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Assessing the effects of the cytostatic drug 5-Fluorouracil alone and in a mixture of emerging contaminants on the mussel *Mytilus galloprovincialis*

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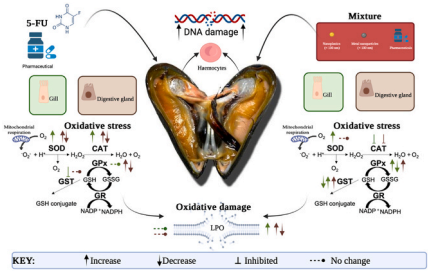
HIGHLIGHTS

- Mussels exposed to 5FU and a tertiary Mix (nAg + nPS + 5FU).
- 5FU and Mix cause genotoxicity in mussel's haemolymph.
- Negative effects of 5FU and Mix are tissue specific.
- Mix is more toxic than 5FU individually.
- In Mix, the interaction of ECCs have a synergistic effect.

GRAPHICAL ABSTRACT



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ABSTRACT

The assessment of contaminants of emerging concern, alone and in mixtures, and their effects on marine biota requires attention. 5-Fluorouracil is a cytostatic category 3 anti-cancer medication (IARC) that is used to treat a variety of cancers, including colon, pancreatic, and breast cancer. In the presence of other pollutants, this pharmaceutical can interact and form mixtures of contaminants, such as adhering to plastics and interaction with metal nanoparticles. This study aimed to comprehend the effects of 5-Fluorouracil (5FU; 10 ng/L) and a mixture of emerging contaminants (Mix): silver nanoparticles (nAg; 20 nm; 10 μg/L), polystyrene nanoparticles (nPS; 50 nm; 10 μg/L) and 5FU (10 ng/L), in an *in vivo* (21 days) exposure of the mussel *Mytilus galloprovincialis*. A multibiomarker approach namely genotoxicity, the antioxidant defence system (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx), glutathione – *S* – transferases (GST) activities), and oxidative damage (LPO) was used to assess the effects in gills and digestive gland of mussels. Both treatments cause genotoxicity in mussel's haemolymph, and antagonism between contaminants was observed in the Mix. Genotoxicity observed confirms 5FU's mode of action (MoA) by DNA damage. The antioxidant defence system of mussels exposed to 5FU kicked in and counter balanced ROS generated during the exposure, though the same was not seen in Mix-exposed mussels. Mussels were able to withstand the effects of the single compound but not the effects of the Mix. For oxidative stress and damage, the interactions of the components of the mixture have a synergistic effect.

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1. Introduction

5-Fluorouracil (5FU), a cytostatic agent (IARC Class 3), is one of the most often prescribed cancer drugs for the treatment of solid tumours as well as colorectal, pancreatic, and breast cancer (Mahnik et al., 2007). 5FU is a pyrimidine analogue of uracil with a fluorine atom in the C-5 position in place of the hydrogen (Vermorken et al., 2007), and its main mode of action (MoA) in mammals is misincorporation of its fluoro-nucleotides into DNA (Matuo et al., 2009), and the release of cytochrome *c* from the mitochondria promoting reactive oxygen species (ROS), leading to the generation of superoxide radicals and oxidative stress (Cairns et al., 2011). 5FU enters the cell by active transportation through the uracil transport system, and three metabolites are formed: 1 – Fluorodeoxyuridine monophosphate (FdUMP) which suppresses the function of thymidylate synthase, in turn inhibiting the production of deoxythymidine monophosphate (dTMP); 2 – Fluorodeoxyuridine triphosphate (FdUTP) which can directly induce DNA damage; and 3 – Fluorouridine triphosphate (FUTP) which can combine into RNA rather than uridine triphosphate (UTP) leading to RNA damage (Ghafouri-Fard et al., 2021). Though this is true for mammalian cells, in invertebrates, such as mussels, the MoA of 5FU is still unknown.

Pharmaceuticals are an important group of contaminants of emerging concern (CECs), and cytostatic drugs have received little attention to date. The number of increasing patients receiving chemotherapy treatments based on cytostatic drugs in hospital or at home is concerning, as these cytostatic agents almost never fully metabolize, and are eventually discharged into the water unaltered or modified (Trombini et al., 2016). Furthermore, wastewater treatment plants (WWTPs) have a lack of treatment available in the removal or breakdown of pharmaceuticals, though there are other sources such as surface runoff and/or aquaculture that also contribute to these contaminants ending up in the rivers and in the ocean, leaving biota constantly exposed to them (Thiagarajan et al., 2021). Cytostatic drugs, especially, are not effectively eliminated and are highly persistent in WWTPs (Bürge et al., 2006; Mahnik et al., 2007). Additionally, this class of pharmaceuticals are known to have cytotoxic, genotoxic, mutagenic, and carcinogenic effects on non-target organisms (Kümmerer and Henninger, 2003). In the marine environment, little is known on the effects of 5FU, however, for example, in freshwater systems, 5FU has shown to cause acute toxicity and hamper with the green microalgae, *Scenedesmus vacuolatus*, growth (5.87–187.7 mg/L; 72 h) (Asad et al., 2012), and lead to DNA damage in the aquatic oligochaeta *Limnodrilus udekemianus* at environmentally relevant concentrations (0.004 μM; 96 h) (Kračun-Kolarević et al., 2015). Therefore, the wide use of the not easily biodegradable 5FU (Kümmerer and Al-Ahmad, 1997), and the effects of this anticancer drug should thoroughly be investigated on non-target marine organisms, as the physical-chemical parameters of the marine environment may influence 5FU and its metabolites behaviour.

In European waters, concentrations of 5FU have been estimated to fall within the range between 0.3 ng/L to 2.5 ng/L (Heath and Isidori, 2020) while in the Taipei region, concentrations of 6.2 ng/L were detected with levels reaching a maximum of 160 ng/L in the Gaoping river (Lin et al., 2014). 5FU seems to be rather persistent in the water and likely to stay in the sediments (Besse et al., 2012). Mahnik et al. (2007) suggested a strategy for the removal of this pharmaceutical using membrane reactor systems and showed that 24 h almost completely eradicated 5FU due to biotransformation. However, according to Li et al. (2021), anticancer medication mixtures appear to have additive, or perhaps higher than additive, effects in marine species. Precautionary mitigation approaches should be adopted to prevent the increasing and continuous discharge of these toxic residues into the marine environment, and some promising treatment technologies have already been identified (Zhang et al., 2013).

Pharmaceuticals are not the only emerging contaminants entering the ocean. Contaminants within the nano-sized range are also increasing, such as nanomaterials and nanoplastics, and attention is quickly spinning towards the mixtures of CECs within the scientific community, on how and if they interact, and whether they will have more severe toxic effects towards non-target marine organisms. CECs are defined as a range of chemical compounds, whether anthropogenic or naturally occurring, that are not routinely monitored in the marine environment, and are classified following the criterion of their persistence in the marine environment as well as the harmfulness and ecotoxicological effects they may have. Pharmaceuticals are known as "classical" CECs; and although microplastics and nanoplastics are not yet a part of this list, nanomaterials and nanoparticles have been included (USEPA, 2007; Gatz, 2021). Even so, CECs have the potential to reach and damage ecosystems resulting in negative effects for humans and/or ecology (Nawaz and Sengupta, 2019), and their fate into the marine environment results from land-based and ocean-based sources, such as waste mismanagement, accidents (i.e., spills), antifouling and fishing gear. As previously mentioned for pharmaceuticals, effluents and WTTP are the main sources of CECs. Not only do pharmaceuticals end up inevitably in the ocean, but products such as plastics, metals, and industrial effluents (Holmes et al., 2012) also end up in abundance in the marine environment, creating mixtures of compounds that may be toxic, and the concern of these mixtures is increasing and there is a need to enlighten our knowledge on how these mixtures behave.

There has been a rapid increase in applications of nanoparticles and nanomaterials (1–100 nm) and silver nanoparticles (nAg) account for more than 50% of global nanomaterial consumer products (Pulit-Prociak and Banach, 2016). nAg's are frequently used in personal care products, antimicrobial coatings, photonic devices, and textile industries (Maillard and Hartemann, 2013; Lee & Jun 2019; Pereirados et al., 2020), and a great deal of nAg in the environment is from its release from consumer products. Once released into the sewer systems, nAg is then released into the WWTP and ultimately released into the aquatic environment, where nAg poses a serious environmental risk with the potential toxicity of these nanoparticles (Wang et al., 2018; Vilela et al., 2018). The MoA of nAg is known to directly damage cell membranes, generate reactive oxygen species (ROS) by the nanoparticle itself or by the release of Ag^{+} ions, serving as a "trojan horse" effect (Ghobashy et al., 2021). In marine organisms, nAg has shown to promote oxidative stress responses by impairing the capacity of the antioxidant enzyme defence system (Gomes et al., 2014; Walters et al., 2016; Bouallegui et al., 2017). However, the effects of nAg towards marine organisms whilst in a "cocktail" of emerging contaminants is still unclear.

Additionally, a major ongoing concerning pollutant that has the scientific community rendered to understanding its potential toxicity is plastic pollution. From macro-to microplastics, where many studies have evaluated the effects in the marine environment (Lu et al., 2016; Ribeiro et al., 2017; Calderon et al., 2019; Vasanthi et al., 2021) a class of plastics yet to be fully assimilated are nanoplastics. Nanoplastics derive from macro- and microplastic degradation (primary nanoplastics) and range from 1 to 100 nm in size (Gigault et al., 2018), but also make their way into the marine environment in their manufactured nano-sized form (secondary nanoplastics) (Andrady, 2011; Cole et al., 2011; Bessa et al., 2018; Tamminga et al., 2018). During the synthesis of nano-sized plastic, changes in the physical-chemical characteristics of the plastic particle occur, with the surface area, size, intensity, conductivity, and reactivity of these nano-scaled plastic particles differing substantially from macro/micro sized particles (Klaine et al., 2012; Mattsson et al., 2015, 2018). A major concern in relation to nanoplastics is that their size permits these particles to pass through cellular boundaries, being that the implications of this makes this size-class of plastics even more critical to understand, as biological reactivity increases as size decreases (Mattsson et al., 2018; Ferreira et al., 2019; Peng et al., 2020). Additionally, plastics have the potential to act as vectors for other contaminants found in the aquatic environment by sorption mechanisms. These sorption mechanisms vary with plastic polymer type, emphasising the importance of investigating these processes (Thiagarajan et al., 2021).

Even though it has been challenging to quantify nanoplastics in the marine environment, polyvinyl chloride (PVC), polyethylene terephthalate (PET), polyethylene (PE), and polystyrene (PS) are examples of nanoscale plastic polymers found in the nanoplastic segment in the subtropical North Atlantic gyre (Ter Halle et al., 2017). Furthermore, just 5 min of mechanical abrasion mimicking coastal activity on a PS cup and lid led to the formation of plastic in the nano-range (Ekvall et al., 2019). PS is the fourth most common kind of plastic polymer used worldwide (PlasticsEurope, 2021), and there is extensive data on their effects on marine organisms such as bivalves (Tallec et al., 2018; Cole et al., 2020; Sendra et al., 2021) and fish (Mattsson et al., 2015; Bessa et al., 2018; Maaghloud et al., 2021), while not much is known on the effects of nanoplastics in their virgin component (Gonçalves and Bebianno, 2021). *M. galloprovincialis* exposed to PS nanoplastic (nPS) at a concentration of 10 μg/L (nPS; 50 nm; 21-d) caused inhibition in the antioxidant defence system leading to oxidative damage, where mussel gills were the most compromised tissue, as well as cause genotoxicity in mussel haemolymph (Gonçalves et al., 2022). Compared to PS-microplastics (PS-MPs), nPS exposure to *M. galloprovincialis* (50 nm; 1.5–150 ng/L; 21-d) caused greater effects on lysosomal indicators of general stress, suggesting an interconnected trend with increased antioxidant activity in mussel gills (Capolupo et al., 2021).

It is therefore crucial to understand how these CECs individually affect marine organisms to comprehend the possible interactions they may have as a mixture. For example, if more than one contaminant is ingested by an organism at the same time, there are various possibilities for interactions; the combination may result in the toxic effect of one contaminant being added to the other (additive or non-interaction) or the toxic effect of the combination being significantly less than the harmful effects of the single contaminant (antagonism) or the hazardous effects of the combination may be considerably more than the effects of the individual compound (synergism) (Stenersen, 2004). It is known that pharmaceuticals and nanomaterials interact through adsorption with micro- and nanoplastics, and the interaction of plastics with other CECs can cause substantial change to surface properties, as well as affect the uptake and accumulation in exposed organisms (Zhou et al., 2021). Although the method of interaction for pharmaceuticals has been addressed, few data exist on the interaction of nanomaterials such as metals (Holmes et al., 2012, 2014; Thiagarajan et al., 2021). For these, there are two methods of sorption: a period of fast sorption followed by an approach towards equilibrium or a more prolonged period of gradual sorption (Holmes et al., 2012). Therefore, the presence of CECs and their effects is especially important to investigate in the light of safety for seafood consumers. Thus, this study aimed to evaluate the effects of 5FU (10 ng/L) alone, and in a mixture with silver nanoparticles (nAg; 10 μg/L; 20 nm) and polystyrene nanoplastics (nPS; 10 μg/L; 50 nm) on the marine mussel *Mytilus galloprovincialis* in a 21-day *in vivo* exposure. A multibiomarker-approach was used to evaluate genotoxicity, oxidative stress, and oxidative damage.

2. Materials and methods

2.1. Experimental design

Mussels *Mytilus galloprovincialis* Lamarck, with a shell size of 50 ± 5 mm were collected from the Ria Formosa Lagoon, Southeast Portugal (37°00′30.6″N 7°59′39.6″W) and transported alive to the laboratory. Following the acclimatisation period (four days), 20 mussels each were placed in 15 L tanks with 10 L of seawater in a duplicate design. Mussels were contaminated with 10 ng/L of 5FU and the corresponding mixture of 10 ng/L of 5FU, 10 μg/L of nAg, and 10 μg/L of nPS. Mussels were exposed to these treatments for 21 days, with contaminants re-dosed and seawater exchanged every two days. Mortality was observed in mussels exposed to 5FU on days 3 (six dead mussels) and 10 (two dead mussels) of exposure. Mussels were collected on days 0, 3, 7, 14 and 21 of exposure for a multibiomarker analysis. Mussels were weighed and the gills and digestive gland were dissected and instantly frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.2. Condition index

The condition index (CI) was used to measure the physiological status of mussels (5 per treatment and period of exposure) at the start (day 0), 7-, and 21-days following exposure. The percentage (%) of the ratio between the entire mussel weight (tissue and shell) (g) and the wet weight (g) of the soft tissues was used to determine the CI (Gomes et al., 2013).

2.3. Genotoxicity assay

The alkaline comet test was used to measure DNA damage, which was developed from Singh et al. (1988) and Gomes et al. (2013). Haemolymph of mussels was retrieved by a sterile hypodermic syringe (1 mL) (25 G needle) from the posterior adductor muscle of five mussels after 0, 3, and 14 days of exposure to 5FU and Mix, as well as five unexposed mussels. 100 μL of sub-sample from each experimental condition was stained with 100 μL trypan blue to test cell viability, and the percent of living cells was determined by counting 100 cells at random.

For the comet assay, microscopic slides were cleaned in ethanol/ether (1:1) and coated with 0.65% normal melting point agarose (NMA) in Tris-acetate EDTA for DNA damage assessment. Mussel haemolymph cells were then centrifuged at 3000 rpm for 3 min (4 °C) to extract cells, which were then suspended in 0.65% low melting point agarose (LMA, in Kenny's salt solution) and cast on microscopic slides. Slides containing embedded cells were then submerged in a lysis solution (2.5 M NaOH, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, 10% dimethylsulfoxide, 1% sarcosil, pH 10, 4 °C) for 1 h to allow cellular components to diffuse into agarose and DNA to be immobilized. After the lysis step, the slides were gently put in an electrophoresis containing electrophoresis buffer (300 mM NaOH, 1 mM EDTA, adjusted pH 13, 4 °C) and left aside for 15 min. After that, electrophoresis was performed for 5 min at 25 V and 300 mA. The slides were then removed and immersed in a neutralizing solution (0.4 mM Tris, pH 7.5), before being washed with bi-distilled water and dried overnight. The existence of comets was determined using an optical fluorescence microscope (Axiovert S100) attached to a camera (Sony) after the slides were stained with 4,6-diamidino-2-phenylindole (DAPI, 1 g/mL). Following the assessment of the proportion of DNA in tail, the Komet 5.5 image analysis system was used to score 50 randomly chosen cells for each slide (a total of 200 cells assessed per group) at a total magnification of 400. The mean and standard deviation are used to express the results.

2.4. Tissue preparation and analysis of enzymatic activities

Individual mussel gills and digestive glands were 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) in an ice bath for 2 min, following Geret et al. (2002). The cytosolic fraction was obtained when homogenates were centrifuged (500 g, 15 min, 4 °C) and the supernatant was re-centrifuged (12 000 g, 45 min, 4 °C). The activities of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidases (GPx)) and biotransformation enzyme (glutathione-S-transferases (GST)) were determined using aliquots (150 μL) of the cytosolic fraction.

In addition, the determination of total protein concentrations was accomplished 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 in both tissues 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).

The quantitative evaluation of CAT activity in both tissues follows Greenwald's (1985) approach, which is 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 in both tissues was assessed using a microplate reader (Infinite® 200, Pro-Tecan) at 340 nm with cumene hydroperoxide as a substrate at 28 °C, based on a technique developed from McFarland et al. (1999). The outcomes are presented in mmol min-^{1}mg $protein^{-1}$.

The GST activity in both tissues 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 KH2PO4/K2HPO4 buffer (pH 7.9) and measured at 340 nm in a microplate reader (Infinite® 200, Pro-Tecan) according to the method by Habig and Jakoby (1981). Data are shown in mol CDNB min^{-1} mg^{-1} protein units.

2.5. Lipid peroxidation (LPO)

Individual mussel gills and digestive glands were homogenized on ice with 5 mL of 0.02 M Tris-HCl buffer (pH 8.6) and a 1:10 ratio of butylated hydroxytoluene (BHT). The supernatant from homogenates (3 mL) centrifuged at 30 000 g for 45 min at 4 °C was used to assess total protein concentrations and LPO levels. The absorbance of malondialdehyde (MDA) and (2 E)-4-hydroxy-2-nonenal (HNE) at 540 nm was used to assess LPO levels, using the technique derived from Erdelmeier et al. (1998). The results are expressed in nanomoles per milligram of protein.

2.6. Statistical analysis

A student t-test was used to evaluate the condition index of mussels, and statistical analysis was carried out on Microsoft Excel (©Microsoft, 2022). To understand if significant differences exist between treatments and time, two parametric tests were performed. A Two-Way ANOVA at a confidence level of 95% and a Post-hoc Tuckey test were used allowing a pairwise comparison of the results. A Principal Component Analysis (PCA) was performed to assess the relationship between treatments (control, 5FU and Mix) and variables (antioxidant and biotransformation enzymes, oxidative damage). GraphPad Prism Version 9.1.1 was used to conduct the statistical analyses (GraphPad software, Inc. CA).

3. Results

3.1. Condition index

There is no significant discrepancy in the condition index of *M. galloprovincialis* between treatments nor times of exposure, except between controls and mussels exposed to 5FU on the 7th day of exposure ($p < 0.05$). Values range from 37.30 ± 2.81 (controls), 34.91 ± 2.45 (5FU) and 40.84 ± 3.82 (Mix) (*see* Table 1).

3.2. Genotoxicity

Haemocytes of mussels from unexposed, and exposed to 5FU and Mix, were analysed for DNA damage at the beginning of the trial, as well as after 3 and 14 days of exposure, using the comet parameter % of tail DNA (Fig. 1). Fig. 1A shows a few examples of comets in mussel haemocytes from controls, 5FU and Mix exposure. After 3 and 14 days of exposure, the nucleoid core of unexposed *M. galloprovincialis* haemocytes showed no broken DNA fragments or damaged DNA migrating towards the tail region, whereas the nucleoid core of 5FU- and Mix-exposed mussels presents broken DNA fragments or damaged DNA moving into the tail region (Fig. 1A). At each time of exposure, unexposed mussels showed no significant differences in % tail DNA ($p > 0.05$; Fig. 1B), however significant difference in DNA damage is observed in 5FU- and Mix-exposed mussels compared to unexposed ($p < 0.05$; Fig. 1B). Furthermore, on the 3rd day of exposure, % tail DNA in both treatments is considerably greater than on the 14th day (2-fold difference in both treatments) ($p < 0.05$; Fig. 1B). As a result, both 5FU alone and in a Mix cause genotoxicity in the mussels' haemolymph (see Fig. 1).

Table 1
Condition index (mean ± S.D.) (%) of *M. galloprovincialis* exposed to 5FU and a mixture of emerging contaminants (10 μg/L of silver nanoparticles + 10 μg/L of polystyrene nanoplastics + 10 ng/L of 5-Fluorouracil) at the beginning, after 7 days, and at the end of exposure.

Time (day)	CT	5FU	Mix
0	37.30 ± 2.81		
7	40.67 ± 8.16	32.59 ± 4.35	31.45 ± 5.55
21	37.22 ± 3.03	34.91 ± 2.45	40.84 ± 3.82

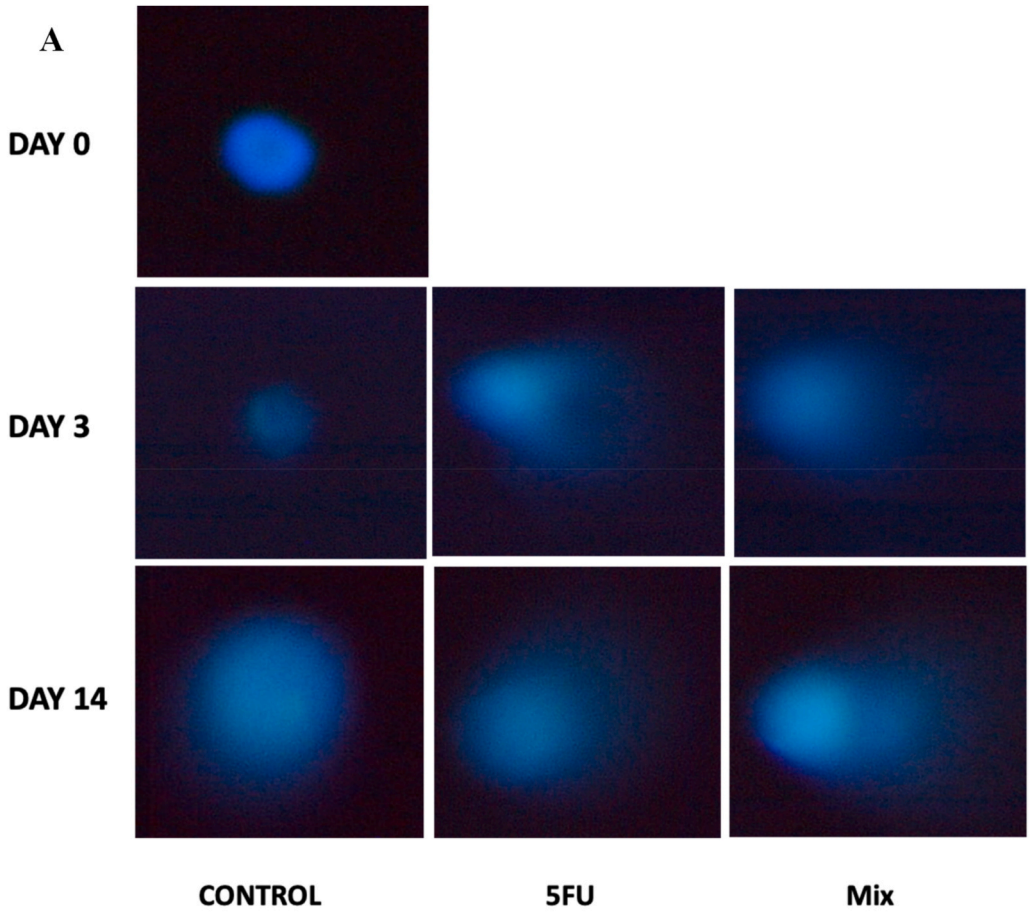
3.3. Enzymatic activities

There were no significant differences for the different enzymatic activities registered over time in the control group ($p > 0.05$, Fig. 2 A – J) except for GST in the gills ($p < 0.05$, Fig. 4). The activity of all enzymes SOD, CAT, GPx and GST changed after exposure to the different types of contamination.

Mussels exposed to 5FU had an increasing trend of SOD activity in gills over the course of time, whereby all exposure times are significantly different from the beginning of the exposure when compared to controls and to Mix-exposed mussels on the 21st day ($p < 0.05$; Fig. 2A). In the gills, after exposure to the mixture of contaminants, a significant increase in SOD activity was observed until day 14, returning to similar levels of controls on day 21 ($p < 0.05$; Fig. 2A). In the digestive gland, SOD activity significantly increased after 3 days compared to the beginning of the exposure for both treatments ($p < 0.05$; Fig. 2B). In the digestive gland exposed to Mix, however, significant increase in SOD, compared to unexposed, is noticeable on the first week of exposure ($p < 0.05$). Overall, the increasing activity of SOD in both tissues after exposure to 5FU or Mix are significantly different from controls after 7 days ($p < 0.05$), whereby in the gills, this significance is maintained until the end of the exposure.

A significant increase in CAT activity is observed in gills of mussels exposed to 5FU compared to the beginning of exposure ($p < 0.05$), within this, a small decrease in activity is observed at day 14 (Fig. 2C). A significant difference for 5FU exposed mussels can be observed at all times of exposure in comparison to control mussels ($p < 0.05$). In gills of mussels subjected to the Mix, an increase is noteworthy after 7 days, maintaining these levels at day 14, with these two days being significantly different compared to day 3 and the initial state of contamination ($p < 0.05$; Fig. 2C). When comparing 5FU and the Mix, it is seen that the CAT activity for gills is significantly higher from the 3rd day throughout to the 21st day of exposure to 5FU. In the digestive gland of mussels, exposure to 5FU led to a sharp significant increase in CAT activity (2.4-fold) after 3 days followed by a decrease ($p < 0.05$; Fig. 2D). In Mix-exposed mussels, a significant decrease in CAT activity occurs throughout the length of exposure, whereby CAT activity at 14 and 21 days of exposure are significantly lower from the other time points compared to unexposed mussels ($p < 0.05$; Fig. 2D). Also noticeable is the significant difference of 5FU and Mix throughout the whole exposure period ($p < 0.05$; Fig. 2D).

For the activity of GPx in gills exposed to 5FU, a slight increase of activity over the course of the experiment is detected, but significant differences are only encountered on the initial day compared to day 21 ($p < 0.05$; Fig. 2E). 5FU-exposed mussel gills were significantly different from the controls on day 3 and day 14 ($p < 0.05$; Fig. 2E). A steep significant increase in GPx activity in gills of Mix-exposed mussels, occurs until the 7th day (4.8-fold and 8.3-fold, day 3 and 7, respectively, $p < 0.05$) followed by a decrease in activity. Significant differences were encountered throughout exposure times when compared to the beginning of the exposure, being the highest significant activity registered at 7



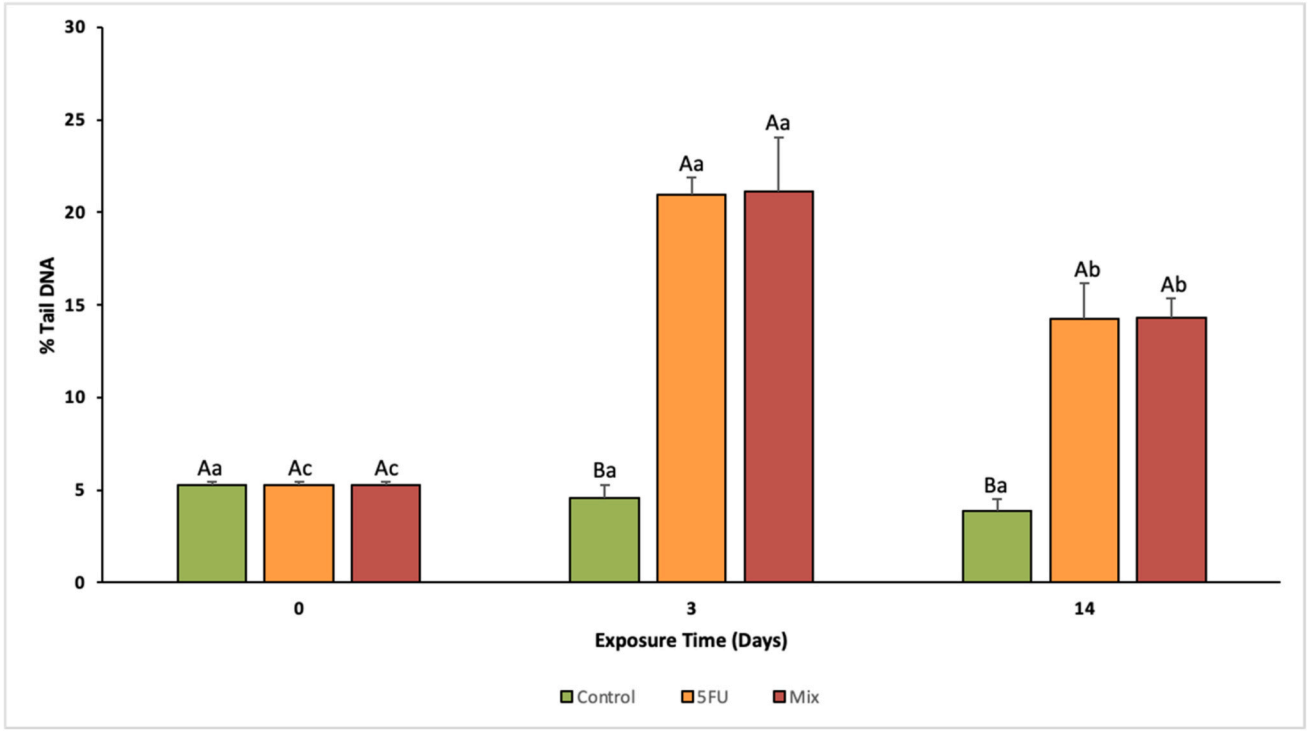


Fig. 1. Genotoxicity effects of *in vivo* exposure of 5-Fluorouracil (10 ng/L) and a mixture of emerging contaminants (10 μg/L of silver nanoparticles + 10 μg/L of polystyrene nanoplastics + 10 ng/L of 5-Fluorouracil) in the haemolymph of *M. galloprovincialis.* (A) examples of comet assay images of unexposed *M. galloprovincialis* haemocytes and (B) DNA damage (% tail DNA). Different upper- and lower-case letters indicate significant differences between treatments for the same time, and between times for the same treatment, respectively ($p < 0.05$).

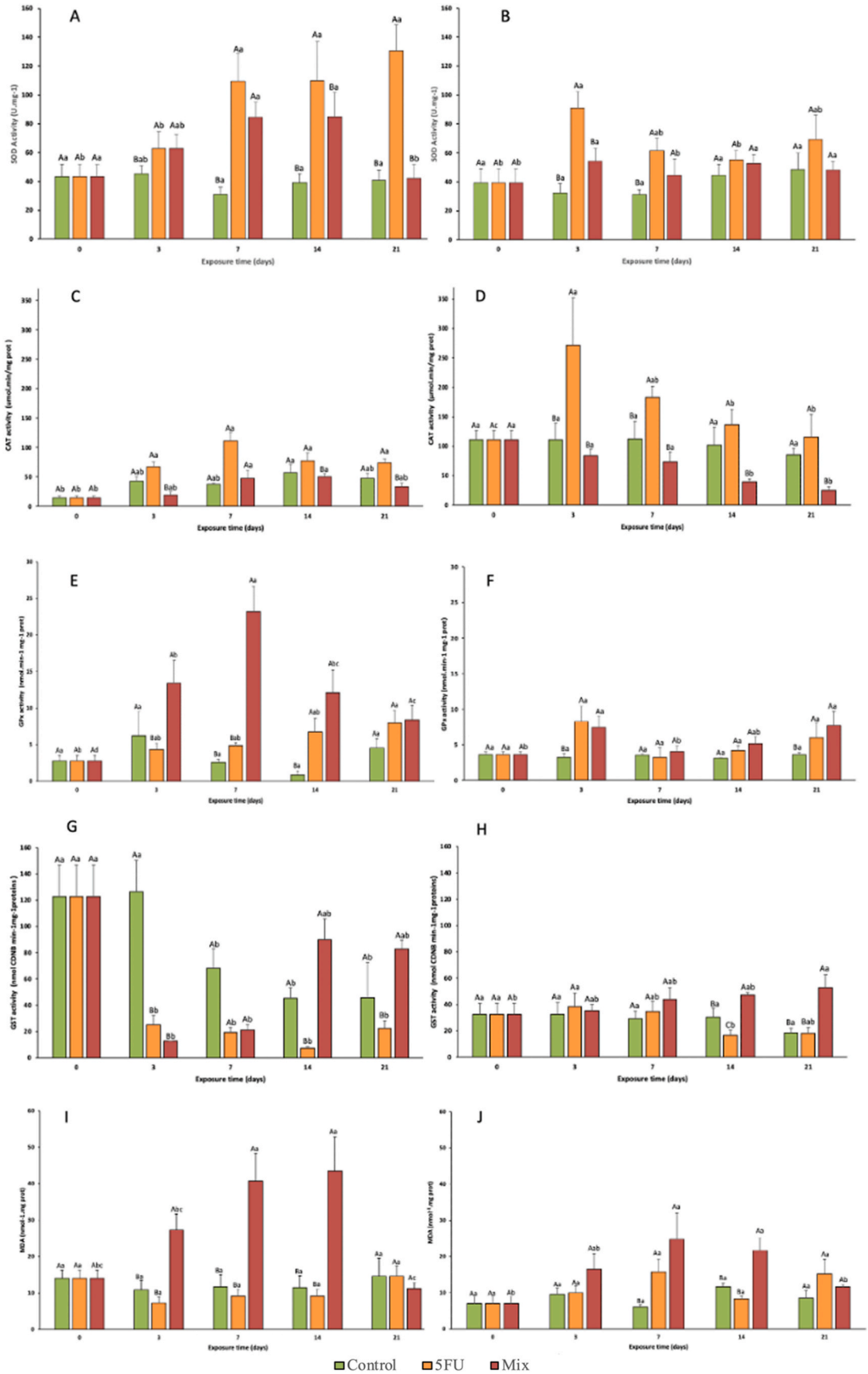


Fig. 2. Biochemical markers in gills (A, C, E, G, I) and digestive gland (B, D, F, H, J) of *M. galloprovincialis* after a 21-day exposure to 5-Fluorouracil (10 ng/L) and a mixture of emerging contaminants (10 μg/L of silver nanoparticles + 10 μg/L of polystyrene nanoplastics + 10 ng/L of 5-Fluorouracil). Different upper- and lower-case letters represent significant differences between treatments at the same time, and between times for the same treatment, respectively ($p < 0.05$).

days of exposure ($p < 0.05$, Fig. 2E). Additionally, significant differences between both treatments are noteworthy during the first week of exposure ($p < 0.05$; Fig. 2E). In the digestive gland of mussels, GPx activity presented no significant differences compared to the initial state of 5FU exposure and unexposed mussels throughout time, except for day 21 ($p < 0.05$; Fig. 2F). In the digestive glands for Mix-exposed mussels, a significant rise in activity is seen on day 3 and day 21 ($p < 0.05$; Fig. 2F). In Mix-exposed mussels, GPx activity represents a similar pattern to 5-FU exposed mussels' digestive gland, with significant differences being reported on day 3 and 21 of exposure ($p < 0.05$; Fig. 2F). Over the course of the exposure, no significant differences in GPx activity were found between 5FU and Mix ($p > 0.05$, Fig. 2F). GPx activity of digestive glands generally shows a low variation for all treatments, except for the 3rd and 21st day of 5FU- and Mix-exposed mussels.

Firstly, it is important to state that significant differences were found in the gills of unexposed mussels on days 7, 14 and 21, where the GST activity is significantly lower ($p < 0.05$) than those measured at 0 and 3 days, showing an overall decreasing trend (Fig. 2G). This may be a result of cyclic behaviour, as it has been seen that GST activity is lower during the summer months because of seasonal variations of temperature and the reproductive cycle (Borković et al., 2005). For gills exposed to 5FU, a significant inhibition of GST activity is noteworthy after 3 days, maintaining lower activity than control until the end of exposure ($p < 0.05$; Fig. 2G). Regarding the gills of mussels exposed to Mix, a significant inhibition ($p < 0.05$; Fig. 2G) of GST activity (9.7-fold) was observed between the initial and 3rd day, whereas after 14 and 21 days of exposure, a significant induction in GST activity was observed. A significant difference in GST activity was observed between the two treatments 5FU and Mix on day 14 and 21 of exposure ($p < 0.05$; Fig. 2G). On the other hand, GST activity in the digestive gland shows a completely different behaviour. Herein, GST activity is not distinctively varying as seen previously in the gills. A significant decrease in GST activity was only observed on day 14 for mussels exposed to 5FU compared to the beginning of the experiment ($p < 0.05$). In the digestive gland, there is a lack of difference in GST activity between times and treatments (Fig. 2H), however, there is a significant difference in Mix-exposed mussels when compared to controls and 5FU-exposed mussels after 14 and 21 days of exposure ($p < 0.05$).

3.4. Oxidative damage: lipid peroxidation

LPO levels in both tissues (gills and digestive gland) of unexposed mussels did not change significantly throughout the 21 days of exposure ($p > 0.05$) (Fig. 2IandJ).

In the gills of mussels exposed to 5FU, no significant differences were found in LPO levels ($p > 0.05$; Fig. 2I). As for the gills of mussels exposed to a Mix, a significant linear increase ($y = 7.66x$, $r = 0.835$) of LPO levels is detected from the 3rd day till the 14th ($p < 0.05$; Fig. 2I). The highest levels of LPO were observed after 14 days of exposure. After 21 days there is a sharp decrease by 3.9-fold reaching control levels (Fig. 2I). LPO levels for gills of Mix-exposed mussels were significantly different from controls and from 5FU-exposed mussels on day 3,7 and 14 ($p < 0.05$).

In the digestive gland of mussels exposed to 5FU, an increase in LPO is after a week, followed by a subsequent decrease and an increase after 14 and 21 days, respectively. Significant differences between unexposed and 5FU-exposed mussels ($p < 0.05$; Fig. 2J) were encountered after one and two weeks of exposure. In the digestive gland, a significant increase ($p < 0.05$; 3.6-fold) in LPO levels was observed after 7 days of exposure to Mix, followed by a decrease to control levels on day 21 (Fig. 2J). Between Mix-exposed and 5FU-exposed mussels, significant differences were detected only on the 14th day of exposure. After 7 and 14 days of exposure, results for the Mix showed to be significantly different from controls ($p < 0.05$, Fig. 2J).

3.5. Principal component analysis

PCA results and applied to help explain the effects of 5FU and the mixture of contaminants on biomarkers response indicate a clear separation between unexposed and those exposed to both types of exposure. The two principal components represent 82.0% in gills (PC1 = 47.7%, PC2 = 34.4%) and 78.2% for digestive glands (PC1 = 49.0%, PC2 = 29.2%) of total variance (Fig. 3A and B). Overall, the results indicate that the effects in both tissues (gills and digestive gland) are time- and treatment-specific. Mussel responses appear to be time- and tissue-dependent regarding 5FU, as with the increase of the time of exposure (21st and 14th day) was most influential for gills and digestive glands, respectively. However, in mix-exposed mussels, the 7th day was most influential for both gills and digestive glands. SOD and GPx are the main loadings affecting the 1st component in gills, whereas GST is negatively correlated (Fig. 3A). On the other hand, GST is the principal loading affecting the 2nd component in the digestive gland (Fig. 3B). This demonstrates that the different tissues exhibit differences in responses to the exposure, and that mussel gills are the tissue most compromised.

4. Discussion

Most of the data available on the effects of 5FU are limited to

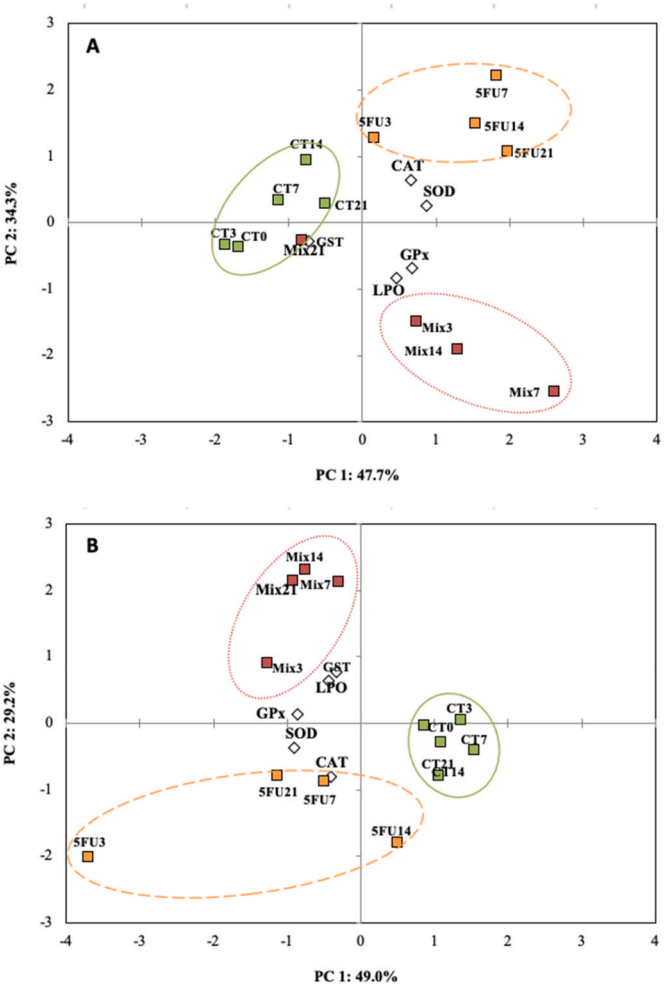


Fig. 3. Principal Component Analysis (PCA) of a battery of biomarkers (SOD, CAT, GPx, GST and LPO) in the gills (A) and digestive gland (B) of mussels *M. galloprovincialis* from controls (CT) and after exposure to both treatments: 10 ng/L of 5FU and a mixture of contaminants (10 μg/L of silver nanoparticles + 10 μg/L of polystyrene nanoplastics + 10 ng/L of 5-Fluorouracil) for 21 days ($p < 0.05$).

freshwater species (Gačić et al., 2014; Parrella et al., 2015; Kleinert et al., 2021). Thus, to the best of our knowledge, this is the first data about the effects of 5FU alone and in a mixture of contaminants on the marine mussel *M. galloprovincialis*, through the assessment of biomarkers of genotoxicity, oxidative stress, and oxidative damage in two different tissues, gills, and digestive gland, as well as genotoxicity in the mussel's haemolymph. The exposure to these contaminants induces genotoxicity and oxidative stress.

5-Fluorouracil MoA is known for mammalian cells (Fig. 4), and a similar MoA of 5FU is suggested by the genotoxicity seen in mussels (Fig. 1). 5FU is an uracil analogue having a fluorine atom at the C-5 position instead of a hydrogen atom (Vermorken et al., 2007), and once it enters the cell, 5FU incorporates itself into the DNA inducing ROS. 5FU's metabolites: FdUMP, FdUTP, and FUTP are known to interfere with both DNA and RNA functions and processing, and though not possible to confirm, the % tail DNA observed in 5FU-exposed mussels is evidence of DNA damage occurring. The haemocyte cells of unexposed mussels revealed an average of DNA damage (5.3 percent fragmented DNA), which is well within normal for *Mytilus* (Mitchelmore et al., 1998). Highest DNA damage observed in both treatments were found on the 3rd day of exposure, with a slightly lower but still significant DNA damage after 14 days, thus 5FU and the Mix are genotoxic towards mussel's immune system (Fig. 1). Comparing 5FU- and Mix-exposed mussels, similar levels of DNA damage were observed, though this effect is not significantly different from the exposure of 5FU alone. It has been proven that NPs could adsorb and transport pharmaceutical compounds (Santana-Viera et al., 2021). Moreover, the ionic strength has an influential factor in the sorption of 5FU and other pharmaceuticals on the surface of plastic polymers, as the most considerable interactions have been observed in fresh water and low salinity conditions (Puckowski et al., 2021). To understand how nAg and nPS are interacting with 5FU, further investigation is necessary to confirm adsorption properties. In HeLa cell lines (human cell lines), 1 h of exposure to 5FU was not sufficient for DNA lesions to be registered, however a low % tail DNA was evidenced (Pinheiro, 2016). In the freshwater mussel, *Elliptio complanata*, the exposure to 5FU caused a decrease in DNA strand breaks whilst LPO levels increased (Kleinert et al., 2021). In this study, the opposite was observed for 5FU-exposed mussels, where DNA strand breaks increased whilst no significant levels of LPO were found. For Mix-exposed mussels however, DNA strand breaks and LPO were positively related in both tissues. Therefore, the physical-chemical parameters of seawater compared to freshwater are highly influential on 5FU's toxicity towards organisms, suggesting that 5FU's toxicity is enhanced in the marine environment. Thus, the genotoxicity observed in Mix-exposed mussels should be further investigated to better comprehend the pharmaceutical-nanoplastic-metal nanoparticle interactions, as according to the antagonistic or synergistic model developed by Ritz et al. (2021), the interactions of these contaminants within the mixture is antagonistic (*see supplementary data*). Moreover, the levels of the different parent compounds of 5FU accumulated in mussel tissues need further investigation to assess 5FU mechanisms in marine species.

The antioxidant defence mechanism of mussels was activated after exposure to 5FU and a mixture of contaminants. After exposure to both treatments, the activity of SOD increased in the gills, and a smaller increase was noticeable in the digestive gland. The gills in filter-feeding organisms, are the first tissue to encounter toxins in the water, causing them to filter and absorb these harmful substances. (Jørgensen, 1996; Gonçalves and Bebianno, 2021). However, in the gills of *M. galloprovincialis* exposed to another anticancer drug – cisplatin (100 ng/L) an inhibition of SOD was observed along the time of exposure (14 d) (Trombini et al., 2016). Nonetheless, a similar trend in the digestive gland (2.1 – fold) was observed in mussels exposed to cisplatin (Trombini et al., 2016) as here seen for 5FU. The metabolic role of SOD activity is critical for removing superoxide radicals formed (Takahashi and Asada, 1983), and as the first antioxidant defence line (Li et al., 2009), it converts the superoxide anion ($\cdot O_2^-$) into hydrogen peroxide (H_2O_2). As more H_2O_2 is generated, CAT activity is expected to increase to ensure H_2O_2 removal. Following observed SOD activity, CAT activity in gills exposed to 5FU present a dumbbell shaped pattern, whereas in the digestive gland, an increased activity is observed after the first week. No changes were found in the activity of CAT in mussels gills exposed to cisplatin (100 ng/L), however, in the digestive gland, mussels present a similar range of activity (272 μmol min/mg protein) confirming that there is an increase in CAT activity due to the exposure to anticancer drugs (Trombini et al., 2016). Although the increase in mussels exposed to cisplatin peaked at day 14 (Trombini et al., 2016), in the present case the highest activity was reached after 3 days and then decreased over the course of the experiment. CAT activity is found to be more active at higher concentrations of H_2O_2 indicating a high percentage of ROS generated in this tissue (Box et al., 2007). The exposure to 5FU shows that GPx activity in the gills significantly increased at the end of the experiment, and a similar trend is observed in *M. galloprovincialis* exposed to cisplatin (100 ng/L; 14 d) (Trombini et al., 2016). The digestive gland of mussels, on the other hand, had a slight increase in GPx activity after exposure to 5FU, whereas the exposure of *M. galloprovincialis* to cyclophosphamide, another anticancer drug, (CP) (1 ng/L, 14-d; Fernandes et al., 2020; and 100 and 1000 ng/L, 28-d, 21 °C; Queirós et al., 2021) caused a significant increase in activity. It is possible to deduce that not all anticancer drugs act in the same way, and that the mussel's antioxidant defence response is dependent on the

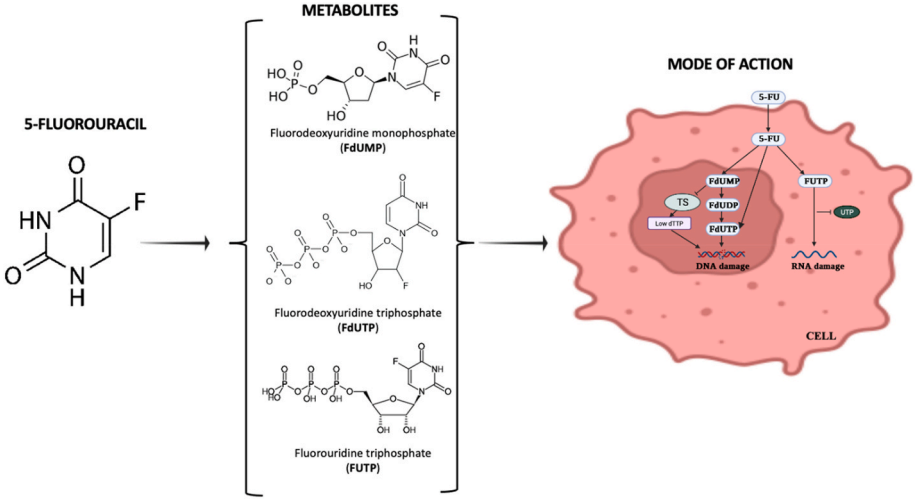


Fig. 4. 5-Fluorouracil pyrimidine analogue, its metabolites and mode of action.

type of anticancer drug and its MoA, as well as the concentration and the pharmaceuticals behaviour in seawater. A significant decrease in activity was observed for the biotransformation enzyme (GST) in mussels' gills and digestive glands after exposure to 5FU. Similarly, GST activity in gills of *M. galloprovincialis* from the north of Portugal, where most residues of pharmaceutically active compounds were detected, also decreased (3.16 μmol/min/mg protein) (Martínez-Morcillo et al., 2020). In the gills of *M. galloprovincialis* exposed to cisplatin (100 ng/L, 14 d), GST activity presented a dumbbell shaped pattern and an increase in GST activity in the digestive gland of mussels on the last day of exposure (Trombini et al., 2016). Here, however, 5FU caused an inhibition of GST after 14 days of exposure. Similarly, GST activities in the digestive gland of *M. galloprovincialis* exposed to CP (1 μg/L, 14 d) decreased after 3 days (Fernandes et al., 2020), whilst Queirós et al. (2021) found a significant increase in GST activity of mussels (*M. galloprovincialis*) exposed to CP (500 and 1000 ng/L, 17 °C, 28 d). All things considered, 5FU caused oxidative stress in mussels, but the antioxidant defence system was able to counteract the toxicity of 5FU as no oxidative damage was observed in both tissues. In contrast, Trombini et al. (2016) found an increase in LPO levels of gills of *M. galloprovincialis* caused by cisplatin exposure (100 ng/L, 14 d) and confirming oxidative damage by increased ROS production. This therefore suggests that the ability of anticancer drugs to cause oxidative stress and damage is highly dependent on concentration, and the chemical composition of the drug. The PCA results (Fig. 3) further confirm the differences observed in 5FU-exposed mussels and those exposed to the mixture. 5FU-exposed mussel response is time and tissue-dependent, and the critical time point for the gills is observed at the end of the exposure period whist for digestive glands it is at 14 days. This confirms 5FU's toxicity towards mussels, despite the lack of oxidative damage, meaning that a longer exposure period could compromise mussel's integrity and should be further evaluated.

Regarding mixtures of contaminants, the effect of a pharmaceutical-nanoplastic-metal nanoparticle mixture (Mix) exposure led to an increase in SOD activity in the first two weeks of exposure in both gills and digestive glands of *M. galloprovincialis*. Comparing to 5FU alone, the increased activity of SOD lasts a week longer in mussels exposed to Mix. In the gills of *M. galloprovincialis* exposed to gold nanoparticles with a corona of zinc oxide (Au–ZnONP; 50 μg/L and 100 μg/L, 14 d), and to Diethyl 3-cyano-1-hydroxy- 1-phenyl-2-methylpropylphosphate (PC, 100 μg/L) led to an increase in activity of SOD, whereas the mixture of both contaminants did not show any significant differences to the control (Sellami et al., 2021). In other mixtures, the combined effects of micro-PS (2 + 6 μm, 32 μg/L) and fluoranthene (FLU, 30 μg/L) on *Mytilus* spp. digestive gland show an increase in SOD activity within the same range as Paul-Pont et al. (2016). SOD activity also increased in the digestive gland of *M. edulis* exposed to either 500 ng/mL PS-MPs (20 μm) or 500 ng/mL polyamide microfibres after 24 h (Cole et al., 2020). In the Mix, individually, CECs present different effects on SOD activity; nPS (50 nm; 10 μg/L; 21 d) led to inhibition of SOD activity in the gills of *M. galloprovincialis* (Gonçalves et al., 2022), followed by inhibition of SOD in the digestive gland; nAg (<100 nm; 10 μg/L; 15-d) led to an increase of SOD activity in both tissues (Gomes et al., 2014). Therefore, there seems to be an interaction occurring within the components of the Mix. In general, the levels for the gills of Mix-exposed mussels have a much lower CAT activity, with an increase between days 3 and 14. As CAT activity is inhibited, a potential increased production of H_2O_2 is mediated by the Mix, and CAT is unable to be counterbalanced the high ROS production, therefore raising concerns about the ROS toxicity towards *M. galloprovincialis*. A dumbbell-shaped pattern, as seen for mussels exposed to 5FU alone, is observable in Mix-exposed mussel gills, though activity values are much lower than that seen for 5FU-exposed mussels. Similarly, CAT activity in *M. galloprovincialis* exposed to nPS (15 ng/L, 21-d) presented a dumbbell-shaped pattern and a similar decrease in activity (Capolupo et al., 2021), suggesting that this activity may be due to the presence of nPS in the Mix. However, CAT activity decreased in both tissues of *M. galloprovincialis* after a 21-d exposure to nPS (50 nm; 10 μg/L) (Gonçalves et al., 2022), whereas nAg exposure to *M. galloprovincialis* (<100 nm; 10 μg/L; 15-d) increased CAT activity in gills and digestive glands (Gomes et al., 2014). This suggests that a synergistic effect between CECs may be occurring in Mix-exposed mussels. For Mix exposure in the digestive gland, a decrease in CAT activity possibly results from the transfer of contaminants onto plastics also observed by Paul-Pont et al. (2016) (micro-PS 2 + 6 μm, 32 μg/L and FLU 30 μg/L) in *Mytilus* spp. and by Almeida et al. (2019) (propanol and nPS, 10 mg/L, 24 h) in a fish cell line of *Sparus aurata,* therefore, supporting the assumption that a Mix of contaminants have an increased toxicity compared to the individual compound, and that plastics may act as carriers of these contaminants. However, we cannot rule out nAg's interaction within the mixture, as adsorption processes may also occur with metal nanoparticles. GPx may operate as a compensation mechanism for CAT activity deficiency. The activity of GPx in the gills of Mix-exposed mussels was induced, and a dumbbell shaped pattern is observed. Thus, the enhancement of GPx activity in the gills when CAT activity was inhibited, indicates that the gills became critically dependent on GPx activity for ROS removal (Regoli and Giuliani, 2014). Moreover, an increase in GPx activity is noteworthy in mussel's digestive gland after two weeks of exposure to Mix, whilst 5FU exposed mussels GPx activity decreased at this same time point. Looking at the effects of the contaminants in the mixture individually, *M. galloprovincialis* gills exposed to nAg (<100 nm; 10 μg/L; 15-d) only the activity of GPx increased (Gomes et al., 2014), and when mussels were exposed to nPS alone (50 nm; 10 μg/L; 21-d) there was low GPx activity in gills and digestive glands (Gonçalves et al., 2022), whereas 5FU shows a decreasing trend in activity similar to unexposed mussels. Thus, the interactions of the different particles in the Mix might explain the effects measured and might suggest a higher ROS production, indicating that the toxicity of these compounds in the mixture is enhanced. Therefore, it is possible that the combined effects of the contaminants are having synergistic effects. In gills and digestive glands of mussels exposed to Mix, GST activity behaved similarly, with a significant increase after two weeks of exposure. However, mussel gills did present an inhibition of GST during the first week of exposure to Mix. The decrease in GST activity in the gills (at day 3) is directly related to an increase in LPO. Therefore, the mussel's antioxidant response to ROS generated is insufficient to prevent oxidative damage during the first week of exposure. However, upregulation of GST activity observed after 14 days in the gills, is also related to the significant decrease in LPO on the 21st day. This indicates that mussels were able to manage the effect of the exposure to the Mix after a longer exposure period. Furthermore, the inhibition of GST activity seen in 5FU alone mussels may suggest adsorption of 5FU onto nAg or nPS in the Mix, making 5FU's toxicity less potent, and may explain the increase observed in the Mix comparative to 5FU alone. However, further evaluation is necessary to confirm this. Moreover, in relation to mussel's digestive gland, a similar trend in increase of GST activity in the digestive gland of *Mytilus* spp. was observed after the exposure to micro-PS and FLU (2 + 6 μm, 32 μg/L; 30 g/L, 14 d) (Paul-Pont et al. (2016), suggesting an increasing toxicity of mixtures over time. Results for Mix-exposed mussels confirm this pattern, seen by an increase in LPO levels for both tissues at first, although with a slight time difference, followed by a decrease in activity after 21 days. Exposure to other metal nanoparticles (e.g., nAg, nCuO) showed to increase oxidative stress in *M. galloprovincialis*, and oxidative damage was higher in the gills than the digestive gland when exposed to nAg (10 μg/L, 15 d), which might indicate that the gills are more affected by nAg (Gomes et al., 2013, 2014), and a comparable behaviour is visible in this experiment. Furthermore, nPS individual exposure (50 nm; 10 μg/L; 21-d) also led to oxidative damage in both tissues of *M. galloprovincialis*, being the crucial time-points for gills and digestive glands, 14 d, and 7 d, respectively (Gonçalves et al., 2022). The exposure to 0.350 ppm of cadmium for 96 h significantly affects both the gills and the digestive gland of *M. galloprovincialis*, as both levels of LPO increased, however the levels for the gills (10 nmol/mg protein) are twice as high as those

for the digestive gland (4.5 nmol/mg protein) (Santovito et al., 2021). These results agree with what was found for Mix-exposure, where the gills had the highest levels of LPO (43.5 nmol/mg protein, 14 d). Moreover, Di Poi et al. (2018) also observed that a mixture of contaminants (carbamazepine, methylparaben, aminomethylphosphonic acid; 0.96, 18.59, 500 ng/L, respectively) is more toxic than the single compound due to synergistic effects, but the toxicity of the single compound did not change. As a result of the overproduction of ROS, the antioxidant enzyme system was overwhelmed, resulting in peroxidative damage to membrane lipids (Fernández et al., 2012). It can be concluded that Mix had a higher effect on LPO levels than 5FU, indicating that mussels were able to withstand the negative effects of the single compound but not the negative effects of the mixture, which Di Poi et al. (2018), also concluded that binary and ternary mixtures (*C. gigas*, carbamazepine, methylparaben, aminomethylphosphonic acid; 0.96, 18.59, 500 ng/L, and methylparaben, aminomethylphosphonic acid respectively) where more toxic than the single compounds.

PCA results (Fig. 3) further confirm the severity of mixtures of CECs, and that mussel responses are mostly time-dependent, being 7 days of exposure, the most critical time point for both tissues. Therefore, the mixture of contaminants toxicity towards mussels is not tissue-specific, and comparing to 5FU-exposed mussels, it seems that mixtures are more toxic than their single compounds. Moreover, using the model defined by Ritz et al. (2021), to assess antagonistic and synergistic effects of binary mixtures was adapted for tertiary mixtures and the effects of pharmaceutical-nanoplastic-metal nanoparticle interactions can be found (see *Supplementary data*). A synergistic effect was confirmed in both tissues. In mussel gills, synergism was found in all biomarkers at the 14th day. In digestive gland of mussels, apart from SOD and CAT, the effects of all other biomarkers were synergistic at days 14 and 21. This model is therefore a useful tool to confirm antagonistic/synergistic effects as suggested, and more crucially that longer-exposure leads to these synergistic effects observed, meaning that the time of exposure is critical when considering the interactions of pharmaceuticals, nanoplastics, and metal nanoparticles.

5. Conclusions

The present data provides evidence that the negative effects of 5FU alone and in a mixture of contaminants in both mussel tissues are time and tissue-specific differentiating in severity. *M. galloprovincialis* were able to counteract the toxicity of 5FU, whilst the opposite was observed in Mix-exposed mussels which consequently led to oxidative damage. An adaptive response observed in both tissues seems to be limited to GST activity, demonstrating the ability of this biotransformation enzyme to activate as a compensating mechanism to manage oxidative stress. Both 5FU and Mix caused genotoxicity in mussel haemolymph, where the interactions between these emerging contaminants are antagonistic. Moreover, it is possible to conclude that single contaminant exposures are not as toxic as a mixture of different contaminants, and, on an important note, these interactions need to be further studied. Data suggests a synergistic effect in the interaction of a mixture of pharmaceutical-nanoplastic-metal nanoparticle in relation to oxidative stress and oxidative damage.

Author 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. Joanna M. Gonçalves, Clara Beckmann, Maria João Bebianno Joanna M. Gonçalves, Clara Beckmann, Maria João Bebianno Maria João Bebianno.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.135462.

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