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Polystyrene nanoplastics enhance the toxicological effects of DDE in zebrafish (*Danio rerio*) larvae

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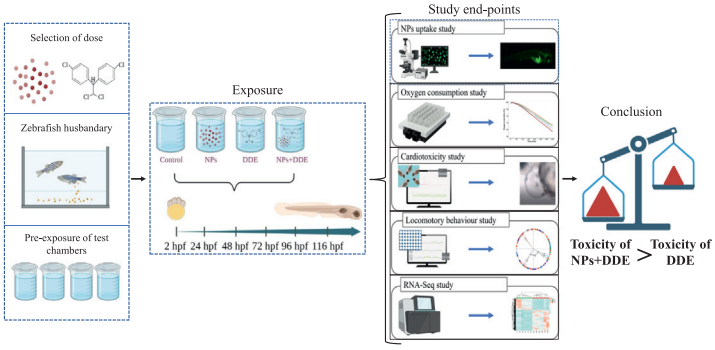
HIGHLIGHTS

- No effect of 50 mg/L PS-NPs on morphology, heart, respiration, or swimming
- 100 μg/L p,p′-DDE caused morphological, cardiac and respiratory alterations.
- PS-NPs and DDE co-exposure altered morphological, respiratory and cardiac endpoints.
- Neither NPs nor DDE exposure alone affected behaviour or inflammation.

GRAPHICAL ABSTRACT



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ABSTRACT

Anthropogenic releases of plastics, persistent organic pollutants (POPs), and heavy metals can impact the environment, including aquatic ecosystems. Nanoplastics (NPs) have recently emerged as pervasive environmental pollutants that have the ability to adsorb POPs and can cause stress in organisms. Among POPs, DDT and its metabolites are ubiquitous environmental pollutants due to their long persistence. Despite the discontinued use of DDT in Europe, DDT and its metabolites (primarily p,p′-DDE) are still found at detectable levels in fish feed used in salmon aquaculture. Our study aimed to look at the individual and combined toxicity of NPs (50 mg/L polystyrene) and DDE (100 μg/L) using zebrafish larvae as a model. We found no significant morphological, cardiac, respiratory, or behavioural changes in zebrafish larvae exposed to NPs alone. Conversely, morphological, cardiac and respiratory alterations were observed in zebrafish larvae exposed to DDE and NPs + DDE. Interestingly, behavioural changes were only observed in zebrafish larvae exposed to NPs + DDE. These findings were supported by RNA-seq results, which showed that some cardiac, vascular, and immunogenic pathways were downregulated only in zebrafish larvae exposed to NPs + DDE. In summary, we found an enhanced toxicological impact of DDE when combined with NPs.

1. Introduction

Over the last 70 years, the annual production of plastics has increased nearly 250 times, from 1.5 million tons in 1950 to 367 million tons in 2020 (Statista, 2021). This increase resulted in an abundance of plastic in

Abbreviations: MPs, microplastics; NPs, nanoplastics; PS-NPs, polystyrene nanoplastics; DDT, dichlorodiphenyltrichloroethane; DDE, dichlorodiphenyldichloroethylene; POPs, persistent organic pollutants; HPF, hours post-fertilization; DEGs, differentially expressed genes; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes.

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the ocean, with an estimated 20 million tons of plastics added to the ocean every year (Borrelle et al., 2020). The threat posed by plastic waste to marine life is enormous and the risk is growing day by day.

A current global concern is an increase of microplastics (MPs) in aquatic ecosystems, with present-day abundance ranging from 1.31 to 43,000 particles/km^2 in the ocean (Eriksen et al., 2013; Lusher et al., 2015). MPs are plastic particles sized <5 mm (Browne et al., 2007). MPs as physical stressors can cause tissue damage, growth impairment, oxidative stress and abnormal animal behaviour (Deng et al., 2020; Missawi et al., 2021; Prokić et al., 2019).

Nanoplastics (NPs) are plastic particles with a size smaller than 1000 nm (Da Costa et al., 2016; Gigault et al., 2018), although some researchers define NPs as plastic particles smaller than 100 nm in size (Besseling et al., 2019; Koelmans et al., 2015). These plastic particles enter the environment due to the mismanagement of bioengineered particles used in cosmetic products (Da Costa et al., 2016). The photolytic disintegration of MPs can also lead to NPs formation (Gigault et al., 2021). Many animal (Brandts et al., 2021; Estrela et al., 2021), plant (Lian et al., 2022; Sun et al., 2020) and human studies (Lehner et al., 2019; Zarus et al., 2021) have revealed the physical and cellular stress caused by NPs. Due to the small size of NPs, they induce more adverse effects than MPs as they can pass through the yolk sac (Lee et al., 2019; Pitt et al., 2018) and can also cross the barrier of the gut-brain axis (Huang et al., 2022; Teng et al., 2022). The gut-brain axis is important in regulating critical functions like immune activation, enteric reflex and neuro-immuno-endocrine signalling (Carabotti et al., 2015). Moreover, the vagus nerve, a major component of the gut-brain axis, controls important physiological functions like digestion, immunity and heart rate (Breit et al., 2018). Due to the large surface area of NPs, they also act as a vector for the uptake of other chemical contaminants like persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), heavy metals and dichlorodiphenyltrichloroethane (DDT) into the body (Abouda et al., 2022; Rist and Hartmann, 2018; Shen et al., 2019).

DDT, the synthetic organochlorine insecticide, was one of the most commonly used pesticides since it was developed in the 1940s. In 1972, a ban was imposed on this chemical because of its capacity to remain in the environment for a long time and bioaccumulate in animal tissues (Turusov et al., 2002). Furthermore, due to its persistence and long half-life, traces of DDT and its metabolites are still found in wildlife (Godfray et al., 2019). Concerning salmon aquaculture; low levels of DDT and its metabolites are year after year documented in fish feed, fish oil, and fish fillet (Bernhard and Hannisdal, 2021; Lundebye et al., 2017; Ørnsrud et al., 2020; Sele et al., 2018). According to annual aquafeed surveillance monitoring conducted by the Institute of Marine Research (IMR, Bergen), 49.4 μg/kg of total DDT was found in fish oil sampled in 2018 (Sele et al., 2019). Moreover, DDT is a known carcinogen, mutagen, endocrine disruptor and neurotoxin, and hence, is a risk factor for humans and other vertebrates (Turusov et al., 2002). The breakdown products of DDT are also harmful. Among them, dichlorodiphenyldichloroethylene or p,p′-DDE (hereinafter called DDE) is the main metabolite of DDT (Kelce et al., 1995). DDE is a potent androgen receptor antagonist and is found to lessen the sperm counts of fishes (Bayley et al., 2002). Furthermore, DDE, like other insecticides, can cause neurotoxicity by interfering with the normal function of neurotransmitters such as acetylcholinesterase and butyrylcholinesterase (Parrino et al., 2021). Despite the known harmful effects of DDT and its metabolites on human health, DDT is produced in India, China and North Korea (Blüthgen, 2009). It is still used in countries like India during agricultural operations and for vector control (Van den Berg et al., 2017) and in malaria-endemic-stricken African countries for vector control (Harada et al., 2016).

This study aimed to examine whether NPs influence the toxicity of persistent pollutants in fish. Polystyrene plastics are among the most frequently manufactured polymer types (PlasticsEurope, 2021), and polystyrene microplastics are reported in marine sediments and water (De Sá et al., 2018). p,p′-DDE, the dominating DDT metabolite found in fish feed and accumulated in the edible part of the farmed fish, was selected as a representative POPs. Employing zebrafish larvae as a model, we hypothesized that PS-NPs and DDE both exert an adverse effect on zebrafish larvae and that PS-NPs enhance the toxicological effects of DDE.

2. Materials and methods

2.1. Chemicals

DDE (CAS number: 72-55-9, Purity: 99 %, Product number: TCIAB0133-5G) was purchased from TCI (Eschborn, Germany). The stock solution of DDE (5 mg/mL) was prepared by dissolving DDE in DMSO (CAS number: 67-68-5, Purity: 100 %).

2.2. Polystyrene nanoplastics

Plain and fluorescent spherical PS-NPs (CD Bioparticles, New York, USA; Cat. numbers: DMP-L124 and DCFG-L120, respectively) of a nominal diameter of 15 nm were used in this study. The PS-NPs were suspended in deionized water containing a small amount of surfactant and 2 mM sodium azide to prevent microbial contamination. The final concentration of the stock solution was 10 mg/mL with 2×10^{16} particles/mL. The density of this solution was 1.05–1.06 g/mL with a negative zeta potential suggesting colloidal steadiness in the exposure solutions. Fluorescent PS-NPs were used to observe NPs uptake in zebrafish larvae, while plain PS-NPs were used in all other experiments. The fluorescent NPs were labelled with dragon green fluorophores (excitation: 460 nm, emission: 500 nm). The NPs were stored in the dark at −20 °C before use.

2.3. Zebrafish husbandry & collection of eggs

Adult zebrafish (AB strain) were raised in a recirculating system (Aquatic Habitats *Z*-Hab System, MBK Installations Ltd., United Kingdom) in the zebrafish facility of Nord University, Bodø. The rearing water temperature was 28.5 ± 1 °C and the photoperiod was 14L:10D. The adult zebrafish were fed 400–600 μm sized Zebrafeed® (Sparos Lda, Olhão, Portugal), twice a day. These adult zebrafish (3:2; M:F) were kept together in the breeding tank. The following morning, eggs were collected and after fertilization, viable eggs were separated from the dead eggs by observing them under a binocular microscope (Leica Z00M 2000). These eggs (<2 hours post fertilization (hpf)) were later employed for the various toxicity tests described in this study. The ethical guidelines of the European legislation governing "the protection of animals used for scientific purposes" (European Directive, 2010/63) were followed in all toxicity tests.

2.4. Selection of exposure dose

The exposure dose for DDE (100 μg/L) was selected based on previous reports on its acute toxicity to zebrafish larvae (Monteiro et al., 2015; Wu et al., 2019) and concentration in aquafeed (Ørnsrud et al., 2020; Sele et al., 2018). Environmentally relevant concentrations of MPs were considered to decide the exposure dose (50 mg/L) of NPs for this experiment because the levels of NPs that are significantly damaging to animals are mostly unknown (Gonçalves and Bebianno, 2021; Koelmans, 2015). The chosen exposure dose for NPs is similar to doses reported in previous studies with zebrafish embryos (Lee et al., 2019; Van Pomeren et al., 2017).

2.5. Exposure groups and exposure protocol

The toxicity tests were performed using zebrafish eggs. Four treatment groups were used in this study: control, 50 mg/L PS-NPs, 100 μg/L p,p′-DDE, and a combination of 50 mg/L PS-NPs and 100 μg/L DDE. These groups will be hereafter referred to as Control, NPs, DDE and NPs + DDE, respectively. The zebrafish eggs in the control group were exposed to only ISO standard fish media (OECD, 2013). The samples for the toxicity assessment were taken from triplicate beakers, but the samples for RNA-seq were obtained from five to six replicates/treatment. Fertilized zebrafish

eggs were exposed to test solutions. The test solution for DDE and NPs was prepared by dissolving their respective stock solution in ISO standard fish media. Before commencing the experiment, each test unit (250 mL glass beaker) received a 24 h pre-treatment with its corresponding test solution. Thereafter, 100 zebrafish embryos (<2 hpf) were exposed to a 100 mL test solution for 96 h. Then, the glass beakers were covered with parafilm and were kept in a climate chamber (Sanyo MIR-154, Sanyo Scientific, Bensenville, Illinois, United States of America) at 28 °C and 14L:10D photoperiod cycles. To maintain the concentration of contaminants in the test solution, 80–90 % of the test solution was replaced every 24 h (OECD, 2013). In all the treatments, test solutions were switched to ISO standard fish media after 96 h of exposure. Zebrafish larvae were examined for NPs uptake at 96 hpf. Larvae morphology, behaviour and heartbeat were recorded at 116 hpf. At the same time point, whole zebrafish larvae samples were collected for a transcriptomic study (RNA-seq).

2.6. Nanoplastic accumulation in zebrafish

To confirm NPs uptake, zebrafish larvae were subjected to fluorescent PS-NPs with similar exposure conditions as applied in the other toxicity tests. Zebrafish larvae exposed to fluorescent NPs were observed under a stereomicroscope Olympus BX61 (Olympus, Shinjuku-Ku, Tokyo, Japan) with a fluorescent filter (excitation: 460 nm, emission: 500 nm) in conjunction with a U-LH100HG halogen lamp (Olympus, Shinjuku-Ku, Tokyo, Japan). Fluorescent images were documented using an Olympus DP74 video recorder (Olympus soft imaging solutions, Münster, Germany).

2.7. Morphological analysis

At 48, 72, 96 and 116 hpf, ten larvae per treatment were assessed for their morphological characteristics using an inverted stereomicroscope Olympus SZX12 (Melville, USA) equipped with an Olympus SC50 (Olympus soft imaging solutions, Münster, Germany) video camera. To prevent the larvae from moving, they were placed on a 50 mL glass petri dish having a layer of 3.5 % methylcellulose. Images were analysed using ImageJ software (http://imagej.net) for body length, eye size, swim bladder area and head-to-trunk angle.

2.8. Respiration assay

An automated microplate-based respirometry (Loligo® Systems, Viborg, Denmark) was employed to measure the zebrafish larval (110 hpf) oxygen consumption (n = 12). Before the start of the study, all the devices involved in the respirometry were sterilized using a mild bleach solution (Yuan et al., 2018). The respirometer was calibrated twice using oxygen saturated and oxygen-depleted (using 0.159 M sodium sulphite) water prepared in ISO standard fish media. Moreover, the respirometer was run overnight without zebrafish larvae to reduce errors due to the possibility of oxygen in the system. Respirometry trials were performed by placing the zebrafish larvae in a 24-well glass microplate (80 μL) on the sensor dish reader (SDR). This SDR was kept beneath the water bath holding the microplate. The temperature (28 °C) was maintained by running the system inside a climate chamber (Sanyo MIR-154, Sanyo Scientific, Bensenville, Illinois, United States of America). The oxygen consumption rate was recorded for 6 h using the software program MicroResp® *v*1.0.4 (Loligo Systems®, Viborg, Denmark).

2.9. Cardiovascular toxicity

At 116 hpf, ten larvae from each treatment group were randomly chosen to measure the heartbeat. Larvae were immobilized by immersing in 3.5 % methylcellulose. Immobilized larvae were acclimated for 5 min before recording. The stereomicroscope Olympus SZX12 (Melville, USA) mounted with a video recorder Olympus SC50 (Olympus soft imaging solutions, Münster, Germany) was used to capture heartbeats videos. The videos were analysed for heartbeats/min using the DanioScope™ software *v*1.1 (Noldus Information Technology, Netherlands). This software works on an algorithm that detects the change in pixel density caused by ventricular contractions. The area of pericardial edema was also calculated using the DanioScope™ software.

2.10. Locomotor assessment

The swimming behaviour of zebrafish larvae (n = 24) was performed in a 24-well plate using the DanioVision system (Noldus Information Technology, Netherlands). Care was taken to keep morphologically impaired larvae out of the behavioural experiment. The well plate was properly randomized across the treatments and their replicates. Larvae were acclimated to plate conditions for 20 min before placing them in the DanioVision observation chamber (Noldus Information Technology, Netherlands) for the recording. The temperature of the well plate inside the DanioVision observation chamber was maintained at 28 ± 1 °C using the DanioVision temperature control unit. The 20 min recording (25 frames/s) consisted of two alternate cycles of light (5 min) and dark (5 min). The experiment was repeated four times to meet the required sample size. Behavioural tests were performed between 10:00 and 13:00 to avoid disturbing the circadian rhythm (Chiffre et al., 2016). Recordings were analysed using the EthoVision XT 16 software (Noldus Information Technology, Netherlands). A smoothing profile of 0.2 mm MDM (minimum distance moved) was applied to reduce the background noise. Locomotory heatmaps and trajectory maps were prepared using the EthoVision® XT 16 software.

2.11. RNA sequencing

Due to the small amount of tissue available from each larvae, ten whole zebrafish larvae (116 hpf) from the same treatment group were pooled. These larvae were quickly frozen in liquid nitrogen and stored at −80 °C. Total RNA from frozen larvae was extracted using the QIAzol reagent (Cat. number: 79306, Qiagen, Hilden, Germany) following the manufacturer's instructions from Direct-zol™ RNA MiniPrep (Cat. number: R2052, Zymoresearch, CA, USA). The extracted RNA was suspended in 25 μL ultra-pure DNAse/RNAse-free water. The quantity and purity of the extracted RNA were assessed using Qubit™ 4 Fluorometer (Cat. number: Q33238, Thermo Fisher Scientific, Waltham MA, USA), NanoDrop™ One UV–Vis Spectrophotometer (Cat. number: ND-ONE-W, NanoDrop Technologies, Wilmington, DE, USA) and TapeStation 2200 (Cat. number: G2964AA, Agilent Technologies, Santa Clara, CA, USA). RNA samples having RIN value > 7.5, concentration > 80 ng/μL and $A_{260–280}$ in the range of 1.95–2.05 were selected for mRNA library preparation. mRNA libraries were prepared using the NEBNext Ultra™ RNA Library Prep Kit (Cat. number: E7760S, NE Biolabs, Ipswich, MA, USA) and poly (A) mRNA magnetic isolation module (Cat. number: E7490S, NE Biolabs) following the manufacturer's instructions. The prepared libraries were then pooled in equimolar ratios for an even representation of each library. The Illumina NextSeq 500 sequencer (Illumina, San Diego, CA, USA) was used to sequence the final pooled library (1.6 pM) using the NextSeq 500/550 High Output Kit (75 bp single-end, Cat. number: FC-404-2005, Illumina). The sequencing was performed at the high throughput sequencing facility at Nord University (Bodø, Norway).

2.12. Bioinformatic analysis

The quality of raw reads was examined using the fastQC command (Brown et al., 2017). Based on the fastQC report, low-quality reads (Phred score < 30) and adapter sequences were eliminated using the fastp software (Chen et al., 2018). Trimmed data was used for further downstream analysis. The reference genome index was built using the Bowtie v2.2.3 (Langmead and Salzberg, 2012). For mapping the processed reads, the reference genome and annotation files were retrieved from the National Centre of Biotechnology Information (https://www.ncbi.nlm.nih.gov/genome/?term=Danio+rerio). Reads were mapped to the reference genome using the HISAT2 v2.2.1 (Kim et al., 2015). To determine the read

counts corresponding to each gene, the reads were annotated using featureCounts v1.6.3 (Liao et al., 2014). DESeq2 v1.30.0 was used to analyse the differential gene expression between the treatments (Love et al., 2014). The term "differentially expressed genes (DEGs)" in the manuscript refers to the transcripts with a Log2 fold change of $> +1$ or < -1 and a p-adjusted < 0.001 (Benjamini-Hochberg multiple test correction method). Gene ontology (GO) and KEGG pathway analysis of DEGs was performed using DAVID v6.8. Gene networking was performed using Cytoscape v3.9.0 (Shannon et al., 2003) and ClueGO v2.5.9 (Bindea et al., 2009).

2.13. Statistical analysis

The normality of the data (except RNA-seq data) was examined by employing Kolmogorov-Smirnov and Shapiro-Wilk tests (Statistical Package 2010, Chicago, IL). P-P and Q-Q plots were visually inspected in addition to the statistical tests to ensure the normalcy of the data. Significant outliers were detected and omitted by Grubbs' method (Graphpad Software, San Diego, CA, USA). For normally distributed data (non-angular data), one-way ANOVA followed by Tukey's posthoc test (HSD) was used to test the effect of the treatment. Non-normal and non-angular data were analysed using Friedman's test followed by the Wilcoxon Signed-Rank test. For circular or angular data, statistical analysis was performed by employing the Rayleigh test (test of uniformity) followed by Stephens Modified Watson's test. R Studio and SPSS v 28.0.1.1 were used to analyse and visualize the data. A significance level of $p < 0.05$ was considered statistically significant (*), and $p < 0.005$ was considered very significant (**).

3. Results

3.1. Nanoplastics uptake

We used fluorescent microscopy to document the uptake of the PS-NPs in the zebrafish larvae at 96 hpf. Fluorescence was observed across the body of the larvae exposed to the NPs and NPs + DDE (Fig. 1B & D). The fluorescence was primarily observed in the gastrointestinal, pericardium, ocular and cranial regions. In contrast, yolk sac protein autofluorescence was observed in larvae from all treatment groups.

3.2. Mortality assessment and developmental alterations

The mortality of zebrafish embryos or larvae was observed at 24, 48, 72, 96 and 116 hpf. We did not observe any significant difference in mortality in any treatment group compared to the control (data not shown). Also, we did not observe any significant difference in the total length of larvae at 72, 96 and 116 hpf (Supplementary Fig. 1A, B & C). Additionally, we did not find any significant difference between the treatment groups in eye size and swim bladder area at 116 hpf (Supplementary Fig. 1D & E). In addition, a significant difference in the head-to-trunk angle was observed in larvae exposed to DDE ($p < 0.005$; Supplementary Fig. 1F) and NPs + DDE ($p < 0.005$; Supplementary Fig. 1F).

DDE and NPs + DDE exposure caused several developmental alterations in zebrafish larvae (representative images are shown in Fig. 2). We observed very few deformities in zebrafish larvae exposed only to NPs (Fig. 2E, F, G & H). Pericardial edema (PE) and lordosis (LO) were observed in larvae exposed to DDE (Fig. 2K & L). In addition to PE (Fig. 2O & P), we observed an uninflated swim bladder (Fig. 2P) and reduced yolk resorption (Fig. 2P) in larvae exposed to NPs + DDE.

3.3. Cardiotoxicity assay

Cardiac ballooning was found in larvae exposed to DDE and NPs + DDE (Fig. 3C & D). A significant increase in the PE area was observed in larvae exposed to DDE ($p < 0.05$) and NPs + DDE ($p < 0.05$) (Fig. 3E). We did not find any significant change in the area of pericardial edema in larvae exposed to NPs (Fig. 3E). Moreover, we also observed a significant reduction in heart rate in larvae exposed to DDE ($p < 0.005$) and NPs + DDE ($p < 0.005$) when compared to the control larvae (Fig. 3F & Supplementary Fig. 2). We did not find any significant change in heart rate in larvae exposed to NPs (Fig. 3F & Supplementary Fig. 2).

3.4. Oxygen consumption assay

Fig. 3G shows the oxygen consumption in larvae at 110 hpf following 96 h of exposure. We found a significant increase in oxygen consumption in the larvae exposed to DDE ($p < 0.005$; treatment effect) and NPs + DDE ($p < 0.005$; treatment effect) compared to the control larvae. Over time, the treatment's impact on larval respiration grew stronger ($p < 0.005$; time effect). Compared to the control, no significant difference in larval respiration was observed in larvae exposed to NPs.

3.5. Locomotor assay

At 116 hpf, behavioural endpoints were assessed with the larval locomotor test. We found a significant reduction in the distance moved, velocity and movement in larvae exposed to NPs + DDE ($p < 0.005$) (Fig. 4A, B and C). We also observed a significant difference in the stasis time in larvae exposed to NPs + DDE ($p < 0.05$) (Fig. 4D). Reduced movement of larvae in this group can also be seen on representative locomotory heatmaps (Fig. 4E) and trajectory plots (Fig. 4F). We found a significant difference in the heading of larvae exposed to the NPs + DDE (Fig. 5A). Angular velocity, an indicator of the speed of change in direction, was significantly affected in larvae exposed to DDE ($p < 0.05$) and NPs + DDE ($p < 0.005$)

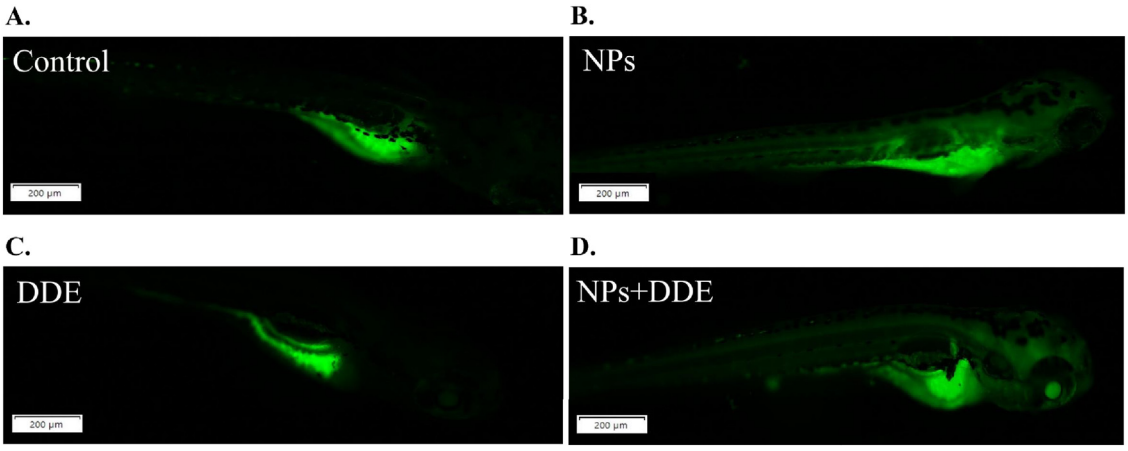

Fig. 1. NPs uptake in zebrafish larvae exposed to fluorescent PS-NPs for 96 h. (A.) Control, (B.) NPs, (C.) DDE and (D.) NPs + DDE. The representative images obtained at 96 hpf were captured using a green fluorescence filter.

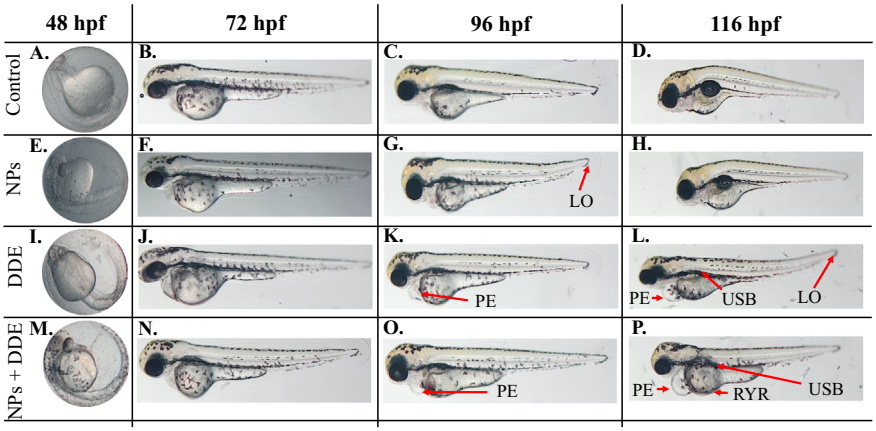

Fig. 2. Morphology of the zebrafish embryos (48 hpf) and larvae (72, 96 and 116 hpf) after exposure to NPs (E, F, G and H), DDE (I, J, K and L) and NPs + DDE (M, N, O and P). PE = pericardial edema, USB = uninflated swim bladder, LO = lordosis and RYR = reduced yolk resorption.

(Fig. 5B). We also found significant changes in the number of clockwise or counterclockwise rotations made by zebrafish larvae after exposure to NPs + DDE. Compared to the control, we did not observe any significant difference in locomotory behaviour parameters in larvae exposed to NPs (Fig. 4 & Fig. 5).

3.6. Transcriptional responses

In total, about 333 million reads from 23 samples were generated and mapped to the zebrafish reference genome with an average of 93.3 % mapping rate (Supplementary Table 1). Exposure to NPs resulted in eight significantly differentially expressed genes (DEGs), all downregulated (Supplementary Fig. 3B; Supplementary Table 2). Exposure to DDE resulted in 1022 significant DEGs (upregulated: 394; downregulated: 628; Supplementary Table 3). Exposure to NPs + DDE resulted in 1915 significant DEGs (upregulated: 652; downregulated: 1263; Supplementary Table 4). The general distribution of DEGs was evaluated with volcano plots (Fig. 6B, Fig. 7B & Supplementary Fig. 3B). The plots demonstrated that co-exposure to NPs and DDE resulted in more significant DEGs than either exposure alone. The majority of the significant DEGs were downregulated (100 % in NPs, 61.4 % in DDE, and 65.9 % in NPs + DDE), indicating a potent inhibitory effect of the treatments on gene expression post-exposure. Principal component analysis (PCA) showed that the NPs group did not cluster differently than the control group (Supplementary Fig. 3A). The DDE and NPs + DDE groups, on the other hand, clearly clustered differently than the control (Figs. 6A & 7A). The separation of differentially up and downregulated genes was studied using hierarchical clustering through a heatmap. We found no clear separation of DEGs between the NPs and control group (Supplementary Fig. 3C). However, a distinct separation of up and downregulated genes was observed in the DDE and NPs + DDE groups (Figs. 6C & 7C). Heatmaps of DEGs associated with cardiac function from the DDE and NPs + DDE groups identified with hierarchical clustering are shown in Supplementary Fig. 4A & B.

Functional analysis using gene ontology (GO) showed that NPs exposure resulted in no significantly enriched GO terms. However, 186 significantly upregulated and 99 downregulated GO terms were observed in

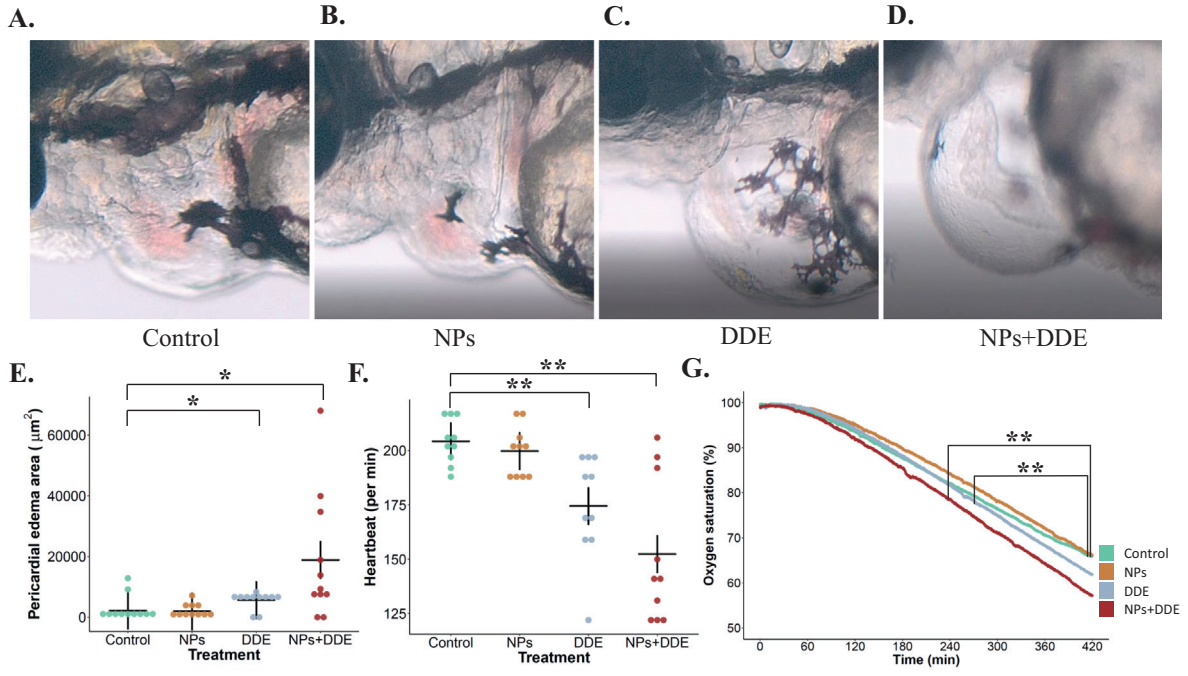

Fig. 3. Effects of different exposure on heart morphology. (A.) Control, (B.) NPs, (C.) DDE and (D.) NPs + DDE. Effects of different exposure on (E.) pericardial edema area, (F.) heartbeat and (G.) oxygen saturation in zebrafish larvae. Data represent the mean ± SE. Asterisks ** signify $p < 0.005$. Plus + represents the mean of the group.

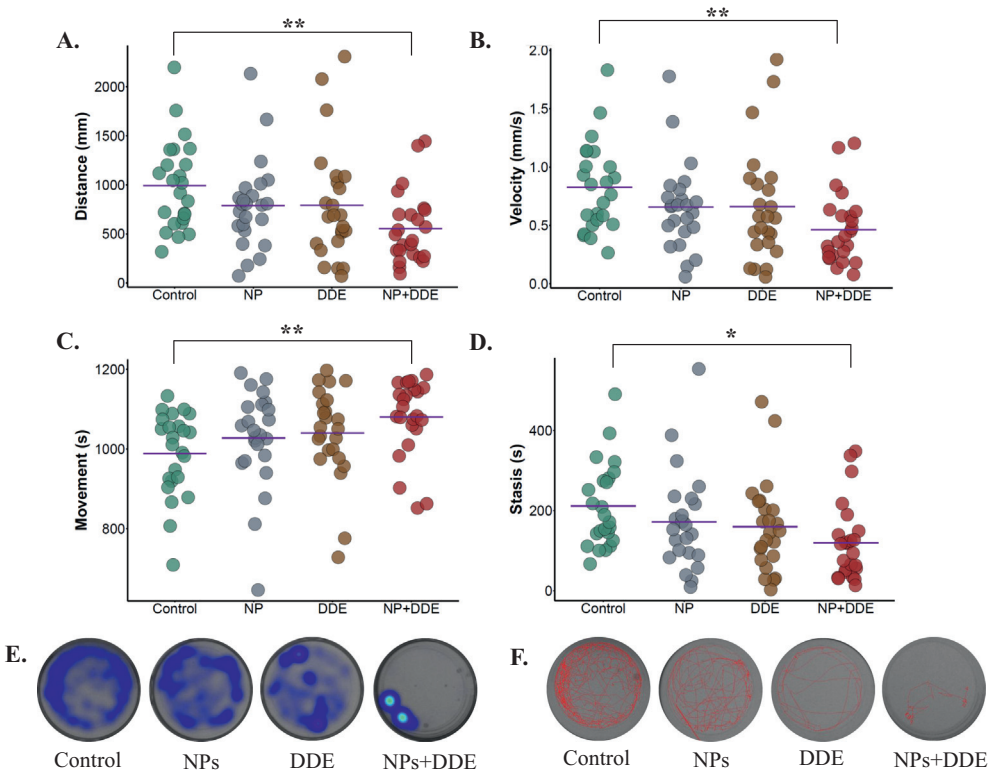

Fig. 4. Behavioural effects of NPs, DDE and NPs + DDE exposure on the zebrafish larvae (116 hpf). (A.) Distance, (B.) velocity, (C.) movement, (D.) stasis, (E.) locomotory heat map and (F.) track visualization. Data represent the mean ± SE. Asterisks * signify $p < 0.05$;** signify $p < 0.005$. Blue traces in locomotory heat maps depicts the time spent by larvae at that position. Red lines in the track visualization plots depicts the path followed by larvae during the 20 min recording.

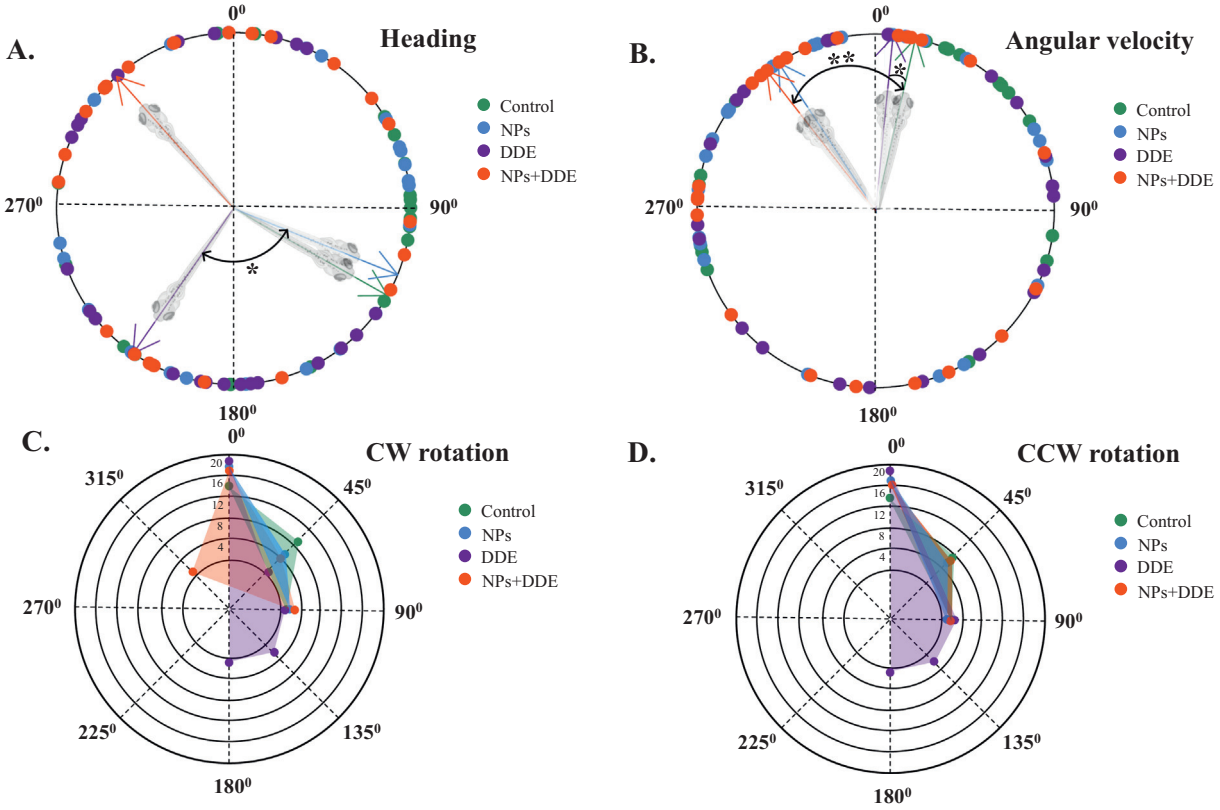

Fig. 5. Behavioural effects after exposure to NPs, DDE and NPs + DDE on the zebrafish larvae (116 hpf). (A.) Heading, (B.) angular velocity, (C.) clockwise rotation and (D.) counterclockwise rotation. Asterisks * signify $p < 0.05$; ** signify $p < 0.005$. Arrow symbol (↑) in the heading and angular velocity plot represent the mean of the group.

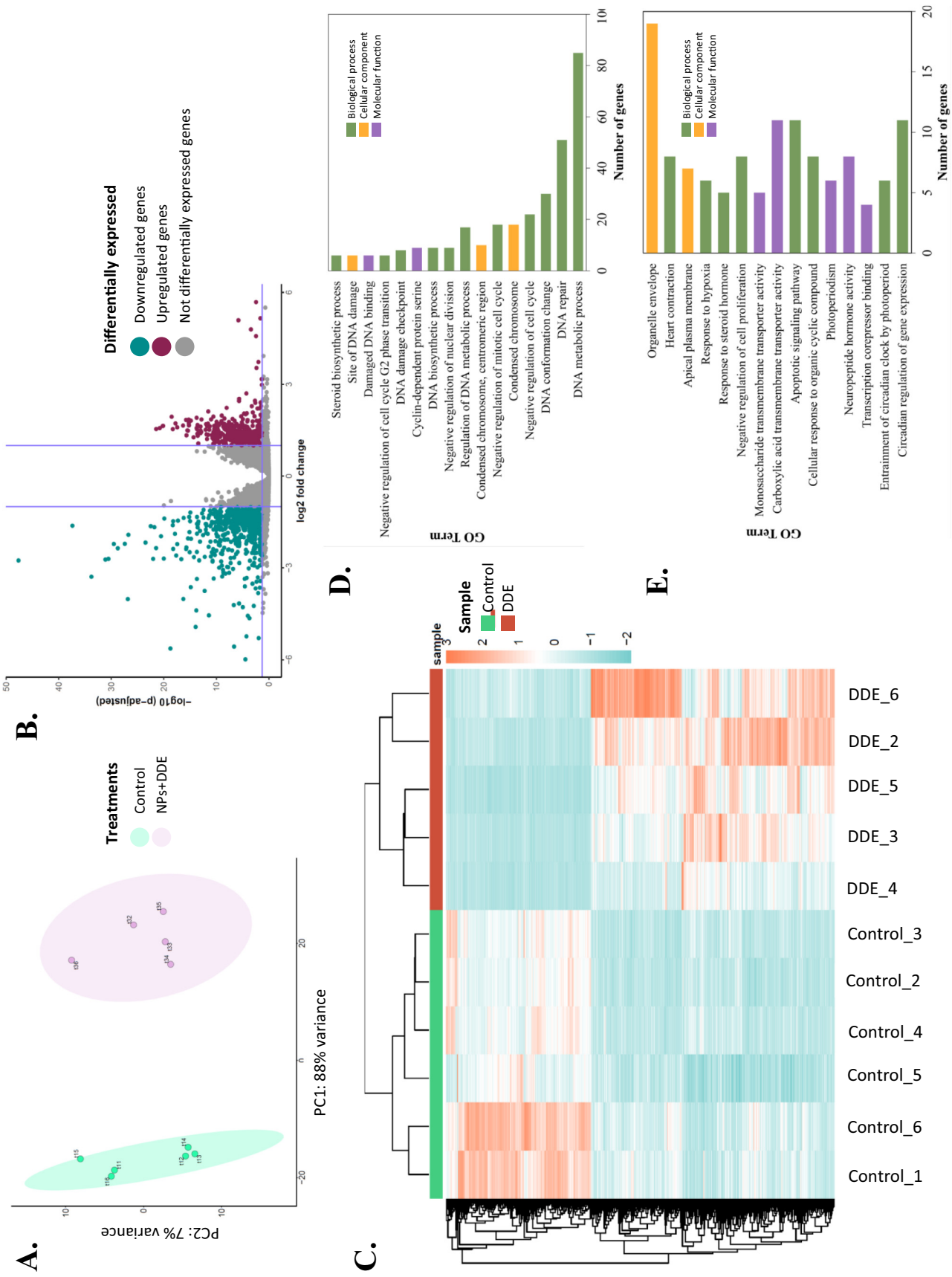

Fig. 6. Transcriptional responses in zebrafish larvae (116 hpf) after 96 h DDE exposure. (A.) Principal component analysis of sample relationship, (B.) volcano plot of DEGs, (C.) heatmap of DEGs, (D.) GO terms associated with upregulated DEGs and (E.) GO terms associated with downregulated DEGs. In volcano plots and heatmaps, transcripts with an adjusted $p < 0.001$ and Log2 fold change of $> +1$ or < -1 were considered as DEGs.

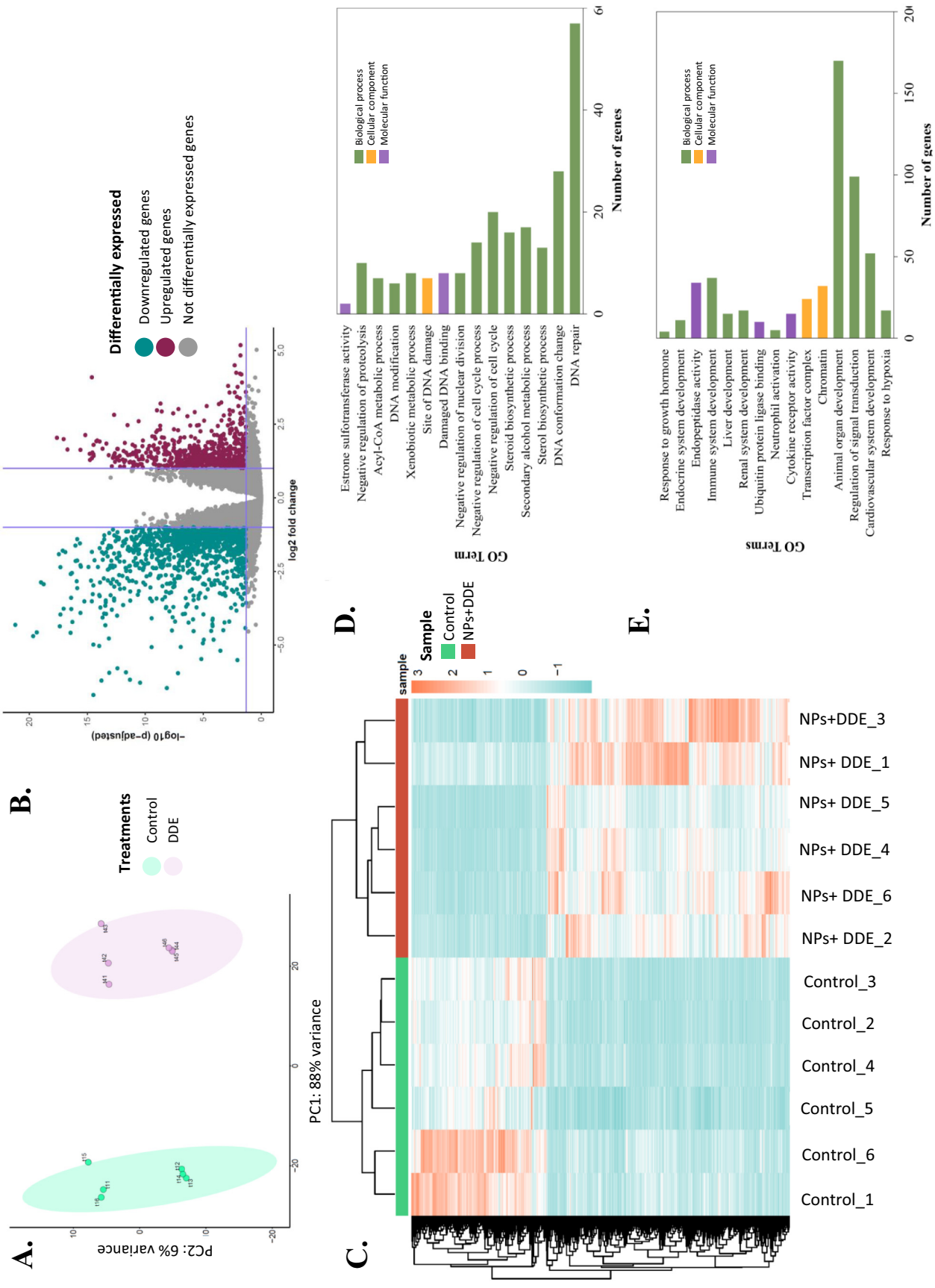

Fig. 7. Transcriptional responses in zebrafish larvae (116 hpf) after 96 h NPs + DDE exposure. (A.) Principal component analysis of sample relationship, (B.) volcano plot of DEGs, (C.) heatmap of DEGs, (D.) GO terms associated with upregulated DEGs and (E.) GO terms associated with downregulated DEGs. In volcano plots and heatmaps, transcripts with an adjusted $p < 0.001$ and Log2 fold change of $> +1$ or < -1 were considered as DEGs.

larvae exposed to DDE (Supplementary Tables 5 & 6). Significantly downregulated GO terms were linked to heart contraction, response to hypoxia, photoperiodism and neuropeptide hormone activity (Fig. 6E), whereas upregulated GO terms were related to the steroid biosynthesis process, DNA damage and DNA repair (Fig. 6D). In larvae exposed to the NPs + DDE, there were 280 significantly upregulated and 255 downregulated GO terms (Supplementary Tables 7 & 8). The most significantly downregulated GO terms were linked to endocrine system development, immune system development, response to hypoxia, cardiovascular system development and signal transduction (Fig. 7E), whereas the upregulated GO terms were linked to the Acyl-CoA metabolic process, DNA modification, xenobiotic metabolic process, steroid biosynthesis process and DNA repair (Fig. 7D).

KEGG pathway analysis showed that exposure to NPs did not significantly impact any pathway. Exposure to DDE resulted in 16 upregulated (Fig. 8A) and nine downregulated (Fig. 8B) KEGG pathways. These pathways were mainly associated with metabolism, signalling, and DNA repair mechanisms. Co-exposure with NPs + DDE resulted in 28 upregulated (Fig. 8C) and 17 downregulated (Fig. 8D) KEGG pathways. The key pathways affected by NPs + DDE co-exposure were associated with apoptosis, signalling, mitophagy and drug metabolism mechanisms. Fig. 9 depicts the network analysis of KEGG pathways following DDE and NPs + DDE exposure. The pathway network analysis results from the DDE group (Fig. 9A) showed fewer terms than the NPs + DDE group (Fig. 9B).

4. Discussion

MPs are today abundant in the aquatic environment. Research on NPs is in its infancy, with early results suggesting they may be even more hazardous than MPs (Fadare et al., 2019; Koelmans et al., 2015). NPs ability to adsorb POPs is one of their characteristic property. Adsorption efficiency depends on the sorption property of NPs and the chemical property of the contaminants (Thiagarajan et al., 2021). Interactions between NPs and contaminants can be either synergistic or antagonistic. Most toxicological studies have suggested that NPs have synergistic effects with other pollutants (Cao et al., 2022; Lee et al., 2019; Li et al., 2021), while a few studies have also documented antagonistic effects of NPs (Verdú et al., 2022; Zhang et al., 2018). Our study provides compelling evidence that NPs exacerbate the toxicological effects of other pollutants. We employed zebrafish larvae as an animal model to study the toxicological effects of NPs, DDE and their combination. Our findings show that spherical 15 nm PS-NPs may not be particularly harmful themselves, but the toxic effects of environmental pollutants such as DDE may be exacerbated when combined with NPs.

Size and concentration are two key determinants for the uptake, bioaccumulation and translocation of NPs into zebrafish larvae (Kögel et al., 2020; Sendra et al., 2021). The other determinants are exposure conditions, polymer type, polymer shape, test animal and its developmental stage (Kögel et al., 2020). A recent study by Manuel et al. (2022) found non-significant mortality in zebrafish larvae after 96 h exposure of 100 mg/L PS-NPs. Another study by Feng et al. (2022) also found that exposure of 100 mg/L PS-NPs (100 nm) had no significant effect on body length, swimming behaviour and ROS generation. In our study, fluorescent microscopy showed that at 96 hpf PS-NPs particles could reach the zebrafish larvae's brain and yolk sac after waterborne exposure. This suggests that the NPs entered the bloodstream and passed the blood-brain barrier. However, we did not find any signs of toxicological effects of PS-NPs on morphological, behavioural or molecular endpoints measured at 116 hpf, probably due to the relatively low exposure dose. In our study, the concentration of NPs used (50 mg/L) is substantially less than the MP/NPs concentrations found in most of the polluted marine environments. It is well understood that particles smaller than 200 nm can enter the brain (Nance et al., 2012; Nowak et al., 2020). Particles smaller than 50 nm have been observed to cross the yolk membrane of zebrafish embryos (Lee et al., 2019). Santos et al. (2022) also did not find any toxicological effects of 44 nm-sized PS-NPs in zebrafish larvae and the results were in line with the findings of present study. Rapid excretion of NPs with feces and urine could be one of the causes. This hypothesis is well supported by a study by Nowak et al. (2020), which documented that particles smaller than 30 nm are easily removed from the body. Moreover, Lee et al. (2022) showed that zebrafish larvae excreted 45 % of NPs (562.15 ± 118.47 nm) within 24 h of exposure. Since most of the toxicological endpoints in our study were evaluated at 116 hpf and the exposure ended at 96 hpf, a considerable fraction of accumulated NPs may have been excreted prior to sampling.

DDE is an organochlorine pesticide that has been banned in most of the world owing to its long-term environmental persistence and associated harmful effects. DDT has been reported to accumulate in sea turtles (Wafo et al., 2005), fish (Hilmy et al., 1983) and vultures (Van Wyk et al., 2001). In the present study, exposure to 100 μg/L DDE caused morphological impairments like pericardial edema, uninflated swim bladder and lordosis in zebrafish larvae. Our results are in line with a study that showed tail and spinal deformity in zebrafish larvae after exposure to the o,p′-DDT and p,p′-DDE (Wu et al., 2019). Moreover, we also found a significant difference in head-to-trunk angle in this group. Wu et al. (2019) concluded that exposure to DDT produces thyroid-disrupting effects via the downregulation of the *dio3b* gene (−1.79-fold). Our study also found significant downregulation of key thyroid genes, including the *dio3b* gene (−2.26-fold). The protein encoded by this gene is also involved in several other processes like swim bladder development and swimming behaviour (Fu et al., 2020).

We also found cardiac ballooning and decreased heartbeat in larvae exposed to DDE. The microscopy results corroborated the transcriptional results. We found significantly affected GO terms related to cardiac functions like heart contraction and heart morphogenesis. KEGG enrichment analysis also revealed the downregulation of the vascular smooth muscle contraction pathway. The primary function of this pathway is to control the contraction of cardiac muscles by regulating the concentration of cytosolic Ca^{2+} ions (Ojamaa et al., 1993). A study by Truong et al. (2020) found that a decrease in Ca^{2+} ions in the sarcoplasmic reticulum is the reason behind the DDE-associated cardiotoxicity in rats. In the present study, we found significantly downregulated DEGs related to Ca^{2+} influx in cardiac muscle cells such as *adma* (−1.26-fold), *rnf207* (−1.86-fold) and *tcap* (−3.13-fold). In short, the exposed animal's heart attempts to maintain an optimal heart contraction and oxygen level by expanding the cardiac area.

Respirometry results showed increased oxygen consumption in zebrafish larvae exposed to DDE, whereas larvae exposed to NPs + DDE had the highest oxygen consumption. The GO term response to hypoxia was significantly downregulated in both the DDE and NPs + DDE groups, while the GO term cellular response to decreased oxygen level was downregulated only in the NPs-DDE group. Hypoxia is defined as a drop in oxygen concentration below normoxic levels (20–30 %), which causes physiologic acclimation at the cellular and organismal levels (Nilsson and Östlund-Nilsson, 2004). The primary way to adapt to hypoxic conditions is by minimizing the metabolic rate by directing AMPK to regulate protein synthesis rather than ATP, as protein synthesis consumes a lot of ATP (Xiao, 2015). We also found downregulated KEGG pathways associated with arginine biosynthesis, proline and glutamate metabolism. Gene mutation is another approach to coping with prolonged hypoxic conditions (Xiao, 2015). In line with this hypothesis, we found several upregulated GO terms associated with DNA damage such as DNA repair, DNA biosynthetic process and DNA modification in larvae exposed to DDE. Furthermore, an increase in oxygen consumption is a direct measure of stress and an indirect measure of metabolism (Varshney et al., 2022). We found enriched GO terms linked to overall stress, such as positive regulation of cell death and positive regulation of programmed cell death in larvae exposed to DDE. The increased oxygen consumption in the DDE-exposed larvae also corroborates the observed reduced heart rate. Taken together, this is the first study describing DDE-associated cardiovascular toxicity in zebrafish larvae.

Furthermore, the cellular response to decreased oxygen levels is a process by which an animal reduces its locomotion, enzyme synthesis and gene expression to cope with the declined oxygen levels (Neiffer and

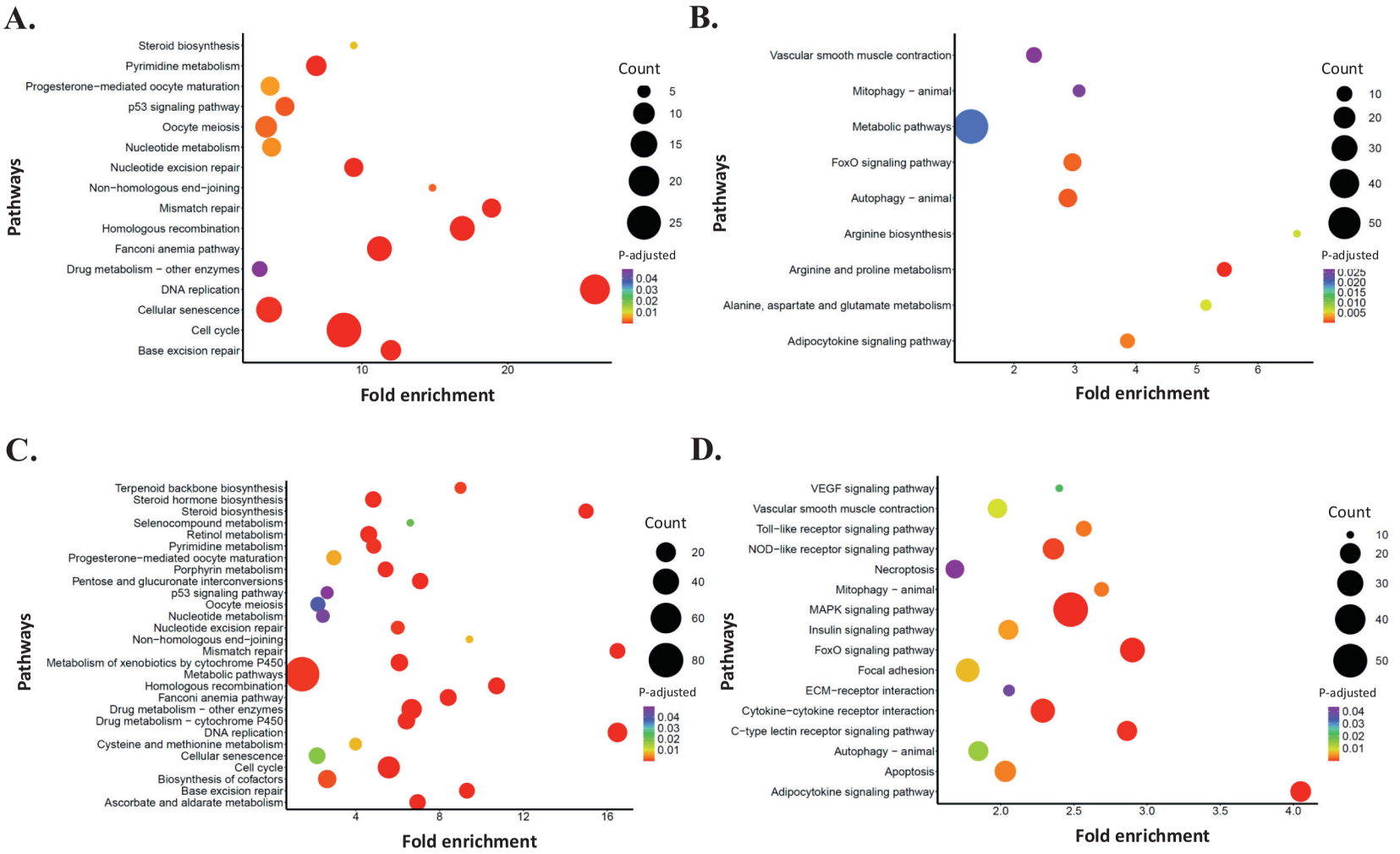

Fig. 8. KEGG pathway analysis. (A.) Enriched KEGG pathways of upregulated DEGs after 96 h exposure to DDE, (B.) Enriched KEGG pathways of downregulated DEGs after 96 h exposure to DDE, (C.) Enriched KEGG pathways of upregulated DEGs after 96 h exposure to NPs + DDE and (D.) Enriched KEGG pathways of downregulated DEGs after 96 h exposure to NPs + DDE. The size of the dots increases with the increase in gene count and vice versa. The intensity of the gradient color bar differs with the adjusted *p*-value (Benjamini-Hochberg method) with each GO term. Transcripts with an adjusted $p < 0.001$ and Log2 fold change of $> +1$ or < -1 were considered as DEGs.

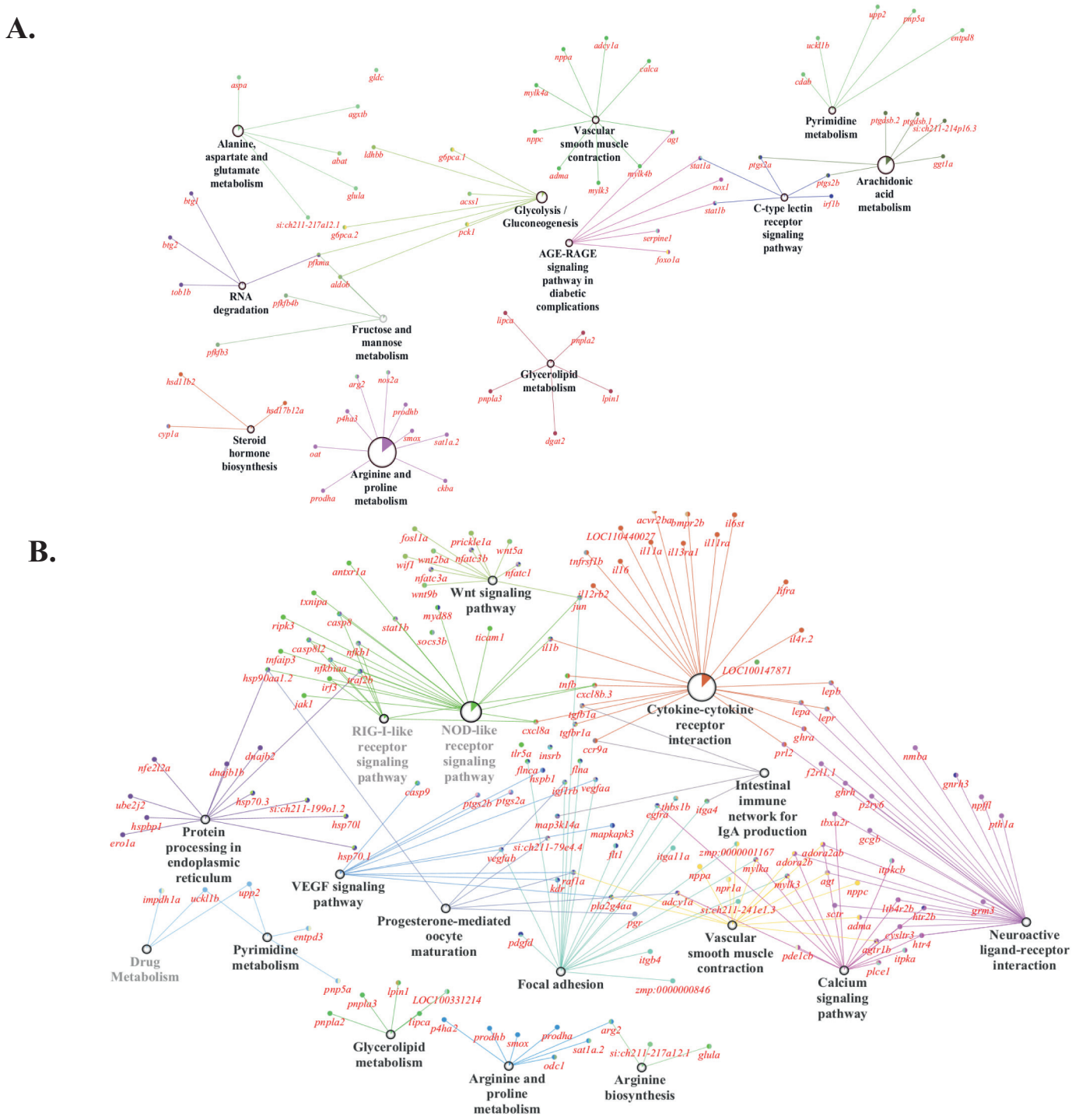

Fig. 9. Network plot showing the connection between the enriched pathways and DEGs that were downregulated after the 96 h exposure of (A.) DDE and (B.) NPs + DDE. The size of the node grows in proportion to the significance (adjusted *p*-value) of the pathway, and vice versa. The color filled in the node represents the pathway's percentage completion. Transcripts with an adjusted $p < 0.001$ and Log2 fold change of $> +1$ or < -1 were considered DEGs.

Stamper, 2009). The GO term response to hypoxia was more strongly enriched in larvae exposed to NPs + DDE (5.09 times) than to the DDE alone (3.74 times). Additionally, significant hypoactivity was observed in NPs + DDE exposed larvae. Moreover, a significant difference in locomotory behaviour (distance, velocity, acceleration, stasis, heading, angular velocity and rotation) was seen in this group only. Numerous studies have found that interference with the neuromotor pathway causes locomotory dysfunction (Kalueff et al., 2013; Pedersen et al., 2020). However, our study's transcriptomic data did not support dysregulation in the neuromotor pathway of zebrafish larvae exposed to NPs + DDE.

Locomotory or swimming behaviour experiments showed non-significant hypolocomotion in zebrafish larvae exposed to DDE. In support of this, the transcriptional results indicated locomotory dysfunction at the molecular level. In fish, locomotion is mainly governed by the central neural system (Zhang et al., 2008). Enriched GO terms for locomotory dysfunction, such as decreased neuropeptide hormone activity and neuron

apoptotic process, were observed in larvae exposed to DDE. Many studies have documented that changes in neurotransmitter levels induce altered locomotion in fish (Dong et al., 2022; Qiu et al., 2022). Other DDE-induced downregulated GO terms included entrainment of the circadian clock by photoperiod and photoperiodism. The circadian clock or biological clock is a time-keeping mechanism within the body that modulates physiological activities such as metabolism, homeostasis and behaviour (Dunlap, 1999). Several studies have demonstrated the importance of circadian rhythms in the swimming behaviour of fish by modulating the expression of two key genes, namely *cry* and *per* (Mech et al., 2022; Sloin et al., 2018). We also found downregulation of genes from these families, such as *cry1*, *cry2*, *cry5*, *per2*, and *per3*. Furthermore, disruptions in circadian rhythms may also cause cardiovascular disease and can even lead to cancer (Thosar et al., 2018).

The presence of 50 mg/L NPs in the mixture group (NPs + DDE) enhanced the toxicological effects of DDE. The exposure resulted in numerous larval deformities such as pericardial edema, reduced yolk resorption, an uninflated swimming bladder, and lordosis. Moreover, we also found a significant difference in the head-to-trunk angle in the larvae exposed to NPs + DDE. Several studies have revealed that variations in developmental regulatory genes like *sox*, *pax*, and *wnt* cause larval deformity (Cermenati et al., 2008; Hayes et al., 2014; Ikenaga et al., 2011). In this experiment, exposure to NPs + DDE but not DDE alone affected the expression of multiple developmental regulatory genes (*sox18, sox11b, pax7a, pax7b, wnt9b, wnt5a, wnt4b,* and *wnt2ba*). In zebrafish, *sox* and *pax* family genes regulate embryo development and cell fate (Krauss et al., 1991; Sarkar and Hochedlinger, 2013), whereas *wnt* family genes play a significant role in cell growth and differentiation (Ueno et al., 2007).

Similar to the DDE group, we also found a significant decrease in heartbeat and a significant increase in the pericardial edema area in co-exposed larvae (NPs + DDE). We observed 199 DEGs associated with the cardiovascular system in this group, whereas only 12 DEGs were observed in larvae exposed to DDE alone. In line with this, more significantly affected GO terms connected with cardiac functions (heart morphogenesis, heart development, heart formation and angiogenesis negative regulation) were observed in co-exposed larvae than in larvae exposed to DDE alone. KEGG enrichment analysis predicted impact on pathways associated with the heart, such as vascular smooth muscle contraction, focal adhesion and vascular endothelial growth factor pathway. The vascular smooth muscle contraction pathway was downregulated in both the DDE and NPs + DDE exposed larvae. In contrast, the vascular endothelial growth factor and focal adhesion pathways were only downregulated in NPs + DDE exposed larvae. The vascular endothelial growth factor pathway plays a crucial role in angiogenesis and heart development (Nasevicius et al., 2000). At the same time, the focal adhesion pathway is required for proper heart valve morphology in zebrafish (Gunawan et al., 2019). A study by Lin et al. (2022) found that 100 μg/mL PS-NPs can cause myocardial fibrosis and autophagy in mice by disrupting the TGF-β1 pathway. This pathway is required for cardiac myocyte development in zebrafish (Peng et al., 2021), and disruption in this pathway can result in myocardial fibrosis and diastolic dysfunction (Kuwahara et al., 2002). In our study, we also found downregulation of key genes (*tgfbr2a*, *tgfb1a*, *tgfbr3* and *tgfbr1a*) associated with the TGF-β1 pathway in the zebrafish larvae exposed to NPs + DDE but not to NPs alone. The plausible reason of no cardiotoxic effect in zebrafish larvae exposed to NPs could be low exposure dose. Overall, the results indicate that NPs aggravate the cardio toxicological effects of DDE in the zebrafish larvae. Moreover our results are in line with a recent study that showed that a combination of NPs with herbicides produces cardiotoxic effects in zebrafish, whereas the herbicide alone did not induce any cardiotoxic effects (Santos et al., 2022).

NPs can also have adverse effects on the immune organs of zebrafish (Cheng et al., 2022). However, we found no differences in the expression of immune-related GO terms or KEGG pathways in NPs and DDE-exposed larvae. Interestingly, downregulation of GO terms associated with immunity, such as immune system development, antimicrobial humoral immune response mediated by antimicrobial peptide, innate immune response-activating signal transduction and activation of the innate immune response, was observed in the NPs + DDE exposed larvae. Co-exposure with NPs + DDE also affected immunity-related pathways such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) signalling pathways. TLRs and NLRs are specific types of proteins that play a vital role in the innate immunity of mammals by recognizing bacterial pathogens (Fritz and Girardin, 2005; Medzhitov, 2001). TLRs and NLRs recognize pathogens and eliminate them by rapidly activating innate immunity and producing proinflammatory cytokines (Majewska and Szczepanik, 2006). The impact on these key pathways might explain the predicted immune response state of impeded immune response in the larvae belonging to the NPs + DDE group. Inflammation is one of the immune system's first responses to a variety of stressors. We also found indications of disrupted inflammatory responses in the larvae exposed to NPs + DDE only. These responses were observed at the transcriptomic level, where we found affected GO terms related to inflammation such as inflammatory response to wounding and regulation of inflammatory response. Pathway analysis also indicated similarly affected pathways such as the MAPK signalling pathway and FoxO signalling pathway.

DDE is a well-known endocrine disruptor and reproductive toxicant (Wu et al., 2019). According to Monteiro et al. (2015), DDE acts as an endocrine disruptor by inhibiting the ability of androgen to bind to its receptors. Surprisingly, no dysregulated endocrine GO terms or pathways were seen in larvae exposed to DDE alone. However, in larvae co-exposed with NPs + DDE, GO terms such as endocrine system development and pancreas development were downregulated. These results indicate that NPs enhanced the endocrine disrupting effect of DDE. A study by Chen et al. (2017) showed that NPs can facilitate the uptake of bisphenol-A in the zebrafish. Similarly in our study, the presence of PS-NPs might have increased the bioavailability of DDE by disrupting the cell membrane, which could explain the increased toxic effects. In line with this, Lin et al. (2021) showed that even noncytotoxic concentrations of PS-NPs (128 μg/mL) when co-exposed with arsenic disrupted the fluidity of the cell membrane and cytoskeleton by inhibiting the activity of ABC (ATP-binding cassette) transporters, resulting in arsenic accumulation in the cells. ABC transporters are membrane-associated ATPases which play a major role in extracellular substrate efflux (Hoffmann and Kroemer, 2004). In our study, we also found downregulation of ABC family genes such as *abcc6b.2* and *abcd1* in zebrafish larvae exposed to NPs + DDE only. This suggests that PS-NPs-induced impairment of the cell membrane could have influenced the normal functioning of ABC transporters, which in turn influenced influx of DDE. Another plausible reason is that the sorption and desorption equilibrium between DDE and PS-NPs in zebrafish might have slowed down DDE metabolism. This could in case lead to higher DDE uptake and exaggerate the toxic effects in the NPs + DDE group only.

Humans are primarily exposed to DDT through consuming meat products, mainly fish. Although environmental DDT levels have been reduced over time, traces of it are still found in fish feed, fish oil and even fish fillet (Garrison et al., 2014). It is imperative to understand whether the consumption of fish with trace levels of DDT can affect humans or not. According to a dietary study, consumption of carp containing traces of DDE (0.028 mg/kg) was deemed safe (Kasza et al., 2020). But even so, the presence of a single pollutant in the environment is extremely rare; it is the presence of multiple pollutants that affects the ecosystem (Weisse et al., 2013). Our study showed that zebrafish larvae exposed to DDE alone had no or minimal toxic effects on some parameters. However, co-exposure with NPs and DDE caused behavioural, cardiotoxicity, and inflammatory responses.

5. Conclusions

The combined toxicity of NPs and pesticide residues in the environment is an unexplored field that necessitates further investigation. Due to their large surface area, NP particles serve as potential carriers for environmental pollutants. In this study, we found that PS-NPs enhanced DDE toxicity, resulting in morphological abnormalities, hypolocomotion, increased oxygen consumption, bradycardia, and inflammation in zebrafish larvae. These

findings are noteworthy as NPs emerge as ubiquitous pollutants. Overall, that the Trojan horse effect of NPs should not be overlooked when assessing the risks of these plastic particles.

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CRediT authorship contribution statement

Shubham Varshney: Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Adnan H. Gora:** Methodology, Formal analysis, Writing – review & editing. **Viswanath Kiron:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Prabhugouda Siriyappagouder:** Methodology, Writing – review & editing. **Dalia Dahle:** Methodology, Writing – review & editing. **Tanja Kögel:** Conceptualization, Supervision, Writing – review & editing. **Robin Ørnsrud:** Conceptualization, Supervision, Writing – review & editing. **Pål A. Olsvik:** Project administration, Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Data availability

The RNA-seq data can be found at the NCBI sequence read archive (SRA) under the BioProject ID: PRJNA869565.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.160457.

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