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Ecotoxicity of emerging contaminants in the reproductive organ of marine mussels *Mytilus galloprovincialis*

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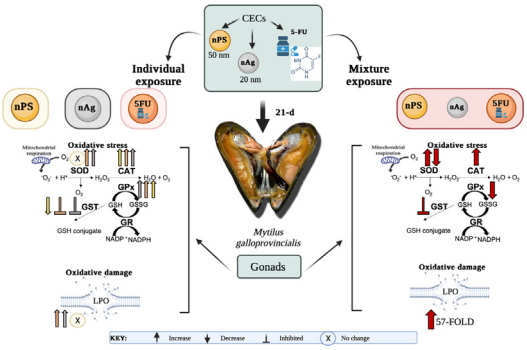
HIGHLIGHTS

- Silver nanoparticle – polystyrene nanoplastics – 5-fluorouracil interaction toxicity
- Mussel gonads antioxidant defence system compromised, leading to oxidative damage
- CECs mixture interactions are synergistic at 3 days of exposure.
- CECs mixture interactions are antagonistic at other exposure times.
- nAg and 5FU most influential in Mix

GRAPHICAL ABSTRACT



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ABSTRACT

Contaminants of emerging concern (CECs) present a new threat to the marine environment, and it is vital to understand the interactions and possible toxicity of CEC mixtures once they reach the ocean. CECs—such as metal nanoparticles, nanoplastics, and pharmaceuticals—are groups of contaminants some of which have been individually evaluated, though their interactions as mixtures are still not fully understood. To ensure a healthy and prosperous future generation, successful reproduction is key: however, if hindered, population dynamics may be at danger leading to a negative impact on biodiversity. This study aimed to understand the effects of silver (20 nm nAg, 10 μg/L), polystyrene nanoparticles (50 nm nPS, 10 μg/L), and 5-fluorouracil (5FU, 10 ng/L) individually and as a mixture (10 μg/L of nPS + 10 μg/L of nAg + 10 ng/L of 5FU) in the gonads of *Mytilus galloprovincialis*. A multibiomarker approach, namely the antioxidant defence system (ADS; superoxide dismutase, catalase, glutathione peroxidases, glutathione – *S* – transferases activities), and oxidative damage (OD; lipid peroxidation) were analysed in the gonads of mussels. All exposure treatments after 3 days led to an increase of enzymatic activity, followed by an inhibition after 14 and 21 days. Thus, ADS was overwhelmed due to the generation of ROS, resulting in OD, except for nPS exposed mussels. The OD in Mix exposed mussels increased exponentially by 57-fold. When CEC mixtures interact, they are potentially more hazardous than their individual components, posing a major threat to marine species. To understand synergistic and antagonistic interactions, a model was applied, and antagonistic interactions were observed in evaluated biomarkers at all timepoints, apart from a synergistic interaction at day 3 relative to LPO. Results indicate that the effects observed in Mix-exposed mussel gonads are mainly due to the interaction of nAg and 5FU but not nPS.

1. Introduction

Contaminants of emerging concern (CECs) has the scientific community rendered with the possible ecotoxicological effects they may have towards aquatic organisms, and consequently, human health. According to the

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criteria—persistence in the environment, and noxiousness and ecotoxicological effects—CECs are a variety of chemical substances (anthropogenic and/or naturally occurring) that are not frequently monitored in the environment (Pastorino and Ginebreda, 2021). These include pharmaceuticals and personal care products, endocrine-disrupting chemicals, nanomaterials and nanoparticles, and persistent organic pollutants (Kumar et al., 2022). A source of most CEC's landing in our seas is primarily due to the lack of treatment and removal of these contaminants by wastewater treatment plants (WWTP). Inevitably, these CECs will end up all together in the ocean and, although crucially important to understand the toxicity that these CECs pose to marine life, it is also of uttermost importance to comprehend how their toxicity differs when the CECs are present all together. Mixtures of CECs are lately getting much attention; however most data focus on mixtures of contaminants belonging to the same group (Affek et al., 2018; Brezovšek et al., 2014; Elersek et al., 2016; Mater et al., 2014; Novak et al., 2017), whilst some focus on microplastics and pharmaceuticals interactions (Martín et al., 2022; Pashaei et al., 2022; Qu et al., 2018; Sellami et al., 2021), and others on micro/nanoplastic interaction with metals and metal nanoparticles (Davranche et al., 2019; Estrela et al., 2021; Yu et al., 2019). So, the question arises to how toxic are mixtures of differently classed CECs, such as engineered nanoparticles, nanoplastic pollution, and pharmaceuticals, towards the marine environment, and whether the interaction of these contaminants are more severe.

With the advancement of nanotechnology, and engineered nanomaterials being most frequently utilised in nano-functionalized consumer goods, silver nanoparticles (nAg) are mostly used in the personal care, antimicrobial coatings, photonic devices, and textile industries (Lee and Jun, 2019; Maillard and Hartemann, 2013; Pereira et al., 2020). Production of nAg products has rocket climbed throughout the years due to its properties — antimicrobial, antibacterial, antifungal, antiviral, anti-inflammatory, and anticancer (Khan et al., 2018) — the global nAg market size, with a revenue of over 2 billion USD in 2021, is projected to surpass 5 billion USD by 2029 (Market Research, 2022). The toxic effects of nAg towards marine organisms have been thoroughly evaluated (Auguste et al., 2018; Gomes et al., 2013; Lorusso et al., 2022; McCarthy et al., 2013; Rezvani et al., 2019) and its mode of action (MoA) is known to directly harm cell membranes; nAg can liberate Ag^+ ions, and both nAg and Ag^+ generate reactive oxygen species (ROS) (Ghobashy et al., 2021). nAg levels in the environment are anticipated to be between 0.03 and 0.08 mg/ L (Fernandes et al., 2017), as waste water treatment plants (WTTP) are not effective in the removal or treatment of nAg. For instance, nAg will primarily partition to the sludge in WWTP (Bolaños-Benítez et al., 2020). Sludge may be sent to landfill/ used as agricultural fertilizer, and through leaching and surface run-off, nAg may enter the marine environment (Blaser et al., 2008).

Moreover, WWTP have a lack in removal of plastic debris, and as global plastic pollution is incrementing — reports from 2021 show an increase in global plastic production to 390.7 million tons in 2022) — the possible adverse effects towards marine organisms is highly concerning. Europe, since 2020, have decreased plastic production by 4 %, whilst China increased their production to almost a third of the world's plastics (PlasticEurope, 2022). Polystyrene (PS), in Europe, accounts for 6.1 % of plastic production, being that its applications, such as packaging and building insulation, accounts for the world's two largest plastic markets, 44 % and 18 %, respectively (PlasticEurope, 2022). Nowadays, within plastic pollution, there is an increasing focus on nanoplastics, whether primary or secondary, as these nano-sized plastic particles could cross cellular boundaries, increasing their potential toxicity towards marine organisms — as the size of the particle decreases, the biological reactivity increases — (Peng et al., 2020). For instance, the antioxidant defence system of *M. galloprovincialis* was inhibited by exposure to PS nanoplastic (nPS) at a dose of 10 μg/L (50 nm nPS; 21 d), resulting in oxidative damage, with the mussel gills being the most severely affected tissue (Gonçalves et al., 2022b). Lysosomal indicators of general stress were more affected by nPS exposure to *M. galloprovincialis* (50 nm; 1.5–150 ng/L; 21-d) than by PS-microplastics (PS-MPs), suggesting a connection between this trend and improved antioxidant production (Capolupo et al., 2021). Moreover, when *M. galloprovincialis* were exposed to different types and shapes of plastic polymers in the micro size range (241.3 ± 32.7 to 3073.8 ± 21.71860.1 μm; 12.5 particles/L & 25 particles/L; polyamide, polyethylene, and polymethyl methacrylate; 7 days) the antioxidant defence in gills and digestive gland was more overwhelmed by smaller particles, that were retained by the digestive tract compared to bigger ones (Provenza et al., 2022a). The glitter particles broke down into smaller and shorter particles which resulted in higher levels of oxidative stress (Provenza et al., 2022a), incrementing the necessity to better understand the toxicity of NPs, as they differ from MPs. Given the absence of appropriate analytical techniques for determining the presence and concentration of NPs in marine matrixes, the detection of NPs in natural environments continues to remain tricky (Cai et al., 2021; Wang et al., 2021), however, Halle et al. (2017) collect from the North Atlantic Gyre a nanoplastic segment containing four plastic polymers, including PS, all in the nano-size range, confirming the presence of nanoplastics in the ocean.

Additionally, pharmaceuticals make up a significant subset of CECs, although anti-cancer drugs have not yet drawn much attention. Concerningly, an increasing number of patients are getting cytostatic drug-based chemotherapy in hospitals or at home. Because these pharmaceutical compounds seldom entirely metabolise, they are eventually released into the water unchanged or modified (Trombini et al., 2016). One of the most popular pharmaceutical compound for the treatment of solid tumours as well as colorectal, pancreatic, and breast cancer is 5-fluorouracil (5FU), a cytostatic agent (IARC Class 3) (Mahnik et al., 2007). 5FU is a pyrimidine analogue of uracil with a fluorine atom (Cairns et al., 2011), and the primary MoA of 5FU in mammals is the incorrect incorporation of its fluoro-nucleotides into DNA (Matuo et al., 2009), as well as the release of cytochrome *c* from the mitochondria, promoting ROS and the production of superoxide radicals and oxidative stress (Gonçalves et al., 2022a). According to estimates, 5FU concentrations in European waterways range from 0.3 ng/L to 2.5 ng/L, and below 160 ng/L in surface waters (Heath and Isidori, 2020). More recent information in the gills and digestive gland of the marine mussel *M. galloprovincialis* (10 ng/L; 21 d) showed that the antioxidant defence system (ADS) was able to counteract ROS production and prevent oxidative damage (Gonçalves et al., 2022a). On the other hand, because of 5FU MoA, genotoxicity did occur in *M. galloprovincialis* haemolymph (Gonçalves et al., 2022a).

Bivalves are a class of species widely used in ecotoxicological assessment, and the Mediterranean mussel *M. galloprovincialis* is an excellent sentinel organism to help understand how these CECs can affect marine organisms. As sessile filter-feeders, *M. galloprovincialis* organisms are effective tools for evaluating the environmental quality because of their characteristics like susceptibility to contaminants, widespread geographic distribution, sedentary lifestyle, and ease of sampling (Provenza et al., 2022b). A more important matter, which is hardly evaluated, are the effects these CECs may have on the reproductive organ, the gonads, of the mussel, as reproductive impairment, or unsuccessful larval-embryo development could consequently affect marine populational dynamics. Therefore, this study's aim is to understand the effects nAg (20 nm; 10 μg/L), nPS (50 nm; 10 μg/L), 5FU (10 ng/L) and a mixture of the three CECs (Mix: 10 μg/L of nAg +10 μg/L of nPS + 10 ng/L of 5FU) in the gonads of *M. galloprovincialis* after a 21-day exposure assay, to evaluate whether CEC's are more toxic individually or as a mixture. A multi-biomarker approach was used to evaluate oxidative stress and oxidative damage, as well as quality indexes and models to comprehend the interactions occurring within the mixture.

2. Materials and methods

2.1. Silver nanoparticles (nAg)

Silver dispersion – nanoparticles of 20 nm in size (TEM) in 0.02 mg/mL aqueous buffer containing sodium citrate as a stabilizer was acquired from Sigma-Aldrich (730793) (JMGS, Portugal). A DLS particle sizer (ZetaSizer

Nano ZS90, Malvern Inc.) was used to measure the hydrodynamic diameter of nAg (in both ultrapure water and filtered seawater (FSW)) and the electrophoresis mobility of nAg to calculate its zeta potential in a disposable polycarbonate capillary cell at 25 °C (DTS1061, Malvern Inc.). The aggregation kinetics were assessed using time resolved DLS measurements. Ag nanoparticles size were measured for a duration of 2 to 12 h. The hydrodynamic diameter (HD) of nAg remained unchanged in ultrapure water (34 ± 0.69 nm) with the ζ -potential under these circumstances (54.8 ± 1.62 mV) and no aggregation occurs in ultrapure water. However, HD increase in FSW (521.9 ± 87.42 nm) indicating an aggregation of nAg in FSW. A concentration of 10 μg/L was used for exposure assays.

2.2. Polystyrene nanoplastics (nPS)

Fluoresbrite® Plain YG spherical polystyrene nanoplastics of 50 nm in size (CAS 9003-53-6) were purchased from Polysciences, Inc. (Germany). 50 nm particles packed as 2.5 % aqueous suspension, with 3.64 × 1014 particles/mL in ultrapure water (7732-18-5) (CV = 15 %, Excitation max. = 441 nm, Emission max. = 486 nm). A concentration of 10 μg/L was used for exposure assays. Characterization of nPS is detailed in Gonçalves et al. (2022b), and shows that nPS hydrodynamic diameter increases when dispersed in FSW (852 ± 103 nm), suggesting that the high concentration of salts in seawater leads to aggregation/agglomeration kinetics.

2.3. 5-Fluorouracil (5FU)

5FU (CAS 51-21-8) was purchased from Sigma Aldrich, Inc. The experimental study was carried out utilizing a class II biological safety cabinet, with suitable clothing, to ensure the safety of medication handling (open-back, impervious chemotherapy protection gown, double powder-free latex gloves, and safety goggles). Sequential dilutions in ultrapure Milli-Q water were prepared, to obtain a final concentration of 100 μg/L. Then a working solution of 10 ng/L of 5FU was prepared in seawater.

2.4. Experimental design

Mussels *M. galloprovincialis* Lamarck (50 mm ± 5 mm) were collected from the Ria Formosa Lagoon, Southeast Portugal (37°00′30.6”N 7°59′ 39.6”W) during the reproduction phase (August 2020) and transported alive to the laboratory. Following a four-day acclimatisation period, a total of 20 mussels were placed into each 15 L tanks, with 10 L of seawater in a duplicate design. Mussels were contaminated with 10 μg/L of nAg (20 nm), 10 μg/L of nPS (50 nm), 10 ng/L of 5FU and the corresponding mixture (10 μg/L of both nAg and nPS, and 10 ng/L of 5FU), for 21 days. Seawater was exchanged every two days and contaminants were re-dosed. Mortality was observed in mussels exposed to nAg on day 6 (one dead mussel), to nPS on day 1 (one dead mussel) and day 3 (one dead mussel), to 5FU on day 3 (six dead mussels) and day 10 (two dead mussels), to Mix on day 0 (one dead mussel) and day 1 (one dead mussel) of exposure. Mussels were collected on day 0, 3, 7, 14 and 21 of exposure for a multibiomarker analysis. Mussels were weighed and gonads were dissected and instantly frozen in liquid nitrogen and stored at −80 °C for subsequent investigation on the effects of nAg, nPS, 5FU and the Mix.

The availability of food resources permits mussels *M. galloprovincialis* to have a quick gonadal recuperation, and therefore, spawning occurs throughout the year with higher rates during the summer months (Ramos, 2017). In August specifically, gonads were either ripe, spawning or in a late spawning stage (Ramos, 2017).

2.5. Quality control and assessment

Each tank had a glass cover to minimise aerial pollution, and aeration was given using glass pipettes to avoid plastic contamination. To prevent further plastic contamination, no gloves or plastic tools or materials were utilised during tissue dissection.

2.6. Antioxidant enzymes

Gonads of mussels were individually homogenized in 5 mL of 20 mM Tris-Sucrose buffer (0.5 M Sucrose, 0.075 M KCl, 1 mM DTT, 1 mM EDTA, pH 7.6) utilizing a VWR Star-Beater (5 min, 20/s shaking, with grinding balls), following Geret et al. (2002). After two centrifugations (1: 500 g, 15 min, 4 °C; 2: 12000 g, 45 min, 4 °C), the cytosolic fraction was obtained. Aliquots were then used to analyse the activities of the antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), and the activity of the biotransformation enzyme: glutathione-S-transferases (GST).

All enzymatic activities were standardized with the determination of total protein concentrations, using the method defined by Bradford (1976). Total protein concentrations (mg protein g^{-1} tissue) were calculated using bovine serum albumin (BSA), as a standard, and optimized for microplate reader.

SOD activity was measured by calculating the reduction in absorption of the substrate cytochrome *c* by the xanthine oxidase/hypoxanthine system at 550 nm, and the responses were represented as U mg^{-1} protein (McCord and Fridovich, 1969).

CAT activity was quantified following Greenwald's (1985) approach, based on spectrophotometric detection of hydrogen peroxide (H_2O_2) consumption at 240 nm. The results are given in mmol min^{-1}mg $protein^{-1}$.

GPx activity, based on McFarland et al. (1999) technique, was assessed using a microplate reader (Infinite® 200, Pro-Tecan) at 340 nm with cumene hydroperoxide as a substrate at 28 °C, and presented in mmol min^{-1}mg $protein^{-1}$.

GST activity, based on Habig et al. (1974) approach, was determined by conjugating 0.2 mM reduced glutathione (GSH) with 0.2 mM 1-chloro-2,4-dinitrobenzene (CDNB) in a reaction mixture of 0.2 M KH_2PO_4/K_2HPO_4 buffer (pH 7.9) and measured at 340 nm in a microplate reader (Infinite® 200, Pro-Tecan). Data is presented in mol CDNB min^{-1} mg^{-1} protein units.

2.7. Lipid peroxidation

Individual mussel gonads were homogenized with the aid of a VWR Star-Beater (5 min, 20/s shaking, with grinding balls) in 5 mL of a 1:10 ratio of butylated hydroxytoluene and 0.02 M Tris-HCl buffer (pH 8.6). (BHT). To measure total protein concentrations and LPO levels, homogenates (3 mL) were centrifuged at 30000 *g* for 45 min at 4 °C. Using a method adapted from Erdelmeier et al. (1998), the absorbance of malondialdehyde (MDA) and (2 E)-4-hydroxy-2-nonenal (HNE) at 540 nm was used to determine the levels of LPO, in units of nm/mg protein.

2.8. Integrated Biomarker Response (IBR)

An efficient index for visualizing the biological consequences of contaminants and the relation between a battery of biomarkers and contamination levels is the integrated biomarker response index (IBR) (Devin et al., 2014). Thus, using a biomarker response index version 2 (IBR) proposed by Sanchez et al. (2013), which was modified from the IBR index defined by (Beliaeff and Burgeot, 2002) and described in Serafim et al. (2012), the biomarker data from gonads of *M. galloprovincialis* exposed to silver nanoparticles (nAg), polystyrene nanoplastics (nPS), 5-fluorouracil (5FU) and the mixture of the three contaminants (Mix) were assessed.

IBR enables the merging of various biomarker responses into a single numerical result. The IBR index was created with the intention of removing the IBR result dependent on the arrangement of the biomarkers as well as the induction and inhibition of each biomarker, and it is based on a disturbed and undisturbed condition (Sanchez et al., 2013). IBR is the sum of the differences between control groups and groups exposed to nAg, nPS, 5FU, or Mix on each sampling day.

In contemplation of reducing variance, the combined data of each individual biomarker (X_i) were compared to the combined data (X_0) of each individual biomarker from the control group and log transformed (Y_i). Y_i's

mean (μ) and standard deviation (σ) were computed, and the input for each parameter was further standardized using the equation:

$$Z_i = \frac{(Y_i - \mu)}{\sigma}$$

The mean of the standardized biomarker response (Z_i) and the mean of the unexposed biomarker (Z_0) were used to generate a baseline centred on controls and show parameter variation relative to this baseline:

$$A = Z_i - Z_0$$

Finally, the absolute value of A for each parameter in each experimental condition was calculated and added to obtain the IBR index:

$$\mathrm{IBRv2} = \Sigma|\mathrm{A_i}|$$

2.9. Model for synergistic or antagonistic effect

This method has been modified for tertiary combinations and is based on the single-dose factorial design described by Ritz et al. (2021) for binary mixtures. In this approach, five treatments: control, nAg, nPS, 5FU, and a mixture of the three (Mix) are created by combining three factors (three contaminants) with two levels. The assertion of an opposing or complementary impact is only true for the concentrations used in this evaluation [10 μg/L of nAg; 10 μg/L of nPS; 10 ng/L of 5FU, and Mix (10 μg/L of nAg +10 μg/L of nPS +10 ng/L of 5FU)]. Values were computed for all sampling times (0, 3, 7, 14, and 21 days) and all biomarkers (SOD, CAT, GPx, GST activities, and LPO levels) examined using the following dose addition and independent action models:

2.9.1. Dose addition

$$E_{add} = (E_{5FU} - E_{control}) + (E_{nPS} - E_{control}) + (E_{nAg} - E_{control})$$

The difference (D_{da}) between the actual response (control) and the previously expected impact can be used to characterise any antagonistic or synergistic effect. The following equation gives the definition of the difference:

$$D_{da} = E_{Mix} - E_{5FU} - E_{nPS} - E_{nAg} + E_{control}$$

2.9.2. Independent action

$$E_{ind} = \frac{E_{5FU} \times E_{nPS} \times E_{nAg}}{(E_{control})^2}$$

Under the presumption of independent action, every antagonistic or synergistic effect can be described as the difference (D_{ia}) between the observed effect and the reference effect. The following equation gives the definition of the difference:

$$D_{ia} = E_{Mix} - E_{ind}$$

2.10. Statistical analysis

According to data distribution and variance homogeneity (Shapiro-Wilk test), parametric tests (ANOVA, followed by Tukey's Post-hoc test) or non-parametric equivalent tests (Kruskal-Wallis and a two-tailed multiple comparisons test) were used to assess the significant differences between treatments and time. If $p < 0.05$, the findings were significant. GraphPad Prism version 9.1.1 (GraphPad software, Inc. CA) was used for the statistical analysis.

A Principal Component Analysis (PCA) was also used to assess the relationship between treatments (unexposed and exposed to nAg, nPS, 5FU, and Mix) and the measured parameters [activities of antioxidant and biotransformation enzymes (SOD, CAT, GPx, GST) and oxidative damage (LPO)] in mussel gonads over the exposure period (21 days). The Statistica 7.0 programme was used to achieve this (Statsoft Inc., 2005; USA).

For IBR, statistical differences were evaluated using a *t*-test on Microsoft Excel (*Microsoft Corporation*, 2018), and results were considered significant when $p < 0.05$.

3. Results

3.1. Antioxidant enzyme activities

The activities of antioxidant and biotransformation enzymes of unexposed mussel gonads did not alter throughout the exposure (21 days; $p > 0.05$; Fig. 1A - D).

SOD activity in gonads of mussels exposed to nAg and 5FU show an increasing trend, being that the activity seen at day 21 was significantly higher when compared to other treatments, as well as significantly different from nAg and 5FU exposed gonads at other time points ($p < 0.05$; Fig. 1A). In Mix-exposed mussel gonads, however, the activity of SOD displays a dumbbell-shaped pattern, with a significant increase on day 7 compared to the beginning and end of exposure, as well as compared to controls and gonads exposed to nAg and nPS ($p < 0.05$). On the other hand, no significant differences were encountered in the gonads of mussels exposed to nPS throughout the time of exposure, nor with controls ($p > 0.05$).

In all exposure treatments, the activity of CAT in mussel gonads increased significantly after 3 days of exposure compared to unexposed and maintain high activity throughout 14 days ($p < 0.05$; Fig. 1B). Mussels exposed to nAg and 5FU have the highest CAT activity at the end of the exposure period (21 days), whereas those exposed to nPS, and Mix show a decrease in activity ($p < 0.05$).

A substantial increase in GPx activity is noticeable in mussel gonads exposed to Mix after 3 days of exposure, and an increase of GPx activity is also noteworthy in nAg and 5FU exposed gonads compared to unexposed ones at the same time point ($p < 0.05$; Fig. 1C). However, gonads exposed to 5FU, and Mix present a decrease in GPx activity on days 14 and 21. Those exposed to nPS, on the other hand, show a dumbbell-shaped pattern in the activity of GPx with significant difference compared to controls from the 7th day of exposure until the end of the trial, and significantly different from all treatment conditions on these days ($p < 0.05$). More significant differences are found between treatments at each time-point, whereby gonads from Mix-exposed are significantly higher than all treatments conditions on days 3 and 7 ($p < 0.05$), and there are also significant differences between nPS-exposed compared to nAg and 5FU-exposed mussel gonads ($p < 0.05$).

All treatments GST activity decrease after 3 days of exposure, being that the decrease in nAg, 5FU and Mix-exposed mussel gonads is significantly different compared to controls and nPS-exposed ($p < 0.05$; Fig. 1D). Low activity of GST remains throughout the exposure period, being nAg, 5FU and Mix-exposed mussel gonads significantly different from unexposed mussels at all time-points, and nPS-exposed mussels GST activity is significantly lower than unexposed mussels on days 7 and 21 of exposure ($p < 0.05$).

3.2. Oxidative damage

Levels of LPO in unexposed mussel gonads did not alter throughout the exposure period (21 days; $p > 0.05$). Besides nPS, all treatments present levels of oxidative damage, wherein, on day 3, the level of LPO in Mix-exposed mussels is exponentially higher than the other treatments, with a 57-fold increase in comparison to unexposed mussels ($p < 0.05$; Fig. 2). On days 7, 14 and 21 of exposure, 5FU-exposed mussel gonads show highest levels of oxidative damage, and nAg-exposed on day 14 compared to controls and nPS-exposed mussels ($p < 0.05$).

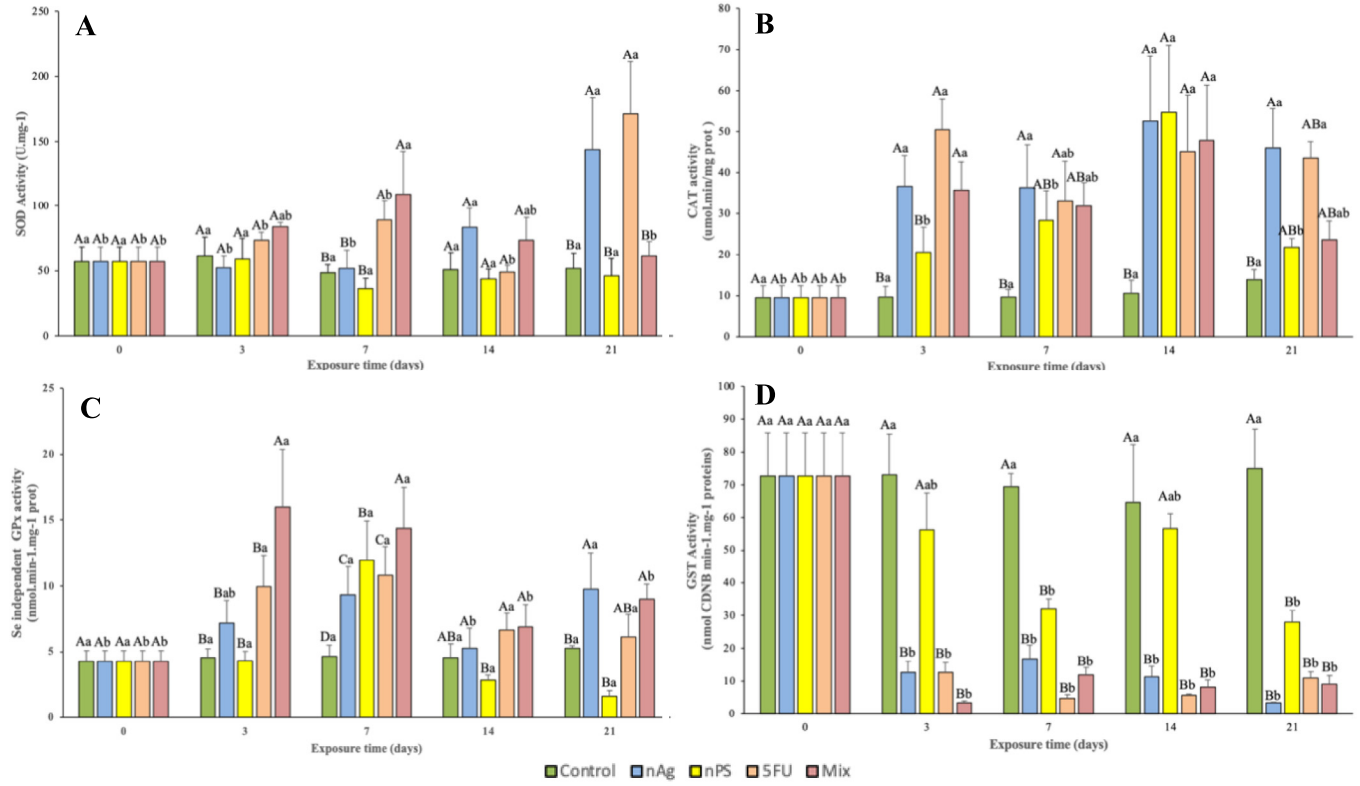

Fig. 1. Activity of superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione peroxidases (GPx) (C), glutathione-*S*-transferases (GST) (D) (mean ± std) in gonads of *M. galloprovincialis* after a 21-day exposure to silver nanoparticles (10 μg/L), polystyrene nanoparticles (10 μg/L), 5-Fluorouracil (10 ng/L), and Mixture of the three contaminants (nAg + nPS + 5-FU). Different upper- and lower-case letters indicate significant differences between treatments for the same time, and between time for the same treatments, respectively ($p < 0.05$).

3.3. Principal component analysis (PCA)

The impact of nAg, nPS, 5FU, and Mix on the response of biomarkers (SOD, CAT, GPx, GST activities and LPO) was described using PCA, which was applied to all the data collected from the gonads of mussels (Fig. 3). In mussel gonads, the two principal components account for 78.8 % of the overall variation (PC1 = 58.8 %, PC2 = 20.0 %; Fig. 3). The PCA displays a distinct difference between exposed and unexposed gonads, whereby unexposed mussel gonads are positioned tightly together, and are negatively related with both principal components. All time periods of nAg-exposure are positively related with the 1st component (PC1) and negatively related with the 2nd (PC2). On the contrary, in nPS-exposed mussel gonads, all exposure times, apart from day 7, are negatively related with PC1, whereby 7 and 14 days are positively related with PC2, and days 3 and 21 negatively related. Like nAg-exposed mussels, all sampling days for 5FU and Mix-exposed mussel gonads are also positively related with PC1. The difference between 5FU and Mix are found in PC2, where, in relation to exposure times, the contrary is observed; day 3 and 21 in 5FU are positively related, as so is days 7 and 14 for Mix-exposed mussel gonads, and negatively related for 5FU-exposed are days 7 and 14, and days 3 and 21 for Mix-exposed gonads. The main biomarker loadings affecting PC1 is LPO followed by GPx, whereas for PC2 it is SOD. This PCA suggests that nAg and 5FU are the two contaminants that are mainly affecting the toxicity effects observed in mussel gonads exposed to Mix, and that day 3 of exposure is the most critical time-point.

3.4. Integrated biomarker response (IBR)

The Integrated Biomarker Response (IBR) was calculated for the data on gonads of mussels biomarkers from all exposure times to nAg, nPS, 5FU and Mix. Graphical representation of IBR and star plots for all treatments are in Fig. 4A – B.

IBR pattern was treatment dependent and time dependent. For nAg-exposed mussel gonads, an increase pattern in IBR score is observed along the time of exposure, with the IBR score on the 21st day significantly different from the other time-points ($p < 0.05$; Fig. 4A). Mussel gonads of nPS-exposed present a substantial increase from day 7 forward, being the score on day 7 and 21 significantly different from day 14 ($p < 0.05$; Fig. 4A). The IBR score for 5FU-exposed mussel gonads increases on day 7 and starts to decline throughout the other days ($p < 0.05$; Fig. 4A). On the contrary, Mix-exposed mussels IBR score increases significantly on the 3rd day of exposure, following a decrease in score until the end of the experiment ($p < 0.05$; Fig. 4A). On day 3 of exposure, Mix-exposed gonads have a significantly higher IBR score on day 3 compared to the individual contaminant exposures, whilst on day 7 it is nPS-exposed, and on day 21 nAg and nPS-exposed mussel gonads ($p < 0.05$; Fig. 4A). Star – plots showed that changes in biomarkers were also treatment and time dependent (Fig. 4B). In nAg-exposed mussel gonads (Fig. 4B), the 21st day is the most critical, with SOD activity being the main contributor to the overall IBR score. There is not one but three critical time-points for nPS-exposed gonads, being these at 7 days with GPx activity and LPO levels main contributors, at 14 days with CAT activity as main contributor, and at 21 days with GPx and GST activities as main contributors to the total IBR score. The star-plot for 5FU-exposed mussel gonads discloses that the 21st day of exposure was the most critical, with SOD activity as its main contributor, followed by day 7, being GPx activity and LPO levels contributing mainly to the overall IBR score. In mussel gonads exposed to Mix, day 3 is the most crucial moment, having GST activity, LPO and GPx activity contributing primarily to the total IBR score.

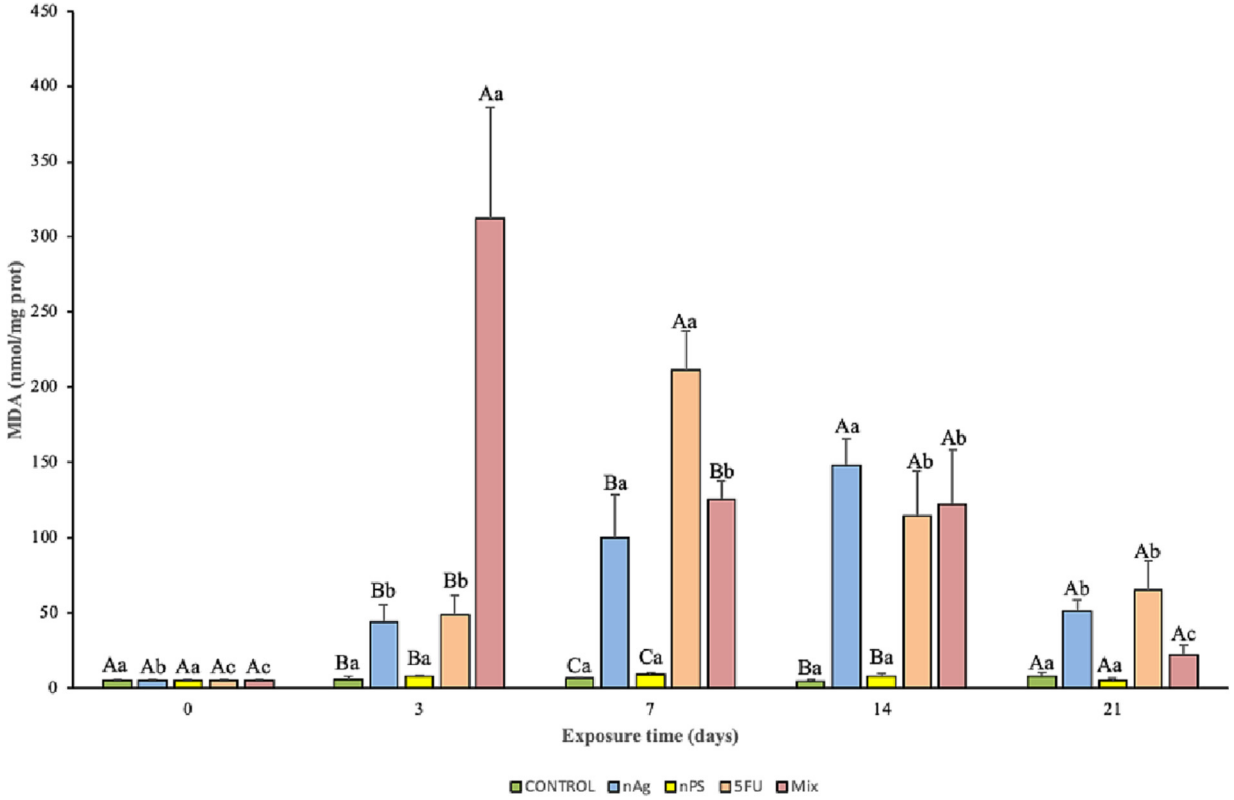

Fig. 2. Lipid peroxidation (LPO) levels (mean ± std) in gonads of *M. galloprovincialis* after a 21-day exposure to silver nanoparticles (10 μg/L), polystyrene nanoparticles (10 μg/L), 5-Fluorouracil (10 ng/L), and Mixture of the three contaminants (nAg + nPS + 5-FU). Different upper- and lower-case letters indicate significant differences between treatments for the same time, and between time for the same treatments, respectively ($p < 0.05$).

3.5. Synergistic or antagonistic effect

3.5.1. Difference of dose addition (D_{da})

The sum of differences relative to the control group for the contaminants when applied alone (assumption of dose addition; D_{da}) was carried out to better understand synergistic or antagonistic effects in Mix-exposed mussel gonads (Table 1). After 3 days of exposure, contaminants in the mixture present synergistic interactions specifically for LPO. At the following sampling time-point (day 7), an antagonistic effect is observed for LPO. On the 14th day of exposure, CAT activity is the main enzyme presenting antagonistic interactions between contaminants in Mix. Whereas, after 21 days of exposure, SOD activity is the main enzyme, and again Mix shows antagonistic interactions.

3.5.2. Difference of independent action (D_{ia})

Furthermore, assuming there is independent action of the contaminants, a difference of independent action (D_{ia}) was also carried out to for Mix-exposed mussel gonads to further comprehend any synergistic or antagonistic interactions between the contaminants (Table 2). Under this assumption, all days present significantly higher negative values for D_{ia}, where both LPO and CAT activity show to have antagonistic interactions between the three contaminants in Mix-exposed mussel gonads from 3 to 14 days of exposure, and SOD activity with the highest antagonistic effect for day 21.

4. Discussion

Mixtures of CECs are gaining much attention; however, metal nanoparticle-nanoplastic-pharmaceutical interactions to this date remain unknown. Therefore, to the best of our knowledge, this is the first data set on the effects of nAg, nPS, and 5FU individually, and as a mixture (Mix) in the gonads of *M. galloprovincialis*.

The increase in use of nAg in everyday lives is mainly down to the properties these nanoparticles possess. In the last decade, the toxicity effects of nAg have been thoroughly evaluated in bivalves, with focus on gills and digestive glands, haemocytes and even embryonic development (Auguste et al., 2018; Carrazco-Quevedo et al., 2019; Gomes et al., 2013; Ringwood et al., 2010), although the effects on oxidative stress in gonads of bivalves remains scarce. The primary mechanism of nAg toxicity when crossing cell membranes is dissolution (McQuillan et al., 2012), and the release of antimicrobial silver ions that may interact with and influence the function of thiol-containing proteins found in the cell wall (Mikhailova, 2020). The reproductive organ of mussels prior to nAg exposure is susceptible to impaire gamete viability, impaire fertilization success and malformations of embryo-larval development and are all possible outcomes of toxicity in mussel gonads. During the 21 day exposure to nAg, the ADS was activated, though the activities of GST (Fig. 1) —which together with GPx enzyme, reduces lipid peroxides and protects against apoptosis (Prabhu et al., 2004) —were inhibited. Consequently, nAg exposure led to oxidative damage in mussel gonads (Fig. 2). With mussel gonads compromised, gametogenesis and fertilization success may be hindered. In the oyster, *Crassostrea virginica*, 1.6 μg/L of nAg (15 ± 6 nm) after a 48 h exposure, a lysosomal destabilization increase in oyster gonads, suggesting a high level of impaired gamete viability and reproductive failure, which was further supported by the decrease in embryonic development (Ringwood et al., 2010). Moreover, the spawning level success of female *M. galloprovincialis* fed with *Isochrysis galbana* exposed to 10 μg/L of PVP/PEI coated nAg (5.08 ± 2.03 nm; 21 d) was affected and triggered reproductive impairment that could impact populational fitness of mussels (Martínez, 2019). According to IBR score, the most problematic time of exposure, as well as the most compromising, is after 14 days for nAg-exposed gonads, therefore, a chronic-exposure to nAg is concerning as reproductive

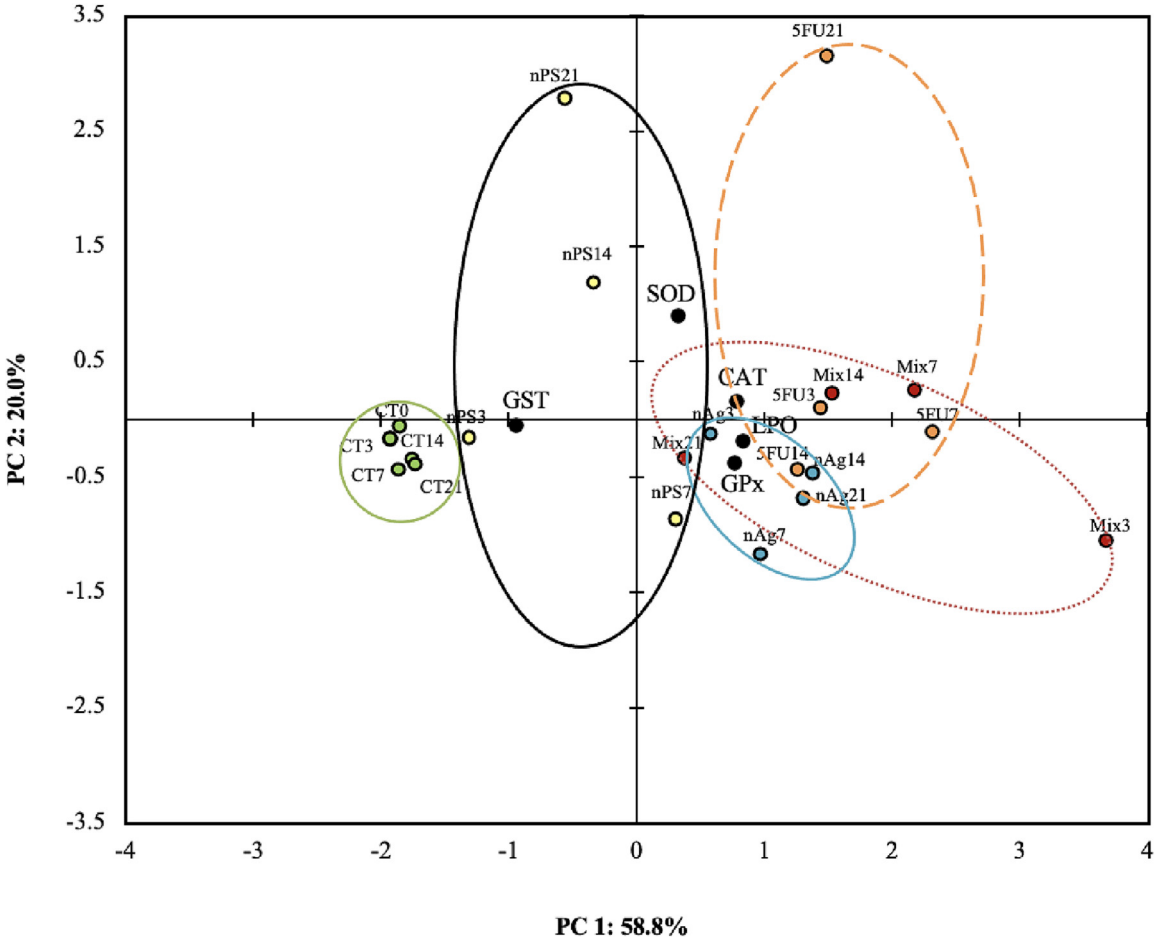

Fig. 3. Principal Component Analysis (PCA) of biomarkers (SOD, CAT, GPx, GST activities and LPO) in the gonads of *M. galloprovincialis* from controls (CT) and those exposed to silver nanoparticles (nAg), polystyrene nanoplastics (nPS), 5-fluorouracil (5FU) and the mixture (Mix) for 21 days ($p < 0.05$).

success of mussels are at stake. Additionally, this time – dependent response has been observed in other studies (Carrazco-Quevedo et al., 2019; Gomes et al., 2013). However, the main mode of action of nAg is still unclear, and further assessments are necessary to confirm whether dissolved Ag^{+} ions released from nAg bind to organic matter present in the FSW.

Translocation of nPS can occur through the bloodstream, but due to nano-size range, they may also cross cellular boundaries and be translocated to other mussel tissues (Sendra et al., 2020), like the gonads. The ADS was not overwhelmed by nPS exposure and prevented oxidative damage from occurring. Compared to other tissues, in mussel's gills and digestive gland ADS is compromised and oxidative damage does occur, being gills the tissue most compromised (10 μg/L; 50 nm; 21 d) (Gonçalves et al., 2022b). Oxidative damage also occurred in digestive glands of *M. galloprovincialis* after 21 days exposure to nPS (50 nm; 150 ng/L) (Capolupo et al., 2021). IBR score for nPS (Fig. 4) suggests that 7 and 21 days of exposure are the most critical timepoints in mussel gonads and is in accordance with the activation and inhibition of the ADS. Moreover, the PCA confirms that nPS effects compared with other treatments are negatively related (Fig. 3).

5FU is known to cause genotoxicity in mussel haemolymph (Gonçalves et al., 2022a) and with its main MoA being substitution of the pyrimidine analogue uracil for its fluoro-nucleotides (Matuo et al., 2009), the damage in DNA is expected. However, in relation to the ADS and oxidative damage, in the gills and digestive gland of *M. galloprovincialis* exposed to 10 ng/L for 21-d showed that the ADS was able to counteract ROS generation and led to no oxidative damage. Although the ADS activated in mussel gonads exposed to 5FU, inhibition of GST activity was sufficient to see an increase in oxidative damage. Cellular membranes in the gonads are imperil after exposure to 5FU, suggesting that this toxicity may affect gonadal development and reproductive success. If robust oocytes and spermatozoa are unsuccessfully liberated into the water column, and embryo-larval development is jeopardized, a cascading effect in populational dynamics will be seen in future years. With the increase in prescription of chemotherapeutical cytotoxic agents, being that breast (8567 cases), prostate (6912 cases) and colo-rectal cancer (5483 cases) were the most diagnosed cancers in Portugal in 2019 (Instituto Nacional de Estatística Statistics Portugal, 2021), the lack of treatment and removal of cytotoxic agents by WTTP is highly worrying for the marine environment, as well as for human health.

Mixtures of CECs in mussel gonads presented both antagonistic and synergistic interactions (Tables 1–2). After 3 days of exposure, synergistic interactions between nAg, nPS and 5FU are noted, coinciding with the 57-fold increase in oxidative damage compared to the individual exposures. However, on the remaining days of exposure, antagonistic interactions were found. This suggests that metal nanoparticle – nanoplastic – pharmaceutical interactions are severely hazardous towards the marine environment at sub-lethal exposures. Nonetheless, the insufficient effort from the ADS and the high levels of LPO indicate reproductive impairment in mussel gonads, as a continuous increase in the activity of the ADS require the energy normally directed towards growth and reproduction (Trestrail et al., 2020). IBR score for the Mix also confirms day 3 of exposure as most jeopardizing (Fig. 4), and it is deducible from the PCA that the most influential contaminants in the toxicity of Mix is nAg and 5FU (Fig. 3). The antagonistic effects on the biomarkers assessed in mussel gonads exposed to Mix suggests that nAg anti-septic properties may be cancelling out the toxicity of 5FU, though binomial mixtures are necessary to fully

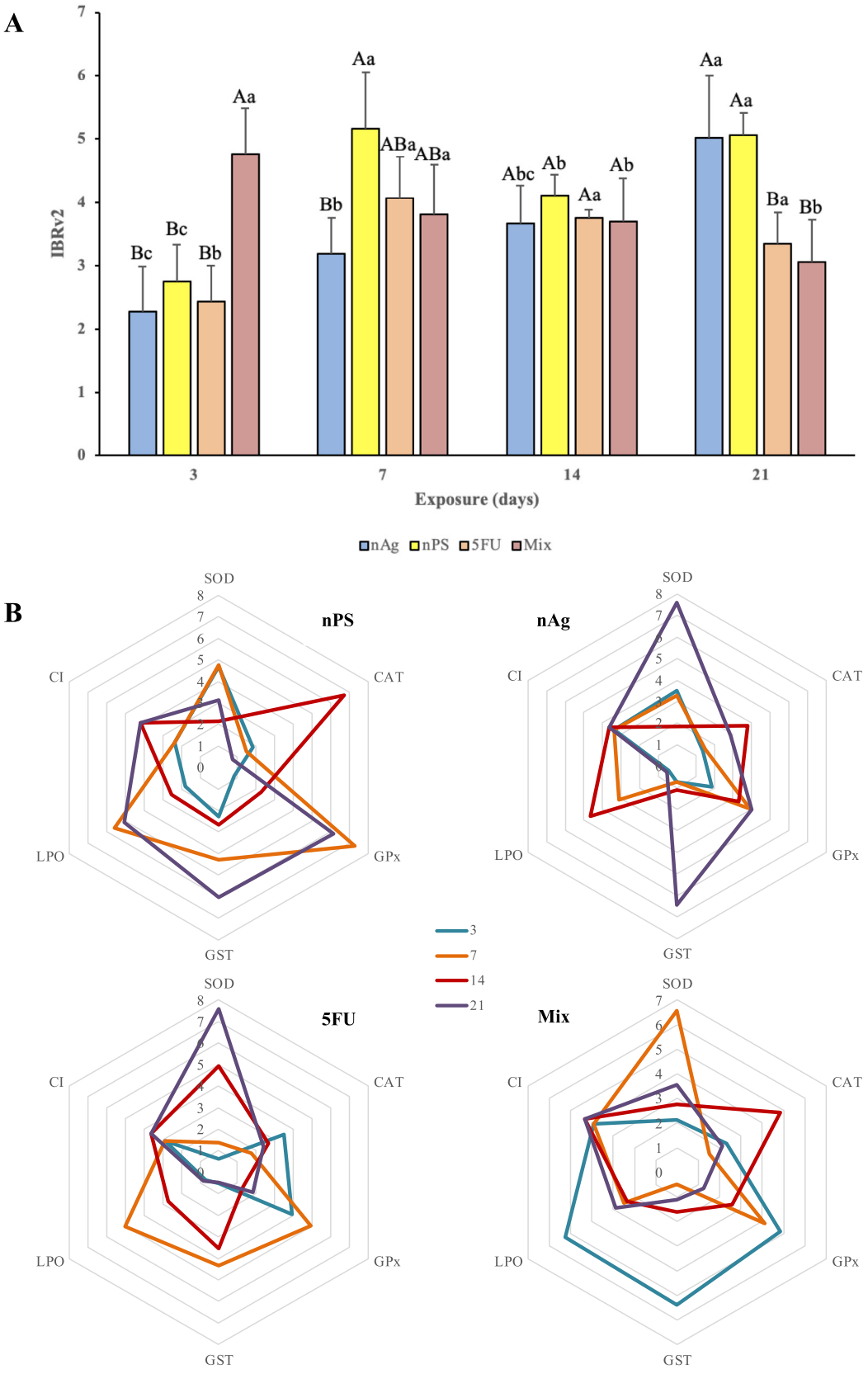

Fig. 4. (A) Integrated biomarker response index version 2 (IBR) for all treatment exposures (nAg, nPS, 5FU and Mix) (mean ± SD), and (B) star plots of each treatment exposure in the gonads of *M. galloprovincialis*. Different upper- and lower-case letters indicate significant differences between treatments for the same time, and between time for the same treatments, respectively ($p < 0.05$).

Table 1
Antagonism or Synergism for tertiary mixtures: Difference of dose addition (D_{da}). *Dda* is under the assumption of dose addition (sum of differences relative to the control group) for the contaminants when applied alone (adapted from Ritz et al., 2021). If the difference D_{da} is below or above 0 it indicates an antagonistic or synergistic effect, respectively.

Exposure time (days)	SOD	CAT	GPx	GST	LPO
0	0	0	0	0	0
3	−40	−62	−1	−5	217
7	−20	−56	−13	28	−188
14	31	−41	2	11	5
21	−104	−28	7	45	−40

establish this result. However, in relation to genotoxicity, the ability of 5FU to incorporate its fluoro-nucleotides is not diminished by the presence of either nAg or nPS (Gonçalves et al., 2022a). Knowingly as vectors (Xiang et al., 2022), the adsorption of other contaminants, such as pharmaceuticals and nanoparticles, by nanoplastics can possibly explain the synergistic interaction encountered after acute exposure. Other studies have looked at the interaction of CECs in a binomial complex (Almeida et al., 2019a, b; Essawy et al., 2021; Sun et al., 2023). Almeida et al. (2019a, b) found that effects in CAT activity were possibly due to the transfer of contaminants onto nPS (propanol + nPS, 10 mg/L, 24 h) in a fish cell line of *Sparus aurata*. On the other hand, antagonistic interactions between nPS and nAg were noted in human undifferentiated Caco-2 cells (0–5 nAg μg/L + 0–100 nPS μg/L; 24 h), whereby the nAg/nPS complex modulated the cell's uptake of nAg and slightly changed nAg toxicity (Domenech et al., 2021). Whilst, synergistic interactions of biosynthesized nAg, rutin (R), and heliomycin (H) demonstrated a dose-dependent antimicrobial efficacy against two fish pathogens (*Aeromonas hydrophila* and *Pseudomonas fluorescens*) (24 h; 56 nm; 1 μg/L nAg + 64–512 μg/L of R and H) (Essawy et al., 2021). Therefore, understanding metal nanoparticle-nanoplastic-pharmaceutical interactions its highly dependent on the contaminant, concentration, size, and exposure time. Specifically, results suggest that Mix at sub-lethal exposures were most toxic in mussel gonads, whereby CECs in the mixture present synergistic toxicity. Moreover, although antagonistic interactions occurred on remaining exposure days, levels of oxidative damage remain high compared to controls and nPS-exposed. Based on the present results, the implications on gonadal development, gamete viability, successful fertilization, and embryo-larval development are concerning. If true, populational dynamics and ecosystem balance is in jeopardy. Therefore, an improvement on risk-assessment strategies and development of new technologies for treatment and removal of CECs in WTTP is highly recommended. In Europe, mussels have high social – economic importance, and for example, in Portugal, a 47.5 % increase in mollusc aquaculture was reported in 2020 (9863 tons), whereby the aquaculture production of mussels increased by 37.7 % (2007 tons) (INE, 2022). In Portugal, the aquaculture of mussels is mainly in coastal areas where CECs may be present in the surrounding waters. Therefore, not only may CEC mixtures affect the reproductive success of mussels and impact aquaculture of mussels but can also have detrimental effects on marine biodiversity and on human health from shellfish-batch consumption.

Table 2
Antagonism or Synergism for tertiary mixtures: Difference of independent action (D_{ia}). D_{ia} is under the assumption of independent action of the contaminants (adapted from Ritz et al., 2021). If the difference *Dia* if below or above 0 it indicates an antagonistic or synergistic effect, respectively.

Exposure time (days)	SOD	CAT	GPx	GST	LPO
0	0	0	0	0	0
3	−3	−392	−10	71	−448
7	13	−330	−40	3	−4278
14	39	−1116	10	11	−5801
21	−350	−179	3	8	−144

5. Conclusion

The data set obtained herein suggests that the mixture of silver nanoparticles, polystyrene nanoplastics and 5-fluorouracil is more toxic than the individual counterparts. The mixture has high acute toxicity towards the reproductive organ of mussels, with synergistic interactions, whilst antagonistic interactions between CECs occur during chronic exposures. However, oxidative damage is inevitable despite synergistic or antagonistic interactions. Therefore, further investigation into complex mixtures and their toxicity is necessary to fully establish the mechanism of how these CECs interact. Also, toxic effects of CECs should be evaluated in mussel gonads with female and male differences for more insight into how reproduction may be affected. Moreover, with the increase in production of nanosilver-based consumer products, plastic pollution, and chemotherapeutical agents prescribed with increase in cancer diagnosis, technologies for treatment and removal of CECs in WTTP need to be revised and improved. Development of novel risk – assessment strategies and regular monitorization of CECs is also recommended.

CRediT authorship contribution statement

All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

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Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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