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From nanoplastics to chemical pollutants: Exploring mixture toxicity in zebrafish

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FACULTY OF BIOSCIENCES AND AQUACULTURE

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Preface

This thesis is submitted in fulfilment of the requirements for the degree of Philosophiae Doctor (PhD) at the Faculty of Biosciences and Aquaculture (FBA), Nord University, Bodø, Norway. The studies included in this dissertation represent original research that was carried out over a period from 01.12.2020 to 01.12.2023 at Nord University, Bodø.

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The core project team consisted of the following members:

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Kiron Viswanath, Professor, FBA, Nord University: Co-supervisor

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Robin Ørnsrud, Program director, Institute of Marine Research, Bergen: Co-supervisor

Shubham Varshney

Bodø, 7 December 2023

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There are so many others whom I may have inadvertently left out. I sincerely thank all of them for their help.

Shubham Varshney

Bodø, Norway

Contents

List of abbreviations

6PPD	-	n-(1,3-dimethylbutyl)-N’-phenyl-p-phenylenediamine
7PPD	-	n-(1,4-dimethylpentyl)-N'-phenyl-p-phenylenediamine
Ach	-	acetylcholine
As	-	arsenic
CAS	-	chemical abstracts service
Cu	-	copper
DDD	-	dichlorodiphenyldichloroethane
DDE	-	dichlorodiphenyldichloroethylene
DDT	-	dichlorodiphenyltrichloroethane
DEGs	-	differentially expressed genes
DVS	-	daniovision system
ERTMA	-	European Tyre and Rubber Manufacturers Association
EVs	-	electric vehicles
EVS	-	ethovision system
GABA	-	gamma-aminobutyric acid
GO	-	gene ontology
HCB	-	hexachlorobenzene
HPF	-	hours post-fertilization
IFN-γ	-	interferon gamma
IGFBP3	-	insulin-like growth factor binding protein 3
IL	-	interleukins
IPPD	-	N-Isopropyl-N’-phenyl-1,4-phenylenediamine
KEGG	-	kyoto encyclopedia of genes and genomes
LC50	-	lethal concentration 50
LOQ	-	limit of quantification
MAPK	-	mitogen-activated protein kinases
MPs	-	microplastics
MPs/NPs	-	micro/nanoplastics
NF-κB	-	nuclear factor-kappa B
Ni	-	nickel
NOXs	-	nicotinamide adenine dinucleotide phosphate hydrogen oxidases
NPs	-	nanoplastics
OTC	-	oxytetracycline
p,p’-DDE	-	*p,p’*-Dichlorodiphenyldichloroethylene
PA	-	polyamide
Pb	-	lead

PCBs	-	polychlorinated biphenyls
PCDD	-	polychlorinated dibenzo-p-dioxins
PE	-	polyethylene
PES	-	polyester
PET	-	polyethylene terephthalate
PFOS	-	perfluorooctane sulfonic acid
PM	-	particulate matter
POPs	-	persistent organic pollutants
PP	-	polypropylene
PS	-	polystyrene
PS-NPs	-	polystyrene nanoplastics
RARα	-	retinoic acid receptor α
ROS	-	reactive oxygen species
RTgill-W_1	-	rainbow trout gill cell line
RXRα	-	retinoid X receptor α
Th1	-	type 1 T helper
Th2	-	type 2 T helper
TLRs	-	toll-like receptors
TNF-α	-	tumour necrosis factor-alpha
TWNPs	-	tyre-wear nanoparticles
TWPs	-	tyre-wear particles
URMS	-	urban runoff mortality syndrome
USTMA	-	U.S. Tire Manufacturers Association

List of Figures and Tables

List of Papers

Paper I

Varshney, S., Gora, A.H., Siriyappagouder, P., Kiron, V. and Olsvik, P.A., 2022. Toxicological effects of 6PPD and 6PPD quinone in zebrafish larvae. *Journal of Hazardous Materials*, *424*, p.127623. doi.org/10.1016/j.jhazmat.2021.127623

Paper II

Varshney, S., Gora, A.H., Kiron, V., Siriyappagouder, P., Dahle, D., Kögel, T., Ørnsrud, R. and Olsvik, P.A., 2023. Polystyrene nanoplastics enhance the toxicological effects of DDE in zebrafish (*Danio rerio*) larvae. *Science of the Total Environment*, *859*, p.160457. doi.org/10.1016/j.scitotenv.2022.160457

Paper III

Varshney, S., O'Connor, O.L., Gora, A.H., Rehman, S., Kiron, V., Siriyappagouder, P., Dahle, D., Kögel, T., Ørnsrud, R. and Olsvik, P.A., 2023. Mixture toxicity of 6PPD-quinone and polystyrene nanoplastics in zebrafish.

(Manuscript)

Summary (English)

Plastics pose an environmental threat, contributing to the proliferation of microplastics (MPs) and nanoplastics (NPs) that contaminate ecosystems. Tyre-wear is a significant source of MPs/NPs to both aquatic and terrestrial ecosystems. Globally, over 2.36 billion vehicle tyres are produced each year. Tyre-wear also contains many harmful chemicals. Additionally, today, it is seldom only a single pollutant but rather a multitude of pollutants present in nature. Other common pollutants in the environment are persistent organic pollutants, pharmaceuticals and heavy metals. This thesis presents novel knowledge on the single and combined toxicity of tyre-wear chemicals, NPs and persistent organic pollutants. To achieve this, zebrafish was used as an animal model. Zebrafish is a well-established model in ecotoxicological studies of emerging and legacy pollutants.

6PPD is an antioxidant added to rubber tyres to prevent their cracking. A derivative of 6PPD, 6PPD-quinone (6PPDq), was implicated in the observed salmon mortality in the United States. In the first study, we investigated the impact of 6PPD and 6PPDq in zebrafish larvae. Our findings suggest that exposure to either toxicants at environmentally relevant concentrations did not significantly affect their growth, development, swimming behaviour and heart rate. However, exposure to elevated concentrations did result in noticeable effects. These findings improve our understanding of the toxicity of 6PPD and 6PPDq in the early life stages of fish.

The second study focused on the individual and mixture toxicity of NPs and p,p'- DDE (2,2-Bis(4-chlorophenyl)-1,1-dichloroethylene) in zebrafish larvae at environmentally relevant concentrations. p,p'- DDE is a metabolite of DDT (dichloro-diphenyl-trichloroethane). DDT is a legacy pollutant that can still be found in fish feed and in the fillets of farmed Atlantic salmon and rainbow trout, even though it was banned in the 1970s. The results showed that exposure to either p,p'- DDE or NPs + p,p'- DDE but not to NPs significantly affected development, swimming behaviour, heart rate, and

oxygen consumption rate. Notably, exposure to NPs alone did not result in a significant effect on these parameters. However, the toxic effect was more substantial in the larvae exposed to the combination of NPs and p,p'- DDE. The transcriptomic results showed that few cardiac, vascular, and immunogenic pathways were only affected in the larvae exposed to the combination of NPs and p,p'- DDE.

The third study of the thesis investigated the individual and mixture toxicity of NPs and 6PPDq in adult male zebrafish. No toxic effect of NPs was observed in swimming behaviour endpoints. But, exposure to both 6PPDq and a mixture of NPs and 6PPDq caused significant hyperlocomotion in the zebrafish. However, the effect was more substantial in zebrafish exposed to a mixture of the contaminants. The stronger toxic effects in zebrafish exposed to a combination of substances were also evident at the transcriptomic level. KEGG pathway analysis suggested compromised mitochondrial function in the intestine and metabolism disruptions in the liver.

This PhD project has generated novel knowledge about the toxicological effects of emerging pollutants such as NPs, 6PPD and 6PPDq using zebrafish as an animal model. The PhD project also showed the importance of understanding the toxic effects of a mixture of contaminants.

Summary (Norwegian)

Plastforurensning utgjør en miljøtrussel, og bidrar til spredning av mikroplast (MP) og nanoplast (NP) som kan forstyrre økosystemer. Dekkslitasje er en betydelig kilde til MP og NP både i akvatiske og terrestriske økosystemer. Globalt produseres det over 2,36 milliarder dekk til kjøretøy hvert år. Dekkslitasje-partikler inneholder også mange skadelige kjemikalier. I naturen finner en sjeldent bare enkeltforbindelser. Det er derfor viktig også å studere kombinasjonseffekter av miljøgifter. Andre vanlige miljøgifter en ofte finner i naturen er persistente organiske miljøgifter, legemidler og tungmetaller. Denne oppgaven presenterer ny kunnskap om toksisiteten til dekkslitasjekjemikalier, nanoplast og persistente organiske miljøgifter, alene og i kombinasjon. Sebrafisk ble brukt som dyremodell i disse forsøkene. Sebrafisk er en veletablert dyremodell som ofte brukes i økotoksikologiske studier av gamle og nye miljøgifter.

6PPD er en antioksidant som tilsettes i gummidekk for å forhindre at de sprekker. En studie i USA har vist at et derivat av 6PPD, 6PPD kinon (6PPDq), forårsaket laksedødelighet i USA etter avrenning fra veier. I den første studien undersøkte vi virkningen av 6PPD og 6PPDq i sebrafisklarver. Våre funn tyder på at eksponering for miljørelevante konsentrasjoner av disse stoffene ikke påvirket vekst, utvikling, svømmeatferd og hjertefrekvens signifikant i sebrafisklarvene. Imidlertid resulterte eksponering for forhøyede konsentrasjoner i merkbare effekter. Disse funnene øker vår forståelse av toksisiteten til 6PPD og 6PPDq i tidlige livsstadier hos fisk.

Den andre studien fokuserte på giftigheten av miljørelevante konsentrasjoner av NP og p,p'-DDE (2,2-Bis(4-klorfenyl)-1,1-dikloretylen), alene og i kombinasjon, i sebrafisklarver. p,p'-DDE er en metabolitt av DDT (diklor-difenyl-trikloretan). DDT er et insektdrepende middel som det fortsatt kan finnes rester av i fiskefôr og i oppdrettet atlantisk laks og regnbueørret, selv om det ble forbudt på 1970-tallet. Resultatene fra denne studien viser at eksponering for enten p,p'-DDE eller NPs + p,p'-DDE påvirket

utvikling, svømmeatferd, hjertefrekvens og oksygenforbruk signifikant i sebrafisklarvene. Eksponering for NP alene ga ingen effekt på disse parametrene. Den toksiske effekten var betydelig sterkere i larvene som ble eksponert for en kombinasjon av NP og p,p'-DDE. Resultatene fra genekspresjonsstudiene viste at mekanismer knyttet til hjerte- og vaskulær funksjon og immunsystemet kun ble påvirket i larvene som ble eksponert for kombinasjonen av NP-er og p,p'-DDE.

Den tredje studien i denne oppgaven undersøkte giftigheten av NP og 6PPDq, alene og i kombinasjon, i voksne sebrafisk-hanner. Eksponering for NP alene ga ingen effekt på svømmeatferd. Men eksponering for både 6PPDq og blandingen av NP og 6PPDq resulterte i hyperaktivitet hos sebrafisken. Effekten var betydelig sterkere hos sebrafisken som ble eksponert for begge stoffene samtidig. Genekspresjonsdataene viste også en sterkere effekt hos sebrafisk som ble eksponert for en kombinasjon av stoffene. KEGG pathway analyse viste at mitokondriell funksjon i tarmen og metabolisme i leveren ble påvirket av kombinasjonen av stoffene.

Dette doktorgradsprosjektet har økt kunnskapen om de toksikologiske effektene av nye forurensninger som NP, 6PPD og 6PPDq ved å bruke sebrafisk som en dyremodell. PhD-prosjektet viser også viktigheten av å forstå blandingseffekter av miljøgifter.

1 Introduction

1.1 Environment and pollutants

The term "environment" portrays the intricate interplay of physical, chemical, and biological elements that constitute the surroundings of living organisms. In a superficial sense, it is everything that surrounds living beings and includes external factors and conditions affecting living organisms. However, no environment, whether aquatic, terrestrial, or any other, is devoid of pollutants. According to the United States Environment Protection Agency (EPA), the term "pollutants" refers to any physical, chemical, or biological entity that harms the environment and its living and non-living components (EPA, 2012). These pollutants negatively impact critical ecosystem services such as primary production, biodiversity maintenance, climate regulation, and nutrient cycling (Erisman et al., 2013, Johnston et al., 2015). According to a report from the World Health Organisation, air pollutants have caused 4.2 million premature deaths of humans worldwide through several diseases in 2019 (WHO, 2022). Also, people living in polluted areas have a 20% higher chance of death due to lung cancer than people living in comparatively less polluted areas (Grens, 2011). These environmental pollutants not only affect humans but also affect the inhabiting organisms in their respective environments. A report from the United Nations states that every year pollution kills one million seabirds (United Nations, 2017).

1.2 Major pollutants in the aquatic environment

Over the years, aquatic ecosystems such as marine, riverine or lacustrine were considered as sinks for pollutants. According to Mateo-Sagasta et al. (2017), around 80% of untreated municipal wastewater gets released into waterbodies annually. Similarly, industries release significant amounts of effluents containing substantial amounts of heavy metals in the waterbodies (Okereafor et al., 2020). Apart from this, agriculture utilizes 70% of terrestrial waters and brings pesticides, insecticides, and other chemicals into the aquatic ecosystem (Pimentel et al., 1997). Since agriculture

runoff is not a point pollution source, identifying and mitigating the source becomes very difficult (Xia et al., 2020). The major pollutants in the aquatic environment are diverse and multifaceted, encompassing amongst others plastic debris, heavy metals, perfluorinated compounds, pesticides, herbicides, nutrients and pathogens (Müller et al., 2020, Schwarzenbach et al., 2010).

1.2.1 Plastics

Plastic pollution is an emerging research topic that needs further investigation. Plastic pollutes our environment at every level of its life cycle, from production to disposal. According to PlasticsEurope (2015), plastic production and manufacturing into final goods consumes around 8% of the world's annual petroleum reserves. The era of plastic started with the invention of the first fully synthetic fibre, i.e., "bakelite", in 1907 by Leo Baekeland through polycondensation of phenol with formaldehyde (Baekeland, 1909). Due to its miraculous properties, such as high versatility, light weight and affordability, it soon became popular. However, rising demand for plastics did not occur until the 1950s. But over the next 70 years, the annual production of plastics boosted nearly 200 times, from 2 million metric tons in 1951 to 390.7 million metric tons in 2021 (Statista, 2021). Moreover, the global production of plastics is increasing at an annual rate of 4% (Statista, 2021). Plastics has an almost infinite number of potential applications. According to global plastics consumption data, the packaging sector (36%) consumes the majority of the produced plastics, followed by construction (16%), textiles (15%), consumer goods (10%), transportation (7%), and other sectors (16%) (Statista, 2021). China is the top plastic-producing nation and holds a share of 32% of global plastic production (PlasticsEurope, 2021). The average lifespan of a plastics product is around ten years, whereas it can also take around 450 years to decompose naturally, depending on its chemical composition (LeBlanc, 2017). A report from Boucher and Friot (2017) concluded that per capita release of plastics ranges from 110 to 750 grams/person/year based on geographical region. It is also estimated that the oceans contain 75 to 199 million tonnes of plastic waste, which is expected to triple by 2060 (McGlade et al., 2021). The increasing global plastic production, slow

decomposition and poor disposal management have led to the accumulation of plastics throughout the oceans. This massive amount of plastic waste in the ocean has affected marine life and ecosystems to a great extent. The most noticeable harm caused by plastic waste is ingestion, suffocation, entanglement, and internal injuries to animals. This serious problem is reflected in the fact that 60% of all seabird species have eaten plastic pieces, and this figure is expected to rise to 99% by 2050 if we continue with current trend of plastics production and waste disposal practices (Wilcox et al., 2015). Due to the widespread presence and significant impact of plastic on the Earth's ecosystem, the term "Plasticene" was coined to describe the current era (Rangel-Buitrago et al., 2022). Plasticene underlines the need for urgent and compelling actions to address the challenges posed by plastic pollution. In the midst of this so-called plastic era or plasticene, an associated problem is caused by microplastics (MPs).

1.2.1.1 Microplastics: Types and sources

The first use of the term "microplastics" is attributed to a widely cited paper published in 2004 (Thompson et al., 2004). MPs are generally referred to as plastic particles smaller than 5 mm in length (Barnes et al., 2009). MPs can occur in the aquatic environment as primary or secondary particles (Cole et al., 2011). Primary MPs are tiny particles designed for commercial use, such as cosmetics (Guerranti et al., 2019, Lei et al., 2017, Napper et al., 2015), as well as synthetic microfibers, shed from clothing, and other textiles (Mathalon and Hill, 2014, Remy et al., 2015). Secondary MPs are plastic particles that are produced from the breakdown of or abrasion from substantially large plastic items, such as packaged drinking water bottles, wrappers, bags, and fishing nets, over time (Thompson, 2019). This breakdown is mainly caused by exposure to environmental factors, solar radiation, and ocean waves. MPs are a global challenge in aquatic bodies, reported even in Antarctica (Zarfl and Matthies, 2010). While debris from larger plastic objects are readily noticeable, whereas MPs (particles less than 5 mm without lower limit) often escape detection by the naked eye. Due to this, it is cumbersome to quantify the total amount of MPs in the ocean, but it has been estimated that there could be 170 trillion plastic particles floating in the world oceans

weighing around 1.1-4.9 million tonnes (Eriksen et al., 2023). Dense MPs such as PET (polyethylene terephthalate), PA (polyamide), and PES (polyester) accumulate on the ocean floor, implying that a significant amount of MPs are also present in the bottom sediments (Boucher and Friot, 2017). Like larger plastics, MPs also biodegrade slowly (Miri et al., 2022, Sun et al., 2022). Thus, they persist and may be taken up in organisms once they enter the environment. MPs have been quantified in marine water with reported concentration values ranging from 65 to 433 particles per m^3 (Baltic Sea), 29 to 424 particles per m^3 (Mediterranean Sea) and 21 to 344 particles per m^3 (Atlantic Ocean) (Long et al., 2015). The investigation into MPs in the aquatic ecosystem encompasses two quantification approaches: number-based and mass-based, each shedding light on different aspects of pollution. Number-based studies provide insight into the sheer abundance of MPs, quantifying the particles per unit weight or volume. Meanwhile, mass-based studies focus on the total weight of MPs present, offering a perspective on the potential environmental impact. The global average number of MPs in world ocean water's surface, column and sediment are 0.79, 0.042 and 473.17 particles per kg, respectively (Booth et al., 2017). However, the average number of MPs in Norwegian surface water is 1.8 particles per g (Booth et al., 2017, Magnusson and Norén, 2011). The average number of particles found in Norwegian beaches, shorelines, and sediment samples was 122.62 particles per kg in the samples collected in June 2015 (Sundet et al., 2016). Gomiero et al. (2019) found concentrations exceeding 100 µg/kg of MPs (dry weight) in various coastal water sediments sampled throughout Norway in the year 2016. Predominantly, the identified MPs were polyethylene (PE), polypropylene (PP) and polyvinyl chloride (PVC) (Gomiero et al., 2019). The other polymers which were found in less amounts were PET, polystyrene (PS), polyamide 66 (PA66) and polycarbonate (PC) (Gomiero et al., 2019). A recent report (MIKRONOR) has revealed the presence of a substantial amount of MPs (>1000 µg/g dry weight) in marine sediment samples collected from the Akerhuskaia, Oslo in the year 2023 (Alling et al., 2023). Notably, approximately 80% of these particles fall within the size range of 1-5 mm, with a lower detection limit of 50 µm. In the same report, authors found an

average of 21.2 µg/L MPs in wastewater samples collected from 15 different locations across Norway (Alling et al., 2023). Among them, the highest amount of MPs (250 µg/L) was found in the sample collected from the HIAS water treatment plant in Hamar (Alling et al., 2023). Similarly, around 150 µg/L MPs were found in urban runoff samples collected from Hasle and Hamar (Alling et al., 2023). In this study, 22 distinct types of MPs were analyzed (Alling et al., 2023), The predominant MPs detected in wastewater and urban runoff samples were PE, PP, and PES respectively (Alling et al., 2023).

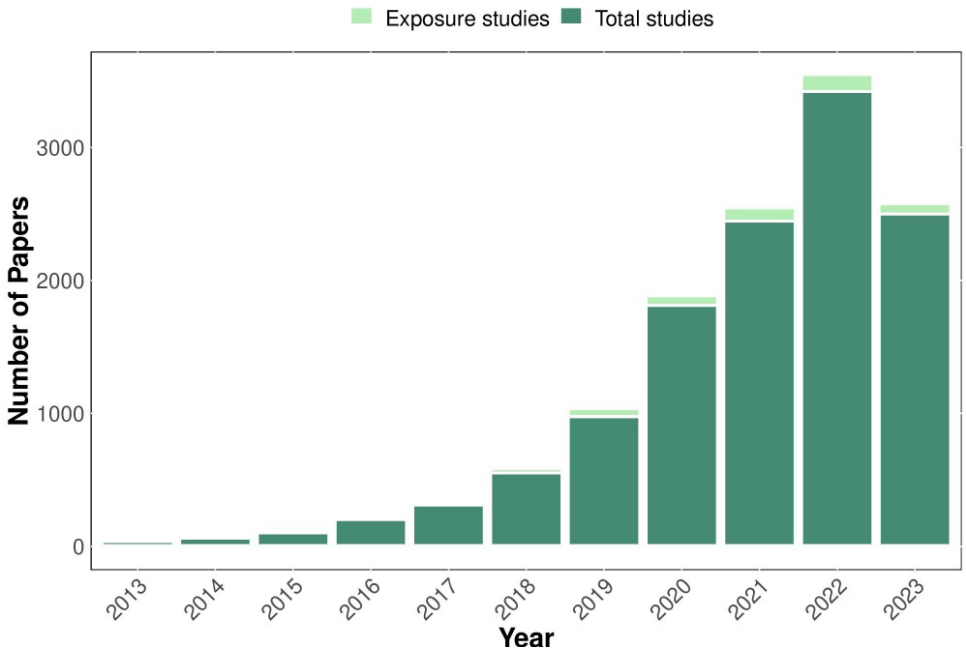

Figure 1. *PubMed literature search results displayed as the number of publications on MPs from Jan 2013 to July 2023. The search was performed on 10 August 2023. The figure was generated by searching for the terms "microplastic" or "microplastics" and classifying the identified publications as exposure studies or not.*

As a research topic, MPs have captured significant scientific attention, evidenced by the increase in scientific publications over the years (**Fig 1.**). There has been a substantial growth in the number of published papers related to MPs in recent years. Most of the studies focused on identifying, characterising, and quantifying MPs in fish, water or soil. Contrastingly, only 4% of total MPs studies focused on the ecotoxicity of

MPs on organisms. Most of these ecotoxicity studies (> 85%) used primary MPs such as plastic pellets, plastic glitter, plastic powder, microbeads, and microfibers for exposure. Only a few studies used secondary MPs, such as tyre wear particles or particles originating from the breakdown of plastic packaging items and fishing gears for exposure.

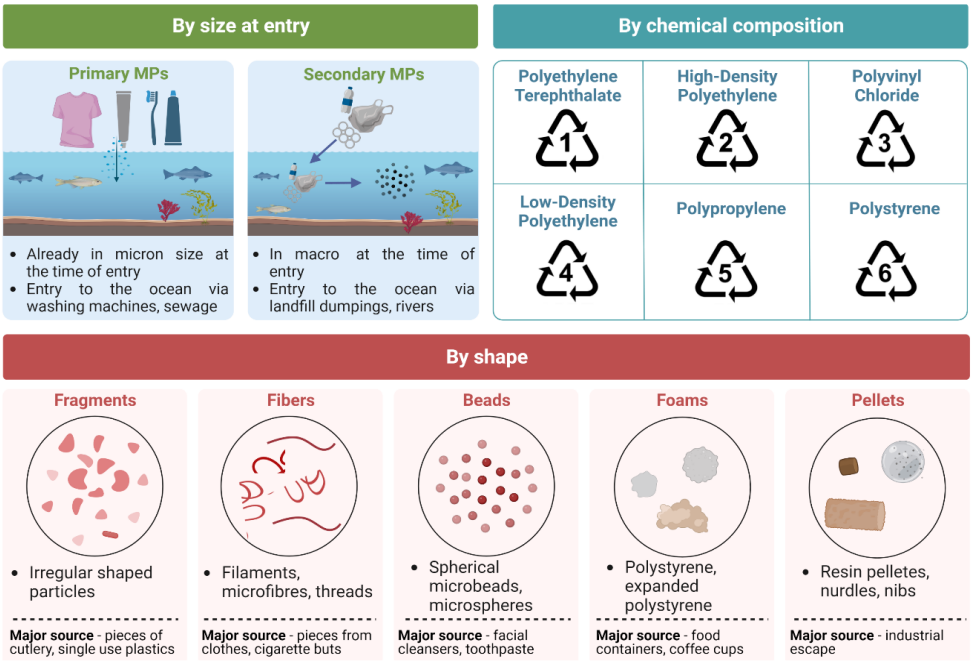

Figure 2. *Classification of microplastics (MPs) based on their size at entry, chemical composition and shape. Primary MPs enters the aquatic ecosystem in the form of micro-sized particulates whereas secondary MPs are formed due to the fragmentation of large debris in the ocean. On the basis of chemical composition, they can be classified as polypropylene (PP), polyethylene (PE), polyamide (PA), polystyrene (PS), polyvinyl chloride (PVC), and polyethylene terephthalate (PET) microplastics. Further, on the basis of their shape they can be classified as fragments, fibers, beads, foams and pellets. Information obtained from (Lusher et al., 2017, Martin et al., 2017). Created with BioRender.com.*

MPs can be classified based on their shape as fibres, fragments, beads, pellets and foams **(Fig 2.)**. Fibres are among the most prevalent MPs in aquatic ecosystems and usually come from clothes, diapers, cigarette butts, fishing nets, and ropes (Burns and Boxall, 2018). Fragment-shaped MPs originate from the breakdown of larger plastic items through mechanical and solar forces (Andrady, 2017). They include tyre-wear

particles (TWPs), pieces of cutlery, lids, and various single-use plastic products. Beads are usually primary MPs and enter aquatic ecosystems through sewage and wastewater (Du et al., 2021). They include tiny pieces from facial cleansers, exfoliating soap products, and toothpaste.

Based on the chemical composition of their parent plastics, MPs can be further classified as PP, PE, PA, PS, PVC, and PET MPs **(Fig 2.)**. Among these, PE and PP are two of the most commonly found MPs in the plastic waste (Kiran et al., 2022). Moreover, PE is the most commonly found MPs in fish (Thiele et al., 2021). PE is one of the most common types of MPs, even in the intensive aquaculture systems such as salmon farming, and one of the primary sources is feeding pipes made of high-density PE materials (Abihssira-García et al., 2022).

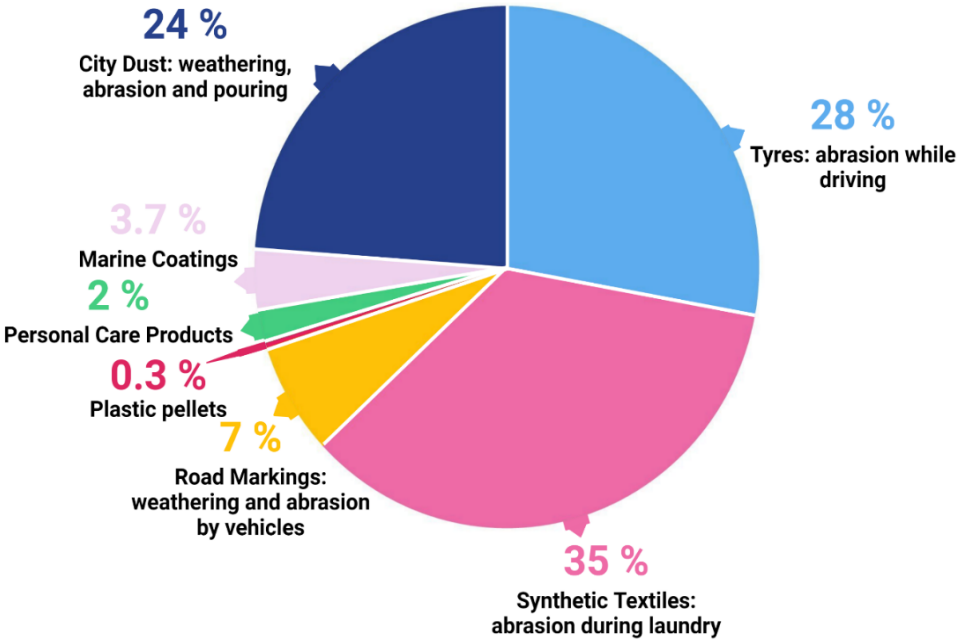

Figure 3. *Classification of the seven major sources of microplastics (MPs) in the ocean. Data obtained from Boucher and Friot (2017). Pie chart was created in R studio and edited with BioRender.com.*

Mismanaged plastic waste is a significant source of plastics pollution for the oceans. According to Jambeck et al. (2015), 4.8 to 12.7 million tons of macroplastics enter the ocean annually. Mismanaged plastics can be managed somewhat easily because they are visible and small-scale ocean cleanups along with adoption of better management practices are possible. Conversely, MPs are making headlines due to their microscopic size and probable harm to the aquatic ecosystem. A single cleanser or facial scrub product can contain 360,000 microbeads (Yurtsever and Yurtsever, 2019). Moreover, Boucher and Friot (2017) estimated that 1.5 million tons of primary MPs annually enter the ocean.

According to the report from Boucher and Friot (2017), there are seven major sources of MPs in the ocean: synthetic textiles, tyres, city dust, road markings, marine coatings, personal care products, and plastic pellets **(Fig 3.)**. Synthetic textiles, tyres and city dust contribute almost to 87% of the total release of MPs into the ocean **(Fig 3.).** Among them, abrasion from synthetic textiles during laundry is the largest major source of the ocean's MPs (35%). These MPs are fibre-shaped and are predominantly made of PES and PE. Abrasion from tyres or tyre-wear particles is the second largest contributor to MPs by mass in the ocean (Boucher and Friot, 2017). Tyres erode over time due to frictional forces between tyres and roads, forming TWPs (Kole et al., 2017). These TWPs end up in the ocean via the atmosphere or runoff water (Siegfried et al., 2017). City dust is the third largest source of MPs (Boucher and Friot, 2017). It includes losses from abrasion of objects (footwear, cooking utensils) and abrasion of infrastructure (household dust, city dust, artificial turfs, harbours, buildings) (Boucher and Friot, 2017). Due to the small size of their individual contributions, these are grouped together under the single category as city dust **(Fig 3.)**.

1.2.1.2 Nanoplastics

Nanoplastics (NPs) are plastic particles less than 1000 nm (diameter) and ubiquitous, like MPs (Alimi et al., 2018, Fang et al., 2023a, Khan and Jia, 2023). Fragmentation of MPs and personal care products like shaving creams and cleansers are the major

sources of NPs in the ocean (Peng et al., 2020). Other sources include effluents from industrial processes, textiles, tyre-wear, and paints (Peng et al., 2020). A study by Yang et al. (2021) found that washing and abrasion of one gram of PES textile releases an average of 2.1 - 3.3 × 10^{11} NPs particles. Boucher and Friot (2017) projected that globally two million tonnes of micro/nanofibers are released into the ocean every year. NPs are distinct from MPs and have different biological effects on animals. Also, as MPs they might serve as vectors for other pollutants (Prüst et al., 2020). Another property of NPs is their large surface to volume ratio, which allows them to easily adsorb other pollutants, such as heavy metals, pesticides, and organic pollutants (Yu et al., 2019). The analysis of publication count shows that there were significantly fewer studies on NPs than on MPs over the years (**Fig 4.**). In 2022, they constituted 19.82% of the studies; in 2021 and 2020, the percentages were 17.74% and 14.50%, respectively (**Fig 4.**). However, there was an increase in the number of studies focusing on NPs as scientists and researchers became more aware of the risks associated with NPs and sought to fill the knowledge gap.

Moreover, numerous analytical methods, such as infrared spectroscopy and Raman spectroscopy, commonly used to examine MPs, do not directly apply to NPs as these methods have size limitations (Yu et al., 2019). One of the main reasons behind this is problem is the process of isolating them, as filtration with such small pore sizes is very difficult, and/or to get enough of them to find them in pyro-GC/MS (Pyro gas chromatography-mass spectrometry) (Ivleva, 2021, Nguyen et al., 2019). Other factors include small size, lack of reference material, background interference, and lack of well-harmonised protocols. Due to these methodological limitations, NPs are not yet adequately studied, and there is a vast knowledge gap in the field of environmental fate and ecotoxicity of NPs.

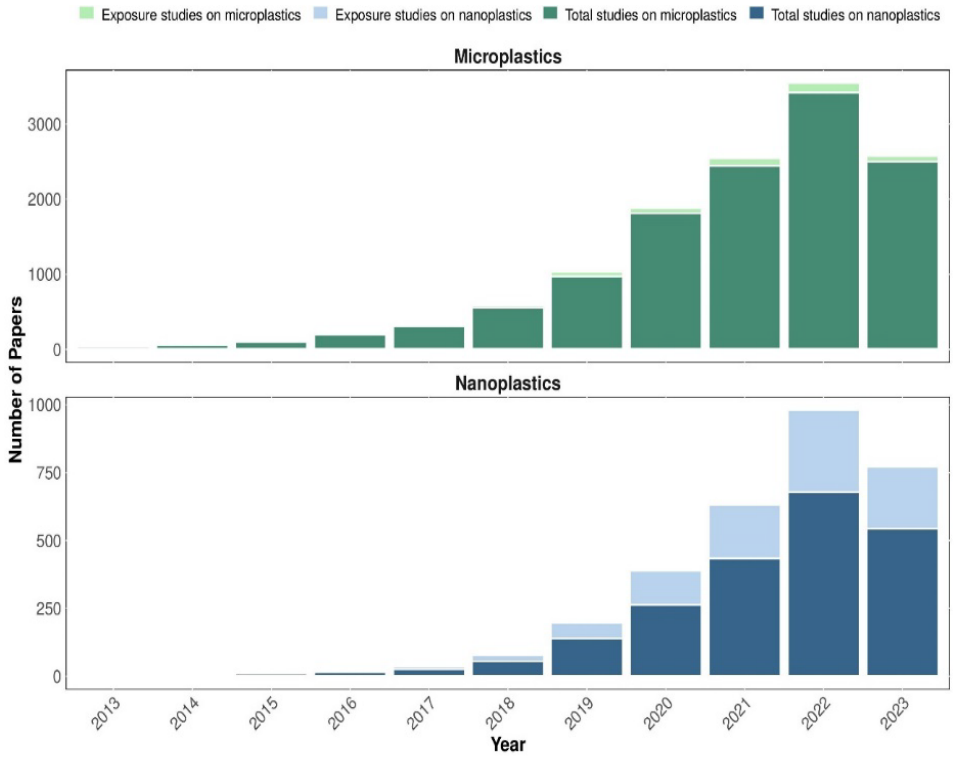

Figure 4. *PubMed literature search results displayed as the number of publications on microplastics and nanoplastics from Jan 2013 to July 2023. The search was performed on 10 August 2023. The figure was generated by searching for the terms "microplastic", "microplastics", "nanoplastic", "nanoplastics" and then further by classifying the publications into exposure studies by searching for "microplastics + exposure", "microplastics + ecotoxicology", "nanoplastics + exposure", "nanoplastics + ecotoxicology".*

1.2.1.3 Harmful effects of micro/nanoplastics: Neurotoxicity and Cardiotoxicity

In aquatic animals, uptake of micro and nanoplastics (MPs/NPs) can occur through gills, skin or ingestion (Ma et al., 2021). Ingestion of these particles can be direct or indirect. Direct ingestion occurs when animals eat them accidentally. In contrast, indirect intake is related to trophic transfer. Several studies have documented ingestion of MPs/NPs, at all trophic levels (Castro-Castellon et al., 2022). These particles can be taken up by small, tiny zooplanktons (Cole et al., 2013, Goswami et al., 2020). They found these particles to be a major cause of concern because their size range mimics the prey size

ingested by many aquatic organisms. MPs/NPs are also known to reduce the organism's feeding rate and alter the individual's growth rate (Sendra et al., 2020, Wang et al., 2021a). MPs/NPs ingestion has been identified in a range of species; it has been predicted that 99% of all seabird species will ingest MPs by 2050 (Wang et al., 2021b). Apart from being a physical entity, these MPs/NPs may also contain different chemicals such as additives (plasticisers, fillers, UV-protection/antioxidants, flame retardants), and surface coatings (PFAS) (Xu et al., 2022). All together, they are known as plasticizers and are added to plastics to improve their flexibility and durability (Godwin, 2017). These plasticizers are known to have potential health effects for both animals and humans (Bui et al., 2016). Phthalates are the most commonly used plasticizer and are known to negatively affect the reproduction and development in all studied animal groups, including fish, crustaceans, birds, and humans (Oehlmann et al., 2009). As we delve deeper into the consequences of MPs, it becomes evident that these plasticizers can have far-reaching effects on wildlife and ecosystems including both aquatic and terrestrial taxa.

The inclusion of behavioural changes in zebrafish when exposed to NPs was a focal point of the thesis. Investigation of behavioural alterations is motivated by the hypothesis that NPs can cross biological barriers such as the blood-brain barrier and gut-brain axis and affect neural functioning, ultimately leading to behavioural dysfunction. MPs/NPs can produce a multitude of adverse effects on fish health, including neurotoxic effects. The extent of toxic effects at the molecular to physiological level can vary significantly based on the polymer type, shape, size and surface properties of NPs (Han et al., 2024, Prüst et al., 2020). For instance, Mazurais et al. (2015) reported that spherical PE MPs were not retained in the intestine of European sea bass (*Dicentrarchus labrax*) larvae, while fibre and fragment-shaped MPs were retained, as reported by Lusher et al. (2013). A study by Gray and Weinstein (2017) reported that the retention time of microspheres varied in the intestine of grass shrimp (116 µm: 27.6 ± 8.57 h, 75 µm: 75.9 ± 13.3 h and 30 µm: 60.6 ± 28.5 h). The authors also found that spherical and fragment-shaped MPs were not acutely toxic, whereas

exposure to fibres-shaped MPs caused significant mortality. Frydkjær et al. (2017) observed that irregular fragments (10-75 μm) exhibited greater retention time and a more pronounced inhibitory effect on the mobility of *Daphnia magna* compared to regular MPs beads (10-106 μm).

However, most studies that focused on the toxicity of MPs/NPs at the molecular level were conducted using PS micro/nanobeads (Prüst et al., 2020). PS micro/nanobeads are widely used in molecular-level toxicity studies due to their uniformity, ease of production, and well-characterized properties (Banerjee and Shelver, 2021). Once ingested, MPs/NPs can enter the bloodstream and circulate to different tissues and organs (Ma et al., 2021). These particles can interact with leukocytes and vascular endothelial cells, generating reactive oxygen species (ROS) (Hu and Palić, 2020). Both MPs and NPs have a similar molecular initiating event: the formation of ROS (Hu and Palić, 2020). Some particles reach the brain and cross the blood-brain barrier to enter the brain parenchyma (Prüst et al., 2020) (**Fig 5.**). In this event, several factors play a key role, such as the size of the particles, the test animal and the life stage of the test animal (Zhou et al., 2018). The interaction of these micro/nanospheres with the brain's cellular biology might reduce the mitochondrial complex enzyme activity and ATP synthesis ability in the mitochondria (Zhang et al., 2023a). This enzymatic dysfunctioning can lead to the leaking of electrons from the electron transport chain, and ultimately generate ROS (Kristanti et al., 2023). Another route of generation of ROS is by activating NADPH oxidases (NOXs). Toll-like receptors (TLRs), as one of the integral parts of the immune system, were responsible for reacting to the endogenic damage to generate superoxide radicals from NOXs (Ogier-Denis et al., 2008). A recent study documented that the TLRs/NOXs signal axis is the brain's main ROS production pathway in response to these PS microspheres (Wu et al., 2022a). The presence of these PS micro/nanospheres and generated ROS can lead to the activation of pro-inflammatory signalling pathways such as NF-κB (nuclear factor-kappa B) and MAPK (mitogen-activated protein kinases) pathways (Chen et al., 2023, Hou et al., 2022). The outcome of these pathways is the production of inflammatory cytokines such as

interleukins (IL) and tumour necrosis factor-alpha (TNF-α) (Deng et al., 2023). These inflammatory cytokines cause microglia to go through phenotypic changes from a resting state to an activated state (**Fig 5.**). The resulting activated microglia further amplify inflammatory responses by generating more cytokines and chemokines, overall causing neurotoxicity (Mahapatra et al., 2023, Yin et al., 2022). The generated neurotoxicity can produce adverse effects at the functional level, such as impaired learning, reduced reproductive capacity and altered swimming behaviour (Mahapatra et al., 2023).

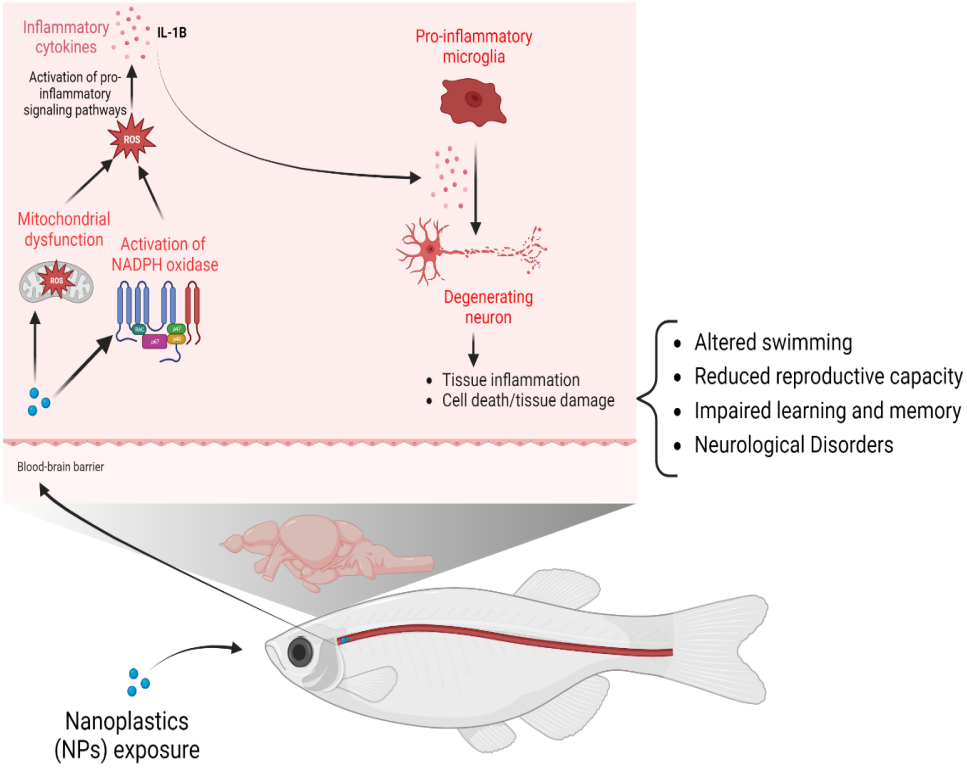

Figure 5. *A schematic diagram illustrating how MPs and NPs can induce neurotoxicity in fishes. MPs/NPs can enter the body via ingestion or absorption and cause damage to the plasma membrane. After crossing the blood-brain barrier, it produces ROS through mitochondrial dysfunction and the activation of NADPH oxidase. This leads to the generation of inflammatory cytokines such as IL-1B which further amplify inflammatory responses by generating more cytokines and chemokines. Information was obtained from (Chen et al., 2023, Ogier-Denis et al., 2008, Yin et al., 2022). Created with Biorender.com.*

Ethology, i.e. the study of animal behaviour, is an important aspect of toxicology. The term behaviour in fish mainly incorporates swimming (Spence et al., 2008). The other aspects are memory, learning, social interaction, avoidance, shoaling, and reproductive behaviour (Spence et al., 2008). The swimming behaviour of fish is mainly controlled by the central nervous system consisting of the brain and spinal cord (Guthrie, 1983). Inside the fish's brain is an intricate web of neurons which communicate through electrical impulses and regulate swimming behaviour (Guo, 2004). Both genetic and environmental factors control behaviour (Richendrfer et al., 2012). Neurotoxic chemicals can have a strong and profound effect on fish swimming behaviour or locomotion. These chemicals can disrupt the nervous system's normal functioning, which might lead to compromised swimming. The exact mechanisms behind these complex responses are not known in detail. There is currently a lack of knowledge on how small-sized NPs affect behaviour in developing fish.

In experimental studies on fish, MPs/NPs have been found to produce cardiotoxic effects (Persiani et al., 2023). TLRs, mainly TLR-4, interact with the particles; this interaction leads to the activation of TLR-4 and the generation of inflammatory cytokines such as IL-1β, IL-6, IL-8, TGFβ (transforming growth factor-beta), and TNFα (Wang et al., 2022). These inflammatory cytokines lead to oxidative stress and trigger ROS production via oxidative stress in cardiomyocytes can damage the intracellular components such as lipids, proteins, and DNA (Cheng et al., 2021). Similarly, ROS disrupts the balance between two types of T helper cells, i.e. Th1 and Th2 (**Fig 6.**). The Th1 cells secrete IFN-γ (interferon gamma), which activates macrophages and causes B-cells to produce IgG antibodies (**Fig 6.**). The cytokine IL-4 causes the mast cells to mature into Th2 cells which secrete IL-4 to stimulate B-cells to produce IgE antibodies. The imbalance between Th1 and Th2 cells triggers pro-inflammatory responses, generating excess ROS (**Fig 6.**). the NOXs pathway within the cardiomyocytes (Wu et al., 2022b). This elevated This state of oxidative stress mediates the activation of insulin-like growth factor binding protein 3 (IGFBP3) and p53 apoptotic pathways. The

accumulation of apoptotic cardiomyocytes may lead to impaired cardiac functioning, such as altered heartbeat and blood flow alteration (**Fig 6.**).

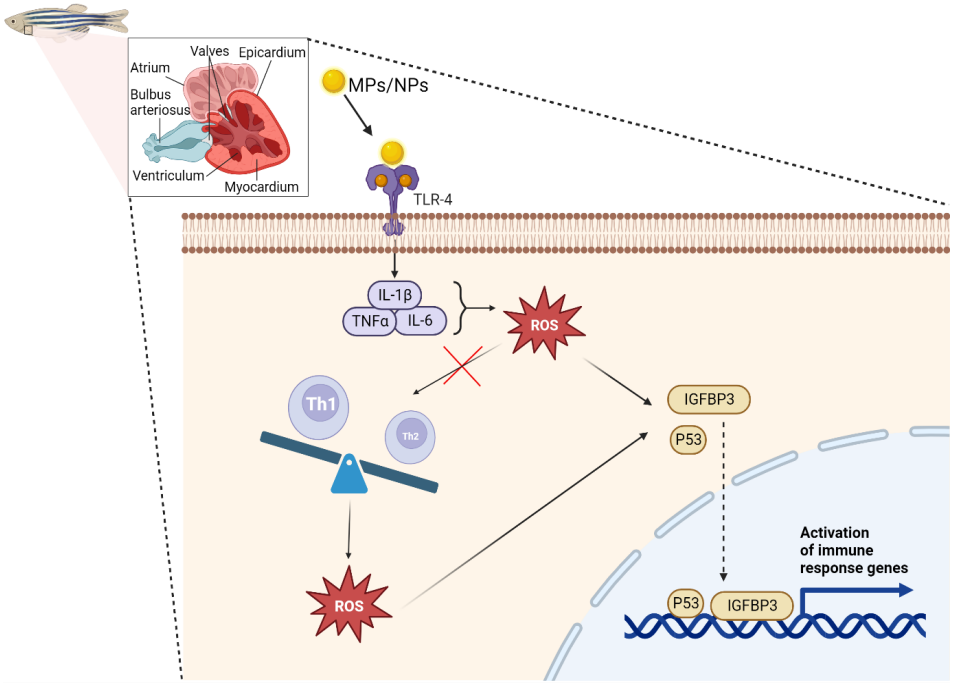

Figure 6. *A schematic diagram illustrating potential MPs/NPs induced cardiotoxicity in fishes. MPs/NPs can enter the body via ingestion or absorption and reaches to the heart via blood. Then, these particles interact with the toll-like receptors (TLRs) of cardiomyocytes and produces pro-inflammatory cytokines such as IL-1B, TNFα, IL-6, etc. The generation these cytokines produces ROS in the cells and disbalances the Th1/Th2. This state of oxidative stress mediates the activation of insulin-like growth factor binding protein 3 (IGFBP3) and p53 apoptotic pathways. Information was obtained from (Cheng et al., 2021, Wang et al., 2022, Wu et al., 2022b). Created with Biorender.com.*

Cardiotoxic effects in zebrafish larvae caused by the NPs exposure was one of the topics investigated in this thesis, focusing on heart morphology and rate employing microscopy, and exploring the associated molecular changes through transcriptomic approaches. The heart is one of the main target organs for toxicity studies due to its critical role in organismal physiology and its sensitivity to environmental stressors (Costa et al., 2013). NPs have the capability to traverse biological barriers, including the intestinal barrier, thereby entering the bloodstream (Barua and Mitragotri, 2014).

Once within the circulatory system, they can interact directly with the heart and other vital organs (Shi et al., 2023). By focusing on cardiotoxicity, the aim was to contribute to the broader understanding of NPs toxicity mechanisms and their potential impacts on aquatic species.

1.2.1.4 Tyre-wear particles and associated chemicals

Tyre-wear is the second largest source of MPs in oceans, following the release of particles from synthetic textiles. The number of vehicles is also increasing over the years. Globally, 2.23 billion tyres were produced in 2022 and is projected to reach to 2.74 billion by 2028 (Smithers, 2023). The automotive industry is still expanding. Earlier tyres were prepared mainly from natural rubber, but nowadays, a mixture of natural and synthetic rubber and several other chemicals is used. A passenger or car tyre is usually composed of fillers (26%), synthetic polymers (24%), natural rubber (19%), antioxidants/antiozonants (14%), steel (12%) and textile (4%) (Macmac et al., 2022). The latex of the *Hevea brasiliensis* tree is the main source of natural rubber (Jacob et al., 1993). Natural rubber has several advantages such as self-reinforcing behaviour and high mechanical strength (Samsuri, 2013). Moreover, natural rubbers need to go through several treatments such as vulcanisation and mixing with carbon black prior to be used for tyre manufacturing (Bockstal et al., 2019). These processes increase its durability, heat resistance, elasticity, and tensile strength (Bockstal et al., 2019). The limited availability and high cost compel tyre manufacturers to utilize synthetic rubber polymers. The two most widely used synthetic rubber in tyre manufacturing industries are butadiene and styrene butadiene rubber (USTMA, 2020). Tyre manufacturers carefully balance the proportions of natural and synthetic rubber to optimize both performance and cost (Myhre and MacKillop, 2002). The friction between the tyre and the road, along with heat, breaks the tyres into TWPs.

According to an estimate by Tan et al. (2023), six million tonnes of TWPs are produced globally. It is estimated that globally 550,000 tonnes of TWPs sized less than 10 µm are deposited on the road surface each year, and almost half of this ends up in the world

oceans (Carrington, 2020, Evangeliou et al., 2020). In Norway, it has been estimated that around 5000 to 11000 tons of road particles are generated, and 80% of these particles are released into the environment annually (Sundt et al., 2020). A study by Rødland et al. (2023) revealed that the average concentrations of TWPs in roadside samples collected from Trøndelag county of Norway ranged from 2.04 to 26.4 mg/g (dry weight). Similarly, high concentrations of TWPs (around 20 mg/g) were found in the sediment samples obtained from the inner Oslofjord (Alling et al., 2023). These particles exhibited higher concentrations near the shoreline compared to offshore locations, and they were also more abundant in sediments than in surface waters (Alling et al., 2023). Rødland et al. (2022) found TWPs in snowmelt water and the concentration of particles ranged between 222 mg/m^2 to 109,000 mg/m^2, with an average of 10,600 ± 2200 mg/m^2 in samples collected from Oslo.

The release of TWPs is affected by four main factors such as tyre features (size and composition), vehicle features (maintenance, engine power and torque), driving features (driving style, braking frequency) and road features (texture, composition and condition of the road) (Gehrke et al., 2023, Wagner et al., 2018, Zhang et al., 2023b). According to the German automobile club, a car releases an average of 120 mg of TWPs per km driven (ADAC, 2024). Nowadays, people and countries are shifting from conventional diesel or petrol vehicles to electric vehicles (EVs). According to statistics from Carlier (2023), there were about 25.9 million EVs globally in 2022. Norway, also known as the EV capital of the world, has around 21% of its vehicles as EVs (Mohanty et al., 2023). These EVs emit very low or no emissions, thereby combating climate change and meeting carbon reduction targets (Miotti et al., 2016). However, EVs are comparatively heavier than diesel or petrol vehicles primarily due to the large and heavy weight of the batteries (Mohanty et al., 2023). This additional weight puts extra pressure on the tyres, resulting in more TWPs (Zhang et al., 2023b). Moreover, the torque characteristic of EVs also contributes to increased tyre wear (Hicks et al., 2023). EVs deliver instant torque with very little lag. This means that the acceleration from a standstill or at low speeds is much more immediate compared to conventional vehicles,

which can lead to more stress on the tyres, especially if the vehicle is not driven carefully (Jekel, 2019, Liu et al., 2022).

The size of these TWPs could be on a macro-, micro- or nanoscale, depending on various factors such as road surface, driving speed, tyre composition, tyre inflation pressure, brake and suspension systems (Kole et al., 2017). TWPs are typically spherical or elongated in shape, which affects their ability to interact with cells (Wik and Dave, 2009). When road runoffs due to rainfall occur, TWPs are entrained with PM (particulate matter) and then deposited into stormwater drains leading to nearby water bodies (Kallenbach et al., 2022). These particles mainly enter the water bodies either through urban or stormwater runoff (Saifur and Gardner, 2021). Once these TWPs enter the aquatic ecosystem, they can undergo various processes influencing their fate and behaviour (Li et al., 2023). Physical processes like sedimentation and settling lead to the deposition of TWPs onto the bottom of water bodies, which may lead to gradual accumulation in sediments over time (Li et al., 2023). Small TWPs may stay in the water column due to currents and turbulences (Bellasi et al., 2020). TWPs can be ingested by aquatic organisms (fish, invertebrates, and plankton) in two ways, i.e. directly by drinking water or by consuming food contaminated with TWPs (Bellasi et al., 2020, Weinstein, 2023). The effect of TWPs has been studied in aquatic organisms such as algae, bacteria, and fish. A study by Shin et al. (2022) found that exposure to TWP leachate in *Brachionus plicatilis* produces oxidative stress and negatively affects its transcriptome and metabolome. Another study by Cunningham et al. (2022) found that micro and nano-sized TWPs negatively affect larval growth, development, and behaviour in Daphnia and zebrafish (*Danio rerio*).

The TWPs can also pollute terrestrial ecosystems. Based on their size in the atmosphere, the particles are classified under the PM_{10} and $PM_{2.5}$ categories (Grigoratos and Martini, 2015, Kim and Lee, 2018). The smaller nano-sized particles, constituents of $PM_{0.1}$, are also known as ultra-fine particles. Studies have shown that these ultra-fine particles are abundantly released from wearing tyres (Piscitello et al.,

2021, Thorpe and Harrison, 2008). The adverse effects of TWPs on humans are a growing concern, with studies indicating that these particles can cause mild issues like chest infections, skin and eye irritation, and vomiting, as well as severe issues like negative cardiopulmonary, developmental, reproductive, and cancer outcomes (Baensch-Baltruschat et al., 2020, Sathicq et al., 2022). The small-sized TWPs can penetrate more deeply into the lungs. Moreover, these particles are also expected to severely impact people with asthma and chronic obstructive pulmonary disease.

In addition, TWPs can also transport tyre-associated chemicals, such as antioxidants, antiozonants, vulcanizing agents, heavy metals, and organic substances (Johannessen et al., 2022). Müller et al. (2022) identified 214 organic chemicals within the TWPs extract. Among these, 145 were classified as leachable, suggesting potential mobility into aquatic and terrestrial ecosystems (Müller et al., 2022). Within this complex mixture of tyre-associated chemicals, two compounds have garnered significant attention, 6PPD (N-(1,3-dimethylbutyl)-N’-phenyl-p-phenylenediamine) and its oxidized counterpart 6PPD-quinone (6PPDq). These compounds have recently received significant attention due to the mass mortality and acute toxicity associated with 6PPDq in Coho salmon (Tian et al., 2021).

1.2.1.5 6PPD-quinone and its toxicity

6PPD is a popular stabilising additive in rubber products such as tyres. This compound acts as an anti-degradant by shielding the rubber from ozone and oxygen reactions, which can cause tyre cracking (Datta et al., 2007). 6PPD is not the only anti-degradant compound that is used for tyre longevity. The other compounds are 7PPD (N-(1,4-dimethylpentyl)-N'-phenyl-p-phenylenediamine) and IPPD (N-Isopropyl-N’-phenyl-1,4-phenylenediamine) (Metherell, 1992). However, the Emissions Analytics company tested more than 200 tyres and found that the 6PPD compound was present in 97% of tyres, followed by IPPD, which was present in 13-14% of tyres (Emissions, 2023). These anti-degradant chemicals are added at an average of 10-20 mg/g tyre (1-2% of tyre weight) (Emissions, 2023). In addition to loss from abrasion, after years of running,

these chemicals slowly migrate to the tyre surface through blooming, increasing 6PPD pollution in the environment. When this 6PPD enters the ocean along with stormwater or urban runoff, it reacts with the atmosphere's ozone, forming a highly toxic compound known as 6PPDq (Tian et al., 2021). This newly formed compound is known to be the reason for the mortality of migrating coho salmon (*Oncorhynchus kisutch*) in the United States (Tian et al., 2021). This mysterious death of coho salmon in freshwater just before spawning is known as urban runoff mortality syndrome (URMS) (Peter et al., 2018). According to recent studies, URMS causes sudden death in 40-90% of adults of coho salmon after rainstorms (Stokstad, 2020, Tian et al., 2021). Chow et al. (2019) found that coho salmon in the freshwater phase are more vulnerable to the URMS than in the marine phase. The authors also claimed that fish exposed to stormwater did not recover to normal behaviour even after transfer to clean water (Chow et al., 2019). This urban runoff contains many pollutants such as polycyclic aromatic hydrocarbons, heavy metals, tyre, and road-wear particles, exhaust emissions and tyre-wear chemicals (Müller et al., 2020). But among them, one of the most toxic pollutants is 6PPDq.

According to an estimate from the Emissions Analytics company, 130 tonnes of 6PPD is released annually in Europe from 250 million cars (Emissions, 2023). Apart from tyres, artificial turf is another primary source of 6PPD pollution, as recycled tyre products are used to prepare artificial turf (Armada et al., 2023, Murphy and Warner, 2022). The enormous amount of release of this chemical made it ubiquitous. Its occurrence was reported in road water (Nedrich, 2022), road dust (Hiki and Yamamoto, 2022a), indoor dust (Deng et al., 2022, Huang et al., 2021), wastewater treatment plants (Johannessen and Metcalfe, 2022, Seiwert et al., 2022), electronic waste recycling workshops (Liang et al., 2022), fish samples (Ji et al., 2022a), honey samples (Ji et al., 2022a) and lettuce roots (Castan et al., 2022).

The widespread presence of this chemical has raised concerns about its probable harmful effects on fish health and the environment. Several studies found that

exposure to 6PPDq affects the development, behaviour, neuronal activity, tissue toxicity and intestinal inflammation in fish and mice, crustaceans and roundworms (Greer et al., 2023, Ji et al., 2022a, Ricarte et al., 2023). Both 6PPD and 6PPDq are known to bioaccumulate in organisms. A study by Grasse et al. (2023) shows that 6PPDq is readily absorbed by zebrafish larvae with concentration factors ranging from 140 to 2500 for 6PPD and 70 to 220 for 6PPDq over 96 h of exposure. In line with this, Hua and Wang (2024) examined the uptake of 6PPDq in *Caenorhabditis elegans*, and found 4.32 µg/g 6PPDq in body tissue when exposed to 10 µg/L 6PPDq. Further studies have demonstrated that 6PPDq upon being absorbed is extensively metabolized in the organisms, thus emphasizing the role of enzymes in causing metabolic alteration of 6PPDq into different metabolites (Hua and Wang, 2024, Montgomery et al., 2023). Grasse et al. (2023) found 22 and 12 transformed products in zebrafish larvae exposed to 6PPDq for 96 h, indicating high efficiency of 6PPDq's biotransformation in zebrafish. This study conclude that zebrafish can metabolize and detoxify 6PPDq via various metabolic pathways. A study by (Hua et al., 2023a) found that exposure to 100 µg/L 6PPDq in *Caenorhabditis elegans* causes intestinal toxicity by disrupting the intestinal lumen. This disruption affects the intestine's morphology and function, i.e., the biological barrier. In the same line, a study found that 21 days of 6PPDq exposure to mice can damage their intestinal jejunum and ileum via disruption of cannabinoid receptors (Yang et al., 2024). Interestingly, the study also showed that the effect was only seen at the jejunum and ileum and not in other parts of intestine (duodenum and colon). Another study by (Hua et al., 2023b) found that exposure to 10 µg/L 6PPDq causes neurodegeneration in *Caenorhabditis elegans* by activating the neuronal receptor DEG-3. This gene encodes a voltage-gated calcium channel, but exposure caused the binding of 6PPDq to DEG-3, leading to the neurodegeneration effect. Similar neurobehavioural adverse effects were seen in zebrafish exposed to this pollutant. Ji et al. (2022b) found that exposure to higher but sublethal concentrations of 6PPDq (1000 µg/L) significantly affects the activity of the neurotransmitter acetylcholine (ACh). Hua and Wang (2024) found enhanced uptake of 6PPDq (10 µg/L)

when co-exposed with environmentally relevant concentrations of PE nanoparticles (0.1, 1 and 10 µg/L). Moreover, the authors also found enhanced neurotoxic effects (locomotion and neurodegeneration) of 6PPDq when co-exposed with PE nanoparticles. Moreover, this pollutant can induce various toxic effects at cellular and sub-cellular levels. Jiang et al. (2024) found that exposure to 6PPDq (>2 µg/L) can induce activation of the aryl hydrocarbon receptor which can further lead to oxidative, DNA damage and cardiac dysfunction in zebrafish larvae via the AHR signalling pathway. A study by Wu et al. (2023) discovered that exposure to 6PPDq enhances the formation of DNA adducts in mammalian lung carcinoma epithelial cells A549 cells. Hua et al. (2023c) showed that exposure to this chemical lowers the reproductive capacity of *Caenorhabditis elegans* by promoting apoptosis in germ cells. A study by Mahoney et al. (2022) showed increased oxygen consumption by uncoupling mitochondrial respiration in the RTgill-W1 cell line when exposed to 1000 µg/L 6PPDq. Besides their own toxic effects, 6PPD and 6PPDq can also enhance the toxicity of other contaminants. A study by Klauschies and Isanta-Navarro (2022) found synergistic toxic effects such as decreased population growth in *Brachionus calyciflorus* in response to combined exposure of NaCl and 6PPD.

Moreover, several studies have documented the presence of 6PPDq in human urine and blood samples, suggesting potential toxicological implications for humans (Du et al., 2022, Mao et al., 2024, Zhang et al., 2024a, Zhang et al., 2024b). Song et al. (2024) found the presence of 6PPD and 6PPDq in healthy human serum with mean values of 0.022 and 0.15 ng/ml. The authors also conducted this analysis in humans with secondary non-alcoholic fatty liver disease (Song et al., 2024). The results indicated elevated concentrations of these pollutants (0.093 ng/ml 6PPD & 0.20 ng/ml 6PPD) in diseased persons. This study further investigated oxidative stress biomarkers in diseased and healthy persons and found a significant and positive correlation between 6PPDq and lipid oxidative damage. Zhang et al. (2024b) found presence 6PPDq in indoor dust samples collected from e-waste recycling area in China. The authors found

a positive correlation between 6PPDq and lower body mass index, influenza and diarrhoea in children (Zhang et al., 2024b).

6PPDq is also known for its species-specific toxicity, i.e., it is highly toxic to some species but only moderately toxic to other species. The toxicity of 6PPDq was initially reported in coho salmon with an LC_{50} of 95 ng/L (Tian et al., 2022, Tian et al., 2021). However, follow-up studies showed that this chemical was not acutely toxic to tropical and sub-tropical fish species, such as zebrafish, medaka (*Oryzias latipes*), white sturgeon (*Acipenser transmontanus*) and Chinese rare minnow (*Gobiocypris rarus*) at similar concentrations (Brinkmann et al., 2022, Hiki and Yamamoto, 2022b). One reason for this discrepancy could be differences in temperature-dependent tolerance between cold water and warm water fishes. Interestingly, the toxicity of 6PPDq varied even among salmonid species (**Fig 7.**). A study by Hiki and Yamamoto (2022b) showed that this chemical is acutely toxic to white-spotted char (*Salvelinus leucomaenis pluvius*) with an LC_{50} of 0.51 µg/L. Still, it did not cause mortality in the southern Asian dolly varden (*Salvelinus curilus*) and landlocked masu salmon (*Oncorhynchus masou masou*), even at 3.8 µg/L (**Fig 7.**). Therefore, there is a species-specific response to the toxicity of 6PPDq (**Fig 7.**), and there are a variety of potential causes for this, including differences in metabolism, detoxification mechanisms, and differences in the abundance or structure of molecules that 6PPDq interacts with, which result in varying degrees of toxicity.

Understanding the toxicity driven by 6PPD and 6PPDq is important to assess their environmental impact. For this reason, we selected the environmentally relevant concentrations as exposure doses. The exposure doses of 6PPDq (1, 10, 25 & 50 µg/L) were selected based on the environmental presence of 6PPDq. Hiki and Yamamoto (2022a) found 116 to 1238 ng/g 6PPDq in road dust samples collected in Tokyo, Japan. Compared to their highest documented 6PPDq concentration, our chosen exposure dose is approximately 25 times lower.

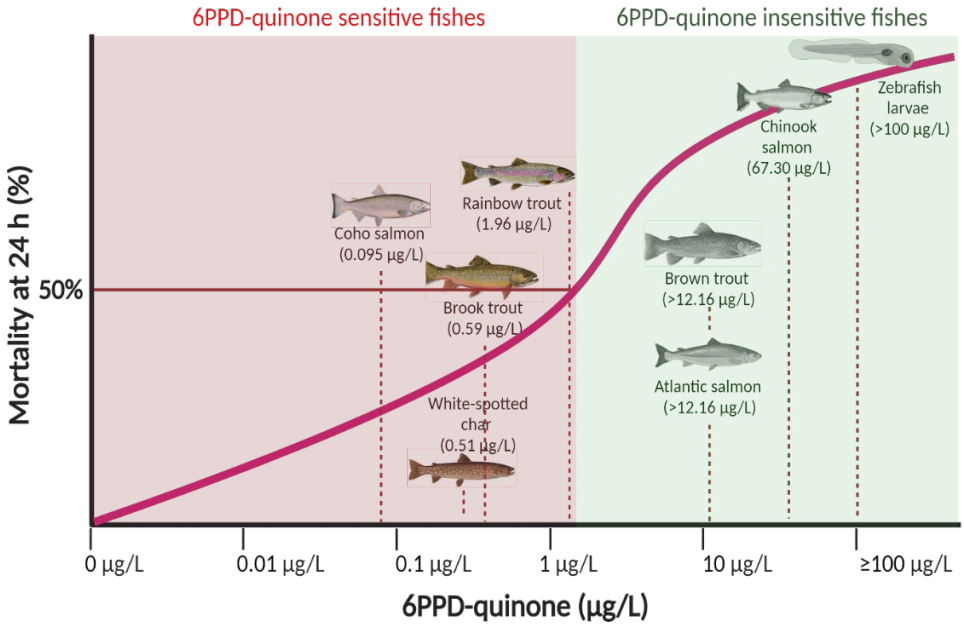

Figure 7. *A schematic diagram illustrating the species-specific toxicity of 6PPD-quinone in various fishes. Coho salmon, white spotted char, brook trout and rainbow trout were found to be highly sensitive whereas other fishes such as Atlantic salmon, brown trout, chinook salmon and zebrafish larvae were found to be less sensitive to the toxicity of 6PPD-quinone.*

Also, to understand the combined toxicity and interaction between TWPs and 6PPDq, we used plain polystyrene nanoplastics (PS-NPs) as a substitute of TWPs. This approach is based on the recognition that TWPs are invariably accompanied by 6PPDq contamination. Therefore, we selected environmentally relevant concentrations of TWPs as exposure doses of PS-NPs (3 mg/L) in the thesis. Rødland et al. (2023) found 2000-26,400 mg/kg of TWPs in the roadside soil sampled in the Trøndelag county, Norway. Similarly, high concentrations of TWPs (around 20000 mg/kg) were found in the sediment samples obtained from the inner Oslofjord (Alling et al., 2023).

1.2.2 Persistent organic pollutants

Persistent organic pollutants (POPs) are diverse organic compounds known for their long persistence or high environmental stability. These compounds can bioaccumulate

in food chains and withstand natural degradation processes. Once they enter the ecosystem, they can endanger humans and wildlife by acting as endocrine disruptors and reproductive toxicants. Studies have shown that POPs can be easily transported by wind and water, affecting people and wildlife far away from their release point (Lohmann et al., 2007, Wöhrnschimmel et al., 2013). To address this issue, 152 countries signed a treaty in May 2001 in Stockholm, Sweden (Stockholm Convention, 2001). The primary aim of the treaty was to eliminate or reduce the production and use of POPs (Stockholm Convention, 2001). As a result of this, many POPs are banned across different countries. But it does not guarantee that people and organisms are no longer at risk, as these chemicals have long persistence and can be easily transported across air or water.

Nickerson (2006) documented the presence of many POPs in breast milk of mothers. Studies have shown that POPs, even at lower concentrations, can cause cancer, reproductive disorders, endocrine disruption and many other health problems (Safe, 2000, Yilmaz et al., 2020). Humans are exposed to POPs through various pathways. These pollutants can enter the human body not only through the consumption of food items, including fish, seafood, crops, and dairy products but also through the air we breathe (Carpenter, 2006). Over 90% of POPs found in humans stems from contaminated food, with seafood being one of the main sources (Çok et al., 2009, Polder et al., 2010). Fish consumption is an important route for the entry of POPs in humans. Moreover, global fish consumption has increased at an average annual rate of 3.1 % from 1961 to 2017 (FAO, 2022). This necessitated the need for careful monitoring of the presence of POPs in fish and associated seafood products. The most common POPs in seafood are organochlorine pesticides such as DDT, polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDD) and various other industrial chemicals (Ashraf, 2017, Rios et al., 2007).

1.2.2.1 DDT

Dichlorodiphenyltrichloroethane (DDT) is one of the most famous, lethal and controversial synthetic insecticides ever made. It was first synthesised in 1874 by Paul Hermann Müller. He was awarded the Nobel Prize in 1948 for Physiology or Medicine for the discovery of DDT and its application to fight against malaria and yellow fever. Since 1940, approximately 0.27 metric million tons of DDT have been produced and used worldwide (Edwards, 2004). DDT is classified as a pesticide in class II, indicating that it is moderately toxic (Pretty and Hine, 2004). This insecticide is known to have serious detrimental effects on non-target species as well. It has caused dramatic declines in many animal populations, such as peregrine falcons (*Falco peregrinus*), gyrfalcons (*Falco rusticolus*) and brown pelicans (*Pelecanus occidetalis*). DDT can cause eggshell thinning, reproductive disorders, endocrine disruptions, and neurological effects. (Beard and Collaboration, 2006). After realising its devastating effects, it was banned in the United States in 1972. The use of DDT was banned worldwide following the 2001 Stockholm convention. However, some countries still use DDT and related compounds to control mosquitoes (Harada et al., 2016, Van den Berg et al., 2017).

Nearly 50 years after DDT was outlawed in Norway, we still deal with its traces today. In Norway, the Institute of Marine Research (IMR) monitors the levels of POPs such as DDT in aquafeed and fish. Monitoring by IMR of aquafeeds and ingredients collected in 2021 showed 4.7 µg/kg total DDT in whole feed, 3.0 µg/kg total DDT in fish meal and 33.1 µg/kg total DDT in fish oil (**Table 1**). Most of the DDT in the whole feed and the feed ingredients is present as p,p'-DDE. Another monitoring report from the IMR found 3.5, 3.7 and 3.9 µg/kg sum DDT in the fillet of Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and arctic char (*Salvelinus alpinus*), respectively (Bernhard et al., 2022).

Table 1. *Average concentration of DDT and its metabolites (µg/kg) in the fish feed, fish meal, and fish oil samples collected in the year 2021. The average concentrations are calculated when the number of observations above the LOQ (limit of quantification) is more than 20%. The data is obtained from Sele et al. (2022). p,p'-DDD: Dichlorodiphenyldichloroethane, p,p'-DDE: Dichlorodiphenyldichloroethylene, Sum DDT: includes op-DDD, op-DDE, op-DDT, pp-DDD, pp-DDE and pp-DDT.*

Samples	p,p'-DDD	p,p'-DDE	op-DDT	pp-DDT	Sum DDT
Fish feed	0.84	2.3	0.31	0.56	4.7
Fishmeal	0.49	1.7	<LOQ	<LOQ	3.0
Fish oil	6.0	20.8	0.35	1.9	33.1

DDT can undergo various chemical processes inside the body and the environment, resulting in the metabolites DDE (dichlorodiphenyldichloroethylene) and DDD (dichlorodiphenyldichloroethane). Among the two metabolites, DDE is the metabolite found in the highest levels in the environment and is formed when DDT is dechlorinated. Several studies have reported that DDE is more stable and persistent than DDT itself (Abou-Arab, 1997, Wolfe et al., 1977). A study by Powers et al. (1975) found that 0.1 parts per thousand million can significantly reduce the growth of a marine dinoflagellate (*Exuviella baltica*). Monteiro et al. (2015) observed a reduced number of mature oocytes through histopathology in female zebrafish exposed to 20 µg/L p,p '-DDE. Song et al. (2008) found that exposure to 30 µM (9.54 µg/L) p,p '-DDE can induce apoptosis in male rats' Sertoli cells, cells of the testis that are essential for testis formation and spermatogenesis. The authors concluded that the generated toxicity is due to the generation of ROS, which further leads to a decrease in mitochondrial membrane potential and release of cytochrome c. Morales-Prieto et al. (2018) also obtained similar results for mitochondrial dysfunction. The authors found 200 differentially expressed genes in mice fed with 112.5 µg/g p,p '-DDE body weight per day. They concluded that apart from mitochondrial dysfunction, it also affected glucose physiology, central signalling pathways and endocrinal regulation. Another study from Olsvik and Søfteland (2018) discovered that 48 h exposure of 10 and 100 µM (31.8 µg/L) p,p '-DDE to primary hepatocytes of Atlantic salmon can produce

cytotoxic and endocrine disrupting effects. The authors also performed metabolic profiling and found that 100 µM *p,p'*-DDE affected several metabolic pathways related to carbohydrate, bile, lipid and amino acid metabolism. However, very little is known about the mixture toxicity of DDE along with other pollutants.

1.3 Types of interactions among pollutants

In nature, multiple contaminants co-exist together. Interaction effects is a critical aspect to consider when evaluating the overall impact of pollution. The combined effects can be significantly similar or different from the individual components (Hernández et al., 2017). The type of interaction depends mainly on the chemical properties of the individual pollutants, such as target molecules, chemical structures, and doses (Altenburger et al., 2003). The combined toxic effects of different mixtures can be significantly influenced by toxicokinetic and toxicodynamic factors as well, which encompass the metabolic pathways and cellular or molecular targets of individual pollutants, respectively (Hernández et al., 2017, Silins and Högberg, 2011). However, sometimes interactions lead to toxic effects that differ from both pollutants (Hernández et al., 2017). There are three kinds of interactions among pollutants in mixture toxicity.

1.3.1 Additivity

Additivity is the interaction between pollutants or contaminants where the combined or joint effect is the sum of their individual effects (Groten, 2000). This model assumes that individual pollutants act through distinct and non-interacting mechanisms (Rider and LeBlanc, 2005). This kind of interaction usually occurs with the pollutants having a similar kind of structure or similar mode of action (Könemann, 1980, Rodea-Palomares et al., 2015). Monchanin et al. (2021) found that heavy metals such as arsenic, copper and lead have additive toxic effects on the cognition in the honey bee. Similarly, Marshoudi et al. (2023) observed additive toxic effects of MPs and Cd in zebrafish embryos.

1.3.2 Synergism

Synergy or synergistic interaction is where the combined toxic effects of pollutants are greater than the individual effects (Singh et al., 2017). It is the amplification of toxic effects of pollutants in the presence of each other. This is the most frequently observed interaction among environmental pollutants such as heavy metals, POPs and MPs/NPs (Holmstrup et al., 2010). Pérez et al. (2013) found synergistic toxic effects of atrazine and terbuthylazine on chlorpyrifos toxicity in zebrafish embryos. There are three main mechanisms of synergistic interactions as biochemical potentiation, receptor-mediated interactions and cellular damage. Biochemical potentiation occurs if one of the pollutants facilitates the uptake, absorption, bioaccumulation or retention of another pollutant (Bhagat et al., 2021). Da Costa Araújo et al. (2022) observed that exposure to MPs (~35 µm sized PE) potentiated the uptake of the mixture of emerging pollutants (pesticides, agro-industrial effluent, pharmaceutics, agricultural fertilizers, surfactant) in zebrafish. Receptor-mediated interactions is observed among pollutants when they target the same cellular receptors (Wilkinson et al., 2016). Several studies observed that combined exposures may disrupt hormonal signaling pathways more significantly than individual exposures after exposure to endocrine-disrupting chemicals (Gao et al., 2018, Pollock et al., 2018). Synergism via cellular damage occurs when simultaneous pollutants are present and may cause intracellular oxidative stress via an imbalance between ROS and the antioxidant defence system (Sun et al., 2018). This oxidative stress cascade can amplify the adverse effects of pollutants on biological systems (Lu et al., 2015, Valavanidis et al., 2013). For example, heavy metals like lead and cadmium can stimulate ROS production through redox cycling, while POPs such as PAHs may generate ROS via metabolic activation (Berntssen et al., 2015, Wang et al., 2004).

1.3.3 Antagonism

Antagonism or antagonistic interaction occurs when the combined toxic effects of pollutants are lower than the individual effects (Preston et al., 2000). In these instances, pollutants counteract each other at the molecular level, mitigating the overall impact.

Antagonism can manifest when pollutants have opposing mode of action or when one pollutant interferes with the absorption, distribution, metabolism, or excretion of another pollutant (Chouhan and Flora, 2010).

2 Objectives

Rapid technological advancements and industrialisation produce new or emerging pollutants, constantly challenging our understanding of environmental risks. Pollutants are pervasive and are found in every corner of the world. Moreover, in nature, a complex mixture of pollutants co-exists together. The overall aim of the thesis was to investigate the individual and combined ecotoxicological effects of tyre-wear chemicals, NPs and POPs in zebrafish, a model organism widely employed in toxicological research. For this, a multi-endpoint approach was used to examine the toxicological profile of the targeted pollutants with development, behaviour, heart rate, respiration and transcriptome as study parameters.

Specific objectives and their respective hypothesis are as follows:

- **Objective 1**: Investigate the ecotoxicological effects of tyre-wear chemicals using zebrafish larvae, to gain insight into the harm caused by these pollutants in aquatic ecosystems (**Paper I**).

  Hypothesis: Tyre-wear chemicals adversely affect the growth, development, heart rate, respiration and behaviour of zebrafish larvae.

- **Objective 2**: Studying single and mixture toxicity of the DDE and NPs in zebrafish larvae, aiming to understand the interaction effects of these pollutants (**Paper II**).

  Hypothesis: The hypothesis is that NPs and DDE both exert an adverse effect on zebrafish larvae and that NPs will enhance the severity of the toxicological effects of DDE.

- **Objective 3**: To understand the toxicological impact of 6PPDq and NPs in adult zebrafish at the molecular level, upon single and mixture exposure to the pollutants (**Paper III**).

Hypothesis: The hypothesis is that both 6PPDq and NPs will adversely affect the swimming behaviour and transcriptome (liver and intestine) even in adult fish, and NPs will enhance the toxicological effects of 6PPDq.

3 Materials and methods

3.1 Behavioural studies

Studying the behaviour of fish or any other animal is of paramount importance as an indicator of underlying physiological or neurological issues caused by environmental pollutants. Thus, behavioural studies are an inevitable tool for the comprehensive risk assessment of the toxicity of any pollutant.

3.1.1 DanioVision system

We employed the DanioVision system (DVS) to study the zebrafish larval swimming behaviour (**Paper I** and **Paper II**). The DVS (**Fig 8.**) is a high-throughput system designed by Noldus Information Technology (Wageningen, the Netherlands). The DVS system track and analyse the swimming behaviour of small fishes like zebrafish, goldfish, medaka, and others (Aliko et al., 2019, Shi et al., 2021, Tao et al., 2022).

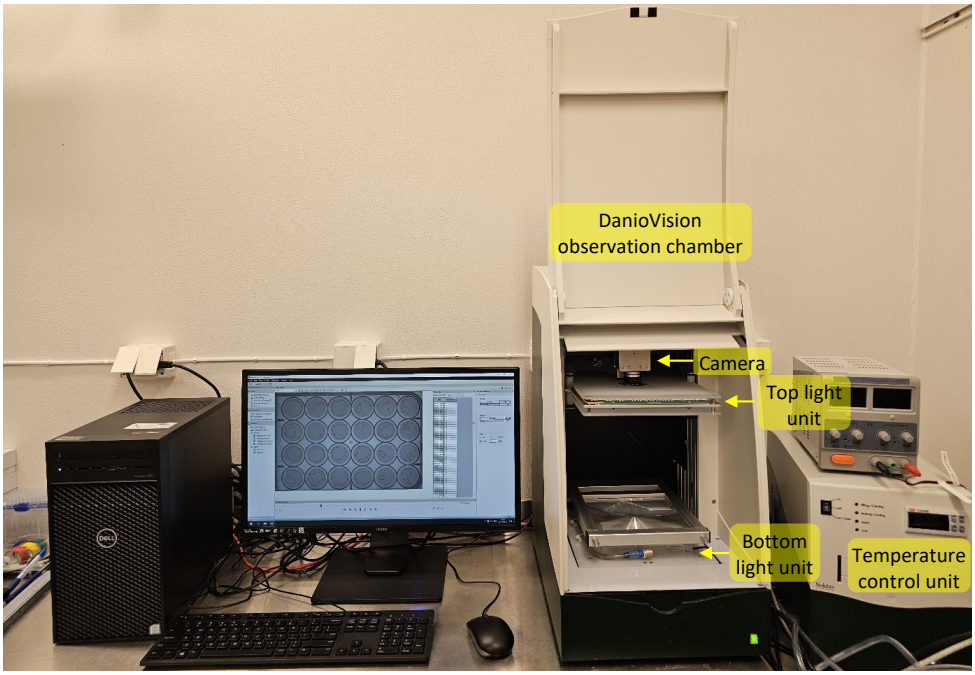

Figure 8. *Setup of the DanioVision system in the physiology lab at the Research Station of Nord University.*

This system offers a non-invasive and automated way to understand fish behaviour in real-time (**Fig 8.**). The DVS has one infrared gigabit ethernet video camera (1280 × 960; 12 mm lens), two white light sources (top and bottom) and an external temperature control unit to maintain the temperature (**Fig 8.**). Using the two light sources, it is possible to change the light levels from 0 to 10,000 lux. The observation chamber of DVS is compatible with petri dishes (diameter up to 90 mm) and well plates (4 to 96 wells). The DVS is equipped with EthoVision® XT software to record, track and analyse the behavioural recordings. The EthoVision® XT software can plot locomotory heatmaps and track visualisation plots just after the recordings.

3.1.2 EthoVision system

We employed the EthoVision® system (EVS) to study the swimming behaviour of adult zebrafish (**Paper III**).

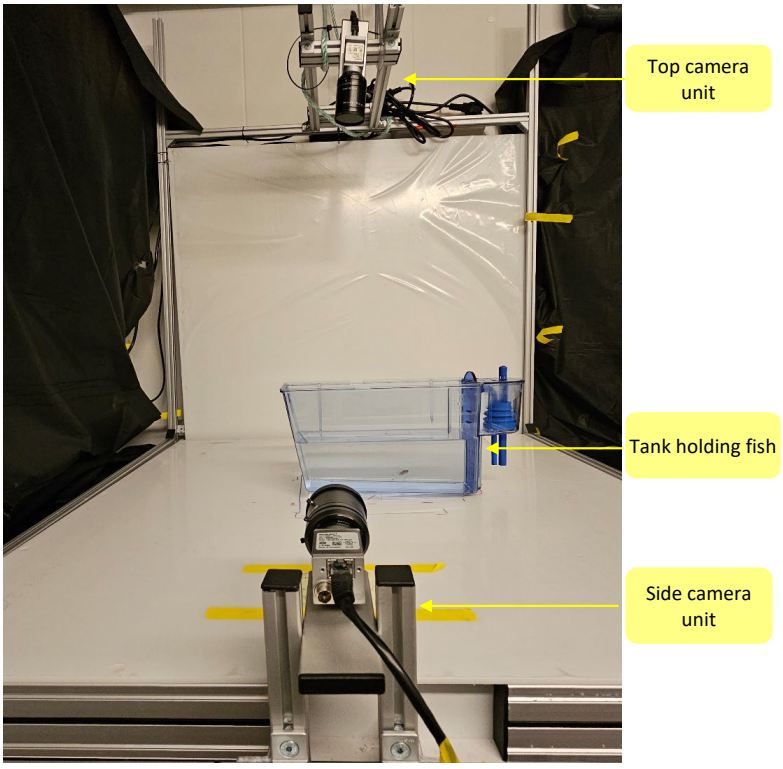

Figure 9. *Setup of the EthoVision system in the physiology lab at the Research Station of Nord University.*

EVS is another high-throughput, non-invasive behavioural recording system designed by Noldus information technology (Wageningen, the Netherlands) (**Fig 9.**). The EVS is designed to track and analyse the swimming behaviour of comparatively large fishes such as juvenile/adult zebrafish, mosquito fish, flying barb, and goldfish (Cachat et al., 2010, Jakka et al., 2007). The EVS has two infrared cameras (Basler acA1300) to record the videos in three dimensions (**Fig 9.**). The recording can be performed in any shaped glass arena or tank. Moreover, the system allows the division of the tank into multiple small arenas such as the top zone, middle zone, bottom zone or corners (**Fig 9.**). EVS can be used for any behavioural test, such as a novel tank test, mirror test, object discrimination test, avoidance learning test, interaction test or shoaling behaviour test (Hawkey et al., 2021). Like DVS, the EVS is equipped with EthoVision® XT software to record, track and analyse the behavioural recordings.

3.2 Respirometry

We employed the Loligo® microplate-based respirometry system (Loligo Systems, Denmark) to measure oxygen consumption or respiration rates through a non-invasive approach in zebrafish larvae (**Paper I** and **Paper II**). This respirometry can measure the respiration rates for zebrafish embryos, daphnia, fish eggs or larvae, drosophila, Xenopus embryo and tadpole (Anderson et al., 2022, Folkerts et al., 2017, Hagedorn et al., 2023, Martin et al., 2020). This respirometry system also sheds light on the metabolic activity of the organism. The respirometry system is equipped with a MicroResp® program to measure and record the available oxygen in each well of the 24-well plate Sensor dish in real-time.

3.3 Heartbeat assay

The assessment of heartbeat or heart rate of zebrafish larvae (116 hpf) was performed by recording heart videos at 45X or 90X through an Olympus SZX12 stereomicroscope, followed by analysis using the DanioScope software (Noldus information technology, Wageningen, the Netherlands) (**Paper I** and **Paper II**). This is a non-invasive video imaging technique to measure the heart rate. To record the heart videos at the best

angle (by placing the larvae dorso-ventrally), the larvae were immobilised using a 3.5% methylcellulose to immobilise the larvae. Methylcellulose is reported to be safe for all animals and does not affect the heart rate (Muntean et al., 2010).

3.4 Transcriptomic studies

Zebrafish have very well-characterised and annotated genome with high similarity to vertebrates (Spence et al., 2008). We employed RNA sequencing or transcriptome studies to elucidate the molecular mechanisms behind the toxicity of pollutants (**Paper II** and **Paper III**). By analysing the transcriptome, we can uncover gene expression changes and identify molecular pathways in response to pollutants.

4 Results

4.1 Paper I

In order to understand the acute toxicity of the tyre-wear contaminants 6PPD and 6PPDq, we followed the guidelines laid by the OECD for the testing of chemicals using the fish embryo toxicity test (OECD, 2013). The LC_{50} values were calculated using probit analysis at 24, 48, 72 and 96 hpf. The LC_{50} values were 1384.93, 816.9, 609.39 and 442.62 µg/L for 6PPD and 308.67, 224.56, 171.63 and 132.92 µg/L for 6PPDq at 24, 48, 72 and 96 hpf, respectively (**Fig 10.**). The LC_{50} results from our study indicate that 6PPDq is not acutely toxic to zebrafish larvae as compared to rainbow trout, brook trout and coho salmon (Hiki and Yamamoto, 2022b, Tian et al., 2021). Then, we selected two environmentally relevant doses (1 and 10 µg/L) and one sub-lethal dose (25 µg/L) for developmental, behavioural and other toxicity tests (**Paper I**). The sub-lethal dose was selected based on the acute toxicity results. The developmental assay results revealed multiple deformities in the larvae exposed to 25 µg/L 6PPD and 6PPDq, while total body length remains unaffected (**Fig 10.**). Also, the exposure to 25 µg/L 6PPD and 6PPDq caused a significant reduction in eye and swim bladder size (**Paper I**). In the locomotor assay, we found a dose-dependent reduction in the swimming performance with a significant reduction in distance moved, velocity and heading in zebrafish larvae exposed to 25 µg/L 6PPDq (**Fig 10.**). We also observed a significant decrease in heart rate in larvae exposed to 25 µg/L 6PPD and 6PPDq (**Fig 10.**). In the respiration assay, we observed a significant increase in oxygen consumption in larvae exposed to even 1 µg/L 6PPD and 6PPDq. This was the only endpoint where we observed significant toxic effects at environmentally relevant concentrations. Together, the results show that exposure to environmentally relevant concentrations did not produce significant behavioural, developmental or cardiotoxic effects in zebrafish larvae. However, exposure to higher concentration (25 µg/L) produced significant toxic effects.

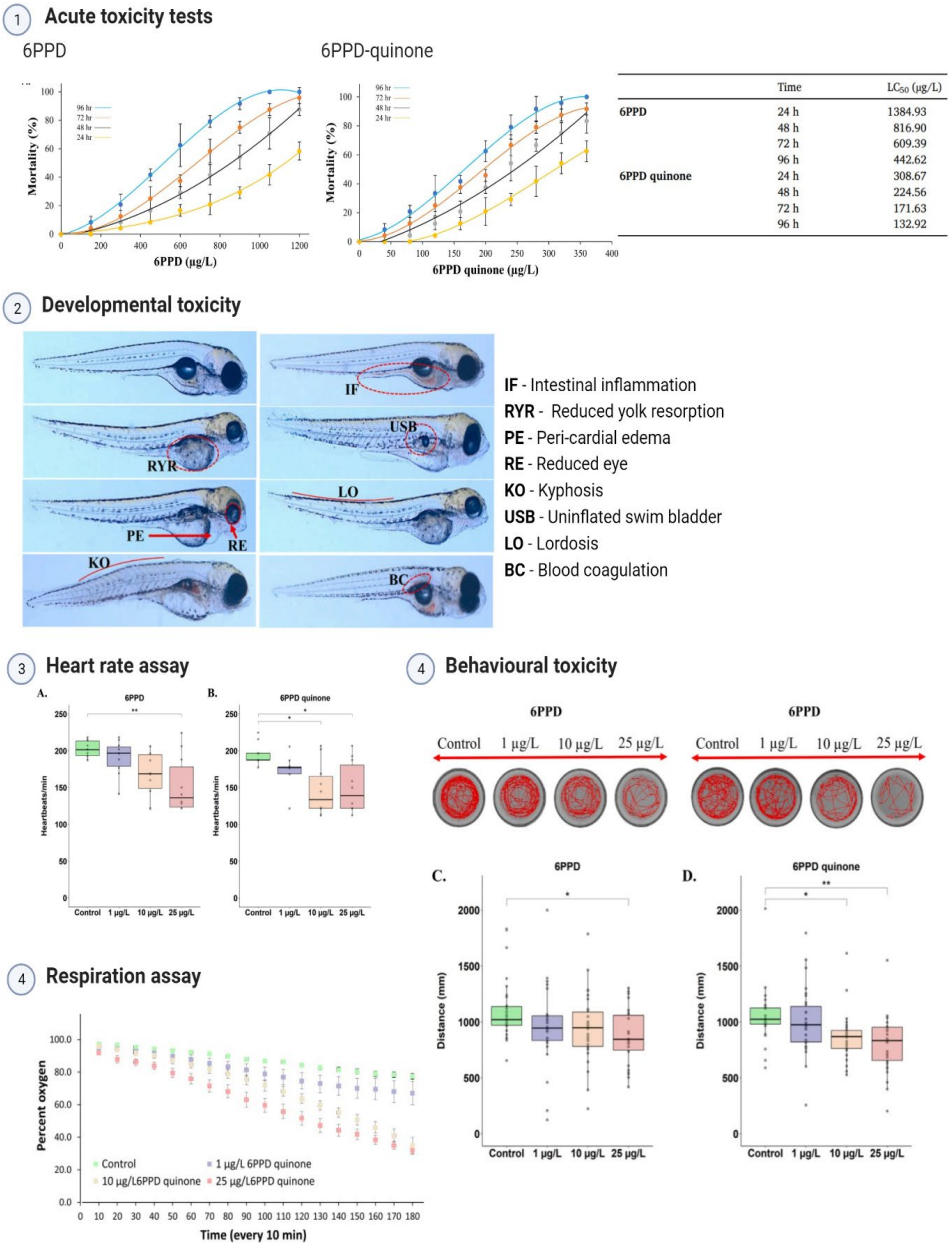

Figure 10. *Overview of the results obtained in Paper I.*

4.2 Paper II

To study the uptake of NPs in zebrafish embryos, we employed fluorescently labelled PS-NPs, and subsequently conducted fluorescent microscopy. The results from this study document uptake of NPs, as fluorescence was observed in the gastrointestinal and cranial regions in the zebrafish larvae exposed to 50 mg/L NPs and a combination of 50 mg/L NPs with 100 µg/L *p,p'*-DDE (**Fig 11.**). After confirming the uptake, we conducted an exposure study with development, behaviour, heart rate, oxygen consumption and transcriptome as study end-points (**Paper II**). The 96 h exposure of DDE and NPs+DDE generated developmental aberrations such as pericardial edema, uninflated swim bladder, lordosis and reduced yolk resorption when assessed at 116 hpf (**Paper II**). However, any exposure (NPs, DDE or NPs+DDE) did not significantly affect the larvae's total body length, eye size, or swim bladder at 72, 96 and 116 hpf. During the microscopic examination of the exposed larvae, we observed cardiac ballooning in the larvae exposed to DDE and NPs+DDE (**Fig 11.**). Along with the cardiac ballooning, we also observed other significant cardiovascular effects in the DDE or NPs+DDE exposed larvae, such as increased pericardial area, decreased heartbeat and increased oxygen consumption (**Paper II**). To understand the molecular mechanism underlying the exposure, we performed transcriptome analysis. We found several affected KEGG pathways or GO terms associated with muscle contraction, neural and non-neural signalling, drug metabolism and immune response in the larvae exposed to DDE or NPs+DDE (**Fig 11.**). However, the pathways were more enriched in the larvae exposed to NPs+DDE than DDE alone. We did not observe any significant KEGG pathways or GO terms associated with the NPs exposure. In summary, while NPs did not induce toxicity on their own, they did exacerbate the toxicity of DDE across the majority of the study endpoints.

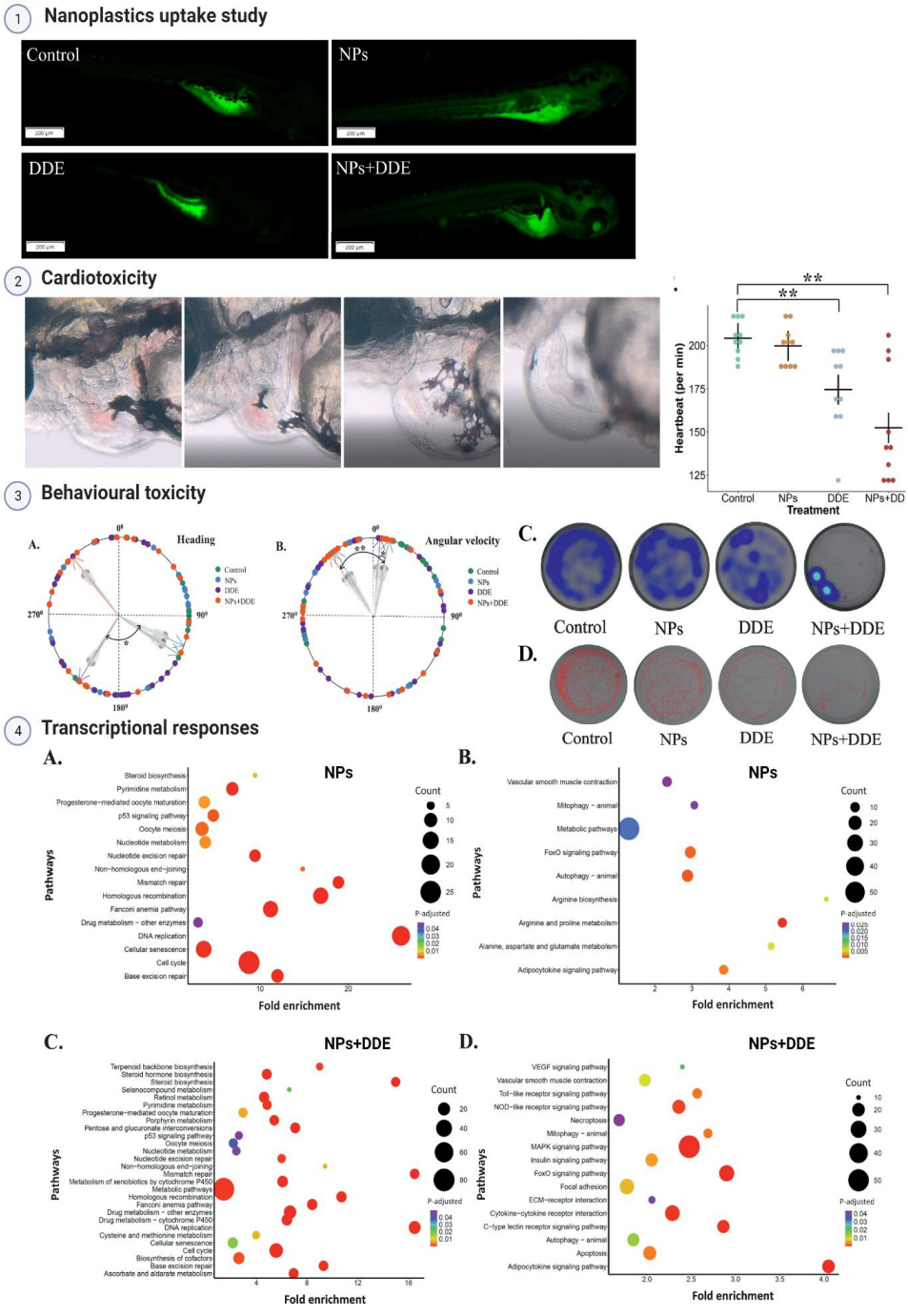

Figure 11. *Overview of the results obtained in Paper II.*

4.3 Paper III

In the behavioural assessment of adult zebrafish, exposure to 6PPDq and the combination of NPs along with 6PPDq significantly altered the swimming behaviour. The zebrafish exposed to 6PPDq and NPs+6PPDq tend to remain either at the bottom or towards one side of the tank (**Fig 12.**). Moreover, the fish exposed to these contaminants also showed short periods of sudden bursts in velocity. However, there were no traces of alterations in the swimming behaviour of zebrafish exposed to NPs alone. Additionally, various other swimming parameters like acceleration, heading, meandering, and angular velocity were unaffected by the exposures. Similarly, no significant effects were noted in the length of the lamina propria, the number of goblet cells, muscularis thickness, area of goblet cells, or villus height in the intestinal histology of zebrafish exposed to 6PPDq, NPs, or a combination of NPs and 6PPDq (**Paper III**). Liver histology also revealed no significant effect on the number of vacuoles and vacuole size (**Paper III**). However, liver and intestinal transcriptomics of the exposed zebrafish presented several key findings. In the NPs group, GO terms indicated mitochondrial dysfunction in the intestinal samples (**Paper III**). In zebrafish exposed to 6PPDq, GO terms related to the disruption of steroid and other metabolic pathways were affected in both liver and intestine tissues (**Paper III**). In the combined exposure group, we observed upregulated GO terms related to the steroid metabolic pathways in the liver, and these GO terms were more enriched than by exposure to 6PPDq alone (**Fig 12.**). Also, several key genes related to the fish's swimming behaviour were significantly affected, and these genes were more strongly affected by the co-exposure than by NPs or 6PPDq exposure alone (**Paper III**). The intestinal transcriptomics results from the combined exposure group indicate mitochondrial dysfunction in the zebrafish (**Fig 12.**). Overall, the results from this study clearly suggest that NPs can exaggerate the toxicity of 6PPDq in adult zebrafish.

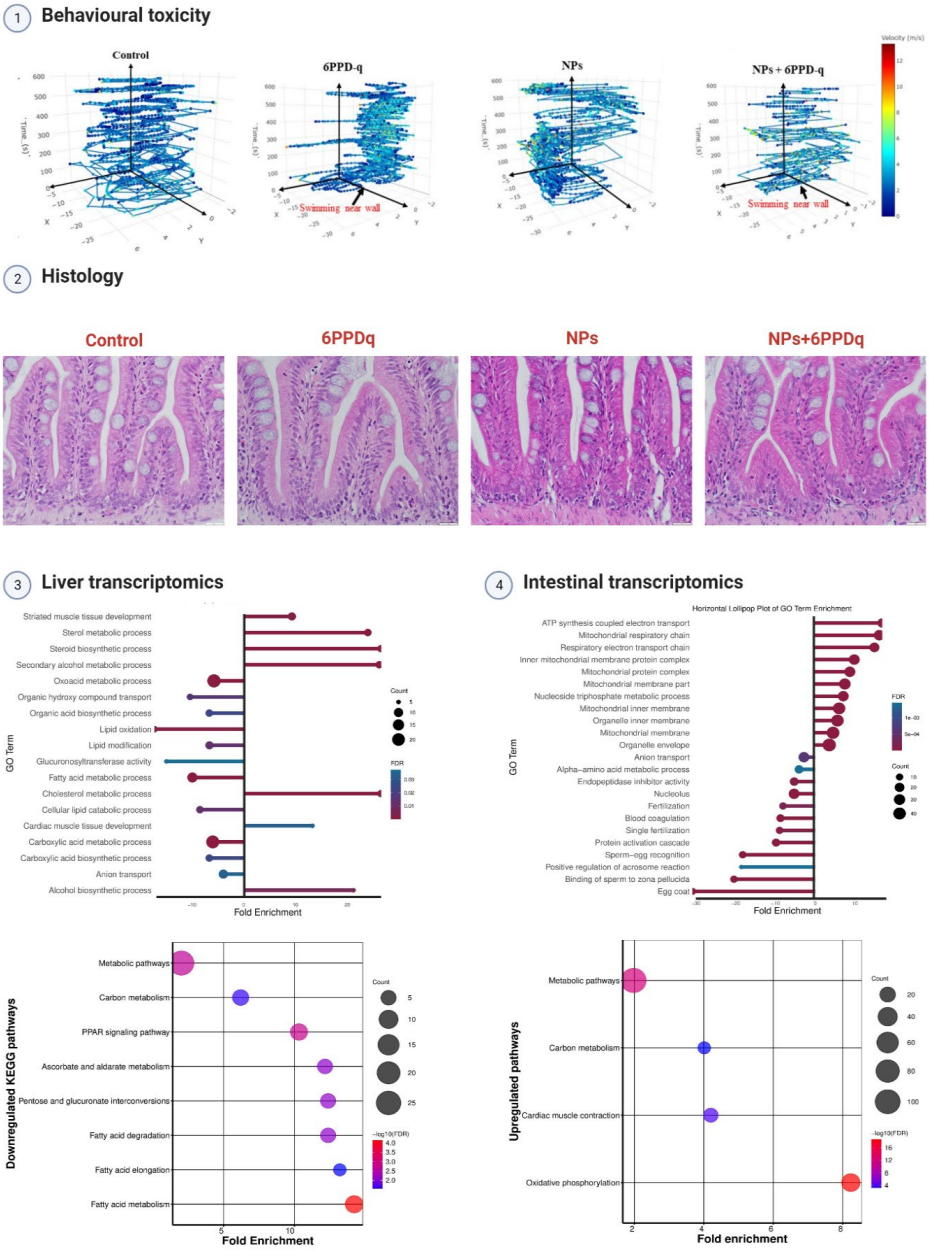

Figure 12. *Overview of the results obtained in Paper II.*

5 General discussion

The ubiquitous presence of plastics, including MPs/NPs, in the ecosystems has raised concern about their potential harm to ecosystem services and human health. The larger-sized plastic waste is comparatively easier to manage than MPs/NPs. These particles in the aquatic ecosystem can cause physical harm and organ-specific toxicity, such as cardiotoxicity, neurotoxicity, and immunotoxicity in experimental studies (Chae and An, 2017). Many exposure studies have shown that MPs/NPs are toxic (Sana et al., 2020, Singh et al., 2022). However, these contaminants co-exist in nature with other pollutants, such as heavy metals, POPs or tyre-wear contaminants (Reddy et al., 2014). As a result, we must not only comprehend the individual toxicity of these contaminants but also better comprehend the actual risks involved with the combined toxicity with other co-contaminants. Additionally, the emergence of novel pollutants presents a pressing challenge. Unlike well-studied legacy pollutants, novel pollutants lack comprehensive toxicity data. Hence, it becomes essential to understand the toxicity of the novel pollutants to assess the risks associated with the ecosystem. The thesis aimed to investigate the toxicity of emerging pollutants (6PPD and 6PPDq) and to fill a knowledge gap in the combined toxicity of the contaminants. Therefore, we firstly studied the toxicity of 6PPD and 6PPDq exposure to zebrafish larvae (**Paper I**). This study provides essential insights into the individual toxicological profiles of 6PPD and 6PPDq. Then, we performed the co-exposure of PS-NPs and DDE in the zebrafish (**Paper II**). This study enabled us to broaden our knowledge of combined toxic effects on zebrafish larvae. The knowledge obtained from the first two experiments was applied to the last chapter of the thesis, where we studied the combined toxicity of PS-NPs and 6PPDq in adult zebrafish (**Paper III**).

5.1 Toxicity of micro- and nanoplastics (MPs/NPs) and factors affecting its toxicity to aquatic organisms

MPs/NPs can reach the body of aquatic organisms through ingestion, ventilation (via gills), dermal uptake and trophic transfer (Cao et al., 2022, Mariano et al., 2021).

Among them, ingestion and ventilation are the main routes of the entry of MPs/NPs in aquatic organisms (Not et al., 2020, Setälä et al., 2018). Ingestion could be direct (ingestion of plastics) or indirect (predation of organisms that have ingested MPs/NPs). Filter-feeding organisms, including zooplankton, mussels, and some fish species, mistakenly take up plastic particles. The toxicity of MPs/NPs can be seen at various levels of biological organisation within an organism, such as organ system, organs, tissues and cells. With this rationale, we employed a multi-endpoint approach to observe the toxic effects of exposure to NPs in the zebrafish larvae (**Paper II**) and adult zebrafish (**Paper III**). The endpoints include development, behaviour, respiration, heart rate and transcriptome. In **Paper II**, we did not observe the toxic effects of NPs at the above said endpoints. We hypothesise that the excretion of the small-sized NP particles could be one of the reasons. In **Paper II**, we used 15 nm PS-NPs, and this was the first exposure study with NPs ≤15 nm in size. A study by Nowak et al. (2020) also found that particles <30 nm are cleared easily from the body. Based on this hypothesis, we designed a follow-up master thesis experiment to investigate whether larger-sized NPs can produce toxic effects in zebrafish under the similar experimental conditions (Hegstad-Pettersen, 2023). The results indicate that 100 nm NPs produced toxic effects at the molecular level (transcriptomics) and exposure also significantly increased the respiration rate (Hegstad-Pettersen, 2023). These findings underscore that particle size is an important factor in determining the toxicity of NPs. In the thesis's last study, we exposed adult zebrafish to 100 nm-sized PS-NPs (**Paper III**). We showed that exposure to 100 nm PS-NPs induced significant toxic effects at the molecular level (**Paper III**). Analysis of the intestinal transcriptome showed that the exposure caused mitochondrial dysfunction and generated ROS which again triggered pro-inflammatory cytokines (**Paper III**). These results are consistent with a study by Félix et al. (2023), which discovered that exposure of 21 days to MPs with a size of 179 ± 77 nm caused mitochondrial dysfunction in adult zebrafish. They found that the exposure significantly decreased mitochondrial respiratory chain complex reactions in the brain. Also, the exposure caused decreased liver mitochondrial respiration and membrane

potential. However, in our study, we did not find any swimming behavioural alterations (**Paper III**). The reason for this discrepancy could be concentration or length of exposure. The accumulation of contaminants increases with exposure time. These elevated doses may exceed the level at which animals can detoxify or excrete the particles (Annabi et al., 2011, Ding et al., 2019). Moreover, higher organisms have well-developed defence systems to counterattack the sudden acute exposure of these contaminants. Nevertheless, prolonged exposure might overload the repair and detoxification systems, making the organisms more vulnerable (Biehl and Buck, 1987). A 7-day exposure study with adult zebrafish using a range of MPs exposure concentrations (0.001, 0.01, 0.1, 0.25, 0.5, 1, 2, 10, and 20 mg/L) documented an MPs-induced effect on swimming behaviour (Chen et al., 2020). The study showed that concentrations >1 mg/L gave histopathological effects. However, the authors performed a swimming behaviour test only at 2 mg/L and found hyperactivity in the exposed fish. Surprisingly, the authors did not find a significant increase in oxidative stress in the exposed fish, as oxidative stress is one of the main reasons for the behavioural alterations in fish exposed to MPs/NPs (Chen et al., 2020). Then, the authors hypothesise that it could be due to a significant increase in the levels of 17 β-estradiol estrogen (E2). This particular estrogen stimulates the body's ability to accelerate, affecting swimming. Félix et al. (2023) observed a significant increase in the anxiety-like swimming behaviour of adult zebrafish exposed to 1 mg/L 179 ± 77 nm sized MPs for 21 days. The researchers did not observe any significant effect of the exposure in the open field test of the swimming behaviour assessment. However, they found that fish exposed to 1 mg/L MPs spent significantly more time in the dark than in the light zone. Another study by Sarasamma et al. (2020) observed hyperactivity in adult zebrafish exposed to 70 nm-sized PS-NPs for seven days at 1.5 mg/L in the novel tank test. The exposed fish were significantly less responsive in the predator avoidance test and showed reduced predator avoidance behaviour. However, there were no significant changes in the conspecific social behaviour in exposed zebrafish. All these above-referred studies exposed the adult zebrafish for more extended periods (≥ 7

days) than our study. The behaviour of zebrafish larvae exposed to MPs/NPs has been the subject of numerous studies, but only a small number of studies have focused on adult zebrafish.

5.2 Tyre-wear particles as toxicants

The size composition of TWPs is non-uniform and depends on the tyre composition, road surface, vehicle load and driving behaviour (weight load, speed and braking style). Kreider et al. (2010) found a size distribution of TWPs between 5 to 280 µm with on-road collected samples from two driving circuits in Clermont Ferrand, France. Similarly, Park et al. (2018) found a particle size distribution between 0.3 to 10 µm. The majority of early-stage studies have used collection and analysis techniques such as filtration, sedimentation, and sieving. These techniques favour capturing larger particles, i.e., MPs, rather than smaller NPs. However, recent studies have shown the vast presence of NPs in the TWPs. An experimental investigation by Kim et al. (2021) concluded the presence of particles < 100 nm in size from the TWPs. These studies show that TWPs contain a mixture of particles that range in size from nano to micrometre. Smaller particles can travel through the air and pose a challenge to humans, while the majority of the larger particles typically end up in the ocean through rainwater or stormwater runoff (Wagner et al., 2018).

In tyre manufacturing, styrene is often added to the rubber compounds to enhance durability. The presence of styrene groups in both rubber and PS suggests a chemical resemblance between the two substances. Given that both materials share some chemical properties, this similarity suggests that PS-NPs may be used to shed light on the toxicity of TWPs. With this hypothesis, we selected PS-NPs as the particle to be studied along with the 6PPDq (**Paper III**). Several studies have shown that larger TWPs are primarily irregular in shapes, whereas smaller particles are round/circular (Ha et al., 2023, Kim et al., 2021). In order to mimic the shape of smaller tyre-wear nanoparticles (TWNPs), we selected round-shaped PS-NPs for the study (**Paper III**).

Aquatic organisms can ingest TWNPs suspended in the water, disrupting biological processes at the physiological, cellular or sub-cellular level. We also discovered that adult zebrafish exposed to PS-NPs experienced toxic effects at the liver and intestinal transcriptome levels (**Paper III**). However, in terms of molecular responses, the toxic effect was more pronounced in the intestine than in the liver (**Paper III**). PS-NPs, as physical contaminants, are known to disrupt the intestinal membrane and induce local inflammation and toxicity. The direct interaction between PS-NPs and the intestinal membrane could have induced a more pronounced effect. Due to their small size and surface characteristics, PS-NPs may easily adhere to the intestinal lining. The intestinal membrane acts as a crucial barrier that controls nutrient absorption and blocks the entry of harmful substances. NPs may jeopardise this barrier's integrity. Several other studies have also observed similar effects of NPs exposure (Teng et al., 2022, Xie et al., 2021, Yu et al., 2022). In the **Paper III**, we found that exposure to PS-NPs can alter mitochondrial function and cellular processes. Upregulated DEGs (*nbufb8*, *ndufa7*, *cox7b*, *cox8b*, *uqcrb* and *ndufb6*) associated with mitochondrial respiratory chain and energy production suggest an adaptive response to the stress of PS-NPs exposure (**Paper III**). These findings emphasize the need for further research to comprehend the broader ecological implications of NPs exposure.

5.3 Nanoplastics (NPs) as vector

NPs are emerging environmental contaminants with a size of less than 1000 nm. The two important sources of these particles are the breakdown of MPs and the release of beads from personal care products. Apart from their own toxic effects, the other most crucial aspect of NPs is their ability to act as vectors for other environmental contaminants such as heavy metals, pesticides, insecticides, antibiotics and other harmful chemicals. There are many reasons why NPs are an ideal vector. Firstly, their small size and large surface area-to-volume ratio make them highly efficient at adsorbing and carrying pollutants (Da Costa et al., 2016, Rocha-Santos, 2018). Although MPs also act as a vector, the difference in their surface area-to-volume ratio is

particularly noteworthy. When comparing this ratio between MPs with a diameter of 5 mm to NPs with a diameter of 100 nm, NPs exhibit 3000-fold more surface area-to-volume than NPs. This makes NPs more effective at adsorption, chemical reactions, and interactions with other pollutants. Yu et al. (2023) discovered a 33.8% increase in oxytetracycline (OTC) accumulation in zebrafish liver when co-exposed with 300 nm NPs compared to OTC exposure alone. They also discovered that when co-exposed with 50 nm NPs, this accumulation increased to 44.5% compared to OTC exposure alone. Secondly, the surface of NPs usually contains functional groups which can bind with hydrophobic environmental pollutants. The surface of the NPs often contains functional groups such as carboxyl, hydroxyl, amino, epoxy and thiol (Wang et al., 2021c). These groups create polarity on the NPs surface, resulting in their increased binding efficiency towards the hydrophobic environmental contaminants (Cao et al., 2022). This phenomenon of carrying pollutants by another pollutant is known as the "Trojan Horse effect" (Zhang and Xu, 2022). Apart from the physical properties of NPs, they are also known to penetrate biological membranes and barriers (Khan and Jia, 2023, Kopatz et al., 2023). This property of NPs results in their increased bioavailability. Our studies found that exposure to environmentally relevant concentrations of NPs can enhance the toxicity of legacy pollutants such as *p,p'*-DDE and tyre-wear chemicals such as 6PPDq in zebrafish (**Paper II** and **Paper III**). In **Paper II**, we did not observe any toxic effect of NPs exposure alone with the selected endpoints (development, heart rate, swimming behaviour and transcriptome). But when co-exposed with DDE, we found a significant decrease in heart rate and swimming performance (distance moved, velocity, movement, stasis, angular velocity and heading). DDE is a known endocrine disruptor (Crews et al., 2000). A study by Monteiro et al. (2015) found that a 14-day exposure to 2 mg/L *p,p'*-DDE significantly increased the levels of vitellogenin in juvenile zebrafish. Vitellogenin is an egg yolk precursor protein that is produced in the liver in response to estrogenic activity and is a widely used marker for endocrine disruption (Hiramatsu et al., 2005). In **Paper II**, we found that exposure to 100 µg/L *p,p'*-DDE did not dysregulate any vitellogenin-related genes in the zebrafish larvae. However, co-

exposure of *p,p'*-DDE with NPs significantly downregulated the *vtg7* gene (3.8-fold). This gene is encoding a protein associated with the cellular response to estrogen stimulus. Similar results were also seen for immune-related genes, with a stronger response in co-exposed larvae (**Paper II**). Overall, this suggests that exposure to low levels or environmentally relevant concentrations of *p,p'*-DDE alone might not affect zebrafish the larvae adversely. However, when co-exposed with NPs, the exposure can lead to adverse toxic effects. Our results align with a study which observed that 5 mg/L PS-NPs have aggravated the toxic effects of 1mg/L PCB77 in the zebrafish embryos (Li et al., 2022). Apart from being the carrier of the chemical pollutants, NPs can also host unique microbial communities. NPs can support the growth of biofilms or colonies of bacteria, algae, and other microorganisms (Rana and Kumar, 2022). This attachment can protect the bacteria or other biological communities from environmental stressors such as UV radiation. NPs are ubiquitous in the environment, so understanding their role as vectors for contaminants is critical for determining the full extent of their environmental and health consequences. In summary, our studies reveal that NPs can act as vectors or carriers to enhance the toxicity of *p,p'*-DDE or 6PPDq in zebrafish. These findings underscore the critical role of NPs as potential carriers of environmental pollutants in the biological system.

In **Paper III**, we found that exposure to 100 nm-sized PS-NPs (3 mg/L) enhanced the toxicity of 50 µg/L 6PPDq. To our knowledge, this is the first study focusing on the combined toxicity of two tyre-wear associated contaminants, i.e. NPs and 6PPDq (**Paper III**). The results of the swimming behaviour assay (open tank test) showed that locomotion is dysregulated in adult zebrafish exposed to 6PPDq or a combination of NPs with 6PPDq but not in zebrafish exposed to NPs alone (**Paper III**). The observed behavioural dysregulation holds significant ecological implications, suggesting impacts on predator-prey interactions and foraging behaviour in higher trophic fishes. It is worthy to note that coho salmon, brook trout (*Salvelinus fontinalis*), rainbow trout and white-spotted char are 6PPDq-sensitive fishes and all are at a higher trophic level where predator-prey interactions are very important. This is the first study to

document enhanced toxicity of combination of tyre-wear-associated pollutants (**Paper III**). In order to effectively manage and protect ecosystems, it is critical to identify interaction among different contaminants.

5.4 Toxicity of 6PPD-quinone (6PPDq)

The synthetic rubber industry has played a significant role in developing the transportation system in modern society. The use of rubber in other sectors, such as infrastructure and consumer goods, can not be underestimated. However, many issues are associated with synthetic rubber products, such as the release of TWPs and leaching of associated chemicals such as 6PPD. Several studies have shown the potential toxicity of these chemicals and particles to aquatic and terrestrial organisms (Page et al., 2022, Peng et al., 2022). Additionally, its oxidised form i.e. 6PPDq was found to be the cause of massive coho salmon mortality during stormwater runoff in the United States over the last 20 years (Tian et al., 2021). Following this breakthrough study by Tian et al. (2021), several laboratories worldwide started assessing the toxicity of 6PPDq and its implications on organisms. Hiki et al. (2021) were the first to assess the toxicity of this compound on four model fish and crustacean species, namely Zebrafish, *Oryzias latipes*, *Daphnia magna*, and *Hyalella azteca*. The study showed that exposure of 6PPDq in concentrations up to 100 µg/L to any of these species did not cause acute high mortality.

In **Paper I**, we studied the toxicity of 6PPDq and its parent compound, i.e. 6PPD, in the zebrafish larvae. We found 24 h LC_{50} in zebrafish larvae as 1384.93 & 308.67 µg/L for 6PPD & 6PPDq, respectively (**Paper I**). This indicates that 6PPDq is less acutely toxic to zebrafish larvae than to coho salmon, with approximately a 1000-fold difference in the 24 h LC_{50}. The plausible cause may be a result of a confluence of multiple factors such as biological (animals' size and life stage), physiological (metabolism and detoxification), genetic (genetic variability), and environmental (water temperature, pH, and exposure conditions) factors. The other possible reason for this discrepancy could be differences in the biology of the tested animals. Apart from the acute toxicity

tests, we also assessed the developmental aberrations, swimming behaviour/locomotor tests, heart rate and respiration rate in the zebrafish larvae exposed to 1, 10 and 25 µg/L 6PPD & 6PPDq (**Paper I**). We found a significant reduction in eye size in zebrafish larvae exposed to 25 µg/L 6PPD and 6PPDq, as well as other developmental abnormalities like jaw deformity, reduced yolk resorption, pericardial edema, reduced eye, uninflated swim bladder, kyphosis and lordosis (**Paper I**). Later, a study by (Zhang et al., 2023c) focused on the developmental toxicity of 6PPD and 6PPDq in zebrafish and found that 100 µg/L 6PPD-exposed larvae had a myopia-like phenotype with a convex eyeball. Zhao et al. (2023) exposed pregnant mice to 6PPD and 6PPDq, using an exposure dose ten times higher than that found in human urines by Du et al. (2022). The authors found that the 6PPDq levels in the placentas and embryos of exposed mice were 1.5-8 times higher than the 6PPD values, suggesting that 6PPDq can easily cross the placenta (Zhao et al., 2023). In addition to these, the authors discovered that exposure to 6PPq can activate the human retinoic acid receptor α (RARα) and the retinoid X receptor α (RXRα) even at an exposure concentration of 0.3 µM 6PPDq. RARα is an important transcription factor that regulates several key processes, such as cell growth, differentiation and embryonic growth (Zhao et al., 2023). Any dysregulation in this transcription factor can cause developmental abnormalities and congenital disabilities (Zhao et al., 2023). This study highlights the potential dangers of 6PPD and its metabolite 6PPDq to pregnant women and their foetuses. In conclusion, our study focused on the toxicity of 6PPD and 6PPDq on zebrafish larvae has revealed a significant difference in acute toxicity compared to other species (coho salmon, white-spotted char, brook trout and rainbow trout), likely influenced by many factors (**Paper I**). Moreover, our study on developmental toxicity in zebrafish larvae revealed significant developmental aberrations, indicating the need for further investigation (**Paper I**).

In **Paper I**, we found intestinal reddening, a clinical sign of intestinal inflammation, in 90% of the larvae examined under the microscope. Our research was the first to suggest that 6PPDq exposure can cause intestinal inflammation (**Paper I**). Later, Zhang

et al. (2023c) observed significant changes in the intestinal morphology in zebrafish larvae exposed to 1.2 mg/L 6PPDq. The changes included an increase in the intestinal area and a thickening of the intestinal wall. Hua et al. (2023a) discovered that chronic exposure to 100 g/L 6PPDq caused intestinal oxidative stress, which resulted in changes in intestinal permeability in the *Caenorhabditis elegans*. However, to our knowledge there has not been conducted any research on intestinal dysfunction at the transcriptomic level after 6PPDq exposure. In order to accomplish this, we designed the third study of the thesis to examine the impact of 6PPDq exposure on the adult zebrafish's intestine (**Paper III**). Based on the transcriptomic data, we observed dysregulated GO terms related to steroid metabolism, lipid metabolism and muscle development (**Paper III**). Steroids can alter the composition of the gut microbiota and the functioning of the intestinal barrier, which may increase the risk of intestinal inflammation. However, the precise effects of steroids on intestinal inflammation are not yet fully understood.

In **Paper I**, we observed that 25 µg/L 6PPD & 6PPDq induced toxic effects on the heart rate. In the 25 µg/L 6PPD group, we also found pericardial edema. Our research was the first to suggest that 6PPD or 6PPDq exposure can generate cardiotoxic effects in fish (**Paper I**). Later, it was discovered in a study by Fang et al. (2023b) that 6PPD can bioaccumulate in zebrafish larvae and that this bioaccumulation can cause oxidative stress. They also discovered that 6PPD induced a downregulation of cardiac function-related genes such as *slc8a2b*, *cacna1b*, *cacna1da* and *pln*. In addition, they found an increase in apoptosis in the pericardial area through acridine orange staining in larval zebrafish. In **Paper III**, we also found that 6PPDq exposure led to a dysregulation of *slc* family genes (*slc26a5*, *slc1a3a*, *slc35a3a*, *slc2a12*, *slc40a1* and *slc4a5*) in the liver. The *slc* (solute carrier) gene family is a large group of membrane transport proteins. These genes are essential in ionic balance and intra and extracellular transport. For example, *slc4a5* encodes a sodium transporter, which is vital in regulating pH in cardiac tissues (Romero, 2005). Alterations in pH and ionic balance can lead to arrhythmias and other cardiac issues.

In **Papers I** and **III**, we observed significant changes in the swimming behaviour of zebrafish larvae and adults when exposed to 6PPDq. We observed hypolocomotion in zebrafish larvae exposed to 25 µg/L 6PPDq (**Paper I**). Our findings are consistent with those of a subsequent study that discovered hypoactivity in zebrafish larvae exposed to 2 µg/L 6PPDq (Ricarte et al., 2023). The behaviour of fish is primarily controlled by the central nervous system; changes in neurotransmitter levels can influence swimming behaviour. Ricarte et al. (2023) observed changes in the levels of neurotransmitters such as ACh, serotonin, norepinephrine, and epinephrine in zebrafish larvae exposed to 0.02 and 0.2 µg/L 6PPDq. Another study by Ji et al. (2022b) also observed a significant decrease in the distance moved and velocity of zebrafish larvae exposed to 1000 µg/L 6PPD. The authors also discovered significant effects on neurotransmitters such as dopamine, gamma-aminobutyric acid (GABA), and ACh, which could cause changes in swimming behaviour. In our study, we found a significant decrease in the distance moved by the larvae exposed to 25 µg/L 6PPD, but we did not find any significant effect on the velocity, and the plausible reason could be the difference in the exposure concentration (**Paper I**). In the third study of the thesis, we observed hyperactivity in the adult zebrafish exposed to 50 µg/L 6PPDq (**Paper III**). The plausible reason for hypoactivity in larvae and hyperactivity in adult zebrafish could be the recording conditions. We used alternate cycles of light and dark to record swimming behaviour of zebrafish larvae, whereas adults were recorded entirely in the dark phase (**Paper I** and **Paper III**). Zebrafish larvae have photophobic swimming behaviour, meaning they move more in the dark phase than the light phase (Burgess and Granato, 2007). Another reason could be that 6PPDq exposure had different toxic effects at the different life stages of the animal.

In **Paper I,** we also found that exposure to both 25 µg/L 6PPD and 6PPDq strongly affected the respiration rate or oxygen consumption in zebrafish larvae. Later, a study by Mahoney et al. (2022) discovered that exposure of 20 µg/L 6PPDq to RTgill-W_1 (rainbow trout gill cell line) cells caused a two-fold increase in oxygen consumption. These findings point to a consistent pattern of increased oxygen consumption

associated with 6PPDq exposure across different experimental models, emphasising the impact on respiratory processes. Despite the fact that 6PPDq research and toxicity are hot topics, our understanding of the mode of action of this TW chemical is still limited. Initial studies have shed light on its toxicity at the organism level, but it is essential to assess it from a larger perspective, i.e., the ecosystem level. In addition, there is a need for further investigation into the effects on human health.

5.5 Pollutants in the fish feed

Aquaculture plays a significant role in meeting the food demands of the increasing human population. Global aquaculture production increased from 21.8 million tonnes in 1990 to 122.6 million metric tonnes in 2020 (FAO, 2022). However, many environmental and health issues are associated with aquaculture practices. Some of these challenges include disease spread, escapes of farmed fish, habitat destruction and pollutants in fish feed (Cole et al., 2009, Naylor et al., 2000). The presence of contaminants in fish feed is an area of focus for food safety authorities since contaminants in feed can be transferred to the food and pose a risk for the consumer. To achieve this in Norway, the IMR, monitors the levels of contaminants in fish feed yearly. The latest report indicates the presence of contaminants such as mycotoxins, metals, PCBs, chlorinated pesticides and DDT (**Table 1**). However, none of the contaminants were above the legal limits. DDT is a synthetic insecticide with a high potential for environmental and human toxicity. Presence of DDT and its metabolites have been documented in fish meal and oil for many years (Ørnsrud et al., 2020, Sele et al., 2019, Sele et al., 2022). Although there have been many studies conducted on the toxicity of p,p'-DDE, none have focused on the combined toxicity of p,p'-DDE along with MPs/NPs.

The results from our study (**Paper II**) demonstrate p,p’-DDE-driven toxicity in zebrafish larvae at multiple endpoints (development, behaviour, respiration and heart rate). The data obtained from these endpoints align with the transcriptomic data, where we

observed dysregulated GO terms such as heart contraction, response to hypoxia, photoperiodism, circadian regulation and neuropeptide hormone activity (**Paper II**).

The results obtained in our study align well with several other studies showing similar developmental and immunotoxic effects (Monteiro et al., 2015, Wu et al., 2019). The concentration used in our study (100 µg/L) was a bit less than four times higher than what is found in fish oil (Ørnsrud et al., 2020). It is very important to recognize that even at this moderate and close to environmentally relevant concentration, p,p'-DDE caused toxicity in zebrafish larvae (**Paper II**). This is just an experimental study with a single DDT congener, in nature there are six congeners occurring simultaneously and that it is important to study mixture effects.

5.6 Mixture toxicity

The results of our study (**Paper II**) hint at the possible synergistic effects between NPs and DDE. The frequency of developmental aberrations was higher in the zebrafish larvae exposed to both NPs and DDE. Similarly, more substantial toxic effects were observed in the swimming behaviour, heart rate, oxygen consumption and gene expression. In **Paper III** of the thesis, we observed probable synergistic effects between NPs and 6PPDq, as we detected elevated hyperlocomotion in adult zebrafish exposed to the mixture of NPs and 6PPDq over their single exposure. The findings from these studies underscore the significance of investigating the toxic effects arising from a combination of contaminants, particularly when they originate from a shared source. It is important to note that while our studies suggest possible synergism between NPs and DDE and between NPs and 6PPDq in zebrafish, further validation is needed to determine whether the chemicals act non-additive. Statistical modelling will provide a more robust understanding of the relationship between these pollutants, offering a solid foundation for drawing reliable conclusions about their combined impact on aquatic organisms.

5.7 Relevance of the findings

Ecotoxicology is a field of science with the prime motto of investigating the effect of pollutants and contaminants on organisms and ecosystems. In the modern world of industrialization, new chemicals are being synthesised to propel human progress. According to Bond (2020), about 25,000 to 140,000 chemicals are in use globally, and many of these chemicals might make their way to the environment during production or post-production stages. Hence, understanding the toxicity of these contaminants at the organism and ecosystem level is the first step in pollutant assessment, and the final step is the development of mitigation methods. The findings from toxicity studies also help to identify bioindicator organisms and develop biomarkers to monitor the impact of pollutants. Another aspect of ecotoxicology is to establish a safe level of pollutants through risk assessment. The results from ecotoxicological studies also help governmental bodies to develop policies and regulations to safeguard ecosystems and human health.

In the first study of the thesis, we assessed the toxicity of 6PPD and its ozone-transformed contaminant, i.e. 6PPDq. The main purpose of the study was to investigate toxic effects at environmentally relevant and sublethal concentrations using zebrafish larvae as an animal model (**Paper I**). The findings suggest that lower or environmentally relevant concentrations of 6PPD or 6PPDq did not cause toxicity to zebrafish larvae (**Paper I**). However, higher concentrations caused toxicity in zebrafish larvae. Subsequent studies on the 6PPDq-driven toxicity in fish supported our findings (Hua et al., 2023a, Mahoney et al., 2022, Wu et al., 2019). Following all these findings, the U.S. Tire Manufacturers Association (USTMA) and the European Tyre and Rubber Manufacturers Association (ETRMA) called for additional research on 6PPDq and looking for greener options (ETRMA, 2023, USTMA, 2021). It becomes of utmost importance to regulate the use of 6PPDq as it has been also detected even in human urine (Du et al., 2022), and studies have shown possible health risks for humans (Zhao et al., 2023). Understanding the route of entry of 6PPDq into humans is not that

difficult because studies have demonstrated the widespread presence of this toxicant in foods like fish, honey and lettuce (Castan et al., 2022, Ji et al., 2022a). Therefore, efficient laws and regulations should be introduced to minimise the environmental release of 6PPDq and encourage the creation of safer alternatives to protect the environment and human health.

In the second paper of the thesis, we investigated the single and combined toxicity of NPs and p,p'-DDE on zebrafish larvae. The relevance of the study lies in two important findings from the study (**Paper II**). First, the study showed that NPs can act as vectors and enhance the toxicity of p,p'-DDE. Secondly, it showed that p,p'-DDE can act toxic at concentrations close to environmental relevance (**Paper II**). The latter finding, i.e., toxicity of p,p'-DDE to fish larvae at relatively low concentration, also underscores the importance of monitoring harmful contaminants in the ecosystem.

In the third paper, we studied the combined toxicity of NPs and the tyre-wear contaminant 6PPDq (**Paper III**). PS-NPs were employed to model nano-sized TWPs (**Paper III**). The combination of these contaminants is highly relevant, as tyres can be a source of both NPs particles and 6PPDq, and runoff from roads can carry these contaminants into waters. The findings from the current study indicate that NPs can act as a vector to enhance the toxicity of 6PPDq. One possible explanation for the more substantial effects of DDE and 6PPDq in co-exposure could be that the NPs disrupt cellular membranes and thus enhance uptake into cells (**Paper II** and **III**). Also, considering the observed toxicity of 6PPD and 6PPDq to zebrafish from our studies (**Paper I** and **III**), there is an urgent need to support the implementation of sustainable practices in the tyre manufacturing sector. The findings also highlight the importance of mixture toxicity and suggest that combination effects of contaminants should be considered when formulating policies.

6 Conclusions

As our urban landscapes grow, new contaminants are emerging, including pharmaceutical drugs, nanoplastics and tyre-wear contaminants. Research on emerging contaminants is vital as it provides data on their source, uptake and toxicity, which is very important to develop mitigation strategies. The first study of the present thesis provides the first insight into the toxicity of two important emerging tyre-wear contaminants, i.e. 6PPD & 6PPD-q. We used several endpoints, such as development, behaviour, heart rate and respiration, to generate the overall landscape of toxicity. We found that higher than environmental relevant concentrations can significantly affect the zebrafish larvae.

The co-existence of pollutants illustrates the paramount importance of the need for research into the combined toxic effects of pollutants. From the latter two studies of the thesis, we discovered that NPs can act as a vector to enhance the toxicity of both legacy (p,p'-DDE) and emerging pollutants (6PPDq). In these two studies, we also used transcriptomics to assess the mechanistic effect at the molecular level along with other endpoints. We found that p,p'-DDE and 6PPDq alone can have toxic effects on zebrafish, but toxicity increases when these chemicals are co-exposed with NPs. The results from our studies emphasise the importance of understanding the effects of complex mixtures of contaminants.

7 Contribution to the field

The present thesis represents a considerable contribution to the field of environmental toxicology, focusing on tyre wear particles and associated chemicals. Through a comprehensive multi-endpoint approach, this thesis has yielded novel insights into the ecotoxicological field through multiple research papers.

The first paper of the thesis focused on the toxicological effects of 6PPD and 6PPDq in zebrafish larvae. This was the first scientific study to be published that suggested that exposure to 6PPDq can have both cardiotoxic and neurotoxic effects. Our findings were based on physiological data obtained by using the DanioVision System to measure swimming behaviour and heart rate through microscopy. After the publication our study, several subsequent studies also shown cardiotoxic, immunotoxic and neurotoxic effects in fish exposed to 6PPDq using transcriptomics, metabolomics and lipidomics data. The findings from our study thus paved the way for more advanced research in the ecotoxicological assessment of tyre-wear contaminants.

The second paper of the thesis focused on the single and combined toxicity of NPs along with *p,p'*-DDE, a breakdown product of the legacy contaminant DDT. To make the study relevant to the present-day situation, we used an exposure concentration close to environmentally relevant. In order to gain a comprehensive understanding of the toxicity of these contaminants, we employed multiple phenotypic to molecular endpoints. Although we did not find any strong toxic effect of NPs at environmentally relevant levels in the second paper, we found that NPs enhance the toxicity of *p,p'*-DDE. This study sheds light on the significance of combined toxicity assessments.

The third study of the thesis delved into the investigation of two important tyre-wear pollutants, namely NPs and 6PPDq, using adult zebrafish as an animal model. Although many studies in the last two to three years have focused on the toxicity of 6PPDq, no studies have examined the combined toxicity of multiple tyre-wear pollutants. **Paper III** is the first study to fill this gap of knowledge. The results indicate that both NPs and

6PPDq have toxic effects at different endpoints. But when combined, NPs enhance the toxicity of 6PPDq.

8 Future perspectives

The present thesis lays out an important aspect of toxicity assessment of NPs and tyre-wear contaminants using zebrafish as an animal model. Overall results at multiple endpoints indicate possible toxic effects of these contaminants at environmentally relevant and sub-lethal concentrations. Additionally, the results suggest that NPs can act as a vector to enhance the toxicity of other pollutants such as *p,p'*-DDE or 6PPDq. This work identified several potential questions that warrant further exploration:

1. We observed significant changes in swimming behaviour in zebrafish exposed to 6PPDq (**Paper I** and **III**). We also identified some key genes and pathways related to locomotor dysregulation in the liver (**Paper III**). Since locomotion in fish is mainly governed by the central nervous system, the brain transcriptome should be examined to get a clearer picture of the molecular pathways affected by 6PPDq exposure.
2. We observed toxicity of 6PPDq on multiple endpoints in the exposed zebrafish (**Papers I** and **III**). However, there is a lack of knowledge about possible intergenerational transmission of effects from exposed parents to offspring and whether this might occur in fish. Investigating the inter and transgenerational effects of 6PPDq on zebrafish at environmentally relevant concentrations might fill this gap.
3. Size plays an important role in controlling the toxicity of MPs/NPs. But there is a lack of knowledge on how shape controls the toxicity especially for NPs. Investigating how shape controls NPs toxicity and vector behaviour will fill this gap.
4. The findings from the third study of the thesis indicate that single and combined exposure of NPs with 6PPDq can cause intestinal inflammation primarily mediated by mitochondrial dysfunction (**Paper III**). In light of these results, a study on the impact of the gut microbiome will indicate the possible effects of 6PPDq exposure on the microbial community.

5. The findings from last two studies of the thesis indicate synergism between the pollutants and NPs (**Paper II & III**). Follow-up studies should use advanced statistical modelling of mixture toxicity to capture the intricate interactions between pollutants, enabling a more comprehensive understanding of their combined effects.
6. There is a pressing need to understand the source, fate and species at risk to 6PPDq. A study with ecosystem-level risk assessment might fill this gap. A comprehensive environmental risk assessment must consider how this contaminant affect entire aquatic ecosystems, including food webs and biodiversity.

9 9. References

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Paper I

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Research Paper

Toxicological effects of 6PPD and 6PPD quinone in zebrafish larvae

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ABSTRACT

N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD) is the most widely used antioxidant in automobile tyres and many rubber products. We investigated the impact of 6PPD and 6PPD quinone on acute toxicity, morphology, swimming behaviour, heart rate, and oxygen consumption in zebrafish larvae. Zebrafish embryos were exposed to 6PPD and 6PPD quinone at concentrations of 1, 10, and 25 μg/L during the development period of 1–96 hpf. In the present study, 6PPD quinone was found to be toxic to zebrafish larvae with a 24 h LC_{50} of 308.67 μg/L. No significant mortality was observed at any of the tested concentrations. A dose-dependent reduction in swimming performance was observed in the exposed larvae at 116 hpf for both toxicants. Overall, our study shows that exposure of zebrafish embryos to 6PPD and 6PPD quinone at environmentally relevant concentrations (1 μg/L) does not affect its behaviour. However, exposure to higher but still sublethal concentrations of 6PPD and 6PPD quinone (10 and 25 μg/L) can affect behavioural endpoints. These findings reveal the toxicity of 6PPD and 6PPD quinone to early life stages of fish.

1. Introduction

N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine or 6PPD is an antioxidant and antiozonant that is widely consumed by the rubber and polymer industry (Datta et al., 2007). This compound is mainly used in rubber tires to protect them from cracking that results from ozone reaction and wear out. 6PPD is also used to prepare dyes (Meyer and Fischer, 2015), lubricants (Liu et al., 2019), and house-hold products. The release of 6PPD into the environment may also occur during rubber manufacturing and recycling of used rubber articles (Hartwig and Commission, 2013). The release of rubber into the air and wastewater is of great concern because air and water-borne pollutants can affect the health of both humans and animals and our natural ecosystem.

The most significant anthropogenic source of 6PPD that enters the aquatic environment is tire wear particles (TWP) (Halle et al., 2021; Wagner et al., 2018). TWPs constitute harmful chemicals and microplastics. According to an estimate, the annual global per capita release of TWP was found to be 0.81 kg (Kole et al., 2017). Studies on the biological effects of TWP on different animal models have revealed that these particles potentially can have harmful effects at the molecular, cellular, and organism level (Halle et al., 2021; Mantecca et al., 2009; Wik and Dave, 2009).

In the Pacific Northwest of the United States, sudden unexplained mortality of adult coho salmon (*Oncorhynchus kisutch*) occurred after exposure to stormwater when they migrated to urban creeks for spawning (Chow et al., 2019; McIntyre et al., 2018; Scholz et al., 2011; Tian et al., 2021). A derivative of 6PPD, namely 6PPD quinone, was implicated for the fish mortality observed in the US (Tian et al., 2021). Ozonation of 6PPD into 6PPD quinone is a chain reaction and involves steps, including (1) loss of oxygen (O_2) to form a 6PPD amine oxide ($C_{18}H_{24}NO^-$), (2) side chain formation, and (3) final dissociation to 6PPD quinone ($C_{18}H_{22}N_2O_2$) (Lattimer et al., 1983). The resulting chemical product, i.e., 6PPD quinone, was found to be highly toxic to coho salmon (Blair et al., 2021; Tian et al., 2021). The mode of action of 6PPD quinone is still unknown.

Zebrafish (*Danio rerio*) has been widely used in eco-toxicological studies evaluating the effects of various environmental contaminants. The zebrafish embryo is a model commonly used to understand acute toxicity, behavioural toxicity, cardiotoxicity, and genotoxicity. The effect of many toxicants to zebrafish embryos is well correlated with those observed for rodents (Ali et al., 2012). Furthermore, the behavioural response is a key measure of toxicity in zebrafish larvae (Brun et al., 2019; Kalueff et al., 2013). Therefore, quantitative analysis of fish behavioural responses to contaminants provides valuable insights into the effects of environmental pollutants.

To date, knowledge about how 6PPD and 6PPD quinone affect the

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Table 1
LC_{50} values in zebrafish larvae following 96 h exposure of 6PPD and 6PPD quinone.

	Time	LC_{50} (μg/L)	95% Confidence limit (μg/L)	Regression equation	R^2
6PPD	24 h	1384.93	1005.06–1908.37	y = 2.7037x - 3.4942	0.9792
	48 h	816.90	649.41–1027.59	y = 3.4521x - 5.0515	0.974
	72 h	609.39	483.70–767.73	y = 3.3111x - 4.2195	0.359
	96 h	442.62	352.71–555.45	y = 3.4758x - 4.2008	0.946
6PPD quinone	24 h	308.67	258.31–368.86	y = 4.2249x - 5.5182	0.9953
	48 h	224.56	189.41–266.24	y = 4.189x - 4.8516	0.9864
	72 h	171.63	138.39–211.60	y = 3.1956x - 2.1421	0.9535
	96 h	132.92	107.65–164.11	y = 3.367x - 2.1564	0.9002

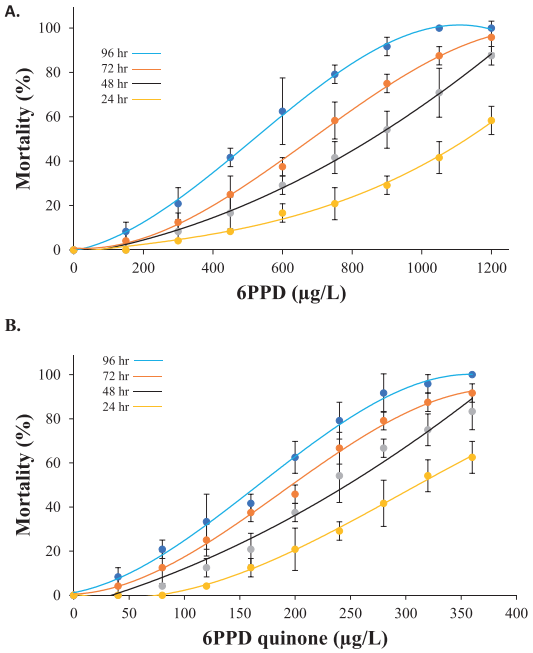

Fig. 1. Dose-response curves of zebrafish embryos exposed to (a) 6PPD and (b) 6PPD quinone following 96 h of exposure (n = 24).

behaviour of fish larvae is not described. The goal of the present study was to investigate the behavioural responses and cardiotoxicity in zebrafish larvae after exposure to 6PPD and 6PPD quinone. Zebrafish eggs were exposed to 6PPD and 6PPD quinone for 96 h post-fertilization (hpf). At 116 hpf, larvae were assessed for behavioural endpoints and cardiotoxicity using the DanioVision system. Moreover, oxygen consumption studies were also performed. Our results suggest that exposure of zebrafish embryos to 6PPD and 6PPD quinone at higher concentrations causes alteration in normal swimming behaviour and oxygen consumption. The study presents new knowledge on the toxic effects of 6PPD and 6PPD quinone on early life stages of fish.

2. Materials and methods

2.1. Chemicals and reagents

The chemicals 6PPD (CAS: 793–24–8, Purity: >98.0%) and 6PPD quinone (CAS: N/A, Purity: >98.0%) were obtained from CymitQuimica Chemicals (Barcelona, Spain). Stock solutions of both the chemicals were prepared by dissolving them in molecular grade ethanol. The concentrations of the prepared stock solutions were 2.5 mg/ml for both 6PPD and 6PPD quinone. Then selected test concentrations for both acute toxicity and exposure experiment were prepared by adding an adequate volume of stock solution to ISO standard fish media (ISO water: ISO 7346–3; 79.99 mM $CaCl_2.2\ H_2O$, 20.00 mM $MgSO_4.7\ H_2O$, 30.83 mM $NaHCO_3$, 3.09 mM KCl, pH = 7.4 ± 0.1). ISO standard fish media was aerated with oxygen 24 h before the exposure. Oxygen saturation of 80% and pH of 7.4 were maintained throughout the exposure study.

2.2. Zebrafish husbandry

Adult zebrafish (AB strain) were maintained and bred at 28 ± 2 °C under a 14 L:10D photoperiod cycle in a recirculating water system (Aquatic Habitats Z-Hab System) at the zebrafish facility of Nord University. Adult zebrafish were fed micro diet Zebrafeed® (Sparos Lda, Olhão, Portugal) at 4% body weight, split into two rations/day. Zebrafish eggs were obtained by random mating between sexually mature individuals, and the fertilization rate was at least 80% for all experiments. Fertilized eggs were separated from the unfertilized eggs using a binocular microscope (Leica Z00M 2000); based on the cleavage irregularities (OECD, 2013). The usage of zebrafish larvae was according to the animal welfare act; since "zebrafish embryos and larvae below 120 h" old are not protected animal stages, no animal test authorization is required according to European legislation Directive 2010/63/EU (Commission, 2010). The final measurements were taken at 116 hpf, and fish were then euthanized by immersing in 200 mg/L of MS222 buffered with 200 mg/L of sodium bicarbonate.

2.3. Zebrafish embryo acute aquatic toxicity (FET) test

The aqueous phase acute toxicity test was conducted according to the OECD guidelines for the two chemicals (OECD, 2013). To find the LC_{50}, firstly we carried out wide range dose-response exposure experiments for both 6PPD (0–1500 μg/L) and 6PPD quinone (0–1000 μg/L), both series with 10 different concentrations. Later, this range was fine-tuned to find the accurate LC_{50} values. For validation of the test, 0.1% ethanol was used as solvent control. FET tests were carried out in clear polystyrene and flat-bottomed 24 well plates, with one embryo in 2 ml of solution per well. These 24 well plates were pre-treated with respective test solutions for 24 h before the FET test. Embryos (<16 cell stage) were exposed to the test solution. Each well plate was covered with a lid and was kept in a climate chamber (Sanyo MIR-154) at 27 ± 1 °C under a 12 L:12D photoperiod cycle. Test solutions were replaced every 24 h to maintain the concentration of the chemical in the test solution, as previous studies have shown that both compounds are present in solution after 24 h (Hiki et al., 2021). The hatching rate of the embryos were observed daily. For calculating lethal concentration (LC) values, mortality was used as the endpoint.

2.4. Exposure experiment

Doses for the exposure experiment were chosen according to the FET tests. Fertilized eggs were exposed to 1.0, 10.0, and 25.0 μg/L 6PPD and

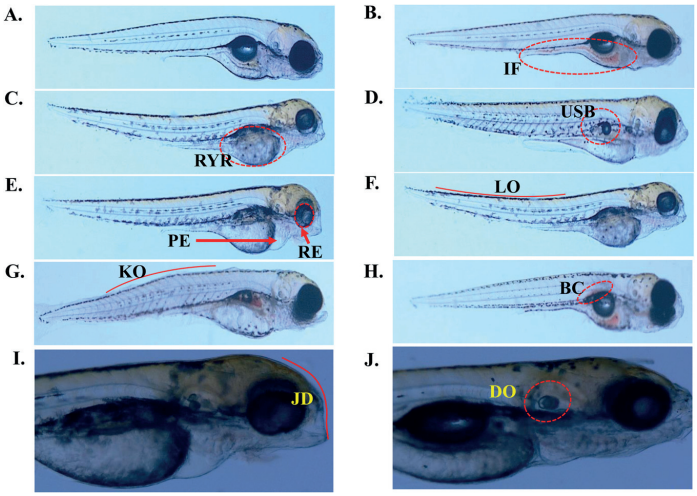

Fig. 2. The deformity was observed in zebrafish larvae (at 116 hpf) due to 96 hr exposure of 6PPD & 6PPD quinone (n = 10). (A.) Normal larvae; (B.) Intestinal inflammation (IF) after exposure of 10 μg/L (9/10), and 25 μg/L 6PPD Quinone (9/10); (C.) Reduced yolk resorption (RYR) after exposure of 25 μg/L 6PPD (4/10); (D.) Uninflated swim bladder (USB) after exposure of 10 μg/L, and 25 μg/L 6PPD (2/10 & 5/10) & 6PPD quinone (3/10 & 6/10); (E.) Pericardial edema (PE) and Reduced eye (RE) size after exposure of 25 μg/L 6PPD (3/10 & 10/10); (F.) Lordosis (LO) after exposure of 25 μg/L 6PPD quinone (4/10); (G.) Kyphosis (KO) after exposure of 25 μg/L 6PPD quinone (3/10); (H.) Blood coagulation (BC) after exposure of 25 μg/L 6PPD (3/10); (I.) Jaw deformity (JD) after exposure of 25 μg/L 6PPD (1/10) & 10 μg/L, and 25 μg/L 6PPD quinone (2/10, 4/10); (J.) Deformed otolith (DO) after exposure of 25 μg/L 6PPD quinone (1/10) (Magnification 25X A-H, 90X I-J). 9/10 means that 9 out of 10 individuals were found with deformities.

6PPD quinone. The experiments were performed with 3 replicates. All the test chambers were pre-treated with their respective exposure contaminant for 24 h before the experiment. Fifty zebrafish embryos (<16 cell stage) were exposed to a 50 ml test solution (1 ml test solution per embryo) in a 100 ml glass beaker for 96 h. The beakers were covered with parafilm (with several holes) to minimize the evaporation of the test medium. Glass beakers were kept in a climate chamber (Sanyo MIR-154) at 27 °C ± 1 °C under a 12 L:12D photoperiod cycle. Test solutions were replaced every 24 h. At the end of 96 h of exposure, test solutions were changed to the ISO standard fish media for the remaining experiment (up to 116 hpf). At 116 hpf larvae were assessed for developmental, behavioural, and cardio toxicities.

2.5. Morphological observation

For morphological analysis, ten larvae per treatment were randomly selected from each treatment group. Larvae were placed on a cavity glass slide containing 3.5% methylcellulose to immobilize the larvae. Images were captured with an Olympus SZX12 (Melville, USA) stereomicroscope equipped with an Olympus SC50 (Olympus soft imaging solutions, Münster, Germany) camera. Morphological pictures were used to quantitatively measure the total body length of larvae (from head to tail), eye size, and swim bladder size. Images were analysed using the DanioScope version 1.1 software (Noldus Information Technology, Netherlands).

2.6. Heartbeat assay

For cardiotoxicity assessment, ten larvae per treatment were randomly selected from each treatment group. Larvae were placed on a cavity glass slide containing 3.5% methylcellulose to immobilize the larvae. Videos were recorded at 45X magnification with an OLYMPUS SZX12 stereomicroscope equipped with an OLYMPUS SC50 camera. Videos were analysed using the DanioScope *v*1.1 software (Noldus Information Technology, Netherlands).

2.7. Locomotor behaviour assessment

Behavioural experiments were conducted using the DanioVision observation chamber (Noldus Information Technology, Netherlands). Each 24 well plate was randomized such that it contained six larvae from each treatment and control. The larvae were allowed to acclimatise to the conditions in the plate for at least 10 min before the behavioural analysis. The plates were then kept in the DanioVision observation chamber for video recording. The temperature of the well plates (27 ± 1 °C) was maintained using the DanioVision temperature control unit. Larvae were acclimated in the dark for 5 min before the video recording. All the behavioural measurements were done between 13:00 and 16:00, the optimal time for the stable basal metabolic activity (Chiffre et al., 2016). The 20 min behaviour analysis included a 5 min dark period followed by a 5 min light period and then a second cycle of 5 min of darkness followed by 5 min of light. The video recordings were analysed to assess the distance moved, acceleration, velocity, meandering and rotation using EthoVision® XT 15 software (Noldus information technology, Wageningen, Netherlands). Angular velocity of larvae is a measure of its erratic or zig-zag movement and is measured in degree/sec. Angular velocity above zero is an indication of hyperactive pectoral fin (Danos and Lauder, 2007). Hyperactive pectoral fin reflects an increase in escape behaviour, which is often correlated with stress (Cachat et al., 2011). Headings are indicators of larval wellbeing as they reflect motility and ample foraging behaviour (Capriello et al., 2019). For this measure, positive values indicate the uniform movement in one direction while negative values indicate the nonuniform and erratic movements. Uniform movements of zebrafish larvae indicate that they are in good health.

2.8. Oxygen consumption analysis

Oxygen consumption of the larvae was measured using the Loligo® Microplate Respirometry System (Loligo Systems, Denmark). The system was calibrated 24 h before the experiment with oxygen saturated and oxygen-depleted ISO fish media at 28 °C. Twelve embryos of each treatment were added to the 24-well plate Sensor dish (PreSens, Germany) with each well containing two larvae. The 24-well plate Sensor dish was submerged in a tank containing ISO fish media. During the 3 h respiration measurement, the tank along with plates was kept at 28 °C in a climate chamber. The amount of available oxygen was recorded using the software program MicroResp® version 1.0.4 (Loligo Systems, Viborg, Denmark).

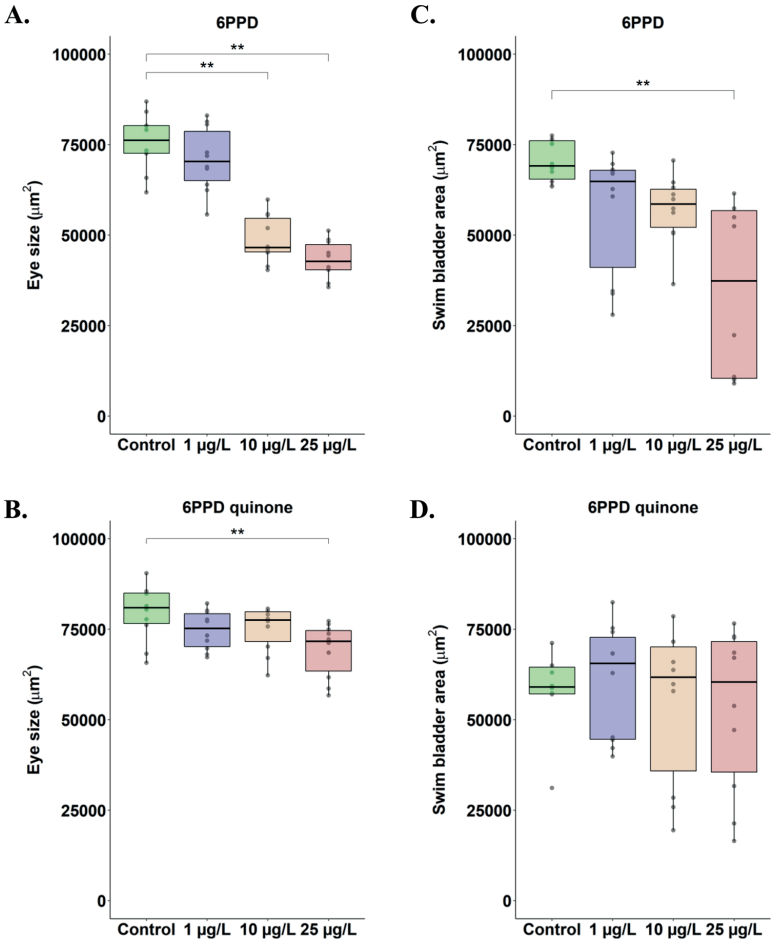

Fig. 3. Effects of 6PPD and 6PPD quinone on the (A.) eye size and (B.) swim bladder area in zebrafish larvae. Zebrafish embryos were exposed to 6PPD (1 μg/L, 10 μg/L and 25 μg/L) and 6PPD quinone (1 μg/L, 10 μg/L and 25 μg/L) for 96 hr. Data represent the mean ± S.E. * *$p < 0.005$; compared with control group (n = 30).

2.9. Statistical analysis

All experiments were performed in triplicate. The acute toxic effect of 6PPD & 6PPD quinone was determined by the use of Finney's Probit Analysis LC_{50} determination method (Finney, 1971) in SPSS version 27.0. Data of behaviour, morphology, and heart rate of 6PPD and 6PPD quinone-exposed larvae were analysed using one-way ANOVA followed by Tukey's post-hoc test (Bartlett's test and Shapiro-Wilk test were employed to confirm the assumptions of one-way ANOVA). $p < 0.05$ was considered to be statistically significant (*), and $p < 0.005$ was considered to be very significant (**). Oxygen consumption data was analysed using Friedman's test followed by Wilcoxon Signed-Rank test to see the difference between treatments.

3. Results

As per the recommendation of OECD (OECD, 2013), all physiochemical properties of water in the test chamber were kept constant. The temperature was 26 ± 1 °C and the pH was 6.81 ± 0.08. Oxygen saturation was about 87.1 ± 2% throughout the experiment (Mean ± S.D, n = 5). All the animals of the control group underwent normal embryonic development and hatched between 48 and 72 hpf.

3.1. Acute toxicity test

The 96 h LC_{50} for 6PPD and 6PPD quinone were found to be 442.62 μg/L and 132.92 μg/L, respectively (Table 1, Fig. 1a and b). Low water solubility and stability, high K_{OW}, and presence of intermediate products represent possible uncertainty factors when estimating LC_{50}-values for compounds such as 6PPD and 6PPD quinone. The LC_{50} values reported here should be considered estimates, made primarily to be able to select doses for later exposure experiments. Based on the results of the acute toxicity test, concentrations of 0 (control), 1 μg/L (low dose), 10 μg/L (medium dose), and 25 μg/L (high dose) 6PPD and 6PPD quinone were selected for further experiments. Low dose was selected based on the occurrence of the 6PPD quinone in the environment whereas medium (2.25% of 96 h LC_{50} for 6PPD, 7.5% of 96 h LC_{50} for 6PPD quinone) and high doses (5.65% of 96 h LC_{50} for 6PPD, 18.8% of 96 h LC_{50} for 6PPD

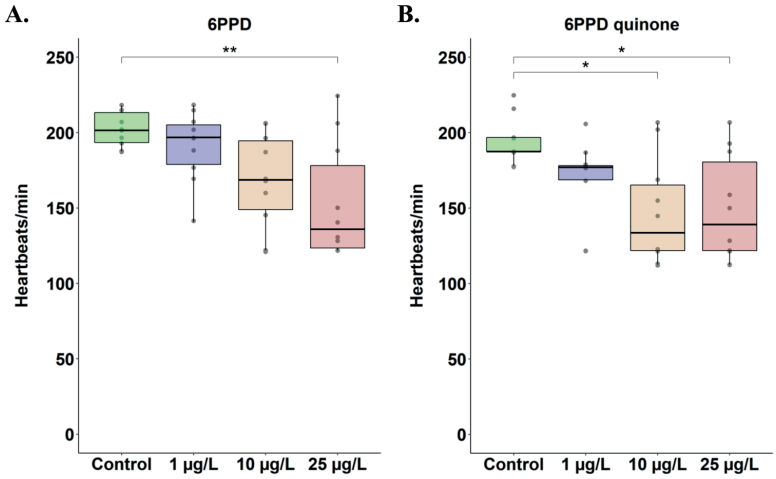

Fig. 4. Effects of 6PPD and 6PPD quinone on the heartbeat in zebrafish Larvae. Zebrafish embryos were exposed to 6PPD (1 µg/L, 10 µg/L and 25 µg/L) and 6PPD quinone (1 µg/L, 10 µg/L and 25 µg/L) for 96 h, their heartbeats were analyzed using the DanioScope™ at 116 hpf. Data represent the mean ± S.E. *$p < 0.05$, * *$p < 0.005$ compared with the control group (n = 10).

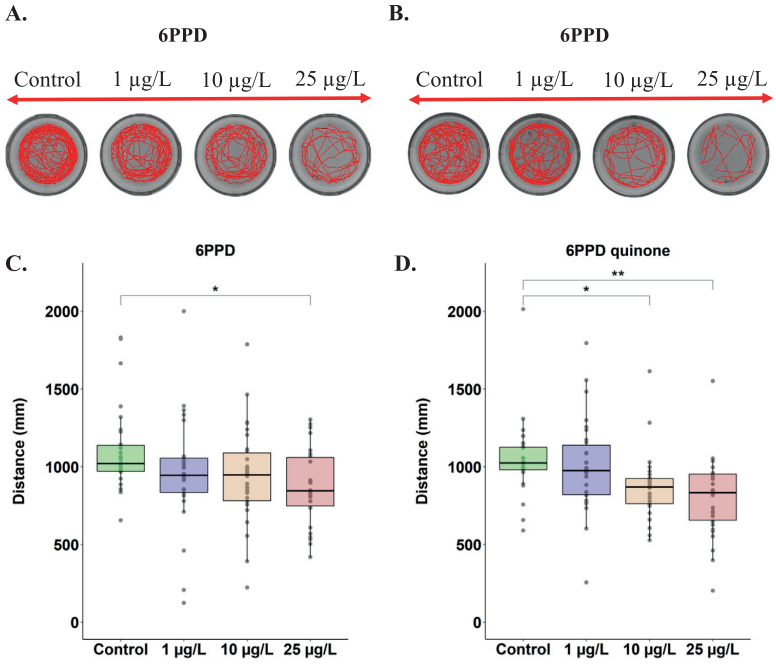

Fig. 5. Effects of 6PPD and 6PPD Quinone on the locomotor behaviour in zebrafish larvae. Zebrafish embryos were exposed to 6PPD (1 µg/L, 10 µg/L and 25 µg/L) and 6PPD quinone (1 µg/L, 10 µg/L and 25 µg/L) for 96 h, their locomotor activity was analyzed using the DanioVision at 116 hpf. (A, B) Respresentative trajectory chart and (C, D) average moving distance were recorded. Data represent the mean ± S.E. *$p < 0.05$, * *$p < 0.005$; compared with the control group (n = 30).

quinone) were selected to see the behavioural changes caused by such doses of the pollutant.

3.2. *Developmental toxicity and morphological abnormalities*

Low concentrations of 6PPD (1, 10 and 25 µg/L)and 6PPD quinone (1, 10 and 25 µg/L) did not affect the zebrafish embryo hatching and survival rate significantly, up to 116 hpf (Fig. S1 and S2; Supplementary

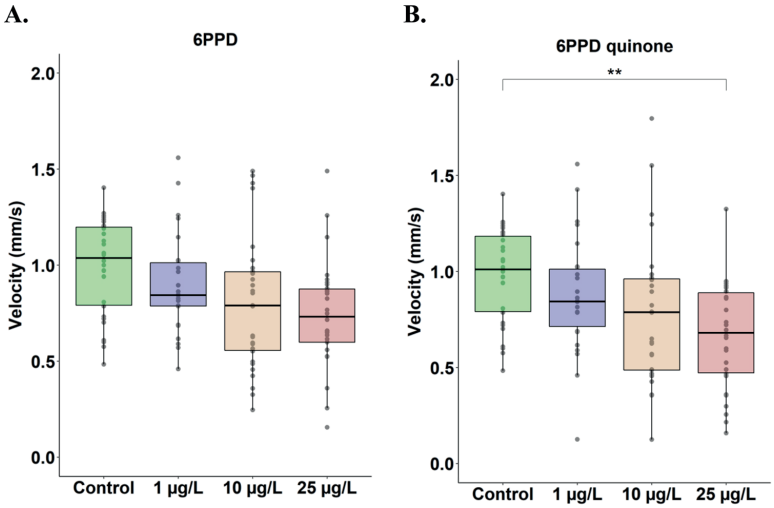

Fig. 6. Graphical representation of the mean velocity (mm/s) of the control larvae and larvae treated for 96 h, respectively, with (A.) 6PPD (1 µg/L, 10 µg/L and 25 µg/L) and (B.) 6PPD quinone (1 µg/L, 10 µg/L and 25 µg/L). Data represent the mean ± S.E. * *p < 0.005; compared with control group (n = 30).

Table S1). Moreover, there were no significant differences in total body length of the larvae (Fig. S3).

Exposure of the larvae to higher concentrations of 6PPD and 6PPD quinone significantly disturbed their normal development. Larvae exposed to 10 and 25 µg/L 6PPD quinone demonstrated intestinal reddening (Fig. 2b). Some of the individuals exposed to the pollutant showed reduced yolk resorption in the 25 µg/L 6PPD groups (Fig. 2c). Physical deformities like lordosis and kyphosis were also observed in individuals of the 25 µg/L 6PPD quinone treated group (Fig. 2 f and g).

Exposure to 10 and 25 µg/L 6PPD resulted in a significant reduction ($p < 0.005$) in eye size of the larvae (Fig. 3a). A significant reduction ($p < 0.005$) in eye size of the larvae was also found in the 25 µg/L 6PPD quinone exposed group (Fig. 3b).

A significant reduction ($p < 0.005$) in the size of the swim bladder was observed in individuals exposed to 25 µg/L 6PPD (Fig. 3c). Exposure to 6PPD quinone had no significant effect on swim bladder size even at the highest exposure concentration.

3.3. Cardiotoxicity

Heartbeat per minute (BPM) was significantly reduced in larvae exposed to 25 µg/L 6PPD ($p < 0.005$), 10 µg/L 6PPD quinone ($p < 0.05$), and 25 µg/L 6PPD quinone ($p < 0.05$) group compared to their respective control groups (Fig. 4).

3.4. Behavioural toxicity

Total distance travelled by the larvae was significantly reduced ($p < 0.05$) in the 25 µg/L 6PPD treatment group (Fig. 5c). Similarly, distance moved by the larvae was significantly reduced when they were exposed to 10 µg/L and 25 µg/L 6PPD quinone (Fig. 5d). Of the two studied contaminants, 6PPD quinone exposure had a stronger effect on the distance travelled by the larvae compared to 6PPD. The trajectory chart was prepared to represent the swimming direction of zebrafish larvae in each well. Trajectory chart of larval locomotion also revealed that locomotion decreased in a concentration-dependent manner for both 6PPD and 6PPD quinone (Fig. 5a and b).

Velocity of larvae in the 25 µg/L 6PPD quinone treatment group decreased significantly ($p < 0.005$; Fig. 6b) compared with the control. A significant increase ($p < 0.005$) in the angular velocity of larvae was found in the 25 µg/L 6PPD quinone treatment group (Fig. 7b).

A significant decrease ($p < 0.005$) in the heading of larvae was found in the 25 µg/L 6PPD treatment group (Fig. 7c). Similar significant results were obtained in the 10 and 25 µg/L 6PPD quinone treatment groups (Fig. 7d). No effects of exposure were seen on acceleration, meandering, turn angle, and rotation (data not shown). Taken together, exposure to the high concentration of 6PPD and 6PPD quinone affected several behavioural endpoints in zebrafish larvae.

3.5. Oxygen consumption

Oxygen consumption at 116 hpf after the 96 h exposure of 6PPD & 6PPD quinone is shown in Fig. 8. We observed increased oxygen consumptions in treated larvae. This effect of 6PPD and 6PPD quinone on larval respiration became stronger with increasing exposure concentrations and also over time. For both 6PPD and 6PPD quinone, Friedman's test followed by Wilcoxon signed-rank test indicated a significant decrease in oxygen level over time ($p < 0.005$), a significant interaction term ($p < 0.005$), and a significant effect of treatment ($p < 0.005$).

4. Discussion

Road dust in urban runoff is a significant source of aquatic pollution. The major part of road dust, which is considered to be microplastic, comes from tyres (Verschoor, 2016). Globally, over 2.36 billion vehicle tyres are produced annually (Smithers, 2019). TWPs have emerged as an environmental contaminant of serious concern. Recent studies have assessed the potential ecotoxicological impact of TWPs and their leachates on the aquatic environment. TWPs contain chemicals such as benzothiazole, 6PPD quinone, 1-indanone, 1-octanethiol, and phenanthrene (Halle et al., 2021; Khan et al., 2019). Among these chemicals, 6PPD quinone is postulated to be present globally because of the worldwide use of 6PPD in tyre manufacturing (Hiki et al., 2021; Tian et al., 2021). Both 6PPD and 6PPD quinone can be considered as highly toxic chemicals since their 96 h LC_{50} values lie in the range of 100–1000 µg/L (according to the US EPA, 1985 classification). The findings from the present study indicated the negative effects of exposure to sub-lethal levels of 6PPD and 6PPD quinone on cardiovascular

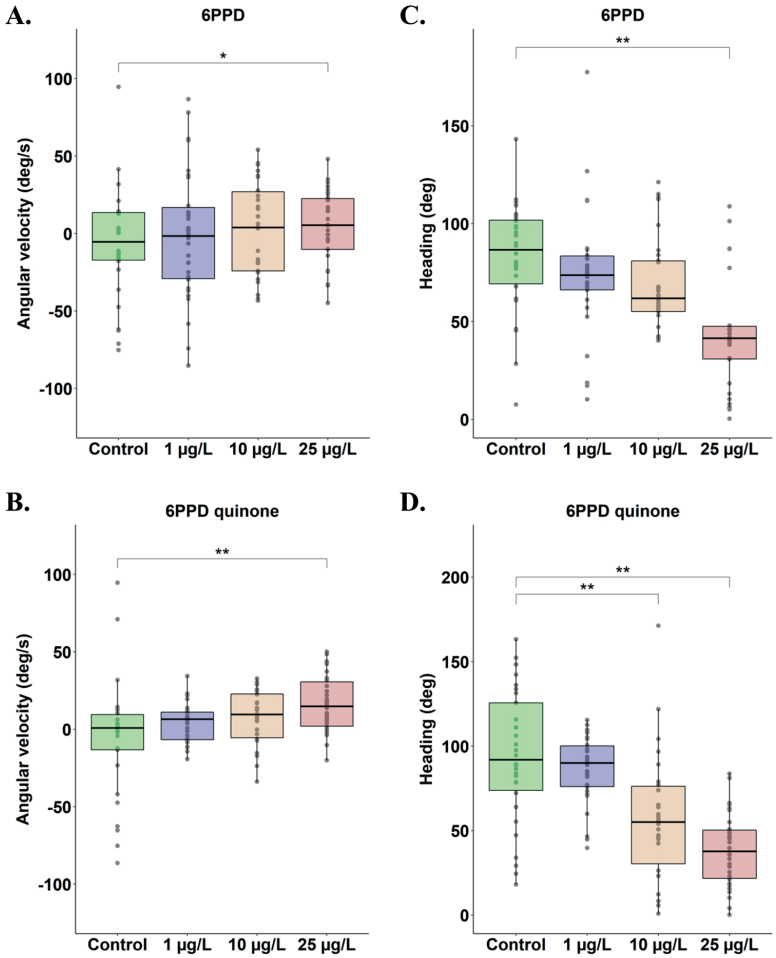

Fig. 7. Graphical representation of the behavioral endpoints of the control larvae and larvae treated for 96 h, respectively, with 6PPD (1 µg/L, 10 µg/L and 25 µg/L) and 6PPD quinone (1 µg/L, 10 µg/L and 25 µg/L). (A, B) Angular velocity and (C, D) Heading were recorded. Data represent the mean ± S.E. *p < 0.05, * *p < 0.005; compared with control group (n = 30).

function and impacted locomotor activity in zebrafish larvae.

6PPD quinone did not induce very high mortality in zebrafish (the 24 h LC_{50} was 308.67 µg/L). In contrast, Tian et al. (2021) found very high mortality in coho salmon even at very low concentrations (the 24 h LC_{50} was 0.79 µg/L). Compared to coho salmon, zebrafish appears to be relatively insensitive to 6PPD quinone exposure. This finding is in line with a study showing no acute toxicity of 6PPD quinone to zebrafish at 54 µg/L (Hiki et al., 2021). Several studies have pointed out the apparent high tolerance of tropical fishes to toxicants compared to temperate fishes (Kwok et al., 2007). The difference in sensitivity to toxicants might be due to several potential factors such as toxic mode of action, temperature-dependent metabolism, and a possible detoxification mechanism present in zebrafish (Eyckmans et al., 2011). The species-specific difference in sensitivity to TWP has also been reported recently (McIntyre et al., 2021). Moreover, the difference in acute toxicity can also be attributed to differences in testing methodology and rearing conditions (mainly temperature). The results suggest that coho salmon has a specific sensitivity to 6PPD quinone, possibly due to a species-specific mode of action of the toxicant. The reason for the observed species-specific tolerance differences is currently unknown.

Stress due to environmental pollutants can cause many abnormalities in the intestine, including those connected to inflammation (Brun et al., 2018), and dysbiosis of gut microbiota (Snedeker and Hay, 2012). The inflammatory response in the zebrafish intestine usually reflects intestinal injury due to environmental pollutants (Xie et al., 2020). We observed reddening in the lumen of the intestine, a typical symptom of inflammation. Similar results were observed in zebrafish after exposure to the imidacloprid (Luo et al., 2021), and chlorpyrifos insecticides (Wang et al., 2019). Oxidative stress might be a possible reason for the 6PPD quinone-induced inflammatory response in zebrafish larvae. However, further research is needed to elucidate the effect of 6PPD and 6PPD quinone on the liver and intestine at the molecular level.

Eye size was reduced in both 6PPD and 6PPD quinone exposure groups, in a concentration-dependent manner. A similar reduction in eye size was seen in zebrafish embryos exposed to heavy metals (Nabinger et al., 2018) and insecticides (Liu et al., 2018). In general, reduction in

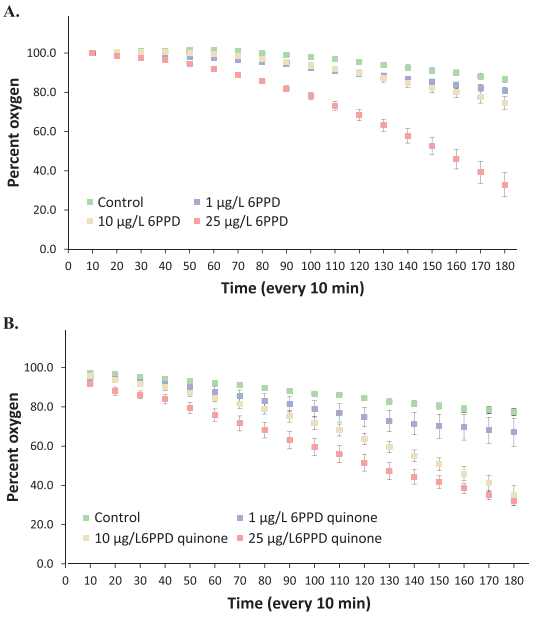

Fig. 8. The effect of 6PPD and 6PPD Quinone on respiration. (A). 6PPD (1 µg/L, 10 µg/L and 25 µg/L), (B). 6PPD quinone (1 µg/L, 10 µg/L and 25 µg/L). Data represent the mean ± S.E. Significant differences ($p < 0.05$) were found between the control and all treatments was determined by Friedman's test ($n = 6$).

eye size is associated with altered or delayed ocular development. For example, phenanthrene was found to retard the retinal development in zebrafish embryos (Huang et al., 2013).

The swim bladder in zebrafish is well developed and inflated at around 108 hpf and plays a crucial role in buoyancy (Winata et al., 2009). Many pollutants are known to affect the developmental morphology and physiology of the swim bladder in zebrafish (Wang et al., 2020; Yang et al., 2021b). Alteration in the morphology of swim bladder leads to erratic movements. Hence swim bladder abnormality is an important marker for assessing the toxicity of any environmental pollutant. In the present study, we observed impaired swim bladder inflation in 116 hpf larvae exposed to 25 µg/L 6PPD. The mechanisms driving the failure in swim bladder inflation due to exposure to 6PPD are still unknown. However, for some other environmental pollutants, proposed mechanisms include altered expression of swim bladder specific genes (Li et al., 2019), transcription of prolactin receptors (Peng et al., 2020), and interaction between swim bladder inflation-related proteins (Yang et al., 2021a).

The heart is the first organ to develop in zebrafish, and it takes just 72 h to attain its full functionality (Stainier, 2001). The heart rate results showed that exposure to both 6PPD and 6PPD quinone induced cardiac toxicity in zebrafish larvae. Heart malformations by pollutants can result in abnormal development and lead to death. We observed a concentration-dependent decrease in heart rate and pericardial edema. The results corroborate with a previous study which documented a decrease in heart rate after exposure to TWP in fathead minnow (Chibwe et al., 2021). Our results suggest that a decrease in heart rate (bradycardia) and pericardial edema was only observed at concentrations higher than the environmentally relevant level. In zebrafish larvae, alteration in cardiac development might thus be a sensitive marker of 6PPD and 6PPD quinone exposure.

Since we observed developmental abnormalities in zebrafish, larval locomotor activity was studied to examine the effect of 6PPD and 6PPD quinone on swimming behaviour. The locomotor activity of zebrafish larvae is considered an essential indicator of neural development. At 96 hpf, zebrafish larvae have a fully developed central nervous system (Legradi et al., 2015). This fully developed central nervous system along with skeletal muscles regulates the locomotion of larvae (Granato et al., 1996). Hence, locomotor activity in larval zebrafish is used to assess the developmental neurotoxicity of environmental pollutants. To our knowledge, the present study is the first to focus on the abnormal swimming behaviour in zebrafish larvae caused by exposure to 6PPD and 6PPD quinone. Our study revealed a decrease in locomotor behaviours (distance moved, velocity and headings) in 10 and 25 µg/L 6PPD and 6 PPD quinone groups. The results showed that both 6PPD and 6PPD quinone exhibited concentration-dependent hypolocomotion responses in zebrafish larvae. Mechanistically, an impact on the neuromotor pathway (Silva, 2020) is the most likely reason for the altered swimming behaviour. However, the molecular modes of action behind these effects need to be explored.

In ecotoxicological studies, oxygen consumption is often used as an indirect measure of metabolic rate. Sublethal exposure of 6PPD and 6PPD quinone in zebrafish larvae caused increased oxygen consumption. These results are in agreement with previous studies which have shown increased oxygen consumption and reduced heart rate in fish following exposure to toxicants, including benzo(a)pyrene (Gerger and Weber, 2015) and crude oil (Pasparakis et al., 2016). Despite reduced heart rate, there was an elevated oxygen consumption. Although we did not investigate the changes in cardiac output in the larvae, we speculate that reduced heart beat might have been compensated by increased cardiac output in the animal, enabling the larvae to meet the increased demand for oxygen. This compensation mechanism has been reported previously (Pasparakis et al., 2016). Future experiments are needed to fully understand the documented increased oxygen consumption in zebrafish larvae exposed to 6PPD and 6PPD quinone.

5. Conclusions

In the present study, we tested 6PPD and 6PPD quinone at three concentrations (1, 10, and 25 µg/L) to understand their possible toxicity to zebrafish embryos. At environmentally relevant concentrations (1 µg/L), there was no significant toxicity based on the selected endpoints. However, we found that both toxicants can induce developmental, behavioural, and cardiotoxicity at higher concentrations. The finding suggests that these toxicants can enter the circulatory system and subsequently affect the neural system of zebrafish larvae. Secondly, the hypoactivity that occurred in the larvae can be due to oxidative stress. The present study further documents how 6PPD and 6PPD quinone affect the early life stages of fish.

CRediT authorship contribution statement

Shubham Varshney: Conceptualization, Methodology, Writing – original draft, Data curation. **Adnan H. Gora:** Methodology, Data curation. **Prabhugouda Siriyappagouder:** Methodology, Data curation. **Viswanath Kiron:** Supervision, Funding acquisition, Writing – review & editing. **Pål A. Olsvik:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced 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.jhazmat.2021.127623.

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

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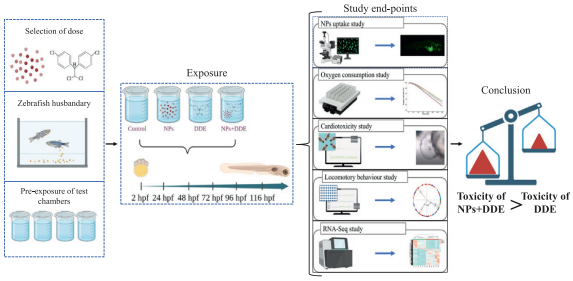
HIGHLIGHTS

- 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 ($p < 0.005$)

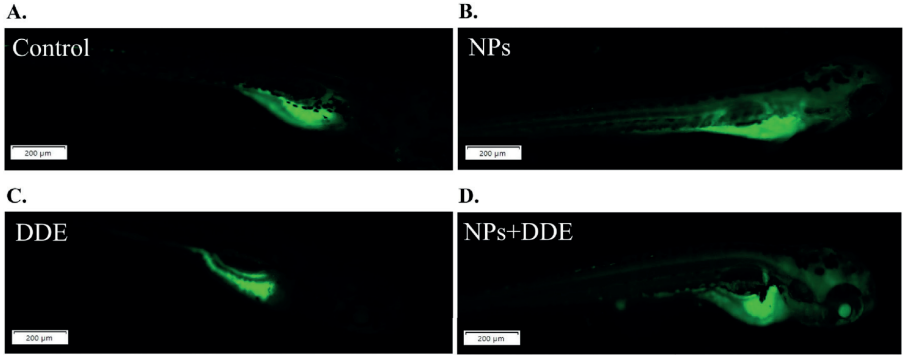

Fig. 1. NPs uptake in zebrafish larvae exposed to fluorescent PS-NPs for 96 h. (A.) Control, (B.) NPs, (C.) DDE and (D.) NPs + DDE. The representative images obtained at 96 hpf were captured using a green fluorescence filter.

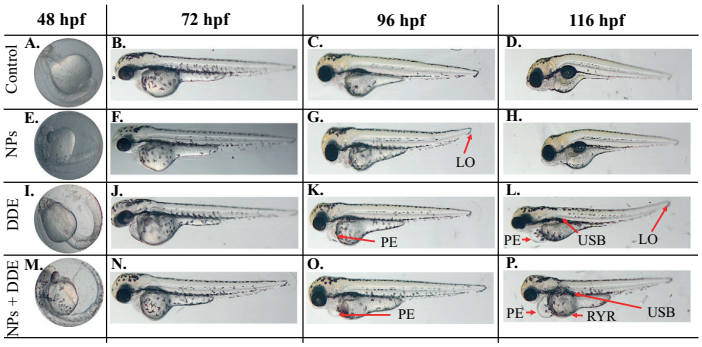

Fig. 2. Morphology of the zebrafish embryos (48 hpf) and larvae (72, 96 and 116 hpf) after exposure to NPs (E, F, G and H), DDE (I, J, K and L) and NPs + DDE (M, N, O and P). PE = pericardial edema, USB = uninflated swim bladder, LO = lordosis and RYR = reduced yolk resorption.

(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

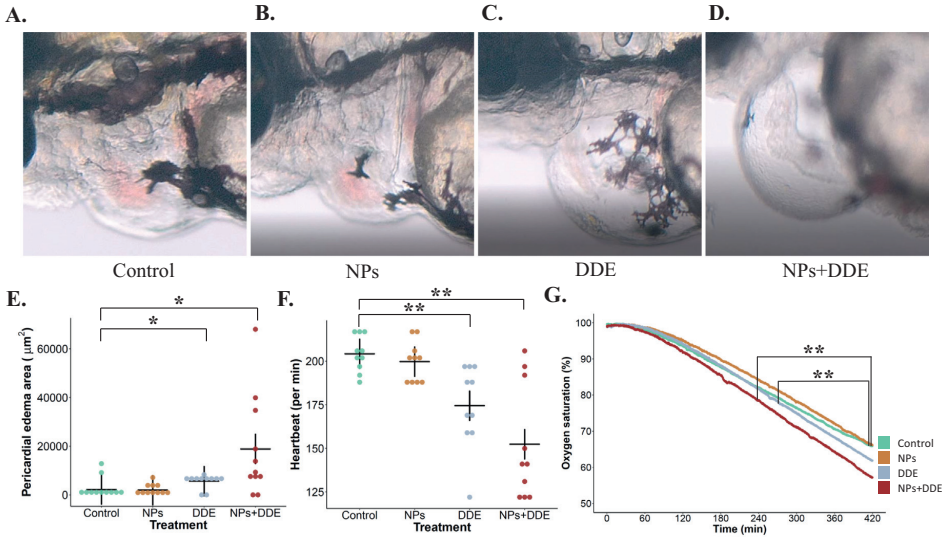

Fig. 3. Effects of different exposure on heart morphology. (A.) Control, (B.) NPs, (C.) DDE and (D.) NPs + DDE. Effects of different exposure on (E.) pericardial edema area, (F.) heartbeat and (G.) oxygen saturation in zebrafish larvae. Data represent the mean ± SE. Asterisks ** signify $p < 0.005$.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

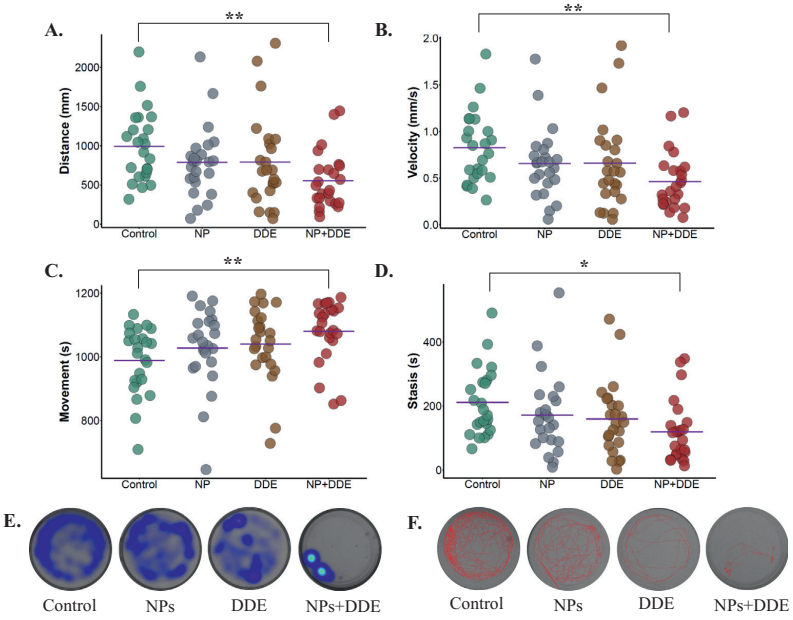

Fig. 4. Behavioural effects of NPs, DDE and NPs + DDE exposure on the zebrafish larvae (116 hpf). (A.) Distance, (B.) velocity, (C.) movement, (D.) stasis, (E.) locomotory heat map and (F.) track visualization. Data represent the mean ± SE. Asterisks * signify $p < 0.05$; ** signify $p < 0.005$. Blue 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.

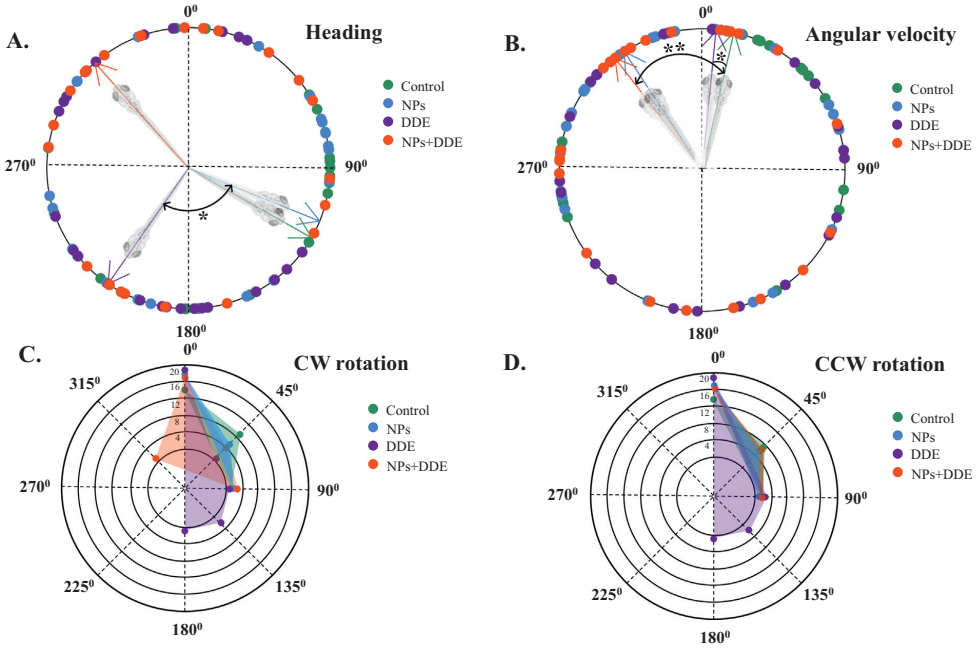

Fig. 5. Behavioural effects after exposure to NPs, DDE and NPs + DDE on the zebrafish larvae (116 hpf). (A.) Heading, (B.) angular velocity, (C.) clockwise rotation and (D.) counterclockwise rotation. Asterisks * signify $p < 0.05$; ** signify $p < 0.005$. Arrow symbol (↑) in the heading and angular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

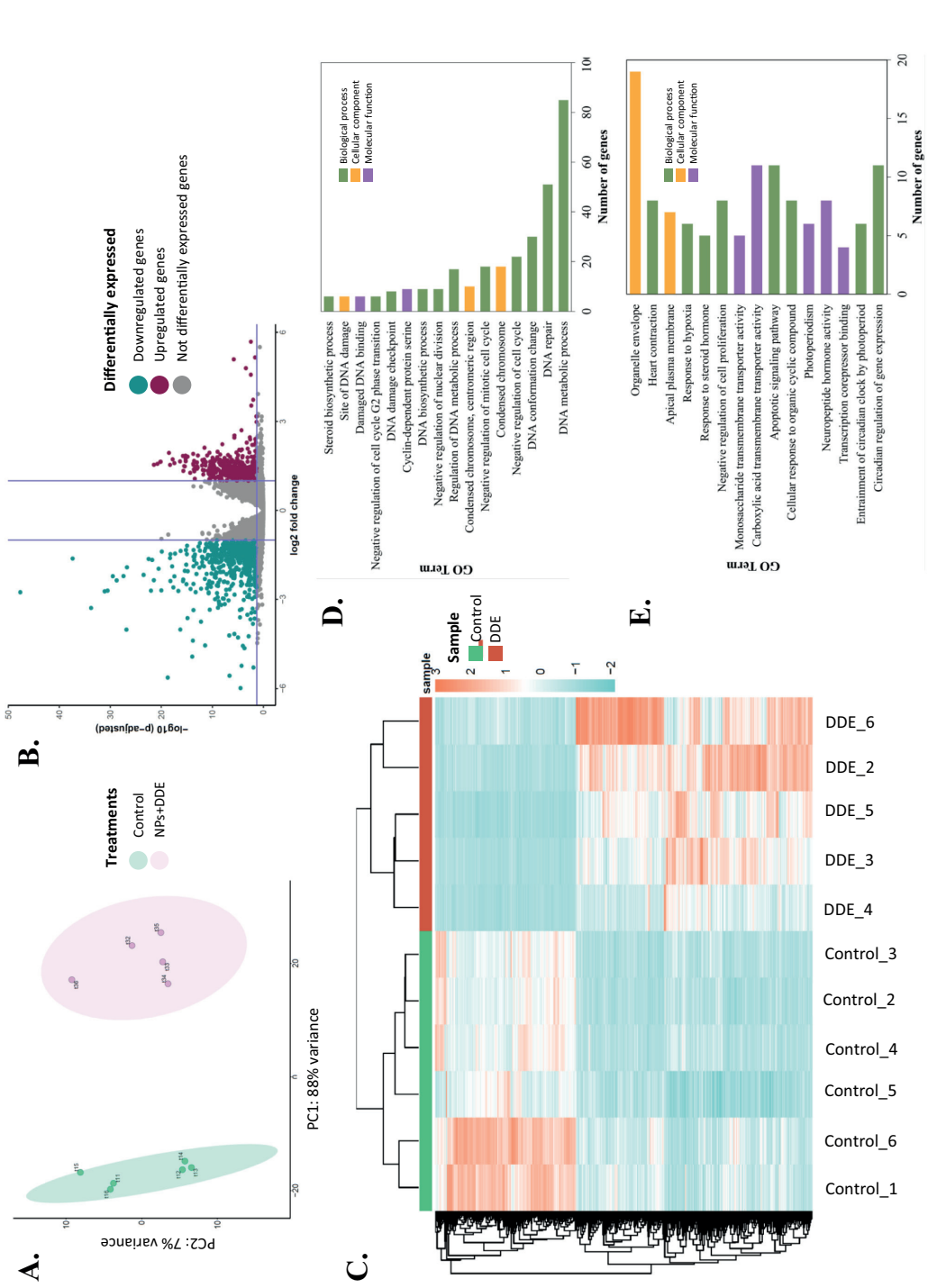

Fig. 6. Transcriptional responses in zebrafish larvae (116 hpf) after 96 h DDE exposure. (A.) Principal component analysis of sample relationship, (B.) volcano plot of DEGs, (C.) heatmap of DEGs, (D.) GO terms associated with upregulated DEGs and (E.) GO terms associated with downregulated DEGs. In volcano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

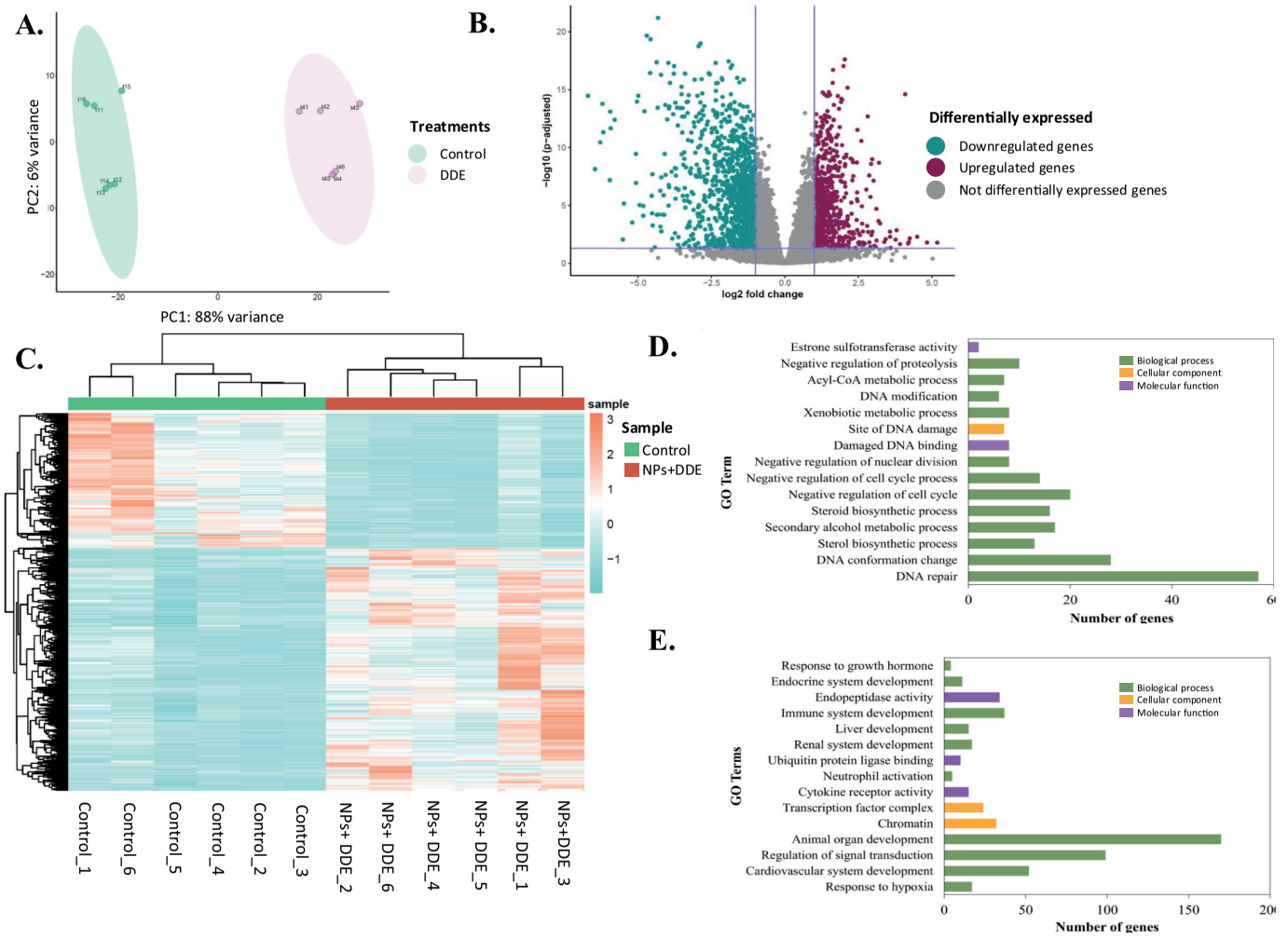

Fig. 7. Transcriptional responses in zebrafish larvae (116 hpf) after 96 h NPs + DDE exposure. (A.) Principal component analysis of sample relationship, (B.) volcano plot of DEGs, (C.) heatmap of DEGs, (D.) GO terms associated with upregulated DEGs and (E.) GO terms associated with downregulated DEGs. 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

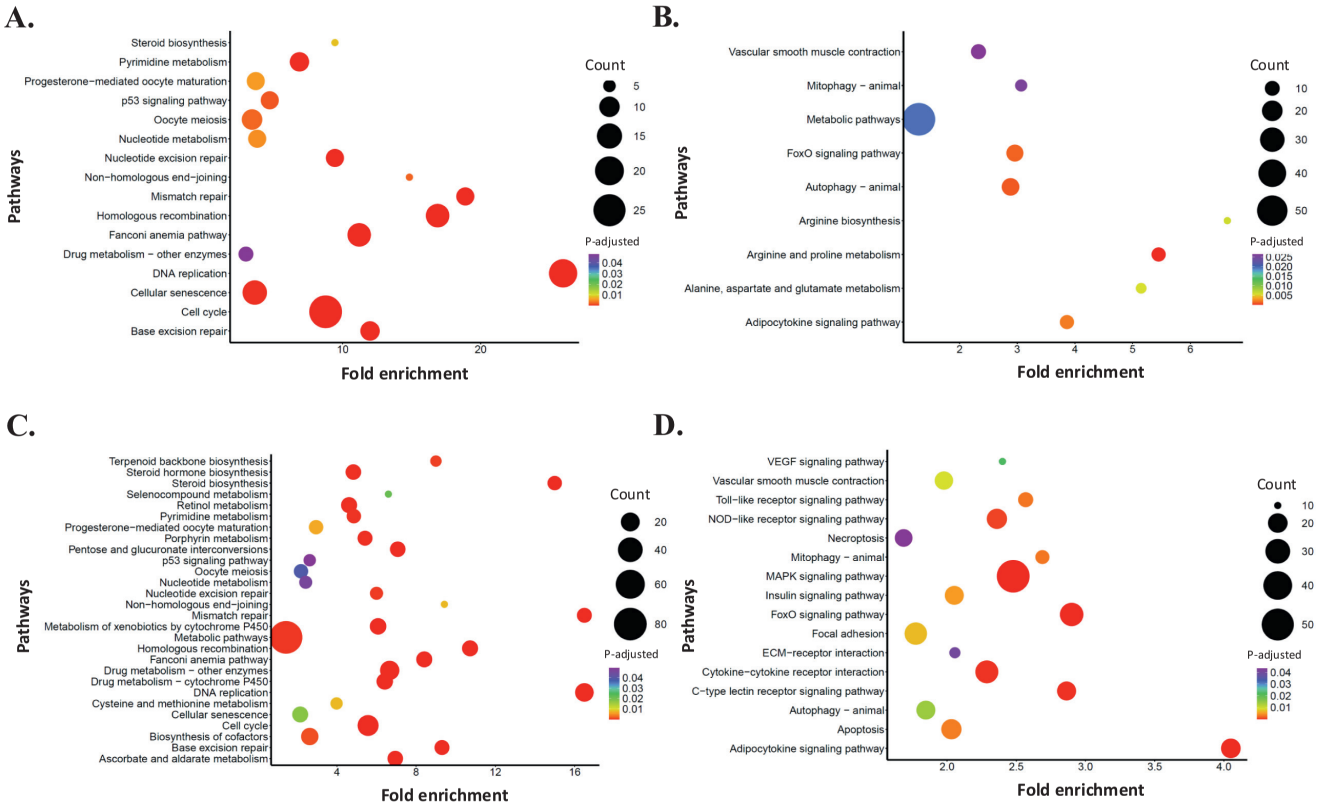

Fig. 8. KEGG pathway analysis. (A.) Enriched KEGG pathways of upregulated DEGs after 96 h exposure to DDE, (B.) Enriched KEGG pathways of downregulated DEGs after 96 h exposure to DDE, (C.) Enriched KEGG pathways of upregulated DEGs after 96 h exposure to NPs + DDE and (D.) Enriched KEGG pathways of 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.

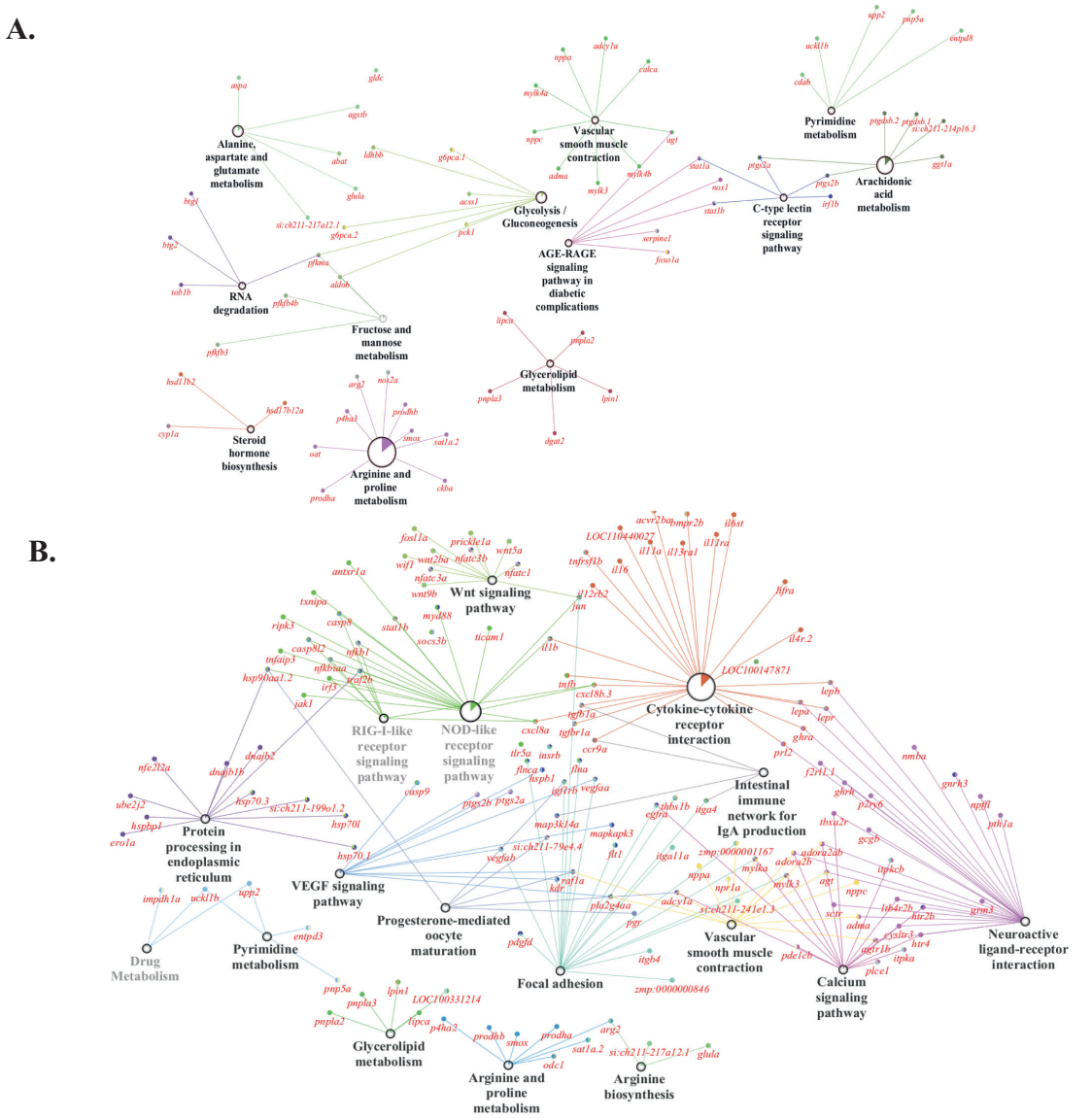

Fig. 9. Network plot showing the connection between the enriched pathways and DEGs that were downregulated after the 96 h exposure of (A.) DDE and (B.) NPs + DDE. The size of the node grows in proportion to the significance (adjusted *p*-value) of the pathway, and vice versa. The color filled in the 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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Paper III

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Mixture toxicity of 6PPD-quinone and polystyrene nanoplastics in zebrafish☆

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ABSTRACT

Plastic pollution, including micro- and nanoplastics, is a growing concern. Tyre-wear particles (TWPs) are the second largest source of microplastics in the ocean following abrasion of synthetic fibres. In addition to the particles themselves, TWPs contain many harmful chemicals, including 6PPD. This chemical reacts with atmospheric ozone and forms the toxic compound 6PPD-quinone (6PPDq), which poses a danger to aquatic life. There is a knowledge gap in understanding risks associated with the combined toxicity of nanoplastics (NPs) and 6PPDq. The present study aimed to investigate the toxicity of NPs and 6PPDq on adult zebrafish using phenotypic (behaviour, histology) and transcriptomic endpoints. Zebrafish were exposed to four treatments: control (contaminant-free), 50 μg/L 6PPDq, 3 mg/L polystyrene (PS)-NPs, and a combination of 50 μg/L 6PPDq and 3 mg/L PS-NPs. We did not observe locomotory dysregulation in zebrafish exposed to NPs. However, we found significant hyperlocomotion in zebrafish exposed to 6PPDq and this effect was even more substantial after co-exposure with PS-NPs. This study explores the molecular mechanisms behind these effects, identifying genes associated with neurotransmitters and fatty acid metabolism that were dysregulated by the co-exposure. Transcriptomic analysis further showed that both 6PPDq and PS-NPs impacted cellular processes associated with sterol biosynthesis, cholesterol metabolism, and muscle tissue development. The effects on these mechanisms were stronger in co-exposed zebrafish, indicating a heightened risk to cellular integrity and mitochondrial dysfunction. These results highlight the significance of mixture toxicity when studying the effects of NPs and associated chemicals like 6PPDq.

1. Introduction

The Earth is facing multiple environmental challenges, such as global warming, deforestation, plastic pollution, biodiversity loss, and waste management. Plastic pollution is a novel challenge that has emerged as a possible threat to our ecosystems' delicate balance. According to a recently published report, the world at present produces 430 million metric tons of plastics yearly (OECD., 2022). A large proportion of this plastic is single-use plastic and soon becomes waste (OECD., 2022). It is estimated that around 20 million tons of plastics are added to the ocean annually (Borrelle et al., 2020). Moreover, it is also forecasted that global plastic production is set to triple by the year 2060 (OECD., 2022). If not appropriately managed, this will further increase the abundance of plastic in the aquatic environment. This upsurge in plastic production and subsequent disposal poses a potential risk to aquatic organisms in the form of both primary and secondary microplastics (MPs). Although actual data on the total amount of MPs present in oceans is missing, an estimate indicates around 170 trillion plastic particles in the world's oceans (Eriksen et al., 2023). The main sources of MPs in the ocean include synthetic textiles, tyre-wear particles (TWPs) and city dust (Allen et al., 2022). MPs endanger human health because their ingestion can result in particle accumulation, raising concerns about their long-term effects on cellular function, inflammatory responses, and the disruption of endocrine pathways (Thompson et al., 2009). Moreover, it has been estimated that an average human seafood consumer swallows

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Abbreviations

TWPs	tyre-wear particles
NPs	nanoplastics
PS-NPs	polystyrene nanoplastics
HPF	hours post-fertilisation
DEGs	Differentially expressed genes
GO	gene ontology
KEGG	Kyoto encyclopedia of genes and genomes

around 11,000 pieces of MPs annually (Van Cauwenberghe & Janssen, 2014).

TWPs are tiny particles (<1 mm) produced due to friction between the tyres and roads (Kreider et al., 2012; Sherrington, 2016). Earlier, there was a debate on whether TWPs should be considered MPs, as they are primarily made of rubber (40–60%), but, due to their similarity in physical and chemical characteristics with MPs, they are often considered as MPs (Knight et al., 2020). These particles are one of the largest subsets of MPs in the environment, with one study estimating that 28% of MPs in the oceans originate from tyres (Sherrington, 2016). Smaller-sized TWPs are part of the particulate matter (PM2.5, PM10) contributing to airborne pollution (Wagner et al., 2018). Larger-sized TWPs get deposited on or near road surfaces and enter the aquatic environment through rain or stormwater runoff (Kole et al., 2017). The TWPs not only bring physical entities, but they also bring organic and inorganic chemicals. Among these chemicals, about 60% can leach and enter the environment (Müller et al., 2022). TWPs consist amongst others of heavy metals (Zn, Fe, Cu, Pb, Cd, Ni, Cr and Cd), tyre-additives substances like DPG (1,3-diphenylguanidine) which is a vulcaniser, 6PPD (N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine) which is an antioxidant, and various PAHs (polycyclic aromatic hydrocarbons) (Halsband et al., 2020; Seiwert et al., 2020). 6PPD is an extensively used additive to protect tyres from wear and tear. When 6PPD enters the aquatic environment with stormwater or urban runoff, it reacts with atmospheric ozone, forming a highly toxic compound known as 6PPD-quinone (6PPDq) (Bohara et al., 2023; Li et al., 2023; Tian et al., 2021).

Tian et al. (2021) found 6PPDq to be the causative toxicant for the mysterious mortality of coho salmon (*Oncorhynchus kisutch*) over many years in stormwater-affected streams in the Western United States. Follow-up studies showed a broad range of species-specific toxicity of this chemical, with a few species such as coho salmon, white-spotted char (*Salvelinus leucomaenis*), brook trout (*Salvelinus fontinalis*), and rainbow trout (*Oncorhynchus mykiss*) being very sensitive (Brinkmann et al., 2022; Hiki & Yamamoto, 2022a; Tian et al., 2022; Tian et al., 2021). In contrast, Atlantic salmon, brown trout and chinook were less sensitive to 6PPDq (Foldvik et al., 2022; Lo et al., 2023). Prosser et al. (2023) found that invertebrate species (*Hexagenia* spp., *Daphnia magna*, *Planorbella pilsbryi* and *Megalonaias nervosa*) were not very sensitive to 6PPDq. Studies also documented that this chemical is bioaccumulative and that it can produce neurotoxic, cardiotoxic and genotoxic effects in animals (Fang et al., 2023; Hua et al., 2023; Varshney et al., 2022; Wu et al., 2023). So far, very few studies have examined the underlying molecular mechanism behind the toxicity of 6PPDq. Moreover, there is a lack of studies showing the combined toxicity of 6PPDq and plastic particles.

This experiment aimed to assess the toxic effects of acute exposure to 6PPDq, polystyrene nanoplastic (PS-NPs), and their combined toxicity in adult zebrafish by examining its swimming behaviour to probe neurological effects, histological endpoints and transcriptomic responses in liver and intestinal tissues. Zebrafish (*Danio rerio*) was selected as a model species due to its well-annotated genome and small size, making it easy to record and assess locomotor behaviour using video-tracking systems. The findings from this study can contribute to our understanding of the potential risks associated with these pollutants and help to develop strategies for mitigating their harmful effects.

2. Materials and methods

2.1. Chemicals

6PPDq ($C_{18}H_{22}N_2O_2$) was purchased from CymitQuimica, Spain (CAS: 2754428-18-5, Purity: >98%). The stock solution of 6PPDq (1 mg/mL) was prepared in the DMSO (CAS: 67-68-5, Purity: >99%). The concentration of DMSO in the final 6PPDq exposure solution was 0.1%. A study by Hoyberghs et al. (2021) showed that DMSO up to 1% are nontoxic to zebrafish and can be used in toxicity assays. The stock solution of 6PPDq was kept in the dark at −20 °C until further use.

2.2. Polystyrene nanoplastics

Plain PS-NPs were purchased from CD Bioparticles, New York, United States (Cat. number: DMP-L019). The PS-NPs were spherical shaped with a diameter of 500 nm. The stock solution of PS-NPs (10 mg/mL) had 3×10^{11} particles/mL with a density of 1.05–1.06 g/mL. The particles had negative zeta potential, suggesting their adsorption behaviour. The particles were suspended in the deionised water with scant amount of surfactant (0.1% Tween 20) and preservative (2 mM sodium azide). The addition of Tween 20 and sodium azide prevents microbial growth in the PS-NPs solution. The stock solution of PS-NPs was kept in the dark at 4 °C until further use.

2.3. Experimental fish

The consent for this study using adult male zebrafish as an animal model was obtained from the Norwegian Food Safety Authority (FDU/FOTS ID: 29963). Adult male zebrafish (4 months, AB strain) were obtained from the zebrafish facility at the Research Station of Nord University (Bodø, Norway). Following the OECD guidelines for zebrafish acute toxicity tests, the fish were acclimatised for ten days to reduce the stress induced by transport (OECD, 2019). The fish were kept in the ZebTEC Stand-Alone toxicological Rack, Tecniplast (Varese, Italy) at 28 ± 1 °C water temperature. During the acclimation period, the fish were fed with 400–600 μm sized Zebrafeed® (Sparos Lda, Olhão, Portugal) twice a day at a feeding rate of 4% of the total tank biomass. A 14:10 (light: dark) photoperiod was maintained throughout the experiment.

2.4. Selection of exposure doses and exposure groups

The exposure dose of 6PPDq (50 μg/L) was selected based on the environmental presence of 6PPDq. Hiki and Yamamoto (2022b) found 116–1238 ng/g 6PPDq in the road dust samples collected in Tokyo, Japan. Compared to their highest documented 6PPDq concentration, our chosen exposure dose was approximately 25 times lower. Additionally, we analysed the concentration of 6PPDq in tunnel wash water from Bodø (Norway), and found a concentration of around 27 μg/L (unpublished data). The chemical analysis of 6PPDq of tunnel wash water from Bodø was performed by the Norwegian Veterinary Institute, Oslo, and due to logistical constraints (transport, shipping), there was a 3–4 day delay from sample collection to analysis. Considering the half-life of 6PPDq of approximately 33 h, the concentration of 6PPDq in the tunnel wash water at the time of collection was likely considerably higher than what was measured. Therefore, our selected exposure concentration of 50 μg/L serves as a relatively conservative estimate.

For NPs, there is a lack of standardised protocols for quantification of concentrations in the aquatic and terrestrial environments. However, a few studies have documented the presence of NPs ranging from ng/L to mg/L in aquatic environments (Kallenbach et al., 2022; Storck et al., 2015). The presence of MPs has been also reported in fish meal in the

range of 50–100 mg/kg (Chen et al., 2022; Wang et al., 2022). TWPs, which are considered as significant source of MPs, were observed in roadside soil in the range of 2000–26,400 mg/kg in Trøndelag county, Norway (Rødland et al., 2023). Hence, to select the exposure dose of NPs for this study, we surveyed environmentally relevant concentrations of MPs. The selected exposure dose of PS-NPs in our study is comparable to doses reported in previous studies with adult zebrafish (Lei et al., 2018; Sarasamma et al., 2020). Thus, we had four treatment/exposure groups: control (without any contaminants), 50 μg/L 6PPDq, 3 mg/L PS-NPs and a combination of 50 μg/L 6PPDq and 3 mg/L PS-NPs. Below, these groups will be referred to as control, 6PPDq, PS-NPs and PS-NPs+6PPDq (combination of PS-NPs and 6PPDq), respectively.

2.5. Exposure protocol

The acute exposure (96 h) was performed in polycarbonate tanks (3.5 L) of the ZebTEC Stand-Alone toxicological Rack, with five tanks per treatment and 12 male zebrafish per tank. During the exposure, water exchange was turned off to maintain a constant level of contaminants. Each tank was connected to an oxygen supply unit to maintain the optimal oxygen level during the exposure. The oxygen supply tubes induced gentle water movement within the tanks. This constant circulation aided in the mixing of the water and ensured that 6PPDq and PS-NPs remained evenly distributed throughout the exposure period. The test solutions were replenished daily at the rate of 80–90% of the total volume, and no feed was added during the 96-h exposure period.

2.6. Sampling

After 96 h of exposure, the fish were euthanised by immersing them in ice-cold water for about 15 s. The fish were dissected to collect the liver and intestine samples. The samples were instantly frozen in liquid nitrogen and stored at −80 °C until further use.

2.7. Locomotor activity

At the end of 96 h exposure, one to two fish from each tank (n = 8 per treatment) were taken out randomly for the locomotor recording using the Noldus EthoVision® system (Noldus Information Technology, Netherlands). The system had one top and one side camera (Basler acA1300 infrared-sensitive). The recordings of individual fish were performed in a 3.5 L ZebTEC glass tank (15 × 24 × 10 cm) and at a water temperature of 28 ± 1 °C. During the behavioural recording, the tank was filled with 1.5 L of system water. Using the side camera recording, two vertical layers of the tank were marked. The upper layer (4 cm) was labelled as the upper arena whereas the bottom layer (4 cm height) was labelled as the lower arena. The behavioural video recording (30 frames per second) was performed in the dark for 10 min per fish. The behavioural videos were analysed using the EthoVision XT® *v*16 software (Noldus Information Technology, Netherlands). In the same software, the 2 mm MDM (minimum distance moved) data filtering option was used, which sets a minimum threshold of movement as 2 mm. 3D swim path reconstructions were created using the plotly package in the R studio *v*2023.09.0.

2.8. Histological analysis of liver and intestine

The liver and middle intestine (n = 8 per treatment group) were dissected and fixed immediately using 10% formalin. After 24 h of fixation, samples were dehydrated in a graded series of alcohol, equilibrated in xylene and paraffin infiltrated using Tissue Processor Leica TP1020 (Leica Biosystems, Nussloch, Germany). Then, samples were embedded in paraffin. Sectioning was performed using rotary microtome Microm HM 355S (Microm International GmbH, Waldorf, Germany). For morphological analyses, the sections of 3 μm were stained by Haematoxylin and Eosin method using Multistainer Leica ST 5020 (Leica Microsystems Nussloch, Germany). The staining procedure was conducted as described by Suvarna et al. (2013). The photomicrographs were captured using an Olympus BX51 microscope equipped with an SC180 video camera. The images were processed with CellSense Entry imaging software (Soft Imaging System GmbH, Munster, Germany). For the quantification of the different parameters, we used ImageJ software (Schneider et al., 2012). The changes in the histomorphological architecture of intestine were investigated by measuring the following parameters: goblet cell number, goblet cell area, villi height, width of lamina propria and muscularis thickness. Liver vacuolisation was assessed by evaluating two parameters-average vacuole area and average vacuole number in a selected area of the liver as described in our previous study by Gora et al. (2022).

2.9. Liver and intestinal transcriptomics

The liver and intestine samples of two fish from the same tank were pooled separately to get enough tissue for total RNA isolation. The samples were homogenised in a 2 mL homogenising tube containing glass beads (size 0.5 mm, Cat. number: SI-BGO5, Scientific Industries, New York) and 500 μL of QIAzol reagent (Cat. number: 79306, Qiagen, Hilden, Germany). Following the manufacturer's protocol, the total RNA was extracted using the Direct-zol RNA MiniPrep kit (Cat. number: R2052, Zymoresearch, CA, USA). The isolated total RNA was suspended in 30 μL DNAse/RNAse-free water (Cat. number: 327390050, ThermoFisher Scientific, MA, USA). The RNA was quantified by Qubit™ 3 Fluorometer (Cat. number: Q33238, Thermo Fisher Scientific, Waltham MA, USA). The quality of extracted RNA was assessed using 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). Then, RNA was stored at −80 °C until the library preparation. The RNA samples (n = six per treatment) were shipped to Novogene (Cambridge, United Kingdom) for library preparation and mRNA sequencing using Illumina NovaSeq 6000 system which generated paired end reads of 150 bp length.

2.10. Bioinformatic analysis of transcriptomics data

The raw reads obtained from the Novogene were filtered using the fastp software *v* 0.12.0 (Chen et al., 2018). After filtering, only good-quality reads (Phred score >30) were taken further for the downstream process. For the mapping, annotation and reference genome files were downloaded from the National Centre of Biotechnology Information (https://www.ncbi.nlm.nih.gov/genome/?term=Danio+rerio). The reference genome index of zebrafish was built using the Bowtie 2 aligner *v* 2.2.3 (Langmead & Salzberg, 2012). The filtered reads were mapped against the indexed reference genome using the HISAT *v* 2.2.1 software (Kim et al., 2015). Following the mapping, featureCounts *v* 1.6.3 software was employed to determine the read counts against the different genes (Liao et al., 2014). DESeq2 *v*1.30.0 software calculated the differential gene expression of the read counts (Love et al., 2014). In the current manuscript, the term "differentially expressed genes (DEGs)" refers to transcripts with a Log2 fold change of ±0 and a p-adjusted <0.05 (calculated through the Benjamini-Hochberg multiple test correction method). The gene ontology and KEGG pathway analysis of DEGs were performed using the DAVID bioinformatics web-based platform (https://david.ncifcrf.gov) (Huang et al., 2009). Gene and pathway networking was accomplished via the ClueGO *v* 2.5.9 software (Bindea et al., 2009).

2.11. Statistical analysis

The normality of the data (except RNA-seq data) was verified with the Kolmogorov-Smirnov and Shapiro-Wilk tests. A significance level of $p > 0.05$ was considered normal data and $p < 0.05$ was considered non-

normal. Following the normality tests, significant outliers (if any) were removed by the Grubbs method. In the normally distributed data, one-way ANOVA combined with Tukey's posthoc test (HSD) was used to test the treatment's effect among different exposure groups compared to the control group. In the non-normal data, Friedman's test coupled with the Wilcoxon Signed-Rank test was employed to test the treatment's effect. For the angular data (heading, meander, turn angle, rotation and angular velocity), Rayleigh test coupled with Stephens Modified Watson's test was employed. In all the statistical tests, $p < 0.05$ was considered statistically significant and was depicted with the single asterisk symbol (*) whereas $p < 0.005$ was considered highly significant and was depicted with the double asterisk symbol (**). All statistical tests and data visualisation were performed in the R-studio *v* 2023.06.1 and Cytoscape *v*3.10.1.

3. Results

3.1. Water quality parameters and mortality

All water quality parameters were within the range of optimal rearing conditions (OECD, 2019). The daily average pH, temperature, and oxygen saturation across 96 h of exposure is provided in the Supplementary Table 1. There was no mortality in any of the tanks during the 96-h exposure period. No developmental aberrations (tail/trunk and fin deformities) were observed in the fish during the 96-h exposure of 6PPDq, PS-NPs, and their combination.

3.2. Behavioural assessment

We investigated the effect of 6PPDq, PS-NPs, and their combination on the swimming behaviour of adult zebrafish. Typically, zebrafish are exploratory and swim throughout the tank (Fig. 1a), but under stressful conditions, they tend to swim near the bottom or along the tank walls. In this study, swimming behaviour was significantly affected in zebrafish exposed to 6PPDq or PS-NPs+6PPDq. In both exposure groups, the fish remained close to one of the side walls of tanks and this can be observed in the 3D swim path reconstruction plots (Fig. 1b & d) and the top view of the heatmaps (Supplementary Figs. 1b and 1d) when compared with the control group. However, swimming behaviour was not affected in zebrafish exposed to PS-NPs alone (Fig. 1c; Supplementary Figs. 1a and 1c). Similarly, significantly increased distance travelled in the lower arena and increased velocity in both the upper and lower arena were observed in zebrafish exposed to 6PPDq ($p < 0.05$) and PS-NPs+6PPDq ($p < 0.05$) (Fig. 2b–d). Significantly increased velocity was observed in zebrafish exposed to PS-NPs+6PPDq ($p < 0.05$) and 6PPDq ($p < 0.05$) in the upper and lower arena, respectively (Fig. 2c & f). Furthermore, fish exposed to 6PPDq ($p < 0.05$) and PS-NPs+6PPDq ($p < 0.005$) spent significantly more time in the lower arena than the upper arena (Fig. 2e–f). However, we did not observe any significant effect on acceleration, heading, meander, turn angle, rotation (clockwise and counter-clockwise) and angular velocity in the fish exposed to 6PPDq, PS-NPs, and their combination (data not shown). Taken together, 6PPDq and PS-NPs+6PPDq exposure significantly increased the movement, velocity, and time spent in the lower arena and near the side walls.

3.3. Histopathological assessment

The liver and intestine histology analyses revealed no gross damage to the tissue microarchitecture in the different treatment groups. Similarly, we did not find any statistically significant differences in the quantified parameters of the intestine sections (Fig. 3a–g). Intra-epithelial leukocyte infiltration was observed in some slides, but there were no significant differences between the treatments compared to the control. Furthermore, the number and size of the vacuoles in the liver tissues did not vary in the different treatment groups (Fig. 4a–f).

3.4. Transcriptional responses

To investigate the molecular mode of action, we performed RNA sequencing of liver and intestinal tissues and compared the responses in each treatment group with the control groups. The sequencing of 46 samples (23 liver samples and 23 intestine samples; five to six samples

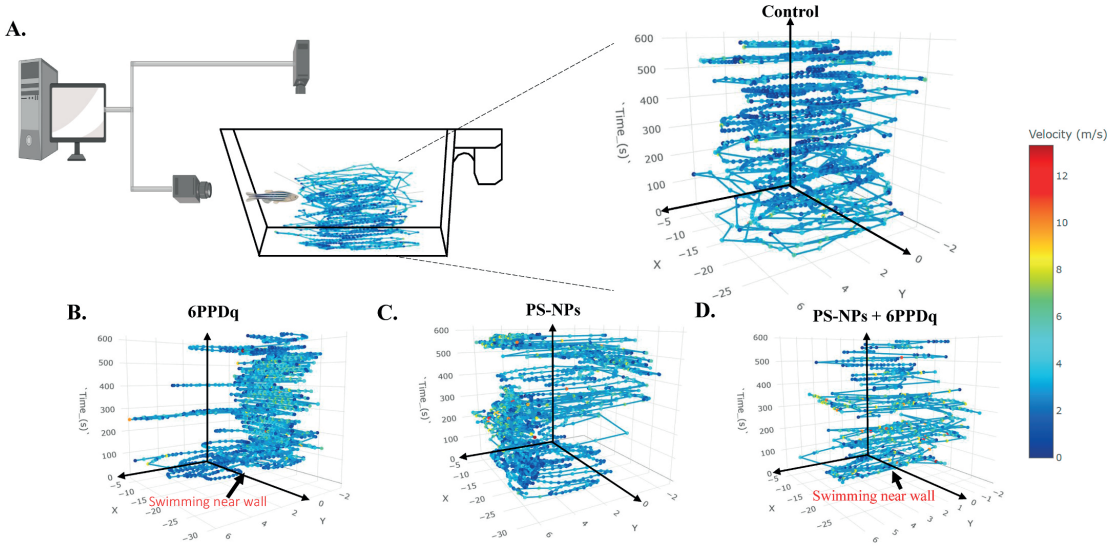

Fig. 1. Representative temporal three-dimensional (3D) reconstruction plots of adult zebrafish exposed to **(a.)** Control, **(b.)** 6PPDq, **(c.)** PS-NPs and **(d.)** PS-NPs+6PPDq. X, Y coordinates represent the position of fish whereas Z axis represent the experimental time. Track colour in the plots (blue to red) represent the changes in velocity from 0 to 12 m/s. Arrow indicates the swimming pattern. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

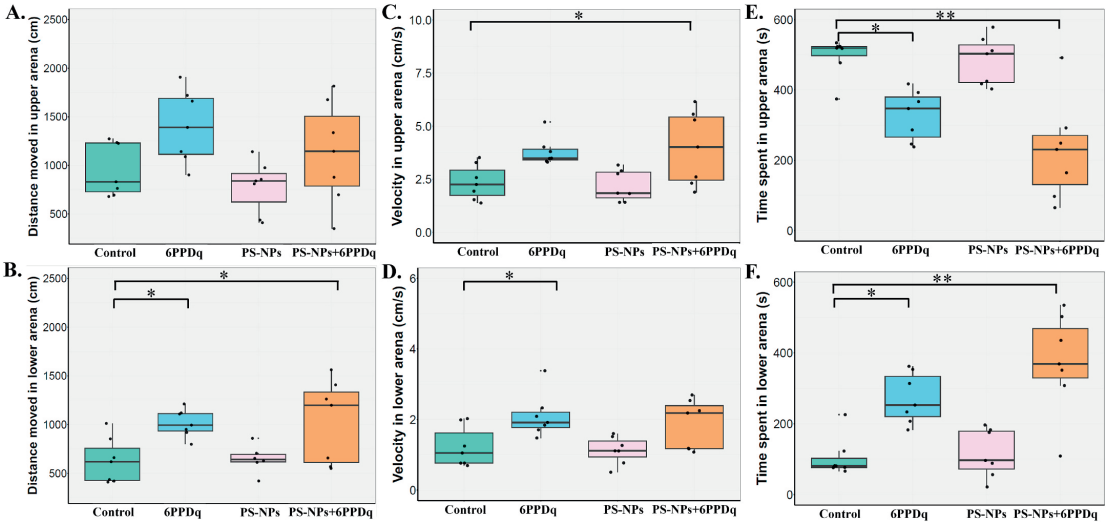

Fig. 2. Behavioural effects following exposure to 6PPDq, PS-NPs and PS-NPs+6PPDq. **(a.)** Distance moved in the upper arena, **(b.)** distance moved in the lower arena, **(c.)** velocity in the upper arena, **(d.)** velocity in the lower arena, **(e.)** time spent in the upper arena and **(f.)** time spent in the lower arena. Data represent the mean ± SE. Asterisks * signify $p < 0.05$; ** signify $p < 0.005$.

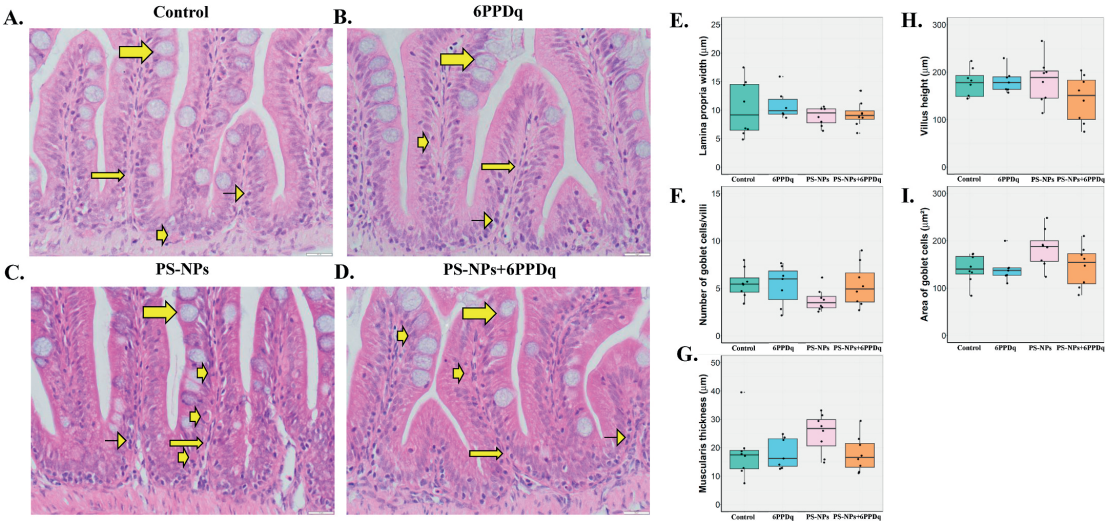

Fig. 3. Intestinal histology of adult zebrafish exposed to **(a.)** Control, **(b.)** 6PPDq, **(c.)** PS-NPs and **(d.)** PS-NPs+6PPDq. Haematoxylin & eosin stains were used for staining and total magnification used was 400x. Boxplots shows **(e.)** length of lamina propria, **(f.)** number of goblet cells per villi, **(g.)** muscularis thickness, **(h.)** villus height and **(i.)** area of goblet cells.

per group) yielded approximately 1207 million raw reads with an average of 26.23 million reads per sample (Supplementary Table 2). Around 99% of the reads were of good quality (>Q30) (Supplementary Table 2). The average mapping rate was around 86.95% (Supplementary Table 2).

The general distribution of DEGs were assessed by plotting the volcano plots (Supplementary Fig. 2). Exposure to 6PPDq resulted in 223 DEGs (96 downregulated & 127 upregulated) in the liver (Supplementary Fig. 2a, Supplementary Table 3). and 180 DEGs (124 upregulated & 56 downregulated) in the intestine (Supplementary Fig. 2b, Supplementary Table 4). Similarly, exposure to PS-NPs resulted 162 DEGs (82 upregulated & 80 downregulated) in the liver (Supplementary Fig. 2c, Supplementary Table 5) and 469 DEGs (257 upregulated & 212 downregulated) in the intestine (Supplementary Fig. 2d, Supplementary Table 6). Combined exposure of PS-NPs and 6PPDq resulted in the highest number of DEGs as compared to the individual exposure of 6PPDq and PS-NPs; 253 (99 upregulated & 154 downregulated; Supplementary Fig. 2e) and 1107 (577 upregulated & 530 downregulated;

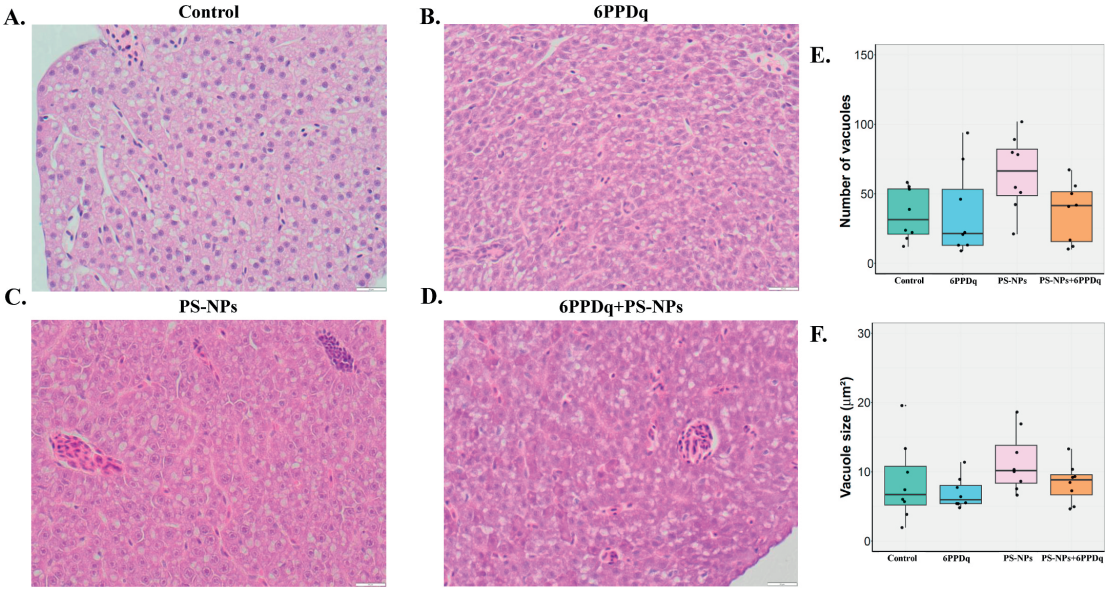

Fig. 4. Liver histology of adult zebrafish exposed to **(a.)** Control, **(b.)** 6PPDq, **(c.)** PS-NPs and **(d.)** PS-NPs+6PPDq. Haematoxylin & eosin stains were used for staining and total magnification used was 400x. Boxplots shows (**e.**) number of vacuoles and **(f.)** vacuole size.

Supplementary Fig. 2f) DEGs in the liver and intestine, respectively. The list of significant DEGs in the PS-NPs+6PPDq group in liver and intestine are provided in the Supplementary Table 7 and 8, respectively. To visually explore the expression patterns of the DEGs, we constructed heatmaps with hierarchical clustering. However, no clear clustering was observed in any of the treatment groups (data not shown).

Gene ontology analysis was performed with gene lists from each treatment group. In liver of zebrafish exposed to 6PPDq, ten significantly enriched GO terms (seven by differentially upregulated, three by differentially downregulated genes) were found (Fig. 5a, Supplementary Table 9). The key GO terms were related to sterol biosynthesis, cholesterol metabolic process, secondary alcohol metabolic process, striated muscle tissue development and oxoacid metabolic process (Fig. 5a). We found eight significantly enriched GO terms (all by upregulated genes) in the intestine samples of the same treatment group (Fig. 5b–Supplementary Table 10). The key GO terms were associated with cholesterol metabolism, sterol biosynthesis and striated muscle development. No significantly dysregulated GO terms were observed in zebrafish exposed to PS-NPs alone in the liver samples. However, we found 62 significantly enriched GO terms (32 by differentially

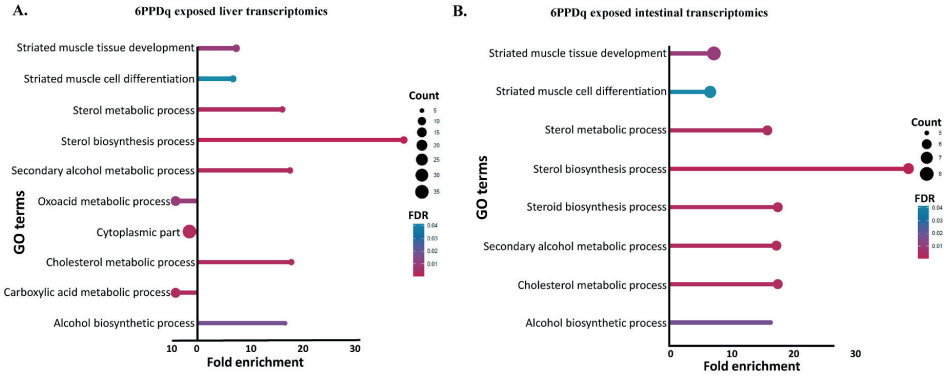

Fig. 5. Dysregulated gene ontology (GO) terms in response to 6PPDq exposure in **(a.)** liver and **(b.)** intestinal samples. The X-axis represents the fold enrichment, while the Y-axis represents the individual GO terms. On the left side of the graph, bars depict significantly downregulated GO terms, while on the right side, bars represent significantly upregulated GO terms. The colour of each bar corresponds to the false discovery rate (FDR) or adjusted p-value (Padj). The magnitude of the 'lollipop' atop each bar varies according to the associated gene count change for the respective GO term. Significant upregulated or downregulated GO terms refer to those with a p-adjusted (Padj) or FDR value of less than 0.05, indicating statistical significance. Lollipop bars on the left side signifies downregulated GO terms whereas lollipop bars on the right side signifies upregulated GO terms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

upregulated, 30 by differentially downregulated genes) in the intestine of the PS-NPs-exposed group (Fig. 6, Supplementary Table 11). Among them, the key enriched GO terms were linked to immune response, mitochondrial respiration, and cytokine-mediated signalling. Combined exposure of PS-NPs and 6PPDq resulted in the highest number of GO terms. In the liver, 26 significantly enriched GO terms (8 by differentially upregulated genes, 18 by differentially downregulated genes) were found (Fig. 7a, Supplementary Table 12), whereas in the intestine, 84 significantly enriched GO terms (37 by differentially upregulated genes, 47 by differentially downregulated genes) were found (Fig. 8a, Supplementary Table 13). In the liver, GO terms were linked to alcohol biosynthetic process, cardiac muscle tissue development, lipid modification, etc. In the intestine, GO terms were linked to mitochondrial respiration, organelle toxicity, platelet activation and phosphorylation.

KEGG analysis was performed to gain further insight into the metabolic pathways affected by the differentially expressed genes. Exposure of 6PPDq alone did not significantly affect any KEGG pathways in the liver. However, 6PPDq exposure significantly upregulated three pathways in the intestine, namely, oxidative phosphorylation, cardiac muscle contraction and metabolic pathways (Supplementary Table 14). Exposure to PS-NPs alone resulted in upregulation of the steroid biosynthesis pathway in the liver (Supplementary Table 15). However, exposure to PS-NPs gave a stronger response in the intestine, with three upregulated (oxidative phosphorylation, metabolic pathways, and cardiac muscle contraction) and two downregulated pathways (cytokine-cytokine receptor interaction and cell adhesion molecules) (Supplementary Table 16). In terms of significant KEGG pathways, the combined exposure of PS-NPs and 6PPDq induced the most severe response. In the liver, co-exposure resulted in nine dysregulated KEGG pathways (one upregulated and eight downregulated). The significantly enriched pathway by differentially upregulated genes was related to steroid biosynthesis, while with downregulated genes were related to fatty acid metabolism, PPAR signalling and other metabolic pathways (Fig. 7b–Supplementary Table 17). Moreover, co-exposure resulted in significantly enriched pathways by differentially upregulated genes in the intestine, namely carbon metabolism, cardiac muscle contraction and oxidative phosphorylation (Fig. 8b–Supplementary Table 18).

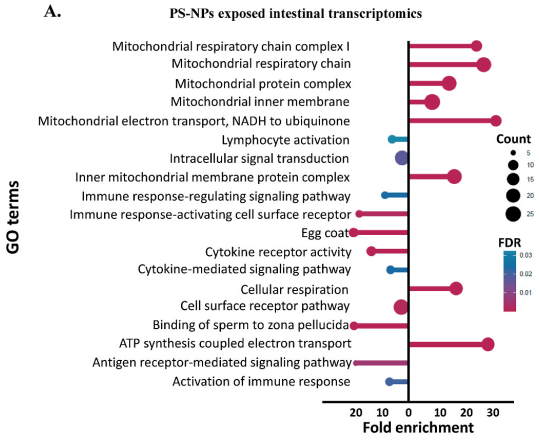

Fig. 6. Dysregulated gene ontology (GO) terms in response to PS-NPs exposure in (a.) intestinal samples. The X-axis represents the fold enrichment, while the Y-axis represents the individual GO terms. On the left side of the graph, bars depict significantly downregulated GO terms, while on the right side, bars represent significantly upregulated GO terms. The colour of each bar corresponds to the false discovery rate (FDR) or adjusted p-value (Padj). The magnitude of the 'lollipop' atop each bar varies according to the associated gene count change for the respective GO term. Significant upregulated or downregulated GO terms refer to those with a p-adjusted (Padj) or FDR value of less than 0.05, indicating statistical significance. Lollipop bars on the left side signifies downregulated GO terms whereas lollipop bars on the right side signifies upregulated GO terms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Environmental pollutants are harmful substances that can have a negative impact on ecosystems and their residing flora and fauna. In nature, these contaminants never occur alone. Most ecosystems contain an array of pollutants interacting with each other. As contaminants, 6PPDq and NPs are interconnected because of their likely shared origin from tyre wear. The PS-NPs used in this study held negative zeta potential, so they are expected to adsorb to the outer surface of the chorion (Li et al., 2022). Moreover, negatively charged nanoparticles are known to attract and adsorb positively charged contaminants such as heavy metals and organic pollutants (Liu et al., 2019; Xin et al., 2023). This adsorption process may facilitate the transport and bioaccumulation of contaminants, posing risks to aquatic organisms and ecosystems. In line with the above hypothesis, a study by Hua and Wang (2024) showed that polyethylene NPs can enhance the neurotoxicity of 6PPDq through adsorption in *Caenorhabditis elegans*. Their study demonstrates that polyethylene NPs can adsorb 6PPDq, with adsorption capacity increasing rapidly within the first 72 h and reaching equilibrium thereafter (Hua & Wang, 2024). A study by Grasse et al. (2023) shows that 6PPDq is readily absorbed by zebrafish larvae and is further metabolized. Given the potential for polyethylene NPs to adsorb and facilitate the transport of 6PPDq, as demonstrated by the findings of Hua and Wang (2024), it becomes crucial to examine the combined impact of such contaminants in the aquatic environment. Hence, understanding of single and combined toxicity of NPs and 6PPDq at the molecular level is pivotal when addressing possible negative effects on aquatic organisms. Based on behavioural and transcriptomics responses, the findings from the present study indicate that mixtures of 6PPDq and PS-NPs might have a stronger toxic effect than the contaminants have individually.

The tyre wear compound 6PPDq, produced when 6PPD is oxidised, is known to have species-specific toxicity. The earliest study on the toxicity of 6PPDq showed a 96 h LC_{50} of 0.79 μg/L for juvenile coho salmon (Tian et al., 2021). Further research revealed that exposure of 6PPDq was acutely toxic to rainbow trout, brook trout, and white-spotted char with a LC_{50} of <2 μg/L. Contrastingly, the LC_{50} was over 67 μg/L for Chinook salmon (Brinkmann et al., 2022; Foldvik et al., 2022; Hiki & Yamamoto, 2022b). In the present study, we found that 96 h exposure to 50 μg/L 6PPDq did not cause mortality in the adult zebrafish. Similar results were obtained with zebrafish larvae; an exposure concentration of 50 μg/L 6PPDq did not cause any significant mortality (Hiki et al., 2021; Varshney et al., 2022). The difference in toxicity of 6PPDq to zebrafish and coho salmon could be due to differences in metabolic and detoxification capacities. One noteworthy point to consider is the temperature-based difference among two species. Salmon, being a cold-water fish, tend to have slower metabolic rates and may have different detoxification mechanisms suited for their lower temperature habitat. Other reasons could be genetic variation and adaptation to pollution. Moreover, the present study found that 96 h exposure to 3 mg/L PS-NPs did not cause mortality in the adult zebrafish. This result is consistent with other studies, showing that exposure to even higher concentrations of PS-NPs does not cause mortality in zebrafish (Lu et al., 2016; Zhao et al., 2021). Furthermore, in the present study, the combined exposure of the two contaminants also did not cause mortality. As intended, the selected exposure concentrations were sub-lethal. These findings indicate a minimal mortality risk at the 6PPDq and PS-NPs concentrations studied in this work.

Behavioural assessments are often applied for suspected neurotoxicants. We have earlier shown that 6PPDq impacts swimming behaviour in zebrafish larvae (Varshney et al., 2022). The locomotory

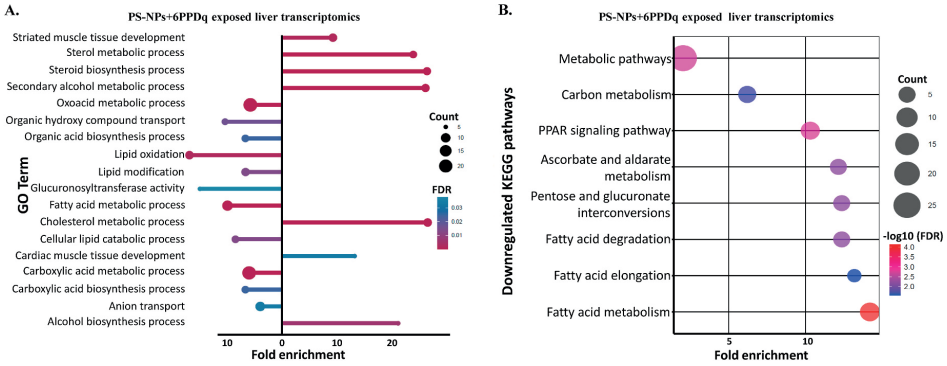

Fig. 7. Biological response in adult zebrafish exposed to PS-NPs+6PPDq in liver samples. (a.) Dysregulated gene ontology (GO) terms, the X-axis represents the fold enrichment, while the Y-axis represents the individual GO terms. On the left side of the graph, bars depict significantly downregulated GO terms, while on the right side, bars represent significantly upregulated GO terms. The colour of each bar corresponds to the false discovery rate (FDR) or adjusted p-value (Padj). The magnitude of the 'lollipop' atop each bar varies according to the associated gene count change for the respective GO term. Significant upregulated or downregulated GO terms refer to those with a p-adjusted (Padj) or FDR value of less than 0.05, indicating statistical significance. (b.) Downregulated KEGG pathways, the X-axis represents the fold enrichment, while the Y-axis represents the individual KEGG pathways. The size of the bubble varies with the gene count whereas its colour varies with the –log10 (FDR). Lollipop bars on the left side signifies downregulated GO terms whereas lollipop bars on the right side signifies upregulated GO terms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

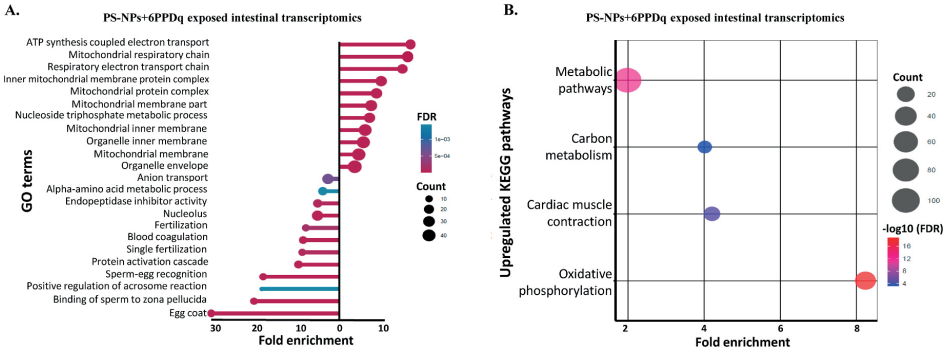

Fig. 8. Biological response in adult zebrafish exposed to PS-NPs+6PPDq in intestinal samples. (a.) Dysregulated gene ontology (GO) terms, the X-axis represents the fold enrichment, while the Y-axis represents the individual GO terms. On the left side of the graph, bars depict significantly downregulated GO terms, while on the right side, bars represent significantly upregulated GO terms. The colour of each bar corresponds to the false discovery rate (FDR) or adjusted p-value (Padj). The magnitude of the 'lollipop' atop each bar varies according to the associated gene count change for the respective GO term. Significant upregulated or downregulated GO terms refer to those with a p-adjusted (Padj) or FDR value of less than 0.05, indicating statistical significance. (b.) Upregulated KEGG pathways, the X-axis represents the fold enrichment, while the Y-axis represents the individual KEGG pathways. The size of the bubble varies with the gene count whereas its colour varies with the –log10 (FDR). Lollipop bars on the left side signifies downregulated GO terms whereas lollipop bars on the right side signifies upregulated GO terms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

behaviour in zebrafish is mainly governed by the neural circuits within the central nervous system controlling muscle contractions necessary for swimming. The present study is the first to investigate behavioural responses in fish following co-exposure with 6PPDq and other contaminants. The behavioural testing suggests that when 6PPDq and PS-NPs are combined, there may be a synergistic or additive effect. We found that although 6PPDq exposure singly affected swimming behaviour in adult zebrafish, the effect was stronger after co-exposure with PS-NPs. Our result is in line with other recent studies that have documented locomotory behavioural dysfunction in invertebrates and fish exposed to 6PPDq (Hua et al., 2023; Ricarte et al., 2023; Varshney et al., 2022). There could be multiple reasons behind the enhanced locomotory dysfunction in the combined exposure group. Firstly, PS-NPs might disturb membranes and enhance the uptake of 6PPDq, leading to increased exposure. PS-NPs have a small size and large surface area, making them an ideal vector for uptake and transport of contaminants in organisms (Alimi et al., 2018; Yu et al., 2019). No other published studies have yet documented enhanced uptake of 6PPDq in the presence of MPs or NPs. However, a study found elevated toxic effects of 6PPD co-exposed with road salt, suggesting an interaction of 6PPDq with other contaminants (Klauschies & Isanta-Navarro, 2022). Secondly, although exposure to PS-NPs alone did not affect behaviour in this study, it might impact the nervous system in a way that intensifies the behavioural responses to 6PPDq. A study by Ricarte et al. (2023) found that exposure of zebrafish larvae to 20 ng/L 6PPDq significantly affected neurotransmitters such as acetylcholine, serotonin, epinephrine and norepinephrine. In the liver transcriptome analysis, we found downregulation of the *synaptotagmin Va* (*syt5a*) gene. The gene encodes the synaptotagmin protein and this protein has a critical role in the release of neurotransmitters at the synaptic junctions (Henry et al., 2022). Notably, zebrafish

exposed to 6PPDq and PS-NPs alone exhibited downregulation of this gene with log2 fold changes of −3.5 and −2.89, respectively, as compared to the control group. Whereas the combined exposure resulted in a more pronounced effect on this gene with a log2 fold change of −4.92, indicating synergistic toxic effects between 6PPDq and NPs. The dysregulation of this gene can lead to neurodegenerative disorders including compromised locomotion (Mitchell et al., 2019). Similarly, we also found downregulation of acetyl-CoA synthetase 1 (acss1) gene. This gene is known to support the energy demands of acetyl-CoA, which is an important neurotransmitter (Castro et al., 2012). The dysregulation of this gene may disturb the neuronal health and associated functions (Kendrick et al., 2010). The log2 fold changes of acss1 gene were −2.94, −3.25 and −3.66 in the zebrafish exposed to 6PPDq, PS-NPs and PS-NPs+6PPDq, respectively. These results again indicate synergistic toxic effects between 6PPDq and PS-NPs. Taken together, the present study shows enhanced behavioural toxicity in adult zebrafish co-exposed with 6PPDq and PS-NPs, a finding that warrants further research to understand the molecular mechanism behind the combined toxic mode of action of 6PPDq and PS-NPs.

Following the behavioural study, we were interested to decipher the effect of exposure on histopathological parameters. Previous studies have indicated that ecologically relevant concentrations of certain pollutants like lead can promote lipid accumulation (steatosis) in liver of zebrafish (Santos et al., 2022). However, in this study, neither of the treatments induced any histopathological changes in terms of gross tissue microarchitecture. A possible reason for this finding could be the short exposure duration, or the relatively low exposure concentrations applied. He et al. (2023) found histopathological changes in mice fed for four weeks with 4 mg/kg 6PPDq. Similarly, Fang et al. (2023) also observed histopathological changes in liver of mice fed with 30 and 100 mg/kg 6PPDq for six weeks. To document similar histopathological changes in fish, longer-term exposure studies are needed.

Transcriptomic analysis showed that exposure to 6PPDq alone significantly affected several GO terms such as sterol biosynthesis process, cholesterol metabolism, and striated muscle tissue development in liver tissue. The sterol biosynthesis process was the most severely exposure-affected GO terms with a 39-fold enrichment. Sterols are an integral part of cell membranes and are involved in membrane structural integrity and signal transduction (Wüstner, 2007). Our findings suggest that 6PPDq exposure substantially affects sterol biosynthesis in the liver, which might have broader implications on cellular integrity. This result is in line with a study which showed a significant upregulation of metabolic sensor genes for steatosis in *Caenorhabditis elegans* exposed to 10 μg/L 6PPDq (Wang et al., 2023). Furthermore, an impact on a GO term associated with striated muscle cell differentiation could indicate possible implications on locomotor abilities. The transcriptomic results also indicate a 17-fold upregulation of the cholesterol metabolic process. Cholesterol is a major component of cell membranes and is involved in membrane fluidity and integrity (Yeagle, 1991). It also acts as a precursor for synthesising steroid hormones and bile (Vlahcevic et al., 1991). The upregulation of cholesterol biosynthesis processes could be a cellular response mechanism in response to 6PPDq. Upregulation of cholesterol biosynthesis processes may reflect the need for enhanced membrane stability and integrity, which could be disrupted due to 6PPDq exposure. Also, cholesterol production may serve as a defence mechanism to mitigate the effects of oxidative stress induced by 6PPDq. Fang et al. (2023) also found dysregulated cholesterol metabolism in liver of mice fed with 100 mg/kg 6PPD. The intestinal transcriptomics analysis also indicated upregulation of GO terms such as sterol biosynthesis, cholesterol metabolic process and striated muscle development. Thus, in terms of enriched GO terms, 6PPDq exposure induced similar responses in liver and intestinal tissues, suggesting a synchronised response to this stressor.

Intestinal transcriptomics revealed that several GO terms associated with mitochondria, cell signalling, and immune function were affected by PS-NPs exposure. Due to the small size of NPs, they are readily taken by zebrafish and can accumulate in the intestinal tract (Bhagat et al., 2020; Varshney et al., 2023). Some of these NPs present in the intestinal tract could be taken up by the intestinal cells and transported to the mitochondria (Hua & Wang, 2022). Accumulated NPs might disrupt the mitochondrial membrane, induce ROS production through mitochondrial dysfunction and activation of NADPH oxidase (Halimu et al., 2022). Newly produced ROS could this way reduce the efficiency of energy production in the mitochondria (Wang et al., 2021). A sudden increase in ROS leads to activation of pro-inflammatory signals such as cytokines and chemokines (Sun et al., 2023). In response to pro-inflammatory signals, immune cells, particularly macrophages and neutrophils, start infiltrating the affected part of the intestine once summoned to the site of inflammation (Huang et al., 2023). In the intestine, we found significant enrichment of GO terms associated with mitochondrial function, such as mitochondrial respiratory chain complex I, ATP synthesis coupled electron transport, mitochondrial respiratory chain, inner mitochondrial membrane protein complex and mitochondrial protein complex, indicating a profound effect of PS-NPs on the intestine. We also found significant GO terms associated with activating pro-inflammatory cytokines in the intestine, such as cytokine receptor activity and cytokine-mediated signalling pathway, suggesting the generation of pro-inflammatory cytokines against PS-NP-induced ROS. Our results align with a study where the authors showed mitochondrial dysfunction in zebrafish larvae co-exposed with NPs and copper (Zhang et al., 2023). In the liver of zebrafish exposed to PS-NPs alone, we found no significant GO terms. The absence of significant GO terms does not necessarily indicate the lack of biological impact of PS-NPs exposure. Upon closer examination of individual DEGs, we found 14 genes such as acss 1, elovl6, me1, b3galnt2, angptl4, sall2, gapdh, slco5a1a, ppp1r15b, gatm, miox, cyp51, slc25a32a, slc40a1 and *alas1* related to fatty acid and lipid metabolism. Deng et al. (2023) also found lipid and fatty acid dysregulation using single-cell liver transcriptomics of adult zebrafish exposed to 100 nm PS-NPs (500 ng/mL). Similarly, Zhao et al. (2020) also found dysregulated fatty acid metabolic pathways in adult zebrafish exposed to 500 nm PS-NPs (100 μg/L). These collective studies highlight the disrupted fatty acid metabolism in response to PS-NPs exposure.

In the liver, exposure to 6PPDq alone and 6PPDq in combination with PS-NPs induced many similar pathways. Both treatments affected striated muscle tissue development, sterol biosynthesis processes and cholesterol metabolic processes. However, these GO terms were more strongly enriched in terms of fold enrichment, number of genes and significance in co-exposed zebrafish. This clearly suggests that PS-NPs enhance the toxic effect of 6PPDq in the zebrafish. Apart from these common GO terms, we also found unique GO terms in the combined exposure group, such as lipid oxidation, lipid modification, and glucuronosyltransferase activity. The multiple GO terms related to lipid processes suggest that co-exposure of PS-NPs and 6PPDq increased the effect on lipid metabolism. Lipids are essential biomolecules and play an important role in energy storage, membrane integrity and signalling (Casares et al., 2019). Many studies have shown that exposure to contaminants can change the lipid profile of the liver, ultimately leading to the dysfunction of normal cellular processes (Arambourou et al., 2020; Shin et al., 2020). A study by Wang et al. (2023) found that exposure to 10 μg/L 6PPDq caused a significant increase in steatosis in *Caenorhabditis elegans*. Increased accumulation of lipids was associated with modification in fatty acid metabolism. In our present study, co-exposure significantly downregulated the fatty acid metabolism pathway, indicating disturbance in the balance of fatty acid metabolism. This can lead to compromise in energy production, altered membrane structure and steatosis in the zebrafish (Mukherjee et al., 2022). Furthermore, we found downregulation of the GO term associated with glucuronosyltransferase activity in the co-exposed group. Glucuronosyltransferase is an important enzyme in the glucuronidation reaction, facilitating the elimination of xenobiotics (Tephly & Burchell, 1990). Any alterations in this enzyme might affect detoxification mechanisms and possibly

excretion.

In the intestine sample from the co-exposed group, we found DEGs related to DNA damage, namely, centrosomal protein 72 (*cep72*), HEAT repeat containing 1 (*heatr1*), growth arrest and DNA-damage-inducible, alpha, b (*gadd45ab*) and isthmin 1 (*ism1*). Centrosomal protein 72 is encoded by *cep72* gene, ensuring proper cell division and chromosome segregation (Oshimori et al., 2009). Studies have shown that alteration in this gene can affect cellular, developmental and immunogenic functions (Corrêa et al., 2019; Maiato & Logarinho, 2014; Stowe et al., 2012). Hua et al. (2023) found that chronic exposure of 6PPDq to Caenorhabditis elegans can cause apoptosis in reproductive cells through DNA damage. They also observed differential gene expression of another gene of the cep family i.e., *cep1*. The gene *gadd45ab* is a known DNA damage-inducible gene (Johnen et al., 2013). The protein encoded by *gadd45ab*, *growth arrest and DNA damage-inducible alpha b* (Johnen et al., 2013), plays an important role in cell cycle regulation and DNA repair, helping cells cope with genotoxic stress (Sharma & Jindal, 2020). The impact of combined exposure of PS-NPs and 6PPDq on cellular health is understood through this GO term enrichment. This reveals that co-exposure resulted in a stronger synergistic effect producing more detrimental outcomes. Furthermore, the identification of DEGs such as *heatr1*, *cep72* and *gadd45ab* highlights the potential risks to genetic stability and cell division caused by combined exposure of PS-NPs and 6PPDq. This knowledge highlights the need for proactive measures to mitigate the harmful effects of these environmental contaminants on both human and ecological health while also providing insight into the molecular mechanisms involved.

5. Conclusion

In summary, we found that PS-NPs alone at a concentration of 3 mg/L did not affect behaviour in adult zebrafish. However, when co-exposed with 50 μg/L 6PPDq, it magnified the toxic effects of the tyre wear contaminant and produced stronger behavioural aberrations which were corroborated by the transcriptomic responses seen in liver and intestine tissues. PS-NPs exposure alone resulted in fewer significant DEGs, GO terms and KEGG pathways compared to the co-exposure in both tissues. Understanding the risks associated with multiple pollutant exposure is essential, especially when contaminants share a common source of origin.

CRediT authorship contribution statement

Shubham Varshney: Writing – original draft, Methodology, Data curation, Conceptualization. **Olivia L. O'Connor:** Methodology. **Adnan Hussain Gora:** Methodology. **Saima Rehman:** Methodology. **Viswanath Kiron:** Supervision, Funding acquisition. **Prabhugouda Siriyappagouder:** Methodology. **Dalia Dahle:** Methodology. **Tanja Kögel:** Supervision. **Robin Ørnsrud:** Supervision. **Pål A. Olsvik:** Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.123835.

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Spatio-temporal dynamics of soft-bottom macrobenthic communities in a rapidly changing Arctic: a case study of the Northwestern Barents Sea
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Plastic pollution, including macro, micro, and nanoplastics (NPs), is an emerging environmental threat to the ecosystems. Particles generated from tyre wear are the second largest source of microplastics in the ocean. In addition, tyre-wear runoff is the primary source of release of several tyre additives such as fillers, antioxidants, and antiozonants to the environment. 6PPD is the most widely used antioxidant in the tyre-manufacturing industries to enhance the durability of tyres. 6PPD quinone (6PPDq), a derivative of 6PPD, has been linked to the mass mortality of Coho salmon in the United States. The primary objective of this PhD thesis was to assess the ecotoxicity of 6PPD, 6PPDq, NPs and p,p'-DDE, a major metabolite of DDT found in the environment, using zebrafish (Danio rerio) as an animal model. We have also assessed the mixture toxicity of NPs with 6PPDq and p,p'-DDE. To achieve this, we employed a multi-endpoint approach including parameters such as development, behaviour, respiration, heart rate and the transcriptome. The results indicate that exposure to higher concentrations of 6PPD and 6PPDq can significantly affect the development, swimming behaviour and heart rate of zebrafish larvae. We also found enhanced toxicity of 6PPDq and p,p'-DDE when co-exposed with NPs. These toxic effects were also observed at the molecular level. Taken together, this dissertation contributes significantly to understanding the ecotoxicity of some novel and legacy environmental contaminants.

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