Development of *in vitro* Toxicological Methodologies for Diesel Fuel Emissions – The EMITTED Study

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Introduction

Diesel exhaust particulate (DEP) exposure studies conducted with humans, animals, and mammalian cell lines show that this pollutant source causes oxidative stress, which culminates in a **pro-inflammatory response**. These biological responses are mediated by pollutant generated **reactive oxygen species** (ROS), but there is little comprehension regarding:

a) DEP attributes (*i.e*. composition, morphology, and volatility) that initiate oxidant production,

b) Time-scale of intra-cellular ROS production induced by toxic DEP components.

This work was conducted as part of the **EMITTED** study (**E**xhaust **M**easurement & **I**nhalation **T**oxicology **T**esting for **E**merging **D**iesel fuels). The overall study is focused on investigating both formation and toxicity of DEP, with the volatile fraction being of most interest. Emphasis has also been placed on identifying toxicologically-relevant DEP characteristics that are responsible for oxidative stress, by using various fuel types and applying different combinations of emission control technologies.

Method Development Aims

1. To develop an *in vitro* pollutant exposure model and experimental protocols for testing indices of DEP-induced cellular activation, and injury, with control particulate matter (PM) for future extension to DEP.
2. To apply these test indices, so the temporal profile of PM-induced intra-cellular ROS generation can be understood across many concentrations and compositions.

Methodology

- *Particle Samples:* **Carbon black** (Arosperse 15V; Orion Engineered Carbons) and **titanium(IV) oxide** (Aeroxide P25 hydrophilic TiO_2*) suspensions were prepared in **Dulbecco's Phosphate Buffered Saline** (D-PBS*), a **serum free media** (SFM) of **Eagle Minimum Essential Medium** (EMEM†) at 3 mg mL^{-1}. The primary particle size of these test samples are 260 and 21 nm, respectively. All media was supplemented with 1 % penicillin streptomycin (PS; Invitrogen) and 150 ng mL^{-1} amphotericin B (Lonza).
- *Cell Culture:* **Calu-3 human bronchial epithelial cells**† were cultured on 6- or 96-well plates until 90 % confluence was reached, using a complete growth media (CGM) of 10 % fetal bovine serum (FBS*), 1 % PS EMEM, and incubated at 37 ˚C in humidified 5 % CO_2 air.
- *Cell Proliferation:* Redox indicator dye **alamarBlue®** (AB; Thermo Scientific) was used to assess cell viability in response to PM challenge. Post-exposure (5 - 160 µg mL^{-1}, for 4 - 20 hr), cells were incubated in the dark for 8 hr with 10 % AB in SFM, and absorbance measured at 570 and 600 nm‡.
- *ROS Production:* Pre-exposure (5 - 160 µg mL^{-1}), cells were protected from light while loaded with 30 µM **2',7'-dichlorfluorescein-diacetate** (DCFH-DA) in D-PBS for 30 min. Excess DCFH-DA solution was then removed by D-PBS wash, and an aliquot of PM suspension was added to each well. DCFH oxidation to DCF was measured as intra-cellular ROS generation.
  - **Live cell fluorescent images** were captured every 5 min over 3 hr with the LumaScope™ (eta luma) in a temperature, CO_2, and humidity controlled environment.
  - ROS production was also assessed over **extended PM exposure periods** (4 - 20 hr) by DCF absorption at 504 nm‡.

*Sigma Aldrich, † ATCC, ‡Molecular Devices VersaMax Microplate Reader

I. Exposure Concentration and Duration Dependent Cell Viability

- Viability was measured to determine the optimal PM exposure time and concentration for measurement of ROS production. Exposure conditions that decreased cell viability below 80 % were considered unsuitable for further experimentation (**Figure 2** - black plane).
- No significant decreases in viability were found for all carbon black exposure times and concentrations (data not shown). However, significant decreases in viability were seen at 12 hr, 120 µg mL^{-1} TiO_2; and at 16 hr for 80, 120, and 160 µg mL^{-1} TiO_2 ($p < 0.05$). Therefore, exposure parameters will be limited to 40 µg mL^{-1} and 8 hr.

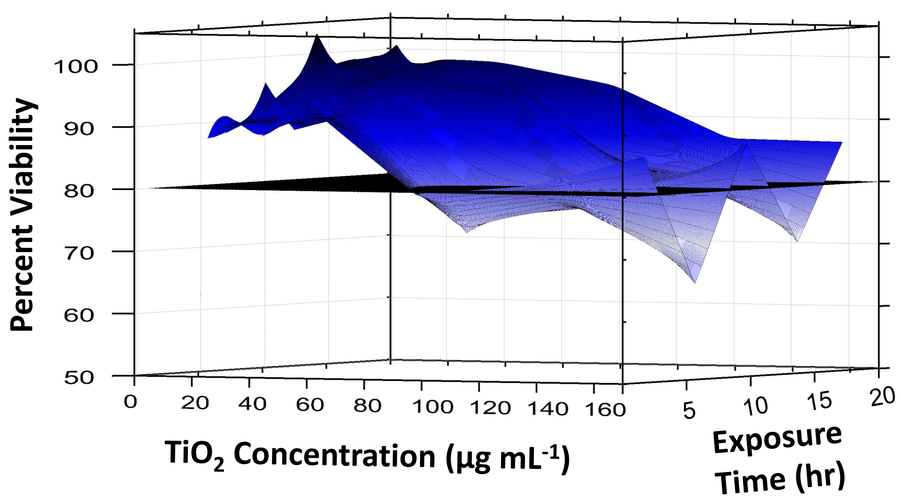

Figure 1. ***Calu-3 cell viability is decreased following exposure to TiO_2 PM in SFM.*** Calu-3 viability, expressed as percent viability relative to controls, was evaluated in response to 5, 10, 20, 40, 80, 120, and 160 µg mL^{-1} PM concentrations following 4, 8, 12, 16 or 20 hour incubation periods. The mean of three biological and technical replicates are shown for each concentration/time point.

II. PM induced ROS Production Over Extended Exposures

- Intra-cellular ROS production, as indicated by levels of DCFH oxidation, was not significantly enhanced by increasing concentrations of TiO_2 and carbon black (5, 20, 80, and 160 µg mL^{-1}), or over multi-hour incubation periods (4, 12, and 20 hr).

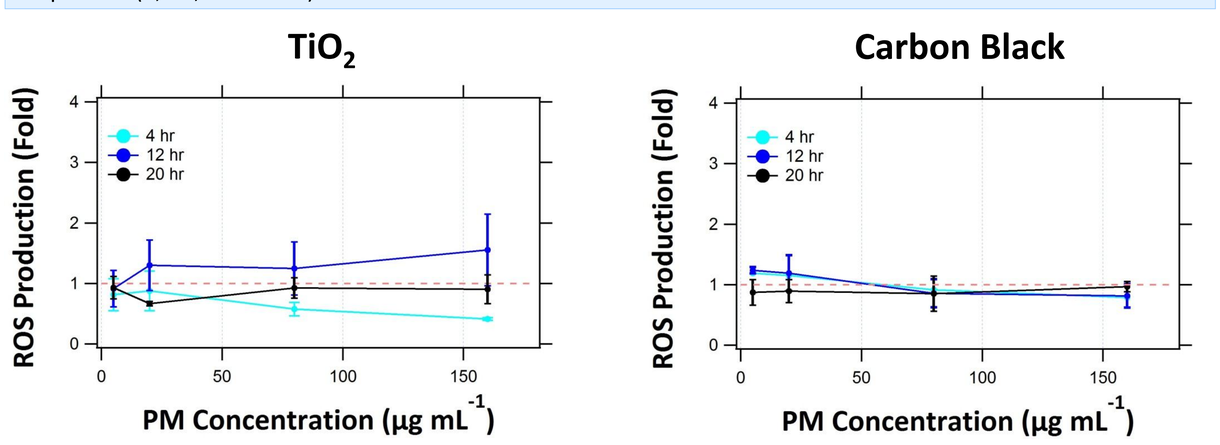

Figure 2. ***Calu-3 cell ROS production is unaffected by TiO_2 or carbon black PM in D-PBS, over extended incubation periods.*** Calu-3 ROS production, measured as DCF absorbance and expressed as a fold change from control, was evaluated in response to 5, 10, 20, 40, 80 and 160 µg mL^{-1} PM exposure concentrations following 4, 8, 12, 16 or 20 hour incubation periods (not all data shown). The propagated error over three biological and technical replicates are shown for each concentration/time point. One-way ANOVAs with post-hoc Tukey testing between experimental PM concentrations and exposure time periods.

III. Real-time PM induced ROS Production

- TiO_2 induced a rapid increase in ROS production (i.e. DCF fluorescence) in the first 50 min of the exposure.
- Carbon black, at the same exposure concentration, caused no significant ROS production.
- Photobleaching and background corrections resulted in a decrease in fluorescence signal over the exposure.

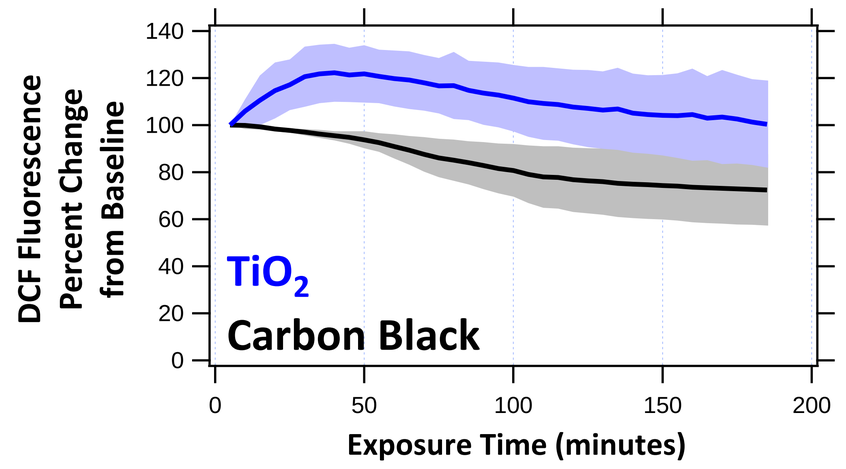

Figure 3. ***TiO_2 PM in D-PBS causes elevated ROS production in Calu-3 cells within the first hour of exposure.*** The percent change of DCF fluorescent was used to evaluate intra-cellular ROS concentrations in Calu-3 cultures in response to 40 µg mL^{-1} TiO_2 or carbon black PM exposure. Fluorescence measurements were collected every 5 min over a 3 hr incubation period. Error bars represent the propagated error over three technical replicates.

The EMITTED Study - Future Research Plan

- Exhaust samples are collected using particle impingers positioned at three sampling ports along the exhaust (**Figure 4**). These units allow direct collection of particles from the exhaust stream into aqueous media, and have been evaluated by examining particle retention over the size range of interest (**Figures 5 & 6**). Cell culture media (D-PBS and SFM) achieved the maximum collection efficiency, over deionised water, between 10 and 200 nm.
- Cultured cells will be exposed to an aliquot of these particle impinged samples for toxicity testing. Use of this sampling setup eliminates the need for filters, which require laborious extraction procedures and are susceptible to losses of semi-volatile compounds.
- Parallel sample collection will enable toxicity of exhaust pollutants to be contrasted across fuel types, engine operating modes, emission control systems.

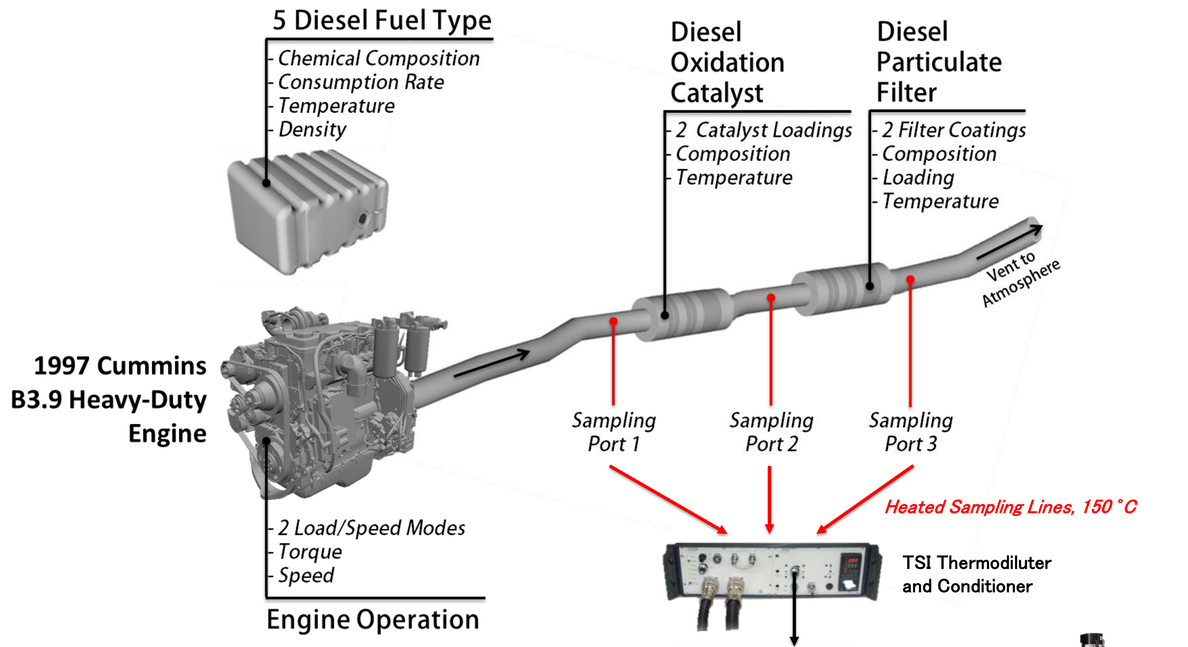

Figure 4. ***Overview of the EMITTED Study engine operation and sampling configuration.***

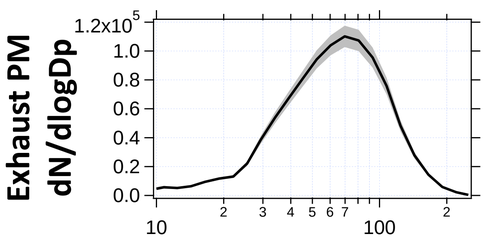

Figure 5. ***Size distribution of raw diesel exhaust number concentration – sampling port 1, upstream of impinger.***

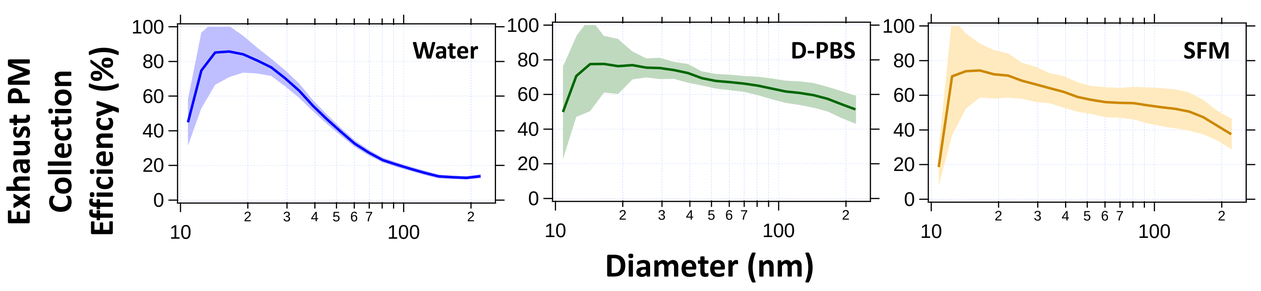

Figure 6. ***Impinging diesel exhaust PM into liquid cell culture media achieves the maximum collection efficiency.*** The collection efficiency of DEP by a standard glass nozzle liquid impinger, operated at 0.6 LPM, was evaluated using three substrates: water and two cell culture media: D-PBS and SFM. Use of FBS media was not possible due to a problem with foaming.

Summary of Conclusions

- Elevated TiO_2 exposures concentrations above 80 µg mL^{-1} and over 12 hr decreased Calu-3 cell viability. DEP exposure times and concentrations will be limited to a max of 40 µg mL^{-1} and up to 8 hr.
- TiO_2 induced peak Calu-3 ROS generation in the first hour of exposure. No response was observed with carbon black. Real-time DEP induced intra-cellular ROS production will be assessed over the first 1.5 hr of exposure.
- Maximum DEP collection efficiencies into D-PBS and SFM will allow us to investigate these responses further with actual DEP. In these future experiments, TiO_2 and carbon black will be used as positive and negative PM controls, respectively.

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