# Application of an optimized system for the well-defined exposure of human lung cells to trichloramine and indoor pool air

C. Schmalz, H. G. Wunderlich, R. Heinze, F. H. Frimmel, C. Zwiener and T. Grummt

## ABSTRACT

In this study an *in vitro* exposure test to investigate toxicological effects of the volatile disinfection by-product trichloramine and of real indoor pool air was established. For this purpose a set-up to generate a well-defined, clean gas stream of trichloramine was combined with biotests. Human alveolar epithelial lung cells of the cell line A-549 were exposed in a CULTEX® device with trichloramine concentrations between 0.1 and 40 mg/m$^3$ for 1 h. As toxicological endpoints the cell viability and the inflammatory response by the cytokines IL-6 and IL-8 were investigated. A decreasing cell viability could be observed with increasing trichloramine concentration. An increase of IL-8 release could be determined at trichloramine concentrations higher than 10 mg/m$^3$ and an increase of IL-6 release at concentrations of 20 mg/m$^3$. Investigations of indoor swimming pool air showed similar inflammatory effects to the lung cells although the air concentrations of trichloramine of 0.17 and 0.19 mg/m$^3$ were much lower compared with the laboratory experiments with trichloramine as the only contaminant. Therefore it is assumed that a mixture of trichloramine and other disinfection by-products in the air of indoor pool settings contribute to that effect.

**Key words** | cell viability, CULTEX®, cytokines IL-6 and IL-8, human lung cells exposure, indoor pool air, trichloramine

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## ABBREVIATIONS

| | |
|---|---|
| A-549 lung cells | human alveolar epithelial carcinoma cell line A-549 (ACC 107, DSMZ, Braunschweig) |
| AOX | adsorbable organically bound halogens |
| DBPs | disinfection by-products |
| DOC | dissolved organic carbon |
| DPD | *N*,*N*-dipropyl-*p*-phenylenediamine |
| DSMZ | German Collection of Microorganisms and Cell Culture |
| GSTT1-1 | glutathione S-transferase theta |
| $IC_{50}$ | 50% inhibitory concentration |
| IL-6 | interleukin 6 |
| IL-8 | interleukin 8 |
| $LC_{50}$ | 50% lethal concentration |
| $NCl_3$ | trichloramine |
| $NO_2$ | nitrogen dioxide |
| PBS | phosphate buffered saline |
| SPW | swimming pool water |
| THMs | trihalomethanes |

## INTRODUCTION

Chlorination is the typical and inevitable method for disinfection of swimming pool water (SPW). Chlorine not only inactivates pathogens but it can also react with the bathers load and with raw water constituents to form partly toxic disinfection by-products (DBPs). Some of the formed DBPs are

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sufficiently volatile to partition from the water to the gas phase. Most measured volatile DBPs in indoor pool air are trihalomethanes (THMs) and trichloramine ($NCl_3$). The quality of indoor pool air depends on the quality of the water, the mass transfer processes dependent on active stripping effects caused by whirls and water attractions and the air exchange rate of the swimming pool hall. The formation, exposure pathways and toxicology of THMs have been studied over the past 30 years. The main uptake of THMs has been identified as the respiratory pathway followed by dermal uptake. Data are based on analyses of urine, plasma and alveolar air samples of swimmers, pool workers and swimmers breathing clean air from scuba tanks (Levesque *et al.* 1994; Fantuzzi *et al.* 2001; Erdinger *et al.* 2004; Caro & Gallego 2007, 2008). Carcinogenicity of chloroform for humans is given by all routes of exposure under conditions that would lead to cytotoxicity; that is, under high-exposure conditions (Richardson *et al.* 2007). Genotoxicity studies showed that brominated THMs were mutagenic after activation by gluthathione S-transferase theta (GSTT1-1) (DeMarini *et al.* 1997; Pegram *et al.* 1997; Richardson *et al.* 2007; Zwiener *et al.* 2007). Dermal and inhalation exposure would permit the activation of selected THMs by GSTT1-1 in target organs, such as the bladder, whereas oral exposure results in the inactivation of these THMs by enzymes in the liver. An increased risk of bladder cancer for swimmers versus non-swimmers was shown in an epidemiological study (Villanueva *et al.* 2007).

Barbee *et al.* (1983) have undertaken a study to determine the acute toxicity of $NCl_3$ from inhalation exposure of 1 h. Besides that, not much is known of its toxicology. The $LC_{50}$ (50% lethal concentration) for $NCl_3$ in rats was determined to be 112 ppm (~550 mg/m$^3$). The acute inhalation toxicity of $NCl_3$ is higher than that of chlorine with a $LC_{50}$ of 293 ppm (Back *et al.* 1972).

Detailed studies of the occurrence of trichloramine in indoor pool halls were performed just recently. Hery *et al.* (1995) developed a method for the determination of trichloramine concentrations in the air. Adverse health effects on respiratory functions and on the risk of developing asthma from attending chlorinated swimming pools were reported in epidemiological studies (Bernard *et al.* 2003; Levesque *et al.* 2006; Nickmilder & Bernard 2007). Often trichloramine has been suggested to cause eye and upper respiratory tract irritations (Massin *et al.* 1998), biomarker changes in the lung and an increase in lung permeability. Hery *et al.* (1995) and Gagnaire (1994) reported on irritative complaints of the eye and throat among pool attendants and irritative effects on mice at trichloramine concentration levels higher than 0.5 mg/m$^3$ in the air. The proposed INRS (French Institute for Occupational Health and Safety) guideline value of 0.5 mg/m$^3$ for $NCl_3$ for indoor pool air is based on these investigations.

Trichloramine is four times more volatile than chloroform and partitions to the air of swimming pools (Sander 1999). Determinations of $NCl_3$ in indoor pool air reveal concentrations between 0.1 and 18.8 mg/m$^3$ (Hery *et al.* 1995; Stottmeister & Voigt 2006). Typical $NCl_3$ air concentrations in German indoor pools are between 0.1 and 0.5 mg/m$^3$ (Zirbs 2008). $NCl_3$ itself is unstable in water and in higher concentrations an explosive compound of penetrating odour. Because of its low water solubility it can easily penetrate into the lower airways of bathers. Here it can cause harm to the non-ciliated bronchiolar Clara cells and to the permeability of the lung epithelium. The results could be dysfunction and manifestation of adverse effects at continued exposure to $NCl_3$. In addition its role as an asthma-causing reagent has been hypothesized (Bernard *et al.* 2003; Lagerkvist *et al.* 2004; Bernard *et al.* 2006). However, at present, there is a lack of substantial information on toxicological effects caused by $NCl_3$ and indoor pool air. Consequently, further investigations are needed to analyse the effects on human health. Conventional *in vitro* studies, in which chemicals are dissolved in culture medium, are of limited value for studies of volatile compounds and cannot be used for $NCl_3$ which is unstable in aqueous solutions. The CULTEX® device allows the exposure of cultured cells to gaseous compounds at an air/liquid interface. This system was tested in several applications for the investigation of tobacco smoke (Wolz *et al.* 2002; Aufderheide *et al.* 2003), volatile chemicals (Pariselli *et al.* 2009a, b; Persoz *et al.* 2010), native gaseous compounds (Ritter *et al.* 2001) and diesel engine exhaust (Knebel *et al.* 2002). The advantage of the system is that the cells are supplied with medium through membrane systems called transwells and that the cultured cells are directly exposed to gaseous samples.

The aim of this study was to establish an *in vitro* exposure test to investigate toxicological effects of gaseous trichloramine. The test system was applied in lab experiments and in

exposure tests with indoor pool air. A configuration for generation of a controlled continuous flow of gaseous trichloramine was adapted to the CULTEX® device. For this study, cells of the human alveolar epithelial cell line A-549 were exposed and cytotoxic and inflammatory effects were evaluated.

## MATERIAL AND METHODS

### Trichloramine generation, analysis and exposure unit

The set-up of the trichloramine exposure unit consisted of six parts: (1) generation of the trichloramine gas; (2) cleaning step; (3) analysing cell; (4) dilution of the gas stream; (5) exposure chamber; and (6) final control of the trichloramine concentration of the diluted gas stream (Figure 1).

### Trichloramine generation and dilution

All chemicals used for trichloramine generation were pro analysi reagent grade and obtained from Merck (Darmstadt, Germany), except sodium hypochlorite solution (12 wt% chlorine) which was purchased from Roth (Karlsruhe, Germany) and 2,2,4-trimethylpentane (iso-octane) from Sigma-Aldrich (Taufkirchen, Germany). Attention: $NCl_3$ is in higher concentrations an explosively unstable compound when heated above its boiling temperature of 71 °C and when catalysed by light or in the presence of heterogeneous catalysts. Isolation of the pure compound and concentrations higher than 2 g/L in water should be avoided (Schlessinger 1966; Savickas *et al*. 1989).

Trichloramine was produced in a stirred 1-L four-neck round bottom flask, which contained 60 ml phosphate buffer (pH 2; 0.17 mol/L) and an initial concentration of 0.3–0.6 mmol/L ammonium chloride and 1.5–3.0 mmol/L sodium hypochlorite. A solution of ammonium chloride (0.5–2 mmol/L) and sodium hypochlorite (2.5–10.0 mmol/L) was continuously dosed from two 100 mL dropping funnels (dropping flow rate of 0.7 mL/min). With a nitrogen flow rate of 100 mL/min the produced trichloramine was continuously transferred to four washing bottles in order to eliminate any chlorine and other chloramines. The first two flasks contained 100 mL amido sulfonic acid solution (2 g/L) each and the last two contained 100 mL distilled water each. The concentrations of free chlorine and combined chlorine were measured in the distilled water of the last washing bottle with the photometric DPD (*N,N*-dipropyl-*p*-phenylenediamine) method (Spectroquant chlorine cell test from Merck) to check the purity of the produced $NCl_3$ gas. The $NCl_3$ gas stream was split into two parts. One partial gas stream was trapped in 10 mL of cooled *i*-octane and analysed UV-spectrophotometrically every 10–30 min.

The second partial flow was diluted with synthetic air to give the required concentration. Dilution factors between 100 and 400 were adjusted with a needle valve and controlled with rotameters in each gas stream. The produced gas then flowed to a distribution system from which a much smaller but defined gas stream was sucked into the exposure unit. At

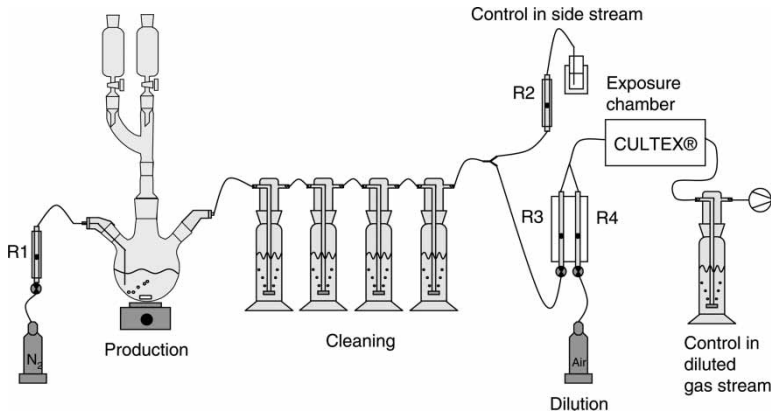


**Figure 1** | Set-up of the trichloramine generation, analysis and exposure method (R = rotameter).

the final gas outlet the $NCl_3$ concentrations of the diluted gas stream were controlled after reduction to chloride. For this purpose a gas stream of 1,000 mL/min was sucked through an impinger filled with 100 mL of an aqueous solution of arsenic(III)-oxide (0.05 g/L) and sodium carbonate (0.4 g/L) by a vacuum pump (PCXR8K universal sampling pump, SKC Inc., Eighty Four, PA). After an initial time period of about 90 min the $NCl_3$ production system reached a steady-state and gas streams of constant $NCl_3$ concentrations between 0.1 and 40 mg/m$^3$ could be produced for a time period of 2 h.

## Monitoring of the trichloramine concentration

The UV-analyses of iso-octane solutions containing the absorbed $NCl_3$ were performed by a Cary 50 spectrophotometer (Varian, Darmstadt, Germany). The concentration of $NCl_3$ was analysed at three wavelengths ($\lambda = 343$ nm, $\varepsilon = 185$ L/mol/cm; $\lambda = 260$ nm, $\varepsilon = 399$ L/mol/cm; $\lambda = 225$ nm, $\varepsilon = 5{,}470$ L/mol/cm). The aqueous chloride solutions in the impinger were concentrated to 10 mL with a rotary evaporator and were analysed after cation exchange (Dowex 50 WX 8) by ion chromatography (IC 790, Metrohm, Herisau, Switzerland; column: Metrosep Anion Dual 2, 75 mm × 4.6 mm, 6 µm particles; eluent: 5 mM phthalic acid with 2% acetonitrile at pH 4.5).

## Cell Exposure unit, CULTEX®

A CULTEX® continuous-flow-module from Vitrocell® (Waldkirch, Germany) with three inserts for 24 mm transwells was used as an exposure unit. Hence three membranes with cells could be exposed in parallel. Figure 2 shows a typical flow chart of the system.

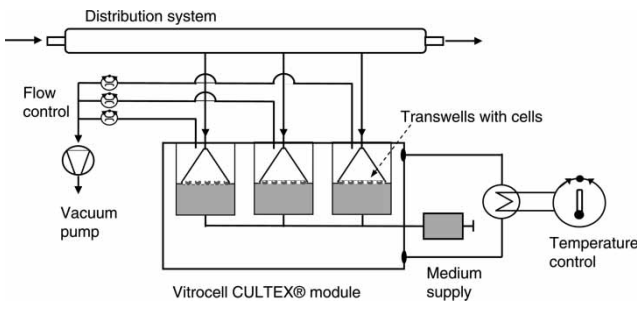


**Figure 2** | Flow chart of the CULTEX® module (Vitrocell® Waldkirch).

During the experiment the cells on the membranes were supplied with medium from the bottom of the wells through the membrane to prevent drying and nutrient deficiency. The conical-shaped tubes of the upper part of the transwells were connected to a vacuum pump to guarantee a continuous gas flow and hence a steady exposure of the cells from the top to the test atmosphere. Flow rates of 5 mL/min in each transwell resulted in tolerable mechanical stress and dehydration of the cells revealed by sufficient viability in control experiments. The cell viability was determined as the ratio of the number of viable cells and the number of total cells (viability (%) = number of viable cells/number of total cells × 100). For $NCl_3$ exposure experiments a viability of 94.1 ± 3.4% ($n = 71$), for the control experiments 95.3 ± 2.9% ($n = 71$) was measured. The total numbers of cells were in an optimal measurement range according to standard protocols (Butler 2004).

The test atmosphere was used without extra humidification or other modifications such as $CO_2$ addition. The gas stream over each transwell was controlled with flow meters. The exposure device was held at a constant temperature of 37 °C with an external water bath. For the exposure to indoor swimming pool air including the control experiment in the office temperature control was not necessary, since the temperature of 29 °C provided sufficient viability of the cells (viability: 88.7 ± 3.6% in pool air, 85.0 ± 2.3% in office air). Positive control experiments were performed by exposure to nitrogen dioxide ($NO_2$), a well-investigated airborne pollutant and a commercially available gas standard already applied in exposure studies with a CULTEX® module (Aufderheide *et al.* 2002).

Test concentrations from 4.2 to 12.4 mg/m$^3$ were obtained by dilution of a $NO_2$ stock gas (10.9 ppm $NO_2$ in synthetic air, 10 L gas cylinder, Linde Gas, Pullach, Germany) with synthetic air using mass flow meters. In parallel to each exposure experiment, control cultures were placed in a second CULTEX® module which was exposed to synthetic air or 'clean' air of an office outside the pool hall in the same building.

## Cell line and culture conditions

Human alveolar epithelial carcinoma cell line A-549 (ACC 107, German Collection of Microorganisms and Cell Culture, DSMZ, Braunschweig) was cultivated in Dulbecco's Modified Eagle's Medium (Sigma, Taufkirchen, Germany),

supplemented with 10% foetal calf serum and 2 mM L-glutamine at 37 °C under a humidified atmosphere with 95% relative humidity containing 5% $CO_2$. This cell line was used within 20 passages from the DSMZ stock received. For exposure experiments, cells were harvested with trypsin-EDTA, counted using an electronic cell counter and analyser system (CASY, Roche, Mannheim, Germany) and seeded at a density of $8 \times 10^4$ cells/mL into 4.2 $cm^2$ cell culture inserts (transwells) with track-etched polyethylene terephthalate (PET) membranes (pore size 0.4 μm, $1.6 \times 10^6$ pores/$cm^2$), which are permeable to the culture medium (Becton Dickinson, Germany). At sub-confluence, the culture medium was removed and replaced with serum-free medium for 18 h to synchronize cells without any loss of viability. Directly before exposure, the cell monolayer on the transwells was washed twice with phosphate buffered saline (PBS) and transferred subsequently to the exposure device. Here, the inserts were only immersed in serum-free medium, the cells were fed and moistened from the basal side through the porous membrane with nutrient mixture F-12 Ham (Sigma) and exposed to the test atmosphere on their apical side for a defined exposure time of up to 2 h. After the exposure experiments, the inserts were transferred into conventional 6-well plates with complete medium and incubated for 2, 24 and 48 h before further measurements.

## Determination of the cell viability and inflammatory biological effects: cytokines

After 2, 24 and 48 h recovery time cytokine release (IL-6, IL-8) in the cell culture medium, the numbers of total cells and viable cells were investigated. The collected supernatants of the three transwells were combined and stored at –20 °C until cytokine analysis. The cells of each transwell were used for cell counting.

### Cell viability

The cells from the membranes of the transwells were trypsinized by adding 500 μL trypsin-EDTA solution. Subsequently the cells were gently suspended and 100 μL of the cell suspension was diluted in 9.9 mL CASYton (Roche). Aliquots were analysed with an electronic cell counter and analyser system (CASY, Roche).

### Cytokines IL-8, IL-6

The cytokines IL-8 and IL-6 were chosen as pro-inflammatory biomarkers. Supernatants were analysed in duplicate with human IL-8 and IL-6 ELISA kits (Diaclone, Besancon, France). Using the calibration curves of the kits the IL-6 and IL-8 concentrations were determined in pg/mL units. The measured IL-6 and IL-8 concentrations in the supernatant of the exposed cells were normalized to the numbers of viable cells of the control experiment.

## Investigations in an indoor pool hall

The indoor pool hall had an air volume of 6,100 $m^3$ and comprises a swimmer pool with a water volume of 850 $m^3$ (water surface area: 418 $m^2$) and a non-swimmer pool with a water volume of 160 $m^3$ (water surface area: 120 $m^2$). The pools were operated with the following treatment scheme: flocculation–ultra-filtration–filtration through activated carbon (15% partial current)–chlorination with chlorine gas. Samples were taken on two consecutive days (25 November 2008, 26 November 2008).

Owing to the instability of $NCl_3$ it is not possible to collect samples. For air measurements the CULTEX® module was directly placed at the pool side of the non-swimmer pool. The air was sucked through 25 cm long polyethylene tubes at the height of 20 cm above the water surface and 10 cm off the pool edge into the CULTEX® module. The sampling time was 2 h. The air flow over each transwell and the cultivation of the cells were the same as described above. The cultivated cells on the transwells in the serum-free medium were transported in thermal isolated boxes to the bath. The transwells with the cell monolayer were washed twice with phosphate-buffered saline (PBS) and transferred to the exposure device directly before the air sampling. After exposure the inserts were again supplied with complete medium and transported in thermal isolated boxes directly to the laboratory. Transport times were at maximum 2 h. In the lab the cells were incubated and analysed as described above. Control samples were exposed in parallel with a second CULTEX®-module in the office area of the building.

Parallel to that the concentration of trichloramine in the air was determined at a height of 20 and 150 cm above the

water surface and 20 cm off the edge of the pool with the method described by Hery *et al.* (1995). Additional water samples were taken to determine the water quality. Free chlorine and combined chlorine were measured spectrophotometrically on site with a chlorine cell test (Merck) after reaction with DPD according to the European Standard method EN ISO 7393-2 (2000). Water samples for the determination of urea, trihalomethanes (THM), dissolved organic carbon (DOC) and adsorbable organically bound halogens (AOX) were taken without headspace in tightly sealed bottles. Sodium thiosulfate was added to quench the residual chlorine. The water temperature was 28 ± 1 °C and the air temperature was 29 ± 1 °C at all measurements. The relative humidity in the pool hall was 60%. THMs were analysed using headspace gas chromatography with electron capture detection on an HP 6890 system according to DIN 38407-30 (2007). Urea was analysed with the enzymatic and colorimetric indophenol blue method (Microquant® urea-test, Merck). AOX measurements were done according to EN ISO 9562 (2004) with an AOX-Analyzer ECS 1200 (Euroglas, Thermo Electron, Dreieich, Germany). Dissolved organic carbon (DOC) was analysed with a TOC-Analyzer 820 (Sievers Instruments, USA).

### Statistics

Results were expressed as arithmetic means ± standard deviations. Statistical analyses were done according to Miller & Miller (1986). Means of samples were compared with means of controls with the *t*-test (independent two sample student *t*-test) for a significance level of $p = 0.01$. The laboratory data represent at least three independent experiments ($n = 3$) each performed in triplicate (three transwells for each experiment). The calculated *t*-values of the regression coefficients were compared with the tabulated *t*-values for $p = 0.01$ using a two-tailed *t*-test for $(n - 2)$ degrees of freedom. For the viability-tests the 95% prediction interval was calculated according to Samuels & Wittmer (2003).

## RESULTS AND DISCUSSION

### Trichloramine generation and dilution

The results of the measurements of UV-absorbance and chloride demonstrate the well-defined generation of trichloramine and steady-state conditions over the experimental period. Constancy of the trichloramine generation was controlled by measurement of trichloramine trapped in isooctane from a side gas stream. All concentrations shown in Figure 3(a)–(c) were normalized to the final exposure concentration after further dilution with synthetic air. Sampling intervals were 30 min for experiments at low concentrations (Figure 3(a)), 20 and 15 min for higher concentrations (Figure 3(b) and (c), respectively). After an initial phase of 90 min a steady-state level with constant trichloramine concentrations could be maintained (standard deviations of 0.02 to 0.09 mg/m$^3$).

The final diluted $NCl_3$ gas concentration used for the exposure experiments was controlled directly at the gas outlet. Differences between the trichloramine concentrations in the side gas stream and at the outlet were at maximum

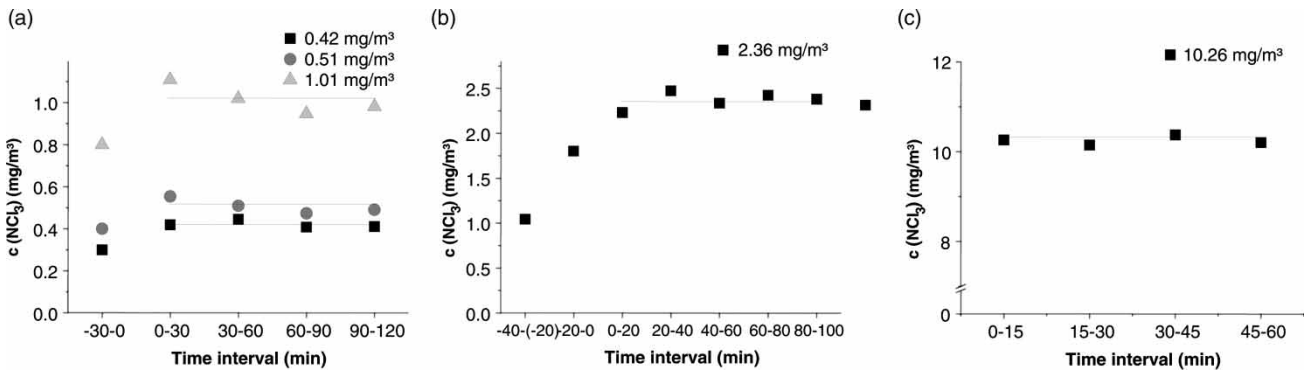


**Figure 3** | Trichloramine concentrations applied to the CULTEX®-chamber. The concentrations reached steady-state conditions after an initial period of about 90 min. Sampling intervals were: (a) 30 min; (b) 20 min; and (c) 15 min.

30% and therefore within an acceptable range to use the side gas stream for continuous monitoring of the generation system over the whole exposure time. The $NCl_3$ gas stream of 0.5 mg/m$^3$ contained less than 0.1% free and combined chlorine compared with the $NCl_3$ concentration (0.36 μg/m$^3$ free and 0.47 μg/m$^3$ combined chlorine), which can be considered as negligible, also at higher concentrated $NCl_3$ concentration. In conclusion the use of the described set-up enabled the generation of a pure and well-defined trichloramine gas stream for exposure experiments.

## Cell viability and cytokine release after exposure to trichloramine

In the next step cytotoxic and inflammatory effects of $NCl_3$ and $NO_2$ to A-549 cells were investigated with the described experimental set-up. The cells, placed in the CULTEX® module, were exposed for a period of 1 h at different concentration levels in the range between 0.01 and 40 mg/m$^3$ $NCl_3$ and 4.2 and 12.4 mg/m$^3$ $NO_2$. The numbers of viable cells and the concentrations of the released pro-inflammatory biomarkers IL-6 and IL-8 in the cell culture medium were analysed. The results of each exposure experiment were directly related to those of the corresponding control experiment performed simultaneously with synthetic air in a second CULTEX® module. The results of the numbers of viable cells were then calculated as a percentage of the number of viable control cells and shown in Figure 4(a) and (b). With increasing concentrations of $NCl_3$ and $NO_2$ a significant decrease in the relative numbers of viable cells could be observed. For $NCl_3$ an $IC_{50}$ (50% inhibitory concentration) of 24 ± 7 mg/m$^3$ and for $NO_2$ an $IC_{50}$ of 11.0 ± 0.8 mg/m$^3$ was determined. The set-up used was more sensitive than the one described by Bakand *et al.* (2007) who measured the cell viability with an ATP assay. The authors found an $IC_{50}$ of 20.7 ± 6.7 mg/m$^3$ for a 1 h exposure of A-549 lung cells with $NO_2$.

The release of IL-6 as an inflammatory response indicator after 1 h of exposure to $NCl_3$ and $NO_2$ followed by two hours of recovery is presented in Figure 5(a) and (b). $NO_2$ at concentrations between 4.2 and 12.4 mg/m$^3$ did not induce any significant IL-6 release compared with the clean air controls. Also $NCl_3$ concentrations between 1.2 and 20 mg/m$^3$ did not induce any IL-6 release compared with the controls. $NCl_3$ concentrations between 20 and 30 mg/m$^3$, however, significantly induced IL-6 release (148.6%; $p < 0.01$) compared with the clean air controls (100%). Further increase of $NCl_3$ concentrations higher than 30 mg/m$^3$ resulted in a decrease of IL-6 induction (52.9%, $p < 0.01$). This can be interpreted by cytotoxicity of $NCl_3$ at higher concentrations ($IC_{50} = 24$ mg/m$^3$).

The induction of IL-8 was chosen as a second inflammatory response indicator. Since IL-8 is one of the major cytokines responsible for inflammatory reactions, a more sensitive response would be expected. Figure 5(c) shows the IL-8 release after 1 h of exposure to $NCl_3$ followed by a 2 h recovery. At $NCl_3$ concentrations between 6.5 and 10 mg/m$^3$ no significant effects on the IL-8 release were observed compared with the controls. However, $NCl_3$ concentrations of 10 to 20 mg/m$^3$ and 20 to 30 mg/m$^3$ $NCl_3$

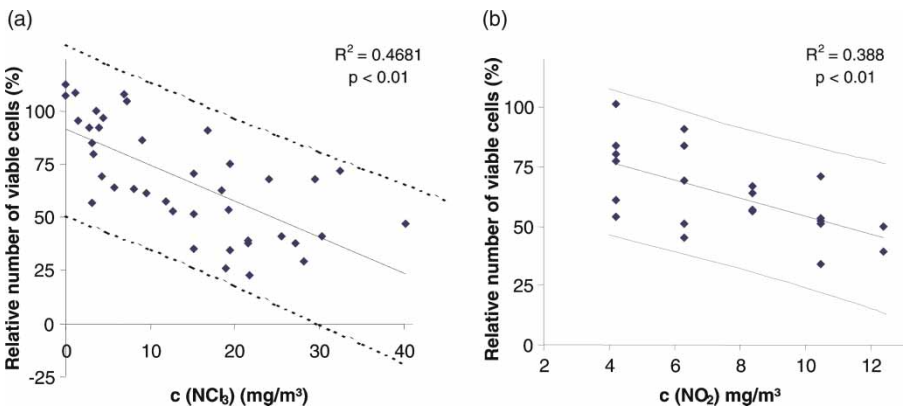


**Figure 4** | Relative number of viable A-549 lung cells after one hour exposure vs. varied concentrations of (a) $NCl_3$ and (b) $NO_2$. Relative numbers of viable cells = number of viable exposed cells/(number of viable control cells) × 100% (prediction interval $p = 0.05$).

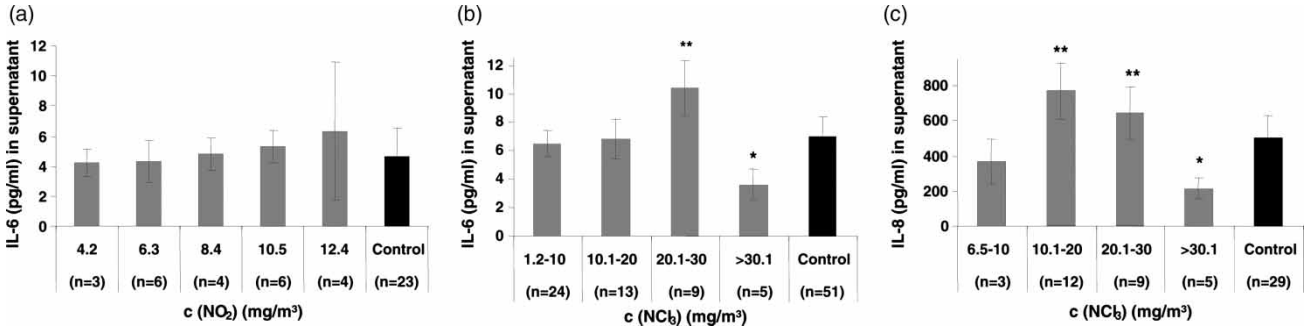


**Figure 5** | Inflammatory cytokine (IL-6 (a, b) and IL-8 (c)) release of lung cells (A-549) after 1 h of exposure to $NO_2$ (a) and $NCl_3$ (b, c) following a 2 h re-cultivation period. The columns in the graph show the means of n independent measurements; error bars show the standard deviation (1 s); significant differences are indicated with an asterisk for a significance level of 0.01 in the *t*-test (* significant inhibition, ** significant stimulation).

significantly ($p < 0.01$) increased the IL-8 induction to 156 and 130%, respectively. At $NCl_3$ concentrations higher than 30 mg/m$^3$ the IL-8 release was inhibited (44%, $p < 0.01$) again because of the strong cytotoxicity of $NCl_3$.

The results clearly demonstrate the suitability of the experimental set-up to investigate toxicological effects of $NCl_3$ on lung cells. The A-549 lung cell line has already been established and characterized in other *in vitro* studies investigating inhalation toxicity (Persoz *et al.* 2010). The properties of the type II alveolar epithelial cells and the differentiation characteristics have been proved for lung-specific toxicity testing.

The critical endpoints, cytoxicity and cytokine release IL-6 and IL-8, have to be considered in the context of the likely mechanisms of the non-polar and oxidative test compound trichloramine. The measured cytotoxic effects of $NCl_3$ which include expected cell membrane damages (Bernard *et al.* 2003) are in the same range as those of $NO_2$, a hazardous substance in the air. Consideration of the cell viability also enabled the cell status of the exposed cells to be monitored in comparison with the controls. The applied exposure flow rate of 5 ml/min/transwell permitted a dynamic and well-defined exposure at sufficient ventilation of the transwells and minimized side-effects due to drying out of the exposed cells. The release of pro-inflammatory cytokines in the supernatant is a typical reaction of A-549 cells after activation by environmental allergens. It is assumed that IL-6 and IL-8 cytokines are responsible for inflammation of the respiratory tract. Properties of IL-6 cytokines are described in the literature (Zhang *et al.* 2005) as: (1) stimulation of the mucin genes which leads to a mucous secretion in the airways; (2) promotion of Th2-type immune deviation which induces and regulates the IgE production; and (3) induction of lung eosinophil airway infiltration. IL-8 is an important mediator for neutrophiles (Zhang *et al.* 2005). Both cytokines IL-6 and IL-8 play a key role in developing asthma.

Increases in IL-8 release and decreases in IL-6 release in *in vitro* lung cell experiments have been shown for aromatic volatile organic compounds at almost cytotoxic concentrations (Fischäder *et al.* 2008; Pariselli *et al.* 2009a). Our investigations clearly demonstrate cytotoxic and inflammatory effects of $NCl_3$ to human lung cells. However, it has to be emphasized that the applied experimental approach cannot completely mimic the complex *in vivo* situation, but offers the possibility to study and understand basic cellular reactions under realistic exposure conditions with human lung cells.

## Investigation of indoor pool air

The CULTEX® device was further applied to study effects of indoor pool air on A-549 lung cells. The determined water quality parameters (Table 1) show a moderately loaded swimming pool water on both sampling days. THMs levels between 6.0 and 7.6 μg/L could comply with the parameter values given in DIN 19643 (1997). Slightly increased concentrations of combined chlorine at 0.24 and 0.20 mg/L were observed.

The results of the IL-6 and IL-8 release as inflammatory response indicators 2, 24 and 48 h after exposure to the indoor pool air are shown in Table 2. After a re-cultivation time of 2 h no IL-6 and IL-8 cytokines in the supernatant medium (detection limit of 2 pg/ml IL-6 and 29 pg/ml IL-8) could be measured for the exposed cells as well as for the

**Table 1** | Typical water and air quality parameters of the investigated indoor swimming pool

| | 25 November 2008 | 26 November 2008 |
|---|---|---|
| Water quality parameters | | |
| Free chlorine (as $Cl_2$) (mg/L) | 0.35 | 0.37 |
| Combined chlorine (as $Cl_2$) (mg/L) | 0.24 | 0.20 |
| Urea (mg/L) | 0.81 | 0.78 |
| DOC (mg/L) | 1.3 | 1.3 |
| THM (sum as $CHCl_3$) (μg/L) | 6.0 | 7.6 |
| AOX (as chloride) (μg/L) | 124 | 136 |
| Air quality parameters | | |
| $NCl_3$ (20 cm[a]) (mg/m$^3$) | 0.19 | 0.16 |
| $NCl_3$ (150 cm[a]) (mg/m$^3$) | 0.17 | 0.16 |

[a]Above water level.

**Table 2** | Release of inflammatory cytokines IL-6 and IL-8 from human lung epithelial cells (A-549) after a 2-h exposure period to indoor pool air followed by a 2, 24 or 48 h re-cultivation period

| | 25 November 2008 | | 26 November 2008 | |
|---|---|---|---|---|
| Re-cultivation time | Indoor pool | Control | Indoor pool | Control |
| | IL-6 (pg/mL) | IL-6 (pg/mL) | IL-6 (pg/mL) | IL-6 (pg/mL) |
| 2 h | <2 | <2 | <2 | <2 |
| 24 h | 7.1 | 2.5 | 10.1 | 3.9 |
| 48 h | <2 | <2 | <2 | <2 |
| | IL-8 (pg/mL) | IL-8 (pg/mL) | IL-8 (pg/mL) | IL-8 (pg/mL) |
| 2 h | <29 | <29 | <29 | <29 |
| 24 h | 315.4 | 190.4 | 631.7 | 386.1 |
| 48 h | 150.4 | 177.3 | 372.9 | 260.4 |

control cells. After 24 h re-cultivation a significant induction of IL-6 release of 284 and 267% and of IL-8 release of 169 and 161% was observed on 25 and 26 November 2008, respectively. After 48 h re-cultivation no IL-6 release could be detected at all. IL-8 was not significantly increased or an inhibition occurred compared with the controls. The results reveal significant effects of indoor swimming pool air on the exposed lung cells. In contrast to the laboratory experiments with pure $NCl_3$ gas streams, the IL-6 and IL-8 release was temporally delayed. The time delay of the cytokine response was observed for the control cells and for the exposed cells. It is reasonable to assume that stress due to handling and transportation of the cells to the pool and back to the lab is responsible for that. For the lower cytokine levels at 48 h compared with 24 h, physiological effects such as cellular adaptation to the environment were assumed to be responsible. Similar effects on IL-8 and IL-1β levels after $NO_2$ exposure of normal human bronchial epithelial cells were demonstrated by Ayyagari *et al.* (2004). However, the IL-6 and IL-8 inductions observed for the exposure to real indoor pool air containing $NCl_3$ at concentrations of less than 0.2 mg/m$^3$ cannot be explained by the presence of $NCl_3$ only, since lab experiments with pure $NCl_3$ required concentrations higher than 10 mg/m$^3$. Therefore, in addition to $NCl_3$, further volatile DBPs contributed to IL-6 and IL-8 induction in the indoor pool air. This result demonstrates that additional, most likely volatile, DBPs affect the lung cells in the applied exposure set-up. Further analyses of volatile DBPs followed by further exposure experiments with DBP mixtures will be necessary to understand toxicological effects of indoor pool air on human lung cells.

## CONCLUSIONS

In this study an experimental set-up was developed to generate a well-defined, pure $NCl_3$ gas stream to study toxicity effects of gaseous trichloramine and indoor swimming pool air on lung epithelial cells.

$NCl_3$ has been found to be an irritating, volatile and non-polar compound with the ability to penetrate into the deeper parts of the human lung (Bernard *et al.* 2003) and is suspected to cause adverse health effects on respiratory functions. Therefore human lung epithelial cells from the line A-549 were chosen for exposure experiments with varied, well-defined $NCl_3$ concentrations between 0.1 and 40 mg/m$^3$ and with swimming pool air. The numbers of viable cells and the inflammatory parameters IL-6 and IL-8 were investigated as biological endpoints for the evaluation of the $NCl_3$ toxicity. In this study a 50% decrease in relative numbers of viable cells could be observed at concentrations of 24 mg/m$^3$ $NCl_3$. Concentrations higher than 10 mg/m$^3$ $NCl_3$ caused an increased release of IL-8; concentrations higher than 30 mg/m$^3$ a decrease of IL-8. The decreased

secretion indicates cytotoxicity at high exposure concentrations. The release of IL-6 by A-549 cells is less sensitive, but behaves in the same manner with an increase at 20 mg/m$^3$ $NCl_3$ and a decrease at 30 mg/m$^3$.

Investigations of indoor swimming pool air revealed those inflammatory reactions at much lower $NCl_3$ concentrations of 0.17 and 0.19 mg/m$^3$. To achieve similar cytokine inductions in laboratory experiments with pure $NCl_3$ gas streams considerably higher concentrations were necessary. Therefore, the results suggest that additional volatile DBPs play a role in cytokine induction and that the complex mixture of pollutants in the swimming pool air may have additive effects on A-549 lung cells.

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