Universidade de Aveiro Departamento de Biologia
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Effects of polymeric nanoparticles on fish: A multiparametric approach

Efeitos de nanopartículas poliméricas em peixe: Uma abordagem multiparamétrica

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica do Doutor Marcelino Miguel Guedes de Jesus Oliveira, Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro, co-orientação da Professora Doutora Maria de Lourdes Gomes Pereira, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro, e co-orientação do Doutor Manuel António Martins da Silva, Bolseiro de Pós-Graduamento do Departamento de Química da Universidade de Aveiro.

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Palavras-chave Nanoplásticos, polimetilmetacrilato, poliestireno, peixe-zebra, robalo, biomarcadores.

Resumo

A contaminação de sistemas aquáticos com vários tipos de detritos é uma crise ambiental emergente. Um dos problemas associados à poluição com plásticos é a sua persistência. As partículas de plástico não desaparecem, degradaram lentamente de tamanhos macro para micro para nano. Embora atualmente haja um número crescente de estudos que avaliem os efeitos dos microplásticos em organismos aquáticos, os efeitos de nanoplásticos são amplamente desconhecidos. O presente estudo tem como objetivo avaliar os efeitos a curto prazo das nanopartículas poliméricas em peixes e na presença de matéria orgânica. Assim, os efeitos sobre o desenvolvimento ontogénico foram avaliados em *Danio rerio*, um peixe de água doce, expondo embriões de peixe por 96h a PMMA (intervalo de concentração de 2,5 a 202,5 mg/L) e PS (intervalo de concentração de 2,5 a 1822,5 mg/L) de partículas (≈ 50 nm). Além do desenvolvimento ontogénico, foram também avaliados efeitos comportamentais (distância e tempo de natação, assim como o tigmotaxia) e os efeitos bioquímicos (NPT, CAT, GPx, GST, GR e LPO). Efeitos no *Dicentrarchus labrax*, uma espécie de peixe marinho, também foram avaliados após a exposição de 96h. Os parâmetros avaliados incluíram genotoxicidade (micronúcleos de eritrócitos e outras anormalidades nucleares e diferenças de forma de equinócitos) e efeitos sobre o estado e dano antioxidante (NPT, CAT, GPx, GST, GR e LPO). No geral, as nanopartículas revelaram a capacidade de ser perniciosas para peixes, com PMMA apresentando maior toxicidade para o peixe do que o PS. *D. rerio* apresentou alterações comportamentais associadas à atividade geral e respostas de stress. Em *D. labrax*, as nanopartículas testadas foram genotóxicas, como demonstrado pelo aumento das anormalidades nucleares dos eritrócitos. As respostas bioquímicas avaliadas foram mais sensíveis nas brânquias e no fígado do que no intestino, com dados que confirmam que os nanoplásticos têm a capacidade de afetar o estado antioxidante.

Os resultados do presente estudo são altamente relevantes, pois demonstram a capacidade dos nanoplásticos testados para afetar o desenvolvimento e o comportamento dos peixes e que eles são citogenotóxicos.

Keywords Nanoplastics, polymethylmethacrilate, polystyrene, zebrafish, sea bass, biomarkers

Abstract

The contamination of aquatic systems with several kinds of debris is an emerging environmental crisis. One of the problems associated with plastic pollution is its persistence. Plastic particles do not disappear, they slowly degraded from macro to micro to nano sizes. Although an increasing number of studies are currently assessing the effects of microplastics in aquatic organisms, the effects or nanoplastics are largely unknown. The present study aims to assess the short-term effects of polymeric nanoparticles in fish alone and in the presence of organic matter. Thus, the effects on ontogenic development were assessed in *Danio rerio*, a freshwater fish, by exposing fish embryos for 96h to PMMA (concentration range from 2.5 to 202.5 mg/L) and PS (concentration range from 2.5 to 1822.5 mg/L) particles (≈ 50 nm). In addition to ontogenic development, behavioural (distance and time swam as well as thigmotaxis) and biochemical effects (NPT, CAT, GPx, GST, GR and LPO) were also assessed. Effects on *Dicentrarchus labrax,* a marine fish species, were also assessed after 96h exposure. Assessed parameters included genotoxicity (erythrocytes micronuclei and other nuclear abnormalities and echinocytes shape differences) and effects on antioxidant status and damage (NPT, CAT, GPx, GST, GR and LPO). Overall, the nanoparticles revealed ability to be pernicious to fish, with PMMA presenting a higher toxicity to fish than PS. *D. rerio* displayed behavioural alterations associated with overall activity and stress responses. In *D. labrax*, the tested nanoparticles were genotoxic, as demonstrated by the increase of erythrocytes nuclear abnormalities. the assessed biochemical responses were more responsive in gills and liver than intestine with data confirming that nanoplastics have the ability to affect antioxidant status.

The present study results are highly relevant as they demonstrate the ability of the tested nanoplastics to affect fish development and behaviour and that they are cytogenotoxic.

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List of Abbreviations

CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
DLS	Dynamic light scattering
DOM	Dissolved organic matter
DTNB	5,5-dithiobis-tetranitrobenzoic acid
FET	Fish Embryo Toxicity
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Glutathione disulphide
GST	Glutathione s-transferase
H_2O_2	Hydrogen peroxide
HA	Humic acid
KPS	Potassium persulfate
LPO	Lipid peroxidation
MMA	Methylmethacrylate
MS-222	Methanesulfonate
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium hydrogen carbonate
NaN_3	Sodium azide
NPT	Non-protein thiols

PMMA	Polymethylmethacrylate
PS	Polystyrene
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
STEM	Scanning transmission electron microscope
Sty	Styrene
TBA	2-thiobarbituric acid
TCA	Trichloroacetic acid
TD	Total Distance
TD%	Total distance out
TEM	Transmission electron microscope
Tris-HCl	2-amino-2-hydroxymethyl-propane-1,3-diol hydrochloride
TT	Total time
TT%	Total time out
V50	2,2'-azobis(2-amidinopropane) chloride
ZP	Zeta potential

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Introduction

1.1. Pollution concerns in aquatic systems

The contamination of aquatic systems with several kinds of debris is an emerging environmental crisis with studies regularly describing its environmental presence and what impacts it has in the organismal. Some of those debris are plastic, since these are durable, versatile and generally used material, with countless applications including in packaging, construction, textiles and electronics (Worm et al., 2017). As society continues to evolve, the benefits from the use of plastic in new and innovate applications increases, increasing their environmental presence and production as well (Conkle et al., 2017, Cole and Galloway, 2015). It is also known that the improperly discarded single-use plastic affects these aquatic systems greatly. These particles can enter the environment through several pathways, including littering, illegal dumping, sewage and storm water runoff and the breakdown of larger plastic litter, affecting the freshwater and marine systems (Hurley et al., 2017). Plastics annual production is estimated to yield a cumulative production of 33 billion metric tons by 2050 (Miller et al., 2017). With this amount of production, the amount of debris will also increase, with estimates that by 2050, the plastic waste that enters the oceans will outweigh fish pound for pound (Conkle et al., 2017).

Plastics debris can fragment through weathering and abrasion into continuously smaller and smaller particles, macro to micro to nano. However, this degradation process is affected by several abiotic factors (e.g., temperature, UV radiation) and the polymers involved. Microplastics (size <5 mm) can be classified in two sub-classes according to their sources: primary and secondary. Primary microplastics include microbeads in cosmetics and personal care products, abrasives used in industrial blast cleaning, microfibers shed from synthetic textiles and virgin resin pellet (Eerkes-Medrano et al., 2015). Secondary plastics are formed by the fragmentation of larger plastics through natural weathering (Bruck et al., 2017). These plastics are known to cause harm to a large range of aquatic organisms. With a decrease in size, particles become more bioavailable to aquatic organisms. Several implications associated with microplastic ingestion by organisms have been reported, including: (i) the retention of microplastics in the gut, causing blockages and reducing nutrient absorption; (ii) transferring absorbed contaminants or plastic additives (Jovanović, 2017); (iii) translocation to other tissues; and (iv) transfer up the food chain, including to human populations (Conkle et al.,2017, Hurley et al., 2017). Recent publications suggest that microplastics will subsequently degrade into nanoplastics (size <100 nm) (Song et al., 2017). Besides the degradation of microplastics, the development of nanotechnology also contributes to the release

of particles of nm range into the environment (accidently or as a consequence of products' degradation). The environmental impacts of these nanoparticles will be different to those presented by microplastics (Lambert et al., 2017). Due to their smaller size, they may enter cells/tissues and accumulate. Furthermore, nanoparticles have been reported to have particular characteristics associated with their nano size, with biological effects different the bulk materials making the prediction of their effects based on bulk materials difficult. Thus, nanoparticles considered as contaminants of emerging concern since there is a lack of adequate data for reliable risk assessment (Blair et al., 2017, Mattsson et al., 2015).

Nanoparticles interact with other substances (e.g. Na^+, Cl^-, Mg^{2+}) present in the aquatic systems. In high ionic strength media, nanoparticles tend to aggregate/agglomerate, modulating their concentration and chemical equilibrium state of functional groups, decreasing their availability and arranging the nanoparticles in a complex manner (Nolte et al., 2017, Mattsson et al., 2015). Dissolved organic matter (DOM) consists of soluble organic materials derived from the partial decomposition of organic materials, including soil organic matter, plant residues, and soluble particles released by living organisms, including bacteria, algae, and plants, found in both freshwater and marine systems. It contains several functional groups, that could serve as heterogenous sites for binding of nanoparticles. Humic acid (HA) is one example of DOM, since they result from the decomposition of plant and animal residues (MacCarthy, 2001). HA are polyelectrolytes with a high density of carboxylic and phenolic functional groups, often preventing aggregation and increasing the transport of polymeric nanoparticles (Chen et al., 2018, Tiraferri et al., 2017).

1.2. Plastic Polymers

Plastic debris are constituted by many typologies of plastic polymers and additives, which can be combined in objects with specific properties and characteristics. Plastics are not one single material, they are a wide family of resource efficient materials derived from organic products such as cellulose, coal, natural gas, salt and crude oil (Europe Plastics, 2016). Two examples of polymers frequently used are polystyrene (PS) and polymethylmethacrylate (PMMA).

1.2.1. Polystyrene

PS is a synthetic aromatic polymer obtained from the polymerization of styrene monomers. PS is one of the most widely used plastics, the scale of its production being nearly two million tonnes during 2015 (Euro Plastics, 2016). PS uses include protective packaging, containers, lids, bottles, trays, tumblers, disposable cutlery and in the making of models. PS nanoparticles offer other numerous possibilities of application as catalysts for industrial usage, fuel additives for catalysis, additives in sunscreens for UV protection or in the textile industry (Loos et al., 2014). One of the most promising fields of nanotechnology is drug delivery and drug targeting. Being slow to biodegrade, PS is therefore a focus of controversy among environmentalists (Nuruzatulifah et al., 2016, Maul et al., 2012).

1.2.2. Polymethylmethacrylate

PMMA, also known as acrylic or acrylic glass, is a transparent thermoplastic often used in sheet form as a lightweight or shatter-resistant alternative to glass. PMMA is an economical alternative to polycarbonate when tensile and flexural strength, transparency, polish ability and UV tolerance are more significant than impact strength, chemical and heat resistance. Additionally, PMMA does not contain the potentially harmful bisphenol-A subunits found in polycarbonate. It is often chosen because of its moderate properties, easy handling and processing, and low cost. PMMA is a versatile material and has been used in a wide range of fields and applications such as rear-lights and instrument clusters for vehicles, appliances and lenses for glasses (Lu et al., 2015). PMMA in the form of sheets affords to shatter resistant panels for building windows, skylights, bulletproof security barriers, signs and displays, sanitary ware such as bathtubs, LCD screens and furniture. It is also used for coating other polymers since methylmethacrylate (MMA) providing outstanding stability against environmental conditions with reduced emission of volatile organic compounds. In the meantime, PMMA is compatible with the human tissue, making these particles an important material for transplants and prosthetics, especially in the field of ophthalmology and orthopaedic surgery, since they also present a similarity of its elastic modulus to natural bone (Cierech et al., 2016, Eslami et al., 2013).

1.3. Biochemical Endpoints

With the growth of pollution, aquatic systems present a complex mixture of contaminants, whose synergistic/antagonistic effects are scarcely interpreted and predictable based on chemical analyses. This led to the use of the responses (e.g. molecular, biochemical, and behavioural) of aquatic organisms, to monitor pollution, as they reflect the integrated effects of exposure to all contaminants, even those that are present at levels below chemical detection limits. Biochemical responses of fish are evaluated as indicators of habitat quality and condition indices, growth estimates and biomarkers of exposure to contaminants in fish are used due to their ability to integrate habitat quality, life-history, inter-specific, temporal and spatial patterns. Biomarkers are normally defined as measures of change in biological responses at the sub-individual level, such as molecular and physiological. These are linked to a potential anthropogenic hazard, which may be physical, chemical or biological, making it important to understand how biomarkers responses to contamination interact with fish growth and condition, and to assess potential deleterious effects at such relevant endpoints (Mieiro et al., 2011, Oost et al. 2003).The organisms exposed to nanoplastics can suffer biological consequences that vary from a moderate alteration of redox status, to the occurrence of lipid peroxidation (LPO) and genetic damage, destabilization of the main cellular functions with the appearance of several pathologies, that can consequently lead to death (Carvalho et al., 2012). Other modifications that can be present are the antioxidant defences, such as non-protein thiols (NPT), catalase (CAT), glutathione peroxidase (GPx), glutathione s-transferase (GST), glutathione reductase (GR) and damage (peroxidative and genetic) responses (Oliveira et al., 2010a, Oliveira et al., 2009).

1.4. Aims

Taking into consideration the high production of plastics, the fact that plastics do not disappear but degrade into smaller particles and the use of nanoplastics in several human applications, it becomes urgent to assess the effects of particles of nm dimensions. However, most of the studies performed focused on microplastics. This study aimed to increase the knowledge on the effects of polymeric nanoparticles on fish. Thus, effects on ontogenic development were assessed in *Danio rerio*, by exposing fish embryos to PMMA and PS particles (≈ 50 nm). The effects on a marine fish were assessed using sea bass juveniles, by studying biochemical alterations associated with antioxidant defences, lipid peroxidation and DNA damage, also assessing the role of humic acids on those effects.

Materials and Methods

2.1. Microemulsion Polymerization

2.1.1. PMMA synthesis

A microemulsion polymerization adapted from Roy and Devi (1996) was performed. The reaction mixture, which contained MMA, sodium dodecyl sulphate (SDS) and ultra-pure water, was heated to the optimized temperature (70°C). The requisite amount of V50 (2,2'-azobis(2-amidinopropane) chloride), dissolved in a minimum quantity of water, was added. The reaction was carried out under nitrogen atmosphere, for 2 hours. The percent conversion was calculated gravimetrically and determined by withdrawing 1 mL of reaction mixture at the end of the reaction and arresting the reaction by adding approximately 0.100 g of hydroquinone. The recipe used for another microemulsion polymerization had the requisite amount of the lipophilic stain Nile Red added to the mixture at the begging of the reaction.

2.1.2. Ps synthesis

A microemulsion polymerization adapted from Rabelero et al. (1997) and Tang et al. (1984) was performed. The reaction mixture, which contained styrene (Sty), SDS, $NaHCO_3$ (sodium hydrogen carbonate), hexadecane and ultra-pure water, was heated to the optimized temperature (70°C). The requisite amount of potassium persulfate (KPS), dissolved in a minimum quantity of water, was added. The reaction was carried out under nitrogen atmosphere, for 4 hours. The percent conversion was calculated gravimetrically and determined by withdrawing 1 mL of reaction mixture at the end of the reaction and arresting the reaction by adding approximately 0.100 g of hydroquinone. The recipe used for another microemulsion polymerization had the requisite amount of the lipophilic stain Nile Red added to the mixture at the begging of the reaction

2.2. Nanoparticles Characterization

Dynamic light scattering (DLS) and the zeta potential (ZP) were assessed using a Zetasizer Nano ZS (Malvern) after three consecutive runs for each sample in ultra-pure water, zebrafish water and seawater (salinity, 34), at 25°C. Average particle size of polymerized microemulsion was determined using scanning electron microscopy (SEM) and scanning transmission electron microscopy (STEM) with a scanning electron microscope SEM, analytical and high resolution Schottky emission (SE), Hitachi brand, model SU-70, equipped with detectors for secondary and backscattered electrons, energy dispersive microanalysis of X-rays (EDS) and S TEM. Also, the size was determined using

transmission electron microscopy (TEM) with the transmission electron microscope TEM H9000-NA Hitachi, with an acceleration voltage of 300KV. The samples were prepared by drying a drop of the polymer suspension in a TEM copper grid. For the samples of polymers with smaller sizes, typically less than 100 nm, the material was stained by a drop of a dilute solution of Uranyl acetate (0.5% wt/vol) for 10 min and then excess was wiped with a paper tissue.

2.3. Zebrafish Experiments

2.3.1. Model Organism

Freshwater systems are recipients of plastic debris and the organisms of those systems a target of potential toxic effects. Zebrafish (*Danio rerio*), is a well-established model for several biomedical fields. It is used in developmental toxicity screening in studies for vertebrate development and gene function since its development and optical clarity during embryogenesis allow for visual analyses of early developmental processes (Dooley and Zon, 2000). The complete genome sequence of zebrafish is known, supporting its use in fields of developmental biology, oncology, toxicology, reproductive studies, teratology, genetics, neurobiology, environmental sciences, stem cell research, regenerative medicine and evolutionary theory (Bugel et al., 2014, Westerfield, 2007, Major and Poss, 2007).

There are other advantages in using zebrafish embryos, such as its low cost, transgenic and *in vivo* genome editing capabilities, conservation of cell signalling and concordance with mammalian developmental phenotypes (Braunbeck et al., 2015). Also, the *Danio rerio* ceases to grow at a maximum of 4 cm turning into an adult, hence becoming sexually mature at approximately 3 months of age (Westerfield, 2007). They have a genetic structure similar to humans, sharing 70% of genes and 84% of genes known to be associated with human disease have a zebrafish counterpart. Zebrafish is the sole regenerative vertebrate organism currently amenable to genetic manipulation, since it exhibits a robust regenerative capacity in several tissues including the fin, spinal cord, retina and heart (Bugel et al., 2014, Major and Poss, 2007).

The zebrafish (*Danio rerio*) facility established at the Department of Biology, University of Aveiro (Portugal) provided all organisms (zebrafish eggs) used in the present study. In the zebrafish facility, organisms are maintained in carbon-filtered water complemented with salt “Instant Ocean Synthetic Sea Salt”, at 27.0 ± 1 °C and under a 16:8 h light: dark photoperiod cycle (conductivity: 550 ± 50 µS, pH: 7.5 ± 0.5 and dissolved oxygen > 95% saturation).

2.3.2. Experimental Design

2.3.2.1. Effects of PMMA and PS nanoparticles in zebrafish

2.3.2.1.1. Experimental Design

The water used to prepare the teste solutions was collected from the zebrafish system. Temperature and photoperiod conditions mentioned above were used in all assays. The test was based on OECD Guideline (1992) for FET Test. Zebrafish eggs were collected within 2h after natural mating, rinsed in water and checked under a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation). Unfertilized or injured eggs or with irregularities during cleavage were discarded.

Zebrafish fertilized eggs were individually exposed in 24-well microtiter plates (20 replicates) to various concentrations of PMMA and PS nanoparticles. Zebrafish eggs were exposed to PMMA and PS at Bioassay 1 and Bioassay 2 for 96h, differing only in the concentrations used. In bioassay 1 zebrafish eggs were exposed to 2.5, 7.5, 22.5, 67.5 and 202.5 mg/L of PMMA and PS, separately. In Bioassay 2, zebrafish eggs were exposed to 22.5, 31.5, 40.5, 49.5, 58.5 and 67.5 mg/L of PMMA nanoparticles and to 22.5, 67.5, 202.5, 607.5 and 1822.5 mg/L of PS nanoparticles. In bioassay 3 and Bioassay 4, in addition to PMMA and PS nanoparticles, the zebrafish eggs were also exposed to PMMA and PS nanoparticles stained with the lipophilic stain Nile Red, at different concentrations, for 96h. In Bioassay 3, zebrafish eggs were exposed to 22.5, 31.5 and 40.5 mg/L of PMMA nanoparticles and PMMA nanoparticles stained with the lipophilic stain Nile Red (separate 24-well microtiter plates). They were also exposed to 67.5, 202.5, 607.5 and 1822.5 mg/L of PS nanoparticles and PS nanoparticles stained with the lipophilic stain Nile Red. Zebrafish eggs were exposed to 7.2, 12.5, 17.5 and 22.5 mg/L of PMMA and PMMA nanoparticles stained with the lipophilic stain Nile Red, separately, in Bioassay 4. They were also exposed to 22.5, 67.5 and 202.5 mg/L of PS and PS nanoparticles stained with the lipophilic stain Nile Red in the presence of 1 and 10 mg/L of HA in separate 24-well microtiter plates (Table 1).

Different concentrations of PMMA and PS were achieved by dilution of a stock solution in the water used for fish maintenance. The test was initiated immediately after egg selection and was continued for 4 days.

Table 1 *– Experiment design for zebrafish assays.*

BIOASSAY 1

Solutions	*Concentrations (mg/L)*				
PMMA	2.5	7.5	22.5	67.5	202.5
PS	2.5	7.5	22.5	67.5	202.5
CONTROL	Zebrafish water only				

BIOASSAY 2

Solutions	*Concentrations (mg/L)*					
PMMA	22.5	31.5	40.5	49.5	58.5	67.5
PS	22.5	67.5	202.5	607.5	1822.5	
CONTROL	Zebrafish water only					

BIOASSAY 3

Solutions	*Concentrations (mg/L)*			
PMMA[(1)]	22.5	31.5	40.5	
PMMA_R[(2)]	22.5	31.5	40.5	
PS[(3)]	67.5	202.5	607.5	1822.5
PS_R[(4)]	67.5	202.5	607.5	1822.5
CONTROL	Zebrafish water only			

BIOASSAY 4

Solutions	*Concentrations (mg/L)*			
PMMA	7.5	12.5	17.5	22.5
PMMA_R	7.5	12.5	17.5	22.5
PS HA 1 mg/L	22.5	67.5	202.5	607.5
PS_R HA 1 mg/L	22.5	67.5	202.5	607.5

PS HA 10 mg/L	22.5	67.5	202.5	607.5
PS_R HA 10 mg/L	22.5	67.5	202.5	607.5
CONTROL	Zebrafish water only			
HA 1 mg/L	Zebrafish water with the concentration of HA mentioned only			
HA 10 mg/L	Zebrafish water with the concentration of HA mentioned only			

PMMA – Nanoparticles of polymethylmethacrylate without the lipophilic stain Nile Red.
PMMA_R – Nanoparticles of polymethylmethacrylate with the lipophilic stain Nile Red.
PS – Nanoparticles of polystyrene without the lipophilic stain Nile Red.
PS_R – Nanoparticles of polystyrene with the lipophilic stain Nile Red.

2.3.2.1.2. Fish Embryo Toxicity (FET)

In the embryo phase the following parameters were evaluated: (i) egg coagulation; (ii) lack of detachment of the tail-bud from the yolk sac; and (iii) lack of heart-beat. After hatching, in the larval phase the following parameters were evaluated: (i) heart-beat; (ii) oedemas; (iii) tail malformation; and (iv) larval behaviour. The observations were done under stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation).

2.3.2.1.3. Behavioural Test

A behavioural test was performed to evaluate the swimming behaviour of the zebrafish larvae. Abrupt changes from darkness to light, was selected as a stressing condition, to study the larvae response. In this test cycle of 2 minutes dark 2 minutes light (8 minutes total) was used. Fish have a startle response to this abrupt change, having an increase swimming activity, moving fast and erratic or they have the opposite response by freezing (Egan et al., 2009, Kalueff et al., 2013). To assess the active avoidance of the centre of the tank (thigmotaxis), two areas of the tank were defined as outside and inside (Figure 1). Fish movements were tracked in the inside and outside areas. The endpoints assessed in this test included total time spent (TT), total distance travelled (TD), percentage of total time spent in the outside area (TT%) and the percentage of total distance travelled in the outside area (TD%).

Swimming behaviour analysis was recorded using a Zebrabox (Viewpoint Life sciences, Lyon, France), equipment that is capable of tracking movement and record it, with automated video, both in light and darkness, since it has internal LED lights and infrared.

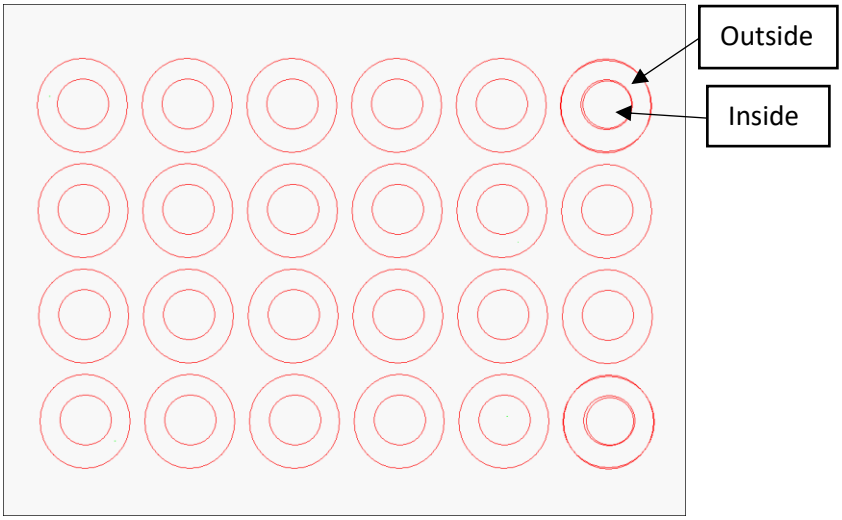


Figure 1 *- Example of a 24-well microtiter plates for the behavioural test.*

2.3.2.1.4. Biochemical Endpoints

A test was performed for collection of larvae for biomarkers analyses that lasted for 4 days. The concentrations used were the same as Bioassay 2, where zebrafish eggs were exposed to 22.5 mg/L, 31.5 mg/L, 40.5 mg/L, 49.5 mg/L, 58.5 mg/L and 67.5 mg/L of PMMA nanoparticles and 22.5 mg/L, 67.5 mg/L, 202.5 mg/L, 607.5 mg/L and 1822.5 mg/L of PS nanoparticles (Table 1). Each concentration was prepared in 6 petri dish with 20 eggs. After 96h of exposure, the 20 larvae were snap-frozen in 1.5 mL microtubes. The four highest concentrations used with PMMA and the highest concentration used with PS were skipped due to high mortality rates previously observed. Samples were stored at −80°C until enzymatic analysis.

Samples (pools of 20 larvae) were homogenized using a sonicator (Branson S-250A) in potassium phosphate buffer (0.1 M, pH 7.4). Two aliquots of the homogenate were collected for the lipid peroxidation and NPT assessment and the remaining centrifuged (Eppendorf 5810R) for 20 min, at 10000 g, at 4 ºC for post-mitochondrial fraction (PMS) isolation. PMS was used to assess CAT, GPX, GST and GR activities. Protein content of the homogenates and PMS fractions were determined by the Bradford method adapted to microplate reader (Thermo Scientific Multiskan Spectrum).

NPT levels

Samples of homogenate were used to assess NPT. To 150 µL of each sample, 150 µL of TCA 10% was added and allowed to incubate for 1h. Samples were centrifuged (4°C, 13400 g, 20 min) and the supernatant was removed and used quantification. The reaction mixture consisted of 230 µL of sodium phosphate buffer 0.1 M, pH=7.4, 20 µL of DTNB and 50 µL of each sample and absorbance was immediately read at 412 nm. (Oliveira et al., 2010b).

CAT activity

CAT activity was measured following the decrease of absorbance at 240 nm due to H_2O_2 (hydrogen peroxide) consumption. The reaction mixture consisted of 0.486 µL of 35% hydrogen peroxide (substrate), 249.514 µL of 0.05 M phosphate buffer, pH=7.0 and 100 µL of each sample. Enzymatic activities were determined in quadruplicate (Oliveira et al., 2010b).

GPx activity

GPx activity was measured at 340 nm during 15 min. The reaction mixture consisted of 200 µL of reaction solution (NaN_3 5 mM, GSH 18 mM, 0.9U/mL GR and phosphate buffer 0.05 M, pH=7.0), 10 µL of NADPH and 10 µL of each sample. Incubate for 5 min. Then add 10 µL of H_2H_2 and read the absorbance. GPx activity was determined in duplicate (Lima et al., 2007).

GST activity

GST activity was measured at 340 nm, each 20 seconds during 5 min. The reaction mixture consisted of 200 µL of reaction solution (CDNB solution 60 mM, GSH solution 10 mM and phosphate buffer 0.1 M, pH=6.5) and 50 µL of each sample. GST activity was determined in quadruplicate for the sea bass and in duplicate for the zebrafish larvae (Oliveira et al., 2015).

GR activity

GR activity of the samples of the liver, gills and intestine of adults of sea bass were measured at 340 nm. The reaction mixture consisted of 190 µL of phosphate buffer 0.1M, pH=7.2, 30 µL of GSSG and 50 µL of each sample. GR activity was determined in quadruplicate (Lima et al., 2007).

LPO levels

LPO levels were determined by the procedure of Ohkawa et al. (1979) adapted by Oliveira et al. (2015). Absorbance was measured at 535 nm and LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per mg of protein.

2.4. Sea Bass Experiments

2.4.1. Model Organism

The estuarine and marine environments are expected to be the ultimate recipients of environmental contaminants, including plastics. The European sea bass is an important species for Mediterranean aquaculture, since it makes up for around 20% of the total fish production (Carbone

et al., 2016). This species has been a model organism for several sorts of studies, such as genotoxic responses to environmental pollutants (Oliveira et al., 2010a), cellular toxicity due to nanomaterials contamination (Picchietti et al., 2017) and stress research (Tsalafouta et al., 2015).

Scientific and technical interest and knowledge about this species has increased since its aquaculture production and commercial importance increased throughout Europe (Louro et al., 2014).

Juvenile sea bass (*Dicentrarchus labrax*) organisms used in the present study, length 14.6 ± 2.4 cm, acquired from an aquaculture facility (Spain), were maintained acclimated for 4 weeks in 1000-L aquaria containing aerated and filtered artificial seawater (salinity, 34) at a 19°C temperature and natural photoperiod. During this period, the experimental fish were fed daily with commercial fish food.

2.4.2. Experimental Design

2.4.2.1. Effects of PMMA and PS nanoparticles in sea bass

2.4.2.1.1. Experiment Design

The procedures adopted in this experiment generally followed the OECD guidelines (1992) for fish acute bioassays. The experiment was carried out in 15-L aquaria, with 3 specimens per aquaria and 2 replicates per concentration, under the conditions described for the acclimation period. Fish were exposed for 96h, without feeding, with 75% medium renewal every 24h, to prevent significant nanoparticles deposition and to reduce the build-up of metabolic residues. Sea bass were exposed to 0.02, 0.2 and 2 mg/L of PMMA nanoparticles in Bioassay 1 and 0.02 and 20mg/L of PS nanoparticles alone or in the presence of 1 mg/L of HA in Bioassay 2 (Table 2).

After 96h exposure, the animals were anesthetized with tricaine MS-222 and a blood sample was collected from the posterior cardinal vein. Liver, gills and intestine were sampled for biochemical endpoints assessment. Samples were stored at −80°C.

Blood smears were prepared for the assessment of micronucleus and other erythrocytic nuclear abnormalities.

Table 2 - *Experiment design for sea bass assays.*

BIOASSAY 1

SOLUTIONS	*Concentrations (mg/L)*		
PMMA	0.02	0.2	2
CONTROL	Seawater only		

BIOASSAY 2

SOLUTIONS	*Concentrations (mg/L)*	
PS	0.02	20
CONTROL	Seawater only	
HA 1 mg/L	Seawater with the concentration of HA mentioned only	

2.4.2.1.2. Erythrocyte Abnormalities Assay

Blood smears were immediately performed for the assessment of micronucleus and other erythrocytic nuclear abnormalities. After fixation in methanol for 15 min, slides were left to air-dry. After, they were Giemsa stained at a concentration of 5%, for 30 min. One thousand erythrocyte cells with complete cytoplasm were scored per fish for cellular abnormalities analysis. Several types of cellular abnormalities visualized: (i) binuclei; (ii) nuclear bud; (iii) lobed nuclei; (iv) notched nuclei; and (v) shape abnormalities.

2.4.2.1.3. Biochemical Endpoints

Samples (liver, gills or intestine) were homogenized using a sonicator in potassium phosphate buffer (0.1 M, pH 7.4). Two aliquots of the homogenate were collected for the lipid peroxidation and NPT assessment and the remaining centrifuged for 20 min, at 10000 g, at 4 ºC for PMS isolation. PMS was used to assess CAT, GPX, GST and GR activities. Protein content of the homogenates and PMS fractions were determined by the Bradford method adapted to microplate reader. The biochemical endpoints were assessed following the methodologies describe in section 2.3.2.1.4.

2.5. Statistical Analysis

Results were expressed as means ± SE (standard error) corresponding to the experimental groups. Statistical data analysis was done using Statistical software (SigmaPlot 12.5). Assumptions of normality and homogeneity of data were verified. One-way ANOVA was performed in order to assess significant effects, followed by post-hoc Tukey test to signal significant differences between groups. The significance of results was ascertained at $\alpha = 0.05$.

Results

3.1. Nanoparticles Characterization

Nanoparticles were characterized by DLS in ultra-pure water, zebrafish water with and without HA and seawater with and without HA, after 1h of the preparation of the dispersions at 25°C. Average particle size of polymerized microemulsion was determined using SEM, TEM and STEM, with uranyl acetate as staining reagent (Figures 1-4).

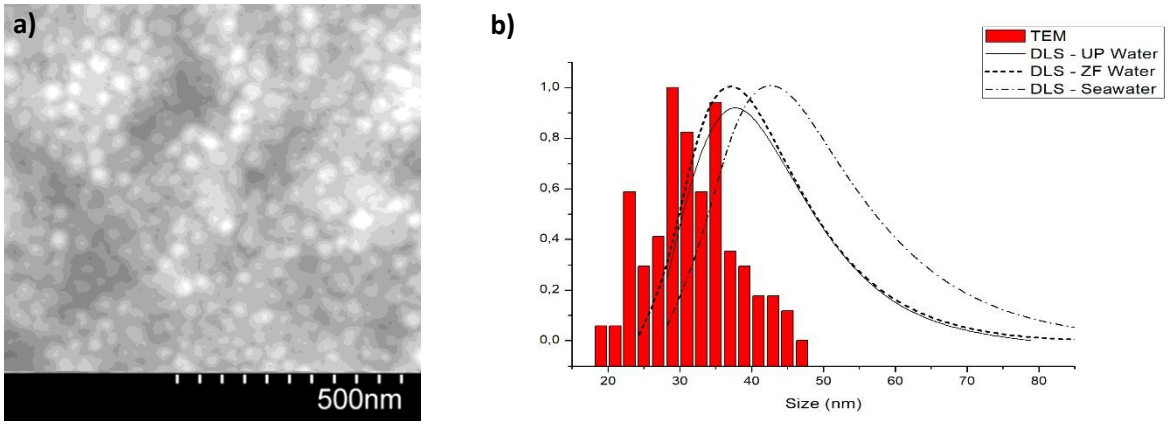


Figure 2 *- Characterization of PMMA nanoparticles. a) TEM image with uranyl acetate as staining reagent. b) Size comparison between measurements from the TEM image using ImageJ and the size measures obtained from DLS.*

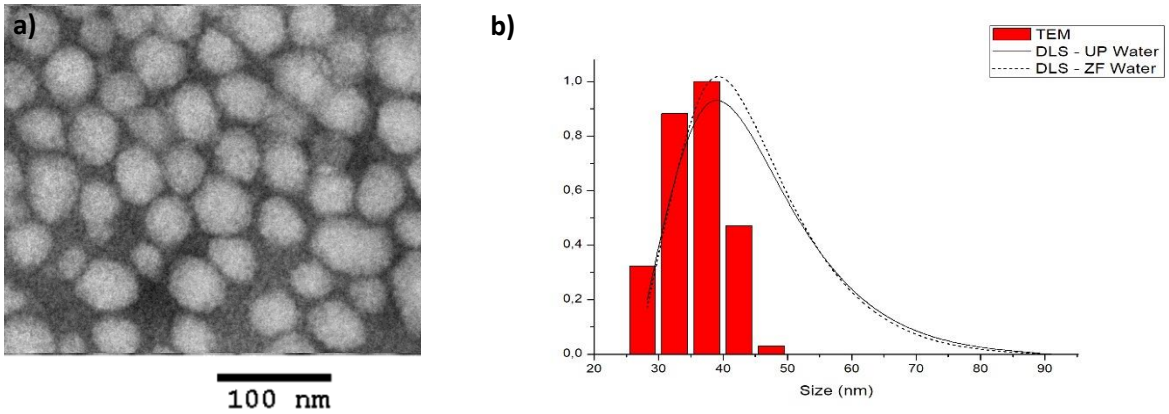


Figure 3 *- Characterization of PMMA nanoparticles with the lipophilic stain Nile Red. a) TEM image with uranyl acetate as staining reagent. b) Size comparison between measurements from the TEM image using ImageJ and the size measures obtained from DLS.*

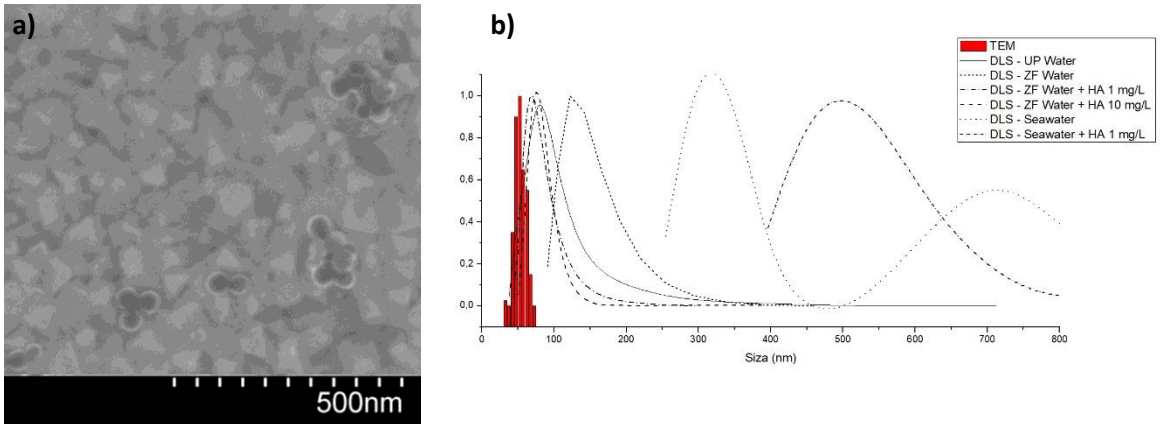


Figure 4 *- Characterization of PS nanoparticles. a) SEM image with uranyl acetate as staining reagent. b) Size comparison between measurements from the SEM image using ImageJ and the size measures obtained from DLS.*

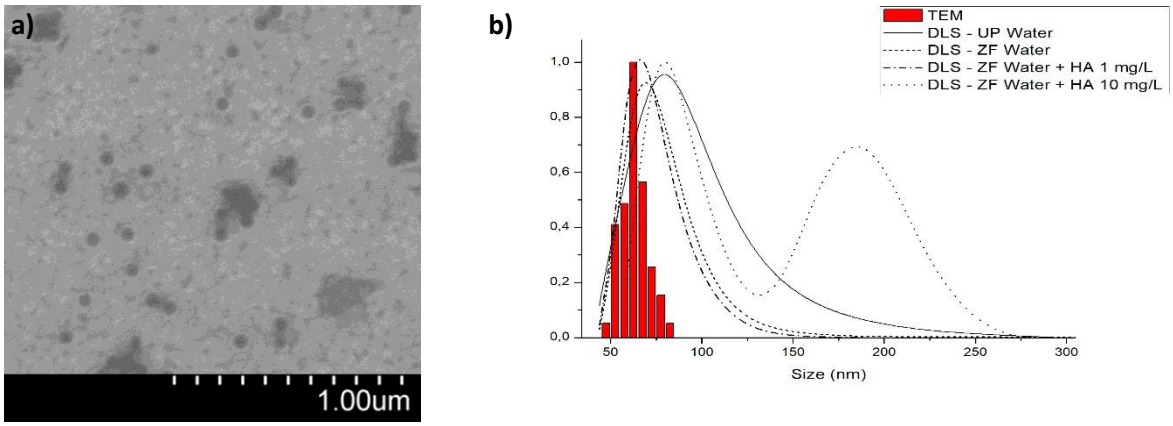


Figure 5 *- Characterization of PMMA nanoparticles with the lipophilic stain Nile Red. a) SEM image with uranyl acetate as staining reagent. b) Size comparison between measurements from the SEM image using ImageJ and the size measures obtained from DLS.*

According to microscopic analysis, PMMA nanoparticles do not have a uniform circular shape (Figure 2a and 3a). PMMA nanoparticles have the smallest size, with an average size of 32 nm (SEM), but from the DLS measurement, they had an average size of 40 nm in ultra-pure water and zebrafish water. PMMA nanoparticles presented a bigger size when in seawater. PMMA nanoparticles stained with the lipophilic stain Nile Red have an average size of 36 nm in ultra-pure water (TEM). From the DLS measurement they had an average size of 42 nm, when measured in ultra-pure water and zebrafish water (Figure 2b and 3b). The zeta potential of the PMMA nanoparticles in ultra-pure water was -26.4 mV, whereas the zeta potential of the PMMA nanoparticles stained with the lipophilic stain Nile Red in ultra-pure water was -31.5 mV.

PS nanoparticles have a circular shape, after observing the microscopic images (Figure 4a and 5a). PS nanoparticles have an average size of 53 nm in ultra-pure water (SEM). From the DLS

measurement, they had an average size of 115 nm, that increased greatly when in seawater and zebrafish water, having shown a smaller size when in the presence of 1 mg/L of HA. The PS nanoparticles stained with the lipophilic stain Nile Red have an average size of 63 nm in ultra-pure water (SEM). From the DLS measurement, they had an average size of 93 nm in ultra-pure water, that increased when the nanoparticles were in the seawater and zebrafish water, presenting a smaller size when HA are present, at 1 mg/L (Figure 4b and 5b). The zeta potential of PS nanoparticles in ultra-pure water was -23.8 mV, whereas the zeta potential of PS nanoparticles stained with the lipophilic stain Nile Red in ultra-pure water was -26.8 mV.

3.2. Zebrafish Experiments

3.2.1. Bioassay 1

3.2.1.1. FET

Table 3 *- FET test with PMMA nanoparticles in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; presence of a pericardial edema.*

		Dead		Hatched		Unhatched		Tail Malformation		Pericardial Edema	
	mg/L	%		%		%		%		%	
24h	Control	4%	*	0%		96%		0%	*	0%	*
	2.5	5%		0%		95%		0%		0%	
	7.5	5%		0%		95%		0%		0%	
	22.5	5%	*	0%		95%		5%		0%	
	67.5	65%		0%		35%		20%		25%	
	202.5	100%		0%		0%		0%		0%	
48h	Control	4%	*	0%		96%		0%		0%	*
	2.5	5%		0%		95%		0%		0%	
	7.5	5%		5%		90%		0%		0%	
	22.5	5%	*	0%		95%		5%		20%	
	67.5	100%		0%		0%		0%		0%	
	202.5	100%		0%		0%		0%		0%	
72h	Control	4%	*	96%		0%	*	8%		0%	*
	2.5	5%		95%		0%		10%		0%	
	7.5	5%		70%		25%		0%		0%	
	22.5	5%	*	80%		15%		20%		15%	
	67.5	100%		0%		0%		0%		0%	
	202.5	100%		0%		0%		0%		0%	
96h	Control	4%	*	96%		0%		0%	*	0%	
	2.5	5%		95%		0%		0%		0%	
	7.5	5%		90%		5%		5%		5%	
	22.5	10%	*	90%		0%		30%		15%	
	67.5	100%		0%		0%		0%		0%	
	202.5	100%		0%		0%		0%		0%	

PMMA nanoparticles affected more the zebrafish embryos at 67.5 and 202.5 mg/L as demonstrated by the mortality found after 24h of exposure, when most organisms were dead. These nanoparticles also delayed the development of the tail at 67.5 mg/L at an early stage and induced a malformation of the tail at 96h in the animals exposed to 22.5 mg/L. There was a

significant number of organisms that presented a pericardial edema at 67.5 mg/L after 24h until the 72h exposure at the concentration 22.5 mg/L (Table 3).

Table 4 *- FET test with PS nanoparticles in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; e) presence of a pericardial edema.*

		Dead		Hatched		Unhatched		Tail Malformation		Pericardial Edema	
	mg/L	%		%		%		%		%	
24h	Control	4%		0%		96%		0%		0%	
	2.5	0%		0%		100%		0%		0%	
	7.5	0%		0%		100%		0%		0%	
	22.5	0%		0%		100%		0%		0%	
	67.5	0%		0%		100%		0%		0%	
	202.5	0%		0%		100%		0%		0%	
48h	Control	4%		0%		96%		0%		0%	
	2.5	0%		0%		100%		0%		0%	
	7.5	5%		10%		85%		0%		5%	
	22.5	0%	*	5%		95%		0%		0%	
	67.5	0%		0%		100%		0%		0%	
	202.5	50%		0%		50%		0%		5%	
72h	Control	4%		96%		0%		8%		0%	
	2.5	5%		95%		0%		10%		10%	*
	7.5	5%		90%		5%		25%		30%	
	22.5	0%	*	100%		0%		5%		0%	
	67.5	0%		100%		0%		0%		0%	
	202.5	50%		50%		0%		20%		0%	
96h	Control	4%		96%		0%		0%		0%	
	2.5	5%		95%		0%		25%		0%	
	7.5	5%		90%		5%		15%		15%	
	22.5	0%	*	100%		0%		5%		5%	
	67.5	0%		100%		0%		0%		5%	
	202.5	50%		50%		0%		15%		15%	

PS nanoparticles affected more the zebrafish embryos at the highest concentration, 202.5 mg/L, since half of the organisms were dead by the end of the test. There was a significant number of organisms that presented a pericardial edema at 7.5 mg/L after 72h (Table 4).

3.2.1.2. Swimming Behaviour

- PMMA Exposure

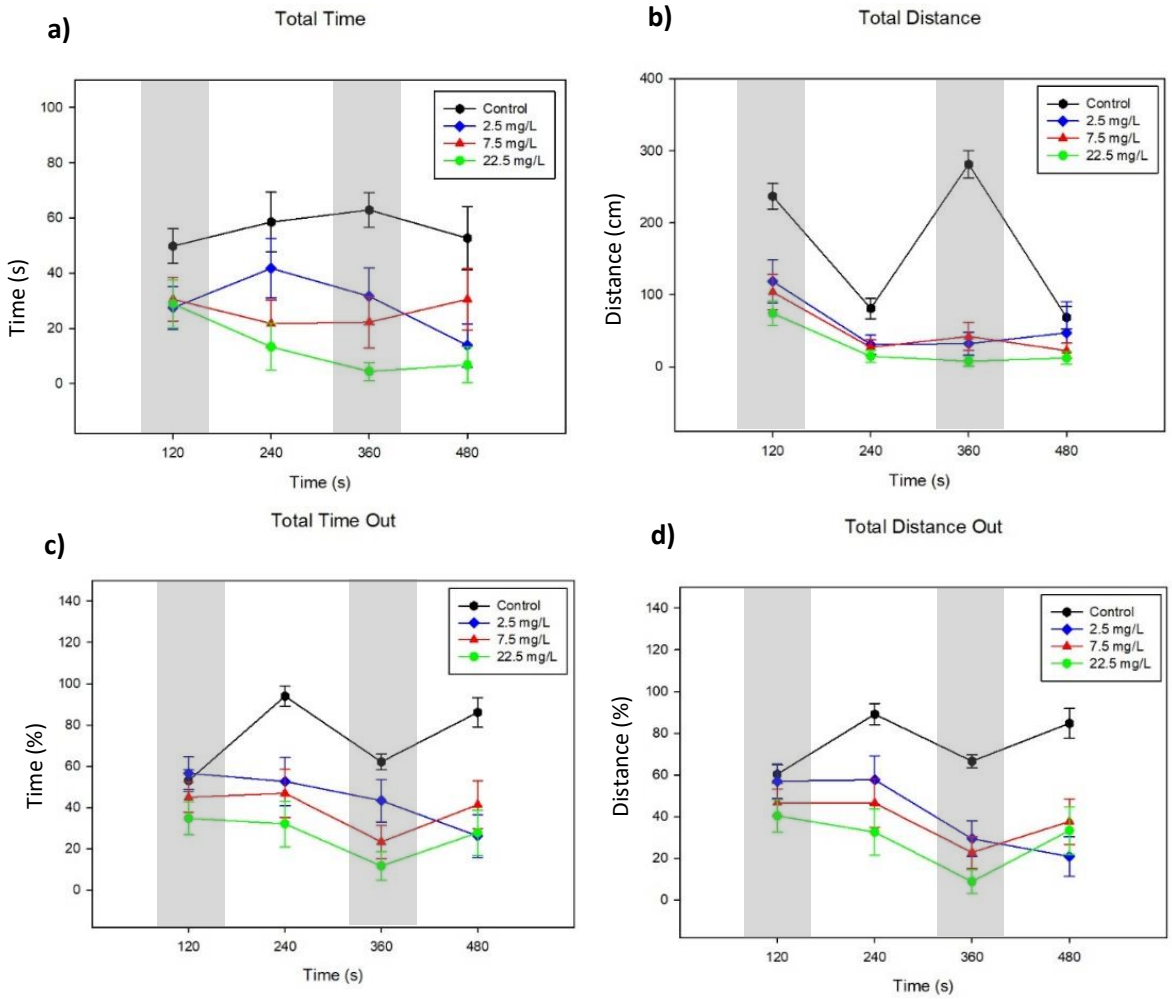


Figure 6 *- Behavioural analysis after 96h exposure to PMMA nanoparticles: a) TT; b) TD; c) TT%; and d) TD%. The total time of the experiment was eight minutes (two minutes dark fallowed by two minutes light, represented by grey and white background, respectively). Each dot represents mean distance swam by zebrafish larvae of each concentration throughout two minutes of experiment and respective standard error. Each symbol and colour represent one concentration group.*

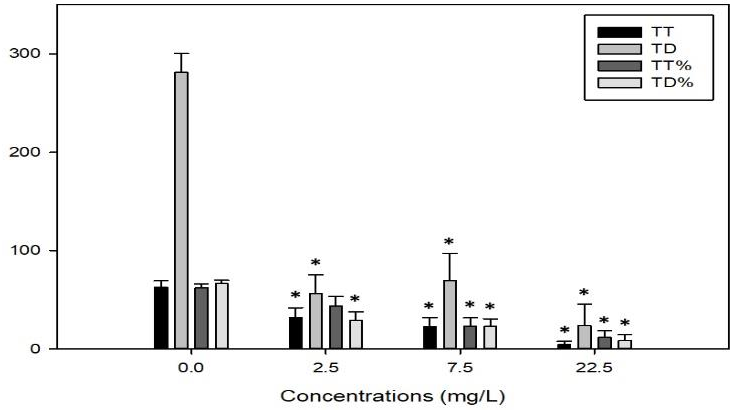


Figure 7 *- Behaviour analysis of zebrafish larvae exposed for 96h to PMMA nanoparticles at the sixth minute of the dark/light cycle stimulus.*

Regarding the swimming behaviour, there were decreases in all parameters (TT, TD, TT% and TD%) compared to the control and 2.5, 7.5 and 22.5 mg/L of PMMA concentrations, being the most significant difference during the dark period, where the zebrafish larvae exposed to PMMA nanoparticles present a decreased activity (Figure 6 a and b). Also, thigmotactic effects, were assessed by calculating the TD% (Figure 6 d) to evaluate the tendency of the larvae to swim at the edges of the wells. Fish exposed to nanoparticles displayed more accentuated differences (decreases) to control in terms of TT, TD, TT% and TD% at 360 seconds, except of TT% at 2.5 mg/L as seen in Figure 7.

- PS Exposure

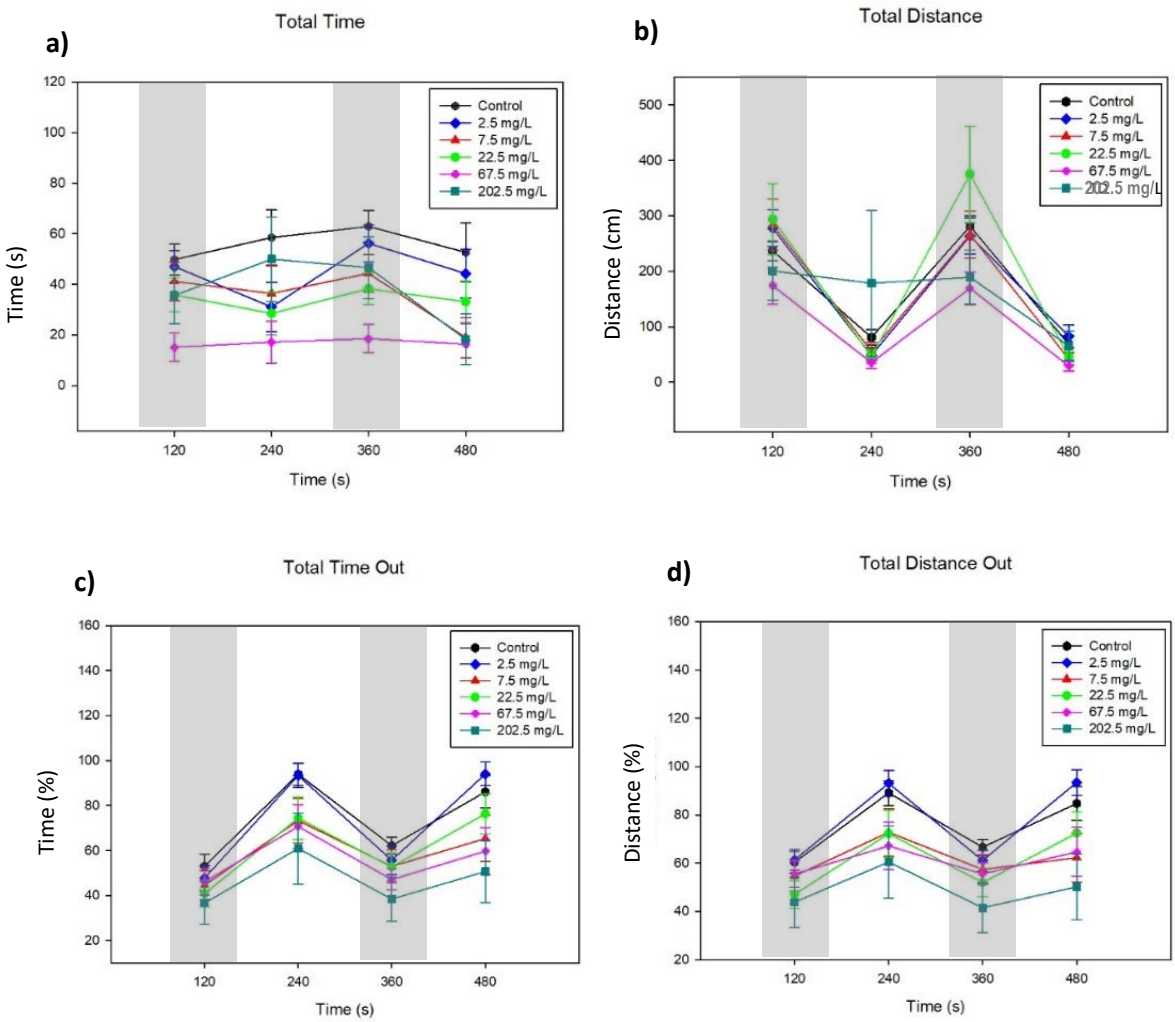


Figure 8 *- Behavioural analysis after 96h exposure to PS nanoparticles: a) TT; b) TD; c) TT%; and d) TD%. The total time of the experiment was eight minutes (two minutes dark fallowed by two minutes light, represented by grey and white background, respectively). Each dot represents mean distance swam by zebrafish larvae of each concentration throughout two minutes of experiment and respective standard error. Each symbol and colour represent one concentration group.*

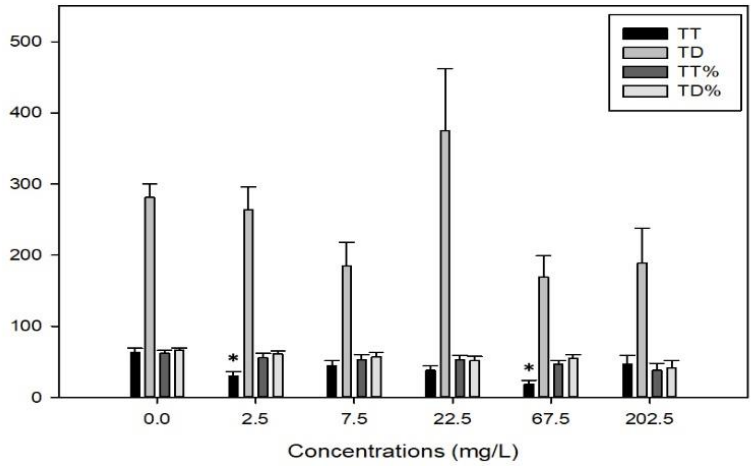


Figure 9 *- Behaviour analysis of zebrafish larvae exposed for 96h to PS nanoparticles at the sixth minute of the dark/light cycle stimulus.*

Concerning the swimming behaviour of larvae exposed to PS nanoparticles, there were small decreases at TT, TT% and TD%, compared to the control at 2.5, 7.5, 22.5, 67.5 and 202.5 mg/L of PS concentrations, whereas 22.5 mg/L has an increase in TD, when compared to the control, being the most significant difference during the dark period (Figure 8a and b). Also, thigmotactic effects were assessed by calculating the TD% (Figure 8 d) to evaluate the tendency of the larvae to swim at the edges of the wells. Fish from the different experimental conditions displayed differences (decreases) to control in terms of TT at 2.5 mg/L and 67.5 mg/L of PS nanoparticles at 360 seconds, as seen in Figure 9.

3.2.2. Bioassay 2

3.2.2.1. FET

Table 5 *- FET test with PMMA nanoparticles in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; presence of a pericardial edema.*

		Dead	Hatched	Unhatched	Tail Malformation	Pericardial Edema
	mg/L	%	%	%	%	%
24h	Control	4%	0%	96%	0%	0%
	22.5	15%	10%	75%	0%	5%
	31.5	20%	0%	80%	0%	0%
	40.5	85%	0%	15%	5%	5%
	49.5	100%	0%	0%	0%	0%
	58.5	85%	0%	15%	5%	5%
	67.5	95%	0%	5%	5%	5%
48h	Control	4%	13%	88%	4%	4%
	22.5	15%	10%	75%	0%	0%
	31.5	20%	0%	80%	5%	5%
	40.5	90%	0%	10%	10%	10%
	49.5	100%	0%	0%	0%	0%
	58.5	90%	0%	10%	0%	5%
	67.5	100%	0%	0%	0%	0%
72h	Control	4%	96%	0%	4%	4%
	22.5	15%	80%	5%	5%	10%
	31.5	20%	75%	5%	10%	30%
	40.5	90%	10%	0%	10%	10%
	49.5	100%	0%	0%	0%	0%
	58.5	90%	10%	0%	5%	5%
	67.5	100%	0%	0%	0%	0%
96h	Control	8%	92%	0%	8%	4%
	22.5	15%	85%	0%	5%	5%
	31.5	20%	80%	0%	30%	5%
	40.5	90%	10%	0%	10%	5%
	49.5	100%	0%	0%	0%	0%
	58.5	90%	10%	0%	5%	5%
	67.5	100%	0%	0%	0%	0%

PMMA nanoparticles affected more the zebrafish embryos at 40.5, 49.5, 58.5 and 67.5 mg/L. After 24h of exposure, most organisms were dead in those conditions. There was a significant number of organisms that presented a pericardial edema at 31.5 mg/L after 72h (Table 5).

Table 6 *- FET test with PS nanoparticles in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; presence of a pericardial edema.*

		Dead		Hatched		Unhatched		Tail Malformations		Pericardial Edema	
	mg/L	%		%		%		%		%	
24h	Control	4%	*	0%		96%		0%		0%	
	22.5	5%		0%		95%		0%		0%	
	67.5	15%		0%		85%		0%		0%	
	202.5	20%		0%		80%		0%		5%	
	607.5	5%		0%		95%		0%		5%	
	1822.5	65%		0%		35%		0%		10%	
48h	Control	4%	*	13%		83%		0%		0%	
	22.5	5%		55%		40%		0%		10%	
	67.5	20%		20%		60%		0%		0%	
	202.5	20%		30%		50%		0%		0%	
	607.5	5%		20%		75%		0%		15%	
	1822.5	65%		5%		30%		0%		0%	
72h	Control	4%	*	96%		0%		13%		4%	*
	22.5	10%		90%		0%		0%		5%	
	67.5	25%		75%		0%		10%		0%	
	202.5	25%		75%		0%		0%		0%	
	607.5	10%		90%		0%		0%		25%	
	1822.5	70%		15%		15%		0%		5%	
96h	Control	8%	*	92%		0%		8%		4%	
	22.5	20%		80%		0%		15%		10%	
	67.5	25%		75%		0%		10%		5%	
	202.5	25%		75%		0%		5%		0%	
	607.5	10%		90%		0%		5%		0%	
	1822.5	80%		15%		5%		0%		0%	

PS nanoparticles affected more the zebrafish embryos at the highest concentration, 1822.5 mg/L, since a significant number of the organisms were dead by the end of the test. There was a significant number of organisms that presented a pericardial edema at 607.5 mg/L after 72h of exposure.

3.2.2.2. Swimming Behaviour

- PMMA Exposure

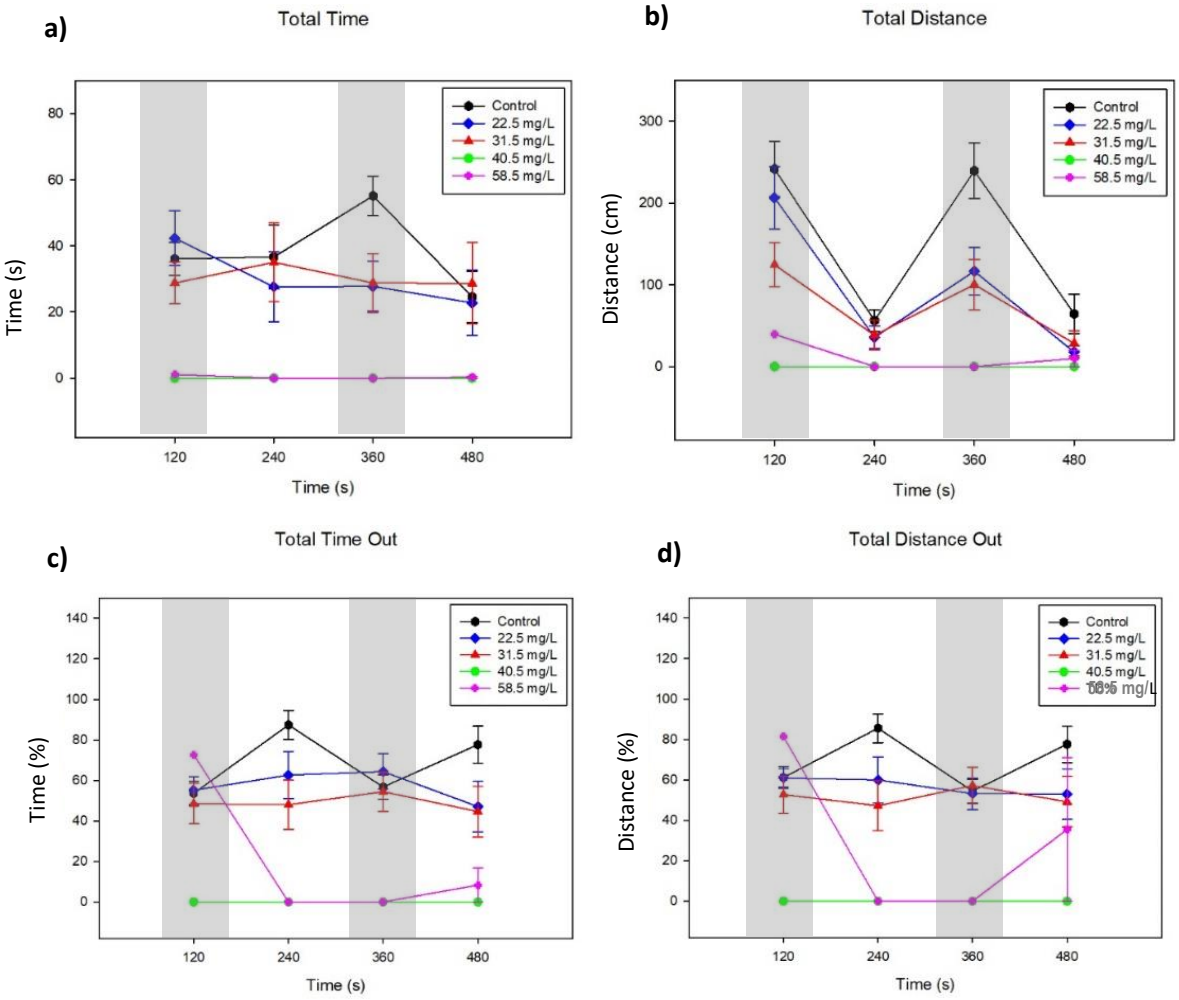


Figure 10 *- Behavioural analysis after 96h exposure to PMMA nanoparticles: a) TT; b) TD; c) TT%; and d) TD%. The total time of the experiment was eight minutes (two minutes dark fallowed by two minutes light, represented by grey and white background, respectively). Each dot represents mean distance swam by zebrafish larvae of each concentration throughout two minutes of experiment and respective standard error. Each symbol and colour represent one concentration group.*

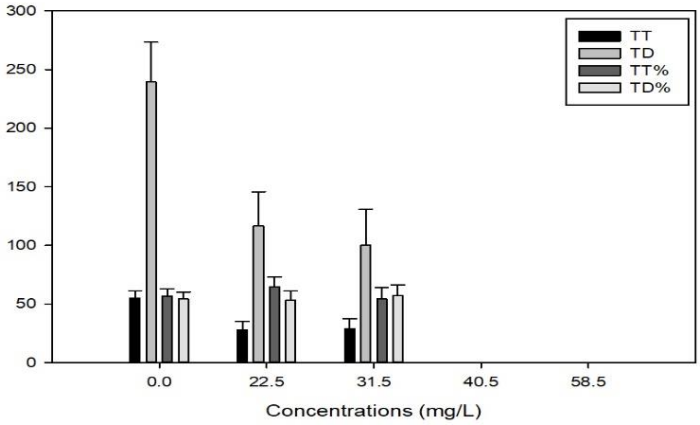


Figure 11 *- Behaviour analysis of zebrafish larvae exposed for 96h to PMMA nanoparticles at the sixth minute of the dark/light cycle stimulus.*

Regarding the swimming behaviour, there were decreases in all parameters (TT, TD, TT% and TD%) compared to the control and 2.5, 31.5 and 40.5 mg/L of PMMA concentrations, being the most significant difference during the dark period, where the zebrafish larvae exposed to PMMA nanoparticles present a decreased activity (Figure 10 a and b). Also, thigmotactic effects, were assessed by calculating the TD% (Figure 10 d). Fish exposed to PMMA nanoparticles displayed no significant difference, as seen in Figure 11.

- PS Exposure

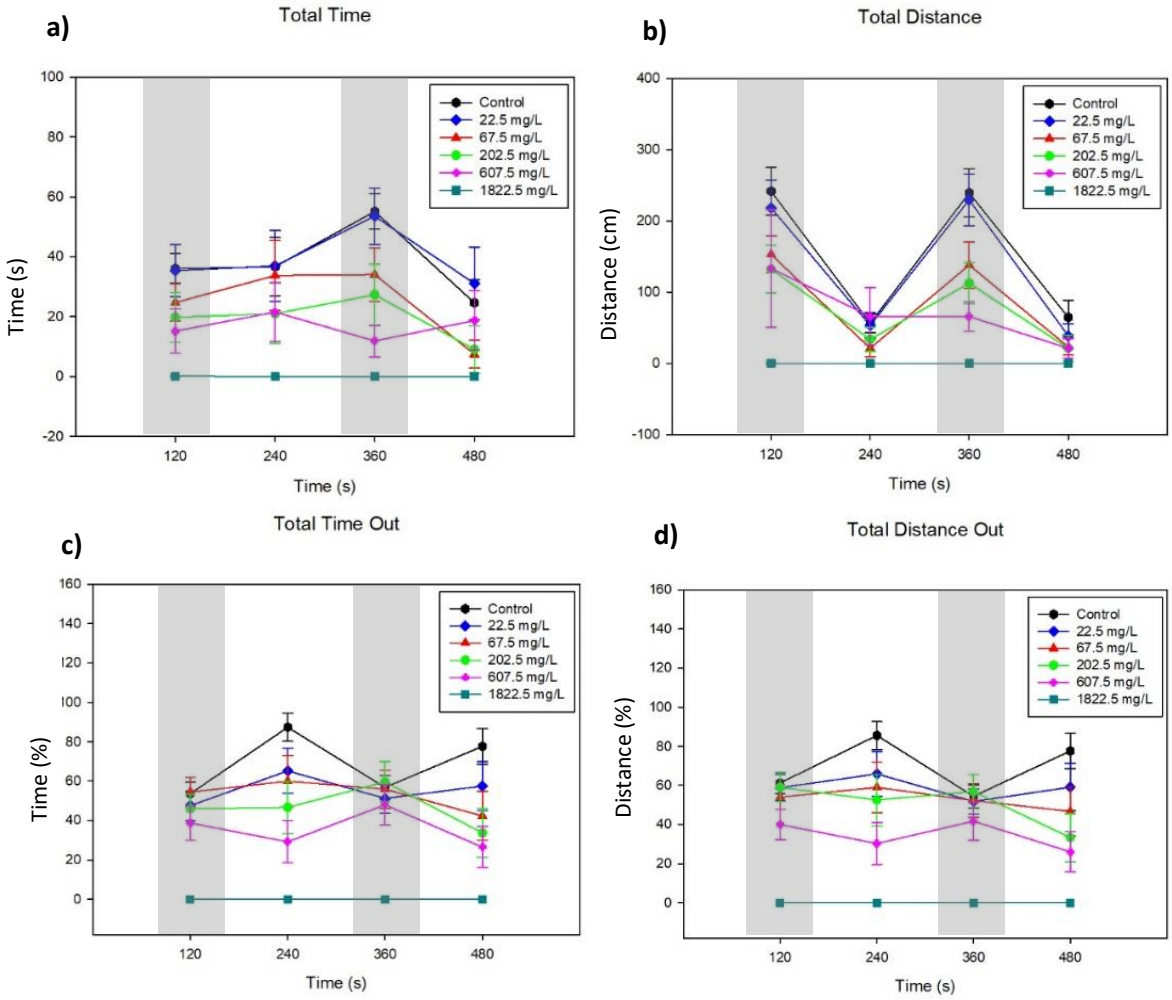


Figure 12 *- Behavioural analysis after 96h exposure to PS nanoparticles: a) TT; b) TD; c) TT%; and d) TD%. The total time of the experiment was eight minutes (two minutes dark fallowed by two minutes light, represented by grey and white background, respectively). Each dot represents mean distance swam by zebrafish larvae of each concentration throughout two minutes of experiment and respective standard error. Each symbol and colour represent one concentration group.*

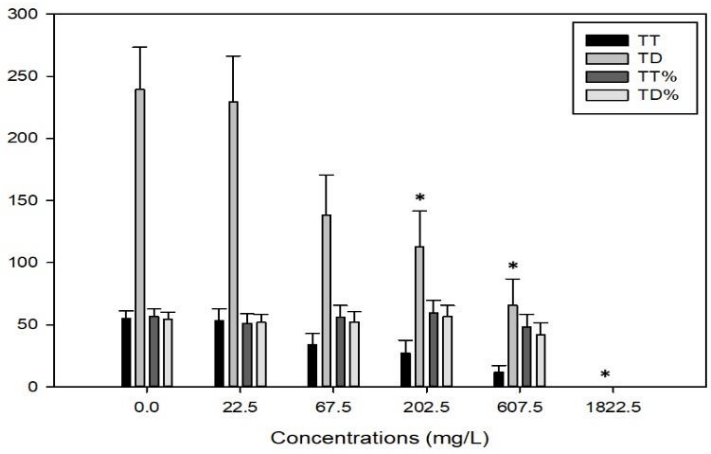


Figure 13 *- Behaviour analysis of zebrafish larvae exposed for 96h to PS nanoparticles at the sixth minute of the dark/light cycle stimulus.*

Zebrafish larvae exposed to PS concentrations, presented a decreased TT and TD swam when compared to the control (Figure 12 a and b). Calculating the TD% to assess thigmotactic effects (Figure 12 d), revealed there were significant differences (decreases) to control in terms of TD at 202.5, 607.5 and 1822.5 mg/L of PS nanoparticles at 360 seconds, as seen in Figure 13.

3.2.3. Bioassay 3

3.2.3.1. FET

Table 7 *- FET test with PMMA nanoparticles in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; presence of a pericardial edema.*

		Dead		Hatched		Unhatched		Tail Malformations		Pericardial Edema	
	mg/L	%		%		%		%		%	
24h	Control	4%		0%		96%		0%		0%	
	22.5	100%	* *	0%		0%		0%		0%	
	31.5	90%	*	0%		10%		10%		10%	
	40.5	95%		0%		5%		5%		5%	
48h	Control	4%		0%		96%		0%		0%	
	22.5	100%	* *	0%		0%		0%		0%	
	31.5	100%	*	0%		0%		0%		0%	
	40.5	95%		0%		5%		5%		5%	
72h	Control	4%		96%		0%		8%		4%	
	22.5	100%	* *	0%		0%		10%		0%	
	31.5	100%	*	0%		0%		0%		0%	
	40.5	100%		0%		0%		20%		0%	
96h	Control	4%		96%		0%		13%		4%	
	22.5	100%	* *	0%		0%		0%		0%	
	31.5	100%	*	0%		0%		0%		0%	
	40.5	100%		0%		0%		0%		0%	

PMMA nanoparticles are highly toxic since all the zebrafish embryos exposed to 22.5, 31.5 and 40.5 mg/L were all dead after 72h of exposure (Table 7).

PMMA nanoparticles stained with the lipophilic stain Nile Red caused 100% of mortality, even at the smallest concentrations, making these nanoparticles the ones with the highest levels of toxicity.

Table 8 *- FET test with PS nanoparticles in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; presence of a pericardial edema.*

		Dead		Hatched		Unhatched		Tail Malformations		Pericardial Edema	
	mg/L	%		%		%		%		%	
24h	Control	4%	*	0%		96%		0%		0%	
	67.5	5%		0%		95%		0%		0%	
	202.5	10%		0%		90%		0%		0%	
	607.5	10%		0%		90%		0%		0%	
	1822.5	100%		0%		0%		0%		0%	
48h	Control	4%	*	0%		96%		0%		0%	
	67.5	10%		5%		85%		0%		0%	
	202.5	10%		10%		80%		0%		5%	
	607.5	10%		15%		75%		0%		0%	
	1822.5	100%		0%		0%		0%		0%	
72h	Control	4%	*	96%	*	0%	*	8%		4%	
	67.5	10%		90%		0%		5%		5%	
	202.5	10%		85%		5%		10%		15%	
	607.5	25%		15%		60%		0%		0%	
	1822.5	100%		0%		0%		0%		0%	
96h	Control	4%	*	96%		0%		13%		4%	*
	67.5	10%		85%		5%		10%		5%	
	202.5	15%		75%		10%		15%		5%	
	607.5	25%		75%		0%		15%		25%	
	1822.5	100%		0%		0%		0%		0%	

PS nanoparticles affected more the zebrafish embryos at the highest concentration, 1822.5 mg/L, since all the organisms were dead by the end of the test. After 72h of exposure, at 607.5 mg/L some larvae presented a delay in hatching compared to the control. There was a significant number of organisms that presented a pericardial edema at 607.5 mg/L after 96h of exposure (Table 8).

Table 9 *- FET test with PS nanoparticles with the lipophilic stain Nile Red in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; presence of a pericardial edema.*

		Dead		Hatched		Unhatched		Tail Malformations		Pericardial Edema	
	mg/L	%		%		%		%		%	
24h	Control	4%		0%		96%		0%		0%	
	67.5	20%		0%		80%		0%		0%	
	202.5	5%		0%		95%		5%		5%	
	607.5	0%		0%		100%		0%		0%	
	1822.5	0%		0%		100%		0%		0%	
48h	Control	4%		0%		96%		0%		0%	
	67.5	20%		0%		80%		0%		5%	
	202.5	5%		0%		95%		0%		0%	
	607.5	0%		0%		100%		0%		0%	
	1822.5	0%		0%		100%		0%		0%	
72h	Control	4%		96%		0%		8%		4%	
	67.5	20%		15%		65%		5%		0%	
	202.5	5%		30%		65%	*	5%		0%	
	607.5	0%		25%		75%		0%		0%	
	1822.5	0%		20%		80%		0%		0%	
96h	Control	4%		96%		0%		13%		4%	
	67.5	20%		75%	*	5%	*	0%		5%	
	202.5	5%		90%	*	5%	*	5%		0%	
	607.5	0%		100%		0%		25%		5%	
	1822.5	0%		100%		0%		20%		5%	

PS nanoparticles stained with the lipophilic stain Nile Red affected more the zebrafish embryos at 202.5 mg/L and 607.5 mg/L, since a significant number of the presented a delay in hatching after 72h and 96h of exposure (Table 9).

3.2.3.2. Swimming Behaviour

- PS Exposure

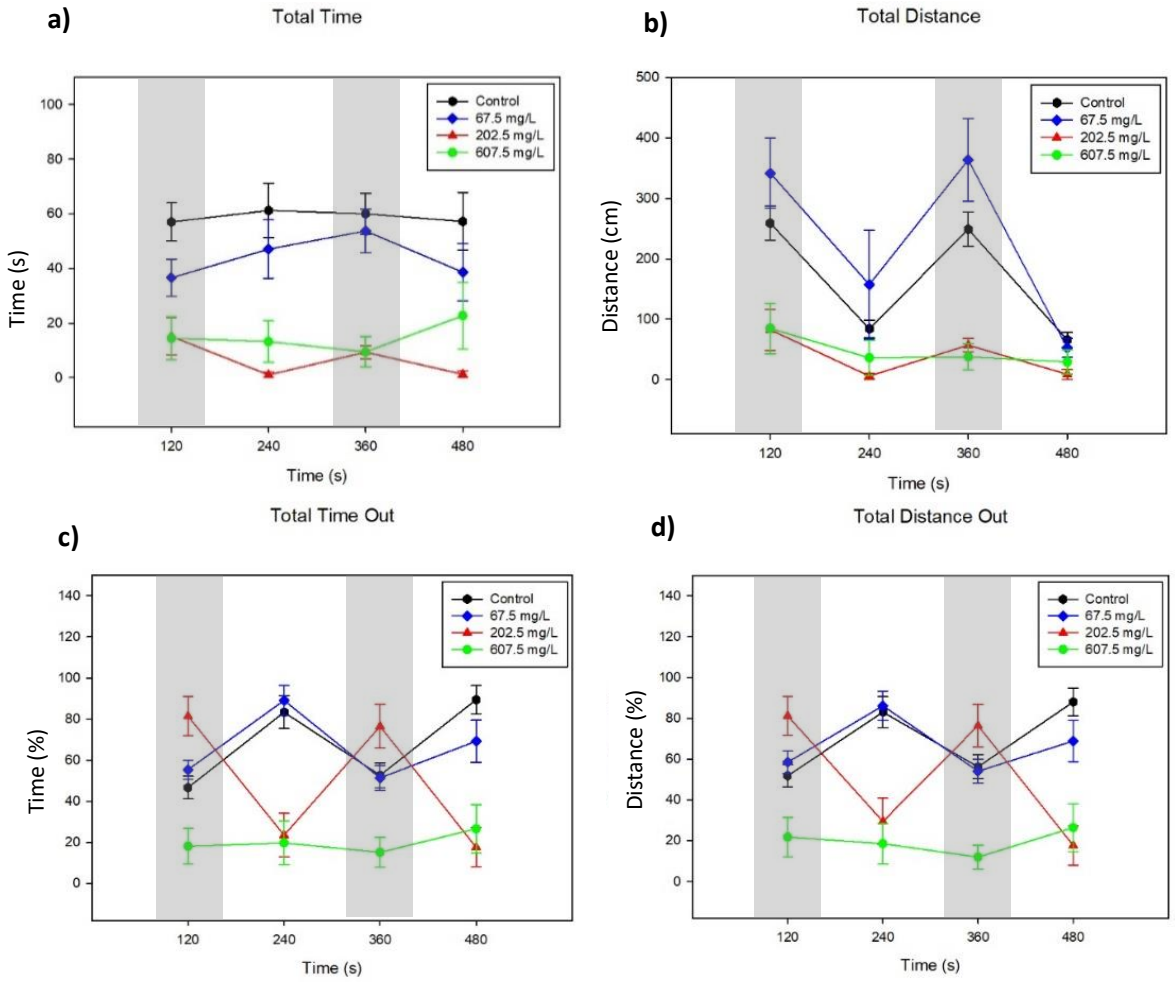


Figure 14 *- Behavioural analysis after 96h exposure to PS nanoparticles: a) TT; b) TD; c) TT%; and d) TD%. The total time of the experiment was eight minutes (two minutes dark fallowed by two minutes light, represented by grey and white background, respectively). Each dot represents mean distance swam by zebrafish larvae of each concentration throughout two minutes of experiment and respective standard error. Each symbol and colour represent one concentration group.*

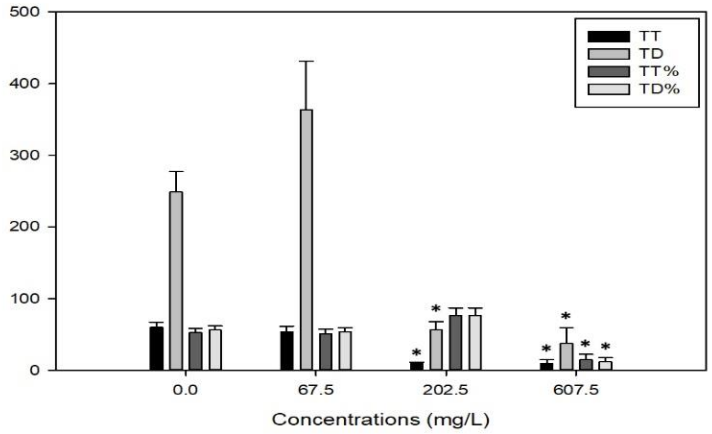


Figure 15 *- Behaviour analysis of zebrafish larvae exposed for 96h to PS nanoparticles at the sixth minute of the dark/light cycle stimulus.*

Larvae exposed to PS concentrations, presented a decreased TT and TD swam when compared to the control, except for 67.5 mg/L which presented an increase TD swam (Figure 14 a and b). Calculating the TD% to assess thigmotactic effects (Figure 14 d), revealed there were significant differences (decreases) to control in terms of TT and TD at 202.5, 607.5 mg/L and in terms of TT% and TD% at 607.5 mg/L of PS nanoparticles at 360 seconds, as seen in Figure 15.

- PS stained with the lipophilic stain Nile Red Exposure

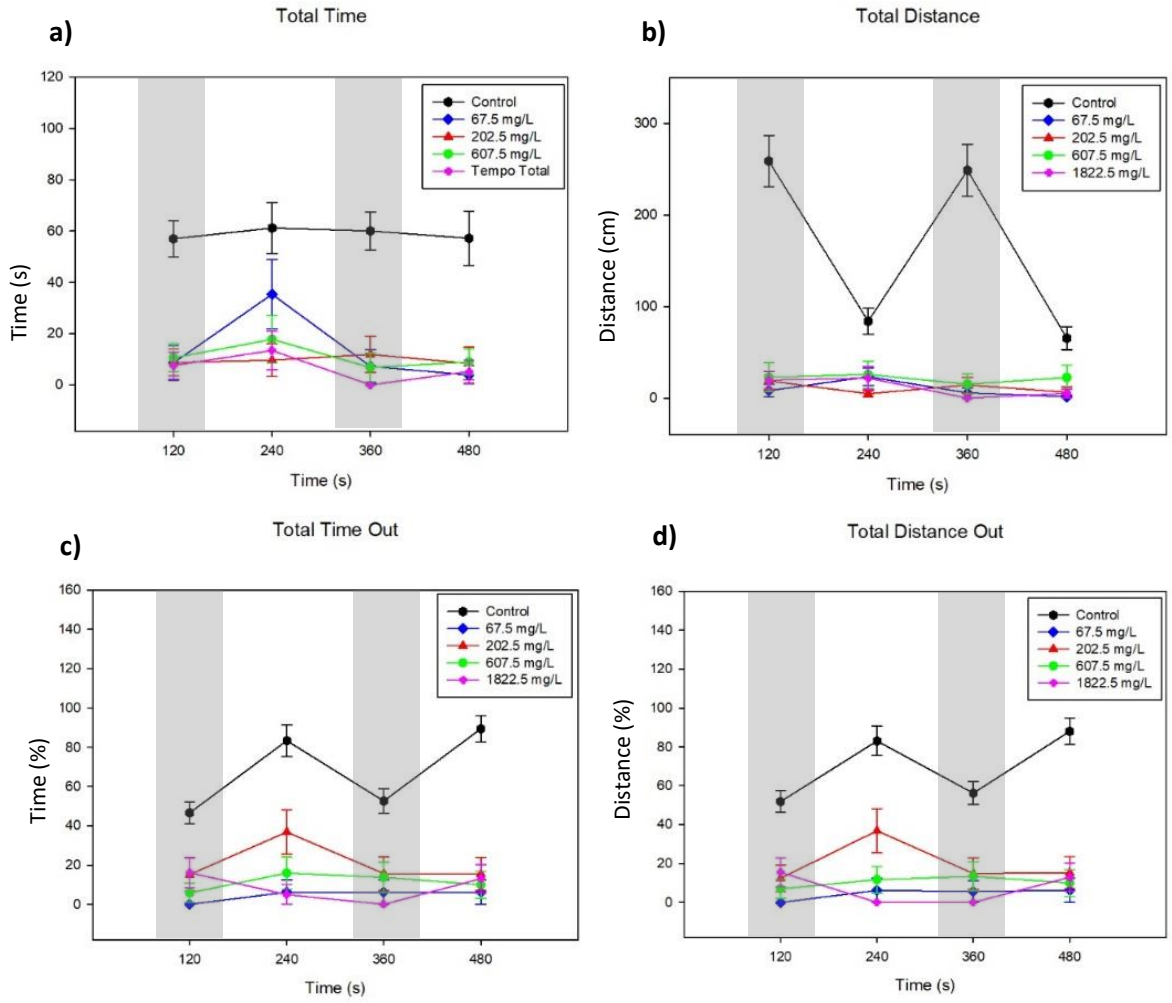


Figure 16 *- Behavioural analysis after 96h exposure to PS nanoparticles stained with the lipophilic stain Nile Red: a) TT; b) TD; c) TT%; and d) TD%. The total time of the experiment was eight minutes (two minutes dark fallowed by two minutes light, represented by grey and white background, respectively). Each dot represents mean distance swam by zebrafish larvae of each concentration throughout two minutes of experiment and respective standard error. Each symbol and colour represent one concentration group.*

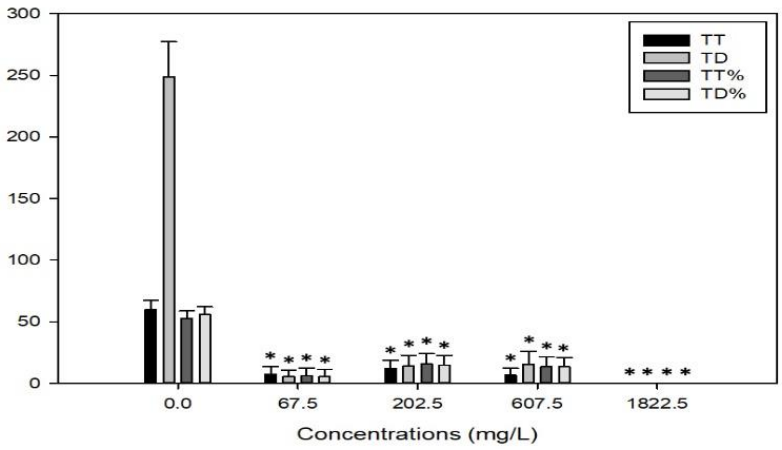


Figure 17 *- Behaviour analysis of zebrafish larvae exposed for 96h PS nanoparticles stained with the lipophilic stain Nile Red at the sixth minute of the dark/light cycle stimulus.*

Zebrafish larvae exposed to stained PS concentrations, presented a significant decrease in all parameters when comparing all concentrations to the control, (Figure 16 a and b), revealing as well significant decreases in all parameters after assessing the thigmotactic effects (Figure 16 d), as seen in Figure 17.

3.2.4. Bioassay 4

3.2.4.1. FET

Table 10 *- FET test with PMMA in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; presence of a pericardial edema.*

		Dead		Hatched		Unhatched		Tail Malformations		Pericardial Edema	
	mg/L	%		%		%		%		%	
24h	Control	4%		0%		96%		0%		0%	
	7.5	15%	*	0%		85%		0%		0%	
	12.5	75%	*	0%		25%		5%		5%	
	17.5	90%	*	0%		10%		10%		10%	
	22.5	95%		0%		5%		0%		0%	
48h	Control	4%		83%		13%		0%		0%	
	7.5	15%	*	0%	*	85%		0%		0%	
	12.5	75%	*	0%		25%		15%		15%	
	17.5	90%	*	0%		10%		10%		10%	
	22.5	100%		0%		0%		0%		0%	
72h	Control	4%		96%		0%		8%		4%	
	7.5	15%	*	70%		15%		10%		0%	
	12.5	75%	*	10%		15%		20%		15%	
	17.5	90%	*	5%		5%		5%		5%	
	22.5	100%		0%		0%		0%		0%	
96h	Control	8%		92%		0%		8%		4%	
	7.5	15%	*	85%		0%		0%		0%	
	12.5	75%	*	20%		5%		15%		15%	
	17.5	90%	*	0%		10%		10%		10%	
	22.5	100%		0%		0%		0%		0%	

PMMA nanoparticles affected more the zebrafish embryos at 12.5, 17.5 and 22.5 mg/L. After 24h of exposure, most organisms were dead at those experimental conditions. There was a significant number of organisms at 7.5 mg/L that presented a delay in hatching (Table 10).

The FET test with the PMMA nanoparticles stained with the lipophilic stain Nile Red caused 100% of mortality, even at the smallest concentrations, making these nanoparticles the ones with the highest levels of toxicity.

Table 11 *- FET test with PS nanoparticles and 1 mg/L of HA in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; presence of a pericardial edema.*

		Dead		Hatched		Unhatched		Tail Malformations		Pericardial Edema	
	mg/L	%		%		%		%		%	
24h	Control	0%		0%		100%		0%		0%	
	22.5	0%		0%		100%		0%		0%	
	67.5	5%		5%		95%		0%		0%	
	202.5	0%		0%		100%		0%		0%	
	607.5	0%		0%		100%		5%		5%	
48h	Control	0%	*	58%	*	42%	* * *	0%		0%	
	22.5	0%		25%		75%		0%		0%	
	67.5	10%		25%		65%		0%		0%	
	202.5	0%		0%		100%		0%		0%	
	607.5	100%		0%		0%		0%		0%	
72h	Control	0%	*	100%	*	0%	*	4%		0%	
	22.5	0%		100%		0%		5%		5%	
	67.5	10%		90%		0%		0%		0%	
	202.5	0%		50%		50%		5%		10%	
	607.5	100%		0%		0%		0%		0%	
96h	Control	0%	*	100%		0%		4%		4%	
	22.5	5%		95%		0%		0%		0%	
	67.5	10%		90%		0%		5%		0%	
	202.5	0%		100%		0%		0%		5%	
	607.5	100%		0%		0%		0%		0%	

PS nanoparticles with 1 mg/L of HA affected more the zebrafish embryos at the highest concentration, 607.5 mg/L, since all the organisms were dead by the end of the test. After 48h of exposure, at 22.5 mg/L, 67.5 mg/L and 202.5 mg/L some larvae presented a delay in hatching compared to the control. 202.5 mg/L, after 72h, still had half of the larvae unhatched (Table 11).

Table 12 *- FET test with PS nanoparticles and 10 mg/L of HA in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; presence of a pericardial edema.*

		Dead		Hatched		Unhatched		Tail Malformations		Pericardial Edema	
	mg/L	%		%		%		%		%	
24h	Control	0%	*	0%		100%		0%		0%	
	22.5	0%		0%		100%		0%		0%	
	67.5	15%	*	0%		85%		0%		0%	
	202.5	0%		0%		100%		0%		0%	
	607.5	10%		0%		90%		5%		5%	
48h	Control	0%	*	88%	*	13%	*	4%		4%	
	22.5	0%		70%		30%		10%		5%	
	67.5	15%	*	40%	*	45%	*	5%		5%	
	202.5	0%		0%		100%		0%		0%	
	607.5	100%		0%		0%		0%		0%	
72h	Control	0%	*	100%		0%		4%		0%	*
	22.5	5%		95%		0%		5%		0%	
	67.5	15%	*	85%		0%		0%		0%	
	202.5	0%		95%		5%		5%		35%	
	607.5	100%		0%		0%		0%		0%	
96h	Control	4%		96%		0%		4%		4%	
	22.5	5%		95%		0%		0%		10%	
	67.5	15%	*	85%		0%		0%		0%	
	202.5	0%		100%		0%		10%		5%	
	607.5	100%		0%		0%		0%		0%	

PS nanoparticles with 10 mg/L of HA affected more the zebrafish embryos at the highest concentration, 607.5 mg/L. After 48h of exposure, at 67.5 and 202.5 mg/L some larvae presented a delay in hatching compared to the control. After 72h of exposure, a significant number of larvae at 202.5 mg/L presented a pericardial edema (Table 12).

Table 13 *- FET test with PS nanoparticles with the lipophilic stain Nile Red and 1 mg/L of HA in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; presence of a pericardial edema.*

		Dead		Hatched		Unhatched		Tail Malformations		Pericardial Edema	
	mg/L	%		%		%		%		%	
24h	Control	0%		0%		100%		0%		0%	
	22.5	0%		0%		100%		0%		0%	
	67.5	0%		0%		100%		0%		0%	
	202.5	5%		0%		95%		0%		0%	
	607.5	0%		0%		100%		0%		0%	
48h	Control	0%		58%		42%		0%		0%	
	22.5	0%		0%	*	100%	*	0%		0%	
	67.5	0%		0%	* *	100%	* *	0%		0%	
	202.5	5%		0%	*	95%	*	0%		0%	
	607.5	0%		5%		95%		0%		0%	
72h	Control	0%		100%		0%		4%		0%	
	22.5	0%		95%		5%		0%		0%	
	67.5	0%		85%		15%		5%		0%	
	202.5	5%		90%		5%		10%		5%	
	607.5	5%		90%		5%		5%		0%	
96h	Control	0%		100%		0%		4%		4%	
	22.5	0%		100%		0%		15%		5%	
	67.5	0%		100%		0%		0%		0%	
	202.5	5%		95%		0%		15%		10%	
	607.5	5%		95%		0%		0%		0%	

PS nanoparticles stained with the lipophilic stain Nile Red and in the presence of 1 mg/L of HA affected more the zebrafish embryos at all concentrations (22.5 mg/L, 67.5 mg/L, 202.5 mg/L and 607.5 mg/L), with a significant delay in hatching after 48h of exposure (Table 13).

Table 14 *- FET test with PS nanoparticles with the lipophilic stain Nile Red and 10 mg/L of HA in zebrafish embryo exposed for 96h with the evaluation of the following parameters: egg coagulation and lack of heartbeat; hatched before and after reaching larval phase; unhatched after reaching larval phase; lack of detachment of the tail-bud before larval phase and tail malformation in larval phase; presence of a pericardial edema.*

		Dead		Hatched		Unhatched		Tail Malformations		Pericardial Edema	
	mg/L	%		%		%		%		%	
24h	Control	0%		0%		100%		0%		0%	
	22.5	5%		0%		95%		0%		0%	
	67.5	0%		0%		100%		0%		0%	
	202.5	0%		0%		100%		0%		0%	
	607.5	0%		0%		100%		0%		0%	
48h	Control	0%		88%		13%		4%		4%	
	22.5	5%		0%	*	95%	*	0%		0%	
	67.5	0%		0%	* *	100%	* *	0%		0%	
	202.5	0%		0%	*	100%	*	0%		0%	
	607.5	0%		5%		95%		0%		0%	
72h	Control	0%		100%		0%		4%		0%	
	22.5	5%		75%		20%		5%		0%	
	67.5	0%		95%		5%		0%		0%	
	202.5	0%		85%		15%		5%		5%	
	607.5	0%		100%		0%		5%		0%	
96h	Control	4%		96%		0%		4%		4%	
	22.5	5%		95%		0%		5%		0%	
	67.5	0%		100%		0%		20%		0%	
	202.5	0%		100%		0%		10%		20%	
	607.5	0%		100%		0%		10%		5%	

PS nanoparticles stained with the lipophilic stain Nile Red and in the presence of 10 mg/L of HA affected more the zebrafish embryos at all concentrations (22.5, 67.5, 202.5 and 607.5 mg/L), with a significant delay in hatching after 48h of exposure (Table 14).

3.2.4.2. Swimming Behaviour

- PMMA Exposure

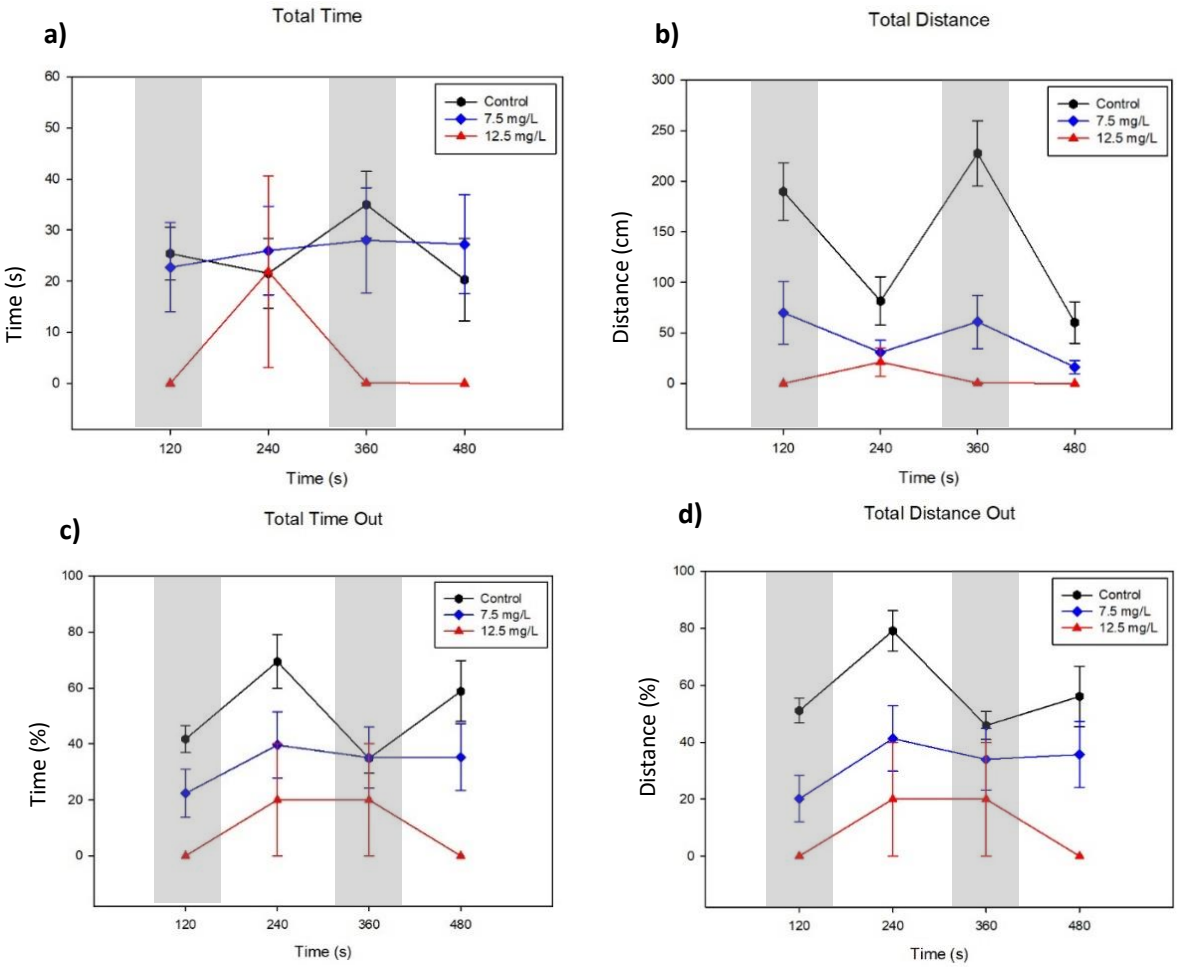


Figure 18 *- Behavioural analysis after 96h exposure to PMMA nanoparticles: a) TT; b) TD; c) TT%; and d) TD%. The total time of the experiment was eight minutes (two minutes dark fallowed by two minutes light, represented by grey and white background, respectively). Each dot represents mean distance swam by zebrafish larvae of each concentration throughout two minutes of experiment and respective standard error. Each symbol and colour represent one concentration group.*

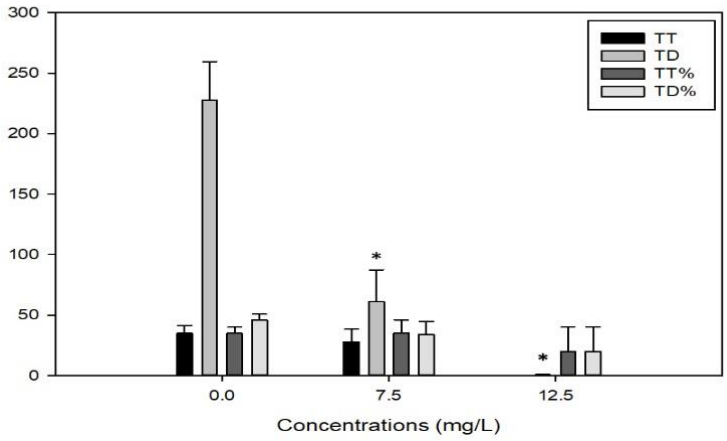


Figure 19 *- Behaviour analysis of zebrafish larvae exposed for 96h to PMMA nanoparticles at the sixth minute of the dark/light cycle stimulus.*

Larvae exposed to PMMA concentrations, presented a decreased TD swam when compared to the control (Figure 18 a and b). Calculating the TD% to assess thigmotactic effects (Figure 18 d), revealed there were significant differences (decreases) to control in terms of TD at 7.5 and 12.5 mg/L at 360 seconds, as seen in Figure 19.

- PS and 1 mg/L of HA Exposure

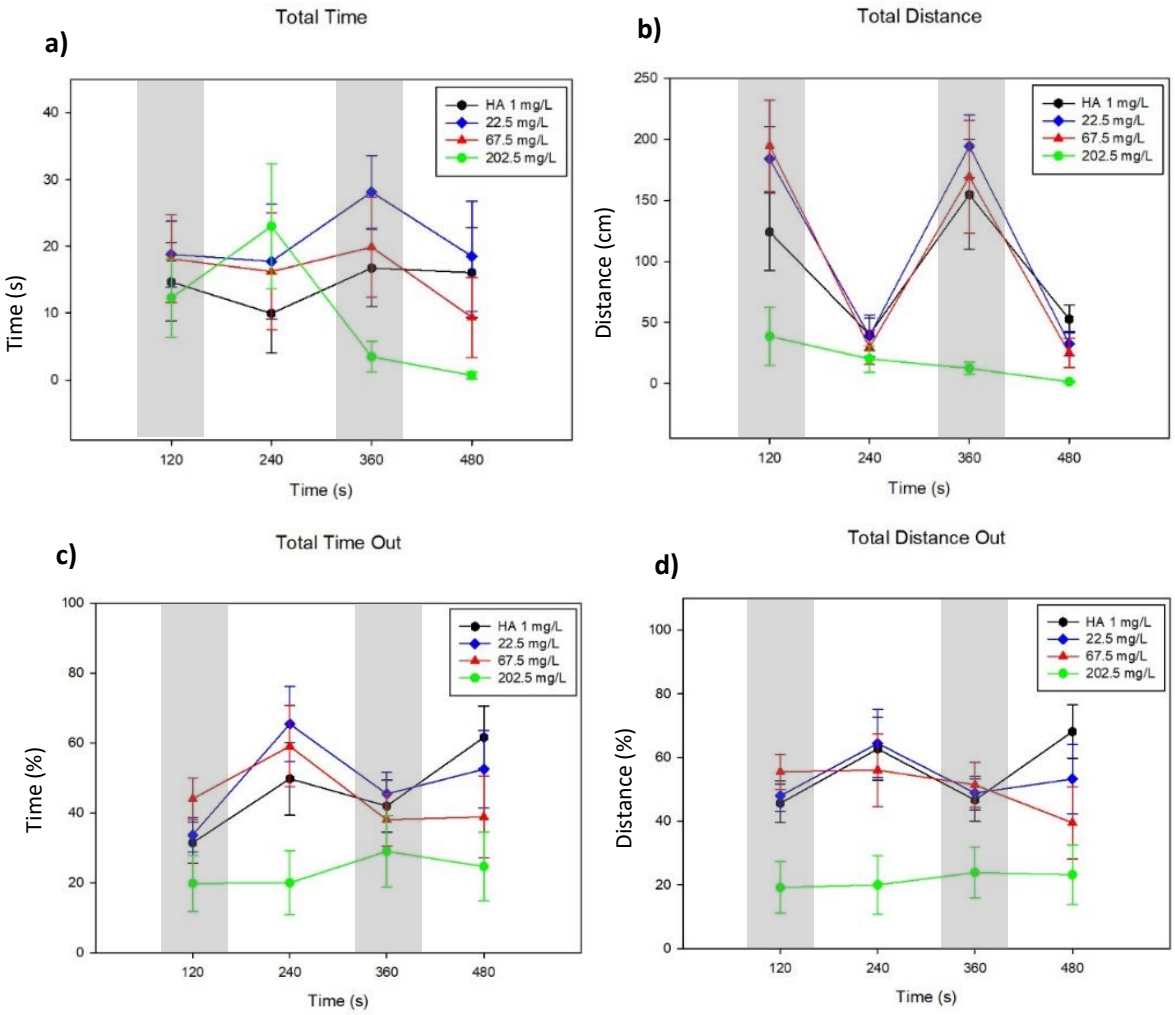


Figure 20 *- Behavioural analysis after 96h exposure to PS nanoparticles and 1 mg/L of HA: a) TT; b) TD; c) TT%; and d) TD%. The total time of the experiment was eight minutes (two minutes dark fallowed by two minutes light, represented by grey and white background, respectively). Each dot represents mean distance swam by zebrafish larvae of each concentration throughout two minutes of experiment and respective standard error. Each symbol and colour represent one concentration group.*

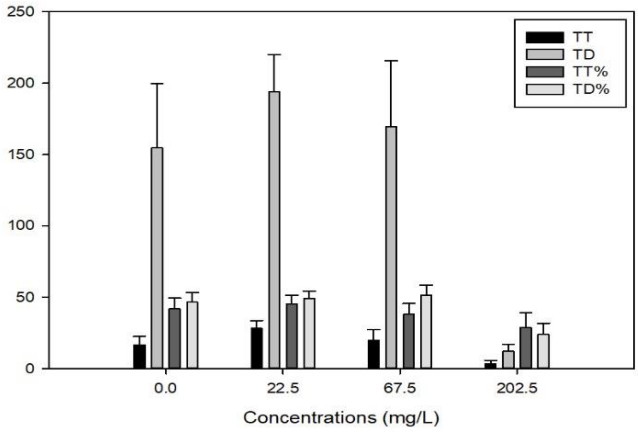


Figure 21 *- Behaviour analysis of zebrafish larvae exposed for 96h to PS nanoparticles and 1 mg/L of HA at the sixth minute of the dark/light cycle stimulus.*

Zebrafish larvae exposed to 22.5 and 67.5 mg/L of PS nanoparticles and 1 mg/L of HA, presented an increase in TT swam when compared to the control, whereas 202.5 mg/L presented a decreased TT and TD swam, especially during the dark period (Figure 20 a and b). Calculating the TD% to assess thigmotactic effects (Figure 20 d), revealed there were no significant differences to control at 360 seconds, as seen in Figure 21.

- PS and 10 mg/L of HA Exposure

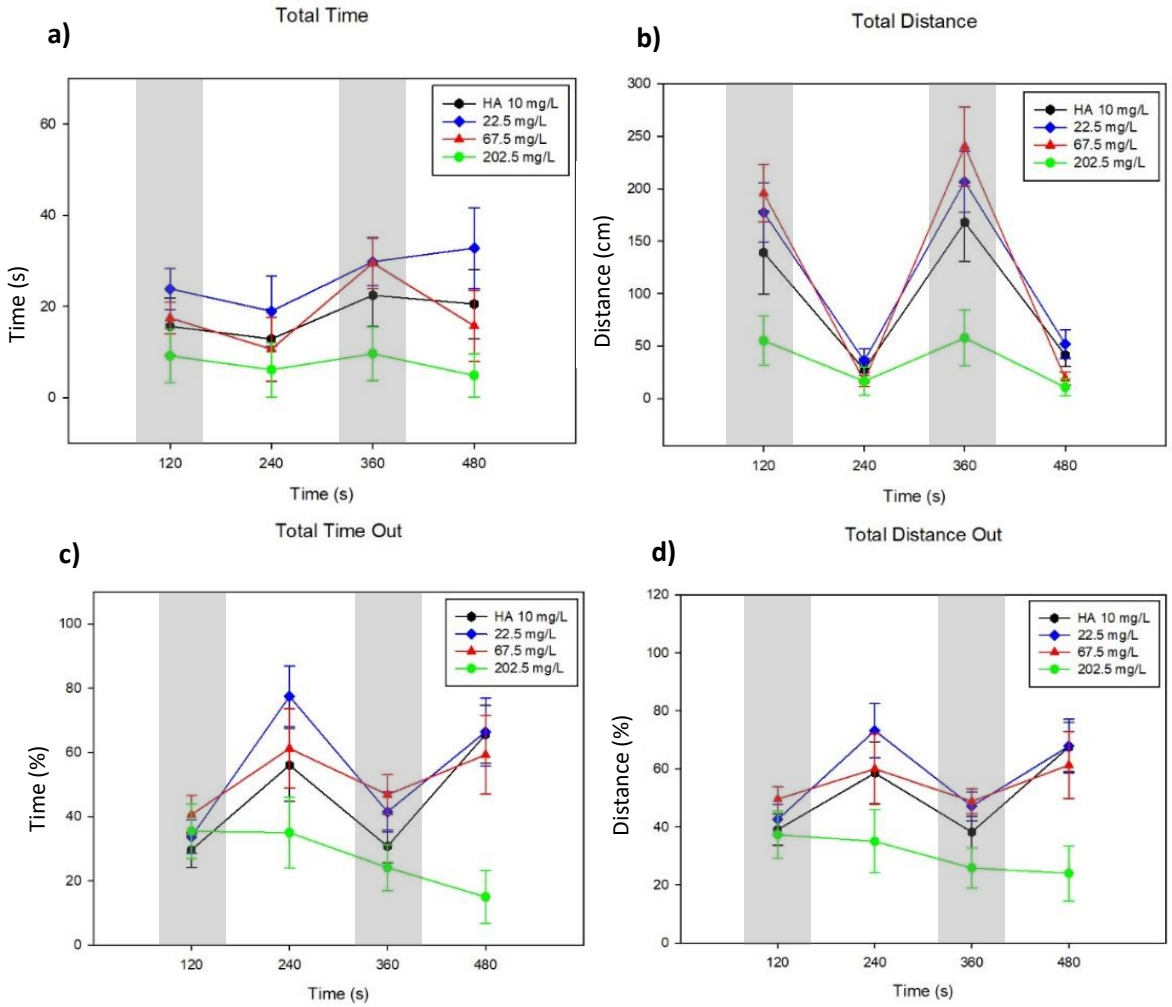


Figure 22 *- Behavioural analysis after 96h exposure to PS nanoparticles and 10 mg/L of HA: a) TT; b) TD; c) TT%; and d) TD%. The total time of the experiment was eight minutes (two minutes dark fallowed by two minutes light, represented by grey and white background, respectively). Each dot represents mean distance swam by zebrafish larvae of each concentration throughout two minutes of experiment and respective standard error. Each symbol and colour represent one concentration group.*

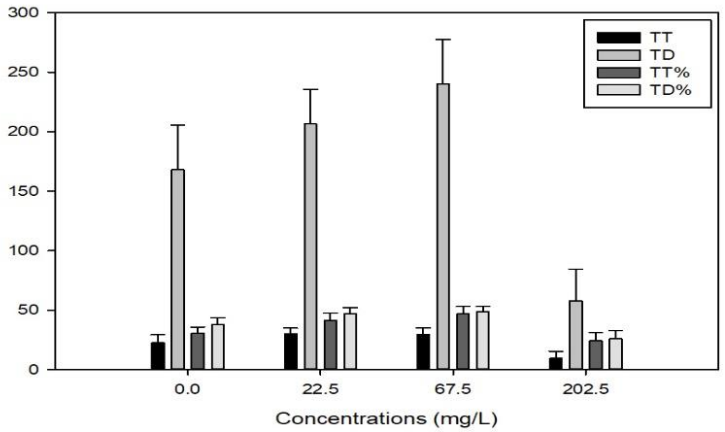


Figure 23 *- Behaviour analysis of zebrafish larvae exposed for 96h to PS nanoparticles and 10 mg/L of HA at the sixth minute of the dark/light cycle stimulus.*

Zebrafish larvae exposed to 22.5 and 67.5 mg/L of PS nanoparticles and 10 mg/L of HA, presented an increase in TT and TD swam when compared to the control, whereas 202.5 mg/L presented a decreased TT and TD swam, especially during the dark period (Figure 22 a and b). Calculating the TD% to assess thigmotactic effects (Figure 22 d), revealed there were no significant differences to control at 360 seconds, as seen in Figure 23.

- PS stained with the lipophilic stain Nile Red and 1 mg/L of HA Exposure

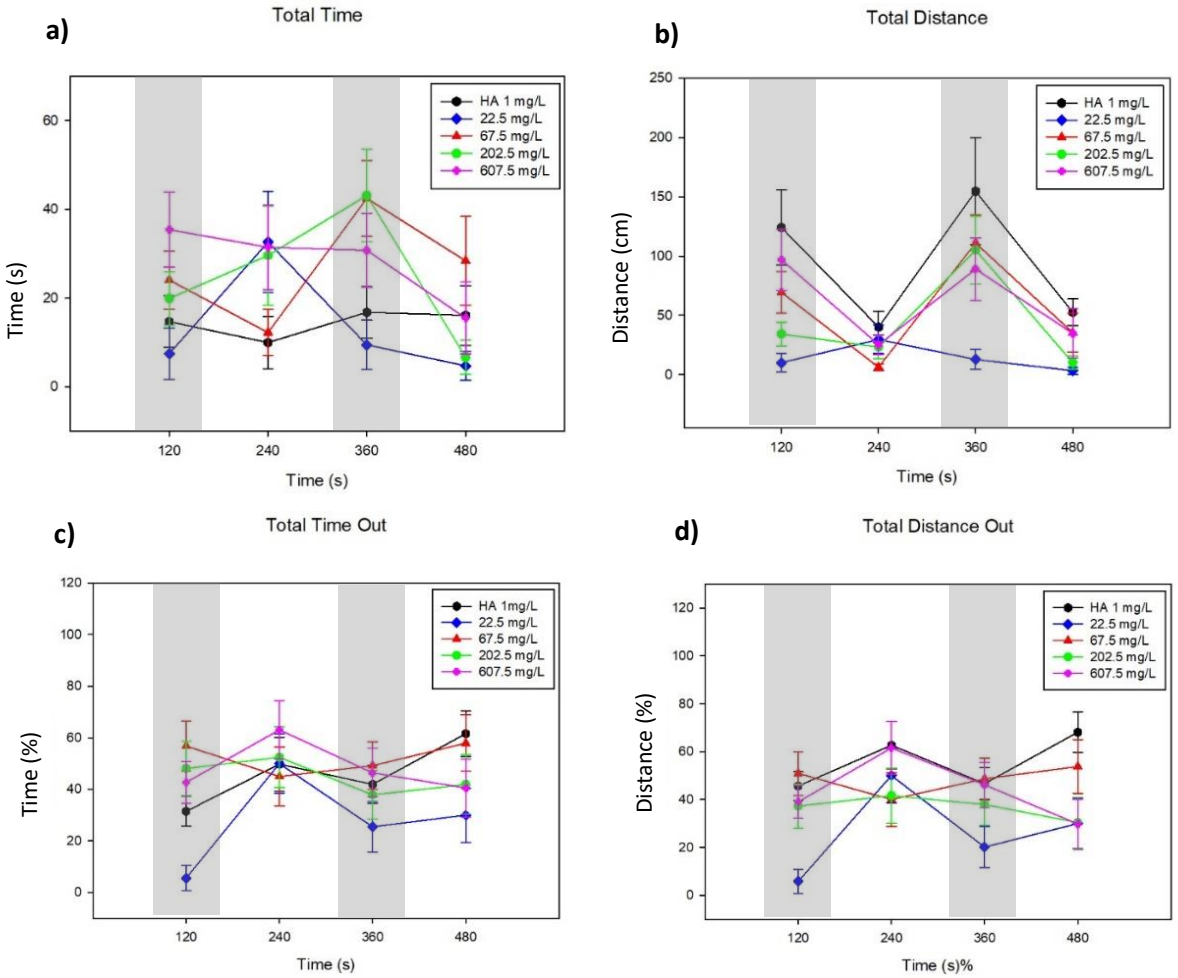


Figure 24 *- Behavioural analysis after 96h exposure to PS nanoparticles stained with the lipophilic stain Nile Red and 1 mg/L of HA: a) TT; b) TD; c) TT%; and d) TD%. The total time of the experiment was eight minutes (two minutes dark fallowed by two minutes light, represented by grey and white background, respectively). Each dot represents mean distance swam by zebrafish larvae of each concentration throughout two minutes of experiment and respective standard error. Each symbol and colour represent one concentration group.*

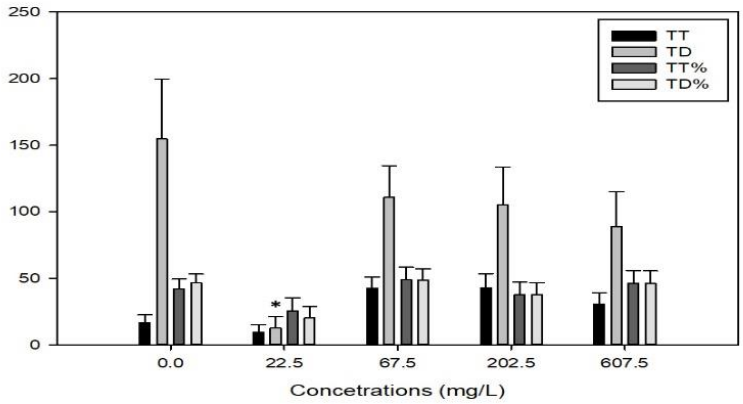


Figure 25 *- Behaviour analysis of zebrafish larvae exposed for 96h to PS nanoparticles stained with the lipophilic stain Nile Red and 1 mg/L of HA at the sixth minute of the dark/light cycle stimulus.*

Larvae exposed to 67.5, 202.5 and 607.5 mg/L of stained PS nanoparticles and 1 mg/L of HA, presented an increase in TT swam and a decrease TD swam when compared to the control, whereas 22.5 mg/L presented a decreased TT and TD swam, especially during the dark period (Figure 24 a and b). Calculating the TD% to assess thigmotactic effects (Figure 24 d), revealed there was a significant differences (decrease) to control in TD at 22.5 mg/L at 360 seconds, as seen in Figure 25.

- PS stained with the lipophilic stain Nile Red and 10 mg/L of HA Exposure

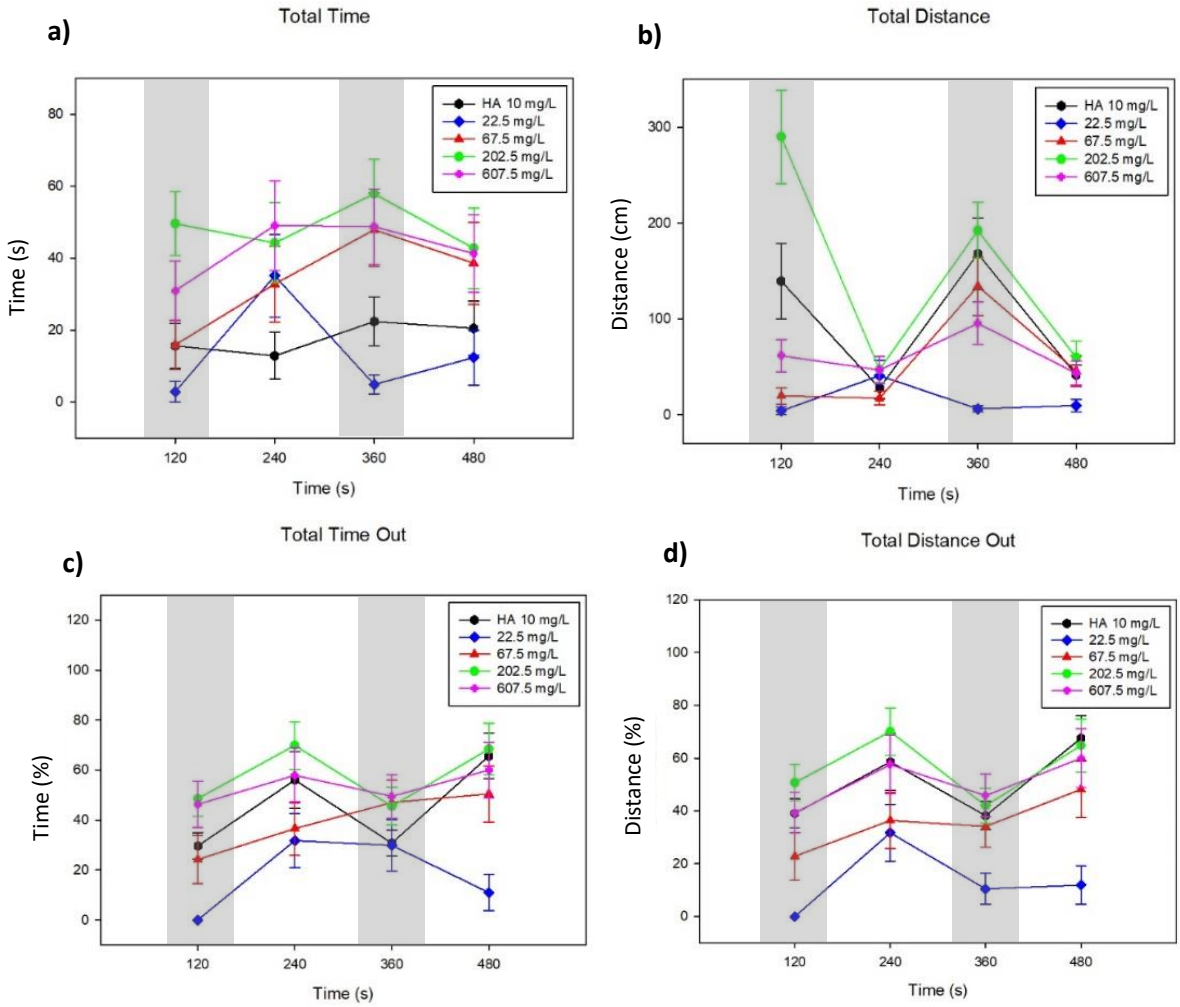


Figure 26 *- Behavioural analysis after 96h exposure to PS nanoparticles stained with the lipophilic stain Nile Red and 10 mg/L of HA: a) TT; b) TD; c) TT%; and d) TD%. The total time of the experiment was eight minutes (two minutes dark fallowed by two minutes light, represented by grey and white background, respectively). Each dot represents mean distance swam by zebrafish larvae of each concentration throughout two minutes of experiment and respective standard error. Each symbol and colour represent one concentration group.*

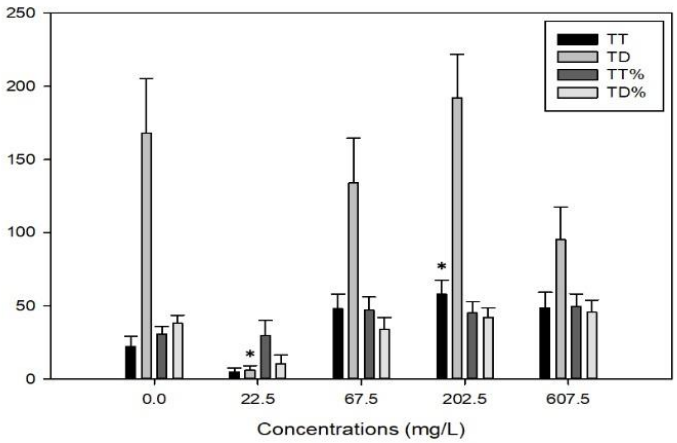


Figure 27 *- Behaviour analysis of zebrafish larvae exposed for 96h to PS nanoparticles stained with the lipophilic stain Nile Red and 10 mg/L of HA at the sixth minute of the dark/light cycle stimulus.*

Concerning larvae exposed to 67.5, 202.5 and 607.5 mg/L of stained PS nanoparticles and 10 mg/L of HA, presented an increase in TT swam and 67.5 and 607.5 mg/L presented a decrease TD swam, while 202.5 revealed an increase TD activity when compared to the control. Stained PS concentration 22.5 mg/L presented a decreased TT and TD swam, especially during the dark period (Figure 26 a and b). Calculating the TD% to assess thigmotactic effects (Figure 26 d), revealed there was a significant differences (decrease) to control in TD at 22.5 mg/L and in TT at 202.5 mg/L at 360 seconds, as seen in Figure 27.

3.2.5. Biochemical Endpoints

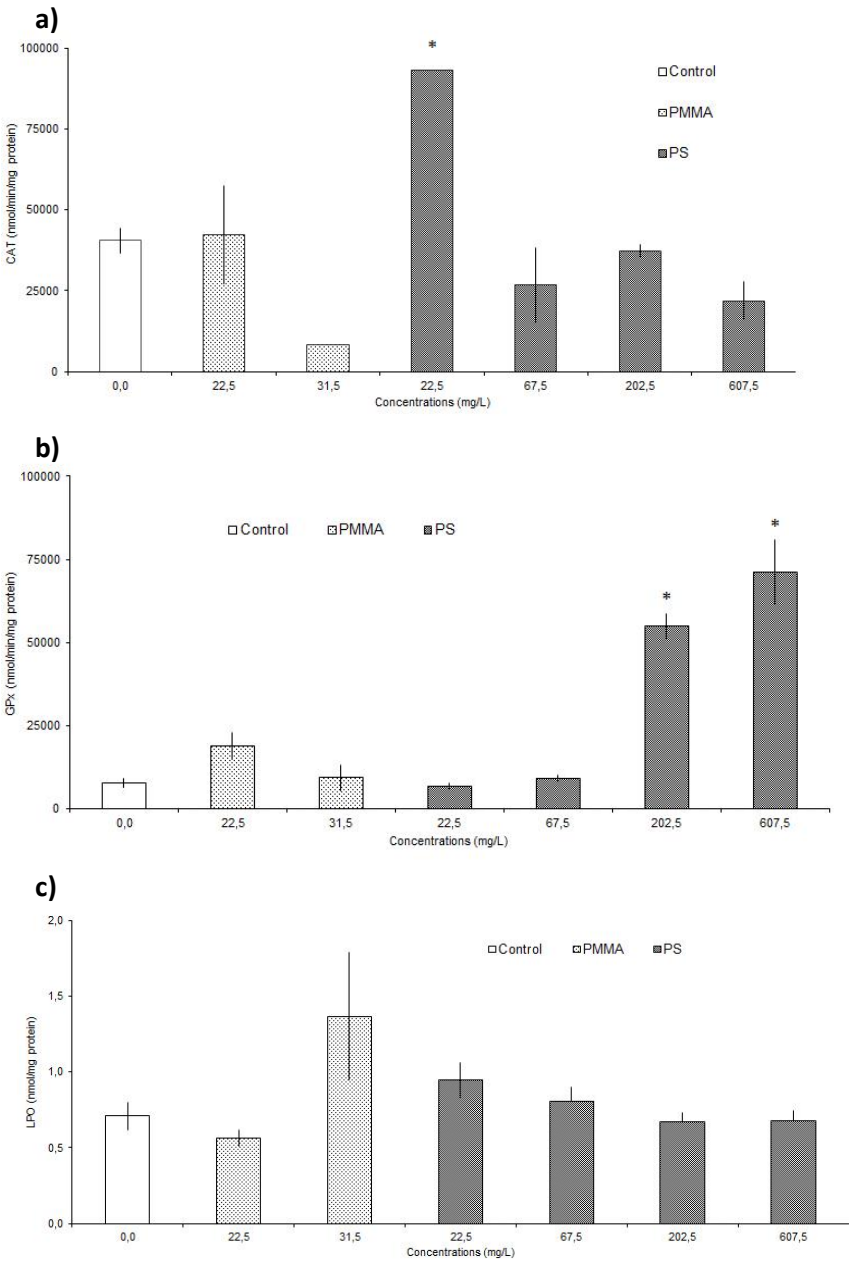


Figure 28 *- Biochemical endpoints in zebrafish after 96h of exposure to PMMA and PS nanoparticles: a) CAT activity; b) GPx activity; c) LPO levels.*

For this analysis, only organisms of 22.5 mg/L and 31.5 mg/L of PMMA were used, since the highest concentrations the organisms ended dead. In this test, PMMA did not have a significant effect in terms of CAT and GPx activity and of LPO levels, when compared to the control. PS nanoparticles caused a significant increase of CAT activity at 22.5 mg/L. In terms of GPx activity, 202.5 and 607.5 mg/L induced significantly higher activities (Figure 28).

3.3. Sea Bass Experiments

3.3.1. Bioassay 1

3.3.1.1. *Physico-Chemical Parameters*

Table 15 *- Physico-Chemical Parameters of the sea bass exposed to PMMA nanoparticles.*

	O_2 (mg/L)	pH	Temp. (°C)
Control	8.30	7.56	16.30
0.02 mg/L	8.44	8.00	16.00
0.2 mg/L	8.66	8.40	15.75
2 mg/L	8.63	7.37	15.80

Physico-Chemical parameters maintained stable throughout the entire test, without having significant differences between the aquarium.

3.3.1.2. *Hepatosomatic Index*

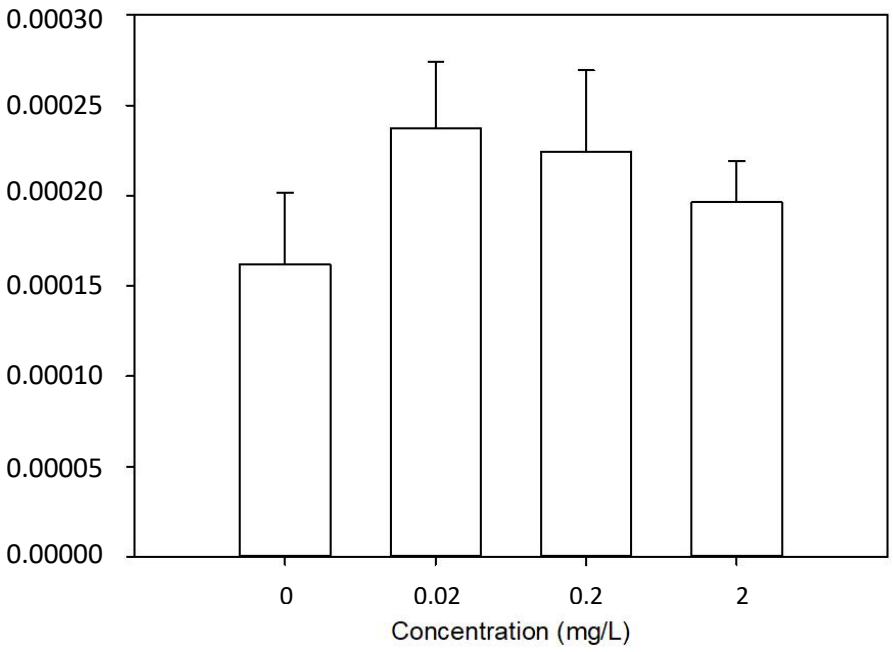


Figure 29 *- Hepatosomatic index of sea bass after 96h exposure to PMMA nanoparticles (Bioassay 1).*

Figure 29 depicts the hepatosomatic index of sea bass when exposed for 96h to PMMA nanoparticles. Although an increase trend was found for organisms exposed to PMMA nanoparticles, no significant differences to control were found

3.3.1.3. Erythrocyte Abnormalities Assay

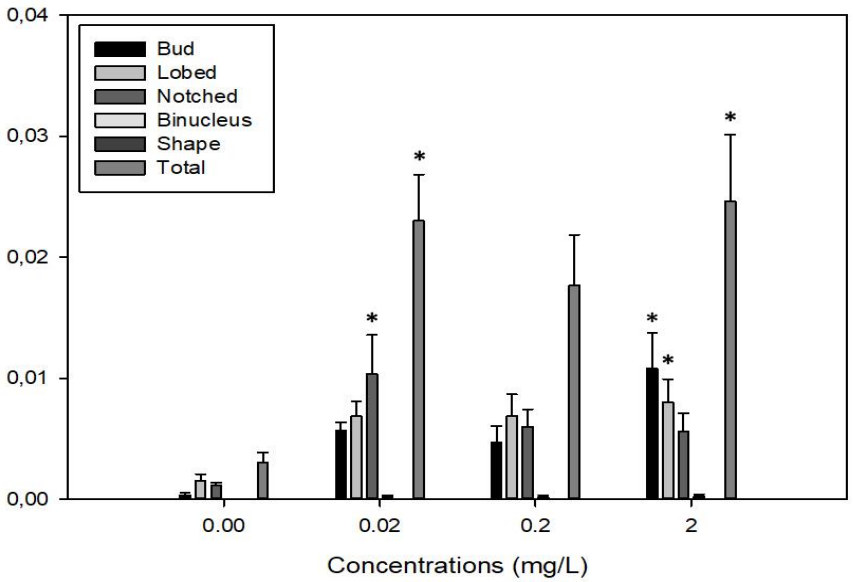


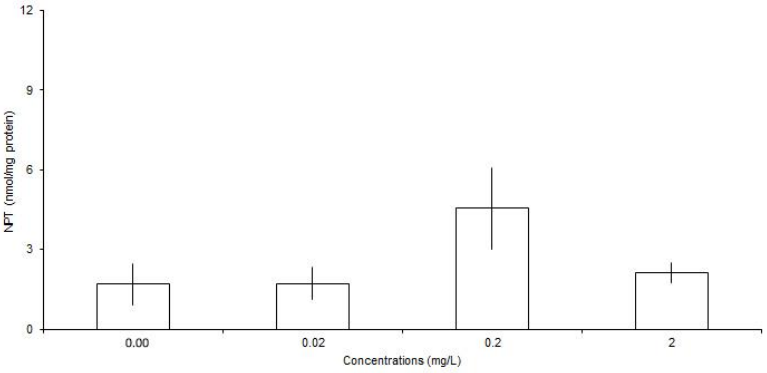
Figure 30 *– Erythrocyte abnormalities in sea bass after the 96h exposure to PMMA nanoparticles.*

Figure 30 shows that exposure to PMMA nanoparticles induces increased nuclear and echinocytes abnormalities. Although only significant for 0.02 and 2 mg/L, the total number of abnormalities was higher than control in organisms exposed to PMMA nanoparticles. The highest tested concentration tested increased all assessed abnormalities and 0.02 mg/L induced a higher number of 138 cells.

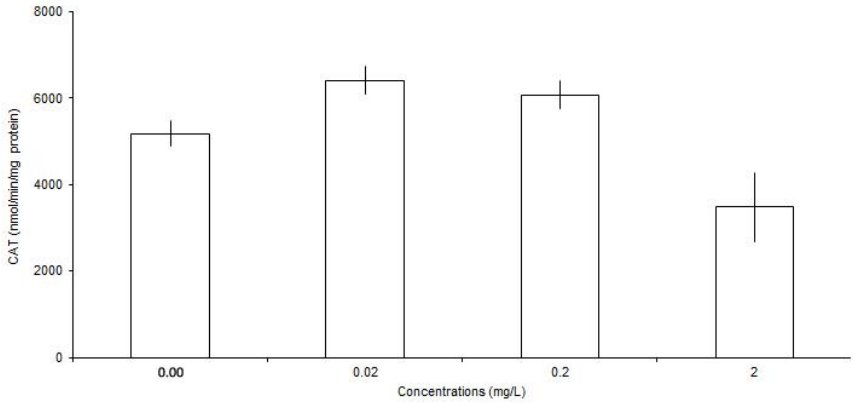
3.3.1.4. Biochemical Endpoints

- Liver

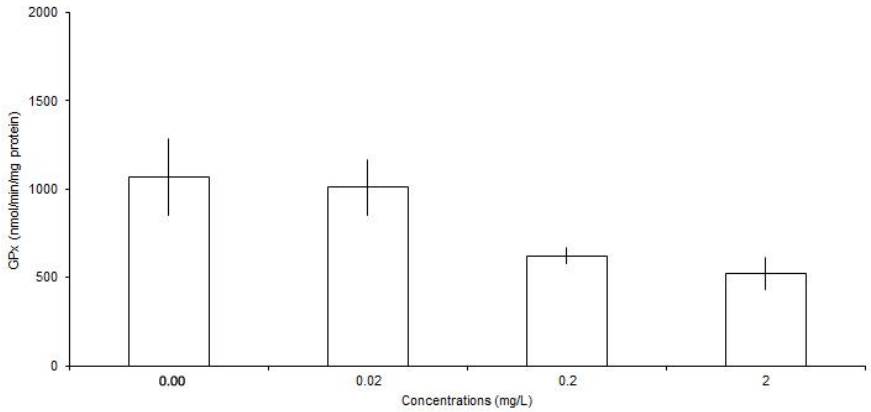
a)



b)



c)



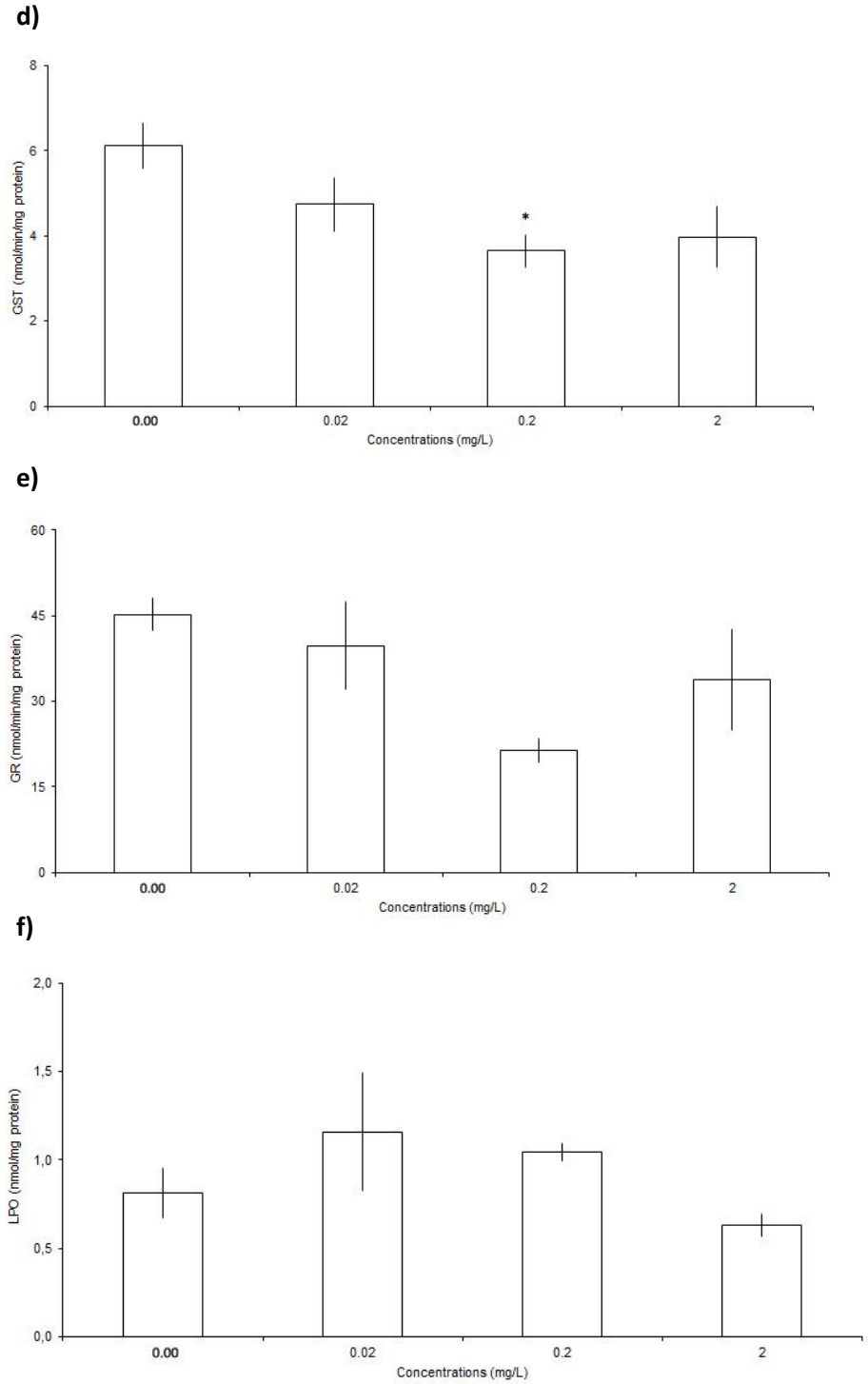
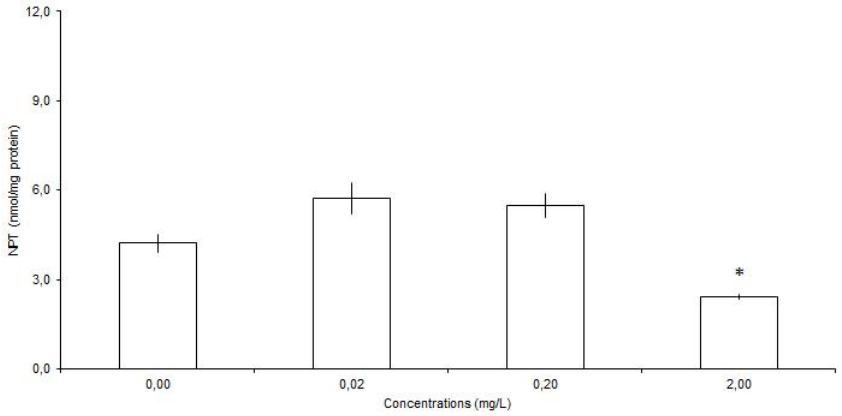


Figure 31 *- Biochemical endpoints in the liver of sea bass after 96h exposure to PMMA nanoparticles (Bioassay 1): a) NPT levels; b) CAT activity; c) GPx activity; d) GST activity; e) GR activity; and f) LPO levels.*

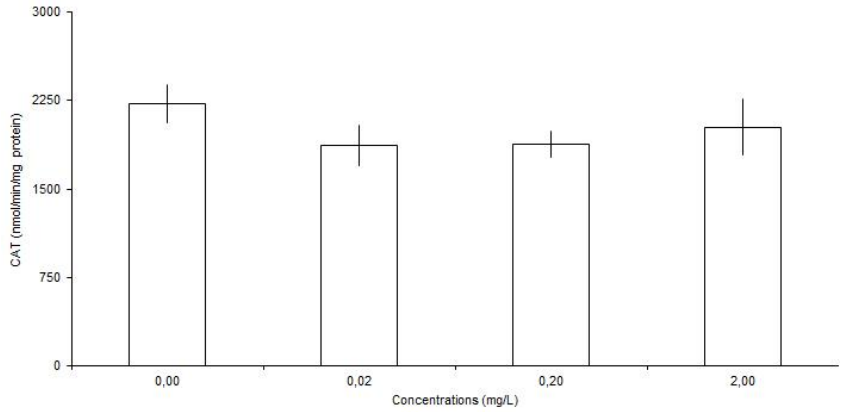
Compared with the control, the only significant difference in the biochemical parameters assessed in the liver was found, in terms of GST activity (Figure 31 d), which was inhibited after exposure to 0.2 mg/L of PMMA.

- Gills

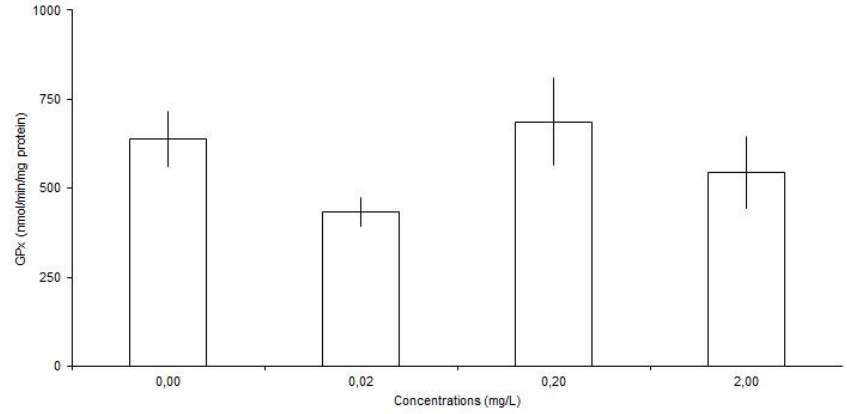
a)


12,0
9,0
6,0
3,0
0,0
NPT (nmol/mg protein)
*
0,00
0,02
0,20
2,00
Concentrations (mg/L)

b)


3000
2250
1500
750
0
CAT (nmol/min/mg protein)
0,00
0,02
0,20
2,00
Concentrations (mg/L)

c)


1000
750
500
250
0
GPx (nmol/min/mg protein)
0,00
0,02
0,20
2,00
Concentrations (mg/L)

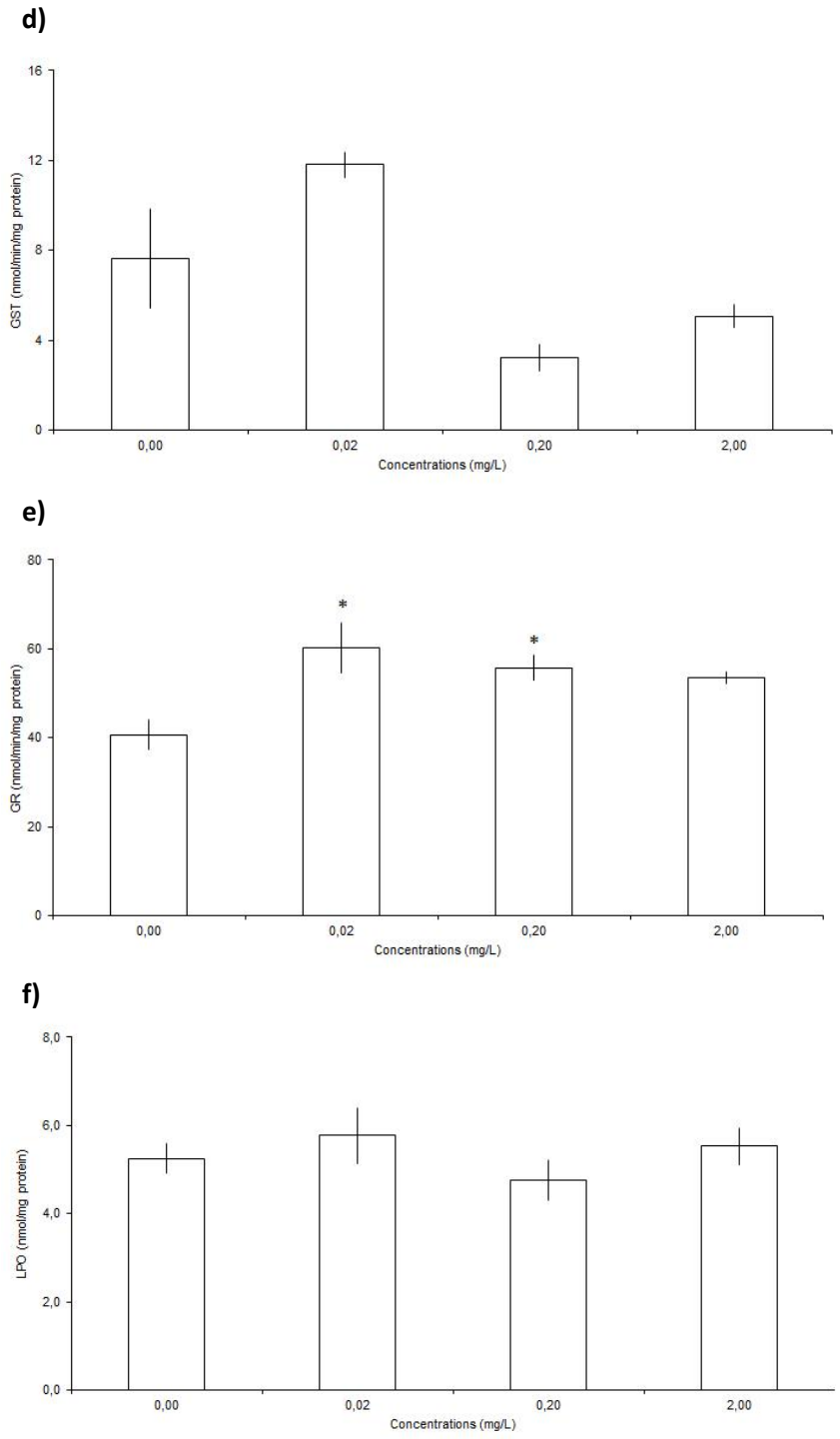
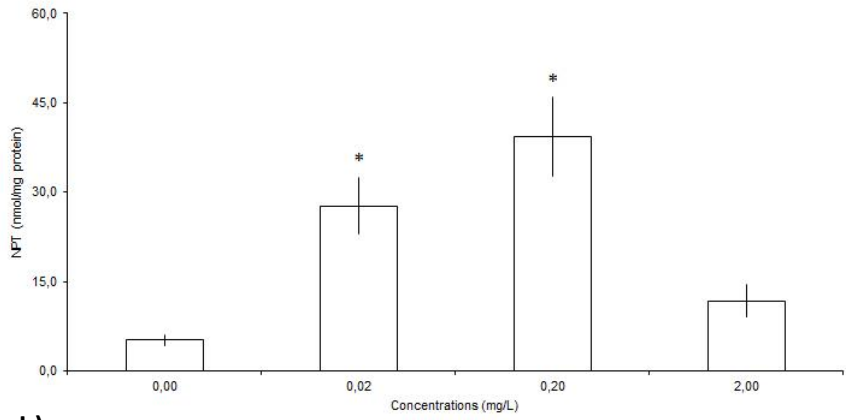


Figure 32 *- Biochemical endpoints in the gills of sea bass after 96h exposure to PMMA nanoparticles (Bioassay 1): a) NPT levels; b) CAT activity; c) GPx activity; d) GST activity; e) GR activity; and f) LPO levels.*

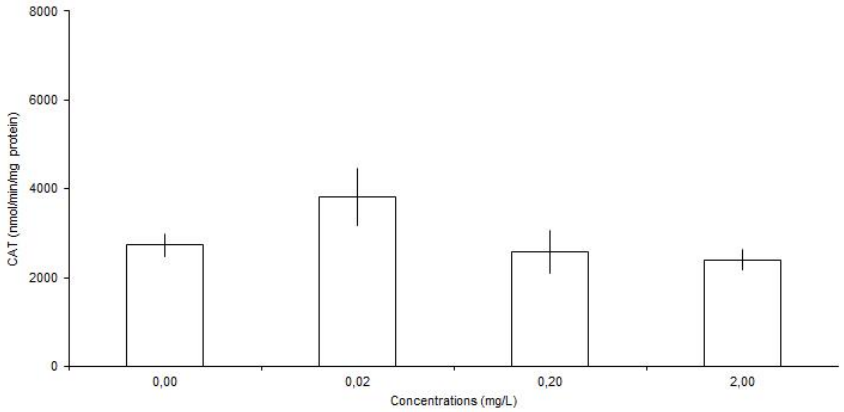
In gills, significant differences to control were found in terms of GR activity (Figure 32), with an increased activity in organisms exposed to 0.02 and 0.2 mg/L of PMMA. Decreased NPT levels, were found in the gills of organisms exposed to 2 mg/L.

- Intestine

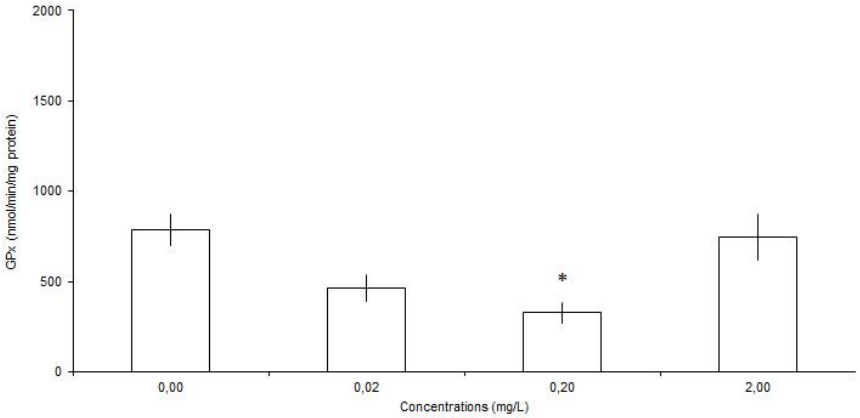
a)



b)



c)



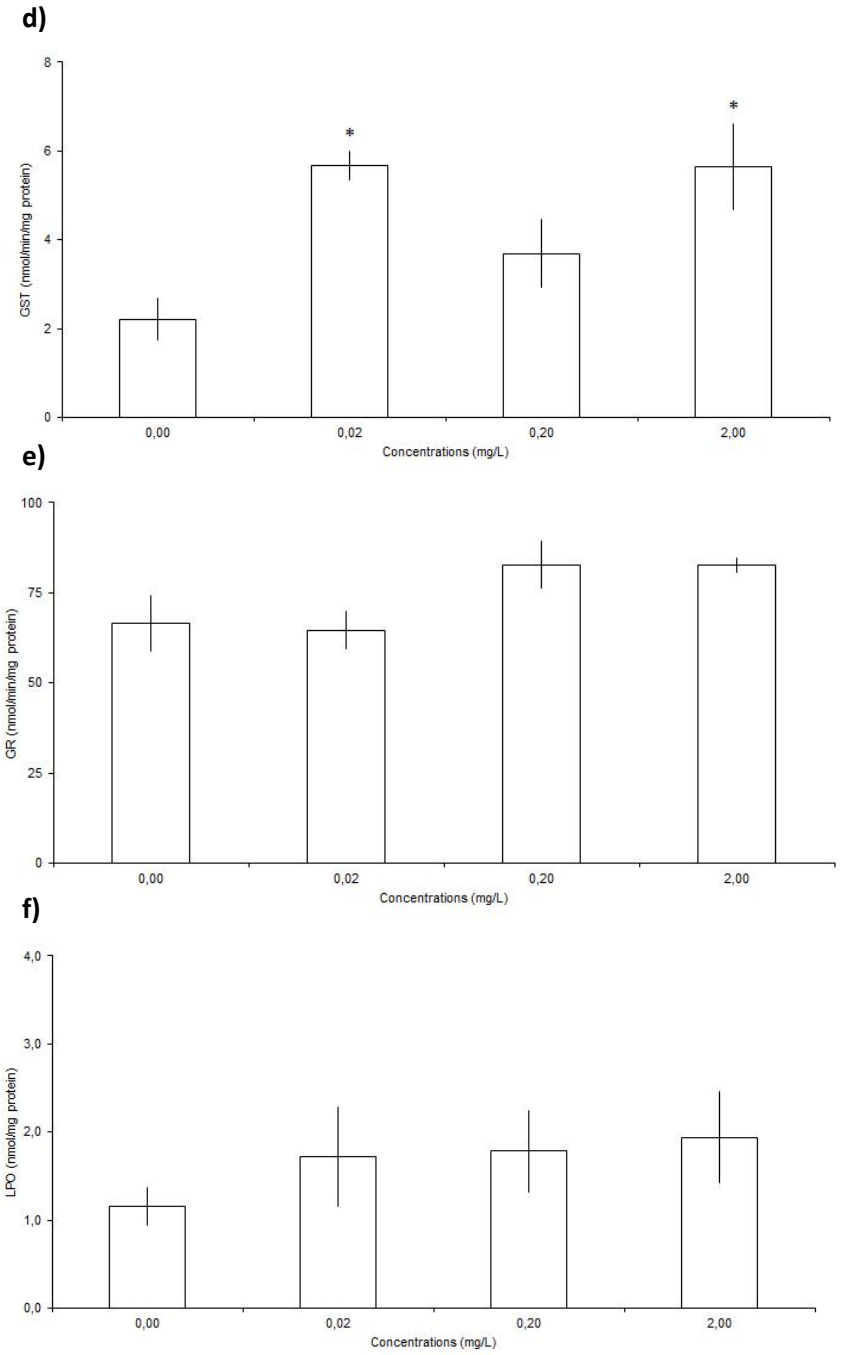


Figure 33 *- Biochemical endpoints in the intestine of sea bass after 96h exposure to PMMA nanoparticles (Bioassay 1): a) NPT levels; b) CAT activity; c) GPx activity; d) GST activity; e) GR activity; and f) LPO levels.*

Compared with the control, intestine GPx activity was significantly increased after fish exposure to 0.02 and 2 mg/L of PMMA (Figure 33). NPT levels were higher than control in organisms exposed to 0.02 and 0.2 mg/L.

3.3.2. Bioassay 2

3.3.2.1. Physico-Chemical Parameters

Table 16 *- Physico-Chemical Parameters of the sea bass exposed to PS nanoparticles.*

	O_2 (mg/L)	pH	Temp. (°C)
Control	8.78	7.75	16.65
H.A. 1 mg/L	8.65	7.73	16.35
0.02 mg/L	8.75	7.79	16.50
20 mg/L	8.78	7.88	16.55
0.02 mg/L + H.A. 1 mg/L	8.85	7.92	16.25
20 mg/L + H.A. 1 mg/L	8.83	7.82	16.25

Table 16 represents the physio-chemical parameters that were accessed in every aquarium used in Bioassay 2.

3.3.2.2. Hepatosomatic Index

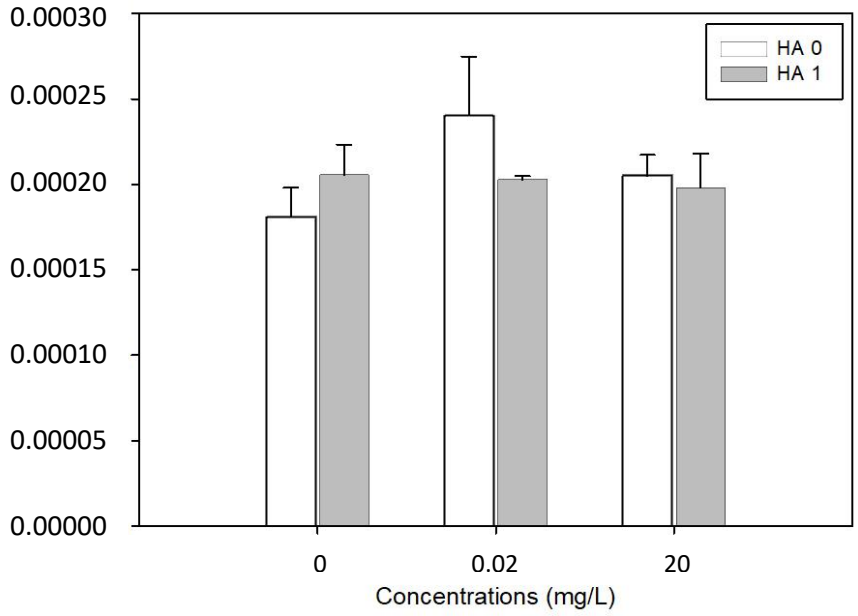


Figure 34 *- Hepatosomatic index of sea bass after the 96h exposure to PS nanoparticles (Bioassay 2).*

Figure 34 depicts the hepatosomatic index of sea bass when exposed for 96h to PS nanoparticles alone or with the presence of 1 mg/L of HA. Although an increase trend was found for organisms exposed to only PS nanoparticles, no significant differences to control were found.

3.3.2.3. Erythrocyte Abnormalities Assay

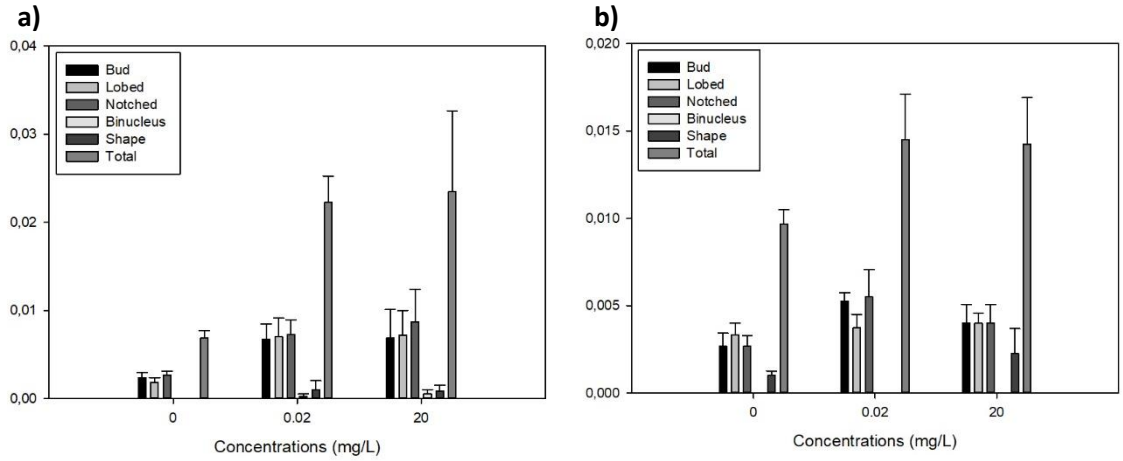


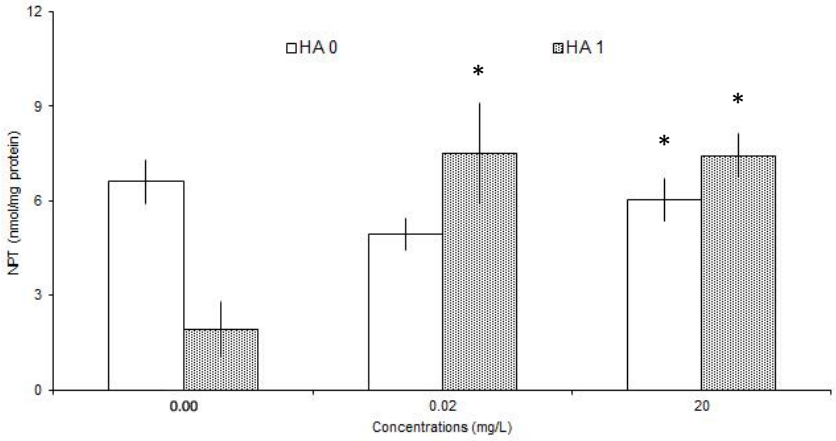
Figure 35 *- Erythrocyte abnormalities in sea bass after the 96h exposure to PS nanoparticles (Bioassay 2): a) with no presence of HA; and b) HA 1 mg/L.*

Animals exposed to PS nanoparticles alone or in the presence of 1 mg/L of HA presented a larger number of anomalies at the highest concentration with the total number of anomalies, although none represents a significant change when compared to the control (Figure 35).

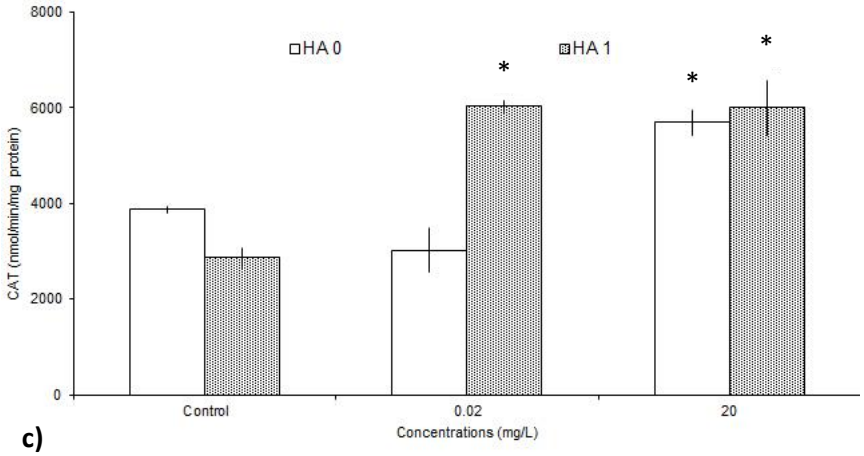
Biochemical Endpoints

- Liver

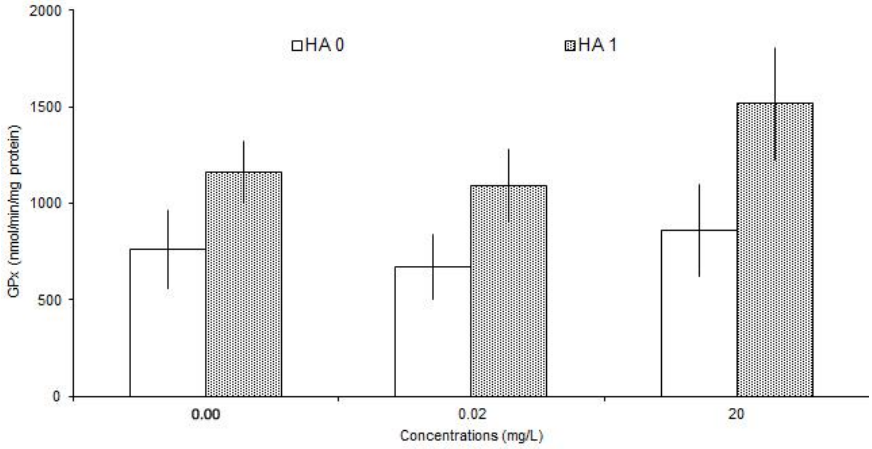
a)



b)



c)



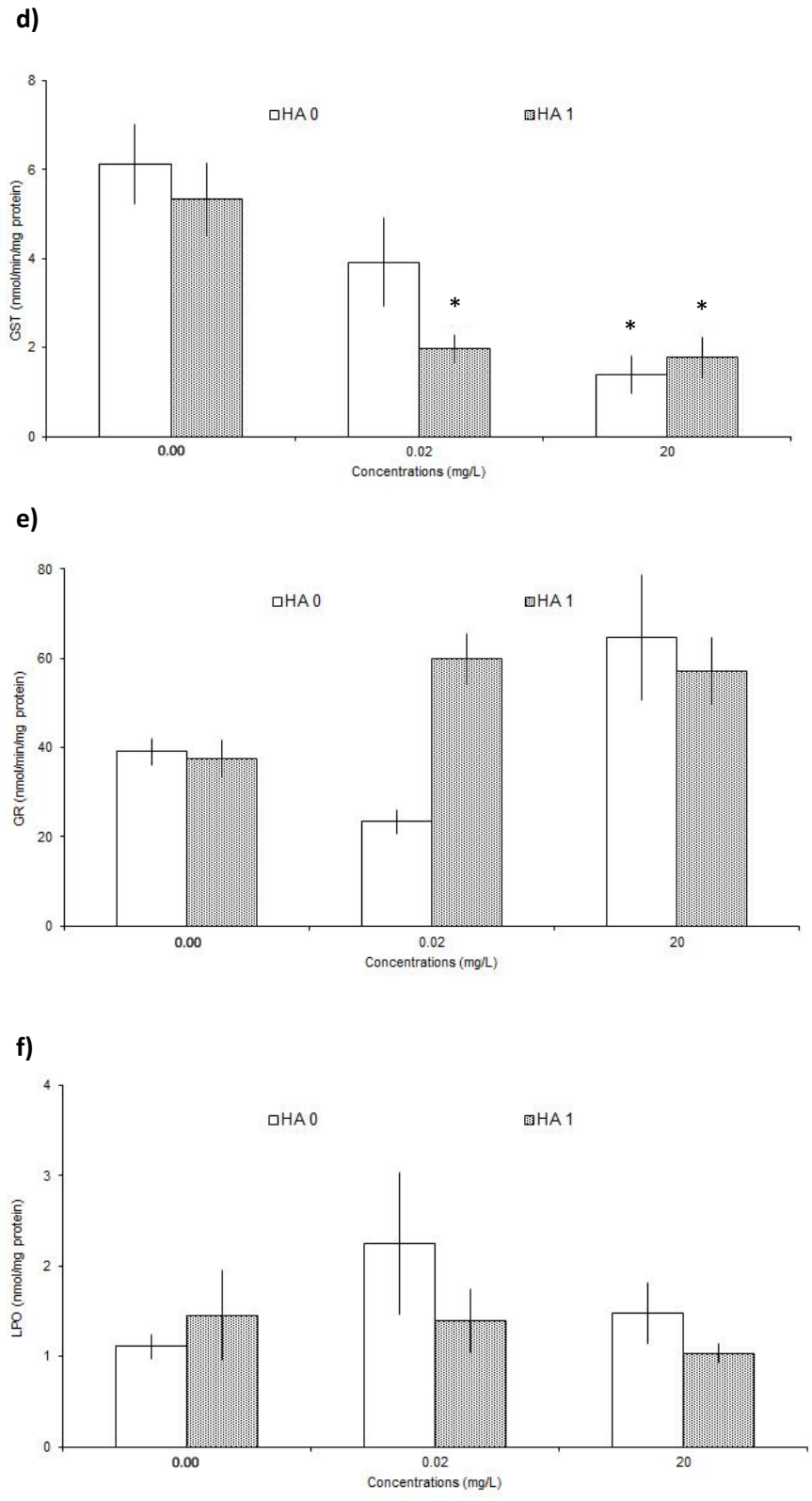
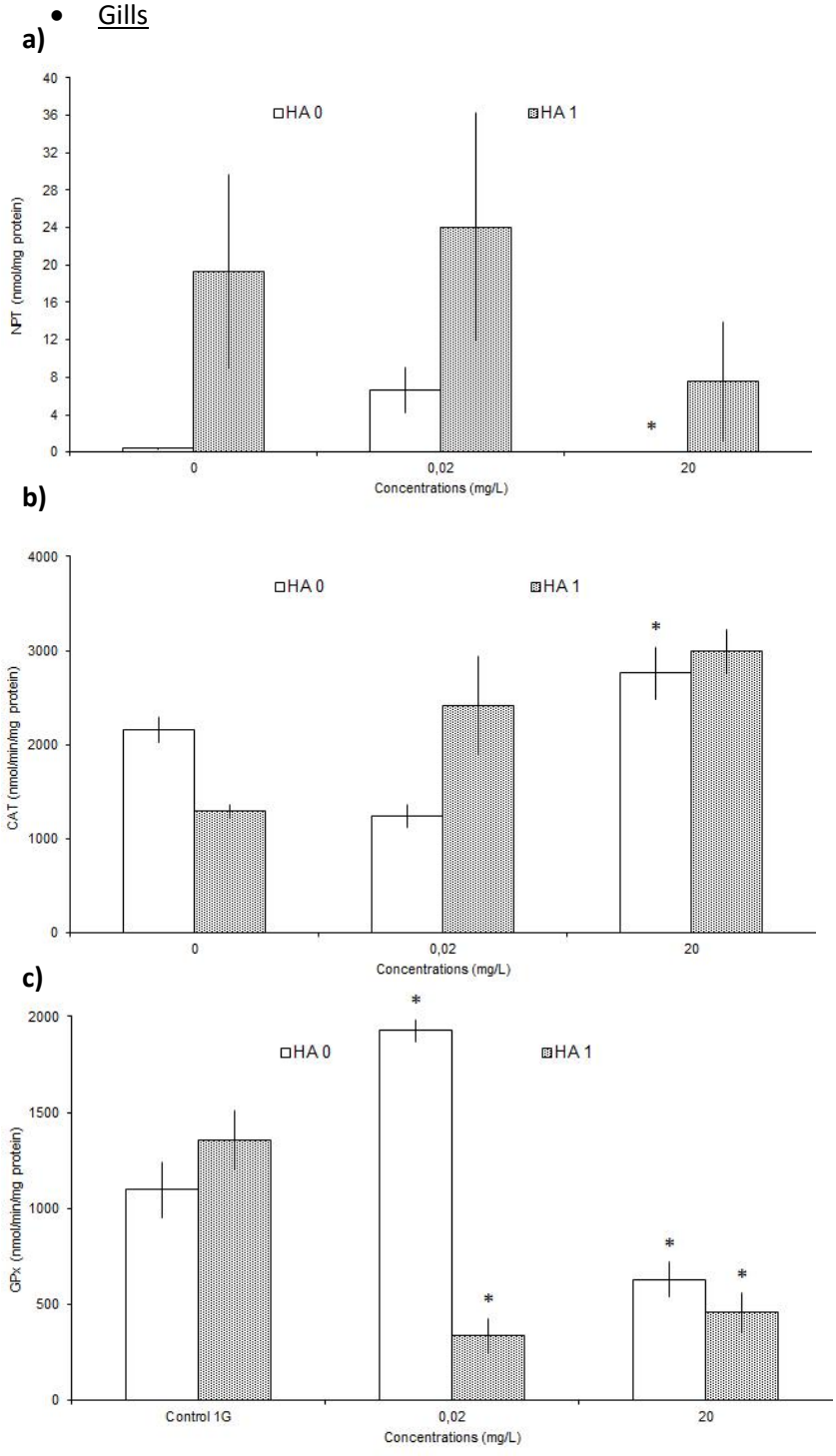


Figure 36 *- Biochemical endpoints in the liver of sea bass after 96h exposure to PS nanoparticles (Bioassay 2): a) NPT levels; b) CAT activity; c) GPx activity; d) GST activity; e) GR activity; and f) LPO levels.*

Significant differences to control were found in hepatic CAT activity, with an increase of activity at the concentrations of 0.02 mg/L of PS in the presence of HA and 20 mg/L of PS alone or in the presence of HA (Figure 36). In terms of GST activity, a decrease was found at 0.02 and 20 mg/L of PS in the presence of HA. NPT levels increased in organisms exposed to 0.02 mg/L of PS in the presence of HA and 20 mg/L of PS alone and in the presence of HA.

- Gills



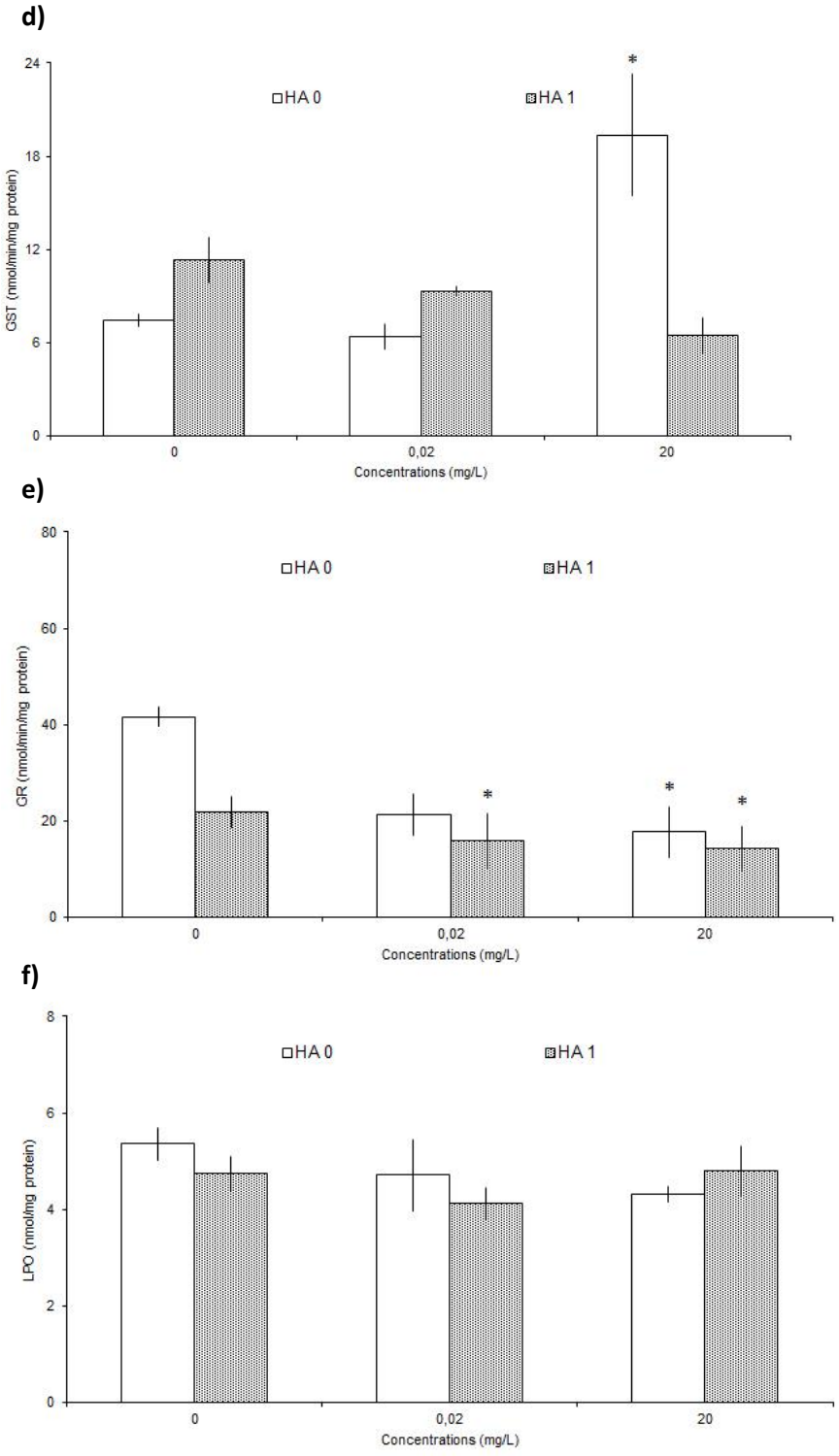


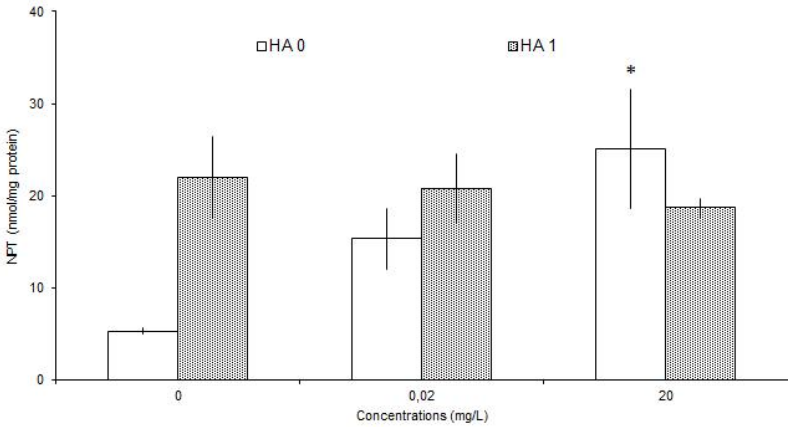
Figure 37 *- Biochemical endpoints in the gills of sea bass after 96h exposure to PS nanoparticles (Bioassay 2): a) NPT levels; b) CAT activity; c) GPx activity; d) GST activity; e) GR activity; and f) LPO levels.*

Gills CAT activity was significantly higher than control at 20 mg/L of PS (Figure 37). GR activity, decreased in organisms exposed to 0.02 mg/L of PS in the presence of HA and 20 mg/L of PS alone

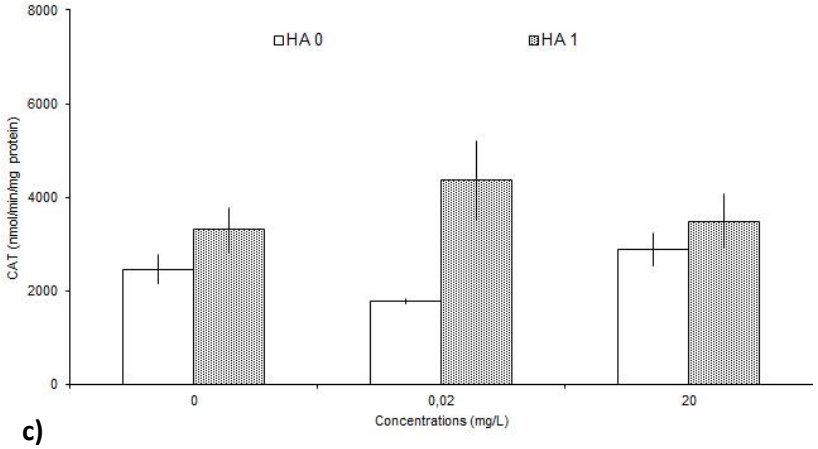
and in the presence of HA. GPx activity displayed an increased activity in organisms exposed to 0.02 mg/L of PS and a decreased activity was found at 0.02 mg/L of PS in the presence of HA and 20 mg/L of Ps alone and in the presence of HA.

- <u>Intestine</u>

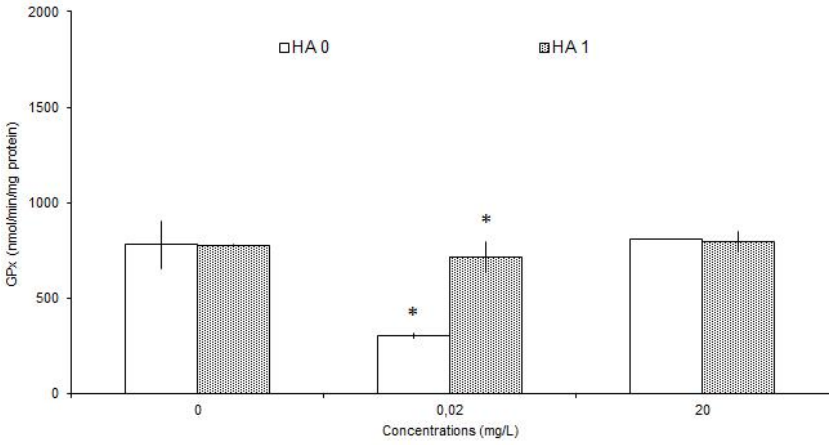
a)



b)



c)



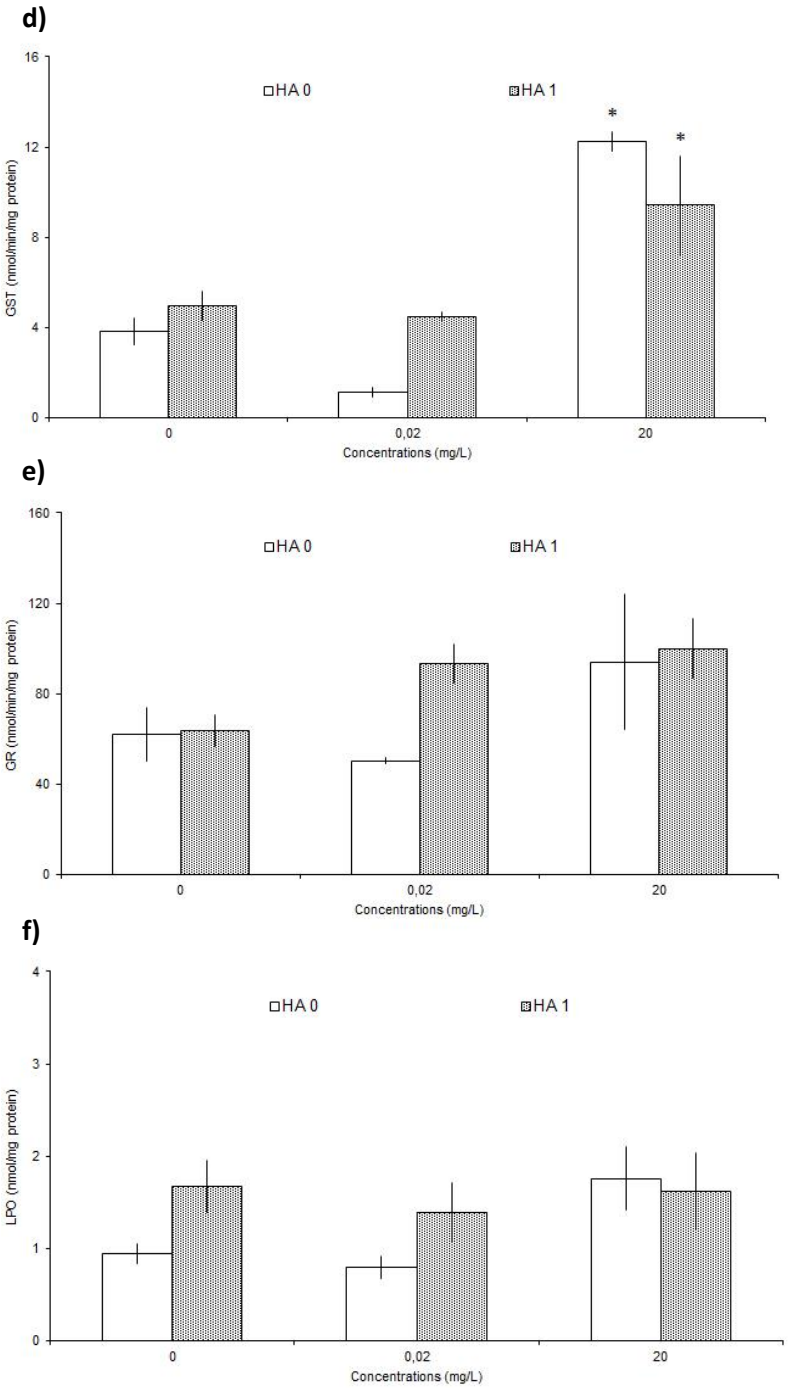


Figure 38 *- Biochemical endpoints in the intestine of sea bass after 96h exposure to PS nanoparticles (Bioassay 2): a) NPT levels; b) CAT activity; c) GPx activity; d) GST activity; e) GR activity; and f) LPO levels.*

After observing, compared with the control, there are significant changes seen in intestine GST activity increased at the concentration of 20 mg/L of PS alone and in the presence of HA (Figure 38). In terms of GPx activity, a decrease was found in organisms exposed to 0.02 mg/L of PS alone and combined with HA. NPT levels were increased at 0.02 mg/L of PS.

Discussion

The chosen methodology to synthesize the nanoparticles proved very effective since it allowed the synthesis of a large number of nanoparticles with a low polydispersion index. This is very important since it has been previously reported batch to batch variations in nanomaterials acquired commercially. The amount produced allowed performing several assays with the assurance the properties of the nanoparticles were the same. Throughout optimization of the polymerization of PMMA and PS, nanoparticles were synthesized with a size range from ≈ 30 nm to ≈ 1 µm. However, for this study, smaller particles were the main goals as there is little information of the effects nanoparticles to fish (Jovanović, 2017, Mattsson et al., 2015). The selection of the polymers was based on the estimated world used, available literature in terms of effects and available methodologies to synthesise nanoparticles. PMMA and PS nanoparticles stained with the lipophilic stain Nile Red were also synthesized, since it would allow to study the distribution of particles in the embryos and fish. However, the obtained data revealed that the chosen synthesis methods was not efficient to that goal as they proved highly toxic.

PMMA had a higher death rate, but PMMA and PS nanoparticles presented a negative value of zeta potential close to what makes a nanoparticle to be considered strongly anionic (> -30 mV), reacting in high ionic strength media such as seawater, that may cause the agglomeration of nanoparticles (Barreto et al., 2015, Clogston and Patri, 2011). PMMA and PS nanoparticles are not stable in seawater and zebrafish water, since agglomeration was witnessed during the tests, especially for PS nanoparticles. These nanoparticles, after 1h aggregated/agglomerated, as seen after DLS measurement. This aggregation/agglomeration leads to a lower bioavailability of the nanoparticles that tend to sediment in the bottom of the tanks. To promote dispersion, HA was added. In the presence of HA, PS nanoparticles did not aggregate, which was expectable as it has been considered a natural dispersant (Chen et al., 2018).

The obtained data provided relevant information on the toxicity of these nanoparticles and is the first study that compares these two nanoparticles. Polymeric nanoparticles significantly affected the ontogenic development of zebrafish. PMMA nanoparticles were more toxic than the PS nanoparticles. In terms of effects in zebrafish, PMMA nanoparticles were more toxic than PS nanoparticles. Sub-lethal concentrations of PMMA nanoparticles, such as 2.5 mg/L, 7.5 mg/L and 12.5 mg/L, had more effect on zebrafish larvae, delaying and affecting their development, which caused some hatching delays and a higher presence of pericardial edema (7.5 and 12.5 mg/L). This

is the first study with PMMA nanoparticles. The effects of this polymer had not yet been tested in micro or macro sizes. Thus, the comparison with other studies is not possible.

PS nanoparticles sub-lethal concentrations 202.5 and 607.5 mg/L, had also an effect in zebrafish larvae development, delaying the hatching at both concentrations. The highest concentration presented more organisms with pericardial edema and tail malformation.

Most parameters tested during swimming behaviour, exposed that the controls demonstrate higher swimming activity during the dark periods, especially the TD, that is considered a direct measurement of activity levels, which may be positively correlated with stress (Kalueff et al., 2013, Champagne et al., 2010). Former studies documented an increase of TD swam during the dark periods, when compared to the light period (Pitt et al., 2018), which was not the case with the zebrafish larvae exposed to 2.5, 7.5 and 22.5 mg/L of PMMA and 202.5 and 607.5 mg/L of PS nanoparticles. The exposure to these nanoparticles may have caused a change in the physiological response of the organisms to react to sudden changes, making them less anxious during dark periods after a rapid change from light. The thigmotaxis, a self-preservation anxiety-related behaviour, is a parameter measured by the outer distribution of the larvae (TD%). Previous studies revealed that is common to observe an increase in the TD% during dark periods after the sudden change from light, suggesting that this change is a stressor. Being near the perimeter of the environment makes it harder for predators to capture them, making this a defence mechanism (Stryjek and Modlińska, 2013). Once again, after the 96h exposure to 2.5, 7.5 and 22.5 mg/L of PMMA and to 202.5 and 607.5 mg/L of PS nanoparticles, zebrafish larvae revealed a decreased activity when compared to the control. This may indicate that the zebrafish larvae are more sensitive at behavioural level, which is environmental relevant.

Biochemical biomarkers did not present significant differences to control, with the exception of increase CAT and GPx activity in the presence of PS nanoparticles. CAT and GPx are enzymes important in cell defence against oxidizing environments, which help organisms to adapt to new conditions (Oliveira et al., 2010b). The increase of these activities may suggest that the presence of PS caused an increase of H_2O_2 and lipid hydroperoxides.

In terms of effects on sea bass, PMMA and PS had no significant effect in the hepatosomatic index, even though there was a small increase seen when the nanoparticles were present. Erythrocyte abnormalities demonstrated that the PMMA nanoplastics used in these tests were genotoxic which is a highly relevant data. PMMA nanoparticles exposure led to significant increases in the total of anomalies in 0.02 mg/L and 2 mg/L, giving emphasis to the notched nuclei at 0.02 mg/L, which may represent problems during the removal of amplified DNA from the nucleus, and

bud and lobed nuclei at 2 mg/L, which may represent problems in segregating twisted and attached chromosomes or gene amplification via the breakage–fusion–bridge cycle (Mohmood et al., 2008). PS nanoparticles did not cause a significant increase in the presence of anomalies although, in the presence of HA, there was an increase in erythrocytes with different shape of echinocytes.

The sea bass revealed to be sensitive to the presence of nanoparticles through changes in biochemical responses. Compared with the control, intestine GPx activity was significantly increased after fish exposure to 0.02 and 2 mg/L of PMMA. NPT levels were higher than control in organisms exposed to 0.02 and 0.2 mg/L. Gills had increased GR activity at 0.02 and 0.2 mg/L, and the liver presented decreased GST activity at 0.2 mg/L. An increased level of NPT suggested that the organism will be more fit to control the levels of pro-oxidants. NPT is a non-enzymatic defence, having an important role in detoxification and excretion of xenobiotics, which may suggest that the organisms were defending themselves against the presence of the PMMA nanoparticles. CAT is responsible for the reduction of hydrogen peroxide, while GPx catalyses the reduction of both hydrogen peroxide and lipid peroxides. The increase of CAT and GPx activities may suggest that the presence of PMMA caused an increase of H_2O_2 and lipid hydroperoxides. GST is a group of widely distributed enzymes that catalyses the conjugation of reduced glutathione (GSH) and induction of GSTs is known to indicate the presence of various xenobiotics. The decrease of GST activity may be related to diminished levels of GSH susceptible of being conjugated (Carvalho et al., 2012, Oliveira et al., 2010b).

Significant differences to control were found in hepatic CAT activity, with an increase of activity at the concentrations of 0.02 mg/L of PS in the presence of HA and 20 mg/L of PS alone or in the presence of HA. In terms of GST activity, a decrease was found at 0.02 and 20 mg/L of PS in the presence of HA. NPT levels increased in organisms exposed to 0.02 mg/L of PS in the presence of HA and 20 mg/L of PS alone and in the presence of HA. Gills CAT activity was significantly higher than control at 20 mg/L of PS. GR activity, decreased in organisms exposed to 0.02 mg/L of PS in the presence of HA and 20 mg/L of PS alone and in the presence of HA. GPx activity displayed an increased activity in organisms exposed to 0.02 mg/L of PS and a decreased activity was found at 0.02 mg/L of PS in the presence of HA and 20 mg/L of PS alone and in the presence of HA. Compared with the control, there are significant changes seen in Intestine GST activity increased at the concentration of 20 mg/L of PS alone and in the presence of HA. In terms of GPx activity, a decrease was found in organisms exposed to 0.02 mg/L of PS alone and combined with HA. NPT levels were increased at 0.02 mg/L of PS. There are studies that try to comprehend the interaction between micro and nanoparticles with dissolved organic matter. This interaction may cause the nanoplastics

to stabilize, becoming more bioavailable, enhancing their toxicity (Chen et al, 2017, Pomeren et al., 2017).

Still there are not known the ambient levels of polymeric nanoparticles and macroparticles, due to the difficulty in assessing the amount of nanoplastic and macroplastic present in aquatic systems.

Conclusions and Future Perspectives

It was demonstrated that the tested nanoparticles are able to affect the ontogenic development of fish. PMMA demonstrated a higher toxicity towards zebrafish embryos with humic acids significantly affecting the toxic effect by PS. These particles demonstrated ability to interfere with fish normal stress response (thigmotaxis).

In terms of effects on marine juvenile fish, it was shown that these particles are able to alter antioxidant status as induce genetic damage in fish. Considering that nanoplastics are expected to be increasing in the environment as a result of the degradation of macro and microplastics, the present study data are very important and support the need for more studies with nanoplastics.

Based on the obtained data, further studies should be performed, promoting longer exposures and different concentrations. Also, studies using fluorescent nanoparticles to study incorporation and distribution.

Analysis of genes associated with immune responses, cell death and energy metabolism (currently being performed) as well as analysis of cortisol levels (also being performed) could also provide valuable data to understand mechanisms of toxicity.

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