

Evaluation on reducing toxicity of fluoxastrobin with doped TiO₂ nanoparticles

Abbas GÜNGÖRDÜ¹, Nesrin ÖZMEN², Sema ERDEMOĞLU³, Duygu ÖZHAN TURHAN¹,
Meltem ASILTURK⁴, Emrah AKGEYİK⁵, Murat ÖZMEN^{1*}

¹Environmental Toxicology Lab, Department of Biology, Faculty of Arts and Science, İnönü University, Malatya, Turkey

²Department of Mathematics and Science Education, Faculty of Education, İnönü University, Malatya, Turkey

³Department of Chemistry, Faculty of Arts and Science, İnönü University, Malatya, Turkey

⁴Department of Materials Science and Engineering, Faculty of Engineering, Akdeniz University, Antalya, Turkey

⁵Science and Technology Application and Research Center, Yozgat Bozok University, Yozgat, Turkey

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Abstract: In this study, toxic effects caused by the degradation of fluoxastrobin, which is a commonly used fungicide where newly synthesized manganese or sulfur-doped TiO₂ nanoparticles exist were evaluated. The characterization study of nanoparticles was performed by scanning an electron microscopy (SEM), X-ray diffractometry, Brunau-Emmet-Teller analysis, X-ray fluorescence spectroscopy, and UV-Vis (ultraviolet-visible) reflectance spectra. Subsequently, the photocatalytic performance of nanoparticles, their toxicity, and the photocatalytic degradation products of fluoxastrobin with the same nanoparticles were tested during the two development stages of *Xenopus laevis*. The LC₅₀s of fluoxastrobin were determined on test organisms, and a 5 mg L⁻¹ fluoxastrobin was selected to evaluate the photocatalytic degradation capacity due to toxicity studies. The sublethal effects of the nanoparticles and the degradation product of fluoxastrobin were assessed with embryonic malformations and biochemical marker responses. Sulfur-doped TiO₂ was found to be more effective compared to manganese-doped TiO₂ for the degradation of fluoxastrobin, photocatalytically. On the other hand, even if the tested nanoparticles were not lethal, they caused effects such as growth retardation and changes in biochemical responses on organisms.

Key words: Doped nanoparticles, fluoxastrobin, photodegradation, toxicity

1. Introduction

Strobilurin components are the fungicides commonly used in various agricultural orchards in recent years. One of the newest strobilurin class dihydro-dioxazine compound is fluoxastrobin (FLX), (E)-{2-[6-(2-chlorophenoxy)-5-fluoropyrimidin-4-yloxy]phenyl} (5,6-dihydro-1,4,2-dioxazin-3-yl) methanone O-methyloxime, which is also effective by inhibiting mitochondrial respiration on fungi such as other strobilurin compounds (EFSA, 2012; Zhu et al., 2015). The commercial formula of FLX is also known as HEC5725. The active ingredients of the pesticide in commercial crystalline formulations range from 20%–50%. It is categorized as having lower toxicity in acute exposure on mammals, while it is moderately toxic to freshwater organisms, such as *Oncorhynchus mykiss* and *Daphnia*, but it is extremely toxic to aquatic crustaceans, such as *Americamysis bahia* (Konwick et al., 2006).¹ Therefore, after agricultural applications, FLX residues can

cause adverse effects on aquatic organisms, mainly due to the production of O₂ and HO· radicals (Uckun and Oz 2020).

Numerous studies have been carried out on the disposal of pesticides from an aquatic environment by biological or physicochemical methods (Kim et al., 2016; Lizano-Fallas et al., 2017). However, existing technologies cannot adequately eliminate toxic pollutants from water sources quickly. In recent years, metal nanoparticles (NPs), like Ag@Mg₄Ta₂O₉, ZnO, CdS, Fe₃O₄, and TiO₂ photocatalysts have been used for advanced oxidation processes of refractory organic compounds (Akbari et al., 2018; Alkayal and Hussein 2019; Amalraj and Pius 2015; Andreescu et al., 2012; Budarz et al., 2019; Zhang et al., 2012). Among these NPs, nanostructured TiO₂ has been used to decompose pollutants due to its highly efficient, chemically stable, and relatively inexpensive materials (El-Shafai et al., 2019; Haynes et al., 2017; Lu and Astruc 2018).

¹ New York State Department of Environmental Conservation Division of Solid & Hazardous Materials. In: http://pmep.cce.cornell.edu/profiles/fung-nemat/febuconazole-sulfur/fluoxastrobin/fluoxas_wth_0907.pdf

* Correspondence: murat.ozmen@inonu.edu.tr

The cause of interest in the use of TiO_2 for photocatalytic activity in organic pollutant degradation is the concern about its high oxidative potential, photostability, and low toxicity (Li and Li 2002). The doping of TiO_2 with metal or nonmetal materials has also been shown to increase its photocatalytic activity for degradation of different pollutants such as pesticides, antibiotics, dyes, and many industrial chemicals (Cruz et al., 2017; Giahi et al., 2019; Jin et al., 2019; McManamon et al., 2015; Piccinno et al., 2012).

In light of all this information, in the present study, we synthesized sulfur-doped TiO_2 (S- TiO_2) and manganese-doped TiO_2 (Mn- TiO_2) NPs, and photocatalytic activities of NPs were determined using the degradation of FLX as an example of commonly used persistent xenobiotic, because FLX is resistant to aerobic biodegradation and their degradation may take several months in aquatic ecosystem. It is reported that azoxystrobin and its metabolites may persist after 21 days in soil, and it affects soil microbiota (Adetutu et al., 2008). It is stable for abiotic hydrolysis in buffer solutions and is relatively stable in water environments. The photolysis in direct aqueous medium can reduce FLX in about four days. Therefore, this period is long enough to cause aquatic organisms to be exposed to toxic substances (USEPA, 2005). For this reason, rapid photodegradation is very important, and photocatalytic nano- TiO_2 particles may provide an advantage for this purpose.

Xenopus laevis are aquatic animals that allow for studies on embryo toxicity, depending on the availability of fertilized eggs in laboratory conditions (ASTM, 2003; Gardner et al. 2016). In this study, the frog embryo teratogenesis assay-*Xenopus* (FETAX) and tadpole toxicity tests have been used to test the photocatalytic capability of the synthesized TiO_2 NPs (ASTM 2003). In addition to determining the possible lethal and teratogenic effects of xenobiotics at the organism level, biochemical markers are important determinants of toxic effects. Studies in the literature have reported the toxicity of strobilurin fungicides including FLX on aquatic organisms (Cao et al. 2019; Zhang et al. 2020; Zhang et al., 2018). There are also studies on the photocatalytic degradation of strobilurin fungicides using nano or bulk metal oxides (Lagunas-Allue et al., 2012; Navarro et al., 2009). However, no previous work has reported the evaluation of the toxicity or photocatalytic degradation of FLX with S- TiO_2 or Mn- TiO_2 NPs. Therefore, the effects of NPs and the toxicity of degradation by-products of FLX were evaluated at the organism level using *X. laevis* stage 8 embryos and stage 46 tadpoles. For this aim, different biomarkers at the suborganism level were used for risk and toxicity assessments (van der Oost et al., 2003). Afterwards, the sub-lethal effects of the NPs and the degradation by-

products were tested with changes in selected biochemical markers on *X. laevis* tadpoles. Therefore, the efficacy and effects of the NPs, as well as the possible effects of the by-products of the pollutant on aquatic environment and health of humans, have been tried to be foreseen.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents for the synthesis of Mn- TiO_2 or S- TiO_2 NPs were purchased from Alfa Aesar (USA), Riedel de Haen, and Merck (Germany). The fluoxastrobin PESTANAL (99.6% HPLC grade) was purchased from Fluka (product no: 33797). Lactate dehydrogenase (LDH) and aminotransferase assay kits were purchased from BioLabo (France). The thiobarbituric acid substance (TBARS) kit provided by Cayman Chemical (10009055, USA). All other chemicals used for the synthesis procedures were analytical quality. The FETAX media was prepared with bi-distilled water according to ASTM standards (ASTM 2003).

2.2. Preparation of the catalysts

The Mn- TiO_2 NPs were previously synthesized by the sol-gel method and characterized as described by Ozmen et al. (2015). S- TiO_2 NPs were synthesized by reflux methods. Synthesized TiO_2 NPs were doped with 1% Mn or % 1 S. Initially, for the precursor solution, titanium (IV) isopropoxide [$\text{Ti}(\text{OPri})_4$] was dissolved in 2-propanol [$2\text{-PrOH}/\text{Ti}(\text{OPri})_4$ molar ratio, $n/n = 10$] at room temperature with stirring for 15 min (solution A). The B media which contained $\text{H}_2\text{O}/\text{Ti}(\text{OPri})_4$ (molar ratio, $n/n = 10$) and $\text{H}_2\text{SO}_4/\text{Ti}(\text{OPri})_4$ (molar ratio, $n/n = 0.01$), was mixed for 30 min at room temperature. Medium A was added dropwise to medium B and the mixture was transferred to reflux at 110 °C during 6 h so that the NPs could be synthesized. The obtained S- TiO_2 NPs were dried at 50 °C in a vacuum oven.

2.3. Characterization of the catalysts

S- TiO_2 was characterized using different techniques. X-ray diffractometry (XRD, Rigaku GeigerflexDMax/B Model) was used to determine the crystal composition and size of crystallites. The elemental contents and morphology of surface of S- TiO_2 were detected using scanning microscope (SEM, LEO-Evo 40), SEM with energy dispersive X-ray (SEM-EDX), and X-ray spectroscopy (XRF, PANalytical Axios). The specific surface area (S_{BET}) was determined using a BET analyzer (Asap 2000). The band-gap energies of synthesized NPs were determined using UV/Vis diffuse reflectance spectra (DRS, Carry UV-Vis-NIR 5000). The synthesized photocatalysts which was 0.1% by mass in 5 mL of distilled water (0.1 % wt v^{-1}) was homogeneously dispersed and then the distributions of the particle size and the hydrodynamic diameters were determined by

using a zetasizer (Nano-ZS model, Malvern Panalytical-DLS method).

2.4. Photocatalytic experiments

To validate the capacity of photocatalytic properties of the S-TiO₂ or Mn-TiO₂ NPs on FLX, studies were carried out in a solar box unit with a Xe lamp (Erichsen 1500, Germany). Stock FLX solution was prepared in distilled water to be 100 mg L⁻¹ as nominal concentration. Due to the problem of solubility of the FLX in the water environment, the solution was also sonified for 2 h in an ultrasonic bath and a dispersed solution was obtained. The prepared solution was used to prepare 5 mg L⁻¹ dilutions for photocatalytic studies. Test medium containing 5 mg L⁻¹ FLX (dissolved concentration was lower than the concentration dispersed in water) and photocatalysts prepared in FETAX solution were kept in the dark at 22 ± 1 °C for 60 min before irradiation in a sunbox. After irradiating the medium containing FLX for specified periods, the mixture was first centrifuged at 10,000 rpm for 20 min to remove as much of the NPs from the medium as possible. It was then passed through a 0.45 µm filter to remove any catalysts that might remain in the water (Ullah et al., 2015). The degradation rate of FLX in the solution during the degradation process was detected with a UV-Vis spectrophotometer (Varian Carry 100) and a TOC analyzer. The degradation efficiency was calculated from the following equation:

$\% = (C_0 - C_t) / C_0 \times 100$ equation, where C_0 represents the initial concentration and C_t is the residual concentration at time t . In addition, LC-MS/MS analysis (Agilent 6140 Triple Quadrupole with an ESI source in positive ion mode) of FLX before and after photocatalytic treatment was performed to determine whether degradation occurred. The analyses were performed with an ACE5C18 S/N-A20810 (250 × 4.6 mm, 5 µm) separation column, and the mobile phase consisted of 70:30 (v/v%) acetonitrile to water.

2.5. Toxicity studies

2.5.1. Test organisms

The toxicities of Mn-TiO₂ and S-TiO₂ and FLX were evaluated on frog embryos and tadpoles. Furthermore, the stage 46 tadpoles were used for biochemical studies after the 96th exposure period. After LC₅₀ values for each tested stage were calculated based on FLX exposure concentrations, it was considered appropriate to evaluate the effects of photocatalytic degradation of 5 mg L⁻¹ FLX. Embryos and tadpoles of frog were provided from our maintained populations under previously described laboratory conditions and the collection of fertilized eggs were executed as defined in the E1439 standard of the ASTM (ASTM, 2003; Birhanli and Ozmen, 2005). The eggs were immediately transferred to the FETAX media. For this aim, the developing normal embryos were

collected up to late blastula (stage 8–11), and those were used for the FETAX-study (Nieuwkoop and Faber, 1956). The stage 46 tadpoles were used for also toxicity tests and for biochemical assays. The laboratory temperature was 22 ± 1 °C, and 12:12 h light:dark photoperiods were used during the studies.

2.5.2. FETAX assay

The FETAX standard medium was used for preparing all exposure media. The pH, conductivity, and temperature of FETAX medium used in the study were determined to be 8.02 ± 0.09, 1.523 ± 0.003 µS/cm², and 21.4 ± 0.1 °C, respectively. The FETAX assay was performed using 24-well microplates. In this application, 2-mL suspensions of NPs or degradation by-products of FLX with NPs were pipetted into microplate wells after 20-min sonication to provide a good dispersion of NPs. For each test group, 8 wells were used and 4 embryos (stage 8–11) were added to each well. Thus, 32 embryos were used in total for each group. Embryos were only retained in the FETAX medium in the control group. Tests were performed at 22 ± 1 °C in a 12:12 h light:dark photoperiod. Embryos were inspected every 24 h for 96 h period. The surviving embryos were euthanized with MS222 (tricaine methanesulfonate-tricaine, 0.05%) and were fixed in 4% formalin at the end of the experiment for observation of developmental malformations. Malformation types of the embryos were determined using a dissecting microscope, and the total body length was measured using a software (Euromex ImageFocus 4.0).

2.5.3. Tadpole toxicity test

The tadpole (at stage 46) toxicity test was performed using 12-well microplates. In this application, 3 mL NP suspensions or degradation by-products of FLX with NPs, were pipetted in the microplate wells after 20-min sonication. For each group, 6 wells were used, and 5 tadpoles were placed in each well (each group contains 30 tadpoles in total). Tadpoles were only retained in the FETAX medium in the control group. Tests were performed at 22 ± 1 °C in a 12:12 h light:dark photoperiod. The tadpoles were inspected every 24 h during a 96-h exposure period, and dead organisms were removed from the plates. The surviving tadpoles were euthanized using MS222 (tricaine methanesulfonate-tricaine, 0.05%) at the end of the exposure period for further studies.

2.5.4. Biochemical assay

The healthy tadpoles (stage 46) were randomly selected as mentioned above and exposed to various concentrations of NPs or degradation by-products of FLX with NPs for determination of biochemical effects. In this application, 10-mL test solutions were transferred to 15-mL capacity polycarbonate cuvettes. For each test group, 5 replicates were used, and 15 tadpoles were added to each cuvette

(75 tadpoles for each concentration in total). Tadpoles were carefully checked every 24 h over 96 h, and dead tadpoles were removed from the cuvettes daily. The surviving tadpoles were collected in microcentrifuge tubes at the end of 96 h and slightly anaesthetized with MS222. Water was removed from the tubes and the residue of MS222 in the tubes was washed thoroughly with water. The samples were transferred to a -80°C ultrafreezer until the biochemical studies were performed. All biochemical assays were performed immediately after preparation of tadpole homogenates, and all selected biomarker assays were completed in the same day without any more freezing of the samples. For preparation of whole-body homogenates, frozen tadpoles were thawed on an icebox and weighed. A hand-type microtube homogenizer with microtube suitable pastels (Bel-Art, USA) was used for sample homogenization in an ice-bath for 10 s with ice-cold homogenization buffer (pH 7.4 in 0.1 M potassium phosphate buffer, 0.15 M KCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.05 mM DL-dithiothreitol (DTT)). For this aim, a 5 $\times$ homogenization buffer was used for the total weight (w/v) of the tadpoles. The post mitochondrial supernatants were collected after centrifugation at 16,000 $\times g$ for 20 min at 4°C (Allegra 64R, Beckman, USA) and the fraction was transferred to clean tubes, and biochemical assays were performed with these samples. The samples were kept on ice at all stages of the studies.

All the selected enzyme activities such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutathione S-transferase (GST), lactate dehydrogenase (LDH), glutathione reductase (GR), and carboxylesterase (CaE) were assayed (Güngördü 2013). The GST activity was determined using postmitochondrial homogenates according to a method specified by Habig et al. (1974). The activity of CaE was assayed using *p*-nitrophenyl acetate (PNPA) as a substrate (Santhoshkumar and Shivanandappa 1999). The GR activity was determined with a reaction mixture containing 0.63 mM NADPH, 0.08 mM DTNB, and 20 μL supernatant. 10 μL of 3.25 mM oxidized glutathione (GSSG) was added into mixture for initiation of the reaction (Stephensen et al., 2000). The aminotransferases and LDH activities were determined according to kit protocols (Biolabo, France). Bovine serum albumin (BSA) was used as a protein standard and the total protein concentration was calculated using the Bradford reagent (Bradford 1976). The specific enzyme activities ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ total protein) were determined from the calculated total protein values. Superoxide dismutase (SOD) activity was assayed using a test kit (Sigma 19160, USA) according to the recommended method. For the measurement, 10 μL of supernatant was added into wells of microplate wells, and the reaction medium was completed

to 120 μL . The mixture was shaken in the microplate reader system three times and incubated at 37°C for 20 min. The absorbance (with the appropriate wavelength for the color product of the WST-1 reaction with superoxide) was read at 450 nm. The enzyme activity (U/mL) was calculated using standard inhibition curves with SOD activities of 0.001–200 U/mL using commercial SOD (Sigma, S2515). SOD activity was then determined as SOD activity/mg protein according to the protein level of each sample.

The reduced glutathione level (GSH_{red}) was detected spectrophotometrically using DTNB. The test method defined by Parihar et al. (1997) was modified to adapt for 96-well plates. Accordingly, 5 μL of the sample was transferred into microplate wells, and 120 μL of phosphate buffer containing 0.5 mM DTNB (pH 6.8, 0.1 M) were added. The mixture was incubated at 22°C for 5 min, and the absorbance was measured at 412 nm. The GSH_{red} levels in the samples were calculated using a calibration curve for reduced glutathione (GSH) standard (0.195–12.5 mM GSH). The TBARS levels were determined according to the test protocol due to the formation of adduct of MDA-TBA from the reaction of malondialdehyde (MDA) and thiobarbituric acid (TBA) at about 100°C . Finally, the product was measured spectrophotometrically at 535 nm under acidic conditions. Lipid peroxidation was expressed as nmol MDA/mg protein.

2.6. Statistical analysis

Analysis of probit was done to determine the mean lethal concentration (LC_{50}) values (USEPA, ver. 1.5). The SPSS program (IBM Corp., Armonk, NY, USA) was used for the analysis of the findings obtained as a result of the biochemical studies. Data were analyzed using the Mann-Whitney U test, followed by a nonparametric one-way analysis of variance (Kruskal-Wallis) to determine whether there was a difference in biomarkers between the control and NP treatment groups. The difference between the groups was determined according to the degree of importance at $P < 0.001$, $P < 0.01$ or $P < 0.05$. Also, the total body lengths of *X. laevis* embryos were compared using ANOVA (Dunnett's post hoc test).

3. Results

3.1. Characterization

The characterization results of the Mn-TiO₂ NPs we synthesized have been given previously (Ozmen et al., 2015), and the characterization results of the S-TiO₂ NPs are given in this section. The XRD data for the S-TiO₂ material is shown in Figure 1A, where S-TiO₂ exhibits a typical anatase crystal structure. The average size of the crystallite was calculated as 9.6 nm using the Scherrer equation. The surface morphology of S-TiO₂ was examined from the SEM images, and S-TiO₂ showed agglomeration, although the particles were spherical (Figure 1B). The EDX

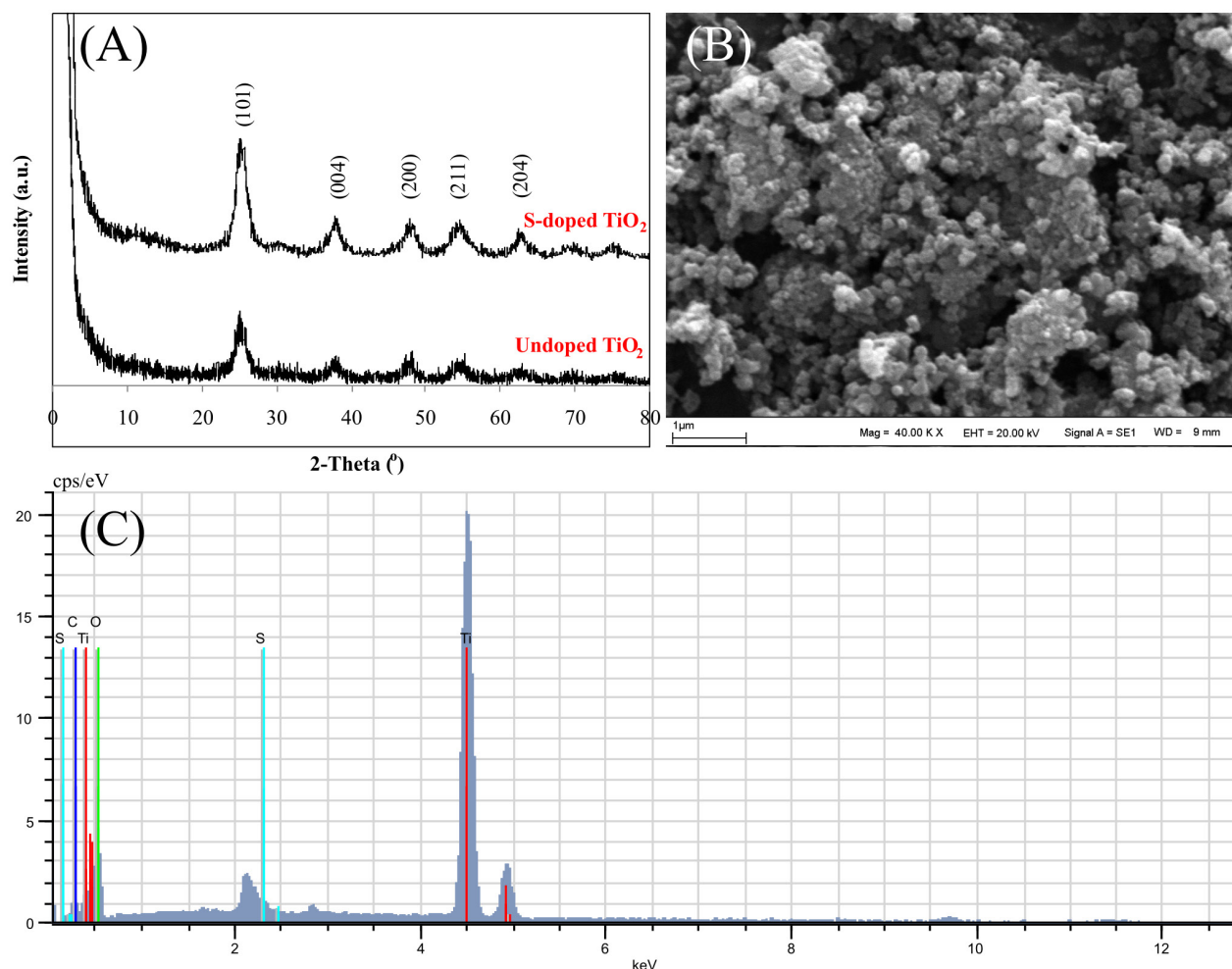


Figure 1. (A) XRD pattern, (B) SEM images of S-TiO₂, and (C) SEM-EDX spectrum.

spectrum of the S peak obtained using SEM-EDX analysis is given in Figure 1C. XRF analysis was also performed to determine the true ratio of S included in the TiO₂ cage. Accordingly, it was determined that the nominal 1 mole % S was present in the TiO₂ with a content of 0.45 wt%. As seen from Table 1, the surface area of S-TiO₂ was 221.33 m²/g, and this value is lower than the surface area of the Mn-TiO₂. It is also seen that the UV/Vis DRS spectrum of the Mn-TiO₂ particles of the absorption edge exhibited red shifts more than S-TiO₂ particles, and the particle size distribution of the Mn-TiO₂ in the aqueous medium appeared larger than S-TiO₂.

3.2. Photocatalytic activities of synthesized S- or Mn-doped TiO₂ NPs

Table 2 shows the optimal conditions for the photocatalytic degradation of FLX with S- TiO₂ and Mn-TiO₂ NPs. After UV-C irradiation, the filtrates were analyzed by UV/Vis spectroscopy and TOC analysis at certain time intervals to determine the degradation products in the

reaction suspensions (Table 3). Similar degradation rates were provided by both methods. Results show that the 10-h degradation process with S-TiO₂ caused about 97% degradation of FLX, while 15-h photodegradation was able to provide similar results with Mn-TiO₂. LC-MS/MS analyses were performed to monitor the photocatalytic degradation process and possible intermediate products. However, it is very difficult to determine which intermediates are formed during the photocatalytic process since they do not have standards for each one. As seen from the LC-MS/MS chromatograms in Figure 2, the peak seen at m/z 458.9 belongs to FLX before irradiation. However, after photocatalytic irradiation the peak intensity of FLX sharply decreased and new peaks with m/z values at low intensities occurred.

3.3. Mortality and developmental effects

The 96 h LC₅₀ values of FLX were determined to be 2.91 mg L⁻¹ and 5.15 mg L⁻¹ for the *X. laevis* stage 8 embryos and stage 46 tadpoles, respectively (Table 4).

Table 1. Comparative sum of the characterization properties of synthesized S- and Mn-doped TiO₂ NPs.

Properties	S-TiO ₂	Mn-TiO ₂
XRD (crystal phase)	anatase	anatase
Average crystallite size, nm	9.6	11.1
S _{BET} specific surface area, m ² g ⁻¹	221.33	284.56
XRF, doping ratio, weight %	0.45 S	0.64 Mn
UV-Vis DRS, E _{bg} , eV	3.14	3.08
Particle size distributions, nm	164	1.9 × 10 ³

Table 2. The optimum conditions for photocatalytic degradation of FLX.

Parameters	S-TiO ₂	Mn-TiO ₂
Initial concentration of FLX, mg L ⁻¹	5.0	5.0
Amount of catalyzer, % wt v ⁻¹ catalyst in sol	0.10	0.15
Time of ads.-des. Equilibrium, min, (in the dark no UV)	60	60
UV light intensity, W m ⁻²	670	670
pH	4.1	4.0
Max illumination time, h (UV-C irradiation-200 to 280 nm)	10	15

Table 3. Photocatalytic efficiency of FLX according to different analytical methods.

Time (hour)	Degradation rate of FLX*										
	S-TiO ₂						Mn-TiO ₂				
	UV-Vis Spect.			TOC			UV-Vis Spect.			TOC	
5	60.2	±	1.30	76.4	±	3.40	16.3	±	1.33	27.5	± 3.13
10	97.8	±	2.10	96.1	±	3.21	56.6	±	1.50	66.0	± 3.90
15	98.0	±	1.90	97.3	±	3.70	94.9	±	1.82	98.2	± 3.46

*These values are expressed as mean ± standard errors (n = 3).

Doped TiO₂ NPs and commercial TiO₂ (Degusa P25; 25% rutile and 75% anatase form) were compared in preliminary studies in terms of lethality, but no difference was observed between them (the data was not given here). Table 5 shows the mortality rates and developmental malformations for exposure to synthesized NPs and photocatalytic degradation by-products tested on *X. laevis*. Both Mn- and S-doped NP exposure of up to 500 mg L⁻¹ did not cause severe mortality on frog embryos. However, S-TiO₂ NPs caused a decrease in the total body length of embryos with increased concentrations (P < 0.05). Exposure to the higher concentrations (≥125 mg L⁻¹) of Mn- or S-doped NPs and photocatalytic

degradation by-products of 5 mg L⁻¹ FLX for 96 h caused low malformation ratios of *X. laevis*, where the highest individual developmental malformation ratio was 9.7%. However, exposure to FLX, exposure to the NP-FLX mixture without any photocatalytic application, and exposure to ≤5-h photocatalytically degraded FLX with Mn-TiO₂ caused 100% mortality during the 96-h period. Photocatalytic degradation of FLX with Mn-TiO₂ NPs for 10 h to 15 h significantly reduced mortality (53% and 97% of individuals survived, respectively). However, the degradation product caused a significant developmental delay in frog embryos (P < 0.001). Similar to Mn-TiO₂, S-TiO₂ NPs also did not cause mortality

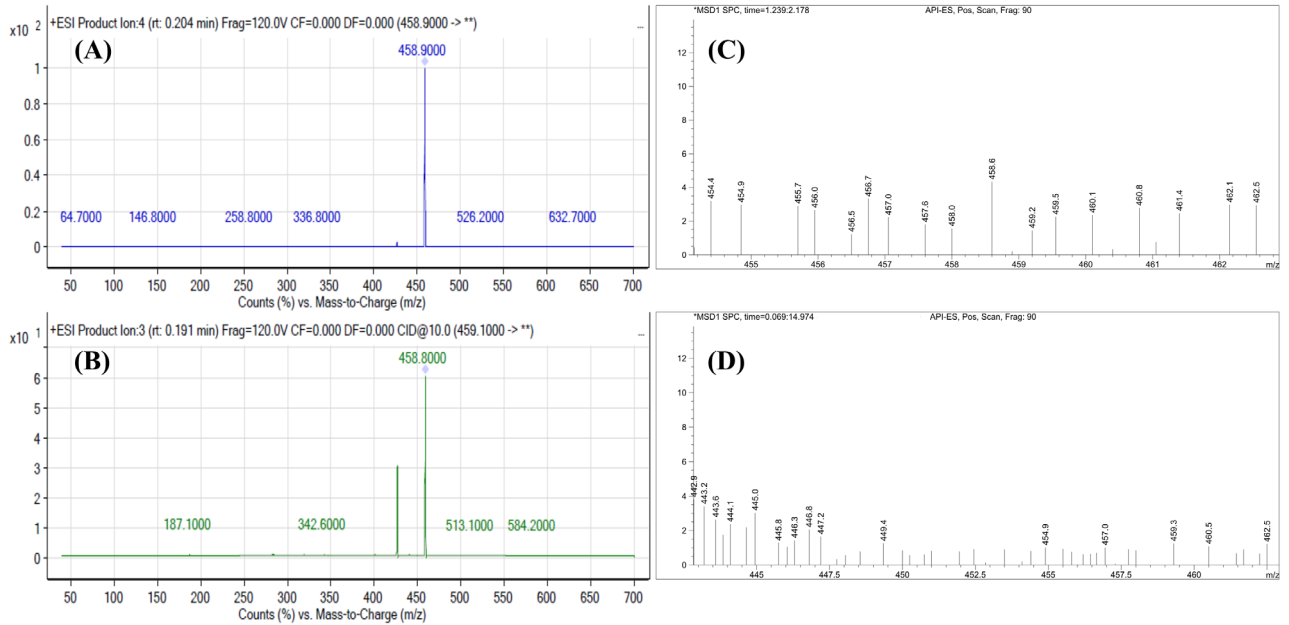


Figure 2. (A) LC-MS/MS spectra of 5 mg L⁻¹ FLX with Mn-TiO₂ in 0.15% wt v⁻¹ catalyst in sol before irradiation and (B) after 15 h of UV-C irradiation. (C) LC-MS/MS spectra of 5 mg L⁻¹ FLX with S-TiO₂ in 0.1%wt v⁻¹ catalyst in sol before irradiation and (D) after 10 h of UV-C irradiation.

Table 4. The calculated LC₅₀ values for FLX.

		LC ₅₀ (mg L ⁻¹)			
		24-h	48-h	72-h	96-h
Embryo	2.71-40	-	7.35 (6.23-8.54)	3.05 (2.57-3.41)	2.91 (2.40-3.28)
Tadpole	3.98-30	5.39 (5.01-5.80)	5.15 (4.81-5.51)	5.15 (4.81-5.51)	5.15 (4.81-5.51)

Values in parentheses represent 95% confidence intervals.

in all tested concentrations. However, 62.5 mg L⁻¹ and higher concentrations caused inhibition of embryonic development ($P < 0.05$). FLX without photocatalytic degradation caused 100% death. However, degradation of FLX with S-TiO₂ NPs for 2, 5, or 10 h significantly reduced the mortality (up to 100% of the embryos lived). While 10-h photocatalytic degradation of FLX with S-TiO₂ NPs did not cause developmental inhibition, the developmental inhibition effect of 2-h or 5-h photocatalytic degradation products was observed.

3.4. Biochemical markers

Selected key biomarkers (GST, GR, GSH_{red}, SOD, TBARS, CaE, LDH, AST, and ALT) were assayed on *X. laevis* tadpoles for determining the toxicity and oxidative stress

response against novel NPs, FLX, and photocatalytic degradation products. The results are presented in Table 6. The 125 and 250 mg L⁻¹ of Mn-TiO₂ NPs caused significant increases in GST and AST activities ($P < 0.05$). Similar to the toxicity tests, the FLX without photocatalytic degradation and 5-h of degradation by-products caused 100% mortality and, therefore, we could not provide enzymatic results. However, 15-h degradation of FLX with Mn-TiO₂ NPs caused significant increases in the activities of GST, GR, and SOD. Furthermore, lower concentrations (5 and 25 mg L⁻¹) of S-TiO₂ NPs caused a significant increase in the activities of SOD and AST and in the GSH_{red} level ($P < 0.05$). Similar to the toxicity test, the FLX and S-TiO₂ NP mixture without photocatalytic degradation

Table 5. Lethality and malformation types of *X. laevis* embryos and tadpoles after 96 h exposure to doped TiO₂ NPs and by-products of FLX.

				Embryo														Tadpole		
						Malformation types														
	Deg.time (h)	Cons (mg L ⁻¹)	n	Dead	Malformed	SV	ST	Gut	AE	CFE	TF	MC	MP	Bl	Length (mm) ^a			n	Dead	
Control						-	-	-	-	-	-	-	-	-	7.95	±	0.05	30	0	
Mn-TiO ₂		5	32	0	-	-	-	-	-	-	-	-	-	-	8.03	±	0.06	30	0	
		25	32	1	-	-	-	-	-	-	-	-	-	-	7.87	±	0.06	30	1	
		62.5	32	1	-	-	-	-	-	-	-	-	-	-	7.88	±	0.05	30	1	
		125	32	0	1	-	-	1	-	-	-	1	-	-	7.76	±	0.08	30	0	
		250	32	2	1		1	-	1	-	1	-	-	-	7.67	±	0.12	30	2	
		500	32	0	-	-	-	-	-	-	-	-	-	-	7.71	±	0.06	30	0	
S-TiO ₂		5	32	1	1	-	-	-	1	-	-	-	-	-	7.70	±	0.07	30	0	
		25	32	0	0	-	-	-	-	-	-	-	-	-	7.68	±	0.05	30	0	
		62.5	32	0	1	-	-	-	-	-	-	-	-	1	7.53	±	0.08	*	30	0
		125	32	1	1	-	-	-	-	-	1	-	-	-	7.58	±	0.09	*	30	0
		250	32	1	1	1	-	1	-	-	1	-	-	-	7.54	±	0.10	*	30	0
		500	32	0	1	-	-	1	1	-	-	-	-	-	7.58	±	0.08	*	30	0
Control		-	32	0	0	-	-	-	-	-	-	-	-	-	7.37	±	0.04		30	0
Mn-TiO ₂ (%0.15 sol)	-		32	1	0	-	-	-	-	-	-	-	-	-	7.40	±	0.04		30	0
5 mg L ⁻¹ FLX+ Mn-TiO ₂ (%0.15 sol)	15		32	1	0	-	-	-	-	-	-	-	-	-	7.11	±	0.04	***	30	1
	10		32	15	0	-	-	-	-	-	-	-	-	-	6.34	±	0.10	***	30	29
	5		32	32	-	-	-	-	-	-	-	-	-	-	-				30	30
	0		32	32	-	-	-	-	-	-	-	-	-	-	-				30	30
Control			32	0	0	-	-	-	-	-	-	-	-	-	8.01	±	0.07		30	0
S-TiO ₂ (%0.1 sol)	-		32	1	1	1	1	1	-	-	1	-	1	-	7.79	±	0.09		30	0
5 mg L ⁻¹ FLX + S-TiO ₂ (0.1%sol)	10		32	0	1	-	1	1	1	1	-	-	1	-	7.91	±	0.08		30	0
	5		32	0	1	1	1	1	-	-	1	1	1	-	7.77	±	0.09	*	30	0
	2		32	1	3	1	1	1	1	1	2	1	1	-	7.54	±	0.14	**	30	5
	0		32	32	-	-	-	-	-	-	-	-	-	-	-				30	30

Malformation types: Severe (SV), Stunted (ST), Gut, Abdominal edema (AE), Craniofacial edema (CFE), Tail flexure (TF), Microcephalia (MC), microphthalmia (MF), Blister (Bl).

^aLengths are expressed as mean ± standard errors. These values were obtained from the lengths of the surviving individuals

n = 32 individuals were used, 8 repeats for each concentration. *P < 0.05, ** P < 0.01, *** P < 0.001 were showed statistical significance compared with control.

Mn-R and S-R indicate that NPs were synthesized with ruflox and TiO₂ was 1% doped with Mn or S.

Table 6. Biomarker levels in *Xenopus laevis* tadpoles after 96 h of exposure to different catalyzers (S- and Mn-doped TiO₂) and photocatalytic degradation by-products of 5 mg L⁻¹ FLX.

				Biomarkers																																		
Catalyzer	Treated pesticides	<i>h</i>	Cons. (mg L ⁻¹)	<i>n</i>	GST ^a			GR ^a			GSH _{red} ^b			SOD ^c			MDA ^b			CaE ^a			LDH ^a			AST ^a			ALT ^a									
Control	-	-		5	145	±	4.1		9.58	±	0.44		0.294	±	0.020		2.78	±	0.11		2.61	±	1.36	190	±	6.6		477	±	33.2		95	±	3.7		18.1	±	0.65
Mn-TiO ₂	-	-	5	5	144	±	4.8		10.07	±	0.38		0.432	±	0.023	**	3.32	±	0.38		1.61	±	0.42	207	±	8.1		690	±	34.8	*	117	±	2.8	**	18.2	±	1.12
	-	-	25	5	151	±	6.3		9.05	±	0.35		0.479	±	0.018	**	2.87	±	0.22		1.62	±	0.42	202	±	4.1		555	±	55.0		113	±	2.2	**	16.8	±	1.00
	-	-	125	5	161	±	5.8	*	8.40	±	0.55		0.479	±	0.040	**	2.95	±	0.67		2.68	±	1.28	218	±	5.1		476	±	23.5		117	±	3.0	**	18.1	±	1.09
	-	-	250	5	161	±	3.9	*	9.74	±	0.47		0.439	±	0.011	**	8.31	±	0.80	**	1.53	±	0.22	199	±	7.6		511	±	19.8		122	±	4.7	**	20.1	±	0.94
S-TiO ₂	-	-	5	5	143	±	8.2		9.17	±	0.27		0.404	±	0.020	*	4.31	±	0.32	**	1.25	±	0.26	203	±	9.9		467	±	17.1		116	±	4.7	**	17.4	±	0.76
	-	-	25	5	136	±	4.8		8.08	±	0.34		0.415	±	0.017	**	3.33	±	0.18	*	1.00	±	0.26	197	±	7.1		461	±	16.8		116	±	2.0	**	17.8	±	0.47
	-	-	125	5	128	±	3.0	**	8.88	±	0.17		0.350	±	0.019		3.14	±	0.14	*	3.75	±	1.34	200	±	6.8		482	±	20.9		110	±	0.8	**	19.6	±	0.52
	-	-	250	5	128	±	7.6		9.37	±	0.28		0.280	±	0.018		3.00	±	0.36		2.19	±	1.23	186	±	9.3		418	±	14.2		105	±	4.6		17.9	±	0.86
Mn-TiO ₂ (%0.15 sol)	-	-	-	5	159	±	8.7		10.91	±	0.40	*	0.319	±	0.018		3.78	±	0.39		1.27	±	0.25	198	±	7.5		518	±	41.3		102	±	3.6		19.0	±	1.00
	5 mg L ⁻¹ FLX	15	-	5	177	±	5.6	**	11.70	±	0.28	*	0.317	±	0.013		4.38	±	0.22	**	1.64	±	0.44	204	±	3.4		626	±	66.1		107	±	2.8	*	19.4	±	0.91
	5 mg L ⁻¹ FLX	10	-	5	210	±	9.0		16.27		-		0.191	±	0.068		-			-			257	±	0.9		469	±	6.2		128	±	8.4		30.2	±	0.66	
	5 mg L ⁻¹ FLX	5	-	5 ^d	-				-			-					-			-			-				-				-				-			
	5 mg L ⁻¹ FLX	0	-	5 ^d	-				-			-					-			-			-				-				-				-			
S-TiO ₂ (%0.1 sol)	-	-	-	5	172	±	4.5	*	10.27	±	0.26		0.437	±	0.026	*	9.05	±	0.94	**	2.10	±	0.33	240	±	7.2	**	793	±	32.9	**	127	±	3.3	**	21.9	±	0.85
	5 mg L ⁻¹ FLX	10	-	5	170	±	6.3	*	9.71	±	0.38		0.344	±	0.029		15.72	±	3.07	**	1.92	±	0.45	206	±	8.2		615	±	21.9	*	116	±	1.4	**	18.3	±	1.02
	5 mg L ⁻¹ FLX	5	-	5	160	±	2.7	*	10.08	±	0.16		0.332	±	0.006		12.97	±	2.43	**	1.16	±	0.19	193	±	3.6		647	±	56.2		121	±	7.0	*	18.2	±	1.06
	5 mg L ⁻¹ FLX	2	-	5	160	±	10.2		12.59	±	0.94	*	0.158	±	0.019	*	23.95		-		2.38	±	0.27	234	±	21.4		452	±	23.4		141	±	10.7	*	27.2	±	2.54
	5 mg L ⁻¹ FLX	0	-	5 ^d	-				-			-					-			-			-				-				-				-			

^a: Enzyme activities were expressed as nmol min⁻¹ mg protein⁻¹ ± standard error.^b: This parameter is expressed as nmol mg protein⁻¹.^c: The activity of this enzyme is expressed as U mg protein⁻¹.^d: All tadpoles died after 96 h exposure.

h: photocatalytic degradation time (hour); n: repeats for each exposure group.

*P < 0.05, **P < 0.0 showed statistical significance compared to control.

also caused 100% lethality. On the other hand, 10- and 15-h photocatalytic degradation of FLX with S-TiO₂ NPs reduced the mortality significantly but caused a significant increase in the activities of GST and SOD.

4. Discussion

Widely used pesticides such as FLX and their residues after degradation in the environment may pose an important risk in embryonic development, especially in aquatic organisms. Half-life of FLX in an aquatic environment may take 40 days (Wightwick et al., 2012). Biological degradation of FLX under aerobic conditions may also take longer than 140 days, depending on environmental conditions (USEPA 2005). It is known that as a result of the degradation of FLX, about 50 metabolites are formed, such as HEC 5725-oxazepine, HEC 5725-E-des-chlorophyll, and HEC 5725-carboxylic acid (EFSA 2007). There are attributions that some metabolites may be highly toxic and/or genotoxic.² Agricultural preparations of FLX have an active substance content of 40.3%, which indicates a very high concentration. Although it has been reported in the literature that FLX has limited water solubility (2.56 mg L⁻¹), its application in very high concentrations in agricultural areas increases the environmental risk considerably (Lewis et al., 2019). Therefore, it is important to use appropriate tools to reduce its environmental risks and to characterize the toxicity of degraded by-products of FLX. If FLX degradation occurs photocatalytically in a short time through NPs, organisms in the ecosystem can be protected from chronic FLX toxicity in the contaminated environment. There are several studies in the literature evaluating the chronic and acute toxicity of various strobilurin fungicides on aquatic organisms (Cao et al., 2019; Chen et al., 2015; Cui et al., 2017; Li et al., 2018; Liu et al., 2013; Zhu et al., 2015). However, studies on FLX toxicity are relatively limited. In zebrafish embryos, 96-h LC₅₀ value is reported as 0.549 mg L⁻¹ (Zhang et al., 2020). The 96 h LC₅₀ of FLX for mature *Oncorhynchus mykiss* and *D. rerio* were reported as 0.44 mg L⁻¹ and 0.51 mg AI L⁻¹, respectively (EFSA 2007; Zhang et al., 2018). It has also been reported that strobilurin fungicides have strong lethal and teratogenic effects on *Xenopus* embryos and other aquatic vertebrates (Zhang et al., 2020; Li et al. 2016).

In this study, FLX was chosen to test the photocatalytic activity of new, synthesized NPs, as it is one of the widely used pesticides in agriculture. It is known that the decrease in the particle size of TiO₂ increases the adsorption rate. Also, the photocatalytic activity is more effective in the light spectrum close to the UV region (Xu et al., 1999; Retamoso et al., 2019). Therefore, we carried out the photodegradation processes with as small particles as

possible and with spectra as close as to the UV light region. We explored the effects of Mn-TiO₂ and S-TiO₂ NPs in embryos and tadpoles of the African clawed frog to evaluate their toxicity and the reduction capacity of toxicity on FLX, after photocatalytic degradation. Comparing different developmental stages with a comparable methodology can provide useful information to estimate potential risks in an ecosystem. The toxicity of a xenobiotic for one developmental stage may significantly differ from another exposed to the same chemicals, and they may also show different physiological responses. The FLX is moderately toxic (>1–10 mg L⁻¹) to marine fish, highly toxic (>0.1–1 mg L⁻¹) to freshwater invertebrates and fish, and very highly toxic (<0.1 mg L⁻¹) to marine invertebrates on an acute basis according to the Environmental Protection Agency (USEPA, 2005). However, degradation period in the water ecosystem can cause additional risks associated with the accumulation of FLX and its degradation by-products. Furthermore, we also found that FLX had a high lethal effect at low water concentrations on *X. laevis* embryos and tadpoles according to calculated 72-h and 96-h LC50 values.

On the other hand, when comparing the toxicity of different concentrations of our novel synthesized NPs, it was determined that both Mn- and S-TiO₂ NPs were not lethal or teratogenic for *X. laevis* embryos (stage 8) and not lethal for tadpoles (stage 46) at concentrations up to 500 mg L⁻¹. It has been shown in previous studies that TiO₂ did not cause significant changes in lethality, malformation, or growth above 1000 mg L⁻¹ on *X. laevis* embryos (Birhanli et al., 2014; Nations et al., 2011; Ohno et al., 2004). However, S-TiO₂ NPs may cause growth retardation in exposed embryos. This may contribute to the production of the superoxide anion, which is a reactive oxygen species (ROS) generated by S-TiO₂ NPs (He et al., 2014; Bacchetta et al., 2011).

In addition to the use of core-shell NPs in applications such as controlled drug transport and bio-labeling, especially the use of metal oxide NPs is increasingly important in applications such as environmental remediation (Chaudhuri and Paria, 2012; Mondal and Sharma, 2016). The TiO₂ core-shell NPs may be useful in the removal of environmental pollutants such as pesticides, especially from the aquatic environment, due to their low toxicological risks and their high photocatalytic degradation capabilities. There are numerous studies on photocatalytic degradation of various toxic organic substances such as organophosphorus (OP) pesticides through NPs. While insufficient mineralization is provided by UV irradiation and H₂O₂ applications in water environments where photocatalysts are not used, it has been reported that a

² New York State Department of Environmental Conservation Division of Solid & Hazardous Materials. In: http://pmep.cce.cornell.edu/profiles/fung-nemat/febuconazole-sulfur/fluoxastrobin/fluoxas_wth_0907.pdf

high level of pesticide mineralization is achieved with TiO_2 photocatalysts (Petsas and Vagi, 2017). However, there is no study on FLX degradation with NPs. Our results show that most organic carbon in FLX is converted to CO_2 after the photocatalytic degradation process and most by-products are expected to be inorganic species. Therefore, it is thought that the active toxic substance may degrade in a short period and its toxicity may be eliminated to a great extent via doped TiO_2 NPs. However, there are some concerns that NPs may also cause adverse effects to aquatic organisms due to biomagnifications at different trophic levels (Yoo-iam et al., 2014). Therefore, for the safe use of TiO_2 and other NPs, their role on the degradation capacity of environmental pollutants should be investigated in terms of potential risks to living organisms (He et al., 2014). By comparing the photocatalytic capacity of both synthesized NPs, we determined that S- TiO_2 may be more effective than Mn- TiO_2 for FLX degradation. The results using spectrophotometry and TOC also exhibited that photocatalytic degradation of FLX by both doped NPs is possible. However, the degradation time may also be important for quick removal of toxic agents from aquatic media. Accordingly, it may be suggested that the S-doped product is more successful because it photocatalytically degrades the FLX in a shorter time. The toxicity leading to a lethal effect and growth retardation for both *X. laevis*

embryos and tadpoles were removed by a 5-h photocatalytic degradation process. Thus, it was shown that short-term photo-degradation of FLX using S- TiO_2 NPs are able to reduce the lethality of embryos and tadpoles of *X. laevis* compared to the degradation of FLX using Mn- TiO_2 NPs (Figure 3). The lethal effect in *Xenopus*, however, was substantially eliminated by degradation of FLX with Mn- TiO_2 for 15 h. On the other hand, the results showed that the by-products or un-degraded parent compounds may continue to limit the growth of organisms, though they are not lethal. This may have been due to some metabolites produced by the photocatalytic degradation of FLX.

In addition to lethality, teratogenicity and growth retardation, some biochemical markers were determined for organism-level evaluation in *X. laevis* tadpoles due to exposure to NPs, FLX and FLX by-products.

Biochemical assays showed that both NPs caused changes in some selected biomarkers at different levels in *X. laevis* embryos, although they were not proportional to concentration (Table 6). The Mn- TiO_2 NP exposure caused to significant increase in GSH_{red} level in all concentrations ($p < 0.01$). AST activity was significantly increased in tadpoles exposed to both Mn- and S- TiO_2 NP ($p < 0.01$). Although it is suggested that most sulfur-containing compounds have a toxic effect, it is known that elemental sulfur does not cause toxicity since it is highly hydrophobic

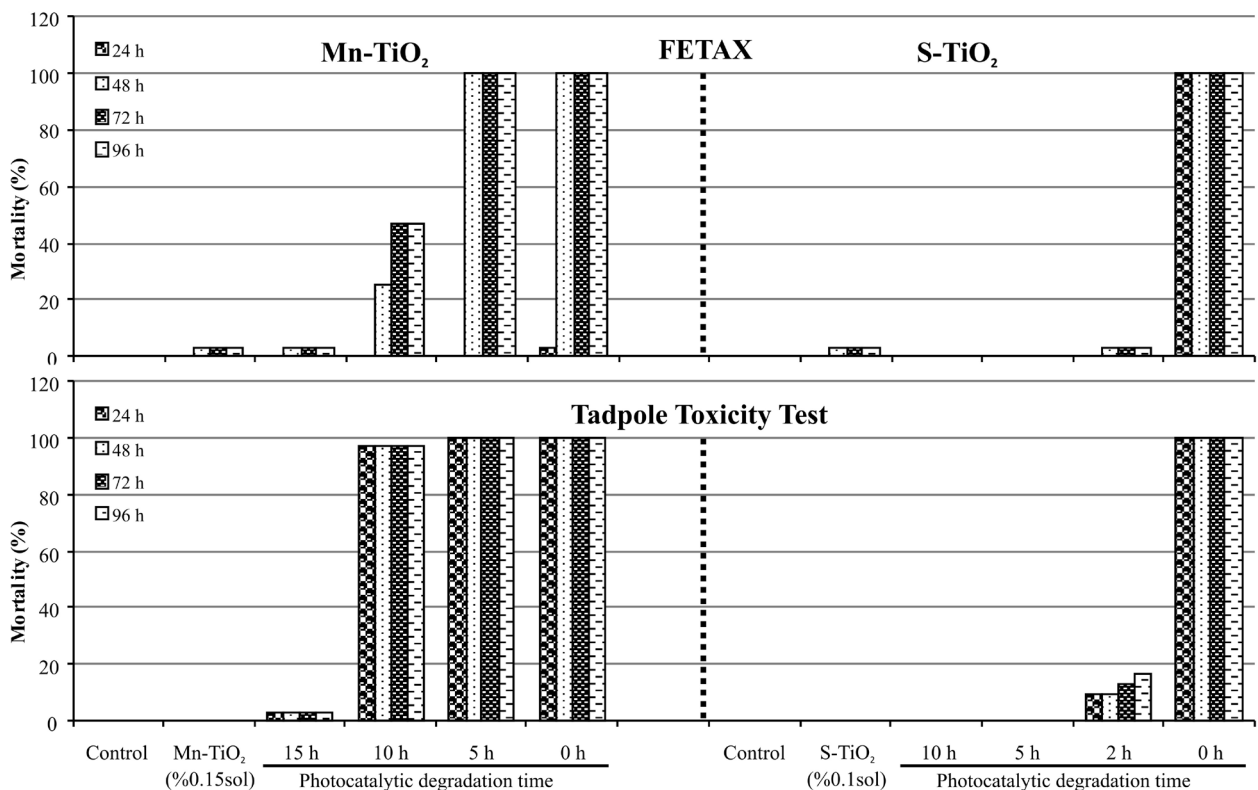


Figure 3. Mortality rates (%) determined in *X. laevis* embryos and tadpoles exposed to degradation products of FLX (5 mg L⁻¹).

(Kuklinska et al., 2013; Pathakoti et al., 2013). Therefore, it may suggest that the toxicity caused by S-TiO₂ may be due to the higher reactivity and their smaller particle size. But, the quantitative analyses made in previous studies showed that the release of the sulfide ion is negligible for S-TiO₂ toxicity. The changes in some enzyme biomarkers, such as increases in GST, SOD, and AST activities and the GSH_{red} level after exposure to all concentrations of Mn-TiO₂, showed toxicological risks of NPs at sublethal concentrations. GSH and SOD activities also increased in relatively low exposure concentrations of S-TiO₂. After photocatalytic degradation of FLX with S-TiO₂ NPs, the increase in GST and AST activities may indicate subcellular adverse effects of sublethal concentrations of NPs and/or the effects of FLX degradation by-products on the organism. Therefore, we assume that the photocatalytic degradation of FLX in aquatic ecosystems may also pose a potential risk due to NP pollution, as well as the toxic effects expected by FLX contamination. These effects may also attribute to the formation of ROS. ROS is an important biomarker for NP toxicity. ROS formation may ultimately a major cause of cellular damage (eg, lipid peroxidation, DNA damage and protein oxidation) and even non-apoptosis cellular death (Dunnick et al., 2014; Pathakoti

et al., 2013). Based on these findings, it is thought that more studies are required to reveal the potential risks of nanotechnological materials and their interactions with chemicals in a versatile and careful way.

Both Mn-TiO₂ and S-TiO₂ NPs used in this study had an anatase structure. The synthesized NPs did not cause significant malformations or lethal effects. With the NPs used, photocatalytic FLX was significantly degraded, and a significant reduction in lethality was achieved. Photocatalytic degradation of FLX could be provided in 10 hours using S - TiO₂, while the same rate of degradation with Mn-TiO₂ was achieved in 15 hours. It shows that S-TiO₂ is a more successful product that can be used for the photocatalytic degradation of FLX. A shorter period of photocatalytic degradation of FLX caused significant toxicity, but a long-term degradation process reduced it. However, ecotoxicological risks of both doped NPs and photocatalytic degradation by-products of FLX should be considered in further studies.

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