SHORT-TERM INHALATION EXPOSURE IN RATS TO ASSESS CARBON PARTICLES EFFECTS ON NAPHTHALENE ABSORPTION, AND PULMONARY TOXICITY

By

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<tr>
<td>ACGIH</td>
<td>American Conference of Governmental Industrial Hygienists</td>
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<td>BALF</td>
<td>Broncho alveolar lavage fluid</td>
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<tr>
<td>BEAS-2B</td>
<td>Human bronchial epithelial cell line</td>
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<tr>
<td>CYP450</td>
<td>Cytochrome P450 enzymes</td>
</tr>
<tr>
<td>DEP</td>
<td>Diesel Exhaust Particles</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EF50</td>
<td>Mid Expiratory Flow</td>
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<tr>
<td>f</td>
<td>Respiratory Rate</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-Macrophage Colony-Stimulating Factor</td>
</tr>
<tr>
<td>GC-MS/MS</td>
<td>Gas chromatography mass spectrometry</td>
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<tr>
<td>IFNγ</td>
<td>Interferon Gamma</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin-1 Beta</td>
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<td>IL-17A</td>
<td>Interleukin-17 A</td>
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<td>IP-10</td>
<td>Interferon gamma-induced Protein 10</td>
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<tr>
<td>KC</td>
<td>Chemokine ligand 1</td>
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<tr>
<td>MSTFA</td>
<td>N-Methyl-N-(trimethylsilyl) trifluoroacetamide</td>
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<tr>
<td>MRM</td>
<td>Multiple Reaction Monitoring</td>
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<td>MV</td>
<td>Minute Ventilation</td>
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<td>NTP</td>
<td>National Toxicology Program</td>
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<td>NIOSH</td>
<td>National Institute for Occupational Safety and Health</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>OSHA</td>
<td>Occupational Safety and Health Administration</td>
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<td>PAH</td>
<td>Polycyclic Aromatic Hydrocarbons</td>
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<td>PEF</td>
<td>Peak Expiratory Flow</td>
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<td>PENH</td>
<td>Enhance Pause</td>
</tr>
<tr>
<td>PIF</td>
<td>Peak Inspiratory low</td>
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<tr>
<td>PPM</td>
<td>Parts Per Million</td>
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<tr>
<td>RANTES</td>
<td>Regulated on Activation, Normal T Cell Expressed and Secreted</td>
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<tr>
<td>SMPS</td>
<td>Scanning Mobility Particle Sizer</td>
</tr>
<tr>
<td>SVOC</td>
<td>Semi Volatile Organic Compound</td>
</tr>
<tr>
<td>TE</td>
<td>Time Expiratory</td>
</tr>
<tr>
<td>TI</td>
<td>Time Inspiratory</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming Growth Factor Beta 1</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor alpha</td>
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<td>TV</td>
<td>Tidal Volume</td>
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Real-life inhalation exposures involve combinations of semi volatile organic (SVOC) chemicals and particles suspended in air. There is almost no information available currently on how particles interact with SVOCs to change their deposition in the lungs and absorption. In this study, the aim was to determine how the presence of carbon particles in air affects the absorption and pulmonary toxicity of naphthalene in vivo. Our approach focused on the recreation of real-life inhalation exposures, organic compounds condensed into a carbonic nucleus, in an attempt to test whether short-term inhalation of combined carbon particles plus naphthalene will affect naphthalene absorption and ultimately pulmonary toxicity in rats. Rats were nose-only exposed to naphthalene vapor at exposure concentrations (5, 10, and 20 ppm) with or without carbon particles (5mg/m$^3$) for 1 h using urinary excretion of 1-naphthol as biomarker. Urine collection happened every 24 h over a 72 h period. Gas chromatography tandem mass spectrometry (GC-MS/MS) analysis was used to measure 1-naphthol concentration in urine. Over 90% of 1-naphthol excretion in urine occurred during the first 24 h in a 72
h collection period. 1-napthol urinary excretion was significantly increased by the presence of particles with naphthalene. However, the test for linear trend in dose was not significant. Also presented here is an investigation of whether acute co-exposure of naphthalene and carbon particles may increase cytotoxicity in pulmonary tissues, and also how may it affect the production of pro-inflammatory cytokines and chemokines in broncho alveolar lavage (BAL) fluid. Rats were exposed nose-only to naphthalene vapor at exposure concentration (20 ppm) with or without carbon particles (5mg/m³) for 4 h. While results for early signs of pulmonary toxicity shows a significant increase in immune cell response, results from the pro-inflammatory biomarkers (cytokines and chemokines) in broncho alveolar lavage (BAL) fluid analysis and histopathology assessment of pulmonary tissues did not provide clear evidence of pulmonary toxicity. However, histopathology assessment of the nasal cavity, used as baseline for inhaled naphthalene toxicity, was significant for epithelial damage. There was no definitive evidence to conclude whether co-exposure had a significant effect in absorption and pulmonary toxicity.
CHAPTER 1
ENVIRONMENTAL AND OCCUPATIONAL EXPOSURES OF NAPHTHALENE AND CARBON PARTICLES (AEROSOLS): IMPLICATIONS TO HUMAN HEALTH

Introduction and Literature Review

Naphthalene Chemistry and Environmental Occurrence

Naphthalene (CAS# 91-20-3; molecular formula C_{10}H_8) is a white crystalline solid that readily sublimes at room temperature. It is the simplest member of the polycyclic aromatic hydrocarbons (PAHs) with a chemical structure consisting of two fused benzene rings. Naphthalene is the most volatile among PAHs with a characteristic smell of mothballs. It has a short half-life of 3-8 hours in the atmosphere. Its physicochemical properties include: molecular weight 120.17 g/mol; melting point 80.2 °C, boiling point 218 °C, relative density 4.42 g/cm at 20 °C and 1 atm; vapor pressure 10 Pa at 25 °C; and diffusion coefficient 7.20 x 10^{-2} cm^2/s at 298 K. It is soluble in alcohol and acetate but not in water (WHO, 2010).

Naphthalene is widely found in the environment and indoor air. It can be carried in particulates from diesel exhaust origin (US EPA, 1998). Naphthalene production is derived from coal tar and petroleum. The most recent estimates place the annual use of naphthalene in the United States (US) in 2000 at more than 100,000 metric ton per year (Jia and Batterman, 2010). Naphthalene is an important intermediate in the production of phthalic anhydride and is used in the manufacturing of several organic products, including plastics and pharmaceuticals. Naphthalene is also an active ingredient in moth balls, carbamate insecticides, resins and tanning agents (ATSDR, 2005; US EPA, 1998).

Naphthalene release to the air occurs from a number of sources including incomplete combustion of biomass and petroleum products, pesticides products and a
number of consumer products (ATSDR, 2005; NTP, 2000; US EPA, 1998). A number of industries including petroleum refining, wood treatment (creosate), and jet fuel formulations also contribute significantly to occupational and environmental settings (ATSDR, 2005; NTP, 2000; US EPA, 1998). Urban atmospheric concentrations are approximately 0.3 ppm (ATSDR, 2005; Dodd et al., 2010; US EPA, 1998). In most outdoor environments, naphthalene is a vapor, and it is found in particulate phase in cigarette smoke. The average daily intake from ambient air was estimated at 19 μg, based on an average naphthalene concentration of 0.95 μg/m³ in urban and suburban air and an inhalation rate of 20 m³/day (ATSDR, 2005). In the vulcanization step of tire manufacturing, naphthalene was measured at concentrations of up to 1.09 mg/m³, resulting in an estimated daily intake of 0.0029 mg/kg of body weight (Durmusoglu et al., 2007). The occupational exposure limits by NIOSH, OSHA and ACGIH are set to 10 ppm time-weighted average (Preuss et al., 2003).

**Naphthalene Toxicity**

Data available from epidemiological studies remain inadequate to establish causality for specific health effects of naphthalene in humans (ATSDR, 2005; US EPA, 1998). However, that does not mean that naphthalene exposure to humans is safe. Based on extrapolation of data from animals to humans, naphthalene is classified as a possible carcinogen to humans (ATSDR, 2005; US EPA, 1998). Accidental acute exposure of naphthalene to humans is known to cause systemic effects (i.e., hemolytic anemia, cataracts, etc.) (ATSDR, 2005; US EPA, 1998). Cancer-related cases in humans have been associated with the use of naphthalene for occupational and medicinal purposes. For example, workers at an East Germany naphthalene purification plant developed laryngeal cancers (NTP, 2000). Patients treated with ‘kafura’ for
anorectal problems in Nigeria were admitted to the hospital with colorectal cancers
(NTP, 2000).

Long-term inhalation studies with naphthalene in mice were positive for toxicity and lung cancer (NTP, 1992). A similar long-term inhalation study with naphthalene using rats developed site and sex-specific respiratory and olfactory epithelial cancers (NTP, 2000). For a long time, naphthalene had been considered to be a non-carcinogenic PAH. However, these studies have raised concerns regarding the possible health consequences associated with environmental and occupational exposure to naphthalene. These studies provided sufficient evidence of carcinogenicity in both mice and rats, and naphthalene is now reasonably anticipated to be a human carcinogen.

**Naphthalene Metabolism and Its Role in Health Effects**

The role of pulmonary metabolism in chemical-induced lung injury in animal models was established over 30 years ago (Buckpitt and Franklin, 1989; O'Brien et al., 1985). Naphthalene toxicity is linked to the production of reactive metabolites by oxidative metabolism (Buckpitt et al., 2002). Naphthalene is toxicologically inert until it converts to the intermediate epoxide by cytochrome p450 monooxygenases. The intermediate epoxide can undergo three major conversions by spontaneous epoxide hydrolase and glutathione s-transferases reactions that can lead to multiple metabolites (Figure 1-5). Furthermore, secondary biotransformations can result in more metabolites that can potentially be toxic to the target tissue. However, it remains unclear as to which of these reactive metabolites may be involved in pulmonary toxicity. CYP2F appears to be the primary P450 involved in metabolism in mice and rats (Li et al., 2011); this protein has high affinity for naphthalene as a substrate (Km ~4 μM) with high catalytic turnover (Baldwin et al., 2004). The rat and mouse differences in metabolism and susceptibility
might be due to substantial differences in quantities of CYP2F protein present (Baldwin et al., 2004). Mice expressed 4-fold greater CYP2F transcript in lungs than in rats. In non-human primates, the quantity of CYP2F transcript appear to be significantly low compared to rodents (Baldwin et al., 2004). Although these findings suggest that naphthalene might not be toxic to humans based on these metabolic differences across species, recently discovered CYP450 enzymes appear to be expressed across the human respiratory tract suggesting other CYP450 enzymes may be functional and potentially involved in the metabolic mechanism of naphthalene toxicity (Lin et al., 2017).

Acute inhalation studies have examined factors such as uptake differences, sex and strain differences, route of exposure, regional metabolic activity, airflow patterns and expression of anti-electrophilic genes that may contribute to the cytotoxic effects of naphthalene in rats. West and colleagues (West et al., 2001) assessed the susceptibility of airways to direct exposure to naphthalene via inhalation and compared the pattern and severity of injury by different route of exposure. Sprague-Dawley male rats and Swiss mice underwent inhalation exposure in a whole-body inhalation exposure system for 4 hours with naphthalene concentrations ranging from 0 to 110 ppm. Another set of animals were administered naphthalene by intraperitoneal injection. While histopathological and morphometric comparison between intraperitoneal injection and inhalation exposure to naphthalene revealed damage to the pulmonary proximal and terminal airways in mice from administration by both routes, rats on the other hand, were not susceptible to injury even at the highest concentration tested, 110 ppm. Another important finding in this study was that Clara cells were more susceptible to
cytotoxicity after inhalation rather than intraperitoneal administration. In a chronic bioassay study in 2000, male rats developed nasal respiratory epithelial adenomas (NTP, 2000). This finding led Lee and collaborators to examine whether mucosal injury to the rat nasal passage can be observed after acute inhalation exposure to naphthalene. Male Sprague-Dawley rats in a whole-body exposure system were exposed to concentrations of 3.4 ppm or 23.8 ppm naphthalene for 4 hours. Not only did the study results confirm that acute exposures to naphthalene can induce olfactory mucosal injury, they also provided evidence of non-uniform patterns of nasal injury that were dependent on both nasal airflow patterns and regional differences in naphthalene bioactivation. They observed that activity of CYP enzymes in the olfactory region of the nasal cavity was significantly greater than in the non-olfactory regions, supporting other research findings that metabolism of naphthalene in the nasal cavity by P450 naphthalene metabolism was a strong contributing factor for naphthalene cytotoxicity (Lee et al., 2005).

Morris and colleagues made an important observation; most of the acute inhalation studies up until 2009 had been limited to males. Their study questioned whether similar naphthalene exposure would have similar or different effects on female rats. Male and female F344 rats in a nose-only inhalation exposure system were exposed to different naphthalene concentrations (1, 4, 10, or 30 ppm) for 1 hr. They found both sexes had similar naphthalene uptake in the upper respiratory tract with nasal cytochrome p450 metabolism contributing to the uptake process (Morris and Buckpitt, 2009). Their study also confirmed the findings Lee and colleagues had made prior — that the presence of nasal cytochrome p450 enzymes contributed to
naphthalene cytotoxicity. Dodd and colleagues also found that rat strain differences were not significant to the effects of naphthalene. Nasal epithelial lesions in F344 and SD rats following naphthalene exposure were similar. They also found that the olfactory epithelial injury was dose-dependent and its severity increased with increasing concentrations of naphthalene (Dodd et al., 2010). In the chronic bioassay study of 2000 (NTP, 2000), female rats developed olfactory epithelial neuroblastoma while male rats developed respiratory epithelial adenomas in the nasal cavity. Cichocki and collaborators looked for markers of oxidant/electrophilic stress or toxicity. Male and female F344 rats in a nose-only exposure inhalation system were exposed to different concentrations of naphthalene for four to six hours. They found a dose-related induction of anti-electrophilic genes in the olfactory mucosa greater in male rats than female rats. This provided an explanation why female rat olfactory mucosa had heightened sensitivity to the carcinogenic effects of naphthalene (Cichocki et al., 2014).

**Naphthalene – Particle Interactions**

Carbonaceous aerosols have emerged as the most common aerosol particles in the atmosphere, especially in large urban settings where most of the world population lives. Carbonaceous aerosols come from anthropogenic and natural sources and are directly emitted into the atmosphere. Carbon in the atmosphere can directly impact human health and influence climate (Riipinen et al., 2012). These urban aerosols are emitted as primary particles and can also be formed as secondary particles in the atmosphere by oxidation of precursor gases such as volatile organic compounds (VOCs), nitrogen oxides, hydrogen sulfides, sulfur dioxides, and other gases (Figure 1-6) (Kulmala et al., 2004; Riipinen et al., 2012). An aerosol is defined as airborne solid or liquid particles. These aerosols consist largely of an organic matter core with less than 1
micron in diameter and are mainly made up of light-scattering organic carbon (OC) and light-absorbing black elemental carbon. They are highly complex, and are major components of particulate matter (PM), in liquid and gaseous form, in the atmosphere (Hinds, 1998; Kulmala et al., 2004; Riipinen et al., 2012).

Carbonaceous aerosols are capable of binding several low molecular weight hydrocarbons such as PAHs. Diesel particulate matter is a good example of carbonaceous aerosols. Emissions of these aerosols affect large urban areas disproportionately and have been on the rise since 1850 (Kanakidou et al., 2018). The main sources of emission include incomplete combustion of fossil fuels, biofuel, power plants, and diesel engines. In addition, inhalation exposure to organic fractions of ambient particles are associated with several human health effects including respiratory and cardiovascular illnesses, lung cancer, and multiple systemic effects (Chan and Yao, 2008; Hinds, 1998; Kulmala et al., 2004; Tie et al., 2009). Carbonaceous aerosols can carry other chemicals, and there is a gas-particle conversion that occurs in the atmosphere potentially providing transport for chemicals to reach lower airways. Studies have shown that particles can provide transport of gas phase chemicals once thought to target upper airways (Chan and Yao, 2008; Hinds, 1998; Kulmala et al., 2004; Oberdorster et al., 2005; Tie et al., 2009; Wexler and Sarangapani, 1998; Wichmann, 2007).

Particulate bound PAHs like naphthalene present in air might be depositing at the lower respiratory tract, potentially targeting sensitive regions where it can elicit toxic responses. There is strong evidence that suggest that adverse health effects from inhalation of combustion-derived particulates are determined by both the particulate and
materials bound to its surface (Asgharian et al., 2003; Bach et al., 2015; Siegel et al., 2004).

Bach et al. experimented with a human bronchial epithelial cell line (BEAS-2B) to analyze the dose-response relationship of diesel exhaust particles’ (DEP) polar and nonpolar soluble constituents. They found that the removal of organics from diesel exhaust particles reduced or almost eliminated their inflammatory properties. On the other hand, the polar and nonpolar constituents extracted from the particles produced pro-inflammatory responses (Bach et al., 2015). Siegel and collaborators performed an *in vivo* study comparing the effects of DEP particulate and the organic soluble components. Rats exposed to DEP and DEP fractions by intratracheal instillation displayed a dose-dependent pulmonary inflammation (Siegel et al., 2004), and rats that received similar DEP exposures were increasingly susceptible to pulmonary infections (Yang et al., 1999). All of these studies confirm how elements bound to particulates induce early signs of biological effects.

**Anatomical Considerations for Particle Inhalation**

The respiratory tract has three main compartments: extrathoracic, tracheobronchial, and alveolar. The extrathoracic compartment comprises the nose, mouth, nasopharynx, oropharynx, and larynx. Air enters the blood containing vessels, nasal cavity for partial filtration as well as interaction with sinuses since they are connected to the nasopharyngeal airways through small openings allowing for irritation when toxic agents are inhaled. This compartment is also comprised of keratinized and stratified squamous epithelial cells (basal cells) as well as respiratory cells. This compartment’s main function includes humidification, heating and filtering of air as it travels to lower airways. It is also responsible for eliminating unwanted chemicals through coughing (Figure 1-2
& Figure 1-3). The tracheobronchial compartment includes the trachea, bronchi, and the terminal bronchial. The trachea carries air from the throat into the lung, covered by cilia, and connects the two main bronchi, which further branch into smaller airways or bronchioles. The inner lining of the bronchi is covered by epithelial cells, which includes ciliated cells, mucus-secreting goblet cells, serous cells, basal cells, and non-ciliated bronchiolar epithelial cells. This compartment initiates branching and exclusively conducts air to the lower airways. Also, it uses the mucociliary apparatus to clear unwanted chemicals from the lower airways as a mechanism of defense (Figure 1-2 & Figure 1-3). The alveolar compartment, part of the respiratory zone of the lungs, consists of the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli. It is in the alveoli that gas exchange between the inhaled air and blood circulatory system occurs. The surface of the alveoli is covered by two epithelial cell types responsible for surfactant secretion, which reduce the surface tension, a key component in pulmonary mechanic movement and function. This respiratory region main function is to exchange gas and houses the alveolar macrophage system to serve as first line of defense in the respiratory tract most sensitive location (Figure 1-2 & Figure 1-3).

Inhaled particles may deposit in the different regions of the respiratory system by the complex action of deposition mechanisms including interception, impaction, sedimentation and diffusion. The deposition of particles along the respiratory regions is dependent on particle size. The smaller the particle the deeper it travels along the respiratory tract. It is also important to highlight that very small particles may deposit in the upper respiratory tract by fast diffusion before they have a chance to flow into deep lungs. Particles with diameters between 3-10 μm may deposit in the nose, throat and
trachea. Particles with diameters between 1-3 μm deposit and may affect the bronchi and bronchioles. Finally, particles with diameters between 0.1-1 μm deposit in the lung and are strongly influenced by sedimentation.

Briefly, the rodent respiratory system including the respiratory tract and lungs is similar to that of most mammals including humans. Rats are widely used for inhalation studies because they are good animal models of lung disease, inexpensive, and allow translation from such studies to humans. Rats have been widely used to study pulmonary toxicity using chemical delivery by inhalation. Several experimental and theoretical approaches have been published on particle deposition in the rat lungs (Asgharian et al., 2003; Raab et al., 1975; Schum and Yeh, 1980; Takenaka et al., 2006; Takenaka et al., 2001).

**Study Rationale and Hypothesis**

The first attempt to investigate whether atmospheric particles can transport compounds to the pulmonary region was published by Wexler and Sarangapani in 1998. They used modeling rather than experimental observations and focused on water-soluble chemicals interacting with particulate water content. Their findings predicted that particles had low carrying capacity to influence the pulmonary deposition and distribution of soluble vapors (Wexler and Sarangapani, 1998). However, there is compelling evidence that some particulates characterized by airborne pollutants adsorbed to carbonaceous surfaces can cause significant inhalation toxicity. Recently, Roberts et al. using experimental observation investigated the gas-particle interaction of how carbonaceous particles influence the deposition of semi volatile organic compounds in lower airways. This was the first study to use an experimental model to assess the extent to which co-exposure to inhaled organic vapors and elemental carbon
particles might influence the deposition of organic compounds within the respiratory tract. Rats were exposed to radioactive naphthalene for 1 h, and sacrificed immediately after exposure. Their findings provided evidence that particles significantly increased the distribution of naphthalene throughout the respiratory tract, more specifically the bronchia, and the right and left lungs (Figure 1-1) (Roberts et al., 2018). Conceivably, these increased lung tissue concentrations could lead to increased systemic absorption. If inhaled naphthalene bound to particulate matter is carried into the lower airways, it could directly or indirectly cause increased pulmonary cytotoxicity in rats and humans, particularly if targeting metabolic active sites like the terminal bronchioles where Clara cells reside in the lungs. These cells are also known to have high rates of CYP450 enzymes with high catalytic activities capable of metabolizing naphthalene although their CYP450 may differ across species (Buckpitt et al., 2013).

Our central hypothesis is that inhalation co-exposure of naphthalene with carbon nanoparticles will enhance naphthalene systemic absorption and pulmonary toxicity. This study was designed to examine the effects of inhaled naphthalene bound to carbon particles as a model compound for carbonaceous aerosols/particulate matter. Biomarkers of naphthalene exposure in human studies include urinary metabolites 1-naphthol and 2-naphthol after inhalation exposure (Sudakin et al., 2013), and systemic absorption will be assessed in this study by using these urinary naphthalene metabolites as biomarkers of exposure.
Figure 1-1. Particles significantly increased the distribution of naphthalene throughout the respiratory tract, especially in bronchia, left and right lung (Roberts et al., 2018)
Figure 1-2. Anatomy and physiology of the respiratory system. Clara cells are located in the lower airways and may be susceptible to naphthalene toxicity (Hinds, 1998).

- Abundant
- High metabolic activity (CYP450 - detoxification)
- Naphthalene metabolism is CYP450 dependent
- Naphthalene metabolites are cytotoxic
Figure 1-3. Predicted fractional deposition of inhaled particles in the nasopharyngeal, tracheobronchial, and alveolar region of the human respiratory tract during nose breathing. Based on data from the International Commission on Radiological Protection (1994). Drawing courtesy of J. Harkema (Oberdorster et al., 2005)
Figure 1-4. Gas-particle interaction model
Figure 1-5. Scheme for naphthalene metabolism and formation of multiple reactive metabolites that may be involved in naphthalene toxicity (Buckpitt et al., 2002)
Figure 1-6. Connections between volatile organic compound emissions, nanoparticle growth and air quality (Riipinen et al., 2012)
CHAPTER 2
EFFECT OF ACUTE INHALATION CO-EXPOSURE OF NAPHTHALENE AND CARBON PARTICLES ON THE SYSTEMIC ABSORPTION OF NAPHTHALENE: USING URINARY EXCRETION OF 1-NAPHTHOL AS A BIOMARKER

Background

Naphthalene is a semi-volatile and toxic air pollutant widely found in the environment and indoor air, (US EPA, 1998). Naphthalene release to the air occurs from a number of sources including incomplete combustion of biomass and petroleum products, pesticides products and a number of consumer products (ATSDR, 2005; NTP, 2000; US EPA, 1998). A number of industries including petroleum refining, wood treatment (creosate), and jet fuel formulations also contribute significantly to occupational and environmental settings (ATSDR, 2005; NTP, 2000; US EPA, 1998).

Naphthalene is a nasal toxicant and carcinogen in the rat. Long-term inhalation studies of naphthalene in both mice and rats resulted in cancer. In the 2-year inhalation study conducted by NTP, rats developed site and sex-specific respiratory and olfactory epithelium cancers (NTP, 2000). Data available from epidemiological studies remain inadequate to establish any causality (ATSDR, 2005; US EPA, 1998). However, based on extrapolation data from animals to man, naphthalene is classified as a possible carcinogen to humans (ATSDR, 2005; US EPA, 1998). There are also concerns regarding possible non-cancer health consequences of exposure to naphthalene. Accidental acute exposure of naphthalene to humans is known to cause systemic effects (i.e., hemolytic anemia, cataracts, etc.) (ATSDR, 2005; US EPA, 1998). Short-term inhalation assessments in rats have been crucial to determine factors that may contribute to both cancer and non-cancer effects. For instance, acute exposure to inhaled naphthalene in rats led to site-specific cytotoxicity; nasal injury, which is also
correlated with local metabolism (Lee et al., 2005). This cytotoxicity may not only explain nasopharyngeal effects of naphthalene, but also contribute to the mode of action for its carcinogenicity.

Because of its high lipophilicity and hydrophobicity, naphthalene tends to deposit in the upper respiratory tract (Morris and Buckpitt, 2009). However, little is known about the influence of co-exposure to particulates in air might affect the toxicity of inhaled naphthalene. To gain insight into how airborne particles can influence the deposition of semi-volatile organics in the respiratory tract, a recent study from our laboratory used a rodent model exposed via inhalation to radiolabeled naphthalene with or without co-exposure of elemental carbon particles. This study showed that co-exposure of naphthalene vapor with particles altered naphthalene distribution in lungs, causing significantly higher concentrations in bronchi, and right and left lung of rats (Roberts et al., 2018) (Figure 1-1) potentially enhancing naphthalene accessibility to deeper parts of the airways (Figure 1-2). Conceivably these increased lung tissue concentrations could lead to increased systemic absorption.

Naphthalene is toxicologically inert until it converts to the intermediate epoxide by cytochrome p450 monooxygenases. The intermediate can undergo three major conversions that lead to urinary metabolites and toxicity (Buckpitt et al., 2002; Warren et al., 1982) (Figure 1-5). Although it is not yet known which reactive metabolite leads to cytotoxicity, urinary excretion of 1-naphthol is very commonly used as indicator of naphthalene exposure in human and animal studies. As a metabolite of naphthalene, its excretion in urine is considered a useful way of assessing external exposure to naphthalene in biomonitoring studies (Sams, 2017).
We report here the results of a study focusing on the effects of carbon particles on the absorption of increasing doses of inhaled naphthalene. We hypothesized that the increased deposition of naphthalene in the lower respiratory tract of rats with particle co-exposure observed previously (Roberts et al., 2018) would result in greater systemic absorption, as indicated by increased 1-naphthol and 2-naphthol urinary excretion. To test this hypothesis, a single inhalation dose of either naphthalene only or naphthalene plus carbon nanoparticles was given to rats. Absorption was evaluated by measuring 1- and 2-naphthol concentrations in urine over time following the inhalation dose. This study represents, to the best of our knowledge, the first attempt to assess the influence of the presence and absence of particles found in air on the absorbed dose of naphthalene using urinary excretion of naphthols as a biomarker.

Methods

Animals

Male Sprague-Dawley rats 8 weeks of age (~200 g body weight) were purchased from Envigo (Indianapolis, IN). The animals were housed individually in polycarbonate cages with free access to water and food (Teklad Rodent Diet 7912, Envigo) in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. Animal rooms had a light/night dark cycle of 12 h and controlled temperature (18-26°C). The rats were kept in cages of two animals with unlimited food and water. At the conclusion of the experiment, animals were euthanized by CO₂, with death confirmed by thoracotomy. Animals were treated humanely according to criteria in the NIH Guide for the Care and Use of Laboratory Animals and all procedures were approved by the Institutional Animal Care and Use Committee.
Animal Treatment

Animals were randomly assigned to receive naphthalene alone, with or without carbon particles.

Particle and Vapor Generation

Naphthalene vapor was generated by passing nitrogen gas through a stainless steel column (30cm x 1 cm) in which 10 g of molecular sieve was impregnated with 0.5 g naphthalene. Temperature of the naphthalene column was maintained at approximately 30°C and adjusted to achieve naphthalene target concentrations of 5, 10, or 20 ppm in the exposure carousel. Carbon particles from graphite were generated using a spark discharge generator (Model GFG 1000, PALAS, Karlsruhe, Germany) with a target concentration in the exposure chamber of approximately 5 mg/m^3 with an approximate particle size of 100-150 nm. Argon and compressed air were passed through the generator with a spark frequency of 300 Hz. A diagram of the exposure system is provided in Appendix A.

Exposure Atmosphere Generation and Characterization

High purity air for the inhalation system was generated with a Zero Air Supply unit (Model Fisher Scientific, Pittsburgh, PA) and was humidified by passing through distilled water. This air was combined with feeds from the vapor column, particle generator, and a 100% oxygen supply and passed through a mixing plenum (Figure A-1) before delivery to a stainless steel, multi-port, nose-only exposure tower (CH Technologies, Westwood, NJ). In the exposure tower, naphthalene, oxygen, humidity, temperature and particle size and concentration were monitored. Oxygen concentration in air was measured with a Biosystems ToxiPro O2 sensor (Honeywell, Smithfield, RI), and oxygen flow into the system was adjusted to achieve a 20% concentration at the
exposure tower. Temperature and relative humidity were monitored throughout the exposure period using a Humicap HMI141 Probe (Vaisala, Finland). Particle size distribution and particle concentration were measured in near-real time from a sampling port in the exposure tower using a Scanning Mobility Particle Sizer (SMPS consisting of Model 3775 Condensational Particle Counter, and Model 3081A Differential Mobility Analyzer, TSI, Shoreview, MN). Air samples taken from a port in the exposure tower were analyzed for naphthalene every 15 min using a custom gas chromatography system (Quantum Analytics, Foster City, CA). This system included an Agilent Model 7890 gas chromatograph (GC) with CP-FID detection. For gas chromatography, Bruker BR1 12.5 M x 0.032 mm and Brucker BR1 30 M x 0.032 mm columns (Brucker, Billerica, MA) were used in tandem with a gas flow rate of 5 ml/min. Naphthalene analyses were run with oven temperatures increasing from 100 to 200°C at 10°C/min. Spatial homogeneity data (particles and vapor distribution across the exposure tower) were measured using SMPS and GC. Temporal stability data (for both particles and vapors) were also obtained using the same methods. Excess air delivered to the exposure carousel, and all air passing through the exposure carousel, was processed through carbon scrubbers and then delivered to a vacuum pump before exhausting in a fume hood. Negative pressure from the vacuum pump was adjusted to maintain neutral pressure in the nose-only exposure carousel.

**Naphthalene Adherence to Carbon Particles**

The extent of binding of naphthalene to carbon particles in this system was described by Roberts et al. (Roberts et al., 2018). Carbon particles and naphthalene vapor were generated in the same manner as the animal exposure experiments, except air from the mixing plenum was passed through two membrane filters in tandem instead
of the exposure chamber for one hour. Membrane filters were weighed to determine the mass of trapped carbon particles on the filter. These values, coupled with the amounts of carbon trapped on the filters, were used to calculate the naphthalene concentrations on the particles.

**Inhalation Experimental Design**

Thirty-six male Sprague Dawley rats with initial weight of 200 g were used. Animals were exposed to naphthalene vapor with or without carbon particulates for 1 h in a six-port, nose-only inhalation system with particulate and chemical exposures capabilities. Rats were divided into six groups (N=6) and were exposed to three different naphthalene exposure concentrations—5, 10, and 20 ppm, with and without carbon particles at a concentration of 5 mg/m³.

**Measurement of Respiratory Parameters**

Respiratory flow signals from each breath (measuring flow and pressure changes during breathing) for each animal were obtained. The six ports in the exposure carousel were fitted with double-chamber plethysmography restraint tubes allowing simultaneous measurement of respiratory parameters and nose-only exposure (EMKA Technologies, Falls Church, VA). Inspiratory time (Ti), expiratory time (Te), peak inspiratory flow (PIF), peak expiratory flow (PEF), tidal volume (TV), minute ventilation (MV), respiratory rate (f), enhance pause (Penh), mid expiratory flow (EF50) were measured throughout exposure in each animal.

**Standards and Reagents**

Naphthalene, 1-naphthol, 2-naphthol, and 1-methoxyfluorene were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-naphthol-d8 and 2-naphthol-d8 were purchased from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada). β-Glucuronidase/
sulfatase (type H-2 from *Helix pomatia*; β-glucuronidase activity, 105,000 U/mL; sulfatase activity, 4,300 U/mL) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Dodecane was purchased from Alfa Aesar (Ward Hill, MA). Methanol, hexane, sodium acetate, sodium bicarbonate, and sodium carbonate were purchased from Fisher Scientific (Fair Lawn, NJ). N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was purchased from Regis Technologies, Inc (Morton Grove, IL).

**Urine Collection**

**Metabolic cage**

After a single 1 h inhalation exposure (nose-only) to naphthalene vapors, rats were placed in a standard circular metabolic cage with a wire mesh floor designed to acquire untainted urine for study. They were allowed free access to food and water. Urine samples were collected in a Nalgene tube at the bottom of a funnel system. All of the cages were washed and dried after every urine collection, every 24 h to ensure accurate urine collection from the rats.

**Biological samples**

The urine samples were collected at 24 h intervals — pre-exposure and, 24, 48, and 72 h post-exposure. Urine was stored at -20 °C.

**Sample Preparation**

In a glass tube (15 mL), 100 μL of the internal standard solution (50 ng/mL 1-naphthol-d8 and 2-naphthol-d8) were added to 2 mL of rat urine. Glucuronide and sulfate conjugates of 1- and 2-naphthol in the urine were hydrolyzed in the presence of 10 μL of β-glucuronidase enzyme and 50 μL of a pH 5.0 sodium acetate buffer solution at 37°C overnight (17 h). The samples were extracted twice with 2.5 mL of hexane by shaking for 1 minute after adding 20 mg of carbonate (ground and mixed sodium
bicarbonate and sodium acetate (1:8)). The samples were centrifuged for 10 min at 3000 rpm to break potential emulsion. To completely remove all remaining water from samples before evaporation, hexane sample extracts were passed through sodium sulfate in thin-stemmed glass funnels with small amounts of glass wool. Dodecane (10 µL) was added to a 16 x 100 mm silanized tube as a “keeper” before the combined extracts were evaporated to ~10 µL in a nitrogen evaporator with a TurboVap LV evaporator from California Life Sciences (Hopkinton, MA), using a gentle stream of nitrogen (5-10 psi, gradually increasing during evaporation) and a water bath (40°C). The samples were reconstituted with hexane (170 µL) and then transferred to silanized amber GC vials 400 µL fused inserts. All samples were spiked with 10 µL of recovery standard solution (1-methoxyfluorene, 1µg/mL). The samples were then derivatized with 10 µL of MSTFA, and incubated at 60°C for 2 h. All sample preparation steps were performed under low light conditions to avoid possible photodegradation of target analytes. Also, to minimize sample loss during processing, silanized glass tubes were used for drying under nitrogen, and hexane extracts were flushed with nitrogen gas to limit oxidation.

Quality Control and Calibration Standards

Pooled rat urine from BIOIVT (Hicksville, NY, USA) vendor samples was spiked with appropriate amounts of 1-naphthol and 2-naphthol and deuterated 1-naphthol-d8 and 2-naphthol-d8 standards, then underwent enzymatic deconjugation overnight. Quality control preparation included duplicates for negative control (no analyte added), low positive control (10 ng/mL) and high positive control (50 ng/mL), and procedural blank (no matrix). A total of nine calibration standards containing 1-naphthol 2-naphthol, deuterated 1-naphthol-d8 and 2-naphthol-d8 were prepared in concentrations between
1-500 ng/mL. Internal standard spiking was set at 100 ppb in the middle of the calibration curve. Samples for quality control and calibration standards were prepared, derivatized and analyzed in parallel with the sample extracts (Figure 2-3 & Table 2-1). The standard operating procedures for this analytical method have been adapted from biomonitoring studies (Gaudreau et al., 2016; Li et al., 2006; Serdar et al., 2012; Smith et al., 2012).

**GC-MS Analysis**

GC-MS analysis was performed using an Agilent 7890B gas chromatography system coupled to an Agilent a 7000C triple-quadrupole mass spectrometer (GC-MS/MS; Agilent 7000C, Palo Alto, CA, USA). The system utilized two Agilent HP-5MS columns (5 m × 250 μm × 0.25 μm, Restek, Bellefonte, PA, USA) as the guard column and a 60 m × 250 μm × 0.25 μm column (Restek, Bellefonte, PA, USA) was used as the analytical column. A 1 μL aliquot of sample was injected to the GC inlet (280°C) in splitless mode. Helium was used as the carrier gas at a constant flow rate of 1.1 mL/min. The oven temperature program was as follows. The initial temperature was maintained at 75°C for 1 min, increased at a rate of 15°C/min to 235°C and held for 3 min, and then increased at a rate of 25°C/min to 300°C and held for 2 min, for a total run time of 19.27 minutes. The transfer line was set to a temperature of 280°C. The mass spectrometer was operated with a collision gas flow of nitrogen (1.5 mL/min) and a quenching gas flow of helium (2.25 mL/min). The ionization was produced with an electronic impact (EI) source set to 230°C, and the naphthols were quantified in the multiple reaction monitoring (MRM) mode. The mass transitions and collision energies for all analytes including internal standards are shown in Appendix B.
Data Normalization

To obtain the final respiration-adjusted 1-naphthol concentration (ng), first, sample concentrations were obtained by multiplying extract concentration (ng/mL) times dilution level (unitless) times extract volume (mL) then divided by the urine volume (mL) used for the extraction. Second, to normalize for total 1-naphthol excreted per animal, the sample concentrations were multiplied by total urine (mL) produced over 24 hr, and finally to adjust for respiration, the 1-naphthol extracted (ng) were multiplied by the respire ratio. The respire ratio is the accumulated volume per animal (obtained during 1 hr inhalation) divided by total accumulated volume of animals in a treated group.

Statistical Analysis

Statistical comparisons among groups were made with a two-factor factorial design with dose, a quantitative factor at three levels – 5ppm, 10ppm and 20 ppm – and particle, a qualitative factor at two levels coded as ‘with’ and ‘without’. A two-way analysis of variance (ANOVA) was used to detect the difference in 1-naphthol concentrations. A one-way analysis of variance (ANOVA) for linear regression was performed to test for linear trend between different dose concentrations. Respiratory parameters among animals treated with or without particle co-treatment were compared using a two-sample independent t-test. Differences with P values < 0.05 were considered statistically significant.

Results

Inhalation Exposures – Delivered Naphthalene Concentrations, Particle Concentrations, Particle Size Distributions

The mean concentration of naphthalene delivered during the experiments was for 5 ppm; 4.80 ± 1.02 ppm, for 10 ppm; 11.05 ± 2.50 ppm, for 20 ppm; 19.70 ± 3.02 ppm
(mean ± SD). Mean particle mass concentrations measured by SMPS for experiments with naphthalene at 5 ppm, 10 ppm, and 20 ppm were 5.00 ± 1.16 mg/m³, 6.86 ± 1.73 mg/m³, and 7.64 ± 1.35 mg/m³, respectively (Table 2-2). The mean concentration of naphthalene delivered during the experiments without particles were in a similar range as the mean concentration of naphthalene delivered during the experiments with particles (5 ppm; 5.20 ± 1.50, 10 ppm; 10.05 ± 1.05, 20 ppm; 18.79 ± 2.72).

**Ventilation Parameters – Comparison of Respiration among Animals Receiving Different Naphthalene Exposures and Particle Exposure**

Several ventilation parameters including inspiratory time (Ti), expiratory time (Te), peak inspiratory flow (PIF), peak expiratory flow (PEF), tidal volume (TV), minute ventilation (MV), respiratory rate (f), enhance pause (Penh), mid expiratory flow (EF50) were measured during the exposure period in each animal. The results are shown in Table 2-1. None of the parameters were significantly different between naphthalene only and naphthalene and particle co-exposure groups (Table 2-1), indicating no sign of bronchoconstriction or other interference with respiration.

**Urinary Excretion of 1- and 2-Naphthol**

1-Naphthol was found above quantitation limits in all urine samples. However, there were several samples in which 2-naphthol was below quantitation limits and, in some cases, below detection limits. As a result, only data for 1-naphthol was used as a biomarker for naphthalene absorption for these experiments. The urinary excretion of 1-naphthol following a 1 h inhalation of naphthalene exposure at 5 ppm, 10 ppm and 20 ppm, with or without carbon nanoparticles is shown in Figure 2-1. The urinary collection occurred over 72 h, capturing nearly all of the elimination (Figure 2-2). On average among all groups, over 90% of 1-naphthol excretion in urine occurred during the first 24
h, and consequently the analysis focused on the mass of 1-naphthol elimination in urine during this period. The pulmonary absorption of naphthalene with or without carbon nanoparticles using urinary excretion of 1-naphthol as a biomarker is shown in Figure 2-1. 1-Naphthol excretion was significantly increased by the presence of particles with naphthalene (P-value <0.001) and the estimated difference is 14600 ± 2600 ng less without particles than with particles, as shown in Figure 2-1. We did not observe a dose-dependent increase in the urinary excretion of 1-naphthol in response to increasing doses of inhaled naphthalene only or naphthalene with carbon particles (P-value = 0.181). The test for linear trend in dose was not significant (P-value= 0.072).

Discussion

The study by Roberts et al. assessed whether inhalation of carbon nanoparticles affected the pulmonary deposition of naphthalene. Their findings showed that particle co-exposure significantly increased naphthalene concentrations throughout the respiratory tract, as a result influencing different deposition patterns of naphthalene in the rat respiratory system (Roberts et al., 2018). If the deposition of naphthalene is significantly increased by the carbon nanoparticles, it is possible that these increased lung tissue concentrations could lead to increased systemic absorption. In this study, we examined the absorption of increasing doses of inhaled naphthalene with or without carbon particles by measuring the excretion of 1-naphthol metabolite in urine following inhalation exposure in rats. We hypothesized that co-exposure of small ~100 nm size carbon nanoparticles would increase the deposition of naphthalene in the lung compared to naphthalene exposure only, and that increased elimination of 1-naphthol in the urine would provide evidence that increased absorption has taken place.
Urinary 1-naphthol is an established biomarker for assessing exposure to ambient and occupational naphthalene levels, and it has been extensively used as urinary biomarker of exposure in animal and human studies (Sams, 2017). Using intraperitoneal injection as a route of administration, Kilanowicz et al. found that after a single dose (20 mg/kg) in rats, the main metabolites (1-naphthol, 2-naphthol, 1,2-naphthalenediol-1,2-dehydro, and methylthionapthalenes) were isolated from urine and feces. Over 88% of the compound was excreted during the first 72 h. The authors concluded that naphthalene had a rapid rate of replacement and little deposition in tissues in rats (Kilanowicz et al., 1999). Moreover, their study identified 1-naphthol as the main metabolite of naphthalene in urine. Despite the differences in approach and focus, the findings of this study are consistent with those of Kilanowicz et al. in showing the rapid elimination of naphthalene and 1-naphthol after initial exposure despite differences in the route of administration.

In the present study, elimination of 1-naphthol in urine occurred primarily in the first 24 h after inhalation exposure and urinary excretion was almost complete within 72 h. Willems and colleagues modeled naphthalene excretion after inhalation exposure in rats and found that the pharmacokinetic model predicts naphthalene to be rapidly absorbed into the blood during inhalation exposure as a result of the high blood:air partition coefficient and unrestricted permeation (Willems et al., 2001). As the simplest form of PAHs, most urinary excretion of naphthalene and its metabolites in rats and humans occurs within the first 24 h (Bieniek, 1994; Turkall et al., 1994; Yang et al., 1999);
1-Naphthol is also a metabolite of the insecticide carbaryl, and when carbaryl exposure is possible, this can confound the use of 1-naphthol as a biomarker for naphthalene exposure. Because naphthalene is metabolized to both 1- and 2-naphthol, both metabolites are typically measured in occupational and ambient naphthalene exposure studies to eliminate misclassification and confounding by co-exposure to carbaryl (Kato et al., 2004; Kim et al., 2001; Kim et al., 1999; Lee et al., 2001; Preuss et al., 2003). However, the present study is an inhalation (nose-only) in rats, controlling for naphthalene exposure only. For that reason, we are not concerned that the 1-naphthol levels are misclassified or confounded by co-exposure to carbaryl. To minimize any remaining concerns with any potential unknown naphthalene exposures in our rats, pre-exposure urine samples were collected and analyzed for 1-naphthol. No detectable concentrations were found in any samples.

Respiratory parameters in rats co-exposed naphthalene or naphthalene plus particles were not significantly different (Table 2-1). These findings were quantitatively in line with those published by Roberts et al. in which similar respiratory parameters were measured and analyzed (Roberts et al., 2018). Although these results indicate no effects of particle co-exposure on the inhalation of naphthalene, urinary excretion values for 1-naphthol were nonetheless corrected for total inhaled volume of air for individual animals to control for inter-subject variability in respiration during the exposure period.

Naphthalene cytotoxicity is dependent upon its metabolism by cytochrome P450 enzymes. Clara cells are known to have high levels of CYP450 enzymes with high catalytic activities capable of metabolizing naphthalene, and are mainly located in the terminal bronchioles. Lower respiratory tract structures varies across species. In rats,
this structure is very short compared to other species with long terminal and respiratory bronchioles.

The changes in naphthalene deposition potentially influencing absorption, especially in the lower respiratory airways, will be dependent upon the naphthalene release from particles. Deposition of particles is highly dependent on particle size. Although our particles are small and are predicted to reach the lower respiratory tract, naphthalene disassociation from these particles might be problematic. There is no evidence in the literature as to how adsorbed naphthalene on a carbonaceous core behave in vivo. A study by Creasia and colleagues looked into the kinetics of elimination of benzo[a]pyrene (BaP), a pulmonary carcinogen and larger PAH, from the lung. Mice were exposed via intratracheal installation to BaP-coated carbon particles and BaP-only without carbon particles. Animals exposed to BaP-only lost 50% of the dose within 1.5 h and more than 95% within 24 h. On the other hand, animals exposed to BaP-coated carbon particles took approximately 36 h to clear 50% of the initial dose, and after 4 days of exposure approximately 10% still remained (Creasia et al., 1976). Their data showed longer retention of carbon particles decreased the elimination rate of BaP from the lung. These results provide one explanation for the increased in 1-naphthol excretion in animals treated with combined naphthalene with carbon particles dose in our study. It is possible that naphthalene to some extent is behaving like BaP in that the elimination rate of naphthalene is decreased from the lung. Hence, prolonging naphthalene availability for metabolism.

Another important observation from this study was the lack of dose-related increase in 1-naphthol. In the NTP 2000 inhalation study, a PBPK model was developed
to describe the processes involved in naphthalene toxicokinetics. Blood time-course data of the parent compound collected from rats were evaluated after a single 6-hour inhalation exposure to 10, 30, or 60 ppm naphthalene. The data showed the rates of naphthalene metabolism did not increase proportionately with increasing exposure concentration, indicating metabolic saturation in the lung (NTP, 2000). It is likely that what we are seeing is the combination of these two phenomena taking place, carbon particles prolonging the presence of naphthalene in the lungs to generate more metabolites than in animals exposed to naphthalene-only. It is also likely that saturation of the metabolism is occurring at lower naphthalene concentrations; hence, 1-naphthol urinary excretion across the different doses remains similar.
Table 2-1. Respiratory parameters in rats during exposure to naphthalene or naphthalene plus particles. Ti: inspiratory time; Te: expiratory time; PIF: peak inspiratory flow; PEF: peak expiratory flow; TV: tidal volume; MV: minute ventilation; f: respiratory rate; Penh: enhance pause; EF50: mid expiratory flow. Data are presented as mean and SD for each treatment group (N=6). No statistical differences in respiratory parameters among animals treated with and without particle co-treatment were observed using a two sample independent t-test.

<table>
<thead>
<tr>
<th></th>
<th>Ti</th>
<th>Te</th>
<th>PIF</th>
<th>PEF</th>
<th>TV</th>
<th>MV</th>
<th>f</th>
<th>Penh</th>
<th>EF50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>msec</td>
<td>msec</td>
<td>mL/s</td>
<td>mL/s</td>
<td>mL</td>
<td>mL</td>
<td>bpm</td>
<td></td>
<td>mL/s</td>
</tr>
<tr>
<td>Naphthalene only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 ppm</td>
<td>N = 40847</td>
<td>175.96</td>
<td>488.07</td>
<td>10.28</td>
<td>9.48</td>
<td>1.12</td>
<td>183.03</td>
<td>163.82</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>26.46</td>
<td>1027.86</td>
<td>4.12</td>
<td>3.39</td>
<td>0.29</td>
<td>66.62</td>
<td>49.05</td>
<td>0.39</td>
<td>3.02</td>
</tr>
<tr>
<td></td>
<td>25.36</td>
<td>397.19</td>
<td>2.5</td>
<td>2.24</td>
<td>0.23</td>
<td>54.16</td>
<td>33.73</td>
<td>0.32</td>
<td>2.14</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.588</td>
<td>0.277</td>
<td>0.976</td>
<td>0.958</td>
<td>0.728</td>
<td>0.629</td>
<td>0.733</td>
<td>0.164</td>
</tr>
<tr>
<td>Naphthalene + Particles</td>
<td>N = 46967</td>
<td>182.68</td>
<td>234.42</td>
<td>10.23</td>
<td>9.4</td>
<td>1.2</td>
<td>200.14</td>
<td>167.11</td>
<td>0.56</td>
</tr>
<tr>
<td>10 ppm</td>
<td>33481</td>
<td>173.84</td>
<td>356.79</td>
<td>10.55</td>
<td>9.53</td>
<td>1.11</td>
<td>181.16</td>
<td>160.67</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>32.27</td>
<td>664.62</td>
<td>4.1</td>
<td>3.32</td>
<td>0.33</td>
<td>76.6</td>
<td>52.1</td>
<td>0.46</td>
<td>3.19</td>
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<tr>
<td></td>
<td>P-value</td>
<td>0.868</td>
<td>0.348</td>
<td>0.824</td>
<td>0.867</td>
<td>0.492</td>
<td>0.404</td>
<td>0.382</td>
<td>0.805</td>
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<td>Naphthalene only</td>
<td>N = 44972</td>
<td>176.08</td>
<td>268.95</td>
<td>10.91</td>
<td>9.79</td>
<td>1.23</td>
<td>210.11</td>
<td>167.72</td>
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<td>20 ppm</td>
<td>40278</td>
<td>171.35</td>
<td>477.79</td>
<td>11.89</td>
<td>10.14</td>
<td>1.31</td>
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<td>3.56</td>
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<td>0.36</td>
<td>3.19</td>
</tr>
<tr>
<td></td>
<td>P-value</td>
<td>0.318</td>
<td>0.399</td>
<td>0.622</td>
<td>0.737</td>
<td>0.771</td>
<td>0.815</td>
<td>0.923</td>
<td>0.235</td>
</tr>
</tbody>
</table>
Table 2-2. Delivered naphthalene concentrations & particle concentrations

<table>
<thead>
<tr>
<th>Exposure chamber</th>
<th>Naphthalene Target ppm</th>
<th>Rat Exposure ppm</th>
<th>Carbon particles Target mg/m³</th>
<th>Rat Exposure mg/m³</th>
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</thead>
<tbody>
<tr>
<td>5</td>
<td>4.80 ± 1.02</td>
<td>5</td>
<td>5.00 ± 1.16</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>11.05 ± 2.50</td>
<td>5</td>
<td>6.86 ± 1.73</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>19.70 ± 3.02</td>
<td>5</td>
<td>7.64 ± 1.35</td>
<td></td>
</tr>
</tbody>
</table>

Mean ± SD
Figure 2-1. Pulmonary absorption and metabolism (1-naphthol) of naphthalene with or without carbon nanoparticles collected during the first 24 h in urine (mean ± SEM, N=6)
Figure 2-2. 1-naphthol urine excretion over time
Figure 2-3. Standard Curve for 1-Naphthol from 1-500 ppb.

Table 2-3. Calibration data and limits of detection of 1-naphthol (in the range 1 to 500 ng/ml) in rat urine (n = 9)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Calibration equation</th>
<th>$r^2$</th>
<th>LOD (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Naphthol</td>
<td>$y = 0.02905 + 0.48399$</td>
<td>0.9982</td>
<td>1</td>
</tr>
</tbody>
</table>

$y = $ area, $x = $ concentration (ng/ml)
CHAPTER 3
CHANGES OF INHALED NAPHTHALENE DEPOSITION FROM CO-EXPOSURE WITH CARBON PARTICLES EXACERBATES PULMONARY TOXICITY IN THE RAT MODEL

Background

Naphthalene is a ubiquitous organic airborne pollutant, and an example of a byproduct of combustion of several petroleum products including diesel fuel known to contribute largely to urban and rural air pollution. Human exposure to naphthalene is ubiquitous, and the potentially negative implications to human health are many, including lung cancer. Long-term inhalation assessments of naphthalene in experimental models resulted in respiratory tract-related cancers. Naphthalene is nasal cytotoxicant and carcinogen in the rat and in mice (NTP, 2000). Based on extrapolation data from animals to man, naphthalene is classified as a possible carcinogen to humans.

In 2000, National Toxicology Program’s in vivo naphthalene chronic toxicity testing found naphthalene to be cytotoxic and carcinogenic to epithelial and neural cells in nasal tissues in rats. This 2-year study found naphthalene to have a dose-dependent increase in tumors that was site and sex specific. For instance, male rats developed nasal respiratory epithelial adenomas and female rats developed olfactory epithelial neuroblastoma (NTP, 2000). Interestingly, intraperitoneal administration of naphthalene in rats also targeted the olfactory mucosa, inducing naphthalene toxicity and injury uniformly across the olfactory region (Lee et al., 2005; Plopper et al., 1992). These studies clearly establish the nasal passage as target for naphthalene exposure in rats.

Some in vivo naphthalene acute toxicity testing studies in rats have been critical to identify potential risk factors including uptake differences, sex and strain differences, route of exposure, regional metabolic activity, airflow patterns and expression of anti-
electrophilic genes that may contribute to the cytotoxic effects of inhaled naphthalene observed in the chronic toxicity testing studies (Cichocki et al., 2014; Dodd et al., 2010; Lee et al., 2005; Morris and Buckpitt, 2009; West et al., 2001). Lee and collaborators found that injury to the nasal mucosal was dependent on both nasal airflow patterns and regional differences in naphthalene bioactivation (Lee et al., 2005), and that genomic responses were related to GSH metabolism, cell cycle, inflammation, and proliferation (Clewell et al., 2014). In the Dodd et al. study, rats had marked cytotoxicity and hyperplasia at 10 and 30 ppm (Dodd et al., 2012). While it is established in the literature that naphthalene causes nasal cancer in rats and lung cancer in mice, the ability of naphthalene to produce cancer in humans is unclear. It is therefore important to understand how the nature of naphthalene exposures as may be encountered by humans affects their potential toxicity within the respiratory tract.

Presented here are the results from a study investigating the morphological differences and pro-inflammatory responses in rats treated with acute naphthalene alone or naphthalene with carbon particles. A previous study from this laboratory found the co-exposure of naphthalene with airborne carbon particles affected the deposition of naphthalene within the lungs of rats, increasing tissue concentrations within the lower respiratory tract (Roberts et al. 2018). This study raised the possibility that particle co-exposure might increase susceptibility of the lungs to naphthalene toxicity.

It was hypothesized that acute co-exposure of naphthalene and carbon particles would increase cytotoxicity in pulmonary tissues and would increase the production of pro-inflammatory cytokines and chemokines. To test this hypothesis we compared early pro-inflammatory responses and morphological differences in the lungs following a
single dose of inhaled carbon particles, naphthalene and naphthalene with carbon particles compared to control rats. Signs of early pulmonary toxicities were evaluated using histopathology and measure of biomarkers of inflammation and immune response (Milliplex assay). To our knowledge, we are the first group to examine how combinations of a semi-volatile chemical and particles suspended in air in rats may contribute to inhalation toxicity in humans.

**Methods**

**Animals**

Male Sprague-Dawley rats 8 weeks of age (~250 g body weight) were purchased from Envigo (Indianapolis, IN). Prior to experimentation, the animals were kept two per polycarbonate cage with unlimited access to food and water (Teklad Rodent Diet 7912, Envigo) in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited facility. Animal rooms had a light/night dark cycle of 12 h and controlled temperature (18-26˚C). Animals were randomized for assignment to treatment groups. Animals were treated humanely according to criteria in the NIH Guide for the Care and Use of Laboratory Animals and all procedures were approved by the Institutional Animal Care and Use Committee.

**Chemicals**

Naphthalene was purchased from Sigma-Aldrich (St. Louis, MO). Phosphate buffered formalin (10%) was purchased from Fisher Scientific (Hampton, NH).

**Particle and Vapor Generation**

Naphthalene vapor was generated by passing nitrogen gas through a stainless steel column (30cm x 1 cm) in which 10 g of molecular sieve was impregnated with 0.5 g naphthalene. Temperature of the naphthalene column was maintained at
approximately 30˚C and adjusted to achieve a naphthalene concentration of 20 ppm in the exposure carousel. Carbon particles were generated using a spark discharge generator (Model GFG 1000, PALAS, Karlsruhe, Germany) with a target concentration in the exposure chamber of approximately 5 mg/m$^3$ with an approximate particle size of 100-150 nm. Argon and compressed air were passed through the generator with a spark frequency of 300 Hz. A diagram of the exposure system is provided in Figure 1-A.

**Exposure Atmosphere Generation and Characterization**

High purity air for the inhalation system was generated with Zero Air Supply unit (Model Fisher Scientific, Pittsburgh, PA) and was humidified by passing through distilled water. This air was combined with feeds from the vapor column, particle generator, and a 100% oxygen supply and passed through a mixing plenum (Figure 1) before delivery to a stainless steel, multi-port, nose-only exposure tower (CH Technologies, Westwood, NJ). In the exposure tower, naphthalene, oxygen, humidity, temperature and particle size and concentration were monitored. Oxygen concentration in air was measured with a Biosystems ToxiPro O2 sensor (Honeywell, Smithfield, RI), and oxygen flow into the system was adjusted to achieve a 20% concentration at the exposure tower.

Temperature and relative humidity were monitored throughout the exposure period using a Humicap HMI141 Probe (Vaisala, Finland). Particle size distribution and particle concentration were measured in near-real time from a sampling port in the exposure tower using a Scanning Mobility Particle Sizer (SMPS consisting of Model 3775 Condensational Particle Counter, and Model 3081A Differential Mobility Analyzer, TSI, Shoreview, MN). Air samples taken from a port in the exposure tower were analyzed for naphthalene every 15 min using a custom gas chromatography system (Quantum Analytics, Foster City, CA). This system included an Agilent Model 7890 gas
chromatograph (GC) with CP-FID detection. For gas chromatography, Bruker BR1 12.5 M x 0.032 mm and Brucker BR1 30 M x 0.032 mm columns (Brucker, Billerica, MA) were used in tandem with a gas flow rate of 5 ml/min. Naphthalene analyses were run with oven temperatures increasing from 100 to 200°C at 10°C/min. Spatial homogeneity data (particles and vapor distribution across the exposure tower) were measured using SMPS and GC. Temporal stability data (for both particles and vapors) were also obtained using the same methods. Excess air delivered to the exposure carousel, and all air passing through the exposure carousel, was processed through carbon scrubbers and then delivered to a vacuum pump before exhausting in a fume hood. Negative pressure from the vacuum pump was adjusted to maintain neutral pressure in the nose-only exposure carousel.

**Animal Treatment**

Four treatment groups of six rats each were exposed for 4 h in the six-port, nose-only inhalation system to filtered air, 20 ppm naphthalene, 20 ppm naphthalene combined with 5 mg/m³ carbon particles, or 5 mg/m³ carbon particles. After 4 h of exposure, animals were kept in cages with two animals each with unlimited access to food and water for 48 h to allow for the observation of early signs of pulmonary toxicity. This time was determined by preliminary experiments and advice from a pathologist. Subjects were euthanized 48 h after inhalation exposure by intravenous injection of pentobarbital (Fatal-Plus, Vortech, Dearborn, MI), with death confirmed by thoracotomy.

**Brochoalveolar Lavage**

Following euthanasia, the animals were placed in dorsal decubitus position to open the chest cage, with incision along the skin upwards to expose salivary glands and trachea. The skin, glands, and other tissues were separated in order to expose the
sternohyoid muscle. The animals were exsanguinated by cutting the abdominal aorta. A small excision of the trachea was made to allow a 24 gauge catheter tube to pass into the trachea. Whole rat lungs were lavaged with 6 mL of cold phosphate-buffered saline and the recovered fluid was collected and immediately placed on ice. BALF volumes were recorded and the total number of cells were quantified by counting under a light microscope using a hemacytometer with 10 μL loaded on both sides. The volume of BALF required for 25,000 cells total per sample was spun onto glass slides using a cytospin (ThermoFisher). The calculated BALF volume was separated into new 1.5 mL tubes and centrifuged for 5 minutes at 1000 rpm at 4°C. The supernatant was removed into new 1.5 mL tubes. The supernatant was stored for cytokine and chemokine analysis (described below). The cell pellet was re-suspended in 450 μL PBS with 5% Bovine Serum Albumin (BSA). The entire volume of cell suspension was loaded into cytospin cartridges and centrifuged for 10 minutes at 600 rpm. All slides were air dried and fixed for 5 minutes in 100% methanol (Fisher Scientific) then stained using a HEMA III kit (Fixative solution, Solution I and Solution II) following the company’s protocol (ThermoFisher). Two hundred cells per slide were counted and identified as monocytes, lymphocytes, neutrophils, eosinophils, or basophils. All samples were counted and the average values were calculated. The remaining lavage fluid for the multiplex assay was collected and stored at -80°C until the assay.

**Quantification of Cytokines and Chemokines in BALF**

In order to evaluate a potential inflammatory response, pro-inflammatory cytokines and chemokines related to acute pulmonary injury were quantified in BALF from animals exposed to filtered air (controls) (n=6), particles (n=6), naphthalene (n=6), or naphthalene plus particles (n=6) using the MILLIPLEX rat cytokine/chemokine
magnetic bead panel (Millipore) following the manufacturer’s protocol. Protein levels of 12 cytokines and chemokines (GM-CSF, GROα/KC/CINC1/CXCL1, IFNγ, IL-1β, IL-6, IL-10, IL-12 (p70), IL-17A/CTLA8, IP-10/CXCL10, RANTES/CCL5, TNFα, TGF-β1) were measured. Prior to analysis of the samples, the rat cytokine and chemokine standards were reconstituted with deionized water. The vials were inverted and mixed evenly several times and vortexed for 10 seconds. The vials were allowed to sit for 5-10 minutes. In preparation of working standards, serial dilutions in deionized water were mixed with assay buffer. In preparation for analysis of samples, assay buffer was added to rinse each well of the plate, and allowed to shake for 10 min, and then discard assay buffer. Assay buffer was added to background and sample wells, and appropriate matrix solution to background, standards, and control wells. Samples diluted 1:2 were added to sample wells, along with beads to each well. Samples were incubated for 2 h at room temperature, followed by removing well contents, and washed twice with wash buffer. The samples received detection antibodies in each well, and then the samples were incubated for 1 h at room temperature. After the incubation, no aspiration or removal of the antibodies was needed. Streptavidin-Phycoerythrin was added to each well, followed by incubation again for 30 min at room temperature. Finally, well contents were removed and washed twice with wash buffer in order to add sheath fluid per well. The samples were acquired on Luminex 200 system (Luminex) and the amount (pg/mL) was calculated using xPONENT software (Luminex).

**Necropsy and Sample Preparation for Histopathology**

Following euthanasia, the head was separated from the cadaver. All tissues were fixed in 10% buffered formalin for at least 72 hours and entire heads were decalcified with 10% EDTA pH 7.4 for at least 1 month before sectioning. We followed Lee et al. as
well as the NTP approach in selecting anatomically defined sites of the nasal cavity with significant airflow patterns. Levels I, II & III are cross sections where airflow studies have provided detailed information of nasal airflow, and where the NTP bioassay reported increased incidence of neoplasms (Lee et al., 2005; NTP, 2000). In order to properly assess the nasal cavity for acute naphthalene toxicity, our histological preparations focused on level I, which is taken immediately posterior to the upper incisor teeth; level II, is the level of the incisive papilla anterior to the first palatial ridge; and level III, is the middle of the second molar teeth. Levels I and II contain the naso- and maxillo-turbinates that, along with the nasal passages (meatuses) and septum, are lined by ciliated respiratory-type epithelium. Level III encompasses the olfactory region of the nose with ethmoid turbinates and meatuses lined entirely by specialized olfactory neuro epithelium (Figure 3-8) (Long et al., 2003; NTP, 2000). The sections were routinely processed, embedded in paraffin, sectioned to 5 microns, and stained with hematoxylin and eosin (H&E). The sections were scanned at 40x with an Aperio/Versa scanner. All photographs were taken from the scanned sections. The high magnification is 400x, the intermediate and low magnification scans are roughly 200x and 100x, respectively.

Histology sections for the nasal cavity were scored for five criteria: epithelial damage, cilia loss, inflammation, fibrinous lining, and goblet cell hyperplasia. Epithelial damage was scored from 0 to 3. 0—essentially normal tissues, 1—small numbers of degenerate epithelial cells (<10%), 2—moderate numbers of degenerate epithelial cell (10-25%) and rare epithelial necrosis, and 3—epithelial cell necrosis or loss with disruption of the mucosal layer. Cilia loss was scored from 0 to 2. 0—essentially normal
tissue, 1 - small, focal to multifocal areas of cilia loss (<25%), 2 - moderate to large multifocal to diffuse areas of cilia loss (>25%). Inflammation was scored from 0 to 3. 0 - essentially normal tissue, 1 - small numbers of lymphocytes and plasma cells within the submucosa, 2 - small numbers of lymphocytes and plasma cells within the submucosa with small numbers extending into the mucosa, 3 - small to moderate numbers of lymphocytes and plasma cells within the submucosa with moderate numbers extending into the mucosa. Fibrinous lining was scored from 0 to 1. 0 - no fibrinous material within or lining the nasal cavity, 1 - fibrinous material within or lining the nasal cavity, and goblet cell hyperplasia was scored from 0 to 3. 0 - essentially normal tissue, 1 - mild goblet cell hyperplasia (<25% of the ciliated epithelium is replaced by goblet cells), 2 - moderate goblet cell hyperplasia (25-50% of the ciliated epithelium is replaced by goblet cells), 3 - severe goblet cell hyperplasia (>50% of the ciliated epithelium is replaced by goblet cells). Histology sections for lower respiratory tissue (trachea, bronchioles, alveoli) were not scored because all of the tissues were considered essentially normal.

**Statistical Analysis**

All statistical analyses were run with n = 6 per group. Data are expressed as mean ± standard error of the mean (SEM). Differences among groups were examined by analysis of variance (ANOVA) and t-tests using GraphPad Prism version 8 (GraphPad Software, San Diego, California). Respiratory parameters among animals treated with or without particle co-treatment were compared using a two-sample independent t-test. Analysis of whether differences in the distribution of naphthalene in the pulmonary tract have any consequences in terms of tissue damage. For the nasal cavity data, each animal was assigned (assumed at random) to one of the 4 treatment groups. Each animal has 4 histology sections that were scored. Histology sections were
assumed to be random selections from a large number of possible sections that could have been scored, hence section was assumed a random effect in the model. Responses that present with only two possible scores (inflammation and fibrinous lining) were analyzed using a generalized linear mixed-effects model with a logistic link function. Responses that present with more than two possible scores (epithelium damage, cilia loss, and Goblet cell hyperplasia) were analyzed using a cumulative link mixed-effects model with a logit link (a type of multivariate analysis on ordinal categorical data). Because so few of the cilia loss data are scores of 2, these 2 scores were recoded as 1 and the data analyzed as a binary response. For the lung and trachea data, each animal was assigned (assumed at random) to one of the 4 treatment groups. Analysis was simpler since only one sample (section) from each animal was scored. Responses that present only zero scores (epithelium damage, bronchi/bronchioles and inflammation) were not analyzed. Epithelium damage in trachea and cilia loss were both analyzed using a generalized linear mixed-effects model with a logistic link function. The one score 2 observation in epithelium damage in trachea was recoded to a 1 for this analysis. All analyses were performed in the stat package R using the glmer() function [lme4 package] for the generalized linear mixed-effects model analyses and the clmm() function [ordinal package] for the cumulative link mixed-effects model analysis. Estimated marginal means (e.g. least-squares means and pairwise comparisons) were computed using the lsmeans() function [emmeans package].

Results

The mean concentration of naphthalene delivered during the experiments during a single dose of 20 ppm was 19.05 ± 2.50 ppm. The mean concentration of naphthalene
during the particle co-exposure was 18.80 ± 3.02 ppm. Mean particle mass concentrations measured by SMPS for experiments with naphthalene at 20 ppm were 4.80 ± 1.16 ppm (mean ± SD) respectively (Table 3-2).

**Ventilation Parameters – Comparison of Respiration among Animals Receiving Different Naphthalene Exposures and Particle Exposure**

Several ventilation parameters including inspiratory time (Ti), expiratory time (Te), peak inspiratory flow (PIF), peak expiratory flow (PEF), tidal volume (TV), minute ventilation (MV), respiratory rate (f), enhance pause (Penh), mid expiratory flow (EF50) measured during the exposure period in each animal and results are shown in Table 3-1. None of the parameters were significantly different between naphthalene only and naphthalene and particle co-exposure groups (Table 3-1), indicating no sign of bronchoconstriction or other impairment of respiration from particle co-exposure.

**Cell Count Numbers and Differential Cell Counts In BALF**

Total cell count numbers and differential cell counts in BAL fluid from rats in all treatment group were quantified. Sprague Dawley (SD) rats were exposed to room air (control), naphthalene (20 ppm) alone, particles (5 mg/m$^3$) alone, and naphthalene (20 ppm) plus particles (5 mg/m$^3$). Forty-eight hours after exposure rat lungs were lavaged with PBS and immune cells were counted. Total cell count examination of BAL fluid showed more than 2-fold significant increase in the recovered immune cells in the animal group treated with the combined naphthalene plus carbon particles compared to carbon particles only treated rats (Figure 3-2). Similarly, differential cell count examination of mainly macrophages and lymphocytes, animals exposed to naphthalene plus particles had significant higher number of recovered cells compared to particles-only treated animals (Figure 3-3). As expected, animals treated with naphthalene only
had less recovered cells than the particles-only group. The number of recovered cells was increased by both exposure to naphthalene and particles, and the increased number of recovered cells among animals exposed to both naphthalene and particles was approximately equal to the sum of the increases from naphthalene and particles alone (Figure 3-2).

**Measurement of Inflammatory Markers in BAL Fluids**

The MILLIPLEX rat cytokine/chemokine magnetic bead panel was used to quantify the levels of pro-inflammatory cytokines and chemokines related to acute pulmonary injury in control, naphthalene, particles, and naphthalene plus particles treated animals. We quantified 12 cytokines and chemokines in BAL fluid 48 h after inhalation exposure. Two proteins: TNFα and KC were not detected in any of the samples analyzed (Figure 3-7). Several of the inflammatory markers were detected in control and treated animals: IL-1β, IL-6, IL-17A, IL-12p70, TGF-β1, IP-10 and IFNγ (Figure 3-6), but no statistically difference was observed among the groups. We observed only two protein markers significantly increased after particle exposure: IL-10 and GM-CSF (Figure 3-6). Overall, we did not observe any significant pro-inflammatory cytokine response – after exposure to naphthalene or co-exposure to naphthalene with particles. These results were consistent with the histopathology data, in which no inflammation and injury were observed in lung tissues.

**Histopathology Analysis**

Nasal cavity tissue was examined for histopathological changes. Histology sections for nasal cavity were scored for five criteria: epithelial damage, cilia loss, inflammation, fibrinous lining, and goblet cell hyperplasia. Histology sections for lower respiratory tissue (trachea, bronchioles, alveoli) were not scored because all of the
tissues were considered essentially normal with respect to histology, including epithelial
damage and inflammatory cell increase.

The sections from the ethmoid turbinates and the olfactory epithelium of the
dorsal meatus (Figure 1A&B, 1E&F) had the most significant pathologic changes. The
study by Lee and collaborators confirmed earlier that olfactory mucosal injury was due
to regional patterns of airflow in the rat nasal passages (Lee et al., 2005). Lesions
related to naphthalene only and naphthalene plus particles in rats included epithelial
damage characterized by extensive fibrinous membrane and necrosis, loss of
epithelium, inflammation characterized by lymphocytes and plasma cells in the
submucosa and epithelium (Figure1E & 1F). Animals treated with particles only and air
only controls did not have significant epithelial damage or inflammation.

Despite the presence of lesions within the nasal cavity in naphthalene exposed
rats, histological scoring of tissues revealed few differences among the treatment
groups. The only statistically significant difference among groups (p-value=0.0005) was
for epithelial damage. No statistically significant difference in inflammation (p-
value=0.82), damage to fibrinous lining (p-value=0.44), cilia loss (p-value=0.32), or
goblet cell hyperplasia (p-value=0.62) was found among groups.

Lung tissues were examined for histopathological changes. The sections from
the trachea (Figure 3-11 A-D), bronchioles (Figure 3-11 E-H) and alveoli (Figure 3-11 I-
L) showed no significant pathology or differences in the epithelial damage or
inflammatory among the groups. Control and exposed lung sections looked very similar
indicating the lack of inflammation and tissue injury compared to nasal cavity sections of
exposed animals.
Discussion

The objective of this study was to determine the acute pulmonary response to the co-exposure of naphthalene and particles after inhalation exposure. We hypothesized that the co-exposure treatment would increase cytotoxicity in lung target tissues, and it would also increase the production of pro-inflammatory cytokines and chemokines. We assessed responses related to inflammation associated with cytotoxicity in the respiratory tissues and for morphological differences between treatments in the rat respiratory target cells.

Inflammation is considered the first pathological sign of injury that leads to morphological changes. It is well-established in the literature that long-term exposure to fine particulate matter (even at the ultrafine particulate size range) elicits inflammation and morphological changes. Acute lung inflammation is characterized by the presence of local macrophages to modulate acute inflammatory response, infiltration of neutrophils as a second-line of defense, and lymphocytes (Moldoveanu et al., 2009). Although we controlled for carbon particle effects in this study, the effects of naphthalene interaction with carbon particles did not significantly influence inflammation and pulmonary cytotoxicity as we had hypothesized. Reed et al. reported that whole-body inhalation of diesel exhaust exposure in rats at occupational levels for either 1 week or 6 months did not lead to persistent lung inflammation and tissue injury (Reed et al., 2004). There appears to be differences in immune reaction and tissue injury response to carbon nanotubes and nanoparticles administration. Intratracheal installation or pharyngeal aspiration of carbon-based nanoparticles leads to significant inflammation, oxidative stress, and tissue injury (Lam et al., 2004; Shvedova et al., 2005; Warheit et al., 2004). However, to our knowledge there are few short-term
inhalation studies assessing the pulmonary inhalation toxicity of either carbon-based nanoparticles or nanomaterials in general. It is clear that inhalation studies, due to their complexity and cost, are less likely be performed despite being the preferred exposure route for the toxicity of respirable substances. The examination of BAL fluid is an excellent method to assess for inflammatory response in the lungs. We quantified the total cell numbers and differential cell counts in BAL fluid from rats in all treatment groups. We anticipated a significant increase in immune cells count (macrophages, neutrophils and lymphocytes), especially in animals treated with the co-exposure to naphthalene and particles. Pulmonary inflammation in rats was evidenced by increased numbers of macrophages and lymphocytes in BALF (Figure 3-3, Figure 3-4 & Figure 3-5). However, we did not observe any neutrophils.

The secretion of pro-inflammatory cytokines and chemokines are associated with injury and tend to serve as chemo attractants to mediate interactions with various immune cells including macrophages, neutrophils and lymphocytes (Saber et al., 2006; Serdar et al., 2012). The toxicity of nanoparticles remains unclear, but there is increasing effort to delineate the potential respiratory toxicity of nanoparticles. Several studies have focused on how nanoparticles might induce pulmonary inflammation through the activation of the pro-inflammatory markers (Card et al., 2008; Reisetter et al., 2011; Yazdi et al., 2010). In order to evaluate for signs of inflammatory response, pro-inflammatory cytokines and chemokines related to pulmonary acute injury were quantified in BAL fluid. Overall, we did not observe any significant pro-inflammatory response after exposure to naphthalene or co-exposure of naphthalene with particles. However, we did observe significant increase of IL-10 and GM-CSF in animal groups.
treated with carbon nanoparticles (Figure 3-7C&F). IL-10 is known to be an anti-inflammatory cytokine that suppresses the immune responses; hence, limiting excessive tissue disruption caused by inflammation (Taylor, 2010). IL-10 is produced by activated macrophages, B cells, and T cells (Mosser and Zhang, 2008), and suppresses the activation and production of TNFα, IL-1β, IL-6, IL-8, IL-12, and GM-CSF (Fiorentino et al., 1991). GM-CSF is also a cytokine involved in hematopoiesis, localized immune cell differentiation that function as a colony stimulating factor facilitating the development of the immune system in response to infections (Root and Dale, 1999).

There is evidence in the literature that IL-10 inhibits the release of pro-inflammatory cytokines including GM-CSF. hence, controlling the inflammatory response of macrophages (Lenhoff et al., 1998). Macrophages secrete the following cytokines when there is an inflammatory stimulus: TNFα, IL-1β, and IL-6. Excessive production of IL-1β and TNFα trigger an acute inflammatory response (Beutler, 1999). Both of these proteins are responsible for septic chock (Arango Duque and Descoteaux, 2014). While IL-1β was detected in all groups at a similar non-significant levels (Figure 3-7A), TNFα was not detected in any of the groups (Figure 3-8B). The primary function of cytokines is to regulate inflammation. They play a vital role in regulating the immune response in health and disease. Although cytokines and chemokines are mainly produced by macrophages and lymphocytes, the increase in immune cell counts observed in BAL fluid in this study does not correlate with the detection of pro-inflammatory cytokines and chemokines, especially those present at the early acute response. Overall, a 4 h inhalation exposure to naphthalene or naphthalene with carbon particles did not result in
significant pulmonary inflammation as shown by the evidence in the pro-inflammatory markers and histopathological data.

We performed nasal morphologic examination to evaluate susceptible regions in the rat nasal passage to airflow patterns and compare the naphthalene only concentration effects versus naphthalene plus particles concentration to the nasal passage epithelial populations, especially respiratory and olfactory cells in order to determine the extent of injury. Nasal olfactory lesions are the most commonly seen in inhalation studies. The study by Lee et al. found that regional patterns of olfactory mucosal injury from acute inhalation of naphthalene correlated with the regions of highest airflow based on previous simulation studies in the rat nasal passages (Lee et al., 2005). Our findings are consistent with previous studies. The gross examination included the identification of signs of nasal inflammation, nasal epithelial lesions or necrosis, and ultimately the atrophy of the turbinates. The severity of nasal epithelium lesions was characterized by one or more of the following: loss of cilia, epithelial cell bleb formation, cellular disorientation, cellular eosinophilia, swelling, loss of cell-to-cell contact or vacuolar changes within the epithelium (Dodd et al., 2010). Nasal epithelial inflammation is frequently seen in conjunction with other lesions such as necrosis and atrophy of the adjacent epithelium. Previous work by our group has shown that co-exposure of naphthalene with carbon particles led to significantly increased deposition of naphthalene in the lower airways (Roberts et al., 2018). We hypothesized that co-exposure of naphthalene with carbon particles would enhance deeper accessibility to the lower respiratory airways, and since naphthalene bio-activation by CYP450 enzymes is prerequisite for cytotoxicity, we expected to see early signs of injury in the
terminal bronchioles, which inner lining are covered by ciliated and non-ciliated bronchiolar epithelial cells including Clara cells. These cells are the second most common cells comprising the respiratory bronchioles (Figure 3-10). Their most important function is to utilize their abundant CYP450 enzymes to detoxify harmful substances inhaled into the lungs, and secrete club cell secretory protein to protect the bronchiolar epithelium population, and act as progenitor cells for themselves and ciliated cells (Figure 3-10) (Broeckaert and Bernard, 2000; Rokicki et al., 2016). Injury to Clara cells can lead to the disruption of their protective role. Compared to mice, rats have a lower rate of naphthalene metabolism in the lung airways (Buckpitt et al., 2013). A closer examination did not show any visible injury or abnormality in the terminal or respiratory bronchiolar cells after the co-exposure treatment.

However, it is likely as shown in several studies in the literature, naphthalene-related injury in the rat model appears to be site-specific determined by CYP450 enzyme levels. This result is consistent with what Lee and collaborators found in rats that acute exposure to naphthalene led to olfactory mucosal injury, which was dependent on nasal airflow pattern and regional differences in naphthalene bioactivation. The activity of CYP enzymes in the olfactory region of the nasal cavity was significantly greater than in the non-olfactory regions, supporting other research findings that metabolism of naphthalene in the nasal cavity by CYP450 naphthalene metabolism was a strong contributing factor for naphthalene cytotoxicity (Lee et al., 2005).

In this current study, pulmonary and nasal toxicities were assessed at a one-time point (48 h after inhalation exposure) in accordance to expert judgement. Although this
time point was enough to induce nasal toxicity as shown in our data, it appears it may have been too late to observe significant changes in cytokines and chemokines as early signs of pulmonary toxicity, and too early to see full effects on histopathology. Lesions were seen in the nasal tissues but not to the extent that there were significant differences in histological scores. In general, \textit{in vivo} toxicity testing for the purpose of pulmonary immunotoxicity testing evaluates adverse effects within 24 h. Also, testing only one naphthalene concentration may have not been enough. Therefore, future studies should test higher naphthalene concentrations though they might be at levels higher than those seen in the ambient environment.
Table 3-1. Respiratory parameters in rats during exposure to naphthalene, naphthalene plus particles, particles only and room air. Ti: inspiratory time; Te: expiratory time; PIF: peak inspiratory flow; PEF: peak expiratory flow; TV: tidal volume; MV: minute ventilation; f: respiratory rate; Penh: enhance pause; EF50: mid expiratory flow. Data are presented as mean and SD for each treatment group (N = 6). No statistical differences in respiratory parameters among animals treated with and without particle co-treatment were observed using a two sample independent t-test.

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<td>bpm</td>
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Mean ± SD
Figure 3-1. Macrophages and lymphocytes in light microscope, 20x.
Figure 3-2. Changes in immune cell profiles in BALF from rats exposed to naphthalene and particles. Sprague Dawley (SD) rats were exposed to Room air (control), Naphthalene (20 ppm), Particles (5 mg/m³), Naphthalene (20 ppm) + Particles (5 mg/m³). On day 2 rat lungs were lavaged with PBS and immune cells were counted; total immune cell counts (number / mL). Letters indicate significant differences between groups (P < 0.05)
Figure 3-3. Changes in immune cell profiles in BALF from rats exposed to naphthalene and particles. Sprague Dawley (SD) rats were exposed to Room air (control), Naphthalene (20 ppm), Particles (5 mg/m³), Naphthalene (20 ppm) + Particles (5 mg/m³). On day 2 rat lungs were lavaged with PBS and immune cells were counted, differential cell counts, numbers of macrophages and lymphocytes (cells/mL). Results are presented as mean ± SEM of 6 rats per group.
Figure 3-4. Changes in immune cell profiles in BALF from rats exposed to naphthalene and particles. Sprague Dawley (SD) rats were exposed to Room air (control), Naphthalene (20 ppm), Particles (5 mg/m³), Naphthalene (20 ppm) + Particles (5 mg/m³). On day 2 rat lungs were lavaged with PBS and immune cells were counted; numbers of immune cell types: lymphocytes (cells/mL). Results are presented as mean±SEM of 6 rat per group. Letters indicate significant differences between groups (P < 0.05)
Figure 3-5. Changes in immune cell profiles in BALF from rats exposed to naphthalene and particles. Sprague Dawley (SD) rats were exposed to Room air (control), Naphthalene (20 ppm), Particles (5 mg/m³), Naphthalene (20 ppm) + Particles (5 mg/m³). On day 2 rat lungs were lavaged with PBS and immune cells were counted; numbers of immune cell types: macrophages (cells/mL). Results are presented as mean±SEM of 6 rat per group. Letters indicate significant differences between groups (P < 0.05)
Figure 3-6. Changes in pulmonary cytokine and chemokine production in BALF from rats exposed to naphthalene and particles. Sprague Dawley (SD) rats were exposed to room air (control), naphthalene (20 ppm), particles (5 mg/m$^3$), naphthalene (20 ppm) + particles (5 mg/m$^3$). On day 2, rat lungs were lavaged with PBS. Chemokines and cytokines in BALF were measured using the multiplex assay. Results are presented as mean ± SEM of 6 rats per group. A two sample independent t-test was used to compare statistical differences between the groups. Asterisk denotes statistically significant values compared to control animals (P < 0.05).
Figure 3-7. Changes in pulmonary cytokine and chemokine production in BALF from rats exposed to naphthalene and particles. Sprague Dawley (SD) rats were exposed to room air (control), naphthalene (20 ppm), particles (5 mg/m³), naphthalene (20 ppm) + particles (5 mg/m³). On day 2, rat lungs were lavaged with PBS. Chemokines and cytokines in BALF were measured using the multiplex assay. Results are presented as mean ± SEM of 6 rats per group. A two sample independent t-test was used to compare statistical differences between the groups. Asterisk denotes statistically significant values compared to control animals (P < 0.05).
Figure 3-8. The location of the three levels of the nose routinely examined by the National Toxicology Program (NTP, 2000)
Figure 3-9. Regional differences in the cellular composition and function of pulmonary epithelium (Rokicki et al., 2016)
Figure 3-10. The sections are from ethmoid turbinates of the dorsal meatus (Level III): A-D; H&E: 100X, and olfactory epithelium of the dorsal meatus (Level III): E-H; H&E: 200X. H&E sections are represented from rats treated with naphthalene (20 ppm) + nanoparticles (5 mg/m³): A&E; naphthalene (20 ppm): B&F; nanoparticles (5 mg/m³): C&G; room air (control): D&H. The sections are from the dorsal nasal cavity in the third section from the nose where the pathologic changes were most significant. Note the significant epithelial damage characterized by extensive fibrinous membrane and necrosis and loss of epithelium and inflammation characterized by lymphocytes and plasma cells in the submucosa and epithelium in the sections from rats treated with naphthalene + nanoparticles and naphthalene alone. Animals treated with nanoparticles only and air only controls do not have significant epithelial damage or inflammation.
Figure 3-11. Trachea: A-D; Bronchiole: E-H; Alveoli: I-L. H&E sections are represented from rats treated with naphthalene (20 ppm) + nanoparticles (5 mg/m³): A-I; naphthalene (20 ppm): B-J; nanoparticles (5 mg/m³): C-K; room air (control): D-L. H&E; 400X. Note the lack of any significant pathology or differences in epithelial damage or inflammatory between the groups.
CHAPTER 4
SUMMARY AND CONCLUSIONS

Burning of fossil fuels is a major cause of manmade air pollution in urban areas, where most people live globally. Air pollution includes ambient nanoparticles and organic vapors like naphthalene that may be targeting sensitive pulmonary regions and be harmful to human health. Naphthalene, a PAH, is a ubiquitous ambient and occupational airborne pollutant. By most part, naphthalene is a byproduct of combustion of several petroleum products including diesel fuel, and this combustion is known to contribute largely to urban and rural air pollution. Human exposure to naphthalene is universal, and the potentially negative implications to human health are many, including perhaps lung cancer. There is considerable interest among inhalation toxicology experts and regulators to understand how the degree and extent of carbon particles in combination with airborne organic vapor exposures may impact public health. Because prolonged exposure to airborne pollutants can cause structural damage to the lungs, this can result in chronic diseases including cancer. In general, since risk is a function of exposure and hazard, understanding the magnitude of naphthalene exposure can be an important stepping stone to implement and develop a reliable human health risk assessment process. We are the first group to study how these combinations of SVOCs and particles suspended in air may contribute to inhalation toxicity. There is almost no information currently available on how particle interactions with organic airborne pollutants like naphthalene may influence pulmonary toxicities.

The work presented here aimed to elucidate whether changes in naphthalene deposition in the respiratory system from co-exposure with particulates results in increased systemic absorption and increased toxicity within the respiratory tract. By
comparing the respiratory parameters among animals receiving different naphthalene exposures, with and without particle co-exposure, we found no effect of naphthalene or particles on ventilation under the conditions of this study. In other words, no acute effects on respiration were observed from inhalation of naphthalene in concentrations up to 20 ppm, or carbon particles at a concentration of 5 mg/m3, for up to 4 hours. This finding eliminates interference with respiration, such as might occur through bronchoconstriction or respiratory depression, as an explanation for any effects of particle co-exposure on systemic absorption or pulmonary toxicity of naphthalene. Similar findings were reported by our group previously in which none of the respiratory parameters were significantly different between naphthalene only and the naphthalene and particle co-exposure groups (Roberts et al. 2018).

We investigated the absorption of increasing doses of inhaled naphthalene (5, 10, and 20 ppm) with or without carbon particles (5mg/m3) by measuring the excretion of naphthalene metabolite (1-naphthol) in urine following inhalation exposure in rats. we were able to demonstrate that 1-naphthol excretion was significantly increased by the presence of particles. At all naphthalene concentrations in air tested, the presence of particles increased the urinary excretion of 1-naphthol 2-3 fold. These results suggest that co-exposure of particles with naphthalene substantially increases its systemic absorption. However, urinary excretion of 1-naphthol was not directly proportional to the inhaled naphthalene dose. It is possible that carbon particles are prolonging the presence of naphthalene in the lungs—preventing the rapid elimination of the parent compound and generating more metabolites. Excretion of the naphthalene was not measured in urine samples, however, so this possibility cannot be evaluated from the
data obtained. The absence of excretion of 1-naphthol in urine proportional to the exposure concentration might also be indicative of saturation of metabolism of the parent compound.

These experiments generated important data that provides a better understanding of the contribution of particles in air on the disposition and toxicity of a model SVOC, naphthalene, relevant to environmental and occupational exposures. If this approach is valid, then it would change the dose and potentially increase its cytotoxicity. This information can also help determine the biologically internal dose for adverse effects.

We aimed to test the hypothesis that the adverse biological effects of inhaled naphthalene are augmented by carbon particles. Inflammation is considered the first pathological sign of injury that leads to morphological changes. It is well-established in the literature that fine particulate matter, even at the ultrafine particulate size range, elicits inflammation and morphological changes. Although we controlled for carbon particle effects, the effects of naphthalene interaction with carbon particles did not significantly influence pulmonary cytotoxicity. The exacerbation of pulmonary cytotoxicity should be consistent with pulmonary inflammation, increase in immune cells counts, increases in pro-inflammatory cytokines and chemokines. While we saw a significant increase in immune cell counts, namely macrophages and lymphocytes, in animals treated with carbon particles, and naphthalene with carbon particles in BAL fluid, the mostly non-significant detection of pro-inflammatory cytokines and chemokines, and the lack of pulmonary injuries as shown in our histopathology data were inconsistent with the increase in immune cell counts.
The terminal bronchioles in rats are vulnerable to low to moderate concentrations of inhaled toxicants because of their morphological and biochemical composition. If our hypothesis was correct, we expected to observe significant increase in morphological changes in regions highly sensitive to naphthalene metabolism, like the terminal bronchioles, in a concentration-dependent manner in animals exposed to the co-exposure treatment compared to animals exposed to either filtered air, naphthalene only, or carbon particles only. We did not observe any significant pathology or differences in epithelial damage or inflammatory between the groups.

This study allowed us to characterize a real-life exposure model that can help us better understand the deposition, pharmacokinetics, and pulmonary toxicity. This initial assessment allowed us to investigate the onset of biological responses in the lower respiratory tract as ambient ultrafine particles provide organic vapors like naphthalene deeper accessibility to pulmonary sites with potentially higher metabolic activity. This approach may provide a new exposure model to examine the effect of a chemical in a mixture of toxins, and the threat it poses to public health in the long-term.
Figure A-1. Inhalation exposure system (Roberts et al., 2018).
APPENDIX B
EXAMPLE OF RESPIRATION SIGNALS USING THE IOX2 SOFTWARE (EMKA TECHNOLOGIES)

Figure B-1. Respiration signal using the IOX2 software
APPENDIX C
EXAMPLE OF CARBON PARTICLE DISTRIBUTION WITH (BOTTOM) AND WITHOUT (TOP) NAPHTHALENE

Figure C-1 Carbon particles distribution with (bottom) and without (top) (Roberts et al., 2018).
APPENDIX D
EXAMPLE CHROMATOGRAPH FOR 1-NAPHTHOL AND 1-NAPHTHOL-D8 IN URINE AND THEIR RESPECTIVE RETENTION TIMES

Figure D-1. Chromatograph for 1-naphthol and 1-naphthol-d8 in urine and their respective retention times
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Edwin Arauz was born in Santa Rosa de Aguan, Colon, Honduras. He graduated from Jesus Milla Selva High School in Tegucigalpa, Honduras in 2001. Following high school he moved to the United States to live with his parents and siblings and went to City College of New York (CCNY), New York to pursue undergraduate degree in biology. During his undergraduate study, Edwin participated in several research projects at CCNY, University of Wisconsin-Madison, and National Cancer Institute/NIH.

Following graduation from CCNY, Edwin spent a year as a post-baccalaureate fellow (IRTA/CRTA) at the National Heart, Lung, and Blood Institute at National Institutes of Health, and two years as a Research Assistant at the Kennedy Krieger Institute at Johns Hopkins University.

To further his graduate education and expand upon his interest in public health, Edwin was accepted into the MPH program in Environmental Health Sciences and Epidemiology at the University of Michigan. He conducted research abroad for his master thesis and received Dean’s scholarship award and Dean’s scholar award for academic excellence.

Following graduation from University of Michigan, Edwin moved to Gainesville, Florida in the fall of 2014 to be a regulatory toxicologist/risk assessor and in the fall of 2015. I joined the Interdisciplinary Toxicology program in the Physiological Sciences Department at the University of Florida’s College of Veterinary Medicine in Dr. Roberts’ lab. While working in Dr. Roberts’ lab, Edwin studied the toxicological and toxicokinetics effects of inhaled carbon nanoparticles interaction with airborne organic chemicals in the rat model. He received his Ph.D. from University of Florida in the summer of 2019.