Addendum to the Research Project of "Testing of Volatile and Nonvolatile Emissions from Advanced Technology Natural Gas Vehicles" (Contract #07-340):

Toxicological Properties of PM emissions from CNG Buses

Toxicological Assays

The toxicological potentials of semi-volatile and non-volatile particulate matters (PM) from compressed natural gas (CNG) buses with three-way catalysts (TWC) were investigated through dithiolthreitol (DTT) chemical, cellular *in vitro* macrophage reactive organic species (ROS), and mutagenicity assays. A DTT assay provides a direct quantitative evaluation of chemical properties that have been proposed as important factors for a sample's inherent toxicity. DTT is a chemical that is easily oxidized in the presence of ROS. Consumption of DTT was measured by ultraviolet (UV) spectroscopy in a cell-free system to determine the oxidative potential of PM samples. A macrophage ROS assay determines the inherent ROS production of a sample and the ROS produced by a cell in response to inflammation caused by a toxic substance. For the Macrophage ROS assay, rat alveolar macrophages were exposed to aqueous suspensions of PM. Intracellular ROS formation was probed though the addition of dichlorofluorescin diacetate (DCFH-DA). DCFH-DA is a fluorescent probe that is de-acetylated by ROS to the fluorescing compound (DCFH). The mutagenicity bioassay was conducted using a microsuspension salmonella/microsome AMES assay that is approximately 10 times more sensitive than the standard plate incorporation test. This assay was conducted using two tester strains, TA98 and TA100, which measure frame-shift and base-pair substitution mutations, respectively. Frame-shift mutations are a result of a deletion or addition of nucleotides in the DNA sequence in the histidine gene, while base-pair substitutions are when a nucleotide base is replaced with another base in the histidine gene.

Sample Collection

For the toxicological assays, PM samples were collected on pre-cleaned Teflon-coated glass fiber filters. Total (semi-volatile + non-volatile) and non-volatile PMs were separately collected by thermal denuders. Detailed sampling procedures can be found in section 4 EMISSIONS TESTING PROCEDURE of the report. The PM samples were properly handled using established sampling procedures and transported to toxicology laboratories. Laboratories at University of California, Los Angeles (UCLA), University of Wisconsin-Madison (UWM) and University of California, Davis (UCD) conducted the DTT (Cho et al., 2005), macrophage ROS (Saffari et al., 2014), and mutagenicity assays (Kado et al., 2005), respectively.

Toxicological Assay Results

DTT assay results for total and non-volatile PM with and without chelation of the metals are summarized in Table 1 and Fig 1. Because chelation of metals in the PM samples reduced the DTT activity substantially, it is implied that the metals could play a major role for oxidative activity from the PM samples. Macrophage ROS assay results for total and non-volatile PM with and without chelation of the metals are summarized in Table 2 and Fig 2. The mutagenicity bioassay results for total and non-volatile PM are summarized in Table 3 and Fig 3

Table 1. DTT Assay Results for Total and Non-Volatile PM with and without Chelation of the Metals

Metal	PM	Cruise (nmole DTT/min/mi)	UDDS (nmole DTT/min/hr)	Idle (nmole DTT/min/hr)
Not Chelated	Total	1.3	12.7	129
	Non-Volatile	3.3	12.3	150
Chelated	Total	0	0.9	26
	Non-Volatile	0.7	2.7	31

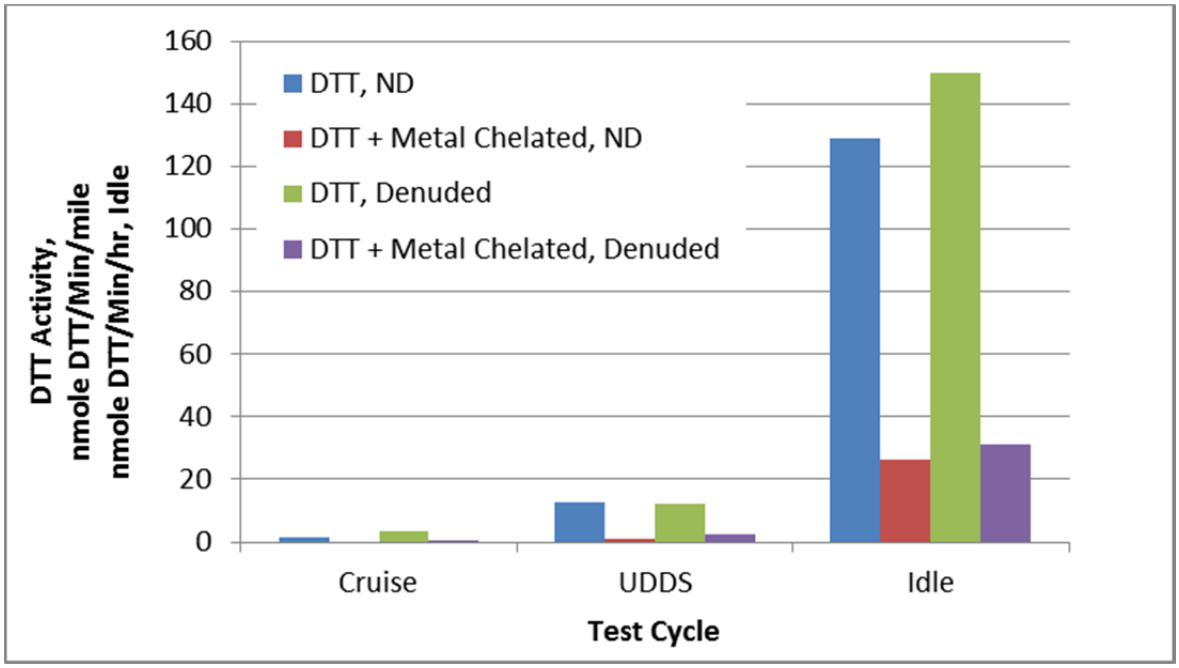

Fig 1. DTT Assay Results for Total (ND) and Non-Volatile (Denuded) PM Samples with and without Chelation of the Metals

Table 2. Macrophage ROS Assay Results for Total and Non-Volatile PM with and without Chelation of the Metals

Metal	PM	Cruise (µg Zymosan units/mi)	UDDS (µg Zymosan units/mi)	Idle (µg Zymosan units/hr)
Not Chelated	Total	268	882	22,641
	Non-Volatile	56	490	53,527
Chelated	Total	na	1,058	9,726
	Non-Volatile	na	60	13,504

na is for not available

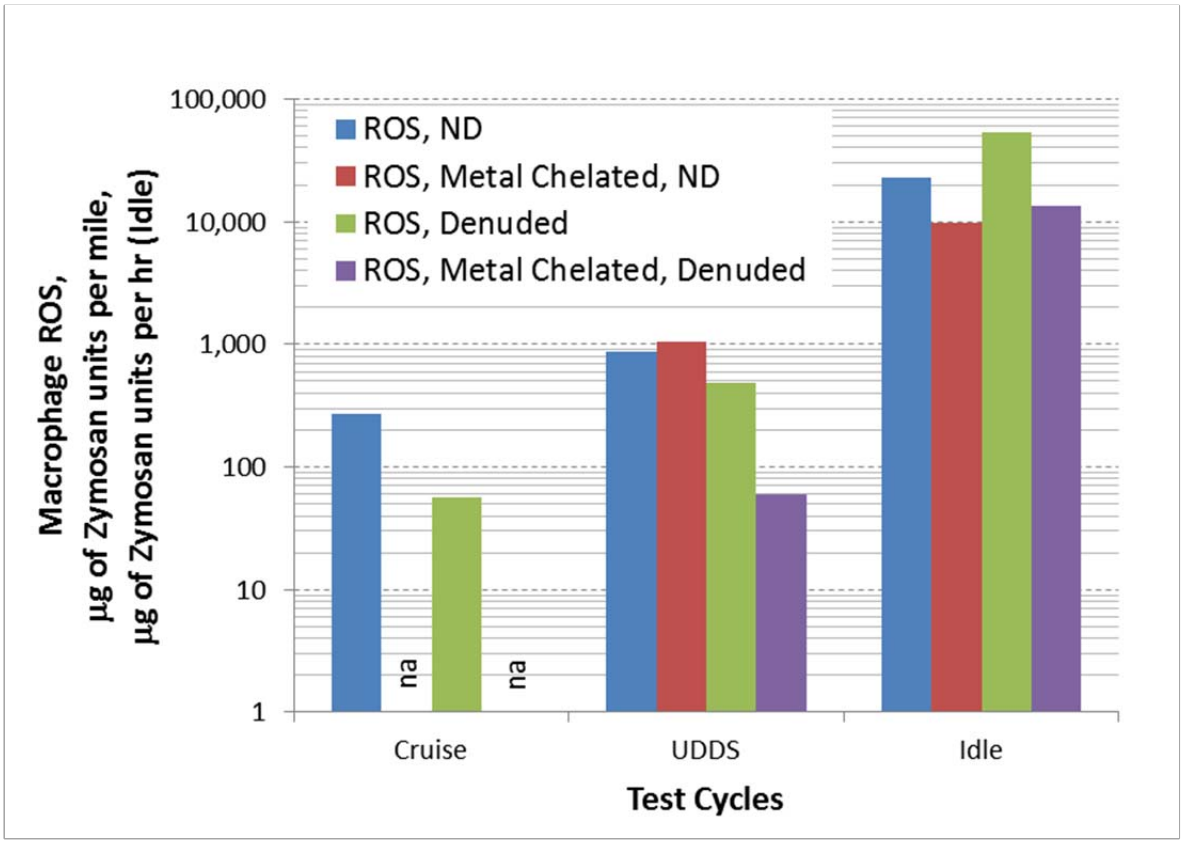

Fig 2. Macrophage ROS Assay Results for Total (ND) and Non-Volatile (Denuded) PM Samples with and without Chelation of the Metals, na for not available

Table 3. Mutagenicity Assay Results for Total (ND) and Non-Volatile (Denuded) PM Samples

Tester Strain	PM	Cruise (revertants/mi)	UDDS (revertants/mi)	Idle (revertants/min)
TA98	Total	175	435	50
	Non-Volatile	139	88	22
TA100	Total	1,744	2,084	831
	Non-Volatile	925	2,619	745

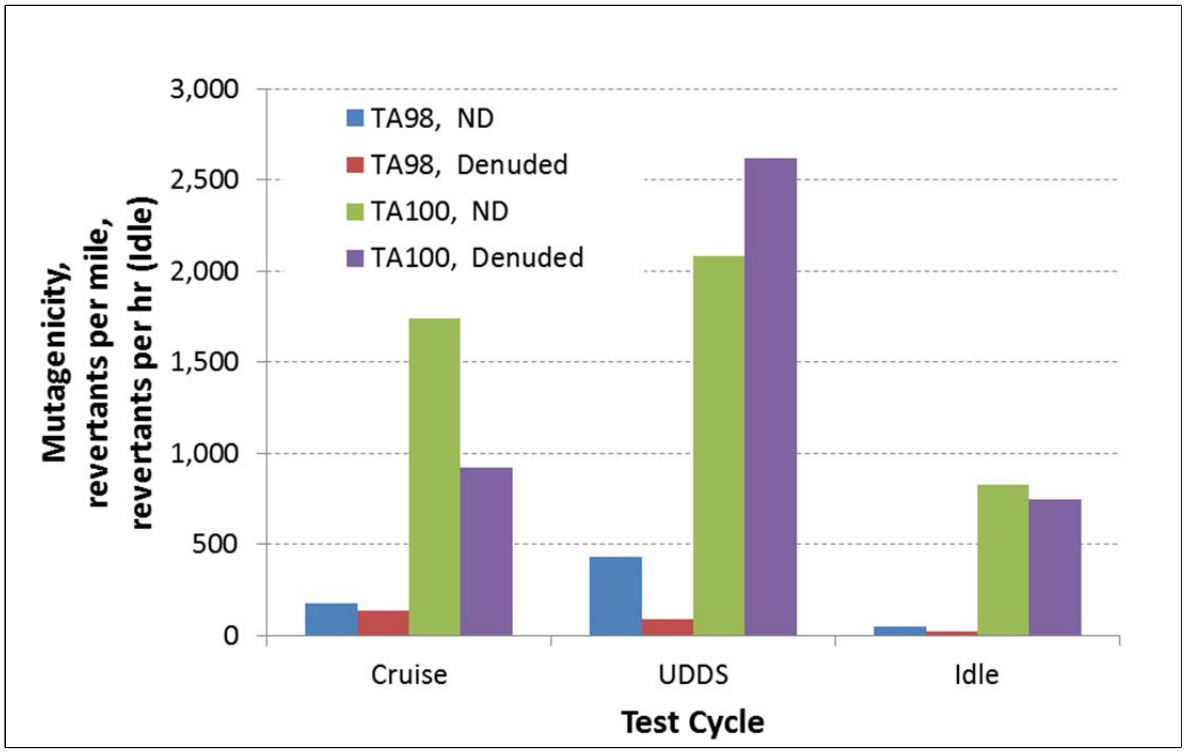

Fig 3. Mutagenicity Assay Results for Total (ND) and Non-Volatile (Denuded) PM Samples

References

Cho, A. K., Sioutas, C., Miguel, A. H., Kumagai, Y., Schmitz, D. A., Singh, M.; Eiguren-Fernandez, A., Froines, J. R., 2005. Redox activity of airborne particulate matter at different sites in the Los Angeles Basin. Environ. Res. 99 (1), 40–47.

Kado, N.Y., Okamoto, R.A., Kuzmicky, P.A., Kobayashi, R., Ayala, A., Gebel, M.E., Zafonte, L., 2005. Emissions of toxic pollutants from compressed natural gas and low sulfur diesel-fueled heavy-duty transit buses tested over multiple driving cycles. Environ. Sci. Technol. 19, 7638e7649.

Saffari, A., Daher, N., Shafer, M. M., Schauer, J. J., Sioutas, C., 2014. Global Perspective on the Oxidative Potential of Airborne Particulate Matter: A Synthesis of Research Findings. Environ. Sci. Technol., 48 (13), pp 7576–7583.