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# Marine ecotoxicity of polystyrene microplastics, imidacloprid, and acyclovir: individual exposure in microalgae, rotifers and crustaceans

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ABSTRACT

Marine ecosystems are increasingly exposed to emerging pollutants such as pharmaceuticals, pesticides, and microplastics, raising concerns over their potential ecological impact. This study investigated the eco-genotoxic effects of the antiviral drug acyclovir (ACV), the pesticide imidacloprid (IMD), and 1.0 μm polystyrene microplastics (PS-MPs) in representative marine organisms from different trophic levels: the microalga *Phaeodactylum tricornutum*, the rotifer *Brachionus plicatilis*, and the crustacean *Artemia franciscana*.

Ecotoxicity tests showed that PS-MPs were the most toxic to *P. tricornutum* (EC50 = 8.30 mg/L), followed by IMD (EC50 = 135.83 mg/L) and ACV (EC50 = 177.83 mg/L). Among consumers, *B. plicatilis* was more sensitive to PS-MPs and ACV (LC50 ~100 mg/L). PS-MPs showed the lowest short-term toxicity in *A. franciscana* (<20 % lethality at 200 mg/L). Genotoxicity was observed starting from 20 mg/L for IMD, 2 mg/L for ACV, and 0.2 mg/L for PS-MPs, the latter being the most environmentally concerning. Environmental risk assessment indicated no immediate ecological threat from IMD and PS-MPs at current marine environmental concentrations.

## 1. Introduction

Marine ecosystem pollution has become a critical global issue, affecting not only the health of oceanic environments but also the well-being of countless species, with long-term consequences for food webs and the human population (Meijide et al., 2018; Amoatey and Baawain, 2019). Pollutants, such as personal care products, microplastics, pesticides, pharmaceuticals, per- and polyfluoroalkyl substances, and organophosphate flame retardants, have increasingly infiltrated marine habitats, disrupting ecological balance and threatening biodiversity (da Costa Araújo et al., 2022).

However, only a small fraction of these substances are currently included in European and international monitoring programs, leaving their environmental fate and ecotoxicological impact largely unknown (Coccia and Bontempi, 2023). Depending on their physicochemical properties, such as solubility and persistence, pollutants can significantly affect aquatic life (Schwarzenbach et al., 2006; Gogoi et al., 2018). Among the most studied, acyclovir (ACV, an antiviral drug), imidacloprid (IMD, a neonicotinoid insecticide), and polystyrene microplastics (PS-MPs) have all been detected in aquatic environments at concentrations ranging from ng to μg/L (Funke et al., 2016; Schirinzi et al., 2019; Vanryckeghem et al., 2019; Badylak et al., 2021; Ghanadi et al., 2024; Liao et al., 2024), and are being studied for their ecotoxicological impact (Minguez et al., 2016; Russo et al., 2017; Wang et al., 2019; Martín et al., 2021; Moeris et al., 2021; Lang et al., 2022; Nugnes et al., 2022a, 2023, 2024a; Liu et al., 2023). ACV is an acycloguanosine that inhibits viral DNA replication through an enzymatic cascade leading to the formation of acyclovir triphosphate, which blocks the synthesis of viral genetic material. Since 45–75 % of ACV is excreted by patients as an unchanged compound, and is poorly retained by wastewater treatment plants (Gupta et al., 2021; Almeida et al., 2023), it may be considered as a pseudo-persistent compound in aquatic environments, although it is highly water-soluble (log Kow = −1.56) (An et al., 2015; Jia et al., 2019). IMD, considered the best-selling insecticide in the world (Tomizawa and Casida, 2005), is a nicotinic acetylcholine receptor (nAChR) agonist that blocks nerve transmission by increasing the influx of $Na^+$ ions and the efflux of $K^+$ ions (Nugnes et al., 2023). With a log Kow of 0.57, IMD reaches surface and groundwaters through atmospheric drift and surface runoff from rainfall or irrigation (Chen et al., 2010; Thuyet et al., 2012; Sánchez-Bayo and Hyne, 2014), and, due to its persistence, it can accumulate in coastal areas and oceans (Liu et al., 2023; Sousa et al., 2020). Polystyrene is one of the most widely

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produced and most commonly found plastics in the environment, entering the marine ecosystems primarily as unintended waste from past and ongoing disposal. Over time, it undergoes fragmentation, forming micro- and nano-plastics, which float and persist in the open-ocean waters (Ali et al., 2025). Due to their small size, these particles are easily ingested by marine organisms, posing a risk to the food chain (Huang et al., 2021). Phytoplankton and zooplankton are among the first organisms to be affected by microplastics and other pollutants in the marine environment. Phytoplankton is highly responsive to environmental changes due to its short life cycle and continuous suspension in the water column, and it directly absorbs dissolved or dispersed substances, including pollutants. Zooplankton, especially filter-feeding taxa, indiscriminately ingest a wide range of particles, facilitating the transfer of MPs and associated contaminants through the food web across different trophic levels. The environmental behaviour and fate of MPs are governed by a combination of physical, chemical, and biological processes. Aging, aggregation, and biofilm formation play key roles in determining MPs' distribution and persistence in the marine environment. Aging alters particle density, surface properties, and reactivity, while the development of biofilms on MPs promotes hetero-aggregation with organic and inorganic particles, influencing their transport and settling dynamics (Gökdağ et al., 2025; Li et al., 2025; Lin et al., 2024). Moreover, salinity and the presence of mineral colloids enhance flocculation, modifying the buoyancy and vertical distribution of MPs in seawater (Mendrik et al., 2023; Gökdağ et al., 2025; Li et al., 2025). These transformations can occur within days to weeks, leading to significant spatial and temporal variability in MP concentrations throughout the water column. Consequently, measured environmental concentrations (MECs) are strongly influenced by these dynamic processes as well as by sampling depth and methodology. Field observations over the past few years have reported surface-to-subsurface levels ranging from tens to thousands of particles per cubic meter (Hoehn et al., 2024; Davidov et al., 2024). Some of the most recent MP-MECs, chosen according to different influencing factors, are reported in Table S1. This heterogeneity governs plankton exposure to MPs, influencing both individual interactions and their transfer within the marine food web.

Microplastics (Yan et al., 2023) together with pharmaceuticals (Duarte et al., 2020), and pesticides (Dupraz et al., 2019), can induce oxidative stress, reduce photosynthetic activity, and inhibit growth in marine algae. Various pharmaceuticals and neonicotinoid insecticides have been found to cause impair egg-laying and larval release in some bivalves (Fong and Ford, 2014) and to induce sublethal effects and changes in gene expression in the marine mussel *Mytilus galloprovincialis* (Mezzelani et al., 2021). Moreover, acute and behavioural effects, including mortality, immobility, and altered swimming speed, have been observed in crustaceans, rotifers, sea urchins, and jellyfish exposed to polyethylene microplastics (Morgana et al., 2020).

To better understand the real effects of pollutants in the environment, it may be helpful to complement conventional ecotoxicological tests with the analysis of genetic material in aquatic organisms. This analysis has emerged as a reliable technique for detecting exposure to low pollutant levels across various species (Frenzilli et al., 2009; Jiang et al., 2023). For this purpose, several assays are employed to observe single and double strand breaks, alkali labile sites, oxidative DNA base damage, DNA-DNA/DNA-protein and DNA-drug crosslinking, chromosomal aberrations, micronuclei (Thirunavukkarasu et al., 2020). The Comet assay is one of the most widely used methods due to its numerous advantages, including the ability to detect genotoxic damage at the single-cell level with high sensitivity, ease of execution, and the rapid induction of DNA strand breaks following contaminant exposure, enabling early assessment of effects on biota (Collins et al., 2023).

Given the limited research on the impact of ACV, IMD, and PS-MPs (1.0 μm, a borderline size between micro- and nanoplastics) in non-target marine species (Booth et al., 2019; Malhotra et al., 2021), this study aims to assess the potential ecotoxic effects of these pollutants in the following marine organisms: the primary producer, diatom *Phaeodactylum tricornutum*, and two primary consumers, rotifer *Brachionus plicatilis* and crustacean *Artemia franciscana*. These sentinel species are recognized as good models for assessing pollutant impacts in marine ecosystems due to their sensitivity, ease of cultivation, rapid life cycle, and widespread distribution (Kim et al., 2022; Varó et al., 2019). To the best of our knowledge, no toxicity data exist on the effects of ACV and IMD on the alga *P. tricornutum* and the rotifer *B. plicatilis*, while data on 1 μm PS-MPs remain limited. *A. franciscana* is also a suitable organism for further assessment of DNA damage due to its ability to produce large numbers of organisms from dormant cysts. *A. franciscana* can exhibit DNA strand breaks and molecular alterations after exposure to various pollutants, which supports its applicability in genotoxicity testing using sensitive endpoints like the Comet assay, which may reveal sub-lethal effects not detectable through conventional endpoints such as mortality or hatching rate (Albarano et al., 2022). In addition, no previous studies have investigated genetic alterations in *A. franciscana* or other marine organisms exposed to ACV and IMD.

Finally, an estimation of marine risk was conducted following standardized guidelines for Environmental Risk Assessment (ERA) frameworks, including those established by the European Medicines Agency (EMA, 2006), the EU Technical Guidance Document (TGD, 2003), and the technical report on marine risk assessment (ECETOC, TR 082, 2001).

## 2. Materials and methods

### 2.1. Test compounds

ACV (CAS: 59 277–89–3, purity ≥99 %), IMD (CAS: 138261-41-3, purity ≥98 %) and an aqueous suspension of PS-MPs (product number: 72938) were purchased from Sigma-Aldrich (Milano, Italy). According to the supplier, the PS-MP suspension contains approximately $3.24 \times 10^{10}$ particles/mL with a particle size of 1.0 μm. This commercial suspension, corresponding to 21000 mg/L, was used as the stock solution from which the test solutions were freshly prepared. Differently, ACV and IMD powders were dissolved in Milli-Q water, obtaining stock solutions of 1000 mg/L and 500 mg/L, respectively, below their solubility limits (1600 mg/L for ACV at 22–25 °C and 610 mg/L for IMD at 20 °C). Test solutions were freshly prepared prior to each assay and handled under controlled laboratory conditions. The pH of the test solutions was measured at the beginning and end of each assay. Differences between the nominal and actual concentrations of ACV and IMD were determined using a Total Organic Carbon Analyzer (TOC-L CSN, Shimadzu, Kyoto, Japan).

### 2.2. Test organisms and exposure media

For the ecotoxicological assay, the algal inoculum of the diatom *P. tricornutum* in steady-state conditions, as well as cysts (dormant eggs) of the rotifer *B. plicatilis* and the crustacean *A. franciscana* were purchased from Ecotox LDS (Milan, Italy). For all bioassays, artificial standard seawater (ASW; NaCl, KCl, $CaCl_2$, $MgCl_2$, $MgSO_4$, $NaHCO_3$, $H_3BO_3$) was prepared at a salinity of 35 ppt and pH 8.0. Regarding the diatom assays, nutrient stock solutions (Solution 1: $FeCl_3$, $MnCl_2$, $ZnSO_4$, $CuSO_4$, $CoCl_2$, $H_3BO_3$, $Na_2EDTA$; Solution 2: Thiamin hydrochloride, Biotin, Vitamin $B_{12}$; Solution 3: $K_3PO_4$, $NaNO_3$, $Na_2SiO_3$) were added to the ASW. Both the ASW and the algal culture medium were prepared according to the respective international standard protocols and the Standard Operating Procedures provided by the Ecotox LDS kits. Test solutions were freshly prepared prior to each assay and handled under controlled laboratory conditions by diluting stock solutions in ASW or algal culture medium. The pH of the test solutions was measured at the beginning and end of each assay.

### 2.3. Toxicity testing

A preliminary range finding test was conducted for each test organism and each compound to identify a suitable concentration range for the definitive tests and to ensure a good concentration-response relationship.

The seawater *P. tricornutum* algal growth inhibition test (ISO 10253, 2024) was performed according to the standard protocol, with the sole modification being the use of 96 well-plates as suggested by Russo et al. (2023) and Nugnes et al. (2024b). 200 μL of each test solutions (ACV from 800 to 12.5 mg/L, dilution factor (DF) equal to 2; IMD from 400 to 25 mg/L, DF = 2; PS-MP from 50 to 0.39 mg/L, DF = 2) was incubated with 100 μL of a $10^4$ cells/mL of algal suspension in six replicates at 20 ± 2 °C for 72 h under continuous illumination (6000 lux) on a microplate shaker (450 rpm). Absorbance was recorded at 750 nm by the microplate reader (Synergy H1, Biotek, Winooski, USA) at time 0 and after 24 h, 48 h and 72 h. Potential interference from PS-MPs scattering and turbidity was controlled by applying a blank correction. Specifically, absorbance readings for each PS-MPs concentration were corrected using blanks containing only particles (ASW with PS-MPs without algae), ensuring that the absorbance values reflected algal cellular density.

*B. plicatilis* cysts (Ecotox LDS, Milan, Italy) were hatched in ASW (only for hatching, at 20 ppt salinity; pH 8.0) under continuous illumination (3000 lux) for 30 h at 25 ± 1 °C (ASTM E 1440-91, 2004). 36-well plates were set up by adding 0.3 mL of each test compound dilution (ACV from 200 to 15.57 mg/L, DF = 1.2; IMD from 200 to 1.56 mg/L, DF = 2; PS-MPs from 172 to 33.4 mg/L, DF = 3.2) or 0.3 mL of ASW in negative control (NC). A stereomicroscope (Olympus SZ-ST, 15x magnification and dark field), and a micropipette were used to transfer five rotifers per well in six replicates. The plates were incubated at 25 °C in darkness for 24 h.

In the absence of an established international standard guideline for *A. franciscana*, the toxicity test was performed according to the Italian Agency for Environmental Protection and Italian Institute for Water Research (APAT IRSA CNR 8060, 2003) except for hatching performed according to the Standard Operating Procedure provided by the Artoxkit M (Ecotox LDS, Milan, Italy). *A. franciscana* cysts were hatched in ASW (35 ppt salinity; pH 8.0) under continuous illumination (3000–4000 lux) for 30 h at 25 ± 1 °C.

24-well plates were set up by adding 1 mL of each test compound dilution (from 200 to 12.5 mg/L, with DF equal to 2) or 1 mL of synthetic seawater in the negative control. Ten brine shrimp larvae/well were transferred to each dilution and the NC in three replicates. The multiwell plates were incubated for 24 h at 25 ± 1 °C in darkness.

Lethality of the rotifers/crustaceans was assessed by counting dead animals in each well. The resulting data were expressed as percentages and subsequently used to estimate the Median Lethal Concentration (LC50)

### 2.4. Genotoxicity testing

*A. franciscana* was used to study the genotoxicity, in terms of DNA damage, induced by PS-MPs, ACV, and IMD tested individually. Single Cell Gel Electrophoresis, also known as Comet assay, was performed according to Sukumaran and Grant (2013), Arulvasu et al., (2014) and Collins and collaborators (2023). The test was performed on the whole organisms hatched from cysts (as reported above) and exposed for 24 h to the toxicants (ACV from 200 to 0.2 mg/L, IMD from 200 to 2 mg/L, PS-MP from 20 to 0.02 mg/L, DF = 10). Specifically, 100 neonates were placed in glass beakers containing the compound at different concentrations, or ASW (NC), in two biological replicates, and incubated at 25 ± 1 °C in darkness. Cell viability was evaluated in all tested dilutions after 24 h of exposure using Trypan Blue staining. Viability remained above 80 % in all samples, confirming their suitability for the Comet assay. Then, the organisms were transferred to 0.4 mL of phosphate-buffered saline containing 10 % dimethyl sulfoxide and 20 mM ethylene diamine tetra-acetic acid (EDTA), homogenized to disintegrate the exoskeleton and to obtain the single cells, and centrifuged at 4500 g for 15 min at 4 °C. The pellet of each biological replicate was resuspended in 85 μL of 0.7 % low-melting-point (LMP) agarose and spread onto two microscope slides (technical replicates) pre-coated with 1 % normal melting point (NMP) agarose, resulting in four slides per concentration. Then, to lyse the membranes, each slide was immersed for 2 h in 100 mM EDTA, 10 mM Tris-HCl, 2.5 M NaCl, 10 % DMSO and 1 % Triton X-100 (pH 10). Subsequently, DNA unwinding was carried out under alkaline conditions (1 mM EDTA, 300 mM NaOH, pH ≥ 13) for 30 min at 4 °C. Electrophoresis was conducted in the same buffer (1L) for 30 min at 25 V(1 V/cm) and 400 mA in a horizontal tank maintained at 4 °C, ensuring that the slides were fully immersed. The slides were dipped in 0.4 M Tris-HCl for 10 min, in 70 % ethanol for 10 min, stained with 50 μL of ethidium bromide (10 mg/L) and finally analysed by fluorescence microscope (400x magnification, Eclipse 50i, Nikon, Kanagawa, Tokyo).

### 2.5. Data analysis

Toxicity assays were carried out in triplicate, while genotoxicity testing was performed in duplicate. Graphpad Prism 8 software (GraphPad Inc, CA, USA) was used to estimate the concentrations giving x% effect (L(E)Cx) by non-linear regression (log agonist *vs.* normalized response-variable slope). L(E)C50, L(E)C20, and L(E)C10, were the concentrations giving 50 %, 20 % and 10 % effect (E), specifically inhibition of growth for algae, and lethality (L) for rotifers and crustaceans. Furthermore, the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC) were estimated by ANOVA and the Dunnett's multiple comparison test (*$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$). Statistical differences among concentrations was estimated by Tukey's HSD multiple comparison test ($p < 0.05$; varying letters).

Regarding the genotoxicity assay, 50 nucleoids were randomly selected and scored on each of 4 slides per concentration, resulting in 200 nuclei per experiment and 400 nuclei in total across the two independent experiments. Scoring was performed using Comet assay IV image analysis software (Perceptive Instrument, UK), applying the software's standard threshold presets for nucleoids detection, which were kept constant across all images to ensure comparability. Overlapping or fragmented nuclei and artefacts were excluded from counting to ensure accurate assessment of DNA damage. It is visualized as a comet-like structure, with the head corresponding to intact DNA and the tail reflecting fragmented DNA (Nugnes et al., 2024b). The percentage of DNA in tail (Tail Intensity) was recorded as a parameter of genotoxicity. Tail intensity is considered the most useful and reliable parameter, since it correlates linearly with the frequency of DNA breaks, is largely independent of threshold settings, and clearly reflects comet morphology (Collins, 2002; Bolognesi et al., 2019). Statistical analysis was performed on the mean values per biological replicate, based on 400 nuclei per concentration to ensure representative measurements; slide-level variation was not modelled separately. The No Observed Adverse Effect Concentration (NOAEC) and the Lowest Observed Adverse Effect Concentration (LOAEC) were estimated by ANOVA and Dunnett's multiple comparison test for significance compared to the negative control (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) while statistical differences among the concentrations was obtained by two-way Anova with Tukey's HSD multiple comparison test ($p < 0.05$; varying letters).

## 3. Results and discussions

No appreciable differences (ranging between 0.2 % and 3 %) between nominal and actual concentrations were detected using the TOC-L CSN analyser. As reported by Nugnes et al., (2022a), actual concentrations that deviated by less than 20 % from nominal values can be

considered effectively equivalent. Therefore, nominal concentrations were used as representative of the actual exposure levels. The pH of each test solution was measured at the beginning and at the end of each assay and remained within 8.0 ± 0.2, consistent with natural seawater conditions and the recommendations of the respective protocols.

### 3.1. Ecotoxicity

The ecotoxicity of ACV, IMD, and PS-MPs was evaluated based on growth inhibition in *P. tricornutum* after 72 h of exposure, and lethality in *B. plicatilis* and *A. franciscana* following 24 h of exposure. Prior to conducting the assays, positive controls were performed using potassium dichromate, a standard reference toxicant in aquatic toxicity assessments, to verify the sensitivity of the test organisms. The results, expressed as EC50 (the effective concentration that inhibits algal growth by 50 %) for producers or LC50 (the concentration causing 50 % lethality) for consumers, were 21.10 (16.55–26.90) mg/L for algae, 313.30 (216.20–449.00) mg/L for rotifers, and 38.45 (36.39–40.63) mg/L for crustaceans, which are in line with the data provided in the specification sheets accompanying the beads or cyst batches.

The results for the xenobiotics investigated in this study showed that PS-MPs were the most toxic to producers, inhibiting algal growth by 50 % at concentrations in the order of units of mg per liter, whereas ACV and IMD determined the same effect at concentrations in the hundreds of mg/L range. For consumers, LC50 values were not always reached (Table 1).

Among the primary consumers, the rotifer was the most sensitive species to the selected xenobiotics. ACV and PS-MPs affected *B. plicatilis* causing 50 % lethality at concentrations in the order of hundreds of mg/L, while IMD induced only about 28 % effect at 200 mg/L. In contrast, none of the tested substances caused a minimum lethality of 20 % in the crustacean up to 200 mg/L, indicating a lower sensitivity of this species to the examined pollutants. According to EU-Directive 93/67/CEE (EU-Directive, 1996), chemicals can be classified based on their L(E)C50 values. Substances are considered very toxic when L(E)C50 $< 1$ mg/L, toxic between 1 and 10 mg/L, harmful between 10 and 100 mg/L, and non-toxic when L(E)C50 $> 100$ mg/L for aquatic species. Based on this classification, after 72 h of exposure, PS-MPs can be considered toxic to algae, whereas ACV and IMD fall within the non-toxic range.

For rotifers and crustaceans, after 24 h of exposure, all tested chemicals showed LC50 values grater than 100 mg/L, indicating a lack of toxicity even at concentrations several orders of magnitude higher than those generally found in the aquatic environment, which generally range from units of ng/L to hundreds of μg/L (Funke et al., 2016; Schirinzi et al., 2019; Vanryckeghem et al., 2019; Badylak et al., 2021; Ghanadi et al., 2024; Liao et al., 2024). Although L(E)C50 is the recommended endpoint in ecotoxicity tests (ASTM E1440-91, 2004; ISO 10253, 2024), in this study it could not always be determined within the tested concentration range. To date, few studies have examined the effects of the selected xenobiotics on the marine species used here (e.g., Minguez et al., 2016; Booth et al., 2019; Wang et al., 2020; Lang et al., 2022; Liu et al., 2023). In particular, no data are currently available on the effects of ACV and IMD on the diatom *P. tricornutum* and the rotifer *B. plicatilis*. Due to the ecological importance of these organisms and the lack of toxicity data, there is a clear need to better understand the risks these compounds may pose to marine ecosystems. For this reason, L(E)C10 and L(E)C20 values were also estimated, as they provide information on lower-level effects (10 % and 20 % response, respectively). The inclusion of these endpoints enhances the robustness of the dataset, facilitates comparisons among substances, and contributes to a more comprehensive evaluation of their ecological impact. The L(E)C10 is particularly important as it represents the threshold below which no significant toxic effect is observed. L(E)C20, on the other hand, serves as an early indicator of toxicity, identifying the onset of measurable adverse effects. Similarly, the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC), defined as the highest no-effect concentration and the lowest observed effect concentration, respectively, were determined through one-way ANOVA and Dunnett's multiple comparison test ($p < 0.05$). NOEC and LOEC are influenced by the concentration intervals selected by the operator and are not statistically extrapolated from the experimental data pool. The aforementioned ecotoxicological data (L(E)C10, L(E)C20, NOEC, and LOEC (expressed in mg/L) are presented in Table 2 to provide a comprehensive overview of the effects of ACV, IMD, and PS-MPs in the marine organisms.

Table 2 shows that the chemicals exhibit distinct behaviours depending on the organisms used. PS-MPs display higher activity than ACV and IMD in algae, with the lowest effective concentrations in the range of units of mg/L. In contrast, the effective concentrations of ACV and IMD for both algae and rotifers fall within the range of tens of mg/L. Similarly, microplastics exhibit their lowest effective concentrations also in the tens of mg/L range towards rotifers and crustaceans. ACV and

**Table 1**
Ecotoxicity data of ACV, IMD and PS-MPs in *P. tricornutum*, *B. plicatilis*, and *A. franciscana*. Data are presented as L(E)C50 values expressed in mg/L, with 95 % confidence intervals in brackets, and as the percentage of effect (mean ± SD, n = 3) at the highest concentration tested (200 mg/L).

| Compound | *P. tricornutum* | *B. plicatilis* | *A. franciscana* |
|---|---|---|---|
| ACV | 177.83 (149.97–210.38) | 105.40 (99.16–112.10) | 5.00 % ± 2.35 at 200 mg/L |
| IMD | 135.83 (120.78–153.11) | 28.35 % ± 2.33 at 200 mg/L | 8.33 % ± 2.30 at 200 mg/L |
| PS-MPs | 8.30 (6.77–10.19) | 110.00 (97.37–128.10) | 18.89 % ± 1.92 at 200 mg/L |

**Table 2**
Ecotoxicological effects of ACV, IMD, and PS-MPs in marine organisms. Data are presented as L(E)C10 and L(E)C20 (mg/L) with 95 % confidence intervals in brackets. NOEC and LOEC values (mg/L) were determined using Dunnett's test.

| | | ACV | IMD | PS-MPs |
|---|---|---|---|---|
| ***P. tricornutum (72h)*** | EC10 | 18.92 (12.79–27.80) | 42.27 (32.80–54.83) | 1.45 (0.94–2.26) |
| | EC20 | 43.25 (32.51–55.72) | 65.01 (53.46–77.62) | 2.76 (1.96–3.73) |
| | NOEC | 25.00 | 25.00 | 1.56 |
| | LOEC | 50.00 | 50.00 | 3.12 |
| ***B. plicatilis (24h)*** | LC10 | 20.94 (17.90–24.32) | 1.84 (1.00–5.13) | 33.65 (33.49–43.35) |
| | LC20 | 38.10 (34.30–41.70) | 25.00 (13.49–43.55) | 46.99 (37.41–56.36) |
| | NOEC | 39.75 | 6.25 | 48.22 |
| | LOEC | 46.51 | 12.50 | 57.87 |
| ***A. franciscana (24h)*** | LC10 | – | – | – |
| | LC20 | – | – | 65.01 (47.31–83.75) |
| | NOEC | 100.00 | 100.00 | 12.50 |
| | LOEC | 200.00 | 200.00 | 25.00 |

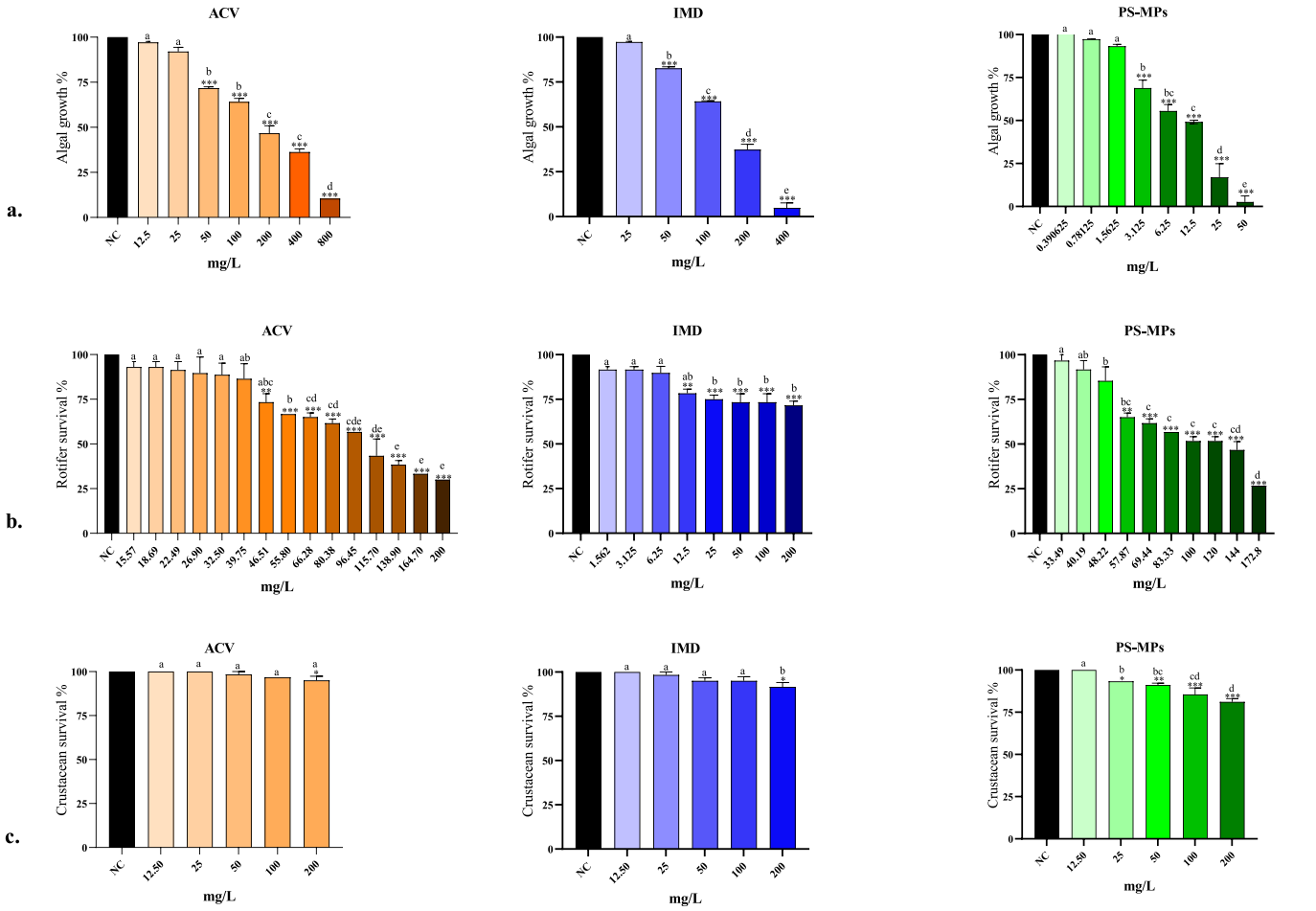


**Fig. 1.** Percentages of algal growth in *P. tricornutum* (a) after 72 h of exposure, survival in *B. plicatilis* (b), and *A. franciscana* (c) after 24 h of exposure to ACV, IMD and PS-MPs. Results are presented as mean ± standard deviation (n = 3). Significant differences between concentrations (mg/L) and negative controls (NC) are indicated by asterisks (ANOVA, Dunnett's test: *p < 0.05; **p < 0.001; ***p < 0.0001). Differences among concentrations (p < 0.05) were assessed using Tukey's HSD multiple comparison test and are indicated by different letters.

IMD induce the weakest activity in crustaceans, with effective concentrations in the range of hundreds of mg/L. To provide a clearer and more comprehensive understanding of the overall impact of the tested xenobiotics, Fig. 1 presents the percentage of effects observed at each tested concentration. The graphs display producers' growth and consumers' survival on the y-axis, and concentrations (mg/L) on the x-axis, allowing for a precise evaluation of concentration-response relationships. They also show a statistical variations in effects, both in comparison to the negative control (ANOVA, Dunnett's test: *p < 0.05; **p < 0.001; ***p < 0.0001), and among different concentrations (Tukey's HSD multiple comparison test, p < 0.05, indicated by different letters). Additionally, the graphs facilitate the visual comparison among different test organisms and exposure conditions, offering insights into potential differences in sensitivity and contributing to a more accurate interpretation of each xenobiotic's toxicological relevance.

In algae, all three xenobiotics caused a significant reduction in growth with increasing concentration, particularly IMD and PS-MPs. For rotifers, a significant reduction in survival was observed for ACV. In crustaceans, only PS-MPs led to a marked decrease in survival. This demonstrates a clear concentration-dependent effect, with statistical variation in response to different concentrations reflected in the assigned letters.

Basically, the concentrations tested in this study (mg/L range) are considerably higher than those typically detected in marine environments (ng–μg/L range, Ghanadi et al., 2024; Liao et al., 2024). However, their selection was guided by range-finding tests and was necessary to obtain measurable responses and to identify short-term toxicity effects that would otherwise remain undetectable at environmental concentrations. Thus, high-concentration assays provide essential effect data that can be interpreted as conservative, worst-case exposure conditions that, although unlikely under average circumstances, may occur transiently in localized hotspots such as wastewater discharges, aquaculture effluents, or agricultural runoff. The toxicological values obtained here contribute to international ecotoxicological databases, fill critical knowledge gaps, strengthen environmental risk evaluation frameworks, and support more accurate regulatory assessments of xenobiotics in marine ecosystems.

At present, there are still few studies where ACV, IMD, and PS-MPs have been tested in the same marine organisms used in this work. Therefore, our results were compared with toxicity data from both marine and freshwater species to provide a broader understanding of ACV, IMD, and PS-MP effects across different aquatic environments. Regarding the base of the trophic chain, Russo et al. (2017) reported no toxic response in the marine bacterium *Vibrio fischeri* exposed to ACV, as luminescence remained unaffected at mg/L concentrations. Moving up the aquatic trophic chain, Nugnes et al. (2024a) tested ACV on *Raphidocelis subcapitata*, a freshwater green alga, reporting an EC50 of 265 mg/L, comparable to the results obtained in the present study for *P.*

*tricornutum* (EC50 = 177 mg/L). In the same freshwater species, Almeida et al. (2021) reported even lower sensitivity (thousands of mg/L). The relatively low toxicity of ACV toward *R. subcapitata* may be explained by the findings of Gomez-Martínez et al. (2023), who propose that the high tolerance observed in the Selenastraceae family, could be linked to physiological and transcriptional mechanisms underlying adaptive responses, although these mechanisms remain only partially elucidated. More recently, Nugnes et al. (2025) expanded the assessment of ACV to three phytoplankton species, *Dunaliella tertiolecta*, *P. tricornutum*, and *Skeletonema pseudocostatum*. ACV induced minimal growth inhibition in *S. pseudocostatum* and *P. tricornutum* (<10 %), whereas *D. tertiolecta* exhibited higher sensitivity (>20 % inhibition), with effects evident at extremely low concentrations (0.00001 mg/L). Although *P. tricornutum* showed no reduction in growth rate, a significant decrease in chlorophyll-a content was detected, greater than that observed in the other two species.

At the level of primary consumers, Minguez et al. (2016) reported LC50 values > 100 mg/L in the marine crustacean *A. franciscana*, consistent with the results of the present study. Similarly, Nugnes et al. (2024a) found LC50 > 200 mg/L for the freshwater crustacean *Ceriodaphnia dubia* and the rotifer *Brachionus calyciflorus* after 24 h of exposure, while rotifers *B. calyciflorus* and *B. plicatilis* displayed slightly higher sensitivity (LC50 ≈ 105 mg/L). In comparison, *Daphnia magna* appeared more vulnerable, with 35 % immobilization at 22.5 mg/L (An et al., 2015) and an EC50 of 64.12 mg/L (Minguez et al., 2016). Nugnes et al. (2025) further observed that ACV exposure in *Amphibalanus amphitrite* nauplii caused mortality rates below 10 % but significantly reduced swimming speed starting at 0.01 mg/L. Conversely, in the echinoderm *Paracentrotus lividus*, developmental anomalies were recorded only at the highest concentration tested (10 mg/L), with no alteration in swimming behaviour.

Finally, at higher trophic levels, Schlüter-Vorberg et al. (2015) found no embryotoxic effects in *Danio rerio* embryos exposed to concentrations up to 100 mg/L. Overall, these results suggest that ACV exhibits generally low toxicity toward algae, although certain species such as *D. tertiolecta* may be more sensitive, while primary consumers show greater susceptibility compared to producers.

As regards IMD, the ecotoxicological profile has been investigated across a wide range of aquatic taxa, revealing substantial variability in sensitivity depending on phylogeny, trophic level, and exposure duration. Tisler et al. (2009) reported that primary producers and secondary consumers generally exhibited lower sensitivity, with effective concentrations in the hundreds of mg/L, whereas reducers and primary consumers were more susceptible, with effective concentrations in the tens of mg/L. Specifically, their study showed EC50 values of 389 mg/L for the freshwater alga *Desmodesmus subspicatus* and 241 mg/L for the fish *Danio rerio*, compared with markedly lower values for the bacterium *V. fischeri* (61.9 mg/L) and the crustacean *D. magna* (97.9 mg/L). In line with these findings, Nugnes et al. (2023) confirmed the low sensitivity of the freshwater alga *Raphidocelis subcapitata*, consistent with the tolerance observed in the marine diatom *P. tricornutum* in the present study.

Marine invertebrates show a similarly broad range of responses. For example, the brine shrimp *A. franciscana* displayed low sensitivity, with LC50 values exceeding 200 mg/L (Booth et al., 2019; Liu et al., 2023), while rotifers, both freshwater (*B. calyciflorus*) and marine (*B. plicatilis*), also exhibited LC50 values above 200 mg/L (Nugnes et al., 2023). Similarly, the copepod *Nitocra spinipes* showed little sensitivity under short-term exposure (Moeris et al., 2021). In contrast, Liu et al. (2023) identified the mysid *Neomysis awatschensis* as the most sensitive marine organism tested, with an acute LC50 of only 0.0265 mg/L and chronic EC50 values as low as 0.0009 mg/L, underscoring the heightened vulnerability of crustaceans to IMD. From a phylogenetic perspective, sensitivity to IMD appears to follow the order Annelida > Arthropoda > Chordata > Mollusca > Bacillariophyta, with annelids and arthropods showing the greatest susceptibility (Liu et al., 2023; He et al., 2022). This pattern reflects both the mode of action of IMD as a nicotinic acetylcholine receptor agonist, which is absent in algae, and differences in detoxification and antioxidant defence capacity among taxa. Chronic exposure studies further highlight species-specific vulnerabilities. De Marchi et al. (2025) observed no acute effects or growth inhibition *in P. tricornutum* or the mussel *Mytilus galloprovincialis* up to 3000 μg/L. However, significant chronic effects were reported in the copepod *Acartia tonsa*, with EC10 and EC20 values in the order of units of μg/L and EC50 in the tens of μg/L. Moreover, *M. galloprovincialis* exhibited oxidative stress and neurotoxic responses when exposed to 0.5–5 μg/L, as indicated by increased lipid peroxidation and elevated acetylcholinesterase activity in the digestive gland. Similar sublethal effects were also described in the Pacific oyster *Magallana gigas*, where IMD altered physiology and gene expression (Kuchovská et al., 2021).

Taken together, these results demonstrate that while primary producers and higher consumers such as fish generally display low sensitivity to IMD, certain invertebrate taxa, particularly crustaceans and annelids, are highly vulnerable, especially under chronic exposure. This highlights the importance of considering taxon-specific responses and long-term effects when evaluating the ecological risks of IMD in aquatic environments.

Polystyrene microplastics have been widely investigated for their ecotoxicological effects across aquatic trophic levels, revealing strong variability in sensitivity among taxa and life stages. In bacteria, Martín et al. (2021) reported limited effects on the marine decomposer *V. fischeri*, with a maximum inhibition of 27 % at 400 mg/L. Moving to primary producers, PS-MPs caused significant growth inhibition and impairment of photosynthetic functions in microalgae. For example, Lang et al. (2022) observed in *P. tricornutum* exposed to 200 mg/L of 0.1 μm PS-MPs a 53.5 % reduction in cell growth, 25.5 % decrease in chlorophyll content, and 12.5 % decline in photosynthetic efficiency. In the present study, 1 μm PS-MPs induced 50 % algal growth inhibition at concentrations as low as 8.3 mg/L, consistent with Nugnes et al., (2022a), who reported a comparable EC50 of 4.02 mg/L in the freshwater alga *R. subcapitata.* These results indicate a relatively conserved sensitivity to PS-MPs among algal taxa, regardless of their ecological niche.

Among primary consumers, rotifers showed contrasting responses depending on habitat. The marine rotifer *B. plicatilis* tolerated high concentrations with no median toxic effects up to 10 mg/L (Gambardella et al., 2018), and LC50 values in the hundreds of mg/L were confirmed in the present study. In contrast, the freshwater rotifer *B. calyciflorus* displayed much higher sensitivity, with 50 % lethality at concentrations in the tens of mg/L (Nugnes et al., 2022a), suggesting a greater vulnerability of freshwater species, possibly related to physiological or environmental differences (Dahms et al., 2011). Similar habitat-related differences were observed in crustaceans: while freshwater species such as *C. dubia* and *Daphnia pulex* exhibited LC50 values in the tens of mg/L (Liu et al., 2019; Nugnes et al., 2022a), the marine brine shrimp *A. franciscana* showed no lethal effects up to 100 mg/L in this study, in agreement with Wang et al. (2019), who also reported no impacts on survival, growth, or development in *A. parthenogenetica*, though cellular alterations such as microvilli disorganization and mitochondrial proliferation were observed.

More broadly, nearly 700 aquatic species worldwide have been reported to be adversely affected by microplastics, including crustaceans, fish, turtles, and seabirds, highlighting the ubiquity of this stressor (Marn et al., 2020; Yang et al., 2025). Evidence indicates that microplastics exert both physical and chemical toxicity, with particularly strong effects on early developmental stages and lower trophic levels. In copepods such as *Tigriopus japonicus*, 0.05–0.5 μm MP caused acute mortality and long-term reproductive impairment (Choi et al., 2020). Barnacle larvae (*A. amphitrite*) and brine shrimp (*A. franciscana*) showed swimming inhibition at ≥1 mg/L (Li et al., 2024), while echinoderm embryos such as *P. lividus* experienced developmental arrest at 30 mg/L of PVC-MPs, with additional toxic effects linked to plastic leachates (Oliviero et al., 2019). Bivalves like *M. galloprovincialis* and *Ruditapes*

*philippinarum* accumulated MPs in tissues, resulting in immune suppression, genotoxicity, and oxidative stress, with enhanced toxicity when MPs carried chemical contaminants (Avio et al., 2017). Fish also displayed significant pathological effects: in *Dicentrarchus labrax*, MPs induced histopathological lesions in liver and intestine, while in *Pomatoschistus microps*, PE-MPs reduced acetylcholinesterase activity and caused oxidative stress (Grattagliano et al., 2025). Chronic exposure of *Oryzias melastigma* to MPs at 200 μg/L produced intestinal damage despite no significant alterations of microbiota composition (Wen et al., 2024).

Overall, the ecotoxicological evidence shows that PS-MPs can impact aquatic organisms at multiple trophic levels, with effective concentrations ranging from a few μg/L to tens of mg/L. Lower trophic levels and early developmental stages appear particularly sensitive, raising concerns about long-term ecosystem stability and the cascading impacts of microplastic pollution (Grattagliano et al., 2025).

Although this study focused on the effects of single pollutants, it is important to recognize that in natural environments organisms are simultaneously exposed to complex contaminant mixtures. Interactions among compounds can lead to additive, synergistic, or antagonistic effects, potentially altering toxicity outcomes compared to single-compound exposures (Lagunas-Rangel et al., 2025). Although mixture experiments were beyond the scope of the present work, future studies should aim to assess combined effects under environmentally realistic exposure scenarios.

It is well known that microplastics are ubiquitous in marine environments, acting as both pollutants and carriers of marine contaminants through the so-called "Trojan horse effect".

In a previous study on the freshwater crustacean *C. dubia* (Nugnes et al., 2022b), the combined exposure to ACV, IMD, and 1-μm PS-MPs in binary and ternary mixtures was evaluated for both genotoxic and chronic effects. After 24 h of exposure of neonates to the mixtures, predominantly antagonistic genotoxic interactions were observed, whereas prolonged (7-day) exposure resulted in additive chronic toxicity. These effects occurred at concentrations comparable to, or even overlapping with, environmentally relevant levels in freshwater systems, thereby highlighting their potential significance and raising environmental concern.

Microplastics can adsorb hydrophobic organic contaminants at concentrations up to six orders of magnitude higher than in surrounding water, depending on polymer type, surface aging, and environmental conditions (Giroux et al., 2024; Prajapati et al., 2022; Wang et al., 2024). Environmental factors such as salinity, pH, temperature, and natural organic matter further influence this sorption behaviour (Sun et al., 2023; Tang et al., 2021). Several studies have highlighted the synergistic toxicological effects between microplastics and co-occurring contaminants, which can amplify adverse biological outcomes (Swathi and Tanushree, 2025; Sun et al., 2022a). Significant adsorption of pharmaceuticals and personal care products (PPCPs), particularly antibiotics, onto microplastic surfaces has been widely reported (Al-Jandal et al., 2022; Atugoda et al., 2021; Zhuang and Wang, 2023). The co-occurrence of microplastics with pollutants such as persistent organic pollutants, heavy metals, and PPCPs can markedly enhance contaminant bioaccumulation and toxicity in aquatic organisms, by up to 31 % and 18 %, respectively (Sun et al., 2022b), thus exacerbating physiological disturbances beyond single-compound exposures.

The effects of polyethylene microplastics in combination with the insecticide chlorpyrifos (CPF) on the marine copepod *A. tonsa* were evaluated by assessing survival, fecundity, feeding activity, and egg viability. The study demonstrated that CPF exhibited higher toxicity when co-exposed with MPs than when tested alone for all biological endpoints examined, indicating a synergistic effect between the two stressors (Bellas and Gil, 2020). Similarly, a study investigating the combined effects of polystyrene microplastics and nanoplastics with the pharmaceutical sertraline on the bivalve *Tegillarca granosa* revealed a pronounced synergistic immunotoxic response between sertraline and nanoplastics (Shi et al., 2020).

### 3.2. Genotoxicity

In the comet assay, several descriptors of DNA migration can be extracted, including Tail Length, Tail Moment, and Tail Intensity (T.I.). The latter can be defined as the percentage of DNA in the comet tail, and it is considered the most informative and reliable parameter for assessing DNA damage. As highlighted by Collins et al. (2023), the relative amount of total DNA in the tail reflects the frequency of breaks, underscoring the fact that tail intensity provides a direct and quantitative measure of the fraction of fragmented DNA relative to the total cellular DNA. Unlike Tail Length, which can vary considerably depending on nucleus size, chromatin organization, and electrophoretic conditions, or Tail Moment, which integrates different variables but is often less consistent across scoring systems, Tail Intensity is normalized to the total DNA content of the cell. This normalization minimizes morphological bias and contributes to its higher sensitivity and reproducibility, confirming it as the preferred endpoint for the comet assay. Thus, the results obtained in this study are reported as T.I.

Genotoxicity induced by ACV, IMD, and PS-MPs was assessed by exposing *A. franciscana* nauplii to various concentrations of each chemical for 24 h.

Positive controls using potassium dichromate and hydrogen peroxide were first performed to assess whether the nauplii responded adequately to genotoxic damage induced by these compounds. These control results (Table 3) are essential for validating the test system's sensitivity. T.I. was measured starting from a concentration corresponding to 1/10 of the LC50, as described by Parrella et al. (2015). The LC50 values obtained were 38.45 mg/L (95 % CI: 36.39–40.63) for potassium dichromate and 641.18 mg/L (95 % CI: 591.51–695.26) for hydrogen peroxide. From these starting points, serial 10-fold dilutions were prepared and tested until the resulting concentrations produced no statistically significant difference in T.I. compared to the negative control (Dunnett's test).

After confirming that nauplii were sensitive to genotoxic damage, the tests on ACV, IMD, and PS-MPs were conducted. The results are reported in Figs. 2 and 3, and Table 4.

PS-MPs induced the most severe damage, with a median tail intensity of 30 % at the highest concentration tested (20 mg/L). A similar effect was observed for ACV at 200 mg/L, whereas at the same concentration of IMD, the median effect was approximately 15 %. Statistical analysis using Dunnett's multiple comparison test revealed that the LOAEC for the three xenobiotics, compared to negative controls, was 0.2 mg/L, 2 mg/L, and 20 mg/L for PS-MPs, ACV, and IMD, respectively (Table 4). The effects of the three xenobiotics at different concentrations were statistically analysed using Tukey's HSD multiple comparison test, with significant differences ($p < 0.05$) indicated by different letters, highlighting the corresponding variation in DNA fragmentation. As concentrations increased, the percentage of DNA in the tail also increased, indicating a clear concentration-effect relationship. This trend was particularly evident for PS-MPs and ACV. To the best of our knowledge, no previous studies have investigated the genotoxic effects of ACV and IMD on DNA in marine organisms. However, Nugnes et al. (2023, 2024a) reported increased DNA strand breaks and elevated reactive oxygen species (ROS) production induced by ACV and IMD in freshwater species. Similarly, Alak et al. (2023) observed DNA lesions linked to IMD-induced oxidative stress in freshwater secondary consumers at concentrations ranging from units to tens of mg/L. This damage was associated with the activation of the Nrf-2/GSH/NF-κB pathways. Regarding PS-MPs, Gambardella et al. (2017) exposed marine crustaceans, *A. amphitrite* and *A. franciscana*, to concentrations ranging from 0.001 to 10 mg/L (concentrations comparable to those used in the present research) for 24 and 48 h, reporting alterations in enzyme activities, and oxidative stress. In the same year, Ribeiro et al. (2017) exposed the bivalve *Scrobicularia plana* to 1 mg/L of 20 μm PS-MPs for 14

**Table 3**
Tail Intensity (T.I., mean value ± standard deviation, n = 2), representing the percentage of DNA fragmentation in the comet tail obtained after exposing organisms to different concentrations of potassium dichromate ($K_2Cr_2O_7$) and hydrogen peroxide ($H_2O_2$). Significant differences from negative control were determined with Dunnett's test (***p < 0.0001).

| $K_2Cr_2O_7$ | 0.00 mg/L (NC) | 0.00038 mg/L | 0.0038 mg/L | 0.038 mg/L | 0.38 mg/L | 3.8 mg/L | |
|---|---|---|---|---|---|---|---|
| T.I. | 2.08 ± 0.89 | 2.36 ± 0.99 | 7.19 ± 2.60*** | 16.87 ± 2.33*** | 25.26 ± 4.03*** | 28.36 ± 4.58*** | |
| $H_2O_2$ | 0.00 mg/L (NC) | 0.00064 mg/L | 0.0064 mg/L | 0.064 mg/L | 0.64 mg/L | 6.4 mg/L | 64 mg/L |
| T.I. | 2.08 ± 0.89 | 2.38 ± 0.75 | 15.11 ± 1.91*** | 26.21 ± 3.44*** | 29.92 ± 4.26*** | 33.72 ± 3.68*** | 44.04 ± 3.10*** |

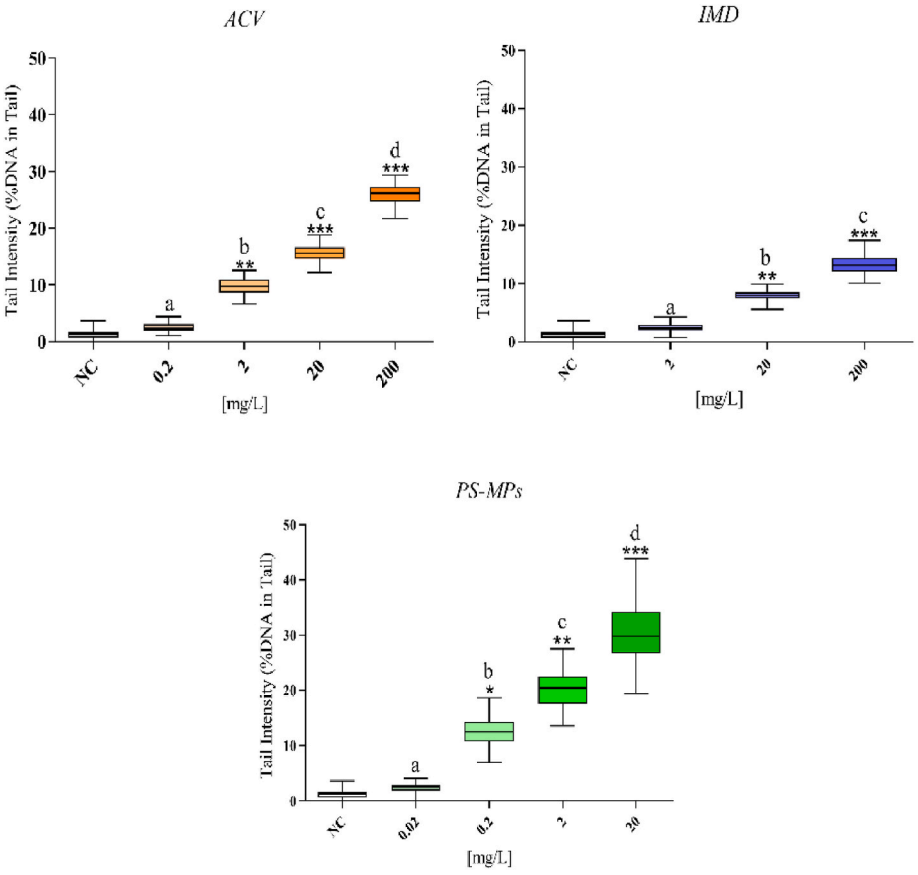


**Fig. 2.** Quartile box plot of DNA in tail (%) in *A. franciscana* after exposure for 24 h to different concentrations of ACV, IMD and PS-MPs. Significant differences from negative control were determined with Dunnett's test (*p < 0.05, **p < 0.01, ***p < 0.001), while difference among concentrations (p < 0.05; different letters) were determined with Tukey's HSD multiple comparison test.

days, observing DNA damage, neurotoxicity, and oxidative stress. Recently, Chelomin et al. (2024) detected DNA damage in the haemocytes of the marine mussel *Mytilus trossulus* when exposed to 0.9 μm PS-MPs.

To gain a broader understanding of the effects of these xenobiotics in aquatic environments, it is crucial to compare responses across different species and ecosystems. In particular, the results obtained from *A. franciscana* were compared to those from a suitable freshwater counterpart, *C. dubia*. Both species serve as standard sentinel species in aquatic toxicology, with *A. franciscana* representing the marine ecosystem and *C. dubia* the freshwater environment. Their differences in physiology, life cycle, and habitat-specific adaptations provide valuable insights into how aquatic organisms with distinct ecological traits respond to genotoxic stressors.

Thus, the genetic damage results obtained for ACV, IMD, and PS-MPs in *A. franciscana* were depicted together with those from previous studies on *C. dubia* (Nugnes et al., 2022a,b, 2023, 2024a).The respective LOAEC values (expressed in mg/L) were reported in Fig. 4.

This comparison provides insights into species-specific susceptibility and offers a broader perspective on the potential risks of pollutants across different aquatic environments. The data indicate that *C. dubia* exhibits greater sensitivity to all tested substances, with LOAEC values of 0.007, 0.008, and 0.2 mg/L for IMD, PS-MPs, and ACV, respectively. Notably, the LOAEC values for ACV differ by only one order of magnitude between *A. franciscana* and *C. dubia*, whereas for IMD and PS-MPs, the differences are more pronounced, spanning five and three orders of magnitude, respectively. *A. franciscana* is highly resistant to genotoxic stress due to its ability to recover from DNA damage, as demonstrated by the genomic template stability investigated by Sukumaran and Grant (2013). *A. franciscana* reproduces sexually, leading to inherent genetic variation that enhances its resistance to genetic damage induced by environmental stressors (Sukumaran and Grant, 2013). In contrast, parthenogenetic species like *C. dubia*, exhibiting genetic uniformity, are more vulnerable to such stress and lack the capacity to withstand genotoxic damage.

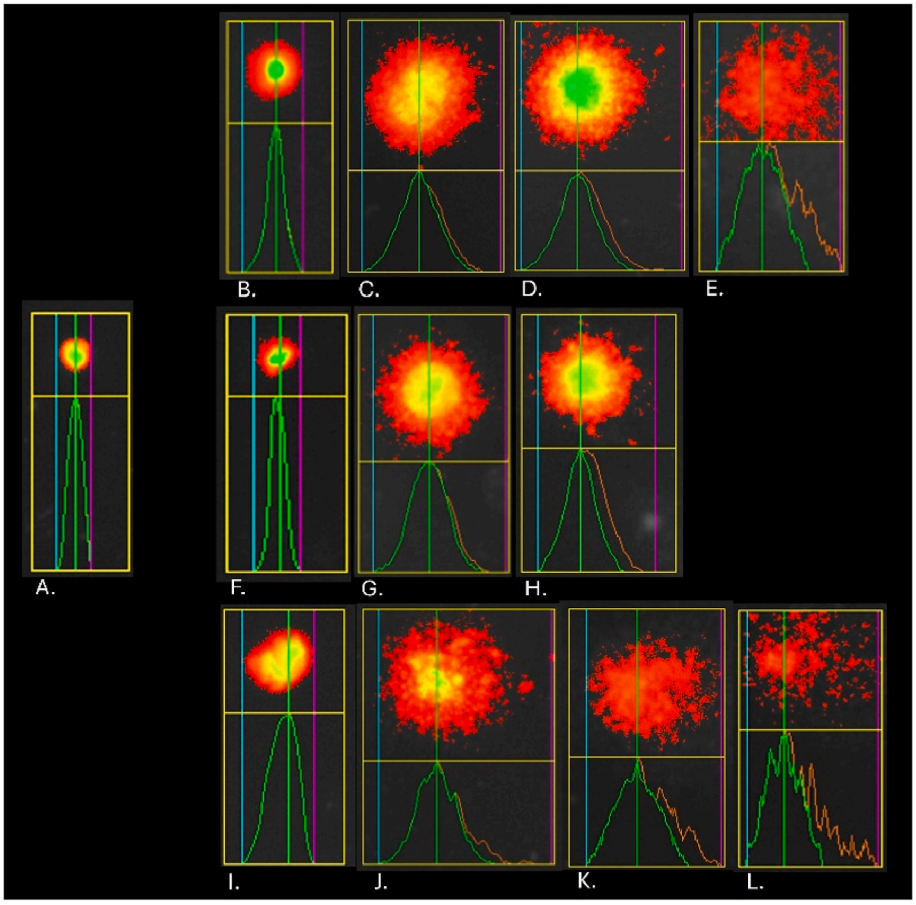


**Fig. 3.** Examples of the most representative nuclei observed exposing *A. franciscana* to NC (A), ACV (B-E, 0.2–200 mg/L), IMD (F-H, 2–200 mg/L) and PS-MPs (I-L, 0.02–20 mg/L) for 24 h. Comets (fragmented DNA in tail) were observed using Comet assay IV image analysis software (Perceptive Instrument, UK) using a fluorescence microscope (Eclipse 50i, Nikon, Kanagawa, Tokyo) after staining with ethidium bromide (10 mg/L).

**Table 4**
Effects of ACV, IMD and PS-MPs (mg/L) on induction of DNA damage in *A. franciscana* single cells. Results are expressed as Tail Intensity (T.I., % DNA in tail), mean ± SD (n = 2). Concentrations were selected up to levels that did not produce statistically significant differences in T.I. compared with the negative control; significance *versus* control was assessed using Dunnett's test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

| [mg/L] | ACV | IMD | PS-MPs |
|---|---|---|---|
| **0 (NC)** | | 1.28 ± 0.86 | |
| **0.02** | – | – | 2.37 ± 0.76 |
| **0.2** | 2.49 ± 0.72 | – | 12.50 ± 2.63* |
| **2** | 9.76 ± 1.45** | 2.37 ± 0.72 | 20.25 ± 3.02** |
| **20** | 15.56 ± 1.41*** | 7.95 ± 0.80** | 30.33 ± 5.22*** |
| **200** | 26.01 ± 1.55*** | 13.21 ± 1.57*** | – |

- concentration not tested.

### 3.3. Risk assessment in the marine environment

Based on the findings from this study, we aimed to evaluate the risk quotient (RQ) as an indicator of the potential risk posed by ACV, IMD, and PS-MPs in the marine environment. This assessment followed standard guidelines (ECETOC, TR 082, 2001; TGD, 2003; EMA, 2006). Additionally, we considered the approaches proposed by Everaert et al. (2018) and Zhang et al. (2020) for specifically assessing the risks associated with microplastics. The RQ value was determined by calculating the ratio between the Measured Environmental Concentration (MEC) of the tested xenobiotics, which refers to the most recently detected concentration (as far as we are aware), in the marine environment, and the

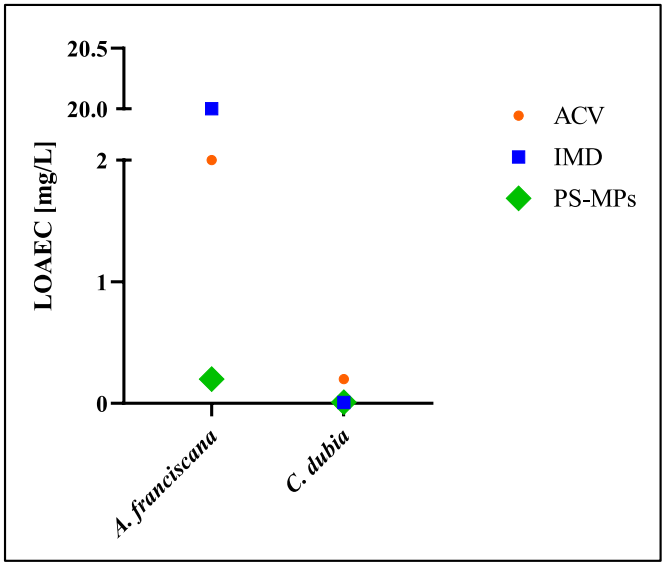


**Fig. 4.** LOAECs (mg/L) for DNA damage in the marine crustacean *A. franciscana* from this study compared with results obtained from freshwater crustaceans (*C. dubia*) in our previous studies (Nugnes et al., 2022a, 2023, 2024a).

Predicted No Effect Concentration (PNEC), which indicates the concentration below which no adverse effects are anticipated, ensuring environmental protection. The PNEC is calculated by dividing a specific endpoint (the lowest L(E)C50) by an assessment factor (AF of 1000), which is determined based on available toxicity data, the number of tested trophic levels, and taxonomic groups. Based on the available literature, no data are currently available on marine concentrations of ACV. The selected MECs were chosen considering sampling data that, to the best of our knowledge, are the most recent available. For IMD, based on average sampling values, is 153.13 ng/L in Shandong, China (Liao et al., 2024). In the case of PS-MPs, the concentration of 28.5 ng/L in Auckland, New Zealand (Ghanadi et al., 2024), was derived by converting particle concentration (0.58 p/L, explicitly reported) into mass units, considering particle density and size as suggested by Everaert et al. (2018). For the mass conversion, a geometric mean particle size of 45 μm was applied, representing the average value within in size distribution within the 20–100 μm range, which contained the majority of the detected microplastic particles in samples taken from the water surface at a depth of approximately 30 cm (Ghanadi et al., 2024). Both in the case of IMD and PS-MPs, the RQs are far lower (three orders of magnitude) than the threshold value of 1, indicating no concerning risk for the marine environment.

Regarding the RQ calculated for IMD, both Liao et al. (2024) and Naumann et al. (2022) estimated marine concentrations of IMD based on ecotoxicological data from the ECOTOX database (http://www.epa.gov/ecotox), which do not specifically refer to marine species. In our study, using exclusively marine organisms with their respective L(E)C50 values, the RQ remains below the threshold value of 1, indicating no potential risk, while in both Liao et al. (2024) and Naumann et al. (2022), the RQ exceeded the threshold of 1.

It should be noted that short-term L(E)C50 values are traditionally used as the standard basis for calculating risk quotients; however, they may not be sensitive enough to detect sublethal, chronic, or genotoxic effects. Thus, in the absence of long-term toxicity data, genotoxicity endpoints may nonetheless provide valuable insights, as they reflect lower-level effects occurring at concentrations well below those causing lethality or inhibition of growth. Hence, although current European guidelines recommend the calculation of the Risk Quotient (RQ) for toxicity data, they do not include provisions for assessing genotoxic RQ. Nevertheless, following the approach adopted in a previous study (Nugnes et al., 2023), we propose a genotoxic risk assessment based on observed DNA damage. Importantly, DNA damage is a relevant biomarker with potential implications at the population level, particularly for sensitive and keystone species such as *A. franciscana*, where impaired genomic integrity could affect reproduction and long-term viability. No Observed Adverse Effect Concentrations (NOAECs) were used in the calculations: 2 mg/L for IMD and 0.02 mg/L for PS-MPs, applying an assessment factor (AF) of 1000, as a measure of maximum uncertainty when data are available only for a single taxonomic group, when a large safety factor is needed to ensure protection of the entire aquatic ecosystem.

The resulting genotoxic RQ values for both IMD and PS-MPs were found to be below the threshold value of 1. Furthermore, in the risk characterization, even when considering the application of a more conservative and refined Assessment Factor (e.g., AF = 100), the resulting RQ would still be below 1, thereby confirming that the risk remains negligible.

Although current risk assessments do not indicate significant concerns for the marine environment regarding PS-MPs and IMD, the increasing production and release of these substances could lead to unforeseen ecological effects. In evaluating of risk related to MPs, it is important to consider that defining representative MECs is particularly challenging due to the heterogeneous vertical distribution of particles, influenced by factors that also affect sampling and analytical methods. Further testing should involve a broader range of marine organisms, trophic levels, and ecological endpoints to provide a more comprehensive and representative understanding of long-term impacts.

This is crucial to identify potential risks that may otherwise remain undetected and ensure the health of marine ecosystems. These data will contribute to future ERA refinements, which will include broader coverage of marine organisms, multiple trophic levels, and chronic NOEC/LOEC data as they become available, reducing uncertainty and allowing a more comprehensive assessment of sublethal and long-term effects.

## 4. Conclusions

This study provides novel insights into the eco-genotoxic effects of ACV, IMD, and PS-MPs in representative marine organisms from different trophic levels. The alga *P. tricornutum* was most the sensitive to PS-MPs, while *B. plicatilis* showed the highest susceptibility among consumers. Despite the low acute toxicity observed in *A. franciscana*, significant DNA damage was detected, especially following exposure to PS-MPs and ACV, indicating sublethal effects.

Environmental risk assessment based on both toxicological and genotoxicological endpoints revealed that IMD and PS-MPs exhibited Risk Quotient values below the regulatory threshold of concern (RQ < 1), suggesting no immediate ecological threat under current environmental concentrations.

Future research should include long-term studies, additional trophic levels, and endpoints to better elucidate the ecological consequences of these contaminants and strengthen environmental risk assessment frameworks.

## CRediT authorship contribution statement

**Roberta Nugnes:** Writing – original draft, Investigation, Data curation, Conceptualization. **Chiara Russo:** Writing – original draft, Investigation, Data curation, Conceptualization. **Elena Orlo:** Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Margherita Lavorgna:** Writing – review & editing, Writing – original draft, Conceptualization. **Marina Isidori:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

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## Declaration of the use of AI and AI-assisted technologies

During the preparation of this work, the authors used Generative AI and AI-assisted technologies (ChatGPT) in the writing process solely to improve readability and language of the work. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

## 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.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2025.107774.

## Data availability

Data will be made available on request.

## References


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