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An original device to assess the respiratory impact of indoor air VOCs mixture using an *in vitro* approach

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ABSTRACT

Exposure to indoor air pollution, particularly volatile organic compounds (VOCs), has been recognized as a risk factor in the development of respiratory and allergic diseases. VOCs are mainly emitted continuously at low concentrations from construction furniture and decoration products. Measurement campaigns carried out in new dwellings in France have shown that aldehydes predominate with a tendency to decrease formaldehyde concentrations and to increase those in hexanal. As the main route of VOCs exposure is inhalation, this project assessed the impact of a mixture of 17 VOCs representative of indoor air (in quality and quantity) on respiratory health using an *in vitro* approach. This original work was based on the set-up of an experimental device, combining a gas generation and dilution bench and exposure to the air-liquid interface (ALI) adapted to the reconstructed human airway epithelium model. The VOC mixture was enriched with formaldehyde or hexanal in different proportions (from 20 to 240 $\mu\text{g}\cdot\text{m}^{-3}$) to study the biological impact of these aldehydes after repeated exposures of airway epithelium. After examination of the stability of the VOC concentrations in generated mixtures and the found of the optimal operating conditions for the dynamic gas generating system, the gaseous mixtures were distributed to the epithelium using the ALI exposure system providing direct contact between the epithelium and the tested mixtures. Our device lead to reproduce real conditions of human exposure. The results showed that the inflammatory response, assessed by the production of four cytokines, varied according to the nature of the aldehyde present in the VOC mixture (formaldehyde or hexanal), its concentration, and the duration and number of exposures applied. The most original and innovative results concern those obtained with hexanal, pollutant under-researched. Our results showed that this aldehyde could pose risks to respiratory health.

1. Introduction

In response to the ethical principles of the 3Rs (Replace, Reduce, Refine) [1], *in vitro* models are increasingly used as alternative methods to animal experiments to study the adverse effects and associated biological mechanisms of various environmental pollutants, such as

volatile organic compounds (VOCs) [2]. To reproduce human exposure to gaseous pollutants by inhalation using an *in vitro* approach, it is necessary to develop a concept combining exposure chambers adapted to respiratory cell/tissue cultures, and a gas generation system that enables pollutants to be tested alone or in mixtures at levels representative of human exposure. Generating the standard gas mixtures,

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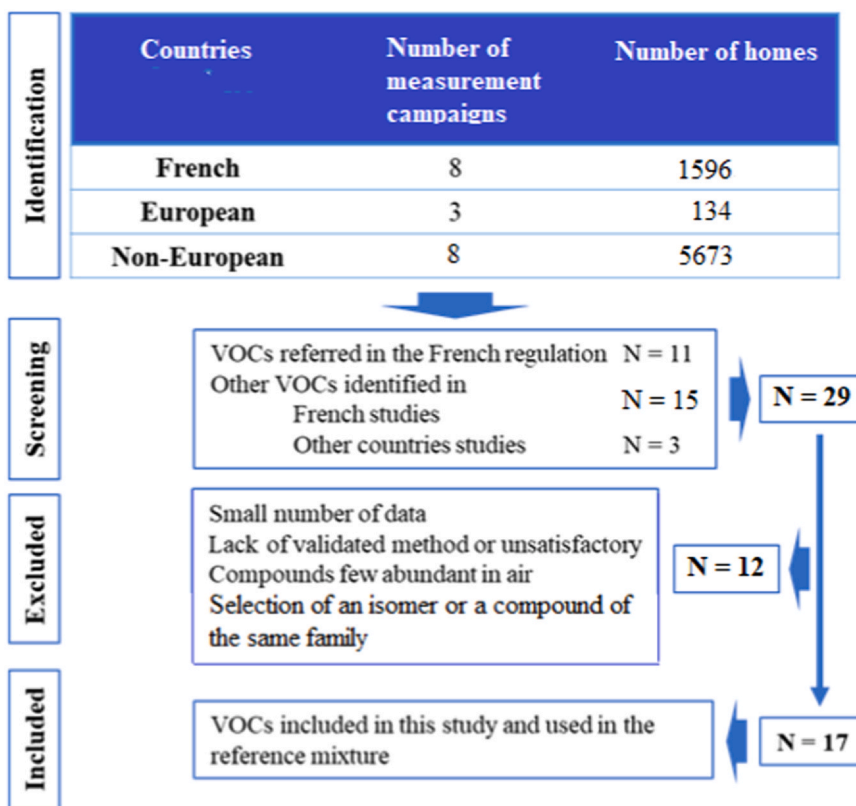


Fig. 1. Flow chart for the selection of Volatile Organic Compounds (VOCs) and concentrations for the referent mixture (N: number of VOCs).

in which concentration levels are stable and accurately known, is a crucial factor in *in vitro* studies carried out to assess the impact of air quality on biological activity. Exposure of cells to gaseous mixtures can be performed by two ways: static or dynamic [3]. In static mode, mixtures are generated by introducing a defined volume of gas, or volatile liquid, into a specified volume of air. Static methods are simple and inexpensive. However, they suffer from many drawbacks, such as analyte losses caused by adsorption and condensation on the parts of experimental device, a dilution *ratio* limited by the volume of the exposure device and the amounts introduced which restrict the application of these static methods to exposures at ppm levels [2]. These static methods are rather used in toxicology studies for which the required concentrations are high, exceeding the environmental VOC levels found in indoor air.

Dynamic generating gas mixtures require a production of a continuous flow of air containing compounds made by mixing and diluting gases. With these dynamic techniques, it is possible to produce multi-component mixtures at $\mu\text{g}\cdot\text{m}^{-3}$ level and to replicate different gas atmospheres by changing the dilution *ratio* [4]. At low concentration levels, the adsorption or release of VOCs on parts of the laboratory installation can lead to significant changes in the composition of the air flow generated depending on the nature of the compounds [3,5,6]. It is necessary to measure the concentrations in the air stream during the exposure of cells to VOCs and to examine, prior to exposure tests, the stability of VOC concentrations in the mixtures (stabilization time and variability). Dynamic generation of VOC mixtures appears to be the most relevant solution when the inflammatory effects are investigated with an *in vitro* model at low concentrations characteristic of indoor air.

Classical cultures of airway epithelial cells have evolved into more realistic *in vitro* models. In addition to monocells in monolayers cultures (primary cells and cell lines), co-cultures integrate the notion of cell cooperation [7,8]. However, the main limitation of these models to assess the impact of air pollutants is that cells are grown and exposed under submerged conditions, i.e. at the liquid-liquid interface, which

does not reflect the reality of the contact between pollutants and epithelial cells. Therefore, human reconstituted airways models have been developed with different anatomical sites (Nasal, Tracheal, Bronchial or Alveolar) [9]. These models are constituted of multilayers of different epithelial cells (ciliated, goblet cells and basal cells), which allow them to exhibit many characteristics of the human epithelium, such as active cilia-beating, production of mucus or surfactant, active ion transport, tight junctions, and metabolic activity [10]. In addition, these models are maintained at the air-liquid interface (ALI) and have a relatively long lifespan [11,12]. To mimic the inhalation pathway, direct contact between epithelial cells and aerosols is made possible using exposure chambers allowing dynamic ALI exposure mode. In this context and using an *in vitro* approach, it seemed important to us to apply such dynamic ALI exposure system to evaluate the respiratory impact of specific pollutant mixtures encountered in indoor air, the volatile organic compounds (VOC), with a focus on formaldehyde (FA) and hexanal (HEX).

While the effects of formaldehyde exposure on the respiratory system are now well established [13], those of hexanal are much less well described. Short-term exposures of healthy volunteers to low levels of volatile irritating chemicals, such as hexanal, have shown a down-regulation of the inflammatory marker C reactive protein (CRP) which might be a response mediated by the cholinergic anti-inflammatory pathway [14]. Other studies have shown that hexanal can modify the gene expression of pro-inflammatory cytokines [15] or lead to epigenetic events [16]. Thus, it is essential to improve scientific knowledge on the potential effects of hexanal, for which there is unfortunately very little scientific information. This was the context of our work.

A gas generating system allowing the continuous production of VOC mixtures at levels representative of indoor air, enriched in FA or HEX, has been developed and combined with the ALI exposure system. The objectives of the present work were 1/ to validate a dynamic gas generating system producing synthetic air containing low-level VOCs allowing the modulation of individual FA and HEX concentrations while

keeping the other compounds constant, 2/ to examine the stability of VOCs concentrations under the conditions required for *in vitro* exposures, and 3/ to assess the feasibility of coupling a gas generating system to an ALI exposure system to evaluate the impact of FA or HEX on the inflammatory response of the 3D-model of reconstructed bronchial epithelium.

2. Materials and methods

2.1. Selection of VOCs and concentration levels of the referent mixture

The identification of VOCs representative of indoor air and their concentration levels was carried out from a review of existing data in French, European and non-European measurement campaigns according to the process summarized in Fig. 1. Details on its application that led to the choice of referent VOC mixture are given later in Section 3.1 and completed by Supplementary material (Tables S2-S4).

According to the two French studies carried out in 2006 and 2016 by the indoor air quality observatory (OQAI) [17,18], FA and HEX are systematically found in all French houses studied in 2006 ($n=567$) and in 2016 ($n=72$) (see Tables S2 and S4).

The medians of FA concentrations were $19.6 \mu\text{g}\cdot\text{m}^{-3}$ in 2006 and $17.2 \mu\text{g}\cdot\text{m}^{-3}$ in 2016, exceeding the French guide value for long-term exposure of $10 \mu\text{g}\cdot\text{m}^{-3}$ [19]. In these same studies, HEX was systematically detected in the samples. A raise of the concentration medians observed by the OQAI between 2006 and 2016, going from $13.6 \mu\text{g}\cdot\text{m}^{-3}$ to $21.3 \mu\text{g}\cdot\text{m}^{-3}$ [17,18]. This trend was also confirmed by Dallongeville [20] in another French measurement campaign carried out in 2013 ($n=147$) for which the median was $25.4 \mu\text{g}\cdot\text{m}^{-3}$. According to this last study, high levels of aldehydes were associated with respiratory diseases, including the development of asthma [20].

2.2. Coupling the dynamic gas generating system combined to the air-liquid interface exposure system

The gas generating system is described in Fig. 2a. Three compressed

gas cylinders manufactured by Air Liquide (Paris, France) and Linde (Saint-Priest, France) constitute it. The first one contained the mixture of 15 VOCs as follows: acetaldehyde (14 ppm), benzaldehyde (0.4 ppm), propanal (2 ppm), acetone (24 ppm), benzene (1.2 ppm), toluene (2 ppm), ethylbenzene (1 ppm), p-xylene (2.8 ppm), styrene (0.4 ppm), 1,2,4-trimethylbenzene (1.2 ppm), n-decane (1.8 ppm), 2-butoxyethanol (0.8 ppm), 1-methoxy-2-propanol (0.6 ppm), limonene (8 ppm) and α -pinene (8 ppm) in nitrogen, the second one formaldehyde (FA, 40 ppm) in nitrogen and the third one hexanal (HEX, 10 ppm) in nitrogen. The total volume available for each mixture was 2 m^3 corresponding to a generating duration of more than 250 days under the operating conditions of this study, the volume needed to ensure long and repeated exposure of our *in vitro* model. FA and HEX were independently produced to be able to increase only their concentrations. Their concentrations were adjusted manually by modifying the set points of the mass flow controllers. These three high concentration mixtures were diluted with a clean dry air supplied by a compressor associated to an air purifier AZ 2020 manufactured by Claind (Marcq-en-Baroeul, France). A part of clean air passed through two bubblers filled with demineralized water to regulate relative humidity (RH) of generated atmospheres containing VOCs. All the ways of the gas generating system were regulated by airflow controllers. Another air channel (noted as "Clean Air" in Fig. 2) was also installed, in order to carry out control tests in the absence of pollutants.

Airflows were adjusted by successive trials until the target concentrations and relative humidity of 40–45% at 23°C were achieved. Tests were carried out in a laboratory room where the temperature was maintained at $23 \pm 3^\circ\text{C}$. Temperature and relative humidity of generated atmospheres were continuously monitored by a probe (Kistock KH 210 manufactured by Kimo, Montpon, France) placed at the outlet of the exposure device.

After airflow adjustments, the gas generating system was connected to the ALI exposure system distributed by Vitrocell® Systems (Freiburg, Germany) and described by Bardet [21]. Briefly, this exposure module includes three exposure chambers, each one corresponding to a culture chamber (insert to receive cells/tissues) and a trumpet to allow the

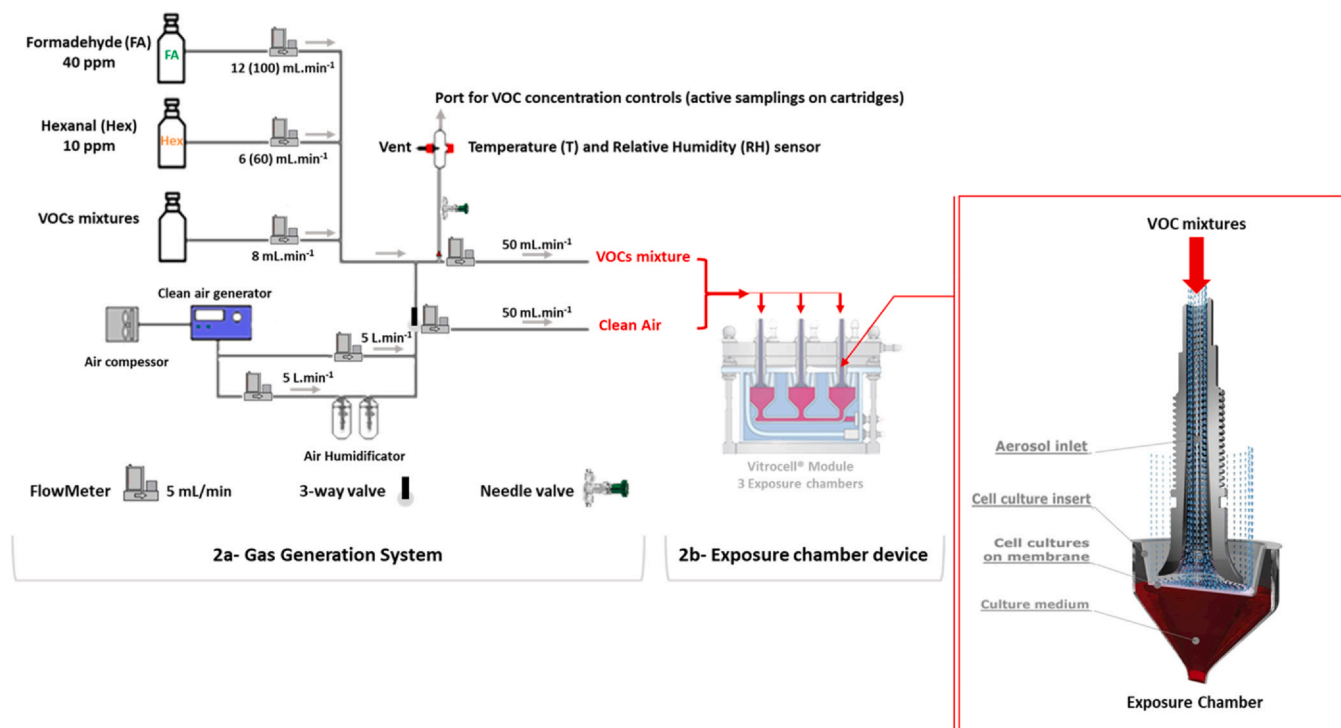


Fig. 2. Dynamic gas generating system (2a) coupled to ALI exposure chambers (2b). The flow rates values noted in brackets correspond to mixtures enriched in FA and HEX.

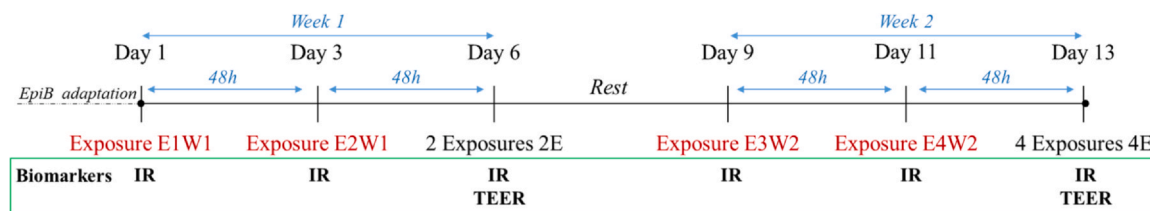


Fig. 3. Exposure design and biomarkers assessments (E, Exposure; W, Week; IR, Inflammatory response; TEER, Trans Epithelial Electrical Resistance).

distribution of the polluted air, at an airflow rate of 2 mL/min, to the apical side of the epithelium (Fig. 2b). The trumpets were previously adjusted at 3 mm above the bottom of the insert corresponding to the apical side of the tissue. A water bath was connected to the exposure module to maintain each exposure chamber at 37°C.

The complete experimental set-up with the dynamic gas generating system coupled to the ALI exposure device is represented in Fig. 2.

2.3. Sampling and analysis of VOCs

The VOC concentrations were monitored by two active sampling methods using a BPE/DNPH cartridge (Sigma-Aldrich, Saint-Louis, MO, USA) for formaldehyde, acetaldehyde, propanal and acetone and a stainless steel tube filled with 400 mg of 40–60 mesh Carbograph 4 supplied by Tera Environment company (Crolles, France) for the other 13 VOCs. Active sampling with BPE/DNPH cartridge was performed at 300 mL.min⁻¹ for 2 hours. For active sampling with Carbograph 4 tube, the flow rate was 100 mL.min⁻¹ for a sampling time of 2 hours. The flow rates were kept constant using a mass flow controller and checked before and after each pumped sample with a gas flow meter (DryCal DC-Lite). A guard tube was systematically added to the sampling device for each test gas atmosphere and showed that no breakthrough occurred under these sampling conditions. The BPE/DNPH cartridges were then extracted and analyzed by HPLC-UV according to the protocol described in previous articles [22,23]. The Carbograph 4 tubes were analyzed with a thermal desorber (TD) (Turbomatrix 350 from Perkin-Elmer, USA) interfaced with a gas chromatograph (GC) equipped with a flame ionization detector (FID) following the method previously described by Plaisance et al. [24].

Quantitative analysis was based on an external calibration with at least six standard levels covering the concentrations of test gas atmospheres produced by dynamic gaseous generating system. For the compounds analyzed by HPLC, standard solutions were prepared by dissolving commercially available 2,4-dinitrophenylhydrazone derivatives of formaldehyde, acetaldehyde, propanal and acetone (Supelco, Saint-Quentin-Fallavier, France) in acetonitrile. For the thirteen other VOCs analysed by TD-GC-FID, standard solutions were prepared from the mixtures of pure compounds diluted in methanol. Carbograph 4 tubes were then loaded with 2 µL of standard solutions using a GC syringe and purged for 10 min with a helium flow of 35 mL.min⁻¹ to remove the solvent. Standards were analyzed under the same analytical conditions as the samples and calibration curves with at least six levels of standards and a R > 0.99 as validation criterion (R: correlation coefficient).

The main performances of the two methods obtained for the measurements of 17 VOCs are given in Supplementary material (Table S1). Sensitivity, linearity range and analytical repeatability are suitable for monitoring concentrations of all compounds. A degradation of the repeatability is noted for 2-butoxy-ethanol (RSD > 10 %) (RSD: Relative Standard Deviation). Control charts (not reported in this article) were drawn up for the 17 VOCs based on a monthly analysis of a standard solution. Maximum response deviations of ± 20 % had been shown.

2.4. In vitro model

The study was conducted with human reconstituted epithelia

bronchial origin co-cultured with human airway fibroblasts (HF) (MucilAir-HF™, Epithelix®, Geneva, Switzerland) based on primary culture of human bronchial epithelial cells from healthy donor (without respiratory pathology, non-smoker) and differentiated at the air-liquid interface on Costar Transwell insert (0.33 cm², pore size of 0.4 µm, Corning, NY, USA) during 48 days [10].

Reconstituted epithelia (named EpiB in this work), were maintained at the air-liquid interface in an incubator at 37°C (5 % CO₂ and 95 % constant relative humidity) with 700 µL of MucilAir nutrient medium (Epithelix®, Geneva, Switzerland) in the basal side. To preserve tissue homeostasis, the culture medium was changed every two days and the apical side was washed to remove mucus and dead cells, once a week according to the supplier's instructions. Morphology of EpiB was controlled using a light inverted microscope (magnification 100X, Nikon Eclipse Ts100) before and after exposure throughout the experiment period. In order to assess the tissue integrity (tight junctions and ionic fluxes), Trans Epithelial Electrical Resistance (TEER) measurement (Ω.cm²) was performed on tissue using an EVOM² VoltOhmmeter (World Precision Instruments, USA). According to Epithelix®, tissue integrity is considered good if the TEER value is greater than or equal to 150 Ω.cm².

2.5. Repeated ALI exposure design

After reception, EpiB were maintained one week in the incubator for adaptation. Then, EpiB were exposed to VOC referent mixture (Ref) enriched or not with formaldehyde (FA) or hexanal (HEX). VOC concentrations present in the referent mixture were fixed. Only FA and HEX concentrations levels could be modulated. Experimental protocol for exposure is summarized in Fig. 3.

Three levels of FA or HEX were tested: 20 µg.m⁻³ corresponding to the FA and HEX concentrations present in the VOCs referent mixture (FA20 and HEX20 respectively), 120 µg.m⁻³ (FA120, HEX120), and 240 µg.m⁻³ (FA240, HEX240). At the same time, and for each experiment, two controls were considered: Incubator control, EpiB without exposure maintained in the incubator during two weeks and Air control, EpiB repeatedly exposed to air without pollutant during two weeks. For each experimental condition and exposure time, duplicates were carried out.

Exposures (E) were performed twice a week with 48 h of interval for two consecutive weeks (E1W1 – E2W1 for week 1; E3W2 – E4W2 for week 2). Each exposure lasted 1 h. At the beginning of each week, before exposure (Day 1 and Day 9), and at the end of each exposure time (Day 3 - Day 6 for week 1; Day 11 - Day 13 for week 2), culture media were collected to evaluate inflammatory response (IR). At the end of each week (Day 6 and Day 13), mucus was collected to evaluate the apical pro-inflammatory cytokine productions and, tissue integrity was assessed by TEER (Trans Epithelial Electrical Resistance) measurement.

2.6. Biological activity assessment

2.6.1. Trans epithelial electrical resistance (TEER) measurement

In order to assess the tissue integrity (tight junctions and ionic fluxes), once a week apical side of EpiB was washed with 3*200 µL of warmed medium to remove mucus and surface desquamating cells. TEER measurement (Ω.cm²) was performed on tissue with 700 µL of

Table 1

Volatile organic compounds (VOCs) and concentration ranges retained for the referent mixture (Ref).

Carbonyl compounds	Target level range in $\mu\text{g.m}^{-3}$	Hydrocarbons and other oxygen compounds	Target level range in $\mu\text{g.m}^{-3}$	Terpenes	Target level range in $\mu\text{g.m}^{-3}$
Formaldehyde	11–27	Benzene	0.6–2.8	Limonene	7.3–205
Acetaldehyde	11–18	Toluene	4.5–13.5	α -Pinene	1.3–22.6
Benzaldehyde	0.7–2.6	Ethylbenzene	0.9–3.1		
Propanal	1.5–5.3	p-Xylene	2.4–12.4		
Acetone	14–75	Styrene	0.4–1.1		
Hexanal	2.4–27.6	1,2,4-Trimethylbenzene	0.6–4.8		
		n-Decane	1.3–15.6		
		2-Butoxy-ethanol	0.1–2.1		
		1-Methoxy – 2-propanol	0.9–1.9		

warmed medium on the basal side and 200 μL on the apical side using electrodes connected to the EVOM² epithelial Voltohmmeter (World Precision Instruments, England).

TEER was calculated as following: $\text{TEER} = (\text{TEERs} - \text{TEER0}) \cdot A$, with TEERs for the experimental value measured of EpiB, TEER0 for the background value of the insert membrane without tissue (100 $\Omega\cdot\text{cm}^2$), A: Surface area of the insert (0.33 cm^2).

2.6.2. Inflammatory response (IR) assessment

The levels of cytokines produced by the epithelial cells of the EpiB were assessed using Human DuoSet ELISA from R&D systems (Bio-Techne SAS, France).

Out of the seven cytokines/chemokines initially chosen (IL-6, IL-8, IL-1 α , IL-1 β , IL-13, MIF, and GM-CSF) to assess the inflammatory response after repeated EpiB exposures, only three cytokines Interleukin 6 (IL-6 # DY206), Interleukin 8 (IL-8/CXCL8 # DY208), Macrophage migration Inhibitory Factor (MIF # DY289) and one chemokine Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF # DY215) were considered as relevant.

Absorbance was determined with a microplate-reader (Multiskan-EX, Thermo Scientific, MA, USA) set to 540/450 nm. Calibration curves were established using Ascent Software® (Thermo Scientific, MA, USA). Cytokine concentrations were expressed in pg/mL.

2.6.3. Statistical analysis

The results are expressed as arithmetic means \pm standard deviation (SD) of two independent experiments performed in duplicates. The number of experiments conducted is indicated in the legend of each Figure. Results were analyzed by one-way analysis of variance (ANOVA) followed by a t-test of Student (Air control *versus* VOC-exposed). Statistical analysis was performed using GraphPad Prism 7 software. Data were considered as statistically different when * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 2

Stability study of the referent mixture produced by the dynamic gaseous generating system (9 samples taken over 24 days).

	Target concentration range for the referent mixture ($\mu\text{g.m}^{-3}$)	Average concentration \pm Standard Deviation ($\mu\text{g.m}^{-3}$)	RSD (%)
Benzene	0.6–2.8	2.4 \pm 0.11	4.6
1-Methoxy – 2-propanol	0.9–1.9	1.4 \pm 0.16	11.2
Toluene	4.5–13.5	4.7 \pm 0.2	4.4
Hexanal	2.4–27.6	18.1 \pm 1.5	8.0
Ethylbenzene	0.9–3.1	2.3 \pm 0.12	5.2
p-Xylene	2.4–12.4	6.8 \pm 0.3	5.1
Styrene	0.4–1.1	0.98 \pm 0.08	8.3
2-Butoxy-ethanol	0.1–2.1	2.9 \pm 0.9	29.8
α -Pinene	1.3–22.6	26.9 \pm 1.5	5.6
Benzaldehyde	0.7–2.6	0.94 \pm 0.08	8.4
1,2,4-Trimethylbenzene	0.6–4.8	3.1 \pm 0.16	5.2
n-Decane	1.3–15.6	5.4 \pm 0.28	5.2
Limonene	7–205	23.8 \pm 1.6	6.8
Formaldehyde	11–27	17.9 \pm 0.9	4.9
Acetaldehyde	11–19	16.8 \pm 0.9	5.5
Propanal	1.5–5.3	3.4 \pm 0.3	9.1
Acetone	14–75	40.2 \pm 2.3	5.7

removed from the final list due to a small number of data on these compounds and a lack of validated analytical method available for ethanol and methanol;

- With regard to terpenes, only the two most abundant compounds, α -pinene and limonene, were kept. β -pinene and camphene were therefore removed from the final list;
- For the alkanes (n-decane and n-undecane), only n-decane was retained. Likewise, p-xylene was chosen as the only representative of the xylene isomers. The n-decane and p-xylene concentrations were increased to offset this reduction in the number of compounds from the same family;
- 1,4-Dichlorobenzene, 2-ethylhexanol and acrolein were removed from the final list because none of compressed gas cylinder manufacturers offered a satisfying technical solution for these three compounds.

At the end 17 VOCs representative of the compounds encountered in residential indoor air were selected and the concentrations chosen for the referent mixture (named Ref) basing on the medians reported in the studies are summarized in Table 1. As for the list of compounds of interest, the definition of concentrations prioritized French data when they were sufficient. In a recent review, Halios [31] listed 65 individual VOCs that were the most commonly measured in European homes and provided their average concentrations from the results of 39 studies. The 17 VOCs retained in our study belong to the list defined by Halios [31] and the concentration ranges given in Table 1 are in agreement with their average ranked values. 11 of the 17 selected chemicals are part of most health relevant VOCs indoors according to their adverse-effect endpoints (i.e. respiratory, irritation of the upper airway system and eyes, cardiovascular, neurological and carcinogenic) [32]. These chemicals are: benzene, ethylbenzene, p-xylene, styrene, toluene, 1,2,4-trimethylbenzene, formaldehyde, acetone, acetaldehyde, limonene, α -pinene. Hexanal is classified as having only irritant effects and is not on the list of priority compounds for its impact on health [31,33].

3.2. Stability of dynamic gaseous generating system for a mixture characteristic of indoor air

Table 2 gives the average values and standard deviations of concentrations of the 17 VOCs for 24 days of operating the gaseous generating system. In addition, Fig. 4 shows the generated concentration profiles over time obtained for this same operating period.

As shown in Fig. 4, the time required to obtain stable concentrations in the airflow is about 3–5 days. A slight increase in concentrations is observed between 3 and 5 days for certain compounds like limonene, α -pinene and toluene. This rather long time could be due to the VOCs sorption on the surfaces of device delaying the steady state. This was already observed for VOC mixtures generated at low concentrations (a few $\mu\text{g}\cdot\text{m}^{-3}$) [4,6].

For ten VOCs, the concentrations vary from $\pm 10\%$ around the average. The variation ranges are higher comprised between $\pm 20\%$ for six other VOCs which are alkenes and oxygenated compounds (limonene, hexanal, propanal, benzaldehyde, 1-methoxy-2-propanol and styrene) and $\pm 40\%$ for 2-butoxy-ethanol. This latter compound is known to be complex to control in multicomponent mixtures at low concentration levels [5]. Despite these technical limits, the concentrations measured at the outlet of gaseous generating system (Table 2) meet in the most cases the target value ranges defined as characteristics of indoor air, apart from α -pinene and 2-butoxy-ethanol, which have values slightly above the target.

3.3. Generation of mixtures with increased concentrations in formaldehyde and hexanal

Formaldehyde and hexanal concentrations were individually increased in the generated mixture by changing the setpoints of mass flow

controllers associated to the compressed gas cylinders of the two compounds. A setting time was at least 2 hours before the measurements of concentrations with active sampling methods. As shown in Table 3, increasing the setpoint by a factor of 10 led to an expected increase in the concentration in the mixture for HEX and FA. The changes in the airflows had a limited impact on the concentrations of other VOCs with a downward trend limited to less than 20% except for 2-butoxy-ethanol. These results are consistent with the concentration variations recorded in the gas generation stability test.

3.4. Control of VOC concentrations upstream and downstream of exposure device

As described in Section 2.2, the gas generating system was coupled to the Vitrocell® exposure system. A continuous air stream containing VOC mixture was supplied the exposure device and was directed to the modules containing the reconstituted tissues. In order to carry out repeated exposures of the epithelia to VOC mixtures, a sterile filter was placed at the outlet of the gas generating system to ensure the sterility of the mixture arriving in contact with the apical side of the epithelial cells without risk of bacterial or fungal contamination.

To check the influence of sampling location on the concentrations measured in VOC mixture, controls were carried out upstream and downstream of the exposure device. As shown in Table 4, no difference between the concentrations measured in upstream and downstream of the exposure system was observed when the VOC referent mixture was generated. These test results showed no loss of compounds between the generation and exposure systems, loss which could result from adsorption on the walls and/or pipes along the VOC pathway.

However, it can be noted that the concentrations measured for most compounds were lower than expected when the referent mixture was pre-generated, particularly for the two glycol ethers, i.e., 2-butoxy-ethanol and 1-methoxy-2-propanol. It is likely that not all concentrations have reached stability at the time of sampling in these tests. As previously described, a long period of 3–5 days was required to reach stable concentrations for all compounds in this low-level mixture.

3.5. Biological impact of VOC mixture enriched with formaldehyde or hexanal on reconstituted airway epithelium

3.5.1. Reconstituted bronchial epithelium integrity

According to Epithelix© tissue integrity is considered good if the TEER value is between 150 and 600 $\Omega\cdot\text{cm}^2$. TEER values not exceeding the 150–600 $\Omega\cdot\text{cm}^2$ bounds were observed from week 1 to week 2 for control epithelia, reflecting the maintenance of good tissue integrity under our experimental conditions, with a high quality of junctional complexes (Fig. 5). Out of any stress, a light increase of the TEER values seems appear. This mechanism leading to such observation, except for tissue remodeling, are not always well-understood [33].

The tight and adherent junctions of the airway barrier are dynamic structures responding to various stimuli such as environmental or household exposures. In our study, enrichment of the VOC referent mixture with FA or HEX did not lead to an upward or downward modulation of TEER values, regardless of the experimental conditions (dose and exposure time) (Fig. 5). However, borderline TEER values (close to 150 $\Omega\cdot\text{cm}^2$) were observed for the series of epithelia exposed to FA in week 1. The same epithelia presented TEER values above 150 $\Omega\cdot\text{cm}^2$ in week 2 indicating a good tissue homeostasis. This phenomenon often is associated with the epithelial reconstruction process. Overall, no impact of the FA- or HEX-enriched VOC reference mixture on epithelial tissue integrity was observed over the 3 weeks of exposure (data not shown for week 3 - data similar to results obtained in week 2).

3.5.2. Inflammatory response

Inflammatory response was evaluated by the assessment of cytokine/chemokine secretion in the culture medium (basal side of the

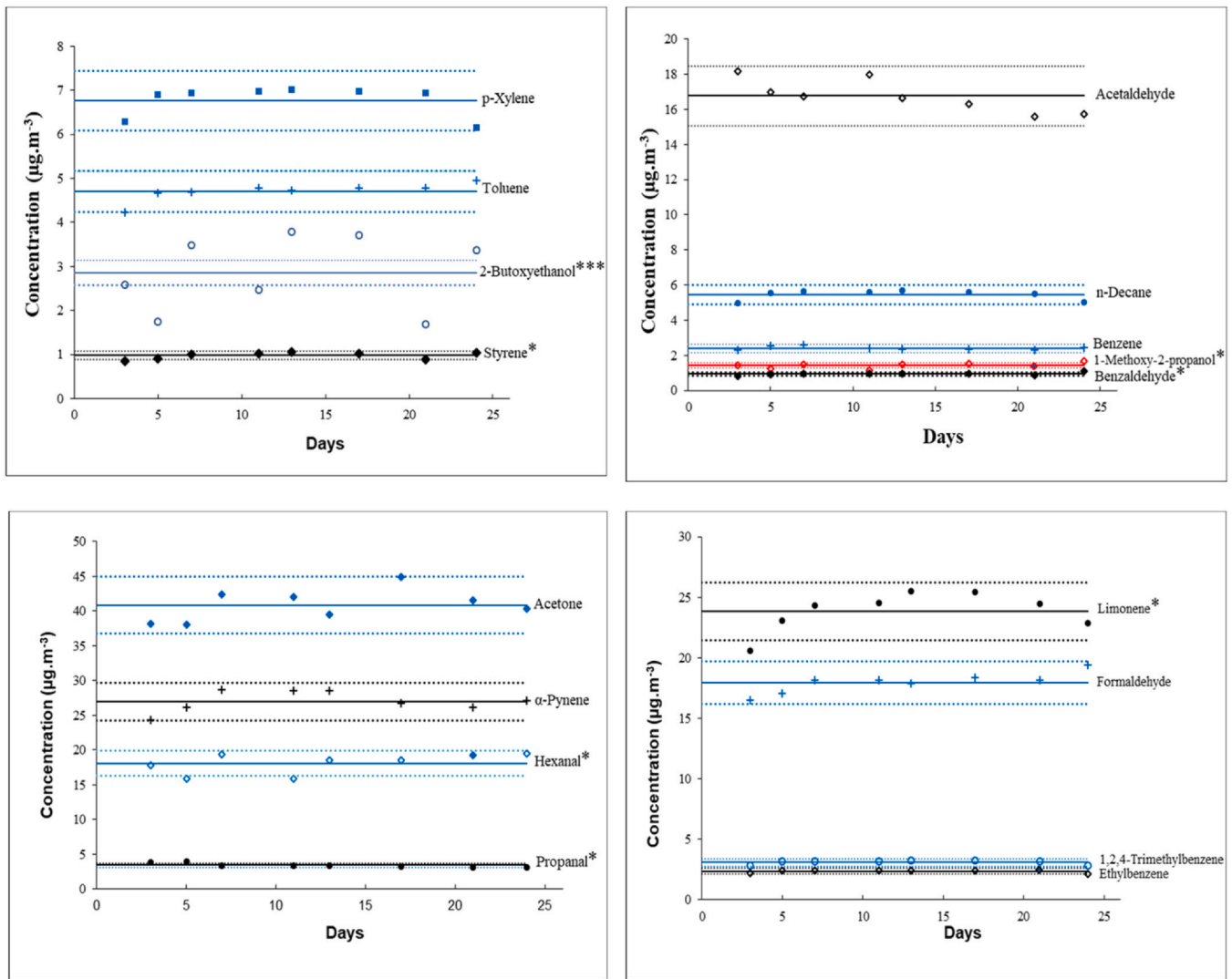


Fig. 4. Changes in the generated VOC concentrations over time for a 24-day follow-up. The solid lines indicate the mean concentrations of compounds and the dotted lines are the intervals of $\pm 10\%$ around the mean values. *: the values are in an interval of $\pm 20\%$, **: the values are in an interval of $\pm 40\%$ and ***: the values exceeded the interval of $\pm 40\%$.

Table 3

Results obtained after increasing the concentration of hexanal (HEX) or formaldehyde (FA). SD Standard deviation; HEX: hexanal; FA: formaldehyde; T mov = 22.5°C (T min = 22.2°C and T max = 22.9°C); HR mov = 41.3% (HR min = 40.7% and HR max = 41.9%).

	Mean Concentration \pm SD in the referent mixture ($\mu\text{g}\cdot\text{m}^{-3}$)	Concentration ($\mu\text{g}\cdot\text{m}^{-3}$) measured in the mixture with the air flow of <u>HEX</u> cylinder increased by a factor of 10	Concentration ($\mu\text{g}\cdot\text{m}^{-3}$) measured in the mixture with the air flow of <u>FA</u> cylinder increased by a factor of 10
Benzene	2.4 \pm 0.11	2.0	2.2
1-Methoxy – 2-propanol	1.4 \pm 0.16	1.3	1.6
Toluene	4.7 \pm 0.2	4.0	4.3
Hexanal	18.1 \pm 1.5	191	18.5
Ethylbenzene	2.3 \pm 0.12	2.1	2.2
p-Xylene	6.8 \pm 0.3	6.0	6.4
Styrene	0.98 \pm 0.08	0.9	0.9
2-Butoxy-ethanol	2.9 \pm 0.9	2.0	1.4
α -Pinene	26.9 \pm 1.5	25	26
Benzaldehyde	0.94 \pm 0.08	0.85	0.9
1,2,4-Trimethylbenzene	3.1 \pm 0.16	2.7	2.9
n-Decane	5.4 \pm 0.28	4.9	5.1
Limonene	23.8 \pm 1.6	22	23
Formaldehyde	17.9 \pm 0.9	17.7	186
Acetaldehyde	16.8 \pm 0.9	13.9	17.0
Propanal	3.4 \pm 0.3	2.8	3.4
Acetone	40.2 \pm 2.3	34	38

Table 4

VOC concentrations in the referent mixture measured in upstream and downstream of the Vitrocell® exposure device. SD Standard deviation; T mov = 22.5°C (T min = 22.2°C and T max = 22.9°C); HR mov = 41.3 % (HR min = 40.7 % and HR max = 41.9 %).

	Mean Concentration ± SD in the referent mixture ($\mu\text{g}\cdot\text{m}^{-3}$)	Concentration ($\mu\text{g}\cdot\text{m}^{-3}$) <u>upstream</u> of Vitrocell®	Concentration ($\mu\text{g}\cdot\text{m}^{-3}$) <u>downstream</u> of Vitrocell®
Benzene	2.4 ± 0.11	2.0	2.0
1-Methoxy-2-propanol	1.4 ± 0.16	0.8	0.6
Toluene	4.7 ± 0.2	3.7	4.0
Hexanal	18.1 ± 1.5	19.3	19.8
Ethylbenzene	2.3 ± 0.12	1.9	1.9
p-Xylene	6.8 ± 0.3	5.5	5.6
Styrene	0.98 ± 0.08	0.8	0.8
2-Butoxy-ethanol	2.9 ± 0.9	0.1	0.2
α -Pinene	27 ± 1.5	26	26
Benzaldehyde	0.94 ± 0.08	0.8	0.8
1,2,4-Trimethylbenzene	3.1 ± 0.16	2.5	2.5
n-Decane	5.4 ± 0.28	4.4	4.6
Limonene	24 ± 1.6	19.9	20
Formaldehyde	17.9 ± 0.9	24	23
Acetaldehyde	16.8 ± 0.9	14.9	14.6
Propanal	3.4 ± 0.3	3.1	2.6
Acetone	41 ± 2.3	36	34

epithelium) as well as in the mucus (apical side of the epithelium) after repeated exposures to various VOC mixtures. Of the seven selected cytokines/chemokines (IL-6, IL-8, IL-1 α , IL-1 β , IL-13, MIF and GM-CSF), the secreted levels obtained for IL-1 α , IL-1 β , and IL-13 were very low with a very large variability (data not shown). The results obtained with the other biomarkers of inflammation (IL-6, IL-8, MIF and GM-CSF) presented in Fig. 6, indicated a significant increase in the inflammatory response. This response was dependent on the levels of FA or HEX enriching the reference VOC mixture and was function of the number of exposures.

Concerning IL-6, a pro-inflammatory cytokine that stimulates protein secretion during the innate immune system response to a stressor like pollutants, a dose-dependent increase is observed in the culture media after repeated exposures to the referent VOC mixture enriched with FA or HEX (Fig. 6A). A time effect can also be noted, with higher production in week 2 after three (E3W2) and four (E4W2) exposures. IL-6 secretion is significantly increased in the mucus on the apical side after FA-exposure but not in a dose-dependent manner, whereas after HEX-exposure, IL-6 concentration augmented strongly in a dose-dependent manner at the end of week 2.

Interleukin 8 (IL-8) is a cytokine produced primarily by epithelial cells following the action of some chemical agents and ensure the recruitment of neutrophils. After one or two FA-exposures during the first week (E1W1 and E2W1, respectively), and after one FA-exposure during the second week (E3W2), no statistical difference in IL-8 secretion was observed in the culture media whatever the FA concentration (Fig. 6B2). A discrete but significant dose-dependent effect appears at the end of the week 2 after four FA-exposures. A significant

dose-dependent increase in IL-8 secretion progressively takes place as a function of time and number of exposures to the referent mixture enriched in HEX (Fig. 6B3). Conversely, IL-8 levels measured in mucus are higher after FA- or HEX-exposures when compared to those obtained before exposure; a significant dose- and time-dependent relationship is to be underlined for both pollutants tested.

The impact of HEX enrichment of VOC mixture on IL-6 production from airway epithelium EpiB, is greater (up to 2–4 fold) than with FA enrichment (up to 1.5–3 fold). Furthermore, regardless of the nature of the enrichment of the VOC mixture, the IL-8 release profiles after repeated exposures are similar.

The two other cytokines assessed are GM-CSF (Granulocyte-macrophage colony-stimulating factor), a granulocyte growth factor essential for pulmonary homeostasis, mediator of tissue inflammation mainly to recruit macrophages and MIF (Macrophage migration inhibitory factor) playing a central role as a regulator of innate immunity and inflammatory responses. Following repeated exposures to the referent VOC mixture enriched in FA or HEX (Figs. 6C and 6D), a strong dose and time-dependent release of GM-CSF and MIF was noted. For these two biomarkers, a dramatically exacerbation of the inflammatory response is observed after the third exposure applied in week 2 (E3W2) with a greater response for GM-CSF after enrichment of the mixture with HEX (to 6.4-fold), and for MIF after enrichment of the mixture with FA (to 16-fold). If the apical production, evaluated in the mucus, of these two cytokine/chemokine is not negligible, it becomes significant after 2 weeks of exposure to the referent mixture enriched in each of the two VOCs; the effect is much more marked after exposure to HEX whatever the concentration applied to the epithelia.

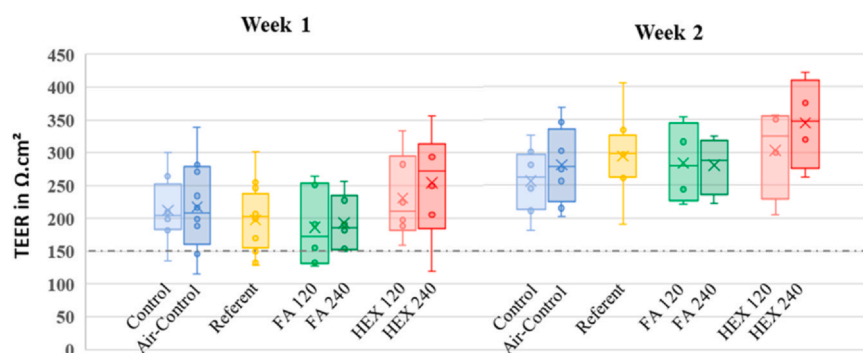


Fig. 5. TEER values taken after one (week 1) and two weeks (week 2) of no exposure (Control, light blue bars) or exposure to air (Air Control, dark blue bars), Referent (VOC referent mixture, yellow bars) enriched with formaldehyde (FA, green bars) or hexanal (HEX, orange bars). (n = 4–8).

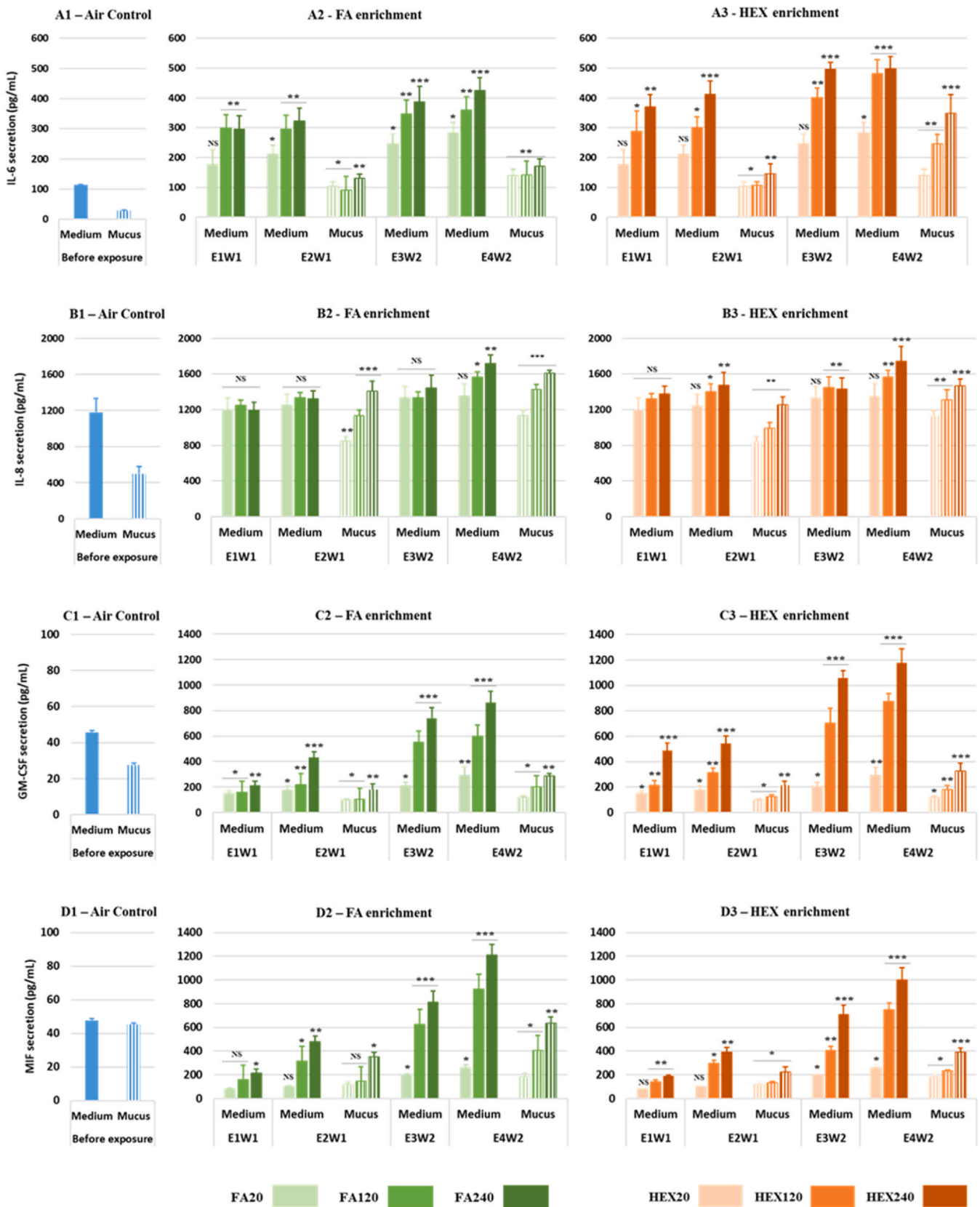


Fig. 6. Cytokines (A: IL-6; B: IL-8; C: GM-CSF; D: MIF) secreted in culture medium (solid bars) and in mucus (hatched bars) before exposure (blue bars), after one or two exposures during the first week (E1W1 and E2W1) and three or four exposures during the second week (E3W2 and E4W2) to VOC referent mixture enriched with Formaldehyde (FA green bars) or Hexanal (HEX orange bars). Culture media samples: n = 8; Mucus samples n = 6. Statistically different with * p < 0.5; ** p < 0.1; *** p < 0.01).

The results obtained in the biological part of this work indicated that repeated exposures to the referent mixture of VOCs enriched with FA or HEX resulted in a dose- and time-dependent inflammatory response. Overproduction of inflammatory mediators in the basal side (culture medium), where macrophages and dendritic cells are located in the organ *in situ* and in the apical part (mucus) towards the lumen of the bronchial ducts promoting mucus production and tissue remodeling correlates with the severity and progression of multiple respiratory diseases [34]. In addition, our data indicated that the levels of cytokine/chemokine secretion differed depending on the pollutant, FA or HEX, suggesting different underlying signaling pathways and therefore different human health consequences [35,36].

As previously mentioned, GM-CSF and MIF are two chemokine/cytokine that were secreted in a very significant way after repeated exposures to the referent VOC mixture enriched with HEX and FA respectively in our experimental conditions. GM-CSF plays a pivotal role in the regulation of pulmonary homeostasis and alveolar macrophage/epithelial cells tandem working into their immune function [37]. This chemokine is responsible for bronchial hyperreactivity, leukotriene release, endothelial cell migration, production of multiple cytokines including IL-6, and eosinophil activation [38]. Once activated, eosinophils could contribute to the development of inflammatory disease such as asthma, in turn, releasing a number of mediators such as GM-CSF and IL-8 [39]. Our results show the early secretion of GM-CSF and its increase over time with the repeated exposures to the different VOC mixtures generated, but they also indicated the later production of the cytokines IL-6 and IL-8. Consistent with our findings, previous *in vitro* studies have reported that FA exposure increases production of various chemokines/cytokines including GM-CSF and IL-8 [7,21,40]. The MIF cytokine is much less described than GM-CSF or IL-6 and IL-8. It is thought to be involved in the induction of cell proliferation and survival by inhibiting apoptosis and in the induction of cell migration.

A mechanistic interpretation of the increase in cytokine expression following exposure to formaldehyde of human cell lines of bronchial (BEAS-2B) [41] or alveolar (A549) [42] origin was reported as could be under the control of the p38 mitogen-activated protein kinase (MAPK) pathway. The interaction of FA with membrane receptors of epithelial cells triggers intracellular signaling cascade, such as MAPK leading to the synthesis and release of pro-inflammatory cytokines, including TNF- α , IL-1 β , IL-8 and IL-6, with the aim of recruiting immune cells [13]. Additionally, FA causes the production of reactive oxygen species (ROS) in respiratory tissues via mitochondrial failure, activation of NADPH oxidase, and depletion of antioxidant defenses. Such oxidative damage, mediated by ROS, disrupts cellular homeostasis and intensifies inflammation [13].

While a clear dose-dependent increase in MIF after exposure to FA- and HEX-enriched referent VOCs mixtures was observed in our study, no effect on epithelial proliferation or remodeling (*via* TEER measurement) was demonstrated. Perhaps if we had considered the effects over a longer period after exposures such a phenomenon could have been envisaged.

Very few studies have examined the impact of HEX on human respiratory health and even fewer considering an *in vitro* approach. However, hexanal is recognized as an indoor air irritant in the same way as other aldehydes such as FA. A population-based study reported that after two hours of exposure to hexanal at 10 ppm, irritation of the eyes and nose as well as headaches appeared rapidly [43]. *In vitro* studies investigating inflammatory responses following exposure of human respiratory cells to hexanal concluded that there was a significant increase in the secretion of inflammatory mediators such as IL-6 and IL-8 supporting our results [15,44]. Thus like formaldehyde, hexanal might also be involved in bronchial hyperactivity, eosinophil activation and apoptosis, contributing to the development of adverse airway events.

4. Conclusions

Exposure to volatile organic compounds in indoor air is associated with effects ranging from simple discomfort (irritation of the eyes, nose, throat, headaches ...) to more significant adverse effects (worsening of asthma symptoms ...). Studies on the impact of these compounds on respiratory health evaluated from experimental *in vitro* studies are numerous for formaldehyde but too few for hexanal. In addition, very few studies evaluate the impact of these two aldehydes in realistic mixtures at concentrations close to those encountered in indoor air. Most studies test these compounds one by one, in liquid or gaseous form and at concentrations in the ppm range. Finally, data concerning cellular and molecular mechanisms are lacking for these two aldehydes, whereas it is recognized by the scientific community that repeated exposures to gaseous pollutants, at low doses, trigger different signaling pathways contributing to the development or exacerbation of respiratory pathologies such as asthma.

In this current study, we have developed an innovative and original tool leading the evaluation of the cellular and molecular impact of repeated exposures to a VOCs mixture enriched in FA or in HEX. This tool combined a bench for the generation of VOC mixtures, at low concentrations (a few $\mu\text{g}\cdot\text{m}^{-3}$) close to those encountered in indoor air, and an exposure system at the air-liquid interface, adapted to cellular/tissue models of the airways.

The strength of our tool is to have associated three essential elements: the generation of a realistic mixture in quality but also in quantity, the use of an *in vitro* model also realistic because physiologically close to the *in situ* epithelia and, an exposure mode at the air-liquid interface allowing to put in direct contact the mixture of pollutants to be tested with the target cells at low concentrations. However, our tool has certain limitations, mainly linked to the difficulty of metrological monitoring. Indeed, it is necessary to perform many controls before, during and after exposure of the cells/tissues in order to assess the levels of pollutants generated, as well as their stability over time. Our system remains an *in vitro* approach. As such, both duration and frequency of exposures are limited in time, so as not to observe cell death. While real-life conditions of chronic human exposure cannot be experimentally reproduced, our system enables us to assess the impact of realistic environmental mixtures in terms of both quality and quantity, by performing repeated air-liquid interface exposures of human airway epithelia with a morphology close to *in situ* epithelium physiology.

Our approach is consistent with the process developed by the OECD demonstrating how an *in vitro* 3D human respiratory model, such as MucilAir™, can be used to replace the *in vivo* repeated-dose inhalation toxicity study [45]. Consideration could be given to proposing our tool for validation by the ECVAM (European Centre for the Validation of Alternative Methods), a tool which enables the toxic risk of a realistic gas mixture to be assessed, thus making a real breakthrough in reducing the use of laboratory animals to test the harmfulness of chemical compounds.

Our tool could be used for other applications and areas of life (occupational exposure, transport, etc.), for other physiological barriers (skin, ocular surface, etc.) or for more in-depth mechanistic studies (genotoxicity, epigenetics, etc.) to improve our understanding of the cellular and molecular effects of a large number of environmental mixtures (VOCs or other emerging pollutants such as SVOCs). Finally, in terms of standardization, regulation and ethics, there is a great need to develop alternative methods to animal experimentation, methods that meet the 3Rs principle, i.e. that reduce, replace or refine the use of animals in research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.indenv.2024.100037](https://doi.org/10.1016/j.indenv.2024.100037).

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