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Article

Toxicokinetics and Particle Number-Based Trophic Transfer of a Metallic Nanoparticle Mixture in a Terrestrial Food Chain

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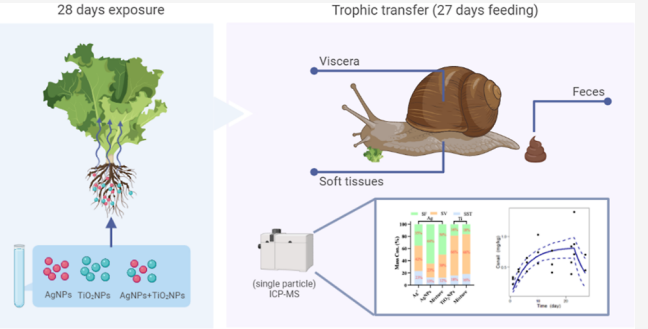
ABSTRACT: Herein, we investigated to which extent metallic nanoparticles (MNPs) affect the trophic transfer of other coexisting MNPs from lettuce to terrestrial snails and the associated tissue-specific distribution using toxicokinetic (TK) modeling and single-particle inductively coupled plasma mass spectrometry. During a period of 22 days, snails were fed with lettuce leaves that were root exposed to $AgNO_3$ (0.05 mg/L), AgNPs (0.75 mg/L), TiO_2NPs (200 mg/L), and a mixture of AgNPs and TiO_2NPs (equivalent doses as for single NPs). The uptake rate constants (k_u) were 0.08 and 0.11 kg leaves/kg snail/d for Ag and 1.63 and 1.79 kg leaves/kg snail/d for Ti in snails fed with NPs single- and mixture-exposed lettuce, respectively. The elimination rate constants (k_e) of Ag in snails exposed to single AgNPs and mixed AgNPs were comparable to the corresponding k_u, while the k_e for Ti were lower than the corresponding k_u. As a result, single TiO_2NP treatments as well as exposure to mixtures containing TiO_2NPs induced significant biomagnification from lettuce to snails with kinetic trophic transfer factors (TTF_k) of 7.99 and 6.46. The TTF_k of Ag in the single AgNPs treatment (1.15 kg leaves/kg snail) was significantly greater than the TTF_k in the mixture treatment (0.85 kg leaves/kg snail), while the fraction of Ag remaining in the body of snails after AgNPs exposure (36%) was lower than the Ag fraction remaining after mixture exposure (50%). These results indicated that the presence of TiO_2NPs inhibited the trophic transfer of AgNPs from lettuce to snails but enhanced the retention of AgNPs in snails. Biomagnification of AgNPs from lettuce to snails was observed in an AgNPs single treatment using AgNPs number as the dose metric, which was reflected by the particle number-based TTFs of AgNPs in snails (1.67, i.e., higher than 1). The size distribution of AgNPs was shifted across the lettuce-snail food chain. By making use of particle-specific measurements and fitting TK processes, this research provides important implications for potential risks associated with the trophic transfer of MNP mixtures.



KEYWORDS: *food chain, terrestrial environment, biodistribution, toxicokinetics, mixture exposure*

INTRODUCTION

Metallic nanoparticles (MNPs), including silver nanoparticles (AgNPs) and titanium dioxide nanoparticles (TiO_2NPs), are used in a broad spectrum of consumer, industrial, and agricultural sectors.[1,2] The extensive and increased application of products containing MNPs has raised global concerns regarding the risk of their release into the environment, especially in soil.[3] As reported, the concentration of the nano-fraction of AgNPs and TiO_2NPs in sludge-treated soil is expected to reach 2.3 μg/kg and 61 mg/kg, respectively.[4] Over the past few decades, the bioaccumulation of MNPs in plants has also been widely confirmed,[2,5,6] and it is verified that bioaccumulation might make dietary uptake and trophic transfer an important uptake route for high trophic level predators, including human beings.[7]

Information about the trophic transfer of MNPs along terrestrial food chains is currently limited. The few published studies addressing the trophic transfer of MNPs along the terrestrial food chain have mainly focused on the influence of physicochemical properties of single MNPs, for instance, the size of MNPs.[7−9] In addition, other factors, including the coexistence of pollutants (such as MNPs, metals, and organic pollutants), can play a major role in affecting their fate in exposure media, bioavailability, and trophic transfer from producers to predators.[10] Haghighat et al.[11] demonstrated that the co-exposure to AgNPs and TiO_2NPs enhanced the bioaccumulation of Ag in the liver and intestine of common carp (*Cyprinus carpio*). Tong et al.[12,13] reported that TiO_2NPs adsorbed dissolved the Zn released from ZnONPs and hence reduced the bacterial toxicity of ZnONPs. Wilke et al.[14] found that the coexistence of TiO_2NPs and AgNPs formed a new

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composite Ag/TiO_2 nanomaterial which exerted a synergistic effect on *Escherichia coli*. Previous studies of exposure to multiple MNPs are mostly limited to bacteria and aquatic organisms.[15,16] Less attention has been paid to the bioaccumulation and impacts of mixtures of MNPs on terrestrial organisms and the potential of MNPs to traffic through different trophic levels along a terrestrial food chain. As exposure scenarios of multiple MNPs are relevant to agricultural ecosystems, which are the basis for our human food basket, there is an urgent need to investigate the interactions between multiple MNPs at all levels of the terrestrial food chain for food safety and human health.

In a recent study, we evidenced the trophic transfer of AgNPs and TiO_2NPs from lettuce (*Lactuca sativa* L.) to terrestrial snails (*Cornu aspersum*). We found that the biomagnification and biodistribution of Ag and Ti in snails did not differ between the treatment of the mixture of AgNPs and TiO_2NPs and the treatments of individual NPs.[17] However, in our case and in previous trophic transfer studies, the accumulated concentration of MNPs in consumers is generally quantified as total metal concentrations, including both the nanoparticulate and ionic forms, at a fixed exposure time.[9,18,19] Uptake and translocation of MNPs in organisms, including plants and animals, could induce complex transformations of MNPs, e.g., dissolution and aggregation, during the internalization processes.[20,21] These transformations may not affect the total metal concentration of MNPs in consumers upon trophic transfer but may potentially alter the size distribution and particle number of MNPs in organisms of different trophic levels. Additionally, interactions between coexisting multiple MNPs potentially influence their respective transformation processes in organisms, which have not been explored so far. More importantly, the dynamic nature of the transformation process of MNPs likely makes the trophic transfer and biomagnification of MNPs in consumers time-dependent.[22] While the importance of time on the fate of MNPs in the environment and their bioavailability to certain organisms has been well documented,[23,24] studies of the trophic transfer of MNPs along multiple-level food chains ignore this time-dependent effect.[7] Altogether, evaluating the biomagnification of MNPs in consumers following trophic transfer based on the total mass concentration of metals at a single duration of exposure may not reflect the realistic environmental risks. Nevertheless, experimentally monitoring the biodynamics of MNPs and directly identifying the biodistribution and size distribution of intact MNPs in consumers following trophic transfer are challenging, which is now possible with new analytical techniques such as single-particle inductively coupled plasma mass spectrometry (spICP−MS).

Toxicokinetic (TK) models can capture the time course of absorption, distribution, and elimination (including biotransformation and excretion) of toxicants in an organism. This allows us to make predictions under non-tested or dynamic exposure scenarios.[25,26] Considering that the aim of this study is to compare the difference of the trophic transfer and the associated TKs between single MNPs and the MNP mixture along a terrestrial food chain, we therefore conduct the experiments as a function of exposure time instead of exposure concentration. This makes us use TK models to assess the dynamic bioaccumulation of single MNPs as well as a mixture of MNPs in snails following trophic transfer from lettuce. We do so by deriving their respective uptake and elimination rate constants from experimental data. A reference experiment with $AgNO_3$ was performed to cover the impact of ionic Ag. The dissolution and agglomeration kinetics of AgNPs and TiO_2NPs alone and in combination were determined to investigate how interactions between AgNPs and TiO_2NPs alter their fate and bioavailability. Additionally, extraction methods of AgNPs/TiO_2NPs from lettuce and snails were developed and integrated with spICP−MS to monitor the size distribution and particle number of MNPs in organisms across the lettuce-snail food chain. Finally, the trophic transfer and biomagnification of MNPs along the lettuce-snail food chain were determined by comparing the particle number-based and metal-mass-based trophic transfer factors.

MATERIALS AND METHODS

Preparation and Characterization of MNPs. A suspension of AgNPs [NM-300K, 100 g of Ag/L stabilized in 4% (w/w) polyoxyethylene glycerol trioleate, 4% ammonium nitrate, and 4% (w/w) polyoxyethylene sorbitan mono-laurate] with a nominal primary diameter of 15 nm was obtained from RAS AG (agpureW10, Regensburg, Germany). TiO_2NPs powder (anatase/rutile = 80:20) with a nominal primary particle size of 25 nm was purchased from the European Commission's Joint Research Centre (Ispra, Italy). $AgNO_3$ was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). The morphology and particle size of AgNPs and TiO_2NPs were characterized using transmission electron microscopy (TEM, JEOL 1010, JEOL Ltd., Tokyo, Japan). The hydrodynamic diameters and zeta potential of AgNPs and TiO_2NPs alone or in combination in 1/4 Hoagland solution (pH 6.0 ± 0.1) were determined after 1, 5, and 24 h on a Zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., Royston, U.K.). The composition of the Hoagland solution is provided in Table S1 in the Supporting Information.

Dissolution and Sedimentation Experiments of MNPs. To compare the dissolution and sedimentation kinetics, suspensions of AgNPs, TiO_2NPs, and the mixture of AgNPs and TiO_2NPs were prepared in 1/4 Hoagland solution (pH 6.0 ± 0.1) at a nominal concentration of 0.75 mg/L for AgNPs and 200 mg/L for TiO_2NPs by sonication in a water bath for 15 min at 60 Hz. Aliquots from each suspension were taken in duplicate from the top 8 cm of the tubes at each time point (0, 2, 6, 24, and 48 h). For each suspension, one of the aliquots was centrifuged for 30 min at 30,392*g* at 4 °C (Sorvall RC5Bplus centrifuge, Bleiswijk, The Netherlands). The supernatant was subsequently filtered via a syringe filter of 0.02 μm pore diameter (Anotop 25, Whatman, Eindhoven, The Netherlands). Several publications have confirmed that the particles in suspensions of NPs can be removed after centrifugation at around 10,000*g*.[27,28] The above steps yielded supernatants with negligible amounts of particles,[29] in which the concentration of Ag/Ti represented the dissolved fraction of the MNPs. Changes in Ag/Ti concentration in the supernatants over time allow us to examine the dissolution kinetics of MNPs alone or in combination.

The other aliquot was digested by aqua regia to determine the total concentration of Ag/Ti. For TiO_2NPs-containing suspensions, the aliquot was diluted using Milli-Q water to obtain a final concentration of around 1 mg/L prior to the addition of aqua regia. As we measured the total concentration of Ag/Ti and the dissolved ionic concentration in the aliquots at 0, 2, 6, 24, and 48 h, respectively, the particulate fraction of MNPs at 0, 2, 6, 24, and 48 h can be obtained by calculating

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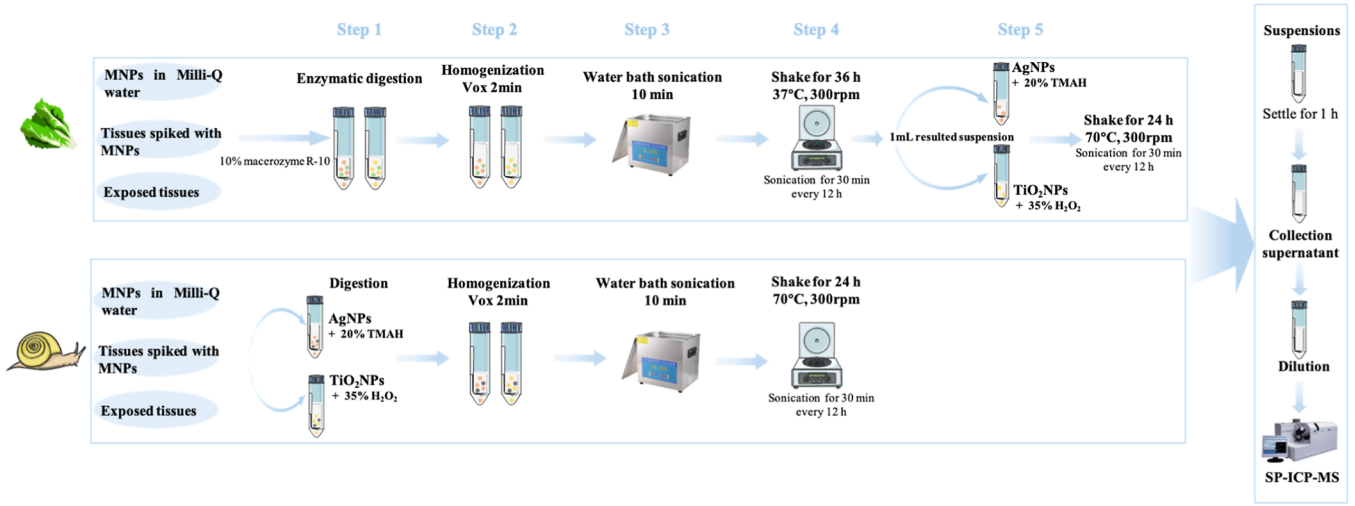

Figure 1. Schematic diagram of the stepwise procedure to separate/extract MNPs employed in this study.

the difference between the total concentration of Ag/Ti and the concentration of dissolved Ag/Ti at each time point. Afterward, the sedimentation kinetics of MNPs over 48 h can be derived on the basis of the time dependence of the residual particulate fraction of MNPs in water from the top 8 cm of the tubes. The assessments of the dissolution and sedimentation of MNPs at each time point were performed in triplicate. The concentration of Ag/Ti was determined by ICP−MS (PerkinElmer, NexION 2000).

Lettuce Cultivation and MNPs Exposure. The germination and cultivation of lettuce seedlings (*L. sativa*, Floveg GmbH, Kall, Germany) were described in our previous study.[17] Three week old seedlings were root exposed to either the Hoagland solution (non-exposed control), Ag^+ (0.05 mg/L), AgNPs (at a nominal concentration of 0.75 mg/L), TiO_2NPs (at a nominal concentration of 200 mg/L), or a mixture containing 0.75 mg/L AgNPs and 200 mg/L TiO_2NPs prepared in Hoagland solution. The exposure concentrations of MNPs were chosen based on the no-observed-effect concentration for lettuce growth. These concentrations are likely to be higher than the concentrations typically present in the pore water of sludge/biosolids-amended soil.[4,30,31] The seedlings were grown in tubes with a height of 15 cm, containing 22 mL of the exposure suspensions or Hoagland solution (one seedling per tube). Each treatment had 30 seedings/replicates to collect sufficient mass of leaf for feeding the snails. All the tubes were covered with aluminum foil in order to minimize the impact of light-induced transformations of AgNPs and TiO_2NPs (Figure S1). Each suspension was refreshed every 2 days and refilled to a volume of 22 mL on the day in between the days of refreshment of the suspensions. Lettuce growth and MNPs exposure were performed in a climate room with a light/dark cycle of 16/8 h and 60% relative humidity at a 25/20 °C day/night temperature regime. After exposure to each suspension for 28 days, the plants were harvested. The plants (including controls) were washed with tap water, HNO_3 (10 mM), ethylenediaminetetraacetic acid (EDTA) (10 mM), and Milli-Q water to remove the attached metal ions/MNPs.[19,32] Afterward, the plants were kept at 4 °C until they were used to feed the snails.

Snail Feeding Experiment. Juvenile snails (*Cepaea hortensis*) were collected from a garden (52°09′39.4″N 4°28′36.8″E, Leiden, The Netherlands; no known use of artificial fertilizers or pesticides) and acclimated in the laboratory under a 25/20 °C day/night temperature regime and 70% relative humidity for 6 weeks. Snails were fed unexposed lettuce *ad libitum* during these 6 weeks. The acclimated snails were not fed for 48 h prior to the start of the experiment to ensure their maximum consumption upon feeding contaminated lettuce leaves. The pre-selected snails (fresh weight of 83 ± 4 mg and diameter of 0.71 ± 0.02 cm) were randomly assigned to five groups of control (CK, fed with unexposed lettuce leaves), Ag^+ (fed with lettuce leaves collected from root exposure to Ag^+), AgNPs (fed with lettuce leaves collected from root exposure to AgNPs), TiO_2NPs (fed with lettuce leaves collected from root exposure to TiO_2NPs), and AgNPs + TiO_2NPs (mixture: snails fed with lettuce leaves collected from root exposure to the mixture of AgNPs and TiO_2NPs). The fresh leaves were cut into small pieces, mixed thoroughly, weighed, and introduced into the bottles as diet every 2 days (~1 g per bottle per day, per bottle containing 10 snails). During the exposure period (the first 22 days), the unconsumed leaves were collected from each bottle and weighed before the next feeding of new leaves to calculate the total leaf consumption. Similarly, feces produced by snails in one bottle were collected before the next feeding of new leaves, weighed, and stored cumulatively for each sampling time at 4 °C.

After feeding contaminated leaves for 22 days, the snails were continuously fed with clean leaves over a period of 5 days and sampled at days 23, 25, and 27, respectively, to assess the depuration of Ag/Ti from the snails. Three snails from each treatment were randomly collected in three different bottles at 1, 3, 6, 10, 15 and 22, 23, 25, and 27 days after the start of the exposure. The collected snails were transferred into clean bottles to fast for 24 h (the associated feces were not collected) and then sacrificed by freezing using liquid nitrogen.

Determination of the Total Metal Concentration. After washing with the 10 mM solutions of HNO_3 and EDTA and with Milli-Q water, a small fraction of the plants from each treatment was separated into root and shoot, and the root tissues and the leaf tissues were divided into two groups. One group was oven-dried at 70 °C for at least 72 h to a constant weight, weighed, and digested to determine the total concentration of metals. The other group was frozen using liquid nitrogen and stored at −80 °C for further analysis of the

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size distribution of the MNPs and the particle number concentration. The snails collected at each sampling time were frozen using liquid nitrogen, and the shell was removed. The whole body was oven-dried, weighed, and digested for the analysis of total metal concentrations.

For the snails collected at day 23, each treatment was run with six replicates to get enough mass for TK and tissue-specific analysis. After removal of the shell, the snail was dissected into the viscera (SV, including the intestine, digestive gland, and stomach) and soft tissues (SST, including the foot, head, eyes, tail hermaphroditic duct, and mental). Similar to the plant samples, the SV and SST were divided into two groups: one for the total metal concentration analysis and another for the analysis of particle number concentration and MNP size distribution. Thereafter, the dried and weighed root tissues and leaf tissue were first digested with HNO_3 (65%) at 120 °C for 4−6 h for plant samples. The dried and weighed snail viscera, snail soft tissues, and snail feces were predigested with HNO_3 (65%) at room temperature overnight. Subsequently, aqua regia was used to fully digest the MNPs in the pre-treated suspensions by sonicating in an ultrasonic bath for 2 h at 60 °C and further kept in a water bath at 80 °C for 3−5 h. Finally, the Ag/Ti concentration in the solutions was determined with an ICP−MS after dilution. Spike experiments conducted with known concentrations of TiO_2NPs (1−2 mg/L) following the acid-digestion-based ICP−MS analysis showed 87−107% recovery, respectively. The spike recovery of Ag for the digestion procedure was 98 ± 6%. The detection limits of ICP−MS for ^{107}Ag and ^{47}Ti were 0.006 and 0.031 μg/L (3 × standard deviation of the blanks), respectively.

Quantification of MNPs Size Distribution and Particle Number Concentration by spICP−MS. *Extraction Procedures.* The extraction scheme of the MNPs from plant and snail samples is provided in Figure 1. First, the frozen plant and snail samples (collected at day 25) were ground into powder using TissueLyser II (QIAGEN, Mainz, Germany). For plant samples, the powdered plant root or shoot tissues (0.1 g) were homogenized in 4 mL of 10% macerozyme R-10 prepared in citrate buffer (2 mM, pH 5.8) for 2 min and sonicated for 10 min. Then the tubes were shaken at 300 rpm at 37 °C for 36 h.[33] The tubes were taken out from the shaker every 12 h and sonicated for 30 min to speed up the breaking down of the tissue and to prevent particle aggregation. Next, 3 mL of 20% tetramethylammonium hydroxide (TMAH, for AgNPs containing plant samples) or 35% H_2O_2 (for TiO_2NPs containing plant samples) was added into 1 mL of the macerozyme R-10-treated suspensions. Subsequently, the resulting samples were shaken at 400 rpm and 70 °C for 24 h to further extract the MNPs.

For snail samples, the powdered snail viscera or snail soft tissues (0.02 g) were directly digested with 20% TMAH (for AgNPs-containing treatments) or 35% H_2O_2 (for TiO_2NPs treatments) without an enzymatic digestion procedure. After homogenization with 20% TMAH or 35% H_2O_2, the suspensions of snail samples were shaken at 400 rpm and 70 °C for 24 h, accompanied by 30 min of sonication every 12 h. Finally, the suspensions were allowed to settle for 1 h, and the obtained supernatants were diluted with ultrapure water for spICP−MS analysis. To assess the influence of the extractants on the stability of the MNPs, the AgNPs, or TiO_2NPs dispersed in Milli-Q water were extracted following the same procedures as used for plants and snails. To assess the MNPs recovery of the extraction methods, spike recovery experiments were conducted by adding MNPs suspensions into plant samples (0.1 g) or snail samples (0.02 g), and then the mixture was immediately extracted with the same extraction procedure as used for plant samples or snail samples. The particle number recovery was calculated by dividing the particle number concentrations of AgNPs/TiO_2NPs in the digested samples to the particle number concentrations of equivalent AgNPs/TiO_2NPs dispersed in Milli-Q water. The particle number recoveries of AgNPs in digestion blanks and matrix spikes (plant or snail) were 66−108%. However, the extraction method employed in this study yielded a poor recovery for TiO_2NPs (25−43%). The particle size detection limits for AgNPs and TiO_2NPs were 11 and 44 nm (3 × standard deviation of the blanks), respectively.

spICP−MS Measurement. The size distribution and particle number concentration of MNPs in the extractions of plant samples or snail samples and in the exposure medium were determined using single particle ICP−MS (PerkinElmer, NexION 2000 ICP−MS operating in a single particle mode). The settings of the instrument are presented in Table S2. The transport efficiency was determined using AuNPs dispersions by diluting stock standards of AuNPs with known particle size and number concentrations under the same instrument conditions. Calibration curves were prepared using five concentration levels by diluting the corresponding metal standard stocks in Milli-Q water. For the measurements of the samples, the prepared extracts were diluted 100−10,000-fold using Milli-Q water to obtain a final number concentration in the range of 5000−500,000 particles/mL. This allows us to minimize the matrix effects on the determination.

Data Analysis. A classical first-order association model was used to assess the dynamic dissolution process of the MNPs alone and in combination

$$[\text{metal}^+]_t = (P_1 - [\text{metal}^+]_0) \times \exp^{-k_d t} + P_2 \quad (1)$$

Also, the sedimentation process of MNPs alone or in combination was examined using a classical first-order decay model as follows

$$[\text{MNP}]_t = ([\text{MNP}]_0 - P_2) \times \exp^{-k_s t} + P_1 \quad (2)$$

where $[\text{metal}^+]_t$ and $[\text{MNP}]_t$ are the concentrations of dissolved Ag/Ti and particulate Ag/TiO_2 at a given time point with the unit of mg/L; P_1 and P_2 are the concentrations of particulate Ag/Ti and dissolved Ag/Ti at equilibrium; and k_d and k_S are the first-order rate constants of dissolution and sedimentation (d^{-1}) of MNPs, respectively. $[\text{Metal}^+]_t$, $[\text{MNP}]_t$, $[\text{metal}^+]_0$, and $[\text{MNP}]_0$ were measured experimentally. Afterward, the dissolution rate constants and sedimentation rate constants of MNPs can be calculated by fitting the corresponding data to eqs 1 and 2, respectively.

The experimental data were fitted to a one-compartment model to describe the TKs of the MNPs in snails following trophic transfer. The uptake rate constant (k_u, kg leaves/kg snail/day), depuration rate constant (k_e, 1/d), and the kinetic trophic transfer factor (TTF_k, kg leaves/kg snail) were estimated according to the following equations

$$\frac{dC_{\text{snail}}(t)}{dt} = k_u C_{\text{leaves}}(t) - (k_e + k_g) C_{\text{snail}}(t) \quad (3)$$

Integration of this equation yields the following equations

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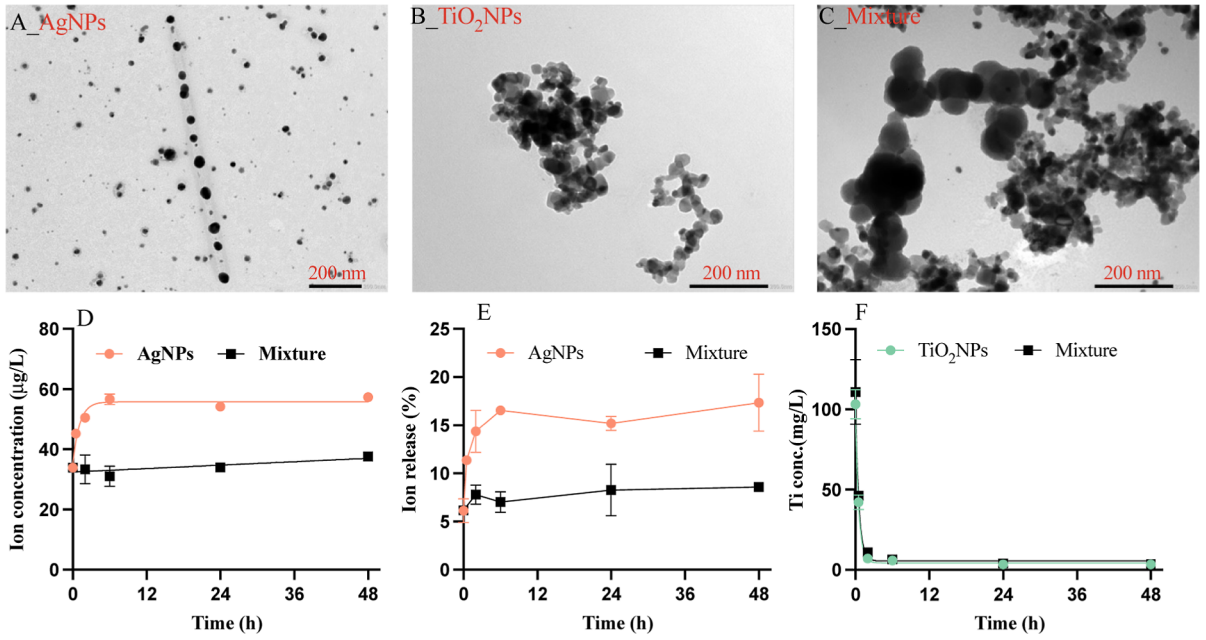

Figure 2. TEM micrographs of MNPs (A−C), ion release profile of AgNPs (D,E), and the sedimentation of TiO_2NPs (F) over time.

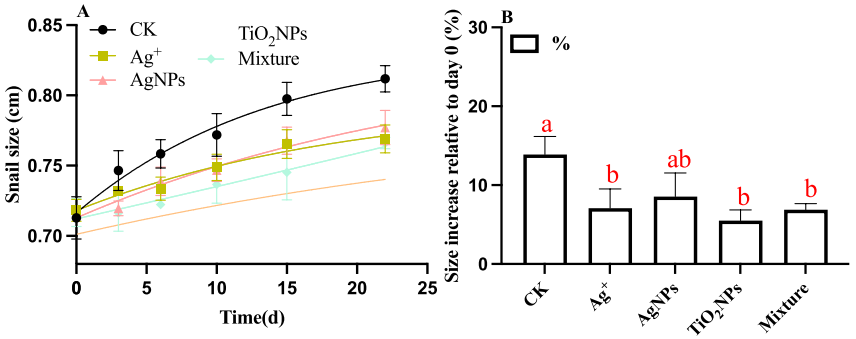

Figure 3. Changes of snail size (diameter) in different treatments over time (A) and the size increase at the end of the feeding (B). The different letters indicate significant differences among different treatments at $p < 0.05$.

$$C_{snail}(t) = C_{snail}(0) + \frac{k_u}{(k_e + k_g)} \times C_{leaves} \times (1 - e^{-(k_e+k_g)t}) \quad 0 \le t \le t_n \tag{4}$$

$$C_{snail}(t) = C_{snail}(0) + \frac{k_u}{(k_e + k_g)} \times C_{leaves} \times (e^{-(k_e+k_g)(t-t_n)} - e^{-(k_e+k_g)t}) \quad t > t_n \tag{5}$$

where $C_{snail}(t)$ and $C_{snail}(0)$ are the concentrations of Ag/Ti in the snails at time t with the unit of mg/kg, C_{leaves} is the concentration of Ag/Ti in lettuce leaves fed to snails (mg/kg, constant value), t represents the feeding time (d), and t_n is the final day of the uptake phase. Equation 4 is used for the uptake phase, and eq 5 is used for the depuration phase. Since the snails grow during the feeding period, a specific growth rate constant, k_g (1/d), obtained by fitting a logistic-growth model on the growth data was included in the model (Figure 3A).

The kinetic trophic transfer factor of Ag/Ti was derived from the TKs as follows

$$TTF_k = \frac{k_u}{k_e + k_g} \tag{6}$$

The trophic transfer factor of Ag/Ti from lettuce leaves to a specific part of the snail (viscera, soft tissue, or feces) at a given time was calculated as

$$TTF = \frac{\text{mass concentration of Ag/Ti in each part of snail (mg/kg)}}{\text{mass concentration of Ag/Ti in plant leaves (mg/kg)}} \tag{7}$$

The particle number concentration-based trophic transfer factor of MNPs from lettuce leaves to specific snail tissues (viscera or soft tissue) was calculated as

$$TTF_N = \frac{\text{number of AgNPs/TiO}_2\text{NPs in each snail tissue (particles/kg)}}{\text{number of AgNPs/TiO}_2\text{NPs in plant leaves (particles/kg)}} \tag{8}$$

The modeling of dissolution kinetics and sedimentation kinetics of MNPs was performed using GraphPad Prism (version 9.0). The TK modeling of trophic transfer of MNPs in snails was carried out using R with the "bcmfr" package (version 0.4-18, OECD TG305). The data of TiO_2NPs were ln transformed to improve their fit to the model. The *t*-test and one-way ANOVA followed by Duncan's post-hoc test were

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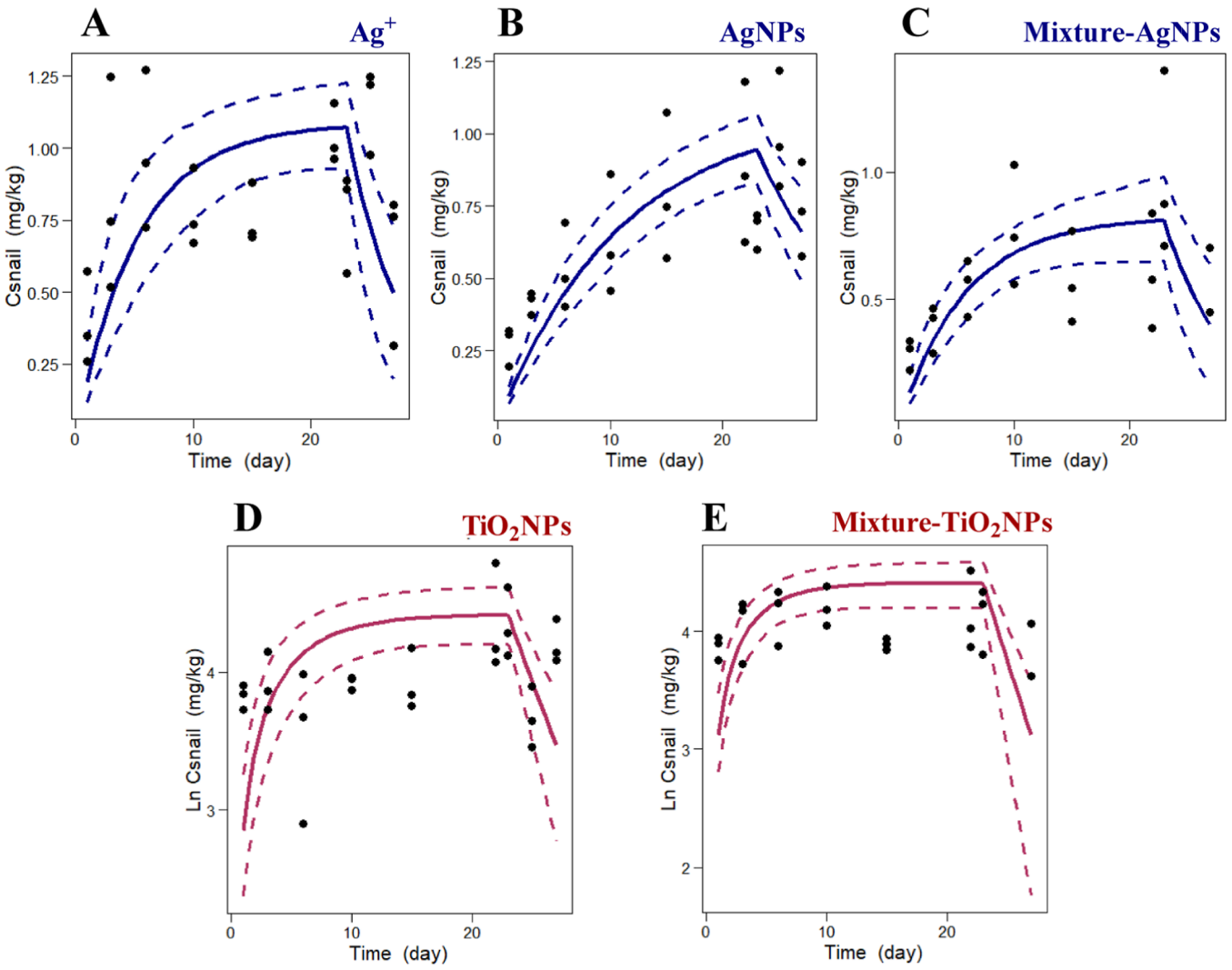

Figure 4. Uptake (days 1−22) and elimination (days 23−27) kinetics of Ag/Ti in snails of single AgNPs/TiO_2NPs treatment or mixture treatment following trophic transfer from lettuce as measured (black circle points) and predicted by the model (lines) over a 27 day exposure/elimination cycle. The individual points indicate the measured concentrations of Ag/Ti in snails, the solid lines indicate the fit of a one-compartment TK model to the measured data, and the dashed lines indicate the confidence limits of the fit. Note: the scaling of the *y*-axes differs among panels (driven by concentration differences in snails).

performed to determine the statistically significant differences between/among treatments at $p < 0.05$ using IBM SPSS Statistics (version 25). Normality and homogeneity of the variance of the data were checked by the Shapiro−Wilk test and the Bartlett test, respectively. All results are expressed as mean ± standard error.

RESULTS AND DISCUSSION

Characterization and Stability of MNPs. The TEM images showed that AgNPs were spherical and that they dispersed well in the exposure medium with an average diameter of 21 nm (n = 100, Figure 2A), while TiO_2NPs agglomerated into angular structures with a diameter of 16−41 nm (n = 50, Figure 2B). DLS results also revealed that the TiO_2NPs formed aggregates as their hydrodynamic diameter increased over time (Table S3). The coexistence of AgNPs and TiO_2NPs induced the formation of co-aggregates with sizes well beyond their initial nanoscale in the exposure medium (Figure 2C). Zeta potential measurements indicated that both AgNPs and TiO_2NPs are negatively charged in the exposure medium (Table S3).

The concentration of dissolved Ag^+ in the AgNPs treatment reached a maximum at 6 h but nevertheless remained relatively constant for 48 h with a dissolution extent of 17%. However, the concentration of released Ag^+ in the mixture treatment was stable (6−8%) during the whole incubation period and lower than the Ag^+ concentration in the AgNPs treatment (Figure 2D,E). This indicates that Ag^+ formed in lower quantities when the AgNPs were in coexistence with TiO_2NPs or, alternatively, that TiO_2NPs can adsorb the dissolved Ag^+. Wilke et al.[14,15] also found a lower concentration of dissolved Ag when AgNPs and TiO_2NPs are present together in a mixture. These authors also attributed this finding to the adsorption of dissolved Ag by TiO_2NPs and determined the maximum adsorptive capacity of TiO_2NPs for dissolved Ag (1.81 ± 0.36 μg Ag/mg TiO_2NPs).

Ti-ions were not detected in the supernatants of the TiO_2NPs treatment and in the mixture treatment. This indicates that the dissolution of TiO_2NPs in this exposure medium was negligible, which is consistent with the results of a

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Table 1. Overview of Uptake and Elimination Rate Constants and Kinetic Trophic Transfer Factors of Ag/Ti from Lettuce Leaves to Snails in Different Treatments[a]

elements	treatment	k_u(kg leaves kg^{-1} snail day^{-1})	k_e (day−^{1})	TTF$_k$(kg leaves/kg snail)
Ag		Un-Transformed C_{snail}		
	$AgNO_3$	0.47 ± 0.11a	0.13 ± 0.05a	3.51 ± 0.57a
	AgNPs	0.08 ± 0.02b	0.05 ± 0.02a	1.15 ± 0.15b
	mixture	0.11 ± 0.03b	0.13 ± 0.05a	0.85 ± 0.16c
cTi		Ln-Transformed C_{snail}		
	TiO_2NPs	1.63 ± 0.36a*	0.20 ± 0.05a	7.99 ± 1.01a
	mixture	1.79 ± 0.35a*	0.28 ± 0.06a	6.46 ± 0.76a

[a]The different letters in the same column indicate statistically significant differences of the same element between treatments at $p < 0.05$; *indicates statistically significant differences between k_u and k_e in the same treatment.

previous study in which it was found that TiO_2NPs exhibit very slow and limited dissolution (up to 3000 h) in an aqueous nutrient solution under normal environmental conditions.[34] The sedimentation curves of TiO_2NPs in the single particle and mixture treatment almost overlapped (Figure 2F), and the sedimentation rate constants of TiO_2NPs in both treatments were comparable (1.91 d^{-1} for the TiO_2NPs treatment and 1.87 d^{-1} for the mixture treatment). These findings indicate that AgNPs have a very limited impact on the sedimentation of TiO_2NPs. This may be due to the much higher concentration of TiO_2NPs than that of AgNPs in the mixture, causing the collision efficiency between TiO_2NPs themselves to be largely higher than between TiO_2NPs and AgNPs. The homo-aggregates of TiO_2NPs thus control the sedimentation of TiO_2NPs rather than the hetero-aggregates of AgNPs−TiO_2NPs. This assumption needs further experimental evidence. Similar results have been reported by Fang et al.[35] who found that the coexistence of 10 mg/L ZnONPs (the solubility of which is comparable to that of AgNPs) had a very limited impact on the sedimentation of TiO_2NPs (15 mg/L), but the sedimentation of TiO_2NPs was significantly affected when the concentration of ZnONPs (25 mg/L) was higher than the concentration of TiO_2NPs.

Impacts on Snail Growth. The impact of dietary exposure to AgNPs, TiO_2NPs, and their combination by consumption of contaminated lettuce leaves on the growth of snails was investigated by monitoring the size changes of the snails over time (Figure 3). The determinant role of bioaccumulation of MNPs in their toxicological effects has been extensively evidenced with respect to the direct exposure scenarios.[32] However, the toxicity of MNPs, especially for the MNPs mixture to higher trophic-level consumers through trophic transfer, is still limited. The size of snails in all treatments increased over the feeding period of 27 days, but the size increase (%) of snails in the Ag^+, TiO_2NPs, and mixture treatments was significantly lower in comparison to that of the control. Notably, the snail growth between the exposure treatments was not significantly different (Figure 3B), and this means that the uptake rates and kinetics can be compared to each other. Similar to the direct exposure, the responses of higher-trophic level consumers to the MNPs' exposure via the food chain could also be determined by species-sensitivity as well as exposure.[7,36] More attention needs to be paid to evaluate the toxicological responses of MNPs associated with food chain transfer in order to capture a more complete picture of their environmental risks.

Uptake and Depuration Kinetics of Ag or Ti from Lettuce to Snails. No mortality was observed for snails in all treatments throughout the entire experiment. The mass concentrations of Ag in the leaves of Ag^+, AgNPs, and mixture treatments were 0.40, 1.2, and 1.3 mg/kg, respectively. In addition, the Ti contents in the leaves of TiO_2NPs and mixture treatments were 12 and 15 mg/kg, respectively (Table S4). An increase of the Ag/Ti concentration in snails occurred over the 22 days of feeding contaminated leaves in all treatments (Figure 4). Similarly, both the Ag and Ti concentrations decreased in the snails in all treatments during the 5 days of clearance in which the snails were fed clean leaves (Figure 4), showing the elimination ability of the organisms.

In this study, the data of the body concentration of total Ag/Ti in snails along the food chain fitted well to the one-compartment TK model. This suggests that using uptake and depuration rate constants is a feasible method to describe the TK processes of MNPs in snails following trophic transfer.[37,38] As summarized in Table 1, the k_u of Ag in snails of the Ag^+ treatment was significantly higher than the k_u from AgNPs and mixture treatments. This can also be seen in Figure 4, specifically in the slope of the first three measured points in time (Figure 4a, vs Figure 4b,c). Similarly, the kinetic TTFs of Ag in the Ag^+ treatment were higher than those in the AgNPs and mixture treatments. The higher k_u and TTFs suggested that the bioavailability of dissolved Ag from lettuce to snail was higher in comparison to that of AgNPs. This is consistent with the results of our previous study performed with the snail *Cornu asperum*.[17] The finding is likely due to the different mechanisms employed by the snails and by other biota to deal with different forms of Ag. As evidenced by a previous study, the translocation mechanism of ionic Ag in snails is mainly through ion transport channels, e.g., the proton-coupled Na^+ channels.[23] This mechanism does not include any size-exclusion phenomena. However, the translocation inside invertebrates is more difficult for AgNPs[39,40] as they need to cross the epithelium in the body.[37,41] The k_e for Ag was not significantly different among treatments (Table 1) and thus meets the assumption to be form dependent and exposure concentration independent.

The values of k_u of Ag in the treatments of AgNPs and the mixture were comparable to the values of corresponding k_e in the treatments (Table 1). It thus showed that a steady-state level in organisms could be reached and that body concentrations decline rapidly under clean conditions. Even though no obvious biomagnification of Ag occurred in the snails of both the AgNPs treatment and the mixture treatment on the basis of mass concentration, the kinetic TTF of Ag from lettuce to snails in the AgNPs treatment was significantly higher than that in the mixture treatment (Table 1). These suggested that the trophic transfer of AgNPs from lettuce to snails was affected by the presence of TiO_2NPs. Two possible

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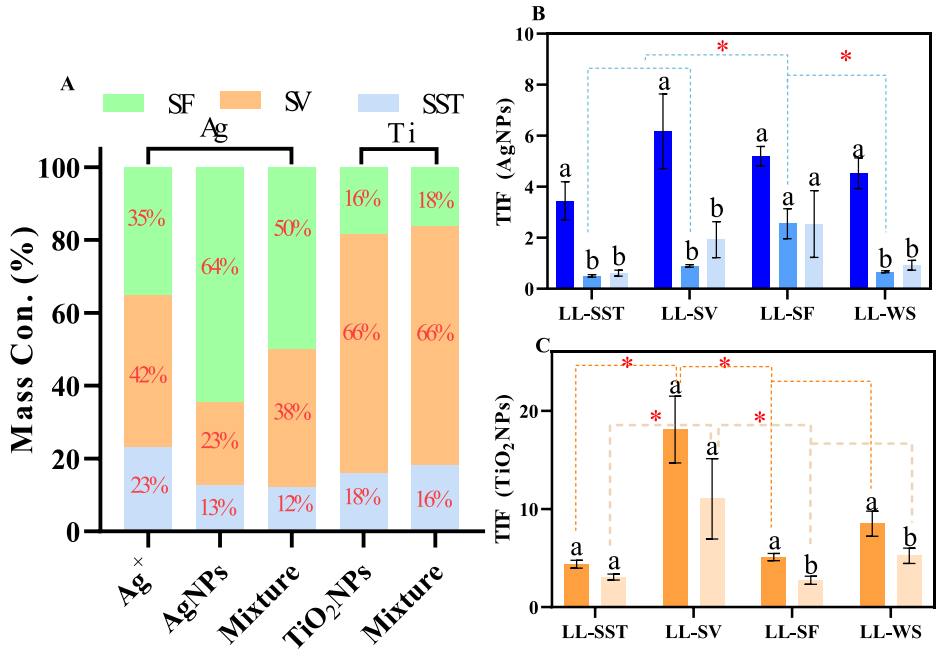

Figure 5. Biodistribution of Ag/Ti in different tissues or the feces of snails in different treatments along the food chain after 23 days of feeding (A), the tissue-specific TTFs of Ag (B) and Ti (C) from lettuce leaves to snail tissues, and the TFs of Ag (D) and Ti (E) from snail viscera to snail soft tissues and feces. LL represents lettuce leave, SST snail soft tissue, SV snail viscera, SF snail feces, and WS the whole snail (SST + SV). The different letters indicate statistically significant differences of the same parameter among different treatments; * indicates the statistically significant differences of different parameters in the same treatment.

explanations can be put forward for this observation. First, as we evidenced, the presence of TiO_2NPs induced lower releases of Ag from AgNPs in suspension by the adsorption of dissolved Ag^+ onto the surface of TiO_2NPs or the formation of TiO_2NPs−AgNPs complexes (Figure 2). This can therefore attenuate the bioavailability and transfer of AgNPs in snails. Numerous studies have reported that TiO_2NPs can affect the fate and bioavailability of concurrent dissolved ions, NPs, and organic pollutants as TiO_2NPs provide a high-surface area for adsorption.[12,15,42] For instance, TiO_2NPs were found to absorb Zn ions released from ZnO and also co-aggregate with ZnONPs, which contributed to the reduction of the bioavailability of ZnONPs.[43] Second, the presence of TiO_2NPs may influence the AgNPs-bio interfacial interactions and hence changes the transfer and bioavailability of AgNPs.[44] Since a previous study has demonstrated that the binding affinity of nanoparticles to biological molecules varies from one to another,[45] the competitive binding of TiO_2NPs and AgNPs with biomolecules may modify the bioavailability of AgNPs in snails. This was supported by our findings that the k_u and TTF_k of Ti were both much higher than those of Ag in the mixture treatment (Table 1).

Notably, the uptake rate constants of total Ti largely exceeded the corresponding elimination rate constants in snails of the TiO_2NPs and mixture treatments, resulting in high kinetic TTFs of TiO_2NPs in snails in the TiO_2NPs (7.99) and mixture (6.46) treatments (Table 1). This finding suggests that TiO_2NPs are biomagnified along the lettuce-snail food chain based on the total mass concentration of Ti. Also, there is a MNPs type-specific phenomenon driving their uptake and depuration kinetics as the TK parameters in snails for stable TiO_2NPs-containing treatments were significantly different from the parameters of the slowly dissolving AgNPs[46]-containing treatments. However, the TK parameters of Ti in snails were not significantly different for the single TiO_2NP exposure compared to the exposure to the mixture of MNPs. This means that the joint presence of AgNPs and TiO_2NPs did not affect the transfer from leaves to snails.

Once the residuals of C_{snail} do not follow a normal distribution, the data was ln-transformed to improve their fit to the model.

Tissue-specific Transfer of Ag/Ti in the Lettuce-Snail Food Chain. Ag/Ti were detected in the viscera, soft tissues, and feces of snails in all treatments, providing clear evidence that the AgNPs and TiO_2NPs internalized in lettuce leaves can be transferred to higher trophic-level snails and subsequently be biodistributed in different organs of the snails. Regarding the AgNPs-containing treatments, more than 50% of the ingested Ag was excreted into the feces of the snails (64% for the AgNPs treatment and 50% for the mixture treatment). The fractions of Ag excreted into the feces of snails in AgNPs-containing treatments were higher than those in the Ag^+ treatment (35%) and TiO_2NPs-containing treatments (16−18%) (Figure 5A). Additionally, the soft tissue of the snails contained 12−13% of the total accumulated metals for all AgNP treatments, but the fraction of Ag retained in soft tissue in the case of the Ag^+ treatment was twice as much (Figure 5A). The findings indicate that ionic Ag is more easily assimilated by snails, which is consistent with the results of our previous study performed with the snail *C. asperum*.[17]

The fraction of Ag retained in the viscera of snails in AgNPs treatment was lower than that in mixture treatment, but the fraction of Ag excreted with the feces in snails of AgNPs treatment was higher than that in mixture treatment. Together with the higher kinetic TTFs observed for AgNPs treatment (Table 1), the results indicate that the presence of TiO_2NPs inhibited the trophic transfer of AgNPs from lettuce to snail but enhanced the retention of AgNPs in snail. Interestingly, the

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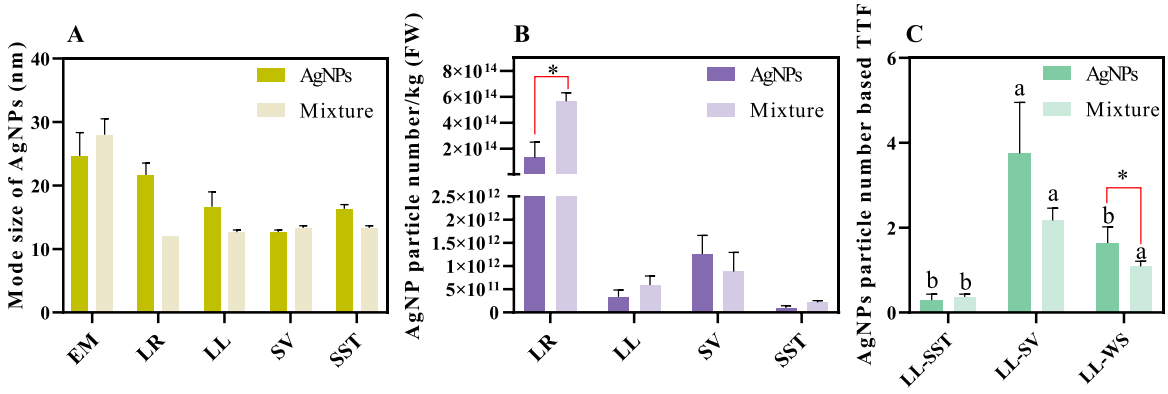

Figure 6. Mode size distribution (A) and particle number (B) of AgNPs in different trophic levels across the food chain and the number-based trophic transfer factors of AgNPs from lettuce leaves to different tissues of snails and the number-based translocation factors of AgNPs from snail viscera to snail soft tissues (C). EM represents exposure medium, LR lettuce root, LL lettuce leave, SST snail soft tissue, SV snail viscera, and WS whole snail (SST + SV).

fractions of Ag in snail viscera and feces and the tissue-specific TTFs of Ag in snails (Figure 5B) observed in this study differed from our previous study performed with the snail *C. asperum*.[17] This indicated that the influence of TiO_2NPs on the trophic transfer of AgNPs is likely consumer species-dependent.[7] The morphological characteristics (weight and size), growth stage, and the growth rate of these two species are different, which thus affect the amount of nanoparticles ingested and their ability to assimilate and eliminate NPs. Several studies have demonstrated that the trophic transfer efficiency of nanoparticles can be affected by the physiology, biology, and life traits including growth stage of the predators involved in the food chain.[7,8,47]

For the TiO_2NPs-containing treatments, the proportions of Ti excreted into the feces of snails for the TiO_2NPs treatment (16%) and the mixture treatment (18%) were similar (Figure 5A), indicating that the elimination of TiO_2NP in snails was not affected by the mixture exposure. More than 65% of the Ti ingested by the snails was retained in the viscera of snails, and the fraction of Ti translocated to the soft tissues of the snails was comparable to the fraction of Ag in the soft tissues of the snails for the AgNPs-containing treatment (Figure 5A). The tissue-specific TTFs of total Ti from lettuce leaves to snails in the TiO_2NPs-containing treatments followed the order of $TTFs_{viscera}$ (11−18) > $TTFs_{soft\ tissues}$ (3−4) (Figure 5B,C). This finding is consistent with the biodistribution patterns of total Ti in snails, which showed that a large amount of Ti was accumulated in the snail viscera. Similar findings were reported in previous studies where the viscera is the main retention tissue of Au in bullfrogs[47] and Ce in snails[48] and chickens[9] following dietary exposure to AuNPs or CeO_2NPs.

Size Distribution of MNPs in Each Trophic Level and Particle Number-Based Trophic Transfer. With the aid of spICP−MS analysis, we quantified the size distribution of MNPs in organisms at each trophic level, as this is of paramount importance for understanding the biotransformation and biodistribution of MNPs along the food chain.[49,50] Although the extraction procedure used in this study did not shift the size distribution of TiO_2NPs measured by spICP−MS in comparison with the size distribution in Milli-Q water prior to particle extraction (Figure S2), and although we obtained a high total mass recovery of Ti (around 90%), the particle number recovery of TiO_2NPs was very low. Hence, we focused on the size distribution and particle number-based trophic transfer of only AgNPs along the food chain in the following discussion (recovery of 66−108%). Abdolahpur Monikh et al.[50] also reported a poor particle number recovery of TiO_2NPs when using proteinase K + H_2O_2 as extractants followed by measurement with spICP−MS. These authors concluded that the obtained poor recovery of TiO_2NPs is due to the limitation of spICP−MS in measuring TiO_2NPs instead of the impact of the extraction procedure.[50] The poor number recovery of TiO_2NPs possibly is due to the addition of H_2O_2 as an extractant in the employed method since Lu et al.[51] found the extraction method using enzymatic digestion without H_2O_2 can recover 94.5 ± 3.5% of TiO_2NP number concentration from fish tissues.

The size distributions of the MNPs in Milli-Q water before and after extraction were compared, and the stability of the MNPs was not altered by the extraction procedures used in this study (Figure S2). The mode (means the most frequent size value) of the size distribution of AgNPs in lettuce roots and in leaves of the AgNPs treatment shifted to a smaller size compared to AgNPs in the exposure medium (Figures 6A and S4). The mode size of AgNPs in snail viscera in AgNPs treatment was smaller than the mode size in lettuce leaves and snail soft tissues (decreased by around 24%, Figure 6A). Collectively, the shifts of mode size of AgNPs between two subsequent trophic level suggested the biotransformation of AgNPs along the food chain. The in vivo dissolution and re-precipitation of AgNPs might be one possible explanation for the observed alterations of the particle size of AgNPs in organisms, as reported by Abdolahpur Monikh et al.[49] For the mixture treatment, the uptake of AgNPs by the lettuce root also shifted the mode size of the AgNPs to a smaller particle size (from 28 to 12 nm), while the mode size of the AgNPs did not change significantly after translocation to lettuce leaves and ingestion by snails (Figures 6A and S4). The mode size of AgNPs in different snail tissues in the mixture treatment was similar to the mode size in lettuce leaves. The findings also support the explanation that the in vivo dissolution and re-precipitation of AgNPs may alter the size distribution of AgNPs as TiO_2NPs can affect the dissolution behavior of AgNPs. Besides dissolution, biological transformation of AgNPs may also occur in organisms, such as the in vivo sulfidation and chlorination to form Ag_2SNPs and AgClNPs[33,52] and the complexation with the exudates/excretes of organisms (e.g., the formation of Ag-ligand complexes and

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the formation of NPs-protein corona).[53,54] These processes may also contribute to the size shifts of AgNPs along the food chain.[33,49] We were unable to verify the formation of sulfurized and chlorinated AgNPs in the current study as this is not possible in our spICP−MS setting. Clearly, further studies incorporating multiple techniques to explore the mechanism underlying the alterations of size distribution of MNPs along the food chain transfer are needed.

In addition to the mass-based trophic transfer of AgNPs from lettuce to snails, we also measured the number concentration of AgNPs in the AgNPs treatment and the mixture treatment across the food chain using spICP−MS to provide insight into the particle number-based trophic transfer of AgNPs. As seen from Figure 6B, the concentrations of AgNPs in lettuce leaves of AgNPs-containing treatments were lower compared to those in the lettuce root, indicating that only a small proportion of the AgNPs taken up by the lettuce root was translocated upward into the lettuce leaves. The AgNPs concentrations in the snail viscera were slightly higher than the concentrations in lettuce leaves and snail soft tissues. This is not surprising as the NPs are difficult to be assimilated and subsequently translocated to the soft tissues due to size exclusion phenomena. As a result, a large fraction of the AgNPs ingested by snails following chronic dietary exposure was retained in the snail viscera. Accordingly, the number-based TTFs (NTTFs) of AgNPs from lettuce leaves to snail viscera were higher than the NTTFs from lettuce leaves to snail soft tissues, and the NTTF in the AgNPs treatment (3.8) was about two times as high as the NTTF in the mixture treatment (2.2) (Figure 6C). The NTTFs of AgNPs were greater than 1, indicating that the biomagnification occurred in snail viscera as expressed on the basis of the particle number concentration. This is in contrast with the observations expressed on the basis of the total mass concentration of Ag (no biomagnification with the corresponding value of the TTFs in the snail viscera of 0.89). Additionally, the NTTFs of AgNPs from lettuce leaves to snail soft tissues and to the whole body of the snail were different from that of the corresponding total Ag mass-based TTFs regardless of treatment (Figures 5B and 6C). These findings suggested that using the total mass of metal as the sole dose metric is not per se the best method to quantify the bioaccumulation and trophic transfer of MNPs because it cannot reflect the particle-specific transfer phenomenon and may lose the information on the transformation of MNPs along the food chain transfer.[49,50]

IMPLICATIONS

In practice, the projected increased use and inevitable environmental release of various MNPs will result in ecosystems being subject to a wide range of mixtures of MNPs in varying compositions and varying concentrations.[14,55] Therefore, this study contributes important insights to better understand the bioaccumulation of MNPs via the food chain and the associated environmental risks under a more realistic exposure scenario. Our findings showed large variations in the MNPs' transfer from plants to consumers. This large variations on biomagnifications between trophic levels is similar to what has been reported for metals,[56] and the discussion on the implications for risk assessment can be held analogue.[57] The risk of MNPs to higher trophic-level consumers not only depends on the species' sensitivity as well as the exposure scenario (such as the concentrations in environment or the mixture exposure), but also the secondary toxicity from biomagnifying MNPs through the food should be taken into account. Further studies should incorporate more exposure concentrations and types of MNPs, and more plant and animal species, in order to understand patterns being concentration-specific, metal(nano)type-specific, species-specific, or scenario-specific, e.g., environmental factors. With the aid of spICP−MS analysis, we noticed that the trophic transfer of MNPs along the food chain can alter their size distribution, and the evaluations based on the total mass concentration of MNPs solely cannot provide sufficient information regarding the bioaccumulation/biomagnification of MNPs. The findings encourage the application of the method of spICP−MS in assessing and quantifying the impact and biological fate of nanoparticles in organisms as it can provide considerable information about the transformation undergone in organisms by monitoring the particle number and size distribution of NPs.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c07660.

Additional information including figures of the size distribution of standard MNPs before and after extraction, comparison of size distribution of MNPs in single and mixture in the exposure medium, size distribution of MNPs in each trophic-level organisms; Hoagland's solution composition, instrumental parameters of ICP−MS for single particle analysis, the hydrodynamic diameter, and zeta potential of MNP suspensions in the exposure medium, and mass concentrations of Ag/Ti in each trophic-level (PDF)

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Notes

The authors declare no competing financial interest.

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