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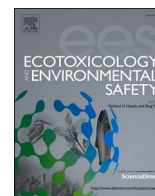
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Inhalation bioaccessibility of polycyclic aromatic hydrocarbons from reference combustion-derived particles: Methodological aspects and toxicological impact on bronchial epithelial cells

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ABSTRACT

Combustion-derived particles are a significant concern due to their nanometric sizes and complex chemical compositions, which can negatively impact health. These particles contain polycyclic aromatic hydrocarbons (PAH) and their oxygenated derivatives (OPAH), which can dissolve in biological lung fluids. As a result, they form complex mixtures whose toxicity is often poorly studied. Therefore, evaluating inhalation bioaccessibility is crucial for accurately assessing the health risks associated with these particles. We determined the inhalation bioaccessibility fractions (IBAF) of 16 PAH and 5 OPAH from particles generated under controlled conditions. Several critical parameters were evaluated, including simulated lung fluids (SLF), extraction times, and solid-to-liquid (S/L) ratios. Our results indicated that eight compounds - NAP, ACY, FLU, 9-FLUO, PHE, ANT, FLT, PYR - were systematically extracted by the different SLF, namely Gamble's solution (GS), artificial lysosomal fluid (ALF), modified GS (MGS), and simulated epithelial lung fluid (SELF) after 24 h of extraction time with an S/L ratio of 1/2000. The evaluation of the toxicological response, based on cytotoxicity and the AhR-dependent pathway, demonstrated variability in the toxicological profiles, depending on the extraction conditions used. Notably, the extraction condition yielding a biological response equivalent to that assessed with the total particulate fraction was the use of MGS as the extraction fluid under our experimental conditions. From a toxicological perspective, these approaches appear relevant for assessing the toxicity mechanisms of PAH mixtures, closely mimicking the in vivo conditions found in the human respiratory tract. This study outlined that understanding whether the bioaccessible fraction of particulate-bound PAHs is a reliable predictor of toxicity is central to advancing inhalation risk assessment.

1. Introduction

Airborne particulate matter (PM) is at the forefront of air quality deterioration and its associated health effects. There are many sources of emissions, but road transport and residential and tertiary heating are major contributors of PM linked to the combustion of fossil fuels and biomass. Combustion-derived PM is nanometric in size and can penetrate deeply into the respiratory tract. These ultrafine particles (UFP) represent complex matrices composed of soot carbon (black carbon) and organic and inorganic compounds, capable of interacting with cellular constituents and inducing health effects. However, the composition can

vary considerably depending on the emission sources, leading to contrasting impacts, as shown in various studies carried out on PM sampled near industrial sites (Thomson et al., 2016), in urban areas (Boogaard et al., 2012), or from biomass or diesel engine combustion (Longhin et al., 2016). This variation in effects may be linked to the variable chemical composition, which is, however, not taken into account in air quality measurements. Polycyclic aromatic hydrocarbons (PAH) or their oxygenated derivatives (OPAH) represent a complex group of chemical molecules present in combustion-derived PM. Numerous PAH are considered key components of soot-induced health effects, and some of them are classified either as carcinogenic or probably carcinogenic for

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humans (IARC et al., 2010). PAH can also cause non-cancerous diseases such as asthma exacerbation or chronic obstructive pulmonary disease (COPD) (Låg et al., 2020). Certain populations are particularly sensitive to the effects of inhaling PAH and OPAH. Children, the elderly, pregnant women, and individuals with pre-existing respiratory or cardiovascular conditions consistently emerge as populations at heightened risk from inhaled PAHs and OPAHs (Låg et al., 2020; Norlén et al., 2019; Jin et al., 2025; Xiao et al., 2023). OPAHs may contribute disproportionately to risk in these groups, but direct evidence remains limited due to measurement and analytical constraints (Hao et al., 2023; Alves et al., 2017).

In the context of PAH-induced adverse effects, the AhR pathway has been identified as playing a significant role. The binding of PAH to the Aryl hydrocarbon receptor (AhR) triggers the transduction of a signal, enabling the induction of phase I xenobiotic metabolism enzymes as CYP1A1 and CYP1B1. While these enzymes are considered to be important in xenobiotic detoxification, they are also involved in the formation of reactive metabolites, such as B[a]P-7,8-dihydrodiol-,10-epoxide (BPDE), the ultimate carcinogenic metabolite of benzo[a]pyrene (B[a]P), capable to bind to the DNA and form adducts. Thus, the biotransformation of PAH can generate metabolites that are more reactive and dangerous than the parent PAH. Induction of this pathway is commonly used as a hallmark of exposure to inhaled PAH, particularly in studies of the PM toxicity in bronchial epithelial cells (Ferecatu et al., 2010; Badran et al., 2020; Holme et al., 2023; Yu et al., 2025).

After inhalation and deposition in the respiratory tree, some of the PM-bound pollutants may be released into extracellular fluids or internalised and induce localised effects. The fraction of compounds that is readily accessible to cells is defined as 'bioaccessible' and is useful in the risk assessment of PM-bound pollutants. Indeed, inhalation risk assessment approaches have shifted from considering total contaminant levels to assessing the maximum fraction that could be leached into extracellular pulmonary fluids (i.e., the bioaccessible fraction). Consequently, bioaccessibility studies, in conjunction with assessments of the cellular toxicity of the resulting inhaled fractions, are becoming relevant to obtain a more accurate assessment of exposure to PM-bound pollutants. This is a crucial consideration when assessing the risks to human health associated with air pollution. However, various methods have been developed to measure inhalation bioaccessibility, making it difficult to compare results among studies. These methods utilize simulated lung fluids (SLF) to simulate the conditions in the human respiratory system. The Gamble's solution (GS) and the artificial lysosomal fluid (ALF) are the most used. The GS mimics the *in vivo* condition of a healthy, non-smoking human and simulates the ionic composition of the lung lining fluid (pH 7.4). In contrast, ALF, with a pH of 4.5, represents the acidic lysosomal environment that occurs after the phagocytosis of PM (Besis et al., 2022a; Tang et al., 2023). The GS has also been adapted and modified (MGS) to mimic the real composition of the pulmonary environment by adding biogenic surfactant dipalmitoylphosphatidylcholine (DPPC) (Xie et al., 2018). DPPC plays a role in preventing particle aggregation and will increase the wettability of hydrophobic particles (Ren et al., 2020; Shi et al., 2022). The simulated epithelial lung fluid (SELF) is a model of the lung's extracellular environment. It contains salts in similar concentrations as other fluids along with the most abundant compounds present in the respiratory tract fluids of healthy human subjects, such as organic reagents (ascorbic acid, uric acid and glutathione), lipids (DPPC), proteins (mucins and albumin) and organic components (glycine and cysteine).

In addition to the composition of the SLF, the literature describes numerous other methodological parameters that may influence bioaccessibility (Badran et al., 2020; Holme et al., 2023; Besis et al., 2022a; Tang et al., 2023). Indeed, parameters such as the SLF volume per mass of PM (i.e., the solid-to-liquid ratio, S/L), incubation time, temperature, agitation, and others can affect the percentage of molecules dissolved in SLF. This is why there is significant variability in literature values for the inhalation bioaccessibility of PM-bound PAH (Badran et al., 2020; Tang et al., 2023). Besides the experimental parameters mentioned above,

variability factors may also include differences in sample collection methods and the physico-chemical properties of PM. These factors can be controlled to examine the influence of other assay parameters by using a source that provides relevant emissions of combustion-derived PM, which can be easily produced in a reproducible manner. The mini-Combustion Aerosol Standard (mini-CAST) is a reference soot generator in which soot particles are produced from a propane diffusion flame quenched by N₂ (Xie et al., 2018). By altering the operating conditions of the mini-CAST, combustion-derived PM may exhibit different microphysical and chemical properties, while maintaining a stable and repeatable profile (Juarez et al., 2022; Juarez-Facio et al., 2022; Juárez-Facio et al., 2023; Das et al., 2024; Jing, 1999). Originally used as a calibration device for combustion-derived PM and to understand their formation, it has recently been employed as a pertinent tool for toxicological studies of UFP, taking into account their organic composition and PAH content (Tang et al., 2023; Xie et al., 2018; Ren et al., 2020).

Consequently, the main objective of this research is to assess and compare the influence of some parameters on measuring the inhalation bioaccessibility of PAH from combustion-derived particles. The parameters investigated were the composition of the SLF, the influence of the S/L, and the particle contact time. To assess the impact of these parameters on bioaccessibility, this study was conducted using UFP generated in a standardized manner. For this purpose, the mini-CAST generator was used with a selected operational condition to produce combustion-derived UFP with a high content of PAH, as previously characterized (Juarez et al., 2022; Juarez-Facio et al., 2022). To explore the toxicological responses associated with these different methodological approaches used for determining bioaccessibility, human bronchial epithelial lung cells (BEAS-2B) were exposed to selected bioaccessible fractions and UFP in their entirety. Toxicological results are discussed in conjunction with the chemical analysis of PAH and OPAH to assess their contribution to the observed effects on cytotoxicity and the AhR pathway.

2. Materials and methods

2.1. Chemicals and reagents

Sixteen US EPA priority polycyclic aromatic hydrocarbons (PAHs), including naphthalene (NAP), acenaphthylene (ACY), acenaphthene (ACE), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), chrysene (CHR), benz(a)anthracene (BaA), benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), indeno(1,2,3-cd)pyrene (IcdP), diben(a,h)anthracene (DahA), benzo(g,h,i)perylene (BghiP), five oxy-derivatives (OPAHs), 1,4-naphthoquinone (1,4-NQ), 9-fluorenone (9-FLUO), 9,10-anthraquinone (9,10-AQ), benzo(a)fluorenone (BaFone), 6H-benzo(c,d)pyren-6-one (6HBPone), and three internal standards (ISTD)—naphthalene-d8 (NAP-d8), phenanthrene-d10 (PHE-d10), and perylene-d12 (PER-d12)—as well as 1,2-dipalmitoyl-rac-glycero-3-phosphocholine (DPPC), magnesium chloride, sodium chloride, potassium chloride, disodium hydrogen phosphate, sodium sulfate, calcium chloride, sodium acetate, sodium hydrogen carbonate, sodium citrate dihydrate, sodium hydroxide, citric acid, glycine, sodium tartrate dihydrate, sodium lactate, sodium pyruvate, ascorbic acid, uric acid, glutathione, albumin, L-cysteine, mucin, hydrochloric acid, and MTT were acquired from Sigma-Aldrich (Saint-Quentin Fallavier, France). The full names and physico-chemical properties of PAH and OPAH are presented in Table S1 of the Supplementary Information (SI). HPLC-grade solvents and DMSO were purchased from VWR (Fontenay-sous-Bois, France). Ultra-pure water was supplied by the Barnstead MicroPure system (Thermo Scientific, Les Ulis, France). The human epithelial bronchial cell line BEAS-2B was purchased from the American Type Culture Collection (ATCC CRL-3588). LHC-9 culture medium, plastic flasks, 96-well plates, 12-well plates, and collagen were acquired from Thermo Fisher Scientific

(Thermo Scientific, Les Ulis, France). The AffinityScript cDNA Synthesis Kit and the Brilliant III Ultra-fast SYBR Green QPCR Master Mix were purchased from Agilent Technologies. The NucleoSpin® TriPrep was acquired from Macherey-Nagel.

2.2. PM sampling

Ultra-fine particles (UFP) were produced in a controlled and reproducible manner in the laboratory using a soot generator (miniCAST; 5201c, Jing LTD, Switzerland). This device allows the production of particles with variable size distribution, organic compound content, and morphology, achieved by modifying the propane and air flow (Juárez-Facio et al., 2023). Flow rates of propane, nitrogen, oxidation air, and dilution air were set at 60 mL.min⁻¹, 7 mL.min⁻¹, 1 mL.min⁻¹, and 20 mL.min⁻¹, respectively. For all experiments, the operating conditions remained constant. The experimental conditions were chosen to obtain UFP with a high organic carbon content and a modal diameter of 58.9 ± 1.53 nm, as previously described in detail (Ren et al., 2020; Shi et al., 2022; Juárez et al., 2022; Juárez-Facio et al., 2023). The UFP surface area (142.1 m²/g) was measured in a previous study using an innovative non-linear optical method and compared with more traditional methods (Joret et al., 2025). UFP were collected on cellulose filters, which were scratched after collection to obtain free particles and then stored at -20°C until their use.

2.3. Total and bioaccessible concentration of PAH in UFP

PAH and OPAH in bioaccessible fractions and UFP extracts were analyzed using gas chromatography-mass spectrometry with an Agilent 7890 gas chromatograph coupled to an Agilent 5975 C mass spectrometer operating in electron impact ionization and selected monitoring mode. Chromatographic separation was achieved with an HP-5-MS capillary column (Agilent, 30 m x 0.25 mm i.d., 0.25 µm film thickness). The injector and transfer lines were maintained at a temperature of 280 °C. High-purity helium with a flow rate of 1 mL.min⁻¹, served as the carrier gas. The initial temperature of 140 °C was held for 1 min, then increased to 250 °C at a rate of 15 °C.min⁻¹, followed by a further increase to 280 °C at a rate of 5 °C.min⁻¹, held for 8 min (Table S2). One microliter was injected in split mode (1/20).

For the quantification of PAH and OPAH in UFPs, briefly, 5 mg of UFP was extracted with 1 mL of an acetone/dichloromethane mixture (V/V) and sonicated for 20 min at 40 kHz. Extracts were filtered through 0.45 µm Nylon filters. Afterwards, extracts were spiked with ISTD mix prepared in hexane (NAP-d8, PHE-d10, PER-d12) and stored at -20°C. The acquisition was performed in selected ion monitoring (SIM) mode; the acquisition parameters are detailed in Table S3.

For the quantification of PAH and OPAH in the bioaccessible fraction, after incubation, the samples were filtered using 0.45 µm Nylon filters, and the fluids were subjected to a liquid-liquid extraction (LLE) twice, assisted with ultrasonication using hexane. The pooled extracts were concentrated, spiked with ISTD, and analyzed by GC-MS.

2.4. Inhalation bioaccessibility

Four types of simulated lung fluids (SLFs), four solid-to-liquid ratios (S/L), and two extraction times were studied.

2.4.1. Simulated lung fluids

This research aims to evaluate and compare the influence of certain parameters on the measurement of the inhalation bioaccessibility of PAH from UFP. For this purpose, the four most commonly described simulated fluids were selected (Thomson et al., 2016) The Gamble's solution (GS, pH 7.4), which simulates extracellular lung fluid with a neutral pH; (Boogaard et al., 2012) the modified Gamble's solution (MGS), supplemented with pulmonary surfactants by adding 1,2-dipalmitoyl-rac-glycero-3-phosphocholine (DPPC) at 100 mg.L⁻¹ (Badran

et al., 2020); (Longhin et al., 2016) the artificial lysosomal fluid (ALF, pH 4.5), which simulates the phagocytosis of alveolar and interstitial macrophages at acidic pH; and (IARC et al., 2010) the simulated epithelial lung fluid (SELF), which contains electrolytes, antioxidants, proteins, and surfactant. All SLF were freshly prepared by dissolving the constituents detailed in Table S4 in 1 L of ultrapure H₂O. The pH was adjusted using 1 M HCl and 1 M NaOH.

2.4.2. Solid to liquid ratios

There is notable heterogeneity in the literature regarding the S/L ratios, and few studies have examined their influence on the bioaccessibility of organic compounds. The solid (g) to liquid (mL) ratios (S/L) tested in this study were 1/2000, 1/20,000, 1/100,000, and 1/200,000. A volume of 20 mL of SLF, representing the total volume of epithelial fluid, was used in the experiments (Das et al., 2024; Jing, 1999). Briefly, the mass of UFP was introduced into amber glass bottles and incubated with 20 mL of SLF at 37 °C for 24 h or 96 h (in the dark) under agitation (100 rounds per minute). After incubation, the supernatant (15 mL) was extracted using the protocol described in section 1.3.

2.4.3. Data analysis

The bioaccessibility of PAH and OPAH in UFP was calculated as follows:

$$\text{Bioaccessibility}\% = \frac{\text{Concentration in SLF}}{\text{Concentration in UFP}} \times 100$$

2.5. Toxicological analyses

2.5.1. Cell culture and exposure conditions

The human epithelial bronchial cell line BEAS-2B is grown in LHC-9 culture medium at 37 °C in an atmosphere of 95 % humidity and 5 % CO₂. The medium was changed every 2 days, and the cells were maintained in a 75 cm² plastic flask until confluence (80–85 %). For the cytotoxicity test and gene expression experiments, cells were seeded in culture plates that had been previously coated with type 1 collagen and acetic acid to enhance adhesion to the surface. For cell exposure, UFP suspensions were prepared in ultrapure water at a concentration of 1 mg.mL⁻¹ and then diluted in cell medium to the exposure concentrations. Reconstituted inhalation bioaccessible fractions (rIBAF) were prepared in cell culture medium from the concentrations obtained in the bioaccessibility test. To reconstitute the IBAF, PAH, and OPAH in 100 % DMSO were used and added to the cell culture medium for a final concentration of DMSO around 0.1 %.

2.5.2. Cytotoxicity

After exposing the cells to increasing concentrations of UFP, ranging from 1.25 to 100 µg.mL⁻¹, and to rIBAF, the cytotoxicity was assessed using the MTT assay. This assay relies on the cleavage of the tetrazolium ring of MTT by dehydrogenases in the active mitochondria of living cells to estimate the number of viable cells. Briefly, cells were seeded in collagen-coated 96-well plates at a density of approximately 20,000 cells per well and then exposed 48 h later to UFP or rIBAF for 3 and 24 h. The cells were then incubated with an MTT solution at 500 µg/mL in cell culture medium for 3 h, after which the formazan crystals were dissolved with DMSO. The absorbance was measured at 570 nm (ClarioStar, BMG Labtech) and normalized to the control for the exposed cells.

2.5.3. qRT-PCR

mRNA expression levels of AHRR, CYP1A1, and CYP1B1 were measured after 3 and 24 h of exposure. Forty-eight hours after seeding the cells in collagen-coated 12-well plates at a density of approximately 250,000 cells per well, the cells were exposed to two concentrations of UFP (20 and 50 µg/mL) and three rIBAF (GS, MGS, and SELF) for 3 and 24 h. Total RNA was then extracted from the cells using the NucleoSpin

TriPrep kit. RNA integrity was verified using the Agilent RNA 6000 Nano Kit, ensuring an RNA Integrity Number (RIN) of 8 or higher for the tested conditions. RNA concentration was then measured with a microplate reader (ClarioStar, BMG Labtech). Reverse transcription of 0.4 μg of total RNA to cDNA was performed with the AffinityScript cDNA Synthesis Kit. mRNA expression levels were determined by real-time quantitative polymerase chain reaction (RT-qPCR) with the Brilliant III Ultra-fast SYBR Green QPCR Master Mix using an AriaMx Real-time PCR System (Agilent Technologies). Relative mRNA expression levels were determined by the $2^{-\Delta\Delta\text{CT}}$ method, normalized against the expression level of the housekeeping gene GAPDH (Joret et al., 2025). Primer sequences were determined using BLAST (National Institutes of Health, NIH) and synthesized by Eurogentec (Table S5).

2.6. Quality assurance and control

Chemical analyses were performed in quadruple, and cell tests were conducted 6 times. The spiked recoveries for PAH and OPAH using the total concentration extraction method and the bioaccessible extraction method were 84–109 % and 88–145 %, respectively. The detection limits (LODs) and quantification limits (LOQs) were determined by extrapolating the concentration that would yield a signal-to-noise ratio of 3 ($S/N = 3$) and 10 ($S/N = 10$), respectively, using the lowest concentration from the calibration curve. The LOQ values for PAH and OPAH were 0.001–0.220 $\mu\text{g}\cdot\text{mL}^{-1}$ (Table S6).

Filter blanks, incubated in SLF, did not show detectable PAH and OPAH.

2.7. Statistical treatment of data

Graphical representations and statistical analyses of the data were conducted using GraphPad Prism (version 10.3.1, San Diego, CA, USA). Results are presented as mean \pm standard deviation (SD). Statistical analysis utilized the non-parametric Mann-Whitney U test. Data with a p -value less than 0.05 were considered statistically significant ($* = p < 0.05$).

3. Results and discussion

3.1. Individual PAH and OPAH total content

In the present study, standard UFP were generated with a high content of organic matter (OC/TC: $87 \pm 5\%$), characterized by a wide range of PAH, as previously described (Juarez et al., 2022; Juárez-Facio et al., 2022; Juárez-Facio et al., 2023; Bescond et al., 2016). Moreover, this experimental approach allowed for the control of the mass of particles employed. Under these conditions, the total concentration of PAH was first assessed to subsequently determine the bioaccessible fractions. Total PAH and OPAH concentrations are summarized in Fig. 1 and Table S7. All 21 analytes, 16 PAH, and 5 OPAH were quantified in UFP. The total concentration of 16 PAH and 5 OPAH adsorbed in UFP ranged from 16.72 to 7424.14 $\text{ng}\cdot\text{mg}^{-1}$ particle and 18.56–1166.09 $\text{ng}\cdot\text{mg}^{-1}$ particle, respectively. Six PAH—ACY, FLU, PYR, BaP, IcdP, and BghiP—accounted for 70 % of the total mass of PM-bound PAH and OPAH. These results are in line with previous studies.

3.2. Influence of SLF and S/L ratios on inhalation bioaccessibility assessment

Then, the influence of several parameters on the determination of inhalation bioaccessibility was investigated. A commonly used extraction time of 24 h was chosen to study the effect of the nature of the simulated lung fluid (SLF) and the solid/liquid ratio (S/L). Fig. 2 shows the PAHs and O-PAHs bioaccessible concentrations extracted from increasing concentrations of UFP in four SLFs: the GS, the ALF, the MGS, and the SELF. Particle concentrations of 5, 10, 50, and 500 $\mu\text{g}\cdot\text{mL}^{-1}$ correspond to S/L ratios of 1/200,000, 1/100,000, 1/20,000, and 1/2000, respectively. According to the literature, S/L ratios were defined to represent a wide range of concentrations (Bescond et al., 2016; Shahpoury et al., 2024). In addition, this range of concentrations avoided reaching a saturation of the solution that could reduce bioaccessibility. Indeed, a higher mass loading may limit the exposure of the particle surfaces to the SLF or may induce agglomeration phenomena.

Out of particle-bound 21 compounds, only 3 were not detected in SLF, 1,4-NQ, ACE and DahA. As shown in Fig. 2, bioaccessible PAH and

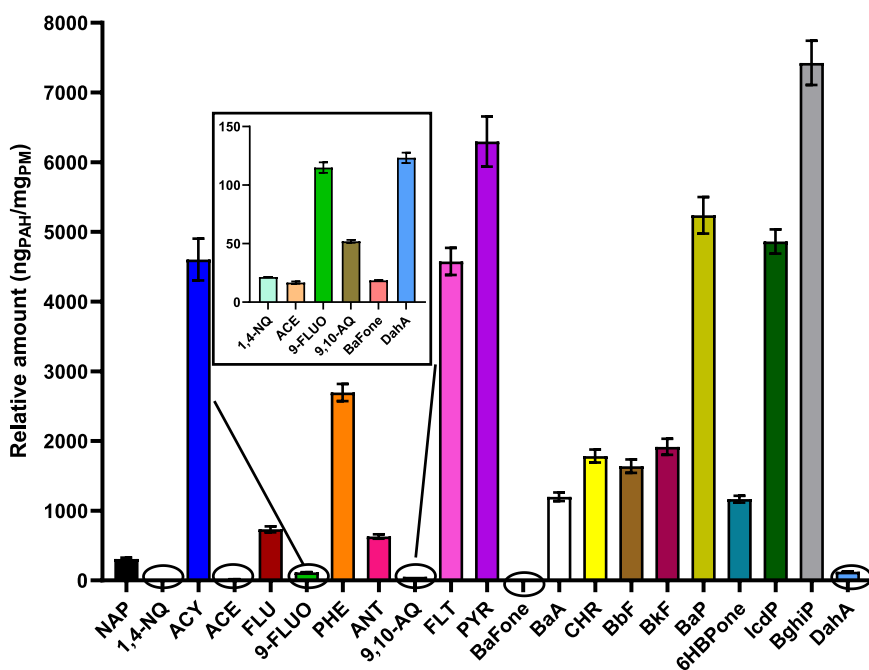


Fig. 1. Concentrations of PAH and OPAH in UFP. Data represent mean \pm SD, $n = 4$.

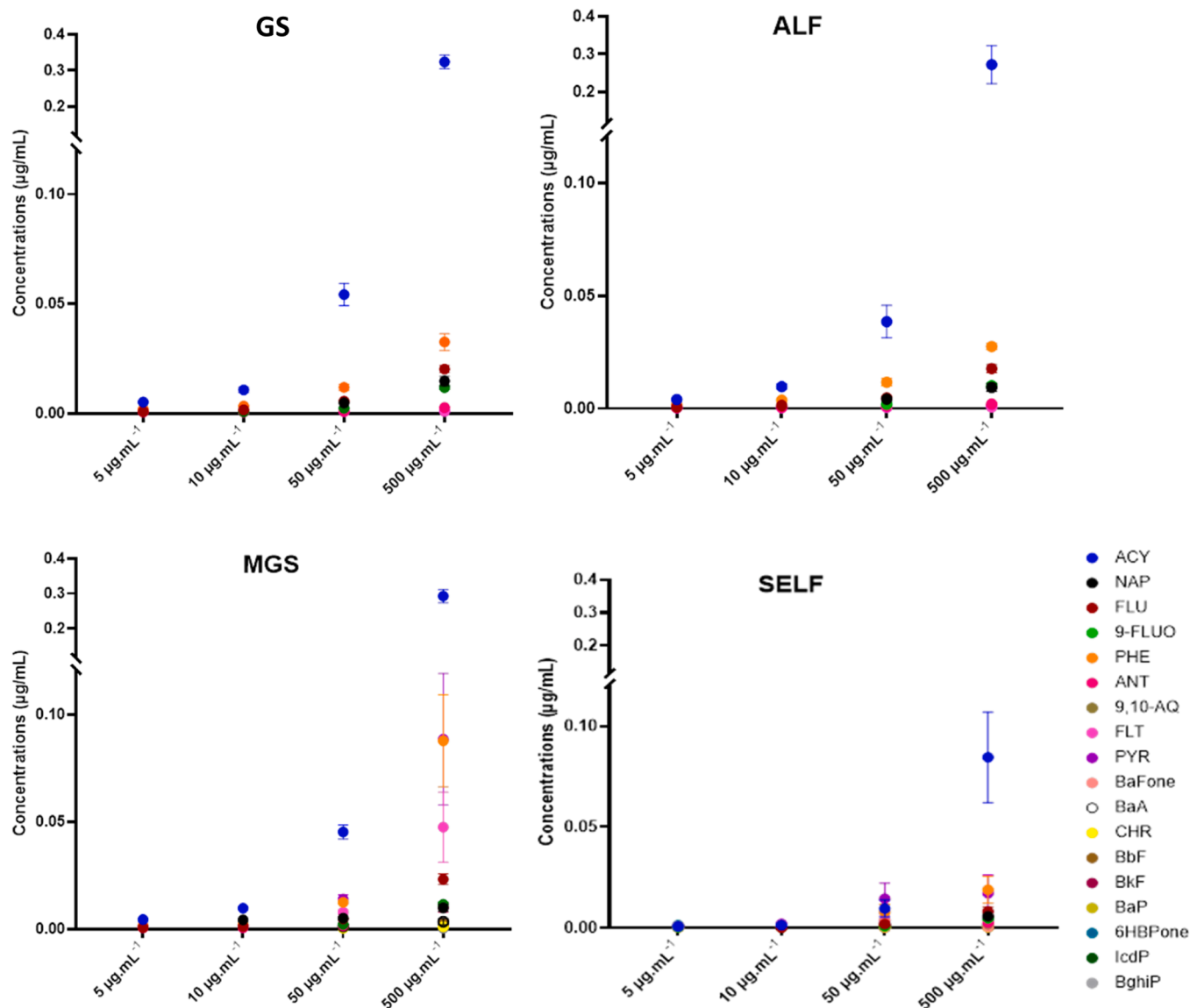


Fig. 2. Bioaccessible concentrations of PAH and OPAH in SLF corresponding to UFP concentrations of 5–500 $\mu\text{g mL}^{-1}$. Data represent mean \pm SD, $n = 4$.

OPAH concentrations increased nonlinearly with particle concentration, regardless of SLF. Similar response profiles were obtained in the study of Shahpoury, who determined the bioaccessible concentrations of PAH from urban PM (SRM-1649a) in cell-free culture medium (Shahpoury et al., 2024). These profiles included non-linear increases in bioaccessible concentrations over the same concentration range. This response is related to the low water solubility of PAH.

The normalization of the bioaccessible concentrations of PAH and OPAH to their total concentrations adsorbed on UFP was calculated to obtain the bioaccessible fractions, as often reported in the literature for inhalation bioaccessibility studies. In these studies, the S/L ratio parameter is considered to be the concentration of inhaled pollutants in lung fluid (Alves et al., 2017). A summary of the inhalation bioaccessible fractions (IBAF) thus calculated for each compound with four S/L ratios (1/2000; 1/20,000; 1/100,000; and 1/200,000) during a 24-hours extraction time in each SLF is presented in Table S9. The IBAF of the majority of PAH and OPAH, namely NAP, ACY, FLU, 9-FLUO, PHE, ANT, FLT, PYR, and PHE are shown in Figure S1. The S/L ratio did not influence the total sum of extracted PAH (\sum PAH), whereas the sum of OPAH was more variable and appeared to be influenced by this parameter. With regard to the SLF, the SELF showed the lowest IBAF for

the \sum PAH, while for the \sum OPAH, this extraction fluid yielded the highest IBAF, depending on the S/L.

In qualitative terms, the number of compounds extracted varied according to the S/L and the fluid. Although the ratio 1/2000 did not allow achieving high IBAF, it produced the highest number of bioaccessible compounds. For example, in the MGS, only 6 compounds (ACY, FLU, PHE, ANT, FLT, PYR) were identified with a 1/200,000 ratio versus 14 compounds (NAP, ACY, FLU, 9-FLUO, PHE, ANT, FLT, PYR, BaA, CHR, BbF, BkF, BaP, BghiP) with a 1/2000 ratio. The same applied with the SELF: 9 compounds (ACY, 9-FLUO, PHE, ANT, FLT, PYR, BaA, CHR, 6HBPone) versus 18 compounds (NAP, ACY, FLU, 9-FLUO, PHE, ANT, 9,10-AQ, FLT, PYR, BaFone, BaA, CHR, BbF, BkF, BaP, 6HBPone, IcdP, BghiP, DahA), 1/200,000 versus 1/2000, respectively. However, a less pronounced effect of the S/L ratio on the number of compounds extracted was observed with GS or ALF.

Eight compounds out of a maximum of 18 detected (NAP, ACY, FLU, 9-FLUO, PHE, ANT, FLT, PYR) were systematically extracted using different SLF, but their IBAFs depend on the S/L ratio. To account for both parameters, SLF and S/L ratio, surface analysis plots were created for these significant compounds (Figure S2).

The differences in IBAF can be attributed to the varying compositions

of the fluids. With GS or ALF, only PAH with 2, 3, or 4 condensed rings could be detected, whereas with MGS and SELF, it was possible to extract PAH with more than four condensed rings. Furthermore, as shown in Tables 1 and S1, the IBAF in GS and ALF decreased with the molecular weight. As expected, the low molecular weight PAH have higher IBAF, whereas the high molecular weight PAH have relatively lower IBAF. Physicochemical properties play a role in the bioaccessibility of organic compounds. This has been demonstrated in numerous studies (Besis et al., 2022a; Xie et al., 2018; Shi et al., 2022; Liu et al., 2023). The downward trend of IBAF has often been described as increasing with molecular weight and log K_{ow}, suggesting that lower molecular weight and less hydrophobic compounds are more easily desorbed from PM into the SLF. The PAH and OPAHs studied have a wide range of log K_{ow} (1.71–7.10) (Table S1). This observation confirms that hydrophobicity plays a crucial role in the ability of organic compounds to transfer from PM to lung fluids. Thus, the less hydrophobic compounds would desorb more easily from the inhaled particles into the respiratory tract than the hydrophobic compounds. Kademoglou *et al.* have observed a decrease in the IBAF of plasticizers with increasing hydrophobicity, independently of the extraction fluid (MGS and ALF) (Kademoglou *et al.*, 2018). Our results are consistent with this study. In contrast, Besis *et al.* found higher IBAF values with ALF than with MGS, and Gao *et al.* demonstrated that higher levels of PAH were extracted from PM_{2.5} by GS than by ALF (Gao *et al.*, 2019; Besis *et al.*, 2022b). The addition of a surfactant DPPC in GS, to reduce aggregates and to increase the wettability of organic PM, did not improve the IBAF of small compounds such as NAP, ACY, FLU, 9-FLUO, PHE, ANT, FLT, and PYR (3–4

condensed rings). However, 6 PAH with high molecular weight (4–6 condensed rings) could be quantified in MGS. This result is consistent with the study of Shi, which suggests that DPPC does not appear to be a determining factor for inhalation bioaccessibility determination (Shi *et al.*, 2022). However, Tang *et al.* and Xie *et al.* found that ALF solution extracted more PAH from PM than MGS (Tang *et al.*, 2023; Xie *et al.*, 2018). Sanchez *et al.* suggest that acidic pH or use of additives (surfactant, protein, lipid, etc.) could increase the IBAF as reported in studies on metals (Kastury *et al.*, 2018; Sánchez-Piñero *et al.*, 2022).

Overall, these results highlight that the bioaccessibility of PAH depends on the solubilisation capacity and chemical composition of the simulated fluid, as well as on the S/L ratio, which establishes a concentration gradient and the kinetics of PAH transfer from particles to fluids. Decreasing the S/L ratio reduces the fluid's solubilisation capacity, while increasing the ratio could promote PAH desorption. The differences of IBAF between the simulated fluids are related to variations in their composition, and therefore their solubilising power. ALF and GS facilitate electrostatic interactions, thereby promoting the solubilisation of more polar compounds. In contrast, adding surfactants increases the solubilisation of PAH through hydrophobic interactions and micelle formation, which facilitate the desorption of weakly polar compounds.

3.3. Influence of extraction times on inhalation bioaccessibility assessment

Several extraction times are described in the literature (Liu *et al.*, 2023; Besis *et al.*, 2022b; Sánchez-Piñero *et al.*, 2022; Meng *et al.*, 2024).

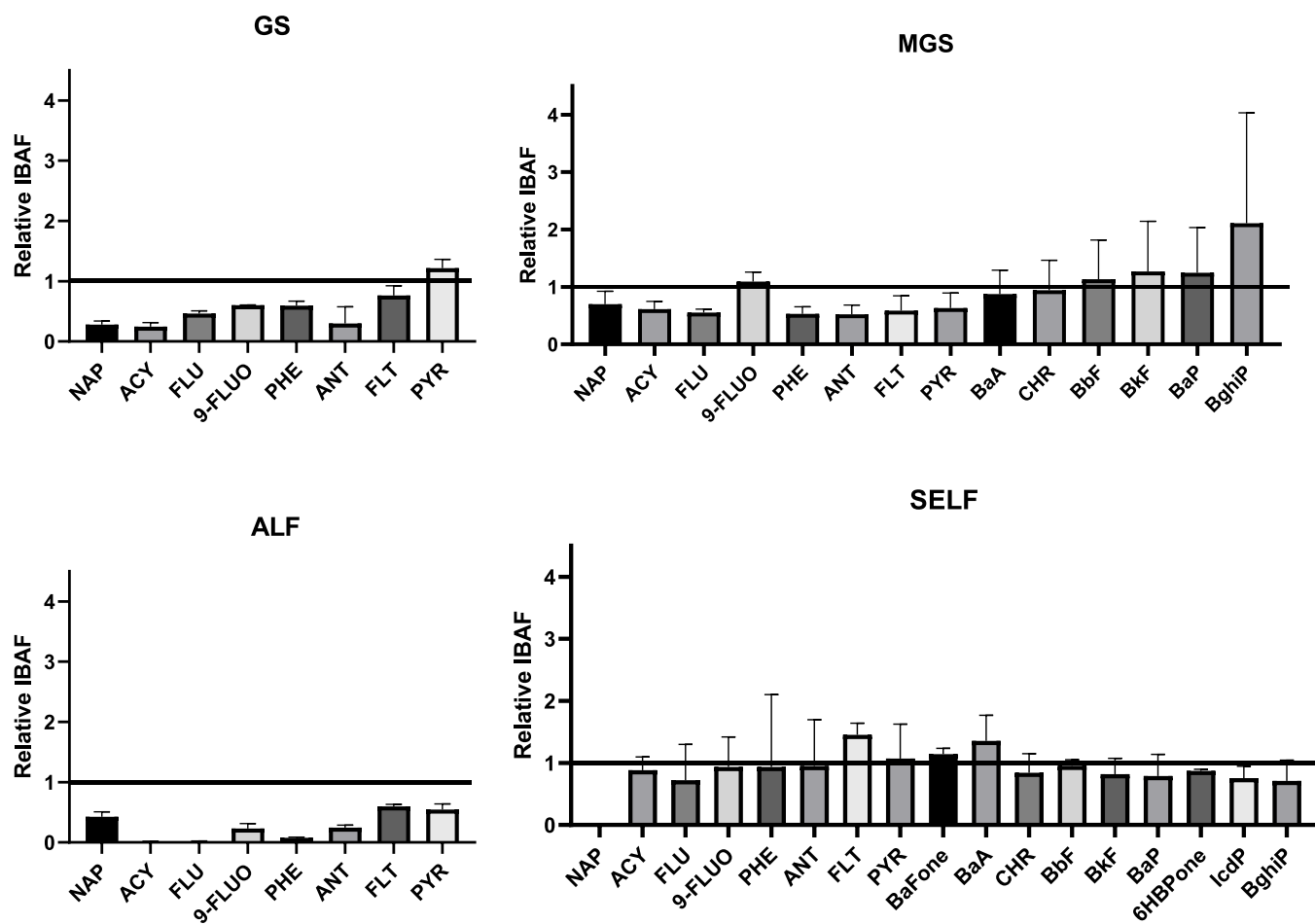


Fig. 3. Influence of extraction time. Relative IBAF of PAH and OPAH in UFP were obtained from four SLF with an S/L ratio of 1/2000. The bold line represents the IBAF with an extraction time of 24 h. Relative bioaccessibility was obtained by dividing IBAF 96 h by IBAF 24 h. Data represent mean \pm SD, n = 4. The 9.10-AQ was extracted only at 96 h with MGS (IBAF = 20.2 %) and 24 h with SELF (IBAF = 7.2 %, data not shown).

The extraction time is related to the particle residence time in the respiratory tract after inhalation exposure (Ren et al., 2020). In the present study, two extraction times were investigated: 24 h, which appears to be the most common, and 96 h, a time point relevant to the human alveolar clearance capacity (Shi et al., 2022; Kademoglu et al., 2018; Li et al., 2019; Sánchez-Piñero et al., 2021). The evaluation of the influence of these two times has been achieved by setting a parameter, the S/L ratio, at 1/2000. As presented above, this ratio is optimal for most bio-accessible compounds. Relative IBAF of PAH and OPAH (96 h/24 h) as illustrated in Fig. 3, bioaccessibility fractions appeared optimal from 24 h for many compounds. A decrease in IBAF was observed at 96 h with GS and ALF, suggesting a potential degradation of the compounds. The decrease seems less pronounced for MGS and SELF fluids with a relative IBAF close to 1. However, the 9.10-AQ was extracted only at 96 h with MGS (IBAF = 20.2 %) and 24 h with SELF (IBAF = 7.2 %, data not shown). Short extraction time of 24 h seems the most relevant, although in theoretical models, clearance of particles deposited in the bronchial airways takes days; mucociliary clearance is mainly completed within 24 h of exposure (Sturm, 2021).

3.4. Toxicological analyses of bioaccessible fractions after exposure of BEAS-2B cells

As demonstrated in the previous section, bioaccessibility was maximized after a 24-hour extraction with an S/L ratio of 1/2000 in terms of the number of extracted compounds, at least with MGS and SELF (Table S9). We chose to test these two fractions along with the GS condition, which represents a more restricted mixture of molecules, primarily low-MW PAHs consisting of 2–3 rings. Consequently, we compared PAH and O-PAH mixtures reconstructed based on IBAFs (rIBAF) obtained from GS, MGS, and SELF after a 24-hours extraction with an S/L ratio of 1/2000. These reconstituted fractions were named according to the corresponding SLF.

Cell viability was first assessed using the MTT assay (Fig. 4). After three and 24 h of exposure, a significant decrease in cell viability of 12.7 % (± 7.4) and 16.5 % (± 4.2), respectively was observed in cells exposed to the SELF condition. Therefore, only the fraction with the highest number of different PAH, i.e., "SELF", affected cell viability. This fraction is composed of low-MW and heavy PAH, such as BbF, BkF, BaP, IcdP, and BghiP. In a study by Jiang et al. on ambient air in Guangzhou city, increasing concentrations of BbF, BaP, IcdP, and BghiP bound to fine particles were associated with rising numbers of hospitalizations for

respiratory diseases (Jiang et al., 1 sept, 2023). These authors also showed that exposure of BEAS-2B cells to each of these 4 PAH for 24 h resulted in acute inflammation and apoptosis. The MGS condition did not affect cell viability after three hours or 24 h, despite three of the four compounds being present at higher concentrations than in the SELF, namely BbF, BaP, and BghiP (Table S8). This result suggests that some compounds, present in the SELF-fraction and absent in the other fractions, may induce cellular toxicity. The SELF-condition is also distinguished from the others by the presence of three OPAH, namely, 9.10-AQ, BaFone, and 6HBPone. OPAH, including 9.10-AQ, are known to induce oxidative stress and have genotoxic and mutagenic potential (Wang et al., 2015; Clergé et al., 2023), which can lead to decrease cell viability.

To evaluate the impact of the rIBAF on BEAS-2B cells related to the AhR pathway, gene expressions of AhRR, Cyp1a1, and Cyp1b1 were examined (Fig. 5). The induction of CYP1A1 and CYP1B1 is a key AhR-dependent adaptive response to environmental chemicals such as PAH and halogenated aromatic compounds (Wang et al., 2015; Clergé et al., 2023), while AhRR functions as a negative feedback regulator that modulates AhR activity. The results were represented as relative quantification (RQ) reflecting the level of gene induction in cells exposed to rIBAF, compared to the same gene expression in control cells. According to the literature, RQ values below 0.5 or above 2.0 indicate significant expression inhibition or induction, respectively.

Pronounced inductions of Cyp1a1 and AhRR, and to a lesser extent Cyp1b1, were observed in Beas-2B cells following a 3-hours exposure to MGS. These inductions were less pronounced after a 24-hours exposure period for Cyp1a1 and Cyp1b1, with no further induction observed for AhRR. The absence of induction with the SELF-condition is noteworthy, given that, as previously mentioned, this fraction comprises a more diverse molecular composition, including molecules recognized as AhR agonists. Among these, BaA, CHR, BbF, and BaP, have been reported to be carcinogenic in rodents and classified as possibly carcinogenic to humans (BaA, CHR, BbF) or carcinogenic to humans (BaP) (IARC et al., 2010), and they weakly induced the lung mRNA expression of CYP1A1 and 1B1 in AhR (+/+) mice (Shimada et al., 2002). BkF has also been demonstrated to be a potent AhR inducer, as evidenced by the research conducted by Libalova et al (Libalová et al., 2014). Their findings revealed that a dose of 10 nM BkF and 100 nM BaP resulted in the upregulation of all the AhR target genes in alveolar epithelial cells. In the present conditions, the SELF-mixture contains 1.9 nM BkF and 3.9 nM BaP, which is below a threshold concentration that does not allow induction to occur. Another explanation is the presence of antagonists of the AhR pathway in a more complex mixture, as represented by the SELF-condition. For example, OPAH have been shown to both activate and inhibit AhR signalling and CYP1 expression and function (Wincent et al., 2016). In this study, performed with human keratinocytes, OPAH inhibited TCDD-induced gene expression of CYP1A1 and CYP1B1. Among them, 9.10-AQ and 6HBPone are inhibitors, whereas BaFone is a potent inducer (Wincent et al., 2016).

Results obtained for BaP, which was used as a positive control, and for UFP confirmed the activation of the AhR signalling pathway (Fig. 6). To note that the induction of Cyp1b1 is modest in your experimental condition after 24 h of exposure with the positive control BaP. Cyp1b1 induction by BaP depends on different parameters, as the time and the dose, with a maximal induction often around 6–12 h and decline thereafter. For example, in the article of Boulé et al (Gualtieri et al., 2011), the induction was observed only after 6 h exposure to 10 μM of BaP in Beas-2B cells exposed at air-liquid interface. Consequently, it would have been interesting to use exposure at the air-liquid interface, that better recapitulate lung physiology (Juarez et al., 2022; Juarez-Facio et al., 2022). The concentrations of UFP did not show any cytotoxicity (Figure S3), and correspond to relatively low doses ranging from 1 to 10 $\mu\text{g}/\text{cm}^2$ as usually used in other experiments (Badran et al., 2020; Gualtieri et al., 2011; Sotty et al., 2019). Previous studies also reported that the AhR signalling pathway was activated in BEAS-2B

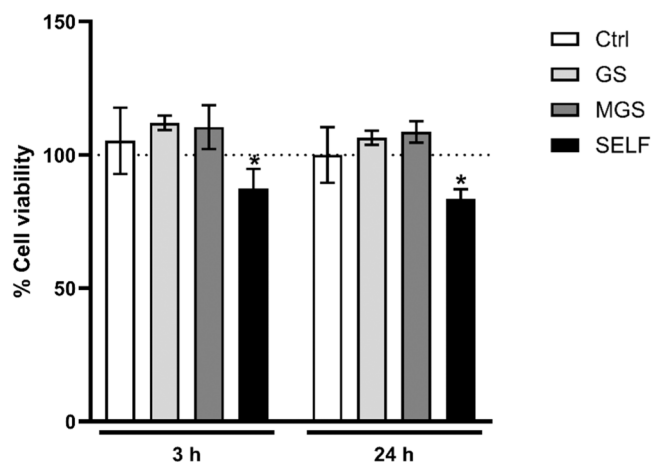


Fig. 4. Effects of rIBAF on cell viability. Results are expressed as percentages of cell viability for BEAS-2B cells after 3 and 24 h exposure to reconstituted inhalation bioaccessible fractions (rIBAF). Data are presented as the mean of cell viability percentage \pm SD from four independent experiments performed in triplicate for the 3 h exposures ($n = 4$) and four to five independent experiments performed in triplicate for the 24 h exposures ($n = 5$). *: $p \leq 0.05$.

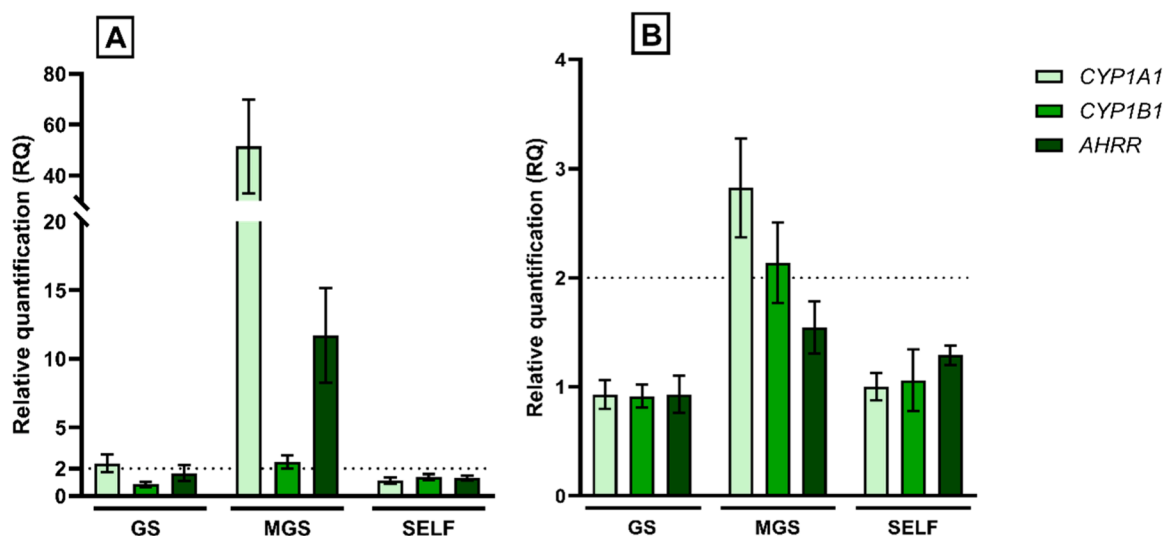


Fig. 5. Effects of rIBAF on the AhR pathway. Gene expression measured by RT-qPCR of the cytochrome P450 1A1 (*CYP1A1*), cytochrome P450 1B1 (*CYP1B1*), and the aryl hydrocarbon receptor repressor (*AHRR*) in BEAS-2B cells after an exposure of 3 h (A) or 24 h (B).

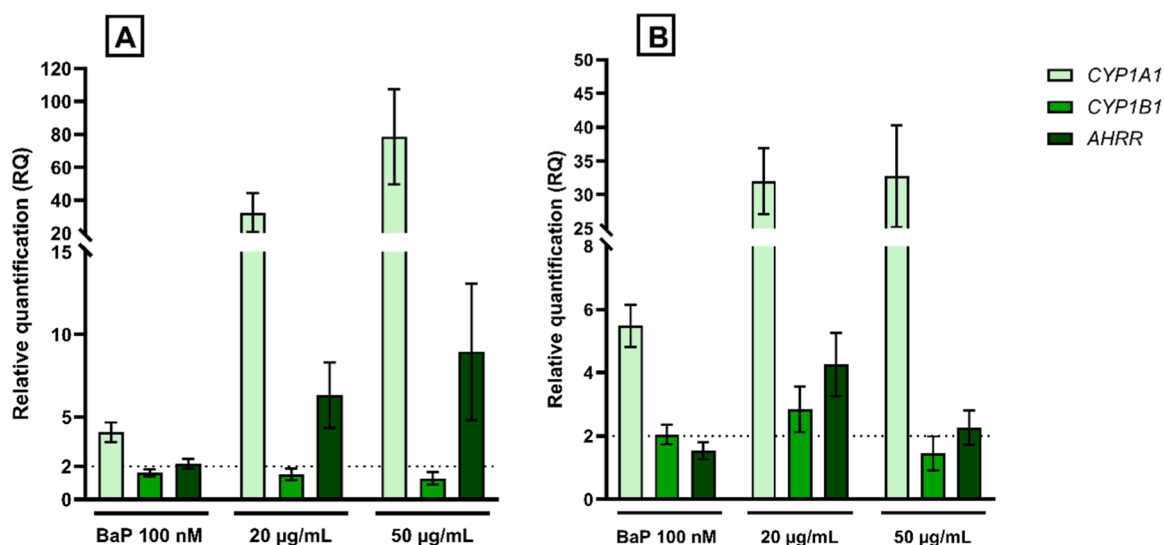


Fig. 6. Effects of BaP and UFPs on the AhR pathway. Gene expression measured by RT-qPCR of the cytochrome P450 1A1 (*CYP1A1*), cytochrome P450 1B1 (*CYP1B1*), and the aryl hydrocarbon receptor repressor (*AHRR*) in BEAS-2B cells after an exposure of 3 h (A) or 24 h (B).

cells exposed to PM in relation to their organic composition (Badran et al., 2020; Gualtieri et al., 2011). However, total PAH measurements can significantly overestimate the actual biologically effective dose. Thus, Gauggel-Lewandowski et al. found that the PAH CALUX (AhR reporter) response to wood combustion particulates correlates more closely with the desorbed PAH fraction rather than the total PAH content in PM samples (Gauggel-Lewandowski et al., 2013). In Nováková's study, the total and bioaccessible concentrations of PAHs from samples collected at urban and background sites in the Czech Republic were analyzed using organic solvent extraction and simulated lung fluid extraction, respectively (Nováková et al., 2020). The particulate-phase sample extraction using simulated lung fluids mobilized only a portion of the biological effects attributable to the total extract, highlighting the importance of examining the bioaccessible mass fraction to assess PM toxicity (Nováková et al., 2020). Although few studies have examined the relationship between in vitro lung bioaccessibility of PAHs and toxicity outcomes, the fact that only a small proportion of particle-bound PAHs can be extracted under lung-like conditions has led to the view that bioaccessibility is a more meaningful metric for

assessing inhalation hazards. Thus, Li et al. collected PM_{2.5} samples from Nanjing, China, and assessed PAH inhalation bioaccessibility using SELF as SLF (Li et al., 2019). The cancer risk probability was calculated both on the basis of total PAH concentration or of PAH inhalation bioaccessibility, which highlighted differences between these two approaches. Their toxicity study, conducted in vitro using A549 alveolar cells exposed to the particles, suggested that bioaccessible concentration of PAHs can better predict toxicity posed by PM_{2.5}-associated PAHs (Li et al., 2019). In our study, the induction of the AhR response was observed only with MGS when we compared the response profiles obtained across our different rIBAFs and the UFP in their entirety. Our results clearly show that the fractions obtained respond differently. Thus, understanding the relationship between bioaccessibility and toxicity is essential for improving the predictive effectiveness of these approaches.

4. Conclusions

The findings of this study indicate that the inhalation bioaccessibility

of PAH is governed by their physico-chemical properties. Indeed, there is a positive relationship between bioaccessibility and PAH hydro-solubility, but a negative relationship with molecular weight or hydro-phobicity. The evaluation of the toxicological response confirms the variability of the profiles obtained as a function of the extraction conditions used. The extraction condition that produced a biological response equivalent to that evaluated with the total particulate fraction was that using MGS as the extraction fluid, under our experimental conditions. Thus, *in vitro* lung bioaccessibility, as measured by extraction in physiologically relevant SLF, is a valid and informative proxy for the biological availability of PAHs in the respiratory tract, particularly for endpoints mediated by AhR activation. It will be interesting to extend these assessments to other molecular events, in order to apply these approaches to a wide range of bioaccessible chemicals. This work opens up interesting prospects for assessing the inhalation bio-accessibility of PAH from combustion particles that may have variable physico-chemical properties, such as size or PAH richness. From a toxicological perspective, these approaches appear relevant for assessing the toxicity mechanisms of PAH mixtures to be as close as possible to the *in vivo* conditions found in the human respiratory tract.

CRedit authorship contribution statement

Mbina Albana: Software, Methodology, Investigation. **Rogez Flor-ent Tiphaine:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Formal analysis, Data curation. **Christelle Monteil:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Jérôme Yon:** Methodology. **Edwige Votier:** Methodology, Investigation, Data curation. **Clémence Bouzar:** Software, Methodology, Conceptualization.

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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.ecoenv.2025.119371](https://doi.org/10.1016/j.ecoenv.2025.119371).

Data availability

Data will be made available on request.

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