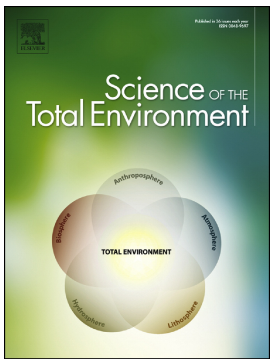

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In vitro toxicity of indoor and outdoor PM_{10} from residential wood combustion

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Abstract

Particulate matter with aerodynamic diameter < 10 μm (PM_{10}) was collected, indoors and outdoors, when wood burning appliances (open fireplace and woodstove) were in operation. The PM_{10} ecotoxicity was assessed with the *Vibrio fischeri* bioluminescence inhibition assay, while the cytotoxicity was evaluated by the WST-8 and lactate dehydrogenase (LDH) release assays using A549 cells. Extracts of PM_{10}-bound polycyclic aromatic hydrocarbons (PAH) were tested for their mutagenicity through the TA98 and TA100 Ames test. The bioluminescent inhibition assay revealed that indoor particles released from the fireplace were the most toxic. Indoors, the reduction in A549 cell metabolic activity was over two times higher for the fireplace in comparison with the woodstove (32 ± 3.2% and 72 ± 7.6% at the highest dose, respectively). Indoor particles from the fireplace were found to induce greater cytotoxicity than the corresponding outdoor samples. Combined WST-8 and LDH results suggest that PM_{10} exposure induce apoptotic cell death pathway in which the cell membrane integrity is maintained. Indoor and outdoor samples lacked direct and indirect mutagenic activity in any of the tester strains. For indoor-generated PM_{10}, organic carbon and PAH were significantly correlated with cell viability and bioluminescence reduction, suggesting a role of organic compounds in toxicity.

Keywords: Bioluminescence inhibition, Cytotoxicity, Mutagenicity, Particulate matter, Residential wood combustion

1. Introduction

Over the years, a wealth of publications has focused on the quantification and characterisation of particulate matter (PM) emissions arising from residential biomass combustion (Vicente and Alves, 2018). PM has been a focus of special research attention because adverse health outcomes associated with exposure to this pollutant have been observed in epidemiological studies (Atkinson et al., 2015; Dockery, 2009; Pope, 2000). Particularly, exposure to wood smoke has been linked to a vast array of adverse health effects (Naeher et al., 2007; Sigsgaard et

al., 2015; Zelikoff et al., 2002). Naeher et al. (2007) reviewed epidemiological observations associating the use of woodstoves and fireplaces and respiratory symptoms (e.g. congestion, lung function decrement, bronchiolitis or pneumonia) in children and in women living in non-smoking households. While epidemiological studies reveal associations between health outcomes and exposure to PM, *in vitro* and *in vivo* models are useful to study the mechanisms involved in the PM-related health effects (Cho et al., 2018; Nemmar et al., 2013; Schlesinger et al., 2006).

The toxicity assessment of biomass burning particles produced under controlled laboratory conditions (source characterisation studies) has been conducted *in vivo* with rodents (e.g. Danielsen et al., 2010; Uski et al., 2012) and *in vitro* using different cell lines, such as epithelial cells of the respiratory tract and alveolar macrophages (Danielsen et al., 2009; Dilger et al., 2016; Kocbach et al., 2008; Totlandsdal et al., 2018), as well as different bacterial strains (Canha et al., 2016; Turóczi et al., 2012; Vu et al., 2012). Many studies underlined that the type of combustion appliance has an important role on the toxicological effects of PM emissions (Canha et al., 2016; Corsini et al., 2017; Jalava et al., 2012; Tapanainen et al., 2011; Vu et al., 2012). Additionally, the fuel burned (Arfi et al., 2017; Canha et al., 2016; Corsini et al., 2017; Kasurinen et al., 2017; Vu et al., 2012) and the combustion conditions (Canha et al., 2016; Jalava et al., 2010; Uski et al., 2014; Vu et al., 2012) were also investigated.

In addition to being a recognised major source of ambient PM (Vicente and Alves, 2018, and references therein), residential biomass burning has also a noticeable impact on indoor air quality (Castro et al., 2018; de Gennaro et al., 2015; Guo et al., 2008; McNamara et al., 2013; Salthammer et al., 2014; Vicente et al., 2020). Furthermore, people spend most of their time in indoor environments (e.g. Brasche and Bischof, 2005; Schweizer et al., 2007), meaning that it is where most of human exposure occurs (Morawska et al., 2013). Despite its importance, the *in vitro* toxicity of indoor particles arising from the use of biomass combustion appliances has been less studied. Marchetti et al. (2019) investigated the toxicological properties of indoor PM_{10} from an open fireplace fuelled with different biomasses (pellets, charcoal and wood). The authors performed *in vitro* assays using human lung cells (A549) and reported that the effects on

the biological endpoints were strongly related to the biomass fuel burned, which generated particles with distinct chemical composition. Ke et al. (2018) collected particulate samples from the combustion of cornstalk in a stove and evaluated their cytotoxicity in human skin keratinocytes. After exposure to biomass combustion particles, signs of mitochondrial damage, changes in the cytoplasmatic membrane and increased vacuolisation in the cytoplasm were observed by transmission electron microscopy. Combustion particles were found to reduce the cellular viability and induce apoptosis.

Despite the knowledge provided by the mentioned studies, particulate matter from residential biomass combustion has distinct physicochemical properties as a result of fuel type, combustion appliance and household behaviour (e.g. Lamberg et al., 2011; Vicente et al., 2015). These properties are key features triggering different biological effects, such as inflammatory responses, cytotoxicity, genotoxicity and oxidative stress (Corsini et al., 2019; Happo et al., 2013; Jalava et al., 2008, 2007). Thus, a toxicological profile of a relevant indoor pollution source is of utmost importance to better understand the potential health risk posed by wood burning emissions and to develop appropriate control strategies.

Taking into account the ethical issues related to *in vivo* testing, as well as its higher cost and time-consuming nature, *in vitro* assays were selected to carry out the present study. A battery of tests, based on different endpoints and cellular action mechanisms, can provide a first screening of toxicity of complex environmental mixtures. The potential of short-term *in vitro* testing, such as genotoxicity and cytotoxicity assays, has been recognised by the scientific community and regulatory agencies. Cytotoxicity and cell viability can be measured based on various cell functions, such as cell membrane permeability, mitochondrial function, enzyme activity and ATP production. The 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) colorimetric assays are widely employed (Aslantürk, 2018; Mahto et al., 2010) allowing the evaluation of mitochondrial function and cell membrane integrity, respectively. Assays based on water soluble tetrazolium salts (WST) are alternatives to MTT with significant advantages (Mahto et al., 2010). The cytotoxicity assays provide a starting point before exploring other biological mechanisms of interest (Peixoto et al., 2017).

Genotoxicity assays have different endpoints, such as single- and double-strand breaks, point mutations, chromosomal aberrations and micronuclei formation. The most commonly applied method for detecting genotoxicity include the Ames test (Dusinska et al., 2012). Toxicity assays using luminescent bacteria have been widely employed to evaluate the PM toxicity (Abbas et al., 2018; Girotti et al., 2008; Kováts and Horváth, 2016; Ma et al., 2014). These assays represent simple and efficient methodologies, useful as a screening tool (Tositti et al., 2018). One of the most commonly used bioluminescence inhibition assays is based on the *Vibrio fischeri* bacterium, which has been reported to show good correlations with other standard acute toxicity assays (Parvez et al., 2006).

This work aimed to evaluate the toxicity of indoor PM generated from Portuguese combustion appliances, which are common in the Mediterranean region, using different *in vitro* tests. Furthermore, outdoor sampling was also carried out in order to compare the results with those from indoor generated particles. The PM overall ecotoxicity was assessed with the *Vibrio fischeri* bioluminescence inhibition bioassay. The PM cytotoxicity was determined by the WST-8 and LDH release assays using a human lung epithelial cell line (A549). The mutagenicity of PM-bound polycyclic aromatic hydrocarbons (PAHs) was evaluated through the *Salmonella* reverse mutation assay. Given the evidence that coarse particles ($PM_{2.5–10}$) may play a role in generating adverse health effects (e.g. Adar et al., 2014; Brunekreef and Forsberg, 2005; Chen et al., 2019; Cheng et al., 2016; Sandström et al., 2005; Strickland, 2018; Zanobetti and Schwartz, 2009), particulate matter with aerodynamic diameter < 10 μm (PM_{10}) was sampled to carry out the analysis.

2. Materials and Methods

2.1. Particle collection and characterisation

A detailed description of the sampling sites and strategy can be found in a previous study (Vicente et al., 2020). Briefly, the PM_{10} sampling was carried out in two unoccupied houses equipped with traditional wood burning appliances, one with an open fireplace and the other with a woodstove. Each combustion appliance was operated for about 8 hours per day for three

and four days (woodstove and fireplace, respectively) under minimum ventilation conditions. No concurrent activities took place during the sampling period. Background concentrations were determined over four days in each room. Particulate matter (PM_{10}) was collected onto quartz filters (Pall Corporation, Ann Harbor, USA) with a high-volume air sampler (MCV, CAV-A/mb, Barcelona, Spain) working with a constant flow of 30 m^3 h^{-1}. During the wood burning period, concurrent samples were collected outdoors in order to evaluate the atmospheric PM_{10} in a rural area highly exposed to emissions from biomass burning for residential heating in the cold months of the year and also characterised by the inexistence of other major pollution sources (e.g. industry, traffic).

After the PM_{10} gravimetric quantification, the samples were analysed for organic (OC) and elemental carbon (EC), water soluble ions, metals and detailed organic composition. The full description of the analytical techniques and the PM_{10} chemical composition can be found in a previous work (Vicente et al., 2020).

2.2. Sample preparation for toxicological assays

Ecotoxicity: Sample preparation followed the protocol developed by Kováts et al. (2012). Briefly, one filter punch of 1.9 cm from each PM_{10} sample was ground in an agate mortar and then transferred into pre-cleaned glass vials. Suspensions were prepared adding 2 ml of high-purity water.

Cytotoxicity: Two filter punches of 47 mm from each PM_{10} sample were firstly extracted by refluxing dichloromethane (125 ml) for 24 h and then two times with methanol in an ultrasonic bath (25 ml for 10 min, each extraction). After each extraction, the total organic extracts were filtered and then concentrated to a volume lower than 1 ml using a Turbo Vap® II concentrator (Biotage) and finally evaporated under nitrogen flow. The final extract was reconstituted in dimethyl sulfoxide (DMSO, Sigma Aldrich).

Mutagenicity: Samples were firstly extracted as described above for the cytotoxicity assay. After drying, the total organic extract was then transferred onto activated silica gel columns and fractionated using solvents of different polarity. After each elution, the different organic

fractions were dried following the procedure described above. Target compounds (16 EPA priority PAHs and some other aromatics - benzo[e]pyrene, perylene, p-terphenyl, carbazole and retene) in the concentrated extracts were analysed by gas chromatography - mass spectrometry (GC-MS). The results were reported elsewhere (Vicente et al., 2020). Afterwards, the dried extracts from each sample were resuspended into DMSO and then tested for mutagenicity.

2.3. Ecotoxicity testing

The *V. fischeri* bioluminescence inhibition assay has been widely used for ecotoxicological screening and assessment of diverse potentially toxic substances including atmospheric pollutants (Abbas et al., 2018). The ecotoxicity testing was carried out using the direct contact test system (referred to as Flash system), which is standardised (ISO 21338:2010: water quality – kinetic determination of the inhibitory effects of sediment, other solids and coloured samples on the light emission of *Vibrio fischeri*/kinetic luminescent bacteria test). Briefly, the lyophilised bacteria were rehydrated with the reconstitution solution and stabilised at 12 °C for 30 min before the measurement using a luminometer (Luminoskan Ascent, Thermo Scientific). Duplicated series of eleven two-fold serial dilution in 2% NaCl were prepared for each sample in 96 well plates. The dilutions were performed following the manufacturer protocol with the supplied diluent reagent (2% NaCl solution in water), to ensure optimal osmotic conditions for the bacteria. After the bacterial suspension was added to the sample, bioluminescence intensity was continuously recorded for the first 30 seconds. After the pre-set exposure time (30 minutes), luminescence intensity was read again. The peak value observed immediately after the addition of the bacteria into the sample was used as a reference for calculations in order to take into account the turbidity or colour of the sample (Lappalainen et al., 2001). The light inhibition (INH%) was calculated based on the following equations:

$$KF = \frac{IC_{30}}{IC_0} \quad (1)$$

$$INH\ (\%) = 100 - \frac{IT_{30}}{KF \times IT_0} \times 100 \quad (2)$$

where KF is the correction factor, IC_0 and IC_{30} are the luminescence intensities of the control at the beginning and after 30 min, IT_0 and IT_{30} are the luminescence intensities of the sample at the beginning and after the 30 min contact time. EC_{50} (concentration that causes 50% reduction in the bioluminescence output of the test organisms relative to the control under the given experimental conditions) values were calculated from the dose-response curves on serial dilutions of the extracts using the Ascent Software provided by Aboatox Co., Finland. The EC_{50} values were then used to calculate the Toxicity Units (TU, unitless), which are defined as follows (Aammi et al., 2017; Romano et al., 2020):

$$TU_{50} = \frac{100\ (\%)}{EC_{50}\ (\%)} \quad (3)$$

Four toxicity levels were proposed on the basis of the TU values: $TU_{50} < 1$ non-toxic, $1 < TU_{50} < 10$ toxic, $10 < TU_{50} < 100$ very toxic and $TU_{50} > 100$ extremely toxic (Romano et al., 2020).

2.4. Cell culture and cytotoxicity assays

In the present study, the human adenocarcinoma alveolar epithelial cell line A549 was used to perform the cytotoxicity tests. This lung cell line is a useful model and widely used to assess the biological effects of PM samples (Cho et al., 2018; Jia et al., 2017; Peixoto et al., 2017).

A549 cells were cultured in 25 cm^2 flasks (Corning®) with 5 ml of Kaighn's Modification of Ham's F-12 Medium (F-12K), supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco), 1% of penicillin-streptomycin (Gibco) and 1% Fungizone (Gibco) at 37 °C, 5% CO_2 in humidified atmosphere. Cell confluence and morphology were observed under an inverted microscope (Nikon® Eclipse TS100). Subculture was performed every 2-3 days, when culture reached approximately 90% confluence. After removal of the culture medium, cells were washed with 2 ml of phosphate buffered saline (PBS, Gibco) and incubated with 1.5 ml Trypsin-EDTA (0.25% trypsin, 1 mM EDTA) for 5 min, to cleave proteins that enable cell adherence to the flask and cell-cell adhesion. After cell detachment from the culture flask,

trypsin was inactivated by adding 3 ml supplemented medium to the culture. Cells were harvested and seeded in a new flask with complete culture medium.

The PM_{10} cytotoxicity was examined using two complementary methods: WST-8 assay to evaluate the cell metabolic activity and lactate dehydrogenase (LDH) activity assay to assess the integrity of the cell membrane.

The WST-8 assay (CCK-8 kit, Sigma-Aldrich®) was performed by following the manufacturer instructions. Briefly, cells were seeded in a 96 well plate at 4×10^3 cells/well and incubated 24 h for adhesion. The PM_{10} suspension was diluted in complete medium to obtain the final concentrations of 0.1, 0.5, 1, 5, 10, 50, 100, and 150 μg ml^{-1} (corresponding to 0.03, 0.16, 0.31, 1.6, 3.1, 16, 31, and 47 μg cm^{-2}). The DMSO concentration in the culture medium was kept below 1.2% (v/v). The lowest range of doses at the alveolar epithelium (0.03 to 0.31 μg cm^{-2}) was determined based on modelling (particle dosimetry model ExDoM2) (Vicente et al., submitted for publication) using the PM_{10} measurements from the field campaign, while the highest range of doses exceeds environment concentrations. The highest doses were selected to generate differences in toxic responses between PM_{10} samples and control exposures in order to evaluate mechanisms of action. Additionally, higher doses allow comparison with results of previous studies. Cellular exposure was achieved by replacing the culture medium in each well with fresh PM-containing medium. The final volume for exposure was 0.1 ml per well. The outer peripheral wells of the 96-well plates were also filled in to reduce medium evaporation from the exposure wells. After 24 h exposure, the wells were emptied and filled with culture medium and 10 μl of WST–8 reagent. Then, the plate was incubated for 2 h at culture conditions and the absorbance was measured at 450 nm in a microplate reader (Biotek® - Gen5™ software). Two independent assays were performed with five technical replicates each and the results compared with the control (no exposure). After the subtraction of the background absorbance (culture medium without cells), the cellular viability was calculated as the ratio between the absorbance from wells exposed to particle suspensions and the absorbance of the control group (unexposed).

The LDH assay was carried out using the cell free supernatants after cell exposure using the LDH assay kit (Cytotoxicity Detection Kit LDH, Roche Diagnostics, France). The A549 cells were seeded and treated with PM_{10} extracts as in the WST-8 assay. After 24 h exposure, the supernatants were collected from each well and added to a new 96-well plate. The LDH activity measurement was conducted according to the instructions in the assay kit. Briefly, the reagent mixture was added to each well and incubated for 30 min at room temperature and protected from light and finally the absorbance was measured at 490 nm (Biotek® - Gen5™ software). The positive control was obtained exposing the cells to Triton-X and the background was culture medium without cells. Two independent experiments with three technical replicates were performed to test each sample. The cellular viability in relation to the control group (unexposed) is calculated from equation 4 (the background absorbance was corrected as described for the WST-8 assay):

$$Cell\ viability\ (\%\ of\ control) = \frac{LDH_{lysed} - LDH_{exposed}}{LDH_{lysed} - LDH_{control}} \times 100\% \quad (4)$$

where LDH lysed is the absorbance from wells treated with Triton-X (maximum LDH release), LDH exposed is the absorbance from wells exposed to particle suspensions, and LDH control is the absorbance from cells in the control group (spontaneous LDH release). Field blanks and solvent controls (DMSO) were also included in the assays. The viabilities of blank samples and solvents were not significantly different from the unexposed control.

2.5. Mutagenicity assay

Mutagenicity of the PM_{10} -bound PAHs was evaluated by the *Salmonella* reverse mutation assay (Ames test, pre-incubation method) (Mortelmans and Zeiger, 2000; OECD, 1997). Although the reverse mutation test relies on the use of prokaryotic cells, hampering the direct extrapolation of the results to human health effects, it is a useful initial screening tool for genotoxic activity and has been widely employed to assess atmospheric PM (Claxton et al.,

2004, and references therein; OECD, 1997). Despite the differences between prokaryotic and mammalian cells (uptake, metabolism, chromosome structure and DNA repair processes), many compounds that are positive in this test are mammalian carcinogens (OECD, 1997).

In the present study, two *Salmonella typhimurium* strains, TA98 and TA100 (Trinova Biochem GmbH), were selected, which are used to determine frameshift mutations and base pair substitution mutations, respectively (Mortelmans and Zeiger, 2000; OECD, 1997). The direct and indirect mutagenic potential was determined in the absence and presence of an exogenous activating metabolising enzyme system (S9 pooled from liver pooled from rat, Sigma Aldrich) to detected mutagens that require metabolic activation to form DNA-reactive intermediates (Ames et al., 1975; OECD, 1997). For assays performed without metabolic activation system, the positive controls were sodium azide (Acros Organics) and 2-nitrofluorene (Sigma Aldrich) for TA100 and TA98, respectively. For assays performed with metabolic activation system, the positive control was 2-aminoanthracene (Sigma Aldrich). The spontaneous mutant frequency was evaluated by negative controls exposing bacteria to DMSO and distilled sterile water. The *Salmonella typhimurium* strains were grown in nutrient broth for 15-18 h at 37 °C. After the incubation period, the tester strains were exposed to the chemical for 20 min in 0.5 ml of either buffer or S9 mix, prior to plating on glucose agar minimal medium. After 48 h incubation at 37 °C, the number of revertant colonies was determined. The solutions used for the assay were prepared according to Mortelmans and Zeiger (2000). Taking into consideration the limited volume of sample, each extract was tested at its maximum concentration (range from 10 to 150 ng per plate) in order to assess the mutagenicity. Three technical replicates were performed for each concentration.

2.6. Statistical analysis

Data analysis was carried out with SPSS software (IBM Statistics software v. 24). Shapiro-Wilk and Levene's tests were firstly applied to evaluate the normality of data and homogeneity of variances, respectively. The results from the cytotoxicity analysis (WST-8 and LDH assays) were compared to control by the non-parametric Kruskal Wallis test followed by Dunn's post

hoc tests and Bonferroni adjustment to the p-value. The results from exposures to indoor particulate samples (fireplace and woodstove) obtained from the WST-8 assay were tested against the corresponding background samples (obtained in the absence of indoor sources) for each particle dose. Additionally, comparisons between the samples collected simultaneously indoors and outdoors simultaneously were also made for different particle doses. Statistical relationships were sought between the EC_{50} obtained either from the bioluminescent inhibition assay or from the A549 cellular metabolic activity assessed with the WST-8 assay, and the chemical composition of the particulate material using parametric Pearson correlation coefficients. The results of the mutagenicity were analyzed through a one-way analysis of variance (ANOVA) followed by the Dunnett's post hoc test, to identify significant differences between the negative control and the PM_{10}-bound PAHs samples. Moreover, the mutagenicity ratio (MR: ratio between the average number of revertants in the sample and the average number of revertants in the solvent control plates) above 2 was used as criteria to identify mutagenic effects (Mortelmans and Zeiger, 2000). All differences were regarded as statistically significant at $p < 0.05$. No significant differences were detected between the number of revertent colonies of the DMSO and sterilised water plate controls, whereby the DMSO control was used as negative control in the statistical analysis.

3. Results and Discussion

3.1. Bioluminescence inhibition assay

Several authors have supported the use of *V. fischeri* bioluminescence inhibition assay as a first screening to examine the particulate matter toxicity (Aammi et al., 2017; Kováts and Horváth, 2016; Roig et al., 2013). The assay is sensitive and performs well regarding the display of false toxicity results (Kováts et al., 2012).

The overall toxicity of PM_{10} samples was assessed using the aqueous extracts and expressed as percentage of bioluminescence inhibition after 30 min of exposure time. PM_{10} collected when the fireplace was lit showed the highest inhibition with EC_{50} values ranging from 6.6 to 17 µg ml^{-1}. The PM_{10} extracts resulting from the use of the woodstove caused a less pronounced effect

on the bacterial suspensions with EC_{50} values ranging from 15 to 81 μg ml^{-1}. Regarding outdoor samples, the concentrations causing 50% reduction in the *V. fischeri* bioluminescence relative to the control after 30 min of exposure ranged from 26 to 72 μg ml^{-1}.

Aammi et al. (2017) collected coarse PM ($PM_{2.5-10}$) samples using a passive sampling method on a monthly basis, in twelve sampling sites from three districts in Istanbul, Turkey. The samples were extracted using DMSO and the toxicity was evaluated using the Microtox bioassay. The researchers suggested that seasonal activities, such as space heating, and meteorological factors (e.g. lower levels of atmospheric mixing and higher stability more likely during winter) were possibly responsible for the higher toxicity of samples collected in winter.

In this study, the indoor TU_{50} values varied from very toxic to extremely toxic (15 to 103) for samples obtained during the operation of the fireplace (Figure 1A). For samples of the room equipped with woodstove, TU_{50} values varied within the range 1.4 (toxic) – 14 (very toxic) (Figure 1B). Despite being equipped with a front door, an increase in PM_{10} concentrations and associated contaminants was still noticeable during the woodstove operation due to the opening of the door to start combustion and for refueling (Vicente et al., 2020). Indoor air background samples were collected in both rooms, one equipped with a fireplace and the other with a woodstove. For these background air samples, the TU_{50} values varied from 0.79 to 1.3. Outdoors, the TU_{50} values were lower than those recorded for samples collected in the rooms when wood burning appliances were used and were in the range from 0.58 (non-toxic) to 5.2 (toxic).

Previous studies have underlined the capability of the assay to display a comprehensive range of toxicity values for PM samples collected at sites impacted by dissimilar sources and pollutant loads. Aammi et al. (2017) found remarkable differences in the TU_{50} recorded at different sites in Istanbul. In a heavily polluted site (local industry) TU_{50} values of 85.7 and 106 were reported, while lower values, in the range from 0.05 to 0.09, were registered in the "clean air" site. The authors pointed out that samples collected in central urban areas impacted by traffic and sites impacted by industry were significantly more toxic than the others. TU_{50} ranging from 1.5 to 3.1 (it was not possible to calculate the TU_{50} for all samples due to the low toxicity) were

documented by Romano et al. (2020) for PM_{10} samples collected during moderate and warm seasons at a coastal site of the Central Mediterranean, away from large pollution sources. In the study of Roig et al. (2013), it was also possible to observe varying degrees of toxicity in PM_{10} samples collected in Catalonia (Spain) in contrasting seasons and monitoring sites (industrial, urban, and rural), which were impacted by different emission sources (cement plant, waste landfill, and municipal solid waste incinerator).

The comparison of the results of the present study with those published in the literature should be viewed with caution due to the array of sampling methods, sample extraction procedures and protocols for carrying out the bioluminescent inhibition assay. Regarding this latter, most of the studies have followed the Microtox bioassay, which differ in several aspects from the protocol used in the present study, for example in relation to the inhibition calculation method (Kováts et al., 2012).

3.2. Cellular metabolic activity and cellular membrane integrity

The effects of the PM_{10} samples (concentration range from 0.1 μg ml^{-1} to 150 μg ml^{-1}) on the metabolic activity of human epithelial cells were investigated with the WST-8 assay and displayed as a percentage of viability in comparison with that from unexposed cells (control). Figure 2 documents a decrease in cell viability for all samples collected either inside or outside. For indoor samples collected when the fireplace was in use (Figure 2A), a significant reduction of cell viability in comparison with control was reached at a concentration of 50 μg ml^{-1} for almost all samples (one sample displayed a significant reduction from 10 μg ml^{-1}). Particles from the operation of the woodstove (Figure 2B) also caused a decrease in cellular metabolic activity, with significant differences compared to control starting from 100 μg ml^{-1}. The metabolic activity of the cells declined down to 31.7 ± 3.19% at the highest dose (150 μg ml^{-1}) for particles generated when using the fireplace, whereas the same PM_{10} dose from the woodstove decreased the metabolic activity down to 71.6 ± 7.60%. Thus, the combustion technology had a remarkable effect on the cytotoxic potency of the particulate samples. A comparison between the cytotoxicity of indoor PM_{10} and the respective background (absence of

source), was also carried out. The reduction in cell viability induced by PM_{10} samples collected indoors when the fireplace was in operation was significantly higher than that of samples obtained when the source was inactive (starting at 0.5, 50, 100, and 10 μg ml^{-1} for days 1, 2, 3, and 4, respectively). In turn, only one PM_{10} sample collected during the use of the woodstove presented significantly higher cytotoxicity than the background at the highest dose (150 μg ml^{-1}).

Previously, investigations focused on the characterisation of emissions at source have underlined the role of combustion appliances on the cytotoxicity of particles from small scale devices using the MTT assay (Jalava et al., 2012; Tapanainen et al., 2011).

In the present study, outdoor samples caused a decrease from 21 to 48% in A549 metabolic activity. A decrease in cell viability, with significant differences compared to control starting at doses ranging from 5 to 150 μg ml^{-1}, depending on the sampling day, were recorded. The daily variability in the cytotoxicity of outdoor particles was noticeable (Figure 2C and D) and might be ascribed to distinct weather conditions in different monitoring days.

The comparison of indoor and outdoor PM_{10} cytotoxicity, for matched pair data, was also carried out. The reduction in cell viability induced by PM_{10} samples collected when the fireplace was in operation was significantly higher indoors than the parallel samples collected outdoors (starting at 5, 50, 100, and 10 μg ml^{-1} for day 1, 2, 3, and 4, respectively), whereas such effect was not seen with the wood stove samples. Figure 3 displays the comparison of indoor/outdoor cytotoxicity at the highest dose tested (150 μg ml^{-1}). The higher cytotoxicity observed for indoor particles during the fireplace operation might be ascribed to the higher organic mass fraction (Table 1). In fact, while the particulate matter content in water-soluble inorganic ions and elements was higher outdoors, biomass burning organic tracers and PAHs showed a greater contribution to the PM_{10} mass indoors during wood burning in the open fireplace (Vicente et al., 2020).

The effects of the PM_{10} samples (concentration range from 0.1 μg ml^{-1} to 150 μg ml^{-1}) on the membrane integrity of A549 cells, investigated with the LDH assay, are displayed in Figure 4 as a percentage of viability in comparison with that from unexposed cells (control). The results

revealed no significant increase in the release of the cytoplasmic enzyme LDH into the culture supernatant after cell exposure, reflecting the maintenance of the cell membrane integrity. This is in agreement with the results obtained by Kocbach et al. (2008). The researchers observed no decrease in the monocyte cell line THP-1 viability (measured as LDH release) after exposure to the extracts of wood smoke particles collected from a conventional Norwegian stove. Similarly, Kasurinen et al. (2017) obtained size-segregated PM emissions from two wood-fired appliances and tested different cell viability endpoints (metabolic activity, membrane integrity, and lysosomal damage) in order to investigate the mechanisms behind the cytotoxicity of wood combustion-generated particles. The authors found no significant reduction of the membrane integrity nor in lysosomal integrity after PM exposure compared to unexposed cells. However, all samples caused a significant reduction in the A549 metabolic activity. The results obtained by the researchers suggested that the mechanism of cell death was apoptosis in which the integrity of the plasma membrane is maintained. The results obtained by Marchetti et al. (2019) also indicated apoptosis behind the cell (A549) viability impairment after cell exposure to indoor particles arising from wood combustion in an open fireplace.

3.3. Mutagenicity assay

The number of revertant colonies obtained from the mutagenicity tests with PAHs extracted from PM_{10} and MR are presented in Table 2.

PAH extracts from PM_{10} samples collected indoors and outdoors showed no direct- or indirect-acting mutagenic effect towards both strains under the test conditions. The significantly higher ($p < 0.05$) number of revertants in the positive control plates in comparison with the number in the solvent control and in sample containing plates, as well as the MR between positive and negative controls, demonstrate the effective performance of the assays.

Several researches have pointed out the importance of PAH metabolic activation into primary and secondary metabolites on the toxicological effects observed. On the other hand, the toxicity of the parent PAHs is, in general, considered negligible (Mesquita et al., 2014 and references therein).

Despite the proved importance of metabolic activation during the organism detoxification process, studies focusing on PM-bound PAHs from biomass burning emissions have reported a mutagenic effect in the absence of a metabolic agent using specific *Salmonella* strains (Canha et al., 2016; Vu et al., 2012). Vu et al. (2012) tested $PM_{2.5}$-bound PAH extracts from the combustion of different biofuels (seven wood species and briquettes) in two appliances (fireplace and woodstove) under two operating conditions (cold and hot start) for mutagenic activities using the Ames test with *Salmonella typhimurium* TA98 and TA100. The authors reported a direct-acting mutagenicity for almost all biofuels and concluded that combustion in a fireplace seems to favour the emission of mutagenic compounds. When S9 was introduced to the test, the mutagenic effect disappeared, suggesting that the samples contained direct-acting base-pair and frameshift mutagens that lose their mutagenicity after being metabolised by enzymes from the S9 liver fraction. The same conclusion was drawn by Canha et al. (2016) who reported a decrease of mutagenicity of the extracts of PM_{10}-bound PAHs from small scale residential combustion of different biofuels in a woodstove and pellet stove when metabolic activation was added. Oanh et al. (2002) assessed the mutagenic potency (Ames test) of particles released from three different cookstoves burning different fuels. The results for the TA98 strain indicated the presence of both direct and indirect mutagenic activity in PM samples from sawdust and wood. On the other hand, the TA100 strain only detected direct mutagenic activity of PM samples. Galvão et al. (2018) collected PM_{10} samples during intense and moderate biomass burning periods in the Brazilian Amazon region. The extractable organic matter was used to assess the mutagenic potential of the samples using two different bacterial strains (TA98 and YG1041). The researchers reported that the mutagenic potencies were higher in the absence of metabolic activation, regardless of the strain used, showing a large contribution of direct acting mutagens.

In addition to different protocols (e.g. standard plate incorporation assay, pre-incubation assay, microsuspension assay) and bacterial strains applied to assess the PM mutagenicity, distinct sample preparation procedures have been described in the literature. For example, the studies of Canha et al. (2016) and Vu et al. (2012) were performed with the PAH extracts, while Oanh et

al. (2002) and Galvão et al. (2018) carried out the Ames test with the total extractable organic matter. Moreover, the test concentrations evaluated through the Ames assay are highly variable, which may have contributed to the discrepancies in the results. PAH emissions, as well as PAH composition profiles, from residential solid fuel combustion are greatly affected by the fuel burnt (Du et al., 2021), which can also affect the results obtained.

3.4. Correlations between biological responses and PM_{10} chemical composition

The impairment of cellular (A549) metabolic activity was highly correlated with the *V. fischeri* bioluminescence inhibition, both indoors ($r = 0.792$, $p < 0.05$) and outdoors ($r = 0.972$, $p < 0.01$).

Correlations between the PM_{10} chemical composition and the EC_{50} values, obtained from the dose-response curves, were studied (Table 3). Detailed information on the chemical composition of PM_{10} has been described in a previous study (Vicente et al., 2020). No correlation was recorded (indoors and outdoors) between the decrease in cell viability or bioluminescence inhibition and PM_{10} concentrations. This may result from the variability in the chemical composition of the particulate matter, suggesting that the toxicity is related to specific compounds.

Indoors, organic carbon (OC) displayed negative correlations with the EC_{50} determined from the WST-8 and bioluminescent inhibition assays ($p < 0.05$), indicating that the increase in PM_{10} organic content decreases the EC_{50}, i.e., enhances its ability to induce toxicity in each target cell. Indoors, the sum of PM-bound PAHs was significantly associated with PM toxicity ($r = -0.930$, $p < 0.01$ and $r = -0.811$, $p < 0.05$ for WST-8 and *V. fischeri* bioluminescence inhibition, respectively).

Over the years, discordant conclusions have been drawn regarding the role of PAHs on the cytotoxicity of biomass burning particles. While some source characterisation studies, aiming at assessing the toxic potential of PM emissions, reported significant correlations between cytotoxicity and PM-bound PAHs (Kasurinen et al., 2016; Sun et al., 2018), others recorded no correlation (Arif et al., 2017; Jalava et al., 2012). The role of PAHs in the *V. fischeri*

bioluminescence inhibition has also been previously highlighted (Alves et al., 2021; Evagelopoulos et al., 2009).

The bivariate correlations between the PM toxicity and individual PAH compounds also revealed statistically significant relationships. Among the studied compounds, several 3-ring (retene and phenanthrene, $p < 0.05$) and 4-ring (fluoranthene $p < 0.01$, chrysene $p < 0.05$, benzo[a]antracene $p < 0.05$) congeners were significantly correlated with the reduction in A549 cell viability (Table 3).

Previous studies, aiming at assessing the cytotoxic potential of retene and other polyaromatic compounds in biomass burning emissions, reported the ability of this alkylated phenanthrene to significantly decrease cell viability (A549) at a dose of 30 $\mu g\ ml^{-1}$ after 72 h of exposure (Alves et al., 2017; Peixoto et al., 2019). In the present study, the sum of polyaromatic compounds associated with PM_{10} showed no correlation with the toxicity of the outdoor samples.

In the present study, anhydrosugars were the dominant group of organic compounds in samples collected both indoors and outdoors (Vicente et al., 2020). Indoors, significant correlations were recorded between increased PM_{10} toxicity towards A549 cells and monosaccharide anhydrides (both their sum and each isomer individually). Outdoors, monosaccharide anhydrides showed no association with the toxicity of the samples, contrarily to results of previous studies assessing PM collected at sites impacted by biomass burning (Van Den Heuvel et al., 2018; Van Drooge et al., 2017). Van Den Heuvel et al. (2018) evaluated the biological effects of PM_{10} sampled in ambient air at an urban traffic site and a rural background location in Belgium. The researchers exposed BEAS-2B cells to PM_{10} to study the cell damage and death, reportig that reduced cell viability was associated with biomass burning markers (levoglucosan, mannosan and galactosan). Van Drooge et al. (2017) evaluated the toxicity of organic extracts from outdoor PM_1 samples from rural and urban locations in JEG-3 human placental cells. The researchers correlated the cytotoxicity of the samples collected in winter at the rural site with biomass burning tracer compounds (levoglucosan, mannosan, galactosan and dehydroabietic acid), and with incomplete combustion products (benzo[b+j+k]fluoranthene, benzo[e]pyrene, benzo[a]pyrene and indeno[1,2,3-cd]pyrene).

Indoors, several other biomass burning markers, such as resin acids, phenolic compounds and sterols, displayed significant correlations with toxicity. The resin acids pimaric and isopimaric, as well as the oxidised derivative dehydroabietic acid, were significantly ($p < 0.05$) correlated with PM cytotoxicity (WST-8). Additionally, isopimaric was also found to correlate negatively with the EC_{50} derived from the bioluminescence inhibition assay ($p < 0.05$).

β-sitosterol, the most abundant compound among sterols and triterpenoids (Vicente et al., 2020), was correlated with the EC_{50} values determined using the WST-8 for indoor samples. For outdoor samples, β-sitosterol correlated significantly with the *V. fischeri* bioluminescence inhibition. Several guaiacol and syringol type methoxyphenols (e.g. vanillin, acetovanilone, 3-vanilpropanol, vanillic acid, syringaldehyde, sinapic acid and syringic acid) linked to PM_{10} from the indoor air were significantly correlated with cytotoxic effects (WST-8). Also, in indoor samples, vanillic acid was significantly correlated ($p < 0.05$) with both cytotoxicity and bioluminescence inhibition. For the outdoor samples, 4-hydroxybenaldehyde was significantly associated with the toxicity measured by both assays ($p < 0.05$).

When applied to indoor samples, the WST-8 assay displayed significant correlations with a higher number of organic compounds than the *V. fisheri* inhibition assay. This can be attributed to the WST-8 sample preparation method, which favours the bioavailability of organic compounds in the extracts (Danielsen et al., 2009; Landkocz et al., 2017).

No significant negative correlations between toxic endpoints and water-soluble ions were observed. It is interesting to note that the bivariate correlations between several ionic species and the bioluminescence inhibition and cytotoxicity assays EC_{50} values were statistically significant, but positive, implying a decreasing effect for increasing ionic PM_{10} mass fractions. On the contrary, a number of studies suggested that several ionic species (e.g. NO_3^-, Cl^-, SO_4^{2-}) may participate in cytotoxicity induced by ambient particles (Chen et al., 2006; Happo et al., 2014; Perrone et al., 2010; Velali et al., 2016). Thus, further investigation is needed to shed light into the role of these species regarding the cellular death induced by combustion derived particles.

It must be mentioned that correlations with the elemental composition of the particles was not undertaken since pooled samples were used for the analyses by inductively coupled plasma (Vicente et al., 2020) due to sample shortage. Despite the relatively low contribution of major and trace elements to the PM_{10} mass (2.20 and 14.1 wt% of the PM_{10}) (Vicente et al., 2020), it is possible that some of these elements may have contributed to the toxicity of the samples. In fact, previous *in vitro* studies involving particles from biomass burning have underlined the importance of PM-bound metals in particle-mediated cytotoxicity (Arif et al., 2017; Kasurinen et al., 2017, 2016; Uski et al., 2015). Studies assessing the cytotoxicity of atmospheric PM also highlighted the role of metals on the results, pointing out significant correlations between decreased cell viability and PM-bound elements, such as cadmium, zinc, copper, chromium, lead, vanadium, tin and arsenic (Happo et al., 2014; Perrone et al., 2010; Roig et al., 2013; Van Den Heuvel et al., 2016; Velali et al., 2016). Roig et al. (2013) also found that several of these PM-bound elements correlated with the decrease in bioluminescence of bacterial suspensions (*V. fischeri*).

The apparent inconsistency between the results of the current work and literature data may be associated with several factors, which might have a pronounced effect on results: i) different cell lines (Arif et al., 2017; Corsini et al., 2017; Danielsen et al., 2009; Gualtieri et al., 2010); ii) sample preparation procedures (e.g. extraction solvent and method, resuspension solvent); iii) PM size fraction under analysis; iv) cytotoxicity test selected (e.g. Alamar Blue, MTT, WST-1,8, LDH); v) test conditions (e.g. incubation time, exposure doses, among others) (Cavanagh et al., 2009; Danielsen et al., 2009; Gualtieri et al., 2010; Happo et al., 2013; Hiebl et al., 2017; Landkocz et al., 2017; Peixoto et al., 2017).

3.5. Limitations

This study has some limitations caused by the limited number of experiments, which might increase the level of uncertainty with respect to source toxicological profiles. Therefore, more complex studies, covering more dwellings and combustion appliances, should be considered in

future studies to account for effects of household characteristics and equipment design on the results.

The bioassays used in the present study are useful as a first screening of the potential toxicity of particulate matter and to find out which constituents contribute the most to toxicity. They can be indicated as efficient methods to routinely complement chemical analysis. Despite the insights given by these tests, other complementary bioassays are needed to make inferences about impacts on human health. *In vitro* studies involving lung cells can be improved by the use of cell co-cultures in which the *in vivo* cell interactions, which can exacerbate or inhibit the toxicological response, can be simulated. Additionally, to overcome the limitations of submerged cell culturing, future work should focus on air-liquid interface systems, in which the exposure is performed through aerosolised particles mimicking more closely physiologic conditions in the lung and therefore creating more realistic conditions of exposure via inhalation. Moreover, it should be borne in mind that *in vivo* exposure is affected not only by the exposure concentrations but also by the deposition rate of particles, clearance mechanisms and retention of particles within the respiratory system, which are not represented in these bioassays.

Given the dissimilarities regarding the cellular uptake and genomic complexity between prokaryotes and eukaryotes, genotoxicity data from the Ames assay should be interpreted carefully and supplemented with additional assays. Some chemicals that are positive in *in vivo* mutagenicity tests are negative or weakly positive in Ames tests because of inefficient metabolic activation of the chemicals *in vitro* even with the inclusion of the S9. Contrarily, some nitro compounds, which are effectively activated by bacterial nitro reductases, are strongly positive in the Ames test but mostly negative or weakly positive in the *in vivo* tests (Nohmi and Tsuzuki, 2016). For certain chemicals, suspected of interfering specifically with mammalian cell replication system, a mammalian mutation test may be more appropriate than the bacterial reverse mutation test. Some mammalian carcinogens might not be positive in the Ames test since some chemicals can act through non-genotoxic mechanisms or mechanisms absent in bacterial cells (OECD, 1997).

Conclusions

In the coldest months of the year, biomass burning is a major source of atmospheric pollutants. It was also proven to be a major indoor source of hazardous compounds, including particulate matter. The present study aimed at investigating the toxicity induced by wood burning particles, in indoor and outdoor environments, using various bioassays (*V. fischeri* bioluminescence inhibition assay, WST-8, LDH and Ames test). The results evidenced different toxic potentials for particles emitted when using the fireplace or the woodstove. Indoor-generated particles by the fireplace were the most ecotoxic and cytotoxic, whereas mutagenicity was not detected in any of the tested samples. PM_{10} samples collected during the operation of the fireplace induced greater metabolic activity impairment in A549 cells than the samples obtained when the source was inactive, while only one PM_{10} sample collected during the use of the woodstove presented significantly higher cytotoxicity than the background at the highest dose. The outdoor samples were significantly less cytotoxic than their corresponding indoor air counterparts during the operation of the fireplace, whereas no such effect was observed with the woodstove samples. After the exposure period, no damage in the cellular membrane was observed at all tested concentrations for both indoor and outdoor samples.

Indoors, several combustion-related organic compounds, such as PAHs and biomass burning tracers, displayed significant correlations with the increase in toxicity (WST-8 and *V. fischeri* bioluminescence inhibition assays). These findings add to the growing body of evidence that toxicological responses are related to the particle chemical characteristics.

Taking into account the results of the present study, homeowners should be encouraged to upgrade the wood burning technology in order to reduce the products of incomplete combustion bound to PM.

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Table 1. PM_{10}, carbonaceous species and PAHs concentrations. Modified from Vicente et al. (2020)

	Indoor		**Outdoor**	
	Fireplace	Woodstove	Fireplace	Woodstove
N	4	3	4	3
PM_{10} (μg m^{-3})	319 ± 173	78.5 ± 24.0	80.0 ± 19.8	32.1 ± 16.7
OC (μg m^{-3})	128 ± 85.2	15.9 ± 7.93	14.4 ± 0.985	7.03 ± 3.11
EC (μg m^{-3})	17.6 ± 9.99	3.69 ± 3.1	3.30 ± 0.442	1.48 ± 0.515
Σ PAHs (ng m^{-3})[a]	92.0 ± 31.5	8.83 ± 6.49	28.3 ± 5.36	6.02 ± 2.65

[a] PAHs -16 EPA priority PAHs plus benzo[e]pyrene, perylene, p-terphenyl, carbazole and retene.

Table 2. Mutagenicity of PAH extracts of particles collected indoors and outdoors to *S. typhimurium* TA98 and TA100 strains in the absence (-S9) and presence (+S9) of metabolic activation.

			TA100 -S9		TA100 +S9		TA98 -S9		TA98 +S9	
		ng PAHs/plate	Rev/plate	MR	Rev/plate	MR	Rev/plate	MR	Rev/plate	MR
Fireplace										
Indoor	Day 1	100	162 ± 30	1.2	179 ± 15	1.1	21 ± 2	1.3	17 ± 5	0.89
	Day 2	150	136 ± 8	1.0	191 ± 26	1.2	15 ± 3	0.94	16 ± 1	0.84
	Day 3	150	164 ± 8	1.2	181 ± 14	1.1	20 ± 3	1.3	16 ± 5	0.84
	Day 4	50	124 ± 20	0.94	143 ± 4	0.89	16 ± 4	1.0	16 ± 1	0.84
Outdoor	Day 1	30	146 ± 15	1.1	138 ± 23	0.86	21 ± 2	1.3	22 ± 11	1.2
	Day 2	30	111 ± 31	0.84	169 ± 7	1.0	22 ± 3	1.4	18 ± 6	0.95
	Day 3	30	137 ± 20	1.0	154 ± 14	1.0	21 ± 2	1.3	16 ± 4	0.84
	Day 4	30	104 ± 27	0.79	146 ± 25	0.91	23 ± 4	1.4	26 ± 5	1.4
Woodstove										
Indoor	Day 1	55	129 ± 7	0.98	169 ± 21	1.0	15 ± 8	0.94	28 ± 8	1.5
	Day 2	10	132 ± 14	1.0	180 ± 26	1.1	12 ± 1	0.75	28 ± 6	1.5
	Day 3	25	125 ± 8	0.95	140 ± 27	0.87	9 ± 2	0.56	21 ± 6	1.1
Outdoor	Day 1	30	124 ± 23	0.94	145 ± 12	0.90	11 ± 4	0.69	18 ± 3	0.95
	Day 2	20	124 ± 11	0.94	148 ± 7	0.92	8 ± 4	0.50	16 ± 2	0.84
	Day 3	15	151 ± 15	1.1	159 ± 22	0.99	12 ± 3	0.75	13 ± 2	0.68
	PC		3077 ± 681*	**24**	524 ± 83*	**3.3**	131 ± 24*	**8.2**	268 ± 78*	**14**
	DMSO		132 ± 23		161 ± 15		16 ± 6		19 ± 3	

Values are means ± standard deviation of 3 plates. MR = mutagenicity ratio; Rev/plate = revertants per plate; PC = positive control. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparison test. Asterisks indicate statistical significance compared to negative control ($p<0.05$). Mutagenicity ratios above 2 are marked in bold.

Table 3. Pearson correlation coefficients between EC_{50} (μg ml^{-1}) obtained in this study from the WST-8 and *Vibrio fischeri* and PM_{10} chemical composition from a previous study (Vicente et al., 2020).

	Indoor (N=7)		Outdoor (N=7)	
	WST-8 assay	*Vibrio fischeri* inhibition assay	WST-8 assay	*Vibrio fischeri* inhibition assay
Carbonaceous compounds				
OC	**-0.870***	**-0.863***	0.301	0.422
EC	-0.393	-0.592	0.239	0.422
Monosaccharide anhydrides				
Levoglucosan	**-0.863***	-0.723	-0.288	-0.136
Mannosan	**-0.871***	-0.725	-0.167	-0.043
Galactosan	**-0.888****	-0.744	-0.465	-0.409
Σ Monosaccharide anhydrides	**-0.870***	-0.728	-0.324	-0.181
Resin acids and derivatives				
Dehydroabietic acid	**-0.868***	-0.751	-0.291	-0.169
Hydroxydehydroabietic acid	0.190	0.183	-0.673	-0.579
Oxodehydroabietic acid methyl ester	-0.713	-0.639	-0.715	-0.621
Pimaric acid	**-0.819***	-0.704	-0.504	-0.365
Isopimaric acid	**-0.872***	**-0.775***	-0.606	-0.513
Podocarpic acid	0.313	0.163	-0.474	-0.393
Σ Resin acids and derivatives	**-0.862**	-0.747	-0.389	-0.271
Sterols				
Cholesterol	0.559	0.344	**0.813***	**0.809***
Dihydrocholesterol	-0.627	-0.570	-0.174	-0.129
β-Sitosterol	**-0.822***	-0.719	-0.731	**-0.768***
Σ Sterols and triterpenoids	**-0.836***	-0.724	-0.708	-0.745
Substituted benzenes and phenolic compounds				
4-Hydroxybenzaldehyde	-0.342	-0.268	**-0.847***	**-0.838***
Sinapic acid	**-0.838***	-0.724	-0.426	-0.522
Vanillin	**-0.855***	-0.741	-0.560	-0.369
Acetovanillone	**-0.771***	-0.683	0.145	-0.010
3-Vanilpropanol	**-0.784***	-0.692	-0.449	-0.466
Vanillic acid	**-0.886****	**-0.774***	-0.124	-0.179
Syringic acid	**-0.867***	-0.720	-0.382	-0.506
Syringaldehyde	**-0.782***	-0.618	-0.268	-0.123
Sinapaldehyde	**-0.792***	-0.623	-0.239	-0.201
Acetosyringone	**-0.815***	-0.695	-0.529	-0.411
Coniferyl aldehyde	**-0.766***	-0.645	-0.445	-0.430
4-Hydroxycinnamic (p-coumaric) acid	**-0.816***	-0.704	-0.200	-0.201
3,4-Dihydroxy-cinnamic (caffeic) acid	**-0.857***	-0.726	-0.488	-0.491
4-hydroxy-3-methoxycinnamic (ferulic) acid	**-0.798***	-0.693	-0.473	-0.508
3-Hydroxybenzoic acid	**-0.896****	**-0.849***	-0.623	-0.593
Protocatechoic acid	**-0.910****	**-0.779***	-0.537	-0.552
Isoeugenol	**-0.798***	-0.696	-0.124	-0.075
PAHs				
Phenanthrene	**-0.843***	-0.658	-0.371	-0.411
Fluoranthene	**-0.877****	-0.675	-0.568	-0.609
Chrysene	**-0.784***	-0.595	-0.476	-0.534
Benzo[a]anthracene	**-0.815***	-0.619	-0.444	-0.523
p-Terphenyl	**-0.908****	-0.726	-0.544	-0.584
Retene	**-0.766***	-0.668	-0.508	-0.459
Σ PAHs[a]	**-0.930****	**-0.811***	-0.455	-0.507
Water soluble ions				
Chloride	0.417	**0.804***	**0.940****	**0.974****
Nitrate	**0.795***	0.695	-0.142	-0.191
Sulphate	**0.757***	**0.834***	-0.611	-0.492
Phosphate	**0.864***	**0.912****	0.376	0.489
Sodium	0.721	**0.930****	**0.905****	**0.951****

Potassium	0.344	0.343	-0.594	-0.645
Magnesium	0.636	0.421	0.667	0.746
Calcium	0.241	0.112	-0.469	-0.424
Σ Water soluble ions	**0.891****	**0.971****	**0.841***	**0.903****

Significant correlation coefficients at the p-level < 0.05 and 0.01, are marked with * and **, respectively. OC: organic carbon; EC: elemental carbon. [a] PAHs -16 EPA priority PAHs plus benzo[e]pyrene, perylene, p-terphenyl, carbazole and retene.

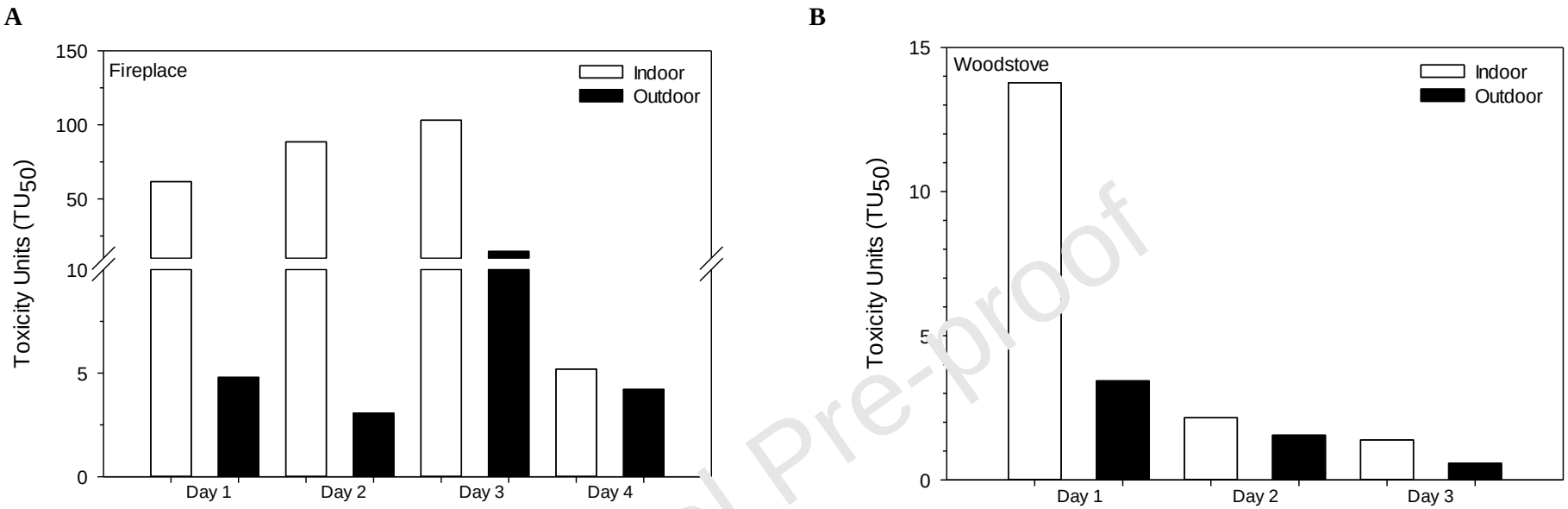
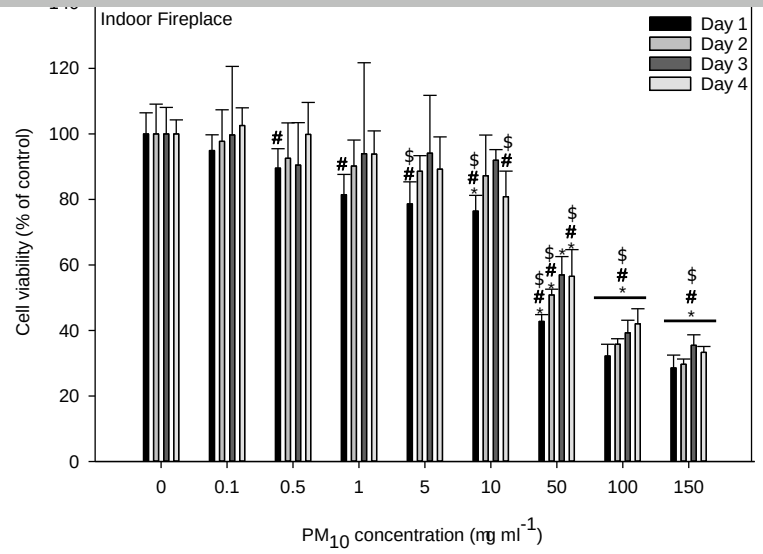
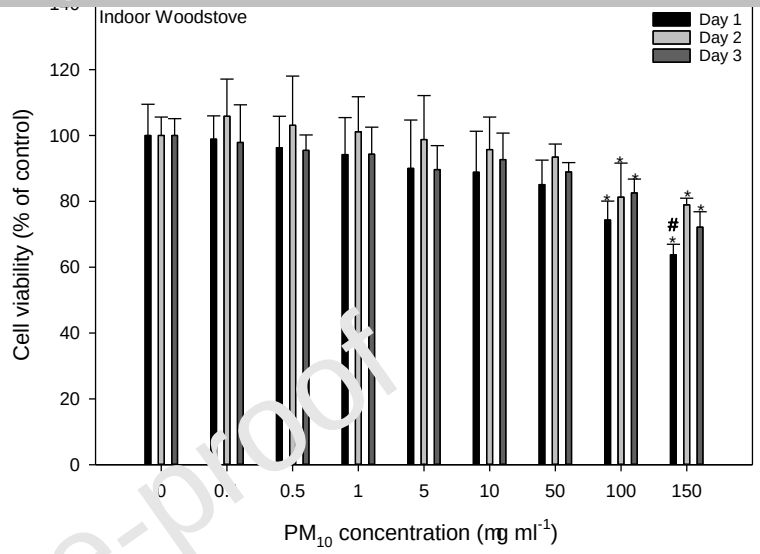
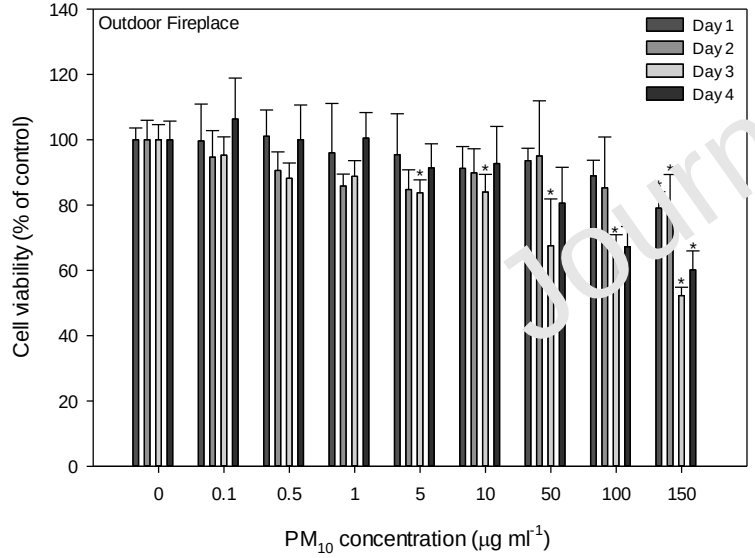


Figure 1. Toxicity Units (TU_{50}) calculated from EC_{50} values obtained from the *Vibrio fischeri* bioluminescent inhibition assay for the samples collected indoors during the operation of the fireplace (A) and the woodstove (B) and corresponding outdoor samples.

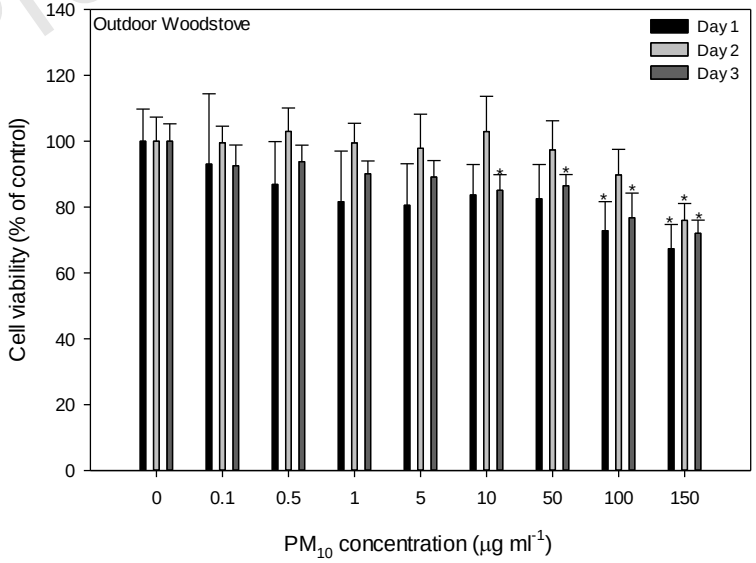




C



D



fireplace (A) and the woodstove (B) and corresponding outdoor samples (C and D for fireplace and woodstove, respectively): 0 (control), 0.1, 0.5, 1, 5, 10, 50, 100 and 150 μg ml^{-1}. Bars represent the mean ± standard deviation of two independent experiments with five technical replicates each. Statistical analysis was performed by Kruskal-Wallis followed by Dunn's multiple comparison test. Asterisks indicate statistical significance compared to control (n = 10, $p<0.05$). Statistically higher responses ($p < 0.05$, Kruskal-Wallis followed by Dunn's multiple comparison test) compared to background and outdoor PM samples are marked with # and $, respectively.

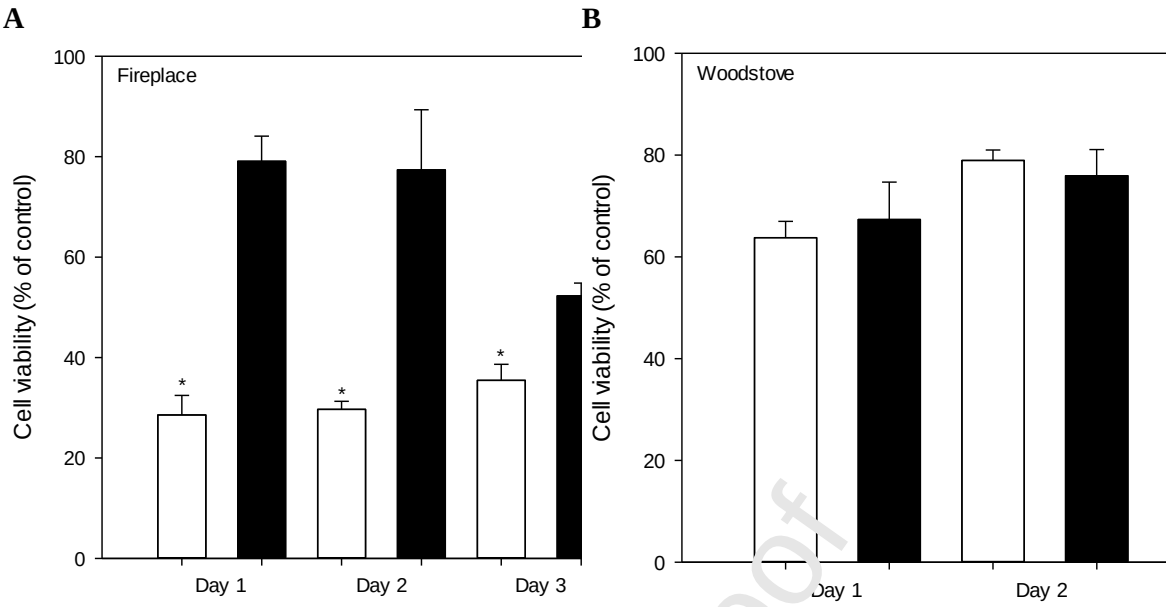


Figure 3. Cell viability assessed with WST-8 assay after 24 h exposure to the highest PM_{10} concentrations (150 µg ml^{-1}) from samples collected indoors during the operation of the fireplace (A) and the woodstove (B) and corresponding outdoor samples. Bars represent the mean ± standard deviation of two independent experiments. Statistical analysis was performed by Kruskal Wallis. Asterisks indicate statistical significance compared to outdoor PM_{10} samples (n = 10, $p<0.05$).

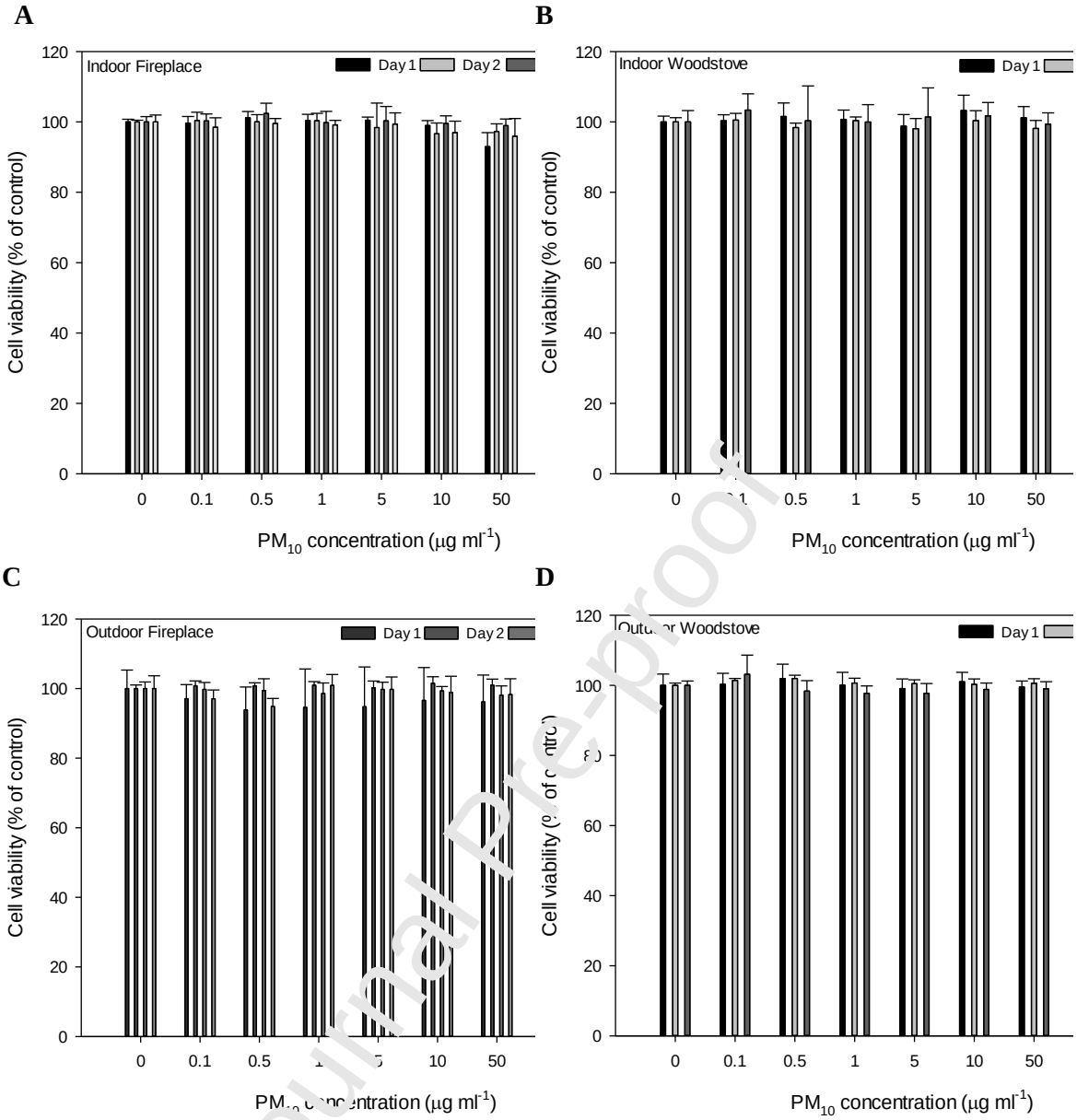


Figure 4. Cell viability assessed with LDH assay after 24 h exposure to increasing PM_{10} concentrations during the operation of the fireplace (A) and the woodstove (B) and respective outdoor samples (C and D for fireplace and woodstove, respectively): 0 (control), 0.1, 0.5, 1, 5, 10, 50, 100 and 150 μg ml^{-1}. Bars represent the mean ± standard deviation of two independent experiments with three technical replicates each. Statistical analysis was performed by Kruskal-Wallis. Asterisks indicate statistical significance compared to control (n = 6, $p<0.05$).

Authors' contributions

Conceptualisation: Estela D. Vicente, Célia A. Alves; **Formal analysis:** Estela D. Vicente; **Investigation:** Estela D. Vicente, Daniela Figueiredo, Cátia Gonçalves; **Writing - original draft preparation:** Estela D. Vicente; **Writing - review and editing:** Estela D. Vicente in collaboration with all co-authors; **Funding acquisition:** Célia A. Alves; **Resources:** Célia A. Alves, Isabel Lopes, Helena Oliveira, Nora Kováts; **Supervision:** Célia A. Alves, Isabel Lopes, Helena Oliveira, Nora Kováts; **Validation:** Isabel Lopes, Helena Oliveira, Nora Kováts; **Project administration:** Célia A. Alves.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

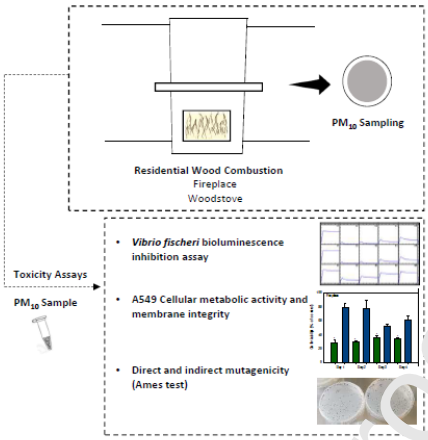
☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Estela D. Vicente

(On behalf of all co-authors)

Aveiro, 09/02/2021

Graphical abstract



Highlights

- The toxicity of indoor PM_{10} from fireplace and woodstove operation was studied
- Samples from fireplace operation were more ecotoxic than those from woodstove
- Apoptosis appears to be the mechanism behind the A549 cell death
- Direct and indirect samples mutagenicity undetectable by the tester strains
- PAHs and biomass burning tracers correlated with increased toxicity