

***In vitro* Evaluation of Oxidative Stress Caused by Fine Particles (PM_{2.5}) Exhausted from Heavy-Duty Vehicles Using Diesel/Biodiesel Blends under Real World Conditions**

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In this work, the redox activity of fine diesel/biodiesel particulate matter (PM_{2.5}) was studied in order to approach its toxicity from reactive oxygen species, due to adverse effects it may cause to human health. The oxidative potential was measured by the dithiothreitol (DTT) assay in order to study the relative contribution of water-soluble transition metals, polycyclic aromatic compounds (PAH), nitro-PAH, and quinones. It was analyzed a total of 24 samples collected from primarily diesel/biodiesel-exhausted particles from buses. The rate concentrations of PM_{2.5} redox activity ranged 0.020-0.069 nmol min⁻¹ μg⁻¹, with median at 0.040 nmol min⁻¹ μg⁻¹ (on average, 0.042 ± 0.005 nmol min⁻¹ μg⁻¹ for morning, 0.033 ± 0.007 nmol min⁻¹ μg⁻¹ for afternoon and 0.045 ± 0.009 nmol min⁻¹ μg⁻¹ for night). The transition metals appear to dominate the DTT response, since they were responsible up to 89% of redox activity measured in the samples. Apparently, the metal fraction contained in PM_{2.5} demonstrated a greater ability to catalyze reactions that promote the formation of reactive oxygen species when compared to organic compounds. It was observed that the oxidative potential of PM_{2.5} particles emitted from diesel/biodiesel (B7) is similar to diesel-emitted particles.

Keywords: dithiothreitol assay, redox activity, ambient particulate matter, reactive oxygen species, diesel/biodiesel particles

Introduction

The most significant source of particulate matter (PM) loadings in urban atmosphere is the exhaustion of both light- and heavy-duty vehicles during daily commuting activities. Inhalation of diesel-emitted particles, mainly in the fine particulate fraction, is a matter of significant concern¹⁻³ since both environmental and occupational exposures to diesel exhaust particles (DEP) are considerable^{1,2} and may lead to diverse health-related endpoints, such as cancer and pulmonary or respiratory disorders,²⁻⁷ as well as morbidity and mortality.^{8,9} When also considering premature deaths according to inhalation of fine particles in outdoor environments, there were 3.3 million deaths worldwide in 2010 and it is estimated to be more than 6.5 million

premature deaths by 2050.¹⁰ It is likely the health concerns about urban atmosphere and DEP is aggravated.

Indeed, the International Agency for Research on Cancer (IARC)¹¹ has recently reclassified DEP as carcinogenic to humans (Group 1) according to sufficient and convincing evidences that DEP exposure increases the risk of lung cancer. As biodiesel has increasing its popularity together with the perceptive reductions of fossil fuel reserves worldwide, it has been used to partially replace fossil diesel as fuel, used mostly in unmodified diesel engines.¹²⁻¹⁹ In regard to regulated emissions, the diesel/biodiesel blends bring some environmental advantages, such as the reduction in the emissions of PM, carbon monoxide (CO), carbon dioxide (CO₂), sulfur oxides (SO_x) and hydrocarbons (HC), even though there is an increase in nitrogen oxides (NO_x) emissions.⁵ But when taking into account unregulated pollutants (such as carbonyls and

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polycyclic aromatic compounds (PAHs), among others), it was found biodiesel may increase emission levels of some of these substances,^{13-16,18,19} although contradictory results are reported,²⁰⁻²² probably due to the differences in the engine technology, fuel composition, and driving regime. However, it is still uncertain if biodiesel-exhausted particles (BEP) would be more or less toxic than DEP. Despite the fact some studies have tried to address this issue,^{4,16,17,23,24} the toxicity of traffic-related urban PM is still not completely understood and more studies are needed.

Plausible reasons for DEP or BEP toxicities are associated with their abilities of inducing oxidative stress and/or inflammation processes, provoking cell alterations. Oxidative stress is generally mediated by reactive oxygen species (ROS), such as hydroxyl radical (OH), hydrogen peroxide (H₂O₂), superoxide (O₂⁻), organic hydroperoxides and other organic radical species.^{1,6,16,25-28} Although the actual mechanisms of how ROS damage cells are unknown, it is believed they trigger multiple biological signaling, which result in inflammation and other cellular distresses.^{6,29,30} ROS may either be primarily transported on particles and/or be catalytically generated by other particle components through redox chemistry producing health-related issues.^{24,25} For instance, some transition metals (such as Fe and Cu) and organic species (e.g. polycyclic aromatic compounds and quinones or quinone-like compounds) as well as humic-like substances (HULIS), black and brown carbon (BC and BrC) within PM are known to increase the production of ROS either *in vitro* or *in vivo*, especially if they are present in fine and ultrafine size range,^{4-6,24,30-35} since they are able to go more profoundly in the respiratory system. Meanwhile, considering diesel/biodiesel blends exhausts are substantially composed by ever small size PM, such as nanoparticles and/or ultrafine particles rich in semi-volatile organic compounds and trace metals,^{1,7,16} it is likely ROS may be contributing to the adverse health effects caused by DEP or even BEP. In this way, it becomes necessary more investigations in this area.

It is available a number of different assays for measurement of fine or ultrafine particulate matter redox properties. However, the dithiothreitol (DTT) assay generally is a good choice since it correlates well with oxidative stress biomarkers such as heme oxygenase 1 (HO-1) and inflammatory markers (e.g. interleukin-6 and interleukin-8)^{36,37} and granulocyte macrophage colony-stimulating factor (GM-CSF).³⁸

This research reports results of prooxidant capacity from PM_{2.5} (fine diesel/biodiesel particulate matter) emitted from buses using diesel/biodiesel blends under real world circumstances, with the engines operating mainly in the stationary mode or even in low acceleration mode. The

first part of the study was focused to measure the relative contribution of selected water-soluble trace elements (Na⁺, K⁺, Mg²⁺, Ca²⁺, Ba²⁺, V³⁺, Cr³⁺, Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Al³⁺, Mo³⁺, Ag⁺, Cd²⁺, Hg²⁺, Sn⁴⁺, Sb³⁺ and Pb²⁺) and organic species (PAHs, nitro-PAHs and quinones) in the catalytic generation of ROS by PM_{2.5} by using SRM 1649b as a representative of real urban PM. After adjusting our analysis protocol, we applied the same reasoning to measure oxidative stress in real diesel/biodiesel emitted particles collected in a bus station.

Experimental

Sample collection and site characteristics

The Lapa Station³⁹ is a bus station located in the city of Salvador, state of Bahia, Northeastern Brazil (12°58'S, 38°30'W, altitude 52 m above sea level). It is the largest bus terminal in the Salvador Metropolitan Area, which occupies 150,000 m², being 30,000 m² of built area. The Lapa bus station receives more than 80 urban bus lines, 21 metropolitan transportations and about 430 thousand passengers *per* day, with an average flow of 325 buses *per* hour. The fuel used by the buses during the present study was B7, a mixture of 93% diesel/7% biodiesel. The diesel was S50 (50 ppm of sulfur) and considering that biodiesel does not contain sulfur, the final content of sulfur was 46.5 ppm. The Brazilian laws regarding to buses emissions was equivalent to the Euro IV and the average age of the fleet was five years old. The Lapa bus Station operates 24 h a day, 7 days a week.

In order to collect representative diesel/biodiesel-emitted PM_{2.5} samples, we placed our sampling system in the underground floor, where no ventilation system is available for dissipating bus exhausts. In turn, in this nearly closed place, buses remain with their engines on, at idle point while waiting for passengers.^{12,40,41} PM_{2.5} samples were collected at PTFE membrane filters 47 mm diameter and pore size of 1.0 μm (Merck Millipore, Ireland), using a low-volume sampler cyclone type (Casella CEL, Bedford, UK), equipped with a filter holder, operated at a flow rate of 15 L min⁻¹. The aerosol collections were done each day in the morning (07:00 AM to 02:00 PM), afternoon (02:00 PM to 07:00 PM) and night (07:00 PM to 07:00 AM). A total of 24 samples, 3 *per* day, were collected for this study. Together to the collected PM_{2.5} samples, one blank filter was used for each sampling period. After sampling, both the sample and blank filters were stored into polyethylene plastic petri dishes and were PTFE tape-sealed and identified, put into a cooler box then transported to the laboratory. All the procedures were strictly quality controlled in order to avoid

any possible sample contamination. For measurement of sampled particle mass, filters were weighed before and after each field collection using a Mettler 5 Microbalance (MT 5, Mettler-Toledo Inc., Hightstown, NJ, USA), under controlled relative humidity (40-45%) and temperature (22-24 °C) conditions. After weighing, filter samples and blanks were stored in a freezer (−20 °C) prior to analysis in order to prevent losses of volatile components.

Reagents and standards

All solutions were prepared with analytical grade reagents and ultrapure water generated from a Milli-Q system (Millipore, Bedford, MA, USA), at resistivity of 18 MΩ cm⁻¹. The reagents used were dithiothreitol (DTT) (99%), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (99%), trichloroacetic acid (TCA) (99%), trizma hydrochloride (99%), dimethyl sulfoxide (DMSO) (99.9%), diethylenetriamine-pentaacetic acid (DTPA) (98%), being all obtained from Sigma-Aldrich (USA). The DTPA solution in concentration 10 mmol L⁻¹ was prepared in potassium phosphate buffer 0.5 mol L⁻¹ and pH 7.4, 2 mmol L⁻¹ DTT, and 10 mmol L⁻¹ were prepared before the execution of each test. Endotoxins-free water (LAL Reagent Water, Endotoxin Content < 0.005 EU mL⁻¹). A standard reference material PAH mix containing the 16 EPA priority PAHs: acenaphthene (ACE), acenaphthylene (ACY), anthracene (ANT), benz[*a*]anthracene (BaA), benzo[*a*]pyrene (BaP), benzo[*b*]fluoranthene (BbF), benzo[*ghi*]perylene (BgP), benzo[*k*]fluoranthene (BkF), chrysene (CRY), dibenz[*a,h*]anthracene (DBA), fluoranthene (FLT), fluorene (FLU), indeno[1,2,3-*d*]pyrene (IND), naphthalene (NAP), phenanthrene (PHE), and pyrene (PYR) at 2000 μg mL⁻¹ each, in methanol:methylene chloride (1:1) was purchased from Supelco (St. Louis, USA). Solutions of nitro-PAHs standards were prepared from two standard reference materials, SRM 2264 (nitro aromatic hydrocarbons in methylene chloride I) and SRM 2265 (nitrated polycyclic aromatic hydrocarbons in methylene chloride II) from the National Institute of Standards and Technology (NIST, USA). The SRM 2264 contains the following compounds: 1-nitronaphthalene (1-NNap), 2-nitronaphthalene (2-NNap), 1-methyl-4-nitronaphthalene (1-methyl-4-NNap), 1-methyl-5-nitronaphthalene (1-methyl-5-NNap), 1-methyl-6-nitronaphthalene (1-methyl-6-NNap), 2-methyl-4-nitronaphthalene (2-methyl-4-NNap), 2-nitrobiphenyl (2-NBP), 3-nitrobiphenyl (3-NBP), 4-nitrobiphenyl (4-NBP), 5-nitroacenaphthene (5-NAce), and 2-nitrofluorene (2-NFlu). In turn, SRM 2265 is composed of the following compounds: 2-nitrophenanthrene (2-NPhe),

3-nitrophenanthrene (3-NPhe), 9-nitrophenanthrene (9-NPhe), 2-nitroanthracene (2-NAnt), 9-nitroanthracene (9-NAnt), 2-nitrofluoranthene (2-NFlt), 3-nitrofluoranthene (3-NFlt), 1-nitropyrene (1-NPyr), 2-nitropyrene (2-NPyr), 4-nitropyrene (4-NPyr), 6-nitrochrysene (6-NCry), 7-nitrobenz[*a*]anthracene (7-NBaA), 3-nitrobenzanthrone (3-NBA), 6-nitrobenzo[*a*]pyrene (6-NBaPyr), 1-nitrobenzo[*e*]pyrene (1-NBePyr), and 3-nitrobenzo[*e*]pyrene (3-NBePyr). The quinones used in this work were 1,4-benzoquinone (1,4-BQ) (98%), 9,10-phenanthraquinone (9,10-PQ) (95%) and 9,10-anthraquinone (9,10-AQ) (99.4%), which were purchased from Sigma-Aldrich (St. Louis, USA); 1,2-naphthoquinone (1,2-NQ) (90%) and 1,4-naphthoquinone (1,4-NQ) (96.5%) were purchased from Fluka (St. Louis, USA). Standard solution containing the PAH was prepared in high-performance liquid chromatography (HPLC) grade acetonitrile. In the case of nitro-PAH and quinones the solvent used was tetrahydrofuran.

High-purity standard solutions at 1.000 mg L⁻¹ (Merck, Darmstadt, Germany) were used to prepare analytical solutions multielemental reference standards (Na⁺, K⁺, Mg²⁺, Ca²⁺, Ba²⁺, V³⁺, Cr³⁺, Mn²⁺, Fe²⁺, Co²⁺, Ni²⁺, Cu²⁺, Zn²⁺, Al³⁺, Mo³⁺, Ag⁺, Cd²⁺, Hg²⁺, Sn⁴⁺, Sb³⁺ and Pb²⁺).

The urban dust standard reference material SRM 1649b from NIST (Maryland, USA) was also used in order to test the applicability of the DTT assay. According to the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA), the SRM 1649b is an atmospheric particulate material collected in an urban area (Washington, DC, USA).

Redox activity assessment

PM_{2.5} samples were extracted with 10 mL endotoxin-free water reagent under mixing vortex at 1000 rpm during 20 min followed by two cycles of 10 min each in ultrasonic bath with controlled temperature 23 °C. The same procedure was applied to the SRM 1649b Urban Dust.

After the extraction procedure, 200 μL aliquots of the extract were transferred to test tubes and then were added 200 μL of potassium phosphate buffer pH 7.4 and 500 μL of Milli-Q water. Subsequently they were added to the test tubes 100 μL DTT 2 mmol L⁻¹ under vortex and inserted into the incubator with controlled temperature at 37 °C with agitation, then it was waited the pre-defined reaction times at 0, 15, 30 and 45 min. After the reaction times were done, the reactions were stopped by adding 1000 μL of 10% TCA under vortex. Then the extracts were transferred to Eppendorf tubes and centrifuged at 1200 rpm for 15 min for removal of any suspended

solids in order to avoid any possible interference in the spectrophotometric measurements. Aliquots of 500 μL from Eppendorf tubes were collected and transferred to new test tubes, followed by the addition of 1000 μL of pH 8.9 Tris-HCl buffer and 25 μL of 10 mmol L^{-1} DTNB solution with subsequent homogenization by vortex. The resulting yellow 2-nitro-5-thiobenzoic acid formed was further measured at 412 nm using a UV-Vis spectrophotometer (model Q898U, QUIMIS, São Paulo, Brazil). The rate of DTT consumption was obtained by calculating the slope of the linear regression of absorbance against time. The average DTT consumption rate of the loaded filters (done in duplicates) was corrected with the results obtained from a blank filter treated in the same way.

Sample enrichment tests

The DTT assay is a commonly used cell-free measure of airborne particle oxidative potential. Within this assay, any redox-active substance present in particulate matter (PM), either being ROS or not, oxidize the added DTT to its sulfide form, which results in a linear DTT decrease. This linear decay of the DTT concentration in the reaction medium is directly proportional to the PM oxidative potential. However, this assay does not respond to any specific ROS individually, it rather responds to the overall effect of every pro-oxidant species on DTT composing the airborne particles.

Although the DTT assay is widely used and relatively well accepted by the scientific community, very little is known about which species oxidize DTT and how these species interact with each other. Indeed, some metals interact with DTT in a non-linear relation. Other metals do not contribute at all for the DTT oxidation.²⁷ On the other hand, the DTT molecule itself may act as a soft Lewis' base and may coordinate to selected metals (e.g. Zn^{II} , Cd^{II} , Pb^{II} , Ni^{II} , and Cu^{I}), as suggested by Krezel *et al.*⁴² Since in DTT assay we actually measure the remaining DTT after pre-established reaction times, this residual DTT would be virtually available to coordinate to any transition metal ion normally and generally present in appreciable quantities in any PM sample. This would, in turn, anomalously decrease the concentration of unreacted DTT in the medium. If there is less unreacted DTT in the reactional medium, less TNB would be formed so it would give us a wrong idea of a higher PM oxidative potential than it actually is. This could, therefore, potentially give us an erroneous measurement of oxidative potential of a given sample and would lead us to draw misinterpretations about PM characteristics.

In order to evaluate relative contributions of different chemical species (both organic and inorganic compounds) to oxidative stress, as well as if would have some

contribution of coordination complex DTT-metal in the anomalously decrease DTT measurements, we designed a set of experiments using SRM 1649b prior to the analysis of real samples. DTT assay tests were performed with some combinations among a known amount of SRM 1649b, a mix of PAHs, a mix of nitro-PAHs, individual quinones and a mix of trace elements, as following:

- (i) 1.0 mg SRM 1649b by itself;
- (ii) an addition of PAH mix containing 16 priority species, at final concentration of 6.5 $\mu\text{g L}^{-1}$ per compound ($\Sigma\text{PAH} = 104 \mu\text{g L}^{-1}$) to 1.0 mg SRM 1649b;
- (iii) an addition of nitro-PAH mix containing 27 species, at final concentration of 6.5 $\mu\text{g L}^{-1}$ per compound ($\Sigma\text{PAH} = 175.5 \mu\text{g L}^{-1}$) to 1.0 mg SRM 1649b;
- (iv) an addition of 1,2-naphthoquinone (1,2-NQ) at final concentration of 6.5 $\mu\text{g L}^{-1}$ to 1.0 mg SRM 1649b;
- (v) an addition of 1,4-naphthoquinone (1,4-NQ) at final concentration of 6.5 $\mu\text{g L}^{-1}$ to 1.0 mg SRM 1649b;
- (vi) an addition of 9,10-anthraquinone (9,10-AQ), at final concentration of 6.5 $\mu\text{g L}^{-1}$ to 1.0 mg SRM 1649b;
- (vii) an addition of 9,10-phenanthraquinone (9,10-PQ) at final concentration of 6.5 $\mu\text{g L}^{-1}$ to 1.0 mg SRM 1649b;
- (viii) an addition of 1,4-benzoquinone (1,4-BQ) at final 6.5 $\mu\text{g L}^{-1}$ per compound at final concentration of 6.5 $\mu\text{g L}^{-1}$ to 1.0 mg SRM 1649b;
- (ix) an addition of trace metal mix containing 21 species at final concentration of 0.01, 0.1, 1.0, and 10 $\mu\text{g L}^{-1}$ per metal, totalizing Σmetals as 0.21, 2.1, 21, and 210 $\mu\text{g L}^{-1}$, respectively.

In each above-mentioned case, we ran experiments with and without addition of DTPA, used as a metal chelating agent, in order to distinguish the relative contribution of organics to trace metals in the PM oxidative potential. Each experiment was run in triplicates.

Results and Discussion

Our discussion is divided in two parts: (i) sample enrichment results and (ii) real sample results. In the first part, we discuss the DTT assay applicability in face to possible formation of stable DTT-metal complexes as well as the relative contribution of organics to metals for the measured PM oxidative potential. In turn, in the last part we discuss the oxidative stress of real diesel/biodiesel-emitted PM_{2.5} samples released from buses in real emission episodes, collected in the Lapa Bus Station.

It is noteworthy to mention two previous studies in the Lapa Bus Station done by our group. In Sousa *et al.*,⁴¹ we found the same five quinones within the range 0.32 to 3.38 ng m^{-3} . Additionally, in Santos *et al.*,⁴⁰ we found the same species considered in the present study, ranging from

0.06 to 15 ng m⁻³ (PAH), < LOD (limit of detection) to 69.4 ng m⁻³ (nitro-PAH), and 0.27 to 115 ng m⁻³ (quinones).

Redox activity of enriched SRM1649b portions

In this step, we carried out experiments employing the DTT assay to measure redox activity of 1.0 mg SRM 1659b with enrichments of known amounts of groups of organic compounds (PAH, nitro-PAH, and quinones) and also trace metals (Tables 1 and 2). In every experiment, we measured DTT loss with and without addition of DTPA. When DTPA was added, any metals originally present in the SRM 1649b matrix was chelated and they further were not available to contribute to redox activity. This gave us the organic compounds (in test in a given experiment) redox activity contribution. On the other hand, when DTPA was not used, the resulting redox activity reflected the contribution of both trace metals and organics (total redox activity). By subtracting the latter result from the former we find the contribution of “trace metals only” to redox activity. Results from Tables 1 and 2 are expressed in terms of PM mass in $\mu\text{g per nmol}^{-1} \text{ min}^{-1}$.

In Table 1 we observe trace metal contribution to total redox activity is about 5× higher than the organic compounds

contribution. Indeed, even when we add organics only, the relative contribution of trace metals originally present in the SRM 1649b is around 70-85% of the total redox activity. When comparing organics only, the addition of PAHs and nitro-PAHs increased organic redox potential 116 and 165%, respectively. Individual quinones increased organic redox potential more efficiently than Σ PAHs and Σ nitro-PAHs, although their relative contribution to total redox activity were much lower than metal contributions. From the five different quinones added, the 1,2-NQ apparently did not contribute to total redox activity (0.253). Meanwhile, the contribution of 1,2-NQ addition, in the redox activity of organics measured in this experiment (0.0068), was 184% higher when compared to SRM 1649b (0.0037). Nevertheless, in the case of the metals activity contribution there was a decrease of 10%, when the value measured for SRM 1649 (0.0205) was compared to the obtained after the addition of 1,2-NQ (0.0185). This decrease of 10% in the metals contribution does not have statistical significance. Hence, when applied the *t*-test, at a level of confidence of 95%, the *t* calculated is 0.31, smaller than the *t* critical 1.94. The apparent discrepancy is due to the contribution of the metals to redox activity (0.0185), which represents, roughly, 10× more the contribution of the organics (0.0068).

Table 1. Reported rates of dithiothreitol (DTT) loss from organic compounds enrichment of SRM 1649b Urban Dust

	DTT activity / (nmol min ⁻¹ μg^{-1})		
	Metal (average ^a \pm CI ^b)	Organic (average ^a \pm CI ^b)	Total (average)
1.0 mg SRM ^c 1649b	0.0205 \pm 0.0012	0.0037 \pm 0.0010	0.0242
1.0 mg SRM 1649b + 104 $\mu\text{g L}^{-1}$ Σ PAH ^d	0.0219 \pm 0.0016	0.0043 \pm 0.0003	0.0262
1.0 mg SRM 1649b + 175.5 $\mu\text{g L}^{-1}$ Σ nitro-PAH ^e	0.0212 \pm 0.0029	0.0061 \pm 0.0006	0.0273
1.0 mg SRM 1649b + 6.5 $\mu\text{g L}^{-1}$ 1,2-NQ ^f	0.0185 \pm 0.0005	0.0068 \pm 0.0009	0.0253
1.0 mg SRM 1649b + 6.5 $\mu\text{g L}^{-1}$ 1,4-NQ ^g	0.0253 \pm 0.0012	0.0084 \pm 0.0008	0.0337
1.0 mg SRM 1649b + 6.5 $\mu\text{g L}^{-1}$ 9,10-AQ ^h	0.0227 \pm 0.0012	0.0037 \pm 0.0007	0.0264
1.0 mg SRM 1649b + 6.5 $\mu\text{g L}^{-1}$ 9,10-PQ ⁱ	0.0224 \pm 0.0032	0.0087 \pm 0.0022	0.0311
1.0 mg SRM 1649b + 6.5 $\mu\text{g L}^{-1}$ 1,4-BQ ^j	0.0238 \pm 0.0017	0.0073 \pm 0.0006	0.0311

^an = 6; ^bconfidence interval; ^cstandard reference material; ^dpolycyclic aromatic compounds; ^enitro polycyclic aromatic compounds; ^f1,2-naphthoquinone; ^g1,4-naphthoquinone; ^h9,10-anthraquinone; ⁱ9,10-phenanthraquinone; ^j1,4-benzoquinone.

Table 2. Reported rates of dithiothreitol (DTT) loss associated to trace metals additions to SRM 1649b Urban Dust

	DTT activity / (nmol min ⁻¹ μg^{-1})	
	Metal (average ^a \pm CI ^b)	Organic (average ^a \pm CI ^b)
1.0 mg SRM ^c 1649b	0.0205 \pm 0.0012	0.0037 \pm 0.0010
1.0 mg SRM 1649b + 0.21 $\mu\text{g L}^{-1}$ Σ metals	0.0304 \pm 0.0004	0.0031 \pm 0.0004
1.0 mg SRM 1649b + 2.1 $\mu\text{g L}^{-1}$ Σ metals	0.0310 \pm 0.0003	0.0030 \pm 0.0006
1.0 mg SRM 1649b + 21 $\mu\text{g L}^{-1}$ Σ metals	0.0314 \pm 0.0005	0.0022 \pm 0.0007
1.0 mg SRM 1649b + 210 $\mu\text{g L}^{-1}$ Σ metals	0.0323 \pm 0.0007	0.0034 \pm 0.0011

^an = 3; ^bconfidence interval; ^cstandard reference material.

Another important point observed is when adding a known amount of organics, except for 1,2-NQ, the relative contribution of the originally present trace metals in SRM 1649b also increased. This is indicative of a synergic effect between organics and trace metals, which results in the increase of the total redox activity, despite the fact the relative contribution of organic themselves accounts for only 15-30% of the total activity. This synergy would be a helpful mechanism to better understand oxidative potential of real samples.

There may have some useful considerations for explaining why the relative contribution of organics to total PM oxidative potential is minor. There are actually some evidences that complex and multiple redox reactions, which includes formal electron transference among species, actually happens during oxidative processes. In this way, trace metals, which are species able to naturally assume different oxidation states, would be species of excellence to take part or even facilitate the transference of electrons. In turn, some organics such as PAHs, nitro-PAHs and quinones take part of oxidative stress, they depend on specific requirements in the reaction media for giving the necessary conditions for distortions of electron π clouds with or without the presence of electronegative heteroatoms in their structure to induce redox reactions. Since they may probably be not able to formally transfer electrons so easily or in the same way transition metals do, their relative contribution to overall oxidative stress is limited.

Another point to be addressed is about the solubility of organics in phosphate buffer used in the DTT assay. Since PAHs are essentially non-polar, their solubility in aqueous media are poor, resulting in a very low contribution of these species in the DTT assay. When considering nitro-PAHs and quinones, because they possess oxygen or nitrogen atoms in their structure, it would proportionate an increased solubility in aqueous solution (when compared to PAHs). This could explain, at least partially, their higher relative contribution to oxidative potential in relation to their parent PAHs in the DTT assay. On the other hand, trace metals generally present a bioavailable fraction, which is water-soluble.

In Table 2 we show results of DTT loss associated to different amounts of trace metals additions. Our intention in this part of the study was to evaluate if the possible DTT-metal complex formation would be significantly important in a way it would compromise the DTT assay results. According to Krezel *et al.*,⁴² DTT coordinates to Zn^{II}, Cd^{II}, Pb^{II}, Ni^{II} and Cu^I, forming stable complexes in the metal-ligand (ML) stoichiometry. Depending on which extension this reaction occurs and if it is relevant in typical trace metals atmospheric concentrations, it could lead us to a super-estimative of PM-induced oxidative stress by

measuring it through the DTT assay. In the attempt to evaluate this possibility, we measured the rate of DTT loss after adding Σ metals of 0.21, 2.1, 21, and 210 $\mu\text{g L}^{-1}$.

When comparing the average DTT activity of trace metals originally present in SRM 1649b prior to any trace metals addition ($0.0205 \text{ nmol min}^{-1} \mu\text{g}^{-1}$) with the activity after addition of $0.21 \mu\text{g L}^{-1} \Sigma$ metal ($0.0304 \text{ nmol min}^{-1} \mu\text{g}^{-1}$), we noticed an increase of 67%. After addition of higher levels of trace metals, we noticed negligible increments in the DTT activity. Considering the DTT assay is generally done in short reaction times (up to 45 min), the experiments are normally finished with a large amount of unreacted DTT. It is also noteworthy to mention several trace metals present solubility in a very narrow pH range. For instance, Fe^{II}, Co^{II}, Cu^{II}, Ni^{II}, Mn^{II}, Zn^{II}, Cd^{II}, Hg^{II}, and Pb^{II}, to name a few, are mostly precipitated in pH 7.4 phosphate buffer, according to their solubility product constant ($\text{p}K_{\text{sp}}$) and acidic constant ($\text{p}K_{\text{a}}$).⁴³⁻⁴⁶ Furthermore, trivalent metal cations, such as V^{III}, Cr^{III}, Al^{III}, Mo^{III} and Sb^{III} hydrolyze in aqueous solution,⁴³⁻⁴⁶ therefore they are also not available in the DTT reaction medium for having any chance to bind to the DTT molecule. Alkaline and/or alkaline earth cations present in the standard mix (Na⁺, K⁺, Mg²⁺, Ca²⁺, and Ba²⁺) will also ineffectively coordinate to DTT since it is energetically more advantageous for them to form non-geometrically-oriented ionic bonds. So, if a small portion of trace metals coming from the PM matrix during aqueous extraction actually binds to DTT molecules, this would happen in a very small extension under typical atmospheric conditions, in a way it should be assumed to be negligible. This effect may be observed in the results since after ten-fold rises of Σ metals concentration three more times after the first addition, it was not followed by significant increases in the DTT activity (Table 2).

Redox activity of diesel/biodiesel emitted fine particles from a bus station

In this part of the study we focused on estimating the oxidative potential of real fine diesel-biodiesel particles collected in a bus station, under real world conditions. In spite of the fact there have been recent efforts to better understand diesel/biodiesel emitted particles toxicity,^{4,5,16,23} several studies consider samples collected on dynamometers, which facilitates to run repeatable experiments and subsequent studies. However, in these experiments it is not taken into account the differences in the driving cycle or engine technology under typical daily commuting around urban regions.¹²

Redox activity of the PM samples was measured by means of the dithiothreitol (DTT) assay. The results

have been expressed as mass (of PM) based activity in $\text{nmol min}^{-1} \mu\text{g}^{-1}$ (Table 3). Every day three samples were analyzed, at morning, afternoon and night periods. However, on the first Friday (of the sampling period) there was a fire on a bus near the sampling site, and with this incident these samples presented increased DTT redox activities when compared with DTT activities of the other days. Thus, in order to eliminate this confounder in our results, we decided to express average DTT activities excluding the results of this day. The DTT redox activities, except for this Friday, ranged 0.020-0.069 $\text{nmol min}^{-1} \mu\text{g}^{-1}$, with an average of 0.040 $\text{nmol min}^{-1} \mu\text{g}^{-1}$. When considering average results in every 24 h as well as in different periods in the days, the DTT activities seemed very similar. Through statistical test analysis of variance (ANOVA) as shown in Table 4, we can infer for a confidence level of 95% $F_{\text{calculated}} < F_{\text{critical}}$, there were no statistically significant differences in DTT activities among those days. In Figure 1 it is presented a ternary correlation of the DTT activities among morning, afternoon and night. This correlation is high ($r = 0.89$, $p < 0.05$), indicating samples from these periods of the day are mostly alike in terms of chemical composition and, therefore, they have also similar oxidative potential. In other words, it seems only one specific source is contributing to the emissions of diesel/biodiesel particles, which may also be governing their toxicities.

The similarities found among results (Table 3 and Figure 1) is probably due to the fact nearly one type of source

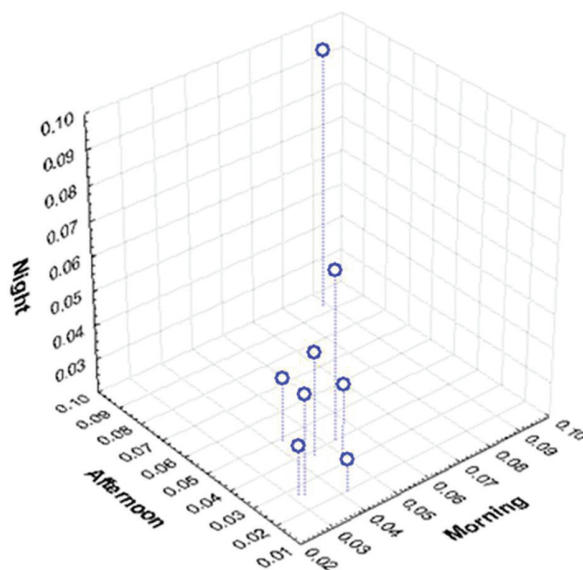


Figure 1. Correlation between ternary total redox activity of the samples collected in the morning, afternoon, and night. Correlation coefficient $r = 0.89$ ($p < 0.05$).

emitted fine particles (which is the fuel burning in buses). Another interesting point is, since the bus station is a place practically almost closed with any ventilation or pollutant dissipation system, as well as the lack of natural light (our sampling took place in the underground floor), the particles exhausted from buses were accumulated and suspended in the air, with virtually no photochemical modifications or particle aging processes. These characteristics make this

Table 3. Total dithiothreitol (DTT) activity from the bus station diesel/biodiesel particles sampled at different periods of the day ($n = 24$)

Day	Total DTT activity / ($\text{nmol min}^{-1} \mu\text{g}^{-1}$)		
	Morning	Afternoon	Night
Thursday	0.033	0.026	0.049
Friday	0.089	0.094	0.095
Saturday	0.045	0.038	0.050
Sunday	0.043	0.049	0.038
Monday	0.048	0.030	0.043
Tuesday	0.053	0.040	0.069
Wednesday	0.032	0.027	0.034
Friday	0.042	0.020	0.029
Average \pm confidence interval	0.042 ± 0.005	0.033 ± 0.007	0.045 ± 0.009

Table 4. Analysis of variance (ANOVA) from the results of diesel/biodiesel particles DTT activity

Source of variation	SS	df	MS	$F_{\text{calculated}}$	F_{critical}
Between groups	0.000462	2	0.000231	0.517	3.47
Within groups	0.009392	21	0.000447		
Total	0.009854	23			

SS: sum of square; df: degrees of freedom; MS: media of square.

bus station an ideal place to study freshly emitted diesel/biodiesel particles under real world conditions.

The DTT activities in terms of metals and organics contributions to the total oxidative potential of diesel/biodiesel particles collected in the bus station are presented in Figure 2. The results are expressed in terms of mass-based activity, in nmol min⁻¹ μg⁻¹. For obtaining the relative contributions of trace metals to organics for the total DTT activities of real samples, we used the same procedure done for SRM1649b (first part of this study). By the Figure 2 we observe metal contribution for oxidative potential was about 89% of the total DTT activity. The metal content in these diesel/biodiesel exhausted particles seems to largely contribute to the ROS production and total oxidative

potential measured by the DTT assay. This is also in accordance to the results reported by Shen and Anastasio,⁴⁷ Charrier and Anastasio²⁷ and Aust *et al.*⁴⁸ They reported in these studies that transition metals may represent a major determinant of the toxicity of PM. Charrier and Anastasio²⁷ found a similar result for DTT loss measured in a small set of PM_{2.5} samples. They estimated that for typical PM_{2.5} samples approximately 80% of DTT loss is from transition metals, while quinones account for approximately 20%.

In Table 5 and Figure 3, DTT loss rate from the present study is compared to previously reported values. So far, we have not found reported DTT loss rate values for diesel/biodiesel exhausted particles. However, as it can be observed from Table 5, other studies used similar particle

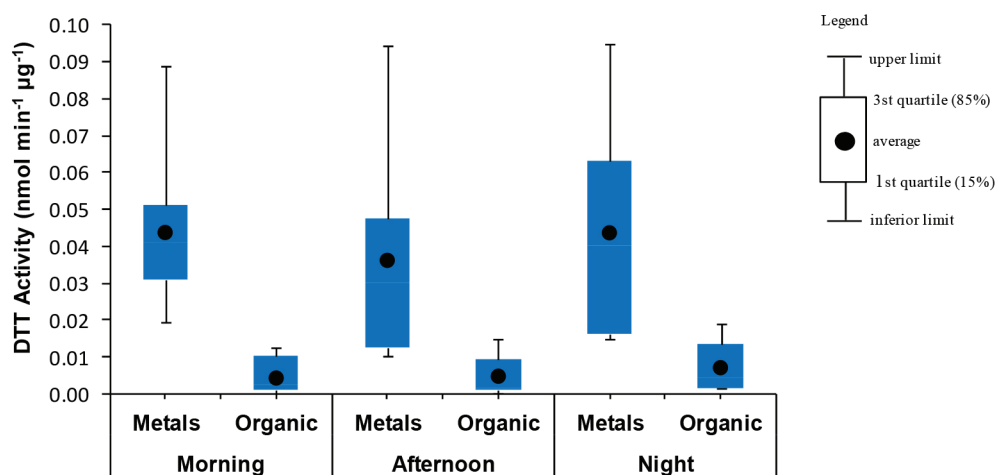


Figure 2. Average of redox activity (n = 24) expressed as nmol of DTT min⁻¹ μg⁻¹ of particulate mass collected at bus station.

Table 5. Comparison of concentration rates of DTT loss from different locations around the world

Reference	PM size fraction	Sampling site	Rate of DTT loss / (nmol min ⁻¹ μg ⁻¹)		Location
			Range	Median	
This study	PM _{2.5}	BDEP, bus station	0.020-0.069	0.040	Salvador, Brazil
Bates <i>et al.</i> ²⁴	PM _{2.5}	gasoline vehicles	0.090-0.130	0.110	Atlanta, USA
		biomass burning	0.049-0.089	0.069	
		diesel vehicles	0.042-0.062	0.052	
Verma <i>et al.</i> ³⁰	PM _{2.5}	urban and rural	0.010-0.070	–	Atlanta, USA
Verma <i>et al.</i> ³⁵	PM _{2.5}	urban and rural	0.019-0.055	0.037	Atlanta, USA
Senthikumar <i>et al.</i> ⁴⁹	PM ₁₀	industrial	0.030-0.130	0.080	Tamil Nadu State, India
McWhinney <i>et al.</i> ⁵⁰	DEP	diesel engine on dynamometer	0.023-0.061	0.042	Ottawa, Canada
Daher <i>et al.</i> ⁵¹	PM _{2.5}	urban	–	0.028	Los Angeles, USA
Verma <i>et al.</i> ⁵²	PM _{0.18-2.5}	urban	0.020-0.120	0.070	Los Angeles, USA
Mugica <i>et al.</i> ⁵³	PM _{2.5}	industrial, roads, and residential	0.039-0.042	0.041	Mexico City, Mexico
	PM ₁₀		0.015-0.020	0.018	
Hu <i>et al.</i> ⁵⁴	PM _{0.25}	urban	0.031-0.055	0.034	Los Angeles Port, USA
	PM _{0.25-2.5}		0.014-0.024	0.040	

DTT: dithiothreitol; BDEP: diesel/biodiesel emitted particles.

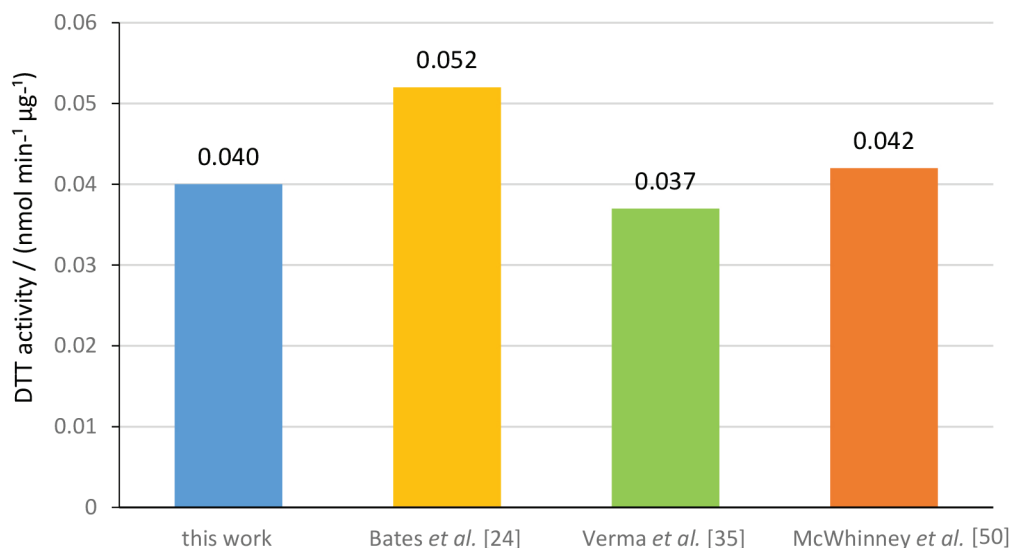


Figure 3. Comparison of DTT activities among the present study and Bates *et al.*,²⁴ Verma *et al.*,³⁵ and McWhinney *et al.*⁵⁰

size fractions and type of samples. Indeed, our results are very similar to the results of other studies, namely the diesel vehicles DTT rate from Bates *et al.*,²⁴ and the reported results from Verma *et al.*,³⁵ and McWhinney *et al.*⁵⁰ (also see Figure 3). We can also conclude oxidative potential of particles emitted from the burning of diesel/biodiesel blends is comparable to fossil diesel-emitted particles.

Conclusions

Biodiesel/diesel particles exhausted from heavy-duty vehicles under real world situations showed oxidative potential levels similar to diesel-emitted particles, with a trace metals contribution at 89%. The metals exhibited significant contributions to the oxidative potential of PM.

Indeed, it became clear that the metal content in atmospheric fine particulate matter have higher oxidative potential relative to the potential induced by organic compounds. The execution of DTT assay for identifying the species that generate greater oxidative potential are important to better assess the potential human health risks, to which the population is exposed daily.

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