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Dynamic *in-vitro* Exposure of Human Derived Cells to Indoor Priority Pollutants

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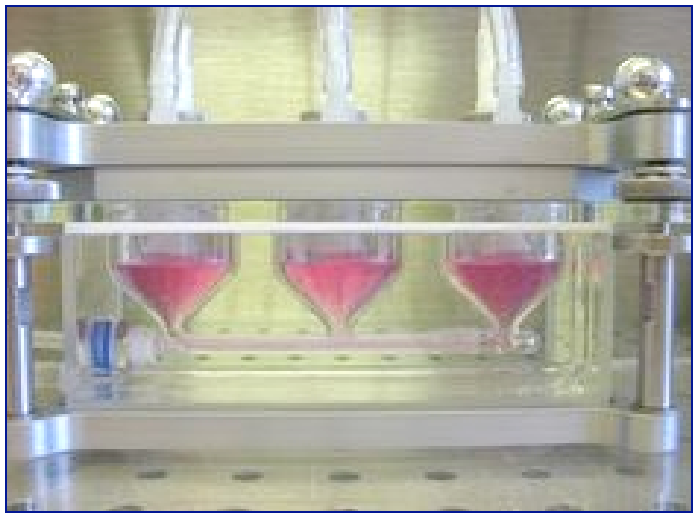

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The mission of the Institute for Health and Consumer Production (IHCP) is to provide scientific support to the development and implementation of EU policies related to health and consumer protection. The IHCP carries out research to improve the understanding of potential health risks posed by chemicals, biocides, genetically modified organisms, contaminants released from food contact materials and consumer products.

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Executive summary

There is abundant evidence among experts and policy makers that human exposure data represent a major bottleneck in the risk assessment process. Hundreds of volatile components like volatile organic compounds (VOCs) are detected in the indoor environment at concentrations often much higher than outdoors. These substances are potential causes of acute symptoms such as allergies, asthma, mucous irritation, headaches and tiredness and may substantially contribute to the increase of cancer incidence in the population.

The aim of this work was to investigate toxicity of single volatile chemicals and their mixtures. We developed a reproducible *in vitro* dynamic exposure model where human tumor lung epithelial (A549) and human keratinocytes (HaCaT) cell lines representative of VOC's target tissues (lungs and skin) were exposed in an airlifted interface to air pollutants.

The CULTEX® device was selected for the exposure of cells grown on porous membranes to individual air pollutants or in mixtures as a simulation of *in vivo* inhalation exposure. The studies started with the application of priority air pollutants, which were selected in the frame of the INDEX project ("Critical appraisal of the setting and implementation of EU INDoor EXposure limits").

Several parameters on cell culture conditions before and during the exposure (airflow, pH buffer of culture medium, moistening) were optimized to achieve conditions for the exposure decreasing mechanical effects that could lead to cell damage. We considered as the minimum requirement for acceptable control samples (synthetic air exposure) culture conditions allowing a survival above 90% (evaluated with LDH cytotoxicity and WST-1 proliferation assay). This was achieved for A549 cell line, while further improvements are necessary for HaCaT cell line which reaches until now viability around 80%.

Toluene was chosen as the first air pollutant to set-up the exposure technique as it is known to induce lung and dermal toxicity. One-hour exposure experiments performed with toluene up to 0.6 ppm, showed reproducible direct toxic effects on cell cultures with an average cytotoxicity of 25% for A549 and 38% for HaCaT cell lines, determined with LDH cytotoxicity assay. At the concentrations tested, toluene as hydrophobic compound directly targets and destroys cell membrane. The increasing LDH cytoxicity observed in A549 cell line with toluene concentration ranging from 0.1 ppm to 0.6 ppm, might give first indications of a dose related response of this cell type. Further exposure experiments with different toluene concentrations have to be worked out for validating this hypothesis.

Benzene is a well-known carcinogen that has been causally linked to leukaemia. The major source of exposure to benzene arises from cigarette smoke which accumulates inside homes, offices and vehicles. With the exposure of lung cells (A549) to benzene concentrations ranging from 0.1 ppm to 0.3 ppm we observed an average toxicity of 29 %.

High VOCs concentrations mainly cause toxic effects, whereas moderate VOC exposure levels trigger inflammatory reactions in particular in the airways, and low exposure levels induce alterations in immune reactivity resulting in a subsequent higher risk for the development of allergic reactivity and asthma. It is demonstrated that both keratinocytes and lung epithelial cells show an inflammatory response after VOCs exposure. High VOCs concentrations induce interleukin-8 (IL-8) production. For these reasons IL-8 release was chosen as a biomarker of inflammatory response after treatment with priority pollutants. In our experimental conditions we were able to demonstrate the ability of toluene and benzene to induce an inflammatory response by IL-8 release.

Skin is a main target of combined exposure to both chemical and physical stressors which could modulate the effects of the single compounds either chemical or physical. In preliminary experiments we exposed *in vitro* human keratinocytes (HaCaT) to both chemical (formaldehyde, toluene) and physical (UVB radiations) agents. Several endpoints were evaluated: cytotoxicity (LDH), proliferation (WST-1), CFU (Colony Forming Units), inflammatory response (IL-8) and oxidative stress (ROS). The effects observed with several single agent treatments seem to be enhanced by their combination. These results will be the basis to perform combined exposure treatments of UV irradiation and priority pollutants alone or in mixtures applied to airlifted cells in the CULTEX device.

The results obtained so far, show the sensitivity and specificity of the overall *in vitro* exposure set-up and suggest that this *in vitro* device is now ready to use and will allow us to extend the work to air pollutants mixture effects, e.g. acetaldehyde, formaldehyde, benzene, toluene and their combination (in the case of dermal cells) with UV radiation.

1 Introduction

Volatile organic compounds (VOCs) are important indoor air contaminants which are evaporated into the atmosphere at room temperature.

The monocyclic aromatic hydrocarbons benzene, toluene, ethylbenzene, xylene (collectively BTEX), and chlorobenzenes (ClB) are potential harmful substances commonly found in occupational or non occupational environments. BTEX are present in fuel, paint, rubber products and adhesives. Benzene and ethylbenzene are present in commercial gasoline at concentrations of 3–5% and up to 15%, respectively. ClB are current intermediates in dye and pesticide industrial synthesis.

Recent studies highlight the fact that exposure levels to these volatile organic compounds (VOCs) are more important indoors that outdoors. The major source of exposure to benzene arises from cigarette smoke and which accumulates inside homes, offices and vehicles (1;2). Other emission sources of theses VOCs are stored household products (white spirit, paint, glue…). Common household activities (e.g., painting, staining, varnishing) can produce air concentrations of VOCs as high as 1000 mg/m^3. Benzene is a well-known carcinogen that has been causally linked to leukaemia. Although toluene and xylene are not currently classified as carcinogens, it was reported an increase of cancer incidence in paint industry workers with long-term exposure to these organic solvents. Furthermore, increased risk for esophageal, rectal and colon cancers was suspected to be related to occupational exposure to toluene and xylene. Besides, a decrease of semen quality was observed in workers after occupational exposure to respective concentrations of toluene and benzene of 200 and 40 mg/m^3 (3).

The potential toxicity of these compounds is increased by the fact that they are strongly lipophilic with a capacity to concentrate in fat deposits throughout long-term exposures (4) and to accumulate in the lipid bilayer of the cellular membranes. Hence, there is a pressing need to find "stress indicators" to rigorously evaluate the impact of these xenobiotics on biological processes (5).

High VOCs concentrations mainly cause toxic effects, whereas moderate VOC exposure levels trigger inflammatory reactions in particular in the airways, and low exposure levels induce alterations in immune reactivity resulting in a subsequent higher risk for the development of allergic reactivity and asthma.

The aim of this work was to set-up an exposure model *in vitro* with priority air pollutants in order to evaluate toxicological properties of single volatile chemicals and their mixtures characteristic of indoor-air environment.

Since the target tissues for the VOCs toxicity are the lungs and the skin, the cell lines chosen for this purpose were human tumor lung epithelial cells (A549) and human keratinocytes (HaCaT).

Skin is the main target for UV radiation which might lead to manifold health effects. HaCaT cell line has been used as *in vitro* model to evaluate how UV radiation could interact with indoor chemical stressors such as VOCs.

2 An *in vitro* model for the exposure of air pollutants

The CULTEX® system (*Vitrocell*, Germany)(6) is based on the use of culture insert system which offer the unique possibility of culturing cells on membranes permeable to culture media. The cultures on the insert are run as conventional submersed cultures in 6 wells plates at 37°C in an incubator (5% CO2, 100% humidity) and use in the CULTEX® system. Here cells are only immersed in medium, the apical medium being removed; they are maintained and exposed to the air/liquid interface until the end of the experiment. Each CULTEX® module has three wells for cell culture inserts which can be filled by medium maintained at 37°C by a regulated flow of temperature-controlled water in the inner space of the module. The CULTEX® system allows exposing the cells maintained in the air/liquid interface to different mixtures of gases via negative pressure through the module. The gases enter in the well by a trumpet-shaped inlet leaving each well from corresponding holes, guaranteeing an equal distribution of the gases in the three wells and on the entire surface of the culture.

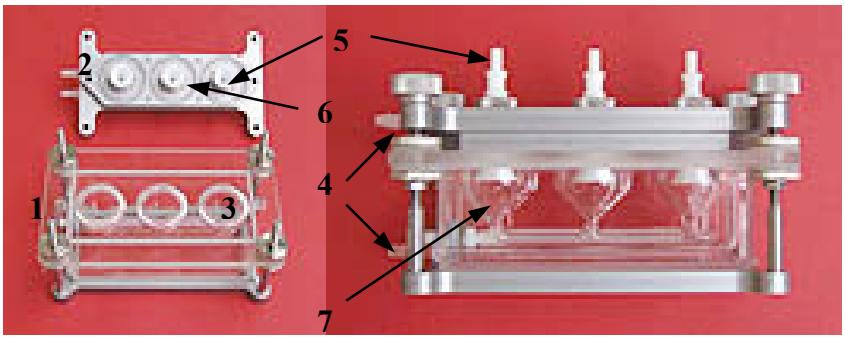

Fig 1: CULTEX® exposure chamber design – 1.Culture chamber, 2.Gas supply top, 3.Holder for the insert, 4.Inlet and outlet of the regulated flow of temperature-controlled water, 5.Gas inlet, 6.Gas outlet, 7. Medium supply.

This *in vitro* exposure system has several advantages:

1. Toxic agents that cannot be used in man or animal for ethical reasons, can be tested in an *in vitro* system,
2. Individual cell type response to pollutants permits better determination of the independent contribution of this cell type to a particular response,
3. A large range of human cell lines, tissues or primary cell lines can be used,
4. *In vitro* exposure conditions are rigorously controlled and can easily be reproduced (7).

Two types of cells are being studied in order to evaluate the health effects of priority air pollutants with this *in vitro* system. They are representative of human tissues which can be in contact with these gases and are able to develop some chronic inflammatory diseases. A pulmonary epithelial cell line, named A549, derived from a patient with alveolar cell carcinoma of the lung, is used as a model of human type II cells. These granular pneumocytes are thought to play a minor role in gas exchange compared to the membranous pneumocytes (type I) but their main functions are the synthesis and secretion of pulmonary surface-active material. Type II cells can regenerate in continuous the epithelium after exposure to air pollutantsin order to modulate lung inflammatory processes.

The other human cell line used to evaluate effects of priority pollutants is derived from a skin carcinoma. These keratinocytes, named HaCaT, have long been regarded as the structural backbone of the epidermis only, and it is indubitable that they play an active role in the pathogenesis of allergic diseases.

Several requirements must be met for the exposure experiments (7):

1. The system should be designed in order to keep cells in optimal conditions during their maintenance in an air/liquid interface; i.e. temperature and pH of the medium should be constant and the atmosphere must be humid, so that extended exposure are possible;
2. Precise control of pollutant generation and measurements: attention must be paid on the purity of the air supply,
3. The gases should be in contact with cells as closely as possible, avoiding the presence of medium, since gases could react with cell culture media components and toxic effects may be masked,
4. Sterile conditions should be maintained during exposure. Gross bacterial or fungal contaminations could easily affect cellular metabolism and lead to erroneous conclusions.

Figure 2 and 4 show the two CULTEX® exposure devices in their respective flow laminar chamber:

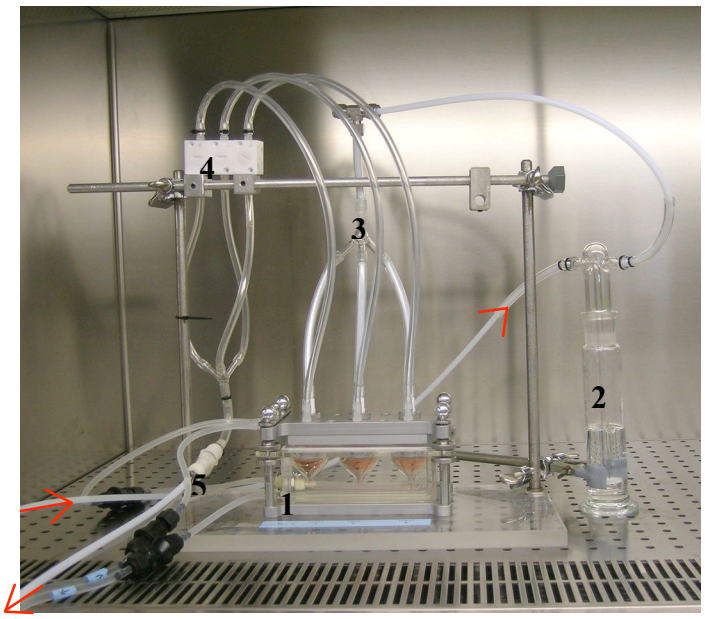

Fig 2: CULTEX I exposure device. 1.Exposure chambers, 2.Moistening device of the inlet gas (air synthetic), 3.Inlet gas divider, 4.Outlet gas flow regulator, 5.Outlet gas. (red arrows show flow directions)

We have two CULTEX® devices set-up for the exposure of air-pollutants: this first one (*Fig 2.*) is dedicated to synthetic air (oxygen/nitrogen 20/80 – *Air Liquid*) which was used in a first time to set up all the exposure conditions and then as a control for all gas mixtures in the successive steps. The other is dedicated to the gas mixture exposure in order to avoid any contamination of the first exposure device. For all the exposure experiments the flow range of the atmospheres is set between 2 and 8 mL·min^{-1}.

CULTEX® I device is directly connected to the gas delivery system at its inlet and to a vacuum pump at its outlet. Two mass flow controllers FC1 and FC2 (*Fig 3*) (*Bronkhorst*, Netherlands) regulate the gas flow before and after the device from 1 to 4 mL·min^{-1}. Three valves (*Fig 2.4 – Fig 3* V-1, V-2, V-3) are placed at the outlet of the device in order to guaranty the same flow in each insert.

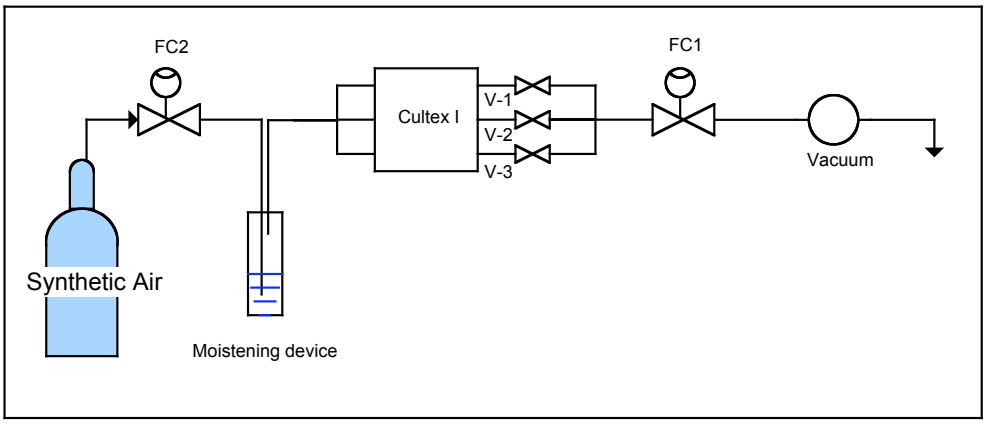

Fig 3: Scheme of the CULTEX I device. FC. Flow Controllers, V. Valves.

The second device set-up is different from the one dedicated to synthetic air. In fact, the mixture of indoor air pollutants used for cell exposure is taken, by negative pressure, from a high level flow (~100 mL·min^{-1}) through a glass tube (*Fig 5.2*).

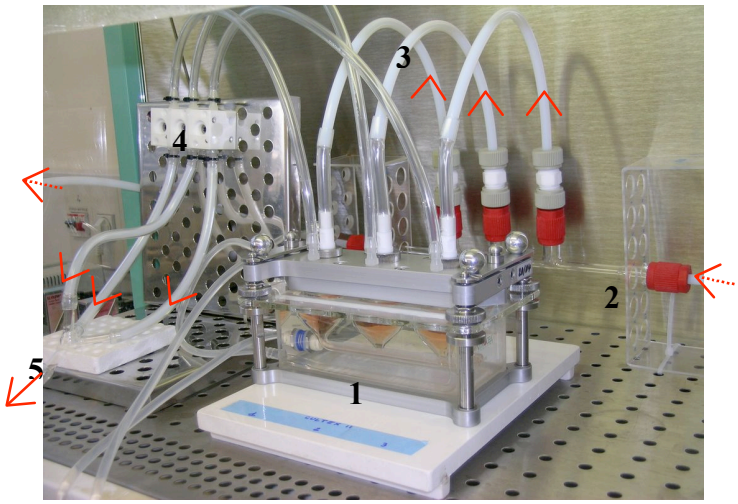

Fig 4: CULTEX II exposure device. 1.Exposure chambers, 2.High flow of the inlet gas, 3.Inlet gas, 4.Outlet gas flow regulator, 5.Outlet gas. (red arrows show flow directions, dot arrows show the high level flow)

Here, only one mass flow controller regulates the negative pressure obtained by a vacuum pump at the outlet of the exposure system (FC1 *Fig 5*). This device will allow us to work in the future with an electronic gas mixture system that requires high flow range and to avoid concentration phenomenon of gas particles that could appears at the flow reducers in order to have a constant concentration of air pollutants in the balance gas.

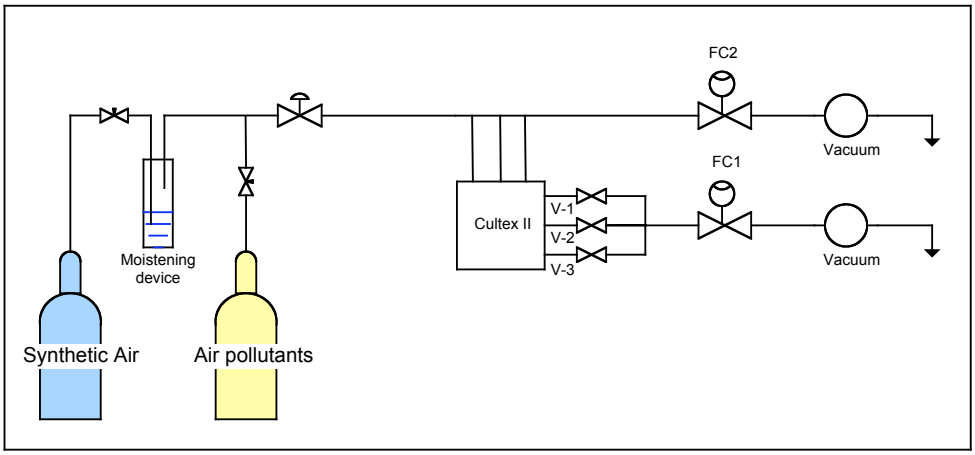

Fig 5: Scheme of the CULTEX II device. FC. Flow Controllers, V. Valves.

3 Choice and set-up of the endpoints

- WST-1 (Proliferation test)
- LDH (cytoxicity test)
- IL-8 (inflammation test)
- Glutathione (oxidative stress test)
- ROS (oxidative stress test)

3.1 WST-1: Proliferation test

Proliferation assays have become available for analyzing the number of viable cells, measured by the detection of cleaved tetrazolium salts added into a medium. Wash and collection of cells are not required, and all procedure, from the culture in small scale to the data analysis with ELISA reader, can be carried out in the same microtiter plate.

Tetrazolium salt (WST-1) is cleaved to soluble formazan dye by the succinate-tetrazolium reductase which exists in mitochondrial respiratory chain and is active only in viable cells.

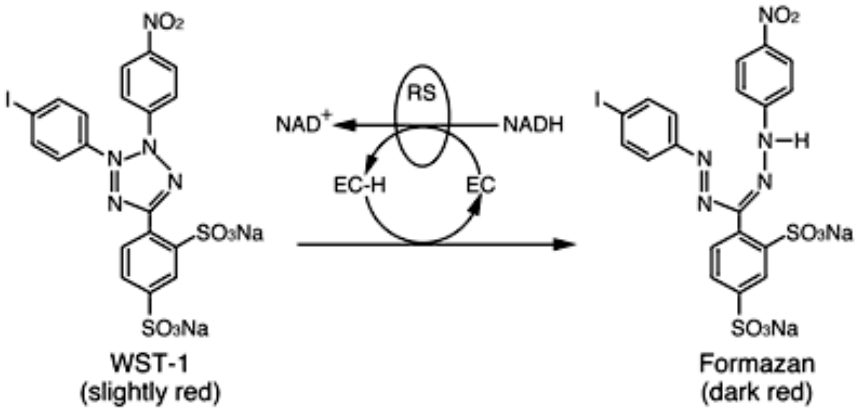

Fig 6.: WST-1 test is an index of mitochondrial function and cell viability/proliferation

Total activity of this mitochondrial dehydrogenase in a sample rises with the increase of viable cells. As the increase of enzyme activity leads to an increase of the production of formazan dye, the quantity of formazan dye is related directly with the number of metabolically active cells in the medium. Measuring its absorbance by a spectrophotometer can quantitate the formazan dye formed by metabolically active cell. The absorbance of formazan dye solution is in direct proportion to the number of viable cells. The cell proliferation reagent WST-1 is a ready-to-use solution (*Roche Applied Science*, USA) that is directly diluted in the cells culture medium at different time after air pollutants exposure, and the formazan dye being quantified after 3 hours of incubation at 37°C.

3.2 Determination of LDH release: cytotoxicity test

Some standard cytotoxicity assays are based on the measurement of cytoplasmic enzyme activity released from damaged cells. The amount of enzyme activity correlates to the proportion of the damaged cells. Alkaline and acid phosphatase, or glutamic-oxalocetic transaminase (GOT), or Glutamic Pyruvic transaminase (GPT) have been conventionally used for the methods. However, this method is not widely used because in many cells only low amounts of these enzymes are present and the quantification of the kinetic assays for these enzymes is elaborative.

Instead the lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme which is present in most cells. This enzyme catalyses the conversion of lactate and pyruvate:

$$\underset{pyruvate}{CH_3COCOO^-} + NADH + H^+ \xleftrightarrow{LDH} \underset{lactate}{CH_3CHOHCOO^-} + NAD^+$$

This is an important reaction of the energetic metabolism of the cell: in fact, the pyruvate links the glycolysis to the Krebs cycle. Because it is released on the cell culture supernatant only upon damage of the cytoplasmic membrane, it is an index of cell membrane integrity and for extension an index of cytotoxicity.

LDH is usually measured by spectrophotometric method reading the kinetics of NADH oxidation as a decrease of absorbance at 340 nm. One unit of LDH is also defined as the amount of enzyme able to oxidize one mole of NADH in one minute. This is an index of LDH activity released from cells and should be proportional to the number of dead cells.

The **Units/Liter (U/L)** were determined with the following formula:

$$U_{LDH} \cdot L^{-1} = \frac{\Delta A \times 1000 \times 1}{\varepsilon \times t \times d \times v}$$

_A = difference of absorbance during the reaction.
ε = micromolar extinction coefficient for NADH (6.3 μmol·L^{-1}·cm^{-1})
t = time of the kinetic
d = dimension of the cuvette in cm (1 cm)
v = sample volume (0,1 mL)

We found that in our conditions an interval of 6 min was the optimal to determine the decrease of absorbance.

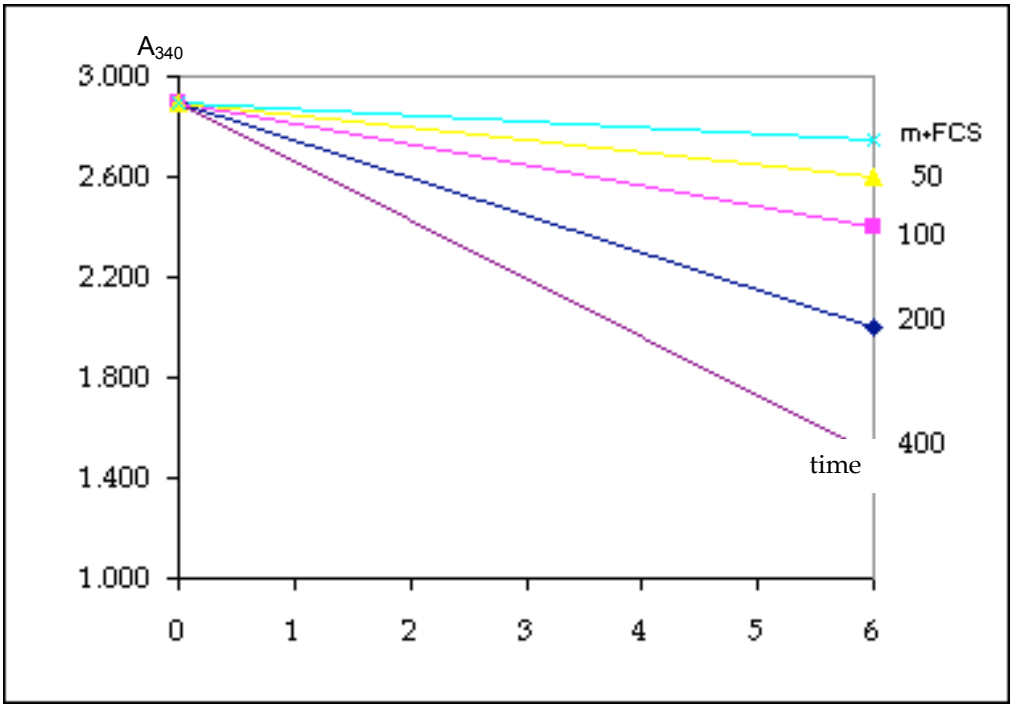

Fig 7.: Kinectics of the oxidation of the NADH during 6 minutes with differents amount of cells (A549 50, 100, 200, 400×10^3 cells); m+FCS=medium+1%FCS

In several experiments we correlated the LDH concentration to the protein (Units/g) content for every sample. After the standardization of the preparations and of the methods, we reached a very good reproducibility.

All the data were then expressed as **U/L** or **Total Units** of **LDH.**

- Correlation between number of cells and LDH

We performed preliminary experiments in which a known number of cells were lysed and the correspondent amount of LDH was measured. There was a very good correlation (R^2=0.9961) between cell number and LDH release. This give us the possibility to determine the amount of damaged or death cells releasing LHD in the supernatant of every sample.

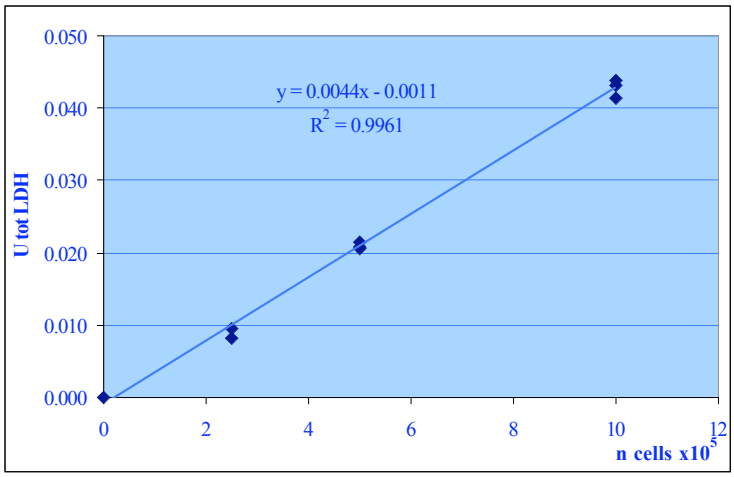

Fig 8.: Relationship between the amount of LDH calculated and the original amount of cells lysed (A549).

- Determination of LDH activity on cellular extracts and supernatants

LDH activity was determined at 24h incubation after the exposure period on 4 fractions for every sample:

- LDH Basal: supernatant in the well,
- LDH Apical: supernatant in the insert,
- LDH Pellet Apical: detached unhealthy cells present in the supernatant, separated by centrifugation and lysed for 3 hours with 1% TritonX-100 in PBS at 37°C,
- LDH Pellet: cell layer lysed as described.

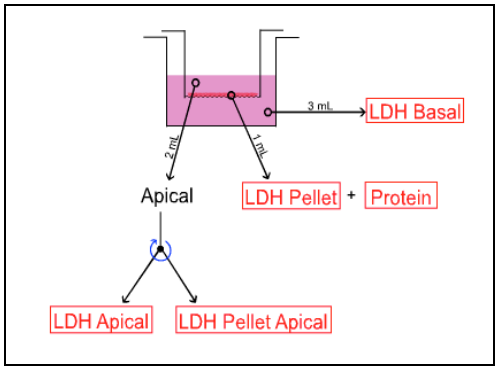

Fig. 9: Fractions for LDH determination

The **Units/L** of LDH calculated in every fraction were multiplied by the volume of every sample to have the total unit of LDH released. The sum of the four fractions gives the total amount of the LDH (Utot) per cell insert culture. The **percentage of toxicity** was calculated as percentage of LDH released (i.e. present in the 3 fractions apical, basal and pellet apical) on the total LDH (sum of the 4 fractions).

3.3 IL-8: mediator of the inflammatory response

Many aromatic compounds and VOCs are known carcinogens with the ability to cause DNA damage, hematopoietosis, neurotoxicity, immunotoxicity (8) and inflammation (9).

Aromatic hydrocarbons are considered to have the highest dermal toxicological potential (10). In addition they are a significant risk for systemic toxicity secondary to inhalation exposure. (11)

In view of the numbers of priority pollutant of toxicological concern to be considered in our study, initially only one cytokine (IL-8) was chosen as a proinflammatory biomarker.

Interleukin-8 (IL-8) is a member of low molecular weight proinflammatory factors family known as chemokines. Although it was initially described as a chemotactic factor produced by activated monocytes, it has later been found to be produced from a variety of different cell types such as lymphocytes, endothelial cells, epithelial cells, fibroblasts.

The selection of IL-8 as such a biomarker has been fully justified elsewhere (9). IL-8 has been shown to significantly increase following the activation of primary proinflammatory cytokines interleukin-1 (IL-1 α, IL-1β) and tumor necrosis factor-α (TNF- α). The non-specific nature of IL-8 and its consistent release (12) by various external stimuli makes it an ideal biomarker, over many other secondary cytokines, for the assessment of early inflammatory responses to chemical irritants.

It was also demonstrated that both keratinocytes and lung epithelial cells show an inflammatory response after VOCs exposure. High VOCs concentrations induce interleukin-8 (IL-8) production.

For these reasons IL-8 release was chosen as a biomarker of inflammatory response after treatment with priority pollutant.

3.4 Intracellular Glutathione determination, an oxidative stress marker

Glutathione is a tripeptide made up amino acids _-glutamic acid, cysteine, and glycine. It is the most abundant low-molecular-mass thiol compound present in mammalian cells.

The primary biological function of glutathione is to act as a non-enzymatic reducing agent, to keep cysteine thiol side chains in a reduced state on the proteins' surface. The reduced form of glutathione (GSH) is also used by cells in the protection against oxidative stress, detoxification of xenobiotics. It also helps them to trap free radicals which can damage DNA and RNA. This protective mechanism results in increased formation of the oxidized form of Glutathione: Glutathione disulfide (GSSG).

Physiologically, the intracellular glutathione is present in the reduced state in most tissues, accounting for more than 99% of the total amount and it is maintained in this form by a NADH-dependent reductase.

Therefore, analysis of changes in GSH status produced by toxic compounds can provide important informations about the toxicity mechanism of a compound. Simultaneous determination of GSH and GSSG is needed because the amount of GSH may decrease and that of GSSG may increase in response to oxidative stress *and this rapidly*. Then the expression of the GSSG/GSH concentration ratio is considered to be one of the markers of oxidative stress.

Numerous methods have been developed for the quantification of glutathione in biological samples, based on a variety of detection and separation techniques. *But* GSH and GSSG determination is difficult because of their very low concentrations and *the case* of spontaneous oxidation of GSH to GSSG in biological extracts.

We first used a HPLC based method for the determination of both GSH and GSSG levels in the cells extract able to measure picomolar concentration of GSH and GSSG with high specificity. This method, which is a modification of Reed et al. method (13), is based on the derivatization of GSH and GSSG with 2,4-dinitroflurobenzene (DNFB) and analyzed by reversed-phase ion exchange HPLC technique with UV detection.

The first results with standard samples show us a linear response of the level of glutathione reduced and oxidized in the sample. Moreover, the value of the resolution (R) indicates that the chromatography system is well adapted to the separation of the two compounds.

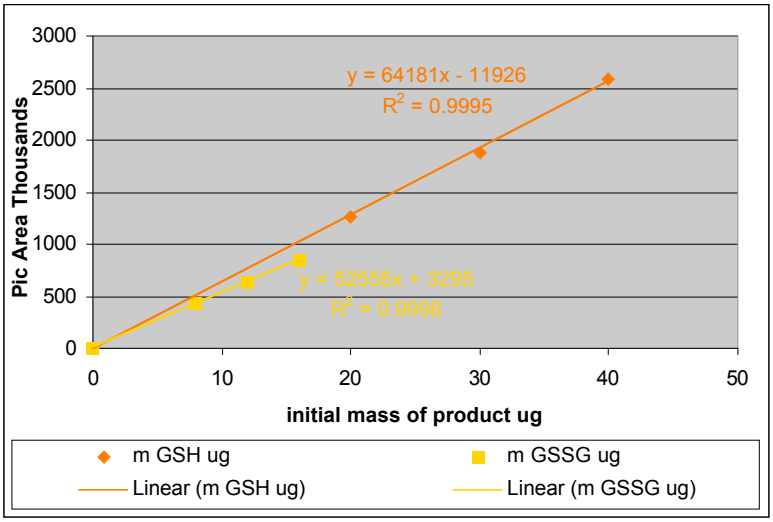

Fig 10: Calibration curves of GSH and GSSG dosage by HPLC.

m GSH µg	m GSSG µg	tr GSH min.	tr GSSG min.	pick area GSH u.A	pick area GSSG u.A	Resolution
0	0	/	/	0	0	/
20	8	11.102	12.811	1263009	430284	2.89
30	12	11.100	12.810	1883147	634044	3.02
40	16	11.095	12.805	2582401	840841	2.53

Table 1: Calibration results of GSH and GSSG dosage by HPLC

GSH and GSSG in the cell culture sample were determined by standard addition method due to the very low concentration of the glutathione's forms in the sample. Theses concentrations were calculated by extrapolation form the linear least-squares regression line of the calibration plot of peak areas of GSH and GSSG, from standard solution, versus those added to the sample.

This method shows its limits when, after several tests, it was impossible to determine the amount of glutathione oxidized in the cell sample, even after an oxidative stress where it is supposed to be increased. This could be due to the fact that this cell line has a very low basal amount of glutathione disulfide which is lost or altered by the chemicals used during the sample preparation.

Another method to detect and measure both glutathione and glutathione disulfide is described by Giustarini D. team (14). Their method involves the use of a rapid quenching agent of thiol groups N-ethylmaleimide (NEM) before DNFB derivation instead of iodoacetic acid used first. This method is still in development and no data can be shown at the moment.

3.5 Reactive Oxygen Species

In aerobics cells, during the course of normal metabolism, there is a continuous generation of reactive oxygen species (ROS), including these free radicals: superoxide ($O^{\cdot-}_2$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$) peroxyl ($ROO^{\cdot}$) and alkoxyl ($RO^{\cdot}$), which may be involved in the initiation and propagation of free radical chain reactions and which are potentially highly damaging to cells (15) by their ability to react with macromolecules (DNA, proteins, lipids, sugars). There are many intracellular sources for the production of ROS, such as the mitochondria, where electron leak out to generate superoxide anion, as well as several enzymes such as lipoxygenase, cyclooxygénase, microsomal cytochrome P450 and NADPH oxidase used by phagocytes to kill invading microorganisms. Exogenous agents such as radiations, chemicals and environmental pollutants, also induce ROS generation (16;17) or could have a cytotoxicity effect increased by them (e.g. formaldehyde.) as describe by Saito and co. (18).

Mechanisms have been evolved to restrict and control such processes, partly by compartmentation, and partly by antioxidant defenses such as chain-breaking antioxidant compounds able to form stable free radicals (e.g. ascorbate, α-tocopherol) and the evolution of enzyme systems (e.g. superoxide dismutase, catalase, peroxidases) that diminish the intracellular concentration of the ROS.

Nevertheless, the generation of ROS inside the cells, also defined by an imbalance between oxidants and antioxidants, is an important contributing factor in several chronic human diseases, inflammation injury, mutagenesis and cancer, neurodegeneration and aging process.

We examined ROS generation induced by various free radical generators (such as tert-butylhydroperoxide) or by air pollutants mixture by using a fluorometric assay. We used the 6-carboxy-2', 7'-dichlorofluorescein diacetate (DCFH-DA) (*Molecular Probes*, USA) which is cell-permeant until the esterases inside the cell remove the diacetate group. Then, the dichlorofluorescein (DCFH) product can be oxidized by reactive oxygen species to highly fluorescent probe dichlorofluorescein (DCF). Therefore, the intracellular DCF fluorescence can be used as an index to quantify the overall oxidative stress in cells. This method has the advantage to be used with a fluorometric plate reader as well by flow cytometry (19).

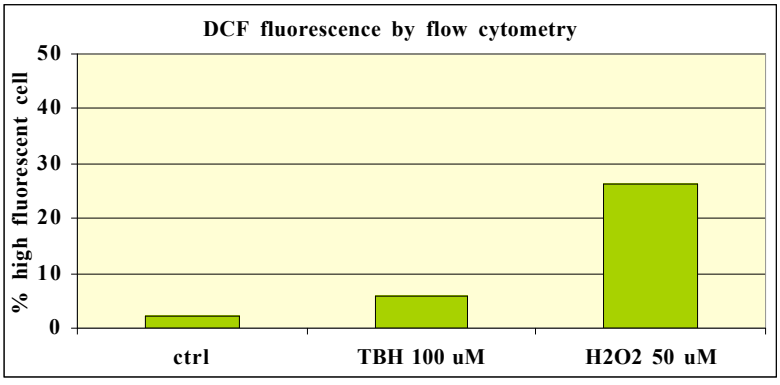

Fig 11: Oxidative Stress quantification in A549 by flow cytometry cell loaded with 50µM DCFH-DA and 3h incubated with tert-butylhydroperoxide (TBH) 100µM as ROS generator or H_2O_2 50µM as ROS control.

4 Exposure conditions optimization.

Work done from April to December 2005

The possibility to use healthy cells in optimal culture conditions is a pre-requisite absolutely necessary for exposure experiments with environmental pollutants in order to obtain reproducible results.

1. Optimization of different cell culture and exposure conditions (pH, humidity).
2. Evaluation of different flow rates and exposure times.
3. Measurements of reproducibility of the system with synthetic air.
4. Comparison of two CULTEX set up by parallel run.

Two end points have been evaluated for the entire set-up of culture and exposure conditions:

- LDH (cytotoxicity test)
- WST-1 (proliferation test)

4.1 Optimization of different cell culture and exposure conditions.

As the bigger CULTEX® system, used since January, has a new design (see before), the fumigation regimes and cell culture and conditions had also to be set up new. In the view of the inconstant results that we had, several points have been changed from previous experiments, and more attention is now made on the cell integrity before and during the experiments.

Cells (A549 or HaCaT, 4 to 7×10^4) were seeded into cell culture insert (4,2 cm^2 of growth area – *Becton Dickinson*, USA) and left to grow 2 to 4 days with complete medium RPMI 1640 (*Invitrogen*, USA) with 10% Foetal Calf Serum (*Invitrogen*, USA) and antibiotics Penicillin/Streptomycin. The medium was changed every two days. At sub-confluence, cells were starved for 12 hours by serum deprivation to coordinate cell cycle. After this, the inserts were used for exposure in the CULTEX® system without apical medium. By this protocol we let the cells adapt to the new support (i.e. insert membrane) and make their inter-connections to have a better distribution of the media nutrients in all the cells even during the exposure time.

As controls of cell integrity, we used unexposed inserts left in normal culture conditions (incubator 5% CO_2, 100% humidity) named **CO_2 +AP** (APical medium), and unexposed inserts left for 1 hour without the supernatant apical (**CO_2 -AP**) to mimic the air/liquid interface conditions.

Another very important parameter to be considered, in order to guaranty optimal control exposure conditions, is the pH of medium below the insert. During the exposure time, with a CO_2 dependent medium the pH can increase from 7.2-7.4 to 7.8, which may cause a stress to the cells. For this reason, F12K medium, the CO_2 dependent medium used in the previous experiments, as suggested in literature, was substituted with RPMI 1640 medium with 25mM HEPES (which should maintain the pH stable in presence or in absence of CO_2 atmosphere).

To validate these new parameters, cells growing on inserts were left without apical medium in the incubator (5% CO_2, 100% humidity) for different times, 1, 2, 3 and 24h. With both cytotoxicity and proliferation tests (LDH and WST-1) performed after 24 hours no significant differences were observed versus controls with apical medium (data not shown).

Finally, the addition of a moistening device at the inlet of the CULTEX devices guarantied the humidification of the air lifted. This particular point was suggested to keep conditions for cells integrity as good as possible and to avoid cell dryness.

4.2 Evaluation of flow rates and exposure times.

The experiments set–up were worked out until now with the same flow rate as it has been done in the preliminary experiments with the small CULTEX® system, meaning a flow rate of 8 mL·min^{-1} (as suggested in literature). In a few experiments, a more manifested damage of the cells has been observed in the centre of the insert.

By getting more experience with the handling of the CULTEX® exposure system, we already have developed a few suggestions for modification. Some of our suggestions have been kindly worked out by the Vitrocell Company (CULTEX® supplier). In order to prevent a mechanical detachment of cells from the inserts during the exposure, we progressively reduced the flow rates of synthetic air from 8 to 2 mL/min for 1 hour of exposure.

First of all, it is very important that in all three small exposure chambers of the CULTEX® system the same flow regime will be achieved, that means not only the same low continuously flow, but also a pressure free flow. In figure 12 is shown the set up of the flow controllers of each exposure device; the flow in each exposure chamber was adjusted by the valves at the outlet of the CULTEX®. Thanks to this set up we are allowed to use different fluxes by changing the value of the mass flow controllers.

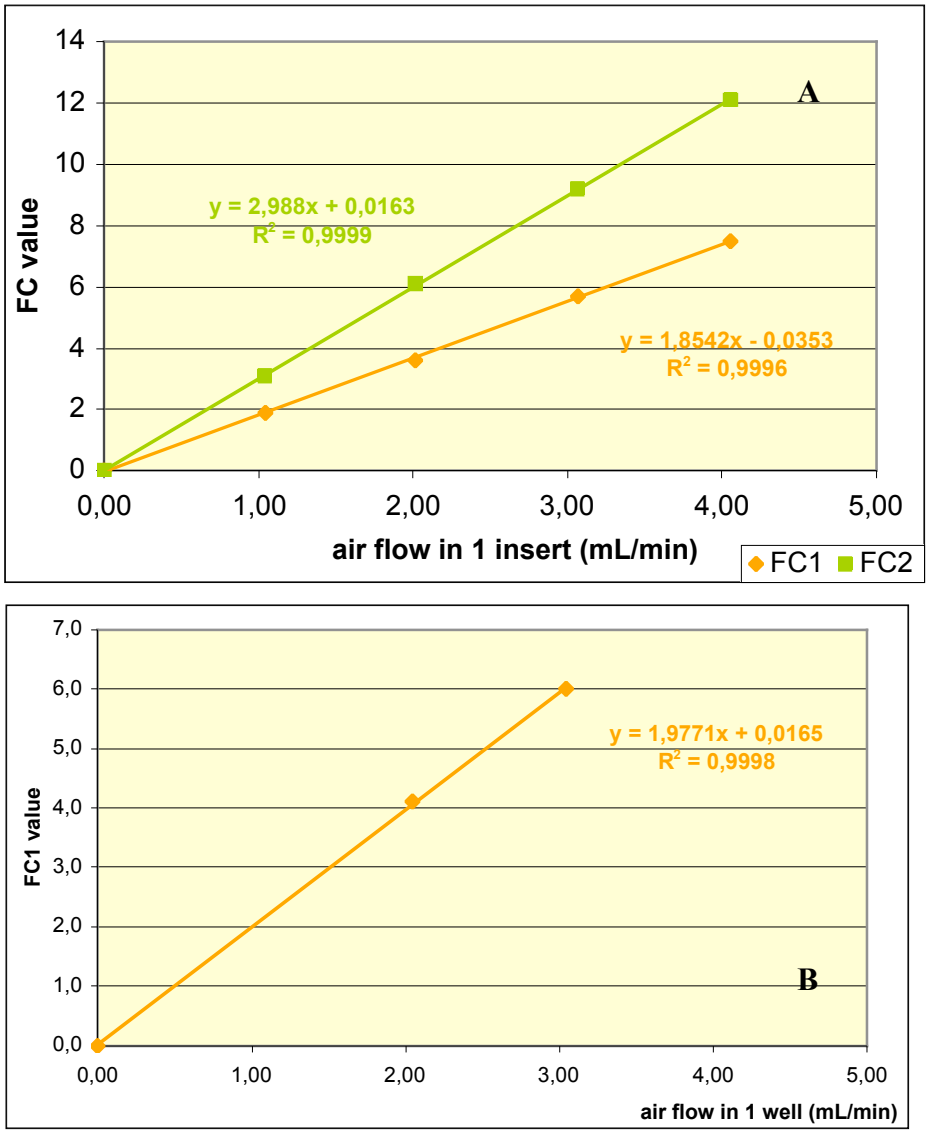

Fig 12: Correlation between Flow Controllers values of the and the air flow in one insert. A. CULTEX I, B. CULTEX II

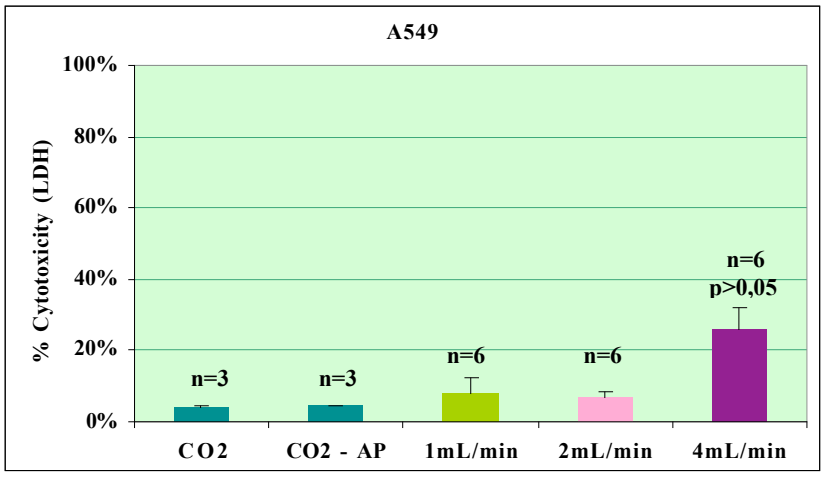

Fig 13: Cytotoxicity effect of the airflow (synthetic air) on insert cell culture of A549 after 24h incubation.

The figure 13 shows the effect of synthetic airflow on the A549 viability (determined with LDH evaluation) in the conditions previously described. The best results were obtained with a flow set between 1 and 2 $mL \cdot min^{-1}$ in each exposure chamber. A flow rate of 2 $mL \cdot min^{-1}$ was chosen for the following experiments.

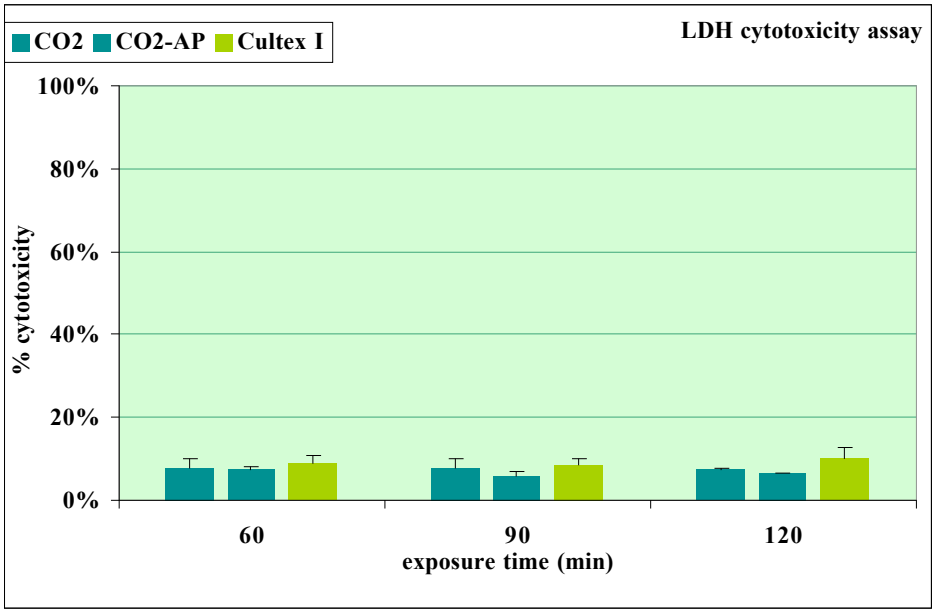

Fig 14: Cytotoxicity effect of the airflow (synthetic air) on insert cell culture of A549 after 24h incubation.

Experiments on exposure time have been performed on the A549 cell line. As shown in figure 14, it is possible to extend the exposure time from 60 minutes to 2 hours without a consistent increase of cell death.

4.3 Reproducibility of exposure in CULTEX device with synthetic air.

To validate the culture and exposure conditions in the CULTEX® device we performed repeated exposures with synthetic air with the CULTEX I exposure system with the parameters described below:

Cell lines	A549 / HaCaT
Culture conditions :	Sub-confluence before exposure
Medium :	RPMI 1640
Flow (Synthetic air) :	2 mL·min^{-1}
Exposure time	1 hour
Moistening :	yes
Tests	LDH, WST-1 after 24h

Moreover, in order to compare the homogeneity of the fluxes from the three trumpets of the CULTEX device, we evaluated the same endpoint (LDH or WST-1) in triplicate for every exposure experiment. Data are expressed as the average ±SEM. Statistical analysis was carried out with Student *t* test.

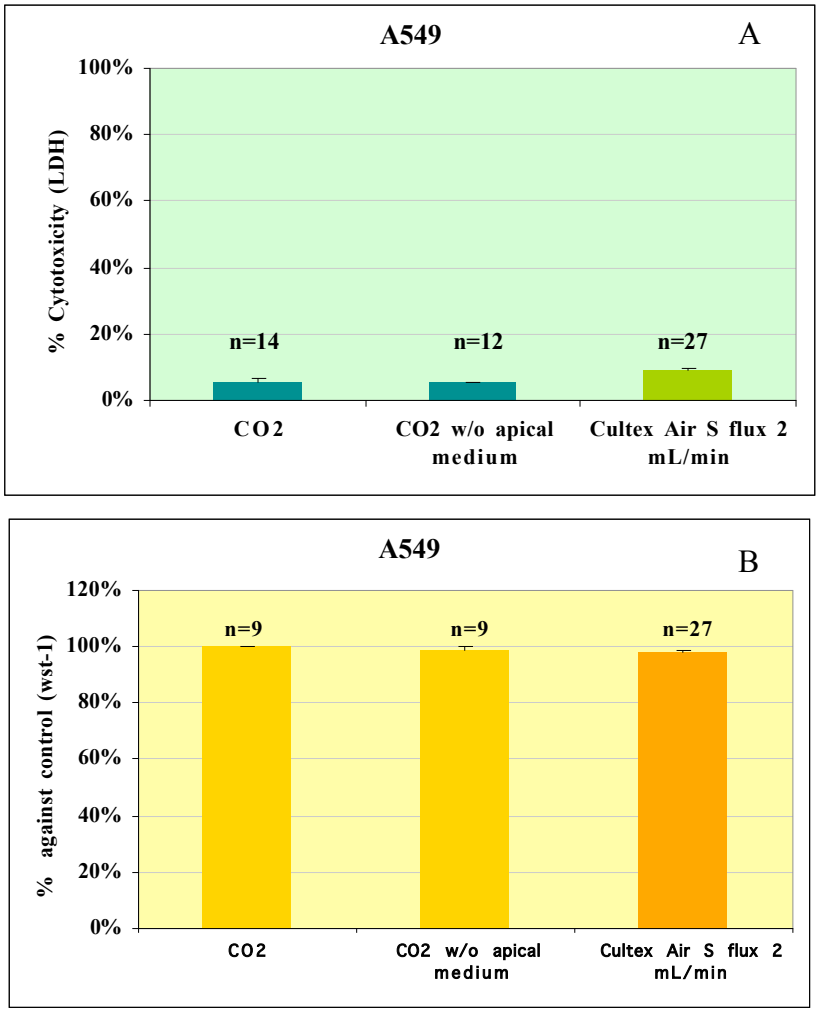

Fig 15: LDH cytotoxicity assay (A) and WST-1 proliferation assay(B) of A549 exposed in CULTEX I to synthetic air after 24h incubation.

As shown in figure 15, we obtained no statistically significant differences ($p>0,05$) with both WST-1 and LHD assay for A549 cell lines after one-hour exposure to synthetic air in the CULTEX I after 24h incubation.

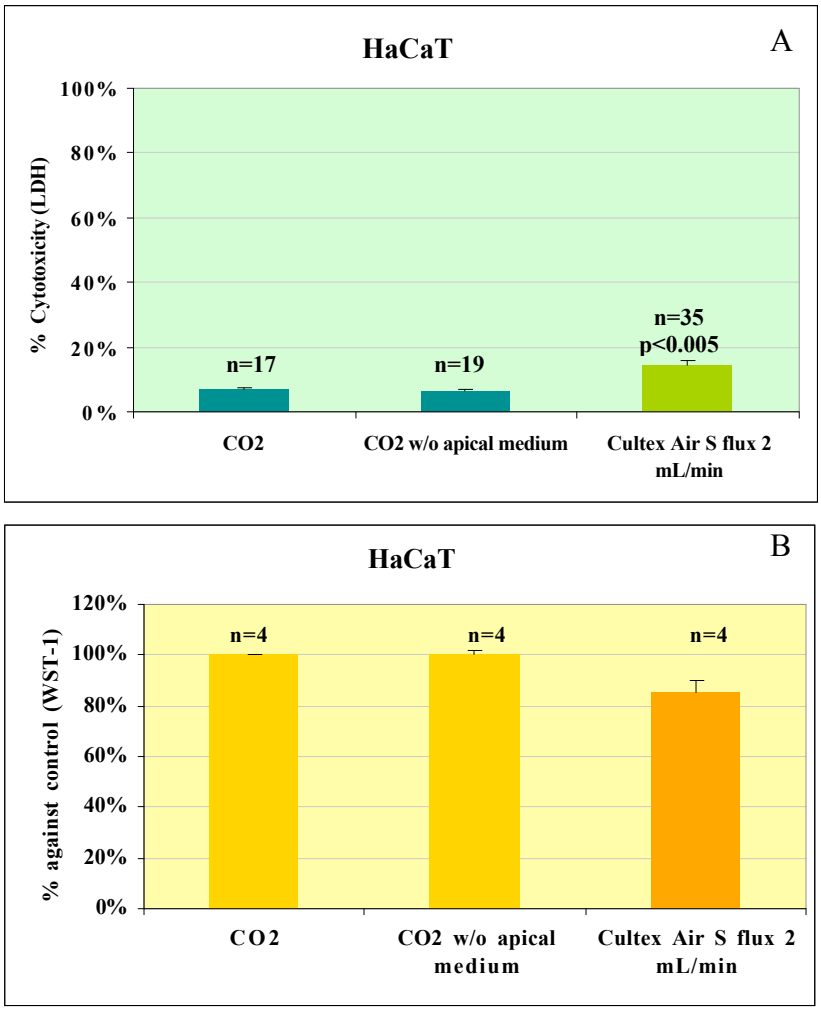

Fig 16: LDH cytotoxicity assay (A) and WST-1 proliferation assay(B) of HaCaT exposed in CULTEX I to synthetic air after 24h incubation.

As shown in figure 16, for the HaCaT cell line we reached about 85% of survival for cells exposed to synthetic air; moreover, the results obtained with LDH assay show statistically significant differences between controls and exposed cells. It will be possible to improve their culture conditions by adding some nutrients in the culture medium and by allowing their better anchorage to the inserts surface by a pre-coating with a collagen matrix.

The results obtained with LDH and WST-1 assays were overlapping.

We decide to choose LDH determination to evaluate the cytotoxicity/survival of treated cells, because it gives more information about the fractions present in our samples. Moreover, for the future, it will be possible to determine the cytotoxicity of treatments on the supernatant fraction (which is proportional to a known number of cells) and use the pellet for other determinations (GSH, ROS, etc).

4.4 Comparison of two CULTEX set up by parallel run.

The second CULTEX® apparatus (CULTEX II), dedicated to the toxic exposure, was compared to the first apparatus by exposing A549 cells to synthetic air in the same conditions.

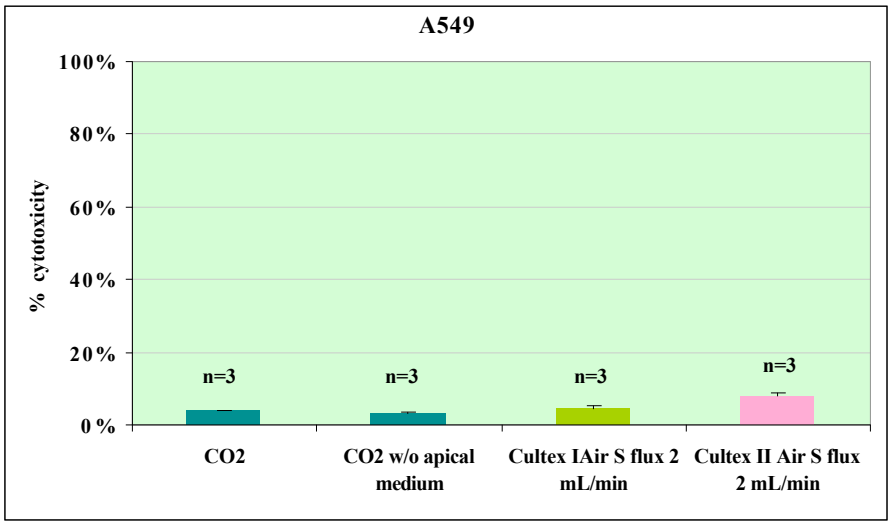

Fig 17: LDH cytotoxicity assay of A549 exposed in CULTEX I and II to synthetic air after 24h incubation.

As shown in figure 17, good results were also obtained for CULTEX II, where the percentage of toxicity was not statistically different from control conditions. In order to avoid contamination, CULTEX I will always be used as control exposure chamber with synthetic air, while CULTEX II will be dedicated to air pollutants exposure.

5 Preliminary results on air pollutants exposure experiments

5.1 CULTEX exposure to toluene with A549 and HaCaT cell lines.

A549 cells were exposed under air-lifted conditions with a mixture of synthetic air/toluene (~50/50 flow equivalent with 5 ppm of toluene in nitrogen) with a 2 mL min^{-1} flow for 1 hour in the CULTEX® device. At the end of the exposure, cells were left on the inserts to recovery for 24 hours in the CO_2 incubator with fresh medium (with 1% FCS, apical and basal). The range of toluene concentration determined with Tenax® sampling and gas chromatography determination was between 0.01 and 0.6 ppm. The sampling was made at the outlet of the CULTEX II before and after exposure.

Cells	A549 / HaCaT Sub-confluence before exposure
Medium :	RPMI 1640 + antibiotics
Gases:	Synthetic air and synthetic air/toluene mixture
Exposure conditions	2 mL·min^{-1} for 1 hour
Moistening :	yes
Tests	LDH after 24h

Control inserts (with or without apical medium) were prepared in triplicate for every exposure experiment. Figure 18 shows in details 2 independent A549 cells exposure results with 2 different toluene concentrations: 0.13 ppm for panel A, and 0.61 ppm for panel B.

Fig 18. A549 toluene 1h exposure. LDH cytotoxicity assay after 24h incubation. CO_2: 3 inserts left in CO_2 incubator with apical medium; CO_2 w/o AP: 3 inserts left in CO_2 incubator without apical medium; CULTEX I: 3 inserts exposed to synthetic air; CULTEX II: 3 inserts exposed to toluene (A: toluene 0.13 ppm; B: toluene 0.61 ppm)

The LDH concentration was determined twenty-four hours after exposure in the adherent cells (alive) and supernatants (death) fractions as previously described. As show, in the figure 18, the percent of LDH released by cells not treated with toluene (CO_2, CO_2 w/o AP and CULTEX I) into the medium was less than 10%, while toluene exposure induces on A549 cells a release that seems to be dose dependent. The treatment with the lower toluene concentration (0.13 ppm) increased LDH release into the medium from 18 to 30% whereas the exposure to the higher toluene concentration (0.61 ppm) led to LDH release from 60 to 65%.

The results of exposure experiments with toluene concentration ranging between 0.1 and 0.6 ppm performed with both A549 and HaCaT cell lines are described in the table below.

Cell line	LDH cytotoxicity				Toluene (ppm)
	CO_2	CO_2 w/o AP	CULTEX I Synthetic air	CULTEX II Toluene	
~ A549 ~					
	5%	5%	7%	10% - 29%	**0.11 - 0.17**
	7%	5%	10%	25%	**0.13**
	9%	6%	8%	61%	**0.61**
~ HaCaT ~					
	10%	8%	33%	51%	**0.21**
	11%	9%	29%	52% - 57%	**0.12 - 0.31**

Table 1: LDH cytotoxicity assay results from independent A549/HaCaT exposures to different toluene concentrations in CULTEX device.

The percentage of cytotoxicity was expressed as the percentage of LDH released in the medium (i.e. present in the 3 fractions apical, basal and pellet apical) on the total LDH (sum of the 4 fractions, see Figure 9).

With the A549 cell line, the range of cytotoxicity in all the three control groups (CO_2, CO_2 w/o apical medium and synthetic air) is about 5 to 10% and reach 10 to 61% when cells are exposed to toluene. Moreover, these experiments indicate a dose related cytotoxicity of the A549 cell line to toluene exposure.

For HaCaT cell line experiments, only cells left in the incubator with or without the apical medium show an acceptable viability (about 10% of cytotoxicity). The exposure to synthetic air in the CULTEX I raise the cytotoxicity to 30% and demonstrate that with this cell line the exposure parameters are not yet set-up and the results obtained with toluene exposure cannot be validate at this point.

All the toluene exposure experiments for the A549 bronchial cell lines are summarized in figure 19. The average of cytotoxicity percent in all control groups is about 10% without statistically significant differences among them. Only the toluene treated samples were statistically different when compared with the 3 control groups with a 25% average toxicity.

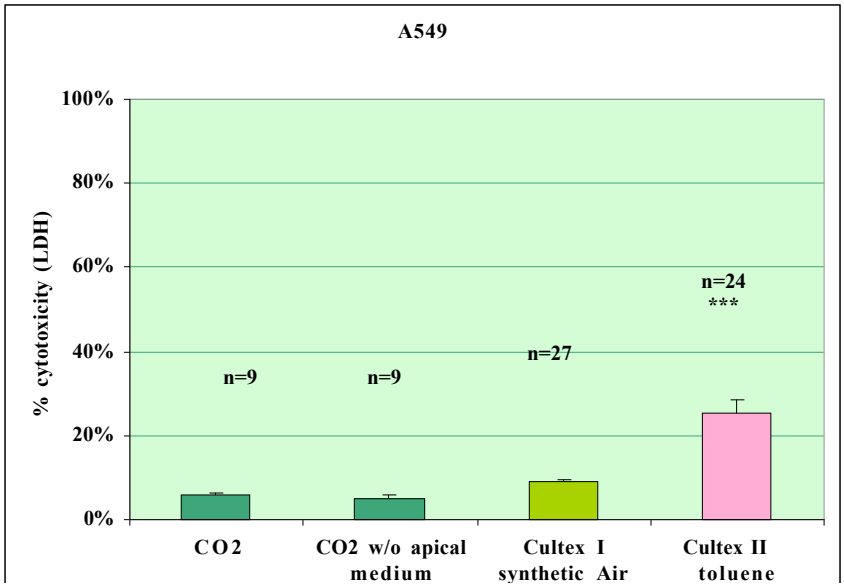

Fig 19: LDH cytototoxicity assay after 24h incubation of A549 exposed to toluene (0.1-0.6 ppm) in CULTEX device. Data are the average of at least 3 independent experiments .Every exposure (with 3 inserts in parallel) was carried out in duplicate or triplicate. Statistical analysis was carried out with Student t Test. The p value was respectively 0.0012, 0.0006 and <0.00001 with respect to the 3 control groups.

The same series of exposure experiments have been performed with HaCaT cell line (see Figure 20). Airlifted cells exposed to synthetic air show an average of cytotoxicity percent of 18% which was significantly different from the control groups (CO_2 and CO_2 w/o AP). This result of the control exposure of HaCaT cell line could be due either to the different adhesion properties of the two cell lines (A549 and HaCaT) when cultured on membranes, or to a higher sensitivity of air-lifted HaCaT cells to mechanical effects of synthetic air exposure. Nevertheless, the average cytotoxicity induced by toluene exposure was 38% and was significantly different to all the control groups (CO_2 and CO_2 w/o AP, synthetic air).

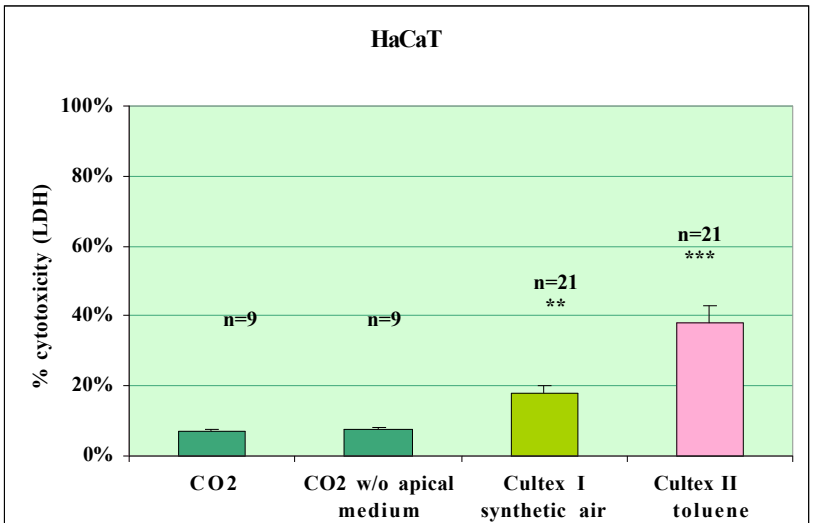

*Figure 20: LDH cytototoxicity assay after 24h incubation of HaCaT exposed to toluene (0.1-0.6 ppm) in CULTEX device Data are the average of at least 3 independent experiments .Every exposure (with 3 inserts in parallel) was carried out in duplicate or triplicate. Statistical analysis was carried out with Student t Test. **The p value for CULTEX I was <0.005 and <0.0001 vs CO2 and CO2 w/o AP. *** The p value for toluene exposure was <0.0005 vs all the control groups.*

5.2 CULTEX exposure to benzene with A549 cell lines.

A549 cells were exposed to benzene with the same experimental conditions used for toluene. The range of benzene concentration determined with Tenax® sampling and gas chromatography determination was between 0.1 to 0.3 ppm. The sampling was made at the outlet of the CULTEX II before and after exposure.

All the benzene exposure experiments for the A549 bronchial cell lines are summarized in figure 21. The average percent of cytotoxicity in all control groups is about 10% without statistically significant differences among them. Only the toluene treated samples were statistically different when compared with the 3 control groups with a 29% average toxicity.

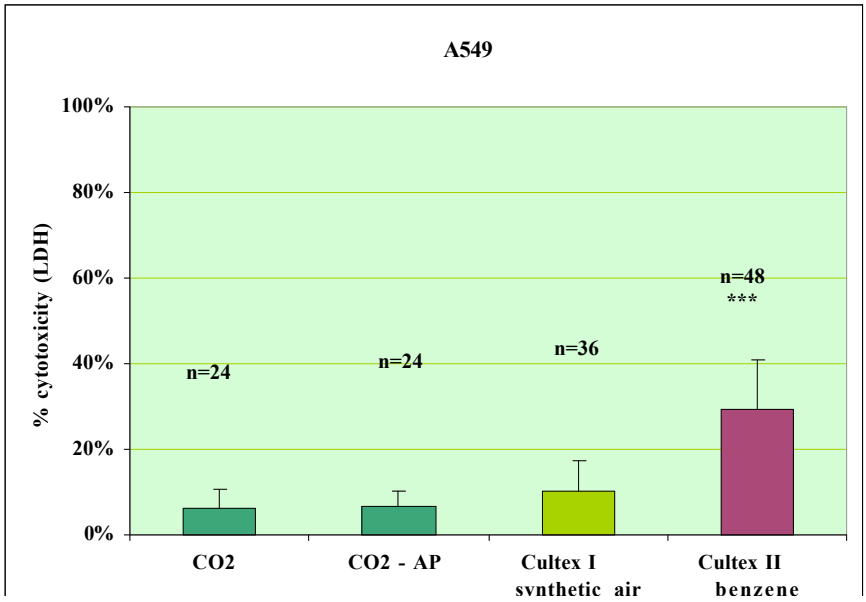

Figure 21: LDH cytototoxicity assay after 24h incubation of A549 exposed to benzene (0.1-0.3 ppm) in CULTEX device. Data are the average of at least 3 independent experiments .Every exposure (with 3 inserts in parallel) was carried out in duplicate or triplicate. Statistical analysis was carried out with Student t Test. The p value was <0.00001 with respect to the 3 control groups.

- IL-8 determination as inflammatory biomarker

The apical medium of cells exposed for 1 hour with synthetic air or toluene, and left in culture for 24 hours, was recovered, centrifuged and frozen at –80°C. Supernatants were thawed and analyzed in duplicate. The amount of IL-8 released from control or treated samples was determined with an ELISA test (*BD Pharmingen*, USA).

IL-8 levels were expressed as percentage of increase with respect to control samples (100%). We considered as control (ctrl) IL-8 released by cells left in CO_2 incubator, as CO_2 w/o AP gave the same response (data not shown).

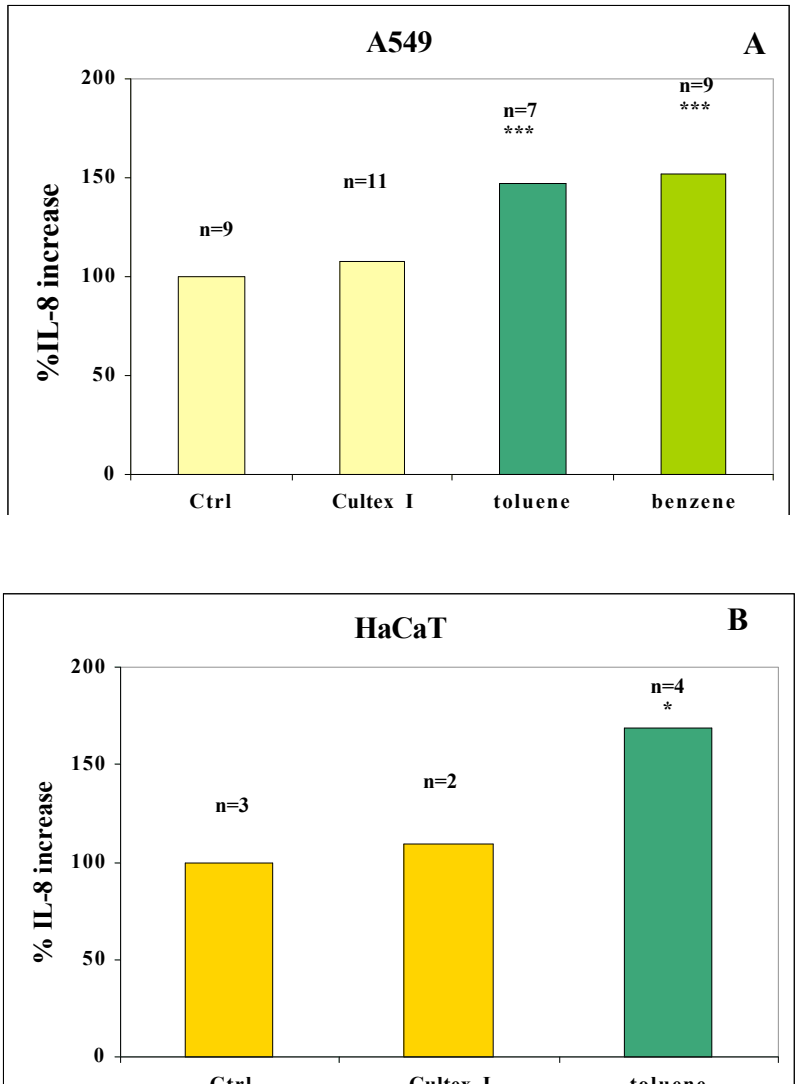

*Figure 22. IL-8 determination with ELISA test on A549 and HaCaT cells exposed with CULTEX to syntetic air, toluene or benzene. IL-8 levels were expressed as percentage of increase with respect to control samples (100%). The determinations were carried out in duplicate for every sample. n=number of determinations. Statistical analysis was carried out with Student t Test. Panel A: A549 cell line: ***p=0.005 (toluene vs Ctrl); p<0.05 (toluene vs CULTEXI); ***p<0.0005 (benzene vs Ctrl); p<0.0005 (benzene vs CULTEXI); Panel B: HaCaT cell line:*p=0.024 (toluene vs Ctrl); p=0.021 (toluene vs CULTEXI)*

Toluene exposure stimulated significantly the IL-8 release for both cell lines (Figure 22). In details, the IL-8 release was increased in average of 36% for A549 cell line and of 44% for HaCaT cell line, when compared with synthetic air exposure. Benzene exposure of A549 cell line stimulated IL-8 release by 30% when compared with synthetic air exposure. These results are in agreement with IL-8 levels described in literature, even in different experimental set-up (exposure system and toluene concentration) (9). From these preliminary results, we demonstrate that CULTEX exposure with toluene and benzene in vapor phase is able to induce an inflammatory response.

5.3 Combined exposure to physical and chemical agents with HaCaT cell line.

Dermal cells are exposed to both, chemical and physical stressors. For this reason, we performed preliminary experiments in order to find the experimental conditions to expose in vitro human keratinocytes to both chemical (VOCs) and physical (UVB radiations) agents.

All these preliminary experiments were carried out on dermal cells growing on Petri dishes, with chemicals dissolved in the medium, in order to set up the endpoints evaluation, in view of future combined exposure experiments with the CULTEX exposure set up

HaCaT cells, growing to subconfluence in 12 well plates, were irradiated with 5.1 W/m^2 UV (280 nm – 400 nm) for 15, 30 or 60 min. The effects of treatments were evaluated after 24 hours recovery with LDH cytotoxicity assay (figure 24, panel A) and WST- proliferation assay 1 (figure 23, panel B).

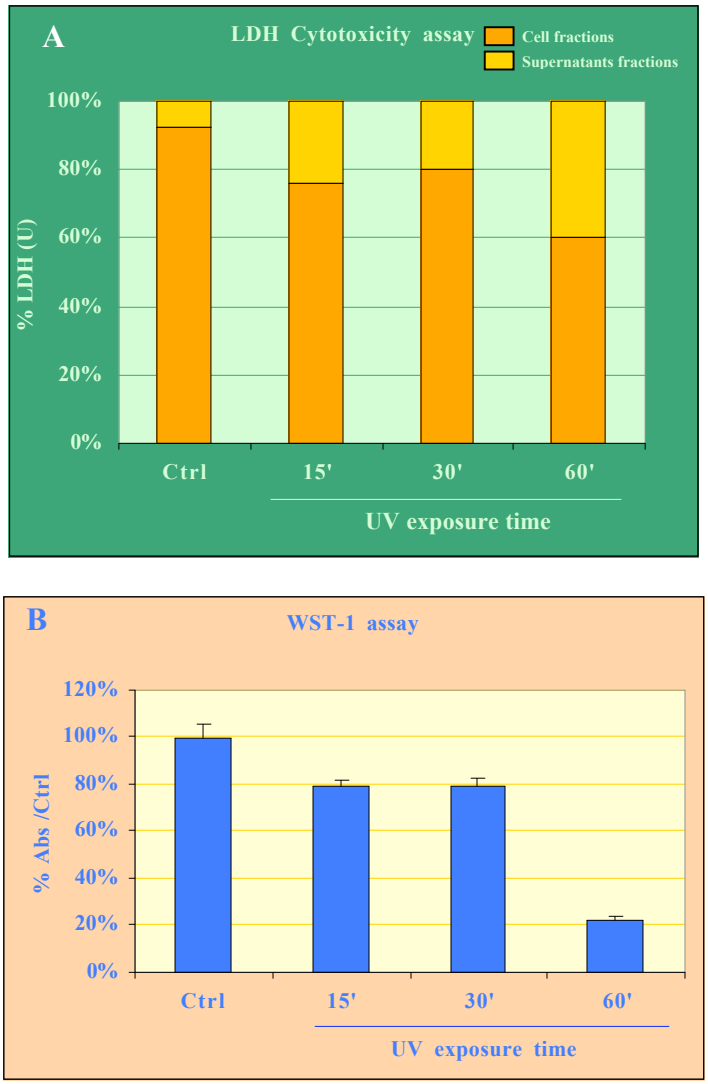

Fig 23: LDH cytotoxicity assay (A) and WST-1 proliferation assay (B) of HaCaT exposed to UV radiations for 15, 30, 60 min

We also performed a proliferation test by plating 100 cells of every treatment in 35mm plates in triplicate. The colonies formed were stained with 20% GIEMSA for 20 min. and counted.

The results in figure 24 were expressed as percentage of colonies grown from every treatment group versus control and show that all 3 treatments reduced considerably the colonies formation compared to the control. While the exposure to 15 and 30 minutes of UV reduced the colonies formation by more than 60%, a reduction of more than 95% was determined for cells exposed for 60 minutes.

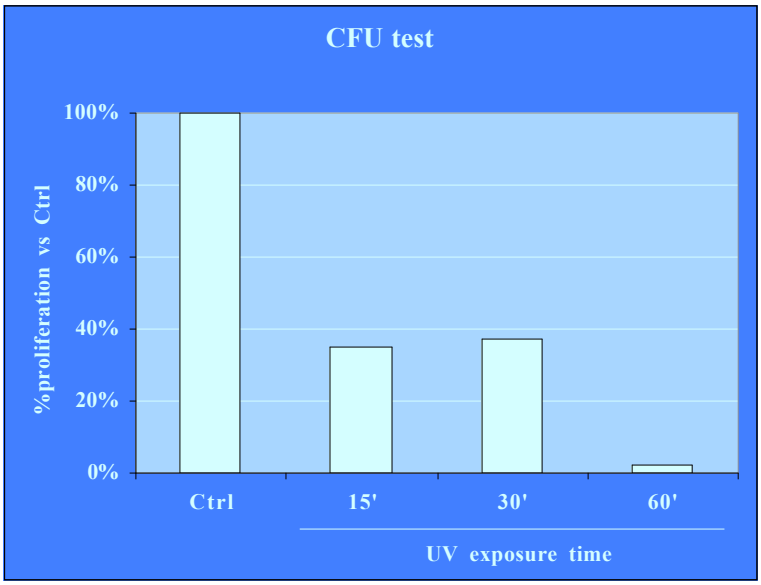

Figure 24. CFU (Colony forming units) proliferation test after UV radiation exposure. Colonies grown for 1 week in 35 mm plates were stained and counted. The results are expressed as percentage of proliferation vs ctrl.

The oxidative stress induced by UV radiation was evaluated as previously described. The results (figure 25) are expressed as percentage of fluorescence increase at 3 or 24 hours after exposure for every treatment group. All three UV treatments were able to generate more Reactive Oxygen Species (ROS) compared to untreated cells.

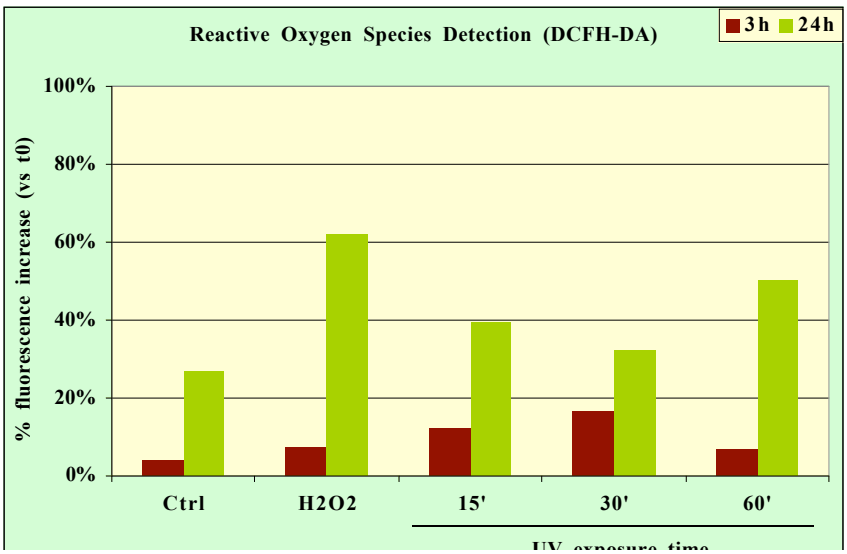

Figure 25. Oxidative stress evaluation on HaCaT cell line 3 and 24 hours after UV exposure for 15, 30, 60 min. H_2O_2 (100 μM) was chosen as ROS positive control

From this preliminary experiment we selected 30 min UV exposure as a starting condition for combined treatments.

As first combined exposure set–up we evaluated the effects of two priority pollutants, formaldehyde or toluene (100μM), before or after UV treatment. The results of the 2 tests, as shown in figure 26 (A and B) are almost overlapping. The association between these chemical and physical agents seems to have an additive or even a synergic effect.

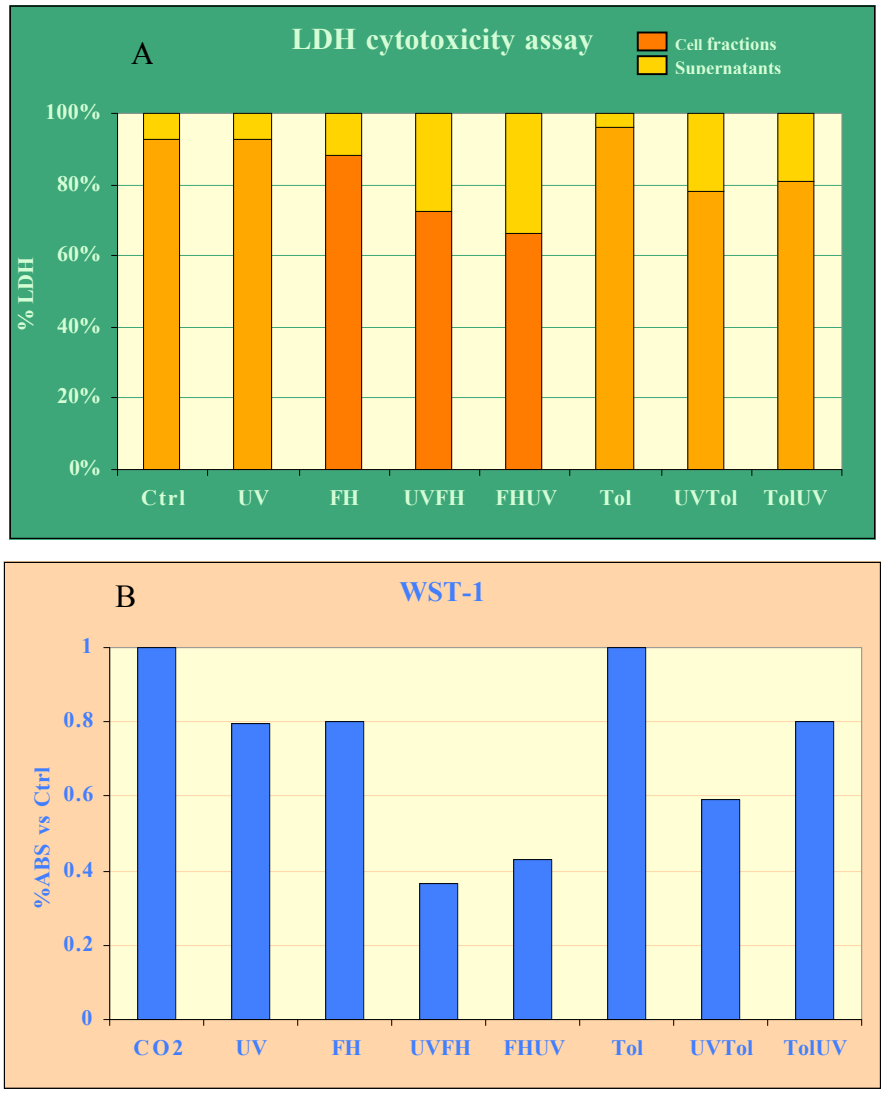

Figure 26. Single or combined exposures of HaCaT cells to UV radiations for 30 min, formaldehyde (FH) and toluene (Tol). LDH cytotoxicity assay (A) and WST-1 proliferation assay(B)

The inflammatory response following the treatment with UV radiations for 30 min., formaldehyde (100 μM for 3 hours) and their association was evaluated (Figure 27). All the treatments stimulated IL-8 release at levels significantly different when compared to control, but not among each other.

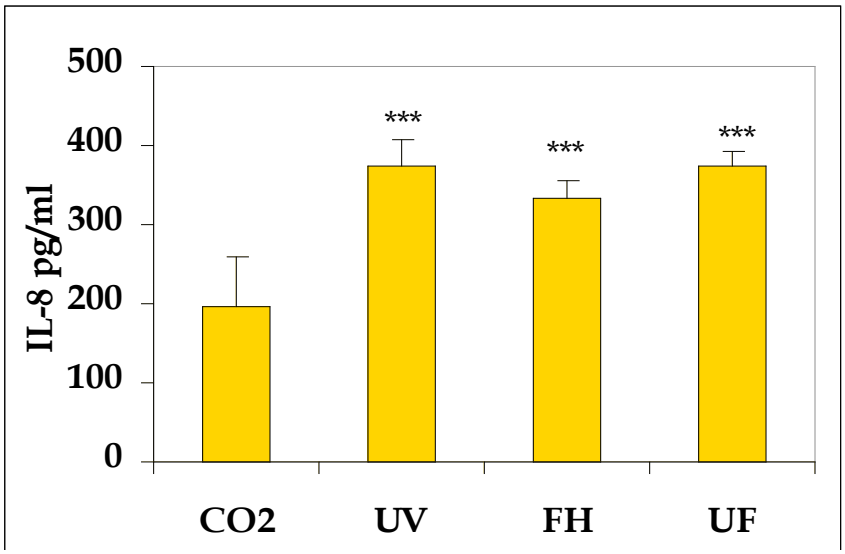

*Figure 27. IL-8 determination with ELISA test on HaCaT. Cells were exposed to UV radiations for 30 min, to formaldehyde 100 μM for 3 hours and to their association. Supernatant was analized after 204 hours of recovery with control medium; ***p<0.05*

The combined treatments (UV and formaldehyde) induced on HaCaT an oxidative stress shown by a dose dependent increasing of ROS generation (see Figure 28) while single treatments show comparable results to the control.

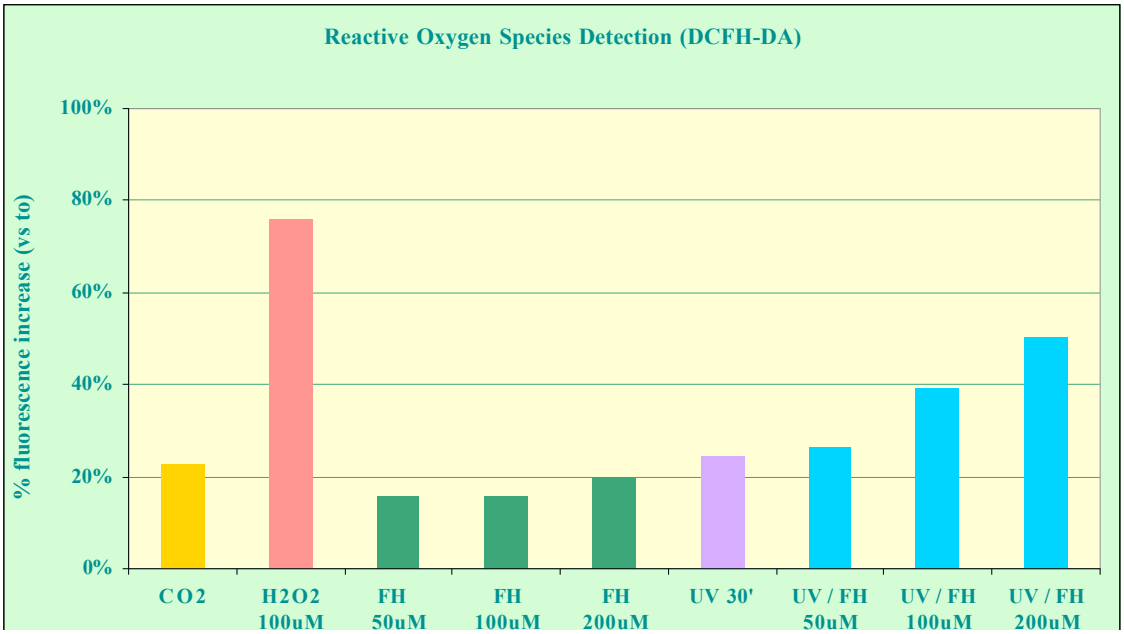

Figure 28. Oxidative stress evaluation on HaCaT cell line. Cells were exposed to UV radiations for 30 min and/or to formaldehyde 50, 100 or 200 μM for 3 hours. H2O2 (100 μM) was chosen as ROS positive control

These results will be the basis to perform combined exposure treatments of UV irradiation and priority pollutants alone or in mixtures applied to air-lifted cells in the CULTEX device.

6 Conclusions

The aim of this work was to set-up an *in vitro* exposure model in order to evaluate toxicological properties of priority air pollutants (VOCs) as single components, in mixtures and combined with physical stressors like UVB radiation.

Human lung and dermal cell lines (A549 and HaCaT) representative of VOCs' target tissues, were exposed to chemicals in air/lifted interface device named CULTEX®. In addition, skin is also a target organ for UV radiation; therefore we performed combined exposures on HaCaT cell line.

The main part of the work done until now was done with CULTEX® device in order to obtained reproducible control exposures conditions to pure air (e.g. synthetic air oxygen/nitrogen – 20/80). As shown in the table below, several parameters have been changed since the installation of the new device.

Several modifications on cell culture conditions before exposure and on the exposure parameters (airflow, pH buffer, moistening) allow us to keep cells in better conditions during the exposure, and to decrease some mechanical effects which can damage them. We considered as the minimum requirement for acceptable control samples (synthetic air exposure) culture conditions allowing a survival above 90% (evaluated with LDH cytotoxicity and WST-1 proliferation assay). This was achieved for A549 cell line, while further improvements are necessary for HaCaT cell line which reaches until now viability around 80%.

Preliminary exposure experiments performed with toluene up to 0.6 ppm; showed reproducible direct toxic effects on cell cultures with 25% of cytotoxicity for A549 and 38% for HaCaT cell lines (with LDH assay). At these concentrations, toluene as hydrophobic compound directly target and destroy cell membrane. The increasing LDH cytoxicity with o.6 ppm toluene exposure compared to 0.1 ppm in A549 cell line might give first indications of a dose related response of this cell type. Similar results were obtained for A549 cells exposed to benzene (0.1 to 0.3 ppm) with 29% of cytotoxicity. Further exposure experiments with different toluene and benzene concentrations have to be worked out for validating this hypothesis. Moreover, we demonstrated the ability of toluene and benzene to induce an inflammatory response (IL-8 stimulation), according to data reported in literature.

Dermal cells are often exposed to a combination to chemical and physical stressors. In preliminary experiments we exposed *in vitro* human keratinocytes (HaCaT) to both chemical (formaldehyde, toluene) and physical (UVB radiations) agents.

Several endpoints were evaluated: cytotoxicity (LDH), proliferation (WST-1, CFU), inflammatory response (IL-8) and oxidative stress (ROS). The effects observed with single agents treatment (statistically significant compared to controls) seems to be enhanced by their combination.

Summarizing, all these results show the sensitivity and specificity of the over all CULTEX exposure set-up and this will be the basis to perform combined exposure treatments to UV irradiation and priority pollutants alone or in mixtures.

7 Acknowledgments

We acknowledge the technical assistance of Mr. T. Krebs from the Vitrocell company, Mr. A. Collotta for his skillful technical assistance and Mr. P. Leva, A. Katsogiannis and O. Geiss from the PCE unit for all air pollutants determination. For the determination and calibration of the UV lamp we thank Mr. J. Schreder. We are also very grateful for the fruitful co-operation with the Nano-Biotechnology Action of the BMS Unit.

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Abstract

This work was to investigate toxicity of single volatile chemicals and their mixtures. We developed a reproducible *in vitro* dynamic exposure model where human tumor lung epithelial (A549) and human keratinocytes (HaCaT) cell lines representative of VOC's target tissues (lungs and skin) were exposed in an airlifted interface to air pollutants.

The CULTEX® device was selected for the exposure of cells grown on porous membranes to individual air pollutants or in mixtures as a simulation of *in vivo* inhalation exposure. The studies started with the application of priority air pollutants, which were selected in the frame of the INDEX project ("Critical appraisal of the setting and implementation of EU INDoor EXposure limits").

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