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Metrological Characterization of an Aerosol Exposure Chamber to Explore the Inhalation Effects of the Combination of Paraquat and TiO_2 Nano-objects

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ABSTRACT

Agriculture emits a significant quantity of airborne contaminants, and the prospective environmental release of nanopesticides, a new type of agrochemical that employs engineered nanomaterials (ENMs) as either active substances or additives in a pesticide formulation, raises concerns about the risks of inhalation which are still unknown. Although the adverse effects of pesticides have been studied extensively, the potential synergistic toxicity between these substances and ENMs has rarely been investigated. To this end, toxicological models are essential to estimating the health consequences of such aerosols. Thus, to assess the respiratory hazards of titanium dioxide nano-objects (specifically, AEROXIDE® TiO_2 P25 nanopowder [nTiO_2]) in combination with paraquat (PQ), we developed a dynamic whole-body exposure chamber for rodents in compliance with guidelines for inhalation toxicity testing (Organization for Economic Cooperation and Development (OECD)) and animal welfare. First, we metrologically characterized the generated test aerosols by determining their mass and number concentrations, size distributions and atmospheric homogeneity at the laboratory. Then, we evaluated the reproducibility and proper functioning of the chamber during a preliminary field campaign, which validated the consistency of the aerosols' mass and number concentrations between the laboratory characterization and the rodent exposure sessions. Finally, we examined the inhalation effects on the rodents.

Keywords: Aerosol, Inhalation paraquat, TiO_2 nano-objects

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1 INTRODUCTION

Epidemiological studies highlight converging evidence suggesting a link between the professional and residential exposures to specific pesticides and chronic illnesses such as neurodegenerative diseases and cancers (INSERM, 2013; Kim *et al.*, 2017). Pesticides represent an occupational risk for farmers, pesticides applicators and manufacturers, but also for the general population who may be indirectly exposed to pesticides during their application or because of their persistence in the air (Lu *et al.*, 2000; Coscollà *et al.*, 2013; Brouwer *et al.*, 2017; Mattei *et al.*, 2019). Engineered nanomaterials (ENMs) are generally defined as novel materials designed at the nanoscale, with external or internal structures under the size of 100 nm (e.g., ISO/TR 18401:2017). ENMs could be used either as active ingredients or co-formulants, to provide nanopesticides (NPEs) proposing a reformulation of conventional pesticides. They are expected to enhance the efficiency of agrochemicals and to mitigate the environmental footprint of modern agriculture. Despite the lack of data on their current use, the number of granted patents associated with ENMs in agriculture grew significantly starting in the 1990s to reach 1,254 patents in 2016 (Kah *et al.*, 2019).

Moreover, some NPEs have already been identified on the market (Kumar *et al.*, 2019), showing that those products are likely to emerge in a near future. As the fate of NPEs is still not understood, some authors have already emphasized the knowledge gap surrounding the environmental impact of these emerging products (Kah *et al.*, 2018) and some research agencies have integrated these new compounds as a healthcare priority (USDA, 2015).

Several studies analyzed the presence of pesticides in the atmosphere (Coscollà *et al.*, 2010; Estellano *et al.*, 2015; López *et al.*, 2017; Désert *et al.*, 2018). Their air concentrations ranged from few picograms to hundreds of nanograms per cubic meter. Nevertheless, the air concentration of pesticides can be locally much higher in treated parcels during the seasonal spreading. For instance, a study with paraquat (PQ) sprayers showed that the PQ mass concentration can reach 125 μg m^{-3} in the breathing zone during work (Morshed *et al.*, 2010). Another study assessed the size distribution of the pesticide residues in a French rural area (Coscollà *et al.*, 2013). Most pesticides were accumulated in the particulate phase, especially in the fine size range (0.1–1 μm), which represent an inhalable fraction, whose size distribution enables particles to be deposited in the respiratory tract. Accordingly, inhalation toxicology studies appear necessary to assess the risk associated with NPE exposure. However, to our knowledge no toxicological data are available on NPE inhalation effects, involving both nano-objects and their aggregates and agglomerates (NOAAs), and pesticides.

A validation of the inhalation facility with characterized aerosol parameters is needed to conduct animal inhalation studies that will provide toxicity data for the risk assessment of nanopesticides. Among the variety of inhalation devices used for animal experimentation, there are two main exposure modalities, namely nose-only and whole-body devices (OECD TG 403; Yeh *et al.*, 1990). The nose-only strategy facilitates the control of the exposure dose for each animal. Animals are not able to move during the aerosol exposure, as they are restrained in a tube to be exposed individually by limiting other routes of exposure, such as the dermal and oral routes (Dorato and Wolff, 1991). However, the stress induced by restraint during gestation could be problematic, notably in case of repeated exposures. By contrast, in whole-body devices, subjects are immersed within the chamber atmosphere, which reflects environmental or occupational exposure scenarios, which are less restraining for animals since they are able to behave naturally. Whole-body exposure chambers encompass various types of chambers, using single-animal holders or large-sized compartments for long-term studies using numerous subjects (Wong, 2007). They are particularly suitable for chronic studies or exposure during the gestation to minimize the stress induced by treatments. It must be noted that animals may be exposed by skin, as they collect particles on their fur during the whole-body exposures (Griffis *et al.*, 1979), which can lead to the particle ingestion during grooming. Nevertheless, as inhalation and dermal routes are considered as the main occupational exposure pathways to pesticides (Damalas and Eleftherohorinos, 2011), this additional source of exposure could be relevant to consider.

To comply with the Organization for Economic Cooperation and Development (OECD) test guidelines 413 on subchronic inhalation toxicity (OECD TG 413), the physicochemical characterization of ENMs is necessary prior to the aerosol exposures. Currently, AEROXIDE® TiO_2 P25 nanopowder ($nTiO_2$) is one of the most studied ENMs in toxicology and it has already been characterized in a previous study in terms of crystalline structure, size of primary particles, specific surface area and chemical composition (Motzkus *et al.*, 2014). Additionally, $nTiO_2$ appears as a good candidate to be integrated as a nano-additive in NPEs for its valuable properties in agriculture (Wang *et al.*, 2016), such as its photocatalytic activity, which can be used to degrade PQ (Florêncio *et al.*, 2004). PQ is one of the most worldwide used herbicide, despite the fact it is suspected to be associated with the occurrence of the Parkinson disease among farmers (Kamel *et al.*, 2007; Costello *et al.*, 2009; Tanner *et al.*, 2011). PQ and $nTiO_2$ under the form of a colloidal suspension may constitute a model of NPE with interesting features based on the technological inputs brought by $nTiO_2$ in agriculture and its ability to decrease the environmental PQ half-life in soils which could reach several years (Sartori and Vidrio, 2018).

Given the fact that the nanopesticide inhalation effects are still not addressed in the literature, the present study is the first necessary step to establish a proof of concept of a transdisciplinary methodology combining aerosol metrology and toxicology to address properly this question. The present paper is dedicated to the metrological characterization of an aerosol exposure chamber to explore the inhalation effects of the herbicide PQ combined with $nTiO_2$ used as reference

materials. The second step consisting in the neurotoxicological assessment of these aerosols, will be addressed separately in upcoming dedicated articles. Thus, a new whole-body exposure chamber was developed to explore the inhalation effects of aerosols. It is a simple device which can fit easily in any animal facility to be operated safely by conforming with animal welfare. As a complete metrological set-up cannot be implemented permanently in an animal facility for long-term studies, the characterization had to be made prior to the animal exposure. The characterization was made in the LNE (Laboratoire National de métrologie et d'Essais) laboratory, in terms of size distribution (Scanning Mobility Particle Sizer [SMPS] + Aerodynamic Particle Sizer [APS]), mass concentration (Tapered Element Oscillating Microbalance [TEOM] + indirect measurements of the sampled mass on 47-mm filters), number concentration (Condensation Particle Counter [CPC]), temperature, pressure and relative humidity. In addition, based on the particle size distribution, the chamber geometry and the airflows, computational fluid dynamics (CFD) modeling was used to investigate the homogeneity of the particle concentration within the cage. The main goal for the CFD simulation was to investigate the fill-up process of the chamber, the possibility of error induced by the relative positions of the injection tube and the measurement sampling tube. These parameters were assessed in accordance with the standard method on the reproducibility measurement (ISO 5725-2:2020).

Because it is essential to validate the functioning of the characterized aerosol exposure chamber on the field, here we also report the comparison of the measurements made in the LNE metrological lab (without animal) in regard to the first preliminary data obtained in the animal facility (with animals) for each of the three aerosols. During the animal exposure, a reduced monitoring of the aerosol was used to check the proper aerosol generation, to enable a precise metrological follow-up of the exposures. The mass concentration and the particle number concentration were therefore used to validate animal exposure sessions in accordance with the metrological characterization phase.

2 MATERIALS AND METHODS

2.1 Inhalation Exposure Chamber

The exposure chamber is composed of a rodent cage used as a whole-body exposure system (Fig. 1). The chamber was sealed and customized with antistatic silicon pipes to be integrated within the generation device. This cage, initially dedicated to rodents hosting (rats, mice, hamsters or others) in animal facilities, is made out of polysulfone (internal volume of 19.8 L, overall dimensions of 395 × 346 × 213 mm [W × D × H]; GR900; Tecniplast). It was selected for the chemical resistance and the transparency of this material (Tuttle *et al.*, 2010), which allowed a visual follow-up of animals during the exposure sessions. Coupled with this chamber, an aerosol nebulizer (Model 3076; TSI Inc.) was operated to generate three aerosols of interest, i.e., $nTiO_2$, PQ and $nTiO_2$ with PQ produced from daily prepared colloidal suspensions and solutions with bulk powders of $nTiO_2$ (75% anatase, 25% rutile; AEROXIDE P25; Evonik [Reference 718467 Sigma-Aldrich]) and PQ dichloride hydrate (Reference 856177; Sigma-Aldrich, France). All colloidal suspensions and solutions were prepared using ultrapure water (18.2 MΩ cm resistivity; Milli-Q; Millipore) with $nTiO_2$ and PQ concentrations of 3 g L^{-1} and 28 mg L^{-1} respectively and were kept under constant stirring during the nebulization process using a magnetic stirrer. These concentrations were used in order to produce aerosols achieving the target concentrations. Concerning the $nTiO_2$ use in nanopesticides, it is not possible to know the precise mass concentration to which people could be exposed in link with professional or residential exposures. Nevertheless, some authors investigated the mass concentrations of $nTiO_2$ in Korean production sites, thanks to personal sampling and real-time monitoring using SMPS (Lee *et al.*, 2011). As a result, they highlighted that TiO_2 mass concentrations can reach 4.99 mg m^{-3}, involving a particle size range of 15–710.5 nm. In addition, to be comparable with other studies dealing with $nTiO_2$ inhalation that showed relevant toxicological endpoints using rodents (Bermudez *et al.*, 2004; Disdier *et al.*, 2017; Chézeau *et al.*, 2019), a target concentration of 10 mg m^{-3} was chosen.

Concerning PQ, the minimum lethal mass concentration in rats from a single 4-h inhalation exposure was reported to be 0.6–1.4 mg m^{-3} (McLean *et al.*, 1985). Moreover, a 3-week inhalation study in rats reported the lowest observed adverse effect level (LOAEL) of 100 μg m^{-3}, based on

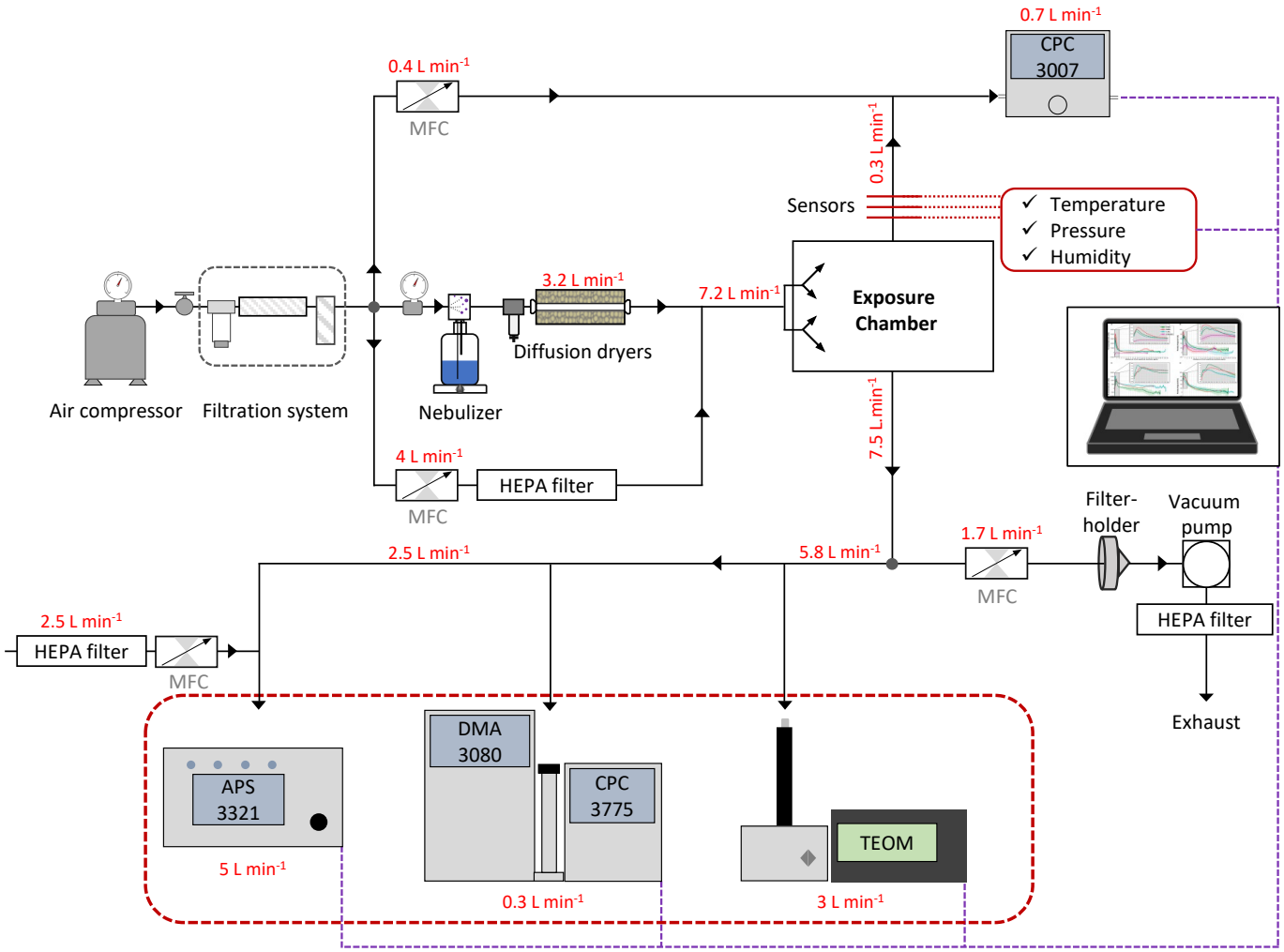

Fig. 1. Experimental set-up of the inhalation exposure chamber. The part surrounded by a red dotted line corresponds to the instruments used only for the metrological characterization phase; the different flowrates are presented in red. MFC: mass flow controller.

the histopathological changes in the upper respiratory tract (Grimshav *et al.*, 1979). Thus, the target concentration of 100 μg m^{-3} corresponding to the National Institute for Occupational Safety and Health (NIOSH) occupational limit was chosen to avoid pain and distress. This concentration is also in line with the 125 μg m^{-3} occupational exposure reported by Morshed *et al.* (2010). The mixture being composed of both $nTiO_2$ and PQ aerosols, its target concentration was assumed to be 10.1 mg m^{-3}. The doses were chosen to be both relevant doses and realistic to cause slight adverse effects in the lungs due to inflammation and oxidative stress, in order to investigate any potential synergy effects due to the mixture. Briefly, these concentrations can be extrapolated to human beings based on the aerosol characterization using the Multiple-Path Particle Dosimetry Model (MPPD), and they are comparable with the NIOSH recommendations for $nTiO_2$ and PQ. The target concentration choice was made to highlight any possible cocktail effects between $nTiO_2$ and PQ within a toxicology protocol involving chronic exposures.

The nebulizer (Model 3076; TSI Inc.) produced stable particle concentrations using compressed air (Pujalté *et al.*, 2017b). It was operated at 2.4 bar which supplied a constant flowrate of 3.2 L min^{-1}. The compressed air was supplied by a silent air compressor coupled with a filtration and drying device (Model 3074B; TSI Inc.). The produced airflow enabled a controlled dilution of the generated aerosols entering the exposure chamber. It was also dedicated to the dilution of a CPC to prevent the instrument upper range (10^5 particles cm^{-3}) to be exceeded. The instrument airflow was checked for each aerosol generation to adjust concentrations with the proper dilution factor. Two downstream diffusion dryers were used to obtain aerosols with a steady relative humidity

during the assays. The aerosols were injected horizontally thanks to a four-branched input in the top of the chamber in order to optimize the dispersion of particles. APS, SMPS and TEOM, which were present only during the characterization phase, needed a total flowrate of 5.8 L min^{-1}. SMPS and TEOM airflows were not diluted while a dilution of the APS flowrate was set at 2.5 L min^{-1}. The sample outlet flowrate of the exposure chamber was therefore set at 7.8 L min^{-1} thanks to the global instrumental flowrate (5.8 L min^{-1}), the filter holder sampling (1.7 L min^{-1}) and the CPC (0.3 L min^{-1}). In absence of the instruments used for the metrological characterization phase, a 7.5 L min^{-1} flowrate was set for the filter-holder sampling line. To comply with OECD TG 413 guidelines, temperature, pressure, relative humidity and airflows were monitored in the chamber as environmental parameters during the characterization.

2.2 Aerosol Characterization and Monitoring

To characterize particle number size distributions (PNSDs), mass and number concentration, a multi-instrumental set-up composed of a real-time monitoring and off-line aerosol samplings was used (Fig. 1, part surrounded by a red dotted line). PNSDs of submicronic aerosols were measured thanks to an SMPS (TSI Inc.) composed of a differential mobility analyzer (DMA; Model 3081; TSI Inc.) and a CPC (Model 3775; TSI Inc.). An APS (Model 3321; TSI Inc.) monitored the 0.6–20 μm particle size range. At the top of the exposure chamber, a CPC (Model 3007; TSI Inc.) was set up to measure the total particle number concentrations in real time during the metrological characterization and during the exposure sessions. Total mass concentrations were measured in real time using a TEOM (Model 1400; Thermo Fisher Scientific Inc.) set at 50°C during the characterization, and a 47-mm filter holder allowed a gravimetric monitoring of aerosols during the exposure phase. This latter gravimetric measurement was performed to assess the mass concentrations, by using the mass difference between pre- and post-sampling filters, to be compared with the real-time aerosol mass concentrations assessed with TEOM. The aerosol filtration was made by a filter holder with 47-mm filters (Pallflex® Emfab TX40HI20WW; Pall Corp.) made out of borosilicate glass microfibers reinforced with woven glass cloth and bonded with PTFE. The filter holder was used to enable a complementary indirect gravimetric measurement during the characterization phase as well as the exposure phase. Gaie-Levrel *et al.* (2018) reported the use of these filters, which filtration efficiency was measured to characterize the most penetrating particle size (MPPS). For the size range of 100 nm particles, the filtration efficiency was 99.97%. Consequently, the sample's fraction of particles on 47-mm filters is representative of the total mass concentration. This filter holder was also coupled with a downstream high-efficiency particulate air (HEPA) filter to prevent any potential particle release during the experiment. Filters may adsorb water, which would alter the quality of particle mass concentration assessment. Nevertheless, our experimental procedure was compared with the reference values of mass concentrations measured with TEOM during the metrological characterization phase to check the consistency between both measurement techniques. A maximum deviation of 3% was obtained by comparing TEOM results with gravimetric analysis on filters.

The TEOM mass concentration measurements were not possible to achieve on a daily basis during the exposures of animal. Therefore, thanks to the 47-mm filter holder, the mass concentration was measured for each experiment, based on the mass difference between pre- and post-sampling filters. These concentrations were used to check the steady functioning of aerosol generation during animal exposure, using as a reference the TEOM characterization data made in real time during the characterization.

For the metrological characterization, 10 experiments of 2 h were performed on five days without animals, in order to characterize each generated aerosol, i.e., PQ, $nTiO_2$ and PQ + $nTiO_2$. To assess the reproducibility of the generation method, the relative reproducibility standard deviation (S_R) according to the ISO 5725-2 standard (ISO 5725-2:2020) was reported. Due to the containment requirements of the animal facility (biosafety levels 1, 2 and 3), all objects entering the building must be sterilized and only workers accredited in animal experimentation can use the facility. As a consequence, it was technically impossible to use all the metrology instruments *in situ* within the animal facility. Therefore, the characterization phase was performed prior to animal experimentation without animal, to use the average number and mass concentrations as tracking parameters to validate the proper aerosol generation during the animal exposure. The exposure campaign, in presence of mice, consisted in 11 exposure sessions of 1.5 h for each aerosol,

during which the mass and number concentrations were measured to assess the reproducibility of the aerosol generation procedure, in order to validate the operating device on the field.

2.3 Aerosol Dispersion Modeling

To study the atmosphere homogeneity in the exposure chamber, simulations of the aerosol dispersion were performed for PQ and $nTiO_2$ aerosols using COMSOL Multiphysics software (version 5.4). A computer-aided design (CAD) model of the test chamber was built and imported in COMSOL. Using the symmetry of the device, half of the chamber was modeled in order to reduce computational time. A preliminary simulation was run to characterize the flow behavior inside the exposure chamber without particles. Reynolds number in the injection tube is evaluated around 1000; hence, a laminar flow formulation of Navier-Stokes equations was used. As the exposure chamber is small, and the ambient temperature is maintained for the duration of the test, it can be assumed that Brownian diffusion will not play an important part in the dispersion of the particles inside the chamber. An order of magnitude computation using the AeroCalc spreadsheet based on Willeke and Baron (2001) shows that diffusion losses are expected to reach a maximum of 2%, given chamber dimensions, flow rate and ambient parameters. It must be noted that such a computation gives a general order of magnitude of the diffusion contribution, which can still be significant at small spatial scale and yield local non-uniformities. Similarly, the small size of the particles leads to negligible inertial effects. Hence, the dispersion analysis was conducted using a passive scalar method. Navier-Stokes equations are resolved on a tetrahedral unstructured mesh with dedicated boundary layers meshing. The mesh includes around 568 k elements, with an average element quality (skewness) of 0.67. Initial conditions are set with no velocity and no particles inside the chamber. At time point T = 0, the fluid flow is ramped up on 3 s, with a target velocity of 0.64 m s^{-1} at both inlets using the experimental flow rate and an input concentration of 1000 particles m^{-3}. The outlet was set at a constant pressure difference of –60 Pa. The simulation was run for 900 s (15 CPUs, 32 GB RAM) of simulated time with a total computation time around 80 min.

2.4 Paraquat Stability

Thanks to a UV–visible spectrophotometer (LAMBDA 25; PerkinElmer), suspensions of PQ with $nTiO_2$ were analyzed at different times, i.e., T = 0, 1, 2 and 24 h, to assess the possible photocatalytic degradations of PQ due to $nTiO_2$. For PQ, the absorbance peak was selected at 257 nm and by-products of degradations can be potentially observed in the 200–230 nm range (Cantavenera *et al.*, 2007). For instrumental calibration, aqueous solutions of PQ with concentrations ranging from 6.25 to 40 mg L^{-1} were used. As $nTiO_2$ absorbs in the UV-visible wavelength, suspensions were filtered (pore diameter of 0.22 μm; Millex-GS Syringe Filter Unit; Millipore) prior to analysis in order to prevent $nTiO_2$ aggregates to interfere with measurements.

3 RESULTS

3.1 Environmental Parameters

Temperature (T, °C) and relative humidity (RH) were monitored for 2-h sessions within the exposure chamber during the characterization phase (Fig. S1 in the supplementary data). Average T°C and RH were characterized to be 24.5 ± 0.4°C and 20.1 ± 0.7% respectively with external laboratory conditions of 22.5 ± 1.2°C and 42.5 ± 7.8%. The difference in temperature was induced by the compressor operating temperature, which was comprised between 50–70°C, explaining the slight increase of temperature along the duration of nebulization, and the temperature difference with the lab conditions. At the outlet, a vacuum pump enabled a slight depression in the chamber (–60 ± 4 Pa) preventing the aerosol leakage in the laboratory. Based on the outlet airflow and the chamber volume, the air renewal was 23 volumes h^{-1}.

3.2 Particle Number Size Distribution

Figs. 2(A) and 2(B) present the average PNSD measured in the exposure chamber during the characterization phase using SMPS and APS. They were unimodal and broadly ranged from 10 to

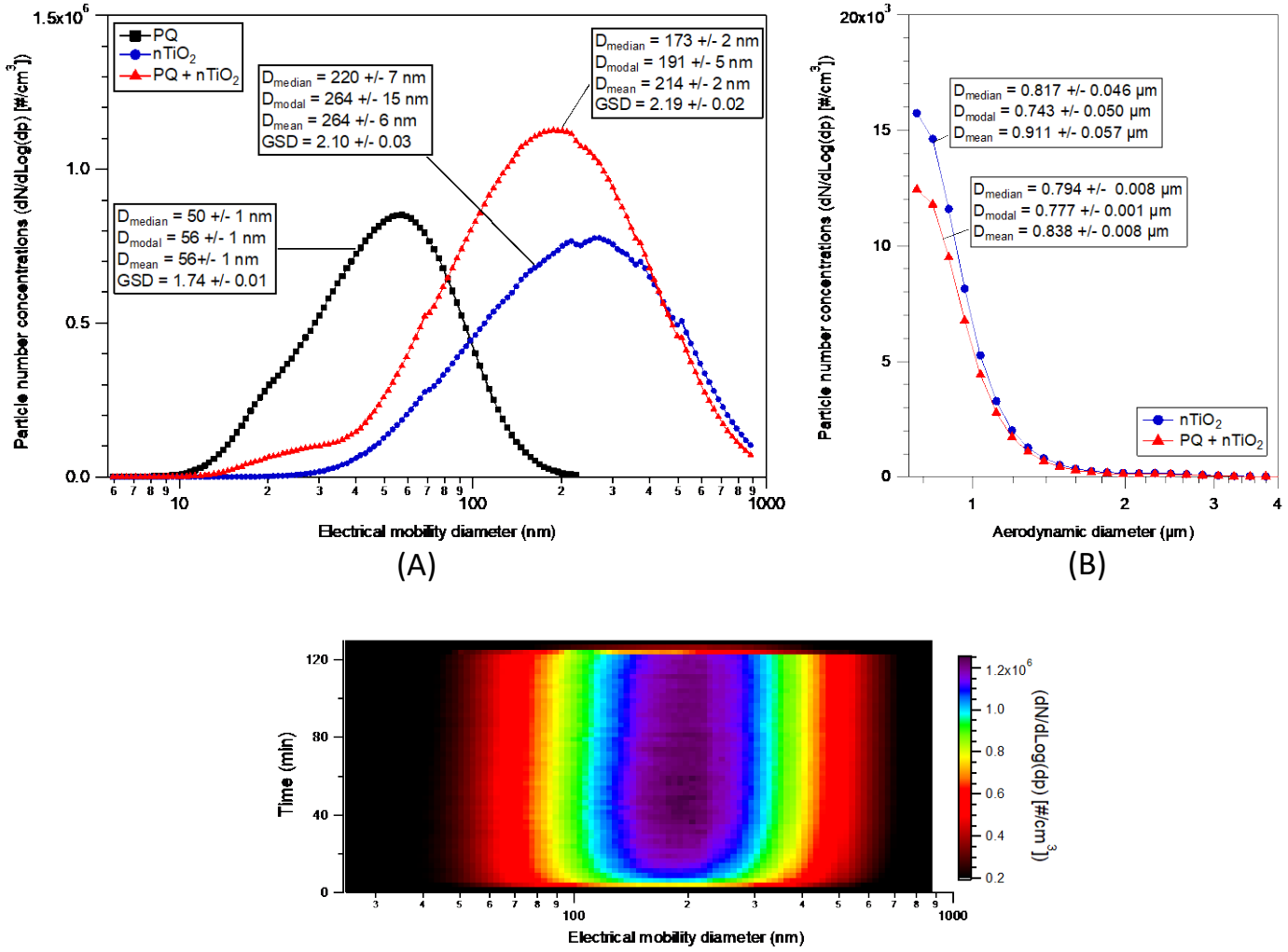

Fig. 2. Average PNSD measured in the exposure chamber during the metrological characterization phase using (A) SMPS and (B) APS without animals, for each generated aerosol, i.e., PQ, $nTiO_2$ and PQ + $nTiO_2$. (C) PNSD temporal stability based on SMPS measurements for the PQ + $nTiO_2$ aerosol.

200 nm for PQ and from 15 to 2000 nm for $nTiO_2$ and PQ with $nTiO_2$. The count median mobility diameters (CMMDs) were respectively 50 ± 1 nm, 220 ± 7 nm and 173 ± 2 nm for PQ, $nTiO_2$ and PQ with $nTiO_2$ aerosols respectively, with uncertainties corresponding to reproducibility standard deviation (S_R) in agreement with ISO 5725-2 standard. Modal diameters for each aerosol were respectively 56 ± 1 nm, 264 ± 15 nm and 191 ± 5 nm; mean diameters were respectively 56 ± 1 nm, 264 ± 6 nm and 214 ± 2 nm. PNSDs were stable for 2 h of aerosol generation, as shown in Fig. 2(C), which presents the average PNSD evolution over time for the PQ with $nTiO_2$ aerosol. PNSDs reached an equilibrium state around 20 min (T_{20}) after the aerosol generation beginning and dropped at 120 min (T_{120}), which corresponds to the end of aerosol generation. In the 0.8–20 µm particle size range monitored using APS (Fig. 2(B)), no particles were detected for the aerosol of PQ.

3.3 Particle Number Concentration Monitoring

The temporal evolution of the average particle number concentrations during both phases is presented in Fig. 3. The real-time monitoring is shown in Figs. 3(A) and 3(B), while the evolution of the average concentrations for each aerosol generation is presented in Figs. 3(C) and 3(D). The real-time measurements during the metrological characterization (Fig. 3(A)) showed that average concentrations were 258 × 10^3 particles cm^3 ± 11%, 205 × 10^3 particles cm^3 ± 10% and

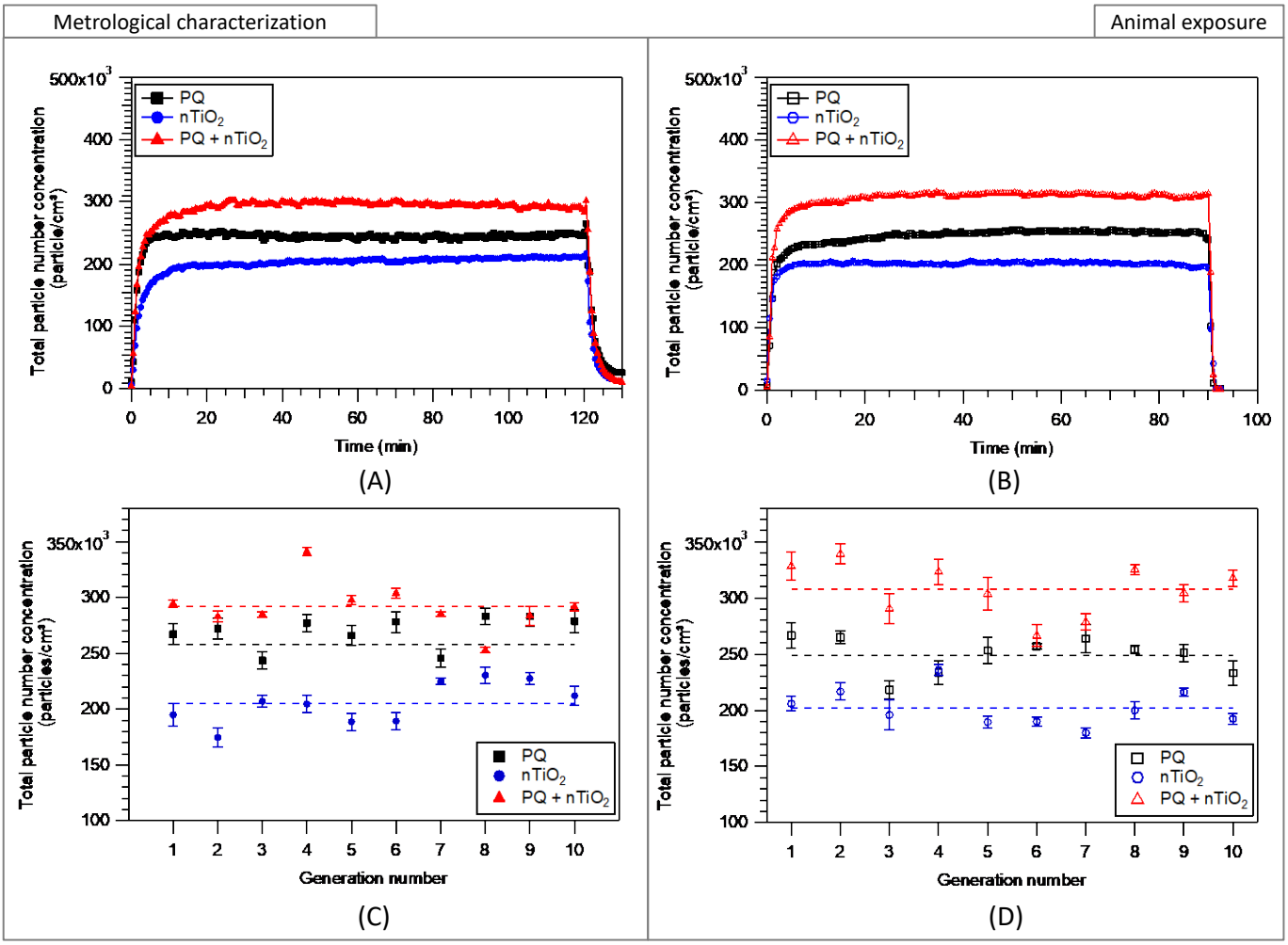

Fig. 3. Particle number concentrations measured with CPC during (A, C) the metrological characterization phase and (B, D) the animal exposure sessions. Mean results presented (A, B) in function of time or (C, D) as individual aerosol generations ± standard deviation, the dotted line corresponding to the mean value for each aerosol.

292 × 10^3 particles cm^3 ± 8% for PQ, $nTiO_2$ and PQ with $nTiO_2$ respectively. Comparatively, these concentrations were respectively 249 × 10^3 particles cm^3 ± 7%, 202 × 10^3 particles cm^3 ± 8% and 308 × 10^3 particles cm^3 ± 9% during the animal exposure phase (Fig. 3(B)). The fact that particle number concentration in the mixture do not correspond to the sum of PQ and $nTiO_2$ number concentrations could be explained by an internal mixing between both components during the aerosol nebulization process. Number concentrations in both conditions reached a steady state between 5 and 10 min; they remained stable over time until the end of the aerosol generation. By taking the metrological characterization phase as reference, the real-time measurements during the first step of the animal exposure phase allowed the validation of the aerosol generation for each inhalation exposure. The results and their S_R are summarized in Table 1.

3.4 Total Mass Concentration

Fig. 4 presents the average mass concentrations measured by TEOM in the exposure chamber during the metrological characterization phase with their corresponding S_R. The total mass concentrations reached a steady state 10 min after the generation beginning for 2 h of aerosol generation. For PQ, $nTiO_2$ and PQ with $nTiO_2$ they were respectively 98.7 ± 3.2 µg m^{-3}, 10.2 ± 0.9 mg m^{-3} and 10.2 ± 0.4 mg m^{-3}. These values comply with the target concentrations of 100 µg m^{-3} and 10 mg m^{-3} for PQ, $nTiO_2$ and PQ with $nTiO_2$. Comparatively, during the exposure sessions, the average mass concentrations were 10.1 ± 0.4 mg m^{-3} for $nTiO_2$ and 9.9 ± 0.8 mg m^{-3} for PQ with $nTiO_2$.

Table 1. Mass and number concentrations measured during the metrological characterization phase and the animal exposure sessions. Results are presented as averages ± S_R with their corresponding relative S_R in brackets.

Aerosol	Average mass concentration [mg m^{-3}]		Average number concentration [× 10^3 particles cm^{-3}]	
	Metrological characterization	Animal exposure*	Metrological characterization	Animal exposure
PQ	98.7 [10^{-3}] ± 3.2 (3%)	–	258 ± 29 (11%)	249 ± 17 (7%)
$nTiO_2$	10.2 ± 0.9 (9%)	10.1 ± 0.4 (4%)	205 ± 21 (10 %)	202 ± 17 (8%)
PQ + $nTiO_2$	10.2 ± 0.4 (4%)	9.9 ± 0.8 (8%)	292 ± 22 (8%)	308 ± 27 (9%)

–: Data not available. * Off-line gravimetric measurements.

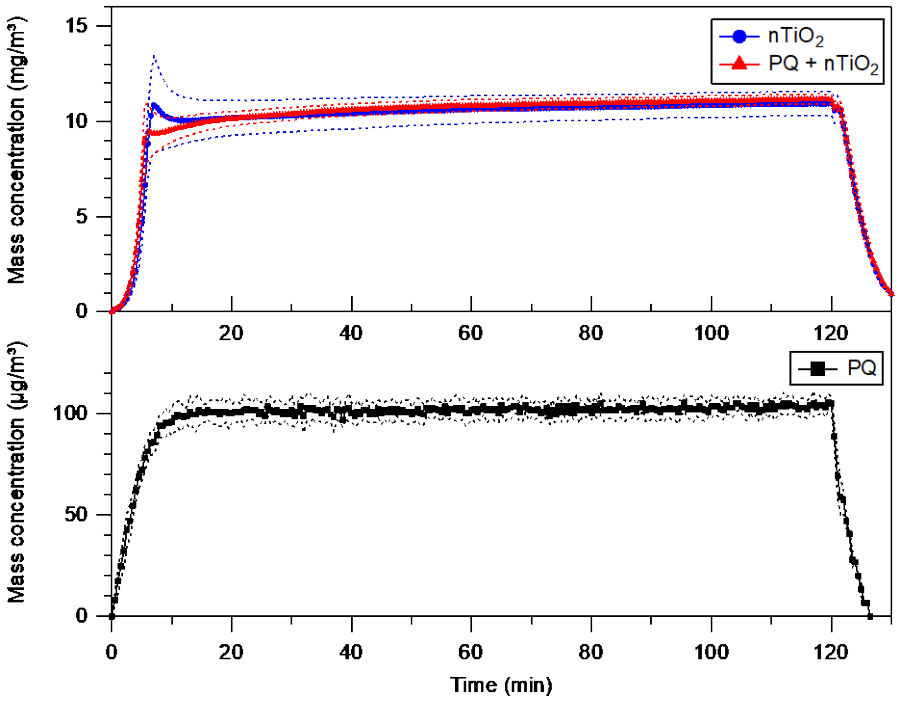

Fig. 4. Average total mass concentration in the exposure chamber using TEOM for 2 h of aerosol generation without animals. Results are presented as averages ± standard deviation (dotted lines) in function of time.

3.5 Aerosol Dispersion Modeling

According to the PNSD values assessed during the prior characterization, no difference was observed by simulation in the particle dispersion between PQ and $nTiO_2$ (data not shown). Consequently, $nTiO_2$ aerosol was selected according to their characterized mean diameter (264 nm). Fig. 5(A) presents the chamber and its associated CAD model used for the simulation. Fig. 5(B) shows the particle number concentration simulation at T = 10 min and T = 15 min. At T = 10 min, the atmosphere was still heterogeneous, since the top area of the exposure chamber presented a slightly higher particle concentration, whereas a lower concentration was observable in the middle zone and at the bottom corner below the injection site. This atmosphere heterogeneity decreased over time and the atmosphere became mostly homogeneous at T = 15 min. Such a result is expected as the simulation was made using a passive scalar method (no diffusion, no inertia). As the characterization was made using aerosol sampling from the top of the chamber, the atmosphere homogeneity was assessed to check if the aerosol characterization reflected what animals were really breathing during exposures. To this end, the simulated number concentrations were specifically obtained in the transverse plane the chamber floor (at a height of 1 cm) corresponding to the breathing area of mice. In addition, particle size distributions were compared using SMPS in both top and floor areas and no difference in the particle size distribution were visible between sampling points (data not shown) highlighting the aerosol spatial homogeneity of particles. Fig. 5(C) compared the simulated and the experimental average number concentrations

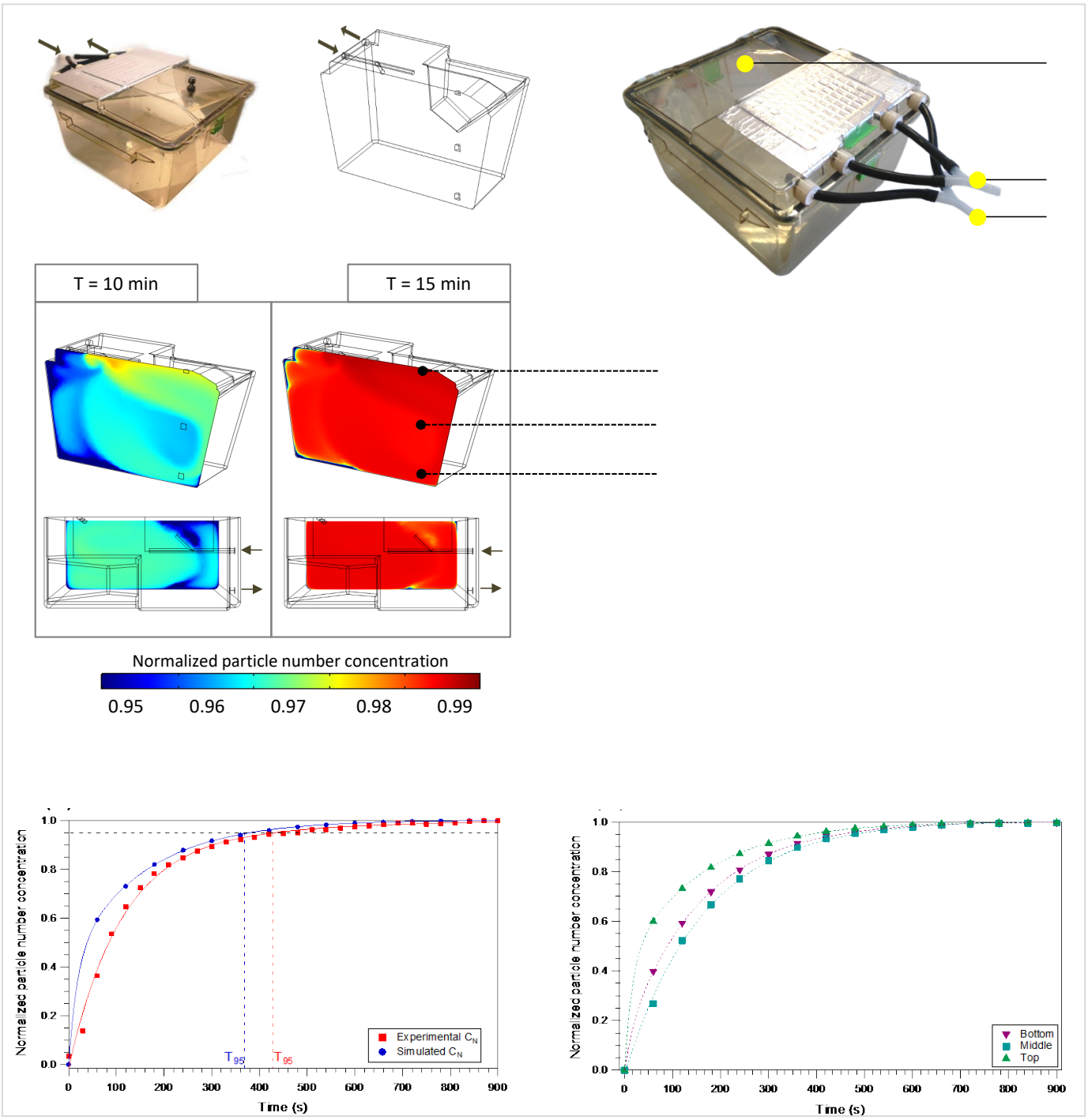

Fig. 5. (A) Chamber pictures and their associated CAD model used for simulation; 1: aerosol sampling point used for CPC measurements; 2: inlet; 3: outlet. (B) Results of the particle number concentration simulation at 10 min and 15 min within the sagittal plane (at the top) and a transverse plane (at the bottom) that represents the chamber floor (+1 cm) corresponding to the breathing area of mice. (C) Comparison of the experimental and simulated normalized particle number concentrations within the exposure chamber. (D) Normalized particle number concentrations assessed by simulation in planes located at different heights: 20 cm (top), 10 cm (middle) and 1 cm (bottom). T95: needed time to reach 95% of the atmosphere equilibration in the chamber.

in the chamber over time. According to the guidelines for subchronic inhalation toxicology studies (OECD TG 413), T_{95} can be defined as the time required to reach 95% of the atmosphere steadiness in the exposure chamber. It was reached at T = 377 s by simulation and T = 428 s

according to the experimental data, which highlighted the consistency between both datasets. In the exposure chamber, the breathing area of mice was in the range of 1–10 cm height, while the CPC measurement spot was located at the top of the chamber (20 cm height). To check if the sampling location could affect the particle concentrations, three transverse planes were defined inside the chamber at different heights (1 cm, 10 cm and 20 cm). Based on the simulated data, average number concentrations were calculated for each plane and T_{95} were respectively 382 s, 475 s, and 446 s (Fig. 5(D)). At 8 min, a plateau corresponding to the atmosphere steady state was reached, showing the representativity of the aerosol sampling location at the top of the chamber compared to the mice breathing area at the bottom.

3.6 Paraquat Stability

Regarding the stability of PQ + $nTiO_2$ suspension, no significant decrease of PQ concentration was detectable up to 24 h after the solution preparation (Fig. S2). The concentration at T_0, T_{120} and T_{24} hours were respectively 24.0 mg L^{-1}, 23.6 mg L^{-1} and 23.1 mg L^{-1}. This slight decrease correlated with an increase in the 200–230 nm range corresponding to by-product formation (Cantavenera *et al.*, 2007). PQ concentration was expected to decrease, by the $nTiO_2$ filtration process if adsorbed, which was not the case.

4 DISCUSSION

In this article, an experimental set-up complying with OECD TG 413 guidelines, dedicated to inhalation toxicology studies with rodents is presented. The nanopesticide inhalation effects are still not addressed in the literature. Besides the novelty of this field of research, the insights of the article rely mainly in the fact that no whole-body chamber has already been used and characterized in this context. The cocktail effects associating nanomaterials and toxic substances have already been reported in numerous ecotoxicological studies, for instance as reviewed by Naasz *et al.* (2018), but not in toxicological studies. Thus, it seems essential to extend this novel topic of research to human health, by beginning to investigate the underlying mechanisms of toxicity, namely on the nervous system which is known to be a specific target of some nanosized toxics (Bencsik *et al.*, 2018). Consequently, the protocol was established by generating inhalable particles whose size is characterized, in order to model the deposit fraction in the airways in upcoming article involving this device, to provide health data related to characterized exposure concentrations and conditions, for regulatory and toxicology purposes. In addition, nanopesticides represent a variety of different products, requiring a case-by-case toxic assessment depending on the conditions of use. The facility could be used to investigate other kind of nanosized substances, on the condition that a minimal characterization of aerosol is reported (size distribution, mass and number concentrations).

Stringent requirements are needed for chronic studies, like stated in the OECD TG 413 guidelines, as this facility is meant to be able to perform subchronic inhalation study lasting more than 30 days. The choice of a nebulizer reflects agricultural use of pesticides, which are typically sprayed using hydraulic nozzles (Hilz and Vermeer, 2013). The humidity control is essential to avoid the generation of too-large aggregates and agglomerates, and to avoid the proliferation of microorganisms within the cage. In comparison to dry powder generation process, wet aerosol generation is more similar to sprayings. Two diffusion dryers were required to obtain a steady humidity for generations of 2 h, by making a fast evaporation of the droplets before entering the exposure chamber. Nevertheless, this is indeed a model of generation and not an exact representation of the droplet size distribution generated with a hydraulic nozzle. In addition, some authors suggested that this type of nebulizer could represent a most versatile choice, suitable for inhalation toxicology studies (Schmoll *et al.*, 2009; Pujalté *et al.*, 2017b). The stability of the exposure conditions and the test atmosphere were characterized in order to comply with the OECD requirements for the testing of chemicals by inhalation. Environmental parameters (exposure chamber airflows, temperature and relative humidity) and test atmosphere were characterized in terms of number size distribution, number and mass concentrations and spatio-temporal aerosol stability. To be consistent between both phases of this study, the animal exposure session includes a real-time concentration monitoring and a gravimetric sampling that are used in a complementary manner, in order to confirm the generation stability of aerosols. As a result, no significant difference was found between both

phases. Between all aerosol generations, the chamber average concentrations did not deviate from the mean by more than ± 20%. Moreover, the time to reach the chamber equilibration (T_{95}) was around 7 min, which is short in comparison with the total duration of exposures. Three different aerosols were generated with a homogeneous and unimodal number size distribution, characterized by a CMMD of 50 nm for PQ, 173 nm for $nTiO_2$ and 220 nm for PQ with $nTiO_2$, with respective geometric standard deviations (GSDs) of 1.7, 2.1 and 2.2. The CMMD differences between the mixture and the compounds alone could be explained by the internal mixing between PQ and $nTiO_2$ during the aerosol nebulization process. This can be clearly observed on the corresponding size distribution which is a convolution of PQ and $nTiO_2$ size distributions. The reproducibility standard deviation was comprised between 3–9% for mass concentrations and 7–11% for particle number concentrations. The size of primary $nTiO_2$ particles used in the study is 22 nm as previously described (Motzkus *et al.*, 2014). However, the generation in the aerosol phase of individual primary particles is impossible due to high Van der Waals forces which do not allow dissociation of aggregates. Thus, the nebulization process enables a deagglomeration but not a disaggregation, explaining the mean size of 264 nm, as it was already previously discussed (Gaie-Levrel *et al.*, 2020).

The characteristics comparison of existing exposure chambers may be inappropriate, because each system has its own requirements, depending on the protocol to achieve. The device presented in this article is in accordance with our expectations and the system was validated for our experimental conditions. It has general advantages; it is easy to transport, economical, compact, and thus adaptable to the constraints of animal facilities. It is also simple to operate and easily put in place, and it offers versatility to work with various rodent species (mice, rats, hamster or others). Moreover, the atmosphere homogeneity was assessed temporally and spatially, and both experimental and simulated data were consistent during the characterization and the exposures. These important features (mass concentration and size distribution) enabled a precise assessment of the exposure dose of animals, which is an essential requirement in chronic inhalation toxicology studies. Regarding the accuracy of data, our device compares advantageously to others. Cosnier *et al.* (2017) reported the generation of P25 $nTiO_2$ aerosols using a rotation brush generator coupled with a nose-only device. Based on the target mass concentration of 10 mg m^{-3}, the calculated CMMD was 347 nm with a GSD of 2.29. The mean intra-experiment precision was comprised between 14% and 22% over six inhalation campaigns. Focusing on studies involving $nTiO_2$ nebulizers, Pujalté *et al.* (2017a) generated aerosols at a mass concentration of 15.57 mg m^{-3} (min–max of 9–21 mg m^{-3}) using a six-jet collision nebulizer with PNSD characterized by a GSD of 1.87 and a geometric mean diameter of 76.91 nm. Using also a six-jet collision nebulizer, Grassian *et al.* (2007) generated aerosols of $nTiO_2$ using a whole-body device for different exposure scenarios, which involved a subacute exposure (10 days, 4 h per day) at a mass concentration of 8.88 ± 1.98 mg m^{-3} with PNSD characterized by a geometrical mean diameter of 128 nm (GSD of 1.7). Yi *et al.* (2013) used an innovative nebulizer to produce aerosol of $nTiO_2$ with PNSD characterized by a CMMD of 145 nm and a GSD of 2.3 in a 500 L whole-body inhalation exposure chamber. In their article, the atmosphere spatial homogeneity was assessed, by measuring the concentrations at different spots within the chamber, thereby showing a spatial maximum relative deviation from the mean concentration < 6%.

In this study, specific care was taken concerning animal welfare to reduce stress. This is a matter of prime importance, because a stress undergone during gestation is critical for the fetal development, and then prenatal stress can have long-term consequences for the offspring (Weinstock, 2005). Consequently, the maternal stress in rodents, possibly due to restraint of poor exposure chamber design, may exacerbate negative developmental effects (Rasco and Hood, 1995). In this regard, we selected a whole-body inhalation device to expose animals, as this cage allowed daily exposures with a direct visual follow-up without constraint. It also enables animals to move freely, which simulates a physical activity enhancing the pulmonary ventilation of mice to reflect more accurately occupational exposures. Glass and stainless steel are the classical material used in whole-body devices, because of their ability to age slowly compared to plastics (Dorato and Wolff, 1991). Some authors used stainless steel exposure chamber with a vertical injection flow (Kimmel *et al.*, 1997) or a combination of vertical and horizontal flows input/output to reach a uniform aerosol concentration (Oldham *et al.*, 2004). In our study, we chose a polysulfone cage dedicated to rodent hosting, because it is an inexpensive alternative, which is easy to accommodate in a rodent facility. It can be used to expose mice or other species continuously in

chronic toxicity studies because this product is standard-equipped to provide water and food. Moreover, it is easily washed and it can be used under a hood if necessary. A large number of animals can be exposed at the same time in a convenient way, which can significantly save time. Complications could arise from an important animal loading in the cage, which can increase the temperature, the relative humidity and the amount of air pollutants inside the chamber. Indeed, the ammonia coming from animal feces may be an issue since ammonia could affect animals and may also react with the test aerosols (Barrow and Steinhagen, 1982). According to Silver *et al.* (1946), the overall animal volume should not exceed 5% of the chamber volume to prevent this phenomenon (OECD TG 403). Therefore, we chose an important air renewal (23 volumes h^{-1}) and a low animal loading ($<$ 1% of chamber volume) to prevent particles and metabolites to be accumulated in our device. However, while air renewal within the cage is a key factor in the aerosol dispersion and homogenization, it must be limited to avoid a cold stress.

Some studies also took into consideration the environmental conditions associated with their exposure chambers (Kimmel and Kirk, 1997; Jeon *et al.*, 2002; Oldham *et al.*, 2004; Lucci *et al.*, 2019), while other studies did not (Barrow and Steinhagen, 1982; Cheng *et al.*, 1989; Phillpotts *et al.*, 1997; Bhaskar and Upadhyay, 2003; O'Shaughnessy *et al.*, 2003). It is indeed necessary to perform a deeper aerosol characterization, before following limited metrological endpoints during animal exposures. This characterization is necessary to enable an accurate assessment of the exposure concentration delivered to each animal. This assessment is based on the simulated deposited dose of particles in the lungs, which is a function of the aerosol mass concentration and size distribution. In addition, a homogeneous atmosphere is required, in order to minimize the variability of deposited dose between animals. For all these reasons, the minimum requirements are the mass and number concentrations, the size distribution, and also the environmental parameters assessments (temperature, air renewal, humidity and pressure), which are mandatory to get the experimentation authorization from the ethics committee. Moreover, it is important to note that only few studies validated their exposure chamber thanks to aerosol dispersion modeling (Kimmel *et al.*, 1997; Oldham *et al.*, 2004). The homogenization process results mainly from an adequate air renewal within the chamber, with proper inlet flows and chamber geometry (Kimmel *et al.*, 1997). The analysis of the chamber structure and mass transfer forces such as diffusion is crucial to understand the test material distribution. Rajabi-Vardanjani *et al.* (2019) suggested that numerical simulation techniques should be used to predict flow velocity and aerosol dispersion in function of the chamber geometry in order to optimize an exposure chamber and the associated aerosol generation. Kimmel *et al.* (1997) found a clear agreement between assessment of chamber performance by CFD modeling and the analysis of performances by more conventional methods, which is in accordance with the results of this study. Nevertheless, their apparatus was much larger than in this work (700 L versus 19.8 L), which consequently required a more complete CFD modeling to be conducted. Cheng *et al.* (1989) and O'Shaughnessy *et al.* (2003) assumed that spatial and temporal uniformity were independent from each other in their whole-body exposure chambers. Oldham *et al.* (2004) did not make this assumption, taking in consideration the size of their chamber (20 L), which is relatively small in comparison with others, but similar to this work. The results we reported tended to conform with their statement. Uniformity assessment seemed not as good for whole-body chamber compared to those reported in nose-only exposure systems (Yeh *et al.*, 1990; Cheng and Moss, 1995). However, it is consistent with the characterization reported for other large stationary whole-body exposure chambers (Schreck *et al.*, 1981; MacFarland, 1983; Yeh *et al.*, 1986; Cheng and Moss, 1995; O'Shaughnessy *et al.*, 2003). The aerosol uniformity in several transverse planes ($\pm$ 5%) was judged acceptable, and since exposures occur with animal moving freely in the totality of the exposure chamber, the stability requirements are less stringent compared to nose-only devices. Nonetheless, variations may be caused by changes in small inward air leaks, mice movements and induced thermal convection, or other any unknown factors.

5 CONCLUSIONS

To expose rodents to submicrometer-sized airborne particles, we developed a simple and versatile whole-body inhalation chamber that can be used with various exposure testing protocols

and animal species. In this chamber, stable and reproducible generations of PQ, $nTiO_2$ or PQ–$nTiO_2$ aerosols were characterized in terms of particle number size distributions, mass and number concentrations with reproducibility standard deviations comprised between 1–11% for up to 2 h in the laboratory. We then validated the reliability of these measurements by conducting inhalation exposure sessions in the field, which exhibited a reproducibility standard deviation of 4–9%. These results confirmed the homogeneity of the chamber's atmosphere and hence the accuracy of the exposure assessment. Our project facilitates the collection of toxicological data, which are essential to evaluating the health risks of emerging substances, such as NPEs.

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DISCLAIMER

The authors declare no conflicts of interest.

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