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Can the impact of micro- and nanoplastics on human health really be assessed using *in vitro* models? A review of methodological issues

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Abstract

Because of the many advantages they offer (strength, low cost, durability, lightweight, resistance, etc.), plastics are integral part of our daily life with a production constantly rising. However, their waste management is still inadequate, resulting in their release and accumulation in the environment, representing a main source of pollution. Their degradation results in debris of variable size including microplastics (0.1 μm –5 mm) and even nanoplastics (< 0.1 μm), whose potential impact on ecosystems and human health have raised concerns. The potential adverse effects they may cause have been evaluated using both *in vitro* and *in vivo* models. However, due to some specific characteristics of micro- and nanoplastics, there are challenging questions about whether conventional *in vitro* tests are appropriate for evaluating their toxicity. For example, low-density plastics float on the surface of the culture medium and cannot come into contact with cells adhering to the bottom of the culture plates, which prevents proper evaluation of potential adverse effects and leads to misinterpretation of toxicological assays. In this review, we discuss the main issues related to the evaluation of micro- and nanoplastics toxicity using conventional *in vitro* assays. A literature survey has allowed to propose some solutions to circumvent these issues including the use of mathematical models to accurately determine the dose of particles delivered to cells, advanced 3D models (organoids), inverted cell culture models, cell cultures at the air-liquid interface or under dynamic conditions. Finally, we propose some perspectives and recommendations for further research on the *in vitro* evaluation of micro- and nanoplastics toxicity, underlining the importance of using standardized protocols for comparison purposes and samples and experimental conditions more representative of real-life exposure.

Key-words: Micro- and nanoplastics; Buoyancy; Low-density particles, In vitro toxicity assessment; Human health, Particle dosimetry

1. Introduction

Due to their tremendous characteristics, plastics are integral part of our daily life. Inexpensive, strong, durable, lightweight, resistant to degradation, exhibiting thermal and electrical insulation properties, they are used in many applications (Banerjee and Shelver, 2021; Facciola et al., 2021; Llorca and Farré, 2021). A wide variety of plastics exists; they are mainly made of several types of polymers such as polyethylene terephthalate (PET), polycarbonate (PC), polyvinyl chloride (PVC), polystyrene (PS), polypropylene (PP) or polyethylene (PE) (Facciola et al., 2021; Revel et al., 2021). Global production of plastic has dramatically increased from 1.7 million tons in the 1950s to 390.7 million tons in 2021 (Plastics Europe, 2022; Revel et al., 2018). After a stagnation in 2020 due to the Covid-19 pandemic, the European production increased to 57.2 million tons in 2021 (Plastics Europe, 2022).

Although efforts are being made to recycle these materials, waste management remains inadequate, resulting in the release and accumulation of plastics in the environment, which are a major source of pollution. It is estimated that 71% of the plastic waste ends up in aquatic or terrestrial environments (Busch et al., 2023). This concern has recently led the United Nations to forge an international legally binding agreement by 2024. The resolution aims at ending plastic pollution and addresses the full lifecycle of plastic, including its production, design and disposal (UN Environment, 2022). Indeed, due to their limited biodegradable nature plastics present an enormous environmental burden with about 8 million tons of plastic litter reaching the oceans every year (Banerjee and Shelver, 2021). In the nature, plastics then undergo photo and thermo-oxidative degradation processes, mechanical fragmentation (physical abrasion, exposure to UV light...) and, to a minor degree, biodegradation (*i.e.*, microbial degradation) resulting in debris of variable size including macroplastics (size >5 mm), microplastics (0.1 μm –5 mm) and even nanoplastics (< 0.1 μm) (Banerjee and Shelver, 2021; Busch et al., 2023; Duan et al., 2021; Facciola et al., 2021; Pulvirenti et al., 2022; Revel et al., 2021).

In addition to these secondary micro- and nanoplastics, which are formed by the degradation of macroplastics, primary micro- and nanoplastics can be produced specifically for various commercial

applications. For example, they can be found in air-blast technologies, detergents, cosmetic products (e.g., exfoliants or toothpaste), drug formulations, paints, adhesives, and electronics (Banerjee and Shelver, 2021; Bradney et al., 2019; Busch et al., 2023; Wright and Kelly, 2017).

Finally, other potential sources of micro- and nanoplastics exist including wear and tear of clothing (release of fibers from synthetic textiles), carpets, furniture, cigarette filters, abrasion of car tires (synthetic rubber is a variation on plastic) and fishing gears, accounting for both indoor and outdoor pollution (Banerjee and Shelver, 2021; Busch et al., 2023; Winkler et al., 2022; Wright and Kelly, 2017).

Because of their persistence and continuous release, micro- and nanoplastics have become ubiquitous, present in many environmental compartments (terrestrial, aquatic, and atmospheric systems). Indeed, they were detected in the oceans, freshwater bodies, sediments, soil, air, and even in our food (Banerjee and Shelver, 2021; Facciola et al., 2021; Kumar et al., 2022). This has raised societal concerns, especially in terms of impact on ecosystems and human health as we are continuously exposed to these pollutants.

The aim of this narrative review is to draw attention to the evaluation of the toxicity of micro- and nanoplastics using conventional *in vitro*, cell-based assays and the main problems associated with them (e.g., buoyancy of plastic particles and the consequent poor contact between particles and *in vitro* cell system, and the difficulty of separating toxicity resulting from the intrinsic properties of micro- and nanoplastics, chemical additives, and contaminants derived from environmental matrices). We have also performed a bibliographic search to highlight the proposed solutions to these problems, and finally propose some perspectives for further research in this area.

2. Human exposure to micro- and nanoplastics

Human exposure to micro- and nanoplastics can occur through three main routes: ingestion, inhalation or dermal contact (Revel et al., 2018; Xu et al., 2022). Once entered in the human body, the particles,

especially those in the submicron range, can enter cell membranes and cross different biological barriers, possibly reaching the blood stream and then be distributed to other organs where they can exert toxic effects (Xu et al., 2022). These physiological barriers include the blood-brain barrier (Han et al., 2023; Kopatz et al., 2023; Prüst et al., 2020; Shan et al., 2022; Windheim et al., 2022), the gastrointestinal barrier (Deng et al., 2017; Hesler et al., 2019; Lu et al., 2018; Xiao et al., 2022), and the placental barrier (Aghaei et al., 2022; Cary et al., 2023; Dusza et al., 2023, 2022; Hesler et al., 2019; Medley et al., 2023).

Many investigations focused on oral ingestion as it has been reported that micro- and nanoplastics were present in different food and beverage sources, and were also detected in human stools (Chain (CONTAM), 2016; Hernandez et al., 2019; Llorca and Farré, 2021; Rubio et al., 2020; Schwabl et al., 2019; Xu et al., 2022). Plastics ingested by marine organisms can reach human beings through trophic transfer in the food chain, especially seafood. In addition, food contamination can occur during storage and transportation in plastic containers (Banerjee and Shelver, 2021; Lickly et al., 1995; Petersen et al., 1995; Revel et al., 2018; Tawfik and Huyghebaert, 1998; Whitt et al., 2016; Xu et al., 2022).

Micro- and nanoplastics have been detected in significant quantities in the atmosphere and human exposure to airborne plastic particles has gained attention. Various sources of airborne micro- and nanoplastics have been reported; they can originate from wave action in aquatic environments or the application of wastewater treatment sludge used as fertilizer in agriculture, they have been detected in atmospheric fallout; they can also result from the wear-and-tear of synthetic textile fibers and rubber tires. Other sources include upholstery and household furniture, buildings, incinerators, landfills, industrial emissions (Banerjee and Shelver, 2021; Gasperi et al., 2018; Llorca and Farré, 2021; Revel et al., 2018; Wright and Kelly, 2017). Among these different sources, the release of synthetic textile fibers occurring during clothes washing and drying is considered as a major contributor (Facciola et al., 2021; Gasperi et al., 2018; Prata, 2018; Rubio et al., 2020). It is now acknowledged that micro- and nanoplastics particles represent a source of both indoor and outdoor pollution. In outdoor

environments, meteorological and geographical factors will deeply influence the atmospheric persistence of micro- and nanoplastics (Facciola et al., 2021; González-Acedo et al., 2021). The indoor presence of airborne micro- and nanoplastics is dependent on room layout and ventilation. In this context, occupational exposure need special attention as it has been reported, especially in the synthetic textile industry, and the vinyl chloride (VC) and polyvinyl chloride (PVC) industries (Facciola et al., 2021; Xu et al., 2022).

Finally, exposure to micro- and nanoplastics can occur through skin contact. As it seems to be less relevant, this route of entry has been poorly documented. However, it has been reported that nanoplastics could cross the dermal barrier (Rubio et al., 2020; Xu et al., 2022). This route can be relevant especially for skin contact with contaminated water, cosmetics and personal care products, like shaving creams, wrinkle creams, toothpaste, eye shadows, facial wash, etc. (Kumar et al., 2022; Revel et al., 2018).

Taken together, these observations show that we are constantly exposed to plastic particles and we may fear that this chronic exposure to plastics may lead to bioaccumulation and subsequent biological effects, as discussed later. In addition, concerns about potential adverse effects of the plastics on human health are directly related to their chemical composition.

3. Composition of the micro- and nanoplastics and potential sources of hazard

As mentioned before plastic is made of synthetic organic polymers, the major ones being PE (high and low density), PS, PP, PVC, PET and polyurethane (PUR) (Revel et al., 2018). Moreover, chemical additives are often intentionally introduced in these matrices during plastic production to improve plastic characteristics such as strength, color and transparency, resistance to degradation (by ozone, temperature, light radiation, mold, bacteria and humidity), and mechanical, thermal and electrical resistance (Campanale et al., 2020; Revel et al., 2018). These additives include inert or reinforcing

fillers, plasticizers, antioxidants, UV stabilizers, lubricants, coating finishers, dyes and flame-retardants (Campanale et al., 2020; Llorca and Farré, 2021; Turner and Filella, 2021).

Therefore, potential toxic effects of micro- and nanoplastics can originate either from the intrinsic hazard of the particles (based on polymer type, size, charge, morphology, dose, leachables) or from the hazard of the additives (Banerjee and Shelver, 2021; Llorca and Farré, 2021). Regarding the plastic nature, PUR, polyacrylonitriles, PVC, epoxy resins and styrene-based have been classified as category 1A or 1B mutagen or carcinogen (Banerjee and Shelver, 2021; Lithner et al., 2011). Regarding additives, because they are not covalently bound to plastic polymer, they can easily be released, leaching in the external medium (Llorca and Farré, 2021; Wright and Kelly, 2017). Some additives have already been reported to have toxicological properties harmful to the environment and human health. Of particular concerns are bisphenol A and phthalates (used as plasticizers), as well as brominated flame-retardants. Indeed, their ability to disrupt the endocrine function has been shown, they can also act as carcinogens and cause respiratory diseases (Banerjee and Shelver, 2021; Facciola et al., 2021; Llorca and Farré, 2021; Rubio et al., 2020).

Another potential source of hazard lies on the ability of plastic particles to serve as a vehicle for toxic environmental pollutants as well as pathogens (Banerjee and Shelver, 2021; Campanale et al., 2020). Indeed, because of their hydrophobic surface, micro- and nanoplastics can adsorb and concentrate hydrophobic organic contaminants (HOCs) such as polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides, dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), persistent organic pollutants (POPs), etc. They can also adsorb heavy metals (Al, As, Ba, Br, Cd, Co, Cr, Cu, Hg, Mn, Sb, Sn, Ti, Zn) or micro-organisms such as pathogenic *Vibrio spp* (Banerjee and Shelver, 2021; Campanale et al., 2020; Kumar et al., 2022).

Although further research is needed to better understand the potential impact of micro- and nanoplastics on ecosystems and human health, some data are available in the literature. We will focus our attention on human health.

4. Health effects induced by micro- and nanoplastics

As previously mentioned, the exposure of humans to plastics have raised concerns about the potential adverse effects they may elicit. Thus, the toxicological profile of micro- and nanoplastics have been assessed using both *in vitro* and *in vivo* models. So far it is acknowledged that they are able to induce cellular toxicity through oxidative stress, inflammation, membrane damage, immune response neurotoxicity, neoplasia, changes in metabolism and energy homeostasis and genotoxicity. It is especially the case for particles of small size and of positive surface charge. High particle concentration and the presence of toxic additives or pollutants in the micro- and nanoplastics also greatly impact their toxicity (Banerjee and Shelver, 2021; Bhattacharjee et al., 2014; Brown et al., 2001; Dong et al., 2020; Feng et al., 2019; Fröhlich et al., 2012; Hwang et al., 2019; Jeon et al., 2018; Lim et al., 2019; Llorca and Farré, 2021; Paget et al., 2015; Pulvirenti et al., 2022; Rubio et al., 2020; Xu et al., 2019; Zheng et al., 2019). Based on findings obtained in mice, disruption of the gut microbiome has also been suspected upon chronic ingestion of micro- and nanoplastics (Banerjee and Shelver, 2021).

As previously discussed, this toxicity may be due to: i) the plastic particle intrinsic toxicity (causing physical damage after cell contact), ii) its chemical composition (leaching of additives), and iii) its ability to serve as a vector for environmental contaminants (Bradney et al., 2019; Llorca and Farré, 2021; Revel et al., 2018).

The investigation of the effects of micro- and nanoplastics on human health is mainly carried out using *in vitro* cell culture or *in vivo* mouse models (Xu et al., 2022). Animal models may not be suitable for exhaustive testing in all cases because of the wide variety of existing types of micro- and nanoplastics (chemical nature, shape, size, exposure scenarios...)(Busch et al., 2023; Rubio et al., 2020). However, there is ongoing research in this area and many scientists are exploring ways to use animal models for testing micro- and nanoplastics (Keinänen et al., 2021; B. Li et al., 2020; Z. Li et al., 2020; Lu et al., 2018; Rawle et al., 2022; Sun et al., 2022; Xiao et al., 2022).

This is why, *in vitro* assays are usually preferred. Indeed, they are fast, easy to perform, inexpensive, practical, do not involve ethical issues, allow a rapid screening of effects of pollutants (defining which should be prioritized for further *in vivo* testing), allow mechanistic studies and are compatible with multi-parametric or high throughput screening (HTS) assays... (Barbosa et al., 2020; Forest, 2022; Fröhlich, 2018; Revel et al., 2021; Rubio et al., 2020; Schröter and Ventura, 2022; Upadhyay and Palmberg, 2018; Xu et al., 2022).

While cell monocultures are basic and simplistic models very useful for screening purposes, co-culture models combining different cell lines better recapitulate a physiological environment and the interactions occurring between the different cell types. It is thus considered that co-cultures strongly improve the predictivity of *in vitro* models (Bredeck et al., 2022).

Most of the studies assessing micro- and nanoplastics toxicity have initially focused on the gastrointestinal system. Indeed, this system is exposed to ingested contaminated food and water, which was considered the predominant route of human exposure to micro- and nanoplastics (Facciola et al., 2021). In this regard, most *in vitro* studies use the Caco-2 cells, representative of the human small intestine, either as a monoculture or in association with other cell lines such HT-29 cells and/or M-cells to recapitulate the intestinal barrier (Rubio et al., 2020; Xu et al., 2022). To assess the potential hepatotoxicity the HepG2 cell line, derived from the liver tissue, is preferentially used (Xu et al., 2022).

But adverse effects observed after exposure to airborne micro- and nanoplastics are increasingly considered. Indeed, depending on their size, inhaled particles can reach different areas of the tracheobronchial tree and subsequently cause a wide range of disorders such as respiratory distress, dyspnea, decreased lung capacity, coughing, fibrosis, and granulomatous lesions. These symptoms have already been observed amongst workers of some industries (Banerjee and Shelver, 2021; Wright and Kelly, 2017). To better characterize the *in vitro* effects of inhaled micro- and nanoplastics, the A549 cell line, representative of human lung epithelial cells, is a popular cell model (Halimu et al., 2022; Xu et al., 2022). THP-1 and Raw264.7 cells representing macrophages are also widely used (Xu et al.,

2022). Other cell models have also been used to assess the potential toxicity of micro- and nanoplastics on skeleton (using the MC-3T3-E1 cell line) (Pan et al., 2023) or the brain (BV-2 cells) (Shan et al., 2022).

However, despite their many advantages, *in vitro* models have some limitations. Even advanced cell models cannot replicate the entire organism and its various levels of interaction (cells/tissues/organs), so they do not allow for absorption and toxicokinetics studies. Similarly, *in vitro* models are not suitable for chronic studies because the duration of exposure is limited, whereas in the environment we are exposed to micro- and nanoplastics on a daily basis and over long periods of time (Rubio et al., 2020; Schröter and Ventura, 2022; Upadhyay and Palmberg, 2018).

In addition to these general drawbacks, another major issue specifically related to micro- and nanoplastics is their buoyancy and the subsequent bias in dosimetry they induce. This raises the question of whether conventional *in vitro* models are appropriate for assessing the toxicity of micro- and nanoplastics.

5. Buoyancy of micro- and nanoplastics is a major issue for their toxicity assessment with standard *in vitro* models

By their chemical nature, plastics may greatly vary in terms of density. For instance, PE, PP, PEST and PU have densities $< 1 \text{ g cm}^{-3}$, PS density is around 1 g cm^{-3} , while some polymers such as PVC, PA or PET are heavier (Busch et al., 2023; Kumar et al., 2022; Schröter and Ventura, 2022; Stock et al., 2020). When plastic density is lower than that of the culture medium in which plastics are suspended for toxicological assays (which is typically around 1 g cm^{-3}), they will float at the medium surface and won't be able to establish contact with the cells which are usually adherent cells located at the bottom of the culture dish (Campanale et al., 2020; Schröter and Ventura, 2022; Stock et al., 2020; Watson et al., 2016), as illustrated in Table 1. Consequently, the toxicity of low density particles cannot be adequately assessed using standard *in vitro* models because of the bias in the dosimetry induced by the buoyancy of these particles (Busch et al., 2023; Stock et al., 2020; Watson et al., 2016).

Indeed, returning to the possible causes of plastic particle toxicity, this may be due to: i) the intrinsic toxicity of the particles, ii) their chemical composition (leaching of additives), and iii) their ability to serve as vectors for environmental pollutants (Banerjee and Shelver, 2021; Campanale et al., 2020; Kumar et al., 2022; Llorca and Farré, 2021; Revel et al., 2018). Without physical contact between particles and cells, we can assess the second and third components, but not the toxicity of the particles themselves, which may lead to an underestimation of the hazard of plastic particles.

Still in order not to neglect the contribution of the intrinsic toxicity of the particles to the overall toxicity, the *in vitro* evaluation, to be accurate, must determine the dose of plastics particles that actually reaches the cells (*i.e.*, the delivered dose), which may differ significantly from the administered dose (Böhmert et al., 2018; Forest et al., 2022; Liu et al., 2015; Xu et al., 2022). Otherwise, results can be misinterpreted, especially toxicity can be underestimated, compromising the meaningfulness of *in vitro* approaches (Böhmert et al., 2018; Watson et al., 2016). Indeed, even using very high exposure concentrations, the dose of plastics particles delivered to cells can remain very low. Therefore, consideration of sedimentation is of paramount importance when assessing concentration-dependent cellular response and uptake (Busch et al., 2023; Watson et al., 2016).

In addition, misinterpretations can result from the fact that the toxicity observed can originate from additives that leach from the particles and reach the cells through dilution in the cell culture medium, despite no physical contact between cells and particles (Banerjee and Shelver, 2021; Campanale et al., 2020; Facciola et al., 2021; Kumar et al., 2022; Llorca and Farré, 2021; Revel et al., 2018; Wright and Kelly, 2017). In this case, we only observe the influence of the chemical composition of the particles but once again, we miss the intrinsic toxicity of the particles, which can lead to a biased toxicological profile.

Although the buoyancy of micro- and nanoplastics is often overlooked, there are several approaches to address this issue.

6. How is this limitation circumvented in the literature?

We conducted a literature review to understand how *in vitro* assessment of micro- and nanoplastic toxicity has been performed and how conventional cell tests have been adapted to address the issue of plastic particle buoyancy or what alternative tests have been used. We searched PubMed for the following terms: (((Micro OR nano) and plastic) OR microplastic OR nanoplastic) AND Toxicity AND In vitro. The search yielded 402 references. We included in our analysis 53 papers, based on their relevance to our topic, *i.e.* these papers were addressing the micro- and nanoplastic toxicity using *in vitro* models, were considering the issue of buoyancy of plastic particles, and were proposing a solution to this problem.

First, our observations confirmed that this issue is often neglected and effective density (*i.e.*, the density of the particle agglomerates) is rarely measured when *in vitro* cellular studies are performed (Watson et al., 2016). Usually, researchers consider the exposure (*i.e.*, the administered) particles dose (Domenech et al., 2021; Hesler et al., 2019; Huang et al., 2022; Poma et al., 2019; Schirinzi et al., 2017; van den Berg et al., 2022; Zhang et al., 2022). Traditionally this administered dose is expressed as particle mass, number, or surface area per unit volume (Liu et al., 2015).

We also observed that many studies used substitution of materials. Since PP and PE are buoyant and therefore difficult to study with conventional *in vitro* cell systems, studies are mainly conducted using PS as model plastic particles, although this type of polymer is not very representative since it only accounts for about 7% of total plastic production (Busch et al., 2021a; Xu et al., 2022).

However, some alternatives have been proposed to face the challenge of buoyant plastic particles as summarized in Table 1.

6.1. Dosimetry taken into account by using mathematical modeling

The delivered particle dose can be estimated using *in silico* calculations. Indeed, the dose of particles delivered to cells can be predicted using mathematical models that take into account both the physicochemical properties of the particles and the setup of the *in vitro* exposure scenario (Böhmert et al., 2018; Busch et al., 2023; Liu et al., 2015).

Examples of such numerical models include the *In vitro* Sedimentation, Diffusion and Dosimetry model (ISDD), the *In vitro* Sedimentation, Diffusion, Dissolution and Dosimetry model (ISD3) and the Distorted Grid model (DG) (Busch et al., 2023; DeLoid et al., 2015; Hinderliter et al., 2010; Thomas et al., 2018; Watson et al., 2016).

The ISDD model was the first computational model to be developed. It can be considered as an *in vitro* counterpart to the Multipath Particle Deposition Model (MPPD) for inhaled particles. It takes into account variable parameters such as particle sedimentation, diffusion, temperature, media height, particle size in solution, agglomeration state and particle density to calculate the delivered dose and rate of transport of particles *in vitro* (Hinderliter et al., 2010).

This model was successfully used by Busch *et al.* when assessing the effects of PS and PVC particles in a co-culture model mimicking the human intestine. The ISDD model was used to estimate the delivered dose for particle exposure experiments. The ISDD simulation showed that the plastic particles settled over time and could therefore interact with the cells. Whereas the administered dose was $50 \mu\text{g cm}^{-2}$, the ISDD model calculated that the doses delivered to the cells corresponded to $8.5 \mu\text{g cm}^{-2}$ and $17.5 \mu\text{g cm}^{-2}$ for PS and PVC particles respectively (Busch et al., 2021a).

In most cases, the ISDD model is sufficient for predicting nanoplastic particles dosimetry. However, ISD3 and DG are better suited for modelling buoyant particles such as PE or PP, as well as polydisperse or degradable materials (Busch et al., 2023; Watson et al., 2016). Indeed, while the ISDD model estimates a delivered particle dose based on the particokinetics taking into account particle sedimentation and diffusion, the ISD3 model includes an additional parameter: the particle dissolution. The DG model considers polydispersity, dissolution, and also adsorption it predicts the biokinetics at

the particle-cell interface as using a Langmuir isotherm (DeLoid et al., 2015; Hinderliter et al., 2010; Thomas et al., 2018). For a detailed comparison of the ISDD, ISD3 and DG models in terms of considered nanoparticle dynamics and requested input parameters please see the *in vitro* dosimetry modelling and experimental design report from the European PATROLS (Physiologically Anchored Tools for Realistic nanOMaterial hazard aSessment) project (PATROLS, 2020).

The degree of sedimentation of particles can be assessed experimentally by determining the concentration of particles remaining in suspension after a given sedimentation period. However, it represents a fastidious task when taking into account the wide range of particles and the possible variable experimental conditions. Therefore, validated predictive models can help save time and enable proper toxicity assays interpretations (Liu et al., 2015).

6.2. Cell culture models grown in dynamic conditions

Cell culture models grown under dynamic conditions using microfluidic devices (*i.e.*, organ on-a-chip) might improve the distribution of particles and help cope with the buoyancy issue, increasing the correspondence between the applied and delivered doses (Busch et al., 2023).

Gupta *et al.* developed a model of microfluidic-chip with BEAS-2B cells (human bronchial epithelial cells) where cells were stationary, but the nutrient supply was dynamic (Gupta et al., 2021). Cell were exposed to PS particles and the cell uptake was assessed. For comparison, tests were also performed using static, multi-well cultures. Exposure under dynamic conditions resulted in higher cellular uptake of the PS nanoparticles when compared to static conditions confirming that conventional cell cultures may not accurately reflect the uptake of low-density (buoyant) particles, potentially leading to an underestimation of their cellular impact (Gupta et al., 2021).

Similarly, Liu *et al.* dynamically exposed mouse hippocampal neuronal HT22 cells to PS micro- and nanoparticles using a microfluidic device (Liu et al., 2022). They observed that the dynamic exposure

to PS particles resulted in much less cell viability than the traditional static exposure. They attributed it to the fact that a dynamic flow allows a better dispersion of the particles and fewer aggregates, which could consequently allow a more efficient cellular uptake (Liu et al., 2022).

Other examples of organ-on-a-chip used to investigate the response to PS nanoplastics include the development of a Caco-2/HT-29-MTX co-culture on a microfluidic device (Esch et al., 2014), and a kidney-testis microfluidic platform (Xiao et al., 2023).

Although adaptations of protocols and optimizations of settings are required, microfluidic devices appear as a promising tool in the evaluation of particle toxicity.

6.3. Inverse culture models

To circumvent the issue of low density of polymers and foster the contact between cells and buoyant particles, inverted cell culture models were developed. The principle is to seed cells on coverslips and after adhesion of the cells, the coverslips are transferred upside down onto wells filled with culture medium added with the particles. In these systems cells are therefore oriented above the particle suspension, so that suspended buoyant particles move toward, rather than away from the cells, as illustrated in Table 1 (Busch et al., 2023; Schröter and Ventura, 2022; Stock et al., 2020; Watson et al., 2016).

For instance, Watson *et al.* developed an inverted 96-well cell culture platform to assess the toxicity of buoyant PP nanoparticles on human macrophages (Watson et al., 2016). It was then compared to the toxicity observed using a conventional cell culture model. While no adverse effects were observed in the standard cell culture, irrespective of the nanoparticle concentration, cell viability decreased and reactive oxygen species production increased in a dose-dependent manner in the inverted system, once again arguing for the fact that the toxicity of low-density particles may be overlooked when using conventional models.

Similarly, Stock *et al.* developed an inverse cell culture model based on HepG2 cells to investigate the toxicity of floating PE particles (Stock et al., 2020). With this system PE particles triggered a dose-dependent cytotoxicity while in standard cell culture conditions (not inverted), no toxicity was reported. The same method was used for the assessment of the toxicity of PE, PP, PVC and PET particles on the Caco-2, HepG2 and HepaRG (Stock et al., 2021).

Still using inverted cell systems, more complex co-culture models have been established. For example, Busch *et al.* used a triple culture of Caco-2, HT29-MTX-E12 and THP-1 as a model of intestine to investigate the effects of polymeric particles with a density of $<1 \text{ g cm}^{-3}$. PE particles induced cytotoxicity and pro-inflammatory effects in the inverted model, which were absent in non-inverted cultures (Busch et al., 2021a).

6.4. Air-liquid interface (ALI) systems

ALI systems can be an alternative to expose cells to buoyant plastic particles. Indeed, because of the absence of medium on the apical side of cells, particles can be brought by aerosolization, *i.e.* cells are exposed to particle-containing aerosols that deposit on the cells through sedimentation, irrespective of the particle density (Busch et al., 2023). This approach has been used in an advanced *in vitro* model of the intestine (Lehner et al., 2020), however, exposure via aerosols is obviously more relevant for *in vitro* models mimicking human airways and for the toxicological assessments of airborne pollutants (Busch et al., 2023; Upadhyay and Palmberg, 2018).

In this context, ALI systems have been used to investigate the uptake and toxicity of various PS particles in monocultures of A549 and Calu-3 cells as well as in co-cultures of A549 and THP-1 (Meindl et al., 2021).

Either cells (mono or co-cultures) or tissues can be exposed to airborne pollutants in ALI systems. Some models are commercially available such as EpiAirway developed by MatTek's (MatTek Corporation,

MA, USA), OncoCilAir™ and MucilAir™ (Epithelix Sarl, Switzerland). One of their advantage is their long lifetime (3–12 months), allowing the possibility for chronic exposure studies. However, the commercially available systems are limited in terms of flexibility (cell manipulation, genetic manipulation) and customization (disease-specific donor for primary cells) (Upadhyay and Palmberg, 2018).

Regarding the exposure systems, the use of exposure chambers is widely shared, especially using commercially available systems such as VITROCELL and CULTTEX. One major concern is to allow an homogenous distribution of the particles over the whole cell surface and reduce agglomeration of particles to avoid the alteration of their physicochemical properties (Upadhyay and Palmberg, 2018).

6.5. Advanced *in vitro* cell models

As previously discussed, standard cell cultures are not suitable for the assessment of low-density particles. However, using advanced cell models such as three-dimensional (3D) cultures such as organoids may be more adapted as they could enhance particle/cell contact.

Organoids are 3D cellular structures generated from induced pluripotent stem cells, embryonic stem cells, or adult tissue-resident stem cells. Recently, organoids of different types have been established including kidney, brain, intestine, liver, pancreas and lung organoids. Their main advantage is to recapitulate *in vivo* tissue and organ complexity (Bredeck et al., 2022; Winkler et al., 2022).

Winkler *et al.* used human airway organoids to investigate the effects of microplastic fibers and provided a proof of the suitability of the model for hazard assessment of airborne micro- and nanoplastics (Winkler et al., 2022).

However, the use of organoids as cellular models for *in vitro* toxicity testing is still insufficiently documented and is associated to technical challenges. For instance, as exposure to particles occurs from the apical side, it is not accessible without manipulation. Similarly, diffusion of particles is

hindered in matrices such as Matrigel, which is often used as a scaffold for organoid construction. Therefore, some adaptations will be required to overcome these challenges, including luminal microinjection or the application of flow after puncturing the organoid, reverse cell polarization (*i.e.*, "apical-out" organoids), or resolution of the 3D structure of the organoids into a 2D structure (Bredeck et al., 2022; Busch et al., 2023).

6.6. Other approaches

To control the buoyancy of the particles, Green *et al.* suggested embedding PE particles in the surface layer of agarose and placing macrophages on them (Green et al., 1998). The problem with this approach is that it is applicable only to phagocytizing cells as the particles are immobile.

It was also proposed to make the particle denser, this approach was used by Ammar *et al.* by using mineral talc as filler for PP to increase its density (Ammar et al., 2017).

An alternative approach was to use solvents such as dimethyl sulfoxide (DMSO) (Hwang et al., 2019). However, it was later demonstrated that the use of DMSO was not sufficient to ensure a stable particle dispersion as particles floated up again after a short time. In addition, the DMSO concentrations that were necessary were above the range recommended for cell culture to avoid cytotoxic effects from DMSO itself (Stock et al., 2020). More generally speaking, the addition of any stabilizing agent can affect either the particle properties or incubation conditions and therefore lead to changed effects on the cells.

Although some challenges remain to be overcome, requiring further developments, the above-discussed adaptations brought to standard *in vitro* models for the testing of low-density micro- and nanoplastics have been shown to be promising, opening new perspectives in the field.

7. Conclusion



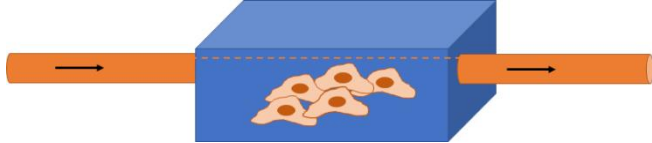
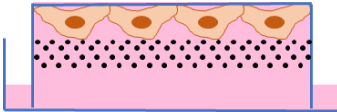
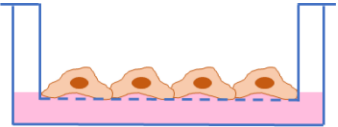
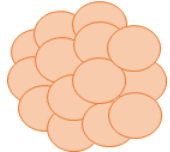
Research on the toxicity of micro- and nanoplastics is growing rapidly. However, conventional *in vitro* assays are not suitable for evaluating the toxicity of buoyant samples. This challenging issue has generally been overlooked, but we have shown, using several examples from the literature, that alternatives or adaptations are possible to account for the buoyancy of particles in *in vitro* models and to obtain accurate data.

8. Perspectives for further research on the assessment of micro- and nanoplastics toxicity

Despite the need for specific adaptations of the standard *in vitro* models for the evaluation of buoyant materials, further recommendations can be made for further research in this area, as shown in Table

1.

Table 1 – Summarizing table of the issues raised during the *in vitro* assessment of micro- and nanoplastics toxicity and the possible solutions.

Issue	Proposed solutions/ perspectives	References
<p>Buoyancy of low-density particles</p>  <p>High-density particles → sedimentation: contact with cells at the bottom of the culture plate</p>  <p>Low-density particles → buoyancy: no contact with cells at the bottom of the culture plate, model not suitable for toxicity assessment</p>	<p>Dosimetry taken into account by using mathematical modeling</p> $(x + a)^n = \sum_{k=0}^n \binom{n}{k} x^k a^{n-k}$ $(1 + x)^n = 1 + \frac{nx}{1!} + \frac{n(n-1)x^2}{2!} + \dots \quad x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$ $f(x) = a_0 + \sum_{n=1}^{\infty} \left(a_n \cos \frac{n\pi x}{L} + b_n \sin \frac{n\pi x}{L} \right)$ <p>Cell culture models grown in dynamic conditions</p>  <p>Inverse culture models</p>  <p>Air-liquid interface (ALI) systems</p>  <p>Advanced <i>in vitro</i> cell models such as organoids</p>  <p>...</p>	<p>(Böhmert et al., 2018; Busch et al., 2023, 2021b; DeLoid et al., 2015; Hinderliter et al., 2010; Liu et al., 2015; Thomas et al., 2018)</p> <p>(Esch et al., 2014; Gupta et al., 2021; Liu et al., 2022; Xiao et al., 2023)</p> <p>(Busch et al., 2021b; Stock et al., 2021, 2020; Watson et al., 2016)</p> <p>(Meindl et al., 2021; Upadhyay and Palmberg, 2018)</p> <p>(Bredeck et al., 2022; Winkler et al., 2022)</p>

Lack of representativity/relevance of the materials tested and of the exposure scenarios	<p>Assessment of a larger and more relevant variety of plastics (types of polymers, shapes, functionalization...).</p> <p>Use of more relevant cell models and exposure scenarios (advanced cell models, chronic exposure, exposure to mixtures of micro- and nanoplastics...)</p> <p>Consideration of the particle environment (corona, contact with fluids from the organism as occurs <i>in vivo</i>...).</p> <p>...</p>	
Lack of identification of the factors responsible for adverse effects	<p>Better characterization of factors driving toxicity (<i>i.e.</i>, intrinsic toxicity of the particles, toxicity of additives, dispersants...), inclusion of appropriate experimental controls.</p>	
Heterogeneity of results observed in the literature	<p>Need of the development of standardization protocols to allow comparisons and the drawing of firm conclusions.</p>	

8.1. Using samples and experimental conditions more representative of real-life exposure

8.1.1. Assessing more relevant micro- and nanoplastic samples

As mentioned above, there is a wide variety of micro- and nanoplastics (different polymer types, shapes, colors, sizes, additives, states of aggregation, charges, etc.), while experimental studies focus only on some model particles. As an example, although PS is less relevant as a pollutant compared to other plastics such as PE and PP in terms of production volume and frequency, it is most commonly used for research. This is due to the fact that PS particles are commercially available and can be provided with a range of particle sizes, fluorescent labelling, and various surface modifications, and are also easy to disperse (Busch et al., 2023; Revel et al., 2021; Schröter and Ventura, 2022; Stock et al., 2021, 2020; Xu et al., 2022). Only few studies have considered alternative polymer types or shapes or weathered particles (Busch et al., 2023).

In addition, instead of using engineered produced particles, it would be much better to isolate them from the environment. While this has already been accomplished for macroscopic plastics, it is not possible for nanoplastics. There are, however, protocols for producing micro- or even nanoparticles from everyday objects such as PET bottles (Schröter and Ventura, 2022). Methods like laser ablation, cryo-cutting or milling allow to produce much more relevant polymer types, shapes and size distributions, but might not allow comparisons between different studies (Busch et al., 2023). For instance, Magrì *et al.* used laser ablation of polymers to form PET nanoplastics, which mimics real environmental nanopollutants, unlike synthetic samples obtained by colloidal chemistry (Magrì et al., 2018). Similarly, Tolardo *et al.* produced PC and PET nanoparticles by laser ablation (Tolardo et al., 2022).

Further investigations regarding particle sampling, identification, and analysis will help better take into account the heterogeneity and relevance of samples (Schröter and Ventura, 2022).

8.1.2. Using more relevant cell models and exposure scenarios

As previously discussed, although monocultures are convenient for screening purposes, more sophisticated cell models are more predictive of *in vivo* biological responses. Models with increasing complexity (3D models, under dynamic conditions, etc.) should therefore be preferred. In addition, the exposure pathway should also be as realistic as possible. For example, ALI systems should be recommended when assessing the respiratory effects of airborne micro- and nanoplastics.

Also, we are not exposed to only one type of micro- and nanoplastics but to a mixture. Consequently, either homo- or hetero-aggregation can occur, thereby affecting the toxicity (Wang et al., 2021). Indeed, the adverse effects elicited by particles are usually assessed focusing on individual particles, whereas their interaction with co-contaminants can deeply affect their biodistribution, fate in the organism and toxicological profile (additive, synergistic or antagonistic responses) (Forest, 2021). The investigation of such combined effects should not be neglected to better reflect real-life exposure scenarios.

8.1.3. Consideration of the particle environment

When particles come into contact with a biological medium or a cell culture medium, the macromolecules present in the fluid (*i.e.*, lipids, carbohydrates but mainly proteins) will adhere to the particle surface forming the so-called “protein corona” (Cedervall et al., 2007; Forest, 2019). This protein layer alters the physicochemical properties of the particles as well as the interactions with biological systems, therefore influencing the subsequent biological response (Forest, 2019; Monopoli et al., 2012). While it can have a deep impact on toxicity assessments, except in few exceptions, this aspect has been often neglected in the case of micro- and nanoplastics and should be more carefully considered henceforth (Busch et al., 2023; Kihara et al., 2021).

Similarly, to reproduce what occurs *in vivo*, it should be interesting to pre-incubate micro- and nanoplastic particles with biological fluids that are encountered in the organism, such as pulmonary surfactant for inhaled particles or gastric fluids for ingested particles. In this regard, DeLoid *et al.*

performed a 3-phase digestion of PS particles to simulate oral, gastric, and small intestinal digestion. The digesta were then applied to an *in vitro* model of small intestinal epithelium (DeLoid et al., 2021).

8.1.4. Chronic exposure

More realistic exposure scenarios should be considered. Indeed, *in vitro* models are better suited for short-term, acute exposure. However, this kind of exposure does not represent what occurs in real-life conditions. Indeed, due to the biopersistence of plastics in biological environments and the continuous human exposure, inhaled or ingested plastic particles can accumulate in tissues and organs leading to long-term effects. This argues for an urgent need to consider repeated or chronic exposure (Rubio et al., 2020). Although they are still rare, some attempts have been made in this direction. For instance, Domenech *et al.* exposed PS nanoparticles to Caco-2 cells for 8 weeks (Domenech et al., 2021). Barguilla *et al.* exposed mouse embryonic fibroblasts to PS nanoparticles for 12 weeks (Barguilla et al., 2022).

8.2. Considering standardization for comparison purposes

Currently, there are no standardized methods for assessing the toxicity of micro- and nanoplastics, resulting in studies differing in terms of types of polymers, particle concentrations, particle sizes, particle functionalizations, cell models, types of biological media, studied endpoints... (Banerjee and Shelver, 2021; Revel et al., 2021; Schröter and Ventura, 2022). In addition, detection methods can vary (Rubio et al., 2020). Taken together, the heterogeneity of samples and experimental conditions make it challenging to compare the particle effects between studies and draw firm conclusions.

Also, for the sake of standardization and to allow relevant comparisons of studies using different exposure models (standard cell culture, inverse cell culture, ALI...), we recommend not to consider the use of similar exposure concentrations but the use of target concentrations. Indeed, depending on the

nanoparticle's characteristics (such as their buoyancy), only a part of the administered dose will reach the cells to induce adverse effects and this is this part of particles interacting with cells that should be considered. It is of utmost importance as for a similar administered nanoparticle concentration, the target concentration can be different because of the specificities of each experimental model.

8.3. Better characterization of the driving factors of toxicity

Future research should also allow to determine if the observed adverse effects are due to the micro- and nanoplastics themselves or originate from other compounds such as additives or a combination of both. This is essential for a correct interpretation of the toxicity data, avoiding overestimating the impact of the plastic particles (Busch et al., 2023; Xu et al., 2022). To that purpose, a proposed approach is to include a filtrate control in experiments, *i.e.*, exposing cells to chemicals extracted from the investigated micro- and nanoplastic particles (Petersen et al., 2022). The biological effects observed are then compared to those induced by the native particles (Xu et al., 2022). Similarly, care should be taken with the dispersants often used in ready-to-use particle suspensions commercially available. The composition of these solvents is rarely given by the manufacturers but they may cause toxicological effects that can be misinterpreted as a particle effect. As an example, Stock *et al.* have shown that some dispersants (separated from the particles by centrifugation) were able to cause a more pronounced cytotoxic effect than the PS particles themselves. Therefore this bias needs to be addressed with proper controls (Stock et al., 2022).

These perspectives should allow better characterization of the potential toxicity of micro- and nanoplastics, which is urgently needed given the ongoing and increasing human exposure to these environmental pollutants.

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