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Short-term effects of nanoplastics in fish: analysis of gene expression and biochemical endpoints in model species|

Brandts Busom, Irene

Universitat Autònoma de Barcelona

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Analysis of gene expression and biochemical endpoints in model species

Irene Brandts Busom

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DIRECTORS

MARIANA TELES

Institute of Biotechnology and Biomedicine
Department of Cell Biology, Physiology and Immunology

Universitat Autònoma de Barcelona

ibb

JOAN CARLES BALASCH

Department of Cell Biology, Physiology and Immunology

Universitat Autònoma de Barcelona

Toda compreensão intensa é finalmente
a revelação de uma profunda incompreensão

Clarice Lispector

Contents

Resum

La contaminació per plàstic és una preocupació ecològica actual, que està rebent creixent atenció per part de la societat, la comunitat científica i els governs. La major part de la contaminació plàstica és causada per petits fragments de plàstic, els microplàstics i nanoplàstics (NP), actualment considerats contaminants emergents. La disminució de la mida de les partícules de plàstic augmenta la biodisponibilitat i els possibles efectes nocius que aquestes poden tenor sobre els organismes. Tot i que el medi aquàtic està especialment en risc de contaminació per NP, actualment es coneix poc sobre els seus potencials efectes tòxics, especialment en organismes de nivells tròfics alts com els peixos. A més, fins ara pocs estudis han tractat els efectes dels NP sobre espècies de peixos marines d'interès per a l'aqüicultura marina. Els estudis s'han dut a terme durant en aquesta tesi pretenen investigar l'ecotoxicologia dels NP utilitzant peixos com a models vertebrats. Per tal d'entendre millor l'absorció i els efectes dels NP en els peixos, individus de les especies *Danio rerio* (peix zebra), *Sparus aurata* (orada) i *Dicentrarchus labrax* (llobarro) han sigut exposats a NP, per tal d'avaluar respostes a curt termini en l'expressió de gens i marcadors bioquímics relacionats amb el metabolisme,l' estrés oxidatiu i el sistema immune.

En peix zebra, vàrem estudiat hepatòcits (cèl·lules ZFL) com a model *in vitro* i es va trobar acumulació de nanoplàstics de poliestirè (PS-NPs) als lisosomes, juntament amb una expressió alterada de gens antivirals en resposta a poly(I:C). En larves de peix zebra també s'hi van acumular PS-NP, principalment a intestí i pàncrees, però l'exposició a PS-NP no va afectar la supervivència de les larves a una infecció per *Aeromonas hydrophila*. En orada, l'exposició a curta (96 h) a través de l'aigua a nanoplàstics de polimetilmetacrilat (PMMA-NP) va provocar estrès oxidatiu i inflamació lleu a les tres barreres mucoses que actuen com a portals d'entrada de NPs en peixos (pell, brànquies i intestí), amb l'intestí com a teixit més afectat. Al fetge no es va trobar cap resposta inflamatòria i només una lleu resposta d'estrès oxidatiu. Tot i no trobar una resposta pronunciada d'estrès oxidatiu, es van observar efectes genotòxics als eritròcits de *S. aurata*. El metabolisme lipídic és la via molecular més afectada al fetge, segons els resultats de l'expressió gènica i paràmetres bioquímics. En el múscul, s'ha obervat una resposta antiinflamatòria transitòria, sense efectes immediats a curt termini sobre el creixement i la diferenciació muscular. En llobarro, l'exposició (96 h) a PMMA-NP va alterar els gens relacionats amb el metabolisme dels lípids, mentre que no es van trobar canvis importants en els paràmetres bioquímics avaluats.

Considerant els resultats en conjunt, trobem que hi ha canvis rellevants tant en les vies moleculars com en els paràmetres bioquímics relacionats amb el metabolisme dels lípids i l'estrès oxidatiu. També s'ha trobat una lleu activació de les respostes inflamatòries i immunes; tanmateix, els mecanismes d'acció i els efectes específics en diferents teixits i espècies s'han d'investigar més a fons. Avaluant globalment tots els estudis presentats en aquesta tesi i valorant l'efecte de la concentració de NPs, trobem que les concentracions d'exposició més baixes semblen ser les que causen més efectes potencials. Això suggereix que una dosi-resposta no monotònica i efectes a dosis baixes són un possible escenari en la toxicologia de NPs. Globalment, els estudis presentats en aquesta tesi contribueixen a augmentar el coneixement en el camp de l'ecotoxicologia de NPs en peixos.

Abstract

Plastic pollution is a major ecological concern that is receiving increasing attention from the general public, the scientific community and governments. The bulk of plastic pollution is mainly caused by small plastic fragments, microplastics and nanoplastics (NPs), currently considered widespread contaminants of emerging concern. The decrease in plastic particle size increases the bioavailability and the potential harmful effects that these particles have on organisms. The aquatic environment is particularly at risk of exposure NPs, yet there is currently little known about their potential toxic effects, specifically in organisms of higher trophic levels such as fish. To date, few studies have addresses the effects of NPs on marine aquaculture fish species. The studies that were conducted during this work sought to investigate the ecotoxicology of NPs using fish as a vertebrate model. In order to gain a better understanding of the uptake and effects of NPs in fish, *Danio rerio* (zebrafish), *Sparus aurata* (gilthead seabream) and *Dicentrarchus labrax* (European seabass) individuals were exposed to NPs to assess the short-term responses of metabolic, oxidative and immune-related genes and biochemical endpoints.

In zebrafish, hepatocytes (ZFL cells) were examined as an *in vitro* model, and accumulation of polystyrene nanoplastics (PS-NPs) was found in lysosomes, together with an altered expression of antiviral genes in response to poly(I:C). Zebrafish larvae also accumulated PS-NPs, mainly in gut and pancreas, but the exposure to PS-NPs did not affect larvae the survival to *Aeromonas hydrophila* infection. In gilthead seabream, a short-term (96h) waterborne exposure to polymethylmethacrylate nanoplastics (PMMA-NPs) caused oxidative stress and mild inflammation in the three mucosal barriers that act as portals of entry of NPs in fish (skin, gills and intestine), with intestine as the most affected tissue. In liver no inflammatory response was found and only a mild oxidative stress response. Despite finding no pronounced oxidative stress response, genotoxic effects were observed in *S. aurata* erythrocytes. Lipid metabolism is suggested as the most affected molecular pathway in liver, as shown by gene expression and biochemistry results. In muscle, a transient anti-inflammatory response was noted and no immediate short-term effects on muscle growth and differentiation. In European seabass, exposure (96h) to PMMA-NPs altered transcripts related to lipid metabolism, while no major changes were found in the assessed biochemical parameters.

Taking all results together, we can say that there are relevant changes both in the molecular pathways and biochemical parameters related to lipid metabolism and oxidative stress. Low-level activation of inflammatory and immune responses was also found; however, mechanisms of action and tissue and species-specific effects

should be further investigated. Globally looking at all the studies presented in this thesis and assessing the NPs' concentration effect, we find that the lower exposure concentrations appear to be the ones causing more potential effects. This suggests that a non-monotonic dose response and low-dose effects are a plausible scenario in NPs toxicology. Altogether, the studies presented in this thesis contribute to increase knowledge in the field of NPs' ecotoxicology.

Abbreviations

AChE: Acetylcholinesterase activity
ADA: Adenosine deaminase
ALP: Alkaline phosphatase
ALT: alanine transaminase
ANOVA: Analysis of variance
AST: Aspartate aminotransferase
ASW: Artificial seawater
cDNA: Complementary DNA
CECs: Contaminants of emerging concern
CK: Creatine kinase
DLS: Dynamic light scattering
DNA: Deoxyribonucleic acid
EA: Esterase activity
ENAs: Erythrocytic nuclear abnormalities
FAO: Food and Agriculture Organization
HIS: Hepatosomatic index
IBR: Integrated biomarker response
IU: International unit
IUCN: International Union for Conservation of Nature
MDA: Malondialdehyde
MPs: Microplastics
MNPs: Micro(nano)plastics
mRNA: Messenger RNA
MS222: Tricaine methanesulfonate
MTT: 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-2H-tetrazolium bromide
NMDR: Non-monotonic dose response
NOAEL: No observed adverse effect level
NPs: Nanoplastics
NTA: Nanoparticle tracking analysis
OECD: Organisation for Economic Co-operation and Development
OSI: Oxidative stress index
PBS: Phosphate buffered saline
PCO: Principal Coordinates
PDI: Polydispersity index
PE: Polyethylene
PET: Polyethylene terephthalate
PolyIC: Polyinosinic:polycytidylic acid
PMMA: Poly(methylmethacylate)
POPs: Persistent organic pollutants
PP: Polypropylene
PS: Polystyrene
PUR: Polyurethane
PVC: Polyvinylchloride
RNA: Ribonucleic acid
ROS: Reactive Oxygen Species
RTGut: Rainbow trout (*Oncorhynchus mkiss*) intestinal epithelial cell line
RT-qPCR: Quantitative reverse transcription polymerase chain reaction
TAC: Total antioxidant capacity
TEAC: Trolox equivalent antioxidant capacity
TOS: Total oxidative status
UNEP: United Nations Environment Programme
WGA: Wheat germ agglutinin
WHO: World Health Organization
ZFL: Zebrafish liver cells

Introduction

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1. Introduction

1.1 A (very) brief history of plastics

Less than a century ago, in our same very own planet earth…nobody knew what plastic was. Today, our present days have been defined by some as the Plasticene, "an era in Earth's history within the Anthropocene commencing in the 1950s, marked stratigraphically in the depositional record by a new and increasing layer of plastic" (Haram et al., 2020). In fact, evidence that plastics are becoming a relevant component of the present-day geological cycle is increasing, as they are so ubiquitous in the environment that they are being used as markers to characterize the sedimentary deposits where they are found (Zalasiewicz et al., 2016).

In 1907, Leo Bakeland, invented "Bakelite", the first entirely synthetic plastic, marking the start of the modern plastics industry. During the period preceding and surrounding World War II a great number of innovations in plastic materials emerged (e.g. polyethylene (PE), polystyrene (PS) and nylon) and in the 1940s, the first industrial production of plastics took place. Nevertheless, mass production of plastics started only in the 1950s, and since then has had an exponential growth, increasing by 100-fold by the 2000s **(Fig. 1)** and reaching values of 368 million tons of world production in 2019 (PlasticsEurope, 2020). The demand and production of plastics is still increasing, with predictions of more than 1800 million tons being produced in 2050, if the tendency were to continue undisturbed (Geyer et al., 2017).

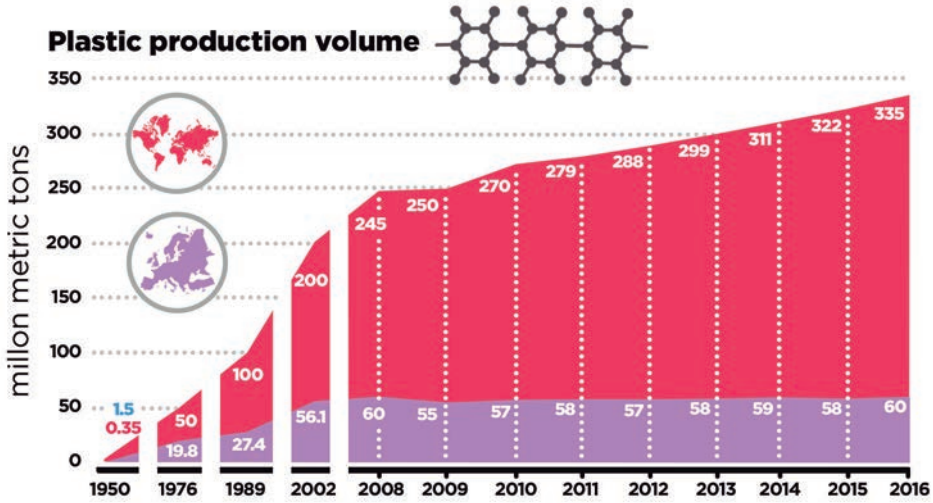

Figure 1. Evolution of plastic production volume worldwide and in Europe.
Adapted from Lehner et al. (2019), data from PlasticEurope.

But what is plastic? Plastic is the name given to a synthetic material that can be made from a wide range of organic polymers, capable of being shaped or molded into multiple different shapes. These synthetic polymers (made of identical repeating units – monomers) are mostly derived from fossil fuels and have generally longer chains than naturally occurring organic polymers (such as cellulose or natural rubbers). The length and conformation of these synthetic polymers, together with the additives incorporated in the fabrication process, grant them a wide range of properties, such as strength, durability, flexibility or lightness.

Polyethylene (PE), polypropylene (PP), polyvinylchloride (PVC), polyethylene terephthalate (PET), polyurethane (PUR), polystyrene (PS) and polyester are the largest polymer groups in plastic production, accounting for 92% of all plastics ever made (PlasticsEurope, 2020; Geyer et al., 2017). Altogether, plastics are cost-effective, chemically stable and versatile materials, which has led to their widespread use and sustained massive production.

The exponential growth of production of plastic materials, together with the mismanagement of plastic waste, has resulted in their environmental release for more than 50 years. In a complex analysis regarding the fate of all plastics ever produced, Geyer et al. (2017) computed that plastic waste generated had reached 6300 million tons by the end of 2015 and at least 80% of this waste is calculated to have been discarded and is currently accumulating in landfills or in the natural environment. This plastic mismanagement is exacerbated by the fact that currently the largest application of plastics is in packaging, dominated by the popularity of single-use containers (PlasticEurope, 2020).

Although plastic waste management policies and practices are gradually improving, only 9% of all plastics ever made is calculated to have been recycled, while another 12% will have been incinerated, but even if these two practices, technically avoid the disposal of plastic waste into the environment, they are not without drawback **(Fig. 2)**. As stated by Geyer et al., 2017, recycling practices can be considered to reduce plastic waste generation only if they are displacing primary plastic production, on the contrary, recycling will merely a delay plastic disposal. It must also be noted that the way recycling is currently implemented, collecting contaminated and mixed polymer plastic waste, generates secondary plastics of low technical and economic value. Regarding incineration, the environmental and health impacts of waste incinerators are controversial, as they are highly variable depending on as incinerator design, operation, and emission control technology.

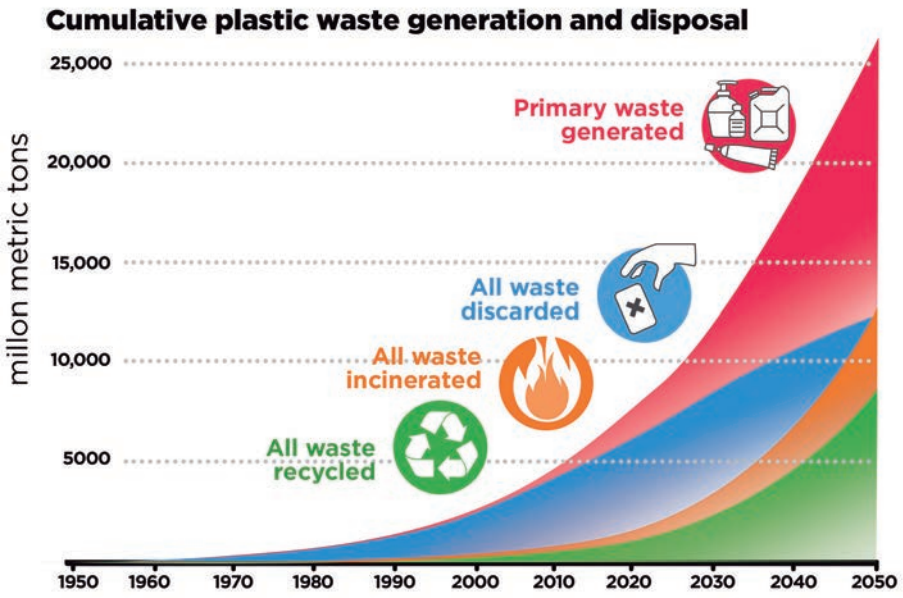

Figure 2. Cumulative plastic waste generation and disposal
as estimated by Geyer et al. (2017).

Considering that their slow degradation rates and pervasiveness guarantee a continuous supply of plastic waste into virtually all ecosystems, plastic pollution has become one of the most pressing environmental issues, as discarded plastic products overwhelm the world's ability to deal with them. Looking into the future, if plastic production maintained the same growth rate, predictions say that by 2050 up to 12000 million tons of plastic will have been discarded into the environment, even assuming that recycling and incineration practices steadily rise (Geyer et al, 2017).

Up to 10% of the discarded plastic is expected to end up in the aquatic environment, with a great proportion of it finding marine waters as a final destination. Looking for precise numbers, Jambeck et al., (2015) estimated that 31.9 million tons of plastic were mismanaged in 2010 and of these, from 4.8 to 12.7 million tons were estimated to have ended up in the ocean. The authors calculated that the mismanaged plastic waste generated by individual countries range from 1.1 to 8.8 million tons and is mostly influenced by population size and economic developmental stage. Regarding the latter, some of the top polluters are middle-income countries where fast economic growth is occurring, but waste management infrastructure is still deficient. Ultimately, if no substantial waste management improvements are adopted at a global scale, the cumulative quantity of plastic waste entering the marine environment from land-based

sources is predicted to have increased by at least an order of magnitude by 2025 (Jambeck et al., 2015).

1.2 Plastics in the environment

Plastic debris can persist in the environment for many years, as its own intended, "beneficial" properties, versatility, stability and durability, ensure that plastic litter remains in the environment long after it has been disposed. The persistence of plastic waste items in the environment is very variable but always longer than its usage time, and it can range from 20 years for a disposable plastic bag to 500 years for a plastic bottle. Since a few years back the question of whether plastic waste ever really disappears has been challenged, as the fragmentation of large plastic objects into gradually smaller pieces has been "discovered".

Once in the marine environment, bulk plastic objects initiate a degradation process, due to the action of several biotic and abiotic factors. This degradation or weathering is provoked by a combination of chemical and physical processes, such as photodegradation, thermo-oxidation, hydrolytic degradation, mechanical disintegration, and biodegradation (Andrady, 2011). Over time these forces degrade plastic litter, breaking down into smaller fragments **(Fig. 3)**: macroplastics (>2.5 cm), mesoplastics (2.5 cm to 5 mm) and microplastics- (≤5 mm, MPs), as defined in literature and adopted by international organizations and monitoring agencies, such as the Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection of the United Nations (GESAMP), the Marine Strategy Framework Directive of the European Union and the National Oceanic and Atmospheric Administration of the US.

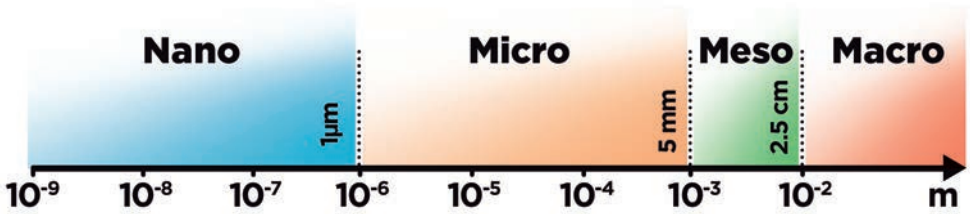

Figure 3. Classification of plastic litter size ranges proposed by GESAMP.

Further degradation to nanosize-range particles, nanoplastics (NPs, <1000 nm (Gigault et al., 2018; Hartmann et al., 2019), has been proven to occur,

both from everyday use plastic objects (Lambert and Wagner, 2016; T. Ekvall et al., 2019), from plastic fragments collected from the environment (El Hadri et al., 2020) and inside organisms (Dawson et al., 2018). Relevantly, although NPs detection techniques are still under development, the presence of NPs has already been confirmed in environmental samples. Ter Halle et al., (2017) isolated polymeric particles from 1 to 1000 nm in size from the colloidal fraction of a water sample obtained from the North Atlantic Gyre, reporting wider variety of polymer types in the NPs fraction than in the larger MPs. In a posterior study, Llorca et al., (2021) detected the presence of NPs in water samples from the Ebre River Delta, documenting PS, PE, and PP among the detected polymers.

Nowadays, plastics have been detected in all habitats and ecosystems around the planet, from terrestrial to aerial to surface waters to soils and sediments of rivers and oceans (Eriksen et al., 2014; Suaria et al., 2016; Alimi et al., 2018; Anbumani and Kakkar, 2018; Strungaru et al., 2019). Eriksen and colleagues (2014) estimated at least 5.25 trillion plastic particles currently floating at sea, with a total weight of 268,940 tons. A similar weight of plastic particles, 236,000 tons was calculated by van Sebille et al., (2015), but this time considering only particles 200 mm or smaller and estimating the particle number to be between 15 and 51 trillion. In a more recent study, Lebreton et al., (2018) focused solely on the plastic pieces forming the "Great Pacific Garbage Patch" and determined that 79,000 tons would be floating in this area of 1.6 million km2, reporting numbers up to 16 times higher than previous studies. Paradoxically, looking at the data from both the estimated total plastic waste entering the ocean and the reported mass of floating plastic debris calculated by monitoring studies, numbers differ by several orders of magnitude. This circumstance has been designated as the "missing plastic paradox", which accounts for the uncertainties and underestimations of the lower size-range plastic particles and is probably due to two or more factors (Isobe and Iwasaki, 2022). On one hand, global ocean estimates usually represent only plastics pieces that are buoyant in seawater, therefore missing those that will be floating lower in the water column or will have sunk to the bottom. The presence of deep-sea plastic debris has been extensively proven (Chiba et al., 2018) and the ingestion of plastic objects or particles by organisms living in benthic zones has also been documented (Fang et al., 2018). On the other hand, the sampling techniques available for monitoring expeditions are probably leading to greatly underestimating the lower size-range of plastic waste, as the mesh nets employed are usually not lower than 300 μm, being unable to detect the presence of smaller plastic particles.

Altogether, plastics represent approximately 80% of total marine debris, and one of the most relevant threats to marine ecosystems nowadays (Cózar et al., 2014; Mattsson et al., 2015). Moreover, 80% of this litter comes from terrestrial sources, while only 18% is attributed to waste from the fishing industry or other marine activities (Auta et al., 2017).

Five large areas of plastic waste floating in the middle of the oceans have been described **(Fig. 4)**, corresponding to each of the subtropical ocean gyres (Cozar et al., 2014): North Pacific, North Atlantic, South Pacific, South Atlantic, and Indian Ocean. These areas, popularly named the "great garbage patches" or "garbage islands" are large expanses of the ocean with diffuse and changing borders, and a large heterogeneity of plastic fragments distributed throughout the water column, composed essentially by MPs and NPs. Concentrations of plastic particles of the order of kg per km2 have been reported in these areas, while oceanic concentrations outside the gyres reach only a few grams per km2 (Eriksen et al., 2014).

The Mediterranean Sea is one of the most polluted marine areas, with MPs concentrations comparable to those found in oceanic gyres de Haan et al., (2019). Cózar et al., (2014) modelled plastic pollution in the Mediterranean Sea, finding that the average density of plastic waste is comparable to the accumulation of waste described in the five oceanic gyres. According to this study, the concentration of plastic present on the surface of the Mediterranean Sea is 423 g/km2, comparable to the average concentrations measured in the internal areas of accumulation of the subtropical ocean gyres, between 281 - 639 g/km2. Furthermore, the total load of floating plastic waste in the Mediterranean is comparable to that of the gyres, as it has an area of 2.5 million km2, similar to the spatial range of the oceanic litter accumulation areas of internal accumulation of oceanic turns (ranging from 1 to 5 million km2 in global models of circulation (van Sebille et al., 2012). This makes the Mediterranean a major area for the accumulation of floating plastic waste worldwide.

Factors such as a high plastic input load, limited export to the Atlantic Ocean, high recreation use of the coastal areas and to the intense aquaculture production contribute to this fact. Given the great biological richness an-d concentration of economic activities in our Mediterranean Sea, the effects of plastic pollution on marine and human life could be especially intense in this area. Although the Mediterranean comprises less than 1% of the total area of the world's oceans it has an enormous ecological, hosting up to 18% of all known marine species and

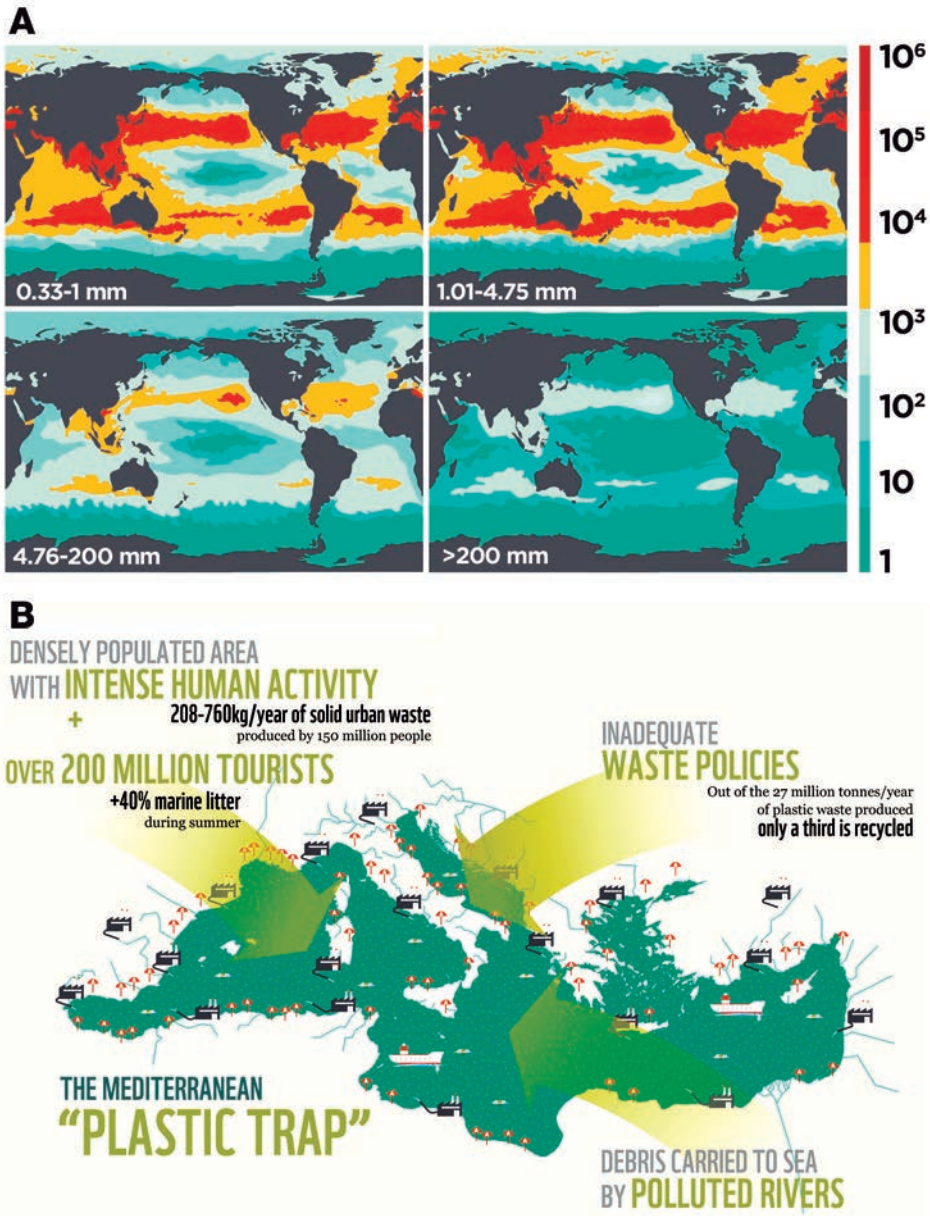

Figure 4. A. Prediction model of the density of plastic particles (pieces km–2), for each of four size-classes: 0.33–1.00 mm, 1.01–4.75 mm, 4.76–200 mm, and >200 mm. Adapted from Ericksen et al. (2014). **B. Representation of plastic pollution inputs in the Mediterranean.** Data from Cózar et al. (2014) and Alessi et al, (2018).

the planet's highest rate of endemic species (UNEP, 2021). Moreover, it is also one of the most densely populated coastal areas, as well as a region with a strong tradition of see food consumption.

It is now recognized that the bulk of plastic pollution is caused by MPs and NPs, and both size-ranges are considered widespread contaminants of emerging concern (CECs) (Rochman et al., 2018). CECs are defined as pollutants that have been detected in the environment with uncertainty as to their effects. Accordingly, the risk they pose to human or environmental health is not yet fully understood and are not regulated under current environmental law. Nevertheless/Encouragingly, scientific efforts dedicated to MPs research in the last 20 years have contributed to changing product regulations (e.g., the Food and Drug Administration or European Chemicals Agency) as well as enabled Governmental Agencies to discuss wastewater treatment policies (e.g., Southern California Coastal Water Research Project). Research concerning NPs is also growing, with the aim of finally better understanding these CECs and their effects and hopefully contribute to future environmental and human health regulations.

Environmental NPs (and MPs) can be divided into two types, based on their origin: primary and secondary **(Fig. 5)**. Primary NPs are those that enter the environment in their original small size associated with consumer products (e.g., cosmetics, and personal care), specific technological or industrial applications (e.g., drug delivery, 3D printers). Secondary NPs are those formed due to the degradation of larger plastic pieces (Lehner et al., 2019), mentioned previously for marine plastic litter or originated terrestrial sources such as transportation (i.e., plastic particles released from tire abrasion) or the release of synthetic fibers during the washing of clothes (Costa et al., 2016). The decrease in plastic particle size increases the bioavailability and the potential harmful effects that these particles have on organisms (Mitrano et al., 2021).

1.3. Size-related effects of plastic debris

Plastic debris is the most abundant litter collected in studies monitoring marine debris, both in water and in beach surveys (Law, 2017). It contaminates a wide range of habitats such as coral reefs (Donohue et al., 2001), estuaries (Browne et al., 2010), the open ocean (Cózar et al., 2014). Moreover, it can be found at all levels of the water column; floating on the ocean surface, buoyant in the water column and the deep sea (Galgani et al., 2015; Law, 2017) The potential interactions of marine biota with plastic litter are multiple, attaining

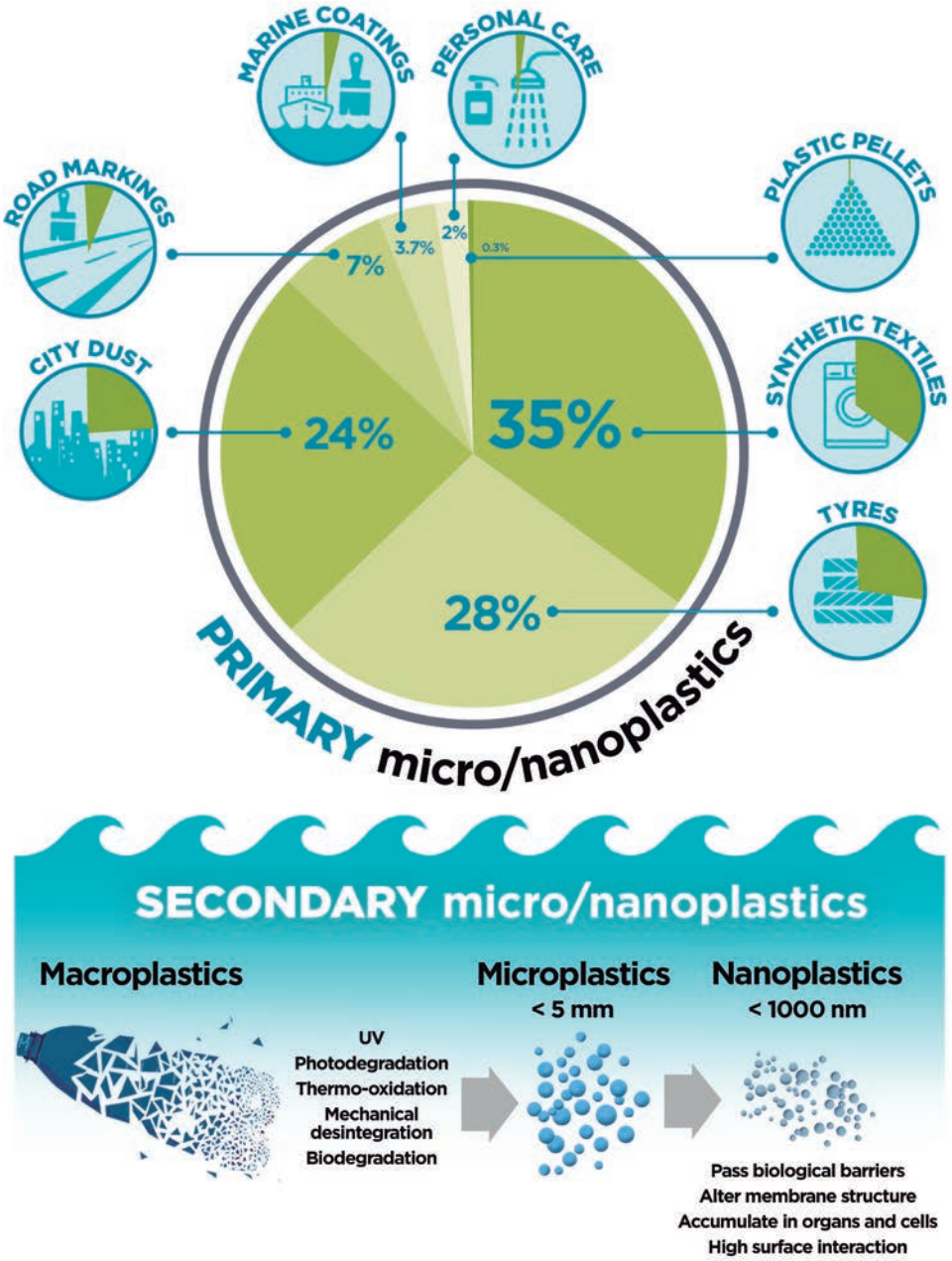

Figure 5. Sources of primary and secondary MPs and NPs found in marine waters. Adapted from Boucher and Friot (2017), IUCN.

organisms from all types of habitats and trophic levels. The frequency of encounter, quantity of debris and type and size of plastic litter will determine the consequences for marine wildlife and ecosystem implications. Mounting evidence has documented contact between macroplastic debris and more

than 600 species of marine wildlife (Gall and Thompson, 2015), either by entanglement or ingestion of plastic objects. Macro- and mesoplastics have been reported in over 350 species of mostly large sizes, including marine turtles, seals, whales, sharks, and seabirds, with approximately 17% of affected species categorized as threatened to critically endangered by the IUCN list (Kühn et al., 2015). This entanglement and/or ingestion of macroplastic debris will inevitably alter the biological and ecological performance of individuals, compromising their health and often ending in death.

As they decrease in size, turning into MPs, plastic particles become more bioavailable to aquatic organisms, as well as potentially harmful for a wider range of species **(Fig. 6)**. That is, in the same way that large amounts of plastic objects have been found in the stomachs of fish, seabirds, turtles and whales, the presence of MPs has been documented in multiple species of crustaceans, mollusks, zooplankton, echinoderms, fish, mammals and birds (Neves et al., 2015; Bellas et al., 2016; Hermsen et al., 2017). As a general rule, the smaller the particle the smaller the organism that can interact with it, which is of special concern, as fito- and zooplankton form the base of the marine food web.

The ingestion of MPs has also been studied in laboratory exposures in a wide range of species of rotifers, annelids, echinoderms, mollusks, crustacean and fish (de Sá et al., 2018), with the aim of discovering hazardous effects associated with the ingestion of MPs. Several effects have been described under these experimental conditions, indicating that MPs could exert both sublethal and lethal effects on natural populations. Some of the most reported effects are retention of MPs in the intestine causing blockage and reduction of nutrient absorption (Wright et al., 2013; Lu et al., 2016; Lusher et al., 2017), neurotoxicity (Oliveira et al., 2013; Ribeiro et al., 2017; Barboza et al., 2018), decreased growth rate (Della Torre et al., 2014; Au et al., 2017; Redondo-Hasselerharm et al., 2018), reproductive alterations (Lee et al., 2013; Au et al., 2017), transfer of absorbed contaminants or plastic additives (Jovanović et al., 2018) and decreased survival (Lee et al., 2013; Li et al., 2016; Au et al., 2017; Mazurais et al., 2020). Altogether, there is general consensus that MPs are able to cause adverse effects on marine and freshwater species (reviewed by (Chae and An, 2017).

The potential harmful effects of plastic litter are not limited to the polymer itself, as they can leach additional chemicals derived from their manufacturing process, incorporated to enhance the material's desired properties (Fries et al., 2013). The leaching of these, such as phthalates and bisphenol analogues, could

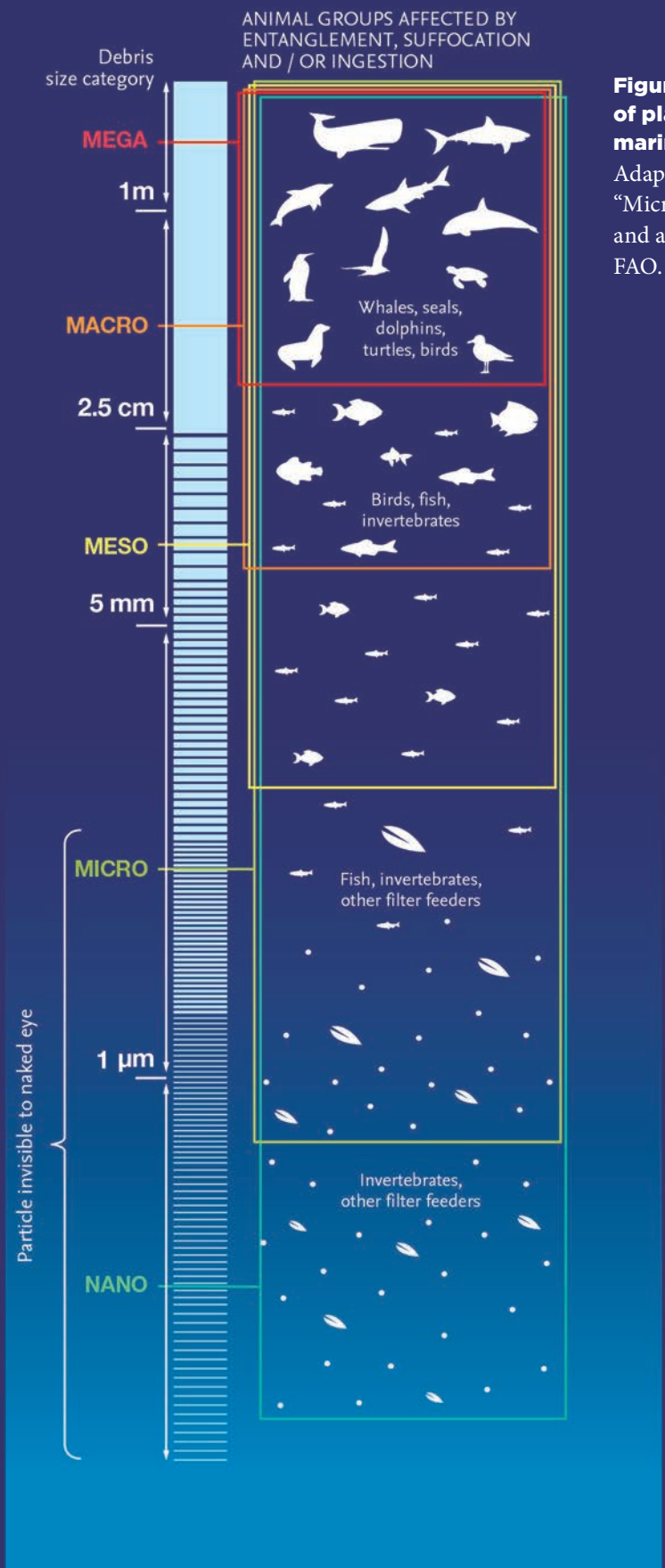

Figure 6. Size-ranges of plastic debris in the marine environment. Adapted from "Microplastics in fisheries and aquaculture", FAO. 2017.

pose a reasonable concern and even more so if the plastic particles are small enough to be ingested by biota or even translocated to their internal tissues (Rochman et al., 2013).

1.4. Nanoplastics

There are still many unanswered questions regarding the effects of NPs, the least studied of plastic particles, yet potentially the most hazardous of marine litter due to their specific properties. The nano-size of NPs confers them specific nano-scale properties regarding their physicochemical characteristics. With their size decrease in particle size, both their bioavailability and their biological reactivity increases (Ferreira et al., 2019). Moreover, their surface area, size, strength, conductivity and reactivity are substantially different from macro/micro-sized particles (Klaine et al., 2012; Mattsson et al., 2015, 201), affecting the NPs interaction with the surrounding media and organisms. As MPs, NPs may interact as well as be easily ingested by organisms, attaining all sizes due to their dimensions (Cole and Galloway, 2015). Thanks to these nano-scale dimensions NPs can pass through biological barriers and enter cells (Kashiwada, 2006), change membrane structure and affect its function (Rossi et al., 2014, 20), penetrate and bioaccumulate in organs and tissues (Ward and Kach, 2009). Therefore, the potential of NPs to have a biological impact is considered to be greater than MPs, as their smaller dimensions and nanoparticle properties allow them to enter cells and affect their components and metabolism (El Hadri et al., 2020).

Due to their high surface area, NPs naturally bind to available macromolecules from their surroundings (Lynch et al., 2014). In biological fluids this means that a corona of biomolecules forms on nanoparticles, the so-called protein-corona, and the presence of this corona plays a crucial role in further interactions with other biological entities (Lundqvist and Cedervall, 2020). The characteristics of the protein corona formed around NPs in different biological fluids is still deeply understudied, although the knowledge of the molecular composition of the protein corona around NPs will provide valuable information to assess the potential biological risks at an organism and ecosystem level (Cedervall et al., 2012). Dissolved organic matter and inorganic colloids will also interact with the surface of NPs, with the ability to alter its characteristics and charge (Zhang et al., 2022) and therefore condition aggregation or dispersion in the media (Oriekhova and Stoll, 2018). The aggregation is relevant, as aggregates will tend to sedimentation, while particles in colloidal suspension are more mobile and able to diffuse, being more dispersed bioavailable and potentially more harmful

to biota (Ramirez et al., 2019). Nevertheless, it might not always be true that aggregation of NPs decreases their toxic potential as PS-NPs aggregated with human immunoglobulin had the same effects as non-aggregated NPs in a study with *Daphnia magna*. (Frankel et al., 2020). Their aggregation or dispersion is a dynamic, non-stationary equilibrium, influenced by multiple factor such as additional suspended matter and molecules, surface charge weathering, UV-light or water temperature (Bergami et al., 2019). Moreover, their high surface ratio also increases the absorption of environmental contaminants to the polymer surface (Velzeboer et al., 2014), such as polycyclic aromatic hydrocarbons (Liu et al., 2016), metals (Davranche et al., 2019), potentially facilitating the internalization of these contaminants in aquatic organisms. Nevertheless, research methodology and relevant conclusions in the field of NPs and NPs toxicology are still at an initial stage, due to the complications presented by these particles' size, surface characteristics dependent effects and the difficulty of quantification and characterization.

1.4.1. Effects of nanoplastics in aquatic organisms

NPs have shown to have potential interactions and effects with aquatic organisms of all sizes and ecological niches. Starting with bacteria, NPs have shown to provoke alterations in bacterial biofilm formation (Okshevsky et al., 2020), decreased efficiency of photosynthesis in photosynthetic bacteria, induction of oxidative stress and alteration of cell metabolism (Miao et al., 2019; Fringer et al., 2020). Effects on algal communities, key in the aquatic environment as source of oxygen and base of food webs, have also been found, with inhibitory effects on growth and development, changes in lipid composition, and alteration of the photosynthetic capacity therefore nutritional quality documented in different species of microalgae (Sjollema et al., 2016; Venâncio et al., 2019; Gomes et al., 2020). Several studies have been done in species that are part of marine zooplankton (e.g. *Brachionus sp.*, *Artemia sp.*), reporting accumulation of NPs, increased mortality, oxidative stress and altered growth and locomotion, among others (Jeong et al., 2017; Manfra et al., 2017; Venâncio et al., 2019; Bergami et al., 2020).

Multiple studies have been conducted in echinoderm and bivalve species, finding effects both at the cellular and organism levels. Among others, altered cellular phagocytosis and apoptosis, embryonic malformations, oxidative stress and inflammatory responses have been documented in echinoderms (Della Torre et al., 2014; Pinsino et al., 2017; Bergami et al., 2019). Sessile filter-feeders such bivalves are specially interesting for the monitoring of NPs,

as they are considered sentinel organisms for ecotoxicological evaluations. NPs have shown to compromise the integrity of the immune response, alter biological membranes, induce cytotoxicity, and impair reproductive success (compromising future populations) in bivalve species such as *Mytilus galloprovincialis* or *Crassostrea gigas* (Canesi et al., 2015; Balbi et al., 2017; Tallec et al., 2018).

Globally, studies investigating the toxicity of NPs in marine biota focus mainly on invertebrates, with the crustacean *A. fransiscana* and the mollusk *M. galloprovincialis* as the most studied species (Gonçalves and Bebianno, 2021) and the knowledge of NPs effects regarding different species of marine biota is still scarce (Gaylarde et al., 2021). The toxicity of NPs to organisms in the environment depends on factors such as exposure concentration and period, potential adsorbed pollutants, species, age and food availability (Kögel et al., 2020).

Effects of nanoplastics in fish

More studies on the effects and mechanisms of action of NPs are needed in higher trophic level organisms such as fish (de Sá et al., 2018). To date, very few studies have addressed the effects of NPs on marine fish, with most available data focusing on freshwater species and mostly in laboratory model species such as *Danio rerio* (zebrafish) or *Oryzias latipes* (medaka) (Chae and An, 2017; Chen et al., 2017; Skjolding et al., 2017; Pitt et al., 2018), which justifies the approach adopted in the present PhD thesis.

In teleost fish, NPs can be absorbed through different routes, mainly the gastrointestinal tract, gills, and skin (Bhagat et al., 2020) and their uptake pathway may condition their effects in the organism (van Pomeren et al., 2017). In studies evaluating the uptake of NPs', mostly done with zebrafish, the intestinal track appears to be the dominant uptake pathway (Bhagat et al., 2020). The absorption of NPs as well as their translocation to tissues has been documented, both after waterborne and dietary exposures (Mattsson et al., 2017; Brun et al., 2018; Chae et al., 2018). NPs have been found to distribute throughout the fish body reaching the circulation as well as distant tissues, such as the brain or the eye (Kashiwada, 2006; Mattsson et al., 2017; Skjolding et al., 2017; van Pomeren et al., 2017; Brun et al., 2018; Pitt et al., 2018).

Detrimental effects of NPs in fish **(Fig. 7)** have been documented ranging from a broad general stress response to behavioural alterations (Pitt et al., 2018; Brun et al., 2019). As other marine pollutants, NPs can potentially overload fish

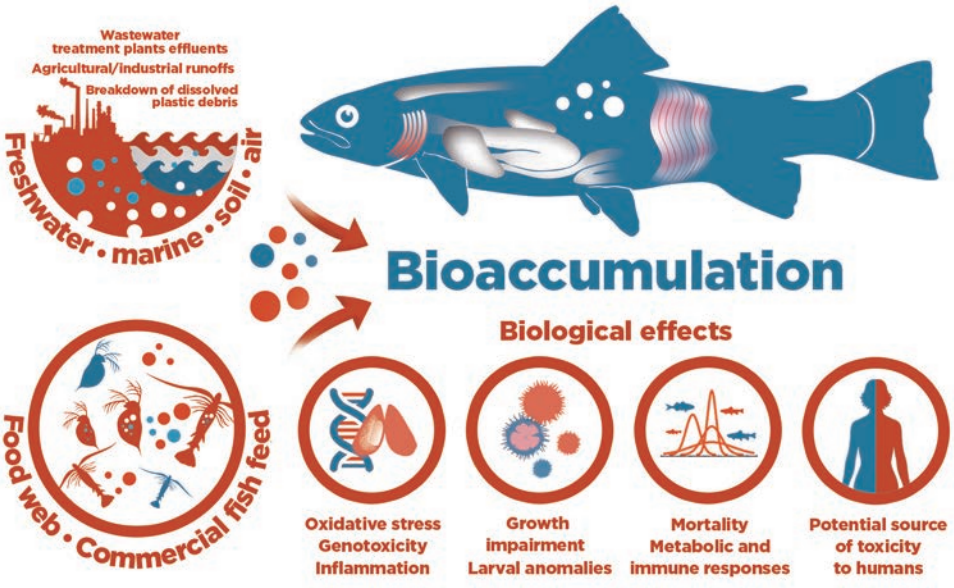

Figure 7. Summary of the reported effects of nanoplastics in fish species.

antioxidant capacity, by inducing the production of reactive oxygen species (ROS) (Lu et al., 2016; Veneman et al., 2017). Oxidative stress has been reported as the most frequent mechanism linked to NPs toxicity (Veneman et al., 2017; Brun et al., 2018; Jacob et al., 2020), consequently leading to genotoxicity (DNA damage), damage of biomolecules (e.g. lipid peroxidation) and ultimately and apoptosis (Sökmen et al., 2020). Changes in glucose metabolism and cortisol levels (Brun et al., 2019), lipid metabolism and accumulation (Lu et al., 2016), histopathological changes liver (Chae et al., 2018) have also been reported. Exposure to NPs has also been seen to interfere with the immune system in fish, for example inducing the expression of pro-inflammatory cytokines (Brun et al., 2018), activating the complement system (Veneman et al., 2017) or altering neutrophil function (Greven et al., 2016). Potential neurotoxic effects in terms of neurotransmission, documented through the decrease in acetylcholinesterase activity have also been reported in fish after the exposure to NPs (Chen et al., 2017).

The trophic transfer of NPs through dietary exposure have also been investigated, addressing their effects on fish as final consumers. NPs ingested through the food chain have shown to provoke behavioral disorders in the top consumer, such as decreased locomotion and predatory activity (Mattsson et al., 2015, 2017), and alterations in lipid metabolism related to impaired used of the animal's fat reserves (Cedervall et al., 2012).

Altogether, NPs have shown to alter relevant functions in fish, such as the immune system, antioxidant/oxidant balance or lipid metabolism. However, more studies need to be carried out to fully understand how the toxicity of NPs affects fish. Still poorly studied are the effects of NPs on fish species besides model laboratory species, which is of great importance to understand the global effects of NPs in biota and ecosystems, as well as to their potential effects on fisheries and aquaculture, and consequent effects on human-beings. Moreover, besides the studies on effects on individual species, it has been demonstrated that NPs can easily enter aquatic food webs and escalate trophic levels, which has greater ecological significance and could also potentially affect humans (Mattsson et al., 2015, 2017; Peng et al., 2020). Although research evaluating the effects of NPs have multiplied in the last years, there are still many notable knowledge gaps in the field, such as the modes of action of NPs, the effects of different plastic polymers or their effects in chronic exposures.

1.5. Species of study

The development of a knowledge base on the effects NPs might have on aquatic organisms is urgently needed to be able to assess the associated risks. A mixed strategy was adopted in this thesis, using a combination of a classical laboratory model species (*D. rerio*) and two commercial marine fish species relevant for human consumption (*Sparus aurata* and *Dicentrarchus labrax*) and model species of stress in marine fish (Cerqueira et al., 2020; Raposo de Magalhães et al., 2020). Both are valuable approaches and a combination of them is probably the best strategy to attempt to fill all the knowledge gaps that this novel field still has. On one side, model species can be used to understand principles phenomena that are not specific to this organism but are of a more general nature (Segner and Baumann, 2016). They also present easy maintenance, reproduction and manipulation in a laboratory setting, and provide biological systems at the cell, tissue, organ, and system level. Moreover, the fact that the same organisms (with similar or identical population traits) are used by multiple research teams allows better comparability of studies. Nevertheless, a central issue in ecotoxicology is diversity, both of toxicological and ecological effects and of target species (Segner and Baumann, 2016). Furthermore, one of the ultimate questions in NPs ecotoxicology is whether these CECs pose a concern for human health. In this direction, investigating their effects in organisms that will be directly ingested by humans is a relevant research topic and where we have directed the focus of our research. To the best of our knowledge, no studies on marine fish species directed to human consumption had been done prior to the beginning of this thesis.

Danio rerio (zebrafish)

Zebrafish is a classical species for aquatic ecotoxicological studies as well as to understand ecophysiological adaptations in vertebrates. This species has several important advantages as are its small size, ease of maintenance and breeding, short life cycle, high fecundity and year-round spawning. The availability of molecular and cellular tools in this species, together with its versatility, has popularized the use of zebrafish in multiple fields, becoming a powerful tool in genetics, development, environmental toxicology, human diseases, and pharmacology (Zhang et al., 2003). In ecotoxicology and environmental monitoring zebrafish have been extensively used for pollutant evaluation (such as toxic heavy metals, endocrine disruptors, and organic pollutants, Magyary, 2018), as a tool to detect toxins in water samples, and to investigate the mechanisms of action of environmental pollutants (Bambino and Chu, 2017). Particularly, the easy obtention of a large number of transparent embryos and rapid embryonic development into semi-transparent larvae, provides a practical and cost-effective tool to study the potential toxicity of environmental contaminants. Moreover, zebrafish have external embryo development, allowing non-invasive techniques that reduce animal suffering and lead to accurate results in a short time span. Accordingly, zebrafish has recently started to be used as a model to assess NPs toxicity, with about 20 papers published since 2016, focusing on different stages of the life cycle (Bhagat et al., 2020).

The fact that zebrafish embryo and larvae are transparent is a huge advantage to study the uptake and localization of fluorescently labelled NPs. Through this methodology, accumulation of NPs has been studied in zebrafish embryo and observed in chorion (Batel et al., 2016; Pitt et al., 2018; Lee et al., 2019), intestinal tract (van Pomeren et al., 2017; Parenti et al., 2019), brain (Sökmen et al., 2020), epidermis and eye (van Pomeren et al., 2017). In larvae, NPs have been located in brain, pericardium (Pitt et al., 2018), gastrointestinal tract (Batel et al., 2016; Pitt et al., 2018; Brun et al., 2019; Qiang and Cheng, 2021), pancreas and gall bladder (Brun et al., 2019). Finally, in adults the accumulation of NPs has been observed in almost all tissues, with studies reporting NPs in head, brain, gills, muscle, viscera, gonads, liver and intestine (Chen et al., 2017; Qiao et al., 2019; Gu et al., 2020; Sarasamma et al., 2020). An extensive list of effects have been reported in zebrafish after exposure to NPs in all life stages, such as increased mortality, DNA damage, development abnormalities, reduced/altered hatching, increased ROS production and oxidative stress, inflammation, altered swimming behaviour, altered gene expression, increased

pollutant (Bisphenol A) uptake, disturbed lipid metabolism, intestinal damage or disfunction, altered microbiome and altered hormone activity (Veneman et al., 2017; Jin et al., 2018; Lei et al., 2018; Pitt et al., 2018; Lee et al., 2019; Parenti et al., 2019; Gu et al., 2020; Sarasamma et al., 2020; Sökmen et al., 2020; Qiang and Cheng, 2021).

Aquaculture species – Sparus aurata and Dicentrarchus labrax

The gilthead seabream (*S. aurata*) is a fish from the Sparidae family that can be found in coastal regions of the north-eastern Atlantic Ocean (from Great Britain to Senegal) and in the Mediterranean Sea. It's a euryhaline and eurythermal species that adjusts to both marine and brackish waters, generally depending on the life cycle stage. Wild seabream can be found in sandy or rocky bottoms and in seagrass meadows and have a carnivorous diet, with crustaceans, mollusks, polychaetes, teleosts and echinoderms as the major dietary groups and a trophic level of 3.3–3.5 (FishBase, 2018). It is extensively cultured, especially in the Mediterranean area, in floating net pens both in coastal lagoons and in open sea installations. The gilthead seabream was chosen as the main human consumption species to investigate, as one of the most relevant commercial fish in the Mediterranean region. It had global joint aquaculture and capture fisheries production of over 250 thousand tons in 2019 (FAO, 2021). Seabream aquaculture production started to raise in the 1980s and nowadays aquaculture accounts for 96.4% of the total supply of this species (APROMAR, 2020).

The European seabass (*Dicentrarchus labrax*) is a carnivorous species, feeding mainly on zooplankton as a juvenile and other fish species as an adult, and is a top predator in its environment, with an overall trophic level of 3.8 (FAO, 2021). Like seabream, seabass is a eurythermic and euryhaline species, capable of adapting to a wide range of salinities and found in marine waters but often in shallow coastal waters and migrating towards estuaries in juvenile stages (FishBase, 2021). The European seabass, present both in the Atlantic and Mediterranean, with a very similar distribution to S. aurata. It is a commercial species, consumed both from capture fisheries and from aquaculture production, considered to be the one of the most important fish currently cultured in the Mediterranean, where it reaches values of over 220 tonnes (APROMAR, 2020). Although annual catches from capture fisheries are relatively low, this species is under increasing pressure from commercial fishing, which has led to a decline in its total wild population biomass and has therefore become focus of conservation efforts in some countries (ICES, 2013).

Both *S. aurata* and *D. labrax* have been previously used in ecotoxicology to assess the effects of environmental pollutants, studying alterations at the molecular, cellular, and physiological levels. The gilthead seabream has been used in research investigating the effects of pharmaceuticals (Teles et al., 2016a; Rodrigues et al., 2018), heavy metals (Guardiola et al., 2013) and nanoparticles (Teles et al., 2016b). In turn, *D. labrax* has been a target species in studies regarding organochlorine compounds (Carubelli et al., 2007), polycyclic aromatic hydrocarbons (Ferreira et al., 2010), heavy metals ((De Domenico et al., 2013; Maulvault et al., 2017) and nanomaterials (Picchietti et al., 2017). The estuarine and marine environments where both seabream and seabass develop their life cycle or alternatively where aquaculture production systems are emplaced coincide with areas of high plastics presence, where high NPs concentrations are expected (Cózar et al., 2014; Lehner et al., 2019), supporting the need of studies assessing the effects of NPs in these fish species. Moreover, the fact that they are both carnivorous species at a high level in the trophic chain could lead to bioaccumulation of NPs in their tissues and a higher risk of transfer to the final consumer. As stated beforehand, the transfer of NPs trough the trophic chain reaching the top predator has already been demonstrated in laboratory exposures (Mattsson et al., 2015; Chae et al., 2018).

The fact that the Mediterranean Sea is one of the most polluted marine areas highlights the need to investigate the effects that NPs may have on the two most important commercially cultured fish in the Mediterranean: the gilthead seabream and the European seabass. The global aquaculture production of seabream plus seabass was estimated to be 465 thousand tons in 2019, with the first sale value of both species together amounting to more than 2,000 million euros (APROMAR, 2020).

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Objectives

2

The main aim of these PhD thesis was to contribute to the knowledge on ecotoxicology of NPs using teleost fish as a vertebrate model. Moreover, we chose to put specific focus on two marine fish species relevant for human consumption. As mentioned in the introduction, the two plastic polymers chosen are known to have been found in the marine environment and can therefore be available to fish in their habitat. No studies had previously been done on the effects of NPs in (marine) human consumption species although one of the ultimate unanswered questions of NPs ecotoxicology is if they pose a risk for humans. Therefore, investigating the effects of NPs in fish species that are ingested by humans can give relevant information, both for human health and for fish production.

The **general objective of this thesis** was to investigate the short-term effects of NPs in fish, using a gold standard laboratory model (*Danio rerio*) and two marine fish species relevant for human consumption (*Dicentrarchus labrax* and *Sparus aurata*), widely used to model ecotoxicological and stress-related immune disturbances.

The **specific objectives** are as follows:

Objective I

To gain a better understanding on the internalization and accumulation of NPs in cells and fish.

Objective II

To explore potentially altered molecular pathways by an exposure to NPs in fish, through the study of gene expression.

Objective III

To evaluate potential biomarkers altered in relevant commercial fish species as a consequence of the exposure to NPs.

Objective IV

To assess which tissues might be more sensible to NPs and if the exposure can be monitored through non-invasive sampling matrices.

Outlined below are the studies that were conducted to address the specific objectives and that conform the chapters of this thesis. They are presented in the form of scientific papers that have been published in peer-reviewed scientific journals.

Study 1. Polystyrene nanoplastics accumulate in ZFL cell lysosomes and in zebrafish larvae after acute exposure, inducing a synergistic immune response in vitro without affecting larval survival in vivo

Study 2. A baseline study on the impact of nanoplastics on the portals of entry of xenobiotics in fish

Study 3. Waterborne exposure of gilthead seabream (*Sparus aurata*) to polymethylmethacrylate nanoplastics causes effects at cellular and molecular levels

Study 4. Short-term exposure to polymethylmethacrylate nanoplastics alters muscle antioxidant response, development and growth in *Sparus aurata*

Study 5. Effects of polymethylmethacrylate nanoplastics on *Dicentrarchus labrax*.

Polystyrene nanoplastics accumulate in ZFL cell lysosomes and in zebrafish larvae after acute exposure, inducing a synergistic immune response *in vitro* without affecting larval survival *in vivo*

Irene Brandts, M. Garcia-Ordoñez, L. Tort, M. Teles and N. Roher

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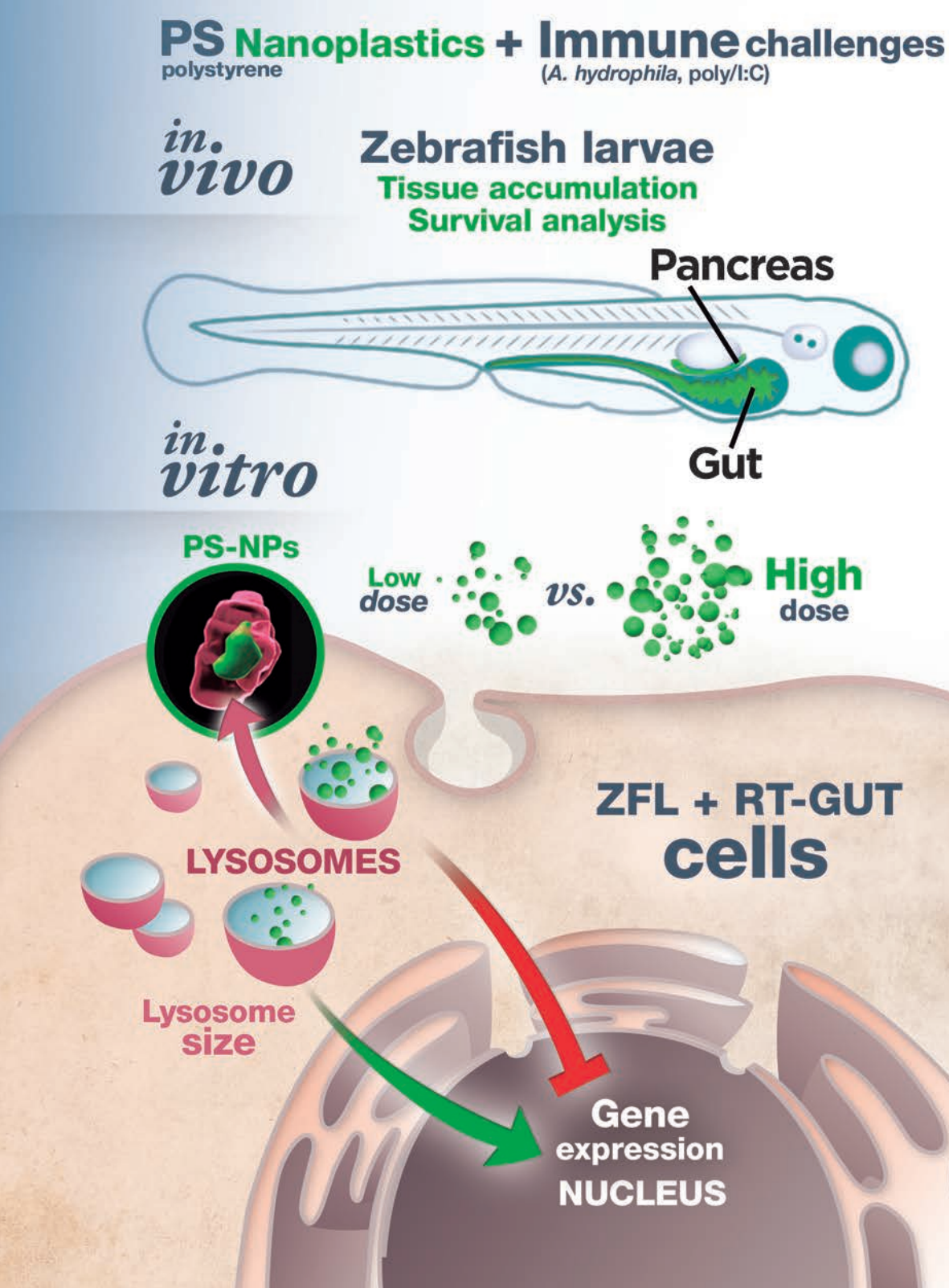
Abstract

The presence of small-sized plastic particles in marine and freshwater environments is a global problem but their long-term impact on ecosystems and human health is still far from being understood. Nanoplastics (<1000 nm) could pose a real and uncontrolled ecological challenge due to their smaller size and sharp ability to penetrate living organisms at any trophic level. Few studies evaluate the impact of nanoplastics *in vivo* on the immune system of aquatic organisms, while most of them assessed the impact on indirect markers of immune response such as regulation of gene expression, ROS production or DNA genotoxicity, among others. Moreover, the study of the effects of nanoplastics on aquatic vertebrate species *in vivo* is still scarce. In this context, we seek to shed light on the underlying effects of polystyrene nanoplastics (PS-NPs) on the immune response in a model fish species (*Danio rerio*, zebrafish) after an acute exposure, with a combination of *in vitro* and *in vivo* experiments. Our results show that PS-NPs (65 nm) are efficiently taken up by zebrafish liver cells, accumulating mainly in lysosomes. Furthermore, the expression of immune genes presents a synergy when cells were simultaneously exposed to PS-NPs, at a low dose and early time point (12 h) and challenged with a viral stimulus (poly(I:C)). Moreover, zebrafish larvae also internalize PS-NPs, accumulating them in the gut and pancreas. However, at concentrations of up to 50 mg l^{-1} in an acute exposure (48 h), PS-NPs do not interfere with the survival of the larvae after a lethal bacterial challenge (*Aeromonas hydrophila*). This study addresses the relevant environmental question of whether a living organism exposed to PS-NPs can cope with a real immune threat. We show that, although PS-NPs can induce an immune response, the survival of zebrafish larvae challenged with a bacterial infection after an acute exposure to PS-NP is not decimated with respect to unexposed larvae.

Graphical abstract



Environmental significance

Small sized plastic particles have been reported around the planet in all kinds of environments and it is generally accepted that microplastics are able to cause adverse effects on organisms. However, the understanding of the effects and possible implications of nanoplastics on vertebrate species is still scarce. The impact of polystyrene nanoplastics (PS-NPs) on indirect markers of immune response (such as regulation of gene expression, ROS production or DNA genotoxicity) has been to some extent previously assessed. Nevertheless, a key question remains unexplored: can an organism exposed to nanoplastics successfully cope with an additional immune challenge? This is the first study to provide data on how the immune system of a vertebrate species might respond to a combined challenge of nanoplastics and a pathogen in the surrounding environment. We show that an acute exposure of zebrafish larvae to PS-NPs at high concentrations has no effect on the survival rate of the larvae after a bacterial infection. Thus, this study provides novel insight beyond the current understanding of the potential interplay between pathogen and nanoplastic contamination.

1. Introduction

After more than 50 years of plastic production, with 360 million tonnes manufactured solely in 2018,[1] and the subsequent accumulation in the natural environment, the impact of plastics in ecosystems worldwide is just starting to be understood. Although nanoplastics, defined as particles ranging from 1 nm to 1000 nm resulting from degradation of plastic objects,[2,3] have already been detected in the environment,[4] their precise quantification in natural aquatic systems and the establishment of environmentally relevant concentration are still under debate.

There is a general consensus on how microplastics are able to cause adverse effects on marine and freshwater species (see Chae *et al.*[5] for a review). However, there are still many unanswered questions in nanoplastic research, regarding for example their final destiny once absorbed/ingested, their long-term effects or their interaction with microbial communities. One of these relevant knowledge gaps is whether nanoplastic pollution can impair the capacity of organisms to fight against pathogens. This publication aims to start the unravelling of this last question, combining an *in vitro* and *in vivo* approach.

Zebrafish (*Danio rerio*) is considered a good model for eco-toxicological studies as well as a tool to understand eco-physiological adaptations in vertebrates. The versatility of this model, together with the availability of molecular and cellular tools, has popularized the use of zebrafish. Previous studies exploring the effects of polystyrene nanoplastics (PS-NPs) on zebrafish have described a broad physiological response, not particularly specific to a given tissue or cell type, but more typical of a general stress response (*e.g.* oxidative stress, complement system or glucose metabolism). Alterations in behaviour, measured by a decrease[6] or increase[7] in locomotor activity, changes in glucose metabolism and cortisol levels,[7] intestinal and skin damage triggering a pro-inflammatory response[8] and production of oxidative stress and lipid accumulation,[9] have been documented in zebrafish after waterborne exposure to PS-NPs. Other studies have evaluated the effects of direct injection of PS-NPs on zebrafish embryos, describing changes in the transcriptome[10] or production of reactive oxygen species (ROS) and apoptosis.[11]

Moreover, PS-NPs have been previously found to interfere with the immune system of zebrafish[8,10,12] as well as other aquatic species.[13,14] In *D. rerio*, PS-NPs have been shown to upregulate the expression of inflammatory cytokines,[8] activate the complement system and oxidative stress mechanisms[10] and provoke

DNA damage, induced by ROS and apoptosis.[11] In this context, we seek to address the question of whether their interaction with the immune system can affect the survival of populations in their natural environments, where organisms are frequently exposed to pathogens. To our knowledge, barely any studies on the combination of PS-NPs and an immune challenge have been performed. In a recent study, Sendra *et al.*[14] evaluated the response of *Mytilus galloprovincialis* haemocytes *in vitro* when infected with *Vibrio splendidus*, following an exposure to PS-NPs. They reported that the invertebrate's haemocytes showed resilience when infected with the bacteria, being able to recover their phagocytic capacity. In the present work, we focus the attention in this direction, to determine whether the combination of an acute exposure to PS-NPs and a subsequent lethal bacterial challenge can shed light on the underlying toxic effects of PS-NPs on aquatic organisms.

At the present time, assessment of environmental concentrations of nanoplastics is largely speculative, primarily due to the lack of efficient analytical methods.[15] In one relevant study by Gallego-Urrea *et al.*[16] measured 10^7–10^9 particles per ml (100–250 nm size) in solution, using nanoparticle tracking analysis (NTA), in different sample sites of Scandinavian waters.[16] The exposure concentrations used in our study fall within this particle range and were chosen taking into account the concentrations used in relevant studies with PS-NPs published in the recent literature[6,8,14,17] and our own initial *in vitro* results, aimed at assessing the interaction of the zebrafish liver (ZFL) cell line with PS-NPs. Even though the tested concentrations could be in the higher range of estimated environmental nanoplastic concentrations, they allow an approximation to the potential response of zebrafish to a double challenge with PS-NPs and a pathogen.

Our results present evidence that PS-NPs are toxic to ZFL cells at doses higher than 75 mg l^{-1} and can accumulate rapidly inside liver cells, specifically in lysosomes. We show that PS-NPs at low doses (5 mg l^{-1}) and early stimulation times (12 h) synergistically activated the expression of anti-viral genes, while low doses and a longer exposure time did not have any effect. Moreover, we did not observe this synergistic interaction at a high dose (50 mg l^{-1}) and early stimulation time.

Similarly, in the *in vivo* experiment, PS-NPs accumulated in the zebrafish larvae intestine and pancreas, but this accumulation did not impair the larvae's defence mechanisms against a bacterial lethal challenge with *Aeromonas hydrophila*. Thus, zebrafish larvae pre-exposed to PS-NPs had the same levels of survival as unexposed larvae.

2. Materials and methods

Characterization of polystyrene nanoplastics (PS-NPs) by DLS and electronic microscopy

Fluorescently labelled PS-NPs (FSDG001 Dragon Green, Bangs Ltd.) were purchased from Bang Laboratories (Fisher, IN, USA). PS-NPs were provided as a 1% (w/w) suspension in water with 2 mM NaN_3. The particle size distribution and zeta potential were measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, UK), under different incubation conditions: pure water (Sigma), PBS (Sigma), cell culture medium (DMEM, Gibco) and E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ and 0.1% methylene blue).

Emission scanning electron microscopy (FESEM, Zeiss Merlin) was used to determine the external morphology and physical dimensions of the PS-NPs in pure water and cell culture medium. Exposure solutions were prepared by resuspending the stock solution of PS-NPs in PBS and further diluting in each medium, at 100 µg ml^{-1}. For electron microscopy, 20 µl of each solution were pipetted onto silicon chips and air-dried O/N. A palladium–gold coating was applied prior to observation. Images were analysed using the Fiji open source image processing package,[18] measuring the dimensions of a minimum of 150 particles for each condition. Size distribution histograms were generated using Prism 7.01 (GraphPad software).

Decay of PS-NP fluorescence *in vitro* and in cell culture

Fluorescently-labelled nanoplastics could leach fluorophores, since they are not covalently linked to the PS-NPs.[19] Catarino *et al.*[20] suggested careful testing of the fluorescent particle uptake in order to avoid wrong conclusions. To establish the true internal accumulation of fluorescent PS-NPs, the following experiment was set up: 1 ml of PS-NP suspension (1000, 100 and 50 mg l^{-1}) was centrifuged at 50 000 × *g* for 60 min, then the supernatant (SN) was transferred to a new Eppendorf tube and the pellet (P) resuspended in 1 ml of PBS. The fluorescence intensity in the different fractions (P, SN and stock solution) was evaluated in solution using a Cary Eclipse fluorescence spectrophotometer (Agilent). At the lowest tested concentration, approximately 25% of the fluorescence remained in SN and we can therefore assume that it leached from the PS-NPs. To further confirm that the fluorescence inside the cells was not due to leached fluorophore, ZFL cells were treated with the PS-NP original suspension, SN and P and the uptake was monitored by cytometry as explained below.

Zebrafish liver (ZFL) and RTGut cell culture

ZFL cells (CRL-2643, ATCC) were cultured at 28 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) 4.5 g l^{-1} glucose, supplemented with 0.01 mg ml^{-1} insulin, 50 ng ml^{-1} EGF, 5% (v/v) antibiotic/antimycotic solution, 10% (v/v) heat inactivated fetal bovine serum (FBS) and 0.5% (v/v) heat-inactivated trout serum (TS) as described in Torrealba *et al.*[21] RTGut cells were obtained from Dr. Carolina Tafalla's laboratory and cultured as described by Kawano *et al.*[22] Briefly, cells were kept at 20 °C in Leibovitz's L-15 Medium GlutaMAX™ (Gibco) #31415029), supplemented with heat-inactivated FBS 10% (v/v) and 1% antibiotic/antimycotic.

PS-NP cytotoxicity studies in ZFL

Cytotoxic and cytostatic effects of PS-NPs on ZFL were assessed using the MTT assay. After 2.5 h in minimal medium (0–0.5% FBS; 2% antibiotic/antimycotic), cultures were incubated with PS-NPs at 0, 0.05, 0.5, 5, 10, 25, 50, 100, 250, 500 and 1000 mg l^{-1} for 20 h at 28 °C. A 0.2 mM sodium azide control was also included. Cells were then washed in PBS and the MTT substrate (Sigma-Aldrich) was added to 10% of the total volume and further incubated at 28 °C for 1–2 h. The solution was removed, the cells were solubilized in DMSO and the lysate was read on a Victor 3 (PerkinElmer) at 550 nm. The experiment was repeated three times. Data were normalized using Prism 7.01 (GraphPad) such that the control readings were set at 100%. One-way ANOVA was performed with Dunnett's multiple comparison test, comparing treatment and control means.

Uptake of PS-NPs by ZFL assessed using flow cytometry and confocal microscopy and in RTGut by microscopy

To test cellular uptake, fluorescently labelled PS-NPs were added to ZFL cultures at 70% confluence after 2 h of incubation in minimal medium, at the doses and times indicated below. Representative cytometry plots with the gating strategy and the threshold of fluorescence are shown in **Supplementary Fig. 1**.

For dose–response assays, cultures were incubated for 20 h with PS-NPs at 0.05, 0.5, 5, 10, 25, 50, 100, 250, 500 and 1000 mg l^{-1}, at 28 °C. For time-course assays, cultures were incubated with PS-NPs at 25, 50 and 75 mg l^{-1} for 6, 12, 24, 48 and 72 h, at 28 °C. For positive control of uptake, Atto-488 conjugated TNFα was used, as its uptake has been previously characterized by the research group in this cell line.[21] Both dose–response and time-course experiments were

performed in triplicate. Post treatment, cells were washed in PBS and incubated at 28 °C with 1 mg ml^{-1} trypsin (Gibco) for 15 min. This strong trypsinization step aimed to remove PS-NPs attached to the cell surface.[21] Then, two volumes of complete medium were added, and cells were retrieved by centrifugation at 300 × *g* for 5 min. Pellets were resuspended in PBS for flow cytometry (FACSCalibur BD), and 10 000 events were counted. Data were analysed using Flowing Software 2.5.1 (University of Turku, Finland) and plotted with Prism 7.01 (GraphPad). One-way ANOVA followed by Dunnett's multiple comparison test was performed, comparing treatment and control means.

To confirm that the fluorescent PS-NPs were inside the cells, confocal microscopy was performed (Leica SP5). ZFL cells were seeded on ibidi 35 mm glass bottom dishes (Ibidi GmbH, Germany). The next day, cells at approximately 60% confluence were placed in minimal medium. PS-NPs were added 2–3 h later at a concentration of 5 or 50 mg l^{-1} and cells were incubated for 24 h at 28 °C. Cells were then washed and the medium replaced with new minimal medium. RTGut cells were treated in the same way for confocal microscopy but incubated with PS-NPs at 10 and 25 mg l^{-1}, at 20 °C. The cells were stained with Hoechst (nuclei), either WGA Alexa Fluor555 or Cell Mask Deep Red (membrane) and LysoTracker Red (lysosomes). Images were analysed using Imaris software v9.3 (Bitplane) and Image J.

Lipid peroxidation

ZFL cells at 60% confluence were cultured in minimal medium (0.5% FBS; 2% A/A) for 2–3 h and then exposed to PS-NPs at 5 mg l^{-1} and 50 mg l^{-1} for 24 h. After the exposure, cells were stimulated with poly(I:C) (25 μg ml^{-1}), a synthetic analog of dsRNA virus, for 16 h. Controls were: poly(I:C) 25 μg ml^{-1} (Sigma-Aldrich) and control cells with neither viral stimulus nor PS-NP exposure. Moreover, each PS-NP exposure time and concentration had a respective control group, with no viral stimulus. After completing the exposure, ZFL cells were washed with PBS and total lipid peroxidation was assessed using the lipid peroxidation (MDA) assay kit (Sigma) following the manufacturer's instructions. The experiment was repeated twice.

RNA extraction and gene expression analysis of ZFL cells treated with PS-NPs

ZFL cells were plated and grown to 60% confluence, cultured in minimal medium for 2–3 h and then exposed to PS-NPs ± poly I:C as described above, for the lipid peroxidation assay. For gene expression analysis, an additional

exposure time to PS-NPs of 12 h was added, together with the 24 h exposure previously described. Total RNA was extracted using TriReagent (Sigma-Aldrich) following the manufacturer's instructions. RNA was quantified using a nanodrop ND-1000 (Thermo Fisher Scientific) and integrity was checked on an Agilent 2100 Bioanalyser using the RNA 6000 Nano Lab-Chip kit (Agilent Technologies). The experiment was repeated, and four complete sets of high-quality RNA from two independent experiments were selected for cDNA synthesis using 1 µg of total RNA and the iScript cDNA synthesis kit (Bio-Rad). RT-qPCR was performed in a CFX384 touch real-time PCR detection system (Bio-Rad) using the iTaq universal SYBR green supermix kit (Bio-Rad) following the manufacturer's instructions. In brief, each PCR mixture consisted of 5 µl SYBR green supermix, 0.4 µM specific primers (**Supplementary Table 1**), 2 µl diluted cDNA and 2.6 µl water (Sigma-Aldrich) in a final volume of 10 µl. Primers for the assessed genes had been previously designed and tested by the research team in the ZFL cell line.[23]

A reference gene (*elongation factor 1alpha* (*ef1*-α)) and three gene markers of the innate immune response to viral infection (*IFN-induced protein Mx* (*mx*), *viperin* (*vig1*) and *grass-carp-reovirus-induced gene 2* (*gig2*)) were used. All the samples were run in triplicate, and data were analysed using the Livak method.[24] For statistical analysis used, a one-way ANOVA test, followed by Dunnett's multiple comparisons for each treatment *versus* control, was conducted; $p < 0.05$ was considered statistically significant in all analyses (GraphPad Prism v7.0).

Zebrafish husbandry and breeding

Wild type zebrafish (*D. rerio*) were kept in a re-circulating aquarium with water temperature maintained between 26 and 28 °C. The lighting conditions were 14 : 10 h (light : dark) and adult fish were fed twice a day at a rate of 2% bodyweight. Ammonia, nitrite, pH and nitrate levels were measured once a week. Ammonia and nitrite levels were kept below the detection level and pH maintained between 6.8 and 7.5. The nitrate levels were maintained to be <100 mg l^{-1}. For in-tank breeding, one female and three males were transferred to a breeding tank in the late afternoon. The divider was removed on the next morning after the onset of light. Embryos were collected after 1–2 h and cultured in embryo medium (E3 medium) in a Petri dish (Deltalab). Fertilized eggs were separated from unfertilized eggs using a plastic pipette (Deltalab). All experiments involving zebrafish (*D. rerio*) were performed following International Guiding Principles for Research Involving Animals (EU 2010/63) and previously authorized by

the Ethics Committee of the Universitat Autònoma de Barcelona (UAB, CEEH number 1582).

Uptake of PS-NPs by zebrafish larvae assessed by fluorescence microscopy

Groups of 14 larvae (n = 14 per condition) were distributed on 96-well plates (ThermoFisher) with one larva per well containing 200 µl E3 medium or fluorescent PS-NPs at 5, 50 and 100 mg l^{-1}. Mortality was recorded for 96 h and zebrafish larvae were observed using a fluorescence stereomicroscope (Nikon SMZ800) coupled with a camera (Nikon DS-Fi2).

A. hydrophila culture and zebrafish larvae infection

Experimental infections were performed as previously described.[25] Briefly, bacteria were grown on LB agar plates overnight at 28 °C, collected from the plates in an Eppendorf tube, washed with PBS and finally resuspended with E3 to obtain a stock solution containing approximately 10^{10} colony-forming units (CFUs) per ml (OD_{620nm} = 1.3). Dilutions at the desired concentration were all prepared from the stock solution. *A. hydrophila* infection was carried out by bath immersion according to Ji *et al.*[25] Groups of 72 larvae (n = 72 per condition) were distributed on 96-well plates (ThermoFisher) with one larva per well containing 200 µl E3 medium or PS-NPs. For infection, the bacteria were diluted from the stock solution using E3 medium in serial 100-fold dilutions from 10^{-1} to 10^{-9}. The bacterial dilutions of 10^{-7}, 10^{-8} and 10^{-9} (100 µl of each) from the stock solution were inoculated on LB plates and incubated overnight at 28 °C to calculate the real CFUs during the infection. The survival curves were analysed, and statistical differences were assessed using the log-rank test (GraphPad Prism v7.0).

3. Results and Discussion

Characterization of PS-NPs in biological relevant buffers

The size and shape of fluorescently labelled PS-NP particles were evaluated under different conditions by scanning electronic microscopy and DLS (**Fig. 1a–c**). We performed incubations at 24 and 72 h in different biological media: phosphate buffer saline (PBS), cell culture medium (DMEM) and larvae water (E3) (**Fig. 1a**). DLS results showed a particle size of 65.1 ± 0.6 nm in water, 61.3 ± 1.4 nm in PBS, 67.2 ± 1.2 nm in DMEM and 59.6 ± 0.4 nm in E3 after 24 h and 72 h. No major changes were observed using DLS under any conditions

with respect to pure sterile water. No aggregation of the particles over time was found (PDI less than 0.09) and the zeta potential was stable and did not change significantly over time, ranging between –20 and –32 mV and thus indicating a stable dispersion of the PS-NPs in suspension. Further confirmation of their characteristics was performed by direct sizing of FESEM images, showing values of 65.2 ± 6.5 nm (water) and 64.9 ± 7 nm (DMEM) (**Fig. 1b and c**). Moreover, the expected spherical morphology was observed by FESEM under both conditions (**Fig. 1b and c**). As shown in **Fig. 1**, the mean size, aggregation and morphology did not change significantly after 24 and 72 h of incubation in DMEM nor E3. Overall, common biological buffers and media did not change the physico-chemical properties of PS-NPs. The same commercially available PS-NPs were used by Cui *et al.*,[26] who reported a similar zeta potential and mean size in a slightly different zebrafish incubation medium. Pitt *et al.*[6] reported a similar zeta potential but different mean size (34.8 ± 10 nm). This difference is probably due to the different compositions of the zebrafish incubation media.

To further study PS-NPs' interaction dynamics with living cells, we used PS-NPs labelled with a green fluorophore (Dragon Green). This fluorophore is not covalently linked to the PS-NPs and could be drained from the NPs. In order to discard uncontrolled loss of fluorescence, we evaluated the fluorophore both by fluorimetry (**Supplementary Fig. 2a**) and cytometry (**Supplementary Fig. 2b and c**). The fluorescence signal at three different concentrations (1000, 100 and 50 mg l^{-1}) was stable under the assayed conditions and the amount of leached fluorophore reached a maximum of 25% after dilution and drastic manipulation (**Supplementary Fig. 2a**). Although there is an apparent increase of fluorescence loss as the PS-NP concentration decreases, this can be

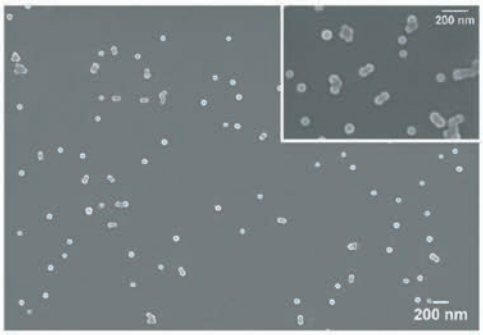
Figure 1. Characterization of fluorescently labelled polystyrene nanoparticles (PS-NPs) in different biological media.

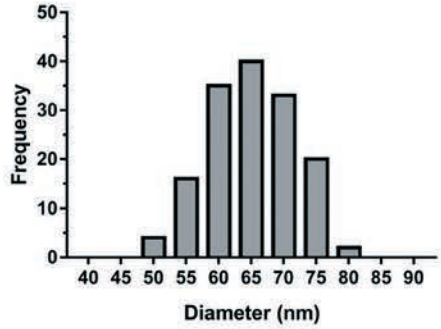
(a) PS-NP properties (size, PDI and ζ potential) assessed by dynamic light scattering, in pure water, PBS, cell culture medium (DMEM) and E3 embryo medium (E3). Field emission scanning electron microscopy representative images and size distribution histograms (n = 150) in **(b)** pure water and **(c)** DMEM. **(d)** Cytotoxicity of PS-NPs in zebrafish liver cells (ZFL). Cell viability after 24 h of incubation with PS-NPs at increasing concentrations (0.05 to 1000 mg l^{-1}). Untreated cells and NaN_3 treated cells were used as controls (C). An additional toxicity control with NaN_3 was included, with the potential maximum concentration of NaN_3 present at the highest concentration of PS-NPs. Data show the mean ± standard deviation (SD) of three independent experiments. Significant differences from the control are indicated as: $^*p < 0.05$.

a

		Tested Media			
		Water	*PBS*	*DMEM*	*E3*
Size (Z-average) (nm)	24 h	65,1 ± 0,6	61,3 ± 1,4	67,2 ± 1,2	59,6 ± 0,4
	72 h	64,4 ± 1,1	60,6 ± 0,8	71,3 ± 0,5	64,4 ± 0,7
Zeta potential (ζ-potential)	24 h	- 23,6 mV	- 19,8 mV	- 20,6 mV	- 29,8 mV
	72 h	- 32,6 mV	- 25,6 mV	- 21,8 mV	- 23,4 mV
PDI	24 h	0,03	0,02	0,05	0,02
	72 h	0,03	0,03	0,07	0,09

b





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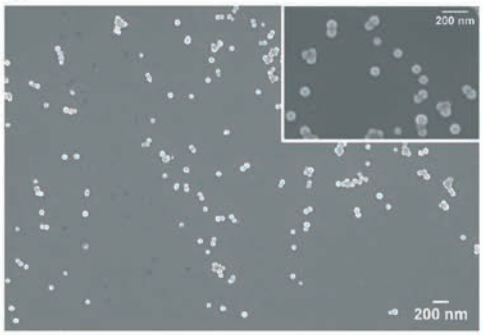

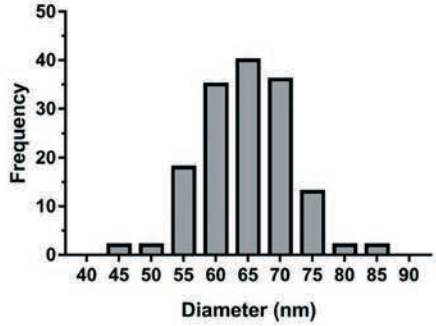

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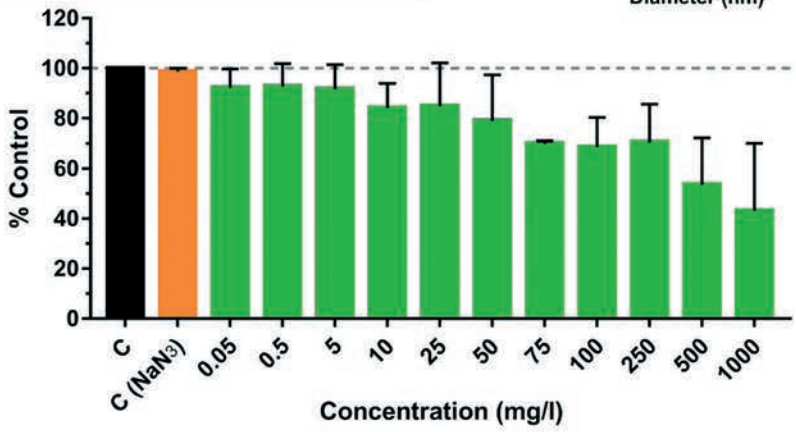


explained by the inner filter effect, a well-known phenomenon that causes a decrease of fluorescence intensity at high doses and a better yield at low doses. Therefore, the fluorescence measured at 50 mg l^{-1} is more precise to obtain real percentages of fluorescence loss. When the distinct fractions (PS-NPs; P, pellet; SN, supernatant) were used to evaluate the internalization of PS-NPs by ZFL cells (**Supplementary Fig. 2b and c**), we observed that a remnant of 24.7 ± 2.4% of cells were fluorescent when incubated with SN, while when incubated with the P fraction positive fluorescent cells were 78.2 ± 1.2%. According to Catarino *et al.*, fluorescent nanoplastics could have a significant loss of fluorescence and this draining has to be characterised using appropriate controls.[20] In the present study, most of the observed fluorescence is stably associated with PS-NPs and the fluorescence signal computed as a positive fluorescence signal by cytometry and confocal microscopy is truly bound to PS-NPs.

In vitro toxicity, uptake and accumulation of PS-NPs in ZFL cells

Liver is the main detoxification organ in vertebrates and accumulation of PS-NPs in zebrafish liver has previously been reported.[6,9] Understanding the interaction of PS-NPs with liver cells would help to better understand how aquatic vertebrates cope with the presence of nanoplastics in their natural environment. Therefore, in order to evaluate the endocytosis and potential toxic effects of PS-NPs, we choose ZFL cells as an *in vitro* model. ZFL cells exposed to PS-NPs showed a significant decrease in viability (68.8 ± 11.6%) after 20 h at concentrations of 100 mg l^{-1} and higher (**Fig. 1d**), although at 75 mg l^{-1} we already observed a non-significant decrease trend in cell viability.

The calculated LD50 of PS-NPs in ZFL cells was 188,8 mg l^{-1} (**Supplementary Fig. 3**), extremely high compared to other toxics or genotoxics such as $KBrO_3$ or MMS.[27] Other studies using different fish cell lines showed that toxicity is highly dependent on the cell line[28,29] but no data was available on any zebrafish cell line. In a recent study by Cortés *et al.*,[27] no toxicity was reported at concentrations of up to 200 mg l^{-1} PS-NPs, after 24 h of treatment. It is worth mentioning that the LD50 calculation methodology is originally designed to calculate lethal doses of drugs or chemicals. Due to this, and since no specific adaptation to nanoplastic toxicity has been made, we sustain that these LD50 calculations should be interpreted with caution. Considering the results of the viability and uptake assays as well as the previous literature, we decided to use 5 and 50 mg l^{-1} as a representative non-toxic low and high PS-NP dose. In all the cytometry experiments, a positive control of fluorescent nanoparticles with a well-characterised internalization behaviour was included.[21]

Results for the PS-NPs endocytosis showed that 100% of ZFL cells took up the fluorescent PS-NPs after 6 h of incubation (**Fig. 2a and c**). Johnston *et al.*[30] showed that 20 nm fluorescent PS-NPs were rapidly internalized in mammalian hepatocytes, as after a 30 min incubation the fluorescence was located inside the cells. The ZFL internalization dynamics follows a dose–response pattern, with cells incubated at higher doses and longer times presenting higher fluorescence (**Fig. 2b–d**).

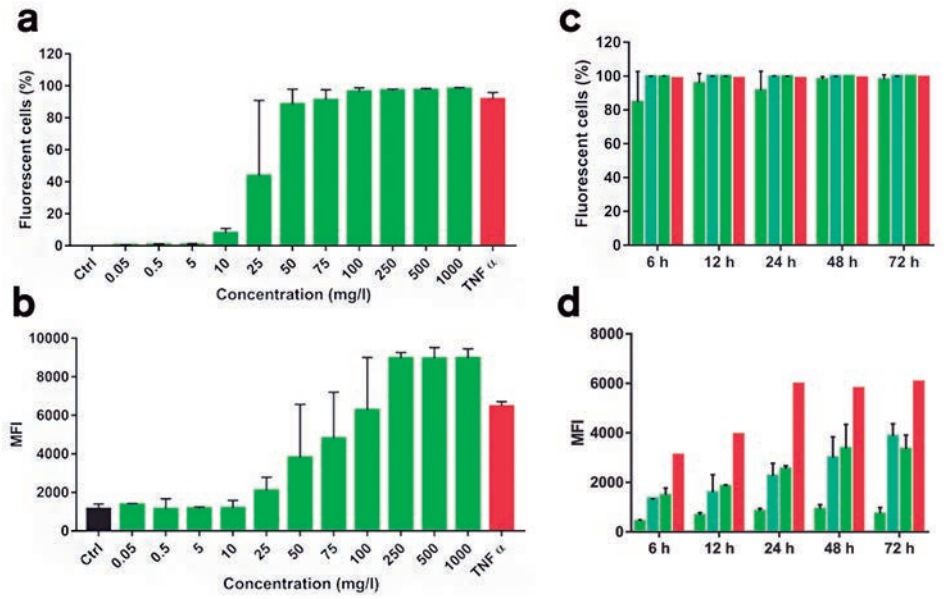


Figure 2 a-d. Uptake of fluorescently labelled PS-NPs by ZFL cells.

Dose–response: (a) percentage of fluorescent positive cells; **(b)** mean fluorescence intensity (MFI). Cells were incubated for 20 h with PS-NPs at an increasing concentration range (from 0.05 to 1000 mg l–1). Control (Ctrl) is ZFL cells without PS-NPs; a positive control of fluorescent nanoparticles (TNFα, in red) was included. **Time-course: (c)** percentage of fluorescent positive cells; **(d)** mean fluorescence intensity (MFI). Cells incubated for 6–72 h with PS-NPs at 25 (light green), 50 (mid green) and 75 (dark green) mg l–1. A positive control of fluorescent nanoparticles (TNFα, in red) was included. Data represent the mean ± SD of three independent experiments. One-way ANOVA was performed with Dunnett's multiple comparison test, comparing treatment and control means. Significant differences are indicated as: *$p < 0.05$; **, $p < 0.01$.

Internalization was confirmed by confocal microscopy at 5 and 50 mg l^{-1} PS-NPs (**Fig. 2e**; see **Supplementary Fig. 4** for the control). PS-NPs were internalized and found in the cytosol as quite large clusters (**Fig. 2e and f,** white arrows). At 50 mg l^{-1}, there was a massive accumulation in the cytosol (**Fig. 2e**) even though the cell viability was barely affected (**Fig. 1d**).

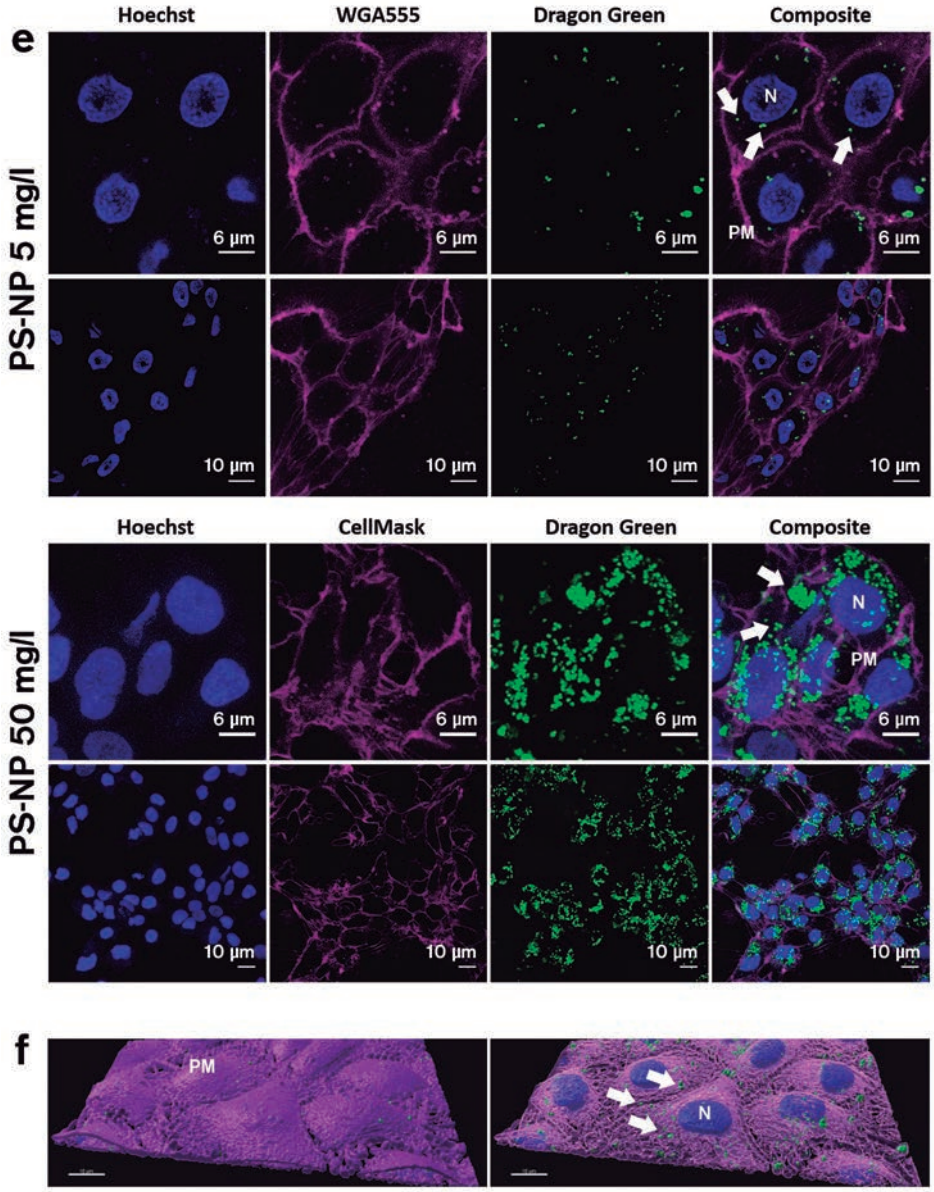


Figure 2 *(cont.)* **e-f. Uptake of fluorescently labelled PS-NPs by ZFL cells.**

(e) Confocal microscopy images of ZFL cells showing the internalization of PS-NPs, after 24 h of incubation at 5 and 50 mg l–1. **Green** fluorescence corresponds to PS-NPs (white arrows), **blue** to Hoechst stained nuclei (N), and **magenta** to WGA555 or Cell Mask stained plasma membrane (PM). **(f)** 3D image analysis of PS-NP uptake (z-stack) and whole-membrane reconstruction.

Co-staining with Cell Mask and 3D reconstruction using Imaris software confirmed that PS-NPs were totally embedded inside the cytosol (**Fig. 2f**). Confocal image analysis indicated that ≈9 PS-NP agglomerates of different sizes were observed per cell (9.23 ± 3.4 agglomerates per cell at 5 mg l^{-1}). Moreover, co-staining with Cell Mask (magenta), Hoechst (blue) and LysoTracker (red), a lysosome specific fluorophore, suggested that PS-NPs (green) accumulated in acidic lysosomes (**Fig. 2g**, white arrows). Lysosomes are dense spherical organelles, but they can display considerable variation in size and shape as a result of differences in the materials that have been taken up[31]. The 3D image reconstruction showed that PS-NPs and lysosomes co-localize in quite large lysosomal structures (**Fig. 2h**).

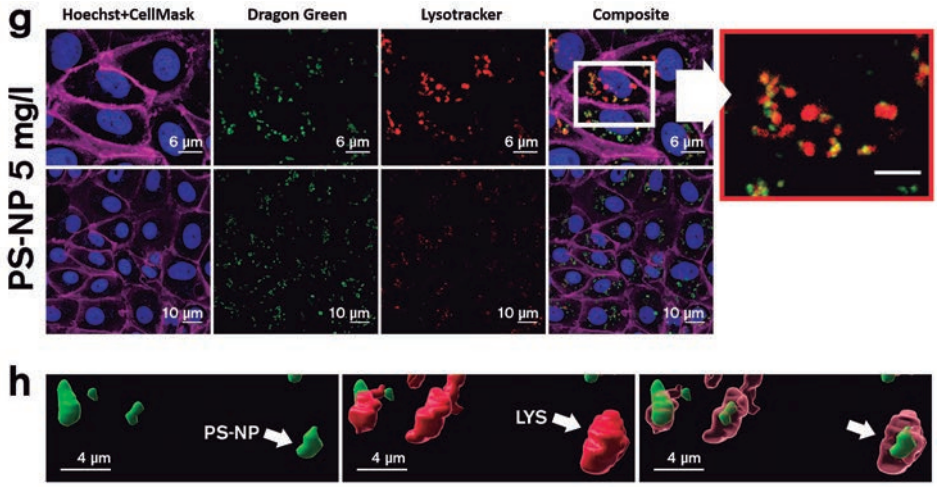


Figure 2 *(cont.)* **g-h. Uptake of fluorescently labelled PS-NPs by ZFL cells.**

(g) Green fluorescence corresponds to PS-NPs; **blue** and **magenta** to Hoechst stained nuclei (N) and Cell Mask stained plasma membrane. **Red** fluorescence corresponds lysosomes (LYS) stained with LysoTracker. White arrows point to colocalization of PS-NPs (green) and lysosomes (red). **(h)** 3D image analysis of PS-NP uptake (z-stack) and lysosome co-localization of PS-NPs.

To our knowledge, few studies have studied the internalization dynamics of nanoplastics in cells of aquatic vertebrates. Different authors have shown that microplastics and larger nanoplastics (larger than 200 nm) are not easily internalised but particles of 100 nm or less are readily taken up by the endocytic cell machinery.[32,33] Johnston *et al.* described that 20 nm PS-NPs did not accumulate in lysosomes but suggested that in human and rat cell lines NPs ended up in the mitochondria.[30] Recently, it has been demonstrated that

granulocytes from the mussel *M. galloprovincialis* internalized 50 nm PS-NPs and accumulated them in lysosomes.[14]

In our work, we showed for the first time the internalization dynamics of PS-NPs in aquatic vertebrate hepatocytes and we demonstrated that they reached the lysosomal compartment (**Fig. 2f**). The final intracellular fate of nanoplastics could be different depending on the species, size, charge and plastic polymer composition, pointing out the need for more studies focused on nanoplastic internalization dynamics in vertebrates.

Transcriptional changes in zebrafish liver cells associated with PS-NP and poly(I:C) treatment

To gain insight into the effects of PS-NPs alone and in combination with an immune stimulus, we used poly(I:C), a synthetic dsRNA compound, to mimic a viral infection. The assessed target genes (*mx, vig1* and *gig2*) are canonical genes related to the antiviral response that codify for interferon induced proteins[34] and their upregulation in ZFL cells in response to poly(I:C), mounting a typical and strong antiviral response, has been previously characterised.[23,35,36] As shown in **Fig. 3**, ZFL cells respond to poly(I:C) by upregulating antiviral genes such as *mx*, *vig1* and *gig2*, at similar levels to those reported previously.[21,23,35] When ZFL cells were pre-exposed for 12 h to PS-NPs and then treated with poly(I:C), a clear synergistic effect was observed (**Fig. 3**). Interestingly, when the cells were pre-exposed to PS-NPs for 24 h instead of 12 h, this synergistic effect was not observed, showing a typical poly(I:C) stimulation pattern.

To further assess whether cells remained in a normal state after 24 h, we assessed lipid peroxidation levels after the PS-NP exposure and subsequent poly(I:C) treatment. No alterations in cell peroxidation status due to PS-NP exposure were observed (**Supplementary Fig. 5**). Lipid peroxidation is a direct consequence of ROS production and we saw that after a 24 h exposure cells can deal with stress and maintain homeostasis. Altogether, data suggest that PS-NPs are internalized, accumulating in lysosomes, and initially provoke a strong and general stress state, thus potentiating the anti-viral response system. It could be speculated that PS-NP aggregation at higher doses could play a role in the uptake dynamics and, in turn, lead to differences in gene expression. However, we discarded this hypothesis, since the PS-NP stability data shown in **Fig. 1** indicate very low aggregation in DMEM after 72 h of incubation (PDI value of 0.07 *versus* 0.03 in water). A second hypothesis is that interferences in the endocytosis and associated mechanisms could be affected

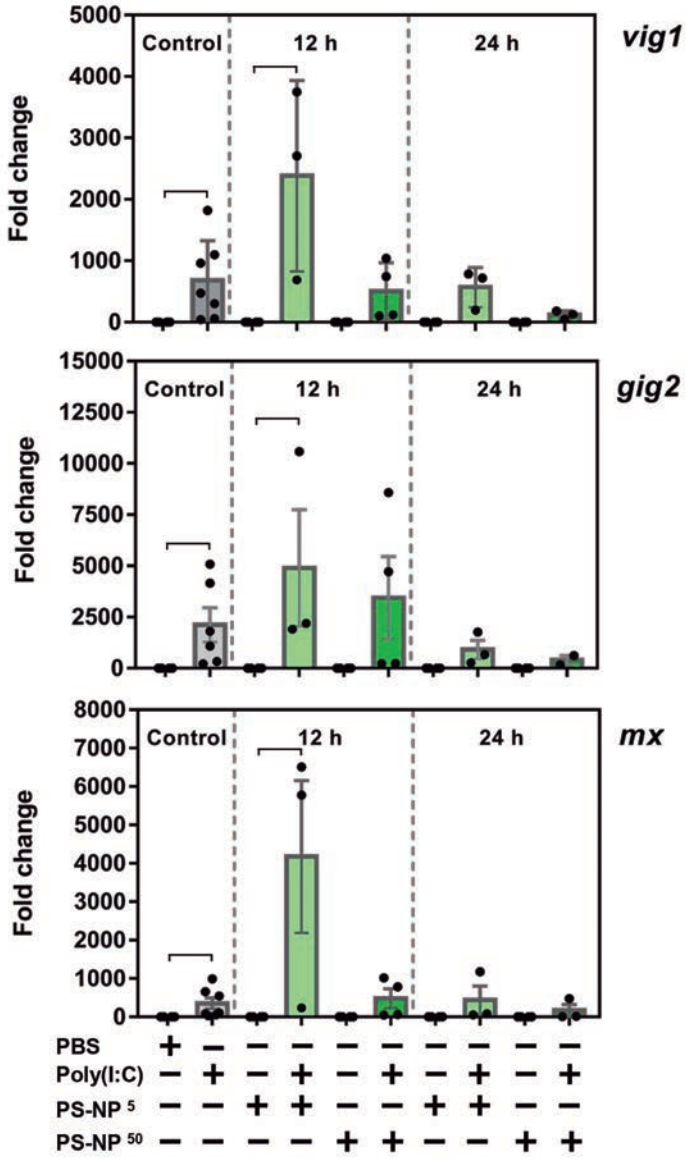


Figure 3. Analysis of gene expression in ZFL cells after PS-NP treatment followed by poly(I:C) treatment.

Cells were incubated for 12 or 24 h with PS-NPs followed by 25 µg ml–1 poly(I:C) stimulation for 16 h. Unexposed control cells (grey), 5 mg l–1 PS-NPs (light green) and 50 mg l–1 PS-NPs (dark green). Viperin (vig1), grass-carp-reovirus-induced gene 2 (gig2) and IFN-induced protein Mx (mx) gene expression is shown. Samples are from two independent experiments and data are expressed as mean ± SD (n = 4). Differences between each treatment mean and control are indicated as *p < 0.05. The legend presented underneath the graphics indicates the presence (+) or absence (–) of the different treatments: PBS ± poly(I:C), 5 mg l–1 PS-NPs ± poly(I:C); 50 mg l–1 PS-NPs ± poly(I:C).

by the PS-NP dose and exposure time. TLR3, the main receptor for poly(I:C) responsible for the initial activation of the immune response, is located in the endosomal membrane,[34] the same subcellular compartment where the PS-NPs traffic and accumulate. We hypothesise that at a low PS-NP concentration the antiviral response signals through TLR3 and synergises with a cell stress response. At a higher dose or longer exposure (leading to an internalization of a higher number of particles), the endosomal architecture might be overloaded and the TLR3 pathway and cell stress response disturbed. Nevertheless, the antiviral response can signal alternatively through RIG-1, LGP-2 or MDA5,[37,38] thus maintaining the poly(I:C) response at high dose or long exposure.

To further explore this hypothesis, we analysed the average size of the PS-NP clusters inside the ZFL cells after exposure to 5 and 50 mg l^{-1} PS-NPs. We found a significant difference in cluster size between the two concentrations (0.2691 ± 0.01395 – low dose *versus* 0.3873 ± 0.01387 – high dose), which could sustain the possibility of a lysosomal overload (**Supplementary Fig. 6c**). In line with this, we also tested RTGut cells, a different fish cell line derived from intestinal epithelia, which could be more conceptually relevant for waterborne absorption. In this cell line, the morphology and size of the endosomal–lysosomal system are massively changed at low *versus* high dose (**Supplementary Fig. 6a and b**). We observed that at high dose: the number of clusters inside the cell increased (n = 464 at high dose *versus* n = 950 at low dose) and their size almost tripled (mean 0.1549 ± 0.0058 – high dose *versus* mean 0.05902 ± 0.001895 – low dose). Coincidentally, Canesi *et al.*[39] found a perturbation on the endolysosomal system of *M. galloprovincialis* haemocytes after exposure to PS-NPs. They observed a decrease in lysosomal membrane stability (LMS) after exposures to 5 and 50 mg l^{-1} PS-NPs.[39] The endosomal–lysosomal system is known to participate in several aspects of cell physiology, besides its classical role in protein degradation. As Neefjes and collaborators[40] say: "endosomal system constitutes a key negotiator between the environment of a cell and its internal affairs". For example, lysosome overload has an impact on the mRNA levels of several lysosomal proteases[41] and is extremely important for a correct regulation of antigen receptor and pattern recognition receptor signalling in the innate immune system.[42]

On the other hand, a cell stress response in which low doses cause a greater impact than high doses has been previously documented in nanoparticle toxicology. For example, Teles *et al.*[43] observed changes in TOS (total oxidative status) and in mRNA levels of antioxidant-related genes after exposure to gold

nanoparticles in *Sparus aurata*. Changes only occurred at the tested low and intermediate concentrations, revealing a non-monotonic dose–response curve, a pattern of response frequently found for endocrine disrupting chemicals. Moreover, Brandts *et al.*[44] found an increased mRNA expression of genes involved in biotransformation (cypp11, pgp) and immune function (cathepsin) and a decrease in ChE in *M. galloprovincialis* only at lower PS-NP doses (0.05 and 0.5 mg l^{-1}). In the same species, Canesi *et al.*[39] found a greater increase in ROS production in haemocytes exposed to PS-NPs at low concentration (1 mg l^{-1}). This suggests that, under some circumstances, low doses of nanoplastics could cause a greater impact than higher doses. Maybe a combination of scenarios (disturbance of the endolysosomal system and/or cell stress response) could explain the gene expression synergy observed at low dose. However, further work would be necessary to understand the precise role of intracellular PS-NP accumulation and how this affects the immune response.

In vivo uptake and accumulation of PS-NPs in zebrafish larvae and effects on survival after bacterial infection

Zebrafish larvae 5 dpf were imaged after 48 h of *in vivo* exposure to fluorescent PS-NPs (5 and 50 mg l^{-1}) (see **Fig. 4a**), to confirm uptake. PS-NPs were taken up by the larvae and accumulated in the intestine and pancreas (**Fig. 4b**). The distribution of the fluorescence intensity normalized by the organ area was 65 ± 8.7% in the gut and 34 ± 8.7% in the pancreas (**Fig. 4c**). The zebrafish pancreas reaches its final position and shape by 6 dpf, the intestine is open to the surrounding environment between 3 and 4 dpf,[45,46] and the larva has a fully functional digestive system at day 5,[47] therefore imaging at 5 dpf is an optimal window of time. The pancreas is located asymmetrically on the right side of the body and has an elongated shape and characteristic head–neck–tail morphology, where the anterior part contains islet tissue surrounded by exocrine tissue, while the neck and tail consist of exocrine tissue.[45]

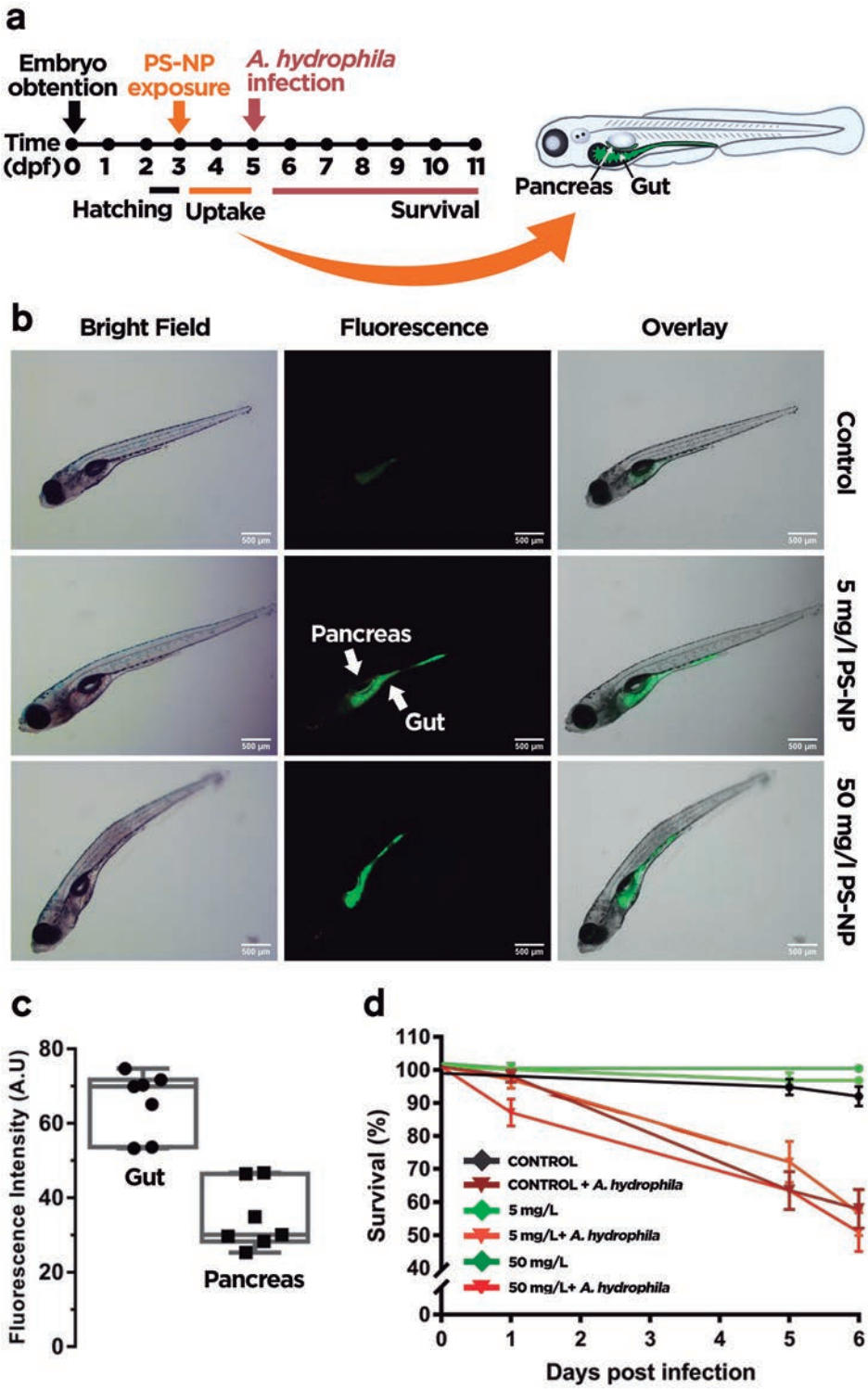
We hypothesise that the larvae immersed in the solution of fluorescent PS-NPs could have ingested the PS-NPs and absorbed them through the intestinal epithelia, distributing them systemically and accumulating also in the pancreas. Nevertheless, we do not have definite proof of this uptake route. We do not discard that PS-NPs may accumulate in other organs such as the liver, spleen or brain at later life stages. We actually expected to find fluorescence in the liver, but even at continuous PS-NP exposure and saturating doses (60 h, 100 mg l^{-1}) we could not detect PS-NPs' fluorescence in the zebrafish liver. This pattern of accumulation has been previously reported although with some

differences, likely due to the specific exposure time, dose, size and larvae age. For example, Brun *et al.* observed accumulation of 25 nm PS-NPs (20 mg l^{-1})[7,8] in the intestine, exocrine pancreas and gall bladder after 48 h of exposure at 120 hpf (5 dpf). It is worth mentioning that there is a considerable variation in between studies, with PS-NPs being located in the intestine and neuromast,[7] in the heart region,[10] in the digestive track and eyes,[48] in the gut and gills region[49] and in the gastrointestinal tract, gallbladder, liver, pancreas, heart and brain.[6] Differences with published data could also be attributed to the use of other ZF strains (*e.g.* AB/TL wild type as in van Pomeren *et al.*,[48] Veneman *et al.*,[10] Brun *et al.*[7] or Sökmen *et al.*[11]), instead of wild type larvae as in our case. We decided to use wild type zebrafish (no strain) to approach the individual variability present in a natural population, in order to better emulate an environmental situation.

In aquatic invertebrates, the scenario is similar, and the accumulation pattern of nanoplastics depends on age, dose, material or size. For example, oyster (*Crassostrea gigas*) larvae have been found to ingest both nanoplastics and microplastics at different ages, being the younger larvae the ones with the highest capacity to ingest 160 nm PS-NPs.[50] Sea urchin (*Paracentrotus lividus*) embryos accumulated PS–COOH-NPs inside the embryo's digestive tract without any toxic effects, while PS–NH_2-NPs induced strong toxic effects.[51] Finally, mussels (*M. galloprovincialis*) can accumulate 50 nm PS-NPs after 24 h of waterborne exposure, in the digestive system, muscle and gills.[14]

Figure 4. Zebrafish larvae PS-NP uptake and survival after PS-NPs and infection with *A. hydrophila*.

(a) PS-Exposure and infection chronogram. Zebrafish larvae (3 dpf) were immersed in 5 or 50 mg ml^{-1} of PS-NPs for 48 h. At 5 dpf, larvae were infected with *A. hydrophila* by bath immersion, at a dose of 1.85 × 109 cfu per ml. Survival was recorded every 24 h for 6 days. E3, 5 and 50 mg l^{-1} PS-NP immersed larvae were used as controls. G, gut and P, pancreas. **(b)** Representative images of biodistribution of fluorescent PS-NPs in zebrafish larvae. Zebrafish larvae (3 dpf) were immersed in 5 or 50 mg ml^{-1} of PS-NPs for 48 h. E3 immersed larvae were used as controls. **(c)** Distribution of the fluorescence intensity (%) in the gut and pancreas. Images were analysed with ImageJ and the measured fluorescence was normalized by the organ area. **(d)** Survival of the PS-NP treated zebrafish larvae (n = 72) challenged with A. hydrophila. Larvae immersed in E3 followed by *A. hydrophila* challenge was used as a mortality control. Cumulative survival curves were constructed using GraphPad. Significant differences were analysed using the log-rank (Mantel–Cox) test.


a
Embryo obtention
PS-NP exposure
A. hydrophila infection
Time (dpf) 0 1 2 3 4 5 6 7 8 9 10 11
Hatching
Uptake
Survival
Pancreas
Gut
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Bright Field
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5 mg/l PS-NP
50 mg/l PS-NP
Pancreas
Gut
c
Fluorescence Intensity (A.U)
Gut
Pancreas
d
Survival (%)
CONTROL
CONTROL + A. hydrophila
5 mg/L
5 mg/L+ A. hydrophila
50 mg/L
50 mg/L+ A. hydrophila
Days post infection

A recent review by Kögel *et al.*[52] pointed out how different parameters such as charge, size or concentration could affect the uptake and toxicity of nanoplastics in aquatic organisms.

In our study, the highest PS-NP concentration tested (100 mg l^{-1}) in a continuous 96 h exposure provoked 100% mortality, while continuous exposure to 50 mg l^{-1} provoked around 35% mortality (**Supplementary Table 2**). However, concentrations of 5 and 50 mg l^{-1} for 48 h did not provoke any mortality different from control larvae (**Fig. 4c**). Low dose (5 mg l^{-1}) and high dose (50 mg l^{-1}) exposures were tested for a subsequent infection with *A. hydrophila*. As shown in **Fig. 4d**, the PS-NP exposure did not affect the survival after infection, as both treated and non-treated larvae had the same survival curves.

Different authors have described how exposure to nanoplastics may affect the immune system by interfering in immune related gene expression, detoxifying enzyme activity (catalase *etc.*), or in ROS production, but this is the first study investigating the effects of nanoplastic exposure on the capacity to fight a lethal infection in zebrafish. At both tested concentrations, zebrafish larvae could cope with an acute PS-NP exposure and the physiological machinery was able to maintain the homeostasis. Only one previous work studied the interaction between nanoplastic exposure and pathogenic infection. In the said study, an *in vitro* infection study using isolated mussel haemocytes,[14] the authors showed that the hemocytes' phagocytic capacity decreased when exposed to PS-NPs but not when the hemocytes were exposed to a combination of PS-NPs and *V. splendidus*. Nevertheless, a chronic or repeated acute exposure could perhaps lead to a failure of the pathogen response mechanisms.

To further understand the effects of PS-NPs on fish, chronic or repeated acute exposure should be investigated, in order to determine if PS-NPs could affect the immune system in the long-term.

4. Conclusions

Our study shows that PS-NPs are efficiently taken up by ZFL cells *in vitro*, tending to accumulate in lysosomes, possibly in an attempt of the cell machinery to degrade PS-NPs. The expression of immune genes was synergistically affected by a viral stimulus (poly(I:C)) at low doses and early time points in ZFL cells. Even though we see this synergistic effect of PS-NPs and poly(I:C) at 12 h and 5 mg l^{-1}, this effect disappears at a longer exposure time (24 h) as well as a higher dose (50 mg l^{-1}). Therefore, it suggests that ZFL cells can regain homeostasis and normal function after the PS-NP challenge, presenting the expected response

to a viral stimulus. Furthermore, zebrafish larvae also incorporated PS-NPs, potentially through ingestion, and accumulated them in the gut and pancreas. Nevertheless, on exposure to PS-NPs at concentrations of up to 50 mg l^{-1}, PS-NPs did not show toxicity nor interference with a normal immune response against *A. hydrophila*. Altogether, although PS-NPs can induce an immune response, the survival of zebrafish larvae challenged with a bacterial infection after an acute exposure to PS-NPs is not decimated with respect to unexposed larvae.

Authorship

IB and NR designed the study. IB and NR designed and performed the experiments. IB, MT, MGO and NR did the data analysis. NR wrote the manuscript and all authors contributed to the preparation of the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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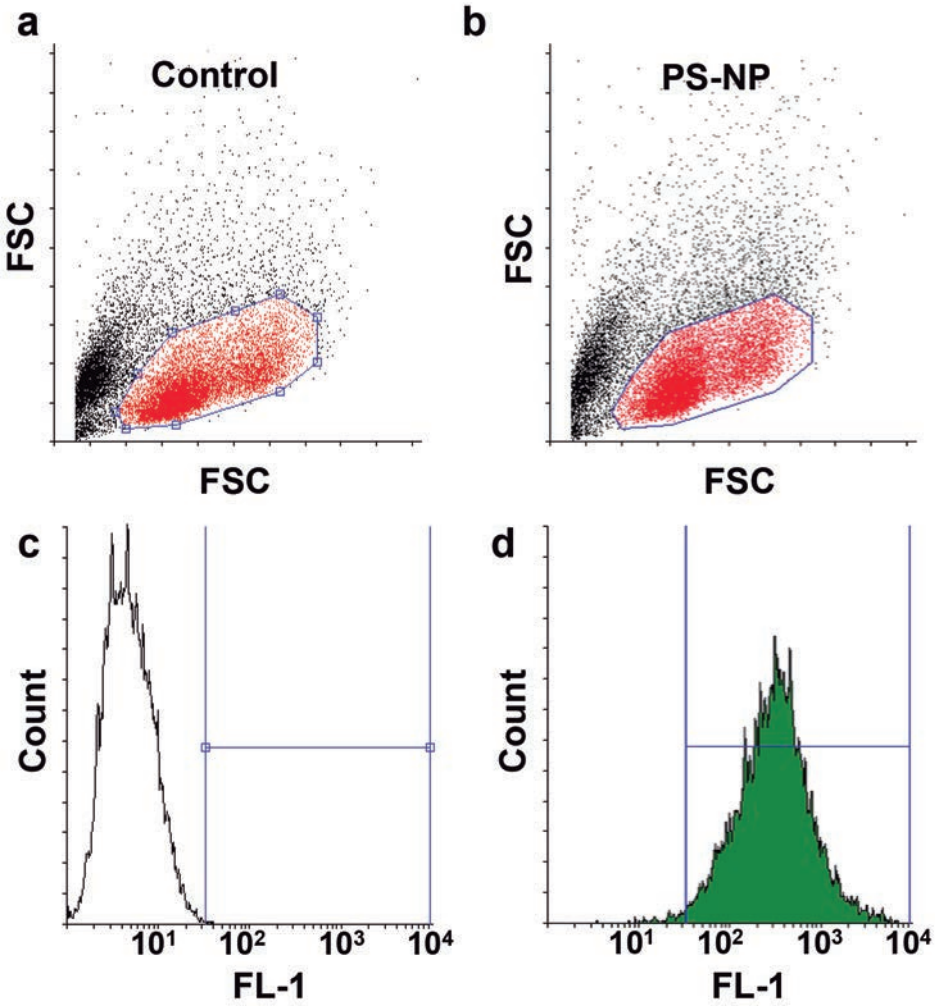
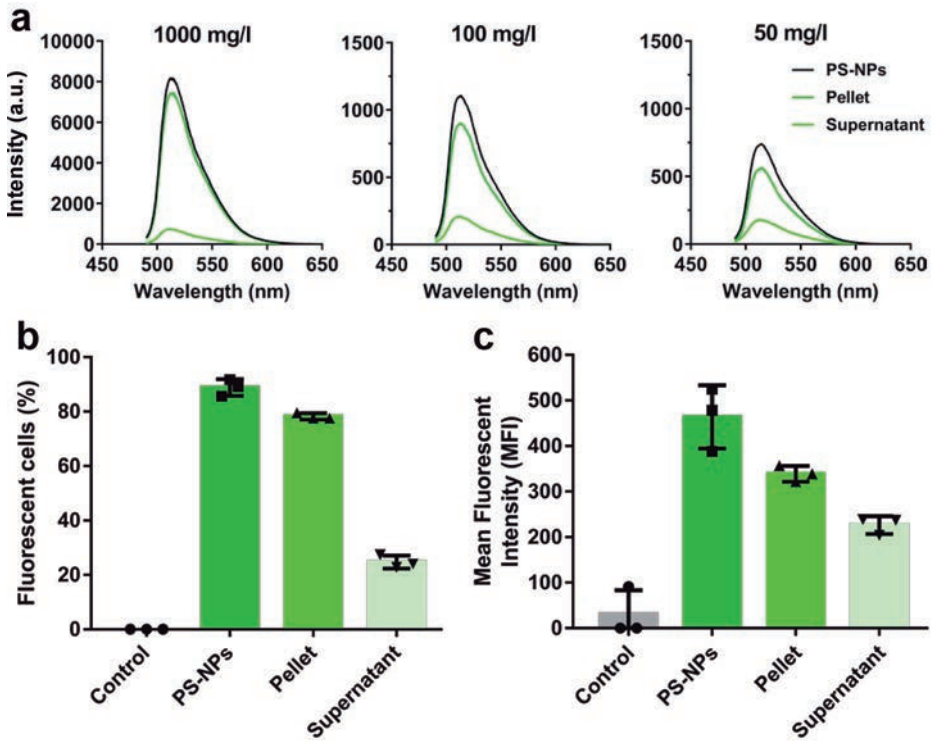
Polystyrene nanoplastics accumulate in ZFL cell lysosomes and in zebrafish larvae after acute exposure, inducing a synergistic immune response *in vitro* without affecting larval survival *in vivo*

Supplementary material



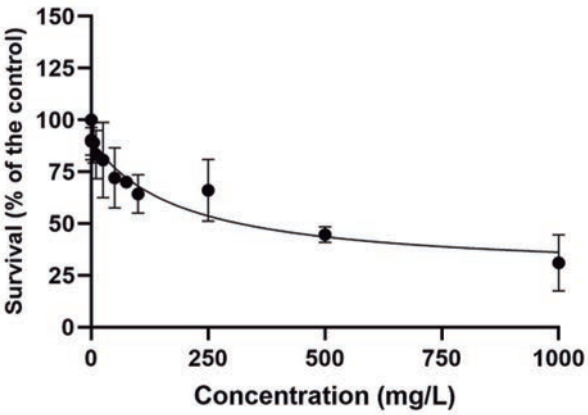
Supplementary Figure 1. ZFL cells Flow cytometry analysis strategy.

Representative dot plots and the gating strategy **(a, b)**, with red dots representing the gated population and black dots showing cellular debris and events outside of the relevant population. Histograms of fluorescence are shown **(c, d)**. Cells were incubated as follows: unexposed control cells **(a, c)** and 50 mg/l PS-NPs exposed cells **(b, d)**.



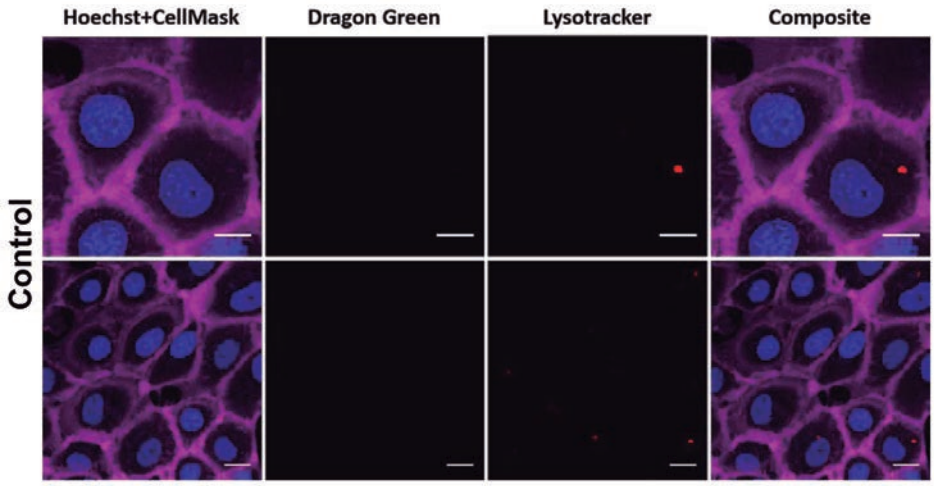
Supplementary Figure 2. Fluorophore leaking analysis from labelled PS-NPs.

(a) Curves of fluorescence intensity of PS-NPs diluted from stock solution, without further manipulation (PS-NPs), and of obtained fractions after manipulation (Pellet, Supernatant). **(b, c)** Cytometry analysis showing the internalization of PS-NPs, Pellet and Supernatant fractions by ZFL cells (in vitro). **(b)** Percentage of fluorescent cells of PS-NPs (dark green), Pellet (green) and Supernatant (light green). **(c)** Mean fluorescence intensity (MFI) of PS-NPs (dark green), Pellet (green) and Supernatant (light green). Data represent the mean ± SD of three samples.



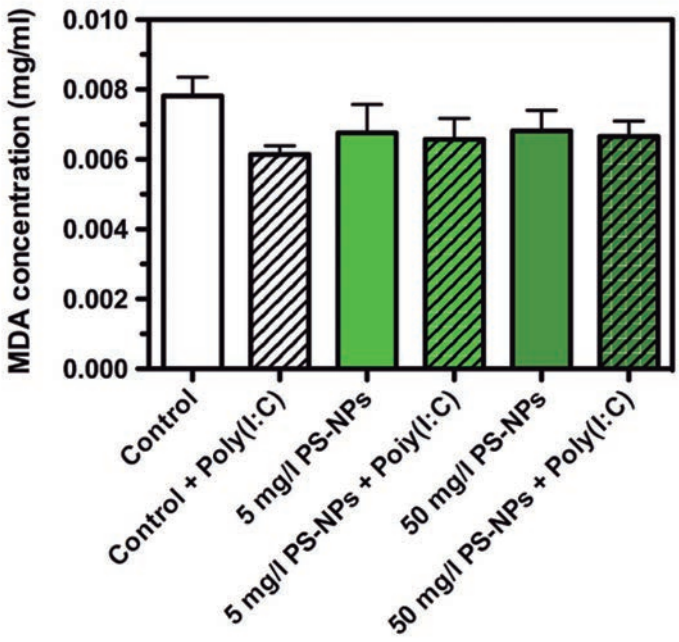
Supplementary Figure 3. Dose-response curve.

ZFL cells were exposed to an increasing concentration range (from 0.05 to 1000 mg/l) of PS-NPs. Lethal doses (LD50) were estimated using a nonlinear regression fitting curve (3 P) using GraphPad Prism 8.0.1 (244). The lethal dose for 50%(LD50) of the cells were estimated at 188.8 mg/l.



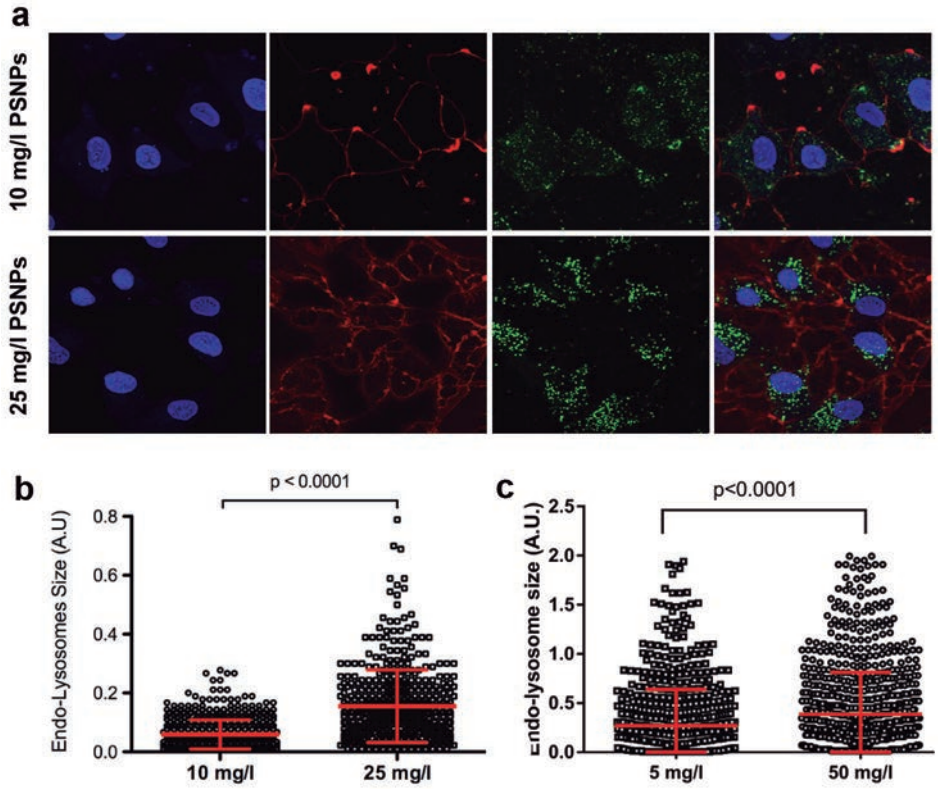
Supplementary Figure 4. Confocal images of control ZFL cells.

Blue fluorescence corresponds to Hoechst stained nuclei, **magenta** to Cell Mask stained plasma membrane and **red** to Lysotracker stained lysosomes.



Supplementary Figure 5. Lipid peroxidation in ZFL cells exposed to PS-NPs.

Lipid peroxidation assessed through the presence of malondialdehyde (MDA) in ZFL cells after exposure to PS-NPs followed by Poly(I:C) stimulus for 16 h. Cells were incubated as follows: unexposed control cells (white), 5 mg/l PS-NPs (light green) and 50 mg/l PS-NPs (dark green).



Supplementary Figure 6.
PS-NPs internalization in RTGut cells and cluster size analysis.

(a) Representative confocal microscopy images of RTGut cells showing the internalization of PS-NPs, after 16 h incubation at 10 and 25 mg/l. Green fluorescence corresponds to PS-NPs, blue to Hoechst stained nuclei and red to WGA555 stained plasma membrane. **(b)**. RTGut cells cluster size analysis at both concentrations performed with ImageJ. **(c)**. ZFL cells cluster size analysis at both concentrations performed with ImageJ from corresponding images in **Figure 2e** and **g**.

Supplementary Table 1. real Time primers.

Gene	Primer sequence (5´-3´)	Product size (bp)	Accession number
ef1-α	FW_CTTCTCAGGCTGACTGTGC RV_ACGATCAGCTGTTTCACTCCC	133	AY422992
mx	FW_ACATCTTGGATCGTTCAGGGGA RV_AACGCAGGTTCCTCCAACAG	163	NM_182942.4
vig-1	FW_CTTATAGGTCGAGCACAGGGC RV_ACGTACTGGATTGAGAGCGGTG	165	NM_001025556.1
gig2	FW_AGGGTACGACACTGCCTGGT RV_ AGGGTCACCAAAGCCACAAT	148	NM_001245989.1

Supplementary Table 2. Zebrafish larvae mortality (%) after continuous exposure to different concentrations of PS-NPs.

Zebrafish larvae mortality (%)

Exposure Group	60 h	96 h
0 mg /l	6,7%	6,7%
50 mg/l	7,1%	35,7%
100 mg/l	71.4%	100%

A baseline study on the impact of nanoplastics on the portals of entry of xenobiotics in fish

Irene Brandts, R. Solà, M.A. Martins, A. Tvarijonaviciute, A. Barreto, M. Teles and M. Oliveira

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4

Abstract

Mediterranean waters are particularly vulnerable to plastic pollution, with plastic particles concentrations comparable to those found in oceanic gyres. This work aimed to assess the impact of polymethylmethacrylate nanoplastics (PMMA-NPs) on the most important mucosal barriers of the gilthead seabream (*Sparus aurata*), a highly consumed fish species in the Mediterranean area. Fish were waterborne exposed to NPs (0.001–10 mg/L) for 24 and 96 h, and biochemical parameters associated with oxidative status (total oxidative status and total antioxidant capacity) and immune function (adenosine deaminase, ADA, acetylcholinesterase activity, AChE, and esterase activity, EA) were assessed in gills, intestine, and skin. In intestine, PMMA-NPs led to oxidative status alterations and decreased ADA and EA. In gills, PMMA-NPs induced EA decrease and AChE activity increase. Total protein values were significantly increased in skin. Overall, more alterations were observed in intestine, suggesting it may be one of the most affected tissues by exposure to NPs.

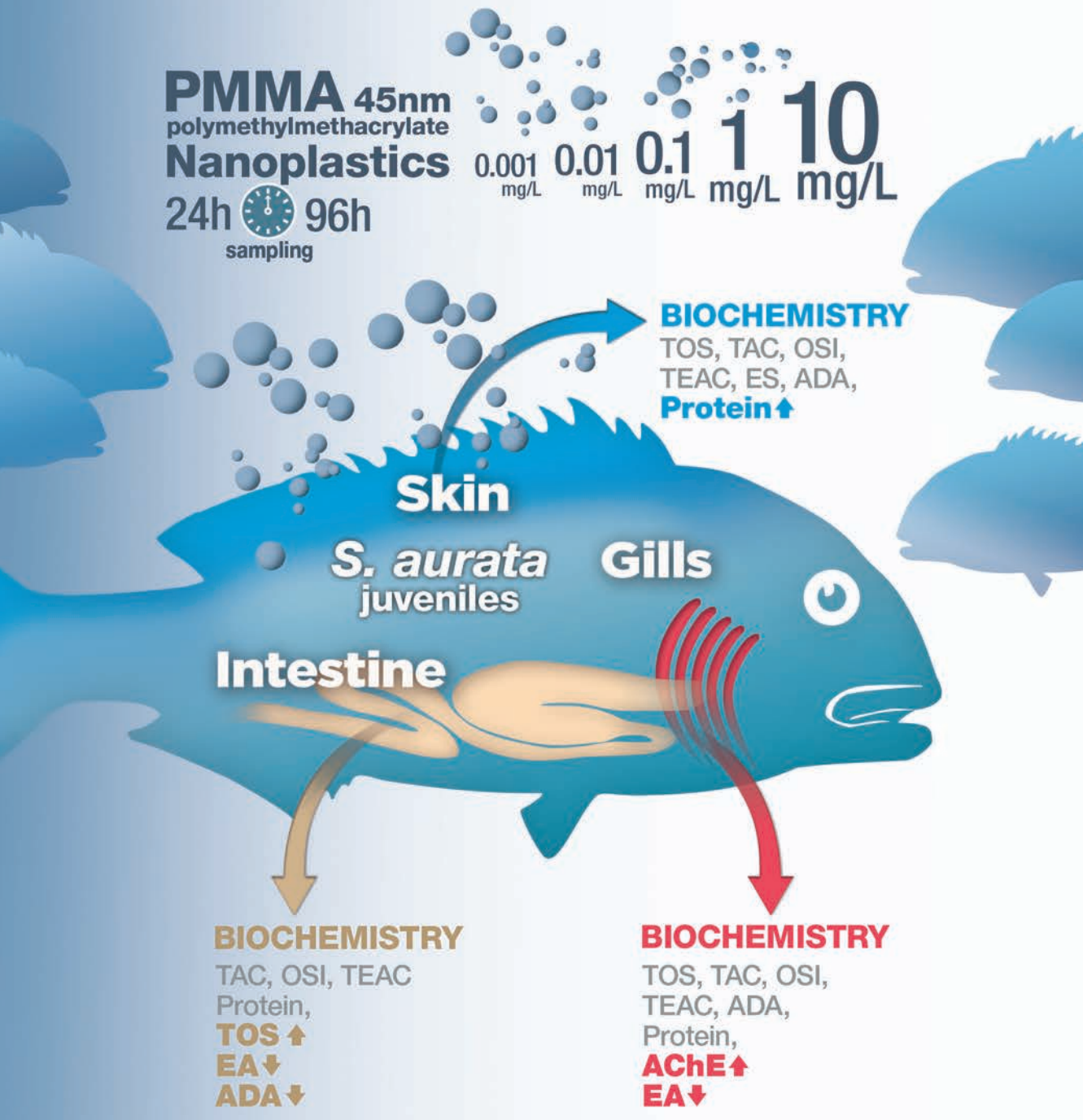
Highlights

- Gilthead seabream were exposed to polymethylmethacrylate nanoplastics (PMMA-NPs).
- Gills, skin and, intestine were analysed in fish after exposure to PMMA-NPs.
- Exposure to PMMA-NPs altered relevant biochemical parameters in tissues.
- Intestine seemed to be the most affected entry organ by a short exposure to PMMA-NPs.

Keywords

Polymethylmethacrylate nanoplastics (PMMA-NPs), 45 nm particles, Edible fish, Marine, mucosal tissues

Graphical abstract



1. Introduction

Nanoplastics (NPs) are contaminants of emerging concern that may induce adverse effects in aquatic organisms (Chae et al., 2018) and disrupt ecosystems worldwide. Their aggravated hazard is due to their capacity to cross epithelial barriers and accumulate in organisms, and be transferred along the food chain to top trophic levels (Gonçalves and Bebianno, 2021). In teleost fish, NPs can be absorbed through different routes, mainly the gastrointestinal tract, gills, and skin, the three main mucosal barriers and portals of entry of xenobiotics into fish (Bhagat et al., 2020).

The effects of NPs in fish species may vary depending on the uptake pathway (van Pomeren et al., 2017). Thus, more information on the main portals of entry of NPs into the organism and the potential consequence for these tissues is vitally needed to advance in NPs' research. Most studies evaluating the uptake of NPs' use zebrafish (*Danio rerio*) as a biological model, where the intestinal track appears to be the dominant uptake pathway (Bhagat et al., 2020). However, gills and skin also interact with the surrounding environment and have a large surface that may allow the absorption of NPs and translocation into inner tissues.

Studies assessing the effects of polymethylmethacrylate, PMMA-NPs in marine biota are scarce. In teleost fish in particular, the effects of PMMA-NPs have been investigated in only two marine species in research works carried out by our research group. We found that *Dicentrarchus labrax* (European seabass) exposed for 96 h to 45 nm PMMA-NPs (0.02-2 mg/L) presented alterations in the expression of genes related to lipid metabolism in liver and specifically in peroxisome proliferator-activated receptors transcripts (Brandts et al., 2018b), as well as decreased esterase activity (EA), an important antioxidant and immune enzyme. Moreover, in a study with *Sparus aurata* (gilthead seabream) exposed to 45 nm PMMA-NPs, we also found an increase in the transcriptional levels of genes related to lipid metabolism and changes in the levels of cholesterol and triglycerides in plasma (Brandts et al., 2021). The same study also reported alterations in oxidative stress biomarkers, such as total antioxidant capacity (TAC) and total oxidative status (TOS). Globally, our data support the evidence that PMMA-NPs may compromise immune function, oxidative status, and lipid metabolism in marine fish species.

The present study aimed to assess biomarkers of oxidative status, immune function and neurotoxicity in gills, skin, and intestine of the gilthead

seabream, a top predator and commercially valuable species destined to human consumption, after a short-time exposure to 45 nm PMMA-NPs. The working hypothesis is that NPs can induce changes in the tissues through which they are absorbed into fish, causing a response that will be reflected in the measured biochemical parameters.

2. Materials and Methods

PMMA-NPs were synthesised and characterized as described in Brandts et al. (2021). Gilthead seabream (8.7 ± 0.4 g) were transported from an aquaculture facility to the experimental facilities at the University of Aveiro (Portugal). Animals were acclimatized 1000 L tanks for 2 months with artificial salt water (salinity 30) at 19 ± 1 °C and natural photoperiod (14 h light: 10 h darkness). All experimental procedures were reviewed and authorized by the Portuguese National Authorities, in accordance with European Guiding Principles for Biomedical Research Involving Animals (EU2010/63) and followed OECD guidelines for chemical testing (OECD, 2019). Fish were randomly assigned to one of six experimental exposure groups, with different concentrations of PMMA-NPs: 0, 0.001, 0.01, 0.1, 1 and 10 mg/L. There were 3 replicate tanks for each condition, with 6 fish in each tank, 3 of which were sampled at 24 h of exposure and 3 at 96 h (n = 9 per condition). The test media (75%) was renewed every day throughout the experiment. Fish were sacrificed by anaesthetic overdose (MS-222/tricaine methanesulfonate) at two-time points (24 h and 96 h). Organs were dissected, snap frozen in liquid nitrogen and kept at -80 °C until further processing. All samples were homogenized in potassium phosphate buffer 0.1 M (pH 7.2), and all the parameters were measured with an automatic analyser (AU 600 automated biochemical analyser, Olympus Diagnostica, GmbH). EA, TOS and TAC were measured as described in Teles et al. (2018). The trolox equivalent antioxidant capacity (TEAC) assay was performed as previously described (Rubio et al., 2016). Acetylcholinesterase activity (AChE) was measured following the methodology described in Tecles et al. (2000). Adenosine deaminase (ADA) activity was determined spectrophotometrically, measuring the ammonia produced in a Berthelot reaction. In all cases, the intra- and inter-assay Coefficients of Variability were < 10%. The Oxidative Stress Index (OSI) was calculated as an indicator of the stress response (Wu et al., 2017). GraphPad Prism 8.0.1 was used to perform a two-way ANOVA, with NPs concentration and exposure time as factors. Post-hoc tests were performed to identify differences between exposure concentrations and control group (Dunnet) and between sampling times for the same concentration (Sidak).

Data were checked for outliers, normality and equal variance and transformed when necessary (Zar, 1999). Analysis of correlations between biomarkers in the three tissues were done using the Pearson correlation coefficient. In all analysis, differences were considered significant when $p < 0.05$. Results are expressed as mean ± standard error ($n = 9$). Endpoints were combined into one general "stress index" (Integrated Biological Response version 2), following the formula described by Sanchez et al. (2013) to combine biomarker data into an integrated biomarker response (IBR) value representing the stress level at each tested concentration. Representative results are shown as a star plot chart indicating the deviation of all biomarkers in relation to the control (0).

3. Results and Discussion

Present results showed that all three studied mucosal surfaces presented altered values of oxidative status and immune related parameters after exposure to PMMA-NPs (**Fig. 1**, **Fig. 2**, **Fig. 3, Supplementary Table 1**). However, in skin few alterations in parameters related to oxidative stress and immune function were found (**Fig. 1**).

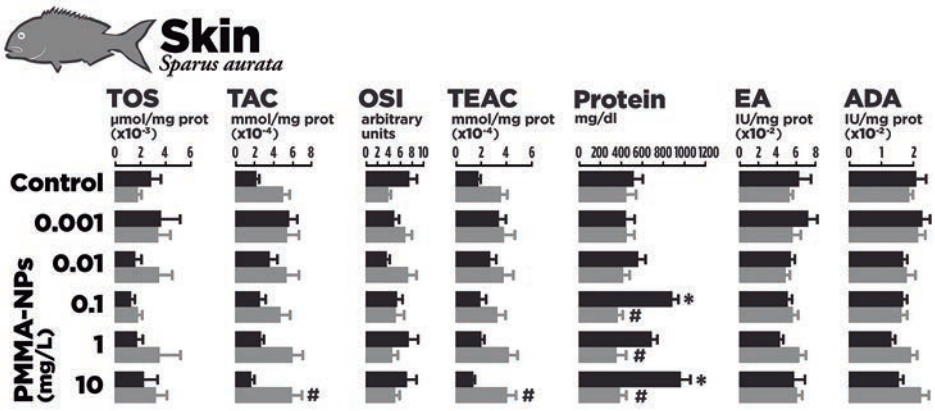


Figure 1. Biochemical parameters in skin of gilthead seabream after 24 (black bars) and 96 h (grey bars) exposure to polymethylmethacrylate nanoplastics (PMMA-NPs).

Values are presented as mean ± standard error ($n = 9$). Data was analysed using a Two-way ANOVA test. All groups were compared to the control group in their respective sampling time-point and significant differences are marked with an asterisk (*). Significant differences between sampling time points are marked at 96 h with a hash (#) *versus* 24 h, $p < 0.05$. TOS (total oxidative state), TAC (total antioxidant capacity), TEAC (Trolox equivalent antioxidant capacity), OSI (oxidative stress index), EA (esterase activity), ADA (adenosine deaminase activity).

Total protein values in skin were significantly increased, when compared to control, after 24 h exposure to 0.1 and 10 mg/L PMMA-NPs. These are interesting results, as there is no alteration of protein values at lower concentrations of PMMA-NPs, suggesting that different exposure concentrations of PMMA-NPs might not interact with the mucosal skin barrier in the same way. The particle dynamics and interactions established in the higher NPs concentrations may promote the formation of PMMA-NPs aggregates in the exposure media (Wu et al., 2019), forming larger clusters of particles that could be trapped by mucus. This would therefore prevent NPs from reaching the epidermal skin layer and explain this apparent difference in the response of skin tissue to the distinct concentrations of PMMA-NPs. Skin mucus acts as a dynamic physical and biochemical barrier that has shown to provide protection against environmental toxins and heavy metal toxicity, with particle trapping properties. Moreover, skin mucus production and composition, such as the level of proteins and immune molecules can vary, responding to exposure to toxic substances (Reverter et al., 2018) and specifically nanoparticle exposure (Oliveira et al., 2018; Torrealba et al., 2019).

TAC levels were significantly altered in fish gills, shown by an increase in TAC after 96 h of exposure to PMMA-NPs in almost all test concentrations (**Fig. 2**).

Although no alteration of TOS levels was registered, the increased TAC levels signify an increase in the total levels of compounds destined to counteract the effects of oxidative species. The production of reactive oxygen species (ROS) after exposure to NPs is one of the most studied as well as most reported mechanisms of toxicity of NPs (Brun et al., 2018; Jacob et al., 2020). However, a decrease in EA was found after 24 h exposure to 0.001 and 0.01 mg/L PMMA-NPs when compared to control group. A significant increase of AChE was observed in gills after 24 h of exposure to 0.001 mg/L PMMA-NPs, which can lead to an increase of hydrolyzation of acetylcholine and this can in turn contribute to a pro-inflammatory response. Thus, the changes in AChE after exposure to PMMA-NPs could be related to inflammatory processes and potentially lead to local tissue damage. The cholinergic anti-inflammatory pathway regulates the innate immune response to injury, pathogens, and tissue ischemia as the neural circuit that responds to and regulates the inflammatory response in mammals (Gallowitsch-Puerta and Tracey, 2005; Czura et al., 2007). This regulation mechanism has also been reported in fish (Baldissera et al., 2016, Baldissera et al., 2019). In this neuro-modulation of inflammation the increase of acetylcholine has a breaking effect on the inflammatory reaction

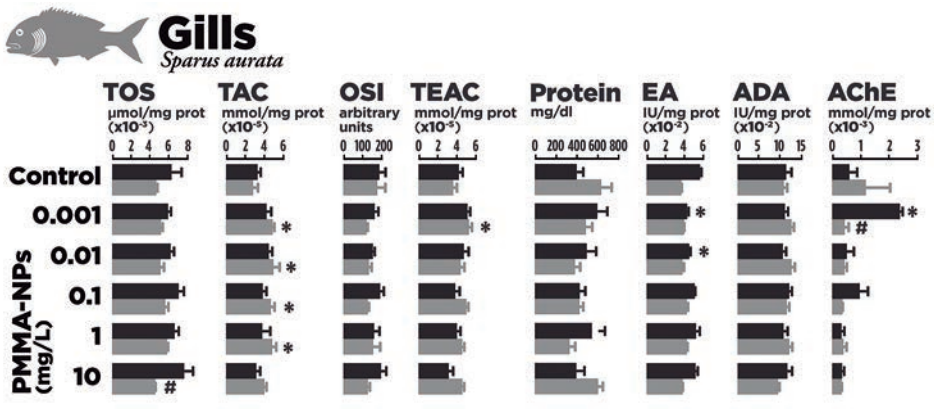


Figure 2. Biochemical parameters in gills of gilthead seabream after 24 (black bars) and 96 h (grey bars) exposure to polymethylmethacrylate nanoplastics (PMMA-NPs).

Values are presented as mean ± standard error (n = 9). Data was analysed using a Two-way ANOVA test. All groups were compared to the control group in their respective sampling time-point and significant differences are marked with an asterisk (*). Significant differences between sampling time points are marked at 96 h with a hash (#) *versus* 24 h, $p < 0.05$. TOS (total oxidative state), TAC (total antioxidant capacity), TEAC (trolox equivalent antioxidant capacity), OSI (oxidative stress index), EA (esterase activity), ADA (adenosine deaminase activity) and AChE (acetylcholinesterase).

of the innate immune system by inhibiting pro-inflammatory cytokines (Rosas-Ballina and Tracey, 2009). On the other hand, AChE activity is often measured to assess neurotoxic effects of contaminants. Inhibition of AChE is considered as a classical biomarker for toxicity in the nervous system induced by a broad spectrum of organic pollutants (Fu et al., 2018) and it has previously been reported in aquatic organisms after exposure to NPs. In fish, decreased AChE activity has been documented in *D. rerio* after 72 h exposure to 1 mg/L of 50 nm polystyrene (PS-NPs) (Chen et al., 2017). However, increases in AChE after exposure to NPs have also been documented in *Ctenopharyngodon idella* after 20 days of exposure to 23 nm PS-NPs (0.04 ng/L, 34 ng/L and 34 µg/L) (Guimarães et al., 2021). Thus, AChE may provide information on inflammatory regulation and neurotoxicity after NPs exposure.

In intestine (**Fig. 3**), increased levels of TOS and in the OSI were observed following a 24 h exposure to 0.001 mg/L PMMA-NPs. These results indicate an increase in ROS that together with the registered decrease in both TAC and EA at the lower NPs concentrations (0.001 and 0.01 mg/L), suggest an incapacity of antioxidant mechanisms to counteract the ROS produced upon exposure to

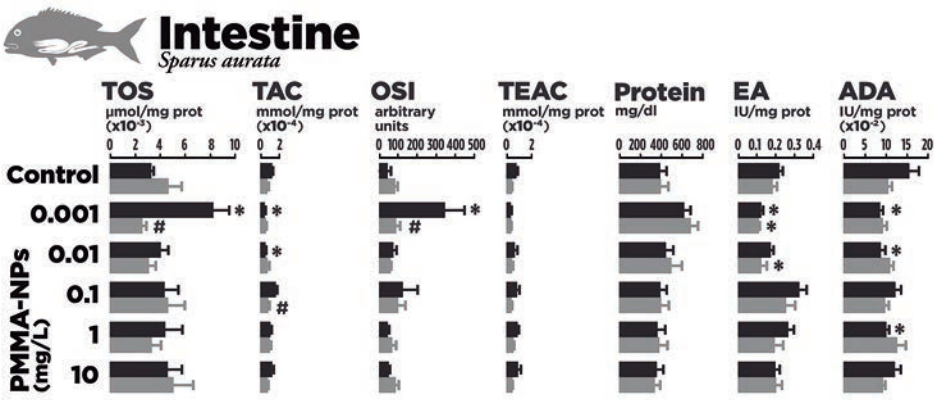


Figure 3. Biochemical parameters in intestine of gilthead seabream after 24 (black bars) and 96 h (grey bars) exposure to polymethylmethacrylate nanoplastics (PMMA-NPs).

Values are presented as mean ± standard error (n = 9). Data was analysed using a Two-way ANOVA test. All groups were compared to the control group in their respective sampling time-point and significant differences are marked with an asterisk (*). Significant differences between sampling time points are marked at 96 h with a hash (#) *versus* 24 h, $p < 0.05$. TOS (total oxidative state), TAC (total antioxidant capacity), TEAC (trolox equivalent antioxidant capacity), OSI (oxidative stress index), EA (esterase activity), and ADA (adenosine deaminase activity).

NPs. TAC, that comprises all specific antioxidant compounds involved in the defence against oxidative stress, and EA, have a protective role against oxidative stress for low- and high-density lipoproteins (Tvarijonaviciute et al., 2012) and their decrease indicates an overload of the antioxidant response at 24 h for lower PMMA-NPs concentrations. The intracellular generation of ROS by NPs has been reported in multiple studies and proposed as a key event for NPs' toxicity to consider in adverse outcome pathways. As an example, a recent study with zebrafish found a substantial increase in ROS generation in gut after exposure to 1 mg/L of 70 nm PS-NPs (Sarasamma et al., 2020). Moreover, there appears to be a strong correlation between particle size and ROS generation potential, with smaller particles having higher ROS generation potential (Hu and Palić, 2020), probably due to the characteristics attributed by their high surface area (Kihara et al., 2021).

Decreased ADA values were observed after 24 h of exposure to 0.001, 0.01 and 1 mg/L PMMA-NPs in intestine. This reduction in ADA activity could be associated with an attempt to constrain an inflammatory response and subsequent cellular damage, by increasing adenosine levels in tissues. Adenosine

is a molecule with immunomodulatory and immunosuppressive properties, that induces the release of anti-inflammatory cytokines and could also contribute to the restriction of inflammatory damage (Whiteside et al., 2011). In zebrafish, a significant downregulation in ADA activity has been observed in fish subjected to stress, both of chronic and acute natures, which was considered an anti-inflammatory profile (Piato et al., 2011; Zimmermann et al., 2016). These authors suggest that purinergic signaling may be part of a compensatory mechanisms to restore homeostasis after a stressful event, which could also be true for a short-time exposure to NPs. Moreover, previous studies in humans have shown that the pharmacological blockade of adenosine deaminase can ameliorate intestinal inflammation (Antonioli et al., 2014). ADA, responsible for adenosine breakdown, is considered one of the key enzymes of purine metabolism with high conservation of the amino acid sequence (Cristalli et al., 2001). The purinergic system is important in the regulation of the innate immune response, to stimulate resolution of inflammation and self-limiting of immune cell activity (Antonioli et al., 2014). Nevertheless, adenosine can exhibit both pro and anti-inflammatory actions, depending on the specific receptor and the cell-type where it is acting, serving as a regulator of immune response upon tissue damage associated with inflammation (Haskó and Cronstein, 2013). Supporting these premises, positive correlations between TAC and ADA (r = 0.76), EA and ADA (r = 0.74), EA and TAC (r = 0.78) were found in the intestine after 24 h exposure to PMMA-NPs. This supports a relation between the antioxidant capacity and the regulation of the inflammatory response. When the antioxidant capacity is surpassed, the purinergic inflammatory pathway appears activated, possibly as a protective defence against NPs' aggression.

When looking at EA values in all analysed tissues, we found a decrease EA in intestine after 24 h and 96 of waterborne exposure to PMMA-NPs at the lowest tested concentration (0.001, 0.01 mg/L) (**Fig. 4**) which coincides with the decrease in EA already mentioned in the results in gills, at 24 h for the lower concentrations. A decrease in EA has been previously documented in plasma of *D. labrax*, after 96 h exposure to 0.02 and 0.2 mg/L PMMA-NPs (45 nm) (Brandts et al., 2018a). Furthermore, Oliveira and co-authors also reported decreased EA in fish skin mucus after exposure to gold-nanoparticles (37 nm) (Oliveira et al., 2018). Therefore, it seems that exposure to nanoparticles (polymeric and others) appears to lead to a decrease in EA values in different fish species and various biological matrices, specifically at lower exposure concentrations, and therefore closer to a realistic environmental scenario.

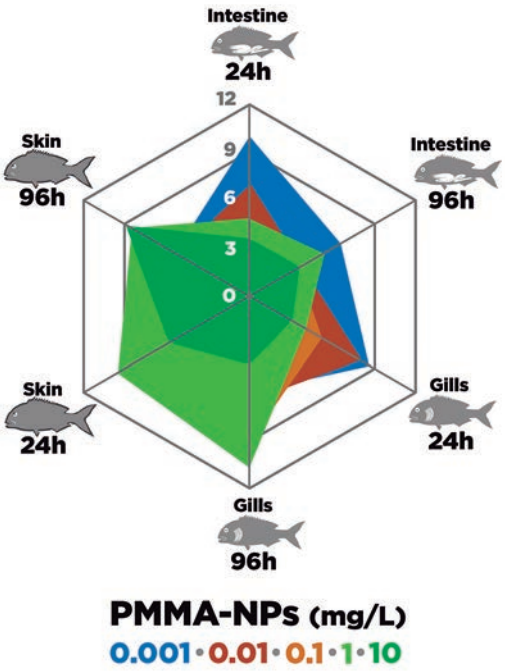


Figure 4. Star plot summarizing responses to the different concentrations of polymethylmethacrylate nanoplastics (PMMA-NPs) and IBRv2 values obtained for each matrix and sampling time-point.

Whether the decrease in EA after exposure to NPs is due to the direct interaction of esterase with the nanoparticles or to its depletion as an antioxidant or detoxification mechanism cannot be easily deduced from these results. Esterase is a relevant immune enzyme in fish external mucosal barriers, and its decrease due to NPs exposure could potentially affect the animal's capacity of response to a concomitant immune challenge (Reverter et al., 2018). Moreover, esterase (arylesterase) is a multifunctional enzyme present in numerous body tissues and therefore implicated in multiple relevant physiological processes, such as both having immune and anti-inflammatory properties (Dullaart et al., 2009) and a protective role against oxidative stress (Tvarijonaviciute et al., 2012). Consequently, its alteration due to NPs' exposure could have systemic implications. Although further studies are needed to clarify the most relevant biological matrix/matrices for its assessment, EA can be proposed as a relevant biomarker for monitoring environmental exposure to NPs in fish.

Globally, oxidative stress indicators returned to control levels after 96 h exposure, in skin and intestine, while gills showed increased TAC levels at this time-point. This points to an activation of the antioxidant defences, possibly leading to a recovery from the initial aggression of exposure to PMMA-NPs. Furthermore, a positive correlation between TAC and TOS was found in gills and intestine after 96 h exposure to NPs (gills – $r = 0.30$; intestine – $r = 0.52$) but none at 24 h.

This suggests that the effective activation of antioxidant mechanism in response to pro-oxidant challenge would be happening somewhere between 24 and 96 h in all tissues, with antioxidant enzymes still active in gills at 96 h, contributing to the return to homeostasis. Nevertheless, EA levels remained decreased in intestine at 96 h, possibly indicating that the activity of this multifunctional enzyme is compromised.

Notably, intestine was the tissue that presented more alterations in biochemical parameters, both for oxidative status and immune markers, suggesting that it is the most vulnerable barrier tissue to the local effects of NPs. Our findings are corroborated by previous findings showing that NPs induce local inflammation in the intestine, produce oxidative stress (Brun et al., 2018; Jin et al., 2018; Gu et al., 2020a), induce dysfunction of local immune cells and affect the intestine microbial structure in fish (Gu et al., 2020a; Gu et al., 2020b). The intestine also appears to be the most favourable route for NPs uptake into the organism and initial (bio)accumulation (Brun et al., 2019; Sarasamma et al., 2020). The fish intestine is a complex multifunctional organ, essential for the absorption of nutrients, acting as barrier for pathogens and xenobiotics, and harbouring complex species-specific microbial assemblages that contribute to the overall animal health status. It has been previously reported that long-term inflammation and elevated oxidative stress in the intestine are closely related to intestinal microbiota dysbiosis and metabolic disorders (Lobionda et al., 2019). Still, little is known about the long-term effects of NPs in intestine linings, but the potential disturbance of intestine immune function and microbial communities is certainly worrisome, as it could ultimately affect digestion and absorption and in consequence reducing fish growth and survival.

Our results indicate that exposure to NPs might affect the correct functionality of fish mucosal barriers. Moreover, the lowest tested concentrations of NPs were the ones that induced more changes in the studied endpoints. This finding corroborates our previous results with NPs where more effects in aquatic organisms, *i.e.*, mussels and fish, were observed at lower doses (Brandts et al., 2018a, Brandts et al., 2018b, Brandts et al., 2020, Brandts et al., 2021) and it could be related to particle interactions and decreased bioavailability at higher exposure doses. Generally, nanoparticles' properties confer them a unique elusiveness of biological defence systems, including barriers such as tissues, mucus, and cell membranes (Kihara et al., 2021). Mucosal barriers could potentially be blocking the entrance of larger particles or aggregates, whilst particles remaining in the sub-micron size-range would continue journey

inside the organism. This ultimately follows the same principle as the proven size-dependent uptake of NPs across biological barriers, as particle aggregates could simply be behaving as larger particles. Particle parameters such as charge, size or concentration can affect the uptake and toxicity of NPs in aquatic organisms (Kögel et al., 2020) and the dynamics and properties of the corona formed around NPs' surface in biological fluids could play a crucial role to their cellular internalization and toxicity mechanisms (Lundqvist and Cedervall, 2020). However, the IBR index (**Fig. 4**) shows that we should interpret results with caution, as there seem to be tissue specific responses to different particle concentrations, with lower doses exerting more responses in intestine and gills, and higher doses in skin. Moreover, the sampling time-point at which we choose to assess the state of each of the tissues also influences the IBR. Taking this into account, we might think that depending on the precise characteristics of the exposure to NPs, time/length, and concentration wise, the answer as to which is the most vulnerable portal of entry could vary. Further studies should consider these variables for a better understanding of the uptake, mechanisms, and effects of NPs in real ecological risk scenarios.

4. Conclusions

Altogether, the present results show that a waterborne exposure to PMMA-NPs alters relevant biochemical parameters in three portals of entry of NPs in *S. aurata*. Oxidative stress as well as activation of antioxidant response after exposure to NPs is suggested in all three mucosal barriers, although general homeostasis seems to be regained after 96 h. Moreover, activation of inflammation and its regulatory mechanisms, potentially involving both purinergic and cholinergic pathways, is suggested. The sensitivity of fish mucosal barriers to NPs exposure appears to be influenced by the particle concentration, probably linked to particle aggregation dynamics influencing perception by the organism. Overall, of the three studied matrices, intestine appears to be the most vulnerable, coinciding with the fact that it has been suggested by other authors as the main uptake pathway for NPs.

CRediT authorship contribution statement

I. Brandts: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. **R. Solà:** Formal analysis, Visualization, Writing – original draft. **M.A. Martins:** Resources. **A. Tvarijonaviciute:** Investigation, Methodology. **A. Barreto:** Investigation. **M. Teles:** Conceptualization, Investigation, Supervision, Visualization, Writing – review & editing. **M.**

Oliveira: Conceptualization, Investigation, Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary material

SKIN	Time	Concentration	Interaction
Protein	p<0.0001	p=0.04818	p=0.0016
TOS	ns	ns	ns
TAC	p<0.0001	ns	ns
TEAC	p<0.0001	ns	ns
OSI	ns	ns	p=0.0281
EA	ns	ns	ns
ADA	ns	ns	ns

GILLS	Time	Concentration	Interaction
Protein	ns	ns	p=0.0275
TOS	p<0.0001	ns	ns
TAC	ns	p=0.0156	ns
TEAC	ns	p=0.0283	ns
OSI	p=0.0116	ns	ns
EA	p<0.0001	p=0.0128	p=0.0044
ADA	ns	ns	ns
AChE	p=0.0045	p<0.0001	p<0.0001

INTESTINE	Time	Concentration	Interaction
Protein	ns	p=0.0029	ns
TOS	p=0.0288	ns	ns
TAC	p=0.0101	p=0.0011	ns
TEAC	p=0.0001	ns	ns
OSI	ns	p=0.0002	p=0.0014
EA	p=0.0240	p<0.0001	ns
ADA	ns	ns	p=0.0224

Supplementary Table 1. Two-way ANOVA results analysing parameters in fish exposed to polymethylmethacrylate nanoplastics with time and PMMA-NPs concentration as factors.

Significance was defined at $p < 0.05$, ns – not significant. TOS (total oxidative state), TAC (total antioxidant capacity), TEAC (trolox equivalent antioxidant capacity), OSI (oxidative stress index), EA (esterase activity), ADA (adenosine deaminase activity) and AChE (acetylcholinesterase).

Waterborne exposure of gilthead seabream (*Sparus aurata*) to polymethylmethacrylate nanoplastics causes effects at cellular and molecular levels

Irene Brandts, C. Barría, M.A. Martins, L. Franco-Martínez, A.Barreto, A.Tvarijonaviciute, L. Tort, M. Oliveira and M.Teles

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5

Abstract

This study evaluated the effect of a short-term exposure to 45 nm polymethylmethacrylate nanoplastics (PMMA-NPs) on the gilthead seabream (*Sparus aurata*), by assessing biomarkers at different levels of biological organization in liver and plasma. Fish were exposed *via* water to PMMA-NPs (0, 0.001, 0.01, 0.1, 1 and 10 mg L^{-1}) and sampled after 24 and 96 h. Results showed a general up-regulation of mRNA levels of key genes associated with lipid metabolism (*e.g. apolipoprotein A1* and *retinoid X receptor*). Together with the modulation of the lipid pathway genes we also found a global increase in cholesterol and triglycerides in plasma. Antioxidant-related genes (*e.g. glutathione peroxidase 1*) were also up-regulated after 24 h of exposure, but their expression levels returned to control afterwards. Total antioxidant capacity (TAC) was increased throughout the experiment, however at 96 h the antioxidant capacity became less efficient, reflected by an increase in the total oxidative status (TOS). Concomitantly, we found an increase in the erythrocytic nuclear abnormalities (ENAs) throughout the trial. Altogether, PMMA-NPs activated the organism's antioxidant defenses and induced alterations in lipid metabolism pathways and genotoxicity in the blood cells of gilthead seabream.

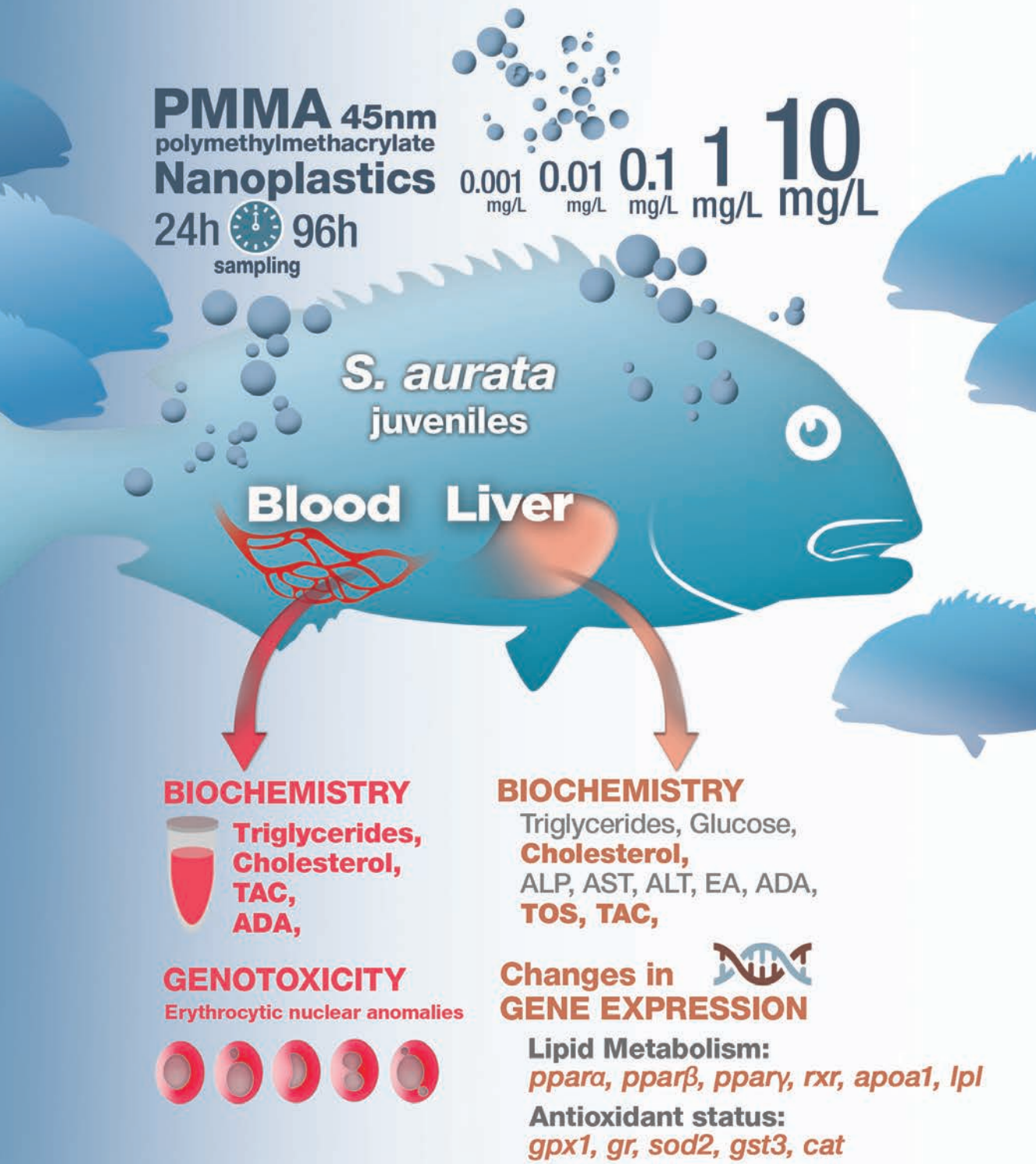
Highlights

- The effects of polymethylmethacrylate nanoplastics (PMMA-NPs) were evaluated in fish.
- Parameters both at the molecular and biochemical levels were modulated by PMMA-NPs.
- A short-term exposure to PMMA-NPs increased the expression of antioxidant genes.
- An increase in both total antioxidant capacity and total oxidative status was found.
- PMMA-NPs induced an increase in the frequency of erythrocyte nuclear abnormalities.

Keywords

Nanoplastics (NPs), Gilthead seabream (Sparus aurata), Gene expression, Lipid metabolism, Genotoxicity

Graphical abstract



1. Introduction

Plastic production has been increasing since the 1950s, with a total estimated amount of plastic produced in the world up to 2017 of 8300 million metric tons (Geyer et al., 2017). The increased use and production of plastic materials has resulted in their environmental release and plastic particles are currently ubiquitous pollutants, found in all kinds of ecosystems around the planet (Alimi et al., 2018) as well as in multiple different species (de Sá et al., 2018). It is now widely recognized that plastic materials released in the environment may go through different biotic and abiotic processes leading to their fragmentation into smaller plastics, called microplastics (<5 mm, MPs). Moreover, the further breakdown to nanoplastics (<1,000 nm, NPs) (Gigault et al., 2018; Hartmann et al., 2019) as a result of fragmentation of daily use plastic objects (Ekvall et al., 2019; Lambert and Wagner, 2016) has been demonstrated, supporting the idea of a continuous formation of small plastic particles from bulk materials.

Although the available data in terms of the levels of NPs in the environment is scarce due to analytical limitations, Gigault et al. (2016) documented the degradation of MPs collected directly from the North Atlantic Gyre in environmentally realistic conditions. Moreover, Ter Halle et al. (2017) reported the isolation of several populations of highly polydisperse particles (1 to 1,000 nm) from the colloidal fraction of a water sample obtained from the same area, with a wider range of polymers in the nanometric plastic fraction than in the larger MPs one (Ter Halle et al., 2017).

NPs are considered emerging pollutants of global importance (Patil et al., 2019), as they have the potential to enter the environment and cause adverse ecological effects (Geissen et al., 2015). These carbon-based nanoparticles may be taken up by a wide range of organisms and potentially cross biological barriers (Ng et al., 2018). Nevertheless, the scientific community's understanding of their effects on biota and ecological impact is still limited (Barria et al., 2020).

Recent studies have addressed the effects of NPs on organisms at the bottom of the food web with relevant role on aquaculture activities (*e.g.* microalgae, rotifers, polychaetes) demonstrating particle and species specific responses. The study of Venâncio et al. (2019) revealed that primary consumers like rotifers (*Brachionus plicatilis*) could be more sensitive to PMMA-NPs exposure than producers like microalgae (*e.g. Tetraselmis chuii* and *Nannochloropsis gaditana*) (Venâncio et al., 2019). In the polychaeta *Hediste diversicolor*, polystyrene NPs demonstrated ability to impact ecologically relevant endpoints like burrowing

behaviour (Silva et al., 2020a) and the regenerative capacity of these organisms (Silva et al., 2020b). Nevertheless, more studies on the effects and mechanisms of action of NPs are necessary, particularly in higher trophic level organisms such as fish, as most studies focus on invertebrate species (de Sá et al., 2018).

In teleost fish, the absorption of NPs as well as their translocation between tissues has been documented, both after waterborne and dietary exposures (Brun et al., 2018; Chae et al., 2018; Mattson et al., 2017; Skjolding et al., 2017), although the precise uptake pathways are not yet clarified. Studies suggest that these particles can be found throughout the fish body (Kashiwada, 2006; Brun et al., 2018, 2019; Pitt et al., 2018; Skjloding et al., 2017) reaching the circulation as well as distant tissues, such as brain or eye (Kashiwada, 2006; Mattson et al., 2017; van Pomeren et al., 2017). Detrimental effects of NPs in fish have been documented ranging from a broad general stress response to behavioural alterations (Pitt et al., 2017; Brandts et al., 2018; Brun et al., 2019). NPs can potentially overload fish antioxidant capacity, as they have been found to induce reactive oxygen species (ROS) production (Sökmen et al., 2020) and oxidative stress (Lu et al., 2016; Veneman et al., 2017). Effects have also been reported in terms of neurotransmission (decreases in acetylcholinesterase activity, Chen et al., 2017), genotoxicity (DNA damage) and apoptosis (Sokmen et al., 2020). Changes in glucose metabolism and cortisol levels (Brun et al., 2019), lipid accumulation (Lu et al., 2016), histopathological changes liver (Chae et al., 2018) and up-regulation of mRNA transcripts related to lipid metabolism (Brandts et al., 2018) have been previously documented in different teleost species. Exposure to NPs has also been seen to interfere with the immune system in fish, inducing the expression of pro-inflammatory cytokines (Brun et al., 2018), activating the complement system (Veneman et al., 2017), decreasing the activity of immune enzymes (Brandts et al., 2018) or altering neutrophil function (Greven et al., 2016). Moreover, the potential effects of NPs through dietary exposure have been investigated, addressing the trophic transfer of NPs and their effects on fish as final consumers. In this scenario, NPs ingested through the food chain have shown to provoke behavioural disorders in the top consumer, such as decreased locomotion and predatory activity (Mattsson et al., 2015, 2017), and alterations in lipid metabolism related to impaired used of the animal's fat reserves (Cedervall et al., 2012).

The Mediterranean Sea is a region of high plastic load and limited export to the Atlantic Ocean (Cózar et al., 2015; Pedrotti et al., 2016), as well as an area of intense aquaculture production. The concentrations of MPs sampled in this

area range from 0.10 to 0.87 particles per m^{-2} (Collignon et al., 2012; Cózar et al., 2015; de Haan et al., 2019; Pedrotti et al., 2016; Ruiz-Orejón et al., 2016; Schmidt et al., 2018; Suaria et al., 2016), deemed comparable to those found in oceanic gyres (Cózar et al., 2014). PMMA has a growing demand, associated with emerging applications in the medical and health sectors, as well as in coatings and emulsions. Moreover, PMMA is a hydrophobic material, which favours the union to cells (Abdel-Fattah, 2019), making it capable of transmitting pathogens in the aquatic environments.

Due to the many knowledge gaps still present in NPs research, assessment of risk of exposure and potential effects of NPs in humans is still limited (SAM, 2018). Therefore, there is a pressing urge to better understand human and environmental exposure to smaller MPs and NPs (SAPEA, 2019), since reports on their detection in environments worldwide keep increasing and the generation of global plastic waste does not seem to be diminish. One thing that is clear is that ingestion constitutes one of the potential exposure routes of humans to NPs (Prata et al., 2020), both through polluted water and through the consumption of animals previously exposed to these particles.

The gilthead seabream (*Sparus aurata*) is a top predator and one of the most relevant fish species for human consumption in the Mediterranean region, with a joint aquaculture and capture fisheries production of over 195 thousand tons in 2016 (FAO, 2020). These circumstances, taken together with the fact that more than 8 million tons of plastic end up in the ocean each year (Jambeck et al., 2015), highlight the need to investigate the effect that NPs may have on commercially cultured fish such as gilthead seabream. In the present study, we aimed to investigate the effects that a short-term exposure (24 and 96 h) to polymethylmethacrylate (PMMA) NPs might have on the gilthead seabream, using biomarkers of different levels: gene expression endpoints in liver, biochemical markers in liver and plasma and erythrocyte nuclear anomalies (ENAs) in blood.

2. Materials and Methods

2.1. Synthesis and characterization of polymethylmethacrylate nanoplastics (PMMA-NPs)

PMMA-NPs were synthesized by polymerization of methyl methacrylate (MMA), generally following the procedure described by Roy and Devi (1996). Briefly, MMA was polymerized in water, at 80 °C, in a nitrogen atmosphere,

using potassium persulphate as initiator and sodium dodecyl sulphate as stabilizer. Dynamic light scattering (DLS) was employed to characterize the NPs' hydrodynamic size and zeta potential (Zetasizer Nano ZS, Malvern) in ultrapure water. Their hydrodynamic size in ultrapure water was 40 nm and the surface charge –26.4 mV. When transferred to artificial seawater (ASW, salinity 30), the particles experimented an instant increase in their hydrodynamic size (58.6 nm). When left in ASW for 1 h the average hydrodynamic size reached 97.3 nm and after 24 h the particles showed an average size of 120.3 nm. Transmission electron microscopy (TEM) (Hitachi, H9000 NAR) was applied to confirm synthesized particles' morphology and size. NPs showed an average size of 45 nm and quasi-spherical shape.

2.2. Bioassay, experimental design and sampling

S. aurata juveniles weighing 8.7 ± 0.4 g (average ± SE) were obtained from an aquaculture facility in Spain (Tinamenor- SonRíoNansa, S.L.). Fish were acclimatized in 1,000 L aquaria with aerated ASW (Ocean Fish, Prodac, salinity: 30) at water temperature of 19 ± 1 °C and natural photoperiod (14 h light: 10 h darkness) for 2 months. During this time, commercial fish feed was supplied daily, *ad libitum*, up until 48 h before the start of the bioassay. Fish were then randomly distributed in six different experimental groups: 0, 0.001, 0.01, 0.1, 1 and 10 mg L^{-1} of PMMA-NPs. Exposure suspensions were prepared by dilution of the stock suspension in ASW.

Each experimental condition consisted of replicate 3 tanks with 15 L of experimental media each and 6 fish in the tank. For each time-point, 24 and 96 h, 3 animals per replicate tank were sampled (n = 9). Exposure concentrations were selected according to the available studies that evaluated the effects of NPs on aquatic organisms (Chae et al., 2018; Barría et al., 2020) and the reported environmental concentrations of MPs, since there are currently no data available on the environmental levels of NPs (Greven et al., 2016). The animals were exposed to NPs generally following OECD Guideline 203 regarding acute toxicity tests (OECD, 1992) and sampled at two different time points, 24 and 96 h.

All experimental procedures involving fish were approved by Portuguese National Authorities (authorization N421/2013 of the legal authority "Direção Geral de Alimentação e Veterinária"), following International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63) and a certificate of approval is available upon request. During the experiment, general conditions were kept as described for the acclimatization period and 75 % of the

test media was renewed every 24 h. At the end of each exposure (24 h and 96 h), fish were euthanized by immersion in a tricaine methanesulfonate (MS222) bath (1 g L^{-1}). Fish were then weighed and measured before proceeding to tissue sampling. Immediately after, heparinized syringes were used to draw blood from the caudal vein and a droplet of blood was used to produce a smear for each sampled fish. The liver was dissected, weighed, halved, instantly frozen liquid in nitrogen and stored at −80 °C. Blood was shortly kept at 4 °C, subsequently centrifuged for plasma isolation (211 g, 10 min, 4 °C), and samples were then immediately frozen at −80 °C.

The liver was selected as it is the target organ for most xenobiotics, playing a crucial role in their metabolism, storage and redox metabolism in fish (Teles et al., 2016a). Plasma was selected due to its ability to reflect the general physiological state of the animal (Teles et al., 2016b), being widely used to diagnose health status in multiple situations, such as inadequate feeding, chronic pathology or stress conditions. Moreover, blood shows high sensitivity to the toxic effects of chemical products, due to its role in the transfer of substances (Oliveira et al., 2007), and has been proposed as a valuable biological sample for the study of contaminants, since it does not require the sacrifice of animals (Brandts et al., 2018).

The Fulton condition factor (K) and the hepatosomatic index (HSI) were calculated with the following formulas:

$$K = \frac{\text{Weight}}{\text{Length}^3} * 100 \qquad HSI = \frac{\text{Liverweight}}{\text{Bodyweight}} * 100$$

2.3. Gene expression analysis

2.3.1. Extraction of RNA and synthesis of complementary DNA

Dissected livers were homogenized individually in 1 mL of TRI Reagent® (Sigma) and total RNA was extracted following the manufacturer's recommendations. Briefly, after homogenization, 100 µL of 1-bromo-3-chloropropane was added to each sample, mixed with a vortex shaker and incubated at room temperature for 10 min Samples were centrifuged (12,000 g; 15 min; 4 °C), and then the aqueous phase was transferred to a new tube, 500 µL of 2-propanol were added and samples left at room temperature for 10 min. Samples were then centrifuged (12,000 g; 10 min; 4 °C) and 2-propanol was removed, leaving the sedimented RNA. The pellet was washed twice with 1 mL of 75 % ethanol, centrifuging at 7,500 g for 5 min at 4 °C during each wash.

The pellet was air dried for 10 min at room temperature and resuspended in 150 µL of water with diethyl pyrocarbonate, to finally incubate in a thermoblock at 55 °C for 10 min. RNA quantification and purity evaluation were performed using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA). RNA quality was verified with Experion (Automated Electrophoresis Station, Bio-Rad), using the Experion Standard Sens RNA chip (Bio-Rad). Reverse transcription was performed using 1 µg of the total RNA using the iScript ™ cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions.

2.3.2. Quantitative polymerase chain reaction (qPCR)

The set of genes studied include nuclear hormone receptors (*pparα, pparβ, pparγ* and *rxr*), markers of lipid and lipoprotein metabolism (*lpl*, *apoa1*), antioxidant status markers (*gpx1*, *sod2*, *gr*, *cat* and *gst3*). The full gene names and primer sequences are provided in **Table 1**. The efficiency of amplification was tested in each primer pair as follows: 5-fold serial dilutions of cDNA pool were analysed, and E = 10 (–1 / s) was used as the formula for efficiency, where s is the slope generated by the serial dilutions. The qPCR was performed in a Bio-Rad CFX384 real-time PCR detection system (Bio-Rad Laboratories, USA). The reactions were performed using iTaqTM Universal SYBR® Green Supermix (Bio-Rad Laboratories, USA), following the manufacturer's instructions. Expression data, obtained from two independent technical replicas, were used to calculate the threshold cycle value (Ct). After verifying the efficiency of the primers, the RT-qPCR analysis of all individual samples was determined following the same protocol described above.

2.4. Standardization strategy

In the RT-qPCR analysis, the expression levels of the target genes are calculated by comparison to the levels of a single constitutively expressed reference gene, whereby the reliability of the relative quantification method is based on the stability of the reference genes used (Pffaffl, 2001). In the present study, we used NormFinder to identify the most appropriate normalization gene among a set of candidates, which employs and algorithm considering intra and intergroup variation and provides a classification of the expression stability of each gene in a set of sample and experimental design (Andersen et al., 2004).

The candidate reference genes were *elongation factor 1 alpha - ef1α*; *alpha tubulin - tub* and *glyceraldehyde-3-phosphate dehydrogenase - gapdh*. Relative gene expression was calculated according to Livak and Schmittgen (2001).

Table 1. Primers used for quantitative real-time PCR amplification of the genes studied in liver of *Sparus aurata*.

Gene name	Acronym	GenBank	Forward	Reverse	Efficiency (%)
Elongation factor-1 α	*ef1α*	AF184170	CCCGCCTCTGTTGCCTTCG	CAGCAGTGTGGTTCCGTTAGC	105.33
α-tubulin	*tub*	AY326430	AAGATGTGAACTCCGCCATC	CTGGTAGTTGATGCCCACCT	117.97
glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	DQ641630	TGCCCAGTACGTTGTTGAGTCCAC	CAGACCCTCAATGATGCCGAAGTT	95.12
Peroxisome proliferator-activated receptor Alpha	*pparα*	AY590299	GCAGCCTGTGAGTCTTGTGAGTGA	CTCCATCAGGTCTCCACACAGC	97.36
Peroxisome proliferator-activated receptor beta	*pparβ*	AY590301	CGTGTTCGGGATTCGGGACT	CACCCTGTCGTGCTGCTCTGTA	96.07
Peroxisome proliferator-activated receptor gama	*pparϒ*	AY590304	CGGAGAGAGAAGCAAGAACAAGAA	GAGGAGGAGGAGATGGAGGTGTA	105.09
Retinoid X receptor	*rxr*	HS092100	GGGCTTCTTCAAGAGGACAGT	TGCACCGCTTCTCTCTTCAT	117.89
Apolipoprotein AI	*apoa1*	AF013120	GAATACAAGGAGCAGATGAAGCAGATG	TGGTGACGGAGGCAGCGATG	83.68
Lipoprotein lipase	*lpl*	AY495672	CGTTGCCAAGTTTGTGACCTG	AGGGTGTTCTGGTTGTCTGC	92.24
Glutathione peroxidase 1	*gpx1*	DQ524992	GAAGGTGGATGTGAATGGAAAAGATG	CTGACGGGACTCCAAATGATGG	105.73
Glutathione reductase	*gr*	AJ937873	TGTTCAGCCACCCACCCATCGG	GCGTGATACATCGGAGTGAATGAAGTCTTG	104.87
Superoxide dismutase [Mn]	*sod2*	JQ308833	CCTGACCTGACCTACGACTATGG	AGTGCCTCCTGATAT TTCTCCTCTG	105.73
Glutathione-S-transferase3	*gst3*	JQ308828	CCAGATGATCAGTACGTGAAGACCGTC	CTGCTGATGTGAGGAATGTACCGTAAC	104.57
Catalase	*cat*	JQ308823	TGGTCGAGAACTTGAAGGCTGTC	AGGACGCAGAAATGGCAGAGG	105.07

2.5. Biochemical analysis

2.5.1. Sample preparation

For the assessment of biochemical endpoints (cholesterol, triglycerides, glucose, ALP (alkaline phosphatase), AST (aspartate aminotransferase), ALT (alanine transaminase), EA (esterase activity), TOS (total oxidative status), TAC (total antioxidant capacity) and ADA (adenosine deaminase)), liver samples were homogenized in potassium phosphate buffer 0.1 M (pH 7.2) at a ratio of 1:4 (weight:volume). The samples were incubated for 30 min at room temperature, centrifuged (20,000 g, 90 min, 4 °C) and the supernatant was then transferred to a new tube and stored at -80 °C until analysis. The plasma was isolated as described in section 2.2 and used to determine cholesterol, triglycerides, TAC and ADA levels. All plasma and liver samples were analysed on the same day in one batch using an automatic analyser (AU 600 automated biochemical analyser, Olympus Diagnostica, GmbH).

2.5.2. Biochemical determinations

TOS was measured as described by Erel (2005) with some modifications (Barbosa et al., 2014), based on the reaction of ferric ions with orange xylenol in an acidic medium. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar (μmol H_2O_2 Equiv L^{-1}). TAC was determined as described by Erel (2004), with some modifications (Tvarijonaviciute et al., 2012, 2018). The assay was calibrated with 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid and the activity expressed in mmol L^{-1}. EA was analysed as described by Haagen and Brock (1992) with modifications described by Tvarijonaviciute et al. (2012). EA was expressed as IU per ml of sample, where 1 IU is equal to 1 μmol of hydrolysed phenyl acetate min^{-1}. Cholesterol, triglycerides, glucose levels and AST, ALT and ALP activities were determined using commercially available kits (Olympus Systems Reagents; Olympus Life and Material Science Europe GmbH, Hamburg, Germany) following the manufacturer's instructions. ADA activity was determined spectrophotometrically, using a Berthelot reaction, measuring the ammonia produced. In all cases, the intra and inter-assay Coefficients of Variability was less than 10 %.

2.6. Erythrocytic nuclear anomalies (ENAs)

Blood smears were prepared on glass slides and fixed with methanol (10 min). Slides were stained using 5 % Giemsa for 30 min. For each sample, 1,000 mature erythrocytes were classified taking into account ENAs, using

an optical microscope (1,000 X magnification) (Pacheco and Santos, 1996). Only erythrocytes with intact nuclear and cytoplasmic membranes were analysed, discarding those overlapping or damaged. Four nuclear anomalies were analysed: micronucleus, reniform nucleus, segmented nucleus and lobed nucleus. The result was expressed as the frequency of ENAs observed (‰) in a total of 1,000 cells.

$$\text{ENAs (‰)} = \frac{\text{Number of cells with ENAs}}{\text{Total number of cells counted}}$$

2.7. Data analysis

Data was analysed using the Sigma Plot 14.0 software package, and results are expressed as mean ± standard error (n = 9). Data was first checked for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test) and transformations were performed when necessary (Zar, 1999). Different treatments were compared using a Two-way analysis of variance (ANOVA), followed by the Tukey test ($\alpha = 0.05$).

3. Results

3.1. Fulton's condition factor (K) and hepatosomatic index (HSI)

K and HSI showed no significant differences between NPs treatments and controls in any of the exposure times (**Fig. 1A**).

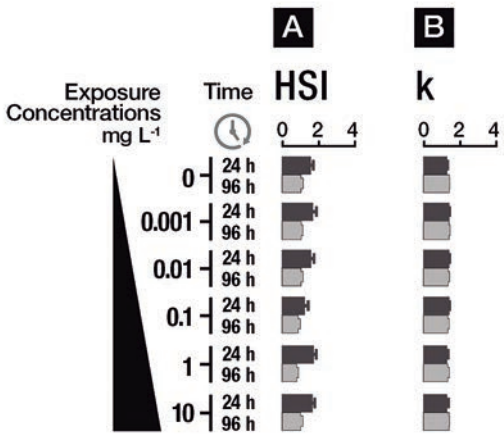


Fig 1. Hepatosomatic index (HSI) (A) and Fulton condition factor (k) (B) of gilthead seabream after 24 and 96 h exposure to polymethylmethacrylate nanoplastics (PMMA-NPs).
Values are presented as mean ± standard error (n = 9). Data was analysed using a Two-way ANOVA test, followed by a Tukey test for Post-Hoc analysis.

3.2. Gene expression

The efficiency of each gene was estimated by a standard curve generated using serial dilutions of a pool of cDNA samples. The correlation coefficients were

highly linear, and the PCR amplification efficiencies ranged from 83.68–117.97 % (**Table 1**). Before the analysis of the target genes, the variation of each reference gene was determined in the liver of *S. aurata* (**Fig. 2**). According to NormFinder, stability values for candidate reference genes were: 0.016 for *tub*, 0.013 for *ef1α* and 0.012 for *gapdh* (**Fig. 2D**). The latter was then considered the most stable reference gene and used for RT-qPCR data normalization.

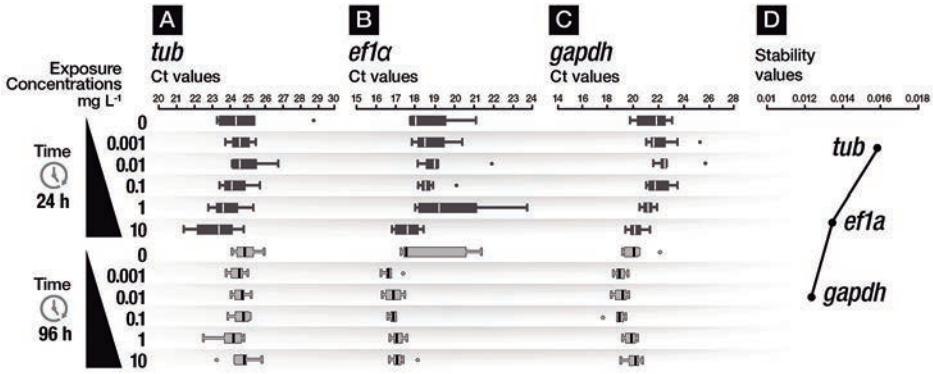


Fig 2. Transcription levels of reference genes in liver of gilthead seabream after 24 and 96 h exposure to polymethylmethacrylate nanoplastics (PMMA-NPs).

Values are presented as mean ± standard error (n = 9). Boxes show percentiles 25/75 and the line represents de average; **(A)** *alpha tubulin* (*tub*), **(B)** *elongation factor 1 alpha* (*ef1α*) and **(C)** *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*). **(D)** Stability values of tested reference genes, as calculated by NormFinder algorithm.

Regarding the expression of target genes in *S. aurata* liver (**Fig. 3**), *pparα* and *pparγ* remained unchanged after 24 h exposure to PMMA-NPs, regardless of the tested concentration. However, after 96 h, a significant increase in *pparα* expression was found after exposure to 0.001, 1 and 10 mg L^{-1} PMMA-NPs when compared to control levels, whereas *pparγ* expression increased significantly only in organisms exposed to 10 mg L^{-1} PMMA-NPs.

On the other hand, all tested concentrations of PMMA-NPs induced a significant increase in *pparβ* and *rxr* expression after 24 h of exposure, which, after 96 h, returned to control levels in the case of *pparβ* expression with *rxr* expression remained up-regulated at 96 h. In terms of genes that encode for proteins involved in lipid metabolism, 24 h exposure to PMMA-NPs induced an up-regulation of *apoa1* (all tested concentrations) and *lpl* (0.01 mg L^{-1} PMMA-NPs) expression. However, after 96 h, expression of *apoa1* returned to control levels and significant differences to control in terms of *lpl* expression were found in organisms exposed to 10 mg L^{-1} PMMA-NPs.

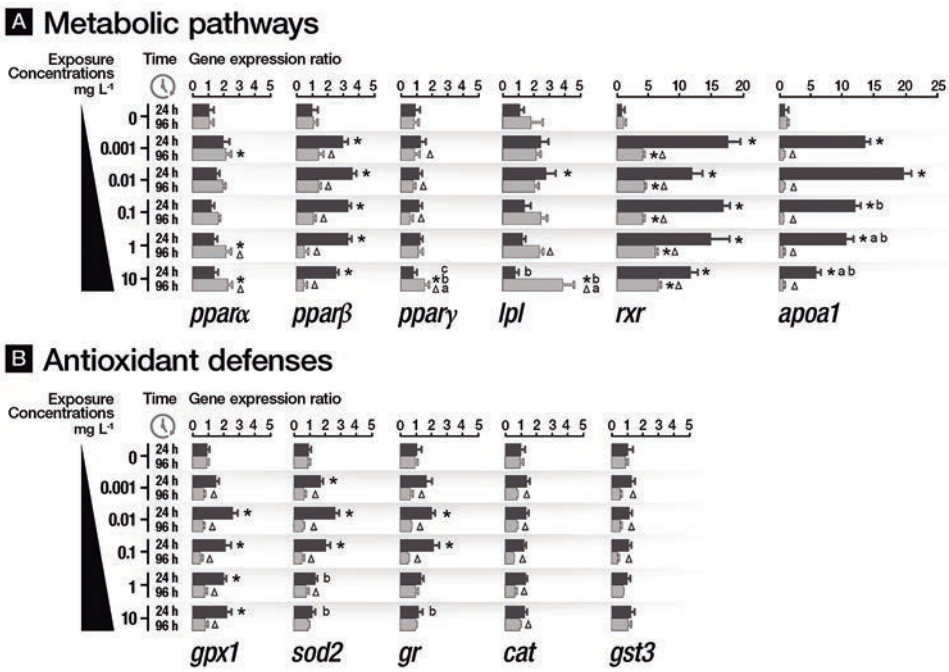


Fig 3. Target genes' mRNA levels in liver of gilthead seabream after 24 and 96 h exposure to polymethylmethacrylate nanoplastics (PMMA-NPs).

(A) Metabolic pathways, **(B)** Antioxidant defenses. Values are presented as mean ± standard error (n = 9). Data was analysed using a Two-way ANOVA test, followed by a Tukey test for Post-Hoc analysis. All groups were compared and significant differences are marked as follows: Asterisk (*) denotes significant differences *versus* control, **a** *versus* 0.001 mg L^{-1} PMMA-NPs, **b** *versus* 0.01 mg L^{-1} PMMA-NPs and c *versus* 0.1 mg L^{-1} PMMA-NPs, within the same exposure time, $p < 0.05$. Significant differences between sampling time points are marked at 96 h with a triangle (Δ) *versus* 24 h, $p < 0.05$.

In terms of antioxidant defence, *gpx1* expression was significantly up-regulated after 24 h exposure to 0.01, 0.1, 1 and 10 mg L^{-1} PMMA-NPs with mRNA levels returning to control levels after 96 h exposure. The expression of *gr*, after 24 h exposure, was significantly higher than control in organisms exposed to 0.01 and 0.1 mg L^{-1} PMMA-NPs, but returned to control levels after 96 h. The mRNA levels of *sod2* increased significantly after 24 h exposure to 0.001, 0.01 and 0.1 mg L^{-1} PMMA-NPs returning to control levels after 96 h. However, the mRNA levels of *cat and gst3* were not altered by PMMA-NPs exposure at both sampling periods.

Concerning the differences among concentrations within one same sampling time-point, 24 or 96 h, the only observed significant differences were as follows. The expression of *pparγ* presented a significant increase at 96 h for the exposure

to 10 mg L^{-1} of PMMA-NPs, when compared to 0.001, 0.01, 0.1 mg L^{-1}. The mRNA levels of *apoa1* at 24 h presented a significant decrease at 1 and 10 mg L^{-1} of PMMA-NPs, when compared to 0.001 as well as a to 0.01 mg L^{-1} and a significant decrease in 0.1 mg L^{-1} of PMMA-NPs when compared to 0.01 mg L^{-1}. Transcription levels of *lpl* appeared down-regulated for the exposure to 10 mg L^{-1} PMMA-NPs when compared to 0.01 mg L^{-1} at 24 h, whereas they were increased after exposure to 10 mg L^{-1} of PMMA-NPs for 96 h when compared to 0.001 and 0.01 mg L^{-1} at the same time-point.

Regarding antioxidant status gene transcripts, *sod2* mRNA levels were down-regulated at 24 h for exposures to 1 and 10 mg L^{-1} PMMA-NPs, when compared to 0.01 mg L^{-1}. The expression levels of *gr* decreased significantly after exposure to 10 mg L^{-1} PMMA-NPs when compared to 0.1 mg L^{-1}, at 24 h.

Addressing the comparison between the data obtained in the two sampling points, 24 h and 96 h, *pparα* presented higher expression at 96 h in organisms exposed to 1 and 10 mg L^{-1} of PMMA-NPs. However, *pparβ* mRNA levels were significantly lower after 96 h exposure than after 24 h, in all tested concentrations. The expression of *pparγ* after 96 h of exposure to PMMA-NPs decreased significantly at concentrations of 0.001, 0.01, 0.1 mg L^{-1} but increased significantly in organisms exposed to 10 mg L^{-1}, when compared to 24 h. The expression levels of *rxr* and *apoa1* appeared significantly down-regulated at 96 h when compared to 24 h, at all PMMA-NPs tested concentrations.

In terms of mRNA levels of *lpl*, exposure to 1 and 10 mg L^{-1} of PMMA-NPs induced significantly higher levels at 96 h, when compared to 24 h. Regarding the expression of genes related to the antioxidant defence, *gpx1* and *cat* mRNA levels were significantly lower at 96 h when compared to 24 h, at all PMMA-NPs tested concentrations. For *gr* and *gst3* transcripts, a significant decrease was found in organisms exposed to 0.001, 0.01 and 0.1 mg L^{-1} PMMA-NPs at 96 h when compared to 24 h. Finally, *sod2* mRNA levels were significantly lower at 96 h for 0.001, 0.01, 0.1 and 1 mg L^{-1} PMMA-NPs exposure concentrations, when compared to the same concentrations at 24 h.

3.3. Biochemical responses

In liver, cholesterol levels were unchanged when compared to control after 24 h exposure to PMMA-NPs but, after 96 h exposure to 10 mg L^{-1} PMMA-NPs, levels increased significantly (**Fig. 4**). Triglyceride and glucose levels and ALP, AST, ALT, EA and ADA activities were not significantly affected by PMMA-NPs exposure at any of the time-points.

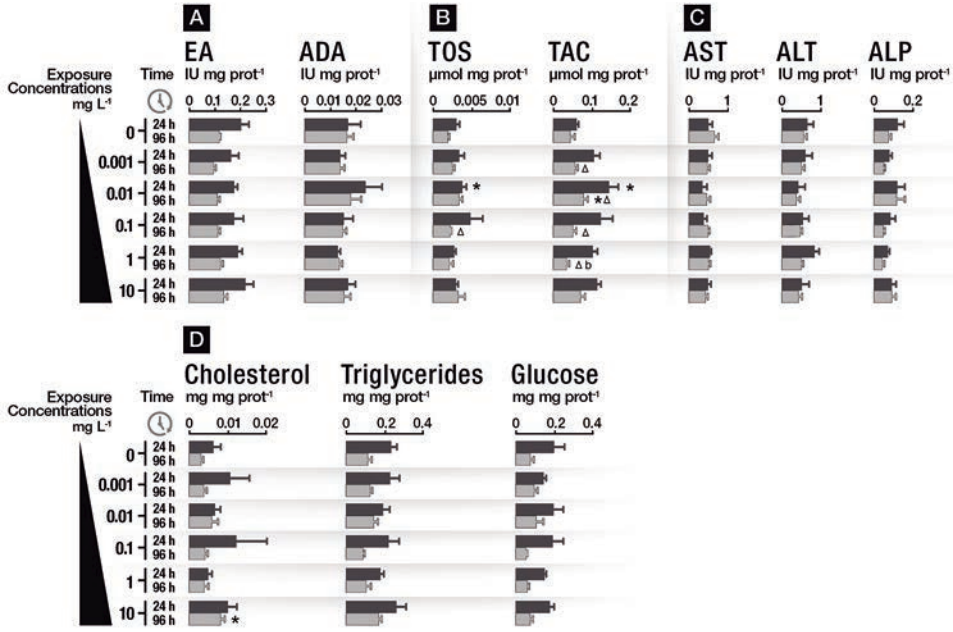


Fig 4. Biochemical parameters in liver of gilthead seabream after 24 and 96 h exposure to polymethylmethacrylate nanoplastics (PMMA-NPs): immune enzymes **(A)**, oxidative status indicators **(B)**, liver enzymes **(C)** metabolism related indicators **(D)**. Values are presented as mean ± standard error (n = 9). Data was analysed using a Two-way ANOVA test, followed by a Tukey test for Post-Hoc analysis. All groups were compared and significant differences are marked as follows: Asterisk (*) denotes significant differences *versus* control, **a** *versus* 0.001 mg L^{-1} PMMA-NPs, **b** *versus* 0.01 mg L^{-1} PMMA-NPs and c *versus* 0.1 mg L^{-1} PMMA-NPs, within the same exposure time, $p < 0.05$. Significant differences between sampling time points are marked at 96 h with a triangle (Δ) *versus* 24 h, $p < 0.05$.

TOS levels, when compared to control, were unaltered after 24 h exposure but presented significantly higher levels after 96 h exposure to 0.01 mg L^{-1} of PMMA-NPs. The same exposure concentration (0.01 mg L^{-1}) caused a significant increase in TAC levels, both after 24 and after 96 h exposure to PMMA-NPs.

In plasma (**Fig. 5**), cholesterol values were significantly higher than control after 24 h of exposure to 0.001, 0.01, 0.1 and 10 mg L^{-1} of PMMA-NPs and remained higher than control after 96 h exposure in organisms exposed to 0.001, 0.01 and 0.1 mg L^{-1} of PMMA-NPs. Triglyceride levels increased significantly after 24 h in fish exposed to 0.1 mg L^{-1} and, after 96 h, in individuals exposed to 0.001, 0.01 and 10 mg L^{-1} of PMMA-NPs. TAC levels were significantly increased after 24 h exposure to 0.1 mg L^{-1} PMMA-NPs, returning to control levels after 96 h.

ADA levels showed no significant differences to controls in any of the PMMA-NPs concentrations.

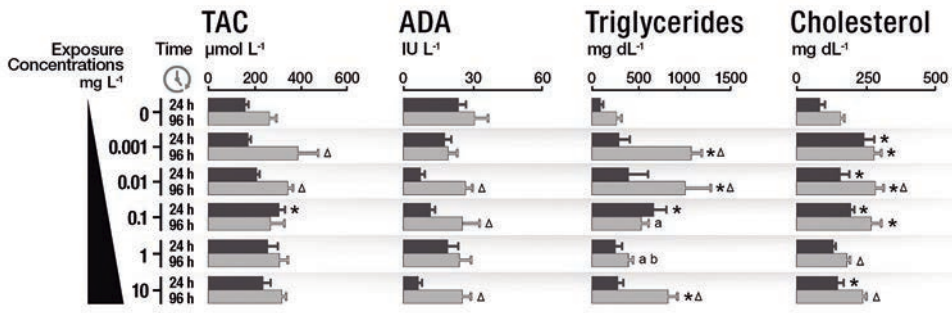


Fig 5. Biochemical parameters in plasma of gilthead seabream after 24 and 96 h exposure to polymethylmethacrylate nanoplastics (PMMA-NPs).

Values are presented as mean ± standard error (n = 9). Data was analysed using a Two-way ANOVA test, followed by a Tukey test for Post-Hoc analysis. All groups were compared and significant differences are marked as follows: Asterisk (*) denotes significant differences *versus* control, **a** *versus* 0.001 mg L^{-1} PMMA-NPs, **b** *versus* 0.01 mg L^{-1} PMMA-NPs and c *versus* 0.1 mg L^{-1} PMMA-NPs, within the same exposure time, $p < 0.05$. Significant differences between sampling time points are marked at 96 h with a triangle (Δ) *versus* 24 h, $p < 0.05$.

Concerning the differences among concentrations within one same sampling time-point, the observed differences were as follows. In liver the only significant change was noted in TAC levels at 96 h, where the exposure of 0.01 mg L^{-1} PMMA-NPs presented an increase when compared to 1 mg L^{-1}. In plasma, the only differences between concentrations were found in triglycerides levels at 96 h, where the concentrations of 0.001 mg L^{-1} PMMA-NPs and 0.01 mg L^{-1} PMMA-NPs showed a significant increase when compared to 0.1 and 1 mg L^{-1} or to 1 mg L^{-1}, respectively. Regarding differences between sampling time-points in liver, TOS levels presented a significant increase for the concentration of 0.1 mg L^{-1} of PMMA-NPs at 24 h when compared to 96 h. TAC levels showed an up-regulation after exposures to 0.001, 0.01, 0.1 and 1 mg L^{-1} of PMMA-NPs at 24 h when compared to their respective exposures at 96 h. In plasma, cholesterol levels were significantly higher after exposure to 0.01, 1 and 10 mg L^{-1} of PMMA-NPs 96 h when compared to the same concentration at 24 h. Similarly, triglyceride levels increased significantly after 96 h exposure to 0.001, 0.01 and 10 mg L^{-1} of PMMA-NPs, when compared to their respective 24 h exposure. TAC levels showed a significant increase at 96 h, when compared to 24 h, after exposure to 0.001 and 0.01 mg L^{-1} of PMMA-NPs. Finally, ADA

levels in plasma presented a significant decrease after 24 h exposures to 0.01, 0.1 and 10 mg L^{-1} of PMMA-NPs, when compared to 96 h.

3.4. Erythrocyte nuclear anomalies (ENAs)

The ENAs levels are presented in **Table 2**. The total ENAs frequency (**Fig. 6B**) was significantly higher than control after 24 h exposure to 0.01 and 0.1 mg L^{-1} of PMMA-NPs and 96 h exposure to 1 and 10 mg L^{-1} PMMA-NPs. Micronuclei were the most frequent ENA, significantly higher than controls after 96 h exposure to 1 and 10 mg L^{-1} of PMMA-NPs (**Fig. 6C**). Regarding different PMMA-NPs exposure concentrations, data showed that at 24 h total ENAs frequency was significantly higher for the 0.01 mg L^{-1} exposure group when compared to 0.001 and 10 mg L^{-1} of PMMA-NPs as well as significantly higher for the 0.1 mg L^{-1} PMMA-NPs exposure when compared 10 mg L^{-1}. At 96 h, total ENAs were increased for the concentrations of 1 and 10 mg L^{-1} PMMA-NPs, when compared to 0.001, 0.01 and 0.1 mg L^{-1}. The frequency of micronuclei at 24 h was significantly higher for exposure to 0.01 mg L^{-1} PMMA-NPs when compared to 0.001 mg L^{-1}.

Table 2. Erythrocytic nuclear anomalies (ENAs) and micronuclei in the blood of gilthead seabream after 24 and 96 h exposure to polymethylmethacrylate nanoplastics (PMMA-NPs).

Values are presented as mean ± standard error (n = 9). Data was analysed using a Two-way ANOVA test, followed by a Tukey test for Post-Hoc analysis. All groups were compared and significant differences are marked as follows: Asterisk (*) denotes significant differences *versus* control, **a** *versus* 0.001 mg L^{-1} PMMA-NPs, **b** *versus* 0.01 mg L^{-1} PMMA-NPs and c *versus* 0.1 mg L^{-1} PMMA-NPs, within the same exposure time, $p < 0.05$. Significant differences between sampling time points are marked at 96 h with a triangle (Δ) *versus* 24 h, $p < 0.05$.

PMMA NPs Concentration (mg L^{-1})	Exposure Time	Nuclear Abnormality Type				
		Micronucleus	Reniform	Segmented	Lobed	Total
0	24h	0.30 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.06	0.40 ± 0.06
	96 h	0.70 ± 0.23	0.50 ± 0.21	0.00 ± 0.00	0.00 ± 0.01	1.20 ± 0.44
0.001	24h	0.20 ± 0.12	0.40 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.70 ± 0.25
	96 h	1.00 ± 0.12	0.60 ± 0.26	0.00 ± 0.00	0.00 ± 0.00	1.60 ± 0.15
0.01	24h	2.50 ± 0.49 a	1.70 ± 0.57	0.00 ± 0.00	0.10 ± 0.06	4.40 ± 0.45 *a
	96 h	0.90 ± 0.10 Δ	0.30 ± 0.10	0.00 ± 0.00	0.00 ± 0.00	1.20 ± 0.17 Δ
0.1	24h	1.80 ± 0.10	1.00 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	2.90 ± 0.15*
	96 h	1.10 ± 0.15	0.80 ± 0.21	0.00 ± 0.00	0.00 ± 0.00	1.90 ± 0.06
1	24h	1.20 ± 0.53	0.80 ± 0.15	0.10 ± 0.06	0.10 ± 0.06	2.20 ± 0.45
	96 h	3.50 ± 0.42*Δabc	0.90 ± 0.00	0.00 ± 0.00	0.40 ± 0.15	4.80 ± 0.44*Δabc
10	24h	0.30 ± 0.00	0.20 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.50 ± 0.06 bc
	96 h	3.30 ± 0.46*Δabc	1.20 ± 0.10	0.10 ± 0.06	0.00 ± 0.00	4.60 ± 0.50*Δabc

The percentage of micronuclei at 96 h were increased for the concentrations of 1 and 10 mg L^{-1} PMMA-NPs, when compared to 0.001, 0.01 and 0.1 mg L^{-1}, equally as for total ENAs.

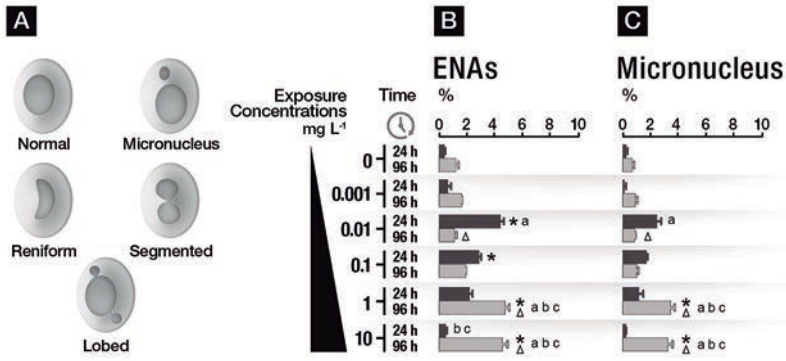


Fig 6. Percentage of erythrocytic nuclear anomalies (ENAs) and micronuclei in the blood of gilthead seabream after 24 and 96 h exposure to polymethylmethacrylate nanoplastics (PMMA-NPs).

Classification of nuclear anomalies (A), total ENAs frequency (B), micronucleus (C). Values are presented as mean ± standard error (n = 9). Data was analysed using a Two-way ANOVA test, followed by a Tukey test for Post-Hoc analysis. All groups were compared and significant differences are marked as follows: Asterisk (*) denotes significant differences *versus* control, **a** *versus* 0.001 mg L^{-1} PMMA-NPs, **b** *versus* 0.01 mg L^{-1} PMMA-NPs and c *versus* 0.1 mg L^{-1} PMMA-NPs, within the same exposure time, $p < 0.05$. Significant differences between sampling time points are marked at 96 h with a triangle (Δ) *versus* 24 h, $p < 0.05$.

Finally, concerning the effect of exposure time, total ENAs frequency decreased significantly at 96 h for the 0.01 mg L^{-1} PMMA-NPs exposure when compared to 24 h, while the concentrations of 1 and 10 0.01 mg L^{-1} increased significantly at 96 h when compared to 24 h. The exact same pattern was observed for the percentage of micronuclei.

4. Discussion

In the present study, the potential impact of a short-term exposure to PMMA-NPs was assessed by evaluating responses at different levels of biological organization. Furthermore, we sought to assess response of two hepatic key functions, lipid metabolism and antioxidant response, particularly relevant considering that previous studies have shown the accumulation of NPs in fish liver and reported alterations in the morphology and metabolism of this organ

(Chae et al., 2018; Kashiwada, 2006). To this end, we evaluated changes in the transcriptional levels of genes encoding the peroxisome proliferator-activated receptors (ppar) in the liver of gilthead seabream, since these nuclear receptors are key regulators of lipid and carbohydrate metabolism and are involved in anti-inflammatory processes (Icre et al., 2006; Varga et al., 2011). In this fish species, three isoforms of peroxisome proliferator-activated receptors (ppar) have been identified (Leaver et al., 2005). We demonstrated that all the tested concentrations induced a significant overexpression of hepatic *pparβ* levels at 24 h, whereas *pparα* and *pparγ* levels remained unaltered at this time point. On the other hand, after 96 h of exposure, expression levels of *pparα* (0.001, 1 and 10 mg L^{-1}) and *pparγ* (10 mg L^{-1}) were up-regulated, while *pparβ* remained at control level.

This response pattern is consistent with previous data obtained with European seabass (*Dicentrarchus labrax*) (Brandts et al., 2018), where a 96 h exposure to the same type of NPs (45 nm PMMA-NPs) induced an increase in the expression of *pparα* (2 mg L^{-1}) and of *pparγ* (0.2 and 2 mg L^{-1}), while the expression of *pparβ* remained unchanged. The *pparα* is a key regulator in lipid metabolism, specifically in the release of energy after the degradation of lipids through β oxidation of fatty acids, while *pparγ* plays role in lipid storage and adipogenesis (den Broeder et al., 2015; Varga et al., 2011). Therefore, alterations in their transcription levels suggest a dysregulation of lipid metabolism and storage after exposure to PMMA-NPs, which could be especially relevant due to the important role of in energy provision in fish (Teles et al., 2016a). The present overexpression of *pparβ* at earlier exposure times (24 h) may reflect an increase in the anti-inflammatory response of the fish in the short term, since it is known that this gene is associated to the anti-inflammatory function (den Broeder et al., 2015; Varga et al., 2011). However, mRNA levels of *pparβ* regained basal levels after 96 h of exposure to PMMA-NPs, indicating a return to homeostasis.

It is known that pparα forms heterodimers with the x retinoid receptor (*rxr*), which can then bind to specific DNA response elements in target genes known as peroxisome proliferator response elements (ppre), generating a transcriptional activation of genes such as apolipoproteins (*apoa1*), lipoproteins lipase (*lpl*) and acyl-CoA oxidase (Rakhshandehroo et al., 2010). In the present study, *rxr* gene presented increased expression levels throughout the experiment after exposure to all the tested concentrations of PMMA-NPs, which is consistent with the up-regulation of apoa1 found at 24 h after exposure to all tested concentrations, since this protein's transcription is regulated by the heterodimer PPARα – RXR.

Although *pparα* levels did not show a significant increase at 24 h of exposure to PMMA-NPs, the concomitant increase in *rxr* and *apoa1* levels suggest an activation of *pparα* before the first sampling time. Similar findings where observed in the same fish species exposed for 96 h to the human pharmaceutical gemfibrozil, a lipid regulating agent (Teles et al., 2016b). In this study, the authors found an up-regulation of *apoa1* and *lpl* without the concomitant activation of the *pparα* pathways. Nevertheless, in the present study, *lpl* levels only showed an increase at 24 h after exposure to 0.01 mg L^{-1} PMMA-NPs, and were unaltered afterwards. Despite the different response profile, which could be explained by the different nature of the tested compounds (a pharmaceutical lipid regulator *versus* NPs), PMMA-NPs modulated *apoa1* and *lpl* mRNA transcription, although the effects appeared to be transitory, with both transcripts able to recover subsequently.

ApoA-1 is a functional protein that forms complexes with phospholipids, cholesterol and triglycerides, so that the significant increase in the expression of the *apoa1* gene at 24 h of exposure could indicate the subsequent use of the protein in these complexes. In this direction, Cedervall et al. (2012) found that 28 nm PS-NPs bind to ApoA-1 in plasma, and this interaction could explain the initial increase in apoa1 transcription observed in our study. The *apoa1* mRNA levels returned to control values after 96 h, which could suggest that the organism has been able compensate the putative effects of PMMA-NPs in ApoA-I abundance after a few days. Nevertheless, in the present study, increased cholesterol levels were found in liver and plasma at 96 of exposure to PMMA-NPs, while triglycerides were also altered in plasma.

This changes in values of both lipid compounds, together with the alterations in the expression of genes responsible for regulating lipid metabolism (*pparα, pparγ, rxr, apoa1*) could suggest that PMMA-NPs are still somehow preventing fish from properly using their fat stores. The alterations in plasma lipids could indicate cholesterol and triglyceride mobilization, and suggest that fish could be exploiting alternative pathways in lipid metabolism. Similarly, increased levels of cholesterol in liver and plasma have been previously reported by Chae et al. (2018) *in Zacco temminckii* (dark chub), exposed for 7 days to 51 nm PS-NPs (5 mg L^{-1}). Cedervall et al. (2012) also reported an increase in hepatic cholesterol of *Carassius carassius* (crucian carp), after exposure to 28 nm PS-NPs through a food chain (algae > zooplankton > fish; 0.01 % (w/v) PS-NPs in algal culture) for 6 weeks. An increase in cholesterol levels after longer term experiments may suggest that a sustained exposure to NPs can cause nutritional or

energetic problems in some fish species. Cholesterol and phospholipids are an important source of energy and an essential component of the cell membrane, in addition to playing an important role as messengers of signal transduction and cell recognition pathways. In consequence, any change in lipid metabolism can indicate a deterioration in these communication pathways, while high cholesterol values may also be indicative of liver dysfunction (Javed et al., 2017).

Nevertheless, ALP, AST and ALT were unaltered under the present experimental conditions, indicating that exposure to PMMA-NPs did not cause liver damage, which corroborates earlier results obtained by the group in European seabass exposed to the same type of NPs during 96 h (Brandts et al., 2018). Moreover, the HSI is unchanged, an expected result considering that a short-term exposure was done and a longer exposure period would presumably be required to see any changes in the HSI. Furthermore, the specific size of the NPs could be relevant to their interference with this parameter, based on their ability to enter cells and interact with biomolecules. Further studies are required to understand the role of nanoparticle size on this parameter. Effects on the HSI of European seabass were previously reported after a long-term (80 days) exposure to low density polyethylene MPs (125–250 μm) which made fish mobilize their hepatic lipid storage, leading to a decrease in the organ's size (Granby et al., 2018).

Regarding the genes encoding antioxidant enzymes evaluated in the liver of the gilthead seabream, on one hand, we observed an overall increase in *gpx1, sod2* and *gr* expression levels at an early time-point, which returned to control levels at 96 h. On the other hand, the other two antioxidant genes (*cat* and *gst3*) remained unaltered throughout the exposure trial. This early increase in the mRNA levels of the antioxidant-related genes suggests that NPs are able to induce an activation of the antioxidant system in the liver of the gilthead seabream, aimed at coping with the oxidative stress generated by the exposure to these particles. Overall, our results indicate that NPs activate the transcriptional machinery in liver, leading to *de novo* synthesis of antioxidant enzymes. This is consistent with the results observed by Chen et al. (2017) where GPx enzyme activity increased in *Danio rerio* (zebrafish) after exposure to 1 mg L^{-1} of polystyrene NPs for 48 and 72 h. Overall, our data show that a 24 h exposure to NPs allowed the detection of more alterations in antioxidant gene transcription.

Oxidative damage is one of the main mechanisms associated with the toxic effects of xenobiotics, and biomarkers such as total oxidative status (TOS) and total antioxidant capacity (TAC) can provide an overview of the animal's

oxidative state (Erel, 2004, 2005; Rubio et al., 2016), reflecting oxidative stress caused by environmental pollutants (Teles et al., 2016a). We observed an increase in TAC levels at both 24 and 96 h, and an increase in TOS the longer exposure time. Taken altogether, the alterations in genes and biochemical parameters related to the antioxidant response suggest that an activation of the transcriptional machinery lead to an increase in TAC allowing the organism to effectively combat oxidative species at 24 h. Nevertheless, at a longer exposure time this effort might not have been sufficient, as, although TAC levels are still up-regulated, TOS is also increased, pointing to the inability of the antioxidant defences to completely tackle the oxidative status induced by exposure to PMMA-NPs. In this same species, Oliveira et al. (2017) reported an increase in TAC in plasma after 24 h of exposure to gold nanoparticles; however, this was not associated with an increase in TOS levels. Alternatively, EA, which is both related with antioxidant and immune response, is unaltered in liver. This results differ from those previously described by Brandts et al. (2018), where European seabass presented a decrease in plasma EA levels after 96 h exposure to 0.02 and 0.2 mg L^{-1} of PMMA-NPs (45 nm). The lack of effects on EA activity suggests that this enzyme did not participate in protection against the oxidative effects generated by PMMA-NPs in the present experiment. ADA, which is also an endpoint related with the immune function is unaltered in both liver and plasma under the present experimental conditions, corroborating an absence of effect of the PMMA-NPs in the assessed immune parameters.

Oxidative stress can cause damage to biological molecules such as DNA, therefore potentially inducing the formation of ENAs. In the present study, we saw an increase in the total number of ENAs at both exposure times, with micronuclei being the most frequently recorded ENAs. Micronuclei are a biomarker of chromosomal damage and/or loss, representing a permanent damage that can only be fixed through cell removal. The formation of other nuclear abnormalities has also been associated with cytogenetic damage. Lobed nuclei may be caused by problems in segregating tangled and attached chromosomes or by gene amplification *via* the breakage-fusion-bridge cycle, during the elimination of amplified DNA from the nucleus; segmented cells contain two nuclei, possibly due to blocking of cytokinesis or cell fusion; reniform nuclei are considered by some authors to have a cytological cause whereas others described them as having a genotoxic origin (Barreto et al., 2020). Although the mechanisms responsible for the formation of ENAs are not yet completely understood, some nuclear abnormalities, such as lobed and segmented nuclei, may be interpreted as nuclear lesions analogous to micronuclei that may be induced by

genotoxicants. Morphological nuclear changes in fish erythrocytes are a good indicator of exposure to contaminants and have been used experimentally as an initial indicator of the genotoxic potential of contaminants (Costa et al., 2011; Carrola et al., 2014). The observed increase in ENAs found in our study could derive from the direct interaction of NPs with the nucli of erythrocytes or may be due to increased oxidative stress after exposure to PMMA-NPs. Comparing results obtained at the different sampling times, it is possible that at 24 h the observed ENAs come from the action of NPs on cells, while at 96 h the effects of oxidative species might be added. To the author's knowledge this is the first report assessing the ENAs after exposure to NPs in fish. Nonetheless, altered ENAs frequencies have been previously described in *S. aurata* exposed to nanoparticles (*e.g.* gold nanoparticles (Barreto et al., 2020). Moreover, it is regularly used to asses genotoxicity in different fish species exposed to a variety of contaminants *e.g. Liza aurata* (golden grey mullet) exposed to pyrene (Oliveira et al., 2007), *Anguilla anguilla* (European eel) exposed to copper (Oliveira et al., 2008), *S. aurata* exposed to a human pharmaceutical (Barreto et al., 2017).

When we compare the effect of the concentration for the same exposure time, we didn't find an evident effect of the concentration, as PMMA-NPs did not cause the typical dose-response pattern. Nevertheless, in some of the parameters the results point to a non-monotonic dose response curve, in which low doses of cause a greater impact than high doses, a pattern of response frequently found for endocrine disrupting chemicals. This is consistent with the results previously described by this research group in European seabass, where increased mRNA expression in genes involved in biotransformation (*cyp11, pgp*) and immune enzyme (*cathepsin*) and a decrease in ChE were observed only at lower exposure doses (Brandts et al., 2018). Canesi et al. (2015) found a greater increase in ROS production in haemocytes of the Mediterranean mussel (*Mytilus galloprovincialis*) exposed to PS-NH^2-NP (50 nm) at low concentrations (1 mg L^{-1}). A similar response was reported by Teles et al. (2016a) in gilthead seabream after the exposure to gold nanoparticles, where the authors observed changes in TOS and in mRNA levels of antioxidant-related genes after an *in vivo* exposure. This could be associated with a lower probability of collision of particles at low concentrations that lead to a lower agglomeration/aggregation.

Globally, our results showed a strong modulation of the studied genes at an early time-point (24 h) in contrast to what was observed at 96 h, when a general tendency to a return to homeostasis was seen. This return to control levels of transcription is especially evident for the genes related with the antioxidant

response. Consistently, the analysis of the transcriptional levels of target genes is particularly applied to the detection of early warning signs of damage following an exposure to contaminants (Teles et al., 2016a). Nevertheless, alterations in the assessed biochemical parameters persisted in time, especially in plasma, as did ENAs observed in blood smears, which in fact increased substantially at 96 h. This points in the direction of plasma being one of the most valuable and integrative matrixes for the assessment of the effects of pollutants, although individual organ/tissue assessment cannot be depreciated.

5. Conclusions

According to the present results, we found that a short-term exposure to PMMA-NPs caused alterations in juvenile gilthead seabream biology, assessed at different levels of organization. Increased transcriptional levels of key genes related with lipid metabolism and changes in plasmatic levels of cholesterol and triglycerides indicate an alteration of lipid pathways after a short exposure to PMMA-NPs.

We also found an early up-regulation of genes related with the antioxidant status, indicating that fish were able to activate antioxidant defences, which coincides with elevated TAC values. Nevertheless, increased TOS levels at 96 h suggests that antioxidant defences might not be able to completely counteract the exposure's effects. The increased frequency of ENAs suggests that PMMA-NPs can induce clastogenic/aneugenic damage in fish erythrocytes. Taken as a whole, the results of this study show that the gilthead seabream is affected by the exposure to PMMA-NPs in the short-term, as reflected by the alteration of parameters both at the molecular and biochemical levels.

As future perspectives, it is recommended to increase the periods of exposure to NPs in order to create a more environmentally realistic scenario.

CRediT authorship contribution statement

I. Brandts: Conceptualization, Investigation, Visualization, Writing - original draft, Writing - review & editing. **C. Barría:** Formal analysis, Investigation, Visualization, Writing - original draft. **M.A. Martins:** Resources. **L. Franco-Martínez:** Investigation, Methodology. **A. Barreto:** Investigation. **A. Tvarijonaviciute:** Investigation, Methodology. **L. Tort:** Funding acquisition. **M. Oliveira:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing - review & editing. **M. Teles:** Conceptualization, Investigation, Supervision, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Short-term exposure to polymethylmethacrylate nanoplastics alters muscle antioxidant response, development and growth in *Sparus aurata*

J.C.Balasch, **Irene Brandts**, C.Barría, M.A.Martins, A.Tvarijonaviciute, L.Tort, M.Oliveira and M.Teles

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6

Abstract

Polymethylmethacrylate (PMMA) plastic fragments have been found abundant in the environment, but the knowledge regarding its effects on the physiology of aquatic animals is still poorly studied. Here the short-term (96 h) effects of waterborne exposure to PMMA nanoplastics (PMMA-NPs) on the muscle of gilthead sea bream (*Sparus aurata*) fingerlings was evaluated at a concentration range that includes 0.001 up to 10 mg/L. The expression of key transcripts related to cell stress, tissue repair, immune response, antioxidant status and muscle development, together with several biochemical endpoints and metabolic parameters. Results indicate that exposure to PMMA-NPs elicit mildly antioxidant responses, enhanced the acetylcholinesterase (AChE) activity, and inhibited key regulators of muscle development (growth hormone receptors *ghr-1/ghr-2* and myostatin, *mstn-1* transcripts). However, no effects on pro-inflammatory cytokines (interleukin 1β, *il1β* and tumor necrosis factor α, *tnfα*) expression nor on the levels of energetic substrates (glucose, triglycerides and cholesterol) were found.

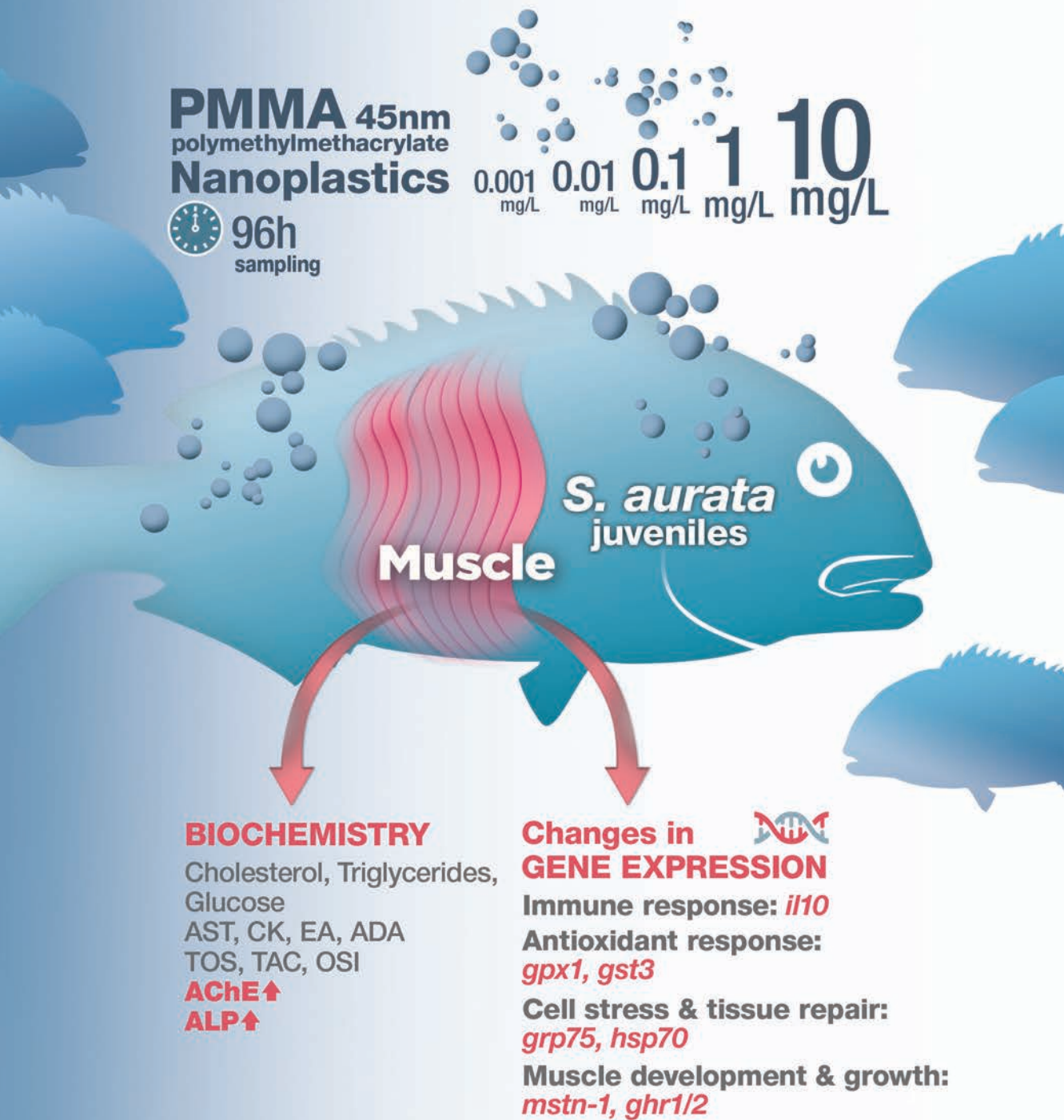
Highlights

- Short-term (96 h) exposure to PMMA-NPs induces oxidative stress in S. aurata muscle.
- No pro-inflammatory responses or metabolic imbalances were observed.
- As a response to PMMA-NPs exposure, myostatin transcripts were inhibited.
- Myogenesis, however, does not seem to be extensively compromised.
- PMMA-NPs may induce mild short-term changes in muscle development and growth.

Keywords

Commercial marine fish, Nanoplastics, Muscle growth, AChE

Graphical abstract



1. Introduction

In the last decades, the pervasiveness and persistence of petrochemical-derived plastic debris in the oceans have turned the problem of local plastic contamination management into a global one. Being extremely versatile, easy to manufacture, inexpensive, chemically stable and moldable, the use of plastic polymers has grown exponentially without effective waste disposal policies having been established (Geyer et al., 2017). It has been estimated that up to 4% of the 350 million tons of plastic produced in 2018 have entered marine environments (Wayman and Niemann, 2021). From these, roughly 80% originate from terrestrial sources, resulting from mismanaged waste removal procedures, sewage systems and indiscriminate dumping, while the remaining 20% can be mainly attributable to tourism, transport and fishing industries (Soares et al., 2020).

The most commonly found plastics in aquatic environments include polystyrene (PS), polyethylene (PE) and polypropylene (PP), and, to a lesser extent, other polymers such as polyethylene terephthalate (PET), polyvinyl chloride (PVC) and poly(methyl methacrylate) (PMMA), among others (Geyer et al., 2017). Each polymer type presents specific properties that determine its dispersion, bioaccumulation and toxicity rates. To date, the vast majority of ecotoxicological studies use PS in controlled setups to analyze the effects of plastic exposure on the physiology of aquatic organisms (Piccardo et al., 2020). Floating plastic fragments deteriorate mechanically, physicochemically and biologically, leading to the formation of small, >1 µm to 5 mm, microplastics (MPs) that will eventually degrade to produce smaller, <1 µm, (though there is still some debate about the precise limits, see Hartmann et al., 2019) nanoplastics (NPs) (Gigault et al., 2018).

The impact of the weathering processes depends strongly on the type of plastic polymer, the additives used for the manufacturing procedures and the aggregated environmental components/pollutants (Oliveira et al., 2019), that constrict both the colloidal and aggregation/sorption indexes with other contaminants and their buoyancy properties, ultimately limiting the dispersal capabilities and deposition of micro(nano)plastic fragments (reviewed in Alimi et al., 2018). Micro(nano)plastics (MNPs) may adsorb other environmental contaminants, such as heavy metals, persistent organic pollutants (POPs) and endocrine disruptors (Campanale et al., 2020; Cortés-Arriagada, 2021; Rochman et al., 2019), enhancing or modifying their bioavailability and potential effects to biota. In the present study, the effects of PMMA NPs (PMMA-NPs) on

fish were tested. PMMA is an acrylic resin used as a protective coating layer in several industrial procedures and products. In surface waters, under the combined effects of photolysis, hydrolysis and elevated temperatures, PMMA may degrade to form micro(nano)plastics within two months (Li, 2020).

NPs are usually viewed as potentially more toxic than MPs, as their reduced size enables the crossing of cellular membranes, and their high surface/volume ratio facilitates the adsorption of POPs (Sharma et al., 2021). However, due to the polymer-specific interaction with other contaminants, biofouling, bioaccumulation, resilience and incorporation in food webs, and also the scalability of physical oceanic patterns and currents, the uncertainties in the detection, quantification, stability and fate of NPs in marine biotopes are still far from being fully resolved (De Sales-Ribeiro et al., 2020; Mao et al., 2020; Piccardo et al., 2020; van Sebille et al., 2020). Nevertheless, even if the resolution of the so-called "missing plastic paradox" (Thompson et al., 2004; Yang et al., 2021) that accounts for these uncertainties and underestimations still faces several analytical challenges, there is a growing consensus on the toxic effects associated to the widespread distribution of NPs in aquatic environments (Banerjee and Shelver, 2021; Chae and An, 2017; Kögel et al., 2020). Larvacean zooplankton, invertebrates (mollusks and crustaceans), and demersal/mesopelagic fish routinely ingest MNPs (Katija et al., 2017; Lusher et al., 2016), contributing to the vertical dispersion of plastic fragments in the water column, and also to their incorporation in the food web, that may escalate the harmful effects of plastic fragments across the trophic levels (Mattsson et al., 2017; Sökmen et al., 2020). The risk assessment of plastic fragments in aquatic environments is still strongly biased towards a few species of crustaceans, mollusks and fish (Huang et al., 2021), usually exposed to high, non-environmental realistic, polymer concentrations in an attempt to elucidate their toxicity range for each model species (de Sá et al., 2018).

Laboratory studies indicate that, even if invertebrate filter feeders may eliminate internalized MPs rapidly after ingestion (Gonçalves et al., 2019), NPs, due to their small size, may cross cellular membranes and translocate from digestive system to other organs, reducing the onset and time course of defensive responses (Cole et al., 2020; Sendra et al., 2021). NPs can then accumulate in tissues such as gills, intestine, hepatopancreas, inducing short-term and medium-term oxidative stress, immune activation and haemocyte genotoxicity (Brandts et al., 2018a; Li et al., 2020; Sendra et al., 2020), and impairing moulting, regeneration and reproductive performance (Bergami et al., 2020; Kelpsiene et al., 2020; Silva et al., 2020; Sun et al., 2021). In addition to size and polymer typerelated polymer,

the physiological effects of NPs seem to be species-specific and dependent on the home range, specific evolutionary constraints, feeding type and morphology, as well as life story traits (Compa et al., 2019; Kögel et al., 2020). A growing literature on fish ecotoxicology attributes to NPs ingestion noxious effects on cellular toxicity, immune stimulation and malfunction, oxidative damage, metabolism alterations, hatching and developmental misdirection's, neurological misbehaviors that affect swimming, navigation and feeding at each life stages (Guimarães et al., 2021; Pedersen et al., 2020; Pitt et al., 2018a; Sarasamma et al., 2020; Wu et al., 2021).

Fluorescence tracing of orally administered NPs in zebrafish showed a webbed distribution of NPs in several organs, even in the embryos, suggesting maternal transfer of NPs to offspring (Pitt et al., 2018b). On the other hand, waterborne exposure of 2-cell stage zebrafish embryos seems to enhance the toxic effects of NPs in the brain of larvae, reducing the growth rates and locomotor activity, specifically when compared with exposure by injection (thus mimicking the maternal exposure and transference of NPs) (Zhang et al., 2020).

Beyond freshwater model species, and in contrast to the sheer number of NPs-related ecotoxicological studies in marine invertebrates, in marine fish the effects of NPs exposure are still poorly understood (Gonçalves and Bebianno, 2021). Short-term exposure to PS-NPs affected cellular viability and oxidative responses in a dose-dependent fashion in cell lines of gilthead seabream (*Sparus aurata*), seabass (*Dicentrarchus labrax*) and mummichog (*Fundulus heteroclitus*) SAF-1, DLB-1, and FuB-1 cell lines, respectively (Almeida et al., 2019; Ruiz-Palacios et al., 2020). Microbiota/enzyme-related digestive processes were impaired in large yellow croaker (*Larimichthys crocea*) after 14-days exposure to PS-NPs (Gu et al., 2020), and short-term hepatic lipid metabolism and oxidative response transcripts were also found to be altered in *S. aurata* waterborne exposed to PMMA-NPs (Brandts et al., 2021). These and other experiments add to the still unresolved complexity of the effects of NPs in fish, suggesting that early and short- to medium exposure to NPs may impair neural wiring, neuromuscular function, motility, feeding and, ultimately, growth in marine fish, all of them processes directly or indirectly related to non-pathological muscle growth and development.

Besides the assessment of larval and adult feeding and locomotor anomalies, the analysis of physiological impairments elicited by NP exposure in fish seems to correlate with several stress-, biochemical/metabolical- and immune-related indicators, such as plasma and mucosal cortisol levels, acetylcholinesterase

(AChE) activity, cholesterol, triglycerides, phospholipids and glucose levels, antioxidant enzymes (CAT, GPx, GSH), and pro/anti-inflammatory cytokines, usually measured by their relative gene expression levels or derived indexes, such as total oxidative status (TOS) or total antioxidant capacity (TAC) (reviewed in Barría et al., 2020). However, it should be noted that the levels and relevance of these indicators for the accurate description of physiological imbalances related to NPs exposure seem to vary between species and within tissues, entangling the assessment of NPs toxic effects. This may be especially relevant in tissues, such as muscle, that may show a lagged or late response to inflammatory reactions induced by NP exposure, as opposed to canonical immune organs (head kidney, spleen, thymus), or portals of entry, including intestinal linings and mucosal skin surfaces, which usually respond to environmental insults with short-term (24–48 h) enhancing of plasmatic stress mediators and exacerbated production of pro-inflammatory cytokines and immune regulators (Valenzuela et al., 2017).

In fish, the muscular system acts as a proxy of imbalances in feeding, foraging, swimming performance and growth. During larval stages, metabolically demanding muscular hyperplasia dominates, and as the myoblasts continue to proliferate and differentiate to myocytes, hypertrophy sums up to normal muscular development (Johnston et al., 2011). Key regulators and hormonal scaffolds, such as the growth hormone/insulin-like factors (GH/IGFs) participate in the balanced interplays between enhancers and inhibitors of muscle fibers (Rius-Francino et al., 2011; Saera-Vila et al., 2009). The most characteristic of these regulators are myogenin and myostatin, which stimulate the differentiation of myoblasts and inhibit muscle formation and growth, respectively (Hernández-Hernández et al., 2017). In indeterminate growers, such as gilthead seabream (Vélez et al., 2017), myogenesis exhibits a pattern of continuous growth that enlarges muscle mass throughout the life cycle, adding muscle metabolic demands to the complexities of the energetic trade-offs between organism and environment.

In this sense, skeletal muscle and its myogenetic regulators are being progressively recognized as modulators of immune reactivity during infection in fish (Balasch et al., 2019; Valenzuela et al., 2017; Wang et al., 2018), which may be aggravated by the effects of NPs exposure in muscle tissues coincident with seasonal pathogen outbreaks, metabolic imbalances or reproductive timing, all of them energy budget trade-offs (Ejsmond et al., 2010). However, the timing and intensity of immune reactivity in muscle may differ from the fast and explosive inflammatory onset observed in canonical immune-related tissues.

In this sense, a certain degree of asynchronicity in immune reactivity may be expected in skeletal muscle compared to other early responding immune tissues, hence the goal of the present study was to restrict the evaluation of the effects of PMMA-NPs exposure to skeletal muscle tissue of gilthead seabream (*Sparus aurata*) fingerlings, an indeterminate grower marine fish species of economic importance in the Mediterranean coastline, by analyzing biochemical and metabolic endpoints, together with transcript expression of immune- antioxidant- stress- and muscle development and growth-related genes.

2. Materials and Methods

2.1. Synthesis of nanoplastic particles

The synthesis of polymethylmethacrylate nanoparticles was done following the procedure described previously (Roy and Devi, 1996), with some modifications. Briefly, methylmethacrylate (MMA) polymerization was carried out in water (80 °C) under a nitrogen atmosphere. Potassium persulphate and dodecyl sulphate were used as initiator and stabilizer, respectively. The hydrodynamic size (40 nm) and surface charge (–26.4 mV) of nanoparticles was analyzed by dynamic light scattering (Zetasizer Nano ZS, Malvern) in ultrapure water. In artificial seawater (24 h, salinity 30) hydrodynamic size reached an average size of 120.3 nm. Synthetized PMMA-NPs had a quasi-spherical shape, as confirmed by transmission electron microscopy (Hitachi, H9000 NAR) and an average diameter of 45 nm.

2.2. Fish husbandry and experimental conditions

Sparus aurata juveniles 8.7 ± 2.5 g (mean ± SD), were obtained from an aquaculture facility in northern Spain (Tinamenor- SonRíoNansa, S.L.). Fish were kept for two months in a 1000 L water tank at the University of Aveiro. Acclimatization conditions were as follows: recirculating artificial saltwater (salinity 30) with continuous aeration, 19 ± 1 °C water temperature and natural photoperiod (14 h light: 10 h dark). Fish were fed a commercial diet (44% protein, 17% fat) *ad libitum* once a day, and feeding was interrupted 48 h before starting the experiment. Procedures adopted in the assay were previously authorized by the Portuguese National Authorities and all experimental procedures involving fish were carried out strictly following ethical animal care legislation: European Guiding Principles for Biomedical Research Involving Animals (EU2010/63). Fish, randomly assigned to one of the 6 experimental groups (n = 9; N = 54), were waterborne exposed to a concentration range of

PMMA-NPs (0 (control), 0.001, 0.01, 0.1, 1 and 10 mg /L) for 96 h, following OECD guideline 203 (OECD, 2019). The exposure concentrations used in our study were chosen considering concentrations used in relevant studies with fish and NPs published in recent literature (*e.g.* Abarghouei et al., 2021; Pedersen et al., 2020; Pitt et al., 2018a, Pitt et al., 2018b). This survey of relevant studies with plastic nanoparticles experiment contributed to the decision of considering 0, 0.001, 0.01, 0.1, 1, 10 mg/L (equivalent to 1, 10, 100, 1000, 10,000 μg/L) as an interesting concentration range composed of "low" and "high" doses to test in our experimental procedures. Although estimates on the environmental concentrations of NPs don't yet have consensus among researchers on the field, some experts suggest that a typical environmental concentration could be found at 1 μg/L and that the environmental concentration range would be between *ca*. 1 pg L/1 and ca 20 μg L/1 (Lenz et al., 2016). Moreover, reports giving information on MPs concentrations found in industrial effluent discharges account for concentrations of ≤30 mg/L or 30,000 μg/L (Lechner and Ramler, 2015).

During the entire assay, fish were monitored for abnormal behavior and lethality and a semi-static renewal procedure was implemented, with 75% of the water renewed every 24 h in all tanks. After the 96 h, fish were euthanized by immersing them in an MS-222 bath at a concentration of 1 g/L. A sample of muscle was excised from the lateral line of each fish, rapidly frozen in liquid nitrogen and stored at −80 °C until further processing.

2.3. Gene expression analysis

Total RNA was extracted from the muscles using Tri Reagent® following the manufacturer's recommendations and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). To synthesize cDNA, 1 μg of RNA was used for each reverse transcription reaction, using the iScript™ cDNA synthesis kit (Bio-Rad), in a Bio-Rad Thermoblock. A Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) was used for the qPCR amplification, with iTaqTM Universal SYBR® Green Supermix (Bio-Rad Laboratories, USA) for fluorescence.

The following protocol was run: 1 cycle at 95 °C for 5 min, 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. To corroborate the specificity of each primer pair, a melting curve analysis was conducted (**Supplementary Fig. 1**), and only primers with a dissociation curve presenting a single peak were used in the subsequent data analysis. Three potential housekeeping genes were

analyzed (*elongation factor-1 α - ef1α*, *α-tubulin - tubulin and glyceraldehyde-3-phosphate dehydrogenase - gapdh*) and NormFinder application (Andersen et al., 2004) indicated *tubulin* as the most appropriate reference gene among the three (**Supplementary Fig. 1**). Selected target genes were representative of immune response (*interleukin 1β – il1β, tumor necrosis factor α – tnfα, interleukin 10 – il10*), antioxidant response (*glutathione peroxidase 1 – gpx1, glutathione-S-transferase3 – gst3, peroxiredoxin 6 – prdx6*), cell-tissue repair (*glucose-regulated protein 75 kDa – grp75, heat-shock protein 70 – hsp70, heat-shock protein 90 – hsp90*) and muscle development and growth (*growth hormone receptor type I – ghr1, growth hormone receptor type II – ghr2, myogenin – myog, myostatin -mstn-1).*

Primers used for quantitative real-time PCR amplification of the genes studied in muscle are shown in **Table 1**. Value for each experimental condition was expressed as normalized relative expression, normalized against those of the reference gene (Pfaffl, 2001). Expression data from nine biological replicates (n = 9) and three independent technical replicates were used to calculate average threshold cycle (Ct) value for each experimental group.

2.4. Biochemical analysis

Muscle samples were homogenized with Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl) at 1:4 weight:volume and then centrifuged (1500 rpm, 10 min, 4 °C). The supernatant was the transferred to a new tube and kept at −80 °C until biochemical analysis of the samples in an automatic analyzer (AU 600 automated biochemical analyzer, Olympus Diagnostica, GmbH). All methodology was previously validated for fish samples and was done in a unique batch.

Glucose, triglycerides, cholesterol, creatine kinase (CK), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and adenosine deaminase (ADA) were determined using specific commercial kits (Olympus Systems Reagents; Olympus Life and Material Science Europe GmbH, Hamburg, Germany; Diazyme Laboratories, Hannover, Germany).

Several biomarkers of oxidative stress and immune function were also analyzed: total oxidative status (TOS), total antioxidant capacity (TAC), esterase activity (EA), acetylcholinesterase (AChE) and Oxidative Stress Index (OSI), an index calculated as the ratio of TOS/TAC (Wu et al., 2017). TOS determination was based on the reaction of ferric ions with orange xylenol in an acidic medium, as described by (Erel, 2005). TAC was determined as previously reported (Erel,

Table 1. Primers used for quantitative real-time PCR amplification of the genes studied in muscle sample of *Sparus aurata* exposed to PMMA NPs for 96 h.

Gene name	Acronym	Function	GenBank	Forward	Reverse	Efficiency (%)
Elongation factor-1 α	*ef1α*	Housekeeping gene	AF184170	CCCGCCTCTGTTGCCTTCG	CAGCAGTGTGGTTCCGTTAGC	105
α-Tubulin	*tubulin*	Housekeeping gene	AY326430	AAGATGTGAACTCCGCCATC	CTGGTAGTTGATGCCCACCT	118
Glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	Housekeeping gene	DQ641630	TGCCCAGTACGTTGTTGAGTCCAC	CAGACCCTCAATGATGCCGAAGTT	95
Interleukin 1β	*il1β*	Immune response	AJ277166.2	TCAGCACCGCAGAAGAAAAC	TAACACTCTCCACCCTCCAC	99
Tumor necrosis factor- α	*tnfα*	Immune response	AJ413189.2	TCGTTCAGAGTCTCCTGCAG	AAGAATTCTTAAAGTGCAAACACACCAAA	99
Interleukin 10	*il10*	Immune response	JX976621	AACATCCTGGGCTTCTATCTG	TGTCCTCCGTCTCATCTG	99
Glutathione peroxidase 1	*gpx1*	Antioxidant response	DQ524992	GAAGGTGGATGTGAATGGAAAAGATG	CTGACGGGACTCCAAATGATGG	105
Glutathione-S-transferase3	*gst3*	Antioxidant response	JQ308828	CCAGATGATCAGTACGTGAAGACCGTC	CTGCTGATGTGAGGAATGTACCGTAAC	104
Peroxiredoxin 6	*prdx6*	Antioxidant response	GQ252684	AGAGACAAGGACGGAATGC	TGTGGCGACCTTCTTCTG	99
Glucose-regulated protein 75 kDa	*grp75*	Cell-tissue repair	DQ524993	TCCGGTGTGGATCTGACCAAAGAC	TGTTTAGGCCCAGAAGCATCCATG	100
Heat-shock protein 70	*hsp70*	Cell-tissue repair	EU805481	AATGTTCTGCGCATCATCAA	GCCTCCACCAAGATCAAAGA	100
Heat-shock protein 90	*hsp90*	Cell-tissue repair	DQ524994.1	GTGGATTCTGAGGACCTGCC	GAGAGTCTTCGTGGATGCCC	100
Growth hormone receptor type I	*ghr1*	Muscle development and growth	AH014067.4	CACGTACTGGCTCCGTCTC	GCCGCTTTCCTGTTGTCAAG	97
Growth hormone receptor type II	*ghr2*	Muscle development and growth	AY573601.2	GACCCCGAACTGCTCAAGAA	TTGTCGCTTTGCTCCTCGAT	97
Myogenin	*myog*	Muscle development and growth	EF462191.1	ATGATGGGCTTGTGTGGGAG	CCGTCTTCCTTTTGCACAGC	100
Myostatin	*mstn-1*	Muscle development and growth	AF258448.1	GTCCGGCAGCAGATCAAAAC	ACCACATCCCTGTTGTCGTC	100

2004) with some modifications (Tvarijonaviciute et al., 2012) and the assay was calibrated with 6-hydroxy-2,5,7,8 tetramethylchroman-2-carboxylic acid. EA was expressed as IU per ml of sample, where 1 IU is equal to 1 µmol of hydrolysed phenyl acetate minute-1 and determined as detailed by Tvarijonaviciute et al. (2012).

AChE activity was measured following the methodology proposed by Ellman et al. (1961), with some modifications (Tecles et al., 2000). The intra- and inter-assay Coefficients of Variability were <10% in all parameters. All analysis was done in the INTERLAB-UMU laboratory of the University of Murcia, Spain.

2.5. Data analysis

All data were analyzed using the Sigma Plot 14.0 software package. Different treatments were compared using the one-way analysis of variance (ANOVA), followed by the Tukey test ($\alpha = 0.05$). Before the analysis, normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test) were verified and transformations performed if necessary (Zar, 1999). Results are expressed as mean ± standard error (SE, n = 9).

In order to analyze the effects of NPs on the gene expression, the data (square root transformed, normalized and the resemblance matrix normalization (Euclidean distance)) were submitted to an ordering analysis performed by Principal Coordinates (PCO), using the PRIMER 6 & PERMANOVA+ (Anderson et al., 2008). Pearson correlation vectors of gene expression (correlation >0.75) were provided as supplementary variables being superimposed on the PCO graph.

3. Results

3.1. Relative gene expression

As gene expression results from PMMA-NPs exposure groups were compared to control group, results are reported as differences from control (**Fig. 1A**). Expression of genes related to the immune function appeared unchanged, except for an upregulation of *il10* mRNA levels in the fish exposed to 0.001 mg/L PMMA-NPs ($p = 0.011$).

Regarding of antioxidant response genes, an upregulation was observed in *gpx1* transcription after exposure to 0.1 mg/L PMMA-NPs ($p = 0.016$) and in *gst3* after exposure to 1 mg/L ($p = 0.037$). Transcriptional levels of *prdx6* remained unchanged. Cell-tissue repair genes showed significant differences after exposure to PMMA-NPs. Transcription levels of *grp75* appeared downregulated

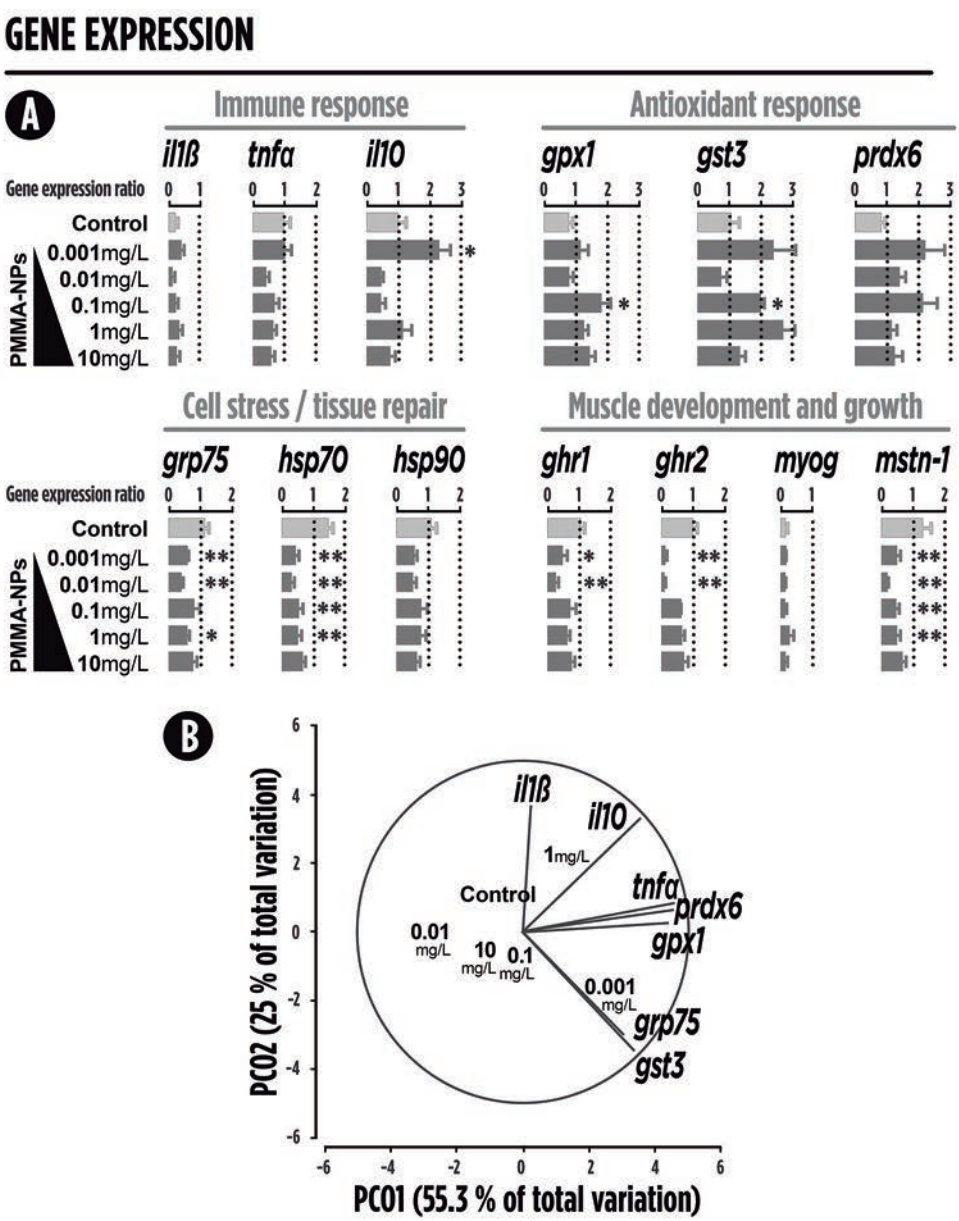


Fig 1. (A) Normalized gene expression of target genes in muscle of *Sparus aurata* juveniles after 96 h of exposure to poly (methyl methacrylate) nanoplastics (PMMA-NPs), and (B) Principal Coordinates (PCO) analysis.

Genes are grouped in the following functions: Immune function, Antioxidant responses, Cell-tissue repair, Muscle development and growth. Exposure groups were compared to the control group and significant differences are marked as follows: Asterisk (*) denotes significant differences *versus* control.

Values are presented as mean ± standard error (n = 9).

in all exposure groups although with statistical significance only for 0.001 ($p = 0.009$), 0.01 ($p = 0.001$), 1 ($p = 0.022$) mg/L PMMA-NPs exposure groups. Moreover, mRNA levels of *hsp70* were significantly downregulated in organisms exposed to 0.001 ($p = 0.001$), 0.01 ($p < 0.001$), 0.1 ($p = 0.007$), 1 ($p = 0.008$) mg/L PMMA-NPs.

Concerning genes related to muscle growth and development, expression levels of both *ghr1* and *ghr2* showed significant downregulation after exposure to 0.001 ($p = 0.019$; $p < 0.001$, respectively) and 0.01 ($p < 0.001$; $p < 0.001$, respectively) mg/L PMMA-NPs. Transcription levels of *myog* remained unchanged. Finally, mRNA levels of *mstn-1* also appear downregulated, with statistical significance for the 0.001 ($p = 0.007$), 0.01 ($p < 0.001$), 0.1 ($p = 0.002$) and 1 ($p = 0.01$) mg/L PMMA-NPs exposed groups. Regarding PCO results, when all endpoints were considered the PCO axis 1 explained 55.3% of total data variation, separating 0.01, 0.1 and 10 mg/L PMMA-NPs at the negative side, from 0.001 and 1 mg/L at the positive side. The PCO axis 2 explained 25% of total data variation, separating 1 mg/L PMMA-NPs, at the positive side of the axis, from 0.001, 0.1 and 10 in the negative side of the axis (**Fig. 1B**).

3.2. Biochemical determinations

The values of both EA and ADA presented no alterations with respect to control in all experimental conditions (**Fig. 2**). Regarding parameters assessing oxidative status, TOS levels were significantly upregulated in fish exposed to 0.01 and 1 mg/L PMMA-NPs. TAC and OSI values showed no significant differences. Glucose, triglycerides and cholesterol levels in muscle were unaltered in all exposure conditions. AChE activity was significantly increased in muscle of *S. aurata* after exposure to 0.01 ($p = 0.016$), 1 ($p = 0.003$) and 10 ($p = 0.011$) mg/L of PMMA-NPs. ALP values were also significantly higher than control in fish exposed to 0.01 mg/L PMMA-NPs ($p = 0.009$). AST and CK levels remained unchanged after exposure to PMMA-NPs.

4. Discussion

Due to the long record of historical settlements in the Mediterranean basin and the partially-closed geographical boundaries that diminish the exchange of water with the Atlantic Ocean, the Mediterranean Sea has become one of the most contaminated waterbodies, with high abundances of micro(nano) plastics, especially in Eastern shores (Llorca et al., 2020; Vlachogianni et al., 2020). A recent survey in the Ebro Delta found NPs in the superficial layer at

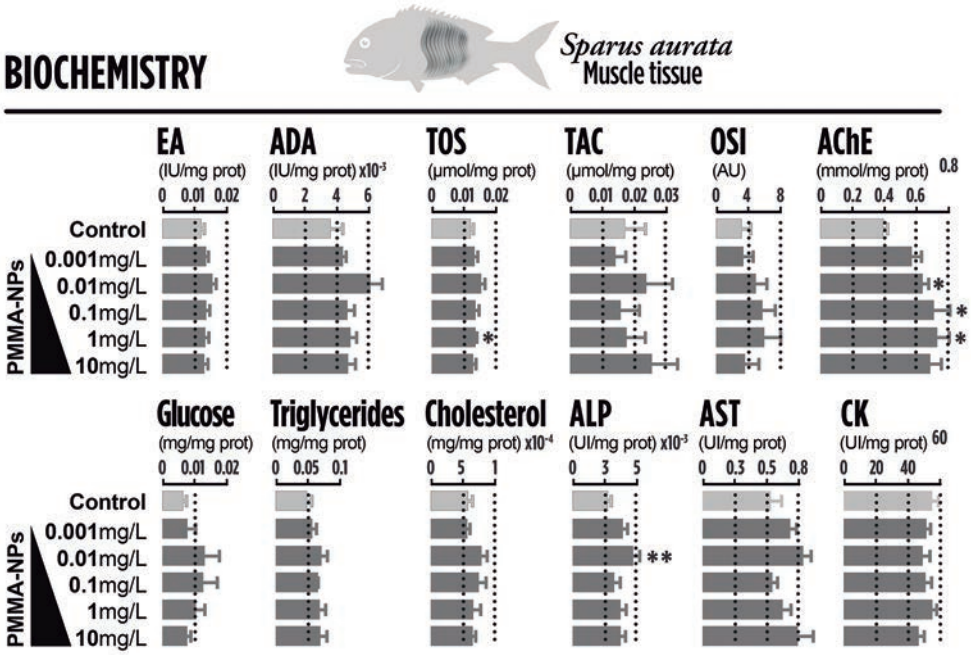


Fig 2. Biochemical parameters determined in muscle of *Sparus aurata* juveniles after 96 h of exposure to poly (methyl methacrylate) nanoplastics (PMMA-NPs).

See the text for abbreviations. Values are presented as mean ± standard error (n = 9). Exposure groups were compared to the control group and significant differences are marked as follows: Asterisk (*) denotes significant differences versus control. Values are presented as mean ± standard error (n = 9).Asterisk (*) denotes significant differences *versus* control.

concentrations up to 7 µg/L in some areas (Llorca et al., 2021). Similarly, to what has been found in global marine surveys (Geyer et al., 2017), polystyrene (PS) and polypropylene (PP) were found to be the typical polymers ingested by several demersal fish species in the Central Mediterranean (Capillo et al., 2020). PMMA, a denser polymer than PS (Annenkov et al., 2021), counts for roughly 25% of MPs polymers found in the Mediterranean seabed (Filgueiras et al., 2019), but its effects on the physiology of organisms are still poorly studied.

The few available studies about PMMA ecotoxicology indicate that PMMA-NPs tend to exert physiological alterations in a very strong species-specific manner. In a recent analysis of short-term (96 h) PMMA effects in marine plankton (Venâncio et al., 2019), several species of microalgae (*Tetraselmis chuii*, *Nannochloropsis gaditana*, *Isochrysis galbana* and *Thalassiosira weissflogii*)

varied greatly with respect to the median effective concentration (EC_{50}) of PMMA –NPs affecting algae growth rate, usually at high concentrations (up to 18.8 mg/L). The same authors found that some subtypes of the rotifer *Brachionus plicatilis* were twice more sensible to PMMA-NPs, with an estimated lethal median concentration (LC_{50}) of 13.3 mg/L.

Interestingly, these concentrations exceed those considered environmentally relevant for PMMA and other plastic nanoparticles (0.01, 0.1 and 1 mg/L), but the lethality also varies with the species and time-course considered. Freshwater crustaceans, such as *Daphnia magna* and *Corophium volutator* showed an estimated $LC_{50\ 48h}$ (*D. magna*) and $LC_{50\ 10d}$ (*C. volutator*) higher than 1000 and 500 mg/L of PMMA-NPs, respectively (Booth et al., 2016). But short-term exposure to PMMA-NPs have been described to induce morphological alterations and slower regeneration rates in the freshwater cnidarian *Hydra viridissima* at estimated 96 h-LC_{50} 96 h of 84 mg/L (Venâncio et al., 2021). In marine gilthead seabream and seabass, waterborne exposure of PMMA-NPs 0.02 to 2 mg/L and 0.01 to 1 mg/L, respectively altered hepatic transcript levels of *pparα/β/γ*, involved in lipid metabolism (Brandts et al., 2021, Brandts et al., 2018b). In the same studies, *S. aurata* also showed enhanced expression of antioxidant transcripts, endured elevated levels of plasma cholesterol and triglycerides, and erythrocyte nuclear anomalies, whereas *D. labrax* displayed elevated plasma levels of alkaline phosphatase, a modulator of inflammation. This indicates that exposure to PMMA-NPs trigger changes in metabolic mediators and elicit antioxidant responses in marine fish. However, the route of internalization, particle size, morphology (variable particle size being determinant) and physicochemical characteristics among other factors, may influence the whole organism response to PMMA nanoparticles. Moreover, the type (innate *vs.* adaptive) and stage of maturation of the immune system will also play a key role. In this sense, species-specific biases are unavoidable, but a common ground may be established relative to the magnitude of physiological alterations elicited by exposure to PMMA-NPs (and in practice, to other NPs) in terms of immune reactivity, including partially or totally, stress-related antioxidant responses, and, as a proxy of overall metabolic performance, growth rates.

All those components of normal homeostasis have been found to be altered, to a greater or lesser degree, in fish as in other vertebrates exposed to NPs (Barría et al., 2020; Yong et al., 2020). Accordingly, our data indicates that the muscle tissue of *S. aurata* fingerlings exposed to PMMA-NPs shows a mild antioxidant

response, absence of pro-inflammatory activity, and a complex modulation of muscle growth. The PCO analysis shows that 55.3% of the total data variation is explained by axis 1, associating organisms exposed to 0.01, 0.1 and 10 mg/L PMMA-NPs with decreased levels of tnfα, prdx6 and gpx1 genes. Axis 2 further explains 25% of variation in our results. This axis highlighted especially the increase in grpt75 in the lowest tested concentrations. Overall, PCO analysis indicated a clear separation between control and 0.001 mg/L PMMA-NPs treatments.

4.1. Effects of PMMA-NPs on immune- antioxidant- and stress-related responses in muscle

In fish, IL-1β is considered a marker for pro-inflammatory activity, able to activate, recruit and orchestrate the coordinated response of cellular components (monocyte/macrophages, neutrophils, several B/T lymphocyte phenotypes, and dendritic cells) and humoral components of the innate and adaptive immune responses (Wang and Secombes, 2013). Tumor necrosis factor α, a key pleiotropic cytokine involved in the cross-talk between phagocytes and the rest of immune cells, also contributes to the onset of inflammation, but may act regulating the progression of inflammation by means of apoptotic-induced clearing of activated T cells (Zou and Secombes, 2016). From the present study data, neither *il1β* nor *tnfα* transcripts presented modified expression levels in seabream fingerlings exposed to PMMA-NPs indicating an absence of pro-inflammatory activity in the muscle. Instead, fish exhibit enhanced expression of anti-inflammatory *il10* transcripts, but only at low dose (0.01 mg/L). Both IL-1β and TNFα cytokines stimulate macrophages to produce reactive oxygen species (ROS) as part of the anti-bacterial defenses (Grayfer et al., 2018), which, in turn, may trigger the expression of antioxidant genes to minimize oxidative damage.

In the regulation of pro-inflammatory processes (besides specialized Th and Treg populations), IL10 is a key component that helps to reduce the production of ROS and activated lymphocytes and facilitates the healing of the inflamed or damaged tissue (Sakai et al., 2021). In addition, reactive oxygen species may also be formed due to accelerated metabolism related to growth or cellular/tissue stress (Hoseinifar et al., 2020). In the present study, of the antioxidant set of genes tested, only *gpx1* and *gst3* were responsive to PMMA-NPs (at 0.1 and 1 mg/L), coincident with the differences observed for the same concentrations in TOS (total oxidative status) but not in other biochemical indicators of oxidative stress, such as TAC and the compound index OSI In this context, both creatine

kinase (CK), a telltale of muscular damage and impaired metabolism with pleiotropic effects in cell and tissue energy transfer and antioxidant processes (Wallimann et al., 2011), and aspartate aminotransferase (AST), a biomarker for tissue necrosis and degeneration (Chen et al., 2020), remained unchanged in the muscle of *S. aurata* fingerlings, indicating tissue integrity. In mammals, CK has been described to enhance muscle cell differentiation as part of an organ-distributed CK/Phosphocreatine (PCr) system of high energy transfer to ATP-demanding organs (Deldicque et al., 2007), and, through the reversible creatine/PCr exchange, enhance the expression of antioxidant enzymes (Young et al., 2010). In fish the effects of CK are much less studied but thought to be similar, considering CK a muscle-related biomarker of early stress (Rojas et al., 2018). Therefore, unaltered muscle CK levels in PMMA-NPS exposed seabream fingerlings may contribute to nurture myogenic growth, potentially reducing oxidative damage or apoptotic processes directly or indirectly by stimulating the expression of antioxidant genes at medium/high environmental concentrations of PMMA-NPs.

These results agree with those of Brandts et al. (2018b) that showed absence of pro-inflammatory response in the liver of *D. labrax* exposed to similar concentrations of PMMA-NPs, and partly with those of Brandts et al. (2021) that indicated a short-term (24 h) antioxidant response to PMMA-NPs in the liver of *S. aurata*. Even if an immune (and an associated increase in oxidative stress reactivity) response can be mounted in 24 h in fish, as part of the innate immunity surveillance (Campos-Sánchez and Esteban, 2021) in *S. aurata* muscle tissues, notwithstanding their immune role and relevance during inflammatory events (Balasch et al., 2019), the immune and associated antioxidant response to waterborne PMMA-NPs are very reduced. In this regard, the low antioxidant activation may be due to an intrinsic mild or reduced stress- and oxidative-related effect of PMMA-NPs itself, and/or a regulation of inflammatory processes in muscle mediated by IL10 and other mediators, including some of the genes linked to muscle growth (see below). Accordingly, the transcripts of heat shock proteins (HSP) remain unaltered (*hsp90*) or downregulated, as observed for *grp75* and *hsp70*. HSPs may promote pro-inflammatory cytokines such as IL-1β and TNFα and has been used as adjuvants in fish vaccines to help induce immunostimulation (Baruah et al., 2017). Moreover, as observed for other HSPs, GRP75 plays a pivotal role in the glucose deprivation management, oxidative stress responses and cell differentiation, and seems particularly sensitive to stress and in species-specific fashion, as demonstrated in stressed gilthead seabream, where *grp75* expression was enhanced to protect against

ROS in the liver (Bermejo-Nogales et al., 2008). The low expression observed in organisms exposed to PMMA-NPs compared to controls of HSPs and the unchanged levels of muscle glucose, triglycerides and cholesterol advocates for a general state of controlled or non-triggered inflammatory processes with minimal metabolic expenditure in short-term exposure to PMMA-NPs.

The unchanged levels of adenosine deaminase (ADA) and esterase activity (EA) across treatments seem to ascribe to this scenario. EA is related to antioxidant and immune response, and variation in ADA activity leads to control of inflammatory processes by virtue of the regulation of adenine nucleoside, an anti-inflammatory molecule (Baldissera et al., 2017), but this study data suggests that the muscle adenosinergic system is not activated by the exposure to PMMA-NPs. In this sense, alkaline phosphatase (ALP) increased levels, but only in organisms exposed to 0.01 mg/L PMMA-NPs, also suggests a mild control of inflammation in *S. aurata* muscle tissue. ALP is a widespread plasma membrane metalloenzyme involved in metabolic trade-offs, nutrient transport and tissue growth, used as a marker of contaminant exposure and pathologies in fish ecotoxicology (Labella et al., 2020), and a component of the innate immune response in mucosal barriers, in what has been suggested as a whole organ distributed alkaline phosphatase "system" of inflammatory control (Lallès, 2019). Evolutionary conserved pathogen-associated molecular patterns (PAMPs) and endogenous tissue/cell damage-associated molecular patterns (DAMPs) trigger the inflammatory responses in vertebrates (Boltaña et al., 2011; Niethammer, 2016), In mammals, ALP dephosphorylate bacterial lipopolysaccharide (LPS) and endogenous polyphosphate (polyP) components of the PAMP/DAMP-related activation of inflammation (Millán, 2006), therefore, high ALP levels have been associated to anti-inflammatory mechanisms of control in several pathologies (Pike et al., 2013). In fish, although less studied, ALP seems to follow the mammalian pattern, especially in tissues such as the gastrointestinal tract and mucosal immune surfaces, in which it may act as an anti-inflammatory component of immune responses (Dawood, 2021; Lallès, 2020). The present study data suggest that muscle may be added to such distributed system of inflammation control mediated by ALP, but an alternative or complementary role in maintaining the energetic balance in muscle under short-term PMMA-NPs exposure cannot be ruled out. Again, the absence of changes in energetic substrates (glucose, triglycerides and cholesterol) may reflect the limited effect of PMMA-NPs on muscle metabolism, or, at certain doses, to the mobilization of metabolism- and membranal transport-related enzymes as observed for ALP.

Interestingly, 0.01, 0.1 and 1 mg/L of PMMA-NPs boosted the levels of acetylcholinesterase (AChE). In fish, as in mammals, enhanced expression of cholinergic system enzymes marks the regulation of inflammatory responses, controlling the immune reaction by means of AChE-mediated hydrolysis of anti-inflammatory acetylcholine (Ach), capable to inhibit the production of IL-1β, TNFα and other pro-inflammatory cytokines (Rosas-Ballina and Tracey, 2009). In fish, increases in AChE activity have been interpreted as a facilitation for pro-inflammatory stages during experimental infections or contaminant exposure (Baldissera et al., 2017; Pretto et al., 2010), but the dynamics of activation/inhibition effects on immune system vary between species and organs and is not fully understood in peripheral tissues or non-neuronal cells in fish. In the freshwater fish *Colossoma macropomum* exposed to the pesticide trichlorfon, the kinetics and half-maximal inhibitory concentration values (IC_{50}) of AChE differ between muscle and brain, a common feature in other teleosts, which indicates that muscle AChE can be considered a marker of mortality in fish ecotoxicology (Duncan et al., 2020), probably due to their pro-inflammatory properties. In silver catfish (*Rhamdia quelen*) infected with *Pseudomonas aeruginosa*, AChE activities in the spleen tissue and lymphocytes increased, reducing the levels of Ach and thus promoting inflammation. However, in homozygous mutant *ache*^{sb55} zebrafish embryos with absence of AChE activity, the normal neuromuscular phenotype is suppressed, indicating that AChE activity is required for muscle development, protection against muscle fiber damage and correct functioning of neuromuscular junction (Behra et al., 2002). Similarly, zebrafish embryos exposed to combined polystyrene NPs and microcystin cyanobacterial toxins showed altered AChE activity together with myogenic regulatory factors (Wu et al., 2021), confirming that interferences in the cholinergic system alters muscle development. This is consistent with what has been described in transgenic murine models related to the expression of AChE: both acetylcholinesterase activity and the numbers of Ach receptors increase during muscle differentiation and growth, and their transcriptional regulation depends on the expression of myogenin (Mutero et al., 1995). In this context, the low to moderate increases of AChE activity in *S. aurata* fingerlings exposed to PMMA-NPs may be interpreted as a mildly inflammatory reaction, but the absence of changes in the pro-inflammatory *il1β* and *tnfα* transcripts and the markers of inflammation discussed above suggest that a protective effect on muscle growth and maintenance may be in order to explain our data. On the other hand, in mammals, half of the Ch present in the neuromuscular junction is estimated to be the result of AChE activity (Hartmann et al., 2008),

and Ch itself has been described to exert anti-inflammatory scenarios by means of enhanced production of IL-10 in fish (Wu et al., 2013; Zhao et al., 2016) and it is used as a dietary complement to reduce apoptosis and promote growth (Yuan et al., 2021). Therefore, it is tempting to speculate about the observed surge of anti-inflammatory IL-10 transcripts in *S. aurata* fingerlings exposed to PMMA-NPs and the coincident increased AChE activity. However, the quantitative immune significance of the relationship between acetylcholinesterase-induced Ach hydrolysis and the role of the choline (Ch) subproduct remains largely unexplored in fish, and no satisfactory conclusion can be drawn to date.

4.2. Effects of PMMA-NPs in muscle growth and development markers

In fish, muscle development results from the concerted expression of myogenic regulators and hormonal axis. The present study data indicates that exposure to PMMA-NPs induce a downregulation of growth hormone receptor transcripts, *ghr1/2* at low doses (0.001–0.01 mg/L) and a pronounced reduction in the expression of myostatin (*mstn-1*) transcripts in all other doses except the maximal (10 mg/L). In fish, as in mammals, the growth hormone (GH)/insulin-like growth factors (IGFs) axis enhance the expression of myogenin transcriptional factors which in turn, promotes muscle hypertrophy and hyperplasia (Codina et al., 2008; Jiménez-Amilburu et al., 2013). Conversely, myostatin regulates hyperplasia arresting the expression of myogenin and inhibiting cell proliferation (Georgiou et al., 2016; Liu et al., 2020).

In addition, GH can activate phagocytes and enhance the proliferation and differentiation of leucocytes in several fish, but in a marked species- and tissue-specific manner (Franz et al., 2016). The acceleration of metabolism mediated by patterned bursts of GH during tissue development may also contribute to the generation of oxidative species (Almroth et al., 2012), and in stressed fish is not uncommon to observe a downregulation of GH, as a means to modulate the production of ROS (Saera-Vila et al., 2009). In gilthead seabream, *ghr1* has been described as a general regulator of IGFs, and *ghr2* as a more stress-sensitive receptor that manages energy substrates and may be affected by oxidative stress (Saera-Vila et al., 2007). Data supports that PMMA-NPs does not impair myogenin or GH-mediated muscle development and growth, but may reduce the anabolic effect of GH at low doses, maybe as a means to reduce the ROS production induced by PMMA-NPs exposure that could overload the animals' antioxidant defenses. Both GH receptors respond in a similar pattern and at the same doses, so no specific targeting of PMMA-NPs for *ghr1* and *ghr2*

transcripts was observed. Instead, *mstn-1* transcripts remained downregulated at all environmentally (and minimal) relevant doses. In seabream, myostatin-1 is expressed in several tissues, suggesting that it may be a pleiotropic gene with multiple functions, including immune responses (Gabillard et al., 2013; Maccatrozzo et al., 2001). *Mst-1* has shown to be upregulated in *S. aurata* infected with *Vibrio anguillarum* high pathogenic serotypes, suggesting an inhibition of muscle growth (Balasch et al., 2019). However, the cross talk between myostatin and the muscle immune system in fish are still in its infancy, and the same authors found no altered expression of pro-inflammatory cytokines in vaccinated seabream. In the present study, an enhanced expression of myogenin transcripts in response to diminished *mstn-1* transcript expression was not observed as described in gene silencing studies (Torres-Velarde et al., 2018). Therefore, an inflammatory related effect of PMMA-NPs in the expression on myostatin cannot be ruled out. The inhibition of myostatin transcripts, as a response to PMMA-NPs exposure, if maintained, may lead to enhanced muscle growth and hypertrophy, but not hyperplasia (Rebhan and Funkenstein, 2008; Torres-Velarde et al., 2020) that, albeit desirable in aquaculture practices, may compromise the balanced expression of myogenic regulatory factors, including GH/IGF axis at medium- to long term.

5. Concluding remarks

Overall, short-term exposure of *S. aurata* fingerlings to PMMA-NPs induced only mildly oxidative stress in muscle, elicited no pro-inflammatory responses or metabolic imbalances, and inhibited key components involved in muscle development and growth, but without dramatically compromising myogenesis. GH-mediated anabolism and energy mobilization were diminished at low doses, but myogenin and several key substrates (glucose, triglycerides and cholesterol) remain unaltered.

Interestingly, the alteration of some key enzymatic systems (AChE and ALP) signal a still unexplored venue for the evaluation of neuro-immune regulation in fish exposed to NPs that deserve more studies to fit the widespread non-neuronal cholinergic systems in the overall regulatory scaffold that limits physiological performance in fish. Additionally, the expression of growth-related genes, such as GHR1 and GHR2 varies seasonally and contextually in fish (Triantaphyllopoulos et al., 2020), which may affect the coupled processes of muscle development, growth, feeding, dispersion and reproduction. Due to its physicochemical properties and rapid degradation rates, PMMA-NPs

may induce mild short-term changes in muscle development and growth, but sustained effluents or resilient sedimentary deposits such as those described in the Mediterranean basin (Filgueiras et al., 2019) may render these physiological changes more pronounced, and divert the energy dedicated to growth to reduce the oxidative stress instead, as demonstrated in aquatic animals (Trestrail et al., 2020). In Mediterranean ecosystems, the meadows of *Posidonia oceanica* seagrasses clear plastic debris in the water column, and in the sediments, by aggregating plastic polymers in lignocellulose fibers, thus reducing the free floating an entrapped sedimentary micro(nano)plastics from entering trophic webs (Sanchez-Vidal et al., 2021). As the continuous receding of *Posidonia* meadows reveals, the bioavailability, bioaccumulation and toxicity of micro(nano)plastics, even if the effects, as described for PMMA-NPs in gilthead seabream, not compromise the normal physiology, are however complex problems that require a multidisciplinary and integrated approach.

CRediT authorship contribution statement

J.C. Balasch and I. Brandts: Investigation, Visualization, Writing - original draft, Writing - review & editing. C. Barria: Technical analysis. M.A. Martins and L. Tort: Resources. C. Barria and A. Tvarijonaviciute: Investigation, Methodology. M. Oliveira: Conceptualization, Investigation, Supervision, Funding acquisition, Writing - review & editing. M. Teles: Conceptualization, Investigation, Supervision, Visualization, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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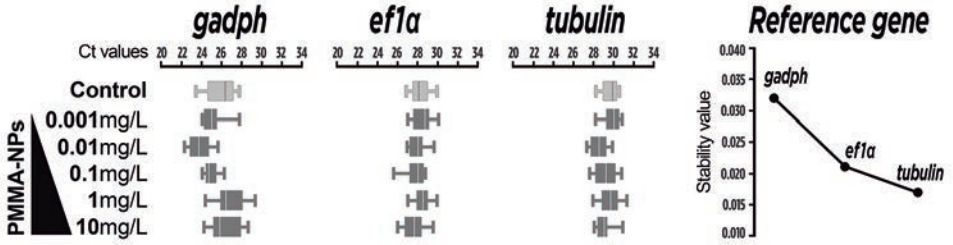
Short-term exposure to polymethylmethacrylate nanoplastics alters muscle antioxidant response, development and growth in *Sparus aurata*

Supplementary material



Supplementary Fig. 1. Candidate reference genes for normalized gene expression calculations in muscle of *Sparus aurata* juveniles exposed to poly (methyl methacrylate) nanoplastics for 96 h.

Cycle thresholds of *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*), *elongation factor 1 alpha* (*ef1α*) and *alpha tubulin* (*tub*). The stability values of tested reference genes, as calculated by NormFinder algorithm, are aslso shown.

Effects of polymethylmethacrylate nanoplastics on *Dicentrarchus labrax*

Irene Brandts, M. Teles, A.Tvarijonaviciute, M.L. Pereira, M.A. Martins, L. Tort, M.Oliveira

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7

Abstract

The present study aimed to evaluate the effects of ~45 nm nanoplastics (NPs) on the marine fish *Dicentrarchus labrax* after a short-term exposure. Animals were exposed to a concentration range of NPs for 96 h and liver, plasma and skin mucus were sampled. Assessed endpoints included biochemical biomarkers and expression of genes related to lipid metabolism, immune system and general cell stress. Abundance of mRNA transcripts related to lipid metabolism, *pparα* and *pparγ*, were significantly increased after exposure to NPs. Biochemical endpoints revealed decreased esterase activity levels in plasma, suggesting that the immune system of fish might be compromised by exposure to NPs. Moreover, significantly lower levels of alkaline phosphatase were found in the skin mucus of animals exposed to NPs. The present results suggest that NPs may represent a hazard to this marine fish, potentially interfering with the metabolism of lipids and the correct function of the immune response.

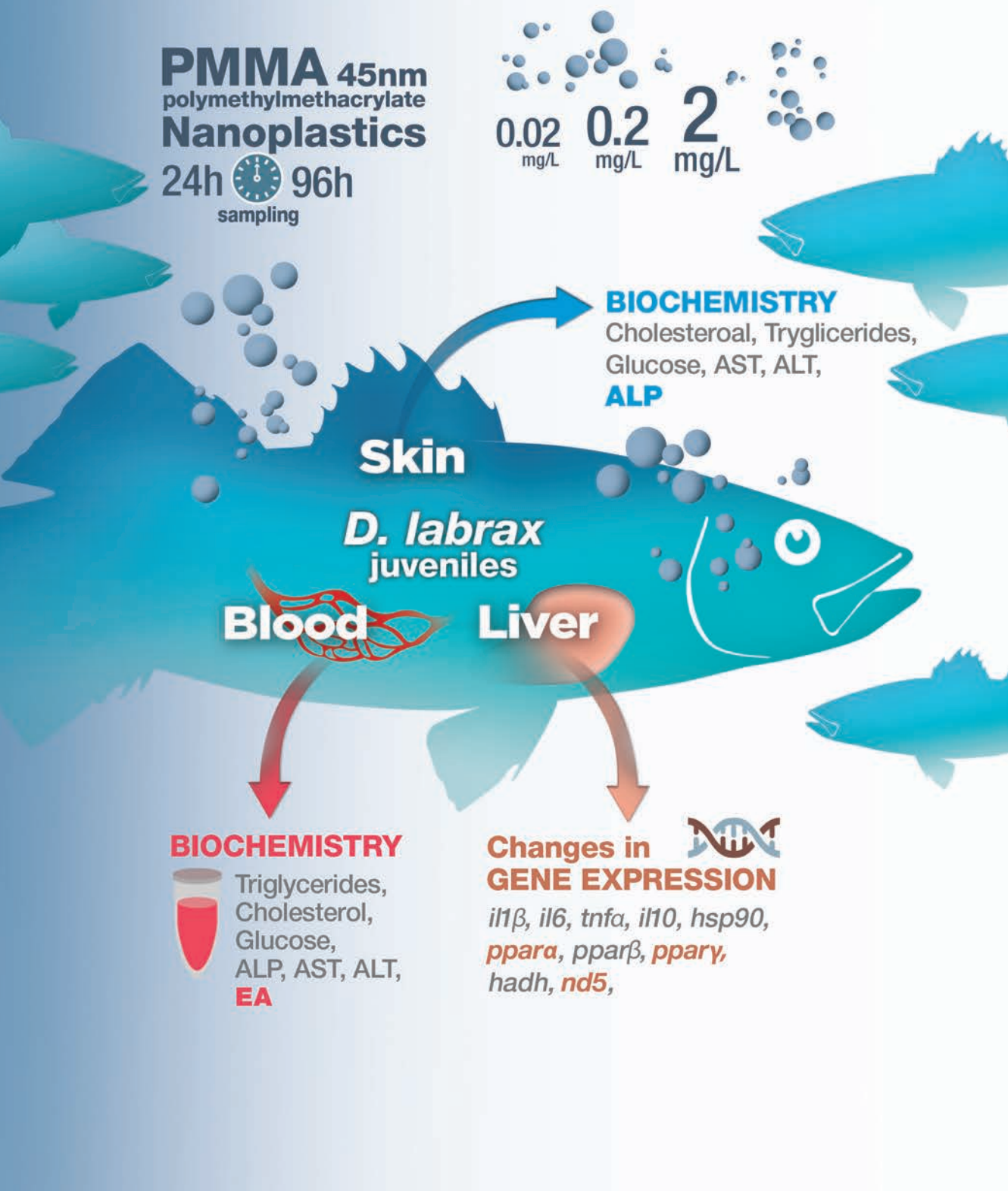
Highlights

- Plastic materials in the environment degrade into nano-size particles
- Effects of nanoplastics (NPs) on *D. labrax* after short-term exposure were assessed
- Molecular signaling pathways related to lipid metabolism were altered in liver
- NPs might interfere with the *D. labrax*'s immune response.

Keywords

Nanoplastics, Sea bass, Lipid metabolism
Gene expression

Graphical abstract



1. Introduction

The existence of small-sized plastic waste in the marine environment and its potential impact on aquatic life has recently become a major concern due to the large quantity of plastic debris released in coastal areas. Once in the water, large plastic debris can be fragmented, through ultraviolet photodegradation, biodegradation, and chemical degradation processes to form smaller-sized plastics, including microplastics (<5 mm) and nanoplastics (<100 nm) (NPs) [1,2]. Even though current environmental concentrations of NPs are not yet available due to analytical limitations [1], their abundance in aquatic environment is strongly hypothesized [3]. With a decrease in size, plastic particles become more bioavailable to aquatic organisms, and potentially more hazardous [4]. Due to their nano-scale properties, NPs may be easily ingested by organisms [5], penetrate tissues [6] and bioaccumulate in organs and tissues [7]. NPs can be taken up by organisms from the environment through direct ingestion [8,9], as well as pass through the food chain *via* trophic transfer [10], affecting the final consumer [[11], [12], [13]]. NPs have been reported to induce oxidative stress [14,15], immune dysfunction [9,16], teratogenic effects [17,18], and altered locomotion [10,11,19]. Furthermore, altered lipid metabolism [11,12,20] and histopathological changes in liver [9,10] of fish exposed to plastic particles have been reported. *Dicentrarchus labrax* (sea bass), a marine fish species and a top predator is a common and valuable aquaculture species in the Mediterranean area. Thus, it may be exposed to NPs *via* water and food web and constitute a potential risk to human health. For these reasons, this species was chosen as model species for the present research.

Liver is a target organ for the majority of xenobiotics, playing an important role in their metabolism and storage, as well as in the redox metabolism [21]. Moreover, previous studies suggest that NPs act as other pollutants and accumulate in liver of fish [10]. For these reasons we chose liver as a target organ for the present study. Following European directive (Directive 2010/63/EU) for the protection of animals used for scientific purposes, the use of less invasive methods to assess the biological effects of xenobiotics on aquatic species is currently essential, both due to ethical and economic reasons. In this perspective, blood and skin mucus have been proposed as valuable biological matrices [22] for the study of aquatic contaminants, not requiring the sacrifice of the animals. Blood has a high sensitivity to the toxic effects of chemicals, playing an important role in the transfer of substances, including absorbed xenobiotics [23]. At this moment, blood plasma is the most used matrix for

the evaluation of the general health status in fish. Skin mucus remains in intimate contact with the surrounding aquatic environment presenting several enzymatic activities that may be affected by environmental contaminants [24]. Fish skin mucus allows an easy and less invasive sampling when compared to blood sampling, and it has been recently proposed as a promising matrix to evaluate fish health parameters in fish [22].

The goal of the present study was to evaluate the effects of NPs on *D. labrax* at different levels of biological organization. Thus, transcriptional levels of target genes associated with lipid metabolism, immune function and cellular stress were measured in the liver in order to investigate NPs' possible effects on the mRNA abundance of these transcripts. Analyzing the transcriptional levels of specific target genes provides insights into the gene regulatory network affected by exposure to pollutants [25], allowing the detection of early warning signs of damage. Biochemical biomarkers related to lipid metabolism (cholesterol, triglycerides and glucose), oxidative stress (EA) and liver health status (aspartate aminotransferase – AST, alanine transaminase – ALT and alkaline phosphatase - ALP) were determined in plasma and skin mucus of *D. labrax*. In carnivorous fish species, dietary lipids are the major provider of energy while carbohydrates play only a minor role due to their low abundance in natural diets [26]. Hence, lipid metabolism and its regulation acquire great relevance in fish.

2. Materials and Methods

2.1. Synthesis and characterization of polymethylmethacrylate (PMMA)

PMMA NPs were prepared by microemulsion polymerization, adapted from Roy and Devi [27] of MMA, with sodium dodecyl sulfate as stabilizer. After polymerization, particle characterization was performed in ultrapure water by assessing hydrodynamic size by dynamic light scattering (DLS) and suspension stability by zeta potential (Zetasizer Nano ZS, Malvern). Morphological characterization was performed by transmission electron microscopy (TEM) (Hitachi, H9000 NAR), where particles did not have a uniform circular shape and presented an average size of 45 nm (**Fig. 1A–B**). The hydrodynamic size determined by DLS was 40 nm in ultrapure water and particle stability was assured by the relatively high surface charge of the particles (–26.4 mV). A stability test revealed that these particles immediately display an increase in the hydrodynamic size to 58.6 nm when placed in artificial seawater (ASW - salinity 30). After 1 h, the average hydrodynamic size reached 97.3 and after 24 h, the particles displayed an average size of 120.3 nm (**Fig. 1C**).

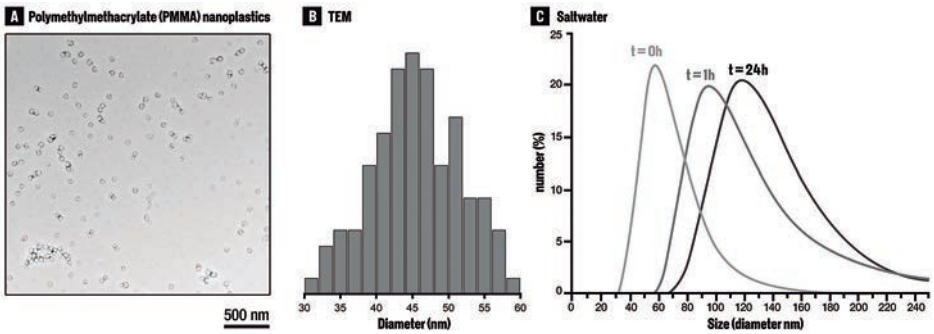


Fig. 1. Characterization of polymethylmethacrylate (PMMA) nanoparticles.

(A) TEM image. **(B)** Particle size histogram in ultrapure water, obtained from the TEM image. **(C)** Particle DLS size characterization in salt water (ASW – salinity 30).

2.2. Animals, experimental design and sampling

Juvenile *D. labrax* specimens (14.6 ± 2.4 cm length and 21.4 ± 6.5 g weight) were obtained from an aquaculture facility (Spain) and acclimatized for 30 days in 1000 L aquaria, with aerated ASW (salinity 30), 19 °C and natural photoperiod (14 h light: 10 h dark). During this period, fish were fed daily with commercial fish food until satiation.

Following the acclimatization period fish were randomly distributed in the following the experimental groups: 0 mg/L (control), 0.02 mg/L, 0.2 mg/L, 2 mg/L and 20 mg/L NPs. Exposure concentrations were chosen based on available studies assessing the effects of NPs on aquatic organisms [28] and reported environmental concentrations of microplastics [29], as no data on the environmental levels of NPs are currently available. Each experimental condition consisted of 2 tanks with 3 animals *per* tank, containing 20 L of experimental media. Animals were exposed to NPs for 96 h, generally following OECD guideline 203 (OECD, 1992), and kept in the conditions described for the acclimatization period (ASW, 19 °C, 14 h light: 10 h dark). The test medium was renewed every 24 h (80%).

At the end of the exposure, skin mucus, blood and liver were sampled. Liver was immediately frozen in liquid nitrogen. Skin mucus was collected following the method described in Guardiola et al. [30]. Briefly, skin mucus was collected by carefully rasping the dorso-lateral surface of the fish, using cell scrapers with enough care to avoid any skin damage and contamination with blood or excretions. Skin mucus samples were homogenized with 1 vol of Tris buffered saline (TBS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl), vigorously shaken and centrifuged (1500 rpm, 10 min, 4 °C). Blood was collected from the caudal

vein with heparinized syringes and plasma isolated (1500 rpm for 10 min, 4 °C). Skin mucus and plasma samples were immediately stored at –20 °C until further analysis.

2.3. Gene expression analysis

Total RNA was extracted from the liver using Tri Reagent® and according to manufacturer's instructions. RNA quantification was done using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA) and RNA quality checked with Experion (Automated Electrophoresis Station, Bio-Rad). Reverse transcription (RT) was performed with 1 µg of RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) was run in a Bio-Rad CFX384 Real-Time PCR Detection System, using iTaqTM Universal SYBR® Green Supermix (Bio-Rad Laboratories, USA) according to the company's instructions.

Potential housekeeping genes were elongation factor 1 alpha (*ef1α*), Glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and cDNA similar to 60S Ribosomal Protein L13α (*l13α*). Target genes were peroxisome proliferator-activated receptor alpha, beta and gama (*pparα, pparβ, pparγ*), hydroxyacyl-CoA dehydrogenase (*hadh*), NADH dehydrogenase subunit 5 (*nd5*), interleukin 1 β (*il1β*), interleukin 6 (*il6*), tumor necrosis factor-α (*tnfα*) and interleukin 10 (*il10*) and heat-shock protein 90 (*hsp90*) (**Table 1**). Efficiency of the amplification was determined for each primer pair using serial 5-fold dilutions of pooled cDNA and calculated as E = 10(–1/s), where s is the slope generated from the serial dilutions [31] (**Table 1**). Expression data, obtained from two independent technical replicates, were used to calculate the threshold cycle (Ct) value. NormFinder application was used to evaluate the most appropriate housekeeping gene and results indicated that most appropriate gene was *gadph* (stability values: 0,012 for *gadph*, 0,013 for *ef1α*, and 0,015 for *l13α*). Thus, expression of target genes was normalized using gadph. Relative gene expression was calculated with the ΔΔCt method [32].

2.4. Biochemical analysis

Cholesterol, triglycerides, glucose, AST, ALT and ALP were determined in the plasma and skin mucus of fish using commercially available kits (Olympus Systems Reagents; Olympus life and Material Science Europe GmbH, Hamburg, Germany) following manufacturers indications. Intra- and inter-CV were below 10% in all cases. EA was analyzed as described by Haagen and Brock

Table 1. Primers used for quantitative real-time PCR amplification of the genes studied in *Dicentrarchus labrax*.

Gene name	Acronym	Endpoint	Accession n°	Forward	Reverse	Efficiency (%)
Elongation factor-1α	*ef1α*	Housekeeping	FM019753	CGTTGGCTTCAACATCAAGA	GAAGTTGTCTGCTCCCTTGG	97
Glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	Housekeeping	AY863148	GTGCCAGCCAGAACATCAT	TGTCGTCATATTTGGCGGGTT	98
cDNA similar to 60S Ribosomal Protein L13α	*l13α*	Housekeeping	DT044539	TCTGGAGGACTGTCAGGGGCATGC	AGACGCACAATCTTGAGAGCAG	90
Peroxisome proliferator-activated receptor alpha	*pparα*	Lipid metabolism	AY590300.1	ACCTCAGCATCAGGTGACT	AACTTCGGCTCCATCATGTC	99
Peroxisome proliferator-activated receptor beta	*pparβ*	Lipid metabolism	AY590300	GCCCTGTTTGTTGCTGCCATTATTCTCT	TCCTGACTCTGCTCCACCTGCTTA	100
Peroxisome proliferator-activated receptor gamma	*pparγ*	Lipid metabolism	AY590303	CAGGACACGCACAACTCAATCA	GGAGAACACGGGACAGTCAGAA	80
Hydroxyacyl-CoA dehydrogenase	*hadh*	Lipid metabolism	KF857303	TGATGGGTGGTCTGCAATGGAT	CTTCTTGTTCAACAGTTCGCTCGG	83
NADH dehydrogenase subunit 5	*nd5*	Lipid metabolism	KF857307	CCCGATTTCTGTGCCCTACTA	AGGAAAGGAGTGCCTGTGA	100
Interleukin 1 beta	*il1β*	Immune response	AJ269472.1	CATGAGCGAGATGTGGAGATCCAAGAT	CATTGTCAGTGGGTGGTGGGTAATC	90
Interleukin 6	*il6*	Immune response	AM490062	ACTTCCAAAACATGCCCTGA	CCGCTGGTCAGTCTAAGGAG	100
Tumor necrosis factor-α	*tnfα*	Immune response	DQ200910.1	TCTACAGCCAGGCGTCGTTCAG	CCGCACTTTCCTCTTCACCATCGT	99
Interleukin 10	*il10*	Immune response	DQ821114	CAGTGCTGTCGTTTTGTGGAGGGTTTC	TCTCTGTGAAGTCTGCTCTGAGTTGCCTTA	75
Heat-shock protein 90	*hsp90*	Cell-tissue repair	AY395632	GAGACCTTTGCCTTCCAGGC	GCCAGACTTGGCGATGGTAC	73

[33], using *p*-nitrophenyl acetate as substrate, with some modifications [34]. All parameters were performed with an automatic analyzer (Olympus Diagnostica, GmbH, Freiburg, Germany).

2.5. Data analysis

Results were expressed as mean ± standard error (SE, $n = 6$ per treatment). Data were tested using the Sigma Plot 12.0 software package. For gene expression data, treatments were compared using non-parametric Kruskal-Wallis test, followed by the Dunnett test to signal significant differences from the control group ($p < 0.05$). For biochemical parameters, different treatments were compared using one-way analysis of variance (ANOVA), followed by Dunnett's method whenever applicable. Data were previously tested for normality (Shapiro-Wilk test) and homogeneity of variance (Bartlett's test).

3. Results

The highest concentration tested resulted in 100% mortality and for that reason this condition is not presented in the results section. Concerning molecular responses (**Fig. 2**), transcriptional levels of genes associated with lipid metabolism presented a global upregulation triggered by exposure to NPs. Peroxisome proliferator-activated receptors', *pparα* expression was significantly increased ($p = 0.018$) after exposure to 2 mg/L NPs, whereas *pparγ* mRNA levels presented a significant upregulation after exposures to 0.2 mg/L ($p = 0.005$) and 2 mg/L ($p = 0.009$) NPs. The expression levels of *pparβ* and *hadh* were not significantly different from control ($p > 0.05$). The mRNA levels of *nd5* were increased ($p < 0.002$) after exposure to NPs (in animals of all tested concentrations).

Regarding immune function, expression of tested genes (*il1β, il6, tnfa, il10)* remained unaltered, when compared to control, after exposure to NPs. The mRNA abundance of cell-tissue repair related gene *hsp90* appeared unaltered as well.

The biochemical responses (**Fig. 3**) assessed in plasma were at control levels in all tested parameters, with the exception of EA, which significantly decreased ($p \leq 0.001$), when compared to control, after exposure to 0.02 mg/L, 0.2 mg/L NPs. In skin mucus, significant differences with respect to control were only found in ALP levels. Significantly lower ALP levels were found in *D. labrax* individuals exposed to 0.2 mg/L ($p = 0.017$) and 2 mg/L ($p = 0.014$) NPs.

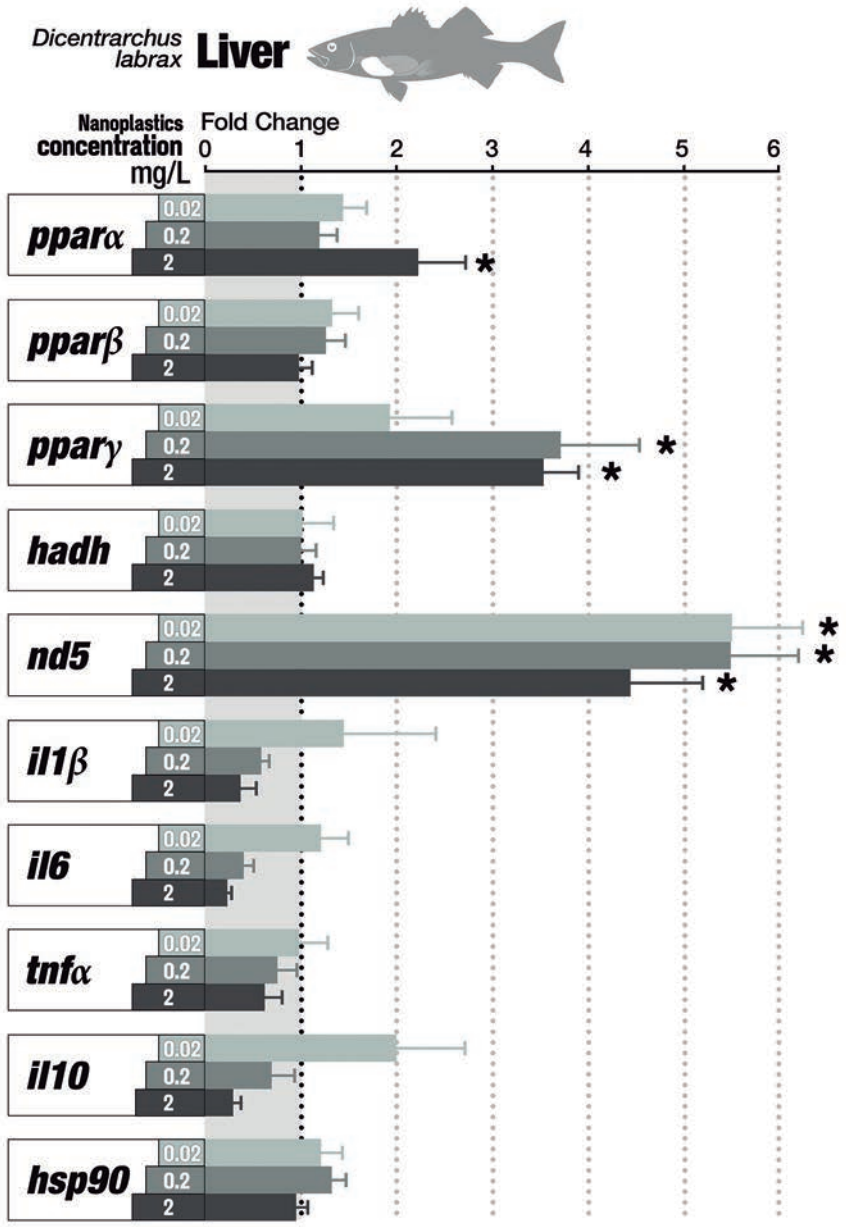


Fig 1. Target genes mRNA levels determined in the liver of *Dicentrarchus labrax* after 96 h exposure to polymethylmethacrylate (PMMA) nanoplastics (NPs).

Values represent the means ± SE (n = 6). Differences were determined Kruskal-Wallis test, followed by the Dunnett test. Statistically significant differences ($p < 0.05$) from control group are marked with an asterisk (*).

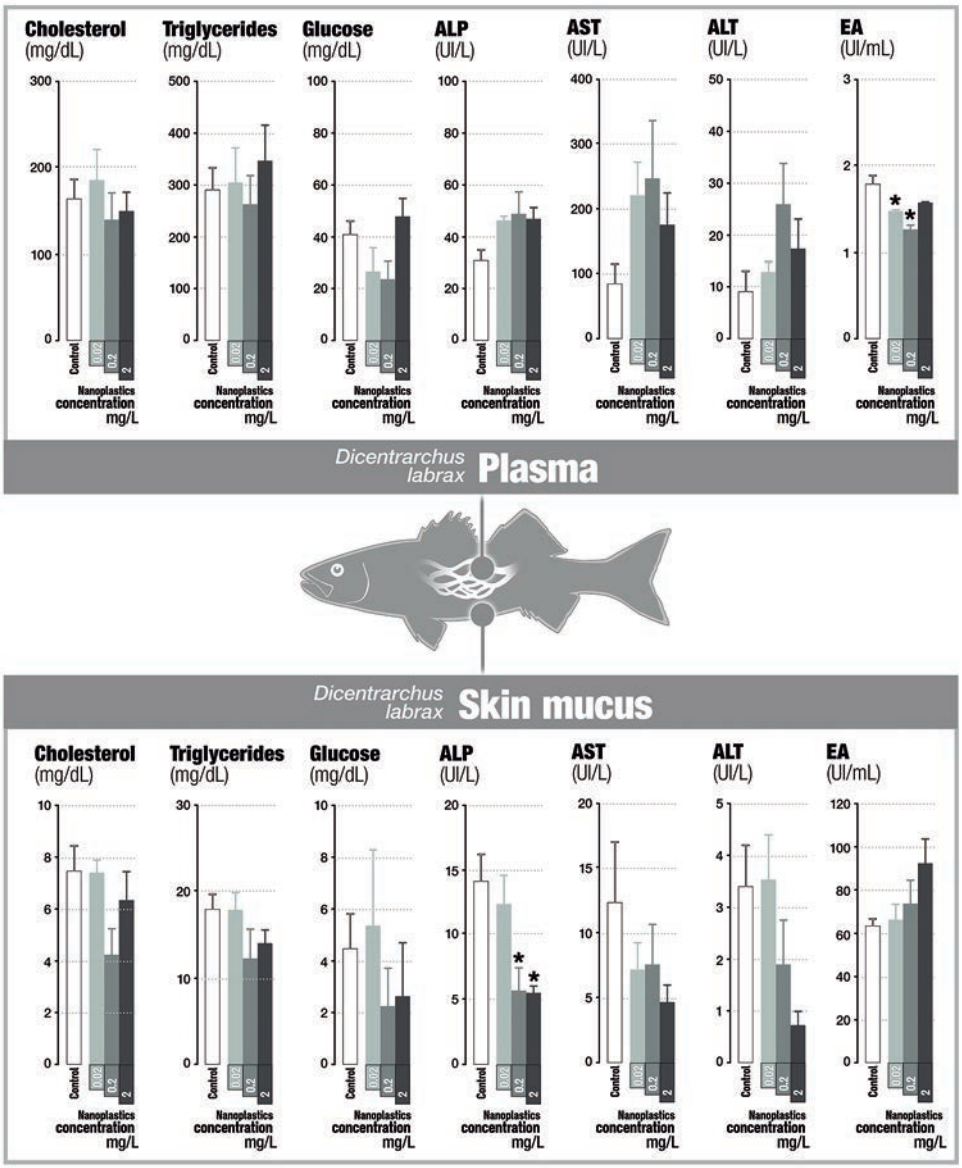


Fig 2. Biochemical parameters in plasma and skin mucus of *Dicentrarchus labrax* after 96 h exposure to polymethylmethacrylate (PMMA) nanoplastics (NPs).

Values represent the means ± SE (n = 6). Differences were determined by one-way ANOVA followed by Dunnett's test. Statistically significant differences ($p < 0.05$) from control group are marked with an asterisk (*).

4. Discussion

Overall, results showed that NPs activate transcriptional machinery in the liver of *D. labrax*, leading to increased expression of genes related to lipid metabolism. The main role of lipids in fish is the storage and provision of energy in the form of adenosine triphosphate (ATP) through the β-oxidation of fatty acids [35], being a major source of energy for many fish species [36]. This β-oxidation occurs in cellular organelles like mitochondria and peroxisomes, catalyzed by a set of different enzymes [37]. Peroxisome proliferator-activated receptors (PPARs) participate on the fatty acids signals as key regulators of lipid metabolism [38]. The peroxisome proliferation has been proposed as a biomarker for environmental pollution assessment [39], as increases in the size, number and volume of peroxisomes in aquatic organisms exposed to contaminants have been documented in marine species [36]. Three distinct peroxisome proliferator activated receptors (ppar) isotypes have been identified in *D. labrax* [26]. Pparα regulates gene expression by binding to specific DNA sequences, resulting in the transcriptional activation of target genes, such as apolipoproteins, lipoprotein lipase and acyl-CoA oxidase, essential for the metabolism of lipids. Moreover, pparα has also been shown to modulate glucose metabolism, liver inflammation and hepatocyte proliferation [40]. In the present study, *pparα* mRNA levels were increased after exposure to 2 mg/L NPs. Cedervall et al. [11] demonstrated that NPs bind to apolipoprotein A-I in fish serum *in vitro*, therefore, possibly preventing fish from properly using their fat reserves. Pparγ is mainly found in adipose tissues, immune cells, intestines, kidney and liver [38]. In the present study, *pparγ* transcript levels were also increased, in fish exposed to 0.2 mg/L and 2 mg/L NPs. Gene *nd5* codifies for the core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I), responsible for the transfer of electrons in oxidative phosphorylation, necessary process for the synthesis of ATP. Alterations in mRNA levels of *ppara*, *pparγ* and *nd5* after exposure to NPs suggest an interference with the ability of fish to mobilize energy reserves.

In order to investigate the immunotoxicity of NPs in *D. labrax*, expression of genes related to the innate immune function in fish were also evaluated. The present findings showed unaltered transcriptional levels of *il1β*, *il6*, *tnfα* and *il10*, after 96 h exposure, suggesting that NPs did not trigger *D. labrax*'s immune function at a molecular level. Under the present experimental conditions, NPs did not induce changes in the expression of *hsp90*, suggesting an absence of cellular stress strong enough to induce *de novo* synthesis of these cell chaperones.

The available pool of HSP might have been sufficient to counteract any oxidative stress induced by NPs. It has been demonstrated that pollutant-related oxidative stress upregulates HSP90 in fish hepatocytes [41], since heat shock proteins are generally expressed at low levels in unstressed cells, and their expression is enhanced following exposure to stressors [42]. Moreover, Espinosa et al. [43] have previously reported upregulated *hsp90* expression in sea bass exposed for 21 days to dietary polyethylene microplastics. The same authors reported *hsp90* upregulation in *Sparus aurata* after dietary exposure to polyvinyl chloride for 30 days [44]. The observed differences in the present study may be due to the different behavior of smaller nanoparticles, to the different exposure times and/or to the possibility of NPs to reach the liver through a different route from the dietary.

Decreased EA levels were found in plasma after exposure to 0.02 and 0.2 mg/L NPs, and levels of ALP in skin mucus also decreased significantly following exposure NPs, at 0.2 and 2 mg/L. Both esterases and alkaline phosphatases are relevant enzymes recognized as part of the natural immunity of fish, even though their role and mechanisms are not yet fully understood [45]. In fish, mucosal surfaces contain numerous immune substances that act as the first line of defense against pathogens. Among them, ALP in skin mucus has been shown to have an important role as an antibacterial agent [30], and has also been associated with skin regeneration in the initial stages of wound healing and infection [45]. Both enzymes could play an important role in sea bass innate immunity, and their decreased levels suggests an impairment of the immune system's function following exposure to NPs. This could lead to an incapacity of the animal's defense mechanisms to perform correctly and, therefore, to defend them from potential external aggressions. Canesi et al. [14] found that NPs exposure induced *in vitro* toxic effects on *Mytilus galloprovincialis* haemocytes. The lack of effects in EA observed at the highest tested concentration may be due to altered bioavailability. The probability of nanoparticles collisions at a given temperature will increase by increasing the number of particles *per* volume (*i.e.*, concentration). In saltwater nanoparticles are expected to aggregate/agglomerate [46] and therefore, differences in terms of decreased bioavailability with increased concentration due to aggregation must be considered.

In summary, the present study demonstrates that NPs can alter molecular signaling pathways related to lipid metabolism in sea bass. Moreover, although no alteration was found in transcripts related to immune function, decreased EA levels suggest that the fish's immune system might nevertheless

be compromised by exposure to NPs. Considering that organisms might be exposed to NPs during long periods of time, the chronic effects of NPs should be assessed in future studies.

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General Discussion

8

With the aim of contributing to the study of the short-term effects of plastic exposure on the physiology of fish, in the present thesis the effects of NPs on three different model fish species were investigated at different levels of biological organization. Here we show that a waterborne exposure to NPs can alter relevant molecular, biochemical, and cellular endpoints in fish, both in a model laboratory species, zebrafish (*Danio rerio*), and in two Mediterranean species used as models for stress and welfare, gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrachus labrax*), (Cerqueira et al., 2020; Raposo de Magalhães et al., 2020). To assess potentially affected molecular pathways, transcriptional levels of relevant genes associated with lipid metabolism, immune function and inflammation, and oxidative stress were evaluated. Alterations in the transcriptional levels of specific genes facilitates the detection of early warning signs of damage, providing insights into the gene regulatory network affected by exposure to pollutant. In parallel, we also measured biochemical parameters related to these three processes, in both gilthead seabream and European seabass.

The widespread contamination of MPs and NPs in marine habitats, evidence of increasing numbers and prevalence of interaction between plastic particles and marine organisms has led to a rising concern about their potential impacts and adverse effects at all levels of biological organization. As contaminants of emerging concern (CECs), NPs are not yet regulated under current environmental laws, and therefore, scientific data reporting their potential effects should aid decision-makers in regarding both nanomaterials and breakdown plastic particles in the update of environmental policies. However, several methodological problems hamper the analysis of NPs effects in aquatic animals and are summarized below concerning the results described in this thesis.

Chosen polymers, environmental relevance, and dose-response

Controlled laboratory exposures allow control variables such as water quality, temperature, salinity, dissolved oxygen, and photoperiod, providing a lower degree of variability than field exposures or samples. They also allow the selection of a certain species in a specific stage of development, control over the number of exposed individuals, the number of replicates, the type of test compound and its concentration, as well as the time and routes of exhibition. As NPs research is still a novel field and many aspects concerning these CECs and nanomaterials in general are still greatly unknown, a high degree of variable control is pertinent. The in vivo exposures to NPs included in this thesis were designed following

recommended OECD guidelines for acute toxicity testing of chemicals (with some adaptations), which are based on experts' recommendations to generate reliable and reproducible data for toxicity testing (OECD guideline 203 - 96 h for acute toxicity testing).

The fish used in the experimental exposures were in a state of sexual immaturity, to reduce the interference of metabolic processes associated with reproduction in other key processes under study. Cell lines are a crucial tool into understanding the effects of emerging contaminants, as are NPs, at the molecular and cellular level (Pannetier et al., 2018; Revel et al., 2021), while complying with European guidelines (Directive 2010/63 EU) to reduce animal use in experimentation. Therefore, they a can be a powerful instrument to explore molecular pathways and modes of action of NPs in organisms and specifically in liver, the main organ both for processing of xenobiotics and metabolism. For this reason, we have included in vitro models in this thesis, as complement to in vivo experimentation.

Polymers

In the marine environment, the most commonly found plastic polymers include PS, polyethylene (PE) and polypropylene (PP), polyethylene terephthalate (PET), polyvinyl chloride (PVC) and PMMA (Geyer et al., 2017). Nevertheless, in practically all published studies with NPs, including our own zebrafish study **(Chapter 3)**, model PS-NPs have been employed, with about 86% of the total number of studies on NPs focusing on this polymer (Barría et al., 2020). The information provided by model PS-NPs in model species is vital for the advance of the field of NPs research, as they provide valuable information regarding for example NPs properties, intracellular localization, modes of action, and affected biological pathways. In the zebrafish study presented in this thesis using model PS-NPs we demonstrated capability of NPs to accumulate in lysosomes of liver cells and in zebrafish larvae pancreas and intestine. Moreover, we also found a synergistic activation the expression of anti-viral genes at low NPs doses, pointing to a possible alteration in immune related pathways in fish. These results contribute to the increasing body of research assessing the effects of model particles, allowing the comparability of results between studies, and facilitating the reaching of conclusions.

Nevertheless, as the adverse effects of NPs can vary depending on particle size, morphology and polymer composition (Piccardo et al., 2020) it is important to have information on polymer-specific effects of NPs to conduct a correct risk assessment. PMMA is a polymer with growing applications in coatings and

emulsions as well as in the medical and health sectors. Moreover, the demand of PMMA or "plexiglas" has exponentially increased in the last years demand in the last years, due to the multiple uses related to the Covid-19 pandemic, such as solid barriers between desks or individual face protections.

When discarded in the environment, the combined processes of photolysis, hydrolysis and thermal degradation in surface waters, can degrade PMMA to MPs/NPs in the span of two months (Li and Guo, 2020). Up to date, studies assessing the effects of PMMA-NPs in marine biota are scarce. This thesis provides the first studies done on the effects of PMMA in fish, proving that it can cause detrimental effects in gilthead seabream and European seabass. Globally, our data supports the evidence that PMMA-NPs may compromise immune function, oxidative status, and lipid metabolism in both Mediterranean marine fish species. Understanding the toxicity and the ecological effects of NPs needs to evaluate if some and particle types may be more or less reactive than others, contributing to the risk assessment for different polymers.

Future research should try also to move closer to environmental reality regarding particle characteristics as well, as heterogeneous NPs can be expected to differ from spherical engineered NPs at least slightly. In this direction, using plastic nanoparticles resulting from breakdown of larger plastic objects can provide this more realistic approach. Secondary NPs are subject to aging processes that might generate reactive oxygen species, roughens the surface and alter material hydrophobicity (Yousif and Haddad, 2013). This will inevitably alter particle dynamics and interaction with organisms and therefore should be considered studies aimed at assessing ecosystem effects.

Concentrations and environmental relevance

NPs particles have been found in the environment but at the present time environmental concentrations assessment is largely speculative, primarily due to the lack of optimized efficient analytical methods (Wagner and Reemtsma, 2019). Considering that secondary NPs are constantly released by the fragmentation and degradation of macro and micro plastic debris, concentrations are bound to be increasing and some authors point to NPs concentrations 1014 times higher than those measured for MPs (Besseling et al., 2019). Furthermore, future predictions visualize increases of at least 50-fold from present-day concentrations during this 21st century (Everaert et al., 2018).

Although estimates on the environmental concentrations of NPs don't yet have consensus among researchers on the field, it has been suggested that the

oceanic concentrations could be of the order of mg/m^3 (Kögel et al., 2020). Moreover, precise estimations of a typical environmental concentration have suggested 1 µg/L and the environmental concentration range varying between ca. 1 pg/L and ca. 20 µg/L (Lenz et al., 2016). On the other hand, reports giving information on MPs concentrations found in industrial effluent discharges account for concentrations of ≤30 mg/L (Lechner and Ramler, 2015). Taking this information into account, the lower dose range used in the in vivo experimental exposures in this thesis (1, 10, 20 µg/L) would fall within the range of estimated concentrations of NPs in the environment. Furthermore, all of the NPs concentrations tested in all of the in vivo studies would fall within the range found in MPs/NPs concentration hotspot, such as industrial effluents, which could very well coincide with the natural habitat or aquaculture area of *S. aurata* and *D. labrax* in the Mediterranean. Moreover, considering that both species are top predator in the food web they could easily bioaccumulate NPs concentrations higher to those estimated in environmental seawater through trophic transfer of NPs, which has already been proved in fish (Mattsson et al., 2015, 2017). Even if some of the tested concentrations could be in the higher range of estimated environmental concentrations, the chosen concentration ranges cover a wide span of possible exposure concentrations while not losing environmental reality.

Altogether, the experiments undergone in this thesis allow an approximation to the potential effects of NPs, both in a model laboratory species and in relevant Mediterranean fish species. It is therefore specifically relevant that effects have been found at the molecular, physiological, and cellular levels in all studied species.

Non-monotonic dose-response

Globally looking at all the studies presented in this thesis and assessing the concentration effect, we don't find a typical dose-response scenario. On the contrary, we find a repeating pattern, where the lower exposure concentrations appear to be the ones causing more potential effects. We can see this in the study done with zebrafish **(Chapter 3)**, where the lower doses of PS-NPs exhibit an exacerbated response in immune genes that is not observed for higher doses. This pattern is also strongly present in the three presented studies with gilthead seabream **(Chapter 4, 5, 6)**, as low doses cause more changes in the measured molecular, biochemical, and cellular endpoints, practically in all tissues. Altogether, these results suggest a non-monotonic dose response (NMDR) after short-term exposure no NPs in the studied scenarios. Moreover,

this pattern of response to NPs exposure is also exhibited in previous studies, both by our and other research groups, in different species (Canesi et al., 2015; Brandts et al., 2018). A NMDR is defined as a dose-response curve whose slope changes direction within the range of tested doses (Lagarde et al., 2015). It is often characterized by responses at low dose (s) and a decreased response at low- and high-exposure levels and seems to contradict the well-accepted hyperbolic or curvilinear dose-response relationship. It is a documented response exhibited by multiple compounds (reviewed by Vandenberg et al., 2012), and is frequently associated to endocrine disrupting chemicals (Kumar et al., 2020). Nevertheless, non-monotonicity presents a challenge for classical toxicology and risk assessment, as regulatory agencies generally operate on the assumption that the hazardous effect to a toxicant increases proportionally with the level of exposure. Based on this, the no observed adverse effect level (NOAEL) is established, a threshold below which a substance is not expected to induce adverse effects or organisms (Slob and Pieters, 1998) and this NOAEL is used to determine regulatory policies. The European Food and Safety Agency recently assessed the biological relevance of NMDR in pollutants, recommending the development harmonised frameworks for identifying and addressing NMDRs in the risk assessment process (EFSA Scientific Committee, 2021).

With the results presented in this thesis, we show that NMDR and low-dose effects are a plausible scenario in NPs toxicology. If experimental evidence pointing to this pattern of response increases, it should be carefully considered when assessing NPs toxicology and elaborating risk assessment protocols and Adverse Outcome Pathways. Paradoxically, the potential effects of NPs specifically at lower exposure concentrations brings us closer to environmentally realistic scenarios. In the case of NPs, the exhibition of NMDR could be related to particle interactions, leading to decreased bioavailability at higher exposure doses. Reportedly, the nanoparticle properties of NPs enable them to escape biological defence systems, crossing physiological barriers such as tissues, mucus, and cell membranes (Kihara et al., 2021). Nevertheless, the interactions between particles established at higher concentrations can promote the formation of aggregates (Wu et al., 2019a), which would enable the physiological defence barriers to block their entrance. Indeed, it has been seen that particle parameters such as size, charge, or concentration can affect the uptake and toxicity of NPs in aquatic organisms (Kögel et al., 2020).

A major challenge in understanding NPs toxicity lies in understanding particle dynamics and interaction with the surrounding environmental and biological

matrices, crucial for predicting NPs' biological impact. As mentioned in the introduction section, NPs naturally bind to available macromolecules from their surroundings, due to their high surface area (Lynch et al., 2014). This interaction can occur in biological fluids and also aqueous environments with dissolved organic matter, which plays an essential role in the aquatic carbon cycle (Chen et al., 2018). The corona of molecules forming on the NPs plays a crucial role in further interactions with other biological entities, affecting their cellular internalization and toxicity mechanisms (Lundqvist and Cedervall, 2020). Despite being crucial for the understanding of NPs dynamics and interaction in media, coronas formed around NPs in both the environment and biological fluids is still deeply understudied. Knowledge of the composition, structure, and full characteristics of the coronas formed around NPs in all scenarios could provide valuable information to elaborate testable hypothesis of the potential biological risks of NPs at an organism and ecosystem level (Cedervall et al., 2012). Moreover, the stability/agglomeration of NPs is also influenced by different environment conditions, as ionic water strength (seawater vs. river water (Singh et al., 2019; Wang et al., 2020) and water temperature (Bergami et al., 2019). Further studies needed to determine the real environmental effects of NPs should consider these variables for a better understanding of the uptake, mechanisms, and effects of NPs in real ecological risk scenarios. Moreover, an ecosystem-specific based risk assessment might be appropriate, in order to establish the habitats more vulnerable no plastic pollution and NPs effects.

Short-term effects of nanoplastics in fish

Lipid Metabolism

Taking all results together, we can say that there are relevant changes both in the molecular pathways and biochemical parameters related to lipid metabolism. In both species, i.e., *D. labrax* and *S. aurata*, the transcripts related to the metabolism of lipids are altered in liver after the short-term exposure to NPs, with significant increases in almost all transcripts measured (namely, *pparα*, *pparβ*, *pparγ*, *lpl*, *rxr*, *apoa1*, *nd5*). Although in *D. labrax* there are no alterations in biochemical parameters related to metabolism neither in plasma nor skin mucus, in *S. aurata* both triglyceride and cholesterol levels increase in plasma (and cholesterol in liver) after the exposure to NPs. Alterations in the mentioned genes together with altered lipid levels in blood suggest a dysregulation of lipid metabolism and storage. Other authors have also found alterations in cholesterol, triglyceride, and lipoprotein levels after exposure to NPs in other fish species, such as *Carassius auratus* (Cedervall et al., 2012; Mattsson et al., 2015) and

Zacco temminckii (Chae et al., 2018), suggesting NPs can cause nutritional or energetic problems in fish species. Moreover, cholesterol and phospholipids are also essential component of the cell membrane and cellular messengers (Javed et al., 2017) and therefore, alterations in lipid metabolism and levels could distort cell recognition pathways. In consequence, any change in lipid metabolism can indicate a deterioration in these communication pathways, while high cholesterol values may also be indicative of liver dysfunction. As dietary lipids are the major provider of energy in carnivorous fish (Boukouvala et al., 2004) lipid metabolism and its regulation acquire great relevance in fish, as their alteration can be detrimental both for the animal's health and for the nutritional quality of fish muscle as a product of human consumption.

In **Chapter 6**, we explored gene expression of growth-related transcripts in muscle of *S. aurata*. We found alterations in gene transcripts related to muscle development and growth with growth-hormone-mediated anabolism and energy mobilization decreased at low doses of NPs. It is not clear, though, if this could actually be detrimental for fish growth, as *myogenin* gene expression, a key regulator of muscle growth, and glucose, triglycerides and cholesterol levels suffered no alteration in this tissue and *myostatin* was downregulated. Consequently, we saw no alterations in growth nor Fulton condition factor of exposed gilthead seabream **(Chapter 5)**, it is relevant to note that changes in these parameters would be difficult to register after only 96 h of exposure and a chronic scenario would be more appropriate. In a 21-day study *Larimichthys crocea*, Lai and coauthors (Lai et al., 2021) reported a decrease in growth with PS-NPs exposure and alterations in lipid metabolism signalling pathways that led to increased lipid accumulation and altered fatty acid composition and texture of fish muscle.

Changes in lipid metabolism caused by diets with different lipid levels or lipid sources can affect the nutritional quality of fish muscle (Suárez et al., 2014; Xu et al., 2016) and the study by Lai and co-authors proves that changes in liver metabolic pathways can also alter muscle quality and potential development. As suggested by this and the previously mentioned studies, sustained exposures to NPs such as those occurring in the Mediterranean basin, could potentiate and expand the alterations found in our 96 h acute exposures, leading to more pronounced physiological changes that may ultimately impair growth, development and swimming performance. Moreover, in particular in *S. aurata* as an indeterminate grower species (Balasch et al., 2019), changes in lipid metabolism and storage could lead to relevant growth alterations in a chronic-

exposure scenario. We might also hypothesize that the lipophilic nature of NPs could provoke that they are somehow partially confounded by lipid substances inside the cell, either by their own nature of by the biomolecules that might be bound to the NPs forming the corona, and therefore activate the cellular machinery and pathway related to lipid metabolism.

Our results show that NPs can accumulate in zebrafish hepatocytes *in vitro*, specifically localizing inside lysosomes **(Chapter 3)**. Other authors have confirmed this colocalization of NPs in lysosomes in different species and cell types, such as mussels and mice (Sendra et al., 2020; Florance et al., 2021; Liu et al., 2021a). The lysosome has been appointed as the cellular metabolic command-and-control centre, with a lipid sorting function derived from its ability to process and sort exogenous and endogenous lipids (Thelen and Zoncu, 2017). Florance et al. (2021), documented that NPs impaired lysosomes and altered lipid metabolism and accumulation in murine macrophages. Overall, we propose lipid metabolic pathways, at the molecular, cellular, and physiological levels as a relevant process to monitor and further explore to understand the effects of NPs in aquatic organisms.

Oxidative stress and inflammatory response

Oxidative damage, often linked to mild or severe inflammatory responses, is one of the main mechanisms associated with the effects of toxicants (Lushchak, 2016). In this sense, molecular and biochemical biomarkers can provide an overview of the oxidative stress caused by environmental pollutants, including nanoparticles (Teles et al., 2016). It has been already proved that NPs interfere with cell metabolism by producing reactive oxygen or nitrogen species that result in oxidative stress and damage in multiple species and models, from mammalian cell lines living fish models (Hu and Palić, 2020). Consequently, oxidative stress has been proposed as one of main mechanisms of toxicity of NPs, as both the production of ROS and oxidative stress have been documented in several species, such as *Daphnia* sp. (Lin et al., 2019; Liu et al., 2020, 2021b), *Caenorhabditis elegans* (Lei et al., 2018; Zhao et al., 2020), *Mytilus* sp. (Canesi et al., 2015; Brandts et al., 2018) or *D. rerio* (Chen et al., 2017; Sarasamma et al., 2020).

In our studies with gilthead seabream **(Chapters 3, 4, 5)**, oxidative stress seems apparent in the three mucosal barriers that can act as portals of entry of NPs in fish, skin, gills and intestine. Moreover, we found a concomitant activation of the antioxidant response, with intestine as the most affected of the

three studied tissues and gills and skin showing a less pronounced response. These differences show that the response to NPs is not necessarily homogenous among mucosal barriers and that it could be influenced by the specific traits and particle dynamics in each tissue. As described previously, multifunctional key organs with mucosal linings, such as gills and intestine, seem to respond and perform differently in pathogenic scenarios, indicating a strong influence of the type and intensity of the stressor in a highly conserved species-specific fashion (Khansari et al., 2018).

In liver of the gilthead seabream, we found an early activation of the antioxidant transcriptional machinery to counteract oxidative stress, but increased TOS levels at 96 h, suggesting that antioxidant production might not have been enough to balance oxidative species produced by the exposure to NPs. Finally, a mild oxidative stress was also suspected in *S. aurata* muscle, as both the activation of myostatin and the downregulation of GH decrease could be a secondary consequence to oxidative stress.

Although in our study with zebrafish liver cells no alterations in lipid peroxidation were documented after 24h of in vitro exposure to PS-NP other studies have found peroxidation in lipids, both in zebrafish and other species (Lu et al., 2016; Brandts et al., 2018; Lin et al., 2019). As stated, oxidative stress has been widely reported as a consequence of exposure to NPs, and it can lead to damage to biomolecules, including lipids and DNA (Juan et al., 2021).

Our study with *S. aurata* did show that an acute exposure to NPs can induce DNA damage in red blood cells, evidenced by the reported increase in ENAs of exposed fish. Multiple studies have documented DNA damage both in vitro (Paget et al., 2015; Gopinath et al., 2019; Ballesteros et al., 2020) and in vivo (Gonçalves et al., 2022; Estrela et al., 2021; Guimarães et al., 2021), including results from our own laboratory showing DNA strand breaks in the hemocytes of *Mytilus galloprovincialis*. Besides oxidative stress, we can hypothesize that alterations caused by NPs could also be due to direct interaction of the particles with the DNA, multiple studies have shown the internalization of NPs by different cell types (eg. Brandts et al., 2020; Rubio et al., 2020; Sendra et al., 2020; Xu et al., 2019). All these results support the idea that NPs can have genotoxic effects, which should be a major concern regarding potential NPs toxicity, as mutagenicity can eventually lead cancer, degenerative conditions, immune dysfunction, and neurodegenerative diseases.

Inflammatory responses related to oxidative stress have been observed in different species after exposure to NPs and are also appointed as one of their

main mechanisms of toxicity (Hu and Palić, 2020). Moreover, it has been suggested that the immune system recognizes NPs as xenobiotics, and different studies have included inflammatory response endpoints to measure NPs effects both *in vivo* and *in vitro* (Brun et al., 2018; Deng et al., 2017; Wu et al., 2019).

In *S. aurata* mucosal barriers, we found activation of inflammation and its regulatory mechanisms, potentially involving purinergic and cholinergic pathways. On the other hand, in liver and muscle, NPs did not appear to elicit a pro-inflammatory response. Decreased EA and ADA activities, found both in gilthead seabream and European seabass, could also confer potential immune deficiencies in the long term if sustained, due to the implication of both enzymes in immune and anti-inflammatory pathways (Dullaart et al., 2009; Antonioli et al., 2014). Nevertheless, in our study with zebrafish **(Chapter 3)**, we observed a synergistic activation of antiviral genes after an immune stimulus (Poly I:C) together with exposure to NPs but the exposure to NPs did not affect larval survival to a bacterial infection with *Aeromonas hydrophila*. On the other hand, a similar study by Sendra et al., (2020) did document a decrease in *D. rerio* larval survival to Aeromonas infection after exposure to NPs, and another recent study found reduced inflammatory and antiviral responses to a Nodavirus infection in *D. labrax* juveniles exposed to polystyrene NPs, affecting the fish' resistance to disease (González-Fernández and Cuesta, 2022).

To date, few studies have detailed the mechanisms underlying the combined effects of NPs exposure and immune performance, and we should consider that different variables could be affecting the immune/inflammatory response to NPs, including the concomitant or successive exposures to NPs and the immune stimulus, the length of the exposure, the polymer and exact particle type, parental exposures, the developmental and sexual stage, the synergistic effects of different stressors and xenobiotics, the tissue-specific and microbiome peculiarities of mucosal lymphoid organs, the seasonally-modulated species-specific immune responses, and the crosstalk between immune responses and neuroendocrine phenotypic outcomes, among others (Khansari et al., 2018, 2019; Brandts et al., 2021; Thomas et al., 2021; Ferrante et al., 2022; Huang et al., 2022; Kim et al., 2022; Santos et al., 2022). In this sense, the short-term inflammatory responses related to NP exposure described here and in other recent studies should be best viewed as a snapshot of a more complex homeostatic landscape, involving major nervous, endocrine and defensive regulatory systems, acting together at the organismal level for each individual species or in assemblies of species in particular ecological contexts.

Concluding remarks: the known unknowns of nanoplastics' effects in fish and its concerns for humans

Research concerning NPs is still in its infancy, with still multiple knowledge gaps in this field, which limits the assessment of risk of exposure and potential effects of NPs in humans (European Commission and Directorate-General for Research and Innovation, 2019). Some of the current drawbacks in NPs research are the lack of standardization both in monitoring and reporting effects in biota, the missing knowledge on the particle's nature and interaction with their surroundings. In order to bridge these gaps, there is a need to better understand NPs' physicochemical complexity, standardize research methodologies and determine environmental relevant concentrations and particles, in order to improve the quality effect assessments (Martin et al., 2022). As formerly mentioned, the quantification of NPs in the environment in order both to establish environmental concentrations in distinct locations and to be able to quantify the bioaccumulation of NPs in organism is a crucial stepping-stone in the field.

Linked with environmental relevance and more realistic scenarios, studies evaluating the sub-lethal effects of chronic exposures to NPs on animal metabolism and on the immune response to pathogens should be pursued, and specifically in fish, where they are mostly lacking. Species-specific effects as well as potential sex-specific effects within each species should also be considered and further investigated in these chronic scenarios. The gilthead seabream, for instance, is a sequential hermaphrodite fish (Pauletto et al., 2018), and the immune and stress-related response that is often different between sexes could also condition the response to NPs (Balasch and Tort, 2019). Finally, the transfer, bioaccumulation and biomagnification in the aquatic food chain is another crucial matter that should be further explored. Trophic transfer has already been demonstrated in laboratory experiments (Cedervall et al., 2012; Mattsson et al., 2017; Chae et al., 2018), as has the accumulation of NPs by invertebrate species that are at the bottom of the marine food web such as *Artemia franciscana*, *Tigriopus japonicus* or *Euphausia superba* (Lee et al., 2013; Bergami et al., 2016, 2020). Extrapolating these facts to the aquatic environment, the health of marine ecosystems could be greatly compromised by NPs as CECs. Moreover, the transference of NPs through the food chain is the basis of the concern for human exposure to NPs (Revel et al., 2018; Barboza et al., 2020).

There is a pressing urge to better understand environmental and ultimately human and exposure to NPs (Science Advice for Policy by European Academies,

2019), as reports predict increasing concentrations, and the generation of global plastic waste does not seem to be diminish. One thing that is clear is that ingestion of contaminated food or drinks is one of the most important exposure routes to NPs for humans (Prata et al., 2020). At least twenty-five of global marine fisheries species have been found to contain MPs (Lusher et al., 2017). MPs, and in consequence NPs, pose an emerging food safety concern that has not yet been properly evaluated by relevant international scientific committees (e.g. Joint FAO/WHO Expert Committee on Food Additives), as there is still a lack of sufficient relevant data to thoroughly evaluate the potential toxicity of NPs. More data is essential for risk assessment, risk management, and risk communication. To assess the potential exposure to NPs from seafood consumption in particular, data about the estimated intake of the most consumed species and NPs concentration are needed, in order to establish the tolerable daily intake, where the worst-case scenario should be projected. Moreover, this risk assessment exercise should also be done for plastic additives and associated pollutants. In fish, it is thought that MPs accumulate in the gastrointestinal, being eliminated with degutting before consumption, minimizing the direct exposure and risk (Lusher et al., 2017). Nevertheless, NPs are known to pass the gastrointestinal barrier, distributing and accumulating in fish tissues (Barría et al., 2020), and could potentially also accumulate in muscle. Therefore, there is a pressing urge to develop methodology to monitor MPs and NPs levels in food products prior to ingestion.

Concerning the fate of plastic in the human body and the possible adverse health effects, much remains unknown, but it is reasonable to think that effects documented in vertebrates could also occur in humans (Teles et al., 2020). It must be said that, based on the available scientific evidence, it is not clear if NPs pose a significant food safety threat and consumers should be aware, that according to the current state of knowledge, the health benefits associated to the consumption of fishery products exceed the potential risks. Nevertheless, precautionary measures to combat MPs and NPs pollution should not wait until science reaches a consensus on the degree of toxicity for biota and humans. The ubiquity of NPs in ecosystems worldwide is a consequence of the unsustainable of plastics and the fact that plastic particles are persistent in the environment should lead to stronger measures against plastic pollution. The high degree of public awareness against plastic in the environment can create momentum to both enforce environmental policies and move towards a more sustainable economy.

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Conclusions

9

The studies performed in the present thesis were conducted with the aim to investigate the short-term effects of NPs exposure in model fish species. Accordingly with the results described in the previous chapters, the following overall conclusions can be drawn:

1. Polystyrene nanoplastics (PS-NPs) were **internalized** in zebrafish hepatocytes (ZFL cell line) in vitro, accumulating in lysosomes and altering the short-term (12h) expression of antiviral genes to an immune stimulus (poly(I:C)). After 24 h, the synergistic effect disappears, suggesting that ZFL cells regain homesotasis. Zebrafish larvae also incorporated PS-NPs, mainly accumulating in gut and pancreas, but the NPs did not interfere with a normal immune response against *Aeromonas hydrophila*.

2. **Gene expression analysis** pointed to lipid metabolism as the potentially more affected molecular pathway in gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) after a short-term exposure (96h) to polymethylmethacrylate nanoplastics (PMMA-NPs). We observed altered transcript expression of peroxisome proliferator-activated receptors related to lipid regulation in both species.

3. Globally, few changes in **oxidative stress-related** transcripts or biochemical biomarkers were observed following NPs exposure in the studied species. In *S. aurata*, short-term (96h) exposure to PMMA-NPs induced early changes in oxidative stress-related transcripts and biochemical endpoints of oxidative responses in liver and muscle. However, genotoxic effects, documented trough the appearance of nuclear abnormalities, were found erythrocytes.

4. Changes in **immune and/or inflammation** related markers were observed in the three studied teleost species. The intensity and extent of the response varied greatly between tissues and species, but overall, we observed a low level of activation of inflammatory responses. In zebrafish, previous exposure to PS-NPs for 48 h did not affect larvae survival to an infection with *Aeromonas hydrophila*, indicating a non-compromised onset of early inflammatory responses. In *S. aurata*, an activation of inflammation in gills and intestine is suggested together with a mild anti-inflammatory

response in muscle in response to PMMA-NPs exposure. In *D. labrax*, short-term PMMA-NPs exposure did not induce changes in immune and cellular stress-related transcripts, but the observed decreased plasma esterase activity EA levels suggest that exposure to NPs may interfere with inflammatory responses.

5 Among the studied tissues, **liver and intestine are suggested as the most appropriate matrices** to analyse the effects of NPs in fish. In *S. aurata*, muscle remains largely unaffected in terms of transcript expression and biochemical biomarker levels. However, due to the observed inhibition of key regulators of muscle development (growth hormone receptor and myostatin transcripts) we cannot rule out further detrimental effects on growth and development if the exposure to NPs becomes chronic. Non-invasive matrices, plasma and skin mucus, appear as promising to monitoring tools.

6 Globally looking at the results from this thesis, **lower exposure concentrations appear to be causing more alterations than higher ones**, in the studied species. These suggests that non-monotonic dose-responses and low-dose effects may occur after a short-term exposure to NPs.

7 **Species-specific effects should be taken into account** when drawing conclusions on the general effects of NPs, as the extent and strength of the response can vary between teleost species. In the results presented in this thesis, we found that *S. aurata* and *D. labrax* presented a similar response to NPs' exposure regarding lipid metabolism but differed in the immune/inflammatory response.

8 **Future studies should investigate chronic toxicity of NPs.** Model plastic nanoparticles can be useful in this process, but a shift to environmentally realistic plastic coming from the degradation of larger plastic objects is needed.

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