Published in final edited form as:

Atmos Environ (1994). 2017 August ; 163: 138–154. doi:10.1016/j.atmosenv.2017.05.019.

Cytotoxic and genotoxic responses of human lung cells to combustion smoke particles of Miscanthus straw, softwood and beech wood chips

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Abstract

Inhalation of particulate matter (PM) from residential biomass combustion is epidemiologically associated with cardiovascular and pulmonary diseases. This study investigates PM_{0.4–1} emissions from combustion of commercial Miscanthus straw (MS), softwood chips (SWC) and beech wood chips (BWC) in a domestic-scale boiler (40 kW). The PM_{0.4–1} emitted during combustion of the MS, SWC and BWC were characterized by ICP-MS/OES, XRD, SEM, TEM, and DLS.

Cytotoxicity and genotoxicity in human alveolar epithelial A549 and human bronchial epithelial BEAS-2B cells were assessed by the WST-1 assay and the DNA-Alkaline Unwinding Assay

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Authors' contributions

ATA, CM, PG, MGK, RGi, RGm, GT, AD, PKh, IN, VMS contributed to the study concept and design. ATA, CM, PG, IN, RGi, RGm interpreted the data and were responsible for writing and reviewing the manuscript. ATA, CM, PG contributed to laboratory research, data acquisition and results interpretation; RGi, RGm and GT were involved as project supervisors and data reviewers; PG collected PM_{0.4–1} during all combustion conditions, and presented confocal images using ImageJ. TB acquired and helped to improve confocal images. AD and GT supervised the work of PG and helped review and improve the manuscript.

(DAUA). $PM_{0.4-1}$ uptake/translocation in cells was investigated with a new method developed using a confocal reflection microscope.

SWC and BWC had a inherently higher residual water content than MS. The $PM_{0.4-1}$ emitted during combustion of SWC and BWC exhibited higher levels of Polycyclic Aromatic Hydrocarbons (PAHs), a greater variety of mineral species and a higher heavy metal content than $PM_{0.4-1}$ from MS combustion. Exposure to $PM_{0.4-1}$ from combustion of SWC and BWC induced cytotoxic and genotoxic effects in human alveolar and bronchial cells, whereby the strongest effect was observed for BWC and was comparable to that caused by diesel PM (SRM 2 975). In contrast, $PM_{0.4-1}$ from MS combustion did not induce cellular responses in the studied lung cells. A high PAH content in PM emissions seems to be a reliable chemical marker of both combustion efficiency and particle toxicity. Residual biomass water content strongly affects particulate emissions and their toxic potential. Therefore, to minimize the harmful effects of fine PM on health, improvement of combustion efficiency (aiming to reduce the presence of incomplete combustion products bound to PM) and application of fly ash capture technology, as well as use of novel biomass fuels like Miscanthus straw is recommended.

GRAPHICAL ABSTRACT

Keywords

Biomass combustion; Miscanthus straw; Wood chips; $PM_{0.4-1}$ emissions; Polycyclic aromatic hydrocarbons (PAHs); Toxicity

1. Introduction

Biomass fuels of either animal or plant origin are becoming increasingly important as a renewable energy source. They can be categorized into liquid, gaseous and solid fuels, whereby the latter comprise wood, crop residues and dung, and are mainly used for domestic needs such as cooking and heating. In the last few years, however solid biomass has become an important substitute for fossil fuels in medium and large-scale power plants (Demirbas, 2004). On a global scale, combustion of biomass fuels is the main domestic source of energy in approximately 50% of urban and 90% of rural households. The associated high concentrations of combustion particles suspended in the emissions are known to be responsible for severe adverse health effects such as acute respiratory infections, cardiopulmonary disease and lung cancer (Lewtas, 2007; Torres-Duque et al., 2008). In developing countries, emissions from combustion of biomass for domestic heat production are the main source of indoor and outdoor air pollution in rural areas, and, increasingly, in urban areas, too (Chen et al., 2016; Sigsgaard et al., 2015). The amount of particulate matter

(PM) generated and its toxicological properties depends on the biomass fuel type and source, the combustion technology used and the combustion conditions (Fachinger et al., 2017; Jalava et al., 2012; Schwarze et al., 2013). Combustion of biomass can create emissions that include solid particles made of crystalline materials, amorphous substances, heavy metals, soot and organic substances like PAHs (Jokiniemi et al., 2008). Three competitive mechanisms have been identified for particle formation during biomass combustion: coarse ash particle entrainment, inorganic vapor (e.g., KOH, KCl, K₂SO₄, Zn) condensation, and pyrolysis product condensation (Kocbach Bølling et al., 2009). The efficiency, i.e. the completeness of the combustion process, is evaluated on the basis of the concentrations of CO and TVOC (total volatile organic compounds) in the exhaust gas, and on identification of polycyclic aromatic hydrocarbons (PAHs) in the condensed phase of PM. As a general rule, the concentration of organic carbon in PM is a function of incomplete combustion (Gauggel-Lewandowski et al., 2013). A comparison of particles emitted from different combustion processes reveals that the size distribution of fine particles from wood combustion and diesel engines is fairly similar, and that their concentration is generally higher than that from combustion of other fuels like coal (Leskinen et al., 2014). The PM emitted from combustion processes can remain in the air for several weeks and undergo long-range transport through the atmosphere, which in turn may influence its potential to induce biological effects in humans (Kocbach Bølling et al., 2009; Lim and Seow, 2012). Many previous studies have demonstrated diesel engine emissions to be harmful for human health (e.g. Danielsen et al., 2008; Totlandsdal et al., 2015).

Both in-vivo (Forchhammer et al., 2012; Muala et al., 2015) and in-vitro (Kasurinen et al., 2015a; Tapanainen et al., 2011) experiments exploring potential relationships between biomass combustion and health effects have confirmed that PAHs can be adsorbed onto the surface of particles, presenting a potential hazard to biota.

Small and medium-scale biomass heating systems may be good alternatives to heating systems fired with fuel oil because overall the emissions from both small and medium-scale controlled wood-fueled heating systems caused fewer cytotoxic effects and DNA damage in a cell model than the emissions from the corresponding oil-fueled heating systems (Kasurinen et al., 2015a). To mitigate the health effects of biomass-derived PM emissions, reduction of exposure to biomass PM emissions through “wise heating”, e.g. using appropriate boilers, dry biomass fuels and complete combustion conditions, combined with cleaner biomass fuels producing limited emissions and exhibiting low toxicity is strongly recommended (Fachinger et al., 2017; Kaivosoja et al., 2013; Leskinen et al., 2014). Giant Miscanthus (*Miscanthus x giganteus*) is a high-yielding perennial grass with the potential to meet biomass fuel production criteria (Atkinson, 2009; Kadžiulien et al., 2014). It is also economically viable and plays an important role in the sustainable production of renewable fuels and chemicals via thermo-chemical conversion (Sørensen et al., 2008). However, there are still challenges to the widespread commercial use of Miscanthus as an energy source, primarily because of its high ash content and low ash-melting temperature, which can cause severe corrosion inside the combustors (Kortelainen et al., 2015; Lamberg et al., 2011).

In-vitro studies suggest that particles produced during incomplete combustion are more toxic than those produced under complete combustion conditions (Forbes et al., 2014; Uski et al.,

2014). For this reason, the aim of the present study was to investigate the influence on various biological endpoints of the physical and chemical properties of smoke particles released during combustion of three different types of solid biomass fuels. We studied the cytotoxic, and genotoxic effects as well as the cellular uptake of $PM_{0.4-1}$ emitted during combustion of commercial Miscanthus straw and commercial softwood and beech wood chips. For this purpose, human epithelial cells (A549 and BEAS-2B) were used as 2D-in-vitro test systems, since the lungs are the main target organ for interaction with air pollution.

2. Materials and methods

2.1. Combustion and PM-sampling procedures

Miscanthus straw was produced locally in Ammertzwiler (Haut-Rhin, France), and the wood chips were obtained from the local municipal forests of Ammertzwiler and Bernwiller (Haut-Rhin, France). Combustion tests at laboratory scale were performed with Miscanthus straw (MS), beech wood chips (BWC), and softwood chips (SWC, mostly spruce) in a multi-fuel boiler (HKRST/VFSK) supplied by REKA® (Aars, Denmark), which can also be used as small-scale domestic boiler (40 kW). The fuels chosen for this study were used as received from commercial biomass fuel suppliers. Combustion conditions and various emission characteristics for the combustion experiments are described in Table 1. Briefly, P_{input} was estimated from the lower heating value of the fuels multiplied by the mass loss during combustion, whereas P_{output} is the energy recovered per unit of time through a heat transfer fluid; see full protocol in (Schönnenbeck et al., 2016). The Dekati Gravimetric Impactor (DGI) system (Dekati, Tampere, Finland) used allows for the collection of particles as a function of their aerodynamic equivalent diameter (D_{ae}) at 50% efficiency at a nominal air-flow rate of 100 L/min. Three particle size fractions ($>2.5 \mu m$, $1-2.5 \mu m$, and $0.4-1 \mu m$) were collected on stainless steel impaction plates. Particles smaller than $0.4 \mu m$ in diameter were collected through a terminal filter stage. The total mass of the four fractions represents the total number of suspended particles (TSP, see Table 1). However, in this study we used the $PM_{0.4-1}$ fraction exclusively, which was scraped from the stainless steel plate of the DGI impactor prior to weighing. For comparison of particle composition, especially for carbonaceous particles we used the Standard Reference Material SRM 2975, a certified Diesel Particulate Matter (DPM) collected from the exhaust of a diesel-run forklift at the U.S. National Institute of Standards and Technology (NIST, 2016).

2.2. Analysis of gaseous emissions and carbonaceous $PM_{0.4-1}$

Gaseous and particulate emissions were measured in the chimneys according to the European standard EN-304. At laboratory scale, O_2 and CO were measured using paramagnetic and infrared cells (Rosemount™ NGA 2000, Emerson, St. Louis, USA). Concentrations of total volatile organic compounds (TVOC) were determined with a Flame Ionization Detector (FID, Cosma graphite 55, Igny, France) and are reported as carbon equivalent (Table 1). The Water Soluble Organic Compound (WSOC) fraction was extracted from the PM using pure water and then quantified using a total organic carbon (TOC) analyzer (TOC-V Series, Shimadzu, Columbia, USA). To quantify the 16 PAHs recommended for analysis by the U.S. Environmental Protection Agency (EPA), we used an Accelerated Solvent Extractor (ASE 300, Dionex, Sunnyvale, USA) followed by High

Performance Liquid Chromatography (HPLC) coupled with two detectors: a Diode Array Detector (DAD, Thermo Finnigan, Spectra System UV6000LP, Waltham, USA) and a fluorescence detector (Thermo Scientific, Finnigan Surveyor FL Plus, USA), following an analytical method (HPLC-FL-DAD) that was recently published (Liaud et al., 2015).

2.3. Characterization of solid particles in $PM_{0.4-1}$ emissions

2.3.1. Scanning electron microscopy (SEM)— $PM_{0.4-1}$ was investigated by SEM combined with energy dispersive X-ray (EDX) spectroscopy to collect data on morphological properties and associated qualitative chemical compositions. The used SEM-EDX device was a LEO 1 525 field-emission scanning electron microscope (LEO Electron Microscopy Inc., Thornwood, New York, USA) equipped with an 80 mm² X-MaxN Silicon Drift Detector (Oxford Instruments, Abingdon, United Kingdom), located at the Institute of Earth and Environmental Science (Crystallography), Freiburg, Germany. Particles were distributed on an adhesive carbon pad mounted on an aluminum stub, and then coated with 10 nm of carbon by a sputter under vacuum. SEM-EDX was conducted with 15 kV acceleration voltage, which allows for determination of the qualitative chemical composition of individual particles, but does not allow to assess whether or not the PM contains water-bearing minerals.

2.3.2. X-ray diffraction (XRD)—Qualitative mineralogical compositions of the $PM_{0.4-1}$ emissions were determined by XRD using a D8 Advance Powder X-ray diffractometer (Bruker Co. AXS, Karlsruhe, Germany) with a Cu target and Cu-K α X-rays and subsequent peak identification at the Institute of Earth and Environmental Science in Freiburg, Germany. Based on the fact that only small amounts were available for each sample (less than 50 mg), the particles were distributed on polished silicon mono-crystal plates with the help of acetone. The setting for the pattern recording was optimized for best peak-to-background ratios and was as follows: angle range of 2–75°2 θ , step size of 0.005°, and 2 s per step. Peak positions of the collected diffraction patterns were matched against the ICDD's PDF 48 Database with the DIFFRAC.EVA V5.0 software by Bruker Co.

2.3.3. Transmission electron microscopy (TEM)—Particle dimensions and shape were determined by TEM after fixing the $PM_{0.4-1}$ emissions on lacey-carbon copper grids. The samples were treated according to a previously described method with some modifications (Kobach et al., 2006). Briefly, $PM_{0.4-1}$ emission materials were dispersed in Millipore water (1 mg/mL) and sonicated in an ultrasonic waterbath (Ultrasonic water bath Badelin electronic, Berlin, Germany) at 37 °C for 10 min. A 1% (v/v) glutaraldehyde solution (Polysciences Europe, Eppelheim, Germany) was prepared by dilution with 1 × HEPES buffer (Bichrom, Berlin, Germany). At the same time, a Petri dish (Greiner Bio-One, Kremsmünster, Austria) was covered with Parafilm® (Bemis, Soignies, Belgium). On this hydrophobic surface, 10 μ L of the dispersion containing the $PM_{0.4-1}$ emission materials were pipetted, and a lacey carbon copper grid (plano-em) was placed on the drop. Next, the 1% glutaraldehyde solution was used for fixation of the $PM_{0.4-1}$ emission materials for 5 min. The grid with the fixed $PM_{0.4-1}$ emission materials was then rinsed four times for 30 s with double-distilled water. Finally, the lacey-carbon copper grid was stained with 1% (v/v) uranylacetate solution (Sigma Aldrich, Taufkirchen, Germany), and the grid was allowed to

dry prior to performing the TEM analysis (Hitachi H600, Tokyo, Japan) at the Institut und Poliklinik für Arbeits-und Sozialmedizin der Justus Liebig Universität Gießen (Germany).

2.3.4. Dynamic light scattering (DLS) analysis—To assess whether the particles in suspension were in an aggregation or a monodisperse state, we determined size distribution, dispersibility, solubility and surface chemistry by DLS (Yi et al., 2015). Therefore, besides hydrodynamic size distribution we measured polydispersity indices (PDI) by DLS according to ISO 22412 (2011). The hydrodynamic diameters of the PM_{0.4-1} samples were measured for dispersions in Millipore water (concentration: 1 mg/mL) using a NANO-flex particle size analyzer (Microtrac, Meerbusch, Germany). After calibration with Milli-Q water (no particles) the dispersions were sonicated in a ultrasonic waterbath (Ultrasonic water bath Badelin electronic, Berlin, Germany) at 37 °C for 10 min. The obtained PM_{0.4-1} dispersions were assessed in triplicate. The viscosity and refractive index were set to those specific to water prior to measurement (Murdock et al., 2008). Zeta potentials of the PM_{0.4-1} samples were measured by a ZetaView® (Particle Metrix, Meerbusch, Germany), whereby the samples were prepared by the same procedure as for measuring the particle size distribution.

2.3.5. Inductively coupled plasma-mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectrometry (ICP-OES)—To investigate the influence of the particle components on health effects, the bulk chemical compositions of the PM_{0.4-1} were determined by ICP-MS and ICP-OES at an analytical laboratory (Medizinisches Labor Bremen, Germany). With ICP-MS, an Agilent 7500ce device (Agilent Technologies, Waldbronn, Germany), concentrations of the following elements were determined in the PM_{0.4-1} samples: As, Ba, Be, Cd, Co, Cu, Hg, Mo, Ni, Sb, Pb, Sn, Sr, and Zr. Concentrations of Al, Ca, Cr, Fe, K, Mg, Mn, Na, Si, Ti, and Zn were determined with an ICP-OES, a Spectro Arcos (Spectro Analytical Instruments, Kleve, Germany). Prior to the ICP analysis, about 0.2 g of each PM sample was digested in aqua regia under pressure-microwave treatment with a Mars microwave device (CEM, Kamp-Lintfort, Germany). Digested samples were diluted with ultrapure water to fill up to 20 mL.

2.4. Biological characterization

2.4.1. Preparation of PM_{0.4-1} emissions for biological assays—Collected PM_{0.4-1} fractions and DPM were suspended in Millipore water (Millipore Elix, Darmstadt, Germany) to prepare stock solutions (1 mg/mL); these were vortexed (Vortex mixer, Heidolph Reax Top Heidolph, Schwabach, Germany) for 1 min and sonicated (Ultrasonic water bath Badelin electronic, Berlin, Germany) for 10 min immediately before cell exposure to disperse the particles and reduce particle agglomeration. Next, the cells were exposed to PM_{0.4-1} for incubation at final concentrations of 1, 3, 10, 30 and 100 µg/cm² in a total volume of 200 µL/well in a 96-well plate (Greiner Bio-One, Kremsmünster, Austria), and 2 mL/well in a 6-well plate. The cells exposed in the 96-well plate were used for the cell viability and apoptosis assays, whereas the cells exposed in 6-well plates were used to investigate cellular uptake of particles on coverslips (18 × 18 mm; R. Langenbrinck, Emmendingen Germany). For genotoxicity testing, cells were exposed in cell culture/ Petri Dishes (TPP, TechnoPlasticProducts, Trasadingen, Switzerland). Cells were kept at 37 °C, 5% CO₂ (v/v) in humidified air in an incubator throughout the entire exposure period (24 h).

These $PM_{0.4-1}$ concentrations were added to a cell culture well in correlation with the total surface area of the well, and they are referred to as the administered dose and not as the delivered dose.

2.4.2. Cell culture and exposure of human lung epithelial cell lines

(BEAS-2B, A-549) to $PM_{0.4-1}$ —The adenocarcinoma human alveolar basal epithelial cell line (A549) was obtained from the American Type Culture Collection (ATCC). Cells were cultivated in DMEM modified with high glucose supplemented with L-Glutamine, pyruvate (Life Technologies, Karlsruhe, Germany), 10% (v/v) heat-inactivated Fetal Calf Serum (FCS) (Gold PAA Laboratories GmbH, Linz, Austria) and U/mL penicillin/streptomycin (Invitrogen, Darmstadt, Germany) at 37 °C, 5% CO_2 (v/v) in humidified air in an incubator.

The human bronchial epithelial cell line BEAS-2B, originally isolated from a non-cancerous patient and immortalized by an adenovirus 12-SV40 hybrid, was also purchased from the ATCC. Cells were cultivated in LHC-9 basal medium (Gibco - Life Technologies, Mannheim, Germany) containing supplements. The cells were cultured in a humidified incubator with 5% CO_2 (v/v), 95% (v/v) air atmosphere at 37 °C.

The cells were thawed and used for the experiments after the fourth to sixth passage. The medium was changed every 2–3 days. At 85–90% confluence, the cells were washed with 1x Dulbecco's PBS (Ca-Mg-free; pH = 7.3; 20 °C; Gibco Life Technologies, Karlsruhe, Germany), harvested using 0.25% (v/v) Trypsin/EDT (PAA Laboratories GmbH, Linz, Austria), and either sub-cultured in T-75 cm^2 flasks (Greiner Bio-One, Kremsmünster, Austria) or plated on 96-well plates (Greiner Bio-One, Kremsmünster, Austria) at a density of 10,000 cells in 200 μ L of medium (added to each well), ensuring that the cells reached a confluent monolayer before they were exposed to $PM_{0.4-1}$. The concentrations of particles and the time of exposure of the cell lines were selected on the basis of published Standard Operation Procedures (SOP) and laboratory protocols from projects of the German BMBF sponsorship program. (SOPs, 2011).

2.4.3. Cell viability measurement—The influence of $PM_{0.4-1}$ emissions on cell viability was determined using the Water Soluble Tetrazolium assay (WST-1, Roche - Mannheim, Germany). This colorimetric assay is designed for detecting living cells based on the enzymatic cleavage of the tetrazolium salt to formazan by cellular mitochondrial dehydrogenases present in viable cells. According to the manufacturer's instructions, cells were washed with 1x PBS after exposure to avoid any interference with light absorption resulting from the $PM_{0.4-1}$. Next, 95 μ L of fresh culture medium and 5 μ L of prepared WST-1 solution were added to each well in an optically clear 96-well flat-bottom plate, and the cells were incubated for 1 h at 37 °C, protecting the plate from light. At the end of incubation, the samples were measured for absorption at a wavelength of 570 nm with a spectrophotometric Microplate reader (Infinite® 200 PRO Tecan Group Ltd., Männedorf, Switzerland). Enzymatic activity correlates directly with the amount of formazan produced by reduction of the tetrazolium salt. The values were determined based on color change, which infers cell viability, and were calculated as percentage by comparing absorption values of the suspensions of cells with PM to a cell suspension without PM (control).

2.4.4. Detection of DNA damage by DNA alkaline unwinding assay (DAUA)—

In this study, the DNA-damaging effects of PM_{0.4-1} emissions and DPM were investigated using the DAUA according to (Hartwig and Schlepegrell, 1995), with minor modifications. Briefly, 1.5×10^5 A549 cells and 2×10^5 BEAS-2B cells were cultured on a 9.2 cm² cell-culture dish in 2 mL DMEM or LHC-9 medium, respectively. The cells were grown for at least 24 h and subsequently exposed as described previously. For the positive control, ethyl methane sulfonate (EMS; Alfa Aesar, Karlsruhe, Germany) was used at a concentration of 5 mmol/L. After exposure, the culture medium was removed and the cells were washed once with cold PBS (4 °C) and stored on ice until performing the DAUA. The PBS was removed and 1.5 mL of alkaline solution (0.06 N NaOH in 0.01 M Na₂HPO₄) at a pH of 12.3 was added to each cell-culture dish. The cells were incubated with the alkaline solution for exactly 30 min at room temperature in red light or in a dark place. After exactly 30 min, the samples were neutralized to a pH of 6.8 by adding 500 µL of 0.1 N HCl to each cell-culture dish. Next, the samples were sonicated (Sonopuls Sonifier, Berlin, Germany) on ice for 15 s. Sonication took place within one minute of neutralization. Directly after sonication, sodium dodecyl sulfate (SDS; Serva electrophoresis, Heidelberg, Germany) was added to a final concentration of 0.05% (w/v). The samples were then stored overnight at -20 °C.

Hydroxyapatite column chromatography was performed in a specially designed heating block (handmade) heated to 60 °C in a water bath (Memmert, Schwabach, Germany). The 1 mL hydroxyapatite columns (Sigma Aldrich, Schnellendorf, Germany) were also prepared at 60 °C. The first step in chromatography is activation of the hydroxyapatite columns. For this purpose, 1.5 mL of 0.5 M potassium phosphate buffer was added. Next, the column was washed with 1.5 mL of 0.01 M sodium phosphate buffer before the samples were added. The columns were then washed a second time with 2.5 mL 0.01 M sodium phosphate buffer. The single-stranded DNA was eluted in a 12-well cell-culture plate with 1.5 mL of 0.5 M potassium phosphate buffer. The 12-well cell-culture plate, where the eluted DNA was collected, was then kept in the dark to protect the DNA because of its light sensitivity. A new 12-well cell-culture plate was placed under the columns and the double-stranded DNA was eluted with 0.35 M potassium phosphate buffer. The fluorescence dye Hoechst 33258 (Sigma Aldrich, Schnellendorf, Germany) was added to each well to a final concentration of 7.5×10^{-7} M and the samples were then placed in the dark for 30 min. After the incubation time, the fluorescence was measured with a spectrophotometer at an excitation wavelength of 360 nm and an emission wavelength of 455 nm. The fractions of double- and single-stranded DNA were calculated as described by Hartwig and Schlepegrell (1995). A blank (buffer only) was prepared for each plate.

2.4.5. Detection of DNA damage by single-cell gel electrophoresis (SCGE, comet) assay—

To detect DNA damage by PM_{0.4-1} emission, the comet assay was carried out according to the protocols of (Tice et al., 2000; Ferk et al., 2016), with minor modifications. The BEAS-2B cells were washed extensively with 1x PBS buffer (Ca-Mg-free; pH = 7.3; 20 °C; Gibco Life Technologies, Karlsruhe, Germany). After trypsination using 0.25% (v/v) Trypsin/EDTA (PAA Laboratories GmbH, Linz, Austria) for 5 min, the cell suspension was centrifuged. The supernatant was removed and the cells were then resuspended in 100 µL of 0.5% (w/v) low-melting point agarose (CAS: 9 012-36-6) from

Serva (Heidelberg, Germany) before being transferred to fully frosted slides and covered with a coverglass. The slides were prepared with a first layer of 1% (w/v) normal-melting point agarose and a second layer of 0.7% (w/v) normal-melting point agarose. They were stored for 15 min at 4 °C to allow for solidification. Following solidification, the cover glasses were removed and the slides immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA-Titriplex, 0.2 M NaOH, 1% (v/v) Triton X-100, pH 10) at 4 °C and stored for 24 h at the same temperature. Next, the slides were rinsed with distilled water and placed in a horizontal electrophoresis tank (VWR, Darmstadt, Germany) filled with ice-cold electrophoresis buffer (0.3 M NaOH, 1 mM EDTA-Titriplex, pH > 13) for 20 min. Electrophoresis was conducted at 25 V and 300 mA for 25 min, followed by 10-min neutralization with Tris buffer (0.4 M Tris, pH 5.7.5) and washing twice in distilled water. To analyze DNA damage in the comet assay, cells were stained with 60 µL of an ethidium bromide solution (0.1 mL/mL). The slides were coded and analyzed immediately by two observers using a fluorescence microscope (DMLS, Leica, Germany) at 400-fold magnification. Slides were randomly scored and 180 nucleoids on duplicate slides were measured using a public domain image processing and analysis program (Scion Image, Scion Corp., Maryland, USA). Data were captured and transferred to a computer by a frame grabber card (LG-3, Scion Corp.). The tail moments, i.e. the product of the fluorescence intensity in the tail and the tail length of selected nucleoids, were calculated by means of a MS Excel-Macro developed by Helma and Uhl (2000).

2.4.6. Determination of apoptosis and necrosis using annexin V and propidium iodide staining

—Staining with annexin V and propidium iodide (PI) was done according to Jalava et al. to detect apoptosis and necrosis (Jalava et al., 2007). The BEAS-2B cells were incubated in the presence of PM_{0.4-1} for 24 h. The extents of apoptosis and necrosis were measured using an Annexin V-FITC apoptosis detection kit (eBioscience San Diego, CA, USA) according to the manufacturer's instructions. In brief, the cells (3×10^5) were harvested and washed with ice-cold PBS. Subsequently, they were stained with Annexin V-FITC in binding buffer in the dark for 15 min; next, propidium iodide (eBioscience, San Diego, CA, USA) was added and the cells were incubated in the dark for 10 min at room temperature; the samples were then measured by flow cytometry (Accuri C6, BD Biosciences). We used 5 µmol/L camptothecin (Tocris, Bristol, UK) and 0.5% (v/v) Triton-X 100 (Sigma Aldrich, Schnelldorf, Germany) as positive controls for apoptosis and necrosis, respectively.

2.4.7. Cell — particle interaction after staining with Phalloidin-FITC and DAPI and confocal microscopy analysis

—Confocal microscopy analysis was applied to observe cell-particle interactions following the protocol by Vranic et al. with some modifications (Vranic et al., 2013). In brief, we used 100,000 BEAS-2B cells on a coverslip, cultivated in 6-well plates for 24 h. The cells were exposed to 30 µg/cm² of the PM in submersed condition. After the incubation time of 24 h, 4% (w/v) paraformaldehyde (PFA; Affymetrix, Ohio, USA) was added for fixation of the cells. The cells were then washed with 800 µL of cold PBS, and Triton X-100 0.1% (v/v) was added to make them permeable in order to ensure access of the stain to the cells. Subsequently, the cells were washed several times with PBS. Next, they were incubated with a Phalloidin-FITC working solution

(Invitrogen, Darmstadt, Germany) for 25 min, rinsed with PBS, and finally incubated with a 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI; Life Technologies, Karlsruhe, Germany) working solution (diluted 1:1 000 in PBS from 1 mg/mL) for 20 min. Next, the cells were washed twice with PBS. All the steps following application of the stain were performed in the dark at room temperature. As a mounting medium for microscopic observation, a saline-based buffered solution (Sigma Aldrich, Schnellendorf, Germany) was used. This was added to the surface; subsequently, the prepared microscope slides were allowed to set overnight at 4 °C. Confocal microscopy investigations on cellular uptake of μm -sized particles are usually performed with artificial particles having a characteristic signal (Gupta and Gupta, 2005). Here, confocal microscopy was performed using a ZEISS LSM 700 upright microscope and a 63 $\times$ objective having a numerical aperture of 1.4. The used immersion oil from Zeiss has a refractive index of 1.518. Blue fluorescence (4', 6-diamidino-2-phenylindole or DAPI) is obtained by exciting the samples under investigation at 405 nm and acquiring from 420 to 480 nm; green fluorescence (fluorescein isothiocyanate or FITC) is obtained by exciting at 488 nm and acquiring from 490 to 555 nm. Reflection is acquired at 405 nm as described by previous users interested in nanomaterial-cell interface (Badique et al., 2013) and was labelled as red LUT in order to enhance contrast. The ImageJ software was used for presentation of the confocal images (Schneider et al., 2012).

2.5. Statistical analysis

All experiments were performed at least three times. Data are expressed as mean standard deviation ($\pm\text{SD}$). Statistical significances were assessed by ANOVA followed by Dunnett's post hoc pairwise comparisons between non-exposed and exposed groups. Correlation analysis between the chemical compounds of the $\text{PM}_{0.4-1}$ samples and the toxicological endpoints in BEAS-2B and A549 cells (WST-1, DAUA, Comet, Apoptosis, Necrosis) was conducted with Pearson correlation coefficient (r) analysis. $\text{PM}_{0.4-1}$ samples resulting from the combustion of the three biomass fuels were tested at the particulate dose of $30 \mu\text{g}/\text{cm}^2$ because at this concentration the effects were significant in all biological endpoints. The correlation coefficient (r) ranges from -1 to $+1$. Effects were considered as statistically significant if $p < 0.05$. Significant levels, $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) are shown in the figures. All the data were analyzed using the GraphPad Prism 6 software system (LaJolla, CA, US).

3. Results and discussion

The goal of this study was to investigate the cytotoxic and genotoxic effects on human lung cells of particles emitted from biomass fuel combustion. To achieve this goal, we characterized the physical and chemical properties of $\text{PM}_{0.4-1}$ generated during combustion of commercial MS, SWC (mainly spruce) and BWC in a small-scale domestic boiler mimicking a real-life combustion scenario.

3.1. Combustion behavior and carbonaceous content of $\text{PM}_{0.4-1}$ emissions

In this study, two types of emissions containing $\text{PM}_{0.4-1}$ particles were generated during combustion of biomass fuel: one with a high content of organics, resulting from significant incomplete combustion (predominantly because of the fuel's high moisture content, see

Table 1, BWC and SWC combustion) and the other with a low content of organics, as a result of nearly complete combustion due to lower residual fuel moisture (MS combustion). The biomass fuels are typically left in storage for a year after harvest, i.e. they only have one year to dry, and depending on storage conditions, their moisture (water content) may therefore be relatively high. The water content of wood chips, for example, can range from 25 wt% to 60 wt%, with an average of 35 wt% (Forest Fuels, 2016). The SWC and BWC used in our study had a water content of 46 ± 2 and 32 ± 3 wt %, respectively. In contrast, the residual water content of the MS used was considerably lower (10 ± 2 wt %). The combustion efficiency, defined as $(P_{\text{output}}/P_{\text{input}}) \times 100$, was comparable for the different fuels, and ranged from 66% to 73% (Table 1).

In detail, nearly complete MS combustion was indicated by the low content of CO (104 mg/Nm^3), TVOC (below the detection limit of 10 mg/m^3) and particle-bound WSOC (1.21 mg/g). In contrast, the substantially higher content of CO ($3\,400\text{--}3\,500 \text{ mg/Nm}^3$) and TVOC ($>210 \text{ mg C/Nm}^3$) in the $\text{PM}_{0.4-1}$ from both SWC and BWC combustion pointed to significant incomplete combustion. As a result, $\text{BWCPM}_{0.4-1}$ showed higher WSOC concentrations (14.6 mg/g) than $\text{MSPM}_{0.4-1}$. Furthermore, the TSP mass emitted was substantially higher for SWC and BWC combustion than for MS combustion (Table 1). Compared with normative references, the CO, TVOC and TSP content produced during MS combustion was far below the threshold levels prescribed by French legislation (NF EN 12809/A1). In fact, the CO and TVOC concentrations were above the legislation thresholds for SWC and BWC combustion, while the TSP concentration was at the threshold value (Table 1). However, this legislation is merely a quality standard and infringement is not punitive.

The total particle-bound PAH concentration in the $\text{PM}_{0.4-1}$ emissions from each biomass fuel used was calculated as the sum of the 16 U.S.-EPA priority PAHs, which were analyzed individually (see Supplementary Material, Table S1). Combustion of MS produced the lowest total PAH content in the $\text{PM}_{0.4-1}$ (2.5 mg/kg). In contrast, the total PAH concentrations in the $\text{SWCPM}_{0.4-1}$ and $\text{BWCPM}_{0.4-1}$ were 914 mg/kg and $2\,458 \text{ mg/kg}$, respectively (Table 1), while for DPM a content of about 69 mg/kg was reported in the certificate of analysis (NIST, 2016) (Table S1).

These data suggest that the high moisture content of SWC and BWC resulted in emissions containing relatively high levels of PAHs. In contrast, combustion of MS produced PAH-poor emissions. Similar results were also obtained for combustion at higher temperatures in studies using dry rice and bean straw. In these studies, PAHs were thermally decomposed, resulting in reduced PAH levels in comparison to combustion of straw with a moisture content of about 30 wt% (Lu et al., 2009; Yang et al., 2006). Our results are also in good agreement with the findings reported in other studies showing that wood smoke particles generally contain considerable amounts of PAHs (Dilger et al., 2016; Jalava et al., 2010; Kaivosoja et al., 2013; Leskinen et al., 2014).

To assess the carcinogenic risk associated with the PAH contained in the $\text{PM}_{0.4-1}$, we calculated the B[a]P equivalent factor (eq.B [a]P) according to equation (1) in the Supplementary Material (B[a] P = benzo[a]pyrene). The eq.B[a]P values of the investigated

PM_{0.4-1} samples (Table S2) range from 0.06 mg/kg for MSPM_{0.4-1} to almost 390 mg/kg for BWCPM_{0.4-1}, whereas about 2.7 mg/kg were observed for DPM.

Our results are not consistent with those of Kasurinen et al. (2015b), who reported that none of the particle-bound PAHs concentrations were significantly lower in the PM₁ emissions from *Miscanthus* sp. pellets (97 mg/kg) than in the PM₁ emissions from softwood (401 mg/kg), poplar (37 mg/kg) or wheat-straw pellets (128 mg/kg). However, should be noted that Kasurinen et al. (2015b) studied the effects of emissions from wood and *Miscanthus* pellets rather than wood chips and *Miscanthus* straw, i.e. the fuel/air interface was distinct. In contrast, the (non-pelletized) MS straw used in our study had low density and high porosity, thus providing optimal oxygen supply during combustion, which in turn further promotes more complete combustion (Daragon et al., 2014). Consequently, the combustion conditions in the study of Kasurinen et al. (2015b) were very different to those used in our experiments; the resulting emissions and their observed toxicity also differed in the two studies. Another study on combustion of *Miscanthus* focused on the physicochemical properties of the emissions from a small-scale multi-fuel boiler (Forbes et al., 2014). The results of the study are in good agreement with the physicochemical data obtained in our experiments, however the authors did not investigate the possible toxic effects of the emissions.

3.2. Physical, chemical and mineralogical characterization of PM_{0.4-1} emissions

BWCPM_{0.4-1} and SWCPM_{0.4-1} showed higher contents of Al, Ba, Be, Ca, Co, Fe, Mg, Mn, Si, Sr and Ti than MSPM_{0.4-1} (Table 2). On the other hand, several metals (Cr, Hg, K, Mo and Ni) and the metalloid Sb occurred in higher concentrations in MSPM_{0.4-1}. The contents of the other elements found and analyzed in the PM_{0.4-1} emissions, including As and Zn were similar, and within the same order of magnitude for all three types of biomass fuel investigated. Concentrations of alkali and alkaline earth metals found in the PM from combustion of the three biomass fuels were relatively high, especially those of K and Ca: MSPM_{0.4-1} contained nearly 330 g/kg K, whereas SWCPM_{0.4-1} and BWCPM_{0.4-1} had a Ca content 63 g/kg (Table 2). It is of note, however, that the concentrations of all the analyzed elements were considerably lower in the DPM reference material with the exception of Si.

SEM-EDX investigations of PM_{0.4-1} from combustion of all three fuel types showed agglomerates of particles smaller than 1 µm across (Fig. 1). The qualitative chemical composition-as derived from EDX spectra -of the three investigated PM_{0.4-1} samples differed: MSPM_{0.4-1} was composed of small particles containing mainly K and Cl, and small amounts of fine carbon particles attached to larger particles, a few micrometers in diameter (Fig. 1B). These larger particles were composed of K and Cl and mostly had crystal shapes. BWCPM_{0.4-1} also consisted of these two particle types; however, the small particles were more abundant, contained mostly carbon, and formed skeleton-like structures encasing the larger particles, which included S in addition to K and Cl (Fig. 1D). SWCPM_{0.4-1} only exhibited particles smaller than 1 µm; these were agglomerated in 'cake-like' structures several micrometers in diameter (Fig. 1C). However, the particle composition was more complex than that of the other two fuel types: in addition to C, K, S and Cl, significant amounts of Ca were present, as well as notable amounts of Na and Mg and traces of Zn.

Phase identification by XRD analysis confirmed that the $PM_{0.4-1}$ from biomass fuel combustion contained crystalline mineral components. The XRD patterns (see Supplementary Material, Fig. S1) also revealed the presence of amorphous substances in the biomass-derived $PM_{0.4-1}$; these do not produce a distinct peak, but rather a characteristic hump in the background. The relative abundance of the amorphous portion could be estimated from the overall strength of the background signal. Of the three samples, $MSPM_{0.4-1}$ produced the lowest background signal and exhibited clear diffraction intensity peaks, which allowed for identification of sylvite (KCl) as the only crystalline phase. The presence of KCl crystals is consistent with the SEM-EDX observations (see Fig. 1B). $BWCPM_{0.4-1}$ exhibited the largest background signal, i.e. the highest content of amorphous particles. However, distinct peaks for KCl and arcanite (K_2SO_4) were present (in agreement with the SEM-EDX data), and smaller peaks revealed that minor amounts of halite (NaCl) were also present in this PM sample. $SWCPM_{0.4-1}$ exhibited a complex pattern showing the presence of the crystalline phases KCl, K_2SO_4 , free lime (CaO), periclase (MgO) and fairchildite ($K_2Ca(CO_3)_2$), which is in good agreement with the elemental data determined by SEM-EDX (Fig. 1). Altogether, XRD intensity patterns for $MSPM_{0.4-1}$ and $BWCPM_{0.4-1}$ showed alkali salts as the only crystalline substances (with crystallite sizes sufficiently large for XRD identification), whereas the XRD pattern for $SWCPM_{0.4-1}$ revealed that other crystalline phases were also present. All identified phases are typical components of biomass-derived combustion products (Maschowski et al., 2016).

The TEM images of the biomass-derived $PM_{0.4-1}$ reveal the presence of both round soot particles and angular, non-carbonaceous mineral matter (Fig. 2B,C,D). The TEM investigations indicated average geometric particle sizes of 200 nm (DPM) and 500 nm ($PM_{0.4-1}$ from biomass combustion; see Table 3) and a heterogeneous composition. The TEM image of DPM (Fig. 2A) shows an agglomerate of small (about 20 nm in diameter), spherical particles, which are typical for soot.

The DLS data show that the reproducible particle size was 0.238 μm for the DPM reference material (Table 3, Fig. 3A). The $BWCPM_{0.4-1}$ samples exhibit three distinct peaks of agglomerate sizes (0.102, 0.368 and 1.008 μm), whereas the $SWCPM_{0.4-1}$ samples show two distinct peaks of agglomerate sizes (0.225, 0.744 μm ; Table 3, Fig. 3C and D). $MSPM_{0.4-1}$ exhibits the sharpest peaks at 0.088 μm , and especially at 0.442 μm (Fig. 3B); thus, it has the most tightly constrained PM size of all studied materials (see also Table 3). PM samples with a low PDI represent a monodisperse sample, whereas particles with a high PDI value represent a poly-disperse sample (Baalousha and Lead, 2012). The $SWCPM_{0.4-1}$ and $BWCPM_{0.4-1}$ showed high PDI values (4.47 and 3.42, respectively), whereas $MSPM_{0.4-1}$ had a low PDI of 0.93 (Table 3). The DPM exhibited very good monodispersity in water (low PDI). Zeta potential measurements were performed to analyze the surface charge of the studied $PM_{0.4-1}$ samples and of the DPM reference material. All investigated samples were highly negatively charged (Table 3). Our SEM-EDX and TEM investigations confirmed the results of the DLS measurements in that almost all $PM_{0.4-1}$ emissions consisted of aggregates of fine-grained minerals and small, round soot particles associated with different concentrations of Si, Na, K, Cl, Ca and Zn. These findings are consistent with previous studies (Kobach et al., 2006, 2005). Table 3 clearly shows the difference in definition of particles sizes: aerodynamic size (normative definition of $PM_{0.4-1}$), the

hydrodynamic size (in solution), and the shape detected by TEM (geometric diameter). $PM_{0.4-1}$ is a particle size fraction defined by the aerodynamic diameter, i.e. 50% of the particles $>0.4 \mu m$ and 50% of the particles $<1 \mu m$ are trapped by the impactor; there is still the probability of trapping particles $<400 \text{ nm}$. Furthermore, the aerodynamic diameter (corresponding to a perfect sphere of 1 kg/L density) is not the same as the morphological size. As PM sampled with the impactor may have complex shapes and densities (as is the case in our study), the aerodynamic diameter may be significantly different (Coudray et al., 2009; Garra et al., 2016).

Size distribution and particle shape are important parameters for full characterization of particles used in toxicological studies. The deposition efficiency of inhaled particles in various parts of the lung varies significantly depending on particle size and shape. The particle size distribution data are important for improving our understanding of the mechanisms by which particles affect human health (Schmid and Stoeger, 2016). Nevertheless, other characteristics such as content of organics and metals, or speciation most likely also contribute to particle toxicity (Dreher, 2000; Gupta and Gupta, 2005).

3.3. Biological investigations

3.3.1. Cytotoxicity and DNA damage in A549 and BEAS-2B cells—Former studies have demonstrated that biological effects induced by wood smoke particles can activate the release of pro-inflammatory cytokines and reduce the number of epithelial cells and macrophages (Danielsen et al., 2009; Yang et al., 2010). The authors of these studies also showed that wood smoke particles can induce DNA damage, and generate reactive oxygen species (ROS). In our study the acute cytotoxicity was investigated by using the WST-1 assay with A549 and BEAS-2B cells after 24-h exposure to PM (concentration range of $1\text{--}100 \mu g/cm^2$). The dose-dependent PM-exposure effects on viability of human lung cells are shown in Fig. 4A (for A549 cells) and in Fig. 4B (for BEAS-2B cells). The diagrams document a concentration-dependent inhibition of cell proliferation compared to unexposed cells for both $SWCPM_{0.4-1}$ and $BWCPM_{0.4-1}$, but not for $MSPM_{0.4-1}$, even at the highest concentration tested ($100 \mu g/cm^2$). The results are very similar for both cell types used, albeit slightly more pronounced for the BEAS-2B cells. It is of note that the observed effects caused by $SWCPM_{0.4-1}$ and $BWCPM_{0.4-1}$ are similar to those induced by the reference DPM material (SRM 2 975). For both cell types, a significant reduction of cell viability is reached at a concentration of $30 \mu g/cm^2$ for $SWCPM_{0.4-1}$, $BWCPM_{0.4-1}$, and DPM.

The PM types studied were also tested in both cell lines (A549, BEAS-2B) for double-strand DNA (ds-DNA) damage (using DAUA). $SWCPM_{0.4-1}$ and $BWCPM_{0.4-1}$ showed similar concentration-dependent genotoxic potency with significant effects starting at a concentration of $30 \mu g/cm^2$ compared to unexposed cells of both types (Fig. 5). Again, the BEAS-2B cells showed a more pronounced effect (Fig. 5B). No genotoxic effect was observed in either of the two cell types when exposed to $MSPM_{0.4-1}$, even at the highest concentration of $100 \mu g/cm^2$. As expected, the reference DPM induced statistically significant DNA strand breaks beginning at a concentration of $10 \mu g/cm^2$ for both studied cell types. Similar results were also obtained with the Comet assay when BEAS-2B cells were exposed to the PM samples for 24 h: DPM, $SWCPM_{0.4-1}$ and $BWCPM_{0.4-1}$ induced

increases in DNA migration in a concentration-dependent manner with significance achieved at $10 \mu\text{g}/\text{cm}^2$, but no induction of DNA migration was observed for MSPM_{0.4-1} (see Fig. S2 and Fig. S3).

PAHs are known to cause genetic damage by forming adducts with DNA (IARC, 2016). Furthermore, PAHs occurring in emissions from combustion of solid biomass fuels can react with NO₂ to form direct-acting nitro-PAHs, which represent mutagens (Wickramasinghe et al., 2012). In our study, the BWCPM_{0.4-1} featured the highest concentrations of ΣPAHs (nearly 2 500 mg/kg) and induced the strongest cytotoxic and genotoxic effects, even though these were only slightly more pronounced than those induced by SWCPM_{0.4-1} (ΣPAH = 914 mg/kg). Dilger et al. (2016) demonstrated that PAH mixtures adsorbed onto wood smoke particles are more potent inducers of gene CYP1A1 expression than B [a]P alone. Furthermore, in-vitro cell studies have shown that PAHs induce inflammation and ROS (Chung et al., 2007; Øvrevik et al., 2010). Experimental animal and epidemiological studies have also confirmed that PAHs are carcinogenic (Claxton, 2014). Moreover, fuel moisture before combustion seems to be an important factor contributing to the high organic content (like PAHs) in the PM and the resulting cell-damaging potential upon interaction with human lung cells (Leskinen et al., 2014; Tapanainen et al., 2011). The high PAH levels in SWCPM_{0.4-1} and especially BWCPM_{0.4-1} (Table 1) may be caused by the high water contents of the two fuels leading to non-ideal combustion conditions. Our results agree with another study (with macrophage cell line RAW 264.7; Jalava et al., 2010), which indicated that particles from “wood combustion” containing organic components are more potent inducers of cell apoptosis than those emitted from “more complete combustion” processes with a larger fraction of inorganic compounds. In summary, our results relate to commercial biomass fuel quality (particularly the moisture content) and the toxicological potential of the PM emitted during combustion.

Another factor contributing to the absence of toxicity of MSPM_{0.4-1} might be its high content of innocuous water-soluble salts (mainly KCl). While the MSPM_{0.4-1} contained relatively high levels of Zn (>2 g/kg), it did not induce toxicological responses in the human lung cells used in this study. This is not in agreement with the results of Kasurinen et al. (2015b), who found that the Zn-rich and PAH-poor PM₁ emissions from combustion of Miscanthus (pellets) induced a strong cytotoxic response. Moreover, Kasurinen et al. (2015b). observed strong genotoxic responses in RAW264.7 cells exposed to PM₁ emissions from Miscanthus pellets, and these responses were significantly more pronounced than those induced by PM emissions from combustion of standard softwood pellets. As stated above (Sect. 3.1), the air-to-fuel interface differs completely when burning Miscanthus straw or Miscanthus pellets, which affects the particles generated (Lamberg et al., 2011) and might be responsible for the observed difference in cytotoxic responses.

Some human and animal inhalation studies (exposure) with wood smoke particles suggest that the toxic effects may be less pronounced than those found during in-vitro tests (Danielsen et al., 2008; Forchhammer et al., 2012; Sehlstedt et al., 2010). Other studies on human exposure have shown that wood smoke particles cause an inflammatory response and signs of increased oxidative stress in the respiratory tract, especially in the lower airways (Barregard et al., 2008). Furthermore, combustion emissions are associated with adverse

health impacts, e.g. cardiopulmonary and lung cancer mortality (Ghio et al., 2012; Lewtas, 2007).

3.3.2. Annexin V/PI staining for apoptotic and necrotic cells—To determine whether or not apoptosis/necrosis induced by exposure to the studied $PM_{0.4-1}$ may be related to the observed genotoxic responses, the apoptosis/necrosis response of BEAS-2B cells was evaluated by using AnnexinV/PI staining after 24 h of exposure to the $PM_{0.4-1}$ materials. For DPM, SWCPM_{0.4-1} and BWCPM_{0.4-1}, a significant increase of both apoptosis and necrosis was found at concentrations of 30 $\mu\text{g}/\text{cm}^2$ (Fig. 6). However, as in the cytotoxicity and genotoxicity tests, no effect was observed in the percentages of apoptosis and necrosis in cells exposed to MSPM_{0.4-1} (Fig. 6 and Fig. S4).

Our study demonstrates that $PM_{0.4-1}$ emissions resulting from combustion of the two investigated wood-chip types induced apoptotic/necrotic responses on a scale similar to those induced by DPM, which is a well-known human Group-1 carcinogen (IARC, 2016). However, none of these effects were observed for MSPM_{0.4-1}. In addition to PAHs, free lime (CaO) identified in the SWCPM_{0.4-1} can play a crucial role in causing the observed cytotoxic and genotoxic effects and in inducing apoptosis in human lung cells: for example, Van Berlo et al. found a significant correlation between activation of macrophage TNF- α production and CaO content during exposure to cement dust (van Berlo et al., 2009).

3.3.3. Correlations between biological responses and $PM_{0.4-1}$ components—

The Pearson correlation coefficients (r) reveal positive or negative correlations in two-tailed rank between the chemical composition of the $PM_{0.4-1}$ samples resulting from combustion of the three biomass fuels and the toxicological endpoints. In our study, we found a significant positive correlation between the Ca and Sr contents in $PM_{0.4-1}$ and all toxicological endpoints in both cell types (Table 4). In addition, Al and Σ PAHs in $PM_{0.4-1}$ from biomass fuel combustion showed a positive correlation with DNA damage; (see Table 4). These results are in agreement with the studies of Kasurinen et al. (Kasurinen et al., 2016, 2015b) who reported a correlation between Al and Ca contents of wood smoke particles and observed toxic effects. Genotoxicity of PAHs contained in PM from biomass fuel combustion was also observed in other studies (Danielsen et al., 2009; Tapanainen et al., 2011). Overall, our results are also in agreement with those of a study highlighting the importance of metals and PAH content in particle-mediated toxicity (Gerlofs-Nijland et al., 2009).

Our data revealed that the MSPM_{0.4-1} was richer in Cr than both SWCPM_{0.4-1} and BWCPM_{0.4-1} (Table 2). This element showed a statistically significant negative correlation with DNA damage (both DAUA and Comet assay) and with induction of apoptosis and necrosis (see Table 4). Therefore, we assume that Cr is not primarily responsible for the observed genotoxic effects induced by wood smoke particles of the investigated wood chips. No significant correlations with the investigated biological end points were observed for the other chemical elements analyzed, including Cu, Ti and Zn (Table 4), although Cu and Ti in their oxide form are known to induce toxic effects in human cells in vitro (Karlsson et al., 2009). However, our TEM and XRD data did not reveal the speciation of these elements.

3.3.4. Cell—particle interaction, confocal reflection and fluorescence

microscopy analysis of particle uptake by BEAS-2B cells—Particle internalization in vitro depends on particle properties, experimental conditions and cell type. Important uptake mechanisms for particles into cells are micropinocytosis, receptor-mediated endocytosis and phagocytosis, as well as diffusion or adhesive interactions (Geiser et al., 2005). Visualization of biomass combustion-derived particles in cells by confocal laser scanning microscopy faces two important challenges: i) a high spatial resolution (ability to distinguish two adjacent objects) of the confocal microscope is necessary to image particles in the size range 0.1–1 μm ; and ii) detection of particles is difficult due to the impossibility of staining them with any marker; they are highly heterogeneous in composition and thus, standard chemical bonding with fluorescent probes is not possible.

Observation of particle–cell interaction was made possible by the simultaneous acquisition of reflected light and classical fluorescence staining. In the present study, we qualitatively investigated and visualized particle internalization (with particles smaller than 1 μm) in BEAS-2B cells. The BEAS-2B cells were cultured as a monolayer in submersed conditions. After 24-h exposure of the cells to a concentration of 30 $\mu\text{g}/\text{cm}^2$ of DPM and of biomass combustion-derived $\text{PM}_{0.4-1}$, the cells were stained with DAPI (nuclei; blue) and with Phalloidin, a fluorescent dye specific for the plasma membrane (cytoskeleton; green) so that they could be studied by classical confocal fluorescence microscopy. One can see from the control image (without particles, Fig. 7A) that reflection of light (red) by cells is very limited and that only the microscope slide and coverslip are seen in the reslice images (the two red lines in plans ZX and ZY). On the contrary, in the images for cells exposed to combustion-derived $\text{PM}_{0.4-1}$ (Fig. 7), small reflecting objects (highlighted in red and acquired in confocal reflection mode) appeared. Therefore, the reflecting objects were interpreted as combustion-derived $\text{PM}_{0.4-1}$. In Fig. 7B–E; numerous particles appeared, documenting that they have been taken up by the BEAS-2B cells.

Our microscopic results suggest that $\text{SWCPM}_{0.4-1}$ and $\text{BWCPM}_{0.4-1}$ induce direct particle–cell interactions, as shown for the first time here by confocal laser scanning microscopy. Individual particles (red) in $\text{SWCPM}_{0.4-1}$, $\text{BWCPM}_{0.4-1}$ and DPM show strong adhesion to the cell membranes (green). With regard to $\text{SWCPM}_{0.4-1}$, it seems that the cells were already altered as a consequence of particle exposure: the circular shape differed from that of normal, unexposed cells (Fig. 7A) with their extended membrane networks. The orthoslice image is a good way to assess the in-depth internalization of $\text{PM}_{0.4-1}$: for example, in the orthoslice reconstructions of Fig. 7B, D, and 7E one can see some particles inside the membrane, suggesting that particles have direct access to intracellular proteins, organelles and DNA. However, no particles were observed inside the nuclei.

4. Conclusions

In this study, three commercial biomass fuels (MS, SWC and BWC) were combusted. The emissions of $\text{PM}_{0.4-1}$ particles resulting from combustion of SWC and BWC induced cell responses, but not the particle emissions from MS combustion. This result seems to be related to the completeness of combustion, which was higher for MS (low residual moisture content) than for SWC and BWC (higher moisture contents).

The results reported here document that the chemical composition of emissions from biomass fuel combustion is highly complex. Concentrations of CO, TVOC, TSP and particle-bound PAHs were high in the BWCPM_{0.4-1} and SWCPM_{0.4-1}, which might be related to incomplete combustion due to the high residual moisture of the biomass fuel. On the other hand, the concentrations of these chemical compounds were low in MSPM_{0.4-1}. In addition, the inorganic crystalline particles present in the PM_{0.4-1} were mostly water-soluble salts (especially KCl) in the case of MS combustion, whereas they were complex mixtures in the cases of SWC and BWC combustion. Based on our results, we conclude that the particle-bound PAHs in SWCPM_{0.4-1} and BWCPM_{0.4-1} are most likely the cause for the toxic effects seen in the human lung cells studied. Since the Zn contents were similar in the PM_{0.4-1} produced from all three biomass fuel types, it seemed that Zn did not have a specific influence on the toxicological responses.

To minimize the possibly harmful effects of PM emissions on health, future research on small- and medium-scale biomass fuel combustion facilities should address the following major issues: a) advanced combustion technology to solve the problem of incomplete combustion products bound to PM, b) development of advanced and efficient fly ash capture systems (cyclones, electrostatic precipitators and baghouse filters) to remove efficiently smoke particles and their toxic constituents generated during combustion of biomass fuels in domestic boilers, and c) increase the use of new biomass fuels (with low residual water content) like *Miscanthus* straw wherever ecologically justifiable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work would not have been possible without financial support from the EU Interreg IV Program (Oberrhein, project C35 BIOCOMBUST; www.biocombust.eu). This work was further supported in part by P30-ES13508 awarded by the National Institute of Environmental Health Sciences. The findings are not the official opinions of NIEHS or NIH.

The authors wish to thank: Céline Liaud and Stéphane Le Calvé (ICPEES - UNISTRA, France) for PAH analysis within the framework of the scientific collaboration between LGRE and the ICPEES laboratory, the Ammertzwiler municipality for the warm welcome to their combustion facilities, and Olivier Allgaier for helpful technical support during the combustion experiments. ATA sincerely thanks the KurdDAAD scholarship program (57076440) for awarding him a Ph.D. Fellowship.

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highlights

- Particles from nearly complete combustion (MS) did not induce cellular response.
- Particles from partial incomplete combustion (SWC, BWC) induced significant cellular response.
- Particles from SWC, BWC contain high levels of PAHs, Si and other compounds.

Fig. 1.

SEM images and EDX elemental characterization of $PM_{0.4-1}$ emissions and the reference DPM (SRM 2 975) (A) DPM (SRM 2 975); agglomerations of very small particles, which consist mainly of C along with small amounts of O, Na, and Si (B) $MSPM_{0.4-1}$; K and Cl were identified as KCl salt particles with a subhedral crystal shape of a few micrometers in diameter embedded in a C-rich porous matrix made of submicrometer-sized particles. (C) $SWCPM_{0.4-1}$; EDX data showed a uniformly distributed mixture of C, K, S, Cl, along with Ca, Mg, Na, and Zn hosted in submicrometer particles forming larger domains. (D) $BWCPM_{0.4-1}$; EDX spectra showed spots of K and Cl, or K and S interpreted as K-salt particles along with a C-rich porous matrix made of submicrometer-sized particles.

Fig. 2.

TEM images of $PM_{0.4-1}$ emissions and the reference DPM (SRM 2 975). **(A)** DPM; image shows distinct spherical particles of the same size (approx. 20 nm in diameter) forming a chain-like domain, a typical feature of soot. **(B)** MSPM_{0.4-1}; soot particles form a matrix hosting larger angular particles. **(C)** SWCPM_{0.4-1}; only agglomerates of similar angular particles are present. **(D)** BWCPM_{0.4-1}; some soot domains are accompanied by agglomerates of slightly larger, angular mineral particles.

Fig. 3.

Hydrodynamic size distributions of PM_{0.4-1} emissions and of the reference DPM (SRM 2975) Size distributions were determined by dynamic light scattering (DLS) (A) DPM; (B) MSPM_{0.4-1}; (C) SWCPM_{0.4-1}; (D) BWCPM_{0.4-1}. PM concentrations used were 1 mg/mL in water.

Fig. 4. Effects of PM_{0.4-1} and DPM (SRM 2 975) exposure on the viability of (A) A549 and (B) BEAS-2B cells, cell viability as a percentage of viable cells compared to untreated control cells (100% $\pm$ SD) was determined by the WST-1 assay in cells following 24 h exposure to different concentrations (1, 3, 10, 30, 100 $\mu\text{g}/\text{cm}^2$) of PM_{0.4-1}. Each bar represents the data of the mean ($\pm$ SD) of 3 independent experiments performed in triplicate. Asterisks indicate statistical significant reductions of cell viability as *p < 0.05, **p < 0.01, and ***p < 0.001

determined by one-way- ANOVA followed by Dunnett's post hoc pairwise to compare between exposed and non-exposed groups.

Fig. 5. Decrease in double-stranded DNA in exposed cells by PM_{0.4-1} and DPM (SRM 2 975) exposure in (A) A549 and (B) BEAS-2B cells. DNA strand breaks were quantified by the DNA Alkaline Unwinding assay after 24 h exposure with PM at different concentrations (1, 3, 10, 30, 100 µg/cm²). Positive control: EMS (5 mmol/L, 24 h exposure). The data represent the mean (±SD) of at least 3 independent experiments, performed in triplicate. Asterisks indicate statistical significance as *p < 0.05, **p < 0.01, and ***p < 0.001

determined by one-way-ANOVA followed by Dunnett's post hoc pairwise to compare between exposed and non-exposed groups.

Fig. 6.

Effects of PM_{0.4-1} emissions and DPM (SRM 2 975) on apoptosis and necrosis induction, the extent of apoptosis and necrosis in BEAS-2B cells was determined by Annexin-VFITC/PI staining and flow cytometric measurement of 3 independent experiments performed in triplicate. Cells were treated with CPT (5 µmol/L), Triton-X (0.5%) or exposed to different concentrations (3, 10, 30, 100 µg/cm²) of DPM and PM_{0.4-1} emissions for 24 h. The amount of apoptotic and necrotic cells was adjusted to unexposed controls (100% ± SD). The data represent the mean (±SD) and significance is indicated with asterisks as *p < 0.05, **p < 0.01, and ***p < 0.001 determined by one-way-ANOVA followed by Dunnett's post hoc pairwise to compare between exposed and non-exposed groups. CPT (camptothecin), Tri-X, (Triton-X).

Fig. 7. Microscopic 3D-reconstruction images of confocal acquisitions after exposure of BEAS-2B cells to $30 \mu\text{g}/\text{cm}^2$ of DPM (SRM 2 975) and $\text{PM}_{0.4-1}$ emissions. (A) Unexposed cells; (B) cells exposed to DPM (SRM 2 975); (C) cells exposed to $\text{MSPM}_{0.4-1}$; (D) cells exposed to $\text{SWCPM}_{0.4-1}$; (E) cells exposed to $\text{BWCPM}_{0.4-1}$.

1 /images: XY plan; on right: ZY plan resliced; below: ZX plan resliced.

- 2** /images: 3D-reconstruction of confocal volume, the volume being focused on the plans between microscope slide and coverslip.
- 3** /images: Orthoslice (simultaneous ZX and ZY plans) reconstruction of the confocal volume.

Staining of the cells was as follows: Green = FITC-Phalloidin-stained actin filaments, Blue = DAPI-stained nuclei. Red LUT = particles (reflected light, no staining). Scale bar shows 5 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Combustion conditions, fuel moisture and emission characteristics for the combustion experiments with Miscanthus straw, softwood and beech wood chips.

Table 1

Fuel	P _{input} [kW]	P _{output} [kW]	(P _{output} /P _{input})*100 [%]	Fuel Moisture [wt %]	CO [mg/Nm ³] ^a	TVOC [mg C/Nm ³] ^a	TSP [mg/Nm ³] ^a	WSOC [mg/g]	ΣPAHs [mg/kg]
Miscanthus straw (MS)	36.6	24.2	66.1	10 ± 2	104 ± 0.2	<10	34 ± 3	1.21	2.5
Softwood chips (SWC)	31.3	22.9	73.2	46 ± 2	3 410 ± 25	310 ± 10	114 ± 3	^b n.d	914
Beech wood chips (BWC)	42.3	30.5	72.1	32 ± 3	3 440 ± 25	210 ± 10	149 ± 3	14.6	2 458
French legislation (NF EN 12809/A1)	<50	^d	^d	^d	3 000	100 ^c	150	^d	^d

^aConcentrations for CO, TVOC and TSP in mg/Nm³ and mg C/Nm³ referred to 10 vol % O₂ in the gas phase fume.

^bNot detected.

^cLegislation is considering non-methane volatile organic compounds (NMVOCs - the experimental data from MS, SWC and BWC combustion include methane in the listed TVOC concentrations).

^dNo requirement.

Elemental composition (in mg/kg PM mass) of the PM_{0.4-1} emissions from combustion of the investigated biomass fuels and of the reference DPM, as determined by ICP-MS and ICP-OES.

Table 2

Chemical element	DPM (SRM 2 975) [mg/kg]	MSPM _{0.4-1} [mg/kg]	SWCPM _{0.4-1} [mg/kg]	BWCPM _{0.4-1} [mg/kg]	Analytical method
Be	<0.025	0.05	0.30	0.67	ICP-MS
Na	<100	1 553	1 398	1886	ICP-OES
Mg	96	6 576	12016	35933	ICP-OES
Al	<1	1 682	4 554	4 461	ICP-OES
Si	46346	19831	29315	57321	ICP-OES
K	<500	329,635	60136	180,744	ICP-OES
Ca	313	17390	63606	81379	ICP-OES
Ti	12	33	227	108	ICP-OES
Cr	<7	164	46	31	ICP-OES
Mn	9	339	2063	4 551	ICP-OES
Fe	183	2 509	4 747	3 285	ICP-OES
Co	0.3	1.9	4.0	2.9	ICP-MS
Ni	2	157	39	96	ICP-MS
Cu	20	131	132	242	ICP-MS
Zn	298	2085	2 996	1 140	ICP-OES
As	2	11	11	11	ICP-MS
Sr	2	61	213	292	ICP-MS
Zr	0.2	3	3	8	ICP-MS
Mo	0.7	30.6	6.2	6.5	ICP-MS
Cd	0.1	25.6	21.1	25.3	ICP-MS
Sn	2.6	4.6	4.4	3.3	ICP-MS
Sb	0.1	2.5	1.3	1.3	ICP-MS
Ba	6	99	1 110	718	ICP-MS
Hg	<0.1	0.52	<0.1	0.21	ICP-MS
Pb	4	112	125	86	ICP-MS

Table 3

Particle size characterization of investigated PM_{0.4-1} emissions and of the reference DPM (SRM 2 975).

Fuel	PM size distribution [μm] ^a	Polydispersity index (PDI)	PM size distribution in solution [μm] ^b	PM size [μm] ^c	Zeta potential (mV) ± SD ^d
Miscanthus straw (MS)	0.4–1	0.93	0.088 and 0.442	≈0.5	-28.31 ± 0.88
Softwood chips (SWC)	0.4–1	4.47	0.225 and 0.744	≈0.5	-24.14 ± 1.74
Beech wood chips (BWC)	0.4–1	3.42	0.102, 0.368 and 1.008	≈0.5	-30.69 ± 1.52
DPM (SRM 2 975)	1.62 ± 0.01 ^e	0.85	0.238	≈0.2	-21.45 ± 4.11

^aGravimetric mass size distribution determined by a Dekati Gravimetric Impactor.

^bHydrodynamic particle size distribution as determined by Dynamic Light Scattering (DLS) analysis.

^cGeometric diameter. Determined by Transmission Electron Microscopy (TEM).

^dSurface charge (Zeta potential) measurement by Nanoparticle Tracking Analysis (NTA).

^eSize distribution according to DPM (SRM 2 975) certification.

Table 4

Pearson correlation coefficients (r) between the chemical components of the PM_{0.4-1} samples from the three investigated biomass fuels and the biological effects in BEAS-2B and A549 cells. Significant r values listed in bold.

Compound	BEAS-2B				A549			
	WST-1	DAUA	Comet	Apoptosis	Necrosis	WST-1	DAUA	
Be	0.961	0.888	0.905	0.937	0.916	0.956	0.905	
Na	0.534	0.356	0.393	0.468	0.416	0.520	0.392	
Mg	0.869	0.755	0.780	0.829	0.796	0.861	0.780	
Al	0.927	0.982[*]	0.974	0.952	0.968	0.933	0.974	
Si	0.902	0.799	0.822	0.866	0.836	0.895	0.822	
K	-0.682	-0.813	-0.790	-0.736	-0.774	-0.694	-0.790	
Ca	0.996[*]	0.993[*]	0.997[*]	1.00^{**}	0.998[*]	0.997[*]	0.997[*]	
Ti	0.532	0.688	0.659	0.595	0.640	0.545	0.660	
Cr	-0.968	-0.998[*]	-0.995[*]	-0.984[*]	-0.992[*]	-0.972	-0.995[*]	
Mn	0.963	0.891	0.908	0.940	0.918	0.958	0.908	
Fe	0.493	0.655	0.625	0.558	0.605	0.507	0.625	
Co	0.616	0.759	0.733	0.674	0.715	0.628	0.733	
Ni	-0.652	-0.789	-0.764	-0.708	-0.747	-0.664	-0.764	
Cu	0.775	0.635	0.665	0.725	0.684	0.765	0.665	
Zn	-0.358	-0.167	-0.205	-0.286	-0.230	-0.343	-0.205	
As	n.c. ^a	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.	
Sr	0.999^{**}	0.982[*]	0.989[*]	0.998[*]	0.992[*]	1.000^{**}	0.989[*]	
Zr	0.770	0.629	0.659	0.719	0.678	0.759	0.659	
Mo	-0.933	-0.985[*]	-0.978	-0.958	-0.973	-0.939	-0.978	
Cd	-0.225	-0.413	-0.377	-0.298	-0.353	-0.241	-0.377	
Sn	-0.853	-0.734	-0.760	-0.811	-0.776	-0.845	-0.759	
Sb	-0.937	-0.987[*]	-0.980	-0.961	-0.975	-0.942	-0.980	
Ba	0.731	0.851	0.830	0.781	0.815	0.742	0.830	
Hg	-0.818	-0.916	-0.899	-0.860	-0.888	-0.828	-0.899	
Pb	-0.519	-0.340	-0.377	-0.452	-0.400	-0.505	-0.376	

Compound	BEAS-2B			A549			
	WST-1	DAUA	Comet	Apoptosis	Necrosis	WST-1	DAUA
Σ PAHs	0.950	0.987 [*]	0.889	0.924	0.900	0.945	0.989 [*]

*
p < 0.05;

**
p<0.01.

^aThe Pearson correlation coefficients (r) for arsenic were not calculable (n.c.) because the As content in all three biomass PM_{0.4-1} samples are equal (11 mg/kg), which precluded calculation by the statistical analysis using the GraphPad Prism 6 software system.