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Running head: Effects of ingested nano-sized TiO_2 on *Porcellio scaber*

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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 terrestrial isopods *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 the food (3000 µg/g food). Exposure concentrations of 0.5, 2000, and 3000 µg of nonsonicated TiO_2/g food decreased CAT and GST activities, but intermediate concentrations (1; 10; 100; 1000 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 set-up with terrestrial isopods designed for dissolved chemicals is also 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 nanoparticles and their specific characteristics.

Keywords–Nanoparticles, Feeding, Biomarkers, Terrestrial, `Titanium dioxide`

INTRODUCTION

Bulk titanium dioxide (TiO_2) in micrometer-size dimensions has been in use for decades in cosmetics, pharmaceutical, paint, and paper industries [1]. Titanium dioxide was labeled by American conference of governmental industrial hygienists as a nuisance dust and it is thus 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 traditionally is often considered to be nontoxic [4].

In the last decade, TiO_2 has been extensively produced in nano-sized form that has been used increasingly in pollution treatment and remediation [4,5], 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 its surface, 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 observed toxic effects of photo-irradiated nano-sized TiO_2 [7].

Nano-sized TiO_2 without photo-activation has also 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 ROS mediated [9, 10, 13]. Increased levels of hydrogen peroxide, increased lipid peroxidation and decreased levels of reduced glutathione were observed

in 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 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]. However, since TiO_2 is used in food production, medicine, and cosmetics, its oral ingestion is also an important exposure route [15]. Toxic effects of orally ingested nano-sized TiO_2 have been demonstrated in mice [11] and rainbow trout [12].

Due to increasing introduction of nano-sized TiO_2 to the environment, it could potentially provoke effects on a variety of organisms in different ecosystems. However, until recently the majority of toxicity studies were focused on laboratory test mammals, such as rats and mice. During the last two years, studies on 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 dose levels (biochemical biomarkers) can aid in the identification of the mechanisms underlying the effects at higher levels of biological organization. Their disadvantage is however often reflected in high variability of the 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 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 during 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, where 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 of 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 paper, the effects of ingested nano-sized TiO_2 on terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea) after a short-term (3 d) exposure are reported. The responses of biomarkers at different levels of biological organization were measured to link the effects of lower-level effects of ingested TiO_2 to responses at higher levels. Based on the reports that nano-sized TiO_2 induces the production of ROS, we measured the activity of two biochemical biomarkers CAT and GST [13, 14]. Weight change, feeding rate and food assimilation efficiency of *P. scaber* were among conventional physiological parameters studied. Other studies provide evidence that biochemical biomarkers are influenced by abiotic and biotic factors [25, 26]. Therefore the effects of laboratory rearing, moult stage, and gender on enzyme activities were systematically evaluated prior to the main experiment with nano-sized TiO_2. 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, 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% or higher.

Characterization of nano-sized TiO_2 particles

The nanoparticles of TiO_2were supplied by Sigma-Aldrich in a 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 value of 5.7. The pH of the dispersions was independent on the concentration of TiO_2 and was the same as in bidistilled water. The same bidistilled water was used in control group and proved not to be toxic to isopods.

The sonicated and nonsonicated dispersions of TiO_2 were inspected with transmission electron microscope analyses and dynamic light scattering technique (DLS). The dispersion

prepared in bidistilled water (0.7 g/L) was sonicated for 30 min using 10 s pulses with 13872 J of the total input of energy (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 and 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 ultra-pure water (Millipore, Billerica, MA, USA; ion free, pH = 5.7) (0.0066; 0.066; 0.222 g/L) were inspected by DLS using a 3D-DLS-SLS Spectrometer (LS Instruments, Firbourg, Switzerland). Other concentrations of TiO_2 prepared for toxicity experiments were also inspected by DLS, but the measurements were not possible, because the signal was either too weak (at 0.00066 g/L) 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 previously used 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 (vicinity of Ljubljana, Slovenia) (locations 1 and 2), and Radlek (Kozjansko, Slovenia) (location 3). All three locations have previously been used as reference sites for isopod collection in toxicity studies, and were found 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 was also analyzed for enzyme activities.

Exposure of P. scaber to TiO_2

Test organisms. Terrestrial isopods (*Porcellio scaber*, Latreille 1804) were collected under the litter layer in an uncontaminated 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. 1**), therefore location 1 was selected as a source of animals for toxicity tests because of the 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), 16:8 h light:dark regime and high humidity.

Food preparation. Hazelnut tree leaves were collected in an uncontaminated woodland and dried at room temperature. Dry leaves were cut up into pieces of similar surface area, and weighed. Pieces of approximately 100 mg were selected for the experiments. Prior to the application of the TiO_2 dispersion, the leaves were kept in humid Petri dishes to facilitate the absorption of applied TiO_2 dispersion. Afterwards, the leaves were dried for 24 h at room temperature. The leaves were not directly exposed to light with the intensity of the illumination:

16 h with 350 lux, and 8 h with 10 lux. 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 was suspended in bidistilled water using a vortex (20 s, 2000 rpm) and prepared freshly each time prior to the experiment. No surfactants were used to disperse the TiO_2, since 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. Prior to the pipetting, the dispersion was each time rotated on vortex for 5 s. Two types of TiO_2 dispersions were applied onto the leaves; nonsonicated and sonicated. 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 the leaves prepared in the same way, but treated with the bidistilled water only.

Experimental design. The adults of *P. scaber* with body weights ranging from 30 to 80 mg, of both sexes and all moult stages, were exposed to TiO_2 within 1 to 11 d after the 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 regime without the direct proximity of the lamp (illumination 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 faecal pellets were counted and weighted after drying in the exsiccator for 48 h. The animals were dissected and the digestive glands (hepatopancreas) were isolated for

248 measurements of CAT and GST activities. Animal mortality was also recorded.

249 Three separate experiments with a different range of tested concentrations were performed
250 (**Table 3**). Since currently no data exists on the environmental concentrations of nano-sized
251 TiO_2, the concentrations of TiO_2 used in the present study were selected based on preliminary
252 long-term studies, in which the effects on feeding and enzyme activities were observed at 1000
253 µg of TiO_2/g leaf (Drobne Damjana, University of Ljubljana, Biotechnical Faculty, Slovenia,
254 unpublished data, preliminary study). In experiment A, the isopods were exposed to high
255 concentrations (1000, 2000, 3000 µg/g leaf) of a nonsonicated dispersion of TiO_2. This
256 experiment was repeated three times, using ten animals per concentration each time. In
257 experiment B, the same high concentrations of sonicated dispersion of TiO_2 were tested and one
258 additional group was exposed to the highest tested concentration (3000 µg/g leaf) of
259 nonsonicated dispersion of TiO_2. This experiment was repeated twice, each time with ten
260 animals per concentration. In experiment C, the animals were exposed in three repeated
261 experiments to various very low concentrations (0.1, 0.5, 1, 10, 100 µg/g leaf) (C1, C2, C3) of a
262 nonsonicated dispersion of TiO_2. The number of animals and concentrations tested in each
263 repetition are described in Table 3.

264

265 *Determination of enzyme activities*

266

267 Animals of both genders and at all moult stages were used for enzyme analyses and a
268 separate enzyme sample was prepared from each animal. The whole digestive gland was
269 homogenized for 3 min in 0.8 ml of 50 mM phosphate buffer pH 7.0, using a Teflon®–glass
270 Elvehjem-Potter homogenizer (Cowie Technology, Middlesbrough, UK). The homogenate was

centrifuged for 25 min at 15000 *g* and 4°C. The activities of both GST and CAT were measured three times in each sample.

Glutathione-*S*-transferase activity was measured on microtiter plates (Bio-Tek® Instruments, Winooski, VT, USA; PowerWave™ XS) [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 is described in 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. Glutathione-*S*-transferase activity was expressed in nmoles of conjugated reduced glutathione/min/mg protein (extinction coefficient $\varepsilon_{340} = 9600$ L/mol/cm).

Catalase activity was determined according to the method of 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 ultraviolet-2101PC spectrophotometer (Shimadzu, Kyoto, Japan). Catalase activity was expressed in µmoles of degraded hydrogen peroxide/min/mg protein ($\varepsilon_{240} = 43.6$ L/mol/ cm).

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

Data analysis

Only animals between the two moults and females without brood chambers were included in the analyses of higher level endpoints, because both the moult and the presence of broods might

294 influence the feeding and animal mass change. The feeding rate and a defecation rate of isopods
295 were calculated as the mass of consumed leaf and mass of faecal pellets per animal wet weight
296 and per day, respectively. The food assimilation efficiency was calculated as the difference
297 between the feeding and defecation rates. The animal mass change was determined as the
298 difference in animal mass at the beginning and at the end of the experiment. The amount of the
299 daily consumed TiO_2 was calculated from the mass of consumed leaf and the corresponding
300 concentration of TiO_2 applied.
301 The significant differences between the control and exposed groups of animals were
302 determined by Kruskal-Wallis analysis and Mann-Whitney *U* test ($p<0.05$) using Statgraphics
303 software (Statgraphics Plus for Windows 4.0, Statistical Graphics, Herndon, VA, USA).
304 Homogeneity of variance was tested using Levene's test.
305
306 **RESULTS**
307
308 *Characterization of nano-sized TiO_2 particles*
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310 The transmission electron diffraction pattern revealed the TiO_2 to be in its anatase phase. The
311 TiO_2 nanoparticles were homogeneous in shape and size, in average 15 nm in diameter, and with
312 up to 1 to 5 aspect ratio between the diameter and length forming elongated spheroidal shapes
313 (**Fig.1**). They were strongly agglomerated. The comparison of sonicated and nonsonicated
314 samples did not reveal any effect of sonication on the intensity of agglomeration. The
315 nanoparticles might also agglomerate during evaporation of water in the process of transmission
316 electron microscope sample preparation, but on average, those clusters are more planar than

317 those formed or retained in ultrasound agitation.

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

321

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

323

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

332 Enzyme activities of animals analyzed after 1 to 3 d, and 5 to14 d were grouped to enable
333 comparison with the values obtained for control animals from toxicity experiments, which were
334 all analyzed within 4 to14 d of the collection from the environment (Table 1).

335

336 *Variability of GST and CAT activities*

337

338 The CAT and GST activities in control animals from different toxicity experiments were
339 grouped in order to investigate the normal range of variability for these biochemical biomarkers

and their dependence on gender, moult and presence of brood chamber. The animals included were dissected within 4 to14 d after the 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, and 83 to 624 nmol/min/mg protein for CAT and GST, respectively (**Fig. 3**). No statistically significant differences in the CAT and GST activities were found between males and females, between animals in the intermoult and moult stage, and between females with and without 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. Both enzyme activities were found to be decreased at 2000 and 3000 µg/g leaf of nonsonicated TiO_2 (experiment A) (**Fig. 5** a,b), but no changes were observed in animals exposed to sonicated TiO_2 (experiment B) **(**Fig. 5 c,d). The decrease in activity of both enzymes was also observed at 3000 µg/g leaf of nonsonicated TiO_2, in the

363 experiment B (Fig. 5 c,d). In experiment C, with low concentrations of TiO_2, the activities of
364 both enzymes were decreased at 0.5 µg/g leaf and again at 3000 µg/g leaf of nonsonicated TiO_2
365 (Fig. 5 e,f).

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

370

371 **DISCUSSION**

372

373 The ingested nano-sized TiO_2 affected the activities of two antioxidant enzymes, CAT and
374 GST, in the digestive glands (hepatopancreas) of terrestrial isopods, *Porcellio scaber*, after 3 d of
375 exposure. The response was not dose-dependent. Only exposure concentrations 0.5, 2000, and
376 3000 µg/g of nonsonicated TiO_2 (corresponding to 0.066, 0.389, and 0.685 mg/kg body wt of
377 total consumed TiO_2, respectively), caused a decrease in the CAT and GST activities;
378 intermediate concentrations failed to provoke significant changes. Higher level responses like
379 feeding rate, defecation rate, food assimilation efficiency, weight change or mortality were not
380 affected up to 3000 µg/g of nonsonicated TiO_2 in the food. When the dispersion of TiO_2 was
381 sonicated prior to application, no effects on enzyme activities or higher level biomarkers were
382 observed.

383 We observed a large range of CAT and GST activities in the hepatopancreas of isopods
384 brought directly from the field. During the cultivation of isopods in the laboratory, these two
385 enzyme activities gradually decreased, and after approximately four weeks remained at a given

level for a longer period. These changes could be explained as a 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 may subsequently result in a lower and narrower range of 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, moult 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 *O. assellus* [31] and 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], CAT activities in marine shrimp *Aristeus antennatus* are higher in females [34], and 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, 2000, and 3000 μg/g of nano-sized TiO_2 in the food-reduced CAT and GST activities; the intermediate concentrations (1; 10; 100; 1000 μg/g TiO_2) had no effect. It is evident that the observed changes of enzyme activities do not depend on the concentration of TiO_2 nanoparticles, but are related to other properties of the

409 nanoparticles. From the present study, it is impossible to deduce the explanation for observed
410 phenomena. However, at very high concentrations of TiO_2, the effect was similar to that of any
411 other dissolved chemical at high doses. The organism cannot cope with large amounts of
412 nanoparticles or disolved chemicals, and the enzyme activities are affected as a result of the
413 impact on the general physiological state. Why low doses of TiO_2 also affected the two enzymes,
414 remains a challenge for further work.

415 Different relationships between the concentration of nano-sized TiO_2 and its effects have
416 been reported previously. For example, more pronounced effects at lower concentrations of
417 nano-sized TiO_2 (20 nm) have been reported for micronuclei sister chromatid exchanges in
418 Chinese hamster ovary cells [10]. An increase of algal growth was observed at 0.01, 0.1, and 10
419 mg/L of TiO_2 (140 nm), followed by a decrease at 100 mg/L [16]. No relationship between the
420 dose and immobility of daphnids was observed when the latter were exposed to TiO_2 (25 nm) in
421 a range from 1 to 3 mg/L [17]. On the other hand, a clear relationship between the dose and the
422 response was observed in different cytotoxicity assays with human cell line A549 cells at
423 concentrations of 0.3, 3, 30, 300, 1500, and 3000 μg/ml of nano-sized TiO_2 [9].

424 Because of the unique surface properties of nanoparticles, mechanisms of toxic action are
425 suggested which are distinct from the effect of soluble chemicals [1, 36]. In studies of
426 mechanism of action, Oberdörster et al. [37] have recommended a careful selection of tested
427 concentrations of nanoparticles. The importance of studying low concentrations of nanoparticles
428 to detect the possible hormetic response was emphasized. In the present study, the changes of
429 enzyme activities were detected at low concentrations, but the observed response was not
430 stimulatory as is typical for hormesis [38].

431 One of the primary problems in toxicity studies with nanoparticles is the preparation of

exposure medium, since 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 where a pH is 6 to 6.5 and high concentration of surfactants is present [39]. Until no standard protocol is established for terrestrial toxicity studies of nanoparticles, we recommend the sonication of the stock dispersions prior to application onto food, because it enables the application of more uniform aggregates than without sonication.

The binary response of CAT and GST activities obtained in the present study differs from the typical dose–response described for soluble chemicals and consequently, the determination of toxicological data, such as the no- or lowest-observed-effect concentration (level) (NOEC, NOEL, LOEC, LOEL) or effect concentrations (ECx, ELx, etc.) is not possible. No similar reports for nano-sized TiO_2 are found in the literature and it will be interesting to see whether the binary response is also 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 (aggregates in water 140 nm) exhibit low concern for the immobility of rainbow trout *Oncorhynchus mykiss* and water fleas *Daphnia magna* (no effect up to 100 mg/L) and medium concern for the growth of algae *Pseudokirchneriella subcapitata* (72 h median effective concentration EC50 = 87 mg/L) [16]. Similarly, no effect on daphnid immobility was observed up to 500 mg/L of sonicated nano-sized TiO_2 (aggregates in water 100-500 nm) [18]. The results of the present study imply, that nano-sized TiO_2 after 3-d of exposure is less toxic for the feeding

455 rate of isopods than some dissolved metals, for instance $ZnCl_2$. Namely, our unpublished data
456 show, that the feeding rate of isopods is affected already after 3-d of feeding with 2000 µg/g of
457 Zn^{2+}, while no effect of 3000 µg/g of TiO_2 was observed in the present study after the same
458 exposure period. After 14 d of exposure, the feeding of isopods was affected at 125 µg/g of Cd^{2+},
459 1200 µg/g of Cu^{2+} and 1800 µg/g of Zn^{2+} [28]. Further studies on the effects of the same nano-
460 sized TiO_2 on isopods after 14-d of exposure are under preparation and will clarify the relative
461 toxicity of nano-sized TiO_2 for isopods.

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

475 The advantage of toxicity tests using the terrestrial isopods lies in the possibility to determine
476 the consumption levels of chemicals and subsequently the effective dose. We recommend the use
477 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 nanoparticles' mode of action and specific characteristics. Since virtually no agreement currently exists as to how to present the toxicity data for nanoparticles, such data will probably have to be evaluated retrospectively and it is therefore very important to provide as much supplementary data as possible. The data should include, for example, a detailed description of organisms (life span, different physiological states, gender, health status), a detailed description of the preparation of dispersions of nanoparticles, characterisation 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 (*Porcellio scaber*) in the laboratory, and that the activities of these two enzymes are not dependent on gender, presence of moult 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 set-up with terrestrial isopods was found to be suitable for testing the effects of ingested nanoparticles and is recommended in future risk characterization of nanoparticles.

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503

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639 **FIGURE LEGENDS:**

640 **Fig. 1**. Transmission-electron micrographs of nanosized titanium dioxide (TiO_2) in bidistilled
641 water. Elongated spheroidal shapes of TiO_2 nanoparticles with an average diameter 15 nm and
642 with up to 1 to 5 aspect ratio between the diameter and length (left), and the typical agglomerates
643 od nanoparticles (right) are shown.

644

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

653

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

658

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

662

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

670

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

676

677

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680

681

Table 1: The number of animals analyzed after a certain period of cultivation in the laboratory (Fig.1)

Days in the laboratory	LOCATIONS		
	1	2	3
	Number of animals analyzed		
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

Table 2: Overview of the testing protocol

<table>
<tr><td colspan="2">Test organism description</td><td>Endpoints evaluated</td></tr>
<tr><td colspan="2">Invertebrate

Isopoda, Crustacea

Terrestrial isopod Porcellio scaber</td><td rowspan="2">Lower level end-points:

Digestive glands:

- Glutathione-S-transferase activity

- Catalase activity</td></tr>
<tr><td>Type of exposure</td><td>Chemical</td></tr>
<tr><td>3 d dietary exposure</td><td>nano-sized titanium dioxide

(anatase, 15 nm)

-Sonicated

-Nonsonicated</td><td>Higher level end-points:

- Feeding rate

- Defecation rate

- Food assimilation efficiency

- Animal mass change

- Mortality</td></tr>
</table>

Table 3: Experimental set-up

Suspension of TiO_2	Final concentrations of titanium dioxide (TiO_2) on leaves (µg/g leaf)	Experiments Total No. of exposed animals					
		A	B	C			
Nonsonicated				C 1	C 2	C 3	ΣC
	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
	1000	30		6			6
	2000	30					
	3000	30	20		7		7
Sonicated	1000		20				
	2000		20				
	3000		20				

ΣC- total amount of animals in experiment C.

Fig. 1.

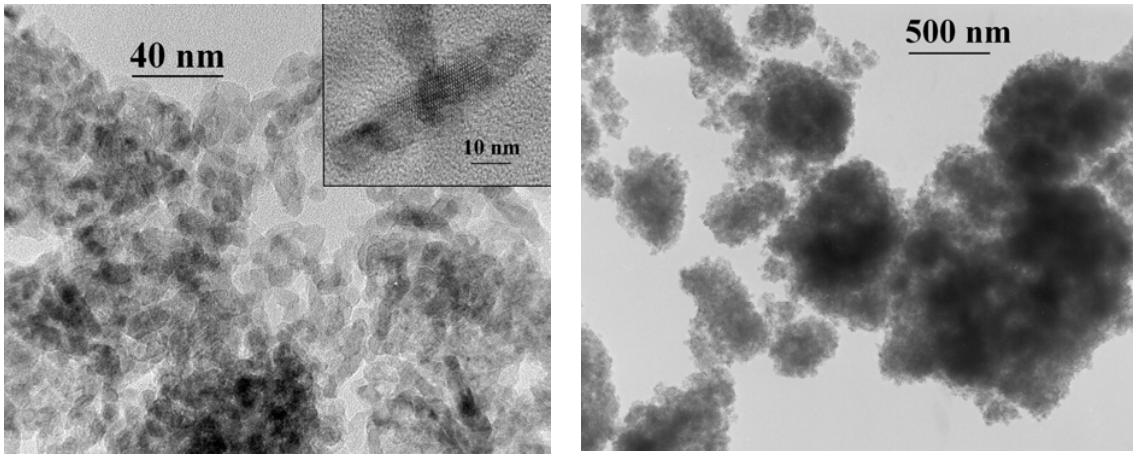
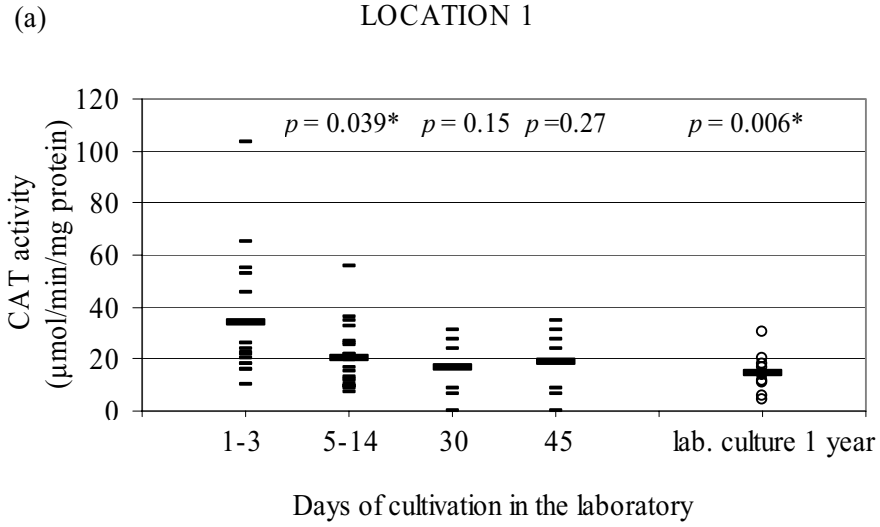
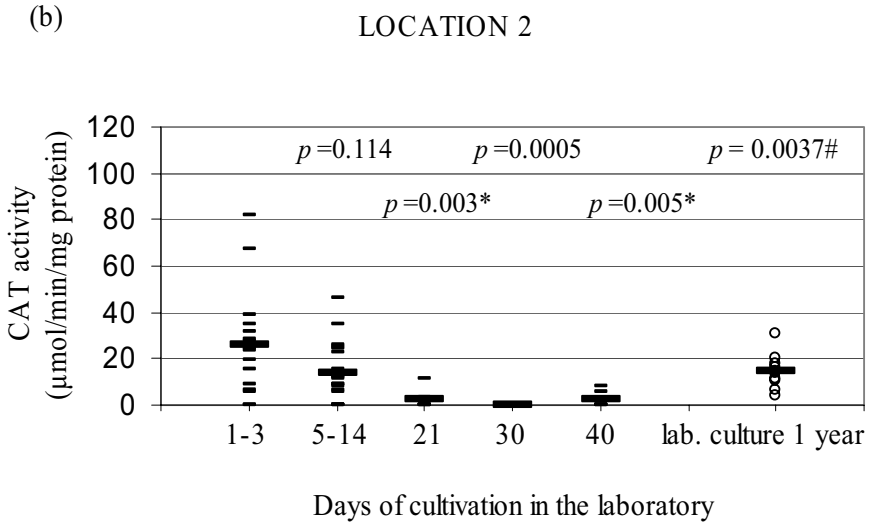
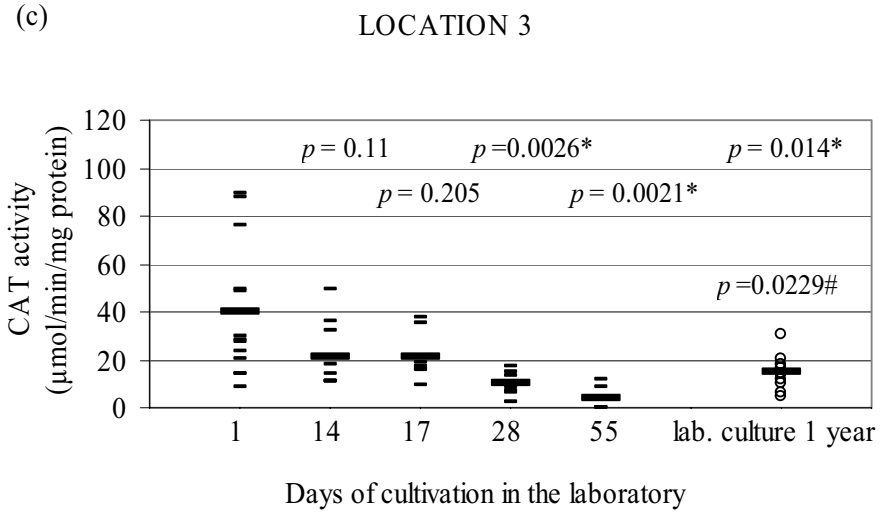


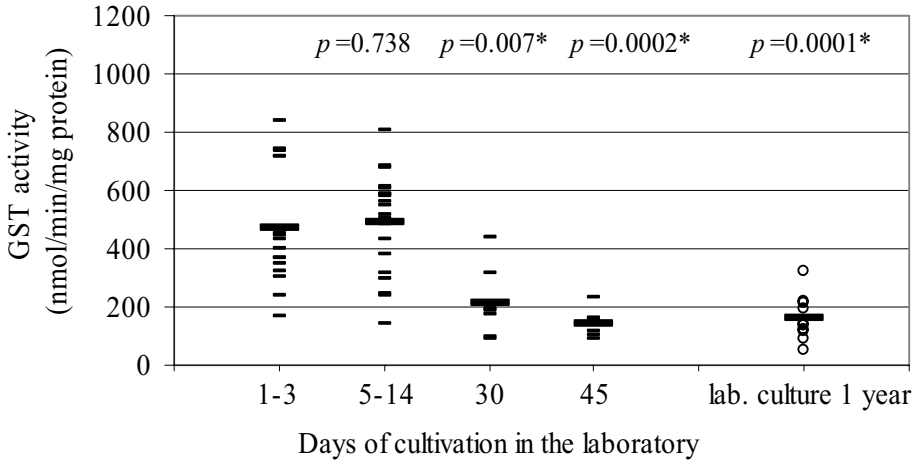
Fig. 2.


(a)
LOCATION 1
p = 0.039* p = 0.15 p =0.27 p = 0.006*
CAT activity (µmol/min/mg protein)
120
100
80
60
40
20
0
1-3
5-14
30
45
lab. culture 1 year
Days of cultivation in the laboratory

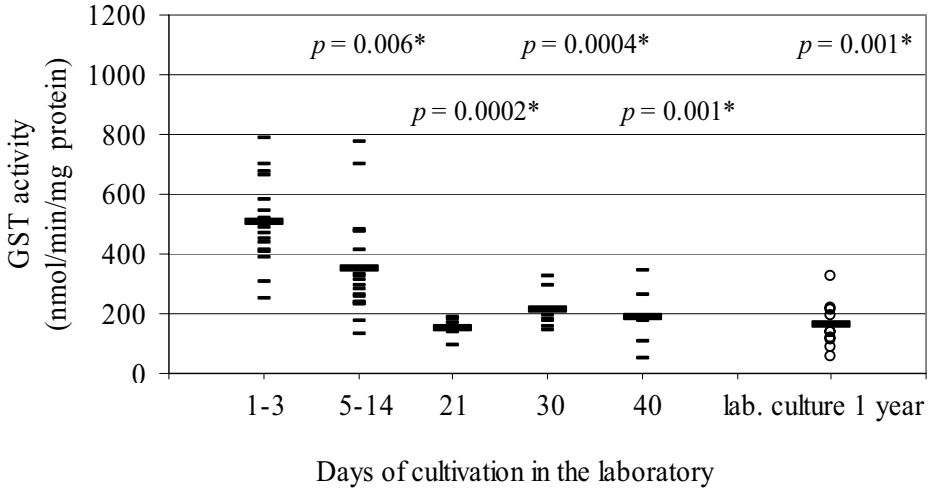

(b)
LOCATION 2
p =0.114 p =0.0005 p = 0.0037#
p =0.003* p =0.005*
CAT activity (µmol/min/mg protein)
120
100
80
60
40
20
0
1-3
5-14
21
30
40
lab. culture 1 year
Days of cultivation in the laboratory


(c)
LOCATION 3
p = 0.11 p =0.0026* p = 0.014*
p = 0.205 p = 0.0021*
p =0.0229#
CAT activity (µmol/min/mg protein)
120
100
80
60
40
20
0
1
14
17
28
55
lab. culture 1 year
Days of cultivation in the laboratory

(d) LOCATION 1



(e) LOCATION 2



(f) LOCATION 3

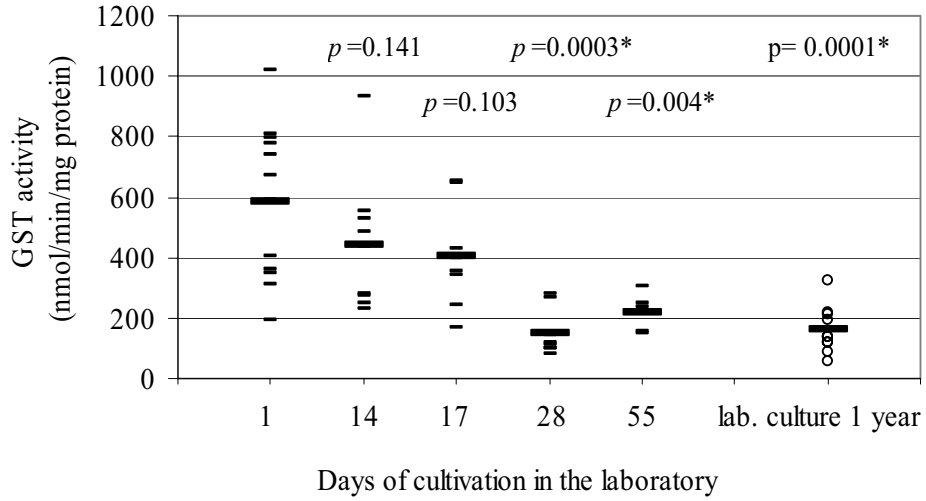


Fig. 3.

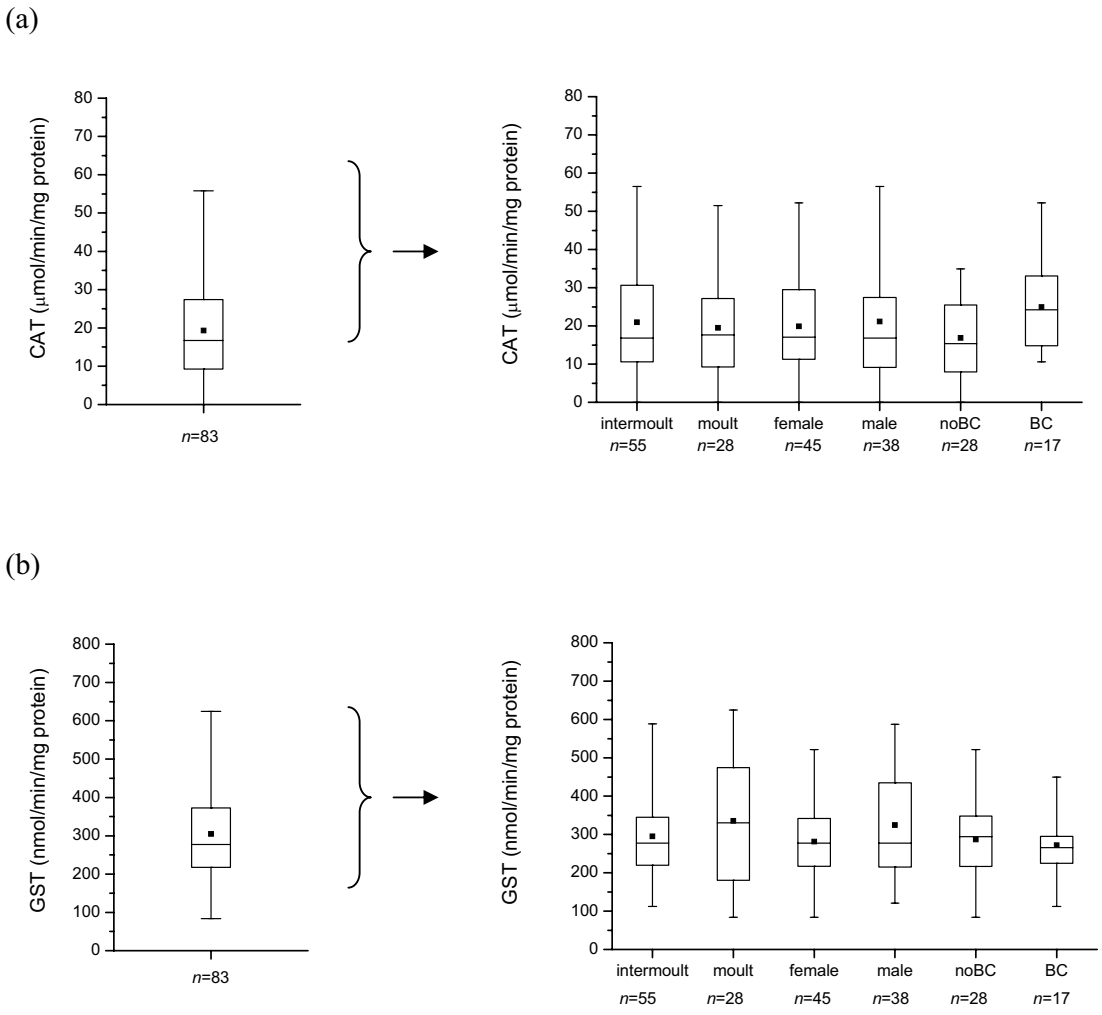

(a)
CAT (μmol/min/mg protein)
0
10
20
30
40
50
60
70
80
n=83
CAT (μmol/min/mg protein)
intermoult n=55
moult n=28
female n=45
male n=38
noBC n=28
BC n=17
(b)
GST (nmol/min/mg protein)
0
100
200
300
400
500
600
700
800
n=83
GST (nmol/min/mg protein)
intermoult n=55
moult n=28
female n=45
male n=38
noBC n=28
BC n=17

Fig. 4.

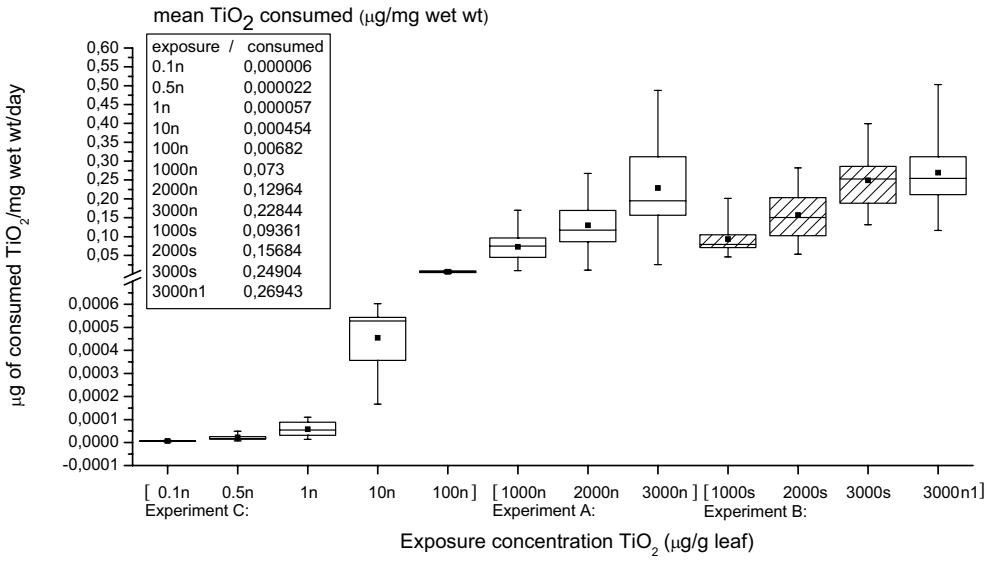

mean TiO2 consumed (µg/mg wet wt)
exposure / consumed
0.1n 0,000006
0.5n 0,000022
1n 0,000057
10n 0,000454
100n 0,00682
1000n 0,073
2000n 0,12964
3000n 0,22844
1000s 0,09361
2000s 0,15684
3000s 0,24904
3000n1 0,26943
µg of consumed TiO2/mg wet wt/day
0,60
0,55
0,50
0,45
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,0006
0,0005
0,0004
0,0003
0,0002
0,0001
0,0000
-0,0001
[0.1n
0.5n
1n
10n
100n]
[1000n
2000n
3000n]
[1000s
2000s
3000s
3000n1]
Experiment C:
Experiment A:
Experiment B:
Exposure concentration TiO2 (µg/g leaf)

Fig. 5.

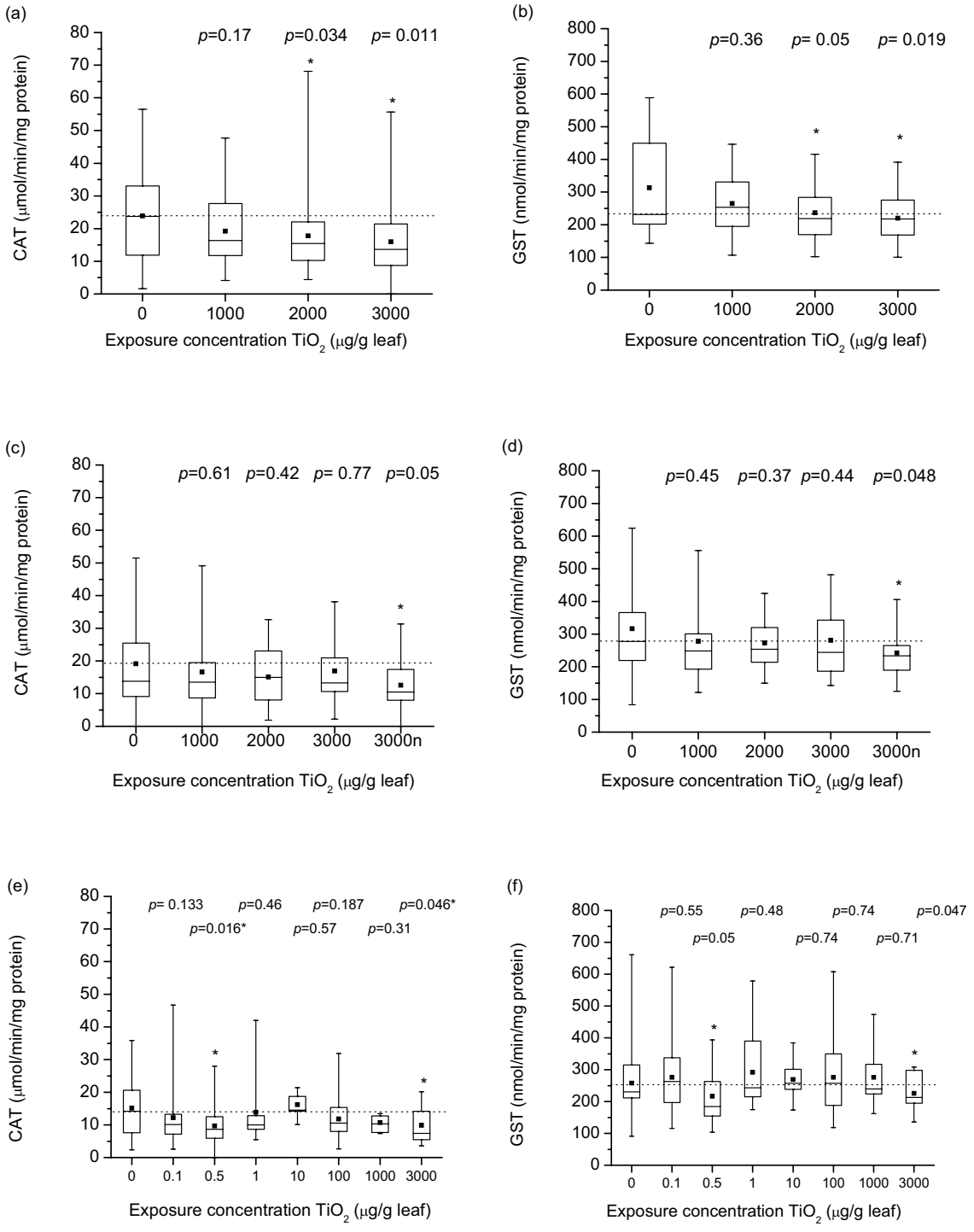
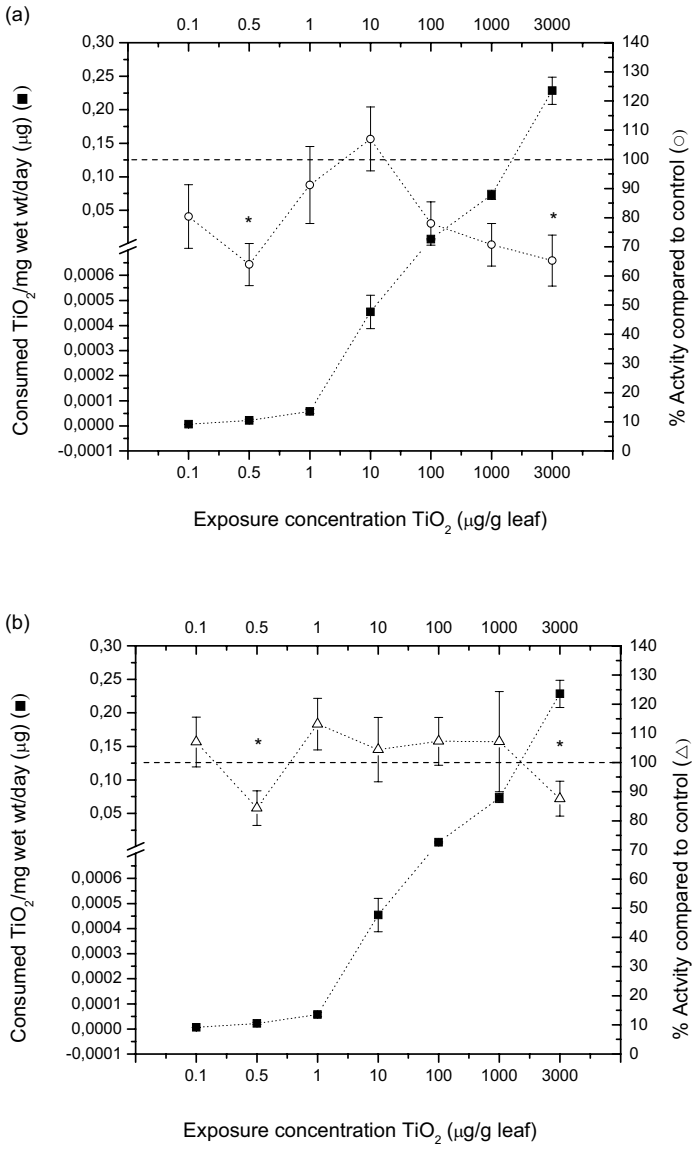

(a)
p=0.17 p=0.034 p= 0.011
CAT (µmol/min/mg protein)
Exposure concentration TiO2 (µg/g leaf)
(b)
p=0.36 p= 0.05 p= 0.019
GST (nmol/min/mg protein)
Exposure concentration TiO2 (µg/g leaf)
(c)
p=0.61 p=0.42 p= 0.77 p=0.05
CAT (µmol/min/mg protein)
Exposure concentration TiO2 (µg/g leaf)
(d)
p=0.45 p=0.37 p=0.44 p=0.048
GST (nmol/min/mg protein)
Exposure concentration TiO2 (µg/g leaf)
(e)
p= 0.133 p=0.46 p=0.187 p=0.046*
p=0.016* p=0.57 p=0.31
CAT (µmol/min/mg protein)
Exposure concentration TiO2 (µg/g leaf)
(f)
p=0.55 p=0.48 p=0.74 p=0.047
p=0.05 p=0.74 p=0.71
GST (nmol/min/mg protein)
Exposure concentration TiO2 (µg/g leaf)

Fig. 6.


(a)
0.1 0.5 1 10 100 1000 3000
0,30 0,25 0,20 0,15 0,10 0,05
0,0006 0,0005 0,0004 0,0003 0,0002 0,0001 0,0000 -0,0001
140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
Consumed TiO2/mg wet wt/day (μg) (■)
% Actvity compared to control (○)
*
*
Exposure concentration TiO2 (μg/g leaf)
(b)
0.1 0.5 1 10 100 1000 3000
0,30 0,25 0,20 0,15 0,10 0,05
0,0006 0,0005 0,0004 0,0003 0,0002 0,0001 0,0000 -0,0001
140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
Consumed TiO2/mg wet wt/day (μg) (■)
% Actvity compared to control (△)
*
*
Exposure concentration TiO2 (μg/g leaf)