

Toxicological mechanisms of nanoplastics: Inflammation as a genotoxicity trigger

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Dedico esta tese a todas as pessoas que têm a coragem
de seguir os seus sonhos e aos meus queridos pais ❤️

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“People won't remember what you said,
but they'll remember how you made them feel.”
Maya Angelou

ABSTRACT

Nanoplastics (NP) pollution poses a significant environmental threat, including to human health due to their capacity to i) bioaccumulate in the food chain, ii) interact with other pollutants in the environment, iii) cross biological membranes, disrupting cellular processes due to their nanoscale diameter. The main goal of this work was to enhance the understanding of the effects and toxicity mechanisms resulting from the exposure of biological systems to polystyrene (PS) NP, with a particular focus on inflammatory responses, genotoxicity, and interactions with a well-known environmental pollutant, benzo[a]pyrene (B[a]P). *In vitro* assays were performed by exposing HT29 and N9 cells to 25 – 500 $\mu\text{g}.\text{mL}^{-1}$ PSNP (25, 50, 100 nm) to assess cell viability, and activation of p38 and p50 NF- κ B signaling pathways, important in regulating the inflammatory response and specific membrane receptor (toll-like receptor 4). These cells were also exposed to NP and B[a]P mixtures to investigate their effects on xenobiotic metabolism, focusing on Phase I and Phase II. Exposure to NP did not significantly alter the viability of exposed cells, however, inflammation pathways and gene expression increased, namely after exposure to smaller NP. The interaction between NP and B[a]P increased oxidative stress and compromised antioxidant defense mechanisms. *In vivo* assays were performed over 28 days exposing *Danio rerio* (Zebrafish) to precontaminated *Daphnia magna* (Dafnias) with NP and B[a]P through water individually and in combination. The results revealed that indirect ingestion of NP significantly affects the intestinal and hepatic systems, influencing overall fish health. These results corroborated *in vitro* findings, showing that NP exposure upregulates proinflammatory genes. Co-exposure of PSNP and B[a]P significantly elevated genotoxicity in zebrafish, surpassing effects from individual exposures. This suggests a "Trojan horse" effect, with NP potentially enhancing B[a]P toxicity, through increased cellular uptake or altered metabolism. The observed upregulation of DNA damage response genes (e.g. TP53) in the liver and the differential expression of genes involved in detoxification (CYP1A) and cellular stress (RAB1A) following co-exposure underscores the severity of the combined impact on cellular homeostasis and DNA integrity and highlights the intricate interplay between NP and B[a]P in zebrafish physiology. This study highlights the urgent need for comprehensive environmental risk assessment and further research to inform regulatory policies aimed at protecting both aquatic ecosystems and human health.

RESUMO

A poluição por nanoplasticos (NP) representa uma ameaça ambiental significativa, incluindo para a saúde humana, devido à sua capacidade de i) se bioacumular na cadeia alimentar, ii) interagir com outros poluentes, iii) atravessar membranas biológicas e perturbar processos celulares, devido ao seu tamanho. O principal objetivo deste trabalho foi aumentar a compreensão dos efeitos e mecanismos de toxicidade resultantes da exposição de sistemas biológicos a NP de poliestireno (PS), com particular foco nas respostas inflamatórias, genotoxicidade e interações com o benzo[a]pireno (B[a]P). Foram realizados ensaios *in vitro*, expondo células HT29 e N9 a 25–500 $\mu\text{g.mL}^{-1}$ de PSNP (25, 50, 100 nm), para avaliar a viabilidade celular, a ativação das vias de sinalização p38 e p50 NF- κ B, importantes na regulação da resposta inflamatória, e de um recetor específico membranar (recetor tipo toll 4). Estas células HT29 e HepG2 foram expostas a misturas de NP e B[a]P para investigar os seus efeitos no metabolismo de xenobióticos, com foco nas fases I e II. A exposição a NP não alterou significativamente a viabilidade das células expostas, no entanto, as vias inflamatórias e a expressão génica aumentaram, nomeadamente após a exposição a partículas menores. A interação entre NP e B[a]P aumentou o stress oxidativo e comprometeu os mecanismos de defesa antioxidante. Estudos *in vivo* revelaram que a ingestão indireta de NP afeta significativamente os sistemas intestinal e hepático, influenciando a saúde geral dos peixes. Estes resultados corroboraram as descobertas *in vitro*, mostrando que a exposição a NP aumenta a regulação de genes pró-inflamatórios. A coexposição de PSNP e B[a]P aumentou significativamente a genotoxicidade nos peixes-zebra, superando os efeitos das exposições individuais. Isto sugere que acontece o efeito "Cavalo de Troia", onde os NP potencialmente aumentam a toxicidade do B[a]P, através de uma maior captação celular ou metabolismo alterado. O aumento da regulação dos genes de resposta ao dano no DNA (ex. TP53) no fígado e a expressão diferencial de genes envolvidos na desintoxicação (CYP1A) e no stress celular (RAB1A) após a coexposição, sublinham a gravidade do impacto combinado na homeostasia celular e integridade do DNA, destacando a complexa interação entre NP e B[a]P na fisiologia dos peixes-zebra. Este estudo realça a necessidade urgente de uma avaliação abrangente do risco ambiental e de mais investigação para apoiar medidas regulatórias destinadas a proteger tanto os ecossistemas aquáticos como a saúde humana.

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ACRONYMS

7-ER	7-ethoxy resorufin
A549 cells	type II pulmonary epithelial cell model
ADME	absorption, distribution, metabolism and excretion
AGS	gastric adenocarcinoma
ANOVA	analyse of variance
AT2	primary human alveolar type 2
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATR-FTIR	attenuated total reflectance Fourier Transform Infrared
Au	gold
B[a]P	benzo[a]pyrene
BDE.47	2,2',4,4'-tetrabromodiphenyl ether
BEAS-2B	lung epithelial cells
BFR	brominated flame retardants
BP	bisphenols
BPA	bisphenol A
BPF	bisphenol F
BPS	bisphenol S
BSA	bovine serum albumin
BSO	L-buthionine-(S,R)- sulfoximine (BSO)
CaCl₂	calcium chloride
CAT	catalase
C-C	carbon carbon ligand
cDNA	complementary DNA
CDNB	chloro-2,4-dinitrobenzene
CENIMAT	centre of materials research
CFC	continuous flow centrifugation
CH₂	two hydrogen atoms attached to a carbon atom
Comet	alkaline version of the single-cell gel electrophoresis
CPE	cloud point extraction
CTRL	control
CYP1A	cytochrome P450 1A
<i>D. rerio</i>	<i>Danio rerio</i>
<i>D. magna</i>	<i>Daphnia magna</i>

DEHP	diethylhexyl phthalate
DGVA	National Directorate for Veterinary
DLS	dynamic light scattering
DLVO	Derjaguin–Landau–Verwey–Overbeek
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-SB	DNA strand breakage
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
ED	endocrine disruptors
EDTA	ethylenediaminetetra acetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
ERK/C-FOS	signal transduction
EROD	ethoxyresorufin-O-deethylase
EU	European Union
FBS	fetal bovine serum
FFF	field flow fractionation
FTH1	ferritin heavy chain 1
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	gas chromatography-mass
GPx1	glutathione peroxidase 1
GPx4	glutathione peroxidase 4
GSH	glutathione
GST	glutathione-S-transferase
hD	hours dark
HDPE	high density polyethylene
HEPES	4-(2-hydroxyethyl)– 1-piperazineethanesulfonic acid
HepG2	human liver cells
HIF-1α	hypoxia-inducible factor 1
hL	hours light
HSI	hepatossomatic index
HSP70	70 kilodalton heat shock proteins
HT29	human colorectal adenocarcinoma cells
HUVEC	human umbilical vein endothelial cells
I3 filter	Leica microscope fluorescence filter

ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IL-1	Interleukin 1
IL1β	Interleukin 1 beta
iNOS	inducible nitric oxide synthase
iq.r	interquartile range
J774A.1	brain astrocytoma and macrophage
JAK2/STAT3	janus kinase 2/signal transducer and activator of transcription 3
L	liters
LDPE	low density polyethylene
LMPA	low melting-point agarose
LPS	lipopolysaccharide
LS174T	human colorectal adenocarcinoma cell line
M1	macrophages
MAC	alveolar macrophages
MALDI	matrix-assisted laser desorption/ionization spectrometry
MALDI-TOF-MS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MALS	multi-angle light scattering
MDA	malondialdehyde
mg.L⁻¹	miligramme per litre
mM	millimolar concentration
MP	microplastics
mRNA	messenger ribonucleic acid
MSFD	Marine Strategy Framework Directive
MTT	3–4,5-dimethylthiazol-2- yl) – 2,5 – diphenyltetrazolium bromide
MTT	3-(4,5-dimethylthiazol-2-yl)– 2,5-diphenyltetrazolium bromide
N9 cells	mouse microglial cells
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NCRNA	non-coding RNA
NH₄	ammonia
NOVA FCT	NOVA School of Science and Technology
NP	nanoplastics
NTA	nanoparticle tracking analysis

O₂	oxygen
°C	centigrades
OECD	Organisation for Economic Co-operation and Development
p38 MAPK	mitogen-activated protein kinase
p50 NF-KB	nuclear factor kappa light chain enhancer of activated B cells
PA	polyamide
PAH	polycyclic Aromatic Hydrocarbon
PB(II)	lead (II)
PBDE	brominated diphenyl ethers
PBS	phosphate buffered saline
PCB	polychlorinated biphenyls
PCR	polymerase chain reaction
PE	polyethylene
PEG	poly(ethylene glycol)
PEG-PLA	poly(ethylene glycol)-d,l-polylactide
PES	polyethersulfone
PET	polyethylene terephthalate
pH	potential of hydrogen
PI3K/AKT	intracellular signalling pathway
PLNP	polystyrene latex nanoplastics
PMMA	polymethyl methacrylate
PMN	polymorphonuclear
POP	persistent organic pollutants
PP	polypropylene
PS	polystyrene
PSMP	polystyrene microplastics
PSNP	polystyrene nanoplastics
PSNP-COOH	anionic carboxylated
PSNP-NH₂	cationic amino-modified
PU	polyurethane
PVA	polyvinyl acetate
PVC	polyvinyl chloride
PY-GC-MS	pyrolysis coupled to gas chromatography and mass spectrometry
PY-GCTOF	pyrolysis coupled to gas chromatography time of flight mass spectrometry
RAB1A	Ras oncogene protein 1 A

RAW	macrophage cell line
REL13	ribosomal proteins L13
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	cell cultured medium
RT-PCR	real time reverse transcription polymerase chain reaction
SDS-PAGE	electrophoresis with acrylamide gel
SEM	scanning electron microscopy
SERS	Surface-enhanced Raman spectroscopy
SLC7A11	cystine/glutamate transporter
spICP-MS	single particle Inductively Coupled Plasma Mass Spectrometry
T0	0 days of exposure
T28	28 days of exposure
TAE	buffer solution containing a mixture of Tris base, acetic acid and EDTA
TD-PTR-MR	thermal desorption–proton transfer reaction–mass spectrometry
TEM	transmission electron microscopy
TGF- B1	transforming growth factor B
TiO₂	titanium dioxide
TLR4	toll-like receptor 4
TNB	5'-thio-2-nitrobenzoic acid
TNF-α	Tumor necrosis factor alpha
TOF	time of flight
TP53	tumor protein p53
TRIS	trisaminomethane
Tris-CI	Tris (triphenylphosphine)rhodium (I) chloride
UV	ultraviolets
w/v	weight by volume
WWTP	watewater treatment plants
XPS	X-ray photoelectron spectroscopy
ZF	zebrafish

CHAPTER 1:

General introduction

Uncovering layer by layer the risk of nanoplastics to the environment and human health

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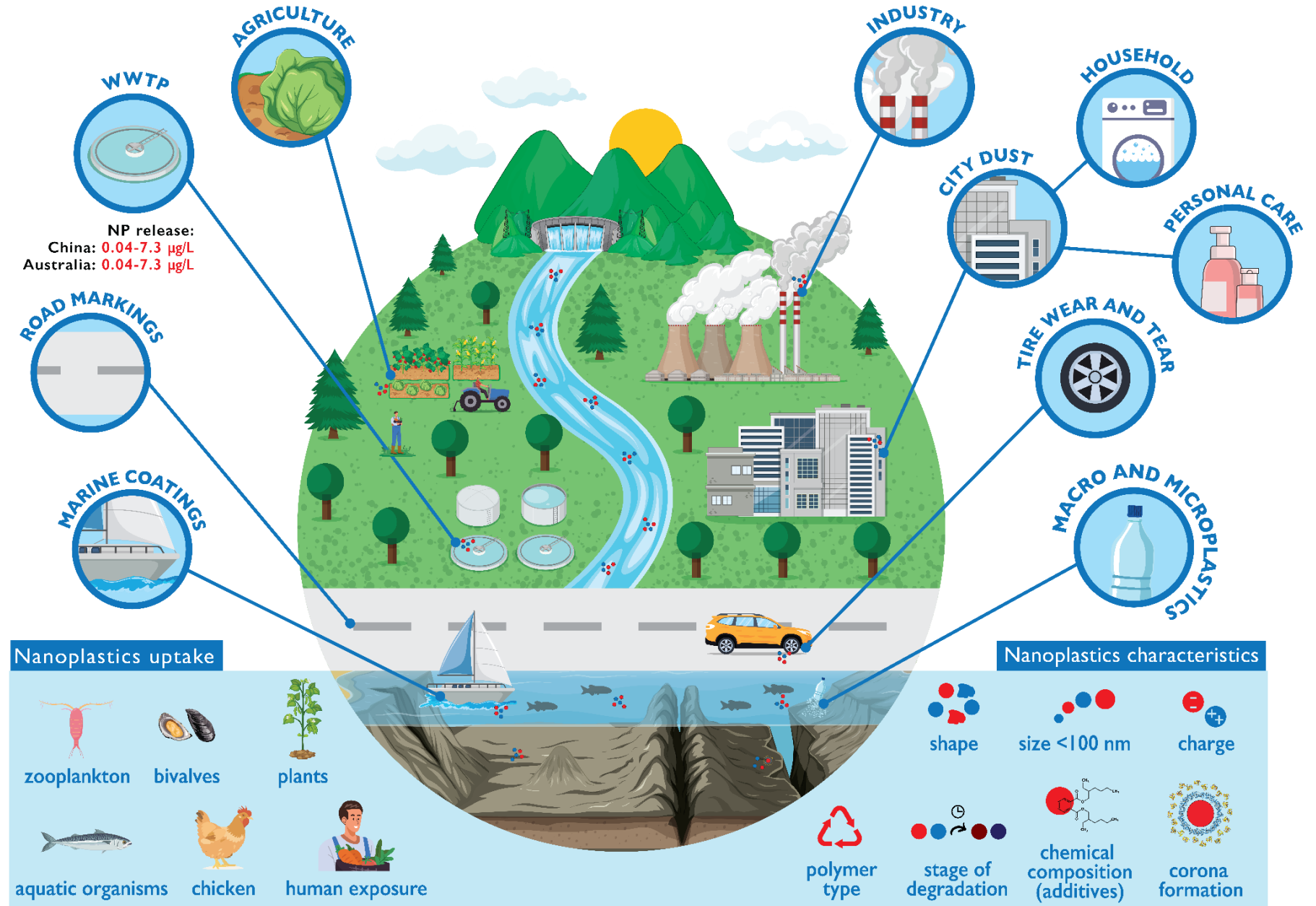
Abstract

Nanoplastics, defined as plastic particles with dimensions less than 100 nm, have emerged as a persistent environmental contaminant with potential risks to both the environment and human health. Nanoplastics can translocate across biological barriers and accumulate in vital organs, leading to inflammatory responses, oxidative stress, and genotoxicity, already seen in several organisms. Disruptions to cellular functions, hormonal balance, and immune responses have also been linked to nanoplastics exposure in *in vitro* assays. Moreover, nanoplastics have been found to adsorb other pollutants, such as persistent organic pollutants, and leach additives potentially amplifying their negative impact, increasing the threat to organisms than nanoplastics themselves. However, nanoplastics toxic effects remain largely unexplored, requiring further research to elucidate potential risks to human health, especially their accumulation, degradation, migration, interactions with the biological systems and the long-term consequences of chronic exposure to nanoplastics. This review provides an overview of the current state-of-art regarding nanoplastics interactions with environmental pollutants and with the biological mechanisms and toxicity inside cells.

Keywords

Nanoplastics detection, plastic pollution, cell damage, trophic level, corona formation

Graphical Abstract



1.1. An overview about plastic pollution in the environment

Plastics have caused widespread pollution concerns due to their increasing worldwide production and inefficient waste treatment (Avio et al., 2017). Their unique physical-chemical properties give them a wide scope of applicability and an essential role in modern lifestyle. However, when combined with inadequate disposal practices and environmental factors, these properties significantly contribute to their dispersion across various environmental matrices, interactions with organisms, and potential risks to global ecosystems. In recent years there has been a significant effort to ban single-use plastics and create environmentally friendly and recyclable packaging solutions, such as those outlined in the European Union (EU) Plastics Strategy and Circular Economy Action (European Commission, 2018). Recent estimates from the Organisation for Economic Cooperation and Development non-packaging applications account for up to 74% of consumption, but only 9% is recycled with 19% being incinerated, and 50% ending up in landfills (OCDE, 2022). Shockingly, 22% of plastic wastes evade waste management systems, especially in developing countries, going into uncontrolled dumpsites and ultimately ending in terrestrial or aquatic environments (OCDE, 2022). The significant amount of plastics that escapes from regulated waste streams in combination with its slow degradation rates contributes to the accumulation of this material in environmental systems. It is estimated that more than 170 trillion pieces of plastic are floating in the oceans (Eriksen et al., 2023), leading some experts to suggest that our current age could potentially be characterised as the 'Plasticene' Era (Reed, 2015).

Plastics are synthetic materials made from a wide range of organic polymers, which are large molecules composed of repeating structural units called monomers. These monomers, typically derived from petrochemicals, are chemically bonded together in a process known as polymerization. During polymerization, monomers react to form long chains or networks, resulting in polymers with diverse properties. Depending on the type and arrangement of monomers used, plastics can exhibit a variety of characteristics, such as flexibility, rigidity, transparency, and opacity. This versatility allows plastics to be molded or shaped during manufacturing processes, making them indispensable in countless applications, from packaging and construction to electronics and medical devices (Andrady & Neal, 2009). Table 1.1 outlines the chemical structures of prominent polymers used in anthropogenic activities, along with essential

information, such as density and plastic production (PlasticEurope, 2023).

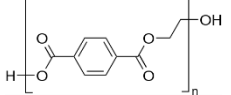
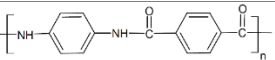
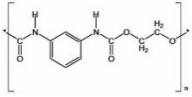
In addition to backbone polymers, plastics often incorporate a variety of additives to enhance their performance and functionality (Wiesinger et al., 2021). These additives include plasticizers, stabilizers, antioxidants, colorants/pigments, flame retardants, UV stabilizers, fillers, biocides, processing aids, impact modifiers, release agents, and foaming agents. Plasticizers, such as phthalates, adipates, and citrates, improve flexibility, while stabilizers, like tin stabilizers and calcium/zinc stabilizers, enhance stability. Antioxidants, such as butylated hydroxytoluene and Butylated hydroxyanisole, are used to prevent degradation (Flieger et al., 2021). Colorants and pigments provide the desired appearance, and flame retardants reduce flammability. UV stabilizers protect against ultraviolet degradation, and fillers reinforce the material. Biocides prevent the growth of microorganisms, while processing aids assist in manufacturing (Sabee et al., 2022). Impact modifiers enhance toughness, release agents aid in demolding, and foaming agents induce the formation of foam structures. This intricate combination of polymers and additives allows plastics to meet a wide range of performance requirements for diverse applications. However, the presence of plastic additives can also contribute to marine pollution, as they can be released from plastics into aquatic environments (Hahladakis et al., 2018a) . These additives can harm marine organisms by disrupting endocrine systems, affecting reproduction and development, and leading to potential bioaccumulation of toxic substances (Hermabessiere et al., 2017a), as will be developed in chapter 1.4.3.

Plastic waste, particularly macroplastics (plastics > 5 mm), exerts detrimental effects on the environment before its fragmentation (Barboza et al., 2018). These larger plastic items, ranging from bottles to bags, pose immediate threats to ecosystems and wildlife. When improperly disposed of, they often end up in water bodies, causing water pollution and endangering aquatic life (Roebroek et al., 2021). The physical presence of macroplastics in terrestrial environments can lead to habitat degradation, changing the soil structure and water infiltration and aeration and obstructing the growth of plants and the movement of animals (Winton et al., 2020). In the marine environment, macroplastics can cause physical harm to marine animals through entanglement (GESAMP, 2015), or be mistaken for food, leading to ingestion and potential injury and death (Roy et al., 2022). Damage to sensitive marine habitats such as coral reefs and seagrass beds (Ajith et al., 2020) and facilitation of transport of invasive species across

oceans have been reported (García-Gómez et al., 2021). In the oceans, macroplastics can accumulate in significant amounts within ocean gyres due to the movement of currents, forming massive patches, frequently called plastic islands. Additionally, the persistence of these large plastic items in the environment exacerbates the overall burden of plastic pollution (van Emmerik, 2021), continuing to cause harm long after they are discarded (Mendes, 2021). Addressing the impact of macroplastics is crucial not only for preserving biodiversity and ecosystem health, but also for mitigating the subsequent stages of plastic degradation, caused by several factors such as ultraviolet radiation, mechanical abrasion, and biological processes contribute to the degradation of macroplastics into smaller sizes (Mattsson et al., 2015a), namely microplastics, plastics with dimensions lower than 5 mm, and nanoplastics, at nanoscale dimensions (1 to 100 nm, according to EU Regulation 2018/1881 nanomaterials), although the size-scale for nanoplastics is not consensual. Understanding the risks associated with these small particles, namely nanoplastics, is challenging due to their tiny size, which makes them difficult to detect and measure accurately (Klein et al., 2015). Their behavior in the environment is complex, as they can interact with other substances and disperse in unpredictable ways (Zeng et al., 2024). Nanoplastics can be easily taken up by living organisms, raising concerns about potential health impacts, but the specifics of these effects are not yet fully understood. Consequently, the long-term impact of nanoplastics are still not well-defined, highlighting the urgent need for further research and more comprehensive strategies to address these emerging environmental and health issues.

This article reviews the current state of knowledge concerning nanoplastics, outlining: i) environmental cycling, deposition, and accumulation in different ecosystems, ii) chemical characteristics and potential interactions with other pollutants before entering biological systems, iii) toxicological properties including effects at the organism, cellular and molecular levels, and, finally, iv) discussing the major knowledge gaps and future research directions to refine risk assessment.

Table 1. 1 - Type of polymers used in anthropogenic activities: abundance production, main applications, density (g.cm⁻³) and chemical structure (Plastics Europe, 2023).

Polymer Type	Abbreviation	Plastic production	Main Applications	Density (g.cm ⁻³)	Chemical structure
Polypropylene	PP	19%	packaging, fabrics, automotive parts	0.90	
Polyethylene	LDPE	14%	packaging, pipes, toys, plastic bags, agricultural films	0.92	
Polyvinyl Chloride	PVC	13%	pipes, coatings, cables	0.88-0.96	
Polyethylene	HDPE	12%	chemical containers, industrial pipes	0.92	
Polyethylene Terephthalate	PET	6%	beverage bottles, fibers	1.38	
Polystyrene	PS	5%	packaging, insulation, utensils, thermal insulation	1.05	
Polyamide	PA	7%	fabrics, toothbrushes	1.14	
Polymethyl Methacrylate	PMMA	7%	glasses, sheets, medical applications	1.19	
Polyurethane	PU	7%	foams, sealants, footwear	0.05-1.7	
Polyester		7%	fabrics, fibers, packaging	1.7	
Polyvinyl Acetate	PVA	7%	shoe soles, toys, coatings	1.7-1.20	

1.2. Nanoplastics: definition, sources and fate

The size definition of nanoplastics remains a subject of uncertainty in scientific literature (El Hadri et al. 2020). Nanoplastics are currently defined as synthetic manufactured plastics with size below $< 1 \mu\text{m}$ by Science for Environment Policy, 2023. However, according to EU Regulation 2018/1881 nanomaterials (including plastics) range between 1 and 100 nm range, and therefore we will focus on data on plastics within this size range. Nanoplastics can be defined according to the production source. Primary nanoplastics are manufactured for specific purposes (e.g. exfoliants in cleaning products, energy-based research, personal care and cosmetic products, biomedical or laboratory applications, and pesticides) (Gigault et al., 2016; Stapleton, 2019). They can enter the environment through wastewater, runoff, or disposal and once in the environment, primary nanoplastics can undergo degradation by physical, chemical, or biological processes, resulting in the formation of secondary nanoplastics (Atugoda et al., 2023; Musa et al., 2024).

There is little information regarding nanoplastics degradation in the environment. Some studies have already proved that the biodegradation of plastic particles, including nanoplastics, involves the breakdown of the polymeric chains, leading to the release of various byproducts, including ions. Biological degradation can primarily occur through microbial action, where bacteria and fungi secrete enzymes that cleave polymer chains, producing smaller molecules. For instance, the bacterium *Achromobacter xylosoxidans* M9 has been identified as capable of significantly reducing nanoplastics particle size (El-Kurdi et al., 2024). Enzymatic degradation by bacteria like *Ideonella sakaiensis*, also showed that can break down polyethylene terephthalate (PET) into terephthalic acid and ethylene glycol, both further degradable into smaller ions (Yoshida et al., 2016). Chemical degradation involves hydrolysis and oxidation, which break down ester bonds and polymer chains, respectively, often mediated by UV light or reactive oxygen species. In this field, specialists are still developing and testing reactors and equipment able to remove and degrade nanoplastics from the environment. One study already showed the efficiency of a photoelectron-Fenton microreactor for efficient degradation of plastic particles with 215 nm used for industrial nanoplastics removal (Chen et al., 2023). Another study showed that photocatalytic degradation with visible light irradiation degraded particles with 350 nm using immobilized copper oxide semiconductors grown by the anodizing

process for the first time (Acuña-Bedoya et al., 2021).

As we know from microplastics studies, during these processes, ions are released from both the polymer and its additives (Yu et al., 2024). Those additives may release metal ions, such as lead, cadmium, and zinc (Sharifi et al., 2023) and photodegradation can lead to metal ion release from marine plastic debris (Hahladakis et al. 2018). Degradation can yield organic acids, such as lactic acid from polylactic acid, and monomeric units like styrene from polystyrene (Thakur et al., 2014). However, no information is yet available regarding nanoplastics in the environment and the release of ions during these degradation processes.

However, not all primary nanoplastics will degrade into secondary nanoplastics, as some may persist for a long time or be removed by other mechanisms, such as sedimentation or ingestion by organisms (Chen et al., 2020a). Secondary nanoplastics are unintentionally produced materials, resulting of the degradation of MP (Rist and Hartmann, 2018). Secondary nanoplastics can result from combustion, abrasion of car tyres, washing of synthetic fabrics, disposal of plastic waste, wastewater from nanomedicine, nanoimaging, nanosensors, and personal care products (Dalela et al., 2015; Panko et al., 2013; Rubio, Marcos, et al., 2020) (Figure 1.1). The fate of nanoplastics is influenced by their size, concentration, material properties, and interactions in aquatic environments (Schiferle et al., 2023). Nanoplastics can either remain dispersed or settle as aggregates with natural colloids, depending on the plastic material and environmental conditions (Alimi et al., 2022)

Wastewater treatment plants (WWTP) are designed to treat and remove contaminants from wastewater before its release into water bodies, however, the removal of nanoplastics can be challenging due to their small size, concentration, and charge (Singh et al., 2022). Besides those difficulties, WWTP are considered one of the main players in the breakdown of microplastics into nanoplastics, during wastewater treatment (Monira et al., 2023). The amount released in the effluent is, thus, dependent on the treatment implemented on the WWTP and the water volume treatment capacity (Yuan et al., 2021). For example, in China, a total of 1.92 and 2.82 $\mu\text{g.L}^{-1}$ polystyrene nanoplastics were detected in river water and the influent of WWTP, respectively (Zhou et al., 2019), while others reported an amount of nanoplastics transported from WWTP to biosolids between 0.1 and 5 g.L^{-1} (Keller et al., 2020), exceeding that found in

seawater (Wei et al., 2019), namely flakes and fibres (Shams et al., 2020). A study in a WWTP with a capacity of 308×10^{12} mL has estimated the release of microplastics associated with each treatment type with values of 4.6×10^8 MP potentially discharged in the primary treatment, 8.1×10^6 microplastics in the secondary treatment and 3.6×10^6 discharged from the tertiary treatment per day into the ocean (Ziajahromi et al., 2017). In another study in the USA, it was estimated that about 500 – 1000 million MP were released daily from three different WWTP (Conley et al., 2019). Similarly, in Germany, a study reported the release of 3000 – 5900 microplastics per cubic meter of treated effluent from a WWTP (Wolff et al., 2019). Microbeads from personal care products and degraded fragments are the most frequent items in the effluent (Talvitie et al., 2017). Recent studies have shown that certain treatment processes can effectively remove nanoplastics by using coagulants, such as AlCl_3 , FeCl_3 , and polyaluminium chloride, reducing more than 95% of certain types of nanoplastics within 30 minutes (Khan et al., 2019), however not all types of polymers or coated nanoplastics are removed, resulting in their release into the environment (Mattsson et al., 2015).

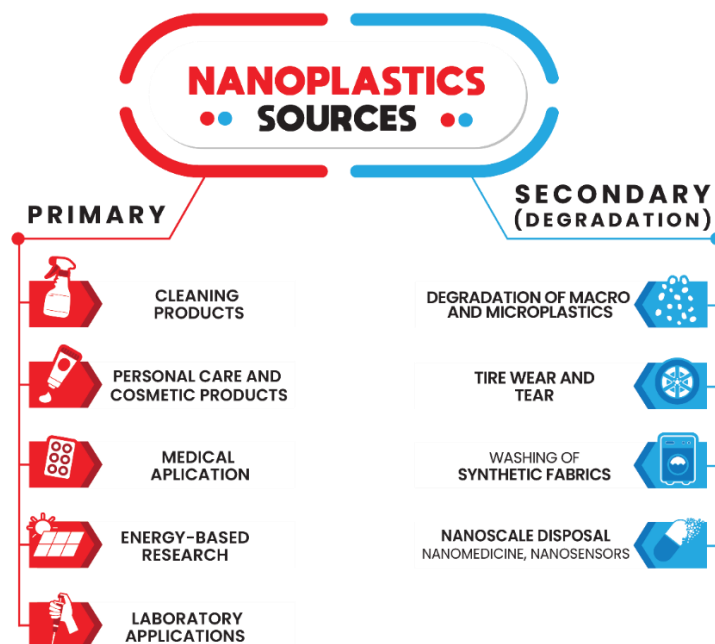


Figure 1. 1 – Sources of primary and secondary nanoplastics. Primary nanoplastics are intentionally produced and added to products (e.g., exfoliants in cleaning products, energy-based research, personal care and cosmetic products, and biomedical or laboratory applications) while secondary nanoplastics are unintentionally produced as byproducts of degradation and disposal of plastic waste.

1.3.Methodologies to identify the presence of NP in environmental samples

The actual major challenge in nanoplastics research is that analytical detection and, especially, quantification in environmental and biological samples is still under development, as there are still major issues in the standardization and validation of methodologies (Oliveira and Almeida, 2019). This handicap results in a scarcity of field studies to discriminate the major nanoplastics sources and pollution hotspots, which conditions the choice of study areas (SAPEA, 2019). Presently, techniques from nanoscience are being used as a reference for nanoplastics research and will be mentioned below.

To accurately identify and quantify nanoplastics, pretreatment is crucial to eliminate impurities while preserving their original properties (Pinto da Costa et al., 2019). Preventing contamination is vital during this step, namely by using non-plastic polymer materials for tools and setups. Minimizing airborne particles and synthetic fibre contamination, by working in a laminar flow hood or air filtration system, is essential. Given the challenging nature of complete contamination avoidance, procedural blank samples and recovery tests must be consistently incorporated into analyses (Oriekhova & Stoll, 2018).

The second crucial step involves matrix digestion. Field samples, including wastewater, organisms, soil, and sediments, often harbor complex organic matrices. To enable further analysis, the extraction or separation of nanoplastics from these matrices is essential. Various methods for digesting organic matrices have been employed in microplastics studies (Filgueiras and Pedrotti, 2019) and are being adapted for nanoplastics. Digestion agents for plastic separation encompass acid treatments, such as 65% nitric acid (Rist et al., 2017), and 30% hydrogen peroxide (Yang et al., 2019), along with alkaline treatments involving sodium hydroxide/potassium hydroxide (Lin et al., 2020). Additionally, a mild and non-destructive enzymatic protocol has demonstrated efficacy in tissue digestion (Löder et al., 2017), and it has been successfully adapted for nanoplastics analysis (EFSA, 2016). Digestion is not included for relatively clean water samples like seawater and drinking water (Schymanski et al., 2018), however, it has been applied in studies including organisms, such as fish, and eggshells (Monikh et al., 2019; Lin et al., 2020; Cai

et al., 2021). Nitric acid (67–69%) and proteinase K (3 mg.mL⁻¹) were used for fish homogenates digestion (Correia & Loeschner, 2018). Alternatives include 10% KOH solution at 50 °C for 36 h (Pequeno et al., 2021) for fish, or 20% of tetramethylammonium hydroxide for eggshells (Monikh et al., 2019).

The third crucial step for the identification and quantification of nanoplastics is the preconcentration of nanoplastics with ultrafiltration, ultracentrifugation, continuous flow centrifugation (CFC), and cloud point extraction (CPE) (Chao et al., 2011a; Fang et al., 2017; Hildebrandt et al., 2020; Philippe et al., 2018). Ultrafiltration, using polyethersulfone (PES)-based cells, concentrates and separates nanoplastics in seawater and drinking water (Mintenig et al., 2018; Ter Halle et al., 2017; Zhou et al., 2019). Ultracentrifugation enhances the detectability of low concentrations of polystyrene nanoplastics in digested eggshell samples (Monikh et al., 2019). CFC efficiently samples fine particulates (1–25 µm) in natural water at high flow rates, successfully enriching small MP (Hildebrandt et al., 2020). Sequential CFC at varying speeds enables size and density-selective preconcentration and separation of nanoplastics in river water samples (Hildebrandt et al., 2020). CPE, a cost-effective technique, concentrates inorganic nanoparticles without altering their original size or morphology, as demonstrated in wastewater samples (Chao et al., 2011b) and efficiently extracts various nanoplastics from aqueous solutions, including PE (Elfgen et al., 2020), PS and PMMA nanoplastics (Zhou et al., 2019). The subsequent step involves separating nanoplastics from the samples, which includes filtration, field flow fractionation (FFF), and size-exclusion chromatography (Strungaru et al., 2019; Hernandez et al., 2019; Wahl et al., 2021).

After the separation of nanosized particles from the environmental matrix, chemical identification is critical to confirm the type of polymer found. In microplastic analysis, vibrational spectroscopy is usually hyphenated with optical microscopy to identify polymer types as well as particle size and morphology. The Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) (detection limit of ~20 µm particles) and Raman spectroscopy (detection limit of ~ 1 µm) are two of the most used methods (Du et al., 2020; Zhang et al., 2020). This methodology, however, becomes increasingly flawed with decreasing particle size (Käppler et al., 2016). For nanoplastics, spectroscopic techniques are the most used chemical identification methods (Cai et al., 2021b). Sobhani et al., 2020 detected nanoplastics with ~200 nm with Raman imaging. However, the

spatial resolution of Raman imaging depends on the wavelength of the laser and the numerical aperture of the spectroscopy (Ossikovski et al., 2008). Hence, Raman imaging cannot detect nanoplastics smaller than the spatial resolution of the spectroscopy, implying that Raman imaging cannot cover the whole nano-range scale (Cai et al., 2021b). A similar method combining scanning electron microscopy and Raman (SEM-Raman) was successfully applied to identify polystyrene nanoplastics, polyethylene, polyamide, and polyvinyl chloride polymers between 11 and 100 nm in size (Sarau et al., 2020). Optical tweezers can also overcome the problem of low Raman scattering; thus, Raman tweezer has realized the analysis of nanoplastics down to ~ 100 nm (Gillibert et al., 2019). Surface-enhanced Raman spectroscopy (SERS) is an approach to increase the electric field intensity of nanostructure and Klarite (commercial SERS substrate) can detect nanoplastics (e.g. PS and PMMA) down to 450 nm extracted from ambient airborne particles (Chen et al., 2020). For water samples, a surface substrate of silver-coated gold nanostars inserted into anodized aluminium oxide nanopores was used to detect particles with dimensions of 400 nm (Lê et al., 2021). Besides FTIR and Raman spectroscopies, X-ray photoelectron spectroscopy (XPS) was also employed for polymer identification complemented with SEM for nanoplastics with 10 to 100 nm diameter (Hernandez et al., 2017). XPS can also observe changes in the surface oxidation of nanoplastics (Y. Lu et al., 2016a; Magrì et al., 2018). Some techniques such as transmission electron microscopy (TEM) are commonly used to characterize nanoplastics (González-Caballero et al., 2024). However, TEM has some disadvantages in terms of cost, complexity and sample preparation, which makes it less suitable for undertaking routine measurements (Zhang et al., 2022).

Advanced mass spectrometry techniques, such as pyrolysis coupled to gas chromatography and mass spectrometry (Py-GC–MS) and pyrolysis coupled to gas chromatography time of flight mass spectrometry (Py-GCToF) have gained significant prominence in the realm of nanoplastics identification. This approach involves treating samples with thermal methods causing the release of gaseous compounds. These compounds are further transferred to gas chromatography-mass (GC) for the identification of chemical compositions (Li et al., 2018). One of the noteworthy advantages of mass spectrometry techniques is its minimal sample pretreatment requirement, as it enables the direct examination of solid polymer samples (Kusch, 2017). Py-GC–MS can identify the presence of nanoplastics associated with the size distribution

(Davranche et al., 2019), despite the abundance of natural organic matter. However, such identification could not give a clear indication of their source and formation pathways. For instance, water samples can be effortlessly placed on a filament or within a furnace of Py-GC–MS for polymer identification. Py-GC–MS has demonstrated its efficacy in identifying nanoplastics in diverse environmental matrices, including drinking water, river water, seawater, wastewater, and sand/soil extraction water (Wahl et al., 2021). Py-GCToF can identify nanoplastics in complex aqueous samples at concentrations as low as 50 parts per billion. The particles contribute to peaks with a greater signal-to-noise ratio and therefore are distinguishable by this method (Sullivan et al., 2020). While Py-GC–MS employs a conventional mass spectrometer to identify volatile compounds generated by pyrolysis, Py-GC-ToF utilizes a high-resolution time-of-flight mass spectrometer for more precise ion separation. This distinction endows Py-GC-ToF with the capability to provide detailed mass resolution, allowing for more accurate and comprehensive identification of nanoparticle compositions. The choice between these methods depends on the analytical needs and complexity of the samples under investigation. Sullivan et al., 2020 utilized pyrolysis–gas time-of-flight mass spectrometry (py-GC-ToF) to successfully identify standard polystyrene nanoplastics ($>0.45\ \mu\text{m}$) that were intentionally introduced into river water samples (Materić et al., 2022; Sullivan et al., 2020).

Expanding the capabilities of nanoplastics identification methods, Materic et al. employed thermal desorption–proton transfer reaction–mass spectrometry (TD-PTR-MS) to identify PET nanoplastics in snow samples, demonstrating the technique's effectiveness even in the presence of complex mixtures of organic compounds. Notably, this method eliminated the need for a preconcentration step, streamlining the analysis process.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is recognized as a powerful technique for (bio) polymer analysis due to its soft ionization and wide mass detection ranges (200 and 400 nm) (Wu et al., 2020). MALDI-TOF-MS was also used for detecting spiked PS and PET nanoplastics in water and fish samples (Lin et al., 2020). The mass analyzer connected with MALDI is usually a time-of-flight mass analyzer which is vacuumed and does not require any field to be added, the ions are separated according to their relative TOF to the detector, so the mass range is unlimited. MALDI MS can be used to determine absolute molar masses of oligomers and molecular structures, it can also analyse copolymers, elucidate polymerization

mechanisms, and identify the repeated units, and end groups of macromolecules (Ren et al., 2018). Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was also considered to be a potential method for nanoplastics identification, however, it is a destructive process that prevents the observation of morphological features of the identified particles. In contrast, an innovative approach known as Single Particle-Inductively Coupled Plasma Mass Spectrometry (spICP-MS) operates on a particle-by-particle basis, providing a non-destructive alternative for nanoplastics samples. Combined TEM and single particle Inductively Coupled Plasma Mass Spectrometry (spICP-MS) can be used to characterize size distributions of nanoplastics and microplastics associated with composite particles (Barber et al., 2020). Jiménez-Lamana et al. 2020 ingeniously conjugated nanoplastics with functionalized gold-containing nanoparticles, rendering them detectable by the highly sensitive spICP-MS (Bolea et al., 2021). This method allows for the preservation of morphological details while achieving precise detection. Barber et al., 2020 extended the application of spICP-MS by developing an analysis method for colloidal-sized nanoplastics, utilizing a gold–polymer nanocomposite (polystyrene-block-poly(acrylic acid), Au–PS/PS-b-PAA). Notably, spICP-MS distinguishes itself from spectroscopic methods by enabling the simultaneous acquisition of information on both the morphology and chemical composition of nanoplastics without the need for extra samples. While mass spectrometry methods provide detailed chemical information, complementary techniques are often required to elucidate morphological features. SEM and TEM serve as invaluable tools for characterizing the morphology of nanoplastics in various matrices (El Hadri et al., 2020b; Mekaru, 2020), including observations of morphological changes during separation procedures (Correia & Loeschner, 2018). The synergy between mass spectrometry and microscopy techniques enriches our understanding of nanoplastics characteristics, offering a comprehensive approach to their analysis.

Urso et al., 2022 introduce a novel method for identifying nanoplastics using multifunctional MXene-derived oxide microrobots in three-dimensional space. The process involves a complex mechanism that makes these microrobots available to move in response to light and have a unique ability to attract and trap nanoplastics, making them effective for collecting nanoplastics from water. As mobile platforms, they also enable the use of simple and portable electrodes to detect nanoplastics.

In the overall process of analyzing environmental nanoplastics, quantification should be

the conclusive step, following the confirmation of polymer types. Quantification methods evolve several techniques, such as SEM, TEM, dynamic light scattering (DLS), nanoparticle tracking analysis (NTA). Cost-effective techniques like NTA and DLS allow for the assessment of nanoplastics quantities without extensive sample preparation. However, challenges arise when directly measuring polydisperse nanoplastics samples using NTA and DLS. Multi-angle light scattering (MALS), and spICP-MS, have been employed to quantify nanoplastics size distribution and concentration. Mass spectrometry methods are also utilized for determining nanoplastics mass concentration. A substantial investment is currently being made to advance the development of optimal methodologies for the identification, characterisation, and quantification of nanoplastics in environmental matrices. This initiative is driven by the critical importance of such information in ecological risk assessment studies and the evaluation of potential risks to human health, as few studies measured nanoplastics in environmental samples (Ter Halle et al., 2017).

1.4.Nanoplastic interaction with the environment and other pollutants

1.4.1. Corona formation and its influence in NP uptake

Plastics have long been considered stable and resistant to degradation due to their strong structures (Guan et al., 2019). However, they can interact with living organisms and the environment, potentially affecting biological systems. When introduced to different fluids, such as blood or intestinal fluids, plastics form a "protein corona" made up of various biomolecules like proteins, lipids, and nucleic acids (Lima et al., 2020; Meng et al., 2023). These biomolecules compete to bind to the plastic surface, leading to a dynamic and evolving corona composition (Wang et al., 2023). Over time, biomolecules with stronger binding affinities replace those initially bound, resulting in a stable equilibrium state (Vroman, 1962). This evolving protein corona significantly influences how cells and tissues interact with the plastic particles.

The adsorbed biomolecules on the surface of nanoplastics are defined as “corona”, consisting of a “hard” and a “soft” layer . The hard corona forms almost immediately (within seconds to minutes) when nanoplastics enter a biological or environmental medium. This layer is characterized by a monolayer of tightly bound molecules that exhibit strong associations with the nanoplastics surface (Wang et al., 2023). Due to their strong attachment, the molecules in the hard corona are difficult to displace, rendering this layer both stable and long-lasting. In a biological context, the hard corona primarily includes proteins, lipids, and other biomolecules that have a high affinity for the nanoplastics surface (ter Halle & Ghiglione, 2021). The stability of the hard corona is attributed to the high affinity of these biomolecules for the nanoplastics surface and their strong tendency to adhere (Schöttler et al., 2016a). While the hard corona is stable, exposure to new environments may only lead to partial replacement of the proteins within it. Consequently, nanoplastics can retain biomolecules from their previous environments within their corona, including those crucial for transport processes. For example, apolipoproteins, which are commonly found in the coronas of a wide range of nanomaterials (such as polystyrene and other polymeric nanoparticles), may persist even after environmental changes (Kim et al., 2007; Lundqvist et al., 2008).

The “hard” corona is often covered by loosely bound molecules, which are defined by

“soft” corona. The molecules in the soft corona are not tightly bound and can rapidly exchange with the surrounding environment, exchanged within seconds (Kihara et al., 2020), once the biomolecules have lower affinity to nanoplastics surfaces. This gives endures dynamic and reversible exchange influenced by the conditions of the surrounding biological fluid (Kokkinopoulou et al., 2017a; Milani et al., 2012) (Figure 1.2).

The retention of specific biomolecules and the different types of corona composition can also change depending on nanoplastics properties (size, hydrophobicity, charge and polymer type) and exposure time and, therefore, its characteristics will influence the cellular internalisation of these small polymeric particles in biological barriers (Florez et al., 2012). Concomitantly, the formation of the protein corona can lead to changes in the properties of nanoplastics, including size, surface roughness, zeta potential, and stability (Zhou et al., 2022). A recent study has found that, apparently, the movement, transformation and impact on cell membranes of nanoplastics are influenced not only by polymer-lipid interaction but the manner of nanoplastic–membrane interactions is strongly dependent on the polymer type. According to Li et al., 2022, in a study that used molecular simulations, polystyrene and polyethylene nanoplastics can rapidly translocate into the cell membrane and attract certain types of lipids to form single-layered coatings. The absorption of these lipids increases the size of nanoplastics, decreasing their hydrophobicity and gives them a negative surface charge. On the other hand, PET nanoplastics, which are less hydrophobic (Min et al., 2020), can easily pass through the membrane with fewer attached lipids. The other conclusion was that while PS, PE and PET nanoplastics remain intact during interactions with the membrane, PP and PVC nanoplastics dissolve in the membrane. Yet, aged nanoplastics has shown to be better able to dissolve, and fibre nanoplastics moved through the membrane more easily, starting from their tips. All these differences in polymers affinities with biomolecules leads to several different possible rearrangements that ultimately affects nanoplastics movement and membrane structure. These unique interactions decrease membrane flexibility and stability, making it more likely to collapse under stress (Li et al., 2023).

The binding mechanisms between the corona and nanoplastics in biological fluids are not fully understood, however, it is believed that the adsorption of the biomolecules to the nanoplastics occurs by non-covalent interactions, such as electrostatic forces,

hydrophobic forces, hydrogen bonding and π – π stacking (Gopinath et al., 2019a; Mahmoudi et al., 2023a; Treuel et al., 2013).

The electric polarity alteration of approaching molecules leads to the establishment of strong bonds (hydrophobic) between them or due to simple dipole against dipole bonding particularly, hydrogen bonds via hydrophilic chemical groups ($-\text{OH}$, $=\text{O}$, $-\text{NH}$, $=\text{NH}$, $\equiv\text{N}$) (Esser, 2014). In a biological context, the spontaneous adsorption of biomolecules to nanoplastics is also influenced by specific aminoacid residues that interact strongly with the surface of polymeric particles through the benzene ring of polystyrene nanoplastics (Adey et al., 1995). Other biomolecules, namely aspartic acid (Asp), arginine (Arg), histidine (His), tryptophan (Trp) and glutamine (Gln) may institute the electrostatic adsorption with polystyrene nanoplastics (Qiang et al., 2017). Considering human serum albumin, the most abundant protein in serum composed of 18 molecules of tyrosine, 31 of phenylalanine and 1 of tryptophan, and 34 disulfide bonds (S-S) that could provide rapid binding (within seconds to minutes) towards nanoplastics and therefore, found as the major proportion of corona in the serum interaction studies (Ramezani & Rafii-Tabar, 2015). A study found that the binding forces of haemoglobin with PS-NH₂ and non charged polystyrene nanoplastics were based on hydrophobic interactions, increased hydrophobicity around aromatic residues (β -Try) and causing structural changes, whereas it was Van der Waals force and hydrogen bonding for PS-COOH (Rajendran & Chandrasekaran, 2023). This corroborated with a previous study that showed protein adsorption pattern is highly influenced by the surface functionalization of nanoplastics (Schöttler et al., 2016b), showing that the absolute number of proteins bound per unit surface area of polystyrene nanoplastics was found to be significantly higher for negatively charged particles ($-\text{COOH}$) compared to both unfunctionalized particles and positively charged particles ($-\text{NH}_2$). In addition, vitronectin, a protein present in the human blood, was shown to highly adhere to negatively charged polystyrene nanoplastics, enriching the protein corona and positively charged polystyrene nanoplastics were specifically adsorbed clusterin, a protein present in several fluids and tissues. Clusterin was also found to be the major hard corona protein of poly(phosphoester) and PEGylated modified polystyrene nanoplastics (Schöttler et al., 2016b).

Additionally, Rits et al., 2015 found that apolipoproteins, which are proteins involved in lipid transport, are preferentially bound to nanoplastics when compared to other

proteins. Apolipoproteins generally act as dysopsonins, helping nanoplastics stay in the bloodstream longer by reducing their uptake by the immune system. Brouwer et al., 2024 showed that in vitro digestion significantly alters the uptake of 50 and 100 nm uncharged polystyrene nanoplastics by THP-1-derived macrophages but polystyrene charged 100 nm or larger than 500 nm did not. Forty proteins were correlated with uptake. The proteins identified here have known roles in phagocytosis, and apolipoproteins such as A-1, A-II, and C-II and complement and coagulation cascade proteins such as von Willebrand factor, complement C3 and fibrinogen may cause differences in uptake. Indeed these proteins can actually increase nanoplastics uptake into human stem cells. In addition, there are other specific proteins (immunoglobulin k, immunoglobulin γ or apolipoprotein AI) that also shown to adhere to polystyrene nanoplastics and enrich the hard corona of these particles (Kokkinopoulou et al., 2017b). Another study compared the adherence of albumin, fibrinogen and apolipoprotein to polystyrene nanoplastics and reported that in the presence of particles with 20 nm at 1 mg.mL⁻¹, albumin attachment time was shorter on the hydrophobic particles. This affinity led to a change in the biological identity of the nanoplastics and consequently physical and chemical modifications (size, aggregation, and surface properties), that finally enhanced the intermolecular interaction between human serum albumin and polystyrene nanoplastics. Indeed, corona formed on the nanoplastics surface have implications in their recognition and internalization by cells (Kihara et al., 2019). A soft corona formation can reach a layer of 70-100 nm of thickness according to (Kokkinopoulou et al., 2017b), in polystyrene nanoplastics unfunctionalized with 140 nm diameter. Zhou et al., 2022 revealed that after 30 minutes a protein corona of a maximum of 60 nm was developed in the surface of polystyrene nanoplastics in the presence of horseradish peroxidase (HRP), an enzyme naturally present in the *Armoracia rusticana* plant.

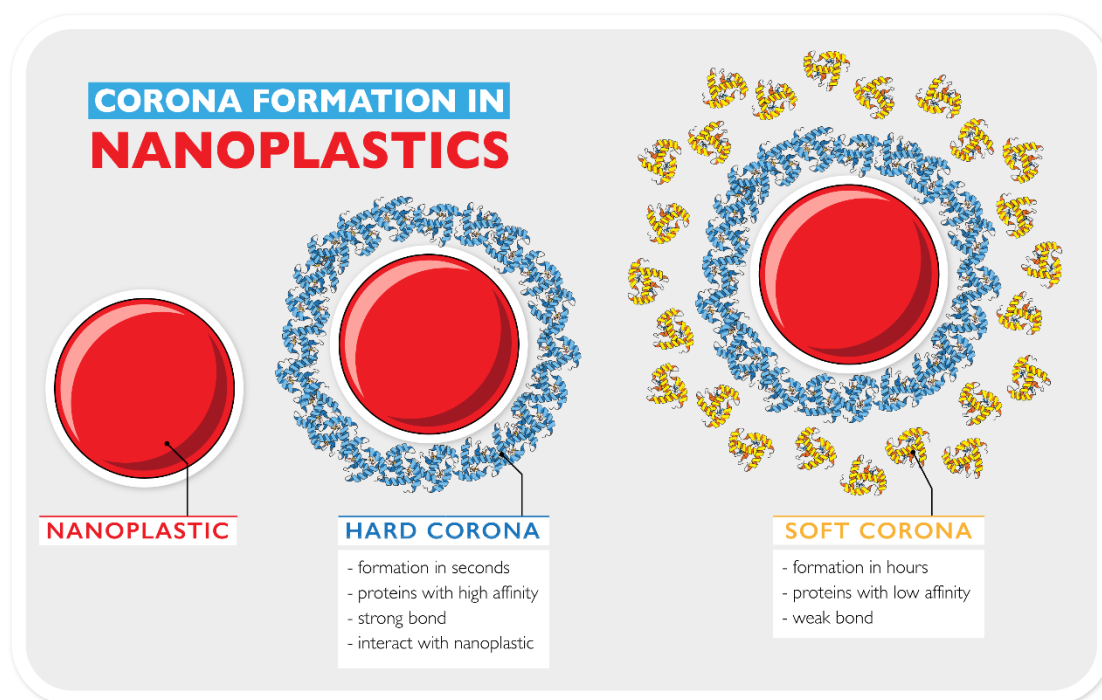


Figure 1. 2 - Temporal Evolution of the Protein Corona on Nanoplastics . Upon exposure to a biological environment, nanoplastics rapidly interact with surrounding biomolecules, particularly proteins, leading to the formation of a "protein corona" that fundamentally alters the nanoplastic's identity. The protein corona is structurally divided into two distinct layers: the "soft corona," comprising proteins with low affinity for the nanoplastic surface that undergo dynamic and reversible exchange; and the "hard corona," consisting of proteins with high affinity that bind irreversibly, providing a stable interface. Understanding the formation and composition of these coronas is essential for predicting the biological behaviour and interactions of nanoplastics in biological environments.

For example, a study concluded that the adsorption of proteins on the surface of 40 nm carboxylated polystyrene nanoplastics (-COOH) ($100 \mu\text{g.mL}^{-1}$) strongly reduced their adhesion to adenocarcinoma human alveolar basal epithelial cells (A549 cells), in comparison to what was observed in neutral polystyrene nanoplastics (Lesniak et al., 2012). Another study observed that increasing the human plasma concentration or increasing the coating temperature leads to a decrease of the uptake of 130 nm polystyrene nanoplastics by HeLa and human umbilical vein endothelial cells (HUVEC) cells (Oberländer et al., 2022). On the contrary, other studies showed that the corona formation led to an increase of cellular absorption, namely in *in vitro* studies with human bronchoalveolar lavage fluid that was exposed to aged polystyrene nanoplastics (more hydrophilic and less electronegative than the pristine polystyrene nanoplastics) (Du et al., 2023).

The corona formation on the nanoplastics surface can also hinder recognition by the immune system (Gopinath et al., 2019b), prolonging circulation and increasing absorption and translocation (Yee et al., 2021a) to all organs, as reported by several studies (Mohr et al., 2014; Pan et al., 2016). As corona facilitates nanoplastics translocation to almost every organ, it increases the potential for negative health hazards (Zhang et al., 2022).

Mechanistically, the protein corona can mask polystyrene nanoplastics surface reactivity, block cell membrane receptors, protect nanoplastics from opsonization and alter blood circulation or enhance cytotoxicity (Gopinath et al., 2019b; Krug and Wick, 2011; Walczak et al., 2015). Inside the cells, the corona formation can also interfere with signalling, metabolism, and gene expression, and cause cellular dysfunction or death (Lin et al., 2017). As coronated nanoplastics trigger immune responses, that can lead to tissue damage and disease (Wang et al., 2023) and autoimmune reactions (Llorca & Farré, 2021a; Rampado et al., 2020).

The same event occur when nanoplastics are introduced in the environment, corona formation occurs by the interaction of nanoplastics with macromolecules available in this media (pollutants, water, soil, organism enzymes) and is called eco-corona. Corona can mediate the release of toxic substances from nanoplastics, such as plastic additives, monomers, or environmental pollutants, which can also cause oxidative stress, inflammation, and genotoxicity (Llorca & Farré, 2021b). Once again, depending on the nature of corona formation, it can affect the transport and mobility of nanoplastics (Cao et al., 2022), impact their toxicity and environmental and health impacts.

Regarding the spatial arrangement of the protein corona surrounding polystyrene nanoplastics, it was shown that proteins are randomly distributed along the polystyrene nanoplastics surface, however these proteins possessed functional epitopes capable of binding to receptors. With the formation of protein-corona, there are high presence of functional epitopes that can enhance nanoplastics uptake in the human body (Kelly & Mcnamee, 2020). The binding affinities of proteins affect the colloidal stability of nanoplastics and depending on which proteins were adsorbed or desorbed to the nanoplastics, conformational changes of proteins occur (Mahmoudi et al., 2023b). External factors such as pH and ionic strength also influence surface-binding-induced

protein unfolding (Ashby et al., 2015; Pan et al., 2012). Protein conformational changes, referring to alterations in the three-dimensional structure of proteins, play a crucial role in influencing their functionality. These changes can lead to the exposure of functional epitopes that were previously concealed (Ha & Loh, 2012). Misfolding events can also occur, which can result in a significant loss of protein function and potentially lead to developing neurodegenerative and systems diseases (Basha et al., 2023). Conformational alterations and denaturation of corona protein, make it a bio-incompatible molecule resulting in genotoxicity and cytotoxicity in human blood cells (T. Yang & Wang, 2023).

Cellular uptake of nanoplastics may promote the production of reactive oxygen species (ROS), which are known to increase the risk of cancer cell growth and proliferation (Poillet-Perez et al., 2015; Roje et al., 2019). Nanoplastics that accumulate in the human body can pose a higher potential risk, as they cannot be easily removed and can persistently exist in the intra-lysosomal membranes (Hornung et al., 2008). Additionally, these plastic particles can induce vascular endothelial leakiness, potentially contributing to adverse effects, especially in cancer patients, where cancer invasion and metastasis can be accelerated (Wei et al., 2022). Hence, the unremoved nanoplastics continues to circulate in the body and can constantly present health risks (Van Cauwenberghe and Janssen, 2014). Although some general principles have been revealed, a few fundamental issues remain unresolved, such as the reversibility and displacement between the adsorbed proteins, the kinetics of corona formation and the prediction of protein corona composition based on the type and properties of the nanoplastics (Hadjidemetriou & Kostarelos, 2017).

1.4.2. Nanoplastics aggregation and surface charge

In addition to the diverse chemical composition and properties that characterize the different plastic polymers (Table 1), nanoplastics exhibit an even more complex composition shaped by environmental interactions. While plastics are initially inert and mostly uncharged, their properties undergo modification upon release into the environment, changing their characteristics and stability after their contact with water, sediment and air matrices. The aggregation of nanoplastics in different environments depends on the chemical and physical parameters of the matrices, including pH, salinity, temperature and presence of ions and environmental contaminants (Oriekhova & Stoll, 2018; Shams et al., 2020). The aggregation of nanoplastics changes their stability and circulation time in the environment and can alter surface properties. Therefore, it is crucial to understand the nanoplastics stability in the environment, to better evaluate their behaviour and residence time in the abiotic and biotic compartments, since it can significantly alter their toxicity (Atugoda et al., 2023).

Temperature and particle concentration have been shown to affect the aggregation and colloidal stability of nanoplastics. For example, in laboratory experiments, negatively charged 70 nm polystyrene nanoplastics were shown to form aggregates up to 2000 nm, particularly in seawater (artificial and coastal), as compared to pure and river waters (Lee & Fang, 2022). This study also showed that the aggregates with increasing size and decreasing colloidal stability were deposited more quickly. Findings from this study suggested that there is an increased potential for nanoplastics exposure in marine benthic organisms, due to the aggregation and deposition, particularly in warmer waters (Lee & Fang, 2022).

Furthermore, the aggregation of nanoplastics in aquatic environments is dependent on water chemistry (dissolved organic carbon, pH, and divalent cations) and particle surface chemistry. When nanoplastics disperse in water, interactions between solid and liquid phases create tension. In aqueous dispersion, the stern layer forms around the particle, developing a negative surface charge (anionic) due to water's high dielectric constant. When cationic ions are present, they partially neutralize this charge, however, a residual anionic charge remains at the nanoplastics surface. This attracts further ions, forming the diffused layer. The boundary between stern and diffused layer (shear plane) provides measurable insights into the dynamic interactions of

nanoplastics within the surrounding medium because it affects the electrical potential, the zeta potential and the mobility of the nanoplastics (Bücker et al., 2019).

Typically, freshwater environments contain higher concentrations of divalent electrolytes like calcium chloride (CaCl_2) compared to seawater, which predominantly contains monovalent electrolytes like sodium chloride (NaCl) (Ma et al., 2023). A study has shown that when polystyrene nanoplastics are positively charged ($-\text{NH}_2$), they exhibit a higher tendency to aggregate with divalent electrolytes (CaCl_2), than monovalent electrolytes (NaCl) (Singh et al., 2019), meaning that in freshwater, positively charged polystyrene nanoplastics tend to agglomerate at higher rates. Another study revealed that polystyrene nanoplastics with ~ 30 nm remained with the same diameter in demineralized water and aggregated and formed larger sizes in seawater. This study also showed that polystyrene nanoplastics negatively charged ($-\text{COOH}$) resulted in large microscale aggregates compared to polystyrene nanoplastics positively charged ($-\text{NH}_2$) aggregates, that still presented nano-size forms (Manfra et al., 2017).

Zhang et al., 2019 showed that the presence of dissolved organic carbon increased the superficial charge of polystyrene latex nanoplastic, however, showed no significant effects on their size in water. When divalent cations (Ca^{2+} and Mg^{2+}) were also present, the surface charge of polystyrene latex nanoplastic decreased and the size increased, which increased the settling of these plastic particles. The augmentation of polystyrene latex nanoplastic aggregation in the presence of dissolved organic carbon and the divalent cation was caused by bridge formation (Zhang et al., 2019). Changes in surface properties and pH affected the stability of polystyrene latex nanoplastic when suspended in water for a long time. Since natural organic matter as well as other suspended solids, including clays and minerals, are ubiquitous in aquatic environments, they have the potential to interact with nanoplastics and influence their aggregation state. These aggregates can occur by self-aggregation (Liu et al., 2019) or by aggregation with other elements (organisms e.g. algae or other materials e.g. clays. Namely clay colloids can also participate in hetero-aggregation with polystyrene nanoplastics under salt influence (Cai et al., 2021b). Additionally, *in vivo*, assays, showed that polystyrene nanoplastics coated with organic matter, such as alginate and humic acid, commonly found in natural environments, significantly reduced the toxicity of polystyrene nanoplastics to zooplankton (Lins et al., 2022). In the presence of algae,

polystyrene nanoplastics aggregation was facilitated and formed larger particles (Liu et al., 2019).

Plastic aging also significantly affects the properties of polystyrene nanoplastics. Liu et al., 2019 simulated aging through UV-irradiation and compared the aggregation kinetics of both fresh and aged polystyrene nanoplastics under aqueous conditions, revealing increased diameter and strong negative surface charge (ζ potential of -43.03 ± 1.1 mV, at pH = 6) in fresh polystyrene nanoplastics (50–100 nm). However, at higher NaCl concentrations, aggregation stabilized due to the absence of energy barrier between polystyrene nanoplastics particles (Chen et al., 2006). The aggregation behaviour follows Derjaguin–Landau–Verwey–Overbeek (DLVO) theory (Yu et al., 2023), balancing van der Waals attraction and electrostatic repulsion (Mylon et al., 2004). Aged polystyrene nanoplastics exhibited a more negative surface charge (ζ potentials decreased from -34.5 to -44.1 mV), likely caused by chain scission during prolonged irradiation, leading to increased organic carbon content. The negative ζ potentials may result from induced O-containing functional groups, reducing hydrophobicity.

The floatability of nanoplastics is also affected after adsorption, desorption, or aggregation of organic and inorganic compounds (Maity et al., 2021; Flury and Yu, 2023), leading to changes in their distribution along the water column. For example, calcium and aluminium ions, which are widely present in natural waters, can adsorb to nanoplastics, causing flocculation and sedimentation of these particles, and consequently influencing their movement within the water column (Pawak et al., 2023). Divalent cations, such as Ca^{2+} , have a greater influence on the aggregation rate of nanoplastics compared to monovalent cations (Singh et al., 2019), as already mentioned in section 1.3.2). The presence of organic substances, such as dissolved organic matter and proteins, can affect the aggregation kinetics of nanoplastics (Yu et al., 2023). Natural organic matter, such as humic acids and sodium alginate, can enhance the aqueous stability of nanoplastics through electrostatic repulsion, steric hindrance, and bridging mechanisms (Pradel et al., 2021). As a result of these interactions, nanoplastics can be bioavailable to several organisms from different water layers and even reach sediments, potentially impact the benthic organisms and, consequently, altering the bioavailability of nanoplastics to aquatic organisms (Sarkar et al., 2022).

1.4.3. Plastic Additives

Plastic particles integrate additives - a vast category of approximately 10,000 chemicals - to provide essential thermoplastic properties essential for achieving desired performance characteristics. Commonly used additives include phthalates, bisphenol A (BPA), nonylphenols, and brominated flame retardants (BFR), such as brominated diphenyl ethers (PBDE) (Fred-Ahmadu et al., 2020a). Plastics can also contain metallic elements, such as chromium (Cr), lead (Pb), and arsenic (As), which are added to plastics for various purposes, including serving as stabilizers, antioxidants, colorants, flame retardants, and catalysts during the manufacturing process (Turner and Filella, 2021). Additives are predominantly distributed among plasticizers (70%) and flame retardants (25%) categories, as outlined in Table 2 (Bianco et al., 2020; Yu et al., 2019; Costa et al., 2023; Deanin, 1975).

The concentrations of additives in the final plastic material may vary widely, between 0.001 and 50.0% (w/w) (Maddela et al., 2023). Despite their crucial role, additives are not chemically bonded with the plastic polymer matrix, rendering them susceptible to leaching from plastics and available for organism uptake (Barrick et al., 2021). In addition, aging, low pH conditions, UV/light irradiation, and microbial degradation can favour the leaching of plastic additives into the environment (Costa et al., 2023; Sarkar et al., 2022). Besides leaching, plastic additives can enter ecosystems through other pathways, such as degradation of plastic waste, and runoff from manufacturing processes.

These chemicals move into the surrounding environment through diffusion, influenced by the porosity and thickness of the polymer, the molecular weight and hydrophobicity of the additives, the characteristics of the surrounding matrix, weathering, and aging processes (Luo et al., 2022; Teuten et al., 2009). The process of leaching from plastic additives occurs by Frick's law, which includes diffusion, desorption, sorption, and adsorption/dispersion (Hahladakis et al., 2018b). In fact, plastic additives have already been registered in every environmental matrix, including river and lake waters (Sun et al., 2018; Ai et al., 2023), estuaries, marine water, marine sediments, municipal wastewater, sludge, landfill leachate, soil, and dust, at several concentrations.

These additives may have been introduced directly in the environment by several industrial processes or they could be the result of the release and lixiviation from

plastics debris.

Examples of plastics additives release from various plastic products include phthalates (Rijk & Ehlert, 2001), BFR (Kim et al., 2006), BPA (Brede et al., 2003; Geens et al., 2010; Olea et al., 1996; Sajiki et al., 2007); bisphenol-A dimethacrylate (Olea et al., 1996), lead, tin and cadmium (Al-Malack, 2001; Mutsuga et al., 2006), formaldehyde and acetaldehyde (Fernandes et al., 2008; Loyo-Rosales et al., 2004; Mutsuga et al., 2006), 4-nonylphenol (Fernandes et al., 2008; Loyo-Rosales et al., 2004), methyl *tert*-butyl ether, benzene (Skjevrak et al., 2003). Irganox 1010, an antioxidant used in plastics such as PP, can be released off-flavors when used as packaging material (Meides et al., 2022). Mato et al., 2001a detected 0.13–16 $\mu\text{g.g}^{-1}$ of nonylphenol in PP resin pellets deployed in Tokyo Bay. Rochman et al., 2014 detected BPA (1.4 ng.g^{-1}), PBDE ($2.2 - 11,182 \text{ ng.g}^{-1}$), alkylphenols ($22.3 - 341.5 \text{ ng.g}^{-1}$) and alkylphenol ethoxylates ($0.8 - 97.6 \text{ ng.g}^{-1}$) in plastic samples collected from the Atlantic Ocean, suggesting that these chemical pollutants were mainly derived from plastic additives. Some plastic additives were found at concentrations up to 6 orders of magnitude higher than the concentrations measured in the surrounding water (from non-detectable to 42.7 pg.L^{-1}). Hirai et al., 2011 detected high PBDE ($0.02 - 9900 \text{ ng.g}^{-1}$), nonylphenol ($0.3 - 3940 \text{ ng.g}^{-1}$), and BPA concentrations (maximum 730 ng.g^{-1}) in PE and PP fragments collected from remote or urban beaches and from the ocean.

A recent study (Fauvelle et al., 2021) found varying rates of leached additives from MP exposed to seawater and deep water in abiotic and biotic conditions. In biotic conditions, deep-sea and surface marine prokaryotes promoted the release of all targeted additives (phthalates, bisphenols, organophosphate esters) from PE and PVC. Additives showed higher release in surface water than in deep seawater (Fauvelle et al., 2021), namely tri-*n*-butyl phosphate ($421 \pm 24 \text{ ng.g}^{-1}$), phthalates (282.8 ng.g^{-1}) and diethylhexyl phthalate ($222 \pm 25 \text{ ng.g}^{-1}$) in PE, and diisononyl phthalate in PVC ($88.2 \pm 24 \text{ ng.g}^{-1}$). Luo et al., 2019 investigated the leaching behaviour of fluorescent additives from MP of PU sponge in stimulated water (e.g., acidic, saline, and basic water) and natural water (e.g., river, wetland, and sea water) and found that the release of additives increased with increasing pH reaching its maximum after 24 hours.

Luo et al., 2019 also found that 1.6 g.L^{-1} of PU MP additives caused notable inhibition

of cell photosynthesis when microalgae were exposed to different MP concentrations (0.1, 0.4, and 1.6 g.L⁻¹, respectively). Another study found that PET showed higher release of plastic additives, particularly from bottles of water, soda, juice and milk. Of the 150 chemicals found in beverages, 18 were measured at levels exceeding EU's regulatory limits (specific migration limit = 40 µg.kg⁻¹), including nickel (48-63 µg.kg⁻¹) and several phthalates (from 42 to 130,000 µg.kg⁻¹) (Gerassimidou et al., 2022). PP and acrylonitrile butadiene styrene can leach out additives, especially when subjected to thermal degradation or environmental stress (Lithner et al., 2012). In several of these studies the concentrations released were low compared to the guideline/legal limit values, but there were also occasions where they were considerably higher. Notably, the guideline values do not consider the low levels at which endocrine disrupting chemicals may be in effect (Vandenberg et al., 2012) and also do not consider the toxic effect of mixtures (Kortenkamp, 2007).

Once introduced into the environment, plastic additives can be absorbed by organisms (e.g. fish, crustacea, molluscs, corals and algae (Hermabessiere et al., 2017b), influencing their metabolism and interactions with surrounding molecules (Maddela et al., 2023; Y. Zhou et al., 2023a). These chemicals exhibit cytotoxicity and some (e.g. BPA) may function as endocrine disruptors (ED) (Campanale et al., 2020), potentially leading to alterations in the reproductive physiology of animals, due to the hormonal activity of these compounds (Marcelino et al., 2022). Notably, ED pose a greater threat than plastic particles, as they have been implicated in DNA mutation and cancer induction (Allouzi et al., 2021), and adverse reproductive effects. Some plastic additives, such as lead, cadmium, and mercury, are known to be toxic to animals, causing damage to their organs, nervous system, and immune system (Kumar, 2018). Plastic additives disrupt oxidative metabolism and cause damage to macromolecules, potentially causing negative consequences on humans and wildlife (Pérez-Albaladejo et al., 2020). Some plastic additives, such as diethylhexyl phthalate (DEHP), are suspected to be carcinogenic to animals, thus increasing the risk of tumors and cancers (Liao et al., 2022).

These substances display environmental persistence, leading to their accumulation in both the food chain and organisms (Maddela et al., 2023) and biomagnification, causing heightened concentrations in higher trophic levels, ultimately affecting humans (Ziani et al., 2023). The leaching of additives into the environment is a source of human

exposure as well as the consumption of animals previously exposed to these additives. *In vitro* assays showed that plastic additives cause adverse effects through the activation of nuclear receptors, peroxisome proliferator-activated receptors, and retinoid X receptor, leading to oxidative stress, cytotoxicity, immunotoxicity, thyroid hormone disruption, and altered adipogenesis and energy production (Manikkam et al., 2013). *In vivo* assays showed that maternal exposure to MP and associated additives altered energy and lipid metabolism in offspring and subsequent generations (Desvergne et al., 2009).

These results, support the hypothesis that MP and their additives are potential obesogens (Kannan & Vimalkumar, 2021). Several of these chemicals, such as phthalic acid esters, and nonylphenols, have been identified in the environment and are known ED (Chakraborty et al., 2022; Fred-Ahmadu et al., 2020a; Koelmans et al., 2015), but several others have been associated with various health effects (detailed in Table 1.2). Still, the adverse effects of over 50% of the plastic additives remain unknown, in spite being evident that there is a significant potential for biomagnification. As these chemicals accumulate in organisms along the food chain, the exposure and potential adverse effects increase at higher trophic levels. This underscores the potential harm to human health posed by these substances.

Table 1. 2 - Effects of plastic additives in marine organisms and human health.

Plastic additives	Function	Potential health implications	References
Bisphenol A	Plasticizers; antioxidant; stabilizer	Endocrine disrupting	Fred-Ahmadu et al., 2020; Hermabessiere et al., 2017
		Epigenetic effects: modification on DNA methylation; histone mark, and non-coding RNA (ncRNA) expression	Deng et al., 2024
		Eryptotic alterations in human red blood cells – human body subacute poisoning	Maćczak et al., 2016
		Structural alterations in the small intestinal tissue, which further affected its functioning; affected the motility of the small intestine and caused its dysfunction	Ambreen et al., 2019
		Alterations of small intestinal morphology; DNA Methylation; Obesity Development	Zulkifli et al., 2022
		Reduce gut permeability Increase pain sensitivity to gut stimuli	Braniste et al., 2010
		Perinatal exposure promotes the development of severe inflammation in adult female	
		Impact on tight junction proteins that affects the integrity of the gut barrier	
		Changes the gut microbiota	Feng et al., 2019
		Increased permeability of the colon; Destroyed the morphology of the colonic epithelium	Silva et al., 2023
		Disrupts the morphology of salivary glands	
Bisphenol F	Plasticizers; antioxidant; stabilizer	Inhibited cell proliferation and secretory cell differentiation; induced inflammation, altered homeostasis	Zhu et al., 2022
		Promoted cell proliferation; alterations in intestinal tissue structure and organization	Zhu et al., 2020
		Oxidative stress and cell damage in the intestine; Inflammation response; disruption in metabolic processes	Wang et al., 2021

Plastic additives	Function	Potential health implications	References
Bisphenol S	Plasticizers; antioxidant; stabilizer	Increased inflammatory markers in colon and ileum samples from F1 and F2 male mice	Brulport et al., 2021
Bisphenol S Phthalates	Plasticizers; antioxidant; stabilizer Plasticizers	Increased the production of pro-inflammatory cytokines	Ao et al., 2022
		Endocrine disruptors	Qian et al., 2020
Phthalates Brominated flame retardants (e.g., PBDEs)	Plasticizers Reduce the rate of combustion and disperse fire	Embryo mortality; uterine fibroids; endometriosis; implantation and placental/developmental development; pregnancy loss/preterm birth	Basso et al., 2022
		Anovulation; reproductive toxicity; Cryptorchidism; hypospadias; Affects fetal growth	Hliseníková et al., 2020
		Testicular toxicity	Rowdhwal & Chen, 2018a
		Ovarian toxicity	Hauser & Calafat, 2005
		Renal; neurotoxicity	Hliseníková et al., 2021
		Hepatotoxicity (hepatocellular carcinoma); cardiotoxicity	Du et al., 2021
		Reproductive toxicity; liver toxicity; kidney toxicity	Brouwer et al., 1999
		Carcinogenic; teratogenicity	Ulbrich & Stahlmann, 2004a)
Brominated flame retardants (e.g., PBDEs) Nonylphenols	Reduce the rate of combustion and disperse fire Plasticizer; antioxidant; surfactants	Neurotoxicity and endocrine disruptors	Muirhead et al., 2006a; Yogui & Sericano, 2009
		Neurotoxicity and endocrine disruptors Endocrine disrupting	Muirhead et al., 2006a; Yogui & Sericano, 2009 Koelmans et al., 2014; Loyo-Rosales et al., 2004
Antioxidants	Inhibit of aging of plastics and delay oxidation	Butylated hydroxytoluene induce apoptosis and promotes tumorigenesis and butylate hydroxyanisole metabolites are involved in carcinogenicity process	Xu et al., 2021
Antioxidants	Inhibit of aging of plastics and delay oxidation	Butylated hydroxytoluene induce apoptosis and promotes tumorigenesis and butylate hydroxyanisole metabolites are involved in carcinogenicity process	Xu et al., 2021

1.4.4. Nanoplastics interaction with environmental pollutants

Nanoplastics interact with environmental pollutants by adsorption, absorption and desorption (Banegas et al., 2023), and act as vectors concentrating inorganic and organic compounds, including metals and metalloids (Pradel et al., 2023; Lee et al., 2019; Singh et al., 2019), polychlorinated biphenyls (PCB), , polycyclic aromatic hydrocarbons (PAH) (Liu et al., 2015) (Sorption of polycyclic aromatic hydrocarbons to polystyrene nanoplastic), bisphenols (BP), such as BPA, bisphenol S (BPS) and bisphenol F (BPF) (Banegas et al., 2023), pharmaceuticals (Thiagarajan et al., 2021) and personal care products, nonylphenol and pesticides (Zhang et al., 2018) (Figure 1.3).

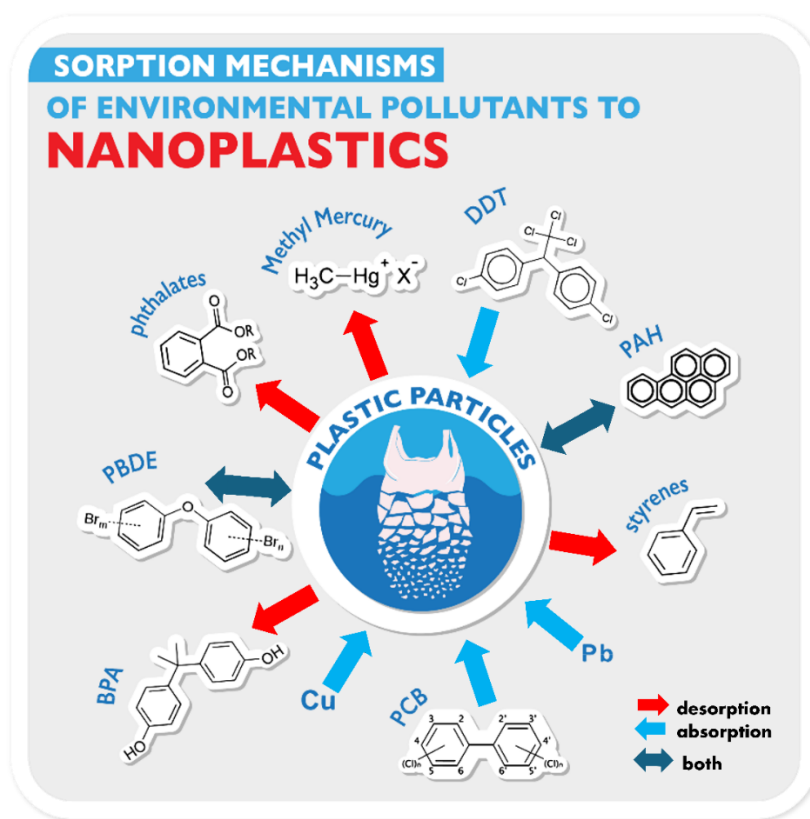


Figure 1. 3 - Schematic representation of sorption mechanisms of environmental pollutants on nanoplastics. Key sorption processes by which environmental pollutants interact with nanoplastics. The mechanisms depicted include leaching, where chemicals previously embedded in the nanoplastic matrix migrate into the surrounding environment; desorption, where pollutants previously adsorbed onto the nanoplastic surface are released back into the environment; and adsorption, where pollutants from the surrounding environment adhere to the nanoplastic surface. Understanding these mechanisms is critical for assessing the environmental impact of nanoplastics and their role as vectors for pollutant transport and accumulation in ecosystems.

These interactions can have detrimental effects on the environment and organisms (Junaid et al., 2023; Khan et al., 2023) and depend on several factors, such as: i) nanoplastics characteristics, such as size, surface area, shape, dose (Mattsson et al., 2015); ii) type of polymer and its sorption capacity (Maity et al., 2021); iii) the presence of additives (Casagrande et al., 2024a; Wang et al., 2020a) and; iv) the hydrophobicity of the contaminants present in the surrounding environment (Atugoda et al., 2023) and medium characteristics, such as temperature, pH, and ionic strength (Gao et al., 2023). The larger surface area of nanoplastics and their smaller size influence the environmental behaviour differently compared to microplastics (Stock et al., 2022).

The main sorption mechanism observed is the hydrophobic interaction force (Pathak & Singh, 2023). These interactions facilitate the uptake of contaminants to living organisms (Llorca & Farré, 2021) and can lead to synergistic, antagonistic, or potentiating effects (Musa et al., 2024; Sharma et al., 2023). For example, the combined exposure of polystyrene nanoplastics with 80 nm diameter and 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) significantly induced hepatotoxicity and neuro-developmental toxicity in zebrafish and remarkably reduced the survival rates of zebrafish embryo (M. Wang & Wang, 2023). The co-exposure of zebrafish to polystyrene nanoplastics with 44 nm and PAH lowered mitochondrial coupling efficiency and increased nicotinamide adenine dinucleotide (NADH) production, implying the impairment of mitochondrial energy production (Trevisan et al., 2019).

Metals are important environmental toxicants known for their ability to accumulate in organisms, transfer through food webs and exert varied toxic effects (Mitra et al., 2022; Peng, 2023). Studying the effects of nanoplastics combined with heavy metals or other environmental pollutants is highly relevant due to their potential for increased toxicity and widespread environmental presence through all environmental matrices. However, while research on nanoplastics adsorption is relatively scarce, studies suggest that the concentrations of metals adsorbed to the surface of nanoplastics may be related to their environmental levels, similar to microplastics (Barletta et al., 2019) and that the metals can enhance or modify the toxicity of nanoplastics, depending on the type, concentration, and interaction of both pollutants (Qi & Qin, 2024). As specific concentrations of metals adsorbed by nanoplastics in natural environments remain largely unexplored due to the challenges of measuring these interactions in situ,

current research often extrapolates results from laboratory assays, such as effects in biological systems. The combined toxicological impacts of metals and nanoplastics include oxidative stress, inflammation, immunotoxicity, neurotoxicity, reproductive damage, and microbiota dysbiosis (Zhang et al., 2022). However, the mechanisms and factors that influence those interactions are still not well understood and need further research. Nevertheless, a study by (Davranche et al., 2019) demonstrated the significant capability of nanoplastics to adsorb cationic lead (Pb(II)). This high adsorption capacity was attributed to the abundance of oxygenated binding sites, which resulted from the weathering of the original plastics due to prolonged exposure to UV irradiation in coastal areas. Another study generated aged polystyrene nanoplastics by UV radiation and investigated the effects of aged nanoplastics on the adsorption and cotransport of Pb-(II) and Cd-(II). The results showed that the UV-aged nanoplastics led to the enhanced adsorption capacity of heavy metals due to the increase in oxygen-containing functional groups, and the promotion of transport by the aged nanoplastics to metals was stronger than that of the pristine nanoplastics (Xi et al., 2022).

Nanoplastics can adsorb metals through electrostatic attraction, pore-filling, complexation, or chelation mechanisms (Blanco et al., 2022). The combination of metals and nanoplastics may affect gene expression, affecting organisms across trophic levels (Sarma et al., 2024). Research has also shown that nanoplastics can increase the toxicity of metals in fish cell lines. Specifically, functionalized nanoplastics have been found to amplify the toxicity of arsenic and methylmercury in a brain-derived cell line from gilthead seabream (González-fernández et al., 2021). Another study observed that polystyrene nanoplastics significantly increased Cu^{2+} accumulation in the liver of fish, which suggested that nanoplastics served as carriers for Cu^{2+} and could increase its bioaccumulation. The results also showed that in a higher concentration Cu^{2+} showed oxidative damage and inflammation. The same study has found congestion and vacuolar degeneration in the liver, as a result of ROS induced by Cu^{2+} affecting the integrity and permeability of the plasma membrane in hepatocytes. Additionally, the study also showed that the diversity and structure of the intestinal microbiota of fish changed, with harmful bacteria increasing in groups exposed to high levels of both copper and polystyrene nanoplastics (Zhang et al., 2022).

In vivo experiments with *Brachionus plicatilis*, a rotifer raised in aquaculture industry used as food for fish larvae, exposed to 1 mg.L⁻¹ Arsenic (As) and different concentrations of nanoplastics (50 nm) and microplastics (6 µm) (1 and 2.5 µg.mL⁻¹) evaluated the reproduction capacity, swimming behaviour, multi xenobiotic resistance activity and antioxidant responses and showed that in individuals exposed to nanoplastics at 2.5 µg.mL⁻¹ there was a strong retardation in population growth, however, the combined exposure to nanoplastics and As exhibited less toxicity than As exposure individually.

Nanoplastics at 2.5 µg.mL⁻¹ caused the highest immobilization rate of these organisms, as well as the nanoplastics combination with As. Moreover, individuals showed higher bioaccumulation when exposed to single As compared to the combination with either nanoplastics or microplastics, however, nanoplastics combined with As increased ROS and higher antioxidant levels (glutathione and glutathione-S-transferase). While combination with microplastics was shown to alleviate adverse effects on *in vivo* endpoints, combination with nanoplastics showed synergistic adverse effects and enhanced toxicity (Kang et al., 2021). Another study showed that polystyrene nanoplastics exacerbated Au ions mortality, deformation, reduced hatching rate, synergized by the production of reactive oxygen species, mitochondrial damage and the proinflammatory responses in zebrafish embryos (Lee et al., 2019).

The heightened affinity between hydrophobic contaminants and nanoplastics, owing to the hydrophobic characteristics of plastic polymers and the extensive surface area of nanoplastics, can disrupt the dynamic distribution of elements in the matrix (Velzeboer et al., 2014). Hydrophobicity is an important factor determining the binding ability of nanoplastics, particularly for non-polar and weakly polar contaminants, greatly affecting environmental transport and distribution compared to polar compounds (Liu et al., 2018). Studies demonstrated that the absorption of PCB and PAH congeners to polystyrene nanoplastics was 2–3 orders of magnitude higher when compared to polystyrene microplastics (Liu et al., 2016; Velzeboer et al., 2014). Therefore, nanoplastics can act as major carriers for hydrophobic contaminants, transporting them to different locations and increasing their bioavailability and toxicity, in a so-called “Trojan horse” effect (Zhang and Goss, 2021).

1.4.5. Microbial biofilms in the surface of nanoplastics

Plastic particles provide abundant superficial area and solid substrate for a variety of microorganisms, namely bacteria, archaea, algae, fungi, protozoa, metazoan and virus (Pinto et al., 2019). The microbial community associated to plastic particles, the plastisphere, are mainly governed by biogeographical and environmental parameters, like availability of nutrients, salinity, and temperature (Monikh et al., 2019; Sharma et al., 2023). Still, it differs significantly from the surrounding environment, with early pioneer communities dominated by primary producers, such as diatoms, cyanobacteria, and green algae. Over time, the plastisphere matures, and the microbial diversity increases, with *Bacteroidetes* and *Alphaproteobacteria* becoming more abundant. Bos et al., 2023 compared early and mature biofilm communities on MP and found that early colonization was dominated by *Alteromonadaceae*, while mature biofilms were predominantly composed of *Rhodobacteraceae*.

Microbial biofilms play a significant role in the interaction between nanoplastics and the environment, once these communities of microorganisms can adhere to plastic surfaces and promote or prevent their aggregation (Sharma et al., 2023). The characteristics of nanoplastics (different surfaces due to size, shape, charge, or type of polymer) play a crucial role in influencing how biofilms interact with and utilize carbon compounds (Liu et al., 2022).

The presence of biofilms on nanoplastics can enhance the desorption and bioaccessibility of pollutants, such as antibiotics in organisms (Moyal et al., 2023), influence their transport (Sooriyakumar et al., 2022a) and act as carriers transferring nanoplastics to higher trophic levels affecting their ingestion by organisms (Baker et al., 2014; Ikuma et al., 2015), influencing on nutrient cycling in aquatic environments (Sooriyakumar et al., 2022). Microorganisms within the plastisphere have been shown to play an important role in plastic degradation, with certain bacterial species capable of degrading microplastics (Zhai et al., 2023). There is a high probability of microorganisms to growth in plastic particles surface (Marsay et al., 2023;), namely in nanoplastics (Miao et al., 2022) and that probably is stimulated by plastic additives as demonstrated by (Lobelle & Cunliffe, 2011). Polystyrene nanoplastics exposure significantly alters the microbial functional diversity (Miao et al., 2022) and can influence the transport and deposition of plastic particles (He et al., 2020).

1.5. Nanoplastics uptake

1.5.1. Cellular uptake

There are different ways that nanoplastics can be internalized by cells (Figure 1.4), depending on their size, shape, surface properties (charge and other elements adsorbed), and exposure route: i) endocytosis or ii) passive diffusion. Despite observations that nanoplastics can enter cells by disrupting cell membrane integrity, namely when aggregated into bigger particles (Khan & Jia, 2023), reports indicate that some nanoplastics employ various molecular pathways for internalization without compromising cell membrane integrity (Xu et al., 2021). For instance, nanoplastics can be internalized by the intestinal mucosa, potentially interacting with membrane transporters, or directly crossing cell membranes (Antunes et al., 2023; Lee et al., 2023).

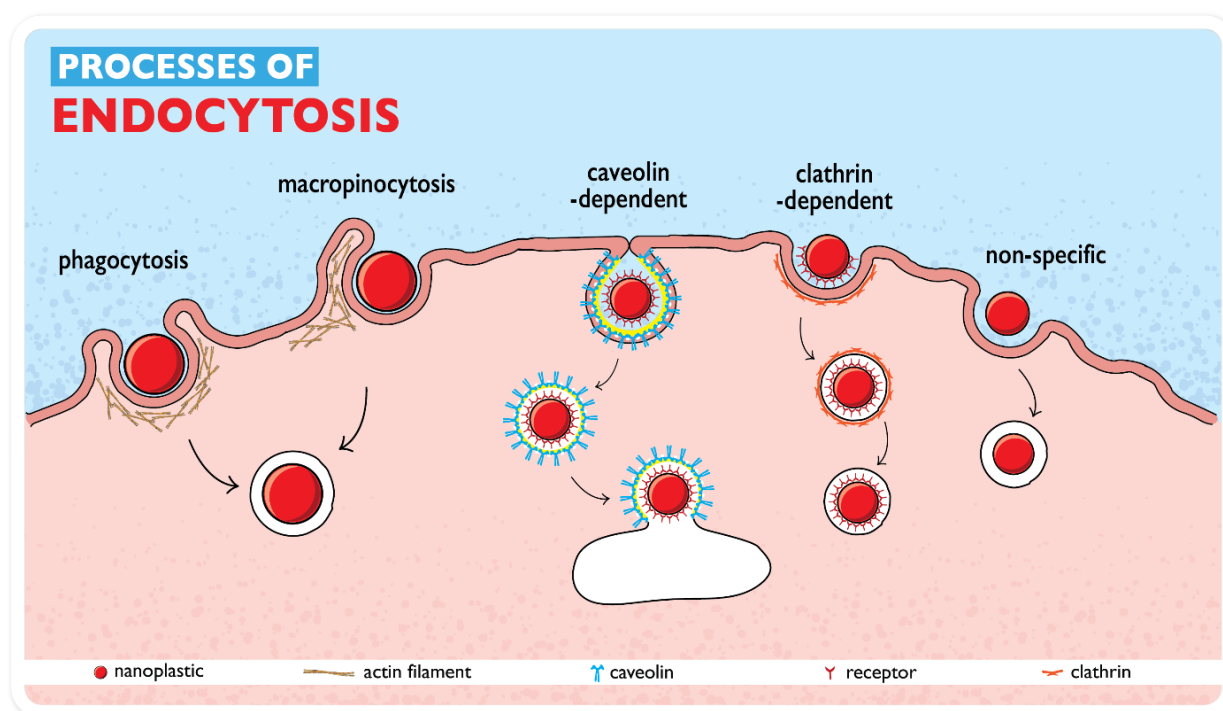


Figure 1. 4 - Mechanisms of nanoplastic uptake in cells. The pathways include endocytosis (phagocytosis, pinocytosis, and receptor-mediated endocytosis), passive diffusion through the cell membrane, and entry via membrane disruptions.

Endocytosis is a process by which cells engulf external particles and form intracellular vesicles. These vesicles facilitate the transport of engulfed particles through the intricate cell membrane trafficking system. This dynamic mechanism is initiated by the invagination or ruffling of the cell membrane followed by the formation of intracellular/endocytic vesicles (Zhao & Stenzel, 2018). Depending on the size, shape,

charge, and surface chemistry of the NP, they can be internalised by different types of endocytosis, such phagocytosis or pinocytosis (Åberg, 2021; Oh & Park, 2014). Agglomerated NP ($\geq 0.5 \mu\text{m} \sim 500\text{nm}$), uptake is done through phagocytosis, as showed by (Kwon et al., 2022) with $0,2 \mu\text{m}$ in microglial cells of the brain. Indeed, this process is performed by specialized cells, such as macrophages, monocytes, dendritic cells, osteoclasts, eosinophils and neutrophils, in which the cell membrane extends and engulfs the particle (Gordon, 2016; Zhang et al., 2015a). In addition, it has been demonstrated that nonspecialized cells such as fibroblasts, epithelial and endothelial cells, also possess phagocytic activity, albeit limited (Rosales & Uribe-Querol, 2017; Swanson, 2008a). This limitation is probably more pronounced when dealing with aggregated NP. Once NP disperse in a physiological fluid such as mucosal, lymph fluid, or blood, they will interact with different proteins that will adsorb to their surface, creating the “protein corona” (Section 1.3). The proteins present in the protein corona (e.g. immunoglobulins) act as opsonins that are recognized by opsonic receptors (e.g. Fc receptors) leading to NP internalization (Gordon, 2016).

Pinocytosis is associated with fluid-phase uptake and includes macropinocytosis, clathrin-mediated endocytosis, caveolin-mediated endocytosis and clathrin/caveolae-independent endocytosis (Doherty & McMahon, 2009a; Kumari et al., 2010). Like phagocytosis, macropinocytosis is an actin-dependent process involved in the engulfment of fluids and micron-sized particles (Geiser, 2010; Swanson, 1995). Depending on the cell–NP interaction, different signalling cascades can occur leading to various structural changes at the cell surface (Sorkin & Von Zastrow, 2009). This mechanism involves the formation of large vesicles called macropinosomes, ranging from 0.2 to $5 \mu\text{m}$ (Swanson, 2008) and cells can engulf NP of various sizes at the same time. Macropinocytosis allows the internalization of larger macromolecules by cells that do not possess phagocytic activity, which would not be possible through other endocytic mechanisms, such as clathrin- and caveolae-mediated endocytosis (Swanson, 1995). Plastic particles with 500 nm were shown to be internalized by macropinocytosis model cell membranes and rat basophilic leukemia (RBL-2H3) cells (Liu et al., 2021). Likewise, macropinocytosis and clathrin-mediated endocytosis were the main routes for the uptake of NP in intestinal cells (Xu et al., 2021). The uptake of NP through macropinocytosis occurs in a non-specific way, meaning that NP are internalized independently of their size and shape, but due to their proximity to the cell

membrane (Reifarth et al., 2018). It is possible that adsorbed proteins on the NP surface (corona protein) can stimulate macropinocytosis. Large particles (>500 nm) are known to be internalized only via phagocytosis and/or macropinocytosis, while the other endocytic mechanisms are limited in terms of cargo size (maximum size of 200–300 nm) (Canton et al., 2013).

Many types of cells use the clathrin- and caveolae-mediated endocytic pathways to internalize nanoscale materials, including NP as shown by Z. Wang et al., 2009. These pathways, characterized by receptor-mediated endocytosis, involve the direct coating of NP with plasma proteins when exposed to physiological fluids. The route of internalization of 40 nm PSNP was studied in different cell types, cervical epithelial (HeLa), lung epithelial (A549), brain astrocytoma and macrophage (J774A.1), revealing the involvement of clathrin- and caveolae-mediated endocytosis in all cells (Santos et al., 2011; Kuhn et al., 2014).

In addition, poly(ethylene glycol)-d,l-polylactide (PEG-PLA) NP have also been shown to be taken up by cells through this mechanism (Luz et al., 2017). NP taken up via clathrin- and caveolae-mediated endocytosis usually end up in the acidic environment of lysosomes (Doherty & McMahon, 2009b). This process is NP size dependent (Cheng et al., 2015; Ho et al., 2018), because caveolae-based vesicles are usually average size ~60 nm and clathrin-based vesicles have an average size ~120 nm. Ho et al., 2018 revealed that 20 and 40 nm PSNP are more dependent on caveolae-mediated endocytosis than 100 nm PSNP, as seen in HUVEC cells. In addition, studies on lung endothelial cells revealed that a single caveolae vesicle was able to engulf up to three 20 nm or two 40 nm albumin-coated polymeric NP (Z. Wang et al., 2009).

Passive diffusion is a type of transport whereby substances move across the cell membrane from an area of higher concentration to a lower concentration without energy consumption. Van der Waals forces and steric interactions can contribute to passive diffusion by influencing how molecules/nanoplastics interact with the membrane. Pore formation in the membrane can also facilitate the passive movement of molecules/NP, especially those that are too large (agglomerations) or polar to diffuse through the lipid bilayer directly (Rimai et al., 2000). NP cannot directly pass through the cell membrane in passive diffusion under normal physiological conditions, unless

they can fit in the pores on the surface (Lai et al., 2022). There are some NP small enough to travel across biological membranes via passive diffusion (Jiang et al., 2008; Zhao & Stenzel, 2018). A study analysed ex vivo human placental perfusion model and showed that PSNP (50 nm) can cross the placental barrier through passive diffusion (Grafmueller et al., 2015). In zebrafish embryos, NP with a size smaller than the chorion pore can also penetrate the cell membrane through passive diffusion and be translocated to the whole body (Lee et al., 2019).

1.6. In aquatic organisms

Planktonic organisms, invertebrates, and vertebrates have been observed to accumulate nanoplastics (Clark et al., 2023; Lee et al., 2022a). Specifically, fish, bivalves, and plankton play crucial roles in the food chain and may ingest organisms that harbour nanoplastics (Monikh et al., 2022). Consequently, these plastic particles can be transferred to other species, including humans (Yuan et al., 2022).

1.6.1. Phytoplankton

Phytoplankton plays a pivotal role in primary production, forming the foundation of the aquatic food chain. An investigation by Liu et al., 2020 using SEM explored the interaction between 300 and 600 nm polystyrene microplastics and the freshwater microalga *Chlamydomonas reinhardtii* (Li et al., 2020). The study revealed that polystyrene microplastics could adhere to the surface of *C. reinhardtii* cells, forming hetero-aggregates. Another study demonstrates that marine phytoplankton *Platymonas helgolandica* var. *Tsingtaoensis* and freshwater phytoplankton *Scenedesmus quadricauda*, exposed particles of 1 µm, internalized these microplastics after 72 h (Chen et al., 2020). Besides that, there is evidence that algal cells are capable of uptaking smaller nanoplastics (Dedman et al., 2022). For example, freshwater green alga *C. reinhardtii*, exposed to polystyrene nanoplastics can accumulate and internalize particles between the size of 40 nm and 85 nm (Yan et al., 2021).

1.6.2. Zooplankton

Zooplankton are significantly affected by plastic pollution, especially nanoplastics. Due to their small size, nanoplastics closely mimic phytoplankton (<2 µm), a primary food

source for zooplankton (Richon et al., 2022). Ingestion is the main pathway for nanoplastic uptake in zooplankton, which exhibit low feeding selectivity (Cole & Galloway, 2015). Additionally, nanoplastics can be transferred maternally, further contributing to their accumulation (Yeo et al., 2023). Zooplankton readily ingest plastic particles of various sizes and surface charges (200 nm), with larger and positively charged nanoplastics tending to accumulate more readily in the gut (M. Wang & Wang, 2023). These particles can also move up the food chain, influencing species interactions, such as disrupting host-parasite dynamics in zooplankton (Mavrianos et al., 2023).

1.6.3. Bivalves

Marine bivalves are reported to accumulate microplastics (including nanoplastics) from all marine environment compartments, being suggested that areas impacted by human activities enhance the uptake of these particles (Saraswati N, 2023), with microfibers and fragments being the most common types. The nanoplastics uptake can occur through the consumption of contaminated biofilms, mucus, plants, algae, and plastic particles with food residues (Mitrano et al., 2021a). They can enter through gills upon contact and inhalation via the siphon (Liu et al., 2024). The uptake of nanoplastics is rapid and influenced by particle size, with smaller particles possibly translocating across epithelial membranes (Al-Sid-Cheikh et al., 2018). Besides gills, studies have recorded nanoplastics in the visceral mass, muscle, hepatopancreas, foot and mantle, in addition to the intestine and stomach (Al-Sid-Cheikh et al., 2018; Sendra et al., 2020). Nanoplastics translocation has been shown to occur faster with smaller particles (50 nm) in comparison to larger ones (1 μm). The internalization of nanoplastics is related to endocytic pathways and has been shown in different types of cells, such as hepatopancreas cells, haemocytes and endothelial cells (Sendra et al., 2020). Different endocytic pathways were confirmed to be involved in the uptake by mussel haemocytes, with phagocytosis being the main uptake mechanism for particles with 1 μm and whereas the caveolae and clathrin routes were shown to play important roles in the uptake of 50 and 100 nm nanoplastics (Sendra et al., 2020).

While no data concerning environmental nanoplastics levels in marine bivalves are known, microplastics were found ranging from 0.05 to 0.83 particles.g⁻¹ wet weight in

marine bivalves from South Korea (Cho et al., 2021), 0.7 to 2.9 particles.g⁻¹ in tissues of wild mussels from United Kingdom (Li et al., 2018) and 0.62 particles.g⁻¹ in tissues of cultivated oysters from China (J. Teng et al., 2019). In three central Italian rivers, the average values of microplastics found was 1953 particles g⁻¹ wet weight, namely in the Aniene River (1615 particles g⁻¹ wet weight), followed by the Marta River (1445 particles g⁻¹ wet weight), and, finally, the Sacco River (980 particles g⁻¹ wet weight). Other studies confirmed that the microplastics concentrations were higher in the aquaculture environment compared to the natural environment (Cesarini et al., 2023).

1.6.4. Fish

Fish are exposed to nanoplastics through diverse pathways, encompassing direct ingestion, indirect transfer via food, exposure via respiration, and absorption through the skin (Shi et al., 2024). These particles have been found to be taken up by fish in various studies. In one study, fluorescent, amino-modified polystyrene nanoplastics (-NH₂) at 10 and mg.L⁻¹ with diameters of 100 and 50 nm were exposed for 96 hours were internalized by fertilized *Danio rerio* eggs and bioaccumulated in the head, especially in the eyes (Contino, Ferruggia, Pecoraro, et al., 2023). Another study found that nanoplastics with 86 and 185 nm were taken up by tilapia, with the intestine exhibiting the greatest relative accumulation (Hao et al., 2023). Polypropylene nanoplastics were also found to be internalized by developing zebrafish embryos and accumulated in the intestine (Lee et al., 2022b). Furthermore, it was shown that 20 nm polystyrene nanoplastics significantly increased the uptake of phenanthrene, a model polycyclic aromatic hydrocarbon, in rainbow trout fingerlings (Zhang and Goss, 2021). Nanoplastics with 80 nm dimensions were also shown to facilitate the transport and uptake of phenanthrene in zebrafish embryos, increasing its bioavailability and potential toxicity (Xu et al., 2022) .

1.7.Nanoplastics toxicity in different trophic levels

The mechanisms underlying nanoplastic toxicity in organisms remain largely unclear, despite a growing body of research over the past decade. In this section, we will

explore the interactions between nanoplastics and biological systems, as well as the potential toxic effects they may pose.

Nanoplastics can affect growth, development, and reproduction across several trophic levels (Cheng et al., 2023; Xie et al., 2023). Their differential surface charges affect accumulation and toxicity in aquatic organisms, which may, in turn, influence their bioavailability and bioaccumulation in the environment (Cunningham et al., 2023). While the majority of studies on the lethal and sublethal effects of nanoplastics in both aquatic and terrestrial organisms have predominantly focused on polystyrene nanoplastics (Masseroni et al., 2022) and adverse effects have been primarily been observed through in vivo and in vitro assays (C. S. S. Ferreira et al., 2023; Hsieh et al., 2023), recent research on freshwater bivalves has demonstrated distinct toxic effects from other types of nanoplastics compared to polystyrene nanobeads. This suggests that different type of nanoplastics polymers may exhibit varying toxicological profiles (Arini et al., 2023). The in vitro findings, however, continue to reveal the complex and multifaceted interactions between nanoplastics and biological systems, emphasizing the need for further research to fully understand the ecological consequences and mechanisms of nanoplastic toxicity in ecosystems.

In aquatic ecosystems, nanoplastics have been shown to affect a wide range of organisms, including bacteria, plankton, bivalves, and fish. The distribution and translocation of these particles across various tissues and organs can have significant health implications for these organisms (Monikh et al., 2022).

For instance, polystyrene nanoplastics have been found to translocate across bacterial cell membranes (Dai et al., 2022), increase intracellular reactive oxygen species (ROS) levels (Sun et al., 2018b), reduce bacterial growth (Mafla-Endara et al., 2023) and even induce gene mutation (Wang et al., 2023). In some cases, nanoplastics can adsorb onto microbial cell membranes, disrupting membrane integrity, inducing oxidative stress, and inhibiting critical processes such as nitrogen cycling (Kim et al., 2022; Wang et al., 2024).

In plankton, Nanoplastics have also been shown to negatively impact planktonic organisms. Exposure to negatively charged nanoplastics has been linked to developmental delays and skeletal defects, including abnormalities in skeleton rods and arms (Annenkov et al., 2023; Bergami et al., 2017). These effects could disrupt

the structural integrity and overall survival of planktonic populations in aquatic ecosystems.

Nanoplastics exhibit varied toxic effects on algal and microalgal species, largely dependent on particle size, concentration, surface charge, and interaction with other contaminants. Smaller nanoparticles, such as 20 nm particles, have been shown to impair algal photosynthesis (Bhattacharya et al., 2010). Polystyrene nanoparticles ranging from 87 to 106 nm have induced growth depression and changes in algal cell morphology (Bellingeri et al., 2019), while smaller polystyrene particles (50 nm) reduced cell density by 45% in *Dunaliella tertiolecta* after 10 days at a concentration of 250 mg.L⁻¹ (Sjollem et al., 2016). Similarly, polystyrene nanoparticles with a 60 nm diameter were shown to affect the growth and microcystin production of *Microcystis aeruginosa* after 30 days of exposure (Zheng et al., 2021). Poly(methyl methacrylate) (PMMA) nanoparticles, 50 nm in diameter and uncharged, caused significant decreases in cell viability in algae, along with increased cell size and complexity, overproduction of pigments, loss of membrane integrity, mitochondrial hyperpolarization, increased ROS and lipid peroxidation (LPO), reduced DNA content, and diminished photosynthetic capacity (Gomes et al., 2020). Zhu et al., 2021 outlined several mechanisms of toxicity in microalgae, including the blocking of substance and energy exchange with their environment, shading effects, increased ROS production, and physical damage to the cells. Nanoplastics can also be trophically transferred through microalgae to higher trophic levels, further amplifying their ecological impact (Liu et al., 2020).

Zooplankton have shown the capacity to fragment microplastics into smaller nanoparticles through digestive processes. This has been observed in Antarctic krill, where digestive fragmentation contributes to the breakdown of larger microplastic particles into nanoscale debris (Dawson et al., 2018). Additionally, exposure to nanoparticles in sea urchins has been linked to larval malformations, suggesting that zooplankton are highly susceptible to nanoplastic toxicity (Pinsino et al., 2017).

Positively charged polystyrene nanoparticles (-NH₂) with a size of 40 nm have demonstrated significant toxicity, causing 50% mortality in zooplankton exposed to concentrations of 13 µg.mL⁻¹ after 24 hours and 7 µg.mL⁻¹ after 48 hours (Manfra et al., 2017).

Nanoplastics significantly impact terrestrial plants, affecting their growth and physiological processes. For instance, nanoplastics with dimensions of 100 nm are readily taken up by plants and translocated to the shoots, leading to negative effects (Li et al., 2021). Bosker et al., 2019 investigated the effects of various sizes of plastic particles (50, 500, and 4800 nm) on the vascular plant *Lepidium sativum*, finding that the germination rate decreased significantly with smaller particle sizes. Moreover, 50 nm nanoplastics induced oxidative stress, increased cytological abnormalities, and decreased root length, biomass, and chlorophyll content in several terrestrial plants (Giorgetti et al., 2020). *Arabidopsis thaliana* can uptake and transport nanoplastics smaller than 200 nm, regardless of their surface charge (Sun et al., 2020). Positively charged polystyrene nanoplastics (-NH₂) exhibited a stronger effect on root systems but showed lower uptake and internalization compared to negatively charged counterparts.

The positively charged nanoplastics can cause root epidermal damage due to electrostatic interactions, which increase local concentrations and disrupt water and nutrient transport, consequently reducing above-ground biomass. Conversely, negatively charged nanoplastics stimulate root exudate production, influencing polystyrene stability and limiting its uptake by *A. thaliana*.

In cucumbers, polystyrene nanoplastics accumulate in the root system before being transported to the leaves, flowers, and fruits through the stems. This accumulation can affect metabolite transformation and mineral element adsorption in cucumber fruits (Li et al., 2021). Weert et al., 2019 showed that nanoplastics sized between 50 and 190 nm reduced the shoot-to-root biomass ratio in *Myriophyllum spicatum* and *Elodea* sp. Studies have also indicated that polystyrene nanoplastics can be taken up by crops like peanuts and rice, accumulating in grains (Jiang et al., 2022). Their presence in grains can negatively affect crop yield and nutritional quality, leading to a decrease in seed-setting rates and changes in mineral elements, amino acids, and unsaturated fatty acids (Vilas Boas & Oliveira, 2023). Additionally, a study confirmed that polystyrene nanoplastics were taken up by the roots of *Oryza sativa* L., enhancing antioxidant enzyme activities and reflecting oxidative stress responses (Zhou et al., 2021).

In molluscs, namely bivalves, nanoplastics anoplastics induce various negative effects,

including malformed and immature embryos, decreased shell length, and reduced swimming speed (Allam et al., 2022; Zhou et al., 2024; Sendra et al., 2021). These particles can adsorb to gills, reducing filtering activity (Wegner et al., 2012). Exposure to negatively charged polystyrene nanoplastics leads to increased levels of reactive oxygen species (ROS), which are associated with oxidative stress and cellular damage (Zhang et al., 2022). This exposure can also result in cytochrome C reduction, decreased phagocytosis, and pronounced lysosomal effects (Balbi et al., 2017; González-Fernández et al., 2018). The reduction in phagocytosis ability may significantly impact the immune response, as it is crucial for clearing pathogens and debris. Significant alterations in lysosomal function could affect cellular homeostasis and function, as demonstrated in *Meretrix meretrix* (Liu et al., 2021).

In crustaceans, such as the brine shrimp *Artemia franciscana*, exposure to 100 nm nanoplastics at concentrations greater than 1 mg.L⁻¹ resulted in aggregation in the gut lumen and decreased swimming speed after 48 hours. Additionally, cholinesterase and catalase activities were significantly affected by exposure to nanoplastics ranging from 0.001 to 10 mg.L⁻¹, indicating induced neurotoxic effects and oxidative stress (Gambardella et al., 2017). Another study evaluated the effects of 40 nm polystyrene nanoplastics negatively charged (-NH₂) and 50 nm polystyrene nanoplastics positively charged (-NH₂) in *A. franciscana*, and showed both acute (72h) and chronic (14 days) toxicity. Moreover, the expression of target genes (i.e. *clap* and *cstb*), having a role in brine shrimp larval growth and molting, was measured in 48 h brine shrimp larvae. polystyrene nanoplastics negatively charged (-NH₂) formed micro-scale aggregates (Z-Average > 1 µm) and did not affect the growth of brine shrimps up to 10 µg.ml⁻¹. However, these negatively charged nanoplastics accumulated and were excreted by brine shrimps after being eaten in a combination with microalgae, suggesting a potential trophic transfer from prey to predator. On the other hand, polystyrene nanoplastics positively charged (-NH₂) formed nano-scale aggregates (Z-Average < 200 nm), caused mortality in brine shrimps after 14 d (LC50 = 0.83 µg.ml⁻¹). Moreover, 1 µg.ml⁻¹ polystyrene nanoplastics positively charged (-NH₂) significantly induced *clap* and *cstb* genes, explaining the physiological alterations (e.g. increase in molting) previously observed in 48 h larvae, but also suggesting an apoptotic pathway triggered by cathepsin L-like protease (Bergami et al., 2017). In *D. magna* 50 nm nanoplastics showed significant toxicity and physical damage, increasing with the concentration of exposure (from 5 to 50 mg.L⁻¹). At 10 mg.L⁻¹ there was an accumulation of nanoplastics

and already severe damage on the thoracopods, which decreased the swimming ability. At the highest concentration (50 mg.L⁻¹), all-body damage was observed and *Daphnia magna* were immobilized. After 14 days, 50 nm nanoplastics (5 mg.L⁻¹) significantly increased the bioaccumulation of ¹⁴C-Phe-derived residues in the daphnid body, indicating that nanoplastics functioned as vectors of hydrophobic POP by adsorbing and concentrating them on the surface. It was also observed that phenantrene (0.1 mg.L⁻¹) can exhibit higher toxicity in the presence of 50 nm nanoplastics (5 mg.L⁻¹) (Ma et al., 2016).

Nanoplastics can negatively affect fish health and performance, with accumulation in tissues, decreased locomotor and foraging activities, effects on growth, immune system, lipid metabolism, and neurotoxicity reviewed by (Barría et al., 2020). It has been demonstrated that nanoplastics tend to specifically accumulate in fish liver (Monikh et al., 2022). Polystyrene nanoplastics with 20 nm in diameter can bioaccumulate and cause oxidative DNA damage in the brain of zebrafish embryos (*Danio rerio*) (Sökmen et al., 2020).

In adult fathead minnows, after 48 hours of exposure to 50 nm polystyrene nanoplastics (*Pimephales promelas*) through intraperitoneal injection (0.1 mL of 50 nm polystyrene nanoplastics at 5 µg.L⁻¹) and ingestion (contaminated *Daphnia* with polystyrene nanoplastics at 5 µg.L⁻¹), nanoplastics were encountered in the liver and kidney. Additionally, there was a downregulation in the gene expression of *ncf*, *mst1*, and *c3* in both organs, suggesting that nanoplastics interfered with the synthesis and function of neutrophils potentially impairing the ability to defend themselves against pathogens and other contaminants (Elizalde-Velázquez et al., 2020). Another study with zebrafish exposed to 70 nm polystyrene nanoplastics (0.5 and 1.5 mg.L⁻¹ during 7 days) showed accumulation in gonads, intestine, liver, and brain. Adult fish exposed to polystyrene nanoplastics showed hyperactivity-behaviour, alteration in exploratory behaviour, aggressiveness, shoal formation, and predator avoidance behaviour, along with a dysregulated circadian rhythm locomotion activity after its chronic exposure. Additionally, ROS levels increased in the muscle of fish, although no significant changes in creatine kinase, a muscle energy marker or Hif-1α, a key marker for hypoxia, were observed. In the liver was observed increased DNA damage, TNF-α expression and a significant immune response, suggesting a synergistic effect on inflammation markers. Furthermore, the lack of anti-inflammatory function observed in

this organ, combined with elevated DNA damage, suggested oxidative stress-related toxicity (Sarasamma et al., 2020). Zebrafish larvae exposed to 0.2, 2 and 20 mg.L⁻¹ of 25 nm polystyrene nanoplastics from 70 to 120 hours post fertilisation also showed dose-dependent disrupted glucose metabolism and increased cortisol levels, leading to increased cortisol secretion and hyperactivity (Brun et al., 2019). A study performed with juvenile *Larimichthys crocea* showed that after 14 days of exposure, polystyrene nanoplastics reduced digestive enzyme activities, impacting negatively the digestion and absorption in this specie. Moreover, the analysis of the intestinal microbiota showed that the proportion of the three-dominant bacterial phyla (Bacteroidetes, Proteobacteria and Firmicutes) in the gut changed significantly, accompanied by a significant increase of potentially pathogenic bacteria (*Parabacteroides* and *Alistipes*). Besides those effects, lysozyme activity was significantly reduced and total mortality of juvenile fish was significantly increased (Gu et al., 2020).

The larval channel catfish *Lctalurus punetaus* exposed to 0, 5, 10, 25, and 50 mg.L⁻¹ of 75 nm polystyrene nanoplastics also showed decreased glucose and triglyceride contents with prolonged exposure duration (maximum 48 hours). Activities of most metabolic enzymes decreased in the larvae after 48 h of exposure, especially with 25 or 50 mg.L⁻¹, suggesting that polystyrene nanoplastics caused a significant increase in energy consumption and disturbed the energy metabolism in larval fish. After 48 hours of exposure to 50 mg.L⁻¹, the expression of genes was altered, namely, genes involved in protein digestion and proteasome response, heat shock proteins, and the genes involved in peroxisome proliferator-activated receptors pathway and biosynthesis of amino acids. also, polystyrene nanoplastics depressed the expression of the genes involved in gonad development or muscle contraction. As observed with other sizes of polystyrene nanoplastics, acute exposure to 75 nm polystyrene nanoplastics also disrupted the energy metabolism by consuming the energy reserves, and affected a series of molecular pathways which may further affect the development and survival of fish (Jiang et al., 2022) (Figure 1.5).

In chickens, exposure to polystyrene nanoplastics (25 nm) during embryonic development caused birth defects, especially leading to problems in cardiac function (Wang et al., 2023). This is related to the disruption of neural crest cells, which are crucial for proper development and migration of cells in the body.



Figure 1. 5 – Impact of Nanoplastics Across Trophic Levels: Decomposers, Producers, and Consumers: summary of the effects of nanoplastic toxicity across various ecological levels, including decomposers (bacteria), producers (plankton and algae), and consumers (plants, invertebrates, fish, mammals, and humans), highlighting alterations in growth, reproduction, and physiological processes. This comprehensive overview emphasizes the broad ecological consequences of nanoplastic pollution and its potential to disrupt entire food webs and impact human health.

1.8. Mechanism of nanoplastics toxicity in humans

Humans are exposed to nanoplastics via multiple sources like through air, food, drinking water and personal care products (Mortensen et al., 2021) and consequently, there are several pathways of human exposure to nanoplastics, namely through inhalation, dermal exposure and ingestion (Yee et al., 2021).

The contribution of each source is poorly understood, as information regarding nanoplastics concentration in these media is lacking. Therefore, as discussed below, exposure to nanoplastics is frequently inferred from data on microplastics, which is necessarily a limited approach, moreover because the data on microplastic exposure also show great variability.

Recent estimates indicate that individuals annually consume a significant number of microplastics through inhalation and food intake, ranging from 74,000 to 121,000

particles, with variations based on age and sex (Cox et al., 2019). Additionally, the consumption of water is suggested to contribute an extra 4,000 to 90,000 particles per year, depending on the preference for tap water or bottled water. The total ingestion, considering microplastics is estimated to be between 39,000 and 52,000 particles annually (Cox et al., 2019), however, the exact number of nanoplastics that contact with the gut epithelium and is absorbed is unknown and is probably lower.

Inhalation of airborne plastic particles can originate from textiles or polluted outdoor air, such as urban dust or rubber tyres (Prata, 2018) (Figure 1.1). Skin contact is also pathway of nanoplastics exposure, whether through contaminated water or direct contact, including the penetration of nanoplastics facilitated by specific ingredients in skin care lotions (cosmetics or personal care products) (Jatana et al., 2016; Xiong et al., 2020).

Micro or nanoplastics were found in the most consumed food in the world (rice, eggs, chickens, wheat) and Europe (milk, pig and sugar). Microplastics were found in rice (4 mg per 100 g in stored package (Dessi et al., 2021), in eggs (average content was 12 ± 4 microplastics.egg⁻¹, mostly polyethylene) (Liu et al., 2022), in chickens gizzards (33 ± 18 microplastics particles per gizzard⁻¹ in Pakistan; 57 ± 41 microplastics particles per gizzard⁻¹ in Mexico; 30 ± 20 microplastics particles per gizzard⁻¹ in Florida; 10 ± 2 in Norway (Bilal et al., 2023; Lwanga et al., 2017; Jonathan et al., 2021; Collard et al., 2022) , and wheat (uptake and translocate 200 nm polystyrene nanoplastics from roots to shoots (Li et al., 2020).

The source of microplastics in chickens is the external natural environment and feed, coming from the soil, earthworms and grains (0.87 and 14.8 particles.g⁻¹, respectively (Lwanga et al., 2017). Therefore, when the chicken is laying eggs, the microplastics in the body will inevitably be excreted in parallel (Liu et al., 2022). Another source of microplastics (mainly PE) can result from packaging, during storage and transportation, which enters the eggs through shell pores (size from 300 – 2000 µm (Zimmermann & Hipfner, 2007). microplastics, including polyethylene, polypropylene, and polyamide have been found in milk (204 - 1004 microplastics per 100 mL) (Da Costa Filho et al., 2021).

Microplastics were already detected in other animals consumed by humans, namely pigs (180,000 MP.kg⁻¹). The contamination likely originates from the farm feeds, which

contain significant levels of microplastics (3.33×10^2 items per kilogram in wet weight (Wu et al., 2021). These findings elicit growing concern, particularly due to contaminants associated with microplastics that were also detected. For instance, concentrations of 2.4 mg.kg^{-1} of DEHP in pigs surpassed the accepted limit stipulated by the European Food Directive 2002/72/EC. This discovery not only raises alarm regarding the potential health repercussions to the animals exposed to microplastics and associated pollutants but also underscores the potential hazards to human health upon consumption of these contaminated animals. According to a recent study, microplastics were also found in commercial salt and sugar. Studies found on average 1653 ± 29 microplastics per kg in commercial salt (Di Fiore et al., 2023) and 343.7 ± 32.08 microplastics per kg in sugar. Although the quantities found in salt have been significantly higher than those in sugar, sugar consumption is much higher than salt and therefore leads to greater exposure to microplastics through this food (Afrin et al., 2022).

Micro and nanoplastics were also found in the most consumed drinks in the world (water, tea, beer, and coffee). In bottled water, microplastics and nanoplastics were detected between 1.08×10^5 nanoplastics per liter (Zhang et al., 2023) to $2.4 \pm 1.3 \times 10^5$ (Qian et al., 2024) and released $1522 \pm 0.21 \text{ ng.mL}^{-1}$ after 240 minutes of irradiation (Râpă et al., 2023). Microplastics ($\sim 700 \text{ }\mu\text{m}$) were also found in the public drinking water network (tap water), with concentrations ranging from 0.7 to $20 \text{ }\mu\text{g.L}^{-1}$, in Barcelona (Vega-Herrera et al., 2022) specifically particles of polyethylene, polypropylene, and polyisoprene. Plastic tea bags reportedly release 12 billion microplastics and 3 billion of nanoplastics into a single cup (Hernandez et al., 2019). In tea leaves 200 to 500 microplastics per gram were found (Li et al., 2022). As in tea, the concentrations of plastics in coffee vary depending on the type of packaging and extraction method used. One study found that a single plastic coffee bag steeped at $95 \text{ }^\circ\text{C}$ for 5 minutes could release more than 10,000 microplastic particles into a cup of coffee (Wang et al., 2023).

Microplastic contamination can also be present in beer cans due to the polymeric coating films used as a barrier between the metal and the product (Nurlatifah & Nakata, 2021), with concentrations ranging from $20\text{-}80 \text{ particles.mL}^{-1}$ (Liebezeit & Liebezeit, 2014). In Croatia, phthalates, have been also detected in beer PET bottles in significant amounts ($219.82 \text{ }\mu\text{g.L}^{-1}$, especially with high concentrations of dibutyl

phthalate ($92.17 \mu\text{g.L}^{-1}$) and bis (2-ethylhexyl) phthalate ($326.81 \mu\text{g.L}^{-1}$) (Habschied et al., 2023).

Nanoplastics have been found in fruits, vegetables, and processed plant-derived foods (Liang et al., 2023) which constitutes a food safety concern (Lazăr et al., 2024). In a study on cucumber plants, polystyrene nanoplastics were found to accumulate in the root system before being distributed to the aboveground parts of the plant, including the fruits (Li et al., 2020). Another study concluded that the estimated daily intakes for adults and children of microplastics ($< 10 \mu\text{m}$) in fruit and vegetables were 223,000 (52,600 – 307,750) and 97,800 (72,175 – 130,500), respectively. In particular, apples were the most contaminated fruit samples, while carrot was the most contaminated vegetable, in contrary to lettuce that was registered the lower levels of microplastics (26,375 – 75,425). Besides the wide variability of microplastics levels, Conti et al., 2020 hypothesized that fruits probably contain high number of microplastics due to their vascularization pulp and the high complex root system and age of the tree that support (several years) compared to the vegetables (maximum 75 days for the carrot).

Since nanoplastics are typically hydrophobic (Yu et al., 2019; Y. Zhang et al., 2020), they may be expected to enter the body through sweat glands, hair follicles, and especially through the skin injury (Bhat et al., 2023). The predominant route of nanoplastics permeation through the skin appears to be the intercellular pathway, where they diffuse through the lipid matrix between corneocytes (Bouwstra et al., 2000). Despite limited evidence on nanoplastics toxicity via skin exposure (Huang et al., 2022), research suggests that nanoplastics may penetrate the skin and induce changes in intracellular reactive oxygen species and thiol levels (Revel et al., 2018; Schmidt et al., 2023). Cosmetic products containing nano-sized plastic particles have been found to adhere to the keratin layer, potentially causing cytotoxic and cytoprotective effects in keratinocytes (Gopinath et al., 2021), while nanoplastics exposure in zebrafish embryos has been linked to apoptosis of skin cells (Kantha et al., 2022). Furthermore, polymethyl methacrylate nanoplastics have been shown to pass into embryos and accumulate intracellularly in fibroblast cells (Simpson et al., 2023). Research on guinea pigs demonstrated that poly(ϵ -caprolactone)-block-poly(ethylene glycol) particles, sized 40 to 130 nm, were able to penetrate the skin, with hair follicles potentially serving as a critical route for nanoplastics entry (Shim et

al., 2004).

The permeability of nanoplastics may vary depending on their polymer type and structure, with more flexible nanoplastics possibly accumulating in specific organs or crossing cellular barriers, slowly releasing chemicals and posing long-term health risks (Zoabi et al., 2021). Although the intercellular pathway seems to be the primary route for nanoplastics penetration, evidence on nanoplastic skin permeation in humans remains scarce. Only one study has investigated nanoplastic penetration in pig skin (20 nm and 200 nm, for 0.5–2 hours), showing accumulation at the hair follicle entrance, but no deep penetration into skin tissue (Alvarez-Román et al., 2004).

Research in the nanomaterial field has shown that the concentration of nanoparticles within cells increases linearly over time, and once internalized, the uptake is essentially irreversible. These particles tend to accumulate in lysosomes (Salvati et al., 2011). In the case of larger nanoplastics (larger than 200 nm), researchers showed that they are not easily internalised, however when nanoplastics have dimensions lower than 100 nm are readily taken up by the endocytic cell machinery (Lee et al., 2023) and the smaller (20 nm) can end up in mitochondria, instead of accumulating in lysosomes (Johnston et al., 2010).

Another study compared particles with 5000, 500 and 50 nm regarding internalization in cells and showed that some particles with 5000 nm dimension did not adhere to the cell membrane or had been internalized due to their large size and weak Brownian motion, however, the 50 and 500 nm nanoplastics were internalized into living cells via both passive membrane penetration and active endocytosis. Passive penetration occurs due to polystyrene nanoplastics partitioning in the water-phospholipid system and endocytosis of 50 nm particles involves the clathrin-mediated pathway, caveolin-mediated pathway, and macropinocytosis, while 500 nm particles mainly use macropinocytosis. Nanoplastics with 50 to 500 nm dimension that were passive internalized seems to be distributed in the cytoplasm and later transported to lysosomes with energy supply and later excreted, while the nanoplastics endocytosed mainly accumulate in lysosomes. Nanoplastics with 50 nm dimensions seems to be easily internalized and excreted than those with 500 nm (Liu et al., 2021). Nanoplastics that accumulated in the lysosomes can lead to lysosomal membrane damage (LMD), causing a DNA mutation in the mitochondria, thus resulting in mitochondrial

dysfunction and further ROS release (Brandts et al., 2023).

Indeed, polystyrene microplastics and nanoplastics have been shown to cause apoptosis, pyroptosis, fibrosis, due to oxidative stress, namely in the granulosa cells of the ovaries after exposure to daily hygienic items (An et al., 2021). As already disclaimed in section 4, when nanoplastics enters the human body, macromolecules such as proteins, lipids, phospholipids, and carbohydrates can form biological protein coronas around them and ease their cellular uptake level (Zhou et al., 2022). Nanoplastics was showed to cause several effects in cells. Figure 1.6 summarises the main cell damage of nanoplastics, such as membrane breakage (Hollóczki & Gehrke, 2019), DNA damage (Møller and Roursgaard 2023), oxidative stress (Zhang et al. 2022), mitochondria damage (Lee et al. 2022) and pro-inflammatory cytokines production (Antunes et al. 2023).

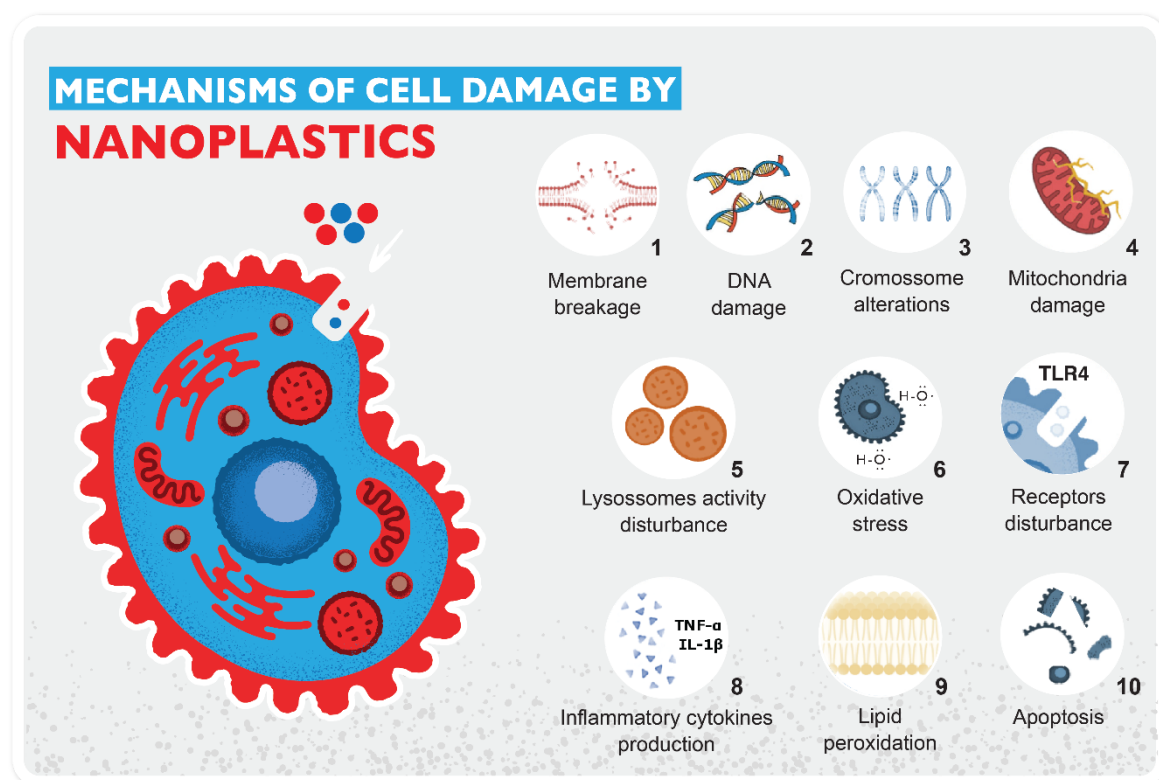


Figure 1. 6 - Mechanisms of cell damage by nanoplastics exposure: 1) membrane damage; 2) DNA damage; 3) chromosome alterations; 4) mitochondria damage ; 5) lysosomes activity disturbance; 6) oxidative stress; 7) receptors disturbance; 8) inflammatory cytokines production; 9) Lipid peroxidation; 10) apoptosis.

1.8.1. Effects on the lung

The lung has a very large alveolar surface area with a very thin tissue barrier with less than 1 μm (Gehr et al., 1978) that allows small plastic particles to penetrate the capillary blood system and being distributed throughout the human body (Lehner et al., 2019). NP have been detected in the lungs, both in lung tissue and alveolar phagocytes, indicating their presence in the respiratory system (Liu et al., 2023) and several studies have shown that exposure to these synthetic polymers can have adverse effects on the health if absorbed by the respiratory system.

A study showed that changes in global gene expression in the lung of ICR mice occurred in response to the inflammation and fibrosis induced by the inhalation of 500 nm PSMP at 4, 8, and 16 $\mu\text{g.mL}^{-1}$ for two weeks (Jin et al., 2023). Significant upregulation was detected in several inflammatory endpoints, including the number of immune cells in bronchoalveolar lavage fluid, the expression of genes associated with inflammatory responses and tissue scarring, mucin secretion, and histopathological changes and accumulation in the lungs of the inhaled ICR mice (average of $13.38 \pm 1.0 \mu\text{g.g}^{-1}$). This study also mentioned upregulation of fibrosis-related factors, such as pulmonary parenchymal area, expression of pro-fibrotic marker genes, and TGF- β 1 downstream signalling (Jin et al., 2023).

Studies have also shown that NP can cause lung injuries mediated by redox imbalance, leading to oxidative stress and tissue damage (Fan et al., 2022). Activation of the JAK2/STAT3 pathway by NP can aggravate pulmonary inflammatory responses and disrupt the balance between M1 and M2 macrophages in the lungs. A study showed that exposure to 100 or 200 nm PSNP ($100 \mu\text{g.mL}^{-1}$, $200 \mu\text{g.mL}^{-1}$ and $400 \mu\text{g.mL}^{-1}$ for 24 h) caused ferroptosis in bronchial epithelial cells by activating the HIF-1 α /HO-1 signalling pathway (Wu et al., 2023) and promote lung injury (Xuan et al., 2023). Another investigation involving A549 cells demonstrated that PSNP of 25 and 70 nm at concentrations of $25 \mu\text{g.mL}^{-1}$, and $160 \mu\text{g.mL}^{-1}$, respectively for 24 hours induced significant up-regulation of NF- κ B and pro-inflammatory cytokines, such as IL-8, and TNF- α , as well as pro-apoptotic proteins such as DR5, caspase-3, 8, and 9, and cytochrome c, consistent with triggering of a TNF- α -associated apoptotic pathway (Xu, Halimu, et al., 2019). Exposure of RAW 264.7 macrophages and BEAS-2B lung

epithelial cells to charged PSNP-NH₂ with 60 nm diameter at 10, 20 or 40 µg.ml⁻¹) for 8 hours, induce autophagic cell death and induced necrosis, likely due to mitochondrial injury followed by rapid ATP consumption (Chiu et al., 2015). It was also shown that aminated NP exhibited higher toxicity compared to carboxylated NP.

Another study (Ruenraroengsak & Tetley, 2015) highlighted the differential effects of surface-modified NP (PS latex NP with 50 and 100 nm) in primary human alveolar macrophages (MAC) and primary human alveolar type 2 (AT2) epithelial cells and human alveolar epithelial type I-like cells (TT1). The amine-surface modified NP induced cell death in all cell types, leading to increased oxidative stress, mitochondrial disruption, and cytochrome C release (indicating apoptotic cell death), while neutral and PSNP-NH₂ exhibited low cytotoxicity. Cellular responses vary between MAC and AT2 epithelial cells and TT1 cells with strong particle internalization observed in MAC and TT1 cells compared to AT2 cells. The bioreactivity of NP also varied with size, with 50 nm NP showing higher reactivity in epithelial cells and 100 nm NP having a stronger effect on phagocytic cells. Mechanisms of cytotoxicity involved oxidative stress, mitochondrial disruption and apoptotic cell death. These findings suggest that inhaled NP can potentially cause respiratory health implications, namely irritation, dyspnea, decreased lung capacity, interstitial fibrosis, and granulomatous lesions (Allen et al., 2022), emphasizing the need for further research.

1.8.2. Gastrointestinal tract

Nanoplastics may enter the human body also through ingestion of contaminated food and water. This route of exposure is particularly significant, as nanoplastics interact with digestive fluids, intestinal cells, and undergo transportation into the intestine and liver (Antunes et al., 2023; Sendra et al., 2021). Studies have shown that exposure to polystyrene nanoplastics with 20 nm and 200 nm diameter at 80 µg.mL⁻¹ can lead to a decrease in human gastric adenocarcinoma (AGS cell line) viability, depolarization of mitochondrial membrane potential, and a decrease in IL10 and p53 protein activity, accompanied by an increase in intracellular ROS and calcium and IL8 levels (Lin et al., 2021), which can contribute to cell damage and inflammation. Polystyrene nanoplastics also caused cellular damage by activating PI3K/AKT, ERK/c-FOS and caspase-3/caspase-9 signalling pathways. This study also showed that there was an enrichment in the Fanconi Anemia pathway, involved in DNA repair and MAPK

signalling pathway, in cell growth and differentiation. At $80 \mu\text{g.mL}^{-1}$, 20 nm sized polystyrene nanoplastics have also been found to disrupt cell membrane integrity and induce cell apoptosis in intestinal cells, namely in Caco-2 cells, an immortalized cell line of human colorectal adenocarcinoma cells (Lu et al., 2022). Furthermore, the long-term exposure (8 weeks) of this type of cell line to 50 nm sized polystyrene nanoplastics at 0.0006, 0.26, 1.3 and $6.5 \mu\text{g.cm}^{-2}$ has been shown to induce changes at the ultrastructural and molecular levels, although no DNA damage or oxidative stress was observed (Domenech et al., 2021). On the contrary, larger particles, namely polystyrene microplastics (500 to 1000 nm) demonstrated no apparent hazardous effects following 24 hours of exposure, as two different coculture models (differentiated Caco-2/HT29 intestinal cells and Caco-2/HT29 + Raji-B cells) were exposed to these particles without significant cytotoxic effects, or effects over the integrity and permeability of the barrier model (Rubio et al., 2020). Nevertheless, microplastics were highly uptake by both barrier model systems, and translocated across the membrane without any genotoxic or oxidative DNA damage induction (Domenech et al., 2020).

The combination of nanoplastics with other elements are also a problem. The combination with arsenic (As), polystyrene nanoplastics, namely 20 nm at $8 \mu\text{g.mL}^{-1}$ and 50 nm at $128 \mu\text{g.mL}^{-1}$ and polystyrene microplastics, namely 200 nm at $128 \mu\text{g.mL}^{-1}$, 500 nm at $128 \mu\text{g.mL}^{-1}$ and 1000 nm at $128 \mu\text{g.mL}^{-1}$ altered the fluidity of the cell membrane and cytoskeleton, inhibiting the activity of ABC transporters and leading to the accumulation of As in cells (Lin et al., 2021). Aged polystyrene nanoplastics (50 nm) with microcistines adsorbed, toxins produced by freshwater cyanobacteria, at 1, 5, 10, 15, and $19 \mu\text{g.mg}^{-1}$ have been shown to accumulate in human hepatoblastoma cells, causing oxidative damage, endoplasmic reticulum stress and reduced protein synthesis (He et al., 2023), aggravated when exposed to a higher concentration of microcistines ($19 \mu\text{g.mg}^{-1}$), that also resulted from the metabolic energy disorder and differences in pathways related to cellular signalling and DNA repair.

Moreover, several studies have demonstrated the impact of nanoplastics on various aspects of cellular physiology. Cortés et al., 2020, after the treatment with 50 - 100 nm size particles at 0 – $100 \mu\text{g.mL}^{-1}$, noticed an increase of mitochondrial membrane potential in a dose-dependent manner. Polystyrene nanoplastics have been shown to compromise cell viability and induced inflammatory events, including the upregulation of inflammatory gene expression in HT29 cells (human colon adenocarcinoma) and

N9 cells (mouse microglia), namely by activating the TLR4/p38-mediated pro-inflammatory response after 25, 50 and 100 nm polystyrene nanoplastics at 25 and 500 $\mu\text{g.mL}^{-1}$ (Antunes et al., 2023). The same events of inflammation seemed to occur in HepG2 after exposure to 10, 50 and 100 $\mu\text{g.mL}^{-1}$, namely by positively charged polystyrene nanoplastics ($-\text{NH}_2$) at 100 $\mu\text{g.mL}^{-1}$. Polystyrene nanoplastics (44 and 100 nm) strongly induced an up-regulation of IL-6 and IL-8 genes, two of the most important pro-inflammatory cytokines associated with gastric pathologies (Forte et al., 2016; Weber et al., 2022).

Antioxidant response are frequently after the exposure to polystyrene nanoplastics. A study showed that polystyrene nanoplastics negatively charged ($-\text{COOH}$) at 10 $\mu\text{g.mL}^{-1}$ led to a significant increase in superoxide dismutase activity, indicating an antioxidant response against oxidative damage. Moreover, treatment with 50 $\mu\text{g.mL}^{-1}$ of polystyrene resulted in a substantial elevation in glutathione content, further highlighting the increase in cellular antioxidant defence mechanisms. Although polystyrene nanoplastics positively charged ($-\text{NH}_2$) exposure did not significantly alter cell viability compared to polystyrene nanoplastics negatively charged ($-\text{COOH}$), exposure to 100 $\mu\text{g.mL}^{-1}$ of polystyrene nanoplastics positively charged ($-\text{NH}_2$) induced severe oxidative stress, as evidenced by elevated levels of malondialdehyde, a marker of lipid peroxidation (He et al., 2020). Also, nanoplastics can perturb the composition of the intestinal microbiome (Teles et al., 2020). Some investigations showed that carboxylated polystyrene nanoplastics with 50 nm can also change intestine cells (goblet cells and M cells) morphology and disrupt iron transport due to disruption of the cell membrane (Mahler et al., 2012). Another study investigated that polystyrene nanoplastics interact with Caco-2, HT29 and LS174T cells, as the mucin layer is the first physical barrier encountered after ingestion, causing alteration in the viability and the apoptotic state. CaCo-2 cells showed increased oxidative stress, while HT-29 cells exhibited reduced cell viability. Positively charged nanoplastics also induced apoptosis in all cell lines independently of their ability of produce mucin (Inkielewicz-Stepniak et al., 2018). Furthermore, nanoplastics exhibit toxicity towards macrophages and epithelial cells (Xia et al., 2008). Their absorption by specialized M-cells that cover intestinal lymphoid tissue, particularly Peyer's patches, depends on adherence to the gastrointestinal mucus, where greater adherence enhances the clearance rate of particles (Ensign et al., 2012).

1.8.3. Other organs

Nanoplastics can cross epithelial and endothelial barriers, facilitating their transportation through the bloodstream and lymphatic system Bai et al., 2024. This phenomenon has been observed in HUVEC cells exposed to polystyrene nanoplastics carboxyl-modified (-COOH) (~20 nm at 0.05, 0.1, 0.25 and 0.5 mg.mL⁻¹) and PMMA nanoplastics (~55 nm at 0.05 and 0.5 mg.mL⁻¹) for 1, 3, and 6 hours, which disrupted vascular endothelial cadherin junctions in a dose-dependent manner. The disruption of these junctions was found to be primarily biophysical-biochemical in nature and unrelated to cytotoxic events, such as reactive oxygen species production, autophagy, and apoptosis (Wei et al., 2022). Additionally, the introduction of nanoplastics into the lymphatic system has been shown to enhance their transport through lymphatic endothelial cells, with surface poly(ethylene glycol) (PEG) density playing a crucial role in determining translocation efficiency (Trac & Chung, 2021).

Another study assessed the cytotoxic and genotoxic potential of polystyrene nanoplastics at higher concentrations (2000 µg.mL⁻¹, 1000 µg.mL⁻¹, and 500 µg.mL⁻¹) on human peripheral lymphocytes and showed an increase in chromosomal aberrations, such as chromosomal breaks and dicentric chromosomes (two centromeres instead of one), and an increase in nucleoplasmic bridge formation (connections between two daughter cells during cell division) and nuclear budding. There was also a significant increase in micronuclei formation, increase of cytostasis and oxidative stress-mediated cytotoxicity, (Sarma et al., 2022). A study exposed white peripheral blood cells: lymphocytes, monocytes, and polymorphonuclear (PMN) cells to 50, 100, 400 and 900 nm polystyrene nanoplastics at 50 and 100 µg.mL⁻¹ for 24, 48, or 72 hours. No cytotoxicity occurred; however, sharp differences were observed between cell types, with limited uptake in lymphocytes, high uptake in monocytes, and intermediate values for PMN cells. Monocytes and PMN cells exhibited a significant increase in DNA damage, while lymphocytes did not. Polystyrene nanoplastics exposure induced changes in the whole blood secretome, and the expression of various cytokines related to the inflammatory, immune, and stress response, as well as cell proliferation, showed a significant increase (Ballesteros et al., 2020).

As nanoplastics can be circulated throughout the whole body via the blood vessels (Lee et al., 2023), they may be capable of crossing the blood-brain barrier and affect

the brain (Hersh et al., 2022). It has already been shown that metal and metal-oxide nanoparticles, such as gold (Au) and titanium dioxide (TiO₂) nanoparticles, can also reach the brain to exert a range of neurotoxic effects (Prüst et al., 2020). Several studies reported the accumulation of nanoplastics (100 nm) in the brain of fish and clearly indicating they can cross the blood-brain barrier (Ding et al., 2018; Mattsson et al., 2017).

A recent study suggested that polystyrene nanoplastics (50 nm at 0.5-50 mg.kg⁻¹ for 7 days) can cross the blood-brain barrier and activate microglia, indicating potential effects on brain health (Shan et al., 2022). Other studies have demonstrated harmful effects on the human reproductive system, namely excessive generation of ROS, mitochondrial dysfunctions, DNA fragmentation and upregulation of protective HSP70 expression in human spermatozoa cells after exposure to 50 and 100 nm polystyrene nanoplastics at concentrations of 0.1, 0.5 and 1 µg.mL⁻¹ after 30 minutes (Contino et al., 2023).

Nanoplastics can accumulate in mammalian reproductive organs and cause toxic effects on the reproductive system for both males and females (Song et al., 2023). In males, the damage caused by micro and nanoplastics includes abnormal testicular and sperm structure, decreased sperm vitality, and endocrine disruption (He & Yin, 2024). In females, 500 nm polystyrene microplastics at different concentrations (0.015, 0.15 and 1.5 mg.d⁻¹ for 90 days) (An et al., 2021) could penetrate in ovarian granulosa cells, increase the number of ovarian follicles, drastically lower anti-Müllerian hormone levels, and cause oxidative stress, which led to cell apoptosis and uterine and ovarian fibrosis (H. Wu et al., 2022), as polystyrene microplastics significantly increased the expression levels of Wnt/β-Catenin signaling pathways-related proteins (Wnt, β-catenin, p-β-catenin) and the main fibrosis markers (transforming growth factor-β (TGF-β), fibronectin, α-smooth muscle actin (α-SMA)).

After continued exposure of ~800 nm at 30 mg.kg⁻¹ body weight polystyrene microplastics for 35 days, these particles accumulated in the uterus, ovary and blood of exposed mice and increased IL-6 level, decreased malondialdehyde level and the survival rate of superovulated oocytes (Liu et al., 2022). The exposure to bigger microplastics (2 and 5 µm) reduced estradiol (Haddadi et al., 2022). Studies have shown that polystyrene nanoplastics (500 nm at concentrations of 0, 0.015, 0.15, and

1.5 mg.d⁻¹) cause apoptosis, pyroptosis, fibrosis, and even reproductive disorders in the rat ovarian granulosa cells due to oxidative stress (Lee et al., 2023). Hence, risks such as oxidative stress and cell death may occur due to cellular uptake, facilitated by the corona formation in the surface of these plastics (mentioned in section 4.1).

Furthermore, the presence of nanoplastics in human reproductive tissues, such as placentas and fetal meconium, has been reported (Hunt et al., 2023), although the association with adverse fertility or pregnancy outcomes is not established (Feng et al. 2023). However, some researchers have suggested that nanoplastics may pose significant risks to the placenta through inhibition of its key enzymes (Enyoh et al. 2023), probably due to associated plastic chemicals that can cause long-term damage and consequently upset the foetus's developing immune system.

No evidence exists regarding the accumulation of nanoplastics in the human heart. However, experimental data have underscored the association between potentially high exposure to MP and cardiovascular risk factors, e.g., abnormal blood velocity (Duan et al., 2020), decreased cardiac output (Sun et al., 2021), altered heart rate (Yang et al., 2020), myocardial fibrosis (Wei et al., 2021), endothelial dysfunction (Shiwakoti et al., 2022), and cardiometabolic disease phenotype (Zhao et al., 2022).

In a recent study, it was demonstrated that polystyrene nanoplastics with 100 nm exposure at 0, 1, 10 µg.mL⁻¹ lead to significant pathological alterations in the gallbladder. Short-term exposure (three weeks) results in gallbladder epithelial hyperplasia, while long-term exposure (six weeks) induces progressive hyperplasia, epithelial-mesenchymal transition (EMT), and fibrosis. Additionally, exposure to nanoplastics facilitates the formation of neutrophil extracellular traps, indicative of an inflammatory response. The study consistently observed epithelial hyperplasia and neutrophil infiltration in gallbladder tissues, correlating with the detection of nanoplastics in bile (Shao et al., 2024).

1.9. Conclusion and future perspectives

Nanoplastics are increasingly recognized as contaminants of emerging concern, yet understanding their interaction with the surrounding biological and chemical environment poses significant challenges. These interactions can alter the physical and chemical properties of nanoplastics, leading to unpredictable toxic effects. The adverse health impacts of nanoplastics, which can range from inflammation to genotoxicity, depend on factors such as concentration, time of exposure, but also biomacromolecules available in the environment and the type of corona formation or yet the presence of other environmental contaminants.

Due to their potential to accumulate throughout the food chain, establishing reliable analytical standards for detecting nanoplastics in animal-derived food products is critical, alongside a comprehensive understanding of their toxicity in humans, particularly by investigating specific mechanisms at the cellular level. However, substantial challenges persist in the accurate quantification of nanoplastics in environmental samples, as standardized protocols for their collection and handling are still under development. This lack of standardization compromises the accuracy and reliability of current data.

Current knowledge gaps include:

- development and refinement of *in vitro* models to study nanoplastics interactions at cellular, tissue and organism levels, providing insights into toxicity and mechanisms of action, namely the use of co-cultured and pathophysiological models that simulate human body physiology (e.g. enzymatic digestion protocols to simulate the effect of the digestive process on nanoplastics; models of the human skin and intestinal barrier);
- understanding the mechanisms by which nanoplastics interact with biological and chemical components across various environmental matrices, including prediction of the several potential corona types and including the diversity of polymer types available in the environment;
- studies on the environmental relevant concentrations of nanoplastics and their associated contaminants, namely of additives and metals.
- developing effective strategies to predict, assess, and mitigate the risks associated

with these small plastic particles on humans and wildlife

A proper exposure evaluation is essential to conclude a proper risk assessment and provide scientific support for decision-makers to take preventive and risk-mitigating measures to respond to the emerging ecological or human health risks and harm specific to nanoplastics. Moreover, there is a critical need for heightened awareness and conscious behaviour, coupled with improved risk mitigation strategies. Proactive measures to reduce exposure to harmful substances in both human and animals are paramount. As we navigate the complexities of nanoplastics interactions in biological systems, addressing these knowledge gaps becomes indispensable for making informed decisions and implementing effective environmental management strategies.

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CHAPTER 2:

Aims and research approach

2.1.Aims and research approach

This thesis is aligned with the needs outlined in the UN's 2030 agenda concerning prevention and reduction of marine pollution in the environment, including marine debris (Goal 14.1) and increase knowledge about the relation between plastic pollution, contaminants and human health (Goal 3.9). With these goals in mind, the present thesis aims to contribute to a better understanding of the effects and toxicity mechanisms resulting from the exposure of biological systems to PSNP, particularly focusing on inflammatory responses, genotoxicity, and toxicological interactions with a very well-known environmental pollutant, benzo[a]pyrene (B[a]P), to improve the basis of risk assessment of these man-made materials to aquatic ecosystems and human health.

The specific goals of this thesis are framed by the following research questions:

- 1) Do PSNP induce inflammatory responses in biological systems, and how do these effects vary based on NP characteristics?
- 2) What are the mechanisms underlying the interaction between NP and environmental pollutants, such as B[a]P, and their individual and combined effects on cellular inflammation and genotoxicity?
- 3) How do alterations in cellular oxidative stress levels contribute to the inflammatory and genotoxic effects induced by exposure to NP and B[a]P?
- 4) Are there synergistic interactions between NP and B[a]P exposure?

What are the effects of indirect ingestion (through food chain) of NP on intestinal and hepatic systems, and how do these interactions influence overall fish health?

2.2.Research approach

Addressing NP toxicity has been a challenge, primarily due to the lack of established methodologies tailored for nanoscale particles and consequently difficulties in identifying and quantifying NP concentrations in the environmental and biological matrices. Thus, this work will focus on hazard assessment testing a wide range of concentrations (25 - 500 $\mu\text{g.mL}^{-1}$) and different particle sizes (25, 50 and 100 nanometres) of PSNP in cellular models. *In vitro* tests will include the human colon adenocarcinoma HT29 cells, mouse microglia N9 cells and the human hepatoma (HepG2) cell lines, all of which have proven value in environmental toxicology. In addition to PSNP exposure, B[a]P will be used as

model carcinogenic pollutant due to its widespread occurrence, well-known mode of action, namely its documented genotoxic and carcinogenic properties.

The results of *in vitro* analysis will be subsequently validated *in vivo*, using zebrafish (*Danio rerio*) as the animal model. Zebrafish serve as a valuable model for research on mutagenesis and neoplasia caused by environmental xenobiotics and is a pivotal model in biomedical sciences. The *in vivo* approach will allow to understand the systemic effects of NP and associated B[a]P. The results from this thesis are expected to contribute to the possible application for Environmental Risk Assessment of NP and future drawing of environmental guidelines (e.g. MSFD).

2.3. Thesis structure

The present PhD dissertation comprises seven chapters, including a general introduction (Chapter 1), which provides a detailed literature review encompassing the definition, sources, and fate of NP in the environment. Additionally, it discusses methodologies for identifying NP in environmental samples, explores their interactions with the environment and other pollutants, investigates their uptake in cells and organisms, and addresses their potential toxicity to humans. Followed by Chapter 2 with the aims and research approach and the structure of the thesis. The first chapter with original data (Chapter 3) addresses the pro-inflammatory mechanisms of NP with different sizes (25, 50 and 100 nm) exposure in the viability of HT29 and N9 cells and their inflammatory responses, regarding pro-inflammatory cytokines. This chapter resulted in a published manuscript. Chapter 4 correspond to the research development *in vitro* to detect the combined effects of NP with B[a]P over intestinal and hepatic human cells regarding antioxidant and detoxification responses. Chapter 5 seeks to validate *in vivo* in zebrafish the results *obtained in* chapters 3 and 4 looking further into systemic effects of NP associated with B[a]P tested in an *in vivo* assay with zebrafish as the animal model. This thesis also contain a chapter with the general discussions (Chapter 6) and the final conclusions and future perspectives (Chapter 7). A summary of the thesis structure and research approach is found in Figure 2.1.

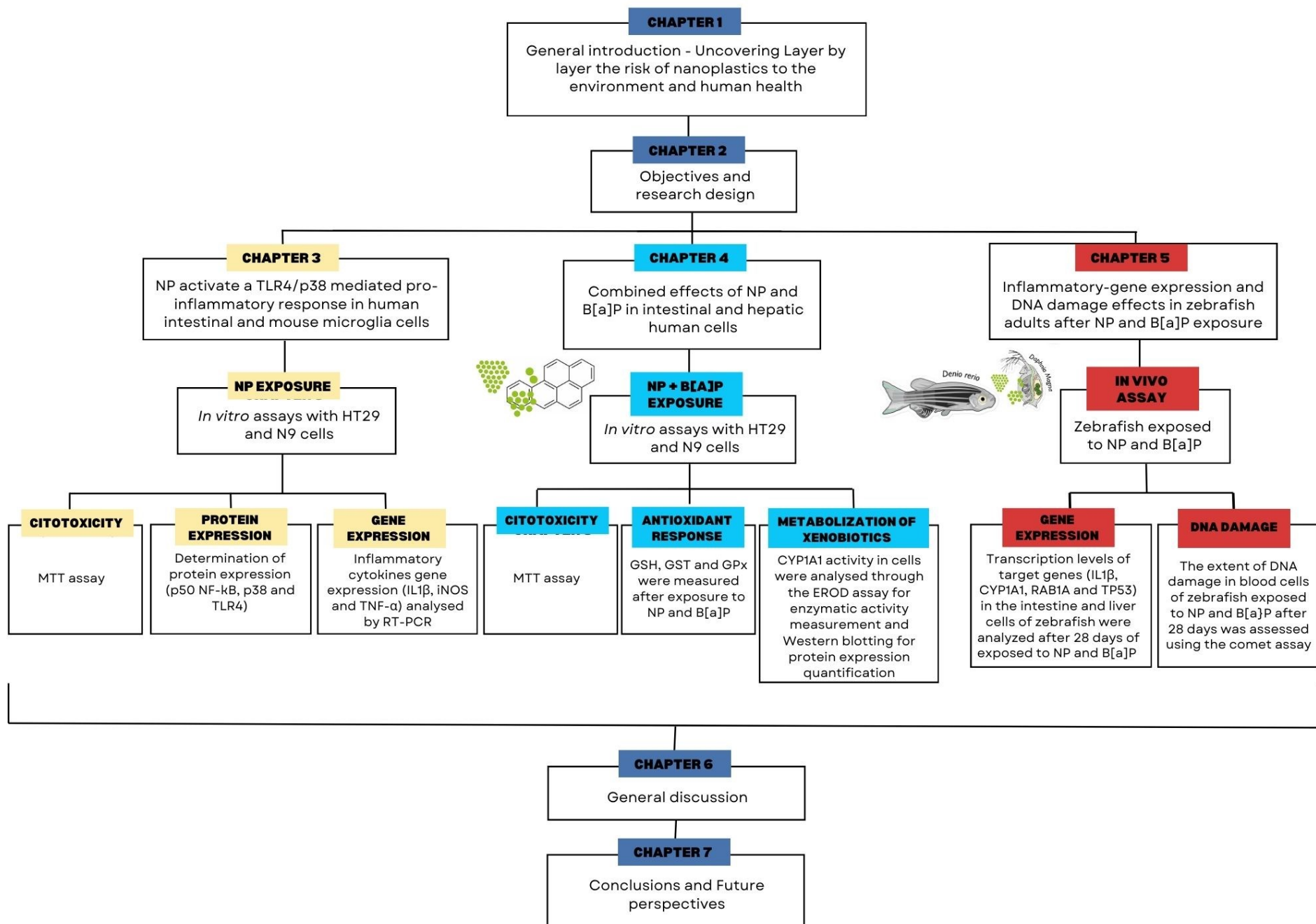


Figure 2. 1 - Diagram of thesis structure.

CHAPTER 3:

Nanoplastics activate a TLR4/p38-mediated pro-inflammatory response in human intestinal and mouse microglia cells

This Chapter is a published Manuscript:

Antunes J, Sobral P, Martins M, Branco V, (2023). "Nanoplastics activate a TLR4/p38-mediated pro-inflammatory response in human intestinal and mouse microglia cells". *Environmental Toxicology and Pharmacology*, 104 (11), 104298. <https://doi.org/10.1016/j.etap.2023>.

Abstract

The crescent presence of nanoplastics in the environment raises concerns regarding their potential impact on health. This study exposed human colon adenocarcinoma cells (HT29) and microglia cells (N9) to nanoplastics (25 nm, 50 nm, and 100 nm polystyrene) to investigate their inflammatory responses, which are vital for body's defence. Although cytotoxicity remained generally low, HT29 cells exhibited a notable upregulation of p50 and p38 expression, concomitant with elevated TLR4 expression, in contrast with N9 cells that showed a less pronounced upregulation of these proteins. Additionally, nanoplastic exposure increased IL-1 β levels, partially attenuated by pre-exposure to TLR4 or p38 inhibitors. Intriguingly, N9 cells exposed to nanoplastics exhibited substantial increases in iNOS mRNA. This effect was entirely prevented by pre-exposure to TLR4 or p38 inhibitors, while TNF- α mRNA levels remained relatively stable. These findings underscore the potential of nanoplastics to activate inflammatory pathways, with response kinetics varying depending on the cell type.

Keywords

Nanoplastics; intestine; inflammation; TLR4; microglia;p38

3.1 Introduction

The overuse and inadequate disposal of plastic polymers have led to a serious pollution problem of worldwide proportions. Polystyrene is one of the most widely used plastic polymers (15.61 million metric tons produced in 2021 and PS), with applications in packaging, insulation, consumer goods, electronics, and construction (Bonanomi et al., 2022). It is lightweight and easily breakable into smaller particles, thus posing a threat to animal and human health (Palmer & Herat, 2021). It is therefore unsurprisingly one of the most found materials in the environment, particularly in marine ecosystems (Pabortsava & Lampitt, 2020), which makes it an important polymer to study.

Among all the environmental challenges posed by plastic particles released by anthropogenic activities (PlasticsEurope, 2022), degradation into nanoparticles by ultraviolet radiation, mechanical abrasion, or biological mechanisms (Mattsson et al., 2015c) to an unknown nanoscale limit (Hadri et al., 2020) raises great concern.

Nanoplastics (NP; diameter < 100 nm) are among the least studied despite their potentially high risk to human health, which adds to the lack of standardized methodologies to assess their levels in the environment and biota (European Commission, 2022).

Due to their small size and the ability to cross biological membranes (Kihara et al., 2021), NP can be easily ingested (Botterell et al., 2019), bioaccumulate, and biomagnified (Heet et al., 2022). Nanoplastic uptake has been associated with oxidative stress (Y. Zhou et al., 2023b) and tissue damage (Larue et al., 2021), metabolic changes (Du et al., 2022; Haldar et al., 2023), changes to the function of both membranes and cells (Han et al., 2024), reduction in reproductive success (Jewett et al., 2022), behavioural disturbances (Ferreira et al., 2023) malformations and changes in feeding ability in fish (Teles et al., 2020). Also, evidence of dermal uptake has been reported (Llorca and Farré, 2021; Ragnarsdóttir et al., 2022; Revel et al., 2018a).

Nanoplastics may potentially interfere with intestinal membrane receptors and intracellular inflammatory signaling cascades (Bouwmeester et al., 2015; Yee et al., 2021b), inducing immune cells to release cytokines thus triggering inflammation (S. Han et al., 2020; J. Hwang et al., 2020). Reported outcomes include microbiota alterations, intestinal barrier permeability, oxidative stress, inflammation, neurotoxicity, and behavioural disturbances (Aljabali et al., 2023). However, the underlying mechanisms are poorly understood. This is especially true for gut cells which are the primary site of contact for NP after oral ingestion. As the gastrointestinal tract is very important in maintaining immune homeostasis, (Vezza et al., 2016), the imbalance of inflammatory processes and cytokine secretion (e.g. TNF- α and IL-1 β) may be linked to disruption of the epithelial-cell barrier which can lead to inflammation and dysregulation of mucosal homeostasis (Y. M. Oliveira et al., 2022).

Toll-like receptors (TLRs) are membrane proteins that belong to the pattern recognition receptor (PRR) family and play an important role in recognizing invading pathogens and triggering an immediate immune response. Activation of TLRs also leads to the synthesis of pro-inflammatory cytokines and chemokines (T. Liu et al., 2017; Zbinden-Foncea et al., 2012). Specifically, TLR4 recognizes bacterial lipopolysaccharide (LPS), the major component of membranes of Gram-negative bacteria (Poltorak et al., 1998) leading to a canonical activation of the NF- κ B pathway and p38 (Sharif et al., 2007);

Wang et al., 2018). The latter is strongly activated by stress and plays an important role in immune responses, such as activation and regulation of kinases, and interaction with other signaling pathways, as well as regulation of cell survival and differentiation (Whitaker and Cook, 2021). This factor is also important in producing cytokines and in return is also strongly activated by proinflammatory cytokines such as interleukin 1 ($IL-1\beta$) and tumor necrosis factor α ($TNF-\alpha$), which play an important role in regulating the inflammatory response (Cuadrado & Nebreda, 2010). Therefore, we hypothesized that exposure of cells to nanoplastic particles triggers inflammatory signaling via the NF- κ B and/or p38 MAPK pathways. To test this hypothesis, we exposed human colorectal adenocarcinoma cells (HT29 cells) and mouse microglia cells (N9 cells) to different concentrations and sizes of PSNP in the presence or absence of either a TLR4 or p38 inhibitor and investigated NF- κ B and p38 activation, TLR4 expression and the levels of pro-inflammatory gene transcripts.

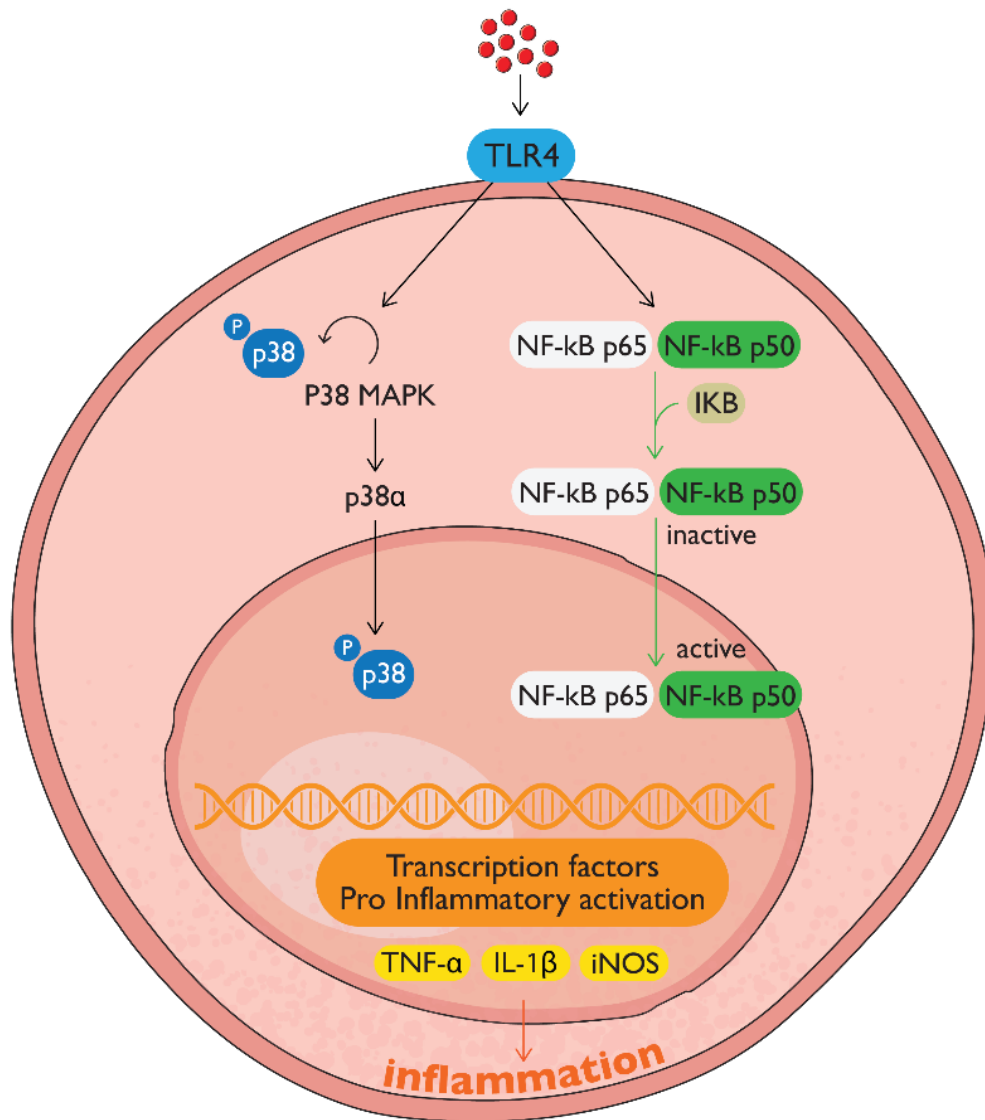


Figure 3. 1 - Activation of inflammatory signaling pathways induced by nanoplastic exposure: focus on NF-κB and p38 MAPK Pathways. Illustration of the activation of inflammatory signaling pathways in response to nanoplastic exposure, specifically NF-κB and p38 MAPK pathways. It depicts how nanoplastics trigger the activation of NF-κB, leading to the expression of pro-inflammatory cytokines and subsequent inflammatory responses. Concurrently, it shows the activation of the p38 MAPK pathway, which is involved in stress responses and further amplifies the inflammatory signaling cascade. Understanding these molecular mechanisms is crucial for elucidating the potential health impacts of nanoplastics and their role in chronic inflammation and related diseases.

3.2 Material and methods

3.2.1 Nanoplastics

Polystyrene fluorescent nanospheres (hereafter NP), with 25, 50 and 100 nm diameter and a density of 1.05 g.cm^{-3} from Thermo Scientific, were purchased from DISTRILAB, Netherlands (catalog references, G25, G50 and G100, respectively. <https://distrilab.nl/dyed-and-fluorescent-particles/>). Fluorescent dyes were incorporated into the polymer mix (not surfaced dyed) and the NP were packaged in deionized water with trace amounts ($<0.1\%$) of surfactant (Triton-X 100) and preservative (sodium azide) to inhibit aggregation and promote stability. The morphology of the above plastics was further characterized using a scanning electron microscope (SEM) (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Briefly, $10 \mu\text{L}$ of well-dispersed NP (100 mg.L^{-1}) were dropped onto glass laminae and then air-dried. SEM images were obtained at the CENIMAT facilities at Nova School of Science and Technology, Portugal. The samples were lightly sputter-coated with gold before observation.

3.2.2 Nanoplastics size and potential zeta

Size and zeta potential of NP were analysed with an HORIBA nano-particle analyser SZ-100. At least three replicates of a NP solution ($500 \mu\text{g.mL}^{-1}$) diluted in either PBS or culture medium were analysed.

3.2.3 Cell culture

Human colorectal adenocarcinoma cells (HT29; ATCC, USA) were cultured at 37°C in Dulbecco's Modified Eagle medium with high glucose (4.5 g.L^{-1}) (DMEM), with GlutaMAX and no sodium pyruvate, (Gibco), 10% of heat-inactivated foetal bovine serum (FBS) (Biochrome) and 1% penicillin/streptomycin ($100 \text{ units.mL}^{-1}$ of penicillin and $100 \mu\text{g.mL}^{-1}$ of streptomycin) (Gibco) in a humidified incubator with a 5% CO_2 atmosphere. N9 mouse microglia were a kind gift from Professor Dora Brites (iMed.Ulisboa) and were cultured in RPMI medium (Gibco), 10% of FBS (Biochrome) and 1% penicillin/streptomycin ($100 \text{ units.mL}^{-1}$ of penicillin and $100 \mu\text{g.mL}^{-1}$ of streptomycin) (Gibco) in a humidified incubator with 5% CO_2 atmosphere at 37°C . Cells

were seeded at 1.0×10^6 cells per 75 cm² culture flask. The culture medium was changed every two days after seeding and cells were harvested after reaching 80% confluency using 0.05% trypsin/EDTA (Sigma).

3.2.4 Cell viability assay

Cell viability was determined by the MTT ((3–4,5-dimethylthiazol-2-yl)–2,5–diphenyltetrazolium bromide) assay described by (Carvalho et al., 2008). Cells (5×10^4 cells per well) were cultured in 96-well plates and allowed to attach for 24 h before the addition of NP (0, 25, 50, 100, 250 and 500 µg.mL⁻¹). Cells exposed to Triton-X 100 (10%) were used as a positive control. After 24, 48 and 72 h of exposure, MTT was added to plates at a final concentration of 400 µg.mL⁻¹ per well followed by incubation at 37 °C for 2 h. Following the incubation period, the MTT solution was removed, and the formazan crystals were dissolved with 4:1 dimethyl sulfoxide/glycine buffer (pH 10.5) (Honey- well, UK). The absorbance was measured at 550 nm and the cellular viability was calculated relative to the control group.

3.2.5 Exposure of cells to nanoplastics

HT29 and N9 cells were seeded in 10 mm culture dishes (1×10^6 cells per plate) and allowed to reach 70–80% confluence. Afterwards, the culture medium was replenished and cells were exposed to 25 and 500 µg.mL⁻¹ of NP (25, 50 and 100 nm) for 24 h. Lipopolysaccharide (LPS) (Sigma-Aldrich) (300 ng.mL⁻¹) was used as a positive control. After exposure, cells were rinsed with PBS and detached from plates with trypsin. The cellular pellet was collected by centrifugation (5 min at 600 g) and washed with PBS (Lonza, BioWhittaker, USA). Following a second centrifugation, the pellet was resuspended in the appropriate lysis buffer, depending on the requirements of each specific protocol.

3.2.6 Total cell lysates

Cell lysates were obtained by disrupting cellular pellets in lysis buffer (25 mM Tris–Cl, pH 7.5; 100 mM NaCl; 2.5 mM EDTA; 2.5 mM EGTA; 20 mM NaF, 1 mM sodium orthovanadate, 20 mM sodium pyrophosphate; 20 mM sodium β-glycerophosphate;

0.5% Triton X-100; and 1 tablet of protease inhibitor cocktail (Roche) per 10 mL). After lysis, samples were frozen at – 20 °C until subsequent analysis.

3.2.7 Nuclear cell fraction

Following exposure to NP (25 and 500 $\mu\text{g.mL}^{-1}$ of 25, 50 and 100 nm NP) and LPS (300 ng.mL^{-1}) for 24 h, cells were washed twice with versene, resuspended in buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl_2 , 10 mM, protease inhibitor tablets - 1 per 10 mL) and incubated on ice for 15 min. Afterwards, Triton X-100 (0.5%) was added, and the lysate was mixed by vortex for 10 s. Samples were centrifugated at 6500 g for 20 s and the cytosolic supernatant was removed. The pellet was washed with 150 μL of buffer A (without Triton) and centrifuged again at 6500 g for 20 s and the supernatant discarded. A volume of 100 μL of buffer B (HEPES pH 7.9, 1.5 mM MgCl_2 , 420 mM NaCl, 0.2 mM EDTA, 25% v/v Glycerol, protease inhibitor tablets - 1 per 10 mL) was added to samples to resuspend the pellet. Samples were incubated on ice with vigorous stirring (using a magnetic bar) for 30 min. Nuclear extracts (supernatants) were recovered after a final 10 min centrifugation at 13,000 g and 4 °C.

3.2.8 Total protein quantification

The total protein content was determined in the soluble fraction of each lysate, using the Bradford method (Branco et al., 2022). After mixing diluted (5x) Coomassie dye (BioRad) with each sample (96-well plates), the absorbance was measured at 595 nm in a microplate reader (Zenyth 3100, Anthos Labtec Instruments). The protein concentration was calculated from a calibration curve (1 – 12 μg of protein per μL) using bovine serum albumin (BSA) (Sigma-Aldrich) as a standard.

3.2.9 Determination of protein expression levels of p50 NF-kB subunit, p38 and TLR4

To evaluate protein expression of p50 NF-K β subunit and p38, total cellular lysates and nuclear fractions of cells were analysed by Western Blot, after 24 h of NP exposure. Protein expression was analysed after separation of total soluble proteins (40 μ g) by SDS–PAGE on a 4–12% Bis–Tris gel (Invitrogen, ThermoFisher Scientific, USA) with MES running buffer under reducing conditions (140 v for 1 h). Proteins were subsequently transferred (40 v for 7 min) to a nitrocellulose membrane using the iBlot 2 dry blotting system (Invitrogen), blocked with a 5% skimmed milk solution (1 h) and rinsed with PBST. Afterwards, membranes were incubated with primary and secondary antibodies for the proteins of interest and detected with chemiluminescence using the Biorad Chemidoc System. Membranes were submerged with shaking for 1 h in a 1% BSA solution containing the respective primary antibody (1/1000). The following antibodies were used: NF-K β p50 (sc-166588, Santa Cruz), p38 (sc-13666588, Santa Cruz). For TLR4 expression, TLR4 Antibody (25): sc-293072 was used (Santa Cruz Biotechnology Inc). Expression levels were normalized for protein loading on the gel, which was assessed by Ponceau S (Sigma-Aldrich).

3.2.10 Pro-inflammatory gene transcription

Cells (HT29 and N9 cells) were seeded in 6-well plates (1×10^5 cells per well) and exposed to 25 and 500 μ g.mL $^{-1}$ of NP (25, 50 and 100 nm) and LPS (300 ng.mL $^{-1}$), as a positive control. Additionally, parallel assays, were performed encompassing either pre-exposure (24 h) of cells to 5 μ M of a TLR4 inhibitor (TAK-242 - CAS 243984–11–4 – Calbiochem) or pre-exposure to 10 μ M of a p38 inhibitor (SB2399063, Sigma-Aldrich). After pre-exposure, the medium was changed, and cells exposed to NP as mentioned above.

RNA extraction was performed using an NZY Total RNA Isolation kit (NZYTech, Portugal) according to manufacturer instructions. Total RNA concentration was determined from spectrophotometric optical density measurements (260 and 280 nm). For each sample tested, the ratio between the spectrophotometric readings at 260 nm and 280 nm (OD260/ OD280) was used to evaluate RNA purity, with the ratio ranging between 1.87 and 2.04 for all samples.

cDNA was synthesized using a NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal) using 1 µg of RNA as a template. Gene expression quantification was performed using NZY qPCR Green Master Mix (NZYTech, Portugal) with 4 µM forward primer and 4 µM reverse primer and 100 ng of template cDNA in a 10 µL reaction volume. All samples were run in triplicate and the output level was reported as the average of the 3 measurements. Amplification conditions included 2 min at 50°C and 10 min at 95°C, and then 40 cycles at 95°C for 15 s and 60°C for 1 min on an Applied Biosystems QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

The primer sequences for human and mouse IL-1β, iNOS, TNF-α and GAPDH were designed with Primer-Blast and synthesis was performed by StabVida (<https://www.stabvida.com>) (Table 3.1). After RT-PCR gene levels were calculated by the relative quantification $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001), using GAPDH as the housekeeping gene.

Table 3. 1 - Primer sequences for target genes IL-1 β , iNOS, TNF- α and housekeeping gene GAPDH for human HT29 cells and for mouse microglia N9 cells.

	mRNA Target		Forward primer (5'->3')	Reverse Primer (5'->3')
Human	Interleukin 1 beta	IL-1β	CTCTTCGAGGCACAAGGCAC	GGCTGCTTCAGACACTTGAGC
	Nitric Oxide synthase 2	iNOS	CAATGGCAACATCAGGTCGG	GTGAATTCCACGTTGGCAGG
	TNF-alpha	TNF-α	TGCACTTTGGAGTGATCGGC	CTCAGCTTGAGGGTTTGCTAC
	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	GGTGGTCTCCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT
Mouse	Interleukin 1	IL-1β	TGCCACCTTTTGACAGTGATG	ATGTGCTGCTGCGAGATTTG
	Nitric oxide synthase 2	iNOS	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGTCAC
	Tumour Necrosis Factor	TNF-α	TAGCCACGTCGTAGCAAAC	GCAGCCTTGTCCCTTGAAGA
	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	GGAGAGTGTTTCCTCGTCCC	ATGAAGGGGTCGTTGATGGC

3.2.11 Statistical analysis

Data in Figures are represented as the mean $\pm$ standard deviation (S.D). Statistical analyses were conducted using GraphPad version 10.0.2. Data normality was evaluated by the Shapiro-Wilks test and variance homogeneity using Spearman's test for heteroscedasticity. When appropriate, data were log-transformed to comply with the assumptions of parametric tests.

The significance of main and interaction effects was evaluated by a Two-way ANOVA, followed by post-hoc assessment with a Tukey test to evaluate differences between exposure groups. When appropriate, data were log-transformed to comply with the assumptions of parametric tests. Differences between groups were classified as significant at $p < 0.05$ and considered very significant at $p < 0.01$.

3.3 Results

3.3.1 Nanoplastics characterization

SEM analysis provided detailed insights into the size and morphology of the PSNP, allowing for precise determination of their dimensions (Figure 3.2).

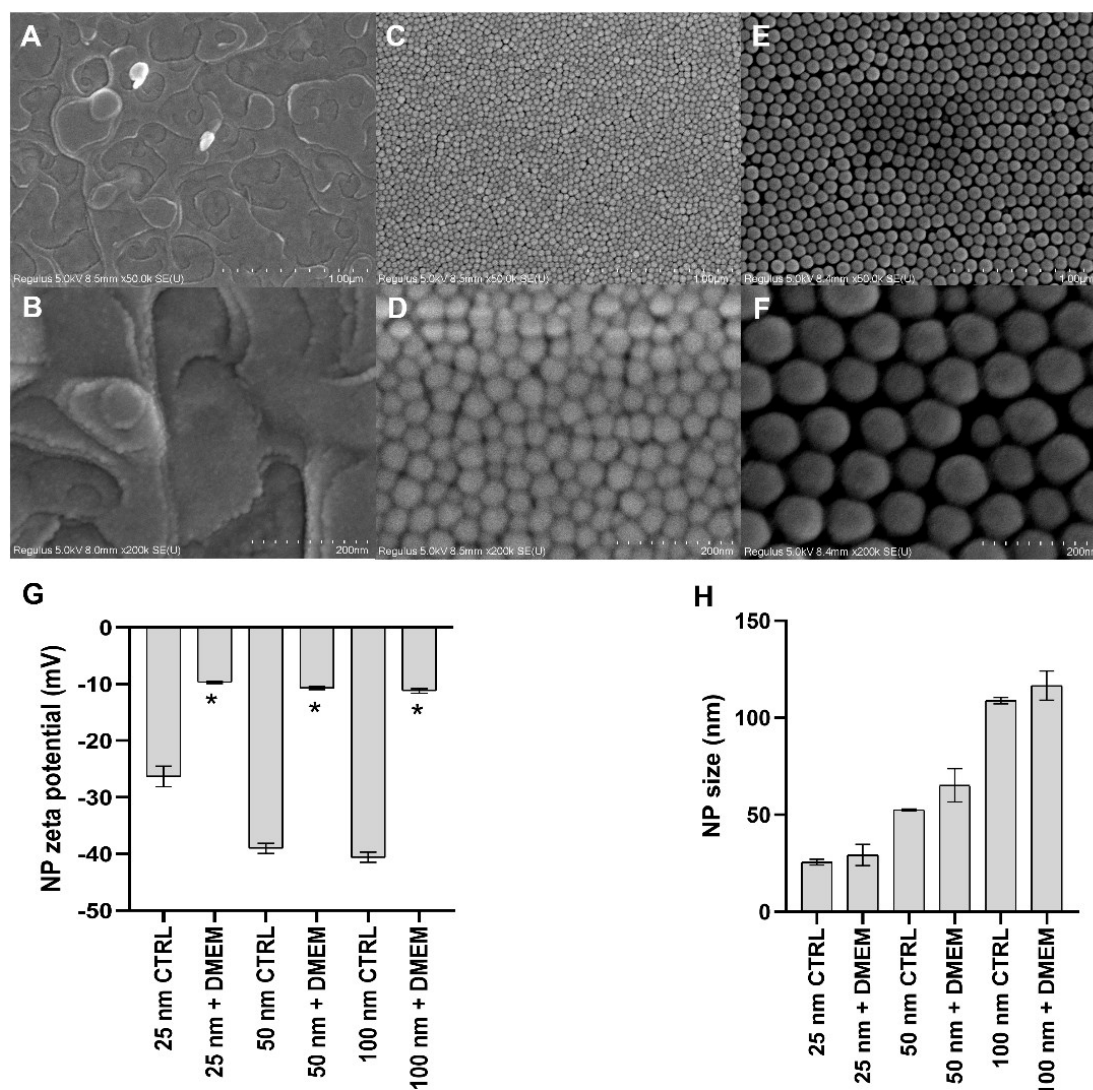


Figure 3. 2 – Characterization of polystyrene nanoplastics: size and surface properties. A and B show scanning electron microscopy (SEM) images of PSNP with a diameter of 25 nm, while C and D display 50 nm and E and F illustrate 100 nm. The size distribution and zeta potential of these particles were analyzed using a HORIBA SZ-100 nanoparticle analyser. G and H depict zeta potential (mV) and size (nm) measurements of PSNP suspended in phosphate-buffered saline (PBS) at a concentration of 500 $\mu\text{g}.\text{mL}^{-1}$, used as a control, and in Dulbecco's Modified Eagle Medium (DMEM) for HT29 cell assays. Statistical differences due to particle size and/or solvent effects were evaluated using a Two-Way ANOVA followed by Tukey's post-hoc test, with ** indicating a very significant difference ($p < 0.01$) between particles of the same size in PBS versus the culture medium.

3.3.2 Effect of NP exposure on viability of HT29 and N9 cells

The viability of HT29 and N9 cells after 24, 48, and 72 h of NP exposure is presented in Figure 3.2. In general, the viability of HT29 was > 80% with no significant differences resulting from increased levels of exposure to 25 and 50 nm (25 nm: Two-way ANOVA, $F(df, 2, 56) = 1.257$, $p = 0.2924$; 50 nm: Two-way ANOVA, $F(df, 2, 52) = 1.372$, $p = 0.2626$). Although a concentration-related effect was observed for the 100 nm (Two-way ANOVA, $F(df, 2, 53) = 8.825$, $p = 0.0005$), pairwise post-hoc comparisons versus the non-treated group did not disclose significant differences (Tukey test, $p > 0.05$).

Regarding N9 cells, viability decreased significantly with exposure denoting a concentration-related effect (25 nm: Two-way ANOVA, $F(df, 2, 42) = 173.7$, $p < 0.0001$; 50 nm: Two-way ANOVA, $F(df, 2, 35) = 27.46$, $p < 0.0001$; 100 nm: Two-way ANOVA, $F(2, 43) = 3.594$, $p = 0.0360$), with NP of 50 nm size causing the highest decrease in viability (>50% decrease). This was most evident after 48 h of exposure (Figure 3.3).

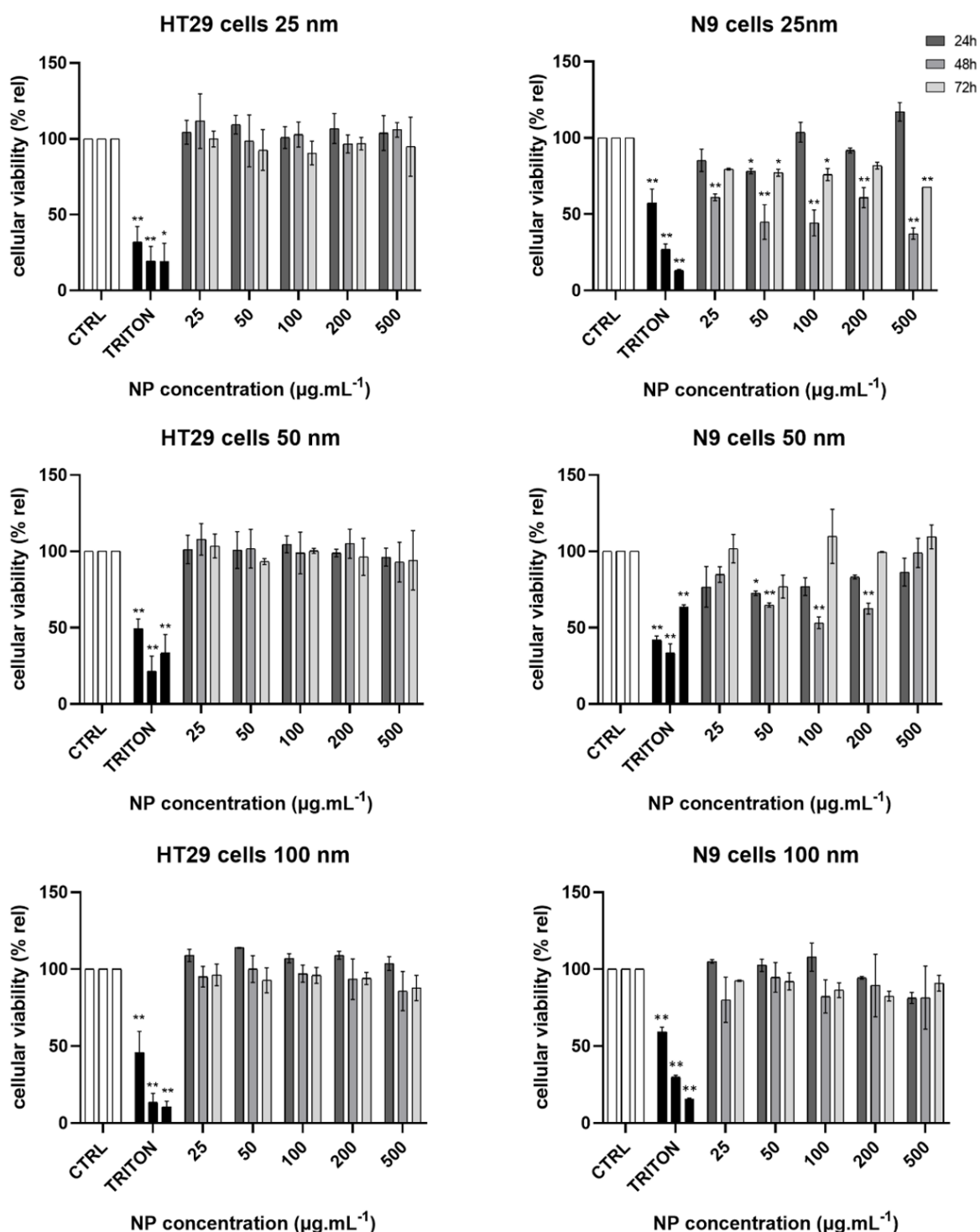


Figure 3. 3 - Effect of polystyrene nanoplastics on the viability of human colon adenocarcinoma (HT29) and mouse microglia cells (N9), as measured by the MTT assay. Viability was analysed after exposure of cells to different concentrations (25, 50, 100, 200, 500 $\mu\text{g.mL}^{-1}$) and particles sizes (25, 50 and 100 nm) for 24, 48 and 72 h. Triton X-100 (10%) was used as a positive control. Results are represented as the mean $\pm$ S.D. of at least three independent experiments. Statistical testing was performed on log-transformed data * significantly different ($p < 0.05$) from the control; ** very significantly different ($p < 0.01$) from the control (Tukey's test).

3.3.3 p50 and p38 expression HT29 and N9 cells

Overall, no changes were observed in the total cellular expression of p50 and p38 following exposure to nanoplastics (data not shown). A significant increase in the nuclear translocation of p50 NF- κ B relative to the control was observed in HT29 exposed to NP (Figure 3.4) in a concentration-dependent effect (Two-way ANOVA, $F(df, 2, 13) = 26.89$, $p < 0.0001$), with cells exposed to $25 \mu\text{g.mL}^{-1}$ of NP showing a higher up-regulation compared to the control, especially for the 25 nm NP (Tukey's test, $q = 7.397$, $DF = 13$, $p = 0.0036$). An overall increase in the nuclear translocation of p38 (up-to 2-fold) was also observable in HT29 albeit the relation to concentration was marginally non-significant (Two-way ANOVA, $F(df, 2, 12)$, $p = 0.055$). In N9 cells, p50 and p38 translocation did not vary significantly compared to the control for any of the treatments (Tukey's test, $p > 0.05$) (Figure 3.4).

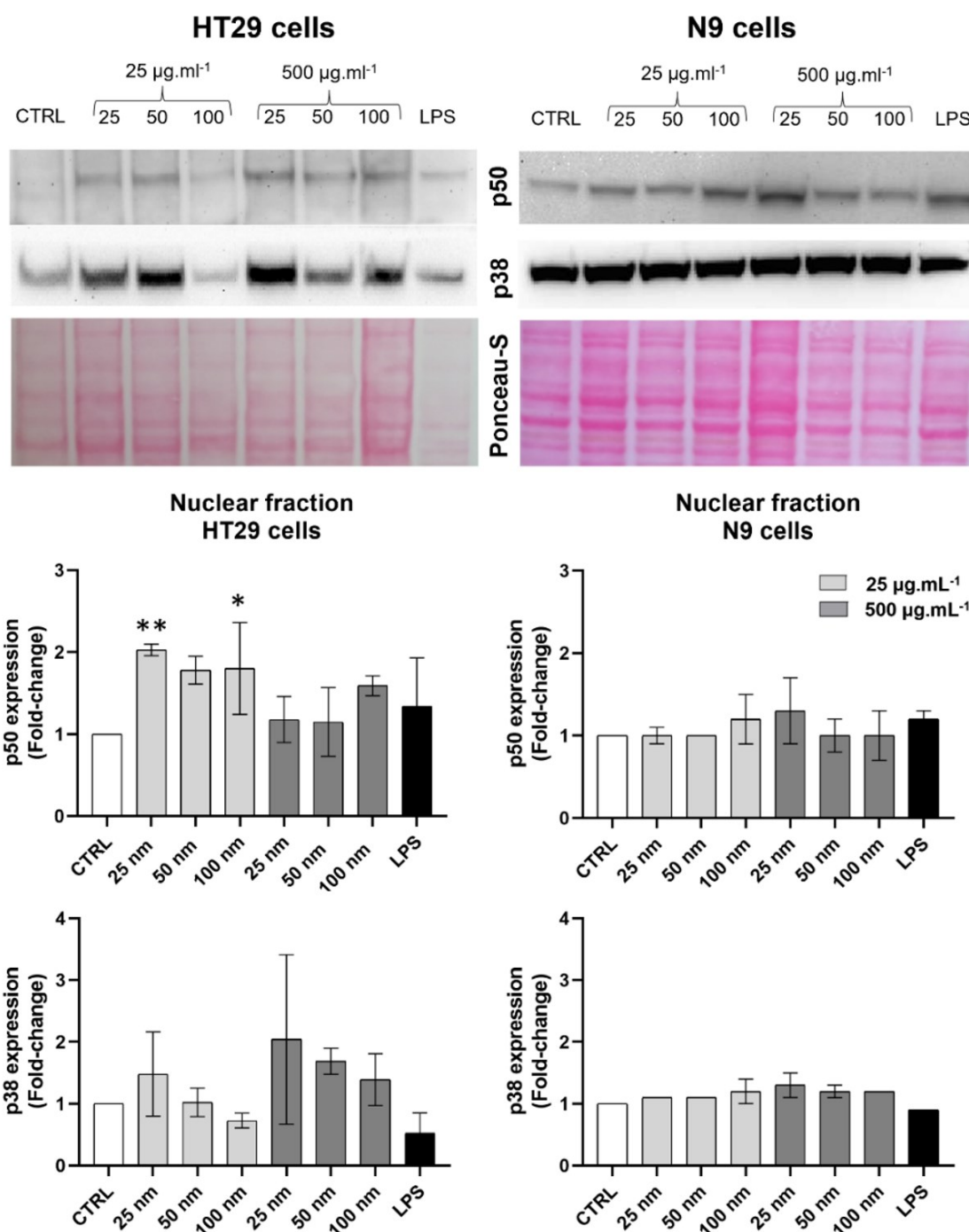


Figure 3. 4 - p50 and p38 expression in the nuclear fraction of HT29 and N9 cells, as determined by Western Blot, after exposure to three different sizes of NP (25, 50 and 100 nm) in two different concentrations (25 and 500 $\mu\text{g.mL}^{-1}$) and LPS (300 ng.mL^{-1}) for 24 h and LPS (300 ng.mL^{-1}). Results were normalized for protein loading using Ponceau-S staining. Statistical testing was performed on log-transformed data. Values are the mean $\pm$ S.D of three independent experiments. * significant differences with a $p < 0.05$ from control (Tukey's test).

3.3.4 TLR4 expression in HT29 and N9 total cell lysates

Analysis of basal TLR4 expression showed that N9 cells expressed higher levels of this receptor compared to HT29 cells (Figure 3.5A). Despite the lower basal levels, HT29 showed an up-regulation of TLR4 of approximately 2-fold relative to the non-treated control when cells were exposed for 24 h to NP (Figure 3.5B). This increase was related to concentration (Two-way ANOVA, $F(df, 2, 9) = 23,02$, $p = 0.0003$) and denoted a significant interaction with size (Two-way ANOVA, $F(df, 4, 9) = 8807$, $p = 0.0035$). Smaller NP (25 and 50 nm) increased TLR4 significantly at $25 \mu\text{g.mL}^{-1}$ (25 nm: Tukey's test, $q = 7.08$, $DF = 12$, $p = 0.0125$; 50 nm: Tukey's test, $q = 7.07$, $DF=12$, $p = 0.0126$) whereas larger particles (100 nm) only at $500 \mu\text{g.mL}^{-1}$ (Tukey's test, $q = 6.662$, $DF = 12$, $p = 0.0183$). This up-regulation was not significant in N9 cells (Figure 3.5B; Two-way ANOVA $p > 0.05$) which had already a high basal expression of TLR4 (Figure 3.5A).

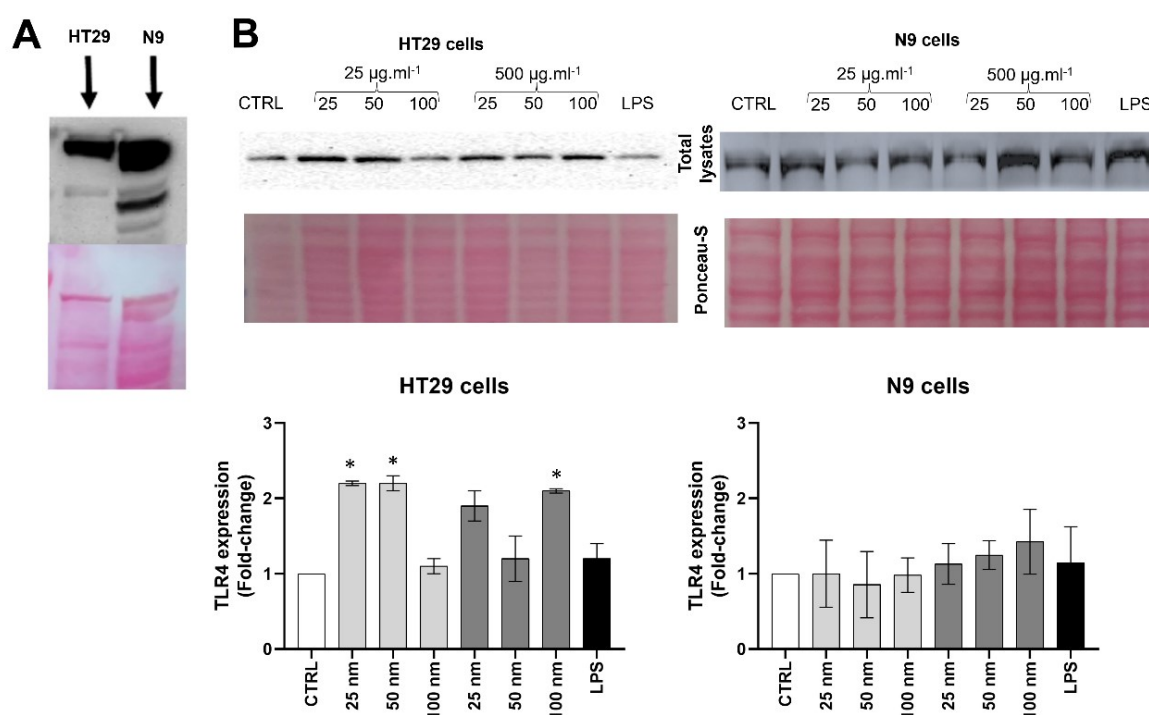


Figure 3. 5 - Basal and nanoplastic-induced TLR4 expression in HT29 and N9 cells. A) Basal TLR4 expression in HT29 and N9 cells. B) TLR4 expression in HT29 and N9 cells exposed to 25, 50 and 100 nm NP at 25 and $500 \mu\text{g.mL}^{-1}$ and LPS (300 ng.mL^{-1}) for 24 h. Values are the mean $\pm$ S.D of three independent experiments. Statistical testing was performed on log-transformed data * significant differences with a $p < 0.05$ from control (Tukey's test).

3.3.5 Effects of nanoplastics over pro-inflammatory gene expression

Effects of NP exposure in gene expression of HT29 and N9 cells are presented in Figures 3.6 and 3.7, respectively. The overall effect of particle size (Two-way ANOVA HT29: $F(df, 2, 25) = 3.903$, $p = 0.0335$; N9: Size $F(2, 16) = 6.031$, $p = 0.0112$) and concentration (HT29: $F(df, 2, 25) = 10.51$, $p = 0.0005$; N9 $F(df, 2, 16) = 28.50$, $p < 0.0001$) over IL-1 β mRNA was very significant in both cell lines (Figures 3.6A and 3.7 A). After 24 h of exposure to NP at 25 $\mu\text{g.mL}^{-1}$, IL-1 β mRNA levels (Figure 3.6A) were increased in HT29 cells exposed to 50 nm NP (Figure 3.7A; Tukey's test, $q = 6.172$, $DF = 25$, $p = 0.051$). When pre-exposed to the TLR4 and p38 inhibitor IL-1 β mRNA expression values of HT29 cells remained unchanged (Tukey's test, $p > 0.05$). When NP concentration rose to 500 $\mu\text{g.mL}^{-1}$ (Figure 3.6 B), a significant increase in IL-1 β (3-fold) relative to the control was observed with exposure of HT29 cells to the smaller NP (25 nm) (Tukey's test, $q = 5.314$, $DF = 25$, $p = 0.0217$), which could be blocked by inhibiting TLR4 or p38 Overall, exposure to NP at both 25 and 500 $\mu\text{g.mL}^{-1}$ caused no significant differences in iNOS mRNA levels in HT29 (Two-way ANOVA, $F(df, 2, 25) = 0.9016$, $p = 0.4187$) (Figures 3.6C and D). It is noteworthy that when pre-exposed to the p38 inhibitor, iNOS transcription increased significantly in cells exposed to 50 nm-sized particles (Tukey's test, $q = 6.298$, $DF = 13$ $p = 0.0068$).

N9 cells showed a significant increase in IL-1 β mRNA levels following 24 h of exposure to 25 $\mu\text{g.mL}^{-1}$ NP relative to the control for the 25 (Tukey's test, $q=5.027$, $DF = 16$, $p = 0.0503$) and 100 nm (Tukey's test, $q = 10.18$, $DF = 16$, $p < 0.0001$) sizes, (Figure 3.7A). Most interestingly, this increase was completely subdued when NP concentration rose to 500 $\mu\text{g.mL}^{-1}$ (Figure 3.7B) denoting a significant interaction between size and concentration (Two-way ANOVA, $F(df, 4, 16) = 7.810$, $p = 0.0011$). A very similar trend was observed for iNOS in N9 cells, again showing increased transcription following exposure to 25 $\mu\text{g.mL}^{-1}$ (Figure 3.7) (up to 25 fold with 100 nm NP), but not at 500 $\mu\text{g.mL}^{-1}$ demonstrating a concentration-related effect (Two-way ANOVA, $F(df, 2, 19) = 52.30$, $p < 0.0001$). Most interestingly, in several cases pre-exposure of N9 cells to TLR4 and p38 inhibitors prevented up-regulation of IL-1 β and iNOS transcription compared to N9 cells exposed only to NP (for Tukey test details) (Figures 3.7A and C). TNF- α expression values in HT29 cells were only significantly increased in cells exposed to 25 $\mu\text{g.mL}^{-1}$ of 100 nm

particles (Figure 3.7E) (Tukey's test, $q = 7.367$, $DF = 24$, $p = 0.0007$). No clear effect of TLR4 or p38 inhibition was observed for all sizes and concentrations (Tukey's test, $p > 0.05$) (Figures 3.7E and F). Overall, no significant differences were registered in TNF- α levels (Figures 3.7E and F) values for N9 cells in any of the exposure conditions (Tukey's test, $p > 0.05$). An exception was observed for 50 nm NP at 500 $\mu\text{g.mL}^{-1}$, where TNF- α levels (Figures 3.7F) were significantly higher relative to the control (Tukey's test, $q = 8.288$, $DF = 18$, $p = 0.0004$), but only for the highest exposure concentration (500 $\mu\text{g.mL}^{-1}$) showing a significant interaction between the effect of particle size and concentration (Two-way ANOVA, $F(df\ 4, 18) = 11.75$, $p < 0.0001$). Pre-exposure to the TLR4 inhibitor (Tukey's test, $p > 0.05$) did not change this effect, but pre-exposure to the p38 inhibitor significantly decreased TNF- α expression values for the 50 nm size (Tukey's test, $q = 11.16$, $DF = 9$, $p = 0.033$) (Figure 3.7F).

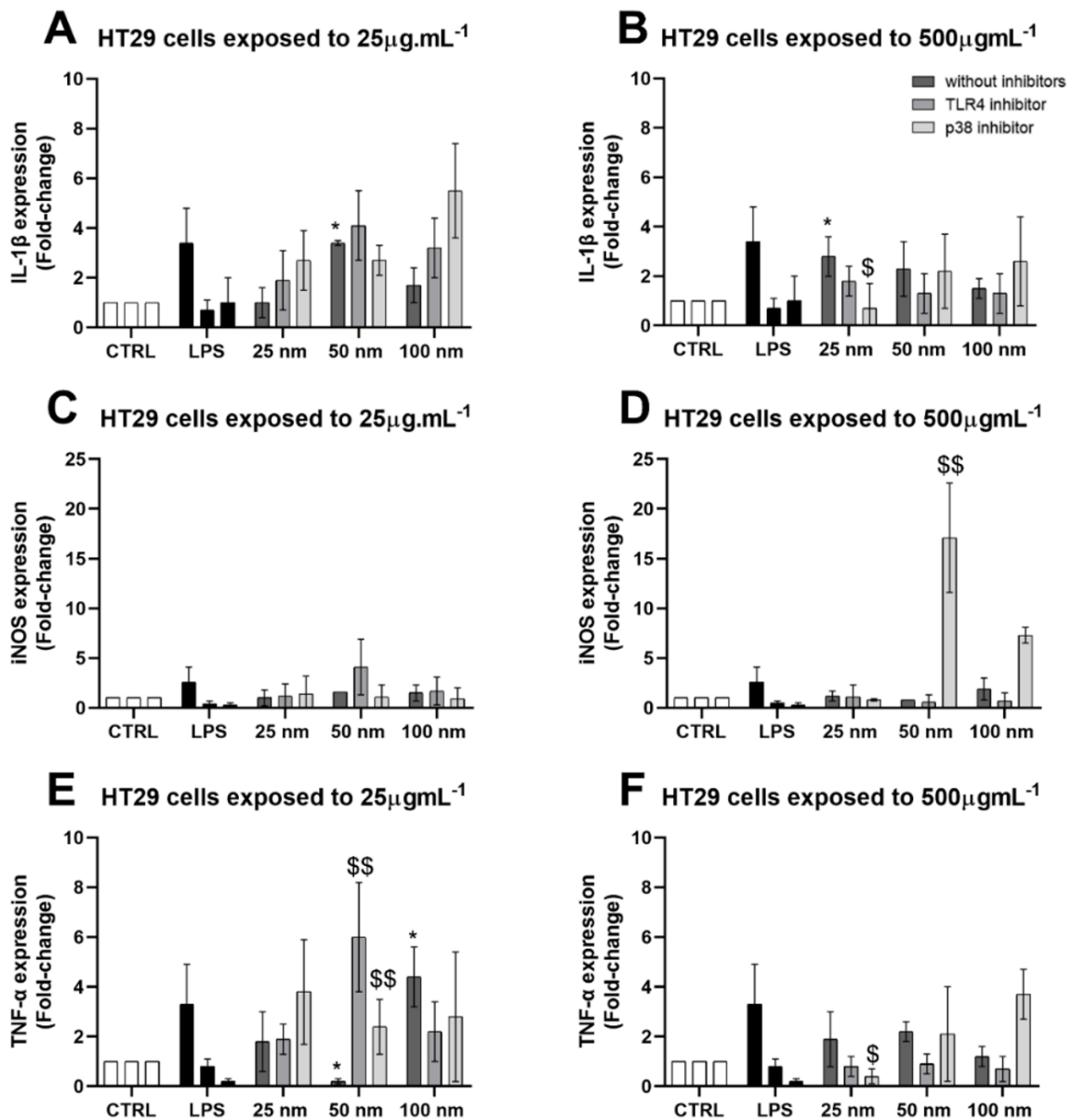


Figure 3. 6 - HT29 cells mRNA expression for IL-1 β (panels A and B), iNOS (panels C and D) and TNF- α (panels E and F) after 24 h of exposure to 25, 50 and 100 nm nanoplastics at 25 and 500 $\mu\text{g.mL}^{-1}$ and LPS (300 ng.mL^{-1}). Data represent the mean $\pm$ S.D of at least 3 independent replicates. Statistical testing was performed on log-transformed data Tukey test: * significantly different from the control ($p < 0.05$); significantly different from the corresponding group without pre-exposure to \$ either the TLR4 or p38 inhibitor ($p < 0.05$). Data represent the mean $\pm$ S.D of at least 3 independent replicates.

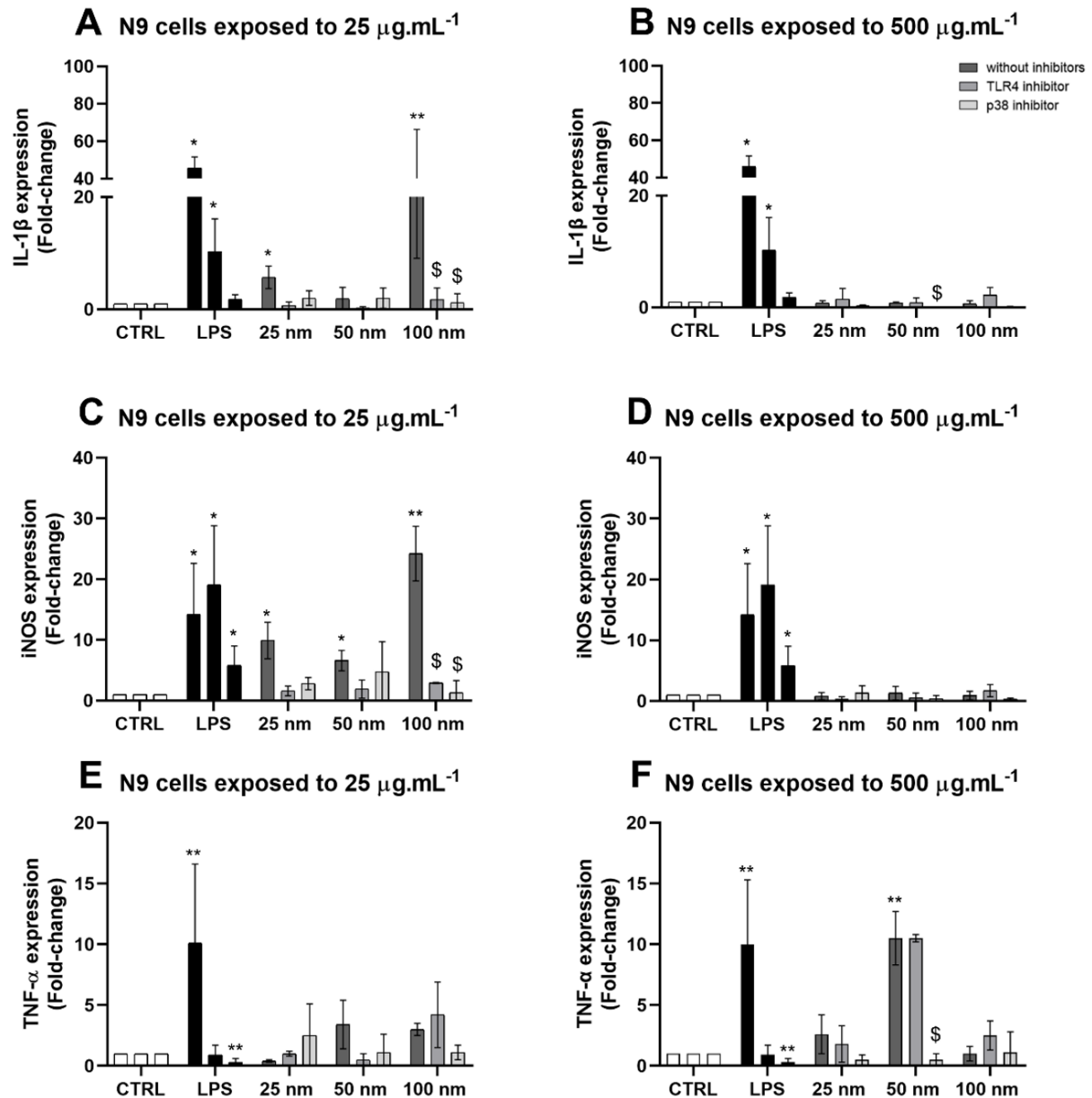


Figure 3. 7 - N9 cells mRNA expression for IL-1 β (A and B), iNOS (C and D) and TNF- α (E and F) after 24 h of exposure to 25, 50 and 100 nm nanoplastics at 25 and 500 $\mu\text{g.mL}^{-1}$ and LPS (300 ng.mL^{-1}). Data represent the mean $\pm$ S.D of at least 3 independent replicates. Statistical testing was performed on log-transformed data. Tukey test: * significantly different from the control ($p < 0.05$); significantly different from the corresponding group without pre-exposure to \$ either the TLR4 or p38 inhibitor ($p < 0.05$). Data represent the mean $\pm$ S.D of at least 3 independent replicates.

3.4 Discussion

Our results showed that NP of different sizes and in a wide range of concentrations (25–500 $\mu\text{g.mL}^{-1}$) did not affect significantly the cellular viability of human colorectal adenocarcinoma cells (HT29) (Figure 3.1). However, the viability of mouse microglial cells (N9 cells) was significantly hindered by exposure to NP, with MTT reduction decreasing to about 50% after 48 h of exposure. This effect was most evident for smaller size nanoplastics (25 and 50 nm) which is in line with previous reports using comparable exposure concentrations in other unicellular models such as microalgae (Hazeem et al., 2020), HepG2 cells (He et al., 2020), splenocytes (Li et al., 2022), neuronal cells (Jung et al., 2020). The effect of nanoplastics on cellular viability is attributed to the easier internalization of smaller size particles (Kihara et al., 2021) which eventually causes cell death (Gopinath et al., 2019; Jung et al., 2020). However, differences in sensitivity between cell lines are often reported, for example between neuronal cells and astrocytes (Jung et al., 2020), which is also in line with the differences we observed between HT29 and N9 cells. Interestingly, Jung et al. (2020) reported that astrocytes, while not showing a decrease in viability, exhibited reactive astrogliosis as seen by an increase in lipocalin-2, showing that NP might induce changes to cellular metabolism regardless of their effect on cellular viability. Indeed, we observed that HT29 cells upregulated the nuclear translocation of p50 and p38 after being exposed to smaller NP (Figure 3.2), even though the overall expression was unchanged (data not shown).

NF- κ B1 is present in the cytoplasm of cells as an inactive heterodimer (p50/p65) bound by I κ B α , which prevents the transcription factor from migrating into the nucleus (Bacher et al., 2021). The activation of NF- κ B1 may result from an inflammatory stimulus, for example, exposure to lipopolysaccharide (LPS), which activates cell surface toll-like receptors, namely TLR4 (Bhagat et al., 2020), and eventually results in I κ B phosphorylation and NF- κ B release (Kawai and Akira, 2007; Viatour et al., 2005). On the other hand, p38 is a MAPK that is involved in multiple signalling cascades including inflammatory response and is responsive to environmental stressors (Cuadrado and Nebreda, 2010; Raingeaud et al., 1995). So, the increase of these two proteins in the nucleus of HT29 cells suggests an activation of an inflammatory response.

In N9 cells no significant increase in the nuclear translocation of either p50 or p38 was observed after 24 h of exposure to NP. This result was unexpected since N9 cells

generally exhibit a strong inflammatory response to environmental stressors (Branco et al., 2022). Moreover, N9 cells did demonstrate an increase in inflammatory gene transcription after 24 h (see discussion below) which could signify that p50/p38 activation might have occurred at an earlier time-point. The lack of an effect observed with LPS exposure seems to corroborate this hypothesis since our previous experience with these cells showed that enhancement of p38 translocation happens after 6 h of exposure (Branco et al., 2022).

In line with the increased nuclear translocation of p50 in HT29 cells, we observed that exposure to NP, particularly to particles with 25 and 50 nm in diameter, upregulated TLR4 levels as seen by the western blot (Figure 3.3B). These cells constitutively express medium-low levels of TLR4 but are known to upregulate its levels upon an inflammatory stimulus (Suzuki et al., 2003). Indeed, we observed levels of TLR4 with as much as a 2-fold increase in HT29 cells exposed to NP, relative to the non-treated control (Figure 3.3B). This result is even more remarkable if we consider that N9 cells, did not up-regulate their levels following 24 h of exposure, perhaps because the basal level was already very high (Figure 3.3A). In fact, LPS, the gold standard of TLR4 activators also did not promote an increase in receptor expression in N9 cells (Figure 3.3).

Analysis of proinflammatory gene expression corroborates these results and suggests activation of inflammatory signalling by exposure to nanoplastics in both cell lines. Also, according to the differences observed for viability and p50 and p38 translocation, HT29 and N9 cells show different profiles of gene transcription after 24 h. Indeed, IL-1 β increase expression in HT29 cells fits well with observations for p50 and TLR4 and suggests a canonical activation of an immune response (Jesus and Goldbach-Mansky, 2014; LaRock et al., 2016). Common activators of a TLR4/NF- κ B mediated inflammatory response include LPS (Elizagaray et al., 2020), which in our HT29 model increased IL-1 β mRNA similarly to what we observed for NP. Likewise, pre-exposure of cells to a TLR4 inhibitor, prevented IL-1 β increase by both LPS and NP, which strengthens the idea that the latter induced a TLR4/p38-mediated inflammatory response as well (Zelova and Hosek, 2013).

Furthermore, the increase in iNOS expression values in HT29 cells, suggests a typical pro-inflammatory response (Sharma et al., 2007). Indeed, increasing NO production

can have beneficial microbicidal, antiviral, antiparasitic, and antitumoral activity (Kleinert and Forstermann, 2007) but at the same time contribute to free radical-mediated tissue injury in inflammatory disorders (Kolios et al., 1995).

Interestingly, we observed a great increase in IL-1 β and, particularly, iNOS mRNA levels when HT29 cells were pre-exposed to a p38 inhibitor before NP exposure (50 nm and 100 nm; 500 $\mu\text{g.mL}^{-1}$ of NP). Although intriguing, this result is in line with previous studies (Lahti et al., 2006) suggesting that inhibition of p38 MAPK leads to iNOS mRNA stabilization and increased iNOS and NO production by a JNK-mediated mechanism.

The response of N9 microglia cells to exposure to NP was quite different. As shown, these cells have a high basal level of TLR4 (Figure 3.3 A) and are promptly activated by exposure to TLR4-receptor agonists, such as LPS, as seen here - by the considerable increase in IL-1 β , iNOS and TNF- α mRNA - and reported elsewhere (Vaure and Liu, 2014). Unlike with HT29 cells, upon exposure of N9 cells for 24 h to NP we did not observe any significant change in the nuclear levels of p50 and p38 (Figure 3.2) or in the overall expression of TLR4 (Figure 3.3B). Nevertheless, IL-1 β and iNOS mRNA levels were significantly increased by exposure to 25 $\mu\text{g.mL}^{-1}$ of NP of all sizes, so inflammatory signalling was definitely present. Indeed, in N9 cells, pre-exposure to either the TLR4 or the p38 inhibitor hindered IL-1 β and iNOS transcription (Figure 3.5 A-D), thus demonstrating that these pathways are in fact involved in the inflammatory response of these cells to NP.

TNF- α mRNA was only increased in N9 cells exposed to the highest concentration of 50 nm NP (Figure 3.5). Increased levels of this cytokine are normally associated to an advanced stage of inflammation. This may explain why we observed a greater effect of NP over N9 viability (Figure 3.1), since TNF- α is known to activate several kinases that induce cell death (Kim et al., 2010). Still, this result lacks consistency throughout the exposure groups to allow the drawing of major conclusions. Most interestingly, the increase in TNF- α mRNA was not suppressed by pre-exposure of cells to the TLR4 inhibitor, suggesting another regulatory pathway. In agreement, p38 inhibition completely prevented the increase in TNF- α mRNA (Figure 3.5 F).

3.5 Conclusions

Our findings demonstrate that nanoplastics induce inflammatory signalling; however, the specific mechanisms and response kinetics may vary depending on cell type and the intricate interplay of different signalling pathways. For instance, in HT29 cells, an intestinal cellular model, the notorious upregulation of TLR4 levels and p50 nuclear translocation suggest that this pathway plays a main role in inflammation. Yet, inhibiting this pathway did not consistently reduce pro-inflammatory gene transcription, implying the involvement of other factors such as p38 in the inflammatory response. The importance of TLR4 was further emphasized as its inhibition substantially reduced IL-1 β and iNOS transcription in N9 microglia cells exposed to nanoplastics. Moreover, N9 cells exhibited a more pronounced response, including reduced viability and cytokine expression, suggesting that nanoplastics may disrupt tissue homeostasis by activating resident macrophages. Since IL-1 β and iNOS have links to immune system dysregulation and inflammation-mediated carcinogenesis, it is imperative to conduct further research to elucidate their involvement in nanoplastic-induced intestinal and tissue inflammation. Furthermore, the absence of knowledge concerning the internal dose of nanoplastics within biological systems hinders the accurate extrapolation of the physiological significance of these mechanisms. It is important to emphasize that the results presented herein show occasional significant variability between replicates which in our assessment may be attributed to the propensity of NP to aggregate in culture medium, as previously discussed. Nevertheless, it is evident from our data that polystyrene nanoplastics are by no means innocuous regarding inflammatory effects.

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Joana Antunes: Writing – original draft, Formal analysis, Investigation. Paula Sobral: Resources, Writing – review & editing, Funding acquisition. Marta Martins: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. Vasco Branco: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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CHAPTER 4:

Combined effects of nanoplastics and benzo[a]pyrene in intestinal and hepatic human cells

This Chapter is a manuscript in preparation for submission:

Antunes J, Sobral P, Martins M, Branco V, (2024). Combined effects of nanoplastics and benzo[a]pyrene in intestinal and hepatic human cells.

Abstract

Polycyclic Aromatic Hydrocarbons (PAH) and nanoplastics (NP) are pervasive environmental pollutants known for their significant health and ecological risks. PAH, characterized by their lipophilic properties, readily infiltrate epithelial cells, resulting in adverse health consequences. These organic compounds stem from diverse sources, including incomplete combustion and industrial pollution, leading to potential bioaccumulation in humans and subsequent carcinogenic and genotoxic responses, such as oxidative stress. NP, emerging as a growing environmental concern, originate from mass plastic production and the degradation of larger plastic debris. Their small size facilitates entry into organisms, potentially impacting marine ecosystems and human health. The co-exposure of NP and PAH represents a relatively unexplored facet of environmental contamination, offering an additional route for harmful chemical exposure. This study aims to assess the impact of NP and benzo[a]pyrene, a representative PAH, contamination on HT29 and HepG2 cells, focusing specifically on the response of CYP450 system. Results showed that PSNP alone did not significantly impact HT29 cell viability, however it significantly increased in HepG2 cells. Our findings suggest that PSNP may have a detrimental effect on HepG2 cells antioxidant capacity, as evidenced by a significant decrease in GPx1 levels. However, when PSNP were combined with B[a]P there GPx1 expression was increased, similarly to HT29 cells. Additionally, the levels of CYP1A1 also upregulated in HepG2 cells when exposed to these combination.

Keywords

Nanoplastics; benzo[a]pyrene; glutathione; oxidative stress; cells

4.1 Introduction

Nanoplastics (plastic particles <100 nm) have emerged as a pervasive environmental pollutant, posing significant threats to ecosystems and human health (Mitrano et al., 2021b). Originating from the degradation of larger plastic debris and the direct release of MP in various industrial and consumer products, NP contamination has reached alarming levels globally (PlasticsEurope, 2022; Thompson et al., 2004).

Understanding the intricate interplay between nanoplastics and co-existing environmental contaminants, along with the potential "Trojan horse" effect they may exhibit, underscores the urgency of further research to mitigate associated risks to ecosystems and human health. B[a]P, a representative PAH, is ubiquitous in the environment due to combustion processes such as vehicle emissions and industrial activities (Bukowska et al., 2022).

NP and B[a]P frequently coexist in environmental matrices, raising concerns about their combined toxicological effects (N. Sun et al., 2023). In this study, we investigated the cytotoxicity of PSNP, B[a]P, and their combination in the HT29 and HepG2 cells, focusing on key cellular mechanisms. Specifically, we analyzed the role of the major Phase I enzyme cytochrome P450 1A (CYP1A1) and its related ethoxyresorufin-O-deethylase (EROD) activity, which relates to PAH metabolic activation, as well as Phase II actors, namely total glutathione and related enzymes such as glutathione-S-transferase (GST) and glutathione peroxidase 1 and 4 (GPx1 and GPx4), all of which play pivotal roles in detoxification and elimination of compounds and in maintaining cellular redox homeostasis (Forman et al., 2009).

By quantifying alterations in total glutathione (GSH), GST, and GPx levels, we aimed to understand how exposure to PSNP, B[a]P, and their combination influences oxidative stress levels and cell viability, shedding light on potential health risks associated with environmental pollutants and nanoplastics exposure.

PROCESSES OF METABOLIZATION AND DETOXIFICATION AFTER EXPOSURE TO PSNP AND B[a]P

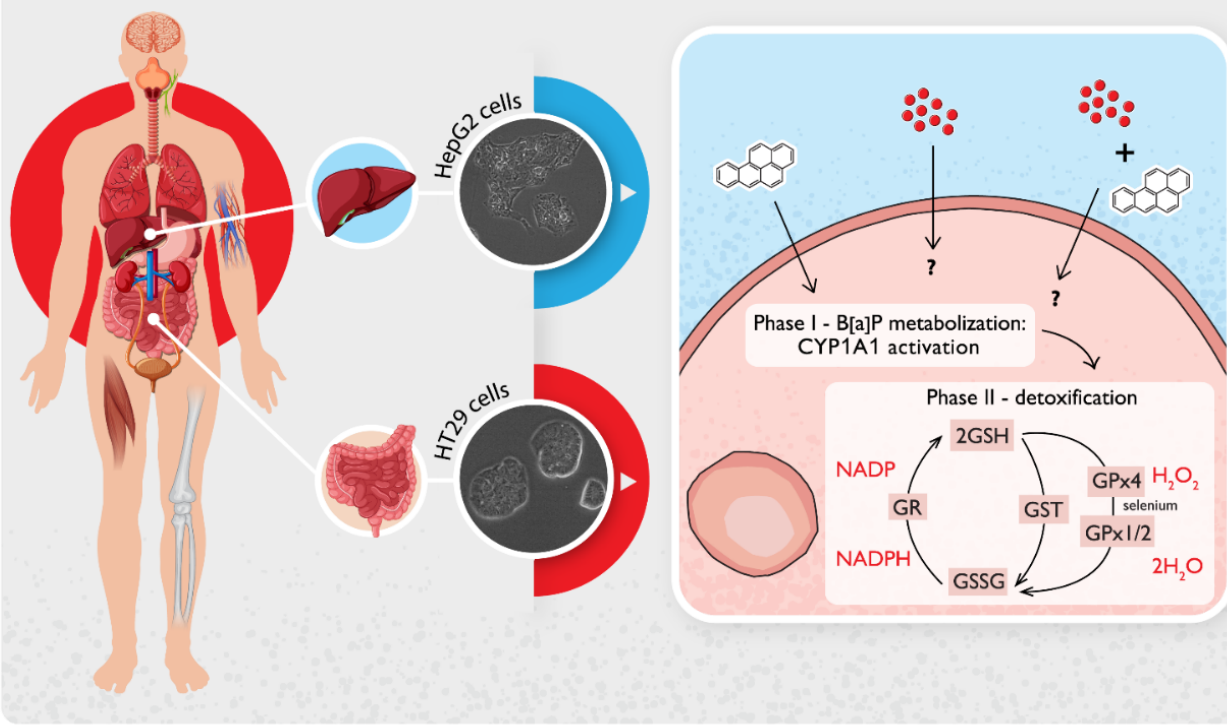


Figure 4. 1 – Analysing processes of metabolism and detoxification after exposure to PSNP and B[a]P.

4.2 Materials and methods

4.2.1 Nanoplastics characterization and preparation

PSNP with 25 nm diameter and a density of 1.056g.cm^{-3} from Thermo Scientific, were purchased from Distilab, Netherlands (catalogue references, G25 (<https://distilab.nl/dyed-and-fluorescent-particles/>)). Fluorescent dyes were incorporated into the polymer mix (not surfaced dyed) and the NP were packaged in deionized water with trace amounts ($<0.1\%$) of surfactant (Triton-X 100), to inhibit aggregation and promote stability, and the preservative (sodium azide). A volume of 10 μL of the initial solution was diluted in 990 μL of ultra-pure water. Intermediate solutions of 1000 and 250 μL were prepared and diluted in PBS x1 solution. Two concentrations were tested for cellular viability (25 and 100 $\mu\text{g.mL}^{-1}$) and the highest concentration was used for further analysis.

The size, zeta potential and morphology of PSNP were characterized. The size and the zeta potentials of the NP were analysed with a HORIBA nanoparticle analyser SZ-100. At least three replicates of a NP solution diluted in either PBS or culture cell cultured medium were analysed at a concentration of 25 $\mu\text{g.mL}^{-1}$. The polymer spectrum was obtained after a 10 μL well-dispersed NP (100 mg.L^{-1}) were dropped onto glass laminae, air-dried, and posteriorly analysed with RAMAN spectroscopy. This was carried out using a Labram 300 Jobin Yvon spectrometer, equipped with a diode laser with an excitation wavelength of 785 nm. The laser beam was focused with a 50x or 100x Olympus objective. The laser power at the surface of the sample was varied with the aid of a set of neutral density filters (optical densities 0.3, 0.6, 1). The sample was analysed using 20 s laser exposure time for 20 scans. The spectrum was recorded as an extended scan. The morphology of the above plastics was further characterized using a scanning electron microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Briefly, 10 μL of well-dispersed NP (100 mg.L^{-1}) were dropped onto glass laminae and then air-dried. SEM images were obtained at the CENIMAT facilities at Nova School of Science and Technology, Portugal. The samples were lightly sputter-coated with gold before observation. The particle size distribution of NP based on SEM results was determined using Image J software (NIH, MD, USA).

4.2.2 Cell culture

HT29 cells were purchased from ATCC® and cultured at 37°C in Dulbecco's Modified Eagle medium with high glucose (25 mM), without sodium pyruvate, but with GlutaMAX (Gibco, Paisley, UK), 10% of heat-inactivated fetal bovine serum (FBS) (Biochrome, Berlin, Germany) and 1% penicillin/streptomycin (100 units.mL⁻¹ of penicillin and 100 µg.mL⁻¹ of streptomycin) (Gibco, Paisley, UK) in a humidified incubator with a 5% CO₂ atmosphere. HepG2 cells were kindly provided by Dr. Elsa Dias (Instituto Nacional de Saúde Doutor Ricardo Jorge – INSA) and cultured at 37°C in complete cell culture medium consisting of a 1:1 mixture of Dulbecco's Modified Eagle medium (MERK, Germany) and Ham's F-12 nutrient mix (Thermo Fisher Scientific, USA) medium supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, 1% (v/v) penicillin/streptomycin (100 units.mL⁻¹ of penicillin and 100 µg.mL⁻¹ of streptomycin) (Gibco, Paisley, UK) in a humidified incubator with a 5% CO₂ atmosphere. The culture medium was changed every two days from the second day after seeding and cells were harvested in the logarithmic phase of growth after reaching 70-80% confluency using 0,05% trypsin/EDTA (Sigma). Cells were seeded at 1.5 x 10⁶ cells per cm² in 75 cm² cultured flasks. In these conditions, cells would be confluent after 24/48 hours of culture.

4.2.3 Cell viability

Cell viability was determined by the MTT assay adapted from Carvalho et al. (2008). Cells (5 × 10³ cells per well) were cultured in 96-well plates and allowed to attach for 24 hours before the addition of NP (25 and 100 µg.mL⁻¹), benzo[a]pyrene (5 and 50 µM) and 5 and 50 µM mixture with 25 and 100 µg.mL⁻¹ of NP. After 24, 48, and 72 hours of exposure, MTT was added to plates at a final concentration of 400 µg.mL⁻¹ per well followed by incubation at 37 °C for 2 hours. Following the incubation period, the MTT solution was removed, and the formazan crystals dissolved with 4:1 dimethyl sulfoxide/glycine buffer (pH 10.5) (Honeywell, UK). The absorbance was measured at 550 nm, after 20 minutes of shaking and the cellular viability was calculated relative to the control group.

4.2.4 Exposure of HT29 and HepG2 cells to nanoplastics and benzo[a]pyrene and cell lysate preparation

HT29 and HepG2 cells were seeded in 10 mm² culture dishes (1×10^6 cell per plate) and allowed to reach 70-80% confluency. Afterwards, the culture medium was replenished and cells were exposed to i) 25 nm PSNP at 25 $\mu\text{g.mL}^{-1}$, ii) B[a]P (5 and 50 μM), iii) combination of both contaminants (5 μM + 25 $\mu\text{g.mL}^{-1}$ of 25 nm PSNP and 50 μM + 25 $\mu\text{g.mL}^{-1}$ of 25 nm PSNP) for 24 hours. For the determination of GSH activity, cells were exposed to 100 μM of L-buthionine-(S,R)- sulfoximine (BSO) for 24h as a positive control. BSO were purchased from Sigma-Aldrich (Deisenhofen, Germany). After exposure, the cell layer was rinsed with PBS and detached from plates with trypsin. The cellular pellet was collected by centrifugation (5 min at 600 g) and washed with PBS (Lonza, BioWhittaker, USA). After undergoing a second centrifugation, the pellet was carefully resuspended in the suitable lysis buffer, ensuring optimal conditions for the subsequent assessment of total cell lysates. Cell lysates were obtained by disrupting cellular pellets in lysis buffer (25 mM Tris-Cl, pH 7.5; 100 mM NaCl; 2.5 mM EDTA; 2.5 mM EGTA; 20 mM NaF, 1 mM sodium orthovanadate, 20 mM sodium pyrophosphate; 20 mM sodium β -glycerophosphate; 0.5% Triton X-100; and 1 tablet of protease inhibitor cocktail (Roche) per 10 mL). After lysis, samples were frozen at -20°C until subsequent analysis. For measurement of glutathione total levels, cells were lysed with a Teflon pestle in cold-ice extraction buffer consisting of 0.1% Triton X-100% and 0.6% sulfosalicylic acid in 0.1 M potassium phosphate buffer containing 5 mM EDTA disodium salt at pH 7.5. Subsequently, the lysates were sonicated for 3 min in ice-cold water and centrifuged at 3000 g for 5 min at 4°C . The resulting supernatant was used for GSH measurements. After lysis, samples were frozen at -20°C until subsequent analysis.

4.2.5 Total protein determination

The total protein content was determined in the soluble fraction of each lysate, using the Bradford method (Branco et al., 2022). After mixing diluted (5x) Coomassie dye (BioRad) with each sample (96-well plates), the absorbance was measured at 595 nm in a microplate reader (Zenyth 3100, Anthos Labtec Instruments). The protein concentration was calculated from a calibration curve (1 – 16 μg of protein per μL) using bovine serum albumin (BSA) (Sigma-Aldrich) as a standard.

4.2.6 Determination of GPx1, GPx4 and CYP1A1 protein levels

To evaluate protein expression of GPx1, GPx4 and CYP1A1, total cellular lysates of cells were analysed by Western Blot, after 24 h of PSNP and B[a]P exposure. Protein expression was analysed after separation of total soluble proteins (40 µg) by SDS–PAGE on a 4–12% Bis–Tris gel (Invitrogen, ThermoFisher Scientific, USA) with MES running buffer under reducing conditions (140 v for 1 h). Proteins were subsequently transferred (40 v for 7 min) to a nitrocellulose membrane using the iBlot 2 dry blotting system (Invitrogen), blocked with a 5% skimmed milk solution (1 hour) and rinsed with PBST. Afterwards, membranes were incubated with primary and secondary antibodies for the proteins of interest and detected with chemiluminescence using the Biorad Chemidoc System. Membranes were submerged with shaking for 1 h in a 1% BSA solution containing the respective primary antibody (1/ 1000). The following antibodies were used: GPx1 (ab22604, ABCAM), GPx4 (sc-13666588, Santa Cruz) and CYP1A1 (AB126887, ABCAM). Expression levels were normalized for protein loading on the gel, which was assessed by Ponceau S (Sigma-Aldrich).

4.2.7 Determination of glutathione S-Transferase activity

GST activity was measured in 20 µL of total cell lysate using a commercial kit (from Sigma-Aldrich) and chloro-2,4-dinitrobenzene (CDNB), according to manufacturer's instructions. Activity was determined by measuring the increase in absorbance at 340 nm during 5 min. Activity was expressed as percentage relatively to control, after normalization for protein content

4.2.8 Measurement of total glutathione

Total GSH was determined using the enzymatic recycling method described by (Rahman et al., 2007). For total GSH analyses, 5 µl of each sample were incubated with 5 mM DTNB, 50 mM NADPH, and 50 nM glutathione reductase (100 U.ml⁻¹ GR) in 0.1 M potassium phosphate buffer with 5 mM EDTA (pH 7.5) and the formation of TNB was monitored at 412 nm for 2 min. Quantification was performed using calibration curves generated with GSH standard ranging from 0 to 500 µM. GSH levels were expressed relatively to the control, after protein normalization.

4.2.9 CYP1A1 activity determined by EROD assay

CYP1A activity was determined using the microplate assay for EROD activity following the protocol described by (M. Ferreira et al., 2014). Cells were seeded in a 24-well plate at a density of 3×10^5 cells per well and incubated for 24 h before exposure to different treatments, including PSNP (25 nm, 25 $\mu\text{g}.\text{mL}^{-1}$), B[a]P (5 and 50 μM) and a combination of PSNP and B[a]P (5 μM + PSNP; 50 μM + PSNP). B[a]P dilutions were prepared in DMSO, with a maximum concentration of 0.1%. DMSO-exposed cells were used as control. Following contamination, the plates were washed with cold PBS and a reaction mixture containing 1 $\text{mg}.\text{mL}^{-1}$ BSA, 5 μM 7-ethoxy resorufin (7-ER), and 0.5 mM NADPH 100 μl . The plates were then incubated for 30 min at $37 \pm 1^\circ\text{C}$ in the dark. Fluorescence was measured at an excitation wavelength of 560 nm and an emission wavelength of 610 nm. To calculate EROD activity, a standard curve of resorufin (ranging from 0.78 to 50 $\text{pmol}.\text{mL}^{-1}$) was used. After EROD activity measurement, the results were normalized to total protein concentration and PSNP background fluorescence.

4.2.10 Statistical analysis

Statistical analyses were conducted using SPSS version 25. Data normality was evaluated by the Shapiro-Wilks test and variance homogeneity using Spearman's test for heteroscedasticity. Significance of main and interaction effects was evaluated by a Two-way ANOVA, followed by post-hoc assessment with a Tukey test to evaluate differences between exposure groups. Differences between groups were classified as significant at $p < 0.05$ and considered very significant at $p < 0.01$.

4.3 Results

4.3.1 Nanoplastics characterization

SEM analysis provided detailed insights into the size and morphology of the PSNP, allowing for precise determination of their dimensions and surface characteristics. Additionally, RAMAN spectroscopy was employed to confirm the composition of PSNP and the inexistence of other elements (i.e., heavy metals), which can affect and negatively influence the consequent toxicity *in vitro* tests., revealing distinctive spectral signatures indicative of polystyrene (Figure 4.2).

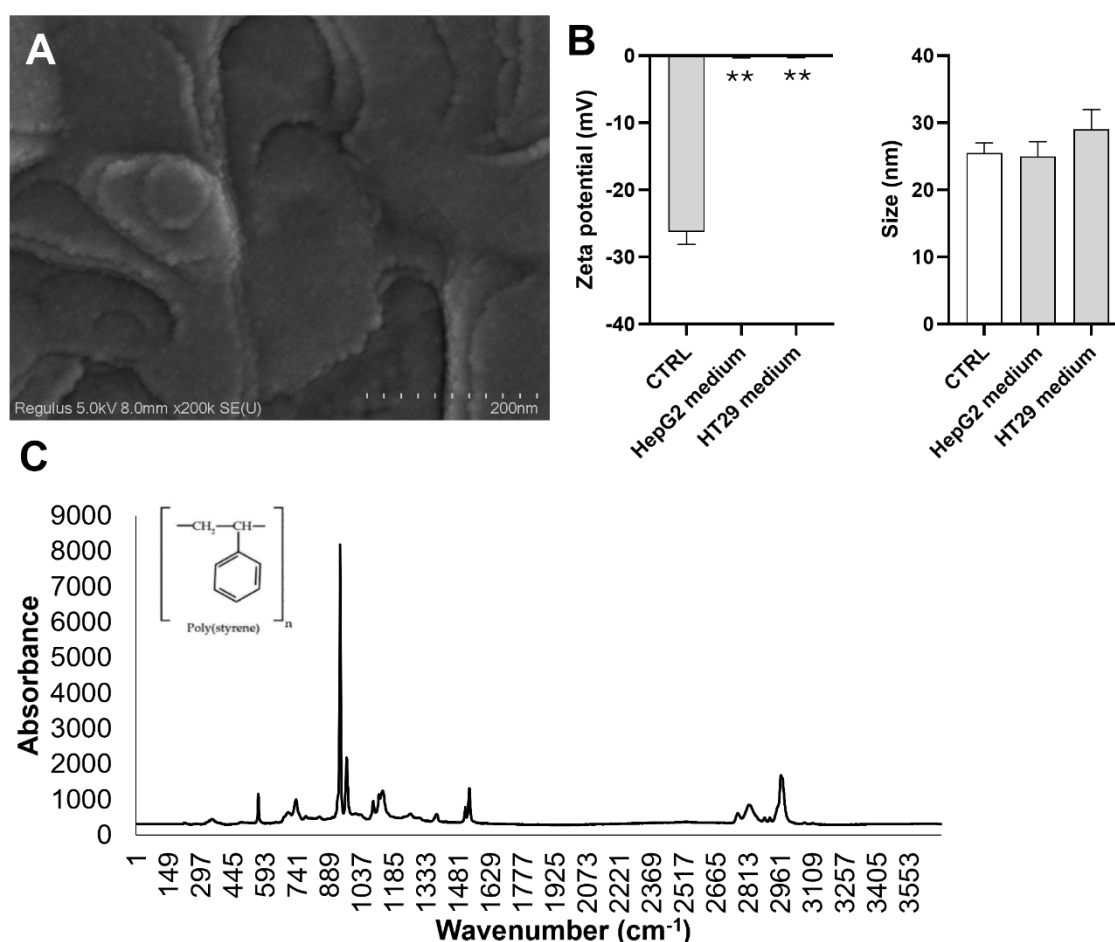


Figure 4. 2 – Polystyrene nanoplastics characterization: A) morphological analysis using scanning electron microscopy (SEM) images; B) zeta potential and size (nm) measurements of PSNP diluted in Dulbecco's phosphate-buffered saline (PBS), used as a control and in cell-cultured mediums used for HT29 and HepG2 cells assays analysed with a HORIBA nanoparticle analyser SZ-100. * indicates the significant differences ($p < 0.05$) from the control (Mann-Whitney test, $p < 0.05$) and C) polystyrene spectrum acquired through a Labram 300 Jobin Yvon RAMAN spectrometer.

Table 4. 1 - Zeta potential and size (nm) measurements of polystyrene nanoparticles (25 nm) diluted in Dulbecco's phosphate-buffered saline (PBS), used as a control, and in Dulbecco's Modified Eagle Medium (DMEM) used in cell culture assays. Data are presented as mean $\pm$ SD of three independent experiments.

Z-average (mV)		
	PSNP	SD
PBS	-26.3	1.80
HepG2 medium culture	-0.2	0.11
HT29 medium culture	-0.2	0.08
Size (nm)		
	average	SD
PBS	25.5	9.60
HepG2 medium culture	25.0	2.18
HT29 medium culture	29.0	2.99

4.3.2 Effect of nanoplastics exposure on viability of HT29 and HepG2 cells

The viability of HT29 and HepG2 cells after 24, 48, and 72 hours of PSNP exposure is presented in Figure 4.3. After 24 hours of exposure, no significant differences were observed in HT29 cells viability in none of the treatments, compared to the control ($p > 0.05$). However, after 48 hours, HT29 cell viability decreased significantly (79 ± 13.1) when exposed to B[a]P at 50 μM , compared to the control ($p < 0.05$). After 72 hours, viability significantly decreased when HT29 cells were exposed to the combination of PSNP (both 25 and 100 $\mu\text{g.mL}^{-1}$) with B[a]P at 50 μM ($p < 0.01$). After 48 hours, the viability of HepG2 cells also decreased significantly when exposed to B[a]P at 50 μM ($p < 0.01$), however, HepG2 exposed to the combination of 25 $\mu\text{g.mL}^{-1}$ of PSNP with B[a]P at 5 μM responded differently compared to HT29 cells, as their viability decreased significantly ($p < 0.01$). The combination of PSNP at 25 $\mu\text{g.mL}^{-1}$ with B[a]P at 50 μM seemed to be toxic after 72 hours ($p < 0.05$). After 72 hours, viability increased significantly after PSNP exposure ($p < 0.01$). This is an unexpected result, as other researchers has observed decreased viability in this cell line after (H. Xie et al., 2023). Generally, the interaction between NP and cells can be quite complex and depends on several factors such as the size, surface charge, and concentration of the NP, as well as the duration of exposure. In this case, it seems that PSNP could interact with biomolecules of the cell cultured medium after 72 hours, formed aggregates with higher dimensions, which probably difficult their uptake in the cells, causing no

cytotoxicity (Z. Yang et al., 2023).

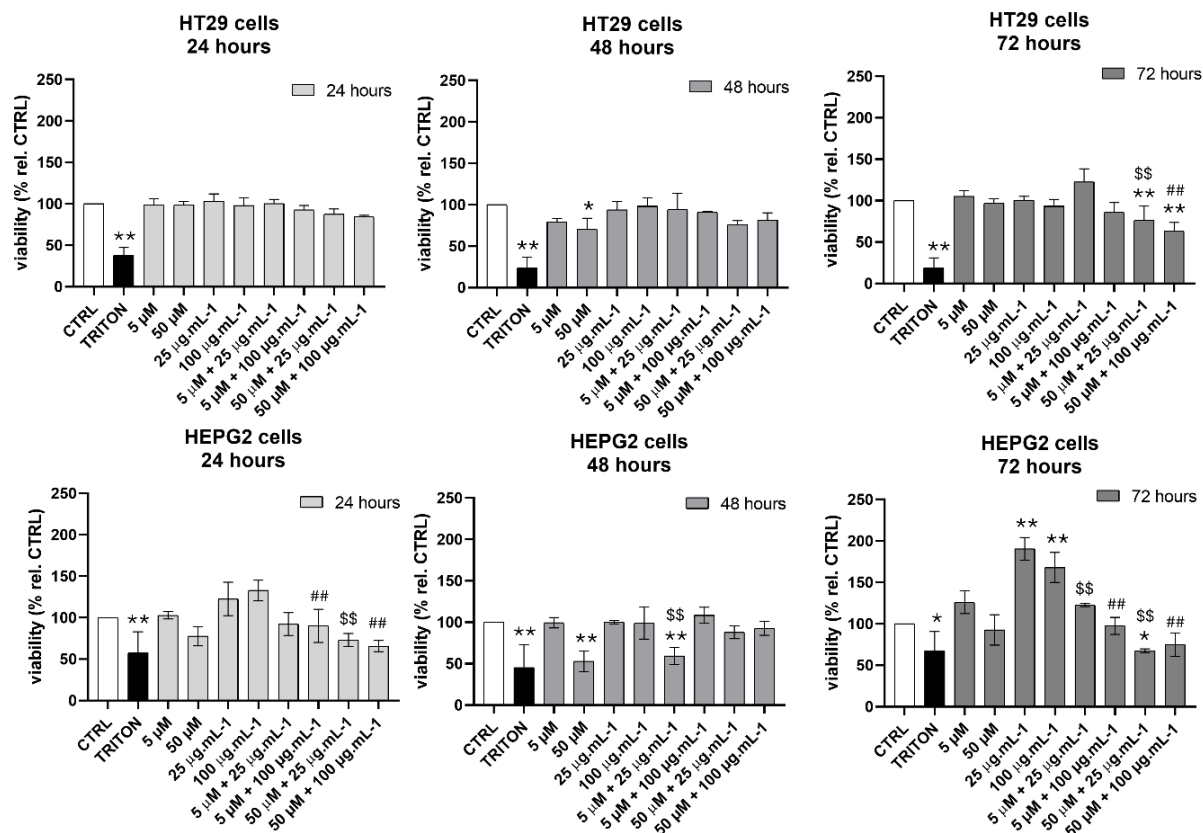


Figure 4. 3 - Viability of HT29 and HepG2 cells, as measured by the MTT assay, after exposure to 25 nm polystyrene nanoplastics at 25 and 100 µg.mL⁻¹, benzo[a]pyrene at 5 and 50 µM and to their combination after 24, 48, and 72 hours. Triton X-100 (10%) was used as a positive control. Results are represented as the mean ± S.D. of at least three independent experiments. * significantly different ($p < 0.05$) from the control; ** very significantly different ($p < 0.01$) from the control (Tukey's test). \$\$ significantly different from the group with PSNP at 25 µg.mL⁻¹ and without B[a]P exposure ($p < 0.01$). ## significantly different from the group with PSNP at 100 µg.mL⁻¹ and without B[a]P exposure ($p < 0.01$).

4.3.3 CYP1A1 protein levels and EROD assay

Figure 4.4 shows CYP1A1 protein expression and EROD activity of HT29 and HepG2 cells after 24 hours of exposure to 25 nm PSNP at 25 $\mu\text{g.mL}^{-1}$, B[a]P at 5 and 50 μM and to their combination. Although CYP1A protein levels in HT29 cells demonstrated no significant differences compared to the control ($p > 0.05$), EROD activity was significantly induced after B[a]P exposure at both concentrations (5 and 50 μM) ($p > 0.01$). For HepG2 results were slightly different compared to HT29. All treatments increased (up to 2 fold) significantly EROD activity above the control (Figure 4.3), except cells exposed to B[a]P at 5 μM ($p < 0.01$). CYP1A1 protein expression, as seen by Western Blot presented higher levels in cells exposed to B[a]P (50 μM) and its mixture with PSNP ($p < 0.01$ and 0.05, respectively).

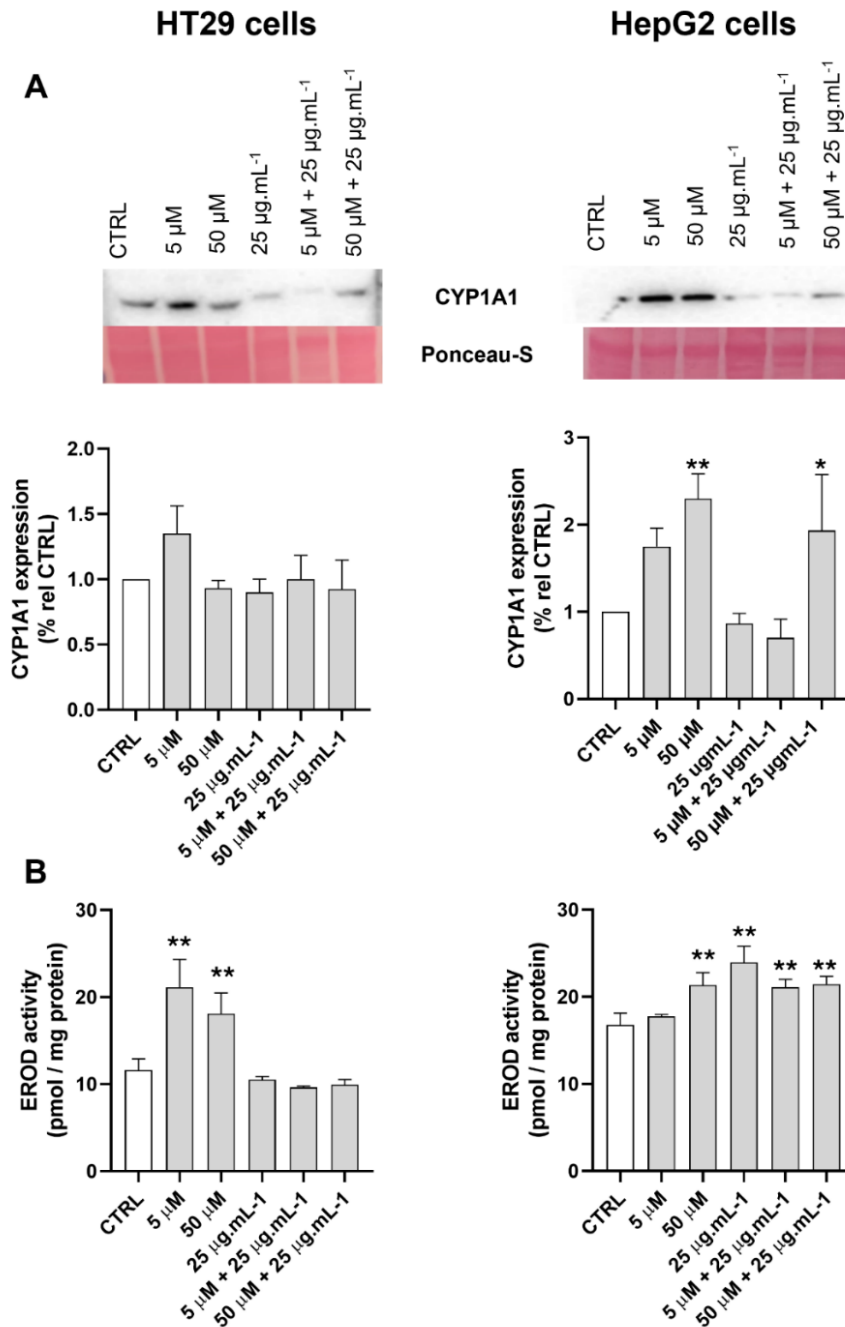


Figure 4. 4 - CYP1A1 expression and erod activity in ht29 and hepg2 cells after exposure to polystyrene nanoplastics, benzo[a]pyrene, and their combination. A) Expression of CYP1A1 by Western blot and B) EROD activity after 24 hours of exposure of HT29 and HepG2 after exposure to 25 nm polystyrene nanoplastics at 25 μ g.mL⁻¹), benzo[a]pyrene at 5 and 50 μ M and to their combination was determined by Western Blot. Results were normalized for protein loading using Ponceau-S staining. Values are the mean $\pm$ S.D of three independent experiments. * and ** represent the significant differences with a $p < 0.05$ and < 0.01 from control, respectively (Turkey test).

4.3.4 Antioxidant response after nanoplastics and benzo[a]pyrene exposure

In HT29 cells, GSH levels were decreased by 50% ($p < 0.01$) following exposure to PSNP and their combination with B[a]P (5 μ M). Interestingly, GST activity was significantly upregulated by exposure this combination and also when cells were exposed to PSNP alone ($p < 0,01$) (Figure 4.5A).

In HepG2 cells, no dramatic decrease was observed in GSH levels, except for cells exposed to B[a]P (5 μ M), where it decreased by approximately 20% ($p < 0.01$). Interestingly, GSH activity was increased by the combination of PSNP with B[a]P (50 μ M) ($p < 0,01$). Similarly to HT29 cells, HepG2 cells exposed to PSNP showed a very significant increase in GST activity ($p < 0,01$) (Figure 4.5B).

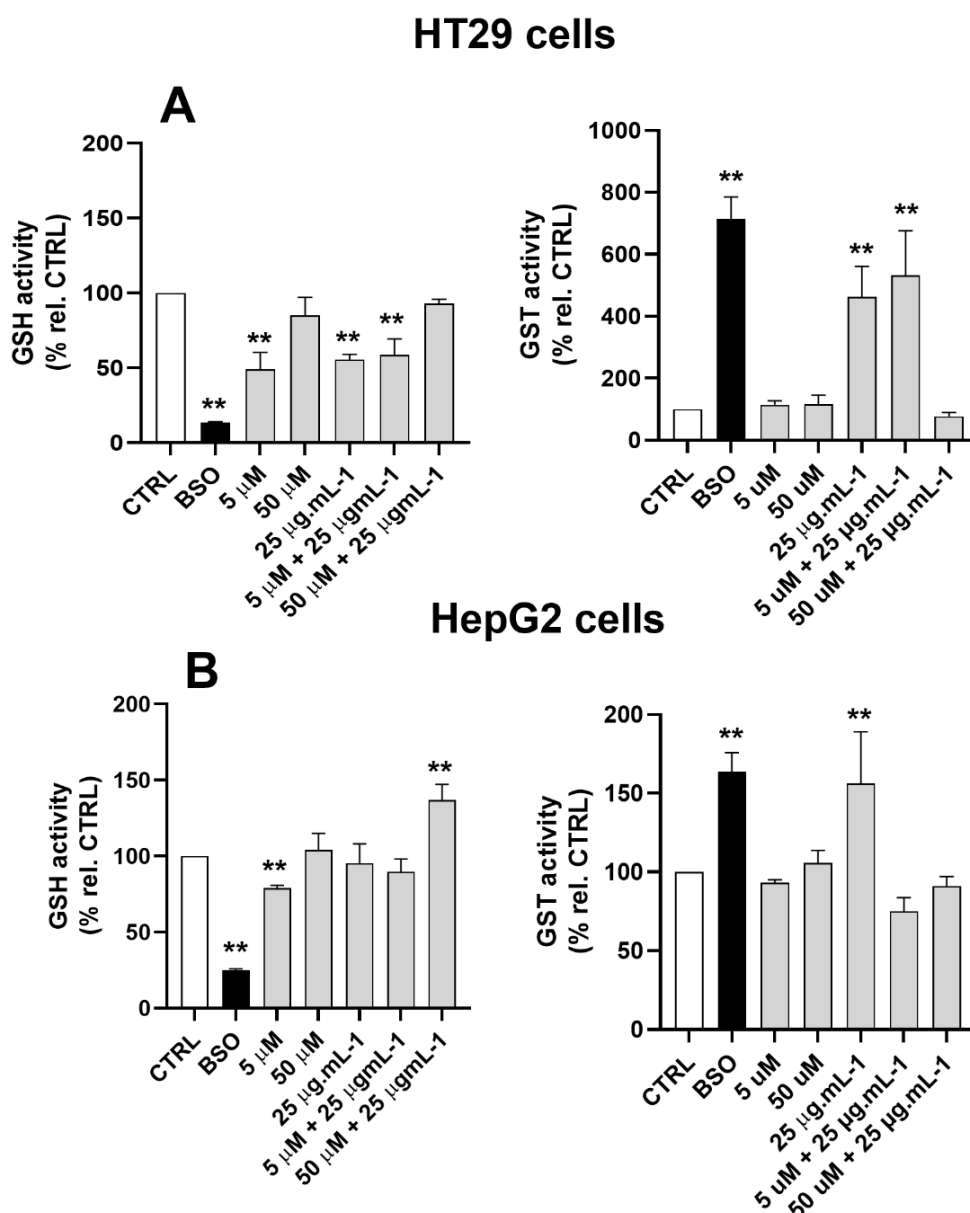


Figure 4. 5 – A) Total glutathione (GSH) and glutathione S-transferase (GST) expression values in HT29 cells and B) HepG2 cells exposed to polystyrene nanoplastics at 25 μ g.mL⁻¹, benzo[a]pyrene at 5 and 50 μ M and their combination for 24 hours. Results are presented by the mean $\pm$ SEM of three independent experiments. * and ** represent the significant differences with a $p < 0.05$ and < 0.01 from control, respectively (Turkey test).

4.3.5 GPx1 and GPx4 protein levels

Figure 4.6 presents GPx4 and GPx1 protein expression levels analysed by Western Blot in HT29 cells after exposure to PSNP and B[a]P. GPx4 protein levels showed a significant decrease in HT29 cells exposed to PSNP individual and combined with both concentrations of B[a]P (5 and 50 μM) ($p < 0,01$) (Figure 4.6A). No significant differences were observed in GPx1 protein levels ($p > 0,05$), except in HT29 cells exposed to the combination of PSNP with B[a]P at 50 μM ($p < 0,01$) (Figure 4.6B).

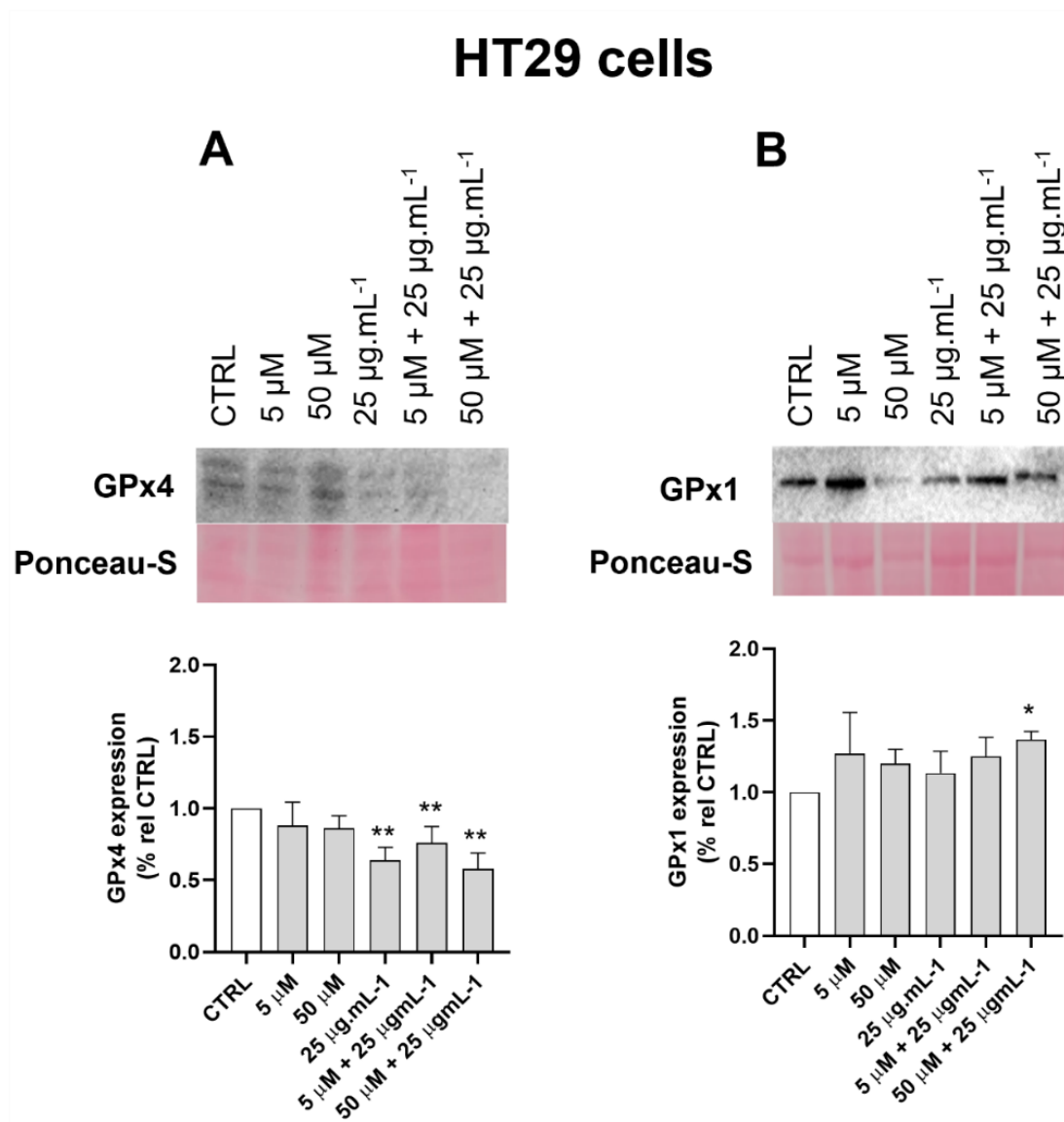


Figure 4. 6 - A) Glutathione peroxidase 4 (GPx4) and B) glutathione peroxidase 1 (GPx1) expression in HT29 cells exposed to polystyrene nanoplastics with 25 nm diameter at 25 $\mu\text{g.mL}^{-1}$ for 24 hours was determined by Western Blot. Results were normalized for protein loading using Ponceau-S staining. Values are the mean $\pm$ S.D of three independent experiments. * and ** represent the significant differences with a $p < 0.05$ and $p < 0.01$ from control, respectively (Turkey test).

Figure 4.7 presents GPx4 and GPx1 protein expression levels in HepG2 cells. While no significant levels of GPx4 were observed in HepG2 cells after exposure to PSNP and B[a]P ($p > 0,05$), there was a general tendency for a decrease in expression. Likewise, GPx1 protein levels showed a significant decrease in cells exposed to PSNP individual and combined with both concentrations of B[a]P (5 and 50 μM) ($p < 0,01$) (Figure 4.7B).

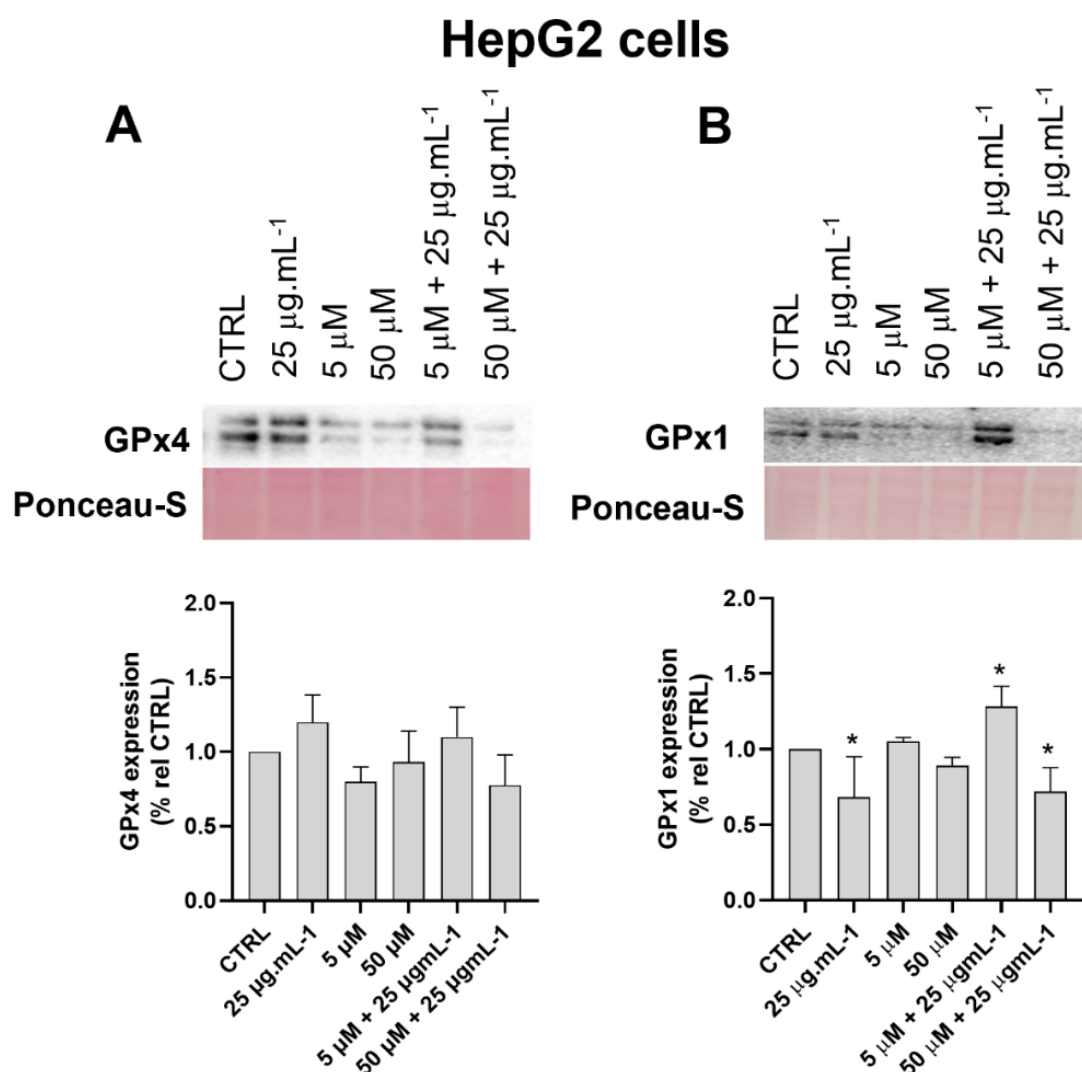


Figure 4. 7 - A) Glutathione peroxidase 4 (GPx4) and B) glutathione peroxidase 1 (GPx1) expression in HepG2 cells exposed to polystyrene nanoplastics with 25 nm diameter at 25 $\mu\text{g.mL}^{-1}$ for 24 hours was determined by Western Blot. Results were normalized for protein loading using Ponceau-S staining. Values are the mean $\pm$ S.D of three independent experiments. * and ** represent the significant differences with a $p < 0.05$ and < 0.01 from control, respectively (Tukey test).

4.4 Discussion

This study aimed to investigate the effects of PSNP and their combination with B[a]P in HT29 and HepG2 cells. Analyses of effects over cell viability and Phase I and Phase II endpoints were used to better understand the mechanisms of detoxification and antioxidant responses. Exposure to PSNP did not affect the viability of both HT29 and HepG2 cells, in line with previous observations in Chapter 3. Curiously, a significant increase in HepG2 cell viability was observed after 72 hours of exposure to PSNP, which could be a response associated to the activation of survival pathways, promoting cell growth, that normally occur under stress conditions as previously described in hepatocarcinoma cells (Tsai et al., 2023). The increase of HepG2 cell viability could also be influenced by the activation of inflammation pathways as a cytoprotective effect to counteract the stress caused by harmful stimuli (Sangaran et al., 2021). Although we did not analysed activation of these pathways here, we previously observed such activation taking place in HT29 cells exposed to PSNP (J. Antunes et al., 2023).

Nanoplastics can adsorb compounds such as B[a]P and serve as carriers that facilitate their entry into cells (Ji et al., 2021). Here we observed that, when in combination, B[a]P and PSNP seemed to enhance negative effects over cellular viability, particularly in HepG2 cells, but no clear time or concentration-related pattern could be noticed. Indeed, HepG2 cells decreased their viability after 48 hours and HT29 cells decreased after 72 hours. This could probably have occurred because the PSNP used in our study tended to agglomerate more when introduced into the culture medium of HT29 cells (PSNP $\varnothing \sim 29 \pm 2.99$ nm) compared to PSNP introduced in the medium of HepG2 that maintained their original size $\sim 25 \pm 2.18$ nm. This increased aggregation was likely to lead to the formation of larger-sized particles in HT29 medium, altering the dynamics of PSNP uptake (Åberg et al., 2021) and consequently, affect cellular responses. Additionally, the difference of cell viability after PSNP exposure could be due to the unique characteristics of each cell line, demonstrating that HT29 cells seem to be more resistant to PSNP effects than HepG2 cells. In other studies it was observed that HT29 cells exhibit higher level of resistance to the cytotoxic effects of gold nanoparticles compared to HepG2 cells (Pavlovich et al., 2017; B. Zhu et al., 2016), because HT29 cells continue to proliferate after exposure of AuNP, while HepG2 cells experience a significant decrease in viability under the same conditions.

The activation of antioxidant and detoxification mechanisms in the presence of PSNP

and in combination with B[a]P can vary depending on the cell type and concentration of B[a]P. The glutathione redox homeostasis system is crucial for the self-protection of cells against oxidative stress. Glutathione peroxidases (GPx) are a key antioxidant enzymes that catalyzes the reduction of H_2O_2 to H_2O by using glutathione (GSH) as a reductant (Ghasemi et al., 2021), while GST catalyze the conjugation of reactive electrophiles and products of oxidative stress with reduced GSH to facilitate elimination (Bessa et al., 2009).

In this study, the cellular response of HT29 cells to both PSNP alone and combined with the low concentration of B[a]P led to a decrease in both GSH and GPx4 levels and an increase in GST activity, indicating a change in cellular redox status already seen in other studies (Forman et al., 2009).

GPx4 is known to protect cells from lipid peroxidation, particularly in the context of ferroptosis, a form of cell death driven by iron-dependent lipid peroxide accumulation (Ran et al., 2007). The decrease in GPx4 levels in HT29 cells may consequently lead to an accumulation of lipid peroxides and increased oxidative stress (R. Yang et al., 2019), suggesting a reduction in the cell's ability to neutralize oxidative damage and protect itself against oxidative stress. PSMP had already been seen to decrease glutathione peroxidase, suggesting cellular stress, in mussels after of virgin PSNP (Magni et al., 2018). Also in bivalves, exposure to PSMP ($20\text{ }\mu\text{m}$, 1 mg.L^{-1}) resulted in an increase in GST activity after 7 days in gills (Ribeiro et al., 2017). Similarly, rats also showed alterations in antioxidant responses, including changes in total glutathione content after exposure to PSNP (Babaei et al., 2020).

Our findings suggest that PSNP may have a detrimental effect on HepG2 cells antioxidant capacity, as evidenced by a significant decrease in GPx1 levels. Additionally, the significant increase in GST levels further signifies potential cellular stress and metabolic perturbations induced by PSNP exposure. However, when PSNP were combined with $5\text{ }\mu\text{M}$ B[a]P there GPx1 expression was increased in HepG2, similar to HT29 cells exposed to PSNP combined with $50\text{ }\mu\text{M}$ B[a]P, indicating a potential protective response. Despite a significant decrease in GPx1 in HepG2 cells, there was a marked increase in GSH, suggesting an adaptative response to oxidative stress (Stolwijk et al., 2020) possibly mediated by the Nrf2 pathway (Branco et al., 2014).

CYP1A expression and/or EROD activity were activated by B[a]P exposure in HT29 and HepG2 cells (Figure 3 A and B), as expected. In HepG2 cells, CYP1A1 activity was increased following exposure to PSNP alone and in combination with B[a]P, suggesting the activation of the aryl hydrocarbon receptor (AhR). HepG2 cells, being liver cells are expected to have an higher activation of AhR signalling due to the liver's role in detoxification and metabolism of xenobiotics (Sturgill & Lambert, 1997). CYP1A1 is a key enzyme responsible in the metabolism of B[a]P converting it into reactive epoxides, such as B[a]P-7,8-epoxide ((Nakamura et al., 2005)). The activation of the CYP1A1 is mediated by the binding of B[a]P to the aryl hydrocarbon receptor (AhR), leading to the translocation of AhR to the nucleus, where it forms a dimer that interacts with xenobiotic response elements to activate transcription and increased expression of CYP1A1 gene. This leads to an upregulation of the CYP1A1 enzyme, thereby increasing its availability to metabolize B[a]P and other substrates (Androutsopoulos et al., 2009a). The upregulation of CYP1A1 in HepG2 cells suggests that the combination of PSNP and B[a]P has probably contributed to the formation of reactive B[a]P epoxides. This phenomenon may explain why we observed an upregulation of GSH and GST levels in HepG2 cells following the combination treatment, since this upregulation could enhance the cells' capacity to conjugate the epoxides formed. The opposite occurred in HT29 cells that showed a decrease in GSH when exposed to B[a]P, although EROD activity, a proxy for CYP1A1 activity, showed increased activity. This downregulation could probably increase the toxic effects of B[a]P in this cells.

4.5 Conclusions

In conclusion, our study sheds light on the intricate cellular responses triggered by exposure to PSNP alone and in combination with B[a]P in HT29 and HepG2 cells. The findings underscore the differential effects of PSNP on cell viability and antioxidant responses between the two cell lines, suggesting varying sensitivities and mechanisms of action.

Notably, while PSNP alone did not significantly impact HT29 cell viability, it significantly increased HepG2 cell viability, possibly through activation of survival pathways and inflammation responses. Furthermore, the synergistic cytotoxic effects observed with the combination of PSNP and B[a]P highlight the potential for enhanced cellular uptake and toxicity promoted by NP.

This study also elucidates the role of antioxidant enzymes such as GPx4 and GPx1 in mitigating oxidative stress induced by PSNP exposure, suggesting that PSNP individually and in combination with B[a]P led to an accumulation of lipid peroxides and increased oxidative stress in HT29 cells. HepG2 cells seem to have a more effective antioxidant response to B[a]P in combination with PSNP, however PSNP individual, similar to HT29, also perturbs antioxidant capacity in these cells.

The observed upregulation of CYP1A1 activity in HepG2 cells underscores their response to the presence of PSNP and B[a]P, possibly via AhR activation. Therefore, the potential for adverse effects cannot be overlooked, since CYP1A1 activation conjugated with a diminished Phase II capacity could enhance toxic effects. Indeed, concurrent with CYP1A1 induction, effects such as genotoxicity may arise, as further discussed in the subsequent chapter.

Future investigations should consider optimizing cell culture conditions to enhance the expression and activities of antioxidant enzymes for more accurate assessments. Namely, it would be of interests to investigate the transcriptional pathways linked to Phase I (AhR) and Phase II (Nrf2) metabolism. Overall, our findings contribute to a deeper understanding of the complex cellular responses to nanoplastic exposure and highlight the importance of considering cell type-specific effects in assessing nanoparticle toxicity.

Funding

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Author contribution

Joana Antunes: Writing – original draft, Formal analysis, Investigation. Paula Sobral: Resources, Writing – review & editing, Funding acquisition. Marta Martins: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. Vasco Branco: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition

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CHAPTER 5:

Inflammatory-gene expression and DNA damage effects in zebrafish adults after nanoplastic and benzo[a]pyrene exposure

This Chapter is a manuscript in preparation for submission:

Antunes J, Bramatti I, Sobral P, Branco V, Martins M, (2024). Inflammatory-gene expression and DNA damage in zebrafish after exposure to polystyrene nanoplastics and benzo[a]pyrene

Abstract

Nanoplastics (NP) and benzo[a]pyrene (B[a]P) represent emerging environmental pollutants that have attracted significant attention due to their potential to accumulate in several environmental compartments and to cause adverse effects on ecosystems. NP, characterized by their small size (< 100 nm) and high surface-to-volume ratio, pose unique challenges in terms of dispersion, transport, and biological impact. On the other hand, B[a]P, a carcinogenic polycyclic aromatic hydrocarbon (PAH), is a well-known ubiquitous and persistent environmental contaminant resulting from incomplete combustion of organic matter, such as fossil fuels and tobacco. The present study examined the toxicological effects of individual and combined sub-acute exposure to NP and B[a]P, in zebrafish adults (*Danio rerio*). Four treatments were considered: a) Control; b) $500 \mu\text{g.mL}^{-1}$ polystyrene nanoparticles (PSNP) with $\varnothing 25$ nm; c) 25 nM of B[a]P; and d) a combination of both contaminants. The exposures of PSNP and B[a]P were performed via diet using *Daphnia magna* and via water, respectively. After 28 days of exposure, DNA damage was analysed in fish blood cells through the Comet assay, and the changes in transcript levels of genes associated with inflammation, detoxification and tumour suppression were assessed in the fish liver and intestine through RT-PCR analysis. Results showed that all treatments significantly increase DNA strand-breaks comparing to control, most notably in the mixture of PSNP and B[a]P (49 ± 16.8). Transcriptomic analysis of IL1 β , CYP1A, RAB1A and TP53 genes revealed variability depending on the organs analysed. However, significant increase in transcript expression was observed following B[a]P exposure for all genes and, also for the mixture in TP53 gene expression. In conclusion, the combination of PSNP with B[a]P can pose higher genotoxic effects than the exposure of nanoplastics itself, but their combination decreased the gene expression response comparing with the exposure to the B[a]P individually, with the exception of TP53.

Keywords

Nanoplastics; benzo[a]pyrene; zebrafish; inflammation; genotoxicity

5.1 Introduction

A nanomaterial is a particle with a diameter between 1 to 100 nm (EUROPEAN COMMISSION, 2022), including nanoplastics, that may pose serious hazards to aquatic ecosystems and marine life. Although bigger plastic particles are known to be present in several environmental matrices, namely in lakes, rivers, coastal and open oceans, deep-sea sediments and wastewater effluents (Eerkes-Medrano et al., 2015), there is still scarce information regarding environmental concentrations of NP, namely due to the lack of standardized analytical methodologies. Nevertheless, it is expected that NP are found in high concentrations in the environment, namely because many results from the fragmentation and degradation of microplastics which are abundant in the environment (Bundschuh et al., 2018).

The hazards associated with NP are intrinsically linked to its nanoscale dimensions, facilitating their interaction with cell surface receptors (Lehner et al., 2019b). The influence of NP size on their behaviour and toxicological properties complicates the straightforward extrapolation of effects from MP to NP. This underscores the necessity of prioritizing research on NP toxicity, emphasizing its significance in both ecological and human health (Lehner et al., 2019). In recent years, experimental studies using model PSNP have emphasized that several organisms such as algae, zooplankton, daphnia, mussels, and fish can actively ingest NP or adsorb them to their surfaces (Ward & Kach, 2009). NP ingestion can alter animal behaviour and interfere with the immune system, causing brain damage, altering gene expression, and affecting DNA integrity already seen in fish (Y. Jin et al., 2018; Mattsson et al., 2017b). Some *in vivo* studies have detected effects of NP exposure in zebrafish guts (Y. Jin et al., 2018), namely the increase in the secretion of mucin and the induction of inflammation. However, the mechanisms underlying an inflammatory response at the gastrointestinal level and the implications of chronic exposure remain unclear. On a mechanistic level, we have shown in HT29 cells that exposure to NP can activate pro-inflammatory cytokine production by a TLR4 receptor-mediated mechanism (J. Antunes et al., 2023). Thus, contact between NP and gastrointestinal tract arises as a relevant route to the development of toxicity. Moreover, the microbiome is also reportedly affected by NP leading to alterations in its composition, which may contribute to enhanced inflammation (Gu et al., 2020b). This is a particularly significant event since inflammation of the gut is largely associated with the triggering of neoplastic events

(Rakoff-Nahoum, 2006). This link is important since plastics can accumulate and adsorb environmental contaminants with genotoxic potential, such as PAH. In fact, according to some studies, the interaction of PAH with NP changes both the absorption, distribution, metabolism, and excretion (ADME) and the molecular interactions of environmental contaminants, leading to altered toxicity and bioaccumulation (Atugoda et al., 2023). Given that direct ingestion and trophic transfer have been recognized as the predominant pathways of exposure to NP (Nelms et al., 2018), the effects of NP and their combination with environmental contaminants demands urgent and comprehensive investigation. This is an essential aspect to investigate to assess NP toxicity and improve the basis of risk assessment. Overall, we hypothesize that PSNP and a carcinogenic PAH, B[a]P, exposed individually may activate inflammatory pathways and cause genotoxic effects.

5.2 Material and Methods

5.2.1 *In vivo* assays

5.2.1.1 Test organisms and acclimatization

Two biological models were used in this study: the crustacean *Daphnia magna* (*D. magna*) as a vehicle of PSNP during exposure and . the fish *Danio rerio* (*D. rerio*) as a model to assess the biological responses to contaminant exposure. *D. magna* specimens were acquired from Aquário Vasco da Gama (Portugal) and were transported to the MARlab laboratory (NOVA FCT, Portugal) acclimatized for one week in a closed system with de-chlorinated tap water, at a pH between 7.5 and 8.2, temperature of $25 \pm 1^\circ\text{C}$, photoperiod: 12hL:12hD and with continuous aeration enough for keeping the dissolved oxygen above 6 mg.L^{-1} . Daphnids were fed every two days with a mixture of *Chlorella* (Iswari, Portugal) and blood gnat (Ocean Nutrition, Europe).

Specimens of *D. rerio* were acquired from Aquaplante (Portugal). Before the beginning of the assay *D. rerio* were acclimatized for one week in a closed circuit system with filtered de-chlorinated tap water, at a pH of 7.9 ± 0.1 , temperature of $25 \pm 1^\circ\text{C}$, photoperiod: 12hL:12hD, with continuous aeration, enough for keeping the dissolved oxygen above 6 mg.L^{-1} . Fish were fed with daphnia. The physical and chemical parameters were checked using an ammonia test kit (API, USA), a dissolved O_2 and pH meter (Hanna Instrumentation, USA), and a manual thermometer. Rigorous procedures were implemented to minimize stress and ensure the well-being of the organisms before their utilization in the subsequent exposure tests. These practices aim to establish a robust experimental foundation, enhancing the reliability and validity of the results obtained from the study.

5.2.2 Determining the optimal concentration of nanoplastics uptake by *D. magna* to use as carrier for zebrafish exposure

These experiments were designed to optimize the exposure time of *D. magna* to PSNP, with the dual objectives of maximizing PSNP accumulation within the gut while minimizing mortality rates. After the acclimatization period, 5 individual *D. magna* were

placed into a 6-well plate (1 per well) and exposed to 500 $\mu\text{g}.\text{mL}^{-1}$ of PSNP added to water. Three replicates of the bioassay were considered. *Daphnia* were sampled at five different time points (30 minutes, 1 hour, 2 hours, 24 hours, 48 hours, and 72 hours). Test conditions were carefully regulated, with water temperature *maintained at* $25 \pm 1^\circ\text{C}$. During the exposure period, the organisms were not provided with food to ensure an accurate assessment of PSNP accumulation in the gut. Furthermore, a balanced light-dark cycle of 12 hours each (12hL:12hD) was implemented to mimic natural environmental conditions. After each time point, the mortality of *D. magna* was recorded. Swimming capacity was also recorded and individuals that were not able to swim were deemed unfit and, for the purpose of this analysis, considered as “dead”. Controls containing only filtered de-chlorinated tap water without NP were included in the experiment. For the observation of the presence of PSNP, microscopy analyses were done using a Leica DMLB microscope equipped with an EL2000 light source, equipped with an I3 filter. The intensity of fluorescence in *D. magna* was quantified using ImageJ software (ImageJ, Bethesda, Maryland, USA) and the final fluorescence concentration was calculated from a calibration curve ($0 - 5000 \mu\text{g}.\text{mL}^{-1}$) prepared with sequential dilutions of PSNP stock solution. For this purpose, calibration standards were placed in capillary tubes (AQCAPS melting point capillaries, Spectrum, India) and fluorescence intensity measured as for *Daphnia*.

5.2.3 Zebrafish exposure to nanoplastics via *D. magna* ingestion and benzo[a]pyrene through water

After one week of acclimatization, *D. rerio* were randomly distributed into 2 liters volume glass beakers (three fishes per beaker) containing filtered de-chlorinated tap water, with controlled temperature ($26.3 \pm 0.5^\circ\text{C}$), pH of 7.98 ± 0.14 , ammonia = $0.6 \pm 0.2 \text{ mgNH}_4.\text{L}^{-1}$ and photoperiod (12 h light:12 h dark) under continuous aeration. Zebrafish were randomly assigned to four experimental treatments: i) Control (CTRL), a control beaker containing filtered and de-chlorinated tap water; ii) PSNP exposure through feeding with *D. magna* previously contaminated with PSNP ($\varnothing$ 25 nm at 500 $\mu\text{g}.\text{mL}^{-1}$); iii) B[a]P at 25 nM; iv) and the combination of PSNP and B[a]P. (Figure 5.1). Each treatment condition was tested in quadruplicate, with four independent replicates per treatment. The experimental duration was 28 days (T_{28}). To ensure constancy of the water parameters and contamination, 75% of the total water volume was replaced

every 48 hours with a freshly prepared exposure medium. Every 24 hours of exposure, fish were fed with contaminated daphnia and the B[a]P was spiked in water. Control animals were fed with daphnia with no PSNP contamination.

All experiments conducted in the present research were carried out in strict accordance with the recommendations of the Portuguese and European Union legislation for animal experimentation following the approval of the National Directorate for Veterinary (DGVA) and according to OECD guidelines (European Union, 2010).

Sampling of fish ($n = 22$) took place at 0 (T_0) and 28 (T_{28}) days of exposure. Morphometric measurements of individuals were analysed, namely total weight, size, liver and intestine weight in both time points. Blood was collected from the caudal artery (above the lateral line system) and processed immediately for genotoxicity assessment ($n \geq 3$). Animals were euthanized by cervical sectioning, and dissected for tissue collection (liver, and intestine). Organs were excised and RNA was extracted with NZY Total RNA isolation kit (NZYtech) for RT-PCR analyses.

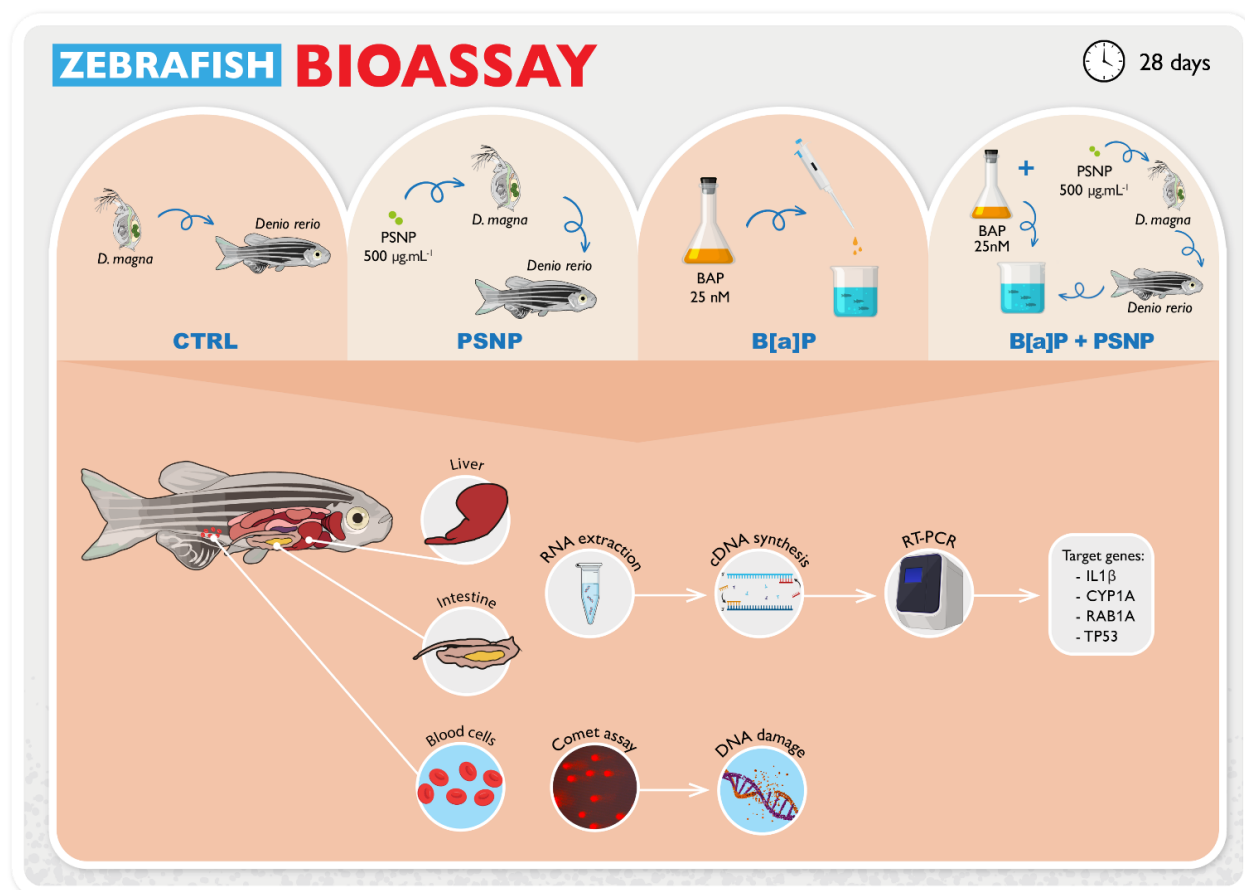


Figure 5. 1 - Schematic representation of the in vivo bioassay with zebrafish (*Danio rerio*). Each experimental treatment consisted of four replicate beakers, with three zebrafish per beaker. In the control group, fish were acclimated in filtered, dechlorinated tap water and fed with uncontaminated *Daphnia*. For the PSNP treatment, fish were fed every 48 hours with *Daphnia* contaminated with PSNP (500 µg.mL⁻¹). The B[a]P treatment involved exposure to water containing B[a]P (25 nM), with fish receiving uncontaminated *Daphnia*. In the combined PSNP and B[a]P treatment, fish were exposed to B[a]P-contaminated water (25 nM) and fed on an alternating schedule every 48 hours, switching between uncontaminated *Daphnia* and PSNP-contaminated *Daphnia* (500 µg.mL⁻¹).

5.2.4 DNA damage – Comet assay

Genotoxicity assessment was assessed by the alkaline version of the single-cell gel electrophoresis (Comet) assay. Blood was collected just above the lateral line system with a syringe previously washed with 0.1 M EDTA to prevent clotting (n = 21; n ≥ 4). Blood aliquots were immediately diluted in cold 50 mM PBS (phosphate-buffered saline) with 0.7% NaCl, pH 7.4 (dilution factor: 200) for the Comet assay, which was performed right after blood collection to ensure maximum cell viability. The alkaline Comet assays were performed as described earlier (Singh et al., 1988) and adapted

for fish erythrocytes (Costa et al., 2008). The Comet assay was performed with 10 μ L of cell suspension in PBS diluted in 180 μ L of liquid (35–40 °C) 1% (w/v) low melting-point agarose (LMPA; Sigma) in PBS. Aliquots (2 $\times$ 75 μ L) of cell-containing LMPA were placed on slides pre-coated with 1% (w/v) normal melting-point agarose in TAE buffer (dried for at least 24 h) and covered with a coverslip.

After agarose solidification (15 min, 4 °C) the coverslip was removed and the slides were dipped for 1 h at 4 °C in lysis solution (2.64% NaCl (w/v), 3.72% EDTA (w/v) and 5 mM TRIS) to which was added 10% (v/v) DMSO and 1% (v/v) Triton-X 100 just before use. Slides were afterwards placed in cold (4 °C) electrophoresis solution (pH=13) for 40 min to allow DNA-unwinding and enhanced expression of alkali-labile sites. Electrophoresis was conducted for 30 min at 25 V, in the cold (4 °C), using a Sub-Cell model 96 apparatus (Bio-Rad). Slides were afterwards neutralized in 0.1 M Tris-HCl buffer (pH 7.5) for 15 min. All preparatory steps were performed under controlled temperature ($\approx$ 16 °C) to avoid gel lifting from the slides and all solutions and electrophoresis apparatus were kept in the dark and in the cold to minimize accessory DNA degradation.

Approximately 100 comets were analysed per slide after staining with 0.02 mg.mL⁻¹ ethidium bromide. Comets were analysed using the software CometScore 1.5 (TriTek Corp., Summerduck, USA). The percentage DNA in the tail was employed as a direct measure of DNA strand breakage (DNA-SB) (Lee et al., 2003). Results are expressed in average percentage of total DNA-SB. A Leica DMLB microscope equipped with an EL2000 light source, equipped with a N2.1 filter (used for ethidium-bromide staining) was used for all microscopy analyses.

5.2.5 Gene expression using quantitative real-time reverse transcriptase PCR

RNA extraction from liver and intestine was performed using an NZY Total RNA Isolation kit (NZYTech, Portugal) according to manufacturer instructions. Total RNA concentration was determined from spectrophotometric optical density measurements (260 and 280 nm). For each sample tested, the ratio between the spectrophotometric readings at 260 nm and 280 nm (OD₂₆₀/OD₂₈₀) was used to evaluate RNA purity, with the ratio ranging between 1.83 and 2.05 for all samples. cDNA was synthesized using

a NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal) using 1 µg of RNA as template. Gene expression quantification was performed using NZY qPCR Green Master Mix (NZYTech, Portugal) with 400 nM forward primer and 400 nM reverse primer and 100 ng of template cDNA in a 10 µL reaction volume. All samples were run in triplicate and the output level was reported as the average of the 3 measurements. Amplification conditions included 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles at 95 °C for 15 s and 60 °C for 1 min on a Applied Biosystems QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

The analysis focused on the following genes: pro-inflammatory cytokine IL-1β; the phase I metabolism CYP1A; neoplasia development, RAB1A; tumour suppressor TP53. After RT-PCR gene levels were calculated by the relative quantification $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), using REL13 as the housekeeping gene.

The primer sequences for zebrafish IL-1β, CYP1A, RAB1A, TP53 and REL13 (Table 5.1) were designed with Primer-Blast and synthesis was performed by StabVida (<https://www.stabvida.com>).

Table 5. 1 - Primer sequences for zebrafish IL-1 β , CYP1A, RAB1A, TP53 and REL13 designed with Primer-Blast and synthesis was performed by StabVida (<https://www.stabvida.com>).

Primers		Forward primer (5'->3')	Reverse Primer (5'->3')
Interleukin 1 Beta	IL1 β	5-GTGGACTTCGCAGCACAAAA-3	5-CACGTTCACTTCACGCTCTTG-3
Cytochrome P450	CYP1A	5-CCTGGGCGGTTGTCTATATA-3	5-TGAGGAATGGTGAAGGGAAG-3
Ras oncogene protein	RAB1A	G-CGATGAATCCCGAATATGAC-3	5-GATTGTGCGAAACCTCTCC-3
Tumor protein p53	TP53	5-ATCATCTGAGCCCAAACAGG-3	5-AAATGACCCCTGTGACAAGC-3
Ribosomal protein L13 a	RPL13A	5-TCCCAGCTG CTCTCAAGATT-3	5-TTC TTGGAATAGCGCAGCTT-3

5.2.6 Statistical analysis

Statistical analyses were conducted using GraphPad Prism software (version 10.0.2, GraphPad Software, Inc., La Jolla, CA, USA). Data normality was evaluated by the Shapiro-Wilks test and variance homogeneity using Spearman's test for heteroscedasticity. The significance of main and interaction effects was evaluated by a Two-way ANOVA, followed by post-hoc assessment with a Tukey test to evaluate differences between exposure groups. When appropriate, data were log-transformed to comply with assumptions of parametric tests. Differences between groups were classified as significant at $p < 0.05$ and considered very significant at $p < 0.01$.

5.3 Results

5.3.1 Nanoplastics characterization

The analyses of the scanning electron microscopy (SEM) images reveal that PSNP present a spherical shape (Figure 5.2A). The nature of the polymer, polystyrene was confirmed by the spectrum acquired through RAMAN spectrometry (Labram 300 Jobin Yvon spectrometer) (Figure 5.2B) (Brun et al., 2013). The acquired spectrum revealed distinctive absorption peaks associated with polystyrene, including bending the prominent peaks in the Raman spectrum of the polystyrene polymer corresponding to the ring deformation mode ($623,12\text{ cm}^{-1}$), ring breathing mode ($1002,92\text{ cm}^{-1}$), C–C stretches (1156.92 and 1184 cm^{-1}), CH_2 scissoring (1455.92 cm^{-1}), and ring-skeletal stretch (1604.30 cm^{-1}) (Itoh and Hanari, 2021). The average size and zeta potential of PSNP diluted in PBS was $25.5 \pm 1.5\text{ nm}$ and the zeta potential was $-26.3 \pm 1.8\text{ nm}$.

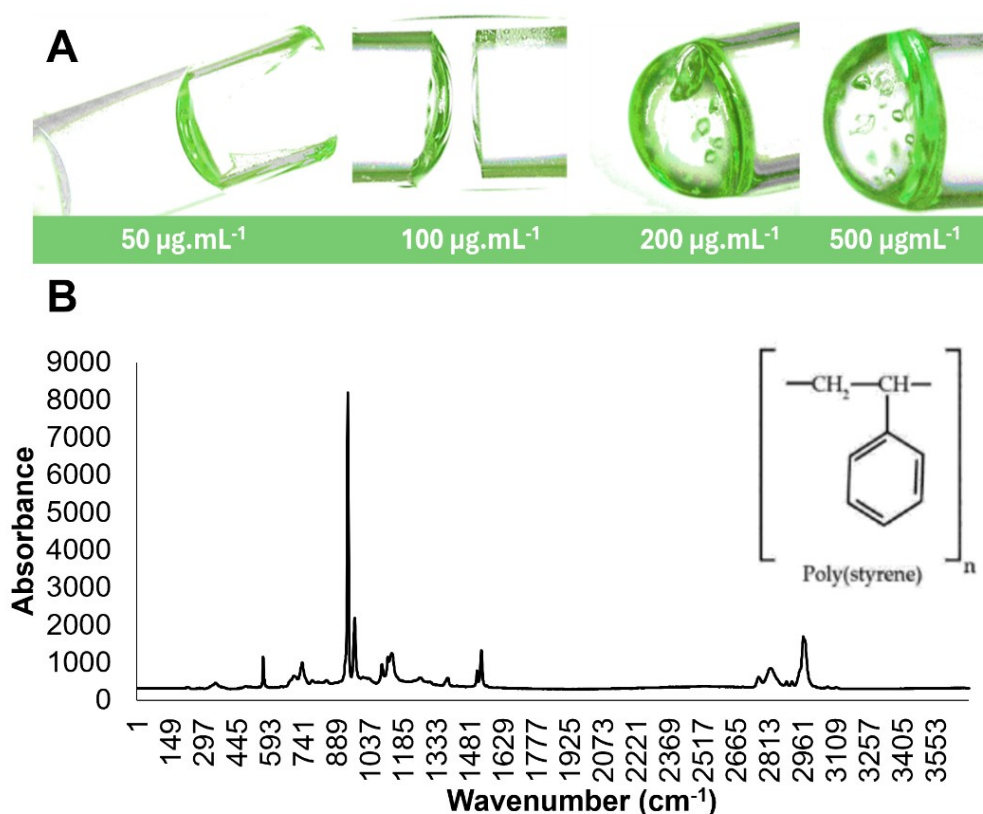


Figure 5. 2 - A) Capillary tubes for fluorescence intensity measurement: Capillary with polystyrene nanoplastics acquired with a Leica DMLB microscope equipped with an EL2000 light source, equipped with an I3 filter with 4x objective. B) Polystyrene nanoplastics (PSNP) characterization: polystyrene spectrum acquired through a Labram 300 Jobin Yvon RAMAN spectrometer.

5.3.2 Optimization of *D. magna* exposure to polystyrene nanoplastics

The concentration of PSNP fluorescence and mortality of *D. magna* exposed to PSNP is presented in Figure 5.3. PSNP fluorescence was observed in all individuals analysed (except control animals, as expected). The concentration of PSNP in the daphnia increased with the exposure time.

After 30 minutes of exposure, *D. magna* exhibited fluorescence in their bodies ($220 \pm 31.4 \mu\text{g.mL}^{-1}$). Notably, the observed fluorescence was the result of PSNP accumulated externally rather than through ingestion. No fluorescence was detected inside the organisms after 1 and 2 hours of exposure (data not shown).

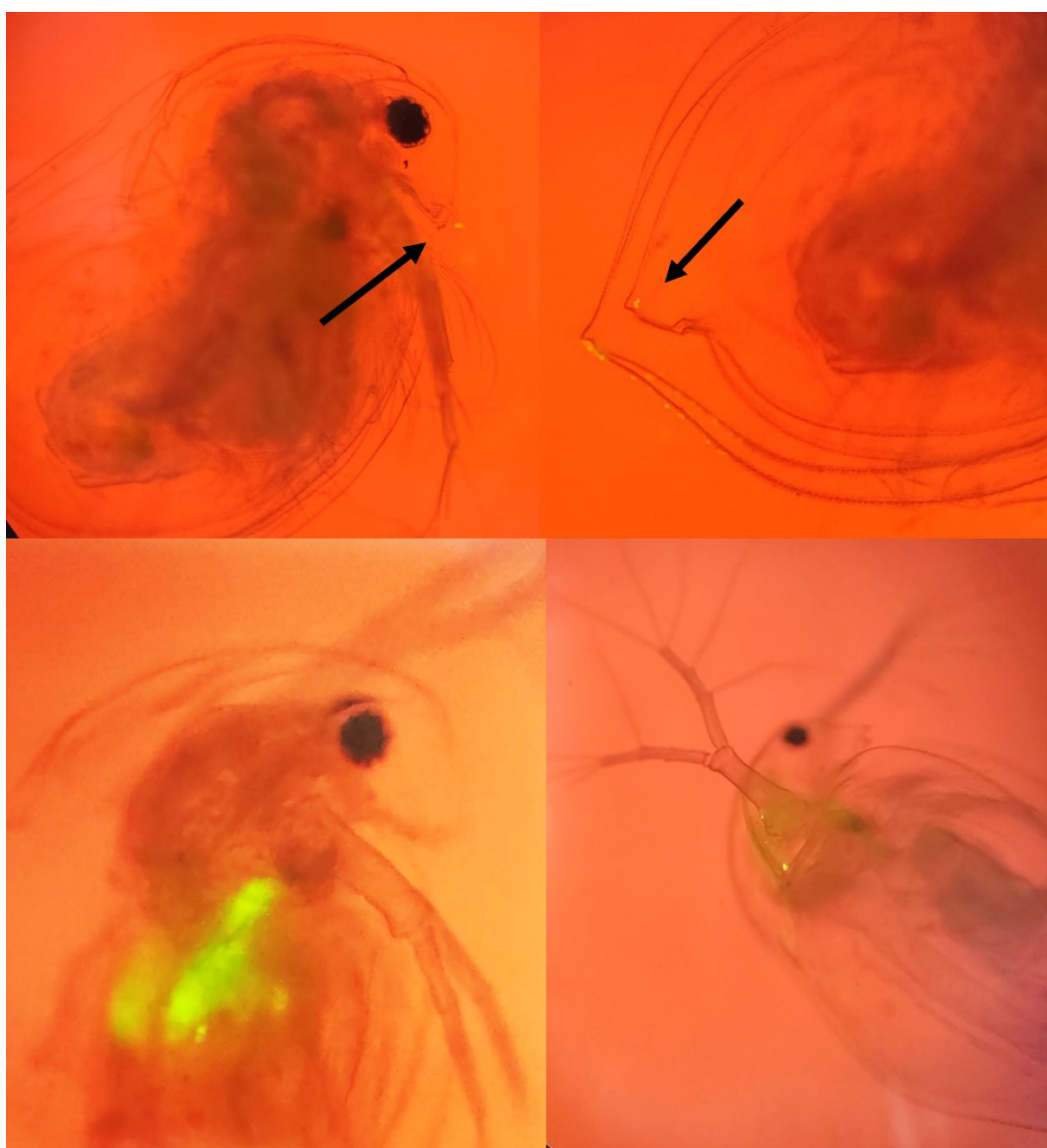


Figure 5. 3 - Polystyrene nanoplastics agglomerated and adhered to the carapace of a *D. magna*.

After 24 hours, fluorescence was observed inside the intestine and in the head with an average of $406 \pm 16.3 \mu\text{g.mL}^{-1}$ PSNP. After 48 and 72 hours, *D. magna* showed the highest fluorescence of PSNP accumulated in their bodies, showing $1245 \pm 43.6 \mu\text{g.mL}^{-1}$ and $1449 \pm 36.5 \mu\text{g.mL}^{-1}$, respectively. After 72 hours, *D. magna* showed 100% of mortality. After 24 hours, the gastrointestinal tract of *D. magna* was notably filled with PSNP, as depicted in Figure 5.4. As the average concentration reached approximately $500 \mu\text{g.mL}^{-1}$, this pivotal observation prompted the selection of this specific time point for the zebrafish feeding in the subsequent bioassay.

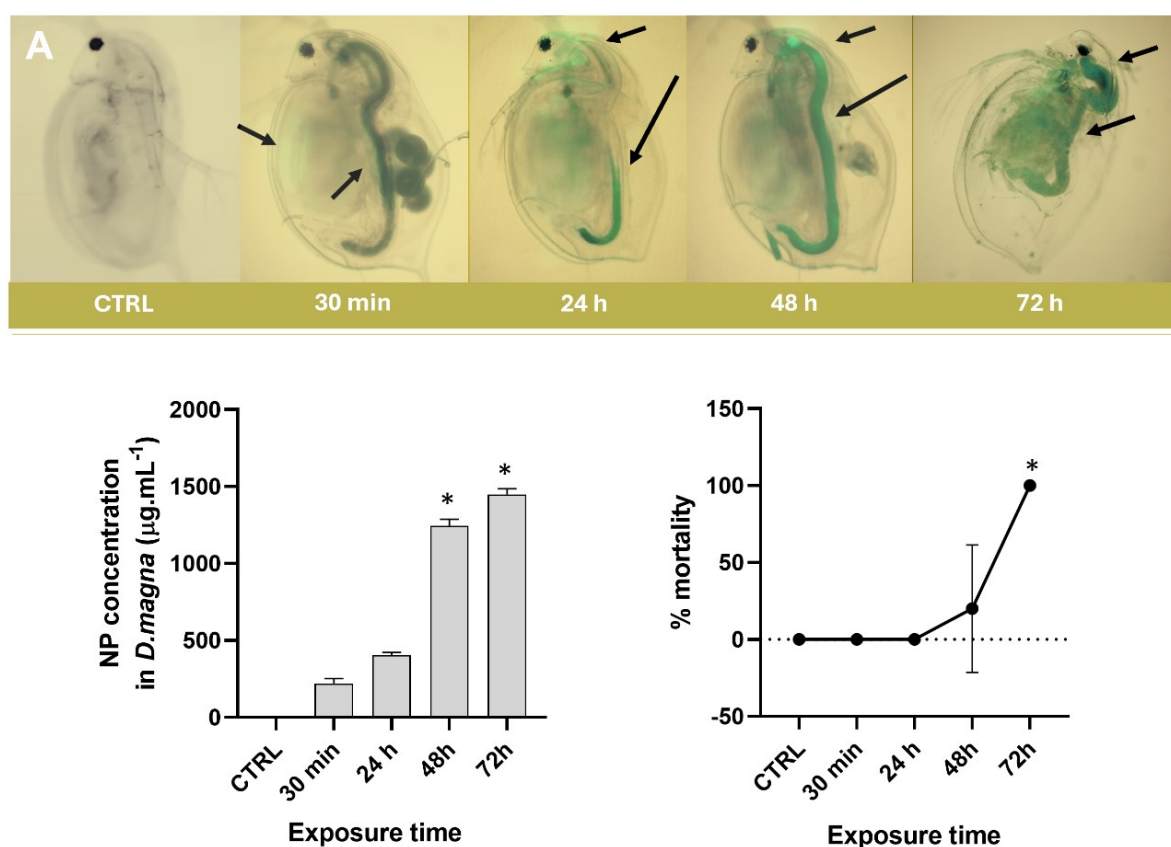


Figure 5. 4 - A) Fluorescence of polystyrene nanoplastics in *D. magna* after 30 minutes, 24, 48 and 72 hours acquired with a Leica DMLB microscope equipped with an EL2000 light source, equipped with an I3 filter with 10x objective. B) Total concentration of fluorescence PSNP ingested by *D.magna* after 30 minutes, 24, 48 and 72 hours of exposure calculated using ImageJ software and determined using the calibration curve. C) % of *D. magna* mortality after PSNP exposure. Presented data are the average and standard deviation of five replicates in each exposure time. * indicates the significant differences ($p < 0.05$) from the control (ANOVA with posthoc Tukey's test).

5.3.3 Zebrafish overall health status

Morphometric measurements of individuals analysed in the *in vivo* bioassay are presented in Table 5.2. Total weight of fish from T₀ was significant higher compared to fish from T₂₈ ($p < 0.01$) for all treatments. However, no significant differences were observed between fish at T₂₈ ($p > 0.05$). No significant differences were observed regarding size of the fish from T₀ and T₂₈, neither between fish from T₂₈ ($p > 0.05$).

Fish liver weight of fish at T₂₈ exposed to PSNP showed significant low values compared to fish from T₀ ($p < 0.05$). The liver weight of fish exposed to B[a]P after 28 days was significantly higher from the control ($p < 0.05$). Also liver weight average from fish exposed to PSNP showed significantly lower values compared to the combination of PSNP with B[a]P ($p < 0.05$) both exposed during 28 days. No significant differences were observed in fish intestine between T₀ and T₂₈ fish. However, significantly higher values were observed in fish exposed to PSNP compared to the control ($p < 0.05$), when exposed to 28 days.

The hepatosomatic index, indicating the weight of the liver as a percentage of body weight, presented a maximum average in fish exposed to B[a]P ($10 \pm 2.4\%$) showing significant higher values compared to the fish at T₀ and the control of the T₂₈ ($p < 0.01$). In T₂₈, fish exposed to the combination of PSNP with B[a]P showed higher values comparatively to fish exposed only to PSNP ($p < 0.01$). Also significant differences were observed in fish exposed to B[a]P (10.1 ± 2.4) and to PSNP (1.6 ± 0.4) ($p < 0.01$).

Table 5. 2 - Weight (mg) and total length (cm) of zebrafish at T₀ and T₂₈ , along with organ-specific weights (liver, intestine), subjected to various treatments: control (CTRL); fish fed with *D. magna* contaminated with 25 nm NP at 500 µg.mL⁻¹ every 48 hours (NP), fish exposed to water containing benzo[a]pyrene at 25 nM (B[a]P), and fish exposed to a mixture of both contaminants (B[a]P +NP). Hepatosomatic index was calculated. * indicates the significant differences (p < 0.05) from the control; \$ indicates significant differences from the T₀ and # indicates significant differences from exposure with individual PSNP (ANOVA with posthoc Tukey's test).

	liver (mg)	intestine (mg)	total weight (mg)	total lenght (cm)	hepatossomatic index (HSI)
T ₀	2.0 ± 0.9	8.8 ±2.4	36.6 ± 5.2	2.8 ± 0.1	4.4 ± 1.0
CTRL	1.2 ± 0.4	5.7 ±1.0	21.6 ± 3.8 (\$)	3.1 ± 0.4	5.3 ± 1.4
B[A]P	2.6 ± 0.7 (*)	7.4 ±1.9	25.1 ±4.5 (\$)	2.7 ± 0.2	10.1 ± 2.4 (*, \$)
PSNP	0.5 ± 0.1 (\$)	10.2 ±3.1(*)	25.7±4.3 (\$)	2.8 ± 0.5	1.6 ± 0.4
B[A]P+PSNP	2.1 ± 0.2 (#)	5.7 ±1.4	25.8±2.6 (\$)	2.7 ± 0.2	8.2 ± 1.6 (#)

5.3.4 Genotoxicity

The DNA-SB (% DNA in tail) in control animals was 28 ± 14.8 %. No significant differences were observed between T_0 and the control after 28 days ($p > 0.05$). In zebrafish exposed to PSNP and their combination with B[a]P exposure led to a significant increase in the levels of DNA-SB compared to the control ($p < 0.05$ and $p < 0.01$, respectively). Notably, PSNP exposure exhibited an average of 43 ± 13.5 % DNA in tail and their combination with B[a]P showed an average of 49 ± 16.8 % DNA in the tail (Figure 5.5). Fish exposed to B[a]P exhibited similar DNA-SB (41 ± 15.6 %) levels with other treatments and higher levels compared to controls (although not statistically different).

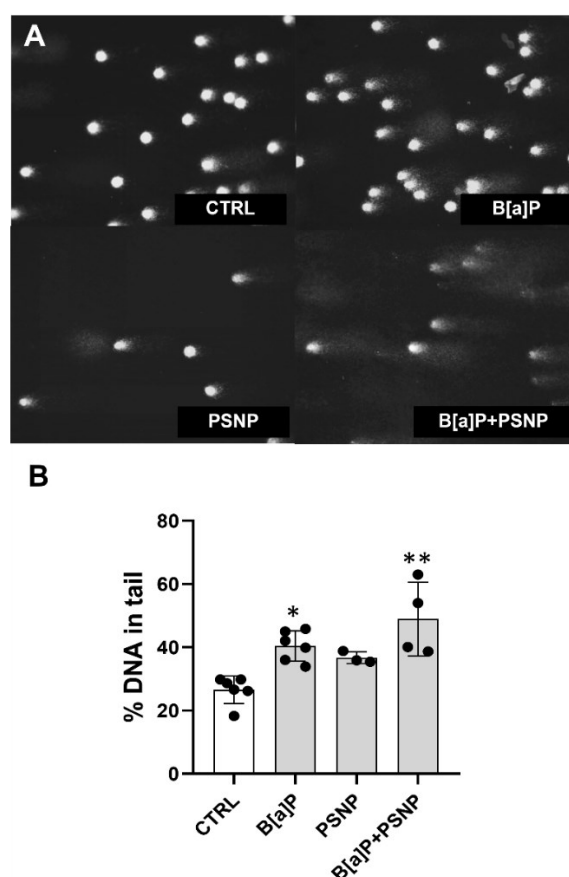


Figure 5. 5 - DNA damage (% of DNA in tail) assessed by the comet assay in zebrafish blood cells after exposure to nanoplastics and benzo[a]pyrene: A) representative comet fields from each tested condition, 200x magnification: control; zebrafish blood cells exposed to 25 nM benzo[a]pyrene; zebrafish blood cells exposed to polystyrene nanoplastics at 500 $\mu\text{g}.\text{ml}^{-1}$ with 25 nm diameter and mixture of benzo[a]pyrene at 25 nM and polystyrene nanoplastics at 500 $\mu\text{g}.\text{ml}^{-1}$. B) Percentage of DNA in tail expressed as means and standard deviation with replicates presented as black dots. * and ** indicate the significant differences ($p < 0.05$ and $p < 0.01$, respectively) from the control (ANOVA with posthoc Tukey's test).

5.3.5 Zebrafish intestine and liver gene expression

Transcriptomic data (mRNA) for the liver and intestine of zebrafish were analysed to survey changes to expression patterns of IL1 β , CYP1A, RAB1A and TP53 genes (Figure 5.6). As shown in Figures 5.6 A and B, CYP1A mRNA registered significant up-to 8 fold expression values in the intestine and up-to 4 fold values in the liver as a consequence of B[a]P exposure ($p < 0.01$), relatively to control. Similarly, IL1 β mRNA showed a significant increase (up-to 3 fold) in the intestine of individuals exposed to B[a]P ($p < 0.01$) (Figures 5.6C). In the liver, all treatments (B[a]P, NP and B[a]P +NP) significantly up-regulated IL1 β ($p < 0.01$) (Figures 5.6 D), compared to the control. RAB1A mRNA registered a significant downregulation in the intestine ($p < 0.05$) and an upregulation in the liver of individuals exposed to PSNP ($p < 0.05$) (Figures 5.6 E and F). Also individuals exposed to B[a]P showed very significant upregulation of RAB1A expression values in the liver ($p < 0.01$), however no significant changes in this gene expression occurred in the intestine. The combination of PSNP with B[a]P did not change RAB1A expression values in any of the exposed organs ($p > 0.05$). TP53 mRNA was significantly higher in the intestine of individuals exposed to B[a]P ($p < 0.05$), however, in the liver, the highest values were observed in individuals exposed to the mixture of B[a]P and NP ($p < 0.001$) (Figures 5.6 G and H).

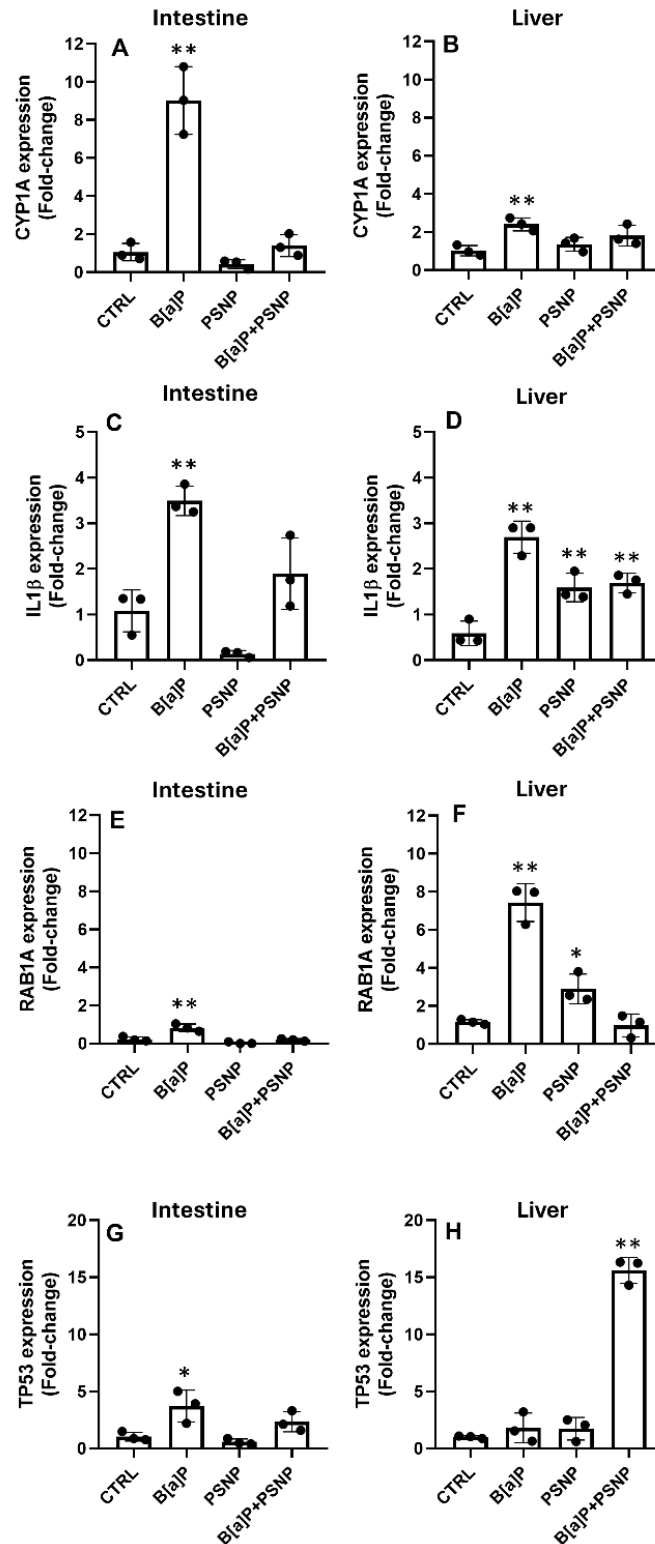


Figure 5. 6 - Target gene expression for all experimental treatments (CTRL, B[a]P, PSNP and B[a]P+PSNP), including the 28 days of exposure, retrieved from intestine and liver mRNAs (n = 3). Data are given as mean ± standard deviation. * and ** indicate the significant differences ($p < 0.05$ and $p < 0.01$, respectively) from the control (ANOVA with posthoc Tukey's test).

5.4 Discussion

5.4.1 *D. magna* ingestion and accumulation of nanoplastics

D. magna is a species that has been used as a model organism in ecotoxicology studies and it is well known as a filter feeder that ingest food based on size (Humphries, 2009). *D. magna* can also ingest different types of plastic polymers (L. Zimmermann et al., 2020) of both micrometric and nanometric size as already demonstrated by other researchers (Jeyavani et al., 2023; Masseroni et al., 2022; Q. Sun et al., 2024). Nano-sized particles, such as quantum dots, were shown to easily adhere to the carapace of *D. magna* and enter the gastrointestinal tract (Lee & An, 2015), respiratory tract (D. Kwon et al., 2012), and brood chamber (Feswick et al., 2013). In the present study, PSNP were found to adhere to the carapace of *D. magna* after 30 minutes, 1 and 2 hours (Figure 5.3). Fluorescence within the body of *D. magna* increased after 24 hours with its intensity continually increasing with prolonged exposure time. These observations confirmed the presence and the accumulation of PSNP over time in the digestive tract of *D. magna* and are in agreement with previous research that has also reported the translocation of PSNP from the gastrointestinal tract to tissues in *D. magna* (Schür et al., 2019). However, it was observed 100% of mortality at 72h indicating possible toxicity effects of PSNP in daphnia during exposure. This is in line with other studies reporting mortality on *D. magna* survival was observed after 24 hours of exposure to 100 and 1000 $\mu\text{g.mL}^{-1}$ (Nasser & Lynch, 2016). Likewise, other studies found that ingestion of PET textile microfibers (length range: 62-1400 μm) by *D. magna* also resulted in increased mortality after 48 hours (Jemec et al., 2016). Daphnids exposed to 1 μm PE particles at concentrations between 12.5 and 400 mg.L^{-1} for up to 96 hours led to immobilisation increasing with dose and time (Rehse et al., 2016). Positively charged 50 nm PSNP exhibit toxicity levels compared to larger PSNP (200 to 500 nm) at 1.4 and 2.7 mg.L^{-1} after 48 hours. Additionally, this study showed that aggregates formed by 50 nm plastic particles, within the same size range as 200 and 500 nm PSNP, exhibited toxicity levels at least as toxic as the dispersed 50 nm PSNP (Frankel et al., 2020).

5.4.2 Effects of nanoplastics ingested by food transfer in zebrafish and B[a]P exposure

The expression of several target genes combined with comet assay results and morphometric measurements were assessed to gain insights into the molecular mechanisms underlying the effects of isolated PSNP and B[a]P exposure and their combination in zebrafish. Although there were no significant differences compared to the control ($p > 0.05$), fish exposed to B[a]P alone registered an average of DNA-SB two times higher compared to control animals. This is in line with previous results in other fish species exposed to similar PAH (benzo[b]fluoranthene) (Bramatti et al., 2023; Martins et al., 2013), which corroborates the severity of this compound as genotoxicant. Additionally, PSNP exposure demonstrated an increase in DNA damage in blood cells, corroborating with previous studies, which as already demonstrated that that PSNP induced oxidative stress, cellular stress, and DNA damage in human neural stem cells after 4 days of exposure when exposed to 30 nm PSNP (Martin-Folgar et al., 2024) and in human peripheral blood mononuclear cells after 24 hours of PSNP with 1000 nm (Martin-Folgar et al., 2024). Most interestingly, the co-exposure of PSNP and B[a]P yielded the most significant increase in genotoxicity, pointing to a possible trojan horse effect (Katsumiti et al., 2021) by NP facilitating B[a]P toxicity.

The increased expression of CYP1A in the zebrafish, known for its role in xenobiotic metabolism, indicates active detoxification processes in response to B[a]P exposure, as expected, corroborating previous findings (Fu et al., 2020; Martins et al., 2018), knowing B[a]P is an inductor of this enzyme (Androutsopoulos et al., 2009b). The absence of significant changes in CYP1A mRNA levels in zebrafish exposed to PSNP have already been observed in other studies that suggested these particles can cause adverse effects in zebrafish without necessarily altering CYP1A expression (A. L. Santos et al., 2024). The results obtained regarding CYP1A expression values after PSNP individually and in combination with B[a]P may signify that the timeline of CYP1A induction precedes that of augmented genotoxicity and, therefore, since we observed high levels of DNA-SB at day 28 of exposure, CYP1A should have happened at an earlier time-point.

While the liver is traditionally considered the primary organ of detoxification, the higher CYP1A mRNA observed in the intestine of zebrafish (Figure 5.6A) suggests that the intestine also plays a significant role, mainly when xenobiotics are ingested orally, namely B[a]P (Tarasco et al., 2021).

Additionally, IL1 β expression values were significantly upregulated in both organs of organisms exposed to B[a]P, in line with the results of several studies that showed the activation of proinflammatory signalling pathways this contaminant (Boraschi et al., 2021; Campos et al., 2023; Tarasco et al., 2022). This result might indicate a cellular response involving programmed cell death (Lopez-Castejon & Brough, 2011; M. Zhang et al., 2015b). No significant changes were observed in IL1 β expression in the intestine of fish exposed to PSNP individually and combined with B[a]P (Figure 6C). Interestingly in our previous study (J. Antunes et al., 2023), we observed a pro-inflammatory response with the production of this cytokine after 24 hours of PSNP exposure in HT29 cells. The absence of IL1 β expression in the intestine could be indicative of an adaptive response, allowing the intestinal tissue to recover after 28 days of exposure, as also observed in other studies with PVC microplastics after 60 days (Xia et al., 2020).

Considering that IL1 β is recognized as an early biomarker in various inflammatory conditions (Gabay et al., 2010), the absence of its expression in the zebrafish intestine suggests the need to explore alternative biomarkers. For instance, genes associated with gastrointestinal disease and tissue damage that respond to chronic exposures could provide valuable insights (Pedersen et al., 2020), namely IL6 (a cytokine involved in inflammatory response and the acute phase reaction, however very important in chronic inflammation, contributing to the progression of inflammatory bowel disease and cancer), IL10 (an anti-inflammatory cytokine that regulates the immune response and can be indicative of solving inflammation mechanisms) or TGF-beta (a cytokine involved in growth regulation and reparation of tissues that can be indicative of cicatrisation processes and tissue remodeling after damage). Additionally, other inflammatory genes could also be analysed, such as C-reactive protein or and TNF alfa (M. Teng et al., 2022). The upregulation of IL1 β gene expression in the liver can be a cellular response of the PSNP mechanical stimulus, causing mechanical stress (D. H. Hwang, 2012) and activating metabolism mechanisms in this organ in response to nanoplastics absorbed proteins/toxics (Yin et al., 2023). IL1 β , traditionally

recognized as a key mediator of inflammation, also plays a significant role in modulating liver metabolism, particularly in the context of xenobiotic detoxification. The upregulation of IL1 β in the liver can influence the expression of various genes, including those encoding cytochrome P450 (CYP) enzymes, which are critical for the oxidative metabolism of foreign substances. By modulating CYP enzyme expression, IL1 β enhances the liver's capacity to metabolize and detoxify xenobiotics, such as environmental toxins or chemical additives, thereby providing an adaptive response that not only addresses inflammation but also bolsters the liver's protective metabolic functions. While the acute upregulation of IL1 β and associated metabolic mechanisms might be beneficial in dealing with short-term exposure, chronic upregulation can lead to sustained inflammation, fibrosis, and even hepatic dysfunction. Chronic inflammation driven by IL1 β is implicated in the development of liver diseases such as non-alcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma (HCC) (Peiseler et al., 2022).

RAB1A gene expression was also upregulated in the liver of zebrafish exposed individually to PSNP and to B[a]P. This guanosine triphosphatase (GTPase) is responsible for the intracellular vesicular transport and protein trafficking between the endoplasmic reticulum (ER) and the Golgi apparatus (M. Xu, Shao, et al., 2019). Generally RAB1A is upregulated as part of a cellular stress response (Tung et al., 2014), namely linked to the cellular strategy to efficiently transport and eliminate xenobiotics from the body (Cui et al., 2022). Recently, RAB1A was showed to be a colorectal oncogene that may play a significant role in the progression of cancer (Z. Cheng et al., 2019), because it can promote cell proliferation and migration. Thus, the upregulation of RAB1A in the liver after exposure to PSNP and B[a]P individually suggests a potential linkage to the proliferation and migration cells and possible tumoral development. Interestingly RAB1A was downregulated in the intestine after PSNP exposure (Figure 5.6E), suggesting a metabolic disorder (Zhang et al., 2021).

Under basal conditions, p53 is kept at low levels in the organism (Bieging et al., 2014), however when cells are exposed to stress, including DNA damage, nutrient deprivation, and cancer cell risk, p53 protein levels rapidly increase (Wei et al., 2006) to either halt cell division until the damage can be repaired or initiate apoptosis if the damage is irreparable (K. M. Kim et al., 2022). P53 protein plays a critical role in

regulating cell cycle and genomic stability and contributes to tumour suppression (H. Wang et al., 2023; Zhou et al., 2023). As B[a]P exposure are associated to the genotoxic effects caused by its metabolization in a reactive form, benzo[a]pyrene-7,8-dihydrodio, the observed upregulation of TP53 following B[a]P exposure suggests a DNA damage response, potentially involving DNA repair processes, cell cycle arrest, and apoptosis, contributing to maintaining tissue homeostasis (Lee et al., 2022). However, the biggest increase was observed in the liver following exposure to PSNP combined with B[a]P (Figure 5.6 H), even though NP alone did not produce any change. Indeed, PSNP combined with B[a]P has reportedly synergistically enhance toxicity in the liver (Batel et al., 2016). The upregulation of p53 levels in the liver following B[a]P + PSNP exposure may indicate a response to the heightened genotoxicity observed.

5.5 Conclusions

The main conclusion from our study is that polystyrene nanoplastics were bioaccumulated by *D. magna* and afterwards, when ingested by zebrafish and also when serving as carrier of B[a]P, caused genotoxicologic effects, after 28 days of exposure. Moreover, the gene expression analysis in the liver and intestine revealed upregulation of IL1 β , CYP1A, TP53, and RAB1A in response to the exposure, indicating a complex array of combined toxic effects. However, B[a]P yielded the highest and significant expressions of genes. Notably, the study suggests that the intestine and liver play distinct roles in responding to environmental stressors, with variations in gene expression levels reflecting their specific functions.

The study contributes to the growing body of evidence on the ecological implications of NP and their interactions with other environmental contaminants. It highlights the need for continued research on the mechanisms of nanoplastic toxicity, considering both short-term and long-term exposures. Furthermore, the findings emphasize the importance of assessing species-specific variations in the response to environmental stressors, recognizing the distinct roles of different organs in the detoxification and adaptation processes.

In conclusion, this research underscores the urgency of addressing plastic pollution in water bodies and calls for comprehensive strategies to mitigate the impact of nanoplastics on aquatic ecosystems.

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Author contribution

Joana Antunes: Writing – original draft, Formal analysis, Investigation. Isabella Bramatti: writing – review, Formal analysis, Investigation. Paula Sobral: Resources, Writing – review & editing, Funding acquisition. Marta Martins: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. Vasco Branco: Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition

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CHAPTER 6:

General discussion

6.1 General discussion

Through rigorous scientific inquiry and interdisciplinary collaboration, this research aimed to contribute with valuable insights to the scientific community and policymakers, thereby facilitating evidence-based decision-making and the formulation of effective strategies to combat plastic pollution and its adverse impacts on marine ecosystems and human well-being. By addressing the complexities of nanoplastic toxicity and its implications for environmental and human health, this thesis seeks to contribute for the urgent needs outlined in the UN's 2030 agenda concerning prevention and marine pollution reduction in the environment, including marine debris (Goal 14.1) and increase knowledge about the relation between plastic pollution, contaminants and human health (Goal 3.9). Overall, the results of these thesis provided answers to the questions outlined in Chapter 2, while pointing forward towards new paths of research.

It was shown that PSNP can induce inflammatory responses in biological systems, as evidenced by our findings in HT29 and N9 cells. The inflammatory responses varied based on the characteristics of the NP, including their size and concentration.

In HT29 cells, exposure to PSNP resulted in an increase in nuclear translocation of p50 and p38, consistent with an inflammatory response (Dinarello, 2009). This was confirmed by the upregulation of proinflammatory genes such as IL-1 β and iNOS. This response in HT29 cells could be attributed to the unique characteristics of the NP and the specific cellular recognition mechanisms involved, namely the activation of TLR4 receptors.

Conversely, in N9 cells, although there was a reduction in cell viability upon PSNP exposure, there was no significant increase in nuclear translocation of p50 and p38 after 24 hours, indicating a different timeline of response compared to HT29 cells. However, there was an clear increase in IL-1 β and iNOS mRNA levels in N9 cells, suggesting the activation of inflammatory pathways (Wang et al., 2020), which was also in line with the observation that TLR4 inhibition decreased inflammatory signalling during exposure to PSNP.

The varying responses observed between HT29 and N9 cells may be attributed to differences in their cellular characteristics, including their regulatory mechanisms for

inflammatory responses. For example, N9 cells, being microglial cells, may have distinct pathways for recognizing and responding to environmental stressors like nanoplastics compared to HT29 cells and have a wide phagocytosis ability (Hazeem et al., 2020; He et al., 2020; Li et al., 2022; Jung et al., 2020). Additionally, the size and concentration of NP can influence their interactions with cellular receptors and internalization pathways, thereby impacting the inflammatory response (Fytianos et al., 2019).

The interaction between NP, like PSNP and environmental pollutants such as B[a]P can lead to complex effects, as showed in this thesis, namely effects on inflammation-related responses and genotoxicity, which involve several underlying mechanisms.

Analysis of antioxidant mechanisms revealed that exposure to PSNP alone or in combination with low B[a]P concentrations resulted in a perturbation of cellular redox balance and antioxidant defence mechanisms, consistent with previous observations of nanoplastic-induced oxidative stress in various cell types and organisms (Babaei et al., 2020; Ribeiro et al., 2017). However, in our case the response was cell-type dependent. Specifically, the cellular response of HT29 cells to both PSNP alone and combined with a low concentration of B[a]P led to a decrease in both GSH and GPx4 levels and an increase in GST activity, indicating a change in cellular redox status. The decreased in GSH and GPx4 levels may indicate a shift towards a pro-oxidative state, while increased GST activity suggests cellular attempts to mitigate oxidative damage through detoxification processes.

In HepG2 cells, exposure to PSNP alone resulted in a significant decrease in GPx1 levels, indicating compromised antioxidant defence mechanisms. However, when PSNP was combined with B[a]P, there was a potential protective response observed in these cells as evidenced by an upregulation of GPx1 expression and GSH levels. This response may reflect a cellular adaptation to oxidative stress induced by the combination treatment, highlighting the complex interplay between nanoplastic exposure, chemical contaminants, and cellular antioxidant responses (Stolwijk et al., 2020).

Furthermore, the activation of CYP1A expression and activity in HepG2 cells upon B[a]P exposure or to PSNP alone or in combination with B[a]P, suggests the involvement of the AhR signaling pathway. Activation of this pathway leads to cellular

stress and epoxide production, which may itself enhance Nrf2-mediated antioxidant defense mechanisms (Androutsopoulos et al., 2009).

Indeed, PSNP and B[a]P exposure seems to alter cellular oxidative stress levels, seen from *in vitro* studies and promote inflammation and genotoxicity through the generation of ROS, activation of pro-inflammatory pathways and induction of DNA damage, as seen by *in vivo* results.

The *in vivo* assay results demonstrated a significant increase in genotoxicity when zebrafish were co-exposed to PSNP and B[a]P, indicating that the combined presence of these contaminants led to more pronounced DNA damage than exposure to either pollutant alone. This phenomenon is often referred to as a "trojan horse effect," where one pollutant, in this case, PSNP, facilitates the toxicity of another, B[a]P (Katsumiti et al., 2021).

Additionally, the increased expression of the TP53 gene in the liver following exposure to both PSNP and B[a]P indicates a cellular stress response. This reaction may represent a defensive mechanism of the liver, aiming to mitigate damage by triggering cell cycle arrest, apoptosis, or DNA repair mechanisms (Mizukawa et al., 2013). Moreover, the substantial elevation in p53 levels observed in the liver after exposure to PSNP combined with B[a]P reinforces the concept of synergistic interactions, signifying an intensified response in this organ due to the combined exposure (Batel et al., 2016).

The results of the *in vivo* assay with zebrafish indicated that exposure to polystyrene nanoplastics (PSNP) through indirect ingestion had significant effects on both the intestinal and hepatic systems, which could ultimately influence overall health outcomes.

In terms of hepatic effects, the liver weight of fish exposed to PSNP showed a significant decrease compared to the control group, which translated in a lower hepatosomatic index. This suggests a potential adverse impact on liver function or metabolism due to PSNP exposure, indicating liver stress or dysfunction.

Regarding the intestinal system, fish exposed to PSNP exhibited significant increases in weight of the intestine compared to the control group. This suggests potential inflammation or other physiological responses in the intestine induced by PSNP

exposure. Indeed, inflammation was apparent as transcriptomic analysis revealed significant changes in gene expression patterns in both the liver and intestine of fish exposed to PSNP. For instance, the upregulation of IL1 β mRNA expression in both organs suggests an inflammatory response triggered by PSNP exposure, which was consistent with finding *in vitro* in HT29 and N9 cells.

Additionally, the downregulation of RAB1A mRNA expression in the intestine and the upregulation in the liver further indicate tissue-specific responses to PSNP exposure.

Overall, these findings highlight the potential adverse effects of PSNP exposure on both the intestinal and hepatic systems, which could have implications for overall health outcomes. Further results, namely histopathological analysis will elucidate the extent of apparent tissue damage caused by exposure.

CHAPTER 7:

Conclusions and Future perspectives

7.1 Conclusion and Future perspectives

In conclusion, this thesis revealed the capacity of PSNP to trigger inflammatory signalling pathways within biological systems. It was observed that the specific mechanisms and response kinetics vary depending on cell type, highlighting the complex interplay of different signalling pathways. For instance, in HT29 cells, an intestinal cellular model, upregulation of TLR4 levels and p50 nuclear translocation suggest a pivotal role of this pathway in inflammation. However, inhibiting this pathway did not consistently reduce pro-inflammatory gene transcription, suggesting the involvement of alternative pathways such as p38. Notably, the inhibition of TLR4 significantly reduced IL-1 β and iNOS transcription in N9 microglia cells exposed to NP, indicating their potential involvement in immune system dysregulation and inflammation-mediated carcinogenesis. Additionally, our findings underscore occasional significant variability between replicates, potentially attributed to nanoplastics' propensity to aggregate in culture medium. CHAPTER 3 underscores the non-innocuous nature of PSNP regarding inflammatory effects, urging further exploration of their implications on human health.

Moreover, this thesis contributed to understand cellular responses triggered by exposure to PSNP alone and in combination with B[a]P in HT29 and HepG2 cells. Our study unveiled the differential effects of PSNP on cell viability and antioxidant responses between the two cell lines, suggesting varying sensitivities and mechanisms of action. Notably, PSNP alone significantly increased HepG2 cell viability, possibly through activation of survival pathways and inflammation responses. Furthermore, it was also observed a synergistic effect after exposure to the combination of PSNP and B[a]P (CHAPTER 4).

Finally, this thesis showed the ecological implications of PSNP, particularly their interaction with other environmental contaminant, B[a]P. Our study demonstrates the bioaccumulation of PSNP by *D. Magna* and their subsequent impact when ingested by zebrafish, leading to genotoxic effects after 28 days of exposure. Moreover, gene expression analysis in the liver and intestine reveals a complex array of combined toxic effects, with B[a]P yielding the highest and significant expressions of genes (CHAPTER 5) These findings underscore the urgent need to address plastic pollution in water bodies and advocate for comprehensive strategies to mitigate the impact of

NP on aquatic ecosystems.

Also, further investigation should be conducted, as the results from this thesis generated several other question left to be answered, namely the effects of organ levels (histopathology), activation of transcriptional pathways involved in Phase I and II metabolism, i.e. AhR and Nrf2, the potential influence of additives present in plastic particles, as these additives may contribute significantly to the overall toxicity of NP. Understanding the role of additives could provide crucial insights into the mechanisms underlying NP toxicity and inform more targeted risk assessment strategies. Furthermore, future studies should explore the long-term effects of NP exposure, considering both chronic and cumulative impacts on human health and the environment. Additionally, testing other plastic polymers are considered important.

Currently, knowledge of NP pollution is limited due to the lack of data on actual concentrations in various environmental compartments. Therefore, conducting studies that focus on measuring and analyzing NP concentrations can significantly enhance the relevance and effectiveness of research in this field. By determining the concentrations of NP in different environmental media such as water bodies, soil, and air, researchers can establish baseline levels and identify hotspots of NP contamination. This information is essential for assessing the magnitude of NP pollution and its distribution patterns, which are crucial for developing targeted mitigation strategies.



2024

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Toxicological mechanisms of nanoplastics: Inflammation as a genotoxicity trigger

