Environmental Nanotechnology, Monitoring & Management 16 (2021) 100572

Contents lists available at ScienceDirect

Environmental Nanotechnology, Monitoring & Management

journal homepage: www.elsevier.com/locate/enmm

The retention of Zr from potential therapeutic silica-zirconia core–shell nanoparticles in aquatic organisms

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A R T I C L E I N F O

Keywords:
Nanoparticle
Silica@zirconia
Accumulation
Daphnia magna
Danio rerio

A B S T R A C T

Model experiments under laboratory conditions were carried out to assess the accumulation tendency of Zr from a silica-zirconia core–shell nanoparticles, synthesised for the assay. Acute exposition tests were conducted with *Daphnia magna* and *Danio rerio* and the accumulation tendency of the nano and the molecular form was compared. Significant elevation of Zr was found in the tissue of the test organisms treated by the NP compared to the control, however, the retention of zebrafish was lower than that of the daphnids. Increased level of bioconcentration factor and the strong correlation of the redundancy analysis data suggest accumulation tendency in *D. magna*, yet long-term experiments are required to prove and further assess the environmental risk of food-chain bioconcentration. The BCF results were under the REACH limit for the *D. rerio* groups indicating the low short-term accumulation tendency of Zr from the NP. However, Zr level was significantly higher in zebrafish individuals originating from the SiO_2@ZrO_2 NP exposed treatments compared to the groups supplemented by the same concentration of $ZrOCl_2$, which did not result in the elevation of Zr in fish tissue. The trace element homeostasis of *D. rerio* was not affected by the acute SiO_2@ZrO_2 NP exposition and no lethality occurred.

1. Introduction

The unique feature of manufactured nano-sized materials is widely utilized in the field of industry, human and animal medicine, agriculture and fabrication of electronics – among many other areas (Lee et al., 2011; Mansha et al., 2016; Ullah et al., 2017; Shaalan et al., 2016; Vance et al., 2015; Zhang et al., 2016; Indoria et al., 2020). The most prevailing inorganic nanoparticles (NPs) are the metallic based ones, such as Ag, Al_2O_3, Au, CeO_2, SiO_2, TiO_2 or ZnO (Asharani et al., 2011; Forgács et al., 2018; Jain et al., 2006; Mahmoud et al., 2017; Pant et al., 2013), but in the global market silver and titanium have the broadest range of applications (Zweck et al., 2008). The widespread utilization of NPs requires the thorough evaluation of their potential health and environmental risk posing directly or indirectly by their processing and usage. The release of these materials into the environment will eventually lead to their accumulation in the different biotic and abiotic components of the aquatic and terrestrial ecosystem resulting them to be considered as emerging pollutants (Johnston et al., 2010; Mukherjee and Weaver, 2010; Richardson, 2009). Emitted NPs reaching surface and underground water, soil and sediment will ultimately appear in the food web by bioaccumulation and bioconcentration presenting health concern to humans, as well.

The main issue is that the particle size may result in different behaviour of the NPs compared to the conventional form of the same material causing unpredictable environmental risk (Williams et al., 2019). It was stated, that the unique feature of these materials being advantageous in the field of application and providing them high market potential is exactly which causes the unknown health-related problems (Hougaard et al., 2011). However, determining their effect, transport routes and fate in environmental media is rather complex and depending on such factors as the physical and chemical characteristics of the manufactured NPs, volume and nature of their application, as well as the way of emission (Kammer et al., 2012; Laborda et al., 2016; Petersen et al., 2016). Still, it is crucial to estimate and determine the toxicological degree and effect of engineered nanomaterials as their use in commercial products is likely to increase continuously (Williams et al.,

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https://doi.org/10.1016/j.enmm.2021.100572
Received 1 February 2021; Received in revised form 8 August 2021; Accepted 9 September 2021
Available online 14 September 2021

2019).

Since nanotoxicology, as a new field of toxicology was proposed in 2004 (Donaldson et al., 2004), several methods of evaluating the potential adverse effect of man-made NMs have been presented. However, there is still no standardized protocol available in regulations for their complex assessment (Shrivastava et al., 2019). Both *in vitro* and *in vivo* studies are essential to be performed, as well as ecotoxicological aspects must be determined (Arora et al., 2012). Even mathematical and computer-based techniques are developed to predict the interactions of disposed NPs with different biological systems such as their transformation trends under oxidative circumstances (Escorihuela et al., 2018; Grammatikopoulos et al., 2019; Yan and Xie, 2013). Toxicological studies using test organisms are also proposed for the investigation of the accumulation tendency of engineered NMs and the importance of model experiments are highlighted for the deeper understanding of their transport routes in aquatic media (Turan et al., 2019; Zhang et al., 2019). Turan et al. in their review published in 2019 concluded, that among all the research assessing the different properties, application and toxicity of NPs, only a few have focused on their end-life and associated consequence of their invasion into the aquatic environment (Turan et al., 2019). Based on such predicting factors as in-use stocks, production volumes and fate mechanism, a probabilistic model estimated TiO_2 to be in the surface waters in an average concentration of 2.2 μg L^{-1}, and AgNP of 1.5 ng L^{-1} (Bundschuh et al., 2018; Sun et al., 2016), emphasising the true concern of aquatic release. Wetlands are not just the most sensitive ones of the Earth's ecosystems but also are the bassinet of high-risk pollution since contaminants can find their way easily via water to get access into the food web and reach higher trophic levels (Harangi et al., 2017; Herman et al., 2020a). Modelling the food chain of these populations under laboratory conditions provide an important possibility to gain experimental data on NP toxicity.

The proper ecotoxicological experiments require not only the applied method to be carefully chosen, but also the test organism (Danabas et al., 2020; Rand, 1995). *Daphni*a species are very commonly applied organisms in acute and chronic toxicology studies due to their favourable features such as sensitivity to the chemical and physical alteration of the surrounding water, simple and inexpensive raise, rapid reproduction (Araujo et al., 2019; Lu et al., 2018; Zhang and Hamza, 2019). In the last few years, these zooplankton organisms were successfully applied in assessments attributed to NP toxicology where behavioural, physiological, biochemical changes, retention tendency and lethality were usually determined (Pakrashi et al., 2017; Xiao et al., 2016, 2015). *Danio rerio*, commonly known as zebrafish, is not only a long-applied indicator organism in aquatic toxicology but its use as a model is recently proposed for screening the toxicity profile of NMs and to determine their feedback (Pecoraro et al., 2017b). The size of the larval and adult zebrafish, the rapid maturation and growth, the transparency of the embryos and their rather surprising genetic similarity to humans resulted in the use of *Danio rerio* as an excellent indicator of environmental pollution studies and also as animal model system for NP toxicity assays (Brundo and Salvaggio, 2018; Jia et al., 2019). In latter, its application is growing exponentially in recent years where hatching-related parameters are determined, as well as embryo toxicity tests are carried out to reveal developmental disorders and pathology analysis in organs upon NP exposition (Asharani et al., 2011; Brundo et al., 2016; Brundo and Salvaggio, 2018; Chakraborty et al., 2016; Chen et al., 2011b; Paatero et al., 2017; Pecoraro et al., 2018, 2017a).

Nanoscale range zirconia particles are utilized in biomedicine as dental fillings, implants and hip replacements as well as they are promising as large catalysts to be used in drug targeting and delivering systems, for loading and labelling thus personalizing medicine (Catauro et al., 2008; Hisbergues et al., 2009; Kohal et al., 2013; Srinivas and Buvaneswari, n.d.). The application of ZrO_2 NPs as drug nanocarriers is a rather new approach (Karthiga et al., 2019), thus very little is known about their ecotoxicology compared to other, more widely applied and investigated NMs (Karthiga et al., 2019). There are information in the literature regarding some biological, biochemical and human toxicological effects of ZrO_2 NPs such as cell cytotoxicology (Abd El-Ghany and Sherief, 2016; Gusarov et al., 2006), but only a few article considers its route, behaviour and fate upon environmental release. However, it was found by Załęska-Radziwiłł and Doskocz that the nano-compound form of Zr was more toxic for the studied aquatic crustaceans than its molecular form. Authors highlighted the importance of further assessing the hazard of Zr NPs on water-based ecosystems by establishing model experiments for the complex determination of exposure related effects. Karthiga et al. also investigated the ecotoxicology of Zr containing NPs where the development of zebrafish embryos was assessed upon contamination (Karthiga et al., 2019). In their paper developmental embryonic toxicity was revealed together with hatching disabilities as well as the malformation of *Danio reiro* embryos and larvae were both observed in some cases leading to their mortality.

The potential biomedical application of Zr containing nanocarriers for therapeutic vaccination is a novel approach (Nagy et al., 2016). While several literature data are available regarding the aquatic and terrestrial ecotoxicological effects of some inorganic nanoparticles, less information is found about the Zr containing ones. Since no previous research has described the accumulation tendency of Zr containing NP used in biomedicine, the aim of the present work is to investigate the retention of Zr released from therapeutic silica-zirconia core–shell NPs by aquatic organisms of *Daphnia magna* and *Danio rerio*. The model experiments were conducted under laboratory circumstances where the bioaccumulation of NP form was compared with the molecular ZrO_2.

2. Materials and methods

2.1. Synthesis of SiO_2@ZrO_2 nanoparticles

The silica-zirconia core–shell nanoparticle used in present study is described by Naszályi Nagy et al. in 2020 as a high capacity and versatile nanocarrier with the prospect of cancer immunotherapy application (Nagy et al., 2020). While details can be found in the above publication, we briefly summarize the steps of the preparation as follows.

2.1.1. Synthetic details of the silica core (batch **SiO_2**) (Stöber, Fink, and Bohn 1968)

250 ml of absolute ethanol (freshly opened, LiChrosolv, gradient grade for liquid chromatography, water content <0.1 %, Merck) was poured in a tall form glass beaker. Stirring was set to 300 rpm, 9.5 ml of ammonia solution was added (freshly opened, 32%, EMPURA, Merck) and the mixture was covered with parafilm for 5 min. 10 ml of tetraethyl-orthosilicate (TEOS, freshly opened, 99%, obcr) was then quickly added to the stirred beaker. The beaker was closed with parafilm and stirring continued till next day. Then ethanol was added to the translucent sol. The temperature was set to 70 °C and stirring continued for 1.5 h without covering the beaker. After cooling down the sol was stored refrigerated.

2.1.2. Synthetic details of the zirconia deposition on the silica cores (batches **SiO_2@ZrO_2 I and II**)

For the first batch (***SiO_2@ZrO_2 I***) 45 ml of ***S13*** was diluted to 450 ml in a three-necked round bottom flask with LiChrosolv ethanol. Septa were put into necks with needles through them. The flask was heated to 55 °C under argon flow and continuous stirring. 50 ml ethanol was put in a dropping funnel adjusted to one of the side-necks. It was also purged with argon. 1.6 ml of TBOZ was put in a plastic syringe (under Ar flow) and mixed to the ethanol in the funnel. Then dropwise addition of TBOZ solution started (~1 drop/s). The reaction was stopped two hours later and aged in a fridge for 3 days (Kim et al., 2009).

The second, parallel batch (***SiO_2@ZrO_2 II***) was prepared with the same procedure. The batches were united after confirmation of the identical particle size distribution (***SiO_2@ZrO_2 I + II***).

2.1.3. Dialysis of the nanoparticles

Cold, quick and basic dialysis was used. 6 * 150 ml of ***SiO_2@ZrO_2 I + II*** were filled into cellulose membrane tubes (CelluSep H1 membrane, high grade, flat width 46 mm, MWCO 3500 Da, Interchim). The tubings were closed with closures and 450–450 ml of them dialysed against 5 * 5–5 L pure water (Sartorius arium 611, 18.2 Ω.cm) basified with 1 M KOH to a pH of 8–9 and precooled to 4 °C.

2.2. Characterization of SiO_2@ZrO_2 nanoparticles

Morphological investigations of the NPs were performed using a JEOL JEM-2200FS transmission electron microscope operated at 200 kV with Cs corrector. Diluted samples were dropped and dried on holey carbon coated copper grids (200 mesh).

FTIR absorption spectrum was measured using a Perkin-Elmer Frontier MIR Spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector and a single reflection attenuated total reflection (UATR) unit (SPECAC "Golden Gate") with diamond ATR element. At 4 cm^{-1} resolution 32 scans were measured.

As the analysis of the samples confirmed that the two batches had very similar physico-chemical properties, they were mixed together, and the solid content, pH and zeta potential of the resulting sample was measured, which was named ***SiO_2@ZrO_2 I + II***.

Sample ***SiO_2@ZrO_2 I + II*** was used for the retention tests. It was stored in the fridge at 4–8 °C prior to the experiments and manipulated at room temperature.

2.3. Experimental setup

The following concentration of treatments were applied for both the experiments with SiO_2@ZrO_2 nanoparticles and with $ZrOCl_2$. Control treatment contained only tap water.

control: no supplementation
treatment 1: supplemented with 0.50 μg ml^{-1}
treatment 2: 5.0 μg ml^{-1}
treatment 3: 10 μg ml^{-1}
treatment 4: 20 μg ml^{-1}

Treatment concentrations were chosen based on the expected level of application of the synthesised NPs as drug delivery systems as well as a recent publication of Karthiga et al. (2018) investigating the effect of zirconium oxide nanoparticles on the embryonic development of zebrafish (Karthiga et al., 2019).

For treatments with Zr nanoparticles, the solution of the ***SiO_2@ZrO_2 I + II*** silica-zirconia core–shell nanoparticle sample was used in water. Treatments with $ZrOCl_2$ were prepared using solutions of solid zirconia oxychloride 8 hydrate (Scharlau).

Daphnia magna were collected from an artificial pond and reared isolated under laboratory conditions prior to their experimental usage. The trials were set in 4 L volume plastic tanks with 6 g of *D. magna* (wet weight) in each. Tanks were filled with continuously aerated tap water with a constant temperature of 22 ± 2 °C. A 16–8 h of light–dark illumination was provided for the model culture of zooplankton. Each treatment was set in triplicate ($n = 3$) and the plastic containers were arranged in a completely randomized design. After the 72 h of treatment *D. magna* was harvested by plankton net of 150 μm mesh size and rinsed with ultrapure water (Millipore MilliQ) to avoid surface contamination of the experimental media.

Juvenile *D. rerio* individuals (zebrafish) were purchased for research purposes at 60 days post hatch and were kept for 48 h in tap water at 23 ± 2 °C prior to the experiments. Rectangular, 40 L volume glass aquaria were used for the trials where the five treatments were set in triplicate and in a randomized design. In each aquarium 10 zebrafish juveniles were placed, with gender equality. The initial individual wet body weight was 0.260 ± 0.04 g and size homogeneity were tested by ANOVA where no significant difference ($p > 0.05$) occurred among the treatments. Aerated tap water was used as a rearing media with an oxygen concentration maintained by individual piped filters. Treatments were added to tap water and the experiment lasted for 96 h in 16–8 h (light–dark) illumination. Most important water quality parameters were monitored during the trial and found to be the following:

- water temperature (HACH HQ30D): 22.85 ± 1.28 °C
- dissolved oxygen (HACH HQ30D): 7.51 ± 0.51 mg L^{-1}
- oxygen saturation: 88.94 ± 4.43 %
- pH (HACH HQ30D): 8.57 ± 0.03
- nitrogen species (HACH DR3900): 0.23 ± 0.13 mg L^{-1} of NH_3^+ and 0.01 ± 0.01 mg L^{-1} of NO_2^-

Fish received no feed during the experiment. Aquarium were checked daily for dead individuals. After the trial period *D. rerio* individuals were collected by fish net and were rinsed with ultrapure water to reduce the positive error in the analytical results. The sacrificed procedure was by physical methods suggested in the AVMA Guidelines on Euthanasia for fish reported by the American Veterinary Medical Association. Fish samples were kept frozen prior to the further laboratory analyses.

2.4. Sample preparation and Zr analysis

D. magna samples were dried in glass beakers in a drying cabinet until constant weight (12 h) and digested in the same vessels by adding 5 ml 65% (m/m) HNO_3 and 1 ml of 30% (m/m) H_2O_2 (both reagent grade, Merck, Hungary) on an electric hot plate. Samples were then diluted with 1% (v/v) nitric acid (reagent grade, Merck and Milli-Q water) in volumetric flasks of 25 ml and kept refrigerated prior to elemental analysis.

Zebrafish individuals were dried until constant weight in a drying cabinet (24 h) and digested with the mixture of 5 ml 65% (m/m) HNO_3 and 1 ml of 30% (m/m) H_2O_2 in glass beakers on an electric hot plate. Samples were then diluted with 1% (v/v) nitric acid (reagent grade, Merck and Milli-Q water) in volume calibrated plastic (PP) test tubes of 10 ml and were stored in refrigerator until the analysis. The dry weight of both zooplankton and fish samples were determined on analytical balance (Precisa 360 ES).

Elemental concentration was determined by inductively coupled plasma optical emission spectrometry (ICP-OES 5110 Vertical Dual View, Agilent Technologies). Auto sampler (Agilent SPS4), Meinhard® type nebulizer and double pass spray chamber were used as well as a five-point calibration procedure was applied (ICP VI, Merck). Certified reference material (ERM-BB422, fish muscle) was treated along with the test organisms to verify the chosen elemental analytical method. The recoveries of the measured elements were within 10 % of the certified values for the metals. The wavelengths and measuring parameters were chosen based on the cookbook of the ICP Expert software.

2.5. Evaluation of experimental data

The statistical analysis of experimental data was carried out in SpSS/PC+ software package. ANOVA was used to compare the Zr concentration results in the applied treatments, as well as the bioconcentration factors and elemental analytical data. The homogeneity of variance was verified by Levene test and significant differences were evaluated by Tukey multi-comparison test where difference was considered to be statistically significant when $p < 0.05$. Redundancy analysis (RDA) was performed in Canoco for Windows 4.5 to investigate the interaction between the studied species (zooplankton and fish) and their environmental background (rearing media): water to *D. magna* and water to *D. rerio*. This test was first mentioned by Rao in 1964, but gained wider use after the publication of Braak & Prentice in 1988 (Ter Braak, 1988; Rao, 1964). It has been used – among others – in environmental assessment and monitoring studies as well as to evaluate the interaction between inorganic contaminants and test organisms regarding aquatic toxicology (Fehér et al., 2013; Herman et al., 2020a; ter Braak and Verdonschot, 1995; Varga et al., 2020). The bioconcentration factor (BCF) for *D. magna* and *D. rerio* was calculated by dividing the Zr content measured in the organisms ($C_{organism}$, mg kg^{-1} dry weight) by the same concentration values of the rearing water media ($C_{organism}$, mg L^{-1}): BCF $= C_{organism}/C_{water}$

3. Results

3.1. Characterization of the prepared $SiO_2@ZrO_2$ I + II sample

The large volume synthesis of the silica-zirconia core–shell nanoparticles were performed in two batches. Before merging it was made sure that they were of identical characteristics.

The size of the particles was monitored throughout the synthesis by dynamic light scattering (DLS) technique (Fig. 1). It showed slight increase after the deposition of zirconia on the surface of silica cores. The resulting mean diameter and size distribution were identical for the two batches. We experienced a second increase in mean size when the particles were transferred into water. This latter was due to the completion of zirconia shell crystallization and also due to a larger hydration sphere of the oxide surface in water than in ethanol. Small amount of aggregates were observed (6% in volume of the total nanoparticle volume).

The FTIR analysis of the samples (Fig. 2) confirmed the formation of identical zirconia shells around the silica core in the two batches (***$SiO_2@ZrO_2$ I*** and ***$SiO_2@ZrO_2$ II***). After dialysis into water, we observed the increase of intensity of hydrogen carbonate vibrational bands at 1640 cm^{-1} in comparison to the bicarbonate species' vibrational bands at 1560–1320 cm^{-1}. The pH, DLS volume mean size (nm), zeta potential (mV) and solid content (mg ml^{-1}) of ***$SiO_2@ZrO_2$ I + II*** are summarized in Table 1. The TEM analysis of the prepared material showed the spherical morphology of the particles presenting a rough surface with some spurs. The formation of some aggregates is also visible.

3.2. Retention of Zr from $SiO_2@ZrO_2$ nanoparticles in Daphnia magna and Danio rerio

The quantitative measurement results proved that the level of Zr increased in parallel with the concentration of the added $SiO_2@ZrO_2$ NPs, as indicated in Fig. 3A. In the control group, no Zr was observed above the limit of detection of the ICP-OES instrument, while the highest concentration was detected in treatment 4 (20 μg ml^{-1}). Thus, significant difference was observed, showing a rapid uptake of Zr in the 72 h of exposition period. Neither vitality nor lethality of zooplankton was observed to be different in the experimental groups suggesting that the

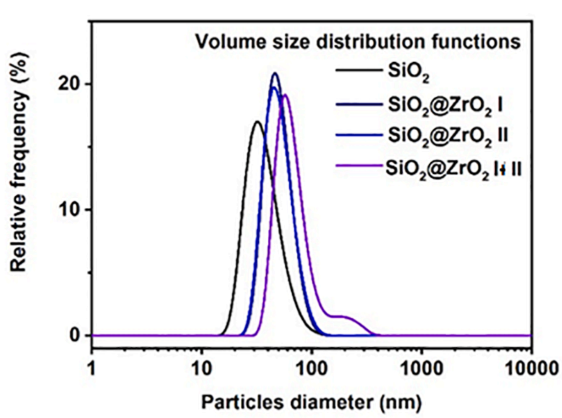

Fig. 1. Progress of DLS size distribution of the nanoparticles during synthesis.

Zr retention did not cause the acute toxicity of the daphnids.

A constrained ordination procedure of RDA was used to determine the effects of environmental variables and model species regarding the Zr level (Fig. 3B). The correlation between the Zr concentration of the rearing media and the concentration of the same elements in *D. magna* was 0.962, while the species-environment correlation was 0.981. The cumulative percentage variance for the species data revealed to be 96.2 and 100.0 for the species-environmental relation. The strong correlation between the Zr content of the rearing media and *Daphnia* organisms shows cause-effect relationship further proving the retention of the nanoparticle in the 72 h of acute toxicity test.

As indicated in Fig. 4A, $SiO_2@ZrO_2$ NPs gained access to the tissues of zebrafish individuals in all treatments compared to the control, as statistically proven difference occurred between the control and the exposed groups ($p = 0.004$; $F = 4.491$). The level of measured Zr in *D. rerio*, however, was lower in dry weight than found in *D. magna* organisms. RDA was applied to evaluate the interaction between the rearing media and the indicator organism of *D. rerio* (Fig. 4B). The correlation between the Zr concentration of the rearing media and the concentration of the same element in *D. rerio* was 0.773, while the species-environment correlation was 0.879. The cumulative percentage variance for the species data revealed to be 96.2 and 100.0 for the species-environmental relation. Statistics indicate correlation between the exposition media and the zebrafish individuals, yet in a lower extent compared to the investigated zooplankton organisms.

The calculated BCFs of the test organisms (Table 2) also prove the higher occurrence of Zr in *D. magna* compared to *D. rerio*. According to the statistical analysis, significant difference was found between treatment 1 and the other experimental groups in both cases: the highest BCF values were observed in the groups where NPs exposition was the lowest (supplemented by 0.50 μg ml^{-1} $SiO_2@ZrO_2$). Treatment 3 (supplemented by 10 μg ml^{-1} $SiO_2@ZrO_2$) and 4 (supplemented by 20 μg ml^{-1} $SiO_2@ZrO_2$) did not differ statistically from each other.

3.3. Elemental content of $SiO_2@ZrO_2$ nanoparticle supplemented Danio rerio

The multielement technique provided the opportunity to analyse the quantity of the most important macro and micro elements in fish tissues. As indicated in Table 3, the elemental concentration of the treatments showed no significant difference according to the ANOVA test either between the treatments or compared to the control ($p > 0.05$). Results suggest that the elemental homeostasis of zebrafish individuals was not negatively affected by the short-term exposition of Zr containing NPs via water.

3.4. Retention of Zr from $ZrOCl_2$ in Danio rerio

The Zr level of zebrafish samples treated by $ZrOCl_2$ in analogous concentration to the NP study provided a completely different accumulation pattern. No significant difference was observed between the Zr content of the experimental groups ($p > 0.05$): low levels were determined with high standard deviation suggesting no retention of Zr in zebrafish individuals from the $ZrOCl_2$ treatments (Fig. 5.)

Results of the Zr retention from the molecular form are demonstrated by the BCF indicated in Table 4: no accumulation of the Zr from $ZrOCl_2$ was observed, and data are lower in each treatment compared to the ones determined for $SiO_2@ZrO_2$ NPs.

3.5. Growing performance of D. reiro

Fig. 6 indicates the average final wet body weight (WBW_f) of *D. reiro* individuals. Compared to the average initial WBW_f (0.260 ± 0.04 g) of the test organisms, no significant different occurred either in the $SiO_2@ZrO_2$ NP supplemented groups or in the $ZrOCl_2$ supplemented ones ($p > 0.05$). The same is concluded when comparing the treated

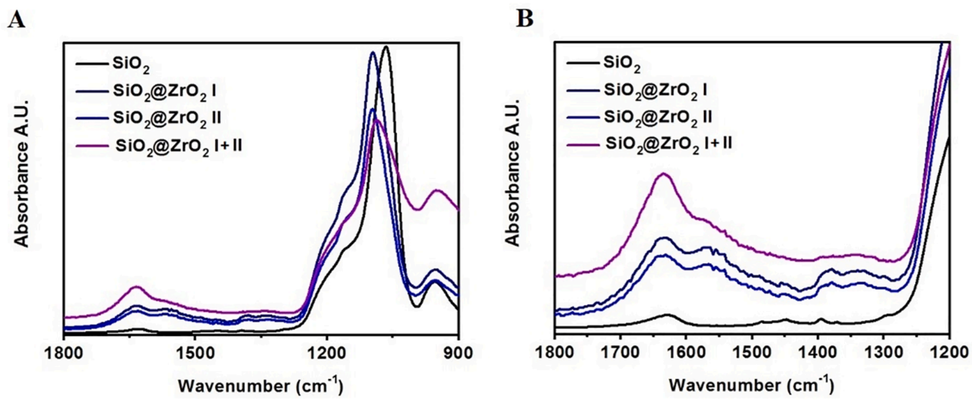

Fig. 2. FTIR spectra of silica and silica-zirconia core–shell nanoparticles in ethanol (A) and in water (B).

Table 1
Characterization results of the synthesized Zr nanoparticles.

Sample	pH	DLS volume mean size (nm)	Zeta potential (mV)	Solid content (mg/ml)
SiO_2		38 ± 14		
$SiO_2@ZrO_2$ I		51 ± 14		
$SiO_2@ZrO_2$ II		51 ± 15		
$SiO_2@ZrO_2$ I + II	8.5	68 ± 24 94%	−19 ± 1	0.72 ± 0.03

groups to the control: no statistically proven difference is observed ($p > 0.05$). The survival of the fish individuals was 100% in each aquarium.

4. Discussion

The industrially controlled, favourable properties of nanomaterials enable their novel commercial application that can directly or indirectly led to their environmental release. Nanocarriers are a relatively new and promising approach for the safe delivery of therapeutic drug products. Zirconia has been studied lately as a material for biomedical applications and as a potential platform for vaccine delivery in nanoparticle form (Huang et al., 2018; Nagy et al., 2020). However, the complex ecotoxicological investigation of Zr containing NPs is yet to be carried out.

Zirconia and its salts are not considered to be particularly hazardous to the environment. While land plants do not tend to accumulate, aquatic plants are shown to adsorb dissolved Zr compounds. However, assays conducted with aquatic vertebrates proved low toxicity of Zr: 96-hr LC50 >20 mg L^{-1} and 96 h minimal stress concentration >20 mg L^{-1}, according to Couture et al (Couture et al., 1989). Zirconia as a NP component thus can be considered a promising initiative from environmental point of view since the element itself does not pose a particular risk to the terrestrial or aquatic ecosystem. At the same time, it was recently shown that the human and ecotoxicology of engineered nanomaterials are dependent of several molecular and physicochemical mechanisms which influence their interaction with cells thus determine their overall potential hazardous features (Huang et al., 2017).

Present study focuses on the aquatic toxicology of Zr containing NPs by adsorption experiments conducted under laboratory conditions. The result proves that retention of Zr from the nanoparticle form occurs in zooplankton via water. *D. magna* is a key species in fresh and brackish water habitats, being primary consumer and prey of many planktivorous fishes; in the early stages of ontogeny, *D. magna* is the exclusive feed of some species. Our findings correlate well with the conclusions of Załęska-Radziwiłł and Doskocz who published an article in 2015 regarding the ecotoxicology of Zr nanoparticles in aquatic invertebrates (Załęska-Radziwiłł and Doskocz, 2016). The research group did not determine the accumulation tendency contrary to the present study, but a toxicology test was conducted on different protozoans (*Tetrahymena thermophila*, *Thamnocephalus platyurus* and *Daphnia magna*) and the greatest effect of Zr containing NPs was observed on *D. magna*, where long treatment period resulted in biodiversity change. However, no organoleptic difference was observed in acute toxicity tests, compared to the control group. Our findings support their conclusion regarding the possible

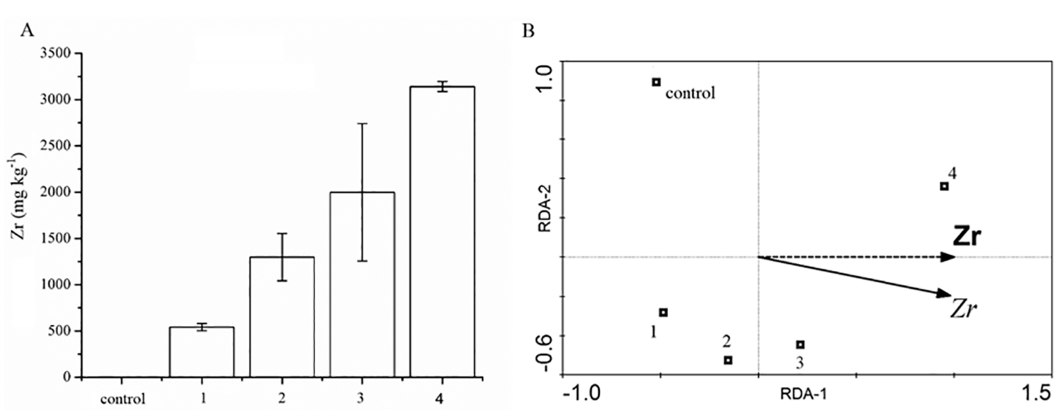

Fig. 3. A. The concentration of Zr in *D. magna* treated by $SiO_2@ZrO_2$ core–shell NPs (mg kg^{-1} ± SD, n = 3) B: Redundancy biplot of rearing water and *D. magna* regarding the Zr concentration (Solid arrow: elemental concentration of water, dashed arrow: elemental concentration of *D. magna*. Squares with numbers indicate experimental groups) Control: no supplementation; 1: supplemented by 0.50 μg ml^{-1} $SiO_2@ZrO_2$; supplemented by 5.0 μg ml^{-1} $SiO_2@ZrO_2$; 3: supplemented by 10 μg ml^{-1} $SiO_2@ZrO_2$; 4: supplemented by 20 μg ml^{-1} $SiO_2@ZrO_2$.

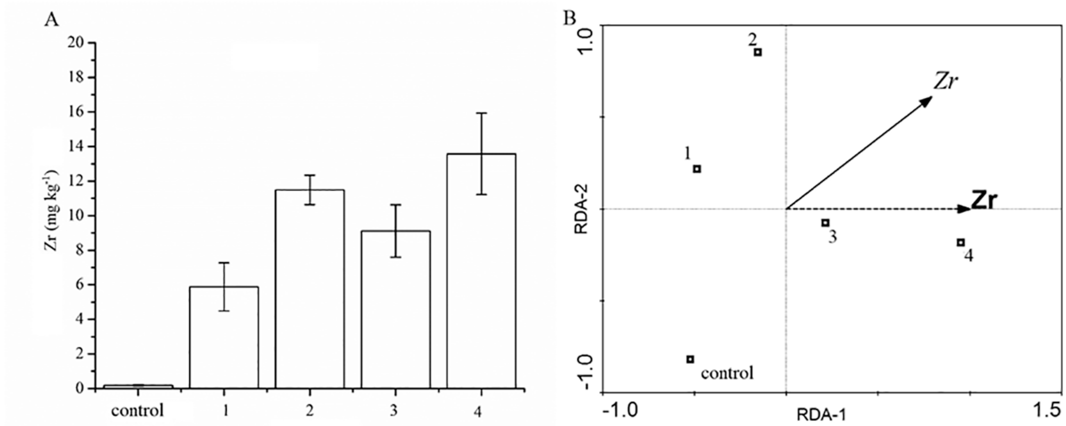

Fig. 4. A. The concentration of Zr in *Danio rerio* treated by $SiO_2@ZrO_2$ core–shell NPs (mg kg^{-1} ± SD, n = 3) B: Redundancy biplot of rearing water and *D. rerio* regarding the Zr concentration (Solid arrow: elemental concentration of water, dashed arrow: elemental concentration of *D. rerio*. Squares with numbers indicate experimental groups) Control: no supplementation; 1: supplemented by 0.50 μg ml^{-1} $SiO_2@ZrO_2$; supplemented by 5.0 μg ml^{-1} $SiO_2@ZrO_2$; 3: supplemented by 10 μg ml^{-1} $SiO_2@ZrO_2$; 4: supplemented by 20 μg ml^{-1} $SiO_2@ZrO_2$.

Table 2
Bioconcentration factors of the test organisms treated by $SiO_2@ZrO_2$ core–shell NPs.

Model organism	Treatment	BCF
Daphnia magna	1	1081
	2	259.3
	3	199.8
	4	157.1
Danio rerio	1	11.76
	2	2.298
	3	0.9111
	4	0.6788

Table 3
Elemental content of *Danio reiro* supplemented by $SiO_2@ZrO_2$ core–shell NPs.

mg kg^{-1} ± SD	Control	1	2	3	4
Ca	9285 ± 607	10614 ± 457	10280 ± 1158	8670 ± 582	9068 ± 494
Cu	1.359 ± 0.1008	1.094 ± 0.05298	1.205 ± 0.1160	1.053 ± 0.09026	1.069 ± 0.03507
Fe	19.93 ± 1.847	17.48 ± 0.7608	19.75 ± 3.015	19.45 ± 1.750	24.35 ± 4.939
K	4086 ± 320.9	3839 ± 129.1	3999 ± 404.1	3480 ± 199.0	3571 ± 79.51
Mg	455.7 ± 35.26	416.9 ± 11.86	416.9 ± 39.69	379.9 ± 23.82	368.7 ± 10.79
Mn	0.959 ± 0.114	0.617 ± 0.084	0.600 ± 0.083	0.574 ± 0.074	0.502 ± 0.043
Na	1175 ± 92.38	1165 ± 53.96	1199 ± 108.0	1048 ± 61.33	1119 ± 24.76
P	7474 ± 584.1	7648 ± 222.3	7636 ± 802.4	6785 ± 398.3	6943 ± 241.7
S	2764 ± 215.6	2593 ± 119.7	2681 ± 219.4	2378 ± 157.6	2439 ± 45.62
Sr	25.52 ± 2.718	25.89 ± 1.842	25.98 ± 3.372	23.59 ± 2.747	18.99 ± 2.772
Zn	68.75 ± 4.717	64.05 ± 3.524	65.74 ± 4.541	58.85 ± 3.541	55.95 ± 6.755

harmful effect of Zr containing NPs to aquatic ecosystems since significant adsorption of Zr was observed in the daphnids after the 72 h of treatment period.

Experiments conducted with zebrafish suggest a lower accumulation tendency of Zr from the NP form, as smaller concentration calculated to dry weight was found in the fish tissues compared to the zooplankton

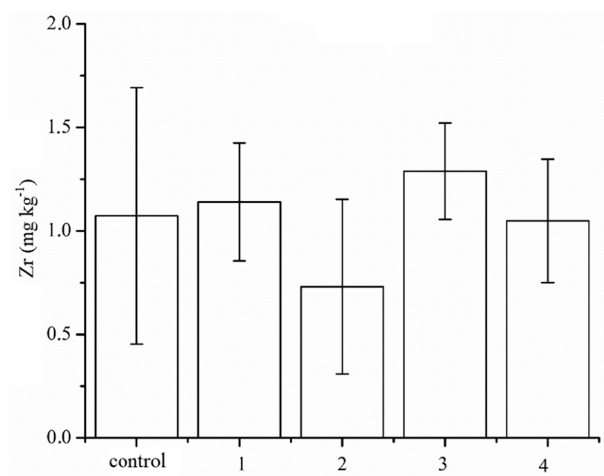

Fig. 5. The concentration of Zr in *Danio rerio* treated by $ZrOCl_2$ (mg kg^{-1} ± SD, n = 3) Control: no supplementation; 1: supplemented by 0.50 μg ml^{-1} $ZrOCl_2$; supplemented by 5.0 μg ml^{-1} $ZrOCl_2$; 3: supplemented by 10 μg ml^{-1} $ZrOCl_2$; 4: supplemented by 20 μg ml^{-1} $ZrOCl_2$.

Table 4
Bioconcentration factors of the *Danio rerio* treated by $ZrOCl_2$.

Model organism	Treatment	BCF
Danio rerio	1	2.280
	2	0.1460
	3	0.1290
	4	0.05200

organisms. However, a significant increase of Zr was determined in zebrafish individuals from the higher dosage treatments than those from the control. The retention of Zr was therefore confirmed by the results in *D. rerio*, yet at a smaller level than in *D. magna*. The observation was further confirmed by the RDA results. Correlation was found between the Zr content of the rearing medium and the zooplankton organism as well as the zebrafish individuals, but the correlation was stronger in the retention route of water to *D. magna*. The decreased extent of correlation can be explained by the fact that different exposure routes result in different accumulation efficiency. *Daphnia* organisms keep a constant whole-body exchange with their surrounding water media as well as being filter feeders, daphnids ingest very small particles: even NP

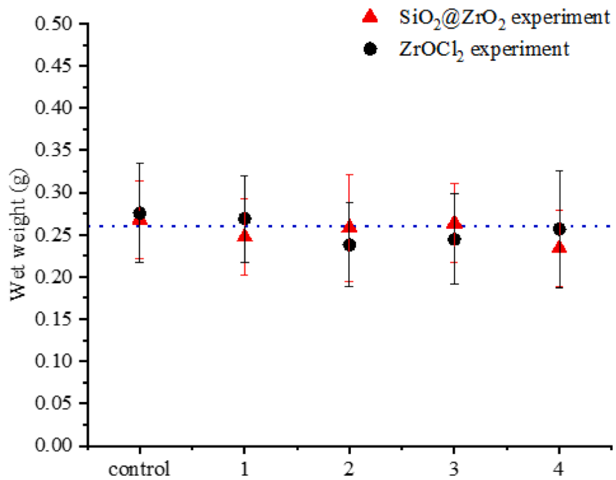

Fig. 6. The average wet weight (mean ± SD, n = 10) of the *D. reiro* individuals in the $SiO_2@ZrO_2$ and $ZrOCl_2$ treatments. (Blue dashed line indicates the average individual wet weight of the fish: 0.260 ± 0.04 g). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

aggregates under 70 μm is readily taken up by them (Xiao et al., 2015). It was shown that the accumulation of NPs usually occurs in the acute time-period of 24–72 h, indicating that the retention tendency in zooplankton organisms is quite fast. In contrast, aquatic vertebrates have more involved excretion routes to efficiently remove toxic metals from their body resulting in the lower accumulation of elements with adverse effects (Kawasaki et al., 1982). Metal retention also occurs in certain organs in higher levels than in others which contribute to a small extent to the total fish body weight (Herman et al., 2021b; Wg and Gr, 1997).

Our model experiment further supports the results of Karthiga et al. by evaluating the exposure and retention level of Zr containing NPs. Their research group published the first results in 2018 regarding the ecotoxicology of Zr containing NP by evaluating its effect on the embryonic development of zebrafish. The group did not carry out accumulation study but conducted exposition experiments in similar dosage to present work and found developmental deformities, hatching delay, malformation and even mortality among the direct effects (Karthiga et al., 2019). The observed adsorption of Zr in zebrafish tissues in present work provides explanation to the negative impact they concluded. Cyto-toxicological processes may arise even at lower uptake level due to the particle size, which is known to contribute NP toxicity (Jiang et al., 2008).

Bioaccumulation factor is successfully used in the assessment of the environmental load caused by different organic and inorganic contaminants to numerically express the retention level of pollutants. If certain limitations of the method are considered, it can be applied for hazard regulation, prediction and to compare the accumulation tendency of the compounds. Fish species can be applied effectively for bioconcentration studies due to the lipid-content of their organs, body fluids and tissues (Adolfsson-Erici et al., 2012; Harangi et al., 2017; Herman et al., 2021b). The BCF data calculated for the treatments suggest that *D. magna* uptake the Zr content of the NPs in a much higher level compared to the zebrafish individuals. Zooplankton species, however, tend to eliminate elements rapidly, thus BCF calculation itself is not completely appropriate for risk assessment. Exoskeleton shedding, for instance, is a known excretion route for daphnids to reduce toxic metal body content and even to regulate the level of essential elements during their molting process. Bioconcentration is still an important indicative in estimating the persistence of chemicals in the aquatic environment and its careful application can improve the ability of environmental health protection (Trowell et al., 2018).

It was also found that the gained BCF values are the highest in the first treatments of the experiments conducted with *D. magna*, while the increasing level of Zr NPs added resulted in the decreasing values of BCF. Similar phenomena were observed for the zebrafish exposures, that the higher dose of treatments lowered the BCF results. This finding also correlates with the conclusion of Karthiga et al. (2018) as they stated, that the lower the concentration of the Zr nanoparticle, the higher the posed hazard to the living organisms (Karthiga et al., 2019). Concentration dependent toxicity has already been observed for other NPs. Naqvi et al. (2010) studied the dosage- and time-affected impact of iron oxide nanoparticles and found that more cell toxicity is observed at lower exposition levels (Naqvi et al., 2010).

It was found that inorganic NPs affected the trace element homeostasis of living organisms. Abdelhalim et al. investigated the distribution of elements in rat tissues after the in vivo treatment of the animals by gold NPs and statistically proven alteration was observed in the elemental level of the GNP exposed animal tissues of liver, kidney, heart as well as blood compared to the control (Wang et al., 2012). Amara et al. studied the elemental content of rat brain after ZnO NP treatment and reported a decreased Fe and Ca level compared to the non-treated animals (Amara et al., 2015). In the acute toxicity assay Zr elevated in the tissue of zebrafish exposed to Zr NPs, yet no significant change in the trace element homeostasis was observed of the organisms as the concentration of the measured elements remained constant.

Our results further suggest that Zr from the molecular form was either not available to *D. rerio*, or being a vertebrate of higher trophic level, have more developed excretion routes compared to the zooplankton species since no statistically proven difference was found between the treatments and the control when $ZrOCl_2$ was added to the rearing media of zebrafish. Our observation is in correlation with the finding of Załęska-Radziwiłł and Doskocz (2015) who carried out the first time in the literature an ecotoxicological study on Zr containing NPs and found that the nano form had higher toxicity for crustaceans compared to the molecular form of the same compound. This observation is further proved by present retention study suggesting that the dissolved salt form of Zr is posing less hazard to the aquatic ecosystem, than Zr containing NPs. The increased surface area of the nano form results in different properties which determine the magnitude of bioavailability and possible environmental risk. However, in contrast to ZrO_2, TiO_2 NPs did not show statistical difference in accumulation tendency compared to the ionic titanium to zebrafish eleutheroembryos in acute toxicity tests (Oliver et al., 2015). The assessment of potential ecological hazard is therefore quite complex since nanomaterials show distinct adsorption behaviour in the aquatic model organisms. While both the ionic form of Ti and Zr are considered to be non-toxic, for instance, the NP form of the two elements show different retention tendency. Titanium is considered to be a safe bio-implant and only a few reports conclude its adverse effect, as well as the TiO_2 NP was observed to be non-accumulative. In contrast, Zr as an ionic compound is known to have very low environmental hazard, yet the NP form shows retention tendency in aquatic organisms. Based on the BCF values the adsorption of Zr from the core shell NP in zebrafish individuals in all treatments was below that of the 100 regulated in REACH, but was significantly higher compared to the BCF values calculated for the same exposition level of the molecular form. Also, the retention of NP ZrO_2 was significantly elevated in the zooplankton experiments. For the safe manufacturing and application of the presented Zr core–shell NP as a biomedical product, more extensive experiments are required to map the environmental aspects of the released material.

5. Conclusion

The thorough investigation of the environmental and health concerns of engineered NPs is an emerging task of nanotoxicology due to the extending worldwide utilization of these materials. The ecotoxicological effects of NPs are usually studied by different indicator species where mortality is the most applied endpoint of the experiments. However, the exact determination of their retention tendency in tissues and body fluids provide further information regarding their toxic mechanisms. Our findings suggest that the NP form of ZrO_2 poses no acute toxicity to the chosen aquatic indicator organisms, however, adsorption of the element was found in both *D. magna* and *D. reiro*. No bioaccumulation was proved for the vertebrates, but statistically higher concentration of Zr was qualitatively measured in the tissues of zebrafish treated by the same exposition doses of the NP compared to the molecular form. The limited knowledge of the ecotoxicology of Zr containing NPs are expanded by present study, however, the long-time effect is important to be further investigated. In contrast to the medium to no toxicity of Ti containing NPs in short term tests, the long-term assay proved the concentration- and time-affected inhibition of zebrafish growth, resulted in decreased liver weight ratio as well as the histopathologic alteration of the gills was observed. Even the translocation of Ti in different organs was concluded (Chen et al., 2011a). In order to gain deeper understanding of the environmental hazard of emitted Zr containing NPs long-term experiments are to be conducted and the biochemical mechanisms should be carefully assessed.

Compliance with Ethical Standards

All experiments and procedures were performed in compliance with relevant laws and institutional guidelines and the appropriate institutional committee have approved them. Animal experiments were carried out in accordance with the EC Directive 86/609/EEC.

CRediT authorship contribution statement

Zsófi Sajtos: Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Milán Fehér:** Conceptualization, Formal analysis, Writing – review & editing. **Áron Molnár:** Data curation. **László Stündl:** Funding acquisition, Supervision. **Livia Naszályi Nagy:** Data curation, Formal analysis, Writing – review & editing. **José C. Martins:** Formal analysis. **Sándor Harangi:** Methodology, Validation. **István Magyar:** Data curation, Formal analysis. **Krisztina Fehér:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing. **Edina Baranyai:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

Acknowledgements

The research was supported by the EU and co-financed by the European Regional Development Fund under the project GINOP-2.3.2-15-2016-00008. We acknowledge the Agilent Technologies, Inc. and the Novo-Lab Ltd. (Hungary) for providing the MP-AES 4200 and the ICP-OES 5100 instruments for the elemental analysis. This project has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 703374. E.D. acknowledges FWO-Vlaanderen for a fellowship (FWO-SB fellowship). K.F. acknowledges the support of the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (BO/004333/18/7) and the New National Excellence Program of Debrecen University (ÚNKP-20-4 Bolyai+). This research was supported by the National Research, Development and Innovation Office of Hungary (grant NKFI/OTKA NN 128368).

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