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*Nanomaterials in the Environment*

# EFFECTS OF INGESTED NANO-SIZED TITANIUM DIOXIDE ON TERRESTRIAL ISOPODS (*PORCELLIO SCABER*)

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**Abstract**—The effects of ingested nano-sized titanium dioxide ($TiO_2$; anatase, 15 nm) on the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea) after short-term (3-d) dietary exposure were studied. Activities of antioxidant enzymes, such as catalase (CAT) and glutathione-*S*-transferase (GST), in digestive glands were affected in a dose-independent manner, but higher-level isopod endpoints, including weight change, feeding rate, food assimilation efficiency, and survival, were not affected up to the highest tested concentration of $TiO_2$ in food (3,000 μg/g). Exposure concentrations of 0.5, 2,000, and 3,000 μg nonsonicated $TiO_2$/g food decreased CAT and GST activities, but intermediate concentrations (1, 10, 100, and 1,000 μg/g food) did not result in significant changes of enzyme activities. When the dispersion of $TiO_2$ was sonicated, no effects on enzyme activities or higher-level biomarkers were observed. The experimental setup with terrestrial isopods designed for dissolved chemicals also is suitable for testing the effects of ingested nanoparticles, but the presentation of toxicity data needs to be adapted according to the mode of action of the nanoparticles and their specific characteristics.

**Keywords**—Nanoparticles Feeding Biomarkers Terrestrial Titanium dioxide

## INTRODUCTION

Bulk titanium dioxide ($TiO_2$) with micrometer-size dimensions has been in use for decades in the cosmetic, pharmaceutical, paint, and paper industries [1]. Titanium dioxide was labeled by the American Conference of Governmental Industrial Hygienists as a nuisance dust, and it thus is considered to be an inert dust not producing significant toxic effects under realistic exposures [2]. Even though adverse effects of micrometer-sized $TiO_2$ have been demonstrated [3], $TiO_2$ is often considered to be nontoxic [4].

During the last decade, $TiO_2$ has been extensively produced in a nano-sized form that has been used increasingly in pollution treatment and remediation [4,5] as well as in disinfection, self-cleaning glass, solar cells, electric devices, food additives, pharmaceuticals, and cosmetic products [6]. Nano-sized $TiO_2$ is a well-known photocatalyst. Namely, the $TiO_2$ crystalline forms are semiconductors, meaning that they can be photo-excited to generate electron-hole pairs on their surfaces, which results in their strong oxidizability [7]. This characteristic enhances the formation of reactive oxygen species (ROS), which is among the main toxic mechanisms proposed for the observed toxic effects of photo-irradiated, nano-sized $TiO_2$ [7].

Nano-sized $TiO_2$ without photo-activation also has been shown to cause adverse effects on a variety of cell types, tissues, and organisms. Examples include the cytotoxicity to rat lung alveolar macrophages [8], human dermal fibroblast and human lung carcinoma cells [9], apoptosis of Syrian hamster embryo fibroblasts [10], hepatic injury in mice [11], and pathologic changes of gills in fish [12]. Similarly, it has been proposed that the toxic mechanism of nonirradiated nano-sized $TiO_2$ is mediated by ROS [9,10,13]. Increased levels of hydrogen peroxide, increased lipid peroxidation, and decreased levels of reduced glutathione were observed in the human bronchial epithelial cell line [13] and rat alveolar macrophages [8] exposed to nonirradiated nano-sized $TiO_2$. The ability of nano-sized $TiO_2$ to induce ROS formation without photo-activation has been related to its crystallinity and electronic configurations [14] and to an indirect effect on the antioxidant system of the cell [15].

Because $TiO_2$ is classified as a dust, a majority of studies have been focused on its uptake by and effects on the lungs [1]. Because $TiO_2$ is used in food production, medicine, and cosmetics, however, oral ingestion also is an important route of exposure [15]. Toxic effects of orally ingested nano-sized $TiO_2$ have been demonstrated in mice [11] and rainbow trout [12].

Because of its increasing introduction to the environment, nano-sized $TiO_2$ could potentially provoke effects on a variety of organisms in different ecosystems. Until recently, however, the majority of toxicity studies were focused on laboratory test mammals, such as rats and mice. During the last two years, studies regarding the effects of nano-sized $TiO_2$ on aquatic organisms have been performed [12,16–18], but terrestrial toxicity studies are still lacking.

Currently, it is believed that a link between lower- and higher-level responses in test organisms will provide the most relevant toxicity data for different organisms. Responses at lower levels (biochemical biomarkers) can aid in the identification of the mechanisms underlying the effects at higher levels of biological organization. Their disadvantage, however, often is reflected in the high variability of response when compared to more integrated level biomarkers [19].

The two biochemical biomarkers investigated in the present study, catalase (CAT) and glutathione-*S*-transferase (GST), are

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very highly conserved enzymes that have been identified in most organisms, including vertebrates, invertebrates, plants, fungi, and bacteria [20]. The main function of CAT is to catalyze the decomposition of hydrogen peroxide derived from the formation of other ROS, such as superoxide or hydroxyl radical. Glutathione-*S*-transferase 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 of lipid peroxidation [20]. The activities of CAT and GST have been related mainly to antioxidant function against ROS produced as a result of chemical stress, but they also have a central metabolic function in the metabolism of ROS during normal cell functioning, in which ROS appear as side products in a number of metabolic pathways [21,22].

In cells stressed by exogenous chemicals, CAT and GST activities are reported to be increased at lower concentrations of the chemical as a response to ROS production [20]. When the chemical is present at higher concentrations, the decrease in enzyme activities has been explained as a consequence of direct enzyme inhibition by the chemical or as a result of cellular dysfunction [23,24].

In the present study, the effects of ingested nano-sized $TiO_2$ on the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea) after a short-term (3-d) exposure were examined. The responses of biomarkers at different levels of biological organization were measured to link the effects of lower levels to responses at higher levels. Based on the reports that nano-sized $TiO_2$ induces the production of ROS [13,14], we measured the activity of two biochemical biomarkers, CAT and GST. Weight change, feeding rate, and food assimilation efficiency of *P. scaber* were among the conventional physiological parameters studied. Other studies have provided evidence that biochemical biomarkers are influenced by abiotic and biotic factors [25,26]; therefore, the effects of laboratory rearing, molt stage, and gender on enzyme activities were systematically evaluated before the main experiment with nano-sized $TiO_2$. Here, we discuss the effects of nano-sized $TiO_2$ on CAT and GST activities and the suitability of the *P. scaber* test for assessing the effects of ingested nanoparticles.

## MATERIALS AND METHODS

### *Chemicals*

The following chemicals were purchased from Sigma-Aldrich (Munich, Germany): Dibasic and monobasic potassium phosphate, hydrogen peroxide (30%), 1-chloro-2,4-dinitrobenzene, and L-glutathione (reduced form). Protein assay reagents A and B were purchased from Pierce (Rockford, IL, USA). All chemicals were of the highest commercially available grade (typically ≥99%).

### *Characterization of nano-sized $TiO_2$ particles*

The $TiO_2$ nanoparticles were supplied by Sigma-Aldrich in the form of a powder with 99.7% purity. The following characteristics were provided by the supplier: Anatase crystalline structure; average particle size, 15 nm; and surface area, 190 to 290 $m^2/g$.

Before applying $TiO_2$ to the leaves, different concentrations of $TiO_2$ were suspended in bidistilled water with pH 5.7. The pH of the dispersions was independent of the $TiO_2$ concentrations and was the same as that in bidistilled water. The same bidistilled water was used in the control group and proved not to be toxic to isopods.

Table 1. Number of animals analyzed after a certain cultivation period in the laboratory (see Fig. 1)

| Days in the laboratory | No. of animals analyzed | | |
|---|---|---|---|
| | Location 1 | Location 2 | Location 3 |
| 1 | 9 | 9 | 13 |
| 3 | 7 | 7 | |
| 5 | 7 | 6 | |
| 8 | | 7 | |
| 10 | 7 | | |
| 14 | 8 | 7 | 9 |
| 17 | | | 7 |
| 21 | | 7 | |
| 28 | | | 7 |
| 30 | 6 | 7 | |
| 40 | | 6 | |
| 45 | 7 | | |
| 55 | | | 5 |

The sonicated and nonsonicated dispersions of $TiO_2$ were inspected with transmission-electron microscopy and dynamic light scattering (DLS) technique. The dispersion prepared in bidistilled water (0.7 g/L) was sonicated for 30 min using 10-s pulses with 13,872 J of total energy input (Sonics Vibra-Cell, Ultrasonic processor VCX 750 Watt; Sonics & Materials, Newtown, CT, USA). Both sonicated and nonsonicated dispersions were put on carbon-coated grids, dried at room temperature, examined with a 200-keV field emission transmission-electron microscope (Philips CM 100; Koninklijke Philips Electronics, Eindhoven, The Netherlands), and analyzed by transmission-electron diffraction to determine the $TiO_2$ phase.

The same concentrations of sonicated and nonsonicated dispersions prepared in ultrapure water (0.0066, 0.066, and 0.222 g/L; ion free, pH 5.7; Millipore, Billerica, MA, USA) were inspected by the DLS technique using a 3D-DLS-SLS Spectrometer (LS Instruments, Firbourg, Switzerland). Other concentrations of $TiO_2$ prepared for toxicity experiments also were inspected by the DLS technique, but the measurements were not possible (the signal was either too weak [at 0.00066 g/L of $TiO_2$] or too strong [at 0.6667 and 2 g/L of $TiO_2$]). The DLS measurements were performed on the $TiO_2$ dispersions without the addition of 0.1% tetrasodium pyrophosphate, as used previously by Warheit et al. [16], to mimic the composition of the dispersion used in toxicity tests.

### *Effects of laboratory conditions on GST and CAT activities*

The effects of laboratory conditions on the CAT and GST activities of isopods collected from the field were investigated. Animals were brought to the laboratory from three different field locations in Slovenia: Two sites near Domžale (locations 1 and 2; vicinity of Ljubljana, Slovenia), and one site at Radlek (location 3; Kozjansko, Slovenia). All three locations have been used previously as reference sites for isopod collection in toxicity studies and were uncontaminated [27,28]. The activities of both enzymes were determined randomly after different periods (up to 55 d) of culturing in the laboratory. The number of animals analyzed at different times is given in Table 1. As a reference, a laboratory culture originating from a completely different location and held in the laboratory for one year also was analyzed for enzyme activities.

### *Exposure of* P. scaber *to $TiO_2$*

*Test organisms.* Terrestrial isopods (*P. scaber* Latreille 1804) were collected under the litter layer of an uncontami-

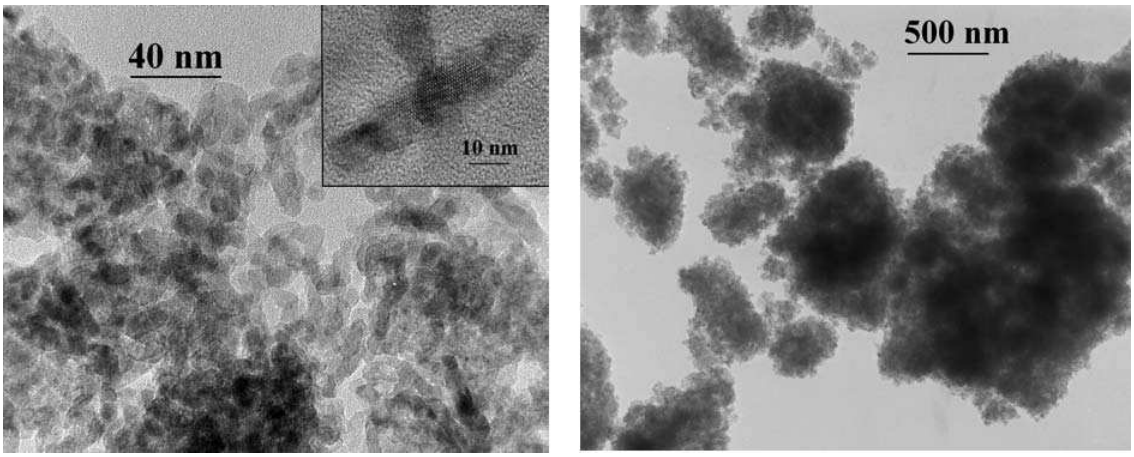


Fig. 1. Transmission-electron micrographs of nanosized titanium dioxide ($TiO_2$) in bidistilled water. Elongated spheroidal shapes of $TiO_2$ nanoparticles with an average diameter of 15 nm and an aspect ratio of up to 1:5 between the diameter and length (left; ×250,000) as well as the typical agglomerates of nanoparticles (right; ×20,000) are shown.

nated location in the vicinity of Ljubljana (location 1). No significant differences in enzyme activities of the animals analyzed within 1 to 3 d after the collection from the field were found between the three locations mentioned above (Fig. 2); therefore, location 1 was selected as a source of animals for toxicity tests because of its close proximity to the laboratory. In the laboratory, the animals were kept in a terrarium (20 × 35 × 20 cm) filled with a 2- to 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), a 16:8-h light: dark photoperiod, and high humidity.

*Food preparation.* Hazelnut tree leaves were collected in an uncontaminated woodland and dried at room temperature. Dry leaves were cut into pieces of similar surface area and then weighed. Pieces of approximately 100 mg were selected for the experiments. Before the application of the $TiO_2$ dispersion, the leaves were kept in humid Petri dishes to facilitate the absorption of applied $TiO_2$ dispersion. Afterward, the leaves were dried for 24 h at room temperature. The leaves were indirectly exposed to light with an intensity of 350 lux for 16 h and 10 lux for 8 h. The periods of maintenance of leaves in humid environment and conditions for drying of the leaves were the same in all experiments.

The $TiO_2$ nanoparticles were suspended in bidistilled water using a vortex (20 s, 18 *g*) and prepared fresh before each experiment. No surfactants were used to disperse the $TiO_2$ nanoparticles, because previous studies have shown that dispersion using solely sonication is adequate [12,16]. We applied 150 μl of the dispersion per 100 mg of leaf onto the lower leaf surfaces. Before pipetting, the dispersion was rotated each time on a vortex for 5 s. Two types of $TiO_2$ dispersions, nonsonicated and sonicated, were applied onto 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). Animals in the control group were fed with leaves prepared in the same way but treated with the bidistilled water only.

*Experimental design. Porcellio scaber* adults with body weights ranging from 30 to 80 mg and of both sexes and all molt stages were exposed to $TiO_2$ within 1 to 11 d after collection in the field. Each animal was placed individually in a Petri dish, to which individual pieces of dry leaves dosed with $TiO_2$ 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 relative humidity close to 100% and a 16:8-h light:dark photoperiod without direct proximity of the lamp (illumination for 16 h with 15 lux and 8 h with 5 lux).

After 3 d of exposure, lower- and higher-level endpoints were evaluated according to the test protocol (Table 2). The animals and leaves were weighed after drying at room temperature for 24 h, and the fecal pellets were counted and

Table 2. Overview of the testing protocol

| Description | Endpoints evaluated | |
|---|---|---|
| | Lower level | Higher level |
| Test organism | Digestive glands: | Feeding rate |
| Invertebrate | Glutathione-*S*-transferase activity | Defecation rate |
| Isopoda, Crustacea | Catalase activity | Food assimilation efficiency |
| Terrestrial isopod (*Porcellio scaber*) | | Animal mass change |
| Type of exposure | | Mortality |
| 3 d of dietary exposure | | |
| Chemical | | |
| Nano-sized titanium dioxide (anatase, 15 nm) | | |
| Sonicated | | |
| Nonsonicated | | |

Table 3. Experimental setup[a]

| Suspension of $TiO_2$ | Final concentration of ($TiO_2$) on leaves (μg/g leaf) | Experiments: Total no. of exposed animals: Exp. A | Exp. B | Exp. C: C1 | C2 | C3 | ΣC |
|---|---|---|---|---|---|---|---|
| Nonsonicated | 0 | 30 | 20 | 6 | 8 | 10 | 24 |
| | 0.1 | | | | 10 | 10 | 20 |
| | 0.5 | | | | 10 | 10 | 20 |
| | 1 | | | 6 | 8 | | 14 |
| | 10 | | | 6 | | | 6 |
| | 100 | | | 6 | 7 | 10 | 23 |
| | 1,000 | 30 | | 6 | | | 6 |
| | 2,000 | 30 | | | | | |
| | 3,000 | 30 | 20 | | 7 | | 7 |
| Sonicated | 1,000 | | 20 | | | | |
| | 2,000 | | 20 | | | | |
| | 3,000 | | 20 | | | | |

[a] Exp. = experiment; $TiO_2$ = titanium dioxide; ΣC = total number of animals in experiment C.

weighed after drying in the exsiccator for 48 h. The animals were dissected, and the digestive glands (hepatopancreas) were isolated for measurements of CAT and GST activities. Animal mortality also was recorded.

Three separate experiments with a different range of tested concentrations were performed (Table 3). Because no data currently exist regarding the environmental concentrations of nano-sized $TiO_2$, the concentrations of $TiO_2$ used in the present study were selected based on preliminary long-term studies in which the effects on feeding and enzyme activities were observed at 1,000 μg $TiO_2$/g leaf (D. Damjana, unpublished data). In experiment A, the isopods were exposed to high concentrations (1,000, 2,000, and 3,000 μg/g leaf) of a nonsonicated dispersion of $TiO_2$. This experiment was repeated three times, using 10 animals per concentration each time. In experiment B, the same high concentrations of a sonicated dispersion of $TiO_2$ were tested, and one additional group was exposed to the highest tested concentration (3,000 μg/g leaf) of a nonsonicated dispersion of $TiO_2$. This experiment was repeated twice, each time with 10 animals per concentration. In experiment C, the animals were exposed in three repeated experiments (experiments C1, C2, and C3) to various very low concentrations (0.1, 0.5, 1, 10, and 100 μg/g leaf) of a nonsonicated dispersion of $TiO_2$. The number of animals and concentrations tested in each repetition are described in Table 3.

### *Determination of enzyme activities*

Animals of both genders and at all molt 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 (Cowie Technology, Middlesbrough, UK). The homogenate was centrifuged for 25 min at 15,000 *g* and 4°C. The activities of both GST and CAT were measured three times in each sample.

The GST activity was measured on microtiter plates (PowerWave™ XS; Bio-Tek® Instruments, Winooski, VT, USA) [29]. 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 provided by Jemec et al. [29]. We added 50 μl of the protein supernatant to start the reaction, which was followed spectrophotometrically at 340 nm and 25°C for 3 min. The GST activity was expressed as nmol conjugated reduced glutathione/min/mg protein (extinction coefficient at 340 nm = 9,600 L/mol/cm).

Catalase activity was determined according to the method described by Aebi [30]. We combined 100 μl of protein supernatant 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 on a Shimadzu (Kyoto, Japan) Ultraviolet-2101PC spectrophotometer. Catalase activity was expressed as μmol degraded hydrogen peroxide/min/mg protein (extinction coefficient at 240 nm = 43.6 L/mol/cm).

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

### *Data analysis*

Only animals between the two molts and females without brood chambers were included in the analyses of higher-level endpoints, because both the molt and the presence of broods might influence the feeding and animal mass change. The feeding rate and a defecation rate of isopods were calculated as the mass of consumed leaf and mass of fecal pellets per wet-weight animal and per day, respectively. The food assimilation efficiency was calculated as the difference between the feeding and defecation rates. 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 $TiO_2$ consumed daily was calculated from the mass of consumed leaf and the corresponding concentration of $TiO_2$ applied.

The significant differences between the control and exposed groups of animals were determined by Kruskal–Wallis analysis and Mann–Whitney *U* test ($p < 0.05$) using Statgraphics software (Statgraphics Plus for Windows 4.0; Statistical Graphics, Herndon, VA, USA). Homogeneity of variance was tested using Levene's test.

## RESULTS

### *Characterization of nano-sized $TiO_2$ particles*

The transmission-electron diffraction pattern revealed the $TiO_2$ to be in its anatase phase. The $TiO_2$ nanoparticles were

homogeneous in shape and size (average diameter, 15 nm), with an aspect ratio of up to 1:5 between the diameter and length, forming elongated, spheroidal shapes (Fig. 1). The nanoparticles were strongly agglomerated. The comparison of sonicated and nonsonicated samples did not reveal any effect of sonication on the intensity of agglomeration. The nanoparticles also might agglomerate during evaporation of water in the process of transmission-electron microscopy sample preparation, but on average, those clusters are more planar than those formed or retained in ultrasound agitation.

The DLS analyses, on the other hand, revealed the difference in the size of agglomerates after sonication procedure. The median particle size of $TiO_2$ was 350 to 500 nm in the sonicated aqueous dispersion and 780 to 970 nm in the nonsonicated dispersion.

### *Effects of laboratory conditions on GST and CAT activities*

The activities of both enzymes gradually decreased during the culturing of isopods in the laboratory. After three to four weeks of laboratory culturing, the span of enzyme activities became narrow, remaining at a certain level, and higher enzyme activities, which were observed in certain specimens during the first 3 d, were no longer detected. After one year, the GST activities of animals from all the tested locations were the same as after three to four weeks of culturing in the laboratory. The same trend was observed for CAT activity at location 1, but at locations 2 and 3, the activity of CAT was significantly higher in the reference laboratory culture kept for one year in the laboratory than in those tested after 40 or 55 d (Fig. 2).

Enzyme activities of animals analyzed after 1 to 3 and 5 to 14 d were grouped to enable comparison with the values obtained for control animals from toxicity experiments, which were all analyzed within 4 to 14 d of collection from the environment (Table 1).

### *Variability of GST and CAT activities*

The CAT and GST activities in control animals from different toxicity experiments were grouped to investigate the normal range of variability for these biochemical biomarkers and their dependence on gender, molt, and presence of brood chamber. The animals included were dissected within 4 to 14 d after collection from the field.

Relatively high coefficients of variability for CAT and GST activities were observed (63.2 and 41.8%, respectively). The range of activities for control isopods were 0 to 55 μmol/min/mg protein for CAT and 83 to 624 nmol/min/mg protein for GST (Fig. 3). No statistically significant differences in the CAT and GST activities were found between males and females, between animals in the intermolt and molt stages, and between females with and without a brood chamber.

### *Effects of $TiO_2$ on* P. scaber

*Effects of $TiO_2$ on physiological endpoints.* After 3 d of exposure to $TiO_2$, no statistically significant effects were observed on the feeding rate, defecation rate, food assimilation efficiency, weight change, and mortality of *P. scaber*. When higher-level endpoints were compared, no differences in the effects of $TiO_2$ between nonsonicated and sonicated $TiO_2$ were observed.

Daily consumed levels of $TiO_2$ were calculated based on consumed food. No differences in the quantity of consumed sonicated and nonsonicated $TiO_2$ were observed (Fig. 4).

*Effects of $TiO_2$ on enzyme activities.* Enzyme activities of both CAT and GST were decreased at 2,000 and 3,000 μg/g leaf of nonsonicated $TiO_2$ (experiment A) (Fig. 5a and b), but no changes were observed in animals exposed to sonicated $TiO_2$ (experiment B) (Fig. 5c and d). The decrease in activity of both enzymes also was observed at 3,000 μg/g leaf of nonsonicated $TiO_2$ in experiment B (Fig. 5c and d). In experiment C, with low concentrations of $TiO_2$, the activities of both enzymes were decreased at 0.5 μg/g leaf and, again, at 3,000 μg/g leaf of nonsonicated $TiO_2$ (Fig. 5e and f).

Based on the changes of CAT and GST activities presented above, it was concluded that the response of both enzyme activities was independent of the dose of ingested nano-sized $TiO_2$. This response is referred to here as binary. The relationship between the consumed levels of $TiO_2$ and the response of enzyme activities is shown in Figure 6.

## DISCUSSION

The ingested nano-sized $TiO_2$ affected the activities of two antioxidant enzymes, CAT and GST, in the digestive glands (hepatopancreas) of the terrestrial isopod *P. scaber* after 3 d of exposure. The response was not dose-dependent. Only exposure concentrations of 0.5, 2,000, and 3,000 μg/g of nonsonicated $TiO_2$ (corresponding to 0.066, 0.389, and 0.685 mg/kg body wt of total consumed $TiO_2$, respectively) caused a decrease in the CAT and GST activities; intermediate concentrations failed to provoke significant changes. Higher-level responses, such as feeding rate, defecation rate, food assimilation efficiency, weight change, or mortality, were not affected up to 3,000 μg/g of nonsonicated $TiO_2$ in the food. When the dispersion of $TiO_2$ was sonicated before application, no effects on enzyme activities or higher-level biomarkers were observed.

We observed a large range of CAT and GST activities in the hepatopancreas of isopods brought directly from the field. During the cultivation of isopods in the laboratory, these two enzyme activities gradually decreased, and after approximately four weeks, activities remained at a given level for a longer period. These changes could be explained as part of a mechanism of acclimation to laboratory conditions. Isopods in the natural environment are constantly exposed to heterogeneous abiotic and biotic conditions, which result in the need for a pool of enzymatic and nonenzymatic compounds, including CAT and GST, related to antioxidant defense. The animals kept in the laboratory are exposed to a less variable environment, and this may result in a lower and narrower range of subsequent antioxidant enzyme activities. The effects of natural factors on the activities of antioxidant enzymes are known [26]. In mussels (*Mytilus galloprovincialis*), for instance, a high seasonal variation in antioxidant enzymes was reported [25]. Oxidative changes have been related to changes in metabolic activity of the organisms, and these changes depend on the intensity of feeding, temperature, and reproduction stage [21,22].

We found no dependence of CAT and GST activities on the gender, molt stage, or presence of neonates in the brood chamber of *P. scaber*. Similarly, no links were found between the gender and GST activities of *P. scaber* and *Oniscus asellus* [31] or between the CAT activities and gender of the marine amphipod *Gammarus locusta* [32]. In other organisms, some inconsistent results have been reported. The CAT activities of mosquitofish (*Gambusi holbrooki*) are higher in males [33], and the CAT activities in the marine shrimp *Aristeus*

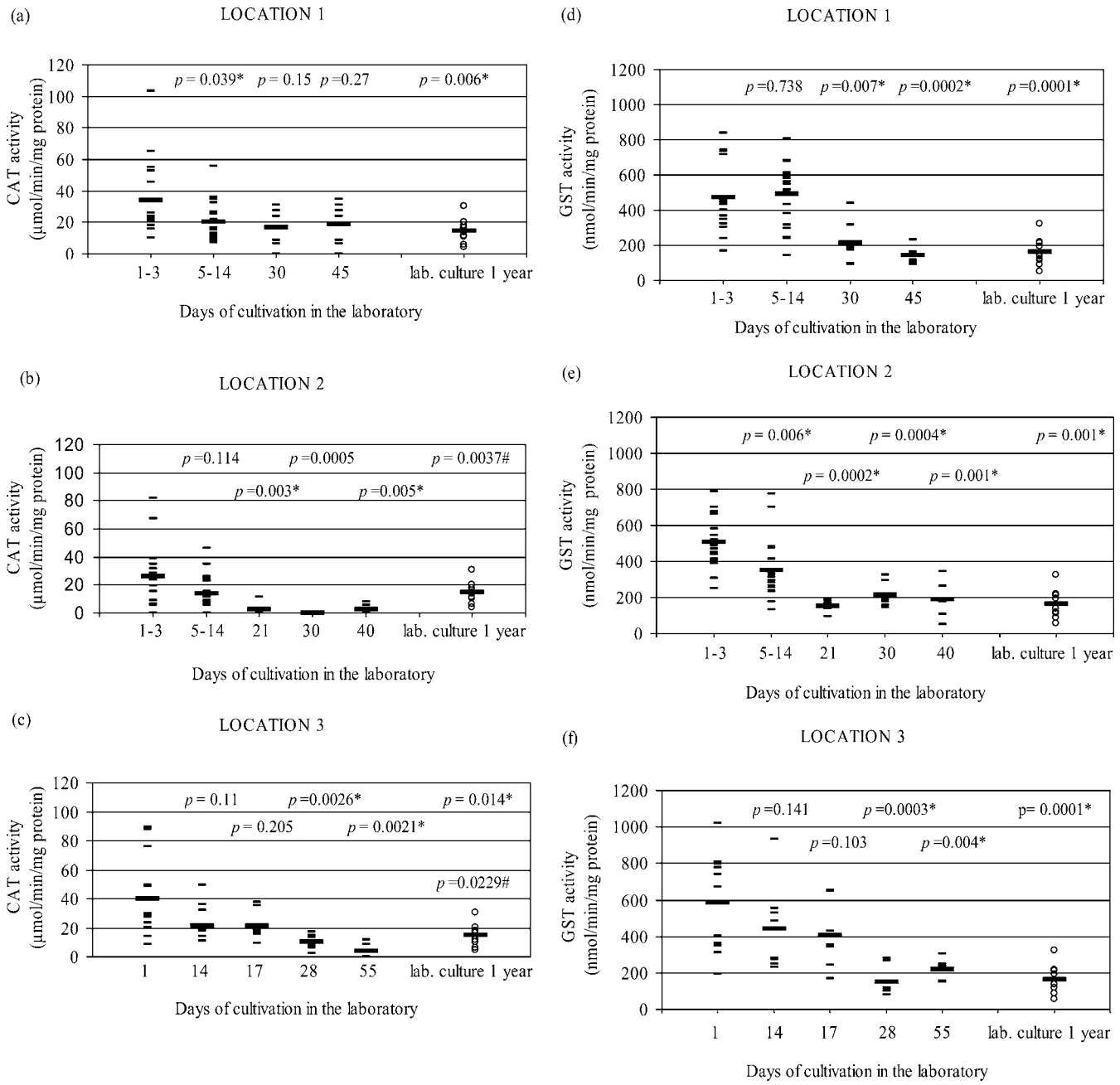


Fig. 2. The effect of laboratory conditions on catalase (CAT) and glutathione-*S*-transferase (GST) activities in isopods (*Porcellio scaber*) collected from three different field locations: (**a** and **d**) Location 1: Ljubljana, Slovenia; (**b** and **e**) location 2: Ljubljana, Slovenia; and (**c** and **f**) location 3: Radlek, Slovenia. The laboratory culture (lab. culture) originating from another location was analyzed as a reference after one year. The data for all animals and the means of a certain time group (thick black line) are shown. Statistically significant differences compared to the first time group (1–3 d; $^*p < 0.05$) and between the lab. culture (one year) and the preceding time group ($^\#p < 0.05$) are shown indicated.

*antennatus* are higher in females [34]. The GST activities in goodeid fish (*Girardinichthys viviparus*) are higher in males [35]. From these data, it would appear that the effect of gender on CAT and GST activities is species specific.

Hepatopancreatic CAT and GST activities in isopods fed with nonsonicated $TiO_2$ for 3 d decreased in a dose-independent manner. Only 0.5, 2,000, and 3,000 µg/g of nano-sized $TiO_2$ in the food reduced CAT and GST activities; the intermediate concentrations of $TiO_2$ (1, 10, 100, and 1,000 µg/g) had no effect. It is evident that the observed changes of enzyme activities do not depend on the concentration of $TiO_2$ nanoparticles but, rather, are related to other properties of the nanoparticles. From the present study, it is impossible to deduce the explanation for observed phenomena. At very high concentrations of $TiO_2$, however, the effect was similar to that of any other dissolved chemical at high doses. The organism cannot cope with large amounts of nanoparticles or disolved chemicals, and the enzyme activities are affected as a result of the impact on the general physiological state. Why low doses of $TiO_2$ also affected the two enzymes remains a challenge for further work.

Different relationships between the concentration of nano-sized $TiO_2$ and its effects have been reported previously. For example, more pronounced effects at lower concentrations of nano-sized $TiO_2$ (20 nm) have been reported for micronuclei sister chromatid exchanges in Chinese hamster ovary cells

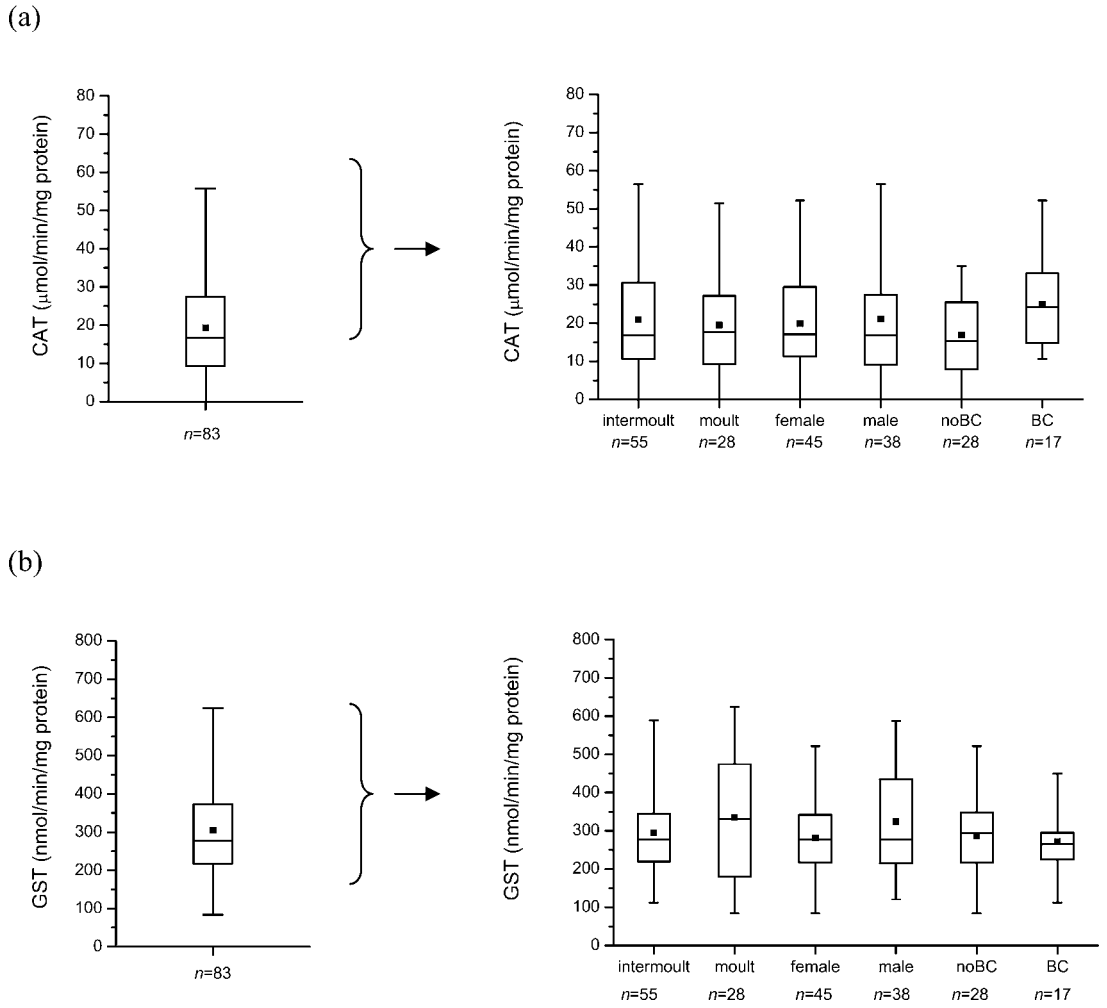


Fig. 3. The variability of (**a**) catalase (CAT) and (**b**) glutathione-*S*-transferase (GST) activities of control animals and their dependence on the physiological state of isopods (*Porcellio scaber*). Symbols on the box plot represent the maximum and minimum value (whiskers: ⊥), mean value (■), females with brood chamber (BC), and females without brood chamber (no BC) ($p < 0.05$).

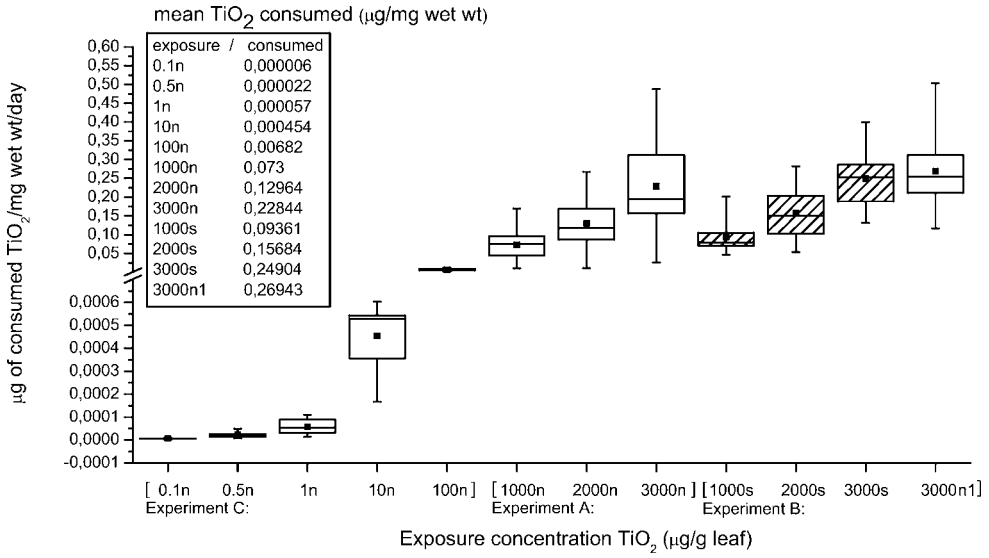


Fig. 4. Daily consumed levels of nano-sized titanium dioxide ($TiO_2$) in all three experiments (experiments A, B, and C). Symbols on the box plot represent the maximum and minimum value (whiskers: ⊥) and the mean value (■). *n* = nonsonicated $TiO_2$; *s* = sonicated $TiO_2$; // on *y* axis = a break.

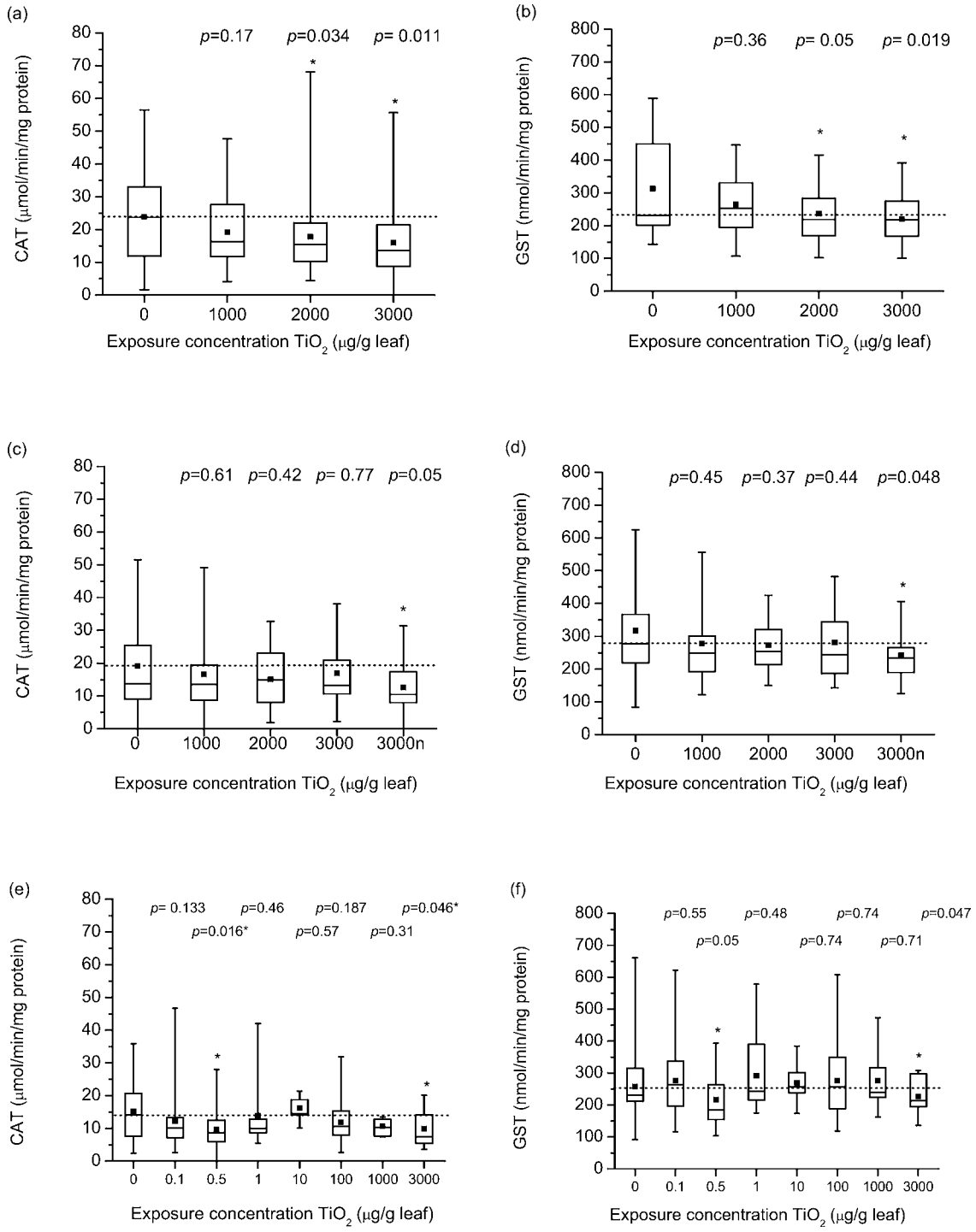


Fig. 5. The effects of high concentrations of nano-sized nonsonicated titanium dioxide ($TiO_2$; experiment A; **a** and **b**) sonicated $TiO_2$ (experiment B; **c** and **d**), and low concentrations of nonsonicated $TiO_2$ (experiment C; **e** and **f**) on catalase (CAT) and glutathione-*S*-transferase (GST) activities in digestive glands of *Porcellio scaber* after 3 d of exposure. Symbols on the box plot represent the maximum and minimum value (whiskers: ⊥), mean value (■), and significant changes compared to control ($*p < 0.05$). The dashed line represents the mean value of the control. $n$ = nonsonicated $TiO_2$.

[10]. An increase of algal growth was observed at 0.01, 0.1, and 10 mg/L of $TiO_2$ (140 nm), followed by a decrease at 100 mg/L [16]. No relationship between the dose and immobility of daphnids was observed when the latter were exposed to $TiO_2$ (25 nm) in a range from 1 to 3 mg/L [17]. On the other hand, a clear relationship between the dose and the response was observed in different cytotoxicity assays with human cell line A549 cells at concentrations of 0.3, 3, 30, 300, 1,500, and 3,000 µg/ml of nano-sized $TiO_2$ [9].

Because of the unique surface properties of nanoparticles, mechanisms of toxic action are suggested that are distinct from the effect of soluble chemicals [1,36]. In studies regarding mechanism of action, Oberdörster et al. [37] have recommended a careful selection of tested concentrations of nano-

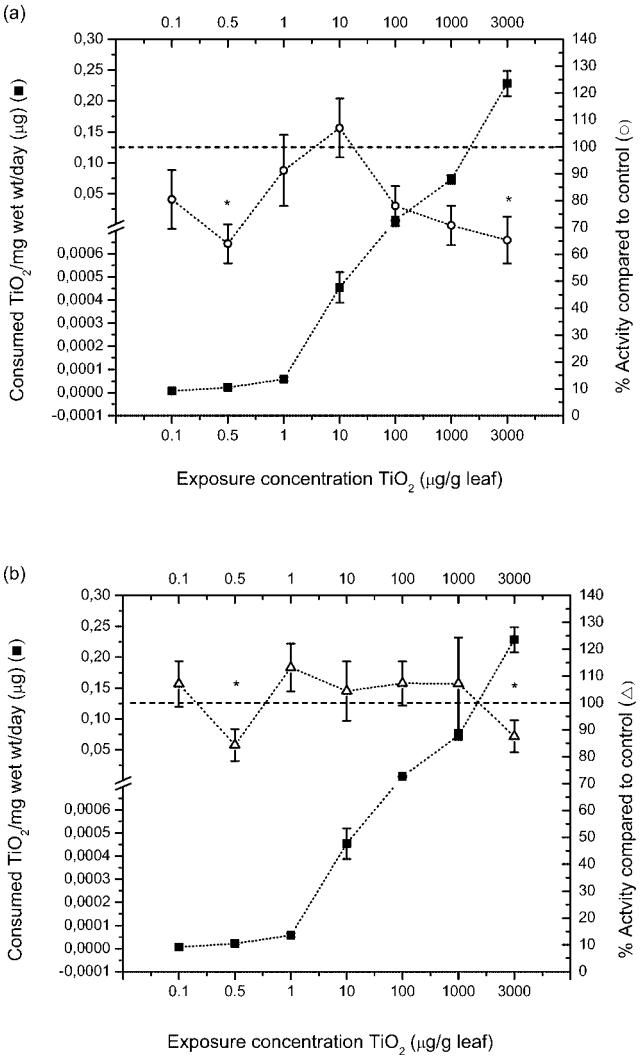


Fig. 6. The relationship between the changes of (**a**) catalase (CAT) and (**b**) glutathione-*S*-transferase (GST) activities and the amount of nano-sized titanium dioxide ($TiO_2$) consumed daily. The data presented are from experiment C and represent the mean ± standard error of the mean. The enzyme activities are expressed as a percentage compared to a mean control value. The dashed horizontal line represents the control (100%).

particles. The importance of studying low concentrations of nanoparticles to detect the possible hormetic response was emphasized. In the present study, the changes of enzyme activities were detected at low concentrations, but the observed response was not stimulatory (as is typical for hormesis) [38].

One of the primary problems in toxicity studies with nanoparticles is the preparation of exposure medium, because the particles tend to form aggregates in water. Most commonly, the dispersion of nano-sized $TiO_2$ is sonicated to diminish the aggregation of particles [9]. The control of the size of aggregates delivered to terrestrial isopods is impossible, because the preparation of the food involves drying of the stock dispersions on the leaves, which results in changes in aggregate formation. Furthermore, the $TiO_2$ ingested by isopods can be further (dis)aggregated in the digestive system with pH 6 to 6.5 and a high concentration of surfactants [39]. Until no standard protocol is established for terrestrial toxicity studies of nanoparticles, we recommend the sonication of the stock dispersions before application onto food, because it enables the application of more uniform aggregates than is possible without sonication.

The binary response of CAT and GST activities obtained in the present study differs from the typical dose–response relationship described for soluble chemicals and, consequently, the determination of toxicological data, such as the no- or lowest-observed-effect concentration (level) or effect concentrations is not possible. To our knowledge, no similar reports for nano-sized $TiO_2$ are found in the literature, and it will be interesting to see whether the binary response also is a characteristic of other nanoparticles and endpoints when a wide range of concentrations is tested.

Previous ecotoxicity studies with aquatic test organisms showed that nano-sized $TiO_2$ (140-nm aggregates in water) exhibit low concern for the immobility of rainbow trout (*Oncorhynchus mykiss*) and water flea (*Daphnia magna*) (no effect up to 100 mg/L) and medium concern for growth of the alga *Pseudokirchneriella subcapitata* (72-h median effective concentration, 87 mg/L) [16]. Similarly, no effect on daphnid immobility was observed up to 500 mg/L of sonicated nano-sized $TiO_2$ (100- to 500-nm aggregates in water) [18]. The results of the present study imply that nano-sized $TiO_2$ after 3 d of exposure is less toxic for the feeding rate of isopods compared with some dissolved metals (e.g., $ZnCl_2$). Namely, our unpublished data show that the feeding rate of isopods is affected already after 3 d of feeding with 2,000 µg/g of $Zn^{2+}$, whereas no effect of 3,000 µg/g of $TiO_2$ was observed in the present study after the same exposure period. After 14 d of exposure, the feeding of isopods was affected at 125 µg/g of $Cd^{2+}$, 1,200 µg/g of $Cu^{2+}$, and 1,800 µg/g of $Zn^{2+}$ [28]. Further studies regarding the effects of the same nano-sized $TiO_2$ on isopods after 14 d of exposure are under preparation and will clarify the relative toxicity of nano-sized $TiO_2$ for isopods.

Data regarding currently detected environmental concentrations of nano-sized or bulk $TiO_2$ in the terrestrial or aquatic environment are not available, because $TiO_2$, which is not included in the priority list of toxic pollutants in the European Union [40] (http://www.mepa.org.mt/index.htm?eu_int_affairs/eu_legislation/mainpage.htm&1), is not systematically monitored. According to the predicted future use of various nanoparticles, including $TiO_2$, they undoubtedly will enter the environment via domestic or industrial wastewaters or direct use for the removal of pollutants from contaminated water and soil, in water treatment filters, and for control of algal growth in water systems. Warheit et al. [16] recently reported a minimum base set of toxicity tests used for nanoparticle risk management, including pulmonary toxicity studies, acute dermal toxicity and sensitization studies, acute oral and ocular toxicity studies, genotoxicity studies, and aquatic toxicity studies. Because of the potential introduction of nanoparticles to the soil, the inclusion of a set of terrestrial toxicity tests in the risk characterization of nanoparticles is necessary.

The advantage of toxicity tests using terrestrial isopods lies in the possibility to determine the consumption levels of chemicals and, subsequently, the effective dose. We recommend use of the same experimental design both for dissolved chemicals and for nanoparticles, but the presentation of toxicity data needs to be adapted to reflect the mode of action and specific characteristics of nanoparticles. Because virtually no agreement currently exists regarding how to present the toxicity data for nanoparticles, such data probably will have to be evaluated retrospectively, and it therefore is very important to provide as much supplementary data as possible. The data

should include, for example, a detailed description of the organisms (life span, different physiological states, gender, and health status), a detailed description of the preparation of dispersions of nanoparticles, characterization of particles, exposure concentrations or ingested dose per day, total ingested dose, duration of exposure, a range of tested concentrations, and as much toxicity data as possible. Such information can be derived from toxicity testing with terrestrial isopods.

## CONCLUSION

The results of the present study show that hepatopancreatic activities of CAT and GST gradually decrease during the cultivation of isopods (*P. scaber*) in the laboratory and that the activities of these two enzymes are not dependent on gender, presence of molt, or marsupium. After short-term (3-d) dietary exposure of terrestrial isopods to 15-nm $TiO_2$ (anatase), the activities of CAT and GST were affected in a dose-independent manner, higher-level responses of isopods were not changed, and the sonication procedure of $TiO_2$ dispersion altered its toxic potential for enzyme activities. The presented experimental setup with terrestrial isopods was suitable for testing the effects of ingested nanoparticles and is recommended in future risk characterization of nanoparticles.

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