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Assessing toxicity of amorphous nanoplastics in airway- and lung epithelial cells using air-liquid interface models☆

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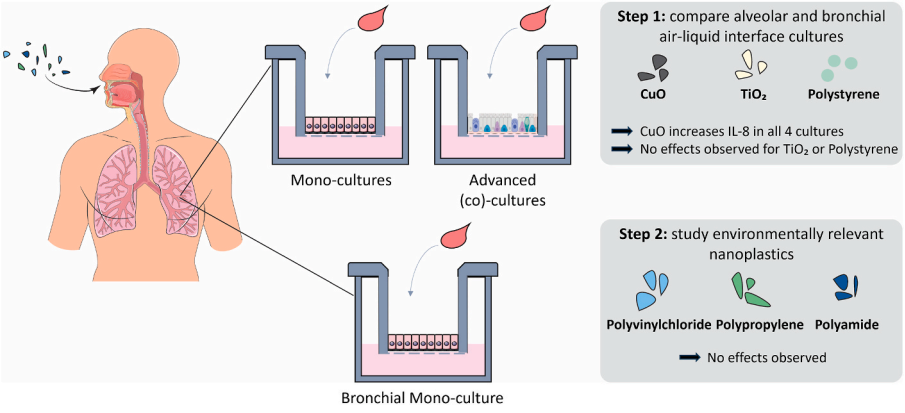
HIGHLIGHTS

- Mono-cultures and advanced (co-) cultures responded similar to nanoparticles.
- None of the tested nanoplastics induced cytotoxicity.
- Polystyrene did not induce inflammation in air-liquid interface cultures.
- Environmentally relevant nanoplastics did not impact inflammation in bronchial cells.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Inhalation is one of the main exposure routes to nanoplastics. Knowledge of the toxicological impact of nanoplastics on the airway- and lung epithelium is limited and almost exclusively based on submerged *in vitro* models using spherical polystyrene (PS) particles.

☆ The authors dedicate this article to the late Ingeborg Kooter, who was both an excellent mentor and good friend.

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Polypropylene
Polyamide
Polyvinylchloride

Methods: Mono-cultures and advanced (co-)cultures of human bronchial- and alveolar epithelial cells, all air-liquid interface (ALI) cultures, were exposed to nanoplastics and reference nanoparticles. Alveolar models included A549 mono-cultures and A549 cells co-cultured with endothelial cells (Ea.hy926) and macrophage-like cells (differentiated THP-1). Bronchial models included BEAS-2B cells and differentiated primary bronchial epithelial cells (PBEC). Cultures were exposed to PS, copper(II) oxide (CuO) or titanium dioxide (TiO_2) nanoparticles (50 nm). Additionally, BEAS-2B cells were exposed to well-characterised, amorphous polyvinyl chloride (PVC), polypropylene (PP), or polyamide (PA) nanoplastics. Cytotoxicity and inflammation (IL-8 secretion and IL-8 transcript levels) were assessed after 24 h of exposure.

Results: Cell viability remained unaffected by all exposures in all models. Unlike PS and TiO_2, CuO exposure dose-dependently induced IL-8 protein secretion and mRNA levels. Although the extent of IL-8 secretion differed between models, the relative response to CuO was similar in both mono-cultures and advanced (co-)cultures. None of the environmentally relevant nanoplastics (PVC, PA or PP) impacted inflammation or cell viability in BEAS-2B ALI cultures.

Conclusion: Although CuO induced inflammation, PS failed to elicit an inflammatory response in any of our models. For the first time, we show that PVC, PA and PP do not induce cell death or inflammation in a BEAS-2B ALI model.

1. Introduction

The world-wide plastic production has increased rapidly and nowadays exceeds 400 million tons annually (Plastics Europe, 2023). This increase in plastic production is accompanied by more plastic waste in the environment. Plastics are designed to be resilient and do not degrade; instead, they fragment in smaller pieces resulting in microplastics (<5 mm) and nanoplastics (<1 μm) (MNPs) by physical or chemical degradation (Gigault et al., 2018; Langknecht, 2022).

MNPs are ubiquitous in the environment and have been detected in diverse environmental compartments including the atmosphere. Atmospheric MNPs have been detected in urban areas, remote areas and indoors (Huang et al., 2021; Li et al., 2020; Zhao et al., 2023). Major sources of atmospheric MNPs include tire wear, agriculture, textile, and paints. Human exposure via inhalation is estimated to be about 0.1 ng per day, with indoor quantities being higher than outdoors (Mohamed Nor et al., 2021; Sharaf Din et al., 2024). However, due to limitations in size detection and sampling complexity it is challenging to estimate the exact amount of MNPs, especially nanoplastics, in the air (Fang et al., 2024; Xie et al., 2022). Also, exposure levels can rise during peak exposures and internal concentrations can accumulate during a lifetime.

Due to their size, nanoplastics can penetrate deep into the lung and even can cross the bronchial/alveolar epithelial barrier (Donkers et al., 2022; Geiser and Kreyling, 2010). Indeed, MNPs have been found in all areas of the human airways and lungs, as well as in blood (Jenner et al., 2022; Leslie et al., 2022). This raises concerns about their potential impact on human health especially since high exposures to nanoplastics have been correlated to respiratory diseases (Atis et al., 2005; Burkhart et al., 1999). *In vivo* exposures to nanoplastics via either inhalation or intratracheal instillation resulted in increased inflammatory cytokine levels and infiltration of immune cells in the lungs of rats and mice (Vattanasit et al., 2023). However, knowledge about the direct effects of nanoplastics on the cells making up the epithelial barrier of the human airways and lung is limited. There are some *in vitro* studies that documented cytotoxic effects of nanoplastics in bronchial- and alveolar epithelial cells (Shahzadi et al., 2023; Wu et al., 2024). Furthermore, exposure of these cells to nanoplastics has been linked to inflammation and oxidative stress (Vattanasit et al., 2023). However, it is important to note that almost all of these studies have been performed with commercially-available spherical polystyrene (PS) particles, which are not representative for the various nanoplastics present in the environment (Wright et al., 2024).

Indeed, nanoplastics are not a single pollutant but they vary widely and can be categorised based on polymer type, size and morphology. Polymers mostly produced by industry include polypropylene (PP), polyethylene (PE) and polyvinyl chloride (PVC) (Plastics Europe, 2023). In addition, PE, PP, polyethylene terephthalate (PET) and PS polymers, mainly fibres and fragments, emerged as the predominant MNPs identified in air samples (Sharaf Din et al., 2024). Other studies pointed out textiles as a main source for airborne MNPs, including PET, acrylic and nylon (polyamide: PA) (Huang et al., 2021). With regard to polymer type of MNPs detected in the human lungs and respiratory tract, PP, PET, PA and PVC have been predominantly identified (Jenner et al., 2022; Jiang et al., 2022). There are only limited studies on the toxicity of MNPs derived from polymers other than PS, especially nanoplastics, in epithelial cells of the airways and lungs. Including a greater diversity of nanoplastics from environmentally relevant polymer types in toxicity studies is urgently needed to perform proper risk assessment of MNPs on human health.

A second reason there are still large gaps in our knowledge of potential toxicity of airborne nanoplastics is the lack of studies using *in vitro* models reflecting the complexity and structure of the human airways and lungs. Studies on airborne nanoplastics toxicity typically focus on mono-cultures of submerged human bronchial- and alveolar epithelial cell lines. While this is a simple and cost-effective approach, submerged cell exposures have several major limitations. First of all, oxygen diffusion in medium is relatively slow, causing hypoxic culture conditions for submerged cells compared to cells exposed directly to air (Kouthouridis et al., 2021; Place et al., 2017). This submersion-induced hypoxia is known to influence cellular responses to nanoparticles (Dabi et al., 2022). Furthermore, nanoplastics can interact with proteins and components in the culture medium, which influences deposition and physicochemical characteristics of the particles. Altogether, a new approach is needed since submerged cell-culture conditions are not realistic. Air-Liquid Interface (ALI) cultures mimic the *in vivo* situation more closely, since apically they are exposed to air and particle deposition is more direct (Lacroix et al., 2018).

Therefore, in this study, we compared four different ALI cultures of bronchial- and alveolar epithelial cell cultures, varying in complexity and cellular composition. Alveolar ALI models included mono-cultures of A549 cells or co-cultures of A549 cells with endothelial cells (Ea.hy926) and macrophage-like cells (differentiated THP-1). Bronchial ALI models included BEAS-2B cells or fully differentiated human primary bronchial epithelial cells (PBEC). We compared the cytotoxic and inflammatory response of alveolar- and bronchial epithelial cells to PS in all four different ALI culture models. Since data on the toxicity of MNPs is scarce, well-studied non-plastic nanoparticles copper(II) oxide (CuO) and titanium dioxide (TiO_2) were included as reference nanoparticles (Kooter et al., 2017). Importantly, since the broad scale of nanoplastics in the environment originate from fragmentation of larger plastic objects, toxicology research should not be limited to the bottom-up produced PS spheres. Therefore, using BEAS-2B cells, we tested environmentally relevant nanoplastics PVC, PP and PA that were synthesised following a top-down procedure by milling larger plastic objects. These particles were produced via a standardised method by the MOMENTUM consortium, a Dutch national collaboration between

academia, government and industry and were thoroughly characterised (Parker et al., 2023).

2. Materials and methods

Full experimental details are available in the supplementary materials.

2.1. Applied nanoparticles

The following commercially available nanoparticles were used: polystyrene Polybead® Microspheres (PS) with a diameter of 50 nm (Polysciences, Warrington, USA), CuO with a diameter <50 nm (Sigma-Aldrich, St. Louis, USA), TiO_2 anatase with a diameter of 50 nm (mkNano, Mississauga, Canada). Environmentally relevant nanoplastics with a diameter <1 μm of PVC, PP mixed with Talc (PP/Talc) and Polyamide 6.6 (PA) were produced by cryo-milling and supplied by the MOMENTUM consortium (Parker et al., 2023). Talc was added to PP as a filler during the production process to increase the density and prevent buoyancy of the particles.

2.2. Preparation of particle suspensions

Stock suspensions of CuO and TiO_2 (2.5 mg/mL) were prepared freshly on the day of exposure in MilliQ-water (MQ) in glass vials. Environmentally relevant nanoplastics were provided by the MOMENTUM consortium already in suspension in 1-propanol (without surfactants) and vortexed thoroughly before use. Stock concentrations were 20.1 mg/mL (PVC), 20.0 mg/mL (PP/Talc) and 6.9 mg/mL (PA). PS particles were delivered in watery suspension (26 mg/mL) by the supplier and vortexed thoroughly before working suspensions were prepared. Working suspensions of all nanoparticles were prepared by first making a predilution of stocks with 0.5% bovine serum albumin (BSA)/Dulbecco's Phosphate Buffered Saline (dPBS) to increase steric repulsion and prevent agglomeration, and then further diluted to exposure concentrations with a final concentration of 0.05% BSA/dPBS (ST1). Cells were exposed to 1.39 μg/cm^2 TiO_2, 0.09–1.39 μg/cm^2 CuO, 1.39–112 μg/cm^2 PS and 0.79–7.14 μg/cm^2 for MOMENTUM nanoplastics. For exposure experiments with MOMENTUM nanoplastics, final concentrations of 1-propanol were set at 1% of the droplet volume and kept equal for all conditions. In this regard, the lower stock concentration of PA nanoplastics limited us in the maximum achievable dose for this material (2.38 μg/cm^2).

2.3. Particle characterisation

Scanning electron microscopy was combined with energy dispersive X-ray spectroscopy (SEM/EDX) to confirm elemental composition, size and shape of the nanoplastics (Parker et al., 2023). Dynamic Light Scattering (DLS) was used to characterise diluted stock suspensions (0.1–0.5 mg/mL) of CuO, TiO_2 and PS in MQ on a Zetasizer nano ZS (Malvern Panalytical, Malvern, UK). Since DLS was not ideal to detect the broader size range of the MOMENTUM nanoplastics, Static Light Scattering (SLS) was used to characterise diluted stock suspensions (0.1 mg/mL) of PVC, PP/Talc and PA. SLS was performed in 1-propanol, using a SALD 7500nano (Shimadzu, Kyoto, Japan) with a 405 nm laser Parker et al. (2024); (Parker et al., 2023). Subsequently, surface charge of diluted stock suspensions was determined by using a Zetasizer nano ZS (Malvern Panalytical). Three independent measurements were performed in triplicate to measure the Zeta potential for CuO and TiO_2 (both in MQ, pH 6.4). Two independent measurements were performed in triplicate for PS (MQ pH 6.4), PP/Talc, PVC and PA (all in 1-propanol pH 6.4).

2.4. A549 and BEAS-2B mono-cultures

Human alveolar lung epithelial cells (A549) and human bronchial epithelial cells (BEAS-2B), both purchased from ATCC, were maintained in growth medium: DMEM + Glutamax with 100 U/mL Penicillin/100 μg/mL Streptomycin and 10% heat-inactivated FBS (all purchased from Gibco). Approximately every 48 h the medium was replaced and cells were passaged at 80–90% confluence using Trypsin-EDTA (Gibco). Passages between 8 and 23 were seeded in 12-well 12 mm Transwells with 0.4 μm pore polyester membrane cell culture inserts (Corning Costar, New York, USA) in growth medium. The medium was replaced after 3 days. 4 days after seeding, around 90% confluency, cells were airlifted by removing the apical medium and replacing the basolateral medium by reduced medium (DMEM + Glutamax with 100 U/mL Penicillin/100 μg/mL Streptomycin (1% P/S), and 1% heat-inactivated FBS (all from Gibco)). Cultures were exposed 24 h after airlifting. Cells were tested for mycoplasma on a regular basis, using the MycoAlert Mycoplasma detection kit (Lonza, Basel, Switzerland).

2.5. Alveolar co-culture

A549/EA.hy926/MΦ-THP1 co-culture was established as previously described, with a few modifications (Klein et al., 2013; Marescotti et al., 2019). Briefly, THP-1 cells were differentiated towards macrophage-like cells (MΦ-THP-1) by 36 h incubation with 20 nM phorbol 12-myristate 13-acetate (Sigma), followed by a 5 d resting period. Co-cultures were established according to the workflow described in Chary et al., 2019, using 1 μm pore membranes of 24-well Millicell® 6.5 mm transwell inserts (Merck KGaA, Darmstadt, Germany). Seeding densities were 36,000 EA.hy926 cells, 25,000 A549 cells and 36,000 MΦ-THP-1 cells per insert). Exposure experiments were performed on cultures that were airlifted for 20–24 h. Immunohistochemistry was performed to characterise the alveolar co-culture (Klein et al., 2013).

2.6. Primary bronchial epithelial cells (PBEC)

PBEC were isolated from tissue obtained from the Maastricht Pathology Tissue Collection (MPTC). Approval for using the tissue for research purposes was provided by both the scientific board of the MPTC (MPTC2010-019) and the local Medical Ethics Committee (METC, 2017-0087). Coded-anonymized tissues were handled compliant with the 'Human Tissue and Medical Research: Code of conduct for responsible use' (2011) (www.coreon.org), and the ethical framework of patient care at Maastricht University Medical Center+ (MUMC+). Isolation, culture and characterisation of cells was performed by the Primary Lung Culture (PLUC) facility at the MUMC + as previously described (van Wetering et al., 2000). Cells were acquired from resected lung tissue of three patients without known history of chronic lung diseases who underwent surgery for solitary pulmonary nodules. In Supplementary Table ST2 demographics and clinical characteristics of the patients are described.

PBEC of passage 1 were thawed, seeded, expanded and seeded in transwells as previously described (Tulen et al., 2022). Upon reaching confluence, cells were airlifted and maintained at ALI for 24–30 days at 37 °C and 5% CO_2 until exposure experiments (Tulen et al., 2022). During differentiation of PBEC, transepithelial electrical resistance (TEER) was determined to monitor differentiation using an epithelial tissue voltohmmeter (World Precision Instruments, Sarasota, USA). Furthermore, differentiation state of PBEC cultures was validated by characterising both mRNA expression and immunohistochemistry staining of epithelial cell type-specific markers (Tulen et al., 2022).

2.7. Exposure of ALI models to nanoparticles

Airlifted cultures were exposed to particle suspensions (see paragraph 2.2 Preparation of particle suspensions) at the apical side via a

single droplet ranging 10–40 μL) (ST1). An unexposed incubator-control was included in all experiments. Cultures exposed to a 10–40 μL droplet (1.79 ng/cm^2) of tumornecrosefactor alfa (TNF-α) (Peprotech, Cranbury, USA) were included as positive controls for inflammation. Immediately after exposure, cells were placed back in the incubator at 37 °C and 5% CO_2, until harvesting after 24 h.

2.8. Polystyrene particle distribution

Airlifted BEAS-2B cultures were exposed to fluorescently Fluoro-Max dyed green aqueous fluorescent polystyrene particles (fluorescent-PS) of 1 μm (Thermofisher, Waltham, USA) (see paragraph 2.2 Preparation of particle suspensions) at the apical side via a single 40 μL droplet for 4 h and 24 h. Pictures were taken at representative locations in the middle of the transwell insert and on the edges of the transwell insert, using an Axio Observer A1 microscope (Zeiss, Oberkochen, Germany) in combination with an AxioCam ICm 1 camera (Zeiss).

2.9. RNA isolation and real-time quantitative PCR

Total RNA from PBEC was extracted by lysis in TRIzol™ reagent (Invitrogen) and processed according to the manufacturer's instruction using GlycoBlue co-precipitant (Invitrogen). Total RNA from other ALI models was extracted using the RNeasy Plus mini kit (Qiagen, Hilden, Germany). Subsequently, cDNA synthesis and Real-time quantitative PCR amplification were performed as previously described (Tulen et al., 2022), primers are listed in Supplementary Table ST3. A fold change between normalised gene expression levels was calculated between the exposed cultures and vehicle control.

2.10. Cytotoxicity assay

After 24 h incubation, an apical wash (dPBS) was collected for analyses of the release of Lactate dehydrogenase (LDH). LDH release by the lung cells reflected cell viability after exposure and was determined using the manufacturer's protocol. A SpectraMax iD3 Multi-Mode Microplate reader (Molecular Devices, San Jose, USA) was used to determine the optical density of all wells. Measurements at 492 nm were corrected with a wavelength correction (650 nm) as well as a correction for background of the medium. Cytotoxicity is expressed as percentage of maximum cytotoxicity (LDH max) which was derived by incubating a non-exposed culture 10 min in 2% Triton-X-100/dPBS in triplicate per experiment.

2.11. Inflammatory protein secretion

Interleukin 8 (IL-8) protein production by ALI cultures was determined in both the apical wash as well as the basolateral medium, using the Human IL-8/CXCL8 ELISA Duoset kit according to manufacturer's specifications (R&D Systems, Minneapolis, USA). A SpectraMax iD3 Multi-Mode Microplate reader (Molecular Devices) was used to measure IL-8 protein production. IL-8 protein levels were calculated based on the standard curve (31.3–2000 pg/mL), corrected for different volumes in the apical and basolateral compartment and subsequently expressed as fold change compared to the vehicle control.

2.12. Statistical analysis

Results were obtained from at least three independent exposure experiments, all performed in technical triplicate. Statistical analyses were performed in GraphPad Prism version 10.2.3. Technical replicates per experiment were averaged and combined in one dataset, where after the assumption of normality and homogeneity of variance is checked using a Shapiro-Wilk test and Brown-Forsythe test respectively. Statistical differences between nanoparticle-exposed cultures versus vehicle-exposed cultures were tested using an ordinary one-way ANOVA with Dunnett's multiple comparisons test (normally distributed data) or a Kruskal-Wallis test (non-normally distributed data). For TiO_2 and TNF-α (both not tested in dose-response) statistical differences compared to the vehicle are tested using a two-tailed unpaired *t*-test with Welch's correction or Mann-Whitney test. Data are presented as mean values for each donor or biological replicate ± SD. p-values<0.05 were considered as significant and indicated as * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$) or **** ($P < 0.0001$).

3. Results

3.1. Characterisation of nanoparticles

PS particles were characterised as uniformly spherical particles whereas the nanoplastics from environmentally relevant polymers (PVC, PP/Talc and PA) displayed a more irregular morphology and heterogeneity in size (Fig. 1). Furthermore, Energy Dispersive X-ray spectroscopy (EDX) confirmed the elemental composition of the PVC, PP/Talc and PA particles (Fig. S1). An overview of physicochemical characteristics of all nanoparticles used in this study is summarised in Table 1. The hydrodynamic diameter (D_H) for CuO, TiO_2, as determined using DLS, was higher than the primary particle diameter (50 nm, the diameter of a single particle in dry state (Centre et al., 2014; Suttiponparnit et al., 2010)), as provided by the supplier) for these particles. For PS, the primary particle diameter (50 nm) and D_H were approximately the same. In addition to hydrodynamic diameter, Particle distribution index (PDI) indicates the width of particles size distribution with values ranging from 0 to 1. DLS measurements of CuO, TiO_2 and PS stock suspensions resulted in low PDI values of 0.21 ± 0.04, 0.16 ± 0.01 and 0.06 ± 0.01 respectively, indicating a relatively monodispersed suspension as can also be observed from narrow size distribution peaks (Fig. S2). In contrast, PP/Talc, PVC and PA stock suspensions displayed a more dispersed size distribution (Fig. S3). The count median particle diameter for these MOMENTUM particles is below 1 μm, and the volume median diameter around 5 μm (Fig. S3). CuO and TiO_2 suspensions showed a positive surface charge in MQ of 33.1 mV ± 3.1 and 21.9 mV ± 1.7 respectively. On the other hand, PS particles showed a negative surface charge in MQ of −38.1 mV ± 6.1. PP/Talc, PVC and PA stock suspensions in 1-propanol all showed a negative surface charge of −11.9 mV ± 0.7, −5.5 mV ± 0.8 and −9.5 ± 4.2 mV respectively (Table 1). In addition to data presented in Table 1, PP/Talc, PVC and PA used in this study were well-characterised by a previous study using various techniques including X-ray fluorescence analysis and micro Fourier Transform Infrared microscopy (μ-FITR) to confirm polymer composition of the nanoplastics (Parker et al., 2023, 2024).

3.2. Characterisation of advanced cell culture models

For all three donors, TEER values displayed an upward trend over time and stabilised before the moment of exposure, although some variability was observed (Fig. S4A). Furthermore, mRNA transcript levels of cell-type specific markers (ciliated-, goblet- and club cells) increased significantly whereas transcript levels specific for basal cells decreased substantially during the differentiation process (Fig. S4B). Moreover, cellular composition was confirmed by immunohistochemistry which showed the presence of these various cell types (Fig. S4C). Mucus production was visible in all differentiated cultures. Characterisation of the alveolar co-culture model with confocal microscopy showed MΦ-THP-1 cells were distributed on top of the A549 epithelial cells (Fig. S5). Furthermore, both the endothelial cells as well as the A549 epithelial cells formed a confluent monolayer, on both sides of the transwell.

3.3. Particle distribution in mono-cultures

Upon application of the single droplet to the mono-cultures, it

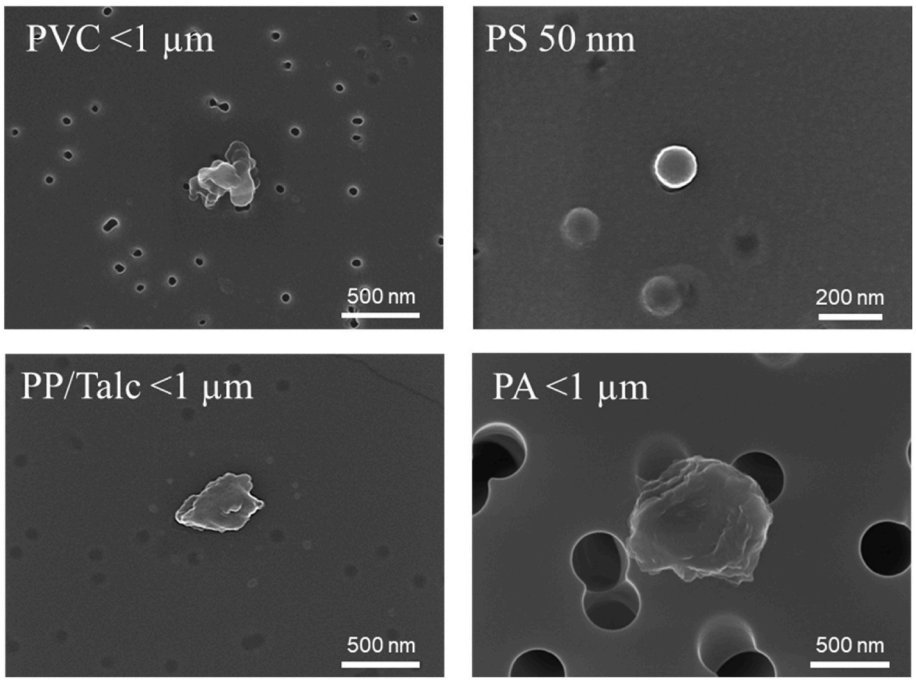

Fig. 1. Characterisation of nanoplastic particles by scanning electron microscopy (SEM). Representative Secondary Electron images of polyvinylchloride (PVC) < 1 µm, polypropylene/talc (PP/Talc) < 1 µm, polyamide (PA) < 1 µm fragments and polystyrene (PS) 50 nm spheres.

Table 1
Physicochemical characteristics of nanoparticles used in this study. The hydrodynamic diameter (D_H) and Polydispersity index (PDI) for copper(II) oxide (CuO) titanium dioxide (TiO_2) and polystyrene (PS) was determined in MQ, using dynamic light scattering. For polypropylene/talc (PP/Talc) < 1 µm, polyvinylchloride (PVC) < 1 µm and polyamide (PA) < 1 µm the count median particle diameter (D_N) was measured in 1-propanol, using static light scattering. For all particles the Zeta potential (in mV) was measured. All values are displayed as mean ± SD. Some values are not determined (n.d.).

	Shape	D_H (nm)	D_N (nm)	Polydispersity Index (PDI)	Zeta potential (mV)
CuO	irregular	228 ± 27	n.d.	0.21 ± 0.04	33.1 ± 3.1
TiO_2	irregular	102 ± 10	n.d.	0.16 ± 0.01	21.9 ± 1.7
PS	sphere	60 ± 11	n.d.	0.06 ± 0.01	−38.1 ± 6.1
PP/Talc	irregular	–	400 ± 80	n.d.	−11.9 ± 0.7
PVC	irregular	–	210 ± 10	n.d.	−5.5 ± 0.8
PA	irregular	–	680 ± 80	n.d.	−9.5 ± 4.2

immediately flattened out and spread out over the cell surface covering a large part of the transwell insert. Indeed, 4 h after exposure, fluorescent 1 µm PS particles were distributed relatively uniformly across the entire insert. However, after 24 h there is a non-uniform exposure of the cell surface in the well with cells at the edges of the well being exposed to larger quantities of (agglomerates) of particles (Fig. S6).

3.4. Polystyrene and reference nanoparticles do not impact cell viability in ALI cultures of bronchial- and alveolar epithelial cells

The response of advanced ALI cultures to PS and reference nanoparticles was compared with the response of the respective mono-cultures of bronchial- and alveolar epithelial cells. As shown in Fig. 2, exposure to PS or non-plastic reference nanoparticles had no significant effect on cell viability in any of the models under the dose ranged studied (<112 µg/cm^2 for PS, <1.39 µg/cm^2 for CuO and TiO_2).

3.5. Impact of polystyrene and reference nanoparticles on transcription and secretion of IL-8

We next explored the potential of nanoparticles to induce an inflammatory response in mono-cultures and advanced (co-)cultures of alveolar- and bronchial epithelial cells. TNF-α, a positive control, did not affect cell viability as assessed by LDH release (S7) and induced significant levels of C-X-C motif chemokine ligand 8 (*CXCL8)* mRNA expression and IL-8 protein secretion in both bronchial- (Fig. 3) and alveolar models (Fig. 4). Interestingly, we observed significant differences between the different culture models in the amount of IL-8 secretion at baseline but also in response to TNF-α (Table ST5). More specifically, at baseline, IL-8 protein levels released by all ALI cultures were substantially higher in the basolateral compartment compared to the apical compartment. We observed biological variation in total IL-8 levels of unexposed PBEC cultures; 1179 pg/cm^2 (donor 1), 1002 pg/cm^2 (donor 2) and 3715 pg/cm^2 (donor 3), most likely as a result of donor variation. As can be observed from comparing unexposed cultures with vehicle-exposed cells, applying a small single droplet had no significant impact on IL-8 protein secretion in any of the cultures (Table ST5).

In bronchial models, compared to the vehicle control, TNF-α exposure resulted in an increase of IL-8 protein secretion, with a higher fold-change in the apical than in the basolateral compartment for BEAS-2B mono-cultures (apical FC: 10.2 ± 11.9, basolateral FC: 3.9 ± 2.3) and PBEC (apical FC: 3.3 ± 2.3, basolateral FC: 2.3 ± 0.7) (Table ST5). Although baseline IL-8 protein levels were lower in alveolar cultures compared to bronchial cultures, a higher response in IL-8 secretion was observed for alveolar cells upon TNF-α exposure. In A549 mono-cultures, similar to bronchial mono-cultures, the fold change in IL-8 protein levels induced by TNF-α versus vehicle control was higher in

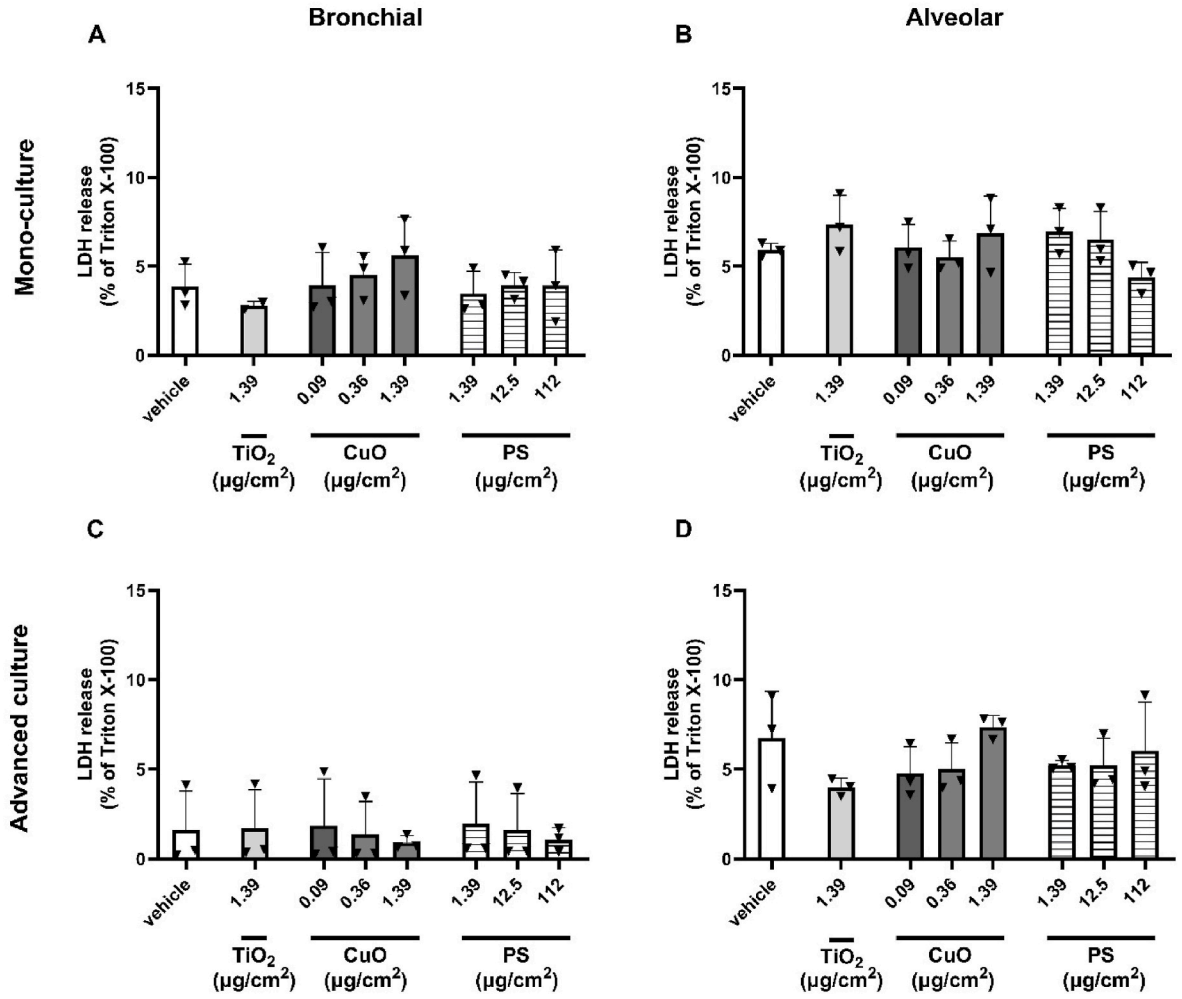

Fig. 2. Nanoparticles do not induce cytotoxicity in alveolar- and bronchial epithelial cells. Mono-cultures of BEAS-2B bronchial epithelial cells (**A**) A549 alveolar epithelial cells (**B**), differentiated cultures of primary bronchial epithelial cells (PBEC) (3 donors) (**C**) and alveolar co-cultures (**D**) were exposed to the vehicle control (dPBS, 0.05% BSA), copper(II) oxide (CuO), titanium dioxide (TiO_2) or polystyrene (PS) nanoparticles in dPBS, 0.05% BSA for 24 h. Nominal doses are displayed on the X-axis, in µg/cm^2. Thereafter, release of lactate dehydrogenase (LDH) was measured at the apical side. Maximum LDH release was achieved by exposure to 2% Triton X-100. Data shown represents the mean of 3 independent exposure experiments (individual experiments represented by triangles), each performed in technical triplicate. Pooled data are presented as mean percentage of the maximum LDH release +SD.

the apical than basolateral compartment. In contrast to the mono-culture, for the alveolar co-culture the fold change between the TNF-α stimulated cultures and the vehicle was similar between the apical (FC: 34.9 ± 11.9) and basolateral compartment (FC: 31.0 ± 3.7) (Table ST4). Baseline IL-8 secretion (unexposed) was higher in the alveolar co-culture, compared to the mono-culture. Although we observed different IL-8 levels for the different model systems, the relative increased total IL-8 protein levels after TNF-α exposure compared to the vehicle were consistent.

We did not observe any change in IL-8 protein secretion or *CXCL8* mRNA levels after PS or TiO_2 exposure, in any model (Figs. 3–4). In contrast, CuO exposure did result in a dose-dependent total increase of IL-8 secretion (FC: 2.2 ± 1.2) and *CXCL8* mRNA expression (FC: 4.1 ± 2.0) in bronchial mono-cultures and advanced PBEC cultures (FC: 2.6 ± 1.7 for protein levels and FC: 4.1 ± 1.8 for mRNA levels). Similarly, CuO exposure of alveolar mono-cultures increased IL-8 protein levels (FC: 2.0 ± 1.1) and *CXCL8* mRNA levels (FC: 1.6 ± 0.3). Similar effects were observed in the advanced alveolar co-culture (FC: 1.7 ± 0.3 for IL-8 protein levels and FC: 2.3 ± 0.3 for mRNA levels).

Altogether, PS exposure of both simple mono-culture-based ALI models and advanced ALI models did not result in an increase of IL-8 protein and *CXCL8* mRNA levels. However, TNF-α and CuO, which were included as positive controls, did induce such effects.

3.6. Nanoplastics interfere with the detection of IL-8

We tested whether or not the PS particles we used, at the doses included in this study, would interfere with the detection of recombinant IL-8 protein. As shown in Fig. S8, in the dose range 0–12.5 µg/cm^2, PS did not interfere with detection of the recombinant IL-8 protein (linearity between optical density and IL-8 standard concentration applied was observed). However, at the highest dose of PS (112 µg/cm^2), we observed significant interference between the PS particles and IL-8 protein evidencing interference of high doses of PS with detection of IL-8. No interference with IL-8 protein detection was observed for PA and PVC (S8). However, the recovery of (low amounts of) IL-8 protein after incubation with PP/Talc was not 100%, which indicate this particle did interfere when used in a dose of 7.14 µg/cm^2.

3.7. Impact of environmentally relevant nanoplastics on viability and transcription and secretion of IL-8 in BEAS-2B ALI cultures

We used the BEAS-2B model to screen for the cytotoxic and inflammatory potential of environmentally relevant nanoplastics. Again, TNF-

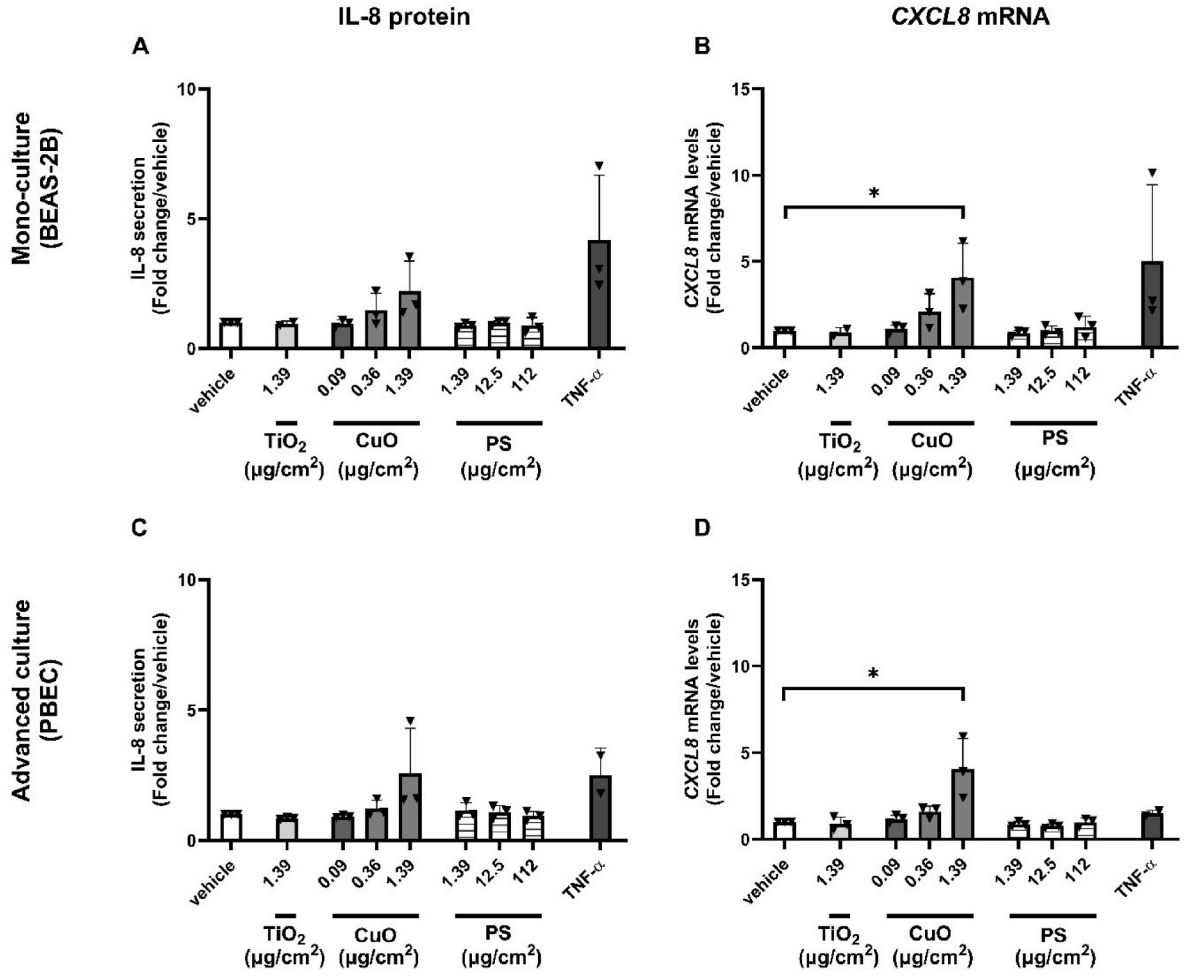

Fig. 3. Effect of nanoparticles on transcription and secretion of IL-8 in bronchial epithelial cells Airlifted cultures of BEAS-2B cells and Primary bronchial epithelial cells (PBEC) (3 donors) were exposed to copper(II) oxide (CuO), titanium dioxide (TiO_2) or polystyrene (PS) nanoparticles in 0.05% BSA for 24 h. Nominal doses are displayed on the X-axis, in μg/cm^2. IL-8 protein levels in both the apical and basolateral compartment were measured with ELISA and total (apical plus basolateral) IL-8 protein levels are expressed as mean fold change compared to the vehicle (0.05% BSA/PBS) (**A, C**). CXCL8 mRNA levels of the same cultures are shown as fold change compared to the vehicle control (**B, D**). Data shown of 3 independent exposure experiments (individual experiments represented by triangles), with exception of TiO_2 exposure of BEAS-2B cells and TNF-α exposure of PBEC that both were performed in 2 independent exposure experiments. Each experiment was performed in technical triplicate. Pooled data are presented as mean +SD. *P < 0.05 compared to vehicle control (ANOVA).

α did not induce significant cell death after 24 h of exposure. Also, none of the environmentally relevant nanoplastics affected cell viability at any of the doses used (Fig. 5A). The highest dose of CuO induced cell death compared to the vehicle control, with a difference of 4 percent (Fig. 5A). Furthermore, CuO exposure dose-dependently increased both *CXCL8* mRNA (FC: 8.1 ± 2.4) and total IL-8 protein secretion (FC: 2.5 ± 0.4). None of the exposures to nanoplastics resulted in a change of IL-8 transcript and protein levels (Fig. 5B–C).

4. Discussion

While airlifted cultures of alveolar and bronchial epithelial cells are widely used to determine nanoparticle toxicity, *in vitro* research on the effects of nanoplastics on human respiratory health has been largely limited to submerged exposure methods (Wright et al., 2024). In the present study, we compared the IL-8 and cytotoxicity responses of mono-cultures of bronchial- and alveolar epithelial cells (all in ALI cultures) to PS nanoplastics and reference nanoparticles to the response of more advanced (co-)cultures. In addition, to the best of our knowledge, this is the first study testing the impact of a range of environmentally relevant nanoplastics in ALI cultures of BEAS-2B cells.

Bronchial BEAS-2B cells and alveolar A549 cells have been extensively used in studies assessing the *in vitro* toxicity of airborne particles (Bessa et al., 2023; Hiemstra et al., 2018). Various studies reported different cellular responses to nanoparticles in ALI cultures compared to conventional submerged exposures (Hilton et al., 2019; Loret et al., 2016; Silva et al., 2023) However, the results between these studies comparing submerged and ALI exposure have been inconsistent. For example, some studies documented enhanced inflammatory responses of airlifted cultures of A549 mono- and co-cultures to zinc oxide nanoparticles compared to submerged cultures (Lenz et al., 2013). In contrast, other studies documented more sensitivity to silica and silver nanoparticles when these cultures were grown and exposed in submerged conditions compared to ALI (Herzog et al., 2014; Holder and Marr, 2013; Panas et al., 2014). The ALI alveolar co-culture used in the present study has previously been shown to be less sensitive to oxidative stress stimuli than when cells were cultured submerged (Klein et al., 2013). Culture methods can influence cellular features, including barrier integrity, mucus production and response to external stimuli in general (Silva et al., 2023). In addition, nanoparticles may deplete medium components in submerged cultures, leading to false positive cytotoxic effects (Casey et al., 2008). This suggests that to more accurately predict *in vivo* effects and avoid either under- or overestimation of cytotoxic effects, it is desirable to study nanoplastics in cells cultured at ALI.

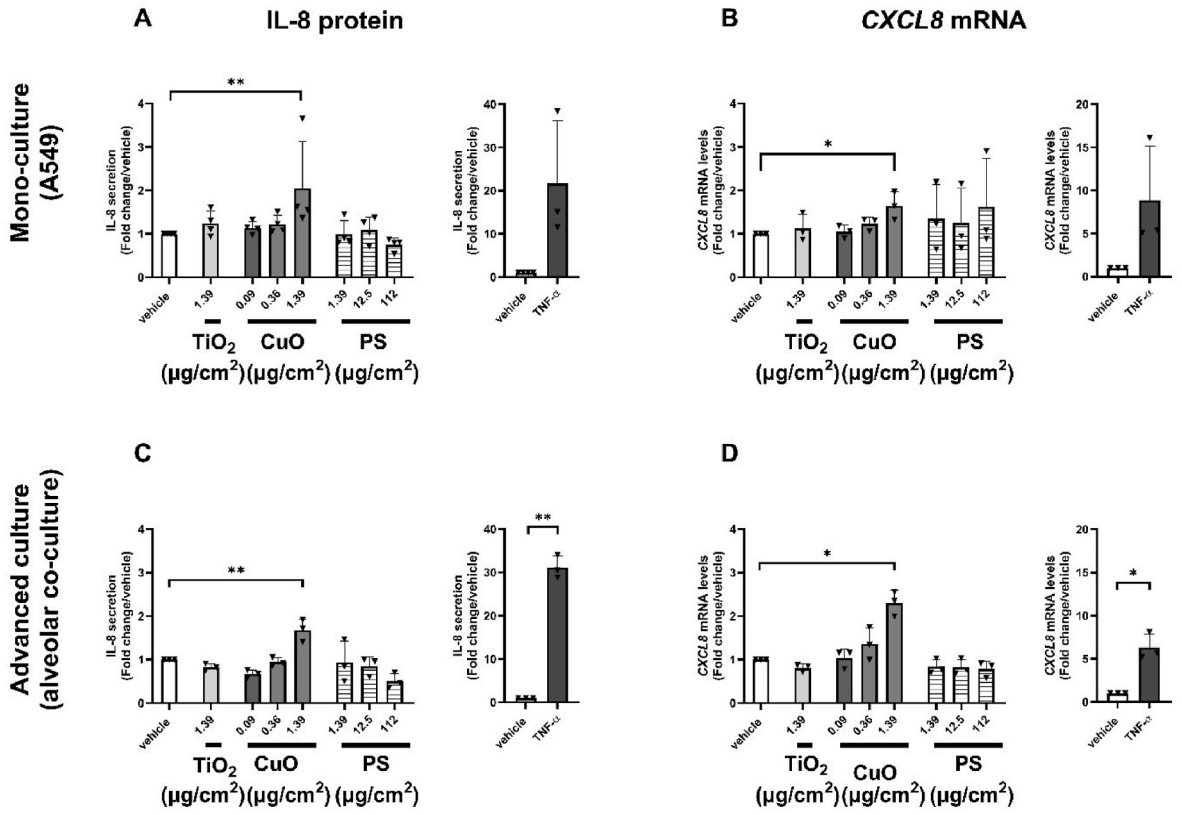

Fig. 4. Effect of nanoparticles on transcription and secretion of IL-8 in alveolar epithelial cells Airlifted cultures of A549 cells in mono-culture and alveolar co-culture with MΦ-THP-1 and EAhy926 cells were exposed to copper(II) oxide (CuO) titanium dioxide (TiO_2) or polystyrene (PS) nanoparticles for 24 h. Nominal doses are displayed on the X-axis, in µg/cm^2. IL-8 protein levels in both the apical and basolateral compartment were measured with ELISA and total IL-8 protein levels are expressed as mean fold change compared to the vehicle (0.05% BSA/PBS). CXCL8 mRNA levels of the same cultures are shown as fold change compared to the vehicle. Data shown of pooled technical replicates of at least 3 independent exposure experiments. Pooled data are presented as mean +SD. *P < 0.05, **P < 0.01 compared to vehicle control (ANOVA or Kruskal Wallis test for nanoparticles, *t*-test for TNF-α).

Obviously, mono-cultures of bronchial- or alveolar epithelial cells do not fully recapitulate the complex cellular composition of the lung and airway epithelium; therefore, we also included more advanced (co-) cultures of both alveolar and bronchial epithelial cells. ALI cultures of PBEC maintain the heterogeneity of cell types present in the bronchial epithelium, include cell-cell interactions, form a protective mucus layer and display cilia beating making these cultures more representative of the *in vivo* human situation. Additionally, for alveolar *in vitro* models, the crosstalk between alveolar epithelial cells and macrophages should be considered, since these tissue-resident immune cells form the first line of defence against inhaled nanoparticles (Marescotti et al., 2019). In this study, we have observed similar IL-8 protein and inflammatory gene responses to CuO nanoparticles in both mono-cultures and more advanced (co-)cultures. This suggests that for CuO, the use of co-cultures is not necessarily warranted for toxicity screening. However, due to the absence of toxicity for the nanoplastics in our study no conclusions can be drawn about the necessity of co-cultures for nanoplastic-toxicity investigation. Overall, both mono-cultures and advanced (co-)cultures offer unique advantages in studying nanoplastic toxicity. The choice of models may vary depending on the specific research questions.

When studying biological responses in mono-cultures or advanced (co-)culture models, it is important to consider the characteristics and dynamics of nanoparticles in ALI systems compared to submerged conditions. Submerged exposure studies often only report the administrated dose, which can differ significantly from the dose that actually reaches the cells (DeLoid et al., 2017). Nanoplastics can interact and agglomerate with media-components, stick to the walls of the well plate or have buoyance issues due to low densities. Applying small liquid volumes in ALI cultures, like the low droplet-volumes (10–40 µL) used in our study, allows for faster particle sedimentation and a more accurate delivered dose compared to traditional submerged exposures. However, a recent study identified that the minimum amount of liquid required for uniform coverage of a 12-well insert is 250 µL sparking concerns regarding the proportion of cells that is actually exposed to particles applied in a small droplet (Mallek et al., 2023). In this regard, we would like to address a few important points. First of all, for differentiated PBEC cultures (where we applied a single 10 µL droplet), it is well established that particles will be distributed across the surface of the culture due to ciliary beating (Khelloufi et al., 2018; Rayner et al., 2019). However, in cultures lacking active cilia beating (BEAS-2B cells), we observed unequal particle distribution across the cell culture surface. Although not uniform, a relatively large proportion of the cell culture surface was exposed, which is in line with previous studies performing single droplet exposures on ALI cultures (Frohlich et al., 2013; García-Rodríguez et al., 2024). Garcia-Rodríguez et al. reported that, in their study, 70% of the bronchial Calu-3 cells was associated with nanoplastics 24 h after applying a single droplet (20 µg/cm^2) with polyactic acid nanoplastics (García-Rodríguez et al., 2024). The localised particle deposition, in combination with agglomeration of particles is a limitation of our study. However, unequal particle distribution is not limited to droplet-exposures since this has also been observed after dry powder dispensers (Tilly et al., 2023) and particle aerosolization (Oldham et al., 2020).

Depending on their size, MNPs can reach different areas of the respiratory tract (Geiser and Kreyling, 2010). Estimating nanoparticle doses is challenging due to technical detection limitations. However, indoor exposure levels to microplastics (>1 µm) are reported to be higher than outdoor levels, significantly contributing to human

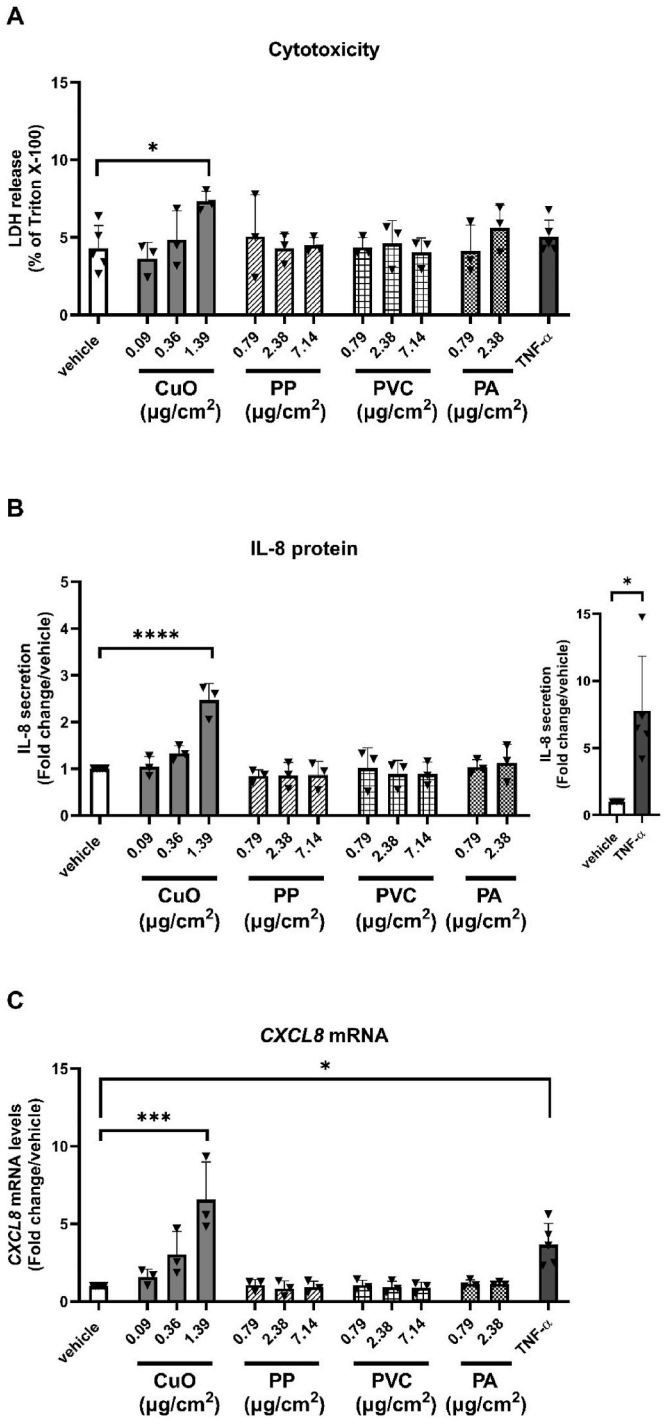

Fig. 5. Effects of environmentally relevant nanoparticles on bronchial epithelial cells Airlifted cultures of BEAS-2B cells (3 biological replicates) were exposed to copper(II) oxide (CuO), polypropylene/talc (PP/Talc) < 1 μm, polyvinylchloride (PVC) < 1 μm or polyamide (PA) < 1 μm nanoparticles for 24 h. Nominal doses are displayed on the X-axis, in μg/cm^2. IL-8 protein levels in both the apical and basolateral compartment are measured with ELISA and total IL-8 protein levels are expressed as mean fold change compared to the vehicle (0.05% BSA/dPBS/1% 1-propanol). CXCL8 mRNA levels of the same cultures are shown as fold change compared to the vehicle. Data shown of 3 independent exposure experiments (individual experiments represented by triangles), each performed in technical triplicate. Pooled data are presented as mean +SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared to vehicle control (ANOVA for nanoparticles, *t*-test for TNF-α).

microplastics exposure since Europeans spend around 90 % of their time indoors (Dris et al., 2017; Schweizer et al., 2007; Torres-Agullo et al., 2022). Daily adult indoor airborne microplastics exposure has been estimated to be 152 microplastics/kg body weight or 507 microplastics/kg body weight, depending on the type of sampling (Eberhard et al., 2024). For a person of 88 kg, with a total airway surface area of 2471±320 cm^2, this would correspond to an exposure of 5–18 microplastics per cm^2 per day (Mercer et al., 1994). Considering the low-weight of the nanoplastics, the lowest dose tested in the present study (0.79 μg nanoplastics/cm^2) exceeds these daily exposure levels of the bronchial epithelium. However, these superficial values do not take into account the regional deposition, particle clearance and/or accumulation (Löndahl et al., 2014). Doses for *in vitro* toxicology studies are usually set based on the maximum tolerated dose (MTD), defined as the highest dose that does not lead to unacceptable toxicity. However, we found that nanoplastics could interfere with absorbance measurements, which are the basis of many toxicological assays. This interference causes significant errors and misinterpretation of results. Due to the high binding capacity of nanoplastics, each *in vitro* assay has to be evaluated for each nanoparticle being studied. Particle interference can be limited by lowering the particle doses and using multiple end-point assays.

Since the health effects of nanoplastics are largely unknown, it is important to compare their toxicity to the effects of well-studied non-plastic airborne nanoparticles, like TiO_2 and CuO nanoparticles (Kooter et al., 2017). We found a dose-dependent increase of IL-8 secretion in response to CuO particles in all cell culture models, consistent with a previous study using similar doses in PBEC (Kooter et al., 2017). In contrast, the same dose of TiO_2 did not result in an inflammatory response in any of our cell culture systems. TiO_2 can have various crystal structures, including anatase and rutile, each found to have different catalytic activity and toxicity. *In vivo* studies have shown genotoxic effects of anatase TiO_2, highlighting the need for thorough analysis, considering these nanoparticles are widely used in food products, cosmetics and pigments (Wani and Shadab, 2020). However, studies on the impact of TiO_2 in ALI cultures of human bronchial- and alveolar epithelial cells do not always replicate the *in vivo* effects (Wani and Shadab, 2020). Furthermore, the dose tested in our study is lower than the calculated maximal accumulated amount of TiO_2 nanoparticles in the alveoli over a full working lifetime (45 years), which is between 10 and 50 μg/cm^2, when exposure is set to 1 mg/m^3 (Gangwal et al., 2011). Anatase TiO_2 nanoparticles (doses up to 26 μg/cm^2), in contrast with CuO nanoparticles, did not alter expression of genes related to oxidative stress response and DNA repair (Hufnagel et al., 2020). Similar results were found in a study on airlifted A549 cells which only observed cytotoxic effects after exposure of 10 μg/cm^2 TiO_2, but not in lower doses (Andersson et al., 2011). In addition, the same study showed these effects were only observed for TiO_2 nanofibers and not for TiO_2 nanoparticles. These findings suggest that not only dose, size and crystal structure are influencing TiO_2 toxicity, but also shape, as it affects interactions with cells and tissue.

Although most research has focused on ingestion routes, an increasing number of studies have recently addressed the effects of inhalation of PS nanospheres *in vivo*. Exposures to PS nanoplastics has been shown to cause structural changes, oxidative stress and inflammation in the lung, as evidenced by histopathological abnormalities, increased cytokine levels and immune cell infiltration (Prata, 2018). Interestingly, PS nanoparticle inhalation also resulted in dysfunction in other organs, including the heart and liver, suggesting translocation across epithelial barriers and blood-born transport (Cary et al., 2024; Ge et al., 2024; Zhang et al., 2023). Limited studies have used *in vitro* models of alveolar- or bronchial epithelial cells to investigate toxicity of PS particles. Most studies have been performed on submerged cultures of bronchial and alveolar cells (Wright et al., 2024). Similar to our results, no cytotoxic effects or increased IL-8 secretion were observed in ALI A549 mono- and co-cultures exposed via a small droplet to PS nanoplastics (200 nm) after 24 h. However, the same study did observe

increased IL-8 secretion after prolonged exposure (14 days) (Meindl et al., 2021). Another study on ALI A549 cultures did observe cytotoxic effects 24 h after PS (20 nm) nebulisation (Frohlich et al., 2013). However, this study used amine-modified PS particles, which have a positive charge, generally correlated with a higher particle uptake (Sukhanova et al., 2018). Surface groups, surface charge, as well as exposure duration might explain differences in toxicity between studies (Jeon et al., 2018; Sukhanova et al., 2018).

In contrast to PS, which has been described in literature previously (as discussed above), studies using other plastic polymers are largely lacking. In our study, we did not observe effects of PA, PVC and PP/Talc nanoplastics on cytotoxicity or IL-8 protein and mRNA levels in BEAS-2B cells. To the best of our knowledge, there are no *in vivo* inhalation studies studying the effect of PP, PA or PVC nanoplastics. Limited studies have focused on *in vivo* inhalation of PP, PA or PVC microparticles. Occupational doses of PA-12 did not result in any acute effect on pulmonary inflammation in rats, although systemic inflammation and impairment of vascular dilation was found (Cary et al., 2023). Intratracheal instillation with PP and PVC microplastics of three strains of mice revealed that inflammatory cell numbers in bronchoalveolar lavage fluid of PVC-instilled mice remained unchanged, whereas PP increased the number of macrophages and neutrophils in one strain (Kwabena Danso et al., 2024).

PP, PA or PVC nanoplastics have not been studied using either submerged or ALI cultures of BEAS-2B cells before, and only a few studies are available that used A549 cells. For better comparison with our study, we converted the doses reported in these studies, usually given as mass per volume (μg/mL), to deposited mass per surface area (μg/cm^2). PP nanoplastics (produced by pyrolysis of spherical beads), only caused cytotoxic effects in extremely high doses (~400–1250 μg/cm^2) in submerged A549 cultures; these effects were not observed at lower doses (Woo et al., 2023). Similarly, the same study reported mitochondrial damage and induction of an inflammatory response only after exposure of the cells to the highest dose of PP. In addition to PP, the only two exposure studies performed on A549 cells with PA nano- and microplastics reported no effects on cytotoxicity or IL-6 or IL-8 levels for doses up to ~62.5 μg/cm^2 (submerged) and ~3.5 μg/cm^2 (ALI) (Chortarea et al., 2022; Irfan et al., 2013). However, a recent study using PA microplastic fibres demonstrated an inhibition of airway organoid differentiation, mediated by compounds released from the fibres (leachate) (Song et al., 2024). The role of compounds leaking out of plastic particles, especially additives, in respiratory toxicity was also studied by H. Xu et al. (2003). The authors demonstrated high doses of PVC particles <3 μm (~1562 μg/cm^2) increased IL-8 and IL-6 secretion in A549 submerged cultures only when additives were present; their additive-free counterparts did not show these effects. PVC microplastics elevated levels of inflammatory proteins and proteins linked to senescence in A549 submerged cultures (Jin et al., 2024).

Research evaluating the effects of environmentally relevant particles in lung and airway epithelial cells at ALI remain scarce. Future research should move away from engineered PS spheres and focus on the effects of nanoplastics in relevant ALI cultures, to better mimic real-life exposure scenarios.

Although this study is the first study testing the effects of environmentally relevant nanoplastics on BEAS-2B ALI cultures and brings novel insights, some limitations should be considered. First of all, extrapolation of these *in vitro* findings to actual risk of nanoplastics for health of the respiratory system in humans warrants caution. Although small droplet exposure of ALI cultures is more realistic than submerged exposure, it is still not fully mimicking *in vivo* exposures. Liquid application can alter physiological characteristics of ALI cultures, such as decreasing barrier integrity, increasing cytokine secretion and affecting gene expression (Mallek et al., 2023). However, these effects have only been studied for 250 μL, not for smaller liquid volumes. IL-8 protein secretion was unaffected by small droplet application (10–40 μL), but further research is needed to explore other impacts of this small volume liquid application on ALI cultures. Aersosol exposures with a cloud chamber have been widely used to investigate inhalation toxicity of nanomaterials and could provide a promising tool for studying the toxicity of nanoplastics (Lacroix et al., 2018). However, this approach is technically more demanding and requires larger quantities of nanoplastics than small volume liquid application since particle deposition efficiency is lower (Bannuscher et al., 2022). In our study, as well as in aerosol exposures, nanoparticles are suspended in liquid, which can alter particle characteristics and consequently toxicity outcomes (Lichtveld et al., 2012). To overcome this limitation, dry powder dispensers can be considered (Tilly et al., 2023). Additionally, it must be considered that microplastics can adsorb and accumulate toxic chemicals, micro-organisms and other biomolecules from the environment. Also, natural weathering processes, like UV exposure, may further exacerbate their potential health impacts on the respiratory system. Although this study did not show acute inflammatory responses to environmentally relevant nanoplastics, prolonged exposure may lead to chronic lung inflammation and tissue damage (García-Rodríguez et al., 2024; Meindl et al., 2021). Future strategies should implement additional readouts, including membrane integrity and cell-specific effects, as well as repeated dosing. Additionally, including PBEC from patient populations could provide an opportunity to identify if certain individuals are more susceptible for detrimental effects of nanoplastic exposure.

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CRediT authorship contribution statement

I.F. Gosselink: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **F.J. van Schooten:** Writing – review & editing, Supervision, Project administration. **M.J. Drittij:** Investigation. **E.M. Höppener:** Visualization, Investigation. **P. Leonhardt:** Investigation. **E. Moschini:** Writing – review & editing, Visualization, Methodology, Investigation. **T. Serchi:** Resources, Methodology. **A.C. Gutleb:** Writing – review & editing, Resources, Methodology. **I.M. Kooter:** Supervision, Conceptualization. **A.H. Remels:** Writing – review & editing, Supervision, Project administration, Conceptualization.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143702.

Data availability

Data will be made available on request.

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