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Nanosilver impacts on aquatic microbial decomposers and litter decomposition assessed as pollution-induced community tolerance (PICT)†

Daniela Batista, *[ab] Ahmed Tlili, [cd] Mark O. Gessner,[ce] Cláudia Pascoal[ab] and Fernanda Cássio [ab]

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The growing proliferation of silver nanoparticles (AgNPs) calls for detailed information on ecotoxicological effects, particularly on diverse communities and key ecosystem processes where impacts remain poorly known. This includes the decomposition of plant litter by fungi and bacteria in streams. Impacts are likely to depend on community composition, because species vary in their sensitivities to stressors. Therefore, our goal was to determine if shifts in microbial communities triggered by chronic exposure to low concentrations of nano (<200 μg L^{-1}) and ionic (20 μg L^{-1}) silver increase community tolerance to these contaminants, as described in the pollution-induced community tolerance (PICT) concept. We used stream microbial decomposers associated with leaf litter in microcosms to assess the applicability of this concept by determining tolerance acquisition towards AgNP and ionic Ag in short-term inhibition assays. Endpoints included fungal sporulation, bacterial production, microbial respiration and the potential activity of a protein-degrading enzyme, leucine aminopeptidase. Analyses of microbial communities showed that chronic exposure to the highest AgNP concentrations led to similar communities, and that these were distinct from the control communities. Most important, chronic exposure of fungi and bacteria to both AgNP and ionic Ag also increased tolerance of the microbes, as revealed by notably reduced adverse effects on bacterial production. Overall, our results demonstrate the usefulness of applying the PICT concept to litter decomposers and decomposition as an approach to assess the risks posed by nano and ionic silver to freshwater ecosystems.

Environmental significance

The rapid proliferation and release of silver nanoparticles (AgNPs) in the environment entails strong impacts on microbial communities. This likely includes fungi and bacteria associated with decomposing leaf litter, with consequences on the decomposition process as a fundamental part of carbon and nutrient cycling in forest streams and many other ecosystems. A promising approach to assess chronic effects of nanoparticles on complex microbial communities and processes is based on the pollution-induced community tolerance (PICT) concept. Here we applied the concept for the first time to litter decomposers and decomposition. Our results show that the approach is effective at detecting tolerance acquisition of microbial decomposers exposed to AgNPs at low concentrations. This finding demonstrates the value of the PICT concept to assess impacts of AgNPs on microbial decomposers and ecosystem functioning by combining structural and functional endpoints.

1. Introduction

The production and use of silver nanoparticles (AgNPs) have been exponentially growing over the last decades, increasing the probability that a fraction of the particles is carried to wastewater and, ultimately, to rivers, lakes and coastal waters. The predicted environmental concentrations (PECs) of AgNPs in surface waters are expected in the range of ng to μg per liter.[1] However, higher concentrations can be attained during accidental spills and other direct releases. Organisms in these aquatic environments are exposed to AgNPs and potentially experience notable consequences in terms of

[a] *Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. E-mail: danimbatista@gmail.com, danimbatista@bio.uminho.pt*
[b] *Institute of Science and Innovation for Bio-Sustainability (IB-S), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal*
[c] *Department of Experimental Limnology, Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB), Alte Fischerhütte 2, 16775 Stechlin, Germany*
[d] *Department of Environmental Toxicology, Swiss Federal Institute of Aquatic Science and Technology (Eawag), Dübendorf, Switzerland*
[e] *Department of Ecology, Berlin Institute of Ecology (TU Berlin), Ernst-Reuter-Platz 1, 10587 Berlin, Germany*

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survival and performance.[2,3] Although AgNPs have been shown to be bactericidal[4] and also toxic to many other species,[5–8] it is unclear whether and to what extent the toxicity is due to nanoparticle properties or to the release of ionic Ag from NPs. While some studies suggest that silver ions released by AgNP dissolution play a key role,[9] others indicate that toxicity caused by intrinsic AgNP properties can also be important, possibly due to the high surface area and reactivity of AgNPs.[8,10,11]

AgNP toxicity has been mainly studied in single organisms.[12,13] However, such studies fall short of capturing impacts on whole communities and ecosystems, where indirect effects mediated by species interactions can superimpose direct damages and lead to complex outcomes.[14,15] Consequently, it has been argued that natural communities and ecosystem processes need to be considered in ecotoxicological assessments, to provide a basis for evaluating environmental impacts of potential contaminants comprehensively, including those of AgNPs.[7,8,14–16]

Plant litter decomposition is a key process in many ecosystems. It is an excellent target to reveal complex impacts of pollutants in ecosystems, because decomposition rates depend on the activity of a broad range of interacting species, including fungi, bacteria and invertebrates.[17] In streams, a group of fungi known as aquatic hyphomycetes have been identified as the dominant microbial decomposers, but bacteria also play a role, especially at advanced stages of litter decomposition.[18,19]

Studies focusing on litter decomposition in streams have shown that decomposition rate, microbial biomass and fungal sporulation decrease as a result of AgNP exposure.[6,8,20,21] Nevertheless, aquatic hyphomycetes occurring in metal-polluted streams[19,22] tend to exhibit greater tolerance to metals[23] and metal nanoparticles[24] than aquatic hyphomycete strains isolated from unpolluted streams, indicating that populations can express resistance mechanisms to cope with metal stress.[23] If so, testing for tolerance acquisition could be a useful approach to assess pollution by nanoparticles and other contaminants in streams.

Microbial communities harbouring species that differ in their sensitivity to stressors constitute the basis of the pollution-induced community tolerance (PICT) concept originally proposed by Blanck *et al.*[25] The rationale behind PICT is that chronic exposure to a toxicant increases community tolerance as a result of adaptation of populations (adjustment of populations to a changing environment involving genetic modifications) or acclimatization (adjustment of individuals to a changing environment to maintain performance) to the stressor, or because a community shift, when tolerant species are replaced by sensitive species. Community tolerance to a given toxicant is quantified by measuring the inhibition of physiological endpoints in short-term acute assays and comparing the responses of both the control and chronically pre-exposed community.[26] A higher sensitivity of the control community compared to the chronically exposed community to the toxicant indicates community tolerance acquisition.

The PICT concept has been tested in terrestrial microbes[27] and stream biofilms,[28] but studies with aquatic microbial decomposers have not yet been conducted, despite the great potential of the concept. Indeed, testing for PICT has been suggested as a powerful approach to establish causal relationships between chemicals and their effects on biota,[29] and could provide valuable information to assess risks posed by AgNPs and other contaminants across taxa and ecosystem processes.

The aim of the present study was to determine if shifts in the structure of microbial decomposers communities triggered by chronic exposure to low concentrations of AgNP and ionic Ag can induce tolerance to silver species. To that end, microbial communities associated with leaf litter in streams were first experimentally exposed to low concentrations (μg L^{-1} range) of AgNPs and $AgNO_3$, before determining community tolerance in short-term assays by establishing dose–response curves for multiple physiological endpoints: fungal sporulation, bacterial production, microbial respiration, and the potential activity of an enzyme involved in protein degradation and nitrogen acquisition. Ionic Ag was used as a positive control to distinguish between the effects of dissolved Ag released from AgNPs and specific nanoparticle effects and the effects of dissolved Ag.

2. Material & methods

2.1. Experimental setup

A mix of leaves (oak, alder and poplar) were placed into 0.5 mm mesh bags (16 × 20 cm) and immersed in mid-September in a forested softwater stream in the Harz Mountains, Germany (51°42′N″, 10°21′E″), to enable microbial colonization. After 7 days, the litter bags were retrieved and transported to the laboratory in a cooling box containing stream water. Dissolved oxygen concentrations and pH of the stream water were measured *in situ* (Multiline F/set 3, no. 400327, WTW, Weilheim, Germany) at these occasions. Water samples were collected and transported to the laboratory in a cooling box with ice to determine inorganic nutrient concentrations (Table S1†). In the laboratory, the colonized leaves were placed in stream water under aeration at 16 °C for 48 h to stimulate the release of fungal spores and the detachment of bacteria, which served to inoculate microcosms (see below).

Freshly abscised and air-dried poplar leaves (*Populus* sp.) were soaked in deionized water until they were pliant before they were cut into 12 mm diameter leaf discs with a cork borer. The discs were dried at 45 °C for 2 days and weighed to determine the initial leaf dry mass. Next, the leaf discs were leached with Volvic mineral water for 24 h (Auvergne Regional Park, France; pH = 7, Ca^{2+} = 11.5, Cl^- = 13.5, NO_3^- = 6.3, K^+ = 6.2, Na^+ = 11.6 mg L^{-1}) and placed in polypropylene microcosms (20 × 15 cm) with 400 mL of Volvic water (130

leaf discs per microcosm) and 40 mL of the above inoculum suspension to ensure microbial colonization of the leaves.

After 5 days, the water was renewed and the microcosms were exposed to four levels of citrate-coated AgNPs (0, 50, 100 and 200 μg L^{-1}; 20 nm nominal diameter, NanoSys GmbH, Wolfhalden, Switzerland) or one level of ionic Ag (20 μg L^{-1}, added as $AgNO_3$, >99%; Sigma-Aldrich, St. Louis, MO, USA). The latter was included as a positive control for dissolved silver. Four replicate microcosm of each treatment were incubated for 25 days at 16 °C on an orbital shaker at 120 rpm. Water was renewed every 5 days, and the spore suspensions collected each time were preserved in 2% formalin for later fungal identification and spore counting. The leaf discs were sampled after 25 days to determine the litter dry mass remaining, Ag accumulation on leaves, microbial biomass and diversity, microbial respiration, and the potential activity of a protein-degrading enzyme, leucine aminopeptidase (LAP). In addition, leaf discs were used to test for tolerance acquisition in short-term bioassays, using increasing concentrations of AgNP and ionic Ag to establish dose–response curves for fungal sporulation, bacterial production, microbial respiration and LAP activity.

2.2. AgNP characterization

AgNP suspensions of 100 and 200 μg L^{-1} were prepared in fresh and conditioned water to determine particle size and surface charge.[20] Conditioned water was obtained from the control microcosms, before each water renewal and spiked with AgNPs (100 and 200 μg L^{-1}). Nanoparticles were characterized in conditioned water to account for biomolecules released from the decomposing leaves, which could affect the colloidal stability of the particles. The AgNPs in suspensions were analyzed for particle size and zeta potential within 15 min, and then again after 5 days. The hydrodynamic diameter of AgNPs was measured by dynamic light scattering (DLS), using a Zetasizer (Nano ZS, Malvern Instruments Ltd., Worcestershire, UK), and by nanoparticle tracking analysis (NTA) using a NanoSight LM10 equipped with a LM14 temperature controller (NanoSight Ltd., Wiltshire, UK). The zeta potential of AgNPs in the suspensions was measured using the Zetasizer.

2.3. Metal analysis

Total Ag concentration in the leaf discs from microcosms containing 50, 100 and 200 μg l^{-1} AgNP were determined after acid digestion (4 mL 65% HNO_3 + 1 mL 35% H_2O_2) in a high-performance microwave (MLS-1200 MEGA, Leutkirch, Switzerland) at a maximum temperature of 195 °C and a maximum pressure of 100 bar. Each solution was then diluted 50 times with nanopure water, resulting in a final concentration of 1.3% HNO_3. The Ag content (isotope ^{109}Ag) was measured by high-resolution inductively coupled plasma-mass spectrometry (HR-ICP-MS, Element 2 high resolution sector field ICP-MS; Thermo Finnigan, Bremen, Germany).[20] The reliability of the measurements was determined by using specific water references (National Water Research Institute, Burlington, Ontario, Canada). Dissolved Ag from AgNP suspensions was determined by ultrafiltration for 30 min at 3220*g* (Megafuge 1.0R, Thermo Scientific Inc., Waltham, MA, USA) using Ultracel 3 k centrifugal filter devices (Amicon Millipore, Darmstadt, Germany) with a molecular cutoff of 3 kDa (pore size <2 nm). Ag in the filtrates was measured as for the total Ag concentration.

2.4. Nutrient quantification

Water samples from each microcosm were analyzed for total phosphorus (TP), soluble reactive phosphorus (SRP), total nitrogen (TN), nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+) and dissolved organic carbon (DOC).[30] Samples were pre-filtered (0.2 μm pore-size Nuclepore membranes) and kept at −20 °C until analysis. TP was first digested with $K_2S_2O_8$ (134 °C for 30 min) and then quantified as PO_4^{3-}. TN was digested to NO_3^- /NO_2^- with Oxisolv® (Merck) and allowed to react at 120 °C for 45 min. Nitrite and nitrate concentrations in water samples were then measured spectrophotometrically (FIAstar™ 5010 analyzer, FOSS GmbH, Rellingen, Germany) according to the manufacturer's instructions. DOC was determined by treating water samples with 2 M HCl and subsequent high-temperature combustion in a TOC analyzer (Multi N/C 3100, Analytik Jena AG, Germany). Data of the nutrient analyses are given in Table S2.†

2.5. Leaf decomposition

Leaf discs retrieved from each microcosm before and after the experiment were freeze-dried (Christ Alpha 2–4, Osterrode, Germany) to constant mass (about 48 h) and weighed to the nearest 0.001 mg.

2.6. Fungal sporulation and biomass

Fungal sporulation rates and community composition were determined by identifying and counting spores of aquatic hyphomycetes released from leaves into the water of the microcosms.[31] Spore suspensions were mixed with 200 mL of 0.5% Tween 80 and filtered (0.45 μm pore size; Millipore, Billerica, MA, USA). The spores retained on the filter were stained with 0.05% cotton blue in lactic acid. At least 300 spores were identified[32] and counted under a light microscope (Leica Biomed, Heerbrug, Switzerland) at 400× magnification.[31]

Fungal biomass on leaves was quantified as ergosterol.[33] Briefly, lipids were extracted from sets of 4 leaf discs by heating (80 °C, 30 min) in 0.8% KOH in methanol and the extract purified by solid-phase extraction. Ergosterol was quantified by high-performance liquid chromatography (HPLC), using a LiChrospher RP18 column (250 mm × 4 mm; Merck, Darmstadt, Germany). The system was run isocratically with methanol at 1.5 mL min^{-1} and the column temperature was 42 °C. Ergosterol was detected and

quantified at 282 nm. Ergosterol standards (≥98%, Acros Organics, Geel, Belgium) were dissolved in isopropanol.

2.7. Total bacterial abundance and production

Total bacterial abundance was estimated from sets of three leaf discs collected before and after the microcosm experiments. Bacteria were detached from the leaf discs by sonication (3 times for 20 s), with the samples being cooled on ice after each cycle.[34] Bacteria were stained with SYBR Green I diluted 10 000× in DMSO (SYBR Green I nucleic acid gel stain, Lonza, Rockland, ME, USA) and counted by flow cytometry. The cells were stained for 15 min at room temperature in the dark. Fluorescent beads (Flow Count Fluorospheres, Beckman Coulter, Inc., Brea, CA, USA) were added to each sample as an internal standard to normalize cell fluorescence emission and light scatter values. All samples were run on a Gallios flow cytometer (Beckman Coulter) equipped with a laser emitting light at 488 nm. The green and red fluorescent signals were collected in the FL1 (368 nm) and FL3 (486 nm) channel, respectively. For each sample run, data for 5000 events were collected. All data were processed with Kaluza for Gallios software (Partek Inc., St. Louis, MO, USA.), which was also used to separate positive signals from noise.

Bacterial production was estimated by following the incorporation of radiolabeled leucine into protein.[35] Sets of three leaf discs per replicate were placed into scintillation vials containing 2.9 mL of filter-sterilized Volvic mineral water. Samples were incubated with leucine at a final concentration of 50 μM: 4.5 μM ^{14}C-leucine plus 2.4 M non-radioactive leucine, for 30 min at 15 °C with gentle shaking. The incorporation was stopped by adding TCA to a final concentration of 5%. Samples were then sonicated for 1 min as described above and filtered on 0.2 μm pore-size polycarbonate filters (Whatman, Maidstone, UK). Both the filter and leaf discs were rinsed twice with 5% TCA, and once with 40 mM leucine, 80% ethanol, and nanopure water. The filter and leaf discs were transferred to a centrifuge tube containing 1.5 mL of an alkaline solution (1.5 M NaOH, 75 mM EDTA, 0.3% SDS) and incubated for 60 min at 90 °C. The samples were cooled down to ambient temperature, centrifuged (10 min at 14 000*g*), and 250 μL of the supernatant was transferred to a scintillation vial containing 5 mL of Ultima gold XR scintillation cocktail. Radioactivity was measured in a Tri-Carb 2810 TR scintillation counter (Perkin Elmer, Waltham, MA, USA) with quench correction.

2.8. Microbial respiration

Microbial respiration was measured with the MicroResp™ approach,[36] a spectrophotometric method based on CO_2 production in a sealed microplate system. One leaf disc per replicate was placed in wells of a microplate (Nunc DeepWell, Thermo Scientific). Glucose was added (25 mM final concentration) as a labile carbon source to maximize respiration. Microplates were sealed airtight, pairing the wells with a second detection plate that contained a pH indicator embedded in agar. Absorbance was measured at 572 nm both immediately before sealing the well plate and after 15 h in the dark at 20 ± 1 °C.

2.9. Leucine aminopeptidase activity

Leucine aminopeptidase (LAP) cleaves peptides bonds and thus plays an important role in protein degradation and nitrogen acquisition of litter decomposers.[37] Its potential activity was assayed by fluorescent-linked artificial substrate L-leucine-4-methyl-7-coumarinylamide hydrochloride (Leu-AMC [7-amino-4-methylcoumarin]; Sigma-Aldrich). One leaf disc per replicate was incubated at saturating concentrations of the substrate (10 mM) at 15 °C in the dark. The reaction was stopped after 1 h by adding glycine buffer (1 M, pH 10.4) and fluorescence was measured at 455 nm upon excitation at 365 nm. The fluorescence data were converted to concentrations of cleaved substrate analogues based on a calibration curve.

2.10. Microbial DNA fingerprints

DNA was extracted from three leaf discs using the MoBio Ultra Clean Soil DNA isolation kit (MoBio Laboratories, Solana Beach, CA, USA), according to manufacturer instructions. The ITS2 region of fungal ribosomal DNA was amplified with primer pairs ITS3GC/ITS4,[38] and the V3 region of bacterial 16S rDNA was amplified with primer pairs 518R/338F_GC.[39] The polymerase chain reaction (PCR) was carried out following the protocol of Duarte *et al.*[40]

Denaturing gradient gel electrophoresis (DGGE) analysis[39] was performed using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Samples of 20 μL from the amplification products of 380–400 bp were loaded on 8% (w/v) polyacrylamide gel in 1× Tris-acetate-EDTA (TAE) buffer with a denaturing gradient from 30 to 70% for fungal DNA and 40 to 70% for bacterial DNA. The gels were run at 55 V and 56 °C for 16 h and stained with Midori Green (GRiSP, Porto, Portugal) for 10 min in a shaker at 40 rpm. Gel images were captured under UV light in a ChemiDoc XRS (BioRad).

2.11. Tolerance assessments

The tolerance of microbial communities induced by AgNPs and ionic Ag was examined in short-term inhibition assays. To this end, sets of leaf discs from each microcosm were collected, and each exposed for 12 h to a range of increasing concentrations of AgNPs or $AgNO_3$ (0 to 16 500 μg L^{-1}, five leaf discs per concentration from each of four replicate microcosms) to determine concentration-response relationships and calculate EC_{50} values for complementary endpoints characterizing the performance of microbial communities: fungal sporulation, bacterial production, microbial respiration and LAP potential activity. Analyses of these endpoints were conducted as described above. Tolerance was assessed by comparing the degree of

inhibition in the short-term assays between control and pre-exposed communities. Higher EC_{50} values indicate a higher tolerance to AgNPs or $AgNO_3$.

2.12. Data analysis

The effects of AgNPs or ionic Ag on leaf mass loss, fungal biomass and sporulation, bacterial production and biomass, microbial respiration and LAP activity were tested by one-way analyses of variance (ANOVA).[41] No data transformation was required since no indications of unequal variances or not normally distributed residuals were found (Shapiro–Wilk and *F* tests).

Dose–response curves obtained from the short-term bioassays were adjusted to four-parameter logistic curves.[41] AgNPs and ionic Ag concentrations producing a 50% effect (EC_{50}) were determined by nonlinear regression of sigmoidal dose–response curves using the Hill slope equation (GraphPad Prism version 6.00 for Windows, San Diego, CA, USA). EC_{50} values were compared by inspecting the overlap of 95% confidence intervals (CIs).

Each DGGE band was considered an operational taxonomic unit (OTU). DGGE gels were aligned and the relative intensity of the bands in the gel was determined with Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analyses of fungal and bacterial DNA fingerprints were performed with PRIMER software (v.6.1.6, PRIMER-E, Plymouth, UK).

3. Results

3.1. Nanoparticle characterization and chemical analysis

The average particle diameter in AgNPs suspensions measured by DLS in fresh water was 67 ± 3 and 76 ± 1 nm for 100 and 200 μg L^{-1}, respectively (Table 1). In conditioned water, the average size tended to increase slightly with exposure time. The maximum of 151 ± 7 nm was reached after 25 days in microcosms with leaves exposed to the highest AgNP concentration (200 μg L^{-1}). Similar particle sizes of AgNPs in suspensions were determined by NTA (Table 1). The zeta-potential of AgNPs in 100 and 200 μg L^{-1} suspensions in fresh water was −16 mV (Table 1). Particle charges in the conditioned water were more negative than in fresh water and slightly decreased with exposure time (Table 1).

The total Ag accumulated in leaves within 25 days increased with increasing AgNP concentrations (3.2 ± 0.7, 11.4 ± 2.6, and 14.2 ± 3.4 μg g^{-1} for 50, 100 and 200 μg L^{-1}, respectively). Moreover, the total Ag accumulated in leaves was a notable fraction in microcosms receiving $AgNO_3$ (12.3 ± 2.9 μg g^{-1} of total Ag). The fraction of dissolved Ag from AgNP suspensions was similar across AgNP concentrations and exposure times (<0.01 μg L^{-1}).

A decrease in DOC concentration, from *ca.* 50 to 19 mg L^{-1}, was observed after 5 days in all microcosms, whereas NO_3^- concentrations tended to increase with exposure time (Table S2†). The dissolved oxygen concentration and pH in the microcosms slightly increased with exposure time in all cases (Table S2†).

Table 1 Particle diameter (mean ± SD, *n* = 3) in suspensions with 100 and 200 μg L^{-1} AgNPs, measured by dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) in freshly prepared water (fresh) and water from control microcosms with leaves colonized by microbes (conditioned). Measurements were performed about 15 min after adding the particles and again after 5 days. Water in the microcosms was renewed and analysed every 5 days during the 25 days of the experiment. The polydispersion index (PdI) and zeta-potential were measured with a Zetasizer

Exposure			Average diameter (nm)			
AgNPs (μg L^{-1})	Water	Time (days)	DLS	NTA	PdI	Zeta potential (mV)
100	Fresh	0	67 ± 3	44	0.35 ± 0.02	−16 ± 2
		5	66 ± 1	39	0.28 ± 0.03	−6 ± 1
100	Conditioned (1st renewal)	0	108 ± 1	73	0.39 ± 0.02	−18 ± 3
		5	107 ± 3	69	0.52 ± 0.01	−15 ± 2
	Conditioned (2nd renewal)	0	100 ± 8	69	0.36 ± 0.03	−16 ± 4
		5	112 ± 6	69	0.56 ± 0.02	−14 ± 3
	Conditioned (3rd renewal)	0	115 ± 9	69	0.34 ± 0.06	−21 ± 3
		5	95 ± 6	76	0.39 ± 0.06	−12 ± 2
	Conditioned (4th renewal)	0	78 ± 9	70	0.40 ± 0.07	−19 ± 1
		5	104 ± 2	78	0.39 ± 0.03	−11 ± 3
	Conditioned (5th renewal)	0	153 ± 5	66	0.32 ± 0.07	−19 ± 1
		5	64 ± 4	66	0.86 ± 0.15	−16 ± 2
200	Fresh	0	76 ± 1	45	0.44 ± 0.06	−16 ± 1
		5	50 ± 2	47	0.42 ± 0.03	−16 ± 1
200	Conditioned (1st renewal)	0	63 ± 1	75	0.38 ± 0.01	−14 ± 1
		5	104 ± 2	76	0.41 ± 0.04	−16 ± 1
	Conditioned (2nd renewal)	0	96 ± 4	73	0.37 ± 0.07	−15 ± 3
		5	108 ± 4	74	0.61 ± 0.06	−14 ± 4
	Conditioned (3rd renewal)	0	77 ± 8	48	0.41 ± 0.09	−17 ± 1
		5	115 ± 5	74	0.53 ± 0.03	−10 ± 2
	Conditioned (4th renewal)	0	101 ± 4	65	0.44 ± 0.05	−21 ± 2
		5	139 ± 7	88	0.55 ± 0.04	−12 ± 2
	Conditioned (5th renewal)	0	77 ± 6	69	0.43 ± 0.10	−18 ± 1
		5	151 ± 7	71	0.49 ± 0.04	−16 ± 1

3.2. Effects on fungal diversity and microbial DNA fingerprints

A total of 15 sporulating fungal species were identified in the suspension used to inoculate the microcosms. After 25 days, 12 sporulating fungal species were found on poplar leaves. In the control microcosms, *Flagellospora curvula* was the dominant species, followed by *Tetracladium marchalianum* and *Tetrachaetum elegans* (Table S3†). *Clavariopsis aquatica* mainly occurred in communities exposed to Ag, whereas *Tetrachaetum elegans* became rare in those microcosms. Cluster analysis of DNA fingerprints suggest that after 25 days in microcosms, bacteria exposed to 100 and 200 μg L^{-1} AgNPs and $AgNO_3$ grouped together, whereas fungi exposed to 50 μg L^{-1} AgNPs grouped with the control (data not shown).

3.3. Effects on leaf decomposition and microbial performance

Poplar leaves had lost 60% of their initial mass after 25 days. This mass loss was not significantly affected by exposure to AgNPs or $AgNO_3$ (Fig. 1a), although the difference approached the conventional significance level of 0.05 (one-

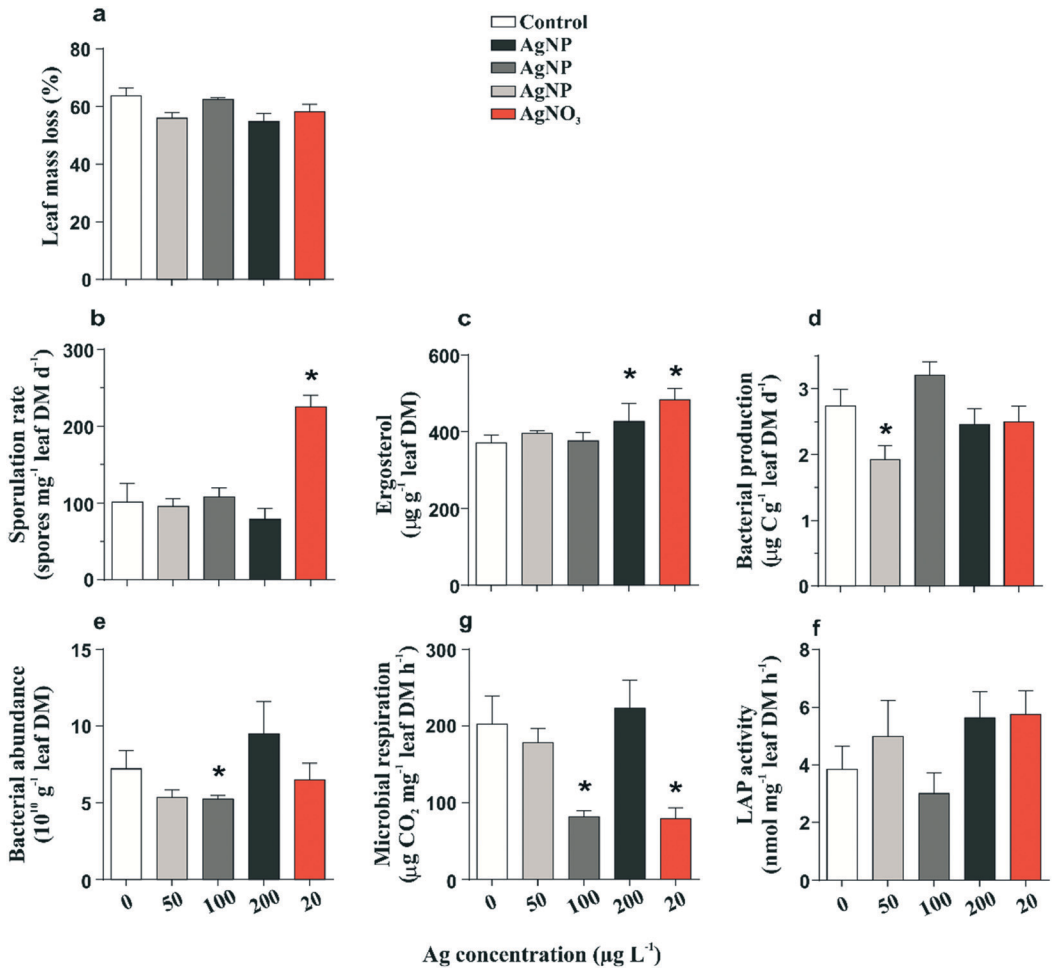

Fig. 1 Mass loss (a), fungal sporulation rate (b), ergosterol concentration (c), bacterial production (d), bacterial abundance (e), microbial respiration (f) and LAP activity (g) associated with decomposing leaves after 25 days of AgNP or $AgNO_3$ exposure. Asterisks indicate significant differences from the control (one-way ANOVA, $p < 0.05$).

way ANOVA, $p = 0.06$). Fungi on decomposing leaves in control microcosms produced 100 spores mg^{-1} leaf dry mass day^{-1}. Exposure to AgNPs had no significant effect on fungal sporulation, whereas $AgNO_3$ stimulated sporulation to 230 spores mg^{-1} leaf dry mass day^{-1} (one-way ANOVA, $p < 0.0001$) (Fig. 1b). Fungal biomass in leaves of control microcosms was 371 µg g^{-1} leaf dry mass. Exposure to 200 µg L^{-1}AgNPs and to $AgNO_3$ significantly increased fungal biomass (one-way ANOVA, $p < 0.0001$) (Fig. 1c).

Bacterial production on leaves was significantly reduced by exposure to 50 µg L^{-1} AgNPs (one-way ANOVA, $p = 0.02$) but not at higher AgNP concentrations or by exposure to 20 µg L^{-1} $AgNO_3$ (Fig. 1d). Bacterial abundance on leaves was significantly decreased in microcosms receiving 100 µg L^{-1} AgNPs (one-way ANOVA, $p < 0.0001$) (Fig. 1e). Microbial respiration on leaves was significantly reduced by exposure to 100 µg L^{-1} AgNPs and to $AgNO_3$ (one-way ANOVA, $p = 0.002$), although no effect was observed at 50 or 200 µg L^{-1} AgNPs (Fig. 1f). The potential LAP activity on leaves was 3.85 nmol mg^{-1} h^{-1} after 25 days in control microcosms and was not significantly affected by exposure to AgNPs or $AgNO_3$ (one-way ANOVA, $p = 0.76$) (Fig. 1g).

ANOVA results on the effects of AgNPs or ionic Ag on leaf mass loss, fungal sporulation rate, ergosterol concentration, bacterial production, bacterial abundance, microbial respiration and LAP activity on decomposing leaves are given in Table S4.†

3.4. PICT responses

The dose–response curves showing short-term effects (12 hours) of AgNPs and $AgNO_3$ on fungal sporulation rates, bacterial production, and LAP activity of microbial communities are presented in Fig. S1.† Fungal sporulation (Fig. S1a and b†) and bacterial production (Fig. S1c and d†) were inhibited by exposure to increasing concentrations of AgNPs and $AgNO_3$. The concentrations reducing sporulation rate and bacterial production by 50% were higher for AgNPs than for $AgNO_3$ (Fig. S1a–d† respectively). The activity of LAP (Fig. S1e and f†) and microbial respiration rates (Fig. S1g and h†) were significantly decreased only by exposure to $AgNO_3$. R^2 values for the fit of each dose–response curve for bacterial production, fungal sporulation rate and potential LAP activity

in control communities and pre-exposed communities to AgNP or $AgNO_3$ can be found in Table S5.†

EC_{50} values for fungal sporulation did not significantly differ among treatments in response to AgNP and $AgNO_3$ exposure during the short-term bioassays (Fig. 2). In contrast, EC_{50} values for bacterial production were significantly higher for communities pre-exposed to 100 and 200 μg L^{-1} AgNPs as well as to 20 μg L^{-1} $AgNO_3$ compared to the control community (Fig. 2). The higher the AgNPs concentration to which the communities had been previously exposed, the greater the community tolerance (Fig. 2). EC_{50} for LAP activity in the AgNP bioassays and for microbial respiration in both AgNP and $AgNO_3$ bioassays could not be calculated because the inhibitions did not exceed 50% (Fig. 2).

4. Discussion

The pollution-induced community tolerance (PICT) concept first applied here to fungi and bacteria on decomposing litter in streams showed that microbial decomposers are capable of acquiring tolerance to AgNPs and $AgNO_3$ upon chronic exposure to these toxicants. This finding supports the idea that the PICT concept provides a valuable framework to assess impacts of AgNPs and other contaminants on microbial decomposers and litter decomposition as a fundamental process in the functioning of stream ecosystems.

Bacterial production has been shown to be an effective endpoint to assess the sensitivity of microbial decomposers to metals, including specific AgNP effects.[20] In the present study, the production of bacteria exposed to 100 and 200 μg L^{-1} AgNPs or to 20 μg L^{-1} $AgNO_3$ exhibited higher EC_{50} than control communities, indicating tolerance acquisition as conceptualized in the PICT concept. For fungi, similar levels of acquired tolerance were not observed, as judged based on the number of fungal spores released from submerged litter. This outcome might be partly related to variability in fungal sporulation rates, which is typically high. However, it could also reflect a greater intrinsic tolerance of fungi than bacteria to silver exposure. Furthermore, fungi tend to have longer generation times than many bacteria and hence are less likely capable of responding rapidly to changing conditions, including to cellular damage caused by AgNPs. Therefore, tolerant bacterial populations could be selected faster than fungal populations, leading to more tolerant communities of bacteria than fungi.[20] Detailed analyses of microbial communities by amplicon sequencing[42,43] would provide deeper insights into such effects on microbial communities, as shown for soils where different bacterial populations were found to differ widely in their sensitivities to Ag.[44]

Another notable observation in our study was that anabolic processes requiring energy, such as fungal sporulation and bacterial production, were more sensitive to AgNP exposure than catabolic processes such as extracellular

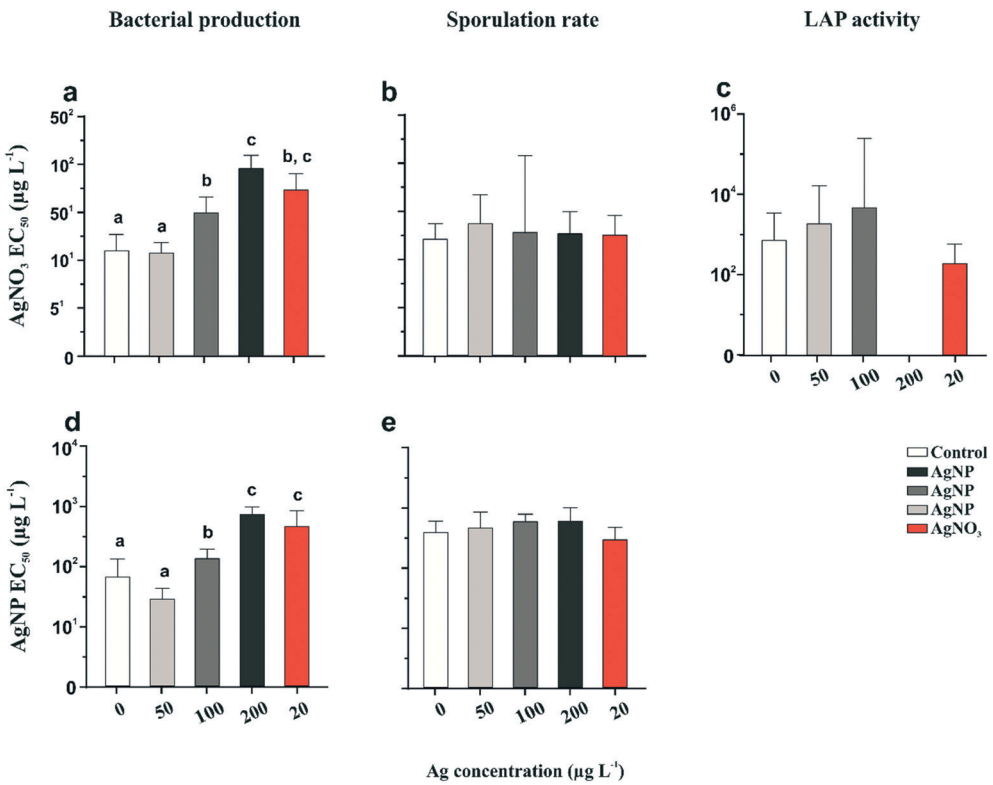

Fig. 2 EC_{50} values for bacterial production (a and d), fungal sporulation rate (b and e) and potential LAP activity (c) in control communities and communities pre-exposed to AgNP or $AgNO_3$, obtained in short-term bioassays with increasing concentrations of AgNP (d and e) or $AgNO_3$ (a–c). Error bars indicate 95% confidence intervals (n = 4). Different letters indicate that EC_{50} values between treatments are significantly different.

enzyme (LAP) activity and microbial respiration. AgNPs toxicity[45] and tolerance acquisition[29] are related to metabolic activities needed for cellular detoxification.[29] This indicates that anabolic and catabolic processes might respond to silver contamination in different ways, and that predictions of AgNP effects can be complex because net outcomes depend on the balance of effects on counteracting anabolic and catabolic microbial responses to a toxicant.[46]

The choice of suitable endpoints is important to determine tolerance in PICT analyses.[11,29,36] LAP is a protein-degrading extracellular enzyme that plays a role in microbial nitrogen acquisition.[47] Therefore and because short-term exposure to AgNPs did not affect LAP activity, our results suggest that metal-resistant microbial species could have maintained the functions of LAP even at elevated metal concentrations. Similarly, although the MicroResp technique has been successful at detecting toxicity in other studies,[36,48] we failed to detect differences in respiration rates between control communities and those pre-exposed to Ag. In the microcosms receiving $AgNO_3$, metabolic activities could have been stimulated by the added nitrate as a nutrient in potentially short supply[36] and that could have confounded estimates of EC_{50} values. In support of this idea, NO_3^- concentrations in microcosms exposed to 20 μg L^{-1} $AgNO_3$ were indeed increased.

One of the most interesting findings of our study is that exposure to 20 μg L^{-1} $AgNO_3$ increased tolerance of the bacterial community on decomposing leaves to AgNPs and *vice versa*. There is evidence that distinct mechanisms govern microbial responses to different silver species.[46] Therefore, our results could indicate that the mechanisms leading to tolerance development towards AgNPs are mainly mediated by Ag particles releasing silver ions. Metal stress responses induced by ionic Ag have been previously reported for bacteria,[27] biofilms[48] and periphyton[49] suggesting that metal ion effects could be common, although not universal, since specific silver particle effects have also been demonstrated.[11,46] In natural environments, exposure to other contaminants could also rise to co-tolerance.[50] However, such mechanisms could not determine outcomes in our experiment, because only a single factor was varied between contaminated and control microcosms.

The chronic exposure to AgNPs and $AgNO_3$ affected the functional endpoints used in our study in distinct ways. AgNPs did not significantly affect leaf mass loss or fungal sporulation rate, even though fungal sporulation can be severely inhibited by both ionic[8,11] and nano Ag.[6,8,16] In contrast, $AgNO_3$ stimulated fungal sporulation in addition to biomass. This unexpected outcome might be explained by the fact that low concentrations of metals can stimulate microbial growth and reproduction, a phenomenon known as hormesis.[51] Alternatively, fungi might have indirectly benefitted from reduced competition by bacteria that exhibit greater sensitivity to Ag than fungal populations.[20] Whatever the mechanism, the varied outcomes highlight the need to assess multiple endpoints for robust assessments of Ag effects in ecosystems.

In conclusion, our results show that PICT is a valuable concept combining structural and functional measures to assess impacts of AgNPs and $AgNO_3$ on microbial communities associated with decomposing litter. All responses in our study were more affected by $AgNO_3$ than by AgNPs, even at 10 times lower concentrations of ionic than nano silver. This implies that at equal effective concentrations dissolved Ag is more toxic than AgNPs, as reported in other environmental systems (algae,[52] periphyton,[49] microbial litter decomposers[6,20]). This outcome was unlikely due to AgNP agglomeration. Although average size of the AgNP slightly increased over time during our experiment, the particles were still well in the nanosize range. Given the central role of litter decomposition in streams, the impacts of silver on microbial litter decomposers can have important repercussions on ecosystem functioning, both in nanoparticle and ionic form.[6,8,16,20] Consequently, studies combining exposure to Ag (or other metal) NPs with approaches such as PICT, hold much potential to establish links between exposure and effects at the community level, facilitating risk assessment for aquatic ecosystems.

Conflicts of interest

There are no conflicts of interest to declare.

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