Science of the Total Environment 1001 (2025) 180527

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Exploring the genotoxic potential of Bisphenol A and its emerging alternatives in an advanced *in vitro* 3D zebrafish hepatic cell model

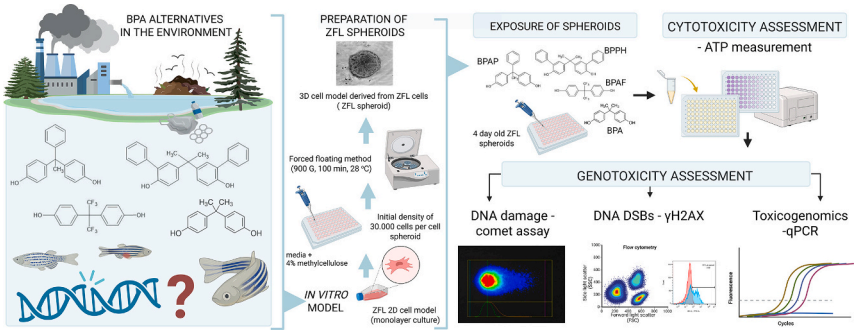
Alja Štern [a,b,*], Katja Kološa [a], Špela Rozman [b], Bojana Žegura [a,b]

[a] National Institute of Biology, Department of Genetic Toxicology and Cancer Biology, Večna pot 121, Ljubljana, Slovenia
[b] Biotechnical Faculty, University of Ljubljana, Jamnikarjeva ulica 101, 1000, Ljubljana, Slovenia

HIGHLIGHTS

- Zebrafish liver cell (ZFL) spheroids are a sensitive *in vitro* model for testing BPs.
- BPAF, BPAP, and BPPH showed higher cytotoxicity in ZFL spheroids than BPA.
- The tested BPs caused transient DNA damage but no DNA double-strand breaks.
- Toxicogenomics indicated induction of the TP53 pathway and NER/BER repair mechanisms.
- The deregulation hormone receptor genes indicated significant endocrine-disruptive potential.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
BPA
BPAF
BPAP
BPPH
Cytotoxicity
Genotoxicity
ZFL spheroids

ABSTRACT

With global restrictions on Bisphenol A (BPA), various BPA alternatives are increasingly found in ecosystems, raising concerns. This study focuses on the genotoxic potential of three emerging BPA alternatives — Bisphenol AF (BPAF), Bisphenol AP (BPAP), and Bisphenol PH (BPPH) — using an advanced *in vitro* 3D model system, spheroids, prepared from a Zebrafish (*Danio rerio*) liver cell line (ZFL). Their cytotoxicity was evaluated using the CellTiter-Glo® 2.0 assay, while their genotoxic potential was assessed using the comet assay, γH2AX assay, and toxicogenomic analysis. The BPA alternatives were more cytotoxic to ZFL spheroids than BPA. Non-cytotoxic concentrations caused transient DNA damage without a significant increase in DNA double-strand breaks (DSBs). The toxicogenomic analysis confirmed these findings, indicating activation of the TP53 DNA damage response pathway, the nucleotide excision repair (NER) and base excision repair (BER) mechanisms, likely in response to bulky DNA lesions and oxidative DNA damage. In addition, the gene expression analysis indicated the influence of the tested BPs on the endocrine system. Our results indicate that BPAF, BPAP and BPPH have considerable genotoxic potential and pose a significant ecotoxicological risk, underscoring the need for further investigation and careful consideration of these chemicals as BPA replacements.

* Corresponding author at: National Institute of Biology, Department of Genetic Toxicology and Cancer Biology, Večna pot 121, Ljubljana, Slovenia.
E-mail address: alja.stern@nib.si (A. Štern).

https://doi.org/10.1016/j.scitotenv.2025.180527
Received 11 April 2025; Received in revised form 7 July 2025; Accepted 15 September 2025
Available online 22 September 2025

1. Introduction

Since Bisphenol A (BPA) has been recognised as an endocrine disruptor and linked to various health issues in multiple organisms, regulatory measures have been implemented to reduce BPA exposure (COMMISSION DIRECTIVE 2011/8/EU, 2011; COMMISSION REGULATION (EU) 2018/213, 2018). Consequently, manufacturers have sought BPA alternatives, perceived as safer with a more favourable toxicity profile. However, emerging research indicates that many of these substitutes may exhibit similar or even more pronounced endocrine-disruptive and toxicological properties than BPA. In fact, most bisphenols are toxic, or even highly toxic, to aquatic organisms and have been found to exert adverse effects on the endocrine, metabolic, reproductive, and immune systems across various species (Adamovsky et al., 2024; Czarny-Krzymińska et al., 2023). Nevertheless, a plethora of BPA alternatives is already in widespread use and is entering ecosystems through industrial discharges, wastewater treatment plants, landfill leaching, and domestic waste. While BPA remains the dominant bisphenol (BP) detected in the environment, several environmental monitoring studies have detected BPA alternatives at varying levels, raising concerns about their ecological impact (Adamovsky et al., 2024). The use and occurrence of BPA alternatives in the environment are only expected to increase in the upcoming years, even surpassing BPA, as BPA and several other BPs, with known adverse effects, are being restricted further. European agencies such as the European Chemicals Agency (ECHA) and the European Food Safety Authority (EFSA) have recognised these concerns and have prioritised research on BPA alternatives (ECHA, 2018).

Among them, bisphenol S (BPS), bisphenol F (BPF), and bisphenol AF (BPAF) are the most common BPA substitutes for the production of epoxy resins and polycarbonate plastics (Bousoumah et al., 2021; Shamhari et al., 2021). BPS and BPF are also most frequently detected in the environment and are therefore also the most commonly studied in terms of toxicity and adverse effects (Adamovsky et al., 2024). Data on other BPA alternatives remain limited. The present study focuses on three less studied and environmentally highly concerning BPA alternatives: BPAF (4-[1.1.1.3.3.3-hexafluoro-2-(4-hydroxyphenyl)propan-2-yl]phenol), Bisphenol AP (BPAP; 4-[1-(4-hydroxyphenyl)-1-phenylethyl]phenol) and Bisphenol PH (BPPH; 4-[2-(4-hydroxy-3-phenylphenyl)propan-2-yl]-2-phenylphenol)(Fig. 1).

BPAF is detected in aquatic ecosystems with the third highest frequency (up to 15.3 μg/L in surface water and up to 155 ng/g dw in sediments) and, in addition, has the highest overall environmental persistence compared to other BPA alternatives (Chen et al., 2016). BPAP is detected less frequently (up to 56 ng/L in surface water and up to 2.3 ng/g dw in sediments); however, it is also less commonly included in monitoring studies (Adamovsky et al., 2024; Chen et al., 2016; Czarny-Krzymińska et al., 2023). There is limited data on the occurrence of BPPH in aquatic ecosystems in the literature. The available studies report no detection or detection of low concentrations up to 9.79 ng/L in water and < 1.18 ng/g dw in sediment samples (Huang et al., 2020; Wang et al., 2024; Zhang et al., 2019). However, quantitative structure−property relationship studies suggest that, among other BPs, this analogue might have one of the highest tendencies to adsorb into sediments and accumulate in tissues (Chen et al., 2016; Czarny-Krzymińska et al., 2023). This raises concerns about the potential environmental presence and ecological risk associated with these emerging BPA alternatives.

BPAF is structurally and functionally similar to BPA, with trifluoromethyl groups replacing BPA's methyl groups (Fig. 1). Because the substituent groups (CF_3) have higher biological activity than the methyl groups (CH_3), previous studies have suggested that BPAF, as a fluorinated BPA homologue, might exhibit greater toxicity than BPA (Shi et al., 2015; Zhu et al., 2020, 2024). It has been shown to exert higher endocrine-disrupting activity (Zhu et al., 2024) and genotoxic potential *in vitro* (Ding et al., 2017; Hercog et al., 2019; Mokra et al., 2017) and *in vivo* (Zhu et al., 2024) than BPA. Ecotoxicological studies have shown BPAF to be toxic to several aquatic cyanobacteria and algae species, affecting the morphology, mortality, and reproduction of *Daphnia magna*, the embryonic development of frogs, and inducing mortality, developmental effects, and estrogenic activity in zebrafish (*Danio rerio*) (Czarny-Krzymińska et al., 2023). However, there is still insufficient data on its hazardous effects for a reliable risk assessment and regulatory actions (ECHA, 2018).

In BPAP and BPPH, the methyl groups of BPA are replaced with one and two phenyl groups, respectively (Fig. 1), potentially increasing the biological activity of the two BPA alternatives. Only a few toxicological and ecotoxicological studies have been conducted on BPAP, and even fewer are studying BPPH. BPAP has previously been shown to induce stronger estrogenic and antiandrogenic effects compared to BPA (Xiao et al., 2018) and to be toxic for certain microalgae species (Czarny-Krzymińska et al., 2023). *In silico* studies on BPPH suggest that it might

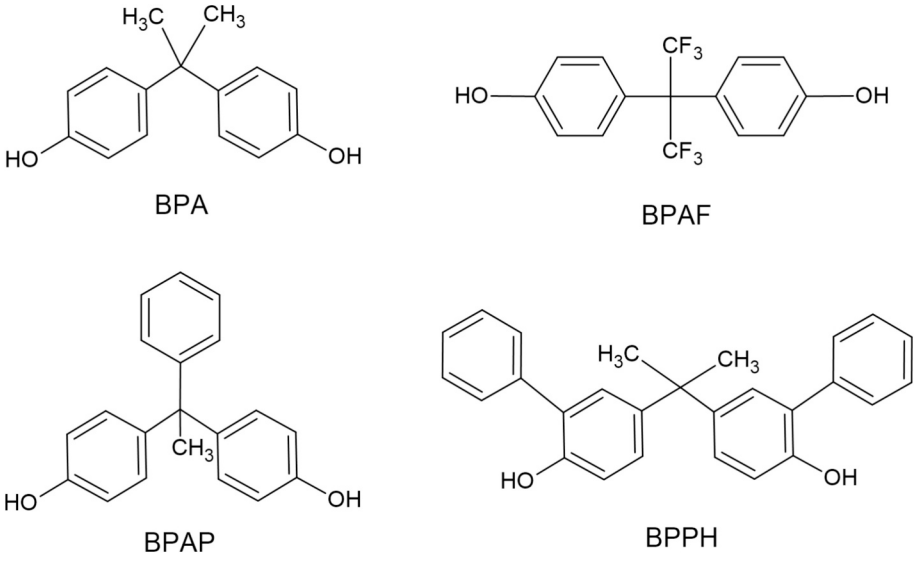


Fig. 1. Chemical structures od BPA and its alternatives BPAF, BPAP and BPPH. The chemical structures of Bisphenol A (BPA; 4,4′-(Propane-2,2-diyl)diphenol), Bisphenol AF (BPAF; 4-[1.1.1.3.3.3-hexafluoro-2-(4-hydroxyphenyl)propan-2-yl]phenol), Bisphenol AP (BPAP; 4-[1-(4-hydroxyphenyl)-1-phenylethyl]phenol) and Bisphenol PH (BPPH; 4-[2-(4-hydroxy-3-phenylphenyl)propan-2-yl]-2-phenylphenol).

induce severe adverse effects due to its potential reactivity with nuclear receptors, steroidogenic enzymes, and proteins involved in the pathogenesis of non-communicable diseases (Franko et al., 2024). However, there are no *in vitro* or *in vivo* data on its adverse effects. Considering the lack of information, research is urgently needed to characterise the potential ecotoxicological impact of these BPA alternatives.

The present study aimed to evaluate the potential genotoxic activity of the emerging BAP alternatives BPAF, BPAP and BPPH in comparison to the well-characterised BPA and assess the potential hazardous impact of these BPA alternatives on aquatic vertebrates. To achieve this, an advanced *in vitro* 3D model system (spheroids), prepared from zebrafish (*Danio rerio*) liver cells (ZFL), was applied. 3D models offer significant advantages over traditional 2D cultures by more accurately replicating *in vivo*-like cell morphology and function through enhanced cell–matrix interactions. This makes ZFL spheroids a highly relevant system for assessing the genotoxic impact of BPs on aquatic organisms (Štampar and Žegura, 2024). The cytotoxicity and genotoxicity of the tested BPA alternatives were evaluated using a comprehensive approach that included cell viability determination through ATP measurement with the CellTiter-Glo® 2.0 assay, assessment of DNA damage induction using the comet and γH2AX assays, and toxicogenomic analysis to gain insight into the cellular responses to BP exposure.

2. Materials and methods

2.1. BPA alternatives

BPA was from Sigma-Aldrich (St. Louis, MO, USA) and its alternatives (Table 1) were purchased from Chiron AS (Trondheim, Norway). The chromatographic purity of all tested BPs, as determined by LC-UV, was 99.9 %. Stock solutions of BPA and the tested alternatives were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0,5 M and stored at −20 °C.

2.2. Cultivation of ZFL cells and spheroids

Zebrafish liver cells, ZFL (American Type Culture Collection, CRL-2634, derived from normal adult zebrafish (Ghosh et al., 1994) were used in the experiments. Cells were grown at 28 °C in complete ZFL growth medium with the composition described in Table S1. Cells at passages between 4 and 13 were used to form the spheroids, which were prepared using the forced floating method (Štampar et al., 2019). Cells were initially seeded onto u-bottom 96-well plates (Avantor, Radnor Township, PA, USA) at 30,000 cells/well, in complete cell growth medium containing 4 % methylcellulose. The plates were centrifuged at 900*g* for 90 min, and the formed spheroids were left to mature for four days at 28 °C before the experiment.

Table 1
Information on BPA and its alternatives evaluated in the present study.

Chemical name (abbreviation)	Molecular formula	Mol. weight (g/mol)	IUPAC name	CAS number
Bisphenol A (BPA)	$C_{15}H_{16}O_2$	228.29	4-[2-(4-hydroxyphenyl) propan-2-yl]pheno	80-05-7
Bisphenol AF (BPAF)	$C_{22}H_{16}F_4O_2$	336.23	4-[1.1.1.3.3.3-hexafluoro-2-(4-hydroxyphenyl) propan-2-yl]pheno	1478-61-1
Bisphenol AP (BPAP)	$C_{24}H_{20}O_4$	290.36	4-[1-(4-hydroxyphenyl)-1-phenylethyl]phenol	1571-75-1
Bisphenol PH (BPPH)	$C_{27}H_{24}O_2$	380.48	4-[2-(4-hydroxy-3-phenylphenyl) propan-2-yl]-2-phenylphenol	24038-68-4

2.3. Cytotoxicity evaluation

Cytotoxicity of BPA and the tested BPA alternatives was evaluated using the CellTiter-Glo® 2.0 Cell Viability Assay (Promega, Madison, Wi, USA), a luminometric method for the measurement of ATP. ZFL spheroids were exposed to BPA (5–250 μM) and the BPA alternatives (5–200 μM) for 24 and 96 h. The assay was conducted in accordance with the manufacturer protocol, with minor modifications. Briefly, the spheroids in 50 μL of cell media were transferred to a white-opaque 96-well plate (Corning, Corning, NY, USA), and 50 μL of the assay reagent was added per well. The content of each well was thoroughly mixed by pipetting, and the reaction was incubated for 20 min at room temperature altogether. The resulting luminescence was measured using a luminometer (Sinergy HTX, BioTek, Winooski, VT, USA). The experiment was repeated three times independently, in five replicates per experimental point. A negative control (NC; cell medium), a solvent control (SC; 0.04 % DMSO), and positive controls (PC; 20 and 10 % DMSO, for 24 and 96 h of exposure, respectively) were included in the experiments.

2.4. The comet assay

Potential DNA damage induction after exposure to BPA and the tested BPA alternatives was evaluated using the alkaline comet assay. ZFL spheroids were exposed to BPA (50–200, and 10–100 μM for 24 and 96 h, respectively), BPAF (10–100, and 5–50 μM for 24 and 96 h, respectively), BPAP (10–100 μM for both time points) and BPPH (5–50 μM for both time points). After 24 and 96 h of exposure, a single cell suspension was prepared from the spheroids, and the comet assay was conducted (comet assay conditions are detailed in Table S2), as previously described (Štampar et al., 2019). Fluorescent microscopy analysis was performed using the software Comet Assay IV (Instem, Philadelphia, USA). In each experiment, a negative (NC; culture medium), a solvent (SC; 0.02 % DMSO) and a positive (PC; 30 μM benzo[*a*]pyren; BaP; Sigma-Aldrich, St. Louis, MO, USA) control were included.

2.5. The γH2AX focus assay

The induction of γH2AX foci, a strong biomarker for DNA double-strand breaks (DSBs), was determined using flow cytometry. ZFL spheroids were exposed to BPA, BPAF, BPAP and BPPH (concentration ranges as described for the comet assay). Etoposide (ET;10 μg/ml; Sigma, St. Louis, MO, USA) served as the positive control. After 24 and 96 h of exposure, spheroids were collected and dissociated into a single-cell suspension by trypsinisation (trypsin; Thermo Fisher Scientific, MA, USA) treatment. The prepared single-cell suspension was washed (1× PBS) and fixed in 4 % paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, USA). Cells were then washed again with 1× PBS, and anti-γH2AX pS139 antibodies were added (dilution 1:250; GENETEX, GTX637288 Lot: 44893) in 1 % BSA and 0.1 % Triton X-100 in 1× PBS for 30 min. After washing with 1× PBS, cells were labelled with a fluorophore-conjugated secondary antibody, goat anti-rabbit AlexaFluor 647 (Thermo Fisher Scientific, A-21244) diluted 1:1000 in 1 % BSA and 0.1 % Triton X-100, and incubated for 30 min at room temperature. To remove unbound secondary antibodies, cells were washed with 1× PBS and analysed using the MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotech, Germany). Data analysis was performed using FlowJo V10 (Becton Dickinson, New Jersey, USA).

2.6. Toxicogenomic analysis

The deregulation of selected genes by the tested BPs was analysed using specifically selected qPCR assays (Applied Biosystems, USA) (Table S3) and the microfluidic One 48.48 Dynamic Array IFC system (Fluidigm, USA). Four-day-old ZFL spheroids were exposed to BPA (50 and 200 μM), BPAF (25 and 100 μM), BPAP (25 and 100 μM) and BPPH

(10 and 50 μM). After 24 and 96 h of exposure, the spheroids were collected, and total RNA isolation was performed using the RNeasy® Mini kit (Qiagen, Germany). The quantification and qualification of the isolated RNA, followed by the reverse transcription using the Reverse Transcription Kit from Applied Biosystems, (MA, USA), the gene pre-amplification using TATAA PreAmp GrandMasterMix (Tataa Biocenter, Gothenburg, Sweden), and the gene expression analysis using TaqMan Universal PCR Master Mix and Taqman Gene Expression Assays detailed in Table S3, were performed as previously described by Štern et al. (Štern et al., 2024). A fold change greater than 1.5 (up/down-regulation - relative gene expression >1.5 or < 0.66, respectively) was considered biologically important.

2.7. Statistical analysis

Statistical analysis was performed using GraphPad Prism v10 (GraphPad Software, San Diego, CA, USA) on data sets from experiments that were repeated three times independently, including technical repetitions. Before statistical analysis, data sets were tested for normality (Shapiro-Wilk for all sample sets and Kolmogorov-Smirnov for flow cytometry data) and heteroscedasticity (Spearman's test). One-Way Analysis of Variance (ANOVA) and Holm-Šídáks's multiple comparison test and nonlinear regression (agonist *vs.* normalised response) were applied for determining statistically significant differences in cell viability between samples, and the calculation of half-maximal inhibitory concentrations (IC_{50}) and concentrations that reduce cell viability for 30 % (IC_{30}), respectively. The data set included at least three technical repetitions per experimental point ($n \geq 9$). The Kruskal–Wallis nonparametric test and Dunn multiple comparison test were performed for the analysis of the comet assay data (a minimum of 50 nuclei per experimental point; $n \geq 150$). A Mixed-effects analysis and Holm-Šhídák's multiple comparison test using median values from a minimum of 5000 cells per experimental point was performed on the data from the γH2AX focus assay ($n = 3$). ANOVA and Dunnett's multiple comparison test were used to evaluate statistically significant differences in gene deregulations in the data (average from two technical repetitions, n = 3) from the toxicogenomic analysis.

3. Results and discussion

Despite limited toxicological data, BPA alternatives are being increasingly used due to BPA restriction measures and are increasingly detected in the environment, raising concerns about their ecological impact. This study explores the genotoxic potential of three emerging BPA alternatives, BPAF, BPAP, and BPPH, in ZFL spheroids, prepared from Zebrafish (*Danio rerio*) liver cells (ZFL). Fish cell lines are frequently used in aquatic toxicology research for assessing toxic and genotoxic impacts, providing a promising alternative to animal testing in initial ecotoxicological evaluations (Žegura and Filipič, 2019). Furthermore, as an advanced *in vitro* model, the ZFL spheroids provide significant advantages over traditional monolayer cultures. There is growing recognition that cultivating cells in a three-dimensional (3D) environment more accurately replicates normal cellular functions, bridging the gap between *in vitro* and *in vivo*. This is due to enhanced interactions between cells and their surrounding matrix, resulting in cell morphology and physiology more similar to that of cells *in vivo,* mimicking the natural structure of tissues (Bloise et al., 2024; Rodd et al., 2017; Urzì et al., 2023). Thus, applying ZFL spheroids as a model system in the present study allows us to generate highly relevant data on the potential hazardous impact of BPAF, BPAP and BPPH on the aquatic ecosystem.

3.1. Cytotoxicity of the BPA alternatives BPAF, BPAP and BPPH in ZFL spheroids

The cytotoxicity of BPAF, PBAP and BPPH was evaluated to assess their toxicity profiles compared to BPA in the ZFL spheroid test system and to identify non-cytotoxic concentrations ($\leq IC_{30}$) suitable for subsequent genotoxicity testing. Identifying non-cytotoxic concentrations is crucial, as cytotoxic levels may produce false-positive results in genotoxicity assessments (Azqueta et al., 2022). Our results showed that BPAF, BPAP and BPPH exhibited higher cytotoxic activity than BPA (Fig. 2). Among the tested BPA alternatives, BPPH was the most cytotoxic, with IC_{50} values of 76.47 ± 18.90 μM (24 h) and 61.98 ± 10.67 μM (96 h), followed by BPAF with IC_{50} values of 135.35 ± 13.21 μM (24 h) and 78.22 ± 15.71 μM (96 h). BPAP was the least cytotoxic among the tested BPA alternatives; however, its IC_{50} values were still approximately 1.5 times lower (164.73 ± 7.00 μM and 117.76 ± 30.15 μM after 24 and 96 h, respectively) compared to the IC_{50} values for BPA (256.37 ± 18.43 μM and 165.75 ± 15.56 μM after 24 and 96 h, respectively).

In line with the observed changes in cell viability (Fig. 2), the exposure to the tested BPs also induced notable morphological changes in ZFL spheroids (Fig. 3). With increasing BP concentration, the spheroids became darker, and their surface became rough, with cells detaching from the spheroid structure. At concentrations above IC_{50} the spheroids became loose, and their structure collapsed.

There is limited data on the cytotoxicity of the studied BPA alternatives *in vitro* and *in vivo*, particularly in fish cell models. The IC_{50} values obtained in this study are 2–3 times higher than those previously reported in human cell lines in 2D culture (60.8–7.1 μM for BPAF and 62.0–3.6 μM for BPAP) (Rifa and Lavado, 2024). Nevertheless, Sendra et al. (Sendra et al., 2024) recently reported that SH-SY5Y cells grown in conventional 2D cultures are much more sensitive to BP-induced toxicity compared to the 3D model, which may be due to differences in drug diffusivity, drug action mechanisms, and cell proliferation capabilities (Bloise et al., 2024). In support of this, no significant cytotoxicity of BPA, BPAF and BPAP was reported in a human hepatic 3D model, HepG2 spheroids, following 24 h and 96 h of exposure to concentrations up to 160 and 80 μM, respectively (Sendra et al., 2023; Štampar et al., 2023). On the other hand, reported LC_{50} values for BPAF and BPAP after 96 h of exposure in zebrafish embryos were significantly lower, 1.16–1.95 mg/L (3.45–5.80 μM) and 1.27 mg/L (3.89 μM) (Gao et al., 2022; Mu et al., 2018), indicating that fish embryo models are much more sensitive to BP exposure than *in vitro* cell models. Generally, BPAF is reported to be more toxic than BPAP, and both alternatives are much more toxic than BPA (Gao et al., 2022; Rifa and Lavado, 2024; Sendra et al., 2024), which is in line with our findings. To our knowledge, no cytotoxicity data for BPPH can be found in the literature.

3.2. Genotoxicity of the BPA alternatives BPAF, BPAP and BPPH in ZFL spheroids

For the assessment of potential genotoxic effects induced by BPAF, BPAP and BPPH in ZFL spheroids, DNA damage and DNA double-strand break (DSB) induction were investigated using the comet assay and the γH2AX assay, respectively. To gain insight into the cellular stress response and response to genotoxic stress after exposure to the tested BPs toxicogenomic analysis was performed.

The comet assay primarily detects single-strand breaks (SSBs) in DNA, but also detects DSBs, alkali-labile sites, apurinic/apyrimidinic sites, DNA-DNA and DNA-protein cross-links, as well as SSBs linked to incomplete excision repair (Collins, 2014). Our results show that all three tested BPA alternatives (at the highest tested concentrations) significantly increased DNA damage in ZFL spheroids after 24 h of exposure (Fig. 4). The lowest observed adverse effect concentration (LOAEC) for BPAP and BPPH was 50 μM, with the latter exhibiting slight cytotoxicity at this concentration. For BPAF, the LOAEC was determined to be 100 μM. BPA on the other hand, induced the most pronounced increase in DNA damage with a LOAEC of 200 μM. Notably, the LOAEC of the tested BPA alternatives was lower than that of BPA, indicating a higher genotoxic potential of BPAF, PBAP and BPPH compared to BPA.

The DNA damage detected after exposure to the tested BPs appeared

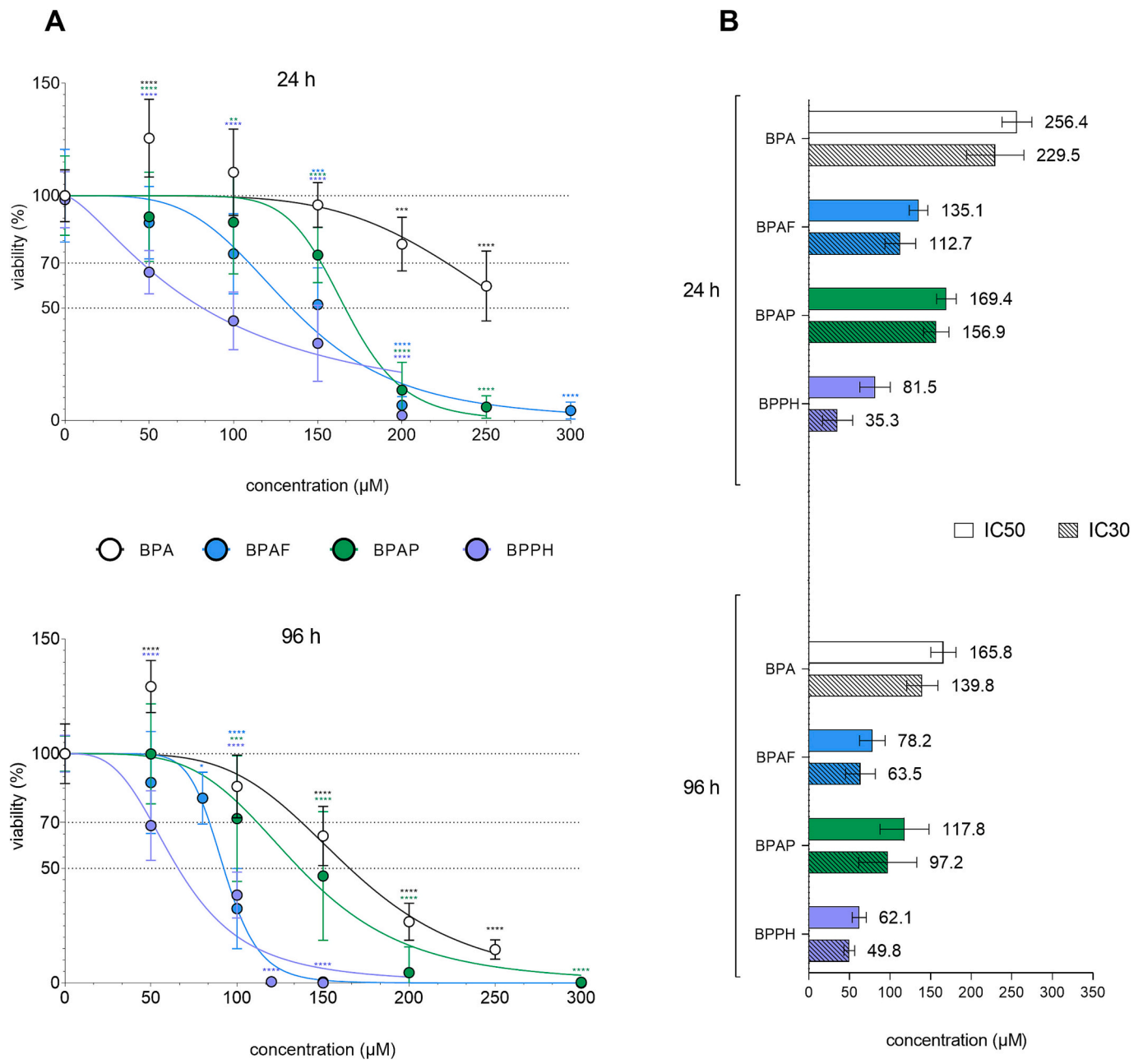


Fig. 2. Cytotoxicity of BPA, BPAF, BPAP and BPPH in ZFL spheroids after 24 and 96 h. Cell viability was determined using the CellTiter-Glo® 2.0 Cell Viability Assay (Promega). (A) Cell viability is presented as the percentage of the solvent control (0; 0.04 % DMSO). The positive control (20 and 10 % DMSO for 24 and 96 h, respectively) performed as anticipated and reduced cell viability to 7.2 ± 10.2 and 11.3 ± 8.9 after 24 and 96 h of exposure, respectively (data not shown in Figure). Asterix denote statistically significant differences (ANOVA and Holm-Šídáks's test) between the samples and 0. ***$p \leq 0.001$, ****$p \leq 0.0001$. (B) Half-maximum inhibitory concentrations (IC_{50}) and inhibitory concentrations, which reduced the cell viability for 30 % (IC_{30}), were calculated using nonlinear regression (log (agonist) *vs.* normalized response—variable slope).

to be largely transient and reversible, as no persistent DNA damage was observed after 96 h of exposure. The exception was BPAP at the highest tested concentration (100 μM), which was borderline cytotoxic and might have contributed to the observed DNA damage through cytotoxicity-related mechanisms. Transient DNA damage, likely reflected initial DNA damage and/or oxidative DNA damage induction and active repair processes (Azqueta et al., 2014). By 96 h, a successful repair might have resolved the damage, antioxidant defences diminished oxidative stress and cells with irreparable damage could have undergone apoptosis. Another plausible explanation for the transient effects is cellular adaptation to BP exposure, through the upregulation of xenobiotic metabolising and detoxifying enzymes, which could have facilitated the breakdown and excretion of the tested BPs, thereby reducing their intracellular concentration over time (Pritchett et al., 2002).

To evaluate whether the tested BPs also induced DSBs, which are considered the most deleterious type of DNA damage, the phosphorylated form of histone H2AX (γH2AX) was quantified using flow cytometry. The phosphorylation of H2AX, one of the earliest cellular responses to DSBs, is rapid and abundant and therefore serves as a reliable biomarker of DSB induction (Kopp et al., 2019). The results of the γH2AX assay showed no statistically significant induction in DSBs after 24 or 96 h, except after 24-h exposure to the highest tested concentration of BPA (200 μM) and the highest tested concentration of BPAP (100

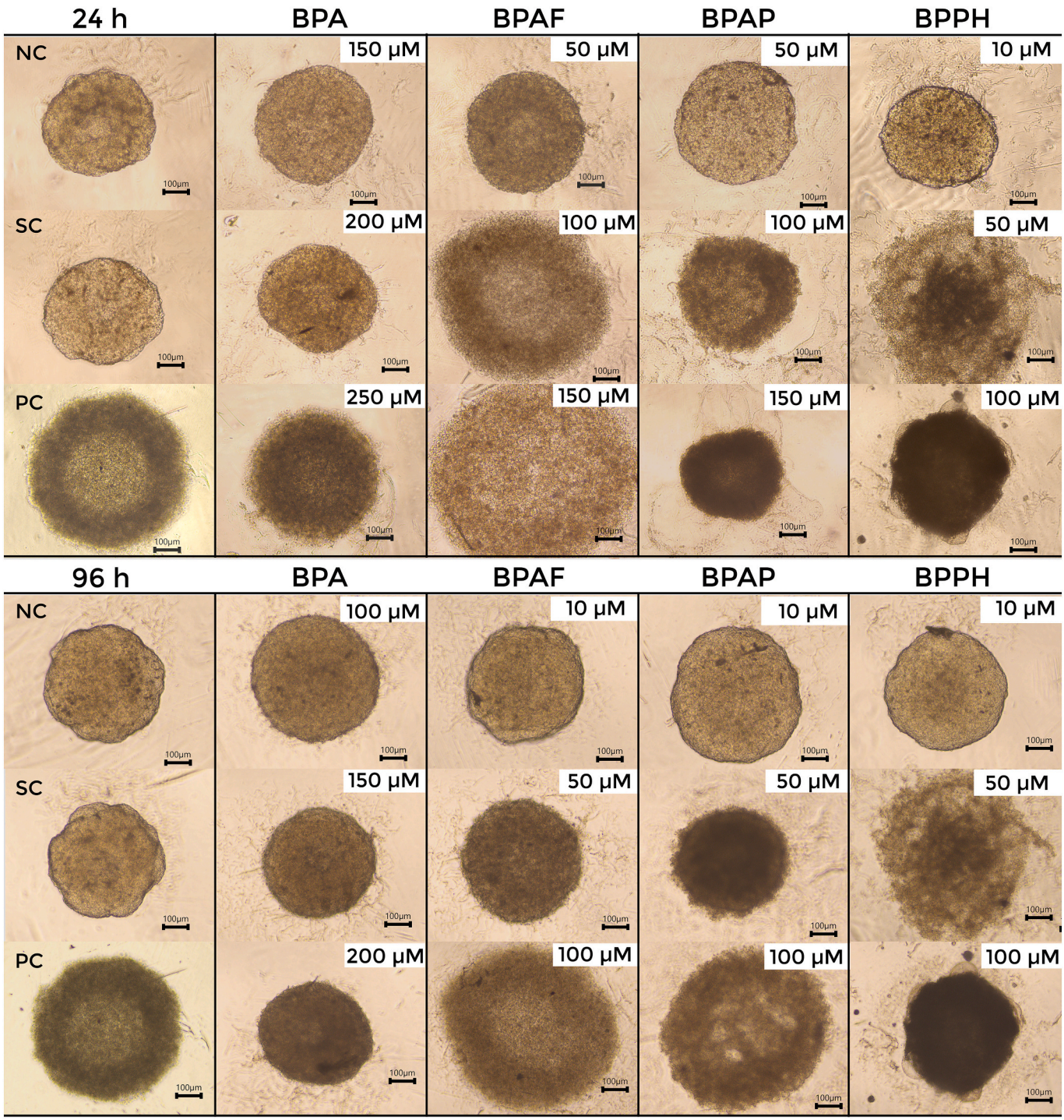


Fig. 3. Morphological changes of ZFL spheroids exposed to BPA, BPAF, BPAP and BPPH for 24 and 96 h. The micrographs were captured using a Nikon Eclipse Ts2R microscope and a BRESSER MikroCam II camera at 4× magnification. NC: negative control (growth medium), SC: solvent control (0.1 % DMSO), PC: positive control (10 % DMSO). The scale in each micrograph represents 100 µm.

µM) (Fig. 5). BPAF (100 µM) slightly elevated H2AX phosphorylation after 24 h of exposure; however, the increase was not statistically significant. These results together indicate that the tested BPA alternatives have a higher genotoxic potential than BPA, and the most potent among them is BPAP.

There is limited data on the genotoxic potential of BPAF and BPAP in aquatic vertebrates. BPPH is the least studied of the tested BPA alternatives, with no genotoxicity data currently reported in the literature. To date, only a few *in vitro* and *in vivo* studies have investigated genotoxicity endpoints induced by BPAF and BPAP, primarily using mammalian test systems involving human or rodent models. BPAF has been shown to induce DNA damage in human peripheral blood monocytes (Mokra et al., 2018), HepG2 cells (Hercog et al., 2019; Sendra et al., 2023), mouse oocytes (Ding et al., 2017), and V79 cells (Pfeiffer et al., 1997), generally indicating a greater genotoxic potential than BPA. BPAF and BPAP (10 µM) have also been reported to induce DSBs in HepG2 spheroids after 96 h of exposure (Sendra et al., 2023). In contrast, no significant increase in cells with chromosomal aberrations

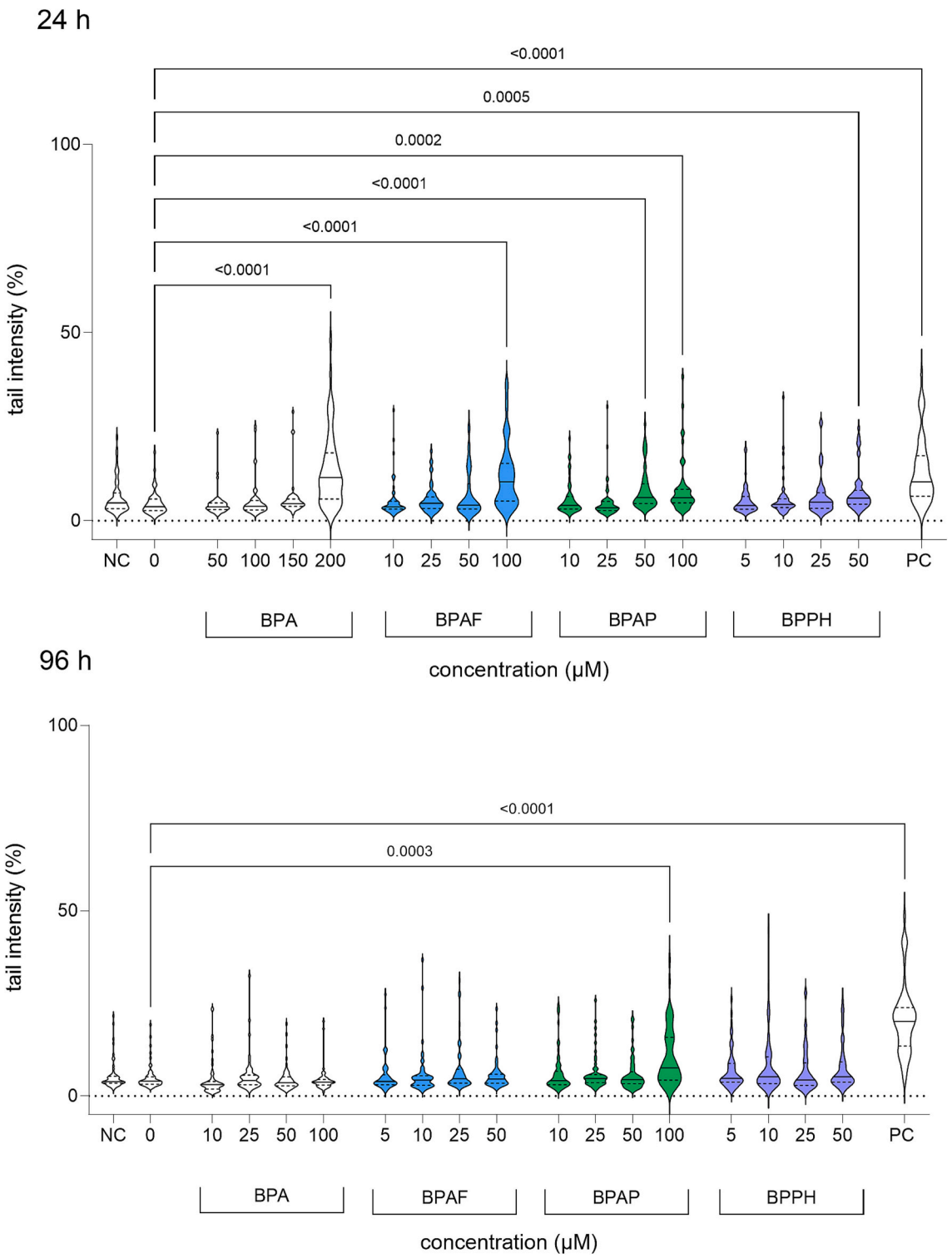


Fig. 4. DNA damage induction in ZFL spheroids after exposure to BPA, BPAF, BPAP and BPPH, for 24 and 96 h, detected with the comet assay. Data are expressed as % of tail DNA and presented as violin plots (with 95 % confidence interval). The solid horizontal line indicates the median, while the dotted upper and lower lines represent the first (25th percentile) and third quartiles (75th percentile). NC: negative control (growth medium), 0: solvent control (0.02 % DMSO), PC: positive control (30 μM BaP). A statistically significant difference (Kruskal–Wallis and Dunn's test) between the 0 and the exposed cells is indicated by *p* values.

or γH2AX foci formation was observed in DT40 cells—either wild-type or homologous recombination-deficient (RAD54$^{-/-}$) mutants—following exposure to BPAF and BPAP (Lee et al., 2013). On the other hand, a recent *in vivo* study demonstrated DNA damage and micronuclei induction by BPAF in male rats, highlighting a similar transient nature of genotoxic damage as observed in the present study, with the damage being repaired after a recovery period (Zhu et al., 2024).

To further elucidate the potential genotoxicity of the tested BPs, toxicogenomic analyses were conducted to identify changes in gene expression associated with (geno)toxicological outcomes. Gene expression profiles have been suggested to be a valuable tool in risk assessments, providing insights into the specific molecular mechanisms

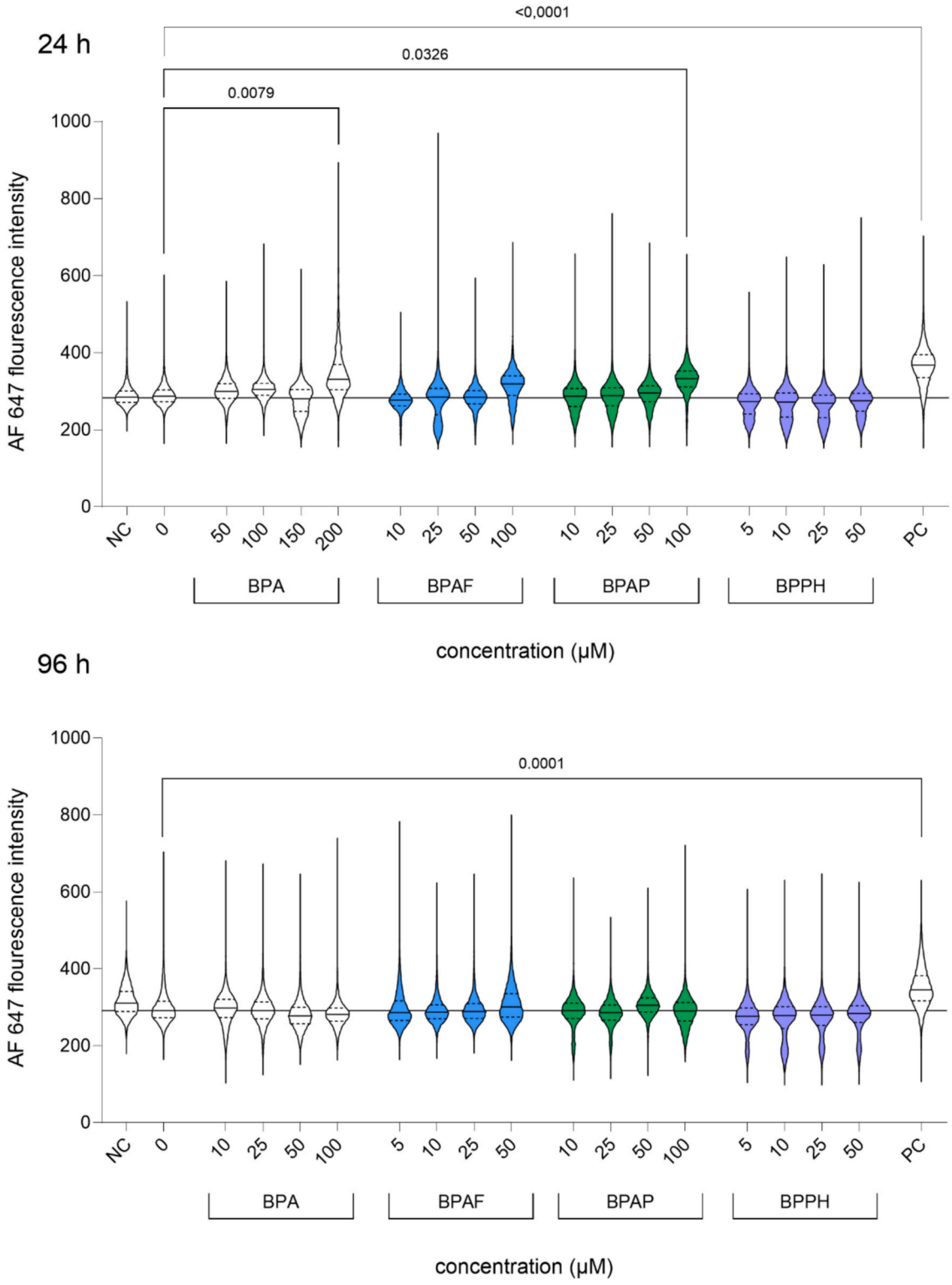


Fig. 5. DNA double-strand break (DSB) induction in ZFL spheroids after exposure to BPA, BPAF, BPAP and BPPH for 24 and 96 h. Phosphorylation of H2AX (γH2AX), a marker of DSBs, was determined using flow cytometry. The γH2AX AF 647 fluorescence intensity of individual cells is presented as violin plots (95 % confidence interval). The solid horizontal line indicates the median, while the dotted upper and lower lines represent the first (25th percentile) and third quartiles (75th percentile). NC: negative control (growth medium), 0: solvent control (0.02 % DMSO), PC: positive control (10 μg/ml ET). Statistically significant differences (Mixed-effects model and Holm-Šhídák's tests using median values) between the solvent control and the BP-exposed cells are indicated by p values.

triggered by test compounds (Corvi and Madia, 2016). This study evaluated the expression of selected DNA damage-responsive genes (DDR genes; *tp53*, *gadd45a*, *mdm2*, *ogg1*, *xrcc5* and *ercc3*) and genes involved in apoptosis (*casp2*, *baxa*, and *bcl2*) in ZFL spheroids following exposure to the tested BPs. In addition, genes involved in hormone signalling (*gnrh2*, *ar*, *esr1*, *esr2a*, *esr2b*, *vtrg4*) were included to confirm the activation of the primary mechanism of action of the tested BPs in the applied test system. For each BP, two concentrations were included

in toxicogenomic analysis: the highest non-cytotoxic concentration for each time point (24 and 96 h) and an additional concentration corresponding to the no observed adverse effect concentration (NOAEC).

Our results show that gene deregulation was more pronounced after 24 h of exposure, compared to the later time point, 96 h (Fig. 6, Table S4 and Table S5), which further confirms the transient nature of the effects induced by the studied BPs. The expression of several genes exhibited more than 1.5-fold change, which is considered a biologically important alteration. Few changes were also statistically significant; nevertheless, deregulation patterns could be observed, indicating the triggering of cellular responses to BP exposure.

The deregulation patterns of the analysed DDR genes indicate that the TP53 pathway was triggered after 24 h of exposure to the tested BPs, probably in response to the initial DNA damage, which was observed in the comet assay. Once DNA damage is detected, TP53 activates a series of downstream targets that lead to cell cycle arrest, DNA damage repair, and other survival pathways, as well as the removal of damaged cells (Helton and Chen, 2007; Hollander et al., 1999). MDM2 regulates TP53 activity and degradation, while GADD45A plays an important role in DNA damage repair and cell cycle control (Hollander et al., 1999). BPA (200 μM) significantly upregulated *gadd45a* and *mdm2*, and increased *tp53* expression by more than 1.5-fold, though not statistically significantly (NS). After 96 h, only *gadd45a* (200 μM) was significantly upregulated. BPAF at 100 μM significantly upregulated *mdm2* and increased the expression of *tp53* and *gadd45a* by over 1.5 times (NS). At 25 μM, BPAF increased *tp53* and *gadd45a* expression by over 1.5 times (NS). BPAP at tested concentrations did not significantly deregulate genes after 24 h, but slightly upregulated *gadd45a* (NS). BPPH caused minimal DDR gene deregulation, with only *mdm2* statistically significantly upregulated after 24 h at 50 μM. No significant changes in DDR gene expression were observed after 96 h of exposure to the tested BPA alternatives.

Interestingly, all tested BPs induced at least a slight upregulation of *ercc3*. The *ercc3* gene encodes an ATP-dependent helicase (XPB), involved in nucleotide excision repair (NER) (Fan et al., 2006). NER is a highly conserved and versatile DNA repair mechanism that addresses a wide range of bulky, helix-distorting DNA lesions, including bulky chemical adducts caused by exposure to various chemicals, intrastrand crosslinks (often induced by chemotherapeutic agents), and oxidative DNA lesions (Fan et al., 2006; Marteijn et al., 2014). The positive control, benzo(*a*)pyrene (BaP), which is known to induce bulky DNA adducts repaired by NER (Henkler et al., 2012), upregulated the expression of *ercc3* by 2.16 ± 0.74 times (NS). In addition, the exposure to the highest test concentration of BPA (200 μM), BPAF (50 μM) and BPAP (100 μM) for 24 h upregulated *ogg1* (≥ 1.5 times; NS), which encodes a protein crucial for base excision repair (BER), that repairs oxidative DNA damage. Specifically, OGG1 is involved in the excision of 8-oxoguanine (8-oxoG), the most abundant type of oxidative DNA damage (Kanvah et al., 2010). On the other hand, exposure to the tested BPs did not influence *xrcc5* expression. This gene encodes a protein involved in the non-homologous end joining (NHEJ) pathway of DNA repair that binds to and initiates DSB repair (Gorre et al., 2014).

Improperly repaired DNA lesions can cause mutations, whereas excessive, unrepaired damage may trigger the TP53 pathway to induce

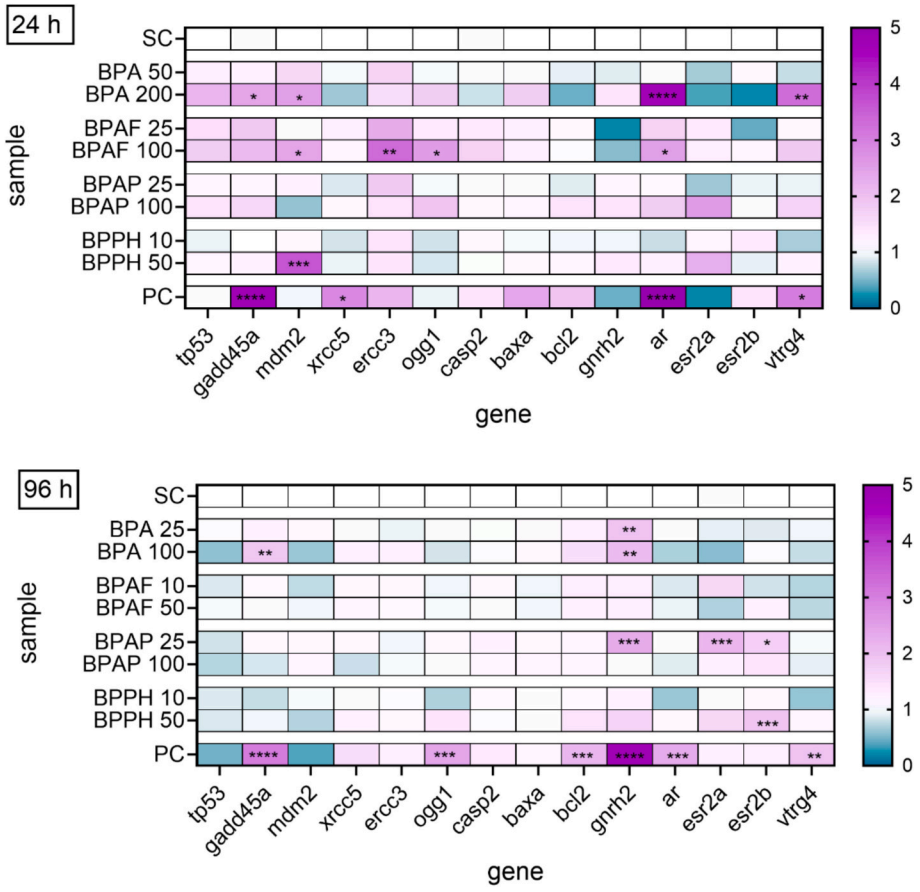


Fig. 6. Deregulation of selected DNA damage-response genes, genes involved in apoptosis induction and the endocrine system, after exposure to BPA and its alternatives BPAF, BPAP and BPPH for 24 and 96 h. Fold change of gene expression of the solvent control (SC; 0.02 % DMSO) is presented as heatmaps. NC: negative control (growth medium), PC: positive control (30 μg/ml BaP). SC fold-change value is set to 1 (white colour), down-regulation (cyan), up-regulation (magenta). The asterisks denote statistically significant differences between the test sample and the SC (Ordinary two-way ANOVA and Fisher's LSD multiple comparison test), * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

apoptosis, in order to remove damaged cells from the population and suppress cancer development (Mu et al., 2018). To investigate whether the chosen non-cytotoxic concentrations of the tested BPs induced apoptosis in the exposed ZFL spheroids, pro-apoptotic (*baxa* and *casp2*) and anti-apoptotic (*bcl2*) genes were included in the toxicogenomic analysis. The results showed that BPA (200 μM) slightly upregulated the pro-apoptotic gene *baxa* and downregulated the anti-apoptotic gene *bcl2* (by ≥1.5, NS), indicating possible apoptosis induction after 24 h of exposure. However, none of the genes involved in apoptosis regulation were significantly deregulated upon 24 and 96 h exposure to the tested BPA alternatives, except for a slight upregulation of *casp2* following 24 h of exposure to BPAF (100 μM).

In addition to genotoxicity-related cellular responses, our gene transcription analysis revealed alterations in hormone signalling pathways, consistent with the known endocrine-disrupting activity of BPs (Fig. 6, Table S4 and Table S5). These findings are not only relevant in confirming the responsiveness of our test system but also provide mechanistic insight, as endocrine-disrupting activity has been linked to secondary genotoxic effects through oxidative stress, disruption of cell cycle control, and modulation of DNA repair mechanisms (Bukato et al., 2024; Jiménez-Salazar et al., 2021; Yedidia-Aryeh and Goldberg, 2022). Therefore, while the primary focus of this study is genotoxicity, the observed endocrine-related transcriptional changes support the plausibility of indirect genotoxic mechanisms and highlight the multifaceted toxicity profile of the tested BPs.

Our results confirmed the deregulation of several genes involved in hormone signalling following exposure to the tested BPs (Fig. 6, Table S4 and Table S5). The genes *gnrh2*, *ar*, *esr1*, *esr2a* and *esr2b* encode for the receptors GNRH2, AR, ERα, ERβ2 and Erβ1, involved in hormone signalling. GNRH2 (gonadotropin-releasing hormone 2 receptor) is involved in the regulation of reproductive hormones (Marvel et al., 2021), AR (androgen receptor) binds androgens, such as testosterone, and is crucial for the development and maintenance of male characteristics (Hossain et al., 2008), and the estrogen receptors ERα (estrogen receptor alpha), ERβ2 (estrogen receptor beta 2) and Erβ1 (estrogen receptor beta 1) bind estrogens and have profound roles in reproductive and other physiological processes (Menuet et al., 2004). The tested BPs deregulated the selected genes from the endocrine system at both exposure time points. While *gnhr2* was not significantly deregulated after 24 h, except for following exposure to BPAF, which induced ≥1.5-fold downregulation of this gene at both tested concentrations. Following 96 h of exposure to BPA (50 and 200 μM), BPAP (25 μM) and BPPH (50 μM), *gnhr2* was statistically significantly upregulated by ≥1.5-fold. The androgen receptor gene, *ar* was upregulated only after 24 h, most notably by BPA (200 μM) and BPAF (100 μM). It was also upregulated by ≥1.5 times after exposure to BPAF (25 μM) and BPAP (100 μM). Among the selected estrogen receptor genes, *esr1* was poorly expressed in ZFL spheroids (data not shown). The expression of the other two estrogen receptor genes, *esr2a* and *esr2b*, was most notably influenced by exposure to BPAP and BPPH. *Esr2a* was upregulated by ≥1.5-fold after exposure to BPAP (100 μM) and BPPH (100 μM), while *esr2b* was downregulated by BPA (200 μM) and BPAF (25 μM) after 24 h. After 96 h, the upregulation of both genes was statistically significant (*esr2a* and *esr2b* – BPAP 25 μM, *esr2b* – BPPH 50 μM).

Additionally, the expression of the *vtrg4* gene was evaluated after exposure to the tested BPs, since previous studies have shown that *vtrg4* is estrogen-inducible and can serve as a biomarker for exposure to estrogenic compounds (Hiramatsu et al., 2006). This gene encodes the vitamin D receptor gamma 4 (VTRG4) that binds vitamin D and is involved in vitellogenesis, the process of yolk protein production in oviparous animals (Zhao et al., 2024). The expression of *vtrg4* is closely linked to exposure to estrogenic compounds, as its promoter region contains multiple estrogen response elements (EREs) (Hiramatsu et al., 2006). It has previously been shown to be upregulated after exposure to a variety of estrogenic compounds, also in zebrafish (Rose et al., 2002; Van Den Belt et al., 2003; Zhao et al., 2024). In ZFL spheroids, *vtrg4* was significantly upregulated following 24 h exposure to BPA (200 μM). It was also upregulated more than 1.5 times after 24 h exposure to BPAF and BPAP (100 μM).

4. Conclusions

In conclusion, our results show that the tested BPA alternatives (BPAF, BPAP and BPPH) were more cytotoxic to ZFL spheroids than BPA. Exposure to non-cytotoxic concentrations of these bisphenols (BPs) induced transient DNA damage, but no significant increase in double-strand breaks (DSBs) was observed. The toxicogenomic analyses revealed a significant impact of these BPs on the activation of the TP53 DNA damage response pathway, followed by the activation of the nucleotide excision repair (NER) repair mechanism, potentially in response to induction of bulky DNA lesions and oxidative DNA damage, and activation of base excision repair (BER) in response to induction of 8-oxoG. These types of DNA lesions, especially the transient single-strand breaks (SSBs) generated during their repair, could have been the cause of the DNA damage detected in the comet assay. Further, the DDR gene expression analysis results suggest no activation of the non-homologous end joining (NHEJ) pathway, confirming the absence of significant DSB induction following BP exposure, which is in line with the results of the γH2AX assay. While apoptosis may have been triggered to some extent by BPA and the highest tested concentration of BPAF, there was no indication of apoptosis induction following exposure to the other tested BAP alternatives.

This study is the first to use zebrafish liver (ZFL) spheroids to investigate the adverse effects of BPs. The toxicogenomic analysis demonstrated the endocrine-disruptive potential of the tested BPs in this test system, confirming its suitability for studying BPs. The comprehensive data from the present study provides valuable insights into the ecotoxicological properties of these emerging BPA alternatives and indicates that they have considerable genotoxic and endocrine-disruptive potential and thus pose a significant ecotoxicological risk. Our findings underscore the need for further investigation and careful consideration of these chemicals as BPA replacements.

CRediT authorship contribution statement

Alja Štern: Writing – original draft, Visualization, Validation, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Katja Kološa:** Writing – review & editing, Visualization, Supervision, Software, Methodology, Formal analysis, Data curation. **Špela Rozman:** Software, Methodology, Formal analysis, Data curation. **Bojana Žegura:** Writing – review & editing, Resources, Funding acquisition, Conceptualization.

Ethics

No human participants and/or animals were involved in the study. The Zebrafish (*Danio rerio*) liver-derived cell line ZFL (CRL-2634), obtained from the ATCC-Cell bank, was used in the study.

Funding

This study was supported by the European HE projects Twinning for Excellence to strategically advance research in carcinogenesis and cancer [CutCancer; GA No. 101079113] and European partnership for the assessment of risks from chemicals [PARC; GA No. 101057014], and the Slovenian ARIS [Research Core Funding No. P1-0245].

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.scitotenv.2025.180527.

Data availability

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials. Generated raw data of this study are available from the corresponding author [AŠ] and at https://doi.org/10.5281/zenodo.17144174.

References

Adamovsky, O., Groh, K.J., Białk-Bielińska, A., Escher, B.I., Beaudouin, R., Mora Lagares, L., Tollefsen, K.E., Fenske, M., Mulkiewicz, E., Creusot, N., Sosnowska, A., Loureiro, S., Beyer, J., Repetto, G., Štern, A., Lopes, I., Monteiro, M., Zikova-Kloas, A., Eleršek, T., Vračko, M., Zdybel, S., Puzyn, T., Koczur, W., Ebsen Morthorst, J., Holbech, H., Carlsson, G., Örn, S., Herrero, Ó., Siddique, A., Liess, M., Braun, G., Srebny, V., Žegura, B., Hinfray, N., Brion, F., Knapen, D., Vandeputte, E., Stinckens, E., Vergauwen, L., Behrendt, L., João Silva, M., Blaha, L., Kyriakopoulou, K., 2024. Exploring BPA alternatives – environmental levels and toxicity review. Environ. Int. 189. https://doi.org/10.1016/j.envint.2024.108728.

Azqueta, A., Slyskova, J., Langie, S.A.S., O', I., Gaivão, N., Collins, A., Wiley, H.S., Ford, J.M., Xu, W., 2014. Comet Assay to Measure DNA Repair: Approach and Applications. https://doi.org/10.3389/fgene.2014.00288.

Azqueta, A., Stopper, H., Zegura, B., Dusinska, M., Møller, P., 2022. Do cytotoxicity and cell death cause false positive results in the in vitro comet assay? Mutat. Res./Genet. Toxicol. Environ. Mutagen. 881. https://doi.org/10.1016/j.mrgentox.2022.503520.

Bloise, N., Giannaccari, M., Guagliano, G., Peluso, E., Restivo, E., Strada, S., Volpini, C., Petrini, P., Visai, L., 2024. Growing role of 3D in vitro cell cultures in the study of cellular and molecular mechanisms: short focus on breast cancer, endometriosis, liver and infectious diseases. Cells 13, 1054. https://doi.org/10.3390/CELLS13121054.

Bousoumah, R., Leso, V., Iavicoli, I., Huuskonen, P., Viegas, S., Porras, S.P., Santonen, T., Frery, N., Robert, A., Ndaw, S., 2021. Biomonitoring of occupational exposure to bisphenol A, bisphenol S and bisphenol F: a systematic review. Sci. Total Environ. 783, 146905. https://doi.org/10.1016/J.SCITOTENV.2021.146905.

Bukato, K., Kostrzewa, T., Gammazza, A.M., Gorska-Ponikowska, M., Sawicki, S., 2024. Endogenous estrogen metabolites as oxidative stress mediators and endometrial cancer biomarkers. Cell Commun. Signal 22, 1–11. https://doi.org/10.1186/S12964-024-01583-0/TABLES/1.

Chen, D., Kannan, K., Tan, H., Zheng, Z., Feng, Y.L., Wu, Y., Widelka, M., 2016. Bisphenol analogues other than BPA: environmental occurrence, human exposure, and toxicity - a review. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.5b05387.

Collins, A.R., 2014. Measuring oxidative damage to DNA and its repair with the comet assay. Biochim. Biophys. Acta, Gen. Subj. 1840, 794–800. https://doi.org/10.1016/j.bbagen.2013.04.022.

COMMISSION DIRECTIVE 2011/8/EU, 2011. As Regards the Restriction of Use of Bisphenol A in Plastic Infant Feeding Bottles (Text With EEA Relevance).

COMMISSION REGULATION (EU) 2018/213, 2018. COMMISSION REGULATION (EU) 2018/ 213 - of 12 February 2018 - on the Use of Bisphenol A in Varnishes and Coatings Intended to Come Into Contact With Food and AMENDING REGULATION (EU) No 10 / 2011 as Regards the Use of That Substance in Plastic Food Contact.

Corvi, R., Madia, F., . In vitro Genotoxicity Testing Can the Performance Be Enhanced?-NC-ND License. http://creativecommons.org/licenses/by-nc-nd/4.0/. https://doi.org/10.1016/j.fct.2016.08.024.

Czarny-Krzymińska, K., Krawczyk, B., Szczukocki, D., 2023. Bisphenol A and its substitutes in the aquatic environment: occurrence and toxicity assessment. Chemosphere 315, 137763. https://doi.org/10.1016/J.CHEMOSPHERE.2023.137763.

Ding, Z.M., Jiao, X.F., Wu, D., Zhang, J.Y., Chen, F., Wang, Y.S., Huang, C.J., Zhang, S.X., Li, X., Huo, L.J., 2017. Bisphenol AF negatively affects oocyte maturation of mouse in vitro through increasing oxidative stress and DNA damage. Chem. Biol. Interact. 278, 222–229. https://doi.org/10.1016/J.CBI.2017.10.030.

ECHA, 2018. Inclusion of Substances of Very High Concern in the Candidate List for Eventual Inclusion in Annex XIV (Decision of the European Chemi- cals Agency). Helsinky, Finland.

Fan, L., Arvai, A.S., Cooper, P.K., Iwai, S., Hanaoka, F., Tainer, J.A., 2006. Conserved XPB core structure and motifs for DNA unwinding: implications for pathway selection of transcription or excision repair. Mol. Cell 22, 27–37. https://doi.org/10.1016/j.molcel.2006.02.017.

Franko, N., Kodila, A., Sollner Dolenc, M., 2024. Adverse outcomes of the newly emerging bisphenol A substitutes. Chemosphere 364, 143147. https://doi.org/10.1016/J.CHEMOSPHERE.2024.143147.

Gao, Y., Li, A., Zhang, W., Pang, S., Liang, Y., Song, M., 2022. Assessing the toxicity of bisphenol A and its six alternatives on zebrafish embryo/larvae. Aquat. Toxicol. 246, 106154. https://doi.org/10.1016/J.AQUATOX.2022.106154.

Ghosh, C., Zhou, Y.L., Collodi, P., 1994. Derivation and characterization of a zebrafish liver cell line. Cell Biol. Toxicol. 10, 167–176. https://doi.org/10.1007/BF00757560/METRICS.

Gorre, M., Mohandas, P.E., Kagita, S., Annamaneni, S., Digumarti, R., Satti, V., 2014. Association of XRCC5 VNTR polymorphism with the development of chronic myeloid leukemia. Tumour Biol. 35, 923–927. https://doi.org/10.1007/S13277-013-1120-5/TABLES/5.

Helton, E.S., Chen, X., 2007. p53 modulation of the DNA damage response. J. Cell. Biochem. 100, 883–896. https://doi.org/10.1002/jcb.21091.

Henkler, F., Stolpmann, K., Luch, A., 2012. Exposure to polycyclic aromatic hydrocarbons: bulky DNA adducts and cellular responses. In: Luch, A. (Ed.), Molecular, Clinical and Environmental Toxicology. Springer, Basel.

Hercog, K., Maisanaba, S., Filipič, M., Sollner-Dolenc, M., Kač, L., Žegura, B., 2019. Genotoxic activity of bisphenol A and its analogues bisphenol S, bisphenol F and bisphenol AF and their mixtures in human hepatocellular carcinoma (HepG2) cells. Sci. Total Environ. 687, 267–276. https://doi.org/10.1016/j.scitotenv.2019.05.486.

Hiramatsu, N., Matsubara, T., Fujita, T., Sullivan, C.V., Hara, A., 2006. Multiple piscine vitellogenins: Biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments. Mar. Biol. 149, 35–47. https://doi.org/10.1007/s00227-005-0214-z.

Hollander, M.C., Saeed Sheikh, M., Bulavin, D.V., Lundgren, K., Augeri-Henmueller, L., Shehee, R., Molinaro, T.A., Kim, K.E., Tolosa, E., Ashwell, J.D., Rosenberg, M.P., Zhan, Q., Fernández-Salguero, P.M., Morgan, W.F., Deng, C.-X., Fornace, A.J., 1999. Genomic instability in Gadd45a-deficient mice. Nat. Genet. 23, 176–184. https://doi.org/10.1038/13802.

Hossain, M.S., Larsson, A., Scherbak, N., Olsson, P.E., Orban, L., 2008. Zebrafish androgen receptor: isolation, molecular, and biochemical characterization. Biol. Reprod. 78, 361–369. https://doi.org/10.1095/BIOLREPROD.107.062018.

Huang, Z., Zhao, J.L., Yang, Y.Y., Jia, Y.W., Zhang, Q.Q., Chen, C.E., Liu, Y.S., Yang, B., Xie, L., Ying, G.G., 2020. Occurrence, mass loads and risks of bisphenol analogues in the Pearl River Delta region, South China: urban rainfall runoff as a potential source for receiving rivers. Environ. Pollut. 263, 114361. https://doi.org/10.1016/J.ENVPOL.2020.114361.

Jiménez-Salazar, J.E., Damian-Ferrara, R., Arteaga, M., Batina, N., Damián-Matsumura, P., 2021. Non-genomic actions of estrogens on the DNA repair pathways are associated with chemotherapy resistance in breast cancer. Front. Oncol. 11, 631007. https://doi.org/10.3389/FONC.2021.631007/BIBTEX.

Kanvah, S., Joseph, J., Schuster, G.B., Barnett, R.N., Cleveland, C.L., Landman, U.Z.I., 2010. Oxidation of DNA: damage to nucleobases. Acc. Chem. Res. 43, 280–287. https://doi.org/10.1021/AR900175A.

Kopp, B., Khoury, L., Audebert, M., 2019. Validation of the γH2AX biomarker for genotoxicity assessment: a review. Arch. Toxicol. 93, 2103–2114. https://doi.org/10.1007/s00204-019-02511-9.

Lee, S., Liu, X., Takeda, S., Choi, K., 2013. Genotoxic potentials and related mechanisms of bisphenol A and other bisphenol compounds: a comparison study employing chicken DT40 cells. Chemosphere 93, 434–440. https://doi.org/10.1016/J.CHEMOSPHERE.2013.05.029.

Marteijn, J.A., Lans, H., Vermeulen, W., Hoeijmakers, J.H.J., 2014. Understanding nucleotide excision repair and its roles in cancer and ageing. Nat. Rev. Mol. Cell Biol. 15, 465–481. https://doi.org/10.1038/nrm3822.

Marvel, M., Levavi-Sivan, B., Wong, T.-T., Zmora, N., Zohar, Y., 2021. Gnrh2 maintains reproduction in fasting zebrafish through dynamic neuronal projection changes and regulation of gonadotropin synthesis, oogenesis, and reproductive behaviors. Sci. Rep. 11, 6657. https://doi.org/10.1038/s41598-021-86018-3.

Menuet, A., Le Page, Y., Torres, O., Kern, L., Kah, O., Pakdel, F., 2004. Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ERalpha, ERbeta1 and ERbeta2. J. Mol. Endocrinol. 32, 975–986. https://doi.org/10.1677/JME.0.0320975.

Mokra, K., Kuźmińska-Surowaniec, A., Woźniak, K., Michałowicz, J., 2017. Evaluation of DNA-damaging potential of bisphenol A and its selected analogs in human peripheral blood mononuclear cells (in vitro study). Food Chem. Toxicol. 100, 62–69. https://doi.org/10.1016/J.FCT.2016.12.003.

Mokra, K., Woźniak, K., Bukowska, B., Sicińska, P., Michałowicz, J., 2018. Low-concentration exposure to BPA, BPF and BPAF induces oxidative DNA bases lesions in human peripheral blood mononuclear cells. Chemosphere 201, 119–126. https://doi.org/10.1016/J.CHEMOSPHERE.2018.02.166.

Mu, X., Huang, Y., Li, Xuxing, Lei, Y., Teng, M., Li, Xuefeng, Wang, C., Li, Y., 2018. Developmental effects and estrogenicity of Bisphenol A alternatives in a zebrafish embryo model. Environ. Sci. Technol. 52, 3222–3231. https://doi.org/10.1021/ACS.EST.7B06255/SUPPL_FILE/ES7B06255_SI_001.PDF.

Pfeiffer, E., Rosenberg, B., Deuschel, S., Metzler, M., 1997. Interference with microtubules and induction of micronuclei *in vitro* by various bisphenols. Mutat. Res. Toxicol. Environ. Mutagen. 390, 21–31. https://doi.org/10.1016/S0165-1218(96)00161-9.

Pritchett, J.J., Kuester, R.K., Sipes, I.G., 2002. Metabolism of bisphenol A in primary cultured hepatocytes from mice, rats, and humans. Drug Metab. Dispos. 30, 1180–1185. https://doi.org/10.1124/dmd.30.11.1180.

Rifa, R.A., Lavado, R., 2024. Cytotoxic impacts of seven alternative bisphenols on human in vitro cellular models. Chemosphere 366, 143408. https://doi.org/10.1016/J.CHEMOSPHERE.2024.143408.

Rodd, A.L., Messier, N.J., Vaslet, C.A., Kane, A.B., 2017. A 3D fish liver model for aquatic toxicology: Morphological changes and Cyp1a induction in PLHC-1 microtissues after repeated benzo(a)pyrene exposures. Aquat. Toxicol. 186, 134–144. https://doi.org/10.1016/J.AQUATOX.2017.02.018.

Rose, J., Holbech, H., Lindholst, C., Nørum, U., Povlsen, A., Korsgaard, B., Bjerregaard, P., 2002. Vitellogenin induction by 17β-estradiol and 17α-

ethinylestradiol in male zebrafish (*Danio rerio*). Compar. Biochem. Physiol. Part C: Toxicol. Pharmacol. 131, 531–539. https://doi.org/10.1016/S1532-0456(02)00035-2.

Sendra, M., Štampar, M., Fras, K., Novoa, B., Figueras, A., Žegura, B., 2023. Adverse (geno)toxic effects of bisphenol A and its analogues in hepatic 3D cell model. Environ. Int. 171, 107721. https://doi.org/10.1016/j.envint.2022.107721.

Sendra, M., Cavia-Saiz, M., Múñiz, P., 2024. Are the BPA analogues an alternative to classical BPA? Comparison between 2D and alternative 3D in vitro neuron model to assess cytotoxic and genotoxic effects. Toxicology 502, 153715. https://doi.org/10.1016/J.TOX.2023.153715.

Shamhari, A. 'Afifah, Hamid, Z.A., Budin, S.B., Shamsudin, N.J., Taib, I.S., 2021. Bisphenol A and its analogues deteriorate the hormones physiological function of the male reproductive system: a mini-review. Biomed 9, 1744. https://doi.org/10.3390/BIOMEDICINES9111744.

Shi, J., Jiao, Z., Zheng, S., Li, M., Zhang, J., Feng, Y., Yin, J., Shao, B., 2015. Long-term effects of Bisphenol AF (BPAF) on hormonal balance and genes of hypothalamus-pituitary-gonad axis and liver of zebrafish (Danio rerio), and the impact on offspring. Chemosphere 128, 252–257. https://doi.org/10.1016/J.CHEMOSPHERE.2015.01.060.

Štampar, M., Žegura, B., 2024. In vitro hepatic 3D cell models and their application in genetic toxicology: a systematic review. Mutat. Res. Genet. Toxicol. Environ. Mutagen. 900, 503835. https://doi.org/10.1016/J.MRGENTOX.2024.503835.

Štampar, M., Tomc, J., Filipič, M., Žegura, B., 2019. Development of in vitro 3D cell model from hepatocellular carcinoma (HepG2) cell line and its application for genotoxicity testing. Arch. Toxicol. 93, 3321–3333. https://doi.org/10.1007/s00204-019-02576-6.

Štampar, M., Ravnjak, T., Domijan, A.M., Žegura, B., 2023. Combined toxic effects of BPA and its two analogues BPAP and BPC in a 3D HepG2 cell model. Molecules 28, 3085. https://doi.org/10.3390/MOLECULES28073085/S1.

Štern, A., Novak, M., Kološa, K., Trontelj, J., Žabkar, S., Šentjurc, T., Filipič, M., Žegura, B., 2024. Exploring the safety of cannabidiol (CBD): A comprehensive in vitro evaluation of the genotoxic and mutagenic potential of a CBD isolate and extract from Cannabis sativa L. Biomed. Pharmacother. 177. https://doi.org/10.1016/j.biopha.2024.116969.

Urzì, O., Gasparro, R., Costanzo, E., De Luca, A., Giavaresi, G., Fontana, S., Alessandro, R., 2023. Three-dimensional cell cultures: the bridge between in vitro and in vivo models. Int. J. Mol. Sci. 24, 12046. https://doi.org/10.3390/IJMS241512046.

Van Den Belt, K., Verheyen, R., Witters, H., 2003. Comparison of vitellogenin responses in zebrafish and rainbow trout following exposure to environmental estrogens. Ecotoxicol. Environ. Saf. 56, 271–281. https://doi.org/10.1016/S0147-6513(03)00004-6.

Wang, P., Li, J., Xie, M.Y., Wu, C.C., Wong, C.S., Zeng, E.Y., 2024. Utility of a modified o-DGT passive sampler for measurement of bisphenol analogues in freshwater and coastal waters. Sci. Total Environ. 931, 172978. https://doi.org/10.1016/J.SCITOTENV.2024.172978.

Xiao, X., Li, J., Yu, T., Zhou, L., Fan, X., Xiao, H., Wang, Y., Yang, L., Lv, J., Jia, X., Zhang, Z., 2018. Bisphenol AP is anti-estrogenic and may cause adverse effects at low doses relevant to human exposure. Environ. Pollut. 242, 1625–1632. https://doi.org/10.1016/j.envpol.2018.07.115.

Yedidia-Aryeh, L., Goldberg, M., 2022. The interplay between the cellular response to DNA double-strand breaks and estrogen. Cells 11, 3097. https://doi.org/10.3390/CELLS11193097.

Žegura, B., Filipič, M., 2019. The application of the comet assay in fish cell lines. Mutat. Res. Toxicol. Environ. Mutagen 842, 72–84. https://doi.org/10.1016/J.MRGENTOX.2019.01.011.

Zhang, H., Zhang, Y., Li, J., Yang, M., 2019. Occurrence and exposure assessment of bisphenol analogues in source water and drinking water in China. Sci. Total Environ. 655, 607–613. https://doi.org/10.1016/J.SCITOTENV.2018.11.053.

Zhao, Z., Peng, L., Zhao, Q., Wang, Z., 2024. Genome-wide identification, expression and response to estrogen of vitellogenin gene family in Sichuan bream (*Sinibrama taeniatus*). Int. J. Mol. Sci. 25, 6739. https://doi.org/10.3390/IJMS25126739/S1.

Zhu, X., Wu, G., Xing, Y., Wang, C., Yuan, X., Li, B., 2020. Evaluation of single and combined toxicity of bisphenol A and its analogues using a highly-sensitive microbiosensor. J. Hazard. Mater. 381, 120908. https://doi.org/10.1016/J.JHAZMAT.2019.120908.

Zhu, X., Cao, L., Liu, Y., Tang, X., Miao, Y., Zhang, J., Zhang, L., Jia, Z., Chen, J., 2024. Genotoxicity of bisphenol AF in rats: detrimental to male reproductive system and probable stronger micronucleus induction potency than BPA. J. Appl. Toxicol. 44, 428–444. https://doi.org/10.1002/JAT.4552.