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Can increased production and usage of metal nanoparticles be a threat to freshwater microbial decomposers?

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Abstract

The extensive use of nano metal-based products increases the chance of their release into aquatic environments, raising the question whether they can pose at risk aquatic biota and the associated ecological processes. Aquatic microbes, namely fungi and bacteria, play a key role in forested streams by decomposing plant litter from terrestrial vegetation. Here, we investigated the effects of nano copper oxide and nano silver on leaf litter decomposition by aquatic microbes and the results were compared with the impacts of their ionic precursors. Alder leaves were immersed in a stream of Northwest Portugal to allow microbial colonization before being exposed in microcosms to increased nominal concentrations of nano metals (CuO, 100, 200 and 500 ppm; Ag, 100 and 300 ppm) and ionic metals ($CuCl_2$, 10, 20 and 30 ppm; $AgNO_3$, 5 and 20 ppm) for 21 days. Results showed that rates of leaf decomposition decreased with exposure to nano and ionic metals. Negative effects of nano and ionic metals were stronger on bacterial biomass than on fungal biomass. Fungal sporulation rates strongly decreased with increased concentrations of nano and ionic metals. These effects were accompanied by shifts in the structure of fungal and bacterial communities based on DNA fingerprints and fungal spore morphology. The impacts of metal nanoparticles on leaf decomposition by aquatic microbes were less pronounced compared to their ionic forms, despite metal ions were applied at one order of magnitude lower concentrations. Overall, results indicate that the increased release of nano metals to the environment may affect aquatic microbial communities with impacts on organic matter decomposition in streams.

Keywords: silver nanoparticles; copper oxide nanoparticles; ionic metals; streams; litter decomposition; microbial communities

Introduction

Owing to the rapid growth of nanotechnology-based industries enormous amounts of nanomaterials are being manufactured and utilized since the past decade [1]. Nano metal-based products have become part of our regular life in the form of cosmetics [42], antimicrobial paints [21], textile fabrics [56] and electronic devices [26]. Nano metals are also employed in biomedical and pharmaceutical applications, like cancer therapy, protein detection, tissue engineering, drug delivery and gene therapy [46]. With the accelerated usage of nanoparticles, aquatic ecosystems most likely will serve as terminal repository for the discharged nanomaterials. For instance, the engineered nanoparticle TiO_2 was detected in aquatic environments as a consequence of being leached from the paint of house facades into the neighbouring stream [21]. Hence, the research pertaining to impacts of nanoparticles and its ionic forms on aquatic biota has become a priority.

Ionic metals are used as precursors for production of many nanomaterials; for instance, silver nitrate and copper chloride are known to be the ionic precursors of nano silver and nano copper oxide, respectively [47, 52]. A number of studies have reported toxicity of metal ions against aquatic organisms ranging from microbes to vertebrates [3, 5, 14], but less is known about the effects of their nanoparticle forms (but see e.g., [2, 32]).

Nano silver and nano copper oxide are used widely (e.g., medical research/applications [31, 43] and textiles [56]) and are becoming the focus of toxicological investigations. These nanoparticles can have toxic effects on various organisms, including yeasts [23], bacteria [24], fungi [25], the marine diatom *Thalassiosira weissflogii* [28], *Chlamydomonas* [45] and fish, like zebrafish [17], and may also pose risks to human health [22, 36]. However, the existing data on the effects of nano-sized silver and copper oxide are mainly based on individual responses of organisms and are clearly insufficient to predict its

impacts on biotic communities (but see reports from Bradford et al. [6] and Shah et al. [48] for estuarine and soil bacterial assemblages, respectively) and ecosystem processes.

In freshwaters, plant-litter decomposition is a key ecosystem process associating riparian vegetation with microbial and invertebrate activities [41, 39]. Fungi, mainly aquatic hyphomycetes, have been distinguished as dominant microbial decomposers [37] and are responsible for transferring carbon and energy from plant litter to higher trophic levels in streams [15]. Bacteria have been recognized to play a role after partial breakdown of plant material [37]. Previous studies demonstrated that litter decomposition is sensitive to changes in water chemistry [12, 37, 41] and this integrative process was proposed as a functional measure to assess the health of freshwater ecosystems [38, 41].

Even though the ionic forms of metals have been reported to affect litter decomposition and the associated communities in freshwaters (e.g. [9, 33, 50]), studies exploring the impacts of nano metals on this ecosystem process are virtually unknown. The aim of this study was to investigate the effects of nano copper oxide and nano silver, and their ionic precursors, on leaf litter decomposition by freshwater microbial communities. We used stream-dwelling microbial communities in microcosms to mimic the natural environment under controlled conditions, and the measured parameters were leaf mass loss, fungal and bacterial biomass and diversity, and fungal reproduction.

Material and Methods

Field experiment

The sampling site is located at the Maceira stream (N 41°45'58.79", W 8°08'49.39", altitude 867 m) in the Peneda-Gerês National Park (Northwest Portugal). At the sampling site, the stream is 0.3-0.5 m deep and 0.5-1.0 m wide and the geological substratum is

constituted by granitic rocks, pebbles, gravels and sand. The dominant riparian vegetation includes *Quercus pyrenaica* Wild, *Quercus robur* L., *Chamaecyparis* sp. and *Ilex aquifolium* L.

Leaves of *Alnus glutinosa* (L.) Gaertn. (alder) were collected from a single Autumn tree and air dried at room temperature. The leaves were soaked in deionised water and cut into 12 mm-diameter disks. Sets of 40 disks were placed into each of 105 fine mesh bags (15 × 15 cm, 0.5-mm mesh size, to prevent invertebrate colonization) that were immersed in the stream for 7 days to allow microbial colonization. After 30 min of leaf immersion, 3 randomly selected leaf bags were retrieved and transported to the laboratory to determine initial leaf mass.

Conductivity and pH of the stream water were measured *in situ* with field probes (Multiline F/set 3 no. 400327, WTW, Weilheim, Germany). Stream water samples were collected into sterile dark glass bottles, transported in a cold box (4°C) to the laboratory to determine the concentrations of nitrate (HACH kit, programme 351), nitrite (HACH kit, programme 371) and phosphate (HACH kit, programme 490) using a HACH DR/2000 photometer (HACH, Loveland, CO).

Microcosm experiment

After retrieval from the stream, leaf disks from each of 102 bags were rinsed with deionised water and placed into 150 mL sterile Erlenmeyer flasks with 80 mL of sterile stream water (121°C, 20 min). Stream water had a pH of 5.9, a conductivity of 16 µS cm^{-1}, and contained 40 µg L^{-1} $N\text{-}NO_3^-$, 2 µg L^{-1} $N\text{-}NO_2^-$ and 20 µg L^{-1} $P\text{-}PO_4^{3-}$. Stream water was supplemented with increasing nominal concentrations of nano metals or ionic metals as follows: 0, 100, 200 and 500 ppm of nano copper oxide (CuO nanopowder <50 nm, 99.5%); 0, 100 and 300 ppm of nano silver (Ag nanopowder, <100 nm, 99.5%); 0, 10, 20 and 30 ppm

of ionic copper ($CuCl_2.2H_2O$, > 99%); and 0, 5 and 20 ppm of ionic silver ($AgNO_3$, > 99%). Nano and ionic metals were purchased from Sigma-Aldrich (St. Louis, MO). Stock suspensions of the two nano metals were sonicated (Branson 2510 sonication bath, Danbury, CT, USA) for 30 min in dark before used [18]. The pH of stock suspensions of nano metals and stock solutions of ionic metals were adjusted to 6.0 ± 0.2.

All microcosms were kept under shaking (150 rpm) at 13°C, and nanoparticle suspensions and ionic metal solutions were renewed every 7 days. After 7, 14 and 21 days of exposure, a set of 33 microcosms (3 replicates of each treatment per time) was sacrificed and leaf disks were freeze dried to determine leaf mass loss, microbial biomass and diversity as described below. In addition, the content of 3 leaf bags was used to determine leaf mass loss and microbial parameters at the beginning of microcosm experiment.

Fungal sporulation rates

After 21 days of exposure to the nano and ionic metals, suspensions of released fungal conidia from each replicate microcosm were mixed with Triton X-100 (40 µl of 15%), to avoid conidial adherence to the flask, and the conidia were fixed with 2% formaldehyde. Then, appropriate aliquots of conidial suspensions were filtered (5 µm pore size, Millipore, Billerica, MA) and stained with 0.05% cotton blue in lactic acid. Conidia of aquatic hyphomycetes were identified and counted under a light microscope (Leica Biomed, Heerbrug, Switzerland) at 400× magnification.

Microbial biomass

Concentration of ergosterol was measured to estimate fungal biomass associated with decomposing leaves [13, 37]. Lipids were extracted from sets of 6 leaf disks per replicate by heating (30 min, 80°C) in 0.8% KOH-methanol and the extract was purified by solid-phase

extraction and eluted in isopropanol. Ergosterol was quantified by high-performance liquid chromatography (HPLC) using a LiChrospher RP18 column (250 × 4 mm, Merck) connected to a Beckmann Gold liquid chromatographic system. The system was run isocratically with HPLC-grade methanol at 1.4 mL min^{-1} and 33°C. The peaks of ergosterol were detected at 282 nm and standard series of ergosterol (Sigma) in isopropanol were used to estimate the ergosterol concentration in the samples. Ergosterol concentration was converted to fungal biomass assuming 5.5 µg ergosterol mg^{-1} mycelial dry mass [13].

To estimate bacterial biomass, sets of 4 leaf disks per replicate were placed into 15 mL falcon tubes with of 10 mL phosphate buffered formalin (2% final concentration) and kept at 4°C until processed. Bacterial cells were dislodged from leaf disks by sonication for 5 min. Aliquots of 2 mL of appropriate dilutions of bacterial suspensions were incubated with 4′,6-diamidino-2-phenylindole (DAPI, 50 µL of 0.1 mg mL^{-1}; Molecular Probes) for 10 min in the dark, before filtered through black polycarbonate membranes (0.2 µm pore size, GTTP, Millipore Billerica, MA). Filters were mounted between two drops of immersion oil on grease free slides, covered with cover slips and bacterial cells were counted using a fluorescence microscope (Leitz Laborlux Heerbrug, Switzerland) at magnification of 1000×. Bacterial numbers were converted to bacterial biomass considering a mean bacterial biomass of 20 fg $cell^{-1}$ [34].

Denaturing gradient gel electrophoresis

DNA was extracted from 3 leaf disks (pooling 2 half disks of each replicate) using the UltraClean Soil DNA kit (MoBio Laboratories, Solana Beach, CA, USA). The ITS2 region of fungal genomic rDNA was amplified with the primer pair ITS3GC and ITS4 [9, 53] and the V3 region of bacterial 16S rDNA was amplified with the primer pair 338F_GC and 518R [9]. The 40-bp GC tail on the 5′ end of the forward primers ensured the amplicon separation by

denaturing gradient gel electrophoresis (DGGE). All primers were purchased from MWG Biotech AG. For polymerase chain reaction (PCR), 1x Go Taq Green Master Mix (Promega), 0.8 µM of each primer and 2 µL of extracted fungal or bacterial DNA were mixed gently with nuclease free water in a final volume of 50 µL. PCR was carried out in the iCycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). DNA amplification programme was started with a denaturation for 5 min at 94°C, followed by 36 cycles of denaturation for 30 s at 94°C, primer annealing for 30 s at 55°C and extension for 1 min at 72°C, concluding with an extension for 3 min at 72°C [9]. The PCR products were separated by DGGE using the DCode™ Universal Mutation Detection System (BioRad Laboratories, Hercules, CA, USA). For fungal DNA, 20–40 µL from the amplified products of 380–400 bp were loaded on 8% (w/v) polyacrylamide gel in 1x Tris–Acetate–EDTA (TAE) with a denaturing gradient from 30% to 70% (100% denaturant corresponds to 40% formamide and 7 M urea). For bacterial DNA, 20 µL from the amplified products of 200 bp were loaded on 8% (w/v) polyacrylamide gels in 1x TAE with a denaturing gradient from 35% to 80%. DNA mixtures of 5 species of fungi or bacteria were used to calibrate the gels. The gels were run at 55 V for 16 h at 56°C and stained with 1x GelStar (Lonza Rockland, Inc., USA). The gel images were captured under UV light in a transiluminator Eagle eye II (Stratagene, La Jolla, CA, USA).

Leaf mass loss

To determine leaf mass loss, freeze-dried leaf disks from each replicate before and after stream colonization, and after microcosm exposure were weighed to the nearest 0.001 mg.

Nano metals in stock suspensions

The nano metals in suspensions were analysed by UV-visible spectrophotometry (UV – 1700 PharmaSpec, Shimadzu, Kyoto, Japan) and by scanning electron microscopy (SEM, Leica Cambridge S 360, Cambridge, UK) coupled to an energy dispersive X-ray microanalysis setup (EDX, 15 KeV). For SEM analysis, 20 µl of stock suspension of each nanometal was mounted on a clean grease free slide in dark, air dried and coated with gold in vacuum by using a Fisons Instruments SC502 sputter coater. Nano silver and nano copper oxide showed plasmon picks at 416 nm and 359 nm, respectively. Scanning electron microscopy confirmed the size of copper oxide nanoparticles (30 to 50 nm) and silver nanoparticles (near to 100 nm). The presence of Cu and O in copper oxide nanoparticles and Ag in silver nanoparticles was confirmed by EDX (Fig. 1A and B). Additional picks were found: Au from the coated gold, Si probably from the glass slide, and Na, Ca and Mg probably from the stream water.

Statistical analyses

Rates of leaf decomposition (k) were estimated according to the exponential model as follows: $W_t = W_0 \times e^{-kt}$, where W_t is the leaf dry mass remaining at time t, W_0 is the initial leaf dry mass and t is the time in days. Regression lines of ln-transformed values of leaf dry mass against time were compared by analysis of covariance (ANCOVA [55]). Three-way analysis of variance (3-way ANOVA [55]) was used to determine the effects of time and concentration of nano and ionic forms of each metal on fungal and bacterial biomass. Two-way ANOVA was used to test the effects of concentration of nano and ionic forms of each metal on fungal sporulation rate, fungal diversity and contribution of aquatic hyphomycete species to the total conidial production after 21 days of exposure. Significant differences between control and treatments were analysed by Bonferroni post-tests [55]. When needed, data were ln-transformed to achieve normal distribution and homocedasticity [55]. Univariate

analyses were performed with Statistica 6.0 (Statsoft, Inc., Tulsa, OK, USA).

Cluster analyses of fungal and bacterial communities based on relative intensity of each DGGE band was done by Unweighted Pair-Group Method Average (UPGMA) using the Pearson correlation coefficient [12]. Each band in the gel was considered one operational taxonomic unit (OTU). Gel and cluster analyses were done with the GelCompar II program (Applied Maths, Sint-Martens-Latem, Belgium).

Results

Nano and ionic metals reduce microbially-mediated leaf litter decomposition

The decomposition rate of alder leaves was high corresponding to 0.037 day^{-1} (Table 1). The exposure to nano or ionic metals led to a significant decrease in leaf decomposition rate (ANCOVA, $P<0.05$). The lowest decomposition rates were found at the highest concentrations of nano and ionic silver (k=0.021 day^{-1} for 300 ppm nano silver and 20 ppm ionic silver) or nano copper oxide and ionic copper (k = 0.017 day^{-1} and 0.015 day^{-1} for 500 ppm of nano copper oxide and 30 ppm of ionic copper, respectively).

After 7 days of colonization in the stream, fungal biomass on decomposing leaves was 10 mg g^{-1} leaf dry mass and increased to 53 mg g^{-1} leaf dry mass after 21 days in control microcosms (Fig. 2A and 2B). Concentrations of nano and ionic forms of silver or copper and exposure time had negative effects on fungal biomass (three-way ANOVAs, $P<0.05$; Table 2). After 21 days of exposure to the highest concentration of nano silver (Fig. 2A) or nano copper oxide (Fig. 2B) a 40% inhibition of fungal biomass was found (Bonferroni tests, $P<0.05$). Fungal biomass was inhibited earlier by exposure to the highest concentrations of ionic silver (15 and 9 mg g^{-1} leaf dry mass at 20 ppm for 14 and 21 days, respectively; Bonferroni tests, $P<0.05$; Fig. 2A) or ionic copper (24 and 18 mg g^{-1} leaf dry mass at 30 ppm

for 14 and 21 days, respectively; Bonferroni tests, *P<0.05*; Fig. 2B). A significant decrease in fungal biomass was also observed at the longest exposure time to the lowest concentrations of ionic silver (17 mg g^{-1} leaf dry mass at 5 ppm; Bonferroni test, *P<0.05*; Fig. 2A) or ionic copper (30 and 18 mg g^{-1} leaf dry mass at 10 and 20 ppm for 21 days, respectively; Bonferroni test, *P<0.05*; Fig. 2B).

Before microcosm exposure, bacterial biomass on leaves was 0.02 mg g^{-1} leaf dry mass and increased to 0.26 mg g^{-1} leaf dry mass after 21 days in microcosms (Fig. 3A and 3B). The exposure to all concentrations of nano and ionic silver led to a significant decrease in bacterial biomass at all times (three-way ANOVA; Bonferroni test, *P<0.05*, Table 2, Fig. 3A) with strongest effects for silver ions (0.01 mg g^{-1} leaf dry mass). Nano copper oxide and ionic copper concentrations and time also had significant inhibitory effects on bacterial biomass (three-way ANOVA, *P<0.05*; Table 2). The exposure to the highest concentrations of nano copper oxide (200 and 500 ppm) or ionic copper (20 and 30 ppm) led to a significant decrease in bacterial biomass at all times, whereas the lowest tested concentrations of these toxicants (100 ppm of nano copper oxide and 10 ppm of ionic copper) decreased the biomass only after 14 and 21 days of exposure (Bonferroni test, *P<0.05,* Fig. 3B).

In control microcosms, sporulation rate of aquatic hyphomycetes attained $245x10^3$ spores g^{-1} leaf dry mass day^{-1} and was significantly inhibited by exposure for 21 days to all concentrations of nano and ionic forms of silver or copper (two-way ANOVA, *P<0.05*; Fig. 4A and B).

Nano and ionic metals affect the structure of microbial decomposer community

Based on conidial morphology, a total of 11 aquatic hyphomycete species were identified on decomposing leaves after 21 days in control microcosms (Table 3). The exposure to nano and ionic metals decreased fungal species richness, particularly in the case

of copper (5 species in treatments with concentrations ≥ 200 ppm of nano copper and 4 species with 30 ppm of ionic copper; Table 3). In addition, nano and ionic metals led to shifts in fungal species composition (Table 3). In control, *Articulospora tetracladia* (51.2%) was the dominant species followed by *Flagellospora* sp. (32.8%) (Table 3). The exposure to nano silver, nano copper and ionic copper significantly increased the contribution of *A. tetracladia* to overall conidial production (two-way ANOVAs, Bonferroni tests, $P<0.05$), whereas ionic silver did not lead to any significant change (Table 3). The exposure to nano and ionic metals significantly decreased the contribution of *Flagellospora* sp. (Bonferroni test, $P<0.05$; Table 3) but increased that of *Heliscus lugdunensis* (Bonferroni test, $P<0.05$; Table 3).

DNA fingerprinting based on DGGE showed that fungal and bacterial communities were affected by nano and ionic metals (Fig. 5A and B, Table 3). Thirty one fungal OTUs and 36 bacterial OTUs were found in control communities (Fig. 5A and B, Table 3). The number of fungal or bacterial OTUs decreased with increasing concentrations of nano or ionic metals, particularly in the case of the latter form of the toxicants, with maximum reduction at the highest concentration of ionic silver (Fig. 5A and B, Table 3). Cluster analysis of fungal communities exposed to the highest ionic silver concentration formed an outgroup (Fig. 5A). Further, fungal communities exposed to nano silver clustered together and were separated from control communities or communities exposed to other treatments. Cluster analysis of bacteria discriminated 3 groups: control communities, communities exposed to silver and communities exposed to copper (Fig. 5B). Bacterial communities exposed to nano and ionic forms of each metal were further separated.

Discussion

Due to the small size and reactive surface characteristics of nanoparticles, they are

prone to aggregation and sorption onto organic materials [19], such as submerged plant detritus in streams. Therefore, a close interaction between nano metals and benthic microbes involved in plant litter decomposition is expected to occur. Our study depicted that nano and ionic metals can have impacts on microbial communities and reduce leaf litter decomposition in freshwater ecosystems. The negative effects of metal ions, such as zinc, copper and cadmium, on freshwater microbial decomposers have been often reported [9, 11, 12, 27, 29]; however, very little is known on the putative impact of nano metals. In our study, the exposure to elevated concentrations of nano and ionic metals affected microbial biomass on decomposing leaves. The biomass of bacteria was strongly inhibited even at short exposure times (7 days). Indeed, many nano metals are known to have anti-bacterial properties (< 100 ppm in axenic cultures [54]). However, negligible effects of nano silver or nano copper on bacterial abundance, diversity (as fatty acid methyl-ester or DGGE profiles) or activity (as metabolic profile) are reported in soil [48] and estuarine [6] communities. The discrepancy between our results and the latter studies may be explained by the two order of magnitude lower concentrations of the nano metals used in the two cited studies. Also, differences in environmental conditions, such as pH, temperature and oxygen availability [44], as well as nanoparticle properties (e.g., size [8]) might account for different results in different studies and is worthy of further investigation.

Here we found that biomass of bacteria was more sensitive to nano and ionic silver or copper than that of fungi. This agrees with earlier reports showing that, contrarily to bacterial biomass [10, 11], fungal biomass is not very sensitive to moderate metal stress and decline only under high stress levels [10, 11, 33]. This might be due to the higher growth rates of bacteria on decomposing leaves than those of fungi [37, 39]. The structure of bacterial communities, based on DNA fingerprint, also appeared to respond better than fungal communities to the presence of these toxicants, because bacterial communities discriminated

well the stress imposed by ionic and nano forms of silver and copper. It is also conceivable that the response pattern of fungal communities become clearer at longer exposure times, as shown by Duarte et al. [9] in microbial communities exposed to copper and zinc ions. Moreover, a differential response of aquatic microbial communities to each nano metal and respective ionic form was found, suggesting different modes of action of these toxicants. This is supported by distinct gene expression profiles in zebrafish gills after exposure to nano or ionic metals [17].

The analysis of aquatic hyphomycete communities based on identification of released spores from decomposing leaves also showed shifts in community composition after exposure to nano and ionic metals. For instance, the dominant fungal species *A. tetracladia* increased its contribution to the total released conidia after exposure to nano silver and ionic copper, but not to ionic silver or nano copper oxide. Moreover, the co-dominant species *Flagellospora* sp. was inhibited by exposure to nano or ionic metals, being replaced by *H. lugdunensis* at the highest concentrations. *Articulospora tetracladia* and *H. lugdunensis* are reported to occur in metal contaminated streams [20, 40] and some strains of these species were found to be resistant to high concentrations of metals [7]. The shift in species composition in this study probably indicates a change towards a better-adapted community, which may play an ecological role under the stress imposed by nano metals and/or their ionic precursors.

The significant reduction in leaf decomposition rate by stream-dwelling microbes in the presence of nano or ionic metals was probably due to the observed decrease in fungal and bacterial diversity and activity on leaf litter. Although biomass of fungi was apparently less affected by these stressors than that of bacteria, fungal biomass on decomposing leaves was two orders of magnitude higher. This agrees with previous studies pointing to a dominant role of fungi during litter decomposition in freshwaters [37, 39]. Therefore, it is conceivable that

the observed reduction in litter decomposition was mainly due to the negative effects of nano and ionic metals on fungal activity. Unfortunately, we do not have data on the activity of plant litter degrading enzymes in aquatic fungi under nano or ionic metal stress. However, in the white rot fungus *Trametes versicolor*, the production of lignocellulose degrading enzymes, such as ß-glucosidase, cellobiohydrolase and ß-xylosidase, decreased by the presence of ionic copper and aggregated nanoparticles [49].

In our study, the impacts of nano and ionic metals were more pronounced on fungal sporulation than on fungal biomass or diversity. This has ecological implications because if fungal reproductive output is affected, it may further compromise fungal dispersal and survival in freshwaters with impacts to leaf eating invertebrates that dependent on fungal activity. Moreover, sporulation rate was one of the most sensitive microbial parameters to nano or ionic metals. Also, other studies point to reproduction of aquatic hyphomycetes as a sensitive measure of water quality [9, 11, 27] with possible applications in stream monitoring programmes.

Even though we used concentrations of ionic metals one order of magnitude lower than those of nano metals, the negative effects of ionic forms were more pronounced compared to their nano forms. Also, the toxicity of nano copper oxide to the protozoa *Tetrahymena thermophila* ($EC_{50,14h}$ = 128 ppm) was 120 times lower than that of the ionic copper [30]. This may be attributed to the low bioavailability of nano forms in water. The toxicity of nano metals to bacteria [18], aquatic algae [2], and to the eukaryotic model yeast *Saccharomyces cerevisiae* [23] was attributed to soluble metal ions originating from the metal oxide particles. Conversely, others found that the toxicity of nano copper and silver in zebrafish and *Daphnia pulex* is unlikely to be merely explained by particle dissolution [16]. Therefore, more investigation on the mechanisms of action of nanoparticles is needed to clarify this aspect.

The effects of nano copper oxide seemed to be stronger than those of nano silver on leaf decomposition rate, bacterial biomass, fungal diversity and reproduction. However, it should be taken into account that the size of metal nanoparticles used in our study was lower for nano copper oxide (30-50 nm) than for nano silver (near to 100 nm). Data from literature have shown that nano metal toxicity to several cell lines [35, 22] and organisms, including aquatic species of different trophic levels, tend to increase with the decrease of particle size [51, 18]. However, the toxicity of nano metals does not appear to be a generic response to exposure to nanosized particles; rather, it seems that particular nano metals have an intrinsic property that confers toxicity [22].

Overall, results provide the novel information that nano metals are potent to pose risks to microbial communities that drive plant litter decomposition in streams by reducing diversity and activity of fungi and bacteria. Moreover, our findings suggest that biomass of aquatic bacteria and sporulation of aquatic fungi might be useful tools in ecotoxicological studies to assess nano or ionic metal stress. Although the negative effects of ionic forms were more pronounced compared to their nano forms, accumulation or adsorption of nano metals to microbial cells is conceivable to occur [4]. If so, nano metals may enter aquatic detritus foodwebs with impacts to higher trophic levels. This study clearly indicates emerging risks to aquatic microbiota and associated ecosystem processes due to increasing industrial scale production and usage of nano and ionic metal-based products.

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Table 1 Effects of nano and ionic metals on decomposition rates (k) of alder leaves

Treatments	k (day^{-1}) ± SE	W_0 (%)	r^2
Control	0.037 ± 0.004	105.6	0.86
AgNP1	0.024 ± 0.003*	98.6	0.86
AgNP2	0.021 ± 0.003*	99.4	0.81
Ag1	0.022± 0.003*	97.3	0.79
Ag2	0.021 ± 0.003*	97.1	0.84
CuONP1	0.021± 0.002*	98.0	0.87
CuONP2	0.019 ± 0.002*	98.5	0.86
CuONP3	0.017 ± 0.002*	98.7	0.87
Cu1	0.022 ± 0.003*	97.8	0.83
Cu2	0.018 ± 0.002*	96.9	0.88
Cu3	0.015 ± 0.002*	95.8	0.75

AgNP1: 100 ppm nano Ag; AgNP2: 300 ppm nano Ag; Ag1: 5 ppm Ag^{+}; Ag2: 20 ppm Ag^{+}; CuONP1: 100 ppm nano CuO; CuONP2: 200 ppm nano CuO; CuONP3: 500 ppm nano CuO; Cu1: 10 ppm Cu^{2+}; Cu2: 20 ppm Cu^{2+}; Cu3: 30 ppm Cu^{2+}; Control: without addition of any form of metals nd, not detected. *, treatments that differ significantly from control (ANCOVA, Bonferroni test, $P < 0.05$). SE: standard error; r^2: coefficient of determination; W_0: initial leaf dry mass.

Table 2 ANOVAs of effects of exposure time, nano and ionic metal concentrations on fungal biomass, bacterial biomass and fungal sporulation (only significant effects are shown)

Parameter		Effect	d.f.	*F*	*P*-value
Fungal biomass					
	Silver				
		Time	4	30.2	<0.0001
		Nano Ag	2	3.4	<0.05
		Ag^+	2	26.4	<0.0001
	Copper				
		Time	4	41.7	<0.0001
		Nano CuO	3	3.1	<0.05
		Cu^{2+}	3	7.5	<0.0005
Bacterial biomass					
	Silver				
		Time	4	191.8	<0.0001
		Nano Ag	2	31.4	<0.0001
		Ag^+	2	101.1	<0.0001
	Copper				
		Time	4	280.2	<0.0001
		Nano CuO	3	82.4	<0.0001
		Cu^{2+}	3	82.0	<0.0001
Fungal sporulation rate (21 days)					
	Silver				
		Nano Ag	2	371.0	<0.0001
		Ag^+	2	646.6	<0.0001
	Copper				
		Nano CuO	3	360.5	<0.0001
		Cu^{2+}	3	364.9	<0.0001

Table 3 Microbial diversity on decomposing leaves as number and composition of fungal sporulating species and number of fungal and bacterial OTUs from DGGE fingerprints after 21 days of exposure to increasing concentrations of nano or ionic metals in microcosms

Species	% of conidia in treatments										
	Control	AgNP1	AgNP2	Ag1	Ag2	CuONP1	CuONP2	CuONP3	Cu1	Cu2	Cu3
Alatospora acuminata Ingold	0.2	0.4	nd	nd	nd	nd	nd	nd	nd	nd	nd
Anguillospora filiformis Greath	4.3	1.4	2.2	6.3	nd	nd	nd	nd	nd	nd	nd
Articulospora tetracladia Ingold	51.2	76.0	76.1	53.2	55.5	51.6	56.1	60.7	66.7	68.0	73.3
Culicidospora aquatica R.H. Petersen	0.1	0.4	nd	nd	nd	nd	nd	nd	0.4	nd	nd
Flagellospora sp.	32.8	9.3	6.7	15.6	15.9	9.1	11.4	7.5	10.9	11.8	6.5
Fontanospora eccentrica (R.H. Petersen) Dyko	0.2	0.4	nd	nd	nd	nd	nd	nd	nd	nd	nd
Fontanospora fusiramosa Marvanová, P.J. Fisher, Descals & Bärlocher	3.9	2.4	2.2	6.3	5.4	5.3	4.0	4.6	3.6	2.4	nd
Heliscus lugdunensis Sacc. & Therry	1.8	6.1	8.1	12.5	17.8	27.5	24.4	22.6	12.3	11.7	15.2
Lunulospora curvula Ingold	0.9	2.0	2.2	nd	nd	1.6	nd	nd	1.7	2.4	nd
Tricladium splendens Ingold	0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Varicosporium elodeae W. Kegel	4.5	1.7	2.2	6.3	5.4	4.8	4.0	4.6	4.4	3.9	5.1
Nº of fungal morphotypes	11	10	7	6	5	6	5	5	7	6	4
Nº of fungal DGGE OTUs	31	26	24	20	11	26	25	23	19	17	16
Nº of bacterial DGGE OTUs	36	28	24	24	19	27	25	24	26	25	24

AgNP1: 100 ppm nano Ag; AgNP2: 300 ppm nano Ag; Ag1: 5 ppm Ag^{+}; Ag2: 20 ppm Ag^{+}; CuONP1: 100 ppm nano CuO; CuONP2: 200 ppm nano CuO; CuONP3: 500 ppm nano CuO; Cu1: 10 ppm Cu^{2+}; Cu2: 20 ppm Cu^{2+}; Cu3: 30 ppm Cu^{2+}; Control: without addition of any form of metals. nd, not detected.

Figure legends

Figure 1 Scanning electron microscopy (SEM) with energy dispersive X-ray (EDX) microanalysis (insight) of nano silver (A) and nano copper oxide (B) in stock suspensions.

Figure 2 Fungal biomass on decomposing alder leaves exposed to nano or ionic silver (A), nano copper oxide or ionic copper (B) in microcosms. AgNP1 and AgNP2: 100 and 300 ppm nano Ag; Ag1 and Ag2: 5 and 10 ppm Ag^{+}; CuONP1, CuONP2 and CuONP3: 100, 200 and 500 ppm nano CuO; Cu1, Cu2 and Cu3: 10, 20 and 30 ppm Cu^{2+}, respectively; Control: without addition of any form of metals. Mean ± SEM, n=3

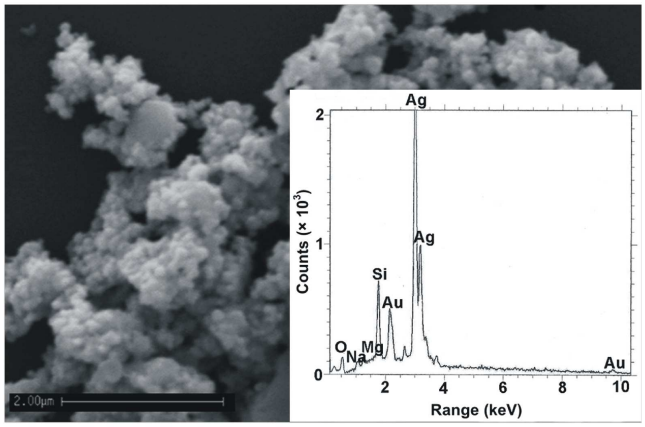
Figure 3 Bacterial biomass on decomposing alder leaves exposed to nano or ionic silver (A), nano copper oxide or ionic copper (B) in microcosms. AgNP1 and AgNP2: 100 and 300 ppm nano Ag; Ag1 and Ag2: 5 and 10 ppm Ag^{+}; CuONP1, CuONP2 and CuONP3: 100, 200 and 500 ppm nano CuO; Cu1, Cu2 and Cu3: 10, 20 and 30 ppm Cu^{2+}, respectively; Control: without addition of any form of metals. Mean ± SEM, n=3

Figure 4 Fungal sporulation from decomposing alder leaves after 21 days exposure to nano or ionic silver (A), nano copper oxide or ionic copper (B) in microcosms. AgNP1 and AgNP2: 100 and 300 ppm nano Ag; Ag1 and Ag2: 5 and 10 ppm Ag^{+}; CuONP1, CuONP2 and CuONP3: 100, 200 and 500 ppm nano CuO; Cu1, Cu2 and Cu3: 10, 20 and 30 ppm Cu^{2+}, respectively; Control: without addition of any form of metals. Mean ± SEM, n=3. *, treatments that differ significantly from control (Bonferroni tests, $P<0.05$).

Figure 5 DNA fingerprints from DGGE and cluster analysis of fungal (A) and bacterial (B) communities exposed to nano and ionic silver or nano copper oxide and ionic copper for 21 days

in microcosms. Dendograms were constructed from UPGMA analysis based on the Pearson coefficient of similarity. AgNP1 and AgNP2: 100 and 300 ppm nano Ag; CuONP1, CuONP2 and CuONP3: 100, 200 and 500 ppm nano CuO; Ag1 and Ag2: 5 and 10 ppm Ag^{+}; Cu1, Cu2 and Cu3: 10, 20 and 30 ppm Cu^{2+}, respectively; Control without addition of any form of metals.

A


Ag
Ag
Si
Au
O
Na
Mg
Au
Counts (× 10³)
Range (keV)
2.00µm

B

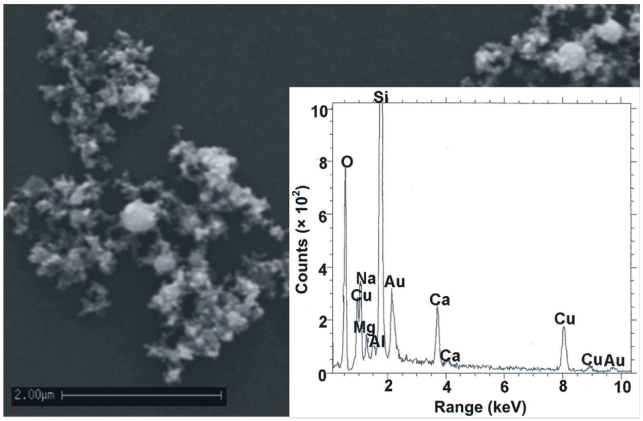

Si
O
Na
Cu
Mg
Al
Au
Ca
Ca
Cu
CuAu
Counts (× 10²)
Range (keV)
2.00µm

Fig. 1

Fig1

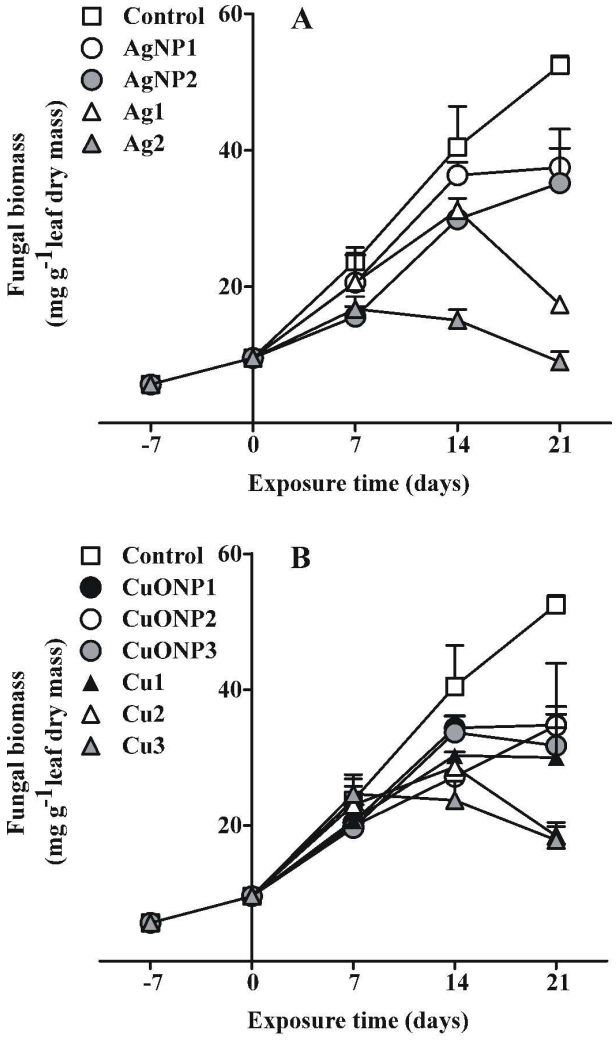

A
Control
AgNP1
AgNP2
Ag1
Ag2
Fungal biomass
(mg g-1leaf dry mass)
60
40
20
-7
0
7
14
21
Exposure time (days)
B
Control
CuONP1
CuONP2
CuONP3
Cu1
Cu2
Cu3
Fungal biomass
(mg g-1leaf dry mass)
60
40
20
-7
0
7
14
21
Exposure time (days)

Fig. 2

Fig2

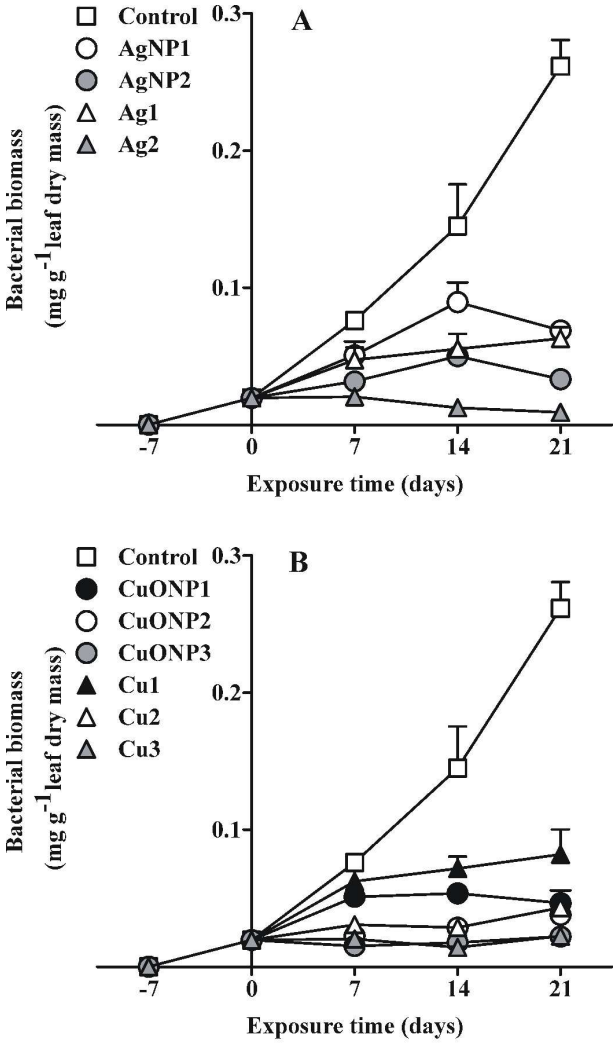

A
Control
AgNP1
AgNP2
Ag1
Ag2
Bacterial biomass
(mg g-1leaf dry mass)
0.3
0.2
0.1
-7
0
7
14
21
Exposure time (days)
B
Control
CuONP1
CuONP2
CuONP3
Cu1
Cu2
Cu3
Bacterial biomass
(mg g-1leaf dry mass)
0.3
0.2
0.1
-7
0
7
14
21
Exposure time (days)

Fig. 3

Fig3

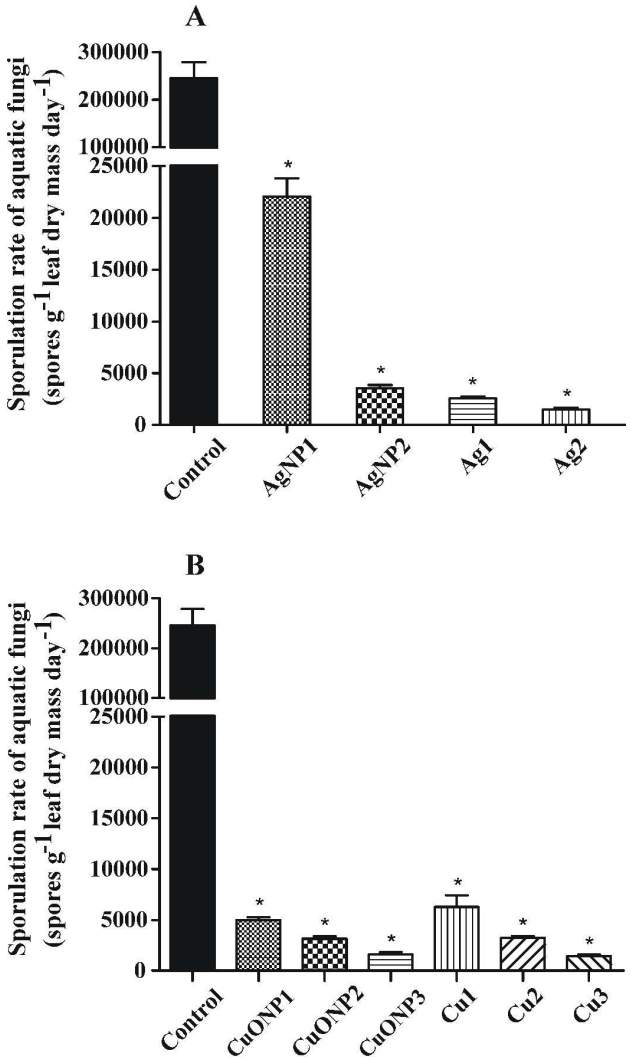

A
Sporulation rate of aquatic fungi (spores g-1 leaf dry mass day-1)
300000
200000
100000
25000
20000
15000
10000
5000
0
Control
AgNP1
AgNP2
Ag1
Ag2
B
Sporulation rate of aquatic fungi (spores g-1 leaf dry mass day-1)
300000
200000
100000
25000
20000
15000
10000
5000
0
Control
CuONP1
CuONP2
CuONP3
Cu1
Cu2
Cu3

Fig. 4

Fig4

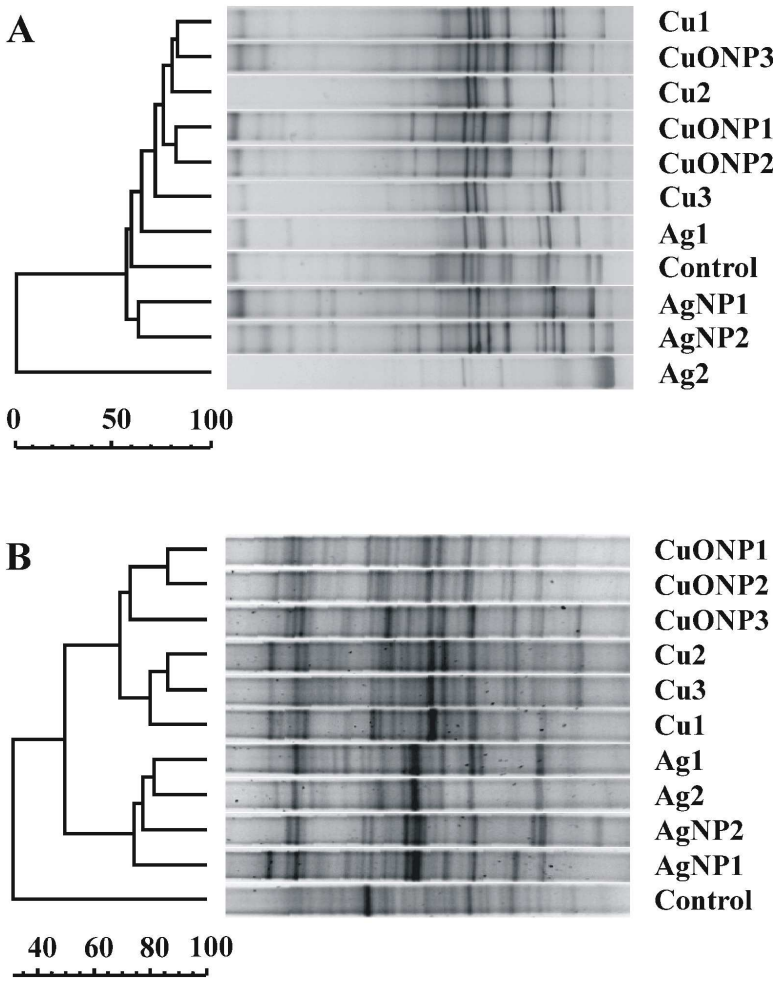

A
Cu1
CuONP3
Cu2
CuONP1
CuONP2
Cu3
Ag1
Control
AgNP1
AgNP2
Ag2
0
50
100
B
CuONP1
CuONP2
CuONP3
Cu2
Cu3
Cu1
Ag1
Ag2
AgNP2
AgNP1
Control
40
60
80
100

Fig. 5

Fig5