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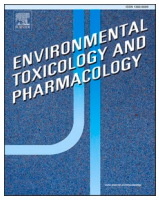

Detrimental effects of individual versus combined exposure to tetrabromobisphenol A and polystyrene nanoplastics in fish cell lines

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ABSTRACT

The potential interactions between the diverse pollutants that can be released into the environment and the resulting outcomes are a challenging issue that needs to be further examined. This in vitro study was aimed to assess potential toxic effects caused by combined exposure to tetrabromobisphenol A, a flame retardant widely used and frequently detected in aquatic matrices, and commercially available polystyrene nanoparticles as reference material to evaluate nanoplastics risks. Our results, using freshwater fish cell lines and a set of relevant cytotoxicity endpoints including cell viability, oxidative stress, and DNA damage, provide additional mechanistic insights that could help to fully characterize the toxicity profiles of tetrabromobisphenol A and polystyrene nanoparticles. Furthermore, we describe subtle changes in cell viability as well as the generation of oxidative DNA damage after coexposure to subcytotoxic concentrations of the tested pollutants.

1. Introduction

Tetrabromobisphenol A (TBBPA) is a brominated flame retardant widely employed as either reactive or additive in a number of marketed products including textiles, plastics, and electronic circuit boards (Covaci et al., 2011). Due to its high production and prevalent use, TBBPA has been ubiquitously detected in environmental (Hou et al., 2021; Malkoske et al., 2016) and human samples (Abdallah and Harrad, 2011; Cariou et al., 2008), raising concerns about the possible detrimental effects on natural ecosystems and human health. As summarized in recent reviews (Feiteiro et al., 2021; Zhou et al., 2020), numerous studies both *in vivo* and *in vitro* have well documented that exposure to this persistent chemical may induce reproductive and developmental defects, nephrotoxicity, hepatotoxicity, neurotoxicity, and endocrine disorders in various experimental models. Based on the available experimental data, TBBPA has been classified as probably *carcinogenic to humans* (Group 2 A) by the International Agency for Research on Cancer (IARC, 2018) *and very toxic to aquatic life* (H400), according to the European Chemicals Agency (ECHA, 2016). However, the potential risks of this environmental pollutant remain questioned since controversial results have been reported in the literature (Zhou et al., 2020). Moreover, most previous studies evaluated the toxic effects caused by individual exposure to the compound but the potential interactions with other environmental chemicals and the resulting outcomes have been underestimated. This is a challenging issue that needs to be addressed because chemical pollution results from a mixture of hazardous substances at low doses, that can potentially be released into the environment (Kortenkamp and Faust, 2018). Among contaminants of emerging concern in aquatic ecosystems, small plastic pieces generated from both primary and secondary sources are currently receiving particular attention (Uddin et al., 2022). Microplastics (MPs, < 5 mm) and nanoplastics (NPs, < 100 nm) may cause negative impacts in the aquatic environment due to their inherent toxicity (Rist and Hartmann, 2018), but also as a result of their ability to adsorb and transport other coexisting pollutants (Bhagat et al., 2021; Yu et al., 2019). To date, most research on the potential risks of MPs and NPs has focused on the marine environment (Ganesh Kumar et al., 2020; Peng et al., 2020) while data are still limited regarding plastic pollution impacts in freshwater ecosystems (Boyle and Örmeci, 2020; Meng et al., 2020). In particular, the mechanisms underlying NPs toxicity to freshwater biota including the

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combined effects with other chemical contaminants remain to be fully elucidated, although significant progress has been made in the last few years (Masseroni et al., 2022; Zhang et al., 2021).

It is now well established that *in vitro* methods play a central role in water quality monitoring (Brack et al., 2019), but also as part of integrated testing strategies for risk assessment of environmental chemicals and nanoparticles (Rehberger et al., 2018; Savage et al., 2019). Currently, fish cell-based assays using a variety of reliable endpoints are recognized as one of the most suitable alternatives to animal experimentation in aquatic toxicology (Goswami et al., 2022; Kolarova et al., 2021; Tan and Schirmer, 2017). This multiparameter study was aimed to assess potential toxic effects in freshwater fish cell lines after combined exposure to the flame retardant TBBPA, frequently detected in aquatic matrices (Kotthoff et al., 2017), and commercially available polystyrene nanoparticles (PSNPs) commonly used as reference material to evaluate the toxicity of nanoplastics (Lehner et al., 2019). RTgill-W1, RTL-W1, and RTgutGC cell lines representative of gills, liver, and gut respectively were selected based on their physiological significance and prediction power of the obtained data (Fischer et al., 2019; Schug et al., 2020). Our results provide additional mechanistic insights that could help to fully characterize the toxicity profiles of TBBPA and PSNPs, describing subtle changes in cell viability as well as the generation of oxidative DNA damage after combined exposure to both environmental pollutants.

2. Methods

2.1. Materials

Unless otherwise stated, cell-culture sterile disposable materials were from Fisher Scientific (Fisher Scientific Spain, Madrid, Spain), and culture media and reagents from Gibco (Life Technologies Corporation, New York, USA) or HyClone (HyClone Laboratories, GE Healthcare Life Sciences, Utah, USA). All chemical reagents were from Sigma (MilliporeSigma, Munich, Germany) or Panreac-Applichem (Barcelona, Spain).

2.2. Cell cultures

Three rainbow trout (*Oncorhynchus mykiss*) cell lines, RTgill-W1(Bols et al., 1994) (CRL-2523, American Type Culture Collections, Virginia, USA), RTL-W1 (Lee et al., 1993), and RTgutGC (Kawano et al., 2011) (both provided by the Swiss Federal Institute of Aquatic Science and Technology-EAWAG under an MTA agreement) were selected for this work as representative cellular models of fish gills, liver, and gut respectively. They grew under a normal atmosphere at 20 °C in an incubator (IPP110, Memmert GmbH + Co. KG, Schwabach, Germany) using Leibovitz L-15 medium (HyClone) supplemented with 10% fetal bovine serum (FBS, Gibco), and a mixture of 100 U/mL penicillin + 100 mg/mL streptavidin (HyClone). Ventilated F75 vessels were used for the routine growing of all cell lines, and multiwell plates of 6, 12, or 96 wells for the different experimental procedures. Daily control of cell cultures was conducted with a phase-contrast microscope (Leica DMi1, Leica Microsystems, Wetzlar, Germany). Culture media was renewed twice a week and cells were subcultured before reaching 100% confluence using trypsin 0.25% and Versene (Gibco).

2.3. Experimental design

A 0.1 M stock solution of TBBPA (CAS 79–94–7, Sigma) was prepared in dimethyl sulfoxide (DMSO, Panreac-Applichem) and kept in the dark at - 20 °C until use. The 10 mg/mL stock of carboxylated polystyrene nanoparticles (PSNPs, CML Latex Beads) with a nominal size of 40 nm was acquired from Sigma. We established a wide range of concentrations for TBBPA (1 – 150 μM; 0.54 – 81.6 μg/mL) in order to adequately fit a dose-response model and determine subcytotoxic conditions in our experimental setup. Likewise, concentrations of PSNPs covering two orders of magnitude (1 – 200 μg/mL) were assayed to explore potential changes in PSNPs behavior.

The experimental design includes a first step of cytotoxicity evaluation of TBBPA with the three cell lines. Afterward, further cytotoxicity evaluations of TBBPA, PSNPs, and selected combinations of TBBPA + PSNPs were performed with the most sensitive cell line. In the second set of experiments, relevant endpoints of cellular damage were evaluated under selected conditions.

2.4. Nanoparticles characterization

Hydrodynamic light scattering studies were performed at the Department of Chemical Engineering (Universidad de Alcalá) under the supervision of Dr. Roberto Rosal. Size distributions were obtained using dynamic light scattering in a Malvern Zetasizer Nano ZS apparatus (Malvern Instruments Ltd., Worcestershire, UK) in MilliQ water and different culture media. Zeta-potential (ξ) measurements were conducted at 25 °C at the prescribed pH employing electrophoretic light scattering combined with phase analysis light scattering in the same Zetasizer Nano ZS instrument using disposable folded capillary cells (Malvern Instruments).

Transmission electron microscopy (TEM) studies were carried out at the CBMSO facilities using a JEM1400 Flash microscope (Jeol, Tokyo, Japan) equipped with a CMOS Oneview camera (Gatan, Pleasantville, USA). One drop of PSNPs suspension was placed onto carbon-coated grids and air-dried prior to observation.

2.5. Cytotoxicity evaluation

All the viability assays were performed in 96-well plates at a density of 2×10^5 cells/mL. When appropriate confluence was obtained, usually the following day, cell cultures were exposed to TBBPA ranging from 1 to 150 μM (0.54 – 81.6 μg/mL) in cell media with reduced FBS (2%), to minimize the binding to albumin (Wang et al., 2014). For the exposure to PSNPs (0.1–200 μg/mL), the stock solution was sonicated 30 min before the preparation of fresh working solutions. Combination exposures of selected TBBPA – PSNPs were prepared likewise.

Three independent experiments were performed using cells of different passage number, which was always below 20. At experiment termination, cell cultures were assayed for metabolic activity (Alamar blue, AB), and plasma membrane integrity (carboxyfluorescein diacetate acetoxymethyl ester, CFDA-AM), following the method of Schirmer et al. (1997) with slight modifications. Briefly, after a washing step in phosphate buffer saline (PBS), cells were incubated for 30 min with a combination of 5% AB v/v (Invitrogen, ThermoFisher Scientific, Massachusetts, USA), and 4 μM CFDA-AM (Invitrogen) in DMEM without phenol red. Fluorescence was measured at appropriate excitation/emission wavelengths (530 / 595 nm for AB, 485 / 530 nm for CFDA-AM) using the plate reader Synergy HT (Biotek, Vermont, USA).

2.6. Determination of reactive oxygen species and mitochondrial membrane potential ($\Delta\Psi$)

Quantitative evaluation of reactive oxygen species (ROS) and the inner mitochondrial membrane potential ($\Delta\Psi$) was performed on black 96-well plates, incorporating the appropriate cell-free controls. The presence of ROS was assessed by the 2′, 7′ – dichlorofluorescein diacetate (H_2DCFDA, Sigma) probe, a non-fluorescent compound hydrolyzed by intracellular esterases and readily oxidized to the highly fluorescent DCF in the presence of intracellular ROS. Briefly, cells were incubated with 5 μM H_2DCFDA for 40 min at 20 °C, washed in PBS, and treated as required. Fluorescence was monitored on the Synergy HT microplate reader with excitation and emission wavelengths of 488 and 530 nm, respectively, for up to 24 h.

Mitochondrial status was assessed with tetramethylrhodamine, methyl ester (TMRM, Invitrogen) staining, which incorporates into the

mitochondrial matrix proportionally to the inner mitochondrial membrane potential (ΔΨ). After the selected treatments, cells were loaded with TMRM 150 nM for 30 min, washed three times with PBS, and fluorescence was measured at 530 nm excitation / 588 nm emission in the Synergy HT plate reader. The protonophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Sigma) was used as a positive control (50 μM, 24 h).

2.7. Genotoxicity assessment

Potential DNA damage was determined by the alkaline comet assay combined with an enzymatic treatment with the bacterial repair enzyme FPG (formamidopyrimidine-DNA glycosylase). Thus, in addition to single and double DNA strand breaks, the assay can measure the oxidation product 8-oxo-7,8-dihydroguanine.

Cells were gently trypsinized, centrifuged (1200 rpm, 7 min), and washed twice in cold PBS. Pellets were stored in a freezing buffer (85.5 g/L sucrose and 50 mL/L DMSO prepared in 11.8 g/L citrate buffer at pH 7.6) at - 80 °C until used. Comet assay was conducted following the protocol of Huarte et al. (2021) with minor modifications. An estimated 10^4 cells were embedded in 0.75% low melting point agarose and deposited on pre-coated slides with 1% agarose to produce 2 mini-gels per slide and 3 slides per condition. Immediately after agarose solidification (10 min on ice), samples were incubated for 1 h at 4 °C in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X- 100, pH 10). Two slides were washed in cold PBS, one was exposed to FPG (1 U, New England Biosciences, Massachusetts, USA), the other to the enzyme buffer alone (NEBuffer™ + 10 mg/mL BSA, New England Biosciences), and incubated for 30 min at 37 °C in a humid chamber. The third slide remained in the lysis buffer. Subsequently, DNA was allowed to unwind for 30 min in the cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) and electrophoresis was carried out for 20 min at 21 V (0.73 V/cm) in a compact-50 vertical electrophoresis tank (Fisher Scientific). Slides were neutralized in PBS at 4 °C, washed in distilled water, and air-dried. Staining was performed with GelRed (Biotium, California, USA) and images of at least 50 nucleoids/gel were acquired under green excitation light with a Leica DMI 3000B microscope, equipped with an EL6000 compact light source. CometScore Pro (Tritek corp., Virginia, USA) was used to quantify the % of tail DNA. For each experiment, the median value of the comets scored for each sample (100 comets) was calculated. The level of FPG sensitive sites was obtained by subtracting the % tail DNA of the samples incubated with FPG buffer from those of FPG treated analogs. Cells incubated 24 h with $KBrO_3$ 500 μM were used as positive controls for FPG treatments, and H_2O_2-treated cells for the alkaline comet assay. Three independent experiments were carried out and the results were plotted as the mean ± standard deviation (SD).

2.8. Statistical analysis

Quantitative fluorescence measures are expressed as % of control untreated cells once the fluorescence of blanks was subtracted. Box plots and bar charts were created using GraphPad Prism 9.0 (GraphPad Software, San Diego, USA). The same program was used to perform all the statistical analyses including normality and homoscedasticity evaluation, appropriate tests for ANOVA or Kruskal-Wallis and multiple comparisons, and sigmoidal dose-response curves with variable slope to estimate effective concentration (EC) 50. Statistically significant differences were considered for $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3. Results and discussion

3.1. RTgill-W1 is the most sensitive of the three cell lines employed

In the first set of experiments, we evaluated the cytotoxicity of TBBPA using three different fish cell lines, observing a similar trend (Fig. 1). After a 24 h exposure, a sudden reduction in cell viability between 25 and 50 μM TBBPA could be noted in all the experimental conditions. Interestingly, metabolic activity (AB) and plasma membrane integrity (CFDA-AM) showed equivalent results, suggesting an unspecific cell injury with TBBPA concentrations above 50 μM.

These results are in good agreement with other cytotoxicity studies performed with TBBPA in different cell lines such as human colon (Huang et al., 2021) and rat kidney (Strack et al., 2007), although differ from the only other study performed on fish cell lines (zebrafish liver cell line) (Yang and Chan, 2015) that showed much lower EC_{50} values than our results. This remarkable disparity could be explained by the absence of serum on their exposure media, a circumstance that we have also observed in preliminary studies on the cytotoxicity of TBBPA using an exposure media without FBS and obtaining comparable EC_{50} values (data not shown).

The different fish cell lines we have chosen represent three relevant potential targets for environmental pollutants in fish: gills (RTgill-W1), intestine (RTgutGC), and liver (RTL-W1). Our results indicate that, despite the similar trend, RTgill-W1 cell line was the most sensitive to TBBPA exposure, as confirmed by the EC_{50} values for both AB and CFDA-AM. This outstanding performance and sensitivity of RTgill-W1 cell line in cytotoxicity studies is well documented (Lee et al., 2009; Tanneberger et al., 2013; Yue et al., 2015) and further supported by their recent incorporation in a new OECD testing guideline for fish acute toxicity (OECD, 2021). The lower sensitivity of hepatic and intestine-derived cell lines could be related to the susceptibility of TBBPA to enzymatic detoxification mechanisms in fish (Nos et al., 2020; Shen et al., 2012). Thus, the higher biotransformation capacity of RTL-W1 and RTgutGC cell lines could explain our results, as they both exhibit higher cytochrome P450 enzymatic activity than RTgill-W1 (Stadnicka-Michalak et al., 2018).

Therefore, we selected the gill cell line to continue our studies. We have further analyzed the progression of cytotoxicity using a wide timing, from 2 to 72 h with different concentrations of TBBPA (Fig. 2). Our results indicate that the cytotoxic effects occur very fast, and there is a remarkable drop in viability after 8 h exposure with TBBPA ≥ 50 μM. In addition, the extended period for up to 72 h showed no changes when compared with the initial 24 h, suggesting that the cells that can cope with TBBPA exposure in the first hours are no further disturbed. Other experimental systems, such as mussel hemocytes (Canesi et al., 2005) and human erythrocytes (Jarosiewicz et al., 2021), have also reported fast effects after TBBPA treatments. In addition, our viability results with AB and CFDA-AM agree with Jarosiewicz et al. (2021) which describe plasma membrane disturbances after TBBPA treatments leading to changes in its fluidity and also in the cellular metabolic activity, measured as ATP levels.

3.2. TBBPA cytotoxicity is slightly modified by polystyrene nanoplastics

After establishing the basal cytotoxicity of TBBPA, we further investigated its effects upon combined exposure with PSNPs. To that end, we selected two non-cytotoxic concentrations of PSNPs (1 and 10 μg/mL, Fig. 3) to assess potential modifications of tetrabromobisphenol A effects on RTgill-W1 cells. These PSNPs concentrations have also shown low cytotoxicity in different mammalian (Domenech et al., 2020; He et al., 2020; Hesler et al., 2019; Kihara et al., 2021; Xia et al., 2008) and fish (Almeida et al., 2019; Jimeno-Romero et al., 2021) cell lines.

The characterization of PSNPs in our experimental conditions (Table 1, Fig. 4) indicated no relevant aggregation, with sizes below 100 nm even after 24 h incubation in the exposure media. The addition of a low percentage of FBS could have helped to avoid the formation of large aggregates, as has been previously suggested for other nanomaterials (Lammel and Sturve, 2018). The Z-potential value measured (-36.37 ± 1.19 mV) is consistent with the carboxylated surface coating of

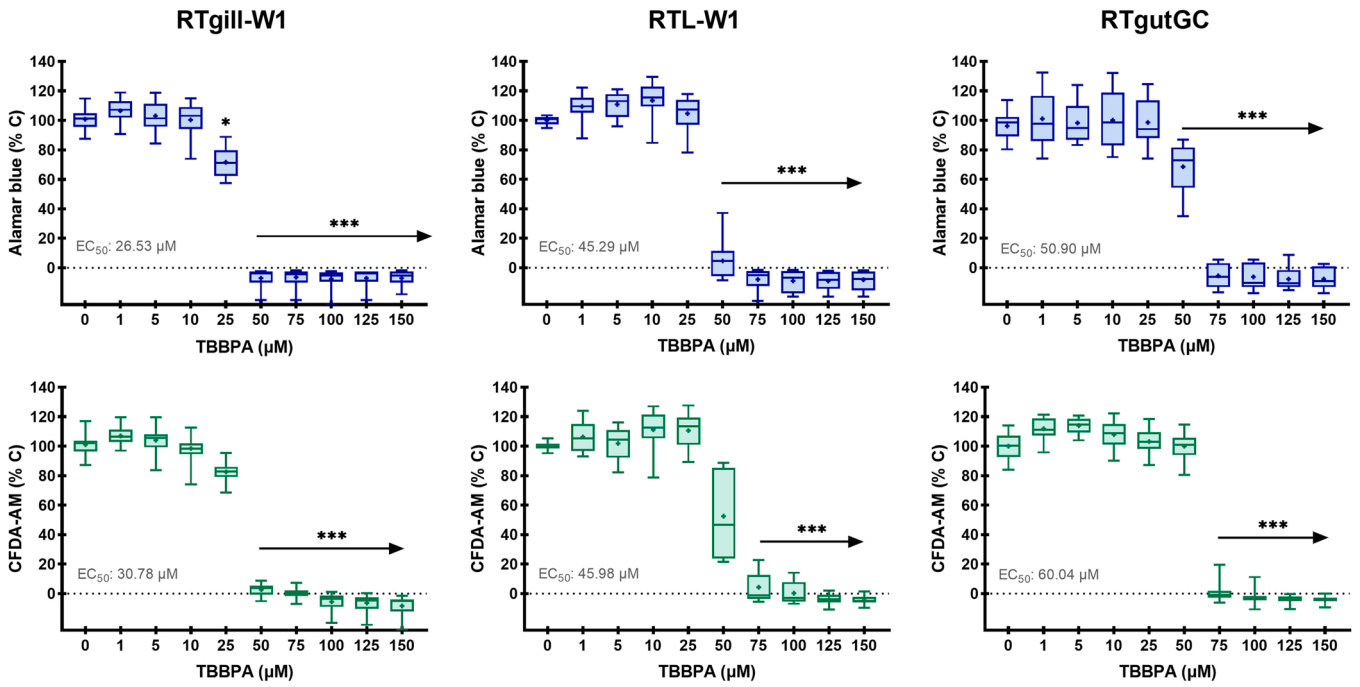

Fig. 1. Cytotoxicity of TBBPA upon fish cell lines. Metabolic activity (AB) and plasma membrane integrity (CFDA-AM) of RTgill-W1, RTL-W1, and RTgutGC cell lines after 24 h exposure to TBBPA. Box-plot representations show the mean (+), median (line), interquartile range (box limits), and maximum and minimum values (whiskers). n = 18, 3 independent experiments. Asterisks indicate statistically significant differences with untreated cells after Kruskal – Wallis + Dunn's tests or Brown-Forsythe and Welch ANOVA + Dunnett's T3 tests (AB RTgill-W1).

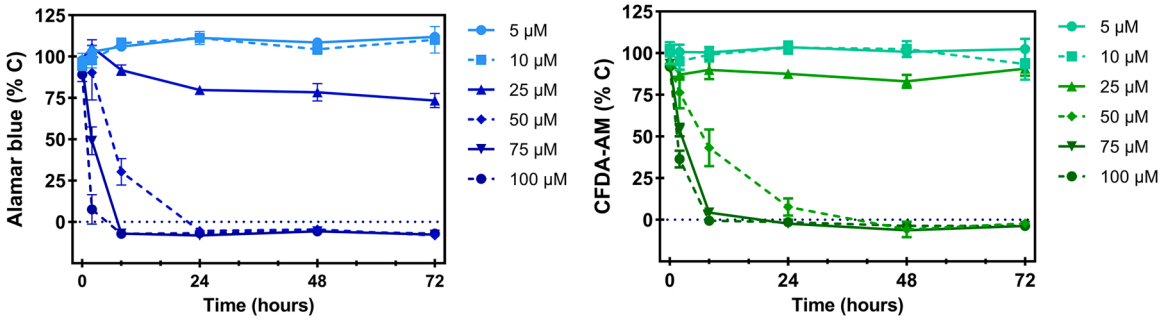

Fig. 2. Cytotoxicity timing of TBBPA on RTgill-W1 cell line. Values (mean ± SD) of metabolic activity by AB (left) and plasma membrane integrity by CFDA-AM (right) at different time points (2 – 72 h). n = 18, 3 independent experiments. For both parameters, statistically significant differences with control cells (two-way ANOVA mixed-effects model with Geisser-Greenhouse, and Tuckey's multiple comparisons test) were found with TBBPA concentrations ≥ 75 μM (2 h), ≥ 50 μM (8 h), or ≥ 25 μM (24, 48, 72 h). No asterisks were added to the graphs to improve clarity.

the nanoparticles that we have employed, and their behavior is equivalent to that determined by other authors in a variety of cell culture media (Hesler et al., 2019).

The joint exposure of low or non-cytotoxic TBBPA concentrations (5, 10, and 25 μM) with PSNPs (1 and 10 μg/mL) did not show a clear concentration-dependent pattern (Fig. 5). Nevertheless, statistically significant differences for AB and CFDA-AM were found between the exposure to TBBPA alone and almost all the combinations with PSNPs. Interestingly, even though similar results were obtained with the two concentrations of PSNPs, both cytotoxicity endpoints indicate higher toxicity for the combinations with PSNPs 1 μg/mL. Overall, the influence of polystyrene nanoparticles on TBBPA toxicity was slight, with no clear potentiation, synergistic, or antagonist effects. The sorption of the compound onto microplastics has been recently evaluated, reporting that hydrophobic partition and electrostatic interactions were the main driven forces (Li et al., 2021). If these same interactions were working on our experimental system, the negative charge of the PSNPs could minimize the potential adsorption of TBBPA.

There is a very limited amount of scientific literature addressing the combined exposure of TBBPA and other environmental pollutants, particularly micro and nanoplastics, and no clear consensus regarding their combined exposure has been reached. As an example, the study of Huang et al. using human intestinal cells indicates that the individual cytotoxicity of TBBPA showed no relevant differences when co-exposed with polyethylene microplastics (< 1000 μg/mL) (Huang et al., 2021). On the other hand, a recent study using commercial clams has reported a higher food safety risk when TBBPA was co-exposed with polystyrene microplastics, due to increased accumulation of TBBPA in these mollusks via the Trojan horse effect (Zhang et al., 2022). The disparities reported are difficult to evaluate due to the distinct experimental system utilized (whole marine organism vs human established cell line) and the different characteristics of the polystyrene employed (micro vs nano-sized). Nevertheless, other studies have described non-significant modifications in the toxicity of other environmental pollutants when jointly exposed to polystyrene microbeads (Bussolaro et al., 2019).

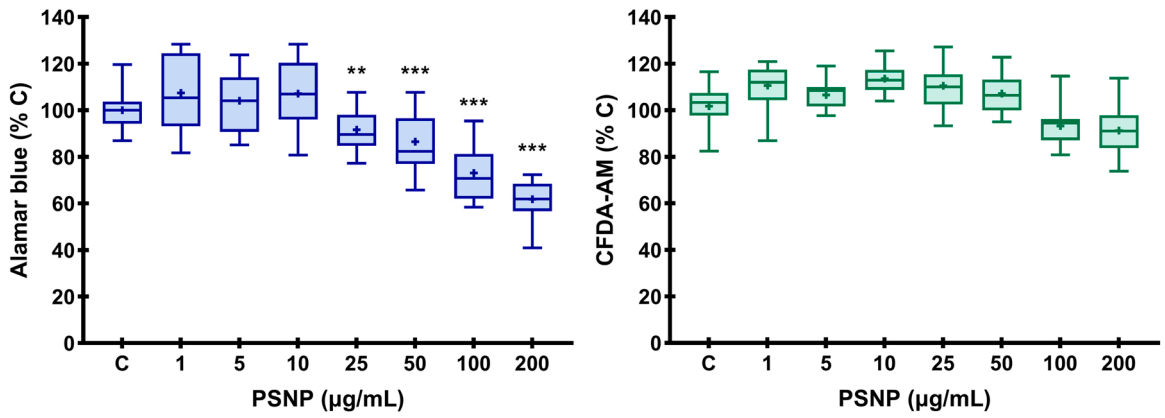

Fig. 3. Cytotoxicity of PSNPs on RTgill-W1 after 24 h exposure. Box-plot representations show the mean (+), median (line), interquartile range (box limits), and maximum and minimum values (whiskers). Asterisks indicate statistically significant differences with control cells ($p < 0.05$) after Brown-Forsythe and Welch ANOVA + Dunnett's T3 multiple tests.

Table 1
Size distribution of the PSNPs used in our experimental conditions. Hydrodynamic diameter distribution values obtained by DLS. Data shows mean and SD of n = 3.

Concentration	Solvent	t (h)	Size (nm)
10 mg / mL	H_2O	–	57.18 ± 22.23
1 µg / mL	L-15 + 2% FBS	0	72.35 ± 5.76
10 µg / mL	L-15 + 2% FBS	0	68.61 ± 3.64
1 µg / mL	L-15 + 2% FBS	24	84.09 ± 0.95
10 µg / mL	L-15 + 2% FBS	24	83.76 ± 0.94

3.3. Mitochondria are a relevant target for the coexposure of TBBPA and PSNPs

We selected relevant exposure conditions to analyze potential subcellular interactions of TBBPA and PSNPs. Two TBBPA concentrations (10 and 25 µM) and one of PSNPs (10 µg/mL) were chosen for that purpose, based on the previously presented results.

Mitochondria are the main intracellular source of ROS due to their intrinsic metabolic activities. Thereby, perturbations in metabolic functioning may lead to a redox imbalance and associated oxidative stress, one of the hallmarks of environmental insults (Peters et al., 2021). In this line, we assessed whether a specific disturbance of mitochondrial activity was related to the cytotoxic effects detected with joint exposure to TBBPA and PSNPs.

Fig. 6 shows the results obtained with H_2DCFDA to evaluate ROS production (Fig. 6A), and the perturbations of ΔΨ using TMRM fluorescence (Fig. 6B). Both endpoints presented a similar trend, where exposure to 10 µg/mL PSNPs alone showed no differences with control untreated cells. Similar results were obtained for 10 µM TBBPA exposure, either with or without PSNPs. For both endpoints, statistically significant results were only obtained after 24 h exposure to 25 µM TBBPA with or without 10 µg/mL PSNPs. These results indicate that TBBPA is the main factor determining the outcome for redox imbalance and mitochondrial inner membrane potential, undermining the influence of PSNPs co-treatment, even though exposure to nanomaterials has been extensively linked to oxidative damage of cellular structures (He et al., 2020).

Despite the difficulties to assess ROS production by TBBPA (Szychowski et al., 2016), this compound has been reported to stimulate ROS production and oxidative stress in peripheral blood mononuclear cells (Włuka et al., 2020) and endometrial cancer cells (as a result of NADPH oxidase activity) (Su et al., 2020). Other authors have described mitochondrial effects of TBBPA on human microglia (Bowen et al., 2020) and the lowering of mitochondrial inner membrane potential on human hepatocytes (Zhang et al., 2019), so our results with a fish gill cell line confirm a universal response after TBBPA exposure.

3.4. Oxidative DNA damage is induced by combined exposure of TBBPA and PSNPs

Finally, we used the comet assay to evaluate the genotoxic potential of individual and combined exposure of TBBPA and PSNPs on RTgill-W1 cell line. Classical alkaline assay (Fig. 7A) showed no statistically significant differences with control untreated cells, indicating that no single or double DNA strand breaks have been produced in our experimental system. On the other hand, the incubation with FPG (Fig. 7B) showed a significant increase in oxidative DNA damage only after the joint exposure to 25 µM TBBPA and 10 µg/mL PSNPs. Different enzymatic modifications of the comet assay have been proposed so far (Cordelli et al., 2021; Muruzabal et al., 2021). Among them, the FPG enzymatic treatment is particularly interesting, allowing to reveal the presence of the base 8-ox-7,8-dihydroguanine due to oxidative DNA damage. Although not routinely used, the comet assay has been successfully performed with fish cell lines (Žegura and Filipič, 2019). In RTgill-W1 cells the use of the classic alkaline version (Amaeze et al., 2015; Bussolaro et al., 2019; Zeng et al., 2016a, 2016b), as well as the FGP-modified protocol have been reported (Amaeze et al., 2015; Bussolaro et al., 2019; Kienzler et al., 2012), with different results on the performance of the assay. In our experimental conditions, the incorporation of the enzymatic treatment allowed us to describe a mild pro-oxidative effect, enhancing the sensitivity of the genotoxicity study.

Furthermore, our results are the first to show DNA oxidative damage after TBBPA exposure, either alone or in combination with other emerging contaminants. This injury could be derived from the redox imbalance and mitochondrial malfunctioning that we have previously described. Nevertheless, the fact that we have found positive results for oxidative DNA damage only with the combination of 25 µM TBBPA and 10 µg/mL PSNPs and not the individual TBBPA exposure suggests a novel combined effect that could not be detected by the rest of our experimental approaches.

The oxidative DNA damage revealed does not straightforwardly imply perturbation of the DNA, because the oxidized bases could be later repaired by different cellular mechanisms. Anyway, the fact that our combined exposure to TBBPA and PSNPs induces oxidative DNA damage to fish cells imposes an extra stressful condition, predisposing them to other potential mutagenic or carcinogenic stimuli.

4. Conclusions

In conclusion, our results indicate that the detrimental effects of

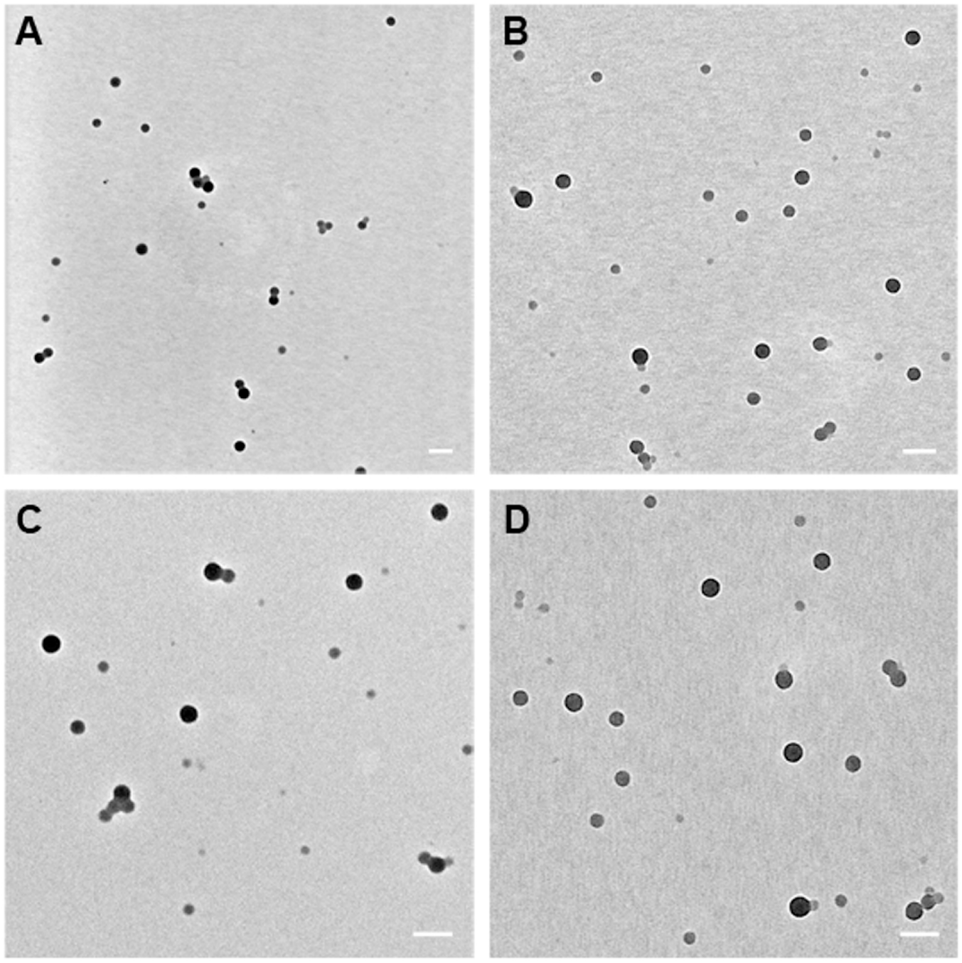

Fig. 4. TEM characterization of PSNPs. Representative images of PSNPs dispersions in exposure medium (L-15 + 2% FBS). **A**: 1 μg/mL freshly prepared; **B**: 10 μg/mL freshly prepared; **C**: 1 μg/mL after 24 h incubation at 37 °C; **D**: 10 μg/mL after 24 h incubation at 37 °C. Scale bar = 100 nm.

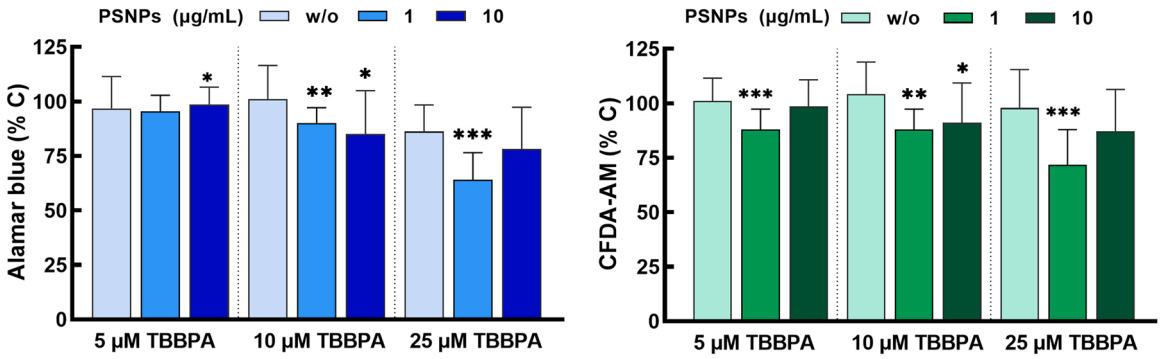

Fig. 5. Cytotoxicity of the combined exposure to TBBPA and PSNPs on RTgill-W1 cells for 24 h. Bar chart (mean ± SD) showing the effects of the combined exposure of different concentrations of TBBPA with 0, 1, or 10 μg / mL PSNPs for Alamar blue (left) and CFDA-AM (right). n = 18, 3 independent experiments. Asterisks indicate statistically significant differences between combined versus individual TBBPA exposures. Brown-Forsythe and Welch ANOVA (AB) / One way ANOVA (CFDA-AM 5 μM) + Dunnett's T3 tests, and Kruskal – Wallis + Dunn's tests (CFDA-AM 10 and 25 μM).

TBBPA on a rainbow trout gill cell line are only subtly modified by the co-exposition with PSNPs. The detection of oxidative DNA damage associated only with the combined exposure to subcytotoxic concentrations of TBBPA and PSNPs gives further credit to the potential environmental health risks related to the exposure to complex mixtures of pollutants.

Funding sources

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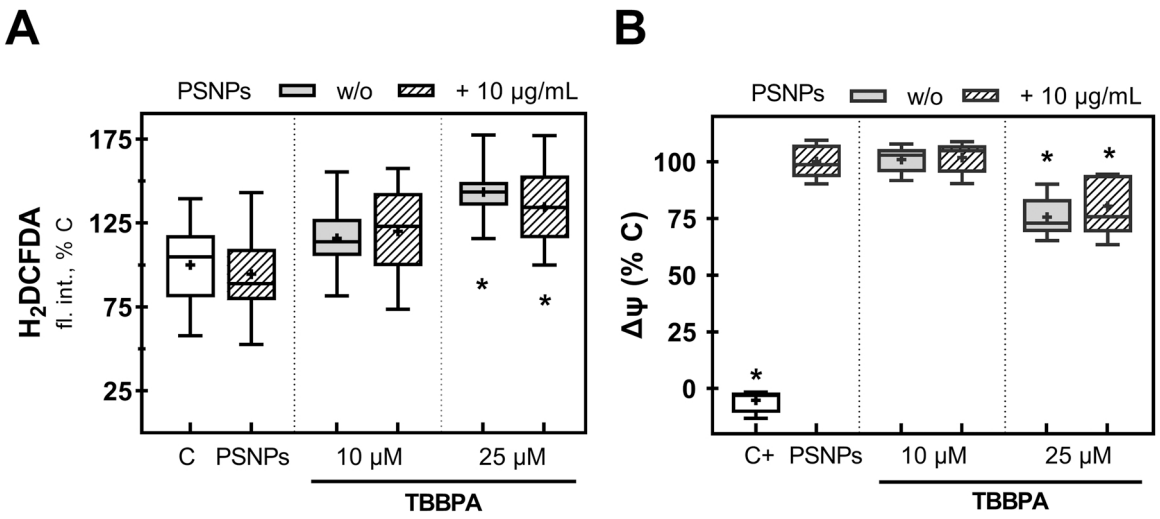

Fig. 6. Box-plot representation of ROS production (**A**) and mitochondrial membrane potential measured by TMRM fluorescence intensity (**B**) in RTgill-W1 cells after selected treatments with TBBPA and NPs, showing the mean (+), median (line), interquartile range (box limits), and maximum and minimum values (whiskers). CCCP was used as a positive control in TMRM measures. n = 3 independent experiments, * indicates statistically significant differences with control cells ($p < 0.05$, Brown-Forsythe and Welch ANOVA tests in A, and ordinary ANOVA in B).

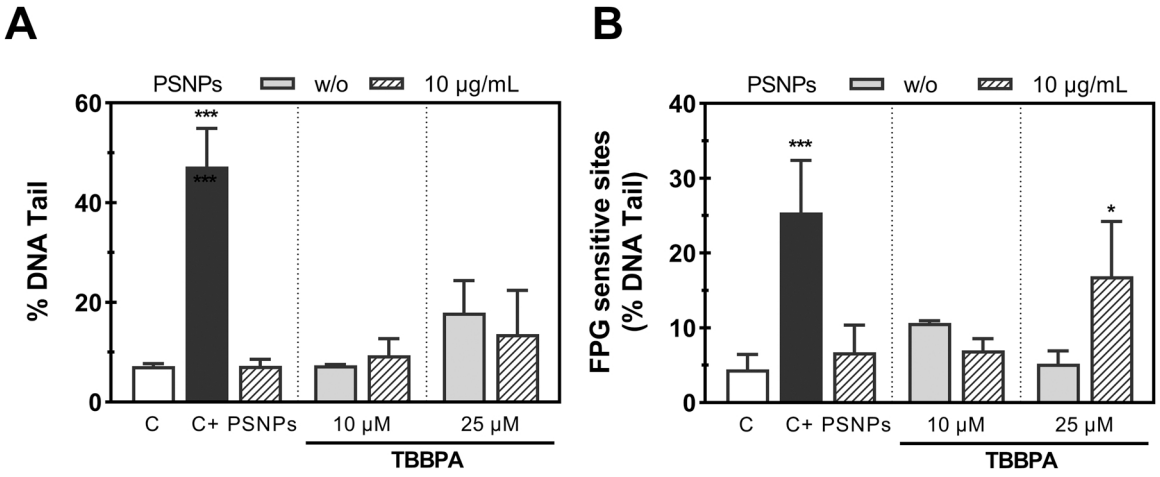

Fig. 7. Genotoxicity of RTgill-W1 cells exposed to TBBPA, PSNPs, or their combination during 24 h. Comet assay results showing % of DNA in tail after alkaline (**A**) or FPG procedures (**B**). Bar charts show the mean ± SD of three independent experiments. Asterisks indicate statistically significant differences with control cells ($p < 0.05$) after Brown-Forsythe and Welch ANOVA (A) or ordinary ANOVA (B) tests followed by Dunnett's multiple comparisons test.

CRediT authorship contribution statement

Conceptualization: PFF, MJH, AP. Data curation: IT, AP, PSB. Formal analysis: IT, PSB, PFF. Funding acquisition: PFF, MJH. Investigation: IT, PSB, AP. Methodology: PSB, IT, AP. Project administration: PFF, MJH. Resources: AP, PFF. Supervision: PFF, AP, MJH. Validation: PSB, AP. Visualization: PSB, AP, PFF. Writing: PFF, MJH, AP. Review & Editing: MJH, AP, PFF.

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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