

**Gold nanoparticles do not induce adverse effects on terrestrial isopods
Porcellio scaber after 14-day exposure**

Nanodelci zlata nimajo negativnih učinkov na kopenske rake vrste *Porcellio scaber* po 14-dnevni izpostavitvi

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Abstract: Despite the anticipated environmental release of anthropogenic gold nanoparticles (AuNPs), there is currently not enough data on their potential impact on terrestrial environment. In the current study, we investigated the effects of considerably low concentrations of AuNPs on terrestrial isopods (*Porcellio scaber*) after 14 days of exposure. The effects on mortality, weight change, feeding rate, avoidance/preference feeding behavior, and cell membrane destabilization of digestive gland cells were followed. In parallel, the accumulation of Au in the digestive glands was measured. Our results show that none of the tested parameters was affected in isopods under given exposure doses (10 and 60 µg Au/g dry leaf) and exposure duration. No Au was assimilated in the digestive glands. Also, the same doses of the reference chemical, AuCl₃, showed no effect. We conclude that these concentrations of AuNPs are safe for terrestrial isopods. We encourage reporting the results showing no adverse effects of nanoparticles to balance the prevailing publication of their adverse effects. This will help to build a realistic public perception of the environmental risk of nanomaterials.

Keywords: nanoparticles, Au³⁺, avoidance behavior, bioaccumulation, safety

Izveček: Kljub naraščujoči uporabi nanodelcev zlata (ND Au) trenutno še vedno ni dovolj podatkov o njihovih potencialnih negativnih učinkih na kopenske organizme. V tej študiji smo proučevali vpliv relativno nizkih koncentracij ND Au na kopenske rake vrste *Porcellio scaber* po 14-dnevni izpostavitvi. Proučevali smo vpliv na smrtnost, maso živali, stopnjo prehranjevanja, izogibalno vedenje in destabilizacijo membrane celic prebavnih žlez. Izmerili smo tudi asimilacijo Au v prebavnih žlezah. Rezultati so pokazali, da testirane koncentracije ND Au (10 in 60 µg Au/g lista) pri kopenskih rakih

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niso vplivale na nobenega izmed testiranih parametrov po danem času izpostavitve. V prebavnih žlezah nismo izmerili Au. Enake rezultate smo dobili tudi z referenčno kemikalijo, soljo AuCl_3 . Zaključujemo, da so testirane koncentracije ND Au varne za kopenske rake. Naše mnenje je, da se na področju nanotoksikologije trenutno objavlja predvsem rezultate, ki kakorkoli nakazujejo na negativni vpliv nanodelcev na organizme. Zato želimo stimulirati tudi objavo rezultatov, ki kažejo nasprotno. Na ta način bomo pripomogli k bolj uravnoteženemu in realističnemu mnenju javnosti o okoljski varnosti nanomaterialov.

Ključne besede: nanodelci, zlato, izogibalno vedenje, bioakumulacija, varnost

Introduction

Gold is a trace element in lithosphere, hydrosphere, and biosphere. It occurs naturally at concentrations around 4 $\mu\text{g}/\text{kg}$ in the Earth's crust, <200 $\mu\text{g}/\text{kg}$ in rocks, <2 mg/kg in soils, <1 $\mu\text{g}/\text{L}$ in freshwaters and <5 $\mu\text{g}/\text{L}$ in marine waters, while waters from auriferous deposits may contain up to 1 mg/L of gold. In living organisms, such as microorganisms from ore fields and marine invertebrates, gold was found at concentrations 30–750 $\mu\text{g}/\text{kg}$ (Korobushkina et al. 1983). Gold has no known physiological functions and it is usually considered as inert in the elemental form, Au^0 (Sadler 1976, Eisler 2004). However, exposure to gold jewelry, gold dental restorations, gold implants, and beverages containing flake gold has been linked to contact allergy in susceptible individuals, mostly in the form of dermatitis. It has been proposed that dark discolorations of skin in contact with gold jewelry and dermatitis are associated with formation of the more reactive Au^+ and Au^{3+} species due to extra- or intracellular dissolution of Au^0 , e.g. by sweat or in lysosomes (Rapson 1984, Eisler 2004).

Gold nanoparticles (AuNPs) – or the so-called “colloidal gold” – may be of either natural or anthropogenic origin. In the nature, AuNPs are formed by weathering of origin rocks or by microbial precipitaton in auriferous soils (Southam et al. 2009). Production of anthropogenic AuNPs has been increasing due to their use in consumer, industrial and medical products. AuNPs are used for numerous applications, ranging from biosensors to catalysts, electronics, cosmetics, and cancer treatment (Unrine et al. 2010). According to the Consumer Products Inventory, there are currently 25 products (out of 1331 in total) listed containing

AuNPs, mostly dietary supplements and cosmetics (Project on Emerging Nanotechnologies, 2015, retrieved on June 21st, 2016). As a result of a variety of uses, environmental release of anthropogenic AuNPs is anticipated and there is a need of further research regarding the impacts of AuNPs on the environment. At the moment, no measured environmental concentration data for AuNPs are available. However, according to Mahapatra et al. (2015), the mean annual predicted environmental concentration of AuNPs in sludge is estimated at 0.120 and 0.150 $\mu\text{g}/\text{g}$ for UK and US, respectively, and in sludge-treated soil at 300 and 150 ng/kg yearly for UK and US, respectively. Although yearly concentration is considerably low, it may increase due to continuous application over years.

The bioavailability of AuNPs and their effects on terrestrial invertebrates have been studied in earthworms (*Eisenia fetida*; Unrine et al. 2010, 2012), fruit flies (*Drosophila melanogaster*; Pompa et al. 2011; Sabella et al. 2011, Vecchio et al. 2012a, 2012b) and tobacco hornworms (*Manduca sexta*; Judy et al. 2010, 2012). However, for terrestrial isopod crustaceans, no data on bioavailability or effects of gold ions or AuNPs exists to date. Terrestrial isopods (*Porcellio scaber*) are suitable model terrestrial organisms for testing effects of chemicals, since they enable precise monitoring of the exposure dose together with its consequences on different levels of biological organization (Drobne 1997). Being hyperaccumulators of various metals (Hames and Hopkin 1989), they are especially convenient in studying the biological effects of metal salts and nanoparticles (Pipan-Tkalec et al. 2010, Golobič et al. 2012, Novak et al. 2012).

In isopods, the most sensitive biological endpoints for studying the effects of chemicals are biochemical, histological, and physiological (Drobne 1997). These endpoints can be simultaneously investigated in the digestive glands (hepatopancreas), which are in direct contact with the substances in food. Isopod digestive glands consist of four blind-ending tubes with intestinal, hepatic and pancreatic functions. The digestive gland epithelium is built of two cell types: larger B cells with secretive and absorptive functions, which contain lipid droplets and glycogen, and smaller S cells, which predominantly accumulate metals (Hames and Hopkin 1989). After consumption of metal salt- or NP-spiked food, the digestive gland epithelium can be often found containing elevated concentrations of metal ions accumulated in storage granules (Pipan Tkalec et al. 2010, Golobič et al. 2012). Accumulation of metals in digestive glands in insoluble form is considered as a means of detoxification under exposure to elevated concentrations of metals in food (Hopkin 1990). Destabilization of the digestive gland cell membrane by NPs is a measure of cytotoxicity; however, damaged cell membrane also permits cellular internalization of NPs, which may lead to further cytotoxic effects (Novak et al. 2012). The biological endpoints at the cellular and tissue level can be combined with organism-level endpoints, such as body mass change, mortality, and avoidance behavior (Škarková et al. 2016) to elucidate a broad picture of the effects of the tested NPs on isopods.

The aim of our present study was to investigate the effects of ingested AuNPs and AuCl₃ (10 and 60 µg Au/g dry leaf) on terrestrial isopods as well as potential bioaccumulation of gold into digestive gland cells. AuCl₃ was used as a positive control to account for potential dissolution of AuNPs inside the isopods' digestive tract (Eisler 2004; Golobič et al. 2012) and to differ between the effects of AuNPs and Au³⁺ ions. We related the data on Au bioaccumulation in digestive gland tissue to the data on the effects of Au exposure, such as mortality, weight change, feeding rate, and cell membrane destabilization of digestive gland cells. A 14-day food selection behavior test was also done to investigate their selection of the Au-spiked food.

Materials and methods

Test chemicals

AuNPs were synthesized by the INMETRO (National Institute of Metrology, Quality and Technology; Rio De Janeiro, Brazil) as a part of the EU FP7 NanoValid project under project label NNV-004. The data on particle size, shape, ζ-potential, and metal content was provided by the supplier. Gold (III) chloride (AuCl₃, ≥99.99% trace metals basis, CAS Number 13453-07-1) and gold standard for AAS (1 mg Au/mL, TraceCERT[®]) were purchased from Sigma-Aldrich (Steinheim, Germany). Water used throughout the work (dH₂O) was first deionized and then further purified using Elix 10/Milli-Q Gradient unit (Millipore, Bedford, Massachusetts, USA [pH = 5.7, ρ = 18.5 MΩ·cm]). Physiological solution for *P. scaber* was prepared according to the protocol published in Hagedorn et Ziegler (2002). Tris(hydroxymethyl)amino-methane, NaCl, KCl, MgCl₂, and glucose were purchased from Merck (Darmstadt, Germany). All chemicals were of the reagent grade (EMSURE[®]). For the hepatopancreatic cell membrane stability assay, acridine orange solution (2% in dH₂O) and ethidium bromide solution for fluorescence (~1% in dH₂O) were used, both from Sigma Aldrich. Microwave acid digestion was performed with the reagent grade 65% HNO₃ (Fischer Scientific, Loughborough, Leicester, UK).

Test organisms

Isopods *P. scaber* originated from the synchronized laboratory culture at the Department of Biology, University of Ljubljana, Slovenia. Cultures of *P. scaber* were derived from individuals collected from an unpolluted site in Polhov Gradec, Slovenia (46° 3' 0" N, 14° 18' 0" E). Animals were kept in a climate chamber at 22 ± 1 °C with a 16/8 h light/dark period (120 and 16 lx, respectively; measured using LI-1000 Data Logger, LI – COR, Nebraska, USA), caged in glass containers with moist loamy sand and peat at the bottom. They were fed with fallen leaves from various trees, with periodical additions of potatoes, fresh vegetables, and apples.

Feeding exposure

Partially decomposed common hazel leaves (*Corylus avellana*) were collected in the Karavanke region, Slovenia (46° 21' 32.29" N, 14° 16' 36.12" E), for the purpose of the experiment. Leaves were air-dried at room temperature (24 ± 1 °C) and stored in a cardboard box until use. Bigger leaves with minimally damaged leaf lamina were straightened and the serrated leaf edge was cut off. Leaf laminae were cut into pieces of 100 ± 10 mg.

The AuNPs or AuCl₃ were suspended in dH₂O using a vortex (20 s, 2000 rpm) to obtain concentrations 10 and 60 µg Au/mL. The higher concentration corresponded to the Au concentration in the original AuNP suspension provided by the supplier (0.006 % w/w), and the lower one was chosen for comparison to the results of our previous studies, e.g. Pipan-Tkalec et al. (2011). No stabilizers were used, and the chemicals were prepared freshly for each experiment. 100 µL of Au NP dispersion or AuCl₃ solution per 100 mg of leaf was applied onto the abaxial surfaces of dry leaves. This resulted in two final concentrations of 10 and 60 µg Au/g dry leaf for both sources of Au. Control leaves were spiked with dH₂O only. Spiked leaves were allowed to dry for 24 hours at room temperature. After 24 hours, dry leaves were re-weighed and this data was then used for further calculations.

Only adult isopods of both sexes, and with 30–60 mg body mass were chosen for the experiments. Moulting animals (Zidar et al. 1998) and gravid females were excluded in order to keep the investigated population as homogenous as possible in terms of its physiological state. Each animal was placed individually in plastic Petri dishes (Æ 9 cm), to which individual pieces of Au-treated dry leaves were added. Two experiments were carried out. The first experiment (**Experiment 1**) consisted of the control group and the groups exposed to AuNPs. The second experiment (**Experiment 2**) consisted of the control group and the groups exposed to AuCl₃. In both experiments, each experimental group comprised 12 animals. The exposure conditions were the same for both experiments. Petri dishes were placed in a large, plastic-covered glass container and their humidity was maintained by periodical spraying of the internal side of the lids with dH₂O. The experi-

ments were maintained for 14 days in controlled and stable conditions at 22 ± 1 °C, 80% relative humidity (TFA, Dostmann GmbH et Co.KG, Wertheim, Germany), with a 16/8 h light/dark period (120 and 16 lx, respectively) and monitored on a daily basis. The food was not replaced during the exposure period, and fecal pellets were collected weekly.

Post-experimental sample preparation and analysis

After the 14-day exposure period, the animals were transferred to new Petri dishes and fed with uncontaminated hazel leaves for 24 h to deplete Au from their digestive system. The leaves and fecal pellets from the experiments were collected and weighed after drying at room temperature for 24 h. On the 15th day, animal mortality was recorded, and the survived animals were weighed. The experiments were considered valid if the mortality of controls did not exceed 20 % (Hornung et al. 1998). The animals were decapitated, and the hepatopancreas and gut were isolated with tweezers. One gland tube was used for the cell membrane stability assay, and other three gland tubes, the gut and the 'rest' of the body were further processed for the measurements with flame AAS.

Hepatopancreatic cell membrane stability assay

Cell membrane stability was tested with a modified method for the assessment of cell membrane stability, previously described by Valant et al. (2009). A single isolated hepatopancreatic tube was incubated for 5 minutes in a mixture of the fluorescent dyes acridine orange and ethidium bromide and then put on a microscope slide. Fresh samples were photographed and examined by the Axioimager.Z1 fluorescent microscope (Carl Zeiss, Jena, Germany) with two different sets of filters. The excitation filter of 450 to 490 nm and the emission filter of 515 nm were used to visualize acridine orange- and ethidium bromide-stained nuclei, and the excitation filter of 365 nm and the emission filter of 397 nm were used to visualize nuclei stained with ethidium bromide only. Cell membrane integrity was assessed by visual examination of micrographs and classified

from 1 to 10 according to a predefined scale. On the basis of preliminary experiments, the control animals showing less than 10 % of nuclei stained by ethidium bromide were classified as 1 or 2. The animals exposed to AuNPs, AuCl₃, but showing less than 10 % of nuclei stained by ethidium bromide, were also classified as 1 or 2 (Valant et al., 2009).

Gold content determination

Each isopod body part was placed on a separate small piece of a filter paper (approximately 4 mm×7 mm size) and stored in a plastic tube. Prior to analysis, samples were acid digested in concentrated HNO₃ in the Milestone Ethos E (Bergamo, Italy) microwave lab station equipped with SK-10 high-pressure segmented rotor and 3 mL quartz microsampling inserts. Digestion was conducted at 180°C and 600 W power, with step 1 (heating) lasting 15 min, step 2 (constant temperature) lasting 10 min, and 45 min cooling to 60°C. Total Au concentrations in the three parts of each animal (one digestive gland, the gut and the ‘rest’ of the body) were measured by flame AAS (Perkin-Elmer AAnalyst 100, Waltham, Massachusetts, USA). Metal spiking recovery was determined by measuring the Au concentrations on the remnants of leaves after the experiment.

Food selection behavior test

The food selection behavior test (**Experiment 3**) was carried out according to the protocol by Zidar et al. (2004). The preparation of spiked food and the selection of animals were conducted in the same way as in the toxicity endpoint tests. Particular attention was paid to include only the animals with intact antennae. Each of the five experimental groups included 15 animals, which were caged individually in plastic Petri dishes (Æ 9 cm) and offered two hazelnut leaf pieces (approximately 100 mg each) of different shape (square and triangular) for 14 days. The two leaf pieces had been treated differently. The first piece was control (spiked with dH₂O only) and the second one was spiked with a test compound. The tested Au sources and concentrations were the same as in the toxicity endpoint tests, i.e. AuCl₃ and AuNPs at concentrations 10 and 60 µg Au/g dry leaf. As a positive control, CoCl₂×6H₂O with the

nominal exposure concentration 2000 µg Co²⁺/g dry leaf was used. In our previous work (data not published), isopods *P. scaber* significantly avoided Co²⁺ contaminated food at the same nominal exposure concentration, therefore we used only 10 animals in this group for ethical reasons. This collectively resulted in 6 combinations of test compounds: “0 (control) vs. 0”, “+control (Co²⁺) vs. 0”, “AuNPs 10 vs. 0”, “AuNPs 60 vs. 0”, “AuCl₃ 10 vs 0” and “AuCl₃ 60 vs. 0”. The experimental conditions and post-experimental procedure were the same as in the toxicity endpoint tests, except that animal dissection and tissue processing were not performed. The total food consumption rate was calculated as the amount of food consumed (both leaves offered) during 14 days, per fresh animal weight. The consumption rate of a single leaf was calculated as the percentage of the total food consumption rate. This data was then used for the calculation of food selection response. Dead animals were excluded from the calculations.

Data analysis

In all experiments, 12 or 15 animals per each tested group were exposed, but the number of analyzed animals after the experiments was lower due to mortality caused by moulting and due to development of marsupia in females; all such animals were excluded from further data processing. The numbers of analyzed animals are presented in the Figures as part of the x-axis labels. Data is presented as mean values, and uncertainties are expressed as standard deviations (SD).

Average animal fresh body mass (per individual) during the experiment was calculated as an arithmetic average of fresh body masses recorded before and after the experiment. Feeding rate (per individual) was calculated by dividing the total mass of consumed leaves during the experiment with the average animal fresh body mass. Since the animals collected for **Experiments 1** and **2** belonged to the same population, the corresponding control groups were tested for homogeneity of variances with the Flieger-Killeen test and their average body masses and feeding rates were compared by the Mann-Whitney *U*-test using R statistical package (R Development Core Team 2015). Because no significant differences were found ($p > 0.05$), the controls were pooled before

further data analysis. Statistical significance of differences between the pooled control and the animals exposed to Au compounds was assessed by the Mann-Whitney *U*-test using OriginPro 8.0 software (OriginLab, Northampton, MA, USA).

Results

Nanoparticle characteristics

Gold NPs were received from the INMETRO as a reddish dispersion in water. As stated by the manufacturer's specifications, the Au NP dispersion contained 0.006 % (w/w) of Au, the nominal average particle size was 15.7 nm, and ζ -potential was -30.4 mV. The shape of nanoparticles was spheroidal (Fig. 1).

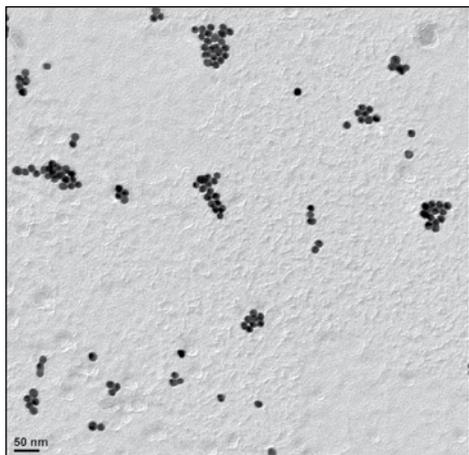


Figure 1. Transmission electron micrograph of Au nanoparticles; the photograph was provided by the INMETRO (National Institute of Metrology, Quality and Technology; Rio de Janeiro, Brazil).

Slika 1. Presevna elektronska mikrofografija nanodelcev Au; sliko je posnel INMETRO (Nacionalni inštitut za metrologijo, kakovost in tehnologijo; Rio de Janeiro, Brazilija).

Mortality, body mass change and food consumption of isopods

Fourteen days of exposure to Au compounds had no statistically significant effect on mortality, average body mass (Fig. 2a) or feeding rate (Fig. 2b) of the test animals in comparison to the control in any of the exposure groups (Mann-Whitney *U*-test, $p > 0.05$, not marked on Fig. 2).

Digestive gland cell membrane stability

Valant et al. (2009) demonstrated that the digestive gland cell membrane stability value was rarely higher than 2 in the animals from the stock culture, which are in good physiological condition, and this was taken as a benchmark. The higher the value, the more the membrane is destabilized, and the cell membranes are considered completely destabilized when the value is 10. In the present study, in the animals from the stock culture, in control animals and in those exposed to Au-spiked food, the values were never higher than 2, which indicates that neither AuCl₃ nor AuNPs affected membrane stability of the digestive gland cells (data not shown).

Gold content in food and animal tissues

The measured metal concentrations on spiked leaves were within 10 % of the nominal values for both tested Au sources at both tested concentrations. No Au was found in the hepatopancreas, the gut or the rest of the body of the animals from any of the exposure regimens, regardless of the Au source or concentration (data not shown).

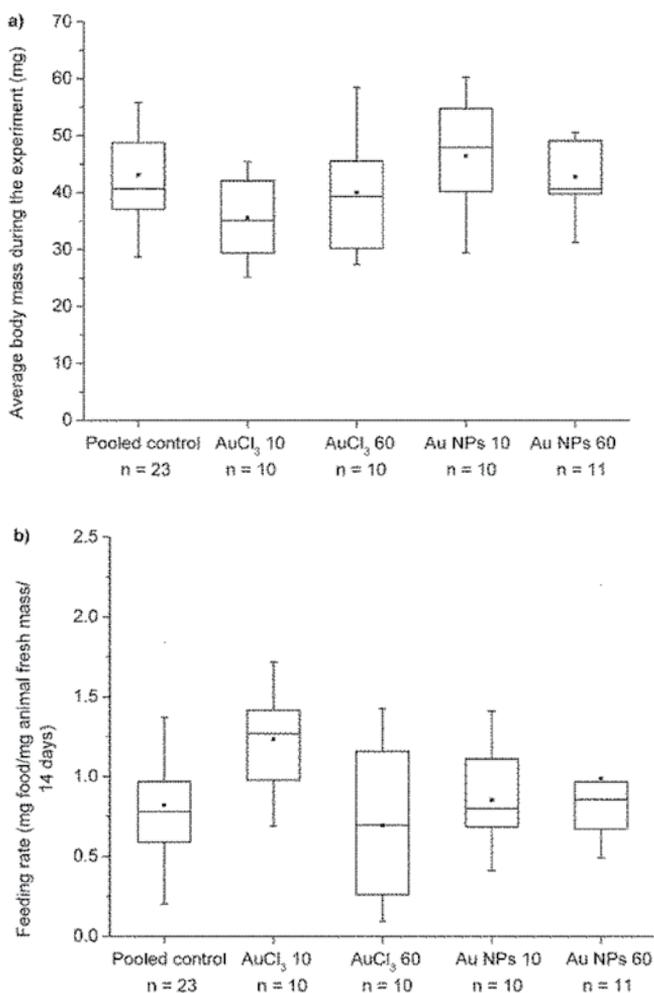


Figure 2. Average body mass (A) and feeding rates (B) of *P. scaber* isopods during 14-day exposure to Au-spiked food. The animals were fed non-spiked food (control) or food that was spiked with AuCl₃ salts (AuCl₃ 10 and AuCl₃ 60, per nominal Au concentrations) or Au nanoparticles (AuNPs 10 and AuNPs 60, per nominal Au concentrations). The controls from Experiment 1 (with AuNPs) and Experiment 2 (with AuCl₃) were pooled because no significant differences between them were found in any of the tested parameters. The nominal exposure concentrations of Au are provided on the x-axis. The symbols on the box plot represent maximum and minimum values (whiskers: ⊥), mean values (■), outliers (–); n = number of specimens in each test group.

Slika 2. Povprečna telesna masa (A) in stopnja prehranjevanja (B) enakožcev *P. scaber* med 14-dnevno izpostavitvijo hrani, tretirani z Au. Živali smo hranili z netretirano hrano (kontrola) ali hrano, na katero smo nanesli AuCl₃ (AuCl₃ 10 in AuCl₃ 60, za nominalne koncentracije Au) oziroma nanodelce Au (AuNPs 10 in AuNPs 60, za nominalne koncentracije Au). Kontrolni skupini iz poskusa 1 (z nanodelci Au) in poskusa 2 (z AuCl₃) smo združili, saj med njima ni bilo statistično značilnih razlik v nobenem izmed testiranih parametrov. Nominalne izpostavitvene koncentracije Au so navedene na x-osi. Simboli na okvirjih z ročajmi predstavljajo minimalne in maksimalne vrednosti distribucije (ročajji: ⊥), povprečja (■) in osamelce (–); n = število osebkov v vsaki testni skupini.

Food selection behavior test

In the **Experiment 3**, avoidance or preference behavior was not demonstrated (Fig. 3). The masses of consumed leaves did not significantly differ (Mann-Whitney *U*-test, $p > 0.05$) between the two leaf pieces (control and Au-spiked) for both tested Au compounds (AuNPs and AuCl₃)

and at both exposure concentrations (10 and 60 µg Au/g dry food). Significant preference for non-spiked over CoCl₂-spiked leaves was found in the positive control group (Fig. 3; Mann-Whitney *U*-test, $p < 0.001$).

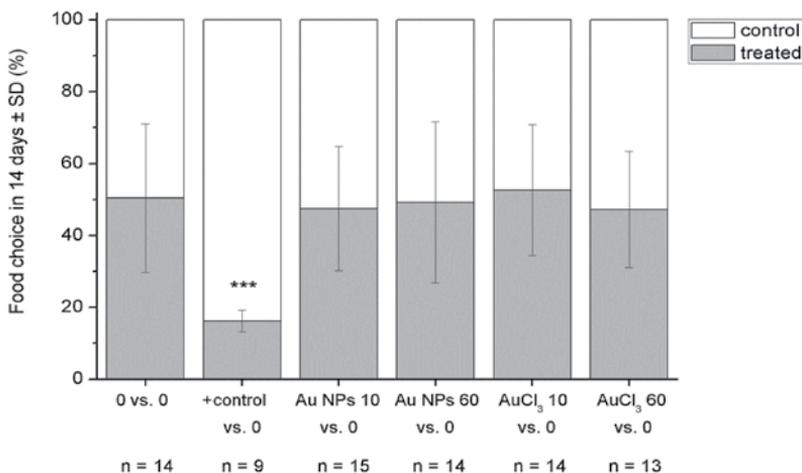


Figure 3. Food selection of *P. scaber* isopods. Animals were exposed to untreated food (0) and food treated with AuNP or AuCl₃ simultaneously for 14 days. The nominal exposure concentrations of Au are provided on the x-axis (in µg Au/g dry leaf). CoCl₂×6H₂O with the nominal exposure concentration 2000 µg Co²⁺/g dry leaf was used as a positive control (+control). The food choice (%) is presented as the ratio between consumption rates of the two offered leaves, expressed as mean percentages of the total food consumption rate for each exposure group. Uncertainties are expressed as standard deviation (SD). Significant differences: $p < 0.001$ (***) ; n = number of specimens in each test group.

Slika 3. Izbira hrane pri *P. scaber*. Živali so bile 14 dni hkrati izpostavljene netretirani hrani (0) in hrani, na katero smo nanесли AuCl₃ oziroma nanodelce Au. Nominalne izpostavitvene koncentracije Au so navedene na x-osi (v µg Au/g suhe mase lista). Kot pozitivno kontrolo (+control) smo uporabili CoCl₂×6H₂O v nominalni izpostavitveni koncentraciji 2000 µg Co²⁺/g suhe mase lista. Izbira hrane (%) je predstavljena kot razmerje med stopnjama prehranjevanja z dvema ponujenima listoma ter izražena kot povprečna vrednost stopnje prehranjevanja za vsako testno skupino posebej. Negotovost je izražena s standardnimi deviacijami (SD). Statistično značilne razlike: $p < 0.001$ (***) ; n = število osebkov v vsaki testni skupini.

Discussion

In the present study we assessed the influence of AuNPs and AuCl₃ on different biological endpoints in the model organisms, terrestrial isopods *P. scaber*. We tested the adverse biological effects of AuNPs and AuCl₃ as detectable by common toxicological parameters (body mass change, food consumption rate, metal feeding preference/avoidance behavior, and mortality) and cell membrane integrity assay, which provides information on the cell membrane destabilization. In parallel, we assessed the Au³⁺ assimilation in digestive glands.

Our results show that AuNPs did not affect *P. scaber* mortality, body mass (Fig. 2a) or food consumption (Fig 2b) when exposed through food at concentrations 10 and 60 µg Au/g for 14 days. In other words, we showed that AuNPs and AuCl₃ do not affect the organism-level endpoints in *P. scaber* at relatively low exposure concentrations. Our results are in line with previous reports on earthworms (Unrine et al. 2010). In *E. fetida*, exposure to AuNPs (20 and 55 nm, 5 µg Au/g dry mass spheres) in soil for 28 days did not affect their mortality or growth (Unrine et al. 2010). In contrast, feeding with 15 nm citrate-capped AuNPs at concentrations 3 and 27 µg AuNPs/g food per day reduced the lifespan of *D. melanogaster* for 24 and 41 %, respectively, in comparison to control (average control population half-life was 80 days; Sabella et al. 2011). In the same experimental setup and with the same AuNPs, the lifespan was reduced for 62 % when *D. melanogaster* were exposed to 12 µg AuNPs/g food per day (average control population half-life was 37 days; Pompa et al. 2011). However, the exposure doses for *D. melanogaster* (Pompa et al. 2011, Sabella et al. 2011) were higher than in our study (0.7 and 4.3 µg Au/g food per day for nominal exposure concentrations 10 and 60 µg Au/g dry leaf, respectively), which may partially explain why mortality in *P. scaber* in our study was not elevated in comparison to *D. melanogaster*.

In line with this, we also showed that isopods show no preference/avoidance towards Au-spiked hazelnut leaves for both tested Au compounds (AuCl₃ and AuNPs; Fig. 3). This is in agreement with the observation that feeding rate of isopods was unaffected upon Au exposure (Fig. 2b). Avoidance behavior of isopods towards metal-

contaminated food has previously been shown (Zidar et al. 2004; Škarková et al. 2016).

Nanoparticles may pass from the gut into the lumen of the *P. scaber* hepatopancreas during digestion, as it has been demonstrated for TiO₂ NPs (Novak et al. 2012) and WO_x nanotubes (Novak et al. 2013), and cause cell membrane destabilization (Novak et al. 2012, 2013). However, neither AuNPs nor AuCl₃ caused the reduced cell membrane integrity of hepatopancreatic cells in our current study. The results of cell membrane integrity assay match the data on the Au assimilation. Namely, neither of the *P. scaber* body parts contained any gold, which indicates that AuNPs were not internalized into tissues and Au³⁺ ions were not assimilated into the digestive gland cells. In general, we cannot exclude the possibility that *P. scaber* possesses the assimilation capacity for Au³⁺, because Au has the affinity for sulphur-bearing ligands (Korobushkina et al. 1983), which are present in the type B granules of the hepatopancreatic S cells (Hopkin 1990) and enable the assimilation of other metals with affinity for sulphur, such as cadmium, copper, lead and mercury (Hopkin 1990), silver (Pipan-Tkalec et al. 2011) and cobalt (Novak et al. 2013). Nor we can exclude the possibility for the dissolution of AuNPs inside the *P. scaber* digestive tract (Golobič et al. 2012), since the dissolution of gold can be induced by biological macromolecules, most notably amino acids and proteins, as well as bacteria (Sadler 1976, Rapson 1982, Korobushkina et al. 1983). However, under employed exposure conditions, the internalization/assimilation of Au into *P. scaber* digestive glands clearly did not occur.

Our results contrast those for *E. fetida* (Unrine et al. 2010), *M. sexta* caterpillars (Judy et al. 2010, 2012), and *D. melanogaster* (Pompa et al. 2011) where AuNPs, administered *via* food, were found in tissues surrounding the digestive tract (including the digestive tract epithelium in *E. fetida*) as well as in the reproductive organs in *D. melanogaster*. The presence and localization of gold was corroborated by transmission electron microscopy (TEM) coupled to energy dispersive spectrometry ([EDS] Unrine et al., 2010), X-ray absorption near edge spectroscopy (µXANES) and synchrotron X-ray fluorescence microprobe ([µXRF]; Judy et al. 2010, 2012) or high angle annular dark field scanning transmission electron

microscopy ([HAADF-STEM]; Pompa et al. 2011). No evidence of dissolution of AuNPs was observed in the quoted studies, so NPs were likely assimilated intact (Unrine et al. 2010, Judy et al. 2010, Pompa et al. 2011).

In conclusion, our results denote that concentrations of Au salt and AuNPs tested in this work (10 and 60 $\mu\text{g Au/g}$ dry leaf) do not induce adverse effects on terrestrial isopods after 14 days of exposure. These concentrations are higher than currently predicted environmental levels (Mahapatra et al. 2015), which further confirms the finding that AuNPs are safe for isopods. In general, AuNPs are among NPs with the least toxic potential for test organisms commonly employed in environmental studies. Namely, Bondarenko et al. (2016) have used a set of assays to screen seven NMs using 14 different test species and cell lines. The toxicity decreased in the following order: $\text{Ag} > \text{ZnO} > \text{CuO} > \text{TiO}_2 > \text{MWCNTs} > \text{SiO}_2 > \text{Au}$ (Bondarenko et al. 2016). According to our perception, the majority of authors in nanotoxicity research encourage the publication of those results that show some kind of effects of nanoparticles on organisms. We therefore suggest that also those data with no documented effects should be published in equal proportion to help build a realistic public perception of the nanomaterial environmental risk.

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Povzetek

Nanodelci zlata se čedalje pogosteje uporabljajo za različne aplikacije, kot je npr. v kozmetiki, barvah in elektrokemijskih senzorjih ter v detekciji, diagnostiki in zdravljenju tumorjev. Kljub temu še vedno ni dovolj podatkov o njihovih potencialnih negativnih učinkih na kopenske organizme. V tej študiji smo proučevali vpliv okoljsko relevantnih koncentracij ND Au na kopenske rake vrste *Porcellio scaber* po 14-dnevni izpostavitvi. Živali smo izpostavili listom, na katere so bili nanešeni nanodelci zlata. Proučevali smo vpliv na smrtnost, maso živali, hitrost prehranjevanja, izogibalno vedenje in destabilizacijo membrane celic prebavnih žlez. Izmerili smo tudi asimilacijo Au v prebavnih žlezah. Rezultati so pokazali, da nobena izmed testiranih koncentracij ND Au (10 in 60 $\mu\text{g Au/g}$ lista) po 14-dnevem hranjenju ni imela vpliva na kopenske rake. Prav tako nismo izmerili prisotnosti Au v prebavnih žlezah, kar kaže, da se ND Au niso niti raztapljali v prebavnem soku niti vstopali v celice prebavnih žlez. Enake rezultate smo dobili tudi z referenčno kovino, soljo AuCl_3 . Zaključujemo, da so testirane koncentracije ND Au varne za kopenske rake. Naše mnenje je, da se na področju nanotoksikologije trenutno objavlja predvsem rezultate, ki kakorkoli nakazujejo na negativni vpliv nanodelcev na organizme. Zato želimo stimulirati tudi objavo rezultatov, ki kažejo nasprotno. Na ta način bomo pripomogli k bolj uravnoteženemu ter realističnemu mnenju javnosti o okoljski varnosti nanomaterialov.

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