



## Quantifying the trophic transfer of sub-micron plastics in an assembled food chain



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### ABSTRACT

Sub-micron plastics (SMPs, size < 1  $\mu\text{m}$ ) are potentially taken up by plants. Serious concerns arise that how far SMPs can transfer from plants into food webs. Here, we show that lettuce takes up 250 nm gadolinium labelled polystyrene (PS) and polyvinyl chloride (PVC) SMPs from the soil. The polymer type influences the biodistribution of the particles in lettuce (roots and leaves) and the number of particles transferred from the plants to insects feeding on the treated lettuce. The SMPs were further transferred from insects to insect-feeding fish to accumulate mostly in the fish liver. No Gd was released from the particles upon bio-transformation (formation of protein corona on the particles) in the plants or insects. However, Gd ion was detected in fish fed with PS-SMP treated insects, indicating the possible degradation of the particles. No biomagnification in fish was detected for either type of SMPs. We conclude that plastic particles can potentially transfer from soil into food webs and the chemical composition of plastics influences their bio-distribution and trophic transfer in organisms.

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### Introduction

Plastics are produced and used in exponentially increasing amounts [1]. Plastic wastes have been found almost everywhere [2]. The concern about plastic pollution has become widespread after it was realized that mismanaged plastics in the environment break down into smaller pieces known as microplastics (1  $\mu\text{m}$  < size < 5 mm) and, recently, sub-micron plastics (SMPs < 1  $\mu\text{m}$ ) [3], mostly as a result of weathering processes such as thermal degradation, oxidation and abrasion [4]. The small size of SMPs likely allows them to pass through physiological barriers [5] and enter organisms [6]. Despite the growing body of evidence on the potential toxicity of

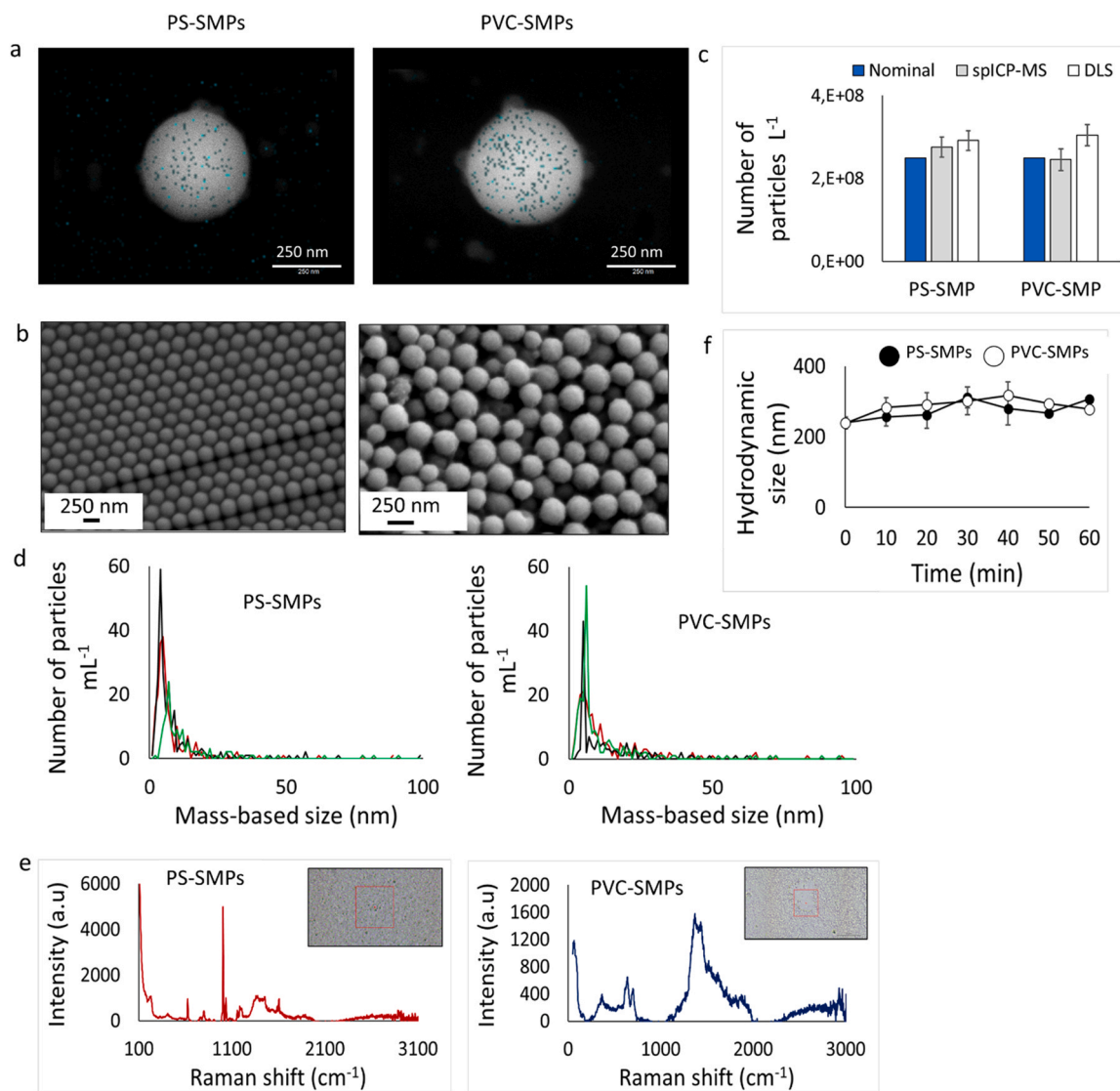
SMPs to plants [7], invertebrates [8] and vertebrates [9], our understanding of plastic transfer in food webs is limited. It has been already reported that the small size of engineered metallic nanomaterials such as gold allows the particles to penetrate organisms' tissues and transfer up food chains [10]. These data might not be directly transferable to plastic particles with a size smaller than 100 nm, analogous to engineered nanomaterials, because there are dramatic differences between engineered nanomaterials and SMPs e.g., in terms of chemical composition and density [11]. It is, thus, critical, to understand whether and to what extent SMPs transfer in food webs.

Trophic transfer is often considered to be a hazard of persistent pollutants [12]. Unlike dissolved chemicals, the uptake and trophic transfer of particulate materials such as SMPs could be influenced by the physicochemical properties of the particles, such as their size, shape, and chemical composition. Since SMPs represent a highly diverse types of materials, including polystyrene (PS), polyvinyl chloride (PVC) and polyethylene (PE), one could expect that the

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**Fig. 1.** Characterization of the SMPs. a) SEM-EDS elemental mapping of PS-SMPs and PVC-SMPs showing the distribution of Gd in the particles. b) SEM images of the PS-SMPs (left) and PVC-SMPs (right) dispersed in MQ water. c) To ensure that most of the SMP particles have a measurable amount of Gd, we compared the nominal number of particles with the number of particles measured by single particle Inductively coupled plasma mass spectrometry (spICP-MS) and dynamic light scattering (DLS). d) The number of particles is determined based on the amount of Gd in each particle, which shows that the quantity of Gd in most of the particles is about equal. The colors (black, red and green) represent the replicate samples. Note that the graphs do not show the size distribution of the SMPs, but the distribution of the Gd per particle. The graphs indicate that most of the SMPs have a mass of Gd equal to a Gd particle with 5–11 nm size (by assuming that no agglomeration has occurred), suggesting that an almost comparable amount of Gd is present in the two types of SMP particles. e) Raman spectroscopy was obtained for SMPs agglomerates confirming the particle compositions as PS and PVC by comparison with the database. f) Hydrodynamic size ( $h_{size}$ , nm) of the particles was measured over 1 h using DLS to show the agglomeration profile of the particles in the water used for culturing the plants.

uptake and trophic transfer of SMPs may differ between various SMP types, even between particles of the same size. Our previous study showed that PS-SMPs can transfer from algae to daphnia through dietary exposure [13]. It is now important to understand how the chemical composition (polymer type) of SMPs influence their trophic transfer in a more complex food chain. Moreover, the biomagnification of plastic particles is still unknown, which needs to be explored.

Little is known about SMPs in soil ecosystems and their uptake by soil organisms, despite the recent discovery of plants being able to take up SMPs and transport the particles to their shoots [14], [7]. Agricultural soil is potentially receiving SMPs from different sources such as atmospheric deposition, irrigation with wastewater, application of sewage sludge for agricultural purposes, and use of mulching film [15]. For example, it has been estimated that farmland in North America and Europe receive annually 63,000–430,000 and

44,000–300,000 tonnes of plastic particles, respectively [15]. Measurement of SMP uptake from the soil by plants, particularly vegetables and fruit in agricultural soils, is thus a critical step to reveal whether and to what extent SMPs can make their way into edible plants and, consequently, into food webs [16]. There is no information about the trophic transfer of SMPs from plants to first consumers, which may indicate a direct hazard to humans via ingestion.

There is no analytical method available that can directly track and measure SMPs in organisms' bodies [17]. We have recently used the approach of entrapping metals in SMPs to facilitate measuring the particles using the metal as a proxy [13]. Particulate materials can be biotransformed in the body of organisms, which dynamically changes the physicochemical properties (e.g. size, shape and surface chemistry) of the particles [10]. For example, the guts of earthworms contain bacteria that potentially degrade low-density polyethylene (PE) [18]. Larvae of the wax moth [19] and mealworms [20] are

capable of degrading both PE and polypropylene. Biotransformation also occurs when SMPs enter the physiological fluids of organisms, such as the hemolymph of insects or the bloodstream of vertebrates. SMPs are immediately covered by proteins after entering into organisms, which forms a so-called protein corona on the surfaces of particles, as extensively reported for nanomaterials [21–23]. These changes in the physicochemical properties of the particles might influence the particle stability [10] and lead to the release of metals from the particles. As a result, the trophic transfer and biodistribution of the metals are mistakenly considered for the SMPs. Thus, when using this approach, the stability of the SMPs and the release of metals in the physiological media must be monitored.

The objective of this study was to reveal how the variation in the chemical composition of SMPs modulates: (a) the particle uptake by plants from the soil, (b) their transfer in food chains, (c) their biotransformation in different organisms, and finally (d) their possible accumulation and biodistribution in predators. We used 250 nm PVC and PS particles as our model SMPs. A rare element, gadolinium (Gd) was entrapped in the matrix of the SMPs and used as a proxy for tackling the challenges associated with tracking and quantification of SMPs in organisms' bodies [17]. The food chain consists of three trophic levels, including a primary producer (lettuce, *Lactuca sativa*), a primary consumer (larvae of black soldier fly, *Hermetia illucens*) and an aquatic insectivorous fish (roach, *Rutilus rutilus*). The reason for selecting each organism is described in the Method. Here, we quantify the number of SMPs taken up from the soil by the lettuce and reveal how the type of SMP influences their biodistribution in the plant tissues. We demonstrate that the type of plastic influences their transfer in a three-level food chain and the biodistribution of the particles in the tissues of predators. We reveal that SMPs in representative physiological media of the test organisms undergo biotransformation due to protein corona formation and that the consecutive biotransformation of the SMPs along the food chain may lead to partial degradation.

## Results and discussion

### Characterization of the traceable SMPs

We applied spherical PS (250 nm, PDI: 0.1) and PVC particles (250 nm, PDI: 0.2) as models of SMPs. To circumvent the analytical challenges associated with SMPs characterization and quantification in biological matrices, particles with a chemically entrapped metallic fingerprint, rare elements, were used as described previously [17]. We selected Gd as the tracer and the reasons for this selection are described in the Method. Scanning electron microscopy (SEM) with energy-dispersive spectroscopy (EDS) analysis images showed the elemental distribution of Gd within the PS-SMPs and PVC-SMPs particles (Fig. 1a). Note that the Gd ions were entrapped inside the particles and not on the surface of the particles to minimize the influence of Gd on the biological interaction of the particles. This was confirmed by analysing the surface of the particles using SEM-EDS (Supplementary, Fig. S1).

The SEM images confirmed that the SMPs are single particles of homogeneous size and of spherical shape (Fig. 1b). The quantity of Gd in the particles was 9.7%–11% as measured by inductively coupled plasma mass spectrometry (ICP-MS). Throughout the study, we used single particle (sp)ICP-MS to measure the number of SMPs. To ensure that the Gd quantity in the particles is detectable by spICP-MS, we compared the number of SMPs measured by spICP-MS with the nominal concentration and with the number of particles measured by dynamic light scattering (DLS). The results showed that there are no significant (ANOVA,  $p < 0.05$ ) differences between the numbers of SMPs measured by different techniques for both particle types (Fig. 1c). Fig. 1d showed that the quantity of Gd in most of the particles is almost equal as measured by spICP-MS.

The chemical composition of the particles was confirmed using Raman spectroscopy and the particles were characterized as PS and PVC when compared to the library spectra of plastic polymers (Fig. 1e). The PS-SMPs and the PVC-SMPs were relatively stable against agglomeration (1 h) in the medium used for watering the lettuces as determined by measuring the hydrodynamic size ( $h_{size}$ ) of the particles over time (Fig. 1f), using DLS. The measured zeta potential ( $\zeta$ ) was  $-16 \pm 2$  and  $-18 \pm 1$  mV for PS-SMPs and PVC-SMPs, respectively. The PS-SMPs (contact angle of  $80 \pm 2$ ) were more hydrophobic than the PVC-SMPs (contact angle of  $60 \pm 1$ ) as determined using their contact angles with water [24].

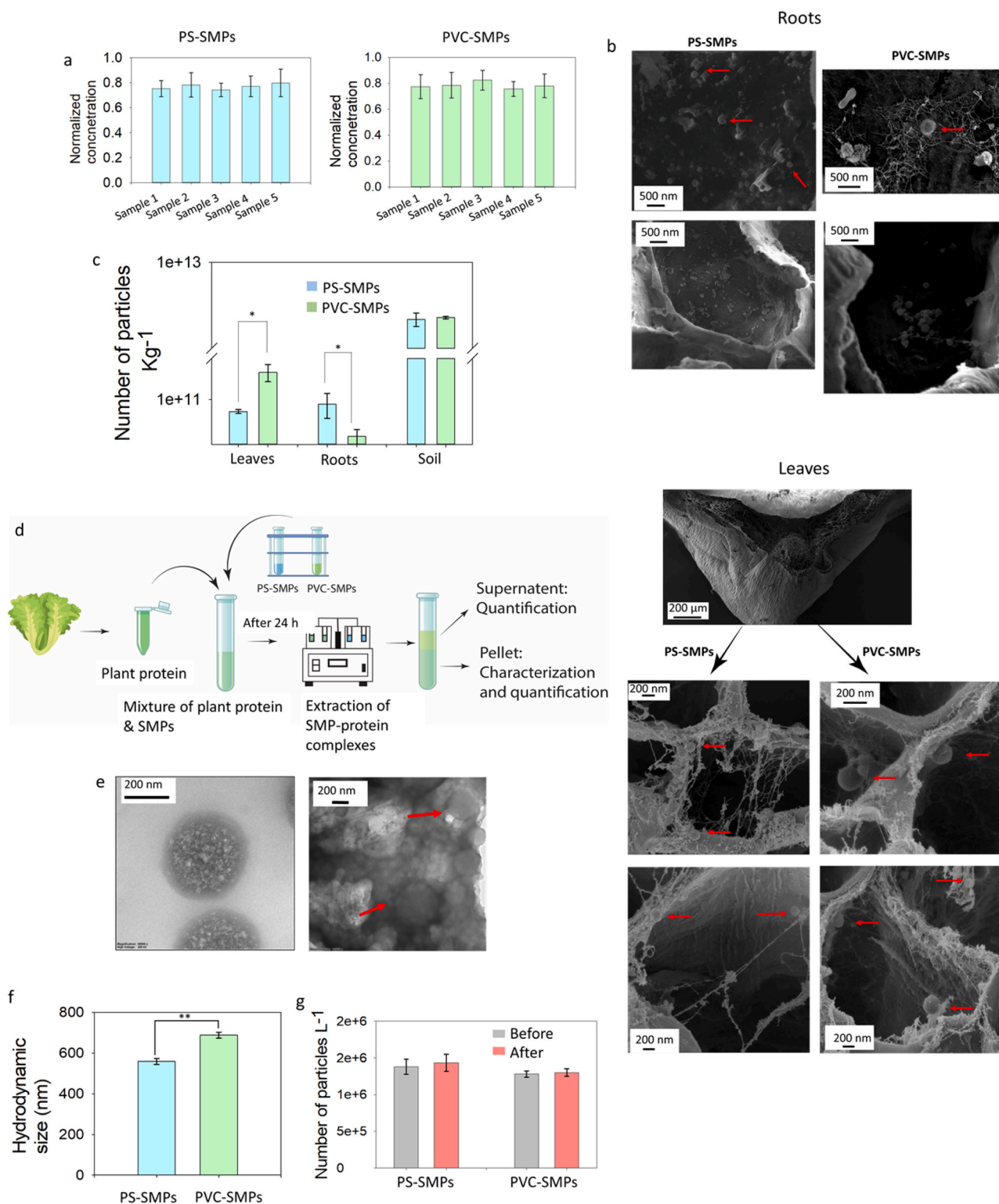
To ensure that the Gd does not leach out of the particles, we first tested the stability of the particles by measuring the Gd ions in the SMP dispersion in Milli-Q (MQ) water after 72 h of incubation at room temperature using spICP-MS (Supplementary, Fig. S2). spICP-MS can differentiate between particles and dissolved ions [10], thus, it allows us to differentiate between Gd ions and Gd in the particles. Moreover, we measured the concentration of the particles and Gd ions in the exposure soil using ICP-MS (Supplementary, S2). No Gd ions could be detected in the supernatants.

### Quantifying the uptake of SMPs from soil by lettuce

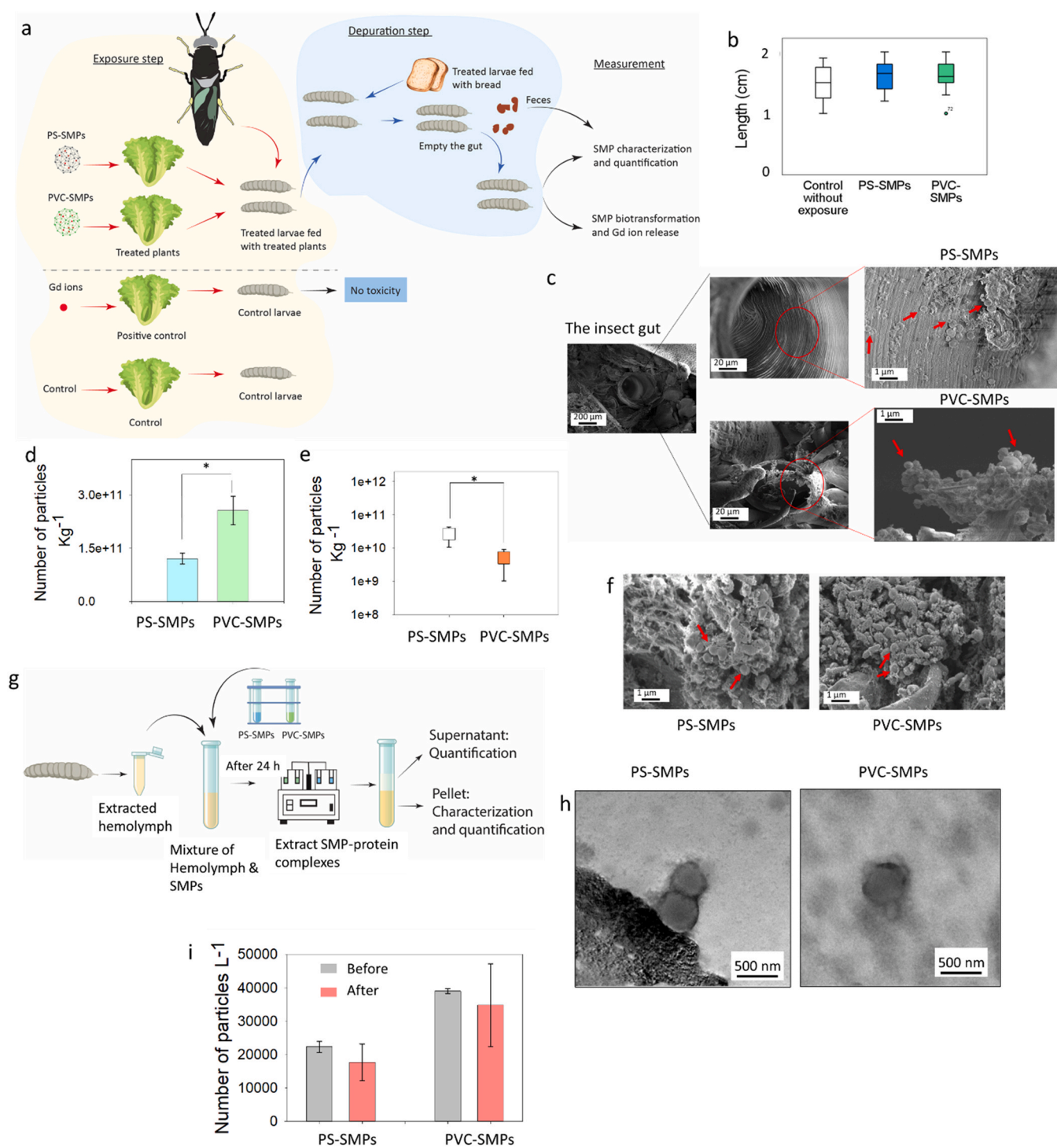
The reported concentration of microplastics in agricultural soil ranges from 6 [25] to 43,000 particles  $\text{kg}^{-1}$  encompassing a multitude of different polymer types [26]. Although the concentration of SMPs in the environment is still unknown, it is expected to be several orders of magnitude ( $10^{14}$ ) higher than their microplastics counterparts [27]. In this study, the SMP concentration was selected based on a literature review of the toxicity of nanoplastics to soil organisms. The nominal concentration of  $\sim 100 \text{ mg kg}^{-1}$  for PS-SMPs and PVC-SMPs in soil was selected. The selected concentration was considerably lower than in a previous study [7] but high enough to allow us to easily track, quantify and characterize the particles in the different organisms used in this study. The measured concentrations of the particles in the dispersions, which were used to spike the soil, were  $\sim 1.3 \times 10^{13}$  particles of PS and  $\sim 1.2 \times 10^{13}$  particles of PVC per liter. The plants were exposed to SMP-spiked soils for 14 days.

The ICP-MS did not detect any background Gd ions in either the water or soil used in this study because Gd is a rare element and not essential in organisms. We ensured that the SMPs were homogeneously distributed in the soil by measuring the concentration of the particles (using Gd as a proxy) in randomly selected samples of the soil (5 samples) (Fig. 2a). Although Gd did not leach out of the particles in the soil, it is possible that biotransformation of the particles in organisms facilitates the release of Gd from the particles. To ensure that the potentially released Gd ions did not induce toxicity to the plant, we spiked the soil with a  $\text{GdCl}_3$  solution (as a positive control) to reach a final concentration of  $10 \text{ mg kg}^{-1}$  soil. The concentration of the positive control is selected to represent the quantity of Gd in the particles (9.7%–11%).

No mortality or changes in the color of the leaves were observed in the positive controls or the plants treated with SMPs, as determined by daily observation. After exposure for 14 days, the lettuce plants were harvested, washed carefully with tap water, and dissected to separate the roots and leaves. The SEM images revealed that the particles were taken up by the roots (Fig. 2b) and transferred to the leaves. This is in agreement with the findings of Li et al. [14] who recently showed that PS-SMPs are taken up by lettuce, although they used hydroponic cultures to investigate the SMPs uptake. Our findings, for the first-time, document that PVC-SMPs are also able to penetrate plants' roots and enter the organisms. This could be due to the small size of the particles which allows them to penetrate the plant cell membrane regardless of their chemical composition. Many studies have reported that soils might be contaminated with SMPs through atmospheric depositions, application of sewage sludge for



**Fig. 2.** Uptake of SMPs from the soil by lettuce and their biotransformation in lettuce. a) To ensure that the SMPs were homogeneously distributed in the soil, the concentration of SMPs was measured in five samples (Sample 1 - Sample 5) randomly selected from the spiked soil (mean  $\pm$  standard deviation). b) SEM images showing the presence of PS-SMPs and PVC-SMPs in the roots and leaves of the lettuces. Two images were presented for each particle in each tissue. Note that the first image of the leaves is just to illustrate where in the plants the images were taken from. The red arrows highlight the positions of some of the particles inside the plants, as examples. c) Number of SMPs in the roots, leaves and soil, as measured by sPLCP-MS [mean  $\pm$  standard deviation, *t*-test, \*:  $p < 0.05$ , degrees of freedom (df): 5]. d) Schematic representation of the extraction of the physiological medium from the lettuces and incubation of the SMPs in the plant proteins for 24 h. The SMP-protein complexes were isolated using centrifugation. e) TEM images showing the formation of the protein corona on the surface of SMPs during the 24 h incubation in the physiological medium of lettuces. f) Number of SMPs before incubation in the plant physiological medium compared with the number of the particles after incubation in the plant physiological medium.



**Fig. 3.** Transfer of SMPs from plants to insect larvae and biotransformation in insects. a) Schematic representation of the experimental design of exposing the insect larvae to SMP-treated lettuces and the depuration period through which the insects emptied their guts. b) The measured length of the larvae in the control samples and the SMP-treated samples, indicating the normal growth for the treated larvae [(box plots indicate median (middle line), 25th and 75th percentiles (box), and S.D. (whiskers)]. c) SEM images of the SMPs in the insect guts. The figure shows an image of the insect gut and two zoomed-in images where the search for the SMPs was performed (note that the first panel of the Image "c" is just to illustrate wherein the insect the images were taken from). The red arrows highlight the positions of some of the particles as examples. d) Measured number of SMPs in the insects after the depuration period (mean  $\pm$  standard deviation, *t*-test, \*:  $p < 0.05$ , df: 5). e) The number of depurated SMPs from the gut of the larvae (mean  $\pm$  standard deviation, *t*-test, \*:  $p < 0.05$ , df: 4). f) SEM images of the larvae feces which were collected after 24 h of depuration period. g) Schematic representation of the extraction of hemolymph from the insect larvae and incubation of the SMPs in the hemolymph for 24 h. h) TEM images showing the formation of the protein corona on the surface of SMPs mixed with hemolymph. i) Number of PS-SMPs and PVC-SMPs before and after mixing with the hemolymph.

agriculture [28], runoff [29], etc. Our findings suggest that there is a potential risk that lettuce and other crops accumulate SMPs from the contaminated soil. The quantity and location of the SMPs in the

plants, however, could be influenced by the chemical composition (polymer type) of plastic, which will be discussed in the next section.

To quantify the number of SMPs in the plant's tissue, the SMPs were isolated from the tissues (approximately 1 g of each tissue)

using tetramethylammonium hydroxide (TMAH) followed by the addition of  $H_2O_2$  (Supplementary, S3). The method for isolation of the SMPs from the tissue was developed and tested. We ensured that the extraction method does not degrade the SMPs using an in house validated method (Supplementary, Fig. S3). The number of SMPs in each tissue was measured using spICP-MS following a method reported previously [13]. The number of both types of SMPs in the plant tissues (wet weight) was lower than the number of each particle in the soil (Fig. 2c). PS-SMPs were accumulated more in the roots while the PVC-SMPs were accumulated more in the leaves. Thus, our findings revealed that the chemical composition of SMPs can influence their biodistribution in lettuce tissues. Since the particles have equal size, this variation in distribution could be related to the difference in the chemical composition and hydrophobicity of the particles. For example, PS-SMPs (contact angle  $80 \pm 2$ ) is more hydrophobic than PVC-SMPs (contact angle  $60 \pm 1$ ) [30]. This could lead to more interaction of PS-SMPs with the cell walls in the plants and a higher retention time compared to PVC-SMPs. These findings might challenge the environmental risk assessment of SMPs as they reveal that each type of SMPs can behave differently and follow different accumulation pathways in plants. In addition to PVC and PS, there are many different types of plastics that are produced and might find their way into soil e.g., polyethylene terephthalate, high-density polyethylene, low-density polyethylene, polypropylene, etc. It is likely that the transfer of SMPs (generated from these plastics) from soil to even one species of plant is different and each type of SMPs accumulates in a specific target of the plant. Since SMPs can have other properties such as shape, size, and surface charge that might influence their transfer from soil and accumulation in plants, it is difficult to extrapolate findings on one type of plastic to other types.

It is likely that SMPs undergo biotransformation (e.g., protein corona formation) in the physiological medium of plants, which may lead to particle biodegradation and, in turn, release of Gd from the particles. To ensure that the particles are stable against biodegradation in the plant tissue, the proteins were extracted from the lettuce leaves (Supplementary, S4) and mixed with the SMPs (Fig. 2d). Note that we did not investigate the influence of protein corona on the biodistribution and trophic transfer of the particles, but the purpose of this test is only to ensure that no Gd ions were released from the particles upon interaction with proteins in organism which is considered as the main biotransformation pathway for particulate materials in organisms bodies [31]. This ensures that the measured Gd is attributed to the particles. After 24 h, the SMP-protein complexes were extracted and characterized. The transmission electron microscopy (TEM) images show that a protein corona has formed on the surface of both SMPs (Fig. 2e) as expected. The spICP-MS results showed that the number of particles after mixing with the plant physiological medium is similar to the number of particles before mixing of the particles with the plant physiological medium (Fig. 2f), which indicates that no particle degradation (fragmentation) has occurred. No Gd ions could be detected in the mixture after 24 h of mixing, indicating that no structural changes occurred in the particles that could lead to the release of Gd from the particles in the plant physiological medium.

#### Quantifying the transfer of SMPs from plants to insects

The leaves of the SMP-treated plants were washed and fed to 6-days old black soldier fly larvae (Fig. 3a) of length around 3.5 mm. Flies in the negative control samples were fed with the control plants (without exposure to any chemicals and materials) and the positive control larvae were fed with plants treated with  $GdCl_3$ . After five days of feeding with lettuce (30 g in total), the larvae were weighed and fed with white bread for 24 h to assist the larvae to entirely empty their guts (Fig. 3a). All the larvae that fed on treated

lettuces grew normally when compared to the controls (ANOVA,  $p > 0.05$ ) (Fig. 3b). No toxicity was observed due to exposure to  $GdCl_3$  treated lettuce.

Both SMPs were transferred from lettuce to the insect larvae. Observation of the digestive systems using SEM imaging shows that both SMPs were present in the mouth (Supplementary, Fig. S4) and in the gut (Fig. 3c) of the larvae even after allowing them to empty their guts for 24 h. The number of PS-SMPs in the larvae was significantly (*t-test*,  $p < 0.05$ ) lower than the number of PVC-SMPs (Fig. 3d). This is consistent with the lower number of PS-SMP in the lettuce leaves. This finding confirms our hypothesis that the chemical composition and hydrophobicity of plastic SMPs determines their bioavailability and their trophic transfer in food chains. For example, due to the variations in the polymer type of PS-SMPs and PVC-SMPs, the particles might undergo different biotransformation in organisms e.g., different proteins attach to the surface of the particles as observed for other nanomaterials [32]. This variation in the adsorbed proteins can influence the biodistribution of the particles in the tissues of organisms [33] and may lead to the accumulation of SMPs in non-edible tissues. Moreover, variation in the hydrophobicity of the particles can critically affect the interaction of the particles with living systems [34]. These in turn can influence the biological fate of SMPs in organisms and food webs.

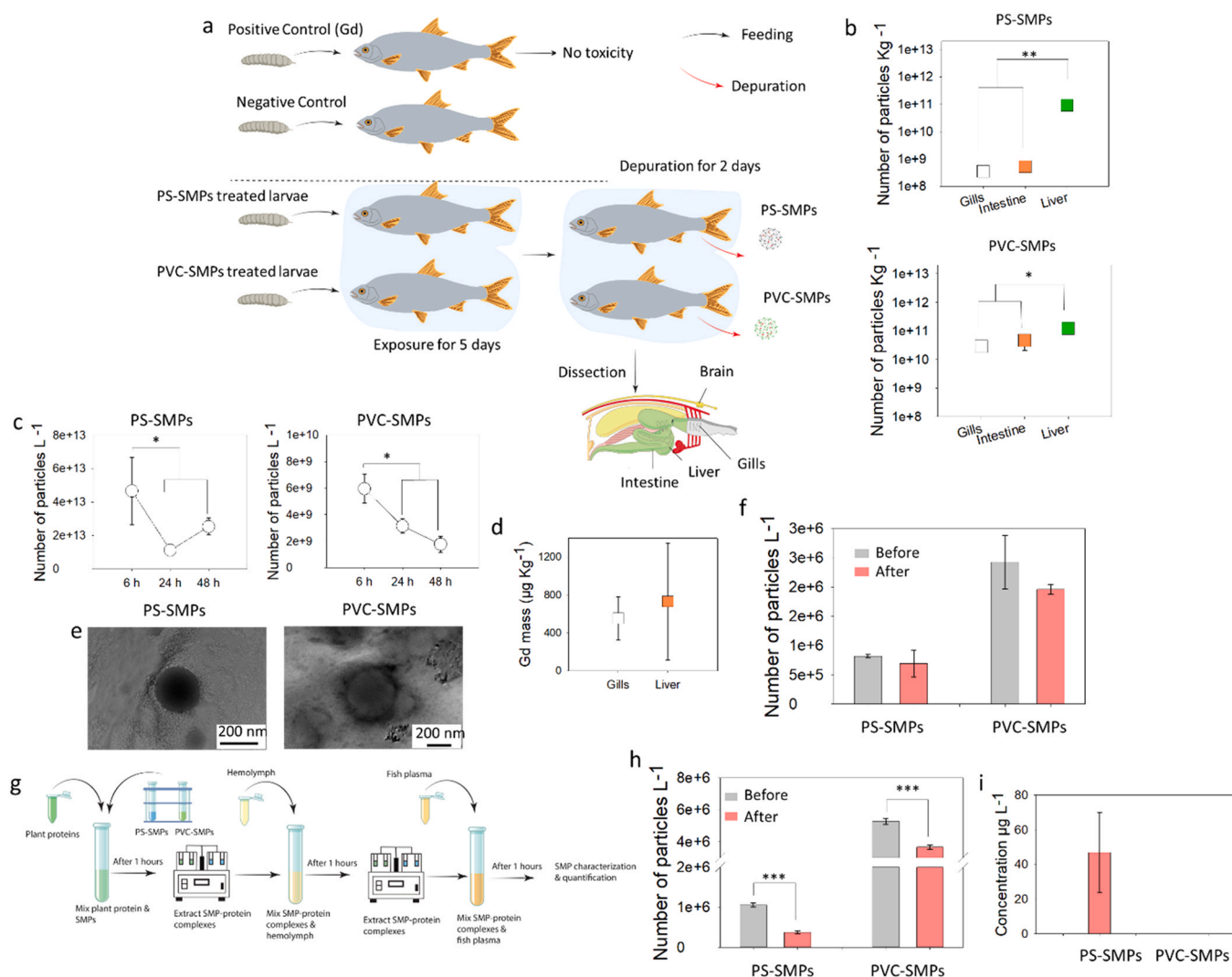
The depuration experiment revealed that a small fraction of the particles was excreted from the gut when the organisms were fed with clean bread (Fig. 3e). This could also be confirmed by imaging the feces of the larvae using SEM (Fig. 3f). The number of depurated PS-SMPs was significantly (*t-test*,  $p < 0.05$ ) higher than the number of depurated PVC-SMPs. This suggests that the absorption of PVC-SMPs in the gut of the insect larvae is higher than that of PS-SMPs.

It is possible that biotransformation of SMPs occurs due to interaction with the insect hemolymph leading to changes in the physicochemical properties of the particles potential release of Gd. The stability of PS-SMPs and PVC-SMPs particles was evaluated after mixing the SMPs with hemolymph extracted from the insect larvae (Fig. 3g). After 24 h of mixing, the formation of a protein corona on the surface of the particles was proven by the TEM images (Fig. 3h, Supplementary S6). No Gd ions, however, could be detected by spICP-MS, suggesting that biotransformation of the particles did not cause Gd release. We quantified the number of particles before and after mixing the particles with the hemolymph. The stability of the particles was also confirmed by almost equal number of the particles before and after incubation in the hemolymph (Fig. 3i).

#### Transfer of SMPs from insect larvae to fish

The experimental design is schematically illustrated in Fig. 4a. Fish were allowed to feed on the lettuce-fed insects for five days, after which they were transferred to clean aquaria for 48 h to empty their guts. We did not feed the fish during the depuration period. The tissues of the fish were dissected to detect the accumulated SMPs in their body. No mortality or abnormal swimming behavior were detected upon feeding the fish with Gd treated larvae as determined by daily observation.

The particles were detected in the gills, liver and intestine tissues, whereas no particles were found in the brain tissue (Fig. 4b). Different tissues accumulated a differential number of particles. The Duncan's post hoc tests revealed that the liver accumulated significantly higher numbers of both SMPs than the gills and intestines (Fig. 4b). This indicated that the liver is a primary target tissue for SMPs entering vertebrates regardless of the chemical composition of the particles, as suggested for other nanoparticles [35]. The liver is a responsible tissue for performing roles in chemical and material detoxification [36], which could remove plastic particles from fish bodies. Critically, SMPs can eventually accumulate in the liver, which



**Fig. 4.** Transfer of SMPs from insect larvae to fish and consecutive biotransformation of SMPs in different physiological media. a) Schematic illustration showing the feeding of fish with insect larvae (fed with SMPs-treated lettuces and control lettuces). The illustration also shows the dissection of the fish tissues (gills, liver, intestine and brain). b) The measured number of PS-SMPs and PVC-SMPs in different tissues of fish (gills, liver, intestine and brain), showing the biodistribution of the particles in different fish tissues (ANOVA, post hoc tests Duncan, \*\*:  $P < 0.001$ ,  $df = 2$ ). c) The number of depurated particles from the treated fish over time (6, 24 and 48 h) (ANOVA, post hoc tests Duncan, \*:  $P < 0.05$ ,  $df = 2$ ). d) Free Gd ions were detected in the gills and liver of the fish with no significant differences. e) TEM images of protein corona formation on the surface of the SMPs incubated in fish plasma. f) Number of SMPs before and after mixing with the fish plasma. Box plots indicate median (middle line), 25th and 75th percentiles (box), and SD (whiskers). g) Schematic representation of the incubation of SMPs in plant physiological medium, insect hemolymph and fish plasma. h) The number of SMPs measured before and after sequential mixing with the various physiological media ( $t$ -test, \*\*\*:  $P < 0.0001$ ,  $df = 4$ ). i) Gd ions were detected in the PS-SMP treatment, but not in the PVC-SMP treatment, indicating some small amount of release.

might lead to profound interactions with hepatic cells and consequently adverse effects on the liver.

A considerable number of the ingested SMPs were released from the gut (Fig. 4c). The number of the SMPs in the depuration medium (water used for the depuration experiment) after the first 6 h was significantly (ANOVA,  $P < 0.05$ ) higher than the number of depurated particles after 24 and 48 h (Fig. 4c). This suggests that a portion of the particles are immediately excreted from the fish bodies into the water, which may lead to the re-uptake of particles via the gills and intestine. This in turn may lead to the decrease in the number of both SMP particles in the depuration medium after 24 h. No Gd ions were detected in the depuration media for either SMPs, indicating that no Gd ions were released from the particles.

The spICP-MS measurements detected free Gd ions in the bodies of fish fed with PS-SMPs treated insects, suggesting that some Gd ions were released from the particles and accumulated in the gills and liver of the fish (Fig. 4d). Since no Gd ions were detected in the insects, the release of ions from PS-SMPs is likely to have occurred in

the fish body due to particle biotransformation. To test this hypothesis, we incubated the SMPs in extracted fish plasma for 24 h (Supplementary, S6). TEM images revealed that a protein corona formed on the surface of the particles (Fig. 4e). The spICP-MS results (Fig. 4f) showed that the numbers of particles before and after incubation of the SMPs in the fish plasma were equal and no Gd ions could be detected after 24 h incubation in fish plasma. This suggests that the fish plasma did not cause the release of Gd ions from the particles. The corona on the surface of SMPs evolves (from lettuce to insects to fish), and this may impact Gd release.

We hypothesized that the consecutive biotransformation of the particles along the food chain (plant, insect and fish) might influence the bulk structure of the particles and thus lead to Gd release. To test these hypotheses, we first incubated the SMPs in plant proteins for 1 h (Fig. 4g). Then, the particles were extracted and incubated with insect hemolymph for 1 h. Finally, we extracted the particles from the hemolymph and incubated them in fish plasma for 1 h. Note that these are not the first contact biofluids for ingested SMPs, but the

particles might be exposed to the lysosomal fluids in fish. The number of SMPs after consecutive incubation in different physiological media decreased significantly compared to the number of particles before incubation in the media (Fig. 4 h). This could be due to the loss of particles during the incubation or extraction processes. The results (Fig. 4i) showed that Gd ions were released from the PS-SMPs after consecutive incubation in the different physiological media.

To test whether the three rounds of consecutive centrifugation influence the particles and drive the Gd release, we dispersed the particles in each medium (plant proteins, insect hemolymph and fish plasma) separately and performed three rounds of centrifugation. No Gd ions could be detected by spICP-MS after three rounds of centrifugation. Our findings suggest that consecutive biotransformation might change the polymeric structure of some SMPs. It is also possible that penetration of biological molecules and enzymes into the polymeric structures weakens the resistance and durability of the material [37].

#### Trophic transfer of SMPs

An overview of the percentage of the applied SMPs taken up from the soil by lettuce and their transfer between organisms of different trophic levels is provided. We determined the mass-balance of SMPs in the tested system (soil and the food chain) and calculated the retention of PVC-SMPs and PS-SMPs in the tested biosphere (i.e., food chain) (Supplementary, Table S1). Out of the  $1.3 \times 10^{13}$  PS-SMPs and  $1.2 \times 10^{13}$  PVC-SMPs that were initially present in 1 kg of soil, 5.5% of PS-SMPs and 7% of PVC-SMPs were transferred in the food chain (Supplementary, Table S1). This finding shows that the retention of SMPs in the biosphere depends on the chemical composition of the particles and the result obtained for one type of SMPs may not be extrapolated to other types of SMPs.

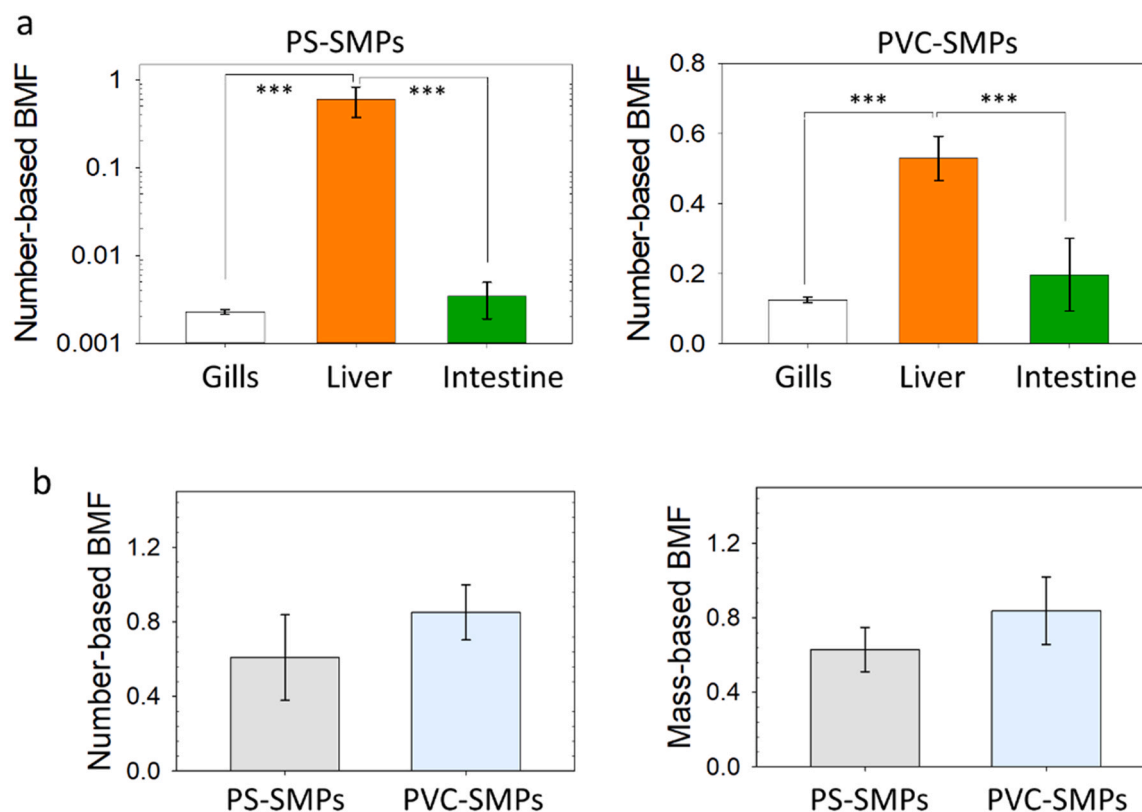
We then calculated the trophic transfer of the particles in the food chain. The total mass concentration of Gd at each trophic level was divided by the total mass concentration of the Gd at the previous level following the approach reported previously [10]. Only a small fraction (< 3%) of the applied particles transferred to the plant shoots, with a significantly (*t*-test,  $p < 0.05$ ) higher quantity of PVC-SMPs compared to PS-SMPs being transferred (Table 1). The particles were further transferred from the leaves to the insect. A high proportion of the PS-SMPs (158%) and PVC-SMPs (132%) transferred from the plant to the insect (*H. illucens*) fed with the treated lettuces. This shows the bioaccumulation of the PS-SMPs and PVC-SMPs in the treated insects (more particles accumulated per unit mass of insect than per unit mass of plant). No significant differences were observed between the transferred PS-SMPs and PVC-SMPs to the insects. A small fraction of the ingested SMPs was excreted from the insect larvae (Table 1). High quantities of both SMPs were transferred to fish (*R. rutilus*) from the insect and only a small fraction of the ingested SMPs was depurated from the organisms' bodies. The quantity of depurated PS-SMPs from the insect and fish was significantly (*t*-test,  $p < 0.05$ ) higher than the quantity of depurated PVC-SMPs. Unlike metallic nanoparticles, which tend to be excreted from organisms' bodies over time [10], PS-SMPs and PVC-SMPs may have a tendency to accumulate in organisms. This could be due to the hydrophobic surface of the plastic particles which increases their tendency to accumulate in organisms, as reported for their analogue hydrocarbon-type chemicals [38]. This highlights the difference between biological fate of SMPs and other nanomaterials. Our findings suggest that plastic particles are likely to enter the biosphere and transfer from one organism to another in food webs and accumulate in different tissue in organisms. For assessing the risk of plastics, thus, it is required to understand what portion of SMPs are already accumulated in the biosphere in different organisms exposed to these contaminants. It is also required to develop analytical

**Table 1**  
Percentage (mean  $\pm$  standard deviation) of the total mass of Gd in soil and in each subsequent trophic level, as determined by ICP-MS. Mass values are normalized to the total mass of Gd in the previous level, in the assembled food chain.

SMPs	Mass of Gd in soil (mg/kg)	Mass of Gd per kg leaves / mass of Gd per kg soil [%]	Mass of Gd per kg insect / mass of Gd per kg leaves [%]	Mass of Gd deputed from insect (per Kg faeces) / mass of Gd per kg leaves [%]	Mass of Gd per Kg fish / mass of Gd per kg insect [%]	Mass of Gd deputed from 1 kg fish / mass of Gd per kg insect [%]
PS-SMPs	9.7 $\pm$ 0.4	1.2 $\pm$ 0.1 <sup>a</sup>	158 $\pm$ 21	35 $\pm$ 22 <sup>b</sup>	78 $\pm$ 23	9.0 $\pm$ 0.9 <sup>b</sup>
PVC-SMPs	9.4 $\pm$ 0.7	3 $\pm$ 0.3 <sup>b</sup>	132 $\pm$ 10	2.2 $\pm$ 1.5 <sup>a</sup>	78 $\pm$ 16	3.3 $\pm$ 0.9 <sup>a</sup>

The letters (a, b) show the significant differences between the treatments (*t*-test,  $p < 0.05$ , df: 4)





**Fig. 5.** Biomagnification factors (BMFs) of the SMPs in fish. a) The number-based (N)-BMFs for each tissue was obtained by dividing the concentration of the SMPs in tissues of fish by the concentration of the SMPs in the lettuces' leaves. Data ( $n=3$ ) were analyzed using one-way ANOVA followed by Duncan's post hoc test ( $***p < 0.0001$ ;  $p < 0.05$ ). b) The calculated total N-BMFs factor and mass-based (M)-BMFs of PS-SMPs and PVC-SMPs in fish. The values were obtained by dividing the total number of the SMPs (N-BMFs) or mass concentration (M-BMFs) of Gd in fish by those in the leaves of lettuces. The  $t$ -test ( $p < 0.05$ ) showed no significant differences between the BMFs of PS-SMPs and PVC-SMPs.

techniques to facilitate biomonitoring SMPs in different organisms including humans and to understand which type of plastics accumulate more in the biosphere compared to other types.

#### Biomagnification in fish

The biomagnification factor (BMF) is often used to quantify the risk of pollutants accumulating in higher animals through dietary uptake [39]. Here, the BMF is expressed as the ratio of either total Gd mass [mass-based (M)-BMFs] or SMP number [number-based (N)-BMF] in fish to total Gd mass and particle number in the treated lettuce (wet mass). A BMF of  $> 1$  indicates biomagnification. We first calculated the BMFs for each tissue of fish (Fig. 5a). In line with the accumulation results in the tissue, the calculated N-BMFs of PS-SMPs and PVC-SMPs for the liver was significantly higher (ANOVA,  $p < 0.05$ ) than the N-BMFs calculated for gills and intestine (Fig. 5a). We also calculated the total N-BMF (Fig. 5b) and M-BMF (Fig. 5b) for fish. Overall, there was no N-BMF in fish (N-BMF  $< 1$ ), i.e., the numbers of SMPs in fish were not higher than those in plants. This, however, does not negate the possibility of biomagnification of SMPs, which may occur over a long timeframe in organisms. The calculated N-BMFs were significantly higher than the values reported for metallic nanoparticles ( $4 \times 10^{-6}$  –  $1.4 \times 10^{-4}$ ) [10].

#### 2.7. Conclusion

Our results show that lettuce can take up SMPs from the soil and transfer them into the food chain. This indicates that the presence of sub-micron sized plastic particles in soil could be associated with a potential health risk to herbivores and humans if these findings are found to be generalizable to other plants and crops and to field settings. Our finding suggests that different types (PS and PVC) of

plastic SMPs have different fates and behavior in organisms and food webs. It is likely that other physicochemical properties of plastic particles such as shape, size, and density can influence the biological fate of these particles in food webs. This implies that the data generated for one type of SMP, for example, PS-SMPs, which is the most commonly investigated plastic-type currently in SMP-related studies, cannot be directly extrapolated to other types of SMPs, and each type and size must be investigated separately despite the complexity of the scenario.

The approach developed in this study can be used to investigate the bioaccumulation and trophic transfer of SMPs in different food webs to elucidate the risk of these persistent materials in the environment. Our study shows that a protein corona can form on the surface of SMPs when they come in contact with physiological media and that this evolves as the SMP move in the environment and up food chains. The detailed evaluation of the corona and its role in biomagnification and food web transport need to be investigated in future studies.

#### Materials and methods

##### Chemicals and materials

All chemicals were reagent grade and were purchased from Sigma-Aldrich, unless otherwise mentioned. The PS-SMPs (250 nm) and PVC-SMPs (250 nm) were designed by our group and were custom synthesized by cd-bioparticles (NY 11967, USA) to our specifications. Accordingly, the Gd were entrapped in the particles (distributed throughout the particles) and after preparation the particles were washed to remove free Gd. We selected Gd because, (a) it is a rare element and does not occur naturally in test plants or organisms' bodies, (b) it allows easy detection and quantification of

the particles using ICP-MS, without interferences, and (c) it can slightly increase the optical density of the particles, facilitating imaging of the particles in biological media using a TEM. The particles were stabilized with Tween 20 (1%). The densities of PS and PVC are 1.05 and 1.38 g cm<sup>3</sup> respectively. Whereas the density of Gd-PS-SMPs and Gd-PVC-SMPs used in this study were 1.3–1.35 and 1.4–1.7 g cm<sup>3</sup>, respectively as reported by the producer. The Milli-Q water was supplied by a Millipore® filtration system (RiOs™ Essential 16 Water Purification System).

#### Particle characterization

The SMPs were dispersed in MQ water for characterization. The  $h_{size}$  of the particles and the particle-protein complexes (using physiological fluids extracted from the various tissues – see Supplementary, S4 for details) as well as the  $\zeta$  were measured using a Zetasizer Nano device (Malvern Panalytical, Malvern, UK). A TEM (JEOL JEM-2100F, JEOL Corp., Tokyo, Japan) operated at 200 kV was used for imaging of the SMPs and SMP-protein complexes. A SEM, (Zeiss Sigma HD,VP, Carl Zeiss NTS, Cambridge, UK) was used with 4 kV for observation of the SMPs in plants and organisms. The sample preparation for the TEM and SEM were performed according to the previous method [40]. The hydrophobicity of the particles was measured after drying a droplet of the particle dispersion of aluminum surface and measuring the contact angle (A KSV Cam 200 contact angle) using Milli-Q water at room temperature. To identify the polymer composition of the SMPs, we used Raman spectroscopy (Supplementary, S8). Raman spectra were measured with a Thermo DXR2xi Raman microscope (Thermo Fisher Scientific, Madison, WI, USA). The concentration of Gd ions and the number of particles in the samples was measured using ICP-MS (PerkinElmer NexION 350D) [10].

#### Soil spiking with SMPs

The PS-SMPs and PVC-SMPs were dispersed in 50 mL of MQ water and sonicated for 1 min using a bath sonicator (35 kHz frequency, DT 255, Bandelin electronic, Sonorex digital, Berlin, Germany). To spike the soil, 50 mL of the dispersion was carefully and homogeneously dropped into the soil to reach a final concentration of ~100 mg per kg of soil. The physicochemical properties of the soil are reported in Table S2 (Supplementary). The spiked soils were mixed in metal bowls using metallic spoons for 10 min to homogenize the soils. Five samples were randomly taken from the soil and inspected to ensure that the particles are homogeneously distributed. The experiment was performed in three replicates for PS-SMPs and PVC-SMPs separately. Control experiments were performed similarly, but without exposure to SMPs. In the positive control, the soil was spiked with GdCl<sub>3</sub> to reach a final concentration of 10 mg per kg soil. The uptake of Gd ions from soil to lettuces and subsequent transfer to other organisms is unknown. Thus, we selected a high concentration for Gd as positive control to ensure no toxicity is induced by Gd to the plants or other organisms even if a high level of Gd is available to plants. The control and spiked soils were kept in the conditions used to culture the plants (see the next section) to allow the aging of the particles for 14 days in the soil matrices.

#### Food chain assembly

As an annual crop, lettuce (*L. sativa*) plays an important role in the diet of humans. The accumulation of SMPs in this plant and the possibility of SMPs transfer from this plant to the higher trophic levels provides a potential pathway for the exposure of humans to SMPs. We used black soldier flies (*H. illucens*) as a model of primary consumers, since they can be easily reared and maintained under

controlled conditions. Moreover, in some countries these insects form a major source of protein, fatty acids, and micronutrients for livestock, including fish [41]. We used the common roach (*R. rutilus*, Linnaeus) as a model vertebrate and aquatic insectivorous predator. This fish species readily feeds on terrestrial insects in the wild [42]. The tissues of this fish species can be accurately dissected, which facilitates investigation of the SMPs biodistribution in the organism.

#### Plant exposure

Lettuce seeds, *L. sativa*, were purchased from Floveg GmbH (Kall, Germany). Detailed information about culturing the plants is described in Supplementary (S10). The same soil as used for culturing of the lettuce plants was spiked with dispersions of SMPs in MQ water to reach a final concentration ~100 mg per kg soil. Similarly, the negative control plants were transferred to clean soil and the positive control plants were transferred to GdCl<sub>3</sub> spiked soil (10 mg kg<sup>-1</sup>). Before transferring the plants to the SMP-spiked soil, the soils with SMPs were kept under the same conditions used for culturing the lettuces for 14 days, to allow aging of the particles. We could not confirm, however, the effect of the aging of the particles on their physicochemical characteristics because separating the particles from the soil to investigate their aging process without influencing the particles was impossible. The plants were cultivated in the spiked soil (150 g soil for each plant, 60 plants in total per treatment) and exposed to PS-SMPs or PVC-SMPs for 14 days. The exposure was performed under the same conditions used to culture the plants in the climate chambers. After exposure, the plants from the controls and the SMP treated samples were harvested [43] and used for SMPs analysis or to feed the insect larvae, as described in the next section.

#### Insect exposure

After thoroughly washing the harvested lettuce plants with deionized water, the shoots and leaves were separated and used as feed for the larvae of black soldier fly (*H. illucens*). Culturing of the larvae is described in the S11 (Supplementary). The 6-day old larvae (ca. 3.5 mm in length) were divided into 4 treatments (the negative and positive control, PS-SMP and PVC-SMPs) with 4 replicates per treatment and 15 larvae per replicate. The larvae were kept under controlled conditions (26 °C, 16 h / 8 h light:dark cycle and 70% relative humidity) and fed with 30 g of the treated plants in total for five days (Supplementary, S12). After exposure, the larvae were counted, and their lengths measured. The larvae were placed on ½ slice of wheat bread (Vaasan Isopaahto Vehnä, Finland) for 24 h to empty their guts by consuming the clean bread. After the depuration period, the larvae were washed and kept at –80 °C for further analysis of the SMPs and to be used as feed for the fish.

#### Fish exposure

All the fish experiments were performed under a fish license from the Finnish Animal Experiment Board (ESAVI/4934/2021). Common roach (*R. rutilus*, Linnaeus) were sampled from Lake Pyhäselkä, Joensuu, Eastern Finland (Supplementary, S13). The fish were maintained for 30 days in the laboratory to become acclimated to the culture water (lake water and dechlorinated tap water with ratio of 3:1). The fish were kept at 17 °C under a 12 h: 12 h (light:dark) cycle. During acclimatization, the fish were fed with unexposed black soldier fly larvae (equal to 500 mg w.w. of larvae per fish per day). Exposures of the fish were conducted in 12 randomly distributed 10 L glass aquaria (with 7 liter of water, Supplementary, Fig. S6). Three replicate aquaria were used for each treatment (negative control, positive control, PS-SMPs and PVC-SMPs) and two fish were used in each replicate. Each fish was fed, for four days, with one SMP-exposed larva per day and the negative control was fed

with unexposed larvae. After exposure, the fish were held for 48 h without feeding to empty their stomachs and the number concentration of the SMPs and the total Gd ions in the medium were measured to estimate the particle depuration time from the fish. After depuration, the intestine, liver, gills, and brains of each fish were immediately dissected, weighed, and kept at  $-80\text{ }^{\circ}\text{C}$ , for later analysis.

#### *Biotransformation of the SMPs in physiological media*

To understand the biotransformation of SMPs and release of Gd ions from the particles inside the organisms, we mixed SMP particles with representative physiological media extracted from the organisms, separately. After mixing, the SMP-protein complexes were separated from the physiological medium (Supplementary, S6). Images of the complexes were made using TEM (JEOL JEM-2100 F) operating at 200 kV accelerating voltage. The number of the particles and the release of Gd ions from the particles were measured using spICP-MS as described in the next section.

#### *Quantification of SMPs and Gd ion release*

The number of SMPs (based on the SMP-bound Gd) and the amount of free Gd ions in the samples were measured using spICP-MS after extraction of the particles from the samples (Supplementary, S3). All spICP-MS measurements were performed on a PerkinElmer NexION 350D ICP-MS operating in single particle mode. The operational parameters for spICP-MS are summarized in the Table S3 (Supplementary). Dispersions of gold nanoparticles with sizes of 10, 60, and 100 nm and mass concentration of 50 mg/L were used to determine the transport efficiency. Particle sizes, particle numbers, and mass concentrations were determined according to the method described in detail and validated in our previous work [13].

#### *Quantification of total mass of Gd*

The total mass of Gd ions in the samples (soil, plants, insect and fish tissues) was measured using ICP-MS after digesting the samples. Accordingly, known quantities of the samples were digested using nitric acid (65%) on a water bath for 2 h (Supplementary, S14). After digestion, the samples were diluted with MQ water and measured using a PerkinElmer NexION 350D. The operational parameters for the ICP-MS are summarized in the Table S4 (Supplementary). Stock standards of Gd ( $100\text{ }\mu\text{g L}^{-1}$ ) were prepared using 0.5% acid nitric solution in MQ water. Calibration standards in the concentration range  $1\text{--}10\text{ }\mu\text{g L}^{-1}$  were prepared by diluting the corresponding ionic stock standards further in 0.5% acid nitric solution in MQ water.

#### *SMPs observation using scanning electron microscope*

To observe the SMPs in the organisms and their tissues, the samples were cut into small and thin sections ( $10\text{--}20\text{ }\mu\text{m}$ ) and fixed using 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 (Supplementary, S15). The sections were dehydrated and coated with a thin layer of gold (30 nm) using an Agar Auto Sputter Coater, in order to ensure electrical conductivity on the sample surfaces and to minimize or eliminate surface charging. The samples were observed using a Field Emission (Schottky type) SEM. During the observation, an acceleration voltage of 4 kV was used under high vacuum conditions (pressure,  $P < 2\text{ mPa}$ ). The micrographs were captured with an InLens secondary electron detector to maximize the spatial resolution and to visualise all the particles of interest.

#### *Data analysis*

The graphs were plotted using SigmaPlot 14. Data were evaluated statistically for normality using a Shapiro-Wilk test in SigmaPlot 14 and the homogeneity of variances was checked. One-way analysis of variance, followed by Duncan's post hoc test, was performed to determine statistically significant (two-sided) differences between tissues of fish and between soil and plants. To obtain the significant differences between two groups, a *t*-test was used. The N-BMF was calculated for each trophic level as described in a previous study [10].

#### **Data Availability**

The authors declare that the data supporting the findings of this study are available within the article and its [Supplementary Information files](#).

#### **Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### **CRedit authorship contribution statement**

F.A.M. designed the experiments, conceptualized, supervised, wrote and reviewed the study. F.A.M., J.K., J.A., R.K. and M.B. performed the soil-plant experiment. F.A.M., S.H. and A.V. performed the plant-insect experiment. F.A.M., R.K., H.H. and L.D. performed the insect-fish experiment. F.A.M., M.B., S.H., R.K. and L.D. studied the biotransformation of the particles in different physiological media. F.A.M., W.J.G. and M.P. designed and characterized the SMPs. F.A.M. and M.B. performed the spICP-MS and ICP-MS measurement. F.A.M., J.L., and A.K. performed the sample preparation for electron microscopy (SEM, TEM) and performed the imaging using SEM and TEM. F.A.M. and A.K. characterized the particles in terms of chemical compositions. J.V.K.K., W.J.G., M.P., I.L. and E.V.J. contributed to conceptualizing, designing the experiment, supervising, writing, and editing the paper. R.K., J.K., J.A. and H.H. contributed to the design of the experiment and editing the paper.

#### **Competing interests**

The authors declare no competing interests.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.nantod.2022.101611](https://doi.org/10.1016/j.nantod.2022.101611).

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