INSTITUTO POLITÉCNICO DE LISBOA

ESCOLA SUPERIOR DE TECNOLOGIA DA SAÚDE DE LISBOA

ASSESSING THE IMPACT OF NANOPLASTICS IN BIOLOGICAL SYSTEMS: A SYSTEMATIC REVIEW OF *IN VITRO* ANIMAL STUDIES

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Master's in clinical-Laboratory Technology

(this version includes suggestions made by the jury)

Lisbon, 2024

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Lisbon, 2024

Acknowledgments

With profound gratitude,

I wish to express my deepest appreciation to my parents for their constant support and encouragement. Their unwavering belief in me, even during moments of self-doubt, has been instrumental in shaping my academic journey. Their willingness to allow me the freedom to carve my own path has been a source of strength and motivation.

I also wish to express my heartfelt gratitude to my partner, whose presence has been a source of solace and support during moments of stress. Their empathy and willingness to lend a helping hand with daily tasks have alleviated the burdens of balancing work and school, allowing me to focus on my studies with clarity and determination.

Lastly, I am indebted to all my teachers for their guidance and wisdom, which have been fundamental to my personal and professional growth. Their belief in my potential and dedication to fostering learning have inspired me to strive for excellence in all endeavors.

Abstract

Nanoplastics (NPs) pollution has emerged as a critical global environmental challenge, with rising concerns about its impact on human health. However, the adverse health effects of NPs on different organ systems are not yet fully understood. This systematic review consolidates current evidence on the biological effects of NPs in *in vitro* animal models. It further examines the impact of NP size, polymer type, and chemical modifications in toxicological outcomes and offers recommendations for future research. Following the PRISMA guidelines, articles were sourced from Web of Science, Scopus, and Pubmed databases, and analyzed to provide a comprehensive analysis of NPs' toxicity on various cellular lines across multiple organ systems. We observed a higher frequency of negative effects from nanoplastic exposure in cellular lines of the respiratory system, and a lower frequency in the immune and hepatic systems. Notably, positively charged polystyrene (PS) nanoparticles are associated with a higher toxicity potential across all organ systems when compared to their pristine and negatively charged counterparts. Size is also a major factor influencing toxicity, with smaller particles demonstrating a higher frequency of negative effects. The prevalence of studies exposing immortalized monocultures to commercially available solutions of PS NPs, coupled with the scarce number of studies utilizing environmentally relevant NPs, and limited adoption of advanced models, such as organoids and organ-on-chip systems, which better simulate physiological conditions constitutes a significant knowledge gap. Furthermore, the lack of standardized materials and methods complicates comparability of studies and should be addressed in future research.

Keywords: nanoplastics; *in vitro*; cellular lines; cytotoxicity; organ system

Abstract (PT)

A poluição por nanoplásticos (NPs) tem emergido enquanto desafio ambiental global, com preocupações crescentes sobre o seu impacto na saúde humana. Porém, os efeitos adversos dos NPs nos diferentes sistemas de órgãos ainda não são totalmente compreendidos. Esta revisão sistemática consolida as atuais evidências sobre os efeitos biológicos dos NPs em modelos animais *in vitro*. Ademais examina o impacto do tamanho dos NPs, tipo de polímero e modificações químicas nos resultados toxicológicos e oferece recomendações para pesquisas futuras. Seguindo as diretrizes PRISMA, os artigos foram obtidos das bases de dados Web of Science, Scopus e Pubmed, e analisados para fornecer uma análise abrangente da toxicidade dos NPs em várias linhas celulares de múltiplos sistemas de órgãos. Observou-se uma maior frequência de efeitos negativos, devido à exposição a nanoplásticos, em linhas celulares do sistema respiratório, e uma frequência menor nos sistemas imune e hepático. As nanopartículas de poliestireno (PS) com carga positiva estão associadas a um maior potencial de toxicidade, comparativamente a partículas não modificadas ou com carga negativa, em todos os sistemas de órgãos. O tamanho é igualmente um fator importante na avaliação de toxicidade, onde partículas menores apresentam uma maior frequência de efeitos negativos. A prevalência de estudos com exposição de monoculturas imortalizadas a soluções comerciais de PS NPs, aliada à escassez de estudos com NPs ambientais, e à limitada adoção de modelos avançados, como organoides e sistemas de órgãos-em-chip, que melhor replicam as condições fisiológicas, constitui uma considerável lacuna no conhecimento científico. Finalmente, a falta de padronização de materiais e métodos complica a comparabilidade dos estudos e deve ser abordada em pesquisas futuras.

Palavras-chave: nanoplásticos; *in vitro*; linhas celulares; citotoxicidade; sistema de órgãos

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Abbreviations list

AOP – Adverse outcome pathway
A-PS – Amine-modified polystyrene
C-PS – Carboxyl-modified polystyrene
DMSO – Dimethyl sulfoxide
DNA – Deoxyribonucleic acid
ECHA – European Chemicals Agency
ER – Endoplastic reticulum
FBS – Fetal bovine serum
HCS – High content screening
LDH – Lactate dehydrogenase
MMP – Mitochondrial membrane potential
MNPs – Micro- and Nanoplastics
MOF – Metal-organic framework
MPs – Microplastics
NPs – Nanoplastics
PET – Polyethylene terephthalate
PE – Polyethylene
PC – Polycarbonate
PLA – Polylactic acid
PMMA – Polymethyl methacrylate
PP – Polypropylene
PS – Polystyrene
PTFE – Polytetrafluoroethylene
ROS – Reactive oxygen species
Sa-PS – Sulfonic acid-modified polystyrene
S-PS – Sulfate-modified polystyrene
UN – United Nations
UPR – Unfolded protein response
uPS – Unmodified polystyrene
wPET – Weathered polyethylene terephthalate
wPS – Weathered polystyrene

1. Introduction

Synthetic items, such as micro- and nanoplastics (MNPs) exhibit resilience, low weight, resistance to deterioration, robustness, affordability, and adeptness in insulating against heat and electricity, thus having widespread utilization worldwide[1–3]. Its manufacturing has experienced exponential growth over the last 7 decades, reaching a staggering 400.3 million tons in 2022[4]. The European Chemicals Agency (ECHA) estimates that, each year, around 42000 tons of intentionally added microplastics end up in the environment[5]. MNPs are small polymeric particles primarily distinguished by the scale of their dimensions[6]. According to some authors, microplastics (MPs) have dimensions between 1 µm and 5 mm[7], while others classify them as being under 5 mm[8,9]. As for nanoplastics (NPs), they are mostly either considered to range from 1-100 nm[10,11] or from 1-1000 nm[7,8], depending on the author or guidelines. However other definitions for plastics according to different size distributions continue to exist in the current literature[12–14]. The definition and classification criteria for NPs have been reviewed, discussed, and proposed, remaining subject to refinement as the field progresses[15,16].

In 2015 nearly 4/5th of the plastic waste generated ended up in either landfill or the environment, while only 9% was recycled[17], and it is suggested that by 2050, another 33 billion tons of plastic will have accumulated on the planet[18]. When combined with the awareness of plastic's established and potential toxicity, these statistics sound a compelling alarm, urging immediate action to mitigate pollution across nations. NPs are particularly problematic due to their small size, which enables them to travel further and more widely in the environment, raising concerns about their pervasiveness. Accurately assessing their quantities in the environment is challenging due to current technological limitations, making it difficult to monitor their impact effectively[19]. Furthermore, their diminutive dimensions allow them to interact with biological systems at organic and cellular levels. These particles can cross the cytoplasmic membrane of cells and interact with cellular organelles, leading to genotoxicity, cytotoxicity, or other forms of cellular damage[20]. Modifications to their chemical surface, created by weathering processes or present since manufacturing, can enhance their potential for cellular damage, with certain surface chemistries being more harmful than others[21]. Particularly, the capability to disrupt metabolic processes raises significant concerns about their impact on human health. Understanding the effects of NPs on biological systems is therefore essential for assessing their potential hazards and informing regulatory decisions.

This project aims to perform a systematic review to map and synthesize the available evidence on the effects of NPs in biological systems, focusing on *in vitro* animal studies. In this study, NPs are considered based on the metric scale, defined as particles below 1000 nm. In terms of sustainable development goals, established by the United Nations (UN), it can be applied to quality education (goal 4), responsible consumption and production (goal 12), good health

and well-being (goal 3), clean water and sanitation (goal 6), life below water (goal 14) and life on land (goal 15), with emphasis on the latter four. To our knowledge, no comprehensive review of this kind exists, highlighting the importance of consolidating the available information, identifying knowledge gaps, and guiding future research.

2. State of the art

2.1. Plastics – an overview

Plastics play a crucial role in various industries, including transportation, food, healthcare, and energy, contributing significantly to modern life. The term "plastic" denotes the ability to be shaped or molded, and as a noun, it colloquially refers to a category of synthetic, organic, high-molecular-weight polymeric materials. Regarding their origin, most plastics are synthesized by using fossil feedstock like petroleum and natural gas[22].

Bakelite, developed in 1907 by chemist Leo Baekeland and used as an electrical insulator, is a thermosetting phenol formaldehyde resin and the first entirely synthetic plastic[23]. The first known research on MPs, based on the smallest plastic debris, was reported by E. J. Carpenter and K. L. Smith in the year 1972[24]. Meanwhile, the first research referring to nano-scale plastic particles/debris as potential hazardous contaminants appeared in 2010[25], even though nanosized plastic particles were already designed, manufactured and sold prior to this date[26].

2.2. Sources of NPs

Over 80% of plastic debris originate from land-based sources, with less than 20% originating from the sea-based activities[27,28]. Broadly, sources of NPs can be categorized into primary and secondary (Figure 1)[29], contingent on their formation mechanisms[30,31].

2.2.1. Primary source NPs

Primary NP particles are intentionally manufactured in their size as raw materials[10] widely used in cosmetics formulations, personal care products containing scrubs and abrasives, paints, industrial abrasives, industrial air blasting, filaments for 3D printing, and drug vectors. The NPs originating during manufacturing or utilization of plastic products, such as tire abrasion, road markings, marine coatings, textiles, and laundry are also included in this category[32,33].

2.2.2. Secondary source NPs

Secondary NP particles can result from the weathering and fragmentation of larger plastic items[28,34,35]. This breakdown may occur through several processes, namely, chemical (photo-degradation), physical (mechanical abrasion) and biological (biodegradation by microbial species)[36] . The presence of secondary NPs in the environment is proportional to the population, number of vehicles and laundering operations[37].

Figure 1 - Common sources of primary and secondary NPs. Adapted from: Sharma, S. et al. (2023).

2.3. Environmental fate

NPs, being extremely small plastic particles, exhibit various behaviors in different environmental media:

i. **Air**: NPs can be transported through the air from sources like industrial activities[38,39], traffic[40], or plastic particles from textiles[41]. Once in the air, they can travel over long distances and potentially settle on surfaces or be deposited into bodies of water, soil, or ecosystems through atmospheric deposition[42].

ii. **Water**: NPs can enter water bodies through various pathways such as runoff from land[43], wastewater discharge[44], or direct release[45]. In aquatic environments, they can undergo processes like suspension, settling, aggregation, or adhesion to other particles[46,47]. NPs can also interact with organisms in water, potentially leading to ingestion by aquatic species.

iii. **Soil**: NPs can reach soil through various routes, including runoff from land, application of plastic-containing products like mulches or fertilizers, or direct deposition[48]. Once in the soil, they can undergo processes like adsorption to soil particles, transportation through soil pores, or interaction with soil microorganisms. NPs in soil may also affect plant growth and soil health[49,50].

iv. **Biota**: NPs can be ingested by different organisms and affect species at a higher trophic level due to being retained and trophically transferred across the food chain[51]. Once ingested, NPs can potentially accumulate in tissues and organs, leading to adverse effects such as inflammation, oxidative stress, or disruption of physiological processes[52]. NPs can also transfer through food webs, potentially impacting entire ecosystems[53].

2.4 Exposure pathways

Most articles published to date have focused on MPs' presence in air, food, beverages, and cosmetics, with only a few articles on NPs specifically. Nevertheless, one can assume that the

exposure pathways will be fairly similar, with differences arising mostly in terms of the values considered. Therefore, it is deemed relevant to describe the three main pathways of MNPs into the human body, according to relevant studies in the sections below.

2.4.1. Inhalation

Inhalation is considered one of the main routes into the human body with NPs originating from industrial dust[54], car tires[55], paints and textile fibers released from synthetic fabrics[56]. Studies estimating how much humans inhale considered values up to 48000 particles/day[57]. In one study it was found that the air outside already contained 500 detectable MNPs per square meter[58], and although that already seems a high amount, multiple studies demonstrated that the amount of MNPs present in the air is higher indoor compared to outdoor[59].

2.4.2. Ingestion

Ingestion is considered the main route of exposure to MNPs. The detection of MNPs in stool confirms their ingestion[60]. Many studies have evaluated the presence of plastic polymers in different food sources, with a high amount focusing on drinking water and seafood. Regarding drinking water, the presence of MNPs may come from pipes, filters, or bottles[61]. In fact, in over 90% of the cases tap water was found to contain MNPs, more frequently polyethylene (PE), polystyrene (PS), and polyethylene terephthalate (PET), although the concentration at which they were found varied between studies[56]. Studies with bottled water also presented a high variability in results, but the concentration of MNPs was significantly higher than in tap water, with PET and polypropylene (PP) being predominant[62].

2.4.3. Dermal contact

Dermal contact might very well be the least acknowledged route of exposure, however one that must be considered due to the potential for NPs to cross dermal barrier[63]. Exposure is predicted to happen due to use of personal care products, as there was a higher content of MNPs in the hands and faces of make-up users[64]. Several European countries have already banned intentional use of MPs in cosmetics, but the size definition is still not consensual leaving gaps in the legislation[65].

2.5. Properties of NPs

The physical characteristics of NPs primarily relate to their size, shape, and color, while their chemical attributes involve factors such as chemical composition, crystallinity, surface properties, presence of toxic metals, and additives adhered to their surfaces[66].

2.5.1. Size

As mentioned previously in this paper, there is currently no consensus on the definition of plastics according to size. Nonetheless, the most used format is 1-5 µm for MPs and 1-1000 nm for NPs (Figure 2)[67].

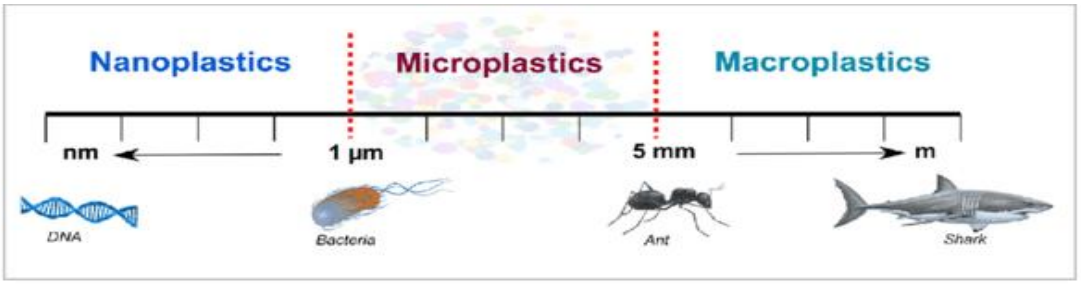

Figure 2 - Plastics' classification according to size, with objects shown for comparison at size delimitations. Source: Ramkumar, Mu. et al. (2022)

2.5.2. Shape

NPs come in a wide range of shapes, such as fragment, foam, paint, pellet, foil, sphere, fiber, film, line, bead, flake, sheet, granule, and nurdle[68]. Primary source NPs typically have more regular shapes, while secondary source NPs can present a more extensive variation, due to mechanical abrasion and chemical weathering[69]. The most common are represented in Figure 3[70].

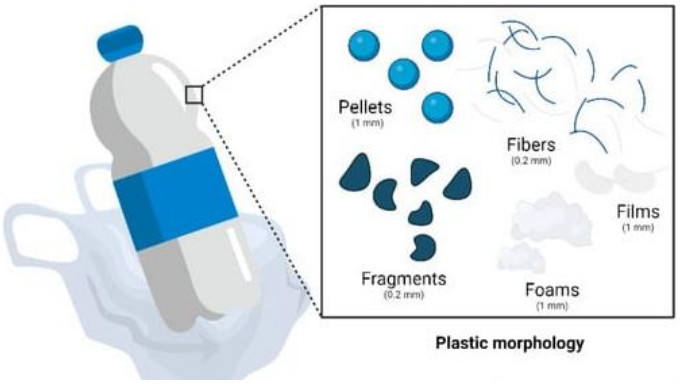

Figure 3 - Plastics' typical morphologies. Image created in BioRender.com, source: Rashed, A. et al. (2023).

2.5.3. Color

Various colored MNPs have been documented including red, orange, yellow, brown, tan, off-white, white, grey, blue, and green[69,71]. Still regarding light transmission, they might also be transparent (PP) or opaque presenting (PE)[68].

2.5.4. Chemical additives

Plastic toxicity is associated with built-in chemicals, used during plastic production to provide the final product with characteristics which allow it to be more resistant to degradation, be it

physical, chemical, or biological[72]. These additives can be distributed in a variety of categories, for example, plasticizers, biocides, flame retardants, stabilizers, oxidants, and organic pigments[45].

2.5.5. Chemical composition

Plastics are comprised of a long chain of polymers, which can be distinguished according to their chemical composition. Some common types of polymers and their uses are as follows:

i. PE is one of the most widely used plastics due to its versatility and low cost. It is used in packaging films, bottles, containers, pipes, and various household products[73].
ii. PP is known for its strength, heat resistance, and flexibility. It is used in packaging, automotive parts, textiles, medical devices, and household products[73].
iii. PS is lightweight and can be molded into various shapes, making it suitable for packaging materials, disposable cups, food containers, and insulation[73].
iv. PET is commonly used in the production of beverage bottles, food containers, and polyester fibers for textiles and clothing[74].
v. Polytetrafluoroethylene (PTFE), commonly known as Teflon, is valued for its non-stick and heat-resistant properties. It is used in cookware, electrical insulation, and as a lubricant[75].
vi. Polycarbonate (PC) is a transparent and durable plastic used in eyeglass lenses, safety goggles, CDs, DVDs, and electronic device housings[76].
vii. Polymethyl methacrylate (PMMA), commonly known as acrylic or acrylic glass, is a transparent thermoplastic with excellent optical clarity and weather resistance used in transparent applications like windows and lenses, as well as dental and medical implants due to its biocompatibility[77].
viii. Polylactic Acid (PLA) is a biodegradable and renewable thermoplastic derived from renewable resources such as corn starch or sugarcane. It is known for its biocompatibility and low toxicity, being employed in biodegradable packaging, 3D printing, medical implants and textiles offering eco-friendly alternatives[78].

2.5.6. Crystallinity

Crystallinity is ordered structural linkages that influence the plastics' density, permeability, and swelling behavior[79]. This property will suffer changes according to atmospheric conditions[80].

2.5.7. Surface properties

The surface property primarily involves surface area and surface chemistry. On one hand, with the decrease in particle size there is an increase in surface area which can lead to adherence of other potentially toxic compounds. On the other hand, surface chemistry affects a particle's interaction with cells, facilitating or hindering its uptake[81,82]. Examples of these different surface chemistries are carboxyl-, amine-, and sulfate-modified PS plastics used in

various toxicological studies.

2.6. Methods of detection

Numerous techniques have been utilized to investigate the physical-chemical properties of NPs (Figure 4).

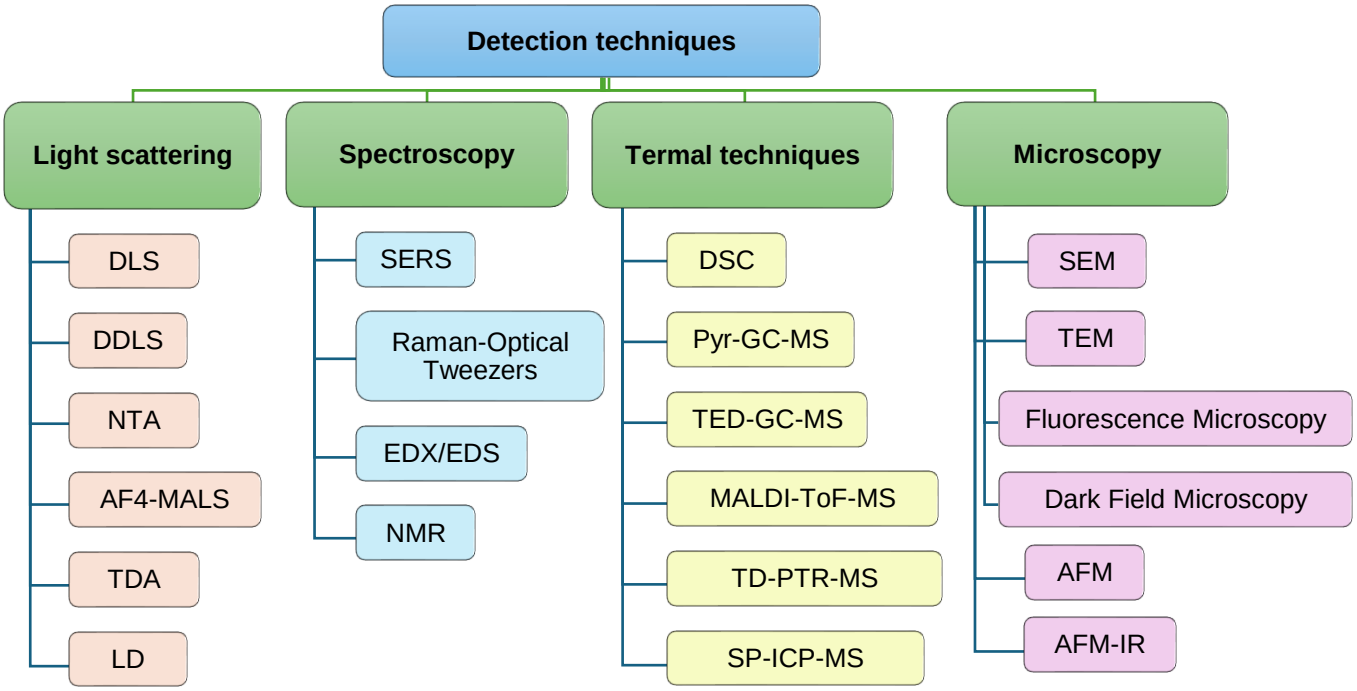

*Figure 4 - Detection and quantification techniques for NPs. **AF4-MALS** – asymmetrical flow field-flow fractionation with multi-angle light scattering; **AFM** – atomic force microscopy; **AFM-IR** – infrared atomic force microscopy; **DDLS** – depolarized dynamic light scattering; **DLS** – dynamic light scattering; **DSC** – differential scanning calorimetry; **EDX/EDS** – energy-dispersive X-ray spectroscopy; **LD** – laser diffraction; **MALDI-ToF-MS** – matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; **NMR** – nuclear magnetic resonance; **NTA** – nanoparticle tracking analysis; **Pyr-GC-MS** – pyrolysis gas chromatography-mass spectrometry; **SERS** – surface-enhanced raman spectroscopy; **SEM** – scanning electron microscopy; **SP-ICP-MS** – single particle inductively coupled plasma mass spectrometry; **TDA** – taylor dispersion analysis; **TD-PTR-MS** – thermal desorption proton-transfer reaction mass spectrometry; **TED-GC-MS** – thermal extraction desorption gas chromatography-mass spectrometry; **TEM** – transmission electron microscopy.*

While feasible for identification, quantification and characterization, these methods often face limitations such as size constraints, high detection limits, and interference from organic compounds in the matrix[83]. Factors like small size, diversity, and low environmental levels, along with the presence of additives and biofilms, can affect particle stability and chemical analysis[84]. These challenges significantly impact the detection of nano-sized plastic particles, underscoring the need for enhanced sample preparation and analytical methods across various matrices. An overview of the different techniques with advantages and disadvantages is presented in Annex 1.

2.7. Health impacts

The complex process of NPs producing toxic effects is influenced by various factors such as the physical and chemical properties described prior, exposure time, and additives. Not only are NPs inherently toxic, but they also serve as carriers for numerous pollutants to infiltrate

biological tissues and organs[85]. This section briefly summarizes the primary effects and mechanisms of toxicity of NPs in current experimental models, including cells, organoids, and animals (Figure 5).

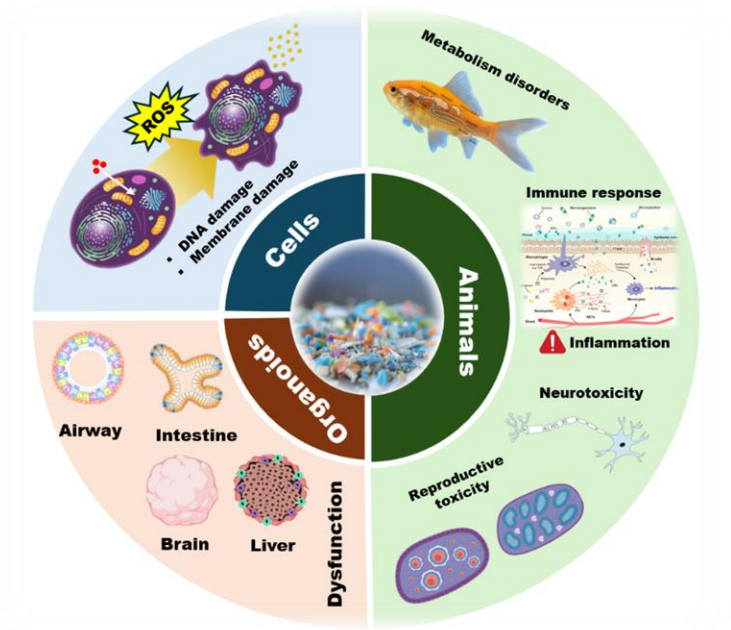

Figure 5 - Toxicity mechanisms of NPs on cells, organoids and animal models.

2.7.1. Factors influencing the toxicity of NPs

The chemical and physical properties of NPs, such as molecular structure of polymers, size, charge, wettability and roughness, combined with potential sorption of other pollutants are just some of the many factors which may influence toxicity[86]. The current knowledge of the role of these factors is reviewed below, with emphasis on pristine plastic particles:

i. **Size and concentration:** NPs toxicity acts in a concentration-dependent manner, being directly proportional to it and inversely proportional to size[87–89], especially because larger particles are less likely to enter cells.

ii. **Shape:** randomly shaped PS fragments with rough and sharp morphologies cause more harmful physical effects that prompt cell toxicity in comparison to their spherical counterparts[90].

iii. **Surface charge:** surface charge is also known to influence toxicity[91], presumably due to their interaction with the cytoplasmic membrane, facilitating their uptake, and other organelles through chemical bounding.

iv. **Weathering process:** Interaction with light during the weathering process can increase the concentration of free radicals in plastic, subsequently increasing its toxicity[92]. However, certain studies showed that the weathering process reduced generation of reactive oxygen species (ROS) instead, being less toxic than their pristine counterparts[93].

2.7.2. Toxic effects in cell experiments

Cytotoxicity of NPs varies with cell type, particle size, dose or concentration, charge, exposure time, polymer type and additives, causing cell death mainly by oxidative stress, membrane damage and DNA damage[94].

2.7.3. Toxic effects in human organoid experiments

Human organoids are the latest development of *in vitro* models and have been used as new exposure models to overcome limitations of static cell culture exposures when it comes to buoyancy. Buoyancy of low-density particles makes static cell culture models not suitable for toxicity assessment since particles stay at the surface of the media culture, having no contact with the cells at the bottom of the plate[95]. In studies utilizing organoid models for MNP exposure, many report functional disorder as an effect associated with said exposure[45,96–98].

2.7.4. Toxic effects in animal experiments

Animal experiments have shown that exposure to MNPs can have negative effects on different organ systems causing, for example:

a. **Metabolic disorders** due to liver and intestinal damage, altering lipid digestion[99], disrupting the energy metabolism[100], inhibiting the activity of digestive enzymes in fish[101] and creating an imbalance of intestinal flora[102];
b. **Inflammation** by triggering an immune response in the body, demonstrated by the increased secretion of pro-inflammatory cytokines and upregulated expression of inflammatory proteins[103].
c. **Neurotoxicity**, which can lead to learning and memory dysfunctions[104], as well as abnormal behavior of nematodes, crustaceans, fish and mammals[105]. Inhibition of acetylcholinesterase stands as the most reported neurotoxic effect caused by MNP exposure thus far[85].
d. **Reproductive and developmental toxicity**, reducing the quality of sperm[106] and oocytes[107] from mice and therefore their fertility and altering the number of live births and body weight of pups in groups treated with PE[108].

2.8. Mitigation strategies

Mitigation strategies must constitute a joint effort, achieved through legislation, technical development, and social awareness or education[109]. Most interventions to prevent plastic leakage are related to removing MNPs from wastewater infrastructure[110], therefore improvements on the infrastructure and management have the potential to reduce environmental plastic pollution. Many studies have reviewed different strategies to reduce or mitigate the presence of MNPs, namely in wastewater treatment plants[80,111–113]. The list of

treatment options is long but not without its own limitations. An example of commonly used and novel treatment strategies (Figure 6) is presented below.

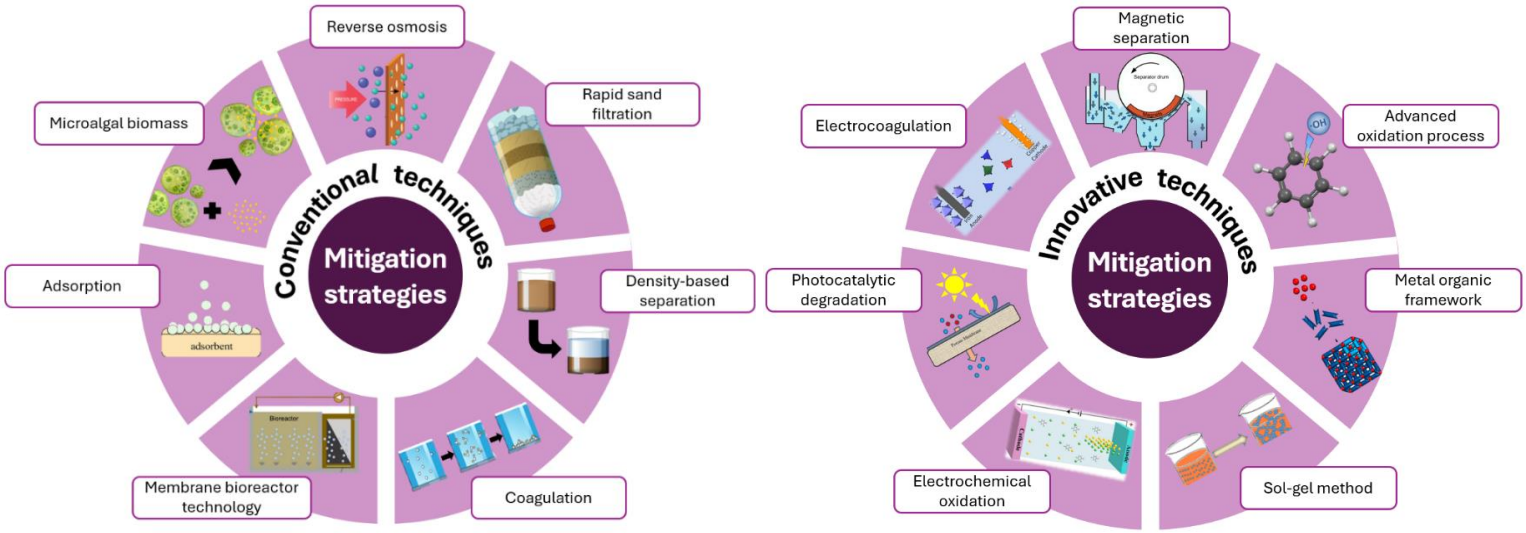

Figure 6 - Representation of conventional and innovative treatment techniques for removal of MNPs in wastewater.

These techniques have proven capable of removing MNPs to a certain degree, but studies aimed at the removal of NPs specifically are now being the focus of the scientific community. Methods like filtration, membrane separation, and coagulation, are available and striving for the removal of NPs in water[114]. Even though treatment processes are available, their effectiveness is highly variable (Figure 7), and they are not without consequences, such as the presence of more organic pollutants mixed with NP contaminants in the wastewater discharge[115]. Despite the development of various procedures for identification, quantification and separation of NPs from the matrix[116], some methods are not suitable due to the difficulty associated with retaining such small particles[117], which makes prevention the best way to mitigate potential risks.

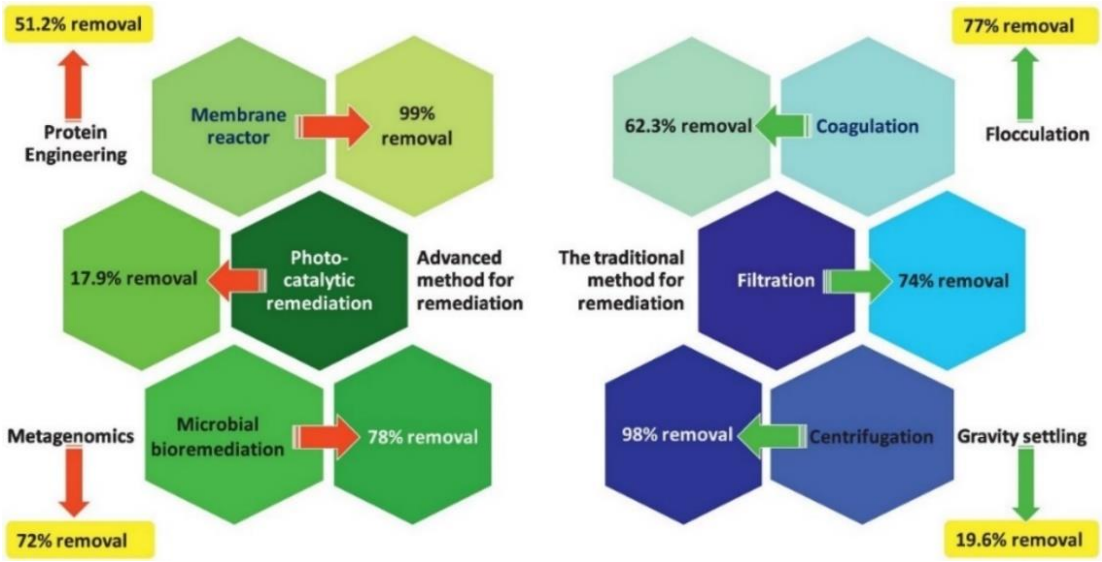

Figure 7 - Effectiveness of various removal strategies for NPs in wastewater. Source: Keerthana Devi, M. et al. (2022)

2.9. Knowledge gaps

While research on NPs has surged in recent years, critical knowledge gaps remain, spanning various aspects of their sources, fate, transport, interactions with organisms, and potential risks to ecosystems and human health.

2.9.1. Type of NP (polymer, size, shape)

To date, most toxicity studies on NPs have focused on commercially available primary PS NPs, accounting for over 90% of reports according to Pelegrini et al. (2023)[6]. Meanwhile only a few studies investigated primary NPs made from a limited selection of other polymers such as PET and PP. Many authors have pointed out that these conditions do not reflect environmental MNPs[6,12,118–123] since PS makes up only around 6,1% of globally produced polymers and a larger proportion of plastics found in the environment are composed of PE and PP[124]. These commercially available solutions have further drawbacks such as being in a solution with additives that might cause artifacts in toxicity studies, having a round shape and a very controlled size range[6,122,125].

2.9.2. Duration of exposure

Most toxicity studies are high-dose and short-term, which is not aligned with the belief that humans are chronically exposed to low concentrations of NPs throughout their lives[6,12,118,126]. For that reason, reports evaluating genotoxicity, mutagenesis, and carcinogenicity of NPs are lacking information, mostly limiting the study to screening genotoxicity via comet assay in monoculture cell lines[94,127–129]. The mutagenesis and carcinogenesis studies required are time-consuming and resource intensive but required to deal with the growing production and, consequently, pollution caused by plastics.

2.9.3. Cell culture

The lack of environmental relevance for NP polymer choice was mentioned in the section above, but it stands together with a lack of physiological relevance seeing that many *in-vitro* studies are using monocultures of immortalized cell lines. These studies are useful to identify and characterize the intrinsic toxicity of substances and provide data for *in vivo* exposure, but do not attain the structural and functional complexity of *in vivo* tissues[6]. Moreover, studies often employ the conventional static methodology for toxicity assessment[130,131]. This strategy can lead to inaccurate results considering the concentration of particles in contact with the cells might differ from the chosen concentration for the protocol due to a phenomenon called buoyancy in which low density particles stay in suspension[95,119]. Several approaches can be used to overcome buoyancy issues such as seeding on inverted coverslips[132], sealing a well with silicone gaskets and inverting the plate[133], seeding the basolateral side of the Transwell®

inserts[134] or using the air-liquid interface system[135] (through aerosol generation). Other options available are to use a dynamic flow condition or microfluidic chips[6,136–138].
In addition, more complex models such as the Caco-2/HT29-MTX co-culture, or 3D models are less studied but offer significant insight for their better representation of tissue conditions[139,140].

2.9.4. Concentration of NPs

The concentration of NPs or applied dose is often deemed unrealistic, given our knowledge of NP presence in the environment[118,121,141]. Additionally, it is also said that reports of environmental presence might be underestimating the number of NPs present due to the limitations of currently employed analytical methods to detect and characterize small nanoscale particles[30,142].

2.9.5. Detection methods and standardization

The lack of tools to characterize NPs both physically and chemically simultaneously presents a challenge to acquire all the information needed for a risk assessment analysis[143]. Standardization of sampling and analysis (detecting and characterizing MNPs) is one of the most pointed out limitations by researchers when it comes to our knowledge of MNPs, especially NPs[11,12,118,121,126]. To bridge this gap, it is fundamental to, first and foremost, create a definition and classification of NPs that is universally acknowledged and accepted.

2.9.6. Study design

Regarding study designs, there is a lack of relevant physiological exposure, for example, simulated gastrointestinal digestion or incubation in lung fluid[119], as well as risk assessment[36]. Moreover, the typical study design uses hypothesis testing to produce single values, such as no/lowest observed effect concentrations is not suitable for modeling risk[36].

2.9.7. Others

There is no direct measurable evidence of human health risk, since the toxicity mechanisms in humans are not fully understood and the process of MNP elimination in the human body lacks sufficient information[12]. Several reviews have been made on the interaction of MNPs with contaminants[144–146], but few with MNPs of environmental origin[147].
Fluorophores, often used to track NPs distribution within organs or cells can leach out[148,149] or be pH sensitive[150] causing difficulty in accessing information for lysosomes. Also, lactate dehydrogenase (LDH), frequently used as a marker of membrane integrity can potentially adhere to the corona formed by NPs while in culture medium, creating a distortion in the results[151,152].

Lastly, a focus on marine and freshwater environments has left a knowledge gap to be filled when it comes to other fields like air and soil[123,153].

3. General objectives and methodology

3.1. Review Question

The aim of this research was to perform a broad systematic review to map and synthetize the available evidence on the effects of NPs in biological systems, namely those assessed by *in vitro* animal studies and describe, whenever possible, the different impacts of these substances according to their size, polymer type, and by cell type.

For such the PEO acronym was be used as follows:

P – cell lines (animal or human);

E – exposed to NPs (plastic particles below 1 µm);

O – studies addressing the effects on cellular parameters (eg.: genotoxicity, cytotoxicity, hazard, damage, viability).

3.2. Objectives

The main objective was to summarize the available evidence on the biological effects of NPs in *in vitro* (cellular lines) animal models.

As for specific objectives, this study intended to describe and organize these effects according to NP type, particle size, exposure concentration and other potentially mentioned factors that can influence internalization/uptake of NPs by the cells/systems. Moreover, we aimed at assessing the methodological quality of the studies to judge the overall strength of evidence.

3.3. Methodology

Studies were searched through PubMed, Scopus and Web of Science databases, using terms related to NPs (e.g., nanoplastics, NPs), their effects (e.g., genotoxicity, cytotoxicity, hazard, damage) and the studies' type (animal model, *in vitro*, cellular line), combined with the boolean operator "AND" or "OR". The specific search string used was as follows: TITLE (nanoplastics) AND TITLE-ABS (damage OR effects OR hazard OR toxicological OR toxicity OR genotoxicity OR cytotoxicity) AND TITLE-ABS ("cell line" OR "cellular line" OR "cell culture" OR "*in vitro*").

For the systematic review at hand, the following inclusion criteria, according to PEO, were considered:

P) any animal or human cell culture model;

E) exposure to NPs at a single concentration or concentration range;

O) biological effects of NPs (e.g. genotoxicity, cytotoxicity, harm and damage) which must include one or more of the following parameters: cell viability, cell proliferation, cytoplasmic membrane integrity, barrier integrity, oxidative stress, apoptosis, necrosis, inflammation, DNA damage, lysosomal damage, autophagy dysfunction, ATP levels, or other bioenergetic parameters.

Selection was restricted to English language articles. *In vivo* studies, observational studies, reviews, commentaries, studies focusing on MPs and those without available data for extraction were excluded.

Two independent reviewers have conducted all steps of the review, and in instances of disagreement, a third reviewer provided the deciding judgement. The first step was the screening based on title and abstract reading, followed by the eligibility step with a full reading of the selected articles. Articles were excluded based on the following prioritization criteria:

1) Not about plastics (e.g. other nanofibers)
2) Different population (e.g. plant or microbial studies)
3) Only MPs or size of particles not disclosed
4) Limited to co-exposure
5) No toxicity analysis or measurement of damage/harm induced by the NPs.

References were managed through Rayyan and MENDELEY and data extraction was performed using a pre-formulated spreadsheet in Excel® with the following information: author names, year of publication, country, cellular line, NP polymer type and size, concentration, time of exposure, methodology/effects evaluated, main findings/results and DOI. The main findings included primary outcomes related to genotoxicity and cytotoxicity, which were measured through parameters such as cell viability, oxidative stress, inflammation, DNA damage, apoptosis, and cytoplasmic membrane damage. Secondary outcomes, which also pertain to cytotoxicity and cellular damage, included measures of cell proliferation, barrier integrity, lysosomal damage, autophagy dysfunction, ATP levels, and other bioenergetic parameters.

The methodological quality of the selected studies was assessed using a checklist by Chierrito et al. (2019)[154] and visually represented through the robvis tool[155] as risk of bias. For the visual representation, each parameter was rated as present, not present, unclear, or not applicable, and transformed into low, high, unclear, or no information, respectively.

A narrative synthesis of the studies was performed, together with tables and figures of the main findings pertaining to toxicity, inflammatory damage, and other hazardous effects, from the included studies. To provide a clear and structured overview of the findings, the included studies have been categorized according to the organ systems they focus on. These were then analyzed according to NP type (polymer and modification) and, when deemed necessary, cellular model. This approach allows for a more detailed and organized presentation of the results, highlighting the specific effects and insights related to each system.

3.3.1. Data Visualization Methods

A violin plot was generated using Microsoft Power BI to illustrate data distribution. Heatmaps were constructed in Microsoft Excel through conditional formatting, employing a gradient of dark teal (Accent 1) for 0%, white for 50%, and dark red for 100%. Percentages were calculated

as the ratio of reported negative effects to total reported effects. The conditional formatting colors were subsequently replicated in Microsoft PowerPoint to develop a corresponding scale, designed with 25% incremental size increases for improved visual distinction. Additional graphics were created using Excel's built-in visualization tools.

4. Results

4.1. Literature search, screening and eligibility

The initial search yielded a total of 434 articles from various databases. Specifically, 183 articles were identified from PubMed, 127 from Scopus, and 124 from Web of Science. After removing duplicates, 237 unique articles remained. The selection process followed the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) 2020 guidelines[156] and is illustrated in the flowchart below (Figure 8). Out of the 237 unique articles, 114 were excluded during the title and abstract screening due to irrelevance to the research question and one article could not be retrieved. The full texts of the remaining 123 articles were then assessed for eligibility. During this stage, 27 articles were excluded for reasons such as lack of information, not about NPs, reviews, wrong study design, and limited to co-exposure protocols. Ultimately, 96 studies met the inclusion criteria. From a manual search through studies' citations, 12 studies not represented in this search string also fit the criteria and were therefore added to the qualitative synthesis, encompassing a total of 108 articles.

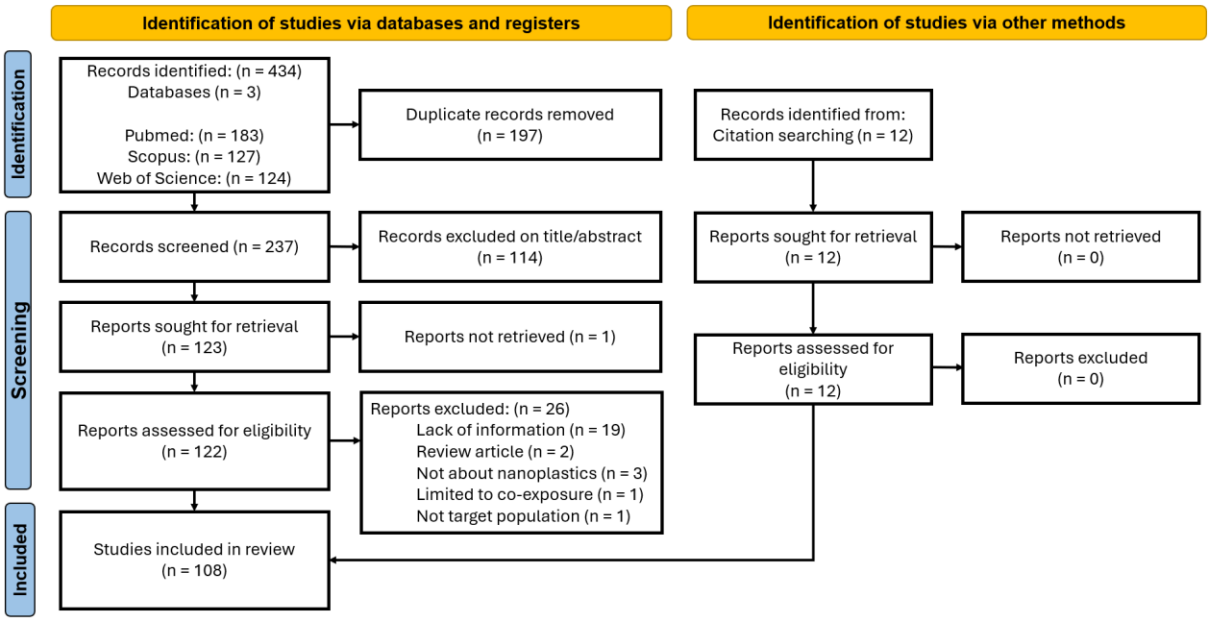

Figure 8 - Flow diagram describing the study selection process and number of studies at each stage according to PRISMA 2020 guidelines.

According to the affiliation of the corresponding author, the included studies were published by more than 20 different countries, with the majority being conducted in China (n=58; 54%), followed by Spain and Italy (14% and 9%, respectively). The studies were published between 2018 and 2024, with a higher prevalence (85%) in the last 4 years (Figure 9). It is worth noting that the last year, corresponding to 2024 only included one month at the time of search, indicating that the number of studies that will be published during this year (2024) will likely surpass the previous year by more than, or close to, twice the amount, once again

demonstrating an increasing scientific interest driven by environmental, health, and regulatory concerns.

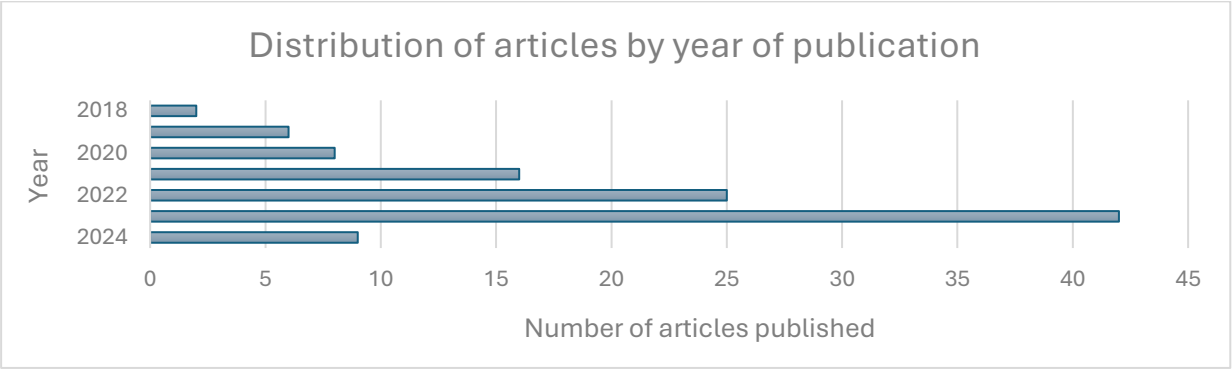

Figure 9 - Distribution of articles by year of publication.

The main NP polymers mentioned in the studies were PS, primarily unmodified or pristine PS (uPS; 58%) but also carboxyl-modified (C-PS; 14%) and amino-modified PS (A-PS; 13%), and PET with a much lower representation of 3%. In total, PS NPs, including all modifications and weathering processes accounted for 90% of NPs described in these studies. NPs' average size in solution varied between 15 nm and approximately 531 nm, and although NPs of higher dimensions were described in two studies[43,157], they constituted a minority in solution.

For this analysis agglomerates were not considered and NP sizes were categorized into specific ranges: <20, [20-40[, [40-100[, [100-150[, [150-200[, [200-300[, [300-500[, and ≥500 nm, with a greater prevalence fitting into the [40-100[category (38%), followed by its closest categories [100-150[and [20-40[(24% and 17%, respectively), as represented in Figure 10.

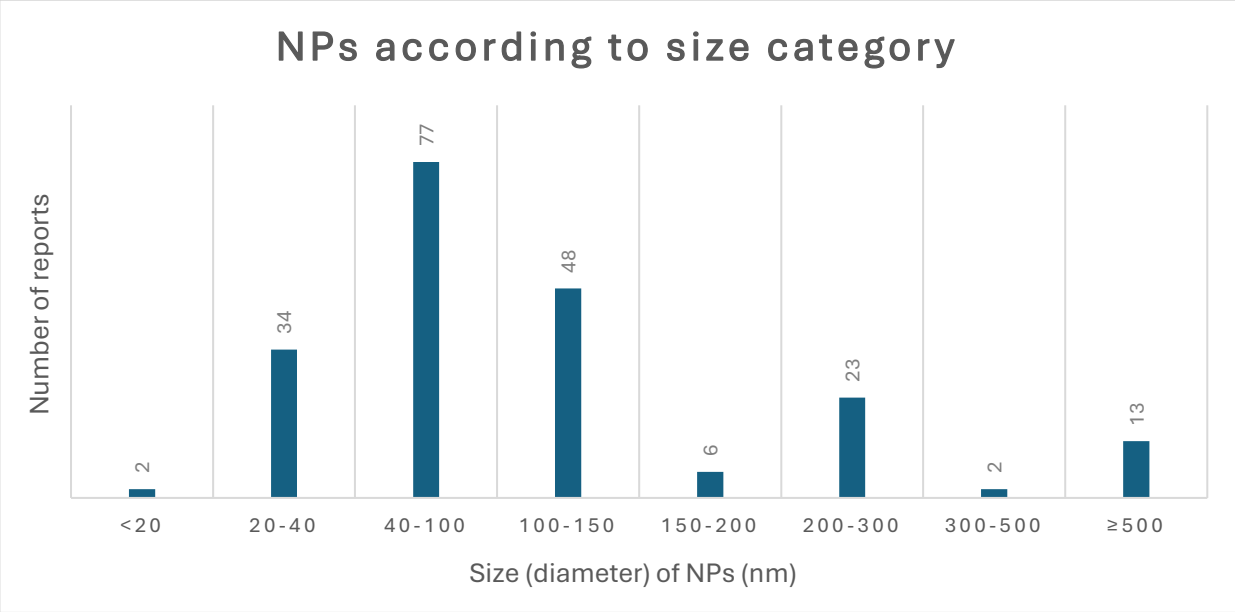

Figure 10 - Distribution of NPs according to size categories.

4.2. Quality assessment

The risk of bias of the included studies was evaluated using the robvis tool, which provides a visual representation of the bias across different domains. The results of this assessment are summarized in Figure 11. Overall, the studies demonstrated low to moderate risk of bias, with over 60% exhibiting low risk. The most common concerns pertaining to risk of bias in this review were pH of growth medium, with only one study reporting it, number of passages with about 12% of studies reporting it, followed by frequency of change of growth medium and confluence with less than 40% of the studies making mention of it in their methods. The substance used for cell collection was mentioned by some studies (≈36%), but the remaining studies made no mention of it or were unclear regarding the concentration/percentage used.

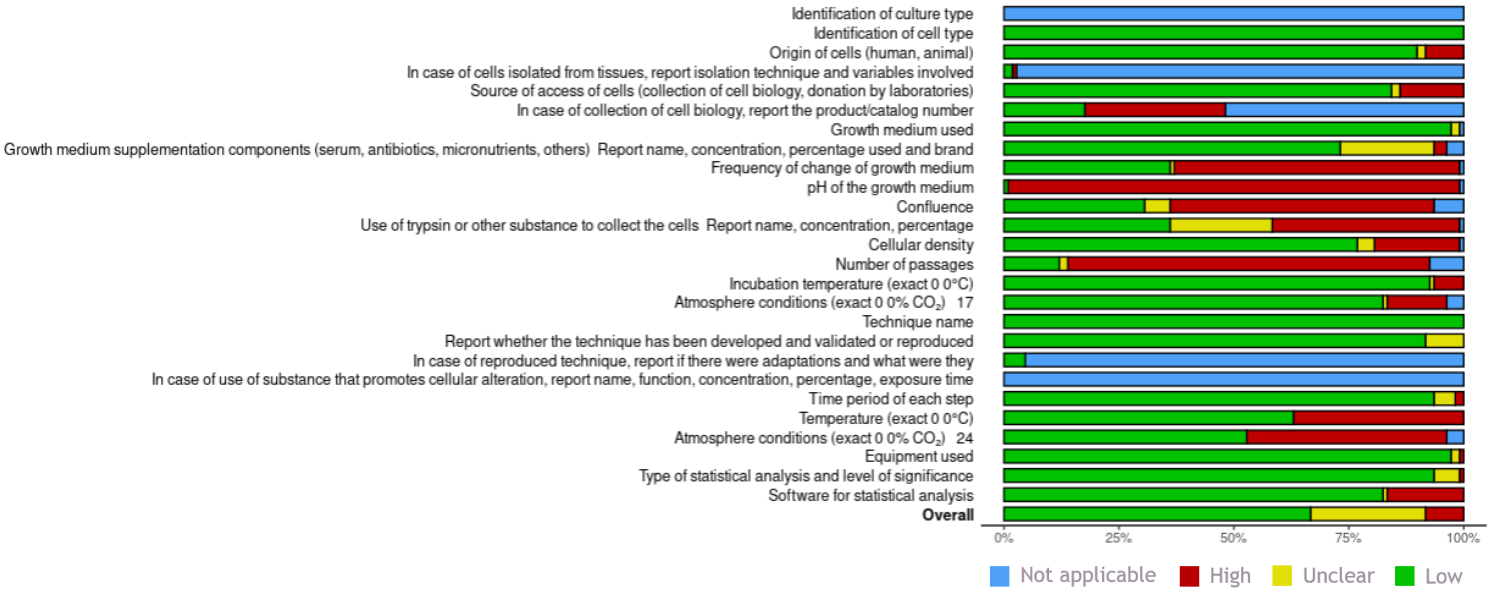

Figure 11 - Summary plot of risk of bias according to Robvis tool.

4.3. Summarized results by system

4.3.1. Overview

In this chapter, we provide an overview of the results by combining data from all systems, pertaining to cell viability, cytoplasmic membrane integrity, barrier integrity, oxidative stress, inflammation, DNA damage, apoptosis, and MMP. To enhance visual clarity and facilitate understanding, a violin plot (Figure 12) illustrates the distribution of sizes for the most commonly used NP polymers. Additionally, heatmaps were generated to depict the relationships between toxicity and polymer types (Figure 13), as well as toxicity according to size categories (Figure 14). The violin plot demonstrates the difference in size distribution

between PS-based NPs, mainly uPS, C-PS and A-PS, which have a higher number of particles studied nearing 100 nm in diameter, and PET with particles closer to the 200 nm value.

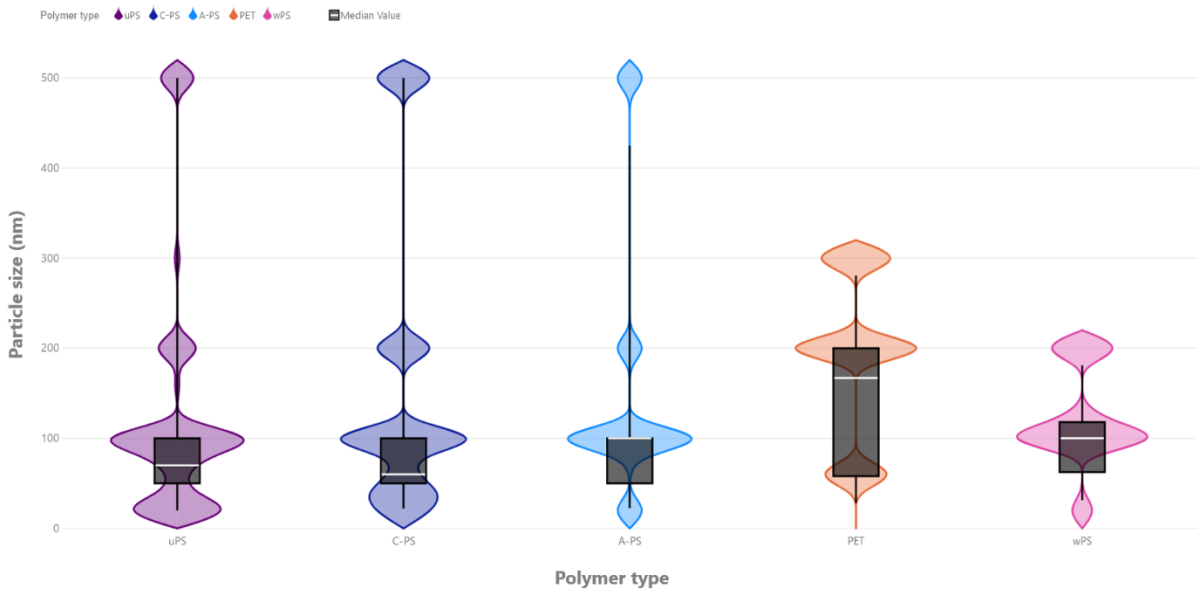

Figure 12 - Size distribution of particles for the most commonly used NP polymers. uPS = unmodified Polystyrene. C-PS = Carboxyl-modified Polystyrene. A-PS = Amine-modified Polystyrene. PET = Polyethylene terephthalate. wPS = weathered Polystyrene.

Figure 13 showcases that uPS, A-PS and C-PS were the most studied polymer, and polymer modifications. For all main parameters assessed, uPS and A-PS seem to present higher toxicity, by having a negative effect in more than 50% of the occasions these were reported. Out of the two, A-PS is represented with a darker red color, which indicates negative effects were reported more frequently for this PS modification in comparison to uPS across all eight main parameters. A few studies included analyzed uPS and A-PS nanoparticles of the same dimension and corroborate this finding. C-PS exhibited some degree of toxicity, although it would appear less impactful on cytoplasmic membrane integrity and barrier integrity. Surprisingly, none of the studies revealed data on inflammation with this modification. PET presented a negative effect on cellular lines on half, or less than half, of the occasions it was reported, except for DNA damage, which was only reported once as having an increase in strand breaks. Only a few studies dedicated themselves to wPS, for which cell viability, cytoplasmic membrane integrity, oxidative stress and inflammation were reported. Oxidative stress was increased in all reports assessed, but the remaining 3 parameters were not affected in more than 50% of the cases. As for the remaining polymer types, PC, Sa-PS, and PTFE were only associated with negative outcomes for the parameters assessed, while PE and PLA mostly caused no significant damage. Finally, PMMA and S-PS had more mixed results. Be that as it may, these studies were reported on few occasions, some only by a single study, making it difficult to draw any conclusions due to the lack of information and other variables involved in these methodologies.

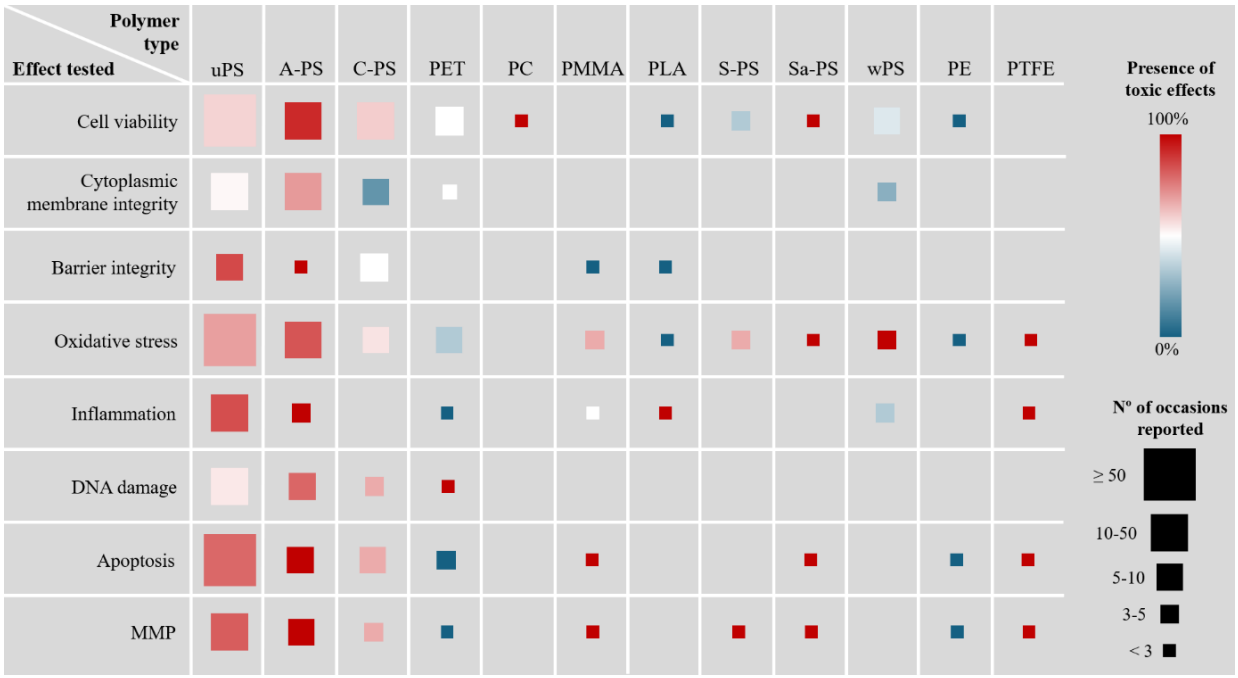

Figure 13 - Presence of toxic effects caused by NPs on eight different parameters, according to polymer type and number of occasions reporting it.

The most used size range for NPs fell between 40 and 100 nm, which was also the range most consistently reporting a negative effect in over 50% of the cases across all eight parameters. On a broader view, Figure 14 reveals that NPs up to 150 nm in diameter cause cellular damage on most, if not all, of the parameters assessed, while NPs over 150 nm had more mixed results. In the latter, cell viability and cytoplasmic membrane integrity or barrier integrity was less frequently affected, but oxidative stress, inflammation, DNA damage, apoptosis and MMP continued to demonstrate the negative effects associated with NP exposure in most or all occasions reported, namely until reaching a particle size of 300 nm. NPs in the two highest sized categories were less studied, but overall had a lower frequency of negative effects reported. The lowest size category (<20 nm) was only reported by one study but presented an important contribute to this debate of size relevancy on toxicity potential. This study exposed HeLa cells to uPS NPs of 10, 15, 25, 40 and 50 nm, and observed that the smallest sizes of 10 and 15 nm could reduce cell viability and increase oxidative stress, while the other NPs of bigger dimensions could not.

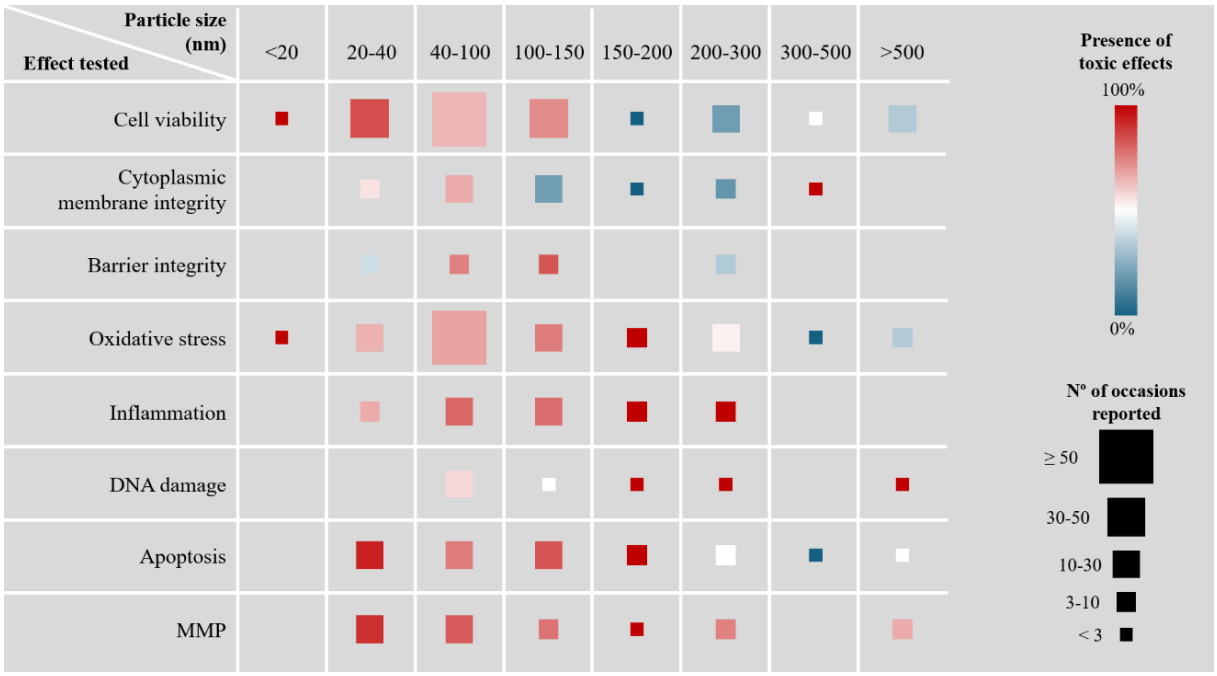

Figure 14 - Presence of toxic effects caused by NPs on eight different parameters, according to size category, and number of occasions reporting it.

Analyzing cellular lines by system, respiratory cell lines appear more susceptible to negative effects from NPs' exposure, since uPS and A-PS collectively reported 68 negative effects across various parameters studied, with no instances of exposure showing absence of toxicity. C-PS and PET still demonstrated some negative effects on cell viability, oxidative stress and inflammation but the results were not as consistent as for the other two polymers. The following systems with a higher frequency of negative effects reported upon exposure to uPS and A-PS were the nervous system, urinary system, and connective tissues, with cell viability decreasing in between 75 and 100% of the occasions it was reported. The reproductive system also exhibited a high frequency of negative effects from uPS NPs' exposure, anywhere from 69% to 100%, depending on the parameter observed. On the other hand, cellular lines belonging to the immune system and the hepatic system appear more resistant, at least in terms of cellular viability, with a frequency of decrease of 38% and 45%, respectively.

4.3.2. Hepatic system

The hepatic system plays a crucial role in detoxification, making it essential to understand how NPs affect liver cells. The table in Annex 2 details studies on hepatic cell lines, focusing on the types of plastic polymers, nanoparticle sizes, concentrations used, and their main findings.

A total of 12 studies investigated the effects of various NP polymers on cell lines belonging to the hepatic system. These studies employed seven different cell lines: LO2, HepG2, second-generation UHHs, HepaRG, ZFL, rat hepatocyte suspensions, and AML-12, out of which human cell lines HepG2 and HepaRG were used more frequently, with 4 and 3 occurrences, respectively. The studies explored the impacts of eleven different polymers or their

modifications, specifically: uPS, C-PS, A-PS, sulfate-modified polystyrene (S-PS), weathered polystyrene (wPS), PET, PC, PMMA, PLA, and PP. The sizes of nanoparticles investigated were distributed as follows: 8 instances of 20-40 nm, 13 instances of 40-100 nm, 7 instances of 100-150 nm, 1 instance of 150-200 nm, 4 instances of 200-300 nm, and 2 instances of sizes greater than 500 nm. None of the 12 studies examined nanoparticles smaller than 20 nm or between 300-500 nm.

As it was previously mentioned, the effects of these nanoparticles were categorized into several key areas: cell viability, membrane integrity, barrier integrity, oxidative stress, inflammation, DNA damage, apoptosis, and mitochondrial membrane potential (MMP).

uPS was the most frequently studied polymer, having 17 occurrences when considering different sizes applied, or 21 if application to different cell lines by size is considered. In terms of cell viability, a negative effect, considered as a reduction in cell viability, occurred 9 out of 20 times this effect was studied (45%), with one study not reporting the effect on cell viability. Interestingly, the negative effect on cell viability was mostly found when particles were under 100 nm (≈78%). Membrane integrity, inflammation and MMP exhibited a negative effect 50% of the time, with a total of 4, 6, and 4 reports, respectively. No data were available for barrier integrity. Oxidative stress was reported on 5 occasions, while 8 occasions found no effect (≈38%). DNA damage was only evaluated once, exhibiting a negative effect. An increase in apoptosis was observed 4 out of 11 times (≈36%).

For C-PS, there were 3 occasions where a decrease in cell viability was reported and one occasion with no effect (75%), and the latter corresponded to the highest sized particle studied (100 nm). There was no data available for membrane integrity, barrier integrity, oxidative stress, inflammation, DNA damage, or MMP, but an increase in apoptosis was reported in one study, while two studies did not find effects (≈33%).

A-PS exposure induced a decrease in cell viability in both studies reporting this effect, for particle sizes of 50 and 100 nm. Once again, there was no data available for membrane integrity, barrier integrity, oxidative stress, inflammation, DNA damage, or MMP, and one study reported an increase in apoptosis. Only one study explored the effect of wPS on cell viability, oxidative stress and inflammation, with particles of 25 and 100 nm, having reported a decrease in cell viability, increase in oxidative stress and no effect on inflammation for both particles[158].

PET was evaluated in two studies, having a wider size dispersion in solution, although the averages were of 58, 89 and 252 nm[157,159]. A reduction on cell viability was reported for all 3 PET NPs, although these were either based on slope differences or high content screening (HCS), unlike most other studies. No effect was found on cytoplasmic membrane integrity or oxidative stress, but one study did report an increase in DNA damage[157]. The other study did not evaluate DNA damage, but nuclear size and intensity were maintained[159].

For PC, both studies reported a decrease in cell viability, having used smaller particle sizes of ≈31.5 nm and ≈ 47 nm[159,160]. One study further reported a negative effect on cytoplasmic membrane integrity, decreased albumin production and the disruption of cytochrome P450 system based on gene expression[160]. None of the studies evaluated barrier integrity, oxidative stress, inflammation, DNA damage, apoptosis, or MMP.

PMMA and PLA were evaluated only once using HepaRG cell lines, and mostly exhibited no effect on barrier integrity, oxidative stress or inflammation, except for an increase in inflammatory cytokines after exposure to PLA[161].

For PP only one study evaluated the effects of particles with an average size of 158 nm, on HepG2 cell line, having found no significant differences to the control group when it comes to cell viability, cytoplasmic membrane integrity, oxidative stress, DNA damage and cell cycle distribution[157].

For S-PS, there was, once again, only one study that exposed HepaRG cell lines to 100 nm S-PS particles. This study reported no significant differences in cell viability or apoptosis, even while potentially using a higher number of particles than its uPS and A-PS counterparts, which exhibited a toxic effect on this cell line[87].

Besides the main effects assessed, one study reported an imbalance in mitochondrial dynamics after exposure of HepG2 cells to uPS, with a decrease in mitochondrial biogenesis and increase in mitochondrial fission[162]. Yet another study, in HepaRG cell lines, found that lipid accumulation increased with uPS25 and uPS100 exposures[158].

4.3.3. Urinary system

Given the urinary system's role in filtering and excreting toxins, it is important to investigate the impact of NPs on renal cells. The table in Annex 3 summarizes key studies, including information on cell lines, plastic polymers, nanoparticle sizes, concentrations, and primary outcomes.

There are only four studies integrated in this review that investigated the effects of NP polymers on cell lines belonging to the urinary system. These studies employed two different human cell lines, namely, HK2 and 293T. The HK2 cell line was used more frequently, with three occurrences while the 293T cell line was only used in one study but tested four different polymer sizes. Despite testing four sizes of uPS between 20 nm and 500 nm, most effects were studied only with uPS20 and uPS60 since they exhibited the highest toxicity based on cell viability. Most NPs investigated were between 20 nm and 150 nm (≈78%), with the remaining two particles tested falling between 150-200 nm, and at 500 nm, none of which influenced cell viability. The studies explored the impacts of a single polymer, specifically uPS, and one study also reported the effects of its weathered counterpart, wPS[163]. Both uPS and wPS presented a toxic effect associated with a decrease in cell viability, cytoplasmic membrane integrity, barrier integrity and/or MMP, or with the increase of oxidative stress,

inflammation and/or apoptosis, with particles below 150 nm. The presence of DNA damage was not evaluated in any of the represented studies.

4.3.4. Respiratory system

As the respiratory system is a primary route of NP exposure through inhalation, understanding its effects on lung cells, and other cells that line the respiratory tract, is critical. Annex 4 outlines studies on respiratory cell lines, detailing the plastic polymer types, nanoparticle sizes, concentrations, and significant findings.

A total of 15 studies investigated the effects of NP polymers on cell lines belonging to the respiratory system. These studies employed seven different cell lines, namely, human cell lines A549, BEAS-2B, HPAEpiC and HNEpCs, murine cell lines MLE-12 and MH-S, and the fish cell line RTgill-W1. Among these, the human cell lines A549 and BEAS-2B were used the most, 7 and 5 times, respectively. The studies explored the impacts of two polymer types, primarily PS, with uPS on 19 occasions, A-PS on 6 occasions, and C-PS on 5 occasions. The other polymer studied was PET, reported on two occasions. Once again, most of the particles employed had a higher representation below the 150 nm diameter size (n= 24; 75%).

Interestingly, respiratory cell lines seem particularly susceptible to NPs, with uPS demonstrating toxicity in all parameters studied with up to 19 reports of decreased cell viability and 11 reports of increased oxidative stress and increased apoptosis. Besides these established parameters, studies also reported mitochondrial impairment[164], an interference with the energy metabolism[165], increased ER stress[165], and autophagy[165,166], or even the halting of the cell cycle at the S phase[167]. A-PS also demonstrated toxicity on all parameters assessed by the studies with a decrease in cell viability and increase in oxidative stress being the most notable, reported 5 and 3 times, respectively. No assessment of barrier integrity or cytoplasmic membrane integrity was done for this modification. C-PS also decreased cell viability on all 6 reports available, however, its effect on oxidative stress and DNA damage was mixed, with one study reporting an increase in these parameters while another study reported no significant differences. It is important to note that the study reporting no significant differences at this level[168] used a much lower concentration than the one claiming the effect[169], not to mention there is a difference in the origin of the cell lines subjected to this exposure, with the first being a fish cell line and the latter a human cell line. In terms of cytoplasmic membrane integrity and MMP, C-PS effects were only reported by the study employing fish cell line RTgill-W1, having demonstrated no significant changes at the concentration of 10 µg/mL[168]. Lastly PET was used for the exposure of A549 cell lines, having demonstrated an increase in oxidative stress in both studies. Cell viability decreased in one of the studies[170] but did not suffer changes in the other[171]. The latter did reveal a significant positive correlation with DNA damage upon PET NP exposure, but no damage to cytoplasmic membrane integrity was

observed. Even though the results for apoptosis are indicated as having no effect, due to a decrease in the combination of early and late apoptosis, PET NP's effect at this level should not be excluded since there was an increase in late apoptosis at the highest concentration studied (196.79 µg/mL)[170]. One study mentioned mitochondrial impairment as an effect caused by exposure to smaller particles, namely uPS20, uPS50 and A-PS20[164]. Predicted increases in autophagy were also mentioned by a couple of studies[165,166]. Not to mention an association between the toxicity caused by uPS exposure (100 and 200 nm) regarding cell viability, apoptosis and inflammation levels, and the accumulation of Fe^{2+}, which has been proposed by a study using BEAS-2B cell lines[172]. In one study utilizing HNEpCs, a significant and steep increase in necrosis was observed after exposure to A-PS50, while its pristine counterpart contributed to an increase in apoptosis above mentioned[173].

4.3.5. Digestive system

The digestive system is another major point of contact for NPs via ingestion. Annex 5 highlights studies examining the effects of NPs on gastric and intestinal cell lines, including co-cultures, tricultures and organoids for the toxicological assessment of these particles. It also describes the types of plastic polymers, nanoparticle sizes, concentrations used, and main results.

In this chapter, a comprehensive review of 33 articles was conducted to assess the impact of various NPs on cell lines related to the digestive system. The studies encompassed 17 different cell lines, including human cell lines such as Caco-2 (n= 17; 41%), HIEC6 (1 studies), HCT-116 (2 studies), HT29, including modification HT29-MTX-12 (3 studies), RKO (1 study), NCM460 (1 study), HET-1A (1 study), HEEC (1 study), SNU-1 (1 study) and GES-1 (3 studies), murine cell line IEC-6, and the fish cell line RTgutGC, each represented by one study. Additionally, two intestinal organoids (one representing mice and one from human HiPSCs), four co-cultures involving Caco-2 and HT29 (with or without modifications such as HT29-MTX and HT29-MTX-12), and two tricultures with Caco-2/HT29 combined with either THP-1 or M-cells were analyzed.

A variety of polymers were examined, primarily PS with uPS, A-PS, C-PS and wPS modifications, representing a combined total of 51 NPs (78%) used for cellular lines within this system. Other polymers studied were PET and its weathered version, PE, PLA, PMMA, PP, PC and PTFE, with occurrences between 1 (PP, PC and PTFE) and 7 (PET and wPET). The size distribution of nanoparticles used was mostly concentrated between 20 and 150 nm (n= 44; 71%), similarly to other systems described in this review. A higher number of nanoparticles between 200-300 nm were utilized in these studies (n=12; 18%), while above or equal to 500 nm there were only 4 particles and below 20 nm there were none. Since this system involves so many NPs of varied polymers and sizes, results are described in the topics below, with the last topic representing polymer types of PLA, PMMA, PTFE, PP and PC as their combined representation corresponds to about 11% of all NPs (n= 7).

4.3.5.1. uPS NPs

Cell viability was the most reported parameter, with a negative effect observed in 61% of the cases (n= 19), followed by oxidative stress, which had an increase after uPS exposure in 68% of the cases (n= 17). Cytoplasmic membrane integrity, DNA damage, apoptosis and MMP were affected in 11, 44, 86 and 73% of reports made, respectively. Finally, barrier integrity, reported in 4 occasions, and inflammation, reported in 8 occasions were the least reported parameters, having a negative effect in 75% and 63% of the cases, respectively. One of the studies focused on the effects of uPS and wPS NPs on bioenergetic levels of the cell[43]. Although a mixture of 100 and 750 nm weathered PS nanoparticles caused no changes at this level, uPS contributed to an increase of the extracellular acidification rate (ECAR), which reflects an increase of glycolysis for energy production.

Gastric cell lines GES-1 and SNU-1 presented either reduced cell viability or reduced cell proliferation when exposed to uPS in four studies. Interestingly, for GES-1 cell line, one study reported a decrease in cell viability after exposure to 40 µg/mL uPS250[174], while a different study using the same cell line and uPS200 observed a decrease in cell viability only at concentrations equal or upwards of 100 µg/mL[175]. Oxidative stress was elevated after exposure to uPS NPs in these cell lines when using particles up to 500 nm, but apoptosis and MMP seem to be negatively influenced by smaller sized particles, such as 50 and 60 nm, and not by uPS250. One study utilizing the SNU-1 cell line aimed at comparing the different polymer modifications, such as uPS against A-PS and C-PS, however the only comparison with the controls was made for cell viability, which was reduced for uPS50 but not for uPS NP particles above 100 nm in diameter[88]. Caco-2 and HT-29 cell lines when used in monoculture were negatively influenced by uPS NP's exposure nearly as many times as, or less than, the moments no effect was observed. For example, cell viability decreased in 50% of the ten occasions it was reported for Caco-2 cell line and of the two occasions it was reported for HT-29 cell line (including modified versions of this line). A similar trend was observed for oxidative stress (n= 4; 44%) and MMP (n= 2; 50%). Cytoplasmic membrane integrity, DNA damage, and inflammation were never altered by uPS NP's exposure, however, barrier integrity was only reported twice for Caco-2 cell lines and considered to be negatively impacted on both occasions, while apoptosis was also increased in all studies made with uPS NPs in these two cell lines. When analyzing the remaining human intestinal cell lines (HCT-116, HIEC-6, RKO and NCM460), together with the two esophageal cell lines (HET-1A and HEEC), the picture shown appears to differ with all parameters having at least one assessment made, except DNA damage, and all having been negatively impacted by uPS NP's exposure. All dual cultures involved Caco-2 and HT-29 and so, as expected, the results were somewhat similar to what

was obtained for these two cell lines in monocultures, with less than 50% of the cases being negatively impacted regarding cell viability, cytoplasmic membrane integrity, barrier integrity, oxidative stress and DNA damage. In the human body, intestinal cells are not present as a monoculture, which can be a setback for many of these studies when trying to predict the impact of potentially toxic particles to human health. To reduce that effect, tricultures, and more so intestinal organoids, can be used in exposure protocols as they come closer to in-body conditions. Since the triculture is also constituted mainly by Caco-2 and HT-29 cell lines, the exposure to uPS NPs did not alter cell viability, cytoplasmic membrane integrity, barrier integrity or oxidative stress, as could be expected when looking at other results of these cellular lines. Nevertheless, the human organoid used did exhibit a decrease in cell viability, increased inflammation, apoptosis and oxidative stress, consistent with the presence of toxic effects stemming from the exposure to uPS NPs[98], as did the mouse intestinal organoid with increases in oxidative stress, inflammation, apoptosis and a decrease in MMP being reported[176]. Finally, fish cell line RTgutGC was exposed to uPS44 and showed no significant differences to control in cell viability, oxidative stress or respiratory capacity[177].

4.3.5.2. C-PS NPs

C-PS NPs had a total of 27 reports across five different parameters: cell viability, cytoplasmic membrane integrity, barrier integrity, oxidative stress and apoptosis. The effects of this modification were investigated in SNU-1, Caco-2 and HT-29 monocultures, as well as in dual cultures and a triculture with M-cells. From all observations, cell viability was affected in 43% (n= 6) of the cases and barrier integrity in 50% (n= 3) of the cases. Oxidative stress was increased in one out of three cases (33%), while cytoplasmic membrane integrity was never found to have significant changes (n= 3; 0%). Contrastingly, apoptosis was increased in Caco-2 cell lines after exposure to C-PS NPs, but only one report for this parameter was present.
The viability of the SNU-1 cellular line was never affected when using concentrations up to 100 µg/mL of C-PS NPs of four different sizes (50, 100, 200 and 500 nm)[88]. One study reported a decrease in cell viability for Caco-2, HT29-MTX and its co-culture when using C-PS20, with higher concentrations needed for the effect to take place in the co-culture, but no changes to either when the particle used had 200 nm (C-PS200)[178]. The same study reported an increase in oxidative stress and mucus secretion in the co-culture after 48h hours of exposure to C-PS20, alongside a decrease in barrier integrity and permeability[178]. Another co-culture experiment noticed a decrease in cell viability with exposure to C-PS50 at the highest used concentration of 100 µg/mL, but no changes to barrier integrity were observed[179]. The triculture demonstrated a decrease in cell viability both with C-PS25 and C-PS100, with the latter taking effect at a lower concentration. The authors also reported an increase in membrane permeability with C-PS100, but no negative effects were observed for barrier integrity, cytoplasmic membrane integrity and oxidative stress for either polymer size[180]. A study using

HT29 monoculture for exposure to C-PS200 at concentrations up to 100 µg/mL observed no difference in cell viability, albeit a decrease in barrier integrity[181]. As for Caco-2 monocultures, one study mentions no effects on cytoplasmic membrane integrity with C-PS particles of 100 nm at 30 µg/mL, although they reported an increase in apoptosis, cells arrested in G0/G1 phase and number of lysosomes, accompanied by a decrease in cell proliferation and barrier integrity[182].

4.3.5.3. A-PS NPs

Amine-modified PS NPs had a total of 16 reports across five different parameters: cell viability, cytoplasmic membrane integrity, barrier integrity, DNA damage and apoptosis. The effects of this modification were investigated in SNU-1, Caco-2, HT29 and HT29-MTX-E12 cellular lines, as well as a triculture of Caco-2, HT29-MTX-E12 and THP1 for cytoplasmic membrane integrity. Overall cell viability was affected 71% of the times it was reported, cytoplasmic membrane integrity in 75% of the cases, barrier integrity in 50%, while DNA damage and apoptosis were significantly elevated in every report made for these parameters (n=2 and n=1, respectively).

When exposed to A-PS, SNU-1 cells experienced a decrease in cell viability, when particles were of 50, 100 or 500 nm, but surprisingly, no statistically significant difference was found upon exposure to A-PS200[88]. Two studies analyzed the effects of this PS modification on Caco-2 cell lines, with particles of either 50 or 100 nm. Although these studies reported different parameters, mostly toxic effects were observed in both, with decreases in cell viability or cell proliferation, increases in DNA damage, apoptosis, cells arrested in G0/G1 phase and lysosome numbers[91,182]. Cytoplasmic membrane integrity was found to be decreased in the study utilizing A-PS50[91] but not in the study utilizing A-PS100[182]. HT29 and its modification HT29-MTX-E12 were also represented twice. In the first case a nanoparticle of 200 nm diameter was used and no negative effects were observed in cell viability or barrier integrity[181]. On the second study, however, particles were smaller (50 nm), which caused toxicity to the cellular line, namely a decrease in cell viability and cytoplasmic membrane integrity, together with an increase in DNA damage[91]. The study employing the triculture above described reported a decrease in cytoplasmic membrane integrity upon exposure to A-PS50[91].

4.3.5.4. PET NPs

PET NPs were used in five different studies, all with monocultures of Caco-2. Sizes used ranged from 26.7 nm to 252 nm using average numbers, but in solution nanoparticles had a high size variability which could reach 600 nm in some cases[43,157]. One of the studies focused on the effects of PET NPs on bioenergetic levels of the cell. Contrary to what was found for PS NPs, the weathered version of this plastic nanoparticle, at 144 nm on average, seemed to shift the energetic trend from aerobic conditions to anaerobic, with increases in ECAR and

glycolytic ATP. The pristine version of PET, with an average size of 197 nm, also enhanced ECAR, however that increase was concomitant with the increase in oxygen consumption rate (OCR) and mitochondrial ATP, suggestive of aerobic conditions[43]. A decrease in cell viability was reported by two studies, using PET NPs of 58, 89 and 252 nm[157,159]. The latter particle (252 nm) also negatively influenced DNA damage and cytoplasmic membrane integrity, although the statistical calculations were made based on slopes instead of each individual concentration[157], therefore a specific concentration at which these effects would be encountered cannot be established. In this same study, cell cycle distribution and oxidative stress were not significantly altered after 3h of exposure. Two other studies, using the lowest sized PET NPs in this entire review, 26.7 and 30 nm, reported no toxicity from Caco-2 cell line's exposure to these NPs, evaluating cell viability, cytoplasmic membrane integrity, oxidative stress, inflammation and apoptosis[183,184].

4.3.5.5. Others – PLA, PMMA, PTFE, PP and PC

The effects of PLA NPs were investigated by two studies. The first study exposed a co-culture of Caco-2/HT29 to PLA280 and reported no changes to barrier integrity, cell viability, oxidative stress or barrier permeability in comparison to the controls[185]. The second study exposed a monoculture of the Caco-2 cellular line to PLA250 and, although the authors also did not find effects that revealed toxicity regarding oxidative stress and barrier integrity, there were signs of an increased inflammatory response, based on the upregulation of genes such as IL8, IL6ST, IL10 and COX2[161]. PMMA NPs were also investigated by two different studies. The first utilized monocultures of HCT116 and mice intestinal organoids for the exposure to PMMA150, reporting a decrease in MMP, increase in oxidative stress, apoptosis and inflammation (only on mice intestinal organoids) when compared to the controls[176]. The same study tested PTFE230 nanoparticles, and similar effects were described[176]. The second study exposed Caco-2 monocultures to PMMA25 and its results were not consistent enough to draw conclusions regarding the parameters evaluated in this review. Regardless, some of the changes seem to indicate a cellular response to oxidative stress or inflammation[161]. Roursgaard et al. (2022)[157] exposed Caco-2 cell line to PP158 and no significant differences to the control were observed in terms of cell viability, cytoplasmic membrane integrity, DNA damage, oxidative stress and cell cycle distribution[157]. Lastly Caco-2 cell lines were also exposed to PC47, which caused a decrease in cell viability[159]. However, it is important to note that in this study the particles' dispersant also caused a significant decrease in cell viability[159] and, for that reason, this negative effect might not be attributed to the plastic nanoparticle.

4.3.6. Immune system

Understanding the immune system's response to NPs is vital for assessing how these particles affect the body's ability to recognize and eliminate threats. Annex 6 details studies on immune

cell lines, highlighting the plastic polymer types, nanoparticle sizes, concentrations, and primary outcomes.

A total of 22 studies investigated the effects of NP polymers on eleven different cell lines originating from mice, fish, sea urchins and humans, and belonging to the immune system. The most frequently used was murine cell line RAW264.7, with 8 studies, followed by human cell lines THP-1 (6 studies), Raji-B (3 studies) and TK6 (3 studies). Mice splenocytes and human PBMCs were studied twice each, and the remaining, including human WBCs, HSPCs and Jurkat cells, sea urchin coelomocytes and fish cell line RT-HKM, were represented once. Three polymers were studied: PS, PE and PET. PS was the most extensively studied polymer, with its unmodified version appearing in 20 instances. Modifications including A-PS, C-PS, S-PS and sulfonic acid-modified (Sa-PS) appeared 4, 3, 2 and 1 times, respectively. Both PE and PET were investigated once, corresponding to two of the highest polymer sizes present, with 531 and 300, respectively. The size range most studied was between 40 and 100 nm (n= 16; 50%), with the other size ranges being more evenly distributed with 4 instances in ranges of [20-40[, [100-150[, [200-300[, and 2 instances in [300-500[and ≥500 nm ranges.

Regarding uPS exposure, immune cell lines seem to be more resistant to negative effects, with cell viability affected in 38% of the cases (n= 11), oxidative stress in 46% (n= 12), cytoplasmic membrane integrity and DNA damage in 50% of the cases (n= 2 and 5, respectively). Inflammation, apoptosis and MMP were negatively affected more frequently, with 75%, 83% and 77%, respectively, and no data were available for barrier integrity. It is important to note that cell line THP-1 was mostly unaffected by uPS exposure in all 6 studies, having only exhibited a decrease in MMP in two of the studies[186,187], and increase in mtROS in one of those studies[186] as potential toxic effects. This event shifts the trend of effects caused by uPS exposure to seemingly lower values. If THP-1 cell line results were to be removed from the analysis, negative effects would increase to 50%, 57%, 63%, 67% and 100% in cell viability, oxidative stress, DNA damage, cytoplasmic membrane integrity and inflammation, respectively. Although these values remain lower than the ones encountered for cellular lines in other systems, it shows the effect a particularly resistant or susceptible cell line may have on the overall results.

A-PS negatively affected all cell lines exposed to it, namely when it comes to cell viability, cytoplasmic membrane integrity, apoptosis and MMP. Oxidative stress was evaluated twice, having suffered an increase with exposure to A-PS100 at concentrations as low as 10 µg/mL in RAW264.7 cell lines[188] but no change with A-PS20 exposure at a concentration of 40 µg/mL in mice splenocytes[189]. Barrier integrity, inflammation and DNA damage were never assessed for this modification.

C-PS had fewer assessments, negatively affecting oxidative stress, DNA damage, apoptosis and MMP in all instances it was evaluated. Cell viability was reported 3 times, having

decreased twice (67%), in RAW264.7 cell lines[188] and mice splenocytes[189], while no differences were observed in sea urchin's coelomocytes[190]. Other parameters have not been reported for this modification.

S-PS was evaluated by two studies[186,191], using a polymer of 200 nm diameter, twice in RAW264.7 cell lines and once in THP-1 cell line. Cell viability was significantly reduced once, when RAW264.7 cell lines were exposed to concentrations equal or above 250 µg/mL[186]. The other study utilizing RAW264.7 cell lines only used concentrations up to 200 µg/mL[191], which may explain the absence of effect in comparison to the first. MMP was found to be reduced upon exposure to S-PS200 both in THP-1 and RAW264.7, while lipidic content was increased[186]. Similarly, oxidative stress was considered to be increased in these cell lines, although it was solely based on mtROS evaluation. Oxidative stress was reported as unchanged in one study[191], depicted as ROS production. On the other hand, that study reported the presence of increased foam cell formations after 24h of exposure to the highest concentration of S-PS (200 µg/mL).

Sa-PS, PET and PE were only investigated by one study each. Sa-PS20 lead to toxicity in mice splenocytes, demonstrated by negative effects to cell viability, oxidative stress, apoptosis and MMP at concentrations of 40 µg/mL[189]. On the other hand, PET and PE did not affect cell viability. PE increased inflammation in RAW264.7 cell line, based on increase production of IL-1β, associated with an increased expression of Pro-IL-1β, as well as increased expression of CD80, suggesting a transition to a pro-inflammatory phenotype[192]. Meanwhile, PET did not influence oxidative stress, apoptosis, nor cell viability as mentioned above[193]. Whether the lack of effect from these NPs stems from chemical properties or the bigger diameter utilized is hard to predict without further studies.

Since some of the cells within the immune system perform phagocytosis as one of their main functions, such as macrophages, neutrophils and dendritic cells, and this process is dependent on the maintenance of lysosomal functions, lysosomal damage and changes to the phagocytic capacity were also reported by some of the studies[190,192,194], revealing another harmful outcome associated with the exposure to NPs of different polymers (uPS, C-PS, A-PS and PE). Furthermore, there is a need for these cells to recognize pathogens in order to bind to them and initiate the phagocytic process. CD206, also known as the mannose receptor, is partially responsible for this function and was found to be decreased upon exposure of RAW264.7 cell lines to uPS80[195]. The induction of different polarization states or differentiation of macrophages towards osteoclast cells has also been reported by two of the included studies[177,196]. Finally, it is worth mentioning that the presence or absence of fetal bovine serum (FBS) in cultures can influence the results, with its absence being correlated with increased uPS20 toxicity by Ilić et al. (2022)[197].

4.3.7. Reproductive system

NP exposure may influence reproductive health and fertility. Annex 7 summarizes studies on reproductive cell lines, including the types of plastic polymers, nanoparticle sizes, concentrations used, and significant findings.

A total of 13 studies investigated the effects of PS NPs on various cells belonging to the reproductive system. These cells included murine cell lines TM3, TM4, GC2, each being reported twice, swine granulosa cells, oocytes from oysters, spermatozoa from oysters and of human origin, as well as human cell lines KGN, HeLa, HEY and NTERA-2, all being reported once. The polymer used for exposure protocols was exclusively PS, mostly the unmodified version (15 instances), followed by 3 instances of the amine-modified and 1 instance of the carboxyl-modified versions. The smallest uPS sizes found in this review belonged to a study on HeLa cell lines, with 10 and 15 nm diameter[198]. Remaining studies used PS NPs of sizes between 20 and 100 nm, and none examined nanoparticles larger than 100 nm.

For uPS, a decrease in cell viability was reported in 9 out of 13 instances (69%). Cytoplasmic membrane integrity, barrier integrity, inflammation, apoptosis and MMP were negatively impacted in all assessments made, which ranged between 1 report for barrier integrity and 5 reports for apoptosis.

For A-PS, cell viability, represented by increased mortality, was negatively affected in the single study that examined this parameter, in oyster's oocytes[199]. Cytoplasmic membrane integrity and DNA damage were evaluated using human spermatozoa and it was determined that A-PS50 had a negative effect while A-PS100 did not exert a significant influence. Oxidative stress increased with A-PS exposure to human spermatozoa[200] but was not altered in oyster's oocytes[199]. No data were available for the remaining parameters for A-PS exposures.

Cell viability and oxidative stress were the only parameters studied regarding C-PS exposure, more specifically in oyster's oocytes, with observed toxicity to both[199].

The main parameters here described are often used to assess the toxicity of certain substances *in vitro*, however, when it comes to cellular lines from the reproductive system, hormonal imbalances or damage to other organelles such as the acrosome are also important descriptors of harm/damage. An example of these has been described by Basini et al. (2021)[201], where an increase in E2 and reduction in P4 was observed for swine granulosa cells exposed to uPS100, as well as by a study where TM3 cells were exposed to uPS20, that revealed a decrease in the steroid hormone biosynthesis[202]. Furthermore, an increase in acrosomal damage, in addition to a decrease in mitochondrial functionality and spermatozoa motility was observed by Contino et al. (2023)[200] for human spermatozoa exposed to A-PS50. A-PS100 did not exert such effects on spermatozoa from either human or oyster origin[199,200]. Hu et al. (2022)[203] observed increased endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in TM4 cells exposed to uPS20. Meanwhile, uPS100 reduced the percentage

healing of HEY cells, presenting 275 differentially expressed genes[204]. Finally, the presence of oncogenic potential from NPs' exposure was described by three different studies, associated with activation of several metabolic pathways, such as MAPK, PI3K-AKT and the Hippo signaling pathways[205–207].

4.3.8. Gestational tissues

The impact of NP exposure on gestational tissues is important for understanding potential reproductive and developmental effects. Annex 8 presents studies on cell lines associated with gestational tissues, including the types of plastic polymers, nanoparticle sizes, concentrations used, and main findings.

In terms of gestational tissues, 4 studies were included in this review, representing 3 different cell lines of human origin: BeWo b30 (2 studies), HTR8/SVneo and HUVEC.

Only PS NPs were studied, with 3 modifications, namely C-PS, A-PS and wPS. Except for the study exposing BeWo b30 cell lines to C-PS, the other two studies employing a modification, also studied their pristine counterpart. In total, that equates to one particle of both C-PS and A-PS being studied, two particles of wPS and 4 particles of PS. The highest particle size investigated was 200 nm (n= 2), with the remaining being either 50 nm (n= 5) or 100 nm (n= 1).

uPS and wPS were unable to cause significant changes in terms of cell viability, while both reports for A-PS and C-PS expressed a decrease after exposure. Cytoplasmic membrane integrity was not affected by most uPS and wPS tested, suffering a decrease only in the case of non-syncytialized BeWo b30 cells upon exposure to the smallest size, 50 nm of both unmodified and weathered PS NPs. Despite having low evidence of toxicity at this level, uPS did lead to an increase in oxidative stress, inflammation, and apoptosis, as well as a decrease in MMP where these effects were studied. A-PS demonstrated toxicity towards HUVEC cells on the parameters of cell viability, cytoplasmic membrane integrity, oxidative stress and MMP, while also contributing to changes in mitochondrial gene expression[208]. Meanwhile C-PS exhibited a toxic effect by reducing cell viability of BeWo b30 cells from concentrations as low as 5 µg/mL, notwithstanding the absence of a significant effect on either barrier integrity or DNA damage[179]. In the HTR8/SVneo cell line, uPS100 exposure also decreased trophoblast migration and invasion, and caused 344 genes to be differentially expressed, with the majority being down-regulated (n= 298; 87%)[209].

4.3.9. Nervous system

Researching the effects of NPs on the nervous system is essential due to potential neurotoxic effects. Annex 9 presents studies on neuronal cell lines, detailing the types of plastic polymers, nanoparticle sizes, concentrations, and main findings.

On this chapter, a total of 12 studies were reviewed, examining the impact of NPs on 10 different cell lines, with murine cell lines HT22 (3 studies) and BV2 (2 studies), and human cell line hCMED/D3 (2 studies) being the most prevalent. Murine cell line C17.2 and primary hippocampal NSCs were also studied, alongside fish cell lines DLB-1 and Fub-1, and human cell lines SH-SY5Y and hNS1, with one study each. A large majority of the studies were performed with particles of diameters between 20 and 100 nm, inclusive (n= 19; 90%), with most of these falling between 40 and 50 nm, inclusive (n= 11; 58%). Only one study used particles of C-PS and uPS with 500 nm each. PS was, as expected, the most used polymer in these studies, with 12 uPS, 5 C-PS and 3 A-PS NPs being employed. PE was used in a single study, exposing SH-SY5Y cell lines to PE NPs of 100 nm diameter[210], with no significant effects in any parameters studied, such as cell viability, oxidative stress, MMP and autophagy.

uPS negatively affected cell viability in 78% of the cases where this parameter was reported (n= 7), and apoptosis in 1 out of 3 instances (33%). Barrier integrity, oxidative stress, inflammation and MMP were negatively affected in all reported cases, while no data were available for membrane integrity and DNA damage. Aside from these parameters, studies also reported increases in autophagy and mitochondrial dysfunction[210], changes to nuclear staining and texture[211] and the activation of the GAPDH/Ac-Tau signaling pathway[212], involved in neurodegenerative diseases. One study examined the effects of uPS on the expression of genes involved in several pathways, such as oxidative stress response, DNA repair mechanisms, inflammation, and regulation of apoptosis, using the hNS1 cellular line, and the alterations observed seem to indicate a multifaceted cellular response to uPS exposure[213]. Furthermore, Liu et al. (2022)[214] demonstrated that a static exposure protocol may not reveal the true consequences of uPS NP's exposure, since toxicity was found at a lower concentration when switching to a dynamic exposure mode. A-PS displayed toxicity on four different cell lines, SaB-1 (A-PS50)[215], C17.2 (A-PS30)[216], hCMED/D3 and HT22 (A-PS100)[212], by analyzing parameters such as cell viability, barrier integrity, oxidative stress, inflammation and apoptosis, which were the parameters studied for this modification. Contrastingly, C-PS and PE did not reveal toxicity according to the parameters of cell viability, oxidative stress, apoptosis, or MMP. SaB-1 and C17.2, which were the cell lines presenting results for C-PS exposure at this level, also demonstrated no significant effect by its pristine counterpart[215,216]. Despite not evaluating any of the main parameters, exposure of primary hippocampal NSCs from E16.5 mice to C-PS NPs of different sizes (50 and 500 nm) caused a reduction in cell proliferation, number of cells, cell diameter and neuron's length, as well as an increase in the number of astrocytes. In this case, a similar effect was observed for the unmodified PS version of these NPs[217]. This study also examined the effect of lower concentrations (0.01 to 1000 ng/mL) of C-PS30 on cell proliferation but no significant differences to control were observed[217].

4.3.10. Connective tissue

Given the potential dermal exposure to NPs, investigating their effects on connective tissues becomes just as important. Annex 10 outlines studies on connective tissue cell lines, detailing the types of plastic polymers, nanoparticle sizes, concentrations, and main findings.

A total of 6 studies investigated the effects of PS NPs on various cell lines belonging to the category of connective tissues. These studies employed seven different cell lines, each appearing once: human cell line Hs27, swine ASCs, murine cell lines MC3T3-E1, and MLOY-4, and fish cell lines ZF4 and SAF-1. Five out of the six studies used uPS and one study[218], used A-PS. Having only one particle size studied per article, there was a representation of 2 instances for the 40-100 nm, and 4 instances for the 100-150 nm NPs. Overall, uPS negatively affected cell viability in 3 out of 4 cases (75%), although the study reporting no changes to cell viability, once again, used a lower uPS concentration of 10 µg/mL[219] when compared to other studies with concentrations reaching up to 200 µg/mL[196,218]. Oxidative stress was reported as having increased in 5 out of 6 instances (83%), with no changes occurring in the human cell line Hs27[128]. uPS also exhibited toxicity by increasing inflammation, DNA damage, and apoptosis on every occasion these parameters were reported. Furthermore, uPS50 hindered the migratory ability of MC3T3-E1 cells, decreasing bone deposition and increasing bone reabsorption[196]. While swine ASCs displayed a decrease in cell viability with no changes to cell proliferation[220], Hs27 suffered a decrease in cell proliferation when exposed to uPS100, using the same concentrations[128]. With the H9C2 cellular line, the exposure to uPS94 increased autophagy and activated the TGF-β1/Smad signaling pathway[221], which has been associated with fibrosis, although it can regulate a wide array of cellular processes. No data were available for cytoplasmic membrane integrity, barrier integrity, or MMP for uPS in these studies. Fish cell line ZF4 was exposed to A-PS100 and these particles demonstrated a toxic effect on all parameters evaluated, namely, cell viability, cytoplasmic membrane integrity, oxidative stress, apoptosis, MMP, and lysosomal integrity[219].

5. Discussion

5.1. Study characteristics

Most scientific research regarding toxicity of NPs in *in vitro* studies originated in China. This is not surprising as China's scientific production has been growing at higher rates than other countries since 1995[222]. In 2012, China became the world's third-largest producer of research articles[223], and by 2022 it achieved first place, by surpassing the United States, according to Nature's index[224]. Simultaneously, China is the world's leading country in plastic waste generation, prior to management, although these numbers are much lower when determined per capita[225] due to the high population density of the country. When it comes to years of publication, most happened in the last four years (n = 92), increasing greatly year over year. The occurrence of microscopic plastic debris in the environment was first demonstrated by researchers from Plymouth University in 2004[226]. Meanwhile, NPs were first catapulted into the public consciousness, 15 years later, by a press coverage of an article written by Hernandez et al. (2019)[227], describing NPs' release in high numbers from plastic teabags, easily correlating with the increase in scientific research around that period.

A clear trend in NP polymer choice can be observed, as 90% of NPs used corresponded to PS, when accounting for surface modifications and weathering processes, or 58% if only uPS is to be considered. This observation has been mentioned before and it may correlate to how easy, or difficult such polymer types are to produce at a nanoscale[6,228]. However, these do not accurately represent environmental NPs, since the most predominant polymer, production wise and in relative abundance in the marine environment, is PE, with 30% and 23% presence, respectively[229,230]. Following PE, PP is also a highly produced plastic at about 19% relative representation[4]. On the subject of size, most NPs employed had a diameter of up to 150 nm, with medians falling at or below 100 nm for the most used PS NPs (uPS, A-PS and C-PS). This finding might be related to the lack of consensus regarding what a NP represents with reference to size, where some authors consider NPs to be plastic particles with dimensions equal to or below 100 nm[8,231].

While most studies showed low risk of bias, insufficient reporting on aspects like pH of the medium, passage number, and medium change frequency hampers reproducibility. It is of utmost importance to work towards standardizing methodologies and reporting protocols to enhance reproducibility and comparability of studies. Furthermore, researchers should reach a consensus regarding the criteria to apply for NP exposure protocols, such as size ranges, concentrations, surface area, or number of particles, stating their reasoning. Discrepancies in these parameters make it challenging to compare results across different studies and draw definitive conclusions. For instance, the same concentration of smaller sized particles represents a higher surface area and a greater number of particles, potentially leading to more

significant biological effects.

5.2. Influence of polymer type and size

The systematic review presented here highlights the significant negative effects of NPs on cellular parameters in *in vitro* studies. A comprehensive analysis reveals that uPS caused damage in more than half of the studies reviewed, affecting cell viability, cytoplasmic membrane integrity, and DNA damage in 50-60% of cases. Parameters such as barrier integrity, inflammation, MMP, and apoptosis were more frequently affected, with over 80% of studies reporting negative outcomes. A-PS exhibited the highest level of damage, impacting over 80% of cases across all parameters, except for cytoplasmic membrane integrity, which still reached 70%. Conversely, C-PS negatively affected cellular parameters in 50-66% of observations. The toxicity and environmental impact of polymeric nanoparticles are widely recognized to depend on several factors, including size, morphology, hydrophobicity, surface area, porosity, surface charge[232–234], material, concentration, and composition[235]. Surface functional groups, such as carboxyl and amine above described, introduce surface charges to the particles, influencing particle-protein interactions, as well as interactions with the cytoplasmic membrane, which may, in turn, facilitate or hinder their uptake and toxicity potential. Given the predominantly negative charge of the cytoplasmic membrane due to the presence of anionic phospholipids[236], positively charged particles like A-PS interact more easily with it, leading to higher uptake compared to negatively charged particles such as C-PS. This finding has been described in different studies and may well contribute to the higher toxicity frequency observed for these particles[87,237]. The zeta potential measures the electrical potential of a particle in fluid and is influenced by surface charge, making it a relevant measurement to be included in nanotoxicity studies.

PET nanoparticles had no effect on inflammation, apoptosis or MMP, with limited negative impacts on other parameters. PET NPs under different conditions exhibit a negative zeta potential of distinct magnitudes[238], which on its own could be related to the lower toxicity associated with this polymer type. More so, the majority of PET NPs employed in these studies had higher average sizes than PS NPs, and MNP's toxicity has been shown to be size-dependent, with smaller particles contributing to more negative outcomes, or doing so at a lower concentration than particles of bigger dimensions. Not only that but the average size of PET NPs is not descriptive of the entire size range of these particles in solution, which could reach values upwards of 500 nm[43,193], influencing their uptake and toxicity.

Sa-PS, PC and PTFE consistently showed negative effects when studied, while wPS, S-PS and PMMA obtained mixed results, though data on these polymers were limited. Conversely, PE and PLA had minimal influence on the parameters studied, with an increase of inflammation for PLA being the only negative outcome described, but again, data were too sparse for a

proper assessment to be made considering all methodological variables involved.

Studies primarily focused on NPs up to 150 nm, with smaller particles being more likely to reduce cell viability. Increased particle size generally resulted in fewer cellular parameters affected, or lower frequency of negative effects reported, particularly on cell viability, membrane integrity, and oxidative stress when particles were bigger than 300 nm. The influence of size on toxicity was heavily focused in a study using HeLa cell lines, demonstrating that uPS NPs below 20 nm were able to cause cytotoxicity while particles above did not present a significant influence on the same parameters[198]. The presence of contradictory results for similar particle sizes in some scenarios indicates that cell susceptibility, polymer type, concentration, along with other, previously mentioned, factors are significant modifiers of cellular response. Notwithstanding, the distribution of sizes across all studies varied, posing difficulties to accurately interpret results, for which a meta-analysis would be recommended.

NPs induce cytotoxicity and genotoxicity through several mechanisms. One primary mechanism is oxidative stress, where NPs generate ROS, leading to cellular damage and apoptosis[12,239,240]. Inflammation is another significant pathway, with NPs triggering pro-inflammatory cytokine release[12,240]. Direct cellular interactions, such as disruption of the cellular membrane and interference with cellular signaling pathways, also play a crucial role in NP-induced toxicity. Regardless of the mechanism, cell viability is one of the main parameters studied to determine at which concentration a substance begins to display toxicity, as seen by EC50 and LD50 values[241].

5.3. System-based impact

Overall, uPS and A-PS demonstrated a higher potential to cause negative effects, associated with higher toxicity. When analyzing these two particles according to their impact on cell viability, by system, we found that respiratory cell lines such as A549 appear particularly susceptible, followed by cell lines from the nervous system, urinary system, and connective tissues. On the other hand, the hepatic and immune systems had the lowest frequency of cell viability decrease upon exposure to uPS NPs, while A-PS exposure continued to demonstrate higher toxicity potential, across different parameters. The physiological roles of liver cells and immune cells in detoxification and defense likely contribute to the lower frequency of negative effects observed with NP exposure. Hepatocytes in the liver are specialized for metabolizing and clearing toxins, leveraging enzymes such as cytochrome P450 to neutralize potentially harmful substances. Similarly, immune cells, including macrophages, neutrophils, and lymphocytes, serve as the body's primary line of defense against foreign contaminants, utilizing phagocytosis and a robust array of immune responses to neutralize and eliminate threats. These inherent protective mechanisms could be responsible for the resilience of liver and immune cells to NPs-induced toxicity, reducing the incidence and severity of adverse

effects compared to other cell types.

5.3.1. Hepatic system

Liver dysfunction and inflammation, metabolism disorders and liver fibrosis were highlighted as potential health risks from NP exposure through an AOP[242]. Similarly to a previous statement for the digestive system, the understanding of hepatotoxicity caused by NPs could benefit from a combination of the AOP and emerging toxicological models, such as liver organoids[97] and liver-on-chips[243], which better simulate the structure and function of the liver *in vitro*. Despite the absence of frequent negative effects on the eight main parameters chosen for this review, it is of vital importance to underline that such observation does not imply the lack of other effects contributing to hepatotoxicity, as some of these effects only surface through interactions that a monoculture cannot replicate. For example, Fan et al. (2024)[244] elucidated a hepatotoxic effect of 20 nm uPS NPs, in which these particles were rapidly internalized by macrophages, accumulating in the mitochondria where they disrupted mitochondrial integrity and increased the production of mitochondrial ROS. The elevated mtROS then triggered necroptosis in macrophages, resulting in enhanced crosstalk with hepatocytes, ultimately leading to hepatocyte damage, which could only be observed in the presence of a hepatic microenvironment. Several studies, often focusing on transcriptomics and metabolomics, have shown that plastic particles affect the expression of genes involved in lipid metabolism and NPs can lead to lipid accumulation[245–251]. Exposure to plastic particles has also been linked to the disruption of the energy metabolism, by affecting the levels of ATP/ADP/AMP[252,253] metabolites or the multiple biological processes such as glycolysis, fatty acid synthesis, glucose transport, and oxidation[251].

5.3.2. Urinary system

Regarding the urinary system, *in vivo* studies often have examined results from oral exposures, where MNPs pass the digestive epithelium through translocation, a process that is affected by particle size[254]. Meng et al. (2022)[255] has demonstrated the bioaccumulation of PS-NPs of 50, 300 and 600 nm in the kidneys by histological investigation, which is consistent with previous research results[256]. Furthermore, the authors revealed that NPs' digestion promoted their aggregation and increased the zeta-potential value. In the case of particles with 600 nm, aggregation exacerbated their biotoxicity[255]. Several studies focusing on the urinary system corroborate our findings, describing the negative effects of PS-NPs with demonstrated nephrotoxicity in mice[255,257] and chicken[258] related to inflammation, as well as changes to several biomarkers responsible for kidney damage, lipid disturbance, increased oxidative stress, and ultimately, increased death rate in mice. Particle aggregation, corona formation, along with other physical and chemical processes along the digestive and respiratory tracts should be studied to better elucidate the effects on the urinary system after exposure through

these primary pathways.

5.3.3. Respiratory system

These results can be particularly worrying regarding the respiratory system since particles this small will readily pass through the airway and interact with these cells. However, it is important to notice that along the respiratory tract, these particles may acquire a pulmonary surfactant corona[259], which alters their intracellular fate and final destinations, constituting a key determinant in the risk assessment that is not represented in the selected studies due to the applied criteria[260].

5.3.4. Digestive system

The digestive system was the most studied, which can be explained by the fact that the main exposure route is through ingestion. Several polymers were analyzed for cellular lines within this system, however, PS NPs, especially uPS, continued to represent the wide majority, making it difficult to determine if the effects of other polymers can be reproduced, or will suffer from variability associated with the lack of standardized materials and methods, as well as to determine the impact of multiple variables affecting toxicity of NPs, due to insufficient data. Future studies should focus on investigating the impact of environmentally relevant NPs and take into consideration their diversity in terms of polymer type, shape, size, and surface chemistries. C-PS had a negative effect on less than 50% of the occasions studied, except for apoptosis, which was only reported once. Damage associated with A-PS and uPS were inconsistent, though as mentioned, most absence of effects could be correlated with Caco-2 and HT-29 cellular lines. Human intestinal organoids and healthy intestinal cell lines, on the other hand, experienced a negative outcome on several parameters from NPs' exposure. Although considerable evidence has supported the digestive toxicity of NPs, results and proposed mechanisms remain ambiguous due to the variety of study types, models, and endpoints. Ding et al. (2023)[261] identified the overproduction of ROS as the molecular initiating event in NPs-mediated injury to the digestive system, using an adverse outcome pathway (AOP) framework. Furthermore, a series of detrimental effects including oxidative stress, apoptosis, inflammation, dysbiosis and metabolic disorders were summarized as key events, most of which were present and identified in this review. *In vitro* studies are useful to identify and characterize the intrinsic toxicity of substances, inclusively to indicate the presence of effects associated with a potential for metabolic disorders, but cannot provide information on gut microbiota, which might contribute or influence the development of several diseases[262]. In this system, we encountered more advanced and physiologically relevant models, such as co-cultures, tricultures, and organoids, though monocultures of immortalized cell lines continued to be the most prevalent. These latter models often fail to accurately simulate the complex biological environments and dynamic processes occurring in living organisms, therefore,

advanced techniques such as organ-on-chip technology and 3D cell cultures are recommended to provide more realistic insights into NP behavior and toxicity, offering a closer approximation to *in vivo* conditions and improving the relevance of the findings. Another key aspect that is being overlooked by most of these studies is that before reaching specific cell types, NPs undergo various transformations, both in the environment and within biological systems. For instance, orally ingested NPs would be subjected to processes in the mouth, stomach and intestinal lumen before interacting with cells from the colon. These processes can alter the physical and chemical properties of NPs, contribute to aggregation, or corona formation, ultimately affecting their toxicity. For that reason, processing NP samples to most accurately represent these conditions is crucial for realistic toxicity assessments. Paul et al. (2024)[263] simulated *in vitro* digestion before exposure of Caco-2 cell lines to NPs. Although this study used two of the least represented polymer types, PMMA and PLA, and toxicity effects were minimal, it provides important context for how particle dispersion, cellular interaction, and transport through the monolayer are affected by processes along the digestive tract.

5.3.5. Immune system

Cellular lines from the immune system appear less affected by NP exposure, especially THP-1, a monocyte cell line isolated from the peripheral blood of an acute monocytic leukemia patient. However, further research is needed to better elucidate these findings and resolve discrepancies found in the literature, as there are at least two other studies describing cytotoxicity from NP exposure in differentiated THP-1 cellular lines, in terms of cell viability[264] or damage to the cytoplasmic membrane[265], through the assessment of LDH release. One of those studies further revealed an increase in necrosis after human macrophages, obtained from monocyte isolation and differentiation, were exposed to PS beads of 500 nm diameter[265]. Nevertheless, the higher resistance leukocytes demonstrate towards NP exposure despite higher internalization rates, has been previously mentioned[266], along with the variability among leukocyte cell lines also present in this review. Wolf et al. (2023)[267] performed a comprehensive study, addressing the effect of PS, PMMA and A-PS MNPs of different sizes on cells of the adaptive and innate immune system, isolated from human PBMCs. The authors highlighted that aminated particles of smaller sizes show the greatest toxicity, with macrophages being the most sensitive to their exposure and T-cells the most robust. Besides, the exposure to plastic particles affected immune checkpoint market expression on all immune cell subpopulations tested and induced a M2 phenotype in macrophages, suggesting a downregulation of inflammatory M1 phenotype. Meanwhile, our findings support the ability of NP exposure to induce different polarization states, as it was found in RT-HKM fish cell line[177] and murine RAW264.7 cell line[196]. Although few aminated particles were investigated in the included immune system studies, the results corroborate the previously stated findings pertaining to

their higher toxicity.

5.3.6. Reproductive system

It is suggested that MNPs could accumulate in reproductive organs and exert toxic effects on the reproductive system. In this review, cellular lines from the reproductive system, along with spermatozoa and oocytes, exhibited negative effects from uPS, A-PS, and even C-PS exposures in a wide majority of the cases. Cell viability and oxidative stress were affected in 69% and 73% of the uPS exposures, respectively, and the occasions where no effect was reported happened almost entirely in a study with HeLa cells with particles of over 20 nm diameter[198]. In animal studies, the damage associated with NPs' exposure consisted of abnormal ovary, uterus, testicular and sperm structure, endocrine disruption, and a decrease in sperm vitality, caused by oxidative stress, inflammation, apoptosis and abnormal hypothalamic-pituitary-adrenal axis for both sexes[268,269]. Changes pertaining to hormonal imbalances and decrease of spermatozoa vitality, based on acrosomal damage, mitochondrial functionality and spermatozoa motility were also observed in studies here included. Alongside the activation of metabolic pathways with oncogenic potential, increased oxidative stress, apoptosis and cases of autophagy, supporting the consistency in results between *in vitro* and *in vivo* studies for this particular system.

5.3.7. Gestational tissues

For gestational cell lines, correlation can be more intricate. Experimental studies in mice revealed various negative effects from NP exposures during pregnancy and lactation, mainly focusing on neurodevelopment[270–272]. In our review, studies were scarce and limited to human cell lines, out of which two studies utilized BeWo b30 cells. This model has been considered an efficient one to get an initial qualitative impression about the capacity of NPs to translocate across the placental barrier[273] and provide worst case-exposure estimates, but requires *in vivo* correlation[274]. A-PS exhibited toxicity signs in parameters such as cell viability, cytoplasmic membrane integrity, oxidative stress and MMP in one study, but a significant effect on cell viability was not present in the 5 reports of uPS exposure. Nevertheless, changes were present regarding oxidative stress, inflammation, apoptosis and MMP, which may adversely affect embryo and fetal development[275,276], influencing other organs and systems in offspring.

5.3.8. Nervous system

Similarly, for the nervous system, these results must be analyzed carefully as cells are often cultivated in a monoculture and used in direct, static exposure protocols. In reality, the probability of these particles crossing the blood-brain barrier (BBB) and interacting with these cells needs to be assessed. For instance, NPs smaller than 5 nm could cross the BBB effortlessly, but particles under 10 nm are more prone to renal clearance[277]. NPs of 100 nm

show minimal BBB penetration (2-6%) without the exposure to an external magnetic field[278], making it less plausible that they would interact with cells from the nervous system. Even particles much smaller, with a diameter of 10 nm only reached a maximum of 18% penetration[278], for which a very high environmental exposure would need to be present to represent the concentration values used in these studies. Nevertheless, some studies seem to indicate PS NPs could pass through the BBB and induce neurotoxicity in mammals potentially by inducing activation of microglia[279,280], and for that reason neurotoxicity should not be discarded and further evaluated through *in vivo* experiments.

5.3.9. Connective tissues

In this review, different connective tissues were studied, including muscular, bone, adipose tissue, and fibroblasts, of human and fish origin. Considering plastic particles may be introduced on the skin through health and beauty products, or through contact with NP-contaminated water, it is surprising that not more studies have used fibroblasts, keratinocytes, and even melanocytes for their exposure protocols. As NPs are hydrophobic, it is predicted that the absorption through the *stratum corneum* is unlikely, though these could enter the body via sweat glands, skin wounds, or hair follicles[281]. Penetration of *stratum corneum* requires particles smaller than 100 nm[63,282]. Even so, studies have demonstrated an inability for NPs as small as 20 nm to permeate this layer and embed themselves into deeper skin tissue[283,284]. Be that as it may, these results are based on interaction with an intact skin barrier. Meanwhile, using atopic dermatitis models, the inflamed skin of both mouse and pig allowed higher accumulation of 70 nm particles compared to 300 nm sized particles[285]. Since animal and human skin show substantial differences, for example, regarding lipid content and hair follicle density[286], data on transdermal penetrability obtained in animal models need to be considered with care. In addition to the size and skin condition (intact or compromised), vehicle solutions seem to be among the most important determining properties for the uptake of NPs via the skin[287]. Zou et al. (2017)[288] used neutrally charged PS nanoparticles of different sizes (25 nm, 50 nm, and 100 nm) and different vehicle solutions (water, dimethyl sulfoxide (DMSO), and ethanol) on *ex vivo* human skin samples from five different donors. When using DMSO as a vehicle, the PS nanoparticles penetrated deeper compared to ethanol and water and have been found in the *stratum granulosum* layer. Not to mention that ingredients such as urea, glycerol and α-hydroxyl acids, widely used in body lotions, can enhance the ability of nanoparticles to permeate the skin barrier[289]. Gopinath et al. (2021)[290] observed adherence and cytotoxicity of PE NPs (400 nm), PS NPs (100 nm), and facial-scrubs isolated NPs (30-300 nm) on HaCaT cells, revealing a concentration-dependent cytotoxic, cytostatic and cytoprotective activity in keratinocytes. Surprisingly, the authors reported no influence of PS NPs on cell viability, but both PE and facial-scrub NPs caused a decrease in cell viability after

24h or 48h. In fact, higher toxicity-related effects were observed for facial-scrub NPs, possibly due to differences in particle shapes and presence of cationic polymers, associated with a positive surface charge.

5.4. Challenges and recommendations

Our understanding of NP toxicity through improved methodologies, standardized protocols, and comprehensive analysis is critical for assessing their potential risks and developing effective regulatory strategies to protect human health and the environment. For that purpose, protocols and methodologies should be standardized to enhance reproducibility and study comparability. Future research should focus on using advanced *in vitro* models that better replicate physiological conditions of tissues and organs, as well as using environmentally relevant plastic particles, such as PE. Protocols should also consider the properties of particles and how they affect buoyancy, adjusting to more dynamic exposure models, accordingly. Existing studies have primarily focused on PS, leaving a significant knowledge gap regarding the effect of other commonly used polymers, which may have different physical and chemical properties that influence their interactions with biological systems and their toxicity profiles. Alongside, most studies utilize commercially available nanobeads, which are spherical. Not only does that leave out particles such as fibers and fragments that may behave differently in terms of toxicity potential, but these come in solutions with a dispersant that may, on its own, cause toxicity, as shown by one of the included studies. NP samples should also be processed to represent as closely as possible the natural state at which they would be present in the biological model studied. That means chemically and/or physically subjecting these particles to processes that mimic functions like digestion. The lack of strong evidence regarding quantitative values of NPs in nature, due to technological limitations, may be reflected in excessively high concentrations of exposure being used, in an attempt to estimate worst-case scenarios. Owing to that, negative effects encountered might not as easily be representative of potential toxicity in animals or humans exposed to environmental NPs. Finally, many studies focus on acute exposure, overlooking the potential long-term effects of NP exposure. Chronic exposure studies can reveal more about the persistence and cumulative effects of NPs, which are critical for assessing real-world risks. However, this can be challenging with cellular lines as their maintenance is limited by the number of passages and immortalized cells can acquire genetic and phenotypic changes over time with potential to influence results.

6. Conclusion

This systematic review highlights the complex and multifaceted nature of NP toxicity, demonstrating that factors such as size, surface charge, and polymer type significantly influence the harmful effects of these particles. Over the past decade, there has been an exponential growth in research on NP toxicity, particularly in countries like China, which has become a leading force in studies in this field. Our findings indicate that the polymer's physical and chemical properties play a crucial role, with PET showing a lower toxicity potential compared to PS. However, the current research predominantly focuses on spherical PS particles, underscoring the need for more studies on other nanosized polymers to form comprehensive conclusions. The surface charge of NPs also emerged as a critical factor, with positively charged A-PS particles exhibiting higher toxicity across various cell lines, while negatively charged particles like C-PS were less toxic. Respiratory cell lines, particularly A549 and BEAS-2B, were more susceptible to NP exposure, in contrast to hepatic and immune system cell lines, which showed greater resilience likely due to their biological detoxification and defense functions. Digestive cell lines had multiple responses, with cancer cell lines demonstrating higher resistance compared to healthy ones, as expected. This review identifies significant knowledge gaps, particularly the reliance on high concentrations of spherical PS particles, which do not accurately reflect real environmental conditions. Also, the lack of standardized methodologies further complicates the comparability of studies, as the same parameter may be obtained through different methods. To address these issues, future research should incorporate diverse polymer types and shapes, environmentally relevant exposure levels, and standardized protocols. Advanced models like organoids and organ-on-chip systems, as well as processing of NP particles to represent the natural state at which they would be present in the biological model studied, are recommended to better simulate physiological conditions and provide more accurate toxicity assessments. These steps are essential to fill the existing knowledge gaps and accurately determine the environmental and health impacts of NPs.

7. References

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Annexes

Annex 1 – Overview of techniques for NP detection and quantification.

Light scattering

Methodology	Range/Limits	Advantages	Disadvantages
DLS	1 nm-10 µm at concentrations of ~10^{-8} 10^{-12} particles mL^{-1}	• Fast • Cheap • In situ • Non-invasive • Aggregation • Direct coupling	• Large particles • Polydispersity • Complex matrix • Non-spherical particles
DDLS		• Non-destructive • Suitable for polydisperse and non-spherical particles	• Differentiate plastic particles from other organic matter
NTA	10 nm-1 µm at concentrations of ~10^{-7}–10^{-9} particles mL^{-1}	• Non-invasive • Single particle tracking	• Limited due to polydisperse population with similar size • Inability of chemical analysis
AF4-MALS	>10 nm at concentrations down to 15–33 µg mL^{-1}	• Large range of sizes • Better for polydisperse samples	• Operational complexity • Possibility of particle interaction with column membrane
TDA	Feasible for particles with sizes <127 nm	• Non-destructive • Less affected by polydispersity • Suitable nanoplastic particles	• Particle size is limited by the diameter of used capillary
LD	10 nm–10 mm conc. 10^{-5} –10^{-1}	• Large size range • Easy • Fast • Automated	• Only spherical models

Microscopy

Methodology	**Range/Limits**	**Advantages**	**Disadvantages**
SEM	>5 nm	• Non-destructive • High resolution	• Chemical insights require complementary procedure • Difficult quantification Expensive • Charging effect
TEM	>0.1 nm		
Fluorescence Microscopy	Spatial resolution >120 nm × 120 nm, depends upon the type of microscope	• Non-destructive • Possibility for 3D imaging • Particle tracking *in-situ* • Sub-diffraction variants	• Environmental plastics are not fluorescent
Stereo Microscopy	>5.9 µm	• Non-destructive • Simple • Rapid detection • Inexpensive	• No information about chemical structure • Imprecise particle concentration
Dark Field Microscopy	>250 nm	• Non-destructive • Simple • Rapid	• Chemical insight only with complementary procedure • Spatial resolution is diffraction limited, • Interference with complex matrices
Hyperspectral Imaging	≥100 µm	• Non-destructive • *In-situ* particle detection • Facile and rapid processing • Label-free imaging, • High reliability and reproducibility	• Spatial resolution is diffraction limited • Interference with organic and inorganic matter in the matrix • Requires calibration or construction of a reference library before analysis

		• Option for semi-automation analysis	
AFM	12 µm to <1 nm	• Non-destructive • High-resolution • Samples might be dried or suspended • 3D imaging	• Time consuming • Small area • Artifacts due to particle movement
AFM-IR	< 1 µm, but >50 nm	• Non-destructive • 3D imaging	

Spectroscopy

Methodology	**Range/Limits**	**Advantages**	**Disadvantages**
FTIR	>10 µm	• One analyzer used for detection of several gases • Non-destructive • Semi-continuous	• Interference from chemicals and other organic matter • Overlapping species made spectra analysis difficult
Raman	1-100 µm	• Fast • Easy sample preparation • Semi-automation possible • Non-destructive • No interference from water	• Fluorescent molecules alter particle signals • Time consuming data collection • Difficult analysis of atmospheric nanoplastics.
SERS	Analyte concentration ≥10 µg/mL	• Non-destructive technique • High sensitivity.	• Substrate specific
Raman-Optical Tweezers	≥100 nm, Concentration of particles >1 mg/ L	• Non-destructive • Detects size and chemical composition concurrently	• Optical trapping due to Brownian motion of particles

		• Well established for polydisperse particle samples	
XPS	Viable to samples containing >0.1 wt% of the element of interest. 10-100 µm by monitored photoemissions.	• Non-destructive • Surface characterization • Able to detect degree of oxidation	• Requires high vacuum environment • Analysis restricted to nanometer range. Laborious
EDX/EDS	Useful for 100 nm PS particles' analysis, spectrum resolution limited to >1 wt% of the element	• Non-destructive technique • Complementary to EM	• Insufficient elemental information
NMR	19–21 µg/mL of the material under study	• Cost effective • Detection limits are size independent	• Lower detection sensitivity • Extended measurement times • Requires pure samples

Thermal techniques

Methodology	**Range/Limits**	**Advantages**	**Disadvantages**
DSC	Limited to a minimum sample mass of >0.2 mg, dependent on the type of equipment used	• Indicate crystallinity • Distinguishes between polymers	• Sample destruction • Unable to analyze plastic nanoparticles within complex environments • Sample pre-treatment required
Pyr-GC-MS	Samples containing >1 µg	• Perfect for identification of polymeric particle content and sample quantification • Less affected by matrix impurities • Little sample preparation	• Destructive technique • Complex data processing

TED-GC-MS	Quantifiable for <20 mg of samples, containing >0.5 wt% of the desired material	• Higher sample masses • Fast • Measurement with matrix	• Destructive technique • Needs calibration before analysis
MALDI-ToF-MS	Useful for samples containing >25 ng of desired material.	• Able to detect unfragmented molecules	• Thermal destructive technique • Sample pre-treatment required
TD-PTR-MS	Viable for samples containing <1 mg	• Sensitive method • Analysis of samples with small volumes • Identification of unique fragmented ions	• Challenging methodology
SP-ICP-MS	135 nm - 1 µm with a concentration of 3.5×10^8 particles/L	• Useful for inorganic nanoparticles	• Background noise from atmospheric CO_2 • Difficult calibration
ToF-SIMS Imaging	Detection of PS microplastics of 20 µm in diameter Lateral resolution limit down to >70 nm and for depth > 9 nm	• Simultaneously data collection	• Destructive technique • Complex data analysis • Unable to differentiate plastics from other organics

Table 1 – Overview of techniques for NP detection and quantification. ***AF4-MALS*** *– asymmetrical flow field-flow fractionation with multi-angle light scattering;* ***AFM*** *– atomic force microscopy;* ***AFM-IR*** *– infrared atomic force microscopy;* ***DDLS*** *– depolarized dynamic light scattering;* ***DLS*** *– dynamic light scattering;* ***DSC*** *– differential scanning calorimetry;* ***EDX/EDS*** *– energy-dispersive X-ray spectroscopy;* ***LD*** *– laser diffraction;* ***MALDI-ToF-MS*** *– matrix-assisted laser desorption/ionization time-of-flight mass spectrometry;* ***NMR*** *– nuclear magnetic resonance;* ***NTA*** *– nanoparticle tracking analysis;* ***Pyr-GC-MS*** *– pyrolysis gas chromatography-mass spectrometry;* ***SERS*** *– surface-enhanced raman spectroscopy;* ***SEM*** *– scanning electron microscopy;* ***SP-ICP-MS*** *– single particle inductively coupled plasma mass spectrometry;* ***TDA*** *– taylor dispersion analysis;* ***TD-PTR-MS*** *– thermal desorption proton-transfer reaction mass spectrometry;* ***TED-GC-MS*** *– thermal extraction desorption gas chromatography-mass spectrometry;* ***TEM*** *– transmission electron microscopy.*

Annex 2 – Summary of hepatic cell line studies

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Brandts, I. et al.	2020	Spain	ZFL	uPS	≈ 65	0.05, 0.5, **5**, 10, 25, **50**, 100, 250, 500 and 1000 mg/L	12, 20, 24h and 36h	↓: Cell viability (20h, >100 mg/L) =: Antiviral response and cell peroxidation status	291
Englert, F.H. et al.	2023	Switzerland	HepaRG	uPS	25 and 100	0.1–1000 µg/mL (**50** µg/mL)	24h and 10 days	=: Cell viability (uPS25/100), oxidative stress (uPS25/100) ↑: Inflammation[#] and lipid accumulation[#] (uPS25/100)	158
				wPS				↓: Cell viability (wPS25/100) ↑: Oxidative stress (wPS25, >3.6 µg/mL; wPS100, >49.8 µg/mL) =: Inflammation[#] (wPS25/100)	
He, Y. et al.	2020	China	HepG2	uPS	50	**10, 50,** and **100** µg/mL	24h	↓: Cell viability[+] (≈75% with 100 µg/mL and ≈84% with 50 µg/mL) Others: Study suggests increase in oxidative stress, but the statistical analysis is not well elucidated. A-PS and C-PS showed more severe cytotoxicity than uPS.	292
				C-PS					
				A-PS					
Huang, J. et al.	2023	China	AML-12	uPS	20, 50, 100, 200 and 500	10, 25, 50, 100, **200**, 400 and 800 µg/mL (**300** µg/mL)	4h	↓: Cell viability (uPS20, > 400 µg/mL) and MMP (uPS20, 200 µg/mL) ↑: Apoptosis (uPS20, 200 µg/mL) and oxidative stress (uPS20, 300 µg/mL) =: MMP (uPS100), cell viability, oxidative stress and apoptosis (uPS50-500)	293
			LO2			5, 10, 25, 50, 100, 250 and 500 µg/mL		↓: Cell viability (uPS100, 500 µg/mL; uPS50, >100 µg/mL; uPS20, >50 µg/mL)	

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Li, Y. et al.	2023a	China	LO2	uPS	**20**, **60**, 100 and 500	1, 5, **25**, 75, and 125 µg/mL	24h	↓: Cell viability[+] (uPS20), cytoplasmic membrane integrity[+] (uPS20/60) ↑: Oxidative stress* (uPS20/60), inflammation[#] and apoptosis[#] (uPS20) =: Cell viability (uPS100/500), inflammation[#] and apoptosis[#] (uPS60)	294
Li, Y. et al.	2023b	China	HepG2	uPS	20	6.25, 12.5, 25 and **50** µg/mL	24h	↓: Cell viability (>6.25 µg/mL), mitochondrial biogenesis (>12.5 µg/mL) ↑: Oxidative stress, apoptosis, and mitochondrial fission (>12.5 µg/mL)	162
Paul, M.B. et al.	2023	Germany	HepaRG	PMMA	25	1×10^9 µm^2 particle surface/mL	24h	No significant negative effects	161
				PLA	250			↑: Inflammation[#] =: Oxidative stress* and barrier integrity	
Roursgaard, M. et al.	2022	Denmark	HepG2	PET	< 600 (x = 252)	1, 2, 4, 8, **16**, **32**, **63** ng/mL	24h	↓: Cell viability[b] ↑: DNA damage[b] (>16 ng/mL) =: Oxidative stress, cytoplasmic membrane integrity and cell cycle distribution	157
				PP	< 700 (x = 158)	3, 5, 11, 22, **44**, **88**, **175** ng/mL		No significant changes were found for cell viability, cytoplasmic membrane integrity, DNA damage and cell cycle distribution	

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Stock, V. et al.	2022	Germany	HepaRG	C-PS	20, 40 and 100	$5x10^9$ - $5x10^{11}$ µm² particle/mL	24h	↓: Cell viability (C-PS20, >$2.5x10^{11}$ µm² particle/mL; C-PS40, $1x10^{12}$ µm² particle/mL) ↑: Apoptosis (C-PS20, >$2x10^{11}$ µm² particle/mL) =: Cell viability (C-PS100) and apoptosis (C-PS40/100)	87
				A-PS	100	$5x10^9$ - $2.5x10^{11}$ µm² particle/mL		↓: Cell viability ($2.5x10^{11}$ µm² particle/mL) ↑: Apoptosis (>$2x10^{11}$ µm² particle/mL)	
				uPS					
				S-PS		$1x10^{10}$ - $5x10^{11}$ µm² particle/mL		=: Cell viability and apoptosis	
Tolardo, V. et al.	2022	Italy	HepG2	PC	≈ 47	**1, 10, 20, 40** or 80 µg/mL	24-48h	↓: Cell viability[a] (PC, >10 µg/mL; PET58, >20 µg/mL; PET89, >40 µg/mL) =: Nuclear size and intensity	159
				PET	≈ 58 and ≈ 89				
Tolardo, V. et al.	2023	Italy	2º Gen UHHs	PC	≈ 31.5	**10, 20, 40**, 60, 80 and 100 µg/mL;	24-48h	↓: Cell viability and cytoplasmic membrane integrity (100 µg/mL), and albumin levels (>40 µg/mL) Others: disruption of cytochrome P450 system.	160
Zheng, T. et al.	2019	China	Rat hepatocyte suspensions (C57BL6-J)	uPS	50	**1, 5, 10, 20,** and **30×10^{-6}** mol/L	24h	↑: Oxidative stress (>$10x10^{-6}$ mol/L) and DNA damage (>$5x10^{-6}$ mol/L)	295

*Table 2 - Summary of hepatic cell line studies: NP polymer type, size, concentration and main findings. In **bold**, values that were carried across in the assessment of different parameters besides cell viability. [+]No statistical analysis. *Based on ROS production. [#]Based on interpretation of mRNA levels and protein levels. [a]Determined by HCS. [b]Based on the slope instead of a single concentration. A-PS = Amine-modified Polystyrene. C-PS = Carboxyl-modified Polystyrene. MMP = Mitochondrial Membrane Potential. PC = Polycarbonate. PET = Polyethylene terephthalate. PLA = Polylactic Acid. PMMA = Polymethyl methacrylate. PP = Polypropylene. S-PS = Sulfate-modified Polystyrene. uPS = unmodified Polystyrene. wPS = weathered Polystyrene.*

Annex 3 – Summary of urinary cell line studies

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
He, S. et al.	2024	China	HK2	uPS	≈ 158	50, 250, **500**, 750, and 1000 µg/mL	12h	↑: Oxidative stress* =: Cell viability and inflammation[#]	163
				wPS	≈ 124			↓: Cell viability (> 750 µg/mL) ↑: Cell viability (250 µg/mL), oxidative stress*, inflammation[#]	
Li, Y. et al.	2023a	China	293T	uPS	**20**, **60**, 100 and 500	1, 5, **25**, 75, and 125 µg/mL	24h	↓: Cell viability[+] (uPS20-100) and cytoplasmic membrane integrity[+] (uPS20/60) ↑: Oxidative stress* (uPS20/60), inflammation[#] and apoptosis[#] (uPS20)	294
Xiao, M. et al.	2023	China	HK2	uPS	50	50, 100, **200**, 500, and 1000 µg/mL	24h	↓: Cell viability[+], barrier integrity[c] ↑: Inflammation, oxidative stress and apoptosis[#]	205
Zhu, Z. et al.	2023	China	HK2	uPS	**20** and 50	**50** and **100** µg/mL	12h	↓: Cell viability (uPS20/50, 100 µg/mL), cytoplasmic membrane integrity (uPS20/50, >50 µg/mL), MMP (100 µg/mL) ↑: Oxidative stress* (100 µg/mL), early apoptosis (>50 µg/mL) and late apoptosis (100 µg/mL)	296

*Table 3 - Summary of urinary cell line studies: NP polymer type, size, concentration and main findings. In **bold**, values that were carried across in the assessment of different parameters besides cell viability. [+]No statistical analysis. *Based on ROS production. [#]Based on interpretation of mRNA levels and protein levels. [c]Based on decrease of claudin-2 protein levels. MMP = Mitochondrial Membrane Potential. uPS = unmodified Polystyrene. wPS = weathered Polystyrene.*

Annex 4 – Summary of respiratory cell line studies

Author	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Alzaben, M. et al.	2023	Denmark	A549	PET	≈ 167	**1.95, 15.6,** and **125** µg/mL	3, 24h	↑: DNA damage[b] and oxidative stress*[b] =: Cell viability and cytoplasmic membrane integrity (≤125 µg/mL)	171
Chen, Y.C. et al.	2023	Hong Kong	BEAS-2B	uPS	≈ 153	**1**, 10, 100 and **1000** ng/cm^2	24h	↓: Cell viability (>10 ng/cm^2) ↑: Oxidative stress* (>1 ng/cm^2), inflammation$^{\#}$ (1 ng/cm^2), apoptosis and autophagy (1000 ng/cm^2)	166
Halimu, G. et al.	2022	China	A549	uPS	50	**40, 80** and **160** µg/mL	24h	↓: Cell viability (uPS50 >40 µg/mL, and uPS20 >20 µg/mL) and MMP (uPS50 160 µg/mL, and uPS20 >40 µg/mL) ↑: Oxidative stress* (uPS20/50) and mitochondrial impairment (uPS20/50)	164
				uPS	20	**10, 20** and **40** µg/mL			
				A-PS	20			↓: Cell viability (>20 µg/mL), MMP (>20 µg/mL) ↑: Oxidative stress* and mitochondrial impairment	
Huang, J. et al.	2022	China	HNEpCs	uPS	20, 50, 100, 200 and 500	10, 50, 125, **500**, and 1250 µg/mL	48h	↓: Cell viability: (uPS20/100/500, >125 µg/mL; uPS50, >10 µg/mL; uPS200, 1250 µg/mL) ↑: Apoptosis^{+} (uPS20-500, 500 µg/mL)	173
				C-PS	50, 100 and 500			↓: Cell viability (C-PS50/500, >500 µg/mL; C-PS100, >125 µg/mL)	
				A-PS				↓: Cell viability (A-PS50/500, >10 µg/mL; A-PS100, >125 µg/mL) ↑: Necrosis^{+} (A-PS50, 500 µg/mL)	
Lim, S.L. et al.	2019	Singapore	BEAS-2B	uPS	50	1, 5, **10**, 25, **50**, 75, 100 µg/mL	24h	↓: Cell viability (>10 µg/mL), ATP levels ↑: Oxidative stress*, ER stress$^{\#}$ (50 µg/mL) and autophagy[a] Others: PS NPs interfered with the energy metabolism[&]	165

Author	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Liu, Y. et al.	2022	China	A549	uPS	20	5, **10**, 20, 40 and 80 μg/mL	24h	↓ Cell viability (uPS20, >5 μg/mL)	297
Shi, Q. et al.	2021	China	A549	uPS	100	10, **20**, 100, **200**, 500 or 1000 μg/mL	24h	↓: Cell viability (>200 μg/mL) ↑: Oxidative stress* and inflammation# (200 μg/mL)	298
Shi, X. et al.	2022	China	A549	uPS	80	2.5, 5, 10, 25, **50, 100, 200** and **400** μg/mL	6, 9, 24h	↓: Cell viability (>50 μg/mL) ↑: DNA damage and oxidative stress* (>100 μg/mL)	169
				C-PS				↓: Cell viability (>2.5 μg/mL) ↑: DNA damage (>50 μg/mL) and oxidative stress* (>100 μg/mL)	
				A-PS					
Soto-Bielicka, P. et al.	2023	Spain	RTgill-W1	C-PS	40	0.1–200 μg/mL (**10 μg/mL**)	24h	↓: Cell viability (>25 μg/mL) =: Cytoplasmic membrane integrity, MMP, DNA damage and oxidative stress (10 μg/mL)	168
Wu, Q. et al.	2024	China	BEAS-2B	uPS	20	0.05, 0.15, **0.2** mg/mL	24h	↓: Cell viability (>0.15 mg/mL) ↑: Oxidative stress*, inflammation# and apoptosis (0.2 mg/mL)	299
Wu, Y. et al.	2023a	China	BEAS-2B	uPS	100 and 200	50, **100, 200** and **400** μg/mL	24h	↓: Cell viability (uPS100/200, >100 μg/mL) ↑: Oxidative stress* (uPS100/200, >100 μg/mL) Others: PS NP exposure might induce ferroptosis in BEAS-2B cells through the HIF-1α/HO-1 signaling pathway	172
Wu, Y. et al.	2023b	China	MLE-12	A-PS	100	**12.5** μg/mL	12h	↑: Inflammation# and apoptosis#	300
			MH-S					↑: Inflammation# and oxidative stress#	
Xu, M. et al.	2019	China	A549	uPS	25	2.5, 5, 10, 15, 20, **25** and 30 μg/mL	24h	↓: Cell viability (uPS25, >25 μg/mL and uPS70, >160 μg/mL) ↑: Apoptosis (uPS25/70) and inflammation# (uPS25/70) Others: Both NPs halted the cell cycle time-dependently at the S phase	167
					70	10, 30, 60, 100, **160**, 220 and 300 μg/mL			

Author	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Yang, S. et al.	2021	China	HPAEpiC BEAS-2B	uPS	40	8, 16, **24**, 32, **48**, 64, 80, **96**, 112 and 128 µg/mL	24h	↓: Cell viability (>48 µg/mL), cytoplasmic membrane integrity (>24 µg/mL) and barrier integrity (>24 µg/mL) ↑: Oxidative stress, inflammation[#] and apoptosis	301
Zhang, H. et al.	2022	China	A549	PET	≈ 122-221	0.098, 0.98, **4.92**, 9.84, 24.6, **49.2**, 98.4 and 196.76 µg/mL	24h	↓: Cell viability (>98.4 µg/mL); apoptosis (early+late and early) ↑: Oxidative stress (>49.2 µg/mL); late apoptosis (196.76 µg/mL) =: MMP	170

*Table 4 - Summary of respiratory cell line studies: NP polymer type, size, concentration and main findings. In **bold**, values that were carried across in the assessment of different parameters besides cell viability. *Based on ROS production. [#]Based on interpretation of mRNA levels and protein levels. [+]No statistical analysis. [b]Based on the slope instead of a single concentration. [a]Based on the increase of LC3-II. [&]Changes in metabolite profiles, primarily glucose, lactate and alanine. A-PS = Amine-modified Polystyrene. ATP = Adenosine Triphosphate. C-PS = Carboxyl-modified Polystyrene. MMP = Mitochondrial Membrane Potential. PET = Polyethylene terephthalate. uPS = unmodified Polystyrene.*

Annex 5 – Summary of digestive system cell line studies

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Banaei, G. et al.	2023	Spain	Co-culture (Caco-2/HT29)	PLA	≈ 280	**50** and **100** µg/mL	48-72h	↓: Barrier integrity (3h, 50 µg/mL) =: Cell viability, oxidative stress*, barrier permeability and barrier integrity (>3h)	185
Banerjee, A. et al.	2021	USA	SNU-1	uPS C-PS A-PS	50, 100, 200 and 500	0.1-**100** µg/mL	24h	↓: Cell viability (uPS50, ≥75 µg/mL; A-PS50, >7.5 µg/mL; A-PS100, >50 µg/mL; A-PS500, >10 µg/mL) =: Cell viability (C-PS50-500, A-PS200 and uPS100-500) Others: No other comparison to control was made; comparison between particles reveals higher apoptotic/necrotic effects in A-PS particles and smaller particles (50 nm)	88
Brandts, I et al.	2023	Spain	RTgutGC	uPS	44	**25** and **50** µg/mL	1, 12 and 16h	No significant differences for cell viability, respiratory capacity and oxidative stress* were observed	177

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Busch, M. et al.	2021	Germany	Caco-2	uPS A-PS	50	1, 5, **10** or **50** µg/cm^2	24h	↓: Cell viability (A-PS, >1 µg/cm^2) and cytoplasmic membrane integrity (A-PS, 50 µg/cm^2) ↑: DNA damage (A-PS, 50 µg/cm^2) =: Cell viability, cytoplasmic membrane integrity and DNA damage (uPS)	91
			HT29-MTX-E12					↓: Cell viability (A-PS, >10 µg/cm^2) and cytoplasmic membrane integrity (A-PS, >10 µg/cm^2) ↑: DNA damage (A-PS, 50 µg/cm^2) =: Cell viability, cytoplasmic membrane integrity and DNA damage (uPS)	
			Triculture (Caco-2/HT29/THP-1)					↓: Cytoplasmic membrane integrity (A-PS, 50 µg/cm^2) =: Cytokine release (uPS)	
Chen, W. et al.	2021	China	Caco-2	uPS	≈ 80	3.125, 6.25, 12.5, 25, 50, **100** and 200 µg/mL	24h	↓: Cell viability (>100 µg/mL) and impaired autophagic flux ↑: Oxidative stress*	302

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Cortés, C. et al.	2020	Spain	Caco-2	uPS	40-100	**1, 25, 50, 100**, 125, 150, 175, 200 µg/mL	24-48h	↓: MMP (1 µg/mL) ↑: MMP and oxidative stress response[#] =: Cell viability, inflammation[#], oxidative stress* and DNA damage Others: At the highest concentration (200 µg/mL) there was a decrease of cell viability to 80% relative to control[+]	303
Cui, M. et. al	2023	China	Co-culture (Caco-2/HT29-MTX)	C-PS	**20** and 200	**1, 10, 100**, 200, 400, 800, **1000**, 1200, 1600 and 2000 µg/mL	24-48h	↓: Cell viability (C-PS20, Caco-2, >1000 µg/mL; HT29-MTX, >400 µg/mL; and co-culture >1600 µg/mL) and barrier integrity (C-PS20, >100 µg/mL) ↑: Oxidative stress* (>100 µg/mL) and model's mucus secretion (>10 µg/mL) =: Cell viability (C-PS200, Caco-2, HT29-MTX and co-culture) Others: HO1/p38/IL-10 axis was involved in the MUC2 induction and the increased mRNA levels of HO-1 and IL10 were due to Caco-2 cells	178
DeLoid, G.M. et al.	2021	USA	Triculture small intestinal epithelial model (Caco-2, HT29-MTX and M-cells)	uPS	25	**0.4** and **1** mg/mL	4 and 24h	=: Cell viability, barrier integrity, membrane permeability, cytoplasmic membrane integrity and oxidative stress*	180
				C-PS	25 and 100			↓: Cell viability (C-PS25, 1 mg/mL; C-PS100, >0.4 mg/mL) ↑: Membrane permeability (PS100C, 0.4 mg/mL) =: Barrier integrity, cytoplasmic membrane integrity and oxidative stress* (C-PS25/100)	

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Ding, R. et al.	2024	China	GES-1	uPS	80, 200 and 500	12.5, 25, **50**, 100 and 200 µg/mL	24h	↓: Cell viability (uPS80, >50 µg/mL; uPS200/500, > 100 µg/mL) ↑: Oxidative stress* (uPS80-500) and DNA damage[#+] (uPS80-500) Others: activation of the β-catenin/YAP cascade	175
Ding, Y. et. al	2021	China	GES-1	uPS	60	**50** µg/mL	2, 4, 6, 12, 24 and 48h	↓: Cell proliferation (24/48h); MMP (12/24h) ↑: Apoptosis ratio (12/24h), autophagosomes and autolysosomes formation (12-48h) =: Apoptosis (48h)	304
Domenech, J. et al.	2020	Spain	Co-culture (Caco-2/HT29)	uPS	40-100	**1, 25, 50, and 100** µg/mL	24h	=: Cell viability, cytoplasmic membrane integrity, DNA damage and oxidative stress*[#]	305
Domenech, J. et al.	2021 a	Spain	Caco-2	uPS	50	0.0006, 0.26, 1.3 and 6.5 µg/cm^2	8 weeks	↑: Oxidative stress response[d] (8 weeks) and DNA damage (8 weeks, 0.26 µg/cm^2) =: Cell viability, oxidative stress* (24h and 8 weeks) and DNA damage (24h)	127
						0.26, 6.5, 13, 26, and 39 µg/cm^2	24h		
Domenech, J. et al.	2021 b	Spain	Caco-2	uPS	40-100	**10**, 25, 50, **100**, 125, 150, 175, and 200 µg/mL	24h	=: Cell viability, oxidative stress* and DNA damage	306
Guanglin, L. et al.	2024	China	HET-1A HEEC	uPS	100	10, **30**, and 50 µg/mL	24h-72h	↓: Cell viability (>10 µg/mL) ↑: Inflammation[#], apoptosis (>10 µg/mL), oxidative stress* (30 µg/mL) Others: Cell inflammation and death may be caused by Fe^{2+} accumulation	307

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
He, Y.J. et al.	2022	China	NCM460	uPS	100	**50** μg/mL	24h	↓: barrier integrity[#] ↑: Oxidative stress* and inflammation[#]	308
Hesler, M. et al.	2019	Germany	Co-culture (Caco-2/HT29-MTX-E12)	C-PS	50	0.01, 0.1, 1, 5, **10**, 25, 50 and **100** μg/mL	24-48h	↓: Cell viability (100 μg/mL) =: Barrier integrity	179
Hou, Z.K. et al.	2022	China	Human intestinal organoid culture from HiPSCs	uPS	50	**10**, 50, 100, 150, and 200 μg/mL	Cell viability: 24-48h; Others: 14 days	↓: Cell viability (48h, >150 μg/mL) ↑: Apoptosis, oxidative stress* and inflammation[#] =: Cell viability (24h, ≤200 μg/mL)	98
Jin, M.H. et al.	2023	China	Caco-2	uPS	80	50, 100, 200, 300, 400, **500**, 600 and 700 μg/mL	24h	↓: Cell viability (>400 μg/mL), MMP, barrier integrity and lysosomal function ↑: Oxidative stress*, apoptosis and autophagy[#]	309
Kaur, J. et al.	2023	Sweeden	HT-29	C-PS	200	0.1–**100** μg/mL	24h	↓: Barrier integrity (C-PS, 100 μg/mL, with or without FBS) =: Cell viability	181
				A-PS				=: Barrier integrity and cell viability	
Li, C. et al.	2023	China	Caco-2	uPS	100	**100** and **200** μg/mL	48h	=: Cell viability, inflammation and oxidative stress[#]	310
Liu, Y. et al.	2022	China	Caco-2	uPS	20 and 100	5, **10**, 20, 40 and 80 μg/mL	24h	↓: Cell viability (uPS20, >5 μg/mL and uPS100, >10 μg/mL) and MMP (uPS20/100, 10 μg/mL) ↑: Oxidative stress* (uPS20/100, 10 μg/mL) =: Cytoplasmic membrane integrity (uPS20/100, 10 μg/mL)	297

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Magrì, D. et al.	2018	Italy	Caco-2	PET	≈ 26,7	**1, 5, 15,** and **30** µg/mL	24, 72, and 96 h	=: Cell viability, cytoplasmic membrane integrity, apoptosis/necrosis, oxidative stress* and inflammation[#]	[184]
Magrì, D. et al.	2021	Italy	Caco-2	PET	10-80 (x = 30)	**30** µg/mL	24-96h	↓: Alanine production and lactate/glucose ratio ↑: Essential and non-essential aminoacids' consumption (phenylalanine, threonine, lysine, valine, methyonine, glycine, tyrosine and glutamine) and lactate/alanine ratio =: Cell viability, oxidative stress* and acetate consumption	[183]
Paul, M.B. et al.	2023	Germany	Caco-2	PMMA	25	**1×10^9** μm^2 particle surface/mL	24h	No consistent significant effects were observed but there was an increase in NFkB and CAT with a decrease in GSTP1 indicating possible response to oxidative stress/inflammation	161
				PLA	250			↑: Inflammation[#] =: Oxidative stress* and barrier integrity	
Peng, M. et al.	2024	Belgium	Caco-2	wPET	<800 (x = 144)	10^2 to 10^7 particles/mL	48h	↑: ECAR and glycolytic ATP	43
				PET	<800 (x = 197)			↑: OCR, ECAR and mitochondrial ATP	
				wPS	100+750			No significant differences were found in OCR, ECAR, mitochondrial or glycolytic ATP	
				uPS				↑: ECAR	

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Roursgaard, M. et al.	2022	Denmark	Caco-2	PET	<600 (x = 252)	1, 2, 4, 8, **16, 32** and **63** ng/mL	24h	↓: Cell viability[b] and cytoplasmic membrane integrity[b] ↑: DNA damage[b] (>16 ng/mL) =: Oxidative stress* (3h) and cell cycle distribution	157
				PP	<700 (x = 158)	3, 5, 11, 22, **44, 88** and **175** ng/mL		=: Cell viability[#], cytoplasmic membrane integrity[#], DNA damage, oxidative stress* (3h) and cell cycle distribution	
Sun, R. et al.	2024	China	GES-1	uPS	50	10, **20, 40** and **80** µg/mL	24-48h	↓: Cell viability (>20 µg/mL) and MMP (80 µg/mL) ↑: Apoptosis (early, late and total), mitochondrial dysfunction[#] and oxidative stress* (>40 µg/mL)	174
					250			↓: Cell viability (>40 µg/mL) ↑: Early apoptosis (80 µg/mL) and oxidative stress* (>40 µg/mL) =: MMP, late and total apoptosis	
Tolardo, V. et al.	2022	Italy	Caco-2	PC	≈ 47	**1, 10, 20, 40** or 80 µg/mL	24-48h	↓: Cell viability (>10 µg/mL) and mitochondrial activity (80 µg/mL) =: Nuclear size and intensity Others: EC50 at 48h was 44.62 µg/mL and the dispersant also caused a significant decrease in cell viability	159
				PET1	≈ 58			↓: Cell viability (80 µg/mL) ↑: Cell viability (10 and 20 µg/mL) =: Nuclear size and intensity Others: EC50 at 48 was 40.06 µg/mL and the dispersant also caused a significant decrease in cell viability	
				PET2	≈ 89			↓: Cell viability (>20 µg/mL) =: Nuclear size and intensity Others: EC50 at 48h was 82.12 µg/mL	

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Xu, D. et al.	2021	China	Caco-2	uPS C-PS A-PS	100	**30**, 60, 120, 240, 480 µg/mL	24, 48 or 96h	↓: Cell proliferation (24h, uPS100, 480 µg/mL; 24h, A-PS100, >60 µg/mL; 96h, all polymers, >30 µg/mL) and barrier integrity ↑: Cell arrest in G0/G1 phase, apoptosis and n° of lysosomes per cell (48h, 30 µg/mL) =: Cytoplasmic membrane integrity (30 µg/mL)	182
Xu, X. et al.	2023	China	RKO	uPS	100	1, **10**, 50, 100 µg/mL	24-48h	↓: Cell viability (48h, >50 µg/mL) ↑: Apoptosis (100 µg/mL) and impaired autophagic flux (due to increase of LC3II and p62)	311
			HT-29					↓: Cell viability (48h, >50 µg/mL) ↑: Apoptosis (100 µg/mL)	
			HCT-116					↓: Cell viability (48h, >50 µg/mL) ↑: Apoptosis (>50 µg/mL)	
			HIEC-6					↓: Cell viability (24h/48h, >10 µg/mL) ↑: Apoptosis (>10 µg/mL) and autophagy (LC3II increase)	
Xuan, L. et al.	2024	China	Mouse intestinal organoids	uPS PTFE PMMA	100 ≈ 230 ≈ 150	**50** µg/mL	3 days	↓: MMP (all polymers, both models) ↑: Oxidative stress, apoptosis and necrosis (all polymers, both models) and inflammation (all polymers on mouse intestinal organoids) Others: Inhibition of the AKT-mTOR signaling pathway; overall uPS had the biggest effect and PMMA the lowest	176
			HCT116			**100** µg/mL	Not disclosed		

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Yan, L. et al.	2023	China	Caco-2	uPS	20	0.1, **10**, 50, 100, 500 µg/mL	24h	↓: Cell viability (>10 µg/mL) =: Oxidative stress*, cytoplasmic membrane integrity, cell membrane fluidity and MMP Others: Complex cellular response to NP exposure that might lead to apoptosis[#]	312
Zhang, Y. et al.	2024	China	IEC-6	uPS	≈ 80	**25, 50**, 75, and 100 µg/mL	24, 48h	↓: Cell viability (48h, >25 µg/mL), MMP (>25 µg/mL) and cytoplasmic membrane integrity (>50 µg/mL) ↑: Oxidative stress*, mitochondrial damage and autophagy[#]	313

*Table 5 - Summary of digestive system cell line studies: NP polymer type, size, concentration and main findings. In **bold**, values that were carried across in the assessment of different parameters besides cell viability. *Based on ROS production. [#]Based on interpretation of mRNA levels and protein levels. [+]No statistical analysis. [d]Based on HO-1 protein levels and SOD activity. [b]Based on the slope instead of a single concentration. [d]Based on gene expressions of HO-1 and SOD2. A-PS = Amine-modified Polystyrene. CAT = Catalase. C-PS = Carboxyl-modified Polystyrene. EC50 = Half Maximal Effective Concentration. ECAR = Extracellular Acidification Rate. FBS = fetal bovine serum. GST = Glutathione S-Transferase. MMP = Mitochondrial Membrane Potential. OCR = Oxygen Consumption Rate. PC = Polycarbonate. PET = Polyethylene terephthalate. PLA = Polylactic Acid. PMMA = Polymethyl methacrylate. PP = Polypropylene. PTFE = Polytetrafluoroethylene. uPS = unmodified Polystyrene. wPET= weathered Polyethylene terephthalate. wPS = weathered Polystyrene.*

Annex 6 – Summary of immune system cell line studies

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Babonaitė, M. et al.	2023	Lithuania	PBMCs	uPS	50-100	15, 20, **25**, 30, 40, **50**, 60, **75**, 85 and **100** µg/mL	3, 24h	**↓:** cell viability (60 and 85 µg/mL) ↑: DNA damage (24h, >15 µg/mL) **=:** cell viability (all other concentrations)	314
Ballesteros, S. et al.	2020	Spain	WBCs	uPS	40-100	1, 10, 25, **50**, and **100** µg/mL	24, 48 and 72h	**↑:** DNA damage (PMNs and monocytes, 100 µg/mL) and inflammation[#] **=:** cell viability; DNA damage (lymphocytes)	315
Brandts, I et al.	2023	Spain	RT-HKM	uPS	44	**25** and **50** µg/mL	1, 12 and 16h	=: Cell viability and oxidative stress* Others: induction of a specific polarization state (different than M1 and M2-like phenotypes)	177
Busch, M. et al.	2021	Germany	THP-1	uPS	50	1, 5, **10** or **50** µg/cm^2	24h	=: Cell viability and cytoplasmic membrane integrity	91
				A-PS				↓: Cell viability (>5 µg/cm^2) and cytoplasmic membrane integrity (>5 µg/cm^2) ↑: expression of IL-1β (5-10 µg/cm^2) =: expression of IL-1β (50 µg/cm^2) and cytokine release (on triple culture with Caco-2/HT29-MTX-E12)	

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Chen, J. et al.	2023	China	RAW264.7	uPS	100	0, 1, 2, 5, **10**, **20**, **50** and **100** µg/mL	6-24h	↓: MMP ↑: Oxidative stress* (>10 µg/mL) =: Cell viability, cytoplasmic membrane integrity and apoptosis	188
				C-PS				↓: Cell viability (>20 µg/mL) and MMP ↑: Oxidative stress* (>10 µg/mL) and apoptosis (>20 µg/mL) =: Cytoplasmic membrane integrity	
				A-PS				↓: Cell viability (>1 µg/mL), cytoplasmic membrane integrity (>2 µg/mL) and MMP ↑: Oxidative stress* (>10 µg/mL) and apoptosis (>10 µg/mL)	
Djapovic, M. et al.	2023	Serbia	PBMCs	PET	≈ 300	0.001, 0.01, 0.1, **1, 10** and **100** µg/mL	4, 24h	**=:** cell viability, apoptosis and oxidative stress*.	193
Florance, I. et al.	2021	India	RAW264.7	uPS	200	1, 5, 10, 25, **50**, **100** and **200** µg/mL	24-96h	↑: Oxidative stress* (200 µg/mL) = Cell viability (96h, ≤ 200 µg/mL)	191
				S-PS				↑: Foam cell formation (24h, 200 µg/mL) = Cell viability (96h, ≤ 200 µg/mL) and oxidative stress*	

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Florance, I. et al.	2022	India	RAW 264.7	S-PS	200	5, 10, 25, **50, 100**, 250 and 500 µg/mL	24h	↓: Cell viability (>250 µg/mL) and MMP ↑: Lipidic content and mtROS (100 µg/mL)	186
			THP-1					↓: MMP ↑: Lipidic content and mtROS (100 µg/mL) =: Cell viability	
Giannandrea, D. et al.	2024	Italy	RAW264.7	uPS	50	1, 10, **50, 100** and 200 µg/ml	4-96h Osteoclastic differentiation assay: 7 days	↓: Cell viability (>10 µg/mL) ↑: apoptosis[#] (>100 µg/mL) and oxidative stress* Others: Exposure increased differentiation of RAW264.7 toward osteoclast cells	196
Guo, X. et al.	2023	China	Human HSPCs	uPS	80	0.05, **0.1**, 0.2, 0.4, and 0.6 mg/mL	12-48h	↓: Cell viability (>0.2 mg/mL), cytoplasmic membrane integrity (>0.1 mg/mL) and colony growth of CFU-GM, CFU-E and BFU-E ↑: Oxidative stress* =: Colony growth of GEMM Others: 10 metabolites altered out of 176 tested	316
Ilić, K. et al.	2022	Croatia	Jurkat cells	uPS	20	1, **10**, 50, **100**, 250, 500 mg/L	24h	**↓:** cell viability; MMP (all except 10 mg/L without FBS) **↑:** apoptosis (with FBS, 500 mg/L; withouth FBS, >50 mg/L) and oxidative stress* (withouth FBS, >10 mg/L) **=:** Oxidative stress* (with FBS)	197

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Li, C. et al.	2023	China	THP-1	uPS	100	**100** and **200** µg/mL	48h	=: Cell viability and inflammation[e]	310
Li, S. et al.	2023	China	RAW264.7	uPS	80	**400** µg/mL;	24h	↓: CD206 ↑: Oxidative stress* and inflammation[#]	195
Li, Y.Q. et al.	2022	China	Splenocytes from female wt BALB/c mice	uPS	20 and 50	PS20: 5, 10, 20, **40**, 80 and 160 µg/mL	6-24h	↓: Cell viability (uPS20 >40 µg/mL and uPS50 >200 µg/mL) and MMP (uPS20/50) ↑: Apoptosis (uPS20 >10 µg/mL and uPS50 >200 µg/mL) and oxidative stress* (uPS20/50)	189
				Sa-PS	20	PS50: 25, 50, 100, **200**, 400 and 800 µg/mL		↓: Cell viability (>40 µg/mL) and MMP ↑: Apoptosis (>20 µg/mL) and oxidative stress*	
				A-PS				↓: Cell viability (>10 µg/mL) and MMP ↑: Apoptosis (>10 µg/mL) =: Oxidative stress*	
Murano, C. et al.	2021	Italy	Primary cultures of coelomocytes	C-PS	60	**5** and **25** µg/mL	4h	↓: Lysosomal membrane stability and phagocytic capacity (25 µg/mL) =: Cell viability	190
				A-PS	50	**25** µg/mL		↓: Cell viability, lysosomal membrane stability, phagocytic capacity and phagocytic index (25 µg/mL)	
Nikolic, S. et al.	2022	Serbia	Splenocytes	C-PS	40+200	**0.01** and **0.1** mg/mL	24-72h	↓**:** cell viability (72h, both concentrations) ↑**:** DNA damage and apoptosis	317

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Rubio, L. et al.	2020	Spain	THP-1 TK-6 Raji-B	uPS	50-100	0-200 µg/mL (**5, 10, 25** and **50** µg/mL)	3, 24, 48h	=: Cell viability, oxidative stress* and DNA damage ↓: Cell viability (Both cell lines >200 µg/mL) ↑: Oxidative stress* (TK6, 24h, >5 µg/mL) and DNA damage (both cell lines) =: Oxidative stress* (Raji-B, 24h)	94
Tan, Y. et al.	2020	China	RAW264.7	uPS	300	50, 100, **150** and 200 µg/mL	24h	↓: Cell viability (>50 µg/mL) and cytoplasmic membrane integrity ↑: Lysosomal membrane permeability Others: NP exposure disrupted the autophagic flow despite the upregulation of LC3B-II	194
Tavakolpourneg ari, A. et al.	2023	Spain	TK6 THP-1 Raji-B	uPS	50, 200 and 500	**50, 100**, 150, 200 µg/mL	3, 24 and 48 h	↓: MMP (THP-1, uPS200/500, >50 µg/mL; THP-1, uPS50, 100 µg/mL; Raji-B, uPS200/500, 100 µg/mL; Raji-B, uPS50, 50 µg/mL) =: Cell viability (all cell lines), oxidative stress* after 48h (all cell lines) and MMP (TK6)	187

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Vela, L. et al.	2023	Spain	TK6	uPS	40-100	50, 100, 150 and 200 µg/mL (**5, 10, 25** and **50** µg/mL)	24-48h	↓: Cell viability (digested and normal uPS) ↑: Oxidative stress* =: DNA damage	318
			THP-1					No significant changes to cell viability, oxidative stress* or DNA damage	
			Raji-B					↓: Cell viability (uPS) ↑: Oxidative stress* =: DNA damage	
Wang, X. et al.	2023	China	RAW264.7	uPS	80	**0.01**, **0.1**, 0.5, **1**, 5 and **10** µg/mL	24h	↓**:** cell viability (>0.1 µg/mL) ↑**:** necrosis (0.1 µg/mL), apoptosis (5 µg/mL), oxidative stress* (>1 µg/mL) and inflammation[#]	319
Yang, Q. et. al	2023	China	RAW264.7	PE	≈ 531	5, 10, **20** and 50 µg/mL	12-24h	↑: Lysosomal damage and inflammation[#] =: Cell viability (≤50 µg/mL)	192

*Table 6 - Summary of immune system cell line studies: NP polymer type, size, concentration and main findings. *Based on ROS production. In **bold**, values that were carried across in the assessment of different parameters besides cell viability. [#]Based on interpretation of mRNA levels and protein levels. [e]Based on cytokine secretion and NF-kB p65 protein levels. A-PS = Amine-modified Polystyrene. C-PS = Carboxyl-modified Polystyrene. FBS = Fetal Bovine Serum. MMP = Mitochondrial Membrane Potential. PBMCs = Peripheral Blood Mononuclear Cells. PE = Polyethylene. PET = Polyethylene terephthalate. Sa-PS = Sulfonic acid-modified Polystyrene. S-PS = Sulfate-modified Polystyrene. uPS = unmodified Polystyrene.*

Annex 7 – Summary of reproductive system cell line studies

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Basini, G et al.	2021	Italy	Swine granulosa cells	uPS	100	**5, 25,** and **75** µg/mL	48h	↓: P4 production (>25 µg/mL) ↑: cell proliferation, E2 production and oxidative stress =: cell viability	201
Chen, G. et al.	2023	China	HEY	uPS	100	0, **1**, 2.5, **5**, **7.5**, **10**, 12.5, 15, **20**, 30, 40 mg/L	24-48h and 16 days	↓: Cell viability and percentage of wound healing (20 mg/L) Others: EC50 was 31,5 mg/L at 48h and 30 mg/L at 16 days. 275 DEGs were found (110 up-regulated and 165 down-regulated)	204
Contino, M. et al.	2023	Italy	Spermatozoa	A-PS	50 and 100	**0.1, 0.5** and **1** µg/mL	30min.	↓: Motility (A-PS50, >0.5 µg/mL); cytoplasmic membrane integrity (A-PS50, 1 µg/mL); mitochondria functionality (A-PS50, >0.1 µg/mL) ↑: Acrosomal damage (A-PS50/100, 1µg/mL); DNA damage (A-PS50); oxidative stress* (A-PS50, >0.5 µg/mL and A-PS100, >0.1 µg/mL) =: motility, cytoplasmic membrane integrity, DNA damage and mitochondria functionality (A-PS100)	200

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
González-Fernández, C. et al.	2018	France	Oysters' spermatozoa	A-PS C-PS	100	**0.1, 1, 10** and **100** mg/L	5h	↓: Single spermatozoa and spermatozoa aggregates (C-PS, 100 mg/L) ↑: cellular relative complexity (C-PS and A-PS, >10 mg/L) =: spermatozoa motility	199
			Oysters' oocytes					↑: Oocyte mortality (C-PS and A-PS, 100 mg/L) and oxidative stress* (C-PS, 100 mg/L) =: Oocyte cell number, relative size and complexity (C-PS and A-PS)	
Hu, R. et al.	2022	China	TM4	uPS	20	0, 12.5, 25, **50**, 100 and 200 µg/mL	0, 3, 6, 12, **24h**	↓: cell viability (24h, >100 µg/mL), cytoplasmic membrane integrity (>25 µg/mL), barrier integrity ↑: ER stress and UPR	203
Li, S. et al.	2022	China	GC2	uPS	80	**400** µg/mL	24h	↓: MMP ↑: Oxidative stress, apoptosis and autophagy[#]	320
Li, S. et al.	2023	China	GC2	uPS	80	**400** µg/mL	24h	↓: MMP (co-culture with RAW264.7) ↑: Apoptosis (co-culture), oxidative stress* (monoculture and co-culture) and inflammation[#]	195
Ma, T. et al.	2023	China	TM4	uPS	100	**30** and **300** µg/mL	24h	↓: Cell viability (300 µg/mL) and autophagy[#]	321
Ruan, Y. et al.	2023	China	HeLa	uPS	10, 15, 25, 40 and 50	0, 1, 10, 20, 40, 80, **100**, 200 and 500 mg/L	1, 4h	↓: cell viability (uPS10, >40 mg/L; uPS15, >80 mg/L) ↑: Oxidative stress* (uPS10, >20 mg/L; uPS15, >10 mg/L)	198

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Sui, A. et al.	2023	China	TM3	uPS	20	0, **40**, 80, 120 and 160 µg/mL	0, 6, **12**, 24h	↓: Cell viability (>120 µg/mL) ↑: Oxidative stress* and translation of HIF-1α mRNA via ERK1/2 MAPK/mTOR/4E-BP1 and AKT/mTOR/4E-BP1 pathways.	207
Sun, Z. et al.	2023	China	TM3	uPS	20	**50, 100** and **150** µg/mL	24h	↓: Cell viability, MMP and steroid hormone biosynthesis/metabolism# (>50 µg/mL), testosterone levels and cytoplasmic membrane integrity (>100 µg/mL) ↑: Oxidative stress* (>50 µg/mL) and apoptosis (>100 µg/mL)	202
Xiao, M. et al.	2023	China	NTERA-2	uPS	50	50, 100, **200**, 500, and 1000 µg/mL	24h	↓: Cell viability (36/48h, >200 µg/mL) ↑: Apoptosis#, oncogenic potential through activation of MAPK and PI3K-AKT signaling pathways	205
Zeng, L. et. al	2023	China	KGN	uPS	20	50 µg/mL, **100 µg/mL** and 200 µg/mL	48h	↓: cell viability (>100 µg/mL) ↑: Oxidative stress* and apoptosis (48h, 100 µg/mL), protein levels in the Hippo signaling pathway.	206

*Table 7 - Summary of reproductive system cell line studies: NP polymer type, size, concentration and main findings. In **bold**, values that were carried across in the assessment of different parameters besides cell viability. *Based on ROS production. #Based on interpretation of mRNA levels and protein levels. A-PS = Amine-modified Polystyrene. C-PS = Carboxyl-modified Polystyrene. DEG = Differentially Expressed Genes. E2 = Estradiol. ER = Endoplasmic reticulum. MMP = Mitochondrial Membrane Potential. P4 = Progesterone. UPR = Unfolded Protein Response. uPS = unmodified Polystyrene.*

Annex 8 – Summary of gestational tissue cell line studies

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Dusza, H.M. et al.	2022	Netherlands	BeWo b30 (syncytialized and non-syncytialized)	uPS and wPS	50 and 200	0.1, 1, **10**, and 100 µg/mL	24h	↓: cytoplasmic membrane integrity (non-syncytialized cells, uPS50 and wPS50, 100 µg/mL) =: cell viability (syncytialized and non-syncytialized cells, uPS50/200 and wPS50/200) and cytoplasmic membrane integrity (syncytialized cells uPS50/wPS50; both cells uPS200/wPS200)	322
Fu, Y. et al.	2022	China	HUVEC	uPS	50	5, **10**, 15, **20**, and 25 µg/mL	0, 24, 48, and 72 h	↓: MMP^+ ↑: Oxidative stress* (>10 µg/mL) No significant changes to cell viability or cytoplasmic membrane integrity	208
				A-PS				↓: Cell viability (>5 µg/mL), cytoplasmic membrane integrity (>10 µg/mL) and MMP^+ ↑: Oxidative stress* (20 µg/mL) Others: mitochondrial gene expression changed mostly with A-PS NPs and/or 20 µg/mL of any polymer tested	
Hesler, M. et al.	2019	Germany	BeWo b30	C-PS	50	0.01, 0.1, 1, 5, **10**, 25, 50 and **100** µg/mL	24-48h	↓: cell viability (>5 µg/mL) =: barrier integrity; DNA damage	179

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Hu, J. et al.	2022	China	HTR8/SVneo	uPS	100	10, 50, or **100** µg/mL	12, 24h	↓: trophoblast migration and invasion (100 µg/mL) ↑: Oxidative stress* (100 µg/mL), apoptosis and inflammation =: cell proliferation Others: 344 DEGs (46 up-regulated and 298 down-regulated)	209

*Table 8 - Summary of gestational tissue cell line studies: NP polymer type, size, concentration and main findings. In **bold**, values that were carried across in the assessment of different parameters besides cell viability. *Based on ROS production. ⁺No statistical analysis. A-PS = Amine-modified Polystyrene. C-PS = Carboxyl-modified Polystyrene. DEG = Differentially Expressed Genes. MMP = Mitochondrial Membrane Potential. uPS = unmodified Polystyrene. wPS = weathered Polystyrene.*

Annex 9 – Summary of nervous system cell line studies

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Almeida, M. et al.	2019	Portugal	DLB-1	uPS	100	**0.001, 0.01, 0.1, 1** and **10** mg/L	24h	↑: GST activity (potential increase in oxidative stress) =: cell viability and CAT activity	219
Bai, H. et al.	2024	China	hCMEC/D3	A-PS	100	3.725, 6.25, 12.5, 25, **40** and **50** µg/mL	12h	↓: Cell viability (>12.5 µg/mL) and barrier integrity[#]	212
			HT22					↓: Cell viability (>20 µg/mL) ↑: apoptosis[#] Others: activation of GAPDH/Ac-Tau signaling pathway	
Ban, M. et al.	2021	Japan	SH-SY5Y	uPS	50	**2, 10** and **50** µL/mL	24h	↓: cell viability (>10 µL/mL); nº and length of processes ↑: cell nucleus staining with DAPI (50 µL/mL) Others: disorderly granular substances were observed in the 10 and 50 µL/mL PS addition groups.	211
González-Fernández, C. et al.	2021	Spain	SaB-1	uPS	50	0.001 to 100 (**1** and **12** µg/mL)	24h	No significant changes to cell viability	215
				C-PS					
				A-PS				↓: Cell viability ↑: Oxidative stress and apoptosis[#]	
Huang, Y. et al.	2023	China	SH-SY5Y	uPS	50	**0.5, 5, 50** and **500** µg/mL	48h	↓: Cell viability (>50 µg/mL) and MMP (>5 µg/mL) ↑: Oxidative stress* (>50 µg/mL), mitochondrial dysfunction[f] and autophagy/mitophagy[#]	210
				PE	100			No significant changes were observed in any of the effects tested	

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Jeong, B. et al.	2022	South Korea	Primary hippocampal NSCs from E16.5 mice	uPS C-PS	50 and 500	5, 10, **25**, 50, 100 µg/mL	7-9 days	↓: Cell proliferation, number of cells, cell diameter and Tuj1+ neuron's length (all polymers) ↑: Number of GFAP+ astrocytes (all polymers) Others: Downregulation of genes related to cell division and proliferation	217
Liu, S. et al.	2022	China	HT22	uPS	100	**5, 25** and **75** µg/mL	24h	↓: cell viability (75 µg/mL from static exposure; >25 µg/mL during dynamic exposure) ↑: Oxidative stress* (24h, >5 µg/mL) =: apoptosis, cell cycle phase distribution.	214
Martin-Folgar, R. et al.	2024	Spain	hNS1	uPS	30	**0.5, 2.5,** and **10** µg/mL	4 days	Multifaceted cellular response to NP exposure, involving alterations in the transcriptional level of genes related to oxidative stress responses, DNA repair mechanisms, inflammation, and apoptosis regulation.	213
Ruiz-Palacios, M. et al.	2020	Spain	FuB-1	uPS	100	10^{-7} up to 10 mg/L	24h	↓: Cell viability Others: Compromised oxidative stress response and detoxification capacity[g] and LD50 of 11.24 mg/L	323
Shan, S. et al.	2022	China	hCMEC/D3	uPS	42	25, 50, **100** and 200 µg/mL	72h	↓: Cell viability (200 µg/mL) and barrier integrity ↑: Oxidative stress and necrosis =: apoptosis	280
			BV2			**100** µg/mL	24h	↑: Inflammation[#]	
			HT22					↓: Cell viability (100 µg/mL)	
Sun, J. et al.	2023	China	BV2	uPS	44	**25, 50** and **100** µg/mL	12-24h	↓: Cell viability (>25 µg/mL) ↑: Apoptosis, inflammation[#] and oxidative stress*[#]	324

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Yang, S. et al.	2023	South Korea	C17.2	uPS	50	100, **200**, or 500 µg/mL	48h	No significant changes in cell viability or oxidative stress were observed	216
				C-PS					
				A-PS	30			↓: Cell viability (>100 µg/mL) ↑: Cellular senescence[#], inflammation[#] and oxidative stress*	

*Table 9 - Summary of nervous system cell line studies: NP polymer type, size, concentration and main findings. In **bold**, values that were carried across in the assessment of different parameters besides cell viability. *Based on ROS production. [#]Based on interpretation of mRNA levels and protein levels. [f]Based on respiratory levels and ATP production. [g]Based on decrease in NPT levels together with CAT and GST activities. A-PS = Amine-modified Polystyrene. CAT = Catalase. C-PS = Carboxyl-modified Polystyrene. GST = Glutathione S-transferase. LD50 = Median Lethal Dose. MMP = Mitochondrial Membrane Potential. PE = Polyethylene. uPS = unmodified Polystyrene.*

Annex 10 – Summary of connective tissue cell line studies

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Almeida, M. et al.	2019	Portugal	SAF-1	uPS	100	**0.001, 0.01, 0.1, 1** and **10** mg/L	24h	↑: Oxidative stress[h] =: Cell viability	219
Basini, G. et al.	2022	Italy	ASCs	uPS	100	**5, 25** and **75** µg/mL	24-72h	↓: Cell viability (72h, >5 µg/mL) ↑: Oxidative stress and inflammation =: Cell proliferation and ATP levels	220
Giannandrea, D. et al.	2024	Italy	MC3T-E1	uPS	50	1, 10, **50, 100** and 200 µg/mL	4-96h	↓: Cell viability (>100 µg/mL), migratory ability (100 µg/mL) and bone deposition[#] (100 µg/mL) ↑: Oxidative stress* (>100 µg/mL), apoptosis[#] (>100 µg/mL) and bone resorption[#] (100 µg/mL)	196
			MLOY-4					↓: Cell viability (>100 µg/mL) ↑: Oxidative stress* (>100 µg/mL), apoptosis[#] (200 µg/mL) and inflammation[#] (100 µg/mL)	
Lin, P. et al.	2022	China	H9C2	uPS	≈ 94	**30** µg/mL	36h	↑: Oxidative stress* and autophagy Others: Activation of the TGF-β1/Smad signaling pathway	221
Poma, A. et al.	2019	Italy	Hs27	uPS	100	**5, 25,** and **75** µg/mL	4, 24, and 48h	↓: Cell proliferation (48h, 75 µg/mL) ↑: DNA damage (>25 µg/mL) =: Oxidative stress*	128

Authors	Year	Country	Cellular line	Polymer type	NP size (nm)	NP concentration	Time of exposure	Main findings	Ref.
Yang, M. et al.	2022	China	ZF4	A-PS	100	10, **20**, 50, 100, and 200 µg/mL	1, 3, 6 and 9h Cell viability: 24h	↓: Cell viability, cytoplasmic membrane integrity, lysosomal integrity and MMP ↑: Oxidative stress* and apoptosis[#] Others: EC50 of 52.8 µg/mL	218

*Table 10 - Summary of connective tissue cell line studies: NP polymer type, size, concentration and main findings. In **bold**, values that were carried across in the assessment of different parameters besides cell viability. *Based on ROS production. [#]Based on interpretation of mRNA levels and protein levels. [h]Based on GST and CAT activities. A-PS = Amine-modified Polystyrene. ATP = Adenosine Triphosphate. EC50 = Half Maximal Effective Concentration. MMP = Mitochondrial Membrane Potential. uPS = unmodified Polystyrene.*