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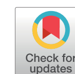
Polystyrene nanoplastics alter the cytotoxicity of human pharmaceuticals on marine fish cell lines



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A R T I C L E I N F O

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A B S T R A C T

There is an increasing concern on the consequences of the presence of micro(nano)plastics to marine organisms. The present study aimed to provide information on the effects of polystyrene nanoplastics (PSNPs) to fish cells alone and combined with human pharmaceuticals, other emerging contaminants, using as biological models marine fish cell lines SAF-1 and DLB-1. Cells were exposed for 24 h to 100 nm PSNPs, starting at 0.001 up to 10 mg/L, to assess effects on viability and activity of catalase (antioxidant defense) and glutathione *S*-transferases (phase II biotransformation and antioxidant defense). The viability of cells was also evaluated after exposure to human pharmaceuticals alone and combined with PSNPs. Overall, PSNPs failed to be cytotoxic but data proved their ability to alter the toxicity of human pharmaceuticals. DLB-1 was the most sensitive cell line to PSNPs. Data support the use of marine fish cell lines in the study of the effects of micro(nano)plastics.

1. Introduction

The problem of plastic pollution in the marine environment, identified in the 1970's (Carpenter and Smith, 1972), has in the recent years attracted the focus of an increased public awareness and scientific research. The pernicious effects of macroplastic debris on wildlife (e.g. entanglement, malformations in development and presence in the stomach of a wide range of organisms) are the most observable consequences of the plastics pollution, but probably not the worst scenario at environmental level. The degradation/fragmentation of plastic particles into microplastics (MPs) and nanoplastics (NPs), less obvious to the general public may, however, constitute a more serious threat to marine biota as they become readily bioavailable and impossible to remove from the environment. Their effects are currently the focus of an increasing number of studies (319 papers in 2018, in Pubmed database) that raised concerns to the risks associated with exposure to MPs (primary and secondary) in the animal welfare (Lots et al., 2017; Oliveira et al., 2013; Silva-Cavalcanti et al., 2017; Wright et al., 2013). These effects are not only manifested by their interference in the feeding of biota (Peters et al., 2017; Wright et al., 2013) but also in key metabolic functions that may affect survival rates of organisms (Jeong et al., 2017; Jin et al., 2018; Ma et al., 2016). One of the main concerns, associated with the presence of small plastic particles in the environment, is their ability to act as carriers of pollutants (Hermabessiere et al., 2017; Teuten et al., 2009) and more recently of potential pathogens (Curren and Leong, 2019; Viršek et al., 2017).

Currently, a wide range of plastic polymers are produced and released to the environment, being polystyrene (PS) among the most produced and reported in marine biota (de Sá et al., 2018). This polymer has a vast range of applications in the industry and degrades to MPs and NPs (Lambert and Wagner, 2016; Zhang et al., 2012), which may significantly enhance the transport of nonpolar and weakly polar compounds (Liu et al., 2018). Although compounds such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) are among the most reported adsorbates found in MPs (Daugherty, 2016; Napper et al., 2015; Wang et al., 2015), other contaminants widely present in the environment such as pharmaceuticals (Heberer, 2002; Li et al., 2018; Sokač et al., 2017) may also interact with plastic particles (Brandts et al., 2018b). Pharmaceuticals represent a potential real risk to the marine biota due to their bioactive nature, which can be incorporated by aquatic biota *via* waterborne exposure and/or adsorbed to MPs or NPs (Goedecke et al., 2017; Li et al., 2018; Razanajatovo et al., 2018; Sokač et al., 2017). These bioactive substances have been included in the category of contaminants of emerging concern. Analgesics, antipyretics, stimulants, antibiotics, lipidemic and cardiovascular drugs (Gaw et al., 2014; Koopaei and Abdollahi, 2017;

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Souza and Féris, 2016) are among the most commonly detected classes, with reported environmental levels oscillating from ng/L (e.g. propranolol - 50 ng/L in surface waters and 142 ng/L in marine waters) to μg/L (e.g. ofloxacin 25 ng/L in surface waters and 5.1 μg/L in marine waters) (Gaw et al., 2014; Sokač et al., 2017). Overall, there is a lack of knowledge on the impact of pharmaceuticals in the marine biota. Considering that organisms might be exposed to environmental NPs and pharmaceuticals, understanding the potential role of these particles in the bioavailability and biological effects of pharmaceuticals is highly desirable (Brandts et al., 2018a). A decrease in the plastic particle size leads to an increase in surface area to volume ratio, making NPs more reactive. Although some studies have assessed the effects of combined exposure to MPs and contaminants such as metals (Luis et al., 2016) and PAHs (Oliveira et al., 2013) on fish, the knowledge of the effects of NPs (alone or in combined exposures) is scarce. Thus, considering that PS is among the most demanded polymers, widely found in the environment and frequently used to assess the effects of plastics on biota (de Sá et al., 2018; Ferreira et al., 2019), it might be considered a good model to study the interaction between plastic particles and pharmaceuticals frequently reported in environmental matrices.

Fish cell lines are emerging as a simple and effective refinement of the use of animal models for toxicology and risk assessment. The majority of *in vitro* studies with fish focus on well-established freshwater fish models and cell lines (e.g. rainbow trout and zebrafish). However, there is the need to establish marine models and cell lines that allow comparison between endpoints on freshwater and marine models. In the recent years, marine continuous fish cell lines have been developed (Langner et al., 2011; Rafael et al., 2010; Villalba et al., 2017; Ye et al., 2006) to aid in the study of molecular and physiological responses to stressors and thus providing valuable tools to study the effects of emerging contaminants at molecular level (Morcillo et al., 2017; Pannetier et al., 2018; Vo et al., 2014). This approach to use fish cell lines in ecotoxicity studies is supported by ethical issues associated with animal welfare and the European directive (Directive 2010/63/EU) for the protection of animals used for scientific purposes, increasing the interest in this research niche. Organisms, such as European sea bass and gilthead seabream, in addition to their high economic importance in Europe, have also been reported as good biological models for marine ecotoxicology (Barreto et al., 2018; Brandts et al., 2018b). In this perspective, the use of marine fish cell lines may provide valuable information in terms of effects and mechanisms of action, and contribute to reduce the need of *in vivo* laboratory studies.

This study aimed to provide data for the research gap existing in the micro(nano)plastics field, namely in their interactions with pharmaceuticals. Thus, we evaluated the effects of PSNPs and pharmaceuticals, either alone or combined, by means of viability assays and two widely used biochemical biomarkers associated with antioxidant defense (catalase and glutathione *S*-transferases activity) and biotransformation (glutathione *S*-transferases activity), using an *in vitro* approach with marine fish fibroblast- and brain-derived cell lines. Cell lines from these fish species were selected because they are considered top predators in the marine environment, with high economic value and greatly consumed by humans. Although very scarcely used in aquatic ecotoxicology despite their promising potential, they represent unique models to study the effects of these xenobiotics, and information on key tissues (e.g. potential impact on brain cells).

2. Materials and methods

2.1. Chemicals

The pharmaceuticals gemfibrozil, propranolol, furosemide, fluoxetine, metformin and nicotine were acquired from TCI chemicals (Japan); paracetamol (acetaminophen) and acetylsalicylic acid from Sigma-Aldrich (Spain). Pharmaceuticals were dissolved at a stock concentration of 250 g/L in dimethyl sulfoxide (DMSO) with the exception of metformin that was dissolved in ultra-pure water and nicotine which was already supplied in liquid form. The stock solutions were diluted in cell line complete culture media to a working-solution of 2 g/L. All other reagents used were analytical grade (Sigma-Aldrich, Spain).

2.2. Nanoplastics

The plastic particles tested in this study, polystyrene nanoplastics (PSNPs), were prepared by mini-emulsion polymerization using sodium dodecyl sulfate (SDS) as a stabilizer (Brandts et al., 2018a). After the polymerization, PS particles were washed several times with ultrapure water. The characterization of the NPs was performed both in ultrapure water and complete culture media, evaluating the hydrodynamic size using dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern). Morphological characterization was performed by transmission electron microscopy (Hitachi, H9000 NAR). To avoid agglomeration of particles and promote homogeneous dispersion, particles were subjected to 30 min treatment in an ultrasonic bath (Sonorex Digitec DT 100 H, Bandelin), prior to dilution in complete culture media and cell exposure.

2.3. Cell culture

The fish cell lines used in this study were SAF-1 (ECACC - 00122301) (Bejar et al., 1997), a fibroblast-like line derived from gilthead seabream (*Sparus aurata* L.) caudal fin, and DLB-1 (Morcillo et al., 2017), a neuronal line derived from European sea bass (*Dicentrarchus labrax* L.) brain. Cells were maintained in an incubator at 25 °C and 85% relative humidity, under atmospheric air. The culture media used was Leibovitz-15 (L-15) (Biowest, France), supplemented with 2 mM L-glutamine (Biowest, France), 100 μg/mL streptomycin and 100 U/mL penicillin G (Biowest, France). SAF-1 required the additional supplementation of 10% fetal bovine serum (FBS; Biowest, France) and DLB-1 required 15% FBS, 10 mM HEPES (Biowest, France) and 0.16% NaCl (Sigma-Aldrich, Spain). Cells were routinely cultivated in 10 cm Ø culture dishes and assays were performed in flat-bottom clear 96-well plates for colorimetric measurements.

2.4. Cells exposure to PSNPs - single exposure

In order to access the effect of the NPs particles in cellular viability, SAF-1 and DLB-1 cells were plated in 96-well culture plates at a cell density of 2.5×10^4 and 5×10^4 cells per well, respectively, and allowed to adhere overnight. These cell densities were based on preliminary assays that assessed cell growth rate and optimal absorbance readings. Cells were exposed for 24 h to 10-fold increased concentrations of PSNPs starting at 0.001 mg/L up to 10 mg/L, to estimate the lethal doses (LD_{50}, LD_{25} and LD_{10}) on both cell lines, based on the thiazolyl blue tetrazolium bromide (MTT) viability assay. Controls consisted in cells incubated with the same reagents used for NPs preparation. The cytotoxicity was determined in at least three independent experiments, in quadruplicates, for all concentrations.A similar setup was made for biochemical biomarker analysis. SAF-1 and DLB-1 cells were plated in 6-well culture plates at 1×10^6 cells per well and allowed to adhere overnight. Cells were exposed for 24 h to increasing concentrations (10-fold) of PSNPs, from 0.001 mg/L up to 10 mg/L. Cells were collected in lysis buffer [20 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride (PMSF)] through scrapping and homogenized in an ultrasonic homogenizer (Branson Ultrasonics Sonifier S-250A). Homogenate was centrifuged at 10,000 g, for 10 min at 4 °C, being the supernatant collected and stored at -80 °C until further analysis.

2.5. Cells exposure to PSNPs and pharmaceuticals – combined exposure

To assess the effects of combined exposure to NPs particles and

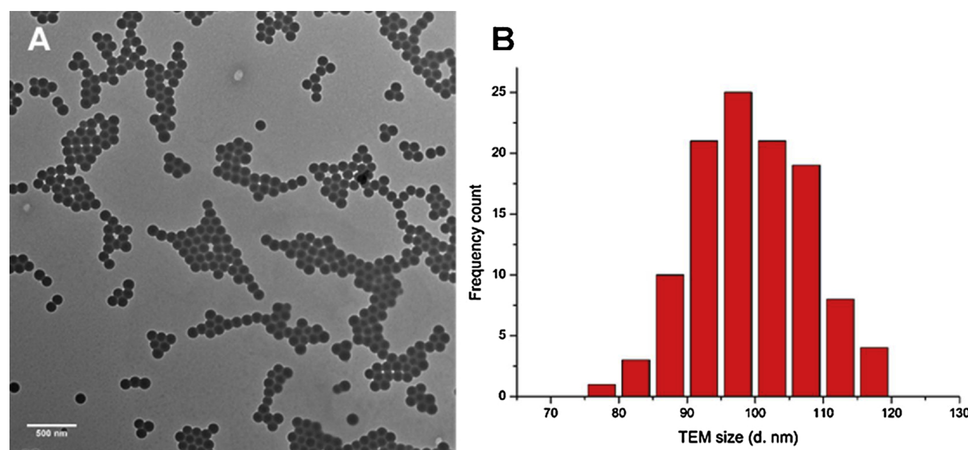

Fig. 1. Characterization of polystyrene particles by transmission electron microscopy (TEM). Particles display a round morphology and an average size of 99 (± 8.3 nm). A – TEM image of PS particles. Bar – 500 nm. B – Graphical representation of size frequencies measured in the TEM.

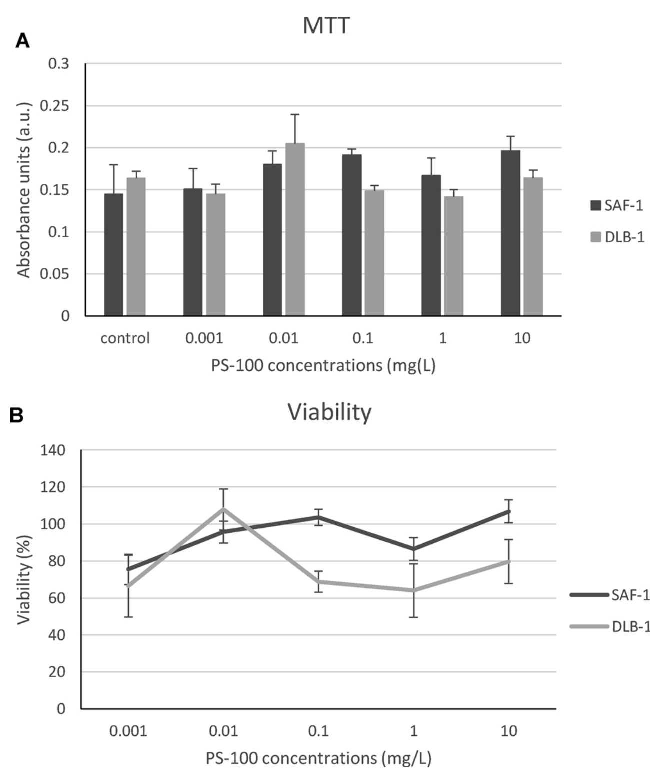

Fig. 2. Viability of SAF-1 and DLB-1 cells exposed to 100 nm polystyrene particles for 24 h. A - Absorbance values of the MTT assay; B – Viability curves of MTT assay. Viabilities are calculated as percentage of the control. Results are expressed as mean ± standard error (n = 3 replicates). PS – Polystyrene.

pharmaceuticals on cellular viability, cells were exposed for 24 h to increasing concentrations (5-fold) of pharmaceuticals starting at 2.56×10^{-6} up to 1g/L alone or combined with 10 mg/L PSNPs (pharmaceutical + NPs) to estimate LD_{50}, LD_{25} and LD_{10}, for single and combined exposures, by MTT assay. Controls consisted in cells incubated with the same reagents used for pharmaceuticals and/or NPs preparation. The cytotoxicity was determined in at least three independent experiments, in quadruplicates, for all concentrations.

2.6. Viability assays

We followed the National Institute of Health (NIH) assay guide for cell viability (Riss et al., 2016) and the Organization for Economic Co-operation and Development (OCDE) guideline 171 for the use of fish-derived cell lines for investigation of environmental contaminants (Bols et al., 2005; Dayeh et al., 2013), optimized for these cells and 96-well plate cultures. To do this, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Spain) was dissolved in ultra-pure water, to obtain a stock solution of 5 mg/mL, sterilized by filtration (0.22 µm pore) and stored at -20 °C, protected from light. After a 24 h exposure to the tested xenobiotics, media was removed and cells washed with phosphate-buffered saline (PBS), pH 7.4. MTT stock was diluted in PBS, pH 7.4 to obtain a 10% MTT solution and used to incubate the cells for 4 h. MTT solution was removed and cells lysed with 100% DMSO to solubilize the formazan crystals formed inside the cells. Samples were analyzed in a microplate reader (Spectra Max M2 - Molecular Devices) at 570 nm, maximum absorbance, and 690 nm, as a baseline. Viability was expressed as a percentage of solvent control. A solution of 1% Triton X-100 in culture media was used as positive control (Riss et al., 2016; Stern and Potter, 2010).

2.7. Biochemical biomarker assessment

Protein content was determined for all samples (Bradford, 1976), by measuring the absorbance at 595 nm, using a microplate-adapted procedure and bovine γ-globulin as the standard.

Catalase (CAT) activity was assayed as described elsewhere (Claiborne, 1985). Changes in the absorbance at 240 nm caused by the dismutation of hydrogen peroxide (H_2O_2) were recorded and CAT activity calculated as µmol H_2O_2 consumed per min per mg of protein ($\varepsilon = 40\ M^{-1}.\ cm^{-1}$).

Glutathione *S*-transferases (GST) activity was determined spectrophotometrically by the method of Habig et al. (1974) adapted to microplate reader (Frasco and Guilhermino, 2002), following the conjugation of the substrate, 1-chloro-2, 4-dinitrobenzene (CDNB, Sigma-Aldrich, Spain), with reduced glutathione. Absorbance was recorded at 340 nm and the GST activity was calculated as nmol of CDNB conjugate formed per min per mg of protein ($\varepsilon = 9.6 \times 10^{-3}\ M^{-1}.\ cm^{-1}$).

2.8. Data analysis

Normality (Shapiro-Wilk test) and homogeneity of variance (Bartlett's test) was tested using the GraphPad 6 prism software. Lethal doses were estimated using a nonlinear regression fitting curve with variable slopes (4 P). Statistical differences between single and combined treatments were verified with a paired *t*-test (significant differences were assumed for $p < 0.05$). For biochemical biomarkers, differences between treatments and controls were tested using one-way analysis of variance (ANOVA), followed by Dunnett's test whenever applicable.

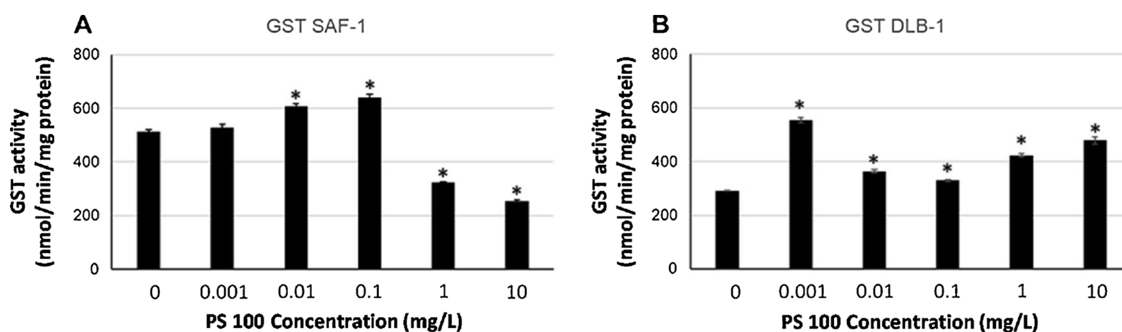

Fig. 3. Glutathione S-transferases (GST) activity of SAF-1 (A) and DLB-1 (B) cells exposed to 100 nm polystyrene nanoparticles for 24 h. Results are expressed as mean ± standard error (n = 3 replicates). *Significant differences to control (Dunnett's test, $p < 0.05$). PS - Polystyrene.

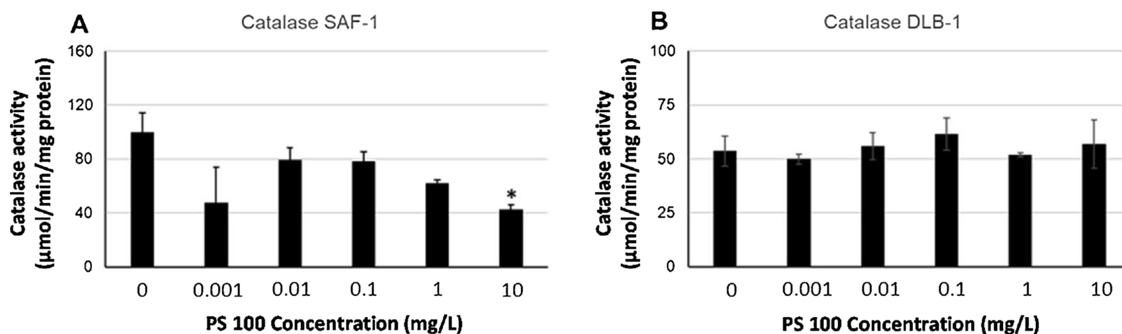

Fig. 4. Catalase (CAT) activity of SAF-1 (A) and DLB-1 (B) cells exposed to 100 nm polystyrene nanoparticles for 24 h. Results are expressed as mean ± standard error (n = 3 replicates). *Significant differences to control (Dunnett's test, $p < 0.05$). PS – Polystyrene.

3. Results and discussion

3.1. NPs synthesis and behavior in test media

The information provided by the transmission electron microscopy confirmed that the particles, in ultra-pure water, displayed a round shape with a medium size of 99.7 ± 8.3 nm (Fig.1). Thus, in this work, they will be referred as PS-100 NPs for uniformization. The behavior of PSNPs was analyzed by DLS in both the cell culture media and ultra-pure water after 1 and 24 h (Fig. S1; Supplementary Information). In ultra-pure water, PS-100 NPs displayed a hydrodynamic size of 125 nm with small variations over time (130.3 nm after 1 h and 130.1 nm after 24 h). In the complete culture media, hydrodynamic size increased to 147.4 nm and 156.4 nm, respectively in the SAF-1 and DLB-1 media (Table S1; Supplementary Information). Despite the initial increase of the hydrodynamic size, it remained stable up to 24 h, which indicates that PS-100 NPs did not agglomerate/aggregate during the course of the experiments. The increase in particle size (up to 50%) may be associated with the adsorption of proteins and lipids, present in the media, onto the NPs surface. One of the constituents of the complete media is FBS (10% and 15%, for SAF-1 and DLB-1 respectively) that is often used as a biocorona in nanoparticles to increase biocompatibility (Izak-Nau et al., 2013; Persaud et al., 2019; Shannahan, 2017). The data show that in the media supplemented with a higher amount of FBS, NPs displayed a higher hydrodynamic size. The formation of many different and variable biological/chemical coronas in the environment increases the complexity of assessing the effects of these materials. Biocorona may change NPs characteristics, e.g. behavior, distribution in biological matrices and toxicity. Although the impact of biocorona formation has been assessed in different nanomaterials such as silica (Izak-Nau et al., 2013; Vo et al., 2014), silver (Persaud et al., 2019) and gold (García-Álvarez et al., 2018) nanoparticles, no study was found concerning NPs. In this study, the authors considered relevant to assess the effects of NPs in the optimal cell culture conditions. The use of lower amount of FBS could compromise the fitness of the cells and the obtained results be a consequence of poorer medium and not a consequence of exposure to NPs. FBS is included in the majority of growth supplements used in the culture of human and animal cells as it provides cells with the factors required for cell growth, proliferation and metabolism. Nonetheless, the role of FBS in terms of potential increased biocompatibility and stabilization of particles cannot be neglected as demonstrated in other studies (Barreto et al., 2015). A corona around NPs may however promote interactions with cells different from those induced by pristine NPs but the formation protein corona in *in vitro* studies is also expected in *in vivo*, where the proteins of the test organisms are always available. A comparison of the behavior of the particles in the culture media and artificial seawater supports the idea that proteins help to stabilize the NPs as these plastics in artificial seawater (salinity 30 ppt) displayed a hydrodynamic size of 134.8 at time 0, which increased to 345.5 after 24 h (Brandts et al., 2018a). However, turbulent waters and a variety of chemicals may act as dispersants and stabilizers in natural conditions.

3.2. Effects of PS-100 NPs exposure on fish cell lines

The two different marine fish cell lines, SAF-1 and DLB-1, were exposed to PS-100 NPs for 24 h to evaluate the NPs effects on cell viability. One of the main limitations of laboratory studies focusing on the potential consequences of NPs and MPs is the lack of environmental data concerning the concentration of particles of sizes bellow 300 µm (Oliveira et al., 2019). In this perspective, environmental relevance of the tested concentrations is still unknown. Nonetheless, the levels of NPs are expected to increase consistently over time as a result of microplastics degradation and continuous environmental release (Oliveira and Almeida, 2019). Although the tested concentrations spanned from µg/L to mg/L no statistical differences between treatments and controls were found for the tested cell lines (Fig. 2). Analysis of the viability curve (Fig. 2B) showed a slight but non-significant decrease of SAF-1 viability with the lowest concentration tested, yielding a 25% decrease in viability when compared to controls. However, at higher concentrations cell

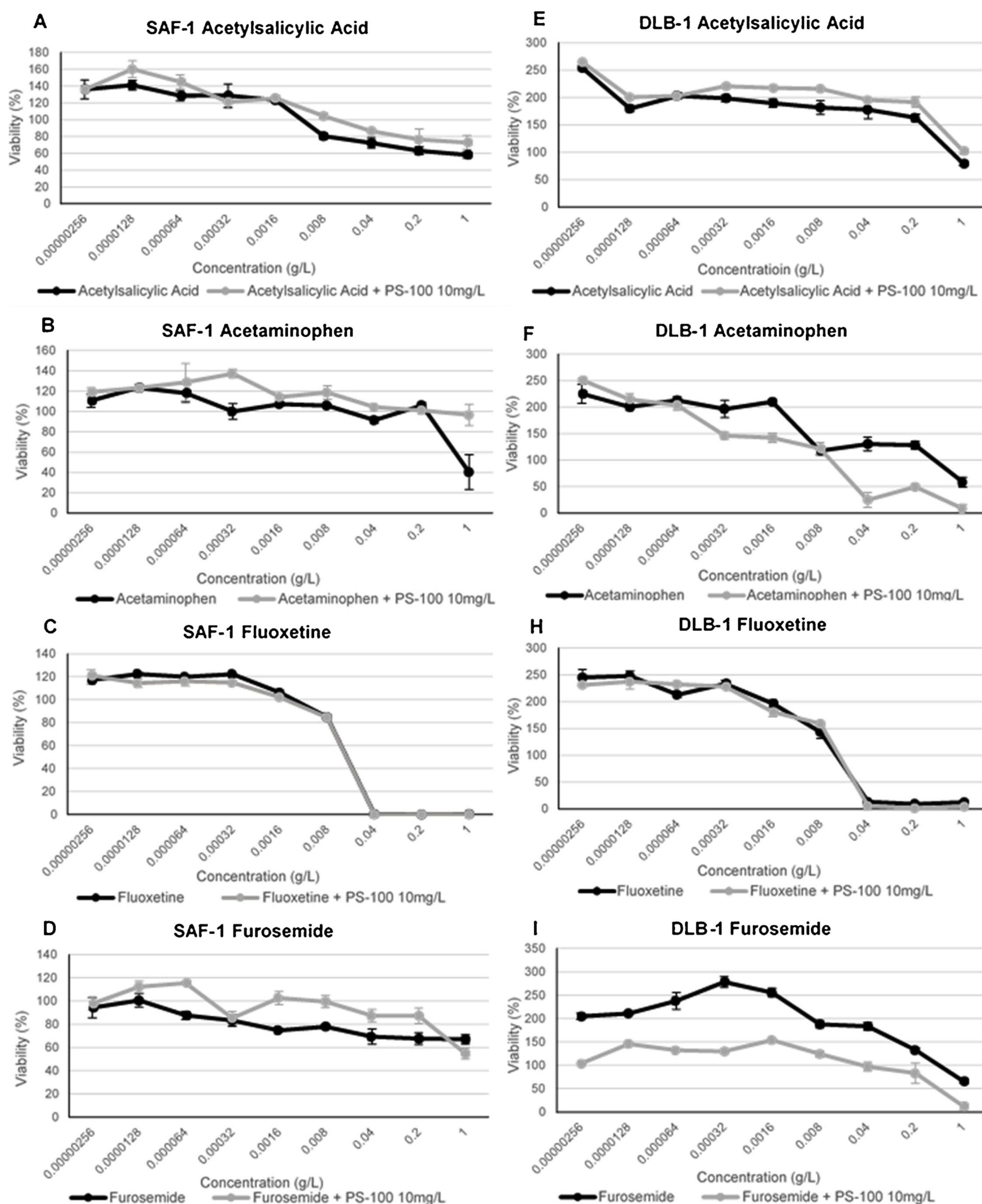

Fig. 5. Viability of SAF-1 and DLB-1 cells exposed to pharmaceuticals (alone and combined with 10 mg/L of 100 nm polystyrene particles) for 24 h. (A, E) – Acetylsalicylic Acid; (B, F) –; Acetaminophen; (C, G) – Fluoxetine; (D, I) – Furosemide; Results are presented as a percentage of control. Results are expressed as mean ± standard error (n = 3 replicates).

viability values were higher, demonstrating mild effects on this cell line. Regarding the DLB-1 cell line, a 66% cell viability was found at 0.001 mg/L of PS-100 NPs, but for higher concentrations viability oscillated with minimum viability (64%) found at 1 mg/L. Data did not allow estimation of LDs that would allow a clear indication of the most sensitive cell line; nonetheless, data suggest that DLB-1 cell line is more susceptible to PS-100 NPs. Based on the scarce available studies with these cell lines, DLB-1 cells have been demonstrated to be more sensitive to metals such as cadmium (Cd), lead (Pb) and arsenic (As) than the SAF-1 (Morcillo et al., 2017, 2016) as this study also supports. Whether this fact is related to the different pollutants, to the fish species or to the tissue of origin merits further investigation.

Based on the lack of significant effects on cell viability, two biochemical endpoints, associated with important cell mechanisms, were assessed. CAT was selected as an important enzyme in the antioxidant defense against reactive oxygen species (ROS), often induced by xenobiotics and associated with mechanisms of toxicity of several emerging contaminants. CAT is known to protect cells by reducing H_2O_2, the main cellular precursor of the most toxic ROS, the hydroxyl radical. Considering the poor efficiency of antioxidants towards this particular ROS, the removal of H_2O_2 has been indicated as an important strategy for counteracting the toxicity of hydroxyl radicals (Oliveira et al., 2009) and CAT considered as the first enzymatic defense to control H_2O_2 (Oliveira et al., 2010a). This enzyme is considered as one of the most

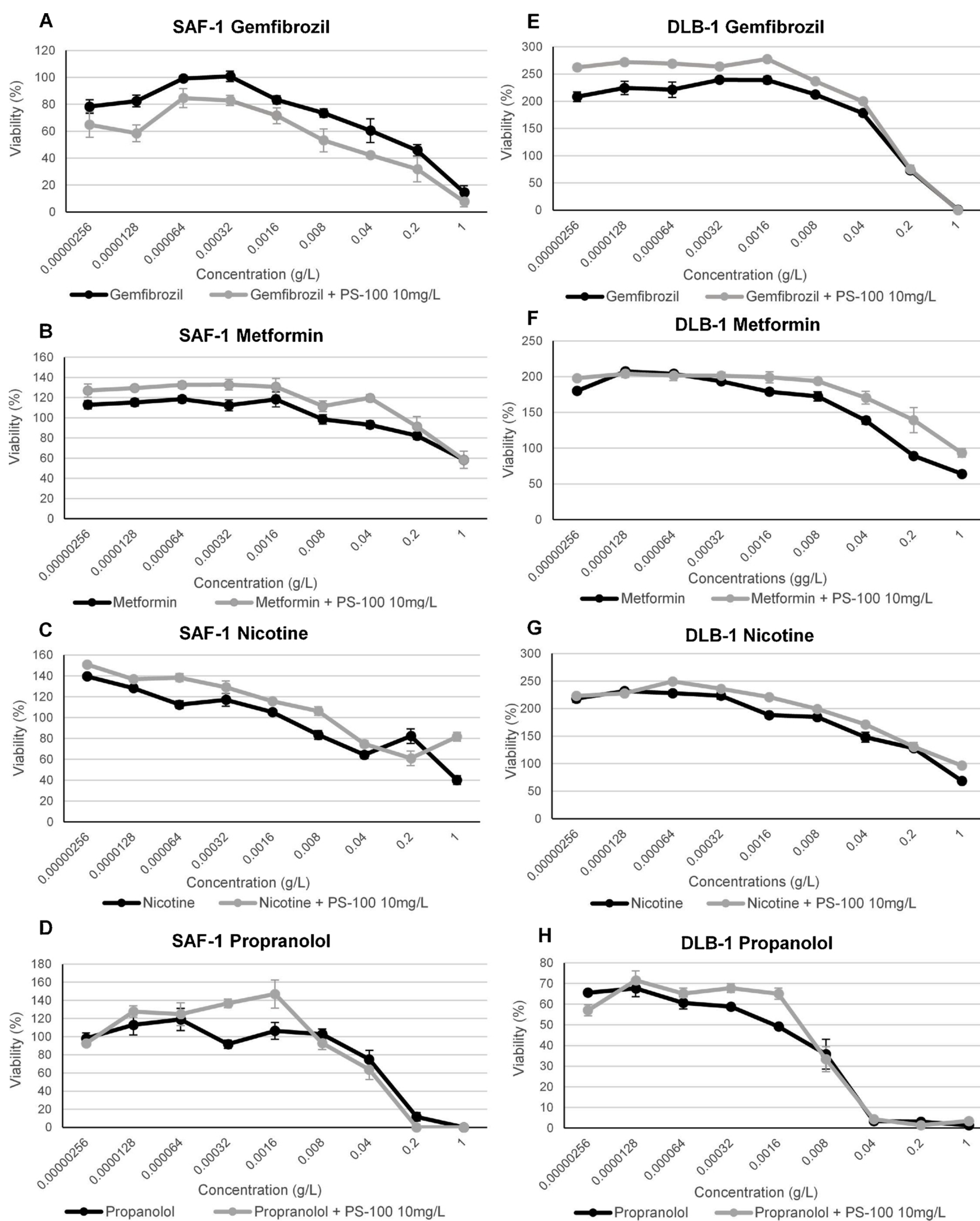

Fig. 6. Viability of SAF-1 and DLB-1 cells exposed to pharmaceuticals (alone and combined with 10 mg/L of 100 nm polystyrene particles) for 24 h. (A, E) – Gemfibrozil; (B, F) – Metformin; (C, G) – Nicotine; (D, H) – Propranolol. Results are presented as percentage of control. Results are expressed as mean ± standard error (n = 3 replicates).

conspicuous and responsive to ROS in vertebrate and invertebrate species (Halliwell and Gutteridge, 1999). Regarding GST this was selected due to its role in phase II of biotransformation, being involved in conjugation reactions of active electrophilic metabolites or their parental compounds with reduced glutathione (GSH), enabling its transformation into more extractable hydrophilic metabolites. But it is also involved in antioxidant defenses. GST, jointly to glutathione peroxidase, decreases the reactivity of lipid hydroperoxides by their conjugation with GSH (Oliveira et al., 2010b).

Our data show that exposure to PS-100 NPs led to a significant increase of GST activity in SAF-1 cells at 0.01 and 0.1 mg/L (Fig. 3A). However, at higher concentrations, GST activity was significantly inhibited. In DLB-1 cells, GST activity was significantly higher than in controls at all PS-100 NPs tested concentrations (Fig. 3B). Regarding the CAT activity, in SAF-1 cells, enzyme activity displayed a decrease trend although significant effects were only found at the highest concentration of PS-100 NPs (10 mg/L) (Fig. 4A). In DLB-1 cells, no significant differences compared to controls were found (Fig. 4B). Overall, the results from the biochemical biomarkers suggest that cells exposed to PS-100 NPs induce the production of hydroperoxides at levels able to induce increased GST at 0.01 and 0.1 mg/L in SAF-1 cells and at all concentrations in DLB-1 cells. However, in SAF-1 cells, PS-100 NPs (1 and 10 mg/L) impaired GST activity. This effect could be caused by a direct enzyme inhibition or depletion of GSH, a non-enzymatic

Table 1
Estimated lethal doses (LD_{50}, LD^{25} and LD_{10}) to SAF-1 cell line after 24 h exposure to pharmaceuticals (alone and combined with 10 mg/L of 100 nm polystyrene particles). LDs were calculated through interpolation of a nonlinear regression with a four-parameter dose-response curve. Values missing indicate that LDs were out of the curve range.

SAF-1	Pharmaceutical			Pharmaceutical + PS		
	LD_{50}	LD_{25}	LD_{10}	LD_{50}	LD_{25}	LD_{10}
Acetylsalicylic Acid	–	0.215	0.048	–	0.676	0.104
Acetaminophen	0.822	0.425	0.299	–	–	–
Gemfibrozil	0.351	0.173	0.075	0.242	0.194	0.082
Fluoxetine	0.013	0.009	0.008	0.014	0.011	0.009
Furosemide	0.103	0.024	0.008	0.850	0.453	0.206
Metformin	–	0.336	0.112	–	0.501	0.145
Nicotine	0.426	0.124	0.032	0.599	0.202	0.092
Propranolol	0.198	0.184	0.174	0.062	0.035	0.025

Table 2
Estimated lethal doses (LD_{50}, LD^{25} and LD_{10}) to DLB-1 cell line after 24 h exposure to pharmaceuticals (alone and combined with 10 mg/L of 100 nm polystyrene particles). LDs were calculated through interpolation of a nonlinear regression with a four-parameter dose-response curve. Values missing indicate that LDs were out of the curve range.

DLB-1	Pharmaceutical			Pharmaceutical + PS		
	LD_{50}	LD_{25}	LD_{10}	LD_{50}	LD_{25}	LD_{10}
Acetylsalicylic Acid	–	0.674	0.486	–	–	0.830
Acetaminophen	–	0.711	0.384	0.081	0.022	0.011
Gemfibrozil	0.262	0.165	0.126	0.249	0.172	0.140
Fluoxetine	0.019	0.013	0.011	0.025	0.017	0.014
Furosemide	–	0.776	0.585	0.727	0.420	0.287
Metformin	–	0.674	0.486	–	–	–
Nicotine	–	0.994	0.718	–	–	–
Propranolol	0.002	–	–	0.005	–	–

antioxidant defense, essential for GST activity. The CAT activity inhibition found in the SAF-1 cells exposed to the highest concentration tested also supports this hypothesis. The results obtained in this *in vitro* study with PS-100 NPs support the information available in the literature that virgin NPs have low toxicity, exhibiting mainly a response by the anti-oxidative cellular platform (Brandts et al., 2018a; Ding et al., 2018; Espinosa et al., 2018). Nonetheless, data suggest that at high concentrations PSNPs may affect the biotransformation of xenobiotics and antioxidant defenses, making cells more susceptible to damage.

3.3. Effects of combined exposure to pharmaceuticals and PS-100 NPs

One the main concerns associated with the presence of small plastics in the marine environment is their potential “Trojan horse” effect. Thus, this study also aimed to evaluate the role of PS-100 NPs on the toxicity of an array of human pharmaceuticals: acetaminophen (analgesic), acetylsalicylic acid (non-steroidal anti-inflammatory), fluoxetine (antidepressant), furosemide (diuretic), gemfibrozil (lipid regulator), metformin (glucose regulator), nicotine (stimulant) and propranolol (betablocker); which are all widely prescribed for human health and their presence in the environment continuously spreading (Gaw et al., 2014; Heberer, 2002; Koopaei and Abdollahi, 2017; Ribas et al., 2014; Sokač et al., 2017). The analysis of the cytotoxicity curves (Figs. 5 and 6; Supplementary Fig. S2, S3) and estimated LD values for the tested pharmaceuticals (Tables 1 and 2) show pharmaceutical specific sensitivity of the tested cell lines. It is important to consider that some of the used pharmaceuticals have cellular proliferative actions (gemfibrozil, acetaminophen) (Teles et al., 2016; Yu et al., 2014), which explain viabilities higher than 100% observed, especially for DLB-1 cell line. But for other pharmaceuticals known to produce oxidative stress (e.g. nicotine, acetylsalicylic acid, furosemide) (Barr et al., 2007; Raza et al., 2011; Silbert et al., 2017), MTT assay might induce false positives as already suggested (Śliwka et al., 2016). Considering the viability curves and the LD_{50} values, it is apparent that both fluoxetine and propranolol were the pharmaceuticals that exhibited the highest toxicity to both fish cell lines. Fluoxetine presented a LD_{50} of 0.013 g/L for SAF-1 and 0.019 g/L for DLB-1 cells, and the combined treatment did not significantly alter the LD_{50} values. Propranolol had similar values in DLB-1 cell line, with a LD_{50} of 0.002 g/L and 0.005 g/L in the combined exposure with PS-100 NPs. For SAF-1 cells, propranolol exerted a lower effect when compared to DLB-1 cells, with a LD_{50} of 0.198 g/L for single exposure. However, in the combined treatment, a lower LD_{50} (0.062 g/L) was estimated, revealing an increase in toxicity towards these cell lines. The results obtained with propranolol in SAF-1 cells support the “Trojan horse” effect assumption of plastics that may act as carriers of pharmaceuticals and increase their toxicity. LD values reported in other studies, for other fish cell lines, vary greatly depending on tissue source and species. The values found for fluoxetine are similar to those registered in PLHC-1 (hepatoma cell line derived from *Poeciliopsis lucida*) and RTG-2 (gonadal cell line derived from *Oncorhynchus mykiss*) cell lines and primary hepatocytes (obtained from *O. mykiss*) (Caminada et al., 2006; Laville et al., 2004). For gemfibrozil, the LD values found in SAF-1 and DLB-1 cells were between those reported for PLHC-1 and RTG-2 cells, but for other pharmaceuticals these levels are dissimilar. The cell lines tested in this study were obtained from fin fibroblasts and brain and thus are expected to present differences in metabolism when compared to cell lines derived from other types of tissue (e.g. obtained from liver). Overall, based on LD_{50} and LD_{25} values, SAF-1 cell line was more sensitive to acetylsalicylic acid, acetaminophen, and nicotine whereas DLB-1 was more sensitive to gemfibrozil, metformin and propranolol. For fluoxetine and furosemide, the estimated LD values were very similar. In general, after simultaneous exposure with PS-100 NPs, the estimated LD_{50} values were altered. For acetaminophen in SAF-1 cells and for acetylsalicylic acid, metformin and nicotine in DLB-1 cells, data did not allow estimation of DL_{50} values. The most altered values in SAF-1 cells were related to gemfibrozil, metformin and propranolol, which presented a lower LD_{50} while for furosemide presented a higher value. In the case of DLB-1 cells, LD_{50} values of acetaminophen and furosemide decreased. The mechanisms behind the altered toxicity by PS-100 NPs should be further explored but may be associated to incorporation rates, sorbing ability and cellular defense mechanisms.

4. Conclusions

The data obtained in the present study clearly support the idea that marine fish cell lines may be a good biological model to assess marine toxicology, including emerging pollutants. Overall, the present study revealed that PS-100 NPs by themselves present low lethality to the tested cell lines, at least after 24 h exposure. Nonetheless, they have the ability to affect key enzymatic activities, suggesting that they may compromise cell responses to additional stressors. This is supported by the fact that combined exposure of the cell lines to pharmaceuticals and NPs altered pharmaceutical toxicity. This effect may be modulated by factors such as cell type and formation of protein corona (e.g. level of FBS). Further studies should explore the effects of these particles as well as smaller particles on marine cell lines after longer exposure periods, also assessing other endpoints such as non-enzymatic antioxidant defenses, DNA and protein damage and expression of genes involved in metabolic pathways. Further studies should be performed to allow a better understanding of the mechanisms involved.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.etap.2019.03.019.

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