Universitat Autònoma de Barcelona

Bioavailability and effects of microplastics and nanoplastics on in vitro gastrointestinal models. A focus on nanopolystyrene as representative nano-sized plastic material

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DOCTORAL DISSERTATION

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2021

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Dissertation respectfully submitted by

Josefa Domenech Cabrera

To Universitat Autònoma de Barcelona in partial fulfillment of the requirements for the degree of Doctor of Philosophy, as per the Doctorate Program in Genetics

Under the supervision of Dr. Ricard Marcos Dauder, Dr. Alba Hernández Bonilla and Dr. Constanza Cortés Crignola

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ABSTRACT

Owing to the wide range of tunable physicochemical properties, and the rapid and low-cost production, plastics present uncountable applications in a variety of fields such as product packaging, agriculture, building, and electronics, among others. This fact leads to the corresponding exponential increase in the generated plastic waste that ends up polluting the environment. Different environmental conditions favor physical, chemical, and biological processes that drive plastic waste to continuous degradation, generating the so-called micro- and nanoplastics (MNPLs). In addition to those, rapid advances in nanotechnology have driven the target industrial production of plastic particles in the micro- and nano-size ranges used in cosmetic or cleaning products that also contribute to plastic environmental pollution.

The increasing presence of MNPLs in nature represents an environmental challenge but, this is also accompanied by potential human exposure that might cause health effects. Nevertheless, the current methodological limitations do not allow for accurate estimations on the levels of human exposure, and the scarcity of data on the effects of MNPLs in mammalian models hampers the understanding of whether the presence of MNPLs in different environmental niches and organisms may pose a risk for humans. Aiming to contribute to expanding the knowledge on human exposure, and the potential toxic and genotoxic effects of MNPLs on human health, in this Thesis we have reviewed the last studies referring to the occurrence of MNPLs in food and airborne, and we have performed extended *in vitro* studies to reinforce our knowledge on the biological effects of polystyrene nanoplastics (PSNPLs) in human cell models.

Our review of literature, comprised in the first Chapter of the present Thesis, revealed ingestion as the major human exposure route to MNPLs and highlighted the knowledge gaps and limitations conditioning the MNPLs hazard assessment. Therefore, we moved forward to evaluate the interactions of PSNPLs with different human gastrointestinal *in vitro* models, as well as the toxicity and genotoxicity of the aforementioned nanoplastics in those models. From our second study, we confirmed the ability of PSNPLs to rapidly internalize into human-derived undifferentiated colorectal adenocarcinoma cells (Caco-2 cell line). Importantly, Caco-2 cells showed signals of general stress induction after the exposure to PSNPLs, namely intracellular structural alterations and increased expression of genes encoding proteins related to cellular stress [*heme oxygenase-1* (*HO1*) and *heat shock protein 70* (*HSP70*)]. However, although PSNPLs reach cell nuclei in only 24 h, Caco-2 cells did not show genotoxic effects.

Because of the heightened interest in the effects of MNPLs after their ingestion, we further analyzed, in our third study, the internalization ability of PSNPLs in Caco-2 based *in vitro* 2D models of the human gut barrier, which include representation of goblet (Caco-2/HT29 model) and microfold cells (Caco-2/HT29/Raji-B model). Although oxidative stress is considered as the main mechanism of MNPLs toxicity, our findings describe PSNPLs as weak toxicants due to their low ability to induce reactive oxygen species (ROS) or other toxic effects. Similar to what was found in the undifferentiated Caco-2 model, PSNPLs did not exert genotoxic or oxidative DNA damage in the 2D models, despite having a great internalization capacity that allowed reaching cell nuclei and even translocating across the intestinal barrier.

To date, results obtained regarding MNPLs' health effects are controversial and consequently, clear conclusions cannot be drawn on the current level of data. Nevertheless, the exposure scenario is aggravated if the potential MNPLs' ability as carriers of other hazard contaminants is considered. Under this hypothesis, MNPLs could prompt human exposure to well-known toxic pollutants and potentially alter their effects on human health. Therefore, MNPLs safety research through a single-material approach leads to an incomplete picture of human exposure and its potentially harmful effects. In our fourth study, we attempted to overcome these limitations by introducing a co-exposure design. Thus, our results demonstrated the physical interaction between PSNPLs and a widespread legacy pollutant, silver nanoparticles (AgNPs), that were selected for that study as environmental metal pollutant model. Silver nitrate ($AgNO_3$) was also included in the study as a surrogate of silver ion releasing agent to confirm that the effects induced by AgNPs were not caused by released ions. Importantly, we proved the adaptability of classical methodological approaches, such as transmission electron microscopy, to prove and visualize the interaction of the heavy metals/MNPLs interplay. Moreover, our results support the hypothesis, since we observed the enhancement of AgNPs internalization into undifferentiated Caco-2 cells, prompted by the AgNPs/PSNPLs co-exposure. The induction of toxic effects by AgNPs remained unaltered after the co-treatment. However, AgNPs/PSNPLs complexes reached Caco-2 cell nuclei, causing an increased genotoxic damage tendency as the AgNPs concentration increases when combined with high doses of PSNPLs.

ABBREVIATION LIST

2D	Two dimensions
Ag^+	Silver ion
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
Caco-2	Human colorectal adenocarcinoma derived cell line
COOH	Carboxyl group
DCFH-DA	2',7'-dichlorofluorescein diacetate
DHE	Dihydroethidium
DLS	Dynamic light scattering
DMEM	Dulbecco's modified Eagle’s medium
DNA	Deoxyribonucleic acid
EFSA	European Food and Safety Authority
FAE	Follicle-associated epithelium
GIT	Gastrointestinal tract
GSTP1	Glutathione-S-transferase pi 1
H_2O_2	Hydrogen peroxide
HO1	Heme oxygenase-1
HSP70	Heat shock protein 70
HT29	Human colorectal adenocarcinoma derived cell line
IL	Interleukin
LDV	Laser Doppler velocimetry
LY	Lucifer yellow
M-cells	Microfold cells

MN	Micronuclei
MNPLs	Micro- and nanoplastics
MPLs	Microplastics
mROS	Mitochondrial-derived ROS
NH_2	Amino group
NPs	Nanoparticles
NPLs	Nanoplastics
$O2^{\cdot-}$	Superoxide radical
ODD	Oxidative DNA damage
$OH^{\cdot-}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PA	Polyamide
PAHs	Polyaromatic hydrocarbons
PC	Polycarbonate
PdI	Polydispersity index
PE	Polyethylene
PET	Polyethylene terephthalate
POPs	Persistent organic pollutants
PP	Polypropylene
PPs	Peyer's patches
PS	Polystyrene
PSNPLs[1]	Polystyrene nanoplastics
PU	Polyurethane

PVC	Polyvinylchloride
Raji-B	Human B lymphocyte cell line
ROS	Reactive oxygen species
RT	Respiratory tract
SOD	Superoxide dismutase
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy
y-PSNPLs[1]	Yellow polystyrene nanoplastics

[1] The abbreviations of the terms polystyrene nanoplastics and yellow polystyrene nanoplastics have evolved throughout the work carried out during this Thesis. Therefore, other abbreviations such as nPS, nPSNPs or PSNPs, and y-nPS, y-nPSNPs or y-PSNPs, respectively, will be found in the different published articles comprised in the Thesis.

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1. INTRODUCTION

1. INTRODUCTION

1.1. MICROPLASTICS AND NANOPLASTICS AS CONTAMINANTS OF EMERGING CONCERN

1.1.1. Plastics: past and present

Plastic is the popularly known term used to name a broad range of synthetic or semisynthetic polymers made of organic compounds. As well as wood, paper or wool, plastics are composed of natural raw products such as cellulose, resin, coal, natural gas, rubber, salt, or petroleum derivatives.

The great variety of synthetic materials that we daily identify as modern plastics, started to appear about 160 years ago. Plastic history began in 1855, when the British chemist Alexander Parkes created the first plastic material, originally known as Parkesine, and named celluloid today. Then, between 1838 and 1872, polyvinylchloride (PVC) was generated. However, the first truly synthetic plastic material produced on an industrial scale was the bakelite, created by Leo Baekeland in 1907. Thenceforth, innovation in plastic continued to develop, generating materials commonly used these days, such as polystyrene (PS) firstly synthesized in 1920, or polyethylene (PE) created in 1933, and it continues to grow (Worm et al., 2017; PlasticsEurope, 2020).

Lightness, thermal and electrical insulation, high durability and resistance, and low-cost production are the main properties that characterize plastic materials. Nonetheless, the industry has developed the ability to modify different attributes such as transparency or electrical conductance by the integration of other materials to meet the desired requirements. Thus, plastic materials can be produced on demand for almost every imaginable use. This versatility had made plastics the best-choice materials for different applications in a full range of areas that include packaging, building and construction, automotive, electronics, household and furniture, agriculture, medical applications, or mechanical engineering, among others. For this reason, world plastic production has rocketed considerably during the last decades, going from about 50 million tons in the ’70s to 368 million tons in 2019 (PlasticsEurope, 2020). Although the contribution to total plastic manufacturing is uneven across the different continents, being Asia the responsible for half of the world's plastic production (Figure 1), the tendency observed in the past years indicates a constant increase (PlasticsEurope, 2020).

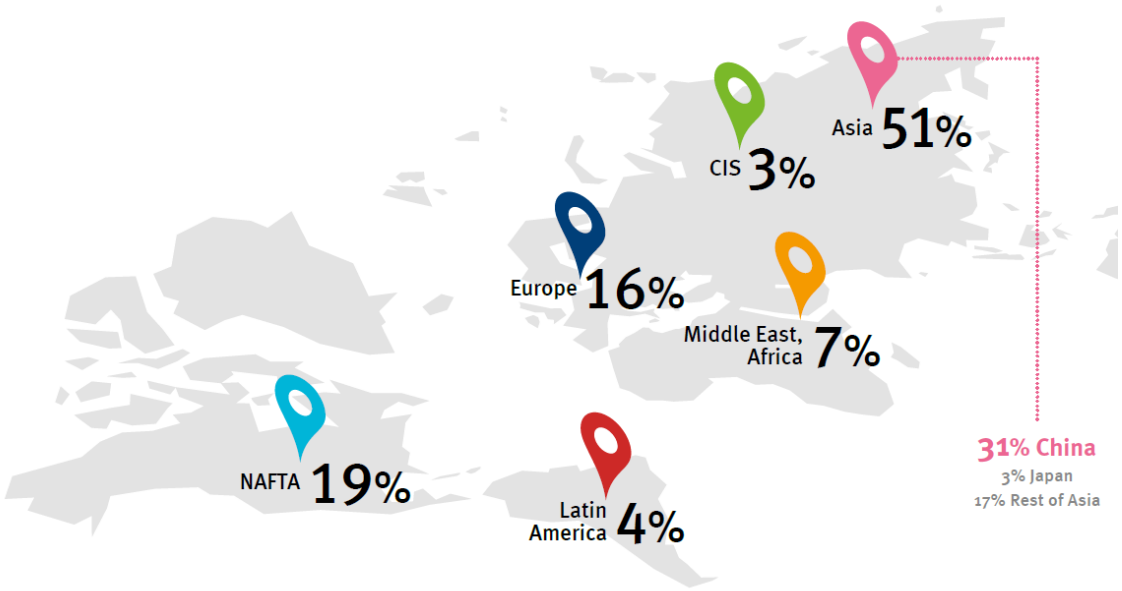

Figure 1. Distribution of global plastic production in 2019**.** Data include the production of thermoplastics, polyurethanes, thermosets, elastomers, adhesives, coatings and sealants, and polypropylene-fibers. Polyethylene terephthalate-fibers, polyamide-fibers, and polyacryl-fibers production are not included. (Source: PlasticsEurope, 2020).

The extensive variety of plastics produced can be classified into two main groups according to their physicochemical properties: thermoplastics and thermosets. Thermoplastics soften with heat and harden with cold [e.g., PVC, PS, PE, polycarbonate (PC), polyethylene terephthalate (PET), polypropylene (PP), expanded polystyrene (EPS), polytetrafluoroethylene (PTFE), polymethylmethacrylate (PMMA), acrylonitrile-butadiene-styrene (ABS)]. Thermosets never soften once molded [e.g., epoxide (EP), phenol-formaldehyde (PF), polyurethane (PU), and unsaturated polyester resins (UP)] (Andrady, 2017). According to the polymer type, the most used plastics are PE, PP, PS, and PET for packaging applications, and PVC for building and construction (Figure 2).

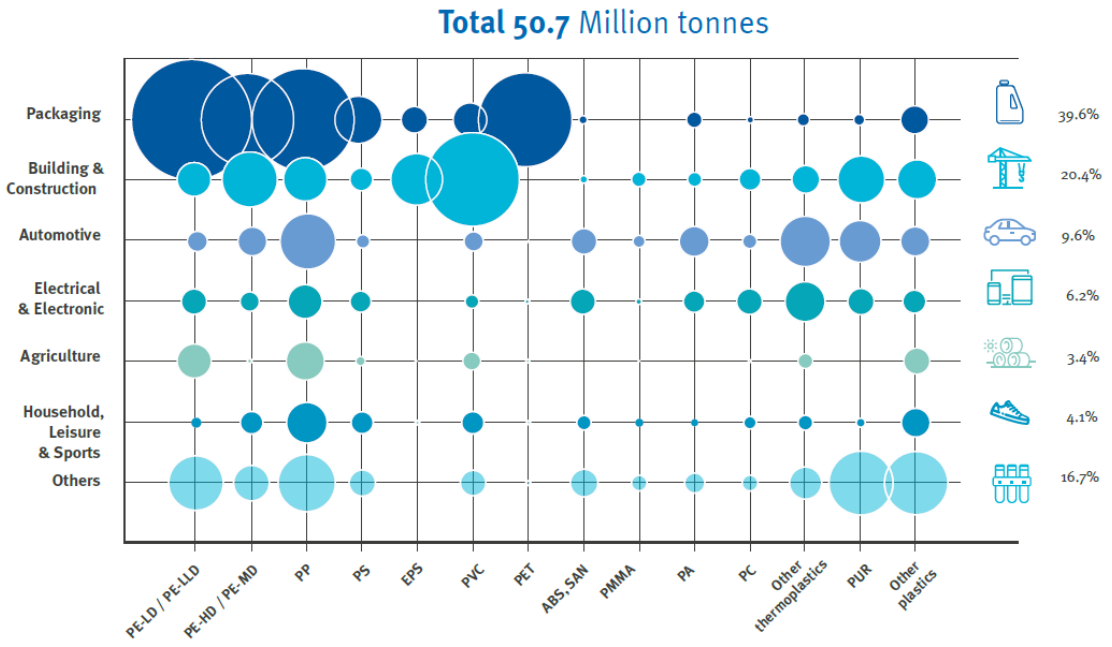

Figure 2. Plastic demand by segment and polymer type in Europe in 2019. (Source: PlasticsEurope, 2020).

1.1.2. Microplastics and nanoplastics: origin and classification

According to the size, plastics have been mostly classified as macroplastics, mesoplastics, microplastics (MPLs), and nanoplastics (NPLs). Despite some authors do not consider it that way, the term NPLs is well-defined by the legacy of the term nanoparticle as particles ranging from 1 to <100 nm (ISO, 2015). Additionally, the European Food and Safety Authority (EFSA), as well as the European Commission agree with this definition detailed by the International Organization for Standardization (ISO) (The European Commission, 2011; EFSA, 2016). Nevertheless, no consensus has been reached regarding the other measurement ranges. Although the microscale is formally defined as 1 to 1000 µm on the conventional units of size, most authors consider particles up to 5000 µm as MPLs, as recommended by the EFSA. In the case of meso- and macroplastics, the most accepted size definitions are <2.5 cm and >2.5 cm, respectively (Hartmann et al., 2019). Limits between size ranges are still a subject receiving intense interest/discussion and continuous review. However, due to the exceptional features that micro- and nanoparticles exhibit, in the context of this Thesis we will refer to a joint category, the so-called micro- and nanoplastics (MNPLs) that includes plastic particles from 1 nm to 5000 µm. As mentioned, MNPLs, unlike their bulk counterparts, exhibit particular chemical, electric, physical, magnetic, and mechanical properties. The fact that small particles present a greater surface-to-volume ratio turns

them into more reactive materials. This is explained because of the higher availability of atoms in the surface that facilitates chemical reactions, so that, better interactions with surrounding inert or alive elements and more toxic effects (Buzea et al., 2007; Lewinski et al., 2008).

Regarding the morphology, different MNPLs shapes have been characterized ranging from small irregular fragments to perfectly rounded beads or irregular spherical granules, porous materials in foam appearance, or fibers (Figure 3) (Andrady, 2017; Zhu et al., 2019a).

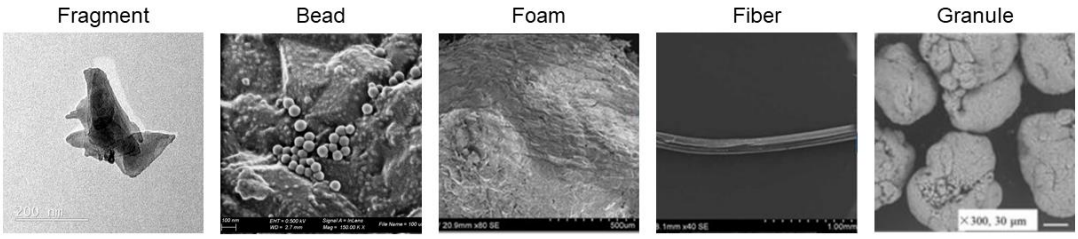

Figure 3. MNPLs morphotypes are represented by PET fragments[1], PS beads[1], foam particles[2], microfibers[2], and PVC granules[3] (Source: [1]Mutagenesis Group, UAB; [2]Liu et al., 2019b; [3]Wang & Wang, 2018).

In addition to size and morphology, environmental MNPLs can also be classified according to their origin. Nanotechnology has led to the targeted industrial production of a variety of plastic particles, the so-called primary plastic particles (Hartmann et al., 2019), to be used in different applications, for instance, personal care products such as scrubs and toothpaste, or sand-blasting media (Andrady, 2017). Moreover, large plastics are, from the very beginning of their production, degraded either by the simple use of the products, as the release of plastic fibers from clothing (Belzagui et al., 2019), or by weathering due to the different environmental factors, especially ultraviolet (UV) light (photodegradation), high temperatures (thermal degradation), water, sediment and wind action (hydrolysis and erosion), or slow oxidation at moderate temperatures (thermo-oxidative degradation) (Liu et al., 2019a; Gerritse et al., 2020; Turner et al., 2020; Wayman & Niemann, 2021) leading to the generation of small plastic debris with different sizes and morphologies referred to as secondary MNPLs (Hartmann et al., 2019).

1.1.3. Microplastics and nanoplastics as contaminants of emerging concern: contextualization

As summarized in Figure 2, about 40% of plastic produced is designed for single-use applications, and most of the plastic generated becomes waste after its useful life. In

2018, only 33% of the plastic post-consumer waste was recycled, and 25% of the plastic residues ended up in landfills over Europe (PlasticsEurope, 2020). The imbalance between the great demands, namely manufactured plastics, and the little recycling rates is leading to an environmental concerning scenario. Representative examples of the environmental impact of plastic pollution are the five well-publicized plastic patches floating in our oceans (two in the Atlantic Ocean, two in the Pacific Ocean, and one in the Indian Ocean). Only the Great Pacific Patch, with a longer extension than three times the area of Spain, accumulates 96,400 tons of plastic waste (Eriksen et al., 2014).

The spread of plastic waste in the environment poses a global challenge. Plastics are persistent materials and remain in the environment fragmenting into smaller particles of a variety of sizes. In addition to these fragments, manufactured primary MNPLs cannot be neglected. Either, due to the runoff during the mass-production or the degeneration of their larger analogs, the generation of plastic particles in the nano and micro range is attracting great attention due to their ubiquitous nature (including the food web) and their exceptional potential to enter the cells and react with biomolecules, as a consequence of their small size. Therefore, MNPLs (independently of their origin) are considered a potential risk for humans, and the scientific community is currently making great efforts to unravel the effects that such exposures can trigger. However, many are the limitations that researchers face and that are challenging the understanding and development of this field of study. As a result, there is a lack of knowledge about the potential interactions and outcomes that MNPLs could arise in humans. Since these limitations govern all the knowledge concerning MNPLs, they will be spelled out in-depth throughout this Thesis.

Attending to all the before mentioned, it is not surprising that MNPLs are considered emergent pollutants, which are defined as synthetic or naturally occurring pollutants that are not commonly monitored in the environment, and from which data related to their fate, behavior, and ecotoxicological and/or human health effects are lacking (Geissen et al., 2015). Such is the attention that MNPLs are attracting that European agencies such as the European Agency for Safety and Health at Work (EU-OSHA, Nanomaterials in Plastic Industry), the European Environment Agency (EEA, Preventing Plastic Waste in Europe), the European Medicines Agency (EMA, Guideline on Plastic Immediate Packaging Materials), the European Chemicals Agency (ECHA, Hot Topics: Microplastics), or the European Food and Safety Authority (EFSA, Plastics and Plastic Recycling) among other examples, have considered MNPLs contamination of global environmental and human concern. The European Union legislation addresses MNPLs by the same regulatory framework for chemicals and mixtures (REACH regulation, EC1907/2006), aiming to improve the protection of human health and the environment

through better and earlier identification of the intrinsic properties of chemical substances. Despite not having specific legislation that regulates the actions of each member of the plastics supply chain, different strategies (COM(2018)28 A European Strategy for Plastics in a Circular Economy and COM(2020) 98 A new Circular Economy Action Plan) are being proposed to limit single-use plastics, promote recycling and circular plastics economy, and regulate the use of intentionally added MNPLs by developing labeling requirements, regulatory measures on waste management, and establishing a policy framework on the use of plastics for producers, which are expected to have a great impact on society, environment, and economy.

1.2. MICROPLASTICS AND NANOPLASTICS OCCURRENCE AND POTENTIAL HUMAN EXPOSURE

1.2.1. Microplastics and nanoplastics environmental distribution

Plastic debris has conquered terrestrial environments, open oceans, and the atmosphere overhead (Petersen & Hubbart, 2021). Almost every daily anthropogenic activity entails MNPLs release to the environment: opening a plastic package, driving a car, doing the laundry, throwing personal care products into the sink, fishing, or walking with footwear are a few examples (Sharma & Chatterjee, 2017; Sommer et al., 2018; O'Brien et al., 2020; Sobhani et al., 2020). In this same line, MNPLs' environmental pollution has aggravated tremendously under the COVID-19 pandemic light due to the popularization of single-use face masks and other personal protective equipment (Ammendolia et al., 2020; Prata et al., 2020; Benson et al., 2021). Besides, direct unauthorized dumping of plastic litter to the environment, blowing from landfills, or plastic incineration, should not be neglected as potential sources of MNPLs (Barnes et al., 2009). Mismanagement of several amounts of plastic debris sources and improper human behavior is worsening a concern that even being stopped today, will persist for years (Worm et al., 2017; Vanapalli et al., 2021).

MNPLs pollution is not static, but it flows through the different environmental niches reaching even the most remote places worldwide. This interconnection between ecosystems is such that it has recently been proposed to be entitled The Plastic Cycle (Figure 4), which is defined by Bank and Hansson (2019) as "the continuous and complex movement of plastic materials between different abiotic and biotic ecosystem compartments, including humans". Nowadays, about 2.7 million tons of plastic and MNPLs are estimated to be floating in the worldwide marine environment (Eriksen et al., 2014). It has been reported that 80% of that, accounts for land-based plastic and MNPLs

waste (Sharma & Chatterjee, 2017). Once MNPLs reach a water body, namely freshwater, the sea, or wastewater, it is a potential contaminant of every aquatic environment due to the interconnection through them. Apart from the secondary MNPLs directly generated in water bodies from large plastic contaminants, there is a direct anthropogenic release by the use of fishing nets and the coastal tourism in the sea (Figure 4A) (Ryan et al., 2009). In addition, the ease of primary MNPLs straight entering water niches from land is alarming. Toothpaste, hand cleansers, and different cleaning products enter the water channels through household and industry drainage systems (Gregory, 1996; Fendall & Sewell, 2009; Duis & Coors, 2016). Secondary MNPLs generated in terrestrial environments, such as laundry residues, overlap with the previous ones through sewage. These primary and secondary MNPLs reaching water bodies through wastewater, are not trapped in wastewater treatment plants (WWTP) and, consequently, are freely transported to rivers and oceans (Gregory, 1996; Browne et al., 2007; Fendall & Sewell, 2009).

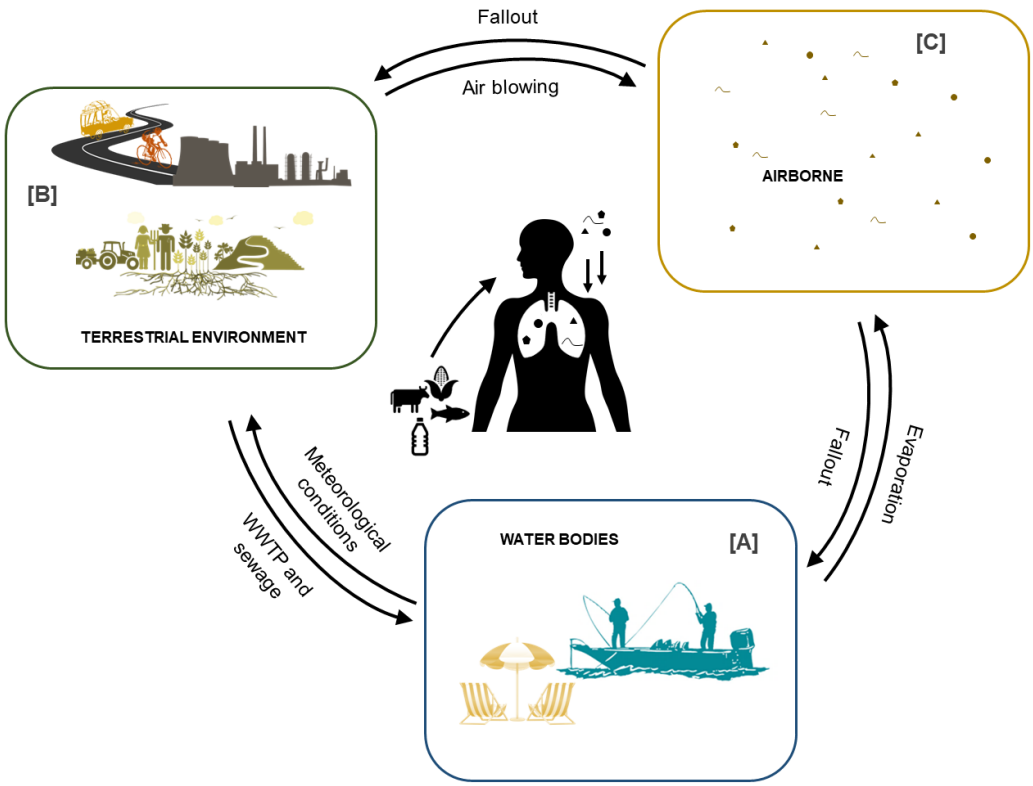

Figure 4. Graphical representation of The Plastic Cycle showing the dynamic MNPLs pollution through the different environmental niches. Humans have a central role in The Plastic Cycle being exposed to MNPLs by inhalation, ingestion, and dermal contact. Letters in brackets refer to what is described in the text.

Not only aquatic systems shape an entwine net, but also terrestrial and aerial environmental niches. MNPLs in aquatic environments can migrate to land by different meteorological conditions including hurricanes, floods, or air drafts (Barnes et al., 2009). Apart from the large amount of MNPLs generated in land by the already described human activity, sewage sludge usually used as agriculture fertilizer, wastewater irrigation, and the extended use of agricultural plastics as mulch films, entail an important contribution of MNPLs to arable soils (Figure 4B) (Weithmann et al., 2018; Corradini et al., 2019; Zhu et al., 2019a). The release from garments and tire abrasion from traffic are the main causes of airborne MNPLs occurrence (Chen et al., 2019), but also many other daily anthropogenic activities contribute to the atmospheric pollution (Figure 4C). Airborne MNPLs can be transported over long distances and it constitutes the major pathway for MNPLs arrival to remote locations (Allen et al., 2019; Evangeliou et al., 2020). The steady exchange of MNPLs between terrestrial or aquatic environments and airborne is a fact. While water evaporation and air blowing lift MNPLs to airborne (Allen et al., 2020), rainfalls or particles fallout due to their density, size, and biofilm formation (Hoellein et al., 2019; He et al., 2020a), drives MNPLs to ground and water bodies (Prata, 2018; Abbasi et al., 2019).

1.2.2. Potential human exposure to microplastics and nanoplastics and body entry pathways

Humans, as part of The Plastic Cycle, unavoidably interact with MNPLs in different ways due to their ubiquitous spread into the environment (Figure 4). Continuous exposure to MNPLs through inhalation is undoubted since indoor and outdoor airborne has been reported to accumulate plastic particles (Gaston et al., 2020). A broad range of circumstances (e.g. anthropogenic activity, population density, industrialization, cleaning habits, or furniture) play a critical role in the amount and types of MNPLs we are exposed to while breathing (Chen et al., 2019; Zhang et al., 2020). Additionally, not only MNPLs suspended in the atmosphere but also the constant particles' fallout increases the human contact with them. This deposition over the human body along with the direct dermal contact with hand and face cleansers, face masks, or toothpaste containing MNPLs, constitutes a potential dermal pathway of human exposure to plastic particles (Revel et al., 2018; Rahman et al., 2020). However, most attention must be paid to the MNPLs ingestion since they can easily enter the human food chain (Waring et al., 2018; Toussaint et al., 2019). As will be discussed in the first article of this Thesis, ingestion accounts for the major route of human exposure to MNPLs. In marine webs, fauna from different trophic positions from plankton to seabirds, turtles, or marine mammals is

exposed to MNPLs by direct ingestion or by predation of species at a lower level in the food chain. Similarly, plastic particles can be unintentionally ingested by livestock (Karbalaei et al., 2018) that feed on crops irrigated and fertilized by contaminated water and sludge. Humans, as top predators, ingest large amounts of plastic particles through trophic transfer (Worm et al., 2017). Thus, MNPLs have been found in different species of edible fishes (Neto et al., 2020; Zakeri et al., 2020), bivalves (Sendra et al., 2021), and crustaceans (Iannilli et al., 2019), as well as evidence of MNPLs in livestock, have been reported (Urbina et al., 2020; Beriot et al., 2021). However, the ingestion of MNPLs is not only due to a meat- and fish-eating diet. Other terrestrial sources of food such as commercially available fruits, vegetables, or cereals, have been informed to be plastically polluted (García Ibarra et al., 2019; Oliveri Conti et al., 2020). In addition to that, contamination of other types of packaged or canned food such as bottled drinks, deliverable food, salt, and sardines or tuna among others is well-documented (Wright & Kelly, 2017; Karami et al., 2018; Akhbarizadeh et al., 2020; Fadare et al., 2020). The sources of MNPLs in these products are both the deterioration of the packages (Hernandez et al., 2019; Fadare et al., 2020), and the contamination during their manufacturing, as in the honey or beer cases (Liebezeit & Liebezeit, 2013; Kosuth et al., 2018).

1.3. POTENTIAL HARMFUL HEALTH EFFECTS ASSOCIATED WITH MICROPLASTICS AND NANOPLASTICS EXPOSURE

1.3.1. Knowledge gaps and limitations conditioning the hazard assessment of microplastics and nanoplastics

There is a dearth of clear evidence on the risk that MNPLs, as emerging agents with environmental/health concerns, could pose to humans. This is mostly motivated by the lack of knowledge on methodological essential procedures, robust protocols, and useful/solid models of study.

1.3.1.1. Lack of reference materials

Plastics used in toxicological assessments are not usually exemplifying their environmental analogs (Prata et al., 2019). This is because of the commercial availability of plastic particles that is very limited in terms of types of polymers, shapes, or sizes. So that, commercial plastic particles are questionable concerning environmental representativeness. In addition, the single-MNPLs-type exposure approach is completely unreal, but the lack of data about the composition of the environmental samples, namely

types and amounts of plastics, and the mentioned limited commercial availability of MNPLs, restrict more representative approaches.

On the other hand, manufactured MNPLs are debatable as models of secondary plastic particles. Therefore, owing to simulate the highly heterogeneous secondary MNPLs spread all over, some authors have proposed methods to artificially produce them under laboratory conditions. von der Esch et al. (2020) established a sonication-based method of fragmentation of plastic materials under hydrolytic conditions to obtain MNPLs. Similarly, PETNPLs have been produced using two different methods reported recently (Magrì et al., 2018; Rodríguez-Hernández et al., 2019). Magrì et al. set up a technique based on ablation of MNPLs from their larger counterparts using a UV-laser beam, while Rodríguez-Hernández and co-workers produced MNPLs by attrition fragmentation and acid conditions. Moreover, many efforts are being addressed to unravel and mimic environmental samples in terms of surface properties, weathering, and adsorbed agents. Although purchased plastic particles perfectly mimic freshly manufactured MNPLs, actual exposure usually occurs after long periods of MNPLs deterioration in the environment. The growing interest among researchers in exploring realistic samples supposes the development of methods to modify commercial MNPLs to get samples resembling environmental debris. In this sense, scientists have been forced to establish aging/weathering *in vitro* techniques, to expose commercial MNPLs samples to different artificial environmental factors. Among the ones typically included are UV photodegradation and thermal decomposition (Müller et al., 2018; Luo et al., 2019; Luo et al., 2020a), oxidation (exposure to H_2O_2, H_2O, or Cl_2, or supervised Fenton reactions) (Lang et al., 2020; Liu et al., 2020b), and abrasion by simulated meteorological or environmental conditions (periodical addition of water to simulate waves or storms, incubation in stream water or faked seawater, and air blowing exposure) (Kalčíková et al., 2020; Sun et al., 2020), albeit most of the procedures include more than one of the mentioned artificial factors (Hüffer et al., 2018; Mao et al., 2020; Wang et al., 2020a; Wu et al., 2020a; Wu et al., 2020b).

However, how environmental plastic particles differ from pristine engineered and laboratory-made MNPLs is largely unexplored. The lack of knowledge about environmental samples' characteristics, composition, and properties means that a consensus on the most appropriate material/materials to explore potential effects cannot be reached at present. In addition to that, the use of labeled MNPLs is mandatory to track their fate in different organs or tissues. Thus, only those plastic particles with commercially available labeled analogs (i.e., PS) are useful for studies focusing on these approaches.

1.3.1.1.1. Polystyrene nanoparticles as the gold standard

Since PS can be easily synthesized in a variety of sizes, it was the only available commercial plastic particle in nano- and micro- dimensions until recently (Loos et al., 2014). Nanotechnology has lately developed new commercial MNPLs, namely PP, PE, and PVC. Nonetheless, unlike PS, their market supply is limited to few sizes, and their functionalized (chemically surface-modified) or labeled counterparts are not available, hampering their study. As a result, the growing interest in MNPLs as pollutants of emerging concern has made PS particles the standard model. This is illustrated when looking at the literature, where a tremendous bias in the studies towards PS MNPLs is found to date. Within these studies having PS as a leading actor are the articles included in this Thesis.

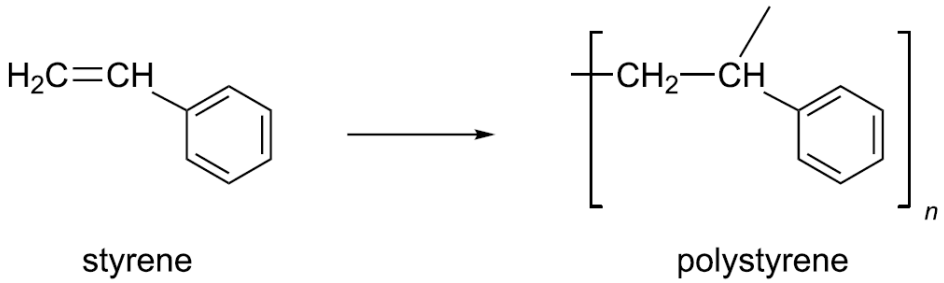

Figure 5. Polystyrene synthesis and chemical structure (Source: Loos et al., 2014).

PS is a non-bio-degradable thermoplastic synthesized by the polymerization of styrene monomers (Figure 5). It is characterized by high translucency, durability, and ease to be dyed. Due to these properties, it is broadly used in a wide range of daily products, for instance, CDs, toys, toothbrushes, laboratory equipment, or biomedical devices (Kik et al., 2020). Rapid heating of PS with foaming agents results in extruded polystyrene popularly known as Styrofoam, which has thermal insulating features and is the main component of food trays, plates, or cups, as well as food storage or packaging products (Wünsch, 2000; Chen et al., 2019). These short-life commodities contribute to the plastic pollution challenge and are responsible for the worldwide spreading of PS MNPLs. Furthermore, PS is not bio-degradable and high temperatures reaching 1000 ºC are required for its incineration. This explains its great persistence that constitutes a major problem for the environment and human health (Kik et al., 2020).

1.3.1.2. Lack of analytical tools and data on exposure levels

One of the major limitations of current perspectives on MNPLs research is the lack of harmonization of analytical methodologies. This is mainly alarming in the identification

and quantification of NPLs. Many are the authors claiming for standardization of operational procedures at different levels of the assessment involving MNPLs, particularly for the collection of samples, isolation of MNPLs, and identification, quantification, and characterization of the different plastic types. Several methods are applied to these aims with great variations among laboratories because of the dearth of established analytical protocols (Enyoh et al., 2019).

The distribution of MNPLs in different environmental matrices is not homogeneous but a valid sampling strategy should be able to represent the original large group from which they were collected (Barbosa et al., 2020). Added to this inherent difficulty due to MNPLs spreading are the sampling methods themselves. Among the most used collection methods are neuston nets or filtering of samples (Bouwmeester et al., 2015; Chen et al., 2019). However, the mesh sizes used widely vary among studies and it is usually settled depending on the scope of the research. Hence, the size detection limit differs from one publication to another, and the most relevant fraction of plastic material from the toxicological point of view, this is NPLs, is usually discarded (Bouwmeester et al., 2015; Dick Vethaak & Legler, 2021). Once collected the samples, MNPLs must be identified, separated from the rest of the sample, and quantified. Separation methods are size-based (sieving, sequential filtration, size exclusion chromatography, or capillary electrophoresis) (Barbosa et al., 2020), which again supposes a bottleneck. In almost all studies reported in the literature, visual examination is the first step to identify and quantify the isolated particulate matter. Depending on the particle's size, it is usually performed by the naked eye or with microscope supplementation (Bouwmeester et al., 2015). In either way, the knowledge and experience of the researcher play an important role since no stipulated guidelines exist for MNPLs identification. So that, human error can lead to discarding or including misidentified particles (Verla et al., 2019a). Moreover, the limit of detection again compromises the analysis of smaller particles. Further chemical composition identification methods are used in most of the studies, ranging from staining techniques with an affinity for plastic to spectroscopy or mass spectrometry-based methods which require certain characteristics of the sample and have their limitations such as low sensitivity at low concentrations, size resolution, or background interferences (Verla et al., 2019a; Barbosa et al., 2020). Notably, limitations to identify/quantify MPLs or NPLs must be addressed differently since most of the approaches proposed for MPLs are not useful for NPLs.

Specific validated guidelines and methods for MNPLs sampling and study are non-existent. Therefore, the real environmental concentrations cannot be cleared up. These difficulties are translated into an impractical capability to estimate certain exposures to

MNPLs (Barbosa et al., 2020). Thus, risk assessment of MNPLs is restricted by the poor understanding of the quantitative levels present in the environment and inside humans. The World Health Organization (WHO) noted the challenge posed by the limitation of accurate and standardized methods when determining the occurrence of MNPLs in drinking water, calling for caution when considering data. In addition, poor standardization, and quality control measures, as well as the neglected detection of small-sized plastics, hamper the interpretation and comparison between studies (WHO, 2019). Altogether, along with the lack of consensus in the units to report quantitative data (Bouwmeester et al., 2015), make the extension of environmental data to toxicological studies a great challenge. For this reason, research results regarding the toxicological impact of MNPLs are limited, speculative, and poorly transferable, and reproducible.

1.3.2. Impact of microplastics and nanoplastics on health

The evaluation of the potentially harmful health effects associated with MNPLs is challenged by the previously discussed factors. Thus, it is not surprising that the review of data from the literature brings to light these limitations conditioning the study of MNPLs and their effects. Therefore, the insufficient and biased knowledge regarding MNPLs will be noticed in the following paragraphs.

MNPLs act differently from their larger counterparts due to their increased surface area. The fact of exhibiting more reactive surfaces may lead to cause cytotoxicity, oxidative stress, disruption of immune function, accumulation, and translocation to other tissues due to their persistent nature that limits the removal from the organism (Prata et al., 2019; Rahman et al., 2020). Hence, through these mechanisms, the potential risks that MNPLs pose to human health are linked to gastrointestinal, liver, and reproductive toxicity, as well as neurotoxicity and metabolic alterations (Chang et al., 2020).

Different *in vivo* studies have shown the impact of MNPLs on metabolic enzymes or energy homeostasis. On the one hand, the direct alteration of metabolic enzymes was described as associated with the MNPLs exposure in fish and mice. These exposures originated the alteration of the anaerobic and lipid metabolism, and adenosine triphosphate (ATP) production (Deng et al., 2017; Brandts et al., 2018; Wen et al., 2018). Further studies have linked MNPLs exposure to the reduction of the energy supply, specifically by decreasing the food intake in clams, crabs, and marine worms (Wright et al., 2013; Watts et al., 2015; Xu et al., 2017), or the predatory performance in fish (Wen et al., 2018). Contrarily, research has also shown the increase of energy demand in response to MNPLs exposure, triggered by inflammatory reactions, the increase of

excretion mechanisms, or the decrease of nutrient absorption due to the digestive capacity depletion (Wright et al., 2013; Watts et al., 2015; Deng et al., 2017; Xu et al., 2017). A significant reduction in liver size and weight was described in mice after exposure to PSMPLs (Deng et al., 2017). Similarly, the depletion of half of the energy reserves was observed following PVC exposure in marine worms (Wright et al., 2013).

The induction of reproductive toxicity has also been related to MNPLs exposures, particularly PS particles, causing the reduction of the reproductive functions in *Daphnia magna* (Besseling et al., 2014), the decrease of the oocytes number and sperm velocity in oysters (Sussarellu et al., 2016), and the accumulation of particles in gonads of *Caenorhabditis elegans* (Qu et al., 2018). These effects are extremely serious from the ecotoxicological point of view since they could draw the decline of a complete population in a given ecosystem (Worm et al., 2017). Particulate matter, which includes plastic particles, has been associated with *in vivo* neurotoxicity due to direct contact of the particles with neurons, or as a result of oxidative stress-mediated responses and the consequent activation of the microglia, and pro-inflammatory cytokines (MohanKumar et al., 2008). The impact of MNPLs in neurotoxicity was confirmed by Barboza and co-workers' findings. They described, in addition to metabolic alterations, a decrease of the brain acetylcholinesterase (AChE) enzyme and the increase of lipid peroxidation in bass fish (Barboza et al., 2018a). Additionally, changes in behavior indicators of neuronal disruption, such as the impact on the swimming conduct, were also observed (Barboza et al., 2018b). Other studies performed in mice demonstrated increased activity of the AChE enzyme and the subsequent affection on neurotransmitters after PSMPLs exposure (Deng et al., 2017).

The great accumulation of MNPLs in the food chain has opened a new field of research focused on the potential gastrointestinal toxicity that they can trigger. Studies analyzing the naturally occurring accumulation of anthropogenic MNPLs in the digestive system have been conducted on different aquatic species (Huang et al., 2019; Park et al., 2019; Zhu et al., 2019b; Wang et al., 2021) and seabirds (Nicastro et al., 2018; Franco et al., 2019). In addition, studies in zebrafish, *Caenorhabditis elegans,* and mice, as *in vivo* models exposed mainly to PS particles (some of them including PE, PP, or PVC), have informed about the MNPLs major impacts in the gastrointestinal tract (GIT), which are an intestinal accumulation of particles, intestinal damage (i.e., morphological, permeability and mucus secretion alterations), oxidative stress, and inflammation progression (Jabeen et al., 2018; Lei et al., 2018; Jin et al., 2019; Li et al., 2019; Qiao et al., 2019a; Sarasamma et al., 2020). The imminent consequence of MNPLs absorption in the GIT and their subsequent distribution in the blood is the accumulation in the liver

(Chang et al., 2020). Recent findings of plastic particle accumulation in the liver of fish, crustaceans, and mice have been published (Collard et al., 2018; Lu et al., 2018; Yu et al., 2018). The effects described regarding MNPLs' liver deposits are related to the disturbance of lipid metabolism originating lipid profile changes, oxidative stress, and inflammation. Moreover, oral exposure to MNPLs in mice led to a decrease in the expression of lipid and triglyceride synthesis-related genes, generating a loss of triglycerides and cholesterol that could induce hepatic lipid disorders (Lu et al., 2018).

On the other hand, some authors have suggested that, in the last term, MNPLs exposure could motive the development and progression of malignancies. According to this hypothesis, the chronic inflammation and oxidative stress promoted by MNPLs would exacerbate the release of pro-inflammatory inducers of angiogenesis, and genotoxic damage would arise (Rahman et al., 2020).

Until now, only speculations based on other organisms' data can be done to depict the potential effects that MNPLs could cause on humans. However, a comprehensive understanding of the toxicity of MNPLs-mediated mechanisms is required to predict the contribution of these particles to an incremented risk of gastrointestinal, liver, metabolic, and neurodegenerative diseases development, as well as reproductive alteration patterns, or other affections in humans (Figure 6). Therefore, the studies included in this Thesis are mainly focused on those mechanisms that could underlie the potential outcomes in humans.

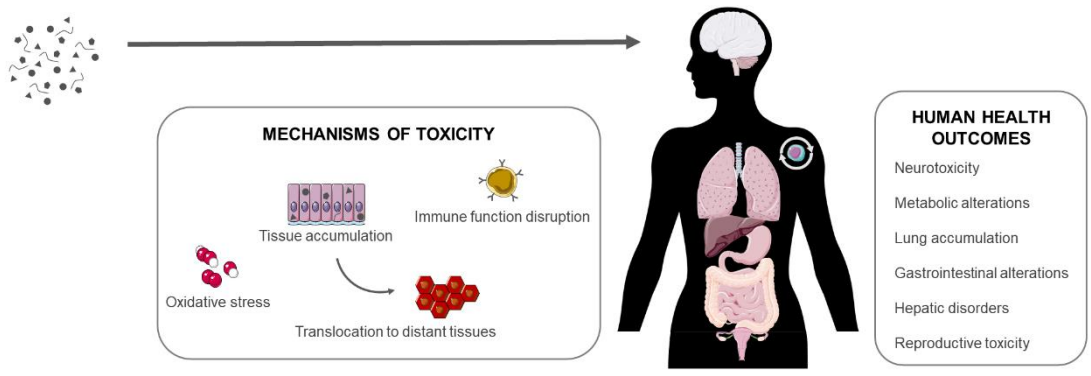

Figure 6. MNPLs mechanisms of action and potential organic outcomes in humans.

1.3.2.1. Cytotoxicity and oxidative stress

One of the well-associated mechanisms of toxicity to MNPLs is oxidative stress. Plastic particles' capacity to cause this response has been linked to their highly reactive surface and the release of oxidizing species adsorbed on it (Kelly & Fussell, 2012; Valavanidis

et al., 2013). Particulate matter has been shown to induce reactive oxygen species (ROS) formation *per se* in a cell-free environment (Wilson et al., 2002). In addition to that, free ROS could be included in MNPLs surface as byproducts of their manufacturing. This inherent ROS holding could be exacerbated by weathering processes. The interaction of plastic particles with different environmental factors provides porous surfaces which add fertile ground for catalyzing the increased generation of ROS (Valavanidis et al., 2013; Gewert et al., 2015). Besides this, the interaction with other environmental pollutants (further described in section 1.3.2.4.), which promotes Fenton-type reactions or has redox properties, can lead to an increase of the free radicals' generation (Gewert et al., 2015). Moreover, MNPLs could induce inflammatory responses that prompt the release of ROS from the host system (Kelly & Fussell, 2012; Valavanidis et al., 2013).

Oxidative stress after the exposure to MNPLs has been described in ecotoxicity models of zooplankton such as *Daphnia pulex* (Liu et al., 2020a), crustaceans (Jeong et al., 2017), mussels (Paul-Pont et al., 2016), and different fish species including European seabass (Barboza et al., 2018a), medaka fish (Choi et al., 2019; Wang et al., 2019), goldfish (Yang et al., 2020) and carp (Xia et al., 2020), among others. In addition, MNPLs-mediated oxidative stress has also been described in typically used *in vivo* models such as zebrafish (Lu et al., 2016; Qiao et al., 2019a; Wan et al., 2019), *Caenorhabditis elegans* (Qiu et al., 2020), and mouse models (Deng et al., 2017; Yang et al., 2019). Cytotoxicity can be the outcome of oxidative stress and inflammation in cells (Prata et al., 2019) and it is usually assessed as the cells' viability faced with the threat. Murine fibroblasts (NIH/3 T3) and murine embryonic stem cells (mES-D3) were used to evaluate the cytotoxicity of PSNPLs and PSMPLs. While PSNPLs did not exert any effect on cell viability, PSMPLs showed a cytotoxic effect in both murine models. However, within the same study, GIT and placenta barrier models did not show cytotoxic effects after 24 h of exposure to PS MNPLs (Hesler et al., 2019). Similarly, Hwang et al. (2019) described *in vitro* cytotoxicity in murine macrophages (Raw 264.7) exposed to PPMPLs, but that decrease in cell viability was not found on human dermal fibroblast under the same experimental conditions. Among brain cells (mixed neuronal cells and primary astrocytes) and mouse embryonic fibroblasts (MEFs) isolated from mice and exposed to PSNPLs for 48 h, only the mixed neuronal cells model showed cytotoxicity (Jung et al., 2020). PS particles have also been reported to cause cytotoxicity in human-derived cell lines, namely lung epithelial cells (BEAS-2B) (Dong et al., 2020), fibroblasts (Hs27) (Poma et al., 2019), hepatocellular carcinoma cells (HepG2) (He et al., 2020b), or different hematopoietic cell lines (Raji-B, THP-1, and TK6) (Rubio et al., 2020a) after

24 h of exposure. Acute and slight cytotoxicity was found on colorectal adenocarcinoma (Caco-2) cells exposed to 1 μm and 4 μm PSMPLs, respectively, but no toxic effects were induced by larger PSMPLs (Stock et al., 2019). Furthermore, other authors showed no changes in Caco-2 cells' viability after exposure to PSNPLs (Wu et al., 2019a) or PET particles (Magrì et al., 2018).

Besides cytotoxicity, human *in vitro* models are also usually chosen to assess oxidative stress induced by MNPLs. For instance, different peripheral blood mononuclear cells (Hwang et al., 2019; Rubio et al., 2020a), glioblastoma cells (T98G), cervical adenocarcinoma cells (HeLa) (Schirinzi et al., 2017), epithelial colorectal adenocarcinoma cells (Caco-2) (Magrì et al., 2018; Wu et al., 2019a), lung epithelial cells (BEAS-2B) (Dong et al., 2020), hepatocellular carcinoma cells (HepG2) (He et al., 2020b), and fibroblast (Hs27 and human dermal fibroblast) (Hwang et al., 2019; Poma et al., 2019) have been selected to this aim. As ROS inductors, the effort has been focused on linking MNPLs-mediated oxidative stress with antioxidant-related activities. The enzymatic activity of catalase (CAT), glutathione (GSH), or superoxide dismutase (SOD) are the most studied, as well as the expression of their encoding genes (Qiao et al., 2019a; Wan et al., 2019; He et al., 2020b; Xia et al., 2020; Yang et al., 2020). As for cytotoxicity assessment, data are variable between models and studies, and consistency of the MNPLs effects on oxidative stress is not depicted in the results available in the literature. Moreover, although multiple mechanisms of oxidative stress have been suggested, there is still a poor understanding of this issue. Further investigation is needed to unravel the most typically produced ROS forms among superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot -}$), peroxynitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2), and the potential involvement of MNPLs in ROS formation due to the alteration of the mitochondrial integrity. Additionally, it is well-known that oxidative species have the potential to damage cellular deoxyribonucleic acid (DNA) through the formation of bulky adducts or strand breaks (Valavanidis et al., 2013). Nonetheless, MNPLs association with these effects remains to be unveiled. Therefore, all these issues are addressed in the work presented in this Thesis.

1.3.2.2. Disruption of immune function

Due to their pervasiveness, MNPLs are easily ingested and inhaled by different organisms. Plastic particles inhaled into the respiratory tract (RT) and ingested through the GIT remain attached and in direct contact with lung or intestine epithelial cells leading to inflammation and the subsequent alteration of the local immune system (Chen et al., 2019; Hirt & Body-Malapel, 2020). The activation of the immune system by inflammatory

responses has been presented as one of the potential effects associated with MNPLs exposure (Prietl et al., 2014; Lehner et al., 2019). Aside from that, and as a consequence of ROS production and oxidative stress progression, systemic immune responses are stimulated. In this context, ROS production would induce the release of immune modulators and the activation of immune cells, which could exacerbate the exposure to self-antigens and the production of autoantibodies favoring an autoimmune disease (Farhat et al., 2011). Nonetheless, the opposite could also be given since MNPLs-induced immunosuppression has been reported in mussels. This was described to be favored by increased production of anti-inflammatory cytokines, suppression of T-helpers, and decreased production of T-effectors (Canesi et al., 2015; Détrée & Gallardo-Escárate, 2018).

As it happens with the other toxicity parameters, MNPLs effects on the immune system are poorly understood. Few publications refer to *in vivo* evaluation of the immune function disturbance in the literature, and they essentially focus on ecotoxicological models. Limonta et al. (2019) described the immune response enhancement induced by PE and PS in zebrafish. Similarly, an *ex vivo* study conducted with neutrophils of fathead minnow fish showed the impact of PSNPLs and polycarbonate NPLs on the immune system by stimulating degranulation of neutrophils' primary granules (Greven et al., 2016). Banaee and colleagues reported, on the contrary, the dysfunction of the immune system marked by the total immunoglobulin (Ig) and serum complement level decrease after 30-days of exposure to PE in carp fish (Banaee et al., 2019). In addition, Li et al. (2019) found colon and duodenum inflammation in mice exposed to PE particles along with the increased secretion of interleukins (IL) (IL-1α, IL-6) involved in inflammation progression and immune function. The literature regarding *in vitro* immune effects of MNPLs exposures is not much more abundant but results mostly agree with an immune system alteration in response to MNPLs. PSNPLs induced a significant up-regulation of pro-inflammatory cytokines [IL-8, nuclear transcription factor-kβ (NF-kβ), and tumor necrosis factor-α (TNFα)] after an 8 h exposure in the human alveolar epithelial cell line A549 (Xu et al., 2019). Similarly, Busch et al. (2021) investigated the effects of PS and PVC particles on the release of pro-inflammatory cytokines in models of the healthy and inflamed intestine. Results did not show acute effects in the healthy gut model, albeit the release of the IL-1β increased after 24 h of exposure in the inflamed model. PPMPLs were reported to induce histamine release in human mast cells (HMC-1), and pro-inflammatory cytokines (IL-6 and TNFα) in human peripheral blood mononuclear cells, in size and concentration-dependent manner (Hwang et al., 2019). Similarly, human whole blood samples *ex vivo*

studied revealed the overexpression of 14 cytokines related to the immune response after the acute exposure of the donors' blood to PSNPLs (Ballesteros et al., 2020).

1.3.2.3. Accumulation and translocation to the circulatory system and distant tissues

Assuming ingestion as the major route of exposure to MNPLs, many studies have described the accumulation of plastic particles in the GIT of various species such as fish (Lei et al., 2018; Huang et al., 2019; Assas et al., 2020), mussels (Fernández & Albentosa, 2019; Wang et al., 2021), or mice (Jin et al., 2019). Despite such studies are limited, they show evidence of the distribution of MNPLs after ingestion. Broadly, while larger particles, namely >150 μm, remain trapped in the mucus layer overlying the intestine, smaller particles manage to cross the mucus shed and are internalized by different mechanisms: (i) endocytosis through enterocytes, (ii) transcytosis through microfold cells (M-cells), (iii) persorption (the passage through gaps at the villous tip), and (iv) paracellular uptake (Powell et al., 2010). Investigations in rodent models have found NPLs uptake by endocytosis, transcytosis, and paracellular diffusion. The translocation of nanoparticles is especially favored by the stuck larger particles since they trigger local inflammation which increases the epithelium permeability (Prata et al., 2019). This fact has been illustrated in zebrafish, where the gut accumulation of PS particles was found to cause mucosal damage, metabolism disruption, inflammation, and therefore, increased permeability of the fish's intestine (Qiao et al., 2019b). Once crossed the gut barrier MNPLs can reach the systemic circulation and disseminate to other organs like the liver, spleen, heart, lungs, thymus, reproductive organs, kidney, and brain (Hirt & Body-Malapel, 2020). A representative example of these effects is the work reported by Deng et al. (2017), where they studied the dissemination and accumulation of PS particles orally administrated in mice. After 4 weeks of exposure, plastic particles were found to accumulate in the liver, kidney, and gut. Similarly, accumulation in the gills, liver, and gut was documented in crabs (Yu et al., 2018) and zebrafish (Lu et al., 2016) after a 7-days exposure to PSMPLs.

In addition to ingested particles, airborne MNPLs can be both accumulated or absorbed and passed through the lung epithelium, depending on the particles' size. MNPLs that remain bound to the RT can move to the GIT by mucociliary clearance, overlapping with those ingested. Here, they undergo the same fate as the previously described swallowed particles (Hirt & Body-Malapel, 2020). As well as the local effects, MNPLs have been reported to cause disturbances in the circulatory system like systemic inflammatory response mediated by blood cell MNPLs internalization and cytotoxicity (Canesi et al., 2015), and vascular inflammation and occlusion (Campanale et al., 2020). *Ex vivo*

studies have demonstrated the accumulation of MNPLs in human blood cells, namely lymphocytes, monocytes, and polymorphonuclear cells, and the impact on their secretomes by altering the expression of cytokines related to immune, inflammatory, stress, and cell proliferation responses after 24 h of exposure to PSNPLs (Ballesteros et al., 2020).

In vitro studies have also been conducted to detect MNPLs uptake in human cell lines, for instance, in leucocytes (Raji-B, TK6, and THP-1) (Rubio et al., 2020a) in which the accumulation of PSNPLs was observed in a concentration-dependent manner, hepatocarcinoma HepG2 cells (He et al., 2020b), colorectal adenocarcinoma Caco-2 cells (Wu et al., 2019a) and bronchial epithelial BEAS-2B cells (Lim et al., 2019), or alveolar epithelial A549 cells in which they found accumulation of PS particles in a time and size-dependent manner (Xu et al., 2019). Attempts to show the internalization and translocation of MNPLs in two dimensions (2D) *in vitro* systems are very scarce but existent, including the work performed within this Thesis. The uptake of PSNPLs by the cells forming 2D *in vitro* models of the intestinal barrier was shown by Hesler et al. (2019) and Stock et al. (2019). Interestingly, a placenta barrier model was additionally used by Hesler and co-workers, where they observed the internalization of PS particles. However, none of these studies demonstrated the translocation of PSNPLs across the corresponding barrier models. Besides, PENPLs were observed to be incorporated by suitably differentiated neurospheres after 24 h of exposure (Hoelting et al., 2013). Overall, *in vivo* and *in vitro* data confirm cellular internalization of MNPLs.

1.3.2.4. Microplastics and nanoplastics as carriers: the Trojan horse effect

Despite the scarcity of available data on MNPLs' effects, plastic particles are raising an increasing concern as potential toxicants owing to their similar behavior to the so-called *Trojan horse* effect, which indicates their behavior as vectors of other agents. Thus, apart from their physical effects described above (fundamentally, bioaccumulation, inflammation, cytotoxicity, oxidative stress, and alteration of the immune system), the spotlight has been placed on the chemical effects that MNPLs can establish due to the intrinsic composition of polymers, the leaching of their additives, and the potential adsorption of toxic contaminants on their surface over time (Bouwmeester et al., 2015; Wright & Kelly, 2017).

Chemical additives are added to plastics routinely during their manufacturing. These extra actors play a role in the strength, durability, rigidity/flexibility, inhibition of photodegradation and microbial growth, or color of the final product. The lack of chemical

bond between these endogenous additives and plastics themselves and their low molecular weight make such additives susceptible to leaching to the surrounding medium. Moreover, fragmentation of MNPLs facilitates the migration and exposure of core additives to the surface (Wright & Kelly, 2017; Verla et al., 2019b). The incorporated agents vary among plastic types, but the vast majority are considered hazardous chemicals (Hahladakis et al., 2018). Representative examples of the threat that these endogenous additives can pose to humans include the male and female reproductive toxicity along with adverse neurodevelopmental and respiratory outcomes in new-born by organophosphate esters (OPEs) or polybrominated diphenyl ethers (PBDEs) used as flame retardants and plasticizers (Zlatnik, 2016; Doherty et al., 2019), and the endocrine disruption by bisphenol A (BPA) or phthalates used as antioxidants and plasticizers, respectively (Zlatnik, 2016; Benjamin et al., 2017).

1.3.2.4.1. Microplastics and nanoplastics as carriers of other environmental pollutants

Along with the inherent toxic factors of MNPLs, their large surface area, their electrostatic charge caused by high-speed manufacturing equipment during plastic production, and their low-polar surface, enables the adsorption of persistent organic pollutants [POPs, also called hydrophobic organic contaminants (HOCs)] such as polyaromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs) or polychlorinated biphenyls (PCBs), and inorganic contaminants such as halogens or heavy metals (Verla et al., 2019b).

Electrostatic charge, which has been reported to increase the pickup of airborne pollutants (Baribo et al., 1966), along with the hydrophobic tendency of MNPLs, facilitates their interaction with other immiscible-in-water pollutants, such as POPs, that are spread over the environment sharing niches with plastics. Further adsorption efficiency can be influenced by the type of plastic (different surface structures or additives) and the weathering processes (Holmes et al., 2014; Hahladakis et al., 2018; Verla et al., 2019b). The alteration of the plastic surface by atmospheric agents and the increased roughness caused by the degradation processes turns low polar MNPLs into more negative surfaces with anionic sites, which increase the interaction of plastic particles with positively charged heavy metals (Figure 7) (Campanale et al., 2020). Besides, different characteristics of the coalescence medium have been reported to alter MNPLs' sorption capacity. Therefore, variations in salinity, temperature, and pH could enhance polymer polarity which benefits better sorption of inorganic molecules onto MNPLs (Wang et al., 2018; Godoy et al., 2019).

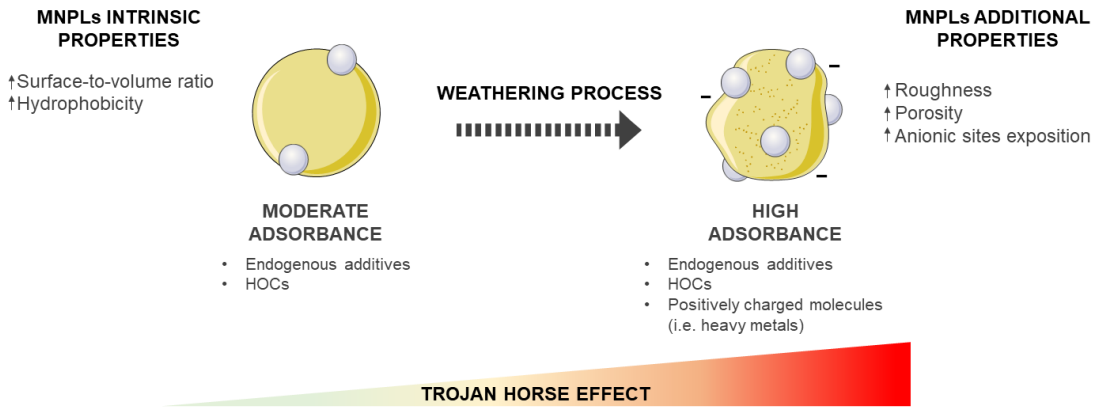

Figure 7. MNPLs' Trojan horse effect. MNPLs inherent features make them ideal carriers of other agents. Through the abrasion of their surface, MNPLs exhibit additional properties which enhance their Trojan horse performance.

Bearing these phenomena in mind, exposure to MNPLs occurs rarely alone but potentially associated with other agents. Though plastic polymers were considered to be inert in the past, greater attention is currently being paid to better understand the interactions of plastic particles with POPs and inorganic pollutants since MNPLs could increase the bioavailability of contaminants in organisms, conferring higher exposure to humans (Barbosa et al., 2020). Hence, exposures to MNPLs/other environmental pollutants could trigger additive, synergistic, or antagonist effects (Sendra et al., 2021). In this context, analyzing the toxic effects caused by MNPLs alone in biological systems might not be biologically relevant enough. Antagonistic interactions between MNPLs and glyphosate, a common herbicide, have been reported by Zhang et al. (2018), which co-exposure led to a lower inhibition of algal growth compared to the inhibition induced by glyphosate alone. Similarly, the mortality induced by bifenthrin in marine microalgae was significantly reduced upon the addition of PEMPLs due to the adsorption of bifenthrin into the plastic particles (Davarpanah & Guilhermino, 2015; Ziajahromi et al., 2019). On the contrary, the toxic effects of triclosan and PAHs have been reported to be enhanced in presence of MNPLs in planktonic crustaceans (Ma et al., 2016; Syberg et al., 2017). An increase of oxidative stress and levels of oxidative biomarkers, higher than the given by the equivalent single exposures, have also been evidenced in mussels and clams exposed to a combination of MNPLs with PAHs (Paul-Pont et al., 2016; Pittura et al., 2018; Tang et al., 2020). Moreover, the co-exposure of zebrafish to MNPLs in combination with a mixture of contaminants revealed potentiated toxic effects of the

contaminants, neurotoxicity, and oxidative stress whereas non-toxic effects were found with single exposures (Rainieri et al., 2018).

On the other hand, MNPLs' ability to adsorb heavy metals has been demonstrated in aquatic and terrestrial environments (Davranche et al., 2019; Li et al., 2020a; Tang et al., 2021), even though there is limited literature evaluating its effects. A potential interplay of cadmium (Cd), lead (Pb), and zinc (Zn) with PSNPLs showed increased toxicity on medaka fish's gut microbiota in comparison with the metals' effects alone (Yan et al., 2020). Also, PS MNPLs enhanced copper (Cu) toxicity in freshwater microalgae (Wan et al., 2021), like PSNPLs raised genotoxic damage caused by metal oxide nanoparticles (CuONPs and ZnONPs) in zebrafish (Singh et al., 2021). Opposite, antagonistic effects of MNPLs with copper were evidenced for oxidative stress-related genes' expression in zebrafish (Santos et al., 2020), as well as to Cd in wheat plants, where the co-exposure to Cd/PSNPLs reduced the Cd-induced toxicity (Lian et al., 2020). ROS production has been proposed as the main mechanism of toxicity triggered by heavy metal/MNPLs interactions. Added to MNPLs inherent capacity to produce oxidative species, metals are oxidized by H_2O_2 generating $OH^{\cdot -}$ via Fenton reaction (Valavanidis et al., 2013). Nonetheless, the lack of studies addressing co-exposures of POPs or heavy metals/MNPLs in *in vivo* or *in vitro* mammal models conduces to total ignorance of the potential effects that they could arise. For this reason, great efforts have been directed towards this topic during the development of Chapter 4 of this Thesis.

1.4. *IN VITRO* HUMAN MODELS OF THE GASTROINTESTINAL SYSTEM

To date, studies regarding MNPLs have emphasized the occurrence over marine, terrestrial, and atmospheric niches, including their abundance or effects on inhabitants of those ecosystems, for example, birds, fishes, and shellfishes, livestock, or microorganisms (Chae & An, 2018; Gunaalan et al., 2020; Rai et al., 2021). The current status of MNPLs toxicity publications are mainly articles referring to ecotoxicology models (plankton, mussel, or crustacean species) and only a few publications include animal models applicable to human health, such as zebrafish, *Caenorhabditis elegans,* and mice (Jeong & Choi, 2019). Unfortunately, when searching for biomonitoring studies in humans, definitely no results come out. Going a step back to the models' level, few *in vivo* studies are found on vertebrates, particularly fish species, and rodents, albeit works investigating the toxicity of MNPLs in fishes are three times more abundant than in *in vivo* mammal models (Yong et al., 2020). On the contrary, *in vitro* research is mainly focused on human cell models (Rubio et al., 2020b; Yong et al., 2020), but the little

abundance of publications and the conflicting results do not allow to draw solid conclusions.

Nevertheless, an increasing body of evidence suggests that humans constantly inhale and ingest MNPLs and, despite the existence of sufficient studies in the accumulation and translocation of MNPLs across the GIT of aquatic organisms, the risk to human health is far from being understood (Chang et al., 2020). Hence, this major research gap needs to be addressed to move forward on the understanding of the hazard that MNPLs pose to humans, which is not possible at the current level of information.

Considering ingestion as the main entrance pathway to the body and its overlap with the inhalation route described previously, a better understanding of the ability of MNPLs to cross human epithelial barriers of the GIT is required. Since ongoing technological advancements for MNPLs analysis in human complex biological matrices, namely body fluids and tissues, are still in their infancy (Dick Vethaak & Legler, 2021), research on *in vitro* human models is mandatory for this assessment. Therefore, the work carried out during this Thesis aimed to help shed light on the effects that NPLs can trigger once swallowed using different *in vitro* human models of the small intestine that will be further developed in the sections below.

1.4.1. Caco-2 cells as a human intestinal epithelium *in vitro* model

Many human colonic-derived cell lines (e.g., HCT116, SW1116, HRA-19, Caco-2, HT29, etc.) have been described and studied for decades (Simon-Assmann et al., 2007). Considering their origin, they have been extensively used under undifferentiated conditions to evaluate the harmful effects of different xenobiotics for those ingestion constitutes the major route of exposure (Demers et al., 2009; Vila et al., 2018a; Zapletal et al., 2019). Typically, under standard cell culture conditions, most cell lines neither differentiate nor simulates the characteristics of the human intestinal epithelial cells (Chantret et al., 1988; Simon-Assmann et al., 2007). However, among all the models Caco-2 cells gained great attention due to their capacity to undergo spontaneous cell differentiation. Similar to what occurs in the *in vivo* context, where the renewal of the human intestinal epithelium occurs through the proliferation in crypts and differentiation of the cells during the migration to the villus tip, Caco-2 cells' growth is characterized by proliferation, confluency, and differentiation (Delie & Rubas, 1997). This cell line has been extensively used as a model of the human intestinal epithelium and remains one of the best choices for the study of intestinal physiology and *in vitro* toxicological assessments.

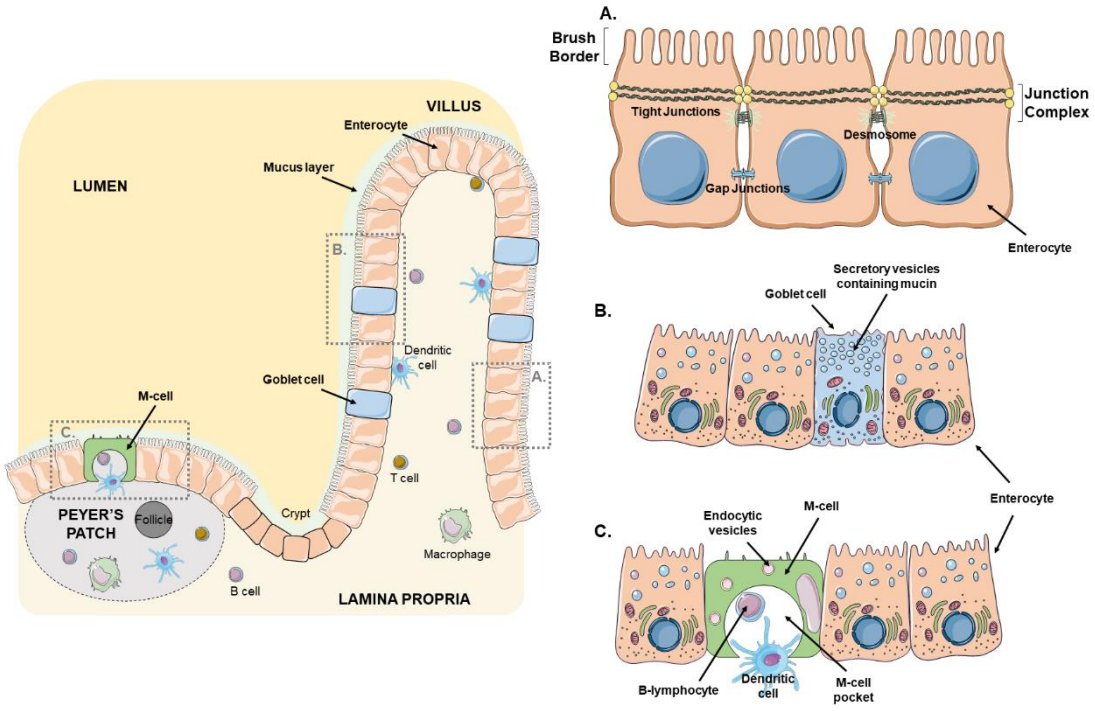

Figure 8. On the left: anatomy of the small intestine. A monolayer of enterocytes and goblet cells lines the intestinal epithelium providing a physical separation between the lumen and the underlying *lamina propria*. The specialized follicle-associated epithelium overlying the Peyer's patches contains the M-cells responsible for mucosal immunity. A mucus shed covers all the intestinal epithelium extension. On the right: detail of the different areas indicated as A, B, and C, respectively in the intestinal organization scheme.

The Caco-2 cell line was first obtained from a colon adenocarcinoma of a 72-years adult Caucasian male in 1974 (Fogh et al., 1977). It has been well documented that the culture of these cells under common cell-culture conditions, without the addition of supplementary differentiating factors, leads to the formation of a cell monolayer that meticulously mimics the human small intestine enterocytes (Figure 8A) (de Angelis & Turco, 2011). Briefly, these differentiated cells form a one-cell-thick layer of columnar absorptive cells with the typical enterocytic polarization, that is, with apical microvilli and a basolateral surface that simulates the cellular bottom in contact with subepithelial tissue in the human intestine. Moreover, despite its colonic origin, enterocyte-like Caco-2 cells' brush border (the apical side with a high density of microvilli) is endowed with transporters, efflux proteins, and metabolic enzymes typically found in the small intestine enterocytes. Among them, specific transporters for sugar, amino acids, peptides, vitamins, micronutrients such as heavy metals and nucleosides, or bile acids have been described and functionally characterized in those cells (Hidalgo & Li, 1996; Delie & Rubas, 1997; Sun et al., 2008). Once formed the monolayer, another similarity to the human epithelium is the presence of junctional complexes, including tight junctions and

desmosomes. In the human intestinal enterocytes, tight junctions play a relevant role in regulating the transport of solutes and immune cells between the lumen and the *lamina propria* domains, enabling paracellular transport across the cellular layer. These junctional complexes mostly consist of zonula occludens, occludin, and claudin family proteins, some of which have been reported to be expressed in Caco-2 cells (Delie & Rubas, 1997).

The protocol for the Caco-2 culture and differentiation has been fully documented and applied by laboratories worldwide. Despite slight variations, all the researchers' community follows the same procedures (de Angelis & Turco, 2011). Shortly, Caco-2 cells are seeded on permeable PET transwells at high density to synchronize the cell differentiation and are left differentiating for 14-21 days. PET transwells allow the cells to grow and form the monolayer acting as a support and, in its turn, separating the apical and the basolateral compartments. The porous surface of the transwell allows the exchange of components present in the cell culture medium between the apical and basolateral chambers (Figure 9A).

Owing to the previously described characteristics and the disposition of the model forming a 2D system that resembles both the intestinal epithelium, and the lumen and *lamina propria* domains, the model has been postulated as the gold standard in pharmacology, toxicity, or nutrition studies (Meunier et al., 1995). However, some limitations of the model have been described. While some studies correlate Caco-2 monolayer permeability values with the human *in vivo* absorption capacity, others conclude that the model cannot be used as a surrogate marker of the *in vivo* absorption (Ren & Lien, 2000), particularly when evaluating paracellular transport (Artursson et al., 2001). The tightness of the resulting Caco-2 monolayers mimics more colonic than small intestinal tissue, this is, the pore radius of Caco-2 cells' tight junctions is tighter than in small intestine enterocytes, which results in a poor permeability for hydrophilic compounds that traverses the epithelium via the passive paracellular route (Artursson et al., 2001; Sun et al., 2008). Moreover, although most of the cells lining the small intestine epithelium are absorptive enterocytes that maintain metabolic and digestive functions, additional specialized cells carry out a diversity of functions in the intestinal epithelium. Goblet cells, which produce the mucus layer, or epithelial M-cells which account for phagocytosis and transcytosis functions, add complexity to the human gut barrier (Figure 8). Hence, these limitations should be highlighted from the use of the Caco-2 monoculture since it does not thoroughly simulate the real small intestine composition and environment.

1.4.2. Caco-2/HT29 as a human intestinal barrier *in vitro* model

The human epithelial layer is not in direct contact with the luminal content. A mucus layer between the intestinal epithelium and the luminal space acts as a physical and biochemical boundary against food particles, chemicals, enzymes, and host products such as bile acids, microbiota, and microbial products (Figure 8) (Johansson et al., 2008). In addition, the presence of the mucus layer plays a role in the estimation of intestinal permeability because it leads to a decreased absorption of certain lipophilic compounds (Behrens et al., 2001). The lack of this defense is a major limitation of the already described Caco-2 monoculture model. Goblet cells are the ones specifically responsible for the mucus secretion in the human intestine (Figure 8B), so that, a cell model mimicking this function is required to better simulate the small intestine microenvironment.

The HT29 cell line was isolated from a 44-years Caucasian female's colon adenocarcinoma in 1964 (Fogh et al., 1977). This cell line has been largely explored due to its potential to imitate both enterocytic and mucus-secreting cells. On the one hand, under cellular stress conditions such as glucose deprivation or the presence of antimetabolites, HT29 cells undergo distinct differentiation processes. Similar to what is accomplished by Caco-2 cells, differentiation to enterocyte-like cells is observed when HT29 cells are grown without glucose supply, showing polarization and expressing brush-border enzymes (Chantret et al., 1988; Simon-Assmann et al., 2007). Although the similarities, the difference between the resulting absorptive Caco-2 and HT29 cells, lies in the longer time course process of differentiation and the lower levels of enzymatic activity of the brush-border enzymes expressed in HT29 cells (Simon-Assmann et al., 2007). Moreover, HT29 subpopulations, namely HT29-MTX and HT29-FU, have been obtained by adaptation to the anticancer drug methotrexate (MTX) and 5-fluorouracil (FU), respectively. The resulting established clones have been presented as highly specialized mucus-secreting goblet cells (Lesuffleur et al., 1993). However, under standard culture conditions (glucose supply and presence of serum), HT29 cells mostly remain undifferentiated, but part of the population develops differentiated characteristics such as the expression of different enzymes, among which are the membrane-bound glycoprotein mucin 1 (MUC1) and other mucins such as the gel-forming mucin 5 (MUC5) (Lesuffleur et al., 1990, 1993). Importantly, previous work carried out in our group confirmed the suitability of HT29 cells as a model of mucus-secreting goblet cells, showing the formation of the extracellular mucus shed that simulates the *in vivo* environment (García-Rodríguez et al., 2018a). Hence, the human adenocarcinoma HT29 cell line has been postulated as the excellent partner for a co-cultivation model

along with Caco-2 cells, which will provide a model constituted by the most represented cell types in a human epithelium: enterocytes and goblet cells (Figure 9B). In this context, our previous results established the proper ratios combination of Caco-2 and HT29 cells (90:10) to better mimic the human intestinal epithelium, maintaining the integrity and compaction values comparable to the *in vivo* barrier (García-Rodríguez et al., 2018a).

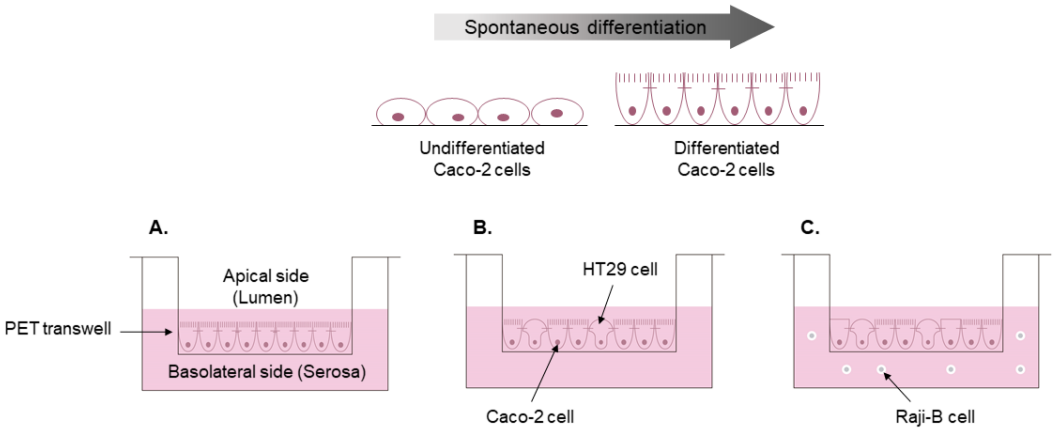

Figure 9. Caco-2 cells' differentiation process is illustrated on the top. Schematic representation of the different Caco-2 based *in vitro* models (A: Caco-2 monolayer; B: Caco-2/HT29 co-culture; C: Caco-2/HT29/Raji-B model) is shown on the bottom.

1.4.3. Caco-2/HT29/Raji-B as a human intestinal barrier *in vitro* model for translocation studies

Along with enterocytes and goblet cells, other structures and cell types are part of the small intestinal barrier. The origin of mucosal immunity and the pathogenesis of infections in the small intestine lies mainly in the Peyer's patches (PPs). These are separated into three domains: the follicular and parafollicular areas which contain lymphocytes, dendritic cells, and macrophages, and the follicle-associated epithelium (FAE), conformed by a monolayer of mainly, enterocytes, specialized epithelial M-cells, and a minor representation of goblet cells (Figure 8) (Gebert et al., 1996; Miller et al., 2007; Corr et al., 2008).

Owing to its highly efficient capacity of transcellular endocytosis (Kraehenbuhl & Neutra, 1992), M-cells have been cataloged as specialized transepithelial transporters, continuously sampling the lumen of the small intestine and transporting intact antigen, particles, and microorganisms across the epithelial barrier (Owen & Jones, 1974; Neutra et al., 1996a). M-cells present an underlying invagination or pocket, where lymphocytes, macrophages, and dendritic cells are located (Figure 8C). Therefore, M-cells play a

crucial role in the processing and initiation of immune responses (Kanaya & Ohno, 2014). This is why, although representing a minor population in the FAE, M-cells are considered the gatekeepers to the mucosal immune system (Neutra et al., 1996b).

While it is accepted that enterocytes and M-cells have a common precursor, it is not clearly understood whether the differentiation of phenotypes commits early in the crypt or it occurs by later-acting factors which trigger the differentiation of enterocytes into M-cells (Corr et al., 2008; Miller et al., 2007). Nevertheless, the confinement of M-cells to the FAE overlaying PPs suggests that immune cells, in particular B-lymphocytes, have a substantial role in M-cell maturation (Kanaya & Ohno, 2014). In fact, *in vitro* experiments carried out decades ago demonstrated for the first time the conversion of human intestine enterocytes into M-cells by murine PPs lymphocytes (Kernéis et al., 1997). This initial proposal of an *in vitro* model of human FAE was constructed with a co-culture of Caco-2 cells on inverted culture inserts and mouse lymphocytes isolated from PPs. Kernéis et al. observed the migration of lymphocytes between epithelial cells, forming the intraepithelial pockets in enterocytes. In addition, the decrease of villin and sucrase-isomaltase, which are key proteins for brush-border organization, led to a disordering of apical microvilli, which in turn prompted the downregulation of their enzymes. Moreover, this incubation did not modify the membrane's polarity or the Caco-2 cells' integrity. According to that described, it is not surprising that human B-lymphocytes have demonstrated the potential to induce Caco-2 differentiation to M-cells. Aiming to further refine Kernéis and colleagues' model, Gullberg et al. (2000) implemented a similar *in vitro* system composed of one-cell-thick Caco-2 layer on normally oriented transwells and the human lymphocytic Raji-B cells. The Raji line of lymphoblast-like cells was established in 1963 from a Burkitt's lymphoma of the left maxilla of an 11-year-old Black male. These cells feature B-cells markers, suggested to be involved in the enterocyte-to-M-cell differentiation induction *in vitro*. Thus, it was demonstrated that the co-culturing of Caco-2 with Raji-B cells made a subpopulation of Caco-2 cells develop towards an M-cell-like morphology, showing the aforementioned typical M-cell features. Furthermore, they showed that the transformation process *in vitro* does not require direct contact between Caco-2 and Raji-B cells, as soluble mediators are suggested to assist the process (Gullberg et al., 2000; des Rieux et al., 2007).

Since M-cells are the less represented cell type in the small intestine (5% in the human FAE and less than 1% of the total intestinal surface) (Giannasca et al., 1999), *in vivo* studies are difficult to perform. However, its transcytosis capacity, which is the main responsible for the gut permeability to microorganisms and micro- and nanoparticles, cannot be neglected (Martirosyan et al., 2012). For this reason, the implementation of an

in vitro cell culture model which includes the M-cell-like performer would be an improved strategy to study intestinal absorption and translocation. Nonetheless, M-cells markers are specie-specific or even location-specific (i.e., small, or large intestine), and no human-specific markers have yet been identified. Thus, the evaluation and discrimination of M-cells within *in vitro* cell monolayers must be performed using morphological criteria, so the establishment of an *in vitro* model which mimics the real context is a challenge (Gebert et al., 1996; des Rieux et al., 2007). Although it is not clear whether the model faithfully reproduces all the features of M-cell function *in vivo*, studies have confirmed that co-cultured Caco-2/Raji-B cells express genes specifically expressed in FAE *in vivo*. These findings were observed along with the loss of the apical microvilli organization and the columnar shape and, therefore, the digestive function as enterocytes (Lo et al., 2004). For this reason, the triple co-culture model Caco-2/HT29/Raji-B (Figure 9C) has been adopted as the model that more accurately mimics the small intestinal epithelial layer and has been postulated as a reliable tool to study, in a mechanistic manner and small-scale size range, absorption and permeability (Antunes et al., 2013). Additionally, the usefulness of the 2D triple co-culture to assess cell uptake and transport of nanomaterials across the barrier was demonstrated in our previously published study (García-Rodríguez et al., 2018a).

2. OBJECTIVES

2. OBJECTIVES

As described in the Introduction section, MNPLs are concerning emergent pollutants to which humans are exposed through different pathways. However, no consensus regarding the exposure levels and the risk that they pose to human health can be reached at present due to the dearth of data. Thus, the general objective of this Thesis is to expand the little knowledge related to human exposure to MNPLs and to evaluate the effects of PSNPLs, as MNPLs surrogate, in different *in vitro* models of the GIT to contribute to the field understanding and clarifying gaps found in the literature.

To achieve this goal, we have set up the following four specific objectives:

1. To determine, from available data in the literature, MNPLs occurrence and human exposure levels to these contaminants of emerging concern. *Objective developed in Chapter 1*.

2. To assess the ability of PSNPLs to be internalized by undifferentiated Caco-2 cells and to evaluate the levels of toxic and genotoxic effects potentially induced by those NPLs. *Objective developed in Chapter 2*.

3. To determine the ability of PSNPLs to internalize and translocate across different *in vitro* 2D models of the intestinal barrier (Caco-2/HT29 and Caco-2/HT29/Raji-B) while evaluating the barriers' stability and integrity, and the potential toxicity and genotoxicity caused by those NPLs, evidencing the potential differences between both models. *Objective developed in Chapter 3*.

4. To explore the potential ability of MNPLs to act as carriers of other environmental pollutants and to unravel the toxic and genotoxic impact of environmentally relevant mixtures of contaminants (silver materials and PSNPLs) in undifferentiated Caco-2 cells. *Objective developed in Chapter 4*.

3. RESULTS

3. RESULTS

The results of this Thesis have been divided into four chapters. These chapters comprise four articles that have already been published in different peer-review journals.

Thus, the four articles arranged accordingly to the objectives of the Thesis are as follows:

3.1. Chapter 1 (Article 1): Pathways of human exposure to microplastics and estimation of the total burden.

3.2. Chapter 2 (Article 2): Nanoplastics as a potential environmental health factor: effects of polystyrene nanoparticles on human intestine epithelial Caco-2 cells.

3.3. Chapter 3 (Article 3): Interactions of polystyrene nanoparticles with *in vitro* models of the human intestinal barrier.

3.4. Chapter 4 (Article 4): Polystyrene nanoplastics as carriers of metals. Interactions of polystyrene nanoparticles with silver nanoparticles and silver nitrate, and their effects on human intestinal Caco-2 cells.

3.1. Chapter 1 (Article 1)

Pathways of human exposure to microplastics and estimation of the total burden

Current Opinion in Food Science, 42: 144–151 (2021)

DOI: 10.1016/j.cofs.2021.01.004

Pathways of human exposure to microplastics and estimation of the total burden

J. Domenech, and R. Marcos

ABSTRACT

Plastic production is continuously growing and their wastes contaminate practically all environmental niches. In the environment, large plastics undergo continuous degradation processes generating a broad amount of microplastics and nanoplastics (MNPLs) that spread through air, land, and seas. Thus, humans suffer chronic exposures to MNPLs through different pathways: ingestion, inhalation, and dermal contact. Here, we have reviewed the recently published data regarding human exposure to MNPLs. The total load of plastic particles that humans are exposed to has been estimated based on these newly reported studies. This analysis of novel literature shows that despite ingestion is the most studied route of exposure, other routes of contact with MNPLs should not be underestimated. At the same time, gaps regarding the investigation of human exposures to environmental MNPLs have been detected, as well as the lack of robust and standardized protocols, operating procedures, and methodologies to detect/quantify MNPL in human/biological matrices.

3.2. Chapter 2 (Article 2)

Nanoplastics as a potential environmental health factor: effects of polystyrene nanoparticles on human intestine epithelial Caco-2 cells

Environmental Science Nano, 7: 272–285 (2020)

DOI: 10.1039/c9en00523d

Nanoplastics as a potential environmental health factor: effects of polystyrene nanoparticles on human intestine epithelial Caco-2 cells

C. Cortés*, **J. Domenech***, M. Salazar, S. Pastor, R. Marcos, and A. Hernández

*Both authors contributed equally to this work.

ABSTRACT

The ubiquitous and increasing presence of micro-/nanoplastics (MNPLs) in our environment demands an urgent hazard assessment, in order to determine the potential risk they pose to human beings. Given the scarce information found in the literature regarding MNPL's effects over human cells, the aim of our work is to evaluate MNPL's ability to penetrate the cells, and their potential toxic/genotoxic effects. To this aim, we used polystyrene MNPLs, as they are a widespread model of synthetic polymer, using nanoparticles with (y-nPS) or without (nPS) a fluorescent label. The human colon adenocarcinoma Caco-2 cell line was used as the cellular target, as ingestion is one of the main entry routes of MNPLs. Different endpoints were analyzed as indicators of nanotoxicity, including cytotoxicity, ROS increase, genotoxicity, DNA oxidative damage and increase in the expression of stress-related genes.

3.3. Chapter 3 (Article 3)

Interactions of polystyrene nanoparticles with *in vitro* models of the human intestinal barrier

Archives of Toxicology, 94: 2997–3012 (2020)

DOI: 10.1007/s00204-020-02805-3

Interactions of polystyrene nanoparticles with *in vitro* models of the human intestinal barrier

J. Domenech, A. Hernández, L. Rubio, R. Marcos, and C. Cortés

ABSTRACT

The universal presence of micro-nanoplastics (MNPLs) and its relative unknown effects on human health is a concern demanding reliable data to evaluate their safety. As ingestion is one of the main exposure routes for humans, we have assessed their hazard using two in vitro models that simulate the human intestinal barrier and its associated lymphoid system. Two different coculture models (differentiated Caco-2/HT29 intestinal cells and Caco-2/HT29 + Raji-B cells) were exposed to polystyrene nanoparticles (PSNPs) for 24 h. Endpoints such as viability, membrane integrity, NPS localization and translocation, ROS induction, and genotoxic damage were evaluated to have a comprehensive view of their potentially harmful effects. No significant cytotoxic effects were observed in any of the analyzed systems. In addition, no adverse effects were detected in the integrity or in the permeability of the barrier model. Nevertheless, confocal microscopy analysis showed that MNPLs were highly uptaken by both of the barrier model systems, and that translocation across the membrane occurred. Thus, MNPLs were detected into Raji-B cells, placed in the basolateral compartment of the insert. The internalization followed a dose-dependent pattern, as assessed by flow cytometry. Nonetheless, no genotoxic or oxidative DNA damage induction was detected in either case. Finally, no variations in the transcription of oxidative and stress genes could be detected in any of the in vitro barrier models. Our results show that MNPLs can enter and cross the epithelial barrier of the digestive system, as demonstrated when Raji-B cells were included in the model, but without exerting apparent hazardous effects.

3.4. Chapter 4 (Article 4)

Polystyrene nanoplastics as carriers of metals. Interactions of polystyrene nanoparticles with silver nanoparticles and silver nitrate, and their effects on human intestinal Caco-2 cells

Biomolecules, 11, 859 (2021)

DOI: 10.3390/biom11060859

Polystyrene nanoplastics as carriers of metals. Interactions of polystyrene nanoparticles with silver nanoparticles and silver nitrate, and their effects on human intestinal Caco-2 cells

J Domenech, C. Cortés, L. Vela, R. Marcos, and A. Hernández

ABSTRACT

Environmental plastic wastes are continuously degraded to their micro and nanoforms. Since in the environment they coexist with other pollutants, it has been suggested that they could act as vectors transporting different toxic trace elements, such as metals. To confirm this, we have assessed the potential interactions between nanopolystyrene, as a model of nanoplastic debris, and silver compounds (silver nanoparticles and silver nitrate), as models of metal contaminant. Using TEM-EDX methodological approaches, we have been able to demonstrate metal sorption by nanopolystyrene. Furthermore, using Caco-2 cells and confocal microscopy, we have observed the co-localization of nanopolystyrene/nanosilver in different cellular compartments, including the cell nucleus. Although the internalization of these complexes showed no exacerbated cytotoxic effects, compared to the effects of each compound alone, the silver/nanopolystyrene complexes modulate the cell's uptake of silver and slightly modify some harmful cellular effects of silver, such as the ability to induce genotoxic and oxidative DNA damage.

4. DISCUSSION

4. DISCUSSION

MNPLs contamination has become a big issue placed in the foreground of mass media, political agendas, and the public. The reason for this great attention lies on the one hand, on the plastic polymers' resistance to degradation and, consequently, their persistence in the environment (Heddagaard & Møller, 2020). As a representative example in PET polymers, which are among the most abundant ones, only a mere 0.1% of the carbon is transformed into CO_2 per year in ideal laboratory conditions. Nevertheless, non-biodegradable plastics can be worn by a variety of naturally occurring mechanisms, namely heat, light, or oxidation, even though a complete conversion to its constituents via environmental degradation could take hundreds of years. This is the reason because biodegradable plastics, which today have a minor share in the plastic market, are gaining their place as a way to reduce the environmental impact of plastic wastes. However, not all of them are completely biodegradables and, in addition, their degradation in nature may alter environmental geochemistry (Hahladakis et al., 2018). On the other hand, due to its poorly monitored ubiquity occurrence and the insufficient understanding of its ecological and health impacts, MNPLs have been stated as contaminants of emerging concern (Geissen et al., 2015). Thus, plastics are attracting the attention of European/international regulatory agencies. An illustration of this is the European Union's chemical policy known as REACH (Registration, Evaluation, and Authorization of Chemicals) (REACH regulation, EC1907/2006) under the guidelines of which the plastic industry works.

Although its presence in nature has been the objective of extensive reviews (Karbalaei et al., 2018; Petersen & Hubbart, 2021; Wayman & Niemann., 2021), scarce data on the quantification of MNPLs in different environmental matrices exist. The scientific community is placing great efforts to adapt and set up existing methodologies to allow MNPLs collection, identification, and quantification; but technical limitations are challenging the development of this field. Therefore, we are far from a reliable estimation of MNPLs' environmental burden. In addition, the expected human exposure to MNPLs must be predicted from the limited data on ecotoxicity-assessing studies (Barboza et al., 2018c; Karbalaei et al., 2018; Toussaint et al., 2019). The lack of harmonization on the reported results hinders this labor and has driven authors to review only one source of potential human exposure, particularly contaminated edibles (seafood, drink water, salt, or beer), or inhaled airborne (Kosuth et al., 2018; Lee et al., 2019; Vianello et al., 2019; Zuccarello et al., 2019; Oliveri Conti et al., 2020). It should be also stated that most of the ecotoxicological approaches evaluated only MPLs, consequently, there is a dramatic

lack of information related to the presence/levels of NPLs that, theoretically, could represent a higher hazard risk for humans. Despite these difficulties, we faced for the first time the daring commitment of estimating the total human exposure to MNPLs, considering the main plausible routes of human contact with plastic pollutants: ingestion and inhalation. According to our review of available data to date, included in the first Chapter of this Thesis, ingestion is highlighted as the major pathway for human exposure to MNPLs. Furthermore, this published review points out the essential limitations and challenges that scientists find when exploring this research line.

The increasing interest of the scientific community, regulatory bodies, and the general population on the potential human exposure to MNPLs has engendered numerous review publications discussing potential health hazards, although they are mainly based on studies describing the impact on wild-life animals (mainly aquatic) and their surroundings (Bouwmeester et al., 2015; Wright & Kelly, 2017; Jiang et al., 2020). Apart from being focused on ecotoxicity models skewed towards the marine organisms, which do not comprehensively mimic human physiology, *in vivo* studies place the spotlight on exploring MNPLs biodistribution and accumulation, while their potentially harmful effects are poorly described. Besides, and as previously indicated, a considerable bias towards MPLs versus NPLs can be identified when exploring the literature sources (Barboza et al., 2018c; Lee et al., 2019; Vianello et al., 2019; Zuccarello et al., 2019; Oliveri Conti et al., 2020). This can be explained basically by the ease of MPLs to be identified in comparison to NPLs. The unaided young-human eye cannot distinguish single particles smaller than 50 µm, which is considered to be notably large from a toxicological perspective since MPLs up to 10 µm could be inhaled and those smaller than 2.5 µm are capable to reach the deep part of the respiratory tree (Heddagaard & Møller, 2020). Conversely, when particles' identification is assisted by using standard microscopy techniques, the limits of detection, which are frequently in the low micron range, are the constraining factor when determining the lower limit size of a complex MNPLs' sample. Thus, *in vivo* approaches are focused on the role of MPLs and poorly take into consideration the misidentified NPLs, which are most probably underestimated both in environmental and wildlife samples due to their escape from conventional collection and identification methods (Bouwmeester et al., 2015).

Facing this current context, it is essential to expand the knowledge on NPLs behavior on human health. To achieve this, mammal *in vivo* models have been used as a surrogate for human beings, albeit these studies are very scarce and essentially provide general information on MNPLs distribution in the whole organism (Deng et al., 2017; Yang et al., 2019). Hence, there is an imperative need to unravel the potential hazardous effects that

NPLs could prompt in the human organism. According to the suggestion stated in our review article (Chapter 1), urgent attention must be placed on GIT exposure to plastic particles by determining MNPLs-induced alterations in the structure/function of the barrier, and MNPLs potential translocation. Given this background, the interest in exploring the effects of MNPLs on intestinal *in vitro* human models is rationalized. Therefore, according to the detected gaps of knowledge and aiming to shed light on the topic, the focus of this Thesis has been placed on NPLs exposure and its entailed toxic effects on GIT *in vitro* models. To this purpose, we used three Caco-2 cell-based models with different levels of complexity that mimics the human small intestine: undifferentiated Caco-2 cells, Caco-2/HT29 bi-cultures, and Caco-2/HT29/Raji-B tri-cultures. It must be remembered that both Caco-2 and HT29 cells are human enterocytic cells, and Raji-B are lymphocytic cells.

Setting and optimization of the cellular models

Beginning from a starting point of uncertainty regarding MNPLs interplay with *in vitro* cultured human cells, this requires a first descriptive experimental approach to provide the methodological and theoretical basis for further exploration. Importantly, it should be noted that our laboratory experience was mainly concentrated on metal NPs. This lack of experience in the MNPLs field required a proof of concept that would evidence the feasibility of the procedures. For this reason, the simplest model of undifferentiated Caco-2 cells was selected for an initial characterization of the interaction between intestinal cells and plastic particles. Undifferentiated Caco-2 cells as a model of intestinal cells have been a suitable and classical choice to evaluate NPs which ingestion constitutes the main body entry route over time. Moreover, further research on the effects encompassing NPLs was performed using the Caco-2/HT29 and Caco-2/HT29/Raji-B intestinal 2D barrier models. These models have been extensively characterized and previously used in nanotoxicological assessment [graphene oxide (GO) NPs and graphene nanoplatelets (GNPs), titanium dioxide (TiO_2) NPs, silica dioxide (SiO_2) NPs, silver (Ag) NPs, or cerium oxide (CeO_2) NPs] in our group (Vila et al., 2017; García-Rodríguez et al., 2018b; Vila et al., 2018b; Saez-Tenorio et al., 2019; Domenech et al., 2020). Unlike undifferentiated Caco-2 cells growing on ordinary plates, the two physically separated chambers (apical and basolateral) used in growing these 2D systems confers an advantage when using the model to assess the stability of the intestinal barrier model, as well as its permeability (Lefebvre et al., 2015). On the other hand, our experience indicates a better intestinal mimicking by the Caco-2/HT29 and Caco-2/HT29/Raji-B co-culture models rather than the Caco-2 monolayer alone (García-Rodríguez et al., 2018a). The absence of the mucus shed, which allows unrealistic easy access of NPs to the

apical cell membrane, along with the limited paracellular transport, make Caco-2 cells monolayer a weak model to determine NPs absorption (Lefebvre et al., 2015). Studies emphasizing the fact that the mucus shed hinders the particles' uptake, bringing co-culture models closer to the *in vivo* situation, have been reported. An example of this is the study conducted by Schimpel et al. (2014), in which they compared the PS particles (50 and 200 nm) uptake in Caco-2 monolayers, bi-cultures (Caco-2/HT29-MTX and Caco-2/Raji-B), and the triple co-culture (Caco-2/HT29/Raji-B) models, with *ex vivo* data obtained from the porcine intestinal mucosa model. Their results support better predictability of the co-culture models, especially Caco-2/HT29/Raji-B, for the MNPLs internalization assessment.

Polystyrene as a reference nano-sized plastic material

To achieve our objectives of determining the potential risks associated with MNPLs exposure, PSNPLs were selected as MNPLs model. Although pristine PS nanoparticles are non-representative of the MNPLs resulting from plastic degradation, in the absence of results from real-life MNPLs' samples and due to its commercial availability in different sizes and with chemical modifications, PS particles are the leading choice in *in vitro* toxicological assessment to date (Loos et al., 2014; Heddagaard & Møller, 2020). In fact, in a review of the literature regarding MNPLs in food, carried out by the EFSA, mainly PS particles were assessed as a surrogate for plastic particles (EFSA, 2016). Contrary to metal NPs which can be distinguished and tracked inside the cells owing to their reflection properties and their atomic constituents that differ from those endogenous to cells, following MNPLs exposures in the selected models is impractical for many methodological designs (Lefebvre et al., 2015). Thus, the availability from vendors of PS particles that incorporate fluorescent labels represents a tremendous advantage in the exploration of cellular uptake and intracellular localization. For this reason, the use of PSNPLs conjugated with a yellow fluorochrome (y-PSNPLs) was exploited to carry out part of the experimental work developed during this Thesis. In addition to these advantages, it should be pointed out that spherical MPLs are found in aquatic and terrestrial environments (Zhu et al., 2019c). This can be explained by the extended use of micro- and nanoplastic beads in the production of cosmetics such as scrub and exfoliating products that end as plastic debris in marine environments (Gouin et al., 2015). According to such authors, the total production of cosmetic products associated with the use of micro/nanobeads for 2012 in Europe was 6.88×10^8 L, representing about 4,130 tons of micro/nanobeads. As the production levels have been increasing, this gives us an idea of the exposure to micro/nanobeads, and their relevance as environmental plastic wastes.

MNPLs, as small size materials, exhibit particular physicochemical properties that could contribute to their biological behavior. The interaction between PSNPLs and its surrounding is a crucial parameter that must be considered. Particularly in the context of this Thesis, agglomeration, changes in the PS surface charges, or chemical modification in culture medium could be key factors for their permeability. Thus, the characterization of different physicochemical parameters, not only on water dispersions but also in the complex biologically relevant culture medium, is required to verify what is being administered to cells (Lefebvre et al., 2015; Bouwmeester et al., 2011). To this intent, we performed a full characterization of PSNPLs and their labeled counterparts, measuring the hydrodynamic size by dynamic light scattering (DLS), and the stability of the suspensions by laser Doppler velocimetry (LDV), taking into consideration the different dispersion media. Additionally, PS particles were visualized by transmission electron microscopy (TEM) to further characterize their morphology and size. TEM visualization confirmed the characterization provided by the supplier: the used PSNPLs were round-shaped particles of about 50 nm in diameter. Moreover, DLS results indicated a tendency to agglomeration of both PSNPLs and y-PSNPLs in Dulbecco's Modified Eagle Medium (DMEM), although this was observed to be enhanced in y-PSNPLs dispersions. This could explain the obtained polydispersity index (PdI) values, which indicates the heterogeneity of the sample based on size, where PdI values close to 0 feature monodisperse samples (Mudalige et al., 2019). Since TEM visualization confirmed a narrow size distribution of the particles, agglomeration would be responsible for those PdI values reached in DMEM by PSNPLs and y-PSNPLs (0.44 and 0.60, respectively). However, high PdI values (> 0.7), which are common when there is a broad size distribution of particles (Mudalige et al., 2019), were not obtained for any of the samples. This slight agglomeration could be prompted by the serum proteins contained in the culture medium, that would interact with the PSNPLs' or y-PSNPLs' surface forming the so-called protein corona, which in turn would promote the interaction between PSNPLs. Protein binding to MNPLs surface has been reported as the natural behavior of plastic particles to remain dissolved in biological fluids (Kik et al., 2020). Moreover, protein corona was described to influence reciprocal interactions between PSNPLs (Mohr et al., 2014), which would explain the agglomeration observed when characterizing PS materials in DMEM. This interpretation is supported by the Z-potential values reported with LDV, which are indicative of the degree of repulsion between particles in the dispersion. Thereby, a nanodispersion with a Z-potential value higher than 30 mV or lower than -30 mV is considered to have optimal colloidal stability (Mudalige et al., 2019). Hence, Z-potential values described for PSNPLs and y-PSNPLs in DMEM (-9.31 and -9.80, respectively) agree with the tendency to agglomeration. On the other hand, we

found a correlation between both PS dispersions results in the tested dispersants indicating their similar behavior. For this reason, we considered their indiscriminate use, depending on the experimental design requirements.

Microplastics, nanoplastics, and adsorbed pollutants

Consonantly to MNPLs, legacy pollutants are widespread. Similar to that described for the medium proteins, the niche-sharing between organic and inorganic contaminants with MNPLs could arouse the sorption of the surrounding toxic trace-elements onto plastic particles' surface (Bradney et al., 2019). The adsorption of trace metals onto MPLs soil samples was studied by Zhou et al. (2019). Different types of MPLs were characterized from the collected samples, including PE, PP, PS, and polyamide (PA) particles, where Ag, cadmium (Cd), chrome (Cr), lead (Pb), copper (Cu), antimony (Sb), mercury (Hg), iron (Fe), and manganese (Mn) metals were found adsorbed onto the particles' surface. The sorption phenomenon has also been shown in marine environments, where the analysis of the MPLs sampled (PE, PP, PU, PS, PVC, and PA) revealed a broad range of metals and other inorganic additives adsorbed (Kühn et al., 2018). The recent acknowledgment of MNPLs acting as *Trojan horses* for environmental pollutants has shifted the perception of plastic pollution to a severe scenario in which plastic particles would rise human exposure to toxic environmental contaminants considerably (Barbosa et al., 2020). Nevertheless, data on the effects of those co-exposures are limited to ecotoxicology models such as fish, microalgae, or plant species (Lian et al., 2020; Yan et al., 2020; Wan et al., 2021). Consequently, information about the potential effects of environmental pollutants/MNPLs on mammal models is not available from the literature. Thus, we aimed to address this prospect in the work presented in Chapter 4, using silver nanoparticles (AgNPs) as a representative inorganic pollutant present in nature and potentially interacting with MNPLs.

Silver is well known for its extensive use. The variety of applications covered by this material ranges from brazing, soldering, and electrical and plating applications to the production of coins, jewelry, tableware, or use in photographic processing. Additionally, the employment of silver in its nanoparticulated form has recently emerged in the medical and commercial fields as a consequence of its antimicrobial properties (Kim & Ryu, 2013). Hence, both the manufacturing process and the array of appliances and products that contain silver, commonly in the AgNPs form contributes to its environmental spread and human exposure, basically through the GIT by the ingestion of contaminated water and food. In addition, particles released in the air during use can be inhaled and trapped in the RT, being transported to the GIT (Vila et al., 2017). Several studies have

demonstrated this exposure to cause toxicity through oxidative stress, DNA damage, and apoptosis in different human-derived cell lines (Kim & Ryu, 2013). Owing to the predicted environmental concentrations of AgNPs in water and air, which are 0.002-0.008 ng/m^3 in European countries (Gottschalk et al., 2009) and supposes a potential niche-sharing between MNPLs and AgNPs, the combined effects of PSNPLs and AgNPs were explored in Caco-2 undifferentiated cells. On the other hand, it is known that the ionization of metal NPs occurs spontaneously when in solution. Silver ions (Ag^+) have been recognized as bioactive species with antimicrobial effects caused by the generation of ROS in bacteria and the inactivation of microbial enzymes. Therefore, the toxicity might be accentuated if the AgNPs are internalized and Ag^+ delivery occurs into the cells or in its surroundings (Kim & Ryu, 2013). For this reason, we included silver nitrate ($AgNO_3$) in our study as a surrogate of an ion releasing agent that allowed the discrimination of the effects caused by AgNPs from those induced by Ag^+.

As well as PS particles, AgNPs were characterized by TEM, showing a mean length of about 5 nm. Despite the visualized small size, the hydrodynamic radius appeared to be larger when measured by DLS, which could be explained by some agglomeration of the particles. Besides, the PdI value (0.76) indicates a wide size range, which agrees with that observed by TEM, and the dispersion stability is lower in contrast to PSNPLs samples. Furthermore, the characterization of the AgNPs/PSNPLs interplay was carried out by TEM. Although TEM is the best way to directly visualize and analyze nanoscale materials and it has been extensively used for this purpose (Mudalige et al., 2019), it had never been used previously to clearly show the interaction between MNPLs and metal nanoparticles until the development of the fourth study included in this Thesis. Additionally, the presence of Ag^+ in the PSNPLs surface was confirmed by energy-dispersive X-ray spectroscopy (EDX), an especially relevant methodology to nanotechnology due to its usefulness to identify and confirm the elements present inside the exposed cells (Mudalige et al., 2019). Interestingly, this methodology has been used to detect the presence of different metals [arsenic (As), nickel (Ni), titanium (Ti), and cadmium (Cd)] in the PE debris from the North Atlantic subtropical gyre (Prunier et al., 2019). Hence, we generated novel methodological data to visualize and certify the interplay between MNPLs and their adsorbed metal pollutants.

Cell viability and barriers' stability

Ideally, the range of concentration used for *in vitro* toxicology studies should be based on the predicted human exposure levels (Lefebvre et al., 2015). Unfortunately, there is a vast gap of knowledge regarding this concern in the MNPLs framework. Thus, the

selection of the experimental doses generally depends on the screening of cytotoxicity in the assayed model. Within the context of this Thesis, uptake and translocation studies must be carried out under non-cytotoxic conditions to avoid the disturbance of the cells'/barriers' integrity, so that, the exploration of toxic concentration ranges is mandatory (de Angelis & Turco, 2011). PS particles (NPLs of 50 and 100 nm, and mostly MPLs), have been reported to exert some toxic effects in a variety of *in vitro* human models, among which there are epithelial cells (BEAS-2B, HepG2) (Dong et al., 2020; He et al., 2020b), or hematopoietic cell lines (THP-1, TK6, and Raji-B) (Rubio et al., 2020a). Nevertheless, the effects reported were detected at concentrations that are unrealistic from a human exposure point of view. Under our experimental conditions, the cell viability remained unaffected after the exposure of the Caco-2 based models (undifferentiated Caco-2 cells, Caco-2/HT29, and Caco-2/HT29/Raji-B) to PSNPLs or y-PSNPLs. These results agree with that observed by other authors after the exposure of Caco-2 cells to MNPLs. Particularly, Wu et al. (2019a) reported no cytotoxic effects of PSNPLs (100 nm) at concentrations up to 200 µg/mL in undifferentiated Caco-2 cells. Similarly, the exposure of undifferentiated Caco-2 cells to PSMPLs (between 1 and 10 µm) did not show alterations in cell viability at environmentally relevant concentrations (Stock et al., 2019; Wu et al., 2019a; Wu et al., 2019b). No toxic effects have also been informed after the exposure of undifferentiated Caco-2 cells to negatively charged (carboxylated and sulfonated) PS particles (50-200 nm) (Abdelkhaliq et al., 2018; Reinholz et al., 2018). However, Wang et al. (2020b) described a decrease in Caco-2 cell viability after exposure to large PSMPLs (300 nm-6 µm). In addition, the results reported in Chapter 4 reveal neither synergistic nor antagonistic cytotoxic effects between PSNPLs and AgNPs (or $AgNO_3$) on undifferentiated Caco-2 cells, since AgNPs/$AgNO_3$ toxicity remained unaltered with the addition of PSNPLs.

Unlike undifferentiated cells, in 2D models, cell viability is usually assessed by measuring the integrity and permeability of the resulting monolayer. The use of the dual-chamber models discussed in this Thesis presents the great advantage of having established tools to evaluate their aptitude when facing a potential risk evaluation. The results described in Chapter 3 include a quality check of the Caco-2/HT29 and Caco-2/HT29/Raji-B barriers' integrity performed by the determination of its transepithelial electrical resistance (TEER). This technique provides information about the integrity of the epithelial tight junctions and the cells' fitness. In addition, markers of paracellular permeability, such as Lucifer Yellow (LY), were further assessed. LY is a small hydrophilic compound which translocation across the barrier occurs via paracellular transport (i.e., through tight junctions) (de Angelis & Turco, 2011). Thus, TEER and LY

assays were used to determine the potential effects of PSNPLs on the structural/functional integrity of the barrier.

Different authors have described MNPLs incapacity to induce the barrier impairment in Caco-2 monolayers, bi-cultures such as Caco-2/HT29, Caco-2/HT29-MTX, or Caco-2/M-cell, and tri-culture models such as Caco-2/HT29/Raji-B or Caco-2/HT29-MTX-E12/THP-1, which mimics the inflamed intestine. These studies included PS particles, namely pristine particles, negatively and positively charged PS particles (COOH-PS and NH_2-PS, respectively) (50, 200, and 500 nm), and PVCMPLs (<50 µm) (Schimpel et al., 2014; Walczak et al., 2015a; Hesler et al., 2019; Busch et al., 2021). As well as what is detailed in the literature, our results indicated that PSNPLs do not exert toxic effects or disturb the barriers' integrity and permeability, as TEER values measured were above 300 Ω/cm^2, and we were not able to detect LY in the basolateral chamber of the bi- and tri-culture (Caco-2/HT29 and Caco-2/HT29/Raji-B, respectively) systems. Moreover, these data, which were not different between the models, clearly show that the presence of M-cells and the mucus shed, do not alter the monolayer consistency when it is exposed to PSNPLs. Despite the advantages of using TEER and LY approaches to determine the effects on the barrier integrity, other authors have used classical methods (metabolic activity assays such as MTS, MTT, or WST-1) to evaluate the effects of different MNPLs (PET and PSNPLs, and PE, PP, PVC, PET, and PSMPLs) on barrier models (Magrì et al., 2018; Hesler et al., 2019; Stock et al., 2021). These results, together with our cell survival studies in Caco-2/HT29 and Caco-2/HT29/Raji-B models, confirm the low capacity of MNPLs to induce cytotoxicity.

Microplastics and nanoplastics uptake and translocation through barrier models

Despite its apparent lack of harmful effects, research on MNPLs uptake in the GIT has received great attention owing to its potential behavior as nanocarriers. Indeed, pristine PS particles (100-500 nm, and 1-6 µm) were described to be easily internalized by undifferentiated Caco-2 cells after 24 h of exposure (Wu et al., 2019a; Wang et al., 2020b). Interestingly, these studies suggest that MNPLs uptake is strongly impacted by the size since smaller particles show greater internalization rates. Abdelkhaliq et al. (2018) explained this uptake size-dependency by the increased deposition of smaller particles (COOH-PSNPLs, 50 nm) on undifferentiated Caco-2 cells' surface, in comparison with larger PSMPLs (200 nm) with the same surface chemistry. Additionally, in this study authors showed that PS particles' cellular association increase upon increasing particle concentrations. According to these findings, the results included in the second Chapter of this Thesis describe the PSNPLs internalization by Caco-2

undifferentiated cells in a concentration-dependent manner, as the single-cells fluorescent signal associated with increasing dose levels of y-PSNPLs detected by flow cytometry, clearly point in that direction. Besides, we identified PSNPLs accumulated in vacuoles and lysosomes through TEM visualization, as well as y-PSNPLs reaching the nuclei of undifferentiated Caco-2 cells by confocal microscopy. Unfortunately, only a few studies, besides ours, specify the intracellular localization of MNPLs (PET and mainly PS particles) in Caco-2 cells and thereof-derived cultures (Abdelkhaliq et al., 2018; Magrì et al., 2018; Reinholz et al., 2018; Wu et al., 2019a). Nevertheless, our results are supported by the published findings, where it is demonstrated the co-localization of PS particles with lysosomes. The Caco-2 cells' internalization of MNPLs has been proposed to occur starting with the uptake, preferably by micropinocytosis, and following with the subsequent intracellular trafficking along the endo-lysosomal or transcytosis pathways (Reinholz et al., 2018). It should be highlighted that among the studies evaluating MNPLs internalization in human cells, TEM as a visualization method to describe MNPLs' cellular localization, has only been chosen by Reinholz and co-workers and ourselves. Despite being a convenient tool in the nanomaterials field, it has been poorly used for this purpose in the MNPLs field. Hence, we have shown the versatility of the TEM technique and its suitability for MNPLs detection and intracellular localization.

Aging and weathering have been demonstrated to modify the particles' surface, endowing them with relative charge and different sorption capacities (Holmes et al., 2014; Verla et al., 2019b; Luo et al., 2020b). Although the aging process was out of the scope of this Thesis, it seems pertinent to understand its effects to contextualize the extended use of functionalized plastic particles that will be discussed in this section. MNPLs are affected by aging time causing C-H bond deformation and so that, skeleton vibration. The C-H bond breaks drive to an increase of oxygen-containing functional groups by forming peroxy radicals (C-O) which can absorb hydrogen atoms from the surroundings and organize hydroperoxide groups (COOH). Then, COOH could be further decomposed into a variety of products (C=O, C-OH, and O-C=O) (Müller et al., 2018; Mao et al., 2020). Therefore, the use of functionalized particles with a positive or negative surface charge for research purposes has been extended aiming to better simulate environmental samples.

The uptake of MNPLs in 2D cultures has been shown to depend on a variety of factors intrinsic to the particles, such as size and surface charge. While the internalization capacity in complex Caco-2 based models seems to indirectly depend on size (Schimpel et al., 2014; Walczak et al., 2015a; Stock et al., 2019), neutral PSNPLs (50 and 100 nm) are suggested to internalize better than their positively or negatively charged analogs

(Walczak et al., 2015a). This seems to be related to the protein corona formation that would depend on the particles' surface chemistry. Indeed, surface chemistry could outweigh the impact of size since the protein corona composition would influence particles' adhesion, uptake, and monolayer transport (Walczak et al., 2015a; Abdelkhaliq et al., 2018). The impact of surface functionalization has also been depicted in exposed rodent models, where different biodistribution and accumulation profiles were detected for neutral and charged PSNPLs (50 nm) in different organs (Walczak et al., 2015b). However, only a minor fraction of orally administrated PSMPLs (1, 4, and 10 µm) to mice was found on the intestinal tissue, and no particles were found in other organs, suggesting the influence of the size in *in vivo* models (Stock et al., 2019).

Most of the studies addressing the MNPLs translocation through barrier models could demonstrate the uptake and accumulation but were unable to show the transport across the intestinal models (Hesler et al., 2019; Stock et al., 2019). Considering the background given, this could be explained because of the use of negatively charged (50 nm and 0.5 µm, COOH-PSNPLs) and/or micro-sized particles (1, 4, and 10 µm, PSMPLs) for those studies. Contrarily, using neutral nanosized PS particles, we have shown not only the concentration-dependent internalization and the cell nuclei reaching in cells conforming the co-culture barriers but also the translocation of PSNPLs through the Caco-2/HT29/Raji-B cells layer and the concentration-dependent internalization into Raji-B cells present in the basolateral compartment, reaching even cell nuclei. Interestingly, as previously discussed no toxic effects were observed after the exposure of the barrier models to PSNPLs, which indicates the inability of PSNPLs to alter tight junctions. Nevertheless, this inability does not avoid the translocation of PSNPLs through the barrier that would indicate that, in humans, PSNPLs may cross the intestinal barrier through a non-paracellular mechanism. Similar to our results, neutral PETNPLs (<80 nm) have been identified to cross the Caco-2 monolayer in a time- and concentration-dependent manner (Magrì et al., 2018).

Moreover, our results informed about a greater ability of the tri-culture model to incorporate y-PSNPLs in comparison to the Caco-2/HT29 model at the higher concentrations tested (50 and 100 µg/mL). This was shown by the greater percentage of fluorescent cells detected by flow cytometry, namely cells incorporating y-PSNPLs, and the increased single-cell y-PSNPLs accumulation. M-cells have been proposed as the major entry route for nanoparticles in the *in vivo* context (Wright & Kelly, 2017). Particularly, the strong impact of M-cells in MNPLs uptake is supported by different studies in which the comparison of PS particles internalization among Caco-2 based co-cultures was conducted (Schimpel et al., 2014; Walczak et al., 2015a; Stock et al., 2019).

Considering the effectiveness of PSNPLs internalization, the way how this internalization affects the uptake of adsorbed metals was investigated in Chapter 4. On the one hand, the intracellular and intranuclear localization of AgNPs/PSNPLs complexes was confirmed by confocal microscopy. It should be noted that along with the Lim et al. (2019) study, where they explored the cellular uptake, cytotoxic effects, and metabolic responses of bronchus epithelial (BEAS-2B) cells exposed to zinc oxide (ZnO) NPs/PSNPLs, these are the only existing studies where the metal/MNPLs complexes are observed inside human cells. In our study, apart from the association AgNPs/PSNPLs, the presence of the ionic metal form and NPLs were also detected inside the cells. Toxic adsorbed pollutants are often attached to MNPLs surface by physical interaction and are held by weak forces involving non-covalent bonds (i.e., hydrogen bonds, van der Waals, and cavity formation). Thus, adsorption/desorption processes occur readily, leading most probably to an equilibrium of association and dissociation rather than a stable and lasting union (Verla et al., 2019b), which would explain our findings.

Aiming to quantify the impact of PSNPLs on the internalization of AgNPs or Ag^{+}, inductively coupled plasma mass spectrometry (ICP-MS) was used to detect the amount of Ag atoms inside the Caco-2 cells after the single- and co-exposures to Ag compounds and/or PSNPLs. In general, the obtained data indicate a Ag concentration-dependent internalization, independently of the PSNPLs presence. Nevertheless, a higher internalization of AgNPs was detected when cells were treated with AgNPs at low environmentally relevant concentrations and PSNPLs, in comparison with the AgNPs treatment alone. Although these results do not show statistical significance when the complete set of data is analyzed, results reach significance if the low concentration AgNPs treatments without and with PSNPLs are analyzed separately, meaning our results are biologically relevant. However, results obtained after exposing the cells to a high concentration of AgNPs or $AgNO_3$ without and with PSNPLs, differ from what would be expected if the Ag materials/PSNPLs association and the effective PSNPLs internalization observed in Caco-2 models is taken into consideration. These results are in line with previous findings showing that AgNPs at high mass ratios block their capture by MNPLs, so that Ag compounds adsorb less efficiently to plastic particles at high concentrations (Wagener et al., 2012; Li et al., 2020b). Nonetheless, the contact of plastic particles with cells could be delayed and/or altered due to the association and dissociation dynamics between plastic and silver (i.e., until reaching the sorption kinetics equilibrium), and the additional changes in the PSNPLs surface composition due to the adsorbed particles or the altered suspension stability after the interactions, which would

be more affected at high concentrations of silver materials (Li et al., 2020b). However, this is a complex process in which detailed reaction mechanisms are still far from being understood. Under such a scenario, longer exposure times should be explored to assess whether PSNPLs acts as a AgNPs or Ag^+ cleanser, or the complexes internalization is just retarded. Unluckily, the heavy metals/MNPLs complex uptake is poorly studied, so few comparisons with other studies can be done. The few publications addressing the topic in non-mammalian models hint contrasting trends. While greater amounts of silver were found in *Daphnia magna* tissues after the co-exposure to Ag^+/PEMPLs or Ag^+/PSMPLs compared with the single exposures (Monikh et al., 2020), the total silver body concentration was significantly reduced when zebrafish were exposed to Ag^+/PEMPLs in contrast to single exposures, and no differences were found in the intestinal silver content between treatments (Ag^+, or the co-treatment) (Khan et al., 2015). In this latter study, the authors suggest that the sequestration of Ag^+ by PEMPLs would decrease Ag^+ bioavailability and therefore, Ag^+ internalization. On the other hand, physiological conditions, namely digestion processes and environment in the GIT, should be further explored as some authors attributed faster contaminants desorption rates from MNPLs to conditions mimicking the gut environment (Bakir et al., 2014; Zhou et al., 2020). These findings do not imply the loss of interest in MNPLs as potential vectors of toxic contaminants since synergistic effects of pollutants and MNPLs are not always accompanied by plastic acting as a carrier but as a magnification of the availability and synergistic effect due to the mixture of pollutants (Sendra et al., 2021).

Cellular stress

After demonstrating the interaction of PSNPLs with Caco-2 models, the induction of potentially harmful effects was evaluated by studying the oxidative stress induction, the inflammatory response activation, and the expression of general stress markers.

Although the intracellular ROS production has been assumed as the MNPLs key toxicological mechanism owing to its inherent capacity to generate oxidative species, the data collected from the studies included in this Thesis point out the contrary. Neither undifferentiated Caco-2 cells nor co-culture intestinal models showed induction of oxidative stress after exposure to PSNPLs. Two techniques using small-molecules fluorescent probes, particularly 2',7'-dichlorofluorescein diacetate (DCFH-DA), and dihydroethidium (DHE), were applied. DCFH-DA allows the detection of a wide panel of oxygen-derived reactive molecules such as H_2O_2, $ONOO^-$, and $O_2^{\cdot -}$ to a lesser extent, while DHE provides detection mainly of the latter (Wang et al., 2013a). The absence of significant changes in the expression of two of the most relevant antioxidant enzymes,

superoxide dismutase 2 (SOD2) and glutathione-S-transferase pi 1 (GSTP1), which can intercept, scavenge, and neutralize radicals and reactive intermediates generated in excess (Valavanidis et al., 2013), confirmed this absence of oxidative stress induction. Interestingly, unlike in barrier models, the expression of the *heme oxygenase-1* (*HO1*) gene, which codifies the HO1 protein that protects cells by reducing $O_2^{\cdot-}$ and other reactive oxygen species, was increased in undifferentiated Caco-2 cells after 24 and 48 h of exposure to PSNPLs. This enzyme is also considered a general stress-induced marker, which upregulated expression reveals an underlying stress-response despite the undetected ROS production. This agrees with the overexpression of the heat shock protein 70 (HSP70), which performs chaperoning functions and protects the cell from physiological stresses. Interestingly, these responses were not evident when bi- and tri-cultures were assayed, indicating greater resistance by differentiated cells in the monolayer disposition. This resistance could be attributed to the reduced particle-to-cell ratio in confluent 2D models, as well as to cell-to-cell contacts, that might confer an additional protective function (Busch et al., 2021).

Special emphasis has been placed on the induction of oxidative stress as a possible hallmark of toxicity caused by MNPLs. Although some authors detected a significant increase in the intracellular levels of ROS using the DCFH-DA assay in undifferentiated Caco-2 cells exposed to PS particles (100, 300, and 500 nm, and 1, 3, and 5 µm), this effect was observed only when high concentrations (120 and 200 µg/mL) of PS MNPLs were used, which do not describe a relevant biological exposure scenario (Wu et al., 2019a; Wang et al., 2020b). Moreover, other studies conducted with the aforementioned cell model exposed to MNPLs agree with our data. Thereby, Magrì et al. (2018) did not found intracellular ROS production after the exposure of undifferentiated Caco-2 cells to PETNPLs (<80 nm) using the DCFH-DA assay, as well as Wu et al. (2019b) who did not find changes in the activity of different antioxidant enzymes, specifically SOD, catalase (CAT) and glutathione (GSH), after the exposure to PSMPLs for 24 and 48 h. In addition, the latter study revealed the downregulation of the nuclear transcription factor-kβ (NF-kβ) and mitogen-activated protein kinase (MAPK) signaling pathways. Surprisingly, contrary to that described for *in vitro* human models, PS particles produce a well-described oxidative response in ecotoxicological models such as the small crustaceans *Daphnia pulex* and *Paracyclopina nana*. The upstream pathways of the antioxidant protection system to MNPLs exposures have been related to the activation of MAPKs, hypoxia-inducible factor 1 (HIF-1), and NF-kβ cascades (Jeong et al., 2017; Liu et al., 2020a). Within this context, it should be remembered that ROS production is associated with the activation of MAPKs, specifically, the extracellular signal-regulated kinase (ERK)

and p38, which play a role in survival responses cause by oxidative stress. Aside, MAPKs pathway induces the expression of HIF-1, which allows for survival and is related to immunological responses and regulation of homeostasis (Ziello et al., 2007). HIF-1 and nuclear factor erythroid 2-related factor 2 (Nrf2), which in its turn is activated by NF-kβ, are described as key factors for genes encoding antioxidant proteins. Importantly, a significant increase in oxidative stress, along with a similar activation of the MAPKs pathway [p38 and c-Jun N-terminal kinase (JNK)] was reported in mice after oral administration of PSMPLs (5-6 μm) (Xie et al., 2020).

The oxidative stress induction was also studied using the DHE method after the single/co-exposure of Caco-2 cells to AgNPs or $AgNO_3$ with PSNPLs in Chapter 4. The generation of ROS at high levels has been proposed as a general mechanism of NPs-mediated cytotoxicity and this is supported by studies demonstrating the generation of intracellular ROS at high levels in human *in vitro* exposures to AgNPs. Within this framework, AgNPs have been reported to elevate ROS levels in lung carcinoma epithelial-like cell lines (A549 and BEAS-2B) (Foldbjerg et al., 2011; Kim et al., 2011), hepatocellular carcinoma cells (HepG2) (Zhu et al., 2016), monocytic cells (THP-1) (Foldbjerg et al., 2009), and colon cancer cells (HT29) (Sanpui et al., 2011), among others. Complementary, some of these studies inform about the behavior and effects of Ag^+ (including $AgNO_3$ in the experimental procedures), showing similar results. Thus, we aimed to cover these latter aspects concerning Ag compounds/PSNPLs co-exposures in Caco-2 cells. Along with what is found in the literature, our results confirm the induction of oxidative stress after exposure to AgNPs or $AgNO_3$. However, this induction was neither intensified nor lessened when silver compounds were combined with PSNPLs. These results confirm the lack of PSNPLs toxicity under our experimental conditions. Nevertheless, synergistic effects of Ag^+ and PSNPLs or modified PSNPLs (with -COOH or $-NH_2$ functionalization, and aged particles) regarding ROS production, were indicated in *Escherichia coli* (Sun et al., 2020).

Humans, as aerobic organisms, generate energy in the mitochondria of their eukaryotic cells, and suitable mitochondrial membrane potential is required to maintain a normal ATP metabolism. The impairment of the mitochondrial membrane potential has been reported in undifferentiated Caco-2 cells after the exposure to PS particles (100 and 500 nm, and 1, 3, 5, and 6 μm), showing significant membrane depolarization (Wu et al., 2019a; Wang et al., 2020b). In healthy conditions, ROS are products of normal mitochondrial metabolism which are necessary for different physiological processes, but altered mitochondrial respiration generates unbalanced levels of mitochondrial-derived ROS (mROS) and antioxidant defenses (Valavanidis et al., 2013). Since the membrane

depolarization and mROS are mutually regulated one another (Suzuki-Karasaki et al., 2014), according to the above-informed results PSMPLs could trigger mitochondrial oxidative damage, such as lipid peroxidation, protein or enzyme oxidation, and DNA oxidative damage (Valavanidis et al., 2013). Although no evidence of intracellular ROS production caused by PSNPLs was illustrated in the work developed during this Thesis, structural mitochondrial alterations were observed by TEM after the exposure of Caco-2 undifferentiated cells to PSNPLs, namely swelling of its cristae and modification of its inner membranes. Hence, we further investigated the potential mitochondrial dysfunction caused by MNPLs by assessing the mitochondrial membrane potential and the production of mROS. Although our results indicated an increase of the mitochondrial membrane potential, mROS accumulation was not detected after the exposure of cells to PSNPLs. Taking together the published data and our results, mitochondrial dysfunction may be related to particles' size, being MPLs more effective in terms of mitochondrial depolarization, than small particles (Wang et al., 2020b), which suggests different modes of toxicity depending on the behavior of the internalized particles. Therefore, long-term accumulation of NH_2-PSNPLs in lysosomes can lead to swelling of those and release of its content (especially cathepsins) into the cytosol, which eventually leads to mitochondrial damage (Wang et al., 2013b), while large particles internalized, would escape from the lysosomes and localize into cytoplasmic vacuoles or free in the cytoplasm, which may disrupt lysosomes, placing it again as the upstream event to other organelles damage (Li et al., 2016). This proposed approach is supported by Auguste et al. (2020) work, suggesting the association between lysosomal disturbances and mitochondrial membrane depolarization in hemocytes of mussels after 24 h of exposure to NH_2-PSNPLs. However, it must be considered that the above-mentioned effects were caused by positively charged NPLs, which are known to cause damage to phospholipid membranes (Wang et al., 2013b). Thus, particle surface functionalization may play a critical role in their intracellular outcomes. Even so, the overexpression of HO1 and HSP70 proteins, as well as the ultrastructural changes visualized by TEM, agree to some extent with the induction of cellular stress in undifferentiated Caco-2 cells.

Cellular stress is confirmed by the activation of pro-inflammatory agents after 48 h of Caco-2 exposure to PSNPLs. Results in Chapter 2 show an increased expression of IL-1β and IL-8, which are inflammation-related proteins. Despite the lack of statistical significance, the expression of the aforementioned ILs reached values about 5 times higher than the untreated controls when cells were exposed to moderate concentrations of PSNPLs (50 and 25 µg/mL for IL-1β and IL-8, respectively). These data are supported by the matching expression pattern of *IL-1β* and *IL-8* genes, among other inflammation-

related genes, in undifferentiated Caco-2 cells after 48 h of exposure to PSMPLs at moderate concentrations (12.5, 25 and 50 µg/mL) (Wu et al., 2019b). Contrarily, PS particles (100 nm and 5 µm) only increased proinflammatory effects after undergoing a simulated digestion process in Caco-2 monolayers (Liu et al., 2020c). Other MNPLs models were not able to induce inflammatory effects. Thus, PETNPLs seems not to affect the production profile of the pro-inflammatory cytokines IL-8 and monocyte chemoattractant protein-1 (MCP-1) in Caco-2 cells (Magrì et al., 2018), as well as PP, PA, and PUMPLs (median sizes from 72 to 282 µm), which did not alter significantly the released levels of IL-1β, IL-8, and tumor necrosis factor-α (TNF-α) by the Caco-2/HT29-MTX/monocyte-derived macrophages and dendritic cells model (Lehner et al., 2020). Moreover, other authors have placed the focus on highly susceptible individuals mimicking and comparing the effects of MNPLs on inflamed and healthy intestine models [Caco-2/HT29-MTX-E12/THP-1 model, primed with interferon-ɣ (IFN-ɣ), a key cytokine for macrophages activation, when imitating the inflamed intestine] (Busch et al., 2021). In this last study, neither NH_2-PSNPLs (50 nm) nor pristine PSNPLs (50 nm) affected the release of IL-1β, IL-6, IL-8, and TNF-α, likewise PVC particles (<50 µm), except for the increased levels of IL-1β detected in the inflamed intestine model. Taken together, these results reveal higher susceptibility of undifferentiated Caco-2 cells to MNPLs than the complex differentiated tri-culture models, which is surprising if the demonstrated leucocyte-epithelial Caco-2 cells crosstalk in inducing cytokine responses is considered (Parlesak et al., 2004). A significant rise in the expression of pro-inflammatory, immune-related, and stress response-related cytokines after the *ex vivo* exposure of lymphocytes, polymorphonuclear cells, and monocytes to PSNPLs (50 nm) was reported by Ballesteros et al. (2020). Considering Parlesak et al. and Ballesteros and colleagues' findings, a greater cytokine release by the Caco-2/HT29/Raji-B model would be expected. But again, the single-cell disposition in undifferentiated Caco-2 cultures may play a relevant role in the more effective inflammatory responses in contrast to those reported in the literature for well-consistent monolayer models.

Genotoxic and oxidative DNA damage

Genotoxicity is not a usual biomarker among the studied endpoints when assessing the potentially harmful effects of MNPLs. However, either direct or indirect interaction of MNPLs with DNA could lead to genotoxic damage. Our results on confocal studies showing the presence of PSNPLs inside the nucleus could suggest a potential genotoxic effect. Some authors have linked inflammation and oxidative stress caused by MNPLs with DNA damage that would have concerning implications for human health, such as human diseases, cancer, or aging (Carriere et al., 2017; Kawanishi et al., 2017). In

addition, the impairment of the replication and/or the repair machinery caused by the MNPLs exposure could lead to unrepaired DNA lesions that would prompt mutagenic processes, which could modulate key genes involved in genomic integrity maintenance or cell cycle control, so that, carcinogenesis might be initiated (Rubio et al., 2020b). Although a strong enhancement of ROS production or considerable inflammatory responses were not detected in our models after the exposure to PSNPLs, the great ability of these plastic particles to internalize and reach the nuclear region of the cells demonstrated in our studies by confocal microscopy and previously discussed makes the evaluation of genotoxic damage mandatory for us. Therefore, the capacity of PSNPLs to induce DNA breaks was investigated by the comet assay in undifferentiated Caco-2 cells, Caco-2/HT29 and Caco-2/HT29/Raji-B as reported in the second and third Chapters of the present Thesis.

Our results revealed the lack of genotoxic damage in both the undifferentiated cells and in the Caco-2/HT29 cells forming the barriers of the bi- and tri-cultures. Additionally, it is well-known that intracellular ROS can interact with DNA driving to oxidation of its bases and generating different types of damage, such as the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) adducts, which is considered mutagenic, since 8-oxodG can pair with adenine and cytosine with the same efficiency (Carriere et al., 2017). Thus, further evaluation of the oxidative DNA damage (ODD) was conducted by the modified comet assay with the addition of formamidopyrimidine DNA glycosylase (FPG) enzyme, which recognizes oxidized DNA bases and cut them, generating transient DNA breaks. As expected, according to the lack of intracellular ROS production previously noted, ODD was not induced in the tested models after being exposed to PSNPLs for 24 h. Despite our negative results, the comet assay has been presented as a robust tool to detect the genotoxic potential of nanomaterials (García-Rodríguez et al., 2019a). This fact is supported by the high sensitivity of the approach to positive controls, indicating its ability to detect genotoxicity and ODD. Despite its advantages, the method has been barely used to detect DNA damage caused by MNPLs. From the literature, only two studies, apart from those included in this Thesis, have addressed the genotoxicity endpoint using this method. Thus, genotoxicity and ODD in different hematopoietic cell lines (Raji-B and TK6) exposed to PSNPLs (50 nm) were reported by Rubio et al. (2020a). In addition, Busch et al. (2021) performed the comet method in Caco-2 based models, and the described results are in line with our data. The latter study failed to detect DNA damage induction in single cultures of undifferentiated Caco-2 and HT29-MTX-E12 cells, as well as in the Caco-2/HT29-MTX-E12 barrier of the Caco-2/HT29-MTX-E12/THP1 healthy and inflamed model, after the exposure to PSNPLs (50 nm), NH_2-PSNPLs (50 nm) and

PVC (<50 µm), excluding the highest concentration of NH_2-PSNPLs, which significantly raised DNA damage in Caco-2 and HT29/MTX-E12 cells after 4 h of exposure. The lack of genotoxicity in our models might be related to the repair capacity of the cells, which would be quickly activated after the potential early damage, and therefore would not be detected after 24 h of exposure. Nevertheless, considering Busch and colleagues' findings, all points to a weak or non-genotoxic capacity of pristine PSNPLs on Caco-2 cells.

Other methods have also been reported to study the genotoxic potential of MNPLs. Hence, the detection of double-strand DNA breaks was confirmed using the γ-H2Ax foci detection in pulmonary epithelial cells (Calu-3) and macrophages (THP-1) after the exposure to NH_2-PSNPLs (50 nm), but no evidence of genotoxic damage was found when cells were treated with PSNPLs or COOH-PSNPLs (Paget et al., 2015). Moreover, the *p53* reporter gene assay was carried out in hepatocellular carcinoma cells (HepG2CDKN1A-DsRed biosensor cells, used for rapid detection of genotoxic agents) exposed to COOH-PS MNPLs (50 and 500 nm) (Hesler et al., 2019). Neither COOH-PSNPLs nor COOH-PSMPLs activated the expression of the *p53* gene, indicating they were non-genotoxic particles. Moreover, the lack of formation of micronuclei (MN) in ovary cells derived from hamster (CHO-K1) was assessed in the same study after the exposure of the above-mentioned particles, confirming the previous results. The MN frequency was also evaluated in human fibroblasts (Hs27) exposed to PSNPLs (100 nm), showing in this case, genotoxic damage induction (Poma et al., 2019). Aiming to further confirm the lack of DNA damage induced by PSNPLs, we also used a complementary genotoxicity test, the flow cytometry micronucleus (FCMN) assay, which is ideally suited to identify aneugenic and/or clastogenic agents, and the high number of scoring events result in a high degree of confidence in the generated data (García-Rodríguez et al., 2019b). As reported in Chapter 2, PSNPLs exposure did not induce chromosomal damage in Caco-2 undifferentiated cells. However, it should be remarked that the basal MN levels (non-exposed cells) for this cell line were enough high (about 600 MN/1000 nuclei), so detecting small increases in the MN frequency would be challenging. Unfortunately, the FCMN assay cannot be used to determine the genotoxic potential of PSNPLs on the bi- and tri-cultures since cells forming the monolayer stop dividing when confluence is reached, thus cell division required for the MN formation detection, does not occur after the exposure to PSNPLs.

These results, along with the above-discussed comet data, are astonishing considering the ability of PSNPLs to reach the cells' nuclei. According to the results obtained and the studies reported in the literature, MNPLs would be classified as weak genotoxic agents.

In addition, the results rarely follow a dose-response trend. This could be related to the variability associated with sampling size, which is extremely important when the changes in the levels of DNA damage are small. Nevertheless, genotoxicity induction has frequently been associated with the cell model chosen, showing similar cell types (lung epithelial cell lines: BEAS-2B and A549, or hematopoietic cell lines: Raji-B, TK6, and THP-1) very different responses to the same threat (García-Rodríguez et al., 2019a; Rubio et al., 2020a). Therefore, the interaction of nanoparticles with the DNA does not seem to imply direct damage but the sensitivity of the selected cell model plays a critical role when evaluating genotoxicity, as well as other endpoints caused by MNPLs, as is illustrated in Rubio and colleagues' work, where authors found that the genotoxic effect was not correlated with PSNPLs uptake, since THP-1 were the cells showing a higher internalization but genotoxicity was not detected in such cells. In this context, Caco-2 cells are resilient cells that are not easily altered by nanomaterials as can be revealed attending to literature. Thus, the model of choice is decisive for the hazard and risk assessment of MNPLs, since the intrinsic nature of plastic pollutants is considered neither safe nor hazardous, but the ability of the system to detect its effects makes a significant difference. Regulatory organizations have emphasized this fact. Although the ISO recommends the use of mammalian cell systems for the genotoxicity testing of nanomaterials (ISO, 2017), and the EFSA highlight the *in vitro* MN test to study structural and numerical chromosome aberrations, as well as the *in vitro* modified comet assay for the detection of genotoxicity and oxidative DNA lesions (Hardy et al., 2018), the Organization of Economic Co-operation and Development (OECD) has developed a guideline to inform about the suitability of different cell lines to test genotoxicity. Thus, human lymphocytes such as TK6, or some rodent cell lines such as CHO, V79, and L5178Y are considered convenient for performing MN assay, while other human-derived cell lines (HT29, Caco-2, HepG2, or A549) have not been extensively validated, albeit having been used in several studies (OECD, 2016).

Although PSNPLs were featured as deficient genotoxicants under our experimental conditions, whether the co-localization of Ag materials/PSNPLs complexes inside the Caco-2 nuclei increase the AgNPs or Ag^+ genotoxic potential was unresolved. Aiming to cover this issue, we evaluated DNA damage in undifferentiated Caco-2 cells after the AgNPs (or Ag^+) and PSNPLs single- and co-exposures. Genotoxic damage caused by the single exposure to AgNPs and Ag^+ followed an increasing trend in Caco-2 cells, albeit only DNA impairment caused by Ag^+ reached significant results. Similar results were found in the alveolar epithelial cell line A549, where a dose-dependent DNA adduct formation was informed after the AgNPs exposure lasting for 24 h, although the results

did not reach statistical significance (Foldbjerg et al., 2011). On the contrary, a moderate AgNPs concentration (5 µg/mL) was required to detect TUNEL positive THP-1 cells (Foldbjerg et al., 2009), while the activation of biochemical markers of DNA damage (p53 and H_2X) were detected at highly cytotoxic concentrations of AgNPs (Zhu et al., 2016). Nonetheless, DNA strand breaks were detected by the comet assay in another lung cell line, BEAS-2B, showing significant differences in comparison to the untreated cells from very low AgNPs concentrations (0.01 µg/mL) in a dose-dependent manner. These results were confirmed in the same study by the MN formation assay (Kim & Ryu, 2013). In our experimental approach, the co-treatment of Caco-2 cells with AgNPs and the highest PSNPLs tested concentration (100 µg/mL), showed a significantly increased linear trend in genotoxic damage. This implies a greater increase in genotoxic damage when high doses of plastic particles are combined with AgNPs, in comparison with the single AgNPs exposure and the co-treatment with a moderate PSNPLs concentration (10 µg/mL). On the other hand, although high concentrations of $AgNO_3$ induced DNA damage, independently of the PSNPLs concentration, the combination of this silver material with the highest dose of PSNPLs did not follow an increasing dose-response relationship. Conversely, a significant genotoxic damage linear trend was similarly illustrated after the exposure of the cells to $AgNO_3$ alone and $AgNO_3$/PSNPLs (10 µg/mL), indicating that PSNPLs do not alter the Ag^+ genotoxic potential. Importantly, the different behavior shown by undifferentiated Caco-2 cells after AgNPs treatments, alone or in combination with PSNPLs, in comparison to treatments including $AgNO_3$, indicates the effects are being produced by the metal NPs, and not by the released ions.

Regarding ODD, while AgNPs induced this effect in BEAS-2B cells (Kim et al., 2011), our results showed that AgNPs are not able to cause ODD in Caco-2 cells. Moreover, the AgNPs/PSNPLs co-exposure not only did not promoted oxidative damage but also decreased as the PSNPLs concentration increased, although in a non-significant manner. Surprisingly, ODD has been described to be caused by AgNPs in Caco-2 monolayers (Vila et al., 2018a). Considering that undifferentiated cells are more sensitive than the well-differentiated cells forming an epithelial layer, AgNPs would be expected to trigger greater ODD to undifferentiated cells. On the contrary, $AgNO_3$ induced a dose-dependent ODD increase, reaching higher values after single $AgNO_3$ exposures than when the co-treatment ($AgNO_3$/PSNPLs) was assayed. Nevertheless, ODD followed a significant increasing trend when cells were exposed to low doses of $AgNO_3$ combined with increasing doses of PSNPLs, indicating that NPLs increase the ODD capacity of $AgNO_3$. Still, the effects of the heavy metals/MNPLs interaction regarding the induction DNA damage are poorly understood and more investigation is required to establish

reliable comparisons and draw conclusions. Generally speaking, genotoxic and ODD effects induced by $AgNO_3$ are more considerable than those of AgNPs, independently of the plastic presence. The toxicological impact of soluble metal-containing NPs has been linked to the impact of toxic metal ions released from the NPs (Carriere et al., 2017). This release of metal ions from metal NPs occurs both in dispersion media and in intracellular media. Therefore, ions release should be well characterized when this type of study is planned. Previous results from our group showed that a mere 12% of the AgNPs dissolve into ions after 24 h and this release is stable for the following 72 h. Thus, such contrasting effects in Caco-2 cells as a response to AgNPs compared to $AgNO_3$ effects supports that the impact caused by AgNPs is the consequence of the particles themselves and not depends on the ions release. This also explains that $AgNO_3$ shows, in general, more severe toxicity than AgNPs.

Concluding remarks

Collectively, our data suggest that PSNPLs do not exert evident short-term toxicity, so under the point of view exposed in this Thesis, PSNPLs would be classified as weak toxicants. The two accepted paradigms for NPs toxicology, which are oxidative stress induction and pro-inflammatory potential (Carriere et al., 2017), have not been demonstrated for PSNPLs along the work developed during this Thesis. Nonetheless, as mentioned in previous paragraphs, other cell models should be considered for the MNPLs risk assessment since Caco-2 derived models showed, in general, high resistance to MNPLs effects. Since ingestion is the major route of MNPLs entry to the human body, intestinal models must be included in the set of analyses exploring MNPLs effects. Hence, further exploration of gut models, such as other intestinal clones, must be carried out to find a more sensitive but fully representative cell model of the human intestine. Despite the limitations of the model, we have shown the outstanding ability of MNPLs to internalize. This important finding cannot be neglected since overcoming the intestinal barrier, PSNPLs could reach the bloodstream. Although the precise mechanisms underlying the interaction of MNPLs and blood cells remain to be elucidated, some studies have provided evidence on the deleterious effects of these interplays (Ballesteros et al., 2020; Rubio et al., 2020a), which might trigger systemic disorders. Linked to this, MNPLs potential long-term effects resulting from the bioaccumulation in other organs, remain to be unveiled.

On the other hand, MNPLs uptake as well as their action as nanocarriers, depend on several factors such as size, surface charge, and hydrophobicity (Wright & Kelly, 2017). Therefore, plastic models intended to study MNPLs effects must be standardized with

reference MNPLs. Nevertheless, the buoyant nature of some plastic particles, such as PE and PP, challenge their study using conventional *in vitro* models. Other issues such as the particles' functionalization to mimic environmental samples, the efficiency of fluorescent labels, or the aging processes must also be thoroughly discussed (Busch et al., 2021). So that, further improvement of reference materials is required to overcome these limitations. In addition to this, while previous studies have determined environmental concentrations of other pollutants such as AgNPs, there is an urgent need to assess environmentally relevant MNPLs concentrations, since as discussed in previous lines, concentrations are decisive regarding levels of exposure and potential interactions between contaminants in nature. This is a relevant aspect that would help to elucidate whether the MNPLs interplay with other legacy pollutants temporally reduces toxicity, but long-term exposures decrease or enhance the toxic potential of other contaminants. Admittedly, MNPLs provide a new pathway for hazardous contaminants to enter organisms, accumulate through the trophic web, and reach humans.

Being an unexplored field, the study of MNPLs is challenged by different factors that have been discussed throughout this Thesis. But some authors begin to point out future directions to be taken, for instance, the development of metal-doped MNPLs to facilitate the tracking (Schmiedgruber et al., 2019; Facchetti et al., 2020), body-on-a-chip models (Esch et al., 2015), or *in silico* approaches (Guo et al., 2019; Li et al., 2021). The urgency to cover the vast knowledge gaps has been evidenced by the increasing number of work programs related to the MNPLs' exposure and their impact on human health. An example is the funding call launched by the EU, Horizon 2020 program: *SC1-BHC-36-2020 Micro- and nano-plastics in our environment: Understanding exposures and impacts on human health*, which is expected to answer many of the questions that remain open providing new methodologies and experimental evidence, as well as human biomonitoring data obtained from highly exposed populations, for policymakers to set the basis for the risk assessment on MNPLs.

5. CONCLUSIONS

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According to the objectives raised in the frame of this Thesis and the obtained results, we conclude that:

1. The understanding of both the exposure levels to MNPLs and their associated hazards is still in its infancy. Thus, it is necessary to focus the efforts on in-depth research on these areas to reach a comprehensive knowledge allowing MNPLs risk assessment approaches.

    a. A set of reference materials representing highly abundant polymers with different sizes, shapes, and chemical modifications, needs to be developed and be used for MNPLs safety assessment.

    b. It is urgent to adapt the existing analytical methods for the detection, identification, and quantification of MNPLs in both environmental and biological samples to determine the actual MNPLs occurrence in environmental matrices, organisms, and food sources.

    c. Human exposure to MNPLs occurs through different routes, and although other body entry routes cannot be neglected, from the current data available ingestion is confirmed as the major route of human exposure to MNPLs.

2. Although PSNPLs do not exert severe toxic or genotoxic effects on undifferentiated Caco-2 cells, there is a close interaction between PSNPLs and these cells, which is linked to cellular stress.

    a. y-PSNPLs are rapidly internalized by undifferentiated Caco-2 cells, being localized inside the cell cytoplasm and the nucleus after 24 h of exposure.

    b. PSNPLs accumulate in vacuoles and induce perinuclear alterations and mitochondrial cristae swelling in undifferentiated Caco-2 cells.

    c. The levels of expression of general indicators of stress, namely *HO1* and *HSP70*, increase after the exposure of undifferentiated Caco-2 cells to PSNPLs.

3. PSNPLs' uptake by the cells forming the barriers of the 2D models (Caco-2/HT29 and Caco2/HT29/Raji-B) is very efficient despite their weak capacity to disturb the barriers' integrity or induce toxicity and genotoxicity.

a. y-PSNPLs enter inside the cells forming the barriers and reach cell nuclei in Caco-2/HT29 and Caco-2/HT29/Raji-B models. In addition, y-PSNPLs translocate through the tri-culture barrier and reach the cytoplasm and the nuclei of Raji-B cells.

b. Both Caco-2/HT29 and Caco-2/HT29/Raji-B models show great resistance to PSNPLs. Results are mostly comparable between models, but the addition of M-cells increases the PSNPLs uptake.

4. PSNPLs can act as carriers of AgNPs and Ag^+ modifying their uptake and genotoxic effects.

a. PSNPLs and AgNPs (or Ag^+) physically interact and the resulting complexes enter undifferentiated Caco-2 cells reaching the cell nuclei.

b. Results hint that AgNPs/PSNPLs interaction, at environmentally relevant concentrations of AgNPs, increases AgNPs bioavailability.

c. AgNPs and PSNPLs combined exposure increases the AgNPs genotoxicity when a high concentration of PSNPLs is present in the medium. On the other hand, low concentrations of $AgNO_3$ induce oxidative DNA damage in a PSNPLs concentration-dependent manner.

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6. REFERENCES

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