Article

Correlation of Oxidative Potential with Ecotoxicological and Cytotoxicological Potential of PM_{10} at an Urban Background Site in Italy

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Abstract: Exposure to atmospheric particulate matter (PM) has detrimental effects on health, but specific mechanisms of toxicity are still not fully understood. In recent years, there has been a growing evidence that oxidative stress is an important mechanism of toxicity; however, when acellular oxidative potential (OP) data are correlated with the outcomes of in vitro (or in vivo) toxicological tests there are contrasting results. In this work, an analysis of PM_{10} health effect indicators was done, using the acellular Dithiotreitol (DTT) assay to retrieve OP^{DTT}, the Microtox® test on *Vibrio fischeri* bacterium to assess the ecotoxicological potential, and the in vitro MTT assay on the human cell line A549 to estimate the cytotoxicological potential. The objective was to evaluate the correlation among acellular OP^{DTT} and the results from toxicological and ecotoxicological bioassays and how these health-related indicators are correlated with atmospheric PM_{10} concentrations collected at an urban background site in Southern Italy. Results indicated that both bioassays showed time-dependent and dose-dependent outcomes. Some samples presented significant ecotoxic and cytotoxic response and the correlation with PM_{10} concentration was limited suggesting that these health endpoints depend on PM_{10} chemical composition and not only on exposure concentrations. OP^{DTT} showed a statistically significant correlation with PM_{10} concentrations. MTT and Microtox outcomes were not correlated suggesting that the two toxicological indicators are sensitive to different physical-chemical properties of PM_{10}. Intrinsic oxidative potential $OP^{DTT}{}_M$ (DTT activity normalised with PM_{10} mass) was correlated with mortality observed with MTT test (normalized with PM_{10} mass); however, it was not correlated with Microtox outcomes.

Keywords: MTT test; oxidative potential; cytotoxicity; ecotoxicological potential; DTT assay; health effects of particulate

1. Introduction

There is increasing evidence that exposure to atmospheric particulate matter (PM) can lead to adverse health effects [1–3]. PM is a complex mixture with physical and chemical properties largely varying in time and space [4] leading to biological effects varying in time, location, and seasons [4–6].

This because different properties of PM determine the biological response, so that even if recent studies demonstrated the statistical association between exposure to PM and health adverse outcomes, they were not able to establish the definitive cause-effect relationship [7]. Several studies suggested that a number of PM health effects could be due to the oxidative or oxidant generating properties of ambient particles [8,9] or that a synergistic effect of inflammation and oxidative stress could be an important aspect of PM toxicity and health effects [10]. Different oxidative mechanisms can act simultaneously, leading to high concentrations of reactive oxygen species (ROS) that, if in excess of the antioxidant capacity to neutralize them, lead to oxidizing other cellular components, which in turn eventually translates into numerous health outcomes [11,12].

The induction of oxidative stress due to the generation of ROS is considered a conceivable paradigm to explain some in vitro toxicity of inhaled PM [13]. As a consequence, in the last years, there has been a growing consensus on the possible use of easy to use, affordable, and relatively fast acellular method to determine the oxidative potential of PM as an indicator of potential health effect [14,15].

However, epidemiological studies and in vitro and in vivo toxicological studies show contrasting results regarding the association of acellular oxidative potential with health outcomes [16–18].

Looking at epidemiological studies, DTT activity was found to be associated with emergency department visits for asthma/wheezing and congestive heart failure [14,19] and for multiple cardiorespiratory health effects [20]. A positive association between OP^{DTT} and respiratory health problems was also found in Yang et al. [21]. Other studies reported little or no association between OP^{DTT} and mortality or hospital admissions [22,23].

Looking at in vitro toxicological studies, an analysis of PM_{10} toxicity in two sites in Belgium showed that OP was not related to the observed cellular response in Beas-2B cells, but it was associated with direct and indirect mutagenic activity [24]. Steenhof et al. [25] found that oxidative potential was significantly associated with MTT-reduction activity in murine macrophages, whereas no association between OP and the production of pro-inflammatory markers was observed.

Looking at in vivo studies, Liu et al. [26] observed a correlation between ROS activity of PM collected in Beijing (China) and inflammatory response in epithelial cells. Instead, OP^{DTT} was found associated with markers of airway and nasal inflammation, but only for specific measurement sites; furthermore, OP^{DTT} was not associated with lung function, inflammatory, and coagulation parameters in blood samples from volunteers exposed to ambient pollution [27].

This poses questions on the efficiency of using OP for the evaluation of health risks, and further research is needed to investigate the association of acellular OP data with health outcomes. The objective of this work was to investigate the correlation between oxidative potential obtained with the acellular DTT assay, which is the most widely used method [14], with the results of two toxicity bioassays: the MTT assay used to evaluate mortality of A549 cells (representative of the alveolar type II pneumocytes of the human lung) exposed to PM and the Microtox® test measuring inhibition of the natural bioluminescence of *Vibrio fischeri* bacteria. These bioassays have been recently proved to be useful tools for the assessment, respectively, of the cytoxicity and ecotoxicity of PM. Compared to the acellular OP measurement, which focuses on a specific aspect of the particulate matter activity, such as the induction of oxidative species, these bioassays provide general endpoint outcomes, representing an integrated response of the multiple effects that the particulate matter can exert at the cellular level. Therefore, the choice of these bioassays arises from the aim to correlate the oxidative potential of particulate matter with more integrated biological effects at the cellular level. The study was performed on water-soluble PM_{10} collected at an urban background site located in Southern Italy.

2. Experimental Methods

The study was carried out on 10 samples of airborne PM_{10} randomly selected among the samples collected between 16/09/2017 and 25/12/2017 at the Environmental-Climate Observatory of ISAC-CNR in Lecce (Southern Italy), regional station of the Global Atmosphere Watch (GAW) network, characterized as an urban background site [28]. The observatory (40°20′8″ N—18°07′28″ E, 37 m asl) is located

inside the University Campus and it is influenced by local emissions (mainly traffic and domestic heating) and by the emission of the town of Lecce and of small villages located around the Campus. Furthermore, it is possible to have transport of pollution from the large industrial settlements of Taranto (about 80 km in the NW direction) and Brindisi (about 30 km in the NNW direction). Daily PM_{10} samples, exposed for 24 h starting at midnight, were collected using a low-volume (2.3 m^3/h) automatic sampler (SWAM, Fai Instruments srl Via Aurora, 15 – 00013 FONTE NUOVA (Roma)) based on β-attenuation for detection of particle concentrations [29]. The sampler was equipped with 47 mm quartz fiber filters (Whatman) pre-fired at 700 °C for 2 h in order to reduce contamination. Average uncertainty on PM_{10} measurements was 2% [30].

The methodology used for sample analysis is schematized in Figure 1. Water-soluble fraction of PM_{10} was obtained, using the whole filter, in 10 mL ultrapure water (Milli-Q) in an ultrasonic bath using four cycles of sonication for a total of 80 min. Successively, the extracts were filtered using PTFE membranes. Three aliquots of each extract were used for the assessment, respectively, of the ecotoxicological potential of PM_{10} using the Microtox® test, of the cytotoxicological potential using the MTT assay, and of the oxidative potential using the DTT (Dithiotreitol) assay. This approach guarantees the comparability of the outcomes of the different assays; all of them start from the same water soluble extract.

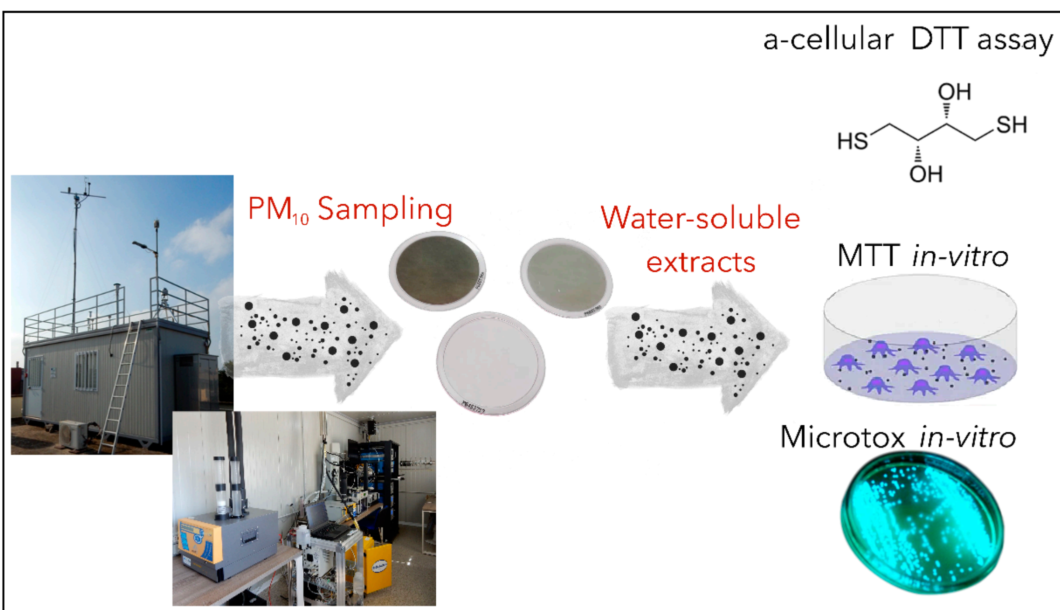

Figure 1. Scheme of the methodology used for the collection and analysis of PM_{10} samples.

The ecotoxicological potential of PM_{10} was assessed by the bioluminescence inhibition assay based on the Gram-negative non-pathogenic bacterium *Vibrio fischeri* (Microtox® test), which physiologically emits light as a result of its metabolic activity. The natural bioluminescence of *V. fischeri* is inhibited by exposure to a number of chemical pollutants, including organic and inorganic compounds [31]. The Microtox test has been successfully applied to measure the ecotoxicity of atmospheric particulate, by exposing the bioluminescent bacterium to aqueous extracts of PM_{10} [32–34]. Roig et al. [35] concluded that Microtox test on *V. fischeri* is a suitable approach as a preliminary test for assessing the effects of particle-phase air pollution. Different exposure times (5, 15, and 30 min) were used in this work and inhibition results were reported as a net effect (percentage of inhibition) corrected using field blanks. Uncertainties were estimated using five repetitions and ranged between 1% and 6% (average 2.5%).

The cytotoxicological potential of PM_{10} was assessed on the aqueous extracts by the MTT assay on the cell line A549. The MTT assay is based on a colorimetric reaction dependent on mitochondrial respiration of the cells and indirectly allows assessing the cellular energy capacity of a cell [36].

The MTT assay was applied to the A549 cell line, representative of the alveolar type II pneumocytes of the human lung [37]. Cell mortality after 24 h exposition is evaluated, in relative terms, considering the net effect of PM_{10} using field blanks for correction. Six repetitions were done to assess the uncertainties that ranged between 3% and 8% (average 5.5%).

The water-soluble fraction of PM_{10} was also used for the analysis of the OP, performed with the dithiothreitol assay (DTT), a surrogate for cellular antioxidants, which analyses the rate of DTT depletion catalysed by chemical species present in the PM [38,39]. An aliquot of the extracts was diluted with deionised water (1:4 factor). Samples were incubated at 37 °C with DTT (0.1 mM) in 0.1 M potassium phosphate buffer at pH 7.4 for times varying from 5 to 90 min. At designated times (specifically at 5, 10, 15, 20, 30, 45, 60, and 90 min), an aliquot (0.5 mL) of incubation mixture was picked up and 10% trichloroacetic acid (0.5 mL) was added to stop the reaction. Then, this reaction mixture was mixed with 2 mL of 0.4 M Tris–HCl, pH 8.9 containing 20 mM EDTA and 25 µL of 10 mM DTNB. Then, this reaction mixture was mixed with a solution containing 10 mM DTNB. The concentration of the formed 5-mercapto-2-nitrobenzoic acid was measured by its optical density absorption at 412 nm using a Eon BioTek Microplate Spectrophotometer). The consumption of DTT over time was determined through the linear fitting of the absorbance with the time in which the withdrawal was done. The DTT depletion rate was used to determine OP values as DTT-activity normalized in terms of sampled air volume ($OP^{DTT}{}_V$) or in terms of mass of collected aerosols ($OP^{DTT}{}_M$). Oxidative potential data reported in this work are corrected for the blanks. Repeatability tests indicate a typical uncertainty of 8–10%.

3. Results

The average and median values of PM_{10} are reported in Table 1 together with the min-max range and the inter-quartile range (between 25th and 75th percentiles). PM_{10} concentrations measured are comparable to the typical values observed in this area [40,41].

Table 1. Statistics of PM_{10} concentrations, Microtox, MTT, $OP^{DTT}{}_V$, and $OP^{DTT}{}_M$ results.

	PM_{10} (µg/m^3)	**Microtox (% Inhibition)**	**MTT (% mortality)**	**$OP^{DTT}{}_V$ (nmol/min*m^3)**	**$OP^{DTT}{}_M$ (pmol/min*µg)**
Average	31.0	55.4	51.1	0.29	10.1
(min–max)	(11.3–53.9)	(30.5–70.2)	(33.7–65.8)	(0.15–0.45)	(6.9–15.7)
Median	32.1	59.7	52.4	0.28	9.0
(25th–75th)	(19.2–40.8)	(46.2–67.7)	(43.3–59.4)	(0.24–0.30)	(8.3–11.8)

3.1. Microtox Test Results

The Microtox test was performed for different exposure times (5, 15, and 30 min) and for different dilutions of the extracts (1:1, 1:2, and 1:4) to test the eventual correlation between dose and effect. The results for one of the samples are reported in Figure 2a.

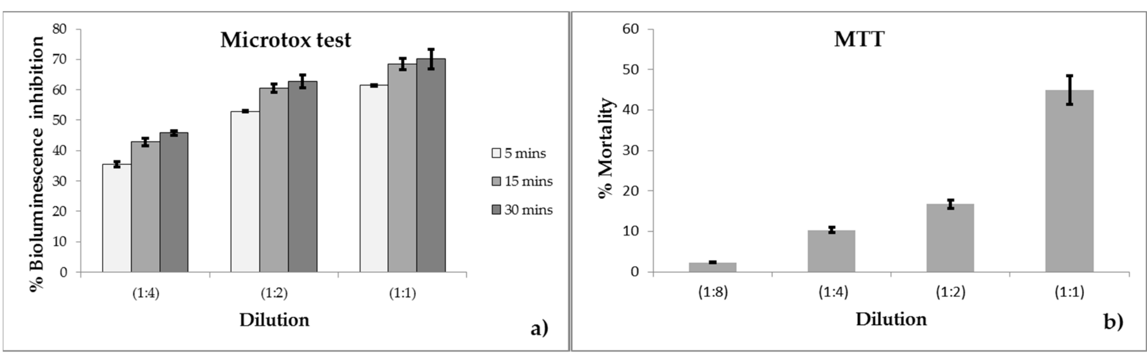

Figure 2. (**a**) Microtox text results for different exposure times and different dilutions executed on sample 1 (25/11/2017). (**b**) MTT results for different dilutions of the extract of the same sample.

They show an increase of the inhibition for low dilutions (i.e., larger concentrations) going from a range of 35%–45% (at the different exposure times) for the (1:4) dilution to a range of 61%–71% when the sample is not diluted (1:1). Furthermore, the percentages of inhibition are increasing when the exposure time increases. These aspects suggest that there is a clear, statistically significant, dose-response relationship in observed percentages of inhibition of the *Vibrio fischeri* bioluminescence. In Table 1 the statistics of the Microtox test results are reported for 30 min exposure. In all the samples analyzed, a significant inhibition of the *Vibrio fisheri* bioluminescence was observed as a result of the exposure of bacteria to the undiluted extracts, suggesting the presence in the PM_{10} of components able to induce an ecotoxic effect. Four samples (samples n. 2, 3, 4, and 7) showed a percentage of inhibition ranging from 30% to 50%, ascribable to a slight toxic effect, while six samples (samples 1, 5, 6, 8, 9, and 10) showed a percentage of inhibition above 50% suggesting the presence of a toxic effect. The correlation analysis between PM_{10} concentrations and the *Vibrio fischeri* bioluminescence inhibition results (Figure 3a) showed a significant positive correlation (Pearson 0.67, $p < 0.05$) for nine of the data pairs, while one sample (sample number 6) was out of trend. Considering that the bioluminescence of *V. fischeri* is sensitive to the exposure to a wide range of inorganic and organic pollutants [42], the obtained results suggest that the chemical composition of the sampled PM can sensibly influence the ecotoxicity of the PM.

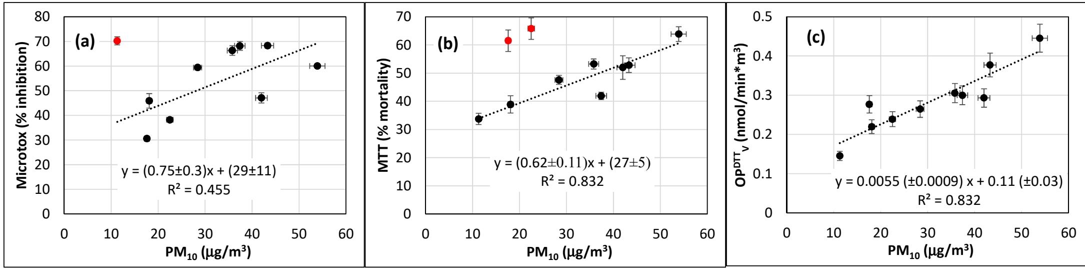

Figure 3. (**a**) Correlation of Microtox text results for with PM_{10} concentrations. (**b**) Correlation of MTT results with PM_{10}. (**c**) Correlation of $OP^{DTT}V$ and PM_{10}. Samples out of trends are highlighted in red. The graphs include linear fits done excluding samples out of trends.

3.2. MTT Test Results

The dose-response relationship was investigated also for the MTT test exposing A549 cells to different dilutions of the same extract. The results for one of the samples are reported in Figure 2b. Results of MTT test showed a statistically significant dose-response relationship with mortality growing from 2% (1:8 dilution) to 45% for the undiluted sample.

The statistics of the MTT results for cytotoxicity are reported in Table 1. In all the samples analyzed, significant cell mortality was observed, ranging from 35% to 65% following exposure of the cells for 24 h to the undiluted samples. Four samples showed slight cytotoxicity (mortality below 50%, sample numbers 3, 6, 8, and 9); while the other showed mortality higher than 50% (the maximum mortality recorded was 65%). These results suggest the presence in the sampled PM of substances able to exert a cytotoxic effect when exposed for 24 h to the cell model utilized. The correlation analysis between PM_{10} concentrations and the MTT results on A459 cells (Figure 3b) showed a highly significant positive correlation (Pearson 0.91, $p < 0.05$) for eight of the data pairs, while two samples (numbers 2 and 7) were out of trend. The cytotoxic nature of PM is related to chemical composition and may be due to the relative abundance of different components such as water soluble metals and organics [43], so that different cytotoxicity could be associated to samples with equal PM_{10} concentrations.

3.3. Oxidative Potential Results

The average and median values of the $OP^{DTT}{}_V$ and $OP^{DTT}{}_M$ values are reported in Table 1. The values found are comparable or slightly lower than the levels observed in other towns in Italy and Europe [38,44–47] and USA [48–50]. The correlation analysis between PM_{10} concentrations and

OP^{DTT}_V shows a significant positive correlation (R 0.91, $p < 0.05$) with no samples out of trend. The OP measured with DTT assay is mainly determined by quinones and transition metals, but several studies indicate a correlation with other major components of PM [51], including primary and secondary organic carbon [38] and water-soluble organic carbon (WSOC) [52]. The correlation with major PM components likely drives the observed correlation between OP^{DTT}_V and PM_{10} at a specific site. It must be mentioned that nonlinear dependence of OP^{DTT}_V with concentrations of specific metals (Cu and Mn) was also observed [53].

3.4. Correlation between OP and Results of Bioassays

As mentioned in the previous paragraphs the endpoints of the toxicity bioassays are likely sensitive to different chemical and physical properties of PM. In Figure 4a, the results of Microtox inhibition and of MTT mortality, normalized by the PM_{10} exposure mass, are compared. Results show a statistically not significant correlation. Normalized Microtox results are compared to intrinsic OP^{DTT}_M, showing a weak (statistically not significant) correlation (Figure 4b). Instead, there was a clear correlation (Pearson 0.91, $p < 0.05$) between normalized MTT results and OP^{DTT}_M (Figure 4c), suggesting that likely similar factors influence the capacity of PM to induce ROS and the reduced mitochondrial functionality, cell damage, and death as measured by the MTT test.

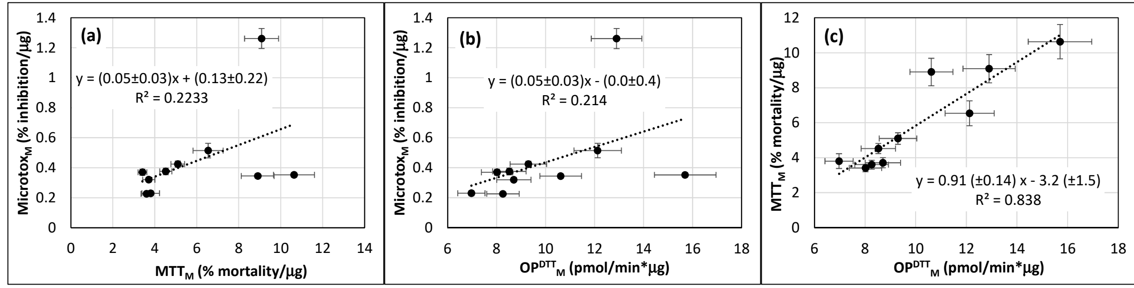

Figure 4. (**a**) Correlation of normalized Microtox results with normalized MTT results. (**b**) Correlation of normalized Microtox results with OP^{DTT}_M. (**c**) Correlation of normalized MTT results with OP^{DTT}_M.

Previous studies on the association of OP^{DTT} and indicators of in vitro or in vivo toxicity of PM are relatively scarce. The results found in this work are in agreement with the observations reported for different sites in the Netherlands during the RAPTEs project by Steenhof et al. [25]. These authors found intrinsic OP^{DTT}_M correlated with MTT-reduction activity in murine macrophages (RAW 264.7 cells), but no association was found between OP^{DTT}_M and the production of inflammatory markers (cytokines IL-6 and TNF-α, and chemokine MIP-2). In Wang et al. [54], cytotoxicity of $PM_{2.5}$, measured in terms of the volume of air that kills 50% (LC_{50}) of the cells (Chinese hamster ovary, cell line K1—AS52, clone 11–4–8), was significantly correlated with DTT consumption. However, other methods for OP estimation had a larger correlation. This was interpreted as a synergistic interaction between organic compounds and metals which are not effectively captured measuring only DTT consumption. Velali et al. [4] found a statistically significant association between mass-based DTT activity and cytotoxicity of water-soluble PM (different size fractions) evaluated using MTT test on MRC-5 cell lines. Furthermore, an association was found between OP^{DTT}_M and LDH release cytotoxicity assay (limited to wintertime samples) in two sites in Greece. Li et al. [8] showed a correlation between in vitro DTT activity and HO-1 induction in RAW 264.7 cells and BEAS-2B cells (a transformed human bronchial epithelial cell). Roig et al. [35] compared the results of the toxicity test of water-soluble PM_{10} collected in Spain using Microtox test on *Vibrio fischeri* bacteria and MTT test on A549 cells and found no correlation similarly to the results reported here. This was interpreted as a consequence of the different influence of the various chemical species on toxicity mechanisms in each cells typology.

4. Conclusions

An analysis of PM_{10} health effect indicators has been performed using the a-celluar DTT assay for the oxidative potential estimation, the Microtox® test on *Vibrio fischeri* bacterium for the ecotoxicological potential evaluation, and the MTT assay on the cell line A549 for the cytotoxicological potential assessment. The objective was to evaluate the correlation among acellular and in vitro tests results and how these health-related indicators are correlated with atmospheric PM_{10} concentrations collected at an urban background site in southern Italy.

Results indicated that toxicity bioassays showed time-dependent and dose-dependent outcomes. Some samples presented significant ecotoxic and cytotoxic response and the correlation with PM_{10} concentration was limited suggesting that these health endpoints depend on PM_{10} chemical composition and not only on particulate exposure concentrations. OP_{DTT} showed values comparable with those observed in other Italian and European urban background sites with and a statistically significant correlation with PM_{10} concentrations.

MTT and Microtox outcomes were not correlated suggesting that the two toxicological indicators are sensitive to different physical-chemical properties of PM_{10}. Intrinsic oxidative potential $OP^{DTT}{}_{M}$ (DTT activity normalized with PM_{10} mass) was correlated with mortality observed with the MTT test (normalized with PM_{10} mass); however, it was not correlated with Microtox outcomes. Further research is needed to fully understand its use; this result could suggest that acellular evaluation of OP using DTT assay is an indicator of cytotoxicological effects of PM, at least on a proxy of the alveolar type II pneumocytes of the human lung.

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