Environmental and Molecular Mutagenesis 53:125–135 (2012)

Research Article

Investigations on Cytotoxic and Genotoxic Effects of Laser Printer Emissions in Human Epithelial A549 Lung Cells Using an Air/Liquid Exposure System

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Exposure to emissions from laser printers during the printing process is commonplace worldwide, both in the home and workplace environment. In the present study, cytotoxic and genotoxic effects of the emission from five low to medium-throughput laser printers were investigated with respect to the release of ozone (O_3), volatile organic compounds (VOC), particulate matter (PM), and submicrometer particles (SMP) during standby and operation. Experiments were conducted in a 1 m^3 emission chamber connected to a Vitrocell® exposure system. Cytotoxicity was determined by the WST-1 assay and genotoxicity by the micronucleus test in human A549 lung cells. The five laser printers emitted varying but generally small amounts of O_3, VOC, and PM. VOC emissions included 13 compounds with total VOC concentrations ranging from 95 to 280 μg/m^3 (e.g., 2-butanone, hexanal, *m*,*p*-xylene, and *o*-xylene). Mean PM concentrations were below 2.4 μg/m^3. SMP number concentration levels during standby ranged from 9 to 26 particles/cm^3. However, three of the printers generated a 90 to 16 × 10^3-fold increase of SMP during the printing process (maximum 294,460 particles/cm^3). Whereas none of the printer emissions were found to cause cytotoxicity, emissions from two printers induced formation of micronuclei ($P < 0.001$), thus providing evidence for genotoxicity. As yet, differences in biological activity cannot be explained on the basis of the specific emission characteristics of the different printers. Because laser printing technology is widely used, studies with additional cytogenetic endpoints are necessary to confirm the DNA-damaging potency and to identify emission components responsible for genotoxicity. Environ. Mol. Mutagen. 53:125–135, 2012. © 2011 Wiley Periodicals, Inc.

Key words: laser printer emissions; micronucleus test; air/liquid exposure system; submicrometer particles

INTRODUCTION

As a result of rapid progress in office and home automation, the number of instruments used for computing, information, and communication has increased worldwide. Among these devices are laser printers, fax machines, and photocopiers that use toner powder for the printing process. Several studies have reported them to emit particles of varying diameter [Kagi et al., 2007; Lee and Hsu, 2007; Destaillats et al., 2008; Wensing et al., 2008; Morawska et al., 2009]. Therefore, laser printer emissions may be one of the most common personal and occupational exposures. Chamber investigations and indoor air measurements have shown that fine particles of toner and paper, as well as submicrometer particles (SMP) are released into indoor air during printing, but also gases such as ozone and various volatile organic compounds (VOC) [Brown, 1999; Bake et al., 2006; Jann and Wilke, 2006; Uhde et al., 2006; Wensing et al. 2006, 2008; He et al., 2007; Kagi et al., 2007; Schripp et al., 2008; Wolkoff, 1999]. An Australian study found that tiny particles are released from some home and office laser printers [He et al., 2007]. The researchers observed that nearly one-third of the 62

Grant sponsors: Viamedica Foundation (''Stiftung Viamedica–Stiftung für eine gesunde Medizin''), Freiburg, Germany, and nano-Control, Internationale Stiftung (International Foundation), Hamburg, Germany.

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Received 12 July 2011; and in final form 23 September 2011

DOI 10.1002/em.20695

Published online 8 November 2011 in Wiley Online Library (wileyonlinelibrary.com).

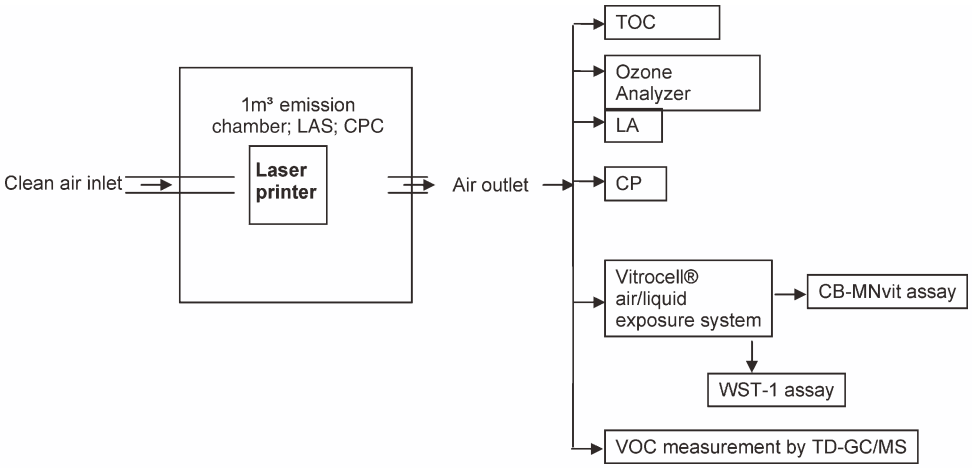

Fig. 1. Scheme of biological chamber emission system (BICES). TOC: total organic compounds; LAS: laser aerosol spectrometer; CPC: condensation particle counter; CBMNvit: Cytochalasin block micronuclei test in vitro; WST-1: viability assay; VOC: volatile organic compound; TD-GC/MS: Thermo desorption-gas chromatography/mass spectrometry.

printers tested emitted high levels of ultra fine particles with diameters <100 nm. Several other investigations have found similar results [Uhde et al., 2006; Wensing et al., 2006, 2008; Kagi et al., 2007]. For some years now, exposure to toner dusts and VOC emitted into indoor air during printing, but also during maintenance of laser printers, has been discussed as possibly being responsible for health complaints, especially regarding effects on the respiratory tract, the immunological and nervous systems [Wolkoff et al., 1992; BGIA and Verwaltungs-Berufsgenossenschaft, 2006; Evers and Nowak, 2006; Gminski and Mersch-Sundermann, 2006]. VOC, particulate matter (PM), and SMP released into indoor air are easily inhaled into the smallest passageways of the lungs, where they may pose a health hazard. Symptoms such as nonallergic rhinitis, sore throat, asthma, and pseudo allergic inflammation of the respiratory tract, irritation of the skin and eyes, headache, and sick building syndrome (SBS) seem to be linked to these emissions [Gallardo et al., 1994; Armbruster et al., 1996; Rabe et al., 2002; Gminski and Mersch-Sundermann, 2006; Jaakkola et al., 2007] Recently, it has been shown that workers with toner dust exposure from laser printers can develop submesothelial deposition of carbon nanoparticles in the peritoneum [Theegarten et al., 2010]. Additionally, copyshop workers possess significantly higher chromosomal aberrations in lymphocytes or DNA-damage in the buccal cells, indicating the influence of either toner dusts or laser printer and photocopier emissions [Goud et al., 2004; Gadhia et al., 2005; Gminski and Mersch-Sundermann, 2006; Kleinsorge et al., 2011].

Therefore, in the present study, we investigated the cytotoxic and genotoxic effects of emissions from five commercial laser printers in vitro on A549 cells using an emission chamber coupled to a Vitrocell® air–liquid exposure system. The A549 cell line represents a well-established model for alveolar epithelium that is presumably the vulnerable area of the respiratory tract for VOC and fine particles [Don Porto Carero et al., 2001; Aufderheide et al., 2003; Schwerdtle and Hartwig, 2006]. In order to correlate the physical and chemical properties of the emissions with biological effects, we determined the release of ozone, VOC, PM, as well as SMP.

MATERIALS AND METHODS

Laser Printers Investigated

The experiments were conducted with five low to medium-output laser printers: Hewlett-Parkard HP-6P (A), Hewlett-Parkard HP-2100 (B), Kyocera mita Ecosys FS-1030 (C), Kyocera mita Ecosys FS-1000+ (D), and Hewlett-Parkard HP-4550DN (E). Printers A to D were monochrome models and printer E was a color printer. All the devices were commercially available and had been used for approximately 5 to 13 years. The printing rates for the five printers A to E were 5, 10, 22, 10, and 16 pages, respectively per minute. To eliminate interference of toner powder quality, only the original toner cartridges were used.

Study Design and Emission Test Chamber

The printers were tested in a 1 m^3 emission chamber (ESPEC, Weilburg, Germany) made of high-grade stainless steel under standardized conditions (T = 23°C ± 1°C, relative humidity = 50% ± 2 %, air exchange rate: 1.0/hr). The size of the chamber needed for the tests was calculated according to the formula developed by the Federal Institute for Materials Research and Testing (BAM, Germany) as a consequence of studies on emissions from laser printers and photocopiers [Jann et al., 2003]:

$$0.01 < V_p/V_k < 0.25$$

with V_P: volume of the laser printer [m^3] and V_K: volume of the test chamber [m^3].

Using commercially available laser printers, i.e., with a volume of about 0.03 m^3 and a chamber volume of 1 m^3, the conditions of the

BAM recommendation were fulfilled. The air exchange rate was 1 hr^{-1}, indicating the time necessary to completely replace the air in the chamber. The chamber was operated in positive pressure mode by introducing medical air from the upper backside and circulating it in such a way so as to allow it to pass over heaters and coolers, thus ensuring uniform air temperature and humidity, called ''forced convection'' (Fig. 1). Air flow velocity around the devices was in the range of 0.1 to 0.3 m sec^{-1}. In accordance with the BAM Research Report [Jann et al., 2003], the model used in our study was well standardized regarding emission conditions (time, temperature, humidity, reference controls, etc.). Air samples for physicochemical analysis were collected via stainless steel sampling devices. Each printer was placed in the center of the emission test chamber and was left there overnight in standby mode. Background concentration measurements for SMP were taken until the concentration in the chamber was lower than 50 particles/cm^3. Standard black text copies with 5% coverage [DIN 33870, 2001] were printed. All the printers operated at normal speed. Air sampling was carried out at 10 min intervals (stand-by and printing). The same brand of standard quality white paper (80 g/m^2) was used in all the experiments.

Ozone

Ozone emissions were monitored by a continuous chemiluminescent-process ozone analyzer (Ozomat LUM, Anseros, Tübingen, Germany). Ozone emissions were monitored continuously during both standby and printing phases and the data generated were stored on a personal computer using the accompanying software offered by the company. Two independent experiments were performed.

Total Organic Carbon (TOC)

TOC was monitored continuously with a photoacoustic field gas-monitor (PAM, INNOVA 1412, LumaSense Technologies, Ballerup, Denmark).

Volatile Organic Compounds (VOC)

VOC measurement was done by active sampling on TENAX-TA® (Supelco Bellefonte, PA; 40/60 mesh, 200 mg, packed thermal desorption tubes with an external diameter of 6 mm and a length of 90 mm) followed by thermal desorption (TD, Series 2 Ultra 50:50 autosampler/thermal desorber, Markes International, Llantrisant, UK) and gas chromatography (HP 7890A, Agilent, Santa Clara, CA) coupled to mass spectrometry (HP 5975C mass selective detector, Agilent, Santa Clara, CA). A dual channel field sampling pump (Model 1067 Tube sampler, Supelco Bellefonte, PA) was used to collect air from the emission chamber. VOC were analyzed in clean air (chamber blank) and in the laser printer emissions during printing. For the printing phase, VOC sampling started at the beginning of the printing process, lasted for 40 min, and was performed with a sample volume of between 2 and 4 L. After sampling, the VOC were subsequently separated on a 5% diphenyl 95% dimethylsiloxane copolymer (DB-5) column (60 m × 0.25 mm × 0.25 μm). An adsorbent tube injector system (ATIS® from Supelco, Bellefonte, PA) was used to spike liquid standard solutions onto Tenax®-TA tubes. The standard solution was pulsed-heated at 90°C and transferred onto Tenax®-TA tubes in nitrogen (purity 4.6) stream at a flow rate of 50 mL/min for 3 min. The calibration range of 4 to 200 ng/tube was acquired by injecting different volumes of standard solutions as well as fluorobenzene (99.7%, Fluka, Taufkirchen, Germany) and 1,4-dichlorobenzene-D4 (99.9%, Fluka, Taufkirchen, Germany) as internal standards (ISTD) using hamilton 10 μL injectors (Hamilton, Bonaduz, Switzerland). Limit of detection (LOD) and limit of quantification (LOQ) were acquired using German standard method DIN 32645 [2008]. Identification of VOC was based on NIST 2008 library searching, retention time (RT) and retention index (RI) matching. Target compounds were quantified according to the response of specific quantification ions to internal standards. Total volatile organic compound concentrations (TVOC) were calculated as the sum of identified VOC concentrations [Jann et al., 2003; Destaillats et al., 2008]. For measurements with concentrations below the LOQ, one-half of the LOQ was taken into consideration to calculate TVOC as described in the ''guidance values for VOC in indoor air'' [AGÖF, 2008]. All VOC standards were purchased from Sigma-Aldrich (Taufkirchen, Germany). Two independent experiments were performed for each printer.

Particulate Matter (PM)

A portable laser aerosol spectrometer (LAS Dust monitor, model 1.108, Grimm Technologies, Inc., Ainring, Germany) was used to continuously measure PM. The measurement principle is based on light scattering to measure mass concentrations. The LAS was set to occupational measuring PM1.0, PM2.5, and PM10.

SMP number concentrations, i.e. particles with diameters of 10 to 1000 nm, were measured by a condensation particle counter (CPC) (Model 3007, TSI Inc. ST. Paul) based on the isopropyl alcohol envelopment of particles in its counting chamber. SMP data were also acquired continuously both at standby and printing phases. Data were analyzed using TSI software, and particle release from each printer during both the standby and printing phase was expressed as mean particle number concentration. The TSI 3007 is able to display particle number concentration from 0 to 500,000 particles/cm^3. Its calibration range works between 0 and 100,000 particle/cm^3. According to the producer's information, measurement exceeding the calibration range is possible but with lower accuracy. Two independent experiments were performed for each printer.

Cell Line and Cell Culture

A549 human lung adenocarcinoma Type-II alveolar epithelial cells were obtained from the ''Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH'' (DSMZ, Braunschweig, Germany). Adherent cells were cultured in plastic flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with low glucose (1 g/L) and with L-glutamine, 10% fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin. Trypsin and phosphate buffered saline (PBS) were purchased from PAA Laboratories (Parsching, Austria). A549 cells were cultured in a humidified incubator at 37°C and 5 vol% CO_2. Cells were harvested with 0.15% trypsin and 0.08% EDTA.

Cell Treatment

Cell exposure, including time, number of exposed cells, procedure for harvesting, and testing of micronuclei induction, etc. were standardized based on former studies dealing with the genotoxicity of VOC, e.g., Gminski et al. [2010]. For the experiments, cells were trypsinized at 80% confluency. The optimal cell density ranged from 3×10^4 cells/insert (for the CB-MNvit test) to 7×10^4 cells/insert (for the WST-1 assay). All cells were seeded on cell culture inserts (Falcon, Heidelberg, Germany) and incubated at 37°C and 5 vol% CO_2 24 hr before exposure. The cells were rinsed twice with 2 mL PBS and then inserted in a Vitrocell® air–liquid exposure system for exposure experiments. This set-up allowed a humidified microclimate around the lung cells and enabled the provision of culture medium from the basal side of the polyethylene terephthalate (PET) membrane (pore size: 0.4 μm). To study possibly toxic effects of laser printer emissions from the chamber atmosphere, the Vitrocell® devices were connected directly to the emission chamber. Air sampling was carried out for each printer during a 1-hr printing interval. The exposure time of 1 hr was chosen based on our experience with the Vitrocell-Exposure system used and that of others. Therefore, regarding this study, we did not need further pretesting. An exposure time of 1 hr is the time period in which the cells show no loss of vitality [Gminski

et al., 2010] without media exchange. For this reason, 1 hr incubation also reflects the worst-case-scenario using the Vitrocell exposure system. The cells were exposed to the aerosols on cell culture inserts at the air–liquid interface using mass flow controllers and an air flow rate of 5 mL/min. The temperature of the Vitrocell® device was adjusted to 37°C by a regulated water circuit.

WST-1 Assay

Cytotoxicity was determined using the WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) assay as an indicator of the metabolic competence of the A549 cells according to the method of Mosmann with some minor modifications [Mosmann, 1983]. After exposing the cells to printer emissions for 1 hr, cells in each insert were incubated with 500 µL WST-1 assay solution (25 µL WST-1 from Roche Diagnostic GmbH, Mannheim, Germany, and 475 µL DMEM medium without phenol red) in a humidified incubator at 37°C with 5 vol% CO_2 for 1 hr. Optical density of the colored supernatants were measured at 450 nm wavelength with 620 nm as reference wavelength using a Tecan® infinite M200 microplate reader (Tecan®, Crailsheim, Germany). In the cytotoxicity experiments, cells were exposed to medical air and to the formaldehyde (100 mg/m^3), which served as negative and positive control, respectively. Data result from six cell culture inserts from three independent experiments and are presented as means ± SD in % viability of control.

Cytochalasin-B Block Micronucleus Test (CB-MNvit Test)

CB-MNvit was carried out with slight modifications according to the OECD guideline [OECD, 2010] and the protocol of Fenech et al. [2003]. Trans-2-octenal (CAS-No. 2548-87-0) used as a positive control was purchased from Sigma-Aldrich, (Taufkirchen, Germany) with a purity of 94%. The cells in the cell culture inserts were washed twice with 2 mL PBS. Cytochalasin-B solution was prepared by dissolving 5 mg cytochalasin-B (Sigma-Aldrich, Taufkirchen, Germany) in 3.33 mL dimethylsulfoxide (DMSO, Sigma-Aldrich, Taufkirchen, Germany). Next, 1 mL of DMEM and 4 µL cytochalasin-B solution were added to the cell culture inserts and incubated for 24 hr. Cells were washed twice with 2 mL PBS and treated with 2 mL trisodium citrate solution (1.5 wt%, all reagents from Sigma-Aldrich, Taufkirchen, Germany) at 37°C with 5 vol% CO_2 for 5 min. After removing the trisodium citrate solution, cells were fixed once with 2 mL of a fixation solution containing 150 mL ethanol, 50 mL acetic acid, and 2.5 mL of 37 vol% formaldehyde (all reagents from Sigma-Aldrich, Taufkirchen, Germany). The bottom membrane of the cell culture inserts was removed and attached to a glass slide. Cells were stained with 50 µL of a 0.2 µg/mL 4′6-diamidino-2-phenylindole-dihydrochloride (DAPI, Sigma-Aldrich, Taufkirchen Germany) solution. The micronuclei were scored using a DML fluorescence microscope (Leica, Wetzlar, Germany) with the filter set to 365 nm excitation and 420 nm emission wave length that was specific for DAPI. All slides were coded before scoring. Scoring was performed at 400-fold magnification by one observer. The binucleated cells (BNC) were selected according to criteria described by Umegaki and Fenech [2000] and Fenech [2007]. For each sample, the number of micronuclei (MNBNC) in at least 1,000 BNC were examined and the MNBNC/1,000 BNC ratio was calculated. To rule out cytotoxic effects, the cytochalasin block proliferation index (CBPI) of each sample was evaluated according to Surralles et al. [1995] by assessing 500 binucleated cells. The CBPI indicates the number of cell cycles per cell during the period of exposure to cytochalasin B, and is calculated as follows:

$$\mathrm{CBPI} = \frac{(\text{No. mononucleated cells}) + (2 \times \text{No. binucleated cells}) + (3 \times \text{No. multinucleated cells})}{\text{total number of cells counted}}$$

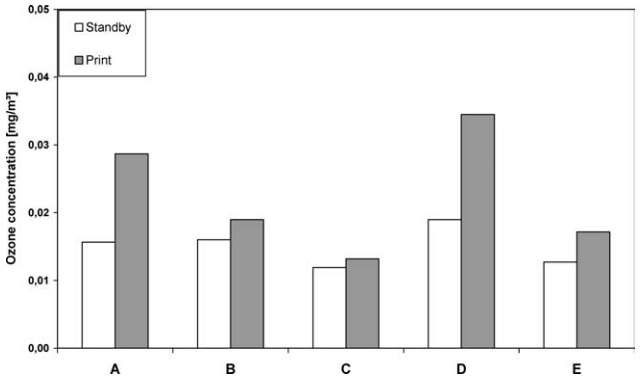

Fig. 2. Ozone concentrations emitted from laser printers A–E during standby and printing phase. Data are mean values obtained from $N = 2$ independent experiments for each printer.

In the in vitro micronucleus test, cells exposed to clean air for medical purposes and to trans-2-octenal (100 mg/m^3) served as clean air and as positive control, respectively. Additionally, ethyl methane sulfonate (EMS, 43 mmol/L)) was included as a positive clastogen control and was tested submerse as a liquid compound in an incubator (37°C and 5 vol% CO_2). Data result from five to six cell culture inserts from three independent experiments.

Statistical Analysis

Treatment-related differences for the WST-1 assay were evaluated using unpaired Students' t-test. Data shown for the micronucleus test are mean values ± SD. The $[chi]^2$ test was applied for comparison of emissions-exposed cell cultures with the clean air exposed cell culture in each set of experiments. For all assays a difference was considered significant at the level of $P < 0.05$. For statistical analysis SPSS Statistics for Windows version 17.0 was used.

RESULTS

Ozone

All five laser printers emitted only small amounts of ozone during the printing phase (Fig. 2). Mean concentrations ranged from 13 to 34 µg/m^3 and were slightly increased compared with the stand-by phases (2–18 µg/m^3). The highest ozone concentration measured during printing for the five laser printers investigated was fivefold below the hourly average ozone concentrations stipulated by EU Council Directive 2002/3/EC for the information threshold of 180 µg/m^3, and sevenfold below the alert threshold of 240 µg/m^3.

VOC and TVOC

Table I shows the concentrations of single VOC and TVOC emitted from clean air and from the emissions of the five laser printers during printing compared with clean

TABLE I. Single VOC and TVOC Concentrations Found in the Emission Chamber for Clean Air and for Emissions of the Five Laser Printers A–E

Analytes	CAS	Clean air (μg/m^3)	Printer A (μg/m^3)	Printer B (μg/m^3)	Printer C (μg/m^3)	Printer D (μg/m^3)	Printer E (μg/m^3)
1-Nonanal	124-19-6	3.03	8.54	5.13	5.93	8.61	5.12
2-Butanone	78-93-3	7.27	127.83	11.74	21.87	10.61	56.99
Acetophenone	98-86-2	3.61	3.61	3.61	3.61	3.61	3.61
Benzaldehyde	100-52-7	7.25	11.35	8.79	10.53	26.54	7.68
Benzene	71-43-2	2.56	2.56	2.56	5.34	2.56	2.56
Hexanal	66-25-1	6.17	70.23	9.71	76.20	11.39	7.44
m,*p*-Xylene	108-38-3 106-42-3	3.78	10.15	15.38	12.39	34.18	8.02
n-Pentadecane	629-62-9	2.48	2.48	2.48	2.48	2.48	2.48
o-Xylene	95-47-6	4.64	3.81	7.66	3.88	66.81	9.19
Phenol	108-95-2	5.68	5.68	5.68	5.79	5.68	5.00
Styrene	100-42-5	4.73	13.63	4.73	4.73	4.73	4.73
Toluene	108-88-3	3.90	3.90	3.90	18.40	3.90	3.90
Valeraldehyde	110-62-3	3.82	5.27	4.03	7.83	4.90	5.83
TVOC		**60.06**	**280.38**	**94.06**	**180.48**	**191.25**	**109.37**

Data represent mean values obtained from two independent experiments ($N = 2$) for each printer.

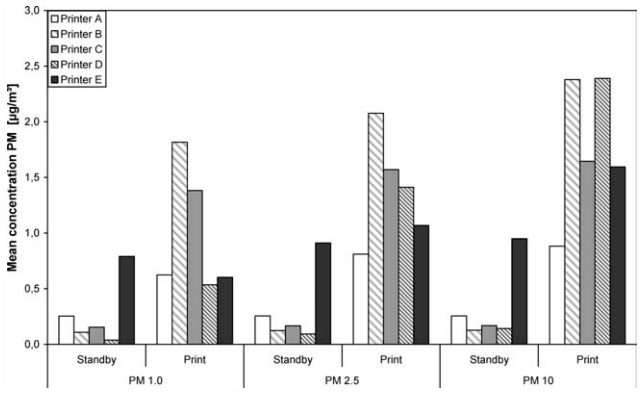

Fig. 3. Mean concentrations of PM1.0, PM2.5, and PM10 of laser printers A–E during standby and print phases. Data are mean values obtained from $N = 2$ independent experiments for each printer.

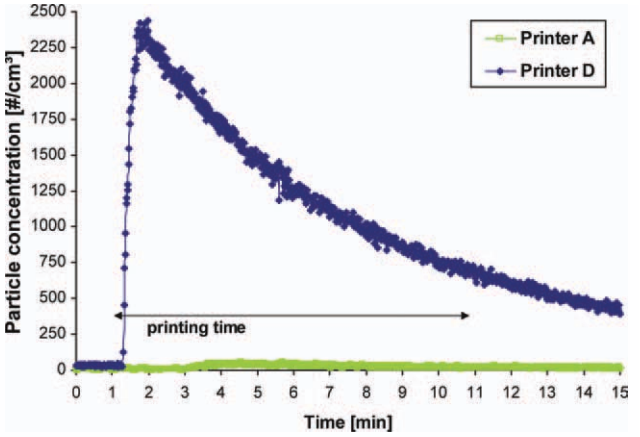

Fig. 4. Representative real-time particle concentration measurements for a nonemitter (A) and a high-emitter (D) (laser printer emission A: showing no micronuclei induction in the CBMNvit test after printing for 1 hr; laser printer emission D: showing micronuclei induction after printing for 1 hr). Printer D exhibits the curve of a typical ''initial-burst'' emitter. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

air. The TVOC concentrations exhibited considerable variability, ranging from 94 to 280 μg/m^3 in comparison with clean air. The concentrations of VOC and TVOC generated by the printing process were always higher than those in clean air. Thirteen different VOC were identified and quantified in the emissions from the laser printers (Table I). The most frequent VOC found at higher concentrations were 2-butanone, *m*,*p*-xylene and *o*-xylene. In two cases (laser printers A and C), hexanal was detected. Beside the listed VOC, further compounds were found in the laser printer emissions such as hexamethylcyclotrisiloxane (D3), octamethylcyclotetrasiloxane (D4), and decamethylcyclopentasiloxane (D5). They exhibited high peaks but were not quantified.

PM and SMP

Figure 3 represents a summary of the mean mass concentration of PM1.0, PM2.5, and PM10 emissions from the five laser printers investigated. PM emission from all five laser printers was usually low (<2.4 μg/m^3). Printers D and E preferentially emitted PM2.5 and PM10, whereas printers B and C emitted relatively high amounts of PM1. The highest release of PM10 was observed for printers B and D. For printer E, a slight increase in mean particle mass concentration was found only for PM10 (from 0.95 to 1.59 μg/m^3). Figure 4 shows exemplary for a nonemitter (printer A) and a high-emitter (printer D) time versus particle concentration profiles for SMP released from laser printers A and D. Particle emission from printer A (nonemitter) increased from background concentrations (13 particle/cm^3) to only 50 particle/cm^3. In contrast, the profile of printer D (emitter) showed a typical

TABLE II. Mean Particle Number Concentrations [#/cm^3] of SMPs (10–1,000 nm in Diameter) Emitted During Standby and Printing Phases of the Laser Printers A–E

	Particle number concentration (particle number/cm^3)			
	Standby		Printing	
Printer	Exp. 1	Exp. 2	Exp. 1	Exp. 2
A	8	8	31	16
B	14	7	15	12
C	6	27	294,460	257,804
D	8	44	3009	1,416
E	7	12	22,178	18,218

Data were evaluated from two independent experiments.

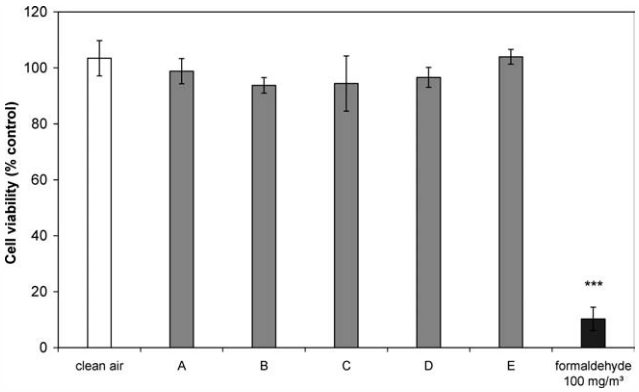

Fig. 5. Cytotoxicity as assessed by the WST-1 assay in human lung cells A549 following 1 hr exposure to clean air, laser printer emissions A–E and to the positive control formaldehyde (100 mg/m^3). Data are mean values ± standard deviation (MV ± SD) obtained in three independent experiments from $N = 6$ cell culture inserts. ***$P < 0.001$ versus clean air cultures (unpaired Students' t-test).

''initial burst.'' In this case, SMP emission increased to about 2,500 particles/cm^3. Table II presents the mean particle number concentration levels for all the investigated printers. Printers C, D, and E showed to be emitters with increased SMP concentrations, whereas printers A and B impressed as nonemitters. For printers C, D, and E, mean SMP concentrations increased by the factors 1.6×10^4, 0.9×10^2, and 2×10^3, in comparison with the respective stand-by phase. The highest emission of SMP was observed for printer C, with a mean particle number concentration of 294,460 particles/cm^3.

Cytotoxicity

Cytotoxic effects in A549 cells caused by laser printer emissions are displayed in Figure 5. No significant effect was observed for any of the printer emissions compared with clean air after 1 hr of exposure. In contrast, cell viability of formaldehyde (100 mg/m^3) as a known cytotoxic and irritant positive control was reduced to 10.27% ± 4.22% compared with clean air ($P < 0.001$, unpaired Students' t-test).

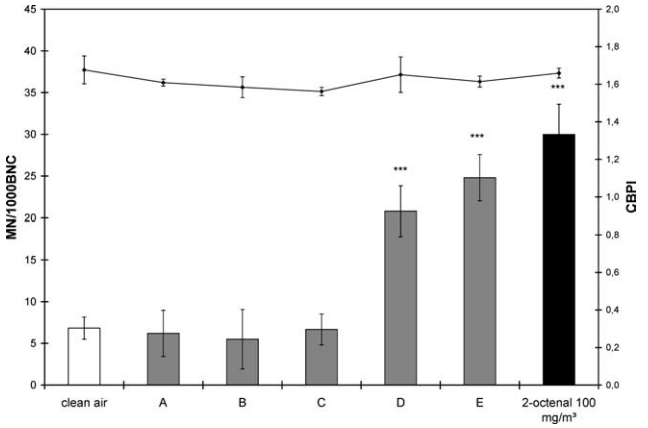

Fig. 6. Micronuclei induction (bars) and cytokinesis block proliferation index (CBPI, lines) after exposure of human lung cells (A549) to emissions of laser printer A–E for 1 hr. Positive control: 2-octenal (100 mg/m^3). Positive incubator control: EMS (43 mmol/L): 19 ± 2 MK/1000 BNC; CBPI: 1.62 ± 0.02). Data are mean values ± standard deviation (MV ± SD) obtained in three independent experiments from $N = 5$–6 cell culture inserts. ***$P < 0.001$ versus clean air cultures ($[chi]^2$ test).

CB-MNvit Test

The results of the MNvit test are shown in Figure 6. The CPBI value as an index of cell proliferation inhibition was similar to the clean air control and ranged between 1.6 and 1.7. From these data it seems that the CBPI was not affected by either clean air, laser printer emissions, or the positive control 2-octenal (100 mg/m^3). Whereas the positive controls 2-octenal (100 mg/m^3, tested as a gaseous compound in the Vitrocell® exposure system) and ethyl methane sulfonate (EMS, 43 mmol/L, tested submerse as a liquid compound in an incubator) induced a significant increase in micronuclei induction ($P < 0.001$, $[chi]^2$ test), the emissions from laser printers A, B, and C did not elicit significant induction of micronuclei ($P > 0.05$, $[chi]^2$ test) in comparison with clean air. In contrast, a significant induction of micronuclei was observed in A549 cells after 1 hr of exposure ($P < 0.001$, $[chi]^2$ test) to emissions from printers D and E. The number of micronuclei induced by printer emissions D and E were 20.8 ± 3.0 MK/1000 BNC and 24.8 ± 2.8 MK/1000 BNC, respectively, as compared with clean air (6.8 ± 1.3 MK/1000 BNC).

DISCUSSION

In the present study, we investigated the ability of laser printer emissions to cause biological effects in a human

Type-II adenocarcinoma cell line (A549) based on the assumption that a correlation exists between cytotoxicity, genotoxicity, and the release of ozone, VOC, PM, and/or SMP. To the author's knowledge, this is the first in vitro experiment of its kind. The idea that laser printer emissions can cause biological effects is based on the fact that commercial toner powders showed genotoxic effects in the same cell line under submerse cell culture conditions [Gminski et al., 2011]. However, despite using laser printers or photocopiers, people are rarely directly exposed to toner powder itself. Direct exposure to toner particles with diameters between 5 and 7 μm normally affects the skin and the upper respiratory tract rather than the lungs. Former studies [Goud et al., 2004; Gadhia et al., 2005] found genotoxic effects in blood cells and significant chromosomal aberrations in lymphocytes as well as micronucleus induction in buccal cells in copyshop workers. Unfortunately, these studies are not suitable to differentiate between toner powder exposure and exposure to emissions from laser printers, which definitely contain no or only very small amounts of particulate toner components [BfR, 2008]. Therefore, the present investigation focused on the biological effects caused by exposure of human lung cells to laser printer emissions, i.e. the complex mixtures of ozone, VOC, and particles, using an emissions chamber connected to an air–liquid exposure system.

The human Type-II alveolar adenocarcinoma cell line (A549) was used in the study presented here. This cell line has already been evaluated in numerous studies and represents a useful model for identification of cytotoxic and mutagenic effects of chemical compounds and emissions [Aufderheide and Mohr, 2000; Don Porto Carero et al., 2001; Kreja and Seidel, 2002; Bakand et al., 2005; Schwerdtle and Hartwig, 2006]. Although limited information is available about the sensitivity of A549 cells to particle exposure, previous studies in our lab showed A549 to respond well to particulate matter [Gminski et al., 2011]. The cultured A549 cells were used in a Vitrocell® air–liquid exposure system that allows to model inhalation exposure by direct exposure of airborne substances to the cell surface. The air–liquid system was first developed at the Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM) in Hannover (Germany) to investigate the toxicological effects of tobacco smoke [Aufderheide and Mohr, 2000; Ritter et al., 2001; Aufderheide et al., 2002, 2003; Knebel et al., 2002; Wolz et al., 2002; Aufderheide, 2008]. The Vitrocell® system has recently been used in several scientific studies and has emerged as a useful instrument for in vitro investigation of VOC and particles [Diabate et al., 2002; Pariselli et al., 2006, 2009; Seagrave et al., 2007; Paur et al., 2008; Gminski et al., 2010]. Although the Vitrocell® system does not reflect the situation in the human respiratory tract, it does provide a useful instrument for estimation of the biological effects in human cells. Former studies evaluating the system showed that biologically active compounds can be successfully examined with the Vitrocell exposure system [Seagrave et al., 2007; Pariselli et al., 2009]. Thus, the Vitrocell® exposure system is sufficiently well evaluated and standardized. Moreover, based on the suggestion that airborne compounds reach the target cells of smaller airways and alveoli in the human lung—which can be assumed of VOC and ultrafine particles—biological effects seen in the Vitrocell system indicate that the compounds tested might also possess biological activity in the human respiratory system.

The cytochalasin block micronucleus test (CB-MNvit) has been used for decades and constitutes a method for detection of cytogenetic effects, i.e. chromosomal changes such as acentric chromosome and chromatid fragments or chromosome lagging in anaphase [Fenech, 1996; Stopper and Müller, 1997; Miller et al., 1998; Fenech et al., 2003]. Additionally, with this assay, cell division kinetics can be estimated as a cytokinesis block proliferation index (CPBI). An OECD protocol [OECD, 2010] exists for the in vitro micronucleus assay; thus, this test has been completely evaluated with regard to the test procedure, the analysis of micronuclei, and the interpretation of the results. However, use of cytochalasin is potentially a problem with regards to the study of ultrafine/nanomaterials because cytochalasin B inhibits endocytosis, which is an important cell uptake mechanism for some nanomaterials [reported, e.g., for single-walled carbon nanotubes, SWCNT; Doak et al., 2009].

Ozone is a well-known compound with DNA-damaging properties. With respect to ozone emissions, all of the printers under examination here emitted only small amounts of ozone ($\leq$34 μg/m^3). These data are in concordance with results obtained by the Federal Institute for Materials Research and Testing (BAM), Berlin, Germany, which examined emissions from laser printers and photocopiers of varying make [Jann and Wilke, 2006]. In that particular study, ozone emissions were not detectable for most of the desktop devices. However, it should be mentioned that ozone levels of $\geq$60 μg/m^3 were found to react with terpenes *d*-limonene and terpinolene forming potentially irritant and toxic compounds such as formaldehyde, glycolaldehyde, acetone, and formic acid [Destaillats and Lunden, 2006]. Compared with the ambient air, most of the reactive compounds are not expected in the chamber air because of the lack of precursors.

Release of VOC from laser printers is probably related to the toner production process and to additives such as thermo-plastic resin (e.g., styrene-based) and charge control agents. Additionally, organic solvents are required to produce toner particles, which may be present in the finished product as residues because of the sorbent character of toner powder. When heat is applied during the printing process, these residues may be released from the toner particles. For example, styrene, *m*,*p*-xylene,

and *o*-xylene have been described as emitting from printer materials [Naoki et al., 2004], and were also detected in our experiments. Benzaldehyde, hexanal, and 2-butanone, which were found in our study, have also been detected in other studies [Wolkoff, 1999; Jann et al., 2003]. To our knowledge the VOC shown in Table I have not been tested before in the micronucleus test or the comet assay using the Vitrocell exposure system. Hexanal was tested in our lab for genotoxicity in A549 cells using the comet assay and an air–liquid exposure system, but was not found to damage DNA even in g/m^3 concentrations [Gminski et al., 2010]. In our study, siloxanes, and especially the cyclosiloxanes D3, D4, and D5, were present in the laser printer emissions. D4 has been tested for genotoxicity in a number of in vitro and in vivo studies, but gave no indication of possessing genotoxic potential [Vergnes et al., 2000]. D4 has been tested in the in vivo micronucleus test where it did not possess genotoxic potential. So far, no trials have been conducted to investigate the ability of siloxanes and cyclosiloxanes to induce micronuclei in A549 human lung cells using the Vitrocell exposure system. This will be the focus of further investigation.

Although the VOC concentrations in the chamber air were rather low, each analyzed printer had its special emission profile, and some of the compounds emitted are on the list of potentially harmful chemicals. No clear correlation was observed between VOC concentrations and biological effects; printers D and E, which induced micronuclei, showed neither a high level of single VOC or TVOC. Altogether, emissions from laser printers display a complex mixture of VOC with so far unknown interactions among each other and other components in the ambient air.

As described in former studies, toner powders and laser printers are a source of PM, which may contribute to the genotoxic effects [Bai et al., 2010; Gminski et al., 2011]. In the study by Gminski et al. [2011] iron oxide-based toner powder was polluted with traces of heavy metals and polycyclic aromatic hydrocarbons (PAH). Therefore, a possible cause of DNA damage by toner powders through either the pro-oxidative effects of iron oxide (magnetite) or genotoxic PAH must be discussed. PM emitted from printers may be partly toner powder that has not completely bound to paper.

Whereas none of the five printer emissions exhibited cytotoxicity, two models (printers D and E) significantly induced micronucleus formation. It was suspected that this effect is associated with the release of particles during the printing process, especially ultrafine/nanoparticles. It is well known, that inhalation of ultrafine particles (UFP) can cause inflammation and generation of reactive oxygen species (ROS). Damage to DNA by ROS is a crucial step in the genotoxicity process by particles [Donaldson et al., 2005, 2010]. However, laser printer C emitted large amounts of SMP without inducing any biological effect in A549 cells. The SMP emissions from laser printer C may possibly differ in quality, or, for so far unknown reasons, did not act on the cells. Already published studies describe how fine and ultrafine particles penetrate the cell membrane and cause oxidative stress resulting in damage to numerous macromolecular structures, e.g., DNA [Møller and Loft, 2010; Könczöl et al., 2011].

It should be mentioned that SMP with diameters ≤ 0.1 μm can also be formed by condensation of VOC or SVOC as secondary particles [Naoki et al., 2004; Namiki et al., 2006]. This observation may therefore lead to the conclusion that SMP that appear during the printing process are mostly not toner particles but condensation products formed inside the printer or in the ambient air [Kagi et al., 2007; Morawska et al., 2009]. This observation is important because when measurement is conducted by CPC SMP are misinterpreted as being compact particles containing compounds other than VOC and water, i.e. fractions or substances of crushed toner particles. Actually, as shown in the BfR study [BfR, 2008] by transmission electron microscopy (TEM), 1% to 10% of the particle number concentrations measured by CPC are indeed compact particles containing elements of the toner powder. The question whether or not secondary particles contribute to the biological effects investigated in the present study must be the subject of further investigations. Additionally, generation of SMP from SVOC, such as flame retardants, i.e. tri-xylyl phosphate, napthalenes, or siloxanes [Wensing et al., 2008] is also feasible, and should also be taken into consideration as possible DNA damaging compounds. Most of the studies performed have concluded that laser printers can be significant sources of ultrafine particles (<100 nm diameter), the particles are of volatile nature, being formed in the air from VOC. They originate from both the paper and hot toner [He et al., 2007, Morawska et al., 2009]. Indeed, in order to evaluate the composition of PM or SMP found in the emissions of the five laser printers investigated, more detailed studies, for example, using scanning mobility particle sizer (SMPS) combined with a differential mobility analyzer (DMA) or transmission electron microscopy (TEM) combined with energy dispersive X-ray spectroscopy (EDX), are necessary.

The ''initial burst'' observed in our investigations has also been reported by other researchers [Schripp et al., 2008]; however, so far, the reason for it has not been identified. During the printing/photocopying process, heat and pressure is applied to bond the toner particles to the paper. Therefore, it is expected that some toner powders, or other accompanying particles, are emitted into the ambient air by heating and ventilation. Additionally, it is suspected that an ''initial burst'' may also be formed

from components of the paper (water mist, fibers). Another possibility for the ''initial burst'' described in the literature may be a chemical reaction of VOC with other substances [Kamens and Jaoui, 2001]. Lee and Hsu also reported a correlation between ozone and ultrafine particles (<50 nm) generated from photocopy machines [Lee and Hsu, 2007]. Even low levels of ozone can react with other indoor pollutants to produce secondary pollutants of ultra fine aerosol particles [Destaillats et al., 2008]. Printer C showed high SMP concentrations in CPC measurements but no potential to induce micronuclei. A possible explanation for this phenomenon is emission of a higher amount of very volatile organic compounds (VVOC) and water mist detected for printer C using a photoacoustic monitor (data not shown). This suggestion must be proved in further studies.

Although only five laser printers were investigated, our results clearly demonstrate that laser printer emissions have the potential to induce genotoxic effects in A549 lung cells. It should be noted that the quality and concentration of VOC and particles generated during printing in the 1 m^3 emissions test chamber was higher than is to be expected in an office room situation. Moreover, a high exposure time of 1 hr was chosen to achieve biological responses in the human lung cell assay used. In order to evaluate the relevance of our findings in the micronucleus assay, more detailed studies, especially more mechanistically oriented analyses, are necessary.

ACKNOWLEDGMENTS

The authors thank Deborah Lawrie-Blum for language assistance.

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Accepted by—
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