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Perfluorooctanoic acid and plastic additive Bisphenol A induce developmental impairments and oxidative stress–mediated apoptosis in *Daphnia magna*: An integrated toxicological assessment

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ABSTRACT

Perfluorooctanoic acid (PFOA) and plastic additive Bisphenol A (BPA) are considered as persistent emerging organic pollutants due to their ubiquitous and degradation resistant nature and toxicological health effects on aquatic species. Assessing their combined toxicity is critical for understanding potential chemical interactions and associated ecological risks. Therefore, the present study investigates the individual and combined effects of PFOA and BPA in *Daphnia magna* at environment-relevant concentrations (ERCs) of 10 μg/L and 20 μg/L for 7 days. The study focuses on developmental toxicity, apoptosis induction, enzymatic activity inhibition, and molecular docking interactions with antioxidant enzymes. Results showed higher mortality and deformity rates ($P < 0.05$) in a dose-dependent manner in the combined PFOA+BPA group than single BPA and PFOA-treated groups compared to the control. Predominant malformations included loss of tail and antennae, blood clots, and carapace deformities, most evident between days 3 and 7 of exposure. Apoptosis, detected through acridine orange staining, was observed in the abdominal claw, mid-gut region, and thoracic appendages. Enzymatic assays revealed substantial inhibition of CAT, GSH-Px, and SOD activities across most treatment groups, except for GSH-Px in PFOA-exposed groups. Molecular docking further confirmed stronger binding affinities of BPA with SOD (-9.2 Kcal/mol) and GSH-Px (-9.1 Kcal/mol) than PFOA (SOD; −8.5 Kcal/mol and GSH-Px; −6.3 Kcal/mol). In conclusion, individual PFOA and BPA showed higher toxicity potential than the combined PFOA+BPA exposure, suggesting antagonistic interactions. These findings highlight the need for further mechanistic studies to better understand the toxicological impacts of PFOA and BPA on aquatic ecosystems.

1. Introduction

In recent years, emerging pollutants, notably per- and polyfluoroalkyl substances (PFAS) and plastic associated bisphenol-A (BPA) drawn increasing scientific and regulatory attention due to their persistence, bioaccumulation potential, and mechanistic toxicity across biological systems [1,2]. Among these, perfluorooctanoic acid (PFOA) and BPA represent two high-priority chemicals frequently detected together in freshwater environments because of widespread consumer, industrial, and household applications [3,4]. Specifically, they are widely used in firefighting foams, cosmetics, plastic industry, and food packaging materials. PFOA is a stable, bioactive compound characterized by strong carbon-fluorine bonds, which confer resistance to both biological and chemical degradation [5].

In freshwater ecosystems, PFOA and BPA frequently co-occur at environmentally relevant concentrations, and both readily accumulate

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in aquatic biota [6,7]. For example, the combined toxicity of PFAS, including PFOS, PFBA, PFHxA, and PFOA, has been demonstrated to exert significant developmental effects on *Daphnia magna* (water flea) [8]. Similarly, the bioaccumulation potential of PFOA in *Salmo salar* (Atlantic salmon) has been associated with elevated somatic indices in the heart, thymus, liver, and kidneys [9]. Naveira et al. [10] found the acute toxic effects of BPA, including behavioural alterations, immobilization, and decreased reproduction in *Daphnia magna* [10]. It is worth mentioning that both PFOA and BPA target similar molecular pathways, most notably recognized as endocrine disruptor chemicals (EDC) that interfere with the endocrine system by mimicking or altering normal hormone functions, leading to various developmental and reproductive consequences [11]. However, their combined toxicological effects remain insufficiently characterized.

Numerous aquatic organisms also serve as model systems to investigate cellular damage, reactive oxygen species (ROS) generation, and antioxidant enzyme activities after EDC exposure [12]. Kim et al. reported that exposure to PFOA resulted in elevated levels of catalase (CAT) and vitellogenin (VTG), which were associated with significant DNA damage [13]. BPA exposure also induced notable histological alterations and oxidative damage in the liver and kidneys of the freshwater cyprinid *Ctenopharyngodon idella*. Furthermore, CAT activity and glutathione S-transferase (GST) levels were significantly reduced across all treatment groups. [14]. Similarly, chronic exposure to PFOA at 3, 10, or 30 mg/L for 28 days led to an upregulation of the *cyp4t11* gene expression in the liver and intestine of *Gobiocypris rarus* [15].

Combined toxicological assessment is a challenging and realistic approach to evaluating chemical interactions and associated health risks. Previously, Seyoum et al. [3] investigated the combined effects of PFOA+PFOS in *Daphnia magna* and found altered lipid metabolism. Key biomarker genes *vtg2*, *vasa, EcRA, EcRB, usp, jhe, HR3, ftz-F1, E74* and *E75* for reproduction and development were also significantly downregulated, implying high toxicity [3]. Likewise, co-exposure to PFOA+BPA at 10 ng/mL BPA and 100 ng/mL PFOS for 14 days inhibited cardiomyocyte growth and perturbed rat's mitochondrial functions [16]. Low environmentally relevant concentration (1 ppb) of PFOA and its alternative GenX for 21 days induced reproductive toxicity in male guppies (*Poecilia reticulata*) [17]. The overlapping mechanistic targets of PFOA and BPA, particularly endocrine signaling, ROS balance, apoptosis, and detoxification pathways, suggest the potential for additive or synergistic interactions. Yet, the mechanistic basis of their combined toxicity remains poorly understood, with limited studies investigating how these chemicals jointly influence antioxidant enzymes, developmental trajectories, or molecular receptor binding (Table 1).

Integrated toxicological assessment combines traditional *in vivo* endpoints with computational *(in silico)* tools to provide a more comprehensive understanding of chemical toxicity. *In silico* modelling, including quantitative structure-activity relationship (QSAR) approaches and molecular docking, allows rapid prediction of toxicological properties, prioritization of hazardous chemicals, and identification of ligand binding proteins before or alongside *in vivo* testing [18]. This integrated framework strengthens mechanistic interpretation, reduces experimental uncertainty, and supports 3 R principles by minimizing reliance on animal testing [19]. Molecular docking is a technique widely used to determine the ligand-receptor amino acid binding interactions. [20]. Numerous studies have shown the androgen receptor and estrogenic binding interactions with PFAS [5,20]. A recent study highlighted the strongest molecular docking potential of the human retinoic acid receptor (RARα), also known as the endocrine receptor with BPA, tetrabromobisphenol A (TBBPA), 4-tert-octylphenol (4-t-OP), and 4-n-nonylphenol (4-n-NP) [18].

The zooplankton *Daphnia magna* is a widely used freshwater model organism in ecotoxicological studies because of its transparency, small size, high fecundity, and easy maintenance [3]. It is also a bioindicator species commonly found in freshwater ecosystems [21]. Various studies previously recognized *Daphnia magna* as an ideal mechanistic model for evaluating developmental, oxidative and molecular responses to environmental contaminants [22,23]. Therefore, this study aims to elucidate the individual and combined toxicity potential of PFOA and BPA at environmentally relevant concentrations (ERCs) in *Daphnia magna*. It examines developmental impacts, morphological abnormalities, apoptosis induction, antioxidant enzyme activity, and interactions with key endogenous antioxidants using *in silico* molecular docking. This integrated approach provides novel insights into how individual and co-exposure to PFOA and BPA disrupts critical biological pathways, advancing our understanding of the risks posed by co-occurring emerging contaminants in freshwater ecosystems.

2. Materials and methods

2.1. Ethics statement

Experiments involving *Daphnia magna* were conducted in accordance with the protocols approved by the Animal Care and Use Committee at the University of Malaysia Terengganu (UMT), Malaysia (Approval ID: UMT/JKEPHMK/2023/107).

2.2. Chemicals, reagents and experimental design

PFOA (Perfluorooctanoic acid (C8HF15O2), CAS no: 335–67–1), and BPA (Bisphenol A (C15H16O2), CAS no: 85–05–7) were purchased from AccuStandard 99.6 % purity (New Haven, CT, USA) for the toxicity experiments. PFOA and BPA were dissolved in 0.01 % dimethyl sulfoxide (DMSO) as a dissolving solvent to make a stock solution (1 mg/L). Then, each stock solution was diluted using distilled water to make

Table 1
Comparative studies on the toxicity of PFOA and BPA *in vivo*.

Chemical	Exposure Concentration	Exposure duration	Model species	Toxicity Endpoints	References
PFOA	0, 3.125, 6.25, 12.5, 25, 50 mg/L	21 days	*D.magna*	Decrease the body length	[31]
PFOA	1, 3.2, 10, 32,100 mg/L	21 days	*D.magna*	Effect the reproduction rate	[32]
PFOA	5, 7.50, 11.25, 16.88, 25.31 mg/L	21 days	*D.magna*	Decrease in the brood count	[56]
PFOA	0.84–97 mg/L	42 days	*Hyalella azteca (amphipod)*	The growth, reproduction & development were decreased	[57]
BPA	6.0,6.5,7.0,7.5,8.0, 9.0,10.0,15.0,20.0 mg/L	21 days	*D.magna*	50 % mortality of females, the number of offspring is reduced at 10.0 mg/L	[58]
BPA	50,500,5000 nM	21 days	*D.magna*	Decreased in molting number and body length, increased swimming speed	[24]
BPA	0.9, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.5, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0 mg/L	48 h	*Danio rerio*	Delays in hatching time, edema, and hemorrhage were observed in the embryos	[58]
PFOS and PFOA	PFOS (12.5 mg/L) & PFOA (10.35) mg/L	14 days	*D.magna*	PFOS showed higher mortality after day 7 than in PFOA	[3]

designed exposure concentrations of low-dose (LD) and high-dose (HD), such as PFOA (LD: 10 μg/mL and HD: 20 μg/mL), BPA (LD: 10 μg/mL and HD: 20 μg/mL), and PFOA+BPA (LD: 10 μg/mL and HD: 20 μg/mL). Then, both single and joint chemicals were exposed to *Daphnia magna* at the ERCs. The concentration of PFOA, BPA, and PFOA+BPA used in the current study was in accordance with the previous toxicological studies [3,24] (Table 1).

2.3. Daphnia magna culture and chemical exposure

Daphnia magna was cultured and maintained in a flow-through system at a 14:10 light-dark cycle at the hatchery department of the University Malaysia Terengganu. 5 mL of Green algae (*Chlorella spp.*) with a density of 1×10^6 cells m/L was fed to daphnids once a day. All the experiments were performed according to OECD Test No. 211 [25]. The exposed solution was replaced daily with a freshly prepared stock solution added to the designated final mixture concentration. Three replicates were used for each treatment with the codes (C1, C2, C3, LD1, LD2, LD3, HD1, HD2, HD3). A total of N = 40 healthy *Daphnia magna* were placed in each beaker containing 300 mL of freshwater and exposed to a single PFOA, BPA, and combined PFOA+BPA for 7 days at ERCs. After exposure, all the daphnia from the control and treated groups were stored at −80 °C for subsequent experiments.

2.4. Developmental toxicity

During the exposure period, developmental toxicity parameters, including mortality, heartbeat rate, body weight, and morphological deformities, were observed at 12-hour intervals. The heartbeat rate and deformities in *Daphnia magna* were assessed using an inverted microscope. Similarly, due to the high-speed heartbeat rate of *Daphnia magna*, a stopwatch was employed to record the number of heartbeats per minute. Morphological abnormalities, such as alterations in body structure, antenna length, tail deformation, and carapace disruption, were also observed and documented under the microscope.

2.5. Acridine Orange (AO) staining

Acridine orange (AO) staining, a nucleic acid-selective metachromatic dye, was utilized to examine cellular apoptosis patterns in *Daphnia magna* [26]. AO staining was performed according to the method described to assess apoptosis induced by the single and combined exposure of BPA, PFOA, and PFOA+BPA [27]. Initially, an AO stock solution was prepared and adjusted to a final concentration of 100 μg/mL. *Daphnia magna* were then incubated in this solution for 30 min at 28 °C. Following incubation, the specimens were washed four times with 1 × PBS at pH 7.4. The stained samples were subsequently transferred to glass slides and observed for apoptotic changes using an inverted fluorescence microscope with 10 × CLSM magnification.

2.6. Enzymatic assays

Frozen *Daphnia magna* samples were utilized to assess the enzymatic activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). Sample preparation and measurement were conducted on ice to maintain sample integrity. Each pooled sample was homogenized in 1.5 mL of phosphate-buffered saline (PBS) solution (0.01 M, pH 7.4) using a motorized pellet pestle (Fisher Scientific). The homogenates were centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was collected for the measurement of CAT, SOD, and GSH-Px activities, as well as protein content. Microplate assay kits for CAT, GST, and protein were obtained from Elabscience, and the assays were performed according to the manufacturer's instructions.

2.7. Molecular docking analysis-ligand and receptor preparation

Two-dimensional molecular structures of PFOA and BPA were initially extracted from the PubChem compound database. Hydrogen atoms were added to each ligand, with a standardized energy force gradient set at 0.05. Given the empirical evidence of interactions between PFAS, BPA and antioxidant proteins such as SOD and GSH-Px, three-dimensional structures were obtained from the Iterative Threading Assembly Refinement I-TASSER server [28,29]. The alignment quality of these models was assessed using the Z-score following various threading procedures. A Z-score more significant than 1, with a confidence interval between −5 and 2, indicated optimal alignment quality. Similarly, a template modelling score (TMS) above 0.5 suggested accurate topology, while a TMS below 0.17 indicated random topology. The receptor models with the best Z-score, confidence interval, and TMS were selected for further analysis.

The receptor molecules were optimized using the AMBER99 force field, with energy minimization and 3D protonation at a force gradient of 0.05. Subsequently, the ligand and receptor structures were imported into docking software, which employs a free energy force field to calculate the binding energy between micromolecules and macromolecules. Receptor preparation involved removing excess hydrogen atoms, adding missing hydrogens and charges, and refining the hydrogen bonding network to address missing loops and side chains. The geometry was optimized to achieve a maximum root mean square deviation (RMSD) of 0.05 Å.

Due to the absence of Protein Data Bank (PDB) structures for SOD and GSH-Px, pocket residues were identified using the 'PocketFinder' algorithm [30]. To determine the optimal pocket residues, the Lamarckian genetic algorithm was employed in conjunction with a grid-supported energy evaluation method. The number of genetic algorithm runs varied between 10 and 100, while other docking parameters were maintained at default settings. Docking simulations of PFOA and BPA against the ligand-binding pocket of the SOD and GSH-Px receptor protein of *Daphnia magna* yielded multiple docking positions for each ligand. The optimal docking pose was selected for subsequent *in silico* analysis. Complexes were ranked based on their binding energy values (S), with lower S values indicating higher ligand affinity for the receptor protein's active pocket residues. The binding energies and docking poses generated were the outcomes of these simulations.

2.8. Statistical analysis

The Kolmogorov-Smirnov test was utilized to assess the data's normal distribution. Developmental toxicity and enzyme assay results were examined using GraphPad Prism version 10, applying two-way analysis of variance (ANOVA) to determine statistical significance, with a threshold of $P < 0.05$. Spearman correlation analysis was conducted using the Heatmapper online tool (http://www.heatmapper.ca/pairwise/). Molecular docking studies were performed using Molecular Operating Environment (MOE) software.

3. Results

3.1. Survival and developmental toxicity

3.1.1. Mortality rate

In this study, individual and combined exposure to PFOA and BPA at ERCs resulted in significant developmental toxicity, mainly after 7 days of acute exposure. Mortality rates increased in a dose-dependent manner across both treatment groups (Fig. 1A). For example, in the low-dose BPA group (10 μg/L), mortality rates were observed to be 6.8 %, 7.6 %, and 8.3 % on 5–7 days of exposure, respectively. Similarly, in the high-dose BPA group (20 μg/L), mortality rates were 7.1 %, 10.2 %, and 10.3 % over the same period. Among all treatment groups, the low-dose PFOA treatment (10 μg/mL) exhibited the highest mortality rates with

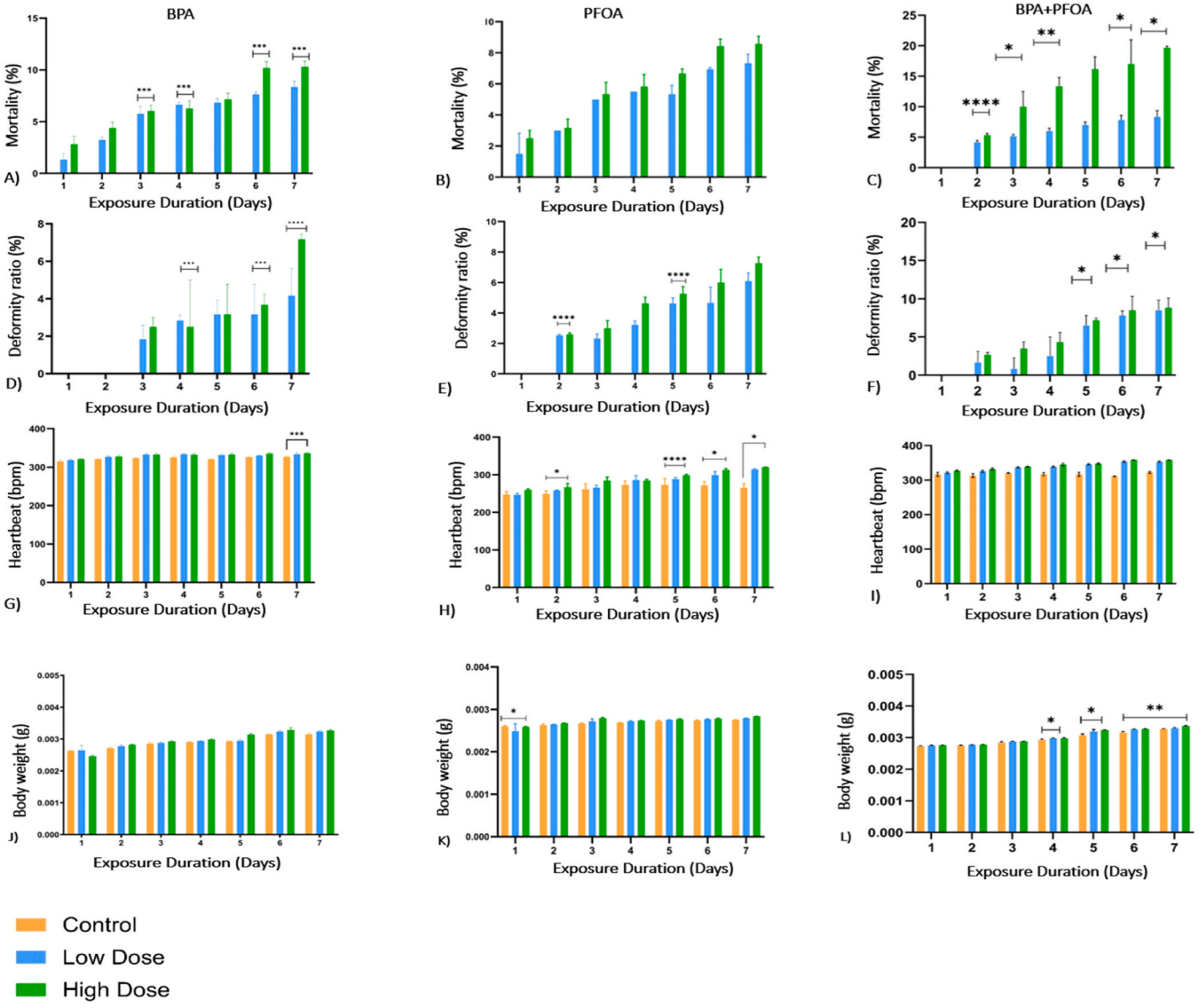

Fig. 1. Developmental toxicity indices in *Daphnia magna* after 7 days post-fertilization (hpf) exposure to single and combined PFOA and BPA mixtures at environmentally relevant concentrations (ERCs) comprising low-dose and high-dose PFOA (10 μg/mL and 20 μg/mL), BPA (10 μg/mL and 20 μg/mL), and PFOA+BPA (10 μg/mL and 20 μg/mL). The data presented are from three replicate groups. Two-way ANOVA significance level: $^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$ and $^{****}P < 0.0001$.

values of 5.3 %, 6.9 %, and 7.3 % (Fig. 1B). A similar trend was observed in the high-dose PFOA group (20 μg/L), with mortality rates of 6.6 %, 8.4 %, and 8.5 % showing a dose-dependent increase. The combined exposure to PFOA+BPA also resulted in dose-dependent mortality across all treatment groups (Fig. 1C). In the low-dose group (10 μg/L), mortality increased from day 5 to day 7, with mean values of 7 %, 7.8 %, and 8.3 %, respectively. The trend in the high-dose group (20 μg/L) was similar to the low-dose group, with the mean values of 16.6 %, 17 % and 19.6 %. ($^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$ and $^{****}P < 0.0001$).

3.1.2. *Deformities ratio*

Individual and combined PFOA and BPA exposure induce notable morphological changes in *Daphnia magna* among all treated groups. (Fig D, E, F). On days 6 and 7 of the individual BPA exposure, the low-dose treatment showed deformities with a mean value of 3.1 % and 4.1 %. Similarly, for the high-dose treatment, the major deformities were 3.6 % and 7.1 % on days 6 and 7, compared to the control group (Fig. 1D). Similarly, individual PFOA revealed maximum morphological changes at day 6–7 with the ratio of 4.6–6.1 % (low dose groups) and 6–7.2 % (high-dose group). (Fig. 1E). A Similar trend was observed for the joint PFOA+BPA, where the deformities were maximum from 7.8 % to 8.5 % on the exposure day 6–7 for low-dose treatment. Likewise, high-dose treatment found high deformities on the same days (8.5–8.8 %) compared to the control (Fig. 1F).

3.1.3. *Heartbeat rate*

After individual low-dose BPA exposure, the heartbeat rate increased to 318 bpm, to 333 bpm on days 1–7, compared to the control group (Fig. 1G). Similarly, the BPA high-dose heartbeat increases with a mean value of 335 bpm and 336 bpm, which was a slight rise in heartbeat rates on days 6 and 7, significantly different from the control group ($^{***}P < 0.001$). The individual PFOA low-dose group showed the highest heartbeat rate on day 7 (314 bpm), followed by day 6 (229 bpm), day 5 (288 bpm), day 4 (286 bpm), day 3 (266 bpm), day 2 (258 bpm), and day 1 (247 bpm) (Fig. 1H). Similarly, the PFOA high-dose treated group exhibited the highest heartbeat rate with mean values of 320 bpm, 313 bpm, 298 bpm, 284 bpm, 284 bpm, 267 bpm, and 259 bpm on day 7, day 6, day 5, day 4, day 3, day 2, and day 1, respectively. For the joint

PFOA+BPA low-dose group, the heartbeat rate was increased from day 1–7 with mean values of 320 bpm, to 352 bpm, compared to the control (Fig. 1I). In the high-dose PFOA+BPA group, heartbeat rates exhibited an exponential increase from day 1 to day 7, with mean values of 321 bpm and 353 bpm, respectively. Similarly, for high doses of joint PFOA+BPA, there was a gradual rise in heartbeat rates on days 1–7, reaching mean values of 327 bpm and 358 bpm, respectively, significantly different from the control group.

3.1.4. Body weight

Individual exposure to BPA resulted in an insignificant reduction in the body weight of *Daphnia magna*. Both low-dose and high-dose groups exhibited a gradual increase on days 4–7, with similar mean values of 0.029 g and 0.0032 g, respectively, compared to the control group (Fig. 1J). Moreover, individual PFOA low-dose exposure revealed consistent body weight from days 1–7, with the mean value of 0.0027 g. Whereas, in the high-dose exposure, the body weight showed a slight increase from day 1 (0.0025 g) to day 7 (0.0028 g), compared to the control. (Fig. 1K). The joint low-dose exposure of PFOA+BPA exhibited an increase from day 1–7, with the mean values of 0.0027 g to 0.0032 g. In the high-dose combined exposure group, body weight increased from day 1–7, with mean values of 0.0027 g to 0.0033 g, compared to the control (Fig. 1K). In conclusion, all the developmental toxicity parameters showed a dose-dependent increase in all treatment groups.

3.2. Morphological deformities

Acute individual and combined exposure to PFOA and BPA caused severe morphological changes in *Daphnia magna* in all treatment groups compared to the control group (Fig. 2, I, II, and III). After a single BPA exposure, the obvious physical malformation observed was blood clotting (BC).(Fig. 2l-B-I). In addition, short tail (ST), swollen carapace (SC) (Fig. 2l-B), undeveloped tail (UT) (Fig. 2l-D), deformed antenna (DA) (Fig. 2l-E), air bubble under carapace (AB) (Fig. 2l-F), and bioaccumulation (BA) (Fig. 2l-H). All the deformities were taken by comparing the daphnid in treatment with the control group. Similarly, after PFOA exposure, significant physiological changes were shorter tail (ST), carapace disruptions (CD), accumulation (AC), agglomerate (AG), ocular abnormalities (OA), and blood clots (BC) (Fig. 2ll-E-M). Nevertheless, on day 2 and day 4, the high-dose treatment group started to show more apparent deformities, such as a shrinking body, carapace disruption (CD), and bioaccumulation (BA) in the midgut region of the *Daphnia magna* body. Interestingly, low-dose exposure on day 5 showed blood clots in the mandible region, while the HD treatment group had blood clots in the abdomen region. On days 6 and 7, the bioaccumulation and blood clots are more prominent in the midgut for the LD treatment group, while for the HD treatment group, *Daphnia magna* deformed as the carapace was disrupted (Fig. 2II K-J).

The joint toxic effects of PFOA+BPA also caused severe effects on *Daphnia magna* (Fig. 2 III B-H). Among all treatment groups, the HD group showed more morphological changes. For instance, on days 4 and 5, the LD group showed a shortened tail, a deformed antenna, and the growth of the tail spine. Meanwhile, for days 6 and 7, they showed deformed abdominal setae (DABS), a shrinking body, blood clots, bioaccumulation in the shell gland, a long neck (LN), and a growth of tail spine (GT) for all treated groups.

3.3. Elevated apoptosis

The AO-stained *Daphnia magna* under a stereomicroscope revealed the induction of apoptosis after the individual and combined exposure to PFOA+ BPA (Fig. 3I-III). Mostly, BPA-treated samples showed increased apoptosis in the gut region, both at the dorsal and ventral sides (Fig. 3I). The fluorescence intensity of the apoptosis area was calculated for the

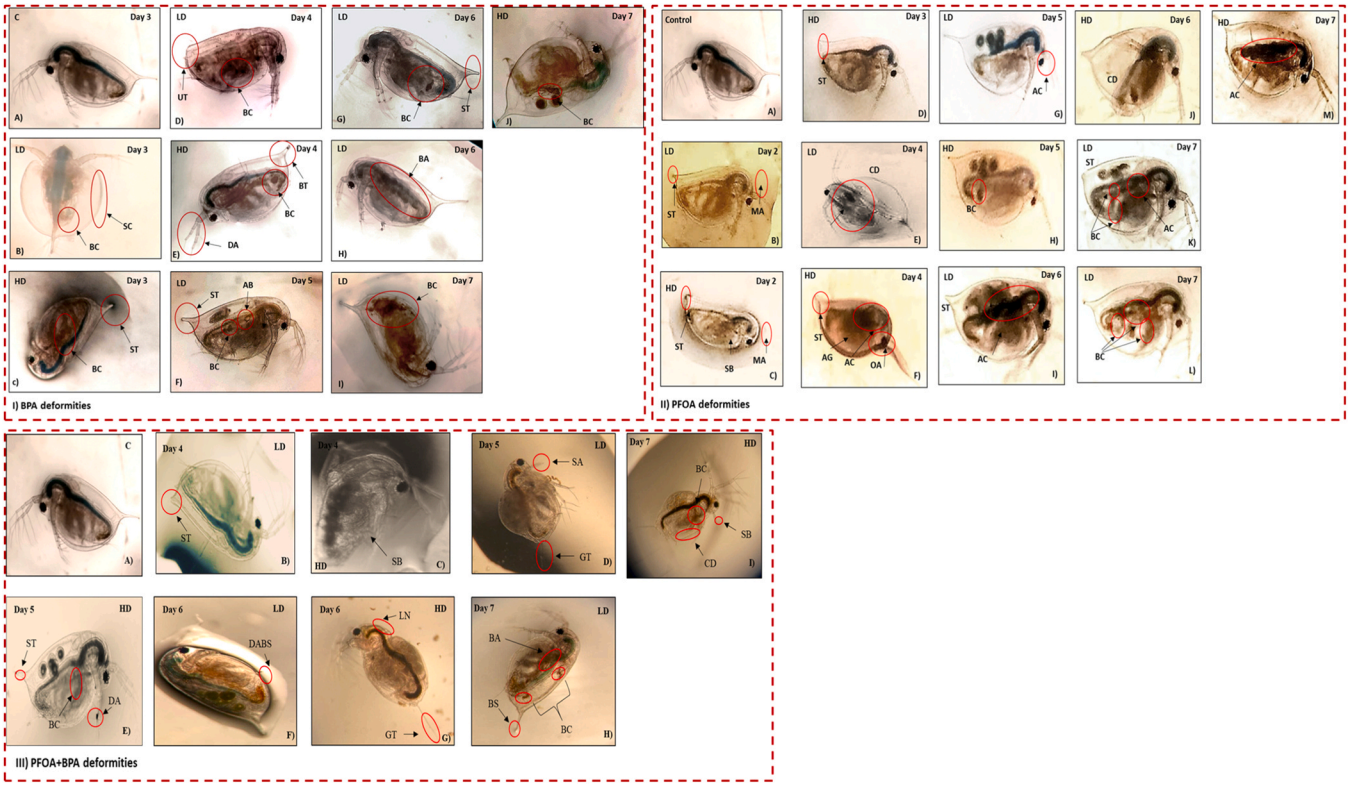

Fig. 2. (I, II, III): Morphological deformities in *Daphnia magna* after 7 days of acute exposure to individual and joint PFOA and BPA at ERCs. UT: Undeveloped tail, BC: Blood clot, ST: Short tail, DA: Deform antenna, BT: Broken tail, BA: Bioaccumulation, AB: Air bubble under carapace, CD: Carapace disruption, MA: Missing antenna, SB: Shrinking body, AG: Agglomerate, OA: Ocular abnormalities, GT: Growth of Tail Spine, SA: Short antenna, BS: Bend spine, LN: Long Neck, DABS: Deformed abdominal setae. LD: low dose; HD: high dose; C: control. Scale: 400 × magnification.

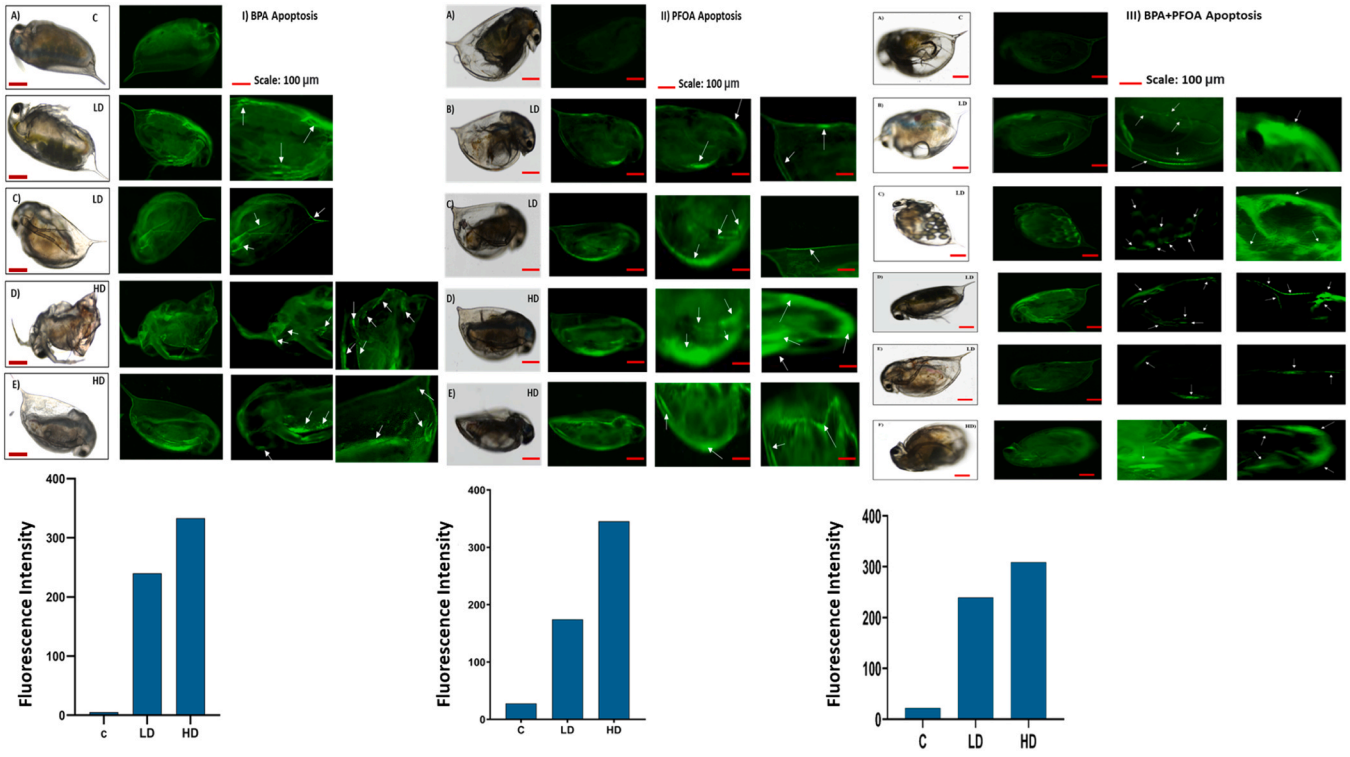

Fig. 3. (I, II, III): Acridine orange staining displays apoptosis with green fluorescence after single and joint exposure of PFOA and BPA at environmentally relevant concentrations (ERCs). Photographs were captured in the bright and dark fields with a scale of 100 µm. (F) Fluorescence intensity values of the apoptosis were calculated through ImageJ.

low-dose and high-dose BPA were 240 and 333, compared to the control (22). Similarly, the majority of the PFOA-treated samples in low-dose showed high apoptosis at the abdominal claw, antenna, and carapace region (Fig. 3II). However, the high-dose treated group shows elevated apoptosis at the midgut, antenna, carapace, and thoracic appendages region (Fig. 3II-D, E). The fluorescence intensity values were also in accordance with the staining results for low-dose (174) and high-dose (345), compared to control (27), suggesting dose-dependent apoptotic

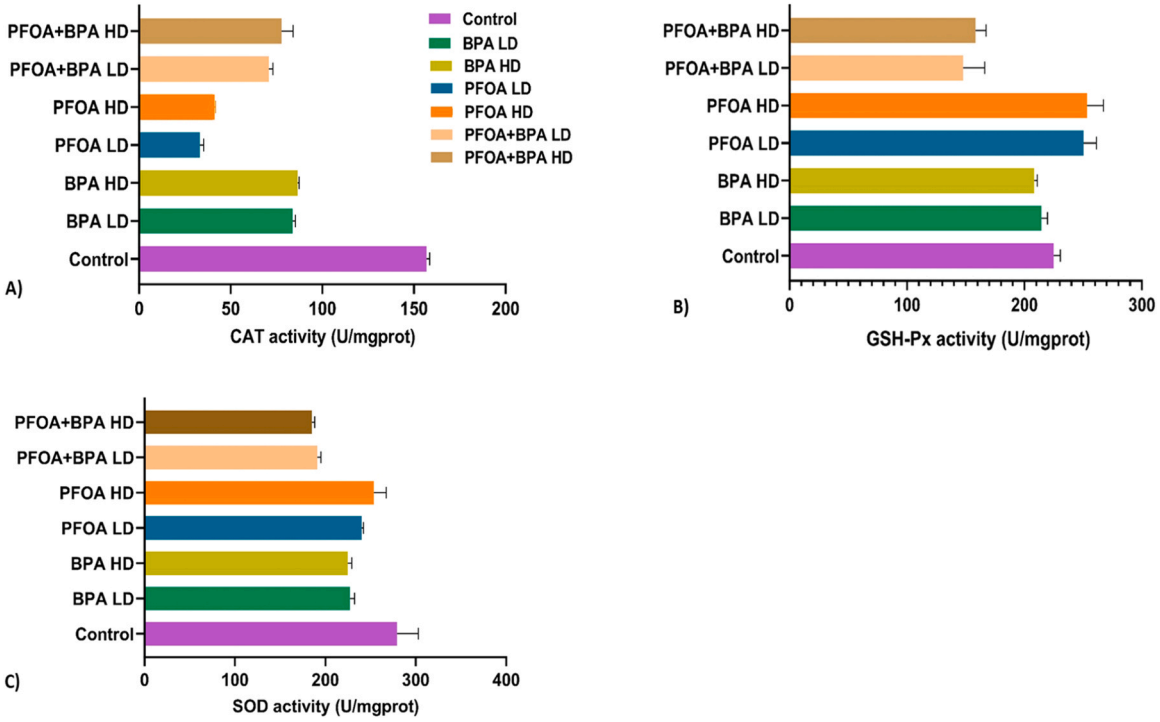

Fig. 4. (a, b, c): The endogenous enzymatic activity of CAT U/mg/Prot, GSH-Px U/mg/Prot and SOD U/mg/Prot was observed after 7 days of exposure to single and joint PFOA and BPA exposure to *Daphnia magna*. LD: low dose; HD: high dose; C: control.

effects of individual PFOA. The joint exposure of PFOA+BPA also induces high apoptosis at the carapace region (Fig. 3lll). Notably, most treated groups showed apoptosis in the head, ephippium, antennal muscle, and midgut area (Fig. 3III B, C, D, F). The maximum fluorescence intensity values for the control, low-dose, and high-dose were 22.3, 293, and 308, respectively.

3.4. Antioxidant enzymatic assays (CAT, GSH-Px and SOD)

The impact of individual and combined exposure to PFOA and BPA on antioxidant enzyme activities, specifically catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), in *Daphnia magna,* is depicted in Fig. 4. To further validate apoptosis findings, CAT activity was assessed following a 7-day exposure to individual BPA, PFOA, and their combination (PFOA+BPA). Apoptosis is closely associated with oxidative stress and CAT activity, indicating the organism's response to toxicants. CAT activity was inhibited in a dose-dependent manner, with mean values of 83.7 U/mg protein and 86.5 U/mg protein for low-dose and high-dose BPA exposure, respectively, compared to the control group (Fig. 4 A). Among all exposure groups, PFOA resulted in the greatest suppression of CAT activity, with mean values below 33.0 U/mg protein for low-dose and 41.1 U/mg protein for high-dose treatments. Moreover, combined exposure to PFOA+BPA also significantly inhibited CAT activity, with mean values of 70.8 U/mg protein and 77.6 U/mg protein for low-dose and high-dose treatments, respectively, compared to the control (Fig. 4 A).

The GSH-Px activity in *Daphnia magna* was inhibited following exposure to BPA, PFOA, and joint PFOA+BPA (Fig. 4B). BPA exposure showed insignificant changes in GSH-Px inhibition, with the mean values of 219 U/mgprot for the low dose and 210 U/mgprot for the high dose, compared to the 220 U/mgprot in the control group. In contrast, exposure to PFOA led to an increase in GSH-Px activity, with mean values of 245 U/mg protein for the low-dose treatment and over 254 U/mg protein for the high-dose treatment, compared to 224 U/mg protein in the control group. Notably, the combined exposure to PFOA+BPA resulted in the highest inhibition of GSH-Px activity in *Daphnia magna* with 140 U/mgprot (low dose) and 162 U/mgprot (high dose). (Fig. 4B).

The highest inhibition of SOD activity was observed after joint exposure to PFOA+BPA, followed by BPA exposure and PFOA exposure. For example, PFOA+BPA exposure had the lowest mean value of 191 U/mgprot for the low dose and 185 U/mgprot for the high dose, compared to the control 279 U/mgprot. (Fig. 4 C). However, BPA has a lower mean value (227 U/mgprot; low dose, 224 U/mgprot; high dose) compared to PFOA (240 U/mgprot; low dose, 253 U/mgprot; high dose), which indicates PFOA does not interfere much with the SOD activity in *Daphnia magna* (Fig. 4 C).

3.5. Spearman correlation among developmental indexes

Spearman correlation analysis was performed to elucidate the relationship among developmental toxicity parameters. Fig. 5 A shows the BPA correlation color gradient from green to pink, representing a positive and negative correlation between the toxicity endpoints. Results showed that mortality positively correlates with all the developmental parameters, including deformities ($R^2 = 0.69$) and heartbeat ($R^2 = 0.65$), except with body weight ($R^2 = -0.11$). Moreover, body weight negatively correlates with all other developmental toxicity parameters (Table S1). In addition, there was a significant positive correlation observed with the heartbeat ($R^2 = 0.82$), deformities ($R^2 = 0.75$), and body weight ($R^2 = 0.71$), after being treated with PFOA (Fig. 5B). This shows that heartbeat rate, deformities percentage, and body weight influenced the mortality rate in each sample size. Moreover, the deformity rate positively correlated with heartbeat, with $R^2 = 0.94$. The combined PFOA+BPA Spearman correlation with developmental toxicity parameters also revealed a similar trend with a positive correlation between mortality and deformity ($R^2 = 0.73$) (Fig. 5 C). Contrarily, heartbeat and deformity rates also showed a negligible correlation with the ($R^2 = 0.56$) in all samples compared to the control. Meanwhile, the heartbeat rate and body weight showed a significantly negative correlation ($R^2 = -0.37$) among all samples.

3.6. Molecular docking

The successful computation of the docking simulation of PFOA and BPA against the ligand-binding pocket of GSH-Px and SOD resulted in multiple binding sites (Fig. 6). Similarly, various parameters, such as docking score (ΔG), energy affinity (Kcal/mol), and confirmation energy (Kcal/mol), were calculated (Table 2). However, the docking score (ΔG) representing the highest ligand binding was chosen as the primary inference parameter for determining ligand docking efficiency. For GSH-Px, the docking complexes revealed BPA and PFOA binding interactions with −9.14 Kcal/mol and −6.37 Kcal/mol. For BPA, the dominant amino acids interaction residues networking with GSH-Px via hydrophobic and hydrogen bonding interfaces were lys-178, Glu-184, and IIe-156. Similarly, the crucial amino acids for PFOA and GSH-Px interactions were Gly-181, Glu-184, Lys-178, Leu-157, Gly-155, Tyr-180, IIe-156, and Arg-179.

Similarly, SOD results showed the docking binding score of −9.2

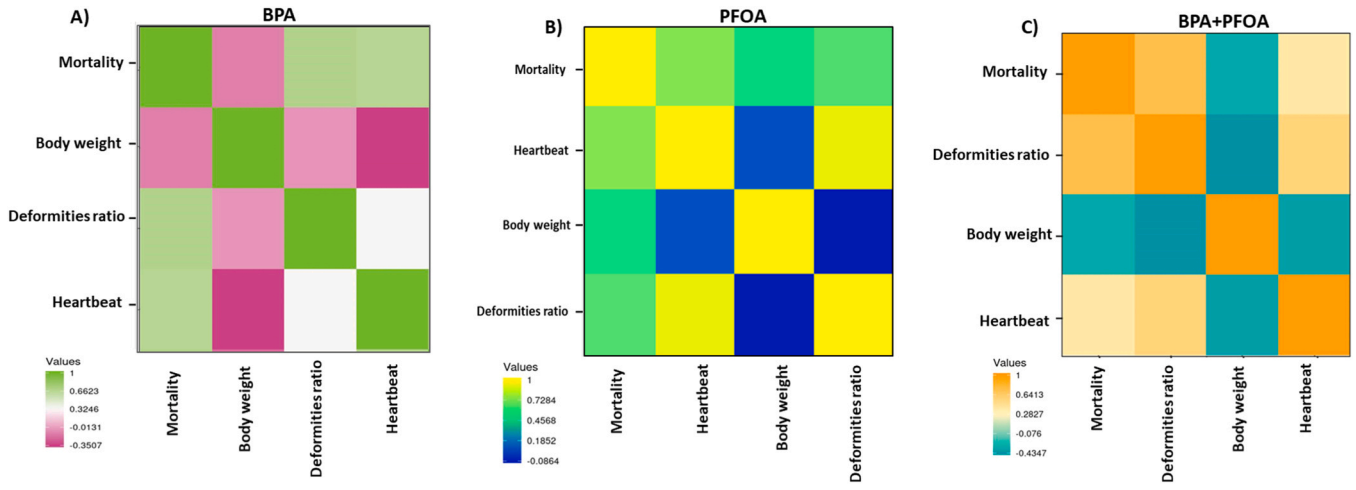

Fig. 5. (A, B, C): Heat maps showing the Spearman's rank correlation among developmental toxicity parameters (mortality, body weight, deformity ratio, and heartbeat rate) observed after 7 days of treatment. Significance levels: $^*P < 0.05$. The rainbow color gradient represents the positive correlation coefficient from dark color to light color.

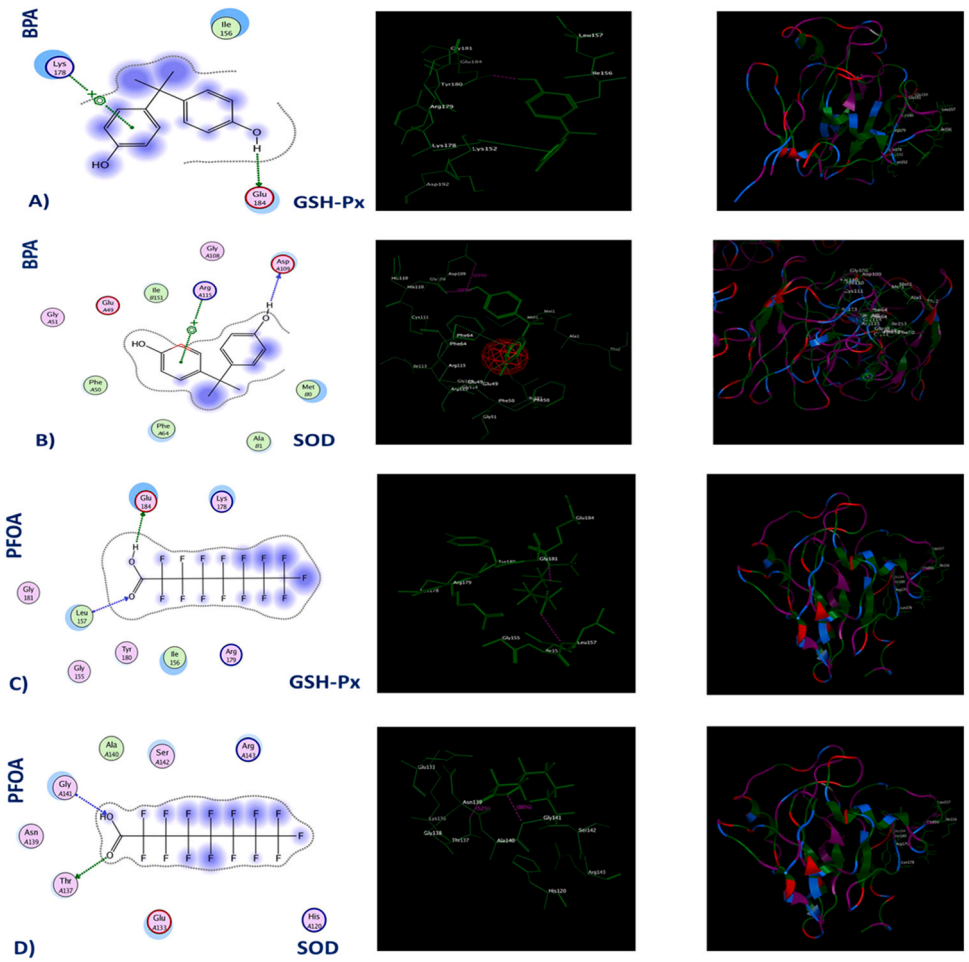

Fig. 6. Molecular docking analysis represents amino acid residues in the binding pocket of endogenous antioxidant superoxide dismutase (SOD) (B, D), and glutathione peroxidase GSH-Px (A, C) protein receptors with ligands PFOA and BPA in the left panel, molecular binding complexes in the middle panel, and ribbed presentation in the right panel.

Table 2
Molecular docking simulation of the docking score (Kcal/mol), interacting residues, energy conformation (Kcal/mol) and energy affinity (Kcal/mol) of BPA and PFOA ligands with *Daphnia magna* SOD and GSH-Px protein receptors.

Ligand	Interacting amino-acid residues number		Docking Score (S) (Kcal/mol)		Energy of conformation (Kcal/mol)		Energy place/affinity (Kcal/mol)	
	SOD	GSH-Px	SOD	GSH-Px	SOD	GSH-Px	SOD	GSH-Px
BPA	10	3	-9.2	-9.14	-76.49	-29.05	-9.2	-50.04
PFOA	8	8	-8.5	-6.37	-54.42	-77.35	-8.51	-39.98

Kcal/mol for BPA and −8.5 Kcal/mol for PFOA, which is comparatively similar to the GSH-Px protein receptor. The binding pocket amino acid residues indicating hydrophobic and hydrogen bonding interfaces for BPA were Gly-A108, Asp-A109, Arg-A115, IIe-B151, Glu-A49, Gly-A51, Phe-A50, Phe-A64, Ala-B1, and Met-B0. Likewise, the specific amino acid residues involved in binding differed between the two receptors. Predominantly, residues such as Arg-A143, Ser-A142, Ala-A140, Gly-A141, Asn-A139, Thr-A137, Glu-A133 and His-A120 were identified for SOD with PFOA (Fig. 6).

4. Discussion

In the environment, various industrial byproducts, such as PFAS and BPA, often coexist depending on the source and history of contamination. This study evaluated the sub-chronic toxicity effects of individual and combined PFOA and BPA in freshwater *Daphnia magna.* Our findings indicate that both single and joint PFOA and BPA exposure at ERCs induce significant developmental toxicity, characterized by morphological deformities, increased apoptosis, and inhibition of enzymatic activity. Additionally, these experimental results were confirmed by *in silico* molecular docking, which revealed strong binding affinities of PFOA and BPA with antioxidant enzymes in *Daphnia magna*.

Developmental toxicity parameters, including mortality, deformity ratio, heartbeat rate, and body weight, showed a significant dose-dependent increase in most samples. This is consistent with previous studies on PFOA exposure in *Daphnia magna*, which demonstrated a decrease in survival rate with increasing exposure concentration [31, 32]. A previously reported study showed similar findings, where the binary effects of the PFOS+BPA mixture in zebrafish resulted in the highest mortality after 300 μg/L exposure. [33] The highest deformity ratio was observed in the group exposed to the combined BPA and PFOA treatment, as compared to the individual exposures of BPA and PFOA. This finding suggests that the co-exposure of these chemicals may result in synergistic interactions, exacerbating developmental toxicity in

Daphnia magna. Similarly, acute exposure to BPA and Zn exhibited significant 40.0 % morphological alterations in *Daphnia magna*. [34]. It is worth mentioning that single PFOA and PFOS did not exhibit acute mortality and deformity (48 h) in *Daphnia carinata* at ERCs (0.001–10 mg/L), while chronic exposure (21 d) induced developmental and reproductive toxicity [3]. Previously, the chronic exposure of BPA and its metabolites also resulted in reproductive toxicity to *Daphnia magna* [35].

The BPA exposure found a negligible difference in the heartbeat rates, except for the individual PFOA and combined PFOA+BPA, compared to the control group. The gradual increase in the heartbeat rate was observed at days 4–7, consistent with the high bioaccumulation trend in all treatment groups. Liu et al. found that heart rate is an important indicator to determine the chemical stress levels, related to the feeding, respiratory and metabolic functions in *Daphnia magna* [36]. Similarly, Qian et al. found that BPA and its analogue caused an increase in the heartbeat of *Daphnia magna* after 21 days of exposure. [37]. The body weight revealed an insignificant increase in all treatment groups, compared to the control. Similarly, body weight exhibited a consistent trend across most treatment groups, with no significant reductions observed. However, in the PFOA+BPA co-exposure group, a slight increase in body weight was noted post-exposure when compared to the control group. This aligns with a previous study documenting reductions in body length and head width of zebrafish exposed to 4 μg/L PFOA [38].

Interestingly, even at low ERCs, significant morphological deformities were observed following individual and combined exposures to PFOA and BPA. The major deformities included carapace disruption, ocular abnormalities, body shrinkage, agglomeration, PFOA accumulation, and blood clot formation. These observations are consistent with previous findings, such as those reported by Grzesiuk et al., who noted similar abnormalities in antennae and ocular regions in multigenerational *Daphnia magna* following exposure to ibuprofen at ERC (4 μg/L) [39]. In our study, the exposure to PFOA, both individually and in combination with BPA, resulted in a higher incidence of deformities and greater bioaccumulation, indicating a substantial toxicity potential. This aligns with the previous findings where PFOS exposure led to significant body flexure [33], and severe skeletal deformities and spinal curvature in zebrafish following PFOS exposure [40]. Another multigenerational study in *Daphnia magna* reported that sublethal BPA exposure over six generations caused delayed oogenesis, reduced body size, and impaired reproduction by generation six, highlighting the latent and cumulative effects of BPA [41]. Blood clotting and bioaccumulation were predominantly observed between days 3 and 4 for individual BPA and PFOA exposures and on day 5 for the PFOA+BPA combined treatment group. This result was consistent with the previous study, which reported blood clots on the gills and body surface of *Clarias batrachus* following acute exposure to copper sulfate [42]. Thus, such responses may be a common physiological reaction to various chemical stressors in aquatic species. Overall, our study highlights the significant developmental toxicity effects of both individual and combined exposures to PFOA and BPA at ERCs, emphasizing the need for in-depth research on mechanistic toxicity pathways.

Apoptosis, or programmed cell death, is a crucial process in regulating growth and development [43]. AO staining is a commonly used method to detect apoptotic cells by binding to chromatin and emitting green fluorescence, providing a visual marker of apoptosis [27]. In this study, individual and combined exposures to PFOA and BPA resulted in significantly elevated apoptosis levels, characterized by increased fluorescence intensity in a dose-dependent manner after 7 days of exposure. Notably, the highest fluorescence intensities were observed following individual exposures to PFOA and BPA, as opposed to combined PFOA+BPA exposure. This could be attributed to chemical competition for active binding sites, reducing the overall fluorescence signal in combined exposures.

Apoptotic activity was most prominently observed in the midgut, antenna, thoracic appendages, and carapace regions across both low-dose and high-dose treatment groups. Additionally, deformed antennae and carapaces were noted, potentially impairing the normal function of locomotory appendages. A recent study demonstrated that PFOA exposure induces apoptosis in multiple regions, including the eyes, head, heart, and tail of zebrafish embryos, causing DNA damage and eliciting an inflammatory response [44]. Wu et al. previously demonstrated apoptosis in zebrafish larval eye by employing AO staining with green fluorescence following exposure to F–53B (PFOS alternative) at concentrations ranging from 0.15 to 15 μg/L [45]. Moreover, Seyoum et al. reported that PFOA exposure at concentrations of 1 μM, 10 μM, and 25 μM downregulated the C1q-domain-containing gene (*C1qdc*), which plays a crucial role in modulating the inflammatory response, further linking PFOA exposure to inflammation and apoptosis. [3]. The combined exposure to PFAS and microplastics produced both additive and synergistic toxic effects on growth, survival, and reproduction across life stages of *Daphnia magna* [46]. Importantly, studies on the combined impacts of PFOA and BPA are elusive; therefore, we compared our results with the literature available on the effects of PFOA/PFOS and other chemical pollutants on freshwater organisms.

The morphological deformities observed in *Daphnia magna* following exposure to PFOA and BPA, including carapace disruption, deformed antennae, body shrinkage, and agglomeration, were closely associated with elevated apoptosis in exposed tissues (Figs. 2 and 3). Apoptotic activity, particularly in the midgut, thoracic appendages, and carapace regions, was markedly increased in PFOA- and BPA-treated groups, suggesting that these chemicals directly disrupt normal cellular differentiation and tissue development, resulting in structural deformities. Mechanistically, oxidative stress induced by PFOA and BPA triggers programmed cell death, impairing growth and organogenesis.

Antioxidant enzymes are critical in protecting organisms from oxidative stress induced by xenobiotics and chemical pollutants [47]. Previous studies showed that the PFOS and BPA interaction induces oxidative stress and causes genotoxic effects in *Daphnia carinata* [14,48]. In the present study, the enzymatic activities of CAT, SOD, and GSH-Px were significantly inhibited following both individual and combined exposure to PFOA and BPA in *Daphnia magna*. These findings were aligned with the previously reported decreased CAT levels (13.8 mg/L) after 21 days of BPA exposure to *Daphnia magna* [49]. Similarly, combined exposure to Zn and PFOS exposure induces oxidative stress by altering SOD, GPx, and malondialdehyde (MDA) levels in *Limnodrilus hoffmeisteri* [50]. Notably, the highest suppression of CAT activity was observed in groups treated with single PFOA, compared to BPA and combined PFOA+BPA groups. In contrast, SOD and GSH-Px revealed the maximum inhibition after joint PFOA+BPA exposure. This is consistent with the findings of Lu et al., who reported a decrease in SOD activity in *Daphnia magna* at a concentration of 0.8 mg/L PFOA [51]. Similarly, a recent study examined the effects of PFOA, PFHxS, and PFOS on zebrafish embryos, revealing not only malformations and mortality but also altered lipid-metabolism behavior and increased oxidative stress, strengthening the evidence that PFAS induce physiological and developmental disruption [52]. The reduction of SOD and CAT activities may be attributed to the increased generation of reactive oxygen species (ROS), which overwhelms the antioxidant defense mechanisms [53]. Given these contrasting results, further investigation is required to explore the response of molecular pathways and apoptosis-related genes following exposure to PFOA and BPA.

In silico molecular docking was employed to characterize the QSAR-based interactions between the ligands (PFOA and BPA) and key antioxidant enzymes. In this study, both PFOA and BPA demonstrated notable binding affinities toward the antioxidant proteins SOD and GSH-Px. Moreover, BPA was found to have higher docking scores with SOD (-9.2 Kcal/mol) and GSH-Px (-9.1 Kcal/mol) than PFOA (SOD; −8.5 Kcal/mol and GSH-Px; −6.3–9.2 Kcal/mol). Due to limited studies on the topic, a recent study found similar binding levels of CAT (-10.0 kcal/mol) and GST (-6.7 kcal/mol) enzymes, indicating strong C-H bonds and

~ alkyl interactions [54]. Further, exposure to BPA and nonylphenol also exhibited strong binding pockets with the antioxidant enzymes such as CAT, SOD, GST, and GSH-Px. [55]. These findings indicate that PFOA and BPA can substantially amplify the toxicological effects in *Daphnia magna*.

5. Conclusion

The present study evaluates the toxicological effects of individual and combined BPA and PFOA on developmental and physiological deformities, apoptosis, and antioxidant enzymatic activity in *Daphnia magna* following sub-chronic exposure at ERCs. Experimental results were further extrapolated *in silico* molecular docking. Higher doses and prolonged exposure (7 days) were associated with increased mortality and deformities. Combined PFOA+ BPA revealed higher developmental toxicity than individual PFOA and BPA, suggesting a synergistic toxicity. The predominant malformations include missing antenna and tail, bioaccumulation, blood clots, carapace changes, and shrinking organs, with higher deformations observed from days 3–7 in all treated groups compared to the control. Following AO staining, apoptotic cells were detected in the abdominal claw, mid-gut region, and thoracic appendages. Enzymatic assays revealed significant inhibition of CAT, GSH-Px, and SOD activities in most treatment groups, except for GSH-Px activity in PFOA-exposed groups. Molecular docking further demonstrated higher binding affinities of BPA to endogenous antioxidants SOD and GSH-Px compared to PFOA. In summary, both individual and combined low-level exposure to PFOA and BPA can cause developmental toxicity, malformations, cellular apoptosis, and inhibition of antioxidant enzyme activities in *Daphnia magna*. Future research should focus on elucidating the molecular mechanisms underlying these toxic effects, particularly in the context of combined exposures in freshwater organisms, to gain a deeper understanding of their mechanistic toxicity.

CRediT authorship contribution statement

Naima Hamid: Supervision, Funding acquisition, Formal analysis, Conceptualization. **Nurnajiha Binti Mazeli:** Investigation. **Marcella Steffany Ann Anak Amoi:** Methodology, Investigation, Data curation. **Noor Azhani Wafiqah Binti Mohd Norrosman:** Methodology, Investigation. **Rakia Manzoor:** Visualization, Validation, Software. **Ong Meng Chuan:** Writing – original draft, Visualization, Project administration. **Stuart Cairns:** Writing – review & editing, Investigation. **Iain Robertson:** Resources, Methodology, Formal analysis. **Muhammad Junaid:** Writing – review & editing, Visualization, Resources.

Conflicts of interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.hazmp.2025.100020.

Data availability

Data will be made available on request.

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