



# **In vitro toxicological evaluation of emissions from catalytic oxidation removal of industrial VOCs by air/liquid interface (ALI) exposure system in repeated mode**

Clémence Méausoone, Rebecca El Khawaja, Gauthier Tremolet, Stéphane Siffert, Renaud Cousin, Fabrice Cazier, Sylvain Billet, Dominique Courcot, Yann Landkocz

## **► To cite this version:**

Clémence Méausoone, Rebecca El Khawaja, Gauthier Tremolet, Stéphane Siffert, Renaud Cousin, et al.. In vitro toxicological evaluation of emissions from catalytic oxidation removal of industrial VOCs by air/liquid interface (ALI) exposure system in repeated mode. *Toxicology in Vitro*, 2019, 58, pp.110 - 117. 10.1016/j.tiv.2019.03.030 . hal-03486078

**HAL Id: hal-03486078**

**<https://hal.science/hal-03486078>**

Submitted on 20 Dec 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

***In vitro* toxicological evaluation of emissions from catalytic oxidation removal of industrial VOCs by air/liquid interface (ALI) exposure system in repeated mode**

Clémence MÉAUSOONE<sup>1</sup>, Rebecca EL-KHAWAJA<sup>1</sup>, Gauthier TREMOLET<sup>1</sup>, Stéphane SIFFERT<sup>1</sup>, Renaud COUSIN<sup>1</sup>, Fabrice CAZIER<sup>2</sup>, Sylvain BILLET<sup>1</sup>, Dominique COURCOT<sup>1\*</sup>, Yann LANDKOCZ<sup>1</sup>

<sup>1</sup>UCEIV - EA4492, Unité de Chimie Environnementale et Interactions sur le Vivant, SFR Condorcet FR CNRS 3417, Université du Littoral Côte d'Opale, Dunkerque, France.

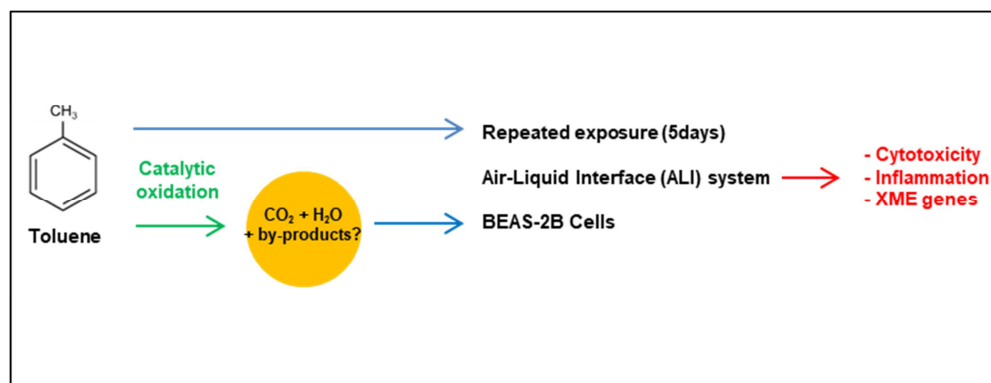
<sup>2</sup>Centre Commun de Mesure, Université du Littoral Côte d'Opale, Dunkerque, France.

**Abstract**

Toxicity of toluene and by-products formed during its catalytic oxidative degradation was studied in human bronchial BEAS-2B cells repeatedly exposed. BEAS-2B cells were exposed using an Air-Liquid Interface (ALI) System (Vitrocell®) for 1h per day during 1, 3 or 5 days to gaseous flows: toluene vapors (100 and 1,000 ppm) and outflow after catalytic oxidation of toluene (10 and 100%). After exposure to gaseous flow, cytotoxicity, inflammatory response and **Xenobiotic Metabolism Enzymes (XME)** gene expression were investigated. No significant cytotoxicity was found after 5 days for every condition of exposure. After cells exposure to catalytic oxidation flow, IL-6 level increased no significantly in a time- and dose-dependent way, while an inverted U-shaped profile of IL-8 secretion was observed. XME genes induction, notably *CYP2E1* and *CYP2F1* results were in line with the presence of unconverted toluene and benzene formed as a by-product, detected by analytical methods. **Exposure to pure toluene also demonstrated the activation of these XMEs involved in its metabolism.** Repeated exposure permits to show *CYP1A1*, *CYP1B1* and *CYP2S1* expression, probably related to the formation of other by-products, as PAHs, not detected by standard analytical methods used for the development of catalysts.

\*Corresponding author: dominique.courcot@univ-littoral.fr

## Graphical abstract



## Highlights

- Toxicity of toluene increases with time when cells are repeatedly exposed
- Toxicological analysis helps for validation of catalysts used in treatment of VOCs
- Cellular response supports the identification of chemically undetected by-products

## Keywords

Air-Liquid Interface system, repeated exposure, toxicological validation, catalysis, toluene

## Acknowledgements

This work was supported by the European Interreg V DepollutAir project (No. 1.1.18). UCEIV EA4492 participates in the CPER research projects Climibio and IRENE, which are financially supported by the Hauts-de-France Region, the French Ministère de l'Enseignement Supérieur et de la Recherche and the European Regional Development Fund. All authors declare no conflict of interest.

## 1. Introduction

Indoor and outdoor air pollution kill 7 million people prematurely every year (WHO, 2016). Volatile Organic Compounds (VOCs) significantly contribute in this pollution. In 2016, almost 8,000 Gg of Non-Methane Volatile Organic Compounds (NMVOCs) were emitted in the atmosphere in Europe (EEA, 2018). Industry, residential, agriculture and road transports were the main emission sectors with respectively 3,689 Gg, 1,296 Gg, 1,318 Gg and 669 Gg of European NMVOC emissions. Toluene is one of the VOCs widely used as solvent in many industrial processes, such as printing, shoemaking, painting and car industries. Indeed, this VOC is manufactured and imported in European Economic Area between 1 and 10 Gg every year (ECHA, 2018). The chronic toxic effects of toluene are well known (INRS, 2012; Tormoehlen et al., 2014). It was classified as Carcinogenic, Mutagenic and Reprotoxic (CMR), category 3, by the European Union. Psycho-organic syndromes as memory and learning deficit, insomnia or personality changes are the other major effects after a chronic inhalation of toluene.

Different remediation techniques have been developed to reduce VOCs emissions, in particular recovery processes such as adsorption, and destruction processes, such as thermal or catalytic oxidation. The latter consists in the total oxidation of VOCs into water and carbon dioxide in a reaction chamber containing a catalyst. However, by-products in low concentration can potentially be formed during reaction depending on the catalyst used and the molecule treated. Some of these by-products are toxics in the human respiratory tract. It is therefore important to evaluate the possibly harmful effects of the gaseous emissions obtained after catalytic treatment.

Toxicological analyses can be performed using different models, animal or cellular ones. The use of alternative methods to animal experiments is a challenge (Doke and Dhawale, 2015) and corresponds to the Reduction, Refinement and Replacement (3R) strategy. Thus, tests using animals can be replaced by *in vitro* models like lung derived cells. [The human bronchial BEAS-2B cell line consists in a relevant model for the toxicological study of atmospheric pollutants \(Garcia-Canton and Minet, 2013; Persoz et al., 2012\). Indeed,](#)

bronchi are one of the first targets of inhaled substances, since they allow the air circulation toward the lung. Moreover, epithelial cells cover the surface of the organs and are in direct contact with the inhaled air and xenobiotics.

Toxicological studies quality also depends on the choice of the exposure system (Gałęzowska et al., 2016). Exposure to pollutants can be performed either in submerged conditions or using an Air-Liquid Interface (ALI) system. The submerged exposures to VOCs generally do not correspond to realistic conditions (Upadhyay and Palmberg, 2018). On the contrary, the ALI method allows to expose the apical side of the cells directly to pollutants contained in the gaseous phase whereas the basolateral side of cells is still in contact with the culture medium (Aufderheide, 2005). This method is known for high reproducibility of exposures and simulate quite well the real *in vivo* exposure conditions (Gałęzowska et al., 2016). BEAS-2B cells are cell lines often cultured and exposed using an ALI System (Anthérieu et al., 2017; Hiemstra et al., 2018; Persoz et al., 2012).

Most of *in vitro* toxicological studies have been performed up to now following an acute design of exposure. This method helps in the understanding of the implied mechanisms of action but cannot mimic cumulated effects observed when people are repeatedly exposed to air pollutants. Consequently, more and more studies are performed considering repeated exposures either in submerged mode (Zhou et al., 2016) or using an ALI system (Bardet et al., 2014). Thus, subacute exposure of BEAS-2B allows to highlight new cellular responses to xenobiotics, such as cells morphology modification (Vales et al., 2015) or alterations in gene expression (Van Der Toorn et al., 2018). Moreover, it can allow the detection of delayed mechanisms of toxicity.

After exposure to airborne pollutants, pulmonary cells can transform xenobiotics to decrease their toxicity and to eliminate their hydrophilic metabolites (Castell et al., 2005). This metabolization is performed by various Xenobiotic Metabolism Enzymes (XMEs) in two steps: functionalization by cytochromes P-450 (CYP) and conjugation. The implied XMEs depend on the cell and on the nature of the xenobiotics (Castell et al., 2005). Consequently, gene induction of XME can be considered as a relevant biomarker of exposure to

104 xenobiotics. In the case of cell exposure to the gaseous flows after catalytic treatment of  
105 pollutants, the determination of the XME gene expression could thus appear as a  
106 toxicological validation method of the tested catalyst.

107 This study focused first on the assessment of the subacute toxic effects of toluene on BEAS-  
108 2B cells. The second aim was to study the toxicity of the gaseous flow emitted after catalytic  
109 oxidation of toluene. Two catalysts were used: a commercial one composed of 0.4%  
110 Pd/ $\gamma$ -Al<sub>2</sub>O<sub>3</sub> and a mixed oxide of Co<sub>6</sub>Al<sub>2</sub>-O. BEAS-2B cells were exposed repeatedly using an  
111 ALI system for 1h per day during 1, 3 or 5 days. *In order to assess a potential dose-response  
112 effect, two concentrations of exposure to toluene or catalytic gas flows were chosen. At first,  
113 cells were exposed to 100 and 1,000 ppm of toluene: 100 ppm is the concentration  
114 corresponding to the occupational limit value of toluene for a short term exposure; 1,000 ppm  
115 is ten-fold more concentrated and corresponds to the catalytic gaseous input. This factor ten  
116 was also used for the cell exposure to 10% and 100% of the gaseous flow emitted after  
117 catalytic removal of 1,000 ppm of toluene.* After cell exposure, inflammatory response and  
118 induction of Xenobiotic Metabolism Enzymes (XMEs) genes were assessed to evaluate the  
119 potential subacute toxicity of the components present in the gaseous streams.

## 120 **2. Materials and methods**

### 121 **2.1. Catalytic oxidation of toluene**

122 The total oxidation of toluene was studied using two solid catalysts: a commercial catalyst  
123 Pd/ $\gamma$ -Al<sub>2</sub>O<sub>3</sub> (Acros Organics, 0.5wt% Pd) and a mixed oxide Co<sub>6</sub>Al<sub>2</sub>-O catalyst. The latter was  
124 synthesized by a coprecipitation method using metal salts with a molar ratio Co/Al of 6/2, as  
125 described elsewhere (Genty et al., 2015).

126 The capacity of catalysts to convert toluene was evaluated in a fixed-bed reactor considering  
127 a toluene/air mixture (1,000 ppm) with a flow of 100 mL.min<sup>-1</sup>. The temperature of the reactor  
128 was increased from room temperature to 250°C (Pd/ $\gamma$ -Al<sub>2</sub>O<sub>3</sub>) and 350°C (Co<sub>6</sub>Al<sub>2</sub>-O). The latter  
129 temperature values were selected to obtain a total conversion of toluene and avoid the  
130 formation of possible by-products. The tests were then followed under isothermal conditions  
131 for 5 days. The outflow gases were analyzed with a micro-gas chromatograph (Varian CP-

4900). Catalytic performances of both catalysts were compared from T50 and T100 which represent respectively the temperatures at which 50% and 100% of toluene was converted. The toluene conversion was calculated considering products, by-products and carbon number for each compound (Brunet et al., 2015).

## 2.2. Cell culture conditions

BEAS-2B cells (ATCC® CRL-9609™, ECACC 95102433) are human bronchial epithelial cells isolated from a healthy donor, transformed by a hybrid virus and cloned. This cell line allows to perform subacute exposure (Zhou et al., 2016) and are metabolically active (Uppstad et al., 2010). This cellular model has also been shown to be suitable for ALI exposures (Hiemstra et al., 2018; Vales et al., 2015). Cell culture conditions were optimized for 5 days exposure. Morphology of cells and mortality were assessed and given in supplementary material.

Cells were exposed to a gaseous flow of toluene (>99.9% purity, VWR Chemicals) diluted in air and to a gaseous stream resulting from the catalytic oxidation of toluene using Vitrocell® Air-Liquid Interface (ALI) system (Vitrocell GmbH, Germany). Three Transwell® inserts, containing BEAS-2B cells, were placed into each 6/3 CF Stainless module containing 17.5 mL of LHC-9 culture medium. The upper cellular supernatant was removed from Transwell® inserts before the exposure and kept to be added back after the exposure. VOCs are delivered to cells in the gaseous form by a trumpet-shaped interface. For each exposure time, five gas mixtures were tested: (i) purified air corresponding to the negative control, (ii) 1,000 ppm and (iii) 100 ppm of toluene in air, (iv) 100% and (v) 10% of gaseous flow after catalytic treatment. In order to study the toxicological effects of a repeated exposure, cells were exposed each day for 1h over 5 days period and then harvested in day 1, day 3 and day 5. In parallel, 3 Transwell® inserts were kept in the incubator to serve as negative control without exposure. Five hours after the cell exposure to toluene or to catalytically treated gaseous flow, supernatants were collected, then Transwell® inserts were rinsed with Phosphate Buffer Saline before storage at -80°C.

## 2.3. Toxicological analysis

### **2.3.1. Cytotoxicity and cell proliferation**

The cellular cytotoxicity detection was performed by LDH assay (Roche Diagnostic, Merck-Millipore), according to the manufacturer protocol. This colorimetric method allowed to evaluate cell membrane integrity with the quantification of the lactate dehydrogenase (LDH) released by loss of cells integrity into the culture medium. 100  $\mu$ L of supernatant were collected into every Transwell® insert, five hours after exposure and transferred in triplicate in a 96-well plate. 100  $\mu$ L of reaction mixture was added and the plate was incubated during 30 minutes in the dark, at room temperature, before the measurement of optical density at 490 nm. AlamarBlue® test was used to evaluate cell proliferation, according to the manufacturer instruction (Invitrogen, ThermoFisher Scientific). Living cells can indeed convert resazurin to fluorescent resorufin (Excitation: 530 nm / Emission: 590 nm). Briefly, a tenth of alamarBlue® reaction solution was added in the culture medium. After 4 hours of incubation at 37°C, the fluorescence intensity was measured.

### **2.3.2. Inflammatory response**

ELISA method was used to study the inflammatory response of BEAS-2B cells. IL-6 and IL-8 cytokines were quantified in the Transwell® insert supernatant using ELISA MAX<sup>tm</sup> kit (BioLegend, Ozyme) according to the manufacturer instruction.

### **2.3.3. Gene expression**

The induction of Xenobiotic Metabolism Enzymes (XMEs) genes was evaluated by Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR). Total RNA was extracted by Nucleospin® TriPrep kit (Macherey-Nagel) and quantified by spectrophotometry and capillary electrophoresis ( $\mu$ dropplate, ThermoScientific and Bioanalyzer 2100, Agilent). cDNA was then synthesized by Reverse Transcription (RT) using High Capacity cDNA RT kit (Applied Biosystem, ThermoScientific) according to the manufacturer protocol. The relative expression of 12 XMEs gene (table 1) was quantified using Taqman® probe assay. The 12 XMEs gene were selected according to benzene and toluene metabolism (INRS, 2012; Rappaport et al., 2009) and according to their induction previously shown in BEAS-2B cells (Courcot et al., 2012). The results have been analyzed by the  $\Delta\Delta C_t$  method (Livak and



Schmittgen, 2001) using air-exposed cells as reference and normalized by the geometric mean Ct of three genes : 18S ribosomal RNA (18S), Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and Beta-2 microglobulin (*B2M*). The results were expressed in the form of relative quantification (RQ) value. The latter represents the gene induction level in cells exposed to diluted toluene or to the gaseous flow after catalytic oxidation of toluene, compared to the same gene induction level exposed to the purified air.

## **2.4. Chemical analysis**

During cell exposure, toluene concentration was monitored by  $\mu$ GC (Agilent Technologies GC 5975C). For cells exposed to the gaseous flow after catalytic treatment, components were collected by a Tenax<sup>TM</sup> TA cartridge (Perkin Elmer) for 15 minutes. After cartridge desorption by a thermal desorber (TurboMatrix, Perkin Elmer), by-products were detected and quantified by chromatography coupled with mass spectrometry (Perkin Elmer GC Clarus 680–MS Clarus 600S).

## **2.5. Statistical analysis**

Statistical analysis was performed using Prism 7.05 (GraphPad Software, La Jolla California USA) for EC<sub>10</sub> cytotoxicity/proliferation assay (Hill slope model) and for ELISA assay (Mann-Whitney U test,  $p < 0.05$ ). For qPCR assay, data treatment was performed on QuantStudio software (Applied Biosystem, ThermoScientific).

# **3. Results**

## **3.1 Chemical analysis**

Monitoring the composition of the gaseous flow emitted after total oxidation of toluene because by-products components could possibly be formed and could be more harmful than the initial VOC. Brunet et al. have proved that the main by-product of toluene oxidation is benzene due to its low chemical reactivity (Brunet et al., 2015). The reaction route is initiated by an attack on the methyl group causing direct dealkylation of toluene. A profile of benzene emission (Figure 1) highlighted the production of few ppm of benzene only with Pd/ $\gamma$ -Al<sub>2</sub>O<sub>3</sub> catalyst. Benzene formation increased with toluene conversion to reach a maximum and then decreased at higher temperature.

Under isothermal conditions, non-converted toluene and by-products formation was evidenced (Table 2). The highest concentration of non-converted toluene was quantified for the commercial catalyst, Pd/ $\gamma$ -Al<sub>2</sub>O<sub>3</sub>. From day 1 to day 5 of exposure, Pd/ $\gamma$ -Al<sub>2</sub>O<sub>3</sub> toluene concentration increased slightly (from 239 ppb to 265 ppb). Lower emission of toluene occurred using the Co<sub>6</sub>Al<sub>2</sub>-O catalyst and the concentration in the gaseous flow was divided by ~2 between the day 1 and the day 5 (96 ppb and 46 ppb respectively).

The main by-products detected were benzene and acetic acid. Benzene was detected after catalytic oxidation using Pd/ $\gamma$ -Al<sub>2</sub>O<sub>3</sub> only and the concentration in the flow decreased over time (from 549 ppb after day 1 to 396 ppb after day 5). After treatment using Co<sub>6</sub>Al<sub>2</sub>-O catalyst, concentration of acetic acid reached 659 ppb and decreases rapidly over time.

### **3.2. Cytotoxicity**

The quantification of LDH release in culture medium did not show any significant changes in the membrane integrity of cells exposed to different gaseous flows (purified air, diluted toluene or flow after catalytic oxidation). Indeed, we observed a LDH release lower than 10% after cell exposure to catalytic oxidation flow after 5 days and lower than 20% for cells exposed to 1,000 ppm of toluene after 5 days (data not shown). This absence of a major cytotoxicity allows the study of the cellular response.

### **3.3. Inflammatory response**

The inflammatory response was evaluated by measuring the relative secretion level of IL-6 and IL-8 (Figure 2). For IL-6, the relative secretion level increased dose-dependently after cell exposure to toluene and to the gaseous flow after catalytic oxidation, and whatever the catalyst used.

Concerning the relative secretion of IL-8, an inverted U-shaped profile over time was observed for the cells exposed to the flow after treatment using Co<sub>6</sub>Al<sub>2</sub>-O (10 and 100%). The cytokine was up-secreted the day 3 beside days 1 and 5. However, a U-shaped profile was revealed for the cells exposed to the flow after treatment using Pd/ $\gamma$ -Al<sub>2</sub>O<sub>3</sub> (100%). Cells exposed to 100 ppm and 1,000 ppm of toluene showed a slight increase for the IL-8 chemokine extracellular secretion during the 5 days. However, the results obtained for IL-6

and IL-8 were not significant compared to their relative secretion in cells exposed to purified air. In parallel, the cells were exposed to LPS ( $0.1 \mu\text{g}.\text{ml}^{-1}$ ) under the same conditions and a significant increase in secretion of IL-6 and IL-8 was observed with induction levels up to 7.21- and 2.47-fold compared to the non-exposed control for IL-6 and IL-8, respectively.

### 3.4. Gene expression

The results obtained for the 12 XMEs were summarized in table 3 and the relative expression level of 6 XMEs genes (*CYP1A1*, *CYP1B1*, *CYP2E1*, *CYP2F1*, *DHDH* and *ALDH3B1*) is illustrated in figure 3.

After toluene exposure, three genes were up-regulated: *CYP1A1*, *CYP2F1* and *CYP2E1*. Cells exposed to 100 and 1,000 ppm of toluene showed a significant induction of *CYP1A1* and *CYP2E1*. *CYP1A1* was induced the day 5 for the two tested concentrations (RQ = 2.3). On the contrary, *CYP2E1* was induced over time and its relative expression decreased between the days 1 and 5. A decrease was also observed for *CYP2F1* from day 1 to day 5, but this gene was only induced at the highest toluene concentration.

Cell exposure to the gaseous outflow using  $\text{Pd}/\gamma\text{Al}_2\text{O}_3$  highlighted a significant induction of *CYP1A1*, *CYP1B1* and *CYP2E1* for cells exposed to 10% of the outflow during 5 day (2.7, 3.0 and 2.7 respectively). The same trend was observed for *CYP1A1* and *CYP1B1* genes when cells were exposed to 100% of the outflow. However, *CYP2E1* was inducible over time with RQ values higher than 4.0 for days 3 and 5. The other tested genes were only induced for 100% of the outflow. At Day 5 of exposure, *CYP2S1*, *EPHX1*, *NQO1*, *GSTM1*, *DHDH* and *ALDH2* were induced with a RQ upper to 2.0 and *ADH1A* with a RQ of 8.7. Finally, *CYP2F1* was significantly over-expressed from day 1 to day 5, with a RQ of 3.4.

The second tested catalyst was  $\text{Co}_6\text{Al}_2\text{-O}$ . Cell exposure to 10% of the outflow induced the gene expression of all the XME, excepted *CYP2F1* and *ADH1A*. The other genes were over-expressed only the day 5. *CYP1A1*, *CYP1B1*, *GSTM1* and *ALDH2* levels were higher than 4.0 (4.9, 5.4, 4.4 and 4.1 respectively). Cells exposed to 100% of the outflow did not show any significant induction of *CYP2F1*. An inverted U-shaped profile was observed for the relative expression of the following 8 genes: *CYP1A1*, *CYP1B1*, *CYP2S1*, *GSTM1*, *EPHX1*,

*DHHDH*, *ADH1A* and *ALDH2* (data not shown). However, *NQO1* was up-regulated with a U-shaped profile. Finally, *CYP2E1* and *ALDH3B1* were also induced when cells were exposed to 100% of the gas resulting from the catalytic oxidation. *CYP2E1* was over expressed from day 1 to day 5 with RQ of 2.1, 3.2 and 3.4 respectively. *ALDH3B1* showed a significant induction after day 3.

#### **4. Discussion**

Repeated exposures to atmospheric xenobiotics contribute to the development of chronic pulmonary diseases, allergies and neurologic disorders. However, mechanisms of the chronic toxicity of air pollution, including the gaseous chemical components, like VOCs, are still not well known (Upadhyay and Palmberg, 2018). Thus, further consideration should be given to toxicological studies using repeated exposures on human cells. In the context of the 3R strategy, performing chronic exposures remains a challenge in *in vitro* studies. Persoz et al., studied single exposure effects of formaldehyde on alveolar and bronchial cells (Persoz et al., 2012). Without cells pre-sensitization, the authors did not observe significant inflammatory response and results were linked to the choice of cells and to the exposure conditions, no representative of real conditions. Cell response after repeated exposures to airborne chemicals using ALI systems was also investigated (Bardet et al., 2014; Gostner et al., 2016; Kastner et al., 2013). To our knowledge, Kastner et al. were one of the first to evaluate the effect of gaseous chemical components after repeated exposure, by comparison of ALI and submerged modes (Kastner et al., 2013). Lung epithelial cells Calu-3 were exposed with an ALI system to synthetic air or to formaldehyde and/or nitrogen dioxide during 30 minutes, 1 hour or 2 hours. Cells showed an increase of IL-6 secretion after ALI exposure to purified air in comparison with non-exposed cells, evidencing the need to optimize the exposure time (Kastner et al., 2013). Bardet et al., studied the effects of human nasal cells exposure to  $200\mu\text{g.m}^{-3}$  of formaldehyde for 1 hour during 4 days, using a commercial ALI system. Indeed, no significant cellular mortality and modification of cellular alteration were observed after the exposure. Only a significant increase of IL-8 production was measured the day 3, in comparison with air-exposed controls and cells exposed for 1

hour only, showing a chronic effect in the cells repeatedly exposed to formaldehyde (Bardet et al., 2014).

In the present study, human BEAS-2B cells were chosen since bronchial epithelial cells are one of the target of atmospheric pollutants (Hiemstra et al., 2018). This cell line is metabolically active and permits repeated exposures. Considering seeding concentration of 1,500 cells.cm<sup>-2</sup>, the cytotoxicity measured by the LDH assay was less than 10% of mortality for day 5, corresponding to the EC<sub>10</sub>, but higher than 20% from day 6. Therefore, no mortality was significantly observed after purified air exposure during 1h/day for 5 days. This exposure time was longer than the one considered in the studies mentioned above (Bardet et al., 2014; Kastner et al., 2013). In addition, the absence of cell death observed after exposure to toluene vapor or to gaseous outflows after catalytic oxidation of toluene allowed us to characterize the cell response until day 5.

The use of remediation techniques directly at the source is recommended to decrease VOCs emissions. The efficiency of these methods, as catalytic oxidation, should be validated before their use at an industrial scale. The efficiency of the catalyst, evaluated following the conversion of VOC and the selectivity to form carbon dioxide and water, is the most commonly measured parameter. ALI experiments could be used in addition to chemical analysis as a complementary investigation to assess the toxicity effects of residual VOCs and their related by-products (Al Zallouha et al., 2017).

Concerning the inflammatory response of cells, we observed a not significant increase of IL-6 and IL-8 secretion level in a time- and dose-dependent way either after exposure to toluene vapor or to the gaseous outflow after catalytic oxidation. The inflammatory response of BEAS-2B cells has already been assessed with LPS and highlight the ability of cells to trigger an inflammatory response (Longhin et al., 2018). No significant changes in IL-6 secretion level in nasal epithelial cells exposed to formaldehyde for 3 days was also observed (Bardet et al., 2014). Secondly, the extracellular secretion of IL-8 was also monitored, while this interleukin participates in pulmonary pro-inflammatory response and is considered as a primary inflammatory signal (Fischäder et al., 2008). After cells exposure to

100 and 1,000 ppm of toluene, an increase level of IL-8 secretion was observed in comparison with cells exposed to air. Extracellular secretion of IL-8 due to VOCs exposure has been previously observed in acute exposure of BEAS-2B to toluene and benzene (Pariselli et al., 2006, 2009b). Repeated exposure of nasal epithelial cells to formaldehyde also pointed out an increase level of IL-8 after 3 days (Bardet et al., 2014). The inverted U-shaped profile of IL-8 secretion observed after catalytic oxidation flow exposure is not clear so far. Modification of intracellular glutathione level by VOCs especially benzene and toluene is observed in human epithelial lung cells A459 exposed in ALI system (Pariselli et al., 2009a). Adequacy between variation of GSH/GSSG and regulation of inflammatory process in lung have been established (Rahman and MacNee, 2000). Furthermore, an increase of mucin protein secretion would promote the decrease of IL-8 secretion in *in vivo* models (Fuentes-Mattei et al., 2010). Mucin secretion allows the decrease of inflammatory injury of cells which dependent of IL-8 secretion (Turner et al., 2014) and the regulation of mucin gene expression in lung by IL-8 (Bautista et al., 2009) can participate as a feedback regulation. The variation of IL-8 profile could be related to the presence of very low concentration of acetic acid in the gas flow after catalytic oxidation. The increase of IL-8 secretion after acidic compounds exposure has already been pointed out in A549 lung cells exposed to acetic acid (Okubo et al., 2016) or in BEAS-2B cells exposed to hydrochloric acid (Chen et al., 2013). The evaluation of the secretion of IL-6 and IL-8 highlights minor modification between cells exposed to toluene or to the flow after catalytic oxidation and the purified-air exposed ones. Further investigation should be carried out to evaluate the intracellular level of these interleukins and the activation of the mucus pathway.

The gene expression evaluation was conducted by considering 12 XME genes selected according to benzene and toluene metabolism (INRS, 2012; Rappaport et al., 2009) and their induction in BEAS-2B cells (Courcot et al., 2012). After BEAS-2B cells exposure to 1,000 ppm of toluene, significant increases in the expression of *CYP1A1*, *CYP2E1* and *CYP2F1* were observed. Contrary to *CYP2F1*, the 2 other XME genes were also induced. For the lowest concentration (100 ppm), only *CYP1A1* and *CYP2E1* genes were induced. For the 2

356 concentrations, *CYP2E1* and *CYP2F1* induction levels decreased over time. The 3 genes  
357 belong to the cytochrome P450 family and participate in the first step of toluene metabolism,  
358 by oxidizing it to benzyl alcohol (Al Zallouha et al., 2017; Pohl and Scinicariello, 2011). An  
359 induction of *CYP2E1* enzyme was observed in tannery workers exposed to toluene  
360 (Jiménez-Garza et al., 2015) and the monitoring of *CYP2E1* expression is considered as a  
361 good marker for toluene exposure in toxicological studies (Mendoza-Cantú et al., 2006).  
362 However, these 3 genes cannot be considered as specific of cell exposure to toluene.  
363 Indeed, they are known to be also involved in PAH metabolism for *CYP1A1* (Savary et al.,  
364 2018) and benzene metabolism for *CYP2E1* (Kim et al., 2007; Pohl and Scinicariello, 2011)  
365 and *CYP2F1* (Castell et al., 2005; Sheets et al., 2004).

366 In a second step, we have evaluated expression of these genes after cell exposure to gas  
367 outflow after catalytic oxidation of toluene. The presence of low amount of unconverted  
368 toluene and the formation of by-products could be evidenced by monitoring the chemical  
369 composition of the outflows. In our study, a significant induction of 4 different CYP450 were  
370 observed. *CYP1A1*, *1B1* and *2E1* were over-expressed for the two catalysts used whereas  
371 *CYP2F1* was significantly expressed for the catalyst Pd/ $\gamma$ -Al<sub>2</sub>O<sub>3</sub> and *CYP2S1* for the catalyst  
372 Co<sub>6</sub>Al<sub>2</sub>-O. According to literature, *CYP2E1* and *CYP2F1* induction can be due to the  
373 presence of unconverted toluene and benzene formed as a by-product (Al Zallouha et al.,  
374 2017; Kim et al., 2007; Sheets et al., 2004). Thus, the induction of *CYP2E1* and *CYP2F1*  
375 corroborate the presence of benzene and unconverted toluene measured by chemical  
376 analysis after catalytic oxidation of toluene. We found that the gene *CYP1A1* was more  
377 induced after exposure to gas outflow after catalytic treatment compared to toluene vapor.  
378 Excepted the presence of unconverted toluene, Polycyclic Aromatic Hydrocarbons (PAHs)  
379 are known to induce *CYP1A1* expression. In addition, *CYP1A1*, *1B1* and *2S1* have been  
380 already used as biomarkers of exposure to PAHs, such as naphthalene (Saarikoski et al.,  
381 2005; Shimada and Fujii-Kuriyama, 2004).

382 In addition to the CYP450 genes expression analysis, non-P450 enzymes were also tested.  
383 The induction of *NQO1*, *EPHX1* and *GSTM1* was investigated. As seen for *CYP2E1* and

384 *2F1*, these 3 genes participate in benzene metabolism (Kim et al., 2007). *NQO1* and  
385 *EPHX1* are XMEs of the phase I whereas *GSTM1* is involved in the conjugation phase  
386 (Courcot et al., 2012). Our results showed that *NQO1*, *EPHX1* and *GSTM1* are significantly  
387 over-expressed after cell exposure to the gas outflow after toluene catalytic oxidation. More  
388 precisely, *NQO1* was only induced the day 5 for Pd/γAl<sub>2</sub>O<sub>3</sub> 10% and for Co<sub>6</sub>Al<sub>2</sub>-O 10% and  
389 100%. *EPHX1* and *GSTM1* were also induced from the day 3 for Co<sub>6</sub>Al<sub>2</sub>-O 100%. Moreover,  
390 induction level for the 3 genes was higher after cell exposure for Co<sub>6</sub>Al<sub>2</sub>-O catalyst. However,  
391 the benzene concentration was lower when using Co<sub>6</sub>Al<sub>2</sub>-O compared to Pd/γAl<sub>2</sub>O<sub>3</sub> catalyst.  
392 This over-expression could be probably explained by the presence of other benzenic  
393 chemical compounds. Indeed, according to literature date, *NQO1* can be induced by B(a)P  
394 (Rossner et al., 2016) and *EPHX1* by butadiene, styrene or PAHS (Václavíková et al., 2015).  
395 Further gene expression investigation showed that Dihydrodiol Dehydrogenase (*DHHDH*)  
396 gene was induced after cell exposure to gas outflows. This observation could be linked to the  
397 presence of PAH in low amount. Indeed, toxicity of PAH by their reactive metabolites can  
398 occur following two different pathways. First, the combination of CYP enzymes and Epoxide  
399 hydrolase generates molecules interacting with DNA and subsequently leads to the formation  
400 of to DNA adducts. The activation of the *DHHDH* pathway results in the formation of o-quinone  
401 by catechol oxidation and leads to the formation of ROS, which could also induce oxidative  
402 DNA damage (Moorthy et al., 2015; Penning et al., 1999). Thus, it is suggested that *DHHDH*  
403 induction could be explained by the formation of PAHs as by-products after catalytic  
404 oxidation of toluene, in agreement also with *CYP1A1*, *1B1* and *2S1* induction.

405 The alcohol dehydrogenase gene *ADH1A* was over-expressed after cell exposure to gas  
406 outflow at higher concentration (100%). This gene was induced the day 5 for Pd/γAl<sub>2</sub>O<sub>3</sub> and  
407 the day 3 for the catalyst Co<sub>6</sub>Al<sub>2</sub>-O. Alcohol dehydrogenases participate in phase I of  
408 xenobiotic metabolism and catalyze benzyl alcohol (INRS, 2012). A decrease in the alcohol  
409 dehydrogenase activity after mice exposure to benzene or toluene has been previously  
410 shown (Bird et al., 2010). In contrast, after exposure to a toluene and benzene mixture, no  
411 significant change in expression level of ADH genes was observed (Bird et al., 2010). Thus,



it could be suggested in our case that the exposure to benzene and/or to toluene generates a modification in ADH genes family induction.

Finally, two aldehyde dehydrogenases were tested: *ALDH2* and *ALDH3B1*. *ALDH* genes are involved in the metabolism of toluene after the formation of benzyl alcohol by *ADH* genes. They also participate to the benzene metabolism. *ALDH2* is the first enzyme involved in the acetaldehyde oxidation (Marchitti et al., 2008). In our study, *ALDH2* was over-expressed when cells were exposed to the gas outflow after catalytic oxidation of toluene. This induction attests the presence of non-oxidized toluene measuring by chemical analysis. *ALDH3B1* belongs to the *ALDH3* family. This family plays a role in the cells defense against oxidative stress and a link between the expression level of *ALDH3B1* and exposure to aldehydes, such as benzaldehyde, hexanal or acetaldehyde, has been reported (Marchitti et al., 2010). In the present work, this gene was expressed only after exposure to gas components formed from the  $\text{Co}_6\text{Al}_2\text{-O}$  catalyst. *ALDH3B1* overexpression could be linked to the presence of by-products formed after catalytic oxidation, such as aldehyde compounds. However, the contribution of toluene metabolism, which generate benzaldehyde, cannot be excluded to explain *ALDH3B1* induction.

## Conclusion

Toluene is one of the most commonly used solvents while it is known as a harmful substance inducing acute and chronic toxicity especially in workers. While acute mechanisms of toxicity of toluene are known, how subacute and chronic effects appear is still unclear. Recent progress in *in vitro* toxicology, in particular, the use of ALI exposure system, can help to increase the knowledge about the subacute toxicity of volatile organic compounds. In this study, normal human lung epithelial BEAS-2B cells were exposed until 5 days to toluene. Only the repeated exposure permitted to detect the gene induction of *CYP1A1* that was not previously showed in acute studies. The second strength of this study was the coupling of the ALI exposure device to two different catalytic systems developed for the abatement of toluene. The XME genes induction measured was not only explained by the presence of

440 unconverted toluene and benzene formed as a by-product. It also revealed the presence of  
441 other catalytic oxidation by-products, as PAHs, not detected by the standard analytical  
442 method used for the development of catalysts. The repeated exposure mode allowed us to  
443 evidence the presence of very low concentration of by-products due to the late expression of  
444 gene, as *CYP1A1* and ALDH family. Therefore, this *in vitro* approach with ALI system pointed  
445 out first the importance of assessing the impact of repeated exposure to VOCs, and secondly  
446 the relevance of performing a toxicological validation of catalytic systems in addition to the  
447 standard chemical analysis. To complete this work, it would be interesting to study other  
448 cellular mechanisms induced by VOCs exposure like mucus production or cellular death.  
449 Secondly, the use of pulmonary tissue, such as organotypic cultures, should allow to  
450 increase the exposure time and period and to investigate the VOCs effect on other cells of  
451 the lung.

- Al Zallouha, M., Landkocz, Y., Brunet, J., Cousin, R., Genty, E., Courcot, D., Siffert, S., Shirali, P., Billet, S., 2017. Usefulness of toxicological validation of VOCs catalytic degradation by air-liquid interface exposure system. *Environ. Res.* 152, 328–335. <https://doi.org/10.1016/j.envres.2016.10.027>
- Anthérieu, S., Garat, A., Beauval, N., Soyez, M., Allorge, D., Garçon, G., Lo-Guidice, J.-M., 2017. Comparison of cellular and transcriptomic effects between electronic cigarette vapor and cigarette smoke in human bronchial epithelial cells. *Toxicol. In Vitro*. <https://doi.org/10.1016/j.tiv.2016.12.015>
- Aufderheide, M., 2005. Direct exposure methods for testing native atmospheres. *Exp. Toxicol. Pathol.* 57, 213–226. <https://doi.org/10.1016/j.etp.2005.05.019>
- Bardet, G., Achard, S., Loret, T., Desauziers, V., Momas, I., Seta, N., 2014. A model of human nasal epithelial cells adapted for direct and repeated exposure to airborne pollutants. *Toxicol. Lett.* 229, 144–149. <https://doi.org/10.1016/j.toxlet.2014.05.023>
- Bautista, M.V., Chen, Y., Ivanova, V.S., Rahimi, M.K., Watson, A.M., Rose, M.C., 2009. IL-8 Regulates Mucin Gene Expression at the Posttranscriptional Level in Lung Epithelial Cells. *J. Immunol.* 183, 2159–2166. <https://doi.org/10.4049/jimmunol.0803022>
- Bird, M.G., Wetmore, B.A., Letinski, D.J., Nicolich, M., Chen, M., Schnatter, A.R., Whitman, F.T., 2010. Influence of toluene co-exposure on the metabolism and genotoxicity of benzene in mice using continuous and intermittent exposures. *Chem. Biol. Interact.* 184, 233–239. <https://doi.org/10.1016/j.cbi.2010.01.012>
- Brunet, J., Genty, E., Landkocz, Y., Zallouha, M.A., Billet, S., Courcot, D., Siffert, S., Thomas, D., De Weireld, G., Cousin, R., 2015. Identification of by-products issued from the catalytic oxidation of toluene by chemical and biological methods. *Comptes Rendus Chim.* 18, 1084–1093. <https://doi.org/10.1016/j.crci.2015.09.001>
- Castell, J.V., Teresa Donato, M., Gómez-Lechón, M.J., 2005. Metabolism and bioactivation of toxicants in the lung. The in vitro cellular approach. *Exp. Toxicol. Pathol.* 57, 189–204. <https://doi.org/10.1016/j.etp.2005.05.008>
- Chen, Q., Huang, Y., Yang, Y., Qiu, H., 2013. Acid-induced cell injury and death in lung epithelial cells is associated with the activation of mitogen-activated protein kinases. *Mol. Med. Rep.* 8, 565–570. <https://doi.org/10.3892/mmr.2013.1537>
- Courcot, E., Leclerc, J., Lafitte, J.-J., Mensier, E., Jaillard, S., Gosset, P., Shirali, P., Pottier, N., Broly, F., Lo-Guidice, J.-M., 2012. Xenobiotic metabolism and disposition in human lung cell models: comparison with in vivo expression profiles. *Drug Metab. Dispos. Biol. Fate Chem.* 40, 1953–1965. <https://doi.org/10.1124/dmd.112.046896>
- Doke, S.K., Dhawale, S.C., 2015. Alternatives to animal testing: A review. *Saudi Pharm. J.* 23, 223–229. <https://doi.org/10.1016/j.jsps.2013.11.002>
- ECHA, 2018. Toluene [WWW Document]. Eur. Chem. Agency. URL <https://echa.europa.eu/fr/substance-information/-/substanceinfo/100.003.297> (accessed 11.12.18).
- EEA, 2018. Air pollutant emissions data viewer (Gothenburg Protocol, LRTAP Convention) 1990-2016 [WWW Document]. Eur. Environ. Agency. URL [1]<https://www.eea.europa.eu/data-and-maps/dashboards/air-pollutant-emissions-data-viewer-1> (accessed 11.12.18).
- Fischäder, G., Röder-Stolinski, C., Wichmann, G., Nieber, K., Lehmann, I., 2008. Release of MCP-1 and IL-8 from lung epithelial cells exposed to volatile organic compounds. *Toxicol. In Vitro* 22, 359–366. <https://doi.org/10.1016/j.tiv.2007.09.015>
- Fuentes-Mattei, E., Rivera, E., Gioda, A., Sanchez-Rivera, D., Roman-Velazquez, F.R., Jimenez-Velez, B.D., 2010. Use of human bronchial epithelial cells (BEAS-2B) to study immunological markers resulting from exposure to PM2.5 organic extract from Puerto Rico. *Toxicol. Appl. Pharmacol.* 243, 381–389. <https://doi.org/10.1016/j.taap.2009.12.009>
- Gałęzowska, G., Chraniuk, M., Wolska, L., 2016. In vitro assays as a tool for determination of VOCs toxic effect on respiratory system: A critical review. *TrAC Trends Anal. Chem.* 77, 14–22. <https://doi.org/10.1016/j.trac.2015.10.012>

- Garcia-Canton, C., Minet, E., 2013. Metabolic characterization of cell systems used in in vitro toxicology testing: Lung cell system BEAS-2B as a working example. *Toxicol. In Vitro* 27, 1719–1727. <https://doi.org/10.1016/j.tiv.2013.05.001>
- Genty, E., Brunet, J., Poupin, C., Casale, S., Capelle, S., Massiani, P., Siffert, S., Cousin, R., 2015. Co-Al Mixed Oxides Prepared via LDH Route Using Microwaves or Ultrasound: Application for Catalytic Toluene Total Oxidation. *Catalysts* 5, 851–867. <https://doi.org/10.3390/catal5020851>
- Gostner, J.M., Zeisler, J., Alam, M.T., Gruber, P., Fuchs, D., Becker, K., Neubert, K., Kleinhapfl, M., Martini, S., Überall, F., 2016. Cellular reactions to long-term volatile organic compound (VOC) exposures. *Sci. Rep.* 6. <https://doi.org/10.1038/srep37842>
- Hiemstra, P.S., Grootaers, G., van der Does, A.M., Krul, C.A.M., Kooter, I.M., 2018. Human lung epithelial cell cultures for analysis of inhaled toxicants: Lessons learned and future directions. *Toxicol. In Vitro* 47, 137–146. <https://doi.org/10.1016/j.tiv.2017.11.005>
- INRS, 2012. Toluène (FT 74) - Fiche toxicologique - INRS [WWW Document]. URL [http://www.inrs.fr/publications/bdd/fichetox/fiche.html?refINRS=FICHETOX\\_74](http://www.inrs.fr/publications/bdd/fichetox/fiche.html?refINRS=FICHETOX_74) (accessed 4.20.17).
- Jiménez-Garza, O., Baccarelli, A.A., Byun, H.-M., Márquez-Gamiño, S., Barrón-Vivanco, B.S., Albores, A., 2015. CYP2E1 epigenetic regulation in chronic, low-level toluene exposure: Relationship with oxidative stress and smoking habit. *Toxicol. Appl. Pharmacol.* 286, 207–215. <https://doi.org/10.1016/j.taap.2015.04.016>
- Kastner, P.E., Le Calvé, S., Zheng, W., Casset, A., Pons, F., 2013. A dynamic system for single and repeated exposure of airway epithelial cells to gaseous pollutants. *Toxicol. In Vitro* 27, 632–640. <https://doi.org/10.1016/j.tiv.2012.11.011>
- Kim, S., Lan, Q., Waidyanatha, S., Chanock, S., Johnson, B.A., Vermeulen, R., Smith, M.T., Zhang, L., Li, G., Shen, M., Yin, S., Rothman, N., Rappaport, S.M., 2007. Genetic polymorphisms and benzene metabolism in humans exposed to a wide Range of air concentrations: Pharmacogenet. *Genomics* 17, 789–801. <https://doi.org/10.1097/FPC.0b013e3280128f77>
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods* 25, 402–408. <https://doi.org/10.1006/meth.2001.1262>
- Longhin, E., Holme, J.A., Gualtieri, M., Camatini, M., Øvrevik, J., 2018. Milan winter fine particulate matter (wPM<sub>2.5</sub>) induces IL-6 and IL-8 synthesis in human bronchial BEAS-2B cells, but specifically impairs IL-8 release. *Toxicol. In Vitro* 52, 365–373. <https://doi.org/10.1016/j.tiv.2018.07.016>
- Marchitti, S.A., Brocker, C., Orlicky, D.J., Vasiliou, V., 2010. Molecular characterization, expression analysis, and role of ALDH3B1 in the cellular protection against oxidative stress. *Free Radic. Biol. Med.* 49, 1432–1443. <https://doi.org/10.1016/j.freeradbiomed.2010.08.004>
- Marchitti, S.A., Brocker, C., Stagos, D., Vasiliou, V., 2008. Non-P450 aldehyde oxidizing enzymes: the aldehyde dehydrogenase superfamily. *Expert Opin. Drug Metab. Toxicol.* 4, 697–720. <https://doi.org/10.1517/17425255.4.6.697>
- Mendoza-Cantú, A., Castorena-Torres, F., de León, M.B., Cisneros, B., López-Carrillo, L., Rojas-García, A.E., Aguilar-Salinas, A., Manno, M., Albores, A., 2006. Occupational Toluene Exposure Induces Cytochrome P450 2E1 mRNA Expression in Peripheral Lymphocytes. *Environ. Health Perspect.* 114, 494–499. <https://doi.org/10.1289/ehp.8192>
- Moorthy, B., Chu, C., Carlin, D.J., 2015. Polycyclic Aromatic Hydrocarbons: From Metabolism to Lung Cancer. *Toxicol. Sci.* 145, 5–15. <https://doi.org/10.1093/toxsci/kfv040>
- Okubo, T., Suzuki, T., Hosaka, M., Nakae, D., 2016. Effects Induced by Organic Acids in a Human Lung Alveolar Carcinoma Cell Line A549. *YAKUGAKU ZASSHI* 136, 1433–1438. <https://doi.org/10.1248/yakushi.16-00039>
- Pariselli, F., Sacco, M.G., Ponti, J., Rembges, D., 2009a. Effects of toluene and benzene air mixtures on human lung cells (A549). *Exp. Toxicol. Pathol.* 61, 381–386. <https://doi.org/10.1016/j.etp.2008.10.004>

- Pariselli, F., Sacco, M.G., Rembges, D., 2009b. An optimized method for in vitro exposure of human derived lung cells to volatile chemicals. *Exp. Toxicol. Pathol. Off. J. Ges. Toxikol. Pathol.* 61, 33–39. <https://doi.org/10.1016/j.etp.2008.05.008>
- Pariselli, F., Sacco, M.G., Rembges, D., 2006. Set-up of a dynamic in vitro exposure device for the study of indoor air pollutant effects on human derived cells, in: *Environmental Toxicology*. Presented at the ENVIRONMENTAL TOXICOLOGY 2006, WIT Press, Mykonos, Greece, pp. 231–239. <https://doi.org/10.2495/ETOX060231>
- Penning, T.M., Burczynski, M.E., Hung, C.-F., McCoull, K.D., Palackal, N.T., Tsuruda, L.S., 1999. Dihydrodiol Dehydrogenases and Polycyclic Aromatic Hydrocarbon Activation: Generation of Reactive and Redox Active *o*-Quinones. *Chem. Res. Toxicol.* 12, 1–18. <https://doi.org/10.1021/tx980143n>
- Persoz, C., Achard, S., Momas, I., Seta, N., 2012. Inflammatory response modulation of airway epithelial cells exposed to formaldehyde. *Toxicol. Lett.* 211, 159–163. <https://doi.org/10.1016/j.toxlet.2012.03.799>
- Pohl, H.R., Scinicariello, F., 2011. The impact of CYP2E1 genetic variability on risk assessment of VOC mixtures. *Regul. Toxicol. Pharmacol.* 59, 364–374. <https://doi.org/10.1016/j.yrtph.2011.01.013>
- Rahman, I., MacNee, W., 2000. Oxidative stress and regulation of glutathione in lung inflammation. *Eur. Respir. J.* 16, 534–554.
- Rappaport, S.M., Kim, S., Lan, Q., Vermeulen, R., Waidyanatha, S., Zhang, L., Li, G., Yin, S., Hayes, R.B., Rothman, N., Smith, M.T., 2009. Evidence That Humans Metabolize Benzene via Two Pathways. *Environ. Health Perspect.* 117, 946–952. <https://doi.org/10.1289/ehp.0800510>
- Rosner, P., Strapacova, S., Stolcpartova, J., Schmuczerova, J., Milcova, A., Neca, J., Vlkova, V., Brzicova, T., Machala, M., Topinka, J., 2016. Toxic Effects of the Major Components of Diesel Exhaust in Human Alveolar Basal Epithelial Cells (A549). *Int. J. Mol. Sci.* 17, 1393. <https://doi.org/10.3390/ijms17091393>
- Saarikoski, S., Rivera, S., Hankinson, O., Husgafvelpursiainen, K., 2005. CYP2S1: A short review. *Toxicol. Appl. Pharmacol.* 207, 62–69. <https://doi.org/10.1016/j.taap.2004.12.027>
- Savary, C.C., Bellamri, N., Morzadec, C., Langouët, S., Lecureur, V., Vernhet, L., 2018. Long term exposure to environmental concentrations of diesel exhaust particles does not impact the phenotype of human bronchial epithelial cells. *Toxicol. In Vitro* 52, 154–160. <https://doi.org/10.1016/j.tiv.2018.06.014>
- Sheets, P.L., Yost, G.S., Carlson, G.P., 2004. Benzene metabolism in human lung cell lines BEAS-2B and A549 and cells overexpressing CYP2F1. *J. Biochem. Mol. Toxicol.* 18, 92–99. <https://doi.org/10.1002/jbt.20010>
- Shimada, T., Fujii-Kuriyama, Y., 2004. Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer Sci.* 95, 1–6.
- Tormoehlen, L.M., Tekulve, K.J., Nañagas, K.A., 2014. Hydrocarbon toxicity: A review. *Clin. Toxicol.* 52, 479–489. <https://doi.org/10.3109/15563650.2014.923904>
- Turner, M.D., Nedjai, B., Hurst, T., Pennington, D.J., 2014. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. *Biochim. Biophys. Acta BBA - Mol. Cell Res.* 1843, 2563–2582. <https://doi.org/10.1016/j.bbamcr.2014.05.014>
- Upadhyay, S., Palmberg, L., 2018. Air-Liquid Interface: Relevant In Vitro Models for Investigating Air Pollutant-Induced Pulmonary Toxicity. *Toxicol. Sci.* 164, 21–30. <https://doi.org/10.1093/toxsci/kfy053>
- Uppstad, H., Øvrebø, S., Haugen, A., Møllerup, S., 2010. Importance of CYP1A1 and CYP1B1 in bioactivation of benzo[a]pyrene in human lung cell lines. *Toxicol. Lett.* 192, 221–228. <https://doi.org/10.1016/j.toxlet.2009.10.025>
- Václavíková, R., Hughes, D.J., Souček, P., 2015. Microsomal epoxide hydrolase 1 (EPHX1): Gene, structure, function, and role in human disease. *Gene* 571, 1–8. <https://doi.org/10.1016/j.gene.2015.07.071>

- Vales, G., Rubio, L., Marcos, R., 2015. Long-term exposures to low doses of titanium dioxide nanoparticles induce cell transformation, but not genotoxic damage in BEAS-2B cells. *Nanotoxicology* 9, 568–578. <https://doi.org/10.3109/17435390.2014.957252>
- Van Der Toorn, M., Sewer, A., Marescotti, D., Johne, S., Baumer, K., Bornand, D., Dulize, R., Merg, C., Corciulo, M., Scotti, E., Pak, C., Leroy, P., Guedj, E., Ivanov, N., Martin, F., Peitsch, M., Hoeng, J., Luetlich, K., 2018. The biological effects of long-term exposure of human bronchial epithelial cells to total particulate matter from a candidate modified-risk tobacco product. *Toxicol. In Vitro* 50, 95–108. <https://doi.org/10.1016/j.tiv.2018.02.019>
- WHO, 2016. OMS | 7 millions de décès prématurés sont liés à la pollution de l'air chaque année. WHO.
- Zhou, W., Tian, D., He, J., Wang, Y., Zhang, Lijun, Cui, L., Jia, L., Zhang, Li, Li, L., Shu, Y., Yu, S., Zhao, J., Yuan, X., Peng, S., 2016. Repeated PM2.5 exposure inhibits BEAS-2B cell P53 expression through ROS-Akt-DNMT3B pathway-mediated promoter hypermethylation. *Oncotarget* 7, 20691–20703. <https://doi.org/10.18632/oncotarget.7842>

## Supplementary material

### Optimization of cell culture and exposure conditions

#### Methods:

BEAS-2B cells were cultured in Corning® Cellbind® sterile flasks with LHC-9 culture medium (Gibco, Fisher Scientific) and incubated at 37°C, in a 5% CO<sub>2</sub> and 100% humidity environment. Firstly, BEAS-2B cells seeding concentration were optimized in order to achieve repeated exposures, for a minimum of 8 days. Six seeding concentrations were tested (190, 375, 750, 1,500, 3,000 and 6,000 cells.cm<sup>-2</sup>) in Cellbind® 6-well plates for 14 days and microscopic observation were daily realized. Cell morphology and cellular division were used as phenotypic criteria. The cell cultures confluence was also limited to 80% according to the recommendations of the American Type Culture Collection standard. Optimal culture conditions on Transwell® insert were determined in order to obtain 500,000 cells on the last day of experiment. So, three cellular concentrations were used: 500,000, 195,000, and 76,000 cells/Transwell® insert for 1, 3 and 5 days respectively. Cells were seeded on 4.7 cm<sup>2</sup> Corning® Transwell® inserts with 0.4 µm pore size (Sigma-Aldrich) and exposed to purified air for 1h during 8 days. Cytotoxicity and proliferation were evaluated considering as a control, unexposed cells placed in similar inserts inside the incubator at 37°C.

Morphology of cells were also evaluated by microscopic observation of cells after exposure to toluene during 1h each day for 1, 3 or 5 days at 100 and 1,000 ppm of toluene. According to Feldman et al., inserts were fixed in formaldehyde (2% solution in PBS) for 30 minutes and stained with hematoxyline and eosine (Feldman and Wolfe, 2014). Then, each insert were put between two microscope slides and visualized with a photonic microscope (Zeiss AxioScope) at x100 of magnification.

## Results:

Cells concentration for repeated exposures was first optimized. 80% of confluence was achieved before 8 days for 3,000 and 6,000 cells.cm<sup>-2</sup>. For the other seeding concentration (190, 375 and 750 cells.cm<sup>-2</sup>), cellular behavior and phenotype modifications were observed from day 5. Indeed, adherent cells had grown in clusters instead of covering uniformly in the well bottom. The confluence for a seeding concentration of 1,500 cells.cm<sup>-2</sup> was reached on the 8<sup>th</sup> day of culture. Then, the exposure period with the Vitrocell® system was optimized (Figure S1). LDH assay (grey curve) and alamarBlue® assay (black curve) were used to measure cytotoxicity and proliferation of cells exposed to purified air, respectively. Curve-response fitting used for cytotoxicity determination highlight that EC<sub>10</sub> (corresponding to 10% of cytotoxicity in cells exposed to purified air) is obtained between the 5<sup>th</sup> and the 6<sup>th</sup> day of exposure. For every tested condition, a decrease of the proliferation index was also observed from day 5. From 1<sup>st</sup> to 5<sup>th</sup> days of culture, exposed cells proliferation was stable in comparison with control.

Secondly, microscopic observation did not shown an effect on BEAS-2B cells morphology caused by toluene repeated exposure (figure S2).

In conclusion, these optimizations allowed to validate the optimal culture and exposure conditions of BEAS-2B cells for repeated ALI exposure.

Feldman AT and Wolfe D. (2014). Tissue processing and hematoxylin and eosin staining. *Methods Mol Biol.* 1180:31-43.



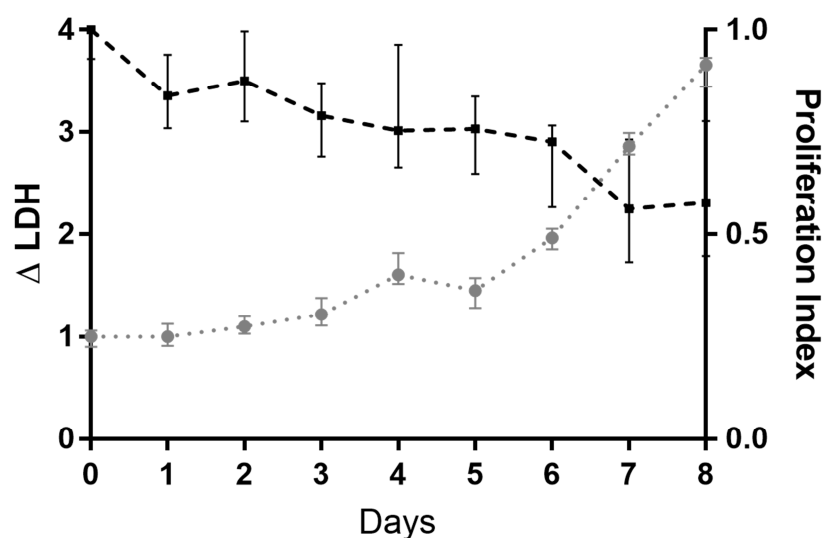


Figure S1: Cytotoxicity (LDH release assay, in grey dot), and relative proliferation index (alamarBlue® assay, in black square), after cell exposure to purified air for 1h/day for 8 days. For each day, obtained values (n=3) were compared to Day 0. Curve-response fitting was performed using Hill equation model, in order to determine EC<sub>10</sub>.

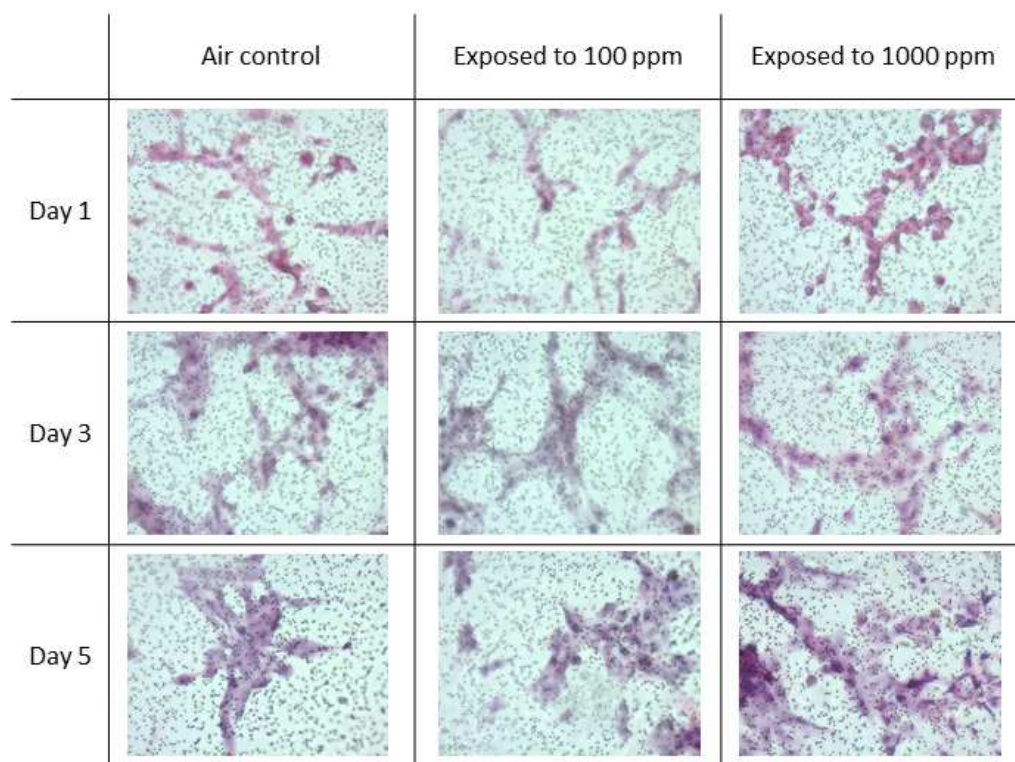
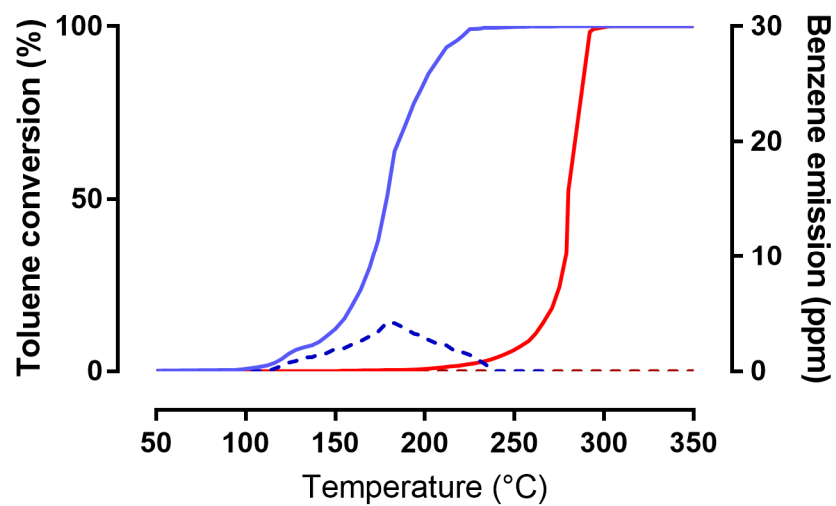


Figure S2: morphologic evaluation of BEAS-2B by photonic microscopy (x100 of magnification) after cell exposure to toluene during 1h/day for 5 days.

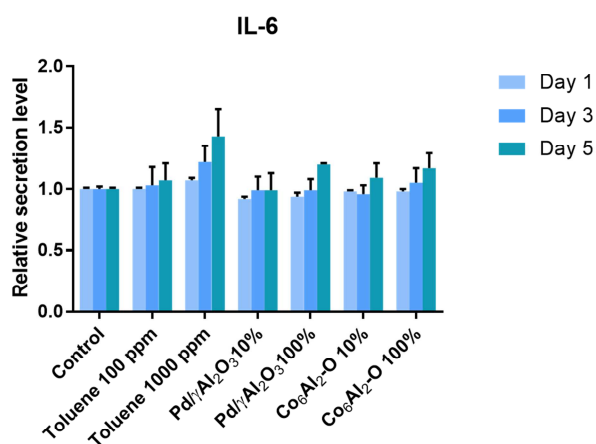


1

2 Figure 1: Light-off curve of toluene conversion (%) represented in continuous lines, and  
3 benzene emission (ppm) represented in dotted lines, as a function of temperature (°C) using  
4 Pd/γAl<sub>2</sub>O<sub>3</sub> (blue) or Co<sub>6</sub>Al<sub>2</sub>-O (red) catalysts.

5

A.



B.

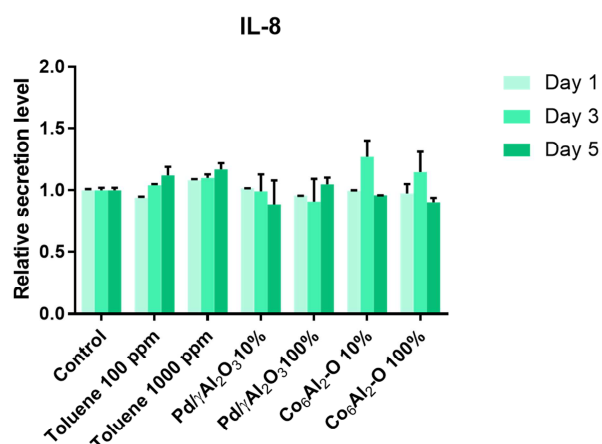
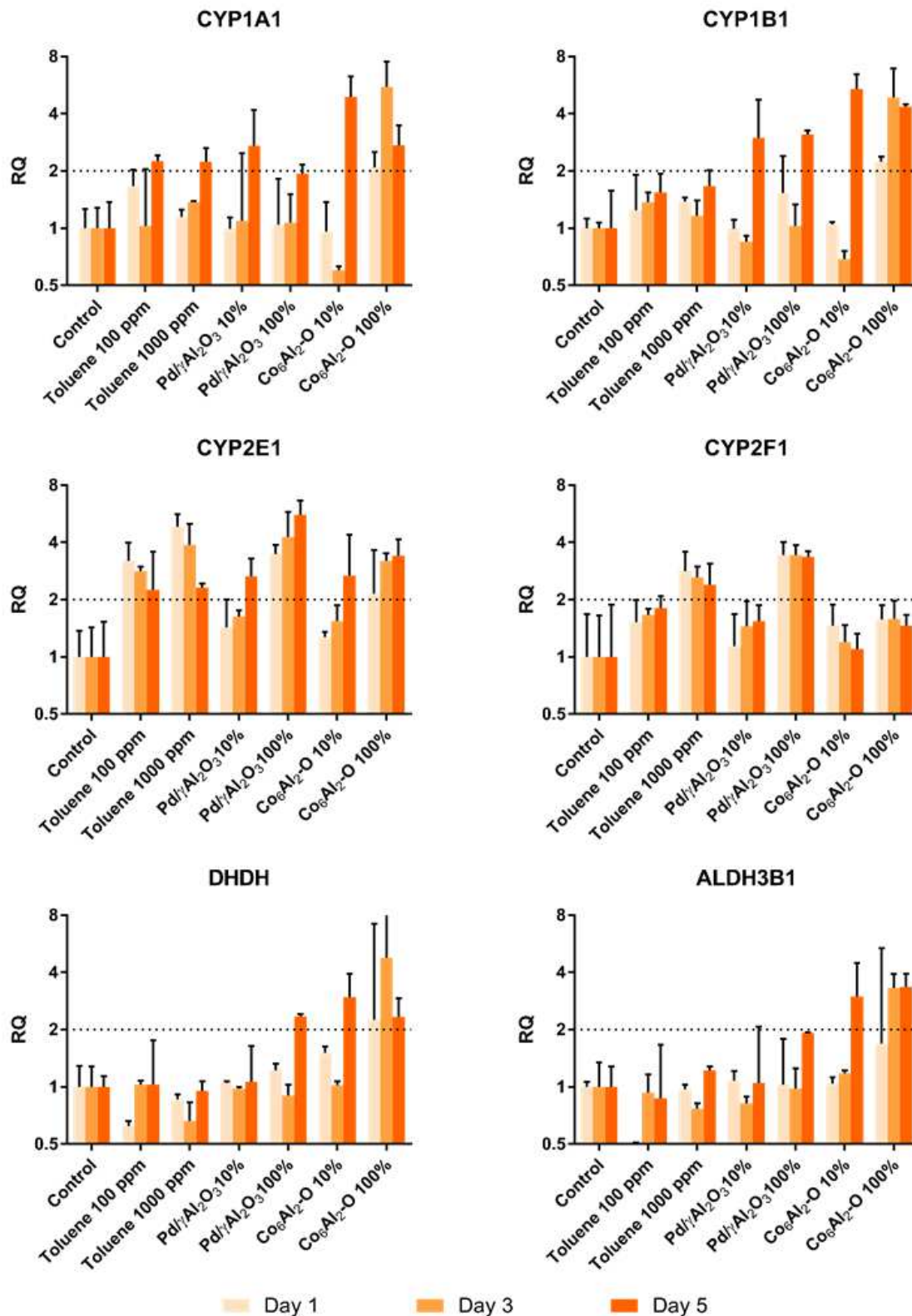


Figure 2: Relative secretion of IL-6 (A) and IL-8 (B) after cell exposure to toluene or to the gaseous flow after catalytic oxidation during 1h/day for 5 days. The cytokine concentrations were measured by ELISA in the supernatant of biological replicates (n=3) and values were compared to air exposed cells. No statistical difference was observed using Mann Whitney U test ( $p < 0.05$ ).



12

13 Figure 3: Relative gene expression of 6 selected XME (*CYP1A1*, *CYP1B1*, *CYP2E1*,  
 14 *CYP2F1*, *DHDH* and *ALDH3B1*) after cell exposure to toluene or to the gaseous flow  
 15 catalytic oxidation during 1h/day for 5 days. Gene induction was measured by RT-qPCR  
 16 (n=3) and analyzed by  $\Delta\Delta C_t$  method (*Livak and Schmittgen, 2001*) using air-exposed cells as  
 17 reference and the geometric mean Ct of *18S*, *GAPDH* and *B2M* as normalization genes.  
 18 Significant up- or down-expression was considered if RQ is higher than 2.0 or below 0.5.

Table 1: TaqMan Gene expression assays used for RT-qPCR

| <b>Genes</b>   | <b>Taqman Assay ID</b> |
|----------------|------------------------|
| <b>18S</b>     | Hs99999901_s1          |
| <b>GAPDH</b>   | Hs02786624_g1          |
| <b>B2M</b>     | Hs00187842_m1          |
| <b>CYP1A1</b>  | Hs01054797_g1          |
| <b>CYP1B1</b>  | Hs00164383_m1          |
| <b>CYP2E1</b>  | Hs00559368_m1          |
| <b>CYP2S1</b>  | Hs00258076_m1          |
| <b>CYP2F1</b>  | Hs00167949_m1          |
| <b>EPHX1</b>   | Hs01116806_m1          |
| <b>NQO1</b>    | Hs02512143_s1          |
| <b>GSTM1</b>   | Hs01683722_gH          |
| <b>DHDH</b>    | Hs00205528_m1          |
| <b>ADH1A</b>   | Hs00605167_g1          |
| <b>ALDH2</b>   | Hs01007998_s1          |
| <b>ALDH3B1</b> | Hs00997594_m1          |

Table 2: Chemical components detected in the gaseous flow after oxidation of toluene using Pd/ $\gamma$ -Al<sub>2</sub>O<sub>3</sub> or Co<sub>6</sub>Al<sub>2</sub>-O catalysts (<LOD: under limit of detection, <LOQ: under limit of quantification).

|   | Toluene (ppb) |       |       | Benzene (ppb) |       |       | Acetic acid (ppb) |       |       |
|---|---------------|-------|-------|---------------|-------|-------|-------------------|-------|-------|
|   | Day 1         | Day 3 | Day 5 | Day 1         | Day 3 | Day 5 | Day 1             | Day 3 | Day 5 |
| <b>Pd/<math>\gamma</math>-Al<sub>2</sub>O<sub>3</sub></b> | 239           | 228   | 265   | 549           | 447   | 396   | <LOD              | <LOD  | <LOD  |
| <b>Co<sub>6</sub>Al<sub>2</sub>-O</b>                     | 96            | 90    | 46    | <LOD          | <LOD  | <LOD  | 659               | 170   | <LOQ  |

Table 3: Relative gene expression of Xenobiotic Metabolizing Enzymes after cell exposure to toluene or to gaseous flow of catalytic oxidation during 1h/day for 5 days. Gene induction was measured by RT-qPCR (n=3) and analyzed by  $\Delta\Delta C_t$  method (*Livak and Schmittgen, 2001*) using air-exposed cells as reference and the geometric mean Ct of *18S*, *GAPDH* and *B2M* as normalization genes (=: no significant induction; +: RQ > 2; ++: RQ > 4; -: RQ < 0.5).

| Days           | Toluene<br>10% |   |   | Toluene<br>100% |   |   | Pd/ $\gamma$ Al <sub>2</sub> O <sub>3</sub><br>10% |   |   | Pd/ $\gamma$ Al <sub>2</sub> O <sub>3</sub><br>100% |    |    | Co <sub>6</sub> Al <sub>2</sub> -O<br>10% |   |    | Co <sub>6</sub> Al <sub>2</sub> -O<br>100% |    |    |
|----------------|----------------|---|---|-----------------|---|---|--|---|---|---|----|----|---|---|----|--|----|----|
|                | 1              | 3 | 5 | 1               | 3 | 5 | 1  | 3 | 5 | 1   | 3  | 5  | 1   | 3 | 5  | 1  | 3  | 5  |
| <i>CYP1A1</i>  | =              | = | + | =               | = | + | =  | = | + | =   | =  | =  | =   | = | ++ | +  | ++ | +  |
| <i>CYP1B1</i>  | =              | = | = | =               | = | = | =  | = | + | =   | =  | +  | =   | = | ++ | +  | ++ | ++ |
| <i>CYP2E1</i>  | +              | + | + | ++              | + | + | =  | = | + | +   | ++ | ++ | =   | = | +  | +  | +  | +  |
| <i>CYP2S1</i>  | =              | = | = | =               | = | = | =  | = | = | =   | =  | +  | =   | = | +  | =  | ++ | ++ |
| <i>CYP2F1</i>  | =              | = | = | +               | + | + | =  | = | = | +   | +  | +  | =   | = | =  | =  | =  | =  |
| <i>EPHX1</i>   | =              | = | = | =               | = | = | =  | = | = | =   | =  | +  | =   | = | +  | =  | +  | +  |
| <i>NQO1</i>    | =              | = | = | =               | = | = | =  | = | = | =   | =  | +  | =   | = | +  | =  | =  | +  |
| <i>GSTM1</i>   | =              | = | = | =               | = | = | =  | = | = | =   | =  | +  | =   | = | ++ | =  | ++ | +  |
| <i>DHDH</i>    | =              | = | = | =               | = | = | =  | = | = | =   | =  | +  | =   | = | +  | +  | ++ | +  |
| <i>ADH1A</i>   | =              | - | = | =               | = | = | =  | = | = | =   | =  | ++ | =   | = | =  | =  | ++ | +  |
| <i>ALDH2</i>   | -              | = | = | =               | = | = | =  | - | = | =   | =  | +  | =   | = | ++ | +  | ++ | =  |
| <i>ALDH3B1</i> | -              | = | = | =               | = | = | =  | = | = | =   | =  | =  | =   | = | +  | =  | +  | +  |