 5, the ratio of their responses is given by:

$$\frac{0.83}{3.21} = 0.26$$

This means that the response of cells exposed to TOLALD + MOA by the gas-only method was roughly one quarter the response observed from cells exposed to TOLALD alone (or, the response for TOLALD was 3.85 times greater than was observed for TOLALD + MOA). All results and further discussion is given below.

Statistical Analysis

All calibration curves were tested by fitting a linear least squares line through the data. Calibration factors were calculated for each species to be analyzed, with appropriate propagation of uncertainty. All instrument measurements are reported as the mean ± 95% confidence interval, unless otherwise stated.

For toxicity endpoints, replicate tests of the same sample were averaged, and cell samples exposed to the same air mixture (i.e., incubator control, clean air control, GIVES exposure, etc.) were grouped for one-way analysis of variance (ANOVA - GraphPad Prism Software, La Jolla, CA). ANOVA analysis was used to determine whether there were statistically-significant differences between the cellular responses to each exposure mixture and their corresponding controls. Cellular response to insult is expressed as the mean ± standard error. The independent variable is exposure. Pair wise comparison (treatment vs. control) was performed as a subtest of the overall ANOVA. Group differences were considered significant if the test statistical type 1 error $p \leq 0.05$.

Results

Gas Phase Compositional Analysis

Table 3: Exposure Conditions. All values represent mean value ± 95% CI.

Experimental Condition[a]	Avg. VOC Conc. (ppmV)	PM Conc. (mg m^{-3})	RH Chamber (fraction)	RH Exposure (fraction)
Clean Air	ND [b]	0.001 ± 0.001	0.478 ± 0.003	0.317 ± 0.001
Clean Air + MOA	ND [b]	1.561 ± 0.027	0.692 ± 0.010	0.301 ± 0.004
Clean Air + ACRO	0.937 ± 0.053	0.007 ± 0.001	0.709 ± 0.008	0.394 ± 0.001
Clean Air + ACRO + MOA	0.929 ± 0.095	1.415 ± 0.027	0.926 ± 0.004	0.323 ± 0.003
Clean Air + TOLALD	2.012 ± 0.102	0.001 ± 0.003	0.738 ± 0.023	0.343 ± 0.010
Clean Air + TOLALD + MOA	1.834 ± 0.057	1.317 ± 0.007	0.686 ± 0.022	0.308 ± 0.008

[a] MOA is mineral oil aerosol, ACRO is acrolein, TOLALD is p-tolualdehyde, and RH is relative humidity

[b] none detected

The gas-phase conditions during exposure, as well as particle mass concentrations, are summarized in Table 3. There were no VOCs detected in the chamber during either clean air exposure (with or without MOA). There was a slight variation in the concentration of TOLALD between exposures, though a difference of 178 ppbV should not be enough to cause a significant discrepancy in response from the cells. The ACRO exposures were more- closely matched (a difference of 8 ppbV). Relative humidities (RH) are presented for each exposure mixture in the chamber air (where it might impact partitioning dynamics) as well as in the incubator (where variations in RH might affect cellular response). The uncertainty reported in Table 3 represents the 95% confidence interval.

Particle Phase Characterization

The particle mass concentrations across all of the exposures in which no particles were added were close to the limit of detection for the SMPS instrument (as determined

by the averages and 95% confidence intervals reported in Table 3). The particle mass

concentrations for the exposures in which MOA was added to the chamber were ca.

1500 times higher than the exposures without PM, well above the level of uncertainty

(Table 3). No change in mode particle diameter was observed when VOCs and MOA

were mixed in the chamber (relative to MOA in clean air; Fig. 13).

Analysis of filter samples was only possible for the portion of the study that used

ACRO. Filters taken from the chamber contained no detectable ACRO when 1) MOA was

tested in clean air and, 2) when ACRO was tested without PM. Filter samples taken from

the chamber when it contained ACRO mixed with MOA, however, yielded an ACRO mass

of 1.872 ng per filter (corresponding to 1.06 ng ACRO per mg PM). Further discussion of
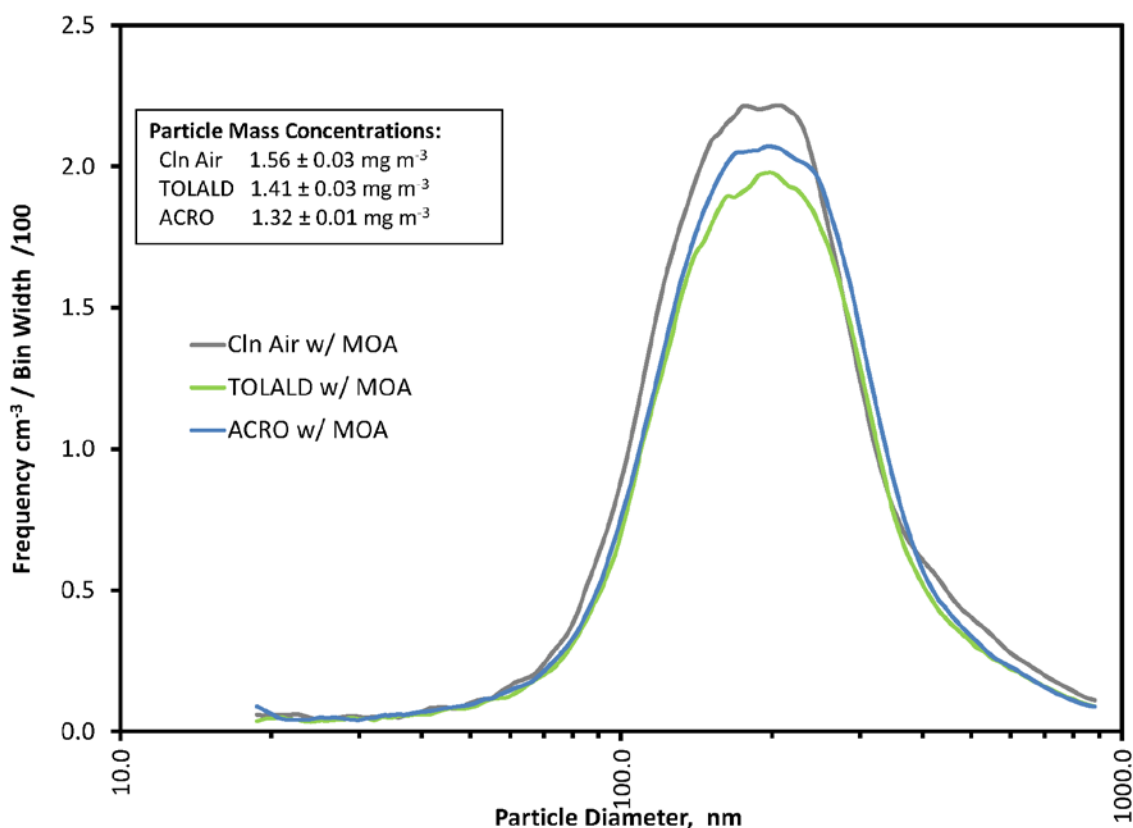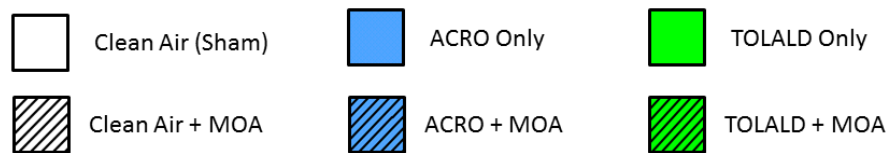
these results is given below.

Figure 13: MOA particle size distributions for Clean Air and single-VOC experiments. Particle mass concentrations reported in the inset were determined from TSP filter measurements.

Toxicological Response

* Indicates a statistically significant difference in response relative to clean air
# indicates a statistically significant difference in response between the VOC alone
    and the VOC + MOA
φ indicates a statistically significant difference in response relative to MOA in clean air

Figure 14: Key for color coding and annotations used in Figs. 15 – 16 MOA is mineral oil aerosol; ACRO is acrolein; TOLALD is p-tolualdehyde

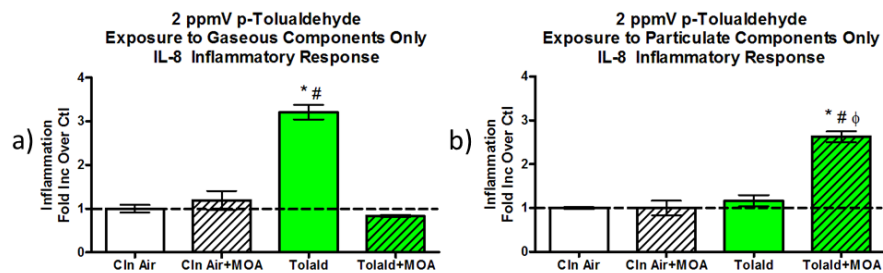Figure 15: The IL-8 inflammatory response of lung cells following exposure to four air mixtures. The response induced by exposure to a) the gaseous components of each mixture and b) the particle phase components of each mixture.
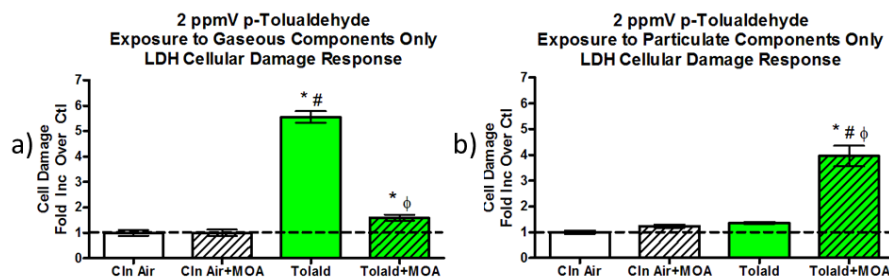

Figure 16: Cellular damage following exposure to four air mixtures. The response induced by exposure to a) the gaseous components of each mixture and b) the particle-phase components of each mixture.

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Table 4: Ratios of measured inflammatory responses (IL-8) for in vitro human lung cell exposures to TOLALD mixtures, (Table 3 and Fig. 15).

Gas-Only Exposure				
Reference Condition[a]	Clean Air	Clean Air + MOA	Clean Air + TOLALD	Clean Air + TOLALD + MOA
Clean Air	1.00	1.19	3.21	0.83
Clean Air + MOA		1.00	2.70	0.70
Clean Air + TOLALD			1.00	0.26
Clean Air + TOLALD + MOA				1.00

PM-Only Exposure				
Reference Condition[a]	Clean Air	Clean Air + MOA	Clean Air + TOLALD	Clean Air + TOLALD + MOA
Clean Air	1.00	1.00	1.17	2.63
Clean Air + MOA		1.00	1.17	2.63
Clean Air + TOLALD			1.00	2.25
Clean Air + TOLALD + MOA				1.00

[a] MOA is mineral oil aerosol, TOLALD is p-tolualdehyde

Table 5: Ratios of measured cellular damage (LDH) for in vitro human lung cell exposures to TOLALD mixtures, (Table 3 and Fig. 16).

Gas-Only Exposure				
Reference Condition[a]	Clean Air	Clean Air + MOA	Clean Air + TOLALD	Clean Air + TOLALD + MOA
Clean Air	1.00	1.00	5.55	1.59
Clean Air + MOA		1.00	5.53	1.59
Clean Air + TOLALD			1.00	0.29
Clean Air + TOLALD + MOA				1.00

PM-Only Exposure				
Reference Condition[a]	Clean Air	Clean Air + MOA	Clean Air + TOLALD	Clean Air + TOLALD + MOA
Clean Air	1.00	1.24	1.37	3.96
Clean Air + MOA		1.00	1.11	3.20
Clean Air + TOLALD			1.00	2.90
Clean Air + TOLALD + MOA				1.00

[a] MOA is mineral oil aerosol, TOLALD is p-tolualdehyde

The relative inflammatory responses to the mixtures that contained TOLALD are given in Fig. 15. The only significant increases in response from cells exposed by the gas-only method were observed when TOLALD was placed in the chamber without MOA (Fig. 15a). This exposure resulted in 3.21 and 2.70 times more IL-8 production than the clean air and clean air with MOA (Table 4). The only significant increase in response observed from cells exposed by the particle-only method was when TOLALD was mixed with MOA in the chamber (Fig. 15b, Table 4). The responses observed from our measure of cellular damage (LDH) resembled the inflammatory response, but with a greater magnitude in measured response (Fig. 16, Table 5).
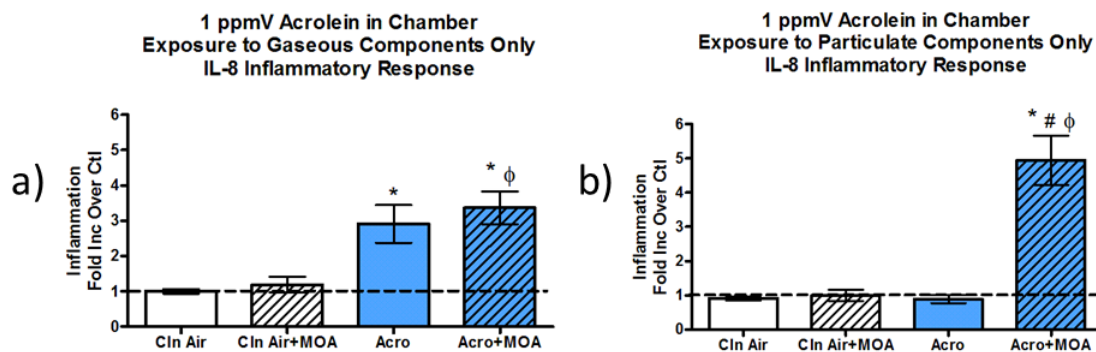
Figure 17: The IL-8 inflammatory response of lung cells following exposure to four air mixtures. The response induced by exposure to a) the gaseous components of each mixture and b) the particle phase
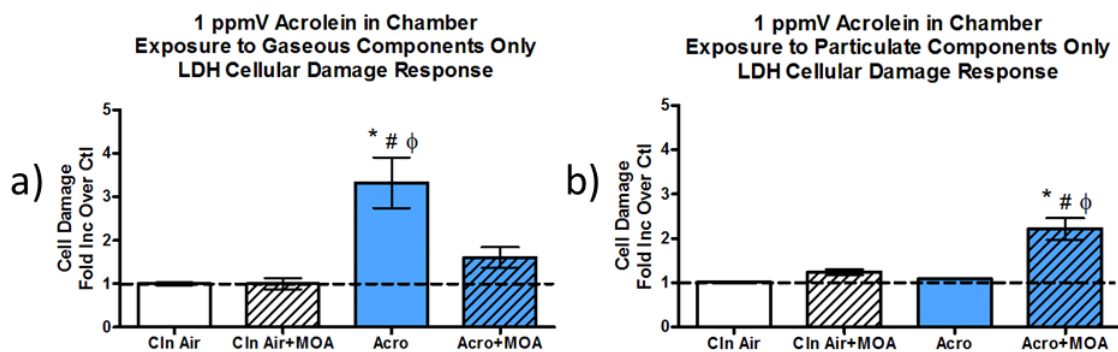
Figure 18: Cellular damage following exposure to four air mixtures. The response induced by exposure to a) the gaseous components of each mixture and b) the particle-phase components of each mixture.

Table 6: Ratios of measured inflammatory responses (IL-8) for in vitro human lung cell exposures to ACRO mixtures, (Table 3 and Fig. 17)

Reference Condition[a]	Gas-Only Exposure			
	Clean Air	Clean Air + MOA	Clean Air + ACRO	Clean Air + ACRO + MOA
Clean Air	1.00	1.19	2.91	3.37
Clean Air + MOA		1.00	2.45	2.83
Clean Air + ACRO			1.00	1.16
Clean Air + ACRO + MOA				1.00

Reference Condition[a]	PM-Only Exposure			
	Clean Air	Clean Air + MOA	Clean Air + ACRO	Clean Air + ACRO + MOA
Clean Air	1.00	1.10	0.98	5.45
Clean Air + MOA		1.00	0.89	4.96
Clean Air + ACRO			1.00	5.57
Clean Air + ACRO + MOA				1.00

[a] MOA is mineral oil aerosol, ACRO is acrolein

Table 7: Ratios of measured cellular damage (LDH) for in vitro human lung cell exposures to ACRO mixtures, (Table 3 and Fig. 18)

	Gas-Only Exposure			
Reference Condition[a]	Clean Air	Clean Air + MOA	Clean Air + ACRO	Clean Air + ACRO + MOA
Clean Air	1.00	1.00	3.33	1.61
Clean Air + MOA		1.00	3.31	1.60
Clean Air + ACRO			1.00	0.48
Clean Air + ACRO + MOA				1.00

	PM-Only Exposure			
Reference Condition[a]	Clean Air	Clean Air + MOA	Clean Air + ACRO	Clean Air + ACRO + MOA
Clean Air	1.00	1.22	1.08	2.20
Clean Air + MOA		1.00	0.88	1.79
Clean Air + ACRO			1.00	2.04
Clean Air + ACRO + MOA				1.00

[a] MOA is mineral oil aerosol, ACRO is acrolein

Figures 17 and 18 show the relative response of lung cells to insult by the four air mixtures in which acrolein (ACRO) was used as the VOC of interest. As with TOLALD, a significant response was only observed when ACRO was present in the chamber. Likewise, the only significant response from cells exposed with the particle-only method was observed when ACRO was mixed with MOA (Fig. 17b). A similar trend was observed with the measure of cellular damage (Fig. 18). The only difference between the two endpoints (IL-8 and LDH) was that the cellular damage observed following exposure to ACRO + MOA was not significantly greater than the control (Fig. 18b). Response ratios are summarized in Tables 6 and 7. Further discussion of results follows.

Discussion

This demonstration study is an effort to create a simplified and abstract model of processes that might occur in the ambient environment and human body. While simple,

it provides us with results that would be difficult to detect directly in the ambient environment with human subjects. The latter conditions are subject to competing processes that, without insight, would likely mask the true causes of observed effects in these complex and open systems. The findings here permit us to apply the same causal reasoning resulting from this study to complex, photochemical systems with urban VOC mixtures and systems with real diesel PM; these additional findings are described in our two subsequent studies (Parts 2 and 3).

Even at the relatively high particle concentration in the MOA exposure in clean air, neither of the in vitro exposure systems registered an increase in toxicity from the clean air exposure with MOA present, demonstrating that MOA itself is not inherently toxic for the endpoints we used (Figs. 15–18). This was true even though ca. 0.2 mg m^{-3} more MOA than the average of the exposures with VOCs was present (Table 3). This lack of inherent acute toxicity will allow us to draw more concrete conclusions about the source of observed cellular responses to particle-phase exposures by removing the chance of false-positive responses due to any inherent toxicity of the particle phase. Moreover, the particle size distribution of each exposure atmosphere was continually monitored (Fig. 13), so we are able to rule out the effects of particle size variation on toxicity.

Efforts were made to exclude as many transformative processes from this test system as possible (i.e., the experiments were carried out at night to prevent the particles themselves from having their toxicity altered by direct reaction with sunlight). As stated

above, this was by design, so that causation of observed effects could be assigned as unambiguously as possible. As there was no chemistry occurring in the chamber, only physical (thermodynamic) processes were present. In addition, any effect that variations in RH might have had on the cells was minimal, as the lowest RH encountered in the study was observed during the Clean Air + MOA exposure. No response was observed from the cells in this exposure, so the likelihood of RH causing the increase in response observed from the VOC-containing exposures is negligible. Any increase in the toxicity of the particle phase was, therefore, solely due to the dynamic uptake of gas-phase toxics. This is a significant distinction to make, since the interplay between gases and particles occurs constantly in the ambient environment. The increase in response (inflammation, as well as, cytotoxicity) observed in the particle exposure for the TOLALD + MOA system was not a surprise, as TOLALD was selected for its likelihood to partition favorably to the condensed phase (Figs. 15 and 16, Tables 4 and 5). When TOLALD was mixed with MOA, the response from the cells exposed with the gas-only method decreased by 71% (relative to TOLALD alone). We have previously observed similar results in other studies involving diesel exhaust (Part 3).

Table 8: Theoretical and observed values for partitioning of TOLALD and ACRO to PM

	Theoretical Concentration		Observed Concentration
	K_p [a]	K_{oa} [b]	
VOC	(ng VOC per mg PM)		
TOLALD	1.87	2.77	missing [c]
ACRO	0.373	0.503	0.93 ± 0.59

[a] Calculated from equations in Pankow 1997
[b] Calculated from equations in Schwarzenbach 2003
[c] all samples were lost

The strong biological response from the cells exposed with the particle-only in vitro method clearly demonstrates that the toxicity of the MOA has been modified by gas-phase TOLALD (Figs. 15b and 16b). Any discussion of the particle uptake of TOLALD, however, is hampered by the loss of the filter samples taken for chemical analysis. We can attempt to compensate for the loss of the filter samples by calculating an estimate of the TOLALD loading of the MOA using K_p equations from Pankow (1997) and K_{oa} equations from Schwarzenbach (2003). Thus, the amount of TOLALD we have calculated to be taken up by the MOA was on the order of 1.87-2.77 ng TOLALD per µg MOA (summarized in Table 8). This means that, using measurements of collection efficiency for the EAVES device (de Bruijne et al., 2009), the amount of TOLALD delivered to each cell culture insert in the particle-only exposure was between 2.82 ng (using K_p) and 4.18 ng (using K_{oa}). As TOLALD is not considered hazardous (it is used as an aroma additive in food), we do not consider that the amount calculated to have been delivered to the cells should have been sufficient to cause such a large increase in the particle-phase toxicity. Likewise, we do not expect that removing 2.82-4.18 ng of TOLALD from the gas-

phase should have been enough of a loss to diminish the response of the gas-phase exposures to the level of the clean air control.

To recap: we have demonstrated that 1) MOA in clean air does not cause a response from exposed cells; 2) MOA, when mixed with TOLALD induced a large response from cells exposed with the particle-only method; and 3) we observed a significant decrease in response from cells exposed to TOLALD + MOA with the gas-only method. All of this suggests that, in a humid environment such as we established in the chamber, these calculations likely underestimate the uptake of TOLALD by MOA (discussed further below).

Given that ACRO is targeted by the EPA for control on its "Dirty Thirty" list(US EPA 2008), the large response from the cells in the gas-phase exposure method is consistent with expectations (Figs. 17a and 18a). Also, given the volatility of acrolein, the lack of change in inflammatory response from cells in the gas-only exposure system when MOA was added was also consistent with expectations (Fig. 17a). The most intriguing results are apparent in the dramatic increase in response from the particle-only exposure method when MOA was mixed with ACRO in the chamber (Fig. 17b). Such a large increase in response (> 5×, Table 6) was unexpected, as ACRO is (according to partitioning theory) even less likely to be found in the particle phase than TOLALD. A similar, though somewhat less dramatic, trend was observed with our measure of cellular damage (LDH in Fig. 18). The one difference between the inflammatory response and cellular damage measurement was observed for cells in the gas-only exposure system when challenged

with the mixture of ACRO and MOA (see Fig. 18a, in which a decrease in response was observed. This result was expected as we have observed this outcome in other systems we have researched, and it could merely indicate that, while the insult was sufficient to elicit an inflammatory response from the cells, it was not enough to result in total failure of the cellular membrane. While it is possible that there is another explanation for the lack of cellular damage from the ACRO + MOA mixture, the large inflammatory response observed for the same exposure makes our explanation highly probable. Future investigations into these phenomena will be able to look at the mechanisms of action for the observed toxicity and RH in greater detail.

The analysis of filter samples taken from the air mixtures of MOA and ACRO yielded surprising results. The mass of ACRO measured on the filters (0.93 ± 0.59 ng ACRO per mg PM) was ca. $1.8 - 2.5$ times greater than predicted by partitioning theory (0.373– 0.503 ng ACRO per mg PM; Pankow, Schwarzenbach; summarized in Table 8). One explanation for the higher-than-predicted concentration of ACRO in the particle phase is given by recent evidence that realistic levels of humidity can facilitate particle uptake for water soluble compounds (Lim, Tan et al. 2010; Parikh, Carlton et al. 2011). While there is always the concern that a higher-than-expected concentration for an analyte might be from contamination, the filter samples were handled in such a way as to completely segregate them from the possibility of absorbing ACRO from the laboratory air. ACRO has, to our knowledge, never entered the lab in which all filter handling and processing was performed. Recent studies have reported detecting species with higher-than-expected volatilities in ambient PM, where they can undergo polymerization processes

(Liggio and McLaren 2003; Hu and Kamens 2007; Cao and Jang 2008; Zhou, Zhang et al. 2011). ACRO, while not included in the study by Liggio and McLaren, has a volatility that falls within the range of those tested (2003). Moreover, ACRO is known to polymerize readily, especially in an acidic environment. Polyacrolein itself has been associated with negative human health outcomes, such as Alzheimer's disease (Seidler and Yeargans 2004; Liu, Lovell et al. 2005; Seidler, Craig et al. 2006). Moreover, whatever the form acrolein may be taking in the condensed phase, the acute inflammatory response ob-served indicates that it is still capable of eliciting a biological effect.

If we assume that particles are more capable of picking up volatile species than expected, it does not necessarily indicate that current partitioning theory is invalid. For instance, if small molecules are easier to dissolve in the condensed phase than currently believed, the calculation of activity coefficients may simply need to be adjusted to ac-count for these effects. In addition, recent findings indicate that humidity may play a larger role in affecting the interactions of PM and water soluble compounds than was previously expected (Li, Hooper et al. 2000; Lim, Tan et al. 2010; Parikh, Carlton et al. 2011). These statements are not meant to be an assertion that solubility will be found to account for the observed uptake, but simply as an illustrative example. This work is meant to be an indication of the direction that future work may go, rather than a defini-tive answer to the problem. The determination of the causes of these observations, as well as the adjustment to the calculation of activity coefficients is, therefore, outside the scope of this work.

Traditionally, the toxicity of PM is estimated by collecting particles and then re-suspending them in a liquid medium that can be instilled onto in vitro cells or into the lungs of test animals (Farina, Sancini et al. 2011; Steenhof, Gosens et al. 2011). Additionally, in toxicology studies, filter samples are routinely left exposed to laboratory air for extended periods of time and/or are autoclaved. These sample handling practices will result in the loss of volatile species from the particle phase and, by extension, most likely diminish effects such as those that we observed during this study.

Conclusions

These results have important ramifications across the air pollution field. This study has shown that atmospheric gases that are toxic can, through physical/thermal process-es, cause non-toxic PM to become toxic. Due to deposition and clearance mechanisms in the human body, the 'newly toxic' PM can act as a pre-concentrator and delivery mech-anism to deliver a dose of the atmospheric gas to a region of the lung that might not normally encounter it. Atmospheric gases delivered to the lung in this way may have an increased biological effect relative to that of the gas-phase toxic species. This may help explain the inability of laboratory-based toxicologists to replicate effects seen in near-roadway epidemiology studies that has confounded the risk assessment field and policy makers alike (Dreher 2000; Schlesinger, Kaunzli et al. 2006). The time scale for these in-ter-phase dynamics is on the order of seconds. What this means in the real world is that vehicle emissions (and other PM) may not be exceptionally toxic as they enter the at-mosphere but, when mixed into an aged or aging air mass, can change dramatically in composition and biological effect by the time they drift off-road and reach the general

population. Thus, an important consideration is the role of PM as a pre- concentrator and carrier of gas-phase toxics into regions of the lungs they may not normally reach, where the toxic load can be released even if the PM mass is mostly expelled.

While the creation of oxidized daughter products has long been investigated for its potential to produce ozone and PM, toxicological work has remained largely focused on single pollutants or primary sources of PM (Tsukue, Okumura et al. 2010; Weldy, Wilkerson et al. 2011). This work demonstrates that the dynamic shifting of toxicity from the gas phase to the PM present in the mixture is likely to be an important component to understanding the response of individuals exposed in the ambient environment. This dynamic shift is an example of what the NRC was referring to when asking for evidence of 'effect modification' (2004). Further, it identifies an important, currently neglected, source of PM toxicity, the reactive atmosphere itself. To fully incorporate the reactive atmosphere into toxicological studies, researchers must have a mechanism by which to photochemically age species in situ. Currently, very few biological exposure facilities have these kinds of transformative capabilities available to them, resulting in exposure studies that miss the full complexity of the entanglements encountered in the ambient environment. What this means for their exposure studies is that they not only miss the full complexity of these entanglements, they likely miss important biological effects as-sociated with PM that has been fully exposed to, and processed by, the atmosphere. It is these dynamic entanglements that we have shown here to be so important for cellu-lar effects. Further demonstrations of these "effect modifications," in more complex and realistic atmospheric systems, are provided in Part 2 and Part 3 of our studies.

Part 2, Complex Urban VOCs and Model PM

Introduction

In its most-recent document summarizing the state of the science related to PM, the US National Research Council (NRC) stated that "a finding that the effect of particles depends on the concentration of another pollutant that is, 'effect modification' would have implications for setting National Ambient Air Quality Standards (NAAQS) independently for the various criteria pollutants" (NRC 2004).

In Chapter 3, Part 1, we demonstrated the existence of PM "effect modification" for the case of a single gas-phase toxicant and an inherently non-toxic PM (mineral oil aerosol, MOA). That is, in the presence of the single gas-phase toxicant in the dark, the initially non-toxic PM became toxic to lung cells in the PM-only-biological indicator exposure system. In the first experiment, the non-toxic PM (MOA) was added to clean air and both biological exposures were performed, which resulted in no response from cells to either gases or PM. In the second experiment, a gas-phase toxicant, either p-tolualdehyde or acrolein, was directly injected into clean air in the dark chamber and both biological effects exposures were performed. The result was an increase in response from the gas-only cells, but not the PM-only cells. In a third experiment both the gas-phase toxicant and the MOA PM were added to the chamber and both biological exposures were performed. In this case, the PM-only-exposures showed 2x to 4x larger responses than did the clean air exposures. The gas-only-exposures, in the PM-added experiment decreased significantly or remained about the same as the gas-only exposure in the absence of PM. That is, for the gas with lower volatility, it appears that much

of the gas-phase mass was removed to the PM, but in the higher volatility gas, it was less clear how the toxicant partitioned. These results showed that a gas from the environment surrounding an initially non-toxic PM could modify the PM so that it became toxic to lung cells due to atmospheric processes only. This is the "effect modification" that the NRC posed would have implications for research and regulation of PM.

This study was designed to extend our demonstration of the existence of "effect modification" to VOC systems that are more like those in large urban areas, but to have an experimental design simple enough to be relatively unambiguous about demonstrating the PM modification by the gas-phase.

Extensive literature exists regarding how gases and particles interact in important and dynamic processes during the aging of anthropogenic emissions in the atmosphere. When PM is present, VOCs can constantly shift between the gas and particle phases of ambient air, and at the same time they can be modified by chemical reactions in each phase. Partitioning theory that describes these processes has recently been coupled to atmospheric chemistry models in an attempt to capture and characterize these interactions in a quantitative way (Kamens, Jeffries et al. 1981; Pankow, Liang et al. 1997; Kamens and Jaoui 2001; Lee, Jang et al. 2004; Donahue, Robinson et al. 2006; Hu and Kamens 2007). What has remained uncharacterized to this point is if—and how—these gas and particle interactions affect the actual toxicity of each phase.

Many toxicological studies focus on single species, or single primary pollutants, in the search an entity that can be assigned causality for health outcomes observed in epi-

demiology studies (Dreher 2000; Schlesinger, Kaunzli et al. 2006; Farina, Sancini et al. 2011; Steenhof, Gosens et al. 2011). While it is useful to examine the role of a single pollutant, these studies ignore atmospheric aging and the photochemical evolution of species that occurs during the day in the ambient environment. The simplified approach of ignoring secondary pollutants, while attractive to permit laboratory studies to be undertaken, also ignores the complexity of the air we breathe in the ambient environment, and thereby misses the contribution of the atmosphere itself as a source of toxicity. "Such approaches are likely to oversimplify the underlying biological processes and how the mixture of air pollutants that is inhaled adversely affects health" (NRC 2004).

The effects of photochemical-aging and gas and particle interactions have recently begun to be evaluated for their impact on the relative risk associated with exposure (Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004; Doyle, Sexton et al. 2007; Lichtveld, Ebersviller et al. 2011). Previous experiments using outdoor irradiation chambers have measured significant changes in chemical composition as air pollution mixtures age in sunlight and, in most cases, have measured an accompanying increase in toxicity. In addition, these studies have indicated that the overall toxicity of an aged pollution mixture may not be completely accounted for by the major daughter products alone, and that significant portions of the observed response can be induced by exposure to minor, and even undetected, products (Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004; Doyle, Sexton et al. 2007).

Therefore, in designing this study, we used sunlit-reactive systems to create a large variety of gas phase toxicants, including those not yet identified or detected, from a complex mixture of oxides of nitrogen (e.g., NO_x, NO, and NO_2) and 54 VOCs that serve as primary reactants in our outdoor sunlit chamber. The daylight reactions in this system consume a large portion of the primary VOCs and create a very large number of secondary oxidized products, with a wide distribution of lifetimes. Some of these products we cannot detect, and others we can detect but cannot specifically identify. This would be a limitation if we were performing a toxicological dose-response study, but we are more interested in having a *fully* complex, urban-like system for exposure to living cells. For these experimental purposes, we are interested in the relative magnitude of cellular responses to reproducible, but not totally characterizable (at least within this study), systems to indicate the "types" of reactive systems that deserve further attention.

To maintain a system with little ambiguity for PM "effect modification" we used the same inherently non-toxic PM from the Chapter 3, Part 1 study. While we are aware of the dynamic interactions of gases and PM in an evolving photochemical oxidizing system with PM present, these processes would introduce ambiguity in interpreting the effects results. Thus, in our experimental design, we exclude PM during the daytime photoactive period and only introduce it when we want to test if some gases present will modify *added* non-toxic PM so that the inherently non-toxic PM becomes toxic to cells. This is "effect modification" as posed by the NAS. Implied in our results is that many urban atmospheres are capable of acting as a source of toxicity via the creation of

photochemically-aged daughter species in situ, and that—even in the presence of non-toxic PM—the uptake of these toxic gases will produce PM that is toxic to lung cells.

Methods

The methods and procedures used in this Part 2 study were essentially identical to those reported in the Chapter 3, Part 1 study. Thus, only brief summaries of these will be given here.

Chamber

The experiments described here were performed in the Gillings Innovation Laboratory 120 m3 chamber on the roof of the four story School of Global Public Health at UNC, the same as used in the Chapter 3, Part 1 study (Fig. 19). The chamber is above all surrounding buildings and receives direct sunshine on its tilted Teflon film faces.
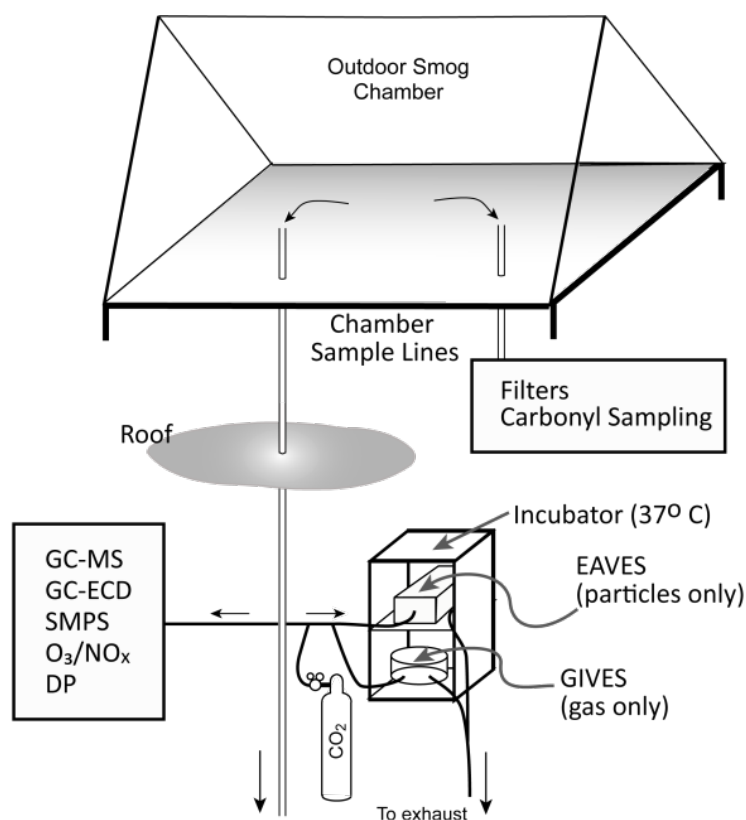
Figure 19: Schematic of The Gillings Innovation Laboratory 120 m^3 Outdoor Chamber located on the roof of the four-story Gillings School of Global Public Health, UNC-CH.
Sample lines pass through the floor of the chamber, the building roof, and then directly into the chemistry and biological exposure laboratory on the fourth floor. Filter and carbonyl-specific sampling are performed on the roof directly through the chamber floor to minimize loss in sample lines.

The chemical and biological analysis laboratories are located directly below the

roof-top chamber on the fourth floor. Pollutants to be studied (VOCs, PM, etc.) can be

injected into the chamber through its floor. Chamber contents are monitored continu-

ously via sample lines that pass through the floor of the chamber, the roof of the build-

ing, and into the laboratory below. For analytes that may be lost in long transfer lines,

samples are collected directly below the chamber on the roof of the building. These

same sample lines provide chamber air to the biological effects exposure systems that

are maintained in an incubator with proper conditions to support living human-derived lung cells.

Gas Phase Compositional Analysis

Direct Sampling of the Chamber by Gas Chromatography

Chamber air was sampled directly with a Varian 3400 gas chromatograph coupled to a Saturn 2000 mass spectrometer (GC-MS). The dilution rate of the chamber was monitored continuously by a Varian 3800 GC with an electron capture detector (ECD). A Varian 3800 GC with Saturn 2200 mass spectrometer (MS) was used to analyze condensed-phase samples (such as function-specific or filter samples). See Chapter 3, Part 1 for more details on components and operating conditions for these systems.

Species-specific Analysis of Carbonyls

Our chemical analysis places a large focus on carbonyl-containing compounds because they are the major oxidation products of primary VOCs, and from earlier research have been shown to have great influence on the toxicity of an aged air mass (WHO 2000; Zhang and Smith 2003; Hauptmann, Lubin et al. 2004; Takigawa, Wang et al. 2009). In this study, selective analysis for carbonyl-containing compounds was performed with o-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine chloride (PFBHA) as described in Part 1. Mixtures of gases and particles were sampled with midget impinger apparatuses similar to those described before (Yu, Jeffries et al. 1995; Yu, Jeffries et al. 1997; Liu, Jeffries et al. 1999; Liu, Jeffries et al. 1999). While we could visually see that some PM was collected in the impinger, we acknowledge that the impingers' collection efficiencies are difficult to accurately quantify for PM. As such, we can be certain that

some of the PM in the mixture was collected, but we cannot determine unambiguously the percentage collected. The PFBHA-derivatized measurements should, therefore, be thought to represent an indication of the species present in the mixture, but not absolute quantification of the total production of a given species by the system. Impinger samples were collected at 1 Lpm for ca. 3 hrs.

Inorganic Gas Phase Analysis/Chamber Condition Monitoring

Ozone (O_3) and nitrogen oxides (NO_x) concentrations in the chamber were monitored continuously (ML9811 Ozone Photometer and ML9841 NO_x Oxides of Nitrogen Analyzer, Monitor Labs, Englewood, CO), as were chamber temperature and dew point (DP). All chamber data was 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.

Particle Phase Analysis

Scanning Mobility Particle Sizer

During all experiments, the size distribution and mass concentration of the aerosols in the test atmospheres were monitored with a Scanning Mobility Particle Sizer (SMPS; TSI Inc., St. Paul, MN). All particle-phase analyses were performed as described in the Chapter 3, Part 1 study.

Teflon Membrane Filters

Teflon membrane filters (2 μm pore size, 47 mm diameter: Pall Corporation, New York) were used to collect particles at an average flow rate of 17.5 Lpm for 3 hours. Filters were collected concurrently with exposure periods. The aerosol mass concentration

in the air was calculated by dividing the mass collected on the filter by the total volume

of air sampled during collection. This value was used as a check against the value calcu-

lated from SMPS data. All particle-phase analyses were performed as described in the

Part 1 study.

Biological Effects Model and Measurement Response

Cultured human epithelial lung cells, type A549, were used as the biological recep-

tor model in this study. A549 human respiratory cells are a commercially available cell

line that has been shown to react in a manner that is representative of the human air-

way in vivo (Lieber, Smith et al. 1976; ATCC 2011). The A549 cells were cultured and

prepared for exposure as explained previously (Jaspers, Flescher et al. 1997; Doyle,

Sexton et al. 2004; Sexton, Jeffries et al. 2004; Ebersviller, Lichtveld et al. 2012). The Gas

In Vitro Exposure System and Electrostatic Aerosol in Vitro Exposure System (GIVES and

EAVES) have also been described in detail previously (Doyle, Sexton et al. 2004; Sexton,

Jeffries et al. 2004; Doyle, Sexton et al. 2007; Lichtveld, Ebersviller et al. 2011;

Ebersviller, Lichtveld et al. 2012). Briefly, these systems allow the cellular toxicity of the

gaseous and particle phase components of the same air pollution mixture to be evaluat-

ed independently of one another, and with no substantive modification to the sample

prior to exposure. This allows cells to encounter pollutants as they might in the ambient

environment. These exposure systems are not attempts to exactly model a human deep

lung. They are models intended to be biosensors – a means by which to further charac-

terize the distribution of species among the gas and particle phases, and to indicate

which systems warrant further evaluation for harmful biological effects. This is meas-

ured by gauging cellular responses to treatment air exposures relative to clean air exposures. Gas-only exposures in GIVES lasted 4 hours in all experiments. PM-only exposures in EAVES lasted 30 minutes. Post-exposure treatment and processing were performed as described in Chapter 3, Part 1.

Biological Effects Endpoints (Cytotoxicity and Inflammation)

Cytotoxicity is expressed as the increase of Lactate dehydrogenase (LDH) levels over clean air controls, as outlined in Chapter 3, Part 1. LDH levels were measured using a Cytotoxicity Detection Kit (TaKaRa Bio Inc., Tokyo, Japan). Interleukin 8 (IL-8) cytokine release was evaluated using an ELISA kit (BD Biosciences, San Diego, CA). Inflammatory response is reported as the increase of IL-8 levels over clean air controls, as described in Chapter 3, Part 1.

Tables of response ratios accompany bar charts of endpoint measurements. Response ratios were calculated by dividing the cellular response to the exposure listed in the column heading of the table by the response to the exposure listed in the first column. Further description of these calculations can be found in Chapter 3, Part 1.

Statistical Analysis

All calibration curves were tested by fitting a linear least squares line through the data. Calibration factors were calculated for each species to be analyzed, with appropriate propagation of uncertainty. All instrument measurements are reported as the mean ± 95% confidence interval, unless otherwise stated. The statistical analysis of toxicity endpoints were the same as those used in the Chapter 3, Part 1 study.

Experimental Design

Urban VOC Mixture and Non-toxic PM

The VOC mixture used in Part 2 experiments was based on canister samples of 06-09 am ambient air collected and analyzed by the US EPA in 39 US large cities (Seila, Lonneman et al. 1989). UNC researchers previously analyzed this database to create a 200 VOC species average composition of urban air (Jeffries 1995). From this analysis, UNC researchers also created a synthetic VOC mixture (consisting of 54 VOC species) that was closely based on the EPA analysis of more than 1000 canisters. The goal of creating this mixture was to permit accurate and reproducible initial injections of urban-like primary VOC emissions into the UNC chambers (Jeffries, Sexton et al. 1985). In the design of this synthetic VOC mixture, care was taken to choose a composition that had more than 70% of the carbon represented explicitly in the mix and to choose the other components to match both the average carbon fractions and average carbon number in each of the VOC groupings (alkanes, alkenes, aromatics, and carbonyls). In addition, the lighter compounds of the designed mixture were able to be produced as a commercially-certified gas cylinder, with 10,000 ppmC of the VOCs mixed in nitrogen. The heavier species are blended as a mixture of liquid hydrocarbons. The result is that only two simple injections into the chamber are required to achieve a highly reproducible total distribution of VOCs. A very large number of chamber experiments have been conducted with this mix—called Synthetic Urban Mixture—and it was also used in some Tennessee Valley Authority indoor chamber experiments and in and the Australian dual outdoor chambers (Johnson, Nancarrow et al. 1997; Simonaitis, Meagher et al. 1997).

The composition of Synthetic Urban Mixture (SynUrb54) is given in Table 9. In this Part 2 study, SynUrb54 and NO_x were used to represent a "typically reactive" gas-only urban environment (i.e., no primary PM). In all Part 2 experiments, SynUrb54 was injected into the chamber in the dark to a total carbon mixing ratio of 2.00 ppmC with ca. 0.30 ppm of NO_x. Furthermore, in the absence of "seed aerosol", this complex and highly-reactive system *does not* create any secondary aerosol in situ. Thus, its use will not introduce any inherent PM (with perhaps inherent toxicity) into the observation of the PM effects. This experimental design for the atmospheric chemistry environment results in a complex and highly realistic, but not totally quantifiable, set of gas-phase potential toxicants that are available for both gas-only exposures, and for potential uptake by test PM we introduce to the chamber to test PM "effect modification".

Table 9: Chemical Composition of Synthetic Urban VOC Mixture, SynUrb54[d].

Alkanes	ppbC[a]	Alkenes	ppbC[b]	Aromatics	ppbC[c]
isopentane	86.4	ethene	26.7	toluene	69.0
n-butane	73.5	2,3,3-trimethyl-1-butene	16.0	1,2,4-trimethylbenzene	56.0
propane	46.1	c-2-pentene	13.5	m-xylene	37.0
ethane	38.5	t-2-butene	11.6	benzene	22.0
3-methylhexane	35.0	propene	8.4	1,3-diethylbenzene	18.0
isobutane	32.9	1-octene	8.0	o-xylene	16.0
n-pentane	31.1	2-methyl-1-pentene	8.0	p-ethyltoluene	15.0
4-methylnonane	23.0	1-pentene	8.0	ethylbenzene	11.0
2-methylpentane	22.0	1-nonene	7.0	n-propalbenzene	11.0
n-decane	20.0	2-methylpropene	4.5	m-ethyltoluene	10.0
2,3-dimethylpentane	17.0	2-methyl-1-butene	4.2	sec-butylbenzene	7.0
n-nonane	16.0	2-methyl-2-pentene	3.0	1,2,3,5-tetramethylbenzene	6.0
n-heptane	16.0	cyclohexene	1.0		
3-methylpentane	16.0				
n-hexane	14.0	Dialkenes	ppbC	Alkenated Aromatics	ppbC
2,2,4-trimethylpentane	13.0	1,3-butadiene	2.1	? -methylstyrene	3.0
n-octane	12.0	isoprene	3.0		
2,5-dimethylhexane	11.0	? -pinene	6.0		
2,3,4-trimethylhexane	10.0			Aldehydes	ppbC
2,3-dimethylbutane	6.0			formaldehyde	10.4
methylcyclopentane	11.0			acetaldehyde	11.2
cyclohexane	10.0				
methylcyclohexane	6.0				

[a] 23 alkanes total 566.4 ppbC;

[b] 13 alkenes total 119.9 ppbC; 3 dialkenes total 11.1 ppbC

[c] 12 aromatics total 278.0 ppbC; 1 alkenated aromatic 3.0 ppbC; and 2 aldehydes total 21.6 ppbC

[d] All 54 species total to 1000 ppbC or 1 ppmC

To maintain a system with little ambiguity for PM "effect modification", we used the same inherently non-toxic PM from the Part 1 study. This PM was created by nebulizing steri-filtered liquid mineral oil (pharmaceutical grade, 100%) into the chamber, thereby creating mineral oil aerosol (MOA). This MOA is a good surrogate for organic-containing ambient PM, as described in Chapter 3, Part 1. The addition of PM to the chamber during experiments was only done in the dark (either before sunrise or after

sunset), to be present during the biological-effects sampling. No experiment had a day-light period in which PM was present.

Experimental Conditions and Sampling Strategy

In Part 2 we simulate the complexity of the ambient atmosphere by performing day-long photochemical experiments in our outdoor chamber. These experiments start in the dark with realistic mixtures of urban hydrocarbons (i.e., SynUrb54) and NOx that undergo oxidation in ambient sunlight and are retained into the dark period after sun-set. The experimental operation can be better understood by examining Table 10 and Figs. 20, 22, and 23 that show time series data for several inorganic and organic species during a particular experiment conducted on June 25, 2009 (named JN2509). On each figure, shading is used to indicate 'dark' periods, and the actual time for sunrise and sunset are marked at the top of each plot. VOCs and NOx were injected at 0230 EDT for this experiment. In these experiments, we have designated the period in the dark just after injection (but before sunrise) as the 'Fresh' condition and the period in the dark after sunset as the 'Aged' condition. These two time windows were used to expose cells and to collect additional chemical characterization samples. The exposures and chemical characterization sampling were performed in the dark to ensure that any chemical reac-tions occurring in the system were proceeding slowly-enough that substantial modifica-tion of the chamber contents would not occur during the sampling windows. On these time-series plots, biological exposure windows are designated by orange bars. The gas-only exposures occurred during the times marked by the solid bars, and the PM-only exposures occurred during the shorter bars with alternating orange and black bands.

Additional sampling to characterize the chemical environment during the 'Fresh' and 'Aged' conditions are designated by the magenta bar and the blue dashed bar. Individual GCMS sampling times are designated by red circles.

Table 10: Chamber Experimental Runs and Relevant Conditions

Id.	Run Name	Experimental Properties				
		Chamber Reactant Initial Conditions				
I.	AP2808	VOC ppmC	NO_x ppm		Type Name	Used in Timeseries Plots
		0.00	0.00		'Blank Run 1'	none
		Exposure Periods				
		Exp. Id.	Time Window	PM Injection mg/ m^{-3}	Name	Used in Figure or Table
		A.	Fresh	0.00	'Clean Air'	Figs. 10, 11; Tab. 6, 7, 8
		Chamber Reactant Initial Conditions				
II.	AU1909	VOC ppmC	NO_x ppm		Type Name	Used in Timeseries Plots
		0.00	0.00		'Blank Run 2'	none
		Exposure Periods				
		Exp. Id.	Time Window	PM Injection mg/ m^{-3}	Type Name	Used in Figure or Table
		B.	Fresh	1.56	'Clean Air, PM added'	Figs. 6, 10 , 11; Tab. 6, 7, 8
		Chamber Reactant Initial Conditions				
III.	JN2509	VOC ppmC	NO_x ppm		Type Name	Used in Timeseries Plots
		2.00	0.30		'SynUrb54/NO_x 1'	Figs. 2, 4, 5
		Exposure Periods				
		Exp. Id.	Time Window	PM Injection mg/ m^{-3}	Name	Used in Figure or Table
		C.	Fresh	0.00	'Fresh, SynUrb54/NO_x'	Figs. 2, 3, 4, 5, 7, 10, 11; Tab. 3, 4, 5, 6, 7, 8
		E.	Aged	0.00	'Aged, SynUrb54/NO_x'	Figs. 1, 2, 3, 4, 5, 7, 10, 11; Tab. 3, 4, 5, 6, 7, 8
		Chamber Reactant Initial Conditions				
IV.	ST2310	VOC ppmC	NO_x ppm		Type Name	Used in Timeseries Plots
		2.00	0.30		'SynUrb54/NO_x 2'	none
		Exposure Periods				
		Exp. Id.	Time Window	PM Injection mg/ m^{-3}	Name	Used in Figure or Table
		D.	Fresh	0.90	'Fresh, SynUrb54/NO_x, PM added'	Figs. 6, 10, 11; Tab. 4, 6, 7, 8
		Chamber Reactant Initial Conditions				
V.	ST1510	VOC ppmC	NO_x ppm		Type Name	Used in Timeseries Plots
		2.00	0.30		'SynUrb54/NO_x 3'	none
		Exposure Periods				
		Exp. Id.	Time Window	PM Injection mg/ m^{-3}	Name	Used in Figure or Table
		F.	Aged	0.87	'Aged, SynUrb54/NO_x, PM added'	Figs. 6, 8, 10, 11; Tab. 4, 5, 6, 7, 8

During this particular experiment (JN2509), no MOA-PM was added at any point. For experiments in which MOA-PM was added, it was injected at the beginning of the 'Fresh' window or at the beginning of the 'Aged' window to permit the gases present to modify the inherently non-toxic PM. Of course, if MOA was added to the 'Fresh' window, the subsequent day time oxidation would be affected due to both evaporation of MOA mass to the gas phase, and perhaps due to gain in PM mass from secondary organic aerosol production on the MOA surface. In this case, the experiment was used only for 'Fresh with MOA' exposure, and the rest of the experiment was discarded. A separate whole-day experiment was performed to produce an 'Aged with MOA' exposure in which the MOA-PM was added only after sunset to the otherwise gas-only mixture.

Within these day-long experiments we used the 'Fresh' and 'Aged' time windows to either sample the gas system without PM, or to add MOA-PM to the gas system and sample, so as to create four different complex gas and PM measurement and exposure conditions. To these four conditions we added two clean air control conditions, resulting in six comparison conditions necessary to demonstrate the existence of PM "effect modification" for complex, urban-like gaseous environments. Due to the need for both initial and final VOC conditions tested with and without PM, five different experiments were necessary to generate these six chamber conditions. The experimental run dates and conditions are described in more detail below.

The final experimental design gave six distinct test atmospheres in the chamber (summarized in Table 11): A) 'Clean, no PM': clean air in the dark; B) 'Clean, PM added':

condition A with non-toxic MOA added; C) 'Fresh, no PM': the SynUrb54/NOx system in the dark after injection and before sun rise; D) 'Fresh, PM added': condition C with MOA added; E) 'Aged, no PM': the SynUrb54/NOx system after a full day of photochemical reaction and with exposure after sunset; F) 'Aged, PM added': condition E with non-toxic MOA added.

Table 11: Six Exposure Conditions During Blank and Complex Mixture Experiments.

Exp. [a]	Treatment			Mixing Ratio				Concentration	Fraction	
	State [b]	VOC/ NO_x Mix	MOA [c] added	VOC ppmC	Ozone ppm	NO ppm	NO_x ppm	PM mg $\overline{m}^3$	RH Chamber	RH Exposure
A.	Clean	none		ND^d	0.009	0.007	0.008	0.001	0.478	0.317
B.	Clean	none	MOA	ND^d	0.009	0.007	0.007	1.562	0.692	0.301
C.	Fresh	SynUrb54/NO_x		1.80	0.002	0.231	0.304	0.001	0.746	0.2988
D.	Fresh	SynUrb54/NO_x	MOA	1.80	0.003	0.232	0.312	0.898	0.611	0.389
E.	Aged	SynUrb54/NO_x		0.86	0.344	0.001	0.085	0.001	0.354	0.296
F.	Aged	SynUrb54/NO_x	MOA	1.07	0.459	0.002	0.052	0.867	0.552	0.310

[a] Exposure Label. All exposures occur in dark before (Clean, Fresh) or after (Aged) daylight period.

[b] Chamber state during exposure. Fresh is injected VOC/NO_x mixture in dark; Aged is dark period after daytime reaction period.

[c] MOA is mineral oil aerosol added only in dark for exposures B, D, and F. MOA was not present during photo-irradiation.

[d] None detected.

All measurements represent the mean value over an entire exposure interval.

As stated above, all biological exposures for gas-only-and PM-only-effects occurred during 'Fresh' or 'Aged' conditions (in the dark, either before sunrise or after sunset).

Results

Table 10 provides the names of the experiments, summarizes the five experiments used to create the six exposure conditions, and gives cross-references to the tables and figures that illustrate the experimental results.

Table 11 provides the names of the exposure conditions, connects these to the experiments, and provides the average values of chemical, PM, and chamber physical environmental variables during the biological exposure windows.

In addition, the data from one experiment (JN2509) was used to illustrate a set of time series conditions, and to highlight the biological sampling windows and the chemical transformation period.

Gas Phase Compositional Analysis

Chromatograms in Fig. 21 show the evolution of the gas-phase composition during the course of photochemical aging for one day in the JN2509 experiment. Observed mixing ratio measurements and peak identities for hydrocarbon species from Fig. 21 are reported in Table 12. Time-series plots for the consumption of NO and VOCs, as well as the resulting production of O_3 and multiple generations of oxidized daughter compounds can be found in Figs. 20, 22, and 23. The species included in Fig. 22 are limited to those found in SynUrb54 (Table 9), with specific (major) components represented explicitly and others grouped by organic function. Species represented explicitly in Fig. 22 are not included in the grouped categories (they are not counted twice). The volume of chamber air needed for the exposures and sampling led to dilution of initial reactants prior to sunrise (Figs. 20 and 22). Significant chemical loss in NO and VOCs began just after sunrise and continued until a given species was consumed, or the oxidant production from photolysis processes stopped (the sun set).

The photochemical aging of SynUrb54 created a clear temporal change in composition through the course of the day (Figs. 21 – 23; Table 12). The relative rates at which

species were consumed were consistent with their kinetic rate constants (determined

by the rate at which they react with photochemically-generated species such as hydrox-

yl radicals and O_3; (Atkinson 2000)). Olefinic compounds (alkenes) reacted at the great-

est rate, and were completely consumed by midday (Figs. 21 and 22; Table 12). The ar-

omatics and alkanes decayed more slowly, and almost all of these compounds were still

detectable at the end of the day (Fig. 22, Table 12).
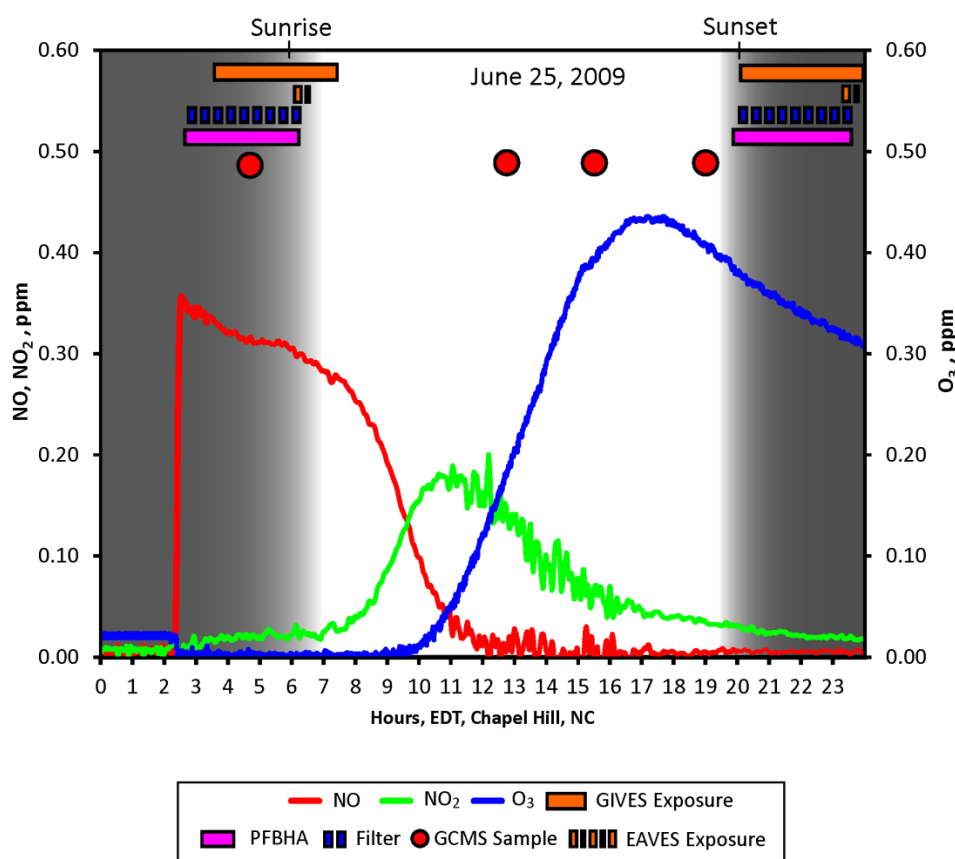
Figure 20: Time series of ozone (O_3), nitric oxide (NO), and nitrogen dioxide (NO_2) during experiment JN2509, performed with SynUrb54 and NO_x injected in the dark at 0230 EDT.
Color bars represent the type and time window for biological exposure sampling (GIVES, EAVES), for filter sampling, and for impinger sampling for PFBHA carbonyl analysis; red dots represent the time of VOC sampling for GCMS direct injection analyses.
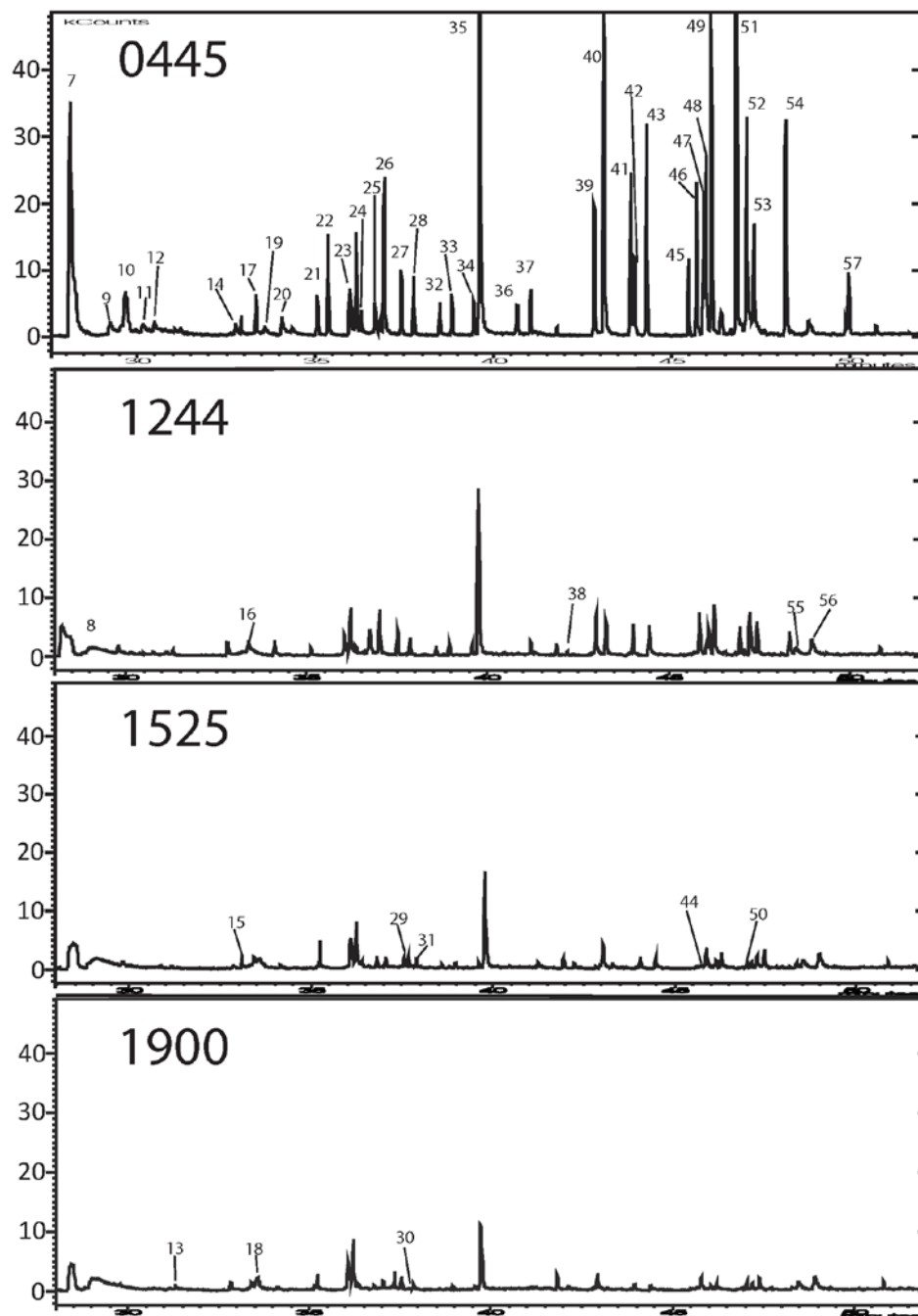
Figure 21: GCMS chromatograms of VOCs during the experiment JN2509 using SynUrb54/NOx mixture. Numbers labeling the species in the chromatograms correspond to the identification numbers in Table 12 and the time of sample corresponds to the red circles in Figs. 20, 22, and 23.
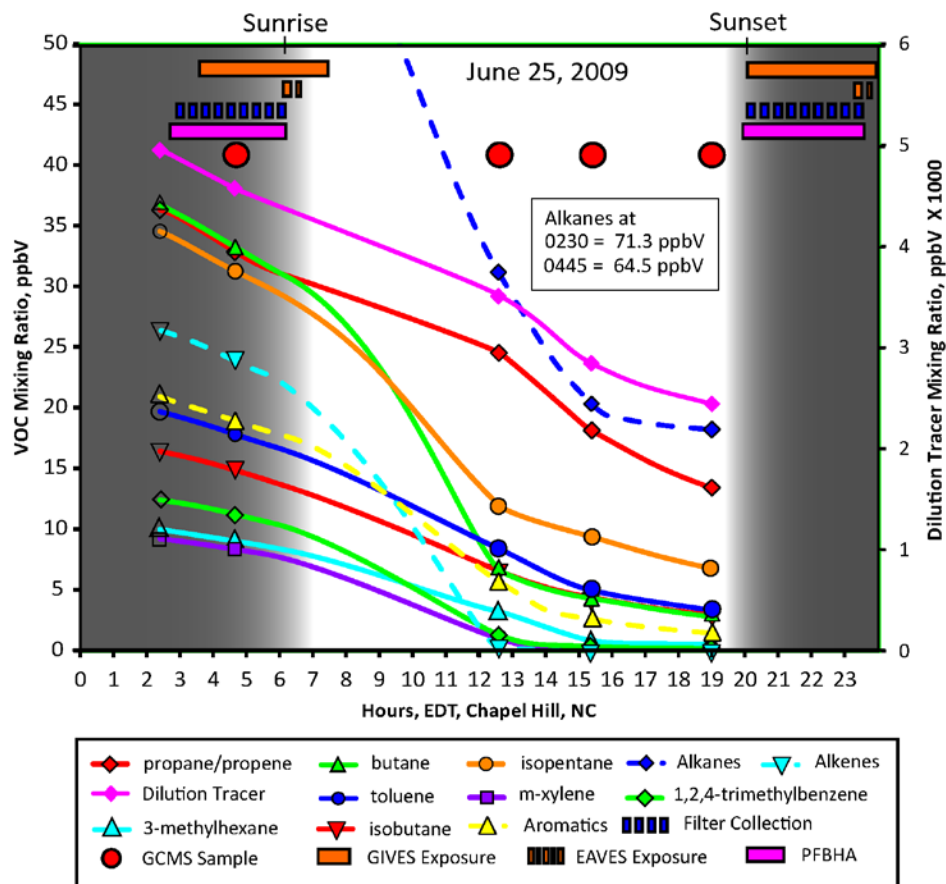
Figure 22: Time series of explicit (solid lines) or functionally-grouped (dashed lines) primary (injected) VOCs mixing ratios during experiment JN2509, performed with SynUrb54 and NOx injected in the dark at 0230 LDT.

Explicitly represented species are not included in their functional group mixing ratio values. Color bars and large red dots are as in Fig. 20. See Table 12 for mixing ratio values at points.
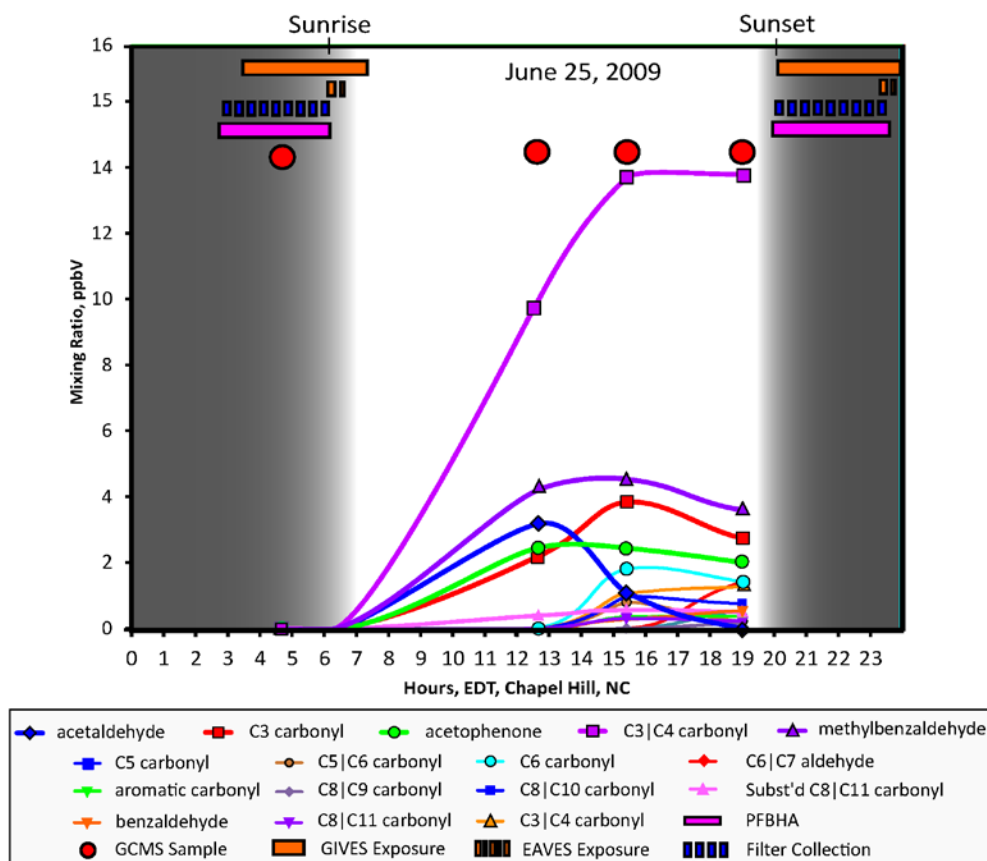
Figure 23: Time series of explicit or type-identified, secondary VOCs produced during experiment JN2509, performed with SynUrb54 and NOx injected in the dark at 0230 LDT.
The symbol between carbon number names means 'or'; the functionality is known, the carbon chain length is estimated by retention times. Color bars and large red dots are as in Fig. 20. See Table 12 for mixing ratio values at point.

Table 12: GCMS Measured VOC species at four times during experiment JN2509 using SynUrb54/NOx in Gillings Outdoor Chamber. See Fig. 21 for the corresponding chromatograms.

Id.	Species	Mixing ratio, ppbV; Time, EDT			
		0445	1244	1525	1900
1	propane/propene	36.33	24.61	18.15	13.46
2	isobutane	16.45	6.62	4.40	2.98
3	acetaldehyde	0.00	3.24	1.13	0.00
4	butane	36.75	6.74	4.33	2.84
5	C3 carbonyl	0.00	2.23	3.91	2.80
6	C3| C4 carbonyl	0.00	0.00	1.09	1.30
7	isopentane	34.56	11.70	9.64	6.78
8	C3| C4 carbonyl	0.00	9.51	13.93	14.03
9	1-pentene	3.20	0.00	0.00	0.00
10	n-pentane	12.44	1.77	0.84	0.45
11	trans-2-butene	5.80	0.00	0.00	0.00
12	cis-2-pentene	5.40	0.32	0.00	0.00
13	C5 carbonyl	0.00	0.00	0.00	0.56
14	2-methylpentane	7.33	7.15	2.58	4.34
15	C5| C6 carbonyl	0.00	0.00	0.80	0.22
16	C6 Carbonyl	0.00	0.00	1.83	1.40
17	3-methylpantane	5.33	5.86	5.04	6.39
18	C6| C7 aldehyde	0.00	0.00	0.00	1.40
19	2-methyl1-pentene	2.67	0.00	0.00	0.00
20	n-hexane	4.67	2.96	0.87	0.68
21	methylcyclopentane	3.67	1.09	2.61	1.47
22	2,3,3-trimethyl-1-butene	4.57	0.00	0.00	0.00
23	benzene	7.33	6.36	5.75	5.87
24	cyclohexane	3.33	1.23	1.13	0.93
25	2,3-dimethylpentane	4.86	2.91	2.64	0.92
26	3-methylhexane	10.00	3.56	0.86	0.57
27	2,2,4-trimethylpentane	3.25	1.53	0.64	0.69
28	n-heptane	4.57	1.74	1.03	1.26
29	aromatic carbonyl	0.00	0.00	0.35	0.38
30	C8| C9 carbonyl	0.00	0.00	0.00	0.17
31	C8| C10 carbonyl	0.00	0.00	0.98	0.77
32	methylcyclohexane	1.71	0.57	0.29	0.14
33	2,5-dimethylhexane	2.75	0.16	0.57	0.37
34	2,3,4-trimethylhexane	2.22	0.85	0.57	0.32
35	toluene	19.71	8.44	4.99	3.37
36	1-octene	2.00	0.00	0.00	0.00
37	n-octane	3.00	1.28	0.93	0.29
38	Subst'd C8| C11 carbonyl	0.00	0.40	0.57	0.52
39	ethylbenzene	2.75	1.23	0.78	0.48
40	m-xylene	9.25	1.05	0.23	0.06
41	o-xylene	4.00	1.04	0.50	0.23
42	1-nonene	1.56	0.00	0.00	0.00
43	n-nonane	3.56	0.89	0.36	0.17
44	benzaldehyde	0.00	0.00	0.28	0.55
45	? -pinene	1.20	0.00	0.00	0.00
46	n-propylbenzene	2.44	0.95	0.47	0.36
47	m-ethyltoluene	2.22	0.00	0.00	0.00
48	p-ethyltoluene	3.33	0.36	0.03	0.00
49	4-methylnonane	4.60	0.57	0.14	0.09
50	C8| C11 carbonyl	0.00	0.00	0.31	0.23
51	1,2,4-trimethylbenzene	12.44	1.35	0.38	0.23
52	n-decane	4.00	0.63	0.11	0.06
53	sec-butylbenzene	1.40	0.62	0.20	0.09
54	1,3-diethylbenzene	3.60	0.78	0.47	0.26
55	acetophenone	0.00	2.48	2.49	2.04
56	methylbenzaldehyde	0.00	4.30	4.62	3.70
57	1,2,3,5-tetramethylbenzene	1.20	0.70	0.17	0.06

The photochemical degradation of parent compounds led to the production of multiple generations of (oxidized) daughter compounds, with some species appearing early in the day and others appearing later (Figs. 21 and 23; Table 12). For many of these oxidized species, direct standards were not available, or there were similarities between the mass spectra of an unknown species and more than one compound. For instances such as these (in which an unambiguous identification was not possible) daughter compounds are identified in the table as "Cn carbonyl" (where n is the carbon number of the compound). For 'Cn' compounds for which more than one carbon number was possible, a | symbol was inserted between possible carbon numbers. Figure 23 illustrates the growth of select daughter and granddaughter compounds, some of which are present at midday (C3|C4 carbonyl, acetophenone, methylbenzaldehyde), while others do not develop until closer to sundown (C6 carbonyl, C6|C7 aldehyde). Moreover, some compounds were produced early in the day and were then consumed by further reaction (acetaldehyde, C3 carbonyl), while others persisted or continued to increase in concentration until the end of the day (acetophenone, C3|C4 carbonyl; refer to Figs. 21 and 23).

Additional sampling using carbonyl-specific analysis (with PFBHA) is summarized in Table 13, and Figs. 24 and 25. It should be noted that the chromatogram at the top of Fig. 24 represents the same chamber condition as the chromatogram at the top of Fig. 25 (i.e., two instances of Exposure condition C: Fresh SynUrb54 without MOA present). Additionally, in the ST1510 1937–2310 sample, particles were not removed from the airstream prior to sampling, meaning that the chromatogram likely contains some

carbonyls that were in the particulate phase. Though it is difficult to accurately measure

PM collection efficiency for this sampling method, the species identified in the chroma-

togram (Fig. 25; 1937 – 2310), and mixing ratios reported in Table 13, likely represent a

mixture of gaseous and particle-borne carbonyls without any distinction as to whether

they were in the gas or the particle phase. The mixing ratios of the unknown carbonyls

were calculated using the carbon number of the species closest to it with a positive

identification in the chromatogram (2-heptanone for both; Table 13).

Table 13: Mixing ratios for carbonyl-specific species by PFBHA derivation and GCMS during selected experiments by exposure condition. See Table 11 for other conditions of these exposures.

		Experiment Name, Time Window		
		ppmV		
Id.	Species	JN2509 Fresh	JN2509 Aged	ST1510 Aged
		Exp. C	Exp. E	Exp. F
1	formaldehyde	16.69	10.97	13.15
2	acetaldehyde	9.10	0.61	1.63
3	acetone	2.79	0.92	1.06
4	hydroxyacetaldehyde	0.87	-	0.75
5	pentanal	-	-	1.04
6	2-hexanone	Detected	Detected	3.11
7	hexanal	-	-	9.11
8	2-heptanone	2.74	Detected	7.31
9	unknown	0.28[?]	-	-
10	unknown	-	-	-
11	glyoxal	-	41.09	2.54
12	methylglyoxal	-	27.22	1.55

[a] see Table 2 for list of experiments and run names.

[?] carbon number estimated from next closest identified peak in chromatogram
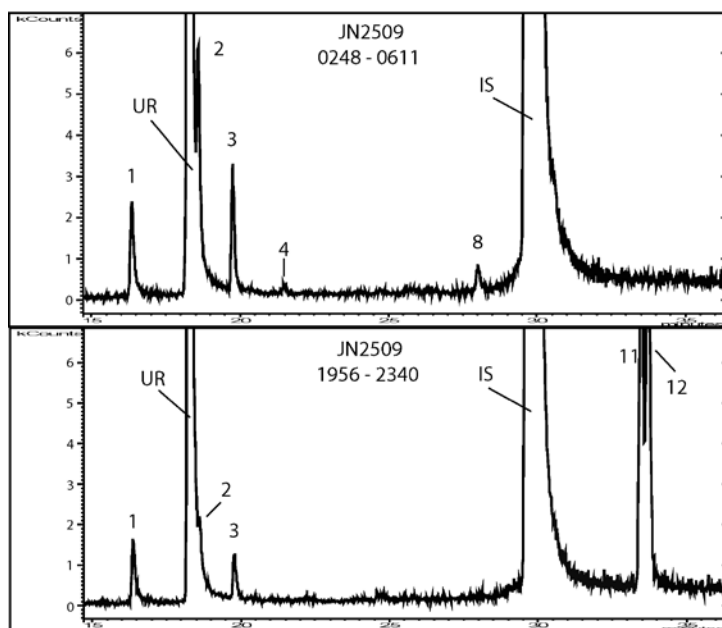
Figure 24: GCMS chromatograms for PFBHA-derivatized samples from time windows for PFBHA sampling shown in Figs. 3 and 5 for experiment JN2509; Top: Exposure C; bottom: Exposure E. See Table 13 for key to numbers labeling species.
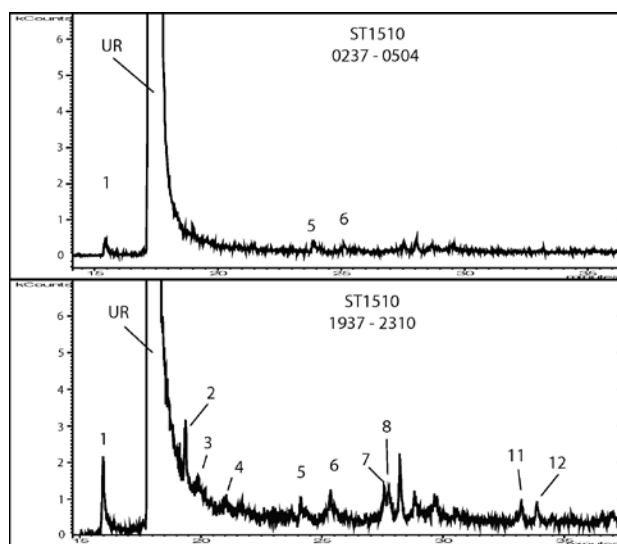
Figure 25: GCMS chromatograms for PFBHA-derivatized samples from time windows for PFBHA sampling in experiment ST1510; Top: Exposure none; bottom: Exposure F. See Table 13 for key to numbers labeling species.

The concentrations of formaldehyde and acetaldehyde were observed to decrease during photochemical reaction. As with the direct sampling technique, other species increased in concentration due to photochemical production (2-hexanone and 2-heptanone in Fig. 24). Still others were absent in the Fresh mixtures, but were significant in the Aged mixtures (glyoxal, methylglyoxal, hexanal, and others; see Table 13). For most of the carbonyl-containing species identified with PFBHA sampling in this study, their highest concentrations were measured in the Aged mixture with MOA (Exp. F, Table 13; 1937 -2310, Fig. 25). The most notable departures from this trend were glyoxal and methyl glyoxal, whose concentrations were observed to be much higher in the Aged mixture without MOA (Exp. E; 16× higher for glyoxal and 17.5× higher for methylglyoxal). This difference may be misleading, however, as the extent of photochemical oxidation (which depends upon the length of day and the solar radiation intensity) was significantly different for JN2509 and ST1510. June 25 is near the longest day of the year (with ca. 14.5 hours of sun), and September 15 is near the day of equal day and night (with ca. 12 hours of sun). Glyoxal and methylglyoxal are both daughter products and grand-daughter products of primary reactants, and they can also act as reactants. Further discussion of these results is given below.

The presence of formaldehyde and acetaldehyde in the Fresh chamber condition were expected, as they are a part of the SynUrb54 mix (Table 9 and Fig. 24). The small decreases in concentration measured for formaldehyde, acetone, and hydroxyacetaldehyde over the course of the day may be somewhat misleading (refer to Table 13). We know formaldehyde and acetaldehyde are products of oxidation, and often approach a

steady state condition in photochemical systems. Also, dilution occurs throughout an experiment (approximately 4% per hour), meaning that we would expect the concentrations of these species to decrease by a greater amount than was observed. What can be inferred from this is that, if the concentrations of these species were corrected for dilution, we would most-likely have observed a sizeable increase in their concentrations during the course of the day due to in situ production. By extension, the concentrations seen to increase (pentanal, hexanal, 2-heptanone, etc.), would likely increase by an even greater margin.

To focus on the differences between the chamber contents during each exposure scenario, concentrations measured by direct GCMS sampling for select species were compiled in Table 14. In this table, species' identification numbers match those from Table 12 and Fig. 21. Chamber conditions match those from Table 11 and Figs. 27 and 28 (Exp. C-F). The exposure concentrations for these species vary greatly by condition, with the greatest difference between the Fresh and Aged mixtures. All of the species present in the Fresh mixtures had much lower concentrations in the Aged mixtures, to the point that some were undetectable (e.g., 2-methyl-1-pentene and 2,3,3-trimethyl-1-butene). In addition, several species were only detected in the Aged mixtures (Table 14). In general, the gas-phase concentration measured for each species was lower when MOA was present in the chamber (Exp. C and F). This observations is true for both the Fresh and Aged mixtures, with the exception of acetophenone in the Aged mixture (Exp. F; species Id. 55 in Table 14).

Table 14: Mixing ratios of select species by exposure condition. Identification numbers for each species are the same as those found in Table 12 and Fig. 21. See Table 11 for other conditions of these exposures.

Id.	Species	Experiment Name, Time Window ppmV			
		JN2509 Fresh	ST2310 Fresh	JN2509 Aged	ST1510 Aged
		Exp. C	Exp. D	Exp. E	Exp. F
3	acetaldehyde	0.00	0.00	0.00	2.49
6	C3| C4 carbonyl	0.00	0.00	1.30	0.76
13	C5 carbonyl	0.00	0.00	0.56	0.34
14	2-methylpentane	7.33	4.70	4.34	3.16
18	C6| C7 aldehyde	0.00	0.00	1.40	1.76
19	2-methyl-1-pentene	2.67	0.41	0.00	0.00
22	2,3,3-trimethyl-1-butene	4.57	3.02	0.00	0.00
29	aromatic carbonyl	0.00	0.00	0.38	0.00
30	C8| C9 carbonyl	0.00	0.00	0.17	0.00
31	C8| C10 carbonyl	0.00	0.00	0.77	0.00
32	methylcyclohexane	1.71	0.97	0.14	0.00
33	2,5-dimethylhexane	2.75	2.24	0.37	0.00
34	2,3,4-trimethylhexane	2.22	0.75	0.32	0.00
40	m-xylene	9.25	6.13	0.06	0.00
49	4-methylnonane	4.60	0.35	0.09	0.00
50	C8| C11 carbonyl	0.00	0.00	0.23	0.00
55	acetophenone	0.00	0.00	2.04	3.82
56	methylbenzaldehyde	0.00	0.00	3.70	2.19
57	1,2,3,5-tetramethylbenzene	1.20	0.74	0.06	0.00

It should be noted that the concentrations and decay rates of the species reported here were not corrected for dilution and should not be taken as direct measurements of the rate of photochemical reaction for the species. The data was tabulated in this way to represent the chamber contents during exposure periods accurately, rather than as measures of the reactivities of the species present.

Particle Phase Characterization

The particle mass concentrations across all of the exposures in which no particles were added were close to the limit of detection for the SMPS instrument (as determined by the averages and 95% confidence intervals reported in Table 11). For the experiment in which SynUrb54 was photochemically aged and no MOA was added to the chamber (JN2509), no measureable mass of secondary aerosol (SOA) was formed *in situ*, though a very small number of particles were observed in the post-nucleation size range after sundown (Fig. 29; Table 11). As stated above, this agrees with previous observations of the photochemical-aging behavior of this mixture. The particle mass concentrations for the exposure periods in which MOA was added to the chamber were ca. 900× to 1500× higher than the exposures without PM (Table 11). A very slight increase in the mode particle diameter was observed when MOA and SynUrb54 were mixed in the chamber (relative to MOA in clean air; from 233 nm to 269 nm for both SynUrb54/MOA mixtures; Fig. 29). The mode particle diameter increased by the same amount regardless of whether the SynUrb54 was at the 'Fresh' (ST2310) or 'Aged' (ST1510) condition (Fig. 29).

The only detectable species present in the filter samples taken during this study were components of mineral oil aerosol. These results are discussed below.
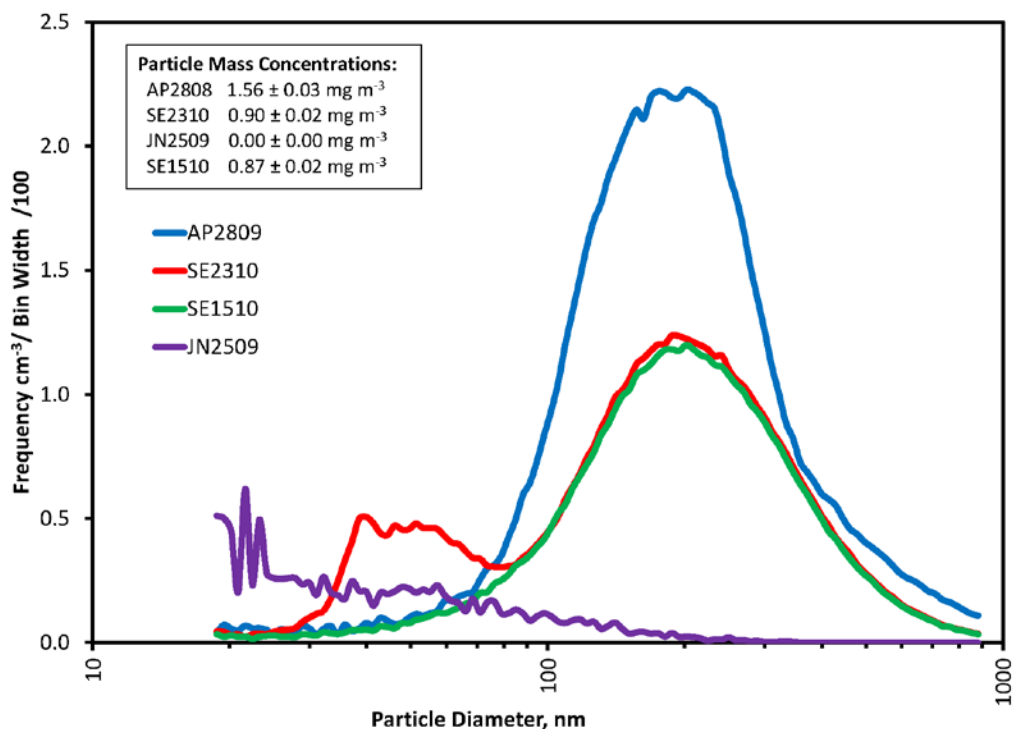
Figure 26: Mineral Oil Aerosol (MOA) size distributions for four exposure periods: AU1909 morning is Exposure B, 'Clean Air with MOA'. ST2310 morning is Exposure D, 'Fresh SynUrb54 with MOA'. ST1510 is Exposure F, 'Aged SynUrb54 with MOA'. JN2509 is Exposure C, 'Aged SynUrb without MOA'. Particle mass concentrations reported in the inset were determined from TSP filter measurements.

Exposure Conditions

The experiments performed in this study resulted in exposures with the gas-phase and particle concentration conditions given in Table 11.

It is important to note that the values presented in Table 11 for VOC mixing ratios are an estimate of the total VOC in the chamber. These estimates were calculated by correcting the initial VOC concentration (2.00 ± 0.04 ppmC) for dilution over time. Measured concentrations of identified species are presented above (Tables 12 and 13).

Biological Exposure Effects Response

Accompanying the compositional changes discussed in the previous sections was a significant increase in toxicity when SynUrb54 was photochemically aged. Fresh SynUrb54 without MOA-PM did not elicit a greater inflammatory response than clean air (Exps. A and C in Fig. 28). Once the mixture was photochemically aged, however, cells exposed to it produced 8× more IL-8 than cells exposed to the Fresh mixture (Exp. E in Fig. 28a and Table 15). But, when MOA was added to the system, a stepwise increase in toxic response was observed, beginning with clean air (with MOA added, Exp. B) and finishing a dramatic increase in response from with Aged SynUrb54 with MOA added (Exp. F; 12.6x and 11.4x more than Exps. B and D; Fig. 28a and Table 15).

The Aged mixture with MOA added (Exp. F; Fig. 28a) induced roughly twice the response observed from the Aged mixture without MOA (Exp. E; Fig. 28a and Table 15). This observation is discussed further below.

* Indicates a statistically significant difference in response relative to Clean Air (no MOA)
# Indicates a statistically significant difference in response relative to Clean Air with MOA
δ Indicates a statistically significant difference in response relative to Fresh (no MOA)
φ Indicates a statistically significant difference in response relative to Fresh with MOA
γ Indicates a statistically significant difference in response relative to Aged (no MOA)

Figure 27: Key for biological effects response plots in Figs. 28 and 29
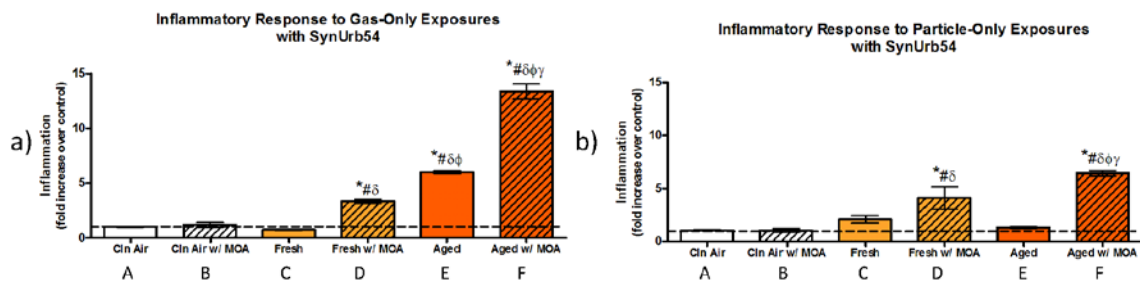
Figure 28: The IL-8 inflammatory response of lung cells following exposure to six air mixtures. The response induced by exposure to a) the gaseous components of each mixture and b) the particle-phase components of each mixture.
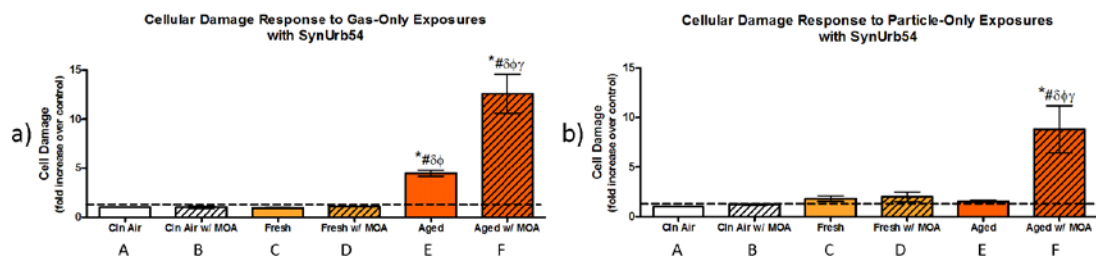
Figure 29: Cellular damage following exposure to six air mixtures. The response induced by exposure to a) the gaseous components of each mixture and b) the particle-phase components of each mixture.

Table 15: Tabular summary of the ratios of measured inflammatory responses (IL-8) for expo-
sures to SynUrb54 mixtures, (Table 11 and Fig. 27).

Gas-Only Exposure				A.	B.	C.	D.	E.	F.
Reference Condition[a]				A.	B.	C.	D.	E.	F.
A.	Clean	Air		1.00	1.18	0.73	3.32	5.95	13.28
B.	Clean	Air	w/ MOA		1.00	0.62	2.81	5.03	11.24
C.	Fresh	SynUrb54				1.00	4.54	8.13	18.17
D.	Fresh	SynUrb54	w/ MOA				1.00	1.79	4.00
E.	Aged	SynUrb54						1.00	2.23
F.	Aged	SynUrb54	w/ MOA						1.00

PM-Only Exposure				A.	B.	C.	D.	E.	F.
Reference Condition[a]				A.	B.	C.	D.	E.	F.
A.	Clean	Air		1.00	0.99	2.05	4.06	1.27	6.36
B.	Clean	Air	w/ MOA		1.00	2.07	4.10	1.28	6.42
C.	Fresh	SynUrb54				1.00	1.99	0.62	3.11
D.	Fresh	SynUrb54	w/ MOA				1.00	0.31	1.57
E.	Aged	SynUrb54						1.00	5.02
F.	Aged	SynUrb54	w/ MOA						1.00

[a] MOA is mineral oil aerosol, SynUrb54 is Synthetic Urban Mix

Figures 28b and 29b show the biological effects measured following exposure with
the PM-only method. The only significant inflammatory responses observed came from
cells exposed when both SynUrb54 and MOA were present in the chamber (Exps. D and
F). This means the cells exposed with the PM-only method do not respond to gas-phase
toxics under normal operating conditions (de Bruijne, Ebersviller et al. 2009; Lichtveld,
Ebersviller et al. 2011; Ebersviller, Lichtveld et al. 2012). This is further supported by the
large increase in response from the cells exposed to the Aged mixture with the gas-only
method when there was no increase in response from cells exposed to the same mix-
ture with the PM-only method (Exp. C in Figs. 28a and 28b).

The damage to the cellular membranes caused by exposure to the gas-phase of the test atmospheres largely followed the trends observed in the inflammatory response (Fig. 29; Table 16). For cellular damage, however, only the Aged exposures resulted in an increase in response (Fig. 29; exposures E and F). Only one test atmosphere (Aged with MOA added, exposure F) caused a significant increase in response from cells exposed with the PM-only method (4.5x to 8x more cellular damage than the other exposures, see Table 16 and Fig. 29b).

Table 16: Tabular summary of the ratios of measured cellular damage (LDH) for exposures to SynUrb54 mixtures, (Table 11 and Fig. 29).

Gas-Only Exposure								
Reference Condition[a]			A.	B.	C.	D.	E.	F.
A.	Clean	Air		1.00	1.00	0.92	1.11	4.48	12.62
B.	Clean	Air	w/ MOA		1.00	0.91	1.10	4.46	12.57
C.	Fresh	SynUrb54				1.00	1.21	4.88	13.75
D.	Fresh	SynUrb54	w/ MOA				1.00	4.04	11.38
E.	Aged	SynUrb54						1.00	2.82
F.	Aged	SynUrb54	w/ MOA						1.00

PM-Only Exposure								
Reference Condition[a]			A.	B.	C.	D.	E.	F.
A.	Clean	Air		1.00	1.23	1.81	1.99	1.53	8.78
B.	Clean	Air	w/ MOA		1.00	1.47	1.62	1.25	7.14
C.	Fresh	SynUrb54				1.00	1.10	0.85	4.84
D.	Fresh	SynUrb54	w/ MOA				1.00	0.77	4.41
E.	Aged	SynUrb54						1.00	5.72
F.	Aged	SynUrb54	w/ MOA						1.00

[a] MOA is mineral oil aerosol, SynUrb54 is Synthetic Urban Mixture

Discussion

We begin this discussion by reviewing the experimental conditions that were designed to permit testing of the hypothesis that PM "effect modification", as initially

posed by the NAS, does occur in complex urban-like oxidized VOC atmospheres. We assess the extent to which we were successful in achieving these conditions and describe any limitations and ambiguities that arise in using them for the biological exposures. Next, we examine the pattern of biological responses relative to the test environment and explore potential causal mechanisms to the extent that our data permits us. We then discuss sampling artifacts and finally we compare our results with others.

Creation of Control Environments

To test the null hypothesis, a set of control conditions had to first be established and characterized. As stated before, the chamber was carefully prepared to remove background contaminants that might alter the behaviors of the test atmospheres (Chapter 3, Part 1). Here we examine the extent to which these "clean" test conditions were achieved and discuss any issues that might limit the conclusions when using them.

Even though the Clean Air exposure with MOA had nearly twice as much MOA present as the Fresh or Aged exposures (both with MOA), neither of the in vitro systems registered an increase in toxicity from exposure (Table 11, Figs. 28 and 29). This demonstrates that MOA itself is not inherently toxic, as we reported previously (Chapter 3, Part 1). This is the major point that we needed to establish and it appears that non-toxic properties of MOA are invariant over a wide range of PM mass concentrations. Moreover, any affect that variations in RH might have had on the cells was minimal (Chapter 3, Part 1), so the likelihood of RH variations causing the increase in response observed from the Aged exposures is negligible. One Clean Air exposure was performed in April (Exp. A), while the other was performed in August, a year later (Exp. B). Since no re-

sponse was measured from either control exposure atmosphere, any chance that our observations were influenced by some unknown, seasonally-variable, inherent quality of the chamber itself can be discounted (Figs. 28 and 29). Therefore, with minimal criteria (described in Chapter 3, Part 1), we were successful in creating stable 'clean' atmospheres in our outdoor chamber that could be used as our baseline ('zero effect' exposure).

Creation of Urban-like Atmospheres

To study the dynamic entanglements of the phases in an urban-like environment, we first needed to create airborne mixtures of VOCs that approached the complexity of the ambient urban environment. The SynUrb54 mixture has been extensively used and well-studied by our research group in the past, so it was an ideal candidate for use in this study. The 'Fresh' conditions in the chamber are a well-designed model for the observed morning atmospheres in large urban areas in the early 1990s. While the average mixing ratios may have changed in the last 20 years, the identities of the VOCs likely have not. These conditions are a model based on generalization, distortion and deletion to create a highly repeatable set of conditions more complex than has been used in other chamber work, which often use only 6 to 10 VOCs in their mixtures. When the SynUrb54 mixture is photochemically oxidized it creates hundreds to thousands of oxidized species in situ, many of which would be unavailable to researchers attempting to evaluate their toxicity in more-traditional ways. This 'Aged' condition in the chamber truly begins to approach the complexity of gaseous pollutants in urban areas.

There were some small differences in composition between the two 'Fresh' chamber conditions without MOA (Exp. C). Specifically, the presence of certain oxidized species in the 'Fresh' condition during the JN2509 experiment (acetone and 2-heptanone; Fig. 24) is from low levels of O_3 that lingered in the chamber from the day before (Fig. 20 before 0200), which likely reacted with the components of SynUrb54 as they were injected into the chamber. This O_3 was titrated with NO, but in this instance the VOCs were added concurrently with the titration, allowing the O_3 to oxidize some of the VOCs in the mix. While this small introduction of oxidized species to the 'Fresh' mixture was not sufficient to alter the biological effects of the chamber contents, it is possible a similar circumstance led to the slight increase in response from the exposures to the 'Fresh' condition with MOA present (Exp. D; Fig. 28 and Table 15).

We also observed some variability in the 'Aged' condition in the chamber. In particular, there were some day-to-day differences in the identities and mixing ratios of the oxidized species present (Table 13), as well as in the O_3 concentration generated by the photochemical aging process (ca. 110 ppb, refer to Table 11). It is important to note that, while some observed VOC mixing ratios in the Aged mixtures were lower when MOA was added, others were higher. The differences in concentration cannot be assigned unambiguously to particle uptake, and may be due to a combination of factors. The presence of MOA in the chamber should, therefore, be thought of as co-incident with these variations in concentration, rather than explicitly causative. Most likely, the reason the observed mixing ratios varied from one experiment to the next had more to do with the differences in the length of the day and, by extension, the extent of reaction

in the system. As stated above, the amount of photochemical aging that occurs in a given system is dependent upon environmental variables such as the intensity of the sunlight, atmospheric pressure, temperature, and humidity. We are able to control the humidity in the system by modifying the dew point in the chamber (as described in Chapter 3, Part 1). We 'control' the remaining environmental variables for the system by performing these experiments during hot summer days with little cloud cover. The length of the day, however, can still be the determining factor for the extent to which chamber contents react. As one experiment was performed shortly after the summer solstice (JN2509) and the other was close to the autumnal equinox (ST1510), the difference in the length the sunlit day from one experiment to the next makes some day-to-day variation in the amount of photochemical products expected.

Some disagreement between direct measurements of the chamber by GCMS and the PFBHA-analysis method is also expected, and should not be seen as a shortcoming in our dataset. The reason that PFBHA-derivative samples are collected directly below the chamber (and not in the laboratory) is that polar compounds can be lost in sample lines. Moreover, direct analysis of carbonyl compounds by GCMS can be unreliable, while numerous studies have used PFBHA to great effect (Yu, Jeffries et al. 1997; Liu, Jeffries et al. 1999; Liu, Jeffries et al. 1999; Liu, Lovell et al. 2005; Seaman, Charles et al. 2006; Ebersviller, Lichtveld et al. 2012).

Due to the dynamic nature of the equilibria between the gas and particle phases, it is not possible to absolutely determine the distribution of a volatile or semi-volatile

species across the phases with current chemical sampling techniques. For instance, when chamber contents are sampled directly with a GCMS, PM is typically removed via an in-line filter to avoid clogging the injection system and/or column. While this may seem to remove particle-borne compounds from the analysis stream, the pressure drop across the filter is likely to lead to off-gassing of volatile species from the back side of the filter, thereby reintroducing them to the sample stream. Other filter sampling artifacts are discussed below.

We have demonstrated that we can create a highly complex, urban-like system that consists of a mixture of primary, secondary, tertiary and beyond oxidized products, even if we cannot totally identify and quantify all of the components. Our systems may have some daily variations that lead to some differences in the concentrations of some products; nevertheless, these systems remain highly representative of urban-like environments and are therefore useful in demonstrating the existence of PM "effect modification", even if the cause of the observed effect cannot be explicitly identified. Furthermore, the totally unambiguous result of the Chapter 3, Part 1 study shows that PM effect modification occurs, and was caused by the uptake of a single VOC by the particle. In this study, even with the variations in the conditions in the chamber, all SynUrb54-type experiments made similar complex mixtures of the same types of compounds that were used in Chapter 3, Part 1 and did not introduce enough ambiguity to the results to alter our conclusions. It is the integration of chemical and biological measurements that allows us to see that species are distributed between the phases, rather than concentrated in the gas phase and absent from the condensed phase.

Discussion of Biological Exposures and 'Effect Modification'

Our results clearly show that when a non-toxic PM is added to these complex oxidized-VOC systems, like in the single-species VOC systems in Chapter3, Part 1, the PM becomes toxic to cells in the PM-only biological exposure system. This is direct proof that in situ generation of gas-phase VOCs that are toxic to cells exposed in the gas-only biological exposure system—just as in the simple systems in the Chapter3, Part 1 study—can, in the presence of non-toxic, PM transform that PM to be toxic to cells exposed in the PM-only biological exposure system. By extension to real urban atmospheres, the atmospheric oxidation of ambient primary VOCs can make otherwise non-toxic PM, become toxic in the lungs of exposed humans. This is an atmospheric chemistry and physics processes phenomena that cannot be ignored when assessing the health consequences of PM being present in these urban environments.

Beyond the direct demonstration shown here, atmospheric scientists would like a mechanistic explanation, or a process identified, that explains this observed phenomena. Our study provides some insight, but was not sufficiently powerful in its design and analytical instrumentation available to answer these casual questions unambiguously. Our results are strongly suggestive that oxygenated, and especially carbonyl-containing compounds, are major components in the transferred toxicity. The following discussion explores suggestions for mechanisms that might account for our observations and discusses and limitations of our work in this regard.

The observed increase in biological effect markers for gas-only exposures when SynUrb54 was photochemically aged was expected, as similar observations have been

made previously (Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004; Doyle, Sexton et al. 2007; Lichtveld, Ebersviller et al. 2011).

In the gas-only exposure system, the Aged condition with added MOA resulted in ca. 2x the response observed from the Aged mixture without added MOA (Exps. E and F; Table 15). While we expect there to be day-to-day variations in composition (and the resulting response from the cells), no clear pattern of causative species is apparent in either the inorganic species analysis or the direct GCMS sampling data (Tables 11 or 14). Of the species identified and quantified by direct GCMS sampling in the two Aged mixtures, only acetaldehyde and acetophenone had a higher observed concentration in the Aged mixture with MOA. The presence of acetaldehyde in the Fresh mixture (Table 9), and the lack of corresponding response from the Fresh exposures (Figs. 28a and 29a) make it an unlikely candidate for the increased response. Moreover, while the acetophenone concentration measured in the Aged mixture with added MOA was approximately twice the concentration measured in the Aged mixture without added MOA, we feel that an increase in concentration of 2 ppbV is unlikely to be the cause of such a large increase in response (observed with both endpoints tested).

Comparing the *in vitro* responses to the species identified from our carbonyl-specific analysis (Table 13), the response of the cells to the Aged mixture with MOA shows a pattern. Of the carbonyl compounds that were identified, nearly all had higher concentrations in the Aged mixture with MOA than in the Aged mixture without MOA. Only glyoxal and methylglyoxal had higher concentrations in the Aged mixture without

MOA (41.09 and 27.22 ppbV in exp. E; 2.54 and 1.55 ppbV in exp. F). Formaldehyde, acetaldehyde and acetone are unlikely to be responsible for the observed increase in response, as the highest measured concentration for all three of these species was in the Fresh mixture (Exp. C; which had the lowest toxicological response other than Clean Air). It is possible that the strong gas-phase toxicological response to the Aged exposure with MOA (exp. F) is due to effects caused by a combination of the measured carbonyl compounds, or the measured compounds and O_3 (i.e., synergism or co-pollutant effects). Based on previous findings, however, it is also possible that undetected species and/or species present at a trace level contributed significantly to the observed response (Doyle et al., 2007). For this reason, we are not attempting to indicate specific causative agents in our results. Further toxicological investigation of the species detected will be necessary for their relative contributions to the total observed responses to be accurately measured.

Occasionally, there were slight variations between the responses observed in the biological effect markers we measured. Specifically, the inflammatory response of cells exposed with the gas-only method to Fresh SynUrb54 with MOA (Fig. 28a) was significantly higher than the cells exposed to clean air (sham). The amount of cell damage induced by these exposures, however, was not significant relative to the sham (Fig. 29a). Similar effects were observed from cells exposed to the Fresh SynUrb54 mixture with MOA in the PM-only method (Figs. 28b and 29b). This result is not without precedent and could merely indicate that the insult was sufficient to elicit an inflammatory response from the cells, but not result in total failure of the cellular membrane.

The particle size distribution of each exposure atmosphere was continually monitored, so we are able to rule out the effects of particle size variation on the observed toxicological responses (refer to Fig. 29). In addition, PM was never introduced to the chamber in the presence of sunlight in any of the experiments presented here, meaning that the particles themselves were never subjected to the possibility of having their toxicity or chemical composition altered by direct reaction with the sun. Any increase in the toxicity of the particle phase was, therefore, most likely due to its uptake of gas-phase toxics.

When taken in combination with the research presented in the Part 1 study in this series (Chapter3, Part 1), we can conclude that gaseous air toxics partitioned to the PM are likely responsible for the observed increase in response, rather than direct oxidation of the PM by ozone (or another component of the Aged mixture).

Discussion of Sampling Artifacts

There are several possible explanations for the lack of detectable species in our filter samples. As we mentioned above, our chemical analysis concentrates largely on carbonyl-containing compounds (specifically, aldehydes and ketones). There is a long history of evidence that species in the condensed phase tend to be organic acids, large hydrocarbons (such as PAHs), and oligomers (Kamens and Jaoui 2001; Lee, Jang et al. 2004; Cho, Sioutas et al. 2005). None of these classes of compounds would be derivatized by PFBHA, which reacts preferentially with aldehydes and ketones (Yu, Jeffries et al. 1995; Liu, Jeffries et al. 1999; Liu, Jeffries et al. 1999). So, by derivatizing our filter samples with PFBHA, we may simply have missed the species that were present. In future

work, multiple filters will be taken at each time point, thereby allowing us to preferentially target more classes of compounds as well as doing more generalized analysis.

It has been a common practice that, to increase the likelihood of having enough material to detect with current analysis methods, researchers typically try to collect as much particulate matter as possible. In the past, sampling artifacts have been documented to alter the chemical composition of particles collected on filters (Volckens and Leith 2003; Volckens and Leith 2003; Sihabut, Ray et al. 2005; Lichtveld, Ebersviller et al. 2011). While these studies were mostly limited to large, low volatility compounds thought to be the mostly likely in the particle phase; it can be extrapolated that these effects would be greatly enhanced for species with higher volatilities—to the point that some species would be completely removed from the particle phase during the sampling process. So, while collecting as much material as possible might be thought to be a move in the right direction, the increased pressure drop across the filter membrane as it becomes plugged with PM may, in fact, impede the improved detection of trace species.

Moreover, to be weighed and prepared for analysis accurately, filters need to be handled multiple times after sampling. Placing particle-laden filters into clean laboratory air will also interrupt the equilibria between the PM and the species partitioned to it—causing partitioned species to leave the filter by off-gassing to the air. Researchers (ourselves included) attempt to mitigate these effects by performing analyses quickly and efficiently, but it is ultimately unlikely that the particles that are eventually analyzed will have exactly the same chemical composition as those that were in the air. In addition,

even though our filters were stored in a freezer at -40°C, prolonged filter storage prior to analysis may have contributed to the loss of species from our samples.

Any and all of these effects could be possible explanations for the lack of detectable species in the filter samples from the SynUrb54 experiments. All of these concerns point toward the need for new particle methods (for both chemical analysis and toxicology) that will not interrupt the equilibria between PM and the species partitioned to it.

Comparison with other work

This research would not have been possible without the tools that we have available to us, especially the in vitro methods. Traditionally, the toxicity of PM is estimated by collecting particles and then resuspending them in a liquid medium that can be instilled onto in vitro cells or into the lungs of test animals (Tsukue, Okumura et al. 2010; Farina, Sancini et al. 2011; Steenhof, Gosens et al. 2011; Weldy, Wilkerson et al. 2011).

Additionally, in toxicology studies, filter samples are routinely left exposed to laboratory air for extended periods of time and/or autoclaved. These sample handling practices are likely to result in the loss of volatile species from the particle phase and, by extension, most likely effects such as those we observed here. The EAVES device has proven to be a sensitive biosensor for particle borne air toxics in this study, as well as previously (de Bruijne, Ebersviller et al. 2009; Lichtveld, Ebersviller et al. 2011; Ebersviller, Lichtveld et al. 2012). If the device (or one similar to it) could be adapted to act as a chemical analysis technique, it is possible that our perception of the interplay between gases and particles would undergo a paradigm shift similar to the conception of partitioning theory.

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In summary, we have demonstrated that test atmospheres with urban-like complexity can be created in our chamber in situ. We also successfully performed in vitro exposures to measure the biological effects induced by each of the complex test atmospheres we generated. Due to the complexity of the dynamic interactions in our systems, we are unable to unambiguously assign observed biological effects to specific species present in the test atmospheres. This causal assignment is not necessary, however, to determine that PM 'effect modification' occurred in our experiments. This not only establishes that the hypothesis for this study is true; it demonstrates that the findings of the Part 1 study can be extended to mixtures of gases with complexity approaching the ambient environment.

Conclusions

These results together have important ramifications across the air pollution field. Our studies have shown that gas phase air toxics can, and are likely to, increase the toxicity of airborne particulate matter by directly modifying the chemical composition of the PM. These findings can help explain the inability of laboratory-based toxicologists to replicate effects seen in near-roadway epidemiology studies, which has confounded the risk assessment field and policy makers alike. The time scale for these inter-phase dynamics is on the order of seconds. What this means in the real world is that vehicle emissions (and other PM) may not be exceptionally toxic as they enter the atmosphere but, when mixed into an aged or aging air mass, can change dramatically in composition and biological effect by the time they drift off-road and reach the general population. Therefore, due to their ability to act as pre-concentrators and delivery mechanisms for

semi-volatiles and SOA compounds (and their associated toxicity), even relatively 'non-toxic' PM (such as salts and minerals) should not be completely discounted from regulation.

Therefore, while studies of the effects of air pollutants that omit a photochemical aging component provide invaluable information for specific exposure scenarios, they may not accurately represent exposures as they happen to the population in general. Moreover, without an understanding of how the environment interacts with materials being studied, researchers cannot ensure that estimates of risk and effect are accurate measures of real-world circumstances. Investigating single pollutants found in Aged air masses (such as O_3) or single point-sources (such as a vehicle's exhaust) can be illustrative of risk, and at other times may be necessary to elucidate the manner in which atmospheric interactions occur (as in the single-VOC experiments presented in Chapter 3, Part 1). With singling out specific sources or pollutants in the search for a 'smoking gun,' however, comes the risk of shifting the focus of the research community away from their overall goal to understand the environment, and to protect human health. Therefore, such simplified systems should not be assumed to be completely representative of, or as a replacement for, pollutant mixtures formed in situ.

What is clear from this work is that the evolution of toxicity with photochemical aging, and the subsequent shift of that toxicity to the PM present in the mixture, is an important component to understanding the response of individuals exposed in the ambient environment. Further, this work reinforces the importance of the atmosphere it-

self as a significant source of toxicity in air pollution. This is true for both gas/particle

interactions and the evolution of toxic species with photochemical atmospheric aging.

These findings are demonstrated for test atmospheres with the full complexity of the

ambient environment in the final study in this series (Chapter3, Part 3).

Part 3, Petroleum Diesel and Biodiesel Oxidations

Introduction

In its most-recent document summarizing the state of the science related to PM, the US National Research Council (NRC) stated that ``a finding that the effect of particles depends on the concentration of another pollutant — that is, `effect modification' — would have implications for setting NAAQS [National Ambient Air Quality Standards] independently for the various criteria pollutants'' (NRC 2004).

In the Part 1 study (Chapter3, Part 1), we demonstrated the existence of "PM effect modification" (NRC 2004) for the case of a single gas-phase toxicant and an inherently non-toxic PM (mineral oil aerosol, MOA). That is, in the presence of a single gas-phase toxicant in the dark, the initially non-toxic PM became toxic to lung cells in the PM-only biological exposure system. In the Part 2 study, we used sunlit-reactive systems to create a large variety of gas-phase toxicants in situ from a complex mixture of oxides of nitrogen and 54 VOCs similar to those measured in US city air (Chapter3, Part 2). When this urban-like mixture of VOCs (SynUrb54) was photochemically-aged, we observed a large increase in gas-phase toxicity. To evaluate ``effect modification'' in these highly complex urban-like atmospheres, and to reduce ambiguity between studies, we used the same non-toxic PM that was used in the Part 1 study (MOA). In all of these test atmospheres, we only added the PM in the dark, either before sunrise or after sunset. As there was no secondary aerosol formed in situ, excluding the non-toxic PM from the reactive aging environment allowed us to ensure that any observed change in the toxicity of the MOA-PM was due to uptake of toxicants from the gas phase. This result al-

lowed us to demonstrate that the findings of the Part 1 study could be extended to highly-complex urban-like mixtures of Fresh and Aged VOCs. In addition, our observations regarding the increase in toxicity of the gaseous components of the test atmospheres gives evidence of the importance of including the atmosphere itself as a dynamic source of toxic species in estimates of the risks associated with air pollution exposures.

The current trend in public opinion and legislation is a push toward renewable, ``green'' alternatives to traditional fossil fuels. Non fossil-fuel-based alternative fuels, including biodiesel, have been increasingly evaluated as possible solutions to the problems presented by gasoline and petrochemical diesel. Biodiesel is the first ``renewable'' diesel fuel to show signs of commercial-market acceptance, and is becoming the default alternative to traditional (petrochemical) diesel fuel. The sources of petrochemical diesel (PCD) and biodiesel (BD) fuels are dramatically different. While PCD is distilled from crude oil, BD is derived from vegetable or animal fats and is, therefore, a renewable resource. While this is attractive for many social, environmental, and economic reasons - the impact of the replacement technology on the health and well-being of the public should be evaluated before any mass-market substitutions occur.

Studies conducted by the Environmental Protection Agency (EPA), and the National Renewable Energy Laboratory have shown significant decreases in VOC, CO, and PM emissions from blends of PCD and BD (B20 – 20% BD), compared to gasoline and B100 (EPA, Division et al. 2002; NREL, Morris et al. 2003; Jung, Kittelson et al. 2006; Yuan, Lin et al. 2007). The gaseous components of BD exhaust were analyzed by Peng *et.al.*

(2006), who observed a high aldehyde fraction (>13%) which they attributed to byproduct impurities from production (predominantly 2-pentanal, 4-pentenal, hexanal, and 2-butenal). In addition, several other studies have referred to observing a high carbonyl content in BD exhaust (Bunger, Krahl et al. 2000; Bunger, Muller et al. 2000; Turrio-Baldassarri, Battistelli et al. 2004; Krahll, Munackl et al. 2006). Therefore, though the reduction in volatile organic compound (VOC) emissions may help decrease ozone production, the high fraction of aldehydes detected in biodiesel emissions raises concerns about an increased risk for direct and secondary health effects due to these air toxics (WHO 2000; Zhang and Smith 2003; Hauptmann, Lubin et al. 2004; Peng, Lan et al. 2006; Swanson, Madden et al. 2007; Takigawa, Wang et al. 2009).

Emissions derived from diesel engines are complex mixtures of gases and particulate matter. Compounding the difficulty of establishing estimates of risk is the manner in which the compositions of conventional diesel and biodiesel fuels are dramatically different. PCD is rich in hydrocarbons with between 8 and 21 carbon atoms that may be paraffinic, olefinic, or aromatic, and typically contains a high concentration of toluene. BD, however, is largely made of alkyl esters, and has been reported to have a larger fraction of aldehydes, many of which may be unsaturated (Bunger, Krahl et al. 2000; Bunger, Muller et al. 2000; Peng, Lan et al. 2006; Swanson, Madden et al. 2007; National Biodiesel Board 2010; Piedmont Biofuels 2010; National Biodiesel Board 2012; Piedmont Biofuels 2012). Increasing the complexity of this issue, nearly any biologically-derived oil can be used as a feedstock for biodiesel. Feed stocks may include sources as varied as pristine oils from soy beans or rapeseed (Canola oil - popular in Europe), or recycled

deep-fat-fryer grease from restaurants. Moreover, the chemical composition of BD is completely dependent upon the feedstock from which it is made (Bunger, Krahl et al. 2000; Bunger, Muller et al. 2000; Peng, Lan et al. 2006; Swanson, Madden et al. 2007; National Biodiesel Board 2010; Piedmont Biofuels 2010; National Biodiesel Board 2012; Piedmont Biofuels 2012). This variation in composition means that significant use of B100 may impact air quality, beneficially or negatively, depending on feedstock. The exact manner in which atmospheric processes affect neat biodiesel (B100) exhaust, and how the outcome compares with conventional diesel exhaust is not known.

While direct emissions are important for estimating the immediate impacts on air quality, as well as for specific exposure scenarios, such emissions will undergo atmospheric processing that begins as soon as the emissions exit the tailpipe. At present, the expected impact on air pollution resulting from a major market replacement of PCD with BD (B100) has not been determined. The largest impediment to this determination remains to be whether the expected contributions of BD can be expected to be less or more harmful than those from PCD. Attempts have been made at speciation of the VOCs in `typical' BD, and application of reactivity metrics and modeling studies has suggested that biodiesel should be beneficial to controlling, or have little impact on, ozone (NREL, Morris et al. 2003). Time-series concentration data from studies of photochemical aging of biodiesel and comparable fuels is, however, sorely needed. Photochemically-aged atmospheres containing BD have not been well tested experimentally, limiting the ability of researchers to compare their modeled outputs to measurement data. Moreover, there are no reliable methods to predict the impact of photochemical aging

on the remaining factors associated with the behavior of biodiesel exhaust in the ambient environment – such as the oxidation of primary PM and the generation of SOA.

Studies to determine the toxicity of BD have used chemical measurements alone (with no direct exposure), blended fuels (such as B20), or have used a different engine for each fuel type, with each engine operating under a different duty cycle (Bunger, Krahl et al. 2000; Bunger, Muller et al. 2000; Peng, Lan et al. 2006; Swanson, Madden et al. 2007; National Biodiesel Board 2010; Piedmont Biofuels 2010; National Biodiesel Board 2012; Piedmont Biofuels 2012). To this point, no consensus has been reached in the literature about whether BD emissions can be expected to have more or fewer harmful biological effects than PCD cycle (Bunger, Krahl et al. 2000; Bunger, Muller et al. 2000; Finch, Hobbs et al. 2002; Peng, Lan et al. 2006; Swanson, Madden et al. 2007; National Biodiesel Board 2010; Piedmont Biofuels 2010; National Biodiesel Board 2012; Piedmont Biofuels 2012).

Previous experiments performed in the UNC Gillings Innovation Laboratory Chamber have shown that photochemical aging in an urban-like atmosphere can impact the toxicity of mixtures of pollutants, including PCD emissions (de Bruijne, Ebersviller et al. 2009; Lichtveld, Ebersviller et al. 2011). Due to the processes involved in atmospheric aging, the compositional differences between PCD and BD may have large consequences for human health. The photochemical oxidation of unsaturated compounds in particular (including unsaturated aldehydes and ketones) are responsible for contributing to ambient levels of ozone, producing secondary air toxics, and modifying primary particulate

matter (Kamens, Jeffries et al. 1981; Atkinson 2000; Kamens and Jaoui 2001; Hu and Kamens 2007). Most biological exposure facilities do not have access to methods capable of creating aged atmospheres similar to those encountered in the ambient environment. What this means for their exposures is that they may have missed the contribution of toxic species from the reactive atmosphere itself. As a result of these omissions, the exposures performed with such systems may not be providing an accurate estimate of the risk posed to the population in general.

We hypothesize that replacing PCD with B100 BD will significantly alter both the atmospheric chemistry and biological effects of a vehicle's emissions. We will investigate our hypothesis by examining the differences between fresh BD and PCD, as well as determining how fuel type influences the photochemical oxidation of vehicle emissions in both a pristine environment, and in a typically-reactive urban-like atmosphere.

Methods

The methods and procedures used in this Part 3 study were essentially identical to those reported in Chapter 3, Parts 1 and 2. Thus, only brief summaries of these will be given here.

Smog Chamber

The experiments described here were performed in the Gillings Innovation Laboratory 120 m^3 chamber on the roof of the four-story School of Global Public Health at UNC, the same as used in the Parts 1 and 2 studies (Fig. 30). The chamber is above all surrounding buildings and receives direct sunshine on its tilted Teflon film faces. The chemical and biological analysis laboratories are located directly below the roof-top

chamber on the fourth floor. Pollutants to be studied (VOCs, PM, etc.) can be injected

into the chamber through its floor. Chamber contents are monitored continuously via

sample lines that pass through the floor of the chamber, the roof of the building, and

into the laboratory below. These same sample lines provide chamber air to the biologi-

cal effects exposure systems that are maintained in an incubator with proper conditions

to support living human-derived lung cells. For analytes that may be lost in long transfer

lines, samples are collected directly below the chamber on the roof of the building.
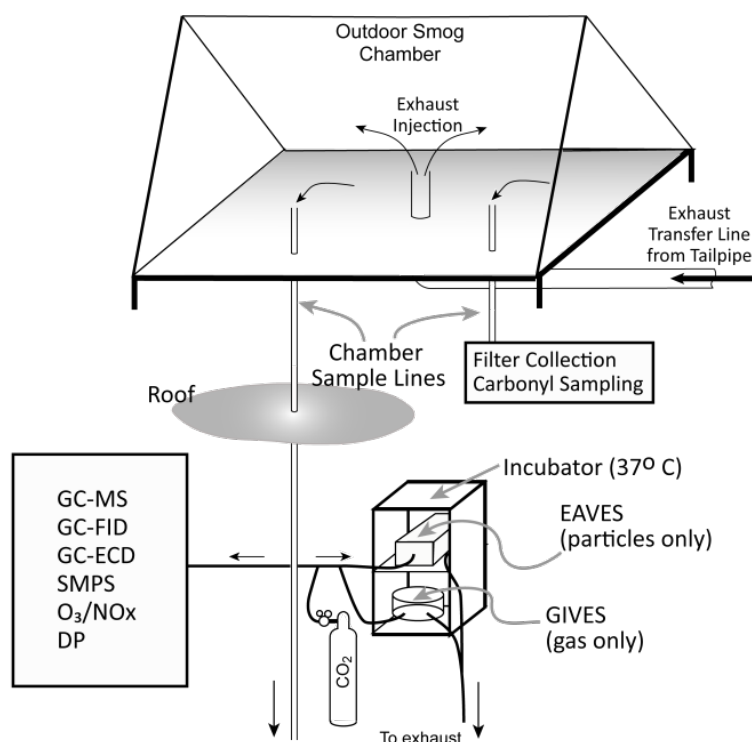
Figure 30: Chamber schematic. Sample lines pass through the floor of the chamber, the roof, and then directly into the lab below. Filter and carbonyl-specific sampling are performed on the roof of the building to minimize loss to the sample lines.

Diesel Engine Vehicle, Engine Conditioning, and Aging Atmospheres

PCD and BD exhaust were collected from the tailpipe of a single-motor 1991 Ford-

350 Diesel pick-up truck, with a 7.3 L engine and equipped with dual fuel tanks. The ve-

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hicle was operated with either commercially-available petrochemical diesel fuel (PCD) or 100% (B100) ASTM grade biodiesel produced from waste vegetable oil (WVO-BD or just BD - Piedmont Biofuels, Pittsboro, NC).

The engine is usually operated using B100 WVO-BD, so the experiments using BD were performed first. Following the completion of the BD experiments, all BD was siphoned from one fuel tank and replaced with low-sulfur PCD from a retail gas station. The vehicle was then operated using PCD fuel until two entire tanks of PCD were run through the engine. The fuel tank was filled a third time with PCD, and the PCD experiments were performed. The vehicle was warmed up under normal idling conditions. Exhaust was transferred from the vehicle's tailpipe into the chamber with a venturi-driven dilution blower as described before (de Bruijne, Ebersviller et al. 2009; Lichtveld, Ebersviller et al. 2011). Exhaust was added to the chamber with the engine running at high-idle conditions (2700 rpm). Rather than attempt to exactly match the particle mass concentration injected into the chamber (or another metric that might be dependent on the type of fuel used), PCD and WVO-BD exhaust were injected into the chamber for an equal time increment (2 min, 15 s).

To determine whether the aging environment in the atmosphere will alter the chemical and particle behaviors, and biological effects, of vehicle emissions, diesel exhaust (DE) was tested in a `pristine' atmosphere as well as a `typically-reactive' urban-like atmosphere. The pristine atmosphere was created as described in the Part 1 and Part 2 studies. To reduce ambiguity between experiments in the three-part study, the

urban-like atmosphere from the Part 2 study was used again in this study (SynUrb54;

Chapter 3, Part 2).

Due to the high NO_x output of the diesel engine with both types of fuel, no addi-

tional NO_x was added to the chamber with the VOC mixture for SynUrb54.

Gas Phase Compositional Analysis

Chamber Characterization by Gas Chromatography

The dilution rate of the chamber was monitored continuously by a Varian 3800 GC

with an electron capture detector (ECD). A Varian 3800 GC with Saturn 2200 mass spec-

trometer (MS) was used to analyze condensed-phase samples (such as function-specific

or filter samples). See Chapter 3, Part 1 for more details on components and operating

conditions for these systems.

Species-specific Analysis of Carbonyls

Selective analysis for carbonyl-containing compounds was performed with o-

(2,3,4,5,6-pentafluoro-benzyl) hydroxylamine chloride (PFBHA) as described before

(Chapter 3, Part 1). In this study, mixtures of gases and particles were sampled with

modified mister apparatuses similar to those described by Seaman e*t.al.* (Seaman,

Charles et al. 2006). Briefly, 'mister' apparatuses pull chamber air through a nozzle that

creates a mist from the PFBHA derivatization solution. The droplets in the mist are large

enough that they either drop directly back into the liquid reservoir that feeds the nozzle

or impact on the walls of the misting chamber and then return to the reservoir. The

high surface area of the mist droplets allows for very efficient collection of water-

soluble species, such as carbonyl compounds. Mister samples were collected at ca. 15

Lpm for 15-20 min. All post-collection processing was completed as described before (Chapter 3, Part 1).

In this study, as in Part 2, we made no attempt to separate the gases and particles prior to sampling in the mister apparatuses. The result is a measure of the overall carbonyl composition of both phases together as a single measurement. We performed all carbonyl sampling directly below the chamber (on the roof of the building) to minimize loss of polar compounds to the walls of the sample lines.

Inorganic Gas Phase Analysis/Chamber Condition Monitoring

Ozone (O_3) and nitrogen oxides (NO_x) concentrations in the chamber were monitored continuously (ML9811 Ozone Photometer and ML9841 NO_x Oxides of Nitrogen Analyzer, Monitor Labs, Englewood, CO), as were chamber temperature and dew point (DP). CO was monitored with a Thermo Environmental Instruments Model 48 (Franklin, MA). The dew point was measured with an EdgeTech Model Dewprime I Tech, Marlborough, MA). Solar radiation was monitored with a Total Solar Radiation Broadband Radiometer Model 8-48, and a Total UV Broadband Radiometer Model TUVR (Eppley Laboratories, Inc, Newport, RI). Temperature was monitored in the chamber with a temperature transducer sensor (Analog Devices, Inc, Part Number AD590JH). All chamber data was 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. CO was calibrated using a NIST standard CO tank.

Particle Phase Analysis

Scanning Mobility Particle Sizer

During all experiments, the size distribution and mass concentration of the aerosols in the test atmospheres were monitored with a Scanning Mobility Particle Sizer (SMPS; TSI Inc., St. Paul, MN). All particle-phase analyses were performed as described in the Part 1 study (Chapter 3, Part 1).

Filter Collection and Analysis

Total suspended particulate filter samples were taken on 47 mm Teflon membrane filters (Pall Corp., East Hills, NY) using mass-flow controllers (Aalborg Instruments & Controls, Inc., Orangeburg, NY). Filter mass collection was measured with a Mettler Toledo MX5 microbalance (Mettler-Toledo, Inc., Columbus, Ohio). The balance was calibrated using standard weights and found to have accuracy of ± 0.9 µg. All TSP mass concentrations reported here are from filter measurements.

Biological Effects Model and Measurements of Response

Cultured human epithelial lung cells, type A549, were used as the biological receptor model in this study. A549 human respiratory cells are a commercially available cell line that has been shown to react in a manner that is representative of the human airway *in vivo* (Lieber, Smith et al. 1976; ATCC 2011).The A549 cells were cultured and prepared for exposure as explained previously (Jaspers, Flescher et al. 1997; Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004; de Bruijne, Ebersviller et al. 2009).

The Gas In Vitro Exposure System and Electrostatic Aerosol in Vitro Exposure System (GIVES and EAVES) have also been described in detail previously (Jaspers, Flescher et al. 1997; Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004; Doyle, Sexton et al.

2007; de Bruijne, Ebersviller et al. 2009). Briefly, these systems allow the cellular toxicity of the gaseous and particle phase components of the same air pollution mixture to be evaluated independently of one another, and with no substantive modification to the sample prior to exposure. This allows cells to encounter pollutants as they might in the ambient environment. These exposure systems are not attempts to exactly model a human deep lung. They are models intended to be biosensors--a means by which to further characterize the distribution of species among the gas and particle phases and to indicate which systems warrant further evaluation for harmful biological effects. This is measured by gauging cellular responses to treatment air exposures relative to clean air exposures. Gas-only exposures in GIVES lasted 3 hours in all experiments. PM-only exposures in EAVES lasted 1 hour. Post-exposure treatment and processing were performed as described in Chapter 3, Part 1.

Biological Effects Endpoints (Inflammation)

All exposed cells were allowed to express for 9 hours post-exposure in an incubator in clean air. Total RNA was then isolated from the cells using Trizol (Invitrogen, Carlsbad, CA) and stored at -40°C until analysis. In this study, the inflammatory response of exposed cells was quantified by real-time, reverse-transcriptase polymerase chain reaction (RT-PCR) for Interleukin 8 (IL-8) expression, as described previously (Jaspers, Zhang et al. 2001). Cellular responses were normalized against a 'house-keeping gene' (one that does not react to the stimuli being tested here, in this case β-actin), and are reported as the fold-increase over clean air control (sham) exposure.

Tables of response ratios accompany bar charts of endpoint measurements. Response ratios were calculated by dividing the cellular response to the exposure listed in the column heading of the table by the response to the exposure listed in the first column. Further description of these calculations can be found in Chapter 3, Part 1.

Statistical Analysis

All calibration curves were tested by fitting a linear least squares line through the data. Calibration factors were calculated for each species to be analyzed, with appropriate propagation of uncertainty. All instrument measurements are reported as the mean ± 95% confidence interval, unless otherwise stated. The statistical analysis of toxicity endpoints were the same as those used in the Part 1 study.

Experimental Design

In this Part 3 study, we simulate the complexity of the ambient atmosphere by performing photochemical aging of PM-containing emissions from a real-world source, both in a `pristine' atmosphere and with the same complex urban-like atmosphere used in the Part 2 study. This inclusion of a real-world source reintroduces the full complexity and balance of the types of atmospheric processes present in the ambient environment. This research will attempt to minimize ambiguity in our results by 1) completely substituting BD for PCD by using B100 BD, 2) using one engine for both fuel types, with the engine under a matched load, 3) using a diesel-engine vehicle with real-world, on-road engine wear, 4) testing both fresh and photochemically-aged emissions, and 5) separating the direct effects of the fuel type used from the influence of its aging environment

by testing emissions in both a `pristine' and `typical' urban-like background of pollutants (SynUrb54).

The experiments described here were performed over the course of two weeks to minimize variations in engine performance due to aging and wear, as well as seasonal variability in the intensity of the sunlight and duration of the day (refer to Chapter 3, Part 2).

While testing exhaust prior to aging allows us to directly compare BD emissions to PCD before any aging occurs, oxidizing the hydrocarbons in situ allows us to include minor and transient products that are not available commercially – thereby providing a more-accurate representation of an aged air parcel in the ambient environment (Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004; Doyle, Sexton et al. 2007; Ebersviller, Lichtveld et al. 2012). Thus, we tested our hypothesis by examining the differences between `Fresh' BD and PCD, as well as determining how fuel type influences the photochemical oxidation of vehicle emissions in both a `pristine' and typically-reactive urban-like atmosphere. After the test atmospheres were established in the chamber, and prior to sunrise, human lung cells were exposed to the `Fresh' chamber contents with EAVES (for 1 hr) and GIVES (for 3 hrs). Chamber contents were then allowed to photochemically age from sun-up to sundown, and the exposures were repeated with new cell culture inserts after sunset (`Aged' condition). The result was eight sets of exposure conditions: PCD only, PCD with SynUrb54, BD only, and BD with SynUrb54 – each with both Fresh and Aged exposures (Tables 2 and 3).

Results

Chamber Conditions

When fuelled with WVO-BD, we observed that the diesel engine we tested produced significantly less particulate matter (PM), carbon monoxide (CO), and formaldehyde than when the same engine was operated with PCD (Table 16 and Fig. 31). The concentration of formaldehyde reported in Table 16 and Fig. 31 are from the Dasgupta instrument (described above) rather than PFBHA analysis. The relatively high uncertainty in the NO and NO_x measurements reported in Table 16 and Fig. 31 represent day-to-day variability in the engine output. Single-day concentrations (with corresponding uncertainty) are reported in Tables 17 and 18. For the sake of continuity, for the rest of this manuscript, analysis of chamber composition will be reported in general terms (Fresh, Midday, and Aged) rather than by absolute time.
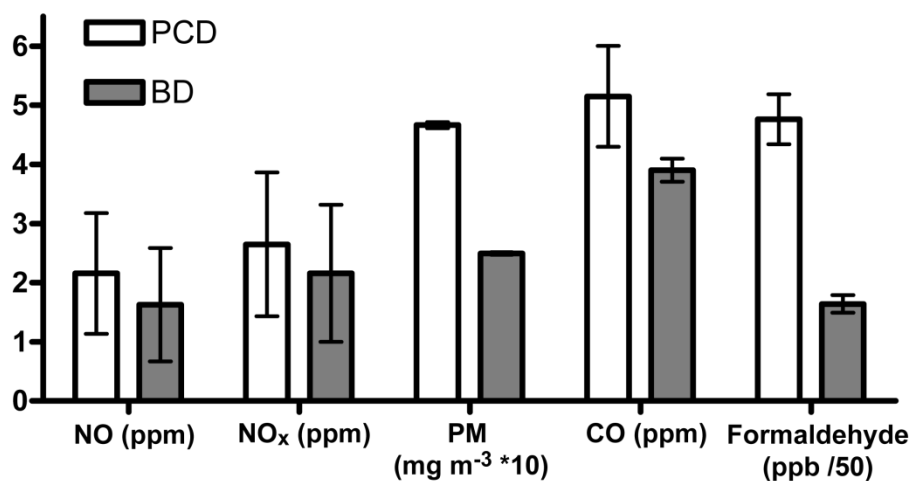

Figure 31: Observed concentrations of select pollutants from diesel emissions when fuelled with PCD and WVO-BD. Numeric values of measurements (and 95% CI) reported in Table 16.

Table 17: Observed concentrations of select pollutants from diesel emissions when fuelled with PCD and WVO-BD. Numeric measurements (reported here as mean ± 95% CI) are represented graphically in Fig. 31.

Fuel Type	NO (ppm)	NO$_x$ (ppm)	PM (µg m^3)	CO (ppm)	Formaldehyde (ppb)
PCD	2.157 ± 0.191	2.648 ± 0.740	0.466 ± 0.005	5.150 ± 0.098	238.24 ± 21.16
BD	1.627 ± 1.301	2.161 ± 1.758	0.250 ± 0.002	3.900 ± 0.195	82.03 ± 7.33

The values reported in Table 17 were measured during the experiments in which emissions were photochemically-aged alone (without SynUrb54 added to the chamber). Measurements reported in Table 18 were observed in the portion of the study in which vehicle emissions were photochemically-aged in the presence of SynUrb54. All measurements are reported as mean ±95% confidence interval.

Table 18: Chamber conditions during exposure periods when emissions were tested without SynUrb54. Uncertainties reported as ± 95% CI.

Condition	O$_3$ (ppm)	NO$_x$ (ppm)	PM (µg m^{-3})	CO (ppm)
PCD Fresh	0.0001 ± 0.0001	2.2708 ± 0.5389	0.3795 ± 0.0105	5.1 ± 0.1
PCD Aged	0.0000 ± 0.0001	1.0189 ± 0.4740	0.0809 ± 0.0105	3.5 ± 0.1
BD Fresh	0.0004 ± 0.0002	1.2635 ± 0.1472	0.2305 ± 0.0043	3.8 ± 0.1
BD Aged	0.0001 ± 0.0001	0.5879 ± 0.1342	0.0337 ± 0.0052	2.6 ± 0.1

Table 19: Chamber conditions during exposure periods when emissions were tested with SynUrb54. Uncertainties reported as ± 95% CI.

Condition	O$_3$ (ppm)	NO$_x$ (ppm)	PM (µg m^{-3})	CO (ppm)
PCD Fresh with SynUrb54	0.0016 ± 0.0004	3.0258 ± 0.6917	0.5532 ± 0.0105	5.2 ± 0.1
PCD Aged with SynUrb54	0.0015 ± 0.0003	1.4425 ± 0.6464	0.1453 ± 0.0105	3.5 ± 0.1
BD Fresh with SynUrb54	0.0008 ± 0.0003	3.0579 ± 0.8231	0.2685 ± 0.0043	4.0 ± 0.1
BD Aged with SynUrb54	0.0016 ± 0.0003	1.4393 ± 0.7594	0.0412 ± 0.0043	2.7 ± 0.1

Carbonyl-Specific Compositional Analysis

Figures 32 – 35 show the carbonyl-containing species present in each mixture throughout the course of the day. Numbers labeling the peaks in Figs. 32 – 35 correspond to the numbers associated with specific species in Tables 19 – 22. For the sake of clarity, the numbering scheme for peak identifiers was integrated across all chromato-

grams and tables (i.e., 2-heptanone is peak id. number 11 in all chromatograms in which it appears, regardless of the number of identified peaks in a given chromatogram). The small peak that is present in all chromatograms, and is located just before acetaldehyde (peak id. number 2), is residual (unreacted) PFBHA reagent (UR). The large peak at ca. 30 min on each chromatogram is our internal standard (IS). No peak quantifications are reported for either the IS or UR here. In addition, no attempt was made to separate the gases and particles prior to sampling. These plots and tables, therefore, represent the species present in the full mixture of gases and particles, rather than in a single phase. It should also be reiterated here that, while the ester functions present in BD *do* contain a carbonyl group, PFBHA preferentially derivatizes aldehydes and ketones.

Figure 32: Carbonyl-specific analysis of chamber conditions during sampling periods when BD emissions were photochemically aged without SynUrb54.

Table 20: Carbonyl-specific analysis of chamber conditions during exposure periods when BD emissions were photochemically aged without SynUrb54.

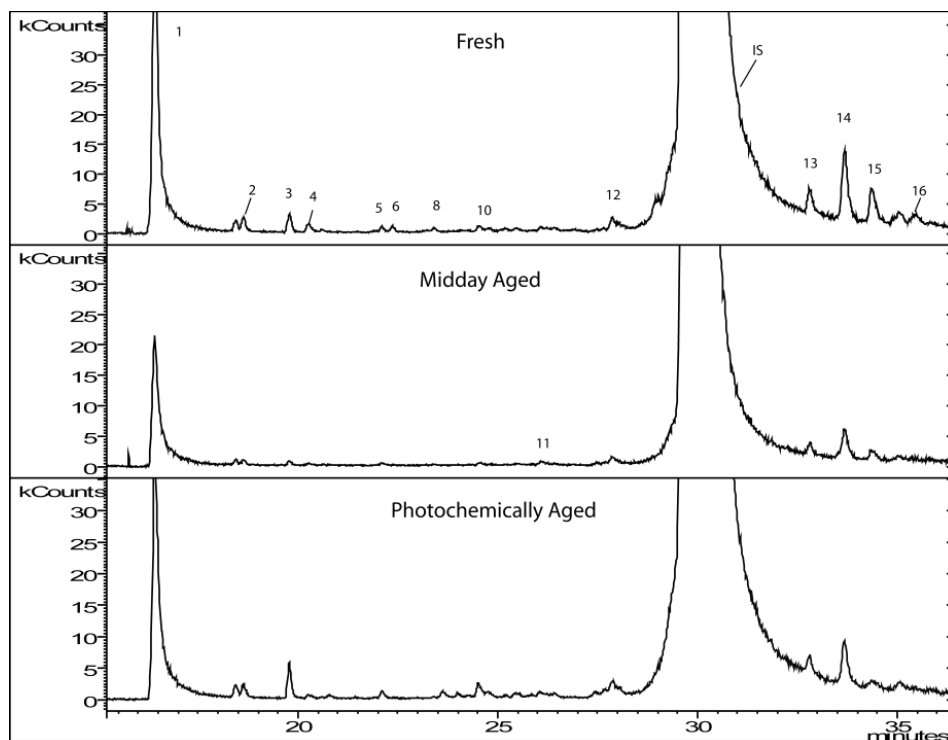| ID No.	Species	Fesh Concentration (ppbC)	Midday Concentration (ppbC)	Aged Concentration (ppmC)
1	formaldehyde	0.499	0.240	0.410
2	acetaldehyde	0.005	0.002	0.005
3	acetone	0.007	0.002	0.012
4	hydroxyacetaldehyde	0.007	0.003	0.004
6	n-butanal	0.004	0.002	0.006
8	2-pentanone	0.007	ND	0.011
10	2-hexanone	0.013	0.011	0.068
11	2-heptanone	ND	0.024	ND
12	unknown	0.034	0.021	0.048
13	unknown	0.048	0.022	0.038
14	glyoxal	0.103	0.042	0.057
15	methylglyoxal	0.056	0.017	0.017
16	biacetyl	0.024	0.005	0.012

ND is Not Detected

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Figure 33: Carbonyl-specific analysis of chamber conditions during sampling periods when BD emissions were photochemically aged with SynUrb54

Table 21: Carbonyl-specific analysis of chamber conditions during exposure periods when BD emissions were photochemically aged with SynUrb54.

ID No.	Species	Fresh Concentration. (ppbC)	Midday Concentration (ppbC)	Aged Concentration (ppmC)
1	formaldehyde	0.510	0.939	0.335
2	acetaldehyde	0.014	0.017	0.007
3	acetone	0.006	0.014	0.005
4	hydroxyacetaldehyde	0.006	0.009	ND
5	unknown	0.013	0.148	0.083
6	n-butanal	ND	0.004	0.004
7	unknown	ND	0.021	0.006
8	2-pentanone	0.007	ND	0.011
9	pentanal	0.024	1.030	0.283
10	2-hexanone	ND	0.189	0.032
11	2-heptanone	ND	0.084	ND
12	unknown	0.027	0.128	ND
13	unknown	0.025	0.050	0.018
14	glyoxal	0.053	0.443	0.036
15	methylglyoxal	0.018	0.070	ND
16	biacetyl	0.010	0.025	0.009

ND is Not Detected

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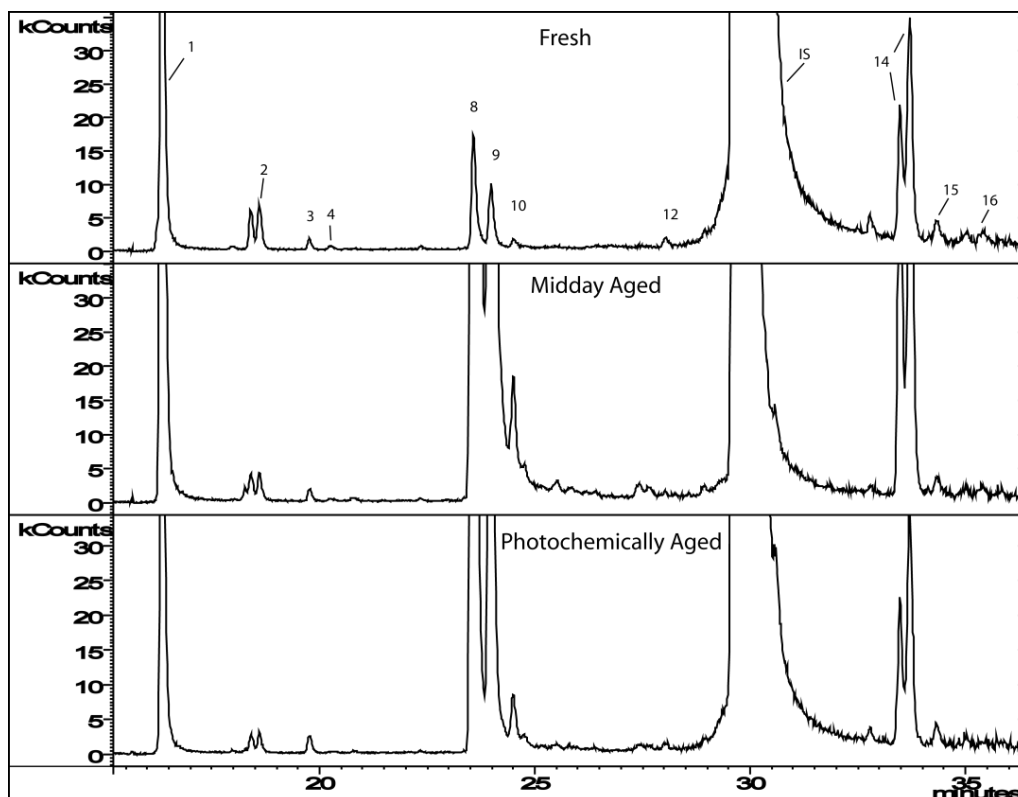
Figure 34: Carbonyl-specific analysis of chamber conditions during sampling periods when PCD emissions were photochemically aged without SynUrb54.

Table 22: Carbonyl-specific analysis of chamber conditions during exposure periods when PCD emissions were photochemically aged without SynUrb54.

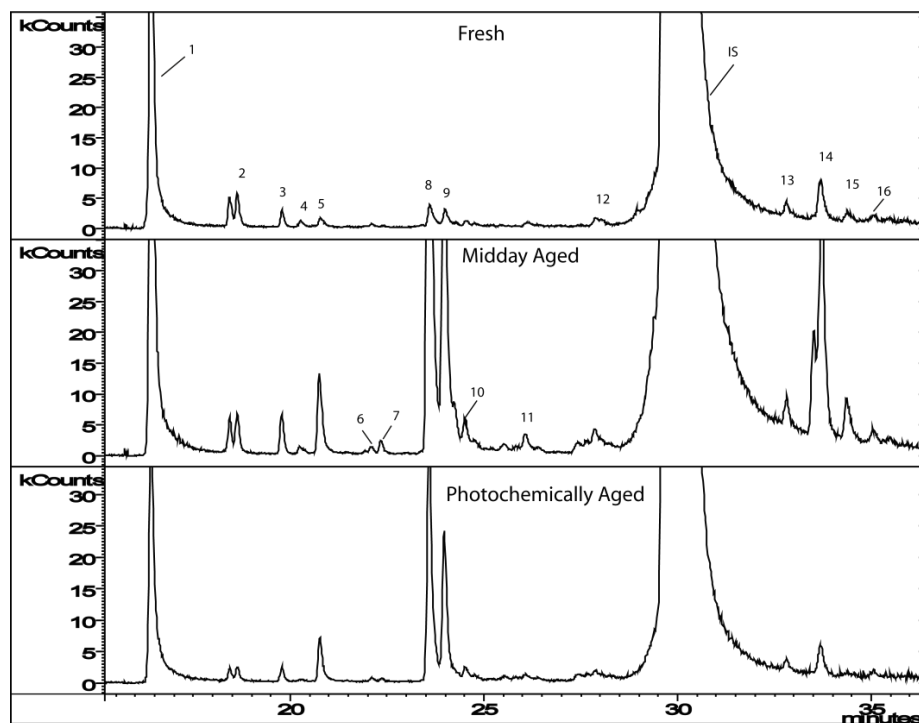| ID No.	Species	Fresh Concentration. (ppbC)	Midday Concentration (ppbC)	Aged Concentration (ppmC)
1	formaldehyde	0.413	1.086	0.504
2	acetaldehyde	0.015	0.010	0.008
3	acetone	0.004	0.004	0.006
4	hydroxyacetaldehyde	0.004	0.003	ND
5	unknown	0.013	0.148	0.083
8	2-pentanone	0.198	9.929	3.014
9	pentanal	0.109	5.428	1.882
10	2-hexanone	0.025	0.592	0.309
12	unknown	0.030	0.046	0.021
14	glyoxal	0.375	1.107	0.354
15	methylglyoxal	0.03	0.024	0.025
16	biacetyl	0.026	0.018	0.016

ND is Not Detected

162
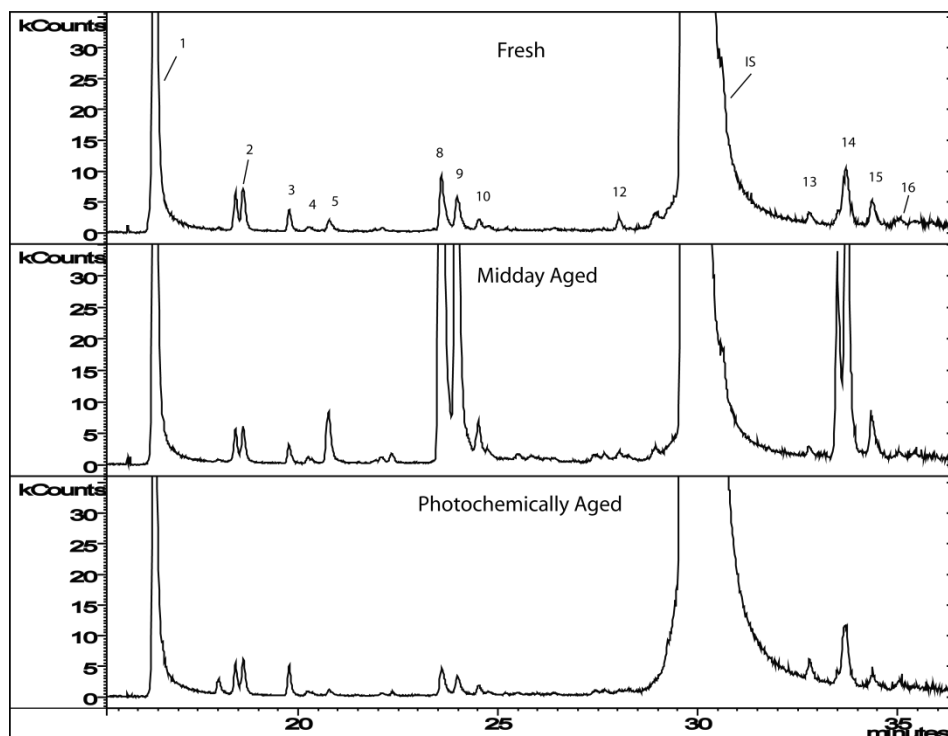
Figure 35: Carbonyl-specific analysis of chamber conditions during sampling periods when PCD emissions were photochemically aged with SynUrb54.

Table 23: Carbonyl-specific analysis of chamber conditions during exposure periods when PCD emissions were photochemically aged with SynUrb54.

ID No.	Species	Fresh Concentration. (ppbC)	Midday Concentration (ppbC)	Aged Concentration (ppmC)
1	formaldehyde	0.679	0.836	0.527
2	acetaldehyde	0.018	0.016	0.017
3	acetone	0.008	0.007	0.011
4	hydroxyacetaldehyde	0.004	0.008	0.006
5	unknown	0.018	0.098	0.083
8	2-pentanone	0.113	1.828	0.059
9	pentanal	0.070	1.069	0.044
10	2-hexanone	0.053	0.151	0.034
12	unknown	0.020	0.023	0.017
14	glyoxal	0.098	0.607	0.099
15	methylglyoxal	0.042	0.062	0.020
16	biacetyl	0.016	0.069	0.020

In the Fresh condition, the carbonyl-containing species present in each mixture are largely the same regardless of fuel type (Tables 19 – 22 and Figs. 32 – 35). The primary difference between these mixtures was not in the identities of the species present, but in their relative concentrations. In particular, 2-pentanone, pentanal, and 2-hexanone had higher concentrations in the PCD mixtures (Figs. 34 and 35). In addition, the PCD emissions without SynUrb54 had a much higher concentration of glyoxal than any of the other mixtures (peak number 14 in Fig. 34 and Table 21). Conversely BD, when it was by itself in the chamber, had the lowest measured concentration of acetaldehyde at the beginning of the day (peak number 3 in Fig. 32 and Table 19).

By midday, however, the chemical composition of the aging BD mixture that contained SynUrb54 appeared to have been altered significantly from that of BD alone (Figs. 32 and 33). By contrast, the midday compositions of PCD and PCD with SynUrb54 are very similar, with the exception of the magnitude of certain species (Figs. 34 and 35). Notably, the composition of the BD mixture with SynUrb54 was also very similar to the two PCD-containing mixtures. In particular, the large increase in concentration for 2-pentanone, pentanal, 2-hexanone, glyoxal, and methylglyoxal (peak id. numbers 8-10, 14, and 15) aligns the composition of BD with SynUrb54 to both PCD mixtures (Figs. 33 - 35), while these species are all but absent from the aging BD alone (Fig. 32).

By Sunset, very little appears to have changed with the BD by itself, indicating a slowly-reacting system. The BD mixture with SynUrb54, however, has clearly evolved in

composition throughout the day and still bears more resemblance to the PCD mixtures than BD by itself (Tables 19 – 22 and Figs. 32 – 35).

Particle Phase Characterization

Figure 36 contains a set of four particle size distribution plots characterizing the Fresh conditions in the chamber (before/concurrent with sunrise). All mass concentrations reported in this study were obtained from TSP filter measurements.

We observed rapid growth of the post-nucleation mode in all systems in which it appeared (Fig. 36a-c). In general, the engine produced a fairly uniform number concentration of PM regardless of the fuel being tested (Fig. 36). The larger diameter of the PM produced by PCD meant that when the engine was fuelled with PCD it produced nearly twice the particle mass concentration than was observed from BD (Tables 16 - 18, Fig. 36). In addition, it appears that the mode diameter of the PCD PM grows more quickly in the dark than PM from BD (the mode diameter of PCD grew by an average of 112 nm, BD grew by 61 nm).

After preliminary examination, the presence of SynUrb54 appeared to have an effect on the observed growth rate (before sunrise) for both types of DE PM tested (Fig. 36). Upon closer inspection, however, the measured change in mode particle diameters between injection and sunrise were mixed. The mode particle diameter of BD PM grew less with the presence of SynUrb54 (BD with SynUrb54 increased by 43 nm, BD alone by 80 nm); while PCD PM was relatively unchanged (PCD PM actually grew slightly less than when it was mixed with SynUrb54, but the difference was only 10 nm).
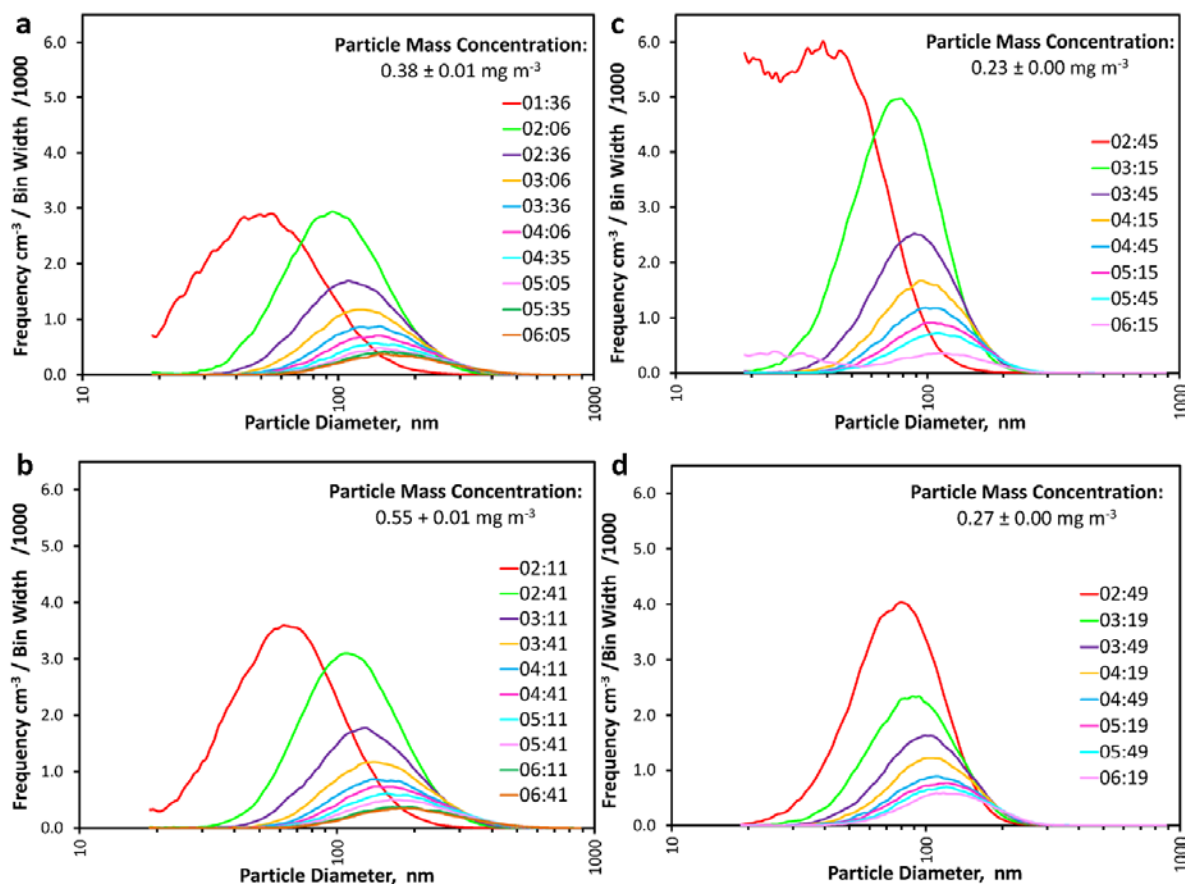
Figure 36: Particle size distribution plots for diesel emissions at the beginning of the day. The emissions in the chamber were a) PCD alone, b) PCD with SynUrb54, c) BD alone, and d) BD with SynUrb54. Mass concentration measurements on each plot match the corresponding measurements reported in Tables 17 and 18, and were obtained from filter samples. Series in each plot are named for the time at which they were measured (EDT, Chapel Hill, NC).

Figure 37 displays midday, averaged distribution of particle diameters for all four experimental systems (distributions were averaged over a period of ca. 2 hrs). By midday, we observed an increase in the mode diameter for particles in all of our experimental atmospheres (Fig. 37). In general, by midday, the diesel exhaust PM had fallen into relatively narrow, single-modal distributions. The notable departure from this trend was BD when it was tested by itself, for which we observed a bimodal distribution (Fig. 37). In Fig. 36c (BD alone), PM in the post-nucleation range was just beginning to reap-

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pear at sunrise (0615), with the bimodal distribution divided equally. The post-

nucleation mode then increased in relative concentration during the day (compared to

the total particle number concentration in the test atmosphere), until it was the domi-

nant mode by midday (Fig. 37). While this is noteworthy, it does not appear that having

such a prevalent post-nucleation mode caused BD PM to form more SOA than the other

mixtures, as BD had the lowest particle mass concentration of any of our test atmos-

pheres (Figs. 36 – 38). No substantial return of particles in this size range was observed

for any of the other mixtures we tested (Fig. 37).
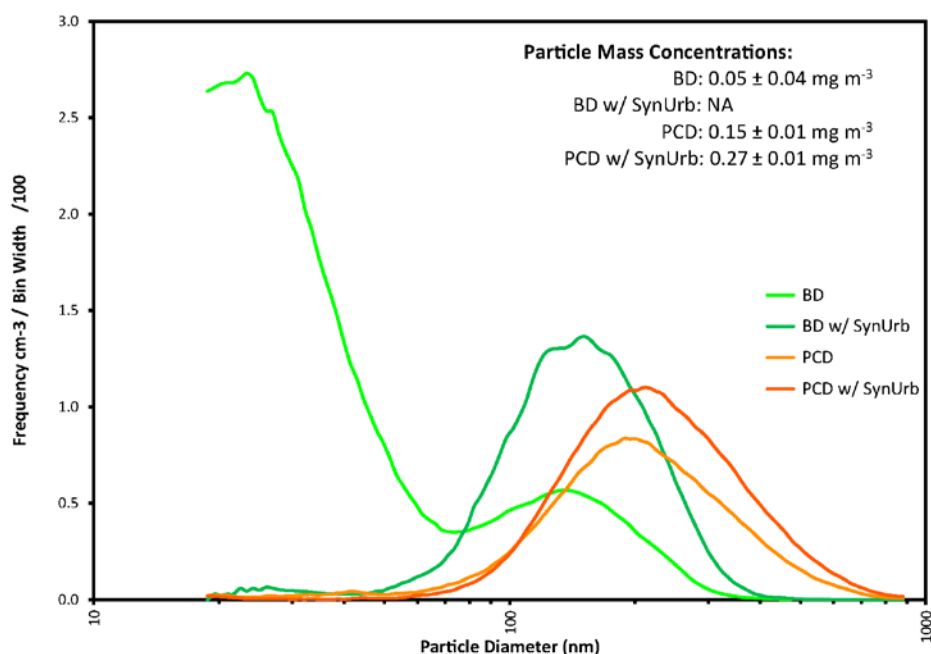
Figure 37: Averaged particle size distributions for diesel emissions at midday. Series are labeled by experiment and represent the distribution averaged over ca. 2 hrs. Mass concentration measurements on each plot match the corresponding measurements reported in Tables 17 and 18, and were obtained from filter samples (NA = Not Available).
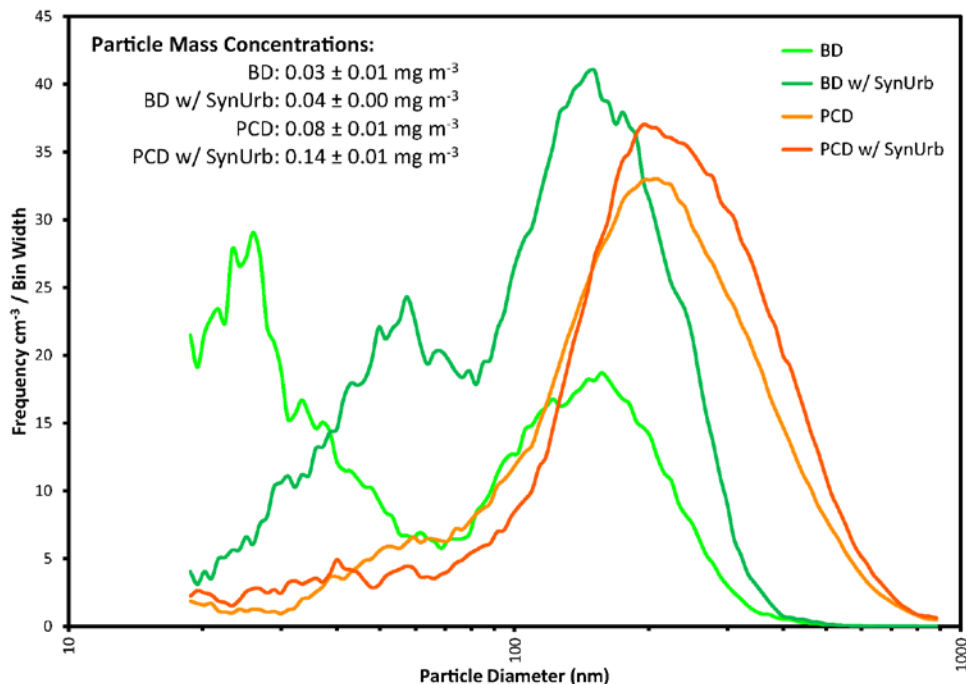
Figure 38: Averaged particle size distributions for diesel emissions at the end of the day. Series are labeled by experiment and represent the distribution averaged over ca. 2 hrs. Mass concentration measurements on each plot match the corresponding measurements reported in Tables 17 and 18, and were obtained from filter samples.

By the end of the day, there was no significant difference among the test mixtures with regards to the growth of particle diameter as a result of photochemical aging. The presence of an urban-like atmosphere appears to have had a slightly greater effect on the size of the PCD PM than BD PM. From sunrise to sunset, the mode particle diameter of PCD increased by 45 nm, PCD with SynUrb54 by 21 nm, BD by 32 nm, and BD with SynUrb54 by 24 nm (Figs. 36 - 38). As observed throughout the day, PCD's relatively larger particle diameters resulted in higher observed particle mass concentrations for the PCD mixtures (Fig. 37).

These distribution plots and mass concentrations have not been corrected for dilution and wall loss. Therefore, they should be taken as a qualitative analysis of SOA

formation/particle growth rather than exact quantitative measurements. Discussion follows.

Toxicological Response

Figure 39 is the legend for the plots of cellular responses (Figs. 40 and 41). Our measurements of IL-8 expression can be found in Fig. 40 for exhaust alone, and Fig. 41 for exhaust mixed with SynUrb54. The relative response ratios for cells exposed with the gas-only and particle-only methods can be found in Table 23.

When cells were exposed to Fresh PCD emissions, they expressed significantly more inflammatory response than the cells that were exposed to Clean Air (Sham; Fig. 40a and Table 23). The observed response to the particle-only exposure, however, was not different than was measured following exposure to Clean Air (Fig. 40b and Table 23). When the PCD emissions were photochemically aged, we observed a large increase in the inflammatory response induced by the gaseous components of the mixture, with a complimentary (though smaller) increase in the response elicited by the Aged particle phase (Fig. 40 and Table 23). We have previously observed these types of modifications to the biological response from our in vitro exposures as a result of aging, both in gas-only systems and with vehicle emissions (Doyle, Sexton et al. 2004; Sexton, Jeffries et al. 2004; Doyle, Sexton et al. 2007; Lichtveld, Ebersviller et al. 2011). Moreover, we observed a nearly identical result when we tested the effects of photochemistry on SynUrb54 alone (Chapter 3, Part 2).

When we photochemically aged PCD with SynUrb54 in the chamber, we observed no difference in the increase in response from the gas-only exposure than was meas-

ured from the aging of PCD by itself (Fig. 41a; Table 23). When we examined the response measured from cells exposed with the PM-only biological exposure system to the Aged PCD with SynUrb54 we observed 2.5 times the response measured from cells exposed to PCD particles that were aged without SynUrb54 (Figs. 41b and 40b; Table 23).

In contrast, the response to the gaseous components of BD exhaust without SynUrb54 in the chamber remained unchanged with aging. Neither the Fresh nor Aged gaseous components elicited a significant response from cells (Fig. 40a, Table 23). Exposure of cells to fresh BD with the particle-only method caused significantly more expression of IL-8 than either the sham or the fresh PCD particles (Fig. 40b; Table 23). When BD was aged in the chamber, a significant *decrease* in response was observed from cells exposed with the particle-only method (a reduction of more than 50%; Fig. 40b and Table 23).

When Fresh BD was mixed with SynUrb54, the gas-only exposure caused 4 times more inflammation than was measured from the control. Unlike our observations of the behavior of PCD, the response measured from the gas-only exposure decreased by 37% with aging (Fig. 41a and Table 23). In addition, when SynUrb54 was included in the chamber, the inflammatory response induced by exposure to fresh BD particulate was not significantly greater than the sham exposure. When BD exhaust particles were aged with SynUrb54, however, the response they elicited increased by approximately 270% (Fig. 41b; Table 23).
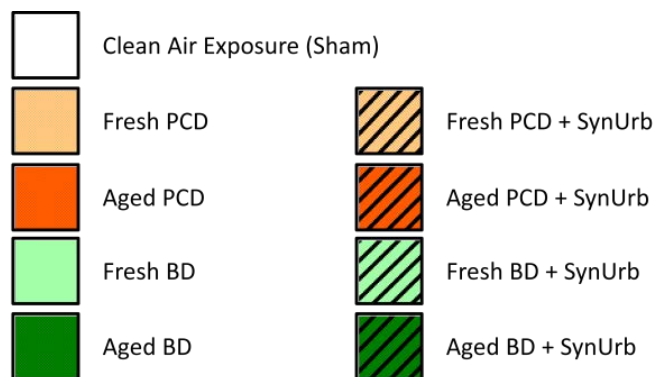
Clean Air Exposure (Sham)

Fresh PCD | Fresh PCD + SynUrb

Aged PCD | Aged PCD + SynUrb

Fresh BD | Fresh BD + SynUrb

Aged BD | Aged BD + SynUrb

* Indicates a statistically significant difference in response relative to Clean Air
\# Indicates a statistically significant difference in response between Fresh and
   Aged exposures with the same fuel type and SynUrb
δ Indicates a statistically significant difference in response between Fresh
   exposures of different fuel types
φ Indicates a statistically significant difference in response between Aged
   exposures of different fuel types

Figure 39: Key for toxicology response plots found in Figs. 40 and 41.
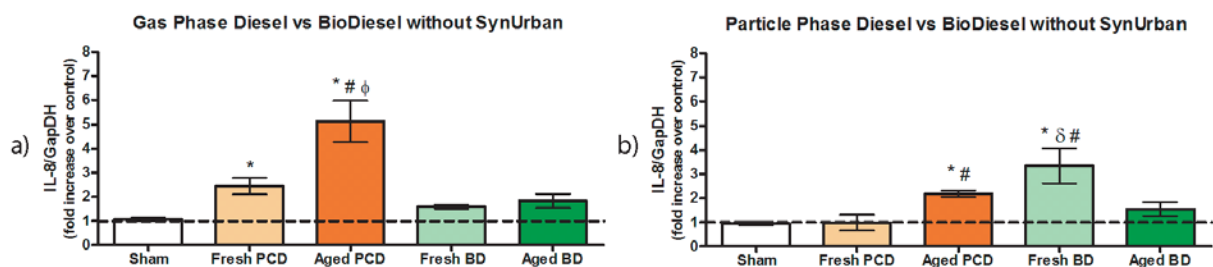
Figure 40: IL-8 inflammatory response of lung cells following exposure to diesel emission mixtures photochemically aged without SynUrb54. The response induced by exposure to a) the gaseous components of each mixture and b) the particle-phase components of each mixture
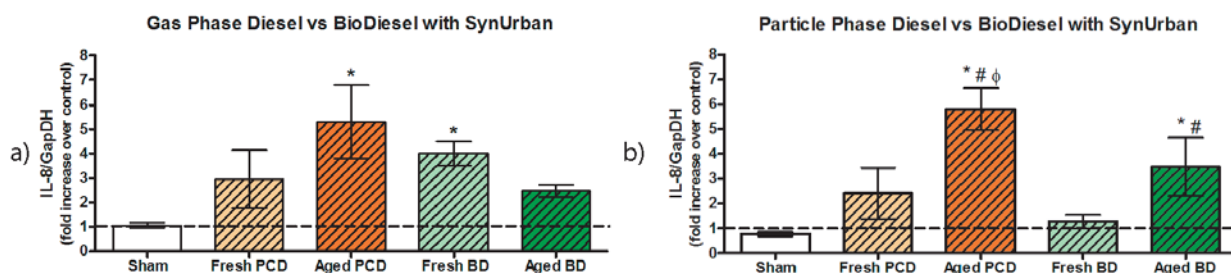
Figure 41: IL-8 inflammatory response of lung cells following exposure to diesel emission mixtures photochemically aged with SynUrb54. The response induced by exposure to a) the gaseous components of each mixture and b) the particle-phase components of each mixture.

Table 24: Tabular summary of the ratios of measured inflammatory responses (IL-8) for exposures to PCD and BD with and without SynUrb54 (see Figs. 40 and 41)

| | | Gas-Only Exposure | | | | | | | |
| | | | Without SynUrb54 | | | With SynUrb54 | | | |
	Condition[a]	Sham	Fresh PCD[b]	Aged PCD[b]	Fresh BD[c]	Aged BD[c]	Fresh PCD[b]	Aged PCD[b]	Fresh BD[c]	Aged BD[c]
Without SynUrb	Sham	1.00	2.27	4.77	1.49	1.71	2.79	5.00	3.78	2.33
	Fresh PCD		1.00	2.10	0.65	0.75	1.21	2.17	1.64	1.01
	Aged PCD			1.00	0.31	0.36	0.58	1.03	0.78	0.48
	Fresh BD				1.00	1.15	1.85	3.32	2.51	1.55
	Aged BD					1.00	1.61	2.89	2.18	1.34
With SynUrb	Fresh PCD						1.00	1.79	1.36	0.84
	Aged PCD							1.00	0.76	0.47
	Fresh BD								1.00	0.62
	Aged BD									1.00

| | | Particle-Only Exposure | | | | | | | |
| | | | Without SynUrb | | | With SynUrb | | | |
	Condition[a]	Sham	Fresh PCD[b]	Aged PCD[b]	Fresh BD[c]	Aged BD[c]	Fresh PCD[b]	Aged PCD[b]	Fresh BD[c]	Aged BD[c]
Without SynUrb	Sham	1.00	1.04	2.31	3.54	1.64	3.09	7.42	1.64	4.46
	Fresh PCD		1.00	2.21	3.40	1.58	2.44	5.86	1.29	3.52
	Aged PCD			1.00	1.53	0.71	1.10	2.65	0.58	1.59
	Fresh BD				1.00	0.46	0.72	1.73	0.38	1.04
	Aged BD					1.00	1.55	3.72	0.82	2.24
With SynUrb	Fresh PCD						1.00	5.86	1.29	3.52
	Aged PCD							1.00	0.58	1.59
	Fresh BD								1.00	2.72
	Aged BD									1.00

[a] The exposure atmosphere in the chamber

[b] Petrochemical Diesel

[c] Biodiesel

Discussion

This discussion starts with a comparison of the Fresh vehicle emissions to evaluate both the composition of the engine's exhaust when operated with each type of fuel and the initial chamber exposure conditions.

Next, we examine the effects of the atmospheric aging environment (`pristine' vs. urban-like) on the composition of the gas and particle phases in each aging environment.

Then, we examine the biological effects associated with each of our eight chamber conditions, and attempt to make causal assignments for observed effects to the extent that our data permits us.

Finally, we discuss ambiguity in our results and the extent to which they limit our ability to draw concrete conclusions from our data.

Comparison of Vehicle Emissions

To evaluate the effect of fuel type on engine exhaust composition, Fresh emissions from the vehicle were compared when it was operated with PCD and BD. We observed that the engine produced significantly more CO, PM, and formaldehyde when it was fuelled with PCD (Fig. 31, Table 16). Biodiesel's effects on NO_x emissions, however, are not so definitive. While the mean observed value for both NO and NO_x were lower in Fresh BD exhaust than they were in Fresh PCD exhaust, large day-to-day variability in engine output caused significant overlap in individual observations (Fig. 31, Tables 16 – 18). Based on our measurements of formaldehyde and NO_x in these test mixtures (Fig.

31, Tables 16 – 18), it is likely that photochemical reactivity will be significantly reduced when WVO-BD replaces PCD (discussed further below).

In general, the diesel engine produced a fairly uniform number concentration of PM regardless of the fuel being tested (Fig. 36). The larger diameter of the PM produced by PCD, however, meant that when the engine was fuelled with PCD, it produced a near-ly twice the particle mass concentration than was observed from BD (Fig. 36 and Tables 16 – 18). Even though the nucleation mode was much more prevalent in BD exhaust, the mode diameter of the PCD PM grew slightly more in the dark than PM from BD (the mode diameter of PCD grew by an average of 84 nm before sunrise, BD grew by 52 nm).

Physical Effects of the Aging Environment (Pristine vs. Urban)
As reported above, we observed that BD emissions, when aged in a `pristine' envi-ronment, retained a secondary particle mode in the nucleation range for much of the day (Figs. 36 - 38). In general, there is concern about the manner in which the nucleation mode might act as `seed' particles for SOA formation. The behavior of the particle phase in the BD photo-reactive system with SynUrb54 provides some indication of whether the concern about SOA formation is warranted. While we did observe an increase in the number concentration throughout the day, relative to BD alone, the mode particle di-ameter did not change appreciably. More significantly, the mass concentration meas-ured at the end of the day in the PCD mixture (in which no nucleation mode was appar-ent) was twice that observed in the BD mixture with SynUrb54. Further, if we compare the two SynUrb54 mixtures, the final mass concentration in the PCD mixture with

SynUrb54 was 3.5 times higher than BD with SynUrb54 (PCD: 0.1453 mg m^{-3} vs.

BD: 0.0412 mg m^{-3}, Fig. 38).

SynUrb54 appears to have had little effect on the oxidized species present for the

PCD mixtures during photochemically aging. The identified species in the two PCD mix-

tures were identical, but had different concentrations at different points in the day

(Figs. 34 and 35; Tables 21 and 22). It does appear, however, that the presence of

SynUrb54 may have increased the rate of the photochemical processing of the PCD mix-

ture. To illustrate, the chromatogram depicting the carbonyl content of the photochem-

ically-aged PCD mixture is very similar to the midday sample for the PCD mixture with

SynUrb54, though the quantified concentrations are not equal (Figs. 34 and 35;

Tables 21 and 22).

By contrast, the presence of SynUrb54 appears to have significantly altered the

chemical composition of the photochemically-aging BD mixture compared to BD alone

(Figs. 32 and 33; Tables 19 and 20). If the aging of BD alone is compared to both of the

aging of the mixtures containing SynUrb54 (Figs. 32, 33, and 35; Tables 19, 20, and 22),

it is clear that SynUrb54 is driving the composition of the BD mixture with SynUrb54 by

midday. In fact, the chemical composition of the aging and aged BD mixture with

SynUrb54 bears more resemblance to the PCD mixtures than BD by itself (Tables 19 – 22

and Figs. 32 – 35). In particular, the large increase in concentration for 2-pentanone,

pentanal, 2-hexanone, glyoxal, and methylglyoxal (peak id. numbers s 8-10, 14, and 15)

aligns the speciation of BD with SynUrb54 to both PCD mixtures (Figs. 33 – 36), while these species are all but absent from the aging BD alone (Fig. 32).

By Sunset, photochemical aging appears to have had little effect on the BD by itself, indicating a slowly-reacting system (Fig. 32), which agrees with our discussion of the NO_x and formaldehyde content of the exhaust (above). The chemical composition of the BD mixture with SynUrb54, however, has clearly evolved throughout the day, and still bears more resemblance to the PCD mixtures than BD by itself (Figs. 32 – 35; Tables 19 – 22). Even with these similarities, the chemical composition of the Aged BD mixture with SynUrb54 mixture is significantly different than both PCD and PCD with SynUrb54, illustrating the importance of the whole atmosphere to the products formed.

Biological Effects of the Aging Environment and "Effect Modification"

One approach in toxicology and risk estimates is to assume `additive' effects (meaning that the individual contribution of each species in a mixture adds up to the total biological effect of that mixture). The additive approach ignores co-pollutant effects (such as synergy and antagonism), but is often used as a `first estimate' approach. As demonstrated previously, when SynUrb54 gases are photochemically aged (without particles present), there is a strong increase in the toxic response to the gases after irradiation (Sexton, Jeffries et al. 2004; Ebersviller, Lichtveld et al. 2012). In fact, it is unprecedented in our research not to see *some* increase in inflammatory response when SynUrb54 is photochemically aged. With this in mind, we expected to see a sizeable increase in the response induced by the gaseous components of the Aged DE exposures with SynUrb54 present (relative to each DE alone).

When we photochemically aged PCD with SynUrb54 in the chamber, however, we observed *no* difference in the increase in response from the gas-only exposure than was measured from the aging of PCD by itself (Fig. 40a; Table 23). Given the similarities between the chemical compositions of the PCD and PCD with SynUrb54 mixtures (discussed above), this lack of increase in the gas-only response may seem intuitive. From a toxicological viewpoint, however, we would expect to observe *some* increase in response simply due to the higher concentration of VOCs overall. When we examined the response measured from cells exposed with the PM-only biological exposure system to the Aged PCD with SynUrb54 we observed 2.5 times the response measured from cells exposed to PCD particles that were aged without SynUrb54 (Figs. 40b and 41b; Table 23). This much greater increase in the response to the particle phase when PCD was aged with SynUrb54 resulted in a net increase in observed response relative to the PCD when it was aged alone.

In contrast, when cells were exposed to BD exhaust without SynUrb54, neither the fresh nor photochemically-aged gaseous components elicited a significant response from cells (Fig. 39a, Table 23). Exposure of cells to fresh BD with the particle-only method caused significantly more expression of IL-8 than either the sham or the fresh PCD particles (Fig. 39b; Table 23). When BD was aged by itself in the chamber, a significant *decrease* in response was observed from cells exposed with the particle-only method (a reduction of more than 50%; Fig. 39b and Table 23). The net decrease in response observed from BD when it was aged without SynUrb54 is without precedent in the aged and aging atmospheres we have tested.

Even with the unusual aging behavior observed for the BD emissions in a pristine atmosphere, we expected addition of the urban-like background of VOCs to alter the photochemical behavior of the BD emissions to more closely resemble PCD with SynUrb54. What we mean by this is, we expected to observe no real change in the toxicity of the Fresh mixture, but see a sizeable increase in the gas-phase toxicity with the Aged mixture with SynUrb54 present (with an accompanying increase in the response from the PM-only exposure). Instead, we observed a significant increase in the toxicity of the Fresh gaseous components of the mixture and a decrease in biological effect response for the particle-phase (Fig. 40). It is not clear which interactions between the BD exhaust and SynUrb54 caused the modification of the response relative to the BD exhaust alone, but given the similarities between the chemical analyses of Fresh BD with SynUrb54 and both Fresh PCD mixtures (Figs. 33 – 35), the similarities in their biological effects is not surprising.

What was surprising, however, was the behavior of the BD exhaust when it was aged with SynUrb54 in the chamber. We observed a decrease in the response to the gas-only exposure (relative to the Fresh BD exposure with SynUrb54 – Fig. 40a; Table 23). While the observed decrease in response initially made us question the success of the experiment, we made an interesting observation when we examined the effect caused by exposure to the particle phase. Contrary to the behavior of BD exhaust when it was aged by itself (Fig. 39), we observed an *increase* in the toxicity of the PM as a result of aging.

In addition, there was never any measureable amount of excess O_3 formed in any of these systems. While this observation may seem trivial, it has important ramifications to the way air pollutants are regulated. In almost all of these systems, we observed significant modifications to biological effects from air pollution mixtures that had no measurable amount of one of the primary Criteria Pollutants used as a surrogate for the amount of reactivity occurring in a system. We understand, of course, that the lack of detectable excess O_3 does not mean that O_3 was not formed at any time during the aging process. We would not, however, have been able to predict the behavior of these systems based on past chemical speciation experience alone. Therefore, without the integrated physical characterization measurements and biological effects monitors we may have misestimated the relative toxicities of the chamber conditions.

It should be reiterated that when SynUrb54 is aged by itself, we observe a significant increase in response from the gas-only exposure to the Aged system, but no response from particle-only exposures (when there are not particles present; demonstrated in Chapter 3, Part 2). The lack of increase in the response with aging from cells exposed using the particle-only method is due mainly to the lack of SOA nucleation in the system without the presence of seed particles. The lack of SOA formation with photochemical aging of SynUrb54 is somewhat counter-intuitive, because ca. 27% of the carbon in SynUrb54 is aromatic (Bloss, Wagner et al. 2005; Hu and Kamens 2007). We began to hypothesize that, in the BD mixture with SynUrb54, the DE particles in the mixture might be providing a sink for potentially-toxic species.

For aerosol scientists and atmospheric chemists, the concept of species moving between phases is not a new one (as discussed above). We observed a slight increase in the mode particle diameters for both fuel exhaust types tested when the urban-like atmosphere was added to the chamber (Fig. 36). It is possible, therefore, that toxic species in the Fresh emissions were taken up by the particles during aging, and simple partitioning between the phases could explain the apparent shift in toxicity between the phases. This assumption, however, completely ignores the evolution and consumption of species that occurs with atmospheric aging. Moreover, while it is possible that the observed increase in particle diameter resulted from SVOCs present in SynUrb54 partitioning to Fresh DE (Fig. 36), the increase was very small (ca. 10 nm). As such, without better understanding of the day-to-day variation in the vehicle's emissions we could not determine without ambiguity that VOCs from SynUrb54 were the cause of the increase.

Another possible explanation of the observed 'shift' is that the gaseous components of the Fresh system responsible for the observed inherent toxicity were rendered biologically inert with aging, and that the PM, conversely, acquired toxicity with aging. There are many iterations of these (and other) processes that might result in the outcomes observed in this study. Therefore, we could not, with our current set of observations, determine unambiguously what was happening in these aging DE systems.

Discussion of Ambiguity

Any conclusions we might draw from these data were complicated by the question of whether DE particles possessed some characteristic that imparted an inherent toxicity to them. In addition, recent evidence has been found for the presence of species

in PM that were previously thought too volatile for particle uptake (Li, Hooper et al. 2000; Volkamer, San Martini et al. 2007; Lim, Tan et al. 2010; Williams, Goldstein et al. 2010; Parikh, Carlton et al. 2011). We already had, therefore, conceptual explanations for our observations. What we required was clear proof to indicate which of the explanations was most feasible. We began to hypothesize that an important component of PM toxicity might be species expected to be in the gas phase that had partitioned to the condensed phase, resulting in the observed shift in toxicity between the phases. Partitioning is a dynamic process, meaning that species causing toxicity in the Fresh mixture can react in the gas-phase prior to partitioning to the condensed phase (and vice versa). Conceptualizing the system to include these exchanges between the phases accommodates what we know about gas-phase photochemical kinetics as well as current partitioning theory. These photochemical aging and gas-particle partitioning phenomena were those that we attempted to capture in the first two studies in this series (Chapter 3, Parts 1 and 2).

While it may be assumed that there were less-complicated ways to investigate the phenomena of interest, neither chemical nor biological measurements alone can provide all of the information necessary to characterize such complex systems. Chemical measurements alone cannot say absolutely whether a measured species is found primarily in the gas phase, the condensed phase, or distributed between the two. Moreover, traditionally, single species of interest (or simple mixtures of species) are tested for their biological effects. Despite extensive work to build databases of carbonyl-containing compounds, however, many WVO biodiesel species (in both Fresh and Aged

conditions) could not be identified using our own chemical libraries, or those obtained from NIST. This lack of positive identification makes it impossible to test individual components of the BD exhaust, even if they are commercially available. Therefore, creating Aged test atmospheres *in situ* was necessary to properly evaluate the biological effects resulting from exposure to PCD and BD exhaust.

While this is Part 3 of our three-manuscripts-set describing our integrated, stepwise research program, the experiments presented here were actually the first performed from the series. The research presented in Parts 1 and 2 of the series was developed to investigate (and attempt to explain) observations made during this (Part 3) work.

This Part 3 study represents another large increase in complexity relative to the mixtures in the first two studies (Chapter 3, Parts 1 and 2). Not only was PM present during photochemical aging (with its associated on-particle aging processes), we incorporated PM from a real-world source while it was operated with two different types of real-world fuels. While we acknowledge that the full complexity of ambient urban mixtures exceeds what we were able to include in this chamber study, we were able to create test atmospheres that have enough types of atmospheric aging processes present to be representative of the processes that occur in the ambient environment.

We are not asserting that these studies, or studies like it, supersede the need for environmental chemical speciation data or traditional toxicological approaches. We are simply saying that the approaches employed here can provide extra information to help

fully characterize the distribution of species across the phases, as well as give indications

of their relative importance to toxicity.

Finally, if either the chemical contributions of, or photochemical processes from,

the atmosphere had been excluded from these studies, our conclusions would have

been very different. Therefore, the integration of chemical, particle, and toxicological

measurements made with the tools employed in this three-part research program pro-

vided us with the evidence needed to prove unambiguously that PM "effect modifica-

tion" as described by the NRC happens, and cannot be ignored.

Conclusions

These studies have important ramifications across the air pollution field. The Na-

tional Academy of Sciences in its 2004, ``Research Priorities for Airborne Particulate

Matter'' specifically stated that a finding like the one we demonstrate in these papers

`would have implications for setting [National Ambient Air Quality Standards] NAAQS

independently for the various criteria pollutants' (NRC 2004). The NAS refers to this

phenomenon as PM `effect modification'.  We have clearly demonstrated such `effect

modification,' and provide clear proof that the atmosphere itself is a source of these

`modifications' that can quickly and dramatically modify primary PM, thereby drastically

transforming its character. Thus, one source of gases and another source of PM can lead

to an entirely different secondary PM that exhibits effects not seen from either the gas

exposure or the source PM exposure when they are alone. Therefore, vehicle emissions

(and other PM) that may not be exceptionally toxic as they enter the atmosphere, when

mixed into an aged or aging air mass, can change dramatically in composition and bio-

logical effect by the time they drift off-road and reach the general population. These rapid changes in composition and effect further illustrates the importance of including the atmosphere itself as a source of toxicity in risk assessment and policy approaches to air pollution.

By extension, studies of the effects of air pollutants that omit a photochemical aging component can provide invaluable information for specific exposure scenarios, but they may not accurately represent exposures as they happen to the population in general. Moreover, without an understanding of how the environment interacts with materials being studied, researchers cannot ensure that estimates of risk and effect are accurate measures of real-world circumstances. Investigating single pollutants found in Aged air masses (such as O_3) or single point-sources (such as a vehicle's exhaust) can be illustrative of risk, and at other times may be necessary to elucidate the manner in which atmospheric interactions occur (as in the single-VOC experiments presented in the Part 1 study; Chapter 3, Part 1). With singling out specific sources or pollutants in the search for a 'smoking gun,' however, comes the risk of shifting the focus of the research community away from their overall goals of understanding the environment and protecting human health. Therefore, such simplified systems should not be assumed to be completely representative of, or as a replacement for, pollutant mixtures formed *in situ*.

It is important to reiterate that, if either the chemical contributions or photochemical processes from the atmosphere had been excluded from this study, our conclusions would have been very different. The integration of chemical, particle, and toxi-

cological measurements made with the tools employed in this overall research study

have provided us with the evidence needed to prove unambiguously that "effect modifi-

cation" as described by the NRC happens, and cannot be ignored.

1. Previously published as:

Ebersviller, S.; Lichtveld, K.; Sexton, K. G.; Zavala, J.; and Jeffries, H. E. (2012) 'Gaseous VOCs Rapidly Modify Particulate Matter and Its Biological Effects: Part 1, Simple VOCs and Model PM', Atmospheric Chemistry and Physics. (in press)

2. Previously published as:

Ebersviller, S.; Lichtveld, K.; Sexton, K. G.; Zavala, J.; and Jeffries, H. E. (2012) 'Gaseous VOCs Rapidly Modify Particulate Matter and Its Biological Effects: Part 2, Complex Urban VOCs and Model PM, Atmospheric Chemistry and Physics. (Submitted, journal is currently soliciting reviewers)

3. Previously published as:

Ebersviller, S.; Lichtveld, K.; Sexton, K. G.; Zavala, J.; Lin, Y-H.; Woodside, R.; and Jeffries, H. E. (2012) 'Gaseous VOCs Rapidly Modify Particulate Matter and Its Biological Effects: Part 3, Petroleum Diesel and Biodiesel Oxidations', Atmospheric Chemistry and Physics. (Reformatted to fit journal style, text under revision)

Not all of the co-authors of the Part 3 manuscript have had time to review the current draft. The manuscript will very likely be revised in several cycles before it is submitted by the end of February 2012

Chapter 4
Overall Conclusions, Limitations, Implications, and Future Work

Conclusion to the dissertation document

Conclusions

Additional Findings

Hypothesis

Limitations

Implications

Future Work

Chapter 4:
Overall Conclusions, Limitations, Implications, and Future Work

Conclusions

To be able to demonstrate 'effect modification' unambiguously, we first had to establish that our chamber and exposure systems would not, by themselves, induce any biological effects. While I was not able to perform a 'Clean Air' exposure prior to each experiment, several Clean Air test atmospheres were created in the chamber throughout this dissertation work. To illustrate, in Chapter 3, Part 2 the clean air exposures were performed in very different seasons (April and August) and a year apart. Since no response was measured from either control exposure atmosphere, any chance that our observations were influenced by some unknown, seasonally-variable, inherent quality of the chamber itself can be discounted. Therefore, with the minimal criteria described in Chapter 3, Part 1, I was successful in creating stable 'clean' atmospheres in our outdoor chamber that could be used as a baseline ('zero effect') exposure.

Also in Chapter 3, even though the Clean Air exposures with MOA had consistently higher PM concentrations than any of the VOC mixtures with MOA, neither of the *in vitro* systems registered an increase in response from exposure. This demonstrates that MOA itself is not inherently toxic, and does not impart toxicity to the gas-phase. This is a major point that I needed to establish, and it appears that non-toxic properties of MOA are invariant over a wide range of PM mass concentrations. Moreover, any affect

that variations in RH might have had on the cells was minimal, so the likelihood of RH variations causing the increase in response observed from any exposures is negligible.

Implicit in these observations is that the response observed during the Clean Air exposures includes any effects that might have been elicited by some sampling artifact of the in vitro exposure systems. The absence of biological effect in any of the GIVES Clean Air exposures illustrates that there are no biological effects elicited by exposure with the GIVES. In addition, the lack of particle-phase response from the mixtures of Clean Air and MOA reinforces the findings of Chapter 2, in which we determined that there are no biologically-relevant artifacts caused by exposure with the EAVES device (for the endpoints used in these studies).

To test 'effect modification,' a series of increasingly-complex test atmospheres had to be created in the chamber. In Chapter 3, Part 1, mixtures of single VOCs and MOA created the simplest condition in which 'effect modification' can be demonstrated. The very limited number of atmospheric processes that were present in the (dark) test atmospheres provided an unambiguous demonstration that 'effect modification' occurs for these simple systems. The step-wise increase in complexity in the gas phase, while keeping the non-toxic PM from Part 1, allowed me to make direct comparisons between the observations of Parts 1 and 2. In addition, by excluding MOA from the photo-reactive systems I was able to ensure that it did not evolve any toxicity of its own exclu-sive of the gaseous toxicants. While explicit determination of the atmospheric process-es responsible for 'effect modification' was not possible in Part 3, 'effect modification'

was evident. This was especially true in the case of the BD emissions with and without the urban-like 'background' atmosphere (SynUrb54).

There was some variability observed in the Aged exposure conditions in Chapter 3, Part 2. 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. Regardless of the composition of the gaseous toxicants, however, in Part 2 the non-toxic PM became significantly toxic when it was mixed with the gas-phase pollutants. This is enough to demonstrate that 'effect modification' occurs.

While I purposefully selected a PM material that should not facilitate/catalyze chemical reactions between absorbed species, condensed-phase reactions are made possible by the simple expedient of bringing moieties in close proximity to one another. The question of condensed-phase chemistry cannot, therefore, be discounted for the biologically-inert PM material used in Chapter 3 (MOA). As discussed in Chapter 3, however, the acute inflammatory and cellular damage responses observed indicate that – whatever form the species in the condensed phase might take - they elicit a toxic re-

sponse. The possibility of condensed-phase chemistry in any of the systems tested here do not, therefore, hinder my ability to conclude that the non-toxic PM material has been made toxic by interactions with the gas-phase.

Throughout the exposures described in this work, dilution of the chamber contents occurred as a result of removing chamber air during sampling. While this intrusion of ambient air should be considered when interpreting the results of these studies, the lack of response from the Clean Air exposures (with and without MOA), as well as the absence of response from the particle-only exposure for the Aged condition in which no MOA was added (JN2509, Chapter 3, Part 2) indicates that any intrusion of gases or PM from the ambient surroundings is unlikely to have significantly altered the observed biological effects caused by exposure to chamber air.

I would like to explicitly state that I am not attempting to assert that VOCs-partitioning-to-PM is the only process important to determining the overall toxicity of PM in the ambient atmosphere. Nor am I asserting that PM uptake of toxic gases is the only atmospheric process by which gases and particles might impart toxicity on one another. For instance, there is extensive and growing evidence that PM, and condensed-phase chemistry in general, can have a significant influence on the composition (and, by extension, the toxicity) of the gas phase. This dependence on the particle phase was also illustrated in this dissertation work by the differences between the BD and PCD systems that included SynUrb54. My intention was not to imply that I am demonstrating the *only* atmospheric process that is important in the interactions of gases and PM, I

have simply placed focus on one atmospheric process in particular that has been largely ignored by the toxicology and risk assessment communities due to a lack of evidence of its importance in the literature.

As stated several times throughout this dissertation, I was not trying to directly model the atmosphere. So the question of "atmospherically relevance," while important to consider, does not negate the impact of the findings reported here. Current chemical measurement technologies are incapable of absolutely determining the distribution of volatile species between the phases in ambient mixtures. This dissertation is, therefore, intended as a demonstration of atmospheric processes that should be included in policy and risk assessment decisions, but are difficult to distinguish in real-world atmospheric mixtures. I believe these processes do occur in the atmosphere, and now that insights have been provided by this preliminary work we can (as a community) begin to focus on creating new technologies that are able to observe these interactions in the ambient atmosphere. Until now, there was no identified need for technologies that can address the sampling artifacts that hinder the observation of these processes in the ambient environment, particularly for species as volatile as acrolein.

Concurrent with the development of these new measurement technologies, test atmospheres for exposure assessments that more-closely mimic actual atmospheric conditions can be created and, in doing so, determine the 'relevancy' and relative contributions of the processes discussed here with regard to the toxicity of ambient PM.

Finally, the findings here have significant ramifications across the air pollution research and policy communities. I concede that no experimental data set is perfect, including mine. This does not, however, invalidate the conclusion that 'effect modification' does occur, and is likely to have a significant influence on the toxicity of the condensed phase in the ambient environment.

Additional findings

The following are findings and observations made during the course of this dissertation work that, while interesting and potentially important, did not directly pertain to the dissertation hypothesis.

Whenever I have described the BD vs. PCD study, invariably my audience asks "Which is better, biodiesel or traditional diesel?" Given the variability of evidence in the literature, and my own experience with other diesel vehicles (i.e., the Mercedes, Passat, and Beetle); I conclude that inter-vehicle variability requires that I qualify my answer to that question with *'for this vehicle.'* Therefore, *for this vehicle*, and in terms of primary pollutants, BD appears to be 'better' than PCD. Also, with regard to the toxicity of the emissions, in every instance but one PCD emissions were significantly more toxic than BD emissions (for the endpoints I tested). The addition of 'the atmosphere' (in the form of SynUrb) appears to diminish this difference.

I observed biological effects without making excess O_3. As I have before, I want to qualify this statement with "that does not mean that there was never O_3 present in the system, just that there was never excess O_3 that the meter was able to quantify." This

has important ramifications for the manner in which air pollution is regulated and monitored by the US EPA. In general, people use the measured concentration of O_3 in the atmosphere as a surrogate estimate for how harmful human exposure can be anticipated to be. As we never made any measureable excess O_3 in the chamber, but still saw significant biological effects, it indicates that O_3 is not sufficient as a surrogate estimate for risk associated with air pollution exposure.

If I had not had access to the in vitro exposure systems used in this study, my conclusions would have been very different. While I was able to detect acrolein in the particle phase in Part 1 of Chapter 3, I was only able to detect components of mineral oil in the filter samples from Part 2 (I was not able to detect any oxidized daughter products from the reaction of SynUrb54). If my conclusions had been based on those measurements alone, I would have most likely concluded that the uptake we observed in Part 1 was solely due to the high concentration of acrolein in the chamber, or was a contamination issue. In addition, based on chemical measurements alone, I would not have been able to predict the behavior of the DE systems with regard to their biological effects. Given the similarities between the chemical speciation data, I would have concluded that we could anticipate the biological effects of exposure to BD with SynUrb to be very similar to PCD, when that was not the case (Chapter 3, Part 3).

Being able to generate oxidized species *in situ* so that they could be included in the chemical speciation and *in vitro* exposures was equally important to the conclusions I drew from this dissertation work. As I demonstrated throughout these studies; the chemical, particle, and biological effect characteristics of the Fresh and Aged mixtures

were very different. Moreover, there were significant differences in the behaviors of the

DE reaction systems when they were tested with and without SynUrb. Therefore, if the

aging processes, contributions of the atmosphere as a source of toxic species (through

the inclusion of SynUrb during aging), or integrated toxicology data had been excluded

from this dissertation, my conclusions would have been very different. The composition

of PM appears to have a significant impact on the behavior of the aging environment, as

demonstrated by the significant differences between the aging and aged compositions

of BD with SynUrb and PCD with SynUrb.

Conceptual Framework

To remind the reader of the conceptual model I used to frame my experiments, I

have repeated it here.

Table 25: Conceptual Gas and Particle States, Processes, and Properties in the Atmosphere

State	Rate of Change	Processes	Toxicity
Fresh	None	• Only dilution	• Inherent
Thermally Transforming	Low Low Energy System	• Particle uptake and off-gassing • On-particle reactions • Depend on composition of both phases	• Transformed Inherent • Acquired • Redistributed
Photo chemically Transforming	Fast High Energy System	• All processes from Thermally Aging • Secondary reactions: ˙OH, O_3, VOC oxidation, etc.	• Transformed Inherent • Acquired • Transformed Acquired • Redistributed
Aged	Low Low Energy System	• Thermal processes • Depend upon composition • Lingering secondary reactions from Photo-Aging	• Transformed Inherent • Acquired • Transformed Acquired • Redistributed

Evolution of State

Hypothesis

The central hypothesis, that 'effect modification' (as defined by the NRC) exists, has been clearly and unambiguously demonstrated in a series of increasingly complex gaseous VOC systems.

I tested the hypothesis (in terms of a non-toxic PM) with regard to how the volatility of the VOC in question impacts uptake (Chapter 3, Part 1). Moreover, Part 1 provided a necessary demonstration that 'effect modification' phenomena exist, in a system for which exceedingly few alternative explanations were available. The Part 1 study focused on the 'Thermally Transforming' State from the conceptual model. For the limited environmental processes available in this State, the most compelling conclusion that could be reached was one admitting only a single likely explanation: PM ``effect modification'' does occur.

In Chapter 3, Part 2, I demonstrated that 'effect modification' can occur in a complex mixture of gases. In addition, this research demonstrated that atmospheric processes can act as a source of toxic species via the creation of a large number of secondary volatile and semi-volatile species in the gas-phase. To limit the number of processes that had to be explained, we excluded from the 'Photochemically Aging' State the atmospheric aging processes that occur on-particle. MOA was retained as a inherently non-toxic model PM, and only introduced to the chamber after sunset (when the chamber contents could be thought of as 'Aged'). By excluding MOA from the photochemical aging processes in the chamber, we maintained its role as a pre-concentrator and deliv-

ery mechanism to bring gas-phase toxic species to the cells, rather than using it as a nucleation site for SOA formation. By our definition, the addition of MOA to the 'Aged' chamber contents created a 'Thermally Transforming' environment analogous to the systems tested in Chapter 3, Part 1.

In Part 3, we expanded upon the complexity of the VOCs included in the study by adding real diesel exhaust to the photochemical aging environment. Further we added primary PM as reactant during the photochemical oxidation process. By choosing two types of fuel, we were able to introduce different primary PM that had differences in inherent PM toxicity. This revealed again how a nearly-non-toxic primary PM can become a significantly toxic PM by acquiring secondary gas toxicity.   Even though 'effect modification' was demonstrated to occur in both the PCD and BD systems, we showed that the 'modifications' varied with fuel type used, and also depended upon whether or not the emissions were in an urban environment (by the inclusion of SynUrb in the chamber). Our observations in Part 3 would have been very difficult to understand and explain without the results from Parts 1 and 2.

Taken together, an overall conclusion that can be reached from this dissertation work is that 'effect modification' does exist, and is important in ordinary urban environments as well as in complex roadway and roadside environments. The second study using urban-like gases in the absence of primary PM during oxidation demonstrated that, upon introduction of a non-toxic PM, this PM became highly toxic. The source of this toxicity was gas-phase photochemical production of secondary VOCs. This demon-

stration clearly establishes the importance of considering the atmosphere itself as a

source of toxic species when performing risk assessments and policy decisions.

Limitations

It is impossible to capture *all* of the complexity of the ambient environment in a

chamber study (refer to Conceptual Model, above). While we attempted to include as

many of the different atmospheric aging processes present in the ambient environment

as possible, I fully acknowledge that our results may have been affected by missing pro-

cesses that may be introduced and/or augmented by species that were not present in

the chamber (such as emissions from biomass burning, etc.). In addition, there is litera-

ture evidence that different PM materials may alter the aging environment. I had simi-

lar findings in Part 3 of Chapter 3, in which we observed significant differences between

the behavior of PCD and BD. As such, PM materials such as ammonium sulfate, acidified

PM (to augment SOA formation), crustal PM (silica, salt, etc.), and emissions from a ve-

hicle with modern emissions controls in place may have yielded different results than

the MOA and diesel exhaust particles that were tested. The missing emissions/species,

however, do not invalidate the findings of this demonstration.

During Parts 2 and 3, in which we tested the effects chemical and toxicological

properties of the Fresh and Photochemically-Aged States, we did not perform exposures

midday (while the chamber contents are in the Photochemically-Transforming State).

Previous studies, however, have indicated that peak toxicity may occur *prior* to sunset

(Doyle, 2004; Doyle 2007). Moreover, within the research described here, peak O_3 con-

centrations were observed prior to sunset (Chapter 3, Part 2; excess O_3 was not present

in any measureable quantities in Part 3). Therefore, in the future, studies evaluating

these States and Processes should be extended to include *in vitro* exposures midday to

accompany chemical and particle composition measurements. Given that this was a

preliminary demonstration, and not an attempt to completely capture and characterize

the entirety of the ambient atmosphere, the missing *in vitro* exposures should not be

viewed as an oversight in the experimental design. This should be taken as a suggestion

of how to produce a more-complete evaluation of the entanglements between the

phases.

In vitro exposures cannot account for all interactions that occur in the human

body. While this is true, *in vitro* techniques are an accepted alternative to human expo-

sures for pollutants whose effects might be too hazardous, or unknown, to risk a human

exposure. Moreover, funding agencies have recently announced that toxicological re-

search should move away from exposures *in vivo*, because of the cost-effectiveness of *in

vitro* exposures (as well as for other reasons).

Using immortalized cell lines rather than primary cells misses inter-personal varia-

bility in response to exposure (as in susceptible subpopulations). Given the increased

cost, lack of access, long preparation time, and relatively short time window during

which primary cells are ready and still viable make their use difficult for studies such as

the biodiesel study presented in Chapter 3, Part 3.

We did not have a particle sampling method for chemical analysis that did not in-

terrupt the equilibria between PM and the species partitioned to it. We were able to

measure ACRO in the particle phase using a hybrid of traditional filter extraction methods and PFBHA analysis methods. If a chemical-analysis sampler analogous to the EAVES device can be implemented, we will have a better assessment of what cells exposed with the EAVES device are actually encountering from the particle phase.

Implications

The findings included in this dissertation work have important ramifications across the air pollution field.

What is clear from these studies is that the evolution of toxicity with photochemical aging, and the subsequent shift of that toxicity to the PM present in the mixture, is an important component to understanding the response of individuals exposed in the ambient environment. Such changes can strongly affect the dose delivered during exposure. This entanglement of species implies that the phases might not be separable without distorting their toxicities. This dynamic entanglement has largely been ignored in the research and policy communities, but its inclusion is necessary to understand a system as complex as the ambient environment. Ignoring the interdependencies of the phases' effects is likely to result in a misestimation of the toxicities of both the primary source under scrutiny, and the complete atmospheric system.

The inability of laboratory-based toxicologists to replicate effects seen in near-roadway epidemiology studies has confounded the risk assessment field and policy makers alike (Dreher, 2000; Schlesinger et al., 2006). This research program has shown that gas phase air toxics can (and are likely to) increase the toxicity of airborne particu-

late matter. Thus, one source of gases and another source of PM can lead to an entirely different secondary PM that exhibits effects not seen by either the gas exposure or the source-specific PM exposure when they are alone. The time scale for these inter-phase dynamics is on the order of seconds. What this means in the real world is that vehicle emissions (and other PM) may not be exceptionally toxic as they enter the atmosphere but, when mixed into an aged or aging air mass, can change dramatically in composition and biological effect by the time they drift off-road and reach the general population. Thus, an important consideration for the policy and risk assessment communities is the role of PM as a pre-concentrator and carrier of gas-phase toxics into regions of the lungs they may not normally reach. The National Academy of Sciences (NAS) refers to these phenomena as "effect modification" of PM.

The NAS, in its 2004 "Research Priorities for Airborne Particulate Matter," specifically stated that a finding that 'effecs modification' exists, "would have implications for setting [National Ambient Air Quality Standards] NAAQS independently for the various criteria pollutants."   We have clearly demonstrated such "effect modification," and provide clear proof that the atmosphere itself can be a source of "modifications" that quickly and dramatically alter primary PM, thereby drastically transforming its character. This indicates that the independent regulation of each source, the current cornerstone of all US air quality regulations, is likely to be ineffective in protecting the health of exposed individuals. This finding has a definite impact on the EPA's policy approach of relying on individual NAAQS verses a "mixture" approach.

Further, this work reinforces the importance of the reactive atmosphere itself as a significant source of toxicity in air pollution. This is true for both gas/particle interactions and the evolution of toxic species with photochemical atmospheric aging. While the creation of oxidized daughter products has long been investigated for its potential to produce ozone and PM, toxicological work has remained largely focused on single pollutants or primary sources of PM (Weldy et al., 2011; Tsukue et al., 2010). Based on previous findings, it is possible that undetected species and/or species present at a trace level contributed significantly to the observed response (Doyle, 2007). This work demonstrates that the dynamic shifting of toxicity from the gas phase to the PM present in the mixture is likely to be an important component to understanding the response of individuals exposed in the ambient environment.

Chemical measurements alone cannot say absolutely whether a measured species is found primarily in the gas phase, the condensed phase, or distributed between the two. When paired with biological sensors that 'see' an airstream without interrupting the equilibria between gases and particles, this differentiation begins to be possible. I do not mean to say that these measurements supersede the need for chemical speciation, simply that they can provide extra information to help fully characterize the distribution of species across the phases, as well as give indications of their relative importance to toxicity. Moreover, if either the chemical contributions or photochemical processes from the atmosphere had been excluded from this study, our conclusions would have been very different. Therefore, without the integration of chemical, particle, and toxicological measurements made with the tools employed in this overall re-

search program, I would not have had the evidence needed to prove that 'effect modifi-

cation' (as described by the NAS) happens and cannot be ignored.

Future Work

- Expand the scope of work to incorporate more types of PM

- Expand scope of atmospheres tested to incorporate regional variability in VOC and PM composition

- Develop new particle sampling method for chemical analysis that will not interrupt the equilibria between PM and the species partitioned to it (based on the EAVES platform)

- Incorporate exposures to primary cells to account for variability that exists among individuals

- Perform exposures midday to better-ascertain the toxicity of transient oxidized species that may be consumed by photochemical processes by sunset

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