# APPENDIX AVAILABLE ON REQUEST

## Research Report 147

## Atmospheric Transformation of Diesel Emissions

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## Appendix D. Detailed Description of the Results Obtained During EUPHORE Campaigns – Toxicity Testing Results

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# APPENDIX D

# DETAILED DESCRIPTION OF THE RESULTS OBTAINED DURING EUPHORE CAMPAIGNS – TOXICITY TESTING RESULTS

## 1. PRESENTATION OF THE RESULTS

Results of the pulmonary toxicity assessment of samples collected from the various atmospheres are reported in several ways. First, the dose-response of each set of samples and all biological endpoints is summarized in Appendix E along with the results of the statistical analyses. Second, the potency estimates (derived from dose-response curves) for all samples are reported in this Appendix D. These samples include all of the measured responses, and include the comparisons between seasons, with and without denuder, etc. In order to simplify the overall interpretation of the toxicity results, and to facilitate the comparison of the relative response among atmospheres, the key samples and a simplified naming scheme are provided in the body of the main report in Section 4.2, Impact of Atmospheric Transformations of Diesel Emissions on Toxicity.

As shown in Appendix E, most of the samples produced reasonably monotonic changes in the responses analyzed, with slopes significantly different from zero as analyzed by linear regression. The figures showing the dose-response curves for each endpoint have been divided into diesel exhaust exposures performed in the dark ("Dark samples") and in the light ("Light samples") for ease of presentation (see Table 3 in the main report for sample description). The response to the positive control DS is included in each figure as a point of reference. The potency estimates are described below for 2005 and 2006. In 2005, samples from the atmospheres were evaluated with rats. In 2006, different atmospheres were evaluated with mice

instead of rats because there was less material available for dosing. The results from each campaign are described below.

## 2. SERIES A: WINTER AND SUMMER 2005, RESPONSES ANALYZED IN RATS.

Potency factors from analysis of the lavage composition for the rats (winter 2005 and summer 2005 samples) are shown in Figures 1 (biochemical parameters) and 2 (inflammatory cells). A summary of the histopathological potency factors is shown in Figure 3. The dose response data (appendix E) show generally dose-dependent responses for all parameters, with the greatest effects of sample D05w_4 and Ld05sF_1-4, with generally smaller responses to the other samples. One exception to the dose dependence is the reduced response of lavage macrophages to the highest dose of D05w_4. Statistical analyses of the differences among pairs are given in Appendix E, Table 1.

This sample set included one pair of replicate samples (D05wN_5 and D05wN_6), both dark exposures without NOx denuder, but with addition of ozone to produce $N_2O_5$. One of these (D05wN_6) was tested only at the highest dose. Although for APase, D05wN_5 gave a slope significantly less than zero, and D05wN_6 did not, this was the result of the linear regression tracking with the intermediate doses and the effect at the single dose tested in both samples was very similar. In contrast, the slope for D05wN_6 was significant for total cells, neutrophils, and lymphocytes, while that for D05wN_5 was not, however both were very weak responses compared to other samples. Importantly, for none of the endpoints was there a significant difference in the calculated potencies between these two samples (highlighted in yellow in Appendix E, Table 1), thus substantiating the ability of the assays to discriminate among samples that are indeed different in composition.

The winter sample of aged diesel exhaust without NOx denuder (D05w_4) was lethal to 4 out of 5 rats at the mid- and high doses, and the remaining rat in each group showed large responses of all cell types in lavage fluid, both lavage parameters of toxicity (LDH and total protein), and histopathological indicators of inflammation. The animals that died generally exhibited severe respiratory distress and expired within 4 hr of instillation, but were not subjected to necropsies. However, as discussed in the chemistry section, the D05w sample was collected while the engine was still very new, and may not be representative of samples from a mid-use engine. Effects on histopathological indicators of cytotoxicity and parenchymal changes were not significantly different from zero based on the remaining animals and fitting to a linear response. However, since four of the rats in this high dose sample died and there is no information available from these animals, this could simply be variation in distribution of the sample into the lungs (lavage being taken from the right lung, and histopathology analyses on the left lung). In this sample set, the denuded sample (Dd05s_1+2), produced responses not very much different from the other samples, and less potent than the most potent samples collected in the light.

## 3. SERIES B: SUMMER 2006, RESPONSES ANALYZED IN MICE.

Given increasing evidence for oxidative stress as a potential mechanism for biological effects of atmospheric aerosols, the studies on the 2006 series of samples included several measures of oxidative stress in addition to the lavage parameters and histopathology reported in the previous section. However, due to very limited amounts of sample, these studies were conducted using Balb/C mice as the rodent model. These potency factors are illustrated in Figure 4 through 12. Dose response curves are provided in Appendix E, figures 4-11 and statistical analyses of the differences among pairs are shown in Appendix E, Table 2.

In the 2005 series, there was excellent correspondence between toxicological potency (Figure 1 lavage LDH and protein [alkaline phosphatase did not correspond well]) and inflammatory responses (Figure 2: total lavage cells and neutrophils, and Figure 3 top panel: histopathological evidence of inflammation). In the 2006 series, the correspondence was less clear: the most potent samples for inducing inflammatory responses were D06s_1+3 and Ld06sV_1, while these samples were relatively low in potency for toxicity parameters. In contrast, the samples that induced the most potent toxicity responses were Dd06s_1+2 and Ld06sT_1.

Similar to the results from the 2005 series analyzed in rats, the 2006 sample of diesel exhaust aged in the dark without NOx denuder (D06s_1+3) was also the most potent sample in the 2006 series tested in mice, significantly more potent than any other sample for neutrophils in lavage (considered the most sensitive indicator of inflammation: Figure 4). This sample also caused the largest increases in oxidized glutathione and a strong increase in HO-1 (Figure 5, lower panel), but had no significant effect on the lung tissue (Figure 6) or plasma (Figure 7) TBARS. In contrast to the 2005 series, however, the dark sample of aged diesel exhaust introduced to the chamber with NOx denuder (Dd06s_1+2) was more potent than the sample without denuder for several indicators of toxicity, significantly greater than the non-denuded sample for total protein (Figure 3). This sample actually caused a small but significant decrease in oxidized glutathione (Figure 4, lower panel), along with a significant decrease in total glutathione (Figure 4 upper panel), a significant increase in plasma TBARS (Figure 7), but no change in lung tissue TBARS (Figure 36), and was not significantly different from the non-denuded sample in its ability to cause increases in HO-1 (Figure 8).

One potentially important exception to the generally monotonic dose-response curves is the change in plasma TBARS (Appendix E, figure 9), where several of the samples (Dd06s_1+2, D06sN, Ld06s_1, and Ld06sT_1) produced changes at low and mid doses, but returned towards the baseline at the highest concentration. Exceptions were the samples from the light exposures with the NOx denuder, with the additions of volatile organics (Ld06sV_1), formaldehyde (Ld06sF_1), or formaldehyde + volatile organics (Ld06sFV_1), all of which produced near-monotonic decreases in this parameter. The non-denuded dark sample (D06s_1+2) also produced a decrease, while the dark, denuded samples with the addition of $N_2O_5$ and volatile organics had little effect (Dd06s_NV). These effects may be of some physiological relevance, since the low doses are probably of more physiological relevance. The mechanisms for the non-linear dose responses, and indeed for decreases at any dose, are not known, but may involve induction of clearance or compensatory mechanisms such as upregulation of antioxidant response genes as previously discussed (Seagrave et al 2005b).

In contrast to the effects in plasma, the effects of the samples on lung tissue TBARS (Appendix E, Figure 8) were relatively small, although trends towards a biphasic response were observed for several samples (Dd06sN, D06sN, Dd06s_1+2, and Ld06s_1). When analyzed by linear regression for all doses (Figure 36), only Ld06sF_1 and Ld06sFV_1 caused significant increases, while LD06s_1 and Ld06sT_1 caused slight, but significant, decreases.

The effects on total lung tissue glutathione were significant decreases for all samples except for the sample exposed in the dark without NOx denuder (D06-s_1+3), the sample exposed in the light with NOx denuder (Ld06s_1), and the sample exposed in the light with NOx denuder and volatile organics (Ld06sV) (Figure 5). The effects of the samples exposed in the light with either formaldehyde or formaldehyde and volatile organics were particularly potent.

The effect on oxidized glutathione (GSSG) were generally increases, with the strongest effect of the sample exposed in the dark without NOx denuder (D06s_1+2). Addition of $N_2O_5$ prevented this effect. However, both the samples exposed in the dark with NOx denuder with or without the addition of $N_2O_5$ (and volatile organics) caused decreases in GSSG. All of the samples exposed in the light appeared to cause increases in GSSG, but the effects of Ld06s_1 and Ld06sT did not reach statistical significance.

HO-1 is considered by some to be a very sensitive indicator of oxidative stress. Both of the samples exposed in the dark with or without NOx denuder, but without any additions to the atmosphere, caused increases in this parameter. However, the addition of $N_2O_5$ suppressed this response. In contrast, the NOx denuded sample exposed in the light (Ld06s_1) had little effect, and the addition of toluene did not change this lack of effect, but the samples with either volatile organics and/or formaldehyde added caused very significant increases. It is interesting that the light samples with the addition of volatile organics or formaldehyde in general caused decreases in plasma TBARS but increase in lung tissue TBARS and HO-1.

Suppression of macrophage phagocytosis has also been implicated as a mechanism for altered innate immune responses following exposure to air pollution. Most of the samples did suppress phagocytosis of fluorescent microspheres as shown in Figure 39. However, Dd05sNV had no significant effect, and there was an apparent stimulation at low doses and suppression at high doses for D06sN, resuting in a potency score not significantly different from zero.

## 4. EFFECTS OF CHAMBER BLANKS

As explained in Section 3, blank chamber samples were collected in summers 2005 and 2006 prior to the beginning of chamber exposure experiments. One dark chamber blank (D05s) and three light chamber blanks (L05s_1b, L06s_1b and L06s_2b) were collected. The light

chamber blank samples were analyzed both for chemistry and toxicology. The chemistry data were reported in Appendix C, and the relative response (per unit of mass) of the material collected from the blanks is shown in Appendix E with the toxicology data. Of note is the chamber blanks contained material, especially SVOC (collected on XAD cartridges following the filter), that yielded toxicity per unit mass that was in many cases on par with the responses from the aging experiments. The presence of chemicals in the chamber blank illustrates the potential for residual material, even after chamber cleaning, to desorb from the walls. In addition to chemicals present in the SVOC fraction of the speciated organics, we observed gas phase reactants in the chamber blank atmosphere that included peroxyacetyl nitrate and formaldehyde. This observation shows that the sunlight and flow of clean air through the chamber serves to first desorb sequestered material from the walls and then initiates reactions that create SVOC. Although some of the chemicals measured in the chamber blanks were also observed in the test atmospheres (they are common), the overall composition was different. Indeed, the chamber blank showed no particle mass and little particle organics such as the PAH. Although the relative toxicity of the chamber blanks was determined to be high (per unit of mass), the mass of the material collected from the chamber during the blank runs was low (PM mass below minimum detection limit), and did not contribute to the interpretation of the ranking among atmospheres.

In other programs, we have observed wall desorption phenomenon that is analogous to the desorption of material from the aging chamber walls observed in this study. In engine studies that use dilution tunnels we often see dilution tunnel blanks that yield concentrations at or higher values than what is observed during operation of engines for emissions studies (Lev-On et al, 2002a; 2002b). In those studies there is an apparent equilibrium that is established between

the emissions and the material that is adsorbed and sequestered to the dilution tunnel walls. When clean air is transported through the tunnel in the absence of emissions this equilibrium is disturbed, and otherwise sequestered material is desorbed from the walls and collected on blanks. The composition of those blanks is typically different than the emissions themselves, although some of the chemical species may be the same in both cases. These types of effects presumably contribute to the blanks from the aging chamber, where material is desorbed in the absence of added reactants. As such, we submit that the observed chemicals and toxicity in the chamber blanks have little impact on the interpretation of the relative composition and toxicity of samples from the atmospheres generated in this program.

# List of Figures

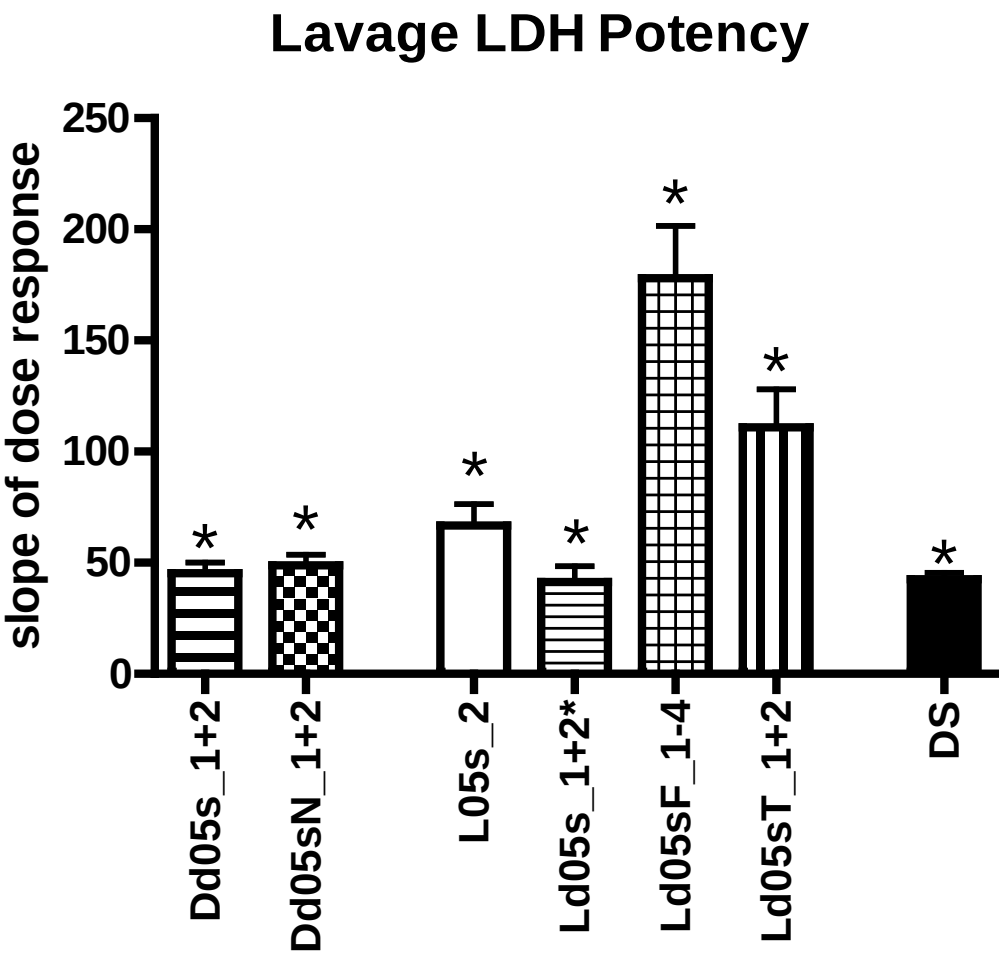


Figure 1. Potency scores for the biochemical results in lavage fluid for samples collected in 2005 and tested by intratracheal instillation in rats. * indicates slope significantly different from zero. Sample identification: D= Dark; L = Light; d = NOx denuder present; 05: samples collected in 2005; s = summer; w = winter; N = added $NO_3$; F = added formaldehyde; T = added toluene; DS = diesel soot standard research material.

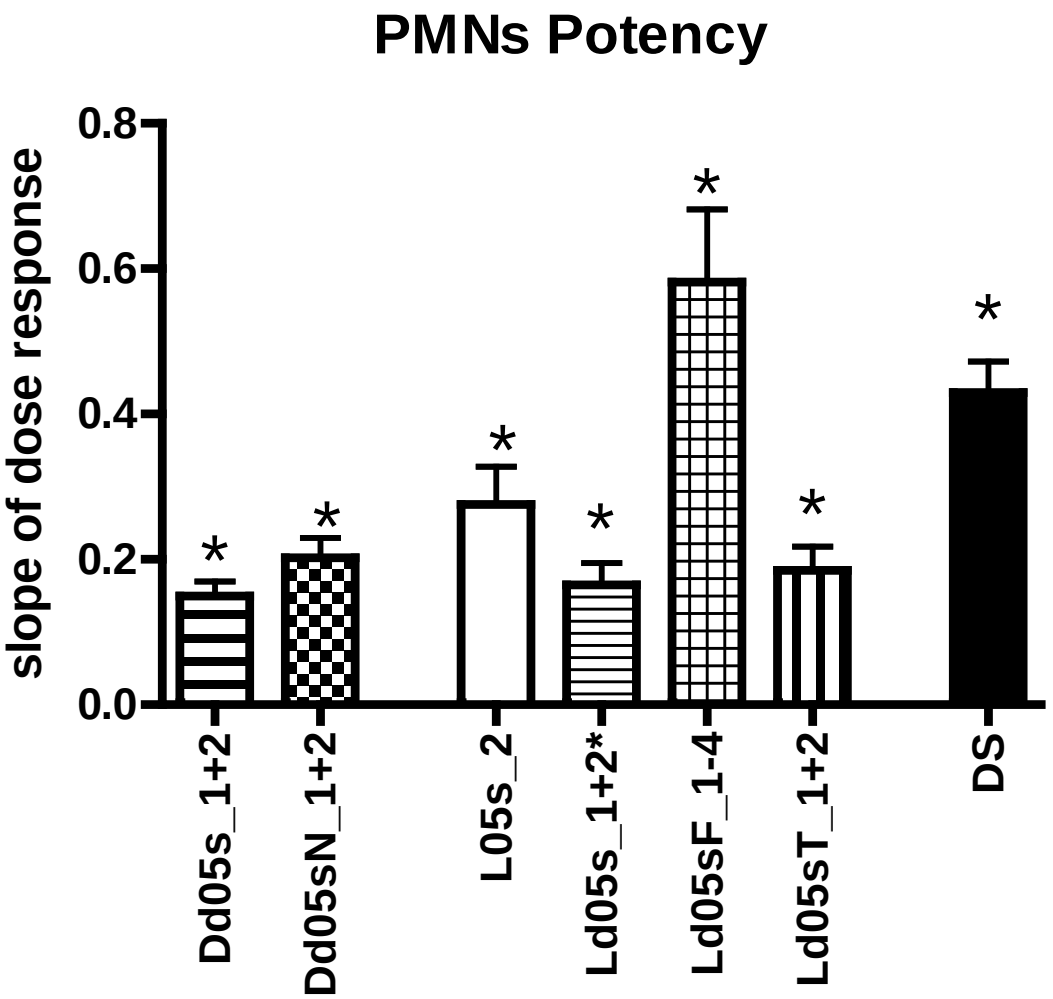


Figure 2. Potency scores for the cellular profiles in lavage fluid for samples collected in 2005 and tested by intratracheal instillation in rats. * indicates slope significantly different from zero. * indicates slope significantly different from zero. Sample identification: D= Dark; L = Light; d = NOx denuder present; 05: samples collected in 2005; s = summer; w = winter; N = added $NO_3$; F = added formaldehyde; T = added toluene; DS = diesel soot standard research material.

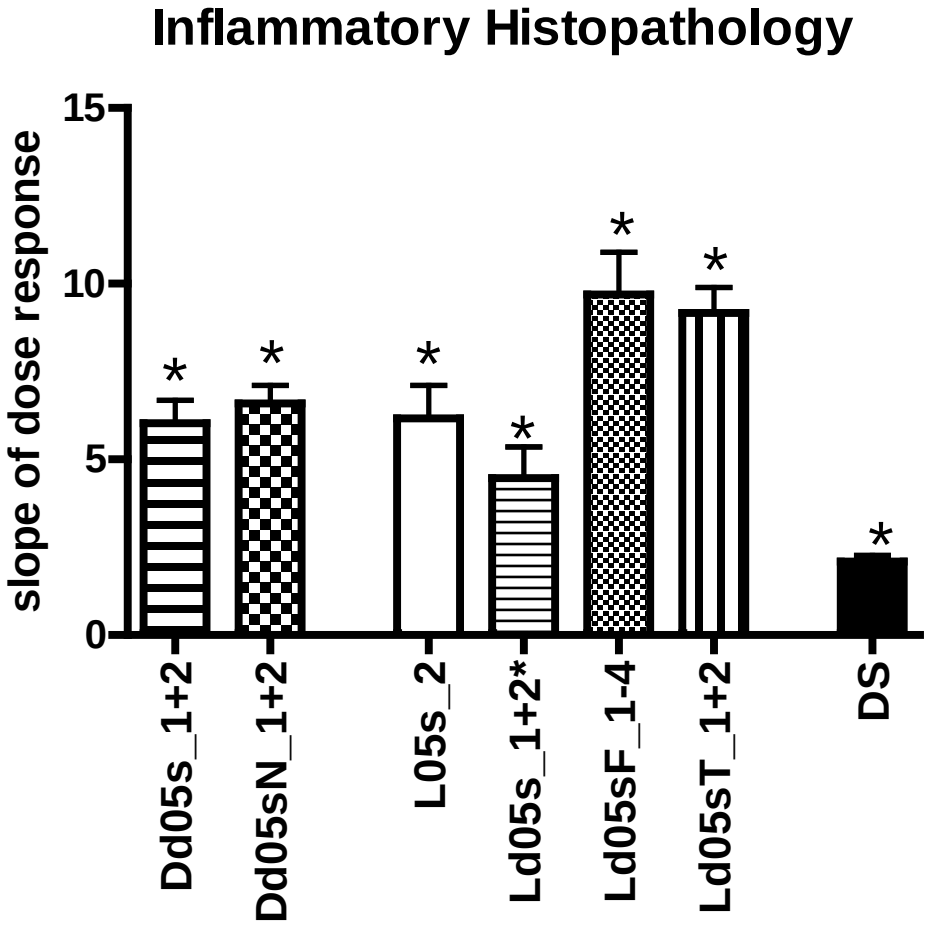


Figure 3. Potency scores for the histopathological results in lavage fluid for samples collected in 2005 and tested by intratracheal instillation in rats. * indicates slope significantly different from zero. Sample identification: D= Dark; L = Light; d = NOx denuder present; 05: samples collected in 2005; s = summer; w = winter; N = added $NO_3$; F = added formaldehyde; T = added toluene; DS = diesel soot standard research material.

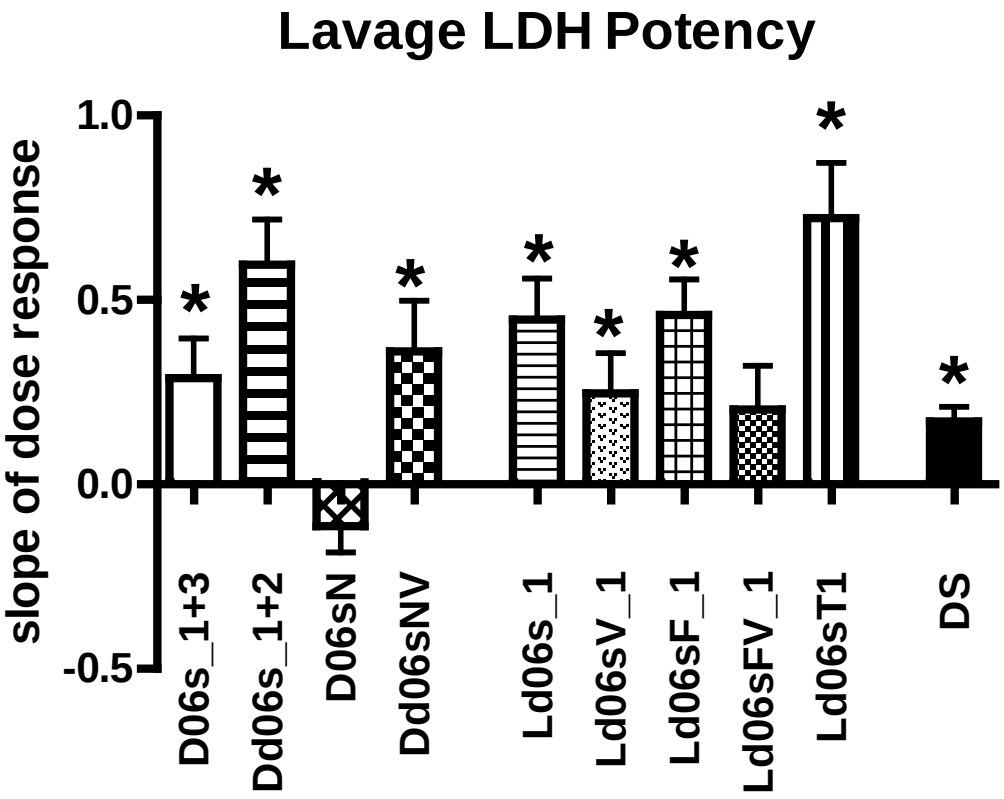


Figure 4. Potency scores for the lavage fluid biochemical responses for samples collected in 2006 and tested by intratracheal instillation in mice. * indicates slope significantly different from zero. Sample identification: D= Dark; L = Light; d = NOx denuder present; 06: samples collected in 2006; s = summer; N = added NO3; V= volatile organic compounds; F = added formaldehyde; T = added toluene; DS = diesel soot standard research material.

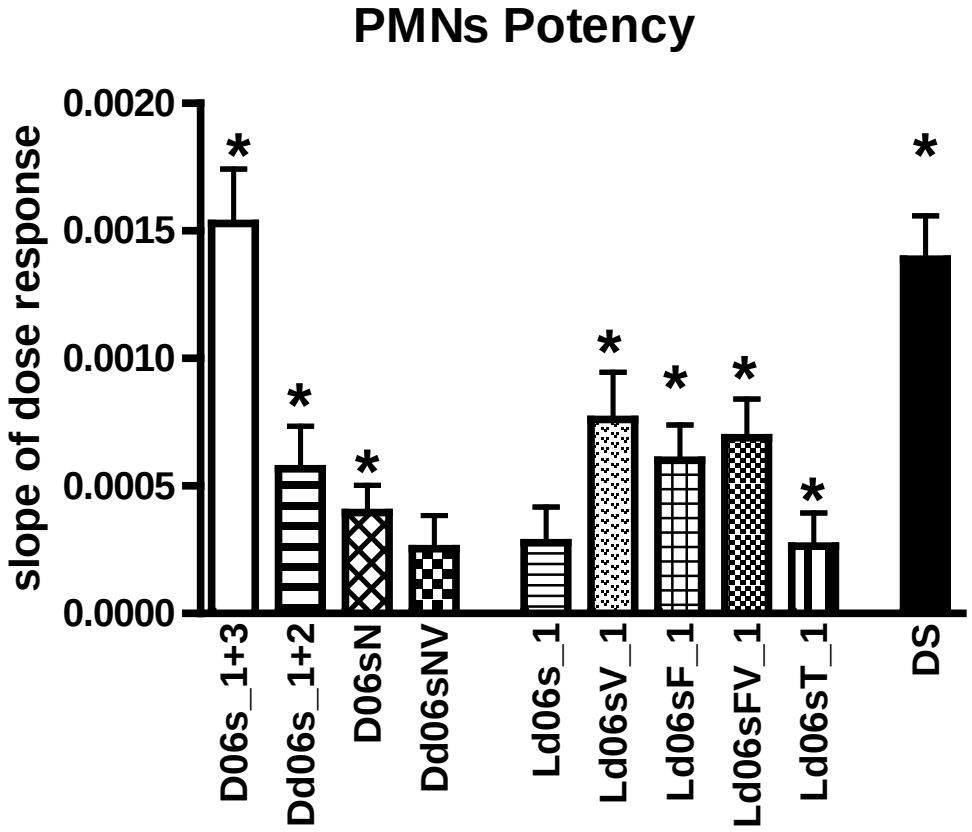


Figure 5. Potency scores for the lavage cell responses for samples collected in 2006 and tested by intratracheal instillation in mice. * indicates slope significantly different from zero. Sample identification: D= Dark; L = Light; d = NOx denuder present; 06: samples collected in 2006; s = summer; N = added NO3; V= volatile organic compounds; F = added formaldehyde; T = added toluene; DS = diesel soot standard research material.

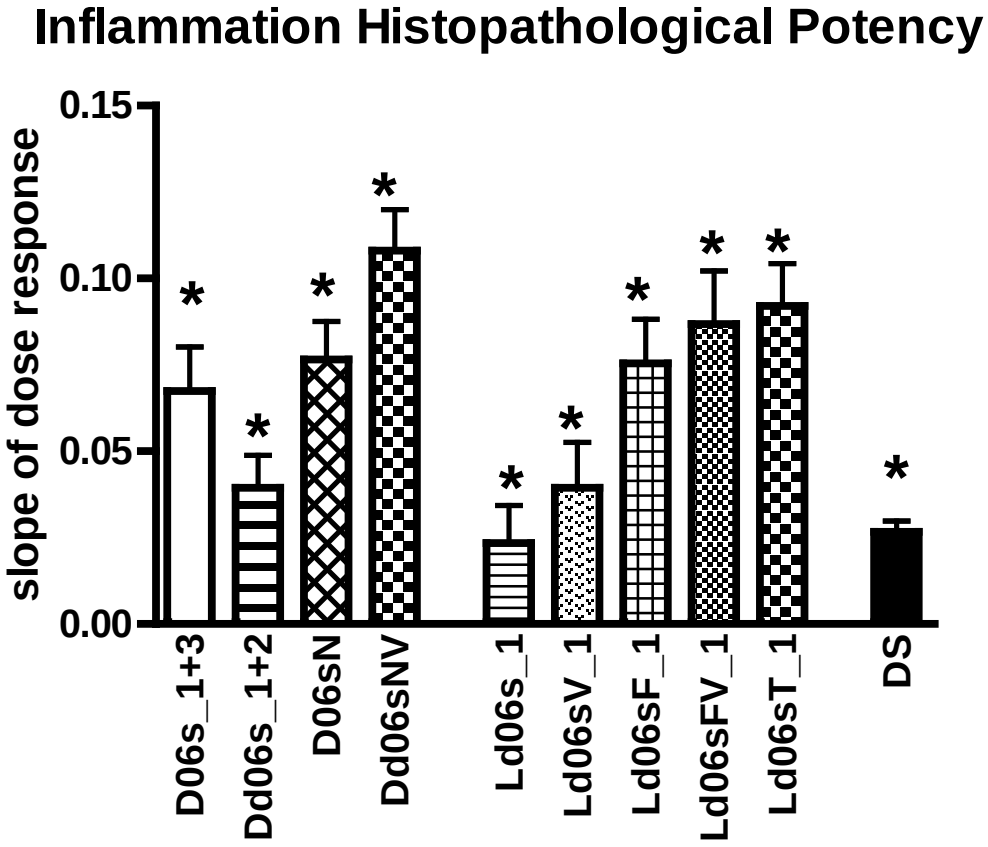


Figure 6. Potency scores for the histopathological responses for samples collected in 2006 and tested by intratracheal instillation in mice. * indicates slope significantly different from zero. Sample identification: D= Dark; L = Light; d = NOx denuder present; 06: samples collected in 2006; s = summer; N = added NO3; V= volatile organic compounds; F = added formaldehyde; T = added toluene; DS = diesel soot standard research material.

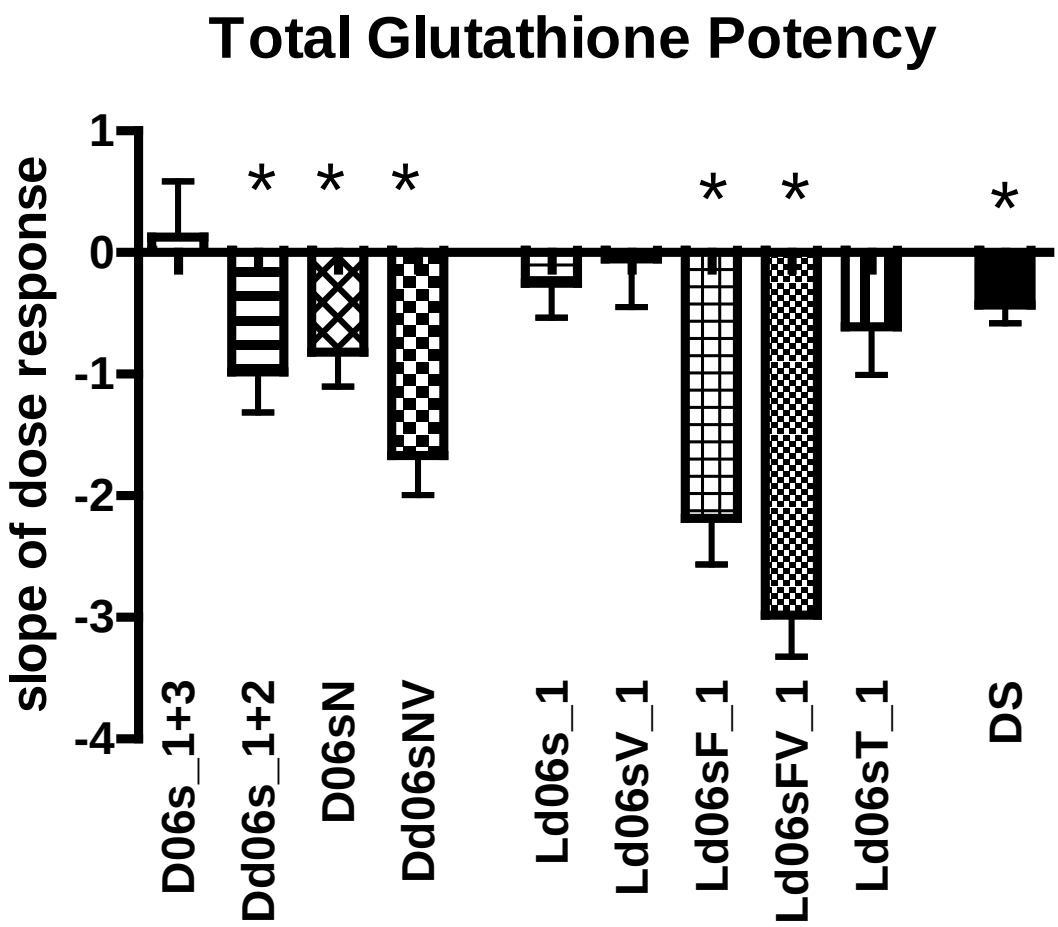


Figure 7. Potency scores for the lung tissue glutathione responses for samples collected in 2006 and tested by intratracheal instillation in mice. * indicates slope significantly different from zero. Sample identification: D= Dark; L = Light; d = NOx denuder present; 06: samples collected in 2006; s = summer; N = added $NO_3$; V= volatile organic compounds; F = added formaldehyde; T = added toluene; DS = diesel soot standard research material.

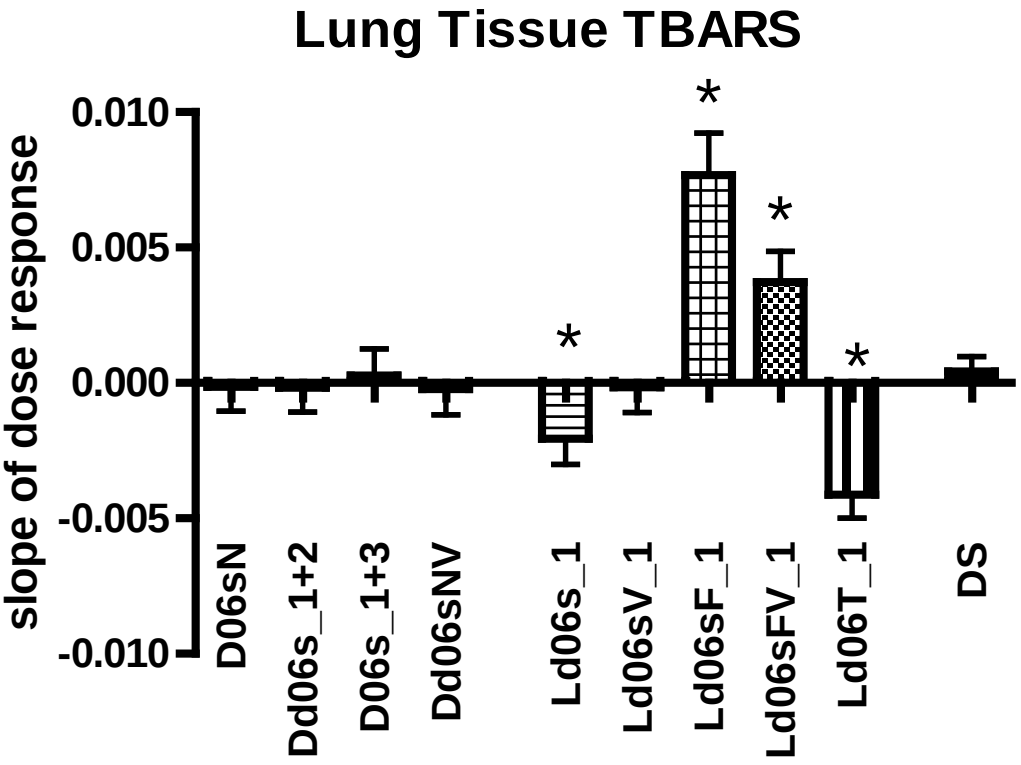


Figure 8. Potency scores for the lung tissue TBARS biochemical responses for samples collected in 2006 and tested by intratracheal instillation in mice. * indicates slope significantly different from zero. Sample identification: D= Dark; L = Light; d = NOx denuder present; 06: samples collected in 2006; s = summer; N = added NO3; V= volatile organic compounds; F = added formaldehyde; T = added toluene; DS = diesel soot standard research material.

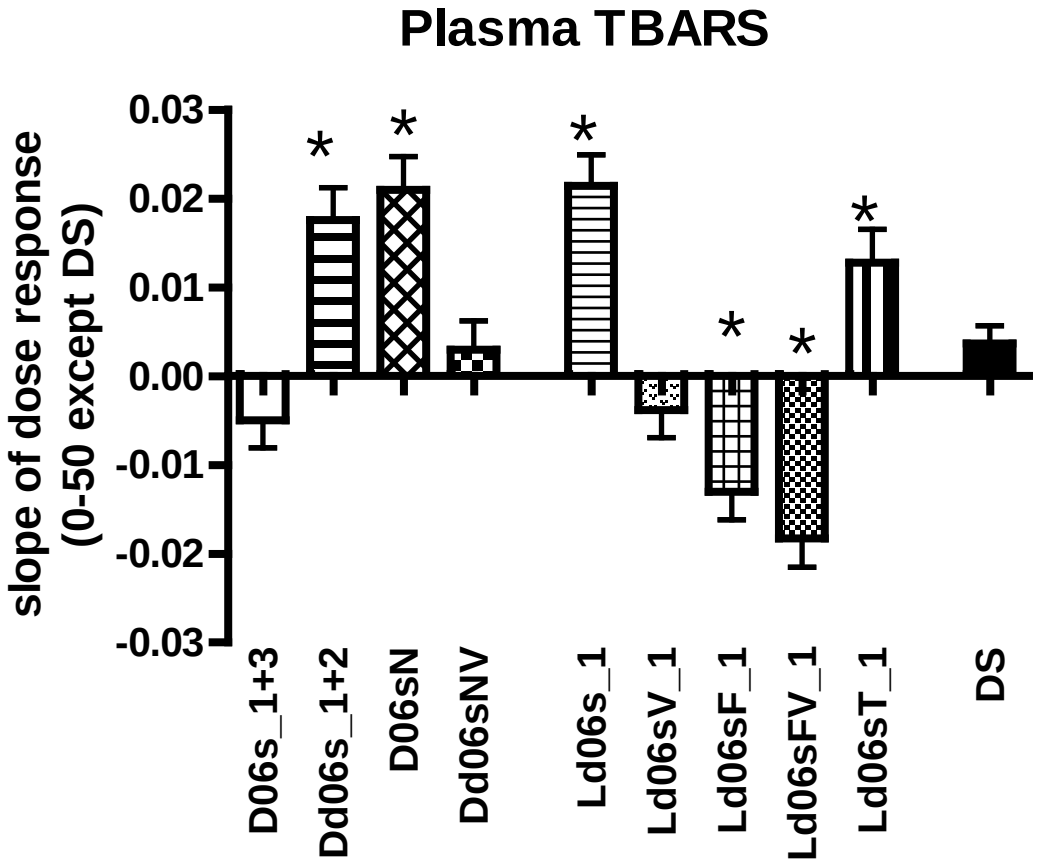


Figure 9. Potency scores for the plasma TBARS responses for samples collected in 2006 and tested by intratracheal instillation in mice. * indicates slope significantly different from zero. Sample identification: D= Dark; L = Light; d = NOx denuder present; 06: samples collected in 2006; s = summer; N = added NO3; V= volatile organic compounds; F = added formaldehyde; T = added toluene; DS = diesel soot standard research material.

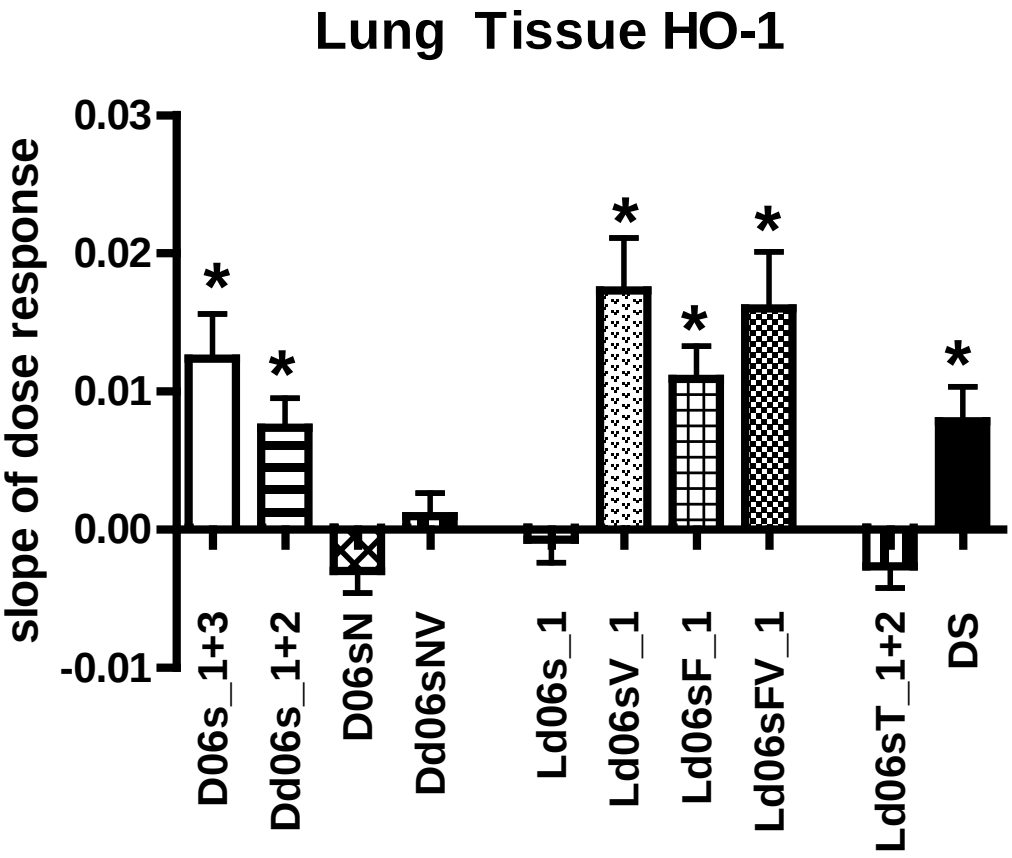


Figure 10. Potency scores for the lung tissue HO-1 responses for samples collected in 2006 and tested by intratracheal instillation in mice. * indicates slope significantly different from zero. Sample identification: D= Dark; L = Light; d = NOx denuder present; 06: samples collected in 2006; s = summer; N = added NO3; V= volatile organic compounds; F = added formaldehyde; T = added toluene; DS = diesel soot standard research material.

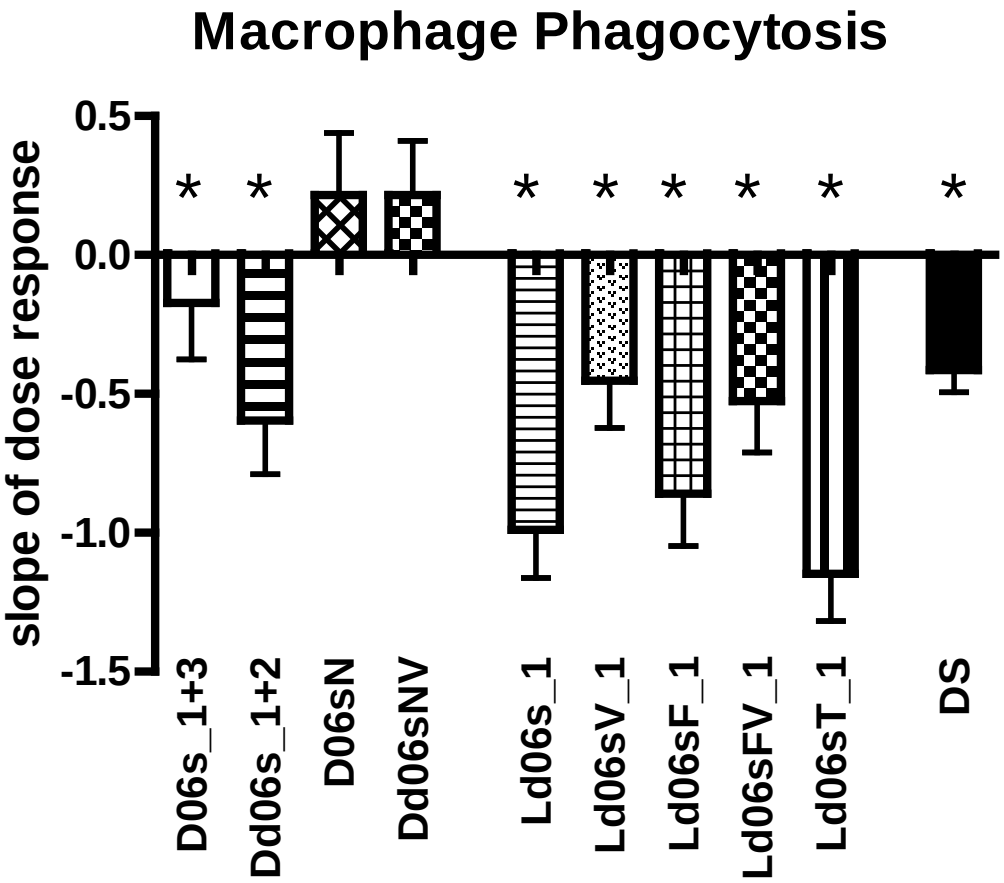


Figure 11. Potency scores for the macrophage phagocytosis responses for samples collected in 2006 and tested by intratracheal instillation in mice. * indicates slope significantly different from zero. Sample identification: D= Dark; L = Light; d = NOx denuder present; 06: samples collected in 2006; s = summer; N = added NO3; V= volatile organic compounds; F = added formaldehyde; T = added toluene; DS = diesel soot standard research material.