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Nanoplastics are bioaccumulated in fish liver and muscle and cause DNA damage after a chronic exposure

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ABSTRACT

The extent of the widespread, planetary contamination by plastic waste is difficult to fully capture. Nanoplastics (NPs) are currently in the center of research concerning plastic litter, both for the analytical challenges they pose and for their potential to provoke hazardous effects in organisms. However, there are still many unanswered questions in this multidisciplinary field, with a crucial missing piece being the quantification of NPs in fish tissues after *in vivo* exposures. Another relevant question that is still greatly unexplored is how a chronic exposure to NPs will affect fish health. This study aims to provide answers to both of these relevant knowledge gaps. To this end, goldfish (*Carassius auratus*) were exposed to 44 nm polystyrene (PS)-NPs via water for 30 days. Following the exposure, gastrointestinal tract, liver and muscle were sampled for PS-NPs analysis by means of size exclusion chromatography coupled to high resolution mass spectrometry. PS-NPs were detected in all liver and muscle samples of exposed fish, with higher concentrations in liver than in muscle, whereas no PS-NPs were detected in the gastrointestinal tract. Nevertheless, exposure to PS-NPs did not induce changes in hematology parameters nor in cortisol and glucose levels in plasma. On the other hand, even a relatively low concentration of PS-NPs was able to cause DNA damage, measured by an increase in erythrocyte nuclear abnormalities, suggesting that PS-NPs can reach the cell nucleus and cause genotoxicity. These results show for the first time that PS-NPs find their way to fish muscle after chronic exposure, where they bioaccumulate, but do not alter fish survival nor hematological or physiological stress indicators. The accumulation of PS-NPs in fish muscle can represent a threat to human health as a possible route of exposure to small-sized plastics. The present results in a model fish species open windows for future studies in edible fish species.

1. Introduction

Plastic pollution is a major ecological concern that is receiving increasing attention from the general public, the scientific community and governments. Plastic waste and fragments of all sizes reach aquatic ecosystems through multiple channels, such as wastewater discharges, littering, illegal dumping, use of plastics for equipment/packaging in marine activities and coastal human activity. All these sources contribute to the accumulation of plastic litter in the aquatic environment, where it can persist for decades and potentially centuries, breaking down to form micro- and nanometric size fragments. In addition, micro/nanometer-size particles are also industrially produced for multiple applications, constituting a second source of plastic particles entering the aquatic environment (Mitrano and Wohlleben, 2020). Paradoxically, with this decrease in size that leads to virtual invisibility in the environment, the concerns on the potential hazardous effects that these particles may have on living organisms increase (Koelmans et al., 2015).

Nanoplastics (NPs), the particle size-fraction under 1000 nm (Hartmann et al., 2019), are potentially one of the most hazardous marine litter, as their physicochemical nanoscale properties allow them to cross biological barriers, including the intestinal wall and the mammalian

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placental barrier (Stapleton, 2019). The detection and quantification of the environmental plastic nanofraction is an ongoing challenge, as the current analytical techniques to detect NPs in complex biological matrices are not fully mature (Cai et al., 2021). Nevertheless, the presence of NPs in marine waters has already been confirmed, both in oceanic (Ter Halle et al., 2017) and in Mediterranean waters (Llorca et al., 2021). Accurately measuring the concentration of NPs is a major challenge, as precise analytical methods are required, accounting for various types of NPs polymers, as well as for a diverse set of complex matrices (soil, sediments, turbid waters and tissues) (Ter Halle et al., 2017).

Studies on the effects of NPs in fish have described alterations in the oxidative status, changes in hormone levels, dysregulation of energy metabolism, interferences with the immune system, gut dysbiosis and behavioral alterations (reviewed by Barría et al., 2020; Jacob et al., 2020). However, knowledge regarding the occurrence and effects of NPs in fish is still in its infancy, as research is mainly focused on acute/short-term exposures with high plastic particle concentrations, whereas chronic toxicity has barely been assessed. To the best of the authors' knowledge, only six studies up to date have evaluated the effects of NPs in fish for exposure periods of more than 28 days, all presented in Table 1. NPs have also been observed to be distributed across food webs (Mattsson et al., 2015), but studies assessing the biodistribution, bioaccumulation and the effects of chronic exposures to NPs are still scarce. Considering that in nature organisms will most probably be exposed to NPs during long periods of time, chronic studies should be prioritized. The mechanisms behind the effects could be different between short- and long-term exposures, and to extrapolate from one to the other might lead the world to wrong conclusions.

In the present study, the model fish *Carassius auratus* (goldfish) was exposed for 30 days to polystyrene (PS)-NPs (PS-NPs), attempting to mimic an environmentally realistic scenario. The main hypothesis of this study was that fish can take up PS-NPs from the water after a chronic exposure and accumulate them in internal organs, causing alterations in fish health. PS-NPs were chosen, as PS contributes to a significant fraction of marine plastic waste (de Haan et al., 2019). Moreover, PS has been identified as one of the polymers present in the nano-fraction of the few real NPs environmental samples (Llorca et al., 2021; Ter Halle et al., 2017). The exposure concentration chosen for this study takes into account predicted environmental concentrations of NPs (Lenz et al., 2016), together with concentrations used in other relevant studies with NPs (Table 1)' as well as on the previous results of the authors (Brandts et al., 2018b, 2020; 2021b), as no solid environmental quantifications of NPs have been performed at present. Goldfish was chosen as a model since it is a sturdy and easily manageable species, with demonstrated utility as a model organism in several areas of research (Blanco et al., 2018; Filice et al., 2022). To test the hypotheses of uptake a protocol for the analysis and quantification of NPs polymers by means of size exclusion chromatography coupled to high-resolution mass spectrometry was set up. In order to evaluate fish general health status, blood and plasma, both non-lethal biological matrices, were collected and hematological parameters were determined using an automatic blood cell analyzer; erythrocyte nuclear abnormalities (ENAs) were counted to assess DNA damage; and key stress parameters, such as cortisol and glucose, were quantified in plasma. Hematological parameters reflect the overall physiological status of the animal and have been widely used to diagnose health status in several situations, including those of contaminant exposure (Witeska et al., 2022). Moreover, there is evidence that exposure to NPs can exert DNA damage after a short-term exposure in blood cells in aquatic species (Brandts et al., 2018a, 2021b), which highlights the importance of evaluating the potential genotoxic effects under a chronic scenario. It is relevant to focus on organisms belonging to higher trophic levels, such as are many fish species, as they are more likely to bioaccumulate xenobiotics and may present a potential risk to humans through their ingestion.

2. Materials and methods

2.1. 1. Fish maintenance, bioassay and sampling

Adult *C. auratus* specimens (11.16 ± 3.23 cm length and 7.07 ± 0.64 g weight) were randomly distributed in the experimental aquariums, and two experimental conditions were considered: **1)** Control group (0 μg/L PS-NPs) and **2)** the group exposed to PS-NPs (100 μg/L PS-NPs). For each experimental condition, fish were randomly distributed in 4 different aquariums (20 L) with 4 fish per tank, n = 16/condition; N = 32. The number of animals used for each parameter was variable due to the small size of the fish and to the variety of parameters determined. Information concerning the number of animals used for each parameter can be seen in the legends of the figures and in Table 2. Fish were exposed to PS-NPs for 30 days, following the Organization for Economic Co-operation and Development's guideline 215 (OECD, 2000). Fish were fed daily *ad libitum* with a commercial diet, the bottom of the aquariums was cleaned, and fish were observed for abnormal behavior. Water physicochemical parameters were monitored daily to ensure water quality, average values were: temperature (16 C°), carbonate hardness (10 dkH), general hardness (6–7 dGH), pH (8.5), dissolved oxygen (4 mg/L), nitrates (<10 mg/L), nitrite (<0.5 mg/L), ammonia (<0.15 mg/L), (<0.25 mg/L). Every 5 days, 75% of the medium was renewed and PS-NPs were added to each aquarium. The concentration of available PS-NPs will vary during the 5 days, due to ingestion by the fish and aggregation with food particles or feces, among others, resulting in a lower number of available particles in the medium over time and affecting the accumulation. After 30 days of exposure, all fish were euthanized by over-anesthetizing them in a tricaine methanesulfonate (MS-222) bath (1 g/L). Fish were then weighed and measured, and

Table 1
Summary of the main effects observed in fish after a chronic exposure to polystyrene nanoplastics (PS-NPs).

Species	Size (nm)	Concentration	Exposure route	Exposure time (days)	Main effects	Reference
Carassius auratus	250	50, 500 and 5000 μg/L	Water	28	Accumulation and histological lesions in gill, liver and intestine, activation of the antioxidant system. Changes in the expression of antioxidant related genes.	Abarghouei et al. (2021)
C. auratus	500	260 and 690 μg/L	Water	28	Inhibition the of olfactory G protein-coupled receptors and ATPases. Olfactory bulb injury and neurotoxicity.	Shi et al. (2021)
Danio rerio	105	10 μg/L	Water	28	Bioaccumulation of EHS and its transfer from parents to offspring. Changes in thyroid hormone levels. Generation of oxidative stress and damage.	Zhou et al. (2021)
Carassius	53, 180	29,000 and 100,000 μg/L	Diet	67	Accumulation in brain and blood. Behavioral changes, reduced locomotor activity and weight of loss. Morphological alteration in the brain (53 nm).	Mattsson et al. (2017)
C. carassius	24, 27	130 mg particles/day	Diet	61	Effects on behavior and metabolism. Weight of loss.	Mattsson et al. (2015)
C. carassius	24	0,01% (w/v)	Diet	39	Decreased locomotor activity and feeding rate. Disturbance of the lipid metabolism and decreased survival rate	Cedervall et al. (2012)

Table 2
Number of fish used for the different analysis performed (n) and *p* value obtained for each parameter (control group *versus* polystyrene nanoplastics, PS-NPs, exposed group. Statistically significant results are marked in bold and considered when $p < 0.05$.

Parameter	n	*p* value
PS-NPs concentration in the liver	9	**0.0057**
PS-NPs concentration in the muscle	9	< **0.0001**
Hematocrit	7	0.5423
Hemoglobin	7	0.7568
Red blood cell count	7	0.5303
Mean corpuscular cell volume	7	0.8329
White blood cells count	7	0.5697
Thrombocyte count	7	0.2571
Lymphocytes count	7	0.6014
Monocytes count	7	0.5934
Neutrophils count	7	0.5594
Eosinophils count	7	0.2231
Fish weight	9	0.5624
Fish length	9	0.3220
Fulton's condition factor	9	0.3711
Hepatosomatic index	9	0.5917
Micronucleus	13	**0.0002**
Reniform nucleus	13	**0.0003**
Segmented nucleus	13	0.8571
Lobed nucleus	13	0.3834
Total erythrocytic nuclear anomalies	13	**0.0002**
Cortisol	7	0.7207
Glucose	7	0.2655

heparinized syringes were used to immediately draw the blood from the caudal vein. Blood smears were made immediately after collection, and the remaining blood was kept in Eppendorf tubes (in ice) with heparin for posterior hematological analysis. Liver, muscle and the gastrointestinal tract were excised, weighted, snap-frozen in liquid nitrogen and kept at −80 °C until further analysis. All experimental procedures involving fish were carried out according to the 3 R's of Animal Experimentation (Replacement, Reduction, and Refinement), following Spanish legislation (law 32/2007 and RD53/2013) that agrees with the International Guiding Principles for Biomedical Research Involving Animals (EU, 2010/63).

2.2. Polystyrene nanoplastics characterization

Polystyrene nanoplastics (PS-NPs, 44 nm size) were purchased from Bangs Laboratories (ref. PS02002). The particles were characterized in ultrapure water (MiliQ) and in the water used in the experiment (AQ water), by dynamic light scattering (DLS) (Zetasizer Pro, Malvern). For this purpose, five dilutions were prepared ($1{:}10^2$, $1{:}10^3$, $1{:}10^4$, $1{:}10^5$ and $1{:}10^6$) from the original stock of PS-NPs (100 mg/mL) in both media and hydrodynamic size, polydispersity index (PDI) and the zeta potential were measured. The hydrodynamic size measures the diameter of a particle by measuring the scattered light from a dispersion containing diffusing particles, while de PDI estimates the width of this particle size distribution (Ragheb and Nobbmann, 2020). The zeta potential measures the electrostatic repulsion/attraction between particles and is an indicator of stability or dispersion in colloidal suspensions (Parupudi et al., 2022). In order to observe changes in PS-NPs through time, nanoparticle hydrodynamic size and the zeta potential were assessed at 0, 24, 48, 72, 96 and 120 h. To further characterize particle size and morphology in MiliQ and AQ water, transmission electron microscopy (TEM, Jeol 1400 120 KV) was used. Following acquisition, images were processed using ImageJ, where a minimum of 150 particles for each condition were measured.

2.3. Extraction of polystyrene nanoplastics from biological samples

Liver, gastrointestinal tract and muscle samples (n = 9; N = 18) were lyophilized using a CRYODOS-80 (Telstar) for 72 h at −75 °C and <0.15 mB. Samples were then homogenized with a Tissulyer II (Quiagen) using 5 mm stainless steel spheres and the following cycle: 1 min at 25 Khz, 1 min in ice, followed by another cycle of 1 min at 25 Khz. subsequently, samples were immediately stored at −80 °C until further analysis. Next, NPs were extracted from fish tissues and purified according to the methodology described by Schirinzi et al. (2020). Briefly, the samples were digested for 6 h with KOH (10%) in a proportion 1:3 (w/v) in a stove at 60 °C and left to rest at room temperature overnight. Afterwards, the samples were filtered through fiberglass filters and the clogged filters were digested with HNO_3 at 20% for 1 h at room temperature, and the acid was eliminated through filtration. These filters were then cleaned with water and dried overnight at 60 °C. Finally, the extraction of NPs from the filters was carried out with 10 mL of toluene by means of ultrasonic assisted extraction (USAE) for 30 min and afterwards collected and transferred to amber glass vials. This procedure was repeated twice, and the final extracts were combined and evaporated under nitrogen stream to concentrate the samples to a volume close to 1.5 mL. The extracts were then vortexed, centrifuged (5376 g, 10 min), and the supernatant transferred to LC-vials and evaporated again until 1.0 mL remained.

2.4. Analysis and quantification of polystyrene nanoplastics using size exclusion chromatography coupled to high-resolution mass spectrometry

The analysis of NPs was performed by size exclusion chromatography (SEC) coupled to high-resolution mass spectrometry (HRMS), equipped with an atmospheric pressure photoionization (APPI) working under negative conditions. The chromatographic system Acquity LC (Waters, Milford) was equipped with an advanced polymer chromatographic column (Acquity APC XT45 1.7 μm) working with isocratic conditions with toluene according to (Schirinzi et al., 2019). The chromatographic system was coupled to a Q-Exactive (Thermo Fisher Scientific) hybrid quadrupole-Orbitrap mass spectrometer. Data acquisition was performed in full scan mode (m/z 500–3000) with a resolution of 17,500 FWHM (Llorca et al., 2021). The whole system is controlled by Xcalibur 3.0 software. The identification was performed by matching with a calibration curve of PS' standard (MW~1200Da, supplied by Polymer Standard Service GmbH (PSS, Mainz, Germany) in toluene. An example of chromatogram and spectra can be seen in Fig. 2a. Finally, the quantification was done by means of equivalent concentration according to the procedure described by Llorca et al. (2021). Calculations were done with respect to the wet weight of the organs.

2.5. Fitness indicators

Fish survival was 100% in both experimental groups during all the exposure trial. At the end of the experiment, fish length was determined (cm) by measuring from the anterior end to the tip of the caudal fin. Fish were also weighted (g) and length and weight were used to calculate Fulton's condition factor (K factor). Afterwards, liver was excised and weighted (g) and the values used to calculate the hepatosomatic index (HSI). K factor and HSI were calculated as follows (Rizzo and Bazzoli, 2020):

$$\boldsymbol{K = \left(weight(g) \,/\, length(cm)^3\right) \times 100}$$

$$\boldsymbol{HSI = (liver\ weight \,/\, total\ weight) \times 100}$$

2.6. Hematological profile

Blood samples were analysed using an automated laser flow blood cell analyzer (Sysmex XN-1000 V) with the veterinary software version 3.04 with a betta version for bird blood analysis, adapted for fish. Hematocrit, red blood cell count, leucocyte count, hemoglobin concentration, mean corpuscular cell volume, and thrombocyte count were determined. Blood smears for differential leucocyte count were stained

with panoptic fast staining. Manual count of lymphocytes, monocytes neutrophils and eosinophils were determined in blood smears counting 100 leukocytes per smear.

2.7. Erythrocytic nuclear abnormalities

Erythrocytic nuclear abnormalities (ENAs) were classified into four categories (micronuclei, reniform-shaped, segmented and lobed nuclei)

A

	Time (h)	Size (nm)	PDI	Zeta potential (mV)
Ultrapure water	0	46.570 ± 1.235	0.168 ± 0.078	-14.190 ± 1.607
	24	47.910 ± 1.160	0.124 ± 0.064	-25.890 ± 2.816
	48	48.840 ± 0.618	0.032 ± 0.008	-26.240 ± 1.135
	72	49.520 ± 1.062	0.032 ± 0.001	-6.385 ± 1.229
	96	48.410 ± 0.863	0.056 ± 0.015	-4.950 ± 0.904
	120	51.510 ± 1.437	0.114 ± 0.020	-9.602 ± 0.674
AQ	0	73.820 ± 3.228	0.303 ± 0.041	11.280 ± 0.942
	24	120.300 ± 37.500	0.376 ± 0.102	-11.840 ± 1.147
	48	109.700 ± 6.118	0.409 ± 0.020	-12.080 ± 0.996
	72	143.400 ± 9.030	0.427 ± 0.061	-11.530 ± 1.067
	96	203.800 ± 30.510	0.485 ± 0.140	-12.620 ± 1.385
	120	349.600 ± 58.120	0.472 ± 0.071	13.960 ± 0.739

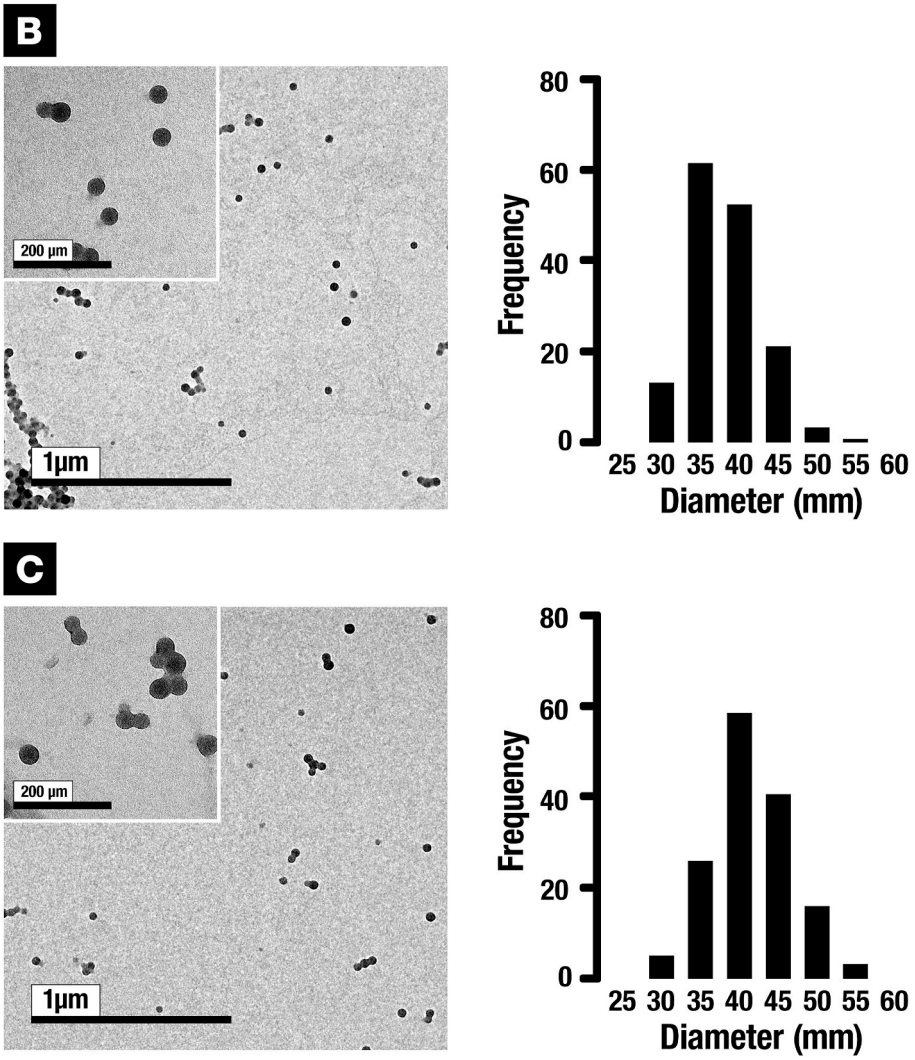

Fig. 1. a) Characterization of polystyrene nanoplastics (PS-NPs) by dynamic light scattering at 0, 24, 48, 72, 96 and 120 h. Hydrodynamic diameters, polydispersity index (PDI) and zeta potential (mean ± standard deviation) of PS-NPs in ultrapure water (MQ) and aquarium water (AQ) over time, at 10^4 µg/L **b)** Transmission Electron Microscopy images of the PS-NPs in MQ and **c)** AQ, with the corresponding size distribution histograms.

according to Pacheco and Santos (1996). Blood smears were prepared on glass slides and fixed with methanol for 10 min. Slides were stained with 5% Giemsa for 30 min. Subsequently, blood smears were stained with panoptic fast staining. A total of 1000 erythrocytes per sample were examined using an optical microscope (100X magnification). Only erythrocytes with intact nuclear and cytoplasmic membranes were considered. The results were expressed as the frequency of ENAs observed (‰) in a total of 1000 cells. Differential anomalies were also presented in the figures. The following formula was used to determine the total number of ENAs:

$$ENAs(‰) = Number\ of\ cells\ with\ ENAs / Total\ number\ of\ cells\ counted$$

2.8. Cortisol and glucose

The levels of cortisol were measured using a commercially available solid-phase, competitive chemiluminescence enzyme immunoassay (COR Cortisol, REF LKC01, Siemens Health Diagnostics, Deerfield, IL) in the automatic analyzer (Immulite® 1000 analyser; Immulite System; Siemens Health Diagnostics, Deerfield, USA) (Franco-Martinez et al., 2019). Plasma glucose levels were determined using the hexokinase G-6-PDH based commercially available method (OSR6121, Beckman Coulter Ireland Inc, Ireland) and following the manufacturer's indications in an automatic analyzer (Olympus Diagnostica, GmbH,

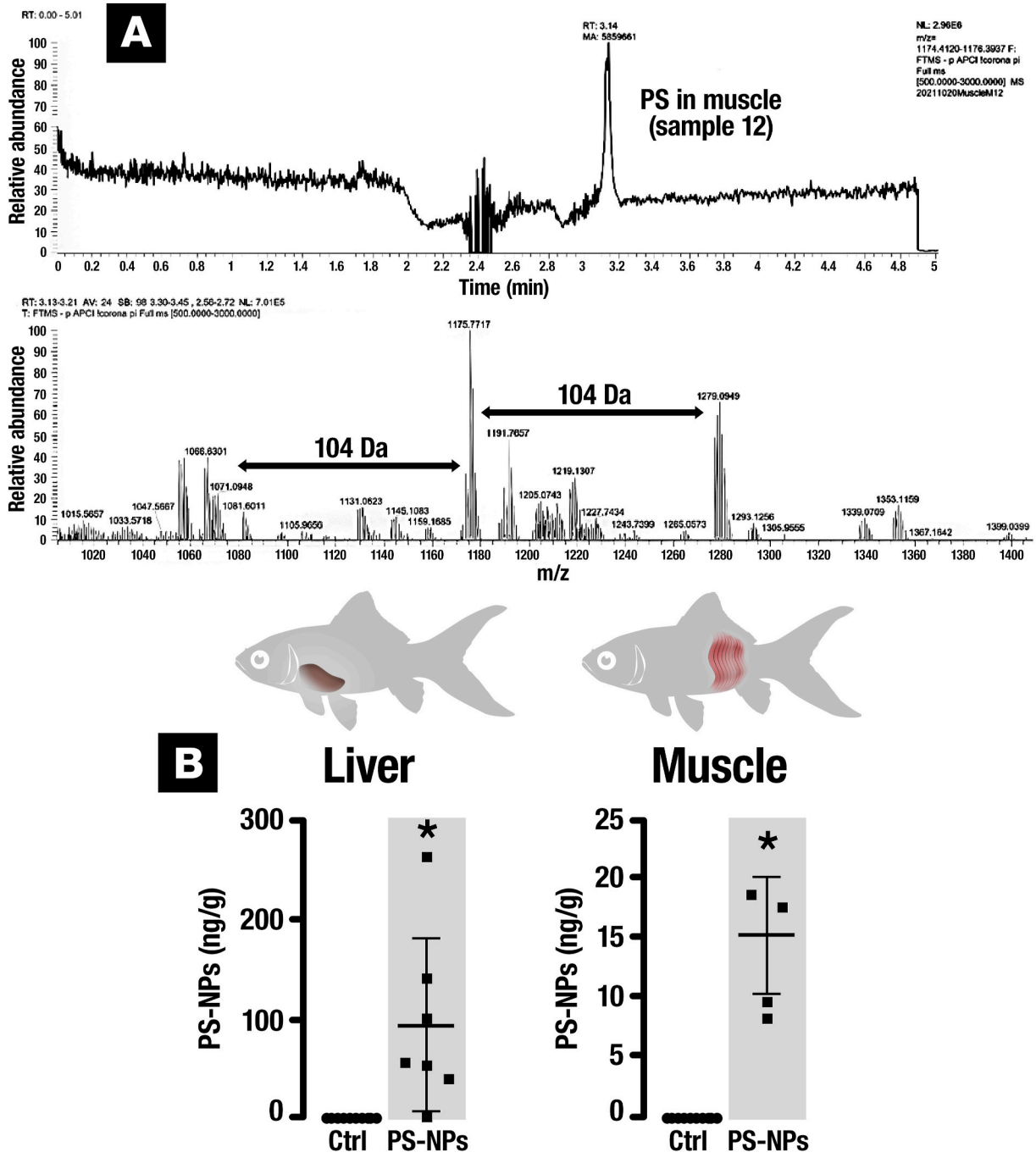

Fig. 2. a) Polystyrene nanoplastics (PS-NPs) chromatogram and mass spectra of the common loss for PS of 104 Da. **b)** PS-NPs concentration in the liver and muscle of goldfish (*Carassius auratus*) after 30 days of exposure to PS-NPs. Values are presented as mean ± standard deviation. Asterisk (*) denotes significant differences *versus* control ($p < 0.05$).

Freiburg, Germany).

2.9. Data analysis

Results are expressed as mean ± standard deviation (for n information see Table 2). The statistical data analysis was done using GraphPad Prism version 8.0.1 for Windows (GraphPad Software, Inc, www.graphpad.com). The data was first checked for normality using the Shapiro-Wilk test. Data that followed a normal distribution was further analysed with a *t*-test and the data that did not follow a normal distribution was further analysed using the Mann-Whitney nonparametric test. Significant differences between the control group and the group exposed to NPs are marked in figures with an asterisk (*) and considered when $p < 0.05$.

3. Results

3.1. Polystyrene nanoplastics characterization

The DLS measurements show that the hydrodynamic diameter of PS-NPs and PDI increased as the concentration of PS-NPs decreased in both tested media. However, in MiliQ, the hydrodynamic diameter of PS-NPs

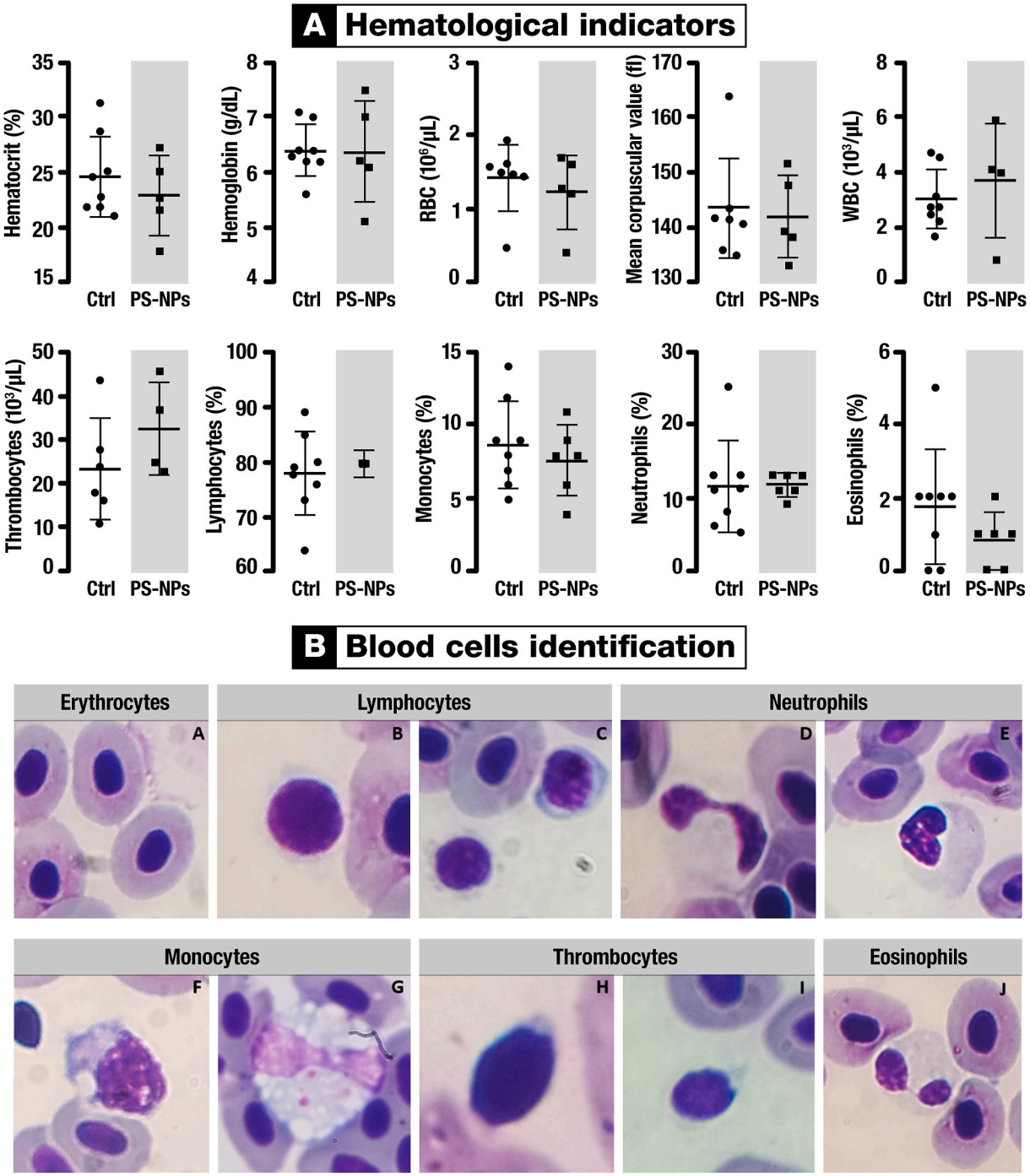

Fig. 3. a) Hematological indicators determined in control group and in goldfish (*Carassius auratus)* exposed to polystyrene nanoplastics (PS-NPs) for 30 days. Values are presented as mean ± standard deviation. **b)** Blood cell identification in goldfish blood using an optical microscope (100 x). **A)** Erythrocytes; **B and C)** Lymphocytes; **D and E)** Neutrophils; **F and G)** Monocytes; **H and I)** Thrombocytes; **J)** Eosinophils.

remained more stable than in AQ water (Fig. 1). As shown in Fig. 1, the hydrodynamic diameter of PS-NPs and PDI also increased over time in both media. In ultrapure water, the hydrodynamic diameter of PS-NPs ranged from 46 to 51 nm. However, in AQ the values ranged from 73 to 349 nm. Zeta potential analysis showed that, in MiliQ water, PS-NPs were negatively charged with values between −14 and −25 mV, but these decreased to −6 to −9 mV after 48 h. At the AQ medium, zeta potential ranged between −12 and 13 mV. This points to a lower stability of dispersion in MiliQ water after 48 h and in AQ water at all measured times; however, PDI values indicate higher polydispersity in AQ water.

3.2. Polystyrene nanoplastics concentration levels in muscle, liver and gastrointestinal tract

PS-NPs were not detected in the gastrointestinal tract of exposed fish. In contrast, PS-NPs were detected in all liver and muscle samples of the exposed fish (Fig. 2b). In liver, 7 out 9 samples had concentrations levels ranging from 2.23 to 265.89 ng/g liver wet weight; in the other two liver samples PS-NPs were below the limit of detection. In 6 out of 9 muscle samples, PS-NPs were detected at concentrations ranging from 9.58 to 18.64 ng/g of muscle wet weight. The concentrations of PS-NPs were significantly higher in the liver and muscle of fish exposed to PS-NPs for 30 days when compared to the control group.

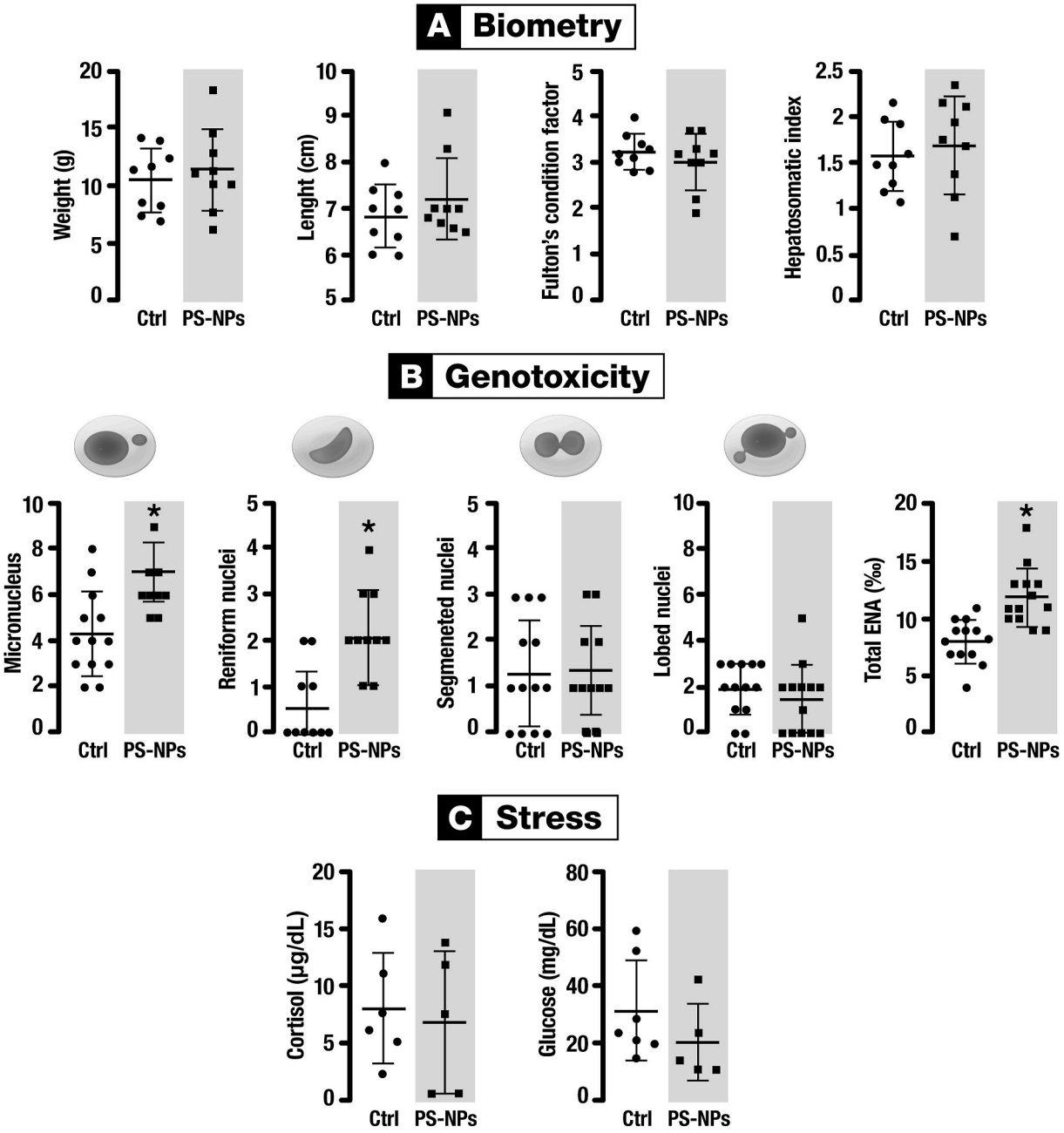

Fig. 4. Biological indicators determined in control group and in goldfish (*Carassius auratus)* exposed to polystyrene nanoplastics (PS-NPs) for 30 days. Values are presented as mean ± standard deviation. Asterisk (*) denotes significant differences versus control, $p < 0.05$. **a)** Biometric indicators. **b)** Genotoxicity indicator. Different erythrocytic nuclear anomalies (ENAs) found and total ENAs (per 1000) scored in the blood of the goldfish. **c)** Stress indicators. Cortisol and glucose levels determined in plasma of the goldfish.

3.3. Biological effects of polystyrene nanoplastics in goldfish

In this study, PS-NPs exposure did not affect fish fitness indicators, as fish survival was 100%, food consumption and fish behavior were similar in both control and exposed fish. The effects of PS-NPs in the hematological parameters (hematocrit, hemoglobin concentration, red blood cell count, mean corpuscular cell volume, leucocyte count, thrombocyte count, and the differential count of lymphocytes, monocytes neutrophils and eosinophils) are shown in Fig. 3a. No statistical differences were observed in the studied hematological parameters in PS-NPs exposed fish compared to the control group. Fig. 3b shows the morphology of the different types of leucocytes found in the blood of goldfish. The exposure to PS-NPs had no significant effects in the body weight, length, condition factor nor liver weight of the fish (Fig. 4a). A significant increase in the total number of ENAs, a genotoxicity indicator, was found in the group of fish exposed to PS-NPs, when compared to control (Fig. 4b). The number of erythrocytes presenting reniform nuclei and micronuclei was significantly higher in fish exposed to PS-NPs than in control fish. However, lobed and segmented nuclei values were similar in both groups of fish. The levels of cortisol and glucose in plasma, were not statistically different between exposed and control fish (Fig. 4c).

4. Discussion

4.1. Polystyrene nanoplastics bioaccumulate in liver and muscle of goldfish

In this study, protocols for the extraction and quantification of NPs in fish tissues were set up. Following these protocols, the present data demonstrates that 44 nm NPs can be taken up from the water, crossing contact surfaces (gills, gastrointestinal tract), translocated and accumulated in inner organs, after a 30-day exposure quantifiable levels of PS-NPs were found in liver and muscle of goldfish. However, no PS-NPs could be found in gastrointestinal tract samples. Although NPs have not yet been detected in tissue samples of animals captured from the environment, microplastics (MPs) have been sampled from the gastrointestinal tracts of multiple fish species (Azevedo-Santos et al., 2019). Furthermore, Dawson et al. (2018) showed that MPs could be turned into NPs inside the digestive tract of Antarctic krill (*Euphausia superba*), a process that could potentially also take place in fish (Dawson et al., 2018). Recently, Clark et al. (2022) documented the translocation of palladium-doped PS-NPs (ca. 200 nm) through an *ex vivo* gut sac exposure system, with the highest passage occurring in the anterior intestine, and up to 700 million particles passing through the intestinal barrier after 4 h. The results from the present study suggest that PS-NPs ingested by goldfish effectively crossed the intestinal barrier, not remaining in the gastrointestinal epithelia but reaching inner tissues, namely liver and muscle, potentially after being distributed through the blood. The translocation of NPs to inner tissues has been documented in teleost fish (Brun et al., 2018; Chae et al., 2018; Skjolding et al., 2017), although mainly through the observation of fluorescence after the administration of fluorescent NPs particles. To the best of the authors' knowledge, the direct quantification of nanometric plastic polymer particles in fish organs has not been previously reported.

Previous studies have documented the presence of NPs in fish liver after controlled laboratory exposures, and several of them have estimated NPs' concentrations by indirect measurement of fluorescence. In a study with red tilapia (*Oreochromis niloticus*), Ding et al. (2020) calculated a concentration of 98,200 ng/g liver of 300 nm PS-NPs after 14 days of exposure to 100 μg/L. With a similar methodology, Sarasamma et al. (2020) estimated a concentration of 2000 μg/g protein of PS-NPs (70 nm) in zebrafish liver after 30 days of exposure to 1500 μg/L of PS-NPs. In a recent study, Guimarães et al. (2021a) estimated the concentration of NPs through indirect fluorescence quantification, obtaining values of 100 ng/g liver wet weight in *Ctenopharyngodon idella* after 20 days of exposure to 34 μg/L PS-NPs (26 nm) (Guimarães et al., 2021a). The concentration values observed in the present study, with an approximate average of 100 ng/g liver wet weight, would match that obtained in *C. idella* and fall lower than the other two estimated quantifications. Nevertheless, the available quantifications for NPs have been estimated by measuring the fluorescence emitted by fluorescently labeled MPs and NPs, which might not always faithfully reflect the mass of plastic particles (Catarino et al., 2019). In some cases, very small MPs (5 μm) have also been found in liver, with Lu et al. (2016), Ding et al. (2020) and Qiao et al. (2019) documenting the presence of 5 μm plastic particles in the fish liver after exposures of 7, 14 and 21 days, respectively. There is contradictory information on the size of plastic particles that could be translocated to the liver, as some studies report that particles over 20 μm cannot be found in the liver (Lu et al., 2016; Su et al., 2019), whereas in other studies particles of up to 90 μm have been found in liver (Ding et al., 2020). The liver is one of the largest internal organs in fish and has a significant role in multiple vital processes, such as nutrient processing and storage, protein synthesis, detoxification of autogenous metabolites, bile production and secretion, as well as drug and xenobiotic metabolism (Olsvik et al., 2007). The accumulation of NPs in fish hepatocytes has been previously documented *in vitro*, with the intracellular localization mainly located in lysosomes (Brandts et al., 2020). The accumulation in this organelle is probably related to its role in xenobiotic metabolism (Gu and Manautou, 2012), but could also lead to alteration in liver metabolisms, due to this organelle's role in lipid metabolism (Thelen and Zoncu, 2017). Changes in liver metabolomics profile have been previously seen in *Carassius carasssius* and in *D. rerio* after exposure to NPs, showing a disruption in lipid and energy metabolism (Mattson, 205; Lu et al., 2016). Moreover, alterations in key transcripts related to lipid metabolism have been found in liver of both *Sparus aurata* and *Dicentrarchus labrax* (Brandts et al., 2018b, 2021b). Oxidative stress, one of the main mechanisms of action of NPs (Hu and Palić, 2020), has also been found in both *Larimichtys crocea* and *S. aurata* livers after exposure to NPs, described as altered gene expression, enzyme activity and lipid peroxidation (Brandts et al., 2021b; Lai et al., 2021; Li et al., 2021). The action of oxidative species generated due to NPs exposure could be related to tissue damage, which has been reported in both goldfish and *Epinepehlus coioides*, following NPs exposure (namely vacuolization, infiltration and necrosis).

Due to its role as part of the digestive system, the liver has an optimal position for gathering, transforming, accumulating, and eliminating substances, as substances absorbed in the gastrointestinal tract have a direct highway to the liver via the portal vein, which transports 70–80% of the blood that reaches the organ (Akiyoshi and Inoue, 2004). Therefore, following this absorption route and considering the role of the liver in xenobiotic metabolism, the accumulation of NPs in this organ, as well as the high levels found in some of the samples, could be expected. Blood coming from the digestive system is processed by the liver as it flows through the sinusoids and is finally collected by the central vein, which will eventually proceed to the systemic circulation. Through this route, NPs that have not been intercepted in the liver could be transported to other organs.

In this study, results show that PS-NPs can find their way to fish muscle, potentially through said circulatory deliverance. To the best of the authors' knowledge, only one laboratory study has quantified NPs in fish muscle, and it was done through the indirect quantification of fluorescent NPs. In that study, Chen et al. (2017) found accumulation of NPs in the muscle of zebrafish after 3 days of exposure to 1000 μg/L of 50 nm fluorescent PS-NPs, with estimated concentrations ranging from 64,000 to 49,000 ng/g of muscle weight. These values are a thousand times higher than those found in the present study, with NPs concentrations ranging from 9.58 ng/g to 18.64 ng/g of muscle weight. A large number of dissimilarities between both studies could explain such difference in order of magnitude; including the fish species, the exposure concentrations, and the quantification methodology, as Chen et al. (2017) estimated the concentration from the emission of fluorescence

while the values presented here directly quantified polymer concentration. The accumulation of NPs in fish muscle can have detrimental effects on this tissue, as shown by Yang et al. (2020) in goldfish larvae, where they found damage and mitochondrial vacuolization in muscle mesenchymal cells, nerve fiber atrophy and decreased acetylcholinesterase (AchE) activity, after a short-term exposure to PS-NPs (70 nm). The generation of reactive oxygen species found by the authors could explain this induced cell injury. In a study with carnivorous fish *L. crocea*, Lai et al. (2021) found alterations in fish muscle flesh quality after an exposure to NPs (80 nm), which could be a consequence of the described tissue damage. Moreover, a previous study by the authors found that a short-term exposure to NPs (45 nm) induce an antioxidant response, altered AchE activity and inhibited key transcripts related to muscle development in muscle of *S. aurata* (Balasch et al., 2021). Effects on fish growth after exposure to NPs have been previously reported in other fish species (Gu et al., 2020; Lai et al., 2021; Wang et al., 2022). The diversion of energy reserves to respond to oxidative stress and inflammation, both systemically and locally in muscle tissue, could partially explain this decrease in growth, together with the alterations in muscle growth regulators and in nutrient metabolism (*i.e.*, lipids and carbohydrates.).

The ingestion of NPs has been suggested as the main route of exposure for humans, as MPs have already been found in edible products, such as seafood, honey, beer, salt and sugar, as well as in both tap and bottled water (Lehner et al., 2019). Nevertheless, fish have not been strongly considered a risk for plastic particle exposure for humans, as it has been assumed that MPs ingested by fish will remain in the gastrointestinal tract and, therefore, be discarded before the meat is ingested. However, when NPs, and possibly small MPs (for example 5 μm) are considered, we cannot assume that particles will stay in the gastrointestinal tract. This study provides evidence that fish species of which only the meat is ingested should be considered as another relevant source of NPs for humans. Fish muscle represents a large proportion of the body mass, of up to 60% depending on the fish species (SäNger and Stoiber, 2001) and, therefore, could potentially store a large amount of the NPs accumulated in fish. Moreover, as trophic transfer of NPs within aquatic organisms has been demonstrated (Cedervall et al., 2012; Mattsson et al., 2015), eating predator fish from higher trophic level might increase the risk of exposure to relevant concentrations of the pollutants, as occurs with heavy metals.

It is relevant to note that most of the detections or approximate quantifications of NPs in fish organs up to date are indirect and based on the use of fluorescently marked MPs and NPs and not on the detection of the polymer itself. Fluorescence is very useful to visualize particles, and it can be used to localize the plastic particles in tissue. However, some authors have reported fluorophore leaching from fluorescent-labeled nanoplastics, which PS can lead to the accumulation of fluorophore alone within in internal tissues, potentially overestimating NPs concentration (Catarino et al., 2019). Moreover, with fluorescence, the smallest particles (or aggregated) can be challenging to be detected and the tissue distribution can also influence the result. The advantage of size-exclusion coupled to high-resolution mass spectrometry is that it allows identifying the different types of polymers and quantifying them in terms of the weight of a polymer by gr of tissue, simultaneously. This technique solubilizes the plastic particles independently of the size or their distribution. Moreover, the method proposed shows high sensitivity, enough to be used with real samples or low concentrations in exposure experiments, as in the present study, in which realistic conditions of exposure are considered. This method has been already validated and applied for real samples (Llorca et al., 2021). On the other hand, some other studies rely on metal-doped NPs, in order to be able to monitor their accumulation in tissues. While both fluorescent and metal-doped particles have proven useful to contribute to understanding the behavior of NPs inside organisms, they do not provide appropriate tools to detect and monitor NPs in real environment of food samples.

4.2. Polystyrene nanoplastics do not alter fish morphological nor hematological indices but induces DNA damage

In this study, the Fulton's condition factor (K) values were higher than 1 in both experimental groups, which indicates that all fish were in good health condition during the experiment and no changes in the nutritional condition were found between control and exposed fish. This was also confirmed by the behavior that the animals presented during the experiment, *i.e.*, no changes in feeding behavior, ventilatory activity, swimming behavior and no signs of aggressiveness. Interestingly, although fish liver accumulated PS-NPs in all exposed fish, no changes in HSI under the present experimental conditions were found. The HSI is a widely known indicator of contaminant exposure due to the critical role of the liver in the detoxification of xenobiotics, which can lead, *e.g.*, to an increase in liver size due to hypertrophy or hyperplasia of the hepatocytes (Ahmed and Sheikh, 2019). HSI, together with K, are also used as indirect indicators of the energy status. Therefore, it seems that under the assessed experimental conditions, the global health condition and energy status of the animal was not affected by the exposure to PS-NPs. These results agree with previous findings obtained for *S. aurata* exposed to a concentration range (0, 0.001, 0.01, 0.1, 1 and 10 mg/L) of 45 nm polymethylmethacrylate (PMMA)-NPs for 96 h (Brandts et al., 2021b), where neither HSI nor K were altered. Even if changes in K and HSI in the present study could have been expected, due to the longer duration of the exposure to PS-NPs (acute *vs.* chronic), this was not the case.

Blood analysis is a reliable and inexpensive tool for the assessment of animal health via non-lethal sampling; therefore, reducing animal sacrifice in experiments as recommended by the European Union (Directive, 2010/63 EU). In the present study, the hematological indicators were unchanged in goldfish blood chronically exposed to PS-NPs. It is difficult to compare the present data with previous results since, to the best of the authors' knowledge, there are no available studies assessing the effects of NPs on fish hematology. A few previous studies where fish were exposed through the water to MPs, showed changes in some hematological parameters (Hamed et al., 2019; Hodkovicova et al., 2021). Hamed et al. (2019) evaluated the effects of a 15-day exposure to MPs through the water on juveniles of the freshwater fish *Oreochromis niloticus* (Nile tilapia). They found changes (increases or decreases, depending on the parameter) in hematological indices, such as the red blood cell count, hemoglobin concentration, hematocrit value and white blood cell count. These authors do not specify the nature of the MPs used in the study. Hodkovicova et al. (2021) also found changes in total red blood cells count and hematocrit in rainbow trout (*Oncorhynchus mykiss*) exposed to 50 μm MP for six weeks. In mammals, mice that received PS-NPs daily during 28 days by oral gavage presented changes in hematological indices, such as a decrease in white blood cell count (Xu et al., 2021). Taking together these findings, changes in blood parameters of the exposed goldfish could be expected in the present study, especially because the exposure time was longer than the exposure duration carried out in the other studies. However, this was not the case. In fact, comparing the effects of NPs with that of MPs could be a mistake, as NPs differ from MPs with respect, for example, to their transport properties, bioavailability and diffusion times, and effects of both contaminants could be different (Gigault et al., 2021). Moreover, other differences between studies, such as the species, animal age (adults *vs.* juveniles), dynamic of the particles inside the organism, concentration or exposure time, are all factors that make this issue extremely complex. In conclusion, based on the available studies it was very difficult to predict the effects of PS-NPs on blood parameters of goldfish and the absence of hematological changes could be due to a capacity of the animal to cope with the stress caused by the absorbed PS-NPs.

Present data suggest that a chronic exposure to 44 nm NPs can induce DNA damage in goldfish red blood cells. This is evidenced by the reported increase in ENAs of exposed fish, with micronuclei being the most frequently recorded abnormality. Micronuclei and other nuclear abnormalities can form during the proliferative phase of the cell cycle,

inducing permanent damage that can only be fixed through DNA repair or apoptosis. ENAs have been extensively used as indicators of exposure to mutagenic or genotoxic contaminants in numerous fish species for a variety of chemicals such as PCBs, PAHs, heavy metals, and pesticides (Braham et al., 2017). It can be hypothesized as to whether the increase in ENAs observed in this study is due to oxidative stress provoked by the PS-NPs or to the interaction of NPs with the chromosomal material, as both are plausible explanations. Oxidative stress has been widely reported as a consequence of exposure to NPs (Jacob et al., 2020), and this imbalance between radical species and the antioxidant defense systems can lead to damage to biomolecules, including DNA; therefore potentially inducing ENAs (Juan et al., 2021). On the other hand, NPs have shown the ability to be internalized by multiple cell types (*e.g.,* Brandts et al., 2020; Rubio et al., 2020; Sendra et al., 2020; Xu et al., 2019), thus, their direct interaction with chromosomes is also a possible scenario. These results corroborate previous findings showing that short-term exposures to a concentration range (from 0.05 up to 50 mg/L) of 100 nm PS-NPs were able to induce DNA strand breaks (comet assay) in the hemocytes of the Mediterranean mussel (*Mytilus galloprovincialis*) and chromosome damage (scoring the ENAs frequency) in erythrocytes of the *S. aurata* exposed to 45 nm PMMA-NPs (0.01–10 mg/L) (Brandts et al., 2018a, 2021b). Therefore, the present data confirm that NPs have genotoxic potential, able to induce DNA damage, after both short and long-term exposures. Results obtained by other authors in mussels (Gonçalves et al., 2022), fish (Estrela et al., 2021; Guimarães et al., 2021b) and human blood cells (Ballesteros et al., 2020; Gopinath et al., 2019) support previous and current findings, reinforcing the idea that PS-NPs are genotoxic contaminants. This is of concern, since the accumulation of DNA damage in cells play a role in their mutagenic potential, which can lead to cancer, as well as to the occurrence of degenerative conditions such as accelerated aging and immune dysfunction.

4.3. Polystyrene nanoplastics do not affect goldfish response to stress

Generally, fish respond to stress with increases in plasma cortisol, their main corticosteroid. In teleost fish, cortisol plays a major role in the stress response and plasma cortisol levels are considered a marker for the intensity of hypothalamo-pituitary-interrenal (HPI) axis regulation under stressful situations (Balasch and Tort, 2019). This hormone is also known to increase the energy availability during stress. This task is accomplished primarily through gluconeogenesis, resulting in higher levels of plasma glucose. Nevertheless, fish responses to stressors demonstrated to be more complex, since different patterns of response have been observed either concerning the cortisol variation (Pacheco and Santos, 2001; Teles et al., 2004) or its relationship with the secondary stress responses (Teles et al., 2017). In the present study, a chronic exposure to PS-NPs resulted in unchanged levels of plasma cortisol, which agrees with previous results concerning a short-term exposure of *D. labrax* to 0.20 and 20 mg/L of PS-NPs (Brandts et al., 2021a). Despite the importance of cortisol, to the best of our knowledge, there is only one more study, where the effects of PS-NPs in cortisol and glucose levels in fish are evaluated. In that study, Brun et al. (2019) exposed zebrafish larvae to three concentrations of 25 nm PS-NPs for 2 days and measured cortisol and glucose levels, finding increased whole-body cortisol levels but decreased glucose (Brun et al., 2019). However, it is difficult to compare a short-term exposure in larvae with a long-term exposure of adult fish from another species. Moreover, under chronic stress sometimes an adaptation to the stressor occurs and the cortisol levels, though initially increased, return to control levels and homeostasis is regained. Plasma glucose levels remained unaltered after the chronic exposure to PS-NPs, corroborating previous data on marine fish after short-term exposures to NPs. Thus, similar results have been found in *D. labrax* and *S. aurata* exposed to PMMA-NPs (Brandts et al., 2021a, 2021b), where glucose levels also remained unaltered after 96 h of exposure. As previously stated, the only other available study is the one referenced above (Brun et al., 2019), where the results differed from the present data. Considering the few available data on the effects of NPs on cortisol and glucose concentrations, variability of these responses may depend on the fish species and experimental protocol; more studies in this direction could be valuable.

5. Concluding remarks

Taken together, present data suggest for the first time that: **1)** fish chronically exposed to 44 nm PS-NPs internalize these emergent contaminants from the water. PS-NPs are then distributed in the organism, potentially through blood, and bioaccumulate in internal organs, such as the liver and the muscle. **2)** Higher levels of PS-NPs were found in the liver when compared to the muscle. No bioaccumulation was found in the gastrointestinal tract which agrees with the main function of this organ, *i.e.*, absorption. Considering that fish consumption is increasing steadily worldwide, the fact that PS-NPs are bioaccumulated in fish muscle, the edible part of the animal, means that these contaminants may be ingested by humans, presenting a potential threat to human health. **3)** Despite the capability of internalization and bioaccumulation, PS-NPs did not induce changes in fish growth, nor in hematological and stress indicators. **4)** PS-NPs induced genotoxicity in fish blood cells, which can escalate to mutagenicity and is related to more serious conditions such as cancer or degenerative conditions.

Credit author statement

I. Brandts: Research conception and design, Data acquisition, Formal analysis, Manuscript preparation, Final approval. **M. Cánovas**: Data acquisition, Formal analysis, Manuscript preparation, Final approval. **A. Tvarijonaviciute**: Resources, Data acquisition, Final approval. **A. Vega**: Data acquisition, Final approval. **M. Llorca**: Data acquisition, Formal analysis, Final approval. **M. Farré**; : Resources, Data acquisition, Formal analysis, Final approval. **J. Pastor**: Resources, Data acquisition, Formal analysis, Final approval. **N. Roher**: Research conception and design, Resources, Data acquisition, Formal analysis, Final approval. **M. Teles**: Research conception and design, Resources, Data acquisition, Formal analysis, Manuscript preparation, Final approval

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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