IIETA International Information and Engineering Technology Association
Advancing the World of Information and Engineering

Journal of New Materials for Electrochemical Systems
Vol. 27, No. 3, September 2024, pp. 236-247
Journal homepage: http://iieta.org/journals/jnmes

Assessing the Toxicological Impact of Exhaust Gas Particulate Matter Across Various Fuels During Cold Temperature Operation

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https://doi.org/10.14447/jnmes.v27i3.a09

Received: 03/01/2024
Accepted: 01/08/2024

Keywords:
In vitro toxicology, Particulate matter, Gasoline, Liquefied natural gas, Diesel, Emissions.

ABSTRACT

The ever-increasingly stringent emission objectives and restrictions have not been able to alleviate the serious danger that road traffic emissions bring to human health. Several EU countries' older vehicle fleets continue to contribute significantly to particulate matter (PM) emissions, despite the new passenger car laws' successfully reduce the PM emissions. It has also been demonstrated that various driving situations, such as sub-zero running temperatures, impact the emissions, and the toxicity of particulate matter (PM) emissions from various novel biobased fuels is still up in the air. Generally speaking, there is a dearth of both theoretical and empirical information regarding the toxicity of various PM emissions and circumstances. This study demonstrates that at sub-zero temperatures, exhaust gas particulate matter (PM) from recently controlled passenger cars powered by various fuels can cause toxicological reactions in laboratory settings. Using exhaust gas volume-based PM doses and an older diesel vehicle, we were able to assess the impact of the new emissions regulations and gain a clearer grasp of the actual exposure. Toxicological reactions and particulate matter (PM) concentrations were highest in E20 gasoline in vehicles that were required to comply with the new standards, but E80, a higher methanol blend, produced exhaust gas PM concentrations that were marginally lower and significantly lower, respectively. Engines that ran on modern diesel and LNG produced the fewest particulate matter (PM) concentrations and toxicological reactions. The current research demonstrates that different fuels have different levels of harmful exhaust gas PM. The increased emissions limits were beneficial, as previous diesel cars produced far more particulate matter (PM), which was both more concentrated and more harmful than what modern cars produce.

1. INTRODUCTION

Over the past decade, the India has prioritized the reduction of greenhouse gas emissions from vehicles on the road. As a first step, the fleet's proportion of more fuel-efficient diesel vehicles has grown significantly [1]. Nonetheless, NO2 emissions have increased as a result, since diesel-powered vehicles are significant NO_2 emitters [2]. One further thing that diesel engines have always had going for them is that they produce more particulate matter (PM) mass emissions than gasoline engines. With the inclusion of CO_2, these parts of car emissions—which are vital to air quality—have been the focus of numerous emission control decisions. As an example of such regulations, consider the emissions requirements for traffic, which were implemented throughout the India in 1992 with the goal of reducing emissions caused by traffic. To comply with the strict exhaust particle emission restrictions set by the emission standard, diesel particulate filters (DPFs) were installed on diesel vehicles. As a result, diesel PM emissions have decreased significantly. Additionally, the most recent pollution regulations have successfully limited NOx emissions. The air quality has not improved enough, even if emission limitations have been tightened [3]. A major contributor to the significant variation in passenger car average ages between countries is the aging fleet,

which contributed to an average age of 11.1 years in 2017 [4]. Carbon dioxide emissions from transportation have increased, despite the goals set for emissions reduction. Reason being, the overall number of vehicles has increased, and there has been a shift from diesel to gasoline-powered cars in recent times [5]. Even without considering other fuels, it is exceedingly difficult to strike a balance between various emissions and the negative health consequences caused by the emissions for the fleet powered by internal combustion engines.

In recent discussions about pollution from traffic and its impact on air quality, diesel-powered vehicles have taken center stage. New evidence suggests that contemporary diesel engines and gasoline-powered engines also emit nanoparticles of comparable size [6]. Thus, if particle filters are not required for gasoline vehicle emissions but only for diesel vehicle emissions owing to NOx, there is a risk that the smallest particles will be released into the air, which could have serious and unexpected consequences for human health. Natural gas and methanol are alternative fuels that have not been adequately explored for their implications on health, air pollution, and automobile emissions. Research has linked pollutants from vehicles powered by liquefied natural gas (LNG) and equipped with a three-way catalyst (TWC) to lower concentrations of PM bound carcinogenic PAHs and lower particle number (PN) emissions overall compared to vehicles powered by gasoline or diesel, according to previous studies [7], [8]. Also, at sub-freezing ambient temperatures, there is less toxicologically active PAH chemicals in the exhaust gases, which means fewer particulate matter (PM) emissions, when gasoline has a greater methanol blend. Emissions of particulate matter (PM) and nitrogen oxides (NOx) from passenger vehicles are known to rise in colder weather [9]. While some research has looked at the link between cold temperatures and emissions toxicity, the exact nature of this effect is still little understood [10]. The Type 6 test, which is used to assess emissions from cold-temperature motor vehicles does not account for particulate matter emissions, which further limits its usefulness and accuracy in projecting emissions in the real life[11].

Many recent articles have demonstrated the negative health impacts of aerosols associated to traffic [12].Authors[13] found that daily mortality in Spanish cities was linked to traffic-related ultrafine particles. In addition, authors[14] assessed PM from various sources using toxicity responses from several toxicological endpoints; the two sources with the most significant toxicological capacity were diesel and gasoline engines. Consequently, it seems that of all the types of air pollution, PM from vehicles produces the most severe toxicological effects. Gasoline exhaust is classed as harmful to humans (Group 1) and diesel exhaust as possibly carcinogenic to humans (Group 2B), according to the International Agency for Research on Cancer (IARC)[15].

Particle mass is now the basis for air quality regulations. The health risks posed by emissions from internal combustion engines have persisted despite the fact that particulate matter levels have declined. We need new ways to find out whether new technologies are safe to use, especially ones that produce little mass-based emissions. The most recent emission guidelines do include the particle countsthey only detect particles larger than 23 nm. The particle number (PN) could have serious negative impacts on human health. There are signs that the combined impacts of particulate matter (PM) and elemental carbon (EC) are harmful to human health, and it is possible that these effects are linked to the emissions of ultrafine particles from vehicles [16].

The current study included dosing RAW264.7 murine macrophages with PM samples obtained from BS VI and one BS II emission controlled automobiles according to the amount of exhaust gas. Particulate matter (PM) concentrations in diluted exhaust gas are directly proportional to PM mass in volume. Due to significantly greater bulk PM concentrations (up to several times higher than BS II cars), the doses for particulate matter (PM) from BS IV vehicles have to be tenths of what they were for BS II diesel cars in order to avoid pernicious cytotoxicity. This method employs a volume-based exposure model that accounts for variations in particulate matter (PM) concentrations in exhaust gases from various passenger car types. This allows us to better understand the real-life revelations and toxicity of particulate matter from street-driving vehicles. Toxicological effects to exhaust gas PM exposures were evaluated using a variety of endpoint studies, including mutagenicity, oxidative stress, cytotoxicity, and inflammatory mediators. Additionally, the PM that was collected was subjected to chemical analysis in accordance with the methods [17].

2. EXPERIMENTAL METHODOLOGY

The study by authors[18] elaborates on all the information about the engines, fuels, and sample procedures. The chapters that follow provide a concise overview of some of the key elements.

2.1 Cars and fuels

Exhaust particulate matter (PM) was collected by the VTT from four vehicles regulated for BS IV emissions and one vehicle regulated for BS II emissions. Diesel vehicles powered by BS II (DE2) utilized winter-grade EN590 diesel fuel, while diesel vehicles powered by BS IV (DE6) used standard EN590 diesel. Various other BS IV vehicles made use of LNG, E20, a gasoline-to-methanol ratio of 80% to 20%, and E80, a high-blend methanol fuel. For E20, E80, and LNG-powered vehicles, BS IV included a diesel particle filter (DPF), turbocharged direct injection engines, an exhaust recirculation (EGR) system, a NOx adsorber catalyst, and a three-way catalyst (TWC). It was standard on BS II diesel vehicles to have a single-injection diesel engine that did not have an exhaust aftertreatment system. The report provides detailed information on the engines and fuels used [19].

2.2 Quantification and sampling of the particles

In accordance with the Bharat Stage regulation, a chassis dynamometer was used to gather PM samples during a climatic test at -8 °C. There were three distinct stages of the driving cycle, which together covered 11.0 km. The test consisted of three phases: an urban driving cycle of 2.026 km for the first two stages and an extra-urban driving cycle of 6.962 km for the third stage.

In order to quantify particulate matter emissions and gather samples for analysis of PAHs, micro-ames, and DTT, a high capacity collection system was employed. A dilution tunnel with

a constant volume sampler, an 80 mm sample probe, two 142 mm parallel filter holders, a blower, a flow meter, and a controller are the parts that make up the system. Using flow rates among 4 and 19 m^3min^{-1}, the dilution tunnel was used to dilute the effluent gas. For every kilometer driven, various vehicles released varying quantities of diluted exhaust gasses. Among many others, E2 released 14.12 m3/km of emissions, LNG 9.86, E20 9.72, E80 9.81, and DE6 9.85 m^3km^{-1}. Leaving out details regarding the quantities of unprocessed exhaust gas and dilution rates (DR) for each vehicle

Using a flow rate of 200-1500 L/min, two PTFE filters measuring 142 mm were employed in parallel following the dilution. Millipore, an American firm, manufactured the filters. Once the sampling was finished, the filters were placed under aluminum foil to shield them from light and then weighed. The particulate filters were refrigerated before making their way to the lab for further testing. The data obtained by the high-capacity equipment was used to calculate the PM mass emissions. The actual PM sample filters were identical to the blank control filters except that they did not gather samples of exhaust gas.The non-volatile PN was determined using a butanol condensation particle counter (bCPC) in accordance with the BS VI specification. The presence of particles measuring between 23 nm and 2.5 μm was detected by the bCPC.

The process of preparing particulate matter samples for toxicological analysis has been exhaustively detailed in prior publications. [20]. Each 50 ml glass tube had four PTFE filters. The specimens were subsequently subjected to extraction via a 30-minute sonication cycle in an ultrasonic water chamber that was controlled to remain below 35 °C or suspended in methanol. Subsequently, glass cylinders were stuffed with the solvent. A rotatory evaporator was subsequently employed to reduce the volume of the suspensions. Using the predicted emission volumes, the necessity for glass tubes for separation was then determined. Following cooling to -20 °C, the specimens were subsequently dried in glass containers containing a 99.5% nitrogen gas flow rate.

2.3 Particle size and chemical analysis

Following the methodology outlined in the study of [21], the components linked with PM, including polyaromatic hydrocarbons (PAH) and various anions, were examined. The ions that were examined were SO_42-, NO_3-, and $Cl-$. Using the methods outlined in ISO 16000-6:2011 and EN 14662-4:2005, a total of twenty-four PAH chemicals were examined in the PM. Numerous PAHs on the US EPA 16 list have been linked to cancer, and these 24 PAHs were among them[22]. In addition to the sixteen PAHs already recognized by the US EPA, six PAHs that are considered to be mobile source air toxics were also discovered [23]. Following sampling, the PM mass was additionally determined from the PTFE filters.

The EC and OC ratio (EC/OC) was calculated by roasting a sample in an oven at 700 °C for 1 hour after it was collected using stabilized Ø 47 mm quartz filters. Using a conventional particle collection technique, EC/OC samples were obtained from DE2, E20, and E80 vehicles and transferred to quartz filters. The present investigation was unable to estimate EC/Organic carbon (OC) ratios from the small sample masses of LNG and DE6 emissions due to the extremely low levels of carbon components in these sources. To summarize, PM were gathered during the whole driving cycle for E20 and E80 and sent to a single filter. However, samples were taken in three stages for DE2 because of its high PM emission level. To achieve the desired level of sample darkness for the EC/OC analysis, the flow rate of the samples was changed to 18.3 $L.min^{-1}$ for DE2 and to 8.2$L.min^{-1}$for DE2.

At VTT, EC/OC was measured with a thermo-optical EC/OC analyzer. The sample is subjected to laser transmission measurements under controlled gas and temperature conditions. The second step involves increasing the temperature after cooling the sample, followed by the introduction of O2/He. In order for the flame ionization detector (FID) to pick up methane, carbon must first be oxidized to CO_2. Constant laser transmission measurement compensates for the organic molecules pyrolyzed into EC. The TC, EC, and OC of the sample were then determined by combining the flame ionization detector response with data from the laser transmission. The calibrating process made use of saccharose and methane.

2.4 Potential oxidation of the particulates

The Dithiothreitol (DTT) assay was used to assess the oxidative potential of the particles that were collected, following the protocol described by Charrier and Anastasio [24]. The sole change made to the Chelex solution was to substitute 200 μM of DTT for 100 μM of phosphate buffer. Occurring at 412 nm, we measured the optical density at5,10,15, and 20 minutes. During the experiment, Chelex was employed three times as a negativity control and 9,10-phenanthrenequinone was used three times as a positive control (30 μM). Oxidation and linear rate of Dithiothreitol loss are the foundations of the cell-free DTT assay. According to authors[25], the DTT-assay usually produces a greater reaction to metals in PM than quinones.

2.5 Culture of cells

This experiment used RAW264.7 murine macrophages as the exposed cells because they have been used in previous research on PM harm[26]. Macrophages, a type of immunological defense cell, are abundant in the lungs and react to invaders in the lower airways. Therefore, they are a good stand-in for the dirt and debris that can be found in vehicle exhaust. Culture conditions included RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 5% carbon dioxide, and a humidified incubator set at 37 °C. Next, the cells were cultivated for 24 hours after being seeded into 12-well plates at a density of 5×10^1 cells/ml/well. The fresh culture medium was changed just before the PM exposure test.

2.6 Exposure experiments

For each PM sample, 1 mL of pyrogen-less H_2Ocontaining 6% dimethyl sulfoxide (DMSO) was added. From 0.075 to 0.3%, the ultimate DMSO content in the wells was dose-dependent. Then, using an ultrasonic water bath, the mixture was sonicated for 30 minutes at a temperature lower than 35 °C. We needed to test this PM pre-treatment to remove PM aggregates so we could be sure the PM was dispersed equally on the cells. BS IV vehicles were required to have diluted exhaust gas dosages of 0.5, 0.75, and 1

m^3 per PM dosage; BS II vehicles were to have doses of 0.01, 0.025, 0.05, and 0.1 m^3. We relied on our knowledge and data from earlier studies and pilot testing to choose the exposure method and dosages in our previous investigations [27], [28], hence we employed volume-based doses instead of mass-based doses. Incubation with 5% CO_2 at 37 °C followed 48 hours of treatment with PM samples. The experiments were conducted with two groups of cells: one that was not treated and another that was subjected to DMSO and pyrogen-free H_2O to serve as a vehicle control. Further testing was carried out on cells using blank filter samples in order to further exclude toxicological effects. The BS II automobiles were subjected to a total of four independent exposure investigations. For the remaining DE2 doses, three separate tests were conducted, increasing the total number of exposures to three. However, for the DIE2 PM dose of 0.01 m^3, only two tests were conducted since there were not enough PM samples. For future cytokine research, the cell culture media was withdrawn shortly after the 24-hour exposure and preserved at -80 °C. After washing the cells with 1 ml of Dulbecco's phosphate buffered saline, scrape them from the well bottoms and resuspend them in PBS: Cell viability, reactive oxygen species (ROS), inflammatory markers, CMA, and CMI were all assessed according to the procedures [29]. Additionally, the potential for mutagenicity was thoroughly investigated.

2.7 Toxicological analyses

In order to ensure that the measurements were not impacted by any PM in the samples, 96-well plates were utilized for CMA and CMI studies. These plates contained equivalent dosages of particulate matter without the cells.

2.8 Analysis of inflammatory mediators

Utilizing 96-well plates and ELISA kits, two potential indicators of inflammation, TNFα and MIP2, were assessed in the culturalstandard. All studies were conducted in accordance with the manufacturer's procedures. A positive control known as lipopolysaccharide (LPS) was utilized to ensure that the procedure was functioning as anticipated.

2.9 Examining the metabolic processes within cells

In order to assess the metabolism of the RAW264.7 macrophages on 96-well plates, the MTT-assay was employed, which involves taking two 110 µl samples of every cell value under investigation. A photometric plate reader and a VICTOR3TM Multilabel Counter were used to measure the absorbance at 570 nm.

2.10 Assessment of the stability of cell membranes

After the DCF assay, CMI, which is an indicator of cell viability, was measured using a propidium iodide (PI) exclusion assay. As part of this process, a PI solution (0.5 mg/ml, w/v in dd H_2O) was added to the wells and the mixture was incubated at 37 °C with 5% CO_2 for 20 minutes. In order to detect fluorescence, the plate reader was utilized. Twenty microliters of Cell Lysis Buffer containing 10% Triton X-100 was subsequently added. Returning to measuring plates for maximum PI fluorescence, incubation was carried out on them after 20 minutes in darkness at room temperature using a plate shaker.

2.11 Analysis of intracellular oxidative stress

Two 100 µl aliquots of each exposure were used to quantify intracellular oxidative stress in a DCFassay on 96-well plates, after which the H2DCF-DA was added to each well. A broad picture of the cellular oxidative stress level can be obtained by using the DCF-assay, which analyzes various intracellular oxidants. Moreover, when DCF was used to measure oxidative stress in vitro, the results may be biased because of the impact of several metabolic products on oxidative stress [30]. A plate reader with 485 nm excitation and 530 nm emission was used to monitor fluorescence before, during, and after incubation at 37 °C with 5% CO_2. To find out how significant something was, researchers looked at the area under the curve (AUC). In order to display the results, the fold change in comparison to the control cells was utilized. Additionally, cells that had been treated to H_2O_2 served as a positive control.

2.12 Investigation of mutagenicity

The microAmes reverse mutation test was used to analyze the mutagenicity potential of the PM samples. This test assesses mutations of histidine utilization capability. The current investigation contrasted the mutagenicity of bacteria with the toxicological reactions observed in cells. On the other hand, bacterial mutagenicity tests can also detect chemicals that aren't directly DNA damaging agents, which is the main difference between cell genotoxicity and bacterial mutagenicity [31]. Using Salmonella typhimurium strain TA98 as the metabolic activator or not, the MicroAmes test was performed. By utilizing the S9 rat liver fraction, we can investigate the various metabolites and their possible impacts on mutations. Two different forms of 2-amino anthracene, one with and one without S9mix, were utilized as positive controls. A shaking water bath was used to incubate the TA98 tester strain culture for 16 hours at + 37 °C in Nutrition Broth *2 containing 10 $mg.ml^{-1}$ ampicillin, to make things simpler. The 24-well plate was then incubated for 2 hours at + 37 °C after the required quantities of different samples were added to the minimum glucose medium. This medium either contained or did not contain the S9 fraction, and the overnight culture of TA98 was also included. After transferring the contents of the 24 well plates to a 384 well plate, they were incubated at + 37 °C for 72 hours. The plate was then sealed in a plastic bag. The pH changed as the tester strain proliferated, causing the color to shift from purple to yellow. This allowed for the detection of mutations. The mutagenic activity of PM emissions from a 1-kilometer driving distance is indicated by the results, which are given as krev/km. This unit represents thousands of revertant.

2.13 Statistical analysis

An analysis of variance (ANOVA) was employed to compare the different responses of RAW264.7 cells to particulate matter exposures with those of the controlling cells. In contrast to traditional ANOVA, this technique preserves accuracy even when variances are not uniform. After Welch's ANOVA showed a statistically significant result, we analyzed the sample means using Dunnett's T3 post-hoc test with correction for multiple comparisons. Every difference was considered statistically significant when $p < 0.05$. Software was used for data analysis.

3. RESULT AND DISCUSSION

3.1 Particulate matterchemical composition and its emissions

Table 1. NOx emissions, particulate matter mass, PN, and organic and inorganic constituents associated with PM analyzed in the exhaust gases of 1E2 vehicle and four BS VI vehicles.

	DE2	LNG	E20	E80	DE6
Organic elements					
Summation of overall PAHs ($\mu g.km^{-1}$)	34.3	0.55	6.55	5.47	0.98
Emissions of particulate matter					
Mass of Particulate matter (mg/km)	63.7	0.51	1.38	0.95	0.67
Particulate number (#/km)	1.31×10^{14}	6.65×10^{10}	7.65×10^{11}	7.51×10^{11}	5.64×10^{9}
Nitrogen oxides					
NO_x (mg/km)	1155	206	36	21	467
Inorganic elements (mg/km)					
NO_3^-	0.127	< Detection Limit	<Detection Limit	<Detection Limit	0.055
Cl^-	0.065	<Detection Limit	<Detection Limit	<Detection Limit	<Detection Limit
SO_4^{2-}	0.127	<Detection Limit	<Detection Limit	<Detection Limit	<Detection Limit

Table 1 displays the PM and NOx emissions from each vehicle's exhaust, together with the total of organic and inorganic components found in the particle phase. Measurements of Elemental carbon/Organic carbon and Total carbon are shown in Table 2. All twenty-four PAHs were analyzed and the results are shown in Figure 1. Owing to the International Agency for Research on Cancer (IARC), we know that a few of the PAHs that were tested are carcinogenic. The International Agency for Research on Cancer has classified indeno [1,2,3-cd] pyrene, benz [a] antracene, chrysene, benzo [b] fluoranthene, and benzo [k] fluoranthene as category 2B PAHs, meaning they may cause cancer in humans. Group 2A also contains the known carcinogen benzo [a] pyrene and the probable carcinogen dibenzo [a,h] anthracene. Samples of various particulate matter revealed concentrations of carcinogenic PAHs in the following amounts: 4.96 µg/km for DE2, 0.07 µg/km for LNG, 2.81 µg/km for E20, 1.63 µg/km for E80, and 0.09 µg/km for DE6. Beta pyrene levels were 0.82 εg/km in DE2, 0.53 µg/km in E20, 0.49 µg/km in E80, and 0.007 µg/km in DIE6

Table 2. Two BS VI vehicles and one BS IIvehicle's OC, EC, and TC values.

	DE2	E20	E80
Organic Carbon (%)	36	50	32
Elemental Carbon (%)	59	50	6
Total Carbon (%)	94	100	34

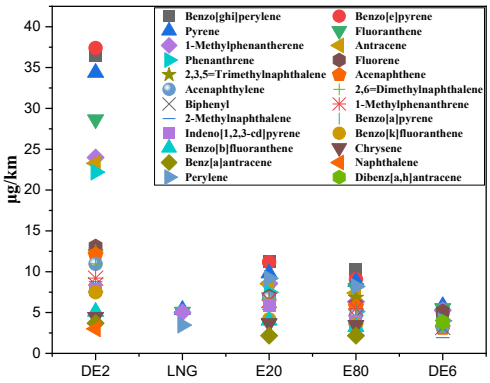

Figure 1. The amounts of polyaromatic hydrocarbons bound to particulate matter in the dilute exhaustible gas produced by 4E6 cars and 1E2 car over a one-kilometer driving distance (g/km).

3.2 The oxidative capacity of PM

The findings of the oxidative potential tests conducted using DTT are shown in Figure 2. At 67.6 µM/min/km, the particulate matterspecimen from 4E6 automobiles had the lowest oxidative potentials, while the PM samples from DE2 cars had the highest oxidative potential.

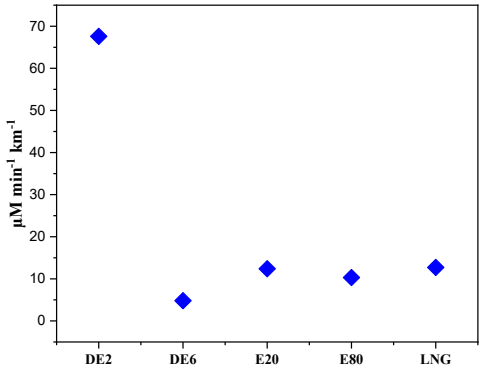

Figure 2. Oxidative potential of Particulate matter samples collected from one BS IIand four BS VI automobiles using the DTT assay.

3.3 The features of toxicology

Toxicological data is derived from untreated control cells because the endpoints for exposures to blank filter, DMSO, and water were highly similar to those of the untreated group.3.4 Mediators of inflammation.

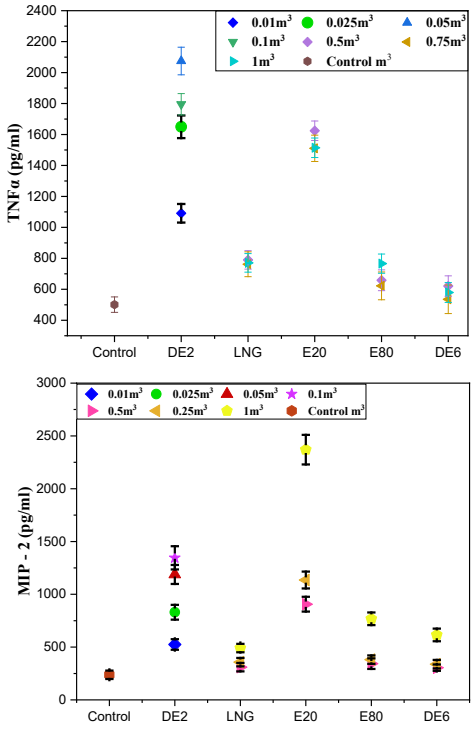

Figure 3. (a) Following a 24-hour exposure to exhaust gas volume-based quantities of particulate matter obtained from 1E2 and 4E6 engines, (b) TNFα and MIP-2 concentrations in RAW 264.7 murine macrophages (pg/ml).

Figure 3 demonstrates that BS II was used for 10% of the doses that BS VI was supposed to be given. The average concentration as well as its standard error (SE) are displayed. Results from Welch's ANOVA and Dunnett's t3 tests, both of which had p-values less than 0.05, show that the variables deviating from the control are statistically significant (asterisks).In response to all exhaust emission PM exposures, the RAW264.7 macrophages secreted more MIP-2 and more cytokine TNFα than the control cells (Fig. 3). When cells were exposed to DE2 PM samples, the levels of TNFα were found to be very high. Compared to control cells, a 221% rise in TNFα (95% CI: 54.1, 257.3) was seen at the lowest of the three concentrations of DE2 PM, and a 458% increase (95% CI: 240.2, 675.0) was seen at the second-highest dose. The response was dose-dependent. The concentration of TNFα in cells exposed to the highest PM dose was, however, comparable to that of cells exposed to the 0.025 m3 dose, with a 386 % difference (CI%95: 220.4, 552.1). Taking BS VI automobiles into account, it was shown that TNFα levels were considerably elevated by all levels of E20 exhaust PM exposure. The control cells showed a 332% difference (95% CI: 324.0, 413.5) at the lowest test dose. With a 95% confidence interval of 119.3-200.8, the amounts of TNFα in the exhaust PM samples from LNG engines were 160% higher than in the control cells, even at the lowest PM dose. In PM-originating cells, the highest dose of E80 increased TNFα by 155% (95% CI: 125.0, 185.1) as compared to control cells. Moreover, just like the control cells, cells exposed to DE6 PM showed TNFα amounts that were similar to what was found in them.

As compared to control cells, the DE2 PM samples in MIP-2 caused a minimum variation of 223% (95% CI: 116.4, 330.4) and a maximum variation of 587% in PM dosage. Compared to the control cells, E20 PM samples subjected to the greatest and lowest PM doses showed the highest concentrations of MIP-2, with 952% and 369% of the total, respectively, compared to 95% CI: 846.5 to 1057.2 and 324.0 to 413.5. At the maximum particulate matter dose, the stages of MIP-2 were 331 % in E80 PM, 255 % in LNG, and 204 % in control cells. In contrast, the lowest and medium doses hardly raised MIP-2 levels.

Activation of cellular metabolism

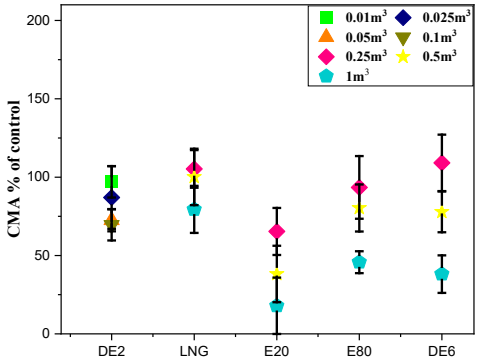

Figure 4. Macrophages isolated from RAW 264.7 mice exhibited a lower CMA related to control cells, as determined by the MTT assay, after 24 hours of exposure to PM samples with concentrations based on exhaust gas volume.

Figure 4 shows that all exhaust gas PM exposures reduced CMA on RAW264.7 macrophages relative to control cells, and the reduction was dose-dependent. With E20 PM samples showing the lowest CMAs compared to control cells, cells exposed to low dosages of PM reduced CMAs by 51% while cells exposed to high doses reduced CMAs by 95%. A 17% decrease in CMA and a 71% decrease in CMA were seen in cells treated with the lowest dose of E80 PM related to control cells. At the maximum dose of DE6 PM, CMA was shown to be reduced by 78%, whereas at the lowest dose, no such impact was noted. Typically, LNG cells showed the exhaust gas particulate matter sample exposures that reduced CMAs the least. As compared to the control cells, the maximum dose reduced CMAs by 33%, whereas the two lowest doses had no effect. At the two highest dosages, 42.3% and 47.0% of DE2 PM were reduced, respectively.3.6 Cell membrane integrity and vitality. Upon exposure to exhaust PM samples, RAW264.7 macrophages exhibited diminished CMI and cell vitality. The highest PM dosage decreased CMI by 28% and the minimum dose decreased CMI by 66%, indicating that the E20 PM exposures were the most effective in reducing CMI. In terms of CMI, the lowest dose of E80 PM exposure was associated with 77% and the maximum dose was associated with 37%. Furthermore, when compared to the control, the reduction in CMI produced by exposure to LNG PM was not statistically significant. The CMI for the highest dosage of DE6 PM was 88% relative to the diesel engines' exhaust gas particulate matter, while the lowest dose produced

76%. The CMI for the lowest dose of DE2 PM was 88% and for the highest dose of DE6 PM it was 68%.

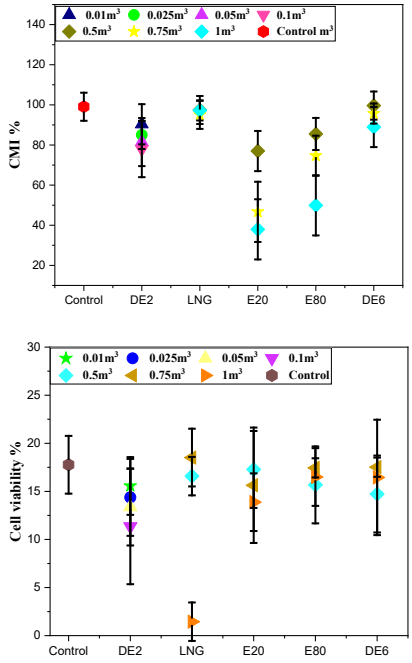

Figure 5. (a) Cell membrane integrity measured by propidium iodide-exclusion; and (b) evaluations of cell viability in RAW 264.7 murine macrophages subjected to PM samples at doses dependent on exhaust gas volume for 24 hours.

Measurements of cell vitality showed that compared to control cells, exposure to E20, E80, and DE6 PM reduced viability in a dose-dependent manner. On the other hand, with regard to DE2 and LNG PM exposures, no significant variations in viability were observed across the various PM concentrations. Clear reductions in cell viability were observed at 0.5 m^3 and 1 m^3 doses of E20 PM, with the greatest dose producing a 32% decrease. Furthermore, it was observed that higher concentrations of E80 and DE6 PM led to viability reductions of nearly 20%.

3.4 Mutagenicity

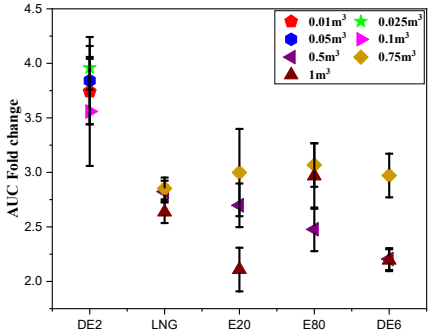

Figure 6. The mutagenicity of PM originating from four distinct BS VI vehicles was assessed using a microAmes assay.

Figure 6 illustrates the diverse levels of mutagenicity that were determined by subjecting distinct BS VI exhaust PM samples to the microAmes test. E20 PM exposures showed the greatest mutagenic potential (14.5 krev/km) in the absence of hepatic S9 supplementation. DE6 and LNG followed with 8.4 krev/km, 3.0 krev/km, and 2.4 krev/km, respectively. Measured mutagenic potentials for E20 (20.1 krev/km), DE6 (12.3 krev/km), LNG (4.1 krev/km), and E80 (13.4krev/km) increased significantly with the addition of liver piece S9.

3.5 Intracellular oxidative stress

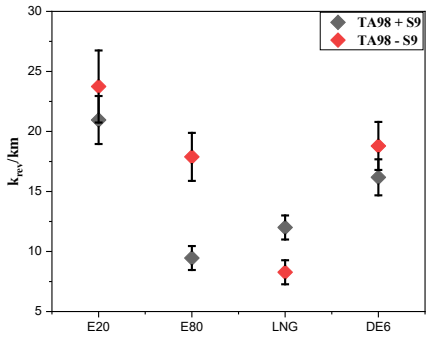

Figure 7. The DCF assay was employed to assess oxidative stress in RAW 264.7 mouse macrophages subsequent to a twenty-four-hour exposure to PM samples with concentrations determined by exhaust gas volume. A tenth of the dosages that were executed at a cost of BS VI were executed at a cost of BSII.

Intracellular oxidative stress was exclusively detected in response to DE2 PM exposures; at all concentrations, the cellular damage was up to two times greater than that of the control cells. An absence of intracellular oxidative stress was observed in control cells exposed to various BS VI automobile PM samples. It is noteworthy that the intracellular oxidative stress was reduced in a dose-dependent manner when DE6 PM was introduced, with the highest PM concentration leading to a 72% reduction in comparison to the control cells.

Although the increased BS VI limit for PN per km is 7 x 10^{11}, it is worth noting that both gasoline-powered BS VI vehicles exceeded this level[32], [33]. Notwithstanding the implementation of this novel regulation in 2020, the petroleum vehicles included in the current investigation continued to comply with the more established PN regulation. Additionally, the reduced temperature (-8 °C) applied during the testing phase might have contributed to the elevated PN observed in gasoline-powered vehicles. Advancements in research have established a correlation between reduced temperatures and elevated levels of PN and PAH. In respect to NOx emissions, the BS VI diesel vehicle might conceivably exhibit the same characteristics. Moreover, an increase in the levels of polycyclic aromatic hydrocarbons (PAHs) discharged into the atmosphere has been documented due to the reduced operating temperatures of petroleum engines [34]. Though not mentioned directly, the LNG vehicle released a great deal of methane, a much more powerful greenhouse gas than carbon dioxide.

Due to differences in sample volume and non-equilibrium PM concentrations, it is not possible to draw firm conclusions about the relative toxicity of PM resulting from different chemical compositions. The PM samples were derived from various engine exhaust gases. Nevertheless, due to the inconsequential

disparity in diluted exhaust gas volumes across different driving distances for BS VI vehicles, it is possible to make an approximation of the toxicological reactions using the PM concentrations from exhaust gas volume. These results therefore demonstrate the toxicity of actual PM emissions from automobiles powered by various fuels and outfitted with various technologies. The various volume quantities of exhaust gas utilized for the BS VI vehicles are approximately equivalent to driving distances of 25, 50, and 100 meters. Furthermore, because these distances were derived from attenuated exhaust gas, they were significantly shorter than those for undiluted exhaust gas. In practice, however, exhaust gases rapidly diluted into the surrounding environment. In comparison to the BS VI PM, the BS IIvehicle produced up to 30% more attenuated exhaust gas per driving distance and was exposed to one-tenth the PM. Conversely, the toxicological reactions exhibited comparable to or exceeded those elicited by various BS VI car PM, suggesting that older diesel vehicles generally produce substantial amounts of PM and possess toxicological potential. The results obtained from the toxicological analyses of various PM samples are elaborated upon in the subsequent chapters.

3.6 DE2

The BS II diesel vehicle produced the most particulate matter (PM) and particulate number (PN) emissions since it lacked exhaust gas aftertreatment equipment. According to earlier research, the DE2 effluent included the highest concentration of carcinogenic PAH chemicals bound to particulate matter. In vitro toxicological reactions have been linked to these substances. Further investigation has demonstrated that diesel exhaust particles (DEP) have the ability to cause oxidative stress, whereas exposure to gasoline exhaust does not appear to increase oxidative stress significantly [35]. DE2 PM contained 58% EC, a value associated with oxidative potential and mutagenicity, as determined by an EC/OC analysis. Additionally, it has been demonstrated that black carbon (BC), which is interconnected with EC, can produce adverse health consequences when added to a PM mixture. Hence, it is logical that the current investigation observed that DE2 PM exhibited the greatest oxidative potential as determined by the DTT assay. Moreover, oxidative stress was induced in vitro through DE2 PM exposure, as determined by the DCF assay, even at the minimum PM concentration, reaching a plateau at higher concentrations. This indicates that oxidative stress in vitro can be severe despite the presence of low DE2 PM concentrations.

The proinflammatory response of RAW264.7 macrophages was induced by the DE2 PM samples, as evidenced by the quantified levels of TNFα and MIP-2.2. This finding is consistent with prior research that has observed elevated inflammatory potential in diesel engine emissions from older technologies. Additionally, the observed decline in TNFα production in response to the highest PM dose exposure could potentially be attributed to the compromised functionality of the cells, which subsequently led to a reduction in TNFα production. This is corroborated by the outcomes of the MTT-assay, which indicated that the highest concentration of DE2 PM led to the lowest CMA in comparison to the lower concentrations. Notwithstanding this, no comparable decline in MIP-2 secretion was detected at the maximum exposure dose.

Regarding the cell viability assessments, it was observed that elevated concentrations of DE2 PM resulted in a marginal decline in cell viability as measured by the CMI. Nevertheless, this pattern was not observed in the viability of cells as determined by vitality measurements, which, on average, indicated greater levels of viability than CMI measurements. This could be attributed to the fact that the measurements were conducted subsequent to the analysis day, when a significant proportion of the deceased cells had already perished, potentially rendering the dye solution incapable of binding to them. Consistent with findings from previous research, the CMI confirms that DEP reduces the viability of cells. Furthermore, the microAmes test for mutagenicity indicates that DE2 PM had a high mutagenic potential, providing further support for the genotoxicity of DEP.

3.7 LNG

The reduced particulate matter emissions exhibited by LNG-powered engines in comparison to gasoline and diesel-fueled engines can be attributed to the absence of carbon-carbon bonds, aromatic and polyaromatic compounds, and carbon-carbon bonds in LNG fuel. In this study, low levels of PM emissions were detected, with LNG exhaust having the second-lowest PN emissions and the lowest mass concentrations of PM. A particulate matter (PM) emissions resulting from the combustion of LNG fuel are most plausibly the product of hydrocarbon pyrolysis in lubrication lubricants.

In relation to the toxicological reactions, it was observed that control cells exhibited minimally altered CMA, oxidative stress, and cell viability results following exposure to PM from LNG exhaust. Inflammatory responses elicited by the LNG PM exposure were quantified by inflammatory mediators; TNFα levels were notably elevated, indicating this; however, the impact on cellular viability and metabolic activity was not detrimental during this process. The microAmes test yielded mutagenicity results that indicated a minimal mutagenic potential as well. In a number of prior investigations, it was similarly noted that the PM emitted from LNG exhaust exhibited minimal toxicity.

3.8 E20

Both the current study and other research have found that PM emissions from modern diesel fleets with DPF are lower than those from modern gasoline fleets. While DEP toxicity has been the subject of much research, PM originating from gasoline engines has received far less attention.

When comparing exposures to several toxicological endpoints in the current investigation, E20 PM exposures performed the best, followed by other BS VI PM samples. BS VI regulated vehicles had the greatest levels of particulate matter (PN), particulate matter mass, and PM-bound PAH chemicals in their exhaust, suggesting that E20 PM is very harmful. E20 PM also had substantial concentrations of both fractions, as revealed by the EC/OC ratio; these two components have been linked to toxicological reactions. In particular, the OC fraction may be very harmful due to the elevated quantities of carcinogenic PMbound PAHs, which were significantly greater than those in

the other BS VI PM samples. Just like in the current study, a prior study found that particulate matter (PM) from gasoline exhaust can trigger inflammatory reactions in a controlled laboratory setting. In that investigation, a range of particulate matter originating from contemporary gasoline automobiles was employed to induce inflammation, as quantified by an increase in tumor necrosis factor alpha (TNFβ).

This led researchers to conclude that PM samples from E20 exhaust had the greatest cytotoxic impact on cells because they also showed the greatest reduction of CMA when exposed to these samples. This is consistent with what was found in an earlier investigation, wherein primary and secondary cells were exposed to aged gasoline PM, which caused cytotoxicity. The liveliness and CMI readings led us to believe that the cells were alive despite the drastically reduced CMA. That the observed cytotoxicity was probably a result of the ongoing process of apoptosis is supported by the data. Gasoline engine PM to carcinogenicity, and E20's mutagenicity was many times higher than that of other BS VI car-originating PM. Emissions particulate matter (PM) from passenger cars powered by E20 engines did not cause oxidative stress in laboratory tests and did not have a greater oxidative potential than PM from other BS VI vehicles, according to DCF and DTT investigations.

3.9 E80

There was a 30% drop in PM mass from E80-fueled vehicles compared to E20-fueled vehicles, but there was a vanishingly little difference in PN numbers, suggesting that E80 combustion generally results in smaller particles than E20 combustion. There is evidence that methanol has an effect on emissions when added to gasoline; adding higher blends of methanol reduces several emissions components, particularly the bigger particulate matter portion. It is worth noting that the EC/OC ratio for E80 was noticeably higher than OC. Since there is evidence that the carbonaceous core of particulate matter serves as a significant carrier of the more harmful molecules, such as PAHs, this could be a contributing factor to the fact that E20 and E80 are poisonous in different ways. So, in contrast to E20 PM, where EC levels were significantly greater, E80 PM's low EC values mean that cells aren't exposed to as many harmful chemicals.

Although the PM mass and PN were not significantly different between E20 and E80 exhaust gasses, the toxicological reactions caused by the PM from these two gasoline types differed significantly. When comparing E20 PM exposures to E80 PM exposures, the latter resulted in significantly diminished inflammatory responses. E80 caused a lesser loss of viability than E20 PM exposure, according to data from cell viability assays, which follow a similar trend. Loss of CMA was the most severe toxicological reaction to E80 PM exposure, suggesting that high concentrations of the compound produce cytotoxicity. In addition, the results of the DTT and DCF tests show that the particulate matter (PM) from E80-fueled passenger cars did not have any significant acellular oxidative potential and could not cause oxidative stress within cells. Previous studies have reported that the levels of genotoxic PAH compounds and the potential for oxidative DNA damage in E80 PM are comparatively lower than those found in gasoline exhaust PM. The inclusion of S9 in the microAmes test showed a multi-fold increase in the mutagenic potential, which is an intriguing finding. This data points to the fact that E80 PM's metabolic byproducts of specific components are more carcinogenic.

3.10 DE6

Out of all the BS VI vehicles tested, the DE6 had the lowest PN content in its exhaust, followed by the lowest amount of particulate matter and PM bound PAH compounds. If the mass-to-PN ratio of the released PM is any indication, the size distribution of the particles is skewed towards larger ones compared to what is seen in LNG exhaust. Although the EGR systems have been demonstrated to minimize particulate matter emissions in exhaust gases, the efficient particle filtration by DPF is the primary cause of low particulate matter emissions from BS VI diesel cars.

Due to the lack of oxidative stress, reduction in cell viability, and production of inflammatory mediators, the majority of the toxicological responses with the DE6 exhaust PM were either nonexistent or very small. On the other hand, the maximum dosage of DE6 PM samples significantly decreased CMA. There was no correlation between the drop in CMA and the RAW264.7 cells' reduced vitality, since cell viability was unaffected in the exposed cells. Consistent with earlier research, the present study found that oxidative stress levels in treated cells decreased relative to control cells after 24 hours of treatment. This was believed to be because of intracellular antioxidant activation caused by increased oxidative stress at timepoints less than 10 hours after exposure. The toxicological tests in this work were performed 24 hours after the first PM exposure; consequently, the fact that the levels of intracellular oxidative stress were lower than in the control cells could be due to temporary oxidative stress and the activation of antioxidative mechanisms. Nonetheless, the DTT test revealed that the oxidative potential of DE6 exhaust PM was extremely low. The DCF-assay results do not seem to take into consideration all of the relevant variables, such as the PM's effect on the readings. Corroborating earlier findings of carcinogenicity in the DEP, a higher mutagenesis potential was noted in the DE6 PM when contrasted with other toxicological effects. The diesel emissions might be even more hazardous when paraffinic fuel content is added. This significantly reduces emissions, particularly soot production. These fuels would likely reduce emissions even further since they are compatible with existing engines and do not necessitate any kind of retrofitting.

3.11 An analysis of the emissions' relative toxicity

The new passenger vehicle emissions laws have effectively reduced exhaust PM emissions, according to the present study's results. Toxicological data from the present investigation corroborate this, showing that particulate matter (PM) emissions from BS IIdiesel engines are equally hazardous as PM emissions from BS VI vehicles, despite the fact that the latter produce substantially lower PM doses based on exhaust gas volume. It can be inferred that the former are five times more detrimental than the latter. To further illustrate the results of this study's comparison, we also performed a numerical analysis of the toxicological outcomes across various particulate matter specimen doses and extrapolated the BS IIoutcomes to a dose of

1 m^3km^{-1}. Exposures to BS IIexhaust PM are many times more harmful than exposures to BS VI PM, as seen in the table. This table is, as the authors point out, merely an approximation of the toxicological findings.

Among the many BS VI vehicles tested, those powered by LNG and newer diesel engines had the fewest particulate matter emissions and the fewest PAHs linked to particulate matter, leading to the mildest toxicological reactions. Additionally, it is anticipated that the quantities of particulate matter (PN) in diesel engine exhaust will be significantly lower upon implementation of a selective catalytic reduction (SCR) catalyst. In this investigation, however, compared to the other BS VI automobiles, the DE6 had relatively high NOx emissions. By starting the creation of the very toxicologically active nitro-PAHs, NOx can significantly add to PM toxicity. While this study did find that NOx emissions from diesel vehicles were significantly lower in newer models compared to older ones, other studies have shown that BS VI-regulated diesel vehicles had NOx emissions that are equivalent to earlier models. The effectiveness of NOx exhaust after-treatment devices also seems to degrade with time, according to the available data. The result is that NOx emissions are still a problem, despite the fact that PM emissions from modern diesel vehicles are very low. As the "diesel scandal" of 2015 showed, one way to reduce NOx emissions is to better monitor exhaust treatment systems for signs of manipulation.

This study's findings suggest that LNG is a viable alternative fuel for passenger automobiles as the PM emissions from these vehicles are the safest. Reduced emissions of greenhouse gases would be an added bonus if LNG were produced using bio-based gas rather than gas derived from fossil fuels, such as biomethane, which could make use of the LNG infrastructure and vehicle fleet already in place. Problems with car-required features, like reduced cargo space and lesser range among refueling, exist, however, due to the gaseous nature of LNG. Additionally, this potent greenhouse gas is leaking from natural gas reservoirs and supply systems at quantities significantly higher than those previously expected. Methane emissions from the study's LNG-powered vehicle were also found to be relatively high.

This study discovered that when compared to gasoline-powered cars, FFVs fueled by high-concentration methanol had lower PAH and PM mass concentrations and exhaust PM toxicity. Identical results have been demonstrated. An encouraging approach to reducing PM emissions from gasoline-powered vehicles—which are substantially greater than those from BS VI diesel and LNG-powered vehicles—one method that has been proven to reduce the toxicity of gasoline exhaust is the use of gasoline particle filters, or GFPs and to decrease PM mass and PN emissions even when operating at cold temperatures. It is astonishing that there are multi-fold variances in PAH concentrations in emissions, considering that all BS VI automobiles contain state-of-the-art equipment.

Emissions of semi-volatile compounds have received less attention in terms of their chemical and toxicological characteristics than those of gases and particulate matter. This is especially surprising considering the abundance of data here about toxicity and emissions. Remember that exposure in humans does not always exhibit the expected toxicological traits. Therefore, the amount of particles deposited in people's respiratory systems and their exposure to toxicological features of particles may be affected by the size distribution of exhaust particles from older diesel vehicles and engines.

4. CONCLUSIONS

This study uses a volume-based strategy to demonstrate that different fuels and technologies cause RAW264.7 macrophages to react toxicologically differently to particulate matter (PM) exhaust from passenger cars. Varied exhaust gas PM masses and PN are likely the primary drivers of this fluctuation. It is likely that the dangerous PAH levels and the EC/OC ratio, which are changes in PM's chemical makeup, also influence the reactions. In addition, prior research has demonstrated that lower operating temperatures have an impact on emissions when concentrations of PM and PM-bound PAHs rise. The study found that the most toxicologically effective and environmentally friendly vehicles were those driven by modern diesel and LNG engines. In contrast, gasoline and high-blend methanol-fueled vehicles' exhaust particulate matter (PM) elicited relatively significant toxicological reactions, with methanol blends dampening reactions. Data from older diesel vehicles shows that the new regulations significantly reduced emissions of PM, PN, and PAHs linked to PM in exhaust gas. Results support the results of the toxicological evaluations. Ironically, when we factor in actual driving times and the total number of vehicles on the road, exhaust particulate matter samples that indicate such a small amount of driving can trigger toxicological reactions. There has to be more regulation and investigation into this issue because it is clear that emissions from passenger vehicles' exhaust gas are harmful.

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