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Ecotoxicity of zirconium oxide nanoparticles in relation to aquatic invertebrates

Monika Załęska-Radziwiłł, Nina Doskocz*

Faculty of Environmental Engineering, Department of Biology, Warsaw University of Technology, Nowowiejska 20, 00-653 Warsaw, Poland, emails: monika.radziwill@is.pw.edu.pl (M. Załęska-Radziwiłł), nina.chrzanowska@is.pw.edu.pl (N. Doskocz)

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ABSTRACT

The purpose of this study was to investigate the ecotoxicity of zirconium oxide nanoparticles to aquatic invertebrates and to compare the effect of activity of the *nano*form with the *macro*form of this compound. The study was conducted using protozoans *Tetrahymena thermophila,* and crustaceans *Thamnocephalus platyurus* and *Daphnia magna*. Acute tests were performed: immobilization assay with the crustaceans and enzymatic assay with *D. magna*, as well as chronic tests; Growth assay with the protozoans and reproduction assay with *D. magna*. Nano-ZrO_2 did not cause the immobilization of crustaceans within 48 h (*D. magna*) and 24 h (*Thamnocephalus platyurus*)—EC_{50} > 400 mg/L. However, the compound influenced enzymatic processes of the crustaceans in sublethal concentrations. EC_{50} in one-hour Fluotox test amounted to 153 mg/L. The largest harmfulness of zirconium oxide nanoparticles was shown in the chronic test with the use of *T. thermophila*. The value of EC_{50} after 24 h amounted to 12.83 mg/L and no observed effect concentrations (NOEC) ≤ 0.19 mg/L. The compound also inhibited the reproduction of crustaceans by 50% in the concentration of 96 mg/L, whereas NOEC was 0.78 mg/L. The conducted assays confirmed that the investigated nanocompound was more toxic for crustaceans than its molecular form.

Keywords: Zirconium oxide nanoparticles; Ecotoxicity; Aquatic invertebrates

1. Introduction

Nanomaterials are more and more often used in many disciplines of life. They are included in different kinds of composites used in aviation or car industry. They are also used in microelectronics, in water purification processes, as catalysts, or even as dietary supplements [1].

With quite a small size (≤100 nm) and a high surface-to-volume ratio, nanomaterials can be quite highly resistant mechanically as well as reactively ideal for many industrial forms of application. These unique features can, however, constitute a great danger for organisms in the environment, especially within aquatic ecosystems.

It is suggested that nanoparticles after penetrating the cells of organisms gather in endoplasmic reticulum, the golgi apparatus, and lysosomes. They

*Corresponding author.

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generate free radicals, which leads to the dysfunction of cell organelle and causes some damage to the DNA, cell membranes, proteins and creates reactive oxygen species. Passing through the blood–brain barrier is a dangerous phenomenon. Nanoparticles can also cause some damage to the barrier integrity by altering endothelial cell membrane permeability. However, the influence of nanoparticles on blood–brain barrier integrity is still not well-known. Sharma et al. studied the effect of Ag, Al, and a Cu nanoparticle on blood–brain barrier permeability in relation to brain edema formation was examined in a rat model. Intravenous (30 mg/kg), intraperitoneal (50 mg/kg), or intracerebral (20 μg/10 μL) administration of Ag, Cu, or Al nanoparticles disrupted the blood–brain barrier function in rats 24 h after administration and induced brain edema formation [2,3].

In extensive studies conducted with the use of cerium nanoparticles in relation to crustaceans carried out by Hoecke et al. [4], their harmful influence toward *Daphnia magna* was shown within a long exposure period $EC_{10} \geq 8.8$ and ≤20.0 mg/L, probably as a result of a decrease in alimentary activity of animals. Lovern et al. proved that *D. magna* mortality increased together with an increase of the concentration of TiO_2 nanoparticles. EC_{50} after 48 h amounted to 5.5 mg/L [5]. On the other hand, the study performed by Adams et al. indicated that nanoparticles caused a 100% rate of mortality of *D. magna* in the concentration of 0.5 mg/L ZnO, 70% mortality in the concentration of 10 mg/L SiO_2, and 40% mortality in the concentration of 20 mg/L TiO_2 [6]. Ud-daula et al. in their study related to the influence of TiO_2 nanocompound on the protozoans *Tetrahymena* sp. obtained EC_{10} equals to 15.03 mg/L after 20 h and 27.82 mg/L after 40 h. In addition, a cytotoxic influence of the studied nanocompound was observed after 20 h [7].

The data referring to subject-related literature prove that different kinds of pollution found in water can be adsorbed on the surface of nanocompounds, which increases their harmfulness for aquatic organisms [8,9]. Sorption of e.g. metals can cause chronic toxicity and can be transferred to higher trophic levels.

Although there is an increasing interest in the harmfulness of nanomaterials in relation to aquatic organisms, there are no ecotoxicological data for most nanoparticles, which makes it impossible to conduct the assessment of risk triggered by the presence of these compounds in the environment.

The purpose of this study was to assess the influence of zirconium oxide nanoparticles (nano-ZrO_2) on aquatic invertebrates. The interest in the nanocompound-chosen results from the fact that its influence on aquatic organisms is virtually unknown.

Zirconium oxide nanoparticles are used to eliminate the pollution of water, *inter alia* the arsenic, as a catalyst and in bioengineering—in the production of prostheses and implants as well as the carriers of medicines (insulin) [10,11]. More and more common use of these nanoparticles can be the cause of their release to the environment and induction of toxic reactions in the organisms of aquatic ecosystems. In this study, the effect of activity of the nanoparticle form of zirconium oxide on aquatic invertebrates was compared to the "macro" form of this compound.

2. Materials and methods

2.1. Chemicals

Zirconium oxide nanoparticles, (nano-ZrO_2), nanopowder <100 nm with a specific surface area >25 m^2/g and zirconium oxide of purity over 98% was obtained from Sigma–Aldrich. Molar mass of ZrO_2 is 123, 22 g/mol (Cas No. 1314-23-4). The stock solutions of nano-ZrO_2 and ZrO_2 (Sigma–Aldrich) were prepared in deionized water. Because tested compounds are able to form aggregates, the stock dispersion was sonicated (0.4 kW, 20 kHz) for 30 min to break aggregates before being diluted to the exposure concentrations. The stock solutions nano-ZrO_2 and ZrO_2 were diluted (using the medium with respect to the procedures of tests) in a descending order with a geometric series of quotient $q = 2$ to obtain final 400–0.19 mg/L.

2.2. Ecotoxicological tests

Acute and chronic tests were performed on crustaceans and protozoa. Ciliates *Tetrahymena thermophila*, crustaceans *Thamnocephalus platyurus, and* neonats of *Daphnia magna* were obtained from dormant eggs in the hatching procedure, according to the appropriate test protocol [12–14].

2.2.1. Acute tests

(1) Crustacean immobilization assays Daphtoxkit F™ and Thamnotoxkit F™ (Microbiotests) were performed, according to the protocols provided with each kit. The organisms were incubated with toxic compounds for 24 and 48 h, respectively, in the temperature of 25˚C. Then, immobilized organisms were counted;
(2) A fluotox fluorescence inhibition assay (IQ toxicity test) was conducted, according to the methodology developed by Espiritu et al. [15].

The organisms showing no fluorescence were counted after one hour of exposure.

2.2.2. Chronic tests

(1) Protozoa growth assay Protoxkit F™ (Microbiotests) was performed, according to the protocol provided with the kit. Ciliates *T. thermophila* were incubated in test vessels, with tested compounds and food suspension, in the temperature of 30˚C. Growth inhibition was determined on the basis of turbidity changes (OD at $\lambda = 440$ nm), at the beginning and at the end of the test;
(2) The reproduction test with *D. magna* crustaceans was conducted according to the OECD methodology 211 [16], in semistatic conditions with a daily solution exchange (21 d, 20–22˚C, 8/16 h dark/light photoperiod). Crustaceans were fed with a unicellular algae suspension. Juveniles were counted daily and removed from the test vessels.

2.3. Calculation procedures

(1) Inhibition of reproduction of *D. magna* was determined on the basis of the following formula:

$$I = 100 - \frac{r_t - r_0}{r_c} \times 100 \quad (1)$$

where r_t – Average reproduction over exposure time *t in the tested concentration*; r_0 – The average number of individuals in a 0 time; r_c – Reproduction in control;

(2) The percentage growth inhibition protozoa *T. thermophila* was determined on the basis of the following formula:

$$\%\ inhibition_{(c1-c12)} = \left(1 - \frac{\Delta OD_{c1-c12}}{\Delta OD_{c0}}\right) \times 100 \quad (2)$$

where OD – Optical density; C1–C12 – Dilution series; C_0 – Control sample;

(3) Lethal and effect concentrations (EC_{50}) were calculated using probit analysis with 95% confidence intervals [17];
(4) No observed effect concentrations (NOEC) were determined using ANOVA and Tukey's test [18].

3. Results and discussions

The results of ecotoxicological tests are presented in Figs. 1–4. The assessment of ecotoxicity of nanoparticles according to the European Union Directive (93/67/EEC) [19] and the US Environmental Protection Agency (USEPA) criteria [20] is presented in Fig. 1. The results of acute tests revealed diversified sensitivity of organisms to the tested compounds.

The greatest impact of zirconium oxide nanoparticles was confirmed in relation to *Daphnia magna* in the Fluotox test. After one-hour exposure to the nano-ZrO_2 in the highest concentration tested (400 mg/L), in the case of 65% of specimens, inhibition of fluorescence was observed, whereas in the remaining concentrations the value of inhibition was maintained on the level of 56–8% (Fig. 1), EC_{50} was: 153.9 mg/L (Fig. 4). Crustaceans *Daphnia magna* and *Thamnocephalus platyurus* were supposed to be less sensitive to the tested nanocompound—48 and 24 h, respectively, in the immobilization test. Only in the highest concentration tested, (400 mg/L) 10% immobilization of the studied organisms was obtained, the value of EC_{50} was >400 mg/L (Fig. 4). Literature data also shows a slight influence of the nanoparticles on the crustaceans *D. magna* and *T. platyurus* in the short period of exposure. Rosenkranz showed that in the acute exposure of *D. magna* to TiO_2 nanoparticles, only 10% mortality was observed in the highest concentration of 100 mg/L after 48 h [21]. On the other hand, in the study carried out by Casado et al., the crustaceans *Thamnocephalus platyurus* proved to be slightly sensitive to the influence of silica nanoparticles. EC_{50} was >1,000 mg/L [22]. Blinova et al. obtained slightly different results in their study. It was proved that silver nanoparticles were highly toxic to both crustaceans: The EC_{50} values in artificial freshwater were 15–17 mg/L for *D. magna* and 20–27 mg/L for *T. platyurus* [23]. Heinlaan et al. observed even lower EC_{50-48h} values, 3.2 mg/L (*D. magna*) and 0.18 mg/L (*T. platyurys*) [24].

The results derived from chronic tests (growth test on protozoa, reproduction test on *D. magna*), lasting from 24 h for the *T. thermophila*, up to 21 d for crustaceans showed some toxic effects of tested nanoparticles. It was stated that the highest zirconium oxide nanoparticles concentration studied in both tests—400 mg/L caused over 70% inhibition of reproduction of the crustaceans (Fig. 2) and growth of the

Table 1
Toxicity profile for nanoZrO_2 and ZrO_2 studies results

Tested organism	Nano-ZrO_2		Ecotoxicity assessment		ZrO_2		Ecotoxicity assessment	
	EC_{50} (mg/L)	NOEC (mg/L)	UE	US EPA	EC_{50} (mg/L)	NOEC (mg/L)	UE	US EPA
Thamnocephalus platyurus (Immobilization)	>400	–	Nontoxic	Slightly toxic	>400	–	Nontoxic	Slightly toxic
Daphnia magna (Immobilization)	>400	–	Nontoxic	Slightly toxic	>400	–	Nontoxic	Slightly toxic
Daphnia magna (Fluorescence)	153.98	–	Nontoxic	Slightly toxic	>400	–	Nontoxic	Slightly toxic
Tetrahymena thermophila (growth)	12.83	≤0.19	Harmful	Moderately toxic	231.3	1.56	Nontoxic	Slightly toxic
Daphnia magna (Reproduction)	95.9	0.78	Harmful	Moderately toxic	>400	3.13	Nontoxic	Slightly toxic

protozoans (Fig. 3). The value of EC_{50} for nanoZrO_2 obtained in the Protoxkit *F* test was 12.83 mg/L, whereas in the reproduction test 95.2 mg/L (Fig. 4). NOEC was: ≤0.19 mg/L (*T. thermophila*) and 0.78 mg/L (*D. magna*), respectively, (Table 1). The data from literature also show that protozoans *T. thermophila* in the growth test and *D. magna* in the reproduction test are highly sensitive to the influence of nanoparticles. In the study of Mortimer et al., EC_{50} for these protozoans amounted to ~5 mg metal/l (nano-ZnO) and 128 mg metal/l (nano-CuO) [25]. Das et al. showed that growth and reproduction *D. magna* were reduced by 35 and 93%, respectively, in the treatments at the highest uncapped nTiO_2 concentration (7.5 mg/L) [26]. The result of the present study is also consistent with Wiench et al. and Zhu et al., which also showed that reproduction was completely inhibited by nanoparticles (nano-TiO_2) [27,28]. Toxicity assessment on the basis of EC_{50} showed that nanoZrO_2 caused some minor damage in relation to most aquatic invertebrates. According to EU criteria, this nanoparticle was harmful to the all studied aquatic invertebrates. However, according to US EPA criteria nanoZrO_2 was slightly toxic to *D. magna* (immobilization and fluorescence) and *T. platyurus* (immobilization), and moderately toxic to *T. thermophila* (growth) and

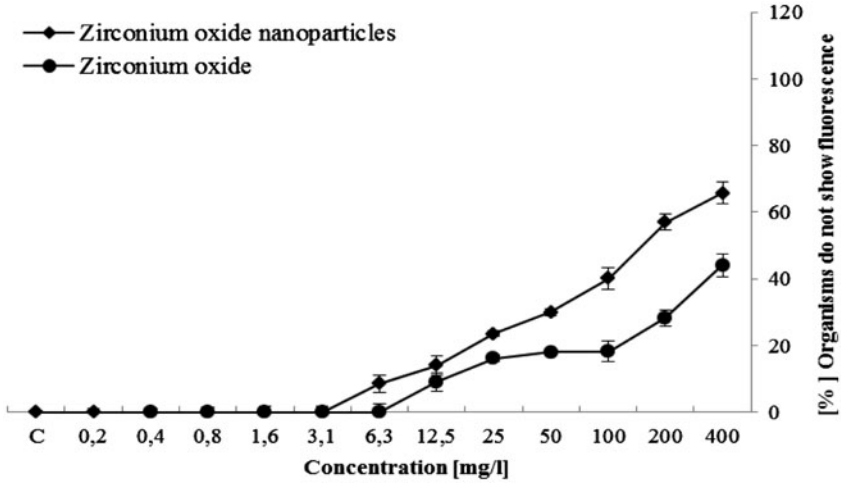

Fig. 1. Effect of nano-ZrO_2 and ZrO_2 on fluorescence *D. magna*. C—means the control.

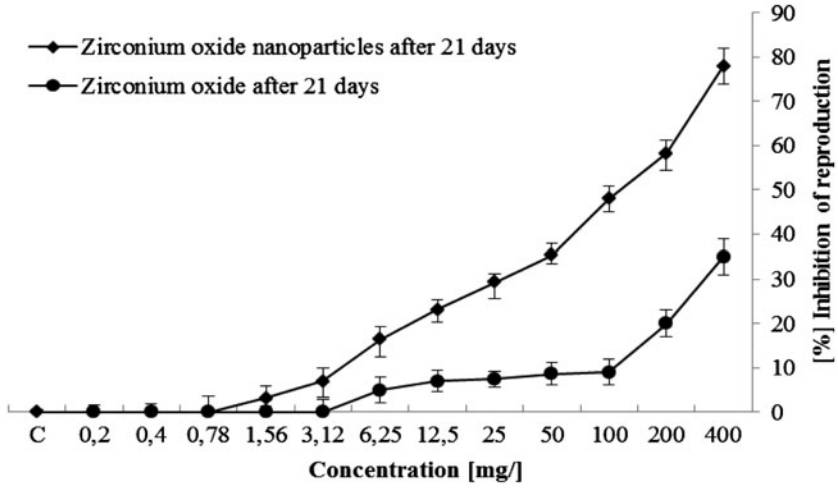

Fig. 2. Effect of nano-ZrO_2 and ZrO_2 on reproduction *D. magna*. C—means the control.

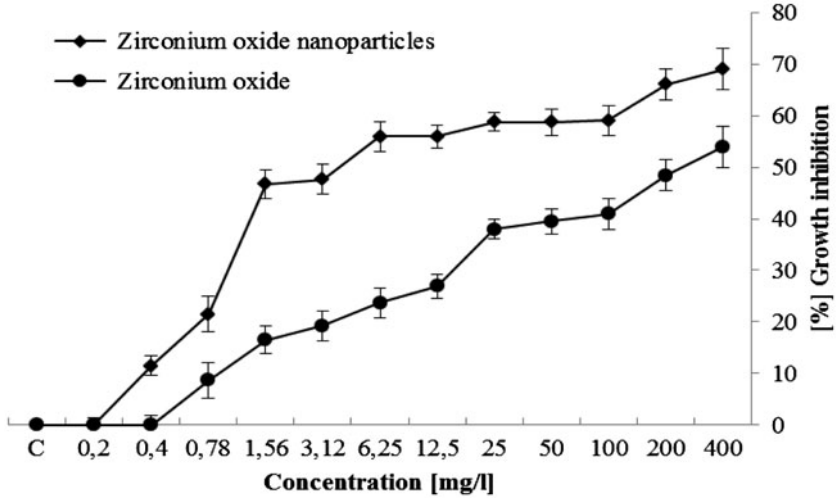

Fig. 3. Effect of zirconium nanooxide and zirconium oxide on growth *T. thermophila*. C—means the control.

D. magna (reproduction) (Table 1). The obtained results also show that the studied nanoparticle is more toxic than the same compound in the "macro" form (Fig. 1–4) (Table 1). This confirms the reports from the literature that the nanoform of a given substance shows different features and may constitute a far greater danger for the environment than the same substance in a larger form. Jośko and Oleszczuk proved that ZnO, TiO_2, and Ni nanoparticles showed completely different ecotoxicity in relation to plants when compared with the same compounds in their traditional form. Also, the study of Chrzanowska and Załęska-Radziwiłł proved that zirconium oxide and aluminum oxide have a significantly smaller influence on the bacteria *P.putida* and *A. hydrophila* in the plankton form as well as the biofilm form than the nanoparticle form of these compounds [29,31]. The study and literature data revealed that long-term presence of nanoparticles in aquatic ecosystems may adversely affect the aquatic invertebrates. This is also confirmed that acute tests used in this study are insufficient for the ecotoxicological assessment of risks and hazards related to nanoparticles in relation to aquatic invertebrates.

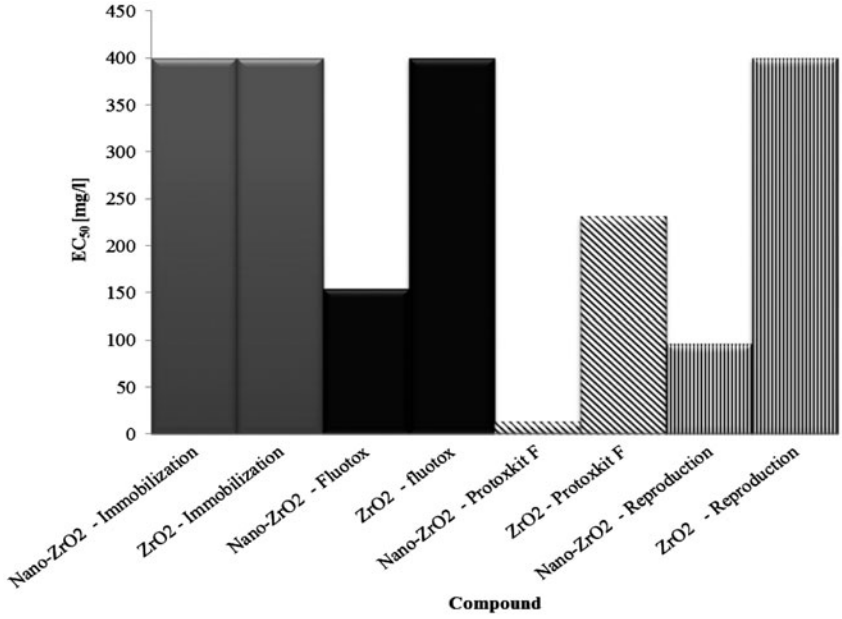

Fig. 4. Comparison of the EC_{50} values of nano-ZrO_2 and ZrO_2 in relation to tested aquatic invertebrates.

4. Conclusions

The conducted studies concerning the ecotoxicity of the zirconium oxide nanoparticles (nanoZrO_2) for *D. magna*, *T. platyurus*, and *T. thermophila* made it possible to formulate the following conclusions:

(1) Zirconium oxide nanoparticles displayed slight harmfulness within the scope of acute toxicity. In the range of concentrations in immobilization tests on *D. magna* and *T. platyurus*, the values $EC_{50-24/48h}$ were >400 mg/L; The crustaceans *D. magna* were confirmed to be the most sensitive to the influence of zirconium oxide nanoparticles in the sublethal enzymatic test—Fluotox;
(2) Considering acute effects, in accordance with the European Union criteria, nanoZrO_2 was harmful for the crustaceans and protozoans in the reproduction and growth assays. However, according to US EPA, it was slightly toxic to *D. magna* (immobilization and fluorescence) and *T. platyurus* (immobilization), and moderately toxic to *T. thermophila* (growth) and *D. magna* (reproduction);
(3) The greatest toxicity was shown by the ZrO_2 nanoparticles in chronic tests; The protozoans *T. thermophila* proved to be most sensitive to the influence of the tested nanoparticles;
(4) NOEC values calculated from chronic tests for zirconium oxide nanoparticles are significantly lower than EC_{50} values. Therefore, extrapolation of NOEC values with EC_{50} is impossible with the use of commonly accepted ACR factor (Acute to Chronic Ratio) = 10 [32];
(5) Toxicity of zirconium oxide nanoparticles in molecular forms was definitely lower than in the case of the nano forms.

Literature data and ecotoxicological research on zirconium oxide nanoparticles carried out in this study showed their harmful effect on aquatic animals, especially within a long period of exposure, which consequently leads to changes in biodiversity. Therefore, reliable evaluation of real hazards involved requires constant monitoring of these pollutants and necessitates to develop some analytical methods and modeling for the purpose of accurate determination of exposure as well as explanation of the mechanisms of the influence of nanoparticles on aquatic ecosystems organisms. This study confirms the fears of ecotoxicologists that standard acute tests required for the procedure by the Chemical Safety Assessment in the REACH regulation for chemical compounds are insufficient to assess the potential danger and the risk in aquatic environment in relation to nanoparticles [33]. Due to specific features which are characteristic for nanocompounds, it is very important to conduct some studies of chronic effects of these compounds, including the molecular level.

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