IN FIELD CONDITIONS, COMMERCIAL PIGMENT GRADE TiO_2 WAS NOT HARMFUL TO TERRESTRIAL ISOPODS BUT REDUCED LEAF LITTER FRAGMENTATION

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Abstract

We investigated the effects of a commercial pigment grade rutile TiO_2 on the terrestrial isopod *Porcellio scaber* in three locations that differed in terms of abiotic and biotic conditions: the laboratory, open air, and the closed barn. Mortality and isopod energy reserves (digestive gland total proteins, lipids and carbohydrates) were not affected following 14 d exposure to up to 1000 mg TiO_2 per kg dry leaves (mg/kg) under any experimental scenario. However, in the field tests, isopods consumption of TiO_2-coated leaves was reduced compared to that of uncoated leaves and the decrease was not dose-dependent. The highest reduction was in the closed barn (45 – 56%) rather than in the open-air (38 – 40%). In laboratory-based food choice tests, isopods neither preferred nor avoided leaves coated with TiO_2, suggesting that rather than sensing the TiO_2 on the leaves directly, the isopods under open-air and barn exposure responded to altered attractiveness and/or palatability of the TiO_2 amended leaves. We propose that this could be due to altered microbial population on the leaves, a hypothesis that requires further investigation. Although short-term exposure to atmospheric deposition of up to 1000 mg/kg commercial TiO_2 is unlikely to pose an immediate threat to isopod mortality and energy balance, reduced leaf feeding may have implications for the decomposition of plant material.

Keywords: *Porcellio scaber*, energy reserves, avoidance behaviour, litter breakdown

1 INTRODUCTION

Titanium dioxide (TiO_2) powders have been commonly used as white pigments from ancient times (Hashimoto et al., 2005). Because of its high refractive index TiO_2 pigment is used in virtually every kind of paint and the volume of TiO_2 pigments produced world-wide is remarkably high (over 4 million tons per year) (Diebold et al., 2003). According to one of the

largest producer of TiO_2 in south eastern Europe (Cinkarna Celje, Slovenia, www.cinkarna.si) the most common form is "pigment grade" TiO_2. This grade has a size range of approximately 200-350 nm with a small fraction of the primary particles < 100 nm.In recent years however, there has been a growing demand for "ultrafine" TiO_2 in which the majority of particles have a diameter of < 100 nm, defined as nano-sized titanium dioxide according to EU Recommendation on the definition of a nanomaterial (EU Recommendation on the definition of a nanomaterial, 2011). Titanium dioxide nanoparticles (TiO_2 NM) are used in a wide variety of materials and applications including self-cleaning surface coatings, light-emitting diodes, solar cells, disinfectant sprays, sporting goods, water-treatment agents, and cosmetics (IARC/WHO, 2010). The upper global annual production of nano TiO_2 in 2010 was estimated at > 88,000 metric tons, 45% of which was expected to be used in coating, paints and pigments (Keller et al., 2013). Emissions of TiO_2 NM in the coatings, paints, and pigments sector in 2010 were calculated to be between 5,000 and 22,000 metric tons/year with 38,200 metric tons/year being released to soil (Keller et al., 2013). Although these quantities reported by Keller et al. (2013) were speculative, they strongly suggest that soil organisms will be exposed to TiO_2 NM. Quite variable values for modelled concentrations of nano TiO_2 have been reported depending on the exposure scenario. For example, mean values in range of approximately 1-80 µg/kg soil/soil treated with biosolid were reported for diffusive TiO_2 emission, and cca. 1000 µg/kg soil/soil treated with biosolid for local site at the highway after 40 years of accumulation (Gottschalk et al., 2013).

A large proportion of the available data on the effects of nanomaterials on terrestrial organisms have been on the effects of uncoated research-quality TiO_2 NM (Hu et al., 2010; Novak et al., 2012, McShane et al., 2012). The primary size of particles used in these studies is commonly close to 100 nm which defines them as nanomaterials (Recommendation on the definition of a nanomaterial, 2011). In reality, however, the TiO_2 particles substantially aggregate in aqueous media to form aggregates with diameters of 200 nm and higher (Hund-Rinke and Simon, 2006; Ma et al., 2012). Furthermore, TiO_2 particles in commercial use usually possess surface functionalization including coating of metals such as Al or Zr or polymers [www. sachtleben.com]. Studies on the non-functionalised TiO_2 particles demonstrated that under laboratory conditions, TiO_2 NMs generally did not induce adverse effects on terrestrial organisms. For example, no effect was observed on juvenile earthworm survival and growth, adult earthworm survival, cocoon production, cocoon viability of earthworms (*Eisenia andrei* and *Eisenia fetida*) exposed up to 10,000 mg TiO_2 NM /kg dry soil (McShane et al., 2012), and there was no evident effect on the feeding rate, weight change and survival of crustaceans isopods *Porcellio scaber* after feeding on 1000 mg TiO_2 NM /kg dry leaves for 14 days (Novak et al., 2012) or on 5000 mg TiO_2 NM /kg dry leaves for 28 days (Srpčič et al., 2015). However, studies with aquatic organisms (for example benthic amphipod, algae, daphnids, and fish) suggested that UV enhances the toxic effect of the anatase/rutile composites of TiO_2 (Li et al., 2014; Hund-Rinke and Simon, 2006, Ma et al., 2012) and other studies have shown that field exposure may modulate the toxicity of nanomaterials (Blinova et al., 2010). For the current study, we postulated that although TiO_2 NMs did not previously induce severe effects on terrestrial organisms in the laboratory, their effect could be different in the field due to significantly different abiotic and biotic conditions, such as variations in the light and moisture regime.

Terrestrial isopods *Porcellio scaber* (Isopoda, Oniscidea, Porcellionidae, Latreille, 1804) are widely distributed, occurring from Iceland to South America and South Africa (Harding and Sutton, 1985). They are abundant and can be found under stones and dead wood, particularly in gardens, marginal grassland and open woodland. Isopods play an important role in plant litter decomposition processes by breaking down organic materials

such as fallen leaves into smaller fragments (Hassall et al., 1987; Gerlach et al., 2014; Špaldoňová and Frouz, 2014), and affect the soil processes by physical transportation of litter materials in soil column, and inducing alteration of microbial activity (David, 2014). They also seem to be able to feed directly on soil, surviving well without the addition of extra food for 14 days, and demonstrate a similar metal uptake rate whether the contamination is supplied on leaves or in the soil (Udovič et al., 2009; Vijver et al., 2006). It has been demonstrated that environmental contamination may decrease the feeding performance of terrestrial isopods (Loureiro et al., 2006) thereby potentially reducing the rate of leaf litter fragmentation. However, the effect of commercial pigment grade TiO_2 on isopod feeding patterns in the field is as yet unknown.

The aim of the current study was to investigate the effects of an industrial pigment grade rutile TiO_2 used in the manufacture of paint on the terrestrial isopod *P. scaber* (Isopoda, Oniscidea, Porcellionidae, Latreille, 1804) under different field and laboratory exposure scenarios. The exposure sites varied in terms of a variety of abiotic factors, such as temperature, humidity, and the illumination, and microbial environment. We also performed two food choice experiments, one with individual animals and one with group exposures, to investigate whether the isopods discriminated between (i.e., avoided or preferred) TiO_2-amended and non-amended leaves. Effects on isopods were evaluated by observations on mortality, feeding behaviour, and measurement of energy reserves in terms of total lipid, protein and carbohydrates content. The study focused on isopod leaves consumption activity as an indicator of invertebrate litter fragmentation potential.

A considerable number of studies have been conducted on pristine TiO_2 NPs but few studies have investigated the effects of TiO_2 materials that are already used in commercial applications and therefore are more likely to be released into the environment. In this study, we exposed isopods to a popular commercial source of TiO_2 collected from a paint factory in Finland, which is commonly used in large amounts to produce high quality solvent and water-borne decorative paints, industrial coatings such as car refinishes, electrodeposition paints and coatings and plastics for exterior rigid PVC applications (Koponen et al., 2015).

2 MATERIALS AND METHODS

2.1 Characteristics of commercial TiO_2 pigment

The TiO_2 material was alumina-zirconia surface-treated rutile TiO_2 pigment (RD3 TiO_2 pigment, Sachtleben Pigment GmbH, Pori, Finland; CAS No. 13463-67-7, www.sachtleben.de). It was collected with a small scoop from bags of pigment prior to their addition to the main mixing chamber of a paint factory in Finland (Koponen et al., 2015). According to the product information data, the pigment was composed of 93% TiO_2 with a 7% coating of Al_2O_3-ZrO by weight (RD3 product information sheet, www.sachtleben.de), with a bulk density of 800 kg/m^3 and a mean crystal size of 220 nm (properties listed by the producer). Scanning electron microscopy revealed that a very small portion of the primary TiO_2 particles in each sample had diameters close to 100 nm (Supplementary information Fig. S1). Despite the material not falling into the strict if arbitrary category of ‘nanomaterial’ (recommendation on the definition of a nanomaterial, 2011), the effect of release of commercial mixtures of nanosized and near-nanosized particles on environmental health warrants investigation.

2.2 Application of TiO_2 in the soil and onto the leaves

The TiO_2 were dispersed in ultra-pure H_2O (MilliQ, Millipore, Bedford, Massachusetts, USA), vortexed and sonicated using probe sonicator for 4 min at 20 kHz using 130 W sonicator probe at 31% of its maximum power (final power 40W) (Sonics, VibraCell, VCX 130 PB). The TiO_2 stock (5 g/L) was stored up to 1 month in the dark at room temperature. This stock was used to prepare the final (nominal) concentrations of TiO_2 applied onto leaves and soil, which were 50, 100, 500, and 1000 mg/L for 50, 100, 500, and 1000 mg/kg dry leaves or soil, respectively. Final concentrations of TiO_2 were not confirmed because of the difficulties involved in preparing TiO_2 for measurement (McShane et al., 2012). In this work we choose lower concentrations of TiO_2 than those previously tested in our laboratory (1000-5000 mg/kg dry leaves) (Novak et al., 2012; Srpčič et al., 2015) where no effect on feeding rate was found, but energy reserves were decreased after 28 days of exposure. However, we expected a higher impact of TiO_2 in this series of tests because we exposed the animals in the field, and because in some tests, both soil and leaves were contaminated.

Application onto leaves. Fallen dry common hazel leaves (*Corylus avellana*) with minimally damaged leaves lamina were used for the experiment. For 100 mg of leaf, 100 µl of freshly prepared TiO_2 dispersion was applied onto abaxial leaf surface and spread over the surface as evenly as possible, using a paintbrush. This resulted in the nominal exposure concentrations 0, 50, 100, 500, and 1000 mg/kg TiO_2 /kg dry leaves (**Table 1)**. Each test included a negative control treatment that received deionized water only. After application the leaves were left to dry at room temperature for 24 h prior to the test.

Spiking of the soil. The agricultural soil was collected from the McGill University farm in Quebec, Canada (45º30'N, 73º35'W). The soil was characterized as a sandy loam with 5% organic matter (loss-on-ignition method), with a pH 5.94±0.03 in water, a cation exchange capacity of 10.37 meq/100 g, and a water holding capacity of 44.9±1.3 g H_2O/100 g dry soil. In the experiments 5 and 6 (Table 1), Lufa 2.2 soil (LUFA Speyer, Speyer, Germany) was used. It was characterized as a loamy sand with 1.77±0.2% organic matter, 7.2±1.2% clay, pH 5.5±0.1 (0.01 M $CaCl_2$), cation exchange capacity 10.1±0.5 meq/100 g, and water holding capacity 41.8±3.0 g H_2O/100 g dry soil.

In all experiments with contaminated soil, only the upper 20% of the total soil in the test container was spiked with the TiO_2. The amended soil was gently placed on top of the remaining 80% unamended soil, so only the top one cm (approximately) of the soil was contaminated. This method of amendment was selected to reproduce aerial deposition of powders released accidentally from factories during paint processing. To amend the soils, the TiO_2 dispersions were added to air-dry soil and mixed thoroughly. Negative control treatments received deionized water only. The final nominal concentrations of TiO_2 were 0, 50, 100, 500, and 1000 mg/kg TiO_2/kg dry soil and are summarised in **Table 1**. The moisture content of each soil was then adjusted to 30% of its water holding capacity (WHC). In the laboratory exposures, moisture loss was determined through mass balance and replenished on a daily basis, whereas under field conditions, the 30% WHC was only adjusted at the beginning of experimental set up.

2.3 Experiments with isopods

2.3.1 Test organisms

The *P. scaber* isopods originated from the synchronized laboratory culture at the Department of Biology, University of Ljubljana (Večna pot 111, Ljubljana), originally derived from individuals collected from a compost heap in a non-polluted garden in Ljubljana, Slovenia in the spring of 2014. Prior to the tests, animals were acclimatised for 14 d in a climate chamber at 22±1 °C with a 16/8 h light/dark period of 120 and 16 lux, respectively (LI-1000 Data Logger, LI – COR, Nebraska, USA), where they were caged in glass containers with moist loamy sand and peat at the bottom, and fed on dry fallen leaves from common hazel (*C. avellana*) and common alder (*Alnus glutinosa).* Only adult animals (30-60 mg fresh body mass) of both sexes with intact antennae were used for the test. Moulting individuals and gravid females were excluded.

2.3.2 Experimental overview

Isopods were exposed to TiO_2 for 14 d via several exposure regimes, either in the laboratory or in the field. Two major types of experiments with different endpoints were conducted: (i) leaf consumption tests (the total amount of consumed leaves), and (ii) food/soil selection tests (the ability of isopods to select TiO_2 spiked versus non-spiked leaves/soil). The experiments are summarised in **Table 1 and Fig.S2 (Supplementary information)**. In all experiments the mass of the consumed food (leaves), the mass of animals prior/after the experiment, and animal mortality were recorded after 14 days of exposure.

Table 1: Experimental design of all experiments with isopods. Tested concentrations, mode of exposure, and the number of test animals exposed per treatment are given.

No. of Experiment	Type of experiment	Contaminated material	TiO_2 concentrations (mg/kg dry weight leaves and/or soil)	No. of animals per replicate	No. of replicates per concentration
	LEAVES CONSUMPTION				
1[#]	Laboratory	Leaves	100, 500, 1000	1	10
2	Laboratory	Leaves and soil[*]	1000	12	2
3	Field, ambient	Leaves and soil[*]	50, 100, 500	12	2
4[#]	Field, barn	Leaves and soil[*]	50, 100, 500	12	2
	LEAVES SELECTION				
5	Laboratory-group	Leaves	1000	10	3
6	Laboratory-individual Petri dish	Leaves	1000	1	15

[*]upper 20% of soil only. See text for details, [#] energy reserves were measured in this experiment.

2.3.3 Leaf consumption tests

2.3.3.1 Laboratory exposure

All laboratory exposures took place in a climate chamber at 21 °C with a 16/8 h light/dark cycle. The UV radiation (UV-R) in the laboratory was close to zero (UVB= 0.001 W/m^2; Radiometer RM 22; Dr.Gröbel, UV-elektronik GmbH).

Experiment 1. Individual isopods in Petri dishes were exposed as previously described to TiO_2 applied onto leaves (Golobič et al., 2012). Ten specimens were exposed per treatment. Each animal was placed individually into a plastic Petri dish (diameter 9 cm) along with the prepared leaves with the contaminated side facing up and covered with a plastic lid. No soil substrate was used. The Petri dishes were placed in a covered glass container and constant moisture was maintained by sprinkling the walls of the container with deionized water. The food was not replaced during the exposure period, and faecal pellets were collected weekly to prevent consumption of faeces (**Table 1, Fig. S3A and S3B**). Tested concentrations are listed in **Table 1**.

Experiment 2. Groups of isopods (12 in each test box) were exposed to both TiO_2-contaminated soil (approximately 1 cm top layer) and TiO_2-contaminated leaves. The soil was placed in plastic test container (15 x 20 x 8 cm; width x length x height) with a perforated lid, and five pieces of TiO_2 spiked hazelnut leaves were evenly distributed over the entire soil surface. One stone was placed in each of the two opposite corners of the test container to provide shelter for the isopods (**Fig. S3C**). The stones had identical surface areas. This test was performed twice. Tested concentrations are listed in **Table 1**.

2.3.3.2 Field exposure

In the field tests, isopods were exposed under two scenarios representing their most common habitats, in groups in either ambient conditions of light and temperature in the vicinity of the resident house (**Experiment 3**), or in a closed barn with mostly dark environment (**Experiment 4**). In both of the field scenarios, groups of 12 isopods were exposed simultaneously to TiO_2-contaminated agricultural soil (approximately 1 cm top layer) and TiO_2-spiked leaves. Also control treatment was applied.

Experiment 3. The experiment in open-air field conditions was performed in the late summer of 2014 at the corner of a building located in the woods surrounding the University of Ljubljana (Biotechnical Faculty, Department of Biology) campus (**Fig. S3D**). The test location was protected from direct rain by an overhanging roof, and was oriented toward east and close to trees to reduce direct sun and reduce risk of animal overheating. The sun had direct access to the location only between sunrise (7 am) and 11 am, during which time the UV radiation was relatively low (UVB = 0.006-0.070 W/m^2, UVA was < 0.006). After 11 am the exposure test containers were in the shadow (UVB and UVB $\leq$ 0.006 W/m^2). The midday temperatures in the experimental units were 21 ± 1°C. During the experimental period (28/8/2014-10/9/2014) the average daily temperature in Ljubljana was 14 - 20°C with a minimum of 11 - 15 °C, and maximum of 20 - 23 °C (ARSO, 2014). Animals were placed in test containers with agricultural soil and TiO_2 amended leaves. The plastic test containers (15 width x 20 length x 8 cm height) were covered with a mesh lid to allow the air exchange and access to sunlight but prevent access to predators and escape of test isopods (**Fig S3E**). The leaves and stones were placed in similar positions to those in the laboratory experiments (**Experiment 2**). The TiO_2 concentrations are given in **Table 1**.

Experiment 4. This test was conducted in a closed barn with limited light (UVB and UVB$\leq$ 0.005 W/m^2) and at a fairly constant temperature (15 ± 2 °C) (Fig S1 F). The animals were exposed to TiO_2 via contaminated agricultural soil and contaminated leaves. Test containers were similar to those used in Experiment 3 (15 width x 20 length x 8 cm height), and groups of animals (n=12 per replicate) were exposed to TiO_2 via contaminated agricultural soil and polluted leaves. The TiO_2 concentrations are given in **Table 1.**

2.4 Food selection tests

Food selection experiments comprised two types of 14 days selection tests: (i) group exposure and (ii) individual exposure.

Experiment 5. The group food selection experiments were conducted in plastic test containers with dimensions of 11 width x 14 length x 5 cm height. In this case we used 100 g of unamended standardised natural soil Lufa 2.2 as substrate. The soil was adjusted to 30% WHC. For each group one whole leaf was cut in half, and the halves were placed on opposite side of the container. In the middle of the test container a slight soil hump was made that allowed passage of animals but prevented the transfer of leaves. In the control group both leaf pieces were treated with deionized water, while in the treated group one leaf piece was contaminated with the TiO_2 dispersion and the other was unamended. Two types of pair choices were performed: control leaves versus control leaves, and control leaves versus contaminated leaves with 1000 mg TiO_2/kg dry leaves. In each group exposure, 10 animals were exposed. The experiment was repeated 3 times. At the end of experiment the mass of consumed leaves from each respective side of the container was recorded and the share of each type of exposure leaves was calculated.

Experiment 6. Individual food selection experiments were performed in addition to group experiments because group exposures are commonly criticized because of the potential for isopod aggregation behaviour, and social interactions to affect their food/soil choice (Broly et al., 2012; Zidar et al., 2012). In the individual exposure, individual animal was placed in Petri dish and offered the choice between two hazelnut leaf pieces (amended or unamended), originally from the same leaf. 15 isopods per concentration were exposed individually. As the leaves could be moved by the isopods, treated and untreated leaves pieces were distinguished by their different shapes (square and triangular). For each individual animal, one whole leaf was cut into 4 pieces, two of which were offered in the first week with the other two offered in the second week of exposure. Otherwise, the leaves were prepared in the same manner as for the group food selection tests. The faecal pellets were collected weekly, and at the end of experiment the mass of consumed leaves was recorded.

2.5 Isopod energy reserves

To compare the physiological status of animals from the laboratory (Experiment 1) with those from the field (Experiment 4), energy reserves in the digestive glands (hepatopancreas) of the isopods were determined. Total lipid and carbohydrate content were measured according to Ferreira et al (2015). Briefly, the isopods were dissected at the same time of the day and the complete digestive gland of each individual animal was homogenised in 650 µL of 50 mM potassium phosphate buffer (pH=7.0) with a probe sonicator (Sonics, VibraCell, VCX 130 PB) at 20 kHz (final power 40 W) for 45 s. All three classes of energy reserves - protein, lipids and carbohydrates - were measured on the same digestive gland homogenate.

Total carbohydrate quantification. 100µL of 15% tricholoroacetic acid (TCA) was added to 300 µl of the homogenate. After vortexing, samples were incubated at -20°C for 10 min followed by centrifugation at 3500 rpm for 10 minutes. The supernatant was separated and further processed as a carbohydrate fraction. The supernatant was then diluted 2 times (300 µL of sample + 300 µL of deionized water). Carbohydrate content was determined via sulfuric acid-UV method according to Albalasmeh et al (2013): sample and concentrated sulphuric acid (H_2SO_4, Merck) were rapidly mixed in ratio 1:3 (150 µL of sample: 450 µL of

H_2SO_4) and the UV light absorbance was measured at 315 nm. Glucose (Sigma-Aldrich) was used as a standard and was processed in the same manner as the sample. Each measurement was done in triplicate.

Total lipid quantification. Total lipid quantification was based on the method by Bligh and Dyer (1959). 500µL of chloroform and 500 µL of methanol were added to 100 µL of the homogenate. After vortexing each sample for 1.5 minutes, 250 µL of deionized water was added. The samples were vortexed again for 1.5 minutes. Subsequently the samples were centrifuged at 2500 g for 10 minutes. The top phase was removed and the bottom phase was used for total lipid measurement. 100 µL of lipid extracts (bottom phase) was mixed with 500 µL of H_2SO_4 and heated for 15 minutes at 200 °C. After cooling down, the total lipid content was measured as the absorbance at 375 nm. Tripalmitin (Sigma-Aldrich) was used as a standard and was processed in the same manner as the sample. Each measurement was done in triplicate.

Total protein quantification. The concentration of proteins in the homogenate was measured using a Pierce® BCA™ Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA), a modification of the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). The homogenates were not centrifuged prior to measurements. Each measurement was made in triplicate. Bovine serum albumine analytical standard (BSA, Sigma Aldrich) was used as a standard and was processed in the same manner as the sample. Each measurement was done in triplicates.

2.6 Data analysis

The experiment was considered valid if mortality in the control treatments did not exceed 20 % (Hornung et al., 1998). All data presented in Figures refer to nominal concentrations of TiO_2 (in mg/kg dry leaves or soil). For the leaf consumption tests, consumption was calculated as the mass of consumed leaves (mg) per individual animal mass (in Petri dish exposure, **Experiment 1**) and as the mass of consumed leaves (mg) per total animal mass (mg) at the end of exposure in case of group exposures. Statistical significance between the control and exposed groups of animals (feeding rate in the case of Experiment 1, and energy reserves in Experiments 1 and 4) was assessed by a Mann-Whitney U test ($p < 0.05$) using Statgraphics software (Statgraphics Plus for Windows 4.0, Statistical Graphics, Herndon, VA, USA). In the case of the 14 days group feeding exposures (Experiments 2, 3, 4) the change in the feeding in the treatment groups was denoted on figures as % change in comparison to controls. In these experiments, the statistical analysis was not feasible due to limited amount of data (two technical replicates were done).

For the food selection experiments (5 and 6), the mass of eaten leaves was recorded after 7 days and 14 days and the percentage of each leaf eaten was calculated. The results were expressed as mean +/- SD. The statistical differences between two pair choices were done using Mann-Whitney U test ($p < 0.05$) using Statgraphics software (Statgraphics Plus for Windows 4.0, Statistical Graphics, Herndon, VA, USA).

The total lipid, carbohydrates and protein contents were calculated from the standard curve of corresponding standards (glucose, tripalmitin and BSA protein). They were expressed as the µg of lipid/protein/carbohydrate per mg animal fresh weight (Donker, 1992).

3 RESULTS

3.1 Isopod 14 days feeding experiments

3.1.1 Laboratory exposure

Experiment 1: Exposure to contaminated leaves in the laboratory (Petri dish). There was no significant difference between mortality in the TiO_2 and control treatments (>20%). No differences in individual specimen food consumption were observed in exposure to up to 1000 mg/kg dry leaves TiO_2 (p>0.05; **Fig. 1A)**.

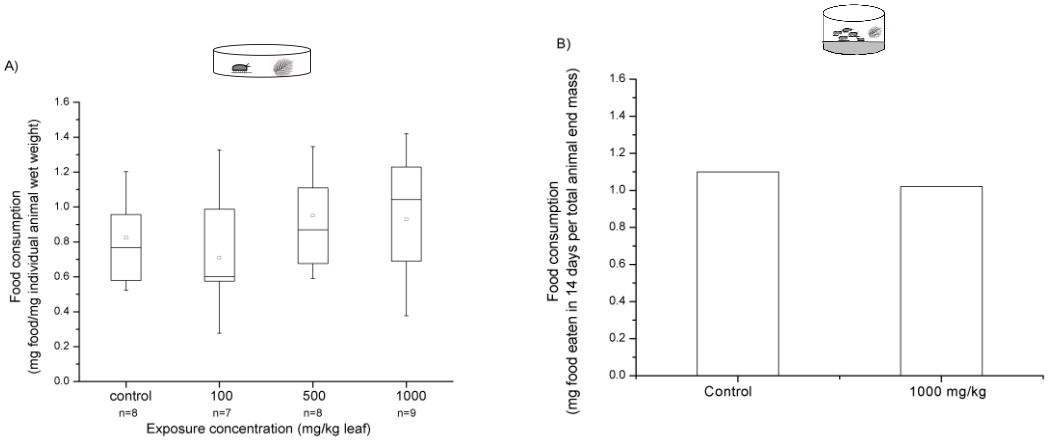
Experiment 2: Exposure to contaminated leaves and soil in the laboratory. There was no significant difference between mortality in the TiO_2 and control treatments. Consumption of TiO_2 amended leaves did not decrease compared to control (**Fig. 1B)**.

3.1.2 Field exposure

Experiment 3: **Open-air exposure to contaminated leaves and soil.** No significant mortality (below 20%) was observed in Experiment 3. However, leaf consumption by animal presented with TiO_2 contaminated leaves was 37 to 40% less than that in control treatments and was not concentration dependent (**Fig 1C)**.

Experiment 4: Exposure to contaminated leaves and soil in a closed barn. There was no significant difference between mortality in the TiO_2 and control treatments. Isopods exposed to TiO_2 contaminated leaves consumed up to 57% less than those in control treatments. Again, the decrease was not concentration dependent (**Fig 1D)**.

LABORATORY EXPOSURE



FIELD EXPOSURE

OPEN-AIR **CLOSED BARN**

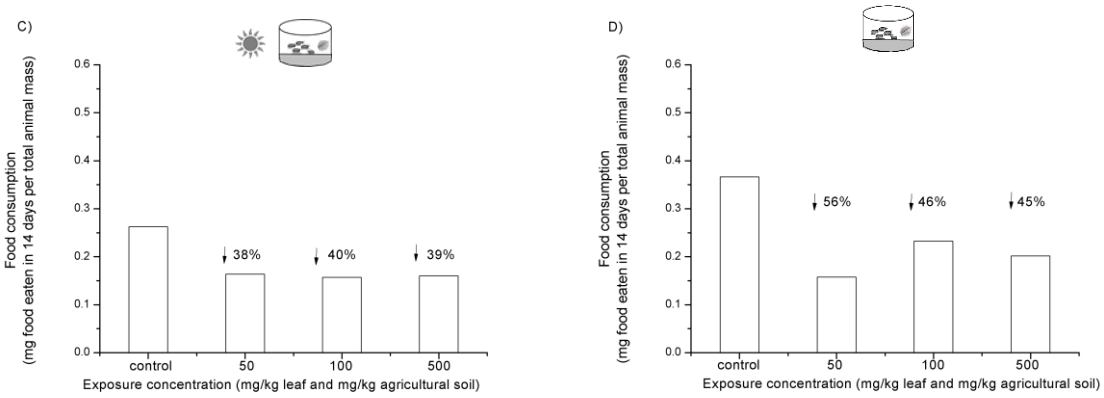
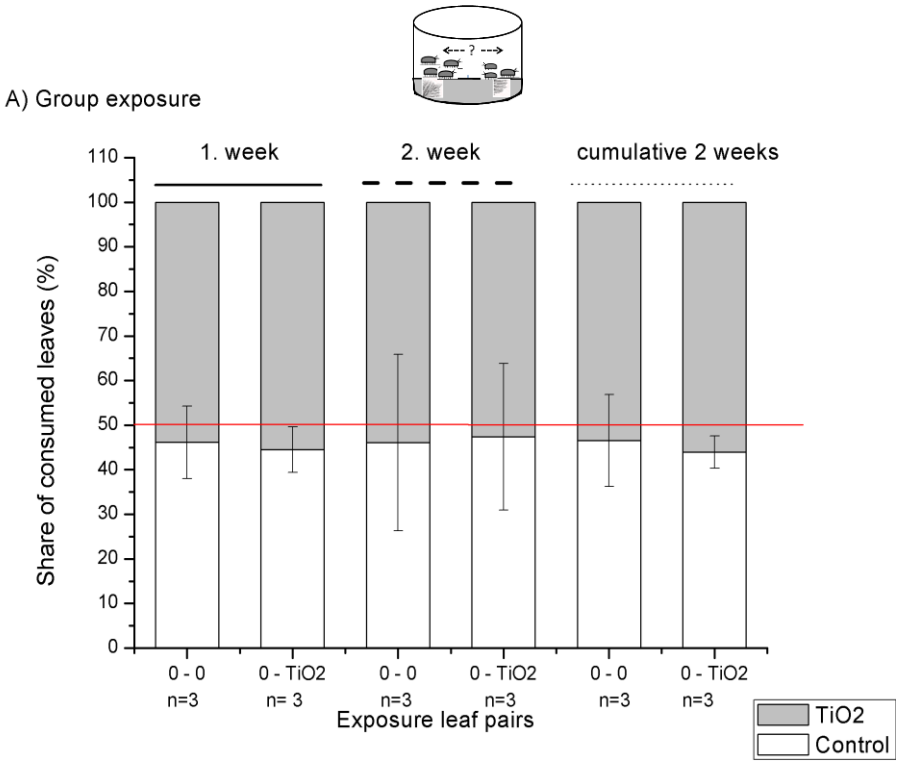


Fig. 1. Results of isopod 14 days feeding experiments on leaves: (A) Experiment 1 (individual animals in Petri dish, contaminated leaves, no soil); **(B)** Experiment 2 (contaminated leaves and soil), **(C)** Experiment 3 (contaminated leaves and soil (exterior exposure), and **(D)** Experiment 4 (contaminated leaves and soil (barn).

3.2 Isopod food selection test

In all paired leaf exposures (Experiments 5 and 6), isopods consumed approximately the same amount of the two offered sets of leaves (approx. 50%) either when control vs control leaves were present or when control vs TiO_2 contaminated leaves were offered ($p>0.05$, not significantly different). This indicates that isopods neither avoided nor selected TiO_2 contaminated leaves (1000 mg/kg dry leaves), either individually or in groups (**Fig. 2**).



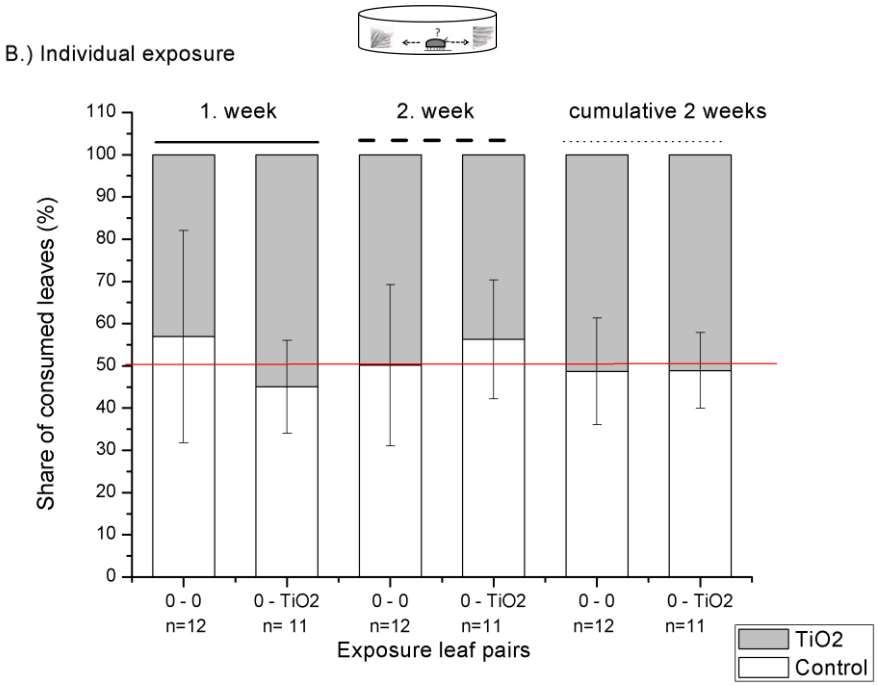
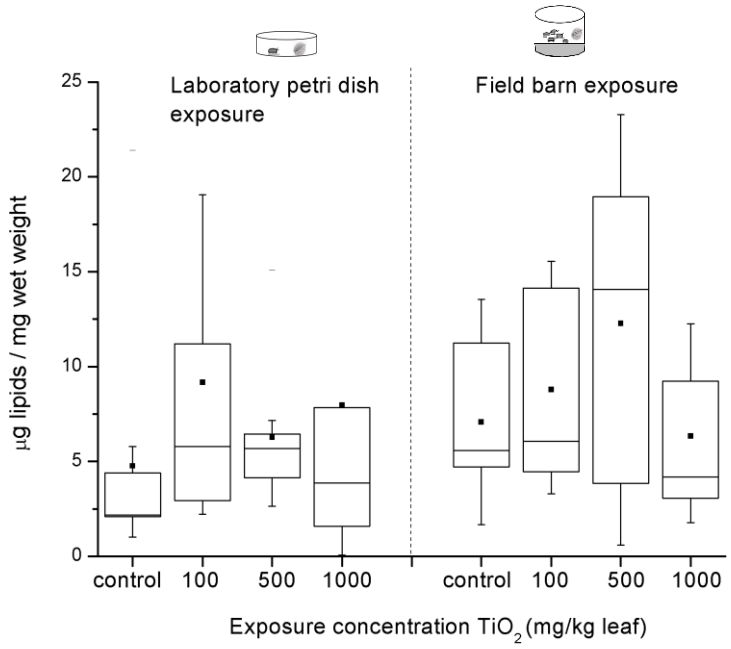


Fig.2. Percentage of consumed leaves isopods exposed to TiO_2 (1000 mg/kg dry soil) (A) group exposure (Experiment 5), and (B) individual exposure in the laboratory (Experiment 6). Two pair choices were conducted: 0-0: control lea-control leaf; 0-TiO_2: control leaf- TiO_2 contaminated leaf. Data are shown for the first week of exposure, the second week of exposure and both weeks cumulative. Data are mean ± SD (n=15 in case of individual exposure and n=3 in case of group exposure).

3.3 Isopod energy reserves measurements

The total lipid, carbohydrates and protein content in digestive glands of isopods exposed to different concentrations of TiO_2 were not significantly different from those in their respective control treatments, either in the Petri dish exposure (Experiment 1) or in the field (barn) exposure (Experiment 4 - **Fig.3 A-C**). Also, there were no significant differences between energy reserves in the different TiO_2 treatments. The isopods in the control treatment in the field tests consumed significantly less (70%) leaf mass than did the equivalent animals in the laboratory exposures (**Fig. 2**). Nevertheless, this difference was not reflected in the digestive gland lipid and protein content, which were similar in control animals in the two exposures (**Fig. 3 A, C**). However, the animals in the field test control treatments had significantly less carbohydrate in digestive glands than did those in the laboratory tests (**Fig. 3 B).**

A)


Laboratory petri dish exposure
Field barn exposure
μg lipids / mg wet weight
25
20
15
10
5
0
control
100
500
1000
control
100
500
1000
Exposure concentration TiO2 (mg/kg leaf)

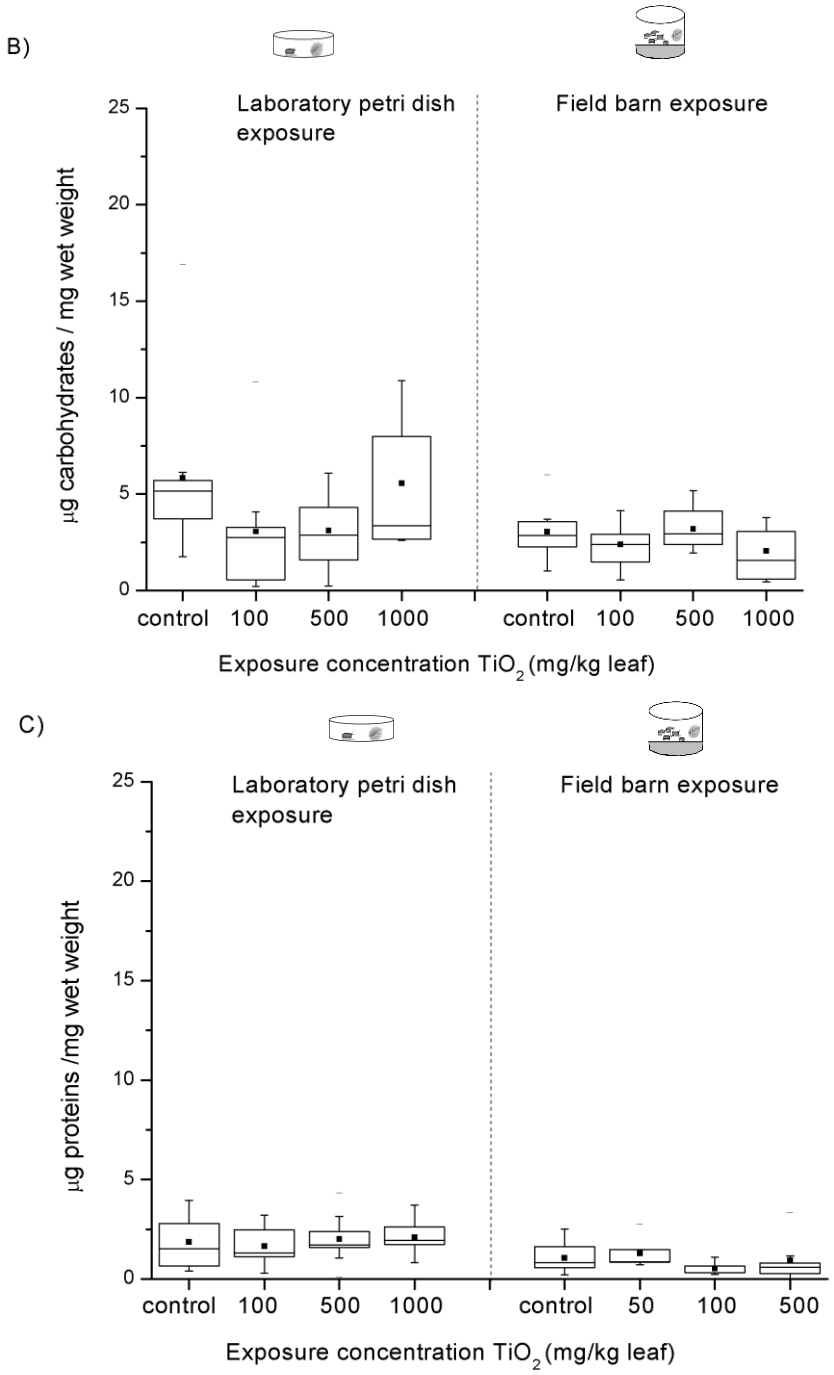


Fig. 3. Total lipid (A), carbohydrate (B), and protein content (C) in digestive glands of isopods *Porcellio scaber* fed for 14 days with TiO_2 spiked food in laboratory Petri dish exposure (Experiment 1) and field barn exposure (Experiment 4). Each group comprised 15 replicates.

4 DISCUSSION

This study investigated the effects of a commercial pigment grade rutile TiO_2 on the terrestrial isopod *P. scaber* in the laboratory and in the field. Isopod consumption of leaves was more

greatly reduced in the barn exposure than in the open-air exposure, where a higher UV was measured, thus there was no evidence that the TiO_2 rutile pigment was phototoxic. This is in line with observations that Al- Zr doped TiO_2 rutile has lower photocatalytic activity than uncoated anatase TiO_2 (Allen et al., 2002; Yin et al., 2012). Since the feeding of isopods was more greatly reduced in the barn than the open-air exposure, and the UV was likely not the main reason for this difference, we suggest that environmental factors other than UV light, such as humidity, temperature, and microbial environment, may also be affecting isopod leaf consumption. We would like to point out that we are unable to determine whether the effects were caused by exposure to TiO_2, to the elements of the particle coating or to a combination of the two.

The food selection experiments (Experiments 5 and 6) demonstrated that in the laboratory, both in groups and individually, isopods neither preferred nor avoided commercial TiO_2 spiked leaves. This observation is in agreement with a previous study where earthworms (*E. andrei* and *E. fetida*) exposed in laboratory conditions neither preferred nor avoided artificial soils amended with less than 1000 mg/kg nano-TiO_2 (McShane et al., 2012). We hypothesised that the decreased consumption of TiO_2-amended leaves in the field tests was due to a reduction in leaves palatability that did not occur under laboratory conditions. While the reason for this reduction was not clear, we speculated that it could result from alterations in microbial colonisation of the leaves due to the presence of TiO_2. Isopods are known to prefer leaves litter with strong microbial colonies over weakly-colonised litter (Kayang et al., 1996), and respond to odours produced by microorganisms colonizing the litter rather than to odours from the leaves themselves (Zimmer and Topp, 1998). Furthermore, *Porcellio scaber* is able to discriminate between different types and species of microbes (Ihnen and Zimmer, 2008). Inhibition of microbial community growth by different nanostructured TiO_2 particles and their larger agglomerates (~1 μm) has previously been reported for natural aquatic environments (Battin et al., 2009). Further investigation of this hypothesis through food selection tests under field conditions was prevented by the onset of cooler weather conditions. As well as further food selection trials under field conditions, novel molecular approaches of microbial species identification can help elucidate whether application of TiO_2 to fallen leaves does affect microbial leaves colonisation.

Decreased leaves consumption by isopods is a common biomarker of effect in laboratory tests where the leaves are the only food source for isopods (Loureiro et al., 2006; Drobne et al., 2008). However, when the isopods are exposed to leaves applied onto soil, they may also feed directly on soil, surviving well without the addition of extra food for 14 days (Vijver et al., 2006; Udovič et al., 2009). This means that the decreased consumption of leaves observed in the field exposure, in which soil was also available, does not necessarily signify adverse effects for isopods. This was additionally supported by the observation that energy reserves in isopod digestive glands following 14 days of exposure to TiO_2 in a barn were unchanged despite significantly reduced leaves consumption compared to animals in 'clean' soil exposed in the barn. Furthermore, lipid and protein contents in digestive glands of isopods from the barn (Experiment 4) were similar to those in isopods from the laboratory tests (Experiment 1), which had consumed a greater mass of similarly-amended leaves. The lack of change in energy reserves in isopods exposed to TiO_2 suggests that the animals did not need to use stored energy. This has previously been shown to indicate stress (Ribeiro et al., 2001; Drobne and Štrus, 1996). A similar exposure to leaves contaminated with 1000 mg TiO_2/kg dry leaves (Srpčič et al., 2015), however, demonstrated that after 28 days, there was a decrease in isopod lipid reserves. The effect on isopod energy reserves of longer exposure to the commercial TiO_2 used in this study would therefore be of interest.

The results of the current study show that laboratory exposures do not entirely represent field exposure scenarios. Namely, in laboratory conditions, isopods consumed a similar mass of TiO_2 spiked leaves as unamended leaves. However, this was not the case in the field exposure. It was previously reported that field conditions altered the properties of TiO_2, thus modifying their toxicity to aquatic species (Blinova et al., 2010) and that field soil properties modulated the toxicity outcome to terrestrial invertebrates (Waalewijn-Kool et al., 2013). The current study demonstrates that unexpected adverse effects, such as decreased leaf consumption not observed in laboratory tests, may be revealed in field tests. We therefore suggest that in addition to standard laboratory testing, hazard assessment of nanomaterials should include field studies are required.

Isopods play an important role in litter decomposition by fragmenting it, and increasing the surface area available for microbial decomposition (Hassall et al., 1987). Decreased consumption of the TiO_2-contaminated leaves observed in the current study points to the potential for a reduction in the role of isopods in nutrient cycling. There are few reports available on the effect of nanomaterials on the decomposition of plant material but impregnating cotton material with TiO_2 nanoparticles decreased the total dehydrogenase activity in the soil and suppressed biodegradation of the material (Lazić et al., 2015). Leaf litter decomposition is an important process in the nutrient cycle of forests (Gerlach et al., 2014), and a reduction in the initial steps of breaking larger organic materials into smaller pieces could have wider implications for nutrient cycling. The effect of atmospheric deposition of TiO_2 on litter fragmentation and its resulting decomposition, even on a local scale, therefore merits more attention.

In conclusion, short-term 14 days exposure to concentrations of a commercial source of TiO_2 as high as 1000 mg per kg food or soil did not pose a hazard to the health of the isopod, *P. scaber*. However, exposure under field conditions resulted in lower leaf consumption, and it is possible that this could result in changes to plant material decomposition. The changes in isopod feeding habits observed under field conditions but not in the laboratory suggest that a combination of environmental factors may be involved in decreasing palatability of TiO_2 -amended leaves.

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