Rat inhalation test with particles from biomass combustion and biomass co-firing exhaust

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Abstract. The health effects of 6 different fly ash samples from biomass combustion plants (bark, wood chips, waste wood, and straw), and co-firing plants (coal, co-firing of coal and sawdust) were investigated in a 28-day nose-only inhalation study with Wistar WU rats. Respirable fractions of carbon black (Printex 90) and of titanium dioxide (Bayertitan T) were used as reference materials for positive and negative controls. The exposure was done 6 hours per day, 5 days per week at an aerosol concentration of 16 mg/m³. The MMAD of all fly ash samples and reference materials in the inhalation unit were in the range from 1.5 to 3 µm. The investigations focused predominantly on the analysis of inflammatory effects in the lungs of rats using bronchoalveolar lavage (BAL) and histopathology. Different parameters (percentage of polymorphonuclear neutrophils (PMN), interleukin-8 and interstitial inflammatory cell infiltration in the lung tissue) indicating inflammatory effects in the lung, showed a statistically significant increase in the groups exposed to carbon black (positive control), C1 (coal) and C1+BM4 (co-firing of coal and sawdust) fly ashes. Additionally, for the same groups a statistically significant increase of cell proliferation in the lung epithelium was detected. No significant effects were detected in the animal groups exposed to BM1 (bark), BM2 (wood chips), BM3 (waste wood), BM6 (straw) or titanium dioxide.

1. Introduction
In the current study, the health effects of six different fly ash samples from biomass combustion and co-firing plants were investigated in a 28-day nose-only inhalation study in Wistar WU rats. Respirable fractions of carbon black (Printex 90) and of titanium dioxide (Bayertitan T) were used as reference materials for positive and negative controls, as long-term experience with these materials has been gained at the Fraunhofer ITEM [1] [2] and has been reported in literature. The health effects of other relevant ambient air particles, like diesel exhaust particles (DEP), have been investigated in the past decades in various studies including animal studies, in vitro cell tests, epidemiological studies, and controlled short-term human exposure. These studies have shown two major endpoints: induction of lung tumors [1] [3], and cardiovascular effects in a sensitive human subpopulation [4]. For both endpoints, inflammatory effects in the lungs were considered to be early indicators in short-term studies. Therefore, investigation of the aerosols from biomass combustion in the present study was focused predominantly on the analysis of inflammatory effects in the lungs of rats by bronchoalveolar lavage (BAL) and histopathology.
2. Materials and Methods

2.1. Test and reference materials

The fly ash samples BM1 (bark) and BM3 (waste wood) have been sampled during pilot-scale test runs at a Mawera combustion unit from the clean flue gas (downstream multi-cyclone) by applying a sampling device consisting of a cyclone and a fabric filter. Fly ash sample BM6 (straw) was sampled directly from the filter fly ash at a grate-fired Elean power station (UK), whereas the fly ash samples C1 (Polish coal) and C1+BM4 (co-firing of coal and sawdust) were sampled at the smallest precipitation stage of the ESP (electrostatic precipitator) during large scale test runs performed at the Dolna Odra power station (Poland). All mentioned samples were subsequently sieved at 40 µm. The fly ash sample BM2 (wood chips) was produced at a small-scale test run and applying a high-temperature sampling device. The chemical composition of the fly ash samples is listed in table 1.

Titanium dioxide was purchased from Bayer AG, Krefeld, Germany (lot # 85/13928). By chemical analysis, the material was 99.5% TiO$_2$; annealing loss 0.22%; 99.5% of the TiO$_2$ was rutile. Its density was 4.26 g/cm$^3$.

The carbon black type Printex® 90 was purchased from Degussa AG. Its density was 1.8-1.9 g/m$^3$. This reference item had been used in two chronic inhalation studies in rats at the Fraunhofer ITEM [1][5]. In these studies, lung tumors had been detected after exposure to Printex 90.

| Sample                  | Ca  | Si  | Mg  | K   | Na  | Zn  | Mn  | Pb  | S   | Cl  | P   | Ti  | Fe  | Al  | Ba  | TOC | TIC |
|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Bark (BM1)             | 18.0| 1.2 | 1.5 | 16.4| 0.6 | 3.9 | 0.7 | 0.2 | 8.0 | 5.8 | 0.7 | 0.0 | 0.5 | 0.4 | 0.2 | 1.0 | 0.9 |
| Wood chips (BM2)       | 0.6 | 0.0 | 0.1 | 37.5| 1.4 | 0.8 | 0.0 | 0.0 | 9.3 | 3.4 | 0.1 | 0.0 | 0.1 | 0.0 | 0.0 | 3.0 |
| Waste wood (BM3)       | 5.7 | 1.3 | 1.0 | 12.1| 3.2 | 12.2| 0.2 | 8.2 | 2.7 | 32.9| 0.3 | 0.2 | 0.3 | 0.2 | 0.1 | 0.6 | 0.1 |
| Coal+ sawdust (C1+BM4) | 3.4 | 19.3| 1.9 | 2.5 | 1.5 | 0.0 | 0.1 | 0.0 | 0.7 | 0.0 | 0.7 | 0.7 | 4.6 | 13.9| 0.0 | 3.1 | 0.1 |
| Coal (C1)              | 3.0 | 20.2| 1.8 | 2.4 | 1.3 | 0.0 | 0.1 | 0.0 | 0.5 | 0.0 | 0.4 | 0.7 | 3.8 | 13.5| 0.0 | 5.8 | 0.1 |
| Straw (BM6)            | 12.7| 2.1 | 0.2 | 31.1| 0.2 | 0.1 | 0.0 | 0.0 | 4.3 | 28.5| 1.1 | 0.0 | 0.0 | 0.0 | 2.7 | 0.3 |

Values are presented in mass % of elements (oxygen not shown)

TOC: total organic carbon; TIC: total inorganic carbon

2.2. Animal model

Female Wistar rats (strain Crl:WU) were purchased from Charles River Deutschland, Sulzfeld, Germany. At the start of exposure, the animals were approx. 9 weeks of age. For a period of 3 weeks prior to exposure, the animals were trained to become accustomed to nose-only tubes.

2.3. Aerosol Generation and Exposure

For each exposure group, the aerosol was generated by dispersing the test item. Dispersion was achieved by a feeding system and a high-pressure, high-velocity pressurized air dispersion nozzle developed at the Fraunhofer ITEM [6]. For each nose-only exposure unit, the aerosol was generated by a high-pressure pneumatic disperser. Air flow, temperature, and relative humidity were measured continuously and recorded as 20-minute means. Limits were set at 22 °C ± 4 °C for temperature and 50% ± 20% for relative humidity. The aerosol was monitored (A) by an aerosol photometer (on-line) and (B) by determination of gravimetric concentration (weighing of filter samples once a week). The aerosol photometer signal was used in the computerized feedback loop to control the test item feeding rate. By this procedure an exposure concentration of 16 mg/m$^3$ was generated for test items and reference items.

The animals were restrained in Batelle type polycarbonate tubes; with this system animals in the supply tubes keep their noses close to the airflow at the opening of the tubes. The exposure of animals...
was performed in identical exposure chambers of cylindrical shape, each housing up to 32 animals. Control animals were exposed in an identical unit to filtered air only. The duration of the exposure was 6 hrs/day on 5 days per week for 4 weeks.

2.4. Gross Pathology/Necropsy
All animals were subjected to complete necropsy, including careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The rats were anesthetized with an overdose of pentobarbital sodium (Narcoren®) and killed by cutting the vena cava caudalis (for BAL of rats, see 2.54). Rats used for histopathological investigations (see 2.6) were killed with an overdose of carbon dioxide. The abdominal cavity was opened and the diaphragm was cut carefully allowing the lungs to collapse. Heart, esophagus, upper half of the trachea, thymus, and lung-associated lymph nodes (LALN) were removed from the lung convolute.

2.5. Bronchoalveolar Lavage (BAL)
Bronchoalveolar lavage was performed in 6 rats per group after end of exposure. The method of Henderson et al. [7] was used with minor modifications. Following preparation, the lungs were lavaged with saline using two lavages of 4 ml each. The lavage fluid was collected in calibrated tubes and the collected volume was recorded. Until processing the lavage fluid was kept on ice. Leukocyte concentration of the lavageate was determined using a counting chamber and two cytoslides were prepared with a cytocentrifuge (Shandon Co., Frankfurt, Germany) for differential cell count (macrophages, neutrophils, lymphocytes). After centrifugation of the lavage fluid, biochemical indicators relevant for diagnosis of lung damage were determined in the supernatant (lactic dehydrogenase - LDH, β-glucuronidase, total protein). These three parameters were analyzed according to routine clinical chemistry protocols using a Randox daytona device (Roche Co., Grenzach, Germany).

The bronchoalveolar lavage fluids were analyzed for tumor necrosis factor-α (TNF-α) and CINC-1 concentrations. Cytokine-induced neutrophil chemoattractant-1 (CINC-1) represents the equivalent molecule for interleukin-8 in the rat system, acting through rat CXCR2, a receptor which shows a > 85% homology to human CXCR2. CXCR2 was formerly termed Interleukin-8 receptor b. Therefore, a commercially available ELISA system for rat CINC-1 was used in this study.

Measurements were performed using the following commercially available ELISA systems:
- TNF-α: Rat TNF-α Duoset, R&D Systems GmbH, Wiesbaden (catalog number: DY510). The lower limit of quantification was 62.5 pg/ml.
- CINC-1: Rat CINC-1 Duoset, R&D Systems GmbH, Wiesbaden (catalog number: DY515). The lower limit of quantification was 15.2 pg/ml.

The bronchoalveolar samples were measured in duplicates using the respective ELISA system following the technical manual of the supplier.

2.6. Histopathology/Cell Proliferation
For 6 animals per group histopathology of lung lobes, including bronchi and the lung-associated lymph nodes (LALN) was performed. A semi-quantitative scoring of fibrotic changes was included.

Lungs were fixed in buffered formalin (10%), embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E). Masson trichrome was applied as special stain for diagnosis of fibrotic changes.

Formalin-fixed tissue of the terminal bronchioles and lung parenchymal cells were examined for cell proliferation using the sensitive S-phase response method. Proliferating cells were labeled by 5-bromo-2'-deoxyuridine (BrdU; 20 mg/ml in phosphate-buffered saline), which was administered to the animals by a minipump (Alzet type 2 ML1; 10 µl/h) 5 days prior to sacrifice. The lung section slides were prepared according to histological routine procedures and stained immunohistochemically following denaturation of the DNA (antibody technique). The antibody to BrdU was labeled by the
chromogen DAB, by which the nuclei of proliferating cells are marked brown. The nuclei of non-proliferating cells were labeled blue by hemalaun staining. The slides were evaluated by analyzing an appropriate number of airway cells and cells of the proximal regions of the pulmonary parenchyma per rat. For at least 4 terminal bronchioles, the number of positive cells and the total length of the analyzed bronchiolar epithelium were measured. From these data, the unit length labelling index was calculated in cells per mm. In four regions around each of these terminal bronchioles, the number of brown coloured cell nuclei (proliferating cells) and the number of blue coloured cell nuclei of the non-proliferating epithelial cells were counted. From these data, the labelling index of the lung parenchymal cells was calculated as percentage of positive cells.

3. Results and Discussion
The MMAD (mean mass aerodynamic diameter) of all fly ash samples and reference materials was in a similar range between 1.5 and 3 µm. Therefore, the deposition of these particles in the respiratory system was similar for all samples.

For different parameters (percentage of polymorphonuclear neutrophils (PMN), Interleukin-8 and interstitial inflammatory cell infiltration in the lung tissue) indicating inflammatory effects in the lung, a significant increase was observed in the groups exposed to carbon black (positive control), C1 (coal) and C1+BM4 (co-firing of coal and sawdust) fly ashes (see all results in table 2). Additionally, for the same groups a significant increase of cell proliferation in the lung epithelium was detected. These effects which were observed already after 4 weeks of exposure could be early indicators for the development of lung tumours. In a 2-year inhalation study with rats using a similar exposure concentration as in the present study carbon black as well as diesel exhaust particles induced lung tumours.

| Parameter                              | Control | BM1 | BM2 | BM6 | BM3 | C1+ | BM4 | C1  | TiO2 | Carbon black |
|----------------------------------------|---------|-----|-----|-----|-----|-----|-----|-----|-----|------------|
| Lung weight                            | 100     | 105 | 100 | 102 | 98  | 103 | 101 | 100 | **120**    |
| BAL results                            |         |     |     |     |     |     |     |     |     |            |
| PMN                                    | 100     | 73  | 138 | 188 | 120 | **1,099** | **1672** | 68     | **6,203**  |
| LDH                                    | 100     | 86  | 84  | 88  | 110 | 151 | 161 | 88   | **396**    |
| Total Protein                          | 100     | 103 | 106 | 97  | 99  | **143** | **148** | 95     | **218**    |
| GSH                                    | 100     | 93  | 94  | 76  | 126 | *136 | 121  | 94   | 128        |
| Interleukin-8                          | 100     | 107 | 106 | 86  | 113 | **318** | **529** | 89     | **1413**   |
| Histopathology                         |         |     |     |     |     |     |     |     |     |            |
| Lung inflammatory cell infiltration^a  | 17      | 50  | 67  | 67  | 50  | 67  | *83  | 17   | **100**   |
| Interstitial fibrosis^a                 | 0       | 0   | 0   | 0   | 0   | 17  | 33   | 0    | **100**   |
| Cell proliferation lung parenchyma      | 100     | 98  | 102 | 102 | 96  | *137 | **172** | 86    | **155**   |

Values are presented in % of controls
^a Values are shown as percentual incidence of lesion

Statistics: Comparison to control group by Dunnett's test: * p < 5%; ** p < 1%

The common characteristic of carbon black, C1 and C1+BM4 is the high content of insoluble particles
whereas the other biomass fly ash samples BM1, BM2, BM3 and BM6 mainly consist of soluble salts. After inhalative exposure these salts are dissolved very fast, can reach the capillary system and are removed by renal clearance. Therefore, the concentration of soluble fly ash particles is very low in the lung after long term inhalation whereas insoluble particles accumulate in the lung. For the insoluble particle samples C1 and C1+BM4 the effects on inflammation and cell proliferation in the lung were statistically significant but lower than the effects induced by the carcinogenic carbon black or diesel exhaust particles. For the soluble fly ash samples no adverse health effects were detected in the present study.

This means, in this rat inhalation study the percentage of insoluble particles in the fly ash samples was the important parameter which influences the potency for the induction of adverse health effects.

It has to be taken into account that the particle samples used in this study originated from state-of-the-art biomass combustion plants respectively from real-scale coal as well as biomass and coal co-firing power plants. In these installations combustion technology and combustion control are highly advanced and therefore, the gas phase burnout is almost complete and the TOC concentrations in the total ashes are <5 wt% d.b.. As a consequence only minor amounts of TOC emissions (<2 mg/Nm³) could be determined and therefore, the fine particulate emissions almost exclusively consist of inorganic salts. Moreover, particle precipitation devices such as baghouse filters or ESPs are applied in order to reduce particulate emissions. In old biomass residential heating systems on the other side, TOC emissions and consequently also soot and organic aerosol emissions can be significantly higher. In this case, the health effects caused by the fine particulate emissions might be more comparable with those of the carbon black samples applied in this study than with the samples from well controlled biomass combustion. Additionally, also polyaromatic hydrocarbons (PAHs) and dioxins may be emitted, which could induce additional health effects.

However, an important aim for future research on this topic should be to investigate the influence of the combustion quality on the health effects of the particulate emissions with special respect to biomass based residential heating systems.

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References
[1] Heinrich U, Fuhs R, Rittinghausen S, Creutzenberg O, Bellmann B, Koch W, Levens, K 1995 Inhal. Toxicol. 7 533.
[2] Muhle H, Bellmann B, Creutzenberg O, Dasenbrock C, Ernst H, Kilpper R, MacKenzie JC, Morrow P, Mohr U, Takenaka S, Mermelstein R, 1991 Fundam. Appl. Toxicol. 17 280
[3] Garshick E, Laden F, Hart J, Rosner B, Smith T, Dockery D, Speizer F 2004 Health Perspect. 112 1539
[4] Mills N, Tornqvist H, Robinson S, Gonzalez M, Darnley K, MacNee W, Boon N, Donaldson K, Blomberg A, Sandstrom T, Newby D, 2005 Circulation 112 3930
[5] Heinrich U, Dungworth D, Pott F, Peters L, Dassenbrock C, Levens K, Koch W, Creutzenberg O, Schulte A 1994 Ann. Occup. Hyg. 38 (Suppl. 1) 351
[6] Koch W 1998 Application of aerosols, in: S Uhlig, AE Taylor (Eds.), Pulmonary Research. Birkhäuser, Basel-Boston-Berlin.
[7] Henderson RF, Mauderly JL, Pickerell JA, Hahn RF, Muhle H, Rebar AH, 1987 Exp. Lung Res. 13 329