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Research Article

Internalization of Consumed TiO_2 Nanoparticles by a Model Invertebrate Organism

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There is little *in vivo* data concerning the fate of ingested TiO_2 nanoparticles (nano-TiO_2). We report here experiments aimed at assessing if ingested nano-TiO_2 accumulates in the digestive gland epithelium or are internalized elsewhere in the body of the terrestrial isopod crustaceans. The animals (Porcellio scaber, Isopoda, Crustacea) fed for 3, 7, or 14 days on food dosed with 100 or 1000 μg nano-TiO_2 showed no evidence of internalization of Ti measured by microparticle-induced X-ray emission method. The effect of ingested nanoparticles was measured by conventional toxicity measures such as feeding rate, weight change, and mortality and did not indicate any toxicity. However, cell membrane of digestive glands, measured with a modified method for assessing cell membrane stability, was affected already after 3 days of exposure to 1000 μg nano-TiO_2 per gram dry weight of food indicating cytotoxic potential of ingested nanoparticles. Our results confirmed hypothesis on low toxic potential and no internalization of consumed TiO_2 nanoparticles by a model invertebrate organism. However, cytological marker unequivocally indicated adverse effect of ingested nano-TiO_2. We conclude that the isopod model system could be used for studying the fate and effect of ingested nanoparticles.

1. Introduction

Titanium dioxide nanoparticles (nano-TiO_2) are among the most commonly manufactured nanomaterials, widely used in a variety of products in cosmetics and pharmaceuticals and in paints, printing ink, rubber, paper, car materials, air cleaning products, biomedicals, and ceramics.

Such extensive production and multiple applications result in both deliberate and unintentional intrusion of nano-TiO_2 into the human or animal bodies by oral or intravenous routes, inhalation, or dermal penetration [1–4].

Oral uptake of nano-TiO_2 demands special attention, because the involvement of nanoparticles in the food industry is increasing rapidly. Nano-TiO_2 is used extensively for photocatalytic disinfectant TiO_2 powder-coated packaging film [5] and as a coloring agent and additive in food [6], and TiO_2 particles are used as ingredients of toothpaste and can enter the body *via* the alimentary tract.

Different authors have reported the effects of ingested nano-TiO_2 on experimental vertebrate and invertebrate organisms. Abe et al. [7] studied internal diffusion and absorption of TiO_2 nanoparticles in the digestive system of mice and reported that nano-TiO_2 fed to mice was detected in the lung, liver, and spleen after 10 days of exposure. They discovered, however, that compared to intravenous injection, the absorption of orally ingested TiO_2 was extremely low. Biodistribution of TiO_2 nanoparticles administered to mice as a single oral gavage has been studied by Wang et al. [8] who reported that TiO_2 nanoparticles migrate into other tissues and organs and induce significant lesions, particularly of the liver and kidneys. They also found that TiO_2 nanoparticles have a wide tissue distribution being found even in the brain.

The effects and accumulation of ingested TiO_2 nanoparticles on juvenile rainbow trout were investigated by Ramsden et al. [9] who observed no major disturbances in red or white blood cell counts, haematocrits, whole blood

haemoglobin, or plasma Na^+. Accumulation of titanium was, however, observed in the gill, gut, liver, brain, and spleen after exposure to TiO_2 in the diet. An investigation of the bioaccumulation of TiO_2 nanoparticles in *Daphnia magna* was reported by Zhu et al. [10] who detected particles only in the gut lumen. No evidence was presented of TiO_2 particle internalization. The same authors also studied tropic transfer of TiO_2 nanoparticles in a simple food chain from *D. magna* to a zebrafish (*Danio rerio*). Zhu et al. [11] reported the first direct evidence that nanoscale TiO_2 particles can be transferred from the plant to *D. magna* and to a fish *D. rerio* by dietary exposure. No biomagnification of nano-TiO_2 was observed, however, in this simplified food chain. Galloway et al. [12] observed TiO_2 particles of diameter <200 nm within the gut lumen in the lugworm *Arenicola marina*, but no uptake of particles across the villi or outer epithelium was evidenced.

All these reports indicated the potential of nano-TiO_2 to pass the digestive system epithelium and to proceed elsewhere in the body, but it is difficult to study ingestion and body distribution of nano-TiO_2 in the absence of simple and reproducible *in vivo* animal models. Currently, work with laboratory experimental vertebrates is limited by law to an absolute minimum (http://www.alternativevet.org/animal_welfare_act.htm). As a consequence, invertebrate *in vivo* animal models or *in vitro* model systems, in which animals are replaced by cell cultures have been used. These techniques are not subject to legal restrictions and they thus could be used to study the potential of ingested nanoparticles to pass the digestive system epithelial barrier penetrating other tissues in the body.

In the work presented here, we used a terrestrial isopod, *Porcellio scaber* (Isopoda, Crustacea) as a model organism to study the fate of ingested nanoparticles. Such organisms have been successfully used in earlier nanotoxicity studies [13, 14].

The aim of the present study was to investigate if a model invertebrate organism, terrestrial isopod *P. scaber* (Isopoda, Crustacea), fed on nano-TiO_2-dosed food shows toxic effects and/or accumulation of Ti either in cells of the digestive system or elsewhere in the body. Animals were fed for 3, 7, or 14 days on food dosed with nano-TiO_2 (100 or 1000 μg per g food). Elemental analyses of entire body cross sections was performed by particle-induced X-ray emission (micro-PIXE) to generate data on Ti distribution in the body. The effects were measured in terms of conventional toxicological responses such as feeding parameters, weight change, and mortality, and cell membrane stability was used as a cytological parameter. We hypothesize

(a) that effect of ingested particles is related to duration of exposure and to the consumed dose,

(b) if nano-TiO_2 is able to pass the digestive gland epithelium, it will be found either in the epithelial cells or elsewhere in the body.

2. Materials and Methods

2.1. Chemicals. Acridine orange (AO), ethidium bromide (EB), and titanium dioxide (TiO_2) were purchased from Sigma-Aldrich. The TiO_2 was the same as was used in our earlier experiments [15] and was supplied as a powder, guaranteed 99.7% pure, with an anatase crystal structure, average particle size <25 nm, and surface area between 200 and 220 m^2/g.

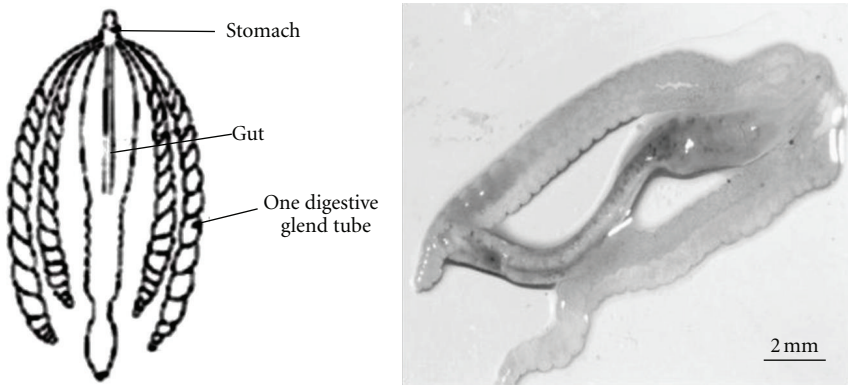
2.2. Model Organisms. Specimens of the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea) were collected in May, 2010, at an uncontaminated location near Ljubljana, Slovenia. The animals were kept in a terrarium filled with a layer of moistened soil and a thick layer of partly decomposed hazelnut tree leaves (*Corylus avellana*), at a temperature of 20 ± 2°C and a 16:8 h light : dark photoperiod. Only adult animals of both sexes and weighing more than 30 mg were used in the experiments. If moulting or the presence of marsupia was observed, the animals were eliminated from the experiment in order to keep the investigated population as physiologically homogenous as possible.

2.3. Anatomy of the Digestive System of the Model Organism. The digestive system of the terrestrial isopod *P. scaber* is composed of a stomach, four blind-ending digestive gland tubes (hepatopancreas), and a gut (Figures 1(a) and 1(b)). Food enters the digestive glands directly *via* a short stomach or after the reflux from the gut, and ingested material is mixed with digestive fluids.

2.4. Characterization of Nanoparticles. Nanoparticles were inspected with transmission electron microscopy (TEM) and by Brunauer-Emmett-Teller analysis of dynamic light scattering (DLS) and X-ray powder techniques. These analyses provide data on the suspension of particles allowing comparison between different studies and among our experiments. TEM micrographs were published in our previous work by Valant et al. [15].

Before the exposure as well as after exposure of isopods to the TiO_2, some selected leaves were dried, attached to mounts with silver paint (SPI), gold-palladium sputtered (Sputter Coater SCD 050, BAL-TEC, Germany), and investigated with a field emission scanning electron microscope (SEM, Jeol JSM-6500F) at the Institute of Metals and Technology, Ljubljana, Slovenia. Scanning electron microscopy reveals that particles remained on the leaf surface and were spread over the entire leaf surface as shown in Figure 2. Energy dispersive X-ray analysis (EDX) was used to confirm their chemical composition and is shown in Figure 3 (EDS/WDS Oxford Instruments INCA, Jeol JSM-6500F) at the Institute of Metals and Technology, Ljubljana, Slovenia.

For DLS analysis, the dispersions of nanoparticles (100 μg nano-TiO_2/mL distilled water) were inspected using a 3D DLS-SLS spectrometer (LS Instruments). This allows the assessment of the hydrodynamic radii of particles in extremely turbid suspensions by a 3D cross-correlation technique that eliminates light scattering. The light source used was an HeNe laser operating at a wavelength of 632.8 nm and scattering was measured at an angle of 90°. At higher concentrations (1000, 2000, 5000 μg/mL) of nanoparticles, measurements were not possible, due to the low transparency of sample [15].



(a) (b)

Figure 1: Schematic diagram of the digestive system (a) and isolated digestive glands of *Porcellio scaber* (b).

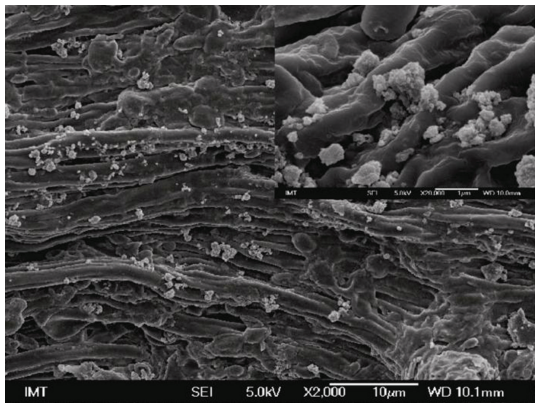


Figure 2: SEM micrograph of TiO_2 particles applied on the leaf surface. The nominal concentration of TiO_2 was 1000 μg nano-TiO_2/g leaf. The insert shows a magnified image of particles attached to the leaf surface.

Brunauer-Emmett-Teller (BET) analysis was performed on the TiO_2 samples, which were dried and degassed with nitrogen prior to analysis (Tristar 3000, Micrometrics Co.) to obtain data on the surface area of the solid material [15].

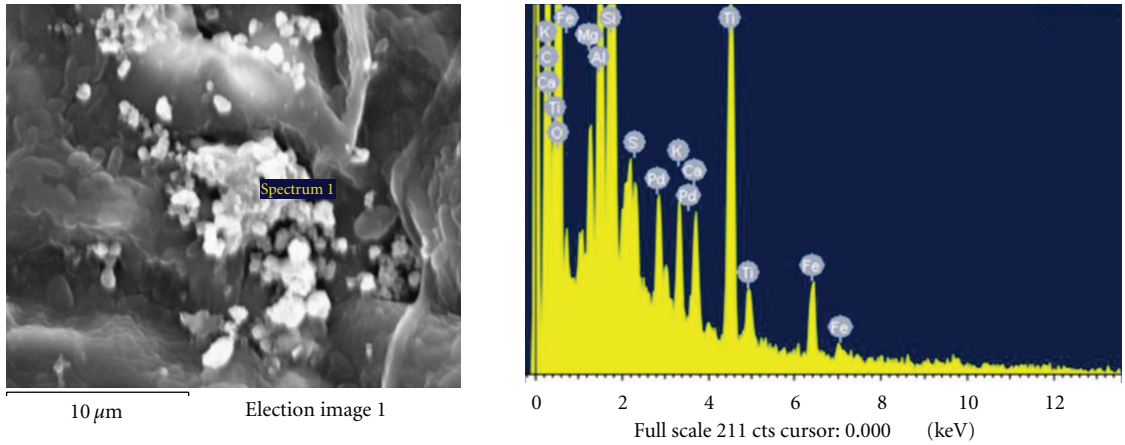
The TiO_2 samples were monitored by X-ray powder diffraction using a Bruker AXS D4 Endeavor diffractometer with Cu-Kα1 radiation and a Sol-X energy dispersive detector within the angular range $20^\circ < 2\Theta < 80^\circ$ with a step size of 0.04° and a collection time of 3 s.

2.5. Food Preparation. In this study, the animals consumed particles that were applied on the leaf surface. Hazelnut leaves were collected in an uncontaminated area and dried at room temperature. Dried leaves were cut into pieces of approximately 100 mg. The TiO_2 nanoparticles were suspended in distilled water to obtain different final concentrations (100 and 1000 μg/mL). The suspensions of nanoparticles were freshly prepared before experiments. In a control group, the leaves were treated with pure distilled water. A suspension of particles was brushed onto the abaxial leaf surface, and the leaf was allowed to dry, giving final nominal concentrations of nanoparticles on the leaves of 100 and 1000 μg nano-TiO_2 per gram (dry wt) of leaf. As mentioned above, the treated leaves were investigated by SEM in order to check the quality of particle application.

2.6. Experimental Details. Each individual animal was placed in a 9 cm Petri dish. A single hazelnut leaf treated with either distilled water or nano-TiO_2 suspension and placed in each Petri dish was the animal's only food source. Humidity in the Petri dish was maintained by spraying tap water on the internal side of the lid every day. All Petri dishes were kept in a large glass container under controlled conditions in terms of air humidity ($\geq$80%), temperature ($21 \pm 2^\circ$C), and light regime (16:8 h light: dark photoperiod). In experiment A, 30 animals per group were exposed for 3 days; in experiment B, 30 animals per group were exposed for 7; in experiment C, 15 animals per group for 14 days. After the exposure, the animals were weighted, anaesthetized by cooling, and decapitated. The digestive glands were isolated and used for AO/EB analyses as described below.

2.7. Feeding Parameters, Weight Change, and Mortality. After 3, 7, or 14 days of exposure of animals to treated leaves, fecal pellets and leaves were removed from the Petri dishes, dried at room temperature for 24 h, and the remaining leaves and the animals were weighed. The feeding rate of isopods was calculated as the mass of consumed leaf per animal's wet weight per day. The animal's weight change in each case was defined as the difference in animal mass at the beginning and at the end of the experiment.

2.8. Digestive Gland Cell Membrane Stability. Cell membrane stability was tested with a modified method for assessing cell membrane stability described by Valant et al. [15]. After isolation, one hepatopancreatic tube was incubated for 5 min in a mixture of acridine orange and ethidium bromide and then put on a microscope slide. Fresh samples were photographed and examined by an Axioimager. Z1 fluorescent



Spectrum	In stats	C	O	Mg	Al	Si	S	K	Ca	Ti	Fe	Pd	Au	Total
Spectrum 1	Yes	36.22	38.81	0.66	3.8	7.57	0.34	0.98	1.04	5.96	2.55	2.07		100

Figure 3: Nano-TiO_2 dispersed over the lower leaf surface (on left) to give the final concentration 1000 μg/g dry wt. of leaf. A location is indicated (spectrum 1) where the spectrum for EDX (on right) was taken to confirm presence of Ti. Proportion of different elements in this spot is provided in the table.

microscope (Zeiss) with two different sets of filters. The excitation filter 450 to 490 nm and the emission filter 515 nm (filter set 09) were used to visualize AO and EB stained nuclei, while the excitation filter 365 nm and the 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 intact 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 (digestive gland cell membrane stability value). On the basis of preliminary experiments, it was concluded that nontreated (control) animals showed <5% of nuclei stained by EB, while severely stressed animals have up to 100% of stained nuclei. 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 [15].

2.9. Micro-PIXE Analysis. To perform microparticle-induced X-ray emission (micro-PIXE) analysis, whole animals were sacrificed and shock-frozen in liquid N_2, using tissue-freezing medium (Jung Tissue Freezing Medium, Leica). Samples were then sectioned with a section thickness of 60 μm using a Leica CM3050 cryotome (Leica) with the temperature of the microtome head and chamber maintained between -25°C and -20°C and. The sections were placed in precooled Al holders, transferred to an alpha 2–4 Christ freeze dryer using a cryo-transfer assembly cooled with liquid nitrogen, and then freeze-dried for 24 h at -30°C and a pressure of 0.4 mbar. Dry sections were mounted between two thin layers of Pioloform foil on the Al sample holder [16, 17].

Two X-ray detectors were used for detection of X-rays between1 keV up to 25 keV. A high-purity germanium X-ray detector (active area 95 mm^2; beryllium window, 25 μm thick; polyimide absorber, 100 μm thick) positioned at 135° with respect to the beam direction was used for the energy range of 4 keV–25 keV. Low energy X-rays in the range of 0.8 keV–4 keV were detected by an Si(Li) detector (active area 10 mm^2) positioned at 125° with respect to the beam direction. The proton dose was assessed with a rotating in-beam chopper. Measurement of micro-PIXE and data evaluation for the biological samples of intermediate thickness at the micro-PIXE laboratory at the Jožef Stefan Institute in Ljubljana has been described previously [16, 18, 19].

In experiments in which animals were exposed for 14 days to TiO_2-containing food, we analyzed sections of two whole animals; one from the control group and one from nanoparticle-exposed group, which were fed on leaves dosed with 1000 μg nano-TiO_2/g of leaf.

2.10. Data Analysis. Data were analyzed using standard statistical methods. The difference in the medians of measured parameter in exposed and unexposed groups was tested with the nonparametric Mann-Whitney U test. All calculations were done using Statgraphics Plus 4.0 statistics software. Statistical differences between exposed and control animals were categorized into three groups with different numbers of stars assigned ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). Digestive gland cell membrane stability was assessed as in our previous work [15] and shown as percentage of animals per group with different degrees of destabilized cell membrane. Tissue distribution of Ti was illustrated by elemental distribution maps.

3. Results

3.1. Characteristics of Nano-TiO_2. Scanning electron microscopy (SEM) revealed the shape and size of tested TiO_2 particles (Figure 2) and EDX confirmed their composition (Figure 3). The DLS revealed the hydrodynamic radii of particles to be 110 nm. The BET method was used to assess the surface area of TiO_2 samples as 144 m^2/g. The size and surface area correspond to the data provided by the supplier, and X-ray powder diffraction confirmed that the TiO_2 was in the anatase crystal form [15].

3.2. Feeding Parameters, Weight Change, and Mortality. Animals were exposed to leaves dosed with nano-TiO_2 providing nominal concentrations of 100 and 1000 μg nano-TiO_2/g of leaf. The animals consumed dried nano-TiO_2 particles spread uniformly over the leaf surface. The number of exposed animals at the beginning of the exposure and that at the end of the exposure failed to correspond because some animals molted during the course of experiment and consequently were excluded from further analysis.

Based on the amount of food consumed it was estimated that when animals were fed on 100 μg nano-TiO_2/g of leaf they consumed approximately 0.01 ± 0.01 μg TiO_2 per day in 3 days, 0.005 ± 0.002 μg TiO_2 per day in 7 days, and 0.005 ± 0.001 μg TiO_2 per day in 14 days. When fed on 1000 μg nano-TiO_2/g of leaf, they consumed approximately 0.07 ± 0.03 μg TiO_2 per day in 3 days, 0.06 ± 0.03 μg TiO_2 per day in 7 days, and 0.04 ± 0.01 μg TiO_2 per day in 14 days.

No significant effect of ingested nano-TiO_2 on survival, weight change, or feeding parameters was observed in animals fed with TiO_2 nanoparticles when compared to control animals fed with untreated food. There was some statistically significant decrease in feeding intensity in animals exposed for 7 or 14 days on food dosed with 100 μg/g nano-TiO_2 when compared with animals fed on similar food (100 μg/g nano-TiO_2) for 3 days only (Figure 4). The feeding rate in a group of animals fed for 3 days on food containing 100 μg/g nano-TiO_2 was variable.

3.3. Digestive Gland Cell Membrane Stability. Our previously published data demonstrate that in animals from a stock culture, which are in a good physiological condition, the digestive gland cell membrane stability value was rarely higher (less than in 5% of animals) than 2 and that was considered to be a benchmark [15]. The higher is the value the more the membrane is destabilized.

Our data show that among the control animals, fed with uncontaminated food, the digestive cell membranes were not affected in more than 6% of animals fed for 3 and 7 days, and in 11% of animals fed for 14 days. In animals exposed to food with the lower amounts of nano-TiO_2 (100 μg/g), cell membranes were affected in 17% of animals fed for 3 days, 33% of animals fed for 7 days and 10% of animals fed for 14 days. However, an exposure concentration of 1000 μg nano-TiO_2/g in the food caused digestive gland cell membrane destabilization in up to 50% of exposed animals fed for 14 days and 42% of animals fed for 3 and 7 days (Figure 5). The cell membrane destabilization pattern was dose dependent. The highest proportion of animals with destabilized membranes was found in a group fed on food dosed with 1000 μg TiO_2/g (50% of animals) in all three exposure durations (3, 7, or 14 days).

3.4. Tissue Distribution and Ti Concentration. In whole body sections that were analyzed (control, and those fed on food dosed with 1000 μg/g nano-TiO_2 dry weight of food), no titanium was detected in the digestive glands or in any other part of the body. As expected, Ti was found only in gut lumen and on surface of animal (Figure 6).

In parallel to analysis for Ti, we also analyzed distribution of other elements. The data concerning copper, for example, shows the location of one cell type in the digestive glands (S-cells) and thus the location of digestive glands on a cross section.

4. Discussion

Our work provides evidence on low toxic potential and no internalization of consumed TiO_2 nanoparticles by a model invertebrate organism at exposure concentrations up to 1000 μg/g nano-TiO_2 and exposure duration up to 14 days. The experimental setup with selected invertebrate organism allowed calculating the exposure dose, which is expressed as consumed amount of nanoparticles either per day or per body mass. Our results showed when animals were fed with TiO_2 particles for 14 days, they consumed up to 0.04 ± 0.01 μg TiO_2 per day. This consumed amount of TiO_2 corresponds to 0.0001% of the body wt, which is between 30 and 50 mg. Ti was not found in the cells of digestive system or any other part of the body. In addition, no toxic effects were noted by conventional toxicity measures, but the cell membranes of digestive glands were destabilized already after 3 days in almost half of exposed animals (40%) fed on 1000 μg/g nano-TiO_2.

These results show that ingested TiO_2 particles, even if consumed as large dry aggregates—the least bioreactive form—destabilize the membranes of digestive gland cells. However, if the cell membrane was destabilized, the effect was not propagated upward to higher levels of biological complexity and no toxic response was apparent after 14 days exposure.

As expected, the toxicological and cytological parameters measured in animals exposed for 3, 7, or 14 days on food dosed with nano-TiO_2 (100 or 1000 μg/g nano-TiO_2) failed to coincide. Cytotoxicity measures are more sensitive than conventional toxicological parameters such as feeding behavior, weight change, or mortality in short exposures. When considering the safety of particles, toxicity measures have to be completed with biomarkers at lower levels of biological complexity.

Cell membrane destabilization is correlated with exposure dose. Since the response appeared to be dose and perhaps also time dependent, it is expected that prolonged exposure or higher doses will lead to effects at the organism level, namely, toxicity.

Micro-PIXE analyses of elemental distribution on a body cross section showed that Ti was not present in the cells

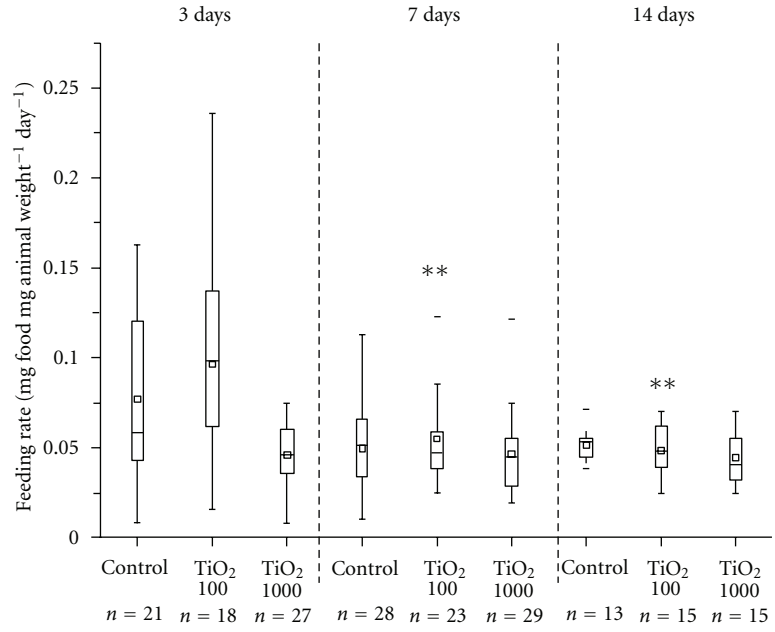


Figure 4: Feeding rate (mg of consumed leaves/animal weight, calculated daily) of animals fed with control, untreated leaves, and leaves dosed with 100 or 1000 μg/g nano-TiO_2 for 3, 7, or 14 days. There are no statistically significant differences within exposure groups treated for different time periods. There are statistical differences between animals exposed to food dosed with 100 μg/g nano-TiO_2 for 3 and 7 days and again between 3 and 14 days ($^{**}P < 0.01$). Symbols on the box plot represent minimum and maximum data values (whiskers), mean value (□), 75th percentile (upper edge of box), 25th percentile (lower edge of box), median (line in box), and max and min value (-).

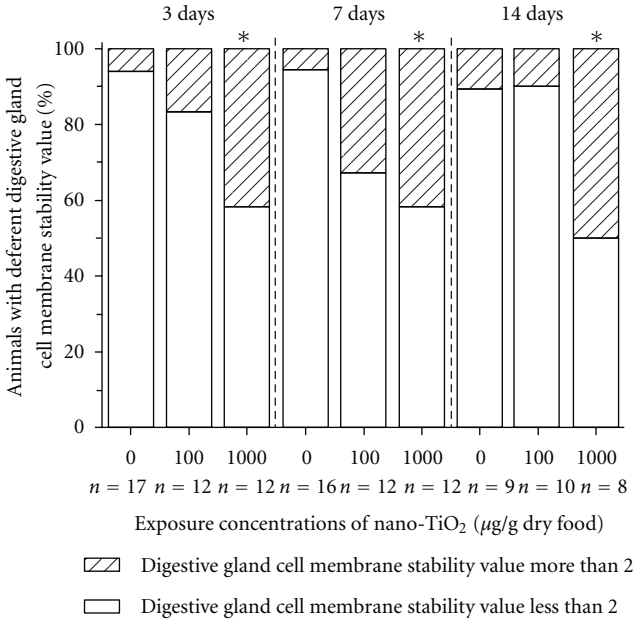


Figure 5: Percentage of animals in each group with different degrees of destabilization of cell membranes. This was assessed visually and classified from 0 to 9 according to the scale defined above in Materials and Methods. Digestive gland cell membrane stability value ≤2 represent animals that had no destabilized cell membrane and digestive gland cell membrane stability values >2 for animals with destabilized cell membrane. Statistical differences between exposed and control animals (within one exposure duration) are marked with an asterisk ($^{*}P < 0.05$).

of digestive glands or anywhere else in the body. Ti was clearly seen in the lumen of the gut and on the cell surface, and the presence of Ti inside the gut tube shows that Ti was ingested but did not pass the epithelial barrier. At the same time, localization of Ti inside the gut proves that sample preparation is adequate and no preparation artefacts occurred.

Our results on low toxic potential of ingested TiO_2 particles are in agreement with data provided by other authors. Adachi et al. [20] reported that no TiO_2 particles were found in viable skin, and the findings of Sadrieh et al. [21] also indicate that there is no significant penetration of TiO_2 nanoparticles through the intact normal epidermis.

Toxicity and cellular internalisation of ingested nano-TiO_2 by environmental organisms have not been previously investigated simultaneously *in vivo*. In this study, we employed micro-PIXE to document the presence of Ti inside digestive gland epithelial cells. The main advantages of this method are high elemental sensitivity and satisfactory lateral resolution which is in the micron range. Micro-PIXE has also been successfully used in skin penetration studies of nano-TiO_2 [22] as well as in studies of some other particles [23] and has the potential to provide important knowledge on tissue level elemental distribution.

Consistent with previously published reports, we confirm the suitability of terrestrial isopods to be used in studies of fate and effects of ingested nanoparticles [23]. The advantages of terrestrial isopods as model organisms are as follows:

(a) on the basis of consumed amount of food, it is possible to estimate the consumed amount of particles;

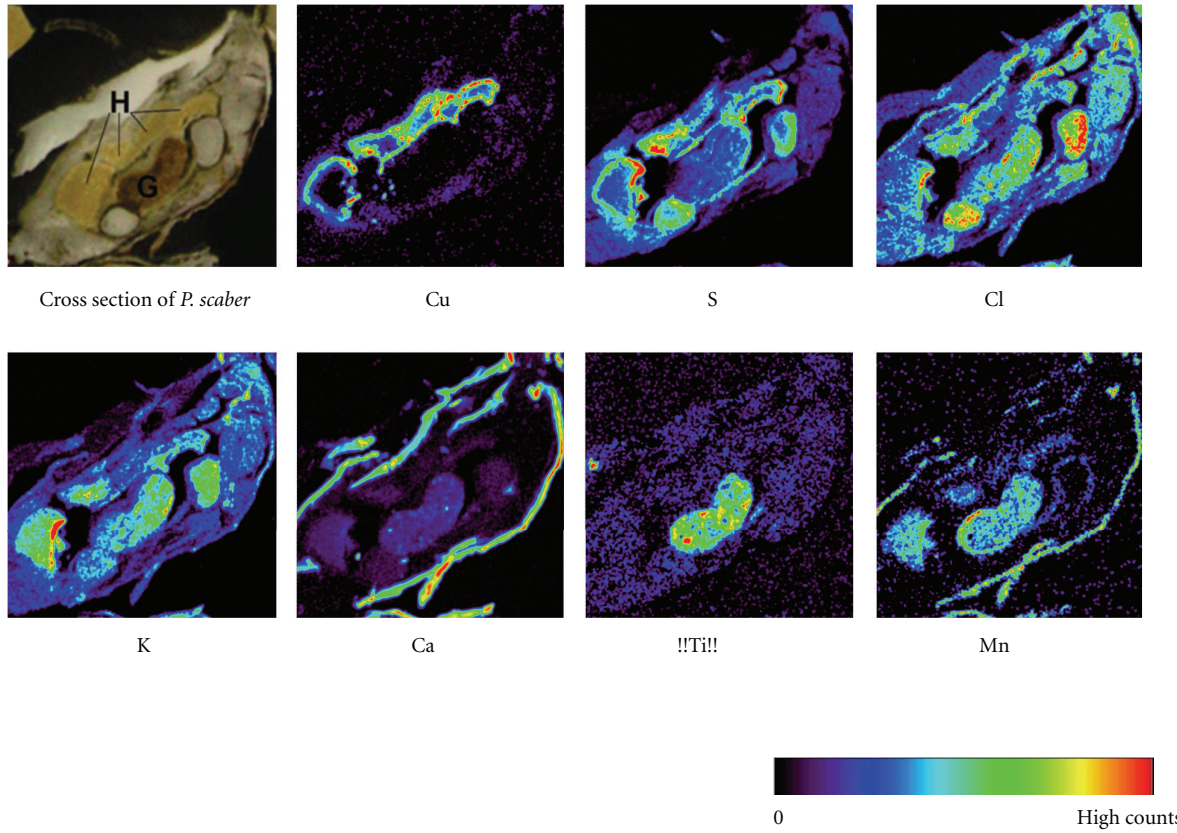


Figure 6: Scanning Transmission Ion Microscopy (STIM) images of body cross section (upper left image) and micro-PIXE qualitative elemental maps of Cu, S, Cl, K, Ca, Ti, and Mn taken on cross sections of *P. scaber* fed on food dosed with nano-TiO_2 (1000 μg/g of dry wt of leaves). The Cu map denotes the location of digestive gland cells (H-hepatopancreas, composed of four digestive gland tubes, G-gut).

(b) protocols to analyze biomarkers at different levels of biological complexity are already available;

(c) animals are large enough to obtain sufficient tissue for chemical, microscopic, or spectroscopic analyses and also small enough to study entire body cross section for elemental distribution in a single cross section;

(d) it is possible to study in a parallel the fate and the effects of consumed nanoparticles and link these data to consumed doses. All these analyses are possible on a statistically significant number of animals per group.

The disadvantages of using invertebrate organisms as laboratory experimental animals are

(a) lack of knowledge concerning handling of invertebrates when one's experience is with vertebrates,

(b) lack of mathematical models with which to interpret the results obtained with invertebrates and relate them to the human health/safety issue,

(c) low levels of similarity with vertebrate physiology. Straightforward comparison and interpretation of fate and effects of particles in vertebrate models is vitiated by this question. When enough data are available on effect of same nanoparticles obtained with *in vitro* and different *in vivo* models, including vertebrates and invertebrates, data on invertebrates will gain importance.

5. Conclusions

A series of *in vivo* laboratory experiments where a model invertebrate organism was fed with nano-TiO_2 dosed food revealed that:

(1) feeding on TiO_2 particles up to 0,0001% of body wt for 14 days did not show cellular internalization or body distribution of Ti. If an organism with 50 kg consumes 0.04 ± 0.01 g TiO_2 per day for 14 days, no toxic effects at organism level could be expected;

(2) feeding with TiO_2-dosed food results in destabilization of digestive gland cell membrane, while no toxic effects were detected by conventional toxicity measures. This means even if no toxic effects at organism level are evident, cell membrane of digestive epithelium cells may be destabilized;

(3) the invertebrate *in vivo* test with a terrestrial isopod is suitable for parallel studies of fate and effects of ingested particles and should be explored further;

(4) if safety of nanoparticles is at issue, toxicity measures must be supplemented with more sensitive cellular markers.

Acknowledgments

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