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

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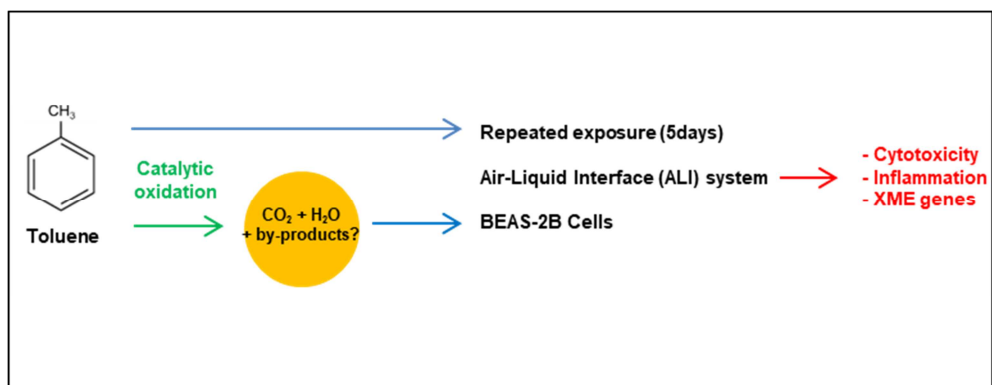
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11
12 **Abstract**

13 Toxicity of toluene and by-products formed during its catalytic oxidative degradation was
14 studied in human bronchial BEAS-2B cells repeatedly exposed. BEAS-2B cells were
15 exposed using an Air-Liquid Interface (ALI) System (Vitrocell®) for 1h per day during 1, 3 or
16 5 days to gaseous flows: toluene vapors (100 and 1,000 ppm) and outflow after catalytic
17 oxidation of toluene (10 and 100%). After exposure to gaseous flow, cytotoxicity,
18 inflammatory response and [Xenobiotic Metabolism Enzymes \(XME\)](#) gene expression were
19 investigated. No significant cytotoxicity was found after 5 days for every condition of
20 exposure. After cells exposure to catalytic oxidation flow, IL-6 level increased no significantly
21 in a time- and dose-dependent way, while an inverted U-shaped profile of IL-8 secretion was
22 observed. XME genes induction, notably *CYP2E1* and *CYP2F1* results were in line with the
23 presence of unconverted toluene and benzene formed as a by-product, detected by
24 analytical methods. [Exposure to pure toluene also demonstrated the activation of these](#)
25 [XMEs involved in its metabolism](#). Repeated exposure permits to show *CYP1A1*, *CYP1B1*
26 and *CY2S1* expression, probably related to the formation of other by-products, as PAHs, not
27 detected by standard analytical methods used for the development of catalysts.

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30 **Graphical abstract**



31

32

33 **Highlights**

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

37

38 **Keywords**

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

40

41 **Acknowledgements**

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46 Fund. All authors declare no conflict of interest.

47

48 **1. Introduction**

49 Indoor and outdoor air pollution kill 7 million people prematurely every year (WHO, 2016).
50 Volatile Organic Compounds (VOCs) significantly contribute in this pollution. In 2016, almost
51 8,000 Gg of Non-Methane Volatile Organic Compounds (NMVOCs) were emitted in the
52 atmosphere in Europe (EEA, 2018). Industry, residential, agriculture and road transports
53 were the main emission sectors with respectively 3,689 Gg, 1,296 Gg, 1,318 Gg and 669 Gg
54 of European NMVOC emissions. Toluene is one of the VOCs widely used as solvent in many
55 industrial processes, such as printing, shoemaking, painting and car industries. Indeed, this
56 VOC is manufactured and imported in European Economic Area between 1 and 10 Gg every
57 year (ECHA, 2018). The chronic toxic effects of toluene are well known (INRS, 2012;
58 Tormoehlen et al., 2014). It was classified as Carcinogenic, Mutagenic and Reprotoxic
59 (CMR), category 3, by the European Union. Psycho-organic syndromes as memory and
60 learning deficit, insomnia or personality changes are the other major effects after a chronic
61 inhalation of toluene.

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

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

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

79 Toxicological studies quality also depends on the choice of the exposure system
80 (Gałęzowska et al., 2016). Exposure to pollutants can be performed either in submerged
81 conditions or using an Air-Liquid Interface (ALI) system. The submerged exposures to VOCs
82 generally do not correspond to realistic conditions (Upadhyay and Palmberg, 2018). On the
83 contrary, the ALI method allows to expose the apical side of the cells directly to pollutants
84 contained in the gaseous phase whereas the basolateral side of cells is still in contact with
85 the culture medium (Aufderheide, 2005). This method is known for high reproducibility of
86 exposures and simulate quite well the real *in vivo* exposure conditions (Gałęzowska et al.,
87 2016). BEAS-2B cells are cell lines often cultured and exposed using an ALI System
88 (Anthérieu et al., 2017; Hiemstra et al., 2018; Persoz et al., 2012).

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

98 After exposure to airborne pollutants, pulmonary cells can transform xenobiotics to decrease
99 their toxicity and to eliminate their hydrophilic metabolites (Castell et al., 2005). This
100 metabolization is performed by various Xenobiotic Metabolism Enzymes (XMEs) in two
101 steps: functionalization by cytochromes P-450 (CYP) and conjugation. The implied XMEs
102 depend on the cell and on the nature of the xenobiotics (Castell et al., 2005). Consequently,
103 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/ γ -Al₂O₃ and a mixed oxide of Co₆Al₂-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/ γ -Al₂O₃ (Acros Organics, 0.5wt% Pd) and a mixed oxide Co₆Al₂-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⁻¹. The temperature of the reactor
128 was increased from room temperature to 250°C (Pd/ γ -Al₂O₃) and 350°C (Co₆Al₂-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-

132 4900). Catalytic performances of both catalysts were compared from T50 and T100 which
133 represent respectively the temperatures at which 50% and 100% of toluene was converted.
134 The toluene conversion was calculated considering products, by-products and carbon
135 number for each compound (Brunet et al., 2015).

136 **2.2. Cell culture conditions**

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

144 Cells were exposed to a gaseous flow of toluene (>99.9% purity, VWR Chemicals) diluted in
145 air and to a gaseous stream resulting from the catalytic oxidation of toluene using Vitrocell®
146 Air-Liquid Interface (ALI) system (Vitrocell GmbH, Germany). Three Transwell® inserts,
147 containing BEAS-2B cells, were placed into each 6/3 CF Stainless module containing 17.5
148 mL of LHC-9 culture medium. The upper cellular supernatant was removed from Transwell®
149 inserts before the exposure and kept to be added back after the exposure. VOCs are
150 delivered to cells in the gaseous form by a trumpet-shaped interface. For each exposure
151 time, five gas mixtures were tested: (i) purified air corresponding to the negative control, (ii)
152 1,000 ppm and (iii) 100 ppm of toluene in air, (iv) 100% and (v) 10% of gaseous flow after
153 catalytic treatment. In order to study the toxicological effects of a repeated exposure, cells
154 were exposed each day for 1h over 5 days period and then harvested in day 1, day 3 and
155 day 5. In parallel, 3 Transwell® inserts were kept in the incubator to serve as negative control
156 without exposure. Five hours after the cell exposure to toluene or to catalytically treated
157 gaseous flow, supernatants were collected, then Transwell® inserts were rinsed with
158 Phosphate Buffer Saline before storage at -80°C.

159 **2.3. Toxicological analysis**

160 **2.3.1. Cytotoxicity and cell proliferation**

161 The cellular cytotoxicity detection was performed by LDH assay (Roche Diagnostic, Merck-
162 Millipore), according to the manufacturer protocol. This colorimetric method allowed to
163 evaluate cell membrane integrity with the quantification of the lactate dehydrogenase (LDH)
164 released by loss of cells integrity into the culture medium. 100 μ L of supernatant were
165 collected into every Transwell® insert, five hours after exposure and transferred in triplicate
166 in a 96-well plate. 100 μ L of reaction mixture was added and the plate was incubated during
167 30 minutes in the dark, at room temperature, before the measurement of optical density at
168 490 nm. AlamarBlue® test was used to evaluate cell proliferation, according to the
169 manufacturer instruction (Invitrogen, ThermoFisher Scientific). Living cells can indeed
170 convert resazurin to fluorescent resorufin (Excitation: 530 nm / Emission: 590 nm). Briefly, a
171 tenth of alamarBlue® reaction solution was added in the culture medium. After 4 hours of
172 incubation at 37°C, the fluorescence intensity was measured.

173 **2.3.2. Inflammatory response**

174 ELISA method was used to study the inflammatory response of BEAS-2B cells. IL-6 and IL-8
175 cytokines were quantified in the Transwell® insert supernatant using ELISA MAXtm kit
176 (BioLegend, Ozyme) according to the manufacturer instruction.

177 **2.3.3. Gene expression**

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

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

194 **2.4. Chemical analysis**

195 During cell exposure, toluene concentration was monitored by μ GC (Agilent Technologies
196 GC 5975C). For cells exposed to the gaseous flow after catalytic treatment, components
197 were collected by a Tenax™ TA cartridge (Perkin Elmer) for 15 minutes. After cartridge
198 desorption by a thermal desorber (TurboMatrix, Perkin Elmer), by-products were detected
199 and quantified by chromatography coupled with mass spectrometry (Perkin Elmer GC Clarus
200 680–MS Clarus 600S).

201 **2.5. Statistical analysis**

202 Statistical analysis was performed using Prism 7.05 (GraphPad Software, La Jolla California
203 USA) for EC₁₀ cytotoxicity/proliferation assay (Hill slope model) and for ELISA assay (Mann-
204 Whitney U test, $p < 0.05$). For qPCR assay, data treatment was performed on QuantStudio
205 software (Applied Biosystem, ThermoScientific).

206 **3. Results**

207 **3.1 Chemical analysis**

208 Monitoring the composition of the gaseous flow emitted after total oxidation of toluene
209 because by-products components could possibly be formed and could be more harmful than
210 the initial VOC. Brunet et al. have proved that the main by-product of toluene oxidation is
211 benzene due to its low chemical reactivity (Brunet et al., 2015). The reaction route is initiated
212 by an attack on the methyl group causing direct dealkylation of toluene. A profile of benzene
213 emission (Figure 1) highlighted the production of few ppm of benzene only with Pd/ γ Al₂O₃
214 catalyst. Benzene formation increased with toluene conversion to reach a maximum and then
215 decreased at higher temperature.

216 Under isothermal conditions, non-converted toluene and by-products formation was
217 evidenced (Table 2). The highest concentration of non-converted toluene was quantified for
218 the commercial catalyst, Pd/ γ -Al₂O₃. From day 1 to day 5 of exposure, Pd/ γ -Al₂O₃ toluene
219 concentration increased slightly (from 239 ppb to 265 ppb). Lower emission of toluene
220 occurred using the Co₆Al₂-O catalyst and the concentration in the gaseous flow was divided
221 by ~2 between the day 1 and the day 5 (96 ppb and 46 ppb respectively).

222 The main by-products detected were benzene and acetic acid. Benzene was detected after
223 catalytic oxidation using Pd/ γ -Al₂O₃ only and the concentration in the flow decreased over
224 time (from 549 ppb after day 1 to 396 ppb after day 5). After treatment using Co₆Al₂-O
225 catalyst, concentration of acetic acid reached 659 ppb and decreases rapidly over time.

226 **3.2. Cytotoxicity**

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

233 **3.3. Inflammatory response**

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

238 Concerning the relative secretion of IL-8, an inverted U-shaped profile over time was
239 observed for the cells exposed to the flow after treatment using Co₆Al₂-O (10 and 100%). The
240 cytokine was up-secreted the day 3 beside days 1 and 5. However, a U-shaped profile was
241 revealed for the cells exposed to the flow after treatment using Pd/ γ -Al₂O₃ (100%). Cells
242 exposed to 100 ppm and 1,000 ppm of toluene showed a slight increase for the IL-8
243 chemokine extracellular secretion during the 5 days. However, the results obtained for IL-6

244 and IL-8 were not significant compared to their relative secretion in cells exposed to purified
245 air. In parallel, the cells were exposed to LPS ($0.1 \mu\text{g}\cdot\text{ml}^{-1}$) under the same conditions and a
246 significant increase in secretion of IL-6 and IL-8 was observed with induction levels up to
247 7.21- and 2.47-fold compared to the non-exposed control for IL-6 and IL-8, respectively.

248 **3.4. Gene expression**

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

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

258 Cell exposure to the gaseous outflow using Pd/ $\gamma\text{Al}_2\text{O}_3$ highlighted a significant induction of
259 *CYP1A1*, *CYP1B1* and *CYP2E1* for cells exposed to 10% of the outflow during 5 day (2.7,
260 3.0 and 2.7 respectively). The same trend was observed for *CYP1A1* and *CYP1B1* genes
261 when cells were exposed to 100% of the outflow. However, *CYP2E1* was inducible over time
262 with RQ values higher than 4.0 for days 3 and 5. The other tested genes were only induced
263 for 100% of the outflow. At Day 5 of exposure, *CYP2S1*, *EPHX1*, *NQO1*, *GSTM1*, *DHDH* and
264 *ALDH2* were induced with a RQ upper to 2.0 and *ADH1A* with a RQ of 8.7. Finally, *CYP2F1*
265 was significantly over-expressed from day 1 to day 5, with a RQ of 3.4.

266 The second tested catalyst was $\text{Co}_6\text{Al}_2\text{-O}$. Cell exposure to 10% of the outflow induced the
267 gene expression of all the XME, excepted *CYP2F1* and *ADH1A*. The other genes were over-
268 expressed only the day 5. *CYP1A1*, *CYP1B1*, *GSTM1* and *ALDH2* levels were higher than
269 4.0 (4.9, 5.4, 4.4 and 4.1 respectively). Cells exposed to 100% of the outflow did not show
270 any significant induction of *CYP2F1*. An inverted U-shaped profile was observed for the
271 relative expression of the following 8 genes: *CYP1A1*, *CYP1B1*, *CYP2S1*, *GSTM1*, *EPHX1*,

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

277 **4. Discussion**

278 Repeated exposures to atmospheric xenobiotics contribute to the development of chronic
279 pulmonary diseases, allergies and neurologic disorders. However, mechanisms of the
280 chronic toxicity of air pollution, including the gaseous chemical components, like VOCs, are
281 still not well known (Upadhyay and Palmberg, 2018). Thus, further consideration should be
282 given to toxicological studies using repeated exposures on human cells. In the context of the
283 3R strategy, performing chronic exposures remains a challenge in *in vitro* studies. Persoz et
284 al., studied single exposure effects of formaldehyde on alveolar and bronchial cells (Persoz
285 et al., 2012). Without cells pre-sensitization, the authors did not observe significant
286 inflammatory response and results were linked to the choice of cells and to the exposure
287 conditions, no representative of real conditions. Cell response after repeated exposures to
288 airborne chemicals using ALI systems was also investigated (Bardet et al., 2014; Gostner et
289 al., 2016; Kastner et al., 2013). To our knowledge, Kastner et al. were one of the first to
290 evaluate the effect of gaseous chemical components after repeated exposure, by
291 comparison of ALI and submerged modes (Kastner et al., 2013). Lung epithelial cells Calu-3
292 were exposed with an ALI system to synthetic air or to formaldehyde and/or nitrogen dioxide
293 during 30 minutes, 1 hour or 2 hours. Cells showed an increase of IL-6 secretion after ALI
294 exposure to purified air in comparison with non-exposed cells, evidencing the need to
295 optimize the exposure time (Kastner et al., 2013). Bardet et al., studied the effects of human
296 nasal cells exposure to $200\mu\text{g}\cdot\text{m}^{-3}$ of formaldehyde for 1 hour during 4 days, using a
297 commercial ALI system. Indeed, no significant cellular mortality and modification of cellular
298 alteration were observed after the exposure. Only a significant increase of IL-8 production
299 was measured the day 3, in comparison with air-exposed controls and cells exposed for 1

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

302 In the present study, human BEAS-2B cells were chosen since bronchial epithelial cells are
303 one of the target of atmospheric pollutants (Hiemstra et al., 2018). This cell line is
304 metabolically active and permits repeated exposures. [Considering seeding concentration of](#)
305 [1,500 cells.cm⁻²](#), the cytotoxicity measured by the LDH assay was less than 10% of mortality
306 for day 5, corresponding to the EC₁₀, but higher than 20% from day 6. Therefore, no mortality
307 was significantly observed after purified air exposure during 1h/day for 5 days. This exposure
308 time was longer than the one considered in the studies mentioned above (Bardet et al., 2014;
309 Kastner et al., 2013). In addition, the absence of cell death observed after exposure to
310 toluene vapor or to gaseous outflows after catalytic oxidation of toluene allowed us to
311 characterize the cell response [until day 5](#).

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

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

328 100 and 1,000 ppm of toluene, an increase level of IL-8 secretion was observed in
329 comparison with cells exposed to air. Extracellular secretion of IL-8 due to VOCs exposure
330 has been previously observed in acute exposure of BEAS-2B to toluene and benzene
331 (Pariselli et al., 2006, 2009b). Repeated exposure of nasal epithelial cells to formaldehyde
332 also pointed out an increase level of IL-8 after 3 days (Bardet et al., 2014). The inverted U-
333 shaped profile of IL-8 secretion observed after catalytic oxidation flow exposure is not clear
334 so far. Modification of intracellular glutathione level by VOCs especially benzene and toluene
335 is observed in human epithelial lung cells A459 exposed in ALI system (Pariselli et al.,
336 2009a). Adequacy between variation of GSH/GSSG and regulation of inflammatory process
337 in lung have been established (Rahman and MacNee, 2000). Furthermore, an increase of
338 mucin protein secretion would promote the decrease of IL-8 secretion in *in vivo* models
339 (Fuentes-Mattei et al., 2010). Mucin secretion allows the decrease of inflammatory injury of
340 cells which dependent of IL-8 secretion (Turner et al., 2014) and the regulation of mucin
341 gene expression in lung by IL-8 (Bautista et al., 2009) can participate as a feedback
342 regulation. The variation of IL-8 profile could be related to the presence of very low
343 concentration of acetic acid in the gas flow after catalytic oxidation. The increase of IL-8
344 secretion after acidic compounds exposure has already been pointed out in A549 lung cells
345 exposed to acetic acid (Okubo et al., 2016) or in BEAS-2B cells exposed to hydrochloric acid
346 (Chen et al., 2013). The evaluation of the secretion of IL-6 and IL-8 highlights minor
347 modification between cells exposed to toluene or to the flow after catalytic oxidation and the
348 purified-air exposed ones. Further investigation should be carried out to evaluate the
349 intracellular level of these interleukins and the activation of the mucus pathway.

350 The gene expression evaluation was conducted by considering 12 XME genes selected
351 according to benzene and toluene metabolism (INRS, 2012; Rappaport et al., 2009) and their
352 induction in BEAS-2B cells (Courcot et al., 2012). After BEAS-2B cells exposure to 1,000
353 ppm of toluene, significant increases in the expression of *CYP1A1*, *CYP2E1* and *CYP2F1*
354 were observed. Contrary to *CYP2F1*, the 2 other XME genes were also induced. For the
355 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/ γ -Al₂O₃ and *CYP2S1* for the catalyst
372 Co₆Al₂-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/ γ -Al₂O₃ 10% and for Co₆Al₂-O 10% and
389 100%. *EPHX1* and *GSTM1* were also induced from the day 3 for Co₆Al₂-O 100%. Moreover,
390 induction level for the 3 genes was higher after cell exposure for Co₆Al₂-O catalyst. However,
391 the benzene concentration was lower when using Co₆Al₂-O compared to Pd/ γ -Al₂O₃ catalyst.
392 This over-expression could be probably explained by the presence of other benzenic
393 chemical compounds. Indeed, according to literature data, *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 (*DHDH*)
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 *DHDH* 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 *DHDH*
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/ γ -Al₂O₃ and
407 the day 3 for the catalyst Co₆Al₂-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,

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

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

428

429 **Conclusion**

430 Toluene is one of the most commonly used solvents while it is known as a harmful substance
431 inducing acute and chronic toxicity especially in workers. While acute mechanisms of toxicity
432 of toluene are known, how subacute and chronic effects appear is still unclear. Recent
433 progress in *in vitro* toxicology, in particular, the use of ALI exposure system, can help to
434 increase the knowledge about the subacute toxicity of volatile organic compounds. In this
435 study, normal human lung epithelial BEAS-2B cells were exposed until 5 days to toluene.
436 Only the repeated exposure permitted to detect the gene induction of *CYP1A1* that was not
437 previously showed in acute studies. The second strength of this study was the coupling of
438 the ALI exposure device to two different catalytic systems developed for the abatement of
439 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.

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623 **Supplementary material**

624

625 **Optimization of cell culture and exposure conditions**

626 **Methods:**

627 BEAS-2B cells were cultured in Corning® Cellbind® sterile flasks with LHC-9 culture medium
628 (Gibco, Fisher Scientific) and incubated at 37°C, in a 5% CO₂ and 100% humidity
629 environment. Firstly, BEAS-2B cells seeding concentration were optimized in order to
630 achieve repeated exposures, for a minimum of 8 days. Six seeding concentrations were
631 tested (190, 375, 750, 1,500, 3,000 and 6,000 cells.cm⁻²) in Cellbind® 6-well plates for 14
632 days and microscopic observation were daily realized. Cell morphology and cellular division
633 were used as phenotypic criteria. The cell cultures confluence was also limited to 80%
634 according to the recommendations of the American Type Culture Collection standard.
635 Optimal culture conditions on Transwell® insert were determined in order to obtain 500,000
636 cells on the last day of experiment. So, three cellular concentrations were used: 500,000,
637 195,000, and 76,000 cells/Transwell® insert for 1, 3 and 5 days respectively.

638 Cells were seeded on 4.7 cm² Corning® Transwell® inserts with 0.4 µm pore size (Sigma-
639 Aldrich) and exposed to purified air for 1h during 8 days. Cytotoxicity and proliferation were
640 evaluated considering as a control, unexposed cells placed in similar inserts inside the
641 incubator at 37°C.

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

648

649

650

651 **Results:**

652 Cells concentration for repeated exposures was first optimized. 80% of confluence was
653 achieved before 8 days for 3,000 and 6,000 cells.cm⁻². For the other seeding concentration
654 (190, 375 and 750 cells.cm⁻²), cellular behavior and phenotype modifications were observed
655 from day 5. Indeed, adherent cells had grown in clusters instead of covering uniformly in the
656 well bottom. The confluence for a seeding concentration of 1,500 cells.cm⁻² was reached on
657 the 8th day of culture. Then, the exposure period with the Vitrocell® system was optimized
658 (Figure S1). LDH assay (grey curve) and alamarBlue® assay (black curve) were used to
659 measure cytotoxicity and proliferation of cells exposed to purified air, respectively. Curve-
660 response fitting used for cytotoxicity determination highlight that EC₁₀ (corresponding to 10%
661 of cytotoxicity in cells exposed to purified air) is obtained between the 5th and the 6th day of
662 exposure. For every tested condition, a decrease of the proliferation index was also observed
663 from day 5. From 1st to 5th days of culture, exposed cells proliferation was stable in
664 comparison with control.

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

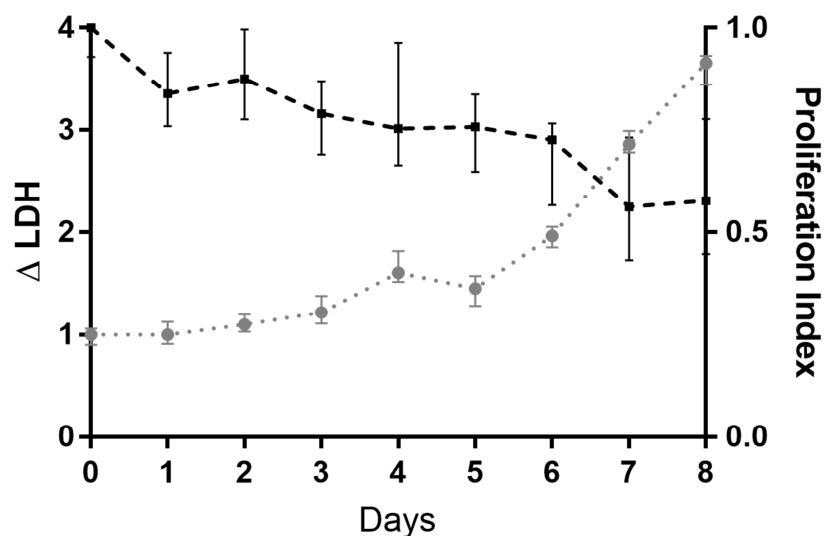
667 In conclusion, these optimizations allowed to validate the optimal culture and exposure
668 conditions of BEAS-2B cells for repeated ALI exposure.

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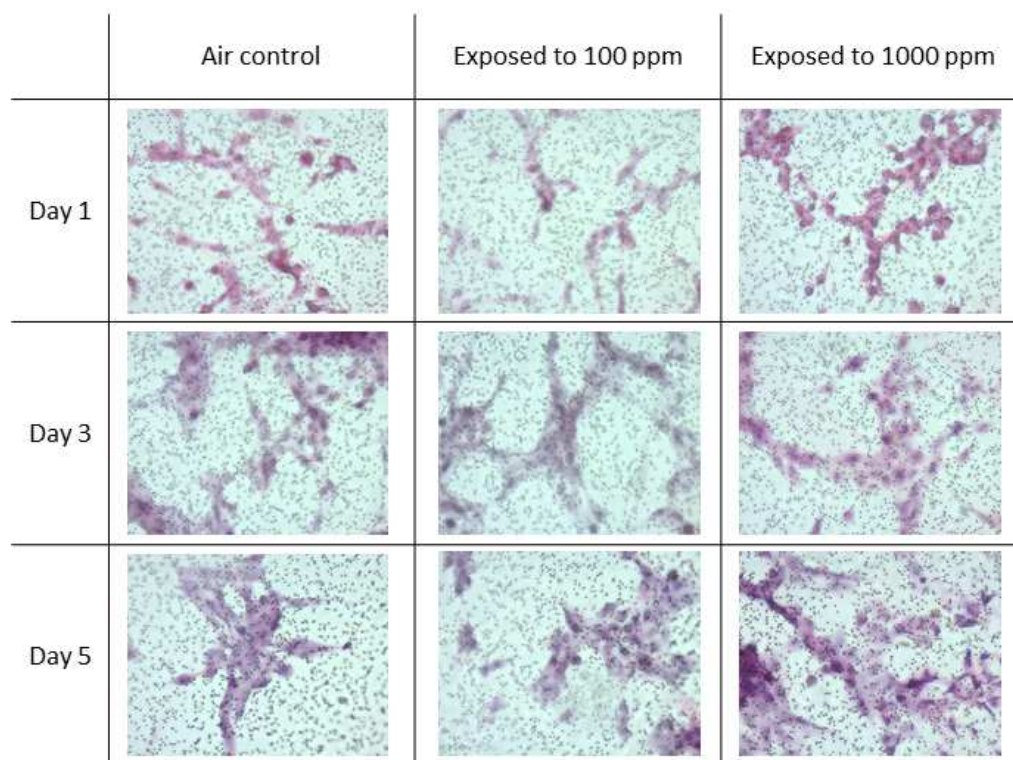
670

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

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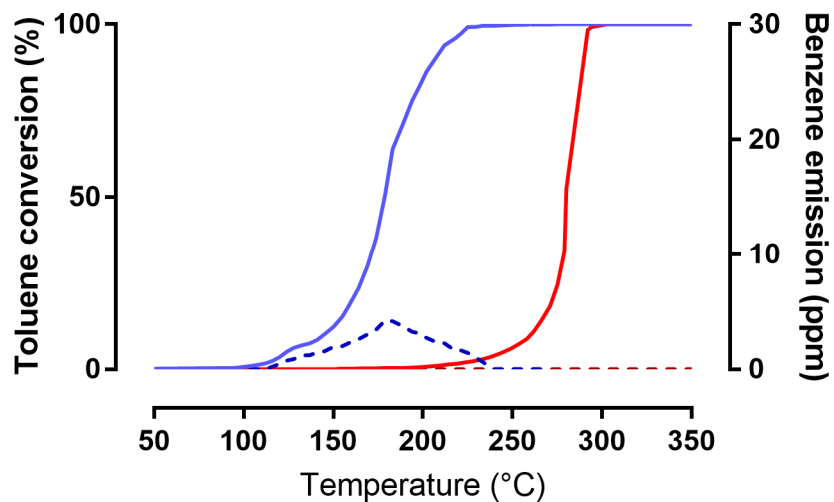


674
 675 Figure S1: Cytotoxicity (LDH release assay, in grey dot), and relative proliferation index
 676 (alamarBlue® assay, in black square), after cell exposure to purified air for 1h/day for 8 days.
 677 For each day, obtained values (n=3) were compared to Day 0. Curve-response fitting was
 678 performed using Hill equation model, in order to determine EC₁₀.



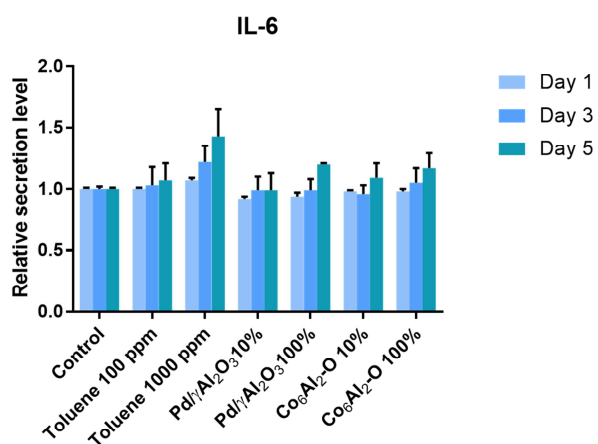
679
 680 Figure S2: morphologic evaluation of BEAS-2B by photonic microscopy (x100 of
 681 magnification) after cell exposure to toluene during 1h/day for 5 days.

682

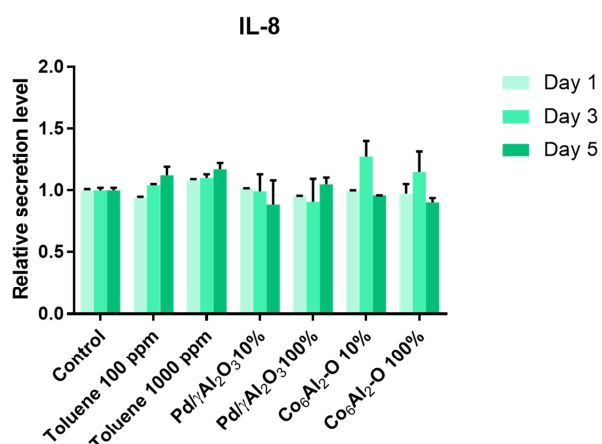


1
2 Figure 1: Light-off curve of toluene conversion (%) represented in continuous lines, and
3 benzene emission (ppm) represented in dotted lines, as a function of temperature (°C) using
4 Pd/γAl₂O₃ (blue) or Co₆Al₂-O (red) catalysts.
5

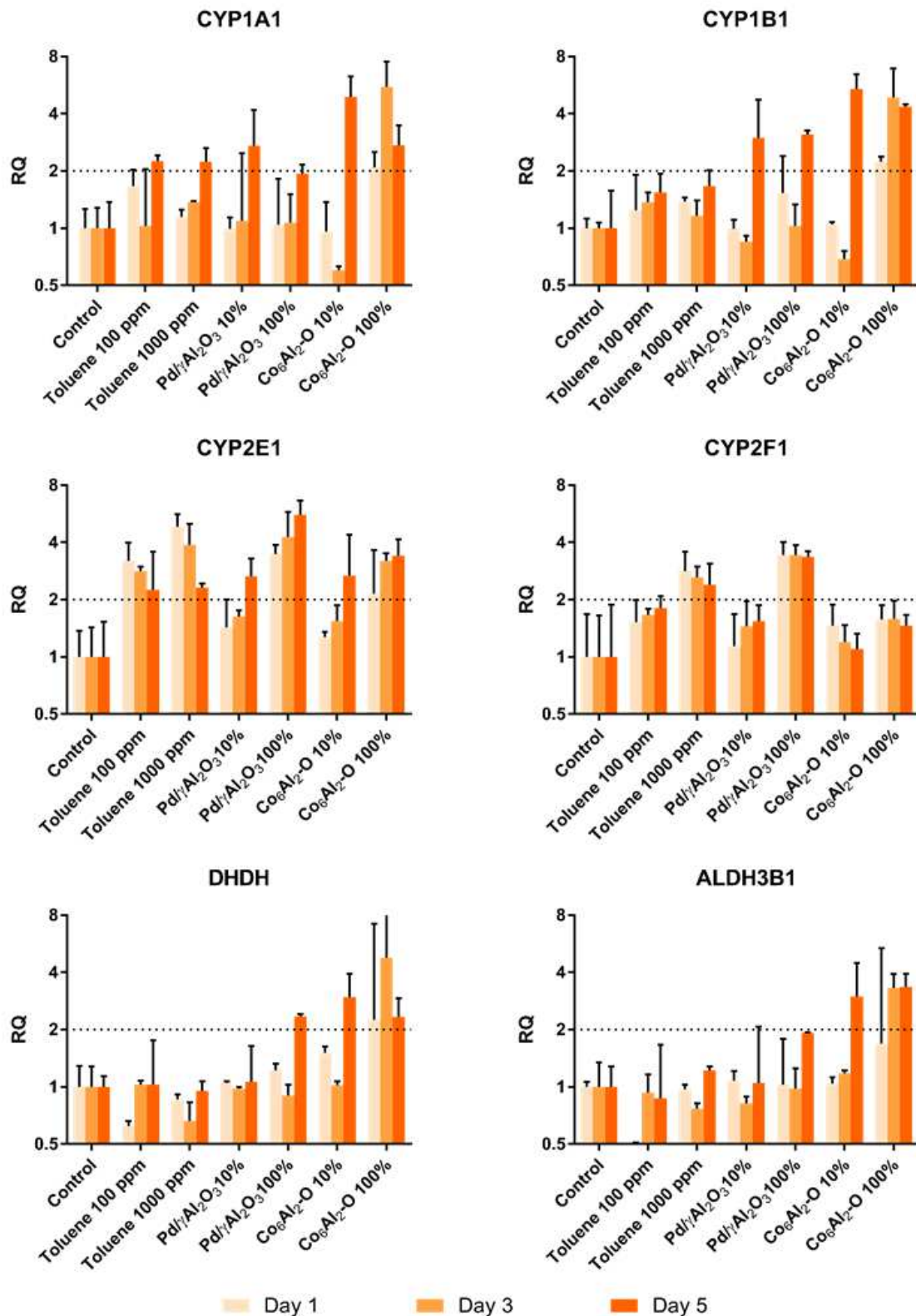
A.



B.



6
 7 Figure 2: Relative secretion of IL-6 (A) and IL-8 (B) after cell exposure to toluene or to the
 8 gaseous flow after catalytic oxidation during 1h/day for 5 days. The cytokine concentrations
 9 were measured by ELISA in the supernatant of biological replicates (n=3) and values were
 10 compared to air exposed cells. No statistical difference was observed using Mann Whitney U
 11 test (p<0.0.5).



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

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

Table 2: Chemical components detected in the gaseous flow after oxidation of toluene using Pd/ γ -Al₂O₃ or Co₆Al₂-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
Pd/γ-Al₂O₃	239	228	265	549	447	396	<LOD	<LOD	<LOD
Co₆Al₂-O	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 $\Delta\Delta\text{Ct}$ 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 10%			Toluene 100%			Pd/ $\gamma\text{Al}_2\text{O}_3$ 10%			Pd/ $\gamma\text{Al}_2\text{O}_3$ 100%			Co ₆ Al ₂ -O 10%			Co ₆ Al ₂ -O 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>	-	=	=	=	=	=	=	=	=	=	=	=	=	=	+	=	+	+