Fate, accumulation and impact of metallic nanomaterials in the terrestrial environment

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Chapter 5

Trophic transfer and toxicity of (mixtures of) Ag and TiO_2 nanoparticles in the lettuce - terrestrial snails food chain

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Abstract: The increasing application of biosolids and agrochemicals containing silver nanoparticles (AgNPs) and titanium dioxide nanoparticles (TiO_2NPs) results in their inevitable accumulation in soil, with unknown implications along terrestrial food chains. Here, the trophic transfer of single NPs and a mixture of AgNPs and TiO_2NPs from lettuce to snails and their associated impacts on snails were investigated. Both AgNPs and TiO_2NPs were transferred from lettuce to snails with trophic transfer factors (defined as the ratio of the Ag/Ti concentration in snail tissues to the Ag/Ti concentration in the lettuce leaves) of 0.2 to 1.1 for Ag and 3.8 to 47 for Ti. Moreover, the majority of Ag captured by snails in the AgNPs-containing treatments was excreted via faeces, whereas more than 70% of Ti was distributed in the digestive gland of snails in the TiO_2NPs-containing treatments. Additionally, AgNPs-containing treatments significantly inhibited the activity of snails, while TiO_2NPs-containing treatments significantly reduced faeces excretion of snails. Furthermore, the concurrent application of AgNPs and TiO_2NPs did not affect the biomagnification and distribution patterns of Ag and Ti in snails, whereas their co-existence exhibited more severe inhibition of the growth and activity of snails than in the case of applying AgNPs or TiO_2NPs alone. This highlights the possibility of nanoparticle transfer to organisms of higher trophic levels via food chains and the associated risks to ecosystem health.

5.1. Introduction

The release of silver and titanium dioxide nanoparticles into agricultural soil is expected to increase through the expanding application of nano-containing biosolids and agrochemicals[55,273]. This raises concerns about their potential adverse side-effects on soil ecosystems and the potential risk to plants and animals. To date, extensive studies have been performed to understand the interactions between metallic nanoparticles and plants because of the crucial role of plants in the terrestrial food chain. Emerging evidence suggests that AgNPs and TiO_2NPs can be taken up by plant roots and subsequently be translocated to leaves[82,187,274,275,276], and even to the fruits/grains[240,277,278] of certain plant species. For example, the uptake and translocation of Ag/AgNPs were observed in rice (*Oryza sativa L.*) with measured translocation factors of 0.11 to 0.21[187], and in lettuce (*Lactuca sativa*) with

translocation factors of 0.002 to 0.01[82], as well as with translocation factors of 0.1 to 0.6 in ryegrass (*Lolium multiflorum*)[61]. Similarly, the accumulation of TiO_2NPs in lettuce[274,275], wheat (*Triticum aestivum*)[279,280] and cucumber (*Cucumis sativus*)[276,278] was confirmed. The considerable evidence of the accumulation of AgNPs and TiO_2NPs in edible parts of plants makes it reasonable to assume the likelihood of their transfer and potential biomagnification to higher-level consumers via the food chain. In contrast to the studies on the trophic transfer of AgNPs/TiO_2NP in aquatic food webs (mostly focused on algae to daphnia[281,282] or daphnia to zebrafish[283]), limited attention has been paid to the trophic transfer of AgNPs/TiO_2NPs within terrestrial food chains, especially for the transfer from plants to animals.

Currently, there are few publications addressing the trophic transfer of metallic nanoparticles from terrestrial plants to primary consumers and the subsequent bioaccumulation in these primary consumers. Judy et al.[284,285] reported the bioaccumulation of gold NPs from tobacco (*Nicotiana tabacum* L. cv *Xanthi*) and tomato (*Lycopersicon esculentum*) to the tobacco hornworm (*Manduca sexta*). CeO_2 NPs have been reported to transfer along several food chains, including: lettuce - snail (*Achatina fulica*)[286], lettuce - hornworm (*Spodoptera litura* F.) - chicken (*Gallus gallus domesticus*)[287], zucchini (*Cucurbita pepo* L.) - cricket (*Acheta domesticus*) - spider (family Lycosidae)[288] and kidney bean (*Phaseolus vulgaris* var. red hawk) - Mexican bean beetles (*Epilachna varivestis*) - spined soldier bugs (*Podisus maculiventris*)[289]. Previous studies also reported on the trophic transfer of La_2O_3 NPs through the lettuce - cricket - mantid (*Tenodera aridifolia sinensis* and *Sphodromantis centralis*)[290] food chain and of CuO NPs via the lettuce - cricket - lizard (*Anolis carolinensis*) food chain[291]. Even though those studies provided evidence of the trophic transfer of NPs via terrestrial food chains, the extent of transfer and biomagnification of NPs to the subsequent trophic level was inconsistent across the food chains. For example, the transfer of AuNPs from tobacco to tobacco hornworm occurred with trophic transfer factors of 6.2 to 11.6[284], while CeO_2-NPs were not magnified at all from lettuce to snail (trophic transfer factor =0.037)[286]. However, in none of the mentioned publications the impacts of trophic transfer of NPs on the behavioural alterations of the consumers was investigated. This information is valuable for assessing their possible risks to the environment and

ecosystem health.

Additionally, another area that is in lack of knowledge is related to the biomagnification and the effects of mixtures of nanoparticles on herbivores that feed on exposed plants. Importantly, once entered into the natural environment, nanoparticles always co-exist with numerous pollutants[292,293] including other nanoparticles[105]. This might result in interactions between the particles. TiO_2NPs are known to have a large specific surface and a strong adsorption ability, which are among the key reasons why TiO_2NPs can affect the biological effects of co-existing pollutants. For example, TiO_2NPs have been reported to decrease the toxicity of ZnO nanoparticles and CuO particles in cress (*Lepidium sativum*), wheat and cucumber[105]. To our knowledge, up till now only one study focused on soil ecosystems concerning the impacts of a mixture of TiO_2 and AgNPs. Specifically, Liu et al.[294] found that TiO_2NPs mitigate the inhibition by AgNPs of the growth of the plant *Arabidopsis thaliana* and the earthworm *Eisenia fetida* as well as the reduction of soil microbial biomass. The mixture of TiO_2NPs and AgNPs significantly decreased the Ag concentration but increased the Ti concentration in plants in comparison with the individual nanoparticles. The differences in Ag/Ti accumulation in plants induced by mixtures of NPs may affect the subsequent trophic transfer of the particles. However, to date, no study is available about the trophic transfer of a mixture of TiO_2NPs and AgNPs along a terrestrial food chain. In addition, the lack of published studies on this topic and the inconsistent biomagnification results highlight the need for further studies on the trophic transfer of nanoparticles in terrestrial food webs. This is especially true for mixtures of NPs, which constitutes a representative environmentally realistic exposure scenario.

In this study, lettuce and garden snails (*Cornu aspersum*) were used to study the trophic transfer of AgNPs and TiO_2NPs and the associated effects on snails. Lettuce is a worldwide cultivated leafy vegetable crop that is suited for evaluating the ecotoxicity of chemicals and soil amendments to higher terrestrial plants, as recommended by various regulations[295]. Similarly, terrestrial snails are recognized as excellent ecological and biological indicators for assessing the ecotoxicity of NPs[150,151]. This is because of the ease of collection and sampling, their global distribution, short

life-cycle, small size, high reproductivity, high adaption to various environmental conditions, and ease of culture under laboratory conditions[150,152]. The lettuce roots were firstly exposed to Ag^+, AgNPs, TiO_2NPs or to a mixture of these NPs, and then the leaves containing internalized Ag/Ti were fed to the snails. Afterwards, the growth and behavior of the snails were monitored over a period of 22 days and the metal accumulation and metal distribution in the snails was determined. The objectives of this study are to investigate 1) the trophic transfer of AgNPs and TiO_2NPs from lettuce leaves to snails, focusing on the biomagnification and biodistribution of Ag/Ti in snails; 2) the effects on snail behavior associated with trophic transfer of AgNPs and TiO_2NPs; 3) the effects of a mixture of AgNPs and TiO_2NPs on the trophic transfer and the behavior of snails. The findings of this study will help to improve the understanding of the trophic transfer of nanoparticles along a terrestrial food chain and the subsequent effects on higher-level consumers. This will provide important information about the potential risk of nanomaterials in ecosystems.

5.2.Materials and Methods

5.2.1 Nanoparticles preparation and characterization

Suspensions of spherical AgNPs (NM-300K, 100 g/L) with a nominal size of 15 nm were obtained from RAS AG (Regensburg, Germany), which is a standard reference of nanosilver for commercial and industrial application as recommended by OECD. TiO_2NPs powder of series NM-105 (mixture of anatase (80%) and rutile (20%) crystal structure, 99.5% purity), with a diameter around 25 nm were purchased from the European Commission's Joint Research Centre (Ispra, Italy). $AgNO_3$ was purchased from Sigma–Aldrich (Zwijndrecht, Netherlands). The size and shape of both AgNPs and TiO_2NPs were characterized by Transmission Electron Microscopy (TEM, JEOL 1010, JEOL Ltd., Tokyo, Japan). The hydrodynamic size and zeta potential of AgNPs and TiO_2NPs suspensions were measured after incubation in 1/4 Hoagland solution for 1 h using a zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., Royston, UK). More details of the physico-chemical properties of the AgNPs and TiO_2NPs are summarized in Reports of the European Commission's Joint Research Centre [255,296].

Suspensions of nominal 0.75 mg/L AgNPs and 200 mg/LTiO_2NPs (based on EC_{25} concentrations for lettuce[274,221]) were freshly prepared in 1/4 Hoagland solution (pH 6.0 ± 0.1; the composition of the Hoagland solution is described in **Table S2.1**) after sonication for 15 min at 60 Hz (USC200T, VWR, Amsterdam, The Netherlands). A mixture containing 0.75 mg/L AgNPs and 200 mg/L TiO_2NPs was prepared by adding a specific amount of AgNPs and TiO_2NPs into 1/4 Hoagland solution and sonicating for 15 min at 60 Hz. The exposure concentration of $AgNO_3$ (used as a reference salt for dissolved Ag ions) was 0.05 mg/L, based on the range of Ag-ion concentrations obtained upon dissolution of AgNPs at the test concentrations indicated above.

5.2.2 Plant cultivation and nanoparticles exposure

Lettuce seeds (*Lactuca sativa*) purchased from Floveg GmbH (Kall, Germany) were sterilized with NaClO (0.5% w/v) for 5 min. After immersing in deionized water for 24 h, the seeds were germinated and allowed to grow in Petri dishes containing wet filter papers (15 seeds per dish). Subsequently, the seedlings were hydroponically grown in tubes (one seedling per tube) containing 1/4 Hoagland solution for three weeks as described by Dang et al.[297] to harvest sufficient leave biomass for feeding the snails. Next, the uniformly pre-grown seedlings were selected and exposed to either Ag^+, AgNPs, TiO_2NPs, the mixture of AgNPs and TiO_2NPs, or the Hoagland solution alone (as negative control) via the roots for 28 days[286]. Each treatment had 30 seedlings/replicates. All the tubes containing a seedling and exposure medium were covered with aluminum foil in order to minimize the impact of light-induced transformations of AgNPs and TiO_2NPs. The exposure media of all tubes were renewed every two days and refilled to a volume of 22 mL on the day in between the days of refreshment of the suspensions. All experiments were performed in a climate room at a 25/20 °C day/night temperature regime with a 16 h light cycle and 60 % relative humidity[287]

After harvesting, the plants were removed from the exposure suspensions and washed with tap water for 10 mins. Afterwards, the plants were kept at 4 °C until they were used to feed the snails. A small portion of the plant tissues (roots and shoots) were immersed into 10 mM HNO_3, 10 mM EDTA for 1 hour each and finally rinsed

with Milli-Q water to remove the attached nanoparticles/metal ions[297,298]. The washed samples were oven-dried at 70 °C for 72 h and digested with *aqua regia* (HNO_3 (65%): HCl (37%) = 1:3)[299]. The total Ag/Ti contents in the plant roots/shoots of each treatment were measured by inductively coupled plasma-mass-spectrometry (ICP-MS, PerkinElmer NexION 300D). The translocation factor (TF) of Ag/Ti from roots to shoots was calculated as follows[298,300]:

$$\mathrm{TF} = \frac{[Ag/Ti]_{shoots}}{[Ag/Ti]_{roots}}$$

Where $[Ag/Ti]_{root}$ represents the concentrations of Ag/Ti in the plants root tissues (mg/kg) and $[Ag]_{shoots}$ represents the Ag/Ti concentrations in plant shoot tissues (mg/kg), respectively.

5.2.3 Snail exposure

The feeding experiments were performed based on the method reported by Ma et al. with a small modification[286]. Specifically, the Juvenile snails (*Cornu asperum*) were collected from a biologically handled garden (52°09'39.4"N 4°28'36.8"E, Leiden, Netherlands) and acclimated for 6 weeks in the laboratory, whilst feeding clean lettuce. Prior to the experiments in which NPs-contaminated lettuce leaves were fed to snails, the acclimated snails were not fed for 48h to ensure their maximum consumption of leaves. The pre-selected snails with the diameter of ~1.1 cm and weight of ~0.4 g were randomly assigned to five treatments cultured in glass bottles and fed with either unexposed leaves (control) or Ag^+, AgNPs, TiO_2NPs, AgNPs+TiO_2NPs (mixture) contaminated leaves. Each treatment had three replicates (bottles) and each replicate contained 3 snails. Immediately before feeding, the fresh leaves were cut into small pieces, weighed, thoroughly mixed and introduced to the bottles as diet (around 1 g per bottle) every two days for a period of 22 days. At each feeding interval, the unconsumed leaves in each bottle were removed and weighed to calculate the leaf consumption rate. During the 22 days of feeding period, faeces produced by snails in one bottle were collected, weighed every two days, and stored cumulatively at 4 °C in order to measure the Ag/Ti contents. After 22 d of feeding, the snails were fed with untreated (clean) leaves for 48 h to depurate the Ag/Ti from the gut before harvest.

5.2.4 Measurement of snail growth and behaviour

During the feeding period, the weight and diameter (the instruction of diameter measurement is given in **Figure S5.1**) of the snails were measured every two days at the same time during the day to monitor their growth. The mobility of snails was analysed by recording the movement of snails in a cylinder glass, and distance was tracked with video using an iPhone 7. The behavioural activity of snails was assessed using the behavioural state score (BSS) system as described previously[301] with some modification. Specifically, snails' activity was scored at 5 levels ranging from 0 to 4 (**Table S5.1**): 0 point for full retraction into its shell; 1 point for being withdrawn without head visible, 2 point for a protruding head without movement, 3 point for an extended foot and head with slight movement, 4 point for full extended with active movement. The feeding and excretion speeds of snails were determined by weighing the consumption of leaves and the production of faeces.

After sacrificing the snails, the shell was removed and snails were divided into the digestive gland (which included the digestive gland, stomach and intestine) and soft tissue (including foot, head, eyes, tail, hermaphroditic duct and mantle) according to the methods provided by University of Florida & United States Department of Agriculture (http://idtools.org/id/mollusc/dissection_snail.php). Thereafter, the dissected snails were stored at -80 °C separately for further analysis. The snail tissues and faeces were oven-dried at 70 °C for 3 days and weighed. The dried and weighed body, digestive gland and faeces were digested with HNO_3 (65%) at room temperature overnight. Subsequently, the pre-treated solutions were further digested with an appropriate volume of *aqua regia* by sonicating for 2 h in an ultrasonic bath at 60 °C and further kept in a water bath at 80 °C for 3-5h. Afterwards, the solutions were diluted and Ag/Ti contents were measured with an ICP-MS.

Trophic transfer factors (TTFs)[286], defined as the ratio of the concentration of Ag/Ti in snails body, digestive gland or faeces (mg/kg) to the concentration of Ag/Ti in lettuce leaves, were calculated with the following formula:

$$\mathrm{TTF} = \frac{[Ag/Ti]_{Snail}}{[Ag/Ti]_{shoot}}$$

5.2.5 Statistical analysis

Statistically significant differences regarding the tested endpoints among treatments at the same time point were analysed by means of one-way ANOVA followed by Duncan's honestly significant difference tests at $\alpha < 0.05$ using IBM SPSS Statistics 25. The Shapiro-Wilk test was used to check for normality and the Bartlett test for homogeneity of the variance of the data. If either of these assumptions were not met, data were log10 transformed to improve their fit. Results are expressed as mean ± standard error of 3 replicates. Besides, the results of prior-calculation of sample size by defining the critical effect size at 25% and the post-hoc calculation of power are provided in **Table S5.2**.

5.3. Results

5.3.1 Characterisation of AgNPs and TiO_2NPs

TEM micrographs showed that both AgNPs and TiO_2NPs formed agglomerates after being dispersed in water (**Figure S5.2**). Both spherical and slightly elongated shape of AgNPs with the diameter ranging from 6 to 45 nm (average 22.6 ± 0.79 nm, n=15) were observed from the TEM images. And the primary TiO_2NPs exhibited a more angular shape having a diameter ranging from 11 to 37 nm (average 21.5 ± 0.57 nm, n=15). The average hydrodynamic diameter of 0.75 mg/L AgNPs and 200 mg/L TiO_2NPs after dispersing in 1/4 Hoagland solution was 239 ± 14 nm and 978 ± 218 nm with the corresponding Zeta potential of -14.5 ± 0.75 mV and -14.4 ± 0.71, respectively. As measured by ICP-MS, the actual exposure concentration of Ag in the AgNPs treatment and the mixture treatment was 0.57 ± 0.05 mg/L and 0.55 ± 0.05 mg/L; the actual exposure concentration of Ti in the TiO_2NPs treatment and the mixture treatment was 103 ± 4 mg/L and 111 ± 8 mg/L, respectively.

5.3.2 Accumulation of Ag or Ti in plants

No significant inhibition of plant growth was observed for all treatments at the selected exposure concentrations when using biomass as the endpoint (data are provided in **Figure S5.3**). As shown in **Figure 5.1**, Ag or Ti were taken up by plant roots and subsequently translocated into plant shoots after exposure to Ag^+, AgNPs,

TiO_2NPs or the mixture for 28 days. The Ag concentration in plants of the Ag^+ treatment was much lower than the Ag concentration in plants of the AgNPs and mixture treatments (ANOVA, $p<0.005$). For example, the average Ag concentrations in plant shoots were 0.21, 1.01 and 1.08 mg/kg for Ag^+, AgNPs and mixture treatments, respectively. Interestingly, exposure to AgNPs alone resulted in a higher Ag concentration in plant roots in comparison to exposure to the mixture, while the differences of the Ag concentration between AgNPs and mixture treatments disappeared in the plant shoots. In contrast, significant differences of Ti concentration between TiO_2NPs and mixture treatments were only observed in the plant shoots (t-test, $p=0.036$) rather than in the plant roots (t-test, $p=0.667$). The average Ti concentrations in plant shoots were 6.15 and 9.07 mg/kg for TiO_2NPs and mixture treatments, respectively. Furthermore, the translocation factors of Ag and Ti in the mixture treatment were both higher than in the treatment of AgNPs or TiO_2NPs alone (**Table 5.1**, $p<0.05$).

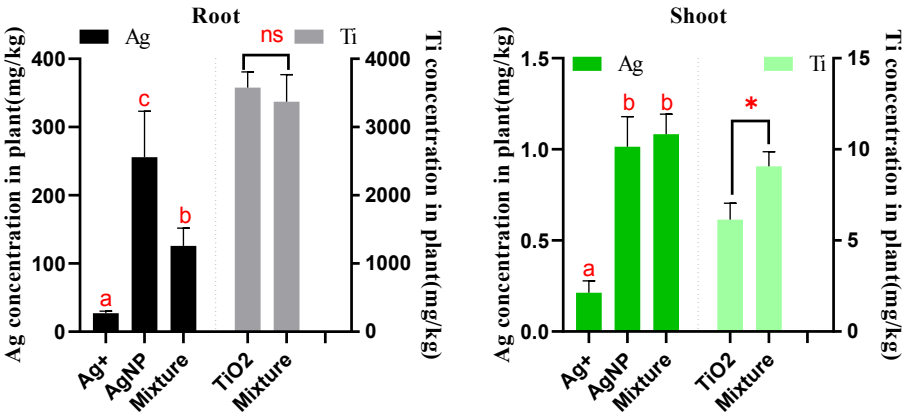

Figure 5.1. The Ag or Ti contents in lettuce root (A) and shoot (B) for different treatments after 28 d of exposure. Both Ag and Ti concentrations displayed in the figures were normalized with the concentrations of Ag/Ti in the control treatment. The different letters indicate significant differences among different treatments within the same tested metal at $p<0.05$.

5.3.3 Ag or Ti content in snails and trophic transfer

As shown in **Figure 5.2**A, either Ag or Ti was detected in the snails in the corresponding treatments. This suggests that both Ag and Ti could be transferred to snails from lettuce leaves when lettuce was exposed to either Ag^+/AgNPs or TiO_2NPs via the root. The Ag concentrations in the soft tissues of the snails in the Ag^+ treatment were higher than in the case of the AgNPs-containing treatments: AgNPs and mixture. The Ag concentration in the digestive gland and the faeces of snails consuming lettuce that were exposed to AgNPs and the mixture were much higher than the Ag-concentration in snails of the Ag^+ treatment (**Figure 5.2**A). In addition, no significant differences were observed for the Ag/Ti concentration in snails between the treatments of single NPs and the mixture regardless of the snail organs (ANOVA, $P>0.05$). This indicates that co-exposure to AgNPs and TiO_2NPs did not affect the trophic transfer of Ag or Ti compared to the trophic transfer following exposure to AgNPs or TiO_2NPs alone.

The Ag concentrations in snails followed the order of digestive gland ≈ faeces > soft tissues, regardless of the consumption of lettuce exposed to Ag^+, AgNPs, or to the mixture. More than 40% of the Ag captured by the snails remained in the digestive gland or was excreted into the faeces in all Ag-containing treatments, while the retention of Ag in snail soft tissues was only 9 – 16 % for any of the Ag-containing treatments (**Figure 5.2**B). The Ti concentration in snails organs and egestion of TiO_2NPs and mixture treatments both followed the order of digestive gland > faeces > soft tissues. More than 70% of Ti was found to be retained in the digestive gland of snails (**Figure 5.2**B).

Additionally, the TTFs of Ag/Ti from lettuce leaves to snails organs were calculated. The TTFs of Ag from lettuce leaves to snail soft tissues and the digestive gland in the Ag^+ treatment were higher than the TTFs calculated from the AgNPs exposure and as calculated from the mixture treatment (**Table 5.1**). The TTFs of Ag in snail organs of the Ag^+ treatment were well above 1, while the TTFs in snail organs of AgNPs or the mixture were below or similar to 1. This suggests that biomagnification of Ag occurred in snails of the Ag^+ treatment whilst it did not occur in the AgNPs and mixture treatments. Furthermore, the TTF of Ti from lettuce leaves to snail soft

tissues in the TiO_2NPs treatment was higher than the TTF in case of the mixture treatment. Finally, the TTFs of Ti from lettuce leaves to the digestive gland of the snails in the TiO_2NPs and the mixture treatments were higher than the TTFs from lettuce leaves to snail soft tissues. This is due to the observation that most of the Ti was accumulated in the snail digestive gland. All the TTFs of Ti from lettuce leaves to snail organs were higher than 4, regardless of the TiO_2NPs or mixture treatment. This suggests that Ti was biomagnified in snails via trophic transfer.

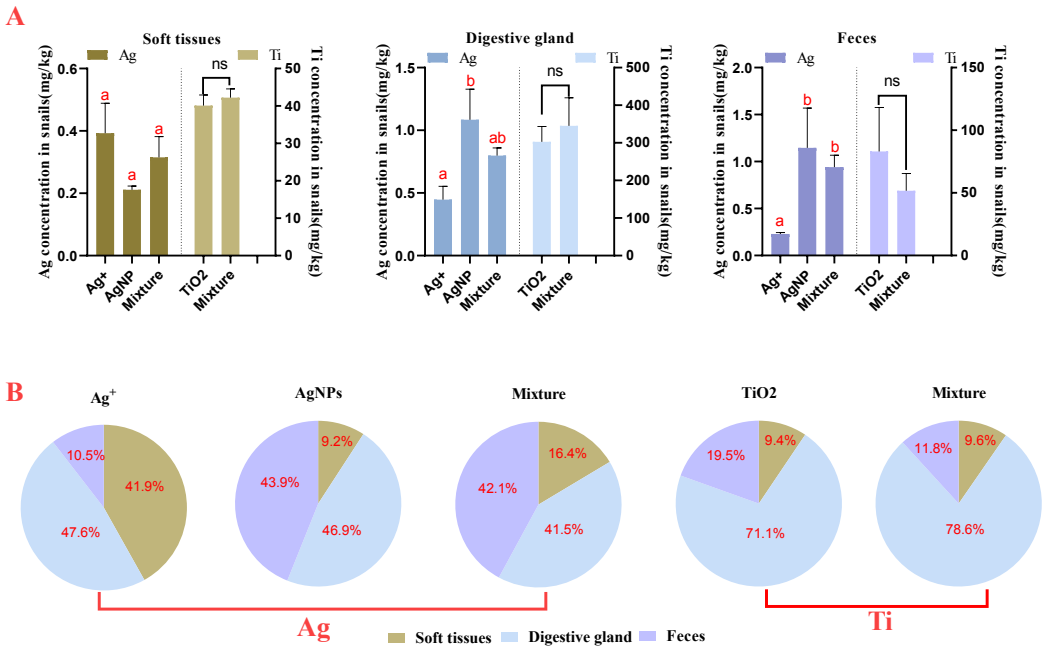

Figure 5.2. Ag and Ti concentrations (A) and distribution (B) in different organs and faeces of snails in different treatments along the food chain. Both Ag and Ti concentrations displayed in the figures were normalized with the concentrations of Ag/Ti in the control treatment. The different letters indicate significant differences of the same parameter among different treatments within the same organs at $p<0.05$.

Table 5.1. Translocation factors (TF) of Ag/Ti from lettuce roots to shoots and trophic transfer factor (TTF) of Ag/Ti from lettuce leaves to snail organs in different treatments. The different letters in the same column indicate statistically significant differences of same element between treatments at $p<0.05$.

Elements	Treatments	TFs (root to shoot)	TTFs (lettuce to snail soft tissues)	TTFs (lettuce to snail digestive gland)	TTFs (lettuce to snail faeces)
Ag	Ag+	0.008±0.001ab	1.8±0.5a	2.1±0.5a	1.1±0.07a
	AgNPs	0.004±0.001b	0.2±0.01b	1.1±0.2ab	1.1±0.15a
	Mixture	0.012±0.002a	0.2±0.05b	0.6±0.05b	0.7±0.10a
Ti	TiO2	0.002±0.0001a	5.3±0.5a	47±7a	11±6a
	Mixture	0.003±0.0004b	3.8±0.3a	37±8a	4.3±1.5a

5.3.4 Impact on snail growth

The impacts of nanoparticles on snails growth following exposure to lettuce leaves for 22 days were evaluated by monitoring the changes of their biomass or diameter (**Figure 5.3**). No snails died during the feeding and depuration period. Feeding with leaves contaminated with Ag^+, AgNPs, TiO_2NPs or the mixture did not result in a significant inhibition of snails biomass in comparison to the control (ANOVA, $p=0.173$). Even though the differences were not statistically significant, a 41.6% decrease in the biomass increase rate of snail in mixture treatment as compared to control treatment should be pointed out. This needs to be interpreted with care (low statistic power as stated in **Table S5.2**). In addition, compared to the control, significant inhibition of the snail diameter was observed for all treatments (ANOVA, $p<0.0001$), with average reductions of 56, 35, 68 and 90 % regarding the diameter increase rate of snail for the treatment with leaves exposed to Ag^+, AgNPs, TiO_2NPs and the mixture, respectively. When comparing the snails consuming leaves contaminated with the mixture to snails consuming leaves contaminated by single nanoparticles, significant differences in diameter increase rate were only observed between the treatments of lettuce with AgNPs and mixture ($p<0.005$).

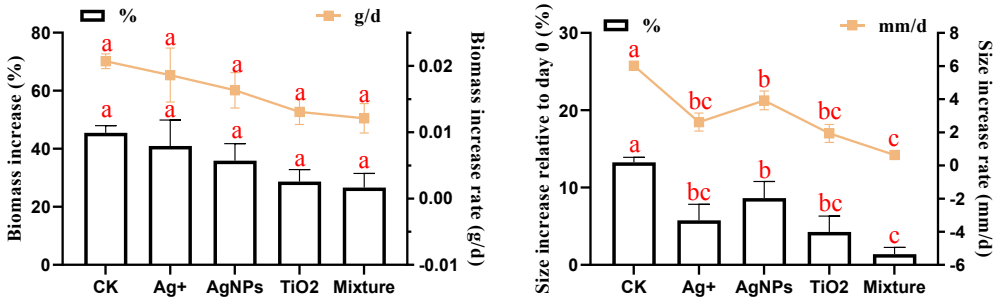

Figure 5.3. Effects of Ag^{+}, AgNPs, TiO_2NPs and mixture on snails growth through food chain transfer: (A) changes of biomass and (B) changes of diameter. CK treatment represents the snails were fed with unexposed lettuce leaves. The different letters indicate significant differences among different treatments within the same tested parameter at $p<0.05$.

5.3.5 Impact on food intake and excretion of snails

No significant differences in food intake were observed after the first two feeding periods (ANOVA, p=0.089 for 0-1 d and p=0.112 for 1-3 d, **Figure 5.4**A). After 6 d of feeding, the food intake rate of snails fed with the leaves exposed to the mixture of NPs was significantly reduced relative to control. By increasing the feeding duration to 10 and 16 d, the food intake rate of snails was significantly decreased for all treatments as compared to the control (ANOVA, p=0.007 for both feeding periods). Notably, although differences from control were observed, food intake did not differ significantly among the other treatments (Ag^{+}, AgNPs, TiO_2NPs and the mixture) regardless of the feeding periods.

Compared to control, excretion of faeces by the snails was significantly inhibited for all exposure scenarios in the first three feeding periods (ANOVA, p=0.018 for 0-1 d, p=0.006 for 1-3 d, p=0.004 for 3-6 d, **Figure 5.4**B). However, the effect on the excretion of snails in the AgNPs treatment disappeared after 10 d of feeding. In addition, a significant lower faeces excretion was observed and occurred in snails of TiO_2NPs treatments compared to AgNPs treatments after 6d of feeding. Nevertheless, no significant differences in snail excretion were observed among the treatments of Ag^{+}, AgNPs and the mixture regardless of feeding period, with the exception in the

period of 10-16 d that the excretion of snails in mixture was much lower than that of Ag^{+} and AgNPs.

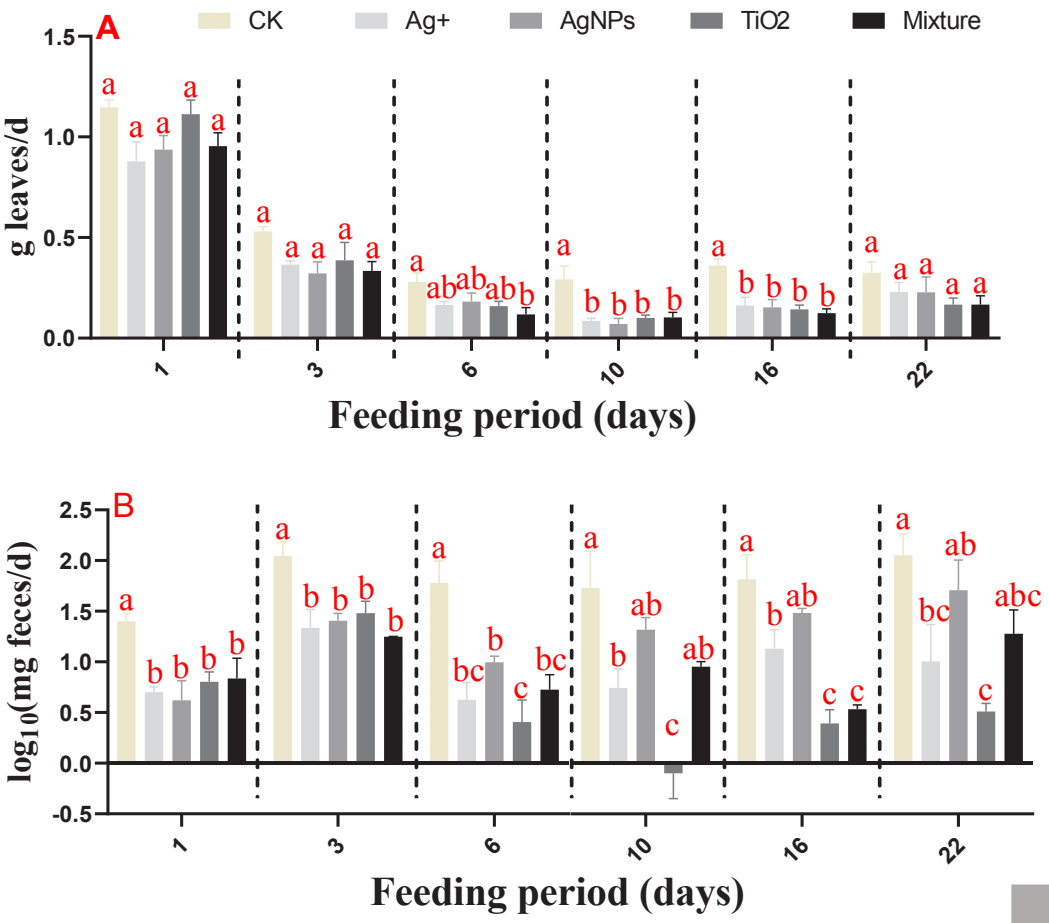

Figure 5.4. Effects of Ag^{+}, AgNPs, TiO_2NPs and mixture on snails food intake (A) and faeces excretion (B) upon trophic transfer. The different letters indicate significant differences between treatments within the same exposure period at $p<0.05$ (intragroup comparison).

5.3.6 Impact on snail activity

After 6d of feeding, significant differences of snail mobility were only detected in the mixture treatment when compared to the control group (**Figure 5.5**A). As the feeding duration was increased to 16 and 22 d, the moving speed of the snails in the TiO_2NPs and the mixture treatments was significantly decreased as compared to the control. In addition, no significant differences of snail moving speed were observed between the mixture and the single nanoparticles (AgNPs or TiO_2NPs) treatments regardless of the feeding period. Notably, the power analysis suggested that the required sample size for this endpoint ranged from 11 to 23 animals under different feeding duration when setting the critical effect size in comparison to the control at 25%. As we used 3 replicates only, our results are indicative only. Including more replicates is needed to properly uncover biological variation and to get more sturdy conclusions regarding this sublethal endpoint.

For the average behavioural state score, only the snails in the mixture treatment showed a reduction during the feeding period from 1 to 6 d. After 10 d of feeding, significant reductions of BSS were observed for the snails in all treatments except for the TiO_2NPs treatment when compared to control. This suggests that prolonged feeding of contaminated leaves induced more severe impacts on snails locomotion. Importantly, the BSS of snails in AgNPs and mixture treatments were similar after 10 d of feeding , but both lower than the BSS of snails in TiO_2NPs treatment.

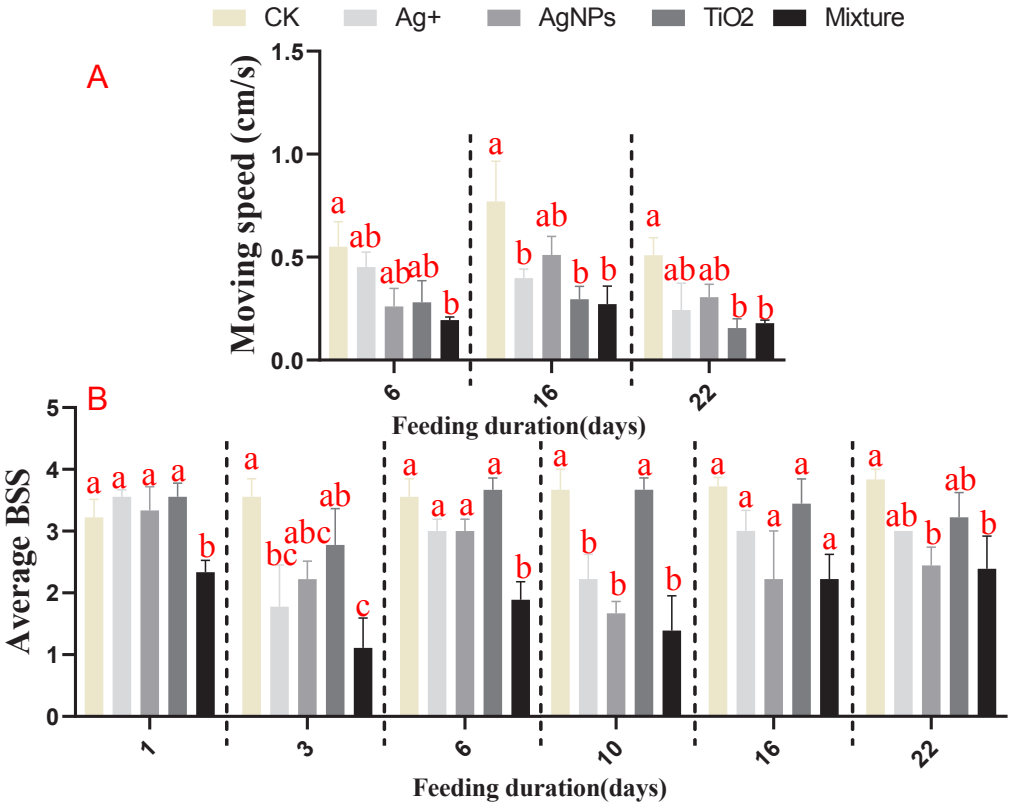

Figure 5.5. Effects of Ag^{+}, AgNPs, TiO_2NPs and mixture on snails moving speed (A) and average behavioural state score (B) upon food chain transfer. The different letters indicate significant differences between treatments within the same exposure period at $p<0.05$ (intragroup comparison).

5.4. Discussion

To our knowledge, this is the first study investigating the trophic transfer patterns of AgNPs, TiO_2NPs and their mixture from lettuce to land snails and the associated effects on various sublethal endpoints. Our results demonstrated that AgNPs, TiO_2NPs and their mixture were transferred along the food chain from the solution into the lettuce roots, to the leaves, and up into herbivorous snails, after which biodistribution occurs over different organs of the snails.

After being ingested into the gastro-intestinal tract of snails, xenobiotics will undergo extracellular and/or intracellular digestion in the digestive gland[302,303]. Subsequently,

size related translocation occurs inside snails[302,303]. Only nanoparticles that can cross the epithelium cell membranes in snails are able to be further transported into the foot, mantle and possibly even the brain and shell of the snails, while the larger nanoparticles will remain in the digestive gland or pass into the intestine for excretion[302,303]. This is why ionic Ag is more readily assimilated and translocated into other organs of snails than the particulate form. This hypothesis was supported by our findings that (1) more than 40 % of Ag was distributed in the soft tissues of snails consuming lettuce exposed to Ag^+, but less than 10 % of Ag or Ti was distributed in soft tissues of snails consuming lettuce exposed to AgNPs or TiO_2NPs; (2) the biomagnification of Ag occurred in the soft tissues and the digestive gland of snails of the Ag^+ treatment (TTFs >1), but no biomagnification was observed in snails organs of the AgNPs treatments (TTFs<1). In addition, as food ingestion was the only pathway for snails to take up Ag^+, AgNPs or TiO_2NPs in the current study, it was not surprising that a large fraction of Ag or Ti was detected in the digestive gland of the snails regardless of ionic or nanoparticles treatments after 2d of depuration. This finding is in agreement with previous studies in which the digestive system was the main site of accumulation of Ce in snails and chickens that were fed CeO_2 nanoparticle exposed plant leaves[286,287].

Importantly, more than 40% of the Ag that was captured by snails consuming the AgNPs-treated lettuce was excreted through their faeces. The same level of Ag excretion was found for snails in the mixture treatment. This results in a high excretion efficiency of Ag and low estimated values of the TTFs of Ag (below 1) in snails of the AgNPs-containing treatments. A conflicting result was reported by the group of Dang et al., who reported the biomagnification of AgNPs from lettuce to snails with TTFs of 2.0-5.9[304]. This discrepancy could be a reflection of differences in experimental conditions and the species, the growth stage and the life history traits of the snails involved[289,299,305]. On the contrary, only a small fraction of Ti (less than 10%) was excreted into the faeces of the snails in the TiO_2NPs or mixture treatments, and more than 70% of Ti was retained in the digestive gland. Additionally, biomagnification of Ti was observed in snails of TiO_2NPs containing treatments as the TTFs of Ti from lettuce to the digestive gland and soft tissue of snails were 38-49 and 4.7-6.5, respectively. The low excretion efficiency and the high estimated TTFs

of Ti in snails suggest that Ti exhibits a higher trophic availability to snails upon consumption of TiO_2NPs internalized lettuce leaves. Furthermore, the TTFs and biodistribution patterns of Ag or Ti in snails were similar between the single nanoparticle treatment and the mixture treatment. This indicates that the concurrent application of AgNPs and TiO_2NPs did not affect the trophic transfer and distribution pattern of Ag or Ti in snails when AgNPs or TiO_2NPs were applied singly.

We also observed that ingestion of leaves contaminated with AgNPs, TiO_2NPs, or their mixture induced adverse effects for the growth and activity (expressed as the average BSS) of snails. After ingestion of either Ag or Ti-containing leaves for 22 d, statistically significant inhibition of snails growth was only observed when using the diameter of the snails rather than the snails biomass as endpoint of assessment. Although not statistically significant, a reduction of 42 % of the biomass increase rate of the snails in the mixture treatment was observed in comparison with the snails in control. The combination of enhanced or reduced mucus secretion, food intake and faeces production could cause high variability in the weight of individual snail[306]. Similarly, up to 50% differences in moving speed of snails between AgNPs treatments and control were detected without statistical significance. We acknowledge that the small sample size of this study could be the reason for the absence of significant effects in terms of the endpoints of biomass and moving speed of snails, thus resulting in low statistical power. The high variability of the tested endpoints requires more replicates (e.g. 11-23 replicates for the endpoint of moving speed) to obtain effective data, thus biomass and moving speed of snails might not be practical indicators for assessing the growth and activity of *Cornu asperum*.

Despite the similar responses of snails to exposure to AgNPs and TiO_2NPs regarding the food intake, treatment of snails with TiO_2NPs contaminated leaves strongly affected their faeces excretion whereas AgNPs strongly affected the activity (expressed as the average BSS) of the snails. This indicates that the behavioural responses of snails to AgNPs and TiO_2NPs are different. The observed strong inhibition in faeces excretion for snails in the TiO_2NPs treatments can be attributed to the high retention of Ti observed in the digestive gland, which may disrupt the functioning of the digestive gland and thus reduce the metabolic activity of snails.

Data on trophic transfer effects of metallic nanoparticles on land snails are scarce, but several studies reported the ingestion of nanoplastics/microplastics, which are also to be considered as insoluble nanoparticles, by land snails[306,307]. These authors demonstrated that ingestion of nanoplastics/microplastics induced damage to the digestive organs of snails such as the digestive gland, intestine or stomach, and thus inhibited the growth and excretion of faeces by the snail *Achatina fulica*[306,307]. In contrast, the BSS of snails in AgNPs treatments was significantly inhibited. Such reduction observed in AgNPs treatments is similar to previous results which show that locomotive activities of springtails (*Lobella sokamensis)* were suppressed when fed with AgNPs exposed earthworms (*Eisenia andrei*)[308]. The energy reallocation or preservation in response to the stressors has been presumed as one explanation for the alterations of locomotion activity in animals[309,310]. Besides the costs of energy in respiration and growth, the snails in the AgNPs treatments may require higher energy for AgNPs excretion as a large fraction of Ag uptake by snails was excreted through their faeces[307], thus resulting in a reduction of the energy available for their locomotive activity. Alternatively, impairment of sensory and nervous system functions in organisms is also widely suggested to explain the alterations of locomotion activity[305,308,310].

Furthermore, the adverse effects were more severe in the snails of the mixture treatments compared to the effects caused by single AgNPs or TiO_2NPs in terms of growth and activity of snails, indicating additive/synergistic effects of AgNPs and TiO_2NPs. So far, knowledge on the mixture toxicity of AgNPs and TiO_2NPs is very limited for land gastropods, which makes the comparison of our results to other published studies difficult. There are two possible explanations for the enhanced toxicity after exposure to a mixture of nanoparticles: one explanation is related to the elevation of the cellular uptake of NPs. First, the presence of TiO_2NPs may change the bioavailability and uptake of Ag by affecting the dissolution and aggregation of the soluble Ag nanoparticles[103,311]. Secondly, TiO_2NPs can work as a carrier to facilitate the uptake of the co-existing nanoparticles[312,313] after the formation of TiO_2-AgNPs complexes, thus affecting the biological effects of co-existing AgNPs. Our results did not support this explanation as the Ag and Ti concentrations in snails were similar between the treatments of single nanoparticle and the mixture. Another

reason for the enhanced toxicity induced by the mixture is the possibility that the presence of TiO_2NPs and AgNPs induced higher oxidative stress, thus leading to more severe adverse effects[311,314]. Last but not least, although the patterns of behavioural changes of snails among different treatments over time are irregular, more severe adverse effects in terms of food intake and locomotion of snails were found at prolonged feeding durations. The observations call for research investigating the long-term effects of the mixture of nanoparticles in consumers through food chain transfer.

5.5. Environmental Implications

This study provided the first report about the trophic transfer and tissue-specific distribution of AgNPs, TiO_2NPs and their mixture along the lettuce-snail food chain, and the associated impacts on the growth and behaviours of snails. Given the increasing likelihood of application of nanoparticles in agriculture and soil remediation, the findings of this study emphasize the importance of considering trophic transfer as a potential pathway for exposure of terrestrial herbivorous to nanoparticles. The concurrent application of AgNPs and TiO_2NPs along the food chain induces additive/synergistic effects on the growth and activity of snails. Nevertheless, understanding the mechanistic underlying such effects remains challenging. More attention should therefore be paid to investigating the combined effects of NPs along the terrestrial food chain. Furthermore, prolonged feeding of contaminated leaves to snails enhanced the adverse effects. This finding highlights the importance of taking long-term application of nanoparticles into account in order to better understand the ecological risks of nanoparticles in terrestrial ecosystems.

5.6 Supplementary Information

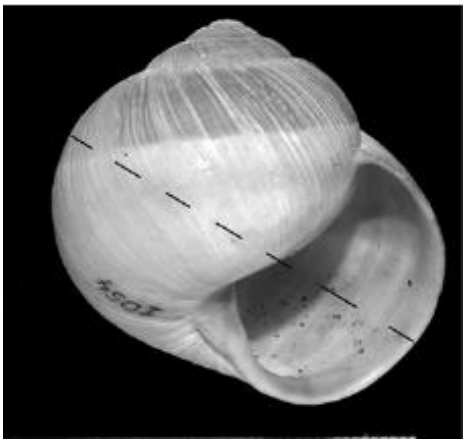

Figure S5.1. The broken line across the shell denotes the diameter measured in the present study[1].

1. Aydın Örstan. A method to measure snail shell. Triton, No 23 April 2011.

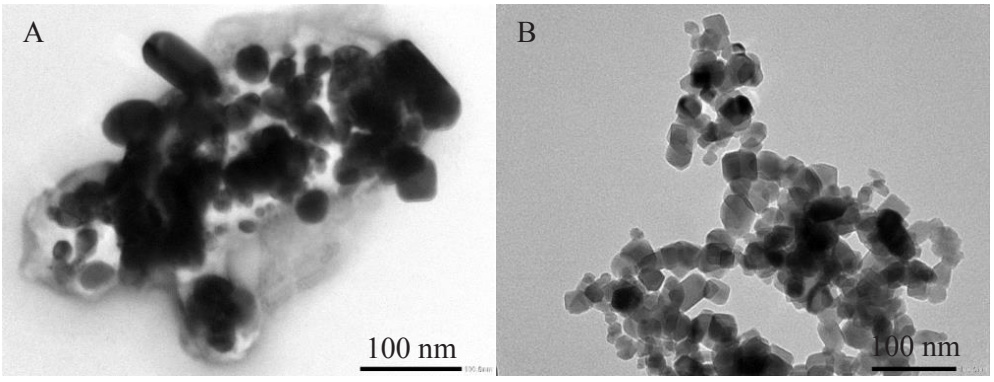

Figure S5.2. TEM picture of AgNPs (A) and TiO_2NPs (B)

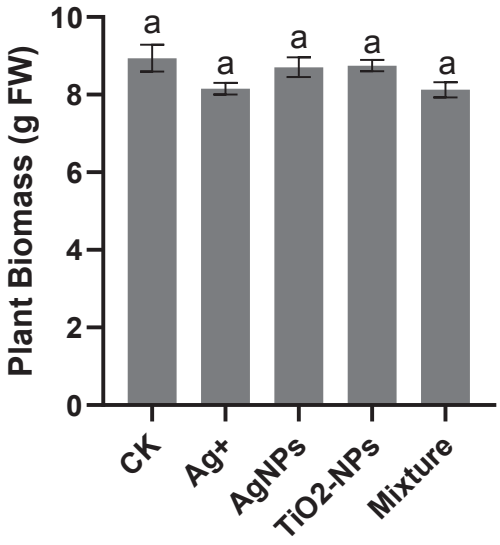

Figure S5.3. Fresh biomass of lettuce exposed to different treatments after 28 days of exposure. Data are the mean ± SE (N= 25). Different letters in the same group indicate statistically significant differences between treatments at $p < 0.05$.

Table S5.1. Summary of the Behavioral State Score (BSS) criteria.

Behavioral state score	Activity description	Picture indication
0	Body is retracted into its shell and operculum is closed	
1	snail withdrawal from the shell, no head visible	
2	protruding head without locomotion	
3	Snail exposes both head and foot but with a slight locomotion	
4	active locomotion of snail	

Table S5.2. Prior-calculation of sample size by defining the critical effect size at 25% and the post-hoc calculation of power

	Endpoints	Feeding duration (day)					
		1	3	6	10	16	22
Prior sample size calculation for each endpoint at power =0.8	Biomass increase (%)						4
	Size increase (%)						3
	Food intake (g leaves/d)	3	3	17	4	5	8
	Feces production (mg feces/d)	5	3	4	4	4	4
	Moving speed (cm/s)			20		11	23
	Behavioral state score	4	4	4	4	3	3
Post-hoc power calculation for each endpoint at n=3	Biomass increase (%)						0.64
	Size increase (%)						0.998
	Food intake (g leaves/d)	0.96	0.85	0.16	0.61	0.55	0.33
	Feces production (mg feces/d)	0.58	0.87	0.77	0.75	0.79	0.66
	Moving speed (cm/s)			0.14		0.23	0.13
	Behavioral state score	0.69	0.77	0.77	0.79	0.81	0.83