Environmental Pollution 157 (2009) 1157–1164

Contents lists available at ScienceDirect

Environmental Pollution

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

In vivo screening to determine hazards of nanoparticles: Nanosized TiO_2

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The response of a biological system to nanoparticles is unique and depends on their physico-chemical characteristics, dose and duration of exposure.

ARTICLE INFO

Article history:
Received 23 May 2008
Received in revised form
6 October 2008
Accepted 21 October 2008

Keywords:
Nanoparticles
Dietary exposure
Terrestrial invertebrates
Biomarkers
Biological activity

ABSTRACT

A single-species laboratory test with terrestrial invertebrates was used to identify the hazard of nanosized TiO_2. Feeding parameters, weight change, mortality, and the activities of catalase and glutathione-S-transferase were evaluated after 3 or 14 days of dietary exposure. The effects of nano-TiO_2 were dependent on exposure concentration and duration, total consumed quantity, size and pre-treatment of particles. The intensity of a response was ruled by duration of exposure and not by consumed quantity of nano-TiO_2 or exposure concentration as expected. The response to nano-TiO_2 is described as threshold-like. The exposure concentrations 10–1000 μg TiO_2/g dry food (1.35–1025 μg of total consumed quantity of TiO_2/g animal wet wt.) were identified as safe for tested species after tested exposure period. We conclude that the response to nanoparticles is different from that of soluble chemicals therefore these two types of data should be interpreted and processed differently.

1. Introduction

The ecotoxicity data on the effects of nanoparticles are in much need for the appropriate environmental risk assessment. Different documents already exist which deal with emerging and newly identified health risks (TGD Document, 2003; NANO Risk Framework, 2007; Scenihr, 2007). Development of a hazard profile is the critical step in characterizing the potential safety of nanoparticles, and the associated health and environmental hazards. A base set of hazard data has been suggested as a reference for characterization and prioritization of nanoparticles (Warheit et al., 2007a).

To characterize nanoparticles and its potential hazards sufficiently, empirical data are necessary. Since the early days of the REACH proposals (REACH, 2006), it has been agreed by all partners that the number of animals used to gain toxicity information on chemicals should be kept to an absolute minimum. There is evidence that in vitro and in silico methods for acute chemical toxicity are able to provide sufficient data to permit classification and labelling. However, for those substances with no available toxicity data a read-across and quantitative structure–activity relationship techniques (QSAR) are not possible, therefore in vivo testing is required to rapidly identify hazardous substances. Tests with invertebrates are suitable for such purposes since they are not subjected to the same legal restrictions as vertebrates.

We present a laboratory single-species toxicity test with the terrestrial arthropod (*Porcellio scaber*, Isopoda, Crustacea) for the purposes of hazard identification of nanosized TiO_2. The experimental design presented in this work provides data on biological responses from several levels of biological organisation; e.g. lower level (enzyme activities) and higher level (feeding, growth and mortality). The two enzymes investigated in the present study were catalase (CAT) and glutathione-S-transferase (GST), both of which are involved in antioxidant defence against reactive oxygen species. The main function of CAT is to catalyze the decomposition of hydrogen peroxide, while GST is a member of a large family of multifunctional enzymes involved in the cellular detoxification of many xenobiotics and physiological substances, including the endogenous products during lipid peroxidation. Our previous work has shown that the advantage of this test is that it provides a variety of toxicity data based on exposure concentration (such as lowest- and no-observed exposure concentration) and also exposure dose (lowest- and no-observed exposure dose). The isopod toxicity test system has been used successfully in metal and pesticide toxicity studies (Drobne, 1997; Stanek et al., 2006).

To validate our test system for testing of nanoparticles we selected nanoparticulate matter for which some toxicity information already exists (Hund-Rinke and Simon, 2006; Federici et al., 2007; Lovern and Klaper, 2006; Warheit et al., 2007a). The nanosized TiO_2 has a number of industrial applications such as a food

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doi:10.1016/j.envpol.2008.10.018

colouring, additive in pharmaceuticals and cosmetics and, due to its photo-physical properties, it is also used in a wide range of other consumer products (Masciangoli and Zhang, 2003). It had been considered biologically inert prior to studies with ultra-fine particles which showed that ultra-fine TiO_2 particles (20 nm in diameter) provoked an inflammatory response in laboratory test organisms (Oberdörster et al., 1994).

The aim of the present work was to investigate the hazard of nanosized TiO_2. We investigated: (a) exposure duration–effect relationship; (b) exposure concentration (dose)–effect relationship; (c) effect–particle size relationship; and (d) the effect of nanoparticle pre-treatment. We compare our toxicity data on TiO_2 with literature reports and discuss suitability of terrestrial isopods for hazard identification of engineered nanoparticles.

2. Materials and methods

2.1. Characterization of TiO_2 nanoparticles

Two sizes of commercially available TiO_2 nanoparticles (Sigma-Aldrich) were investigated: <25 nm in diameter (referred to here as 'smaller') and <75 nm in diameter ('larger'). The characteristics provided by the supplier are described in Table 1. Additional characterization of the test material was performed on the nanoparticles as delivered (either powder or liquid medium) and also dispersed in bidistilled water (pH value 5.7), which was used to prepare the food for isopod toxicity testing.

The commercial material was investigated by BET analysis (Brunauer–Emmett–Teller surface area analysis; Tristar 3000, Micrometrics) (Braunauer et al., 1938) to obtain information concerning the surface area of the solid material. Here, samples were dried and degassed with nitrogen prior to analysis.

Sonicated and non-sonicated dispersions of TiO_2 in bidistilled water were inspected by transmission electron microscopy (TEM) and a dynamic light scattering technique (DLS). The dispersions prepared in bidistilled water (0.0066; 0.066; 0.667 g/L) were sonicated on ice for 30 min using 10 s pulses with 13,872 J of the total input of energy (Sonics vibra-cell, Ultrasonic processor VCX 750 Watt; Sonics & Materials, Newtown, CT, USA). Both sonicated and non-sonicated dispersions were put on carbon-coated grids, dried at room temperature and examined by TEM (Philips CM 100).

The same concentrations of sonicated and non-sonicated dispersions prepared in ultra-pure water filtered through a 0.2 μm sieve (Millipore, Billerica, MA, USA; ion free, pH = 5.7) were inspected by DLS using a 3D-DLS-SLS Spectrometer (LS Instruments, Firbourg, Switzerland).

Table 1
Characteristics of TiO_2 nanoparticles studied in the present work.

	Small nanosized TiO_2	Large nanosized TiO_2
Supplier info (Sigma-Aldrich)	Nanopowder, anatase crystalline structure, particle size <25 nm, surface area 200–220 m^2/g	Amorphous liquid medium, dispersion 5 wt.% in H_2O, mixture of rutile and anatase crystalline structure, particle size <50 nm (XRD), <75 nm (BET), no data on surface area
BET (supplied material)		
Particle size (nm)	10	40
Specific surface area (m^2/g)	145	40
TEM (aqueous dispersion)		
Single particle size within the aggregates	10–20 nm (Fig. 1a)	10–120 nm (Fig. 1b)
Single particle shape	Elongated and round	Round
Description of aggregates	N, dense aggregates; S, net like, loose aggregate	N, loose aggregates
DLS (aqueous dispersion)		
Size of aggregates	N, 750–950 nm; S, 400–460 nm	N, 100–200 nm

Symbols: XRD, X-ray diffraction; BET, Brunauer–Emmett–Teller surface area analysis; TEM, transmission electron micrograph; DLS, dynamic light scatter; N, non-sonicated dispersion, S, sonicated dispersion.

2.2. Exposure of isopods P. scaber to TiO_2

2.2.1. Test organisms

Terrestrial isopods (*P. scaber*, Latreille 1804) were collected under the litter layer in an uncontaminated location in the vicinity of Ljubljana. In the laboratory, the animals were kept in a terrarium (20 × 35 × 20 cm) filled with a 2–5 cm layer of moistened sand and soil and a thick layer of partly decomposed hazelnut tree leaves (*Corylus avellana*). The substratum in the terrarium was heated to 80°C for several hours to destroy predators (spiders) before the introduction of the isopods. The culture was kept at controlled room temperature (21 ± 1 °C), 16:8 h light/dark regime and high humidity. The adults of *P. scaber* of both sexes and with body weights ranging from 30 to 80 mg, and all moult stages, were exposed to TiO_2 within 1–14 d after collection in the field. It has been previously shown, that these confounding factors do not influence the possible toxic outcomes of pollutants on isopods (Jemec et al., 2008).

2.2.2. Experimental design

Each animal was placed individually in a Petri dish, to which individual pieces of TiO_2-treated dry leaves were added. Humidity in the Petri dishes was maintained by regular spraying with tap water on the internal side of the lids. All Petri dishes were placed in a large plastic-covered glass container maintained at approximately 100% relative humidity and a 16:8 h light/dark regime without the direct proximity of the lamp (illumination 16 h with 203 nmol m^{-2} s^{-1} (15 lux), and 8 h with 67 nmol m^{-2} s^{-1} (5 lux)).

After 3 d and 14 d of exposure, lower and higher level endpoints were evaluated according to the test protocol (Table 2). Animal mortality was recorded, the surviving animals were weighed at the end of the experiments, and the leaves were dried at room temperature for 24 h prior to weighing. Faecal pellets were counted and weighed after drying in the exsiccator for 48 h. The animals were dissected and the digestive glands (hepatopancreas) were isolated for measurements of catalase (CAT) and glutathione-S-tranferase (GST) activities.

Altogether, four experiments were performed (Table 3). In two of them (A, B) we assessed the effect of duration of exposure (3d and 14d). In other two (B, C) the main focus was placed on the effect of particle size (<25 nm and <75 nm), and in one simple experiment (D) we tested the possible influence of the pre-treatment of nanoparticles. Based on previous results only one concentration (1000 μg of TiO_2/g dry food) of small size nanoparticles was selected for this purpose. Exposure concentrations presented in Table 3 are nominal concentrations, no actual concentrations on leaves were measured.

The number of animals tested in each experiment depended on the abundance of population collected prior to exposure (Table 3). Namely, our previous work has shown, that the animals investigated for enzyme activities have to be collected at the same field location and exposed immediately after the collection (Jemec et al., 2008).

Since currently no data exists on the environmental concentrations of nanosized TiO_2, the concentrations of TiO_2 used in this study were selected based on preliminary short-term studies, where the effects on enzyme activities were observed up to 3000 μg of TiO_2/g dry food (Jemec et al., 2008).

2.2.3. Food preparation

Food was prepared as previously described (Jemec et al., 2008). Hazelnut tree leaves were collected in uncontaminated woodland, dried at room temperature and the dry leaves were cut up into pieces of similar surface area, and weighed. Pieces of approximately 100 mg were selected for the experiments. Before the application of TiO_2 to the leaves, different concentrations of TiO_2 (0.0066; 0.066; 0.667 g/L) were suspended in bidistilled water with pH value of 5.7. The pH of the dispersions was independent of the concentration of TiO_2 and was the same as in bidistilled water.

Table 2
Summary of the test organism, nanoparticles tested, type of exposure and endpoints evaluated in the present paper.

Description	Endpoints evaluated	
	Lower level endpoints	Higher level endpoints
Test organism	Digestive glands	–Feeding rate
Invertebrate	–Glutathione-S-transferase activity	–Food assimilation efficiency
Isopoda, crustacea	–Catalase activity	–Animal mass change
Terrestrial isopod *Porcellio scaber*		–Mortality
Type of exposure		
3 d and 14 d		
Dietary exposure		
Chemical		
Nano-sized TiO_2 <25 nm; <75 nm		

Symbol: d, days.

Table 3
The total number of animals exposed in each experiment.

Suspension of TiO_2	Final exposure concentrations of TiO_2 (μg/g dry food)	Experiments			
		Total no. of exposed animals			
		A[a]	B	C	D
		3 d	14 d	14 d	14 d
	0	8 + 8 + 8	15	7	10
<25 nm non-sonicated	10	6	15		
	100	8 + 8 + 7	15	9	
	1000	10 + 10 + 10 + 6	15		10
<25 nm sonicated	1000				10
<75 nm non-sonicated	10			7	
	100			9	
	1000			10	

Symbol: d, days.
[a] Experiment A was repeated up to 4 times, each number indicates the number of animals in each exposure.

The TiO_2 was suspended using a vortex (20 s, 2000 rpm) and prepared freshly for each experiment. Surfactants were not used to disperse the TiO_2, since previous studies have shown that dispersion using solely sonication is adequate (Federici et al., 2007; Warheit et al., 2007b). 150 μl of the dispersion per 100 mg of leaf were applied onto the lower leaf surfaces and dispersed using a paintbrush. Dispersions of TiO_2 with concentrations 0.0066, 0.066 and 0.667 g/L resulted in final concentrations of 10, 100 and 1000 μg of TiO_2/g dry food. Prior to sampling of the suspension, the dispersion was each time rotated on a vortex for 5 s. Non-sonicated and sonicated dispersions of TiO_2 were applied to the leaves. The sonicated dispersion was prepared using a sonicator (30 min, 10 s pulses; Sonics vibra-cell, Ultrasonic processor VCX 750 Watt; Sonics & Materials, Newtown, CT, USA). Animals in the control group were fed with the leaves prepared in the same way, but treated with the distilled water only.

2.2.4. Determination of enzyme activities

Animals of both genders and at all moult stages were used for enzyme analyses and a separate enzyme sample was prepared from each animal. The whole digestive gland was homogenized for 3 min in 0.8 ml of 50 mM phosphate buffer pH 7.0, using a teflon–glass Elvehjem-Potter homogenizer. The homogenate was centrifuged for 25 min at 15,000*g* and 4 °C.

GST activity was measured on microtiter plates (Bio-Tek® Instruments, Winooski, VT, USA; PowerWave™ XS) (Habig et al., 1974; Jemec et al., 2007). Final concentrations of both 1-chloro-2,4-dinitrobenzene and reduced glutathione, prepared in 100 mM potassium phosphate buffer pH 6.5, were 1 mM. A detailed description of the preparation of 1-chloro-2,4-dinitrobenzene solution has been described previously (Jemec et al., 2007). 50 μl of the protein supernatant was added to start the reaction which was followed spectrophotometrically at 340 nm and 25 °C for 3 min. GST activity was expressed in nmoles of conjugated reduced glutathione min^{-1} mg $protein^{-1}$ (extinction coefficient, $\varepsilon_{340} = 9600\ L\ mol^{-1}\ cm^{-1}$).

Catalase activity was determined according to a published method (Aebi, 1984). 100 μl of protein supernatant was combined with 700 μl of hydrogen peroxide solution (11.6 mM) in 50 mM potassium phosphate buffer pH 7.0. The final concentration of hydrogen peroxide was 10.2 mM. The reaction was followed spectrophotometrically for 3 min at 25 °C and 240 nm in a Shimadzu ultraviolet-2101PC spectrophotometer (Shimadzu, Kyoto, Japan). Catalase activity was expressed in μmoles of degraded hydrogen peroxide min^{-1} mg $protein^{-1}$ ($\varepsilon_{240} = 43.6\ L\ mol^{-1}\ cm^{-1}$). The activities of both GST and CAT were measured three times in each sample.

Protein concentration was measured using a BCA™ Protein Assay Kit, a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

Table 4
The effects of nanosized TiO_2 on *P. scaber*.

Exposure concentration (μg/g dry food)	Particle size (nm)	AE	Feeding rate	CAT	GST	Weight change	Mortality
Exp. A: 3 d							
10	<25	/	/	/	/	/	/
100	<25	/	/	/	/	/	/
1000	<25	/	/	/	/	/	/
Exp. B: 14 d							
10	<25	**	**	/	/	/	/
100	<25	*	**	*	/	/	/
1000	<25	*	*	*	/	/	/
Exp. C: 14 d							
10	<75	*	/	/	/	/	/
100	<75	/	/	/	/	/	/
1000	<75	±	*	/	/	/	/
100	<25	*	±	*	/	/	/
Exp. D: 14 d							
1000	<25 N	/	/	*	/	/	/
1000	<25 S	*	/	*	/	/	/

The effects at a certain exposure concentration, which are significantly different in comparison to control, are shown. Symbols denote: (/) $p > 0.1$ – no effect, (±) $p < 0.1$, (*) $p < 0.05$, and (**) $p < 0.001$. Symbols: N, non-sonicated dispersion; S, sonicated dispersion; d, days; AE, food assimilation efficiency; CAT, catalase; GST, glutathione-S-transferase.

2.3. Data analysis

At the end of experiment faecal pellets were removed completely from the leaves using a brush, they were counted and weighted. Also, the leaves were weighted. The feeding rate and defecation rate of isopods were calculated as the mass of consumed leaf and mass of faecal pellets per animal wet weight per day, respectively. The food assimilation efficiency was calculated as the difference between the mass of consumed leaf and mass of faecal pellets divided by the mass of consumed leaf. The animal mass change was determined as the difference in animal mass at the beginning and at the end of the experiment. The amount of the daily consumed TiO_2 was calculated from the mass of consumed leaf and the corresponding applied concentration of TiO_2.

Homogeneity of variance was tested with Levene's test. The differences between the control and exposed groups of animals were determined by Kruskal–Wallis analysis and the Games-Howell post hoc test using SPSS for Windows 8.0 (SPSS Inc., USA). The comparison of data was done within a single experiment, no cross statistical comparisons between the experiments (A–D) were performed.

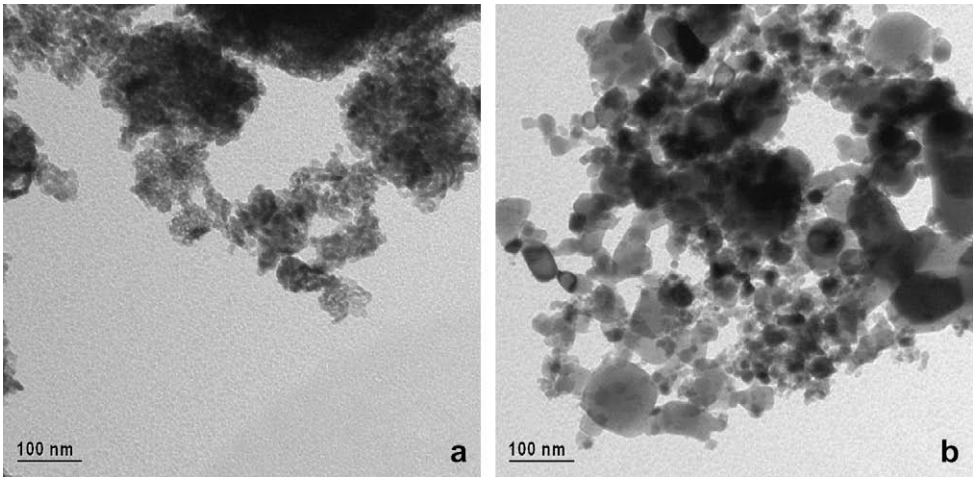

Fig. 1. Transmission electron micrographs of nanosized titanium dioxide (TiO_2) <25 nm (a) and <75 nm (b) in bidistilled water (non-sonicated).

Table 5
Comparison between the effects caused by the exposure concentrations, daily consumed doses and total consumed quantities of TiO_2.

Exposure concentration of TiO_2 (μg/g dry food)	Ex	Daily consumed dose of TiO_2 (μg/g wet wt./day)[a]	Total consumed quantity of TiO_2 (μg/g wet wt.)[a]	AE	Feeding	CAT
<25 nm, 10, 3 d	A	0.45	1.35	/	/	/
<25 nm, 10, 14 d	B	0.58	8.12	**	**	/
<75 nm, 10, 14 d	C	0.53	7.42	/	/	/
<25 nm, 100, 3 d	A	6.8	20.4	/	/	/
<25 nm, 100, 14 d	B	5.82	81.5	*	**	*
<25 nm, 100, 14 d	C	7.05	105	±	±	*
<75 nm, 100, 14 d	C	4.38	61.3	/	/	/
<25 nm, 1000, 3 d	A	73	219	/	/	/
<25 nm, 1000, 14 d	B	64.6	905.5	*	*	*
<25 nm, 1000 S, 14 d	D	55.7	835.5	/	/	*
<25 nm 1000 N, 14 d	D	61.1	916.5	*	/	*
<75 nm 1000, 14 d	C	73.21	1025	±	*	/

The effects at a certain exposure concentration/dose, which are significantly different in comparison to control, are shown. Symbols denote: (/) $p > 0.1$ – no effect, (±) $p < 0.1$, (*) $p < 0.05$, and (**) $p < 0.001$. Symbols: N, non-sonicated dispersion; S, sonicated dispersion; Ex, experiment; d, days; AE, food assimilation efficiency; CAT, catalase.

[a] Expressed per animal wet weight.

3. Results

3.1. Characterization of nanosized TiO_2 particles

The characteristics of nanosized TiO_2 are provided in Table 1. The BET analysis revealed that both sizes of nanosized TiO_2 formulations were in accord with the data provided by supplier (smaller, <25 nm; larger, < 75 nm). BET revealed a specific surface area of 145 m^2/g for the small sized TiO_2 nanoparticles and 40 m^2/g for the larger sized nanoparticles.

The TEM analysis showed that looser aggregates of nano-TiO_2 were formed when the dispersion was sonicated in comparison to non-sonicated small sized TiO_2. Looser aggregates were also formed in the case of larger nanosized TiO_2 in comparison to smaller one (Fig. 1).

Similarly, the size of aggregates as determined by DLS (0.0066 and 0.066 g/L of TiO_2) was lower in the case of sonicated smaller TiO_2 and larger TiO_2 form in comparison to non-sonicated small sized TiO_2. Concentrations of 0.667 g/L of TiO_2 were not examined by DLS, because at such high concentrations the signal was beyond the scale of the detector.

3.2. The effects of nanosized TiO_2 on P. scaber

The results presented in this work demonstrate that nanoparticulate TiO_2 in exposure concentrations 10, 100 and 1000 μg TiO_2/g dry food has no effect on mortality, weight change or GST activity in *P. scaber* after feeding with two sizes of nanosized TiO_2 dosed food for 3 d or 14 d. The activity of CAT and two feeding parameters (food assimilation efficiency and feeding rate) were changed in dependence of duration, dose, nanoparticle size and pre-treatment (Tables 4 and 5, Figs. 2–4).

When the animals were exposed to the same concentrations and size of nanoparticles in two different experiments, the results on AE and feeding rate were not entirely repeatable (at 100 μg and 1000 μg of small size TiO_2/g dry food). We explain this phenomenon in Section 4.

3.2.1. Exposure duration dependence

After three days of exposure, there were no changes in any of measured responses in animals fed on smaller nanoparticulate TiO_2

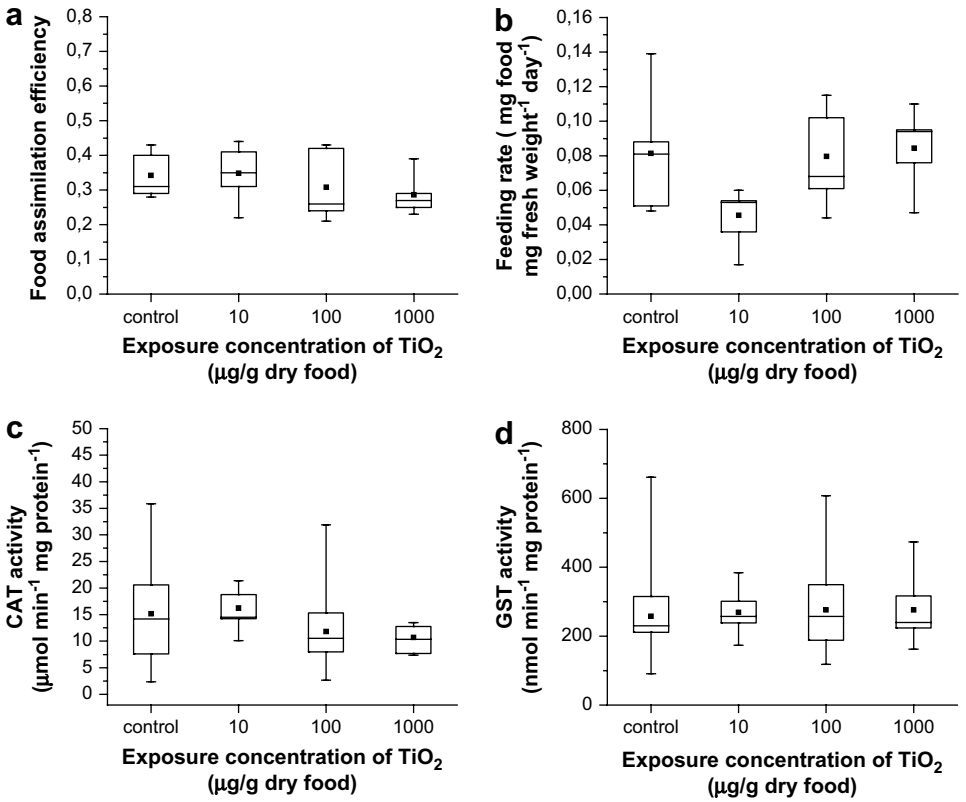

Fig. 2. The food assimilation efficiency (a), feeding rate (b), catalase (CAT) activity (c), and glutathione-S-transferase activity (GST) (d) in isopods fed with small sized TiO_2 (<25 nm) for 3 d (experiment A). Symbols on the box plot represent maximum and minimum value (whiskers: ⊥) and mean value (■).

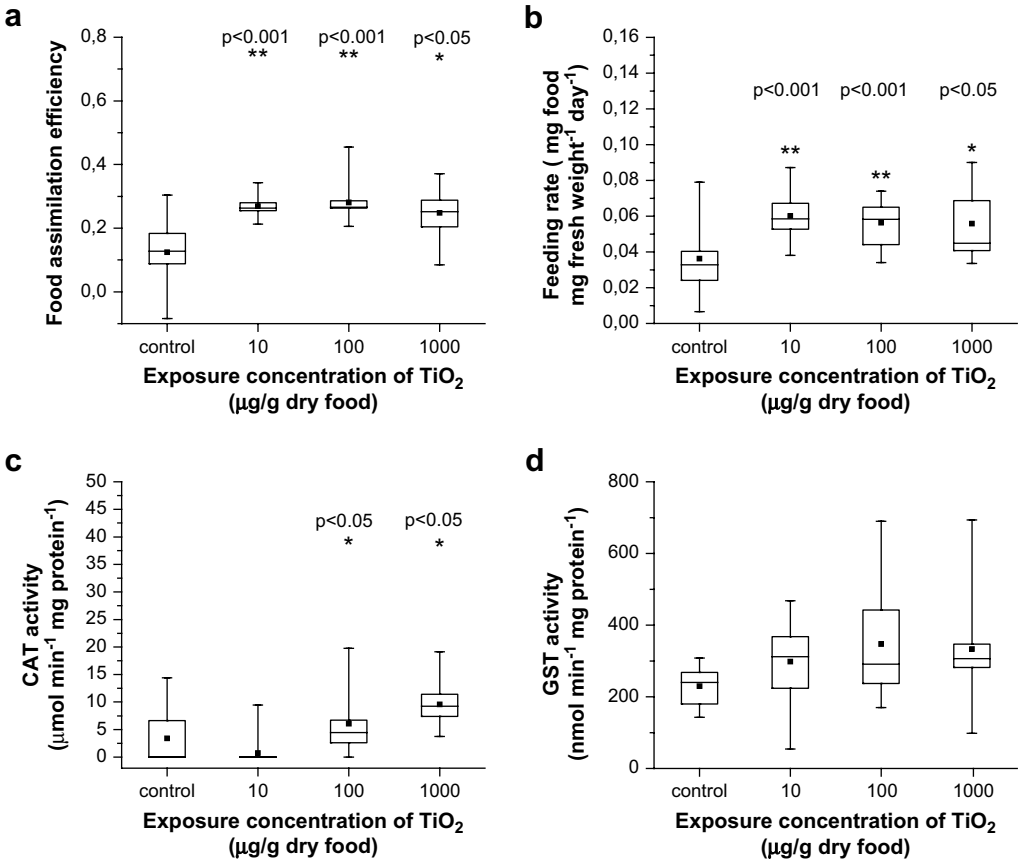

Fig. 3. The food assimilation efficiency (a), feeding rate (b), catalase (CAT) activity (c), and glutathione-S-transferase activity (GST) (d) in isopods fed with small sized TiO_2 (<25 nm) for 14 d (experiment B). Symbols on the box plot represent maximum and minimum value (whiskers: ⊥) and mean value (■). The effects at a certain exposure concentration, which are significantly different in comparison to control, are shown (symbols denote: (*) $p < 0.05$, and (**) $p < 0.001$).

(10, 100, 1000 µg TiO_2/g dry food) when compared to the control (Table 4a, Fig. 2). However, the same exposure concentrations and same type of nanoparticulate TiO_2 significantly affected CAT activity and two feeding parameters after 14 d of exposure (Table 4b, Figs. 3 and 4).

When the total consumed quantities of TiO_2 were compared (experiments A and B), similar total consumed quantities had different effects when ingested in 3 d or in 14 d. For example, a total consumed quantity in the range from 1.35 µg of TiO_2/g animal wet weight (wet wt.) to 219 µg of TiO_2/g wet wt. in 3 d exposure had no effect on measured parameters, while similar total consumed quantity (8.12 µg TiO_2/g wet wt. to 905 µg TiO_2/g wet wt.) in 14 d provoked changes to some of measured parameters (Table 5). These results show that the effect was not primarily related to exposure concentration or total consumed quantity, but was dependent upon duration of exposure.

3.2.2. Exposure-dose dependence

The dose-dependent pattern for feeding parameters and CAT activity was recognised to be threshold-like when animals were fed on small nanosized TiO_2 (Tables 4b and 5, Fig. 3). When animals were exposed to larger nano-TiO_2 no dose–response relationship pattern could be recognised for feeding parameters (Tables 4c and 5, Fig. 4).

3.2.3. Size of nanoparticles dependence

When the biological effects of both sizes of nanoparticles were compared within experiment C, significant differences were observed (Table 4c, Fig. 4). Smaller nanoparticles (100 µg/g dry food exposure concentration) caused induction of feeding parameters and increased CAT activity, while no change was observed at the same concentration of larger nanoparticles.

3.2.4. Pre-treatment of nanoparticles dependence

In a simple test, we compared the effects of the same exposure concentration, dose and size of nanoparticles prepared in different ways, i.e. sonicated or non-sonicated (Table 4d, Fig. 5). Sonicated smaller nanoparticles of TiO_2 enhanced AE, which was unaffected by the same exposure concentration of non-sonicated nanoparticles. CAT activity was increased in both groups of exposed animals independently on pre-treatment of nanoparticles. It is evident that the modification of nanoparticles might affect their biological reactivity potential, however to what extend remains to be further investigated.

4. Discussion

The effects of nanosized TiO_2 on terrestrial isopods depended on the total consumed quantity and exposure concentration of nanoparticles, exposure duration, and the size of particles as well as their pre-treatment.

It was expected that the intensity of a response would reflect the amount of consumed quantity of nanoparticles, but the results show that it was ruled by duration of exposure and not by consumed quantity or exposure concentration of nano-TiO_2. For instance, a total ingested amount of 8.12 µg/g wet wt. of smaller TiO_2 in 14 d led to elevation in feeding parameters (Table 5), but the feeding parameters were not affected when an even higher amount (20.4 µg/g wet wt.) of the same size TiO_2 was consumed in three days.

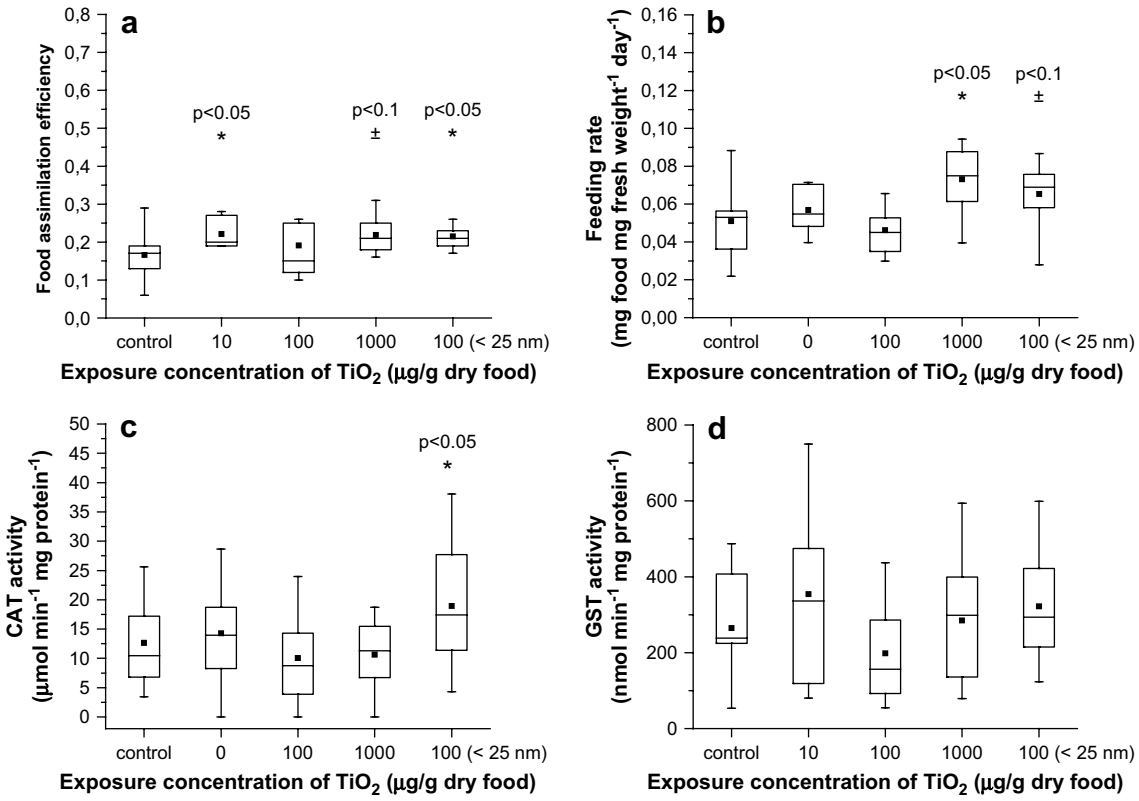

Fig. 4. The food assimilation efficiency (a), feeding rate (b), catalase (CAT) activity (c), and glutathione-S-transferase activity (GST) (d) in isopods fed with large sized TiO_2 (<75 nm) for 14 d (experiment C). Symbols on the box plot represent maximum and minimum value (whiskers: ⊥) and mean value (■). The effects at a certain exposure concentration, which are significantly different in comparison to control, are shown (symbols denote: (±) $p < 0.1$; and (*) $p < 0.05$).

As determined in the present study, the dose–response relationships for nanoparticles are different from those of conventional chemicals (Drobne et al., 2008; Stanek et al., 2006). Nanosized TiO_2 provoked a threshold-like dose–response of parameters studied in *P. scaber*. This was evident in the case of exposure to small nanosized TiO_2. Here, two orders of magnitude different concentrations of nano-TiO_2 had similar effect on feeding parameters and CAT activity.

Contrary to expectations, nanosized TiO_2 enhanced feeding rate of *P. scaber*. On the basis of our previous work, we expected reduced feeding rate as recorded many times upon exposure to metal dosed food (Drobne and Hopkin, 1995). We explain the increase of feeding parameters as a hormetic-like response (Calabrese and Baldwin, 2003), which can have complex time response dynamics. In the present work, this means that after 14 d of exposure we can either detect an increase of the feeding response (experiment B) or miss it due to its cessation (experiment D).

It has been suggested, that small sized particles, whose surface area per unit mass is larger than that of larger particles, are more biologically potent (Borm et al., 2006; Oberdörster et al., 2007; Warheit et al., 2007b). Our results confirm this suggestion. When effects of similar doses of smaller nanosized TiO_2 and larger nanosized TiO_2 are compared, differences in feeding parameters and CAT activity were observed (Table 4c). This might suggest different modes of action and/or toxicodynamics of the two sizes of TiO_2. However, the effect of nanoparticle size remains to be further studied, since the two tested sizes of nanoparticles were of different crystalline phase. Smaller nano-TiO_2 particles were in pure anatase crystalline phase while larger nanoparticles were a mixture of both, anatase and rutile crystalline phase.

The effects of nanoparticles are often linked to their physico-chemical characteristics (Borm et al., 2006; Oberdörster et al., 2007; Warheit et al., 2007b). This was also proven by our results. Sonicated dispersions, which formed smaller aggregates than non-sonicated suspension, resulted in a higher biological potency. Anyway, the measured responses observed in this work cannot be explained straightforward by size and surface area of nanoparticles as analysed in aqueous dispersion. Namely, the aggregation pattern of TiO_2 nanoparticles can be further changed on leaf surface and inside the animal's digestive fluids due to different pH in different parts of the digestive system, the presence of surfactants and other biologically active molecules (Diegoli et al., 2008).

No adverse effects of nano-TiO_2 on isopods, such as mortality, weight change or decrease of feeding, were observed in this study. Therefore the tested concentrations may be considered safe for isopods exposed for 3 d or 14 d to nanosized TiO_2. Furthermore, the concentrations tested in the present study (the lowest concentration was 10 µg/g dry food) are much higher as the recently reported predicted high emission scenario environmental concentrations of nano-TiO_2 in soil (0.0048 µg/g) (Mueller and Nowack, 2008). Other similar studies also report the low toxicity potential of nanosized TiO_2 when compared to dissolved chemicals. Similar studies report the effects of TiO_2 on the mobility of water fleas *Daphnia magna* (no effect up to 500 mg/L) (Lovern and Klaper, 2006; Warheit et al., 2007a), the mortality of crustacea *Thamnocephalus platyurus* (no effect up to 2 g/L) (Heinlaan et al., 2008), the luminescence of bacteria *Vibrio fischeri* (no effect up to 2 g/L) (Heinlaan et al., 2008), the growth of algae *Pseudokirchneriella subcapitata* (72 h median effective concentration EC50 = 87 mg/L) (Warheit et al., 2007a), the growth of algae *Desmodesmus subspicatus* (72 h EC50 = 32–44 mg/L) (Hund-Rinke and Simon, 2006), and the mobility of rainbow trout *Oncorhynchus mykiss* (no effect up to 100 mg/L) (Warheit et al., 2007a). It remains to be further checked whether longer exposure periods,

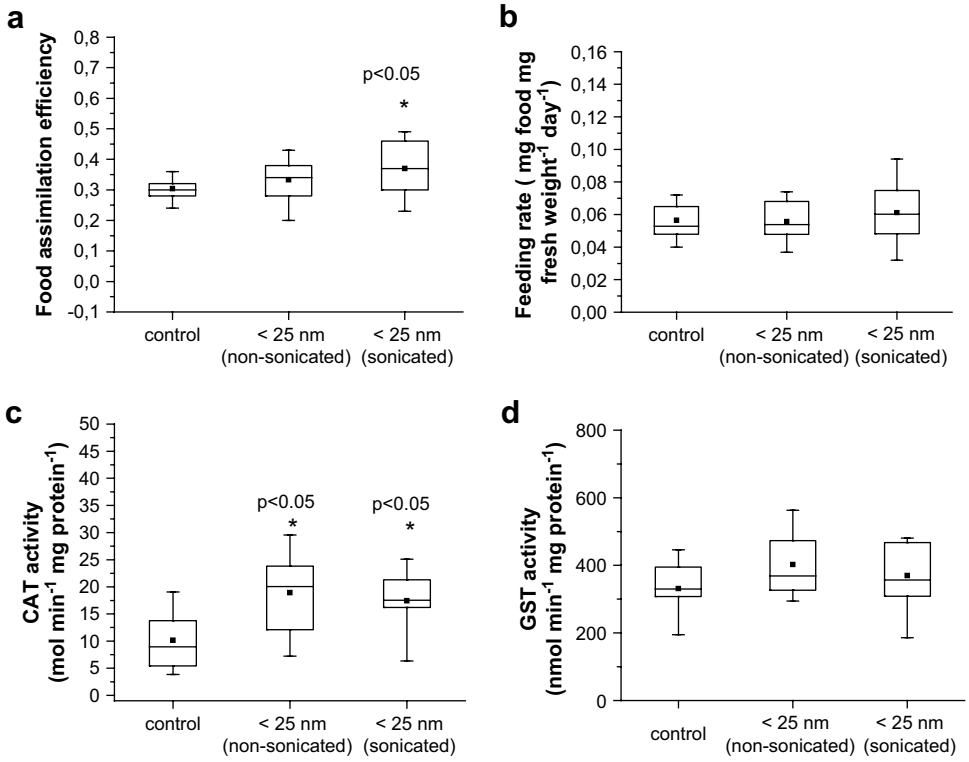

Fig. 5. The food assimilation efficiency (a), feeding rate (b), catalase (CAT) activity (c), and glutathione-S-transferase activity (GST) (d) in isopods fed with non-sonicated and sonicated small sized TiO_2 (<25 nm) for 14 d (experiment D). Symbols on the box plot represent maximum and minimum value (whiskers: ⊥) and mean value (■). The effects at a certain exposure concentration, which are significantly different in comparison to control, are shown ((*) $p < 0.05$).

which are more realistic in the field, would result in more pronounced effect of nano-TiO_2 on terrestrial isopods.

Despite these data, conclusions concerning the safety of nanoparticles must be drawn with great care. Safety data for nanoparticles should be interpreted as a function of dose, exposure period and also size and surface modifications. To collect all these data a lot of testing is needed under varying conditions and with a reasonable set of endpoints. A bioassay with the terrestrial isopod *P. scaber* proved to be suitable for detecting effects of nanoparticles. The suite of analysed biomarkers enables detection of both early non-toxic effects as well as potential adverse effects within changeable duration of exposure. Tests with isopods fit well into a set of tests suited for hazard characterization of nanoparticles (Warheit et al., 2007a).

5. Conclusion

The response of a biological system to nanoparticles appeared to be unique and depends on the physico-chemical characteristics of nanoparticles, dose and duration of exposure. The data from biological tests should therefore be interpreted and processed differently from data for chemicals. This is in line with the recommendations provided by the European Commission scientific committee on emerging and newly identified health risks (Scenihr, 2007). At the present state of knowledge comparative information on the biological activity of nanoparticles would serve best for characterization of hazard and prioritization of nanosized material.

Acknowledgements

This work was financially supported by Slovenian Research Agency project number J1-9475. We thank Ksenija Kogej for DLS analysis and G.W.A. Milne for editorial assistance.

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