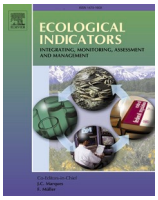
Ecological Indicators 147 (2023) 109966

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind



Evaluation of a chronic exposure to nanoplastics in goldfish (*Carassius auratus*): Analytical validation of automated assays for the measurement of biochemical markers

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ARTICLE INFO

Keywords:
Automated assay validation
Biomarkers
Nanoparticles
Fish

ABSTRACT

The bulk of plastic pollution is mainly composed of small fragments, micro- and nanoplastics (NPs). Although many studies are currently published on NPs, research on the effects of NPs in fish after a chronic exposure is still scarce. The present study aimed to validate a series of automated assays to be used in the monitoring of fish challenged with a chronic exposure to NPs, using *Carassius auratus* (goldfish) as model species. For this purpose, adult *C. auratus* were exposed to 100 μg/L polystyrene (PS)-NPs for a 30-day period. Total oxidative status (TOS), total antioxidant capacity (TAC), esterase activity (EA) and adenosine deaminase activity (ADA) were measured in the gills, brain and muscle of fish. In addition, acetylcholinesterase activity (AChE) and creatinine kinase (CK) were measured in the muscle. All biomarkers were successfully validated in goldfish tissues and consequently used to assess the effects of NPs following a chronic exposure. Results showed that EA and TAC significantly increased in gills, while EA decreased significantly in the brain, and no effects were observed in any of these parameters in muscle. These results indicate that both immune and antioxidant responses were triggered by NPs in gills, but not in the brain nor muscle. This suggests that gills may be a primary target for NPs, potentially leading to a cascading effect on gas exchange, or osmo- and ionic regulation that should be further investigated.

1. Introduction

Plastic pollution in aquatic environments is a worldwide problem and has raised increasing concern amongst the scientific community over the last decades (da Costa et al., 2016). As a result of both biotic (e.g., digestion; Dawson et al., 2018) and abiotic factors (e.g., UV radiation, Lambert and Wagner, 2016), discarded plastics are fragmented into microplastics (MPs, <5 mm, Browne et al., 2007), and eventually broken down into nanoplastics (NPs < 1000 nm, Hartmann et al., 2019). In addition, the presence of NPs in both industrial cleaning and personal care products represents an additional pathway for this emerging contaminant to enter aquatic systems (Hernandez et al., 2017; Saroglia and Terova, 2020). NPs appear as emerging contaminants in both natural and urbanised environments (Cai et al., 2021; Materić et al., 2022), and conventional wastewater treatments have proven unable to effectively remove them from influents (Zhou et al., 2018). One of the most reported plastic polymers in aquatic environments is polystyrene (PS; Cai et al., 2021; Llorca et al., 2021). An increasing number of scientific studies have focused on the effects of PS-NPs on the health and performance of aquatic organisms, such as fishes (Barría et al., 2020; Brandts et al., 2021a; Pitt et al., 2018). Moreover, the increasing usage of Recirculation Aquaculture Systems (RAS) for aquaculture production raise interest on the assessment of NPs in these systems and the impact on fish reared in such systems.

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https://doi.org/10.1016/j.ecolind.2023.109966
Received 20 October 2022; Received in revised form 26 January 2023; Accepted 29 January 2023
Available online 3 February 2023

Most of the published research regarding PS-NPs has explored their effects on the model species zebrafish (*Danio rerio)*, leaving only a small fraction of the literature investigating the response in other fish species (reviewed by Barría et al., 2020). However, when considering relevant factors such as rearing and maintenance costs, commercial availability, genetic distance with farmed species, and facility to handle, the goldfish (*Carassius auratus*) emerges as an ideal model organism to investigate the effects of NPs (Blanco et al., 2018; Filice et al., 2021). On the other hand, when reviewing NPs' ecotoxicology, most of the available literature deals with the response of fish to short-term exposures of no longer than 7 days (Barria et *al.*, 2020). In real-life scenarios, it is more likely that organisms are subjected to long-term exposures, with low but persistent concentrations of NPs present in the ecosystems (de Ruijter et al., 2020; Weis and Palmquist, 2021). Nevertheless, the effects of chronic exposures to NPs in fish are still poorly understood, although ontogenetic, intestinal, metabolic, or behavioural anomalies, amongst others, have already been reported in some species (Gu et al., 2020; Guimarães et al., 2021; Marana et al., 2022; Mattsson et al., 2015). To the best of the authors' knowledge only one previous study has explored the long-term effects of PS-NPs in goldfish, carried out by this research group (Brandts et al. 2022). In this recent study, the presence of NPs in goldfish liver and muscle after a chronic exposure was confirmed, and genotoxic damage in red blood cells was found. Therefore, further investigating the response to a chronic exposure to PS-NPs in *C. auratus* as a model species is of great interest, as it can provide tools key answers that could be relevant to other fish species.

NPs have been observed to enter fish organs mainly through the gills and the gastrointestinal track, under laboratory conditions, (Clark et al., 2022; van Pomeren et al., 2017), being eventually translocated through blood transport into other organs, where they can accumulate (Ma et al., 2022). In fish, the exposure to PS-NPs has been found to elicit immune responses, and to have deleterious effects on the digestive, reproductive, and endocrine systems, as well as to negatively impact behaviour and significantly alter gut microbiota (Brandts et al., 2021a; Marana et al., 2022; Pitt et al., 2018; Yin et al., 2021). Therefore, investigating biological endpoints related to oxidative status, and immune and metabolic activities may provide interesting insights on the toxicity of NPs (Franco-Martinez et al., 2016; Yin et al., 2021). In addition, nanoplastics have been demonstrated to enter organisms through trophic transfer (Chae et al., 2018), indicating an additional exposure source for humans through the ingestion of contaminated food. Furthermore, previous studies have shown the ability of these nanoparticles to cross the blood–brain barrier, not only in fish (Ma et al., 2022), but also in mammals (Shan et al., 2022). Thus, investigating the effects of PS-NPs in fish, particularly their brains may allow for a better understanding of the potential risks that this contaminant may pose to human health and welfare.

Biomarkers, defined as biochemical, physiological, or histological indicators of either exposure to, or effects of, anthropogenic stress (Huggett, 2018), have been applied in ecotoxicology and ecological risk assessment for>20 years (van der Oost et al., 2003). One of their primary strengths is that they can provide early signs of response to exposure, allowing us to determine damage and defence processes at a sub-cellular level (Roméo et al., 2013), measurable before effects on individual performance and population/community dynamics occur (Forbes et al., 2006). Thus, developing biomarkers to monitor NPs' pollution can be extremely valuable to indicate that organisms have been or are being exposed to these pollutants, and could suffer future impairments of ecological relevance. Generally, biomarkers are determined using manual techniques and often without being validated for the species under study (Franco-Martinez et al., 2016; Oliveira et al., 2018). Determining biomarkers via automated analysers and assays presents significant advantages compared to manual techniques, being more time- and cost-effective, eliminating operator-tied errors and, consequently, increasing the repeatability and reliability of the obtained results (Franco-Martinez et al., 2018; Oliveira et al., 2018). However, these must be previously validated in the appropriate species and matrix, to ensure their reliability, accuracy, and precision as indicators for a specific factor (Franco-Martínez et al., 2021). To this end, the specific objectives of the present study are twofold: 1) to achieve the analytical validation of biochemical markers in goldfish tissues; and 2) to assess the effects of a chronic exposure to PS-NPs in the gills, muscle, and brain of *C. auratus*, using the validated biomarkers. We studied a panel of biomarkers, namely, total oxidative status (TOS), total antioxidant capacity (TAC), oxidative status index (OSI), esterase activity (EA), adenosine deaminase (ADA), acetylcholinesterase (AChE) levels, and creatine kinase (CK), with the final aim of providing a set of adequate tools for giving a first assessment of general fish health status after a chronic exposure to NPs.

2. Materials and methods

2.1. Fish husbandry, bioassay and sampling

A total of 32 adult *C. auratus* (11.16 ± 3.23 cm total length and 7.07 ± 0.64 g weight) were selected for the present study. Individuals were randomly allocated to experimental aquaria and subjected to either of two experimental conditions: "Control" (0 μg/L PS-NPs) and "Exposed" (100 μg/L PS-NPs). To this date, no substantial environmental quantification of PS-NPs has been established, therefore the exposure concentration selected for this study took into consideration estimated concentrations in the environment (Lenz et al., 2016), in conjunction with those utilised in previous studies (Brandts et al., 2018; Brandts et al., 2020; Brandts et al., 2021b). Regarding the chosen polymer, PS has been found in real NPs environmental samples (Llorca et al., 2021; Ter Halle et al., 2017) and it has also been documented that this polymer contributed to a significant fraction of plastic waste in monitoring studies (de Haan et al., 2019). For the experiment, we used commercial PS-NPs (44 nm size) obtained from Bangs Laboratories. Dynamic light scattering (DLS) was employed to characterize the NPs' hydrodynamic size and zeta potential (Zetasizer Nano ZS, Malvern) in ultrapure water, and results were as shown in Brandts et al. (2022). Four experimental aquaria were prepared for each treatment, each containing 20 L of water and 4 individuals (n = 16; N = 32). Fish were exposed to the experimental conditions for a 30-day period, as per guideline 215 of the Organization for Economic Co-operation and Development (OECD; Fish, 2000). Throughout the experimental period, fish were fed *ad-libitum* at the same hour every day with a commercial diet (TROPICAL Goldfish Colour Pellet; crude protein 45.0 %, crude fat 7.0 %, crude fibre 3.0 %, moisture 10 %; fish and fish derivatives, derivatives of vegetable origin, oils, fats, and minerals), and the bottom of the aquaria was cleared of impurities which could negatively affect water quality. Fish mortality, as well as behavioural parameters (e.g., aggressiveness, activity rate:

hyperactivity/lethargy/erratic swimming, feeding behaviour/appetite) were recorded daily. The physicochemical parameters of the water were kept at optimal levels for the studied species (Table 1).

Approximately 75 % of the medium was replaced once every fifth day and PS-NPs were added, aiming to maintain the contaminant's level as close to the starting concentration as possible. At the end of the 30-day experimental period, all individuals were over-anesthetized in a tricaine methane-sulfonate bath (MS-222; 1 g/L). Each fish was subsequently measured and weighted, and blood was successively extracted through caudal puncture using heparinized syringes. Brain, gills, and muscle samples were collected from 7 randomly selected individuals from each group (n = 7; N = 14), snap-frozen with liquid nitrogen, and stored at −80 °C until analysis. The remaining fish were either used to test the reliability of the employed techniques or stored at −80 °C until further analysis. The entirety of the experiment was carried out following the 3 Rs of Animal Experimentation (Replacement, Reduction, and Refinement), under Spanish legislation (law 32/2007 and RD53/2013), and in agreement with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63).

Table 1
Parameters of water quality maintained throughout the acclimatisation and experimental period.

Temperature (T°)	Alkalinity	Dissolved Oxygen	pH	Nitrate (NO_3^-)	Nitrite (NO_2^-)	Ammonia (NH_3)	Ammonium (NH_{4+})	Hardness
16 °C	6.7 dkH	4 mg/L	8.5	<10 mg/L	<0.5 mg/L	<0.15 mg/L	<0.25 mg/L	6–7 dGH

2.2. Sample processing prior to analysis

Samples of gill, brain and muscle were homogenized in potassium phosphate buffer 0.1 M (pH 7.2), at a weight:volume ratio of 1:4. Following a 30-min incubation at room temperature, the samples were centrifuged at 15,000 × g for 90 min, at 4 °C, and the obtained supernatant translocated to a clean tube, and placed at −80 °C until further use.

2.3. Validation of the biochemical parameters in goldfish tissues

Precision and accuracy were evaluated for all biomarkers in tissue homogenates. Precision was assessed by the determination of intra-assay coefficients of variation. For this, biomarkers were measured in one sample with high and one sample with low values three times in the same run. Coefficient of variation (CV) was calculated using the following equation (1):

$$CV(\%) = \frac{\sigma}{\overline{x}} * 100 \quad (1)$$

where: σ = standard deviation; $\overline{x}$ = average.

Accuracy was evaluated indirectly by performing linearity and recovery tests. To assess linearity under dilution, samples with high concentrations were serially diluted with a phosphate buffer. To evaluate the ability of the assays to recover the amount of added analyte, spiking recovery was performed by mixing two samples (one with high and one with low concentrations) at different rates. Test recovery (percentage) was calculated for each dilution for comparison of expected versus measured concentrations of each analyte in each tissue homogenate.

2.4. Measurement of biochemical biomarkers

TOS was measured as previously described (Erel, 2005). TAC was assessed by evaluating ferric reducing ability of the samples as described by Benzie and Strain (1996). EA was measured using p-nitrophenyl acetate as substrate (Haagen and Brock, 1992) adapted to automatic analyser (Tvarijonaviciute et al., 2012a). AChE was measured following the methodology described by Tecles et al. (2000). CK and ADA activity were determined using commercially available kits (Creatine Kinase, Olympus Systems Reagents; Olympus life and Material Science Europe GmbH, Hamburg, Germany; Adenosine Deaminase assay kit, Diazyme Laboratories, Poway, CA, USA) following manufacturer's indications. All parameters were determined with an automatic analyser (Olympus Diagnostica, GmbH).

2.5. Oxidative stress index

The oxidative stress index (OSI) was determined through the following equation:

$$OSI = TOS/TAC$$

2.6. Data processing and analysis

Data manipulation and analysis were carried out through GraphPad Prism 8.0.1 (GraphPad Software, Inc.). Homogeneity of variance was tested with an F-test, and the normality of the data was tested with the Shapiro-Wilk test. Data complying with normal distribution and homoscedasticity were analysed with a student's *t*-test. The non-parametric Mann-Whitney *U* test was employed to analyse data that did not follow a normal distribution. The results are presented as bar graphs, displaying the mean ± standard deviation (sd; n = 7), considering the threshold for significance at $p < 0.05$. Ordinary linear regression analysis, comparing measured and expected concentrations of analytes in different samples, was used to evaluate the linearity under dilution, and Runs-test was performed to determine whether data deviated significantly from the linearity.

3. Results

3.1. Parameter validation

The precision and accuracy data of the methods are presented in Table 2. All methods showed a coefficient of variation below 15 %. Serial dilutions of samples resulted in linear regression equations in which correlation coefficients did not differ from 1, the slope and intercept were close to 1 and 0, respectively, and the Runs test revealed no deviation from linearity ($P > 0.05$). Recovery between observed and expected concentrations ranged from 81 to 114 %, in all cases.

3.2. Biochemical parameters in gills, brain and muscle

The analysed biochemical parameters for all three studied tissues are shown in Fig. 1. No significant differences were found in TOS nor OSI

Table 2
Precision and accuracy of employed techniques. Intra-assay coefficient of variation (CV), linearity under dilution and spiking recovery rates of total oxidant status (TOS), total antioxidant capacity (TAC), esterase activity (EA), adenosine deaminase (ADA), acetylcholinesterase activity (AChE), and creatinine kinase (CK) in homogenates from the gills, brain and muscle of the teleost fish Carassius auratus.

Tissue	Analyte	CV, % (Range)	Linearity under dilution*		Recovery, % (Range)
			Linear Regression Equations	R^2	
Gills	TOS	3.6–7.3	y = 0.566x + 0.0177	0.999	90.3–108.7
	TAC	2.2–7.2	y = 1.1713x − 0.0109	0.979	100.0–103.5
	EA	1.9–5.4	y = 0.6892x − 0.0289	0.995	81.5–100.0
	ADA	0.6–2.5	y = 0.6684x + 3.3182	0.999	98.4–100.0
Brain	TOS	7.1–10.3	y = 0.8569x − 0.4469	0.999	90.8–100.0
	TAC	0.5–2.8	y = 1.0883x + 0.0066	0.990	96.2–108.9
	EA	8.0–14.1	y = 0.9623x − 0.0102	0.983	89.8–100.0
	ADA	0.9–1.9	y = 1.0136x − 0.9661	0.998	95.9–100.0
Muscle	TOS	9.1–13.0	y = 1.9064x − 7.4016	0.989	100.0 – 106.5
	TAC	6.5–9.4	y = 0.9191x + 0.0206	0.968	94.9–100.0
	EA	7.7–12.4	y = 0.8232x + 0.0105	0.998	96.3–101.1
	ADA	6.2–11.8	y = 0.9993x − 0.0085	0.996	95.7–106.4
	AChE	4.4–9.1	y = 0.9567x + 1.4078	0.994	87.4–113.3
	CK	1.5–5.3	y = 0.7845x + 12.435	0.998	96.8–101.9

*, Runs-tests were not significant ($P > 0.05$) in all cases.

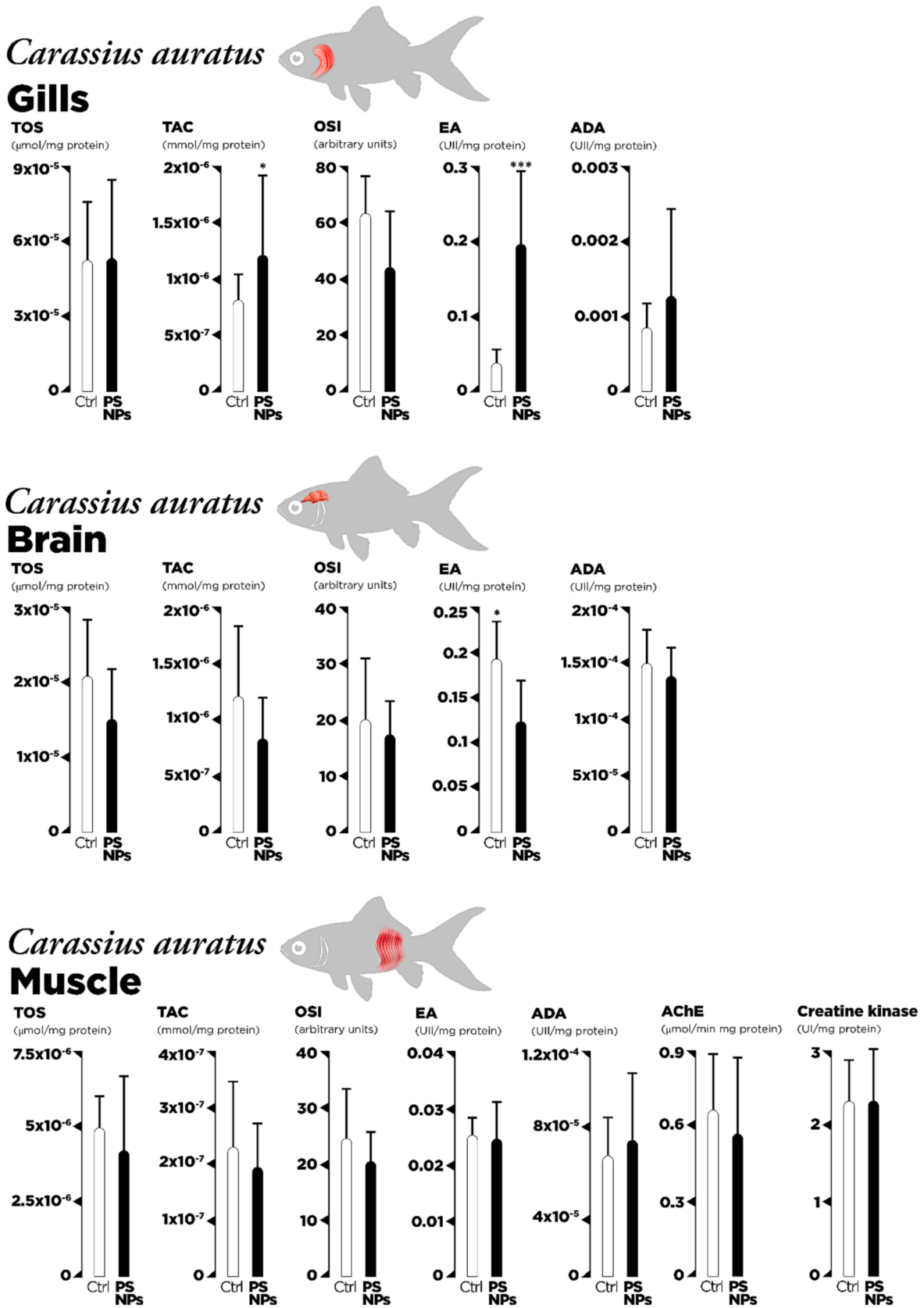

Fig. 1. Effects of PS-NPs on the gills, brain, and muscle of *Carassius auratus* (goldfish). Control conditions (ctrl = 0 µg/L PS NPs; n = 7) are compared to the treatment group (PS NPs = 100 µg/L PS NPs; n = 7). Variations in Total Oxidative Status (TOS), Total Antioxidant Capacity (TAC), Oxidative Status Index (OSI), Esterase Activity (EA), Adenosine Deaminase (ADA), Acetylcholinesterase activity (AChE), and Creatine Kinase (CK) are depicted in the corresponding organs. Significant differences, as well as degree of significance between control and treatment groups are indicated (* = P-value < 0.05, P-value < 0.01 ***).

between the control and the fish exposed to NPs in any of the studied organs. However, gill samples from the individuals exposed to 100 μg/L of PS-NPs displayed significantly higher TAC when compared to the control group (p-value = 0.016). Moreover, EA was also significantly increased in the gills of fish exposed to PS-NPs (p-value < 0.001). In contrast, the results indicated a significant decrease of EA in the brain (p-value = 0.01) of fish exposed to NPs compared to the control group. In muscle, no significant variations were observed in any of the biomarkers assessed.

4. Discussion

Analytical validation of given biomarkers is essential to ensure that test results reflect the condition of an animal, rather than variations caused by the laboratory itself due to method imprecision and inaccuracy, among others (Jensen and Kjelgaard-Hansen, 2010; Tvarijonaviciute et al., 2012b). Therefore, validation studies must ensure that analytical methods can detect the corresponding analyte in a precise and accurate way in a target sample. This practice is of particular importance in novel fields like the effect of exposure to NPs in living organisms, in order to avoid under- or over-estimating the extent of a problematic, and to enable for standardisation of techniques, allowing for the correct comparison between results of different studies (Dor et al., 1999). Furthermore, validating biomarkers for a particular condition is of utmost importance to ensure the accuracy of ecotoxicological studies monitoring nanoplastics pollution and its effect on both wild and cultured populations. In this study, automated methods for oxidative stress markers (TAC, TOS, EA), biomarkers of immune response (EA, ADA), neurotoxicity (AChE), and a biomarker of muscle damage (CK), were validated in gills, brains and muscle homogenates of *C. auratus*. All the evaluated methods presented adequate precision in tissue homogenates with intra-assay CVs lower than 20 %, the limit for an objective analytic performance standard for precision (US Department of Health and Human Services, FDA, CDER, CVM, 2018). In the same line, the accuracy of the methods has fulfilled the established criteria consisting of (1) obtaining a regression equation approximating R2 to 1.0 in the regression analysis of the relationship of the measured and expected analyte values in the linearity under dilution test; and (2) obtaining percentages between 80 % and 120 % in the spiking recovery test (Jensen and Kjelgaard-Hansen, 2010). These findings confirm all the methods to be precise and accurate when quantifying their respective analytes in *C. auratus* tissue homogenates.

This study showed that, in *C. auratus*, immune and antioxidant responses occur in the gills following a chronic waterborne exposure to PS-NPs, but not in brain nor muscle. To the best of the authors' knowledge, no study has investigated the effects of PS-NPs on the considered biomarkers in the gills, muscle, and brain of *C. auratus*, after a chronic exposure. For these reasons, the results hereby presented are mostly compared with previous findings with different species or polymers, with some exceptions.

The increase in TAC detected in gills of *C. auratus* in the present experiment indicates a boosted production of antioxidant compounds aiming to inhibit the effects of reactive oxygen species (ROS), which have been documented in fish exposed to different types of NPs (Jacob et al., 2020). Similarly, a recent study indicates that waterborne, chronic (28-day) exposure to PS-NPs (250 nm, 0.05–5 mg/L) induces oxidative stress in *C. auratus*, although different biomarkers as those hereby considered were investigated (Abarghouei et al., 2021). Indeed, the authors reported significant increases in the levels of superoxide dismutase (SOD) and catalase (CAT) in serum. In addition, PS-NPs appeared to have size-, and concentration-dependent effects on the health of *C. auratus*, with smaller particles at higher concentrations causing severe histopathological changes in gills, liver, and gut. Moreover, a number of previous studies have reported evidence that exposure to PS-NPs, and other NPs, cause oxidative stress in fish (Reviewed by Han et al., 2021). Similarly, Brandts et al. (2021c) detected a significant increase of TAC in gills of *Sparus aurata* exposed to polymethylmethacrylate (PMMA) NPs concentrations ranging from 0.001 to 0.1 mg/L. This suggests a similar effect of PMMA-NPs and PS-NPs, both causing oxidative stress in gills. Nonetheless, in yellow croaker (*Larimichthys crocea*), dietary exposure to PS-NPs did not cause any significant variations in TAC in liver, but did on muscle (Lai et al., 2021), and waterborne exposure led to oxidative stress in the liver, with evident rises in levels of CAT, SOD, and glutathione peroxidase (GPx; Gu et al., 2021). This points towards variations in the effects of NPs linked to species, organ, and exposure type. Furthermore, a significant increase was found in TOS in the muscle and liver of *S. aurata* exposed to similar concentrations of PMMA-NPs (Balasch et al., 2021; Brandts et al., 2021b). Nevertheless, no significant differences in TOS were found in gills nor skin under the same experimental conditions (Brandts et al., 2021c). In addition, Brandts et al. (2021a) reported no significant differences in TAC or OSI when examining skin mucus and plasma of *Dicentrarchus labrax* exposed to PS-NPs. This further indicates inter-organ variations in the response to NPs, although these differences could also be explained by the specificities of each polymer, such as size, composition, and shape, and the way these factors affect the organ-NPs interaction (Guerrera et al., 2021). These results suggest an additional inter-specific component that could explain the observed differences, by which different organs in different species display varying responses to similar NPs polymers and concentrations. The present results also suggest that gills are one of the main pathways for NPs to be internalized in freshwater fish, as shown previously for other xenobiotics (Bhagat et al., 2020). It should be considered that the ingestion of food during chronic bioassays might modify the nutritional status of fish and help to counteract the effects induced by NPs, notably oxidative stress (Sinha et al., 2015), on internal organs; but not in gills, which would serve as a portal of entry and internalization. In addition, the differences in findings regarding the potential of oxidative stress or oxidative damage caused by NPs exposure between the current study and the available literature could be attributed to differences in targeted biomarkers (Han et al., 2021).

ADA has been widely investigated as an indicator for immune response and oxidative stress management (Baldissera et al., 2018; Capiotti et al., 2016; Zhang et al., 2022). This enzyme, involved in purine metabolism (Cristalli et al., 2001), has been reported to play a major role in neutrophil regulation, and in controlling the immune response to bacterial stressors (Kälvegren et al., 2010). The lack of response of this parameter to long-term exposure to PS-NPs in any of the studied organs suggests that the specific mechanisms regulated by this enzyme were not significantly affected. Similarly, Brandts et al. (2021b) and Balasch et al. (2021) did not report significant alterations in ADA in gilthead seabream subjected to short-term exposure to PMMA-NPs. Moreover, Brandts et al. (2021a) did not detect significant differences in ADA in skin mucus nor plasma of *D. labrax* exposed to PS-NPs, further corroborating the present findings. Moreover, the esterase activity (EA) was significantly increased in the gills of fish exposed to PS-NPs (p-value < 0.001), which suggests that the mechanism to counteract the oxidative stress caused by an anti-inflammatory response due to PS-NPs was successfully triggered. EA is also often employed as a proxy for immune activity (Marcos-López et al., 2017), and the observed response could indicate that the gills were developing an anti-inflammatory response to the contact and internalization of NPs. The present results are in contradiction with the findings published by Brandts et al. (2021c), describing a significant decrease of EA in both gills and liver of *S. aurata* exposed to PMMA-NPs, but, once again, this could point not only to inter-specific variations, but also differences linked to the nature of the polymer, the exposure period, and the absence of food throughout the acute tests. The significant decrease in EA observed in brain, could indicate an alteration in the immune capacity of this organ (Oliveira et al., 2018), and could be attributed to the direct effect of the PS-NPs on this enzyme, or to its relocation as a response to oxidative stress in gills (Brandts et al., 2021c). Interestingly, besides the immune and antioxidant responses observed in gills,

previous studies reported a general genotoxic response in blood cells (Brandts et al., 2021b), which might mean that NPs are entering blood circulation through the gills, inducing oxidative stress in blood cells, reaching preferentially the liver due to a fenestrated endothelium, compared to muscle or brain. In accordance with previous experiments carried out in a different species (*S. aurata*) and NPs polymer (PMMA) (Balasch et al., 2021), EA did not result in significant differences in muscles between treatment groups. This absence in variations of EA in muscle indicates that esterase did not take part in counteracting the oxidative stress caused by PS-NPs in this organ.

AChE activity is widely used as a biomarker for neurotoxicity of environmental pollutants (Fulton and Key, 2001), and alterations in this factor are known to lead to motor problems, potentially causing severe paralysis and, eventually, death (Modesto and Martinez, 2010). No abnormal behaviour of any kind was reported in goldfish in the present study, and the lack of alterations in AChE activity seems to match these observations. Previous studies have reported either an upregulation or downregulation in AChE activity following acute exposure of *S. aurata* to PMMA-NPs, and *D. rerio* and *Oreochromis niloticus* to PS-NPs (Balasch et al., 2021; Chen et al., 2017a; Chen et al., 2017b; Ding et al., 2018). However, the differences in previously reported results might be linked to the characteristics of each particular polymer (Guerrera et al., 2021), the exposure period (Atli and Canli, 2011; Kögel et al., 2020), or the species of interest and their developmental stage when subjected to the stressor (Barton, 2002; Fowler et al., 2009).

CK is a reliable indicator for muscle damage (Wallimann et al., 2011; Yousaf and Powell, 2012), and high levels of this protein could cause changes in the swimming behaviour. Therefore, the lack of alterations in the activity of this enzyme supports the fact that the exposed fish did not display any sort of abnormal behaviour throughout the experimental period. This result is in accordance with what Balasch et al. (2021) recorded, stating that no significant effect of PMMA-NPs on CK was observed. The main difference between the present study and the literature described above, other than the exact type of contaminant used and the species of interest, is the exposure period. In a recent study from the authors' research team, it was observed that NPs were preferentially accumulated in muscle of *C. auratus* (Brandts et al., 2022), but the complete absence of response observed in this organ during the present experiment indicates that the number of particles was below the threshold to trigger an immune or antioxidant response.

Altogether, it appears as if the gills sustained greater oxidative damage compared with the brain or muscle, which could be justified by gills, as one of the major entry portals, being subjected to direct exposure to xenobiotics (Arellano et al., 2001). Thus, gills are generally more susceptible to external contaminants than brain or muscle, as it could have been expected. Furthermore, differences regarding the main organs accumulating nanoplastics, and therefore displaying responses to this contaminant, could be expected between freshwater and marine species. Indeed, marine fishes tend to drink large quantities of seawater to compensate for the important osmotic loss through the gills, due to their euryhaline environment (Takei, 2021), making the intestinal tract a greater portal of entry than gills. In contrast, freshwater fishes are subjected to osmotic gain of water through the gills due to their oligohaline surroundings (Edwards and Marshall, 2012), therefore, gills play a major role as a portal of entry for xenobiotics. This should be taken into consideration when selecting organs of interest for investigating both the accumulation potential, and the effects of NPs in fish.

Research concerning NPs still has multiple knowledge gaps, which limits the assessment of risk of exposure and potential effects of NPs in biota and humans (European Commission and Directorate-General for Research and Innovation, 2019). The accurate quantification of NPs in the environment in order both to establish environmental concentrations and to be able to quantify the bioaccumulation of NPs in organism and studies evaluating the sub-lethal effects of chronic exposures to NPs are crucial stepping-stone in the field. Moreover, species-specific effects and potential sex-specific effects within each species should also be considered and further investigated in these chronic scenarios, as both oxidative-stress and general stress related responses have been shown to differ between sexes and could condition the response to NPs (Balasch and Tort, 2019; Niksirat et al., 2021).

Although estimates on the environmental concentrations of NPs don't have consensus among researchers on the field, it has been suggested that the environmental concentration range varying between ca.1 pg/L and ca. 20 μg/L (Lenz et al., 2016). Moreover, to the best of the author's knowledge, the only existing studies that report actual environmental NPs quantifications, report concentration ranging from 7 to 52 μg/L (Llorca et al. 2021; Materić et al. 2022). On the other hand, reports giving information on MPs concentrations found in industrial effluent discharges account for concentrations of $\leq$ 30 mg/L (Lechner and Ramler, 2015). 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 10^{14} 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). Considering this, the dose used in this study, (100 μg/L) would fall close within the range of estimated concentrations of NPs in the environment and only one order of magnitude greater than real environmental quantifications. Future studies should further explore more environmentally realistic chronic-exposure scenarios, leaning towards lower doses of NPs and plastic nanoparticles resulting from breakdown of larger plastic objects instead of model spherical particles, to better understand potential ecosystem effects. More data is essential for risk assessment, risk management, and risk communication.

5. Concluding remarks

New automated methods for biomarkers of oxidative stress (TAC, TOS, EA), immune response (EA, ADA), neurotoxicity (AChE), and muscle damage (CK), were successfully validated in gills, brain, and muscle homogenates of *C. auratus*, showing their adequate analytical performance. These methods enabled the assessment of the impact of PS-NPs on gills, where it possibly caused oxidative stress and subsequently triggered antioxidant mechanisms. It appears as if PS-NPs had a heavier impact in gills when compared to brain or muscle, where they had little to no evident effect. The obtained results, compared with those available in the published literature, confirm strong inter-specific and inter-organ variations. Even though some responses seem generalised, specific organs react differently to the presence of PS-NPs, although some of these differences might be due to the specificities of the particles used (e.g., size, shape). Further research is needed to fully understand the effect of chronic waterborne exposure of fish to PS-NPs. However, although the effects observed in brains are relatively mild, the results could have implications for human health, as humans are in constant exposure to these contaminants, and might suffer from the NPs ability to cross the blood–brain barrier.

CRediT authorship contribution statement

M. Blonç: Writing – original draft, Writing – review & editing. **I. Brandts:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **M. Cánovas:** Investigation. **L. Franco-Martínez:** Methodology. **C.P. Rubio:** Methodology. **L. Tort:** Funding acquisition, Supervision, Writing – review & editing. **A. Tvarijonaviciute:** Funding acquisition, Methodology, Writing – review & editing. **C. Gravato:** Writing – review & editing. **M. Teles:** Conceptualization, Funding acquisition, Supervision, 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.

Data availability

Data will be made available on request.

Acknowledgements

This research was supported by the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie (MSCA) grant agreement No 956481 through the RASOPTA received by MB. MT was supported by a Ramon y Cajal contract (ref. RYC2019-026841-I), and the Plan Nacional de Investigación with reference PID2020-113221RB-I00. IB was supported by a PhD grant from Generalitat de Catalunya (2018FI_B_00711). LFM was granted a Margarita Salas postdoctoral contract by the Ministerio de Universidades of the Government of Spain, financed by the European Union—NextGenerationEU. CPR has a post-doctoral fellowship "Juan de la Cierva Formación" supported by the "Ministerio de Economía y Competitividad" (FJC2019-042475-I), Spain. Thanks are also due to Fundação para a Ciência e a Tecnologia (FCT, Portugal). In addition, the authors acknowledge Joan Carles Balasch for the graphical design of the figures and Jennifer Lima for her technical assistance during the experiment.

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