ABSTRACT

TURLEY, AUDREY THERESE. Physical, Chemical, and Toxicological Characteristics of Combustion Generated Soot-Iron Particles. (Under the direction of William L. Roberts)

Experiments examined soot generated from atmospheric pressure ethylene/air laminar diffusion flames. Without ferrocene addition, pure soot particles were collected. Addition of controlled quantities of ferrocene produced particles containing both iron and soot with iron contents up to 15%. The physical and chemical characteristics of the samples were characterized using a scanning mobility particle sizer (SMPS), X-ray fluorescence spectroscopy (XRF), and carbon aerosol analysis. Results indicated that iron addition caused a substantial reduction in the soot aerosol mass emissions and a notable modification of the particle size distribution when the flame was stable. Carbon aerosol analysis revealed an increase in the organic carbon fraction in the iron-soot particles when compared to the soot only particles or to a physical mixture of soot and commercially purchase γ-Fe₂O₃. Corresponding toxicological experiments involving intratracheal instillation of particles in mice examined indicators of pulmonary inflammation and induced allergic asthma-like responses to samples composed of soot only, soot-iron additive composites, and physical mixtures of soot and commercially purchased gamma-Fe2O3 nanoparticles. The soot only samples overall induced the greatest inflammatory response at a time point of 4 hours post-instillation. The levels of pro-inflammatory cytokines, interleukin-6, macrophage inflammatory protein-2, and tumor necrosis factor-α showed trends of increase as the amount of carbon, not iron, in the samples increased. The results suggest that the toxicity of ultrafine particles, such as though from diesel engines or coal combustion, is correlated to the carbon content of the particles, rather than their transition metal content. Future work should examine the effects of other transition metals such as platinum and cerium in conjunction with carbonaceous particles, as well as the toxicological influence of sulfur as compared to iron in the conjunction with carbonaceous particles.
Physical, Chemical, and Toxicological Characteristics of Combustion Generated Soot-Iron Particles

by

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DEDICATION

This work is dedicated to Ryan for believing in me, encouraging me, and supporting me. I could not have done this without you, and I am so blessed to have you as my husband and best friend.
Audrey Turley is the wife of Ryan, mother of Samuel, dog mom of Lucy and Desi, daughter of George and Ana, sister of Laura and Scott, and friend to Corrie, Ariel, Katie, and Julie. Without the encouragement of these people, this thesis would not exist.

Audrey was raised in Houston, TX and attended Texas A&M University, graduating in 2002. WHOOP! With a degree in mathematics, Audrey taught high school geometry and math models at Bryan High School in Bryan, TX. On July 5, 2003, Audrey married Ryan Turley, also an A&M graduate, and they moved to Durham, NC for Ryan to attend Duke Medical School. Audrey taught Integrated Math 1 and 2 to students in grades 8 through 12 at Durham School of the Arts and coached (with no prior experience) middle school cheerleading. Sometime during that second year of teaching, the desire to go back to school for a higher degree became so pressing that it could no longer be ignored. Audrey took a job as a math content specialist and assistant project manager at a company that creates standardized tests for use in public schools. This enabled her to attend school part-time for two semesters to fulfill the prerequisites necessary to be admitted into the Masters in Mechanical Engineering program at North Carolina State University.

Audrey chose to focus on combustion because of its direct impact on the environment, and she was afforded the opportunity to complete her thesis research under the direction of Dr. Bill Roberts at N.C. State and Dr. Bill Linak at the Environmental Protection Agency. Combustion research turned into toxicology research as well, and Audrey learned much about both subjects from Jong-Ik (Jay) Yoo, Seung-Hyun Cho, Charly King, Dr. Andy Miller, Dr. Jost Wendt, and Dr. Ian Gilmour.

On July 1, 2007, following graduation from medical school, Ryan began his residency in surgery at Duke. On July 12, 2007, with her thesis research complete but her thesis writing incomplete, Samuel McKee Turley was born. He is a distraction but a most wonderful and welcome distraction!

Audrey accepted a position with ICF International, a consulting firm with a focus on environmental issues, among other topics. She now works to develop and apply models for environmental exposure and risk assessment.
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1 INTRODUCTION

The first use of the word “soot” dates back to before the 12th century and is defined in the Oxford English Dictionary as “a black carbonaceous substance or deposit consisting of fine particles formed by the combustion of coal, wood, or other fuel.” Clearly the carbon particulate resulting from incomplete hydrocarbon combustion has been a problem for centuries and remains a problem today. As our knowledge of the soot formation process has increased so to has our insight into the prevention and elimination of soot, as well as our awareness of soot’s harmful health effects.

The primary motivation for this work stemmed from previous experimental results. Coal is composed of a very complex heterogeneous mixture of elements, and thus, the soot produced as a result of the combustion of coal also contains a very complex heterogeneous mixture of elements. Soot collected from the combustion of coal in a drop-tube furnace revealed the presence of iron and specifically iron oxide nanoparticles of the form $\gamma$-Fe$_2$O$_3$ (Linak and others 2006; Cho and others 2008). It has been suggested that the iron may promote oxidative stress within the cardiovascular system by catalyzing the formation of free radicals. Utilizing a diffusion flame, it was possible to produce soot doped with iron in order to examine the resulting particle size distribution and soot composition. In addition, the toxicological effects of this iron-doped soot were studied via intratracheal instillation in mice so that these effects could be compared with the effects of soot generated from coal combustion.

One possible method for reducing potentially harmful soot emissions, especially from diesel-powered internal combustion engines, is the use of metallic fuel additives, such as those derived from iron. A number of commercial diesel fuel additives containing metal catalysts such as ferrocene, platinum, and cerium have been designed to reduce emissions of carbon monoxide, hydrocarbons, and soot as well as to improve engine performance. However, as these metals are not consumed during combustion, they can be emitted into the environment and result in human exposures. The physical and chemical characteristics as well as the toxicological effects of the iron-soot compounds described in this work are secondarily applicable to a beginning study of the health effects of iron-based fuel additives.
1.1 **SOOT FORMATION**

Soot in a flame is a key indicator of the interaction between the fluid mechanics of the flame and the combustion chemistry. First, soot precursors, in the form of acetylene (C$_2$H$_2$) are formed in the flame by pyrolysis, i.e. decomposition of the fuel by heat. The acetylene molecules then recombine to form benzene rings (C$_6$H$_6$). The benzene rings polymerize to form polycyclic aromatic hydrocarbons (PAHs) with a carbon to hydrogen ratio of approximately one. PAHs are thought to be responsible for the mutagenic and carcinogenic characteristics of soot. As the benzene rings condense further, hydrogen is lost and the carbon to hydrogen ratio increases. The hydrogen atoms go on to form hydroxyl and water molecules. Depending on the structure of the parent fuel molecules, these decomposition and recombination steps may be skipped, and thus, the soot formation process is more rapid. Soot nucleation occurs when enough benzene rings combine to form a crystallite, and surface reactions cause the crystallite to increase in size to be classified as a spherule. When the size of the spherule reaches 10 to 50 nm, the growth stops. At this point, the spherules begin to combine, forming agglomerates. Soot, with an empirical formula of C$_8$H, has an emissivity approaching that of a black body (Kuo 2005; Roberts 2005).

1.2 **TRANSITION METALS IN ULTRAFINE COAL FLY-ASH PARTICLES**

In a previous studies (Linak and others 2006; Cho and others 2008) coarse, fine, and ultrafine coal-fly ash particles were collected and examined. Mossbauer spectroscopy of the size-fractionated fly ash revealed iron in all size fractions. In the ultrafine size fraction, nanoparticle sized iron existed in the form of iron oxide, $\gamma$-Fe$_2$O$_3$. Transmission electron microscope (TEM) images revealed that the iron oxide nanoparticles were often bound in the particulate matter with carbonaceous species.

Through intratracheal instillation in mice, it was revealed that the ultrafine particles were more toxic than both the fine and coarse particles. In addition, the chemical toxicity of the ultrafine particles could be correlated with the carbon content of the given particles. However, it was unknown whether this increased pulmonary injury was the result only of the carbon content of the particles or also a result of the presence of trace or transition metals including highly reactive iron oxide nanoparticles. Iron is a major component of coal ash, and theories exist that suggest that transition metals induce oxidative stress. It was speculated that the iron oxide nanoparticles may promote the formation of free radicals in the cardiovascular system and thus lead to oxidative stress (Linak and others 2006).
1.3 **IRON IN THE FLAME**

The behavior of ferrocene in a diffusion flame has been studied by other researchers prior to this work, and the aim of this work was to verify previously reported results while extending those findings with the addition of toxicology results.

Ferrocene dissociates early in the flame, prior to soot inception, and the remaining iron oxide particles act as nuclei for soot formation (Kasper and others 1999). This behavior increases soot inception early in the flame. As the iron oxide particles traverse through the flame, they collect carbonaceous particles until the surface of each iron oxide particle is completely covered. In the oxygen rich regions, towards the tip of the flame, the iron accelerates the soot oxidation process and the iron oxide particles begin to reappear alone, with no carbons attached. Finally, the iron oxide is reduced to iron via the following reaction

\[
\text{Fe}_x\text{O}_y + \text{C} \rightarrow x\text{Fe} + y\text{CO}
\]

(Kasper and Siegmann 1998).

The work of Zhang and Megaridis (1994) emphasized the formation of a “chemically inhomogeneous” soot iron compound in an ethylene-air diffusion flame. The particles they extracted from inside the flame were examined and showed iron inclusion within layers of carbonaceous matter.

Zhang and Megaridis (1994) examined the effects of ferrocene addition on the soot particles inside the flame. In a laminar diffusion flame burning ethylene in air, ferrocene enhanced soot oxidation rates in the flame operating above its smoke point. At the tip of the flame, the soot volume fraction was greatly decreased and the mean particle size was also significantly smaller. However, the ferrocene did not affect the soot volume fractions in regions of the flame where soot growth dominated oxidation. In these regions, the addition of ferrocene did not change the mean particle diameter either (Zhang and Megaridis 1996).

Kasper and co-workers showed in 1999 that in a non-smoking methane or acetylene diffusion flame, ferrocene did not change the total amount of particulate matter produced in the flame. Rather, the ferrocene promoted more efficient burnout of the carbonaceous matter formed (Kasper and others 1999). In regions of an ethylene-oxygen-nitrogen diffusion flame where soot formation dominated soot oxidation, it was shown that the total soot collected was up to an order of magnitude greater in a ferrocene-seeded flame compared to an unseeded flame (Ritrievi and
others 1987). A similar trend was shown in a premixed propane-oxygen-nitrogen flame also operating above the smoke point (Charalampopoulos and others 1992).

Also in a premixed laminar ethylene flame, Hirasawa and co-workers (2004) reported an increased propensity to soot near the origin of the flame in a ferrocene-doped flame when compared to a non-doped flame. This observation supported the theory of others that the addition of ferrocene initially promotes soot nucleation in the flame and then later increases oxidation by acting as a catalyst.

In a laminar premixed ethylene flame, researchers at Massachusetts Institute of Technology found that adding ferrocene to the flame increased the soot volume fraction within the flame but did not change the total number of soot particles within the flame. Instead, the flame, when seeded with ferrocene, produced larger particles. They speculated that the soot-reducing characteristic of ferrocene comes into play in the soot oxidation regions of the flame where the amount of oxygen available is greater (Feitelberg and others 1993).

In a soot-emitting acetylene diffusion flame, it was shown that the addition of ferrocene almost completely eliminated soot particles above the flame. The flame did continue, however, to emit iron oxide particles with a mean particle diameter of around 20 nm (Kasper and others 1999). Another group found similar effects. In a smoking isoctane-air diffusion flame, the addition of 0.3% ferrocene by weight of fuel eliminated the emitted smoke. These researchers recorded that after nucleation, the presence of ferrocene increase the particle size and number density early in the flame and then decreases the size and density later (Bonczyk 1991).

Along the same lines, Yang (2004) reported that iron “promoted soot formation in the flame but it also suppressed soot emission from the flame tip” in an ethylene-acetylene-air diffusion flame. Based on measurements taken from scanning electron microscopy images of samples emitted from the flame, soot particles generated from flames doped with iron pentacarbonyl were one third the size of particles from a non-doped flame (Yang 2004). Similar results were recorded by the same group in 2001 as the result of analysis using a scanning mobility particle sizer above the flame (Yang and others 2001). When doped with iron pentacarbonyl, the largest particles from the flame disappeared and the mean particle diameter shifted to between 50 and 80 nm (Yang 2004). Another group of researchers showed that ferrocene in the flame above a few hundred parts per million loses its initial effectiveness (Linteris and others 2000).

Kasper and Siegmann (1998) extracted gas samples at various heights in both methane and acetylene fueled diffusion flames doped with ferrocene. The study of the effect of ferrocene
on a methane flame was original to this work. Differences in PAH formation between the ferrocene-seeded and the unseeded flames were expected because each methane molecule has only one carbon to contribute to PAH formation. In the methane fueled flame, the ferrocene attracts carbon molecules which are then no longer available for the formation of acetylene, the primary PAH building block. Thus, the concentrations of all PAHs are smaller in the ferrocene-seeded methane flame than they are in the unseeded methane flame. However, they found that in the acetylene flame the PAH concentration did not change with the addition of ferrocene. They speculate that this is because the C$_2$H$_2$ is readily available through the fuel, and thus the iron-acetylene association does not decrease the amount of C$_2$H$_2$ available for PAH formation.

In summary, it has been shown by others that the addition of iron to a flame initially enhances soot growth because the iron particles serve as nuclei for the soot formation. Later in the flame, however, iron acts as a catalyst and stimulates the oxidation of soot before it is emitted from the flame. In general, the particles emitted from a flame doped with iron are smaller and fewer in number. However, above a certain iron concentration, this is no longer the case.

1.4 IRON IN DIESEL ENGINES

The addition of catalytic transition metals to diesel fuel has been shown to reduce the total particulate matter emitted from the engines. However, this reduction in harmful emissions is accompanied by an increase in the amount of iron emitted in the exhaust. Skillas and others reported a significant decrease in total mass of particles emitted from a diesel generator as the mass of iron added to the generator increased (2000). With this overall decrease in total mass, a decrease in particles with diameters greater than 50 nm occurred but was accompanied by an increase in smaller particles (Skillas and others 2000). Another group reported a similar trend of a decrease in overall emissions accompanied by an increase in smaller particles, especially those with iron nuclei agglomerated with carbon and also iron-only nanoparticles. Due to the nano-scale size of the iron-carbon particles, the group suggested further research into the health effects of such particles (Miller and others 2007).

A heavy duty diesel engine, operating on fuel doped with 120 mg of ferrocene per kilogram of diesel fuel, showed a 50% reduction in the number of carbonaceous particles emitted. The emissions contained finely dispersed iron oxide nuclei, but these nuclei did not contain an outer coating of carbonaceous particles as is seen emitted from diffusion flames. The researchers suggested that in turbulent combustion, like in diesel engines, the iron oxide particles and the soot
particles are formed simultaneously rather than in separate steps (as in a diffusion flame where the iron oxide particles are first formed and the carbon particles coagulate on their surfaces leading to increased carbon burnout). This formation mechanism means that the iron oxide particles and the soot particles do not interact and the same degree of catalytic effects of the iron oxide seen in diffusion flames are not seen in diesel engines (Kaspar and others 1999; Matter and Siegmann 1997).

Lee and others presented the results of doping diesel fuel with ferrocene in 2006. They observed the formation of iron-rich nanoparticles that increased in number, as well as in size, as the iron content of the fuel was increased by the addition of increasing amounts of ferrocene. They found higher hydrogen to carbon ratios in smaller particles as compared to the larger particles emitted by the engine. This was indicative of increasing organic carbon percentages as the amount of ferrocene in the fuel increased (Lee and others 2006).

1.5 SOOT-IRON TOXICOLOGY

It has been established that ambient air pollution, composed of carbonaceous particulate matter, ozone, sulfates, and nitrates among other components, causes pulmonary inflammation in humans (Chuang and others 2007; Ghio and others 2000). For example, researchers recently established that those with pre-existing respiratory ailments, such as asthma, experienced decreased lung function and an increase in pulmonary inflammatory markers following a 2 hour walk down a busy street in London (McCreanor and others 2007).

The research community continues to seek an answer to the question of which characteristic of particulate matter makes it toxic: mass, size, composition, or a combination of these factors. Studies have shown that the adverse health effects of particulate matter, specifically PM$_{10}$ particles, is due to the generation of hydroxyl radicals that damage or degrade the DNA of affected cells (Gilmour and others 1996). Many researchers believe that transition metals attached to the surfaces of particulate matter may facilitate the formation of free radicals in the body and lead to inflammation and other toxic effects (Berube and others 1999).

Researchers at several institutions have sought to determine the effect of iron on the toxicity of carbonaceous particles. An in-vitro study using human lung epithelial cells examined the inflammatory response and the increase in reactive oxygen species following exposure to particulate matter of different size fractionations and transition metal contents, specifically iron. The particulate matter, obtained from the combustion of coal, showed that smaller particles
produced greater inflammatory effects and created more bioavailable iron within the lung cells. The researchers hypothesized that the iron content of the particulate matter may be directly responsible for these differences (Aust and others 2002).

A study performed at the University of California at Davis used a diffusion flame burner with ethylene, air, and iron pentacarbonyl (Zhou and others 2003). The experimental set-up as well as the physical and chemical characteristics of the generated soot are presented in articles described above by Yang and others (2001) and also independently by Yang (2004).

As for the toxicology, rather than instillation, groups of three rats were subjected to inhalation of the emitted soot-iron particles, to only soot particles, or to only iron particles for six hours a day for three days. Exposure to soot only (with no iron additive) and exposure to iron only (with no accompanying soot) resulted in no significant changes in oxidative stress, lung injury, ferritin levels, or proinflammatory cytokines. However, exposure to soot with included iron particles with a total mass concentration of 250 μg/m³ (45 μg/m³ iron) showed a 2.6-fold increase in ferritin when the levels were compared to those of rats exposed only to filtered air. In addition, the level of the pro-inflammatory cytokine IL-1β increased in rats exposed to soot with iron while the level of IL-1β in rats exposed to either soot only or iron only did not show a significant increase. Levels of the pro-inflammatory cytokine TNF-α did not change significantly in any of the exposure groups. Based on these findings the authors speculated that exposure to soot plus iron induces a biological response because of the “synergistic interaction between soot and iron particles” while exposure to soot only or iron only does not induce the same response (Zhou and others 2003).

The work described here differs from the UC-Davis work in that ferrocene was used instead of iron pentacarbonyl. Iron pentacarbonyl is explosive, highly flammable, and evaporates in the air at 20°C; therefore, we elected to use a safer alternative, ferrocene. In addition, the UC-Davis work used inhalation with groups of 3 rats as opposed to the instillation with groups of 6 mice that was used in this work. A comparison of the results of this work to those generated at UC-Davis follows in the section titled “Toxicological Characteristics.”
2 Experimental Approach

2.1 Particle Generation

Soot particles containing varying amounts of iron were generated and collected to study their chemical, physical, and toxicological characteristics. Using a Burke-Schumann laminar diffusion flame allowed the study of the basic behavior of metal-based fuel borne catalysts in combustion devices relatively inexpensively. The advantage to this approach was that a larger number of tests could be conducted to evaluate how emissions changed under different conditions at a relatively low cost. Iterative testing was performed in order to identify and resolve potential problems with sampling and elemental and speciated analyses and provided an opportunity to identify possible unexpected behavior.

2.1.1 Diffusion Flame Burner

A co-annular laminar diffusion burner similar to the one used by Santoro, Semerjian, and Dobbins (1983) was used to create the iron-doped flame so that particles could be collected in necessary quantities. The brass fuel tube had an inner diameter of 11.1 mm and was surrounded by the co-annular oxidant tube with an inner diameter of 101.6 mm. To create a uniform velocity profile in the flame, both the fuel and oxidant tubes were packed with 3 mm glass beads. In addition, the oxidant passed through a series of 40 and 70 mesh screens as well as a 2 inch layer of ceramic honeycomb before entering the combustion zone. Additional details of the burner are shown in Figure 1.
Figure 2 on the following page depicts the diffusion flame burner and sampling system. Briefly, the fuel and air for these experiments were delivered to the burner through mass flow controllers calibrated by the metrology lab at the Environmental Protection Agency. Ethylene (C₂H₄) was used as the primary fuel and as the carrier gas for the iron, but it was supplemented by the addition of additional ethylene or acetylene (C₂H₂) in order to insure adequate soot production even in the presence of the soot-reducing iron.

The flame was housed in a clear globe ducted to a 2 inch stainless sampling ductwork with ports. The system remained at negative pressure through the use of a ring compressor fan pulling exhaust and HEPA filtered dilution air. The iron delivery system will be described in a subsequent section.
Figure 2: Diffusion flame burner and sampling system
2.1.2 **IRON DELIVERY SYSTEM**

The iron was added to the fuel using vaporized ferrocene powder. The vapor pressure of ferrocene between 277 and 360 K as a function of temperature can be found using the following equation,

\[
R \ln(p) = 15.196 + 74290 \left( \frac{1}{\theta} - \frac{1}{T} \right) - 71 \left[ \frac{\theta}{T} - 1 + \ln \left( \frac{T}{\theta} \right) \right],
\]

where \( R \) is the universal gas constant in J/K mol, \( p \) is the vapor pressure in Pa, \( \theta = 317.20 \) K, and \( T \) is the temperature in K (Jacobs and others 1983). The fuel line was coiled and placed in a constant temperature water bath so that the fuel would be preheated prior to reaching the ferrocene tube, which is also in the water bath. For the ferrocene tube, layers of ferrocene and 3-mm glass beads were packed in alternating layers in a 13 cm stainless steel tube. Glass wool was used to prevent the ferrocene powder and glass beads from escaping the tube and entering the fuel line. This ferrocene tube design is similar to one used by previous researchers (Zhang and Megaridis 1994).

![Figure 3: A section of the ferrocene vaporizer cylinder](image)

The fuel line after the water bath was wrapped in heat tape to prevent the vaporized ferrocene from condensing and depositing in the fuel tube. The fiberglass heat tape was further insulated, wrapped in sheets of aluminum and controlled using a temperature controller.
2.1.3 **PARTICLE GENERATION PROCEDURE**

The following details the procedure used to generate soot particles containing iron.

1. **Begin heating the water bath.**
   a. The ferrocene tube should not be in the water bath during heating.
   b. Ensure that the bath is filled to the maximum level.
   c. Place the heaters in the bath and turn them both on.
   d. Place the thermocouple in the bath and turn it on also.
   e. Plug the bath in.
   f. The set temperature on the water bath should be 4°C below the desired water temperature.
   g. The water bath takes approximately 30 minutes to reach the set point temperature. It is important to wait until the water bath itself has reached the set point temperature, even if the water is at the correct temperature, before beginning sampling.

2. **Load the ferrocene tube.**
   a. The ferrocene tube should be disconnected on both ends from the fuel line so that it can be refilled.
   b. In the hood located in room H106I, begin by removing the glass wool from one end of the ferrocene tube and allowing the glass beads, ferrocene, and remaining glass wool to empty into a labeled waste container.
   c. When the tube is empty, insert a new piece of glass wool into one end of the tube.
   d. Follow the glass wool with a layer of glass beads (approximately 10 beads).
   e. Add a small amount of ferrocene (approximately 0.5 mL) to cover the glass beads.
   f. Repeat the layers of glass beads and ferrocene until 1 cm of the tube remains empty, ending with a layer of glass beads.
   g. Fill the top end of the tube with glass wool and screw the swage-lock end caps on.
   h. Reinsert the ferrocene tube into the fuel line, making sure the fittings are as tight as possible.

3. **Turn on the exhaust.**
   a. The exhaust scrubber is located at the far end of H106. Control of the system is accessed through the computer on the desk in front of the scrubber system.
   b. Turn on the scrubber by clicking On/Off from the home screen.
   c. Turning on the scrubber will turn on the fan also.
   d. The pressure should rise to 2 inches of water

4. **Turn on the air.**
   a. To the right of the system, mounted on the ceiling support column are the controls for the compressed air.
   b. Slide the yellow handle parallel to floor.
   c. Pressure valve should be set at 20 psi.
   d. Plug in flow controller box and adjust the air flow rate.

5. **Turn on the fan and sampling pump.**
   a. The exhaust from the large fan should go through a hole and into the Rainbow furnace where it is exhausted.
   b. Plug in both the fan and sample pump.
c. Turn the large pump all the way on.
d. Leave the small sampling pump closed (no flow on flowmeter) for the time being.

6. Turn on the temperature tape.
   a. Plug in temperature tape.
   b. Wait for temperature to rise on control box to desired temp, a minimum of 10°C above the water bath set point.

7. Turn on the ethylene
   a. In closet, turn large knob on top of tank to open
   b. Turn small knob to open
   c. Pressure should be 20 psi on exit gauge
   d. Bottle pressure should be above 80 psi – if not, order more

8. Light the flame.
   a. On flow controller box, adjust ethylene to low flow, about 1 volt
   b. Light burner with ignitor
   c. If it won’t light, increase the ethylene flow

9. Adjust flows to sample rates

10. Notes on the use of acetylene as supplementary fuel
    a. Begin the addition of acetylene after the flame is lit and running with ethylene.
    b. Ensure that the acetylene mass flow controller is completely closed.
    c. Open the acetylene bottle, and then open the valve very slowly.
    d. After rotating the acetylene valve at least 3 times but fewer than 6 full revolutions, adjust the acetylene flow rate using the mass flow controller.
    e. Initially, the acetylene will cause the flame to grow in size and intensity. After the addition of the ferrocene with the ethylene reaches steady state, these effects will be less noticeable.

A record of the flow rates used to create the samples for these experiments is found later in Section 3.1.1.

2.2 Real-Time Particle Characterization

When iron is added to the fuel prior to combustion, the size distribution of the emitted particles shifts and the particles have a distinctly smaller size. By monitoring this shift in the particle size distribution, the presence and activity of iron in the flame can be verified. A Scanning Mobility Particle Sizer (SMPS), Aerosol Particle Sizer (APS) and Condensation Particle Counter (CPC) are used to determine the particle size distribution of the emissions.

2.2.1 Scanning Mobility Particle Sizer

The Scanning Mobility Particle Sizer (SMPS) (TSI 3080) operates based on the relationship between electrical mobility and particle size of singly charged particles. Using a
long Differential Mobility Analyzer, the SMPS classifies particles in the range from 10 to 1000 nanometers in diameter (0.01 to 1 \( \mu \)m).

The particles first enter an inlet impactor (pre-separator). In the impactor, particles above the cut-point diameter \( \text{D}_{50} \), generally 1 \( \mu \)m, are removed from the particle stream. The cut-point diameter is a function of the aerosol velocity and the nozzle diameter. The aerosol stream flows through the nozzle of the impactor, and particles with diameters above \( \text{D}_{50} \) impact on the plate because their inertia is too great, and they cannot follow the 90° bend in the stream line that is created by the impaction plate.

It is necessary to remove these large particles from the aerosol stream when they are present because they adversely affect the final charge and thus the determined particle size distribution. These large particles can, however, be counted and sized when the Aerodynamic Particle Sizer (APS) is used in conjunction with the SMPS.

Following the inlet impactor, the particle stream moves into the Kr-85 Bipolar Charger, also called an aerosol neutralizer. In the neutralizer, the particle stream is bombarded with bipolar ions, and the particles in the stream reach a charge equilibrium. From the neutralizer, the particles move to the DMA where they are sorted based on electrical mobility and thus their size. In the DMA, the charged particles come in contact with an electric field that increases the velocity of each particle up to its terminal velocity. The ratio between the terminal velocity and the magnitude of the electrical field is defined as the electrical mobility. The electrical mobility is inversely related to particle size and is proportional to the number of charges on the particle.
The DMA consists of two concentric metal cylinders and the aerosol and a stream of sheath (or excess) air flow between the cylinders. The inner cylinder has a negative charge while the outer cylinder remains neutral. Particles that have a positive charge are attracted to the inner cylinder where they precipitate. The particles with the highest electrical mobilities precipitate closest to the top of the inner cylinder and those with the lowest electrical mobilities precipitate near the end of the high voltage inner cylinder. Through this classification, only particles within a narrow range of electrical mobilities pass all the way through and exit the DMA as a monodisperse aerosol. This monodisperse aerosol moves on to the Condensation Particle Counter (CPC) while the particles collected on the inner cylinder are purged with the exhaust gas.

2.2.2 CONDENSATION PARTICLE COUNTER

The TSI 3775 Condensation Particle Counter (CPC) detects particles as small as 4 nanometers in diameter in concentrations between 0 and $10^7$ particles/cm³. The monodisperse aerosol sample stream exits the DMA and enters a heated saturator (39° C) in the CPC. In the saturator, butanol is vaporized and mixes with the sample stream. The sample stream and the supersaturated butanol move into the cooled condenser (14° C) and the butanol begin to condense on the particles in the aerosol sample stream, forming droplets. In the single particle counting mode, each droplet is counted by a photodetector and its diameter recorded.

2.2.3 AERODYNAMIC PARTICLE SIZER SPECTROMETER

The Aerodynamic Particle Sizer (APS) (TSI APS 3321) records the size distribution of particles between 0.5 and 20 μm (500 to 20,000 nm). In the APS, the sample aerosol flow enters the instrument and is split into two streams, a sample flow and a sheath flow. The sample flow is accelerated through an inner nozzle, while the sheath flow moves through the outer nozzle surrounding the inner nozzle. When the particles are accelerated, the smallest particles will move with greater velocity than the larger particles due to the size and thus inertia of the larger particles.

The inner nozzle deposits the fast-moving sample particles just prior to the exit of the outer nozzle. In this way, the sample particles are trapped in a center stream that is surrounded by the sheath flow. The streams move together though two laser beams and the accelerated particles scatter light as they move. The scattered light is captured by a photodetector and converted to electrical pulses. The velocity of each particle can be determined from the peaks between
electrical pulses. The APS uses the known velocity, or time of flight, for each particle to predict the aerodynamic diameter of the particles.

2.2.4 SMPS/APS CALIBRATION

The APS was factory calibrated for flow and electronics in May 2004. The SMPS was factory calibrated for flows and electronics in September 2001. Further, the SMPS was calibrated for flow in April 2004 using a Gilabrator Bubble Meter.

2.2.5 PARTICLE SIZE DISTRIBUTION PROCEDURE

The following details the procedure used to collect particle size distribution data.

1. Attach the SMPS sample line to the sampling port for the experimental system.
2. Power on the Condensation Particle Counter (CPC).
   a. The green indicator lights next to Optics, Condenser, Saturator, and Laser will light indicating that the temperature for these components is satisfactory.
   b. Connect the butanol line/bottle to the port labeled Liquid Supply on the back of the CPC. For best results when filling, the butanol bottle should be placed above the CPC.
   c. On the front of the CPC, press Shift and then Total to initiate the butanol fill.
   d. When the butanol fill is complete, the green indicator next to Liquid Level will light.
3. Start the pump on the CPC by pressing Pump. Again, the green indicator next to Flow will light.
4. Turn on the Electrostatic Classifier and wait for the initialization process to be completed.
5. Turn on the high voltage collector rod.
   a. On the front of the Classifier, turn the control knob to highlight Panel Control on the LCD display.
   b. Then press the knob to switch from Panel Control to Analog Control.
6. Adjust the sheath flow rate.
   a. Use the control knob to move to Sheath Flow on the LCD display.
   b. Push the control knob to select so that the sheath flow rate can be entered.
   c. Turn the control knob clockwise until it reaches 3.0 LPM.
   d. Push the control knob a second time to accept this sheath flow rate.
7. The sample flow rate should be 0.30 LPM.
8. If the sample flow rate is greater than 0.32 LPM, the impactor needs to be cleaned.
10. Create a new file by selecting New from the File menu.
    a. Enter the name of the new file. Files are generally named with the date of sample collection followed by a hyphen and the sample number.
    b. Click Open to create the new file.
11. Choose Run (or click on the green circle) to begin data collection.
2.3 PARTICLE COLLECTION

Particles generated by the diffusion flame burner were collected on filters for analysis of their chemical and toxicological characteristics. The filters were kept in labeled polyethylene petri dishes both before and after use. Filters were prepared for sampling according to their purpose. Quartz filters used for carbon analysis were baked at 105°C for 12 hours prior to use. Teflon-coated quartz filters were analyzed as blanks using x-ray fluorescence spectroscopy before use. Both types of quartz filters were weighed before and after sampling to a constant weight, and the results were recorded to the nearest 0.1 mg. The weighing protocol can be found in the following section.

<table>
<thead>
<tr>
<th>Filter Type</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycarbonate</td>
<td>Elemental analysis by x-ray fluorescence spectroscopy</td>
</tr>
<tr>
<td></td>
<td>Bulk sample collection for animal instillation</td>
</tr>
<tr>
<td>Quartz</td>
<td>Elemental carbon and organic carbon analysis</td>
</tr>
<tr>
<td>Teflon coated quartz fiber</td>
<td>Elemental analysis by x-ray fluorescence spectroscopy</td>
</tr>
<tr>
<td></td>
<td>Bulk sample collection for animal instillation</td>
</tr>
</tbody>
</table>

Particulate matter was withdrawn isokinetically through a sampling port in the burner duct work and collected on the various types of filters for analysis. By collecting on two filters simultaneously it was possible to collect a sample for elemental analysis during each run so that the soot composition could be verified by x-ray fluorescence (XRF) spectroscopy each time.

A system of ½” stainless steel tubing and Swagelock fittings was attached to the sampling port on the system duct. The exhaust flow from the burner was pulled through the sampling port by a rotary vein pump and two stainless steel 43-mm filter holders were used to collect simultaneous samples. The holder design provided a positive seal against leakage from the outside or around the filter.
Samples for XRF analysis and animal instillation were collected until a significant cake of particulate matter collected on the filter. The build-up of this cake was indicated by a drop in pressure through the filter. Samples collected for EC/OC analysis require only a light coating of particulate matter on the filter.

2.4 SAMPLE ANALYSIS

2.4.1 X-RAY FLUORESCENCE SPECTROSCOPY

X-ray fluorescence (XRF) was conducted using a wavelength dispersive (WD) Philips PW2404 XRF spectrometer (Panalytical, Natick, MA). To characterize the elements present in a sample using WD-XRF, energy greater than the binding energy of the electrons at the lowest
energy level of an atom is applied to the sample. This forces an electron in the lowest energy level to be released from the atom. An electron from the next highest energy level moves down to fill the vacancy in the lower energy level, and in doing so, a photon or quanta of energy, is released. Every element has its own characteristic binding energies necessary to maintain the orbit of the electrons around the nucleus, and thus if the energy of the photon that is released when the higher energy electron drops to the lower energy orbital is known, that amount of energy can be used to identify the element. This unique energy is referred to as the element-characteristic wavelength. Using irradiation from x-rays generated in an x-ray tube, as in a WD-XRF spectrometer, is the safest and most reliable way to perform this qualitative and quantitative elemental analysis (Scholtz and Uhlig 2006).

The excitation source for Philips PW2404 WD-XRF spectrometer is a 4kW rhodium x-ray tube with an end window. The source is capable of producing excitation up to 60 kV. The sealed tube is maintained at a constant temperature through cooling with deionized water and operates under a vacuum. This spectrometer is capable of measuring both quantitatively and qualitatively all elements with atomic numbers greater than 9 (fluorine).

To characterize the energy of the released photons, the WD-XRF employs a collimator, a goniometer for crystal control, a flow detector, a scintillation detector and a data collection and processing system. The WD-XRF uses a single measuring channel spectrometer that measures one element at a time, in a sequential order (Philips-Electronics 1997).
The fluorescent radiation released from the sample after it is bombarded with x-rays passes first through a collimator mask to ensure that only fluorescent radiation will enter the collimator. This fluorescent radiation is a blend of all of the element-characteristic wavelengths from the elements present in the sample. The collimator, or wave guide, filters the fluorescent waves entering it so that only those that will be parallel to the analyzing crystal are allowed to penetrate to the crystal. WD-XRF uses the diffraction pattern produced by the analyzing crystals to separate the wavelengths.

A crystal is a solid composed of a repeating, patterned arrangement of atoms or molecules that extends in all spatial dimensions. The arrangement of the atoms or molecules is called the crystal lattice, and, because of the repeating, patterned arrangement in a crystal, there are many different lattice planes that run parallel to the plane through any given atom or molecule. These planes are equally spaced a known distance apart, called the lattice plane distance, d. If the angle that the wave hits the crystal, \( \theta \), is measured and the lattice plane distance for the crystal is also known, the wavelength can be determined by Bragg’s Law:

\[
n \lambda = 2d \sin \theta
\]

where \( n \) is the reflection order and \( \lambda \) is the wavelength. The WD-XRF uses a goniometer to rotate the crystal and the detectors so that their precise angular position and thus \( \theta \), is always known (Scholtz and Uhlig 2006).

The diffracted element-characteristic wavelengths move into the detectors where the wavelengths are converted to pulses that are proportional to the x-ray radiation and inversely proportional to the wavelengths. The flow detector measures longer wavelengths by utilizing the ionization of argon gas in an electric field to convert the wavelengths to electrical pulses. The scintillation detector converts the x-rays into light and measures them with a photomultiplier. It functions best for shorter wavelengths (Jenkins 1999).

The data collected from the spectrometer is stored in SuperQ (Panalytical, Natick, MA) and can then be analyzed using UniQuant (Omega Data Systems, The Netherlands). Analysis results are considered to be reliable and significant when the signal for the element is greater than 2 times the standard error for that element.

### 2.4.1.1 XRF Sample Preparation

For accurate analysis, samples analyzed by XRF must be representative and heterogeneous. The x-rays from the tube penetrate approximately 20 µm into the sample and
total area irradiated is around 5 cm$^2$. For this reason, it is especially important that small samples be evenly distributed in all directions over the analysis area (Jenkins 1999). In addition, small samples must be distributed to a depth that can be considered infinitely thick. The surface of any sample analyzed should be smooth with no irregularities (Scholtz and Uhlig 2006).

For the diffusion flame burner, samples for XRF were collected in either of two ways. First, particulate matter in the exhaust gas stream was pulled through a Teflon-coated quartz filter. Each 43-mm filter was first analyzed using XRF as a blank filter with a film cover before any sample was collected on it. The filters were weighed pre- and post-collection to determine the mass of collected particulate matter. In addition, the diameter of the area covered with sample on each filter was measured. The filter samples collected from the flame were prepared with 0.1 mm polypropylene film covers (Chemplex Industries, Stuart, FL) to prevent contamination of the spectrometer with particulate matter, and the UniQuant program takes into consideration the film cover in its analysis of the sample. Each filter was then mounted flat in a stainless steel sample holder for analysis.

Other samples were pulled through polycarbonate filters. Using polycarbonate filters allows for analysis of a smaller sample and generally produces a more uniform sample surface since the filters do not have a weave like Teflon coated quartz filters. The polycarbonate filters were weighed and measured just like the Teflon coated filters. The filters were fitted with polypropylene film covers (Chemplex Industries, Stuart, FL) and then mounted in plastic filter cups for analysis.

The XRF procedure used, along with the inputs for analysis by the UniQuant program are included here.

### 2.4.1.2 XRF Calibration

The WD-XRF is calibrated monthly by a trained specialist, and calibration records are available.
2.4.1.3 PROCEDURE FOR XRF

The following details the procedure used to analyze samples with XRF. This procedure is written for the Philips PW2400 spectrometer.

1. This process initializes the spectrometer and warms up the X-ray tube prior to beginning analyses.
   a. Close all Super Q windows that are open.
   b. Open Super Q Manager.
   c. Click on Measure & Analyze.
   d. When prompted, click No, you don’t want to compact.
   e. When prompted, the password is quantitative. Click OK and then wait.
   f. Open the System menu at the top of Measure & Analyze. Choose Spectrometer Status Screen.
   g. The spectrometer will be at 60kV-40 mA 1 minute after opening Measure & Analyze.
   h. Wait 1 hour for the spectrometer to warm-up.
   i. In the green log book, add a new entry:
      
      | Date | Opened SuperQ. At 60 kV-40mA at (time) |
      | Initials | Begin analysis at (time) |
      
2. Load the samples into the XRF sample changer, beginning with position A1. Refer to the procedure titled XRF Sample Preparation for further information.
3. In Measure & Analyze, open the Measure menu at the top of the screen.
   a. Choose Open Sample Changer.
   b. Select Sample Changer from sample list.
   c. Click OK.
4. Add the samples to be analyzed to the Sample Changer.
   a. Double click on position A1 on the Sample Changer Map or select position A1 and then click on Add New Sample on the right side of the screen.
   b. Select the Application UQ5Fast.
      i. Type: NJOB (this is filled in automatically)
      ii. Job #: this should be filled in automatically with the correct Job Number, but it should be verified with the last entry in the log book.
      iii. Description: Enter the name to give the sample.
      iv. Click Add on the right side of the screen and repeat this procedure until all sample have been entered.
   v. After the final sample, click OK, rather than Add.
5. To begin measuring, click Start on the Sample Changer Map.
6. After measurement is complete, click OK.
   a. Then on the Sample Changer Map, click End of List to move the arm of the sample changer back to its resting position.
   b. Again, on the Sample Changer Map, select all of the samples by clicking on them while holding Shift or Ctrl. Then click on Remove Samples on the right side of the screen.
   c. Close the Sample Changer Map, but do not close Measure & Analyze.
7. Back on the desktop, open Uniquant Results Transfer. Wait for the list of exports to appear.
8. Open Uniquant 5.27 from the desktop.
9. Import the data from Super Q into Uniquant so that it can be analyzed.
   a. On the Start-up menu, select Full Mode. The title of the menu will change to Main Menu
   b. Select Import.
   c. On the Monitor & Import Menu, select Import again.
   d. Select the appropriate file (the one with today’s date) by right clicking on that file. A message confirming Import OK will appear.
   e. Return to the Main Menu by clicking Back or pressing F1.
10. Select Job from the Main Menu to move to the Job Menu.
    a. Select Job again to enter the job number of the sample to be analyzed.
11. Select GEN to enter the data about the sample. The following gives the information to be entered in each field for any sample. In brackets is the information to be entered for a soot sample or soot with metal sample that has been collected on a filter.
    a. Remark: any descriptive information to be included such as the nature of the sample, the name of the analyst, etc.
       [Leave blank.]
    b. Chemistry: the form of the elements in the sample
       i. E = elements
       ii. S = sulfides
       iii. O = oxides
       iv. X = oxides + CO2
       v. A = alkalies
       vi. I = ionic and is used only for liquid samples
       [O = oxides]
    c. Shape & Impfc: the matrix to be used for the background, only a few of the matrices are set up so Automatic is usually selected
       i. Automatic
       ii. 21 Fi Steel = Teflon filter with film in steel cup
       iii. 22 Fi Al Cup = Teflon filter with film in aluminum cup
       iv. 23 Tflno mask = Teflon filter with film in steel cup without mask
       v. 24 T filter = Teflon filter with film in steel cup with no mask
       vi. 25 Z filter = Zefluor filter without film in steel cup without mask.
       [Automatic]
    d. Case No.: how much is known about the sample
       i. 0 = All Known (mass, area, rest, dilution)
       ii. 1 = Area Unknown (net diameter unknown)
       iii. 2 = % Rest Unknown
       iv. 3 = Dilution Unknown
       v. 4 = Mono-layer Sample (multi-element monolayer with unknown g/cm2)
       vi. 5 = Multi-layer Sample (unknown mass and area)
       [2 = % Rest Unknown]
    e. Kappa List: most samples should be run using the Any Sample setting, however fly ash is run using the Fly Ash setting
       [Any Sample]
    f. Helium:
       i. 0 = vacuum
       ii. 1 = helium
g. Film: film is used over a particulate sample to protect the spectrometer from loose particulate
   i. 0 = none (if pellet sample is analyzed)
   ii. 1 = 0.1 mm polypropylene film
   iii. 2 = 2.5 mm mylar film

h. Report level: concentrations below this threshold (mg/kg) are reported as less than the specified parts per million
   [20 ppm]

i. Sector: area in degrees covered by the sample. Usually 360°, but can be 30°, 60°, 90°, or 180° if sectored centering rings are used.
   [360]

j. Area: automatically calculated by UniQuant
   [leave unchanged]

k. Diameter: the first number is the diameter of the analyzed portion of the sample for example, the diameter of the sample cup opening. The second number is the total diameter of the sample, even the portions not seen by the spectrometer because they are covered by the sample cup.
   [1st: 25 mm, 2nd: measured diameter of filter covered with soot, usually between 37 and 43 mm]

l. Mass: the first number is calculated by UniQuant and represents the mass of the analyzed portion of the sample. The second number is the total mass of the sample.
   [1st: automatically calculated, 2nd: post-collection filter weight – pre-collection filter weight = mass of sample collected]

m. Rho: density, calculated by the UniQuant
   [automatically calculated]

n. Height: thickness of sample, calculated by UniQuant
   [automatically calculated]

o. Shadow: percent of intensity lost due to uneven surface (for example, if the sample is composed of beads)
   [leave at 0 because no correction is needed]

p. % Known Concentration: the first value specifies the known percentage of the sample composed of an element or material that will not be measured; the second value specifies the element or material
   [leave unchanged]

q. % Rest: the first value is an initial guess at the percentage of the sample composed of a known element that will not be measured; the second value specifies the material that will not be measured but should be used in the final composition analysis
   [1st: automatically changes to 1.00 after 2nd is chosen; 2nd: Mat Code: 6 = Carbon]

r. Diluent/Sample: the first value is the ratio of diluent to sample if the sample was diluted in some way (for example, if a pellet was created from the sample combined with liquid binder \( \frac{mg \text{ liquid binder}}{mg \text{ sample}} \)); the second value specifies the diluent material
12. Press Back to save the information entered into the general data fields and return to the Job menu.

13. For soot samples collected on a Teflon coated quartz filter, it is necessary to subtract the original blank filter from the analyzed filter so that only the sample results are reported.
   a. Choose Intensities from the Job menu.
   b. Press $U$ to specify the background intensities calculated from the initially analyzed blank filter. Enter the Job Number of the initially analyzed blank filter.
   c. The background intensities will be entered in the CalcBg column, followed by a $P$.
   d. To clear the entered background intensities, click in the CalcBg column and press $Ctrl + C$.
   e. Press Back to return to the Job menu.


15. Again, on the Job menu, choose Simple Report. This option is only available after the sample has been calculated.

16. The assumptions entered on the General menu are valid if the Sum of Concentrations before normalization is very near 100%.
   a. Between 95% and 100% is an ideal range.
   b. If the Sum of Concentrations is not within this range, the assumptions entered on the General menu should be changed.
   c. The diameter of the sample is a good place to begin when reconsidering the initial assumptions.

17. On the Simple Report, if three times the Error is greater than the % Weight, then that element or component should be ignored.
Table 2: General job information for XRF analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>Soot Sample</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Remark</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chemistry</td>
<td>Oxides</td>
<td>Elements</td>
</tr>
<tr>
<td>Shape &amp; ImpFC</td>
<td>Automatic</td>
<td>Automatic</td>
</tr>
<tr>
<td>Case Number</td>
<td>2 = % Rest Unknown</td>
<td>0 = All Known</td>
</tr>
<tr>
<td>Kappa List</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Helium</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Film</td>
<td>1 for Prolene</td>
<td>1 for Prolene</td>
</tr>
<tr>
<td>Report Level</td>
<td>20 ppm</td>
<td>20 ppm</td>
</tr>
<tr>
<td>Sector</td>
<td>360</td>
<td>360</td>
</tr>
<tr>
<td>Area</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diameter</td>
<td>25 mm, 38 mm</td>
<td>25 mm, 45 mm</td>
</tr>
<tr>
<td>Mass</td>
<td>W_{\text{final}} - W_{\text{initial}} (mg)</td>
<td>W (mg)</td>
</tr>
<tr>
<td>Rho</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Height</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% Known Conc</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>% Rest</td>
<td>6 = Carbon</td>
<td>-</td>
</tr>
<tr>
<td>Diluent/Sample</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2.4.2 Carbon Analysis

The Sunset Laboratory’s Carbon Aerosol Analysis Lab Instrument was used to determine the total carbon content of the samples as well as the fractions of organic and elemental carbon. By controlling the temperature and the atmospheric composition within the analyzer, the thermal-optical analyzer differentiates between organic and elemental carbon and sums the two fractions to calculate the total carbon in the sample (µg/cm²).

The samples may be prepared in one of two ways. Option one: the samples are collected or deposited on quartz filters. Quartz filters are recommended because of the high temperatures used during the analysis. A representative punch from the quartz filter measuring either 1.0 cm² or 1.45 cm² is inserted into the sample oven so that it is in line with the optical analyzer. Option
two: a bulk sample can be suspended in either methanol or water and then dropped onto a punch from a pre-baked quartz filter. A methanol suspension may not be ideal though because the methanol contains organic carbon and, though it evaporates from the filter, may leave trace amounts of organic carbon on the filter and increase the organic carbon fraction. Although it may be more difficult to obtain a homogenous suspension of soot in water, it may be preferable to use water rather than methanol if a suspension is necessary to deposit the sample onto filters for analysis.

During the first stage of analysis, the sample is heated in incremental steps in a pure helium environment up to a temperature of 820°C. The carbon released during the first stage is withdrawn from the initial oven and is oxidized to CO$_2$ when it passes over a bed of granular MnO$_2$ maintained at 920°C in the oxidizer oven. The CO$_2$ is then reduced to methane, CH$_4$, in the methanator oven at 450°C. During final step of stage one, the CH$_4$ generated is quantified by a flame ionization detector (FID). All carbon generated in the pure helium environment is considered to be organic carbon (OC).

To determine the portion of elemental carbon (EC) in the sample, the oven is cooled and the pure helium environment in the sample oven is replaced with a mixture of 2% O$_2$ and He. Then the temperature is incrementally stepped up to 860°C. The final stage of analysis is a calibration check of the FID using methane gas (Birch and Cary 1996).

Throughout the analysis, the filter transmittance of a red light (633 nm wavelength) shining through the sample is monitored with a He-Ne laser (Schauer 2003). Some OC transforms to pyrolytic carbon, or char, as the environment switches from pure He to He-O$_2$ (Yu and others 2002). The char darkens the filter and so the transmittance through the filter decreases. With the addition of oxygen to the sample oven, the char is first oxidized and the filter returns to its original transmittance level. This return marks the split between OC and EC, and thus the pyrolytic carbon or char is correctly incorporated into the OC result (Birch and Cary 1996). Therefore, OC is defined as carbon that evolves during the He only stage and pyrolytic carbon or char. EC is the carbon that evolves from the sample after the laser transmittance returns to its initial reading (Schauer 2003).

According to Birch and Cary (1996), the pyrolysis correction is unnecessary when the sample analyzed contains a large amount of EC because the char contribution to the EC measurement will be small in comparison to the actual amount of EC. Also, colored inorganics, such as Fe$_2$O$_3$, have little to no impact on the carbon analysis because they maintain a constant absorbance level throughout the analysis, unlike the char (Birch and Cary 1996).
2.5 Toxicology Study

Ambient particulate matter (PM) is a complex mixture of organic compounds, soot, transition metals, sulfates, and nitrates, as well as other trace elements. Among these, specific components of PM composition, such as transition metals, have been implicated in particle-induced adverse health effects. In this experiment, mice were exposed to iron, a predominant transition metal in PM, in combination with soot that was generated by well-controlled combustion in a diffusion flame burner. Sample treatments included soot only, iron only, soot with iron generated by combustion, and soot mixed with iron, not generated by combustion. Two different doses of each sample treatment were administered and the changes in pro-inflammatory cells and cytokines, proteins, antioxidants and antioxidant power in bronchoalveolar lavage fluid (BAL) and cellular and humoral markers in blood were investigated to determine the toxicological effect of the presence of iron in soot and of the differences between combustion generated mixtures of iron and soot and non-combustion generated mixtures.

2.5.1 Animal Procurement

Female CD-1 mice 6 to 8 weeks old and weighing 18-22 g, were purchased from Charles River, Inc. (Raleigh, NC). CD-1 mice are used as a general multipurpose model for biological research. Upon arrival, all animals were housed (5 per cage) in animal facilities with high-efficiency particulate air filters, fed mouse chow and water ad libitum, and handled according to the Animal Care and Use Committee at the Environmental Protection Agency.

2.5.2 Sample Treatments

Seventy two CD-1 female mice were divided into seven groups and exposed by intratracheal instillation to either soot only, a mixture of soot with 2.6% iron created in a flame, a mixture of soot with 13.3% iron created in a flame, a mixture of soot with 15.8% iron created by mixing only, iron oxide only, lipopolysaccharide (LPS), or saline.

Six mice from each treatment group received a 25 µg dose and six received a 100 µg dose. All mice were sacrificed at 4 hours post exposure. Mice were numbered according to sacrifice order as follows.
Table 3: Description of mouse identities

<table>
<thead>
<tr>
<th>Mouse Number</th>
<th>Treatment</th>
<th>Dose</th>
<th>Tail Marking</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 13, 25, 37, 49, 61</td>
<td>Soot Only</td>
<td>25 µg</td>
<td>1 green line at base of tail</td>
</tr>
<tr>
<td>2, 14, 26, 38, 50, 62</td>
<td>Soot Only</td>
<td>100 µg</td>
<td>2 green lines at base of tail</td>
</tr>
<tr>
<td>3, 15, 27, 39, 51, 63</td>
<td>Low Iron (2.6%)</td>
<td>25 µg</td>
<td>1 black line at base of tail</td>
</tr>
<tr>
<td>4, 16, 28, 40, 52, 64</td>
<td>Low Iron (2.6%)</td>
<td>100 µg</td>
<td>2 black lines at base of tail</td>
</tr>
<tr>
<td>5, 17, 29, 41, 53, 65</td>
<td>High Iron (13.3%)</td>
<td>25 µg</td>
<td>1 red line at base of tail</td>
</tr>
<tr>
<td>6, 18, 30, 42, 54, 66</td>
<td>High Iron (13.3%)</td>
<td>100 µg</td>
<td>2 red lines at base of tail</td>
</tr>
<tr>
<td>7, 19, 31, 43, 55, 67</td>
<td>Iron Only</td>
<td>25 µg</td>
<td>1 green line at end of tail</td>
</tr>
<tr>
<td>8, 20, 32, 44, 56, 68</td>
<td>Iron Only</td>
<td>100 µg</td>
<td>2 green lines at end of tail</td>
</tr>
<tr>
<td>9, 21, 33, 45, 57, 69</td>
<td>Mixture (15.8% Fe)</td>
<td>25 µg</td>
<td>1 black line at end of tail</td>
</tr>
<tr>
<td>10, 22, 34, 46, 58, 70</td>
<td>Mixture (15.8% Fe)</td>
<td>100 µg</td>
<td>2 black lines at end of tail</td>
</tr>
<tr>
<td>11, 23, 35, 47, 59, 71</td>
<td>Saline</td>
<td>-</td>
<td>1 blue line at end of tail</td>
</tr>
<tr>
<td>12, 24, 36, 48, 60, 72</td>
<td>Lipopolysaccharide</td>
<td>-</td>
<td>1 blue lines at end of tail</td>
</tr>
</tbody>
</table>

On Day 1 of the study, mice numbered 1 through 24 were necropsied, followed by numbers 25 through 48 on Day 2, and numbers 49 through 72 on Day 3.

The particles for each sample treatment were weighed and diluted with saline to create 50 µL doses containing either 25 or 100 µg of particulate matter for each treatment group. The particle suspension was sonicated for 2 minutes and then vortexed prior to administration. The positive control for the experiment was a dose of 2 µg of LPS diluted in 50 µL of saline. LPS is a toxic, natural compound from the outer membrane of gram negative bacteria that is released when bacteria cells are lysed. LPS causes a known immune response and was therefore used to prove that the mice tested were capable of such a reaction. The negative control for the experiment was a 50 µL dose of saline because saline is not known to cause an immune response when administered.

I prepared the samples for instillation.
2.5.3 INSTILLATION

Mice were anesthetized in a small plexiglass box by isoflurane vapor inhalation and intratracheal-instilled (involuntary aspiration) with a 50 µL saline bolus containing 25 or 100 µg of particulate matter. During intratracheal instillation, the mouse was suspended vertically by its teeth and its tongue was extended. The treatment dose was injected into the back of the mouse’s mouth near the throat, and then the mouse’s nostrils were covered, forcing it to inhale the treatment dose into its lungs. Because of EPA animal protocols, I could only observe the instillation. Other EPA biologists and post-docs performed the actual instillation.

2.5.4 NECROPSY

At the time point of 4 hours post-exposure and following respiratory function testing, mice were euthanized one at a time with 0.2 mL of a 5:1 pentobarbital solution and then each mouse was weighed.

Each mouse was laid ventral side up (on its back), and the fur of the animal was wet with ethanol to prevent contamination of the incision or organs with mouse fur. Next, an incision was made from the chin of the mouse down to the groin and then from the groin to the knees. Liz Boykin, an EPA biologist performed this part of the animal necropsy.

![Figure 7: Incisions for mouse necropsy](image)

Blood was removed from the mouse’s heart and a complete blood panel was performed to determine the white blood count (WBC), the red blood count (RBC), the hemoglobin level (HGB), the hemacrit level (HCT), the mean corpuscular volume (MCV), the mean corpuscular hemoglobin level (MCH), the mean corpuscular hemoglobin concentration (MCHC), the platelet concentration, and the lymphocyte concentration and percentage. The blood plasma was further analyzed for markers of inflammation including α-1-antitrypsin (AAT), alanine aminotransferase
ALT), aspartate transaminase (AST), creatine kinase (CK), c-reactive protein (CRP), fibrinogen (FIB), haptoglobin (HPT), lactate dehydrogenase isoenzyme-1 (LD-1), lactate dehydrogenase (LDH), and total protein.

Then, the left mainstem bronchus (the division of the trachea that connects to the left lung) was isolated and clamped with alligator clips. Following this, a small trachea tube was inserted into the trachea of the mouse; in other words, the mouse was cannulated. The clamping of the left mainstem bronchus and the subsequent insertion of the trachea tube allowed any liquid inserted into the trachea tube to travel only to the right lung and not to the left lung.

This is important because the lobes of the right lung were lavaged with three volumes of 0.6 mL of warm Hanks’ Balanced Salt Solution (HBSS; Invitrogen, NY; body weight × 0.035 × 0.65 mL, 37°C) according to the operating procedure. HBSS is an isotonic solution that preserves the cells. The lavage procedure washes the airway (broncho) and air sacs (alveolar) in order to recover inflammatory cells. The fluid recovered from the wash, called Brochoalveolar Lavage (BAL), was analyzed for inflammatory endpoints. Mary Daniels, an EPA biologist, performed the lung lavage.

The remaining steps of the necropsy are considered sample processing. The processing was completed by several post-docs with my help. We rotated tasks on each of the three necropsy days.

BAL was centrifuged at 800 rpm for 15 min at 4°C to separate the cells recovered from the lung (the pellet) from the fluid used for recovery (the supernatant). The supernatant was stored in vials at 4°C before biochemistry analysis and at -80°C before cytokine and antioxidant analysis.

The cell pellet recovered from the centrifuged BAL was resuspended in 1000 µL of HBSS. Cell counts were performed using a Coulter Counter (Beckman Coulter, Inc., CA) on 500 µL of the resuspended cell fluid in 10 mL isotonic solution. In the Coulter Counter, 500 µL of cell solution is drawn up with a vacuum pump through a small, electrically charged tube with an aperture at one end. As the particles pass through the aperture, they block the electrical field, and thus the frequency of blocks in the current can be matched to the number of cells passing through the aperture (Beckman Coulter).

In addition to the cell pellet resuspension used for cell counts, 200 µL of the cell pellet resuspension was centrifuged in duplicate onto slides using a Cytospin (Thermo Scientific, MA). The Cytospin uses centrifugal force to evenly distribute a single layer of cells in a defined area on a microscope slide. Through this process the cells remain whole and can then be classified and
counted under a light microscope. After the cells were applied to the slides, each slide was
stained with Hema 3 Stain Set (Fisher Scientific, Middletown, VA) for differential cell counts
under the microscope. The slides were first dipped 10 times in a coplin staining dish filled with
the turquoise Hema 3 Fixative solution to insure that the cells remained adhered to the slides.
Then the slides were dipped 10 times in a red Hema 3 Solution I followed by 5 dips in the purple
Hema 3 Solution II. The slides were then rinsed with water to wash away the excess stain and
were left to dry overnight before being counted.

An additional 200 µL of BAL was analyzed for total antioxidant status and biochemical
markers of lung injury and edema including lactate dehydrogenase (LDH), microalbumin (MIA),
N-acetyl-β-D-glucosaminidase (NAG), and total protein.

Three hundred microliters of BAL were analyzed using the biochemical technique of
Enzyme-Linked ImmunoSorbent Assay (ELISA) for the pro-inflammatory cytokines interleukin-
6 (IL-6), macrophage inhibitory protein-2 (MIP-2), and tumor necrosis factor-α (TNF-α) by
testing samples for the presence of specific antibodies or antigens of IL-6, MIP-2, and TNF-α.

The lavaged right lung lobes were divided into three pieces. Two lobes of about 30 mg
each were immediately frozen separately in liquid nitrogen, and then one of the lobes was later
homogenized in 2 mL of 3% PCA to be analyzed for ascorbic acid and uric acid. The other 30
mg lobe was homogenized in 10% PCA/BA and analyzed for GSH and GSSG. The third right
lung lobe weighing about 50 mg was frozen in liquid nitrogen and later homogenized in 20mM
Tris-HCl pH 7.4 buffer of 2 mL and analyzed for cytokines (IL-6, MIP-2, TNF-α), total protein,
lung ferritin and total antioxidant power. The left lobes were frozen in liquid nitrogen and stored
at -80°C for future analysis.

Table 4: Summary of toxicology samples collected

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Markers Analyzed</th>
<th>Sample</th>
<th>Container</th>
<th>Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood cell analysis</td>
<td>White blood count (WBC)</td>
<td>Blood withdrawn from heart</td>
<td>72 biochemistry vials</td>
<td>Permanent marker</td>
</tr>
<tr>
<td></td>
<td>Red blood count (RBC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobin (Hgb)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemaerit (HCT)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean corpuscular volume (MCV)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean corpuscular hemoglobin (MCH)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean corpuscular hemoglobin concentration (MCHC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Platelets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood plasma analysis</td>
<td>α-1-antitrypsin (AAT)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>for inflammatory and</td>
<td>Alamine aminotransferase (ALT)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>systemic</td>
<td>Aspartate transaminase (AST)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Creatine kinase (CK)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-reactive protein (CRP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fibrinogen (FIB)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Haptoglobin (HPT)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

32
<table>
<thead>
<tr>
<th>Purpose</th>
<th>Markers Analyzed</th>
<th>Sample</th>
<th>Container</th>
<th>Label</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung lavage</td>
<td>Lactate dehydrogenase isoenzyme-1 (LD-1) Lactate dehydrogenase (LDH) Total protein</td>
<td>n/a</td>
<td>72 Syringes</td>
<td>n/a</td>
</tr>
<tr>
<td>Initial collection of lung lavage fluid and then resuspension of cell pellet</td>
<td>n/a</td>
<td>n/a</td>
<td>72 - 5 cc polypropylene tubes</td>
<td>Permanent marker</td>
</tr>
<tr>
<td>Cell count using Coulter Counter</td>
<td>n/a</td>
<td>500 μL cell pellet resuspension</td>
<td>72 Coulter cups filled with 10 mL isoton</td>
<td>Permanent marker</td>
</tr>
<tr>
<td>Cell differentials</td>
<td>Macrophages Neutrophils</td>
<td>200 μL cell pellet resuspension</td>
<td>2 x 72 microscope slides</td>
<td>Pencil</td>
</tr>
<tr>
<td>Biochemistry analysis</td>
<td>Lactate dehydrogenase (LDH) Microalbumin (MIA) N-acetyl-β-D-glucosaminidase (NAG) Total Antioxidant Status (TAS) Total Protein</td>
<td>200 μL of BAL supernatant</td>
<td>72 biochemistry vials</td>
<td>Permanent marker</td>
</tr>
<tr>
<td>Pro-inflammatory cytokines using ELISA</td>
<td>Interleukin-6 (IL-6) Macrophage inhibitory protein-2 (MIP-2) Tumor necrosis factor-α (TNF-α)</td>
<td>300 μL of BAL supernatant</td>
<td>72 Eppendorf tubes</td>
<td>Stickers</td>
</tr>
<tr>
<td>Antioxidants</td>
<td>Ascorbic acid Uric Acid</td>
<td>One right lung lobe homogenized with 2 mL of 3% PCA</td>
<td>72 Eppendorf tubes</td>
<td>Stickers</td>
</tr>
<tr>
<td>Antioxidants</td>
<td>GSH GSSG</td>
<td>One right lung lobe homogenized with 10% PCA/BA</td>
<td>72 Eppendorf tubes</td>
<td>Stickers</td>
</tr>
<tr>
<td>Pro-inflammatory cytokines and Biochemistry analysis</td>
<td>Interleukin-6 (IL-6) Macrophage inhibitory protein-2 (MIP-2) Tumor necrosis factor-α (TNF-α) Ferritin Total Antioxidant Status (TAS) Total Protein</td>
<td>Largest right lung lobe homogenized with 2 mL of 20mM Tris-HCl pH 7.4 buffer</td>
<td>72 biochemistry vials</td>
<td>Permanent marker</td>
</tr>
<tr>
<td>Future analysis</td>
<td>n/a</td>
<td>Left lung</td>
<td>72 cryovials</td>
<td>Stickers</td>
</tr>
</tbody>
</table>
3 RESULTS AND DISCUSSION

3.1 PHYSICAL AND CHEMICAL CHARACTERISTICS

After more attempts than I will ever care to recall, samples were generated and collected for the toxicology study. The problems encountered were numerous and are detailed in a later section. The samples were first characterized using XRF to determine their iron content. Carbon analysis was performed on each sample used in the toxicology study. The particle size distributions characterize samples that are independent of the ones used in the toxicology study.

3.1.1 SAMPLES GENERATED

The samples were named based on their iron concentrations as determined by XRF and are shown in Table 5 below.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Iron Concentration (by XRF)</th>
<th>Soot Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron Only</td>
<td>100%</td>
<td>0%</td>
</tr>
<tr>
<td>Mixture</td>
<td>15.8%</td>
<td>77.4%</td>
</tr>
<tr>
<td>High Iron</td>
<td>13.3%</td>
<td>81.0%</td>
</tr>
<tr>
<td>Low Iron</td>
<td>2.6%</td>
<td>96.3%</td>
</tr>
<tr>
<td>Soot Only</td>
<td>0%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Table 5: List of samples created

Iron in the samples was measured using XRF as percent iron oxide, and thus the sum of the iron concentration, the soot concentration, and the remaining oxygen concentration will equal 100%.

The iron only sample was composed of commercially available iron oxide nanoparticles purchased from Nanostructured and Amorphous Materials Inc. (Houston, TX). The γ-Fe$_2$O$_3$ particles had a 20-50 nm spherical particle size. The gamma orientation of particles was chosen to complement and extend previous work by Linak and others at the Environmental Protection Agency. Mossbauer spectroscopy of coal fly ash samples from that work indicated the presence of the gamma orientation of iron oxide (Linak and others 2006).

The mixture sample was created by physically mixing commercially available γ-Fe$_2$O$_3$ with flame generated soot in an approximate ratio by weight of 3 parts of γ-Fe$_2$O$_3$ to 10 parts of soot. A portion of the sample was then mixed with liquid binder and pressed into a pellet prior to verifying its composition using XRF analysis.
The high iron sample was created with a flow of 188 sccm of C\textsubscript{2}H\textsubscript{4} flowing through the ethylene/ferrocene line submerged in the 78.5°C water bath and 48.4 sccm of C\textsubscript{2}H\textsubscript{4} flowing through the supplemental fuel line. The air flow rate was approximately 55.3 slpm. After a total of 8 hours, a 5.6 mg sample was collected on a polycarbonate filter.

The low iron sample was created with a flow of 188 sccm of C\textsubscript{2}H\textsubscript{4} flowing through the ethylene/ferrocene line submerged in the 78.5°C water bath and 16.4 sccm of C\textsubscript{2}H\textsubscript{2} flowing through the supplemental fuel line. The air flow rate was approximately 56.2 slpm. After a total of 30 minutes, a 2.35 mg sample was collected on a polycarbonate filter.

### 3.1.2 TOTAL CARBON RESULTS AND DISCUSSION

A small portion of each of the samples was suspended in either water or methanol for total carbon analysis. A portion of each solution was then pipetted onto prebaked quartz filters. For the first set of carbon analyses, the samples were diluted at a ratio of either 1 to 2 or 1 to 40 with methanol. For all of the samples, at least one of the total carbon analyses performed closed the mass balance, verifying that the samples did indeed contain only soot and iron. The confirming results are highlighted in Table 6.
Table 6: Carbon mass balance: comparison of XRF and carbon analysis results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Est. Sample (μg)</th>
<th>By XRF (%)</th>
<th>Minimum by Carbon Analysis (%)</th>
<th>Maximum by Carbon Analysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Iron</td>
<td>1 : 2</td>
<td>26.00</td>
<td>96.3</td>
<td>86.42</td>
<td>97.96</td>
</tr>
<tr>
<td>Low Iron</td>
<td>1 : 2</td>
<td>26.00</td>
<td>96.3</td>
<td>90.88</td>
<td>102.88</td>
</tr>
<tr>
<td>Low Iron</td>
<td>1 : 40</td>
<td>1.30</td>
<td>96.3</td>
<td>81.54</td>
<td>138.46</td>
</tr>
<tr>
<td>Low Iron</td>
<td>1 : 40</td>
<td>1.30</td>
<td>96.3</td>
<td>149.23</td>
<td>213.85</td>
</tr>
<tr>
<td>High Iron</td>
<td>1 : 2</td>
<td>26.00</td>
<td>81.0</td>
<td>71.65</td>
<td>81.65</td>
</tr>
<tr>
<td>High Iron</td>
<td>1 : 2</td>
<td>26.00</td>
<td>81.0</td>
<td>71.65</td>
<td>81.65</td>
</tr>
<tr>
<td>High Iron</td>
<td>1 : 40</td>
<td>1.30</td>
<td>81.0</td>
<td>98.46</td>
<td>156.92</td>
</tr>
<tr>
<td>High Iron</td>
<td>1 : 40</td>
<td>1.30</td>
<td>81.0</td>
<td>80.77</td>
<td>137.69</td>
</tr>
<tr>
<td>Mixture</td>
<td>1 : 2</td>
<td>22.00</td>
<td>77.4</td>
<td>78.77</td>
<td>89.95</td>
</tr>
<tr>
<td>Mixture</td>
<td>1 : 2</td>
<td>22.00</td>
<td>77.4</td>
<td>93.64</td>
<td>106.36</td>
</tr>
<tr>
<td>Mixture</td>
<td>1 : 40</td>
<td>1.10</td>
<td>77.4</td>
<td>73.64</td>
<td>139.09</td>
</tr>
<tr>
<td>Mixture</td>
<td>1 : 40</td>
<td>1.10</td>
<td>77.4</td>
<td>80.91</td>
<td>146.36</td>
</tr>
<tr>
<td>Soot Only</td>
<td>1 : 2</td>
<td>36.00</td>
<td>100.00</td>
<td>48.53</td>
<td>55.42</td>
</tr>
<tr>
<td>Soot Only</td>
<td>1 : 2</td>
<td>36.00</td>
<td>100.00</td>
<td>116.75</td>
<td>130.81</td>
</tr>
<tr>
<td>Soot Only</td>
<td>1 : 40</td>
<td>1.80</td>
<td>100.00</td>
<td>64.44</td>
<td>106.67</td>
</tr>
<tr>
<td>Soot Only</td>
<td>1 : 40</td>
<td>1.80</td>
<td>100.00</td>
<td>67.22</td>
<td>109.44</td>
</tr>
</tbody>
</table>

Based on the methanol suspension results, it was decided that a 1 to 2 dilution ratio seemed to produce the most accurate results. The variation in the results above can most likely be attributed to a failure to produce a homogenous mixture before the suspension drops were applied to the quartz filter. Because of the charge on the soot particles, they were attracted to the sides of the test tubes and the pipette tip. In addition, the methanol-soot solution was mixed rapidly with the pipette before the sample was extracted to be dropped onto the filter, but it is possible that the solution was not completely mixed.

A second round of carbon analysis was performed using water as the solvent, rather than methanol because of the concern that any methanol that did not evaporate from the filter prior to carbon analysis would alter the carbon results as methanol is an organic solvent. Interestingly, the water and methanol suspensions produced similar results, as shown in Figure 8, indicating that the methanol did fully evaporate prior to carbon analysis.
The percentage of organic carbon measured in all samples using both water and methanol suspensions is shown above. Incorporation of iron in the soot increased the percentage of organic carbon in the sample, but the iron had to be incorporated via combustion. Physical mixing of the iron and soot did not shift the carbon from elemental to organic; thus indicating the role of the iron nuclei in the shift from elemental to organic carbon. The error in the sample is automatically calculated by the carbon analyzer and is presented in this graph.

Simply mixing iron particles with previously generated soot had little effect on the OC/EC results; the OC/EC ratios of the mixture and the soot only samples are very nearly identical. The high iron and low iron samples had similar organic carbon percentages while the mixture and soot only samples were similar to each other in regards to OC percentage. This difference between the mixture sample and the high iron and low iron samples suggests that during combustion, the iron particles were actually incorporated into the soot particles, as shown in work by Zhang and Megaridis in 1994. The incorporation of the iron within the soot matrix possibly shifted the carbon within the soot matrix from elemental to organic. That is, the carbon within the matrix was bonded to more hydrogen and therefore required less energy to oxidize it during carbon analysis. If the soot particles have a greater organic carbon percentage, it is possible that this makes them easier to oxidize in the flame and reveals why the iron inclusion inhibits the soot emitted from the flame.

In addition, the OC/EC results, when compared to other samples generated by diesel engines, suggest that the diffusion flame burner is capable of creating samples that are similar to the diesel exhaust particles (DEP) obtained from an engine. The OC/EC data indicated that the
presence of iron during combustion promotes higher organic carbon percentages that are comparable to DEP.

Lee and others (2006), using a diesel engine and the addition of ferrocene to the fuel, also observed a decrease in the amount of elemental carbon as the amount of iron in the particulate matter increased. They speculated that the iron initially acted as a nucleus for soot formation but later in the flame condensed onto the outer surface of the soot particles prior to their exit from the flame. They observed a decrease in the amount of elemental carbon in the samples as the amount of iron in the samples increased. They suggested that this outer iron oxide partial coating promoted greater initial oxidation during carbon analysis which immediately resulted in an increase in organic carbon (since it, by definition, is the carbon that is initially oxidized during carbon analysis) (Lee and others 2006). Their results were similar to those reported by Bonyzik in 1991.

In future work, this idea will be tested by determining the percentage of extractable organic material (EOM) before carbon analysis is performed. Then the EOM percentage can be compared to the OC percentage to determine if the OC/EC ratio is changed during carbon analysis.
3.1.3 PARTICLE SIZE DISTRIBUTIONS

Particle size distributions were obtained under two different experimental conditions: using acetylene as the supplemental fuel and then later using ethylene as the supplemental fuel. The first results presented and discussed were obtained using acetylene.

The graph in Figure 10 shows a decrease in the amount of soot collected from the flame in milligrams per minute as the calculated amount of iron in the sample increases. The amount of iron in the sample had to be calculated rather than measured for some of the samples because there was not enough sample to analyze for accurate XRF results. Also, the x-ray tube in the XRF spectrometer burned out and the XRF machine could not be used. However, the calculated iron concentrations very nearly match the measured iron concentrations for the samples in which both methods were employed.

Park and others (2005) reported the necessity of supplementing the fuel flow when iron, in the form of iron pentacarbonyl, was added to the flame. They increased the supplementary acetylene flow rate by 20% to accommodate for the decrease in soot production with the addition of iron. The goal of their diffusion flame work was to maintain a constant soot production rate while adding iron to the flame. The results shown above in Figure 10 indicate that soot production does in fact decrease with the addition of iron to the flame, in agreement with the work of Park and others (2005).
Figure 11: Increasing iron leads to decreasing volume concentration emitted

The volume concentration (nm$^3$/cm$^3$) decreased as the percentage of iron in the sample decreased. The range on the $y$-axis of the smaller graph is from 0.0 to 2.0x10$^3$ nm$^3$/cm$^3$ while the range on the larger graph is from 0.0 to 5.0x10$^{12}$ nm$^3$/cm$^3$.

The amount of iron in the sample, as shown in Figure 10 and Figure 11, was calculated based on the concentration of ferrocene saturating the fuel, assuming that all iron that entered the flame was later emitted from the flame. This iron concentration was translated into grams of iron per minute and multiplied by the sample collection time to give the grams of iron in the sample. The percentage of iron in the sample could then be determined by dividing the grams of iron by the grams of sample.
Sample 1

Mean = 96.97 nm
Total Conc. = 5.14 × 10^6 #/cm^3
Total Volume = 1.81 × 10^{13} nm^3/cm^3
Fe by XRF = 2.66%

Sample 2

Mean = 28.56 nm
Total Conc. = 6.92 × 10^6 #/cm^3
Total Volume = 4.94 × 10^{12} nm^3/cm^3
No XRF

Sample 3

Mean = 27.36 nm
Total Conc. = 9.09 × 10^6 #/cm^3
Total Volume = 2.96 × 10^{12} nm^3/cm^3
Fe by XRF = 10.48%

Figure 12: Shift in particle size distributions
In Figure 12, the iron concentration in the soot increases from Sample 1 to Sample 3. The particle size distributions pictured here were collected using acetylene as the supplementary fuel and show a shift towards smaller particles with an increase in the iron concentration. The size distributions shown correspond with the 2 previous graphs and show a decrease in the mean particle diameter while the soot emitted and the volume also decreased. Between Sample 1 and Sample 2, the iron in the sample increased by approximately 2% while a 60% decrease in soot collected and a 75% decrease in volume concentration occurred. However, the number concentration increased as the iron in the sample also increased.

Interestingly, the particle size distribution changed from being bimodal to being nearly unimodal between Sample 1 and Sample 2, although there still exists a small remnant of the bimodal behavior in Sample 2. Between Sample 1 and Sample 2, the mean particle diameter decreased from 96.94 nm to 28.56 nm. This is likely because of the catalytic action of the iron since the particles are so much smaller and the mean particle size is very close to what others have reported when only iron is present in the sample (Kasper and others 1999). Other researchers reported a significant shift in mean particle diameter following the addition of iron pentacarbonyl to the flame along with the disappearance of most large (>80 nm diameter) particles when producing soot with approximately 15% iron (Yang and others 2001). Researchers have also noted that along with the shift in particle size comes an increase in the number concentration (Lee and others 2006). This may be a consequence of the iron addition as the organic iron is liberated from the ferrocene as it enters the combustion zone and nano-sized iron particles await the deposition of carbonaceous material on their surfaces. Lee and others observed that the emitted particles also contained a partial iron coating, indicating that some of the iron condenses on the soot particles after they are formed.

The first set of samples clearly demonstrated, in agreement with the work of others, that even a small amount of iron added to the flame has a large soot-reducing effect.
The particle size distributions show the effects of adding iron to the flame at 400 and 450 sccm of fuel. The iron was added by submerging the ferrocene tube in water at 80°C and waiting approximately 30 minutes before sampling to ensure that the ethylene was fully saturated with ferrocene before collecting the particle size distributions.

Figure 13: *Particle size distributions: effects of iron I*

For the second set of particle size distributions, presented in Figure 13 and Figure 14, ethylene, rather than acetylene, was used as the supplementary fuel. The data was taken to show the differences in particle size distributions with and without ferrocene added at the same fuel and air flow rates. At 400 sccm of fuel, the particles collected when iron was combusted have a greater mean diameter than those collected without iron in the system. At 450 sccm, the difference between the distributions with and without iron was even greater.

Figure 14: *Particle size distributions: effects of iron II*

With more fuel added to the flame, the flame became more unstable and the oxidation region of the flame decreased. This resulted in larger particles being emitted from the flame.
Though the mean particle diameter of the sample containing iron was greater, this indicated that there were less particles smaller particles. This result is contrary to what Skillas and others (2000) reported as a result of combustion of diesel fuel supplemented with an iron additive in a diesel engine. However, other researchers have suggested that ferrocene actually promotes soot growth initially and later acts as a catalyst during the oxidation of the soot particles later in the flame. It has been suggested that the iron initially acts as a nucleus for the growth of the soot matrix around it, thus promoting increased soot formation earlier in the flame. As these particles move from the soot formation region of the flame to the oxidation region, they are oxidized more quickly than particles in flames with no additives (Feitelberg and others 1993; Hirasawa and others 2004). During the collection of the second set of particle size distributions (Figure 13 and Figure 14), the flame was very unstable, indicating that the oxidation region of the flame was diminished. Thus, these particle size distributions may reveal, as reported by others, the initial increase in soot formation as a result of adding iron to the flame. In addition, it has been suggested that during diesel combustion of fuel mixed with ferrocene, the iron oxide particles and the soot particles are formed simultaneously, rather than first the iron oxide particles and then the coagulation of carbon particles on the iron nuclei, because of the turbulent nature of diesel engines (Kaspar and others 1999, Matter and Siegmann 1997). The particle size distributions in Figure 14 may support this observation.

This flame and the corresponding particle size distributions cannot be compared to the ones reported by Park and others (2005), Yang and others (2001), or Yang (2004) because the difference in fuel flow rates is too great.

3.1.4 PROBLEMS ENCOUNTERED

“Results? Why, man, I have gotten lots of results! If I find 10,000 ways something won't work, I haven't failed. I am not discouraged, because every wrong attempt discarded is often a step forward....”

Thomas Edison

I have learned that being successful in research requires perseverance, more perseverance, and a little luck.
The first problem encountered in this project was the matter of the ferrocene vaporizer. I first tried a glass tube that had to be cut before it could be used. The fragility of the glass did not lend itself to accommodation within the experimental apparatus. The glass, because the ends could not be cleanly cut, allowed water to seep into the vaporizer, soaking the ferrocene and glass wool and essentially stopping up the entire tube. On the next attempt, the glass broke when the fittings were tightened to prevent the infiltration of water into the vaporizer. We elected to use a stainless steel tube that was successful with enough tightening of the connectors and Teflon tape.

The next difficulty lay in particle collection. This was the major problem of the entire experiment. How do we efficiently collect ultrafine particles for research? The diffusion flame burner generated ultrafine particles that easily penetrated the weave of Teflon coated glass fiber filters. In order for the particulate matter collected to be recoverable, it was necessary to collect particles for an extended period of time to first fill the pores of the filter before it would build a layer on the top of the filter. Initially, I collected filter on a giant flying saucer looking filter holder that was part of a dilution sampler. With no ferrocene being added to the fuel, it took a week of sampling to collect 14 grams of soot only particles off of one 24 inch filter. When ferrocene was added to the fuel, it was impossible to use the giant filter to collect the particles because they were way too small to even begin to build up a particle layer in any reasonable amount of time.

We moved on to smaller 4 inch filters with different pumps and vacuums and then to even smaller filters with an even larger pump. The problem remained pulling enough particles through the filter before the weave of the filter was plugged. As a last resort I tried polycarbonate filters and found some success. I had avoided them because they are so flimsy, tend to roll up when they get hot, and tear easily so that scraping the particulate matter off is very difficult. But in the end, they effectively collected particles. The bonus of using polycarbonate filters for XRF analysis was that the filters were undetectable by the spectrometer and therefore gave more reliable results.

Another problem involved the water bath. If left on overnight at 80°C, the water in the water bath completely evaporated. Turning it off at the end of the day meant that I wasted two to three hours the next morning waiting for the water to equilibrate at 80°C. The solution to this problem came in the form of two electric horse trough heaters that, when submerged in the water bath, were able to heat the bath to 80°C in 30 minutes.

At one point, the flow of ethylene through the primary fuel tube was completely shut off because the fuel tube had clogged with ferrocene that condensed onto the sides of the tube. After
replacing the fuel tube, I ensured that the heat tape covered all exposed sections of the fuel tube to prevent the problem from occurring again. Despite my best efforts, the fuel tube clogged again and had to be replaced in order to complete sample collection.

After many attempts at particle collection across the span of a few months, it was very apparent that having only one fuel line left me unable to control both the amount of ferrocene flowing through the flame and the amount of soot produced by the flame. In order to have a smoking flame, the ethylene flow had to be greatly increased but this meant that the ratio of iron to soot in the samples decreased. By adding a supplementary fuel line, I was able to use the fuel line passing through the ferrocene vaporizer to control the amount of iron in the sample and the other fuel line to control the amount of soot produced by the flame.

This fix would have been great, but we first used welding grade acetylene as the supplementary fuel. XRF on sample after sample kept reporting phosphorous in the range of 10 to 20% of the sample. The iron in the samples was in the right range, between 10 and 30%, but phosphorous contamination meant that these samples could not be used for the toxicology study. The problem was not with the XRF spectrometer or with the filters or with the ferrocene. It turns out that the primary contaminant of bottled acetylene is phosphorous (Long and Boss 1981). After changing the supplementary fuel back to ethylene, the problem immediately disappeared.

Persistence finally paid off, and I was able to collect samples for toxicology, XRF, and carbon analysis as well as obtain particle size distributions.

3.2 TOXICOLOGICAL CHARACTERISTICS

3.2.1 BALF CELL COUNTS

Macrophages and neutrophils are both types of phagocytes that specialize in the ingestion and digestion of unwanted microorganisms within the body. In the blood, macrophages are called monocytes, are immature, and await full development depending on their ultimate destination and function. In addition to the destruction of unwanted pathogens, macrophages also stimulate further immune response by secreting interleukin-6 and tumor necrosis factor-α as well as other cytokines (Kobayshi and others 2002). These cytokines recruit other immune system cells, like neutrophils, to the site of infection. The accumulation of macrophages and neutrophils in an area represents an inflammation (Parham 2000).

Neutrophils are white blood cells that respond to signs of inflammation and infection. Generally 11 to 14 µm in diameter, neutrophils comprise 50 to 70% of the body’s circulating
white blood cells. At times of infection, the number of neutrophils in the body increases (Kobayshi and others 2002).

Counting of cells from the bronchoalveolar lavage fluid cell pellet provided a percentage of macrophages and neutrophils for each mouse. Combined with the total cell counts obtained from the Coulter Counter, it was possible to determine the total number of macrophages and neutrophils in the lung fluid of each mouse.

<table>
<thead>
<tr>
<th>Average Macrophage Percent</th>
<th>Average Neutrophil Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low Iron</strong></td>
<td><strong>Low Iron</strong></td>
</tr>
<tr>
<td><strong>High Iron</strong></td>
<td><strong>High Iron</strong></td>
</tr>
<tr>
<td><strong>Mixture</strong></td>
<td><strong>Mixture</strong></td>
</tr>
<tr>
<td><strong>Iron Only</strong></td>
<td><strong>Iron Only</strong></td>
</tr>
<tr>
<td><strong>Soot Only</strong></td>
<td><strong>Soot Only</strong></td>
</tr>
<tr>
<td><strong>Saline</strong></td>
<td><strong>LPS</strong></td>
</tr>
</tbody>
</table>

**Figure 15: Macrophage percent and neutrophil percent**

As seen most dramatically in the neutrophil data, the inflammatory response seems to correlate with the amount of soot in the sample. For both doses, the percentage of neutrophils responding decreases as the amount of soot in the sample decreases, and thus, the percentage of macrophages increases as the amount of soot in the sample decreases.

In all of the toxicology graphs, one asterisk (*) indicates that the treatment was statistically significant when compared to the saline treatment. Two asterisks (**) indicates that the treatment was statistically significant when compared to the soot only treatment of the same dose.

For the 25 µg dose, the percentage of macrophages for the soot only treatment (79.73% with outliers, 91.66% without outliers) was lower than the percentage for all of the other treatments because the significant increase in neutrophils caused by the soot only treatment reduced relative percentage of macrophages in the sample. The same was true for the 100 µg dose (72.33%, 82.9%). For both treatments, the percentage of macrophages increased as the amount of soot in the sample decreased, although this increase was slight. As expected, the percentage of neutrophils responding to the LPS treatment was quite high (80.13%) and the percentage responding to the saline treatment was low (1.50%). The decrease in the percent of macrophages for the soot only 100 µg dose was statistically significant when compared to the
saline treatment both with and without outliers. Without outliers, the low iron 100 µg dose was also statistically significant when compared to the saline treatment. When compared to the soot only treatment at 100 µg, the low iron treatment showed a statistically significant lower response.

For the 25 µg dose, the number of macrophages in the low iron and high iron doses was greater than for the mixture, the iron only, and the soot only. This increase was not statistically significant compared to either the saline response or the 25 µg soot only response. However, for the 100 µg dose, only the high iron dose had a greater number of macrophages responding while all the other doses invoked relatively the same response. The high iron response was statistically significant when compared to the soot only response. As expected, the number of macrophages responding to the LPS treatment was lower than the number responding to the saline treatment, since LPS is intended to elicit an immediate immune response, signified by an increase in neutrophils, rather than macrophages.

The number of neutrophils responding was greatest for the soot only treatment as both doses and least for the iron only treatment. For both doses, the number of neutrophils responding to the high iron treatment was 35 – 40% lower when compared to the soot only treatments of the same dose while the low iron treatment showed a 70% decrease. The mixture showed an 85% decrease when compared to the soot only treatments, and the iron only treatment was approximately 95% lower than the soot only treatment.
As seen in Figure 17 below, the number of neutrophils measured following dosing with particulate matter samples from coal combustion showed a similar trend. For the ultrafine size fractionation, the number of neutrophils responding increased as the amount of carbon in the samples increased, not as the amount of iron in the sample increased (Cho and others 2008). This is similar to the diffusion flame soot samples in that the soot only sample generated by the diffusion flame induced a greater neutrophil response than any of the samples that included iron.

Zhou and others reported no significant changes in the percentages of macrophages or neutrophils in their inhalation work with soot generated from iron-pentacarbonyl and ethylene in a diffusion flame burner. They did not, however, report trends observed, and their quantitative results have not been published (Zhou and others 2003). Had they reported on the trends they observed, the results presented here could have been compared with theirs to determine if similar trends were observed.
3.2.2 BALF BIOCHEMICAL ANALYSIS

Lactate Dehydrogenase (LDH) in BAL

Microalbumin (MIA) in BAL

Figure 18: Lactate dehydrogenase and microalbumin
There are no trends in the data for LDH or MIA. In the precursor coal fly-ash study, the elevated levels of MIA induced by doses of ultrafine particles suggested that the ultrafine particles caused a greater inflammatory response than did the coarse or fine particles (Linak and others 2006). This data, however, does not show an elevated MIA level to indicate the same type of inflammatory response. The coal fly-ash study data was taken at 18 hours post-instillation, while this data was taken at 4 hours post-instillation. It is possible that not enough time elapsed between dosing and necropsy to prompt a full response.

Lactate Dehydrogenase, LDH, is an enzyme found in a variety of tissues including the lungs, kidneys, heart, liver, and blood. Elevated levels of LDH in plasma indicates tissue damage as more of the enzyme is released. Enzymes are protein catalysts that increase the rate of a reaction without being changed themselves (Champe and Harvey 1994).

There were no noticeable trends in LDH levels for the sample treatments. In fact, LDH levels for all sample treatments decreased when compared to saline levels. After the outliers were removed from the data, the difference between the saline treatment and the soot only 25µg dose as well as between the saline treatment and the low iron 100 µg dose were found to be statistically significant. Others have reported no significant changes between the iron-soot samples and controls in LDH (Zhou and others 2003).

The microalbumin, MIA, concentration is measured as an indicator of vascular leakage into alveolar region of the lung, which is part of the inflammatory process (Singh and others 2004). There were no noticeable trends in MIA concentrations for the various sample treatments.
and none of the treatments produced statistically significant results when compared to either saline or soot only at the same dose. In the coal fly ash study that was a motivation for this work, the ultrafine portions of both Montana and Illinois coal produced a 2- to 3.5-fold increase in microalbumin in the lavage fluid at 18 hours post-instillation (Linak and others 2006).

![N-acetyl-b-d-glucosaminidase (NAG) in BAL](image)

**Figure 19: N-acetyl-b-d-glucosaminidase and protein in BAL**

The NAG results show that the iron only treatment at 100 µg was statistically lower than the soot only treatment also at 100 µg. However, the 25 µg results do not reveal the same trend. The protein measurements taken from the BAL do not indicate any significant responses for either dose.

N-acetyl-d-glucosaminidase, NAG, is a lysosomal enzyme, which breaks down large molecules that have been taken up by the cell. Since NAG is present in inflammatory cells such as macrophages and neutrophils, it is an indicator for inflammation (Metzger and Peterson 1988). For the 25 µg dose, there were no significant differences or trends within the treatments. However, for the 100 µg dose, the iron only treatment was statistically lower than the soot only treatment when the outliers were included in the data.

The biochemical analysis of the BALF also included a measure of total protein. Proteins located throughout the body participate in all processes within the cells. They can function as enzymes to act as a catalyst for biochemical reactions; participate in cell signaling and immune responses; or have structural and muscular functions. The protein response measured in the BALF did not show any trends or statistically significant differences.
Finally, the total antioxidant status was measured from the BALF. Total Antioxidant Status, TAS, is a measure of the effect of antioxidants, which protect cells from damage caused by reactive oxygen species. Oxidation occurs by the increase in positive charge on an atom or loss of negative charges, commonly achieved by the removal of a pair of hydrogen atoms. No significant differences in means or trends were seen in the TAS measurements.

### 3.2.3 Lung Homogenate Biochemical Analysis

The biochemical analysis of the lung homogenate included measures of ferritin, total antioxidant status, and total protein.
The total antioxidant status was measured from the lung homogenate, but the data collected was weak and resulted in many missing data points as well as a large variance in the data collected. Therefore, the TAS (LH) results are unreliable.

Ferritin is a protein and an acute phase reactant. It stores iron and regulates the amount of iron in the blood. To transport oxygen from the lungs to other cells in the body, iron atoms are needed to bind with oxygen in hemoglobin. Free iron is toxic to cells, so iron must be stored in cells bound to ferritin as Fe(III). When higher iron levels are needed in the blood, the Fe(III) is released as Fe(II). High levels of ferritin indicate an excess amount of iron in the body. If ferritin levels measure high and C-reactive protein levels are normal, then the elevated ferritin indicates too much iron rather than an injury, inflammation, or infection (Donlin and others 1998).

Although there were no statistically significant differences to report, two interesting measurements of ferritin levels were taken. For the 25µg dose, the low iron treatment had a lower ferritin level than all of the other treatments. However, the same was not true for the 100 µg dose. In the 100 µg dose group, the iron only treatment produced a greater ferritin response than the soot only.

Following inhalation of soot and iron particles, a 2.6-fold increase in ferritin was reported when compared to animals inhaling only filtered air (Zhou and others 2003). The results from our instillation study did not show the same trend. Zhou’s results do not show a change between soot only samples and the filtered air control nor between the iron only samples and the filtered
air control. The differences seen following our instillation study were not nearly as dramatic as theirs. The data can be compared side by side in the graphs below.

**Figure 22: Comparison of ferritin results**
The ferritin levels measured in each of the sample treatments were compared to the level measured in the saline treatment. Although all sample treatments showed an increase over the saline level, none of the differences were statistically significant and none of the increases were of the same magnitude as reported by Zhou and others in 2003.

**Figure 23: Protein in lung homogenate**
The protein levels measured in the lung homogenate were all relatively similar to each other and not statistically different from the levels measured from the saline and LPS doses.
Both doses and all treatments produced similar total protein numbers when the lung homogenate was analyzed.

### 3.2.4 Plasma Biochemical Analysis

The plasma separated from the blood taken from the mice prior to dissection was analyzed for several biochemical markers of inflammation.

![Figure 24: α-1-Antitrypsin and alanine aminotransferase](image)

For both the AAT and the ALT, the level measured for each treatment was similar to the saline and LPS responses, indicating no pronounced induction of these reactants.

First, α-1-antitrypsin, AAT, is an Acute Phase Reactant. AAT is also known as α1 proteinase inhibitor. This protein normally inhibits serum elastase, which is a protease that destroys the cell walls of lungs by digesting the connective tissue if not controlled. This occurs especially during inflammation. If AAT is oxidized, then it can no longer function, which results in lung damage. Analysis of AAT in the plasma showed no significant differences or trends in either dose, indicating a lack of destruction of lung cells.

Alanine aminotransferase, ALT, is a transaminase enzyme that is responsible for the transfer of an amino group and acts as a catalyst. It catalyzes the transfer of an amino group from alanine, an amino acid, to a-ketoglutarate, which is not an amino acid. This reversible transamination reaction produces pyruvate and glutamate. ALT is active in amino acid and carbohydrate metabolism. Although it can be found in other locations, its presence usually indicates liver damage. All treatments for both doses produced similar ALT results.
Aspartate Transaminase (AST)

Creatine Kinase (CK)

Figure 25: Aspartate transaminase and creatine kinase

The AST results indicate no red blood cell or heart muscle tissue damage. The CK levels were relatively similar for all treatments, with the exception of the 25 µg high iron and iron only treatments. However, the errors associated with these means negate any possible statistical significance. The CK results also indicate the absence of muscle damage or cardiac tissue damage.

Aspartate transaminase, AST, facilitates the conversion of aspartate, an amino acid, to α-ketoglutaric acid, which is not an amino acid but can participate in oxidation. In the next reaction step, the aspartate converts to oxaloacetate, which is not an amino acid, and the α-ketoglutaric acid converts to glutamate, an amino acid. AST is present in elevated amounts in the liver, red blood cells, and cardiac muscles at times of tissue damage. No significant differences or trends were found upon AST analysis.

Creatine Kinase, CK, is an enzyme. This catalytic protein is active in muscle tissue, and elevated amounts indicate muscle damage and/or heart attack. For the 25 µg dose, all treatments resulted in levels less than soot only, but the trend did not seem to depend on the amount of soot in the treatment. For the 100 µg dose, all treatments except the low iron treatment were less than soot only, but again the trend did not seem to depend on the amount of soot.
Figure 26: C-reactive protein and fibrinogen

The CRP levels measured do not indicate an intense inflammatory response. However, CRP levels for all mice could not be measured, and so the results are not reliable. For the fibrinogen levels measured, the iron only 100 µg dose was statistically significant when compared to the soot only dose. The results for 25 µg and 100 µg, though, do not have the same trend.

Next, the c-reactive protein, CRP, is a plasma protein and is considered an Acute Phase Reactant. CRP assists complement, which is a set of plasma proteins that attack extracellular forms of pathogens, in binding to foreign and damaged cells (Parham 2000). The pathogens become coated with complements and can be killed directly or be engulfed and destroyed by phagocytosis. Phagocytosis is enhanced by CRP. During the inflammatory process, CRP levels rise. Weak CRP data was collected and many of the treatments were missing data points making the results unreliable.

Fibrinogen, FIB, is a blood plasma protein and an Acute Phase Reactant. FIB is active in blood clotting, and it is a nonspecific indicator of inflammation. The soot only treatment for the 25 µg dose had a lower fibrinogen level than all of the other treatments. The other treatments produced relatively equal fibrinogen levels. For the 100 µg dose, however, the soot only treatment was about equal to all of the other treatments, with the exception of the iron only treatment. Iron only dosed at 100 µg produced a fibrinogen level that was much lower than the soot only treatment. This difference was statistically significant.
Haptoglobin (HPT) and Lactate Dehydrogenase Isoenzyme-1 (LD-1)

The haptoglobin results do not necessarily indicate damage to tissue or red blood cells. It is interesting to note that the soot only treatments for both doses registered the highest haptoglobin levels. For LD-1, the results do not show any trends or statistically significant differences.

Haptoglobin, HPT, is an acute phase reactant that binds to free hemoglobin in the blood. Free hemoglobin in the blood is a sign that red blood cells have been lysed, and its presence increases with tissue damage. For both doses, the soot only treatment resulted in a higher haptoglobin level than the other treatments, although for both doses, the mixture produced nearly similar results. This result potentially emphasizes that the carbon content of the treatment has a greater effect on the toxicology than the iron content.

The presence of Lactate Dehydrogenase Isoenzyme-1, LD-1, in the plasma indicates tissue damage. Isoenzymes are different structural forms of enzymes that function as the same as the regular enzymes but may have different kinetic or regulatory parameters. The LD-1 results for the two doses show opposite trends. For the 25 µg dose, the high iron and iron only treatments were equal and higher than the roughly equivalent soot only, low iron, and mixture treatments. But for the 100 µg dose, the high iron and iron only were less than the soot only, low iron, and mixture treatments.
The lactate dehydrogenase levels were also measured in the plasma. For the 25µg dose, the high iron and iron only treatments had greater LDH levels than the soot only treatment. However, for the 100 µg dose, all treatments were less than the soot only treatment. The difference between the 100 µg soot only response and the 100 µg iron only response was statistically significant.

3.2.5 COMPLETE BLOOD PANEL

A complete blood panel was performed on blood taken from each mouse’s heart. However, all measurements taken were relatively equivalent.
Although the white blood cell count for the mixture at 100 µg treatment appears to be higher, the increase is due to a single data point that when excluded brings that white blood cell count down to be roughly equivalent to the others. The red blood cell count was unchanged between any of the samples or controls.

White blood cells, WBC, or also called leukocytes, are cells in the immune systems that defend the body against infection and foreign materials, and they include neutrophils, eosinophils, basophils, lymphocytes, monocytes, and macrophages. WBC are produced in bone marrow and transported in the blood and lymphatic system. No differences in the WBC count were recorded in either dose or in any of the treatment groups.

Red blood cells, RBC or also called erythrocytes, contain the protein hemoglobin, which transports oxygen from the lungs to other body tissues. The RBC count for all treatments was approximately the same.
No changes were recorded in the levels of hemoglobin, HGB, an iron-containing protein in red blood cells that is used to transport oxygen from the lungs to the rest of the body.

Hematocrit, HCT, is a measure of the fraction of the fraction of blood volume that is occupied by red blood cells. The remaining fractions are plasmas and white blood cells. The HCT values were roughly equivalent for all treatments in both doses.

Mean corpuscular volume, MCV, is the average red blood cell volumetric size. It is equivalent to the hematocrit divided by the red blood cell count. Because there were no recorded differences in red blood cell count or hematocrit, none can be expected or were found for the MCV levels.

Mean corpuscular hemoglobin, MCH, is a measure of the mass of hemoglobin contained by a red blood cell. It is equal to the amount of hemoglobin over the amount of red blood cells. Again, no differences were found.

Mean Corpuscular Hemoglobin Concentration, MCHC, is a measure of concentration of hemoglobin in given volume of red blood cells, and it is calculated by dividing the amount of hemoglobin by the hematocrit. All treatments produced similar MCHS values.
Platelets are principally responsible for blood clotting, but they are also present at sites of infection or injury and interact with leukocytes by secreting cytokines and other inflammatory mediators. However, no significant differences in mean values of platelets were observed.

Lymphocytes are a type of white blood cells. Small lymphocytes are either B lymphocytes, B cells, or T lymphocytes, T cells, and they are responsible for adaptive immune responses since they have cell-surface receptors for antigens. An antigen is a molecule that can bind specifically to an antibody, which is a secreted form of the immunoglobulin made by B cells. Immunoglobulin is the antigen-binding molecules in B cells. Large lymphocytes are natural killer cells, NK cells. For the 25 µg dose, the lymphocyte percentage for all treatments was higher than for soot only, although none of these differences were statistically significant. For the 100 µg dose, all treatments produced similar results. The number of lymphocytes in each sample was also measured. The treatments administered with 25µg doses produced similar results, but the treatments of 100 µg doses did not show significant differences or trends.

3.2.6 PRO-INFLAMMATORY CYTOKINES

The presence of pro-inflammatory cytokines interleukin-6 (IL-6), macrophage inflammatory protein-2 (MIP-2), and tumor necrosis factor-α (TNF-α) were measured in both the bronchoalveolar lavage fluid and in the lung homogenate. For each of these inflammatory markers, the soot only treatment induced the greatest response with the exception, as expected, of the positive control, LPS.
3.2.6.1 **INTERLEUKIN-6**

Interleukin-6, IL-6, is a pro-inflammatory cytokine released by macrophages that stimulates a wide range of responses at early times of infection (Parham 2000). It induces an immune response to trauma or tissue damage, which leads to inflammation. IL-6 acts as a mediator of acute phase response by signaling the acute phase reactants that there is an infection or inflammation. Therefore, elevated levels of IL-6 are often the first signs of inflammation.

![IL-6 in BAL](image1)

![IL-6 in LH](image2)

**Figure 32: Interleukin-6**

The trends and differences in IL-6 are most pronounced in the BAL fluid data. Here, it can be seen that the IL-6 response increases as the amount of carbon in the sample increases. In the lung homogenate, the LPS produced less of an IL-6 response, so it can be expected that the trends in IL-6 would be more pronounced in the BAL data.
In the BAL fluid, the soot only treatment at both doses produced the greatest response. The trend through the treatments at both doses reveals that the level of IL-6 measured in the BAL fluid decreases as the amount of soot (carbon) in the sample decreases. The difference between the soot only response at both 25 µg and 100 µg was statistically significant when compared to the iron only response. For the 100 µg dose, comparison of the high iron and mixture treatments indicated that the same amount iron in the sample induced a greater inflammatory response when it was mixed with soot in the combustion process than when the iron and soot were physically mixed outside of the flame.

When IL-6 was measured in the lung homogenate of the 25 µg dose group, all treatments with the exception of iron only produced the same response, and the iron only response was much lower than the other IL-6 levels. For the 100 µg dose group, the soot only treatment induced the greatest response, although the low iron and high iron induced levels were quite similar. In fact, the soot only treatment at 100 µg was statistically significant when compared to the negative control, saline. The mixture and iron only treatments produced significantly lower IL-6 levels in the lung homogenate. Again, the data shows that the same amount of iron caused greater inflammation when mixed with soot in the combustion process than when physically mixed.

Figure 33, below, shows a measured increase in IL-6 levels resulting from the instillation of size fractionated coal samples. For the ultrafine portion, the IL-6 response increased as the amount of iron in the sample decreased, and most importantly, as the amount of carbon in the sample increased (Cho and others 2008).
The results presented in the work of Cho and others from 2008 show a similar trend in IL-6. The amount of IL-6 measured as a response to exposure to coal with differing carbon contents increases as the amount of carbon in the ultrafine fraction increases and the amount of iron decreases.

### 3.2.6.2 Macrophage Inflammatory Protein-2

The macrophage inflammatory protein-2, MIP-2, is another pro-inflammatory chemotactic cytokine. This chemokine, a chemical stimulus that induces the movement of bodily cells, is produced by macrophages to stimulate the response of neutrophils, basophils, and eosinophils. It can also induce the release of other pro-inflammatory cytokines, such as IL-6.
For the MIP-2, the soot only treatment again induced the greatest response and the iron only treatment the least pronounced response. The response of all treatments was greater than that of the saline, but none of these differences were statistically significant.

In the BAL fluid, the soot only treatment for both doses again illicited the greatest MIP-2 response. For the 25 µg dose, the iron only treatment produced a statistically significant, lower response than the soot only treatment. The low iron, high iron, and mixture treatments at 25 µg had responses similar but lower to that of the soot only treatment. The 100 µg low iron, high iron, mixture, and iron only doses produced similar results and all were lower than the soot only treatment.

The 25 µg dose of soot only and low iron produced similar results in the lung homogenate, and these results, while not statistically significant, were greater than the other treatments. In the lung homogenate, the 100 µg dose of soot only induced the greatest response,
and the response level to the other treatments decreased as the amount of soot in the sample decreased. This result suggests that the ultra-fine carbon in the sample was responsible for the inflammatory response, rather than the carbon-iron complexes that were formed when ferrocene was combusted.

### 3.2.6.3 TUMOR NECROSIS FACTOR-α

Tumor Necrosis Factor-α, TNF-α, is a cytokine produced by macrophages and T cells, and it is a prototype of the TNF family of cytokines. TNF-α has several functions in the immune response, such as functioning as cell-associated or secreted proteins that interact with receptors of the tumor necrosis factor receptor, TNFR, family (Parham 2000).
Figure 35: Tumor necrosis factor-α
The TNF-α shows similar results to both the IL-6 and MIP-2 in that the soot only treatment produced the greatest immune response though it was significantly less than that of the LPS.

In the BAL fluid, the soot only treatment at 25 µg was statistically significant when compared to both the saline treatment and the iron only treatment. Soot only at 25 µg and at 100 µg was greater than all other responses. However, there was no trend in the data like there was for MIP-2 or IL-6. In the lung homogenate, the soot only 25 µg dose produced the greatest response, but the low iron treatment was very close. For the 100 µg dose, soot only again produced the greatest response, but when outliers were excluded from the data, this was no longer the case.
Table 7: Pro-inflammatory cytokine summary
For the pro-inflammatory cytokines measured, the soot only treatment had the greatest measured response for all cytokines measured, at both doses, in both the BAL and the lung homogenate, with the exception of IL-6 in lung homogenate.

<table>
<thead>
<tr>
<th>Soot Only Greatest Response?</th>
<th>25 µg</th>
<th>100 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6 in BAL</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>IL-6 in LH</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>MIP-2 in BAL</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>MIP-2 in LH</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>TNF-a in BAL</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>TNF-a in LH</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

The levels pro-inflammatory cytokines measured in both the bronchoalveolar lavage fluid and the lung homogenate, show, in general, that the soot only treatment, at either dose (25 µg or 100 µg) elicited a greater inflammatory response within the lungs that any sample containing iron. This indicates that the pulmonary toxicity of soot-iron particles is linked to their soot content rather than their transition metal content. However, because pro-inflammatory cytokines are “first responders” at times of inflammation additional analysis at longer time points post-instillation might support a different conclusion. Additional experiments are recommended to confirm the conclusion that soot alone is more toxic than combination of soot and iron also because most of the differences observed in pro-inflammatory cytokines were not statistically significant.
4 SUGGESTIONS FOR FUTURE WORK

Future work could focus on several aspects of this work to offer clarification, confirmation, and/or extension of the results:

- How do particles generated with ferrocene compare to those generated with iron pentacarbonyl, both physically and chemically? Most importantly, the structure of the ferrocene-soot particles should be compared to the structure of the iron pentacarbonyl-soot particles by examination with a transmission electron microscope. These results could potentially help to explain the differences between the work presented here and the work by Zhou and others in 2003.

- How does the inflammatory response following inhalation of soot-ferrocene particles compare to the response following instillation of the same particles? How do these *in vivo* results compare to *in vitro* results obtained using lung epithelial cells? Again, these results could potentially explain differences between this work and that of Zhou and others in 2003 and Aust and others in 2002.

- What characteristics of the iron-carbon particles lead to their increased organic carbon percentage when compared to carbon only particles? These results could confirm the analysis reported by Lee and others in 2006.

- Does the analysis of the extractable organic matter from iron-carbon particles produce similar results to carbon aerosol analysis of the same samples? These results would offer further support to the carbon analysis results presented here and to the results of Lee and others in 2006.

- Does increasing the time-point for necropsy change the results and show a more pronounced inflammatory response?

- Can particles be generated containing only sulfur and carbon? Sulfur, iron, and carbon? What type of pulmonary inflammatory response do these particles induce? These results would provide further insight into the toxicity of ultrafine particles generated during coal combustion.

- Can particles be generated using a different transition metal such as platinum or cerium? These results would help to characterize the toxicological impact of using metal-catalysts to reduce emission from combustion in engines and could be combined with an effort to combust diesel fuel with the same metals in a generator or an engine.
5 CONCLUSION

The primary focus of this work was to determine if prototype particles similar to those generated in diesel combustion or coal combustion could be generated using a diffusion flame. Prototype particles containing varying amounts of iron within the soot structure were successfully created by combusting ethylene saturated with ferrocene in a diffusion flame burner. The physical and chemical characteristics of the generated particles, as reported in this work, appear to be similar to the results of other researchers; however, the toxicological data is very different than what other groups have found.

This work shows that the combustion of iron in a flame along with the fuel reduces the total amount of soot emitted as a function of the iron concentration when the flame is stable. An approximately 60% decrease in mass and 75% decrease in volume were observed. A decrease in mean particle diameter resulted from the addition of iron to the flame, as observed using SMPS. Interestingly, the particle size distribution shifted from being unimodal with large particles (mean >80 nm) to being bi-modal with both small and large particles to being unimodal with small particles (mean <30 nm) as the amount of iron increased.

The iron content of the generated particles, varying from 0% to 13% was verified using XRF, and the mass balance was closed following carbon analysis. In addition to closing the mass balance, carbon analysis showed that the incorporation of iron in the soot increased the percentage of organic carbon likely because of the inclusion of an iron-partial outer shell on the soot particles which facilitates the oxidation of a greater amount of soot immediately after beginning carbon aerosol analysis.

The original hypothesis that the inclusion of iron within the soot particles causes a greater inflammatory response was not supported by the experimental results. In fact, the inflammatory response instead corresponded to the amount of carbon in the sample, although at a time point four hours post-instillation, only the proinflammatory cytokines measured showed this trend. Neither the soot nor the soot-iron complexes induced the inflammatory response seen in the ultrafine fractionation of coal fly-ash particles leading to the conclusion that a different component of the coal fly-ash, not the iron content, is responsible for the inflammatory response.
6 REFERENCES


