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Ano 2019

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EMBRIÕES DE PEIXE-ZEBRA COMO MODELO BIOLÓGICO PARA O ESTUDO DOS EFEITOS DE NANOPLÁSTICOS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica do Doutor Marcelino Miguel Guedes de Jesus Miguel Oliveira, Equiparado a Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro e coorientação do Doutor Manuel António Martins da Silva, Investigador em Pós-Doutoramento do Departamento de Química da Universidade de Aveiro.

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palavras-chave

Peixe-zebra, nanoplásticos, poliestireno, polimetilmetacrilato, pireno, toxicidade.

resumo

O lixo plástico é atualmente reconhecido como fator crítico para a saúde ambiental. Este problema ambiental é agravado pelo fato de o plástico se ir degradando em partículas cada vez mais pequenas, atingindo dimensões nanométricas, o que aumenta a sua biodisponibilidade e potencialmente os seus efeitos tóxicos. Há, no entanto, poucos estudos sobre os efeitos nanoplásticos (NPs, definidos como partículas de plástico de tamanho inferior a 100 nm), principalmente em vertebrados de água doce. Assim, este trabalho teve como objetivo estudar o potencial efeito deletério de nanopartículas de dois polímeros diferentes, o poliestireno (PS) e polimetilmetacrilato (PMMA), com um tamanho aproximado de ~30 nm, numa gama de concentrações entre 0,001 e 100 mg/L. De forma a garantir que os NPs a testar apresentavam as características desejadas (e.g. tamanho e forma), diferentes metodologias de síntese foram testadas. Após a síntese, os NPs foram purificados com recurso a mangas de diálise, e caracterizados, em água ultra-pura, em termos de tamanho hidrodinâmico (com recurso a espalhamento de luz dinâmico (DLS), tamanho e morfologia (com recurso a microscopia eletrónica (SEM)). Após a caracterização, foram efetuados ensaios experimentais com embriões de peixe zebra que envolveram exposição a apenas NPs e exposições combinadas com um poluente ambiental com uma ampla distribuição em sistemas aquáticos, o pireno, hidrocarboneto aromático policíclico de quatro anéis aromáticos) Nos ensaios apenas com NPs foram avaliados parâmetros como alterações no desenvolvimento, comportamento natatório e resposta de stress; e respostas bioquímicas associadas a neurotransmissão (colinesterase - ChE), metabolismo energético (glicogénio, atividade da isocitrato desidrogenase – IDH e lactato desidrogenase - LDH), resposta antioxidante (glutationa S-transferase - GST, glutationa peroxidase – GPx e a catalase - CAT) e dano oxidativo (peroxidação_lipídica). Nos ensaios em que os embriões foram sujeitos a exposição combinada a NPs e pireno, foram avaliadas apenas alterações no desenvolvimento, comportamento natatório e resposta de stress. De uma forma geral, verificou-se que nas condições experimentais testadas os NPs (redondos, de tamanho médio de 30 nm) não tiveram impacto significativo em termos de mortalidade nem em termos do desenvolvimento dos embriões. Contudo, foram observadas alterações no comportamento natatório das larvas e em parâmetros bioquímicos. Assim, nos organismos expostos a NPs PS, a distância total nadada aumentou nos organismos expostos a 0,001, 1 e 100 mg/L. Em termos de NPs PMMA, a distância total nadada aumentou significativamente nos organismos expostos a 0,001, 0,01 e 1 mg/L. No entanto, a distância nadada na zona externa da área de teste e o tempo passado nessa área (expresso em percentagem) diminuíram nos organismos expostas a 0,001 e 10 mg/L.

Em termos de respostas bioquímicas, verificou-se que NPs PS têm a capacidade de afetar a neurotransmissão (aumentou a atividade da colinesterase na concentração 1 mg/L e diminuiu nas concentrações 0.01, 0.1 mg/L (PS NPs). Nos organismos expostos a PMMA NPs, foi igualmente observada inibição da colinesterase nos organismos expostos a 0.01 mg/L. Em termos de parâmetros associados à resposta antioxidante, NPs PS inibiram a GST (0.01,10 e 100 mg/L) e CAT (0.001 e 10 mg/L), enquanto que a GPx foi induzida (0.001 mg/L) e inibida (0.1 e 1 mg/L). Relativamente ao PMMA, verificou-se que os organismos expostos a estas partículas apresentaram uma indução da GPx (10 mg/L) e aumento da atividade da CAT (0.001, 0.1 e 10 mg/L) e, também se observou uma diminuição da sua atividade na condição 0.01 mg/L. Os dois polímeros demonstraram a capacidade de induzir peroxidação lipídica sendo esse efeito mais evidente para as partículas de PMMA (0.001, 0.01 e 0.1 mg/L) que PS NPs (1 mg/L). Em termos de metabolismo energético, verificou-se que a IDH foi inibida nos organismos expostos a NPs PS (0,001, 0,01, 0,1 e 100 mg/L) e um aumentou a sua atividade nos organismos expostos a NPs PMMA (0,001 mg / L). Os resultados da exposição simultânea a NPs e pireno desmontaram que o efeito de toxicidade (edema do pericárdio e curvatura da cauda) do pireno (0.01 e 1 µg/L) decresceu. Alem disso, a combinação NPs e pireno também reduziu o efeito dos NPs (0.1 e 100 mg/L) no comportamento dos dois polímeros. Assim, demonstra-se que a exposição de curta duração a NPs pode causar alterações a nível bioquímico e comportamental que podem comprometer o bem-estar animal e ter consequências a nível ambiental (e.g. capacidade de adquirir alimento e escapar a reprodutores). Os embriões de peixe zebra demonstraram grande sensibilidade a concentrações baixas de NPs, justificando a sua aplicação em estudos ecotoxicológicos de contaminantes emergentes.

keywords zebrafish, nanoplastic, polystyrene, polymethylmethacrylate, pyrene, toxicity

abstract

Plastic waste is currently recognized as a critical factor for environmental health. This environmental problem is aggravated by fact that plastics fragment to smaller and smaller particles, reaching nanometric dimensions, increasing their bioavailability and potentially their toxic effects. There are, however, few studies of nanoplastics effects (NPs, less than 100 nm plastic size reduction), especially in freshwater vertebrates. Thus, this work aimed to study the potential effect of nanoparticles of two different polymers, polystyrene (PS) and polymethylmethacrylate (PMMA), ~ 30 nm, in a range of concentration between 0.001 and 100 mg / L. In order to ensure that NPs has desirable characteristics (size, shape, different analysis methods have been tested for reproducibility of the final product).After synthesis, the NPs were purified using dialysis sleeves and characterized in ultrapure water, in terms of hydrodynamic size (using dynamic light scattering (DLS), size and morphology (using electron microscopy). Following characterization, experimental assays with zebrafish embryos that involved exposure to NPs (in single exposures and combined with an environmental pollutant with a wide distribution in aquatic systems, pyrene, four rings polycyclic aromatic hydrocarbon). In the NPs single assays, parameters such as developmental changes, swimming behaviour and stress response and biochemical responses associated with neurotransmission (cholinesterase - ChE), energy metabolism (glycogen - Gly, isocitrate dehydrogenase- IDH and lactate dehydrogenase - LDH) and antioxidant response (glutathione) were evaluated. S-transferase - GST, glutathione peroxidase - GPx and catalase - CAT) and oxidative damage (lipid peroxidation - LPO). In trials where embryos were subjected to combined exposure to NPs and pyrene, only developmental changes, swimming behaviour and stress response were evaluated. In general, it was found that under the experimental conditions tested, that NPs (round, average size ~30 nm) had no significant impact on mortality or embryo development. However, changes in larval swimming behaviour and biochemical parameters were observed. Thus, in organisms exposed to PS NPs, the total swim distance increased in organisms exposed to 0.001, 1 and 100 mg/L. In terms of PMMA NPs, total swim distance increased significantly in organisms exposed to 0.001, 0.01 and 1 mg/L. However, the swimming distance and time in outside of the test area (expressed as a percentage) decreased in the exposed organisms at 0.001 and 10 mg/L. In terms of biochemical responses, it was found that PS NPs could affect neurotransmission (causing increased cholinesterase activity at 1 mg/L concentration and decreased at 0.01.0.1 mg/L (PS NPs) and 0.01 mg/L (PMMA). In terms of parameters associated with the antioxidant response, PS inhibited GST (0.01,10 and 100 mg / L) and CAT (0.001 and 10 mg / L), while GPx was induced to (0.001 mg / L) and inhibited at (0.1 and 1 mg / L). PMMA induced GPx (10 mg / L), increased CAT activity (0.001, 0.1 and 10 mg / L). Also, CAT was decreased by PMMA at 0.01 mg/L.

abstract continuation

There was lipid peroxidation in the exposure by PS NPs (1 mg / L) and PMMA NPs (0.001, 0.01 and 0.1 mg / L). In terms of energy metabolism, IDH was found decreased in organisms exposed to PS NPs (0.001, 0.01, 0.1 and 100 mg / L) and an increase for PMMA (0.001 mg / L). Results of simultaneous exposure to NPs and pyrene disassembled that the effect of pyrene toxicity (pericardial oedema and tail curvature) (0.01 and 1 µg / L) decreased. In addition, the combination of NPs and pyrene also reduced the effect of NPs (0.1 and 100 mg / L) on zebrafish swimming behaviour of both polymers. Thus, it is demonstrated that short-term exposure to NPs may cause biochemical and behavioural changes that may compromise animal welfare and have environmental consequences (e.g. ability to acquire food, breeding and escape from predation). Zebrafish embryos demonstrated sensitivity to low concentrations of NPs, justifying their application in ecotoxicological studies of emerging contaminants.

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List of abbreviations

ChE	Cholinesterase
BPA	Bisphenol A
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
D out	Distance out
Dbio	Department of Biology
DLS	Dynamic light Scattering
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
EEA	European Environment Agency
FD	Food and Drug Administration
FET	Fish Embryo Toxicity
Gly	Glycogen
GPx	Glutathione peroxidase
GST	Glutathione S-Transferase
HD	Hexadecane
IDH	Isocitrate Dehydrogenase
KPS	Potassium persulfate
LDH	Lactate dehydrogenase
MMA	Methyl methacrylate
NPs	Nanoplastics
NPs+Pyr	Nanoplastic + pyrene
OECD	Organization for Economic Cooperation and Development
PAHs	Polycyclic Aromatic Hydrocarbons
PBDE	Polybrominated Diphenyl Ether
PMMA	Polymethylmethacrylate
PMMA+Pyr	Polymethylmethacrylate + Pyrene
PS	Polystyrene
PS+Pyr	Polystyrene + Pyrene
Pyr	Pyrene
SDS	Sodium Dodecyl Sulphate

SE	Schottky Emission
SEM	Scanning Electron Microscope
Sty	Styrene
T out	Time out
TBARS	Thiobarbituric Acid Reactive Substance
TD	Total Distance
TT	Total Time
US EPA	United State Environment Protection Agency
USA	United State of America
UV	Ultraviolet
V50	2,2'-Azobis(2-methylpropionamidine) dihydrochloride

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Chapter I

General introduction

1. Plastic use and environmental pollution

Plastics are nowadays a predominant component on Human daily routines (da Costa, 2018; Oliveira, Ameixa and Soares, 2019), having contributed to a considerable improvement of life quality. Described as organic, synthetic or semisynthetic polymers, plastics are easily moulded into various features and artefacts (PlasticEurope,2018). Numerous natural resources (e.g. crude oil, cellulose, coal, natural gas) can be used as precursors for plastic synthesis (Parker, 2018). The plastic polymer chain can achieve mainly through two chemical processes polymerization and polycondensation, in the presence of distinct catalysts (Pavel, 2004; Rabelero, et al.,1997; Wang, et al.,2015). Currently, a wide range of plastic polymers are available in the market (e.g. polycarbonate, polyethylene, polymethylmethacrylate, polystyrene polypropylene, polyurethane, epoxide, polytetrafluoroethylene) presenting different characteristic like resistance and durability. In this work, attention will be given to polystyrene and polymethylmethacrylate polymers, which are plastics extensively used with emerging applications in different fields (PlasticEurope, 2018).

Since the 1950's, an accelerated increase of plastic production has been observed, reaching, in 2017, an estimated world production of 350 million tons (PlasticEurope,2018). The packaging industries demands a large part of the annual production, mainly for products made to assure efficient conservation of food, prolonging their shelf lives, and aseptic condition for medical products. Plastics are also used in other sectors like construction, production of medical devices, (e.g. gloves, syringes), automobile industry (allowing the reduction of the weight of the cars and promote lower carbon dioxide emissions), agriculture, clothing and technological equipment, among others (PlasticEurope,2018).

In order to provide plastics with specific characteristics, needed for specific applications (e.g. colour, durability, heat resistance), chemicals like phthalates, polybrominated diphenyl ether (PBDE), nonylphenols (NP), bisphenol A (BPA), among others, are frequently added during their synthesis processes (Revel, Châtel and Mouneyrac, 2018; Chow et al., 2013; Hermabessiere et al., 2017).

These have been reported to induce toxic effects on different organisms. PBDE, for example, can collapse the digestive system and antioxidant defence in *Daphnia magna* (Zhao et al., 2019); phthalates have been reported to cause malformations and decrease acetylcholinesterase activity in zebrafish embryos and larvae (Roy, Zambrzycka and Santagelo, 2017; Zhang et al., 2014).

The continued increased use of plastics has also led to their environmental release (Jambeck et al.,2015). According to Geyer et al. (2017), in 2015, plastic waste produced was around 6300 million tons and, major part of this (4900 million tons) accumulated in the environment, with greater emphasis to the fresh and seawater bodies. Plastic particles may be dispersed through different environmental compartments by winds, rivers and sea waves/currents (Oliveira, Ameixa and Soares, 2019). As a result, the marine debris is mainly composed by plastic particles (Kaza et al.,2018).

The concerns about the presence of plastic in the environment was raised, for the first time, in 1970s (Carpenter and Smith, 1972). Environmental factors like biotic and/or abiotic (e.g. waves, UV radiation, oxidation) conditions can promote plastics fragmentation into increasingly smaller particles (Coe et al.,2019; Gallo et al., 2018), reaching micro (size comprised between 5 mm and 100 nm) and nano (size lower than 100 nm) (Lambert and Wagner, 2016). The decreased size promotes an increased bioavailability and may also be associated an increased toxicity.

The levels of microplastics in the environment vary considerably. As an example, Desforges et al. (2014) reported particles from 64.8 to 5810 µm at concentrations ranging from 8 to 9200 particles/m^3 in subsurface waters of four oceanographic regions (West coast of Vancouver Island, Northeast Pacific Ocean, Queen Charlote, Soound and Georgia Strait). The study of Peng et al. (2017) reported, in the substrates of Changjiang Estuary (China), levels of microplastics (ranging from 46.8 to 4968.7 µm), between 20 to 340 particles/kg dry weight. Concerning particles of sizes below 80 µm, there are considerable methodological and analytical limitations that do not allow an accurate estimation of their environmental levels (Oliveira and Almeida, 2019). Furthermore, the

complexity associated the behaviour of nanosized particles in natural waters also makes the evaluation of their effects a challenge (Hartmann et al.,2019).

In the nano size range, particles have an increased surface area when compared with bigger particles, leading to a higher potential to adsorb other environmental contaminants (Cooper and Corcoran, 2010), mainly those with hydrophobic characteristics. Recent studies reported toxicity of NPs in organisms like the freshwater organisms *Daphnia pulex* (Liu et al., 2019), *Scenedesmus obliquus* and *Danio rerio* (Liu et al., 2019), as well as in saltwater organisms such as *Mytilus galloprovincialis* (Brandts et al., 2018) *and Crassostrea gigas* (González-Fernández, et al., 2018).

A considerable number of ecotoxicological studies concerning the impact of microplastics has been performed (e.g., Catarino, et al.,2018: Oliveira et al.,2013), but the number of studies focusing on nanoplastics (NPs) is still limited, particularly in the freshwater environment (Liu, et al., 2019). Thus, becomes highly important to provide more data concerning the potential effects of NPs to freshwater organisms. Although it is important to know the effects of NPs and, also important to know their effects in the presence of other environmental contaminants as NP particles in the environment will coexist with other harmful substances. This issue has raised the concern of the scientific community (Riva et al., 2019). Among the different types of contaminants likely to interact with NPs, a concern can be addressed to hydrophobic substances like polycyclic aromatic hydrocarbons (PAHs). Pyrene is a PAH with four aromatic rings with a wide distribution in the aquatic environment, representing a major portion of the total PAHs found in contaminated sites and among the 16 PAHs included in the list of priority pollutants of the United States Environmental Protection Agency (Oliveira, Gravato & Guilhermino, 2012). A previous study has demonstrated that a combined exposure of this PAH with polyethylene microplastics results in altered biological effects on fish, suggesting a toxicological interaction of the mixture (Oliveira et al., 2013). In this study to simulate this environmental reality, we´ll perform a combined exposure with pyrene (PAH with four rings), a pollutant frequently detected in the environment. However, few studies have addressed this issue.

2. Zebrafish (*Danio rerio*) taxonomy and use as a model organism in ecotoxicological studies

Zebrafish is an organism that inhabits freshwater bodies and is widely distributed throughout lakes and rivers of the Indian Northeast (Ganges region), Bangladesh and Myanmar regions (Hock, F.J.,2015). Included in the *Cypriniforms* order, *Cyprinidae* family and *Danio* genus, this fish species is commonly found in ornamental aquaria (Hock, F.J, 2015). This fish has anatomical and physiology characteristics that made it a valuable model organism for scientific studies. Zebrafish use as a model organism started in the 1960s and since then it has become a model organism in different areas of biomedical research (Kimmel at al.,1995; Braunbeck, et al., 2014; Beekhuijzen, et al., 2015), like genetics, development, behaviour, and toxicology. Its genome is already sequenced, and these organisms have a significant physiological and genetic homology to humans (Howe et al., 2013).

The embryonic stage has been receiving an increasing attention in ecotoxicology studies. The eggs and larvae are transparent, allowing to observe embryogenesis states and internal malformations without the need to sacrifice them. The fish embryo test (FET), introduced in 2005 (Braunbeck, et al.,2005) was, after 8 years, accredited by OECD (OECD, 2013). The use of *Danio rerio* embryo as model in ecotoxicological experiments is supported by its proven sensitivity to a wide range of toxic substances and characteristics of the species like production of high number of eggs, rapid development and embryonic transparency (Kimmel et al.,1995). The use of *Danio rerio* embryos has been proposed as a good alternative to the use of adult animals (Braunbeck, et al., 2014; Beekhuijzen, et al., 2015; Busquet et al., 2014; OECD, 2013; Nüßer, et al., 2016; Yan et al., 2019) due to its high sensitivity to contaminants (Awoyem, et al.,2019; Balbi, et al., 2017; Della et al., 2014; Embry, et al., 2010; Oxendine, et al.,2006; Yan et al., 2019) and the fact that non-feeding developmental stages of fish are not categorized as protected stages according to the European Directive 2010/63/EU on the protection of animals used for scientific purposes (Braunbeck et al., 2014). These tests allow the assessment of different anatomical, physiological, biochemical and behavioural endpoints (Pitt et al., 2018, Awoyemi, et al.,2019, Velki, et al., 2019).

3. Objectives and the thesis justification

Water is a fundamental component for life existence on earth, which has been permitting ecosystems sustainability. The presence of plastics in oceans, and rivers became an environmental stress factor that has the potential to compromise environmental and human health (Araujo et al.,2015). The potential impact of plastic can be modulated by different factors like type of polymer, shape and size which can range from macroplastics to nanometric dimensions (Andrady, 2017; Gallo et al., 2018; Lambert and Wagner, 2016). The interactions of large plastic particles with biota are comparatively well known but the effects of small plastic particles that become available to a wide range of organisms from planktonic (Moore et al., 2001) to benthic (Oliveira et al.,2013). The concern related to the potential effects of NPs, have been increasing considering that its behaviour and effects are modulated by environmental factors that alter their behaviour and bioactivity. Researchers are trying to understand the kinetics and properties of NPs of different polymers in different environments and their effects on aquatic organisms (Ribeiro et al., 2019; Mattsson, Doverbratt, and Hansson, 2018; Zhang et al.,2019).

Thus, the general aim of the work was to increase the knowledge of the potential short-term effects, of nanoparticles polymers on freshwater organisms. To achieve this general goal, we performed the nanoplastic synthesis with a specific characteristic like size ~30 nm of two polymers (polystyrene -PS and polymethylmethacrylate - PMMA). *Danio rerio* embryos were exposed to a concentration range of PS and PMMA nm NPs (~30 nm of diameter), selected due to its increasing application in different areas of human activity. Assessed endpoints included parameters associated with ontogenic development, antioxidant status, neurotransmission, energy metabolism and behaviour related endpoints. The potential interaction with environmental pollutants was assessed using pyrene as model contaminant assessing ontogenic development and behaviour. This work was divided into five chapter. In chapter I, a general contextualization of the research problematic is presented; Chapter II presents data the study of the effects of PS NPs; Chapter III, presents the effects of PMMA NPs; Chapter IV presents the effects of the combined exposure of NPs and

pyrene; Chapter V presents a general discussion aiming to integrate all the obtained data and a critical discussion on potential future studies.

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Chapter II

Effects of polystyrene nanoparticles on zebrafish embryos

Abstract

The contamination of water bodies by plastic waste of nanometric dimensions is currently an emerging concern. The present work aimed to investigate the effects of polystyrene nanoparticles (PS NPs ~30 nm) on zebrafish embryos. Thus, zebrafish embryos (2 hpf) were exposed to six concentrations of polystyrene nanoplastics (PS NPs; 0.001, 0.01, 0.1, 1, 10 and 100 mg/L) and parameters associated with development, swimming behaviour and biochemical endpoints related with neurotransmission, oxidative status and energy reserved were assessed. Results showed that PS NPs can alter swimming behaviour of fish larvae. A significant increase in terms of total distance moved by larvae was found in organisms exposed to 0.001, 1 and 100 mg/L. However, the distance swam and, time spent in the outer zone of the test well, decreased in all test conditions. In terms of biochemical endpoints, PS NPs exposure caused variations on cholinesterase activity (ChE) inducing increases (1 mg/L) and decreases (0.01 and 0.1 mg/L after 96h exposure, suggesting modulation of neurotransmission parameters. Considering antioxidant defences and damage biomarkers, PS NPs demonstrated ability to alter the oxidative status causing decreases of glutathione S-transferase (GST, 0.01, 10 and 100 mg/L), glutathione peroxidase (GPx; 0.1 and 1 mg/L) and catalase (CAT; 0.001 and 10 mg/L) activities. However, despite the inhibited defences, oxidative damage assessed as lipid peroxidation was only found in organisms exposed to 1 mg/L PS NPs. In terms of energy reserves, glycogen content increased with in all PS NPs tested concentrations, except 0.001 mg/L. Concerning energy metabolism, PS NPs generated decrease of isocitrate dehydrogenase (0.001, 0.1 and 100 mg/L) and lactate dehydrogenase (0.01 mg/L) activities. Overall, data showed that short-term exposure to PS NPs may compromise the health status of fish as demonstrated by the effects on neurotransmission and behaviour, considered as the outcome of many molecular and physiological responses.

Keywords: nanoplastics, polystyrene, toxicity, behaviour, zebrafish.

1. Introduction

The increased plastic demand, production, use and poor management of plastics resulted the environmental presence and accumulation of plastic debris in the environment. The environmental presence of plastics has raised numerous concerns about eco-sustainability leading to new approaches in the use and management of plastics, involving the 4Rs policy (reduction, reuse, recycling and recovery). The reuse, recycling and recovery strategies has allowed the recovery of more than 8.4 million tonnes of plastic waste collected inside and outside the European region (PlasticsEurope, 2018). However, plastic continues to represent the largest portion of solid waste accumulating in the environment (Achilias et al., 2007; Šilc et al., 2018) already recognized by European Union Marine Framework Directive (2008/56 / EC), what stressed the urgent need for studies on quantity, distribution, type, properties and possible impacts caused by marine litter on aquatic ecosystems (Galgani et al., 2010).

Polystyrene (PS) represents one of the most common types of plastic used in the world (PlasticEurope,2018). It is an aromatic polymer, synthetic thermoplastic, resulting from the polymerization of the styrene monomer, known since the mid-nineteenth century, a transparent organic liquid with a strong odour (Ho, Roberts and Lucas, 2018). Synthesized for the first time by the German organic chemist, Staudinger (Scheirs and Priddy, 2003), polystyrene is considered chemically inert (PlasticEurope, 2015). It has a peculiar consistency, can be produced as foamed plastic and usually is colourless (Ho, Roberts & Lucas, 2018). PS can be easily moulded into various articles, it is widely used as raw material for diverse areas like manufacturing of packaging, electrical appliances, electronic products, construction (e.g. insulation foam, plumbing, lighting equipment), medical equipment (e.g. syringes, needles, cannulas, Petri dishes) (PlasticEurope, 2017). To improve its characteristics and appearances, some additives chemicals are frequently added and, many of them are recognized as an environmental threat (Ho, Roberts and Lucas, 2018). Like

other types of plastic polymers, PS particles are accumulating in large amounts at different natural environments (Mor &Sivan, 2008; Zhang et al., 2018).

Under the presence of biotic (e.g. bacteria) and abiotic (e.g. wind, UV radiation, photo-oxidation) factors macroplastics may break into smaller particles reaching the micro and/or nanometric sizes (Gallo et al., 2018; Lambert and Wagner, 2016; Oliveira; Ameixa and Soares, 2019). Therefore, the study about PS degradation (reaching nanometric sizes), has been performed under laboratory condition (Lambert and Wagner, 2016), so, the nanoparticles can be more bioavailable and bioactive than other plastic size particles. However, currently, the unavailable methodologies/equipment make extremely hard to study the behaviour of NPs in complex media and perform its quantification in natural waters and biota. There are no known environmental levels of NPs and, even for microplastics, known levels refer to the particles usually larger than 80 µm (Oliveira and Almeida, 2019). Nevertheless, the available studies support that there may be considerable consequences of the presence of NPs in the environments (as demonstrated by NPs ability to impact reproduction, behaviour and growth of *Mytilus galloprovincialis, Caenorhabditis elegans, Halomonas alkaliphile*, as reported by (Balbi, et al., 2017; Dong, et al., 2018;; Sun, et. al., 2018).

Thus, this study aimed to increase the scarce knowledge in terms of the impact of NPs to biota, particularly freshwater vertebrate organisms. In this perspective, the effects of PS NPs were assessed using zebrafish embryos as biological model, assessing mortality, effects on development (e.g. hatching, pericardial oedema and tail deformations), swimming behaviour (distance and time spent swimming) and biochemical parameters associated with neurotransmission (cholinesterase - ChE), antioxidant status (glutathione S-transferase - GST, glutathione peroxidase – GPx, catalase – CAT) and), oxidative damage (lipid peroxidation – LPO) as well as endpoints related as energy reserve and metabolism (glycogen - Gly levels , isocitrate dehydrogenase – IDH and lactate dehydrogenase – LDH activities).

2. Materials and methods

2.1. PS NPs synthesis and characterization

The PS NPs synthesis was achieved based on the microemulsion polymerization process based on procedures described by Rabelero, et al. (1997) and Tang, et al. (1984). Briefly, PS NPs were obtained by i) mixing the anionic sodium dodecyl sulphate surfactant (SDS - $NaC_{12}H_{25}SO_4$) and sodium bicarbonate (NaHCO3) in ultrapure water (ultrasonic bath); ii) adding the polystyrene monomer (PS) with hexadecane (HD) and leaving under normal stirring for 30 minutes; iii) sonicating the mixture for seven minutes to obtain a stable emulsion; iv) transferring the contents to a three-arms round bottom flask containing an oval magnetic stirrer; v) Stirring under a nitrogen (N_2) atmosphere for 20 min at 70 °C; vi) Adding the polymerization initiator potassium persulfate (KPS - $K_2S_2O_8$); vii) Allowing the reaction to occur for a maximum of 4 hours. viii) The degree of conversion was calculated after dehydration of the emulsion by calculating the weight of the dry portion. The particles purification performed by washing the synthesized particles through a dialysis sleeve (Bangs, 2013) in an aqueous medium (ultra-pure water) over a period of seven days, with two replacements of the medium per day.

The nanoparticles structural characterization was performed by scanning electron microscopy with a voltage of 500V to 30Kv (SEM - Hitachi SU-70), analytical and high resolution, Schottky emission (SE), at Aveiro Institute of Materials Facilities (CICECO). The analyte was prepared by drying a small amount of the material (approximately one drop) on a copper grid that is attached to the grate holder with the aid of tweezers. Particle characteristics like shape, diameter, size dispersion (average size) were analysed. The hydrodynamic size was analysed using a Malvern zeta sizer Nano ZS equipment. The dynamic light scattering (DLS) technique, was performed only in at zero time, to analyse the hydrodynamic behaviour of the particles. NPs´s samples were prepared from the stock suspension through dilutions in ultrapure water and zebrafish breeding water to achieve concentrations of 0.001, 0.01, 0.1, 10, and 100 mg/L.

2.2. Test organism

Zebrafish (*Danio rerio*) embryos used in this study were obtained from the laboratory culture in the Department of Biology of the University of Aveiro, Portugal. Adult fish are kept under appropriate breeding condition, in a recirculating system (ZebTEC; Tecniplast) with reverse osmosis and activated carbon filtered tap water, complemented with salt (Instant Ocean Synthetic Sea Salt) (Spectrum Brands, USA).

Briefly, fish are maintained at the following conditions: I) Aquariums tanks with 5 pairs per aquarium; II) water temperature of 26 ± 1 °C; III) pH of 7.5 ± 0.5 IV) conductivity of 750 ± 50 µS / cm; V) with dissolved oxygen ≥ 95%; VI) salinity 0.35; VII) photoperiod of 16: 8 (light: dark).

To obtain embryos, fish were in the end of the afternoon transferred to new aquariums with an artificial substrate (marbles) and placed again in circulation system. In the following day, at 9 am the aquaria were removed from the system and allowed to stand. After 2 hours (period of egg deposition and possible fertilization stimulated by the light period) the eggs from each aquarium were collected and viable eggs sorted under a stereomicroscope (Stereo Zoom Microscope-SMZ 1500, NiKon Corporation).

2.3. Experimental design

The tests were performed generally following OECD guideline 236 (OECD, 2013). Seven experimental conditions were selected for the test (0 – control, 0.001, 0.01, 0.1, 1, 10 and 100 mg/L). Organisms were exposed in 24 wells plates, with 2 ml of test medium per well (20 embryos per each test concentration and 4 used as the internal control), kept at a controlled temperature room 27 ± 1 C, with morphological analysis every 24h (Fig. 1). The test suspensions were achieved by dilution of the stock suspension with zebrafish maintenance media water.

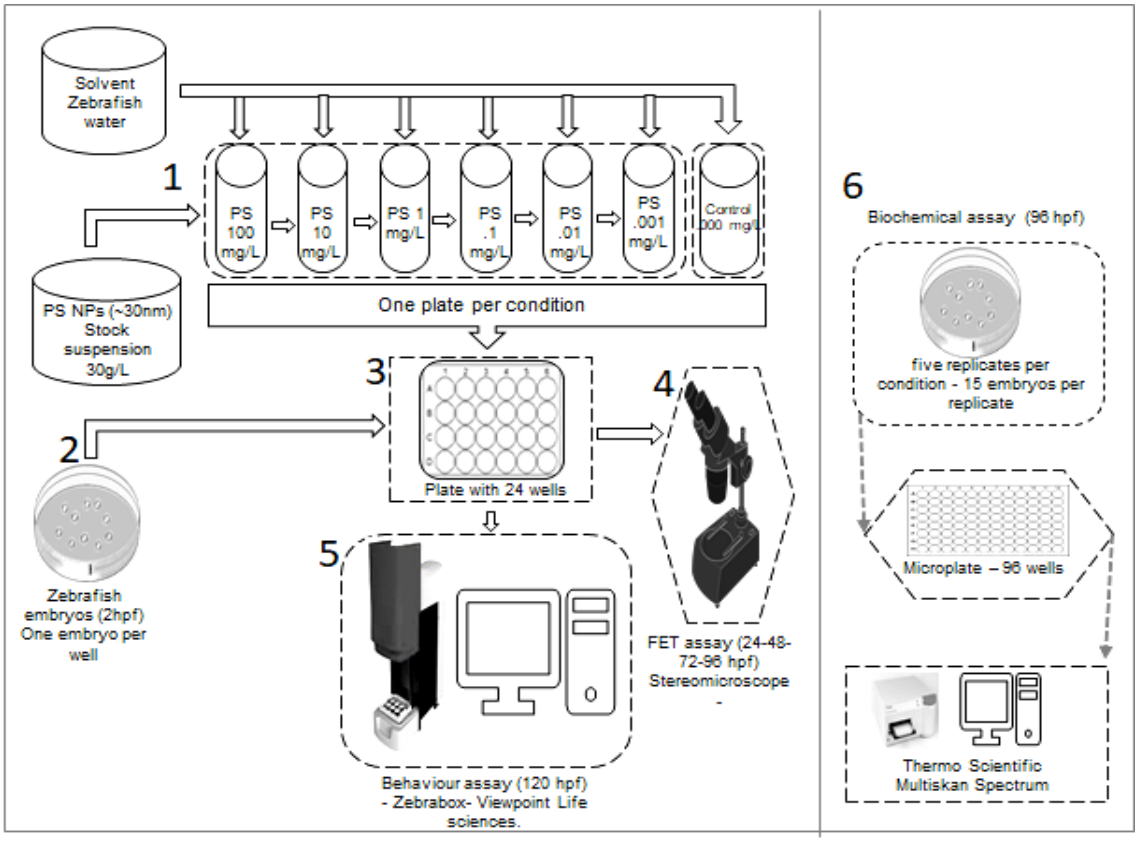

Figure 1. Schematic representation of experimental design. 1) Preparation of test suspensions from stock suspension. 2) Fertilized (viable) eggs (embryos) collection. 3) Filling the plates with the test suspensions, one concentration per plate (2 ml per well - 20 wells and 4 wells filled with the control solution as an internal control). 4) Observations of exposed embryos to analyse lethality or effects on development (24, 48, 72 and 96 hours) – using a stereoscope. 5) behavioural analysis, performed through using Zebrabox, 120 hpf. 6) biochemical test, exposure conditions, sample treatment and analysis method.

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2.4. Fish embryo toxicity (FET) test

FET was performed generally following OECD guidelines (OECD, 2013). Embryos (2 hpf) were exposed to the selected concentrations (0.000 – control, 0.001, 0.01, 0.1, 1, 10 and 100 mg/L) of PS NPs and the effects monitored using a stereomicroscope (Stereo Zoom Microscope-SMZ 1500, NiKon Corporation). The main parameters during the 96h period were: (a) coagulated egg; (b) hatching rate; (c) tail malformation; (d) pericardial oedema.

2.5. Swimming behaviour assessment

The behavioural analysis test was performed when larvae were able to swim freely (120 hpf). Thus, 20 organisms were analysed per condition, following the experimental design described for FET, using Zebrabox - ZEB 478 (Viewpoint Life sciences, Lyon, France), an (automated) tool that allows tracking the movement of the fish in light and dark conditions. For the behaviour analysis two areas of the well were defined (internal and external) which allows the study of potential effects on thigmotaxis. Thus, the following parameters were analysed Total Time spent moving (TT), Total distance (TD), Time and distance travelled in each part of the well (expressed as percentage). Time was defined in seconds and distance in mm.

2.6. Biochemical Endpoints

The biochemical analysis was performed with the same concentrations used in the FET and behavioural tests (0.000, 0.001, 0.01, 0.1, 1, 10 and 100 mg/L). Fifteen embryos per replicate (total of five replicates per condition) were exposed in Petri dishes. After 96 hours exposure, the larvae were collected and transferred to Eppendorf microtubes (2000 µl), frozen in liquid nitrogen and stored at -80 ° C. Samples were homogenized in 0.1 M phosphate buffer solution (pH = 7.4), using a ultrasonic machine (Branson ultrasonic S-250A). The homogenised was divided in portions for lipid peroxidation (LPO) levels estimation, the cholinesterase (ChE) activity determination and antioxidant defences and energy metabolism determination. The samples for ChE determination were centrifuged at 3000 g for three minutes (4ºC), and the supernatant collected for the determination. For antioxidant defences and energy metabolism determination samples were centrifuged for 20 minutes at 10000 g (4 °C) and stored until further processing.

2.6.1. Protein estimation

Protein quantification was performed according to the Bradford method (Bradford, 1976), in 96-well plates, using four technical replicates, at 595 nm (Thermo Scientific Multiskan Spectrum).

2.6.2. Neurotransmition

The ChE activity was determined according to the method described by Ellman's (Ellman's et al., 1961), in 96-well plates. Acetylthiocholine was used as a substrate, and the increase in absorbance (yellow colour increase) caused by the conjugation of acetylthiocoline degradation product, thiocoline, with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), measured at 412 nm, for 5 minutes.

2.6.3. Antioxidant defenses

GST activity was determined according to Habig et al. (1974) assessing the product of conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). Results were expressed as nmol CDNB conjugate formed per minute per mg of protein.

2.6.3.1. Glutathione peroxidase (GPx)

GPx activity was determined according to the method described by Floh é and Günzler (1984), measuring the oxidation of NADPH, at 340 nm. Results were expressed as nmol per minute per mg of protein.

2.6.3.2. Catalase (CAT)

The CAT activity was measured based on the procedure described by Claiborne (1985), adapted to microplate. Absorbance was recorded at 240 nm and results expressed as mmol H_2O_2 consumed per minute per mg protein.

2.6.4. Oxidative damage

Lipid peroxidation (LPO) was determined generally following the procedure described by Oliveira et al, (2010). Absorbance was measured at 535 nm and results expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per mg of protein.

2.6.5. Energy reserve and metabolism

Glycogen concentration was determined according to the method described by Dubois et al. (1956), which basically consists on dehydration of

sugars by sulfuric acid and production of a derivative that reacts with phenol to form a coloured product. Absorption was analysed at 490 nm and results expressed as nmol per min per mg protein.

2.6.5.1. Isocitrate dehydrogenase (IDH)

IDH activity was determined according to Ellis & Goldberg (1971), adapted in 96-well plates as described by Lima et al., (2007). Absorbance was measured at 340 nm. Results were expressed as nmol NADPH regenerated per minute per mg of protein.

2.6.5.2. Lactate dehydrogenase (LDH)

LDH was determined according to the method described by Vassault, (1983) by evaluating NADH oxidation at 340 nm. Results were expressed as nmol of NADH oxidized per minute per mg of protein.

2.7. Data analysis

Results were reported as means ± standard error. The statistical analysis was performed using SigmaPlot software (V12.5). Data was previously tested for normality (Kolmogorov–Smirnov normality test) and homogeneity of variance (Bartlett's test). For each endpoint, different treatments were compared using two-way analysis of variance (ANOVA), followed by Dunnett's comparison test whenever applicable. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. PS NPs characterization

The analyses of the electron microscopy images reveal that PS NPs are not uniform in size and present a spherical shape (Fig.2-a). PS NPs presented a normal distribution and an average size of 22 nm in ultra-pure water. The hydrodynamic size of the synthesized PS NPs (Fig.3-a), in ultrapure water, was

59.25 nm. In zebrafish water (Fig. 3-b), DLS results showed an immediate increase of PS NPs hydrodynamic size, with an average size of 93.87 nm.

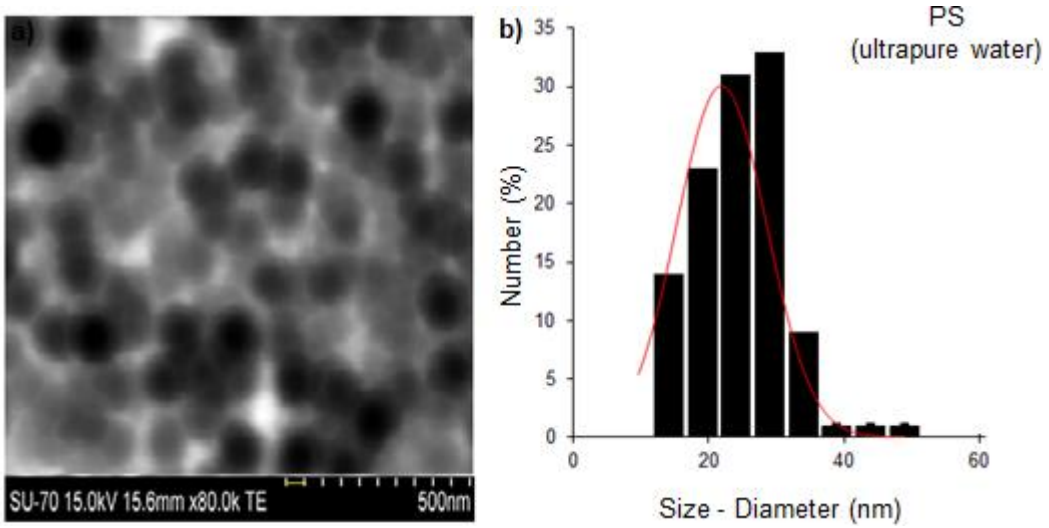

Figure 2. Characterization of polystyrene nanoplastics (PS NPs). a) SEM image of particles in ultrapure water. b) Histogram of size distribution.

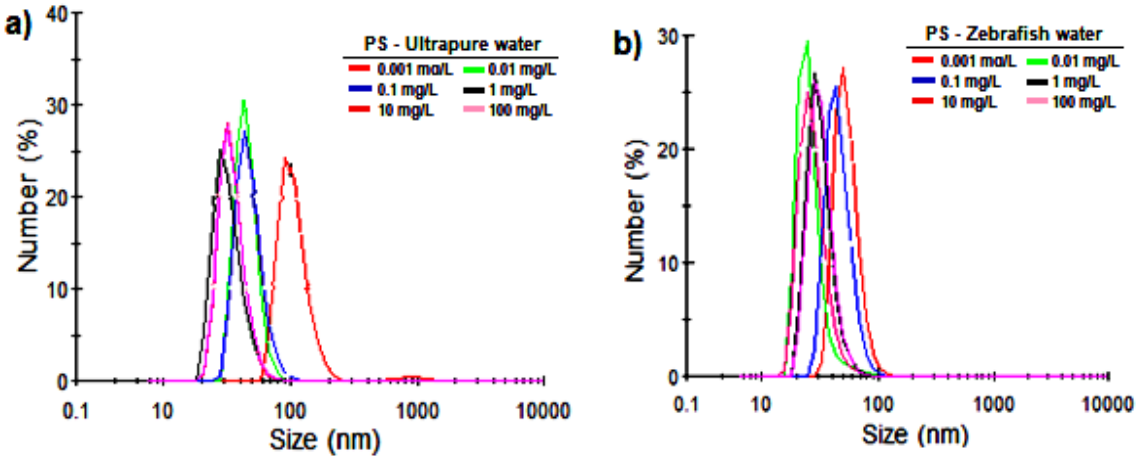

Figure 3. Characterization of polystyrene nanoplastics (PS NPs). a) Hydrodynamic size in ultrapure water. b) Hydrodynamic size in zebrafish maintenance water.

3.2. Effect of PS NPs on embryo development (2 to 96 hpf)

Regarding development, parameters defined in this study were not significantly affected ($p<0.005$) by PS NPs exposure, when compared to control, under the used experimental conditions.

Table 1. Effects of PS nanoparticles (~ 30nm) in the embryonic development stage of zebrafish (96 hours exposure). Results are expressed as a percentage. Parameters assessed were mortality, hatching, pericardial edema and tail malformation.

Time (h)	Concentrations (mg/L)	Parameters			
		Mortality (%)	Hatch (%)	Pericardial edema (%)	Tail deformation (%)
24	0.000	0	--	--	0
	0.001	5	--	--	0
	0.01	5	--	--	0
	0.1	5	--	--	0
	1	0	--	--	0
	10	5	--	--	0
	100	5	--	--	0
48	0.000	0	0	0	0
	0.001	5	0	0	0
	0.01	5	0	0	0
	0.1	5	0	0	0
	1	0	0	5	5
	10	5	0	0	0
	100	10	0	0	0
72	0.000	0	87.5	0	0
	0.001	5	85	0	5
	0.01	5	85	0	0
	0.1	5	85	0	0
	1	0	90	5	5
	10	5	70	0	0
	100	10	20	0	0
96	0.000	0	100	0	0
	0.001	5	95	0	0
	0.01	5	95	0	0
	0.1	5	95	0	0
	1	0	100	5	5
	10	5	95	0	0
	100	10	90	0	0

3.3. Effect of PS NPs on swimming behaviour

3.3.1. Total time (TT)

In terms of TT spent moving, no significant differences ($p<0.05$) were found in organisms exposed to PS NPs when compared to control (Fig. 4). No statistic significant interaction was found between concentration and illumination condition $p>0.05$).

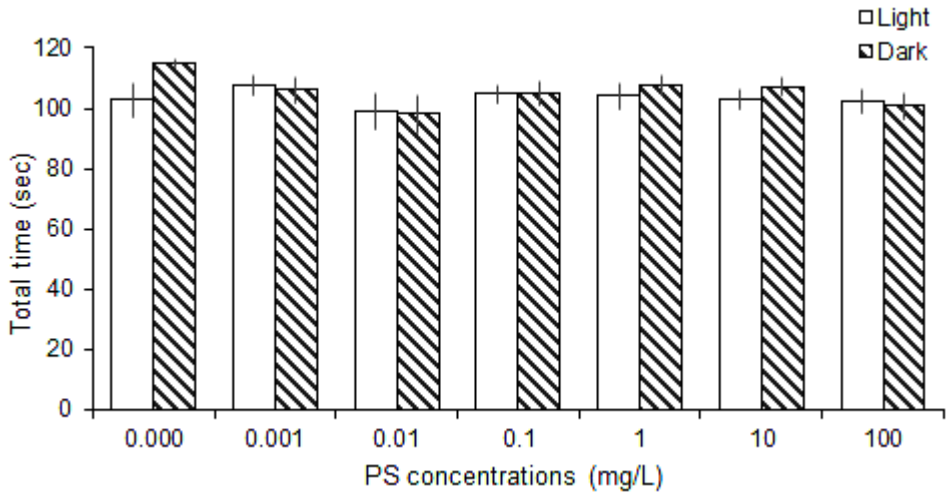

Figure 4. Total time (TT) spent swimming by zebrafish larvae after exposure to PS NPs (~30 nm), under two different light conditions, light period and dark period at 120 hpf,. Results are expressed as means± and standard errors.

3.3.2. Total distance (TD)

TD was increased in larvae exposed to PS NPs at concentrations 0.001 mg/L ($p=0.039$), 1 mg/L ($p=0.041$) and 100 mg/L ($p<0.001$) (Fig.5). However, this effect was only observed during the light period. In dark condition, no significant differences to control were found ($p>0.05$). No statistic significant interaction was found between concentration and illumination condition.

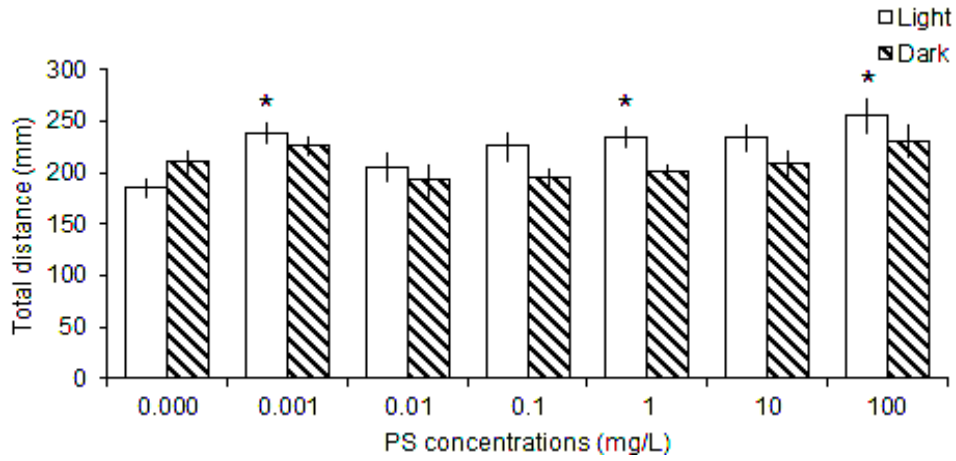

Figure 5. Total distance (TD) moved by zebrafish larvae after exposure to PS NPs (~30 nm), under two different light conditions, light period and dark period at 120 hpf. Results are expressed as means ± standard errors. Asterisks (*) denote significant differences between PS NPs and control within the same light period.

3.3.3. Time spent swimming in the out zone (%T out)

The % Tout data is expressed in Fig. 6. Significant effects of PS NPs exposure were found in this parameter. PS NPs induced a decrease in groups treated with 0.1, 1 and 100 mg/L, in the light period, with a higher effect in larvae exposed to 100 mg/L ($p<0.001$). In the dark period, PS NPs induced significant effects in a higher number of concentrations (0.01; 0.1; 1; 10 and 100 mg/L). The highest significance was recorded in larvae exposed to 1 and 100 mg/L ($p<0.001$), followed by 10mg/L ($p=0.004$) and 0.01 mg/L ($p=0.021$). No significant interaction between concentration and illumination.

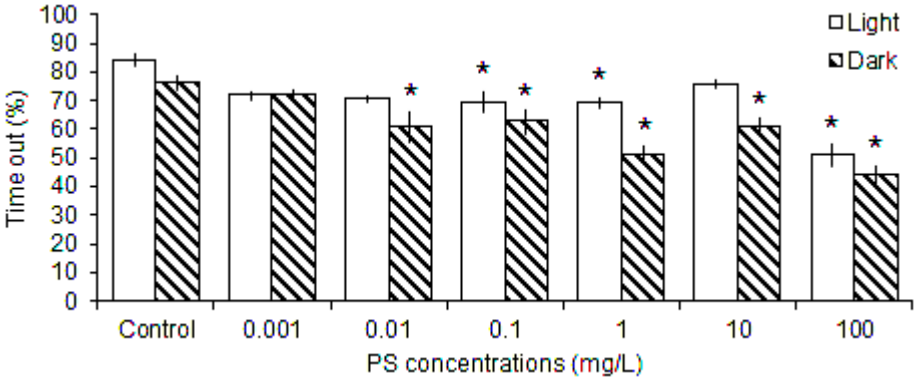

Figure 6. Time out (%Tout) moved by zebrafish larvae after exposure to PS NPs (~30 nm), under two different light conditions, light period and dark period at 120 hpf. Results are expressed as means ± standard errors. Asterisks (*) denote significant differences between PS NPs and control within the same light period.

3.3.4. Distance travelled in the outer area of the well (% D out)

In terms of %D out (Fig.7), significant effects of PS NPs exposure were found. Despite the lack of significant interaction between concentrations and illumination condition, significant differences to control were found in all exposed groups. However, two concentration induced the significant difference ($p<0.005$) only in light (0.001 and 0.1 mg/L) and other remaining conditions (0.01,1,10 and 100 mg/L) at both light and dark.

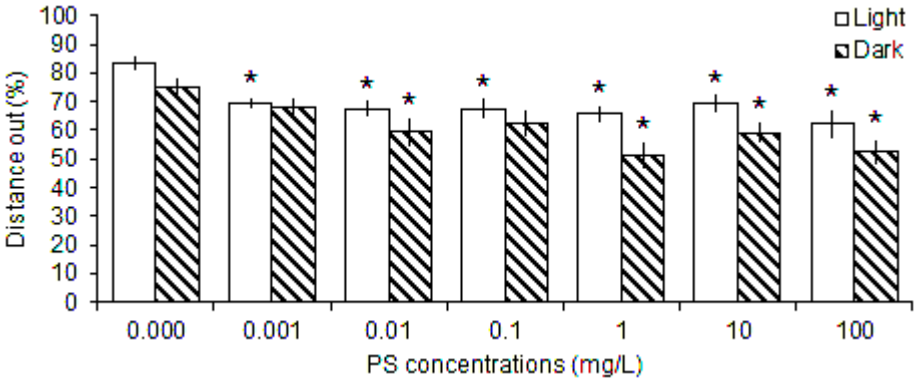

Figure 7. Distance out (%Dout) moved by zebrafish larvae after exposure to PS NPs (~30 nm), under two different light conditions, light period and dark period at 120 hpf. Results are expressed as means ± standard errors. Asterisks (*) denote significant differences between PS NPs and control within the same light period.

3.4. Effect on biochemical Endpoints

3.4.1. Neurotransmission

PS NPs displayed significant effects ($p<0.05$) on ChE activity (Fig.8). However, the reported effects varied with concentration. A decreased activity was observed in organisms exposed to 0.01 and 0.1 mg/L PS NPs. However, a significant increase was found at 1 mg/L.

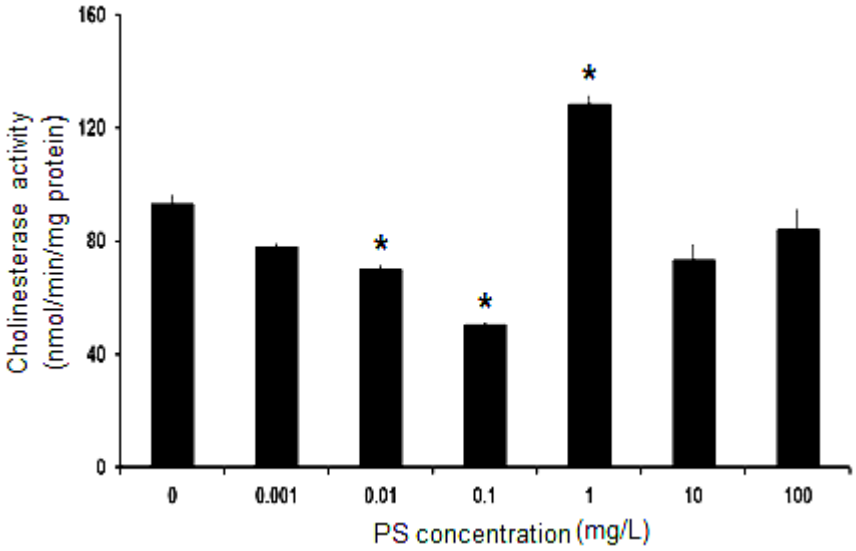

Figure 8. Effect of PS NPs (~30 nm) on cholinesterase (ChE) activity of zebrafish embryos after 96 hours exposure. Results are expressed as means ± standard errors. Asterisks (*) denote significant differences between PS NPs and control.

3.4.2. Antioxidant defences

PS NPs also demonstrated the ability to alter antioxidant related endpoints. Thus, GST activity was significantly decreased ($p<0.005$) in organisms exposed to 0.01, 10 and 100 mg/L (Fig.9).

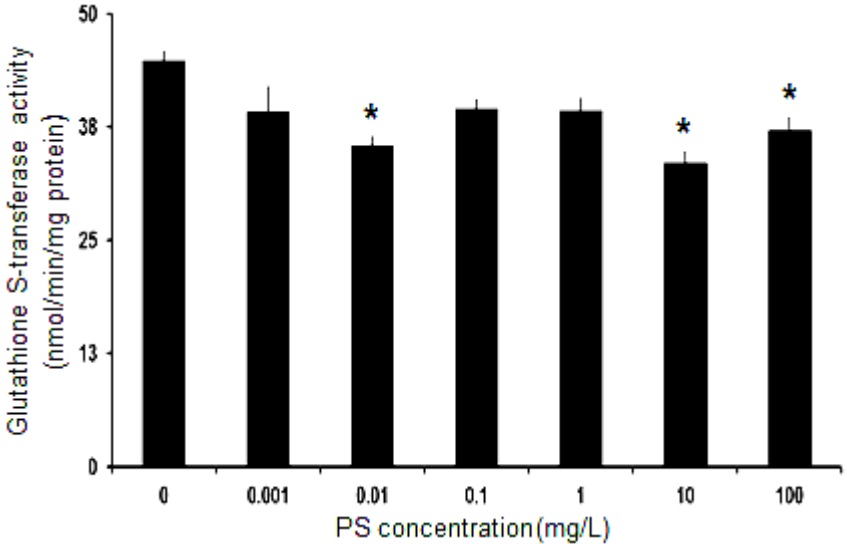

Figure 9. Effect of PS nanoparticles (~30 nm) on glutathione *S*-transferase (GST) activity of zebrafish embryos after 96 hours exposure. Results are expressed as means ± standard errors. Asterisks (*) denote significant differences between PS NPs and control.

3.4.2.1. Glutathione peroxidase (GPx)

Concerning GPx (Fig.10), exposure to 0.001 mg/L PS NPs increased this antioxidant enzyme activity ($p<0.05$) whereas 0.1 and 1 mg/L PS NPs induced significant decreases ($p<0.05$), when compared to controls.

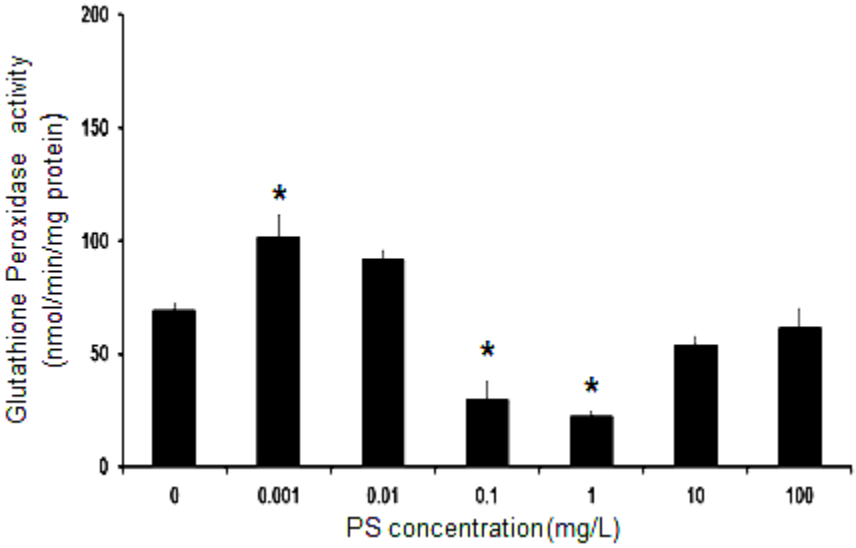

Figure 10. Effect of PS nanoparticles (~30) on glutathione peroxidase (GPx) activity activity of zebrafish embryos after 96 hours exposure. Results are expressed as means ± standard errors. Asterisks (*) denote significant differences between PS NPs and control.

3.4.2.2. Catalase (CAT)

PS NPs caused a down-regulation of CAT activity (Fig. 11) in organisms exposed to 0.001 and 10 mg/L ($p<0.05$).

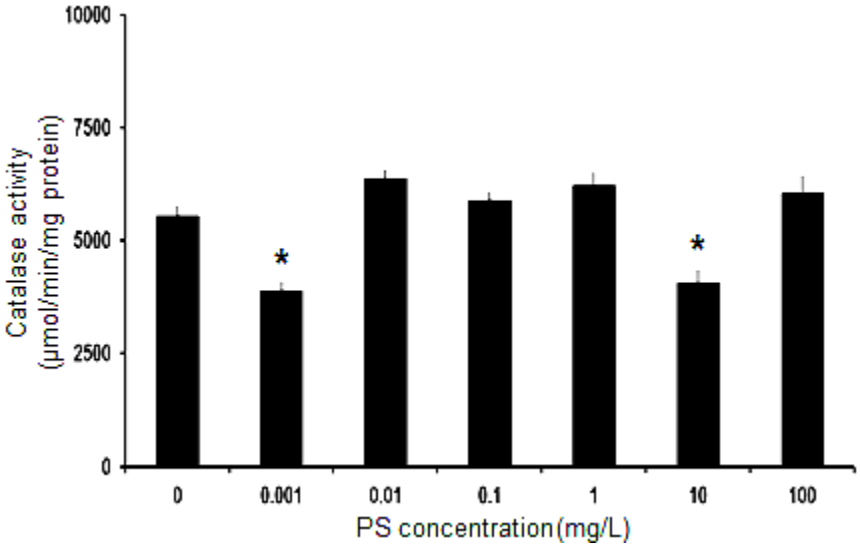

Figure 11. Effect of PS nanoparticles (~30 nm) on catalase (CAT) activity of zebrafish embryos after 96 hours exposure. Results are expressed as means ± standard errors. Asterisks (*) denote significant differences between PS NPs and control.

3.4.3. Oxidative damage

Oxidative damage assessed as LPO (Fig.12) significantly increased in organisms exposed to 1 mg/L ($p<0.05$). However, decreased TBARS levels, when compared to control, were found at 0.1 mg/L ($p<0.05$).

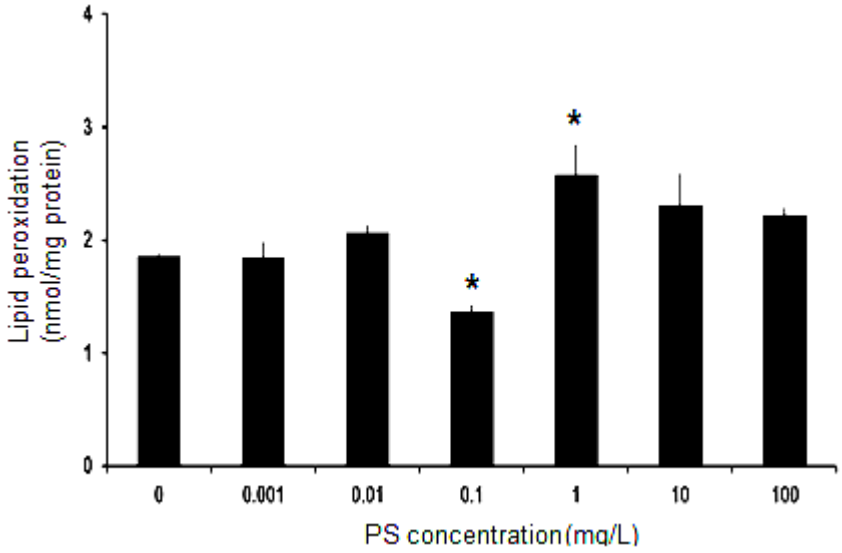

Figure 12. Effect of PS nanoparticles (~30 nm) on lipid peroxidation (LPO) levels of zebrafish embryos after 96 hours exposure. Results are expressed as means ± standard errors. Asterisks (*) denote significant differences between PS NPs and control.

3.4.4. Energy metabolism

Considering the energy metabolism, glycogen displayed an increase trend in organism exposed to PS NPs (Fig. 13) although only significantly for 0.01, 0.1,1,10 and 100 mg/L ($p<0.05$).

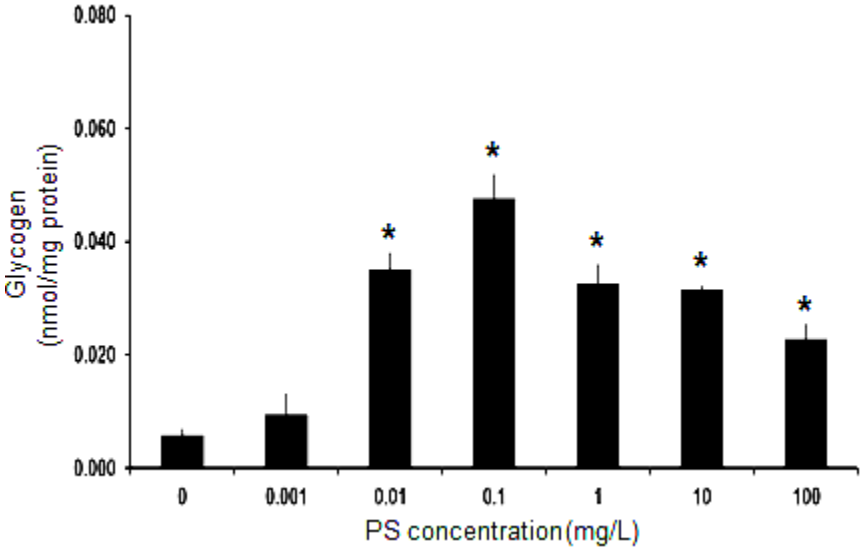

Figure 13. Effect of PS nanoparticles (~30 nm) on glycogen levels of zebrafish embryos after 96 hours exposure. Results are expressed as means ± standard errors. Asterisks (*) denote significant differences between PS NPs and control.

3.4.4.1. Isocitrate dehydrogenase (IDH)

IDH activity was also affected by PS exposure (Fig. 14). Significant lower activities ($p<0.05$) when compared to control were found for organisms exposed to 0.001, 0.1 and 100 mg/L.

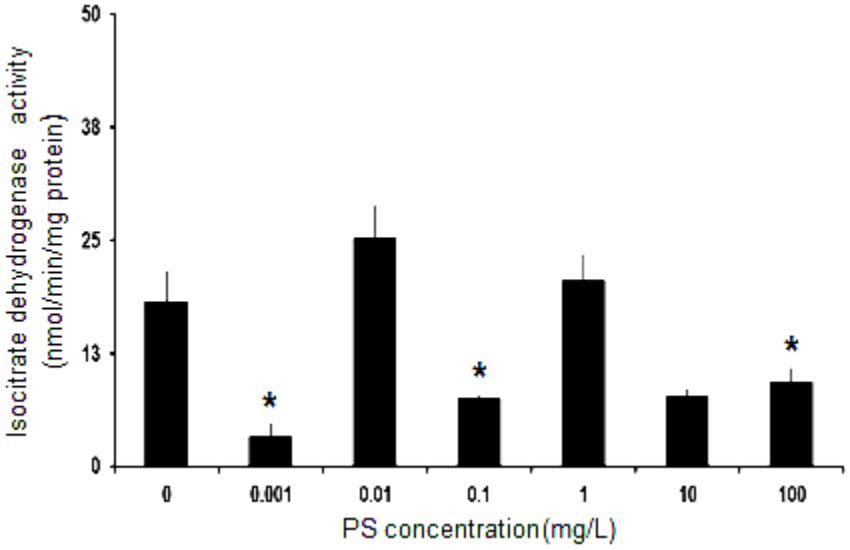

Figure 14. Effect of PS nanoparticles (~30 nm) on isocitrate dehydrogenase (IDH) activity of zebrafish embryos after 96 hours exposure. Results are expressed as means ± standard errors. Asterisks (*) denote significant differences between PS NPs and control.

3.4.4.1. Lactate dehydrogenase (LDH)

LDH activity was also affected in embryos exposed to PS NPs but significant effects ($p<0.05$) were only found in organisms exposed to 0.01mg/L, where a significant decreased activity.

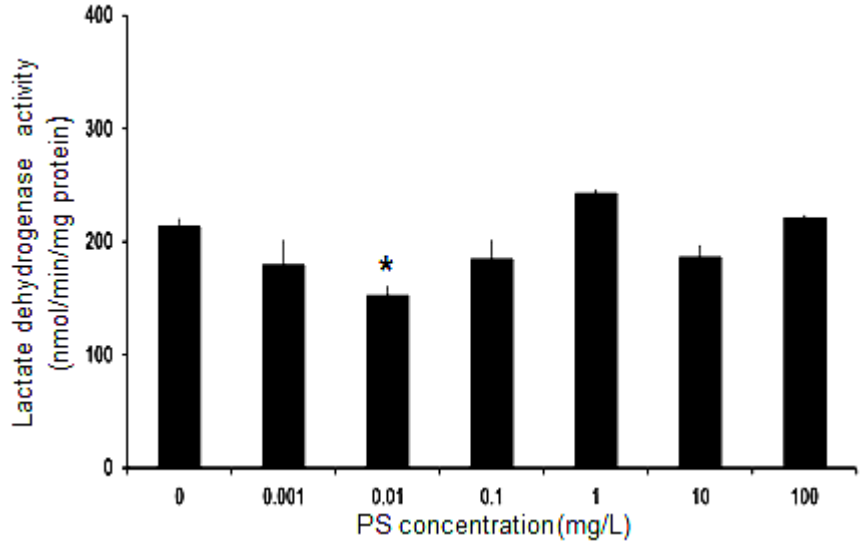

Figure 15. Effect of PS nanoparticles (~30 nm) on lactate dehydrogenase (LDH) activity of zebrafish embryos after 96 hours exposure. Results are expressed as means±standard errors. Asterisks (*) denote significant differences between PS NPs and control.

4. Discussion

The short-time analysis of the effects of the contaminants is important for the environmental risk assessment. Moreover, performing the test in early life stage is fundamental to understanding its impact that can subsequently affect the organism's population. In addition, the biochemical and behavioural endpoints present a promising approach to unravel the potential mechanisms of contaminants.

The present work aimed to provide data in terms of the potential consequences of PS NPs to aquatic organisms. Thus, zebrafish embryos were exposed to PS NPs during 96 h to assess biochemical endpoints and effects on development and 120 h to assess effects on swimming behaviour. Previous studies have reported the ability of nanoparticles to accumulate in the yolk sac of the zebrafish embryo and, subsequently migrate to other parts like heart, brain, liver (Pitt et al., 2018; Rossi, Barnoud & Monticelli). Acute exposure to PS NPs at different sizes (50, 500 and 34.5±10.8 nm) has been reported to cause diverse negative effects on zebrafish embryos, (e.g.: decreasing growth rate, inducing oxidative stress and causing swimming behaviour alterations (Lee et al., 2019; Parenti et al., 2019; Pitt et al.,2018)

In this work, PS NPs did not induce significant effects on zebrafish embryo development, supporting the previous findings of Pitt et al. (2018) with the particles size of 34.5±10.8 nm. Concerning swimming performance, PS NPs significantly increased the zebrafish TD after exposure to different concentrations (0.001, 1 and 100 mg/L), but the time and distance travelled by zebrafish larvae in outer zone of the test well were significantly decreased by PS NPs at all concentrations. This later finding seems to suggest that larvae are not displaying a thigmotaxis behaviour. The obtained data support the need for more studies considering that the altered behaviour may affect fish daily routines like feeding or escaping from predators.

Regarding the nervous system function, PS NPs altered ChE activity although effects were antagonic depending on concentrations. Thus, ChE activity was decreased at the lower concentrations (0.01 and 0.1 mg/L) and increased in the group exposed a 1 mg/L.

The ChE activity alteration in zebrafish, exposed during 120 hours to PS NPs with a seize of ~30 nm, was also reported by Chen et al., (2017). The reported effects of PS NPs on ChE is a highly relevant effects considering the important functions of ChE which may compromise the survival of fish. The link between altered ChE levels and the observed altered behaviour is, however, not straightforward as no decreased movement was found in organisms presented decreased ChE.

The assessed antioxidant defenses were sensitive of PS NPs exposure as reported in other studies (Chen et al., 2017). GST activity, a phase II of biotransformation enzyme, also involved antioxidant defense, was significantly decreased (0.01, 10 and 100 mg/L), suggesting a decreased metabolism and defense against hydroperoxides. GPx increased in the lowest tested unlike CAT that was inhibited. Organisms exposed to 0.1 and 1 mg/L displayed a decreased GPx, whereas inhibition of CAT activity was also found in organisms exposed to 10 mg/L. Chen at al., (2017) study reported no effect on GPx and CAT activities, on zebrafish (~30 nm). In this study, oxidative damage, assessed as LPO, was only found in organisms exposed to PS NPs 1 mg/L, despite the observed inhibition of antioxidant defences. A previous study with PS NPs of 100 nm also reported oxidative damage in the marine mussel *Mytilus galloprovincialis* exposed to at 0.05 mg/L (Brandts et al., 2018). In this study, at 0.1 mg/L decreased TBARS levels were found which seems to suggest activation of defences and repair mechanisms.

The effect of xenobiotics on energy metabolism has been used as a tool to study potential mechanisms of action (Oliveira et al.,2010; Almeida et al.,2019). In this study, glycogen levels, LDH and IDH activities were assessed. Unexpectedly Glycogen levels increased in the presence of PS NPs suggesting a potential activation of synthesis or decreased ability to mobilize this reserve, despite the increases in TD. However, LDH which is involved in anaerobic metabolism and IDH, involved in aerobic metabolism were significantly reduced suggesting that the ability of larvae to produce energy may have compromised by PS NPs.

Overall, this study demonstrated that PS NPs do not alter developmental abnormalities in zebrafish embryos but can have other serious consequences like altered energy metabolism, neurotransmission and behaviour. Further studies should be performed to understand the reversibility of these effects and the size dependent effects of PS NPs.

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Chapter III

Effects of polymethylmethacrylate nanoparticles on *D. rerio* embryos

Abstract

An extensive production associated with the lack of policies that guarantee the responsible use of plastics led to its ubiquitous presence in the environment. Plastics have been detected in areas beyond human regular presence and plastic litter is now considered as a big threat for ecosystems. A problem associated with plastic litter is that particles tend to degrade into smaller particles reaching the nanometric size range, where particles become highly bioavailable and reactive. At this size range, particles have been reported able to interact with organic endogenous components, posing a big threat for biota. The effects of nanosized particles are largely unknown particularly for polymers like polymethylmethacrylate (PMMA), a polymer that has been increasingly used. Thus, the effects of PMMA NPs was assessed in terms of lethal and sublethal effects (developmental, behavioural and biochemical alterations) on zebrafish embryos. Results revealed that PMMA NPs can alter fish swimming behaviour. A significant increase was observed in terms of total distance moved (0.001,0.01 and 1 mg/L).However the time spent and distance moved in the out zone of well (test area to analyse the thigmotaxis effects) were a significant decreased (0.001 and 10 mg/L). PMMA NPs demonstrated the ability to inhibit the cholinesterase activity (0.01 mg/L). Antioxidant defences were also altered after PMMA NPs exposure. Catalase (CAT) activity had a significant increase (0.001, 0.1 and 10 mg/L) and decrease (0.01 mg/L), whereas glutathione peroxidase (GPx) was significantly increased (10 mg/L). PMMA NPs induced increased oxidative status that was reflected in peroxidative damage assessed as lipid peroxidation (0.001, 0.01 and 0.1 mg/L). Concerning energy metabolism, isocitrate dehydrogenase (IDH) activity was induced by PMMA NPs (0.001 mg/L) whereas lactate dehydrogenase (LDH) activity was inhibited (0.01 mg/L). Overall, this study demonstrated the ability of PMMA to induce toxic effects on zebrafish larvae supporting the need of further studies to understand mechanisms of toxic action.

Keywords: Nanoplastic, polymethylmethacrylate, behaviour, biomarkers, toxicity

1. Introduction

The benefits of using plastic has boosted its large-scale production. The 1950s is considered as the start of its global production in large quantities. For several decades production has been increasing and, between 2016 and 2017, production increased 13 million tons (PlasticEurope, 2018). Because of this and due to the lack of applicability of the policies that guarantee its responsible use, plastic has become ubiquitous in the environment. Plastics have been detected in locations of wide human activity, terrestrial (Chae & An, 2018) and aquatic environments (Andrady, 2011; Burji, et al., 2019; Cincinelli, et al., 2017), as well in areas considered untouched, like in the Arctic Antarctic region (Munari, et al., 2017; Peeken, et al., 2018; Waller et al., 2017). Thus, the contamination of water bodies all over the globe by solid plastic waste is currently an emerging concern.

Among the widely available polymers, polymethylmethacrylate (PMMA) has been used in several applications, including biomedical (Puppi et al., 2018). This polymer is produced by the polymerization reaction of methylmethacrylate monomer (MMA), a volatile liquid mainly derived to crude oil (Harrison, 2007). Discovered in the early 1930s, by British chemists John Crawford and Rowland Hill, it had its first major use during World War II, when it was employed in aircraft windows (The Editors of Encyclopaedia Britannica, 2018). Presenting different characteristics like resistance and durability, polymethylmethacrylate has been used extensively in emerging applications of different fields (PlasticEurope, 2018). Due to a reported biocompatibility (Linan et al., 2018), is used as raw material for prostheses manufacture, for substitution of living organs (e.g.: bone implant of the facial contour) (Puppi et al., 2018). As demonstrated by Lambert and Wagner, (2016), plastic (PS) can degraded into tiny particles reaching a nanometric size (100 nm), rising the hypothesis that PMMA can also degraded to smaller particles.

In nanometric dimensions, plastics become more complex and potentially more toxic. Its potential ecological impact has been supported (Dong, et al., 2018; Li, et al., 2019; Sun, et al., 2018). In this context, despite the existence of

studies, reporting nanoplastics toxicity (Dong, et al., 2018; Sun, et al., 2018), they are still considered scarce when compared with those performed for other classes of nanomaterials like metallic (Hu et al., 2018; Qin, et al., 2018), carbon (He et al., 2018; Yi, et al., 2018).

Therefore, this work aimed to analyse the deleterious effect of PMMA nanoparticles, on zebrafish during its embryonic and larval phase in terms of embryonic development, larval behaviour and biochemical endpoints associated with oxidative stress (glutathione S-transferase - GST, Glutathione peroxidase - GPx, catalase - CAT and lipid peroxidation - LPO); neurotransmission (cholinesterase activity - ChE), and energy metabolism (glycogen levels - Gly, Isocitrate dehydrogenase – IDH and lactate dehydrogenase – LDH activities).

2. Materials and methods

2.1. Synthesis and characterization of polymethylmethacrylate (PMMA) nanoparticles

PMMA nanoparticles were obtained by a microemulsion polymerization reaction, following a procedure adapted from Roy and Davi (1996). First, the anionic surfactant, sodium dodecyl sulphate (SDS - $NaC_{12}H_{25}SO_4$) was dissolved in ultrapure water (sonicator) then, mixed with the methyl methacrylate monomer (MMA). The reaction occurred at constant stirring under a nitrogen atmosphere (N 2) at 70 °C. Then, 2,2'-Azobis (2-amidinopropane) dihydrochloride (V50), previously dissolved in ultrapure water, was added as initiator.

The reaction occurred for 2 hours. The conversion rate was calculated by calculating the weight of the dry portion. The product was purified by a dialysis sleeves (Bangs, 2013) in aqueous medium (ultrapure water) over a period of 7 days with two medium replacements per day.

The characterization of PMMA nanoparticles was done through two known and commonly used methods, such as dynamic light scattering (DLS) (Brandts et al., 2018) which gave a description of the particle size and its hydrodynamic distributions. Another method was the scanning electron

microscope (SEM) technique (Ahluwalia, et al., 2018). It should be noted that the equipment, as well as the samples number, were the same as described in the previous chapter, specifically in the characterization of polystyrene nanoparticles.

2.2. Test organism

The adult zebrafish (*Danio rerio*), was obtained from the laboratory culture in the Department of biology at the University of Aveiro, Portugal. Adult fish are kept under appropriate breeding condition, in a recirculating system (ZebTEC; Tecniplast) with reverse osmosis and activated carbon filtered tap water, complemented with salt (Instant Ocean Synthetic Sea Salt) (Spectrum Brands, USA). The culture is kept in aquariums with a water temperature of 26± 1 ° C; pH 7.5 ± 0.5, conductivity 750 ± 50 µS / cm; dissolved oxygen ≥ 95%; salinity 0.35 ppt; 16: 8 photoperiod (light: dark) and fed daily.

2.3. Experimental design

The tests were performed generally following OECD guideline 236 (OECD, 2013). Seven experimental conditions were selected for the test (0 – control, 0.001, 0.01, 0.1, 1, 10 and 100 mg/L). Organisms were exposed in 24 wells plates, with 2 ml of test medium per well (20 embryos per each test concentration and 4 used as the internal control), kept at a temperature-controlled room 27 ± 1 C, with morphological analysis every 24h (Fig. 1). The test suspensions were achieved by dilution of the stock solution with zebrafish maintenance media water.

2.4. The fish embryo toxicity (FET) test

Embryo toxicity testing was performed according to the OECD guidelines (OECD, 2013). Embryos were exposed two hours post fertilization (2 hpf) for a period of 96 hours. Periodic observations were performed using a stereomicroscope (Stereo Zoom Microscope-SMZ 1500, NiKon Corporation) which allowed the analyses of the following parameters: (a) coagulated eggs (mortality), (b) hatching, (c) pericardial oedema; (d) tail malformation.

2.5. Swimming behaviours Assessment

The behavioural analysis test was performed when larvae were able to swim freely (120 hpf). Thus, 20 organisms were analysed per condition, following the experimental design described for FET, using Zebrabox - ZEB 478 (Viewpoint Life sciences, Lyon, France), an (automated) tool that allows tracking the movement of the fish in light and dark conditions. For the behaviour analysis two areas of the well were defined (an internal and external) which allows the study of potential effects on thigmotaxis. Thus, the following parameters were analysed: total time spent moving (TT), total distance (TD), time and distance travelled in each part of the well (expressed as percentage). Time was defined in seconds and distance in mm. The periods were divided into light and dark. So, first, the larvae were exposed in light for 180 seconds and following by suppression of light (dark stimulus) also 180 seconds.

2.6. Biochemical Endpoints

To perform the biochemical analysis six experimental concentrations were tested (0.001, 0.01.0.1,1.10 and 100 mg/L). Embryos were divided into five replicates with fifteen embryos per replicate. After the exposure period (96 hpf), the larvae were transferred to microtubes (2 ml), frozen in liquid nitrogen and kept at -80 ° C.

Samples were homogenized in 0.1 M phosphate buffer solution (pH = 7.4), using a ultrasounds homogenizer (Branson ultrasonic S-250A). The homogenised was divided in portions for lipid peroxidation (LPO) estimation, cholinesterase (ChE) activity determination and antioxidant defences and energy metabolism determination. The samples for ChE determination were centrifuged at 3000 g for three minutes (4ºC), and the supernatant collected and stored at -80ºC until determination. For antioxidant defences and energy metabolism determination samples were centrifuged for 20 minutes at 10000 g (4 °C) and stored at -80ºC until further processing.

2.6.1. Protein estimation

Protein quantification was performed according to the Bradford method (Bradford, 1976), in 96-well plates, using four technical replicates, at 595 nm (Thermo Scientific Multiskan Spectrum).

2.6.2. Neurotransmission

The method described by Ellman (Ellman et al., 1961) was used to determine ChE activity in 96-well plates. Acetylthiocholine was used as the substrate, so, was analysed the increase of its absorbance (yellow colour increase) which originated from the conjugation of the acetylthiocholine degradation product (thiocoline) and 5,5-dithio-bis-2- nitrobenzoic acid (DTNB), assessed at 412 nm for 5 minutes.

2.6.3. Oxidative stress

GST activity was determined according to Habig et al. (1974) assessed the product of conjugation of reduced glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB). Results were expressed as nmol CDNB conjugate formed per minute per mg of protein.

2.6.3.1. Glutathione peroxidase (GPx)

GPx activity was determined according to the method described by Floh é and Günzler (1984), measuring the oxidation of NADPH, at 340 nm. Results were expressed as nmol per minute per mg of protein.

2.6.3.2. Catalase (CAT)

The CAT activity was measured based on the procedure described by Claiborne (1985), adapted to microplate. Absorbance was recorded at 240 nm and results expressed as mmol H_2O_2 consumed per minute per mg protein.

2.6.4. Oxidative damage

Lipid peroxidation (LPO) was determined generally following the procedure described by Oliveira et al, (2010). Absorbance was measured at

535 nm and results expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per mg of protein.

2.6.5. Energy reserve and metabolism

Glycogen (Gly) concentration was determined according to the method described by Dubois et al. (1956), which basically consists on dehydration of sugars by sulfuric acid and produces a derivative that reacts with phenol to form a coloured product. Absorption was analysed at 490 nm and results expressed as nmol/mg protein.

2.6.5.1. Isocitrate dehydrogenase (IDH)

IDH activity was determined according to Ellis & Goldberg (1971), adapted in 96-well plates as described by Lima et al., (2007). Absorbance was measured at 340 nm. Results were expressed as nmol NADPH regenerated per minute per mg of protein.

2.6.5.2. Lactate dehydrogenase (LDH)

LDH was determined according to the method described by Vassault, (1983) by evaluating NADH oxidation at 340 nm. Results were expressed as nmol of NADH oxidized per minute per mg of protein.

3. Results

3.1. PMMA NPs Characterization

The electron microscopy images revealed that PMMA NPs are not uniform in size and present a spherical shape (Fig.1-a). PMMA NPs had a normal distribution and an average size of 32 nm in ultra-pure water. The hydrodynamic size of PMMA NPs (Fig.2-a) in ultrapure water was 109 nm. In zebrafish water (Fig. 3-b), an increase of PMMA NPs hydrodynamic size was found with particles presenting an average size of 210 nm.

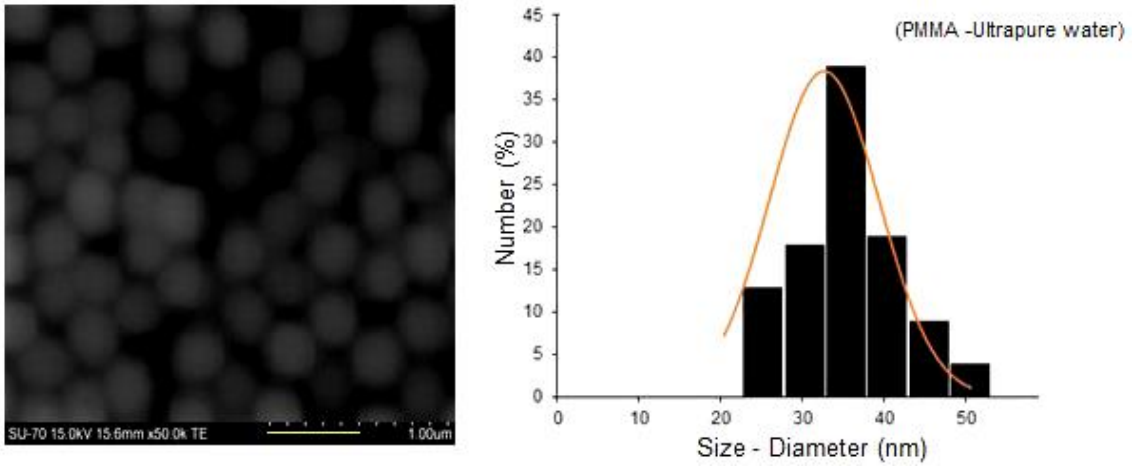

Figure 1. Characterization of polymethylmethacrylate nanoparticle (PMMA NPs). a) Electron microscope (SEM) of PMMA NPs image in ultrapure. b) Histogram of size distribution

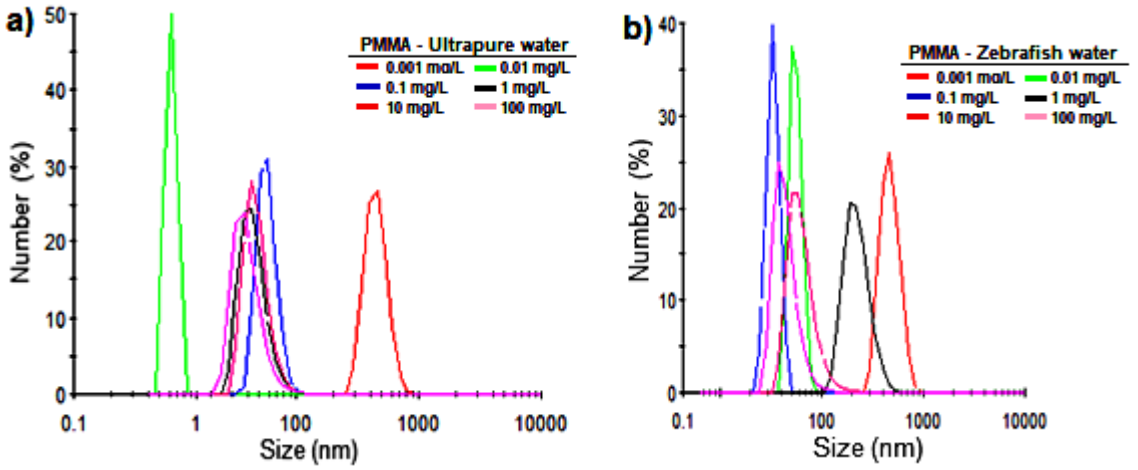

Figure 2. Characterization of polymethylmethacrylate nanoparticles (PMMA NPs) by dynamic light scattering (DLS) in ultrapure water and b) zebrafish water.

3.2. Effect of PMMA NPs on zebrafish embryo development (96 hpf)

In terms of PMMA effect on zebrafish embryos development, there was no significant effect, besides that PMMA induced pericardial oedema at high concentration but wasn´t statistically significant to control.

Table 1- Effects of PMMA nanoparticles (~ 30nm) in the embryonic development stage of zebrafish (96 hours exposure). Results are expressed as a percentage. Parameters assessed were mortality, hatching, pericardial oedema and tail malformation.

Time (h)	Concentrations (mg/L)	Parameters			
		Mortality (%)	Hatch (%)	Pericardial oedema (%)	Tail deformation (%)
24	0.000	4	--	--	0
	0.001	10	--	--	0
	0.01	5	--	--	0
	0.1	10	--	--	0
	1	10	--	--	0
	10	5	--	--	5
	100	30	--	--	0
48	0.000	4	46	0	0
	0.001	10	0	0	0
	0.01	5	0	0	0
	0.1	10	0	0	0
	1	10	10	0	0
	10	5	10	10	5
	100	30	5	40	10
72	0.000	4	96	0	0
	0.001	10	95	0	5
	0.01	5	95	0	5
	0.1	10	85	10	0
	1	10	90	5	5
	10	10	90	5	10
	100	30	65	40	10
96	0.000	5	96	0	0
	0.001	10	95	0	5
	0.01	5	95	0	0
	0.1	10	90	10	0
	1	10	90	5	5
	10	10	90	10	10
	100	30	70	25	10

3.3. Effect of PMMA NPs on zebrafish larval swimming behaviour

3.3.1. Total time (TT)

PMMA NPs did not affect the total time (TT) spent by larvae swimming (Fig.3), during light or dark periods ($p>0.05$).

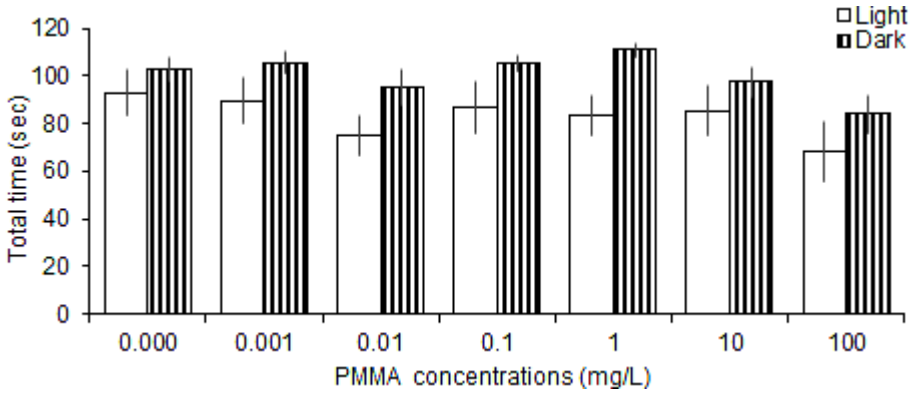

Figure 3. Total time (TT) zebrafish larvae spent swimming after exposure to PMMA NPs (30 nm), under two different illumination conditions, light period and dark period at 120 hpf,. The results are expressed as means± and standard errors.

3.3.2. Total distance (TD)

TD in light period, was increased in larvae exposed to 0.001 mg/L (p=0.022), 0.01 mg/L (p=0.014) and 1 mg/L (p=0.001) (Fig.4). Within dark period, significant effects of PMMA were also found in the same concentrations (0.001, 0.01 and 1 mg/L) with the same value of significance of the light period. However, there was no significant interaction between concentrations and illumination periods.

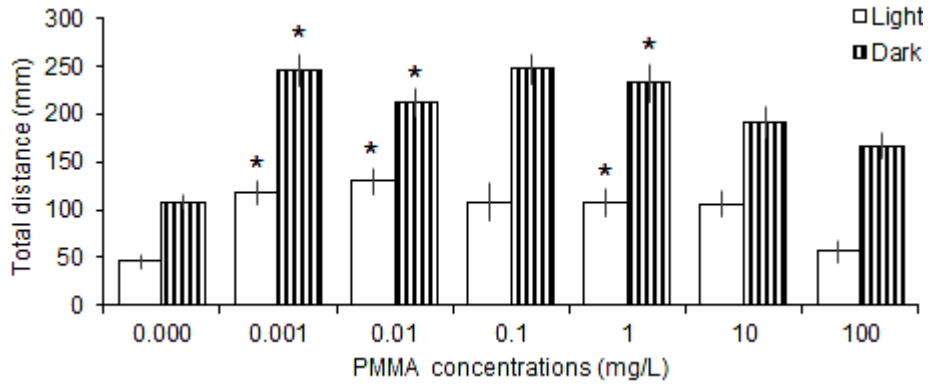

Figure 4. Total distance (TD) swam by zebrafish larvae after exposure to PMMA NPs (~30 nm), under two different illumination periods, light period and dark period at 120 hpf,. The results are expressed as means± and standard errors. Asterisks (*) denote the significant difference to control.

3.3.3. Time out (%T out)

Regarding % T out (Fig.5), PMMA NPs induced a significant decrease in dark period on organisms exposed to 0.001 mg/L (p=0.034) and 10 mg/L

(p=0.040). No statistic significant interaction between concentrations with illumination periods was found.

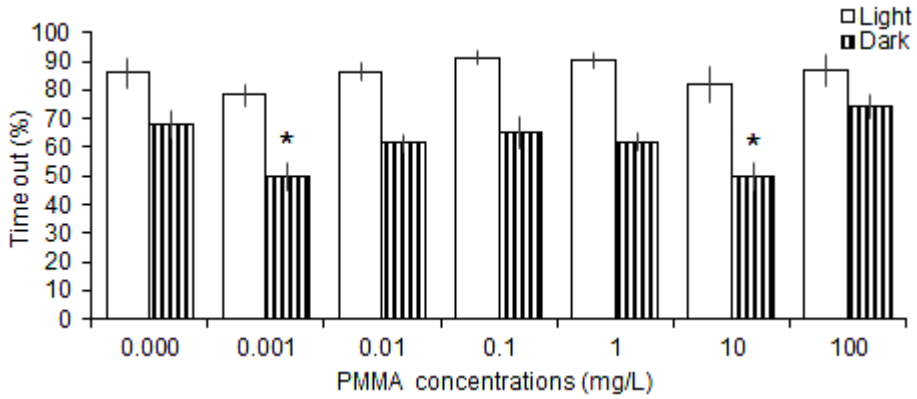

Figure 5. Time out (%Tout) spent swimming by zebrafish larvae after exposure to PMMA NPs (~30 nm), under two different illumination conditions, light period and dark period at 120 hpf. The results are expressed as means± and standard errors. Asterisks (*) denote significant difference to control

3.3.4. Distance out (% D out)

The % D Out (Fig.6), was significantly decreased, in the dark period, in the organisms exposed to 0.001 mg/L (p=0.007). No significant interaction between concentration and periods was found.

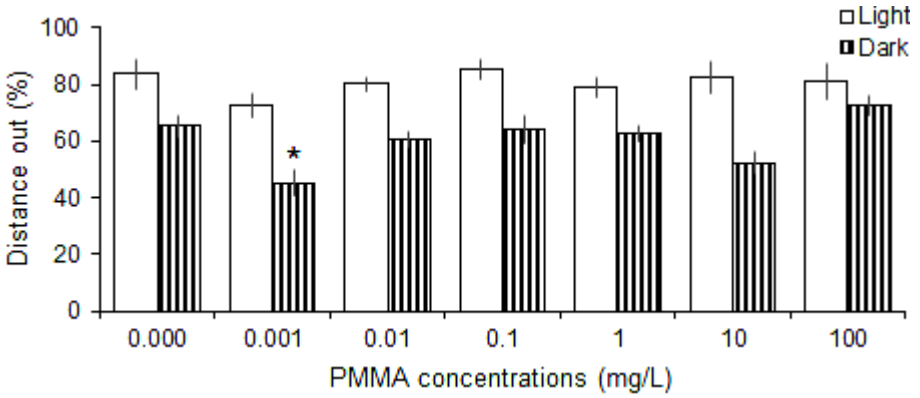

Figure 6. Distance out (%D Out) spent swimming by zebrafish larvae after exposure to PMMA NPs (~35 nm), under two different light conditions, light period and dark period at 120 hpf. The results are expressed as means± and standard errors. Asterisks (*) denote significant differences to control.

3.4. Effect of polymethylmethacrylate on biochemical endpoints

3.4.1. Neurotransmission

Cholinesterase (ChE) activity was significantly inhibited by PMMA NPs in organisms exposed to 0.01 mg/L ($p<0.05$) (Fig.7).

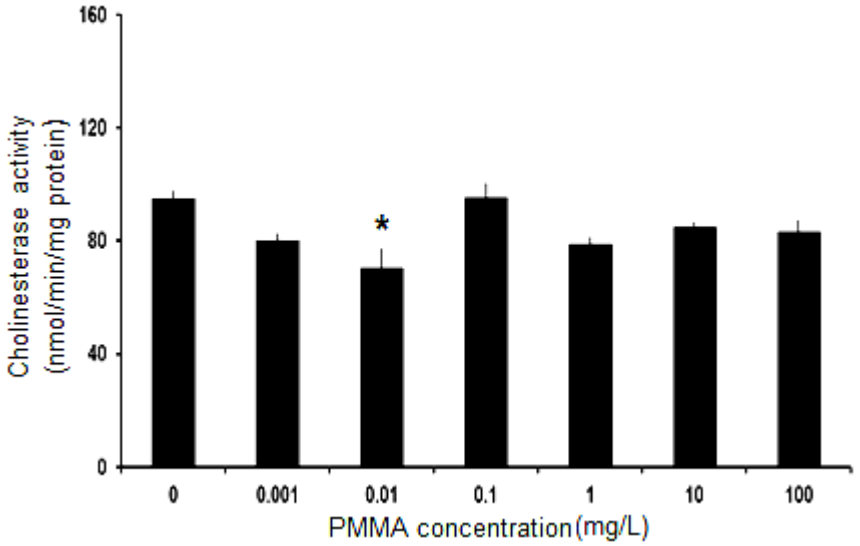

Figure 7. Effect of PMMA NPs (~30 nm) on cholinesterase (ChE) activity of zebrafish embryos after 96 hours exposure. The results are expressed by means ± standard errors. Asterisks (*) denote significant differences between exposed and control groups.

3.4.2. Antioxidant defences

Concerning GST activity, no significant effects ($p>0.05$) of PMMA NPs was found (Fig.8).

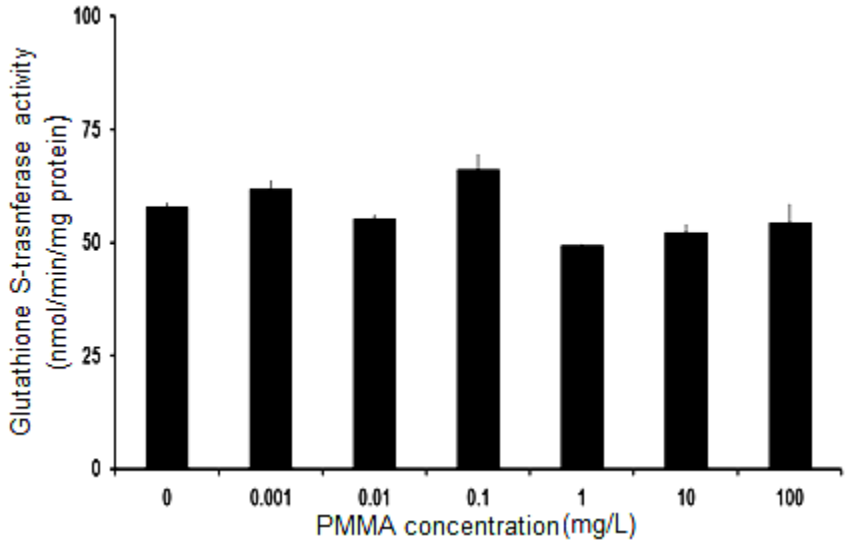

Figure 8. Effect of PMMA NPs (~30 nm) on glutathione S-transferase (GST) activity of zebrafish embryo after 96 hours exposure. The results are expressed by means ± standard errors.

3.4.2.1. Glutathione peroxidase (GPx)

In terms of GPx activity (Fig.9), a significant increase was found in the organisms exposed to 10 mg/L ($p<0.05$).

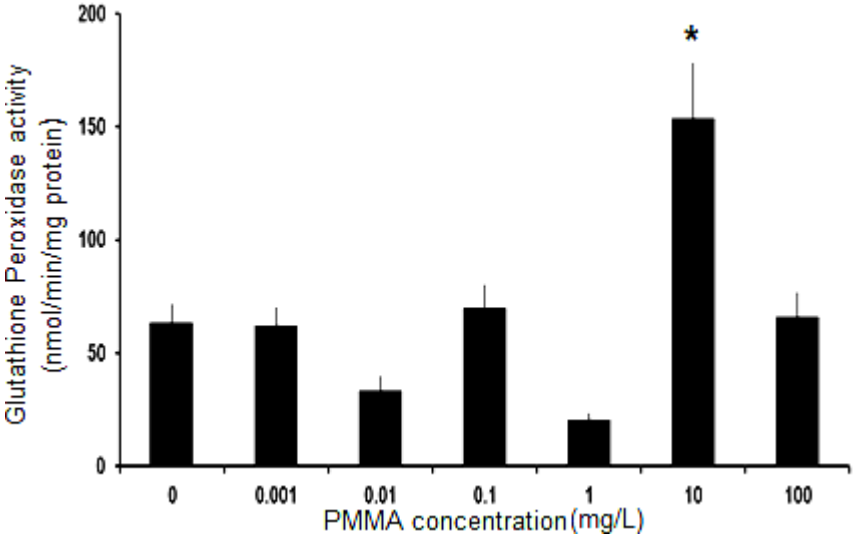

Figure 9. Effect of PMMA NPs (30 nm) on glutathione peroxidase (GPx) activity of zebrafish embryos after 96 hours exposure. The results are expressed by means ± standard errors. Asterisks (*) denote significant differences between exposed and control groups.

3.4.2.2. Catalase (CAT)

PMMA NPs also affected CAT activity (Fig. 10) promoting increased activities in the organisms exposed to 0.001, 0.1 and 10 mg/L ($p<0.05$). However, an enzyme inhibition was found in organisms exposed to 0.01 mg/L ($p<0.05$).

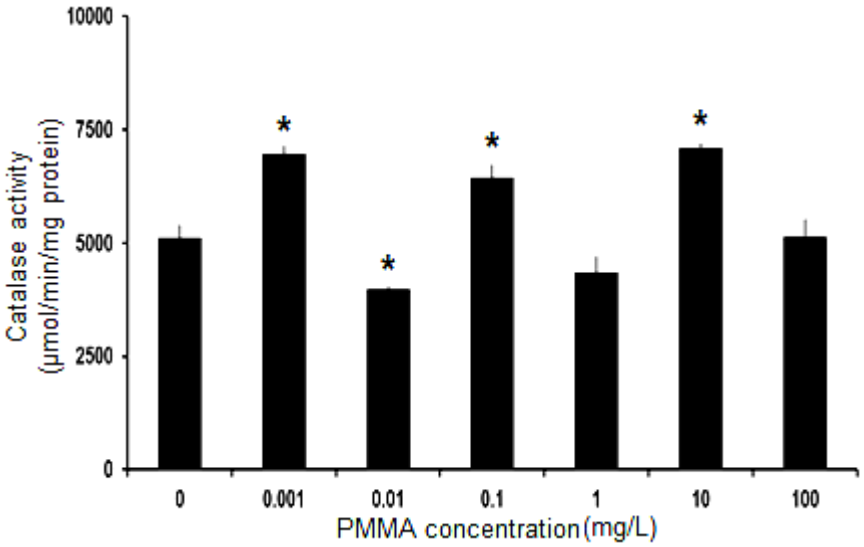

Figure 10. Effect of PMMA NPs (30 nm) on catalase (CAT) activity of zebrafish embryos after 96 hours exposure. The results are expressed by means± standard errors. Asterisks (*) denote significant differences between exposed and control groups.

3.4.3. Oxidative damage

Regarding oxidative damage, lipid peroxidation (LPO) registered statistic significant higher levels in embryos exposed to 0.001, 0.01 and 0.1 mg/L ($p<0.05$) (Fig.11).

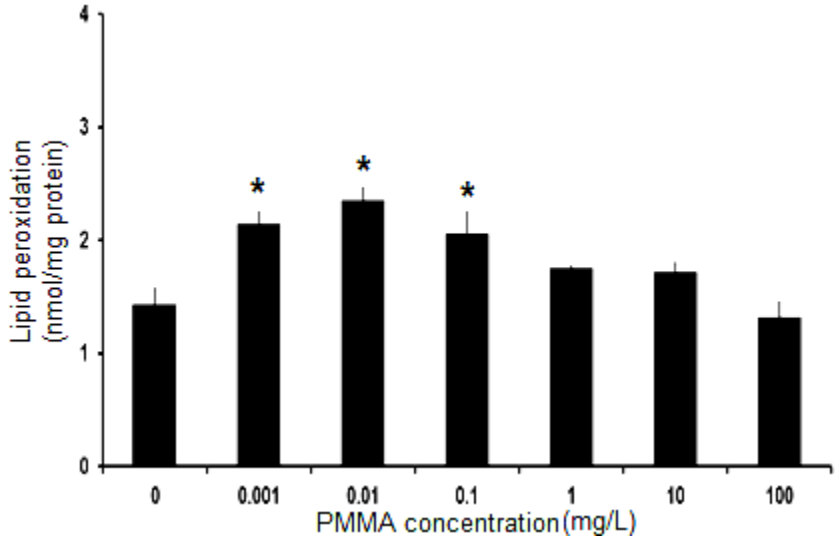

Figure 11- Effect of PMMA NPs (~30 nm) on lipid peroxidation (LPO) levels of zebrafish embryos after 96 hours exposure. The results are expressed by means± standard errors. Asterisks (*) denote significant differences between exposed and control groups.

3.4.4. Energy reserve and metabolism

Considering energy metabolism, the tested conditions did not have a significant effect on Gly levels (Fig.12).

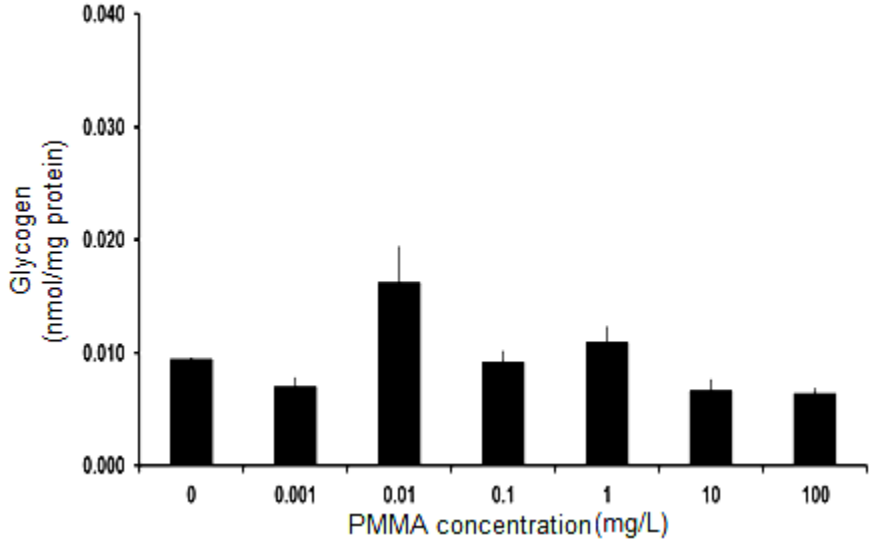

Figure 12. Effect of PMMA NPs (30 nm) on glycogen (Gly) levels of zebrafish embryos after 96 hours exposure. The results are expressed by means± standard errors. Asterisks (*) denote significant differences between exposed and control groups.

3.4.4.1. Isocitrate dehydrogenase (IDH)

IDH activity was significantly increased by 0.001 mg/L PMMA NPS, the lowest tested concentration ($p<0.05$) (Fig. 13).

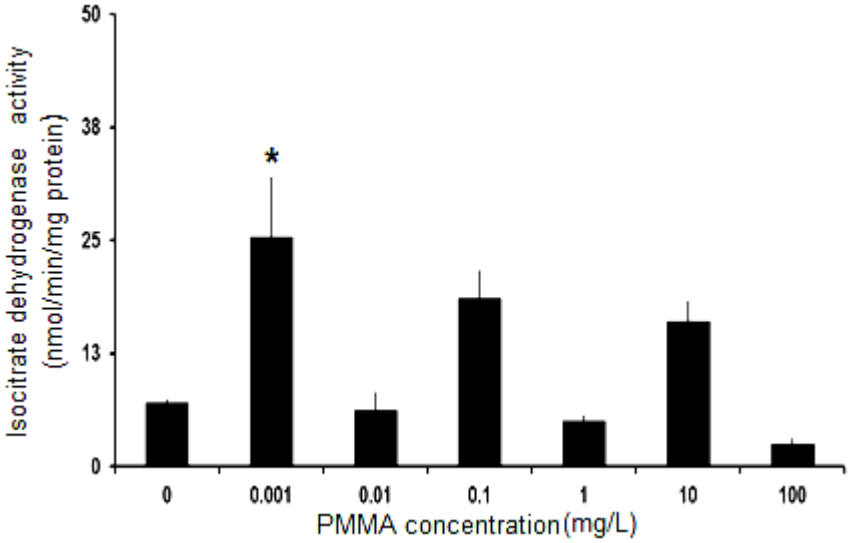

Figure 13. Effect of PMMA NPs (~30 nm) on isocitrate dehydrogenase (IDH) activity of zebrafish embryos after 96 hours exposure. The results are expressed by means± standard errors. Asterisks (*) denote significant differences between exposed and control groups.

3.4.4.2. Lactate dehydrogenase (LDH)

A significant inhibition of LDH activity was found in organisms exposed to 0.01 mg/L ($p<0.05$) (Fig. 14).

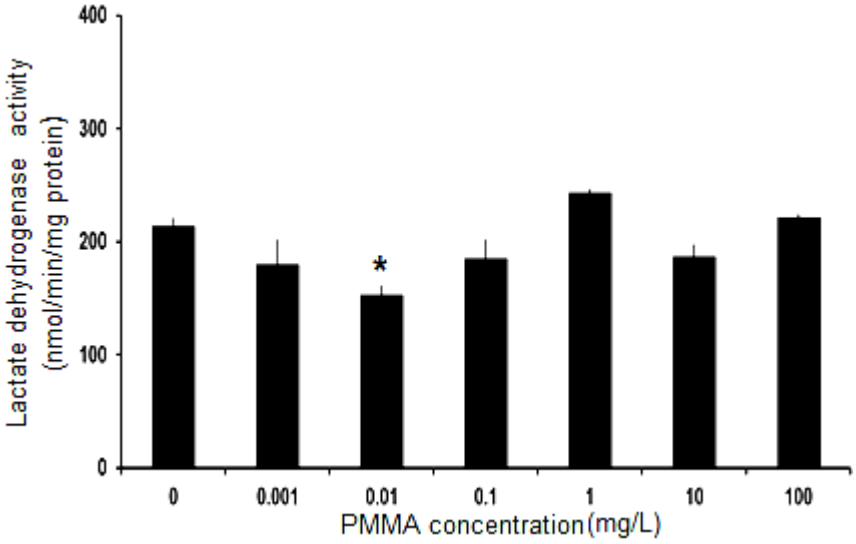

Figure 14. Effect of PMMA NPs (~30 nm) on lactate dehydrogenase (LDH) activity of zebrafish embryos after 96 hours exposure. The results are expressed by means± standard errors. Asterisks (*) denote significant differences between exposed and control groups.

4. Discussion

To explore the effect of PMMA nanoparticles, zebrafish embryo development, behaviour and biochemical endpoints alterations were assessed. Overall, the results demonstrated the PMMA nanoparticles can induce toxic effects on zebrafish embryos. Although no significant effects on the zebrafish development were found, PMMA was able to promote alterations in the swimming behaviour and biochemical parameters. The lack of effects on development was already reported in the study of Pitt et al., (2018) with PS NPs of 34.5±10.8 nm size Concerning swimming behaviour, TD at both light regime periods was significantly affected by 0.001, 0.01 and 1 mg/L PMMA NPs exposure, potentially representing an expenditure of energy that may compromise the ability to respond to additional stressors like escaping from a predator or essential for survival like capturing preys. A previous study with PS

NPs reported no effect on zebrafish TD (Parenti et al., 2019). This highlights the importance of studying the effect of different polymers (PMMA NPs ~30 nm). The time and distance (%) swam in the out area of the well was significantly decreased (0.001 and 10 mg/L). Thus, data suggest that PMMA NPs does not cause a thigmotaxis effect on zebrafish. Nevertheless, the results showed that PMMA NPs altered the well-known pattern of behaviour of fish larvae in light and dark periods, potentially suggesting a stressful situation to the fish and or impairment of light recognition. This is a potentially very pernicious effect. As reported by Shao et al. (2017) light recognition behaviour needed, because it demonstrates demonstrate that larvae are capable to feel and answer the period changes on the day at the natural conditions.

Regarding the biochemical effects, PMMA nanoparticles demonstrated the ability to inhibit ChE activity. A previous study, concerning PS NPs toxicity, also demonstrated the ability of PS NPs (~ 35 nm) to inhibit this enzyme on zebrafish embryos (Chen at al.,2017).

The induction of oxidative stress is a general mechanism of toxicity that can provoke several pernicious effects, including cell death. LPO was found in the groups of animals exposed to three lowest concentrations (0.001, 0.01 and 0.1 mg/L) and not the highest, which may be associated with alterations of particles behaviour and size at high concentrations. There is a lower probability of particles aggregation at the lowest concentrations tested. This result is particularly relevant considering that effects were found at low and more relevant concentrations. In this study, PMMA nanoparticles did not affect GST activity. A similar result was reported for PS nanoparticles by Parenti et al., (2019). However, the same study also reported lack of effects on GPx and CAT whereas the present study found that PMMA NPs were able to inhibit and stimulate both activities.

Energy metabolism plays a key role on organism's survival. In nature, many contaminants have caused damage to this important mechanism (Oliveira et al.,2010). In the present study, PMMA NPs caused a significant increase in the aerobic metabolism as demonstrated by the increased IDH activity at the lowest concentration tested (0.001 mg/L) and a decrease of anaerobic metabolism (0.01 mg/L), reflected by LDH inhibition.

Overall, this study demonstrated that PMMA nanoparticles may induce toxic effects at low concentrations which are not maintained at higher concentrations. The ability to affect neurotransmission, fish ability to respond to light and dark as well as oxidative damage show that PMMA nanoparticles may prove a danger to aquatic organisms. This data supports the need for additional studies.

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Chapter IV

Single and combined effects of nanoplastic particles (PS and PMMA) with pyrene

Abstract

The widespread distribution of plastics in the environment and reported effects make this type of debris a threat to the aquatic environment. The available studies show that characteristics like polymer type, and size play a determinant role on toxicity. Despite the increasing number of studies, particularly with microparticles, the available information may be considered scarce. There is a considerable gap in the knowledge concerning the toxicity of nanoplastics and its association with polymer and sizes. The available information in terms of microplastics reveal its ability to interact and modulate the biological effects of other environmental contaminants. However, little in known in terms of nanoplastics. Thus, the aim of this study was to analyse the effect of polystyrene (PS) and polymethylmethacrylate (PMMA) nanoparticles (NPs), alone and in a combined exposure with the polycyclic aromatic hydrocarbon pyrene, on zebrafish embryos. Assessed endpoint included effects on development and larvae swimming behaviour. Concerning swimming performance, NPs (PS and PMMA) significantly decreased the total time and total distance at both single treatments (0.1 and 100 mg/L), it was also decreased by pyrene (0.01 and 1 µg). The time and distance recorded in the outer area of the well were significantly decreased by PS NPs single exposure (100 mg/L) and pyrene (0.01 µg), While the combined conditions had no significative effect. The results showed that the interaction of NPs and pyrene my influence the nanoplastics and pyrene toxicity effect.

Keywords: nanoplastic, pyrene, zebrafish embryos, combined exposure, toxicity

1. Introduction

Currently plastics are one of the widely produced materials (PlasticEurope, 2018). The packaging industry represents the main demanding activity and main origin of plastic waste present in the environment (Geyer, Jambeck & Law, 2017), reaching freshwater and saltwater bodies (Andrady, 2017; Wang et al., 2017). The presence of plastic debris in these environments has been the subject of different studies that aimed to characterize plastic debris abundance in terms of sizes, shapes and polymers (Blettler et al.,2019; Oliveira and Almeida 2019). These studies have highlighted that these materials can be a stress inducing factor for ecosystems (Basto et al., 2019; Rainieri, et al., 2018; Venâncio et al., 2019; Woods, Rødder & Verones, 2019; Zhang et al.,2020).

Plastics materials in the environment can fragment and originate particles of different sizes that will continue to fragment reaching dimensions like micro and nano sizes (da Costa et al., 2016; Lambert and Wagner, 2016). As a result, the surface area to volume ratio increases and so does the capacity to adsorb substances including environmental contaminants like polycyclic aromatic hydrocarbons - PAHS and metals (Lee et al., 2019). PAHs, as hydrophobics substance, are easily adsorbed into distinct materials (e.g.: plastic) (Fisner et al., 2013; Oliveira et al.,2013; Rios, Moore and Jones 2007), and absorbed by living organisms, having the potential to bioaccumulate (Shao et al., 2010; sun, et al., 2018; Tongo, Ogbeide and Ezemonve, 2017; Wetzel and Van Vleet, 2004). Some PAHs have the teratogenic, mutagenic and carcinogenic properties (Oliveira, Pacheco and Santos, 2007), which raise concerns because of its environment presence.

In this context, this work, aimed to evaluate the effects of combined exposure of nanoplastics and pyrene (Py), one of the most common environmental contaminants in estuaries and coastal areas, known to cause toxicity in several aquatic organisms (Oliveira et al., 2013). Detection of its metabolites has been suggested as a good indicator of PAHs presence in the environment (Van Gestel et al.,2004).

Its single and combined toxicity has been the subject of ecotoxicological studies (El-Kady, Wade, and Sweet, 2018; Oliveira, Pacheco and Santos, 2007; Oliveira et al., 2013). Thus, the main purpose of this work was to analyse the single and combined effects of plastic nanoparticles (polystyrene and polymethylmethacrylate) and pyrene in zebrafish embryonic development (96 hpf) and larval behaviour (120 hpf).

2. Materials and methods

2.1. Test chemicals

Pyrene (purity ≥99) and acetone (purity ≥99) were acquired from Sigma-Aldrich (Spain).The nanoplastic particles (polystyrene and polymethylmethacrylate) were synthesized by the research team from microemulsions (styrene and methyl methacrylate) and purified, as referred in the previous chapters (II and III). All other chemicals were analytical grade.

2.2. Test organism

Adult zebrafish reared in the zebrafish facility of Department of Biology - Aveiro University. Animals are maintained in aquaria with water temperature of 26 ± 1º C, conductivity 750 ± 50 mS/cm, pH 7.5 ± 0.5, salinity of 0.35 and dissolved oxygen equal or above 95% saturation, at a 16:8 h (light: dark) photoperiod. After natural spawning (within 2 hours), embryos were collected, and screened using a stereomicroscope (Stereoscopic Zoom Microscope-SMZ 1500, Nikon Corporation) to exclude unfertilized eggs.

2.3. Experimental design

The bioassay generally followed the OECD guidelines for fish acute bioassays (OECD, 2013), with modifications in the number of test conditions. Stock solutions of pyrene were prepared every 24 h using acetone as solvent (Oliveira et al., 2012). The pyrene concentrations tested were based on levels reported in the environment (e.g. surface water from the Jiulong River Estuary and Western Xiamen Sea (1 µg/L) by Maskaoui et al., (2002). A previous study has reported that pyrene at low concentrations (ng/L) caused cardiac

malformation on zebrafish embryos (Zhang et al., 2012). The concentrations of nanoplastics (0.001 and 100 mg/L) were selected based on a previous study, where the aim was to assess the combined effects of polyethylene microplastics and pyrene (Oliveira et al., 2013). The test suspensions of nanoplastics (0.001 and 100 mg/L) were prepared by dilution of the stock suspension in zebrafish maintenance water. As controls conditions, we used zebrafish maintenance water and zebrafish maintenance water with acetone that was used to dissolve pyrene (0.004%). Thus, the test was performed with five experimental conditions: control, solvent control (acetone), nanoplastics (NPs) (0.001 and 100 mg/L), pyrene (Pyr) (0.01 and 1 µg/L), combined exposure of nanoplastics and Pyr - (0.001 mg/L nanoplastics + 1 µg/L; and 100 mg/L nanoplastics + 0.01 µg/L Pyr). To prevent the significant degradation of Pyr and to reduce metabolic residues, the test media was changed every 24 h (Oliveira et al., 2013). The exposure period was 96 h for embryotoxicity test and 120 h for the behaviour assessment.

2.4. The fish embryo toxicity (FET) test

The FET was performed according to the OECD guidelines (OECD, 2013), so the exposure of the contaminant, embryos with short fertilization period (2 hpf). The effects of PS and PMMA NPs on zebrafish embryonic development was monitored by a stereomicroscope (Stereo Zoom Microscope-SMZ 1500, NiKon Corporation) therefore, the main parameters evaluated were: i) coagulated eggs; ii) hatching rate; iii) tail malformation; iv) pericardial oedema.

2.5. Swimming Behaviours test

The swimming performance monitoring test was performed at 120 hpf. So, 20 organisms per condition were kept in the same plates (24 wells). The computational technology, Zebrabox - ZEB 478 (Viewpoint Life sciences, Lyon, France) was used as (automated) tool, which made possible to track by video recording the larvae movements at two established well regions (internal and external). It was characterized to catch the possible signs of thigmotaxis and, performed within two periods, light/dark as a stimulus. However, we analysed the following parameters, the total time (TT), total distance (DT), percentage of

time and distance travelled outside the well (% T out and% D out). Time was defined in seconds (180 sc) and mm for distance.

2.6. Data analysis

Data were tested using the SigmaPlot 12.5 software. The results are expressed by means ± standard errors. To test the variance within and between groups, two Way ANOVA was performed, with $p<0.05$ as statistical significance. Tukey test permitted the multiple comparison procedures between the groups.

3. Results

3.1. Effect on zebrafish embryo development

3.1.1. PS nanoparticles combined with pyrene

In these experimental conditions, PS NPs didn´t significantly affect any established parameters of zebrafish embryos development. The same values were obtained when combined with pyrene compared to control. However, Pyr induced pericardial oedema and tail malformation (1 µg/L), that was significantly when compared with control, NPs PS (0.1 mg/L) and to combined exposures. The data shows that, the combination of PS NPs (0.1 mg/L) and pyrene (1 µg/L) can, reduce the pericardial oedema on zebrafish embryos.

Table 1. Effects of PS nanoparticles (~ 30nm) after individual and combined exposures with pyrene in the embryonic development of zebrafish (96 hours exposure). Results are expressed as a percentage. Parameters assessed were mortality, hatching, pericardial oedema and tail malformation. (-- not observed).

Time (h)	Concentrations (mg-µg/L)	Parameters			
		Mortality (%)	Hatch (%)	Pericardial oedema (%)	Tail deformation (%)
24	Control	0	--	--	0
	Control solv.	0	--	--	0
	PS (.1)	5	--	--	0
	PS (100)	5	--	--	0
	Pyr 0.01	0	--	--	5
	Pyr 1	0	--	--	0
	PS+Pyr (.1+1)	0	--	--	5
	PS+Pyr(100+.01)	0	--	--	0
48	Control	0	20	0	0
	Control solv.	0	15	0	0
	PS (.1)	5	0	0	0
	PS (100)	5	0	0	0
	Pyr 0.01	0	15	15	10
	Pyr 1	0	0	80	35
	PS+Pyr (.1+1)	0	5	15	0
	PS+Pyr(100+.01)	10	5	0	0
72	Control	0	100	0	0
	Control solv.	0	85	0	0
	PS (.1)	5	85	0	0
	PS (100)	10	20	0	0
	Pyr 0.01	0	85	15	20
	Pyr 1	0	80	100	60
	PS+Pyr (.1+1)	0	80	30	0
	PS+Pyr(100+.01)	10	75	0	0
96	Control	0	100	0	0
	Control solv.	0	85	0	0
	PS (.1)	5	95	0	0
	PS (100)	10	90	0	0
	Pyr 0.01	0	90	20	20
	Pyr 1	0	85	100	95
	PS+Pyr (.1+1)	0	85	35	0
	PS+Pyr(100+.01)	10	75	25	0

3.1.2. PMMA combined with pyrene

In this experimental conditions, PMMA nanoparticles (~30 nm) did not affect the zebrafish embryos development when compared to control

organisms. Regarding to combined condition, PMMA NPs (0.1 mg/L) demonstrate that can inhibit the pyrene (1 µg/L) toxicity effect (pericardial oedema and tail malformation).

Table 2. Effects of PMMA nanoparticles (~ 30nm) after individual and combined exposures with pyrene in the embryonic development of zebrafish (96 hours exposure). Results are expressed as a percentage. Parameters assessed were mortality, hatching, pericardial oedema and tail malformation. (-- not observed).

Time (h)	Concentrations (mg-µg/L)	Parameters			
		Mortality (%)	Hatch (%)	Pericardial edema (%)	Tail deformation (%)
	Control	0	--	--	0
	Control solv.	0	--	--	0
24	PMMA (.1)	10	--	--	0
	PMMA (100)	30	--	--	0
	Pyr 0.01	0	--	--	5
	Pyr 1	0	--	--	0
	PMMA+Pyr).1+1	0	--	--	0
	PMMA+Pyr(100+.01)	0	--	--	0
	Control	0	20	0	0
	Control solv.	0	15	0	0
48	PMMA (.1)	10	0	0	0
	PMMA (100)	30	5	40	10
	Pyr 0.01	0	15	15	10
	Pyr 1	0	0	80	35
	PMMA+Pyr).1+1	0	0	21	0
	PMMA+Pyr(100+.01)	5	17	0	0
	Control	0	100	0	0
	Control solv.	0	83	0	0
72	PMMA (.1)	10	85	10	0
	PMMA (100)	30	65	40	10
	Pyr 0.01	0	85	15	20
	Pyr 1	0	80	100	60
	PMMA+Pyr).1+1	0	83	40	0
	PMMA+Pyr(100+.01)	5	80	0	0
	Control	0	100	0	0
	Control solv.	0	85	0	0
96	PMMA (.1)	10	90	10	0
	PMMA (100)	30	70	25	10
	Pyr 0.01	0	90	20	20
	Pyr 1	0	85	100	95
	PMMA+Pyr).1+1	0	85	50	0
	PMMA+Pyr(100+.01)	5	80	35	0

3.2. Effect on zebrafish larval behaviour (120 hpf)

3.2.1. PS nanoparticles combined with pyrene

The swimming performance of zebrafish larvae has significantly affected after exposure to PS nanoparticles. In light and dark periods, TT was significantly decreased, when compared to control, in organisms exposed to 0.1 and 100 mg/L PS NPs nanoparticles (p=0.017 and 0.025) in light and (p≤0.001) in dark (Fig.1). In the combined exposures, the effects of PS nanoparticles were reversed by the presence of pyrene. However, significant differences to individual exposures were only found for 100 mg/L with pyrene 0.01 µg/L (dark period). No significant interaction between tested concentrations and illumination conditions was found.

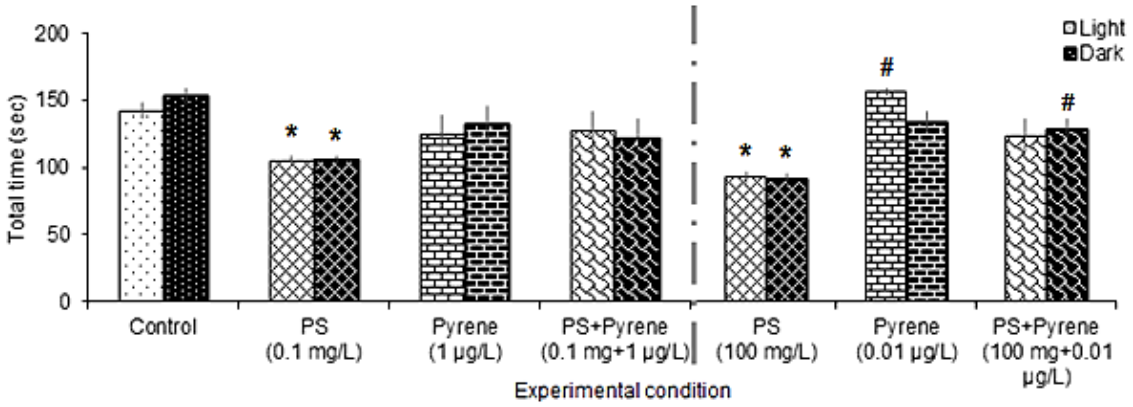

Figure 1. Total time zebrafish larvae (TT) spent swimming by after 120 hours exposure to PS NPs (~30 nm) alone and combined with pyrene, under two illumination conditions, light and dark period. The results are expressed as means± and standard errors. Asterisk (*) denote the significant difference to control and cardinal (#) difference to PS NPs.

Total distance (TD) (Fig.2), was significantly decreased after exposure to 0.1 and 100 mg/L PS nanoparticles (p<0.001) during light and dark periods. In the dark periods, TD was also affected by exposure to pyrene at 0.01 µg/L(p=0.007) and 1 µg/L(p<0.001) compared to control. In the dark, TD moved by larvae exposed to PS nanoparticles was significantly lower than moved by organisms exposed to pyrene at 100 mg/L (p=0.002) and 0.1 mg/L /L (p=0.009) in dark. The combined exposure reverted the effects of induced by 0.1 mg/L (completely in the light condition and partially in the dark period). In the highest concentration of PS nanoparticles pyrene combined exposure had no effect in

the light period but completely reversed the of PS nanoparticles in the dark. Concern the differences combined treatment, PS NPs 0.1 mg/L was significantly decreased to combined with pyrene 1 µg /L (p=0.015) and 100 mg/L to combined to 0.01 µg /L (p<0.001)

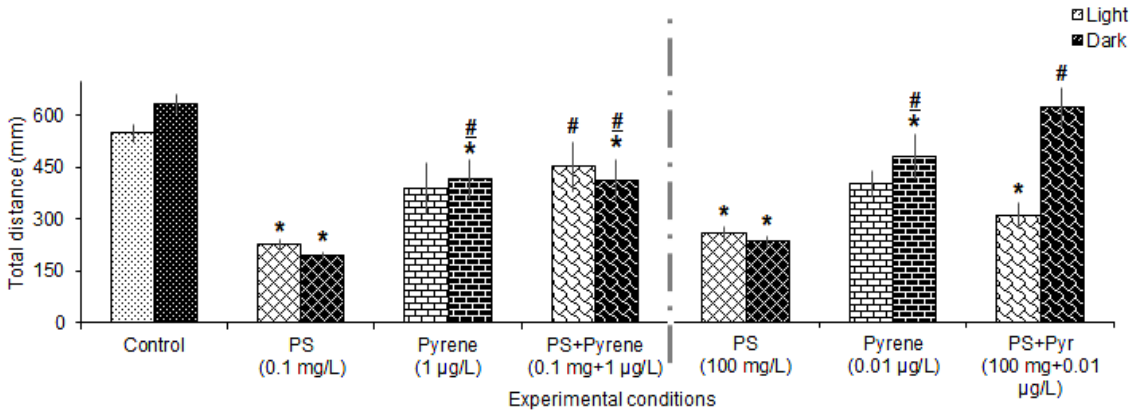

Figure 2. Total distance (TD) zebrafish larvae swam by after 120 hours exposure to PS NPs (~30 nm) alone and combined with pyrene, under two different light conditions, light and dark period. The results are expressed as means± and standard errors. Asterisk (*) denote the significant difference to control and cardinal (#) the difference to PS NPs.

Time in the outer area (Fig.3), was significantly (p<0.001) decreased in organisms exposed to 100 mg/L PS NPs in light and dark, when compared to control. Pyrene (alone) did not cause any effects when compared to controls. However, PS NPs affect significantly at 0.1 mg/L (p=0.039) and 100 mg/L (p<0.001) compared to pyrene solely in light period. In terms of combined condition, PS at 100 mg/L presented a significant (p<0.001) decrease to combined with pyrene 0.001 µg /L.

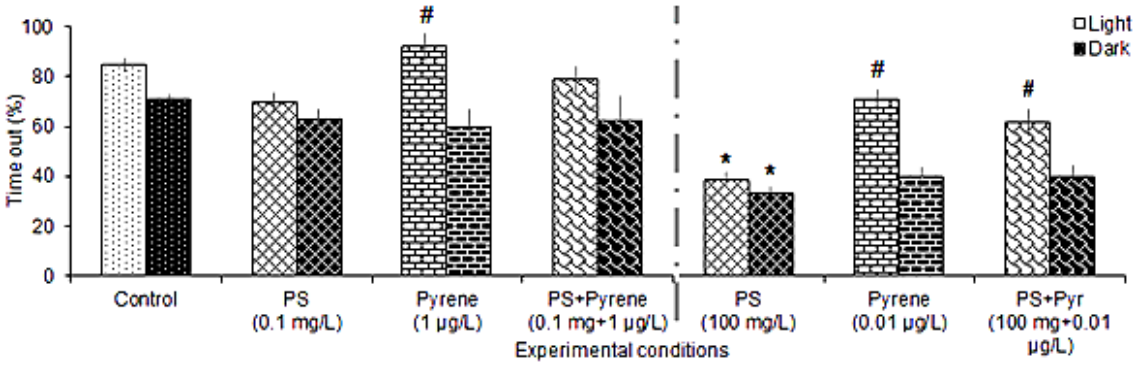

Figure 3- Time out (% Tout) spent swimming by zebrafish larvae after 120 hours exposure to PS NPs (~30 nm) alone and combined with pyrene, under two different illumination conditions, light and dark period. The results are expressed as means± and standard errors. Asterisk (*) denote the significant difference to control and cardinal (#) the difference to PS NPs.

In term of distance moved in the outer area (Fig. 4), 100 mg/L PS nanoparticles induced a significant decrease, when compared to control ($p=0.018$) in light and, ($p=0.014$) in dark to control). Pyrene individual exposure had no effect but, in the combined exposure with 100 mg/L was able to revert the inhibition of PS nanoparticles, although significant effects to individual exposure was only found the light period. Comparing to pyrene effect, PS NPs at 100 mg/L had significant difference at 100 mg/L ($p>0.001$) and at 0.1 mg/L ($p=0.004$).

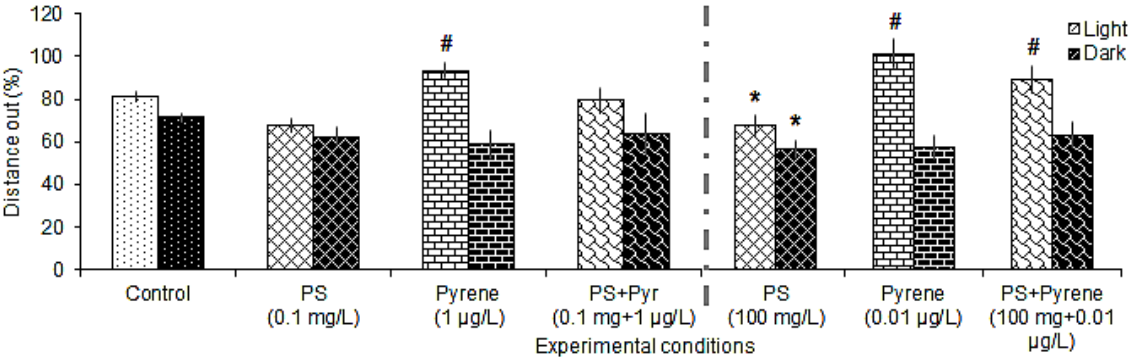

Figure 4- Distance out (% Dout) swam by zebrafish larvae after 120 hours exposure to PS NPs (~30 nm) alone and combined with pyrene, under two different illumination conditions, light and dark period. The results are expressed as means± and standard errors. Asterisk (*) denote the significant difference to control and cardinal (#) the difference to PS NPs.

3.2.2. PMMA NPs and combined with pyrene

PMMA nanoparticles significantly reduced the larvae´s TT spent swimming in the organisms exposed to 0.1 and 100 mg/L ($p<0.001$) at both light

and dark to the control. Pyrene tested concentrations had no effect in this parameter, when compared to control. The presence of pyrene was able to revert the effects of PMMA nanoparticles in the tested conditions (100 mg/L) had a significant decrease to PMMA NPs (100 mg/L) ($p<0.001$) in light and dark. Concern combined exposure, PMMA 100 mg/L decreased significantly than combined with pyrene 0.01 µg /L ($p=0.023$)

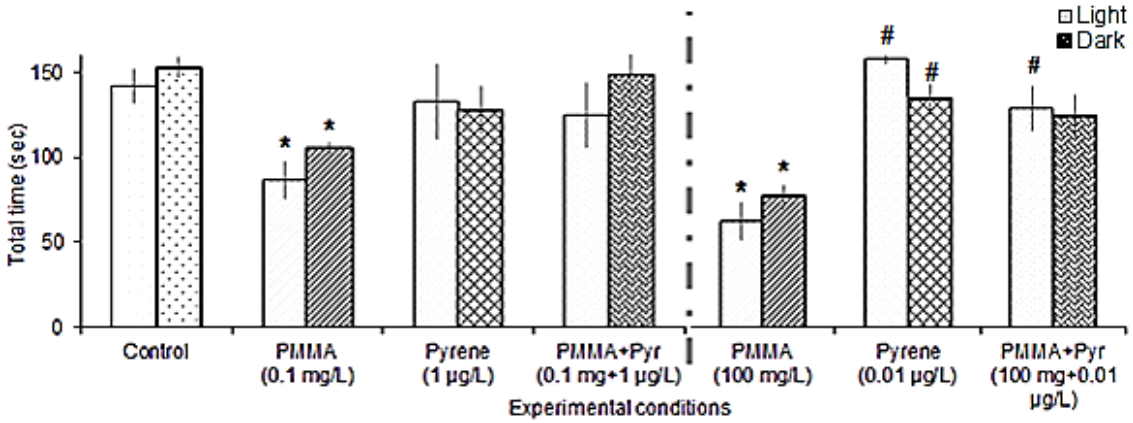

Figure 5. Total time (TT) zebrafish larvae spent swimming by after 120 hours exposure to PMMA NPs (~30 nm), alone and combined with pyrene, under two different illumination conditions, light and dark period. The results are expressed as means± and standard errors. Asterisk (*) denote the significant difference to control and cardinal (#) the difference to PMMA NPs.

The total distance (Fig.6) swam by larvae was significantly decreased in organisms exposed to PMMA nanoparticles ($p<0.05$). Exposure to pyrene, alone, caused a decrease of total distance moved by fish in the dark period. The total distance moved by organisms from the combined exposures presented an overall increase, when compared to PMMA nanoparticles individual exposures (although not significant for 100 mg/L PMMA + 0.01 µg/L pyrene). The presence of pyrene was able to revert the significant decrease induced by 0.1 mg/L PMMA (in the light condition).

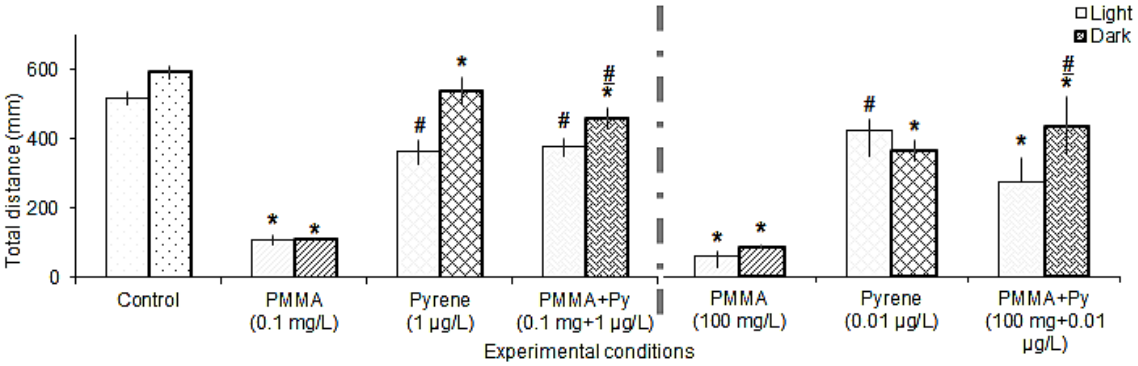

Figure 6-Total distance (TD) swam by zebrafish larvae after 120 hours exposure to PMMA NPs (~35 nm), alone and combined with pyrene, under two different illumination conditions, light and dark period. The results are expressed as means± and standard errors. Asterisk (*) denote the significant difference to control and cardinal (#) the difference to PMMA NPs.

The time fish spent in the outer area (% T out) of the well, in the dark condition, decreased (p=0.006) in organisms exposed to 0.01 µg/L (Fig.7) compared to PMMA NPs. Thereby, PMMA combined to 0.01 µg/L, was significantly decreased also compared to PMMA alone exposure (100 mg/L) (P=0.032) and to control organisms (p=0.009)

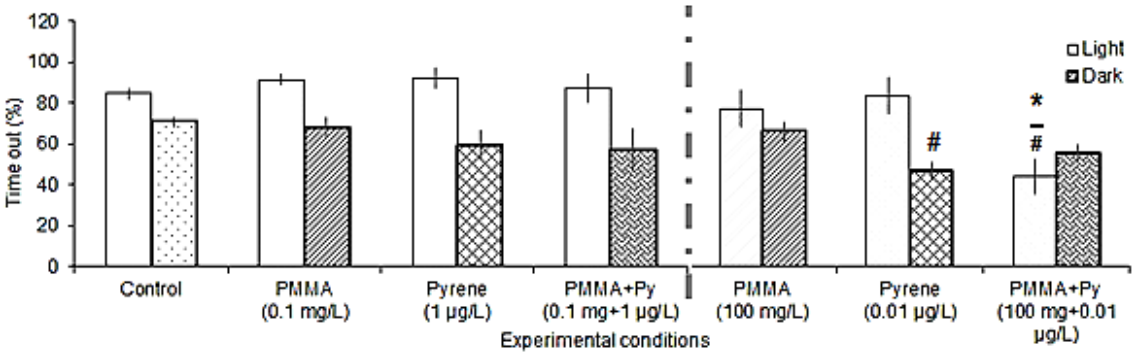

Figure 7-Time out (%) spent by zebrafish larvae after 120 hours exposure to PMMA NPs (~35 nm), alone and combined with pyrene, under two different light conditions, light and dark period. The results are expressed as means± and standard errors. Asterisk (*) denote the significant difference to control and cardinal (#) the difference to PMMA NPs.

Concerning distance moved in the outer area (Fig.8), no significant differences were found in the experimental conditions.

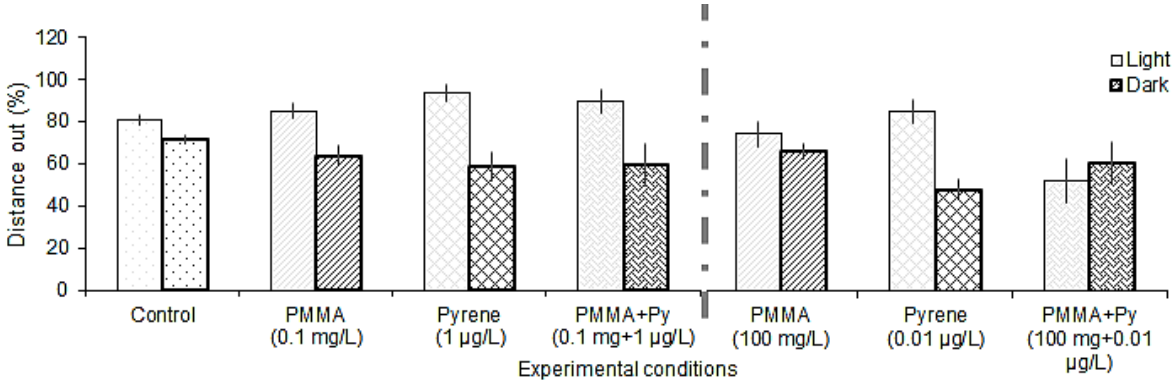

Figure 8. Distance out (%) swam by zebrafish larvae after 120 hours exposure to PMMA NPs (~30 nm), alone and combined with pyrene, under two illumination conditions, light and dark period. The results are expressed as means± and standard errors.

4. Discussion

Embryo toxicity tests and behaviour alteration has been used recognized as an important tool to study the toxicity of many chemical (e.g: Awoyemi et al.,2019; Fraser et al., 2017; Nema, & Bhargava, 2018; Pitt et al., 2019). The polymeric nanoparticles (polystyrene and polymethylmethacrylate) toxicity was assessed in zebrafish embryo development and larvae (120 hpf) behaviour alteration, testing a potentially more environmentally relevant scenario. Two conditions, single and combined exposures with pyrene were tested.

In terms of development embryos were affected by Pyr (0.01 and 1 µg/L) and combined with PMMA (0.1 mg/L) which caused a significant increase of pericardial oedema and tail malformation. Thus, the results represent the possibility of NPs to inhibit the pyrene effects.

Regarding behaviour performance, the results demonstrated a significant effect of nanoparticles, which was more representative at single exposure treatment. Both polymers decreased the total time and total distance TD moved by larvae as we say previously at single conditions. The presence of pyrene promoted a decreased the effect of nanoparticles, except for total time spent in the outer area by organisms exposed to PMMA nanoparticles, where the combined exposure led to significant differences compared to control and did no effect in the individual exposures.

The data demonstrate that NPs can interact with other environmental contaminants and affect its property. Although the assessed endpoints do not allow a clear identification of the mechanisms associated the altered behaviour, it may be hypothesized that the presence of pyrene promoted alterations on the surface of nanoparticles leading to its aggregation and lower bioavailability. This aspect must, however, be assessed (e.g. through dynamic light scattering). Nonetheless, the present study shows that, as observed by Oliveira et al. (2012), pyrene interacts with plastic particles modulating its effects. Further studies should be performed to assess levels able to interact with plastics and their effects on other endpoints.

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Chapter V

General discussion

Considering that each chapter included a specific discussion, this general discussion aimed to provide a critical analysis of the overall dissertation.

Plastics play an important role in human lives, promoting economic and social development. Despite this important role, its high production, linked to a lack of compliance with good solid waste management practises, resulted in the generation of large amounts of waste, which has been accumulating in the environment and have the potential to compromise the environmental health. Despite the increased concern about the effects of plastics, the knowledge regarding the effects of particles in the nanometers size range is extremely limited, particularly in terms of vertebrate organisms. This dissertation aimed to provide relevant data to help to understand if the presence of nanoplastics (NPs) may compromise the fish development and have consequences on its health and fitness.

The effects of nanoparticles of polystyrene (PS) (one the most used and studied polymers) and polymethylmethacrylate (PMMA) (an increasingly used but poorly studied polymer) were assessed in individual exposures and in combined exposures with pyrene, a PAH commonly found in the aquatic environment. Unlike PS, very studies have addressed the effects of PMMA nanoparticles (Venâncio et al.,2019) to aquatic organisms and to the author's knowledge, no studies have reported effects on zebrafish.

The data obtained suggest that ~30 nm PS (studied in chapter II and IV) and PMMA nanoparticles (studied in chapter III and IV) do not have a relevant impact on the zebrafish development. The main effect detected was pericardial oedema in organisms exposed to 100 mg/L PMMA nanoparticles (alone and combined with pyrene (0.01 and 1 µg/L)). However, behaviour demonstrated a high sensitivity to these nanoplastics. PS NPs (0.001, 1 and 100 mg) and PMMA NPs (0.001, 0.01 and 1 mg/L) increased the total distance moved by zebrafish larvae which is a clear sign that organisms recognize the presence of these particles as stressors. However, the time and distance made in the out area of the well, assessed to evaluate thigmotaxis, was decreased, with PS nanoplastics

affecting the larvae almost in all tested concentrations (0.001,0.01,0.1,1,10 and 100 mg/L).

In terms of biochemicals effects, embryos exposure to nanoplastics elicited alterations on neurotransmission, antioxidant status and energy metabolism. The nanoplastics of each polymer demonstrated the ability to alter neurotransmission with inhibitions of cholinesterase activity induced by PS (0.01 and 0.1mg/L) and PMMA (0.01 mg/L). PS NPs demonstrated ability to inhibit the phase II of biotransformation enzyme that is also involved in the antioxidant defence, glutathione S-transferase (GST) (0.01, 10 and 100 mg/L) and catalase (CAT) activity (0.001 and 10 mg/L). The overall impact in the antioxidant status results in peroxidative damage at a concentration where the antioxidant defences were no affected (0.1 mg/L). Considering PMMA, activation of antioxidant defences was found in organisms exposed to 0.001 and 0.1 mg/L, CAT and 10 mg/L (glutathione peroxidase (GPX) and CAT). Despite the reported activation of antioxidant enzymatic activities oxidative damage was found (0.001, 0.01 and 0.1 mg/L) and on organisms exposed to PS NPs at 1 mg/L. The activation of defences and repair mechanisms if frequently associated with an increased energy demand. The energy metabolism associated to endpoints were also affected by nanoparticles exposure, mainly by PS NPs. Higher levels of glycogen were found in the organisms exposed to PS NPs (except 0.001 mg/L) whereas organisms exposed to PMMA did not show significant alterations on this parameter. PS NPs exposure resulted in decreased aerobic metabolism (IDH activity) in organisms exposed to 0.001, 0.1 and 100 mg/L and anaerobic metabolism (LDH activity) in organisms exposed to 0.01 mg/L. PMMA NPs induced a different response profile increasing IDH activity (0.001 mg/L) and decreasing LDH activity (0.01 mg/L).

The present study data clearly demonstrates that NPs may induce alterations on fish larvae that may have serious repercussions at a population level (e.g. altered behaviour) at concentration in the mg/L range. The alteration of fish swimming behaviour may lead to altered performance and compromise reproduction, ability to escape from predators and capture food. However, it must be emphasized that these effects may however be influenced by environmental

conditions and the presence of other environmental contaminants, as demonstrated in the studies with combined exposures.

Factors like the ability of contaminants to adsorb to the particles surface, promote particles aggregation/agglomeration may significantly alter their bioavailability. The characteristics of the polymer are determinant in the toxicity level. At a biochemical level, nanoplastics demonstrated the ability to induce toxic responses through oxidative stress (e.g. lipid peroxidation) and alter the energy metabolism (reserves and aerobic and anaerobic metabolism).

Overall, this dissertation demonstrates that fish embryos are sensitive to nanoplastics and may be used to assess effects and mechanisms of toxicity, and rank polymers according polymer. The data from this study show that PMMA NPs are more toxic to fish larvae than PS. Further studies should be performed with particles of different sizes and other polymers. Furthermore, studies should assess if the behavioural effects detected at these stages of development are maintained in adults. The potential effect of exposure through feeding should also be considered as nanoparticles are expected to be incorporated by plankton which may be a food source for fish.