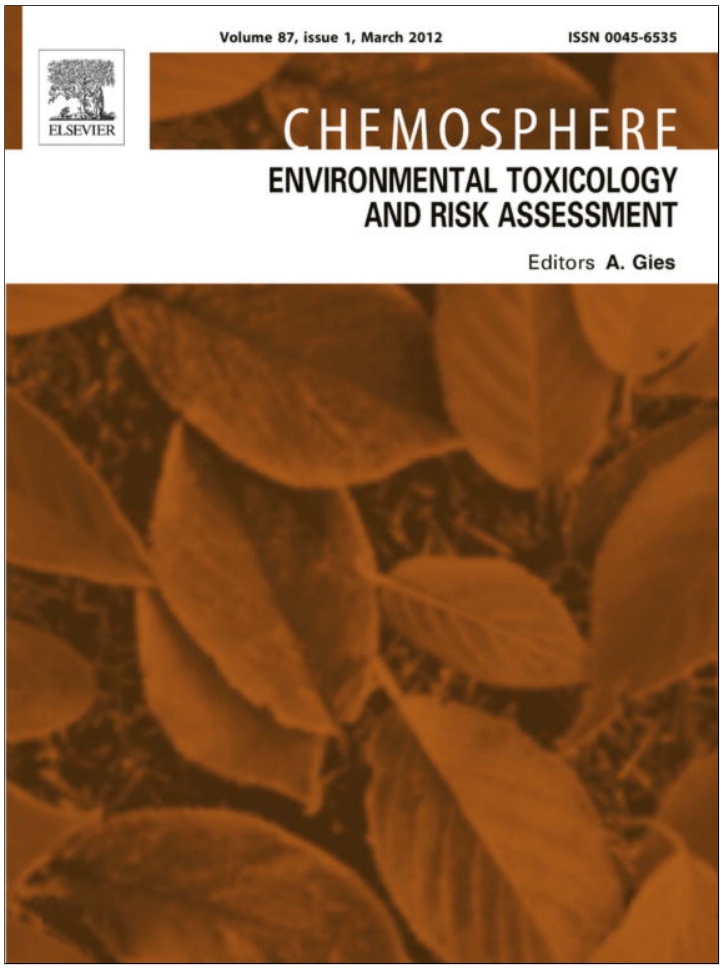

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# Effect of ingested titanium dioxide nanoparticles on the digestive gland cell membrane of terrestrial isopods

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ABSTRACT

The aim of this study was to find out whether ingested titanium dioxide nanoparticles (nano-$TiO_2$) cause cell membrane damage by direct contact or by lipid peroxidation. We assessed lipid peroxidation and digestive gland cell membrane stability of animals fed on food dosed with nano-$TiO_2$. Conventional toxicity measures were completed to determine if cellular effects are propagated to higher levels of biological complexity. An invertebrate model organism (*Porcellio scaber*, Isopoda, Crustacea) was fed with food containing nanosized $TiO_2$ and the result confirmed that at higher exposure concentrations after 3 d exposure, nano-$TiO_2$ destabilized cell membranes but lipid peroxidation was not detected. Oxidative stress as evidenced by lipid peroxidation was observed at longer exposure durations and high exposure doses. These data suggest that cell membranes are destabilized by direct interactions between nanoparticles and cell membrane, not solely *via* oxidative stress.



## 1. Introduction

Nanoparticulate-$TiO_2$ is one of the most commonly used nanoparticles and is expected to become ubiquitous in the environment. Currently, only predictions of the actual occurrence of engineered $TiO_2$ nanoparticles in the environment (Kaegi et al., 2008) or calculations of its potential environmental concentrations (Mueller and Nowack, 2008) are available, but the amount of data on the effects of $TiO_2$ nanoparticles on different species is increasing exponentially (Menard et al., 2011). In this paper, we report here the results of a study of the *in vivo* cellular effects of nano-$TiO_2$ in terrestrial isopods.

Recently some comprehensive reviews on the toxic potential of nano-$TiO_2$ relevant to human exposure (Aschberger et al., 2011; Iavicoli et al., 2011; Valant et al., 2012) or environmental species exposure (Menard et al., 2011) have been published. The aim of these reviews was to provide an overview of the detrimental impact of nano-$TiO_2$ on organisms. These comprehensive reports suggested that the mode of adverse action of nano$TiO_2$ on biological material is not entirely clear and that published reports are sometimes contradictory. Numerous *in vitro* and *in vivo* studies have provided evidence of oxidative stress resulting from nano-$TiO_2$ exposure. They point out that interaction between cells and nano-$TiO_2$ leads to elevated production of reactive oxygen species (ROS) (Shukla et al., 2011) but that otherwise, the mechanisms involved of oxidative stress are unclear (Johnston et al., 2010; Christensen et al., 2011; Iavicoli et al., 2011). The work of Trouiller et al. (2009), who made a comprehensive study of genotoxicity induced by $TiO_2$ nanoparticles *in vivo* in mice is consistent with these reports. These authors explain that genotoxicity is possibly caused by a secondary genotoxic mechanism associated with inflammation and/or oxidative stress.

In addition to indirect effects of nano-$TiO_2$ *via* ROS elevation modification of cell membranes by direct interactions with nanoparticles, possibly leading to stress has also been discussed. Physical interactions between nanoparticles and biological membranes in lipid vesicles serving as surrogates for cells have been reported by different authors (Holl et al., 2006; Leroueil et al., 2007; Wang et al., 2008; Zupanc et al., 2011).

The aim of the work presented here was to determine whether ingested $TiO_2$ nanoparticles damage cell membrane of digestive gland cells either mechanically or by means of oxidative stress. We studied the interactions of cells with nanoparticles *in vivo* in the digestive system of a model invertebrate organism (*Porcellio scaber*, Isopoda, Crustacea).

To elucidate the interactions between nanoparticles and digestive gland cells, we examined digestive gland cell membrane stability and oxidative stress resulting from lipid peroxidation. Conventional toxicity measures such as feeding behavior, weight change, and mortality were undertaken in order to test if nanoparticles' effects at lower levels of biological complexity are propagated upward.

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Cell membrane stability assays rest on the assumption that changes in cell membrane integrity result in changes in the permeability of cells to dyes (Mcgahon et al., 1995). We assessed cell membrane stability with an acridine orange/ethidium bromide (AO/EB) assay adapted for tissue analysis as described by Valant et al. (2009).

It has been reported that lipid peroxidation may result from interactions between nanoparticles and lipids (Kiwi and Nadtochenko, 2004) or from elevated concentrations of reactive oxygen species (ROS) which are capable of peroxidising membranes (Sayes et al., 2005). In the study presented here, we assessed lipid peroxidation by analysis of malondialdehyde (MDA) formation, which is known to be correlated with oxidative degradation of unsaturated lipids.

We investigated time- and dose-dependent effects of ingested nano-$TiO_2$ on digestive gland epithelium cells and assessed the performance of the exposed organisms and addressed the following three questions:

(1) Is the digestive gland cell membrane destabilized directly by nanoparticles or do nanoparticles first create oxidative stress which subsequently leads to cell membrane destabilization?
(2) Are membrane destabilization and lipid peroxidation in digestive gland cells dose- and/or time-dependent?
(3) Is digestive gland cell membrane destabilization and/or lipid peroxidation reflected in toxicity responses at higher levels?

We hypothesize that if nanoparticles interact directly and mechanically with cell membranes, it may be expected that the cell membrane will be affected before evidence of lipid peroxidation is observable. This could be the case at lower exposure concentrations and shorter exposure times.

## 2. Materials and methods

### 2.1. Chemicals

Acridine orange (AO), ethidium bromide (EB), trichloroacetic acid (TCA), hydrochloric acid (HCl), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), *n*-butanol (*n*-BuOH), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), glucose, TRIS, hydrogen peroxide ($H_2O_2$), cupric nitrate [$Cu(NO_3)_2$] and cuprous chloride (CuCl) were purchased from Merck (Darmstadt, Germany). The $TiO_2$ which was used in these and our earlier experiments (7) was supplied as a powder by Sigma–Aldrich (Steinheim, Germany), and was guaranteed 99.7% pure, with an anatase crystalline structure, average particle size <25 nm and surface area between 200 and 220 $m^2\ g^{-1}$.

### 2.2. Model organisms

Individual terrestrial isopods *P. scaber* (Isopoda, Crustacea) were collected during August 2008 at an uncontaminated location near Ljubljana, Slovenia. The animals were kept in a terrarium at temperature of 20 ± 2 °C and 16:8-h light:dark photoperiod. The terrarium was filled with a layer of moistened soil and a thick layer of partly decomposed leaves of the hazelnut tree (*Corylus avellana*). Only adult animals of both sexes and weighing more than 30 mg were used in the experiments. If molting or the presence of marsupia were observed, the affected animals were eliminated from the experiments to keep the investigated population as physiologically homogenous as possible.

### 2.3. Anatomy of the digestive system of the model organism

As shown in Fig. 1a–e, the digestive system of the terrestrial isopod *P. scaber* is composed of a stomach, four blind-ending digestive gland tubes and a gut. The digestive gland (hepatopancreas) with intestinal, hepatic, and pancreatic functions is the isopod's major digestive organ. Food enters the digestive gland directly or after reflux from the gut and ingested material is mixed with digestive fluids in the hepatopancreas and the gut. Hypothetically, ingested nanoparticles can reach the surface of gland cells immediately after consumption.

### 2.4. Characterization of nanoparticles

The primary size of the nanoparticles was assessed by transmission electron microscopy (TEM) and the Brunauer–Emmett–Teller (BET) surface analysis method.

For TEM investigation, dispersions of nanoparticles in bidistilled water were applied 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).

The BET method was used for the surface area analyses of solid material. For this analysis, samples were dried and degassed with nitrogen prior to analysis (Tristar 3000, Micrometrics Co., Norcross, GA, USA).

The $TiO_2$ samples were also examined by X-ray powder diffraction (XRD) using a Bruker AXS D4 Endeavor diffractometer (Karlsruhe, Germany) with Cu $K_{\alpha 1}$ radiation and a Sol-X energy dispersive detector within the angular range $20° < 2\Theta < 80°$ with a step size of 0.04° and a collection time of 3 s.

### 2.5. Food preparation

Hazelnut leaves were collected from an uncontaminated area, dried at room temperature then cut into segments of approximately 100 mg. $TiO_2$ nanoparticles were suspended in bidistilled water to achieve different nominal concentrations of 100, 1000 or 2000 μg $mL^{-1}$. Nanoparticle suspensions were prepared immediately before application. In a negative control group, the leaves were treated only with bidistilled water. In the test samples, a suspension of particles was brushed over the lower leaf surface to give final nominal concentrations on the leaves of 100, 1000 or 2000 μg $TiO_2$ per g (dry weight) of leaf. Before and after the exposure to animals, pieces of leaves dosed with each of the three suspensions were sampled and prepared for scanning electron microscopy (SEM) in order to examine the distribution of particles and their attachment to leaves after drying and after the exposure period. For SEM investigation, remnants of some selected leaves were dried and attached to mounts with silver paint, gold–palladium sputtered (Sputter coater SCD 050, BAL-TEC, Germany) and investigated with a field emission scanning electron microscope (Jeol JSM-6500F, at the Institute of Metals and Technology, Ljubljana, Slovenia). Energy dispersive X-ray analysis (EDX) was used to establish the chemical composition of the particles (EDS/WDS Oxford Instruments INCA, Jeol JSM-6500F, at the Institute of Metals and Technology, Ljubljana, Slovenia).

### 2.6. Experimental procedure

Each individual animal was placed in a 9 cm Petri dish with a single hazelnut leaf treated with either bidistilled water or with a suspension of nanoparticulate-$TiO_2$ as the only food source. All Petri dishes were placed in a large glass container and humidity in the Petri dishes was maintained by spraying tap water daily on the internal side of their lids. Leaves were weighed before and after feeding to calculate feeding rate and nano-$TiO_2$ exposure doses. Feeding rate was calculated as the mass of consumed leaves per animal wet weight. Nano-$TiO_2$ exposure doses were calculated from the mass of consumed leaf and corresponding applied concentration of $TiO_2$.

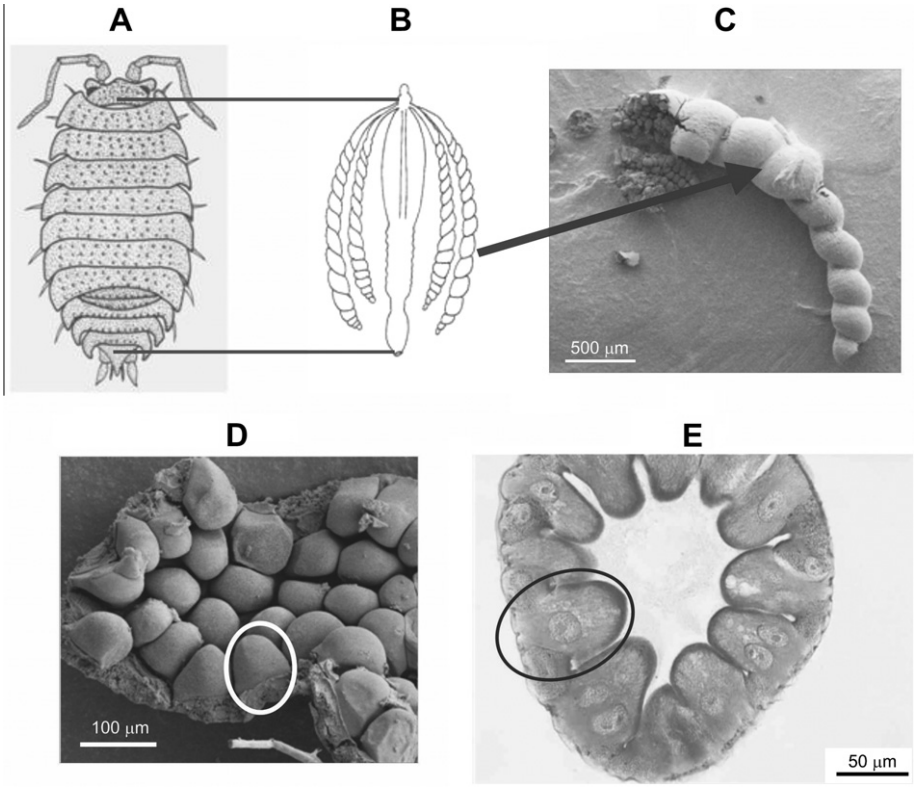


**Fig. 1.** Scheme of a terrestrial isopod: (A and B) digestive system anatomy, (C) scanning electron micrograph of isolated digestive gland tube, (D) interior of digestive gland tube in which one cell is circled, (E) light micrograph of a cross section of the digestive gland tube with one cell encircled.

The numbers of exposed animals per treatment are shown in Table 1. Digestive glands of separate animals were used for AO/EB assay and lipid peroxidation. After 3 or 7 d of exposure, the animals were dissected and all four digestive gland tubes were isolated. In the AO/EB analysis, a solution of $Cu(NO_3)_2$ was used as a positive control (Valant et al., 2009) and in the lipid peroxidation, a mixture of CuCl and $H_2O_2$. A total of 177 animals were used in the experiments.

### 2.7. Cell membrane stability assay

A modified method for assessment of cell membrane stability (Valant et al., 2009) was used. After animals were exposed to $TiO_2$ nanoparticles for a specific period of time, the digestive gland tubes were isolated and treated for 5 min with a solution containing AO/EB. A sample was then placed on a microscope slide and immediately photographed with an Axioimager.Z1 fluorescent microscope (Zeiss) using an excitation filter 450–490 nm and an emission filter 515 nm (filter set 09) to visualize AO and EB stained nuclei. An excitation filter 365 nm and emission filter 397 nm (filter set 01) were used to visualize nuclei stained with EB only. Cell membrane integrity was assessed by examination of micrographs. Photographs of digestive glands were examined by the same observer twice at intervals of at least 24 h. Cell membrane integrity was assessed visually and classified from 0 to 9 according to a predefined scale. On the basis of preliminary experiments, we concluded that non-treated (control) animals showed <5% of nuclei stained by EB, while severely stressed animals have up to 100% of EB-stained nuclei. Approximately 200 cells per animal, derived from two gland tubes, were inspected; the <5% of hepatopancreatic tubes stained with EB were classified as 0, and those with the highest portion (>95%) of EB stained nuclei as 9. Positive controls are described separately (see below).

### 2.8. Lipid peroxidation analysis

The degree of lipid peroxidation was determined from the amount of malondialdehyde (MDA) formed as a by-product of

**Table 1**
Total number of animals ($n$) exposed for 3 or 7 d to control leaves or leaves dosed with nanoparticles. Nano-$TiO_2$ 100 μg $g^{-1}$, nano-$TiO_2$ 1000 μg $g^{-1}$ and nano-$TiO_2$ 2000 μg $g^{-1}$ represent nominal exposure concentrations of 100, 1000 and 2000 of nano-$TiO_2$ μg $g^{-1}$ dry weight of the leaf. AO/EB denotes acridine orange/ethidium bromide staining. There was no mortality of animals during the exposure period.

| Methods, duration of exposure | Treatment | | | | |
|---|---|---|---|---|---|
| | Control | Nano-$TiO_2$ 100 μg $g^{-1}$ | Nano-$TiO_2$ 1000 μg $g^{-1}$ | Nano-$TiO_2$ 2000 μg $g^{-1}$ | Positive control |
| AO/EB assay, 3 d exposure | $n = 20$ | $n = 12$ | $n = 11$ | $n = 11$ | $n = 6$[a] |
| AO/EB assay, 7 d exposure | $n = 17$ | $n = 12$ | $n = 12$ | $n = 6$ | $n = 6$[a] |
| Lipid peroxidation, 3 d exposure | $n = 7$ | $n = 6$ | $n = 6$ | $n = 6$ | $n_1 = 6$[b] |
| Lipid peroxidation, 7 d exposure | $n = 7$ | $n = 6$ | $n = 6$ | $n = 8$ | $n_2 = 6$[b] |

$n_1$ – first group of positive controls, $n_2$ – second group of positive controls.
[a] Feeding experiment, $Cu(NO_3)_2$ dosed food.
[b] Animals taken directly from stock culture, incubated in a mixture of CuCl and $H_2O_2$.

peroxidation, quantified by its reaction with thiobarbituric acid (Ortega-Villasante et al., 2005). Four hepatopancreatic gland tubes were homogenized with homogenization beads in 1 mL of 15% TCA, 0.37% 2-thiobarbituric acid, 0.25 M HCl and 0.01% BHT and 50 μL of the homogenized sample was used to measure by UV analysis at 280 nm the total protein concentration, taken as a measure of the biomaterial in the experiment. Samples in which MDA content was to be assessed were incubated at 90 °C for 30 min, then chilled on ice and centrifuged at 10,000 rpm for 10 min at 4 °C. The absorbance of the resulting chromophore was measured at 535 nm and 600 nm. The two values obtained were subtracted from one other to correct for non-specific turbidity. The concentration of MDA was calculated assuming an extinction coefficient of 156 1 $mM^{-1}$ $cm^{-1}$. For statistical analysis, each MDA concentration was divided by the total protein concentration of the sample and the MDA concentrations are normalized to that of the control group (100%).

### 2.9. Positive controls

Measured biomarkers in *in vivo* tests were validated with relevant positive controls. Cell membrane destabilization was induced by $Cu(NO_3)_2$ in the food. Cupric ions ($Cu^{2+}$) stimulate production of excess levels of ROS which leads to oxidative stress and subsequently cell membrane destabilization (Regoli et al., 1998).

As positive controls in the case of cell membrane stability six animals were fed with $Cu^{2+}$ dosed food for 3 or 7 d. Exposure concentrations were 1000 μg of $Cu^{2+}$ per gram dry weight of leaf. After feeding exposure, cell membrane stability was assessed as described above for nanoparticle-fed animals.

Lipid peroxidation in positive controls was triggered by incubation of digestive glands in a mixture of CuCl and $H_2O_2$. In a Fenton-like reaction, $Cu^{1+}$ ions from the CuCl react with $H_2O_2$ to form hydroxyl radicals, which induce extensive lipid peroxidation and thus formation of MDA (Gunther et al., 1995; Ding et al., 2000). Positive control of peroxidation of cell membrane lipids was achieved with mixture of $Cu^{1+}$ ions (100 μM) and $H_2O_2$ (1%). Six animals were taken directly from the stock culture and dissected. Digestive gland tubes were incubated with $Cu^{1+}$ ions and $H_2O_2$ for 1 h under nitrogen. The level of MDA was assessed as described above.

### 2.10. Statistical analysis

Data were analyzed with standard statistical methods. All calculations were done using Statgraphics Plus 4.0 statistics software for Windows. The difference of the medians of measured parameters in exposed and unexposed groups was tested with the non-parametric Mann–Whitney *U* test. Statistical differences between exposed and control animals were categorized into three groups according to *p* value and denoted as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

## 3. Results

### 3.1. Characteristics of nanoparticles

The surface area of the $TiO_2$ nanoparticles, assessed by the BET technique, was 144 $m^2$ $g^{-1}$ and the primary particle size of the $TiO_2$ nanoparticles as observed by TEM was approximately 10 nm. X-ray powder diffraction (XRD) revealed that the crystal form of the $TiO_2$ sample was pure anatase. The predominant shape of individual nanoparticles was between elongated and spherical.

### 3.2. Nano-$TiO_2$ dosed food

SEM reveals that particles remained evenly spread over the entire surface of the hazelnut leaf (*C. avellana*). Similar distribution patterns were observed immediately after application of a suspension of particles on the leaf surfaces and after 7 d of exposure of animals to the leaves. EDX gave the Ti content in particles shown in Fig. 2A–C.

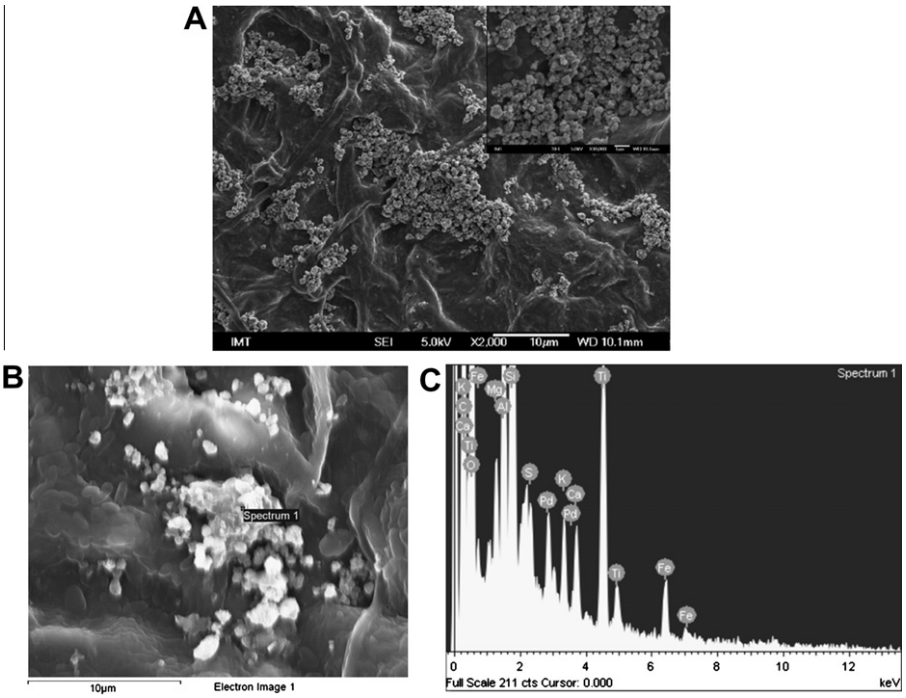


**Fig. 2.** (A) SEM micrograph of nano-$TiO_2$ dispersed over the leaf surface to give a final concentration of 1000 μg $g^{-1}$ dry weight of leaf, (B) location where EDX analyses were performed, (C) results of the EDX analysis.

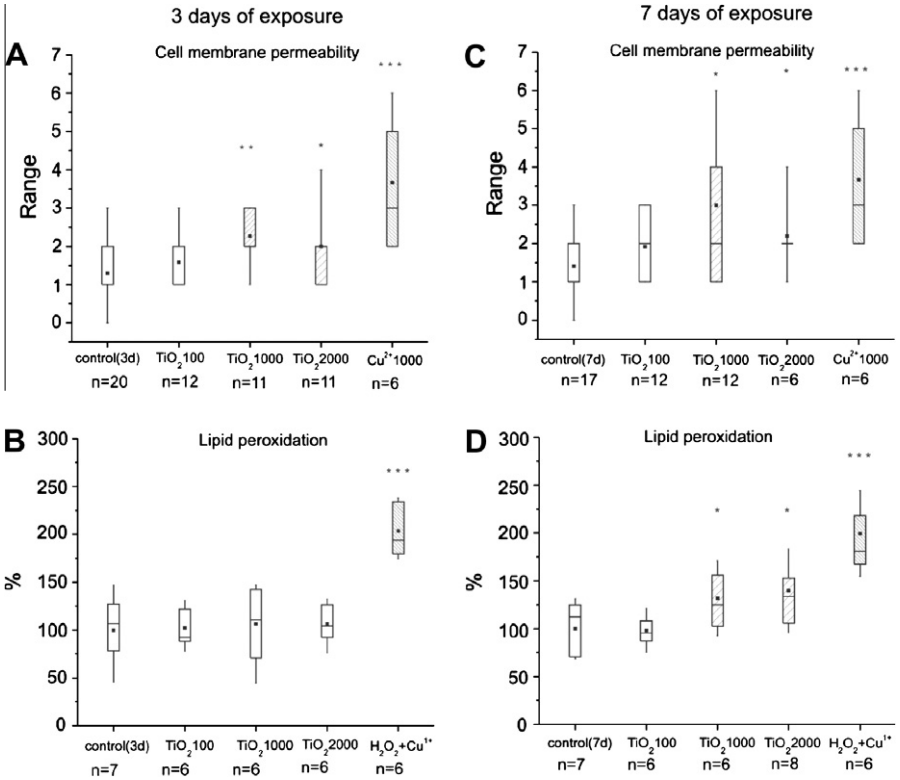


**Fig. 3.** (A and C) Cell membrane permeability and (B and D) lipid peroxidation of digestive glands after feeding for three or 7 d with nanosized $TiO_2$ or $Cu^{2+}$. To assess lipid peroxidation, digestive glands were incubated in $H_2O_2$ and $Cu^{1+}$. The left side of the figure shows (upper) the results from 3 d of exposure and the right side from 7 d of exposure to $TiO_2$ nanoparticles or $Cu^{2+}$ and (lower) $H_2O_2$ and $Cu^{1+}$. The concentration of MDA was taken as a measure of lipid peroxidation. The amount of MDA in each exposed group is presented as a percentage and is normalized against the control group (100%). Points on the *x* axis represent exposure concentrations (100, 1000 and 2000 μg of nano-$TiO_2$ per gram [dry weight] of leaf). *n* – number of animals analyzed (either two pairs or four pairs of glands). The boxes contain 75% of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (■).

### *3.3. Hepatopancreatic cell membrane stability after exposure to nanoparticles or $Cu^{2+}$ as a positive control*

The modified AO/EB assay revealed that after feeding for 3 d on food containing 1000 or 2000 μg nano-$TiO_2$/dry weight of leaf, the digestive gland cell membrane integrity was compromised as can be seen in Fig. 3A. Consumption of food with additional nano-$TiO_2$ (2000 μg $g^{-1}$ nano $TiO_2$) or exposure for a longer period (7 d) did not intensify the effects, and it was concluded that destabilization of the cell membrane has a threshold-like response (Fig. 3C). $Cu(NO_3)_2$-dosed food served as a positive control and, as expected, led to significant destabilization of the cell membrane (Fig. 3A).

### *3.4. Lipid peroxidation of hepatopancreatic cells after exposure to nanoparticles or $H_2O_2$ and $Cu^{1+}$ as a positive control*

Relative to controls and judged by MDA content, no statistically significant changes in lipid peroxidation were detected in digestive gland tissue of nano-$TiO_2$ fed animals after 3 d of exposure (Fig. 3B). After 7 d of exposure (Fig. 3D) to nano-$TiO_2$ at 100 μg $g^{-1}$, the lowest exposure concentration, the extent of lipid peroxidation did not differ from that of controls. Statistically significant differences in levels of lipid peroxidation were seen at higher exposure concentrations of nano-$TiO_2$ (1000 and 2000 μg of nano-$TiO_2$ $g^{-1}$ dry weight of leaf). Incubation of digestive gland tubes in a mixture of $H_2O_2$ and $Cu^{1+}$ ions for 1 h significantly elevated MDA content and served as a positive control.

### *3.5. Toxicity parameters: mortality, weight change, feeding behavior*

We observed no effects of nano-$TiO_2$ dosed food on feeding behavior (Fig. 4A and B), weight change or mortality. Consequently, the selected exposure concentrations of nano-$TiO_2$ could be considered as non-toxic for *P. scaber* at exposures of 7 d.

### *3.6. Exposure doses*

Nano-$TiO_2$ exposure doses were calculated from the mass of consumed leaves and corresponding applied concentration of $TiO_2$. Animals, fed with 100, 1000 and 2000 μg of nano-$TiO_2$ $g^{-1}$ dry weight of leaf for 3 d ingested approximately 29 ± 16, 162 ± 41 and 251 ± 108 μg of $TiO_2$ respectively (Fig. 5A). Animals fed with 100, 1000 and 2000 μg nano-$TiO_2$ $g^{-1}$ dry weight of leaf for 7 d ingested approximately 40 ± 17, 320 ± 144 and 647 ± 177 μg of $TiO_2$ respectively (Fig. 5B).

Consequently, the selected exposure doses of nano-$TiO_2$ are considered to be non-toxic for *P. scaber* at exposures of up to 7 d.

## 4. Discussion

The data from the *in vivo* feeding experiments with the terrestrial isopod *P. scaber* show that ingested nano-$TiO_2$ affects the cell membrane of digestive gland cells (hepatopancreas) before oxidative damage can be detected. Prolonged feeding on nano-$TiO_2$ dosed food however, leads to oxidative stress. No toxicity as assessed by feeding rate, weight change or mortality was observed within 7 d of exposure. The failure of cell membrane destabilization and oxidative stress to coincide indicates that nano-$TiO_2$ interacts more directly with cell membranes and not exclusively *via* oxidative stress.

Our results are in agreement with previously published data which provide evidence that oxidative stress is involved in the effect of nano-$TiO_2$ on cells. Our experimental evidence supports

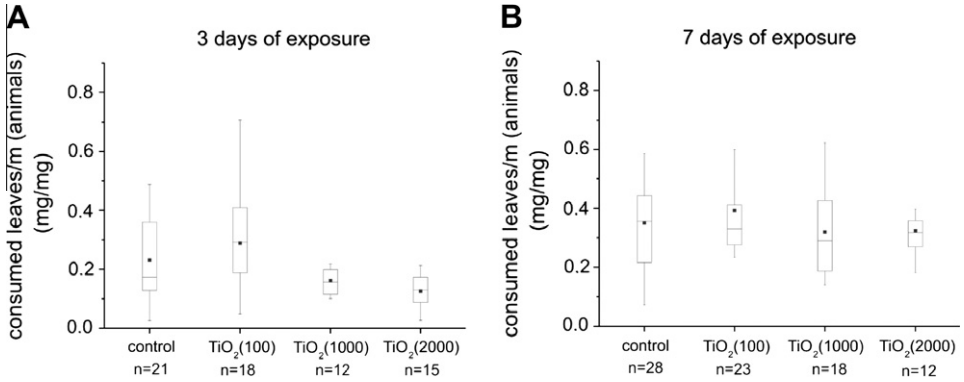


**Fig. 4.** (A) Feeding rates after 3 d of exposure and (B) feeding rates after 7 d of exposure to $TiO_2$ nanoparticles. Points on the *x* axis represent exposure concentrations (0-control, 100, 1000 and 2000 μg of nano-$TiO_2$ per gram dry weight of leaf). The boxes contain 75% of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (■).

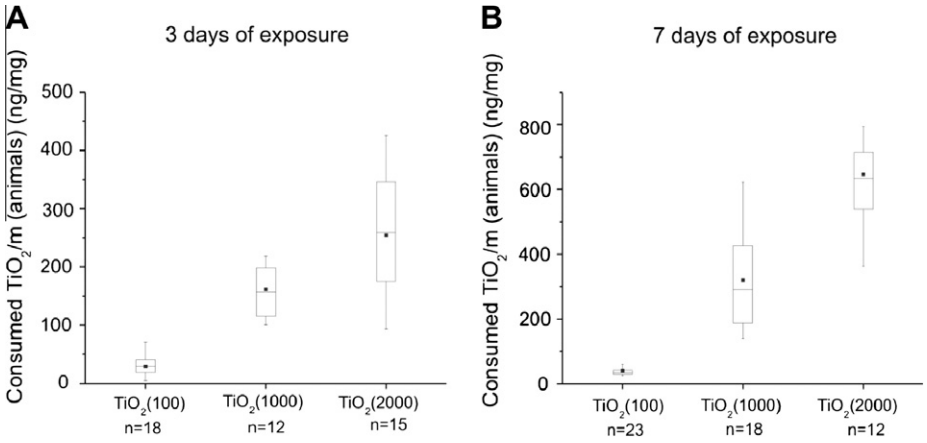


**Fig. 5.** (A) Exposure doses after 3 d of exposure and (B) exposure doses after 7 d of exposure to $TiO_2$ nanoparticles. Points on the *x* axis represent exposure concentrations (0-control, 100, 1000 and 2000 μg $TiO_2$ per gram dry weight of leaf). The boxes contain 75% of all readings, the symbols represent minimum and maximum values (⊥) and the mean value (■).

the suggestion that oxidative stress is not the primary mechanism by which nano-$TiO_2$ affects cells.

A comprehensive review of *in vitro* mammalian studies of effects of nano$TiO_2$ by Iavicoli et al. (2011) revealed that most papers report induction of reactive oxygen species (ROS) followed by different types of cellular effects. Menard et al. (2011), at also reviewed *in vivo* ecotoxicity papers, but those studies failed to reveal a possible mode of nanoparticle action.

Some authors also indicate that oxidative stress due is not the only way by which nanoparticles affect cells. Banaszak (2009) and Pal et al. (2007), report direct interactions between plasmalemma lipid molecules and nanoparticles, but others (Gurr et al., 2005; Wang et al., 2009) explain cell membrane destabilization in terms of lipid peroxidation, which itself is a consequence of oxidative stress.

The ability of $TiO_2$ nanoparticles to destabilize cell membranes after *in vitro* exposure of cell lines has also been noted by some authors (Simon-Deckers et al., 2008; Thevenot et al., 2008; Lee et al., 2009). Nanosized $TiO_2$ was reported to disrupt cell membrane stability as a result of its photocatalytic properties (Amezaga-Madrid et al., 2003). Sayes and Warheit (2008) interpreted affected cell membrane stability after exposure to nanosized $TiO_2$ as result of decreased mitochondrial membrane potential, causing release of lactate dehydrogenase – an indication of 'leaky membranes'. Impairment of mitochondrial activity by $TiO_2$ has been reported also by Barillet et al. (2010) as a possible common mode of action to metal oxide nanoparticles and it was suggested that since a direct parallel could not be drawn between ROS level and occurrence of cytotoxicity, oxidative stress was perhaps not the only mechanism underlying cytotoxicity.

A shortcoming of many *in vitro* test systems used in nanoparticle studies is that cells are exposed to nanoparticles under unnatural conditions. The invertebrate *in vivo* test system with isopods however proves to be well-suited to a study of the cytotoxicity of ingested nanoparticles to cells of the digestive system under realistic exposure conditions in variable micro-environments within digestive media. In biological media nanoparticles absorb biological molecules on their surface and modulate their biokinetics, and the subsequent fate of the biomolecules (Deng et al., 2009; Lynch et al., 2009). It is difficult to replicate such dynamic and versatile conditions *in vitro*. A test system with isopods allows detection of links between cytotoxic effects and higher levels of biological complexity. With prolonged exposure or higher exposure concentrations, effects at biochemical levels are accompanied by responses at organism level, toxic responses (Stanek et al., 2006; Drobne et al., 2008). In addition, realistic exposure doses can be calculated on the basis of food consumed per day or for the entire exposure duration. The use of terrestrial isopods as experimental animals is not subject to legal restrictions, and sufficient experimental data can be generated to draw reliable conclusions on the effects of ingested nanoparticles.

## 5. Conclusions

(1) Exposure concentrations of up to 2000 μg of nano-$TiO_2$ $g^{-1}$ dry weight of leaf for 7 d failed to affect the performance of the organism as measured by feeding behavior, weight change and mortality which are toxicological measures, and therefore that concentration is considered non-toxic.
(2) Exposure concentrations of 1000 and 2000 μg of nano-$TiO_2$ $g^{-1}$ dry weight of leaf caused lipid peroxidation only after 7 d of exposure.
(3) Exposure concentrations of 1000 and 2000 μg of nano-$TiO_2$ $g^{-1}$ dry weight of leaf affected cell membranes after 3 d. Cell membrane stability was more sensitive to ingested nano-$TiO_2$ than was lipid peroxidation and the cell membrane was affected by nano-$TiO_2$ prior to the onset of oxidative stress.
(4) Exposure to nano-$TiO_2$ could be considered safe if the digestive cell membrane remains intact. In our study, the safe concentration was 100 μg of nano-$TiO_2$ $g^{-1}$ dry weight of leaf for up to 7 d of feeding. This corresponds to 29 ± 16 μg in 3 d or 40 ± 17 μg in 7 d.

## Acknowledgements

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