Universidade de Aveiro
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Marlene de Jesus Salvado Soares

Efeitos dos hidrocarbonetos aromáticos policíclicos particulados de emissões de tráfego em células de epitélio alveolar humano

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Helena Cristina Correia de Oliveira Investigadora Auxiliar do Departamento de Biologia do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro e da Doutora Célia dos Anjos Alves, Investigadora Principal do Departamento de Ambiente e Ordenamento, CESAM, Universidade de Aveiro.

“Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth.”

- Jules Verne

(A Journey to the Center of the Earth)

o júri

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agradecimentos

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palavras-chave

PM; PAH; Emissões de Exaustão; Emissões de Não-Exaustão; A549; Citotoxicidade;

resumo

A poluição atmosférica constitui o maior risco ambiental para a saúde na Europa. A matéria particulada (PM) é considerada o poluente mais nocivo para o ser humano. O tráfego rodoviário representa uma das principais fontes emissoras de PM. As emissões desta fonte podem ser subdivididas em emissões de exaustão (cano de escape) e emissões de não-exaustão, as quais abrangem as partículas que resultam do desgaste de pneus e travões e ainda a ressuspensão de poeiras rodoviárias. Contrariamente às partículas de exaustão, as de não-exaustão têm sido muito pouco estudadas. Apesar de contribuírem pouco para a massa das partículas, os hidrocarbonetos aromáticos policíclicos (PAH) constituem um grupo de poluentes orgânicos persistentes com elevado potencial carcinogénico e mutagénico.

Neste trabalho foi utilizada a linha celular A549 de epitélio alveolar humano para estudar os efeitos tóxicos dos PAH extraídos de PM emitido pelo tráfego, incluindo amostras de exaustão obtidas em distintos ciclos de condução, amostras coletadas num túnel rodoviário e paralelamente numa atmosfera de fundo urbano próxima, e ainda amostras resultantes do desgaste de vários tipos de travões. Foram realizados ensaios de viabilidade, ciclo celular e níveis intracelulares de espécies reativas de oxigénio (ROS).

Os extratos com PAH resultantes das partículas do desgaste de travões com baixo teor de aço induziram maiores decréscimos na viabilidade celular. Observou-se que a citotoxicidade é mais dependente da composição individual dos PAH do que da concentração global.

Os resultados obtidos para as amostras de exaustão de veículos pesados, revelaram que os veículos Euro V produzem partículas mais tóxicas. A norma Euro VI impôs um limite de emissão de PM dez vezes menor que os níveis permitidos na norma anterior, contribuindo significativamente para o decréscimo do material particulado na exaustão e correspondente toxicidade. Dentro dos veículos Euro V, o combustível GTL foi o mais tóxico para as A549.

A amostra compósita semanal da atmosfera de fundo urbano da Escola Valsassina revelou ser extremamente tóxica, reduzindo a viabilidade celular para 2.66%, enquanto a redução máxima observada para as amostras diárias do túnel foi de 83.6%. No extrato orgânico da amostra da escola foi encontrado reteno e naftaleno, o reteno não foi detetado e o naftaleno está presente em níveis vestigiais nas amostras do túnel rodoviário.

Embora, para algumas amostras, tenham sido observadas alterações em todas as fases do ciclo celular A549 após 24 h de exposição, as diferenças nos valores médios não foram suficientes para excluir a possibilidade de variabilidade aleatória. Quando os resultados das três fases (G0/G1, S e G2/M) das células expostas a todos os extratos de PM foram comparados com o controlo, não foram detetadas diferenças estatisticamente significativas. Independentemente do tipo de amostra, foram observados alguns incrementos significativos nos níveis celulares de ROS nas células expostas às amostras da exaustão de camiões.

Keywords PM; PAH; Exhaust; Non-Exhaust; A549; Cytotoxicity

abstract

Air pollution consitutes the largest risk to health in Europe. Particulate matter (PM) is considered the most harmful pollutant for humans. Road traffic represent a major source of PM. Emissions from traffic can be subdivided in exhaust emissions (tailpipe) and non-exhaust emissions, which comprise particles from tyre and brake wear, as well as road dust ressuspension. Contrary to exhaust PM, non-exhaust PM has been much less studied.

Despite their low contribution to the PM mass, polycyclic aromatic hydrocarbons (PAH) constitute a group of persistent organic pollutants with a high carcinogenic and mutagenic potential.

In this work the A549 pulmonary epithelial cell line was used to study the toxic effects of PAH extracted from PM emitted by traffic, including exhaust samples obtained under different driving cycles, samples collected in a road tunnel and simultaneously in a nearby urban background atmosphere, and samples from the wear of distinct types of brakes. Tests to assess cell viability, cell cycle and reactive oxygen species (ROS) intracellular levels were performed.

The PM-bound PAH extracts from the wear of low steel brake pads induced greater decreases in cell viability. It was observed that cytotoxicity was more dependent on the individual composition of PAH than on the global concentration.

The results obtained for the exhaust PM samples from from heavy-duty vehicles reveladed that Euro V trucks produce more toxic particles. The Euro VI norm adopted a limit of PM emission that is ten times lower than the levels allowed in the previous norm, contributing to a significant decrease of particulate matter in the exhaust and of the corresponding toxicity. Among Euro V vehicles, the one powered with GTL was the most toxic to A549 cell line.

The composite weekly sample of the urban background atmosphere of the School Valsassina showed to be extremely toxic,decreasing the cell viability to 2.66%, while the maximum reduction observed for the daily road tunnel samples was 83.6%. Retene and naphthalene, which were not detected or were present at trace levels in samples from the tunnel, were found in the particulate extracts of the school sample.

Irrespective of the sample, although some changes were observed in the A549 cell cycle after 24 h exposure in all the phases, the differences in the mean values were not great enough to exclude the possibility of random variability. When the results from the three phases (G0/G1, S and G2/M) from the cells exposed to all PM extracts were compared to control, no statistically significant difference was detected. Regardless of the type of sample, significant increases in cellular ROS levels were observed in cells exposed to the PM extracts of some heavy-duty vehicles.

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Abbreviations

ATP Adenosine triphosphate

DCF 2'-7'- dichlorofluorescein

DCFH-DA 2',7'- dichlorofluorescein diacetate

DEP Diesel Exhaust Particles

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

FBS Fetal bovine serum

HMW High molecular weight

LM Low metallic

LMW Low molecular weight

MTT 3-(4, 5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide

NOx Nitrogen oxides

NAO Non-asbestos organic

PAH Polycyclic aromatic hydrocarbons

PI Propidium iodide

PM Particulate Matter

RNA Ribonucleic acid

RNase Ribonuclease

ROS Reactive Oxygen Species

SM Semi metallic

VTT Virtual Test Track

WHVC World Harmonized Vehicle Cycle

Introduction

1. Particulate Matter

Air pollution affects health at a large scale and even on low air pollution days individual health can be impacted. Short-term exposure to air pollutants is closely related to chronic obstructive pulmonary disease (COPD), cough, shortness of breath, wheezing, asthma, respiratory disease, and high rates of hospitalisation (Manisalidise et al., 2020).

It is known that most environmental pollutants are emitted through large-scale human activities, for example, the use of industrial machinery, power-producing stations, and combustion engines. As these activities are performed at such large scale, they are by far the major contributors to air pollution, with vehicles estimated to be responsible for approximately 80% (Manisalidis et al., 2020).

The World Health Organisation estimates that particulate matter (PM) air pollution aids in approximately 800,000 premature deaths per year, representing the 13th mortality leading cause in the world. PM is a portion of air pollution that is composed by very small particles and liquid droplets that contain organic chemicals, metals, and mineral soil/dust components. PM sources can be natural or anthropogenic. Manmade PM emissions include combustion in mechanical and industrial processes, vehicle emissions, and tobacco smoke. Natural sources encompass volcanoes, fires, dust storms, and aerosolised sea salt (Anderson et al., 2012).

Airborne particles can be directly emitted, named primary, or formed within the atmosphere from the condensation of gases, named secondary particles. PM has an especial characteristic that increases its importance, it both absorbs and reflects solar radiation and therefore affects climate. When components such as carbon black absorb incoming solar radiation, it leads to local heating of the atmosphere, while more reflective particles, like ammonium sulphate, reflect sunlight back to space and cause a cooling effect at the surface (Harrison, 2020).

PM is usually divided based on how the particles are generated and where they deposit in human airways. Particles with the same size or diameter, usually have the same settling velocity. Because of their inhalable sizes, the Air Quality

Directive 2008/50/EC and the WHO guidelines set limit values for PM <10, and PM<2.5 μm (PM_{10}, $PM_{2.5}$, respectively). Particles that have a diameter higher than 10 μm are normally filtered out by the nose and upper airways (Table 1) (Anderson et al., 2012).

Particles with a diameter between 10 and 2.5 μm are classified as “coarse,” between 2.5 and 0.1 μm as “fine,” and smaller than 0.1 μm as “ultrafine”. PM10 samples usually contains ultrafine, fine, and coarse fractions (Figure 1) (Anderson et al., 2012).

Table 1- Penetrability according to particle size (Manisalidis et al., 2020).

Particle size	Penetration degree in human respiratory system
>11μm	Into nostrils and upper respiratory tract
7-11μm	Into nasal cavity
4.7-7μm	Into larynx
3.3-4.7μm	Into trachea-bronchial
2.1-3.3μm	Second bronchial area
1.1-2.1μm	Terminal bronchial area
0.65-1.1μm	Bronchi
0.43-0.65μm	Alveoli

Fine fraction particles are mostly produced by gas-to-particle conversion processes within the atmosphere or from high-temperature emission processes such as vehicle exhaust or industrial combustion. Coarse fraction particles are more normally associated with mechanical break-up through abrasion or wind-driven processes like soil resuspension or the creation of sea spray by breaking waves (Harrison, 2020).

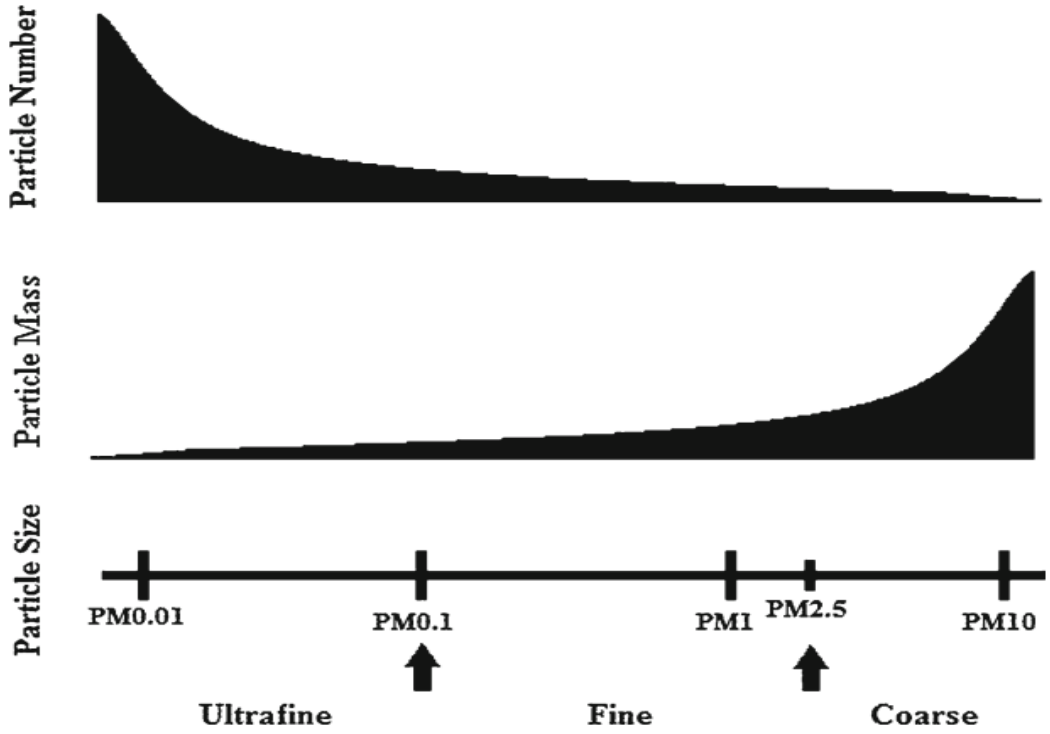


Figure 1 -A hypothetical mixed particle distribution (Anderson et al., 2012).

Traffic is a major source of air pollution. Traffic related PM can be distinguished in two types, exhaust, and non-exhaust PM (Thorpe & Harrison, 2008).

1.1 Exhaust PM

Exhaust particles result from incomplete fuel combustion and lubricant volatilisation during the combusting process. PM from these processes have been studied and characterised a lot over the past few years. Due to technological improvements and the imposition of restrictive emission limits (Euro standards), a significant reduction of exhaust PM emissions have been observed (Amato et al., 2014). In fact, exhaust sources are very carefully watched and regulated, manufacturers have strict emission standards to obey and the majority of diesel vehicles travelling on roads are now equipped with particulate traps (Lawrence et al., 2016).Current emission guidelines applied within the European Union (Euro VI for heavy-duty vehicles and Euro 6 for light-duty vehicles) followed a restriction of PM emission that is ten times lower than the ranges allowed in 2000. Countries where those emission limits are enforced and applied have

registered significant reductions in PM emissions (Posada et al., 2016). These guidelines require large deployment of superior engine tuning, the addition of greater aftertreatment gadgets and usage of low sulphur diesel content; consequently, they represent viable fleet renovations. Selective Catalytic Reduction or Exhaust Gas Recirculation aftertreatment structures continue to be the primary techniques to reduce PM emissions, after the adoption of the new emission standards (Du & Miller, 2017).

A big challenge in exhaust PM measurement is the dynamic character of exhaust PM. When hot exhaust plumes are released, they cool off fast and this leads to repartition of volatile and semi-volatile exhaust species between the gas and particle phases, depending on the dimension of exhaust plume dilution and cooling (Canagaratna et al., 2010). During plume dilution, there is competition between new particle formation (by nucleation), particle growth (by condensation and coagulation), and reduction of particle size and mass (by evaporation). Because of this competition, the PM chemical and physical properties in a diluting exhaust plume is directly influenced by where, when, and how the measurement is made. An example of this is the nanoparticle formation in vehicle exhaust, which has shown to be highly variable and critically dependent on the plume dilution process details, like, dilution ratio, and dilution temperature (Canagaratna et al., 2010; Jacobson et al.,2005; Morawska et al., 2008).

It is expected that the exhaust plume dilution and atmospheric process may lead to continue alterations in emitted PM mass concentrations and composition downwind of the emission source. For example, evaporative repartition provides a mechanism for continued evolution of exhaust PM properties. The soot containing accumulation-mode exhaust particles, which have long atmospheric lifetimes, can also interact with aerosol and gas phase species via coagulation and condensation. These interactions can transform hydrophobic, and fractal soot particles into internally mixed and more spherical particles (Cubison et al., 2006). The times and lengths scales of these transformations are important uncertainties in the prediction of the local, regional, and global impacts of exhaust PM (Canagaratna et al., 2010).

Diesel Exhaust Particles (DEP) have more than forty toxic air pollutants, including nitrogen and sulphur oxides, carbon monoxide, hydrocarbons, volatile organic

compounds, metals, organic and elemental carbon, and its makeup varies by engine load conditions and fuel composition (Costa et al., 2019). DEP are constituted by polycyclic aromatic hydrocarbons (PAH), redox active semi-quinones, and transition metals. They contain large surface carbon areas that absorb chemicals like PAH, aldehydes, quinones and heavy metals (e.g., copper, nickel, iron, and chromium). The organic compounds such as quinones and PAH cover about 30% of the weight of DEP (Lawal et al., 2016).

1.2 Non-exhaust PM

Non-exhaust emissions release mostly PM_{10}, but also $PM_{2.5}$, and are mainly composed of zinc, copper, iron, and lead (Thorpe & Harrison, 2008). Non-exhaust emissions related PM are created through brake, tyre and other vehicle parts wear processes or resuspension of already existing road wear particles (Thorpe & Harrison, 2008). Tyre, brake, clutch, road surface wear and resuspension of material already present on the road are the most important processes that aid the emissions of non-exhaust PM. Other smaller causes can be engine wear, abrasion of wheel bearings and corrosion of other vehicle components, street furniture and crash barriers (Grigoratos & Martini, 2015).

Contrary to exhaust PM, non-exhaust PM have not been enough studied and several questions regarding their health effects still need an answer (Denier van der Gon Hugo et al., 2013). Although non-exhaust PM emissions also contribute to air pollution, no actions are being made to reduce them (Amato et al., 2014).

Tyre wear PM emission result from a complex physicochemical process involving the frictional energy created at the interface between the tyre tread and road surface (Singh et al., 2020). Tyres can release up to 10% of their mass during their use, some of these 10% are emitted as PM_{10} (Boulter et al., 2006).

Road wear appears as consequence of the mass loss from the road surface due to the friction of tires with the road surface. Road wear emissions depend on the type of road, vehicle speed, and tyre pressure. As the temperature decreases, the tyres become less elastic, so, the road surface wear rate increases (Singh et al., 2020).

One of the most intense non-exhaust sources of traffic related PM is break wear, contributing to 55% of PM_{10} non-exhaust emissions, according to Harrison et al. (2012), and 21% to total PM_{10} traffic related emissions (Gasser et al., 2009).

Brake wear emissions are highly dependent on the type of brake used in a vehicle (drum brake or disc brake). Brake wear particles are released when brakes are applied to reduce vehicle speed and contributes to about 11-21% of total traffic PM emissions (Bukowieki et al., 2010; Lawrence et al., 2013). The contribution of brake wear is obviously higher with frequent braking at congested traffic lanes and junctions and smaller at the highways where the braking is not frequent (Bukowieki et al., 2010; Singh et al., 2020).

Brake wear particles are produced from friction among brake pads and disks. Garg et al. (2000) determined that approximately 35% of lost brake pad mass are emitted as airborne PM. Brake wear is a hazard to public health. It is a big source of redox-active and toxic metals, which include Sb, Cu, Zr, Fe, and Ba (Zhang et al., 2020). Cu and Fe are the most common metals in brake linings; however, their contents oscillate broadly among models. Even hard decreased copper brake pads should incorporate much less than they do (Hulskotte et al., 2014).

Brake wear PM chemical composition can rely on the driving behaviour, mainly in the braking frequency and intensity (Kwak et al., 2013). It is additionally crucial to consider the situations under which the braking motion occurred (ambient temperature and vehicle's velocity, for example). Those details can have an impact on the PM traits (Grigoratos & Martini, 2015). In a frictional braking system, activating the braking device makes the pad/disk or shoe/drum to come in touch causing friction, then the rotor decelerates or stops. Heat and brake wear are produced in the process and a part of the brake particles generated are launched into the atmosphere (Wahid, 2018). Passenger automobiles are usually geared up with a disk brake or a drum brake device that differ in the rotating part (a metallic disk or a cylinder). The rotating disc is typically manufactured from iron. However, it may additionally be manufactured from metallic ceramic or aluminium matrix composites. Clippers are manufactured from aluminium. To assure top thermal and mechanical properties, brake linings normally have a greater engineered composition than the rotating parts (Grigoratos & Martini, 2014; Piscitello et al., 2021)

There are many types of brakes differing on brand, fibres, comprising fillers and friction modifiers (Thorpe & Harrison, 2008). During the braking motion, friction among the pad and disc transforms kinetic energy into heat, and this excessive temperature changes the brake additives. Because of those excessive temperatures micro sized PM are launched (PM_{10} and $PM_{2.5}$) collectively with carbon-based particulates derived from condensation of natural compounds (Kukutschová & Filip, 2018).

Brake substances are categorised in 3 categories: non-asbestos organic (NAO), semi metallic (SM) and low metallic (LM). The NAO pads are the softest and produce low brake noise. However, they have a lower friction overall performance than other styles of pads and are more sensitive to increased temperatures. This sort of pads is appropriate for normal street driving in light and compact automobiles. SM pads have the best metal content material (30–60% of low carbon metallic fibre and/or iron powder), better durability and top heat transfer, however they induce an extensive wear of rotor. With those traits in mind, they may be satisfactory for excessive overall performance race motors. LM pads are constituted by a combination of natural compounds with a small part of metal additives. They present high friction performance, durability, and top braking potential at higher temperatures. These functions make them suitable for medium sized cars and trucks (Grigoratos & Martini, 2015; Kukutschová & Filip, 2018; Piscitello et al., 2021).

2. The influence of PM emissions on health

When examining the toxicity of particles, several characteristics must be considered, in particular the size of the particles. The size of the particles determines their deposition in the airways. Coarse particles remain in the upper airways (nose and throat) and ultrafine particles penetrate deep into the lungs (Poepping & Ginda, 2010). PM tends to settle easily at the bifurcation of the bronchial tree. Most inhaled particles can also accumulate in the lymph nodes, which are in line with the lymphatics that drain the lungs, thus involving the immune response (Losacco & Perillo, 2018). Although PM_{10} cannot reach the lungs as it is the largest size class, these fractions also contain ultrafine ($PM_{0.1}$),

fine ($PM_{0.1-2.5}$) and coarse fractions ($PM_{2.5-10}$) in the mixture. In a PM_{10} sample, most of the particles would be ultrafine, but they would represent only a very small fraction of the total mass of the sample (Anderson et al., 2012).

These smaller particles related to oxidative stress and inflammation are a threat to our health (Grigoratos & Martini, 2015). They may also promote early atherosclerosis due to their redox chemicals and ability to promote oxidative stress in tissues (Araujo & Nel, 2009). PM contribute to cardiovascular and cerebrovascular diseases through inflammatory mechanisms, coagulation activation and translocation into the systemic circulation. Respiratory disease is also promoted by PM exposure, which causes respiratory morbidity and mortality, and generates oxidative stress, genotoxicity and inflammation leading to lung remodelling and cell death (Figure 2) (Anderson et al., 2012).

Human airway epithelial cells exposed to PM express inflammatory cytokines (Silbajoris et al., 2011). Alveolar macrophages increase respiratory activity, produce reactive oxygen species (ROS), nitrogen species, and release TNFα and IL1 (Driscoll et al., 1990). The toxicity and health consequences of exposure to exhaust particulate matter have been extensively studied, but on the other hand, there are few studies on the specific health effects of PM from brake wear (Gasser et al., 2009).

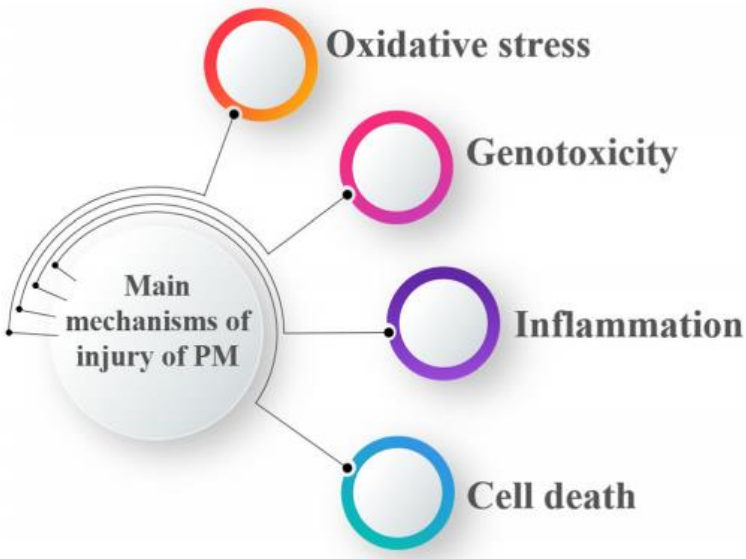


Figure 2- Main mechanisms of cell injury induced by PM (Arias-Pérez et al., 2020).

2.1. Oxidative stress

Part of the point of interest in respirable PM toxicology is directed to oxidative stress. The ability of respirable particles to penetrate the breathing system and reach the lung alveoli leading to ROS generation is usually pointed as the primary component involved of their pathogenic potential. There is numerous research and medical evidences for ROS involvement in lipid peroxidation, DNA mutations and protein oxidative harm (Gurgueira et al., 2002; Li et al., 2008).

ROS are produced through enzymatic and non-enzymatic processes by electron transfer reactions. The principal source of ROS in all cells of aerobic organisms is mitochondria, cytochrome P450 and peroxisomes. There is a constant endogenous production of reactive intermediates of radical species of oxygen and nitrogen that act as regulatory mediators of signalling processes for metabolism, cell cycle, intracellular transduction pathways, cellular redox systems and mechanisms of apoptosis (Valavanidis et al., 2002). The most important ROS include superoxide anion, hydrogen peroxide, the most reactive hydroxyl radical, singlet oxygen, ozone and others (Marrocco et al., 2017).

Oxidative stress is a scenario in the cell in which the redox stability is disturbed and inclines in the direction of a pro-oxidant state. This may also come from increased production of oxidant species or reduced levels of free radical scavengers like ascorbate and glutathione or antioxidant enzymes, like catalase, superoxide dismutase or glutathione peroxidase. The lung lining fluid is the primary antioxidant protection barrier that protects epithelial cells from oxidant harm from inhaled compounds with oxidising potential as it includes quite a few free radicals scavengers and antioxidant enzymes (Kelly et al., 2003). Small quantities of oxidants and antioxidants are crucial for numerous biochemical techniques and intracellular signaling. Redox homeostasis is critical for aerobic organisms, being ruled through the presence of huge pools of antioxidants that absorb and attenuate reductants and oxidants (Valavanidis et al., 2013). Oxidative stress is responsible for the rise of pulmonary pathology caused by airway inflammation, in particular when humans are exposed to inhalable airborne PM (Ghio et al., 2012; Valavanidis et al., 2013).

Airborne PM produce ROS that are implicated in the activation of mitogen-activated protein kinase (MAPK) family and transcription elements NF-κB and AP-1. These signaling pathways take part in processes of inflammation, apoptosis, proliferation, transformation and differentiation (Terzano et al., 2010). Studies confirmed that PM stable free radicals play a crucial function in growing ROS manufacturing, particularly hydroxyl radicals. ROS are tremendously reactive, responsible for damaging the DNA and inducing lipid peroxidation (Knaapen et al., 2002; Valavanidis et al., 2013).

PM from combustion sources contain several constituents that create ROS by a diversity of reactions. The most important are transition metals with redox properties, persistent free radicals and PAH (Risom et al., 2005).

There are several methods to measure free radical levels production in cells: chemiluminescence of luminol and lucigenin, cytochrome c reduction, ferrous oxidation of xylenol orange and dichloro-dihydro-fluorescein diacetate (DCFH-DA). All these methods have been used successfully to detect ROS generation (Eruslanov & Kusmartsev, 2010).

Flow cytometry and fluorimetry can be used to detect ROS through fluorescent probes like DCFH-DA. DCFH-DA is one of the most used forms to directly measure the redox state in a cell, since it has several advantages. It is easy to use, highly sensitive to changes in the cell redox state, it is not expensive and follows ROS changes over time. DCFH-DA is cleaved into two ester bonds, resulting in a polar and cell membrane-impermeable product, H_2-DCF. This non-fluorescent molecule accumulates in the cell and oxidation produces a highly fluorescent product DCF. The redox cell level is directly proportional to the increase in fluorescence. Accumulation of DCF in cells can be measured by an increase in fluorescence at 530 nm when the sample is excited at 485 nm. Fluorescence at 530 nm is measured using a flow cytometer and it is proportional to the concentration of hydrogen peroxide in the cells (Bass et al., 1983; Eruslanov & Kusmartsev, 2010).

2.2. Genotoxicity/Mutagenicity

One of the many consequences of the growing urbanisation with high level of industrial activities and increaed traffic density of automotive vehicles is the production of genotoxic agents that, when released, can combine with other compounds present in the air endangering human health (Silva da Silva et al., 2015). In a review of literature, Claxton et al. (2004) highlighted that studies performed in Asia, Europe, North America and Latin America, have associated the genotoxicity response of airborne particulate matter with the presence of several classes of mutagenic organic particles from anthropogenic sources, and chemical reactions in the air.

PM genotoxicity can be explained by organic substances adsorved on the particle surface, mainly PAH, nitro-polycyclic aromatic compounds, dioxins, and metals. Heavy metals and PAH damage the double helix structure of DNA alone or by synergistic action result in gene mutation (Li et al., 2017). The contribution of these pollutants to PM_{10} genotoxicity may depend on the particles sources and meteorological conditions. The spatial and temporal conditions during the generation of PM_{10} as a genotoxic pollutant is considered important for the evaluation of health risks driven from exposure. The consequences of this exposure may depend on local factors like industrial emissions, traffic and domestic and agricultural ativities (Brits et al., 2004).

To evaluate the changes in the quantity of genetic material several assays can be used, including cell cycle evaluation by flow cytometry. Flow cytometry is a technique that allows analysis of multiple cellular parameters, including DNA content. The technique bases itself on a single cell being passed through a stream into a laser (Chin & Yeong, 2010). DNA content is an indicator of the cellular phase in the cell cycle: cells in G0/1 phase have DNA content equal to 1 unit of DNA, cells in S phase duplicate DNA, increasing its content directly proportional to their progression through S phase and entering G_2, and during M the cells have twice the $G_{0/1}$ phase DNA content (equal to 2 units), as presented in Figure 3. Cellular DNA content analyses allow the discrimination between G0/1 S, and G2/M phases. The cells are stained with propidium iodide (PI), a fluorochrome. When excited with blue light (488 nm), the DNA-bound PI emits red fluorescence. As PI also stains RNA, its removal is necessary by incubation

with RNase. Results of fluorescence levels are presented in cellular DNA content frequency histograms that show the proportions of cells in the respective phases of the cycle based on fluorescence intensity. Apoptotic cells are often distinguished on frequency histograms by their fractional DNA content (Darzynkiewicz et al., 2001).

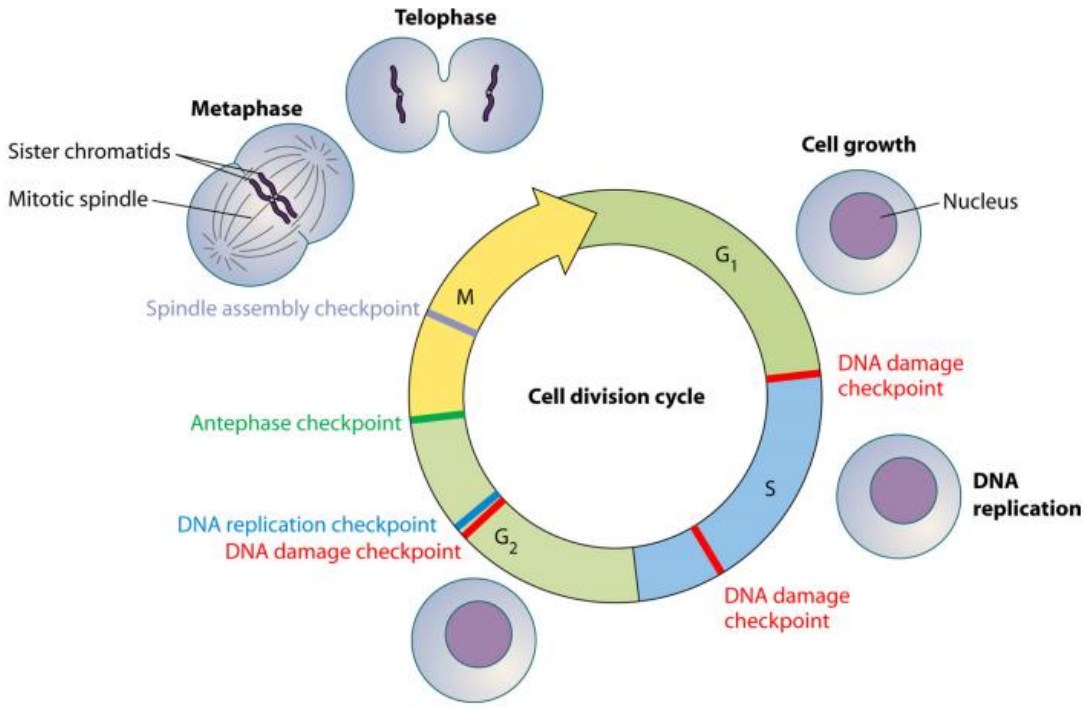


Figure 3- Cell Cycle checkpoint pathways impinging upon the cell division cycle (Chin & Yeong, 2010)

2.3. Inflammation

PM induced inflammation in the lung is highly modulated by alveolar macrophages and their biochemical signaling, including production of inflammatory cytokines, which is the primary mechanism responsible for inflammation initiation and sustention. In vitro experiments have shown that PM can produce damage to the whole respiratory tract, by increasing cellular permeability and reducing the mucociliary activity through ROS production and cytokine releases (Cachon et al., 2014; Huang et al., 2009; Losacco & Perillo, 2018). The inflammatory mediators can activate various pathways, such as MAP kinases, NF-κB (Nuclear Factor-Kappa B), and Stat-1, or induce DNA adducts

(Losacco & Perillo, 2018). Many studies confirmed the oxidative effect of PM due to the increase in NF-κB levels, a nuclear factor that induces the transcription of genes encoding cytokines and interleukins (IL-6, IL-8 and TNF-α) responsible for the on-site recall of neutrophils and subsequent tissue damage (Churg et al., 2005; Shukla et al., 2000; Takizawa et al., 1999).

2.4. A549

The pulmonary epithelium is composed by two major cell types, Type I and Type II. Type I covers approximately 96% of the surface area of the pulmonary epithelium and are unable to divide (Ryan et al., 1994). Type II cells cover a lot less surface area of the alveolus, are more numerous and have distinct functions. Type II cells are believed to be the progenitor cells for Type I pulmonary epithelial cells. The endocytic properties of these cells have been well characterised, what makes them a potential target for drug delivery of macromolecules. The A549 cell line was established in 1972 and has characteristic features of Type II cells of the pulmonary epithelium, including lamellar bodies (Lieber et al., 1976).

A549 is a human adenocarcinoma alveolar epithelial cell line commonly used in toxicity studies. These cells have specific properties such as surfactant production and transport-like AT-II cells in vivo, cytokines secretion, and ability to carry out xenobiotic biotransformation phase I and phase II like healthy cells. For these reasons, this is the most representative cell line for lung tissue and the most used for studying effects of toxicology in inhaled microparticles (Fröhlich & Salar-Behzadi, 2014).

2.5. PM exposure effects on children and adults

Literature shows that PM affect lung function and development. The Childrens Health Study (Gauderman et al., 2004) evaluated 1,759 patients over 8 years, and the results show that children who lived in places with highest PM concentrations had five times more probablility to have low FEV1 (forced expiratory volume in 1 second) than kids in communities with low PM concentrations. Several other studies also show that children that moved from

areas with high PM_{10} concentration to lower PM_{10} concentration increased lung function, and those that moved from areas of lower PM_{10} concentration to higher PM_{10} concentration decreased their lung function (Avol et al., 2001). Children with cystic fibrosis exposed to higher levels of PM_{10} and $PM_{2.5}$ decrease their lung function (Goss et al., 2004). On the other hand, children with good lung function became susceptible to asthma when exposed to higher levels of $PM_{2.5}$ (Islam et al., 2007).

In adults, PM effects on lung function have been found mostly in susceptible populations. Asthmatic Londoners taking walks in areas with higher PM rates had significantly higher reduction in FEV1, and an increase in sputum biomarkers of inflammation (McCreanor et al., 2007). In older patients, high rates of PM_{10} and $PM_{2.5}$ were related to decreases in peak expiratory flow rate (PEFR), which is a person maximum speed of expiration (Lee et al., 2007). In patients with chronic obstructive pulmonary disease (COPD), decrements in lung function were associated with increased $PM_{2.5}$ concentration (Lagorio et al., 2006). Oxidative damage is related to development of asthma and COPD. Long-term PM exposure leads to airway remodeling and chronic inflammation. Several PM controlled human experiments showed negative effects on the pulmonary system (Hogg et al., 2005).

Gasser et al. (2009) suggested that brake wear PM resultant from a car “full stop” braking can cause proinflammatory responses in lung cells through organic compounds and black carbon mechanisms. Although lung cells are the major focus of these studies, long term cardiovascular risk cannot be overlooked, an increase in myocardial infraction was detected on professional drivers particularly and, more generally, people who live next to major roadways have higher mortality rate (Grigoratos & Martini, 2015).

2.6. Respiratory mortality

An investigation that collected data from 20 cities revealed a growth of 0.87% in respiratory mortality for short-term increases in PM_{10} by 10 $\mu g/m^3$. This was expanded for 112 US cities, where researchers found an increase rate of 1.68% in respiratory mortality for every 10 $\mu g/m^3$ increase in $PM_{2.5}$ (Zanobetti &

Schwartz, 2009; Zeka et al., 2005). Also, a study in California revealed an increased respiratory mortality with high rate of PM_{10} emissions (Ostro et al., 2006). Another study in Italy showed that for 275,000 adults in 10 Italian cities, short-term increases in PM_{10} led to a 2.29% growth in respiratory mortality (Stafoggia et al., 2009). Similar results were found in Asia, where investigators came across excess respiratory mortality risk for increases in PM_{10} (Wong et al., 2008). In Europe, across 29 cities, identical results were found (Analitis et al., 2006). Other studies show that ultrafine particles can translocate to other tissues, like liver, kidneys, and brain. This happens when they become blood borne (Grigoratos & Martini, 2015). The World Health Organisation wrote that PM inhalation can be harmful in short periods of time as well in the long term, leading to mortal respiratory and cardiovascular diseases. Several constituents have been pointed out to be responsible for this harm, especially PAH (World Health Organization, 2013).

3. PM main components

PM components consist mainly of a carbonaceous fraction associated with adsorbed organic chemicals and reactive metal(loid)s. Common components of PM are elemental carbon, nitrates, sulphates, PAH and other organic compounds, endotoxins, and elements (Hamanaka & Mutlu, 2018).

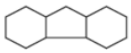
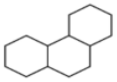
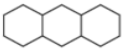
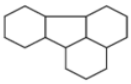
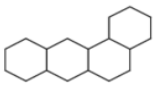
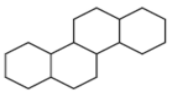
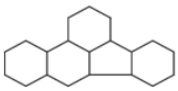
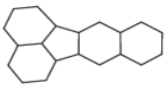
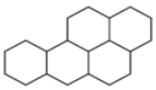
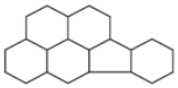
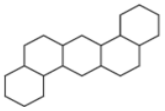
3.1. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are a group of persistent, organic pollutants. The United States Environmental Protection Agency (US EPA) classified 16 PAH as high priority pollutants (Honda & Suzuki, 2020; Joa et al., 2009) (Table 2). These compounds are widely distributed in the environment and enter environmental matrices via natural and anthropogenic sources, especially combustion processes of organic matter (Alegbeleye et al., 2017; Dat & Chang, 2017; Islam et al., 2007; Kim et al., 2013; Lannerö et al., 2008; Samanta et al., 2002). Large PAH concentration ranges are found as a result of organic matter incomplete combustion, emission sources, traffic exhaust and non-exhaust

matter and in food (Finlayson-Pitts & Pitts, 1997). PAH are composed of fused aromatic benzene rings and a system of hydrophobic and lipophilic double bonds throughout their hydrocarbon rings. They appear in significant concentrations in terrestrial, atmospheric, and aquatic ecosystems (Fernández-Luqueño et al., 2011; Sojinu et al., 2011). These compounds are potentially harmful to ecosystems and humans, many of them have already shown to be carcinogenic, teratogenic, and mutagenic, and their removal from the environment has many limitations (Alegbeleye et al., 2017; Samanta et al., 2002; Skupińska et al., 2004). Several approaches and strategies were developed and optimised to mitigate the effects of these contaminants and remediate polluted sites. But these remediation techniques have significant limitations, such as their technological complexity, high cost and the lack of public acceptance. On top of these complications, most of the techniques are invasive and only relocate the contamination problem, often requiring further waste management (Alegbeleye et al., 2017).

PAH rings can be presented in different arrangements (linear, angular or clustered) (Lundstedt, 2003). PAH are lipophilic, have low vapour pressure and water solubility, as well as high melting and boiling points (Skupińska et al., 2004). They are only made of carbon and hydrogen atoms, but nitrogen, sulphur and oxygen atoms can appear as substitutes in the benzene ring to form heterocyclic aromatic compounds that are usually grouped with PAH (Lundstedt, 2003). PAH can be classified as low molecular weight (LMW) compounds if they only have two or three fused rings, and as high molecular weight (HMW) compounds if they have four or more fused rings (Alegbeleye et al., 2017). LMW PAH are more susceptible to degradation and more volatile compared to HMW PAH (Harvey, 1998). The increase of molecular weight leads to increase of hydrophobicity/lipophilicity, which makes the structure more recalcitrant (Alegbeleye et al., 2017). To support this theory, research demonstrated that the average half-life of the tricyclic phenanthrene varies from 16 to 126 days in soil. Contrary to this, for the five-ringed HMW PAH benzo[a]pyrene the half-life may vary from 229 to 1500 days (Sojinu et al., 2011). HMW PAH are more persistent because of low water solubility, low volatility, resistance to leaching and their recalcitrant nature (Alegbeleye et al., 2017).

Table 2 – Selected properties of US EPA 16 priority PAH (Joa et al., 2009).

PAH	Abbrevi-ation	Structure	Molecular mass, Daltons	Boiling point, °C	Vapor pressure at 25 °C, Pa
Naphthalene $C_{10}H_8$	**NA**		128	218	10.4
Acenaphthene $C_{12}H_{10}$	**AC**		154	278	30×10^{-1}
Acenaphthylene $C_{12}H_8$	**ACN**		152	265	9.0×10^{-1}
Fluorene $C_{13}H_{10}$	**FL**		166	295	9.0×10^{-2}
Phenanthrene $C_{14}H_{10}$	**PHE**		178	339	2.0×10^{-2}
Anthracene $C_{14}H_{10}$	**AN**		178	340	1.0×10^{-3}
Fluoranthene $C_{16}H_{10}$	**FA**		202	375	1.2×10^{-3}
Pyrene $C_{16}H_{10}$	**PY**		202	360	6.0×10^{-4}
Benz[a]anthracene $C_{18}H_{12}$	**B[a]A**		228	435	2.8×10^{-5}
Chrysene $C_{18}H_{12}$	**CHR**		228	448	5.7×10^{-7}
Benzo[b]fluo-ranthene $C_{20}H_{12}$	**B[b]F**		252	481	N/A
Benzo[k]fluo-ranthene $C_{20}H_{12}$	**B[k]F**		252	481	5.2×10^{-8}
Benzo[a]pyrene $C_{20}H_{12}$	**B[a]P**		252	495	7.0×10^{-7}
Benzo[ghi]-perylene $C_{22}H_{12}$	**B[ghi]P**		276	N/A	6×10^{-8}
Indeno[1,2,3-cd]-pyrene $C_{22}H_{12}$	**IP**		276	536	N/A
Dibenz[a,h]-anthracene $C_{22}H_{14}$	**D[ah]A**		278	524	3.7×10^{-10}

3.1.1. Exposure to PAH and effects

Exposure to PAH essentially occurs from ambient air, eating food that contains PAH, smoking cigarettes or smoke from open fireplaces (ACGIH, 2005).

A variety of PAH originating from tobacco smoke are suspected to lead to cancer (Lannerö et al., 2008). For non-smokers, the main exposure is through food. Processing and cooking of foods at high temperatures (grilling, roasting, and frying) are big sources of PAH (Chen & Chen, 2001). In general, PAH exposure can occur from contaminated soil via ingestion, dermal (skin) exposure and from inhalation of PAH vapours (Wang et al., 2012).

Reactive metabolites of some PAH have great potential to bind to cellular proteins and DNA, leading to toxic effects. The resulting biochemical disruption and cell damage causes mutations, malformations, tumours, and cancer (Kim et al., 2013). Reactive metabolites of PAH can bind themselves to cellular proteins and DNA with toxic effects, this biochemical perturbation can lead to mutations, tumours, and cancer. Results of primary studies show that the exposure to mixes of PAH can be more harmful than the exposure to just one type (Kim et al., 2013). Effects of PAH on human health depend on exposure time, PAH concentration, and form of exposure (inhalation, ingestion, or skin contact). Other secondary factors can affect the health impacts, like pre-existing health conditions, the presence of other pollutants and age (Alegbeleye et al., 2017). Short-term exposure to PAH has been reported to damage lung function in asthmatics and originate thrombotic effects in people affected by coronary heart disease (ACGIH, 2005). Exposure to high levels of pollutant mixtures that contain PAH usually result in eye irritation, nausea, vomiting, and skin irritation and inflammation (Unwin et al., 2006). Long-term exposure to PAH lead to increased risk of skin, lung, bladder, and gastrointestinal cancers (Kim et al., 2013). Long-term exposure to low levels of PAH was pointed out as a cause of cancer in laboratory animals (Diggs et al., 2012). Exposure to PAH may also cause cataracts and kidney and liver damage and jaundice (Kim et al., 2013).

It is often difficult to associate exclusive health effects to specific PAH because most exposures happen in a combination of different PAH. Some studies have shown that exposure to certain individual PAH during extended periods at high concentrations can be poisonous (Alegbeleye et al., 2017).

Reports suggest that repeated skin contact with a specific PAH (naphthalene) can cause skin redness and inflammation and its ingestion can breakdown red

blood cells, haemolytic anaemia and eye defects (Srogi, 2007). It can also act as an inhibitor of mitochondrial respiration (Falahatpisheh et al., 2001). In cases of acute exposure, it can lead to symptoms such as nausea, vomiting, abdominal pain, diarrhea, headache, confusion, profuse sweating, fever, tachycardia, and tachypnoea. Acute dermal exposure to naphthalene has been associated with mild irritation and, in some sensitive individuals, may cause dermatitis (CDC, 2009). It hasn't been demonstrated that Naphthalene can cause cancer in humans, however, it has been shown to induce tumour in laboratory mice and other animals (Alegbeleye et al., 2017). PAH are lipid-soluble and can be highly absorbed from the gastrointestinal mammalian tract (Cerniglia, 1984). They spread fast in tissues with tendency for body fat location, their metabolism occurs via cytochrome P450-mediated mixed function oxidase system, starting with hydroxylation or oxidation (Stegeman et al., 2001).

Phenanthrene, a major constituent of urban air pollution, is known to have a mutagenic and impair immune function (Liu et al., 2013). Acenaphthene has been known to have harmful effects on skin, body fluids and immune system of animals after short-term or long-term exposure. Some suggest that fluorene induces skin and eye irritation and is a potential carcinogen (Alegbeleye et al., 2017).

Although most studies on this matter investigates PAH potencial to cause cancer, PAH can also interfere with hormone systems, afecting reproduction and immune function. Long-term exposure to PAH is suspected to increase the risk of cell damage by gene mutation and cardiopulmonary mortality (Kuo et al., 2003).

Objectives

Taking into account that both exhaust and non-exhaust emissions from road traffic represent major sources or air pollution, especially in urban areas where most of the population lives, further studies on the toxicity of these emissions must be carried out because automotive technology is constantly changing, fuel quality has improved, and new fuel gas after-treatment technologies have been

adopted. On the other hand, non-exhaust emissions, and their PAH and toxicity have been very little studied.

Since the toxicity of the emitted particulate matter depends on the source, this work focused on samples of PAH-bound PM from different traffic-related emissions: i) wear of distinct types of brakes, ii) exhaust from heavy-duty vehicles tested under various driving cycles, and iii) a road tunnel and the surrounding background air. It should be noted that road tunnels are representative of the vehicle fleet, allowing to obtain characteristic samples of exhaust and non-exhaust emissions under real traffic conditions, with negligible photochemical degradation, since they are semi-enclosed environments. The main objective of this thesis was to evaluate the toxicological profile of these samples and their effects on human lung epithelial A549 cell line by observing cell viability, cell cycle alterations and ROS intracellular levels after 24 h exposure to the extracts.

Methods

1. Sample collection

1.1. Brake wear

PM_{10} from brake pad wear were obtained in a laboratory in Italy equipped with a bench dynamometer with the brake system closed in a chamber. The brake pads tested can be classified as follows: 1) conventional brake pads (low steel) mostly used in Europe; 2) non-asbestos organic brake pads (NAO) with non-ferrous metals, typically used in the USA; 3) pads with higher metallic content (high steel), more frequent in South America. Two types of brake discs were tested for each one of these two categories. Different brake cycles have been tested: 1) the smoothest protocol with 8 braking events, 120–80 kph deceleration, temperature 100 ºC; 2) protocol with 8 braking events, 200–170 kph deceleration, temperature 100 ºC; 3) FADE, with more severe braking and higher temperature and pressure, performing 15 braking events, 100–5 kph deceleration, maximum temperature 550 ºC. Pallflex 47 mm diameter Tissue Quartz 2500 QAT-UP filters were used to collect PM_{10} samples at a flow of 10 L min^{-1} (Alves et al., 2021).

1.2. Exhaust from heavy-duty vehicles

PM_{10} Exhaust samples from diesel (1 and 2) and GTL powered heavy duty vehicles (Euro V and VI) were collected under different driving cycles (Table 3 and Figure 4). The chassis dynamometer test cycles, with hot or cold start, were carried out by the Technical Research Centre of Finland (VTT), whose facility is equipped with a full-flow dilution tunnel and an AVL PSS i60 particulate matter sampler. PM was collected on 47 mm diameter filters (Pallflex® Emfab™). The VTT driving cycle was developed by the Technical Research Centre of Finland. It is an urban cycle with several stops. WHVC (World Harmonized Vehicle Cycle) is a transient engine dynamometer schedule defined by the proposed global technical regulation (GTR) developed by the UN ECE GRPE group. The GTR is covering a world-wide harmonized heavy-duty certification procedure for engine exhaust emissions. The proposed regulation is based on the world-wide pattern of real heavy commercial vehicle use. The Braunschweig driving cycle was developed at the Technical University of Braunschweig. It is a transient driving schedule simulating urban driving with frequent stops.

Table 3 - Details of the heavy-duty vehicles tested.

High duty vehicle	Test cycle	Fuel	Vehicle model year	Emission standard
HDV I	VTT	Diesel 1	2013	Euro V
	VTT	Diesel 1	2013	Euro V
	VTT	Diesel 1	2013	Euro V
	WHVC cold	Diesel 1	2013	Euro V
	WHVC cold	Diesel 1	2013	Euro V
	WHVC cold	GTL	2013	Euro V
	WHVC cold	GTL	2013	Euro V
HDV II	WHVC cold	Diesel 2	2016	Euro VI
	WHVC hot	Diesel 2	2016	Euro VI
	WHVC hot	Diesel 2	2016	Euro VI
	WHVC hot	Diesel 2	2016	Euro VI
	WHVC hot	Diesel 2	2016	Euro VI
HDV III	Braunschweig hot	Diesel 2	2016	Euro VI
	Braunschweig hot	Diesel 2	2016	Euro VI

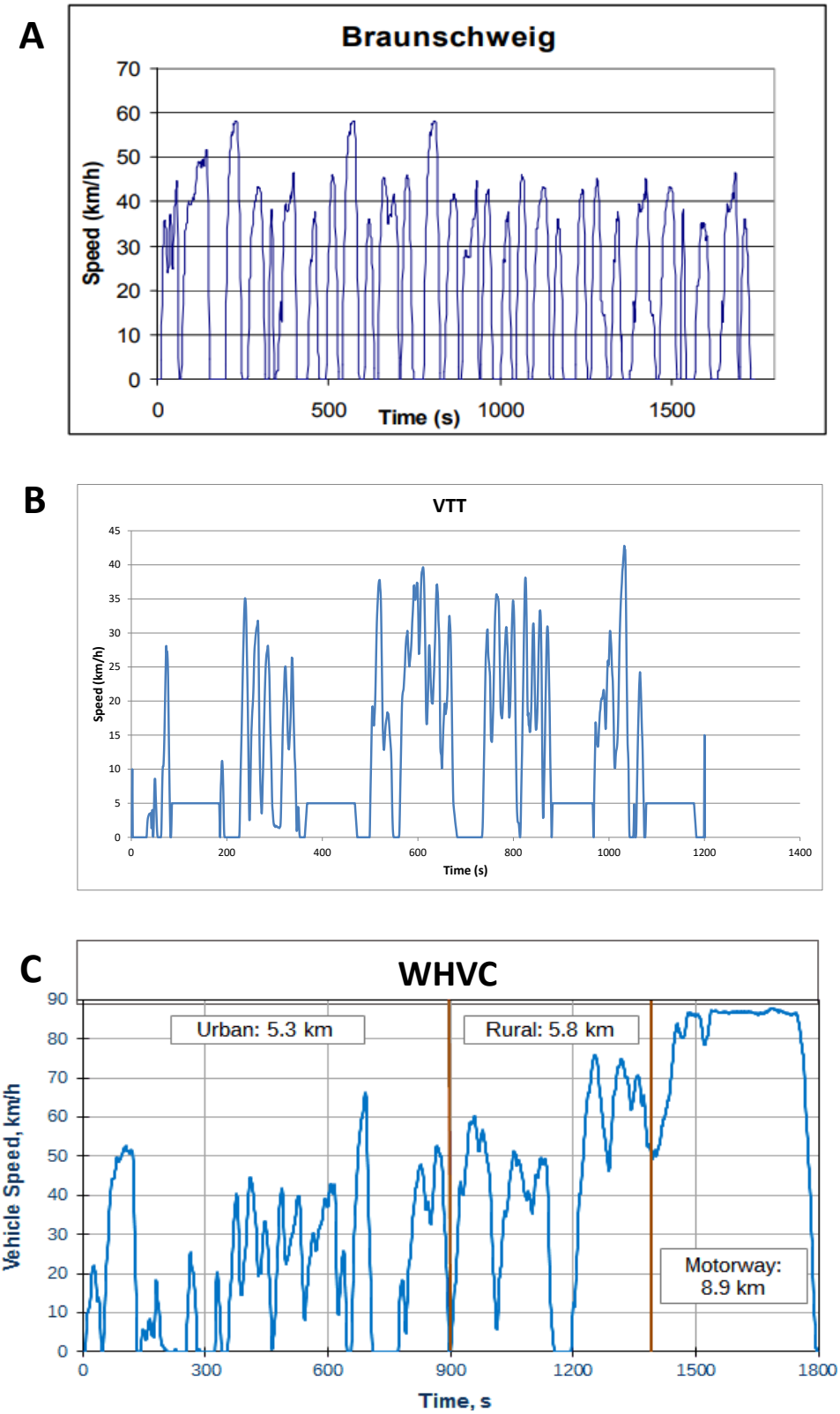


Figure 4 - Driving cycles applied to the heavy-duty vehicles tested in the present study. (A) Braunschweig driving cycle developed at the Technical University of Braunschweig; (B) VTT driving cycle developed by the Technical Research Centre of Finland; (C) WHVC (World Harmonized Vehicle Cycle) based on the world-wide pattern of real heavy commercial vehicle use.

1.3. Tunnel João XXI

A sampling campaign was carried out in a road tunnel (João XXI) in Lisbon for one week. Daily samples were obtained covering the period between 8 a.m and 8 p.m. To obtain samples representative of the urban background atmosphere, PM_{10} samples were parallelly collected at School Valsassina near that tunnel on the same days. Due to the low mass amounts, the extracts of the background air were combined in one vial to obtain a composite sample of the entire week. Low volume samplers from Sven Leckel (model LVS6-RV) were used to collect $PM_{2.5}$ samples onto Pallflex 47 mm diameter quartz filters at a flow of 2.3 m^3/h.

2. Extraction method and PAH analytical determination

After gravimetric determination, each PM filter was sequentially extracted with dichloromethane and methanol. After filtration of both solvents, the combined organic extract was concentrated to a volume of 0.5 mL in a TurboVap® evaporator workstation and then dried under a gentle nitrogen stream. Flash vacuum silica gel column chromatography was employed to separate the extracts into five groups of organic compounds using eluents of increasing polarity. The eluent used to separate PAH was a mixture of n-hexane/toluene (9.6/5.4 mL). After elution, the solvent was concentrated in the TurboVap system and dried with nitrogen. PAH were analysed by gas chromatography–mass spectrometry (GC–MS) in a Shimadzu QP5050A model equipped with a TRB-5MS 30 m × 0.25 mm × 0.25 μm column. Six deuterated compounds (acenaphthene-d10, chrysene-d12, 1,4-dichlorobenzene-d4, naphthalene-d8, perylene-d12, and phenanthrene-d10) contained in the EPA 8270 semi-volatile internal standard mix (Supelco, St. Louis, USA), benzo[a]pyrene-d12 (Supelco, St. Louis, USA) and fluorene-d10 (Aldrich, St. Louis, USA) were used to spike the PAH extracts. Data were acquired in both full scan and selected ion monitoring (SIM) modes at 70 eV. The oven temperature program was as follows: 60 °C (1 min), 60 to 150 °C (10 °C min^{-1}), 150 to 290 °C (5 °C min^{-1}), 290 °C (30 min). Helium was used as carrier gas at 1.2 mL min^{-1}. Calibrations were performed with authentic standards (Sigma-Aldrich, St. Louis, USA) in eight different concentration levels.

3. Preparation of PAH extracts to cytotoxicity assays

The dry PAH extracts were diluted at 1.1% DMSO, according to the results of the DMSO toxicity. PM_{10} from brake samples were diluted to the final concentration of 150 ng/mL and 400 ng/mL, while those from heavy duty vehicle exhausts were all diluted to the concentration of 100 ng/mL. The $PM_{2.5}$-bound PAH extracts from the road tunnel campaign were diluted at 150 ng/mL.

Table 4 - Available masses of PM extracts from the brake extracts and respective dilutions.

PM Sample	Mass of extract (ng)	DMSO (µL)	[ng/mL]	%v/v DMSO
120-80 km/h NAO 1	1453	110	150	1.1%
200-170 km/H NAO 1	1467	110	150	1.1%
120-80 km/h low steel 1	1468	110	150	1.1%
200-170 km/H low steel 1	9666	270	400	1.1%
120-80 km/h NAO 2	1467	110	150	1.1%
200-170 km/H NAO 2	1463	110	150	1.1%
120-80 km/h low steel 2	1462	110	150	1.1%
FADE low steel 2	1463	110	150	1.1%
120-80 km/h high steel	1463	110	150	1.1%
200-170 km/H high steel	9381	260	400	1.1%

Table 5- Available masses of PM extracts from the exhausts of heavy-duty vehicles and respective dilutions.

PM Sample	Mass of extract (ng)	DMSO (µL)	[ng/mL]	%v/v DMSO
Euro V Diesel 1 VTT (1)	1060	120	100	1.1%
Euro V Diesel 1 VTT (2)	1178	110	100	1.1%
Euro V Diesel 1 VTT (3)	896	100	100	1.1%
Euro V Diesel 1 WHVC cold (1)	1593	180	100	1.1%
Euro V Diesel 1 WHVC cold (2)	1182	110	100	1.1%
Euro V GTL WHVC cold	955	105	100	1.1%
Euro VI Diesel 2 WHVC cold	1173	110	100	1.1%
Euro VI Diesel 2 WHVC hot (1)	1162	110	100	1.1%
Euro VI Diesel 2 WHVC hot (2)	1147	110	100	1.1%
Euro VI Diesel 2 WHVC hot (3)	1171	110	100	1.1%
Euro VI Diesel 2 WHVC hot (4)	1754	130	100	1.1%
Euro VI Diesel 2 Braunschweig hot (1)	1187	110	100	1.1%
Euro VI Diesel 2 Braunschweig hot (2)	1747	130	100	1.1%

Table 6- Available masses of PM extracts of samples collected in Tunnel João XXI and surrounding background air and respective dilutions.

PM Sample	Mass of extract (ng)	DMSO (µL)	[ng/mL]	%v/v DMSO
Monday	4499	330	150	1.1%
Tusday	3802	280	150	1.1%
Wednesay	3421	250	150	1.1%
Thursday	4836	350	150	1.1%
Friday	4207	310	150	1.1%
Saturday	4220	310	150	1.1%
Sunday	2902	210	150	1.1%
Background	2366	175	150	1.1%

4. Cell culture

The pulmonary adenocarcinoma cell line A549 obtained from European Collection of Authenticated Cell Cultures (ECACC) and supplied by Sigma Aldrich (St. Louis, MO, USA) was used to evaluate the cytotoxicity and genotoxicity mechanisms of PM-bound PAH extracts. A549 cells were cultured at specific conditions (37 °C and 5% CO_2), and maintained in 25 cm^2 flasks (SPL Life Sciences, South Korea) with Kaighn's Modification of Ham's F-12 Medium (F-12K) (Gibco, Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Grand Island, NY, USA), 2 mM L-glutamine (Grisp, Porto, Portugal), 1% pen/strep (100 µg/mL de streptomycin, 100 U/mL penicillin, Grisp, Porto, Portugal) and 2.5 µg/mL Amphotericin B (Gibco, Life Technologies, Grand Island, NY, USA), at 37 ºC in humidified atmosphere with 5% CO_2. To evaluate the confluence, morphology and possible contaminations, the cells were observed daily with an inverted phase contrast microscope Eclipse TS100 (Nikon, Tokyo, Japan). Subculture was performed when cells reached 80/90% confluence (every 2 or 3 days) (Figure 5).

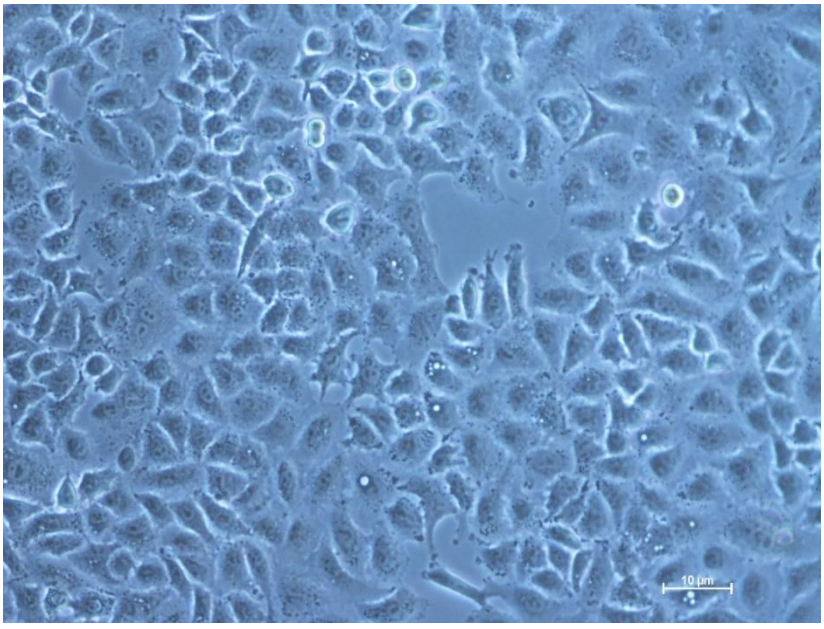


Figure 5 - Light microscopy images of A549 cells at 80/90% confluency.

4.1 Subculture

Subculture process started with medium removal from the flasks, leaning the flask and not dragging the cells attached to the flask interior surface. The old medium was removed, the cells were washed with 2 mL of PBS to clean possible medium leftovers, then the PBS was removed and 1.5 mL Trypsin -EDTA (0.25% de trypsin, 1 mM de EDTA) are added. The flask was incubated at 37 ºC and 5% CO_2 for approximately 5 minutes to detach the cells from the flask surface and from each other. Cell detachment was verified under the microscope. Then, complete medium was added to the flask to inactivate the trypsin. Cells were then counted using an hemocytometer and seeded.

5. MTT Assay

In viable cells, the mitochondrial activity is constant and therefore an increase or decrease in the number of viable cells is linearly related to mitochondrial activity. The mitochondrial activity of the cells is shown by the conversion of the tetrazolium salt MTT into formazan crystals, which are solubilised for homogenous measurement. Thereby, any increase or decrease in the number of viable cells can be detected by assessing formazan concentration reflected in optical density using a plate reader (Van Meerloo et al., 2011).

For the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide MTT assay cells were incubated for 24 h for adhesion and then the medium was replaced by new one, to simulate exposure stress, and the plate incubated for another 24 h. After 24 h exposure, 50 µL of MTT (1 mg/mL in PBS) were added to each well, and cells were incubated for 4 h at 37 ºC, 5% CO_2. The medium with MTT was removed and replaced with 150 µL dimethyl sulfoxide (DMSO) to solubilise the formazan crystals. The plates were then shaken in the dark for 2 h at room temperature to assure the complete dissolution of the formazan crystals. The optical density was measured at 570 nm using a microplate reader (Biotek® - Gen5™ software) and the percentage of viable cells was calculated with Eg. 1. Viability, proportional to absorbance was presented as mean and standard error in three independent experiments. Three independent assays with six replicates were performed for each treatment.

$$\%\ \text{Cell viability} = \frac{(\text{absorbance of sample} - \text{absorbance of DMSO})}{(\text{absorbance of Control} - \text{absorbance of DMSO})} \times 100 \quad \text{(Eq. 1)}$$

5.1 Optimisation of cell seeding densities for 96 well plate 24 h exposure

In order to determine the ideal cell seeding density for the cell viability assays, 100 µl of cell suspension were seeded in a 96 well plate (Corning®), at various concentrations: 20,000 cells/mL; 30,000 cells/mL; 40,000 cells/mL; 50,000 cells/mL; 60,000 cells/mL; 70,000 cells/mL; 80,000 cells/mL for 24 h at 37 ºC and 5.0% CO_2 to allow cell attachment, then the plates were treated as described in section 5.

5.2 Determination of DMSO percent toxicity for 24-h exposure

To determine the toxicity of DMSO A549 cells were seeded in 96 well plates (Corning®) at $4x10^4$ cells/mL (100 µl/well) and incubated for 24 h 37ºC and 5.0% CO_2, to allow cell attachment. Then the cells were exposed to different DMSO solutions: 0.9%; 1.0%; 1.1%; 1.2%; 1.3%; 1.4%; 1.5%. The cells were incubated for another 24 hours and handled as described in section 5.

5.3 Cell viability assay

To determine the toxicity of PM-bound PAH, A549 cells were seeded in a 96 well plate (Corning®) at $4x10^4$ cells/mL (100 µl of cell suspension/e well) and incubated for 24 h at 37 ºC and 5.0% CO_2 to allow cell attachment. Afterward, the medium was removed and replaced by new medium with PM_{10} extracts diluted at several concentrations. 1.1% DMSO solution was used as control. The MTT assay was used to determine cell viability, as described in section 5.

6. Cell cycle determination

For cell cycle dynamics evaluation, A549 cells were seeded in 12 well plates at $4.5x10^4$ cells/mL, with 1 mL solution per well, and incubated for 24 h at 37 ºC and 5.0% CO_2 to allow cell attachment. After 24 h, the medium was removed and replaced by the respective sample solution. 1.1% DMSO solution was used as control. After 24 h exposure, culture medium was removed, each well was washed with 500 µl of PBS, tripsinyzed at 37 ºC and 5.0% CO_2 for 5 min to promote cell detachment. Afterwards, 300 µl of medium were added to inactivate the trypsin and the cells were transferred to microtubes. The microtubes were centrifuged at 700 g for 5 minutes. Supernatant was removed and the cells were washed with 800 µl of PBS and fixed in 1 mL of ethanol 85% at 4 ºC and stored at -20ºC until analyses.

On the analysis day, the microtubes were centrifuged at 700 G for 6 minutes at 4 ºC, then the supernatant was removed, and the cells resuspended in 800 µl of PBS. To avoid clusters, the cell suspension was filtered with nylon mesh (41 µm).

To each tube, 50 µg/mL RNase and 50 µg/mL propidium iodide (PI) were added and incubated for at least 20 min, in the dark and at room temperature. Propidium iodide-stained cells were analysed on a Attune® Acoustic Focusing Cytometer (Applied Biosystems) flow cytometer. Two independent assays with two replicates were performed for each treatment, and for each sample, at least 5000 events were acquired. The percentage of cells at G0/G1, S and G2 phases was analysed using the FlowJo Software (Vermes et al., 1995).

7. ROS assay

Intracellular ROS accumulation in A549 cells was assessed using the probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Upon cleavage of the acetate groups and further oxidation, DCFH-DA transforms into the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS.

Cells were seeded in 12 well plates at $4.5x10^4$ cells/mL, 1 mL per well and incubated for 24 h at 37 ºC and 5.0% CO_2 to allow cell attachment. After the 24 h, the medium was removed and replaced by the respective sample solution.

1.1% DMSO solution was used as control. After 24 h exposure, cell culture medium was removed, cells were washed with 500 µl of PBS (pH 7.2) and a set of wells were incubated with 300 mM H_2O_2 at 37 ºC for 30 minutes to be used as positive controls. Then, H_2O_2 solution was removed, and all cell wells were treated with 500 µl of non-supplemented culture medium with 10 µM de DCF-DA and further incubated for 30 minutes.

After that, cells were washed, trypsinized and collected for analyses on a Attune® Acoustic Focusing Cytometer (Applied Biosystems) flow cytometer in the next 45 minutes. The fluorescence intensity was proportional to the intracellular ROS levels. Three replicates were performed for each treatment, and for each sample at least 20000 events were acquired. The data was analysed using the FlowJo software (Vermes et al., 1995).

8. Statistical analyses

All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed in SigmaPlot version 14.0 software and by one-way ANOVA, followed by Dunn's test, for multiple comparisons ($p<0.05$).

Results and Discussion

1. Cell viability quantification trough MTT assay

1.1 Ideal cell seeding concentrations for 24 h exposure in 96 well plate

Before starting the experiments with the PAH samples, the ideal seeding concentration was optimised, because the 24 h MTT assay is less sensitive at cell numbers below 1000 cells/well (Van Tonder et al., 2015). The initial cell density can oscillate between 5×10^2 to 10^4 cells per well depending on the cell line. The optimal density was determined by selecting the density that gives an absorbance value close to 1, i.e. the closest value above 1 was chosen (Table 7 and Figure 6), because cells tend to decrease replication at a certain stage. So a

few days later, when the viability assays started with the PAH extracts, 40,000 cells/mL gave us absorbances of 1 (Plumb, 2004).

Table 7 - Seeding density test for 24 h exposure assays on A549 cell line.

	Seeding Density (cells/mL)						
	20000	30000	40000	50000	60000	70000	80000
Absorbance (570 nm)	0.735 ±0.0576	0.967 ± 0.0156	1.130 ±0.0250	1.291 ±0.0516	1.406 ±0.141	1.478 ±0.132	1.437 ±0.171

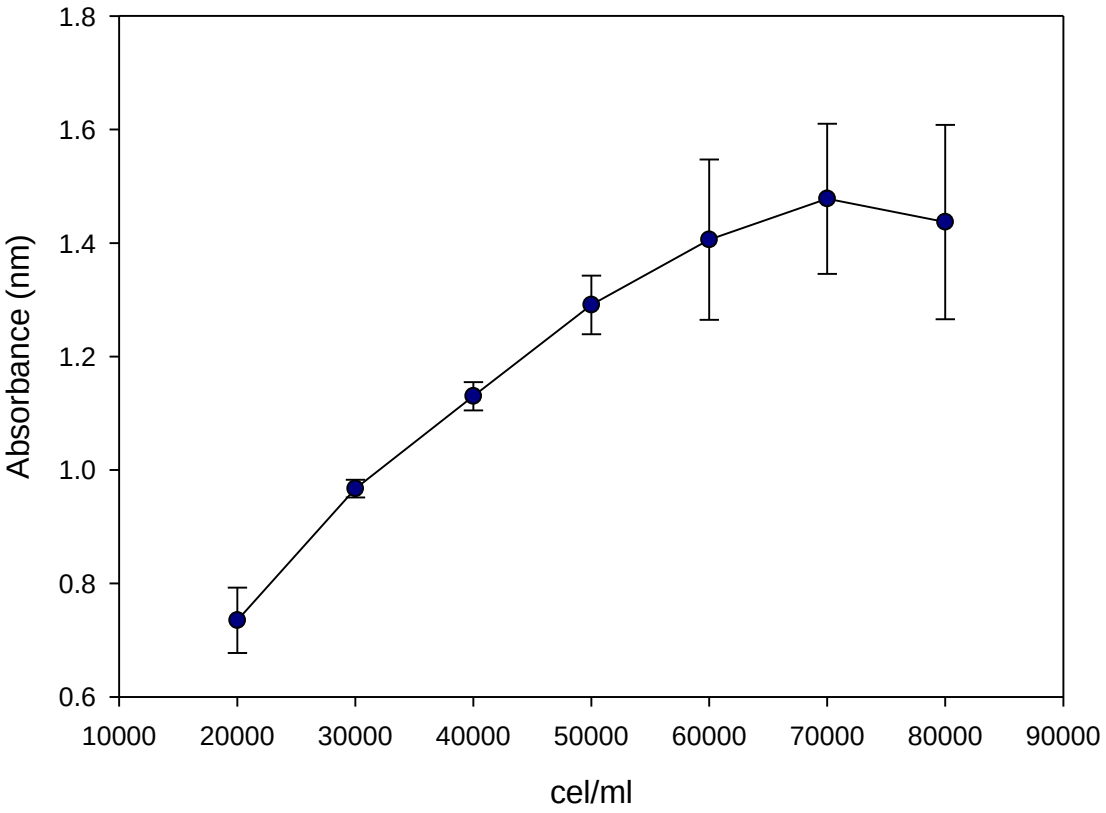


Figure 6- Seeding density test for 24 h exposure assays on A549 cell line. Results are presented with mean and standard deviations from three independent assays.

1.2 DMSO toxicity determination for 24 h exposure

The PM samples were diluted in DMSO to standardise their concentration. DMSO is toxic for cells, being tolerated by the A549 cell line at <1% (Achar et al., 2016). However, due to the low quantity of PM samples available, and to enable the maximisation of the quantity of assays performed, the DMSO quantity was

adjusted to 1.1%. As shown in Table 8 and Figure 7, A549 cell viability was very similar in cells exposed to 1.0% and 1.1% DMSO. Therefore, cells were exposed to a solution composed by medium with of 1.1% DMSO.

Table 8 - Effects on A549 cell line after 24 h exposure to DMSO percentages from 0% to 1.4%.

	DMSO %						
	0	0.9	1.0	1.1	1.2	1.3	1.4
Viability (%)	100 ±0.000	84.5 ± 2.264	85.2 ±1.030	85.3 ±1.511	82.5 ±4.897	81.9 ±2.629	79.3 ±4.294

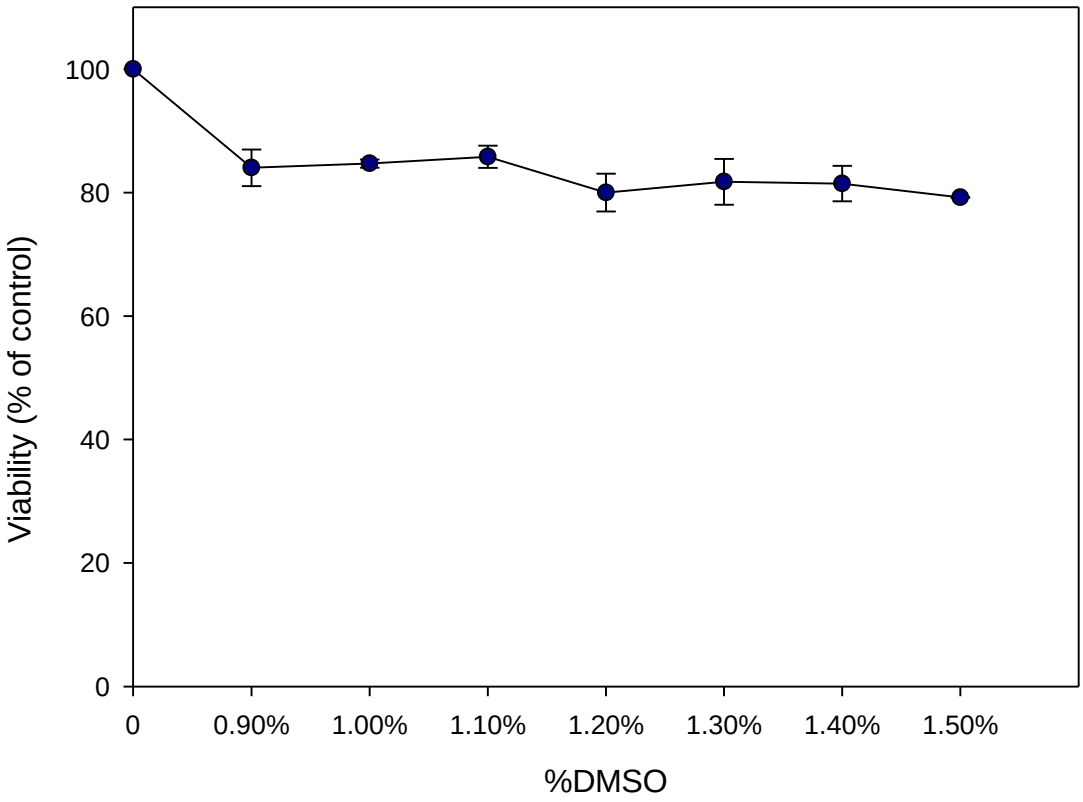


Figure 7 - Effects on A549 cell line after 24 h exposure to DMSO percentages from 0% to 1.4%. Results are presented with mean and standard deviations from three independent assays.

1.3 Cytotoxic effects after 24 h exposure to PM_{10}-bound PAH from brake wear

The A459 cells were exposed to several PAH samples that resulted from different brake materials in different test cycles. These samples presented multiple combinations and quantities of individual PAH as presented in Table 9.

Table 9 -PM_{10}-bound mass (ng) PAH from brake wear present in each exposure extract vial.

Molecular weight	Compounds	120-80 kph NAO1	200-170 kph NAO1	120-80 kph Low Steel 1	200-170 kph Low Steel1	120-80 kph High Steel	200-170 kph High Steel	120-80 kph NAO2	200-170 kph NAO2	120-80 kph Low Steel2	FADE Low Steel2
LMW	Benzothiazole	0.00	0.00	0.00	0.00	0.00	1.75	0.00	0.00	0.00	0.00
	Carbazole	0.00	0.00	0.29	0.18	0.15	0.38	0.00	0.33	0.00	0.30
	p-Terphenyl	0.00	0.00	0.00	0.08	0.00	0.08	0.00	0.11	0.08	0.08
	Retene	0.00	0.00	0.00	0.99	0.47	0.71	0.74	0.73	1.24	0.43
	Naphthalene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Acenaphthylene	0.00	0.00	0.00	0.06	0.13	0.00	0.00	0.11	0.00	0.00
	Acenaphthene	0.20	0.86	1.35	1.22	1.00	0.96	1.31	0.45	0.34	1.01
	Fluorene	1.96	7.66	14.13	8.28	9.63	0.00	12.26	5.98	6.70	8.84
	Phenanthrene	0.07	0.40	0.25	1.21	0.32	0.72	1.08	0.72	0.95	0.30
	Anthracene	0.00	0.00	0.00	0.19	0.07	0.00	0.00	0.00	0.17	0.00
HMW	Fluoranthene	0.07	0.46	0.27	0.57	0.18	0.28	0.43	0.25	0.47	0.30
	Pyrene	0.15	0.51	0.61	0.36	0.31	0.46	0.58	0.51	0.73	0.33
	Benzo[a]anthracene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.49	0.00	0.28
	Chrysene	0.00	0.00	0.00	0.10	0.12	0.08	0.28	0.16	0.22	0.00
	Benzo[b]fluoranthene	0.00	0.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Benzo[k]fluoranthene	0.00	1.23	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Benzo[e]pyrene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Benzo[a]pyrene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Perylene	0.00	0.00	0.00	0.00	0.00	1.55	0.00	1.70	0.00	0.00
	Indeno[1,2,3-cd]pyrene	0.00	1.59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Dibenzo[a,h]anthracene	0.00	1.51	0.00	0.00	0.00	0.00	0.00	0.13	0.22	0.00
	Benzo[g,h,i]perylene	0.00	1.98	0.17	0.00	0.00	0.00	0.00	0.14	0.00	0.00
	Other compounds	63.00	575.22	197.96	42.26	76.79	427.23	105.12	105.22	117.71	126.56

The PM_{10}-bound PAH from brake wear with the test cycles 120-80 kph NAO1, 120-80 kph NAO2, 200-170 kph NAO2, 120-80 kph low steel 1, FADE low steel 2 and 120-80kph low steel 2 steel showed to be more hazardous than the remaining samples. Even in lower concentrations, they caused more damage to the cells than the higher concentrated samples, as shown in Figure 8. PM_{10}-

bound PAH from 200-170 kph high steel brake pads also showed some toxicity, probably caused by the higher masses in the extracts of other compounds, where multiple plasticisers such as phthalates were included, together with the presence of benzothiazole.

The most toxic extract was that of wear particles from 120-80 kph low steel 2. When exposed to this PM_{10} sample, cell viability was reduced to 80.5%. Low steel materials have higher concentrations of Fe and Cu and abrasive materials. Because of these characteristics, the higher friction is coupled to higher brake wear and noise. The PAH extract from low steel 1 displayed a higher retene concentration and lead to a decrease in cell viability to 83.8%. The extract from 120-80 kph NAO1 caused a similar decrease. When exposed to the PM_{10} sample 120-80 kph NAO1 (collected from the NAO brake type, under the protocol with 8 braking events, 120–80 kph deceleration, temperature 100 ºC), A549 cell viability dropped to 83.1%. Although only a few PAH were detected in this sample, cytotoxicity may have been induced by unanalysed compounds, such as other polyaromatics, nitro-PAH, alkyl-PAH or oxy-PAH that are more toxic than their precursors. These constituents may derive from the brake pad material itself or from lubricating brake greases or oils, whose formulations is kept secret by the manufacturers.

Extracts from NAO2, collected under the protocol with 8 braking events, 120–80 kph deceleration, temperature 100 ºC (120-80 kph NAO 2) displayed a high amount of fluorene. Although the brake test cycle is not very aggressive, the PAH present in this sample led to a 12% reduction in cell viability.

PM_{10}-bound PAH extracts from low steel 2 wear, collected under the FADE test cycle, comprising 15 braking events, 100–5 kph deceleration, maximum temperature 550 °C (FADE low steel 2), led to a decrease in cell viability to 89.3%. Comparing to other extracts, this PM_{10} sample present low PAH masses. Probably, severe braking, high temperatures and pressures can cause degradation of these polyaromatic compounds. Although the extract from the brake 200-170 kph high steel has a higher PAH content than the other samples with statistically significant toxicity, this extract did not cause much damage to the cells. The 200-170 kph low steel 1 PM_{10} sample did not cause any toxicity for the highest concentration (400 ng/mL).

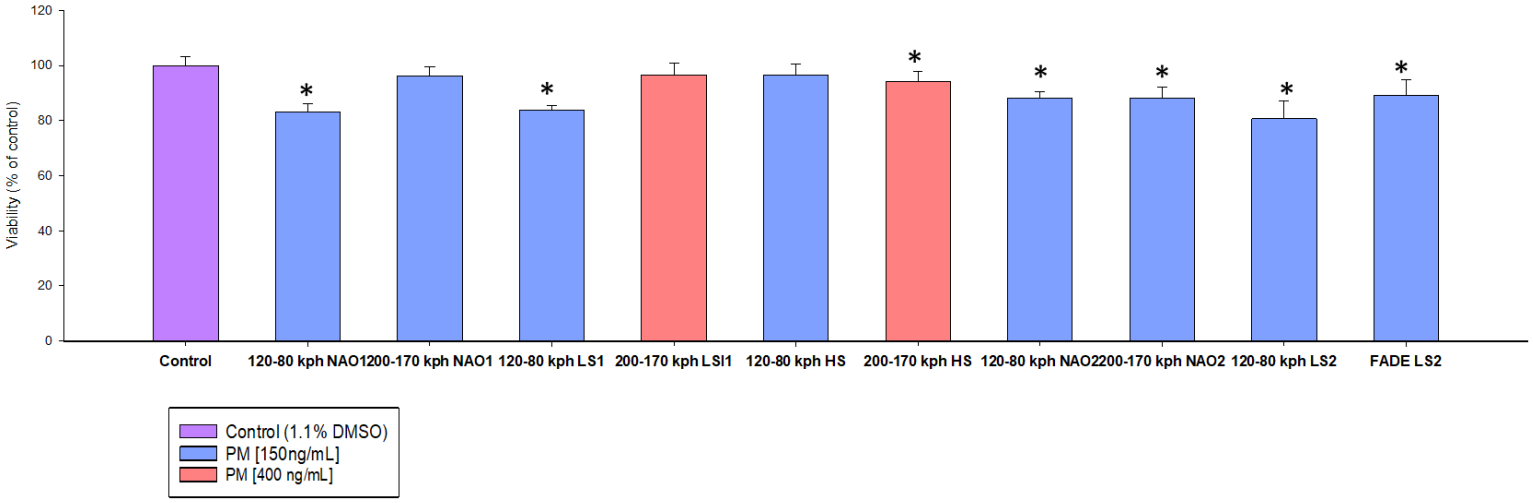


*Figure 8 –24 h exposure effects of PM_{10}-bound PAH from brake wear [150 and 400 ng/mL] on A549 cell viability. Results are presented as mean and standard deviations from three independent assays. * Indicates statistically significant results when compared to control 1.1% DMSO (p<0.05).*

1.4 Cytotoxic effects of 24 h exposure to PM-bound PAH from heavy-duty vehicle exhaust particles

Observing Table 10 and Figure 9 it is possible to relate PAH quantity to viability cell decrees. Regardless of the driving cycle, the only vehicle that caused a statistically significant reduction in cell viability was the 2013 Euro V. The fuel that proved to be the most harmful to the cells was the GTL. The PM sample collected from the exhaust of the GTL heavy-duty vehicle, tested under the WHVC cold start driving cycle, revealed itself the most toxic. Exposure to PAH derived from this PM sample lowered cell viability to 78.7%, while the extract from the exhaust PM from the same vehicle category, but powered by diesel, led to a decrease to 83.8%. The imposition of much tighter emission limits by the Euro VI standard appears to have resulted in a substantial reduction in PM and its toxicity.

Table 10 – Masses (ng) of PM_{10}-bound PAH from heavy duty vehicles present in each exposure extract vial.

Molecular weight	Compound	Euro V Diesel 1 VTT (1)	Euro V Diesel 1 VTT (2)	Euro V Diesel 1 VTT (3)	Euro V Diesel 1 WHVC cold (1)	Euro V Diesel 1 WHVC cold (2)	Euro V GTL WHVC cold	Euro VI Diesel 2 WHVC cold	Euro VI Diesel 2 WHVC hot (1)	Euro VI Diesel 2 WHVC hot (2)	Euro VI Diesel 2 WHVC hot (3)	Euro VI Diesel 2 WHVC hot (4)	Euro VI Diesel 2 Braunschweig hot (1)	Euro VI Diesel 2 Braunschweig hot (2)
LMW	Benzothiazole	5.82	2.14	1.29	4.01	0.38	4.31	0.64	0.36	0.47	0.30	11.48	6.67	1.04
	Carbazole	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.51	0.00	0.00
	p-Terphenyl	0.27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15
	Retene	3.51	0.71	0.47	0.55	1.25	1.60	0.70	0.61	0.00	1.08	0.43	0.00	1.35
	Naphthalene	11.41	0.00	1.73	0.00	1.10	10.68	0.00	0.00	0.00	0.93	0.00	5.80	0.00
	C2-naphthalene	5.44	2.39	0.00	0.00	0.97	1.89	2.59	2.95	0.40	0.89	0.00	1.04	0.00
	Acenaphthylene	0.60	0.00	0.00	0.11	0.00	0.30	0.00	0.00	0.00	0.00	0.21	0.00	0.09
	Acenaphthene	9.70	0.75	0.00	0.86	0.73	4.07	1.21	0.43	0.00	1.24	1.99	2.76	0.00
	Fluorene	33.80	12.06	8.16	0.00	13.10	37.02	19.93	8.31	0.00	16.95	16.06	29.42	8.86
	Phenanthrene	12.15	6.22	2.41	1.51	6.64	9.67	0.78	0.77	0.00	0.88	0.42	0.32	0.88
	C1-phenanthrene	15.25	7.29	2.40	0.00	9.70	8.57	0.97	1.21	0.00	1.30	0.00	0.00	0.00
	Anthracene	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.26
HMW	Fluoranthene	2.10	0.71	0.32	0.28	0.68	0.50	0.35	0.64	0.00	0.61	0.41	0.31	0.31
	Pyrene	1.26	0.78	0.47	0.26	0.71	0.49	0.24	0.50	0.00	0.49	0.58	0.31	0.59
	C1-pyrene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Benzo[a]anthracene	5.13	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Chrysene	0.94	0.14	0.00	0.00	0.00	0.00	0.25	0.67	0.00	0.44	0.19	0.23	0.00
	Benzo[b]fluoranthene	9.66	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	7,12-Dimethylbenz[a]anthracene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Benzo[k]fluoranthene	6.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Benzo[e]pyrene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Benzo[a]pyrene	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.59
	Perylene	0.00	0.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Indeno[1,2,3-cd]pyrene	20.45	0.00	0.00	15.35	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.07
	Dibenzo[a,h] anthracene	16.67	0.00	0.00	20.97	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Benzo[g,h,i] perylene	25.70	0.00	0.00	21.31	0.54	0.00	0.00	0.00	0.00	0.00	0.16	0.00	0.15
	Other Compounds	2512.73	174.58	2028.48	229.41	387.50	2129.11	468.50	116.15	63.03	1031.65	78.75	218.71	749.96

The PM samples from Euro VI vehicles did not show significant toxicity levels, independently from the test cycle.

Although the extract of the sample from the Euro V diesel powered vehicle, driven according to the VTT cycle, presented a greater variability of PAH, the GTL Euro V vehicle under the WHVC cycle with cold start showed the highest particulate mass fractions of fluorene. Mammalian toxicity tests have shown that inhalation of GTL substances induce lung damage (Boogaard et al., 2017).

Although the PM sample from the Euro V diesel 1 vehicle under the WHVC cold start (1) cycle showed a significant toxicity rate, which did not occur with the extract of the replicate sample (Euro V diesel 1 vehicle under the WHVC cold start (2)), which did not present significant cytotoxicity. This may be due to a higher content of heavy molecular weight PAH and benzothiazole in the PM extract from cold start (1).

In addition to fuels, lubricating oils are also a significant source of PAH emissions (Brandenberger et al., 2005). It should be also noted that the organic extracts may contain other PAH congeners that were not quantified. PAH analyses showed differences related to acenaphthylene, indeno[1,2,3-cd] pyrene, dibenzo[a,h]anthracene and benzo[g,h,i]perylene. These last three PAH were mostly present in the exhausts from the Euro V diesel 1 vehicle under the VTT cycle (1) and from the same vehicle and fuel under the WHVC cold start (1). Although the replicate samples of VTT driving cycle for the Euro V Diesel 1 vehicle showed very similar levels of toxicity, their PAH composition is not similar. The particulate extract from the Euro V diesel 1 (1) vehicle has a much higher PAH content that explains why it is the most toxic of the three replicates. The higher PAH amounts of the first replicate may be associated with the fact that this test was more representative of cold start conditions than the following ones.

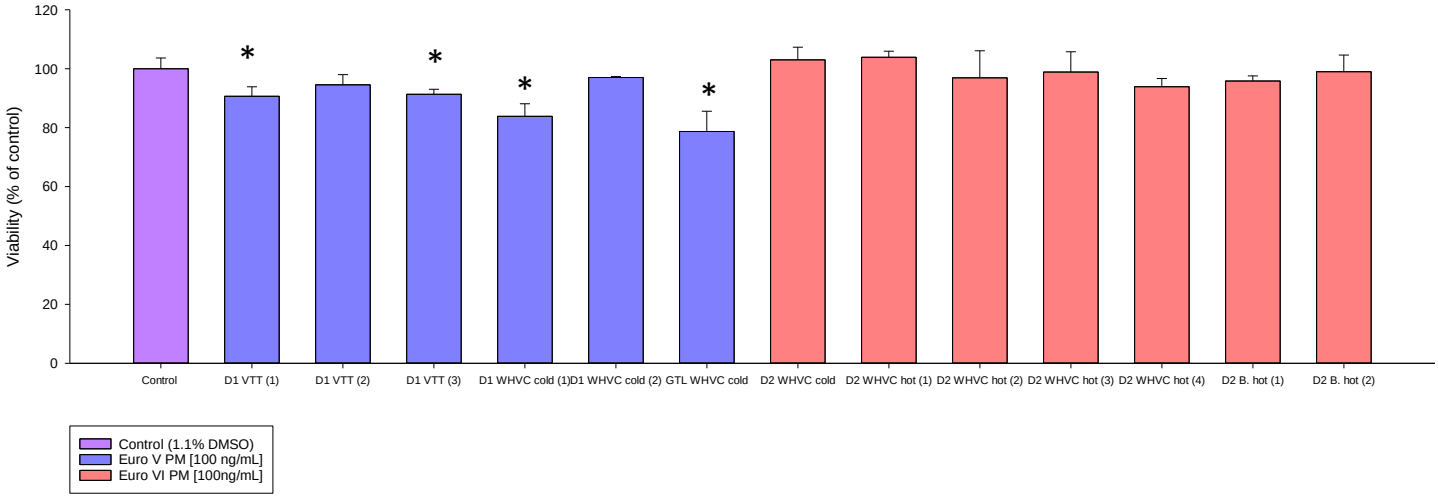


*Figure 9 - Effects of 24 h exposure to PM-bound PAH from the exhaust of heavy-duty vehicles [100 ng/mL] on A549 cell viability. Results are presented as mean and standard deviations from two independent assays. * Indicates statistically significant results when compared to control (p<0.05).*

1.5 Cytotoxic effects of 24 h exposure to $PM_{2.5}$-bound PAH collected in the Tunnel João XXI and urban background air

The A459 cells were exposed to several PAH extracts from $PM_{2.5}$ samples collected over a week simultaneously in the road Tunnel João XXI and at School Valsassina, the latter as representative of the urban background atmosphere. The PAH from Tunnel João XXI collected on Thursday, Friday, Saturday, Sunday, and from the school are more hazardous than the remaining samples, as shown in Figure 10. It should be borne in mind that the background air sample represents a composite extract resulting from the combination of all samples from the week. The particulate extract from the school showed to be extremely toxic. A549 exposure to this extract lowered cell viability to 2.66%, that is, contrary to what was expected. Because of the low individual concentration from every weekday sample, it was not possible to test the extracts individually, so samples from the seven weekdays were combined into one general background sample with a 150 ng/mL final concentration (same concentration of the samples collected in Tunnel João XXI), allowing the dilution in 1.1% DMSO to provide enough sample volume to perform the assays necessary for the study. By evaluating the PAH composition, it was found that retene was present in the background school

sample but not in any of the extracts collected from the Tunnel João XXI (Table 11). Retene forms from wood combustion through the biotransformation of a conifer resin (dehydroabietic acid) (Ramdahl, 1983). Retene is a biomass burning marker and is commonly used as a tracer of wood combustion, especially conifers. It has been shown that retene contributes to genotoxic effects and cell death caused by airborne PM. However, few studies have demonstrated the toxic effects of retene in specific organisms (Peixoto et al., 2019). De Oliveira Alves et al. (2017), in a study carried out in an Amazon region impacted by biomass burning, documented that exposing human lung cells to PM_{10} significantly increased the level of ROS, inflammatory cytokines, autophagy, and DNA damage. Continued PM_{10} exposure activated apoptosis and necrosis. Interestingly, retene was pointed out as the major responsible for the effects of PM_{10}, causing DNA damage and cell death. So, the presence of retene probably contributes to the high toxicity of the extract collected from the school as background air sample.

Naphthalene was also found in the background extract in high concentrations, higher than in any sample collected from Tunnel João XXI. Most airborne emissions result from combustion, and key sources include industry, open burning, tailpipe emissions, and cigarettes. The second largest source is off-gassing, specifically from naphthalene's use as a deodorizer, repellent and fumigant (Jia & Batterman, 2010). Naphthalene is the most volatile PAH. This PAH is a great environmental and health concern, because of this compound carcinogenic effects. Naphthalene is, therefore, assumed to be a substance that considerably contributes to human cancer development (Preuss et al., 2003). Near the School Valsassina exists a gas station. Depending on wind direction, PM from that gas station can be transported towards the school. Other possible sources are biomass burning for heating and charcoal grilling in restaurants nearby.

Table 11 – $PM_{2.5}$-bound PAH mass (ng) from Tunnel João XXI and the nearby School Valsassina in Lisbon present in each exposure extract vial.

Molecular weight	Compounds	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday	Valsassina School (BCK)
LMW	Benzothiazole	9.4	6.0	4.5	7.0	0.0	0.0	7.3	40.0
	Carbazole	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	p-Terphenyl	4.1	3.7	3.2	4.5	4.5	4.4	3.8	5.5
	Retene	0.0	0.0	0.0	0.0	0.0	0.0	0.0	23.3
	Naphthalene	12.1	4.8	0.0	0.0	0.0	66.1	8.4	293.9
	Acenaphthylene	0.0	0.0	5.5	0.0	8.3	0.0	0.0	0.0
	Acenaphthene	0.0	7.1	10.1	11.6	0.0	0.0	0.0	72.3
	Fluorene	8.3	6.0	3.5	7.3	6.9	6.2	5.3	16.9
	Phenanthrene	247.6	185.1	158.6	202.0	200.8	204.2	163.4	83.3
	Anthracene	41.5	35.2	29.1	36.5	36.8	36.9	29.7	28.9
HMW	Fluoranthene	447.6	333.0	321.3	413.0	392.6	379.2	299.9	47.5
	Pyrene	911.5	661.6	638.5	802.2	789.8	729.9	558.2	65.1
	Benzo[a]anthracene	230.2	231.0	189.7	343.8	273.6	278.3	172.2	71.4
	Chrysene	276.9	273.2	235.7	384.4	318.1	322.5	230.3	152.3
	Benzo[b]fluoranthene	14.1	14.0	10.6	23.1	17.0	18.7	11.6	5.8
	Benzo[k]fluoranthene	12.4	12.0	9.6	21.9	16.6	17.3	10.2	4.9
	Benzo[e]pyrene	14.3	15.1	12.6	23.7	17.8	18.7	11.9	7.7
	Benzo[a]pyrene	10.7	11.7	9.1	22.5	13.2	15.3	10.6	14.2
	Perylene	147.8	163.5	130.6	284.7	176.2	209.6	86.5	83.5
	Indeno[1,2,3-cd]pyrene	70.3	104.6	86.5	239.6	102.5	152.4	42.1	171.5
	Dibenzo[a,h]anthracene	8.1	12.8	10.4	19.9	11.6	18.8	6.8	25.5
	Benzo[g,h,i]perylene	192.1	323.4	258.2	511.7	242.2	295.3	106.1	229.9
LMW	C1-naphthalenes	45.8	22.6	4.6	24.3	24.5	72.8	34.4	487.9
	C3-naphthalenes	302.6	196.8	192.3	211.4	171.4	177.3	175.9	292.0
	C2-fluorenes	60.4	65.0	63.1	59.9	86.5	60.6	45.1	0.0
	C1-phenanthrenes/Anthacenes	290.3	244.0	221.9	234.6	255.4	246.9	216.5	95.5
	C3-fluorenes	13.4	45.4	40.1	32.3	37.6	21.2	18.5	0.0
	C2-phenanthrenes/Anthacenes	432.3	304.6	290.6	323.5	366.8	317.6	254.8	47.0
	C3-phenanthrenes/Anthacenes	197.8	142.4	132.9	146.4	170.1	146.7	116.7	14.1
	C1-fluoranthenes/pyrenes	506.3	382.8	353.3	451.2	466.0	402.8	282.5	25.9
	Other compounds	9 550.2	8 288.7	7 891.5	8 946.7	8 942.0	9 758.2	9 441.7	52 658.9

From the samples collected in the tunnel, the one collected on Friday showed to be the most toxic to the A549 cells, leaving their viability in 83.6% after 24 h exposure. This result is likely associated with a higher traffic intensity on this day. The toxicity registered for the Saturday sample can be explained by the higher amount of naphthalene present in the extract. The extract from Thursday's sample presented the highest PAH content, but the amounts of some individual

compounds were lower than those observed in the Friday extract, which seems to be enough to see a small difference in their toxicity rate, although it is very similar.

The remaining $PM_{2.5}$ tunnel samples did not show statistically significant toxicity levels.

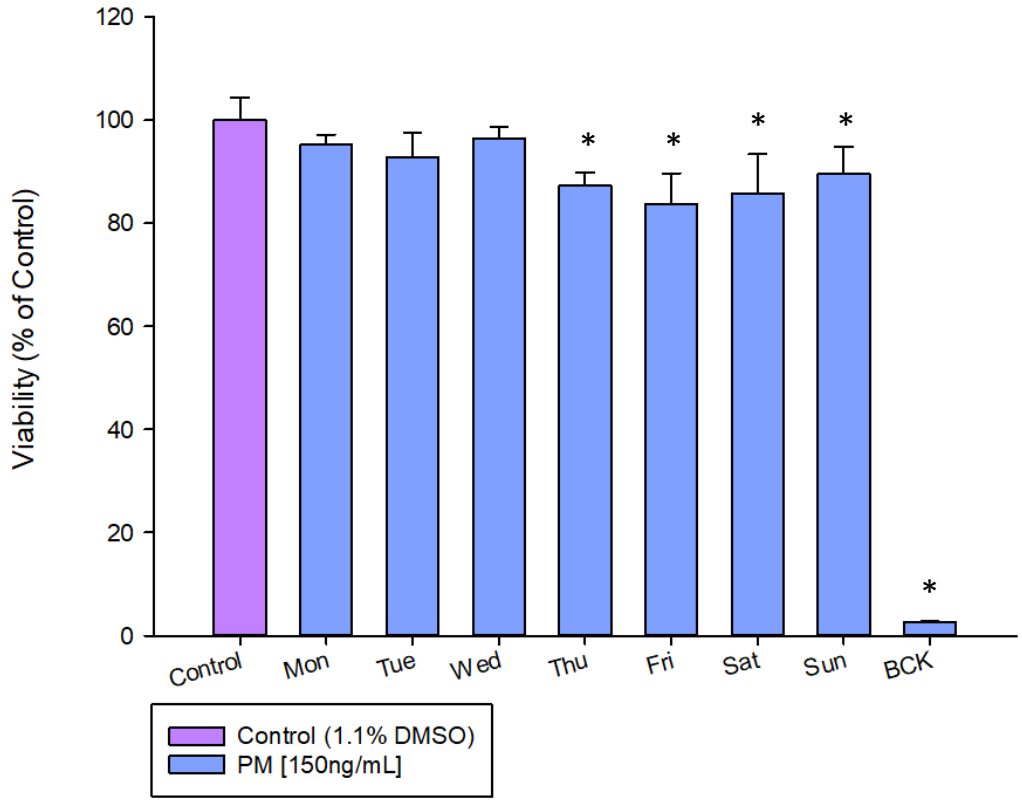


*Figure 10 – Effects of 24h exposure to PAH-bound $PM_{2.5}$ collected from Tunnel João XXI in Lisbon samples [150 ng/mL] 24 hours exposure effects on A549 Cell viability. Results are presented as mean and standard deviations from two independent assays. * Indicates statistically significant results when compared to control ($p<0.05$).*

2. Cell cycle evaluation by flow cytometry

2.1 Effects of 24 h exposure to PAH from PM_{10} samples from brake wear on A549 cell cycle

To study the effects of exposure to PM_{10} samples from brake wear on the cell cycle dynamics of A549 cell line, the cells were exposed to different extracts, at different concentrations (150 ng/mL and 400 ng/mL) and incubated for 24 h at

37ºC and 5% CO_2. Although there were some changes in the A549 cell cycle after 24 h exposure in all the phases, the differences in the mean values between groups were not great enough to exclude the possibility that the difference is due to random variability. The toxicity results from the brake wear extracts cannot be explained by the A549 cell cycle response to these samples (Figure 11).

PAH can be turned into DNA reactive intermediates. Some of these intermediates are known to form stable DNA adducts, this formation will depend on these compounds escape from the particles in a biological environment. To prevent mutagenesis, when the DNA is damaged, processes like cell cycle arrest and induction of apoptosis are normally initiated (Schins & Knaapen, 2007).

PAH at this concentration levels (ng/mL) dis not induce cell cycle arrest, so it is safe to assume that there were no DNS-adduct formations, or the ones that were formed were repaired without the need to induce cell cycle arrest.

The ideal would be to preform tests with higher PAH concentration over longer exposition times.

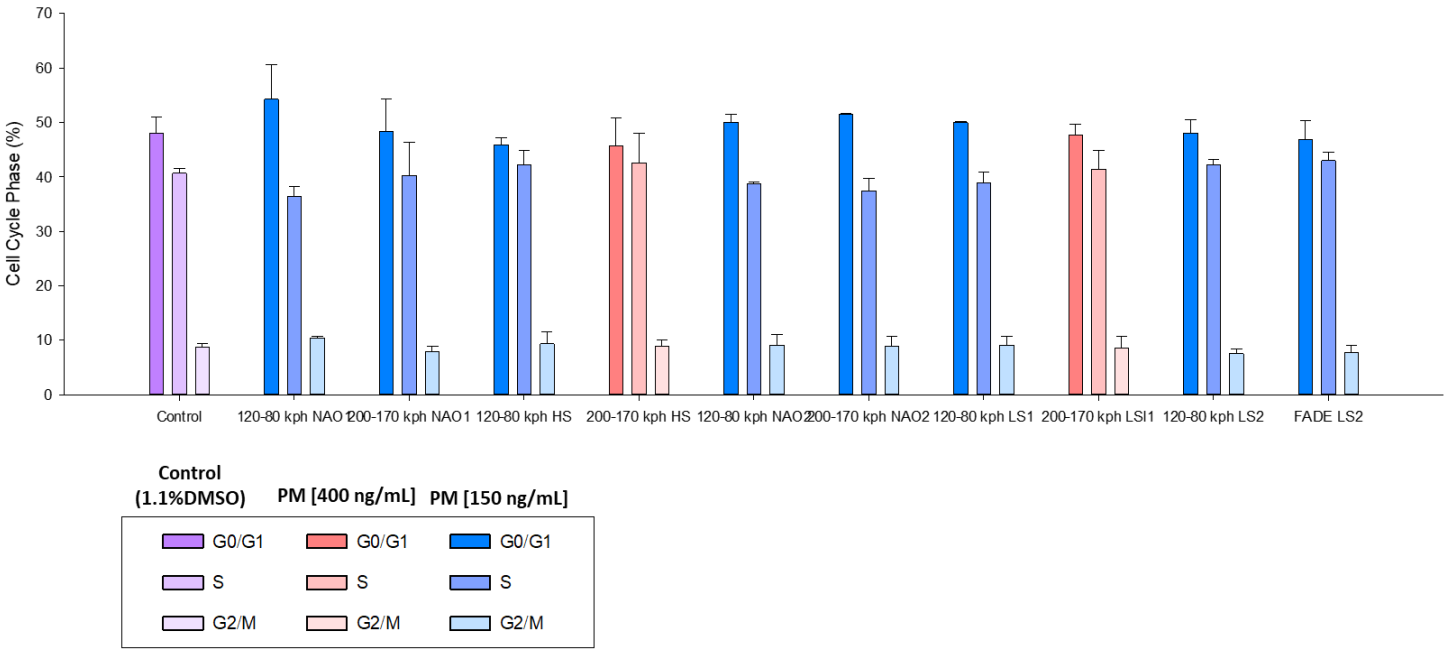


Figure 11-A549 cell cycle profile alterations induced after 24 h exposure to PAH from PM_{10} wear. Results are presented with mean and standard deviations from two independent assays.

2.2 Effects of 24 hours exposure to PAH from PM10 exhaust samples from heavy-duty vehicles on A549 cell cycle

To investigate the possible mechanisms associated with the reduction effect on cell viability, the alterations caused by the treatments on cell cycle dynamics were analyzed. Cells were exposed to extracts of PM_{10} exhaust samples from heavy duty vehicles at a concentration of 100 ng/mL collected from different vehicles and incubated for 24 hours at 37 ºC and 5% CO_2. The A549 cell cycle suffered no significant changes upon exposure to exhaust extracts for 24 h.

As cell cycle arrest is induced to diminish the consequences of damaged DNA, this set of PM-bound PAH extracts, at this concentration was not harmful enough to activate that cell response (Schins & Knaapen, 2007). The toxicity results from the exposure to the PAH extracts from the exhaust of heavy-duty vehicles cannot be explained by the A549 cell cycle response. (Figure 12).

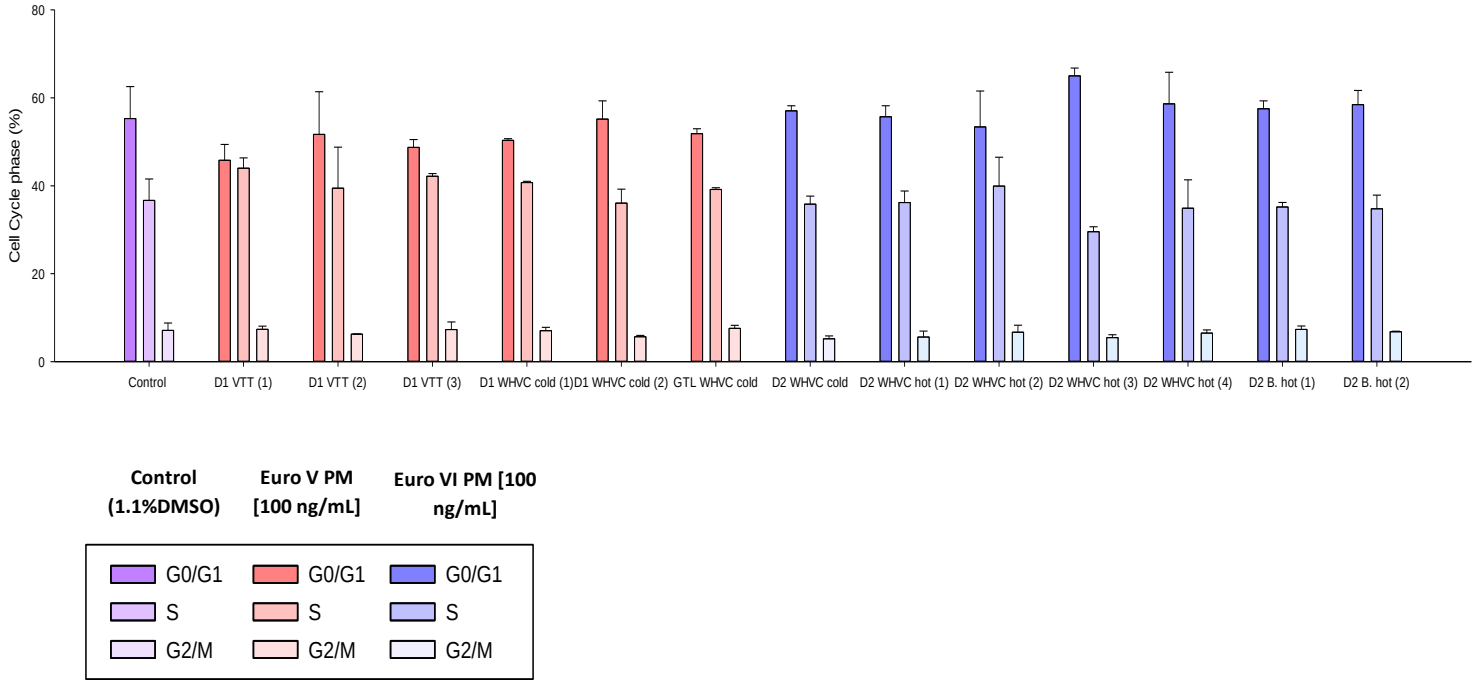


Figure 12- A549 cell cycle profile alterations induced after 24 h exposure to PAH from exhaust PM samples from heavy-duty vehicles. Results are presented as mean and standard deviations from two independent assays.

2.3 Effects of 24 h exposure to PAH from $PM_{2.5}$ samples collected in Tunnel João XXI in Lisbon on A549 cell cycle

To evaluate the effects of exposure to $PM_{2.5,}$ samples collected from Tunnel João XXI in Lisbon on human adenocarcinoma alveolar epithelial cell line (A549) cycle, the cells were exposed to extracts with a concentration of 150 ng/mL and incubated for 24 h at 37 ºC and 5% CO_2 (Figure 13).

Although some changes could be detected in the A549 cell cycle after 24 h exposure to the extracts in all the phases, especially in phase S, it could be observed that, comparing to the control, cells exposed to the tunnel samples showed a trend for higher percentage of cells at S phase, not reaching, however, statistical significance, while G0/G1 phase oscillated depending on the day of the week. G2/M phase showed similar results to control in all days, differing more on Monday.

The toxicity results from the exposure to PAH present in Tunnel João XXI PM extracts cannot be explained by the A549 cell cycle response.

Unfortunately, the background air extract killed most of the cells (around 98%), making impossible for the cytometer to evaluate DNA quantity with near zero events.

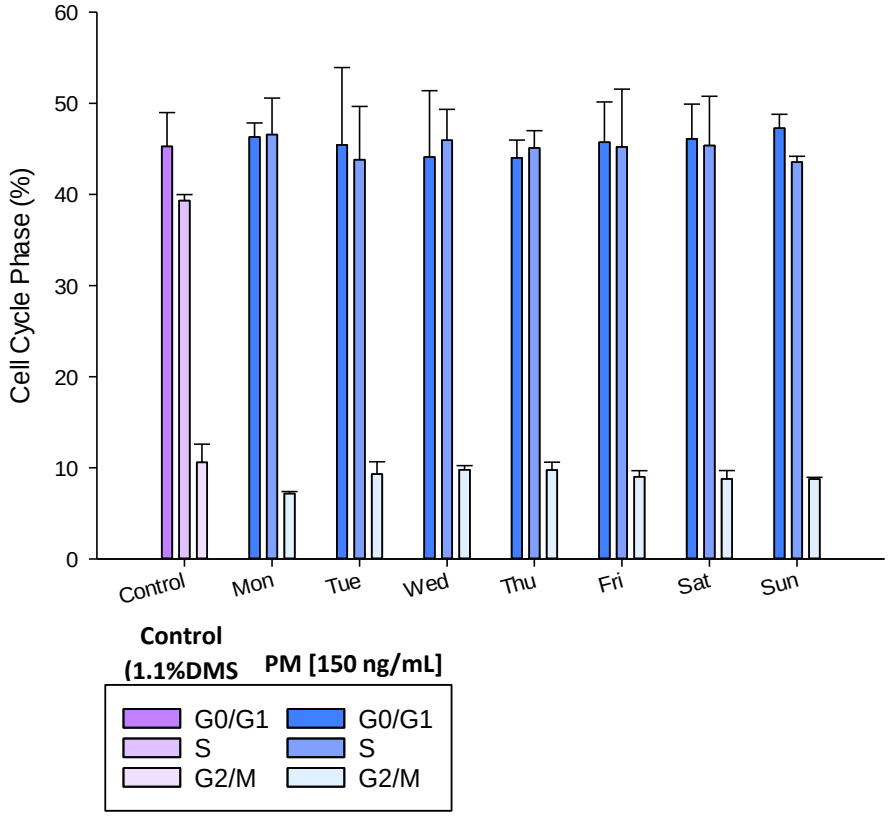


Figure 13 - A549 cell cycle profile alterations induced after 24 h exposure to PAH from $PM_{2.5}$ samples collected in João XXI Tunnel in Lisbon. Results are presented as mean and standard deviations from two independent assays.

3. Reactive Oxygen Species (ROS) Assay

3.1 Effects of 24 h exposure to PAH from PM_{10} samples from brake wear on A549 ROS intracellular level

Although it would be convenient to test extracts from the samples that showed the greatest decrease in cell viability, this was not possible due to the limited amount of sample. Unfortunately, it was only possible to test two samples, one from low steel, and another from high steel pads. Exposure to both samples did not increase the intracellular ROS levels (Figure 14), which is coincident with the MTT toxicity results; although 200-170 kph affected more cell viability it was not

a drastic difference. A small reduction is detectable in both samples, but it did not reach statistical significance.

The importance of ROS in particle genotoxicity is generated by the fact that these oxidants are implicated in the oxidative attack of DNA, that origins base-pair mutations, deletions, or insertions (Schins & Knaapen, 2007). As reported in cell cycle analyses, PAH did not cause cell cycle arrest, so there was no need to stop possible DNA damage, as these results match the ones observed in cell cycle analyses.

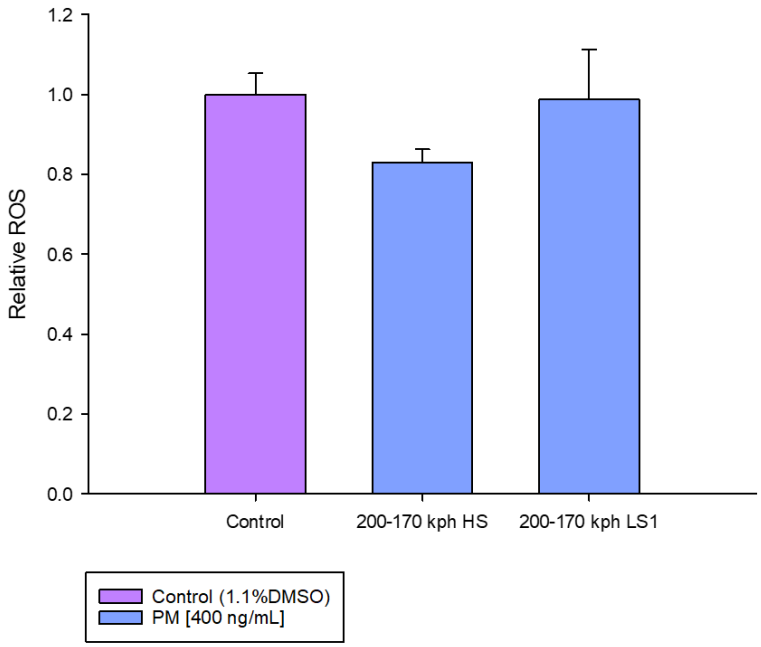


Figure 14- Alteration of intracellular ROS levels in A549 cells after 24 h exposure to e PM_{10}-bound PAH extracts from high steel and low steel brake wear. Results are presented as mean and standard deviations from two independent assays.

3.2 Effects of 24 h exposure to PAH from PM exhaust samples from heavy-duty vehicles on A549 ROS intracellular level

Unfortunately, it was not possible to test two PM extracts from this sample set due to their low volume: Euro V Diesel 1 VTT (3) and Euro V GTL WHVC cold. From the results presented in Figure 15, it is possible to see that exposure to most PAH extracts from the exhaust of heavy-duty vehicles increased the

intracellular ROS levels in cells exposed to three extracts, Euro V Diesel 1 VHVC cold (1) and Euro VI Diesel 2 VHVC cold. Increase in intracellular ROS levels may lead to oxidative stress that may cause damage to DNA, proteins and lipids leading to apoptosis (Zuliani et al., 2005). In the case of Euro V Diesel 1 VHVC cold (1) extract, the increase in ROS levels may have induced cell death, as seen by the decrease in cell viability observed for this sample. For the Euro VI Diesel 2 VHVC, in spite of an increase in the levels of ROS observed for this sample, a decrease in cell viability was not observed. Furthermore, in both samples the potential DNA damage induced by the increase in ROS levels was not reflected in alterations in the cell cycle dynamics of A549 cells for the 24 h exposure period.

The ideal would be to perform more tests with these samples and A549 cells, to assess membrane integrity changes, and metabolites production and release.

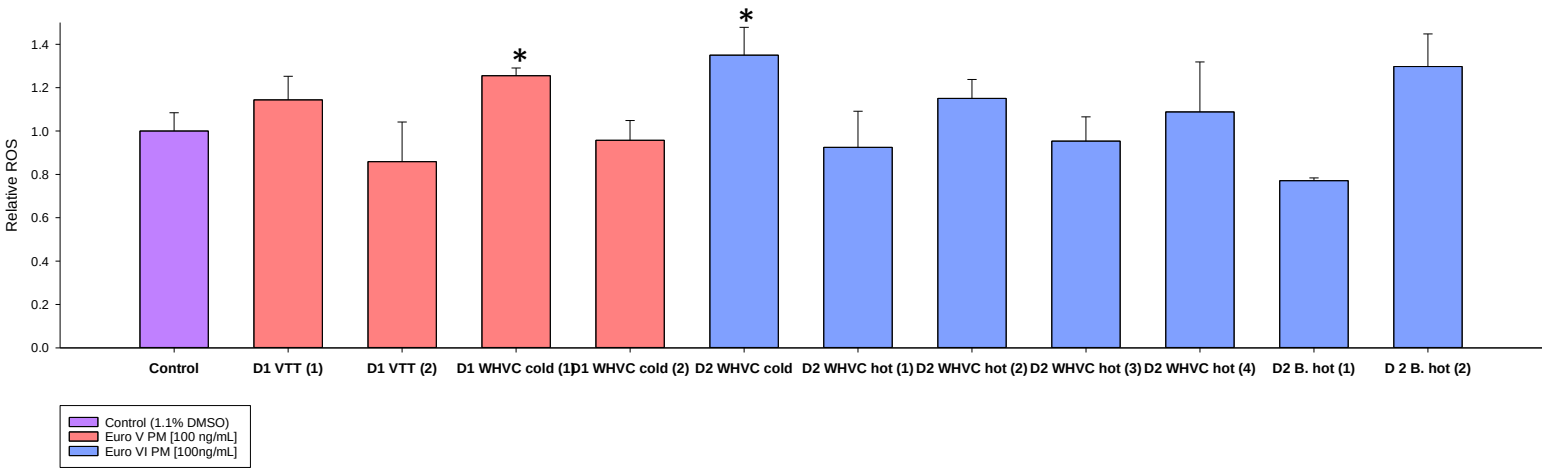


*Figure 15- Alteration of ROS levels in A549 cells after 24 h exposure to PM-bound PAH extracts from the exhaust of heavy-duty vehicles. Results are presented as mean and standard deviations from two independent assays. * Indicates statistically significant results when compared to control (p<0.05).*

3.3 Effects of 24 h exposure to PAH from PM exhaust samples from heavy-duty vehicles on A549 ROS intracellular level

From the results presented in Figure 16, it is possible to see that no extract collected in Tunnel João XXI in Lisbon caused a significant increase in A549 ROS production. Thursday sample showed the lowest ROS levels in comparison to the

other days of the week although this extract did not present statistical difference (p=0.469) comparatively to the control

Unfortunately, the background air extract did not have enough volume for the ROS detection test.

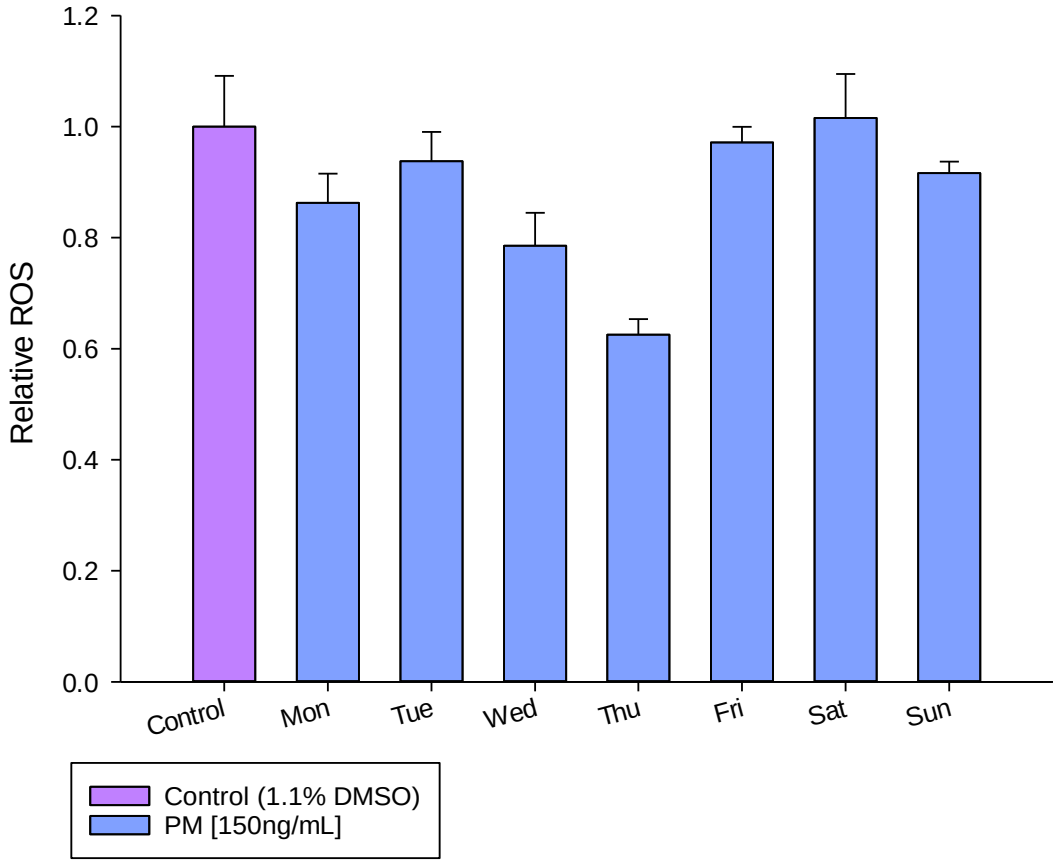


Figure 16- Alteration of intracellular Reactive Oxygen Species (ROS) levels in A549 cells after 24 hours exposure to the PAH extracts from $PM_{2.5}$ collected in João XXI Tunnel in Lisbon. Results are presented as mean and standard deviations from two independent assays.

Conclusions

The cytotoxic effects of several PAH present in PM_{10} from brake wear under different braking cycles, PM exhaust samples collected from heavy-duty vehicles powered by different fuels, also subject to various driving cycles, and $PM_{2.5}$ collected from the road Tunnel João XXI in Lisbon were assessed.

Some extracts from all sets of samples affected A549 cell viability. These results can be influenced by variation in the sample concentration and individual components present in each extract, in particular PAH. Particulate extracts from brake with higher concentration did not cause any significant viability damage in the cells. This implies that concentration alone is not enough to affect the cells, and that several factors must be in action. From the non-exhaust PM_{10} samples from brake wear, it was observed that low steel type brakes, whose emissions contain high concentrations of fluorene, influence the cytotoxic capability of the extract towards human adenocarcinoma alveolar epithelial cells. It can also be concluded that a mixed PAH extract can be more hazardous than a single PAH in high concentrations, although when combined with other factors like high braking temperatures and easy deterioration brake material, the result can be equally damaging.

Regarding the exhaust PM from heavy-duty vehicles, it was noticed that Euro V vehicles produce more toxic extracts, probably because Euro VI adopted a limit of PM emission that is ten times lower than the levels allowed in 2000. Among the PM-bound extracts from Euro V, the ones tested under the WHVC with cold start conditions were the most toxic. The particulate extract from the Euro V vehicle driven over this cycle and fuelled with GTL was the most toxic to human adenocarcinoma alveolar epithelial cells.

The $PM_{2.5}$ from the school representing the urban background atmosphere, collected in parallel with the road tunnel samples, showed to be extremely toxic, the opposite of what is expected from a background sample. A549 exposure to this extract lowered cell viability to 2.66%. The PAH composition of this sample revealed that retene was present in the $PM_{2.5}$ but not in any of the extracts collected from the Tunnel João XXI. Naphthalene was detected in higher concentration in samples from the school compared to those from the tunnel. The presence of retene and naphthalene may have contributed to the increased toxicity of the $PM_{2.5}$ extract from the “background” air of the school. It is unexpected that this two specific PAH appear more in extracts collected from a school than extracts collected from Tunnel João XXI. Inside the tunnel, the only pollution source is traffic, while the school area can be affected by multiple emissions, for example a close gas station and grill charcoal restaurants. From

the samples collected in the tunnel, the one collected on Friday showed to be the more toxic to the A549 cells, leaving their viability in 83.6% after 24 h exposure. Friday is the day of the week with a higher volume of traffic.

The second assay performed was cell cycle evaluation. Although some changes were observed in the A549 cell cycle after 24 h exposure in all the phases, the differences in the mean values between groups of samples were not great enough to exclude the possibility that the difference is due to random variability. When the results from the three phases (G0/G1, S and G2/M) from the cells exposed to all PM extracts (PM_{10} from brake wear, PM from heavy-duty vehicles and $PM_{2.5}$ from Tunnel João XXI) were compared to control (1.1% DMSO) no statistically significant difference was detected.

Regarding ROS intracellular production, exposure to brake wear PM samples did not increase the intracellular ROS levels.

Exposure to PM-bound PAH from heavy-duty vehicles increase the intracellular ROS levels. ROS levels detected in cells exposed to Euro V Diesel 1 VHVC cold (1) extract support the results obtained on the viability assay, ROS levels detected justifies the decrease in the viability. The ideal would be to perform more tests with these samples and A549 cells, to assess membrane integrity changes, and metabolites production and release.

In the case of A549 exposure to $PM_{2.5}$ from Tunnel João XXI, the sample collected on Saturday showed the higher ROS value, but not great enough to be considered by the statistics program.

Although the assays performed in this dissertation granted a higher understanding on how PAH affect A549 cells, more tests should be performed. Larger exposure and higher PAH concentrations should be experimented. Membrane integrity should be tested, and metabolite cell release should be analysed to support these assays and get more accurate and conclusive information.

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