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Research Article

Effects of naproxen and titanium dioxide combination on the early life-stages of Zebrafish (*Danio rerio*): Acute toxicity, morphological defects, and gene expression

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Abstract

Zebrafish (*Danio rerio*) early-life stages of larvae were used to investigate behavioral, genetical and neurochemical changes promoted by the aquatic toxicity associated with the widely used medicines including naproxen (NPX) and titanium dioxide (TiO_2), individually or in combination. Zebrafish as a biological model system genetically very similar to human. This study was carried out with a control (C) group and six treatments including TiO_2 at 2 (2T) and 4 mg/L (T4), 50 µg/L (50N) and 100 µg/L (100N) of NPX, 2 mg/L TiO_2+50 µg/L NPX (2T50N) and 4 mg/L TiO_2 + and 100 µg/L NPX, (4T100N). Approximately 48 hours post-fertilization (hpf), the groups exposed to 4 mg/L TiO_2 individually or in combination of 100 µg/L NPX induced large suites of symptoms in zebrafish (*D. rerio*) early-life stage, including hatching inhibition particularly in 50 and 100 µg/L exposed groups (10.0% and 10.3%, respectively), increased mortality specially in the group 4T100N (39.6%), high heart-beat, and few morphological abnormalities. At approximately 168 hpf, severing of the yolk sac and pericardium oedema, severe swim bladder inflation, short tail with axial malformation, and small eyes were other significant occurrences in *D. rerio* exposed particularly to 100 µg/L NPX, which can be collectively referred to as pigeon chest deformity. The results of mRNA expression of neurogenesis- and growth-associated genes of the targeted ones presented that *gfap* mitigated exception for 2T group compared to the control group. For *mbp*, fish of all groups showed downward gene expression, except for 100N group exhibited a normal expression compared to the control as well as the situation observed for AChE, although the fish showed relatively downward gene expression compared to the control group. The brain showed apoptosis as vacuoles in 4T and 50N groups. It is concluded that TiO_2 had low acute toxicity to the embryos and larvae of *D. rerio* compared to NPX and could be used in different industries with low-risk rate, while it was used at low concentration (2 mg/L or less). although exposure to higher concentrations (4 mg/L or more) resulted in the increase of susceptibility risk of diseases.

Keywords: *Danio rerio*, Naproxen, Titanium dioxide, Axial defects, *gfap, mbp, AchE*

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Introduction

The neurotoxicity of AgNPs (silver nanoparticles) on aquatic organisms is rarely reported. Powers *et al*. (2010) studied the swimming behavior of zebrafish larvae to assess the neurotoxicity effects of AgNPs. Currently, there is very limited information on the neurotoxicity of AgNPs, particularly on the early developmental stages of organisms. These studies can be useful in animal models to extend the result for humans. Zebrafish embryos are transparent and this makes it easy to observe the effects of toxicants on organs (Xin *et al.*, 2015). In recent decades, despite the fact that pharmaceuticals have saved millions of lives, their consumption has increased considerably throughout the world. Nowadays, they are detected in surface water and irregularly in groundwaters. Therefore, they can affect both chronic and acute destructive effects on natural flora and fauna particularly fish and shellfish (Li *et al.*, 2016).

Although the levels of identified pharmaceuticals in surface waters are negligible, chronic exposure of aquatic organisms to these compounds can result in various adverse effects (Ebele *et al.*, 2017). Human drug release into water occurs through sewage, agriculture, or leachate from waste disposal sites, while veterinary drugs often enter water through manure or directly from their use in aquaculture (Farré *et al.*, 2007). The increased production and use of engineered nanoparticles (NPs) inevitably leads to their distribution in the aquatic environment, posing a threat to aquatic organisms (Klaine *et al.*, 2008). Therefore, there is a mixture of NPs and other toxic environmental substances in the environment. Since nanoparticles are highly insoluble and have a high surface area to volume ratio, they are likely to interact with other substances present in the water (Handy *et al.*, 2008). Hence, NPs may affect the bioavailability, metabolism, fate, and toxicity of a toxic substance for aquatic organisms. Among nanoparticles, titanium dioxide nanoparticles (TiO_2NPs) are one of the most popular nanomaterials used in various industrial and environmental applications such as catalysts for the decomposition of organic pollutants (Chen and Mao, 2007). Therefore, TiO_2NPs is inevitably released into the environment and enters aquatic environments. While it has been confirmed that TiO_2NPs exhibits low acute toxicity for fish species and other aquatic invertebrates at concentrations ranging from tens to hundreds of milligrams per liter (e.g., *Daphnia magna*) (Federici *et al.*, 2007), several studies have shown that TiO_2NPs adsorbs organic pollutants and increases the adsorption of non-organic pollutants (such as tributyltin and polybrominated diphenyl ethers [PBDEs]) (Zhu *et al.*, 2011).

On the other hand, among the compounds isolated in the environment, nonsteroidal anti-inflammatory drugs (NSAIDs) are a group of substances with similar pharmacological properties that are widely used for inflammation control in both animals and humans. NPX is a known anti-inflammatory drug that is

commonly used to reduce inflammation (Parolini *et al.*, 2011).

Danio rerio belongs to the Cyprinidae family and it is native to freshwater habitats in tropical regions with 7-22°C water temperature and 5.7-8.6 pH. An important point regarding the maintenance of this fish is its high adaptability (Spence *et al.*, 2008). Due to its physiological and genetic similarities (80% homology with the human genome), *D. rerio* is widely used as a valuable animal model to investigate genes involved in various physiological and immunological functions (Lieschke and Currie, 2007). Furthermore, *D. rerio* has been used to study neurological and behavioral phenotypes such as learning, sleep, drug addiction, and other behavioral traits (Cahill, 2002). Gene expression study could help to analyze the phenotypic occurrences and behavior changes of *D. rerio*. Glial fibrillary acidic protein (*gfap*) is a type III intermediate filament protein essential for maintaining the cytoskeletal architecture of glial cells (Nielsen and Jørgensen, 2003), providing mechanical stability and supporting neighboring neurons (Romero-Sandoval *et al.*, 2008). downregulation of *gfap* expression has been shown to reduce microglial activation (Hussain *et al.*, 2018). Myelin basic protein (*mbp*) is another important gene involved in axon growth and development (Li *et al.*, 2016). AChE is the major neurotransmitter of the cholinergic system, expressed in a number of tissues, including the central nervous system and muscle (Bertrand *et al.*, 2001).

The neurotoxicity and phenotypical effects of TiO_2 and Naproxen on embryos were studied at both the morphological levels and molecular levels. Therefore, the objective of this research was to determine the LC50 of NPX and TiO_2, as well as to investigate their individual and combined effects on phenotypic changes, such as axial malformations, tail shortening, and pathological occurrences, including edema, heartbeat, survival rate, and hatchability of the zebrafish eggs during 168 hpf of the eggs. Additionally, this study aimed to assess the disruption of expression of targeted genes, representing transcription factors that play critical roles in the nervous system development of zebrafish larvae at an early-life stage including *gfap*, *mbp*, and *AchE* were selected as endpoints of biological effect.

Materials and methods

Animal

The broodstocks were obtained from the Royan Institute in Tehran. Newly hatched 48-hour-old wild-type strain zebrafish larvae (*D. rerio*) were obtained from the aforementioned broodstock and used as the animal model for this study. Approximately 5250 zebrafish larvae were transferred to the laboratory and placed in petri dishes pre-filled with clean water at a temperature of 24-26°C with equal dark-light cycle. The petri dishes were then placed in a room with controlled lighting conditions, following the guidelines of Truong *et al.* (2011).

Study design

For this experiment, the prepared zebrafish larvae were randomly distributed among seven groups, each with three replicates of 250 larvae, and exposed to solutions containing either NPX, titanium dioxide nanoparticles alone, or a combination of the two, with each group containing 500 milliliters of the respective solution as assigned in Table 1. The larvae were exposed to these solutions for a duration of 8 days with the initial solution. During the experimental period, the larvae were not fed, and the number of mortalities in each group was recorded.

Preparation of a naproxen stock solution

Naproxen was purchased commercially for use in this test. A solution was prepared by dissolving 20,000 µg/L NPX in acetone, and it was stored in darkness at a temperature of 4ºC. Finally, the desired concentrations were obtained (Table 1) by diluting the stored solution with filtered water (Li *et al.*, 2016).

Table 1: Group assignments and doses of naproxen and titanium dioxide used in the study with Zebrafish Larvae (*Danio rerio*)

Groups	Description
Control	without the presence of NPX or titanium dioxide in the water of the Petri dish containing the larvae
2T	2 mg/L titanium dioxide presented in the water with the larvae
2T50N	2 mg/L titanium dioxide and 50 µg/LNPX presented in the water with the larvae
4T	4 mg/L titanium dioxide presented in the water with the larvae
4T100N	4 mg/L titanium dioxide and 100 µg/L NPX presented in the water with the larvae
50N	50 µg/L NPX presented in the water with the larvae
100N	100 µg/L NPX presented in the water with the larvae

Preparation of a titanium-dioxide stock solution

Commercially purchased 25 nm titanium dioxide nanoparticles were analyzed for particle size using electron microscopy based on the (Ramsden *et al.*, 2013) method. To prepare the stock solution with a concentration of 1 mg/mL of TiO_2NPs, NPs were added to ultra-pure water (Millipore) and sonicated (2 W/L, 50 kHz, 40 min). Test solutions containing titanium dioxide nanoparticles were prepared by diluting the stock solution with filtered water using a filter paper with a pore size of 3 µm and sonicating (4 W/L, 50 kHz, 20 min) immediately before use (Fang *et al.*, 2015).

Determination of LC50 of titanium dioxide nanoparticles and Naproxen

Four hundred and ten almost-hatched eggs of zebrafish were randomly divided into five groups with three replicates each (20 larvae per plate) to determine the LC50 of titanium dioxide, and 480 ones were used for NPX exposure, separately (Jarque *et al.*, 2020). The larvae were exposed to concentrations of 0 (control), 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 milligrams per liter of titanium dioxide nanoparticles in related Petri dishes, and

exposed to 0.0, 0.02, 0.04, 0.06, 0.08, 0.12, 0.14, and 0.16 milligrams per liter of NPX in each separate 10 mL solution Petri dishes for 168 hours. The mortality rate of the larvae was recorded daily up to 96 hpf (Alavinejad *et al.*, 2023).

Target Gene-RNA isolation, and real-time PCR

After 168 hours post-fertilization, thirty randomly selected larvae from each treatment group and their respective replications were randomly selected and stored at -80°C in RNase-free microtubes (Wong *et al.*, 2014). Total RNA was extracted from whole-body samples using Wizol Reagent according to the manufacturer's instructions, and the quality and quantity of the RNA were assessed using a Nano Drop spectrophotometer (USA, Thermo Scientific). Next, cDNA synthesis was carried out using a Thermo Scientific kit, and real-time PCR was performed using the WizPure^{TM} kit following the manufacturer's protocols (Qin *et al*., 2018). Gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method to normalize the results. The primer sequences were designed using Gene Runner software and synthesized by ZistFanavari-e-Pishgam (Table 2). Finally, the mRNA expression levels of genes associated with neurogenesis and growth, including *gfap*, *mbp*, and *AChE*, as well as the control gene β-actin, were analyzed using real-time PCR.

Table 2: Primers of the considered genes used in this study

Primer name	Sequence	Reference
Forward primer *AChE*	CCAAAAGAATAGAGATGCCATGGACG	(Bertrand *et al.*, 2001)
Reverse primer *AChE*	TGTGATGTTAAGCAGACGAGGCAGG	
Forward primer *mbp*	AATCAGCAGGTTCTTCGGAGGAGA	(Xu *et al.*, 2013)
Reverse primer *mbp*	AAGAAATGCACGACAGGGTTGACG	
Forward primer *gfap*	AAATGAATTCGAGCCAGAGCAGG	(Candiani *et al.*, 2020)
Reverse primer *gfap*	CAACCCGCGGCATCACATCCTTGTGCTCCTG	
Forward primer β Actin	GTCCCTGTACGCCTCTGGTCG	(Alavinejad *et al.*, 2023)
Reverse primer β Actin	GCCGGACTCATCGTACTCCTG	

Observed embryonic development criteria and pathological signs

Evidence was found for the rate of certain embryonic development criteria, including hatchability, un-hatched eggs, incomplete hatched eggs, heart rate, and mortality, as well as pathological clinical signs such as changes in swim bladder inflation, yolk sac oedema or resorption, oedema of the head, yolk sac, pericardium, and tail, skeletal malformations such as scoliosis, lordosis, tail curve, and short tail, and a reduction in pigmentation extension. The observation and evaluation of the aforementioned behavior were carried out on 48, 60, 96, and 168 hpf. The equations of some quantitative characters were presented as follows:

$$\text{Hatchability rate (\%)} = 1 - \frac{\text{unhatched eggs}}{\text{total eggs}} \times 100 \quad (1)$$

$$\text{Unhatched rate (\%)} = 1 - \frac{\text{hatched eggs}}{\text{total eggs}} \times 100 \quad (2)$$

$$\text{Incomplete hatched rate (\%)} = \frac{\text{Inc,hatched eggs}}{\text{total eggs}} \times 100 \quad (3)$$

$$\text{Mortality rate (\%)} = 1 - \frac{\text{live eggs}}{\text{total eggs}} \times 100 \quad (4)$$

$$\text{Hear beat (N/Min):} = \text{Beat number in 15 seconds} \times 4 \quad (5)$$

Statistical analysis

Data analysis was performed using SPSS 20 software. Differences among treatments were assessed using one-way ANOVA, followed by Duncan's multiple range test to compare significant differences between groups. The data were presented as mean±standard deviation (SD), and statistical significance was set at $p<0.05$.

Results

The probit analysis for TiO_2 NPs is depicted in Figure 1. Accordingly, LC50 of TiO_2 NPs for *D. rerio* exposed to various concentrations at 96 hpf was 9.58 mg/L with lower and upper bounds of 7.5 and 14.0, respectively at 95% confidence limits. This probit model can be responsible for 75.4% of changes may be observed in the future (Fig. 1). This value for NPX was 130 µg/L with lower and upper bounds of 0.05 and 0.25, respectively at 95% confidence limits. This probit model can be responsible to 59.8% of changes may be observed in the future (Fig. 2).

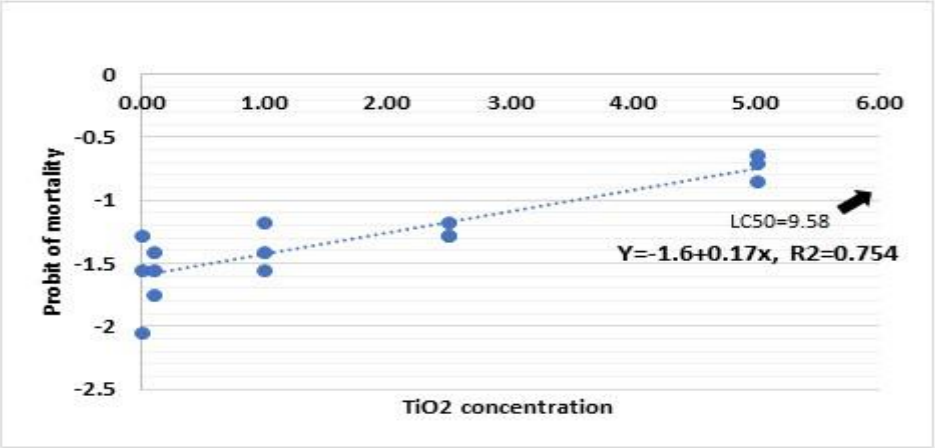


Figure 1: Regression scatter plot between the concentration and probit value of TiO_2NPs (mg/L) for newly larvae of *Danio rerio*) at 96 hours post fertilization exposure time ($p<0.05$).

At approximately 48 hpf, the hatching rate was 50%, 48.3%, and 36.6% in the control, 2T, and 2T50N groups, respectively. This value gradually decreased and reached 10.0% and 10.3% in the groups exposed to 50 and 100 µg/L of naproxen.

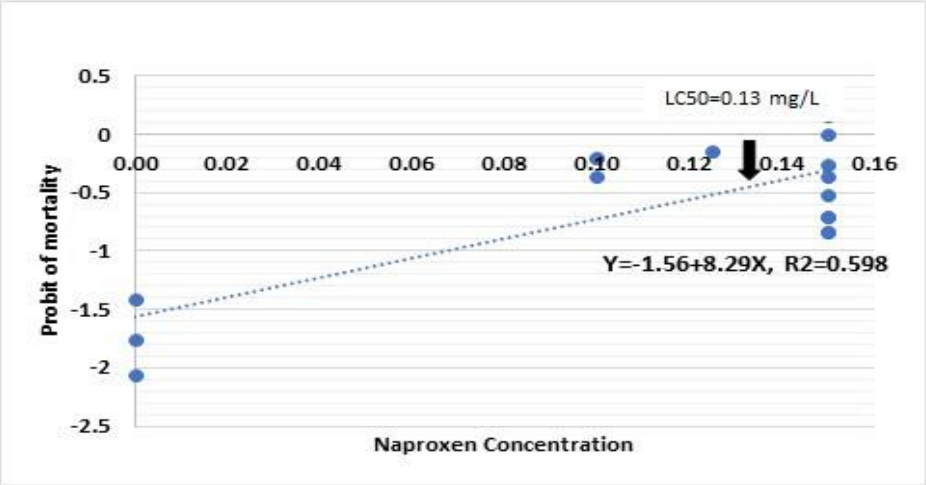
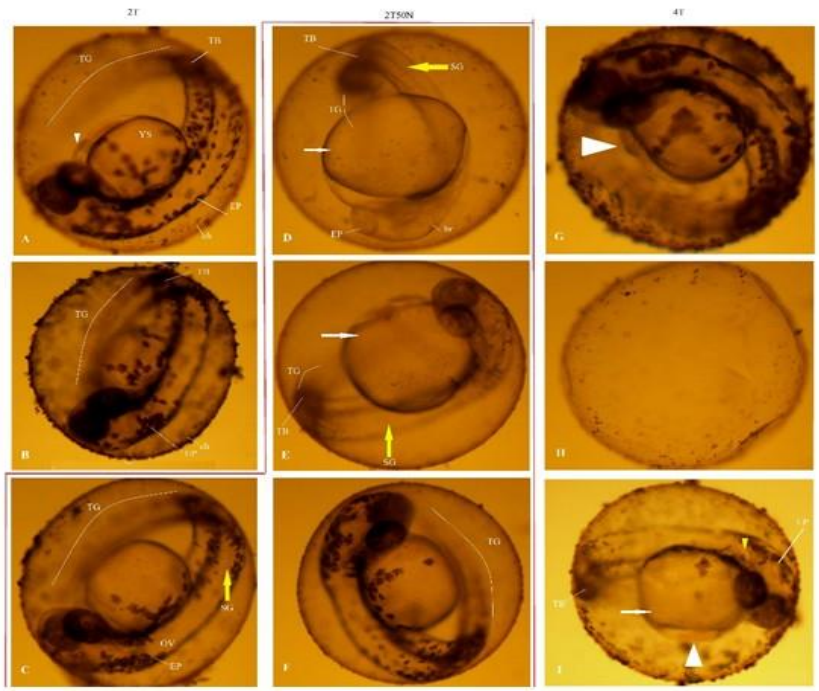


Figure 2: Regression scatter plot between the concentration and probit value of naproxen (mg/L) for new larvae of *Danio rerio*) at 96 hours post fertilization exposure time (p<0.05).

The percentage of un-hatched associated eggs for the first three groups was 49.3%, 47.3%, and 54.3%. The hatching rate decreased with an increase in NPX concentration in the groups. In the 100N group, 10.3%, 37.0%, and 33.0% of the eggs hatched, un-hatched, and had incomplete hatching, respectively. Weak extension of pigmentation, moderate to severe pericardial edema, and short tail were significant phenotypes in the 50N and 100N groups (Fig. 3M-Q). The maximum heart rate was 207.6 beats per second in the 4T100N group, which did not differ significantly (p>0.05) from that of the 100N group. The heart rate was 165.3 beats per second in the control group (Table 3).



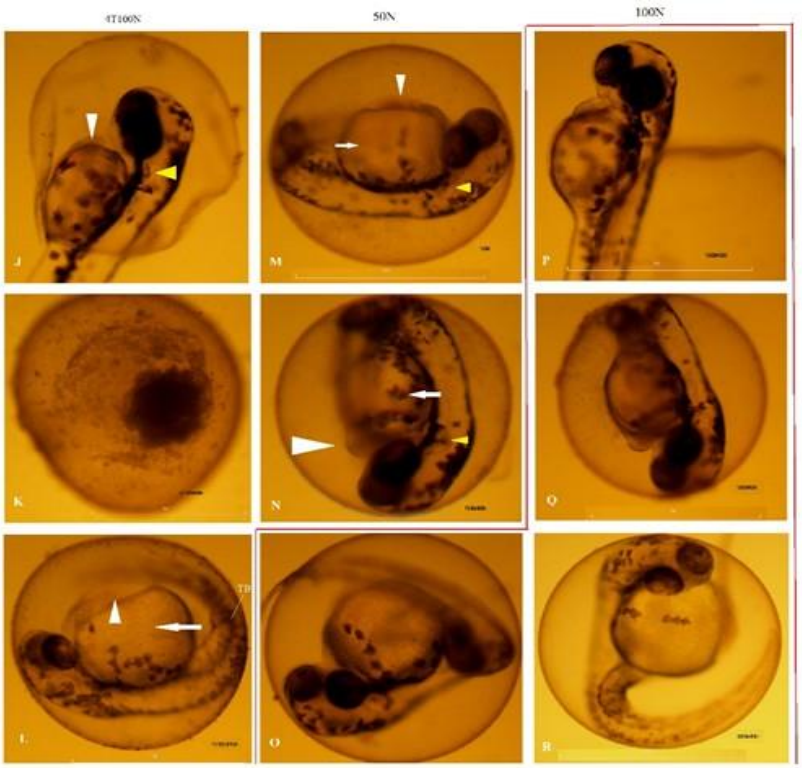


Figure 3: Larval at approximately 48h post-fertilization (hpf). The 2T group showed normal tail and somite growth, but some unhatched eggs exhibited mild pericardial edema (A-B). In the 2T50N group, many unhatched eggs had oedema in the epicardium and yolk sac (C-F) pale pigmentation and short tail (D-E). The 4T group exhibited weak to moderate abnormalities such as yolk sac and pericardial edema, short tail (G, I), and coagulated eggs (H). The 4T100N group showed incomplete hatched eggs (J), coagulation (K), and unhatched eggs with moderate to severe yolk sac and pericardial edema (L). The 50N and 100N groups had worse conditions with moderate to severe oedema of the pericardium and yolk sac eggs, along with short tail (M-R) and otolith relocation to vertical position vs longitudinal axis (N). Incomplete hatched eggs (P), delayed hatched eggs (Q) along with Tail lashing (O) and tail coiling (R) were observed in the 100N group (P). Ch: Chorion, EP: Extension Pigmentation, OV: Otic vesicle, TB: Tail bud, ST: Short tail, Large white arrow: Yolk sac oedema, White three angle: Pericardium oedema, Orange three angle: Otoliths, 2T: 2mg/L TiO_2NPs, 4T:4mg/L TiO_2NPs, 2T50N: 2mg/L TiO_2NPs+50 µg/L naproxen, 4T100N: 4mg/L TiO_2NPs+100 µg/L naproxen, 50N: 50 µg/L naproxen, 100N: 100 µg/L naproxen.

While normal tail length and somite growth were observed, some eggs remained un-hatched. One of the most prominent features of the embryos in the 2T50N group was a delay in tail growth and extension pigmentation at 48hpf compared to the control and the other groups exposed to titanium dioxide (Fig. 3D-E). In contrast to the groups exposed to titanium dioxide, in which spontaneous contractions were strong, the autonomic contractions were absent in the 4T and 4T100N groups, while they were very weak in the eggs of the 50N-100N groups (Fig. 3M-R). Perhaps one reason for the delay in hatching of zebrafish eggs in the 50N and 100N groups is the effect of NPX, which causes a delay or reduction in neurogenesis and a subsequent decrease in spontaneous autonomic contractions in the tail of the fish. This leads to incomplete hatching or a decrease in energy during exit from the chorion, resulting in the death of the embryos.

Table 3: Phenotypic alterations and quantitative evaluation of significant criteria in Zebrafish (*Danio rerio*) embryos 48 hours post-fertilization exposure to different doses of naproxen and titanium dioxide.

	C	2T	2T50N	4T	4T100N	50N	100N
EP	+++++	+++++	+++	++++	++	+++	++
YE	-	-	+++	+++	+++	++	++
PE	-	+	+	+	+++	+++	+++
Short T.	-	-	+++	+	-	+	+
Hatch%	50.0 ± 2.0^{a}	48.3 ± 5.50^{a}	36.6 ± 2.08^{b}	50.6 ± 3.05^{a}	20.3 ± 4.5^{c}	10 ± 2.0^{d}	10.3 ± 4.5^{d}
Un-hatched%	49.3 ± 6.0^{ab}	47.3 ± 4.04^{b}	54.3 ± 9.5^{a}	47.6 ± 12.2^{b}	25.0±3.0	50.6 ± 8.02^{ab}	37.0 ± 4.0^{c}
Inc. H.%	0.0 ± 0.0^{c}	0.6 ± 1.15^{c}	0.3 ± 0.6^{c}	0.6 ± 1.15^{c}	$15.6\pm\pm2.08^{b}$	$29.6\pm\pm4.5^{a}$	33.5 ± 3.05^{a}
Cg /Mortality%	0.0 ± 0.0^{e}	5.3 ± 2.5^{d}	10.3 ± 3.5^{c}	5.3 ± 1.5^{d}	39.6 ± 2.5^{a}	10.3 ± 4.5^{c}	20.0 ± 3.0^{b}
Heartbeat	165.3 ± 4.5^{cd}	174.6 ± 4.2^{c}	155.3 ± 12.5^{d}	160.3 ± 30.6^{cd}	207.6 ± 38.5^{a}	191.3 ± 34.6^{b}	206.6 ± 18.9^{a}

(-): negative, (+): minor positive, (++): mild positive, (+++): moderate positive, (++++): great positive, (+++++): severe positive, EP: Extension in pigmentation, YE: Yolk sac oedema, PE: Pericardial oedema, HE: Head oedema, Inc. H: Incomplete hatch, Cg: Coagulation, Short T.: Short tail. Numerical data was presented as Mean ± SD.

At 60 hours post-fertilization, Embryonic development in all eggs of the 4T100N group ceased with nearly all exhibiting coagulation and subsequent mortality, at approximately 60hpf.

A gradual decrease in pigmentation extension was observed with an increase in NPX concentration, resulting in the group 100N exhibiting less pigmentation extension than the other groups. Moreover, Significant phenotypes, such as short tail, pericardial and head oedema, were observed in the group 100N, similar to those of groups 50N and 4T (with varying degrees of severity). However, the hatching rate in the groups 50N, 100N, and 4T was 25%, 28%, and 15%, respectively, which was lower than the other groups (Table 4). This suggests a potential adverse effect of NPX at concentrations of 50-100 µg/L and TiO_2NPs at 4 mg/L. Additionally, roughly 33% and 25% of embryonic *D. rerio* in the 100N and 50N groups, respectively, exhibited incomplete hatch at 60hpf (Fig. 4). Subsequently, zebrafish larvae that were unable to emerge from the chorion experienced mortality within a few hours, likely due to insufficient energy for chorion rupture (Tables 4 and 5).

The hatching rate was decreased with increase of concentrations of NPX and titanium dioxide while the hatching rate of group 100N was 25% against that of the control group (90%). At 60 hours post-fertilization, the highest mortality (60%) was observed in 4T group (Table 4).

Table 4: Summary of phenotypic alterations in zebrafish (*Danio rerio*) embryos after 60 h exposure

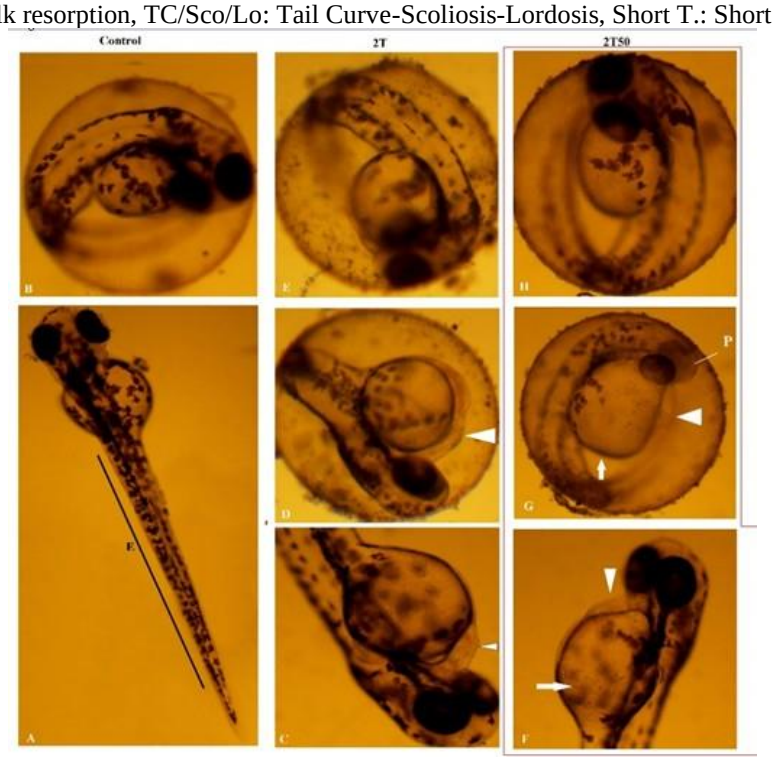
	C	2T	2T50N	4T	50N	100N
EP	+++++	+++++	++++	++++	+++	+
YE	-	-	+	+	++	++
PE	-	+	+	++	++	++++
HE	-	-	-	+++	++	+
TC/Sco/Lo	-	-	-	-	-	+
Short T.	-	-	-	+	++	+++
Hatch%	90.3±2.5^{a}	80.0±3.6^{a}	75.3±1.5^{a}	15.0±4.0^{b}	28.3±0.57^{b}	25.3±1.5^{b}
Un-hatched%	10.33±2.0^{c}	7.5±0.8^{c}	25.6±5.0^{a}	20.3±4.5^{a}	27.6±3.0^{a}	17.0±1.0^{b}
Inc. H.%	0.0±0.0^{c}	0.6±1.15^{c}	0.3±0.5^{c}	0.6±1.15^{c}	25.3±0.6^{b}	33.6±3.0^{a}
Cg /Mortality%	0±0.0^{d}	12.5±1.5^{c}	8.0±3.0^{c}	65.3±1.5^{a}	20.0±2.0^{b}	25.0±2.0^{b}

(-): negative, (+): minor positive, (++): mild positive, (+++): moderate positive, (++++): great positive, (+++++): severe positive, EP: Extension in pigmentation, YE: Yolk sac oedema, PE: Pericardial oedema, HE: Head oedema, Inc. H: Incomplete hatch, Cg: Coagulation, TC/Sco/Lo: Tail Curve-Scoliosis-Lordosis, Short T.: Short tail.

Table 5: Morphological evaluation and mortality percentage of Zebrafish (*Danio rerio*) Larvae at 96 Hours Post-Fertilization Exposure: Summary of Findings

	C	2T	2T50N	4T	50N	100N
EP	+++	+++	+++	+++	++	+
YE	-	+	+	++	++	+
PE	-	-	-	+	++	++
RYR	-	-	+	++	++	++
SE	-	-	-	-	-	-
TC/Sco/Lo	-	-	-	+	++	++
Short T.	-	-	-	+	++	++
Mortality%	0.0±0.0^{a}	2.0±2.0^{a}	10.3±4.5^{a}	30.3±3.5^{b}	45.0±2.0^{c}	60.6±4.0^{d}

(-): negative, (+): minor positive, (++): mild positive, (+++): moderate positive, (++++): great positive, (+++++): severe positive, EP: Extension in pigmentation, YE: Yolk sac oedema, PE: Pericardial oedema, RYR: Reduced yolk resorption, TC/Sco/Lo: Tail Curve-Scoliosis-Lordosis, Short T.: Short tail.



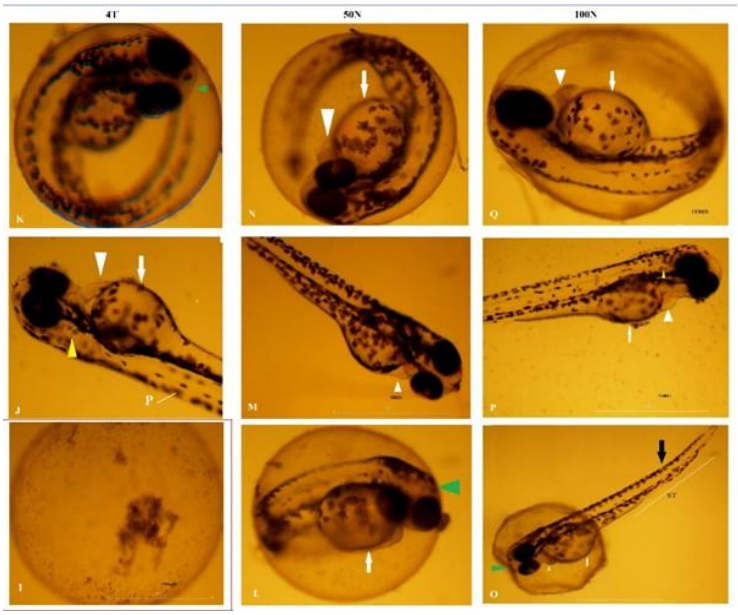


Figure 4: Larval at approximately 60h post-fertilization (hpf). A): The control group exhibited normal and elongated bodies (A) with unhatched eggs (B). The early hatched larvae as well as unhatched embryo in the 2T group showed mild pericardial oedema (C-D) and unhatched eggs (E). The 2T50N group exhibited weak to moderate abnormalities including yolk sac and pericardial oedema (F-G) while a few eggs showed un-hatching condition or early coagulation (H-I). In the 4T group, a few early larvae showed weak pigmentation while moderate yolk sac and pericardial oedema were significant phenotypes (J-K). The 50N group remarkably presented head oedema (L) along with moderate to severe abnormalities including yolk sac and pericardial oedema (L-N). In the100N group, many incomplete hatching eggs were observed, along with cases of tail curvature and unhatched eggs with shrunken chorions. Severe abnormalities including yolk sac and pericardial oedema were the major phenotypes observed. P: Pigmentation, E: Elongated body, ST: Short tail, Large white arrow: Yolk sac oedema, Large black arrow: curve occurred in tail, White three angle: Pericardium oedema, Green three angle: Head oedema, Pale Orange three angle: Otoliths, 2T: 2mg/L TiO_2NPs, 4T:4mg/L TiO_2NPs, 2T50N: 2mg/L TiO_2NPs+50 µg/L naproxen, 4T100N: 4 mg/L TiO_2NPs+100 µg/L naproxen, 50N: 50 µg/L naproxen, 100N: 100 µg/L naproxen. Bar scale 1mm.

In Figure 5 and Table 5 evidences of the changes in the morphological features of larval *D. rerio* at approximately 96 hpf was presented. The control group exhibited no abnormalities and had well-developed pectoral fins with pigmentation extending well along the body (Fig. 5A) similar to the groups exposed to 2mg/L of titanium dioxide and the 4T group (Fig. 5B-E). The groups exposed to NPX (Fig. 5F-L) showed a pale appearance, particularly the group exposed to 100 µg/L of NPX (Fig. 5I-L), which the yolk sac was wrinkled. The presence of a wrinkled yolk sac in the larvae of the 100N group suggests a significant depletion of nutrient reserves compared to both the control group and other experimental groups, indicating a potential nutritional deficiency or metabolic impairment in this particular group. Axial malformation of scoliosis with gray plaques was observed in the tail region of the group 50N. Axial malformation exhibited a high incidence of lordosis in 4T group (Fig. 5E), and scoliosis in 50N and 100N groups (Fig. 5K-L). The presence of gray plaque in the tail region of zebrafish larvae may indicate the occurrence of apoptosis or an immune reaction, suggesting a potential defect in this area.

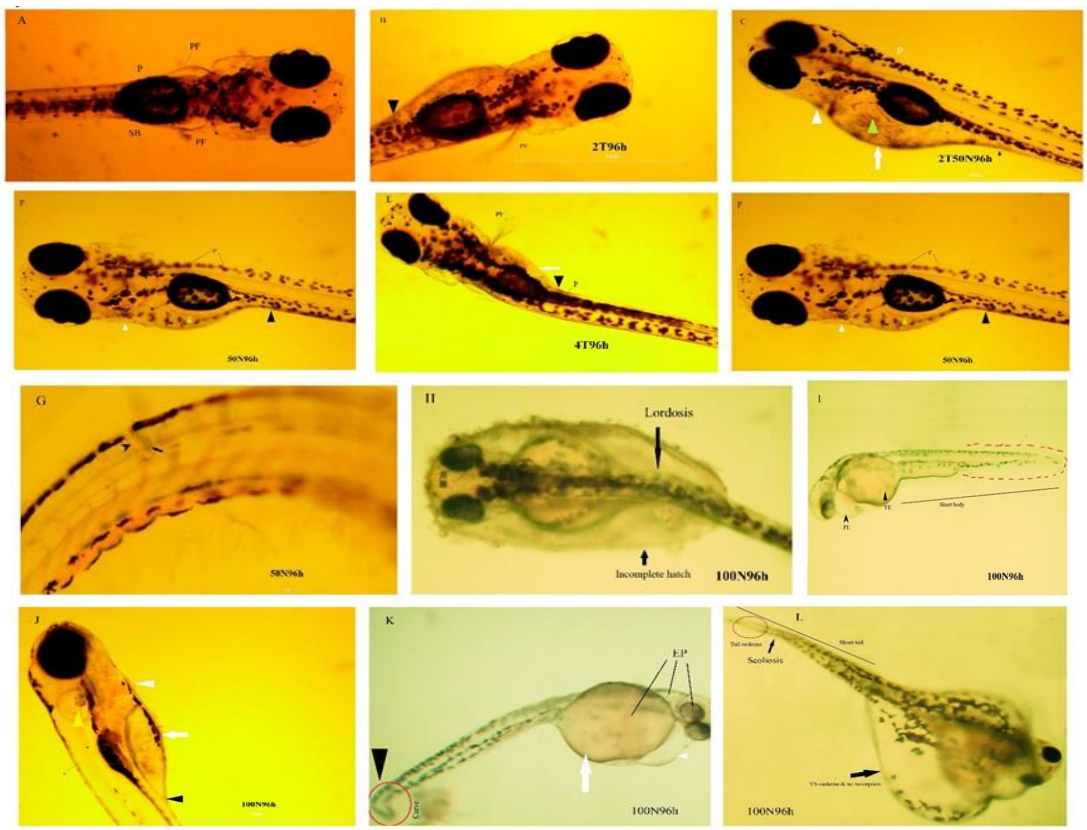


Figure 5: Larval at approximately 96h post-fertilization (hpf). A): The control group exhibited normal and elongated bodies same as 2T and the larvae showed few signs of weak to moderate abnormalities including yolk sac oedema (C-E). As the concentration of naproxen increased, the larvae showed an increase in abnormalities, oedema being more prominent (F-L). PF: Pectoral fin, E: Elongated body, SB: Swim blader, Large white arrow: Yolk sac oedema, Large black arrow: gray plaques or curve occurred in tail, pointed arrow: A defect on body, White three angle: Pericardium oedema, Black three angle: Reduced yolk sac resorption, Bright green three angle: Intestine, Pale Orange three angle: Otoliths, 2T: 2mg/L TiO_2NPs, 4T:4mg/L TiO_2NPs, 2T50N: 2mg/L TiO_2NPs+50 µg/L naproxen, 4T100N: 4mg/L TiO_2NPs+100 µg/L naproxen, 50N: 50 µg/L naproxen, 100N: 100 µg/L naproxen. Bar scale 1mm.

In the 4T100N group, embryonic development was halted at approximately 12hpf, suggesting potential deleterious effects of NPX on zebrafish. Notably, the groups exposed to NPX exhibited more pronounced symptoms, including reduced yolk sac resorption, yolk sac and pericardium edema, as depicted in Figure 5. It is worth mentioning that in the 4T group, a few viable eggs that survived up to approximately 96 hpf ultimately experienced coagulation. About 30% of the initially developed eggs in the 4T group subsequently perished, while the resulting larvae that hatched displayed a range of abnormalities, as shown in Table 5 and Figure 5E. At 96 hpf, the maximum mortality (60.6%) was observed in the 100N group (Table 5), followed by the 50N group (45%), which significantly differed from the groups exposed to TiO_2NPs. The groups exposed to 4T and 50N-100N demonstrated instances of axial malformation, indicating a potential adverse effect of individual exposure to 50-100 µg/L NPX and 4mg/L TiO_2NPs on the skeletal development in *D. rerio*. Our findings suggest that exposure to 50 or 100 µg/L NPX may lead to a decrease in neurogenesis rate and hinder skeletal growth in fish.

At approximately 168 hpf, of common potential sublethal endpoints with regeneration potential in zebrafish (*D. rerio*) larvae were selected and presented in Figure 6 and Table 6. The control group exhibited normal elongated bodies and well-developed pectoral fins with no abnormalities (Fig. 6A). Pigmentation extension did not significantly differ between the control and TIO_2NPS-exposed groups but the 50N and 100N groups showed reduced pigmentation. Yolk sac and epicardium oedema gradually increased from the TIO_2NPS -exposed groups to the NPX-exposed groups (Fig. 6 and Table 6).

Table 6: Assessment of morphological features and mortality rate of Zebrafish (*Danio rerio*) larvae 168 Hours Post-Fertilization: Summary of Findings

	C	2T	2T50N	4T	50N	100N
EP	+++	+++	+++	++	++	+
YE	-	+	+	++	+++	++++
PE	-	-	-	+	++	++++
RYR	-	-	+	+	++	+++
HE	-	-	-	-	+	++
Cg	-	-	-	-	-	-
SBD	-	-	-	-	-	+
SE	-	-	-	-	-	++
TC/Sco/Lo	-	-	-	++	+	++
Short T.	-	-	-	+	++	++
Gray P.	-	-	-	+	-	+
Mortality%	0.0 ± 0.0^a	10.0 ± 2.0^a	12.3 ± 1.5^a	30.3 ± 2.5^b	45.0 ± 2.0^c	60.6 ± 2.0^d

(-): negative, (+): minor positive, (++): mild positive, (+++): moderate positive, (++++): great positive, (+++++): severe positive, EP: Extension in pigmentation, YE: Yolk sac oedema, PE: Pericardial oedema, RYR: Reduced yolk resorption, HE: Head oedema, Cg: Coagulation, SBNI: Swim bladder deviation, SE: Small eye, TC/Sco/Lo: Tail Curve-Scoliosis-Lordosis

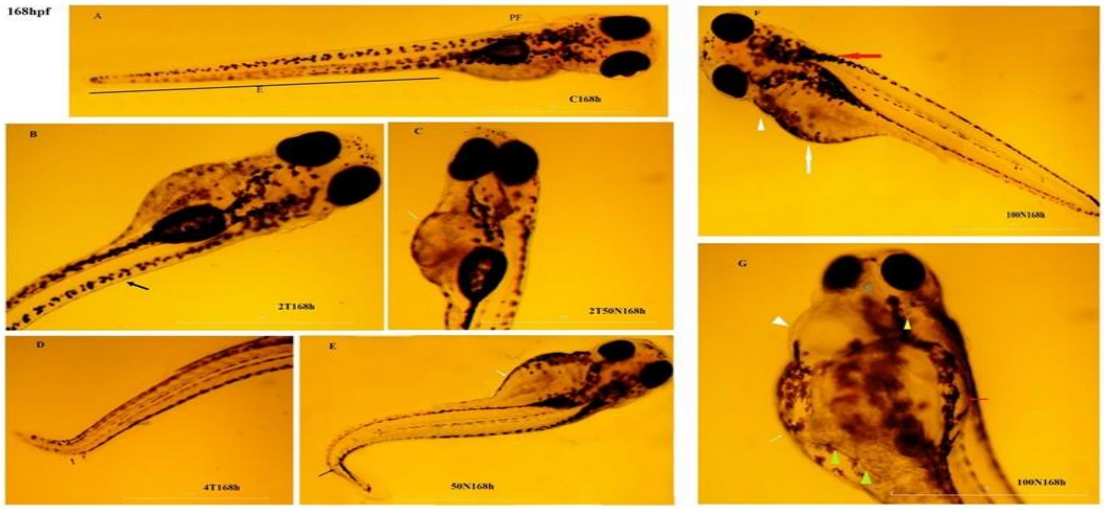


Figure 6: Larval at approximately 168h post-fertilization (hpf). A): Control (B-C) larva showing few signs of abnormalities including yolk sac oedema. B-G). The larvae showing abnormalities with increase of the concentration of TIO_2NPs or naproxen individually or in the combination form, of which oedema was increased with the increase of naproxen. PF: Pectoral fin, E: Elongated body, Large white arrow: Yolk sac oedema, Large blue arrow: Exophthalmia of eyes, Large black arrow: Axial malformation, Large red arrow: Sever inflated swim bladder, White three angle: Pericardium oedema, Red three angle: Hyperplasia intestine, Black three angle: Reduced yolk sac resorption, 2T: 2mg/L TIO_2NPs, 4T:4mg/L TIO_2NPs, 2T50N: 2mg/L TIO_2NPs+50 µg/L naproxen, 50N: 50 µg/L naproxen, 100N: 100 µg/L naproxen. Bar scale 1mm.

Larvae in the 2T and 2T50N treatment groups displayed no significant abnormalities, except for mild yolk sac edema (Fig. 6B, C). In contrast, the 4T group exhibited a short tail with axial malformation in the tail region (Fig. 6D). The 50N group showed a moderate short tail with minor spinal cord defects and mild head edema (Fig. 6E). Exposure of fish to 100 µg/L of NPX resulted in rare features such as intestine hyperplasia and exophthalmia, which are not commonly observed in other NPX poisoning cases. Severing of the yolk sac and pericardium oedema, severe swim bladder inflation, short tail with axial malformation, and small eyes were other significant occurrences in *D. rerio* exposed to 100 µg/L NPX, which can be collectively referred to as "pigeon chest" features (Fig. 6E). Head oedema was also observed in fish exposed to both 100 µg/L and 50 µg/L of NPX but with less frequency (Fig. 6E, G).

After 168 hpf of the study, the results of mRNA expression of neurogenesis- and growth-associated genes of the targeted genes were obtained (Fig. 7). Accordingly, for *gfap* almost the gene expression showed downward compared to the control. Among the treatments, the fish of 2T group showed 2-fold expression. According to the findings of this study, the fish of group 100N showed a minimum change due to titanium dioxide. For *mbp*, fish of all groups showed downward gene expression, exception for 100N group exhibited a normal expression compared to the control after 168 hpf. For AChE, the fish showed relatively downward gene expression compared to control a situation observed for *mbp*. The gene expression was the least for groups 4T and 2T50N with no significant difference (p>0.5) for the three aforementioned genes.

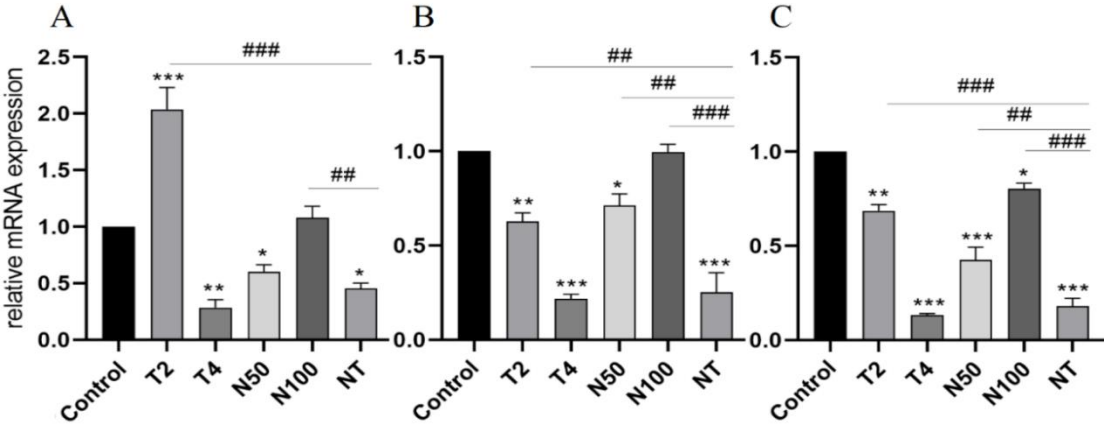


Figure 7: The mRNA expression of neurogenesis- and growth-associated genes (ratio), including *gfap* (A), *mbp* (B), *AChE* (C), and β-actin (as the control group), in the different groups of zebrafish larvae at 168 hpf. Whole-sample RNA was isolated from the groups at 168 hpf, and RT-PCR products were prepared. 2T: 2 mg/L titanium dioxide, T4: 4 mg/L titanium dioxide, N50: 50 µg/L naproxen, N100: 100 µg/L naproxen and NT: 2 mg/L titanium dioxide with 50 µg/L naproxen presented in the water with the larvae. The data are presented as means ± SD. Statistical significance was determined with ANOVA followed by Duncan's post hoc test. *p≤0.05, **p≤0.01, and ***p≤0.001, indicating significant differences from the control group.

Discussion

The present study investigated the acute toxicity, morphological changes, and gene expression alterations in early life-larvae stages of zebrafish (*Danio rerio*) exposed to NPX and TIO_2NPS either individually or in combination. The current study demonstrated that the exposure of *D. rerio* to NPX and TiO_2 resulted in malformations and adverse effects on body growth during the early stages of development. Exposure to the NPX produced a characteristic pattern of stunted growth and malformation. Representative images of incomplete hatch, unhatched, and mortality rates of fish exposed to TiO_2 (4mg/L), and NPX particularly in the fish of 50N and 100N groups ranging from 72% to 75% showed a reduction in overall size, failure to progress through metamorphosis, and specific defects.

The results of the probit analysis revealed an LC50 value of 9.58 mg/L for TiO_2NPs and 130 µg/L for NPX at 96 hpf, indicating that both substances are highly toxic to zebrafish larvae. The value of 96-h LC50 for the NPX was 115.2 mg/L (Li *et al.*, 2016) at the embryo stage less toxic compared to this study. For TiO_2, acute toxicity showed an LC50 of 0.28-0.50 mg/L even more at 96 hpf in zebrafish depending on the NPs size (Carbaugh *et al.*, 2022). This value was 160.54 mg/L after 96 hpf exposure to Caspian trout juveniles, *Salmo trutta caspius* (Kaviyani *et al.*, 2020).

Similar to the findings of Li *et al.* (2016) the hatching rate in this study decreased with an increase in NPX concentration. In a study (Tang *et al.*, 2019), the hatching rate of zebrafish embryos exposed to 10mg/L TiO_2 nanoparticles for 96 hours was approximately 90%, slightly higher than the 80% observed in this study, in group 2T at 60 hpf, with 7.5% of eggs remaining unhatched (Table 4). In concentrations lower than 100 µg/L, exposure to TiO_2 nanoparticles (NPs) did not have a significant effect on the hatching of zebrafish embryos (Vicario-Parés *et al.*, 2014). These findings suggest that acute toxicity of TiO_2 NPs may be limited at lower concentrations.

The delay in hatching of zebrafish eggs exposed to NPX, particularly in the groups, 50N and 100N may be due to the effect of NPX, which causes a delay or reduction in neurogenesis and a subsequent decrease in spontaneous autonomic contractions in the tail of the fish. According to a study (Xu *et al.*, 2019), the bioconcentration of NPX in zebrafish appears to be associated with reduced activity of detoxification enzymes involved in metabolism. This, in turn, may lead to a significant reduction in length.

In the 50N and 100N groups, significant phenotypes included weak extension of pigmentation, as well as moderate to severe pericardial edema and short tail. These findings are similar to those reported by Rangasamy *et al.* (2018) in zebrafish exposed to 1 mg/mL of ketoprofen, which resulted in various malformations as well as delayed hatching and increased mortality rates at higher concentrations. The acute toxicity study showed that exposure to AgNPs affected neurological development, and

the exposed embryos exhibited anomalies such as small head with a hypoplastic hindbrain, small eye, and cardiac defects (Pensado-López *et al.*, 2021).

An increase in naproxen and titanium dioxide concentrations was observed to result in an increase in mean heart beat at 48 hpf, with the greatest rate (206.6 beats/min) observed in fish exposed to 100 µg/L. This finding was in contrast to another study (Li *et al.*, 2016), which showed that heart beat decreased with increasing naproxen concentration, reaching a rate of 106 beats/min at the same time. As a result, the polar groups of naproxen may interact with those present in the embryonic membrane when in solution. Li *et al.* (2007) have suggested that chemicals need to interact with the cell membrane in order to penetrate it and affect the function of the target biomolecule, which leads to toxicity. Consistent with the findings of this study (Table 6), Li *et al.* (2016) showed that pericardial edema was the most sensitive sub-lethal effect, with its incidence rate increasing in a dose-dependent manner with increasing naproxen concentrations. In contrast, the fish exposed to less than 4 µg/L TiO_2 nanoparticles did not display pericardial edema or shortened length at 168 hpf, as was observed in this study in the group of 2T (Table 6). In some studies (Tang *et al.*, 2019; Gu *et al.*, 2021), exposure of zebrafish embryos to nano- and micro-TiO_2 at a concentration of 1 mg/L did not affect hatching or mortality rates. However, zebrafish larvae exposed to 1 mg/L of nano- TiO_2 exhibited significantly reduced body length and weight, similar to this research. These results suggest that short-term exposure to nano- TiO_2 may not pose an acute risk to embryonic development or survival, but can have sublethal effects on the growth and development of zebrafish larvae.

Glial fibrillary acidic protein (*gfap*) is a common sensitive and early biomarker of neurotoxicity (O'Callaghan and Sriram, 2005). The *gfap* expression in the zebrafish brain, was reported to begin at the 12-somite-stage, which is occurred at 15 hpf (Marcus and Easter Jr, 1995), and expression persists in the CNS glia of adult zebrafish (Tomizawa *et al.*, 2000).

An increase in *gfap* expression was observed in less concentration of AgNPs and downregulation of the *gfap* expression in a higher concentration of AgNPs exposed to zebrafish at the early life stage. This finding was in line with the findings of this research that exhibited less concentration of TiO_2 upregulated the *gfap* expression after 168 hpf indicating that in early time of exposure to higher concentration of the neurotoxicant, the *gfap* expression is high same as condition occurred in treatment with less concentration such as 2T in this study exhibited in Figure 7. However, due to the severe injuries caused in 4T, 50N, and 100N, the gene expression couldn't be increased over the time passing of the exposure (Fig. 7). The eye defects occurred in 100 N treated zebrafish embryos (Fig. 6) may be associated with the aberrant expression of neural genes such as *gfap*

(Xin *et al.*, 2015). Thus, based on the accumulated morphological data, the ideal 'biomarker' of all types of nervous system injuries is enhanced expression of GFAP (O'Callaghan and Sriram, 2005). At 48 hpf, the mRNA expression of the gene that encode myelin basic protein (*mbp*) could be detected. This finding suggests that the development of myelin, a crucial component of the nervous system, begins early in embryonic development. Their findings indicate that transcripts of myelin basic protein (*mbp*) can be detected in the central nervous system (CNS), Schwann cells located in the lateral line, cranial nerves, and spinal motor nerves. This suggests that *mbp* may play a critical role in the formation and maintenance of myelin in both the CNS and the peripheral nervous system (PNS), which is responsible for transmitting sensory and motor information between the body and the CNS. According to reports, messenger RNA (mRNA) encoding *mbp* is predominantly localized to the processes of myelinating cells (Brösamle and Halpern, 2002).

In this study, *mbp* gene expression was greatly downregulated in the groups 4T, 50N and 100N compared to the control (Fig. 7) and the fish located in the 2T group, which were less affected by TiO_2 (Table 6), the mRNA relative gene expression showed no significant changes (Fig. 7). Exposure to nano- TiO_2 had a significant impact on the expression of genes associated with axonal growth, including *mbp* and *gap*43 (Gu *et al.*, 2021). The larvae of the 100N group exhibited a wrinkled yolk sac, suggesting a significant depletion of nutrient reserves compared to both the control group and other experimental groups, indicating a potential nutritional deficiency or metabolic impairment in this particular group (Table 6).

During the early developmental stages of zebrafish, locomotor behavior is primarily initiated and regulated by the nervous system, making it a sensitive indicator of any abnormal changes in nerve development. As such, alterations in locomotor activity can provide valuable insights into the effects of various environmental stressors on the developing nervous system (Shaw *et al.*, 2016). Furthermore, analysis using real-time polymerase chain reaction demonstrated that Exposure of zebrafish to nano- TiO_2 during early-life stages has been shown to have adverse effects on neural outcomes, including the inhibition of neurodevelopment and axonal growth in motor neurons. These findings suggest that exposure to nano-TiO_2 during critical periods of nervous system development may disrupt the normal processes of neuronal growth and differentiation, with potential long-term consequences for neural function and behavior (Gu *et al.*, 2021).

It is reported that the opaque regions in the developing zebrafish embryos implicate cell death and could be a consequence of apoptosis (Langheinrich *et al.*, 2002). During early somitogenesis, the apparent opaque region was observed in the head of rhoA morphants zebrafish, and cell corpuses were detected within or at the boundary of deformed somites. By mid-

segmentation stage, the opaque regions were found throughout the morphants, but mostly in the head and tail ('opaque' phenotype or gray plaque). Axial malformation as scoliosis with gray plaques was observed in the tail region of the groups 4T 50N and 100N (Fig.5 and Table 6) indicating definite defects occurred. The aforementioned groups of 4T, 50N, and 100N showed cases of axial malformation, indicating a potential adverse effect of individual exposure to 50-100 µg/L NPX and 4mg/L TiO_2NPs on skeletal development in *D. rerio*.

Exposure to a combination of substances, such as TiO_2NPs and NPX, may result in more severe developmental abnormalities than exposure to either substance alone which exhibited in the group 4T100N. Li *et al.* (2016) reported that such developmental abnormalities, including pericardial edema, axial malformations, and yolk sac depletion, may occur due to the inhibition of Cyclooxygenase (COX), the rate-limiting enzyme for the synthesis of prostaglandins. The observed depletion of nutrient reserves in the yolk sac of the 100N group may be due to the effect of TiO_2NPs and NPX on nutrient uptake and lipid metabolism. Similarly, pericardial edema may result from the effect of TiO_2NPs on the cardiovascular system, which is essential for the proper circulation of blood and oxygen, as noted by Nam *et al.* (2017).

In zebrafish embryos, AChE first appears in the nervous system in the primary motoneurons of the rostral spinal cord when the embryo has nine somites, approximately 14 hpf, revealing significantly earlier than the onset of body movement (Hanneman and Westerfield, 1989; Bertrand *et al.*, 2001). Exposure to certain toxicant has been exhibited to inhibit acetylcholinesterase (AChE) activity, leading to feeding and locomotor alterations in Gammarus fossarum (Xuereb *et al.*, 2009). Expression of AChE can also be detected in many primary neurons of the zebrafish embryo, including both motor neurons and sensory neurons, which differentiate in the nascent central nervous system during early somitogenesis stages (Behra *et al.*, 2002). In our study, decreased expression of this neurotransmitter might be due to injuries faced by neurons and the brain.

In conclusion, the present study provides new insights into the combined toxicity of TiO_2NPs and NPX on zebrafish larvae. The findings suggest that the combined exposure to these substances may result in more severe developmental abnormalities than exposure to either substance alone particularly exhibited in the fish exposed to 4 mg/L TiO_2+100 µg/L NPX. In summary, the results exhibited that waterborne exposure to naproxen and titanium dioxide can cause a series of negative effects on zebrafish at embryonic and larval stages, including delayed hatching, pericardial edema, axial malformations, lower heart beat and yolk sac depletion, are likely due to the disruption of normal developmental processes in zebrafish larvae. The gene

expression analysis revealed significant alterations in the expression of several genes involved in developmental processes, providing a potential mechanism for the observed developmental abnormalities. These findings have important implications for the assessment of the environmental risks associated with the use of TiO_2NPs and NPX. The observed alterations in gene expression suggest that the developmental abnormalities observed in this study may be due to the disruption of normal gene expression patterns in zebrafish larvae. TiO_2NPs and NPX may interfere with the expression of genes involved in developmental processes, leading to abnormal development and growth occurred in phenotypical and genetical changes in early-life stage of *D. rerio.* Further research is needed to fully understand the mechanisms underlying the observed developmental abnormalities and to develop effective strategies for mitigating the risks associated with these substances. The results showed that Nano- TiO_2 had low acute toxicity to the embryos and larvae of *D. rerio*, although exposure to higher concentrations (4 mg/L) resulted in increased mortality rates. However, it was not possible to establish LC50 96h values at the concentrations tested in this study and the value given was prediction.

References

Alavinejad, S., Kakoolaki, S., Kazempoor, R., Anvar, S., Khajehrahimi, A. and Hemati, A., 2023. Effect of dietary supplementation of potential probiotic *Lacticaseibacillus casei* on immune-related genes expression, intestinal microbiota and gut histology of zebrafish (*Danio rerio*) during *Aeromonas hydrophila* infection. *Iranian Journal of Fisheries Sciences,* 22(**1**), 156-177. DOI:10.22092/ijfs.2023.128662

Behra, M., Cousin, X., Bertrand, C., Vonesch, J.-L., Biellmann, D., Chatonnet, A. and Strähle, U., 2002. Acetylcholinesterase is required for neuronal and muscular development in the zebrafish embryo. *Nature neuroscience,* 5(**2**), 111-118. DOI:10.1038/nn788

Bertrand, C., Chatonnet, A., Takke, C., Yan, Y., Postlethwait, J., Toutant, J.P. and Cousin, X., 2001. Zebrafish acetylcholinesterase is encoded by a single gene localized on linkage group 7: gene structure and polymorphism; molecular forms and expression pattern during development. *Journal of Biological Chemistry,* 276(**1**), 464-474. DOI:10.1074/jbc.M006308200

Brösamle, C. and Halpern, M.E., 2002. Characterization of myelination in the developing zebrafish. *Glia,* 39(**1**), 47-57. DOI:10.1002/glia.10088

Cahill, G.M., 2002. Clock mechanisms in zebrafish. *Cell and Tissue Research,* 309, 27-34. DOI: 10.1007/s00441-002-0570-7

Candiani, S., Carestiato, S., Mack, A. F., Bani, D., Bozzo, M., Obino, V., Ori, M., Rosamilia, F., De Sarlo, M. and Pestarino, M., 2020. Alexander

disease modeling in zebrafish: an i*n vivo* system suitable to perform drug screening. *Genes, 11*(**12**), 1490. DOI: 10.3390/genes11121490

Carbaugh, C.M., van der Schalie, W.H. and Widder, M.W., 2022. High throughput embryonic zebrafish test with automated dechorionation to evaluate nanomaterial toxicity. *PloS one*, 17(**9**), e0274011. DOI:10.1371/journal.pone.0274011

Chen, X. and Mao, S.S., 2007. Titanium dioxide nanomaterials: synthesis, properties, modifications, and applications. *Chemical Reviews*, 107(**7**), 2891-2959 DOI:10.1021/cr0500535

Ebele, A.J., Abdallah, M.A.E. and Harrad, S., 2017. Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. *Emerging Contaminants*, 3(**1**), 1-16. DOI:10.1016/j.emcon.2016.12.004

Fang, Q., Shi, X., Zhang, L., Wang, Q., Wang, X., Guo, Y. and Zhou, B., 2015. Effect of titanium dioxide nanoparticles on the bioavailability, metabolism, and toxicity of pentachlorophenol in zebrafish larvae. *J Hazard Mater*, 283, 897-904. DOI:10.1016/j.jhazmat.2014.10.039

Farré, M., Petrovic, M. and Barceló, D., 2007. Recently developed GC/MS and LC/MS methods for determining NSAIDs in water samples. *Analytical and Bioanalytical Chemistry*, 387, 1203-1214.

Federici, G., Shaw, B.J. and Handy, R.D., 2007. Toxicity of titanium dioxide nanoparticles to rainbow trout (Oncorhynchus mykiss): Gill injury, oxidative stress, and other physiological effects. *Aquatic Toxicology*, 84(**4**), 415. DOI:10.1016/j.aquatox.2007.07.009

Gu, J., Guo, M., Huang, C., Wang, X., Zhu, Y., Wang, L., Wang, Z., Zhou, L., Fan, D. and Shi, L., 2021. Titanium dioxide nanoparticle affects motor behavior, neurodevelopment and axonal growth in zebrafish (*Danio rerio*) larvae. *Science of the Total Environment*, 754, 142315. DOI:10.1016/j.scitotenv.2020.142315

Handy, R.D., Owen, R. and Valsami-Jones, E., 2008. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. *Ecotoxicology*, 17, 315-325. DOI:10.1007/s10646-008-0206-0

Hanneman, E. and Westerfield, M., 1989. Early expression of acetylcholinesterase activity in functionally distinct neurons of the zebrafish. *Journal of Comparative Neurology*, 284(**3**), 350-361. DOI:10.1002/cne.902840303.

Hussain, G., Zhang, L., Rasul, A., Anwar, H., Sohail, M. U., Razzaq, A., Aziz, N., Shabbir, A., Ali, M. and Sun, T., 2018. Role of plant-derived flavonoids and their mechanism in attenuation of Alzheimer's and Parkinson's diseases: An update of recent data.

Molecules, 23(**4**), 814. DOI:10.3390/molecules23040814.

Jarque, S., Rubio-Brotons, M., Ibarra, J., Ordoñez, V., Dyballa, S., Miñana, R. and Terriente, J., 2020. Morphometric analysis of developing zebrafish embryos allows predicting teratogenicity modes of action in higher vertebrates. *Reproductive Toxicology,* 96, 337-348. DOI:10.1016/j.reprotox.2020.08.004

Kaviyani, F.E., Naeemi, A. S. and Salehzadeh, A., 2020. Acute toxicity and effects of titanium dioxide nanoparticles (TiO_2 NPs) on some metabolic enzymes and hematological indices of the endangered Caspian trout juveniles (*Salmo trutta caspius* Kessler, 1877). *Iranian Journal of Fisheries Sciences,* 19(**3**), 1253-1267. DOI:10.22092/ijfs.2019.119319

Klaine, S.J., Alvarez, P.J., Batley, G. E., Fernandes, T.F., Handy, R.D., Lyon, D.Y., Mahendra, S., McLaughlin, M.J. and Lead, J.R., 2008. Nanomaterials in the environment: Behavior, fate, bioavailability, and effects. *Environtal Toxicology and Chemistry,* 27(**9**), 1825. DOI:10.1897/08-090.1

Langheinrich, U., Hennen, E., Stott, G. and Vacun, G., 2002. Zebrafish as a model organism for the identification and characterization of drugs and genes affecting p53 signaling. *Current Biology,* 12(**23**), 2023-2028. DOI:10.1016/s0960-9822(02)01319-2

Li, L., Gao, H.W., Ren, J.R., Chen, L., Li, Y.C., Zhao, J.F., Zhao, H.P. and Yuan, Y., 2007. Binding of Sudan II and IV to lecithin liposomes and *E. coli* membranes: insights into the toxicity of hydrophobic azo dyes. *BMC Structural Biology,* 7, 1-9. DOI:10.1186/1472-6807-7-16

Li, Q., Wang, P., Chen, L., Gao, H. and Wu, L., 2016. Acute toxicity and histopathological effects of naproxen in zebrafish (*Danio rerio*) early life stages. *Environmental Science and Pollution Research,* 23, 18832-18841. DOI:10.1007/s11356-016-7092-4.

Lieschke, G.J. and Currie, P.D., 2007. Animal models of human disease: zebrafish swim into view. *Nature Reviews Genetics,* 8(**5**), 353-367.

Marcus, R.C. and Easter Jr, S.S., 1995. Expression of glial fibrillary acidic protein and its relation to tract formation in embryonic zebrafish (*Danio rerio*). *Journal of Comparative Neurology,* 359(**3**), 365-381.

Nam, S.H., Shin, Y.J. and An, Y.-J., 2017. Effects of titanium oxide nanoparticles on *Oryzias latipes* embryos and sac-fry under different irradiation conditions. *Environmental Engineering Research,* 22(**4**), 426-431. DOI:10.4491/eer.2017.054.

Nielsen, A.L. and Jørgensen, A.L., 2003. Structural and functional characterization of the zebrafish gene for glial fibrillary acidic protein, GFAP. *Gene,* 310, 123-132. DOI:10.1016/s0378-1119(03)00526-2

O'Callaghan, J.P. and Sriram, K., 2005. Glial fibrillary acidic protein and related glial proteins as biomarkers of neurotoxicity. *Expert opinion on Drug Safety,* 4(**3**), 433-442. DOI:10.1517/14740338.4.3.433

Parolini, M., Binelli, A. and Provini, A., 2011. Assessment of the potential cyto–genotoxicity of the nonsteroidal anti-inflammatory drug (NSAID) diclofenac on the zebra mussel (*Dreissena polymorpha*). *Water, Air, and Soil Pollution,* 217, 589-601. DOI:10.1007/s11270-010-0612-9

Powers, C.M., Badireddy, A.R., Ryde, I.T., Seidler, F.J. and Slotkin, T.A., 2010. Silver nanoparticles compromise neurodevelopment in PC 12 cells: critical contributions of silver ion, particle size, coating, and composition. *Environmental Health Perspectives,* 119(**1**), 37-44. DOI: 10.1289/ehp.1002337

Pensado-López, A., Fernández-Rey, J., Reimunde, P., Crecente-Campo, J., Sánchez, L. and Torres Andón, F., 2021. Zebrafish models for the safety and therapeutic testing of nanoparticles with a focus on macrophages. *Nanomaterials,* 11(**7**), 1784. DOI:10.3390/nano11071784.

Qin, Z., Babu, V. S., Wan, Q., Zhou, M., Liang, R., Muhammad, A., Zhao, L., Li, J., Lan, J. and Lin, L., 2018. Transcriptome analysis of Pacific white shrimp (*Litopenaeus vannamei*) challenged by Vibrio parahaemolyticus reveals unique immune-related genes. *Fish & shellfish immunology, 77*, 164-174. DOI: 10.1016/j.fsi.2018.03.030

Ramsden, C., Henry, T. and Handy, R., 2013. Sub-lethal effects of titanium dioxide nanoparticles on the physiology and reproduction of zebrafish. *Aquatic Toxicology,* 126, 404-413. DOI:10.1016/j.aquatox.2012.08.021

Rangasamy, B., Hemalatha, D., Shobana, C., Nataraj, B. and Ramesh, M., 2018. Developmental toxicity and biological responses of zebrafish (*Danio rerio*) exposed to anti-inflammatory drug ketoprofen. *Chemosphere,* 213, 423-433.

Romero-Sandoval, A., Chai, N., Nutile-McMenemy, N. and Deleo, J.A., 2008. A comparison of spinal Iba1 and GFAP expression in rodent models of acute and chronic pain. *Brain Research,* 1219, 116-126. DOI:10.1016/j.brainres.2008.05.004

Shaw, B.J., Liddle, C.C., Windeatt, K.M. and Handy, R.D., 2016. A critical evaluation of the fish early-life stage toxicity test for engineered nanomaterials: experimental modifications and recommendations. *Archives of toxicology,* 90, 2077-2107. DOI:10.1007/s00204-016-1734-7

Spence, R., Gerlach, G., Lawrence, C. and Smith, C., 2008. The behaviour and ecology of the zebrafish, *Danio rerio*. *Biological reviews,* 83(**1**), 13-34. DOI:10.1111/j.1469-185X.2007.00030.x

Tang, T., Zhang, Z. and Zhu, X., 2019. Toxic Effects of TiO_2 NPs on Zebrafish. *International Journal of Environmental Research and Public*

Health, 16(**4**), 523. DOI:10.3390/ijerph16040523

Tomizawa, K., Inoue, Y. and Nakayasu, H., 2000. A monoclonal antibody stains radial glia in the adult zebrafish (*Danio rerio*) CNS. *Journal of neurocytology*, 29, 119-128. DOI: 10.1023/A:1007156529390

Truong, L., Harper, S.L. and Tanguay, R.L., 2011. Evaluation of embryotoxicity using the zebrafish model. *Drug safety evaluation: Methods and Protocols*, 271-279.

Vicario-Parés, U., Castañaga, L., Lacave, J. M., Oron, M., Reip, P., Berhanu, D., Valsami-Jones, E., Cajaraville, M. P. and Orbea, A., 2014. Comparative toxicity of metal oxide nanoparticles (CuO, ZnO and TiO_2) to developing zebrafish embryos. *Journal of Nanoparticle Research*, 16, 1-16. DOI:10.1007/s11051-014-2550-8

Wong, D., von Keyserlingk, M.A., Richards, J.G. and Weary, D.M., 2014. Conditioned place avoidance of zebrafish (*Danio rerio*) to three chemicals used for euthanasia and anaesthesia. *PloS one*, 9(**2**), e88030. DOI:10.1371/journal.pone.0088030.

Xin, Q., Rotchell, J.M., Cheng, J., Yi, J. and Zhang, Q., 2015. Silver nanoparticles affect the neural development of zebrafish embryos. *Journal of Applied Toxicology*, 35(**12**), 1481-1492. DOI:10.1002/jat.3164.

Xu, C., Niu, L., Guo, H., Sun, X., Chen, L., Tu, W., Dai, Q., Ye, J., Liu, W. and Liu, J., 2019. Long-term exposure to the non-steroidal anti-inflammatory drug (NSAID) naproxen causes thyroid disruption in zebrafish at environmentally relevant concentrations. *Science of the Total Environment*, 676, 387-395. DOI:10.3390/ijerph16040523

Xu, H., Shao, X., Zhang, Z., Zou, Y., Chen, Y., Han, S., Wang, S., Wu, X., Yang, L. and Chen, Z., 2013. Effects of di-n-butyl phthalate and diethyl phthalate on acetylcholinesterase activity and neurotoxicity related gene expression in embryonic zebrafish. *Bulletin of Environmental Contamination and Toxicology*, 91, 635-639. DOI:10.1007/s00128-013-1101-9

Xuereb, B., Lefèvre, E., Garric, J. and Geffard, O., 2009. Acetylcholinesterase activity in *Gammarus fossarum* (Crustacea Amphipoda): linking AChE inhibition and behavioural alteration. *Aquatic Toxicology*, 94(**2**), 114-122. DOI:10.1016/j.aquatox.2009.06.010.

Zhu, X., Zhou, J. and Cai, Z., 2011. Nanoparticles in the Marine Environment: Impact on the Toxicity of Tributyltin to Abalone (*Haliotis diversicolor* supertexta) Embryos. *Environmental Science & Technology*, 45(**8**), 3753-3758. DOI:10.1021/es103779h