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Investigation of the effects of metal oxide nanoparticle mixtures on *Danio rerio* and *Xenopus laevis* embryos

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ABSTRACT

In this study, the toxic effects of single TiO_2, ZnO and Fe_2O_3 nanoparticles (NPs) and their binary and ternary combinations on the embryonic development of *Xenopus laevis* and *Danio rerio* were evaluated. It was aimed to determine the median lethal concentrations, growth retardation and developmental malformation effects and the effects of mixed nanoparticle exposure to selected enzyme biomarkers resulting from the exposure of embryos to NPs. The results showed that ZnO NPs, which are the most toxic according to the highest lethality level, cause developmental delay and malformations. The Fe_2O_3 and TiO_2 NPs caused growth retardation only in *D. rerio* embryos. It was determined that double and triple combinations of NPs did not cause significant lethality in both species. Biochemical markers showed that mixed NPs could lead to greater toxicity compared to single exposures. The results also revealed that TiO_2 or Fe_2O_3 NPs caused an increase in toxicity when co-existing with ZnO NP due to additive effects. Overall, the results showed that *D. rerio* was more susceptible to NP exposure in terms of growth and mortality than *X. laevis*. However, studies that are more comprehensive are needed to better understand the toxicity mechanism of metal oxide NP mixtures.

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Nanoparticle mixture; zinc oxide; iron (III) oxide; titanium dioxide; toxicity; biomarker

1. Introduction

Nanotechnology is a rapidly developing science due to economic and scientific interests and nanoparticles (NPs) have been used in many fields in recent years [1, 2]. Metal oxide NPs are industrially important materials frequently used as catalysts due to their semiconducting properties [3]. They can be used mostly as single NPs (ZnO, TiO_2, CeO_2, CuO, fullerenes, carbon nanotubes etc.) or as binary mixtures, e.g. to produce materials such as core/shell structures [4, 5]. However, both their increased use and their potential for accumulation in aquatic environments can cause significant environmental risks. Therefore, there are numerous research results in the literature that have been conducted to determine the toxic effects of almost all NPs on various organisms. However, aquatic

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environments can also be contaminated with a variety of NPs from different sources and these contaminants can lead to toxic effects depending on NP mixtures and NP combinations [6]. On the other hand, the toxicity of NPs depends on a wide range of biotic/abiotic factors. For example, the stability and aggregation of ZnO are affected by ions in aquatic environments and the toxicity of NPs may increase in aquatic environment [7]. The potential of Fe_2O_3 to be widely used for medical purposes may lead to its accumulation in aquatic environments and it is also known to show high accumulation potential in different tissues [8, 9]. TiO_2 is one of the most widely used NPs with the highest photocatalytic properties [10] and can cause toxicity even when present alone in aquatic environments due to ROS production [11]. Accordingly, the coexistence of multiple NPs such as TiO_2, ZnO and Fe_2O_3 in aquatic environments may interact with each other, leading to unexpected increases in risk for organisms. However, few studies have evaluated the risks that metallic NPs may pose to aquatic organisms when present in combinations with other NPs and the studies were mostly investigated binary combinations of NPs [12–14]. However, we also need to understand the toxicity of NPs, especially ternary mixtures of NPs, on aquatic organisms. Furthermore, there are no comparative studies in the literature on the effects of NP mixtures on different aquatic organisms. Therefore, the aim of this study was to comparatively evaluate the toxicity of commonly used TiO_2, ZnO and Fe_2O_3 NPs, especially binary and ternary mixtures of these selected NPs, on embryonic developmental stages of aquatic organisms of two different evolutionary classes forming trophically important steps of the food chain using some biomarkers. Thus, it was aimed to reveal the effects of both single and mixed NPs on *Danio rerio* and *Xenopus laevis* embryos.

Differences in the activity of many enzymes involved in metabolic and oxidative stress and in the level of some biomolecules are useful biomarkers to detect the organism's response to environmental toxicants in the laboratory. It is also predominantly suggested that the toxic effects of metal and metal oxide NPs in organisms are caused by the formation of reactive oxygen species (ROS), especially in their environment and in cells [15]. Measurement of carboxylesterase (CaE) activity is recognised as a method to diagnose exposure to different pollutants. CaEs play an important role in metabolism and detoxification of numerous xenobiotics [16]. On the other hand, exposure to xenobiotics, such as exposure to NPs, can cause oxidative stress in aquatic animals due to ROS production. To cope with oxidative stress, cells have antioxidant defenses such as superoxide dismutase (SOD), glutathione reductase (GR) and glutathione S-transferase (GST) enzymes [17]. Therefore, this study aimed to evaluate selected biochemical responses as well as lethal effects and malformations that may be observed during embryonal development.

2. Materials and methods

2.1. The nanoparticles and characterisation

The titanium (IV) oxide tested (TiO_2, Sigma, CAS No: 13463-67-7) was 21 nm in size. The dimensions of the ZnO NPs (Sigma, CAS No: 1314-13-2) and Fe_2O_3NPs (Sigma, CAS No: 1309-37-1) were <100 nm and <50 nm, respectively. Scanning electron microscope (SEM) images of commercial NPs are given in Figure S1 (in the Supplementary Material). The particle size distributions of the NPs were determined from SEM images in addition to

the information provided by the relevant company. The pH of the test samples was determined separately in FETAX medium and fish water medium. In addition, the hydrodynamic diameter (nm) and zeta potential (mV) of the NPs tested were individually measured by dynamic light scattering (DLS; Zetasizer Nano-ZS model, Malvern Ins. Ltd.) in both FETAX medium and fish water. Other characteristics of the NPs tested are given from the manufacturer's information and reports of previous studies [18–20].

2.2. Test organisms

Embryo experiments were approved by the local ethics committee of Inönü University. The work plan was prepared in accordance with the principles outlined in the Manual for the Care and Use of Laboratory Animals (National Academy of Sciences, Washington, D.C., NAS 1996).The zebrafish embryos used in the tests were obtained from wild-type (Singapore origin) colonies in the zebrafish system (ZebTec Active Blue, Tecniplast, Italy) in Inönü University (Turkey) at *D. rerio* unit. The adult fish were kept in a 14:10 h day-night photoperiod with pH 7.25±0.15, conductivity 720±20 µS/cm, and temperature 28.0±0.2°C. Fish were fed twice a day with flake food (Rotifish Premium Food, USA) consisting of a mixture of artemia and spirulina (50:50%). Ten-month-old adult fish from a fish colony were pooled in a spawning system (iSpawn, Tecniplast, Italy) and all eggs were collected 3 h after spawning. Normally developing embryos were selected under the stereo microscope, cultured in fish broth and incubated at 28.5±0.1°C [21]. 96-h embryonic toxicity tests were also conducted using 'fish water' whose conductivity was increased to 450–800 µS/cm by adding 60 mg/L sea salt (Instant Ocean®, Aquarium Systems, France) to reverse osmosis water[21].

Xenopus adult care, reproduction, and embryo collection were performed as described in ASTM-E1439-98 [22]. *X. laevis* embryos were obtained from the colonies where mature male and female individuals in the *X. laevis* unit of Inönü University. During the studies, the temperature and photoperiod of the laboratory were kept at 23±1°C and 12:12 h day-night cycles, respectively. The FETAX media (pH 7.87) contained 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4.2H_2O$ and 75 mg $MgSO_4$ per litre and was prepared using RO water for all studies [22]. Fertilised eggs were obtained according to the standard procedure [23]. Normally developing stage-8 embryos were used for all FETAX tests. The developmental stages of the embryos and their suitability for the test were checked under a stereo microscope. Stage 46 tadpoles were used for biochemical studies.

2.3. Toxicity tests

The species-specific media mentioned above were used for each species to determine the toxic effects of NPs on organisms. The concentrated NP solutions were kept in an ultrasonic bath for 20 min until dispersed well before tests. The different concentrations of the test materials were freshly prepared for the studies. The concentrations selected for the studies were determined by considering the preliminary study results and possible higher environmental exposure concentrations. For single NP toxicity studies, NP concentrations were determined based on the preliminary tests. The NPs were prepared in the range of 1.25–50 mg/L for ZnO NPs. It was ranged 25–250 mg/L for Fe_2O_3 or TiO_2 NPs.

It is important to evaluate the toxic effects in individuals exposed to the effect of substances until this period since embryonic development is largely completed in the first 96-h in both zebrafish and *X. laevis* and the circulation and digestive systems are developed [22, 24]. For the NP mixture tests, the lowest selected concentration of each NP (25 mg/L for Fe_2O_3 and TiO_2 NPs, 1.25 mg/L for ZnO NP) was used. The lethality was recorded daily and body length and abnormalities were determined via a stereo microscope with a camera system using Euromex Image Focus 4.0 software.

The zebrafish embryo toxicity assay was performed using 96-well microplates. The toxicity test was carried out according to previously described protocols [25, 26]. Fish water was used in control groups, at which point the pH of the NP concentrations was determined in a range of 7.16-8.06 (see Table 1). Tests were performed at 28±1°C in a 14:10 h day–night photoperiod in an incubator. At the beginning of the experiment, it was determined that all embryos developed normally at 6–8 h post-fertilization (hpf) [24]. The test setup was arranged to be 32 wells for each concentration and one embryo was placed with 0.250 mL of the medium per well. Embryos in the control group were only retained in the fish water. Embryos exposed to NPs were checked at 24, 48, 72, and 96 h. At the end of the exposure assays, surviving embryos were euthanized with 200 mg/L of MS222 and were fixed in 3% formalin. The types of malformations followed were selected as spinal curvature, eye, caudal fin, pectoral fin, pigmentation, pericardial edema (PE), and yolk sac edema (YSE) in *D. rerio* (Figure 1).

The FETAX assay was performed using 24-well microplates. The pH, conductivity, and temperature of the FETAX solution used in the study were determined in a range of 8.20 ±0.12, 1523±3µS/cm, and 21.4±0.1°C, respectively. Tests were performed in a 12:12 h day–night cycle photoperiod. *Xenopus* embryonic development tests were conducted by placing four randomly selected embryos in each well containing 2 mL of the test medium [27, 28]. All concentrations were tested with eight replicates. Embryos in the control group were only retained in the FETAX solution. Embryos were checked at 24, 48, 72, and 96 h. At the termination of the bioassays, surviving embryos were euthanized with 200 mg/L of tricaine methanesulfonate (MS222) and fixed in 3% formalin for

Table 1. The pH, Hydrodinamic diameter and zeta potential values of NPs in different solution.

			FETAX solution*			Fish water*		
	NPs	Conc. (mg/L)	pH	Hydrodinamic diameter (nm)	Average Zeta potential (mV)	pH	Hydrodinamic diameter (nm)	Average Zeta potential (mV)
Single	ZnO	1.25	8.08	162 ± 18.2	−6.81 ± 2.22	8.06	5023 ± 597.6	−8.31 ± 2.16
		50	8.43	5560 ± 32.2	−9.29 ± 2.47	7.90	759 ± 82.3	−4.89 ± 2.28
	Fe_2O_3	25	8.40	436 ± 30.1	−9.13 ± 1.68	7.16	5206 ± 471.3	−6.82 ± 3.49
		250	8.38	600 ± 68.9	−3.94 ± 1.71	7.38	2136 ± 78.0	−8.28 ± 1.86
	TiO_2	25	7.76	734 ± 66.2	−17.9 ± 3.19	7.34	2324 ± 250.7	−8.05 ± 1.92
		250	8.03	932 ± 81.8	−15.8 ± 1.63	7.59	534 ± 15.8	−3.59 ± 1.54
Mixture	ZnO	1.25	8.12	3411 ± 166.7	−7.90 ± 1.94	7.45	822 ± 84.9	−18.8 ± 2.37
	Fe_2O_3	25		2875 ± 72.2	−8.58 ± 1.37		484 ± 34.5	−14.7 ± 2.72
	ZnO	1.25	7.96	4317 ± 55.6	−14.1 ± 1.87	7.65	295 ± 3.8	−3.39 ± 0.98
	TiO_2	25		2335 ± 163.5	−16.9 ± 2.19		5366 ± 331.0	−11.1 ± 1.45
	Fe_2O_3	25	8.25	4323 ± 93.1	−7.74 ± 1.42	7.61	2477 ± 88.3	−10.2 ± 3.14
	TiO_2	25		5462 ± 31.0	−7.11 ± 2.25		5385 ± 319.5	−12.6 ± 1.86
	ZnO	1.25	7.92	4904 ± 74.8	−21.6 ± 2.10	7.65	620 ± 10.3	−9.67 ± 2.84
	Fe_2O_3	25		7469 ± 25.4	−23.1 ± 1.28		5364 ± 33.2	−13.2 ± 1.67
	TiO_2	25		7849 ± 16.8	−24.3 ± 2.94		5194 ± 47.8	−15.0 ± 3.12

*Particle distribution is monomodal in all single and mixed NP

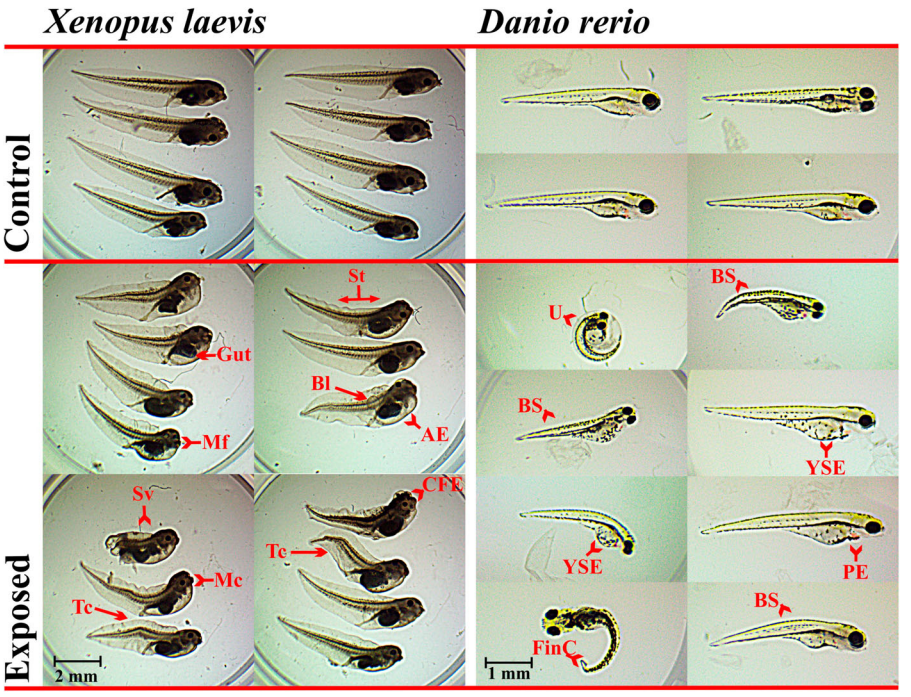

Figure 1. Determined malformations in *Xenopus laevis* and *Danio rerio* embryos exposed to commercial NP for 96 h. Malformation types: Sv: Severe (multiple, severe malformations), CFE: Craniofacial edema (face, head and eye edema), AE: Abdominal edema, TC: Tail curvature, Mc: Microcephaly, Mf: Microphthalmia, Bl: Blister, Gut, BS: Bent spine, PE: pericardial edema, YSE: yolk sac edema. U: unhatched.

observation of anomalies. Malformed embryos and malformation types were determined using a dissection microscope, as suggested by Bantle [29]. In *X. laevis*, malformations were determined as severe, stunted, gut (improper gut coiling, long loosely coiled gut), abdominal edema, craniofacial edema (face, head, and eye edema), microcephaly, microphthalmia, blisters, and curvature of tail (Figure 1).

2.4. Biochemical studies

Using the species-specific test solutions in a continuously ventilated container, the healthy 96-h-old *D. rerio* larvae and stage 46 tadpoles of *X. laevis* were randomly selected and exposed to various concentrations of the NPs for biochemical assays, in separate tests. In this application, embryos were placed in 10 mL test solutions in polycarbonate vessels under static test conditions. Concentrations of 25, 50,125, and 250 mg/L for TiO_2 and Fe_2O_3 NPs and 1.25, 2.5, 5, 12.5, 25 and 50 mg/L for ZnO NPs were tested. For each group, the experiments were set up in 5 different vessels, each containing 20 zebrafish larvae or 10 tadpoles. The larvae/tadpoles exposed to the test solutions were checked at 24, 48, 72, and 96 h. At the end of the exposure period, surviving embryos were collected, euthanized by MS222, and placed in microcentrifuge tubes as soon as possible. Each vial was cooled on ice, and all samples were stored at –80°C until biochemical studies.

Selected lowest concentrations of each NP (25 mg/L for Fe_2O_3 and TiO_2 NPs; 1.25 mg/L for ZnO NPs) were used for NP mixture applications. The experiment was set up with 5 different vessels for each NP combination in double and triple mixtures and with 20 larvae or 10 tadpoles in each vessel. The exposed animals were checked for lethality at 24, 48, 72 and 96 h, and the deceased were removed from the test vessels. Survivors were euthanized with MS222 at the end of exposure and placed in microcentrifuge tubes as soon as possible. The tubes were stored at −80°C until enzyme assays. The samples for each concentration were also evaluated morphologically and their lengths were measured. These values are given as additional data (Figure S3).

The frozen samples of both frogs and fish larvae were thawed on ice and weighed separately. The samples were homogenised before biochemical analysis. Homogenisation was carried out by a hand homogeniser in an ice bath using 5 volumes of ice-cooled homogenisation buffer (0.1 M potassium phosphate buffer with pH 7.4, containing 0.15 M KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.05 mM dithiothreitol) per gram of tissue (w/v). The homogenate was transferred to clean tubes and centrifuged at 16.000 *x*g for 20 min at 4°C (Allegra 64R, Beckman). The supernatant was taken, and biochemical assays were done as soon as possible for the post-mitochondrial fraction. Total protein amount and other biochemical assays were determined using a microplate reader system (Versamax, Molecular Devices Corp).

In the study for GST, the method developed by Habig et al [30]. was used with some modifications. Accordingly, 10 µL of supernatant was added to the medium containing potassium phosphate buffer (0.1 M, 100 µL, pH 6.5), glutathione (2 mM, 100 µL GSH), and 1-chloro-2,4-dinitrobenzene (20 mM, 10 µL) for initiation of the reaction. Absorbance was recorded for 2 min. GSH was used as the cofactor in the reaction.

The GR activity was measured according to Stephensen et al [31]. The reaction solution contained150 µL of 0.1 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), 20 µL of 12 mM NADPH, and 20 µL of the sample. The reaction was initiated by the addition of 20 µL of 3.25 mM oxidised glutathione (GSSG). All solutions were prepared in 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA. The DTNB reduction was monitored at room temperature at 405 nm, and the activity of GR was calculated according to the absorbance values obtained (reaction product: $\varepsilon = 14{,}151\ M^{-1}\ cm^{-1}$ for the 2-nitro-5-thiobenzoate anion).

SOD activity was determined using a SOD Kit (Sigma Aldrich, 19160) according to the manufacturer's method. The percentage of inhibition by the xanthine oxidase and water-soluble tetrazolium dye (WST-1) substrate reaction was measured by the SOD enzyme. For this purpose, 10 µL of the supernatant was pipetted into wells containing 120 µL of the total reaction medium. The mixture was incubated at 37°C for 20 min. At the end of the period, the absorbance was read at 450 nm. Enzyme activity (U/mL) was also calculated using the inhibition curve with standards containing 0.001, 0.01, 0.05, 0.1, 1, 5, 10, 20, 50, 100, and 200 U/mL commercial SOD (Sigma, S2515).

The activity of CaE was determined using the spectrophotometric method described by Santhoshkumar and Shivanandappa [32] adapted to the microplate reader system. P-nitrophenyl acetate (PNPA) was used as the substrate for assaying the enzyme activity. Accordingly, 5 µL of the sample was pipetted into microplate wells, and 250 µL (0.05 M) of trizma buffer (pH 7.4) was added. After 3 min of incubation, the reaction was initiated by adding 5 µL of PNPA (concentration of 0.5 mM in the mixture) and the absorbance was recorded at 405 nm for 2 min.

Total protein amounts obtained from homogenates were determined using a microplate reader according to Bradford et al [33]. Test samples were used after dilution 1/5 and 5 µL of the diluted samples were pipetted into the wells. Finally, 250 µL of Bradford solution was added to the tubes according to the assay procedure to initiate the reaction at room temperature. The absorbance was detected at 595 nm due to colour change. The values were compared with the bovine serum albumin (BSA) standard curve to determine the total amount of protein and used to calculate specific enzyme activities.

2.5. Statistical analysis

The probit regression analysis method (EPA, ver. 1.5) was used to determine the mean lethal and effective concentrations (LC_{50} and EC_{50}), respectively. Malformations observed in embryos were used to calculate EC_{50} concentrations. Graphpad Prism (ver. 5, USA) was used for all other statistical analyses. Data were statistically analysed for normal distribution (Kolmogorov–Smirnov test) and equal variance (Bartlett test). Nonparametric data were analysed using the Kruskal–Wallis test followed by the Dunn's post-hoc test. Parametric data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test.

3. Results

According to the results of SEM imaging and particle size distribution of the tested commercial NPs, values of 21.4±0.3 nm for TiO_2, 40.4±6.6 nm for Fe_2O_3 and 70.0±8.2 nm for ZnO were obtained. Accordingly, while the measurement values determined for TiO_2 and Fe_2O_3 were in agreement with the death values given by the company, the particle sizes obtained for ZnO were measured to be approximately 70 nm and were found to be smaller than the 100 nm value given in the commercial preparation. The zeta potential values of each NP tested in fish and frog embryo-specific test media are presented in Table 1. According to the results obtained, while NPs formed environments with low pH values in pure water, they exhibited a relatively more stable state and formed more dispersed solutions. However, apart from TiO_2, the other two NPs also tended to precipitate more in neutral–basic pH conditions in both fish water and FETAX media. In addition, the dispersion of NPs in the test media did not cause a significant change in the pH of the media. This indicates that there is no NP-induced ion release.

The highest mortality rate for zebrafish embryos was determined in groups exposed to ZnO NPs. All embryos exposed to 12.5 mg/L and higher concentrations of ZnO NPs died at 96 hpf, while 3% mortality was observed in embryos exposed to 1.25 mg/L concentration (Table 2). Depending on ZnO exposure, PE and YSE type malformations were observed, but malformations were rarely found in embryos exposed to Fe_2O_3 and TiO_2NPs (Table 2, Figure S2). The two other tested metal oxide NPs did not cause significant lethality in zebrafish (concentrations up to 1000 mg/L have also been tested for preliminary studies for TiO_2 and Fe_2O_3). On the other hand, the highest 96 h lethality ratios for *X. laevis* have been determined as 25% for 25 and 50 mg/L of ZnO NP exposure, 16% for 25 mg/L of TiO_2 NP exposure, and 13% for 50 mg/L of Fe_2O_3NP exposure (Table 3). The LC50 and/or EC50 concentrations and 95% confidence intervals (CI) of the tested NPs for both test organisms were determined if the lethality levels were available for

Table 2. Time-dependent toxicity in Danio rerio embryos exposed to different concentrations of commercial NPs.

						Details about 96 h exposure period									
		Lethality (%)						Malformation Types							
Exposure	Cons. (mg/L)	24 h	48 h	72 h	96 h	Length (mm)	Malformed Ind. (%)[a]	BS	Eye	FinC	FinP	Otic	Pig	PE	YSE
Control	–	0	0	0	0	3.23 ± 0.04	0	–	–	–	–	–	–	–	–
ZnO	1.25	0	0	3	3	3.21 ± 0.05	23	–	–	–	–	1	–	7	7
	2.5	6	6	9	13	3.23 ± 0.04	46***	1	–	–	–	–	–	9	12
	5	9	9	9	31	2.44 ± 0.04***	41**	–	–	2	–	–	–	2	8
	12.5	0	19	41	100***	–	–	–	–	–	–	–	–	–	–
	25	3	69	88	100***	–	–	–	–	–	–	–	–	–	–
	50	0	66	97	100***	–	–	–	–	–	–	–	–	–	–
Fe_2O_3	25	0	3	3	9	3.11 ± 0.04	0	–	–	–	–	–	–	–	
	50	3	6	6	6	3.10 ± 0.05	3	–	–	–	–	–	–	–	1
	125	6	6	6	6	3.09 ± 0.07	3	–	–	–	–	–	–	–	1
	250	0	0	0	3	2.95 ± 0.05***	3	1	–	–	–	–	–	–	1
TiO_2	25	3	6	6	6	3.12 ± 0.03	3	1		1		–		1	1
	50	3	3	6	6	3.07 ± 0.05*	3	–	–	–	–	–	–	1	1
	125	0	6	6	6	3.12 ± 0.04*	0	–	–	–	–	–	–	–	–
	250	0	0	0	0	3.00 ± 0.05**	0	–	–	–	–	–	–	–	–
$ZnO+Fe_2O_3$	1.25+25	3	3	3	3	3.01 ± 0.06**	26**	–	–	2	–	–	–	6	7
$ZnO+TiO_2$	1.25+25	0	0	6	6	2.97 ± 0.04***	17	1	–	1	–	–	–	2	4
$Fe_2O_3+TiO_2$	25+25	3	3	3	6	3.29 ± 0.05	0	–	–	–	–	–	–	–	–
$ZnO+Fe_2O_3+TiO_2$	1.25+25+25	13	16	16	16*	2.89 ± 0.06***	4	–	–	–	–	–	–	–	1

n: 32 individuals were usedfor each concentration.

[a] malformed individuals in living embryos

Malformation Types: BS: Bent spine, Eye, FinC: Caudal fin malformation, FinP: Pectoral fin malformation,Otic, Pig: Pigmentation, PE: Pericardial edema, YSE: Yolk sac edema

*, **, ***: shows statistical significance compared to control ($p<0.05$)

calculation. Accordingly, LC50 concentrations could be calculated for *D. rerio* embryos exposed to ZnO NPs at 48, 72 and 96 h. These values were calculated as 23.9 mg/L (95% CI: 18.0–34.3), 11.6 mg/L (95% CI: 9.28–14.6), and 5.19 mg/L (95% CI: 4.36–6.24), respectively. The other NPs tested did not cause significant lethality, even at the highest exposure concentrations in *D. rerio*. Therefore, LC_{50} concentrations could not be calculated for the TiO_2 and Fe_2O_3 NPs for zebrafish embryos. On the other hand, for *X. laevis* embryos LC_{50} concentrations of any NP could not be determined since the highest mortality level caused by the applied NP concentrations remained at 25%. However, the EC_{50} of ZnO NPs for *X. laevis* was determined at 96 h from the number of malformed individuals as 10.5 mg/L (95% CI was from 7.98–13.0).

In addition, although the triple NP mixture partially increased the lethal effect, a high rate of malformation was not observed in the zebrafish embryos. On the other hand, high malformation rates were determined in embryos exposed to binary mixtures containing ZnO NPs (26% and 17% with Fe_2O_3 and TiO_2 NPs, respectively; see Table 2). The NP mixtures and the highest concentrations of single NP applications caused growth inhibition, but no growth retardation was observed in those exposed only to Fe_2O_3/TiO_2NP mixtures in zebrafish.

When the malformation ratios of *X. laevis* embryos exposed to NPs for 96 h were examined, Fe_2O_3 and TiO_2 NPs did not cause a high malformation rate (Table 3, Figure S4), whereas ZnO NP exposure increased the number of malformed embryos depending on the exposed concentration. The highest exposure concentration of ZnO NPs (50 mg/L, Table 3) caused single or multiple malformations in all individuals. In addition, growth retardation of 3–21% was detected in *X. laevis* embryos exposed to ZnONPs (Table 3), while the other NPs did not cause growth inhibition. On the other hand, the TiO_2NP (25 mg/L) and ZnO NP (1.25 mg/L) mixtures caused statistically significant growth inhibition (5%) compared to the control group ($p<0.05$). However, other mixtures did not cause growth retardation. The types of malformations that are common in this study are given in Figure S4. Accordingly, the most common malformation types in *X. laevis* embryos exposed to ZnO NPs were identified as tail curvature and gut (Table 3).

To evaluate the sublethal effects of NPs, selected biochemical markers (GST, GR, SOD, CaE) were studied in the test organisms. The results of the biochemical assays are given in Table 4. The biochemical markers mentioned here were assayed in both *D. rerio* and *X. laevis* embryos to determine the effects of the NPs individually and as mixtures. According to the results, both the single concentrations of TiO_2 and Fe_2O_3 NPs as well as the mixtures other than ZnO and TiO_2 NPs binary mixture caused significant GR inhibition. While no single NP exposure at any concentration caused a significant change in SOD activity, all NP mixtures inhibited SOD activity in zebrafish ($p<0.05$).

The highest concentration (250 mg/L) of Fe_2O_3NPs caused a significant increase in the activity of GST ($p<0.05$) in tadpoles. Also, the highest exposure concentration (250 mg/L) of TiO_2NP caused to significant inhibition of SOD and CaE activities ($p<0.05$). On the other hand, in particular, the highest concentration of ZnO NP exposure (50 mg/L) resulted in an increase in GST and GR activities and a significant inhibition in SOD activity ($p<0.05$) in tadpoles. While single concentrations of Fe_2O_3NPs (25 mg/L) and TiO_2NPs (25 mg/L) did not cause a significant change in GST activity and single ZnO NPs (1.25 mg/L) induced GST activity, binary mixtures of ZnO NPs with Fe_2O_3 and TiO_2 NPs caused GST inhibition. All NP mixtures resulted in significant inhibition of CaE activity compared to the control

Table 3. Time-dependent toxicity in Xenopus laevis embryos exposed to different concentrations of commercial NPs.

						Detail about 96 h exposure period									
		Lethality (%)						Malformation Types							
Exposure	Cons. (mg/L)	24 h	48 h	72 h	96 h	Length (mm)	Malformed Ind. (%)[a]	Sv	St	Gut	AE	CFE	Tc	Mc	Mf	Bl
Control	–	0	0	0	0	8.07 ± 0.09	3	–	–	–	–	–	1	–	–	–
ZnO	1.25	3	3	9	9	7.84 ± 0.15	3	–	1	–	–	–	1	–	–	–
	2.5	0	3	3	3	7.80 ± 0.10	10	1	2	–	2	1	1		1	–
	5	3	3	3	3	7.35 ± 0.09***	16	1	1	1	–	1	5	–	1	–
	12.5	6	9	9	9	7.07 ± 0.10***	59***	1	1	14	7	8	12	–	1	2
	25	9	16	25	25**	6.60 ± 0.11***	96***	2	6	19	12	10	22	1	1	4
	50	6	9	16	25**	6.41 ± 0.09***	100***	2	4	21	15	12	23	1	1	3
Fe_2O_3	25	0	0	0	0	7.94 ± 0.07	6	1	1	–	1	–	1	1	1	–
	50	6	9	13	13	8.16 ± 0.08	4	1	1	–	–	–	1	1	1	–
	125	3	9	9	9	7.88 ± 0.11	14	1	1	–	1	3	2	–	–	–
	250	0	0	3	3	7.99 ± 0.09	6	–	1	–	1	2	–	–	–	–
TiO_2	25	3	13	16	16	7.89 ± 0.08	11	3	3	–	1	1	1	–	–	–
	50	3	6	6	6	7.95 ± 0.11	7	1	–	1	1	2	1	1	1	–
	125	9	9	13	13	8.11 ± 0.10	7	–	1	–	1	2	–	–	–	–
	250	9	9	9	9	8.10 ± 0.07	3	–	–	–	–	1	–	–	–	–
$ZnO+Fe_2O_3$	1.25+25	6	6	13	13	8.01 ± 0.09	11	1	2	–	1	–	2	1	1	–
$ZnO+TiO_2$	1.25+25	3	13	16	16	7.70 ± 0.14	11	1	2	2	2	2	2	–	1	–
$Fe_2O_3+TiO_2$	25+25	3	3	6	6	8.00 ± 0.12	10	1	1	–	1	2	1	–	–	–
$ZnO+Fe_2O_3+TiO_2$	1.25+25+25	6	9	16	16	8.10 ± 0.10	4	1	1	–	1	1	1	–	–	–

n: 32 individuals were used for each concentration.
[a]malformed individuals in living tadpoles
Malformation Types: Sv: Severe, St: Stunted, Gut, AE: Abdominal edema, CFE: Craniofacial edema (face, head and eye edema), Ms: Microcephaly, Mf: Microphthalmia, Bl:Blisters, Tc: Tail curvature
*, **, ***: shows statistical significance compared to control ($p<0.05$)

Table 4. Selected biochemical marker for X. laevis tadpoles and D. rerio embryos after 96 h exposure to different concentrations of NPs.

	NP	Conc. (mg/L)	*N*	GST[a]	GR[a]	SOD[b]	CaE[a]
D. rerio	Control	–	6	51.0 ± 2.7	10.08 ± 0.18	1.08 ± 0.03	1121 ± 109
	ZnO	1.25	6	46.8 ± 4.1	9.08 ± 1.22	0.99 ± 0.05	1090 ± 55
		50	6	52.6 ± 2.4	10.18 ± 0.34	1.05 ± 0.12	1096 ± 38
	Fe_2O_3	25	6	54.8 ± 2.8	7.99 ± 0.82*	1.18 ± 0.07	1358 ± 60
		250	6	49.3 ± 3.3	7.74 ± 0.56*	1.01 ± 0.08	1230 ± 79
	TiO_2	25	6	63.0 ± 4.5	8.80 ± 0.48	0.97 ± 0.05**	1279 ± 40
		250	6	52.5 ± 4.8	8.26 ± 0.60*	0.96 ± 0.14*	1093 ± 72
	ZnO+Fe_2O_3	1.25+25	6	61.7 ± 4.3	8.76 ± 0.40	0.79 ± 0.03*	1443 ± 78
	ZnO+TiO_2	1.25+25	6	55.4 ± 3.1	10.28 ± 0.68	0.86 ± 0.03	1325 ± 80
	Fe_2O_3+TiO_2	25+25	6	50.1 ± 2.3	6.94 ± 0.84*	0.80 ± 0.04*	1307 ± 51
	ZnO+Fe_2O_3+TiO_2	1.25+25+25	6	49.4 ± 1.9	8.21 ± 0.51	0.68 ± 0.01***	1224 ± 54
X. laevis	Control	–	5	207 ± 5.1	8.89 ± 0.36	4.89 ± 0.16	253 ± 7
	ZnO	1.25	5	239 ± 7.2	8.65 ± 0.34	5.03 ± 0.50	250 ± 7
		50	5	547 ± 32.1***	12.47 ± 0.63*	3.94 ± 0.30	218 ± 6*
	Fe_2O_3	25	5	205 ± 15.6	8.91 ± 0.59	4.76 ± 0.66	228 ± 13
		250	5	263 ± 9.9*	10.21 ± 0.46	4.22 ± 0.31	241 ± 12
	TiO_2	25	5	225 ± 10.7	8.88 ± 0.46	4.36 ± 0.32	240 ± 9
		250	5	211 ± 9.9	9.09 ± 0.32	4.04 ± 0.15*	226 ± 5*
	ZnO+Fe_2O_3	1.25+25	5	168 ± 7.4*	7.97 ± 0.44	6.20 ± 0.52	207 ± 10
	ZnO+TiO_2	1.25+25	5	186 ± 7.1	7.75 ± 0.48	4.47 ± 0.16	192 ± 4*
	Fe_2O_3+TiO_2	25+25	5	189 ± 6.8	7.76 ± 0.29	6.32 ± 0.40	197 ± 15*
	ZnO+Fe_2O_3+TiO_2	1.25+25+25	5	217 ± 15.3	8.21 ± 0.54	4.16 ± 0.49	198 ±16*

[a]: The activity of this enzyme is expressed as nmol/min/mg protein ± standard error.
[b]: The activity of this enzyme is expressed as U/mg protein± standard error.
*, **, ***: shows statistical significance compared to control ($p<0.05$)

($p<0.05$). Also, Fe_2O_3NPs containing binary mixtures increased the SOD activity ($p<0.05$) in *X. laevis* tadpoles ($p<0.05$).

No statistically significant difference was found when comparing the total body length of the tadpoles in the control groups and exposure groups ($p>0.05$) (for additional data, please see Supplementary Material and Figure S2).

4. Discussion

In this study, single and mixture (binary and ternary) effects of commercial TiO_2, Fe_2O_3 and ZnO NPs with particle sizes of 21 nm, 50 nm and 100 nm, respectively, were evaluated comparatively in *D. rerio* and *X. laevis* embryos. One of the debates in aquatic toxicology research is whether the indicator species used in laboratory tests are sufficiently effective and appropriate to evaluate the environmental effects of chemicals [34]. In aquatic toxicology studies, some researchers suggest that amphibians are less sensitive to the effects of environmental pollutants than fish [35]. However, another group argues that amphibian testing is necessary because amphibians are much more sensitive to certain contaminants due to their unique metamorphosis characteristics [36]. Despite some similarities, there are also biological differences between fish and amphibians that can affect their exposure and response to chemicals [37–39]. For this reason, in this study, it was aimed to evaluate the effects comparatively by selecting two different organisms commonly used in the laboratory. On the other hand, a comparative assessment of fish and frogs as two important living components of the aquatic ecosystem, for the NPs tested was not found in the literature. However, these three different NP pollutants may contaminate aquatic ecosystems at the same time. In this case, we can make more accurate judgments

about the ecotoxicological risks of the pollutant by comparatively evaluating the effects on different living groups in the environment. Furthermore, since fish and frog embryos may be exposed to the effects of NPs, which may be present in relatively lower concentrations in their natural habitats, the assessment of the effect of the relatively low concentration and mixtures will also facilitate our identification of possible ecological risks.

Although there are many studies in the literature on the toxic effects of each of the three metal oxides separately on aquatic organisms including bivalves, shrimps, fish and amphibians [18, 40–45], the number of studies evaluating various ternary NP mixing is quite limited, especially. In addition, existing studies have reported that the combined effect may vary according to the variety of NPs in the binary and ternary mixture and that the toxic effect that may be observed in NP mixtures may be synergistic or antagonistic [46]. For example, it has been claimed that the toxic effect may also be reduced due to the antagonistic effect that may occur if NPs are in a mixture [47]. Therefore, we think that it is important to understand the effects of NPs, which are widely used and whose environmental pollutant potential is likely to increase in the future depending on the amount of use, when they come together in an aquatic ecosystem.

In our study, although TiO_2 and Fe_2O_3 NPs caused very low mortality rates at all applied concentrations, they were considered toxic because they caused significant suppression of total body length in zebrafish embryos ($p<0.05$). On the other hand, when ZnO NPs are mixed with TiO_2 NPs, it is claimed that the toxicity potential of ZnO NPs is significantly reduced in the mixture [48, 49]. In contrast, relatively high exposure concentrations of ZnO NPs (12.5-50 mg/L) caused the death of all embryos at the end of 96 h, while the exposure concentration of 5 mg/L caused a significant lethal effect (31%) as well as a marginal reduction in total body length. The high mortality rates in zebrafish embryos at concentrations of 12.5 mg/L and above within 96 h, compared to the lethality rates observed in *X. laevis* embryos due to ZnO NP exposure, can be attributed to the less protective effect of the chorion protecting the embryos. However, no significant change was detected at concentrations of 1.25 and 2.5 mg/L compared to the control, which may indicate that the toxic effect of ZnO NP alone at low concentrations in aquatic environment may be relatively less. The total body length of frog embryos tested in FETAX medium was significantly ($p<0.05$) reduced by exposure to 5-50 mg/L ZnO NPs, whereas TiO_2 and Fe_2O_3 NPs had no toxic effect in this regard. Interestingly, NPs, except the $TiO2/Fe_2O_3$ mixture, showed suppressive toxicity on total body length for *D. rerio* embryos even when a mixture was formed at low concentrations ($p<0.05$), while no mixture showed such toxicity in *X. laevis*. This may be a consequence of the toxicity observed in *D. rerio* embryos being more sensitive to the synergistic effect resulting from the NP mixture. Furthermore, in our study, toxicity was more pronounced in the presence of low exposure concentrations of Zn and/or iron oxide in the tested NP combinations, as can be seen especially in the biochemical markers discussed below. However, TiO_2 NP combinations did not cause such effects. This may suggest that TiO2 in combination with other NPs may play an antagonist role against toxic effects.

Different factors may affect NP toxicity together, and this may become even more complex, especially with NP mixtures. Depending on their physical states, metal NPs can change their properties due to interaction with ambient pH, proteins, lipids, and genetic materials [50]. This may cause unexpected differences in toxic effects in mixtures of different metal oxides. One of the factors determining NP toxicity is particle size, and,

theoretically it is claimed that those with smaller particle sizes may be more toxic due to their association with the cell [51]. However, although the ZnO NPs used in our study had the largest particle sizes (100 nm) and the TiO_2 NPs had the smallest (21 nm) particle sizes, no size-related trend was observed in terms of toxicity. The pH, hydrodynamic diameter and zeta potential results of commercial ZnO, Fe_2O_3 and TiO_2 nanoparticles used in tests performed in FETAX medium and fish water are given in Table 1. The results show that the test media containing NP have a pH value that embryos can tolerate, with the pH being 8.13±0.08 in FETAX medium and 7.58±0.08 in fish water medium. This also showed that in the presence of NP mixtures, the NPs had no effect on the pH of the medium and no ion release occurred as expected. On the other hand, the hydrodynamic diameter and zeta potential values were changed depending on the medium in which the NPs were dispersed, their concentration and/or the mixtures of NPs in the medium (FETAX medium or fish water). As a matter of fact, the FETAX medium used for the toxicity tests carried out in *X. laevis* embryos in this study is a solution with a high ion load consisting of many different salt mixtures. Therefore, the behaviour of the tested NPs in test environment may have been affected by both the ion charges of the NPs in the environment and the ion charge of the FETAX medium used. The characterisation results for the NPs we tested showed that the NPs exhibited a more stable structure as a triple mixture in the FETAX environment. On the other hand, since all NPs in the mixtures were found to be negatively charged, this also showed that different metal oxide NPs acted independently of each other without forming any bond. According to our results, this may have led to an increase in the hydrodynamic diameter, as the ZnO NPs in the medium are the largest NPs in the mixture and ions in the medium may stratify and cause deposition on the NP surface. Also, the relatively low electronegative change may have led to increased aggregation. However, the fish water we used for *D. rerio* embryonic tests is a medium containing only 60 µg/ml instant sea salt, and the ion load in this solution is much lower than in the FETAX medium. It was determined that the hydrodynamic diameter for all three metal oxide NPs in fish water media increased approximately ten times in parallel with the increase in concentration. On the other hand, the zeta potential values showed a more stable state in fish water medium. The metal oxide NPs exhibited hydrodynamic diameters when present in their mixtures in both binary and ternary combinations, similar to the results when a single variety of NP is present in the fish water. In addition, it was determined that the hydrodynamic diameters of the particles did not increase significantly due to the ion charges in the environment. Zeta potential values also showed that NPs are found without bonding to each other, even in double and triple mixtures in fish water. For this reason, we can assume that the hydrodynamic diameters of NPs, when they are singular or in a mixture, can be bigger in environments with high and variable ion load, especially like in FETAX environment, than in environments with low ionic load [52].

In order to determine the individual effects of NPs on selected test organisms, applications were made both at concentrations where they could be exposed as close as possible to the environmental concentrations recommended by the OECD [26], and at higher concentrations. In previous studies evaluating the toxicity of a single NP, Azevedo et al. [42] and Zhu et al. [53] reported the 96 h LC50 value of ZnO NPs for *D. rerio* embryos as 27.21 and 1.79 mg/L, respectively. On the other hand, for zebrafish embryos, the 96-hour LC_{50} due to Fe_2O_3 toxicity has been reported as 60.17 mg/L [54]. Because of the NP

concentrations exposed in our study, LC_{50} concentrations could be calculated for ZnO based on mortality recorded in zebrafish embryos, whereas a LC_{50} concentration could not be calculated for TiO_2 and Fe_2O_3 due to the very low mortality observed. Even after exposure of zebrafish embryos to a Fe_2O_3concentration of approximately five times the LC_{50} concentration reported in the literature, the lower mortality detected in our studies may be due to the difference in initial exposure time (application at 8 hpf instead of 1 hpf). All embryos exposed to ZnO NP concentrations of 12.5 mg/L and above died at 96 hpf in our tests (Table 2). Accordingly, LC_{50} concentrations of ZnO NPs at 48, 72, and 96 h for *D. rerio* embryos were calculated respectively as 23.9 mg/L (95% GI: 18.0–34.3%), 11.6 mg/L (95 GI: 9.28–14.6) and 5.19 mg/L (95% GI: 4.36–6.24). It is seen that the 96 h EC_{50} value (10.5 mg/L) determined for *X. laevis* in our study is compatible with the values given for ZnO NPs (10.3 mg/L) by Nations [43]. On the other hand, the highest 96 h lethality rates for *X. laevis* were 25% for the 25 and 50 mg/L ZnO NP exposures; The highest mortality rates were 16% for 25 mg/L TiO_2 NP exposure and 13% for 50 mg/L Fe_2O_3 NP exposure (Table 3). Due to the low mortality rates in our tests, the LC_{50} concentration in *X. laevis* could not be calculated for the NPs tested. These results may also be related to the increase in particle sizes and hydrodynamic diameters due to the number of ions and ion diversity in the environment, which are important factors affecting lethality.

On the other hand, Azevedo et al. [42] reported tail malformations as the most common type of malformation in zebrafish embryos exposed to ZnO NPs. In our study, it was determined that ZnO NPs caused significant increases in lethality and developmental malformations (mostly edema) in *D. rerio*. Edema caused by ZnO NP exposure in zebrafish may have resulted from the increase in hydrostatic pressure that may occur due to heart or kidney failure [55]. It may also be thought to be related to changes in osmoregulation due to the disruption of cell membrane lipid layers [56]. The calculated minimum concentration to inhibit growth (MCIG) with ZnO NPs was 5 mg/L for *D. rerio* embryos. However, the relatively high rate of malformations (23%) observed in zebrafish embryos with low ZnO NP concentration (1.25 mg/L) exposure compared to the control group revealed that ZnO NPs have a high risk of toxicity even at low concentrations. These results may also make it easier for us to estimate the ecotoxicological risk to which fish may exposed in their natural environment. While PE and YSE type malformations were observed at higher rates in zebrafish embryos due to ZnO exposure, ratio of the malformed embryos increased in parallel with the increase in concentration. But, malformations were observed rarely in *D. rerio* embryos exposed to Fe_2O_3 and TiO_2 NPs (Table 2, Figure S2). On the other hand, since fish embryos exposed to concentrations of 12.5 mg/L and higher all died during the 96 h test period, malformation types could not be determined for these embryos. Although ZnO exposure did not show high lethality even at the highest concentrations exposed in *X. laevis*, the increase in concentrations caused a high rate of malformations (up to 100%) in embryos. Nations [43] reported that the NPs we used in our study inhibited the development of embryos in *X. laevis* at high concentrations (1000 mg/L for TiO_2 and Fe_2O_3NPs: >10 mg/L for ZnO NPs). However, our results showed that ZnO exposure at concentrations of 5 mg/L and higher also significantly decreased embryonal development (up to 21%) in *X. laevis* compared to control samples ($p<0.05$). Therefore, we can assume that the development of *X. laevis* embryos for ZnO was suppressed when exposed to lower concentrations than those

reported in the literature. It was also noteworthy that multiple types of malformations were common in *X. laevis* embryos exposed to ZnO. The most common types of malformations in *X. laevis* embryos exposed to ZnO NPs were identified as tail curvature and gut (different types of intestinal malformations) (Table 3; Figure S4). When we evaluated the lethality and malformation results together, the most toxic effect for fish and frog embryos exposed to the singly effect of NPs was with ZnO, as expected. On the other hand, although TiO_2 and Fe_2O_3 showed similar toxic effects, TiO_2 NPs were observed to be slightly more toxic, especially for *X. laevis* embryos compared to *D. rerio* embryos. When these results are evaluated together, it can be stated that the tested NPs exhibit significant ecological risks for both *D. rerio* embryos and *X. laevis* embryonal development, depending on the pollution load that may occur in their living environments. In addition, it is conceivable that long-term exposure may also cause a delayed toxic effect in organisms due to the presence of sublethal concentrations of NPs in the medium.

Furthermore, another factor affecting NP toxicity is the reactive oxygen species (ROS) that occur in the environment in relation to the NP properties and the ionic charges of the environments where the NPs are present [57]. Oxidative stress triggered by ROS may be due to many factors such as disruption of cellular membranes, intestinal lesions, lipid peroxidation, activation/inhibition of metabolic enzymes, DNA/RNA damage of developing embryos, destruction of embryonic protective sheaths such as chorion [58]. Although the ZnO NPs used in our study was much larger (100 nm) than the other two metal oxides in terms of particle size, they showed the highest toxic effect for both aquatic organisms. Although previous studies have shown that ZnO ions in the aquatic environment may have an important role in ZnO NP toxicity, it is also known that nZnO particles are more toxic than Zn^{+2} ions. Therefore, the toxic effect may also be due to a more complex sequence of events [59]. It is suggested that ZnO NPs may easily be taken up by aquatic organisms, accumulate, and may cause toxic effects [44]. However, due to the larger particle size, the toxicity of ZnO was not only dependent on particle size and physicochemical properties, but shape and exposure environment may also be determinants of the toxicity. When the literature is examined, it is seen that the individual effects of the tested NPs are evaluated at different concentrations and sizes, albeit with different biomarkers. It has been claimed that zebrafish embryos exposed to iron oxide NPs at concentrations higher than 10 mg/L trigger death, delay in hatching, and various malformations [41]. It has also been reported that OH radicals produced by TiO_2 cause oxidative damage in zebrafish embryos, and related toxic effects may occur [40]. While fishwater, which is the development medium of fish embryos, contains 60 µg/mL NaCl, FETAX solution is a medium consisting of a mixture of many different salts [21, 60]. It is thought that this observed difference may be related to the different environments in which fish and tadpoles live during development. As a matter of fact, it was determined that ZnO NPs dispersed in two different environments were more stable compared to the other two metal oxide NPs (see Table 1). This stability may be a cause of the increased ZnO NP toxicity. Nevertheless, we can say that the possible environmental concentrations of ZnO NPs are at a level that may pose a risk for both species, in line with the findings we obtained as a result of the study. On the other hand, it was found that the toxic effect capacity of TiO_2 and Fe_2O_3NPs was relatively low compared with ZnO NPs. But, although TiO_2 NP toxicity is

higher compared to Fe_2O_3, TiO_2 has a reducing effect on the toxicity caused by ZnO or Fe_2O_3 when present in the tested mixtures. It is thought that this may be the result of an antagonistic effect of TiO_2 NPs in the mixtures.

Table 4 shows the biochemical responses of fish and frog embryos to single metal oxide exposures and mixture combinations. Contrary to our expectations, a more limited change in enzyme activities was observed in zebrafish and *X. laevis* embryos exposed to NPs in our study. Although the selected biomarkers were considered important indicators of oxidative stress, they induced a limited response depending on the concentrations tested. In contrast, developmental malformations and suppression of growth in development revealed more pronounced effects. The decreases in GR activity with exposure to Fe_2O_3 at both concentrations in fish embryos and the suppression of SOD activity by TiO_2 exposure at both concentrations suggest that the effect in the mixtures is due to TiO_2 and Fe_2O_3. Furthermore, the increase in GST activity observed in *X. laevis* due to higher ZnO and Fe_2O_3 exposures can be considered an increase in concentration-dependent toxicity for *X. laevis* embryos. The suppression observed in CaE activity as a result of exposure of *X. laevis* embryos to NP mixtures may be related to the suppression of both metabolic toxicity and detoxification. On the other hand, it may also be possible that organisms have evolved an evolutionary tolerance to Zn ions, since Zn ions act as a cofactor for many enzymes, such as SOD.

Consequently, the fact that metal oxide NPs are more toxic when mixed in the aquatic environment than alone may be an issue with aggregation. An example of this is that when Cu and Zn oxide nanoparticles are combined, they have greater particle aggregation in mixtures, ultimately leading to increased toxicity [61]. In addition, since different vertebrate species can coexist in an aquatic ecosystem, evaluating the effects of the same substance on different groups is particularly important in determining the ecotoxicological risk. Therefore, it is thought that our study may contribute to the evaluation of the effects of the mixture of NPs on fish and frog embryos in the aquatic environment. However, the results of the study suggest that the behaviour and interactions of NPs vary in different test environments and that these effects may be due to other mechanisms besides the biochemical markers tested. More extensive studies are needed to determine this.

5. Conclusions

According to the results of our study, when the growth retardation and mortality levels of fish and frog embryos exposed to the same NP concentrations in single and mixed forms are compared, it is seen that fish embryos are more sensitive to NP toxicity. On the other hand, when the biomarker responses for both species are compared, it is noteworthy that the responses of the species are mostly different. For the NPs tested, a ranking from highest to lowest toxicity is the following: ZnO NPs > TiO_2NPs > Fe_2O_3NPs. Considering that NPs will be used more widely in the future, it is necessary to evaluate more comprehensive studies on the application of NPs to prevent environmentally adverse effects and to understand their mechanism of action.

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